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Review

Determination of vitamin C in foods: Current state of method validation

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ABSTRACT

Vitamin C is one of the most important vitamins, so reliable information about its content in foodstuffs is a concern to both consumers and quality control agencies. However, the heterogeneity of food matrixes and the potential degradation of this vitamin during its analysis create enormous challenges. This review addresses the development and validation of high-performance liquid chromatography methods for vitamin C analysis in food commodities, during the period 2000–2014. The main characteristics of vitamin C are mentioned, along with the strategies adopted by most authors during sample preparation (freezing and acidification) to avoid vitamin oxidation. After that, the advantages and handicaps of different analytical methods are discussed. Finally, the main aspects concerning method validation for vitamin C analysis are critically discussed. Parameters such as selectivity, linearity, limit of quantification, and accuracy were studied by most authors. Recovery experiments during accuracy evaluation were in general satisfactory, with usual values between 81 and 109%. However, few methods considered vitamin C stability during the analytical process, and the study of the precision was not always clear or complete. Potential future improvements regarding proper method validation are indicated to conclude this review.

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1. Introduction

Food analysis represents an important research field in current chemistry, mainly due to the continuous appearance of new foods, supplements, nutraceuticals, etc., which makes the availability of fully validated methods of analysis an important issue. Not only is food composition of paramount importance for quality control purposes, but also for the consumers, who are more aware than ever of the benefits of the different commercial products. Some of the main beneficial components that consumers look for in new food products are vitamins and antioxidants.

In recent years there has been a major consumers' concern regarding the nutritional quality of foods. In the case of vitamin C, this concern is common to both consumers and food manufacturers, since this nutrient is among the most sensitive to processing conditions, and its degradation during the storage process depends on several factors. Considering the potential oxidation of vitamin C, and the high heterogeneity of the food samples, special emphasis has to be paid to the development of analytical methods (storage of samples, sample preparation, etc.) for vitamin C quantification.

Method validation is a fundamental pre-requisite to evaluate the ability of analytical methods to generate reliable analytical data for their routine application. The validation demonstrates that an analytic method measures the correct substance, in the correct amount, and in the appropriate range for the intended samples [1–3]. Without results of adequate quality or reliability, the data obtained in scientific work are untrustworthy [4,5]. Therefore, not only the development and optimization of an analytical method are important, but also its proper validation.

This review focuses on the current state of HPLC methods for the determination of vitamin C in food. The data for this review were collected from the Scopus database, in the period of time 2000–2014. After a brief summary of vitamin C characteristics, a critical discussion is presented regarding method development and optimization and, especially, method validation. The approaches used by different authors are discussed, mentioning the most important aspects to consider, the advantages and disadvantages of the analytical methods currently available, and future trends in vitamin C quantification and validation.

2. Vitamin C characteristics

Vitamin C refers to all compounds exhibiting equivalent biological activity to L-ascorbic acid (L-AA), including its oxidation products (dehydroascorbic acid, DHAA), isomers (isoascorbic acid, IAA), esters (ascorbyl palmitate), and synthetic forms (6-deoxy-L-AA, 2-phosphate-L-AA). It is one of the most important water-soluble vitamins for human health, known by its high antioxidant activity. It participates in many biochemical functions, such as the absorption of iron, the synthesis of collagen and hormones (enzyme cofactor), and the neutralization of free-radicals resulting from cellular metabolism (antioxidant). It prevents the appearance of aging, cataract formation, arteriosclerosis, cancer, and cardiovascular diseases [6–8]. It is also applied by the food industry as an additive, preventing the oxidation of food products. Physical properties of L-AA are given in Table S1.

Supplementary Table S1 related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.chroma.2014.09.087.

L-AA is naturally present in most fruits and vegetables, but humans are unable to synthesize it. The Recommended Dietary Allowances (RDA) are 75 and 90 mg/day for adult women and men, respectively [9,10]. L-AA is rapidly oxidized to DHAA, and its oxidation can be induced by exposure to high temperatures and pH, light, presence of oxygen or metals (Fe^{3+}, Ag^{+}, Cu^{2+}), and enzymatic action. DHAA exhibits equivalent biological activity to L-AA, so it is important to measure both molecules to know the total ascorbic acid (TAA) or total vitamin C content in foodstuffs. The equilibrium between L-AA and DHAA is dependent on the sample pre-harvest (origin, cultivation practices, maturity stage) and post-harvest conditions (sample handling and storage). DHAA represents less than 10% of TAA in fresh horticultural products, but its content tends to increase during storage. The main goal of an analytical method is to measure the actual contents of the sample without artificially shifting the equilibrium of the two molecules [11]. Therefore, care must be exercised throughout the extraction and analysis to prevent degradation and loss of L-AA [8,12]. Irreversible hydrolysis of DHAA produces the biologically inactive 2,3-diketo-L-gulonic acid (DKGA), followed by its degradation to other by-products, including oxalic acid, L-threonic acid, CO_2, L-xylonic acid, and L-xylose. Some reducing agents can convert DHAA back to L-AA in vivo (glutathione dehydrogenase) and in vitro systems (homocystein; DL-1,4-dithiothreitol, DTT; dimercaptopropanol, BAL; and *tris*(2-carboxyethyl)phosphine, TCEP) [6,7,9].

Considering the potential degradation of L-AA depending on the storage, sample preparation, and extraction conditions, it is important to carefully develop and validate the analytical methods used for vitamin C determination in food samples, so the obtained results are reliable.

3. Method development

Method development and method validation are continuous and close processes that are conducted throughout food analysis [3,13]. The implementation of an analytical method in the laboratory undergoes several closely related phases aimed at ensuring the credibility of the generated data (Fig. S1).

Supplementary Fig. S1 related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.chroma.2014.09.087.

The choice of the analytical method should take into account the characteristics of the analyte, the matrix, the purpose of the analysis, and the available analytical resources [3,14]. The three critical components of an HPLC method are sample preparation, HPLC analysis conditions, and standardization. Good reviews on vitamin C determination are available in the scientific literature [6–9,15,16], so only the most important aspects will be covered here.

3.1. Sample preparation

Sample preparation affects nearly all the later assay steps, and is hence critical for unequivocal identification, confirmation and quantification of analytes [1,17,18]. L-AA may be easily oxidized, so optimization of sample extraction when analyzing L-AA in complex samples is very important [9,10]. Careful attention must also be paid to sample handling procedures before the analysis, otherwise L-AA content may not reflect the concentration in food as consumed [12]. The presence of interferences in the matrix can affect the detection/quantification process, and even contribute to L-AA degradation [7,10]. Processing methods involving heat and water, such as canning and bleaching, produce large losses of vitamin C, in the range of 20–60% and 65–90%, respectively, depending on time, methodology, and food matrix [19].

First of all, the storage of the samples previous to the analysis is a key aspect. Fruits and vegetables show a gradual decrease in L-AA content as the storage temperature and/or duration increases. Minimum L-AA losses have been reported in cruciferous vegetables, whereas other vegetables showed large losses during storage [11]. Daily L-AA losses between 2 and 7% were observed in different fruits and vegetables when stored in the refrigerator for 5 consecutive

days, being the results highly dependent on the particular type of sample [20].

In general, it is advisable to use fresh products instead of frozen ones for L-AA analysis, since L-AA stability usually decreases during freezing or freeze-drying processes. Losses of L-AA between 8 and 10% were reported in lyophilized red tomatoes [21]. Garrido-Frenich et al. [22] observed that L-AA content was higher when samples were prepared and analyzed in the same day than when frozen vegetables samples were used (losses of approximately 50%). Rizzolo et al. [23] minimized the oxidative action of enzymes in fresh fruits by avoiding excessive cutting of samples, and by freezing them in liquid nitrogen and/or storing them at low temperatures (−80 °C) prior to extraction. However, other authors did not observe significant differences in L-AA contents in fresh and freeze-dried fruits [24,25]. The influence of different drying methods (air-, oven-, and vacuum-drying) on vitamin C concentration in apples was recently reported [26]. In general, vitamin C losses during the entire freezing process range between 10 and 80% (with averages around 50%).

Other aspect to consider during sample preparation is the acidification of the samples, since freezing increases the stability of L-AA, but does not fully prevent its degradation. The acidification should be done promptly after sampling [8,15]. Meta-phosphoric acid (MPA) is the most common reagent, fulfilling the roles of extractant and stabilizer, inhibiting ascorbate oxidase and metals catalysis, and precipitating proteins, a process that aids in extract clarification. MPA can also be combined with other acids and/or organic modifiers (acetic acid, methanol, acetonitrile, trichloroacetic, citric acids) and stabilizers (ethylenediaminetetraacetic acid (EDTA), monosodium glutamate (MSG)) to increase stabilization [8,9,15]. Extractants that usually limit L-AA oxidation to less than 5% include 3–6% MPA containing acetic or sulfuric acid or 0.005 M EDTA [9].

Hernández et al. [10] compared the efficacy of two extractant solutions and concluded that MPA was more effective than oxalic acid in the stabilization of L-AA, providing better recovery rates. Chebrolu et al. [27] conducted a similar experiment concluding that L-AA was more stable in MPA, since trichloroacetic acid (TCA) extracts showed degradation in 48 h. Failure to assess stability of L-AA in raw products during sample processing and analysis could result in significant errors in analytical results [12], not reflecting the real concentration in food as consumed.

The adoption of quick and simple extraction procedures is important to ensure the efficiency of the extraction [8,9,15,27]. For the analysis of liquid samples, their direct injection in the HPLC after dilution with the initial mobile phase is the most common procedure. For solid samples, solid–liquid extraction (SLE) is normally used. In this case, samples are thoroughly homogenized with the extractant, centrifuged or filtered, and injected in the HPLC after dilution with the initial mobile phase.

3.2. HPLC methods for vitamin C quantification

Many analytical methods, including chromatography, have been reported to measure vitamin C content in foods [7,9,16]. Despite its limitations, classical methods are still common in food analysis. AOAC Official titration method (967.21/2006) is routinely applied for the analysis of fruits and juices, due to its simplicity and low cost [9,10,28]. Comparisons between HPLC and traditional methods have been previously reported [10,29–31]. Even though similar results were observed for several samples, HPLC methods are necessary for the analysis of many food samples due to their high complexity, which demands high selectivity and sensitivity. In addition, only L-AA can be determined by the traditional iodometric titration, whereas L-AA and DHAA can be quantified with appropriate HPLC methods. Different HPLC methods are shown in Table 1 for vitamin C determination, all of them published between 2000 and 2014 in the field of food chemistry, by chronological order. The validation parameters reported in each method are also shown in Table 1.

Because of the non-volatile and hydrophilic nature of vitamin C, reversed-phase (RP)-HPLC is the most common approach [7–9], although ion exchange, ion-pair, ion-exclusion, and hydrophilic interaction liquid chromatography (HILIC) have also been reported (Table 1). Ultra-high performance liquid chromatography (UHPLC) has recently been applied to the analysis of vitamin C in foods [20], observing as key advantages the shorter time of analysis and the much lower solvent consumption when compared to other analytical approaches.

The pH of the mobile phase is usually adjusted below L-AA's pK_a (4.17) to prevent its degradation. Additional information on vitamin C analysis is available in other reviews [7,9,71].

L-AA presents a strong absorption in the ultra-violet region (245–270 nm), making UV absorbance the most popular detection technique [7,9,15]; however, this absorbance is strongly dependent on the pH (Table S1) and this aspect has to be taken into account. Less frequently, fluorescence detection (FD) and electrochemical detection (ECD) have been used [7,8], as well as mass-spectrometry (MS) [7,22,53,65,72–74]. The limits of detection (LODs) ranged between 0.02 and 0.16 μg/mL for ECD, between 1.2×10^{-3} and 7.2 μg/mL for UV, and approximately 0.27 μg/mL for fluorescence detection (Table 1). Usually, ECD is more sensitive than UV, although the wide ranges of LODs make it difficult to perform an accurate comparison. Fluorescence is also more sensitive than UV, although the derivatization procedure required makes it more tedious than UV.

The low levels of DHAA in most samples difficult its quantitative analysis with any HPLC detector. Therefore, DHAA is often reduced to L-AA before its chromatographic separation, and it is measured as TAA, i.e., the sum of L-AA and DHAA contents [7,8]. This procedure demands two chromatographic runs, and DHAA is then calculated by the subtraction of L-AA from TAA [7].

4. Method validation

Once a method has been optimized, it has to be validated to ensure its suitability for the desired applications [2,3]. A number of guidance documents on method validation have been issued by various international reputable organizations and conferences, guidelines that are described in different scientific works [1,4,5,75]. Examples of these associations are AOAC (Association of Official Analytical Chemists), FDA (Food and Drug Administration), FAO (Food and Agricultural Organization/World Health), EURACHEM (A Focus for Analytical Chemistry in Europe), ICH (International Conference on Harmonization), IUPAC (International Union of Pure and Applied Chemistry), and the European Commission, among others [4,13,76–78]. Unfortunately, there is still no single source or final guideline on analytical method validation [76,79].

For the validation of a quantitative analytical procedure, there is a set of key elements that are generally accepted: selectivity, linearity, stability, accuracy, precision, and the lower limit of quantification. Additional parameters which may be relevant include LOD, reproducibility, robustness, and ruggedness [2,4,5]. For analytical methods using LC–MS, the assessment of possible matrix effects (ME) should always be part of the validation process, particularly if electrospray ionization (ESI) is used [5]. There are already good reviews covering in detail the different definitions for each validation parameter [1,75], so this review will focus in the parameters that are usually validated in HPLC methods for vitamin C determination in food samples.

A proper validation of an analytical method not only has to be adequate, but also clear to all the scientific community. Hence, it

Table 1
Overview of validated chromatographic methods for vitamin C determination in foodstuffs.

Analyte	Sample	Sample preparation	HPLC conditions	Method validation	Ref.
L-AA, DHAA, TAA (reduction with DTT)	Green beans	SLE with 4.5% MPA; filtration	Sphereclone ODS (250 mm × 4.6 mm, 5 μm) 1.8 mM H_2SO_4 (pH 2.6) UV (245 nm)	Selectivity: t_R and UV spectra comparison R^2 > 0.9993; 5–20 μg/mL (ES) LOD: 0.097 μg/mL; LOQ: 32.3 μg/mL (*S*/*N*) Repeat: 6.4% L-AA; 7.1% TAA Accuracy: 93.3% L-AA; 89.4% TAA (recovery tests)	[30]
L-AA, DHAA (reduction with DTT)	Fruits and vegetables	SLE with H_2O; filtration	HiChrom C18 (250 mm × 4.0 mm, 5 μm) M KH_2PO_4 (pH 2.4) UV (254 nm)	R^2 = 0.9992; 10–100 μg/mL (ES) Int. precision < 1% (t_R) Accuracy: 81.7–105.9% (recovery tests)	[32]
TAA (reduction with DTT)	Fruit drinks	Dilution with water and centrifugation	JÕsphereÒ ODS-H80 (250 mm × 4.6 mm, 4 μm) 2% (v/v) acetic acid (pH 2.5) UV (243 nm)	Selectivity: t_R and UV spectra comparison R^2: 0.9998; 20–2000 μg/mL (ES) LOD: 0.5 μg/mL (*S*/*N*) Repeat: 1%	[33]
L-AA, β-carotene	Beverages	Dilution with mobile phase	Kromasil NH_2 (250 mm × 4.6 mm, 5 μm) M Acetic acid in water UV (250 nm)	Selectivity: t_R and UV spectra comparison R^2 = 0.9997; 4–600 μg/mL (ES) LOD 1.2 μg/mL; LOQ 4.0 μg/mL (*S*/*N*) RSD: 2.1%	[34]
L-AA	Supplements, juices, teas, vinegar, honey	Dilution with mobile phase + 20 μM L^{-1} methionine	Inertsil ODS-3 (150 mm × 4.6 mm, 5 μm) 0.2% phosphoric acid (pH 2.1) ECD (Ag/AgCl 400 mV)	0.02–2.3 μg/mL (ES) LOD: 0.02 μg/mL (*S*/*N*) Repeat: 2.8%; int. precision: 3.7% Accuracy ≥ 90% (recovery tests) Stability[2]: pH, temperature, light, concentration, metallic ions, stabilizing agents	[35]
L-AA, other water-and fat-soluble vitamins	Powdered drinks and foods	Dilution with mobile phase and centrifugation	MetaChem Polaris C18-A (150 mm × 4.6 mm, 3 μm) 0.010% TFA: MeOH (50:50) DAD (280 nm); ESI-MS	R^2 = 0.9986; 10–1000 μg/mL (ES) LOD: 0.025 μg/mL; LOQ: 0.085 μg/mL (*S*/*N*) Precision: 2.1% Robustness: % TFA and pH of mobile phase, HPLC gradient and temperature,	[36]
L-AA	Fruits and vegetables	SLE with 10% acetic acid–5% MPA–1.5 M H_2SO_4–1 mM EDTA	Hydrobond AQ C8 (3 mm × 100 mm, 5 μm) 1 mM KH_2PO_4, 1 mM Na_2 EDTA (pH 3.0) ECD (250 mV); UV (245 nm)	Selectivity: t_R and UV spectra comparison R^2 > 0.9700; 2–20 μg/mL (ES) LOD: 0.002 μg/g ECD; 0.012 μg/g UV (*S*/*N*) LOQ: 0.3 μg/g ECD; 1.8 μg/g UV (*S*/*N*) Repeat: 5%; int. precision: 7% Accuracy: 95–107% (recovery tests) Robustness: different columns	[37]
L-AA (8 other water-soluble vitamins)	Polyvitaminated premixes	Dilution with water + 1 M phosphate buffer (pH 5.5)	YMC-Pack Pro C18 (250 mm × 4.6 mm, 5 μm); A: 0.025% TFA (pH 2.6), B: ACN (gradient elution) UV (275 nm)	Selectivity: t_R and UV spectra comparison R^2 = 0.9956; 5–200 μg/mL (ES) LOD: 0.2 μg/mL Repeat.: 2.1%; int. precision: 5.9%	[38]
L-AA, carotenoids	Fruits	SLE with MeOH + 3% MPA-8% acetic acid; filtration	Coupled Symmetry C18 (75 mm × 4.6 mm, 3.5 μm) & Atlantis C18 (150 mm × 2.0 mm, 5 μm) 70% MeOH: 0.05% CH_3COOH (70:30) ESI-MS	Selectivity: t_R and UV spectra comparison R^2 = 0.9956; 0.1–1 μg/mL (SA) LOD: 0.01 μg/mL; LOQ: 0.05 μg/mL (*S*/*N*) Repeat.: 8.7% Accuracy: 85% (recovery tests)	[22]
L-AA, TAA (reduction with DTT)	Human milk	Dilution with 0.56% MPA solution; centrifugation and filtration	Spherisorb ODS2 C18 (250 mm × 4.6 mm, 5 μm) 0.1% Acetic acid: MeOH (95:5) UV (254 nm)	Selectivity: t_R and UV spectra comparison R^2 = 0.9990; 0.5–100 μg/mL (ES) LOD: 0.003 μg/mL; LOQ: 0.009 μg/mL (*S*/*N*) Repeat.[a]: 3.09% AA; 2.54% TAA Int. precision[a]: 4.03% AA; 3.63% TAA Accuracy: 95.6% AA; 95.1% TAA (recovery tests)	[31]
L-AA	Wines	Direct injection without dilution	PLRP-S 100A (150 mm × 4.6 mm, 5 μm) A: TFA: H_2O (99:1), B: ACN–solvent A (80:20) (gradient elution) UV (243 nm)	Selectivity: t_R and UV spectra comparison R^2 = 0.9990; 1–200 μg/mL (ES) LOD: 1 μg/mL; LOQ: 5 μg/mL (visual approach) Repeat.[a]: 1.5–2.2% Accuracy > 92% (recovery tests)	[39]
L-AA, DHAA, TAA (reduction with DTT)	Tropical fruits	SLE with 3% MPA-8% acetic acid; centrifugation	Shodex RSpak KC-811 (250 mm × 4.6 mm, 5 μm) 0.2% *o*-phosphoric acid UV (245 nm)	Selectivity: t_R and UV spectra comparison R^2 = 0.9990; 0.5–50 μg/mL (ES) LOD: 0.10 μg/mL (*S*/*N*) Repeat.[a]: 5.9–11.9%; int. precision[a]: 8.9–12.8% Accuracy: 93–104% (recovery tests) Stability[c]: temperature, oxidative agents, thawing	[10]
L-AA, vitamin A	Banana and papaya	SLE (3% MPA-8% CH_3COOH-1 mM EDTA); centrifugation; clean-up (C-18 cartridges)	PLRP-S column (250 mm × 2.1 mm, 5 μm) 0.2 M NaH_2PO_4 (pH 2.14) DAD (254 nm)	Selectivity: t_R and UV spectra comparison ES (25–100 μg/mL) LOD: 0.05 μg/mL (*S*/*N*) Accuracy: 95% (recovery tests)	[40]

Table 1 (*Continued*)

Analyte	Sample	Sample preparation	HPLC conditions	Method validation	Ref.
TAA, iso-AA (reduction with TCEP)	Fortified products: infant formulas, cereals, soup, juice, compotes	Dilution with mobile phase; filtration	Ion-pair LiChrospher RP-18 (250 mm × 4.6 mm, 5 μm) ACN, sodium acetate eluent (pH 5.4), TCEP, and decylamine DAD (265 nm)	Selectivity: t_R and UV spectra comparison R^2 = 0.9999; 1–100 μg/mL (ES) LOD: 1 μg/g; LOQ: 3 μg/g (*S/N*) Repeat.: 0.8–4.6%; reproducibility: 2–8% (CRMs) Accuracy: 93–105% (CRMs)	[41]
L-AA, DHAA, TAA (reduction with DTT or BAL)	Fruits	SLE with 4.5% MPA; centrifugation and filtration	(a) C18 Spherisorb ODS2 (250 mm × 4.6 mm, 5 μm), 0.01% H_2SO_4 (pH 2.6) (b) NH_2 Spherisorb S5 (250 mm × 4.6 mm, 5 μm) 10 mM KH_2PO_4 buffer (pH 3.5): ACN (60:40) UV (245 nm)	$R^2 \geq 0.9936$; 5–50 μg/g (ES; statistics) LOD < 1.8 μg/g; LOQ < 6.1 μg/g (SDRS) Precision[a]: 0.6–3.9% Accuracy: 93.6–104.4% (recovery tests)	[42]
L-AA, iso-L-AA, L-AA-2G, L-AA-2βG	(a) Teas (b) Dried fruits	(a) Dilution with mobile phase (b) USLE; centrifugation	HILIC Interstil Diol (250 mm × 4.6 mm, 5 μm) ACN: 66.7 mM CH_3COONH_4 (85:15) UV (260 nm)	Selectivity: t_R and UV spectra comparison R^2: 0.9996; 1–50 μg/mL (ES) LOD: 0.3 μg/mL (*S/N*) Repeat.: 0.5–2.8%; int. precision:0.5–2.1% Accuracy: 92% (recovery tests) Stability[c]: temperature, concentration	[43]
L-AA, DHAA, TAA (derivatization with OPDA)	(a) Milk (b) Fruit juices (c) Fruits and vegetables	(a) Protein precipitation with TCA; centrifugation and filtration (b) Dilution (1% MPA) (c) SLE with 1% MPA; centrifugation	NovaPack C18 (150 mm × 3.9 mm, 4 μm) 80 mM phosphate buffer (pH 7.8): MeOH (84:16) FD (λ_{ex} = 355 nm, λ_{em} = 425 nm)	Selectivity: t_R and UV spectra comparison R^2: 0.9997; 0.5–8 μg/mL (ES) LOD: 0.27 μg/mL; LOQ < 0.83 μg/mL (*S/N*) Repeat. ≤ 5.4%	[44]
L-AA IS = chlorogenic acid	Tablets	Dilution with mobile phase	ZIC-HILLIC (150 mm × 2.1 mm, 3.5 μm) ACN: 50 mM ammonium acetate buffer (pH 6.8) (78:22) DAD (268 nm)	Selectivity: t_R and UV spectra comparison R^2: 0.9995; 0.1–100 μg/mL Repeat.: 3.04–3.72% Accuracy: 97.8–104.5% Stability[b]: light, temperature, pH, reducing agent, concentration	[45]
L-AA	Fruit juices and soft drinks	Dilution with mobile phase; filtration	Tr-010065 Mediterranea sea18 (15 cm × 0.4 cm, 3 μm) 0.1% (v/v) formic acid UV (245 nm)	Selectivity: t_R and UV spectra comparison $R^2 \geq 0.9991$; 0.2–20 μg/mL; 20–400 μg/mL (ES) LOD: 0.01 μg/mL (*S/N*) Repeat.: 0.4%; int. precision < 2% Stability[c]: temperature	[46]
L-AA, DHAA, TAA (reduction with DTT)	Vegetables	SLE with 3% MPA–8% acetic acid–0.15 M H_2SO_4–1 mM EDTA; filtration and centrifugation	Lichospher 100 RP18 (250 mm × 4 mm, 5 μm) 1 mM KH_2PO_4, 1 mM EDTA (pH 3.0) UV (245 nm)	Selectivity: t_R and UV spectra comparison R^2: 0.9996; 16.5–206.1 μg/mL (ES) LOD: 0.05 μg/mL; LOQ: 4.73 ug/mL (*S/N*) Repeat.: 1.70% Accuracy: 83–99% (recovery tests)	[47]
L-AA, DHAA, TAA (reduction with TCEP)	Grapevines (berries, rachis, leaves, roots)	SLE with 3% MPA–1 mM EDTA; centrifugation	Synergi Fusion (150 mm × 4.6 mm 4 μm) A: 25 mM KH_2PO_4–0.1 mM EDTA (pH 2.5); B: 100% MeOH (gradient elution) UV (245 nm)	Selectivity: ascorbate oxidase R^2: 0.9993; 0.002–0.5 μg/mL (ES) Repeat.: 7.6% L-AA; 5.4% TAA Accuracy: 73% L-AA; 108% TAA (recovery tests) Stability[c]: freeze-thawing	[48]
L-AA and gallic acid	Indian gooseberry juice	Dilution with MeOH:H_2O (70:30, v/v)	Zorbax SB RP-C18 (250 mm × 4.6 mm, 5-μm) A: 0.1% (v/v) acetic acid B: MeOH DAD (278 nm)	Selectivity: t_R and UV spectra comparison R^2: 0.9998; 30–90 μg/mL (ES) LOD: 1.42 μg/mL; LOQ: 4.73 μg/mL (*S/N*) Repeat.: 1.04%; int. precision: 1.48% Accuracy: 99.37% (recovery tests)	[49]
L-AA	Fruit juices	Filtration, centrifugation, and dilution with mobile phase	Hypersil Gold (250 mm × 4.6 mm, 5 μm) KH_2PO_4 buffer (pH 2.8) UV (254 nm)	Selectivity: t_R and UV spectra comparison R^2: 0.9990; 1–300 μg/mL (ES) LOD: 0.5 μg/mL (*S/N*) Repeat. < 2% Accuracy: 95.8–102.1% (recovery tests)	[50]
L-AA, iso-AA	(a) Fruit juices (b) Chestnut, ham	(a) Dilution with 6.25% MPA–2.5 mM TCEP–2.5 mM EDTA (b) SLE with 5% MPA–2 mM TCEP–2 mM EDTA; centrifugation	TSKgel Amide-80 (4.6 mm × 100 mm, 5 μm) ACN: 0.1% TFA (90:10) UV (244 nm)	Selectivity: t_R and UV spectra comparison R^2 > 0.9989; 0.5–25 μg/mL LOQ: 1.5 μg/mL; 3.7 μg/mL (*S/N*) Repeat: 1.43–1.92% (L-AA); 2.59–3.01% (iso-AA) (Horwitz criteria) Accuracy: 96.41–103.49% (recovery tests) Robustness: % organic in mobile phase; HPLC flow rate; column temperature Stability: concentration, temperature	[51]
L-AA, B1, B2, B3, B6	Cereals, cacao powder and fruit juice	SLE with 2% MPA, US, centrifugation, filtration	Restek Ultra Aqueous C18 column (150 mm × 3.2 mm, 5 μm) A: 0.1% (v/v) formic acid in water (pH = 2.55) B: 0.1% (v/v) formic acid in methanol UV: 266 nm	Selectivity: t_R and UV spectra comparison R^2 = 1 (0.002–200 μg/mL) (ES) LOD: 0.006 μg/mL; LOQ[d]: 0.06 μg/mL Repeat.: 0.2–1.9% Accuracy: 90–100% (recovery tests)	[52]

Table 1 (*Continued*)

Analyte	Sample	Sample preparation	HPLC conditions	Method validation	Ref.
L-AA, DHAA	Fruits and vegetables	SLE with 0.05% EDTA; centrifugation; clean-up with C18 cartridges	Prontosil C18 (250 mm × 3 mm; 3 μm) 0.2% (v/v) formic acid ESI-MS	Selectivity: t_R and UV spectra comparison $R^2 \geq 0.9970$ SA LOD: 0.013 μg/mL L-AA; 0.011 μg/mL DHAA (*S/N*) LOQ: 0.044 μg/mL L-AA; 0.038 μg/mL DHAA (*S/N*) Repeat.: 1.6–2.8% L-AA; 1.1–2.7% DHAA Accuracy: 81–109% (recovery tests); ME	[53]
L-AA and 3 other water-soluble vitamins	Honey	Dilution with phosphate buffer 1 M (pH = 5.5)	Alltima C18 (250 mm × 4.6 mm, 5 μm) A: 0.025% TFA; B: ACN (gradient elution) UV (254 nm)	Selectivity: t_R and UV spectra comparison R^2: 0.9989; 0.05–500 μg/mL (ES) LOD: 0.10 μg/g; LOQ: 0.30 μg/g (ULA) Repeat.[a]: 7.3%; int. precision[a]: 3.3% Accuracy: 104% (recovery tests)	[54]
L-AA	Health drinks	Dilution with 0.56% MPA; centrifugation and filtration	Symmetry C18 (250 mm × 4.6 mm, 0.5 μm) Acetic acid in water: methanol 95:5% (v/v) DAD (245 nm); ESI	R^2: 0.9979; 0.1–0.6 μg/mL LOD: 0.01 μg/mL; LOQ: 0.1 μg/mL (visual approach) Repeat.: 0.75–1.34%; int. precision: 0.78–1.25% Accuracy: 97.5–98.6% (recovery tests) Robustness: % MeOH in mobile phase Stability[c]: temperature	[55]
TAA (reduction with TCEP)	Fruits, vegetables, fruit juices and dried spices	LLE or USLE with 5% MPA-1 mMEDTA-5 mM TCEP; centrifugation and filtration	C18 Synergy Hydro RP (250 mm × 4.6 mm, 3 μm) A: 0.05% (w/v) formic acid B: 0.02% (w/v) *o*-phosphoric acid UV (254 nm)	Selectivity: CRMs ES (0.01–50 μg/mL) LOD: 0.6–0.9 μg/g; LOQ: 2 μg/g (*S/N*) Repeat.[a]: 0.8–3.6%; int. precision: 1.1–4.8% (CRMs) Accuracy: 97–103% (recovery tests)	[56]
L-AA, DHAA, TAA, other organic acids (reduction with L-cystein)	Vegetables	SLE with 4.5% MPA	Sphereclone ODS (250 mm × 4.6 mm, 5 μm) 1.8 mM H_2SO_4 (pH 2.6) UV (245 nm)	R^2: 0.9980; 100–2000 μg/g (ES) LOD: 0.8 μg/g (*S/N*) Precision < 5.3% (AOAC) Accuracy: 90.1–94.6% (recovery tests)	[57]
L-AA, DHAA, TAA, carotenoids	Fruits	SLE with 3% MPA-8% acetic acid–0.15 M H_2SO_4–1 mM EDTA; filtration and centrifugation	Lichospher 100 RP18 (250 mm × 4 mm, 5 μm) 1 mM NaH_2PO_4–1 mM EDTA (pH 3.0) UV (245 nm)	Selectivity: t_R and UV spectra comparison $R^2 \geq 0.9970$ (0.204–113.75 μg/mL) ES LOD: 0.050 μg/mL (*S/N*) LOQ: 0.075 μg/mL (*S/N*) Accuracy: 95.7–101% (recovery tests)	[58]
L-AA, DHAA, TA (reduction with TCEP, BME or DTT)	Fruits and vegetables	SLE with MPA (3 g/100 mL); centrifugation	Spherisorb C18 (150 mm × 4.6 mm, 3 μm) M dihydrogen ammonium phosphate (pH 2.6) PDA (254 nm); ESI-MS	Selectivity: t_R and UV spectra comparison R^2: 0.9995 (1.9–62.5 μg/mL) ES (statistical analysis) Precision ≤ 0.003% Accuracy: 89.9–113.3% (recovery tests) Stability[c]: reducing agents, pH, temperature	[27]
L-AA and other organic acids	Fruit juices	Dilution with mobile phase	RP-C18 (150 mm × 4.6 mm, 3 μm) M KH_2PO_4 buffer solution (pH 2.60) DAD (250 nm)	Selectivity: t_R and UV spectra comparison R^2: 0.9998; 0.00001–0.008 μg/mL (ES) LOD: 0.03 μg/mL; LOQ: 0.10 μg/mL (*S/N*) Instr. precision[a]: 1.67–2.04%; int. precision: 2.50–5.0% Accuracy: 82–110% (recovery tests) Stability[c]: temperature	[59]
L-AA	Beverages	MEPS with methanol–water solution (10:90, v/v)	LiChrospher_100 RP-18e (250 mm × 4 mm, 5 μm) A: water acidified with acetic acid (pH 2.94); B: MeOH (80:20) UV: 265 nm	R^2 = 0.9994; 40–1000 μg/mL (ES) LOD: 7.2 μg/mL; LOQ: 24 μg/mL (*S/N*) Repeat. < 10% Accuracy: 97.46–106.88% (recovery tests) Stability: light, temperature, pH	[60]
L-AA, B1, B2, B3, B5, B6, B9, E and provitamin A	Vegetables	USLE with 10 mM CH_3COONH_4/MeOH (50:50, v/v) + 0.1% BHT; centrifugation; USLE with ethyl acetate + 0.1% BHT	ACE-100 C18 (100 mm × 2.1 mm, 3 μm) A: 10 mM CH_3COONH_4 (pH 4.5); B: MeOH with 0.1% CH_3COOH; C: MeOH with 0.3% CH_3COOH (gradient elution) ESI^-/MS	R^2 = 0.9930; 0.070–5.280 μg/mL (ES) LOD: 0.042 μg/mL; LOQ: 0.128 μg/mL (*S/N*) Repeat.: 5.6%; Int. precision: 6.3% Accuracy: 102.8% (recovery tests)	[61]
L-AA, DHAA, TAA (reduction with DTT)	Fruits and vegetables	SLE with 3% MPA–8% acetic acid–1 mM EDTA; centrifugation	Acquity HSS (100 mm × 2.1 mm, 1.8 μm) 0.1% formic acid in water (v/v) PDA (245 nm)	Selectivity: t_R and UV spectra comparison R^2: 0.9999; 0.05–2 μg/mL (ES) LOD: 0.022 μg/mL; LOQ: 0.067 μg/mL (SDRS) Repeat.: 0.9–3.9% Accuracy: 87.0–103.7% (recovery tests)	[20]

Table 1 (*Continued*)

Analyte	Sample	Sample preparation	HPLC conditions	Method validation	Ref.
L-AA, TAA (reduction with DTT)	Strawberries	USLE with 3% MPA–8% acetic acid; centrifugation	Phenomenex Gemini C18 (250 mm × 3 mm, 5 μm) M sodium acetate/acetic acid buffer, 5% MeOH UV (251 nm)	R^2: 0.9960 L-AA; 0.9980 TAA; 4×10^{-3} to 20×10^{-3} μg/mL (ES, statistics) LOD: 1.2×10^{-3} μg/mL L-AA; 8.8×10^{-4} μg/mL TAA (*S/N*) LOQ: 3.4×10^{-3} μg/mL L-AA; 2.5×10^{-3} μg/mL TAA (*S/N*) Repeat.: 1.5% L-AA 1.8% TAA Accuracy: 92.3–100.6% L-AA; 94.3–104.8% TAA (recovery tests) Robustness: % MeOH, pH and flow rate of mobile phase	[62]
L-AA and other 6 water-soluble vitamins	Honey	Dilution with H_2O	μBondapack C18 (150 mm × 3.9 mm, 10 μm) 0.01%/H_2SO_4, 0.01 M CTAB, 0.022% (v/v) MeOH (pH 2.8) UV (254 nm)	R^2: 0.9981; 0.11–276.70 μg/mL (ES) LOD: 0.64 μg/mL; LOQ: 2.13 μg/mL (*S/N*) Repeat.: 0.35%; int. precision: 3.76% Robustness: %MeOH, pH of mobile phase, temperature of column	[63]
L-AA, TAA (reduction with DTT)	CRMs (milk, nutritional formula, cereals)	USLE with 40% MPA; centrifugation	YMC C18 Pro (250 mm × 4.6 mm, 5 μm) A: 0.02 M KH_2PO_4 buffer (pH 3.1) B: ACN (gradient elution) UV (243 nm)	Selectivity: CRMs R^2: 0.9986; 1000–4000 μg/g (IS) LOD: 0.05 μg/g (*S/N*); LOQ: 0.7 μg/g (*S/N*) Repeat.: 2.7–6.5%; int. precision: 3.8–4.5% (CRMs) Accuracy: 93.7–106.4% (CRMs)	[64]
L-AA	Grapes	USLE with 96% acetic acid; USLE with 2% MPA; centrifugation	Kromasil C18 (100 mm × 2.1 mm, 3.5 μm) 0.1% (v/v) acetic acid + MeOH UV (245 nm) ESI-MS	Selectivity: t_R and UV spectra comparison R^2: 0.9990; 0.5–15 μg/mL (ES) LOD: 0.32 μg/mL (*S/N*) Repeat.: 2.3–4.2% Accuracy: 96.9–102.4% (recovery tests)	[65]
L-AA and organic acids	Citrines juices	Dilution with H_2O; filtration and centrifugation	ZirChrom-SAX (150 mm × 4.6 mm; 5 μm) 50 mM ammonium dihydrogen phosphate (pH 5.8) PDA (254 nm)	Selectivity: t_R and UV spectra comparison $R^2 \geq 0.9990$; 32–252 μg/mL (ES) LOD: 3 μg/mL; LOQ: 9 μg/mL (*S/N*) Repeat.: 3.6% Accuracy: 105% (recovery tests)	[66]
L-AA	Peppers	USLE with 3% MPA–EtOH (2:8); centrifugation	C18 Gemini (250 mm × 4.6 mm, 5 μm) A: 0.03 M phosphoric acid; B: MeOH (gradient elution) UV (254 nm)	Selectivity: t_R and UV spectra comparison R^2: 0.9981; 3.91–62.50 μg/mL (ES) LOD: 0.26 ug/mL (*S/N*) Repeat.: 0.86% (t_R); int. precision: 2.93% (t_R) Accuracy: 97.1–98.8% (recovery tests)	[67]
L-AA, TAA (reduction with TCEP)	Exotic fruits, juices and fruits' pulp	Dilution with 10% PCA-1% MPA + TCEP; filtration	Synergi Hydro-RP (150 mm × 4.6 mm, 4 μm) 20 mM $NH_4H_2PO_4$ (pH 3.5) + 0.015% (w/v) MPA PDA (246 nm)	Selectivity: t_R and UV spectra comparison R^2: 0.9995; 1–100 μg/mL (ES; statistics) LOD: 0.035 μg/mL; LOQ: 0.09 μg/mL (*S/N*) Repeat.: 0.43–0.70%; int. precision: 3.67% Reproducibility[a] < 2 Accuracy: 96.6–97.3% (recovery tests); ME Robustness: pH and flow of mobile phase, temperature Stability[c]: temperature	[68]
L-AA, vitamins B_1, B_2, B_3, B_6 and B_9	Breakfast cereals	SLE with 25:75 (v:v) MeOH: 0.3% MPA (200 mg L^{-1} DTT); centrifugation; filtration	ZORBAX HILIC Plus (100 mm × 4.6 mm, 3.5 μm) A: 10 mM CH_3COONH_4 (pH 5.0) in H_2O:ACN (95:5), B: 10 mM CH_3COONH_4 (pH 5.0) in ACN:H_2O (95:5) (gradient elution) ECD (30 mV)	Selectivity: t_R and UV spectra comparison R^2 > 0.9980; 0.16–10 μg/mL (ES) LOD: 0.16 μg/mL; LOQ: 0.55 μg/mL (*S/N*) Repeat.: 1.2% Int. Precision: 5.5%	[69]
L-AA, phenolic acids and flavonoids	Indian gooseberry (*Emblica officinalis*) juice	(a) Dilution, centrifugation, filtration (b) Thermal treatment (c) Pulsed electric field	Zorbax SB RP C-18 (250 mm × 4.6 mm; 5 mm) A: 0.1% *o*-phosphoric acid B: ACN (gradient elution) PDA: 254 nm	Selectivity: t_R and UV spectra comparison R^2 = 0.9930–0.9950 (ES) LOD: 0.129 μg/mL; LOQ: 0.430 μg/mL (*S/N*) Repeat.: 2.64% Accuracy: 99.04% (recovery tests)	[70]

[a]Using Horwitz ratio, [b]using standard solutions; [c]using real samples; [d]LOQ defined as the lowest concentration of L-AA where RSD < 10%; int. precision = intermediate precision; repeat. = repeatability; ACN = acetonitrile; MEPS = microextraction by packed sorbent; USLE = ultrasound assisted solid–liquid extraction; TFA = trifluoroacetic acid; PCA = perchloric acid; TCEP = Tris-[2-carboxyethyl] phosphine hydrochloride (TCEP·HCl); CTAB= hexadecyltrimethylammonium bromide; instr. precision = instrumental precision; precision = includes both repeatability and intermediate precision. All PCA solutions are expressed in % (v:v). All MPA solutions are expressed in % (w:v); they are all prepared in H_2O. RSD = authors do not mention which kind of precision is studied.

is important to mention which guideline has been adopted for the validation, so the method can be easily implemented in other laboratories. Few of the reviewed methods were validated according to official guidelines [31,46,49,54,55,57,63,68,73]. Most of the publications did not mention which guidelines were adopted; instead, the authors used the information provided by some reviews about method validation [2,4,5]. Finally, three of the publications [80–82] did not present any information about method validation at all.

4.1. Selectivity

The most important goal of an analytical method is to obtain a signal free from the influence of other species contained in the

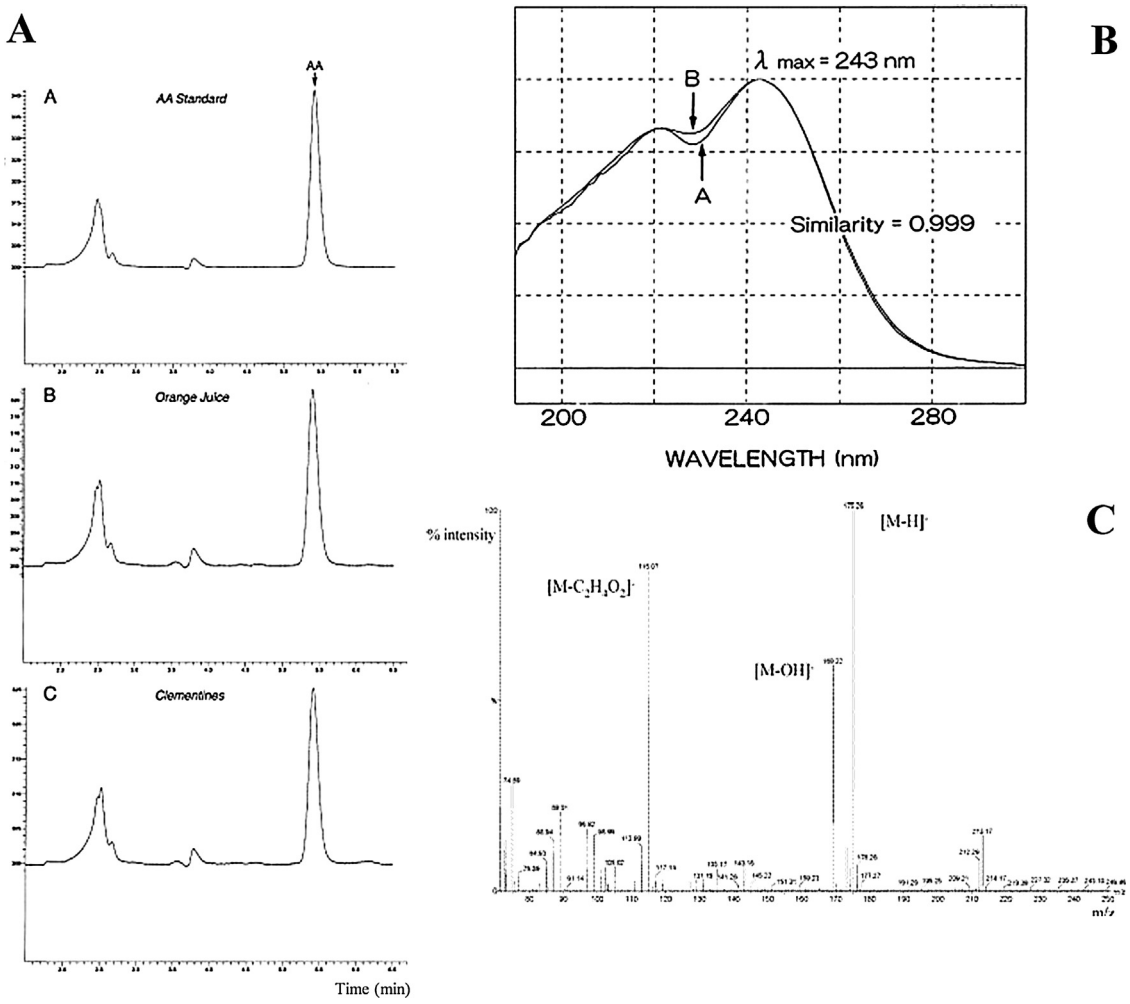

Fig. 1. (A) Representative chromatograms for L-AA in standard solution, in orange juice extract, and in clementines extract; (B) UV-absorption spectra for L-ascorbic acid (L-AA) in a chromatogram (λ_{max} = 243 nm): (A) 100% natural orange juice extract; (B) L-AA standard; (C) mass spectrum of L-AA, $[M-H]^-$ (m/z) = 175.

(A) Reprinted from K.M. Phillips, M.T. Tarragó-Trani, S.E. Gebhardt, J. Exler, K.Y. Patterson, D.B. Haytowitz, P.R. Pehrsson, J.M. Holden, Stability of vitamin C in frozen raw fruit and vegetable homogenates, J. Food Comp. Anal. 23 (2010) 253–259. Copyright (2010), with permission from Elsevier; (B) Reprinted from N. Furuwasa, Rapid high-performance liquid chromatographic identification/quantification of total vitamin C in fruit drinks, Food Control 12 (2011) 27–29. Copyright (2011), with permission from Elsevier; (C) Reprinted with permission from A. Garrido-Frenich, M.E. Hernández-Torres, A. Belmonte-Vega, J.L. Martínez-Vidal, P. Plaza-Bolaños, Determination of ascorbic acid and carotenoids in food commodities by liquid chromatography with mass spectrometry detection, J. Agric. Food Chem. 53 (2010) 7371–7376. Copyright (2005) American Chemical Society.

sample, so this signal can be unequivocally attributed to the analyte. A method must first demonstrate high selectivity, or the following validation parameters may be compromised and the method may not be valid [2,13,83]. A typical example of selectivity can be observed in Fig. 1A, in which the peak corresponding to L-AA is clearly distinguished from the rest of the peaks in the chromatogram.

Demonstrating and documenting method selectivity can be made through several approaches [1,4,84]. In HPLC, we can distinguish between detection selectivity (detection technique) and separation selectivity (chromatographic separation conditions).

The most common detection technique for L-AA determination is UV detection. In many of these methods [10,12,20,27,30,31,33,34,37,41,43,49,54,59,64,67,85–87], the purity of the L-AA peak is confirmed by the comparison of its retention time (t_R) and UV spectrum to those of a pure standard, as seen in Fig. 1B. The use of mass detectors [22,53,72–74] can improve the confirmation of the purity of the peak by also measuring the mass to charge ratio of, usually, the deprotonated molecule (in negative ESI mode as $[M-H]^-$ = 175, Fig. 1C). Fluorescence has also been used for identification purposes [44]; however, a derivatization reaction is needed for L-AA, demanding the use of additional reagents and more time-consuming procedures.

Many methods only checked the purity of the peak as described above, an insufficient procedure for most applications. For instance, UV detection is not very discriminating, so other compounds may be also present at the same t_R. The confirmation of the purity of the peak in this way may be satisfactory for qualitative or semi-quantitative analysis, but more detailed experiments are required to ensure the selectivity of quantitative methods.

The most appropriate way of ensuring the selectivity would be the comparison of the chromatograms obtained after injection of blank samples with and without L-AA. However, this is not an easy task due to the absence of food samples (equal or similar to those intended to be analyzed) that do not contain L-AA. Therefore, other approaches have been described.

The analysis of CRMs for selectivity studies has been reported in several papers [12,56,68,79]. CRMs should match the matrix of the real samples that will be routinely analyzed [83,84]. However, due to the limited range of CRMs for L-AA in food, and to their high price, only few publications have demonstrated the selectivity using this

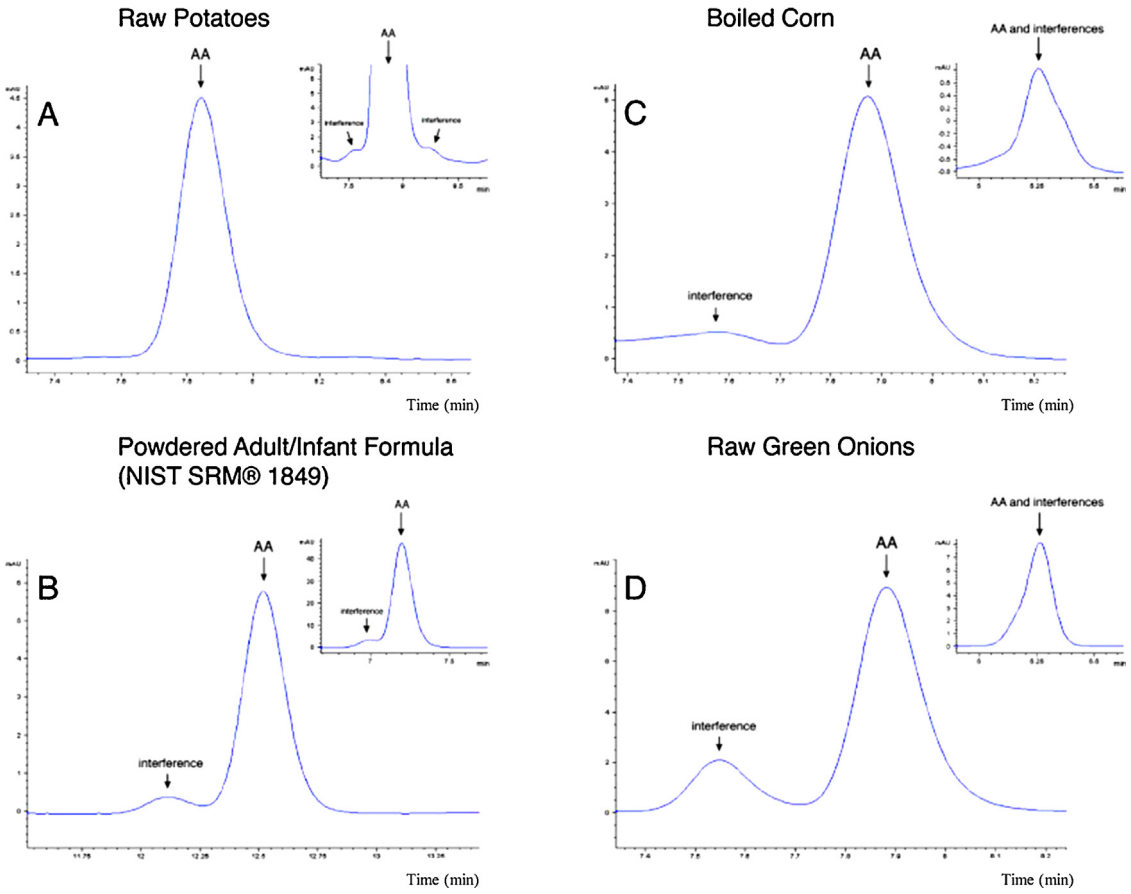

Fig. 2. Representative HPLC chromatograms of matrixes with interferences eluting near L-AA. Main panels show chromatograms using optimized HPLC separation, and insets show chromatograms before method optimization. (A) Raw potato extract at 1:5 dilution (inset) showing small shoulder peaks to the left and to the right of L-AA, and 1:20 dilution (main panel) where those shoulder peaks are barely visible; (B) powdered adult/infant formula (NIST SRM 1849) extract with incomplete separation of L-AA from unknown interference eluting shortly before L-AA (inset), and resolved from interference with optimized method; (C) corn and (D) raw green onion extracts showing broad and asymmetric L-AA peak (insets) using mobile phase validated for analysis of orange juice (isocratic 0.05% formic acid elution at 1 mL/min), and resolution of L-AA with optimized method (isocratic 0.02% ortho-phosphoric acid at 0.7 mL/min).

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approach [41,56,64,68]. Therefore, using blank samples similar to the ones to analyze is an easier way of evaluating the selectivity.

Lopes et al. [39] used a model wine during the validation tests, observing good resolution and selectivity with the different HPLC columns tested. However, poor resolution and selectivity were obtained with real wine samples. To improve L-AA separation, the authors replaced formic acid with TFA in the mobile phase and increased the acidity of the mobile phase (pH 1.4), obtaining a better peak shape. However, an unknown peak was still observed on the tail of the L-AA peak. This example demonstrates the paramount importance of selecting an appropriate matrix to check the selectivity. In some cases, a simple dilution of the samples removes the interferences (Fig. 2A) [12,20,56], but careful experiments are required to confirm the suitability of just a dilution step.

Tarrago-Trani et al. [56] highlighted the importance of matrix-specific method validation. In their work, L-AA peak was well resolved and symmetric in most samples, but for some foods the chromatograms presented unidentified minor "shoulders". To solve this problem, different modifications were made to the initial method, such as sample dilution and change of mobile phase (Fig. 2). Details on method modifications are available in [56]. According to the authors, without matrix-specific method adjustments, the interferences in the L-AA peaks may lead to over- or underestimation of the content of vitamin C. Underestimation was most likely due to incomplete extraction, whereas overestimation was the result of failure in the separation of interferences from the L-AA peak.

A less common approach consists in incubating the samples with ascorbate oxidase, demonstrating the authenticity of the L-AA peak by its disappearance. This has been successfully applied by various authors [48,85,88,89], although the necessity of the enzyme kits and a series of time-demanding steps may be mentioned as handicaps.

The interaction of the extraction solution with the mobile phase can compromise the selectivity of the method. Some extraction solutions have proved to be incompatible with the mobile phase [37,47,68], observing interferences in the L-AA peaks. For instance, Campos et al. [47] showed the chromatograms obtained through consecutive changes in the extractant; by modifying MPA concentration (from 4.5% to 3%) and changing the mobile phase pH (from 2.2 to 3.0) using *O*-phosphoric acid instead of MPA, the selectivity of the method improved; however, L-AA peak resolution became very poor, and the extracting solution was modified to a mixture containing MPA, acetic acid, sulfuric acid and EDTA, observing no loss in the extraction efficiency and improving peak resolution. Hernández et al. [10] used the same extraction solution and mobile phase than the previous authors, obtaining satisfactory results, although differences were observed depending on the kinds of matrixes. Hence,

the results of already published papers can give valuable information to other authors, but the conditions have to be carefully examined and adjusted for new food samples.

The choice of the reducing agents is also important. For instance, in amino columns the homocysteine set for the reduction of DHAA does not interfere with the L-AA peak, whereas in ODS columns it may disturb the separation [15]. Hence, it is always important to check the compatibility between the chromatographic system and the mobile phases and/or reagents.

It can be concluded that the study of the selectivity in HPLC methods for L-AA quantification is still a "trial and error" task. One of the main problems is the absence of appropriate blank samples. Therefore, different approaches have been used, although most of the papers do not present completely adequate selectivity studies. In general, the content of L-AA can be underestimated or overestimated using the same analytical method, but analyzing different matrixes. Hence, further research concerning selectivity validation is advisable to improve the quality of the analytical methods.

4.2. Matrix effect

This parameter is not usually included as mandatory in validation guidelines, and has been omitted in most of the methods here reviewed. However, ME is a very important parameter to obtain a properly validated and accurate analytical method.

ME causes a compound's signal to be different in a real sample than in a standard solution, being this difference induced by other co-eluting compounds. ME can cause a suppression or an enhancement in the analytical signal [77,90,91], although suppressions are more common.

ME is more important when MS detection is used, although it can occur no matter the detection technique. However, the evaluation of ME is usually mentioned only for LC–MS methods [77,84]. The procedures used to evaluate ME are extensively described elsewhere [56,77,90–93]. To minimize ME, sample dilution, modifications in sample preparation and HPLC parameters, or injecting smaller volumes of sample, are common approaches. However, the most efficient and advisable approach is the use of an internal standard (IS) to compensate for the alteration of the analytical signal [77,91]. Unfortunately, this approach is not often used in L-AA assays [7], mainly due to the lack of appropriate IS.

One of the few examples of ME evaluation was carried out in fruits and vegetables by Fenoll et al. [53], using a LC–MS method. In most cases, the authors observed suppression in the analytical signals, with a ME as high as 42% and 89% for L-AA and DHAA, respectively. Considering these results, the standard addition method was used for calibration purposes instead of an external standard calibration. Another example of ME validation was reported by Valente et al. [68], observing negligible ME with UV detection.

Despite its importance, most of the authors did not evaluate the ME, which can seriously compromise the accuracy of the method, leading to over- or under-estimation of the results.

4.3. Linearity

The linearity of a method and the range in which this method is linear, precise, and accurate are very important parameters, indicating at which analyte concentrations the method is applicable. As can be seen in Table 1, most of the methods checked the linearity of their calibration curve by inspecting the correlation coefficient (r) or the determination coefficient (R^2). In the reviewed papers, a method was generally accepted to present proper linearity if the correlation coefficient was greater than or equal to 0.97. Although this approach is a widespread practice, evaluating a calibration model through its coefficients of correlation is only informative and barely relevant from a statistical point of view [1,3,5,79,83,84,94]. The appropriateness of the calibration curve must be confirmed by statistical tests for model fits (analysis of variance of the regression, ANOVA, residual plot, F test, etc.) [1,13,83,95]. In a fine review about method validation and the evaluation of linearity [1], Araújo provided a comprehensive example of statistical analysis, demonstrating that a correlation coefficient close to 1 is not a reliable indicator of linearity, and that the use of statistical methods, such as the F-test, is required for a proper validation.

Unfortunately, few of the revised publications reported the use of statistical methods for linearity evaluation [27,42,62,68], indicating that the validation of this parameter should definitely be improved. Among the reported works in this review, Chebrolu et al. [27], after constructing the calibration curve for L-AA determination in fruits and vegetables, confirmed the linearity by residual plot and normal probability plot (P–P plot) (Fig. S2). Valente et al. used the ANOVA one-way test to prove variance homogeneity in their calibration working range [68]. The analysis of variance of the regression and a residual plot were performed by Odriozola-Serrano et al. [42] for the analysis of fruits, whereas an F-test was carried out by Van de Velde et al. [62].

Supplementary Fig. S2 material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.chroma.2014.09.087.

Another parameter closely related to linearity is the range of analyte concentrations for which the method presents suitable precision, accuracy, and linearity [1,75]. In some cases, the LOD was used as the lower concentration of the range, whereas in other cases the limit of quantification (LOQ) was used. Considering that the precision and accuracy of the method are usually not demonstrated for values lower than the LOQ, using the LOQ in the description of the range would be a better option than using the LOD.

In general, the linearity and working/linear range were reported in most of the reviewed papers, although the use of statistical treatments was missing in most cases.

Finally, a brief discussion of the different calibration procedures that have been used for L-AA determination in food samples follows:

- *External standard (ES)* – it provides limited accuracy and precision because the standards and samples are analyzed at two different times and the detector response might drift [76,96]. Moreover, possible losses of analyte during sample preparation steps and ME are not taken into account. This method should only be applied if it presents sufficient accuracy and precision, and if ME is negligible [91]. Nevertheless, ES calibration has been routinely applied for vitamin C quantification (Table 1) due to its ease, simplicity, and the use of the same calibration curve for each different matrix.
- *Standard addition (SA)* – it is more convenient for complex matrixes and is useful for assessing possible ME. There is a general agreement that the calibrations should be matrix-based and analyte-matched [5,13,83,84,91]. Unfortunately, this method is time consuming and only a small number of works [22,53] have used it.
- *Internal standard (IS)* – it is the most convenient approach, since it takes into account the instrumental drift, sample losses during sample preparation, and ME [76,91,96]. However, the IS has to be carefully selected, so its characteristics and physical/chemical behavior are as close as possible to L-AA. The best way to ensure the suitability of the IS is by using stable isotopically labeled internal standards. To our best knowledge, only Thomas et al. [64] and Engel et al. [52] used IS (4-pyridoxic acid and hippuric acid, respectively). However, L-AA and the IS presented very different retention times in both works, whereas the analyte and IS peaks

should be closer, so the IS can correct instrumental drifts and ME in a proper way.

To sum up, the use of ES calibration should be avoided if possible, and always use matrix-matched standards and/or IS. As mentioned before, the use of isotopically labeled L-AA would be a desirable option, avoiding all the inconveniences that may arise during sample preparation and quantification procedure. However, the price of isotopically labeled standards is a handicap for most analytical laboratories. In addition, it is difficult to obtain a pool of blank matrix samples to prepare matrix-matched standards. Hence, SA calibration is the best option for the accurate quantification of L-AA in foods, and the calibration procedure needs improvement in the future. However, the required precision and accuracy for the intended applications has to be taken into account too, so for some laboratories the use of ES calibration (aqueous standards) may be sufficient if a semi-quantitative analysis provides the required information.

4.4. Limit of detection (LOD) and limit of quantification (LOQ)

There are three main approaches to calculate the LOD and LOQ: "*visual determination*", "*signal-to-noise (S/N) ratio approach*" and "*standard deviation of the response and slope (SDRS)*" [1,3,5,76,84]. For a detailed discussion, the authors recommend the review from Araújo [1].

For a visual evaluation of the LOD and LOQ, relevant chromatograms should be presented [1]. To our best knowledge, this approach has only been applied to L-AA determination in drinks [55]. The main handicap is the lack of accuracy, and the absence of any mathematical model related to the calibration curve.

The *S*/*N* ratio is the most common approach (Table 1), not only because the concept is so well-known, but also because the analyst is able to optimize the *S*/*N* by varying the chromatographic conditions, therefore affecting the LOD and LOQ values [4,79,94]. This approach seems to be more reliable than the previous one since it can use statistical models to confirm its confidence level acceptance.

The third approach is based on the "*standard deviation of the response and the slope*" [1,3,76,79,84,97]. Only three studies [20,42,44] applied it for the calculation of the LOD and the LOQ. It may be related to the fact that, from all the described methods, this is the one that requires more calculations [5].

A less common approach, approved by IUPAC, is the use of the "*upper limit approach* (ULA)", applied by Ciulu et al. [54] for L-AA quantification in honeys. Finally, the lowest concentration of L-AA for which the RSD was lower than 10% was also reported as the LOQ [52]. Uncommon approaches for LOD/LOQ calculations complicate the comparison with other methods, so they should be avoided.

Some papers only reported the calculation of the LOD [10,33,35,38,43,46,50,57,65,67]. Although a better approach would be to indicate both the LOD and LOQ, in accordance to FDA guidance, the LOQ calculation may be omitted [91].

The guidelines on method validation do not express any particular preference for any of the approaches here described. However, the definition used in its evaluation should always be stated [1]. The values of the LOD and LOQ determined by different approaches yield different results, which makes it difficult to compare the LODs of some methods. Moreover, the discussed approaches for the evaluation of the LOQ do not demonstrate that, the method is accurate and precise at that level, so further experiments would be needed. Otherwise, the validation of LOD and LOQ is not fully complete.

Despite its importance, four publications included neither the LOD nor the LOQ on their validation tests [27,32,48,91]. In addition, the approach used for LOD calculation was not mentioned in one work [38]. In general, the LODs observed in the revised works were lower for fluorescence (0.27 μg/mL) and ECD (0.02–0.16 μg/mL) when compared to UV detection (1.2×10^{-3} to 7.2 μg/mL). Although the reported LODs were usually low enough for the required applications, the use of pre-concentration steps prior to analysis, and the improvement of sample treatments (avoiding interferences) could improve the LODs, therefore widening the range of applications of the methods.

4.5. Precision

The validation of the precision has to be performed using real samples, with the same matrix than the samples intended to be analyzed. According to literature, the use of CRMs for precision evaluation is rare [41,56,64,68]. In most of the reviewed publications, spiked samples were used to evaluate the precision, as recommended by FDA and ICH [5,76]. The evaluation of the precision is subdivided into three levels [3,5,84,94,97]: repeatability, intermediate precision, and reproducibility.

Repeatability refers to the precision during one day and under the same conditions of analysis [1,4,5,83], using different preparations of the same sample. Even though most of the reviewed papers have calculated the repeatability in a proper way (Table 1), some mistakes or unclear definitions were observed. For instance, some authors [10,59] measured the same standard solution several consecutive times, and they wrongly reported the data as repeatability, whereas it was instrumental precision. Other authors [46,86] used the expression "within-day repeatability" to refer to repeatability, which is not correct since the term repeatability already implies the same day for the analyses.

Intermediate precision refers to analyses carried out in the same laboratory, but on different days, usually three or more days [1,4,5,83]. Intermediate precision values lower than 13% [10] and 3% [67] were reported for L-AA content during five consecutive days. However, this evaluation is recommended in non-consecutive days, to assess the variation over a longer period of time. The intermediate precision was not evaluated in many works [20,22,32–34,39,44,48,50,53,57,62,63,65,66,98], contrary to what is recommended. Given the poor stability of L-AA, the study of the intermediate precision is imperative to evaluate the method ability to provide the same results in different days.

The reproducibility is used to demonstrate the consistency of results between different laboratories under the same conditions [4,83]. In some publications, this term was used erroneously to express the analyses conducted in the same laboratory [10,31–33,46,48,54,56,57,65,99]. To our best knowledge, few methods [41,68] studied the reproducibility. Satisfactory results were obtained in both works. For instance, Fontanaz et al. obtained reproducibility values (RSD) lower than 8% for ten samples analyzed by nine different laboratories [41].

The evaluation of the precision should be reported in terms of peak area, which is the parameter used to calculate the analyte concentration. However, some authors [32,67] only evaluated the precision for the retention time of the analyte.

Some of the reviewed papers [10,31,39,42,54,56,99] used the Horwitz function [6,13,76,94] for the evaluation of the intermediate precision. However, this function was proposed for reproducibility studies, and not for the calculation of the intermediate precision [100]. To our best knowledge, only Valente et al. correctly used the Horwitz function in the validation of the method [68]. The topic of the Horwitz function is discussed in more detail in the review from Taverniers et al. [13], and the handicaps when applied to intermediate precision can be found in [100].

In general, low relative standard deviation (RSD) values were reported for precision assays (<13%) in the compiled methods. However, according to Rozet et al. [84], the use of common formulas can lead to an optimistic vision of the method quantitative

performances. A statistical approach is therefore recommended to evaluate the variability of an analytical procedure, although these approaches are not usual. As an exception, Odriozola-Serrano et al. [42] applied ANOVA to test the RSD obtained with two different columns, observing that a C_{18} column was significantly better than an NH_2 column for vitamin C determination.

Since precision is influenced by the calibration range, it is recommended to evaluate it at least at three concentration levels (low, medium, high) [1,83], with a minimum of three replicates [1,83,94]. Unfortunately, most of the validations did not mention the concentration levels neither the number of replicates that were analyzed.

Even though most of the reviewed methods included precision assays, there were several methods in which the experiments were incomplete or the data vague and unclear. This aspect should be improved, especially regarding the study of the intermediate precision.

4.6. Accuracy

Accuracy is a mandatory parameter in all regulatory bodies [1,94]. Like precision, it also allows to judge the reliability of the analytical method, and should be inferred after establishing the selectivity, linearity and precision [3,76].

In general, the recommended strategy is to evaluate the accuracy through the analysis of CRMs. The commercially available CRMs for L-AA analysis in food include brussels sprouts, whole milk powder, green beans, powdered adult/infant nutritional formula, powdered milk, cranberry juice cocktail, and fortified breakfast cereals. For L-AA analysis by HPLC, only two publications [41,64] assessed the accuracy of the method using CRMs (milk powder SRM/RM 1846 from NIST, whole milk powder SRM 1549a, infant/adult nutritional formula SRM 1849a, and fortified breakfast cereal SRM 3233). The main problem concerning the use of CRMs is, in addition to their high price, the low availability of CRMs for L-AA analysis in food. Considering that the CRMs should ideally match the matrix of the samples to be analyzed, there are many cases in which the use of CRMs is not possible. However, even if the matrix of the CRMs is not the same, a fine option is to analyze similar CRMs in addition to recovery experiments. In the absence of suitable CRMs, the next approach consists in performing recovery experiments (spiking blank matrix samples with standard solutions of L-AA). In fact, most of the reviewed methods were validated using recovery experiments (Table 1).

Recovery studies are always performed by analyzing a sample pool before and after the addition of a known L-AA concentration. As the recovery data depend on the sample preparation, extraction procedure, and HPLC analysis, samples should always be spiked at the beginning of the sample preparation, so the whole analytical method is taken into account in the recovery experiments. Their main advantage is using exactly the same matrix than the samples to be analyzed in future applications. However, they also present an important handicap: a correct spiking procedure is not always easy. In order to obtain properly spiked samples, it is mandatory that the spiked L-AA and the original L-AA contained in the sample are in the same chemical form and present the same reactivity and behavior. Otherwise, the recovery may differ for spiked and real samples; therefore, the accuracy observed during the validation step would not be the same for real samples. For instance, most liquid samples may be easily spiked with an aqueous L-AA standard solution, and readily homogenized; however, some solid samples may require more carefully designed spiking procedures to obtain homogenized spiked samples. The proper design of the recovery experiments is especially important for L-AA determination, due to its poor stability and potential degradation during sample preparation.

The efficiency of the method varies according to the analyte concentration, so recovery percentages may substantially differ at high and low analyte concentrations [13,76,83]. Therefore, it is recommended to perform assays at three levels (low, medium, high), according to the calibration curve. However, most of the methods do not mention the concentration levels neither the number of replicates assayed, contrary to what is recommended. Some authors carried out recovery experiments at two concentration levels [20,59], whereas others used three levels [43,48,49,54,65–67].

Another critical aspect, omitted in most of the reviewed papers, is to explain in detail how the recovery experiments were carried out, so other authors can mimic those experiments. For instance, most of the papers only mentioned that the samples were spiked prior to the extraction procedure with a L-AA standard solution, but did not include other details, such as homogenization time, precautions to prevent L-AA degradation, etc. Some authors only mentioned the recovery values [50,57], but did not explain anything about how those data were obtained. This lack of information prevents other laboratories from reproducing those experiments.

Most works reported the recovery values, without the use of any statistical analysis. However, a limited number of methods included statistical data in the validation of the accuracy, which improved the reliability of the presented results. On the one hand, Ciulu et al. [54], Odriozola-Serrano et al. [42], and Van de Velde et al. [62] applied a statistical analysis to their recoveries results, using the Student's *t*-test. On the other hand, Valente et al. [68] calculated the standard uncertainty from the method accuracy, at three concentration levels.

Some works omitted the validation of the accuracy (Table 1), especially when liquid samples were analyzed. Although liquid samples were diluted and directly analyzed, the study of the accuracy should have been performed. In general, recoveries in the 90–105 and 85–110% range were observed for liquid and solid samples, respectively. However, lower recovery values were also reported in some works, such as a recovery of 73% for L-AA in grapevines [48]. The good recoveries observed in most works are related to the use of simple and fast extraction procedures, which prevent L-AA oxidation. In general, the validation of the accuracy of the reviewed methods can be considered appropriate. However, more detailed explanations are needed concerning the recovery experiments; otherwise, the recovery values cannot be considered completely reliable, as the spiking procedure is not clearly reported.

4.7. Robustness

Robustness tests are very helpful during the method development/pre-validation phase, so the analyst may know what factors should be paid special attention during normal use [3,5]. Variations in method conditions for robustness studies should be small and reflect typical day-to-day variations [3].

To examine the causes of variability of the results, several factors may be assessed and divided in two categories: sample handling and HPLC conditions. The sample preparation variations include solvents, sample preparation procedure (centrifugation time, different membrane filters) and analyte stability in solution [3,97]. Table 2 provides a summary of chromatographic parameters variations that can be applied for testing robustness. Vander-Heyden et al. [101] presented an excellent discussion of all the steps involved in the study of robustness, including a case-study to illustrate the evaluation of this parameter.

If changes in the conditions of analysis produce results within acceptable limits of selectivity, precision, and accuracy the method can be considered robust; otherwise, these variations must be controlled and precautions included in the procedure [76,79,97].

According to Table 2, the most typical variations were the composition, the pH, and the flow-rate of the mobile phase. The revised methods [36,37,55,62,63,68] proved to be generally unaffected by small variations in the chromatographic conditions assayed.

Table 2
Typical variations in chromatographic parameters for robustness evaluation.

Factor	Recommended limit range	Change	Ref.
Isocratic separations			
Organic solvent concentration	±2–3%	±2% MeOH	[55]
		3–7% MeOH	[62]
		2–7% MeOH	[63]
		0.005–0.030% TFA	[36]
Buffer concentration	±1–2%	–	
Buffer pH	±0.1–0.2 pH	3.4–3.6 pH	[68]
		5.7–5.9 pH	[62]
		2.5–3.7 pH	[63]
		3.2–5.1 pH	[36]
Temperature	±3 °C	29–31 °C	[68]
		25–49 °C	[63]
		15–30 °C	[36]
Flow rate	±0.1–0.2 mL/min	0.70–0.90 mL/min	[68]
		1.10–1.20 mL/min	[62]
Detector wavelength	±2–3 nm	250–252 nm	[62]
Column	2–3 different lots and brands	2 different columns	[37]
		3 different columns	[36]
Gradient separations			
Initial gradient hold time	±10–20% of hold time	2–8 min initial gradient	[36]
Slope and length	Slope determined by the gradient range and time; adjust gradient time by ±10–20% and allow the slope to vary	–	
Final hold time	Adjust to allow last-eluted compound to appear	–	

However, TAA recovery was influenced by the flow rate variation in the work presented by Van de Velde et al. [62].

Robustness is generally not considered in most validation guidelines [37,55,62,63,68], so it is rarely used. Only six of the revised methods studied this parameter. However, it is a very useful parameter to measure how typical daily laboratory variations can affect the results of the method.

4.8. Stability

These assays are a pre-requisite in order to ensure that the stability of the analyte is kept during the storage, preparation, and analysis of the samples [76,79,83,84]. This is especially true for L-AA analysis, due to its instability in aqueous solutions. The stability should be tested at ambient temperature over a period of time that encompasses the typical time required for sample preparation, sample handling and analytical run time [76]. There is no agreement on the acceptance criterion that defines the acceptable stability, only a consensus that the degradation should be small (RSD ≤ 15%) [68,73,76,84].

Even though L-AA stability is a critical issue to obtain reliable results, since it is an optional parameter in method validation [91], few papers considered it as a part of method development and validation studies [43,68,98,99]. Numerous studies have been performed to find optimal conditions for L-AA stability [10,12,23,27,46,55,59,68,73,98,29,102]. An overview of the literature revealed that temperature, time, and pH are the main factors considered during stability studies.

Low temperature is a key factor in preventing the oxidation of L-AA, and stability studies conducted at room or higher temperatures confirmed an increase in L-AA degradation [46,73]. Chebrolu et al. [27] reported no significant loss in L-AA and TAA contents (extracted with MPA and reduced with TCEP) stored at room temperature during 48 h. This may be due to the use of stabilizing agents that prolonged the stability at high temperature. However, the most common and recommended approach to prevent L-AA oxidation is a temperature decrease [7,10,12,68,29]. In a previous work [29], the effect of storage temperature (4, −20 and −80 °C) on L-AA degradation was studied in standard solution and in selected horticultural extracts; stability of samples at laboratory temperature was also assayed. It was found that L-AA was stable at room temperature for ≤2 h. These data were similar to those from Iwase [98], that reported L-AA to be stable at laboratory temperature for 1 h. The degradation study also showed that extracts were stable for at least 24 h at 4 °C, and during 4 weeks when stored at −80 °C. Tai and Gohda [43] performed a stability study to evaluate L-AA degradation, and their results indicated that samples could be processed within 24 h at 4 °C. Hernández et al. [10], Phillips et al. [12], and Valente et al. [68] concluded that storage of acidic samples at low temperatures (≤−60 °C) provided excellent stabilization of L-AA for at least one month.

The stability of L-AA in solution when exposed to other conditions (light, presence of metal ions) and with the addition of stabilizing agents (homocystein, DTT, MSG, EDTA, etc.) has also been studied by many authors. A more detailed overview of stability precautions can be found in [7].

L-AA stability depends not only on general parameters (temperature, pH, etc.) but also on the particular matrix of the samples to be analyzed. As a result, especially when dealing with complex matrixes such as foodstuff, it is necessary to perform a stability study, because it can differ completely from one particular food sample to a different one. Many of the methods here reported omitted the stability study, so the results observed in those works may not be completely reliable without further information.

Finally, it is worth mentioning the effect of the enzymes on L-AA stability, especially in fresh food produce, in which the enzymatic activity may be an important contributor to L-AA degradation. Even though analytical laboratories pay special attention to the storage and handling of samples at low temperature conditions (so inhibiting enzymatic activity), these conditions are obviously not the same for consumers. Therefore, more studies would be required in this area to compare L-AA stability under the ideal storage conditions, and under the normal conditions in which food samples are usually stored and consumed.

5. Final remarks

This review presented and discussed the different aspects involved in the development and validation of HPLC methods for vitamin C quantification in food. The proper validation of these methods is not an easy task to perform. The poor stability of L-AA in solution and the complexity and heterogeneity of food samples demand an additional effort regarding method validation. Proper sample handling, including freezing and acidification, is

mandatory to avoid L-AA degradation. In this sense, even though thorough studies of L-AA stability are already available in scientific literature, new studies have to be performed for each particular sample to be analyzed.

Other aspect to take into account is the absence of CRMs for many types of foods, and the difficulty of obtaining blank matrix samples (as most of the analyzed foods already contain L-AA). In this sense, selectivity and accuracy studies are not always easy to carry out. However, excuses cannot be made to avoid a proper method validation, and more exhaustive experiments in this area would have been necessary in some works. In addition, it is not enough to present the validation data, but also to explain in detail how those data were obtained, which guideline was used, etc., so the method could be implemented in a different laboratory if necessary.

The main goal of this review was not only to discuss the different approaches used by scientists for vitamin C quantification in food, but also to extract conclusions regarding the main aspects that have to be considered to obtain reliable results. In this way, the information that different scientists have provided in the last years concerning vitamin C analysis in food is here summarized, so analytical laboratories and food companies can access this information and improve their analyses and quality control processes. Industry and, therefore, consumers, rely on accurate and comparable data. Food industry needs to be aware of the most important parameters to control during food processing, storage, and analysis, so the final product presents a high-quality regarding its vitamin C content, which, as mentioned thorough this review, is a difficult task due to all the parameters that need to be controlled.

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References

[1] P. Araujo, Key aspects of analytical method validation and linearity evaluation, J. Chromatogr. B 877 (2009) 2224–2234.
[2] S. Bansal, A. DeStefano, Key elements of bioanalytical method validation for small molecules, AAPS J. 9 (2007) E109–E114.
[3] R. Singh, HPLC method development and validation – an overview, J. Pharm. Educ. Res. 4 (2013) 26–33.
[4] H.A. Rogers, How composition methods are developed and validated, J. Agric. Food Chem. 61 (2013) 8312–8316.
[5] F.T. Peters, O.H. Drummer, F. Musshoff, Validation of new methods, Forensic Sci. Int. 165 (2007) 216–224.
[6] J. Zempleni, R.B. Rucker, D.B. McCormick, J.W. Suttie, Handbook of Vitamins, fourth ed., CRC Press, Boca Raton, 2007.
[7] L. Nováková, P. Solich, D. Solichová, HPLC methods for simultaneous determination of ascorbic and dehydroascorbic acids, TrAC Trends Anal. Chem. 27 (2008) 942–958.
[8] L.F. Russel, Quantitative determination of water-soluble vitamins, in: L.M.L. Nollet (Ed.), Food Analysis by HPLC, second ed., Marcel Dekker, New York, 2000, pp. 403–476.
[9] R.R. Eitenmiller, L. Ye, W.O. Landen Jr., Vitamin Analysis for the Health and Food Sciences, second ed., CRC Press, Boca Raton, 2008.
[10] Y. Hernández, M.G. Lobo, M. González, Determination of vitamin C in tropical fruits: a comparative evaluation of methods, Food Chem. 96 (2006) 654–664.
[11] S.K. Lee, A.A. Kader, Preharvest and postharvest factors influencing vitamin C content of horticultural crops, Postharvest Biol. Tec. 20 (2000) 207–220.
[12] K.M. Phillips, M.T. Tarragó-Trani, S.E. Gebhardt, J. Exler, K.Y. Patterson, D.B. Haytowitz, P.R. Pehrsson, J.M. Holden, Stability of vitamin C in frozen raw fruit and vegetable homogenates, J. Food Comp. Anal. 23 (2010) 253–259.
[13] I. Taverniers, M. De Loose, E. Van Bockstaele, Trends in quality in the analytical laboratory. II. Analytical method validation and quality assurance, TrAC Trends Anal. Chem. 23 (2004) 535–552.
[14] C.M.M. Almeida, Métodos de Análise, in: J.F. Pinto (Ed.), Nutracêuticos e Alimentos Funcionais, Lidel, Lisboa, 2010, pp. 143–158.
[15] K. Nyyssönen, J.T. Salonen, M.T. Parviainen, Ascorbic acid, in: A.P. De Leenheer, W.E. Lambert, J.F. Van Bocxlaer (Eds.), Modern Chromatographic Analysis of Vitamins, Marcel Dekker Inc., New York, 2000, pp. 271–300.
[16] S.P. Arya, M. Mahajan, P. Jain, Non-spectrophotometric methods for the determination of Vitamin C, Anal. Chim. Acta 417 (2000) 1–14.
[17] L. Nováková, H. Vlcková, A review of current trends and advances in modern bio-analytical methods: chromatography and sample preparation, Anal. Chim. Acta 656 (2009) 8–35.
[18] Y. Chen, Z. Guo, X. Wang, C. Qiu, Sample preparation, J. Chromatogr. A 1184 (2008) 191–219.
[19] J.C. Rickman, D.M. Barrett, C.M. Bruhn, Nutritional comparison of fresh, frozen and canned fruits and vegetables. Part 1. Vitamins C and B and phenolic compounds, J. Sci. Food Agric. 87 (2007) 930–944.
[20] V. Spínola, B. Mendes, J. Câmara, P. Castilho, An improved and fast UHPLC-PDA methodology for determination of L-ascorbic and dehydroascorbic acids in fruits and vegetables. Evaluation of degradation rate during storage, Anal. Bioanal. Chem. 403 (2012) 1049–1058.
[21] C.-H. Chang, H.-Y. Lin, C.-Y. Chang, Y.-C. Liu, Comparisons on the antioxidant properties of fresh, freeze-dried and hot-air-dried tomatoes, J. Food Eng. 77 (2006) 478–485.
[22] A. Garrido-Frenich, M.E. Hernández-Torres, A. Belmonte-Vega, J.L. Martínez-Vidal, P. Plaza-Bolaños, Determination of ascorbic acid and carotenoids in food commodities by liquid chromatography with mass spectrometry detection, J. Agric. Food Chem. 53 (2005) 7371–7376.
[23] A. Rizzolo, A. Brambilla, S. Valsecchi, P. Eccher-Zerbini, Evaluation of sampling and extraction procedures for the analysis of ascorbic acid from pear fruit tissue, Food Chem. 77 (2002) 257–262.
[24] N.M. Shofian, A.A. Hamid, A. Osman, N. Saari, F. Anwar, M.S.P. Dek, M.R. Hairuddin, Effect of freeze-drying on the antioxidant compounds and antioxidant activity of selected tropical fruits, Int. J. Mol. Sci. 12 (2011) 4678–4692.
[25] S. Georgé, F. Tourniaire, H. Gautier, P. Goupy, E. Rock, C. Caris-Veyrat, Changes in the contents of carotenoids, phenolic compounds and vitamin C during technical processing and lyophilisation of red and yellow tomatoes, Food Chem. 124 (2011) 1603–1611.
[26] A.P.K. Joshi, H.P.V. Rupasinghe, S. Khanizadeh, Impact of drying processes on bioactive phenolics, vitamin C and antioxidant capacity of red-fleshed apple slices, J. Food Process. Pres. 35 (2011) 453–457.
[27] K.K. Chebrolu, G.K. Jayaprakasha, K.S. Yoo, J.L. Jifon, B.S. Patil, An improved sample preparation method for quantification of ascorbic acid and dehydroascorbic acid by HPLC, LWT – Food Sci. Technol. 47 (2012) 443–449.
[28] V. Kabasakalis, D. Siopidou, E. Moshatou, Ascorbic acid content of commercial fruit juices and its rate of loss upon storage, Food Chem. 70 (2000) 325–328.
[29] V. Spínola, B. Mendes, J.S. Câmara, P.C. Castilho, Effect of time and temperature on vitamin C stability in horticultural extracts. UHPLC-PDA vs iodometric titration as analytical methods, LWT – Food Sci. Technol. 50 (2013) 489–495.
[30] M.C. Sánchez-Mata, M. Câmara-Hurtado, C. Díez-Marqués, M.E. Torija-Isasa, Comparison of high-performance liquid chromatography and spectrofluorimetry for vitamin C analysis of green beans (*Phaseolus vulgaris* L.), Eur. Food Res. Technol. 210 (2000) 220–225.
[31] M. Romeu-Nadal, S. Morera-Pons, A.I. Castellote, M.C. López-Sabater, Rapid high-performance liquid chromatographic method for vitamin C determination in human milk versus an enzymatic method, J. Chromatogr. B 830 (2006) 41–46.
[32] V. Gökmen, N. Kahraman, N. Demir, J. Acar, Enzymatically validated liquid chromatographic method for the determination of ascorbic and dehydroascorbic acids in fruit and vegetables, J. Chromatogr. A 881 (2000) 309–316.
[33] N. Furusawa, Rapid high-performance liquid chromatographic identification/quantification of total vitamin C in fruit drinks, Food Control 12 (2001) 27–29.
[34] M. Rodríguez-Comesaña, M.S. García-Falcón, J. Simal-Gándara, Control of nutritional labels in beverages with added vitamins: screening of β-carotene and ascorbic acid contents, Food Chem. 79 (2002) 141–144.
[35] H. Iwase, Routine high-performance liquid chromatographic determination of ascorbic acid in foods using l-methionine for the pre-analysis sample stabilization, Talanta 60 (2003) 1011–1021.
[36] B. Klejdus, J. Petrlová, D. Potěšil, V. Adam, R. Mikelová, J. Vacek, R. Kizek, V. Kubáň, Simultaneous determination of water- and fat-soluble vitamins in pharmaceutical preparations by high-performance liquid chromatography coupled with diode array detection, Anal. Chim. Acta 520 (2004) 57–67.
[37] A.A. Franke, L.J. Custer, C. Arakaki, S.P. Murphy, Vitamin C and flavonoid levels of fruits and vegetables consumed in Hawaii, J. Food Comp. Anal. 17 (2004) 1–35.
[38] O. Heudi, T. Kilinç, P. Fontannaz, Separation of water-soluble vitamins by reversed-phase high performance liquid chromatography with ultra-violet detection: application to polyvitaminated premixes, J. Chromatogr. A 1070 (2005) 49–56.
[39] P. Lopes, J. Drinkine, C. Saucier, Y. Glories, Determination of L-ascorbic acid in wines by direct injection liquid chromatography using a polymeric column, Anal. Chim. Acta 555 (2006) 242–245.
[40] M.M. Wall, Ascorbic acid, vitamin A, and mineral composition of banana (Musa sp.) and papaya (*Carica papaya*) cultivars grown in Hawaii, J. Food Comp. Anal. 19 (2006) 434–445.

[41] P. Fontannaz, T. Kilinç, O. Heudi, HPLC-UV determination of total vitamin C in a wide range of fortified food products, Food Chem. 94 (2006) 626–631.
[42] I. Odriozola-Serrano, T. Hernández-Jover, O. Martín-Belloso, Comparative evaluation of UV-HPLC methods and reducing agents to determine vitamin C in fruits, Food Chem. 105 (2007) 1151–1158.
[43] A. Tai, E. Gohda, Determination of ascorbic acid and its related compounds in foods and beverages by hydrophilic interaction liquid chromatography, J. Chromatogr. B 853 (2007) 214–220.
[44] G. Burini, Development of a quantitative method for the analysis of total l-ascorbic acid in foods by high-performance liquid chromatography, J. Chromatogr. A 1154 (2007) 97–102.
[45] L. Nováková, D. Solichová, S. Pavlovičová, P. Solich, Hydrophilic interaction liquid chromatography method for the determination of ascorbic acid, J. Sep. Sci. 31 (2008) 1634–1644.
[46] A.R.-B. de Quirós, M. Fernández-Arias, J. López-Hernández, A screening method for the determination of ascorbic acid in fruit juices and soft drinks, Food Chem. 116 (2009) 509–512.
[47] F.M. Campos, S.M. Rocha-Ribeiro, C.M. Della Lucia, H.M. Pinheiro-Sant'Ana, P.C. Stringheta, Optimization of methodology to analyze ascorbic and dehydroascorbic acid in vegetables, Quim. Nova 32 (2009) 87–91.
[48] V.J. Melino, K.L. Soole, C.M. Ford, A method for determination of fruit-derived ascorbic, tartaric, oxalic and malic acids, and its application to the study of ascorbic acid catabolism in grapevines, Aust. J. Grape Wine Res. 15 (2009) 293–302.
[49] L. Sawant, B. Prabhakar, N. Pandita, Quantitative HPLC analysis of ascorbic acid and gallic acid in *Phyllanthus Emblica*, J. Anal. Bioanal. Tech. (2010) 111.
[50] V. Nour, I. Trandafir, M.E. Ionica, HPLC organic acid analysis in different citrus juices under reversed phase conditions, Not. Bot. Hort. Agrobot. Cluj 38 (2010) 44–48.
[51] A.I.R.N.A. Barros, A.P. Silva, B. Gonçalves, F.M. Nunes, A fast, simple, and reliable hydrophilic interaction liquid chromatography method for the determination of ascorbic and isoascorbic acids, Anal. Bioanal. Chem. 396 (2010) 1863–1875.
[52] R. Engel, É. Stefanovits-Bányai, L. Abrankó, LC simultaneous determination of the free forms of B group vitamins and vitamin C in various fortified food products, Chromatographia 71 (2010) 1069–1074.
[53] J. Fenoll, A. Martínez, P. Hellín, P. Flores, Simultaneous determination of ascorbic and dehydroascorbic acids in vegetables and fruits by liquid chromatography with tandem-mass spectrometry, Food Chem. 127 (2011) 340–344.
[54] M. Ciulu, S. Solinas, I. Floris, A. Panzanelli, M.I. Pilo, P.C. Piu, N. Spano, G. Sanna, RP-HPLC determination of water-soluble vitamins in honey, Talanta 83 (2011) 924–929.
[55] R.K. Konda, P. Praveen Kumar, N. MallikarjunaRao, Development and validation of RP-HPLC method for the estimation of ascorbic acid in health drinks, J. Chem. Pharm. Res. (2011) 363–374.
[56] M.T. Tarrago-Trani, K.M. Phillips, M. Cotty, Matrix-specific method validation for quantitative analysis of vitamin C in diverse foods, J. Food Comp. Anal. 26 (2012) 12–25.
[57] M.C. Sánchez-Mata, R.D. Cabrera Loera, P. Morales, V. Fernández-Ruiz, M. Cámara, C. Díez Marqués, M. Pardo-de-Santayana, J. Tardío, Wild vegetables of the Mediterranean area as valuable sources of bioactive compounds, Genet. Resour. Crop Evol. 59 (2012) 431–443.
[58] P.C. Cardoso, A.P.B. Tomazini, P.C. Stringheta, S.M.R. Ribeiro, H.M. Pinheiro-Sant'Ana, Vitamin C and carotenoids in organic and conventional fruits grown in Brazil, Food Chem. 126 (2011) 411–416.
[59] R. Scherer, A.C.P. Rybka, C.A. Ballus, A.D. Meinhart, J.T. Filho, H.T. Godoy, Validation of a HPLC method for simultaneous determination of main organic acids in fruits and juices, Food Chem. 135 (2012) 150–154.
[60] M. Adam, P. Pavlíková, A. Čížková, P. Bajerová, K. Ventura, Microextraction by packed sorbent (MEPS) as a suitable selective method for l-ascorbic acid determination in beverages, Food Chem. 135 (2012) 1613–1618.
[61] J. Santos, J.A. Mendiola, M.B.P.P. Oliveira, E. Ibáñez, M. Herrero, Sequential determination of fat- and water-soluble vitamins in green leafy vegetables during storage, J. Chromatogr. A 1261 (2012) 179–188.
[62] F. Van de Velde, M. Pirovani, M. Cámara, D. Güemes, C.d. Bernardi, Optimization and validation of a UV–HPLC method for vitamin C determination in strawberries (*Fragaria ananassa* Duch.). Using experimental designs, Food Anal. Meth. 5 (2012) 1097–1104.
[63] V. León-Ruiz, S. Vera, A. González-Porto, M. San Andrés, Analysis of water-soluble vitamins in honey by isocratic RP-HPLC, Food Anal. Meth. 6 (2013) 488–496.
[64] J. Thomas, J. Yen, K. Sharpless, Characterization of NIST food-matrix standard reference materials for their vitamin C content, Anal. Bioanal. Chem. 405 (2013) 4539–4548.
[65] N. Matei, G.-L. Radu, G. Truica, S. Eremia, S. Dobrinas, G. Stanciu, A. Popescu, Rapid HPLC method for the determination of ascorbic acid in grape samples, Anal. Meth. 5 (2013) 4675–4679.
[66] S. Carballo, F.A. Zingarello, S.E. Maestre, J.L. Todolí, M.S. Prats, Optimisation of analytical methods for the characterisation of oranges, clementines and citrus hybrids cultivated in Spain on the basis of their composition in ascorbic acid, citric acid and major sugars, Int. J. Food Sci. Technol. 49 (2014) 146–152.
[67] H. Bae, G.K. Jayaprakasha, K. Crosby, J.L. Jifon, B.S. Patil, Simultaneous quantification of capsaicinoids and ascorbic acid from pungent peppers, J. Chromatogr. Sci. 51 (2013) 412–418.
[68] A. Valente, A. Sanches-Silva, T.G. Albuquerque, H.S. Costa, Development of an orange juice in-house reference material and its application to guarantee the quality of vitamin C determination in fruits, juices and fruit pulps, Food Chem. 154 (2014) 71–77.
[69] S. Langer, J.K. Lodge, Determination of selected water-soluble vitamins using hydrophilic chromatography: a comparison of photodiode array, fluorescence, and coulometric detection, and validation in a breakfast cereal matrix, J. Chromatogr. B 960 (2014) 73–81.
[70] V. Bansal, A. Sharma, C. Ghanshyam, M.L. Singla, Coupling of chromatographic analyses with pretreatment for the determination of bioactive compounds in *Emblica officinalis* juice, Anal. Meth. 6 (2014) 410–418.
[71] C. André, I. Castanheira, J.M. Cruz, P. Paseiro, A. Sanches-Silva, Analytical strategies to evaluate antioxidants in food: a review, Trends Food Sci. Technol. 21 (2010) 229–246.
[72] Z. Chen, B. Chen, S. Yao, High-performance liquid chromatography/electrospray ionization-mass spectrometry for simultaneous determination of taurine and 10 water-soluble vitamins in multivitamin tablets, Anal. Chim. Acta 569 (2006) 169–175.
[73] M. Szultka, M. Buszewska-Forajta, R. Kaliszan, B. Buszewski, Determination of ascorbic acid and its degradation products by high performance liquid chromatography-triple quadrupole mass spectrometry, Electrophoresis 35 (2014) 585–592.
[74] S.M. Wabaidur, Z.A. Alothman, M.R. Khan, A rapid method for the simultaneous determination of l-ascorbic acid and acetylsalicylic acid in aspirin C effervescent tablet by ultra performance liquid chromatography–tandem mass spectrometry, Spectrochim. Acta A 108 (2013) 20–25.
[75] M. Rambla-Alegre, J. Esteve-Romero, S. Carda-Broch, Is it really necessary to validate an analytical method or not? That is the question, J. Chromatogr. A 1232 (2012) 101–109.
[76] M. Ribani, C.B.G. Bottoli, C.H. Collins, I.C.S.F. Jardim, L.F.C. Melo, Validação em métodos cromatográficos e eletroforéticos, Quim. Nova 27 (2004) 771–780.
[77] A. Van Eeckhaut, K. Lanckmans, S. Sarre, I. Smolders, Y. Michotte, Validation of bioanalytical LC–MS/MS assays: evaluation of matrix effects, J. Chromatogr. B 877 (2009) 2198–2207.
[78] European Commission, Guidance document on analytical quality control and validation procedures for pesticide residues analysis in food and feed, SANCO/12571/2013, 19 November 2013 rev.0.
[79] G.A. Shabir, Validation of high-performance liquid chromatography methods for pharmaceutical analysis: understanding the differences and similarities between validation requirements of the US Food and Drug Administration, the US Pharmacopeia and the International Conference on Harmonization, J. Chromatogr. A 987 (2003) 57–66.
[80] J. Kubola, S. Siriamornpun, N. Meeso, Phytochemicals, vitamin C and sugar content of Thai wild fruits, Food Chem. 126 (2011) 972–981.
[81] P.H. Mattila, J. Hellström, G. McDougall, G. Dobson, J.-M. Pihlava, T. Tiirikka, D. Stewart, R. Karjalainen, Polyphenol and vitamin C contents in European commercial blackcurrant juice products, Food Chem. 127 (2011) 1216–1223.
[82] B. Ribeiro, J. Rangel, P.c. Valentão, P.B. Andrade, J.A. Pereira, H. Bölke, R.M. Seabra, Organic acids in two Portuguese chestnut (*Castanea sativa* Miller) varieties, Food Chem. 100 (2007) 504–508.
[83] G.T.R. Tiwari, Bioanalytical method validation: an updated review, Pharm. Meth. (2010) 25–38.
[84] E. Rozet, R.D. Marini, E. Ziemons, B. Boulanger, P. Hubert, Advances in validation, risk and uncertainty assessment of bioanalytical methods, J. Pharm. Biomed. Anal. 55 (2011) 848–858.
[85] I. Nishiyama, Y. Yamashita, M. Yamanaka, A. Shimohashi, T. Fukuda, T. Oota, Varietal difference in vitamin C content in the fruit of kiwifruit and other actinidia species, J. Agric. Food Chem. 52 (2004) 5472–5475.
[86] G. Shui, L.P. Leong, Separation and determination of organic acids and phenolic compounds in fruit juices and drinks by high-performance liquid chromatography, J. Chromatogr. A 977 (2002) 89–96.
[87] A. Valente, T.G. Albuquerque, A. Sanches-Silva, H.S. Costa, Ascorbic acid content in exotic fruits: a contribution to produce quality data for food composition databases, Food Res. Int. 44 (2011) 2237–2242.
[88] A. Karlsen, R. Blomhoff, T.E. Gundersen, High-throughput analysis of vitamin C in human plasma with the use of HPLC with monolithic column and UV-detection, J. Chromatogr. B 824 (2005) 132–138.
[89] J. Lykkesfeldt, Determination of ascorbic acid and dehydroascorbic acid in biological samples by high-performance liquid chromatography using subtraction methods: reliable reduction with tris[2-carboxyethyl]phosphine hydrochloride, Anal. Biochem. 282 (2000) 89–93.
[90] E. Rogatsky, D. Stein, Evaluation of matrix effect and chromatography efficiency: new parameters for validation of method development, J. Am. Soc. Mass Spectr. 16 (2005) 1757–1759.
[91] L. Nováková, Challenges in the development of bioanalytical liquid chromatography–mass spectrometry method with emphasis on fast analysis, J. Chromatogr. A 1292 (2013) 25–37.
[92] F. Gosetti, E. Mazzucco, D. Zampieri, M.C. Gennaro, Signal suppression/enhancement in high-performance liquid chromatography tandem mass spectrometry, J. Chromatogr. A 1217 (2010) 3929–3937.
[93] P.J. Taylor, Matrix effects: the Achilles heel of quantitative high-performance liquid chromatography-electrospray–tandem mass spectrometry, Clin. Biochem. 38 (2005) 328–334.
[94] D. Stöckl, H. D'Hondt, L.M. Thienpont, Method validation across the disciplines – critical investigation of major validation criteria and associated experimental protocols, J. Chromatogr. B 877 (2009) 2180–2190.

[95] A. Bouabidi, E. Rozet, M. Fillet, E. Ziemons, E. Chapuzet, B. Mertens, R. Klinkenberg, A. Ceccato, M. Talbi, B. Streel, A. Bouklouze, B. Boulanger, P. Hubert, Critical analysis of several analytical method validation strategies in the framework of the fit for purpose concept, J. Chromatogr. A 1217 (2010) 3180–3192.
[96] D.A. Skoog, D.M. West, F.J. Holler, S.R. Crouch, Fundamentals of Analytical Chemistry, Thomson Brooks/Cale, Belmont, 2004, pp. 192–214.
[97] H. Bridwell, V. Dhingra, D. Peckman, J. Roark, T. Lehman, Perspectives on method validation: importance of adequate method validation, Qual. Assur. J. 13 (2010) 72–77.
[98] H. Iwase, Use of an amino acid in the mobile phase for the determination of ascorbic acid in food by high-performance liquid chromatography with electrochemical detection, J. Chromatogr. A 881 (2000) 317–326.
[99] R. Scherer, A.C.P. Rybka, H.T. Godoy, Determinação simultânea dos ácidos orgânicos tartárico, málico, ascórbico e cítrico em polpas de acerola, açaí e caju e avaliação da estabilidade em sucos de caju, Quim. Nova 31 (2008) 1137–1140.
[100] M. Thompson, The characteristic function, a method-specific alternative to the Horwitz Function, J. AOAC Int. 95 (2012) 1803–1806.
[101] Y. Vander Heyden, A. Nijhuis, J. Smeyers-Verbeke, B.G.M. Vandeginste, D.L. Massart, Guidance for robustness/ruggedness tests in method validation, J. Pharm. Biomed. Anal. 24 (2001) 723–753.
[102] M. Romeu-Nadal, A.I. Castellote, M.C. López-Sabater, Effect of cold storage on vitamins C and E and fatty acids in human milk, Food Chem. 106 (2008) 65–70.