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TITLE:

Improved UPLC-UV method for the quantification of vitamin C in lettuce varieties (*Lactuca sativa* L.) and crop wild relatives (*Lactuca* spp.)

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KEYWORDS:

Ascorbic acid, Crop Wild Relatives (CWR), Dehydroascorbic acid, Lettuce, *Lactuca sativa* L., Liquid chromatography

SUMMARY:

We present a fast and reliable method to quantify vitamin C in *Lactuca* spp. using UPLC-UV, potentially transferable to other plants. The key steps are the sample preparation and vitamin C extraction under stable conditions, the reduction of dehydroascorbic acid to ascorbic acid and the optimization of the chromatographic procedure.

ABSTRACT:

Vitamins, especially vitamin C, are important micronutrients found in fruits and vegetables. Vitamin C is also a major contributor to their antioxidant capacity. Lettuce is one of the most popular vegetables among consumers worldwide. An accurate protocol to measure vitamin C content in lettuce and other related species is crucial. We describe here a method using the ultra-high-performance liquid chromatography-ultraviolet (UPLC-UV) technique, in which sample preparation, vitamin extraction and chromatography conditions were optimized.

Samples were collected to represent the entire plant, frozen at -80 °C and lyophilized to prevent undesirable oxidation and make their manipulation easier. The extraction of

vitamin C was carried out in acidic media, which also contributed to its stability. As vitamin C can be present in two different interconvertible forms, ascorbic acid (AA) and dehydroascorbic acid (DHAA), both compounds should be measured for accurate quantification. The DHAA was quantified indirectly after its reduction to AA because AA shows a higher absorptivity than DHAA in the UV range of the spectrum. From the same extract, two measurements were carried out, one before and one after that reduction reaction. In the first case, we were quantifying the AA content, and in the second one, we quantified the sum of AA and DHAA (TAA: total ascorbic acid) in the form of AA. Then, DHAA quantity was indirectly obtained by subtracting AA coming from the first measurement from TAA. They were determined by UPLC-UV, using a commercial AA standard to build a calibration curve and optimizing the chromatographic procedure, to obtain AA peaks that were completely resolved in a short time. This protocol could be easily extrapolated to any other plant material with slight or no changes. Its accuracy revealed statistically significant differences otherwise unperceived. Other strengths and limitations are discussed more in depth in the manuscript.

INTRODUCTION:

Cultivated lettuce (*Lactuca sativa* L.) is one of the most produced and consumed leafy vegetables worldwide, with a total production of about 27.3 million tons in 2018[1]. Lettuce is perceived as healthy by consumers. The nutritional properties are mainly attributed to the source of antioxidant compounds in the crop, such as vitamin C, among others like polyphenols and vitamin E[2]. Vitamin C is an essential micronutrient for humans unlike many other vertebrates, as we are unable to produce it due to mutations present in the gene coding for the last step enzyme in the biosynthetic pathway[3]. It is required for a normal cell metabolism and it also plays an important role in immune responses mainly due to its antioxidant activity[3,4].

Total vitamin C is made up of ascorbic acid (AA) and dehydroascorbic acid (DHAA). AA is the most biologically active form of the vitamin, but DHAA (its oxidation product) also shows biological activity and it can be easily converted into AA in the human body[5]. Therefore, quantifying both forms is important to determine the total vitamin C content of any horticultural crop, lettuce included.

A wide variety of approaches based on different analytical techniques have been used to measure vitamin C in vegetables, such as enzymatic, spectrophotometric, and titrimetric methods[6–8]. Although these methods are simple, they are not chemically specific for AA[9]. Consequently, chromatographic methods are preferred, especially the high-performance liquid chromatography-ultraviolet (HPLC-UV) technique, because of their higher accuracy[10]. HPLC-UV has been used to determine vitamin C in a great diversity of crops, like broccoli, spinach and lettuce[11–13]. However, the simultaneous quantification of AA and DHAA is complicated due to the low absorptivity of DHAA in the UV range of the spectrum. Alternatively, DHAA can be determined indirectly by using a reducing agent that converts DHAA to AA, measuring total ascorbic acid (TAA), and then calculating the difference between TAA and AA. Due to the necessity of a reduction reaction, in some studies, only AA has been quantified[14], which could actually represent an underestimation of vitamin C activity. That additional reduction reaction is also needed to determine DHAA indirectly even when the last advance in liquid chromatography techniques, ultra-high performance liquid chromatography (UPLC), is used. That step also benefits from the advantages that UPLC exhibits when compared to HPLC: higher efficiency and resolution, increased sensitivity, shorter time analysis and lower solvent consumption[15]. In consequence, UPLC-UV technique has been utilized to quantify vitamin C in different crops[16].

In addition, AA is a very labile molecule; thus, it is important to develop a protocol that prevents its degradation during lettuce storage and vitamin C analysis[9]. In this context, the following protocol offers a rapid and improved quantification of vitamin C content in lettuce by UPLC-UV, as well as an efficient extraction procedure. Not only elite cultivars have been included in the present study, but also traditional landraces and some wild relatives due to their potential interest in crop breeding, specifically in the improvement of the nutritional value of lettuce.

PROTOCOL:

1. Plant material preparation

1.1. Sample at least two leaves per plant in 50 mL polypropylene tubes, an outer (older) and an inner (younger) one in order to represent more accurately the whole plant. Collect at least three biological replicates for each sample.

1.2. Freeze them immediately using liquid nitrogen and store them at -80 °C until use. Make sure the liquid nitrogen does not get into the tubes; otherwise they could explode when removed due to the gas expansion during vaporization.

CAUTION: Gloves and a face shield are required due to the potential hazards associated with using liquid nitrogen.

1.3. Remove the caps from the tubes and place them on the trays within the freeze dryer chamber of the lyophilizer (**Table of Materials**) programmed as follows: -25 °C for 72 h, -10 °C for 10 h, 0 °C for 10 h, and 20 °C for at least 4 h. Maintain the condenser temperature and the vacuum constant during the freeze-drying process at -80.2 °C and 112 mTorr, respectively.

1.4. When the material is completely dry (between 4 and 7 days depending on the plant and the degree of compaction into the tube), preserve at 4 °C, -20 °C or -80 °C for short (days to weeks), medium (months) or long (years) storage, respectively. The inclusion of bags containing silica gel beads in the sample-containing tubes is recommended.

1.5. Place the lyophilized samples into 20 mL polypropylene tubes together with 10 mm stainless steel balls and grind them with a multitube vortexer using the intensity and time needed to obtain a fine dust.

NOTE: During the entire process, protect the samples from exposure to direct light.

2. Reagent and solution preparation

2.1. Prepare the solvent extraction solution: 8% acetic acid (v/v), 1% MPA (meta-phosphoric acid) (w/v), 1 mM EDTA (ethylenediaminetetraacetic acid).

2.1.1. Calculate the total volume of solvent needed to process the whole set of samples taking into account that 5 mL will be added to each. To prepare 1 L of the solution, add to a flask: 30 g of MPA, 0.372 g of EDTA dehydrate, 80 mL of acetic acid and 500 mL of ultrapure water (scale volumes and quantities accordingly). Seal the flask mouth with plastic film.

2.1.2. Once dissolved with the help of a magnetic stirrer, use a volumetric flask to accurately measure 1 L, adding the necessary ultrapure water.

2.2. Prepare the reduction reaction: buffer (0.5 M Tris (2-amino-2-(hydroxymethyl)-1,3-propanediol) pH 9.0) and reducing solution (40 mM DTT (1,4-Dithiothreitol) with 0.5 M Tris pH 9.0).

2.2.1. Calculate the total volume of reducing solution needed to process the whole set of samples taking into account that 200 µL will be added to each of them. To prepare 100 mL of the buffer, add to a beaker: 6.055 g of Tris and 90 mL of ultrapure water (scale volumes and quantities accordingly). Seal the beaker mouth with plastic film.

2.2.2. Once dissolved with the help of a magnetic stirrer, adjust the solution to pH 9.0 by adding 2 M HCl and use a volumetric flask to accurately measure 100 mL, adding the necessary ultrapure water.

2.2.3. To prepare 100 mL of the reducing solution, add to a beaker: 0.629 g of DTT (purity: 98%) and 90 mL of the buffer (0.5 M Tris pH 9.0) previously prepared (2.2.1 to 2.2.2). Scale volumes and quantities accordingly. Seal the beaker mouth with plastic film.

2.2.4. Once dissolved with the help of a magnetic stirrer, use a volumetric flask to accurately measure 100 mL, adding the necessary volume of buffer 0.5 M Tris pH 9.0.

NOTE: The reducing solution is very unstable. That is why a freshly made solution is strongly recommended.

2.3. Sulphuric acid (0.4 M H_2SO_4)

2.3.1. Calculate the total volume of 0.4 M sulphuric acid needed to process the whole set of samples taking into account that 200 µL will be added to each. To prepare 100 mL of the solution, add to a beaker: 80 mL of ultrapure water and then 2.22 mL of H_2SO_4 (purity: 96%, density: 1.84 g mL^{-1}). Use a volumetric flask to accurately measure 100 mL, adding the necessary ultrapure water.

CAUTION: Sulphuric acid is very corrosive, so it must be handled using protective equipment and under hood. In addition, the acid should be added to ultrapure water, and not water to acid, to reduce fumes and avoid accidents.

2.4. Hydrochloric acid (2 M HCl).

2.4.1. To prepare 100 mL of 2 M hydrochloric acid, add to a beaker: 80 mL of ultrapure water and then 6.13 mL of HCl (purity: 37%, density: 1.19 g mL^{-1}). Seal the beaker mouth with plastic film. Use a volumetric flask to accurately measure 100 mL, adding the necessary ultrapure water. Scale volumes accordingly.

CAUTION: Hydrochloric acid is very corrosive, so it has to be handled using protective equipment and under hood. In addition, the acid should be added to ultrapure water, and not water to acid, to reduce fumes and avoid accidents.

2.5. AA standard (stock and dilutions)

2.5.1. Weigh exactly 10 mg of AA standard (purity: 99%) using a precision balance and add 90 mL of mobile phase (ultrapure water pH 2.0 with formic acid).

2.5.2. Once dissolved with the help of a magnetic stirrer, use a volumetric flask to accurately measure 100 mL, adding the necessary volume of ultrapure water pH 2.0 with formic acid.

NOTE: Protect this stock solution from the exposure to light.

2.5.3. Prepare five dilutions from the stock of the AA standard to obtain a calibration curve following the instructions in **Table 1** and proceed with step 5.2.

[Place Table 1 here]

3. Extraction of AA and DHAA

NOTE: It is recommended to work under conditions of low light intensity during the extraction steps.

3.1. To a 15 mL polypropylene centrifuge tube, add 50 mg of lyophilized ground sample and 5 mL of the extraction solvent (step 2.1).

3.2. Shake the mixture using a vortex for 5 s and then an orbital shaker for 10 min at 2000 rpm.

3.3. Introduce the tube in an ultrasonic bath for 10 min at room temperature with ultrasound activated.

3.4. Centrifuge at 4,000 x *g* for 10 min at 4 °C.

3.5. Take the supernatant, pass it through a 0.22 µm regenerated cellulose filter and store it in a 5 mL amber vial. This is Extract 1, which contains AA and DHAA.

NOTE: The protocol can be paused here by freezing the extracts at -80 °C and protecting them from exposure to light as AA and DHAA are very unstable and degrade easily in the presence of light, at high temperatures or under oxidizing atmospheres (**Supplemental File 1**).

4. DHAA reduction to AA to extract TAA

4.1. Transfer 200 µL of Extract 1 to a 2 mL amber vial for liquid chromatography and add 200 µL of the reducing solution (step 2.2). Close the vial with a PTFE-silicone plug with pre-opening and shake it with a vortex for 5 s.

4.2. Allow the solution to stand for 30 min at room temperature and protect from light.

4.3. Add 200 µL of 0.4 M H_2SO_4 to stop the reaction and stabilize AA in acidic pH. The resulting solution is Extract 2, which contains only AA and is actually TAA.

5. Determination

5.1. UPLC-UV preparation

5.1.1. Prepare the working solutions described in **Table 2**, suitably filtered through 0.22 µm filters, sonicated for at least 10 min and place them in the UPLC system.

5.1.2. Switch on the three UPLC modules and wait for the internal calibration process to finish.

5.1.3. Open the software (e.g., Empower3) and load the instrumental program described in **Table 2**: **Empower3 | Run Samples | Vitamin C method | UPLC_PDA | Use QuickStart**.

5.1.4. Once the software is loaded with the correct program, access the UPLC management console: **Quaternary Solvent Manager | Click right mouse button | Launch Console**.

5.1.5. Proceed to the preparation and stabilization of the UPLC instrument: **System | Control | Startup**.

5.1.5.1. Purge all UPLC lines for at least 5 min: **Prime Solvents | QSM | Check A, B, C, D and Seal Wash | Duration of prime > 5 min**.

5.1.5.2. Purge and clean the injector: **Prime Solvents | SM | Check Wash solvent (> 45 s) and Check Purge solvent (> 35 cycles)**.

5.1.5.3. Equilibrate UPLC to method conditions: **Equilibrate to Method | QSM | Flow (0.3 mL min^{-1}) | Solvent A (2%) | Solvent B (0%) | Solvent C (98%) | Solvent D (0%)**; **Equilibrate to Method | SM | Sample (5 °C) | Column (35 °C)** and **Equilibrate to Method | Other | Check Lamp On | Press Start**.

5.1.5.4. Wait for at least 1 h (even more time is recommended) for the equipment to stabilize. Stability can be verified checking the pressure in the column in the **Launch**

Console: **System | Quaternary Solvent Manager | QSM System Pressure**. Ensure that there are no identifiable trends in pressure changes (either increases or decreases) and the delta value is less than 10 psi.

5.1.6. In the **QuickStart** screen, fill the matrix with the names of the standards and samples to be analyzed.

5.2. AA determination in the standards

5.2.1. Transfer 1 mL of each of the five AA standards previously prepared (step 2.5.3) to 2 mL amber vials for liquid chromatography. Close the vial with a PTFE-silicone plug with pre-opening and inject 5 µL in the UPLC instrument.

5.2.2. Carry out the chromatography following the procedure described in **Table 2** starting from most diluted to most concentrated.

5.3. AA determination in the samples

5.3.1. Pipette 200 µL of Extract 1 in a 2 mL amber vial for liquid chromatography and add 800 µL of ultrapure water. Close the vial with a PTFE-silicone plug with pre-opening and inject 5 µL in the UPLC instrument.

5.3.2. Carry out the chromatography following the procedure described in **Table 2**.

5.4. TAA determination in the samples

5.4.1. Add 400 µL of ultrapure water to Extract 2. Close the vial with a PTFE-silicone plug with pre-opening and inject 5 µL in the UPLC instrument.

5.4.2. Carry out the chromatography following the procedure described in **Table 2**.

[Place Table 2 here]

6. Quantification of AA and DHAA

6.1. Statistical analysis

6.1.1. Determine the analytical parameters of the chromatographic method as described by Bertolín et al.[18] (**Table 3**).

NOTE: The values of the parameters presented in **Table 3** will need to be defined under specific experimental conditions.

[Place Table 3 here]

6.2. Calculate the AA and TAA concentration.

6.2.1. Open the standard and sample chromatograms: **QuickStart | Browse Project | Channels | "name of standard or sample" | PDA Ch1 245 nm@1.2 nm**.

6.2.2. Integrate the corresponding peak (AA or TAA) in the standards and samples by clicking on its starting point (1.790 min) and dragging it with the mouse to its end point (1.910 min).

6.2.3. Build a calibration curve representing the absorbance values determined chromatographically (step 5.1.) against the concentration of the five AA standards prepared above (**Table 1**).

6.2.4. Interpolate the absorbance values of the samples determined in step 5 and obtain the AA and TAA concentration with the following formula:

$$y = mx + n$$

where y is the integrated peak area, x is the AA or TAA concentration in ppm and m and n are the slope and the y-intercept of the obtained regression line, respectively.

6.2.5. For calculating the concentration of DHAA, apply the following formula:

$$[\mathrm{DHAA}](\mu\mathrm{g\ mL}^{-1}) = [\mathrm{TAA}] - [\mathrm{AA}]$$

NOTE: To obtain the total concentrations of the DHA, AA and TAA in mg g^{-1} of dry weight, the values obtained directly interpolating in the calibration curve will have to be multiplied by the total extract volume and the dilution factor applied, and then divided by the weight of the sample used to carry out the extraction.

REPRESENTATIVE RESULTS:

Vitamin C quantification in *Lactuca* matrixes requires the development of a chromatographic approach that can ensure reliable results. **Figure 1A** shows a chromatogram resulting from a non-optimized protocol (**Supplemental File 2**), which presents an AA peak together with an unidentified minor "shoulder". Nevertheless, after improving the extraction and chromatographic conditions, a resolved AA peak without interferences of unknown compounds was achieved (**Figure 1B**). In addition, the use of UPLC-UV equipment instead of HPLC-UV allowed us to reduce the retention time (RT) for AA: 1.874 min in the optimized chromatograms *versus* 2.980 min in the non-optimized ones (**Figure 1**), as well as the running times, 3 and 7 minutes for the optimized and non-optimized protocols, respectively.

[Place Figure 1 here]

Interferences in AA peaks, like those observed in **Figure 1A,** consistently resulted in underestimation of vitamin C (AA, DHA and TAA) content (**Figure 2**) due to an insufficient separation during the chromatographic process as the overlapping peak areas were integrated by a vertical drop at the deepest point between them. This bias is especially noticeable in the case of the crop wild relatives, especially for DHAA and TAA content (**Figure 2**).

[Place Figure 2 here]

Furthermore, the use of a non-optimized protocol prevented us from extracting any useful conclusion from the results as they showed all samples, both types of lettuces and the wild relatives, having a similar vitamin C content. In contrast, the optimized protocol allowed us to detect statistically significant differences among them for DHAA and TAA content (**Table 4**), the richest ones being the wild species (**Figure 2**).

[Place Table 4 here]

Figure 1. Chromatograms of AA in the same lettuce sample (commercial cultivar 'Begoña'). (**A**) HPLC-UV chromatogram resulting from a non-optimized protocol (conditions described in Supplemental File 2). (**B**) UPLC-UV chromatogram obtained with the optimized protocol (conditions described in **Table 2**).

Figure 2. Distribution of the content of vitamin C. Split violin plots of DHAA, AA and TAA content (mg g^{-1} dry of weight) in commercial and traditional lettuce varieties and some wild relatives using non-optimized and optimized protocols. Black lines show the mean values.

Figure 3: Workflow of the quantification of vitamin C in lettuce and some wild relatives. Schematic diagram of the optimized protocol showing two branches for the determination of only AA or AA + DHAA (TAA).

Table 1: **Protocol to prepare five standards of AA (ascorbic acid).** Volumes of solute and solvent to prepare each of the different concentrations of the standards are indicated.

Table 2: Chromatographic procedure optimized to determine AA (ascorbic acid) in extracts from lettuce and wild relatives. Description of the components, conditions and solutions employed.

Table 3: Optimized analytical parameters for the detection and quantification of AA (ascorbic acid) and TAA (total ascorbic acid). The linear range, the equation and the coefficient of determination of the calibration (R^2) curve, as well as the limits of detection and quantification of AA (the same for TAA), and the repeatability, intermediate precision and recovery obtained with a sample injection volume of 5 µL.

Table 4: Variation in the content of vitamin C. F-ratios (quotients of two variances, the between-group variance and the within-group variance) and significance values from the one-way ANOVA considering the type of *Lactuca* (commercial lettuce varieties, traditional lettuce varieties, and crop wild relatives) for DHAA, AA and TAA content in non-optimized and optimized protocols.

Supplemental File 1. AA and TAA stability at 5 °C over 24 h. (**A**) AA and TAA peak areas throughout 24 h. (**B**) AA and TAA content (mg g^{-1} of dry weight) throughout 24 h. Bars represent the standard deviations of two technical replicates (n=2) kept in the autosampler at 5°C and protected from exposure to light.

Supplemental File 2. Main differences between the optimized and the non-optimized protocol for TAA, AA and DHAA extraction and quantification. The samples used were the same in both cases.

DISCUSSION:

Vitamin C is a very important nutrient, but it is a very labile compound too, so its UPLC-UV quantification is dependent on multiple factors, such as sample storage and preparation, extraction method and chromatographic conditions. Therefore, a fast and simple procedure to prevent AA (with antioxidant power) oxidation to DHAA (without antioxidant properties) was needed. It was also crucial to avoid high pH and temperature conditions, as well as intense light and an oxidizing atmosphere during sample treatment to promote the stability of the compound.

To minimize AA oxidation, the following measures were taken. First of all, lettuce samples were lyophilized as a starting material for both protocols to ensure accurate quantification of vitamin C content and to easily manipulate samples. This option was preferred over fine grinding, commonly found throughout the literature[19], as the dust thaws very quickly so the water becomes available again. During the extraction procedure, a higher volume of a more acidic solution (8% acetic acid and 1% MPA) was used as extractant in the optimized protocol (**Supplemental File 2**), which also acted as a stabilizer by preventing AA degradation. This solution also contained EDTA as a chelating agent to increase stabilization[16], unlike the extractant in the non-optimized protocol (**Supplemental File 2**). Moreover, we tested if the extraction procedure could be enhanced by using two consecutive extractions with 2.5 mL of extractant instead of a single one with 5 mL and under a N_2 atmosphere instead of the standard atmospheric conditions. The best results were reached using only one extraction under an unmodified atmosphere, which simplified the protocol by making unnecessary additional steps (data not shown). Other minor changes were also introduced in the protocol in order to enhance the extraction (i.e., sonication), obtain a clearer extract (finer filtration) and reduce the protocol duration (**Supplemental File 2**). Regarding the chromatographic conditions, the validation of the method was carried out as reported before[18], guaranteeing good analytical parameters (**Table 3**). Besides, the use of ultrapure water with formic acid (pH 2.0) and methanol (98:2 v:v) with a 0.3 mL min^{-1} flow, instead of monopotassium phosphate 30 mM (pH 3.0) at 1 mL min^{-1} as the mobile phase (**Supplemental File 2**), resulted in an improved method. The most important advancement was likely using a UPLC system instead of a HPLC, which allowed us greater control of impacting conditions (like the temperature) and resulting in resolved AA peaks without interferences by unknown compounds, in a shorter time and consuming less volume of extract (**Supplemental File 2**).

Nevertheless, there are two main limitations of this method. The first one is that DHAA cannot be measured directly using an UV detector due to its low absorptivity in the UV range of the spectrum. It is important to quantify the DHAA content because it presents certain biological activity and is easily convertible to AA in the human body[5]. For that, an additional reaction to reduce DHAA to AA is needed, together with a second chromatographic run in order to measure TAA and then determine DHAA indirectly by subtracting AA content from TAA (**Figure 3**). In this sense, the reduction step has been optimized by using a higher concentration of the reducing agent (DTT), increasing the reaction time from 4 to 30 min, and stopping the reaction with sulfuric acid (**Supplemental File 2**). The low stability of AA constitutes the second limitation of the method. As AA starts to degrade 4 h after extraction (**Supplemental File 1**), it is necessary to quantify it in this time interval. So, the number of samples to extract is conditioned by the chromatographic procedure. That is why we propose to freeze them at that step in this protocol, though in that case, not all of them could be placed in the UPLC autosampler to be measured automatically. Fortunately, the reduced RT for AA allowed us to obtain 3 min chromatograms, much shorter than the 7-min chromatograms obtained using HLPC (**Supplemental File 2**). Hence, vitamin C content could be determined in a high number of samples in a 4 h window.

[Place Figure 3 here]

As vitamin C is an essential nutrient for humans and due to its important health benefits, it has become the object of many studies. Therefore, it has been quantified in a great variety of crops, including lettuce, one of the most consumed vegetables worldwide. Simple classical methods have been gradually replaced by liquid chromatography techniques because they are more specific and accurate[10]. However, due to the need of an additional reaction to quantify both, AA and DHAA via HPLC, in some studies on lettuce, only AA[14] or only TAA[11] (without quantifying AA before the reduction of DHAA into AA) have been measured. Furthermore, only a few authors have quantified AA and DHAA, despite the contribution of both molecules to vitamin C antioxidant activity[2]. Nevertheless, UPLC technique has become more important in recent times because of its higher performance when measuring vitamin C in several crops[16]. Comparing the results obtained in this study with the two methodologies, UPLC and HPLC, these advantages have been confirmed: well-defined AA peaks thanks to a higher sensitivity, and in very short times, have been achieved, which also implies fewer resources consumed. Despite of UPLC efficiency, only Chen et al.[20] have applied this technique to measure the vitamin C content in lettuce, which still led to an underestimation as only the AA form was quantified.

In summary, this work represents the first successful attempt to determine the total vitamin C content not only in different lettuce varieties but also in some of their wild relatives. Vitamin C quantification is also essential to select lettuces with higher antioxidant activity within breeding programs. In this sense, the increased total vitamin C content in lettuce wild relatives found here and the increased AA content reported in previous studies[14], as well as other antioxidant compounds[21], broadens the suitable candidates to improve the nutritional value of lettuces.

In conclusion, even with some limitations inherent to vitamin C’s nature, like its gradual degradation few hours after being extracted or the need of a reduction reaction due to the low DHAA UV-absorptivity, it offers a less labor-intense and a less time-consuming method to measure vitamin C content. Additionally, it is also very robust and shows a high sensitivity and power of resolution. Moreover, it is easily transferable not only to other plant materials with slight or no changes, but also to processed products that supply the dietary intake of vitamin C to humans, which gives rise to a wide range of future applications in the emerging field of testing for reliable food quality.

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DISCLOSURES:

The authors have nothing to disclose.

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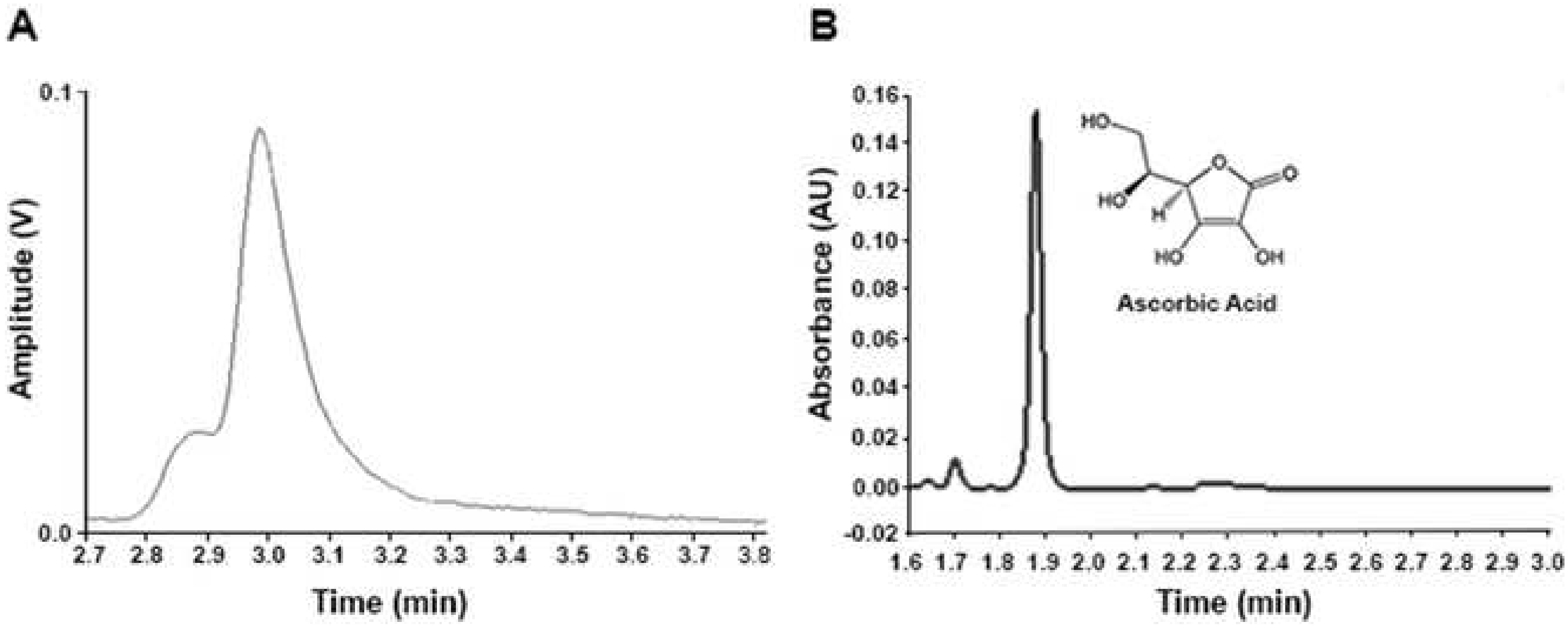
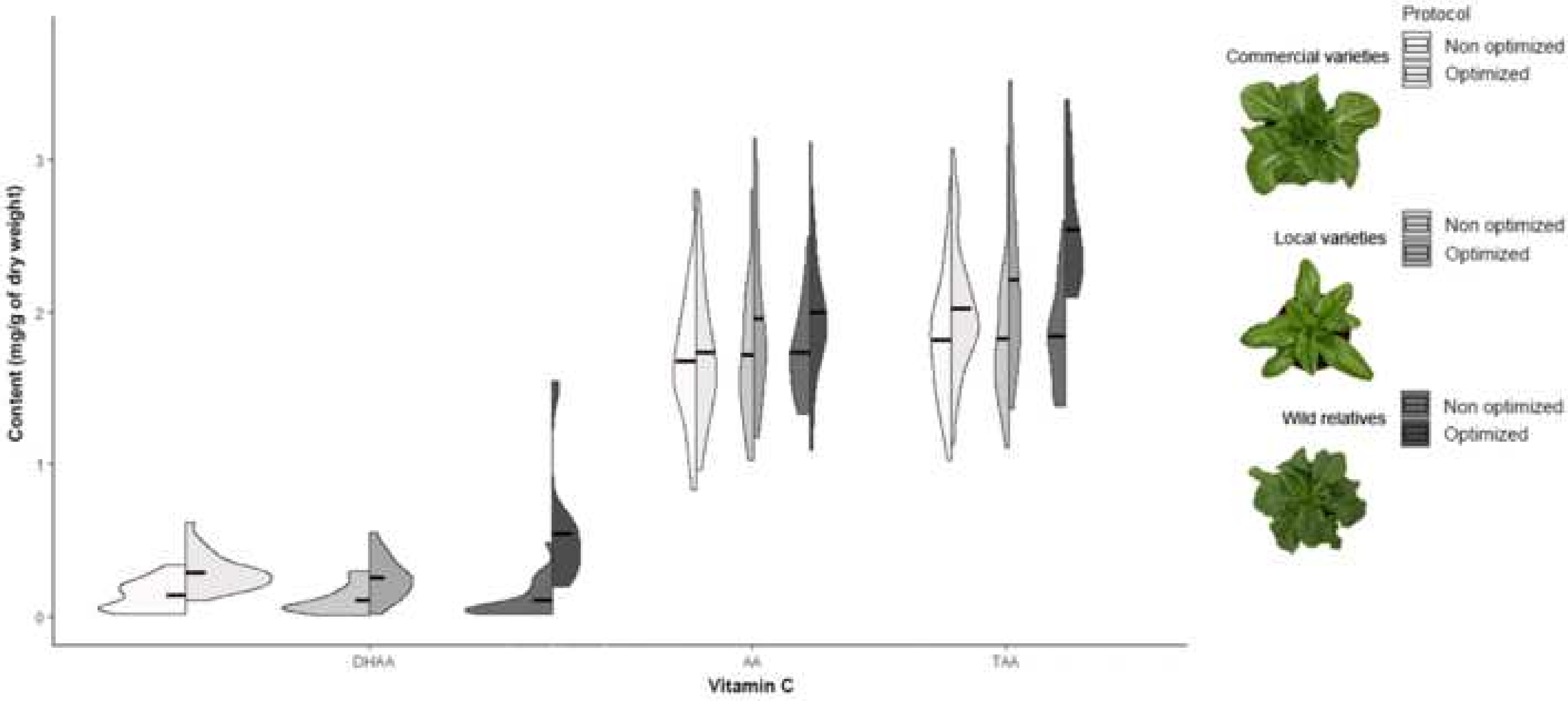
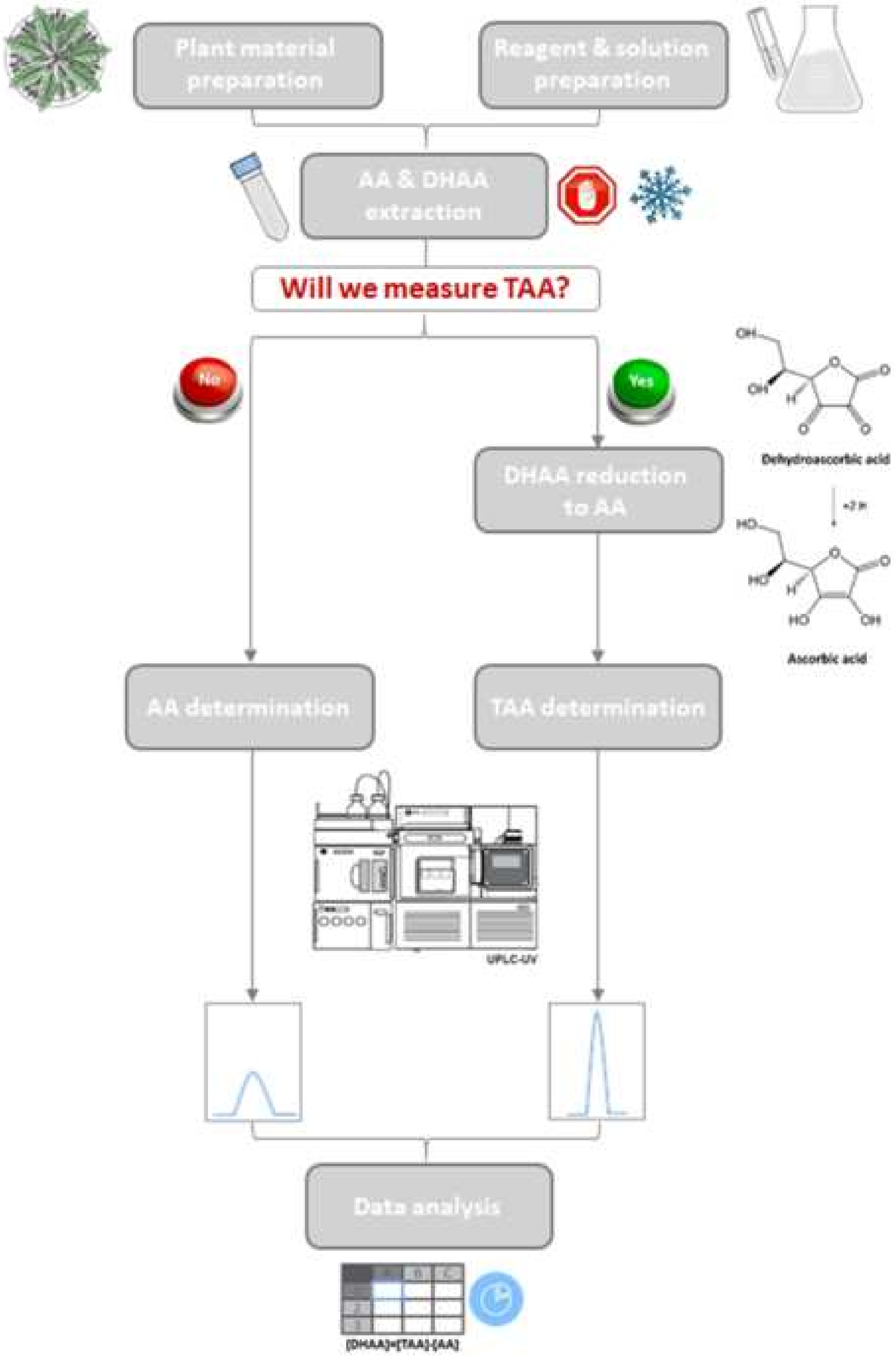

A
Amplitude (V)
0.1
0.0
2.7 2.8 2.9 3.0 3.1 3.2 3.3 3.4 3.5 3.6 3.7 3.8
Time (min)
B
Absorbance (AU)
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
-0.02
1.6 1.7 1.8 1.9 2.0 2.1 2.2 2.3 2.4 2.5 2.6 2.7 2.8 2.9 3.0
Time (min)
HO
HO
H
O
O
HO
OH
Ascorbic Acid


Content (mg/g of dry weight)
0
1
2
3
DHAA
AA
TAA
Vitamin C
Protocol
Commercial varieties
Non optimized
Optimized
Local varieties
Non optimized
Optimized
Wild relatives
Non optimized
Optimized


Plant material preparation
Reagent & solution preparation
AA & DHAA extraction
Will we measure TAA?
No
Yes
DHAA reduction to AA
Dehydroascorbic acid
+2 H
Ascorbic acid
AA determination
TAA determination
UPLC-UV
Data analysis
[DHAA]=[TAA]-[AA]

Standard	[AA] (μg mL^{-1})	AA (100 μg mL^{-1})	Mobile phase (μL)[a]
1	0.5	5	995
2	2.5	25	975
3	5	50	950
4	10	100	900
5	25	250	750

[a] Ultrapure water pH 2.0 acidified by formic acid.

Components and paramethers	Description
Instrument	Acquity UPLC H-Class
Detector	PDA eλ Detector λabs for AA=245 nm
Software	Empower 3
Column	Acquity UPLC HSS T3 (150 mm x 2.1 mm x 1.8 µm)
Channel A	CH_3OH
Channel B/Wash	H_2O:CH_3OH (50:50 v:v)
Channel C	Ultrapure water pH 2.0 acidified by formic acid[a]
Channel D/Seal Wash	Ultrapure water:acetonitrile (90:10 v:v)
Mobile phase	0.3 mL min^{-1} of 2%A + 98%C (isocratic mode)
Column temperature	35°C
Autosampler temperature	5°C
Injection volume	5 µL
AA retention time	1.874 min
Runing time	3 min

[a] Undetermined volume of formic acid used until pH adjustment

Analytical parameters of the method	Values
Linear range (µg mL^{-1})	0.5-25
Linear equation	y=53,143.03x
R^2	0.99998
Limit of detection (mg AA/g of dry matter)	0.013
Limit of quantification (mg AA/g of dry matter)	0.045
Repeatability (CV, %)[a]	1.75
Intermediate precision (CV, %)[a]	4.22
Recovery (Rec, %)[b]	95.6±2.4

[a] CV: coefficiente of variation

[b] The recovery assay was performed with 10 aliquots containing 50 mg of the same sample, 5 spiked with 2 mg of AA/g of dry matter, and 5 non-spiked.
%Rec=([AA]spiked sample-[AA]sample)/([AA]spiked sample)x100.

	Non optimized		Optimized	
	F-ratio	*p* -value	F-ratio	*p* -value
DHA	0.460	0.637^{ns}	5.613	0.009**
AA	0.070	0.932^{ns}	1.020	0.374^{ns}
TAA	0.015	0.985^{ns}	4.438	0.022*

^{ns}, * and ** indicate non significant and significant at p <0.05 and 0.01, respectively.

Table of Materials

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
1,4-Dithiothreitol (DTT) ≥98% (Ellman's reagent)	Roche	10197777001	
10 mm ϕ stainless steel ball	Euro Aznar Supplies S.L.	20112100	
15 mL polypropylene tube for centrifuge	DeltaLab	429946	
2 mL HPLC amber vial	Agilent	5190-9063	
2 mL syringe with needle	DeltaLab	JS2	
20 mL polypropylene tubes	Dealtalab	202840	
2-Amino-2-(hydroxymethyl)-1,3-propanediol (TRIS) ≥99.9% (titration)	Roche	10708976001	
50 mL polypropylene tube	DeltaLab	429951	
Acetic acid (CH_3COOH) ≥99% purity glacial, ReagentPlus	Sigma-Aldrich	A6283	
Acetonitrilo, HPLC super gradient grade (99.9+% CH3CN-0.2 µm filtrated)	Chem-lab	CL00.0189	
Acquity UPLC HSS T3 column (150 mm x 2.1 mm x 1.8 µm)	Waters	186003540	
Beaker 1 L, 200 mL	DeltaLab	191725, 191722	
Dipotassium phosphate (KH_2PO_4)	Panreac	766384	Only in the non-optimized protocol (Supplemental File 2)
Ethylenediaminetetraacetic Acid Disodium salt (EDTA x 2H2O) 99-101% assay, ACS reagent	Panreac	131669	
Fisherbrand Analog MultiTube Vortexer	Thermo Fisher Scientific	15549004	
Formic acid 98-100% for HPLC LiChropur	Supelco	5438040100	
Freeze dryer VirTis genesis 25EL	VirTis	na	
Heidolph Multi Reax mixer	Heidolph	na	
Heidolph Reax top mixer	Heidolph	na	
Hewlett Packard HPLC 1050 equipped with an eλ Detector	Hewlett Packard	na	Only in the non-optimized protocol (Supplemental File 2)
Hydrochloric acid (HCl) 37% purity, ACS reagent	Sigma-Aldrich	320331	
L-Ascorbic acid ≥99%, ACS reagent	Sigma-Aldrich	255564	
Meta-phosphoric acid (MPA) ACS reagent, chips, 33.5-36.5%	Sigma-Aldrich	239275	
Methanol ≥99.9% (HPLC supergradient grade)	ChemLab	CL00.0189.2500	
Micropipettes 10-1000 µL	Socorex	na	
Nucleosil 120 C18 Tracer column (250 mm x 4 mm x 5 µm)	Merck (Sigma-Aldrich)	54919	Only in the non-optimized protocol (Supplemental File 2)
PTFE-silicone cap with preaperture	Agilent	5190-9067	
Refrigerated centrifuge Gyrozen 1248R	Gyrozen	na	
Regenerated cellulose filters 0.22 µm (13 mm)	Agilent	1015190-5108	
Regenerated cellulose filters 0.45-µm (13 mm)	Labbox	SFCA-145-100	Only in the non-optimized protocol (Supplemental File 2)
Spectrophotometer Heλios β	Thermo Scientific Corporation	na	
Sulfuric acid (H_2SO_4) 95.0-98.0% purity, ACS reagent	Sigma-Aldrich	258105	
Ultrapure water	WasserLab	na	
Ultrasons H-D	Selecta	na	
Volumetric flasks 1 L, 100 mL	DeltaLab	191489, 191486	
Waters Acquity UPLC H-Class equipped with a PDA eλ Detector	Waters	na	

Dear Editor and Reviewers,

We very much appreciate your suggestions and corrections. We feel that they have helped to improve the manuscript, particularly the Protocol and Discussion sections.

Before starting with our reply, we would like to point out that precisely two weeks ago, the Government of Aragon (to which our institute belongs) has changed the affiliation format, so we have had to adapt it to the new rules in this manuscript. Basically, they are the same two institutes that originally appeared however we now have to split them (Centro de Investigación y Tecnología Agroalimentaria de Aragón and Instituto Agroalimentario de Aragón).

We have addressed all the changes that the reviewer has requested after accepting the manuscript and most of the suggestions or offered justification. We hope that this final version is satisfactory for you all.

Editorial Comments:

• Please **submit each figure as a vector image file to ensure high resolution throughout production: (.psd, ai, .eps., .svg). Please ensure that the image is 1920 x 1080 pixels or 300 dpi.** Additionally, please **upload tables as .xlsx files**.

Authors' comment: done. All images are at least 1920 x 1080 pixels and all tables are submitted as .xlsx.

• Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammatical errors.

Authors' comment: done.

• **Protocol Detail:** Please note that your protocol will be used to generate the script for the video, and must contain everything that you would like shown in the video. **Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc) to your protocol steps**. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

Authors' comment: done. All the required steps to prepare the instrument have been included (5.1 in the Protocol section), as well as those to integrate the peaks of the chromatograms (6.2.1 and 6.2.2 in the Protocol section). In order to reduce the length of the protocol to meet the journal criteria, we have also eliminated one of the two possible ways of preparing the AA standard (stock). So, we have left the method actually used with the samples and data reported in this manuscript.

- **Protocol Numbering:** Please adjust the numbering of your protocol section to follow JoVE's instructions for authors, 1. should be followed by 1.1. and then 1.1.1. if necessary and all steps should be lined up at the left margin with no indentations. There must also be a one-line space between each protocol step.

Authors' comment: done.

- **Protocol Highlight:** After you have made all of the recommended changes to your protocol (listed above), please re-evaluate the length of your protocol section. There is a 10-page limit for the protocol text, and a 3- page limit for filmable content. If your protocol is longer than 3 pages, please highlight ~2.5 pages or less of text (which includes headings and spaces) in yellow, to identify which steps should be visualized to tell the most cohesive story of your protocol steps.

1) The highlighting must include all relevant details that are required to perform the step. For example, if step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be included in the highlighting.
2) The highlighted steps should form a cohesive narrative, that is, there must be a logical flow from one highlighted step to the next.
3) Please highlight complete sentences (not parts of sentences). Include sub-headings and spaces when calculating the final highlighted length.
4) Notes cannot be filmed and should be excluded from highlighting.
5) Please bear in mind that calculations cannot be filmed.

Authors' comment: done.

- **Discussion:** JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail and in paragraph form (3-6 paragraphs): 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol.

Authors' comment: all those aspects are covered in the discussion section though not exactly in that order (paragraph 1: troubleshooting; paragraph 2: modifications; paragraph 3: limitations and critical steps; paragraph 4: significance with respect to existing methods; paragraph 5: future applications).

- **Commercial Language:** JoVE is unable to publish manuscripts containing commercial sounding language, including trademark or registered trademark symbols (TM/R) and the mention of company brand names before an instrument or reagent. Examples of commercial sounding language in your manuscript are VirTis Genesis 25EL, parafilm,

1) Please use MS Word’s find function (Ctrl+F), to locate and replace all commercial sounding language in your manuscript with generic names that are not company-specific. All commercial products should be sufficiently referenced in the table of materials/reagents. You may use the generic term followed by “(see table of materials)” to draw the readers’ attention to specific commercial names.
2) Please remove the registered trademark symbols TM/R from the table of reagents/materials.

Authors’ comment: brand names and registered trademark symbols have been eliminated.

- If your figures and tables are original and not published previously or you have already obtained figure permissions, please ignore this comment. If you are re-using figures from a previous publication, you must obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure). Please upload the text of the re-print permission (may be copied and pasted from an email/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [citation]."

Authors’ comment: all the figures and tables included in the manuscript are original.

Comments from Peer-Reviewers:

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

This work presents a protocol for the extraction and quantification of vitamin C including both the reduced and oxidized forms, by means of the ultra high performance liquid chromatography (UPLC-UV). In particular, the protocol has been improved for lettuce materials including traditional varieties and also other crop wild relatives. Vitamin C is an essential nutrient for humans which deficiency cause health disorders, and it is also of importance as a bioactive compound with antioxidant activity. Therefore, obtaining crops rich in this compound can help to increase the health status of consumers.

The analysis of vitamin C can be achieved with several methodologies. Within them, the use of LC methodologies has important advantages such as the accuracy of the measurements. However, some steps during the preparation of samples, extraction and analysis can compromise the effectiveness and success of these methodologies. This manuscript identifies and clearly explains most of these critical steps, and therefore I would recommend it to be published.

Nevertheless, there are some specific comments and questions to be solved, reason why I suggest proceeding with a Minor revision of the manuscript. The main point that should be addressed **in the Discussion is a clear comparison among both the optimized and non-optimized protocols**.

Authors’ comment: main differences between the optimized and the non-optimized protocol have been summarized in Supplemental File 2 (as more detailed information was requested below) and profusely discussed (mainly in paragraphs 2 and 3 of the Discussion section).

Minor Concerns:

Specific comments:

Although the title clearly identifies the work performed, I would consider a little modification to focus more on the purpose of the work that is to explain the methodology (more than the study of different lettuce materials). For example: "Improved UPLC-UV method for the quantification of vitamin C in lettuce varieties (Lactuca sativa L.) and crop wild relatives (Lactuca spp.)".
Authors’ comment: done.

Line 43: "-80 ºC"
Authors’ comment: done.

Line 45: "what also contributed"
Authors’ comment: done. “Also” was moved in the sentences as suggested here though “which” was used instead of “what” as recommended by Reviewer 3.

Lines 47-50: Sentence too long, split.
Authors’ comment: done.

Lines 47-48: The sentence suggests that the authors decided to reduce the DHAA to simplify the analysis, whereas the indirect quantification of DHAA is the common procedure. Modify the sentence to make clear that this is the way to proceed. For example: "The DHAA was quantified indirectly after its reduction into AA".
Authors’ comment: done.

Line 53: "obtained by subtracting"
Authors’ comment: done.

Lines 56-59: This sentence is too specific for the materials.

Authors’ comment: We consider it important to highlight that using the right protocol allowed us to detect differences in the content of vitamin C. In our opinion, it is not only one of the strongest conclusions of the study, but it is also its justification (why the protocol needed to be optimized).

Could it be possible to include a final sentence saying how this protocol can become useful for other works (i.e., could it be applied to other crops?)? And also to suggest that other advantages and some limitations are also discussed in the manuscript?

Authors’ comment: done.

Line 63: 27.3 million tonnes yearly?
Authors’ comment: those data are the most recent available (2018). It has been clarified in the text.

Line 66: "like vitamin C, among others". Cite others.
Authors’ comment: done.

Lines 66-68: "Vitamin C is an essential micronutrient (...)". Reference.
Authors’ comment: done.

Line 73: "can be easily converted into AA in the human body". Reference.
Authors’ comment: done.

Line 82: Delete "many others, including"
Authors’ comment: done.

Lines 86-88: Although I see what the authors mean, it seems that some information is missed. As it is written, it can be interpreted that the reduction step is a constraining factor that could be solved by using UPLC. But the step has to be addressed also in UPLC (as indicated in line 93). Therefore, what is constraining is that we have to inject each sample twice, thus increasing the time of analysis and the consumption of solvents.

Authors' comment: we totally agree with the reviewer, it can lead to a misunderstanding. So, we have reworded the paragraph to clarify that point: "That additional reduction reaction is also needed in order to determine DHAA indirectly even when the last advance in liquid chromatography techniques, ultra-high-performance liquid chromatography (UPLC), is used. That step also benefits from the advantages that UPLC exhibits when compared to HPLC: higher efficiency and resolution, increased sensitivity, shorter time analysis and lower solvent consumption".

Line 95: "In this context, the following protocol (...)"
Authors' comment: done.

Line 96: "a rapid and improved extraction and quantification". The authors highlight in the previous sentence the lability of AA. Therefore, this protocol has not been only improved for the quantification but also for the extraction procedure.
Authors' comment: the specification suggested by the reviewer has been added as follows: "In this context, the following protocol offers a rapid and improved quantification of vitamin C content in lettuce by UPLC-UV, as well as an efficient extraction procedure".

The protocol is described very accurately. However, I consider that the form (not the content) could be improved somehow. For example:
Line 105: The authors include too many notes which could be combined as a sentence of the point described. This one in particular could be included within the point 1.1 as a sentence instead of an advice (authors should remember that, according to the authors instructions, each step should include 2-3 sentences. See for example: doi: 10.3791/55425). Example: "(...) more accurately the whole plant. At least three biological replicates should be collected for each sample".
Authors' comment: done.

Similarly, authors split steps that, as I consider, could be combined (e.g., points 2.3.2 and 2.3.3). This would help to reduce the length of the manuscript and to agree with authors instructions (2-3 sentences per step). Please revise along the manuscript.
Authors' comment: done. The following steps have been merged together:
1.3 and 1.4.
2.1.1 and 2.1.2.
2.2.1 and 2.2.2.
2.3.1, 2.3.2 and 2.3.3.
2.4.1 and 2.4.2.
4.1 and 4.2.
5.1.1 and 5.1.2 (now 5.2.1)

5.2.1 and 5.2.2 (now 5.3.1)

Now all the steps consist of a maximum of three sentences.

Line 114: Remove information of the lyophilizer used (brand) and combine points 1.3 and 1.4

Authors' comment: done.

Line 120: What do the authors consider as short, medium and long storage? Indicate.

Authors' comment: it has been specified in the manuscript (lines 125-126) as follow: "…for short (days to weeks), medium (months) or long (years) storage…"

Line 123: Remove "(see Table of Materials)"

Authors' comment: done.

Line 131: Combine steps 2.1.1 and 2.1.2.

Authors' comment: done.

Line 131: For volumes, use L, mL... instead of l, ml... along the manuscript.

Authors' comment: done.

Line 159: According to my experience, I would recommend to add the 80 mL of water firstly, and then the H_2SO_4. By doing it, fumes and accidents can be reduced. Please modify to clearly indicate the proper order.

Authors' comment: done.

Line 166: The same.

Authors' comment: done.

Line 176: µg mL-1

Authors' comment: done.

Line 181: µg mL-1

Authors' comment: done.

Line 190: µg mL-1

Authors' comment: done.

Table 1: I assume that the mobile phase refers to 2% methanol in ultrapure water including 100 µg mL-1 formic acid pH 2. Is it? Please indicate in Table 1.

Authors' comment: the mobile phase in Table 1 is different from the one in Table 2 (chromatographic procedure). Only the one described in Table 2 contains methanol. The mobile phase that appears in Table 1 consists of ultrapure water with pH adjusted at 2.0 with formic acid (as much as needed). Thanks to the reviewer comment, we have detected an error that has now been corrected: formic acid is not added at a final concentration of 100 µg·mL^{-1} in any case, it is added until the pH reach the value of 2.0.

Mobile phase has been described in Table 1 as requested.

Line 214: As a suggestion, did the authors consider to add ice in the ultrasonic bath to cool the water, in order to extract the vitamin C at lower temperature?

Authors' comment: at the time this was not considered as the water temperature in the bath did not reach such a high value that would compromise the vitamin stability. However, we think that is a good idea and we will probably incorporate that "tip" into our protocol in future experiments.

Line 223: Combine 4.1 and 4.2
Authors' comment: done.

Line 228: Instead of "(Extract 2: contains only AA, which is TAA), write it as a complete sentence since it is important for readers to clearly identify such extract.
Authors' comment: done.

Line 232: Combine 5.1 and 5.2
Authors' comment: done. We assume that the reviewer means 5.1.1. and 5.1.2.

Line 236: How long do the authors consider that samples can be kept until analysis without significant degradation? Does the time to consider start at the moment that samples are unfrozen (if stored after extraction)? Even for the TAA extract?

Authors' comment: we performed a time course assay to check the stability of AA and TAA throughout a period of 24 h once the extraction had been carried out (Supplemental File 1). The assay was carried out with the extracts in the autosampler, that is, at 5ºC (this piece of information has been added to the Supplemental File caption). As it becomes clear in those graphs, there is at least a 3-4h window in which the vitamin C content remains stable (from 4h onwards, its quantity decays progressively). So, according to our experience, that is the maximum time in which samples should be quantified after having been extracted. As this could not always be possible, we advise to freeze the extracts at this step (see Figure 3 and lines 595-600 of the revised manuscript). The truth is that, in this particular experiment, we have not checked the stability of vitamin C before and after Extracts 1 and 2 were frozen, as freezing them was not required. However, according to our previous experience with vitamin C and other labile compounds that easily degrade with high intensity light, high temperature and/or the presence of oxygen, like vitamin E, vitamin A and other carotenoids, they remain stable when frozen in darkness.

On the other hand, when samples are collected and frozen immediately (at -20ºC or -80ºC) and/or lyophilized and kept at darkness after being collected, they are not exposed to any of the agents that cause vitamin degradation (no light or high temperatures or available water). That is why we recommend to proceed immediately after the sample has thawed.

Line 244: For running the samples, I would suggest indicating that first of all, the standard curve should be run, starting from diluted to concentrated. And then, the samples.
Authors' comment: we agree with the reviewer that it was not clear that AA standards were run before the samples, so we have included another step under the head "Determination" named "AA determination in the standards" (5.2) prior to "AA determination in the samples" (5.3) and "TAA determination in the samples" (5.4).
Line 253: Instead of Data analysis, I would suggest "Quantification of AA and DHAA".
Authors' comment: done.

Line 259: It is important to indicate that these values are obtained for the specific conditions in which authors worked, but reader will need to obtain these data for their specific conditions.
Authors' comment: a note has been added clarifying this point (NOTE: the values of the parameters presented in **Table 3** will need to be defined under specific experimental conditions).
Line 269: Add the general equation for interpolation: y=mx + n (explaining y, x, m, n)
Authors' comment: done.

Line 273: µg mL-1

Authors' comment: done.

Line 273: Authors provide the equation for obtaining the concentration as µg mL-1 but Fig. 2 provides results as mg g-1. Please provide the adequate equation for obtaining the latter concentration.

Authors' comment: the value obtained directly interpolating in the calibration curve is in ppm, equivalent to ug/mL (1 ug/mL=1 ppm). From there, we multiply by the total extract volume and the dilution factor. Finally, we divide it by the weight of the sample. This is how we obtained the final mg g-1 of dry weight. We think that is important to give the equation as it is (general). So, we have added the following note to indicate how to calculate the final amount referred to the starting dry weight al plant material:

NOTE: to obtain the total concentrations of the DHA, AA and TAA in mg g^{-1} of dry weight, the values obtained directly interpolating in the calibration curve will have to be multiplied by the total extract volume and the dilution factor applied, and then divided by the weight of the sample used to carry out the extraction.

Line 275: instead of providing a list of abbreviations, these should be explained the first time that they appear in the manuscript. Delete.
Authors' comment: done.

Line 287: In the manuscript, authors compare the advantage of the optimized protocol against non-optimized protocols. Could the authors indicate which non-optimized protocol have they used (by a reference if published, otherwise provide the essential information)?

Authors' comment: we have added a Supplemental File (Supplemental File 2) with the main differences between the optimized and non-optimized protocol to identify the changes carried out easily.

Line 291: Authors provide the RT for AA. Which was the total running time in both protocols?

Authors' comment: the running times were 3 and 7 minutes for the optimized and non-optimized protocols, respectively. This has been specified in the first paragraph of the Representative results' section and in the Supplemental File 2 (summary of the main differences between optimized and non-optimized protocol).

Figure 1: Could the authors provide similar scales for axis Y?

Authors' comment: even if in both cases we are showing absorbance (in V or AU), given that they come from two different instruments with different configurations for the detectors, we do not think that the absolute values of the chromatograms are comparable and, even if they would be, it does not add much (apart from the visual aspect). They are two different ways of recording the signal and therefore the scales do not match.
We could manipulate the images (making one of them smaller at the same time that we keep the proportions) to match 1 AU = 1 V but we do not think that is what the reviewer is asking for and it does not seem appropriate. Finally, the purpose of comparing both chromatograms was to show that we managed to resolve the AA peak with the optimized protocol (separating it from the shoulder present when the non-optimized protocol was used). We think that with the actual scale that point is sufficiently clear.

Are both chromatograms representing the same sample?

Authors’ comment: yes, both chromatograms represent the same sample, specifically the lettuce commercial cultivar ‘Begoña’ (biological repeat 1). This has been clarified in the caption on Figure 1.

Apart from the LC conditions, were the samples extracted following the same protocol? Please clarify.

Authors’ comment: there are other differences as commented in the Discussion section. To make the differences more patent and the comparison between both protocols easier, we have added a Supplemental File (Supplemental File 2) as commented above.

Line 299: "with the optimized protocol"
Authors’ comment: done.

Line 312: mg g-1. Dry weight or fresh weight?

Authors’ comment: it is dry weight. As we detailed in section 1 of the protocol (“Plant material preparation”), we lyophilized all the samples.

Line 337: Does the sentence suggest that in non-optimized protocols the extraction is obtained from fresh material? Only in the non-optimized protocol used by the authors or as general terms? Please specify and provide references if it is the case.

Authors’ comment: it is very common to find in the literature that the samples are finely ground while frozen. That is perfectly ok but when the plant material is like a fine dust, it thaws very quickly and then the water becomes available. We have used lyophilized material in both protocols, optimized and non-optimized. We have clarified this point in the text and added a cite (a review of determination methods of vitamin C in food).

Line 342: enhanced by using
Authors’ comment: done.

Line 348: "ultrapure water containing 100 µg mL-1 formic acid (pH 2.0)"
Authors’ comment: done.

Line 359: Delete (previously measured)
Authors’ comment: done.

Line 384: Although it is clear that the protocol is optimized for lettuce, I would recommend writing a Conclusion more focused on the protocol, usefulness, advantages and limitations, instead of in the representative results.

Authors’ comment: we have expanded on the general conclusion as suggested by the reviewer (last paragraph), but, at the same time, Reviewer 3 requested some other references about vitamin C and other antioxidant compounds in lettuce wild relatives to be included there.

Figure 3: It may be placed before since it summarizes how to analyze DHAA.

Authors’ comment: done. We have inserted Figure 3 earlier in the text, right after the paragraph where we talk about the additional reaction to reduce DHAA to AA and ultimately determine DHAA indirectly by subtracting AA content from TAA.

Table 2: Running time? And for channel C, specify the concentration of formic acid.

Authors’ comment: the running time (3 minutes) has been included in the table. The concentration of formic acid is unknown. The necessary volume of formic acid was used to adjust ultrapure water to a pH of 2.0 (a note has been included in the table).

Table of Materials: Acetic acid = CH_3COOH
Authors’ comment: done.

Supplemental File: "Bars represent the standard deviations". Does it represent the analysis of several samples? Or one sample with several biological/technical replicates? If so, how many samples/replicates were performed? Indicate (n = ?). In which conditions were the samples kept between injections? Indicate.

Authors’ comment: two technical replicates (from one biological sample) were analyzed. They were kept in the autosampler at 5ºC and protected from the light. The details have been added to the caption of Supplemental File 1.

Reviewer #2:

Manuscript Summary:

This manuscript developed a fast and reliable UPLC-UV method for the quantification of vitamin C in Lactuca spp. The overall manuscript seems to be well-written, however, there are some questions and/or suggestions to improve the quality of the manuscript.

Major Concerns:

1. Line in 222-229, how to make sure that the reduction reaction was fully completed.
Authors’ comment: the chosen reaction time to fully convert DHAA into AA in this protocol was the recommended by Spínola, V. *et al*. Analytical and Bioanalytical Chemistry. 403 (4), 1049–1058 (2012). Even in that case, longer reaction times, 45 and 60 minutes, were tested without observing significant differences (data not shown).
2. Please specify the spike and recovery experiments, and why you did not use HQC, MQC and LQC concentrations.

The recovery assay was performed with 10 aliquots of 50 mg of the same sample (5 not spiked samples and 5 spiked samples at a concentration of 2 mg AA g^{-1} dry sample adding with micropipette 100 µL of AA standard = 1 mg mL^{-1}). Once the data were obtained, the following formula was applied:

$$\%Rec = \frac{[AA]spiked\ sample - [AA]sample}{[AA]spiked} \text{x}100.$$

We have added all those details to Table 3, in which only the amount of AA used to dope the sample was specified. All the validations and calculations were carried out exactly as in Bertolín *et al*. (2018), already cited in the Protocol section of the manuscript.

We strongly agree that it would have been better to perform the recovery test with different levels of AA, with a low, medium, and high concentration of it. However, we decided to do the doping only with a medium concentration due to the limitations of these recovery assays. That is, the added AA was already dissolved in a solvent which is soluble and highly miscible with the extraction solvent, and the AA itself is also highly soluble in the extraction solvent (there are no liquid-liquid extractions, nor consecutive solid-liquid extractions, nor SPE purifications during the sample treatment). We decided to perform only one level in the recovery assay because the biggest problem associated with recovery could have been AA degradation during sample processing. We believed that, as verification, it would be sufficient with the repeatability tests, the intermediate precision calculation, and that recovery test.

Minor Concerns:

1.Line in 43, the symbol of "℃"is wrong, please correct it.
Authors' comment: done.

2."ml" should be replaced with "mL", "l" should be replaced with "L";
Authors' comment: done.

3.Table 1 should be adjusted to one page;

Authors' comment: done.

Reviewer #3:

Manuscript Summary:

The manuscript describes a method to quantify vitamin C from lyophilized plant tissue. Standardized extraction and quantification methods of secondary metabolites are desirable. The method the authors describe is rapid and accurate (though it needs UPLC-MS, an expensive, specialized equipment). The manuscript is overall detailed and allows for the replication of the experiment. However, the manuscript needs a few changes for clarification, and would benefit from a thorough copy correction.

Minor Concerns:

Instead of "", " ".

1. Line 31, instead of "key steps were", " key steps are".
Authors' comment: done.

2. Lines 37-38. Instead of "The content of vitamins, especially vitamin C, is one of the most important indicators of the nutritional value of fruits and vegetables.", "Vitamins, especially vitamin C are important micronutrients found in fruits and vegetables. Vitamin C is also a major contributor to fruit and vegetable antioxidant capacity. "
Authors' comment: done, though “fruits and vegetables” in the second sentence have been substituted by “their” in order to fulfil the 300 word limit for the abstract.

3. Line 40: instead of "a method using…", "a method using the… ".
Authors' comment: done.

4. Line 44: instead of "make its manipulation", "make their manipulation ".
Authors' comment: done.

5. Line 45: instead of "what contributed also", "which also contributed".
Authors' comment: done.

6. Line 47: instead of "should be measured", "should be measured for accurate quantification".
Authors' comment: done.

7. Line 49: instead of "reaction because", "reaction, because".

Authors' comment: not applicable as that sentence has already been changed by Reviewer 1.

8. Line 56: instead of "procedure to obtain AA peaks completely resolved…", "procedure, to obtain AA peaks that are completely resolved…".
Authors' comment: done.

9. Line 58: instead of "types of samples that had actually gone unperceived", "samples, unperceived".
Authors' comment: that sentence was already changed as a consequence of Reviewer 1's comments.

10. Line 64: instead of "particularly interesting because of its perception as a healthy food", "perceived as healthy by consumers".
Authors' comment: done.

11. Lines 65-66: instead of "provided by the crop, like vitamin C", "in the crop, such as vitamin C ".
Authors' comment: done.

12. Lines 80-81, instead of "because they are regarded as more accurate", "because of their higher accuracy ".
Authors' comment: done.

13. Line 85, instead of "Alternatively, DHAA can be determined indirectly using a reducing agent that converts DHAA in AA to measure total ascorbic acid (TAA) and then calculating the difference between TAA and AA. ", "Alternatively, DHAA can be determined indirectly by using a reducing agent that converts DHAA to AA, measuring total ascorbic acid (TAA), and then calculating the difference between TAA and AA."
Authors' comment: done.

14. Line 90: instead of "some advantages", "advantages".
Authors' comment: done.

15. Line 98: instead of "some of its wild relatives", "some wild relatives".
Authors' comment: done.

16. Lines 159-164 Instead of " add to a beaker: 2.2 ml of H2SO4…", add 80 ml ultrapure water to a beaker, and then add 2.2. ml of H2SO4…" Add warning about adding acid to water, and not water to acid in the caution part, lines 163-164.
Authors' comment: done.

17. Lines 166-172. Same as for lines 159-164.
Authors' comment: done.

18. Line 210: instead of "grounded", "ground".
Authors' comment: done.

19. Lines 210-215: Do you need to protect vitamin C during extraction from light (e.g by covering the tubes with aluminium foil?) Could degradation already occur during extraction?

Authors' comment: we did not cover the tubes with aluminium foil, but we worked under low light intensity conditions during the extraction and the samples were in darkness in the orbital shaker, in the ultrasound bath and in the centrifuge. The following note has been added to the protocol.
NOTE: It is recommended to work under conditions of low light intensity also during the extraction steps.
20. Line 214: instead of "at room temperature.", "at room temperature, with ultrasound activated."
Authors' comment: done.

21. Line 215: instead of "centrifuge it at", "centrifuge at".
Authors' comment: done.

22. Line 260: instead of "liner", "linear".
Authors' comment: done.

23. Line 266: instead of "Built", "Build".
Authors' comment: done.

24. Line 280: instead of "Meta-phosphoric acid", "meta-phosphoric acid".

Authors' comment: it has been deleted from here (as suggested by Reviewer 1) but it has been corrected the first time that appears in the manuscript (line 138 in the version with accepted changes).

25. Line 287: instead of "protocol which presents AA peak", "protocol, which presents an AA peak".
Authors' comment: done.

26. Line 302: instead of "implied", "resulted in".
Authors' comment: done.

27. Lines 312-313: instead of "some of its wild relatives", "some wild relatives".
Authors' comment: done.

28. Line 323: explain what F-ratios are.

Authors' comment: done.

29. Line 330: instead of "quantification is very dependent on different factors", "quantification is dependent on multiple factors".
Authors' comment: done.

30. Line 336: instead of the sentence "Considering this potential… ", "To minimize AA oxidation, the following protocol steps were optimized."
Authors' comment: done.

31. Lines 337-338: instead of "…to prevent possible water interferences with vitamin C content and, in addition, to get an easier sample manipulation", "…to ensure accurate quantification of vitamin C content and to easily manipulate samples ".
Authors' comment: done.

32. Line 340: instead of "extractant but also", "extractant, which also acted".
Authors' comment: done.

33. Line 340: instead of "It also ", "This solution also".
Authors' comment: done.

34. Line 341: instead of "Moreover, it was checked if the extraction could be…", "Moreover, we tested of the extraction process could be…".
Authors' comment: done.

35. Line 343: place comma after extractant.
Authors' comment: we guess that the reviewer means “delete” instead of “place” as there was a comma originally there. Done.

36. Line 347: instead of "guarantying", "guaranteeing".
Authors' comment: done.

37. Line 349: instead of "implied an improvement in the results obtained", "resulted in an improved method".
Authors' comment: done.

38. Line 350: instead of the sentence "While this being the case…", "The most important advance was likely using a UPLC system instead of an HPLC, resulting in resolved AA peaks without interferences by unknown compounds, and in a shorter time".
Authors' comment: done.

39. Line 355: instead of "take into account the", "quantify".
Authors' comment: done.

40. Line 356: remove "also".

Authors’ comment: done.

41. Line 360: instead of "As its content starts to decrease", "As AA starts to degrade".
Authors’ comment: done.

42. Line 368: instead of "objective", "object".
Authors’ comment: done.

43. Line 374: instead of "taken into account", "quantified".
Authors’ comment: done.

44. Line 375: instead of "importance", "contribution".
Authors’ comment: done.

45. Line 375: instead of "to determine", "to".
Authors’ comment: done.

46. Line 384: instead of "So", "In summary".
Authors’ comment: done.

47. Line 386: instead of "here developed", "reported here".
Authors’ comment: done.

48. Line 387: instead of "… and DHAA content that could be…", "and DHAA content. The method could be… ".
Authors’ comment: that sentence has disappeared as it was original because the paragraph has been modified as requested by Reviewer 1.

49. Lines 390-391: There are already publications that have found that wild relatives have high Vit C and antioxidant content. Please rework the sentence, and reference here at least van Treuren et al. 2018 Metabolomics 14:1-14 and Damerum et al. 2015 Horticulture Research 2: 15055 (antioxidants, but not Vit C).
Authors’ comment: done. We have included both works, though van Treuren *et al*. did not measure the total amount of vitamin C (only AA), that is what we were talking about (vitamin C as total ascorbic acid).

We are looking forward to hearing from you.

Best regards,

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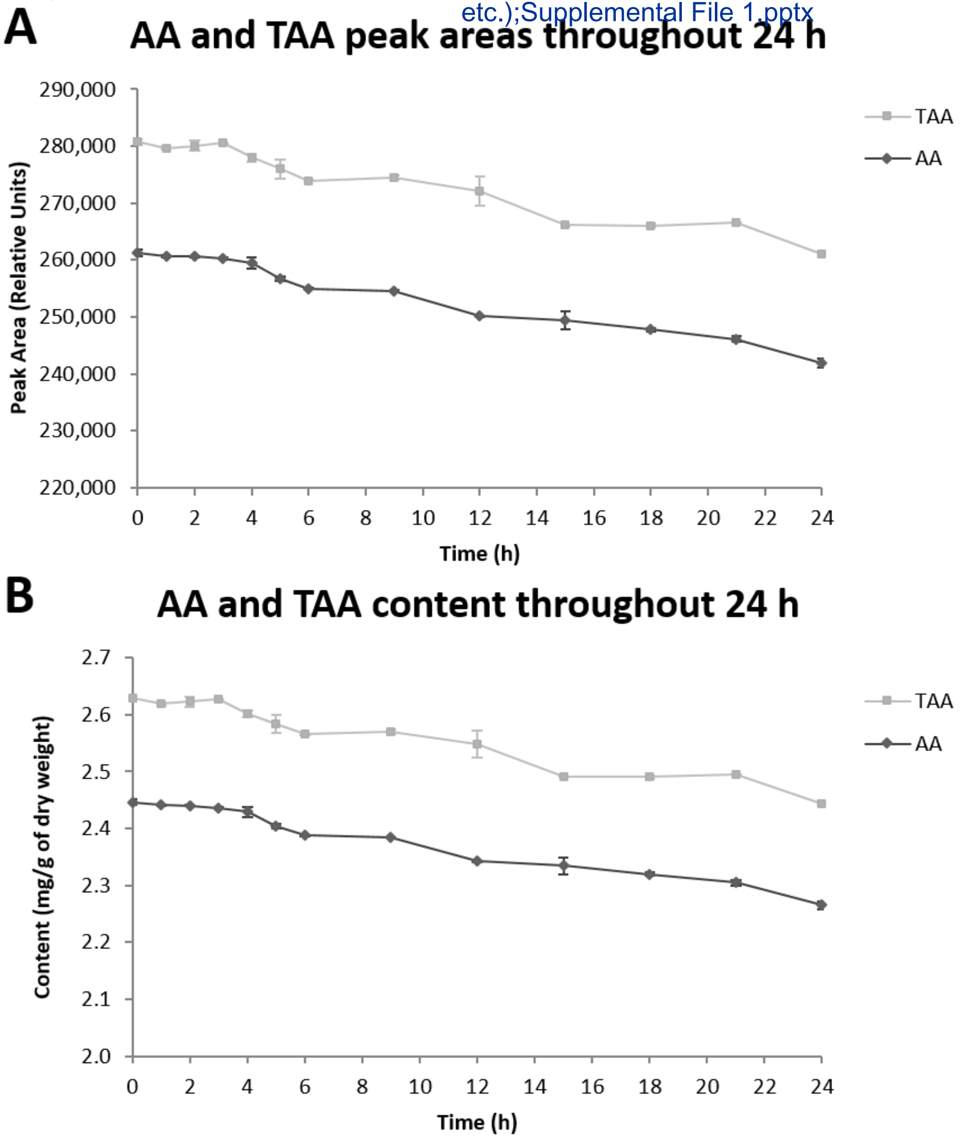

Supplemental File 1. AA and TAA stability at 5°C over 24 h. (**A**) AA and TAA peak areas throughout 24 h. (**B**) AA and TAA content (mg g^{-1} of dry weight) throughout 24 h. Bars represent the standard deviations of two technical replicates (n=2) kept in the autosampler at 5ºC and protected from exposure to light.

Supplemental File 2. Main differences between the optimized and the non-optimized protocol for TAA, AA and DHAA extraction and quantification. The samples used were the same in both cases

Section	Step	Optimized protocol	Non-optimized protocol
Reagent and solution preparation	Extraction solution	8% acetic acid (v/v), 1% MPA (w/v), 1 mM EDTA	5% acetic acid (v/v), 3% MPA (w/v)
	Reducing solution	40 mM DTT in 0.5 M Tris pH 9.0	30 mM DTT in dH_2O
	Sulphuric acid (H_2SO_4)	0.4 M	-
	Hydrochloric acid (HCl)	2M	5M
	Stock AA standard preparation	Solvent: Ultrapure water pH 2.0 acidified by 98-100% formic acid	Solvent: dH_2O
	AA calibration curve	5 points (0.5, 2.5, 5, 10, 25 µg mL-1)	6 points (10, 20, 40, 60, 80, 100 µg mL-1)
		Solvent: Ultrapure water pH 2.0 acidified by 98-100% formic acid	Solvent: 1.5% MPA
Extraction of AA and DHA	Extraction solution volume (mL)	5	3
	Mixing	Vortex 5 sec; orbital shaker (2000 rpm) 10 min at room temperature	Vortex 1 min
	Sonication (ultrasound bath)	10 min at room temperature	No
	Centrifugation (4000 *g*)	10 min at 4 ºC	20 min at room temperature
	Filtration	0.22-µm regenerated cellulose filter	0.45-µm regenerated cellulose filter
DHAA reduction to AA	Incubation at room temperature	30 min	5 min
	Stop reaction	200 µL of 0.4 M H_2SO_4	No
Determination	Instrument	UPLC: Acquity H-Class	HPLC: Hewlett Packard 1050
	Detector	PDA eλ Detector λabs for AA=245 nm	eλ Detector λabs for AA=265 nm
	Column	Acquity UPLC HSS T3 (150 mm x 2.1 mm x 1.8 µm)	HPLC C18 Tracer column (250 mm x 4 mm x 5 µm)
	Channels	A: CH3OH; B/Wash: H2O:CH3OH (50:50 v:v); C: Ultrapure water pH 2.0 with formic acid; D/Seal Wash	No
	Mobile phase	0.3 ml·min^{-1} of 2%A + 98%C (isocratic mode)	1 mL min^{-1} of KH_2PO_4 30 mM ajusted pH 3.0 HCl 5M (isocratic mode)
	Column temperature	35ºC	Room temperature
	Autosampler temperature	5ºC	No (room temperature)
	Injection volume (µl)	5	20
	AA retention time (min)	1.874	2.980
	Total running time (min)	3	7

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Author(s):	Inés Medina-Lozano, Juan Ramón Bertolín, Raquel Zufiaurre, Aurora Díaz

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