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Determination of Organic Acids in *Brassica Rapa* L. Leaves (Turnip Greens and Turnip Tops) Regulated by the Protected Geographical Indication "Grelos De Galicia"

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Abstract A rapid high-performance liquid chromatographic method for the determination of organic acids in *Brassica rapa* L. leaves is reported. Under optimum conditions the detection limits were between 0.26 to 8.00 µg/mL and quantification limits were between 0.28 to 12.50 µg/mL. The precision results showed that the relative standard deviations of repeatability and reproducibility were ≤ 0.84% and ≤ 1.49%, respectively. The recovery of the organic acids varied from 97.21 to 99.57%. Organic acids were determined in 44 samples of *Brassica rapa* var. *rapa* L. protected under the PGI "Grelos de Galicia" in the two plant status (turnip greens and turnip tops). The results showed that all of the samples presented a profile composed of at least four organic acids: citric, malic, oxalic, and ascorbic acids. However, these organic acids were present in different concentrations in turnip greens and turnip tops.

Keywords: *Vitamin C, Organic acids, HPLC, turnip greens, turnip tops*

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1. Introduction

Fruit and vegetable antioxidants are emphasized and recognized as important components which, since they are important sources of vitamins and antioxidants, reduce the risk of several chronic diseases. An increase of fruit and vegetable consumption has been linked to reduced risks of cardiovascular disease, stroke, arthritis, inflammatory bowel diseases, and some cancers [1,2]. This association is often attributed to the antioxidants present in vegetables, such as vitamin C, which prevents free radical damage.

Ascorbic acid is the most widely distributed water-soluble antioxidant in fruits and vegetables. Besides commercial vitamin C products, horticultural products are considered to be a very important source for ascorbic acid intake in the human diet [3]. However, the amount of ascorbic acid in fruits and vegetables depends not only on the species type, but also on genetic, environmental, climate phenology, and growing conditions, location factors, the agricultural techniques applied, and even on the differences in postharvest storage conditions [4,5].

Citric, malic and oxalic acids are commonly found in vegetables. The organic acid content varies among different types of vegetables, and may even show variation in the same plant depending on the degree of development or maturation. Moreover, the environment and agronomic practices may affect the expression of genes in the seeds, thereby determining changes in the organic acid contents [6]. The main organic acids present in vegetables are citric and malic acids; oxalic acid is present in higher amounts in green leaves [7,8,9]. Organic acids have a strong influence on the organoleptic properties of fruits and vegetables; in particular, they are responsible for sourness [6]. Oxalic acid and other carboxylic acids, such as malic and citric acids act as antioxidants, because they also have the ability to chelate metals [9]. Much concern has been given to high oxalic acid concentrations in leaves of plant species, especially those used as green leafy vegetables in the daily diet, because of the harmful health effects associated with a high intake of oxalic acid. Hence, the presence of high oxalic acid hinders the acceptance of newly identified sources of otherwise nutritious vegetables [10]. Oxalic acid can combine easily with Ca^{2+} and Mg^{2+} to form less soluble salts known as oxalates. High levels of oxalates in the diet can lead to the irritation of the digestive system and contribute to the formation of kidney stones. Hence, the detection of the oxalic acid content in food is of great practical significance [11].

The determination of the organic acid profile is important when characterising different genotypes, or when studying the influence of maturity or agronomic

factors on vegetable quality. Several methods have been developed for individually identifying and quantifying organic acids in different vegetal matrixes by using non-enzymatic spectrophotometric, enzymatic, gas chromatography (GC) and high-performance liquid chromatographic (HPLC) with different detectors [7,8,12].

In addition, various methods were reported for the determination of ascorbic acid (AA) in foods, including titration, enzymatic assays, spectrophotometry, fluorometry, voltammetry, electrophoresis and high-performance liquid chromatography (HPLC) or liquid chromatography coupled with tandem-mass spectrometry (LC–MS/MS) [1,13,14]. Martínez-Sánchez et al. [15] indicated that AA was the predominant form of vitamin C in all of the cruciferous species studied.

Brassica vegetables belong to the *Cruciferous* family which includes a variety of economically significant, horticultural crops, such as: broccoli, brussels sprouts, cabbage, collards, kale, turnip greens and leaf rape. They are consumed all year long as ingredients in different salads, or after the processing and/or cooking of these raw or frozen vegetables [16,17].

There is an increasing interest in the relationship between diet and health. In fact, food not only provides vital nutrients which are essential for life, but also other bioactive compounds which are necessary to promote health and prevent disease [17]. The relative contribution of each commodity to human health and wellness depends upon its nutritive value and per capita consumption; the latter is greatly influenced by consumer preferences and degree of satisfaction from eating different fruits or vegetables [6]. In the last few years, the importance of the quality of vegetables for consumers has continuously increased. The main criteria are sensory characteristics and greater health benefits. Varieties of turnip greens and turnip tops from NW Spain are an appreciable source of health-protective compounds [18,19]. Farnham et al. [20] indicated that leafy green *Brassica* (LGB) crops (i.e. turnip greens) are commercially and culturally important vegetables, especially in the southeastern United States where they are included among the most economically important vegetables. Furthermore, leafy forms of *Brassica rapa*, L. crops are very popular for farming and consumption in certain European countries such as: Portugal, Spain or Italy. In Spain, the largest producer and consumer of *Brassica rapa* L. is Galicia (NW Spain). About 100,000 t are collected here each year [21]. Given the nutritional and economic importance of such products, with the Order of December 23 [22], the Galician government approved the regulation of the Protected Geographical Indication "Grelos de Galicia". The products regulated by this PGI are defined as the vegetative parts suitable for human consumption from plants of the *Brassica rapa* L. var. *rapa* species (commonly known as the turnip), from Santiago and Lugo. Turnip greens are the leaves harvested in the vegetative period, while turnip tops are the fructiferous stems including the flower buds and the surrounding leaves, which are consumed before the buds open and while they are still green [18].

The two main aims of the present work were: (1) to develop a rapid method to simultaneously detect and quantify oxalic, malic, citric and ascorbic acids using high performance liquid chromatography (HPLC) in order to establish the organic acid composition of *Brassica rapa* L. leaves; and (2) to check the effect of the plant status (turnip greens and turnip tops), organic acids composition were determined in *Brassica rapa* L. leaves regulated by the Protected Geographical Indication "Grelos de Galicia".

2. Materials and Methods

2.1. Material

Brassica rapa L. samples (turnip greens and turnip tops) were supplied by different local farmers from areas included in the Protected Geographical Indication "Grelos de Galicia" [22] in Galicia (NW Spain). A total of 22 producers were selected and all the plants were analyzed as turnip greens and turnip tops (44 samples).

Plant material was collected and immediately transported to the laboratory for analysis. The samples were cleaned with water, selected, cut, and the edible portion was taken for analysis.

2.2. Chemicals and standards

Milli-Q water was obtained using a Millipore water purification system Milli-Q plus. Ascorbic acid (Cod. 401471000) and malic acid (Cod. 155059-0250) were purchased from Acros Organics. Citric acid, (Cod. 131808), oxalic acid (Cod. 131041.1210), sulphuric acid (Cod. 131058) and anhydrous sodium carbonate (Cod. 131648.1210) were provided by Panreac. Metaphosphoric acid (Cod. 1.00546.05000) came from Merck, and Acetone (Cod. AC0311) from Scharlau.

2.3. HPLC analysis of Organic Acids

Oxalic, malic, citric, and ascorbic acids were determined using a HPLC system equipped with a ternary pump (Jasco PU-1580), a Rheodyne manual injector with 20 µL injection loop, a column oven (Jetstream Plus, 90305-2) and a degasser (Gastor Mod.154). Separation and detection were carried out using a Spherisorb ODS2 250.0 x 4.6 mm C_{18} Waters column, (particle size 5 µm) with a Spherisorb ODS2 10,0 x 4.6 mm C_{18}, Waters pre-column (particle size 5 µm) and a UV/VIS diode array detector (Jasco MD-1515). Elution was isocratic with HPLC-water acidified to pH 2.2 with sulphuric acid at a flow rate of 0.4 mL/min. A wavelength of 245 nm was used for the detection of ascorbic acid and a wavelength of 215 nm was used for the detection of oxalic, malic and citric acids. Data was processed using Borwin-PDA Version 1.0 software. All analyses were carried out in duplicate, and injected at least twice; the results are expressed as mean values.

Sample preparation was performed using the method described by Vázquez et al. [232]. 10 g of a homogenized fresh sample was extracted with a 4.5% solution of metaphosphoric acid, and organic acids were determined by reverse-phase HPLC.

2.4. Statistical Analysis

Samples data is presented as means ± standard deviations. The Student's t-test for independent samples was applied to study the effect of plant status on organic acids content of *Brassica rapa* L samples. Statistical

calculations were performed using SPSS Statistics for Windows ver. 20.0.

3. Results and Discussion

Validation of a HPLC method for simultaneous determination of organic acids in B. rapa L. (turnip greens and turnip tops).

Two reverse phase columns were tested: a Phenomenex Luna C_{18} (5 µm, 250 mm x 4.60 mm I.D.) column and a Spherisorb ODS2 (5 µm, 250 mm x 4.6 mm I.D.) column. Since the Phenomenex Luna C_{18} (5 µm, 250 mm x 4.60 mm I.D.) column yielded overlaps between organic acids, the Spherisorb column ODS2 (5 µm, 250 mm x 4.6 mm I.D.) was selected to carry out the development of the proposed method for the simultaneous determination of organic acids and vitamin C.

The column was thermostated at different temperatures to ascertain the influence of the temperature on the retention time of the acids. The best results (retention time and appropriate peak resolution) were obtained with a column temperature of 25°C.

In previous literature, there are two mobile phases which are used for the HPLC determination of organic acids and vitamin C in food: water acidified with sulphuric acid [24] and water acidified with metaphosphoric acid. Sulphuric acid is more stable and economic than metaphosphoric acid and metaphosphoric acid presents a solvent front which interferes with the determination of organic acids with short retention times, mainly with oxalic acid. For these reasons, water acidified with sulphuric acid was selected as a mobile phase.

To determine the influence of pH, different values between 2.2 and 3.0 were tested using Milli-Q water acidified with sulfuric acid as a mobile phase. Precise pH value was very important since, with a C_{18} column, it is not advisable to work at pH lower than 2.0 because it deteriorates the fill and the C_{18} chains bonded to silica are lost. In contrast, working at high pH values decreases the retention times and hence the resolution; moreover, overlap between the different acids starts to appear. The best results (adaptation to the column and peak resolution) were obtained with a pH of 2.2.

Different flow rates of the mobile phase, between 0.2 and 1.0 mL / min, were tested, and the best results were yielded (retention time and appropriate peak resolution) at 0.4 mL / min.

Sample preparation: The reagents most commonly used in the extraction of ascorbic acid are: metaphosphoric acid, trichloroacetic acid and oxalic acid alone or in combination with other acids or short chain alcohols, such as methanol and ethanol [24]. After several trials, a 4.5% solution of metaphosphoric acid was selected as the extraction solvent [24]. To establish the extraction time 10, 15 and 20 minutes were tested. After 10 minutes, extraction was not complete, and at 20 minutes ascorbic acid was degraded.

Therefore, 15 minutes was selected for the extraction of organic acids in all samples of *Brassica rapa* L. Once the time of extraction of organic acids was selected, it was observed that the concentration of ascorbic acid was stable for up to 24 hours of storage (excluding light and under refrigeration) and the other organic acids for 5 days in the same conditions. Oxalic, malic, citric and ascorbic acids were identified by comparing their retention times with those of their corresponding patterns and were quantified using an external calibration standard.

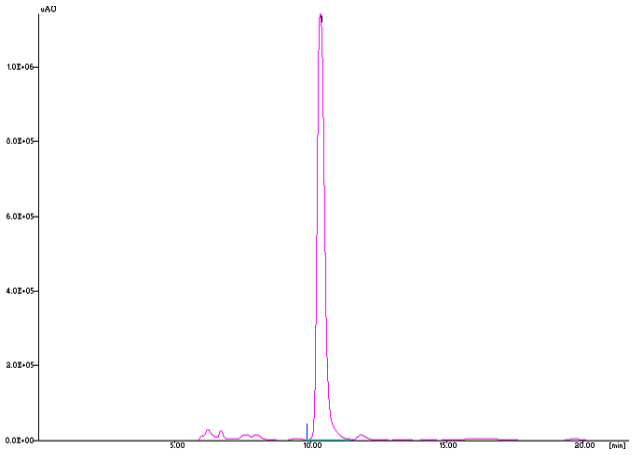

Figure 1. Chromatogram of ascorbic acid (1) in *Brassica rapa* L. at 245 nm

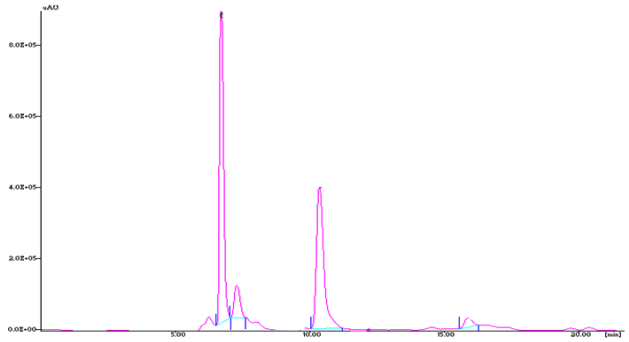

Figure 2. Chromatogram of the oxalic (1), malic (2), ascorbic (3) and citric (4) acid in *Brassica rapa* L. at 215 nm

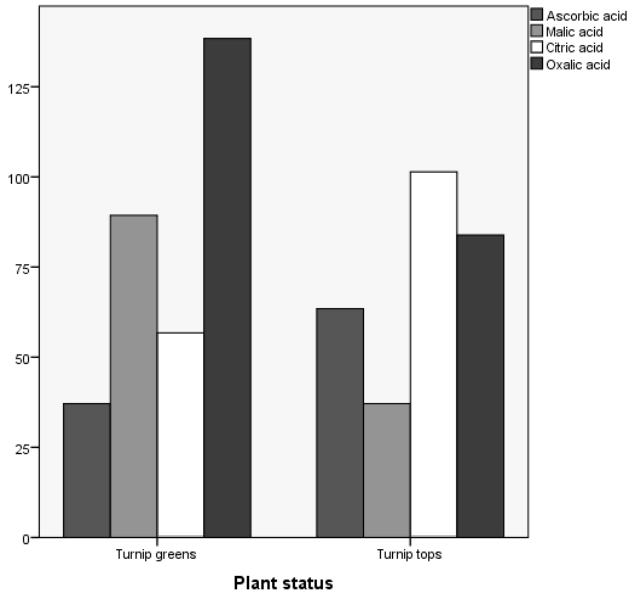

Figure 3. Oxalic, malic, citric and ascorbic acid (mg/100 g fresh weight) of turnip greens and turnip tops analyzed

Figure 1 shows the chromatogram of ascorbic acid in *Brassica rapa* L. at 245 nm and Figure 2 the chromatogram of organic acid at 215 nm. Standard solutions of OAS were analysed by HPLC and a standard

curve was constructed and used in the analytical quantifications. The linearity range for this assay was determined as 4.8-50.4 µg/mL (R^2 = 0.9996) for oxalic acid, 40.5-550.8 µg/mL (R^2 = 0.9975) for malic acid, 24-320 µg/mL (R^2 = 0.9991) for citric acid and 8.8-178 µg/mL (R^2 = 0.9995) for ascorbic acid. All analyses were carried out in duplicate and injected at least twice; the results are expressed as mean values. The detection limits, LOD [25] were 0.26 µg/mL for oxalic acid, 8.00 µg/mL for malic acid, 7.30 µg/mL for citric acid, and 0.25 µg/mL for ascorbic acid. The quantification limits LOQ [25] were 0.29, 12.50, 11.20 and 0.28 µg/mL for oxalic, malic, citric and ascorbic acid respectively. To assess repeatability, the entire process was repeated six times (from the same sample) and coefficients of variation were 0.84, 0.78, 0.72 and 0.59% respectively for oxalic, malic, citric and ascorbic acid. To assess reproducibility, samples were analyzed on three different days in triplicate and coefficients of variation were 1.14, 1.34, 1.49, and 1.39% respectively for oxalic, malic, citric and ascorbic acid.

The recovery of each organic acid was studied to evaluate the extraction efficiency of the proposed methods. Recoveries for oxalic, malic, citric and ascorbic acid were 97.21, 99.57, 99.34 and 97.74% respectively.

The proposed method provides a way to identify and quantify the organic acid contents (oxalic, malic, citric and ascorbic acids) in *Brassica rapa* L. samples.

Organic acid contents in samples of B. rapa L. (turnip greens and turnip tops).

Figure 3 shows oxalic, malic, citric and ascorbic acid average contents (mg/100 g fresh weight) of the 22 samples of turnip greens and the 22 samples of turnip tops analyzed.

The variability in the organic acid content in vegetables is dependent upon many factors, such as farming, the method of collection or the maturity index [4].

The oxalic acid content in the analyzed samples was between 138.40 ± 56.38 and 83.89 ± 46.65 mg/100 g fresh weight for turnip greens and turnip tops respectively. Oxalic acid is an organic acid which is widely distributed in various organisms, especially in plants. It has been found in the roots and/or leaves of rhubarb and buckwheat, potatoes, beans, spinach, beets, tomatoes, cauliflower, onions, mushrooms, and celery root, among the vegetables [26,27]. Wu et al. [27] found that the content of oxalic acid in spinach was 420 mg/100 g fresh weight; these values are somewhat higher than the 324 ± 0.19 mg/100 g fresh weight reported by Zheng et al. [11]. Uusiku et al. [28] collected data of oxalic acid content present in many African leafy vegetables edible portion such as *E. hirta* (1115 mg/100 g fresh weight), *Ipomoea involucrata* (913 mg/100 g fresh weight), *Xanthosomas sp*. (654 mg/100 g fresh weight), *Amaranthus sp*. (40–50 mg/100 g fresh weight), *M. esculenta* (20 mg/100 g fresh weight), *Celosia argentea* (20 mg/100 g fresh weight), *Telfaria occidentalis* (40 g/100 g fresh weight) and *Vernonia sp.* (1-2 g/100 g fresh weight). Morales et al. [29] reported oxalic acid values in edible portion of non-cultivated vegetables whose basal leaves have been traditionally consumed in Spain (581 ± 428.64 mg/100 g fresh weight for *Beta marítima L.*, 93 ± 11.71 mg/100 g fresh weight for *Chondrilla juncea* L., 446 ± 321.56 mg/100 g fresh weight for *Papaver rhoeas* L., and 485 ± 470.45 mg/100 g fresh weight for *Scolymus hispanicus* L.).

The malic acid content in the product varied between 89.34 ± 39.70 mg/100 g fresh weight in turnip greens and 37.12 ± 12.41 mg/100 g fresh weight in turnip tops. Belitz et al. [30] show variations in vegetables between 42 mg/100 g fresh weight in spinach and 910 mg/100 g fresh weight in rhubarb. Ayaz et al. [7], reported lower values of 151 mg/100 dry mater, in kale (*Brassica oleraceae* L.var. acpehala DC) leaf. Flores et al. [12] indicated that leafy vegetables showed a high concentration of malic acid that varied between 190, 83, 81, 575 and 233 mg/100 g fresh weight in green pepper, red pepper, tomato, lettuce and lamb's lettuce, respectively. The values reported by Morales et al. (2014), in non-cultivated vegetables, were 51.36 ± 7.41 mg/100 g fresh weight for *Beta marítima L.* and 147.19 ± 92.49 mg/100 g fresh weight for *Papaver rhoeas* L.

The citric acid content in samples of turnip greens and turnip tops was between 56.75 ± 26.16 and 101.42 ± 38.72 mg/100 g fresh weight. Ayaz et al. [7], reported values of 2213 mg/100 g dry mater in kale (*Brassica oleraceae* L.var. acpehala DC.) leaf. Flores et al. [12] indicated that leafy vegetables showed a high concentration of malic acid that varied between 77, 789, 1064, 118 and 356 mg/100 g fresh weight in green pepper, red pepper, tomato, lettuce and lamb's lettuce, respectively. Morales et al. (2014) reported values of 126.24 ± 11.37 mg/100 g fresh weight for *Beta marítima L.*, 45.65 ± 5.89 mg/100 g fresh weight for *Chondrilla juncea* L., 149.04 ± 106.55 mg/100 g fresh weight for *Papaver rhoeas* L., and 59.51 ± 4.49 mg/100 g fresh weight for *Scolymus hispanicus* L., in edible portion of non-cultivated vegetables whose basal leaves have been traditionally consumed in Spain.

The ascorbic acid content in the analyzed samples was between 37.13 ± 13.20 and 63.47 ± 11.23 mg/100 g fresh weight for turnip greens and turnip tops respectively. The values obtained were within the range indicated by Podsedek [16] who indicated that the content of vitamin C varies significantly among *Brassica* vegetables and within their subspecies. Vitamin C levels varied over a 4-fold in broccoli and cauliflower, 2.5-fold in Brussels sprouts and white cabbage, and twice in kale. Martínez-Sánchez et al. [15] evaluated the vitamin C content in different species of *Brassicaceae* leaves and found that the highest content of AA was in leaves of watercress and wild rocket (81 and 73 mg 100/g fresh weight, respectively) while mizuna and salad rocket had the lowest (52 mg/100 g fresh weight). Olivera et al. [1], reported contents in harvested brussels sprouts of 89 mg/100 g fresh weight. Martínez et al. [3], found contents between 89.90 to 143.4 mg/100 g fresh weight, in whole and leaves of turnip tops respectively. Farnham et al. [19] studied leaf green Brassica crops: 15 collard (*Brassica oleracea* L.), 2 mustard (*Brassica juncea* L.) and 2 turnip (*Brassica rapa* L.) and found that ascorbic acid concentrations were between 58.7 mg and 52.8 mg/100 g fresh weight. The ascorbic acid content, in edible portion of non-cultivated vegetables whose basal leaves have been traditionally consumed in Spain, reported by Morales et al. (2014) were of 9.99 ± 1.62 mg/100 g fresh weight for *Beta marítima L.*, 1.76 ± 1.58 mg/100 g fresh weight for *Chondrilla juncea* L., 14.11 ± 2.25 mg/100 g fresh weight for *Papaver rhoeas* L., and 1.11 ± 0.18 mg/100 g fresh weight for *Scolymus hispanicus* L.

Although turnip greens and turnip tops are the same product, they are differentiated, produced and consumed in different stages of the plant. Thus, the Student t-test for independent samples was applied to study the effect of plant status on the organic acid contents in the samples.

Depending on plant development, there were significant differences (Table 1) for all variables studied.

Table 1. Student's t-test results according to the plant status

Variables	t	p-Value
Ascorbic acid	-10.080	0.000***
Malic acid	8.325	0.000***
Citric acid	-6.342	0.000***
Oxalic acid	4.941	0.000***
	t	Sig
Acido ascórbico	-10.080	0.000
Acido málico	8.325	0.000
Acido cítrico	-6.342	0.000
Acido oxálico	4.941	0.000

*** p<0.001

The oxalic acid content was always less in turnip tops with mean values of 83.89 mg/100 g fresh weight versus mean values of 138.40 mg/100 g fresh weight in turnip greens. Similar behavior occurs with malic acid for the turnip tops that had significantly lower values, with averages of 37.12 versus 39.70 mg/100 g fresh weight for turnip greens.

Moreover, the citric acid content was higher in turnip tops with mean values of 101.42 versus 56.75 mg/100 g fresh weight in turnip greens. This behavior was the same in the case of ascorbic acid; the content was always less in turnip greens with mean values of 37.13 versus 63.47 mg/100 g fresh weight mean values in turnip tops.

4. Conclusions

A fast and reliable HPLC method was developed for the simultaneous determination of organic acids (oxalic, malic and citric acid) and ascorbic acid in *Brassica rapa* L samples. According to the validation study, the proposed method was precise and accurate, and showed appropriate sensitivity for the determination of organic acids in turnip greens and turnip tops. This methodology can be used in routine analysis in order to determine organic acids in *Brassica rapa* L vegetables, and may be extended to other matrices or products.

After application of the proposed method, the results indicate that the turnip tops presented a higher content of citric and ascorbic acids than the turnip greens, whereas the contents of malic acid and oxalic acid were higher in turnip greens.

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