

Free Amino Acids of Tronchuda Cabbage (*Brassica oleracea* L. Var. *costata* DC): Influence of Leaf Position (Internal or External) and Collection Time

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The free amino acid profile of 18 samples of tronchuda cabbage (*Brassica oleracea* L. var. *costata* DC) leaves, harvested at three different months, was determined by HPLC/UV–vis. The tronchuda cabbage leaves total free amino acid content varied from 3.3 to 14.4 g/kg fresh weight. Generally, arginine was the major compound, followed by proline, threonine, glutamine, cysteine, and glutamic acid. This study indicates that free amino acids are not similarly distributed: in external leaves, proline and arginine were the major free amino acids, while in internal ones, arginine was the main free amino acid, followed by threonine, glutamine, and cysteine. Significant differences were observed for valine, proline, arginine, leucine, cysteine, lysine, histidine, and tyrosine contents. The levels of some free amino acids were significantly affected by the collection period. In external leaves, this occurred with glutamic acid, serine, valine, leucine, cysteine, and ornithine contents, while in internal leaves, it occurred with aspartic acid, arginine, and total contents.

KEYWORDS: *Brassica oleracea* L. var. *costata* DC; tronchuda cabbage; internal and external leaves; free amino acids

INTRODUCTION

Experimental, clinical, and population studies confirmed the benefit of diets that are rich in fruits and vegetables for the prevention of cardiovascular diseases, cancer, hypertension, diabetes, and obesity (1). Brassicaceae plants represent one of the major vegetable crops grown, worldwide, constituting an important part of a well-balanced diet (2), such as a Mediterranean one. In Europe, owing to the availability of local markets, inexpensiveness, and consumer preference, cruciferous vegetables, such as cabbage, are among the most important dietary vegetables consumed (1). Several epidemiological studies report an inverse correlation between consumption of Brassicaceae and risk of cancer (1). The cancer preventive properties mainly were attributed to the glucosinolates and their derived products; however, phenolic compounds, such as flavonoids, also contribute to these capacities (3).

Tronchuda cabbage (*Brassica oleracea* L. var. *costata* DC) is especially popular in Portugal, having a determinant role in

the Portuguese diet and agricultural systems (2). It is a hardy crop that is high yielding, less susceptible to pests and diseases, well-adapted to a wide range of climates, and generally grown with little or no agrochemical input. The cabbage plant resembles a thick-stemmed collard with large floppy leaves. The leaves are close together, round, smooth, and slightly notched at the margins and are eaten raw or cooked. The internal and external leaves are considerably different with regard to organoleptic characteristics, which may influence the consumer's preferences. Internal leaves are pale yellow, tender, and sweeter than external leaves, which are dark green (3).

Consumers have increased their awareness concerning food composition, and further comprehensive information has been demanded, beyond that available in food composition tables. In such tables, the amino acid composition is limited and mostly given as both free and bound amino acids. Since free amino acids are involved in secondary plant metabolism and in the biosynthesis of compounds, such as glucosinolates and phenolics, which directly or indirectly play an important role in plant–environment interaction and human health, free amino acid profile determination becomes more relevant (4).

In the past few years, the *B. oleracea* var. *costata* chemical composition and antioxidant potential were studied by our

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research group (2, 3, 5–8). Regarding phenolic composition, external leaves showed high levels of complex flavonol glycosides, while the internal leaves exhibited both flavonol glycosides and hydroxycinnamic acid derivatives (3, 5). The organic acid profile and antioxidant capacity of external and internal leaves against DPPH and reactive oxygen species (superoxide and hydroxyl radicals and hypochlorous acid) also were described, with external leaves exhibiting a higher antioxidant potential (5, 6). The influence of two fertilization regimens (conventional and organic practices) on the amounts of organic acids and phenolic compounds of tronchuda cabbage also was reported (8). The results obtained in this study indicated that, in a general way, tronchuda cabbage from organic cultures presents a higher phenolic content than that from a conventional culture (8).

In this work, we analyzed the free amino acid composition in tronchuda cabbage leaves. We also clarified as to whether there were any differences between internal and external leaves and harvesting months. By these means, it is possible to know if it is a material or a period in which the production of amino acids is clearly higher, which would constitute a nutritional advantage. For this purpose, a methodology based on free amino acid precolumn derivatization with dabsyl chloride and reversed-phase HPLC/UV–vis analysis was applied to tronchuda cabbage leaves.

MATERIALS AND METHODS

Standards and Reagents. All L-amino acid standards, dabsyl chloride reagent, sodium hydrogen carbonate, sodium dihydrogenphosphate, dimethylformamide, and triethylamine were from Sigma (St. Louis, MO). HPLC grade acetonitrile, ethanol, and phosphoric acid were obtained from Merck (Darmstadt, Germany). The water was treated in a Milli-Q water purification system (Millipore, Bedford, MA). All other reagents were of analytical grade from several suppliers.

Samples. The experimental work was carried out in one field located in Bragança, NE Portugal (41°48'N, 6°44'W). Plant sowing occurred in the middle of June 2005, in a greenhouse (22 ± 2 °C, 80% humidity). Young plants were transplanted to the field at the end of August, spaced at 0.8 m × 0.5 m between and within rows, and all agricultural practices followed the traditional practices of the region with respect to fertilization regime, irrigation, and phytosanitary treatments.

Samples were collected on three occasions during the growth season: in mid-November 2005, mid-December 2005, and mid-January 2006. In each harvesting date, six plants were randomly collected in the field. All samples were collected in the morning, at the same hour. After harvesting, the plants were immediately transported to the laboratory, and external (older) and internal (younger) leaves were separated. Care was taken to choose plants and leaves of similar developmental stage: internal leaves, pale yellow and tender, were separated from the external ones, which were dark green and no longer actively expanding, although not yet senescent. Two hours (maximum) after their collection, the samples were frozen at –20 °C and then lyophilized (Labconco 4.5 Freezone apparatus, Kansas City, MO). The freeze-drying yield was ca. 14%. The freeze-dried samples were powdered and kept in a desiccator in the dark until they were subjected to aqueous extraction.

Extraction. Leaf extract was prepared by pouring 600 mL of boiling water on to ca. 30 g of plant material. The mixture was boiled for 1 h and then filtered through a Buchner funnel. The resulting extract was then lyophilized in a Labconco 4.5 apparatus (Kansas City, MO) and ca. 14 g of lyophilized extract was obtained, which was afterward kept in a desiccator, in the dark. Approximately 40 mg of each lyophilized extract was redissolved in 400 µL of 0.1 M HCl.

Derivatization Procedure. Dabsylation was achieved as reported by Silva et al. (9). Aliquots of 20 µL of standard solution (ca. 0.2 mg/mL each amino acid in 0.1 M HCl) or redissolved extract were diluted with 180 µL of the reaction buffer (0.15 M sodium hydrogen carbonate, pH 8.6 with NaOH). After thorough mixing on a vortex-mixer, 200

Table 1. Equations for Regression Lines and Correlation Coefficients, Concentration Range of Linearity, and Detection Limits for Amino Acids

amino acid	equation ^a	linearity (µg/mL)	detection limit (µg/mL)
aspartic acid	$y = 1.42 \times 10^4 x; r = 0.99624$	1.25–10.0	0.85
glutamic acid	$y = 1.28 \times 10^4 x; r = 0.99994$	1.25–10.0	0.94
asparagine	$y = 1.47 \times 10^4 x; r = 0.98967$	0.59–4.68	0.81
glutamine	$y = 2.28 \times 10^4 x; r = 0.99795$	1.28–10.2	0.53
serine	$y = 3.22 \times 10^4 x; r = 0.99846$	1.26–10.1	0.38
threonine	$y = 1.48 \times 10^4 x; r = 0.99709$	1.27–10.2	0.81
glycine	$y = 8.25 \times 10^4 x; r = 0.99738$	1.29–10.3	0.15
alanine	$y = 5.75 \times 10^4 x; r = 0.99855$	1.27–10.1	0.22
valine	$y = 1.16 \times 10^4 x; r = 0.99823$	1.25–10.0	1.04
proline	$y = 4.82 \times 10^4 x; r = 0.99641$	1.25–10.0	0.25
arginine	$y = 5.95 \times 10^4 x; r = 0.99561$	1.26–10.1	0.20
isoleucine	$y = 6.19 \times 10^4 x; r = 0.99486$	1.29–10.3	0.20
leucine	$y = 4.46 \times 10^4 x; r = 0.99796$	1.28–10.2	0.28
tryptophan	$y = 3.32 \times 10^4 x; r = 0.99674$	1.25–10.0	0.36
phenylalanine	$y = 4.21 \times 10^4 x; r = 0.99446$	1.28–10.2	0.29
cysteine	$y = 1.05 \times 10^4 x; r = 0.99337$	1.28–10.2	1.15
ornithine	$y = 5.77 \times 10^4 x; r = 0.99826$	1.34–10.7	0.22
lysine	$y = 6.05 \times 10^4 x; r = 0.99876$	1.26–10.0	0.20
histidine	$y = 3.30 \times 10^4 x; r = 0.99606$	1.29–10.3	0.36
tyrosine	$y = 4.44 \times 10^4 x; r = 0.99816$	1.25–9.97	0.28

^a y : peak area at 436 nm; x : µg of amino acid; and r : correlation coefficient.

µL of the 12.4 mM dabsyl chloride reagent (in acetone) was added, and the vials were mixed again. The resulting solutions were incubated at 70 °C in a water bath for 15 min. The reaction was stopped by placing the vials in an ice bath for 5 min. A total of 400 µL of the dilution buffer [mixture of 50 mL of acetonitrile, 25 mL of ethanol, and 25 mL of 9 mM sodium dihydrogenphosphate 9; 4 % dimethylformamide; and 0.15% triethylamine (pH 6.55 with phosphoric acid)] was added, followed by thorough mixing and centrifugation (5 min, 5000 rpm). The clear supernatants were directly set for injection or stored at –20 °C.

HPLC/UV–vis Analysis. Dabsyl derivatives of free amino acids were separated on a Gilson HPLC unit, using a reversed-phase Spherisorb ODS2 column (25.0 cm × 0.46 cm; 5 µm particle size) (9). The solvent system consisted of 9 mM sodium dihydrogenphosphate, 4% dimethylformamide, and 0.15% triethylamine (pH 6.55 with phosphoric acid) (A) and 80% acetonitrile (B). Elution was performed at a flow rate of 1 mL/min, starting with 20% B until 7 min and installing a gradient to obtain 35% B at 35 min, 50% B at 45 min, and 100% B at 66 min. Detection was achieved with a UV–vis detector set at 436 nm. Free amino acid quantification was accomplished by the absorbance recorded in the chromatograms relative to external standards. Twenty microliters of the derivatized standard solutions or samples was injected.

Under the assay conditions described, a linear relationship between the concentration of amino acids and the absorbance at 436 nm was obtained in the tested range (Table 1). The correlation coefficient for the standard curves invariably exceeded 0.99 for all compounds. The detection limits were calculated as the concentration corresponding to 3 times the standard deviation of the background noise, and the values obtained were low, once they ranged from 0.15 to 1.15 µg/mL (Table 1).

Statistical Analysis. The results are expressed as mean values and standard error for the three sampling periods and type of leaf (internal and external). Analysis of variance followed by a Tukey test with $\alpha = 0.05$ were performed to study the differences between harvesting times and leaf types for free amino acid composition using SAS v. 9.13.

RESULTS AND DISCUSSION

Amino acids are important for human nutrition and affect food quality, including taste, aroma, and color (10). When compared to other varieties, the *B. oleracea* var. *costata* leaf total free amino acid content was high, varying from 3.3 to 14.4 g/kg fresh weight (mean value of 7.9 g/kg fresh weight or 56.4 g/kg dry weight).

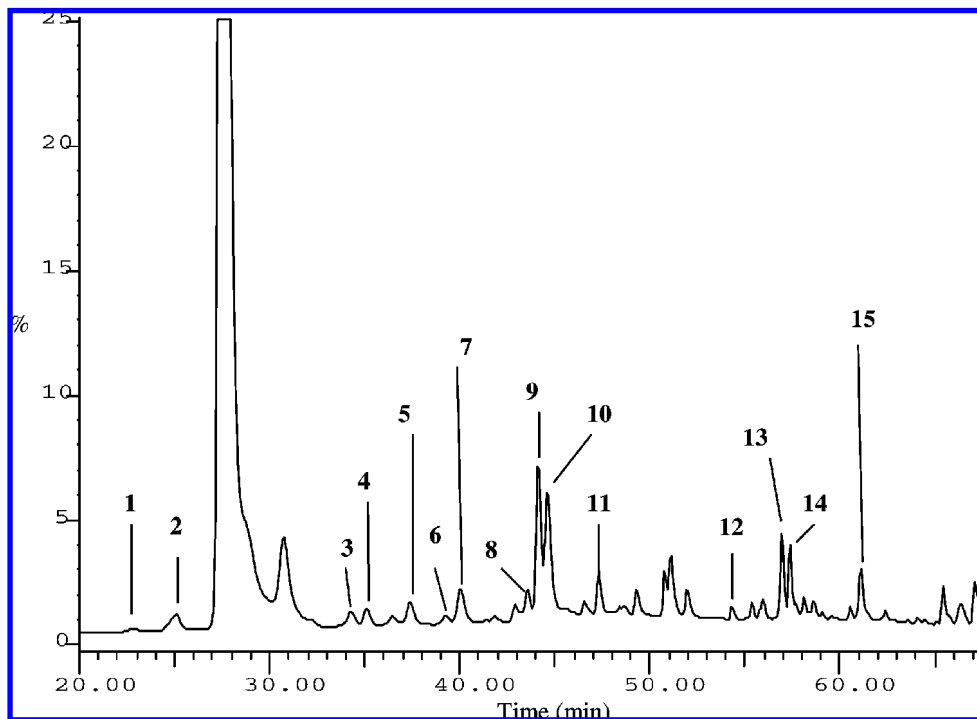


Figure 1. HPLC/UV-vis chromatogram of free amino acids in a tronchuda cabbage external leaf sample, collected in December. Detection was at 436 nm. (1) Aspartic acid; (2) glutamic acid; (3) glutamine; (4) serine; (5) threonine; (6) glycine; (7) alanine; (8) valine; (9) proline; (10) arginine; (11) leucine; (12) cysteine; (13) ornithine; (14) lysine; and (15) tyrosine.

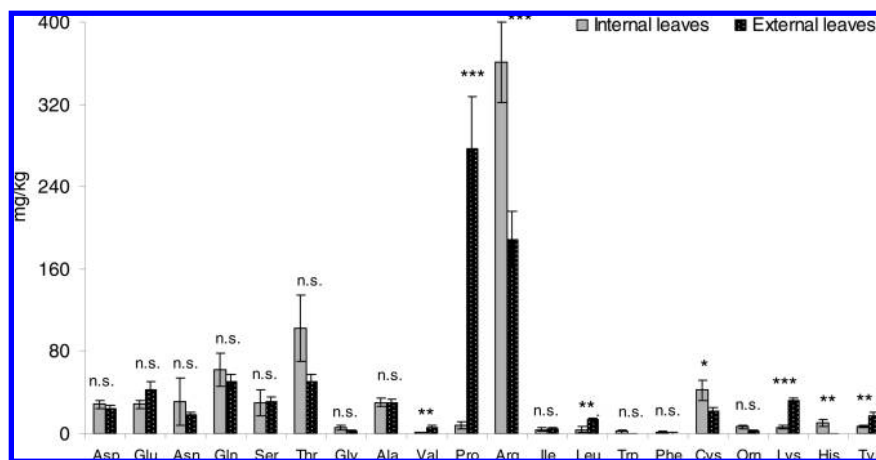


Figure 2. Quantitative free amino acid profile of tronchuda cabbage internal and external leaves samples, using all data combined (mean value, mg/kg). (asp) Aspartic acid; (glu) glutamic acid; (asn) asparagine; (gln) glutamine; (ser) serine; (thr) threonine; (gly) glycine; (ala) alanine; (val) valine; (pro) proline; (arg) arginine; (ile) isoleucine; (leu) leucine; (trp) tryptophan; (phe) phenylalanine; (cys) cysteine; (orn) ornithine; (lys) lysine; (his) histidine; and (tyr) tyrosine. * $0.01 < p \leq 0.05$ (significant difference); ** $0.001 < p \leq 0.01$ (very significant difference); $p \leq 0.001$ (extremely significant difference); and n.s., nonsignificant difference.

Considering all tronchuda cabbage leaf samples, their free amino acid profile was highly dispersed among the 20 constituents (**Figure 1**). Nevertheless, in a general way, leaves were rich in terms of arginine (35.6% total free amino acid content); had medium values of proline, threonine, glutamine, cysteine, and glutamic acid (16.8, 8.9, 7.1, 4.7, and 4.6% total free amino acid content, respectively); presented low contents of alanine, serine, aspartic acid, lysine, asparagine, and tyrosine (4.0, 3.6, 3.4, 2.7, 2.4, and 1.7% total free amino acid content, respectively); and had very small proportions of the other eight free amino acids.

Arginine, the main compound, is a semiessential amino acid for humans, which is required to ensure that liver, joints, muscles (including the heart muscle), and skin are kept healthy. Arginine strengthens the immune system, promotes male fertility, and is

involved in the regulation of many hormonal processes in the body (pituitary gland, pancreas, and human growth hormone). Arginine is semiessential because the body can usually produce enough amounts in normal circumstances, but when submitted to great physical stress or illness, more arginine is required. In addition, babies cannot produce arginine in their first few months. Arginine is also of great importance as an intermediary product in urea synthesis. This amino acid is present in all proteins at an average level of 3–6% (10).

Proline improves skin texture by aiding in the production of collagen and reduces its loss through the aging process. This nonessential amino acid works with ascorbic acid to promote healthy connective tissue. Proline also helps in the maintenance and healing of cartilage and the strengthening of joints, tendons, and muscles (including the heart muscle). Generally, proline is

Table 2. Free Amino Acid Composition of Tronchuda Cabbage External Leaf Samples (Mean \pm SD) (g/kg Fresh Weight) at Different Collection Times^a

amino acid (g/kg)	November 2005	December 2005	January 2006
aspartic acid	0.29 \pm 0.08 a	0.23 \pm 0.06 a	0.22 \pm 0.03 a
glutamic acid	0.67 \pm 0.16 a	0.42 \pm 0.11 a,b	0.19 \pm 0.05b
asparagine	0.17 \pm 0.04 a	0.19 \pm 0.05 a	0.18 \pm 0.06 a
glutamine	0.61 \pm 0.15 a	0.43 \pm 0.10 a	0.48 \pm 0.14 a
serine	0.51 \pm 0.09 a	0.22 \pm 0.03 b	0.20 \pm 0.03 b
threonine	0.56 \pm 0.14 a	0.50 \pm 0.17 a	0.44 \pm 0.08 a
glycine	0.03 \pm 0.00 a	0.03 \pm 0.01 a	0.02 \pm 0.01 a
alanine	0.40 \pm 0.06 a	0.25 \pm 0.03 a	0.26 \pm 0.04 a
valine	0.00 \pm 0.00 b	0.08 \pm 0.03 a,b	0.10 \pm 0.03 a
proline	2.83 \pm 1.23 a	2.97 \pm 0.97 a	2.52 \pm 0.67 a
arginine	1.48 \pm 0.40 a	1.57 \pm 0.49 a	2.61 \pm 0.55 a
isoleucine	0.03 \pm 0.01 a	0.06 \pm 0.01 a	0.05 \pm 0.02 a
leucine	0.09 \pm 0.02 b	0.16 \pm 0.03 a	0.15 \pm 0.02 a,b
tryptophan	0.00 \pm 0.00 a	0.00 \pm 0.00 a	0.00 \pm 0.00 a
phenylalanine	0.01 \pm 0.01 a	0.00 \pm 0.00 a	0.00 \pm 0.00 a
cysteine	0.11 \pm 0.06 b	0.32 \pm 0.06 a	0.21 \pm 0.06 a,b
ornithine	0.08 \pm 0.03 a	0.00 \pm 0.00 b	0.00 \pm 0.00 b
lysine	0.30 \pm 0.04 a	0.30 \pm 0.05 a	0.36 \pm 0.05 a
histidine	0.00 \pm 0.00 a	0.00 \pm 0.00 a	0.00 \pm 0.00 a
tyrosine	0.15 \pm 0.07 a	0.21 \pm 0.04 a	0.17 \pm 0.06 a
total	8.31 \pm 1.39a	7.93 \pm 0.96 a	8.16 \pm 1.06 a

^a In the same row, between different collection times, means with different letters are significantly different ($p \leq 0.05$).

Table 3. Free Amino Acid Composition of Tronchuda Cabbage Internal Leaf Samples (Mean \pm SD) (g/kg of Fresh Weight) at Different Collection Times^a

amino acid (g/kg)	November 2005	December 2005	January 2006
aspartic acid	0.10 \pm 0.05 b	0.42 \pm 0.02 a	0.33 \pm 0.06 a
glutamic acid	0.21 \pm 0.04 a	0.28 \pm 0.09 a	0.36 \pm 0.10 a
asparagine	0.02 \pm 0.02 a	0.08 \pm 0.09 a	0.82 \pm 0.72 a
glutamine	0.44 \pm 0.13 a	0.69 \pm 0.45 a	0.73 \pm 0.24 a
serine	0.10 \pm 0.03 a	0.47 \pm 0.31 a	0.32 \pm 0.27 a
threonine	0.40 \pm 0.17 a	1.42 \pm 0.43 a	1.26 \pm 0.91 a
glycine	0.00 \pm 0.00 a	0.07 \pm 0.07 a	0.09 \pm 0.04 a
alanine	0.26 \pm 0.04 a	0.23 \pm 0.09 a	0.41 \pm 0.07 a
valine	0.02 \pm 0.02 a	0.00 \pm 0.00 a	0.00 \pm 0.00 a
proline	0.19 \pm 0.09 a	0.02 \pm 0.02 a	0.03 \pm 0.03 a
arginine	2.27 \pm 0.42 b	3.61 \pm 0.54 a,b	4.95 \pm 0.67a
isoleucine	0.03 \pm 0.02 a	0.03 \pm 0.02 a	0.06 \pm 0.03 a
leucine	0.02 \pm 0.02 a	0.07 \pm 0.08 a	0.03 \pm 0.02 a
tryptophan	0.01 \pm 0.01 a	0.00 \pm 0.00 a	0.05 \pm 0.051 a
phenylalanine	0.04 \pm 0.03 a	0.00 \pm 0.00 a	0.01 \pm 0.01 a
cysteine	0.24 \pm 0.14 a	0.55 \pm 0.21 a	0.48 \pm 0.18 a
ornithine	0.08 \pm 0.04 a	0.06 \pm 0.04 a	0.07 \pm 0.03 a
lysine	0.06 \pm 0.03 a	0.07 \pm 0.03 a	0.06 \pm 0.03 a
histidine	0.08 \pm 0.03 a	0.16 \pm 0.09 a	0.07 \pm 0.03 a
tyrosine	0.09 \pm 0.03 a	0.06 \pm 0.01 a	0.04 \pm 0.02 a
total	4.64 \pm 0.60 b	8.32 \pm 1.02 a,b	10.16 \pm 1.51 a

^a In the same row, between different collection times, means with different letters are significantly different ($p \leq 0.05$).

obtained primarily from meat sources. Although, according to Sharma et al. (11), it accumulates in plants that are under heavy metal exposure. It seems that this amino acid can effectively protect plants from heavy metal attack. This protective effect may be explained by heavy metal detoxification through the formation of a nontoxic heavy metal–proline complex. *B. oleracea* var. *costata* leaves seem to be well-protected against heavy metals. The lowest heavy metal content that proline detoxification provides is beneficial to consumers' health.

Generally, threonine and lysine contents are limiting factors in the biological value of many proteins, mostly those from plant origins (10). In tronchuda cabbage, free amino acids seem to compensate for its possibly low biological value proteins. Additionally, threonine, an essential amino acid, helps to

maintain the proper balance of protein in the body. It is important for the formation of collagen and elastin in the skin and aids in fighting fatty deposits in the liver, when combined with aspartic acid and methionine. Lysine is also an essential amino acid and is required for growth and bone development in children and assists in calcium absorption and in the maintenance of the correct nitrogen balance in the body, as well as lean body mass. Lysine is also needed to produce antibodies, hormones, enzymes, collagen formation, as well as to repair tissues.

Glutamine, a nonessential amino acid, is found in large amounts in the muscles of the body. Since glutamine passes easily through the blood–brain barrier, it is also known as an excellent brain fuel. This amino acid is converted to glutamic acid in the brain, which is essential for proper brain function, and increases the amount of gamma-amino butyric acid (GABA), which is required for brain function and mental activity. Glutamine is a source of fuel for cells lining the intestines, and it is also used by white blood cells, so it is important for immune function.

Although cysteine is a sulfur-containing nonessential amino acid, it can partly replace methionine (essential), which has the main role of being a methyl donor in many biochemical processes (to detoxify the body and its organs) (10). Cysteine is necessary in the detoxification of the body from harmful toxins, helping to protect the liver and brain from damage. It is required for the production of taurine and is a component of glutathione.

Glutamic acid (nonessential amino acid) is synthesized from a number of amino acids including ornithine and arginine, as it is important in the metabolism of sugars and fats. It also helps with the transportation of potassium across the blood–brain barrier (although glutamic acid itself does not pass this barrier that easily). Glutamate is part of the folate molecule, so that is why it is deemed a nonessential amino acid, as the body can usually get enough of it through adequate folate in the diet.

Aspartic and glutamic acids provide the umami taste or perception of satisfaction, which is an overall food flavor sensation induced or enhanced by monosodium glutamate (12). In humans, the taste receptor is far more sensitive to glutamate than to other amino acids (13). Other amino acids appear to exhibit sweet, bitter, or less intense tastes. Alanine, serine, glycine, and threonine are known to have sweet tastes, while leucine, phenylalanine, isoleucine, valine, histidine, arginine, and tryptophan present bitter flavors. Lysine, cysteine, and tyrosine are considered tasteless amino acids (12).

Several authors described the antioxidant effect of several amino acids in various matrices (14–17), and, for instance, tryptophan, cysteine, alanine, and glycine exert a synergistic effect with ascorbic acid on the antioxidant activity of vitamin E (17). All the referred amino acids were found in the analyzed samples and since tronchuda cabbage contains large amounts of free amino acids, it seems that they can confer or, at least, enhance the antioxidant capacity of this variety. In fact, the previously analyzed aqueous lyophilized extracts of *B. oleracea* var. *costata* leaves revealed a great antioxidant potential (5, 6).

As far as we know, this is the first time that the free amino acid composition is reported for tronchuda cabbage leaves, and this quantitative profile seems to be quite unusual. In 1996, Eppendorfer and Bille (18) determined the free and total amino acid composition of edible parts of beans, kale, spinach, cauliflower, and potatoes and found that free glutamine was dominant in kale and cauliflower. Latter, Gent (19) reported the free amino acid profile of seven salad greens, including

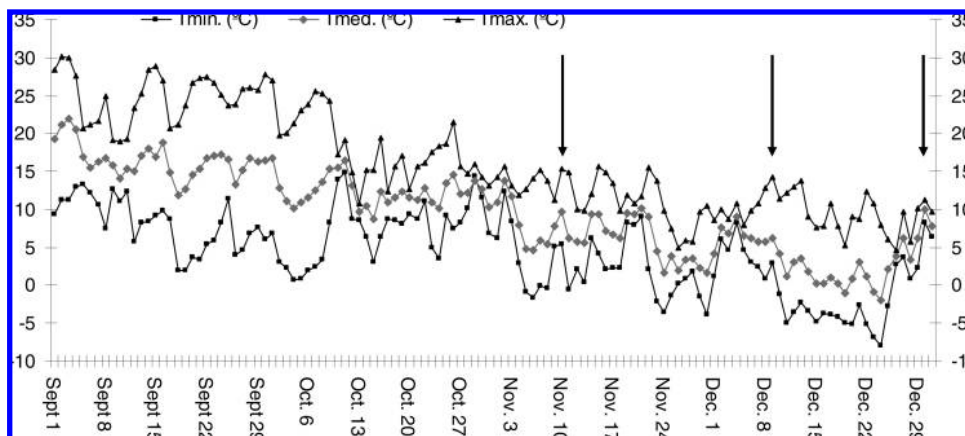


Figure 3. Environmental temperatures during the experiment. T_{\min} : Daily minimum temperature; T_{med} : daily medium temperature; T_{\max} : daily maximum temperature; and \rightarrow : collection date.

leaves of kale (*B. oleracea*) cv. Red Russian, mibuna (*Brassica campestris*) cv. Green spray, and mustard (*Brassica juncea*) cv. Osaka purple. The sum of free amino acids ranged from 40 to 160 mmol/kg dry weight (or 4–16 g/kg dry weight, assuming an average molecular weight of 100), which is lower than that found in tronchuda cabbage leaves, and glutamic acid and/or glutamine were predominant amino acids in the leaves of all species (19). In 2006, Ayaz et al. (20) determined the amino acid profile of kale leaves (*B. oleracea* var. *acephala*). The most abundant amino acids (free plus bound) were glutamic and aspartic acids, arginine, leucine, proline, and valine (12.2, 10.2, 7.6, 7.5, 6.5, and 6.3% of the total amino acid content, respectively). The total (free plus bound) amino acid content was 271 g/kg dry weight. Gomes and Rosa (4) studied the free amino acid profile of primary and secondary inflorescences of 11 broccoli (*B. oleracea* var. *italica*) cultivars and its variation between seasons. These authors reported that the major amino acids were glutamine, glutamic and aspartic acids, alanine, and asparagine. That study indicated that free amino acids were not similarly distributed in the primary and secondary inflorescences since their content tends to be higher in the primary ones in the summer/winter season and in the secondary ones in the spring/summer. The total free amino acid content varied from 158 to 391 mmol/kg dry weight (or 16–39 g/kg dry weight, assuming an average molecular weight of 100) (4), which is lower than that found in tronchuda cabbage leaves.

As described by Gomes and Rosa (4), *B. oleracea* var. *costata* leaf free amino acids are not similarly distributed in internal (younger) and external (older) leaves. Proline and arginine were the major free amino acids, in external leaves, representing ~56% of the total free amino acid content (31.9 and 24.2%, respectively). Threonine, glutamine, glutamic acid, and lysine were present in medium proportions (5–6% of total free amino acid content). However, internal leaves presented a different quantitative profile. Arginine was the main free amino acid (46.9% of total free amino acid content), followed by threonine, glutamine, and cysteine (11.9, 8.2, and 6.5% of total free amino acid content, respectively). Proline was present in a smaller amount (1.7% of total free amino acid content) than in external leaves. Significant differences were observed between leaf types for valine ($F = 9.469$; $d.f. = 1, 34$; $p = 0.004$), proline ($F = 30.249$; $d.f. = 1, 34$; $p \leq 0.001$), arginine ($F = 13.696$; $d.f. = 1, 34$; $p \leq 0.001$), leucine ($F = 12.454$; $d.f. = 1, 34$; $p = 0.001$), cysteine ($F = 4.191$; $d.f. = 1, 34$; $p = 0.048$), lysine ($F = 93.434$; $d.f. = 1, 34$; $p \leq 0.001$), histidine ($F = 12.591$; $d.f. = 1, 34$; $p = 0.001$), and tyrosine ($F = 12.341$; $d.f. = 1, 34$; $p = 0.001$) (Figure 2), although the total free amino acid content

was similar in both kinds of leaves (mean values of 8.1 and 7.7 g/kg fresh weight for external and internal cabbage leaves, respectively).

No statistically differences were observed between the three different harvesting periods for the total free amino acid content in external leaves. However, marked differences were observed for glutamic acid, serine, and ornithine contents, which decreased significantly ($p \leq 0.05$) from November to January, and valine, leucine, and cysteine abundances, which increased significantly ($p \leq 0.05$) during this period (Table 2).

In internal leaves, the total free amino acid content increased significantly ($p \leq 0.05$) from November (4.6 g/kg fresh weight) to January (10.2 g/kg fresh weight). Concerning individual compounds, only aspartic acid and arginine showed significant variations (Table 3).

Gomes and Rosa (4) concluded that the levels of free amino acids of broccoli varieties were affected by climatic conditions, being reduced when the plant was submitted to stress (4). However, according to our results, no differences were observed in tronchuda cabbage external leaves (Table 2), and significant increases were registered in internal ones (Table 3), even though extreme temperatures (less than zero) were observed during various days in December (Figure 3). This fact can most likely be related to genetic factors of this cabbage species. In plant species that are not adapted to low temperatures (below 0 °C), the crystallization of water promotes the rupture of membrane cells and the destruction of plant tissues. Tronchuda cabbage seems to be well-adapted, and its tissues are maintained in good condition for several days at low temperatures (8). Generally, in the north of Portugal, tronchuda cabbage is cultivated in the autumn/winter.

Free amino acids were identified as the precursors of several classes of secondary plant metabolites, namely, phenolic acids, flavonoids, and glucosinolates, although, as far as we know, there are few reports concerning the relationship between those secondary metabolites and their precursors in vivo. Considering that methionine, phenylalanine, tyrosine, and tryptophan are precursors of glucosinolates, Rosa and Gomes (21) investigated the relationship between these two classes of compounds in 11 broccoli cultivars grown early (April–July) and late (August–January). They concluded that in this matrix, there is no correlation between the glucosinolates and their precursors, the free amino acids.

This study attempted to contribute to the knowledge of nutritional properties of an important Portuguese crop. In conclusion, the two kinds of tronchuda leaves (external and internal) in a diet supply different proportions of free amino acids. It is important to consume both kind of leaves to obtain

the appropriate amounts of amino acids and a great diversity of palatable sensations. In addition, these cabbages are of great nutritional importance because they are rich in free amino acids (mean value of 7.9 g/kg fresh weight), which are indispensable in a healthy diet.

In external leaves, proline and arginine are the major free amino acids, while in internal ones, arginine is the main free amino acid, followed by threonine, glutamine, and cysteine. Significant differences between leaf types were observed in the quantitative profile, namely, in the valine, proline, arginine, leucine, cysteine, lysine, histidine, and tyrosine contents. Significant differences among harvesting periods also were observed in the free amino acid profiles (glutamic acid, serine, valine, leucine, cysteine, and ornithine contents and aspartic acid, arginine, and total contents in external and internal leaves, respectively).

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