

ASCORBIC ACID IS VIRTUALLY DEGRADED IN DRIED HERBAL PRODUCTS - AN HPLC ASSESSMENT OF SIX PLANT SPECIES

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Abstract

Ascorbic acid is almost universally present in the living world (humans are a notable exception) and herbal medicinal products are relatively often presented as source of ascorbic acid. We hereby report the results of an HPLC assessment of the ascorbic acid contents in vegetative organs (roots and other subterranean organs, stems, leaves) of six herbal species, the main finding being that dried parts of all six species (derived from culture sources) are virtually devoid of ascorbic acid (under the detection limit). Ascorbic acid is particularly unstable in extractive solutions prepared from these species, despite of the use of conventional stabilization methods such as the use of inorganic or organic acids (ortho-phosphoric, oxalic). Although acetonitrile is considered to act as a stabilizer (reducing fourfold the degradation rate of ascorbic acid), it was ineffective in stabilizing the ascorbic acid during extraction. Using methanol as an extraction solvent it was ensured the stability of this natural compound for about 3 hours.

Rezumat

Acidul ascorbic este prezent în mod aproape universal în lumea vie (omul reprezentând o excepție remarcabilă), iar produsele vegetale medicinale sunt adesea prezentate ca o sursă de acid ascorbic. Prezentăm rezultatele evaluării printr-o metodă HPLC a conținutului în acid ascorbic din organele vegetative (rădăcini și alte organe subterane, tulpini, frunze) de la șase specii vegetale, principala concluzie fiind că părțile uscate ale celor șase specii (provenite din cultură) sunt practic lipsite de acid ascorbic (sub limita de detecție). Acidul ascorbic este deosebit de instabil în soluțiile extractive preparate din aceste specii, în ciuda utilizării unor metode convenționale de stabilizare, cum ar fi utilizarea acizilor anorganici sau organici (orto-fosforic, oxalic). Deși se consideră că acetonitrilul acționează ca stabilizator, reducând de patru ori rata de degradare a acidului ascorbic, acesta a fost inefficient în stabilizarea acidului ascorbic în cursul extracției. Utilizarea metanolului (pur) ca solvent de extracție a asigurat stabilitatea acestui compus natural timp de aproximativ 3 ore.

Keywords: ascorbic acid, HPLC, herbal products

Introduction

Ascorbic acid ("vitamin C") is a natural compound with a large array of physiological functions in the living world, from microorganisms to angiosperms and primates. Ascorbic acid is considered one of the main endogenous antioxidant agents protecting cells, tissues and various biological compounds from the untoward effects of free radicals or other oxidative agents (e.g. it safeguards LDL together with vitamin E against oxidative damage induced by peroxy radicals [3]). However, under specific conditions it may also act as a pro-oxidant, this property giving rise to what has been termed "the ascorbic acid paradox" [11].

It is often reported that herbal medicinal products contain ascorbic acid (in various amounts). For instance, using a spectrophotometric method, Rafaela Guimarães et al. (2011) reported levels of

5-11 mg/g (0.5-1.1%) of vitamin C in decoctions and infusions prepared from mixtures of *Aloysia citrodora* Palau (lemon verbena), *Foeniculum vulgare* Mill. (fennel) and *Mentha spicata* L. (spearmint) [7]. Popular literature often exaggerates the content of certain medicinal herbs in vitamin C. "Mint is especially rich in carotenes and vitamin C", states one website [17], and another popular website asserts in a similar line that "peppermint is a good source of ... vitamin C" [16]. Within a larger research project we assessed the ascorbic acid contents in vegetative organs of six herbal species using an HPLC method, but we concluded that the dried parts of all six species (derived from culture sources) are virtually devoid of ascorbic acid. Moreover, our results showed that ascorbic acid is particularly unstable in extractive solutions prepared from these species, despite the use of conventional stabilization methods such as the use of inorganic acids.

Materials and Methods

A Varian HPLC system, equipped with a Prostar 240SDM quaternary high-pressure pump and diode-array detector (PDA, Prostar 3309) has been used for the separation of ascorbic acid. C18 (Inertsil 5 ODS-2, 250 x 4.6 mm (i.d.)), C8 (Inertsil 5 C8-3, 150 x 4.6 mm (i.d.)), and amino (Microsorb 100-5 Amino, part. 5 μ m) columns were used as a stationary phase. The analytical column was protected by a guard column (Amino, part. 10 μ m). Scharlau gradient-grade acetonitrile and methanol for liquid chromatography, as well as ortho-phosphoric acid 85% and oxalic acid of analytical grade (Merck) were used for the preparation of the mobile phase. Pure pharmaceutical grade ascorbic acid was acquired from NutriVita and its purity assessed against a certified reference substance purchased from Scharlau.

Herbal products: Dried vegetative organs (roots and subterraneous stems, aerial stems and leaves, two or three organs for each species) of *Mentha x piperita* L., *Glycyrrhiza glabra* L., *Medicago sativa* L., *Tagetes erecta* L., *Acorus calamus* L. and *Petroselinum crispum* (Mill.) Fuss (syn. *Petroselinum sativum* Hoffm.) were kindly provided by Hofigal Import Export S.A., Romania, being harvested in 2014 and dried in industrial conditions. Subterraneous organs, aerial stems and leaves or - where relevant - leaflets were separated for analysis. In the case of *Petroselinum crispum*, stems were extracted together with petioles, as on the fragmented dry product it was impractical to separate them and dried roots were not available. Each product was powdered by an electric mill and sifted through a stainless steel sieve of 250 μ m mesh size.

Sample preparation for HPLC: 1 gram of each sample was accurately weighed in a volumetric flask of 25 mL and filled up to the mark with solvent (solution of o-phosphoric acid 0.1% or methanol, as discussed below) and sonicated for 15 minutes. An aliquot of 10 mL was centrifuged 10 minutes at 6000 rpm. The supernatant was filtered through a 0.45 μ m filter and injected into the HPLC system. An infusion of *Menthae folium* was prepared according to the Romanian Pharmacopoeia, using a drug:solvent ratio of 1:25, centrifuged, filtered and injected in a similar way.

Chromatographic conditions: The mobile phase consisted from a mixture of a solution of o-phosphoric acid 0.1% (A) and acetonitrile (B). The separation was carried out in isocratic conditions: 20% A, 80% B. The column was maintained at a temperature of 25°C, a flow rate of 1 mL/min and an injection volume of 20 μ L was used. The chromatograms were recorded at 243 nm.

Ascorbic acid was identified by comparing its retention time (tR) and UV spectrum with those of the reference substance. Spectral purity was

checked by assessing the available diode array detector (DAD) data using the "peak purity" option in the Varian MS Workstation ver. 6.9.1. software.

Stability of ascorbic acid stock solution: The stability of an ascorbic acid stock solution (1 mg/mL) prepared in o-phosphoric acid 0.1% was assessed by successive injections up to 360 minutes. A similar procedure was applied for extractive solutions from herbal products obtained with different solvents (as discussed below) spiked with known amounts of ascorbic acid (the resulting solutions having an ascorbic acid level of about 20 μ g/mL).

Linearity, limit of detection (LOD) and limit of quantitation (LOQ): a calibration curve in 5 points (11.17-55.85 μ g/mL) was prepared from the stock solution by appropriate dilutions. The detection (LOD) and quantification limit (LOQ) for ascorbic acid were estimated as the minimum concentration giving a signal-to-noise ratio (S/N) of 3 and 10, respectively; the computation was based on the standard deviation of the regression line and slope of the calibration curve (LOD = 3 sd/S and LOQ = 10 sd/S, where sd is the standard deviation and S the slope of the regression line) [15].

Results and Discussion

We have initially tested an HPLC method based on a C18 column and using a mixture of o-phosphoric acid 0.1% and methanol in isocratic conditions (95:5) as mobile phase. While this method has been proved appropriate and useful for certain herbal preparations, such as fruits extracts, it was unsuitable for the six herbal products analysed, as it was not able to separate the ascorbic acid from other phytochemicals present in the herbal analytical matrix of the samples. Because of the very high proportion of the polar solvent (95%), there was little space for modulating the chromatographic conditions to improve the separation.

We therefore turned to a method based on an -NH₂ stationary phase, using acetonitrile and o-phosphoric acid 0.1% in a proportion of 80:20. This was shown to ensure a good separation for the majority of the products examined (the ascorbic acid having a retention time around 6.6), the only exception being *Tagetes erecta* leaf (for which there was overlapping in the retention time for an abundant compound from the extract and the ascorbic acid).

The stability study has shown that in o-phosphoric acid 0.1% ascorbic acid is stable for about 5.30-6.00 hours (at 360 minutes there was a slight decrease in peak area, suggesting degradation).

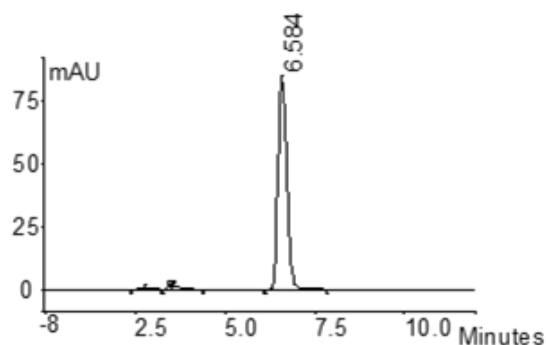
Linearity data for ascorbic acid with the method applied are provided in Table I (the three equations refer to each of the single injections and the average of two injections for the calibration curve).

Table I

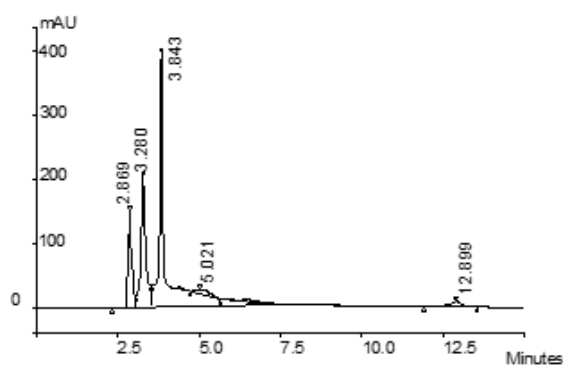
Linearity data for ascorbic acid with the method applied: regression equation (Y corresponds to the peak area, X to the amount in $\mu\text{g/mL}$), correlation coefficient (r), limit of detection (LOD) ($\mu\text{g/mL}$), limit of quantitation (LOQ) ($\mu\text{g/mL}$) and linear range ($\mu\text{g/mL}$)

Regression equation	Regression coefficient (r)	LOD $\mu\text{g/mL}$	LOQ $\mu\text{g/mL}$	Range $\mu\text{g/mL}$
$Y = 57379.15x + 200746.43$	0.9995	2.03	6.75	11.17 - 55.85
$Y = 55151.90x + 224802.16$	0.9987	3.30	11.00	11.17 - 55.85
$Y = 56265.53x + 212774.29$	0.9993	2.42	8.06	11.17 - 55.85

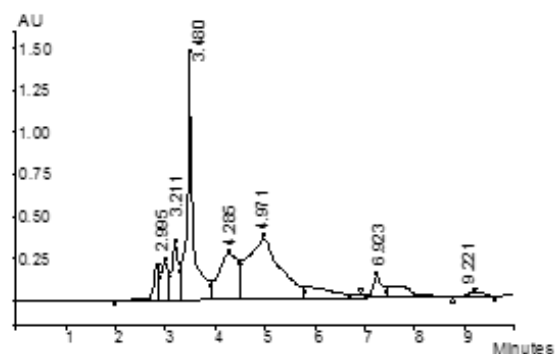
We intended to evaluate the repeatability, precision and accuracy of the method, but we have abandoned this because no amount of ascorbic acid could be detected in any of the tested herbal samples. Although in some cases (e.g. *Medicago sativa* leaf) a peak was present at a similar retention time with that of ascorbic acid, the UV spectrum was completely different, ruling out its presence in the sample. Illustrative chromatograms of the reference substance and of herbal samples are provided in Figures 1-5.

**Figure 1.**

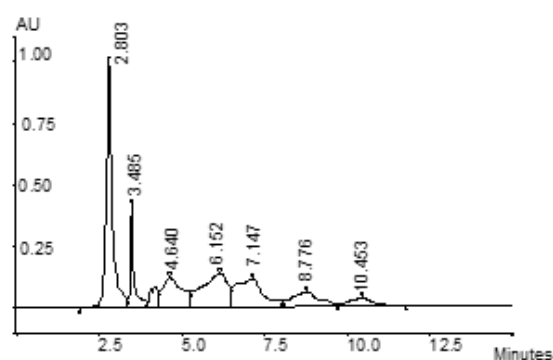
Representative chromatogram of the reference substance (ascorbic acid)

**Figure 2.**

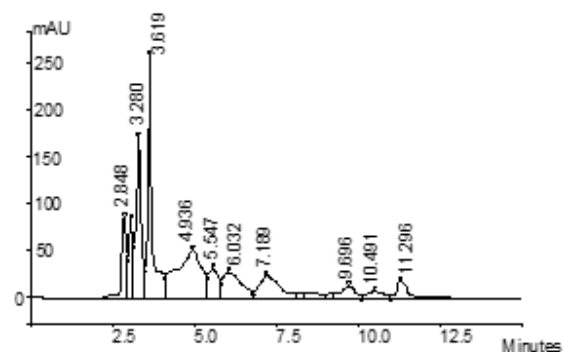
Chromatogram of the extractive solution prepared from *Mentha x piperita* leaf with o-phosphoric acid 0.1%

**Figure 3.**

Chromatogram of the extractive solution prepared from *Medicago sativa* leaf with o-phosphoric acid 0.1%

**Figure 4.**

Chromatogram of the extractive solution prepared from *Acorus calamus* leaf with o-phosphoric acid 0.1%

**Figure 5.**

Chromatogram of the extractive solution prepared from *Petroselinum crispus* leaf with o-phosphoric acid 0.1%.

Not only is ascorbic acid under the detection limit in all herbal parts of the species evaluated, but we found that when spiking herbal extractive solutions (prepared in o-phosphoric acid) with amounts of pure ascorbic acid, the latter is degraded in a very accelerated manner (after immediate spiking to produce about 20 µg/mL of ascorbic acid, no peak is visible, as if no ascorbic acid was added). This suggests that the use of o-phosphoric acid is not sufficient to stabilize the ascorbic acid in the extraction process.

We have extracted the samples with o-phosphoric acid due to its stabilizing effect on ascorbic acid, as evaluated by us in the stability investigation, although it is known that o-phosphoric acid is not the most successful in stabilizing ascorbic acid. Meta-phosphoric acid and oxalic acids are considered most successful for this purpose, but meta-phosphoric acid is not suitable for this chromatographic system, as it has been shown that it reacts chemically with the -NH₂ stationary phase, leading to its degradation [5].

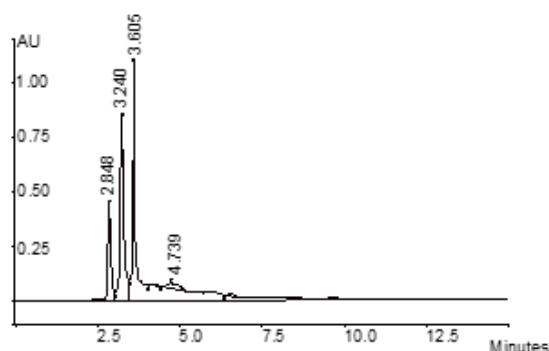


Figure 6.

Chromatogram of the extractive solution prepared from *Mentha x piperita* leaf with oxalic acid 0.5%

We have therefore tested the use of oxalic acid to stabilize the extraction of *Mentha x piperita* leaves, as it has been argued that this acid is one of the best, with a similar efficacy to that of m-phosphoric acid in the stabilizing effect on ascorbic acid [2, 12, 13]. However, when using a solution of 0.5% oxalic acid, not only there was no difference from the use of o-phosphoric acid (Figure 6), but the oxalic acid precipitated with the acetonitrile from the mobile phase (80%), causing temporary clogging of the injection loop. For this reason we have abandoned the use of oxalic acid with this mobile phase.

Although it has been argued in the literature (based on experimental data) that “acetonitrile considerably reduces the rate of ascorbic acid decomposition” [5], we have not observed a perceivable difference: after preparing a sample of *Mentha x piperita* leaf in mobile phase (80% acetonitrile), in the first injection no trace of ascorbic acid was detected.

As o-phosphoric acid was shown to be unable to stabilize ascorbic acid during extraction and since we could not use m-phosphoric or oxalic acids for stabilizing purposes, we investigated whether the use of a non-aqueous solvent, such as methanol would be more efficient for this purpose. And indeed, when the extraction of the herbal products was performed with methanol, ascorbic acid was considerably more stable (spiked solutions were stable for about 180 minutes), although its stability was still only about half of that seen with a solution prepared in o-phosphoric acid 0.1%.

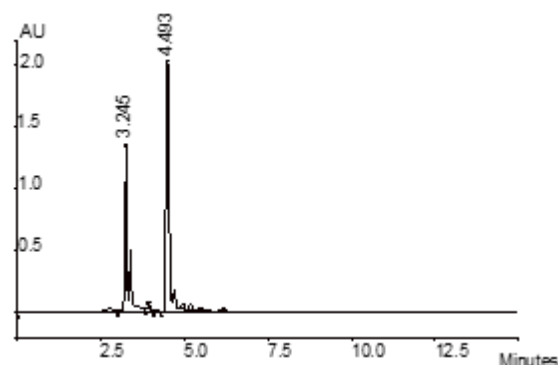


Figure 7.

Chromatogram of the extractive solution prepared from *Mentha x piperita* root with methanol

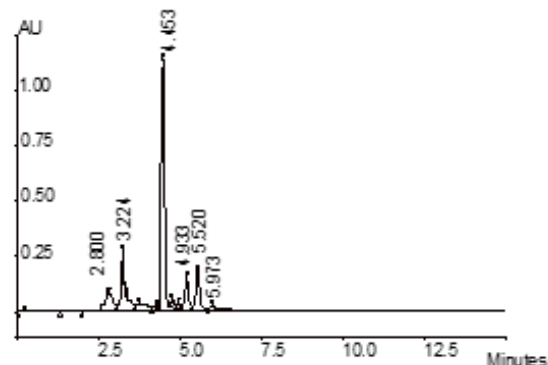


Figure 8.

Chromatogram of the extractive solution prepared from *Mentha x piperita* stem with methanol

Because we hypothesized that the non-detection of ascorbic acid in the species investigated was related to its instability in the extraction environment and not to a complete absence from the herbal products, we repeated the HPLC assessment of the products using methanol as an extraction solvent and injecting each sample immediately after its preparation, so as to avoid any degradation because of potential delays. Because the processing time took about 35-40 minutes and the stability in methanol, in these conditions, was about 3 hours, we should have been able to detect amounts higher than the detection limit of the method. It is

interesting to note that G. B. Golubitskii et al. (2007) [5] reported that ethanol was much less effective than acetonitrile in stabilizing ascorbic acid. Our findings suggest the contrary for a different alcohol (methanol): the alcohol is much more effective than acetonitrile. However, no peak of ascorbic acid was recorded in our sample chromatograms, confirming that it is not only a problem of extraction instability, but of a reduced amount in the dried herbal product. Illustrative chromatograms of the herbal samples extracted with methanol are shown in Figures 7-11.

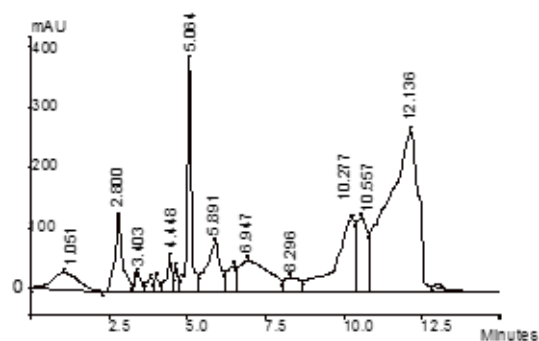


Figure 9.

Chromatogram of the extractive solution prepared from *Petroselinium crispum* stems and petioles with methanol

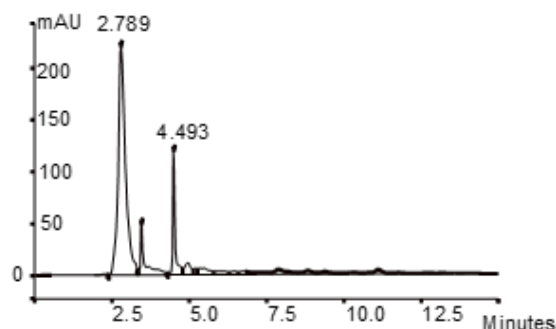


Figure 10.

Chromatogram of the extractive solution prepared from *Glycirrhiza glabra* roots with methanol.

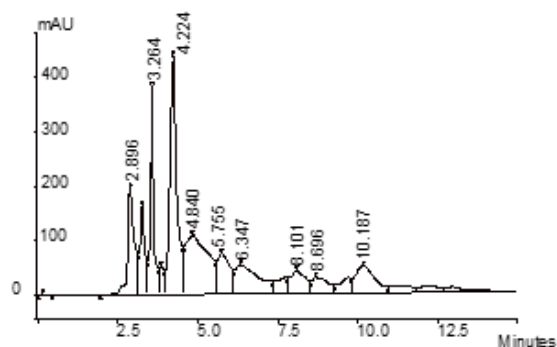


Figure 11.

Chromatogram of the extractive solution prepared from *Tagetes erecta* stems with methanol.

In the scientific literature, for various species of genus *Mentha*, levels, of ascorbic acid varying between 21.24 and 52.6 mg/100 grams of fresh matter have been reported [6]. In *Glycirrhiza glabra* root extracts, a relatively high amount of ascorbic acid (1.762 mg/g) was measured by a spectrophotometric method; it is stated that the product was acquired from the market place and stored in refrigerated conditions after manual grinding [14]. In *Medicago sativa* leaves a relatively elevated concentration (225 mg/100 grams) has been reported [9]. In the fresh leaves of *Acorus calamus* the concentration of ascorbic acid was estimated in an accession at 407.42 µg/100 g [8]. *Petroselinum crispum* leaf is considered a rich source of ascorbic acid, especially the cultivars of the *radicosum* group [1] (150 mg/100g [9]). We have not identified any published data on the ascorbic acid in fresh or dried parts of *Tagetes erecta*.

Considering the lowest level reported for *Mentha* species, in our samples we should have obtained concentrations of about 8.5 mg/mL (in the injected solution), which is over the limit of quantification. However, ascorbic acid is rapidly degraded during the drying process, which could explain why no amount was detected after a few months after harvesting and drying. It is also known that ascorbic acid is found higher in amounts in young plants and decreases considerably during the maturity and aging stages [9, 10]. As the tested plants (including *Mentha x piperita*) were harvested at full maturity, this may also explain the undetectable levels. It is also known that ascorbic acid levels tend to be higher for plants collected from the spontaneous flora, while those cultivated often have a fall in ascorbic acid amounts [4]. The plants investigated were derived from cultivation and this might also have contributed to the undetectable levels. Similar considerations are in principle valid for other species, although for most of them it is likely that ascorbic level in fresh states is higher than in *Mentha x piperita*.

Conclusions

It has been stated that “in dried herbs, the amount of ascorbic acid is much diminished” [9], but it has not been asserted how much. Our findings indicate that in dried products, most of the ascorbic acid is virtually degraded and such products – contrary to the popular opinion - should not be considered as sources of ascorbic acid.

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