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Abstract

Vitamin C is widely studied for its protective role in humans and plants, but its quantification in fresh vegetable matrices is delicate, especially because of the unstable nature of its reduced form. Facing this aspect and with unavoidable extraction constraints, we developed and validated a method for extracting vitamin C from the lyophilized powder of tomato fruits and leaves. Easy and quick to implement by removing most of the cold constraints needed for the use of fresh powder, this method proved to be precise, accurate, and linear at the same time, across a large vitamin C concentration range (160–740 mg 100 g^{-1} for dried fruits; 190–1140 mg 100 g^{-1} for dried leaves). A simultaneous quantification of total and reduced vitamin C levels was performed by spectrocolorimetry, using a microplate reader. The evaluation of the impact of the storage conditions of the lyophilized powders on the vitamin C concentrations made it possible to optimize certain parameters of the method and to evaluate its robustness as well as the remarkable biochemical stability of the lyophilized sample.

Keywords: vitamin C; lyophilized powder; tomato; cold chain; spectrocolorimetry

1. Introduction

Vitamin C is of major interest for its health value, both to the plant (Truffault et al., 2014; Zaoui et al., 2016) and to humans who consume it (Camarena & Wang, 2016; Moser & Chun, 2016; Pearson et al., 2017). These characteristics and quality are largely related to the antioxidant properties of the reduced form of the molecule (ascorbic acid, AsA). In vegetables, vitamin C is naturally present in the form of AsA and in its oxidized form (dehydroascorbic acid, DHA). The sum AsA + DHA constitutes the total vitamin C (T-AsA) and DHA is recyclable in AsA under certain conditions (Truffault et al., 2016; Zaoui et al., 2016). It is essential to determine the proportion of AsA in the plant precisely, so as to be able to calculate the redox ratio (AsA/T-AsA), which is a frequently used indicator to assess its development stage or the impact of different stresses on its metabolism (Bernard et al., 2009; Burkey et al., 2003; El Airaj et al., 2013; Garchery et al., 2013; Gest et al., 2013; Massot et al., 2013). The literature refers to numerous techniques for the extraction and assay of vitamin C, but they are generally long to implement, expensive, analyze only one form, or require specific material (Liu et al., 2010; Munyaka et al., 2010; Tarrago-Trani et al., 2012). Stevens et al. (2006) were thus inspired by the method described by Kampfenkel et al. (1995) to propose a method of extraction and colorimetric assay of vitamin C both fast and inexpensive for assaying simultaneously T-AsA and AsA by microplate in about 20 samples of small size (< 1 g fresh matter). The extraction was performed by homogenization with a solution of trichloroacetic acid at 6% and the determination was based on the spectrocolorimetric detection of the dipyridyl-Fe^{2+} complex formed after the reduction of Fe^{3+} to Fe^{2+} by the AsA present in the extracts. Since then, we have successfully used such methodology on powders of fresh tomato fruit and leaf (Gautier et al., 2008; Gautier et al., 2009; Massot et al., 2010; Massot et al., 2012; Ripoll et al., 2016a; Ripoll

et al., 2016b; Truffault et al., 2015). However, the technique of extraction used always brought along the major disadvantage of the constraints related to the necessary preservation of the biochemical integrity of the fresh sample. Indeed, vitamin C is very sensitive to temperature and AsA tends to oxidize quickly to DHA (Truffault et al., 2014), which complicates the reliability of extraction in fresh vegetables. This led us to search for and then validate a less restrictive alternative, consisting of extracting vitamin C from lyophilized vegetable material (DM). Studies already conducted in this regard have yielded contradictory results on the relevance of this approach. For example, Chang et al. (2006) and Stevens et al. (2006) attributed an 8% to 20% loss of vitamin C content in tomato to the freeze-drying process, unlike George et al. (2011), who observed no significant impact.

Based on the aforementioned context, the present study aims to report the validation of a new method of extraction of vitamin C from lyophilized tomato plant powder, which we named "new method", by verifying its accuracy compared to the results obtained with the fresh plant powder (reference methods), its precision, the linearity of its response, and the absence of a matrix effect. We also tested robustness criteria, namely the influence of the storage temperature of the lyophilized powder (room temperature, −20 °C, and −80 °C), the storage time, or the impact of the mass of the analytical tested sample (TS).

2. Material and methods

2.1. Plant material

The validation of the "New method" was carried out on samples stored at −80 °C, resulting from a 2010 greenhouse experiment on the impact of the genotype on the texture and firmness of the tomato fruit under hydric stress (Aurand et al., 2015).

Samples of leaves (final stage but not senescent) and tomato fruits at mature stage (red ripe stage and seven days after postharvest storage at 20 °C) and at green stage (20 days after anthesis) were selected from six genotypes (Cervil, Levovil, VilB, NIL-L4, NIL-L9, and NIL-V9) described by Aurand et al. (2015). A wide range of vitamin C concentrations was obtained from fruits (around 160–450 mg 100 g^{-1} DM or 10–30 mg 100 g^{-1} fresh matter) and from leaves (around 190–450 mg 100 g^{-1} DM or 25–65 mg 100 g^{-1} fresh matter), in order to highlight a possible matrix effect. Samples were harvested in 2010, cut into pieces (the fruits), immediately immersed in liquid nitrogen, and stored at −80 °C. Then, they were finely ground in the presence of liquid nitrogen and stored at −80 °C. For validation purposes, part of this powder was freeze-dried in 2015 (GENESIS 25ES; Virtis Company, Gardiner, NY) under the following conditions: freezing at −25 °C, followed by a primary freeze-drying at 0.4 mbar to eliminate free water (ending at 3 °C) and by two subsequent cycles of secondary freeze-drying with a pressure variation of 1.6 mbar at 0.001 mbar to remove the bound water (ending at 10 °C). At the end of the freeze-drying process and upon return to atmospheric pressure, the dry powders were stored at −80 °C.

2.2. Reagents and standards

The AsA (ref. A0278-25G) used as standard and the reagents 2,2-dipyridyl (DPD), dithiothreitol (DTT), *N*-ethylmaleimide (NEM), and $FeCl_3$ were provided by Sigma (Saint-Quentin Fallavier, France). The reagents NaH_2PO_4 and Na_2HPO_4 (for phosphate buffer preparation), trichloroacetic acid (TCA), H_3PO_4 and ethanol were obtained from VWR (Fontenay-sous-Bois, France).

2.3. Extraction methods

Two different reference methods were used to validate the accuracy of the "new method" (Figure 1).

2.3.1. "New method"

The sampling steps of each tested sample (TS) and vitamin C extraction took place at room temperature and were dissociable. Samplings consisted of weighing TS of lyophilized leaf (20 mg) or fruit (40 mg) powder in 2 mL microtubes and storing them at −80 °C (preferably) if the extraction and analytical determination for vitamin C was not performed rapidly. After adding the extraction solvent in each microtube (1 mL TCA at 6%), the extraction by homogenization was carried out in three series of eight TS using a vibratory oscillating mill (MM301, Retsch; 30 s; 30 oscillations s^{-1}) and accessories for agitation of up to 10 microtubes. After a first centrifugation (16,110 *g*, 5 min at 4 °C; Model 5415R; Eppendorf, Le Pecq, France), the 24 microtubes (limit of the centrifuge) were again homogenized and centrifuged under the same conditions.

2.3.2. Reference method 1 on fresh leaf powder

Vitamin C was extracted according to the method of Stevens et al. (2006), with the only significant modification consisting of intensifying the cold chain. For this purpose, the samples of fresh leaf powders (−80 °C) were kept on a bed of liquid nitrogen placed inside a cold bank (~−20 °C). Within this low-temperature environment, a TS of fresh leaf powder (approximately 100 to 200 mg) was weighed and introduced into a microtube containing the extraction solvent (0.6 mL TCA at 6%). Vitamin C was immediately extracted by homogenization with a vortex (at room temperature), then the microtube was put back on hold in crushed ice, still in the cold bank. This process was repeated to obtain one series of 24 microtubes. At the end of the series, the microtubes

were again stirred for a few seconds in a vortex, weighed to determine the mass of each TS and centrifuged (16,110 *g*, 15 min at 4 °C; model 5415R; Eppendorf, Le Pecq, France).

2.3.3. Reference method-2 on fresh fruit powder

For this reference method, vitamin C was extracted from the fresh fruit powder according to a modified version of the method of Stevens et al. (2006). The first step was to introduce the TS of fruit powder (approximately 400 to 600 mg) into previously weighed and frozen microtubes. The integrity of the vegetable powders and TS were ensured by the presence of liquid nitrogen in a box placed at room temperature. The microtubes containing the TS were then stored at −80 °C until analysis (extraction and analytical determination), carried out in a series of 24 samples (three series of eight TS) as follows. The first series of eight fresh TS were placed on a frozen rack (~ −20 °C); the extraction solvent (0.6 mL TCA at 6%) was immediately added and vitamin C was extracted simultaneously in the eight TS using a vibratory oscillating mill (1 min; 30 oscillations s^{-1}; MM301, Retsch). After a slight vortex agitation, the microtubes were then placed on hold in crushed ice for the necessary time to carry out the analogous extraction of two new series of eight TS. The 24 microtubes were then weighed to determine the mass of each TS and centrifuged (16,110 *g*, 15 min at 4 °C; model 5415R; Eppendorf, Le Pecq, France;).

2.4. Analytical determination of vitamin C

Regardless of the extraction method applied, the vitamin C analytical determinations by colorimetry with a microplate-reader described by Stevens et al. (2006) were carried out immediately with minor modifications. The determination was

144 based on spectrophotometric detection of the dipyridyl-Fe^{2+} complex formed after the
145 reduction of Fe^{3+} to Fe^{2+} by the AsA present in the extracts. Part of the 96-well
146 microplate allowed dosing the AsA naturally contained in the sample extract. The other
147 part of the microplate allowed determining the T-AsA after the reduction of the DHA
148 contained in the sample extract by incubation with a reducing agent (DTT). The
149 analytical determination was performed by external calibration with a Multiscan Ascent
150 MP reader (Labsystems, Thermo Fisher Scientific, Courtaboeuf, France). The stock
151 solution of AsA (1 mg mL^{-1} in 6% TCA) used to prepare standards for external
152 calibration was stored at −80 °C as 1-mL aliquots in microtubes. In contrast, the
153 standard solutions (seven from 0.02 to 0.4 mg mL^{-1} AsA in TCA at 6% and one without
154 AsA) were prepared at each assay from an aliquot of the stock solution of AsA. In order
155 to assay vitamin C, 20 µL of standard solutions were distributed in the T-AsA part of
156 the microplate and 20 µL of extracts were distributed in both the T-AsA and AsA parts.
157 For all the following steps, the microplate was shaken (~1 min) after the addition of
158 each reagent. For T-AsA determination, 20 µL of 5 mM DTT (in 0.5 M phosphate
159 buffer, pH 7.5) were added into the T-AsA part and the covered microplate was
160 incubated for 20 min at 37–39 °C to reduce DHA. Then, 10 µL of NEM at 0.5% (*w/v* in
161 water) were added to eliminate the excess of DTT. Subsequently, 80 µL of color reagent
162 were added (see below) and the covered microplate was incubated for 60 min at 37–39
163 °C. After incubation, the absorbance was read at 550 nm using a microplate reader. For
164 the simultaneous AsA assay, the DTT and NEM were omitted and replaced by the same
165 volumes of 0.5 M phosphate buffer, pH 7.5, in the AsA part of the microplate and the
166 procedure was carried out as described above. The color reagent consisted of the
167 following: solution **A**: 31% orthophosphoric acid, 4.6% *w/v* TCA, and 0.6% *w/v* iron

chloride; solution **B**: 4% 2,2-dipyridyl (*w/v* made up in 70% ethanol); solutions **A** and **B** were mixed, 2.75 parts **A** to 1 part **B**.

Calculation. The T-AsA and AsA contents (in mg 100 g^{-1} DM) were calculated using the following equation:

$$\text{T-AsA or AsA content} = (A - b)/a \times [V_{TCA} + TS \times (1 - DM_{(\%)}/100)] \times 100/TS \times 100/DM_{(\%)}$$

Simplified equation for lyophilized powder ($DM_{(\%)} = 100$):

$$\text{T-AsA or AsA content} = (A - b)/a \times V_{TCA} \times 100/TS$$

For an extract absorbance, A (for T-AsA or AsA calculation, depending on the part of the microplate); external calibration curve parameters a and b (for $y = ax + b$); a mass of test sample (in g), TS; a percentage of dry matter of the sample, $DM_{(\%)}$; a solvent extraction volume of TCA at 6% (in mL), V_{TCA}.

2.5. Investigating a matrix effect

The internal standards were prepared and added to 20 lyophilized tomato samples (four green fruits, nine mature fruits, and seven leaves). For each sample, the analysis was carried out with no addition and then with addition of three levels of AsA corresponding to 20, 100, and 150 µg per TS (TS = 40 mg for fruits and 20 mg for leaves). The extraction of TS with the internal standards addition was carried out according to the proposed "new method" but replacing the extraction solvent (1 mL TCA at 6%) with 1 mL of standard solution (respectively, 0.02, 0.1, and 0.15 mg mL^{-1} in TCA at 6%), each being prepared by dilution of the stock solution of AsA.

The lowest concentration of added standard was equivalent to the lowest point of the external calibration curve. The highest standard concentration (7.5 times the lowest)

192 was chosen so that the overall concentration of the extracts would remain within the
193 calibration range.
194
195 **2.6. Evaluation of the robustness of the "new method"**
196 The effect of storage temperature on the stability of freeze-dried powders was
197 evaluated by comparing the AsA and T-AsA contents of 40 TS stored for 3.5 months at
198 −20 °C or at −80 °C (eight green fruits, 20 mature fruits and 12 leaves). The long-term
199 stability of mature fruit powders was verified after the storage of 58 TS for 20 months at
200 −80 °C. The stability of the freeze-dried powders was also evaluated after 24 hours of
201 storage of the TS at room temperature and exposed to room lighting (three green fruits,
202 seven mature fruits and three leaves). This test was repeated over a period of 34 days
203 with three samples (one green fruit, one mature fruit, and one leaf).
204 **2.7. Statistical analysis**
205 The "new method" has been validated in T-AsA and AsA by checking its
206 precision, linearity and accuracy. For each test, the series were compared using the
207 Wilcoxon signed rank test and their correlation was verified with the Spearman rank test
208 (Scherrer, 1984). According to the definitions of Kennedy and Neville (1986), the term
209 precision refers to the closeness to which measurements agree with each other, while the
210 term accuracy expresses the closeness of measurements to the true value.
211 In order to study the precision of the "new method", two series of analyses of 44
212 lyophilized tomato powders (eight green fruits, 25 mature fruits, and 11 leaves) were
213 conducted from different TS, with several days apart (between six and 15 days).
214 According to ISO 5725 (1994) standard regarding results obtained under these
215 conditions, the reproducibility value R is the value below which there is 95%
216 probability of finding the absolute difference between two results from a single assay.

The lower the *R* value, the better the reproducibility. This value was calculated using the following equation:

$$S_r = \left(\frac{1}{2q}\sum_{i=1}^{q} w_i^2\right)^{\frac{1}{2}}$$

$$R = 2.8_{S_r}$$

Where q is the double analyzed sample number and w_i is the absolute difference between pairs of results. In order to take into account the order of magnitude of the values obtained during the precision test, we also calculated the relative root mean square error (RRMSE).

The regression parameters obtained using the internal standards addition made it possible to evaluate the linearity of the method and a possible matrix effect was sought by comparing the concentrations of T-AsA and AsA obtained by internal and external calibrations.

The accuracy of the “new method” was checked on the same 44 samples selected to evaluate its precision by comparing the results obtained by this method (lyophilized powders) with those obtained with "reference method 1" for fresh leaf powders and "reference method 2" for fresh fruit powders.

3. Results and discussion

All the validation results of the statistical analysis are presented in Table 1.

As a preamble to the validation of the "Nnew method", here are some observations on its development. The major constraint of the extraction of vitamin C from fresh powder is the cold chain imposed by the high lability of this compound. Very easy to handle and much less sensitive to temperature and oxidation, the “new method” using lyophilized powder reduced this cold chain significantly by making the use of liquid nitrogen and

cold bank unnecessary. The powders were lyophilized without taking any special precautions but respecting a cold chain sufficiently rigorous to conserve their integrity between the −80 °C freezer where they were stored and the freeze-drier. The choice of a vibratory oscillation mill was self-evident to extract vitamin C from the lyophilized TS of fruits and leaves because it allows up to 10 simultaneous extractions as well as a more efficient homogenization compared to vortex (lyophilized powder tends to flocculate with a vortex agitation). It should also be highlighted that an improved version of this mill may handle more extractions. We opted for TS masses of 40 mg for the fruits and 20 mg for the leaves, in order to maintain a ratio dry mass of sample/volume of extract comparable to that obtained by analyzing the fresh powder. However, by doubling the masses of lyophilized TS (an impossible procedure with fresh TS due to the capacity of the microtube), the results were equally satisfactory, giving the possibility of quantifying vitamin C levels up to two times lower than in the fresh TS (data not shown).

3.1. Precision

The two series of analyses are very strongly correlated ($\rho > 0.97$) and no bias was detected ($p > 0.01$) either for T-AsA or for AsA (Figure 2). The reproducibility values reflect the excellent precision of the "new method", with the high values of R (21.6 and 18.6, respectively) being explained by the extent of the concentration range (~170 to 450 mg 100 g^{-1} DM). The RRMSE calculated to overcome this variable are between 3.8% and 3.9%. This result is highly satisfactory because the two series of analyses were performed several days apart and it shows that the conservation of the powders at −80 °C has no impact on the contents of T- AsA and AsA, regardless of the tissue and stage of maturity. The results are only slightly better when the two series of

264 analysis are performed on the same day on the same microplate (RRMSE = 3.3% for T-
265 AsA and 3.6% for AsA; data not shown).
266
267 **3.2. Response linearity and matrix effect**
268 The standard additions carried out on 20 samples (0 to 150 µg per TS), of which
269 three examples (green fruit, mature fruit, and leaf) are presented in Figure 3a, provide
270 linear regression coefficients of the order of 0.99. This fact testifies the very good
271 linearity of the "new method" for both T-AsA and AsA. The measured absorbance is
272 proportional to the vitamin C concentration, irrespective of the tissue (fruit or leaf) and
273 the stage of maturity of the tomato (green or mature fruit). Considering the standard
274 additions, it can be deduced that the "new method" is linear at least over the ranges
275 170–630 mg, 160–740 mg and 190–1140 mg of vitamin C for 100 g DM of green fruits,
276 mature fruits and leaves, respectively.
277 A possible matrix effect was sought by comparing the values obtained by
278 internal and external calibration on the 20 samples (Figure 3b). The excellent
279 correlation between the two series (ρ = 0.97) and the similarity of results (p > 0.01)
280 testify to the absence of a matrix interference.
281 These results are highly important from a practical point of view because they
282 validate the external calibration of the "new method" for both T-AsA and AsA.
283
284 **3.3. Accuracy**
285 The "new method" and reference methods for fresh powder are significantly
286 correlated (ρ > 0.97) for both T-AsA and AsA. The absence of bias (p > 0.01) reflects
287 the accuracy of the "new method" (Figure 4). In order to obtain such an excellent
288 correlation, we mainly improved the performance of fresh powder methods, which tends

289 to show that the most accurate method is the "new method". Indeed, "reference method
290 1" corresponds to the extraction method of Stevens et al. (2006) with an intensification
291 of the cold chain (cold bank), intended to improve the precision of the results on fresh
292 powder. With this change, the CV of fresh powder analysis decreased a lot, from 9.5%
293 to 2.0% for T-AsA and from 16.2% to 1.4% for AsA (data not shown). However, the
294 additional cold constraint made the homogenization step much longer and tedious (risk
295 of musculoskeletal disorder for the operator by prolonged use of the vortex) when TS
296 were greater than 500 mg (a frequent case with fruit powder). Therefore, we developed
297 "reference method 2", which allowed vitamin C to be extracted from 10 TS
298 simultaneously in just 1 min. For this we used a vibratory oscillating mill, whose
299 efficient homogenization is much greater than that of a vortex, especially in a cold
300 environment. Thanks to the essential presence of liquid nitrogen to handle the fresh
301 powders and to collect the TS, we managed to keep a cold chain that would be strong
302 enough to guarantee the reliability of the results of AsA, without resorting to the cold
303 bank. "Reference method 2" was compared to "reference method 1" by analyzing 35
304 samples of fresh fruit powders (20 green and 15 mature). The similarity of the two
305 methods was verified for both T-AsA and AsA (Wilcoxon signed rank test: $p > 0.01$ and
306 spearman correlation: $\rho > 0.95$; data not shown). However, we chose to keep "reference
307 method 1" for the fresh leaf powder because it seems that vitamin C degrades much
308 more quickly in this tissue than in fruits and the extraction time is quite reasonable even
309 in the cold bank (small size of the TS).

310 The "new method" and the reference methods give comparable results over a
311 wide range of vitamin C concentrations (~180–450 mg 100 g^{-1} DM), and the correlation
312 parameters show a very acceptable systematic and proportional bias (< 5%). Thus,

lyophilization and the subsequent extraction procedure do not lead to a loss of T-AsA or AsA.

3.4. Robustness

The TS weighing was carried out without any particular precaution since no degradation of vitamin C was observed in lyophilized TS of fruit or leaf after 24 hours of storage at room temperature and exposure to daylight (data not shown). As we have shown, the possibility of fractioning the preparation steps without any impact, irrespective of the tissue, contributes to the robustness of the “new method”. We also set out to evaluate the stability of the composition of lyophilized powders over time, since the excellent precision of the "new method" had already answered this question for short-term storage. In addition, we assessed long-term precision by re-analyzing 58 lyophilized powders of mature fruits stored for 20 months at −80 °C (Figure 5). After this time, the T-AsA and AsA contents had hardly changed and only a decrease in the AsA content of around 5 to 10% was observed. Nevertheless, we recommend performing the analyses quickly after lyophilization. These results reflect the adaptability of the "new method" with the possibility of postponing analysis for several days or even several months in the case of mature fruits. Storage at −80 °C is therefore, a guarantee of preserving the integrity of the lyophilized sample over the long term. In the short term, however, other preliminary tests showed that storage at −20 °C was satisfactory but that the nature of the sample must be considered (data not shown). Thus, 3.5 months of storage at −20 °C did not seem to affect the T-AsA or AsA contents measured on lyophilized powders of red fruits, whereas these same contents decreased by 10% in green fruits and about 15% in the leaves. This matrix effect related to storage temperature was also verified with lyophilized powders stored for 34 days at room

338 temperature and daylight. Thus, the T-AsA and AsA contents decreased much more
339 rapidly in the leaves (75%) than in the red fruits (20%) or in the green fruits (35%) (data
340 not shown). Therefore, it is essential to adapt the method protocol (and in particular the
341 timeframe of the analytical determination after lyophilization) according to the nature of
342 the sample and storage capacity.
343
344 **3.5. Highlighting interfering absorbance in extracts**
345 Whatever the extraction method used, the results were obtained from the same
346 DPD colorimetric assay technique described by Stevens et al. (2006). However, more
347 recent work (Ueda et al., 2013) has shown a possible overestimation of vitamin C levels
348 with this assay. Garchery et al. (2013) adapted the dosage with DPD to take into
349 account these non-negligible absorbance interferences related to the Fe^{3+} reduction in
350 Fe^{2+} by compounds other than the AsA contained in the extracts. In order to consider it,
351 it must be measured in each extract after oxidation in the presence of ascorbate oxidase,
352 then deducted from the absorbances with and without DTT to calculate the
353 concentrations of T-AsA and AsA, respectively. To verify the existence of this
354 interfering absorbance, vitamin C was extracted with the "new method" from 202 TS of
355 lyophilized powders stored at −80 ° C (41 green, 133 mature, and 28 leaves). The
356 analytical extracts were assayed according to Garchery et al. (2013) to highlight the
357 interfering absorbance specific to each one. From the same microplate, the contents of
358 T-AsA and AsA were calculated both by deducting and without deducting this
359 interfering absorbance. Figure 6 represents the comparison of these results for T-AsA
360 showing a low interference absorbance in fruits, whether mature or not (< 10% in the
361 range of T-AsA values measured (110 to 780 mg 100 g^{-1} DM)). For the leaves, the error
362 is much more significant because if we refer to the regression line, it is similar to a

363 systematic bias that generates an important overestimation of vitamin C (~85 mg 100
364 g^{-1} DM), regardless of the real concentration of vitamin C (range 130 to 400 mg 100 g^{-1}
365 DM). According to Ueda et al. (2013), this additional absorbance would result in
366 particular from a significant amount of iron in the matrix or from the presence of
367 absorbent pigments at the dosing wavelength in the extract. The tomato plants that
368 supplied our samples received an iron dietary intake that covered their nutritional needs.
369 When assaying the AsA with DPD (in reality we are dosing the dipyridyl-Fe^{2+}
370 complex), one mole of Fe corresponds to one mole of AsA. In fact, when measuring an
371 absorbance equivalent to 85 mg of ASA, this corresponds to the measurement of
372 approximately 27 mg of Fe^{2+}. Knowing that the amounts of total iron found in tomato
373 leaves can reach 30 mg 100 g^{-1} DM (Moreno et al., 2005), it is possible that the non-
374 chelated Fe^{2+} present in the leaves alone might explain the essence of the observed
375 interfering absorbance. Tomato leaves sometimes contain anthocyanins (Larbat et al.,
376 2014), pigments absorbing at 550 nm, but the samples we used did not contain them.
377 We have verified in 24 samples that deducting this residual absorbance had no impact
378 on the reliability of the "new method", which remains excellently accurate, in
379 comparison with the reference methods of extraction on fresh powder (data not shown).
380 Therefore, unless an error of 10% is considered negligible (fruits), it seems important to
381 take into account the interference absorbance.
382
383 By developing and validating a method for extracting vitamin C from the lyophilized
384 powder which is both reliable and applicable to fruit and tomato leaves, we have freed
385 ourselves from the constraints of handling fresh powder (working under permanent cold
386 chain with liquid nitrogen and cold bank). The “new method” proved to be precise,

accurate, and linear at the same time over a wide range of concentration of vitamin C and no matrix effect was observed.

The stability of the lyophilized sample contributes to the robustness of the “new method” by bringing much more flexibility in its handling and implementation compared to others. It makes it possible to weigh the TS at room temperature and to dissociate the preparation steps easily (sampling/weighing of TS and extraction) regardless of the tissue. The lyophilized powder is much less sensitive to oxidation and temperature than the fresh powder. As a result, even in the case of breakdown of a freezer or power failure for 24 hours, T-AsA and AsA would still be reliably quantifiable in lyophilized powder. Under such conditions (however plausible), fresh powder would lose its integrity by heating up, causing the oxidation and degradation of vitamin C very quickly, making it impossible to analyze. We have not highlighted the need to recommend specific freeze-drying conditions, which reinforces the robustness of the "new method", and lyophilized tomato powders require less space than fresh powders for freezer storage.

Very easy to implement with the use of a vibratory oscillation mill for simultaneous extractions, this "new method" is about two times faster than the reference methods on fresh powder, making it easy to analyze at least 48 samples per day.

There are other testing methods for vitamin C in the literature. For example, after the oxidation of AsA to DHA with ascorbate oxidase and derivatization with 1,2-*o*-phenylenediamine, T-AsA is quantifiable by HPLC–fluorimetry (George et al., 2011). AsA and T-AsA (after the reduction of DHA to AsA) are also quantifiable by HPLC–UV (Phillips et al., 2016), or by spectrophotometry by measuring the difference in absorbance before and after the addition of ascorbate oxidase (Queval and Noctor,

411 2007). Since vitamin C is also extracted in an acid medium when using all these dosing
412 techniques, it is very likely that the "new method" is compatible with their use.
413 The TS of lyophilized powder are of identical mass (direct weighing), unlike the TS of
414 fresh powder introduced in approximate quantities into the microtubes. The exact mass
415 of fresh powder is determined only after the extraction step, by weighing the total mass
416 of the microtube and deducting the masses of the empty tube and extraction solvent,
417 which increases the risk of error.
418 With fresh powder, the quantification of the T-AsA and AsA contents is closely related
419 to the $DM_{(\%)}$ of each sample because this parameter occurs twice in the calculation (see
420 equations § 2.4). Firstly, to calculate the aqueous volume of each TS (assimilated by the
421 difference between the fresh and dry masses) and adding it to the volume of solvent (0.6
422 mL of TCA at 6%) in order to obtain the total extraction volume, thus differing from
423 one sample to another. Secondly, to express the vitamin C content of a fresh sample in
424 relation to its dry mass, a necessary calculation to compare results from both lyophilized
425 and fresh powders. The influence of $DM_{(\%)}$ is such that an underestimation of only 0.5%
426 DM can result in an overestimation of the vitamin C content of almost 10% in fresh
427 powder. This could also contribute to the differences in concentrations of T-AsA and
428 AsA (−20%) mentioned by Stevens et al. (2006) between the measurements on fresh
429 powders and on lyophilized powders. For all these reasons, the quantification of the
430 vitamin C content seems more reliable with lyophilized powder because in this case, the
431 $DM_{(\%)}$ is always equal to 100 and the calculation no longer depends on this parameter
432 (see equation 2.4).
433 The "new method" prevents the phenomenon of "dilution" of the extract related to the
434 water content of fresh powders and enables the quantification of lower contents of
435 vitamin C. Lyophilized powder ensures reproducible vitamin C extraction conditions

because the parameters are constant for all samples: mass and $DM_{(\%)}$ of the TS, volume of extract, and then its concentration of extraction solvent. The extraction conditions in fresh powders are not as reproducible because the mass and $DM_{(\%)}$ of the TS are different for each sample, which induces non-constant extraction volumes and so non-constant extraction solvent concentrations.

We also confirm the existence of an interfering absorbance due to compounds other than AsA in extracts that varies according to the samples, which we recommend taking into account, especially regarding the leaves.

Finally, the genericity of the "new method" is still to be verified, namely its application to other vegetable matrices, a work that we have begun to do with apple and peach trees.

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570 **Figure Captions**

571

572 **Figure 1.** Procedure for vitamin C micro-extraction on tomato powder: "new method"
573 on lyophilized fruit and leaf; "reference methodv1" on fresh leaf and "reference method
574 2" on fresh fruit. (TCA – trichloroacetic acid).

575

576 **Figure 2.** Precision of the "new method". Double vitamin C analysis in 44 tomato
577 samples with several days apart between sets (◊ green tomato; ○ mature tomato; △
578 tomato leaf; T-AsA - total vitamin C; AsA - ascorbic acid).

579

580 **Figure 3.**

581 **3a:** Internal standard calibration determination of vitamin C with the "new method" in
582 three samples (from the 20): green fruit, mature fruit and leaf (◊ GT – green tomato; ○
583 MT – mature tomato; △ TL – tomato leaf; T-AsA – total vitamin C; AsA – ascorbic
584 acid).

585 **3b.** Comparison between measurements of vitamin C concentrations with external
586 (series 1) and internal standards (series 2) in the 20 samples (◊ green tomato; ○ mature
587 tomato; △ tomato leaf; T-AsA – total vitamin C; AsA – ascorbic acid).

588

589 **Figure 4.** Accuracy of the "new method". Comparison between measurements of
590 vitamin C concentrations in 44 tomato samples analyzed with "reference method 1"
591 (fresh leaves) or "reference method 2" (fresh fruits) as reference, and the "new method"
592 (lyophilized fruits and leaves) (◊ green tomato; ○ mature tomato; △ tomato leaf; T-
593 AsA – total vitamin C; AsA – ascorbic acid).

594

595 **Figure 5.** Robustness of the “new method”. Double vitamin C analysis in 58 mature
596 tomato samples with lyophilized powder storage at −80 °C for 20 months between
597 series 1 and series 2 (♢ green tomato; ○ mature tomato; △ tomato leaf; T-AsA – total
598 vitamin C; AsA – ascorbic acid).
599
600 **Figure 6.** Influence of ripening stage and tomato organ on interfering absorbance levels.
601 Comparison between measurements of total vitamin C concentrations in 202 tomato
602 samples analyzed with the “new method” and assayed without ascorbate oxidase (series
603 1) and with ascorbate oxidase in parallel (series 2) (♢ GT – green tomato; ○ MT –
604 mature tomato; △ TL – tomato leaf; T-AsA – total vitamin C).

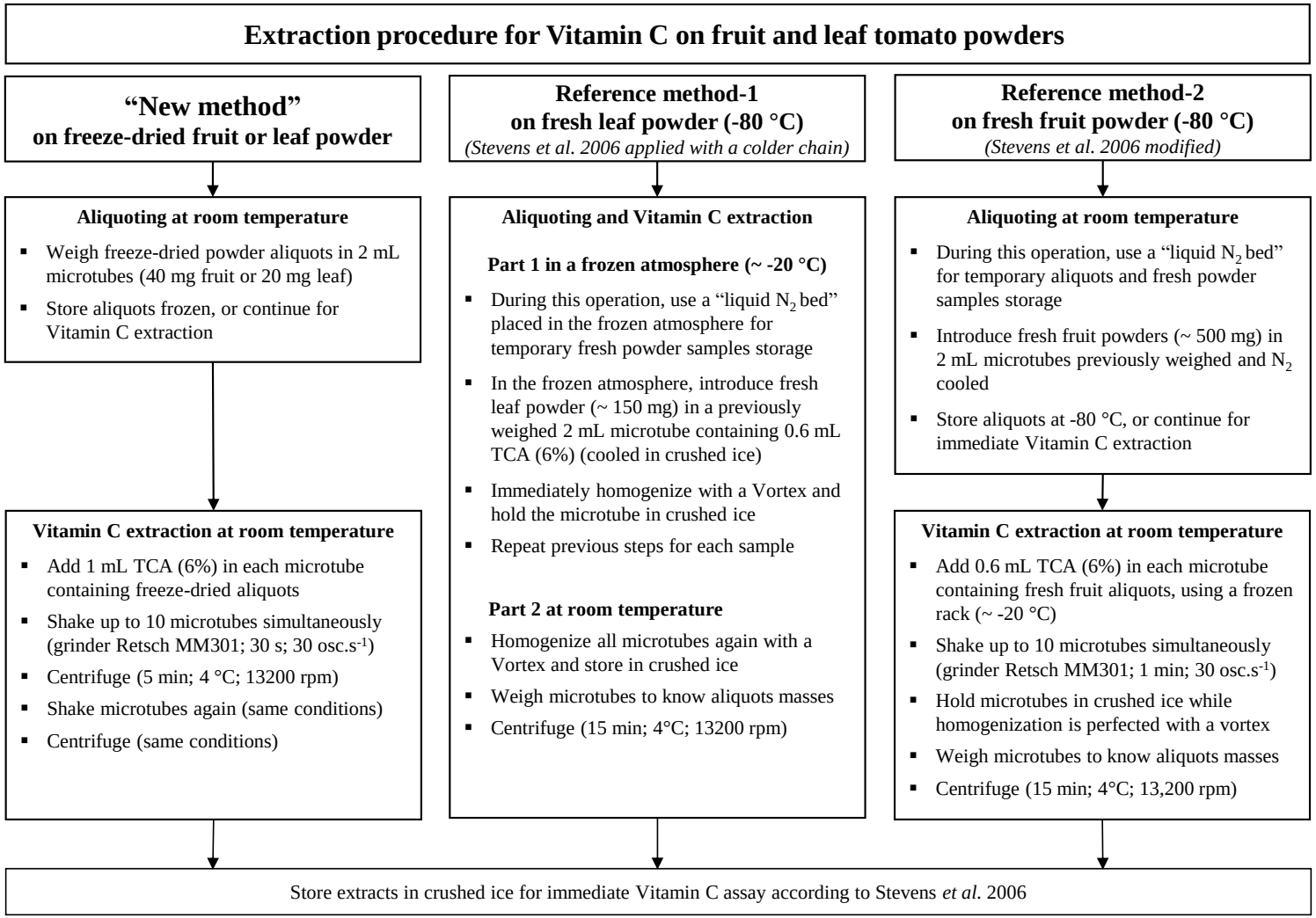

Extraction procedure for Vitamin C on fruit and leaf tomato powders
"New method"
on freeze-dried fruit or leaf powder
Aliquoting at room temperature
Weigh freeze-dried powder aliquots in 2 mL microtubes (40 mg fruit or 20 mg leaf)
Store aliquots frozen, or continue for Vitamin C extraction
Vitamin C extraction at room temperature
Add 1 mL TCA (6%) in each microtube containing freeze-dried aliquots
Shake up to 10 microtubes simultaneously (grinder Retsch MM301; 30 s; 30 osc.s-1)
Centrifuge (5 min; 4 °C; 13200 rpm)
Shake microtubes again (same conditions)
Centrifuge (same conditions)
Reference method-1
on fresh leaf powder (-80 °C)
(Stevens et al. 2006 applied with a colder chain)
Aliquoting and Vitamin C extraction
Part 1 in a frozen atmosphere (~ -20 °C)
During this operation, use a "liquid N2 bed" placed in the frozen atmosphere for temporary fresh powder samples storage
In the frozen atmosphere, introduce fresh leaf powder (~ 150 mg) in a previously weighed 2 mL microtube containing 0.6 mL TCA (6%) (cooled in crushed ice)
Immediately homogenize with a Vortex and hold the microtube in crushed ice
Repeat previous steps for each sample
Part 2 at room temperature
Homogenize all microtubes again with a Vortex and store in crushed ice
Weigh microtubes to know aliquots masses
Centrifuge (15 min; 4°C; 13200 rpm)
Reference method-2
on fresh fruit powder (-80 °C)
(Stevens et al. 2006 modified)
Aliquoting at room temperature
During this operation, use a "liquid N2 bed" for temporary aliquots and fresh powder samples storage
Introduce fresh fruit powders (~ 500 mg) in 2 mL microtubes previously weighed and N2 cooled
Store aliquots at -80 °C, or continue for immediate Vitamin C extraction
Vitamin C extraction at room temperature
Add 0.6 mL TCA (6%) in each microtube containing fresh fruit aliquots, using a frozen rack (~ -20 °C)
Shake up to 10 microtubes simultaneously (grinder Retsch MM301; 1 min; 30 osc.s-1)
Hold microtubes in crushed ice while homogenization is perfected with a vortex
Weigh microtubes to know aliquots masses
Centrifuge (15 min; 4°C; 13,200 rpm)
Store extracts in crushed ice for immediate Vitamin C assay according to Stevens et al. 2006

Figure 1.

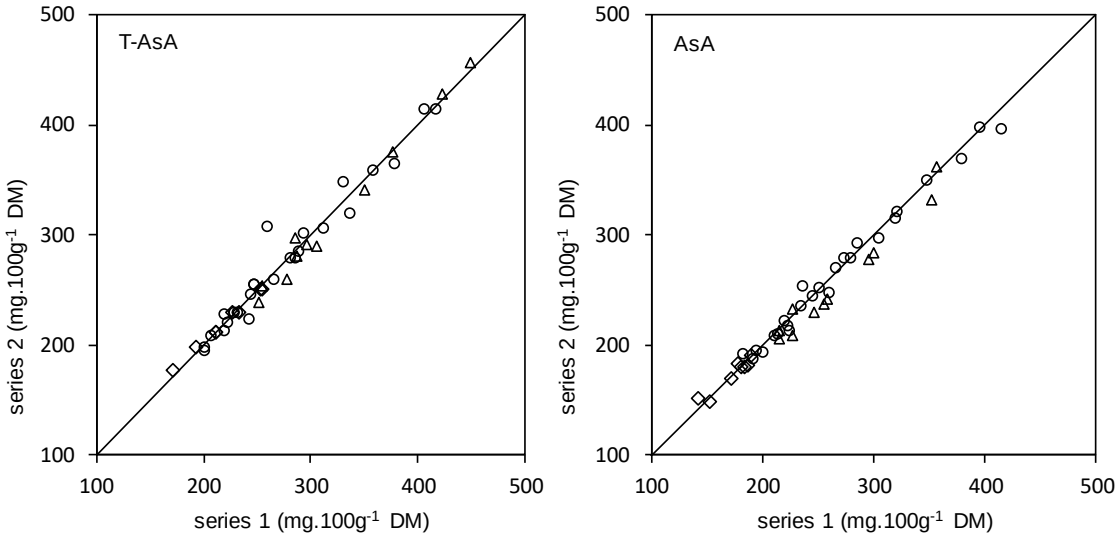

T-AsA
AsA
series 2 (mg.100g-1 DM)
series 1 (mg.100g-1 DM)
100
200
300
400
500

Figure 2.

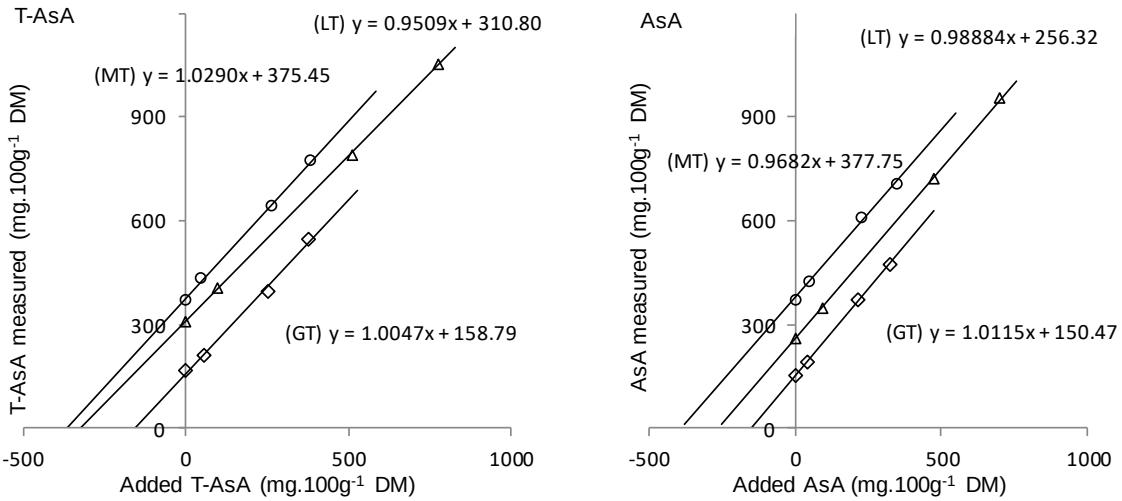
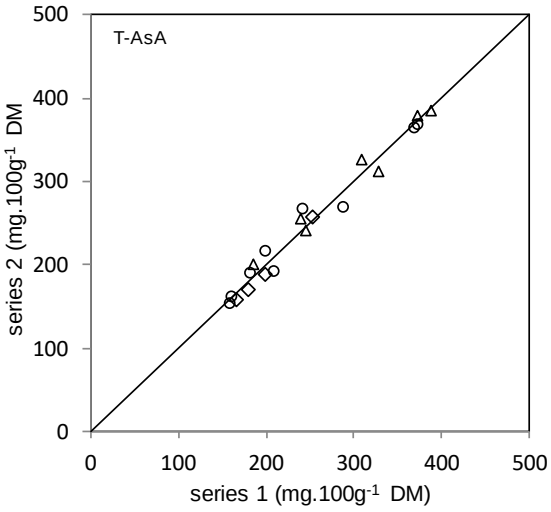

T-AsA
(LT) y = 0.9509x + 310.80
(MT) y = 1.0290x + 375.45
(GT) y = 1.0047x + 158.79
T-AsA measured (mg.100g-1 DM)
Added T-AsA (mg.100g-1 DM)
AsA
(LT) y = 0.98884x + 256.32
(MT) y = 0.9682x + 377.75
(GT) y = 1.0115x + 150.47
AsA measured (mg.100g-1 DM)
Added AsA (mg.100g-1 DM)

Figure 3a.


T-AsA
500
400
300
200
100
0
0
100
200
300
400
500
series 2 (mg.100g-1 DM
series 1 (mg.100g-1 DM)

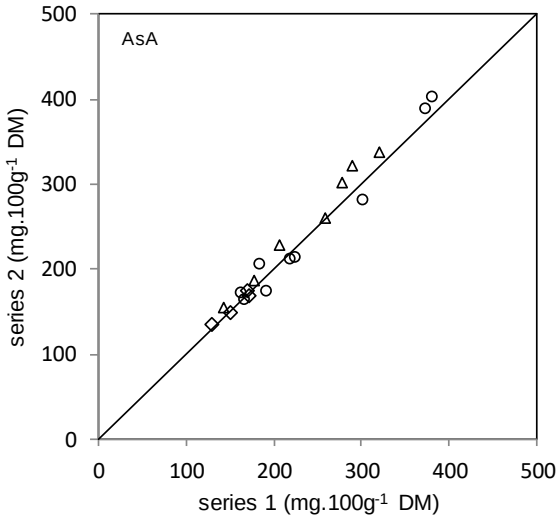

AsA
500
400
300
200
100
0
0
100
200
300
400
500
series 2 (mg.100g-1 DM)
series 1 (mg.100g-1 DM)

Figure 3b.

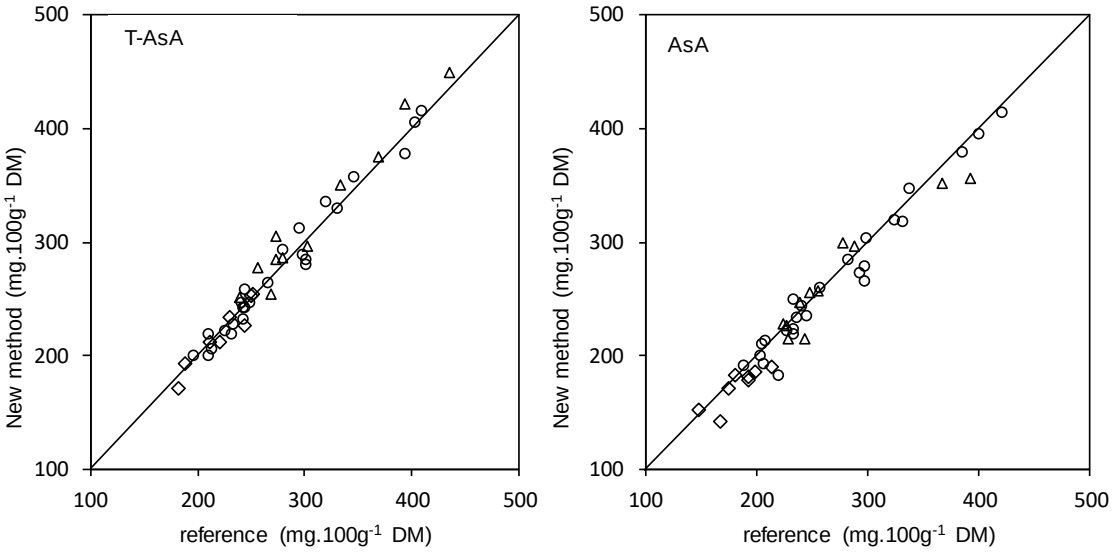

T-AsA
AsA
New method (mg.100g-1 DM)
reference (mg.100g-1 DM)
100
200
300
400
500

Figure 4.

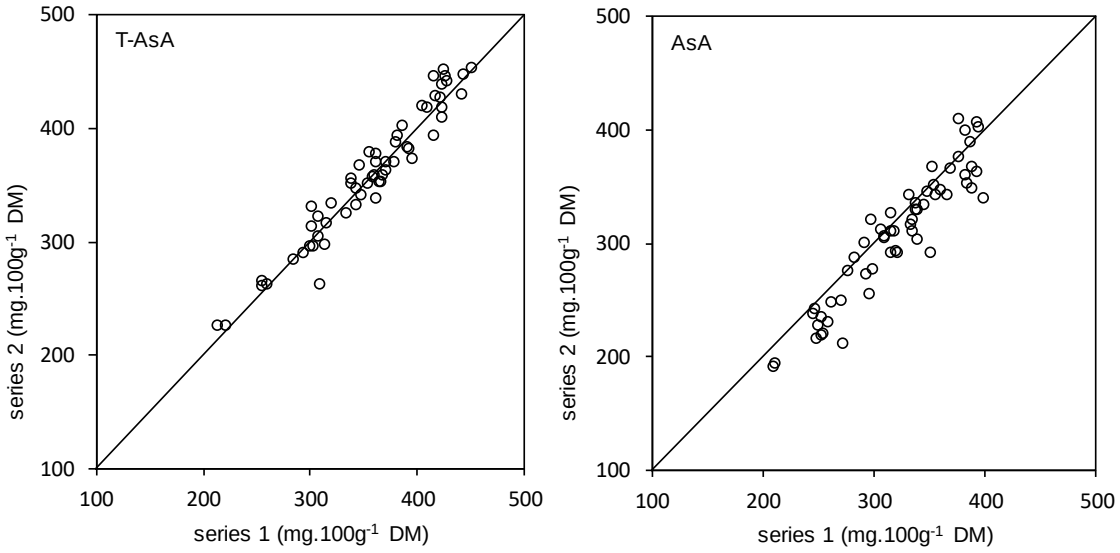
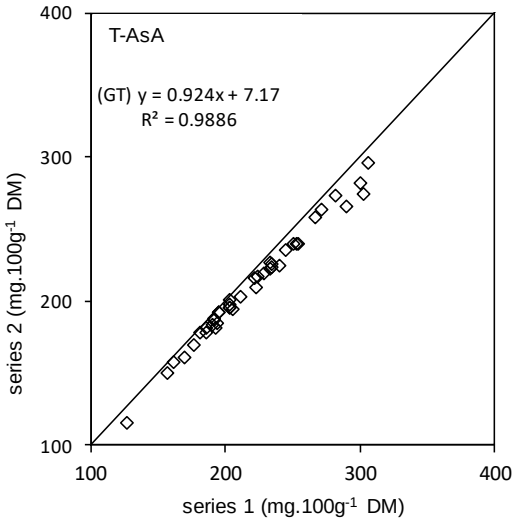
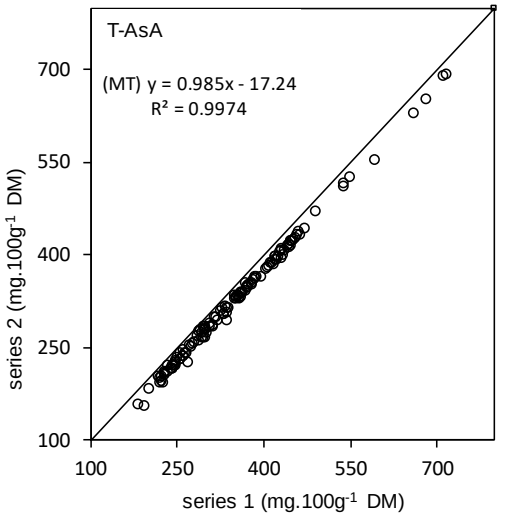

T-AsA
AsA
series 2 (mg.100g-1 DM)
series 1 (mg.100g-1 DM)
100
200
300
400
500

Figure 5.


T-AsA
(GT) y = 0.924x + 7.17
R² = 0.9886
series 2 (mg.100g-1 DM)
series 1 (mg.100g-1 DM)
100
200
300
400


T-AsA
(MT) y = 0.985x - 17.24
R² = 0.9974
series 2 (mg.100g-1 DM)
series 1 (mg.100g-1 DM)
100
250
400
550
700

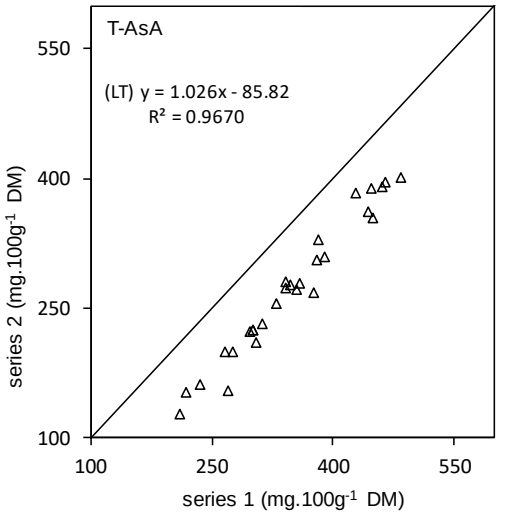

T-AsA
(LT) y = 1.026x - 85.82
R² = 0.9670
series 2 (mg.100g-1 DM)
series 1 (mg.100g-1 DM)
100
250
400
550

Figure 6.

Table 1. Summary of statistical analysis results

Figure	Vitamin C	No.	*p*-value Wilcoxon's test	ρ Spearman's test	Reproducibility test (mg $100g^{-1}$)	RRMSE	
						Series 1	Series 2
2	T-AsA	44	0.4484	0.9790	21.63	0.0393	0.0394
2	AsA	44	0.0409	0.9862	18.61	0.0376	0.0381
3b	T-AsA	20	0.9273	0.9774			
3b	AsA	20	0.0484	0.9714			
4	T-AsA	44	0.2531	0.9758			
4	AsA	44	0.0154	0.9711			

Graphical Abstract