COMPARATIVE HPLC DETERMINATION OF ASCORBIC AND DEHYDROASCORBIC ACIDS IN *"PEPERONI DI SENISE"*, A GEOGRAPHICALLY EUROLABELLED SWEET PEPPER

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SUMMARY

In a small arable area around the town of Senise, Basilicata Region, Southern Italy, sweet pepper (*Capsicum annuum* L.) is traditionally grown under Mediterranean climate, and its consumption is customary by more than hundred years. The prevalent variety cultivated in this zone is the acuminatum cv., which in 1996 received the European label of "Product with a typical Geographic Indication". Sweet pepper assumes a relative importance for the local people`s diet as a source of ascorbic acid (vitamin C). In this study, the amount of total vitamin C in fruits of sweet pepper in 14 local ecotypes and other two commercial varieties was investigated. Analyses were performed by HPLC-UV technique. Results show a wide variation, and high content of vitamin C in pepper fruits ranging from 167 to 246 mg per 100 g fresh weight (FW). Selection of the pepper ecotypes with high levels of vitamin C is very important for identifying the most appropriate strategy and environmental conditions for '*in situ'* preservation of the "Peperoni di Senise" (Senise peppers) germo-plasma and to enhance fruit quality. The information reported in this work could be of practical use for breeders and the development of antioxidant-rich varieties, following genetic improvement of cultivars.

KEYWORDS: Ascorbic acid, Dehydroascorbic acid, Sweet pepper, *Capsicum annuum* L., "*Peperoni di Senise"*, HPLC-UV.

INTRODUCTION

During the last years, great concern has been focused on antioxidant molecules in fruits and vegetables, due to their beneficial role in dietary intake and health prevention [1-6]. Antioxidant molecules control the oxidation-reduction reactions and, when associated with metal or metal-enzymes, show a good protection effect against free radical damages and peroxidation of human and plant tissues [7, 8].

Vitamin C indicates the whole compounds exhibiting the biological activity of ascorbic acid. The ascorbic acid (AA) and the other biologically active form, dehydroascorbic acid (DHA), show the same anti-scorbutic activity. The total amount of vitamin C is expressed as the sum of AA and DHA [9, 10]. Vitamin C is usually considered as an important factor for the assessment of nutritional quality of fresh products [11]. Many factors including agricultural practices, genomic differences, ripening process, climatic conditions, and light have a strong influence on chemical composition and vitamins amount in fruits and vegetables [12].

Pepper (*Capsicum annuum* L.), belonging to Solanaceae family, is a popular vegetable species worldwide cropped. The fruits represent a remarkable source of antioxidants, flavonoids, phenolic acids, carotenoids, and vitamins [13, 14]. About 90 varieties have been described without considering the large number of existing ecotypes. In several regions of Italy, the favourable climate and the soil characteristics favour the survival cultivation of a great number of landraces, specifically adapted to local conditions. These local landraces are very important to prevent the genetic erosion, and to preserve pepper genetic resources [15]. The sweet pepper produced by more than hundred years in Senise arable area, Basilicata Region, Southern Italy (Fig. 1), is a brick-red sweet pepper, acuminatum cultivar, which in 1996 received the European label of "Product with a typical Geographic Indication". Forty-two different ecotypes of *C. annuum* landraces, based on a farmer-made selection of seeds and labelled as "Peperoni di Senise" (Senise peppers), were found in Senise area [16].

Determination of vitamin C can be performed by various methods including titrimetric and fluorimetric [17], spectrophotometric [18], chromatographic [19], polarographic-amperometric [20, 21], coulometric [22], and enzymatic techniques [23], but HPLC is the system with high specificity and sensitivity for vitamin C detection in fruits [24].

FIGURE 1 - Landrace cultivation area in the Basilicata region, Southern Italy (surface devoted to "Peperoni di Senise" = 65 ha; annual production = 850 t).

This study was straightened up to investigate the total amount of vitamin C in 14 local ecotypes compared to sweet and hot commercial varieties, as a first quantification of quality parameters needed to address the breeding selection of "Peperoni di Senise" [16]. Since the presence of heavy metals in the vegetable matrix can produce instability of the ascorbic acid [25], iron and copper contents, together with the applicability of a modified extraction method, tandem HPLC detection of AA and DHA, were also evaluated.

MATERIALS AND METHODS

Plant materials

Plants of sweet peppers (*Capsicuum annum* L., acuminatum cv.) [26] were grown according to recommendations of EU official rules for "Peperoni di Senise" (CE Official Journal, L 163, 02/07/1996) in an arable field of Senise (340 m a.s.l.), Southern Italy (40° 08' N, 16° 17' E), characterized by Mediterranean climate. The soil was a silt-loam type with the following characteristics: sand 320 g kg^{-1}, loam 520 g kg^{-1}, clay 160 g kg^{-1}, pH 7.4. For the experiment, 14 of the major ecotypes cultivated in Senise area were selected, plus one hot pepper and two sweet commercial varieties (Lipari and Zico cultivars). Fruits were collected during the summer at the physiological maturity stage (red), and stored in the dark at -20°C.

Chemicals and reagents

L-ascorbic (AA) and dehydroascorbic (DHA) acids were purchased from Sigma-Aldrich (Milan, Italy). All solvents used were of HPLC-grade, and purchased from Merck (Darmstadt, Germany). The other reagents were of analytical grade. Ultrapure water at 18 MΩ was obtained with a MilliQ system from Millipore Corporation (Milford, MA, USA). Standard solutions of the ascorbic acids were freshly prepared in 135 μM EDTA plus 0.1 M H_2SO_4 at pH 2 by appropriate dilutions of 1.0 mg mL^{-1} stock solution.

Sample preparation

For the extraction, the method suggested by Wimalasiri and Wills [27] was modified substituting citric acid with EDTA. Briefly: 12 pepper fruits of each ecotype were reduced into small pieces (about 1 cm^3) and 25 g of these were extracted with 100 mL of 135 μM EDTA plus 0.1 M H_2SO_4 until pH 2 (dilution factor, F=5). Samples were Vortex-homogenized for 2 min, and the homogenized suspensions were centrifuged at 7,000 rpm for 10 min and supernatants collected. Prior to HPLC analysis, all samples were filtered using 0.45 μm Millex-HV filters (Millipore, Milford, MA, USA). All samples were protected against sunlight during preparation, extraction and investigation.

HPLC equipment

A Hewlett Packard (San Fernando, CA, USA) model 1090 liquid chromatograph, equipped with a Reodyne injector (loop 20 μL) and a Diode Array Detector, was used for analyses. The column was a Lichrosorb C18 (250 x 4.6 mm i.d., 5 μm particle size) (Alltech, Nicholasville, KY, USA) plus a security guard column. The temperature was kept at 25 °C during LC analyses. An isocratic mobile phase was used: 40 mM CH_3COONa, 0.5 mM $[(C_4H_9)_4N]H_2PO_4$ and 2% CH_3OH, adjusted to pH 6.2 with glacial CH_3COOH, flow rate 0.7 mL min^{-1}. Detection of AA and DHA was carried out at λ = 254 and 214 nm, respectively. To obtain stable retention times, the column was preconditioned by washing with water and methanol for 12 h (5:95 v:v) at reduced flow (0.15 mL min^{-1}). Chromatographic peaks were identified and quantified by comparing both retention time and absorbance values to those of standard solutions. Recovery tests were performed by spiking fruit samples with standard solutions before homogenization.

Iron and copper content

Iron and copper contents were determined in clear solutions, after mineralization of homogenized samples, with a Varian (Melbourne, VIC, Australia) model Spectra AA10 atomic absorption spectrophotometer operating in flame mode. The samples were mineralized in a mixture of concentrated nitric and perchloric acids (4:1 v:v). Standard solutions of heavy metals were used to calibrate the atomic absorption signals in the range 0.05-10.0 mg L^{-1}.

Statistical analyses

Statistical significance was determined by one-way analysis of variance using Statistica 5.1 software (Statsoft, Tulsa, OK, USA).

RESULTS AND DISCUSSION

Analytical method

It is well-know that ascorbic acid produces hydrogen peroxide in the presence of metal catalysts, such as Fe and Cu [28]. Ascorbic acid and Fe-salts showed degradation effects on different molecules, such as proteins [29], carbohydrates [30] and synthetic polymers [31]. In the presence of transition metals and light, the enzyme ascorbic acid oxidase is able to activate the reaction with oxygen, which is the real source of AA instability. For this reason, the addition of small amounts of a chelating agent may be required during the extraction phase from vegetable tissues containing heavy metals [25, 32]. Addition of a strong ferric chelant was shown to keep iron in solution (ferric complex-forming) and protect ascorbic acid from being oxidized in standard solutions and sample extracts [33]. AA itself exerts a good chelating action on metals contained in vegetables. Metaphosphoric acid (MPA) and other chelant molecules were proven to be useful dissolving agents for the determination of AA and the stabilization of the HPLC pre-column. MPA is the most efficient AA extraction agent, however, having a retention time similar to that of DHA and absorbing strongly at 214 nm, it can interfere with DHA determination. In addition, MPA can cause the drift of the chromatogram baseline. Finally, pH influences the stability of ascorbic acid that exhibits maximal stability in the pH range 4-6 [34].

In our experiments, the presence of the metals Fe and Cu (Table 1), even though slight, suggested the use of a strong chelating agent (EDTA) and acidic medium (H_2SO_4) to stabilize ascorbic acid in the standard solutions and extracted samples. The HPLC chromatograms of standards (kept in the dark) did not exhibit a significant reduction of peak intensities after 1, 4, 8 and 16 h of storage. The reproducibility of signals, measured on 6 real samples spiked with AA and DHA, showed an average variation coefficient lower than 1% for both acids, and recoveries were 97.2 ± 1.2 and 98.1 ± 1.1 (n = 6), respectively. According to Ashoor *et al.* [35], EDTA was eluted at a shorter retention time than AA and DHA, and the chelating agent was not retained by the LC column, and the chromatographic peaks were well-resolved with no interference. The calibration curves were linear in the concentration range used for AA and DHA standard solutions (R^2 = 0.9949 and 0.9932, respectively). The quantification limits were 0.05 and 0.02 mg per 100 g of fresh weight (FW) for AA and DHA, respectively.

Comparative discussion of data

Normally, there are significant variations in fruit traits of different varieties, however, a given pepper genotype can include only a limited subset of different morphological characters. The close range of morphological variations existing among ecotypes of "Peperoni di Senise" has been documented by several workers [15, 36]. Senise peppers have a slightly elongated form and thin flesh. The typical morphological characters and also flavour are probably a consequence of climate, careful seed selection and cultivation methods used. Mean composition of the edible portion of fruits "Peperoni di Senise" is reported in Table 1. Pepper is not considered to be a significant source of mineral elements because their concentration is influenced by soil content, fertilization and cultural practices. The "Pep-

eroni di Senise" fruits were characterized by a low content of mineral elements, with the exception of potassium, and water content was about 85% FW. The other parameters were in the normal range reported in literature for most of sweet pepper varieties [14].

Table 2 shows the vitamin C contents (sum of AA and DHA) of the different ecotypes of "Peperoni di Senise" in comparison to a hot pepper and two sweet cultivars. Data are reported as means ± standard deviations of triplicate values. In the last column, there are reported significance characters according to Duncan test, and values followed by different letters are statistically different at P = 0.01.

Vitamin C is concentrated in plant regions with high metabolic activity and, particularly, during the ripening phase. For this reason, fruits were collected when they reached the physiological maturity, recognizable by their intense and uniform red colour. Ripening stadium is the main phenological factor influencing the amount of vitamin C in pepper fruit. Howard *et al.* [37] showed that in green and red peppers of several genotypes ascorbic acid increased up to the maturation and ripening stage. On the other hand, the beginning of vitamin C degradation coincides with the increase of the ascorbate oxidase activity at the end of the ripening period, as indicated by the appearance of brown shades on fruits' surface and their colour changing. Yahia *et al.* [38] reported that total ascorbic acid content in pepper fruits increased rapidly during their development, reaching a maximum of 136.1 mg per 100 g FW, 51 days from fruit set, and decreasing up to 50% of the maximum value, when 64 days were elapsed. According to Marín *et al.* [39], our experiments confirmed increasing vitamin C content during pepper ripening, and a depletion up to 38% (mean value) of the maximum amount during the following 4 weeks (data not reported).

TABLE 1
Composition of "Peperoni di Senise" (100 g fresh edible fruit); mean ± standard deviation on 14 triplicate samples.

Character	Unit	Mean	S.D.
Fruit edible portion	(g)	82.3	± 3.1
Dry matter	(g)	15.0	± 1.9
Total fibres	(g)	1.9	± 0.4
Carbohydrates	(g)	4.2	± 0.5
Lipids	(g)	0.3	± 0.1
Proteins	(g)	0.9	± 0.2
Phosphorus	(mg)	28.1	± 1.6
Calcium	(mg)	17.3	± 1.1
Potassium	(mg)	210	± 8
Sodium	(mg)	2.1	± 0.8
Copper	(mg)	0.11	± 0.04
Iron	(mg)	0.73	± 0.11

TABLE 2
Means and standard deviations of vitamin C contents (as sum of AA and DHA concentrations) in 14 different ecotypes of "Peperoni di Senise", as compared to a hot pepper and two sweet cultivars (according to Duncan test, values followed by different letters are statistically different at P ≤ 0.01).

Ecotype	mg/100 g FW	S.D.	Statistical Significance
1	179.9	± 1.6	ghi
2	172.9	± 1.9	hi
3	210.9	± 14.2	cde
4	183.4	± 7.8	ghi
5	209.8	± 13.0	cde
6	169.8	± 7.8	hi
7	194.6	± 4.1	efg
8	203.5	± 4.3	def
9	166.7	± 4.4	i
10	219.7	± 11.1	cd
11	188.6	± 7.2	fgh
12	224.3	± 19.2	c
13	212.7	± 3.9	cde
14	245.9	± 11.3	b
Hot pepper	268.8	± 4.2	a
Zico	120.3	± 5.1	k
Lipari	139.0	± 7.2	j

The total amount of ascorbic acids (AA plus DHA) in the 14 "Peperoni di Senise" ecotypes ranged from 166.7 to 245.9 mg per 100 g FW, with an average value of 198.8 mg (n = 14). The studied cultivar showed a significant level of vitamin C, higher than the other tested sweet cultivars and those reported by other authors. Vanderslice *et al.* [40] and Lee *et al.* [8] referred a vitamin C content of 155 and 134 mg per 100 g FW for red and green pepper varieties at the ripening time, respectively. Wimalasiri and Wills [27] showed values of vitamin C in a red pepper variety of 186.7 mg. Martinez *et al*. [41] reported 154.3 mg for the red pepper named "Fresno de la Vega". A variable amount, in the range 64-168 mg per 100 g FW, measured in five different varieties of red pepper, was reported by Lee *et al.* [5].

As expected, the tested hot pepper showed the highest vitamin C content. Nevertheless, many significant differences were also found among the 14 ecotypes of the acuminatum cultivar selected for investigation. These differences were probably due to environmental and agricultural factors, such as different selection criteria adopted by farmers as well as genetic contamination with commercial cultivars grown in the same area. The lower values of standard deviations, shown by ecotypes 1 and 2, indicate a substantial stabilization of their genetic characteristics, even though the vitamin C content remains within a medium range (170–180 mg). Besides, for ecotypes 3, 5, 10, 12, and 14, which were in the maximum range (210–246 mg) and revealed higher values of the standard deviations, more careful selection of seeds will be necessary to reduce the presumable genetic variability. In any case, the investigation evidenced highly statistically significant differences among the whole population of acuminatum ecotypes and the other cultivars, contributing to set the degree of genetic uniformity existing in the ecotypes of "Peperoni di Senise" with respect to the other pepper varieties.

Fresh pepper is a vegetable with high contents of vitamin C and carotenoids, the last determining the colour of the fruits. The quantification of these substances yields two important quality parameters, particularly influenced by plant genetics and environmental characteristics [40]. The concentration of pigments decreases with longer storage time, but increases with higher concentration of ascorbic acid [42]. Therefore, the nutritional value and chemical composition of peppers is related to the intensity of fruit colour. The "Peperoni di Senise" ecotypes showed not only a very intense colouring capacity, but also a satisfying bio-antioxidant content.

CONCLUSION

The results obtained with the proposed extraction and HPLC methods were satisfactory with respect to rapidity, simplicity and applicability to routine analyses of vegetables.

The differences among ecotypes found in this study might be a consequence of poor selection criteria as well as the seeding procedures adopted by farmers. Considering the popular use of peppers in the Basilicata Region, ascorbic and dehydroascorbic acids, present in a fairly good amount in *C. annuum*, have an evident nutritional relevance. The reported information could be of practical use for pepper breeding, future development of antioxidant-rich varieties, and genetic improvement of fruits. To realize the enhancement of fruit quality, it will be necessary to define the most appropriate strategies and environmental conditions for '*in situ'* preservation of the "Peperoni di Senise" germoplasma.

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