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Influence of Two Fertilization Regimens on the Amounts of Organic Acids and Phenolic Compounds of Tronchuda Cabbage (*Brassica oleracea* L. Var. *costata* DC)

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A phytochemical study was undertaken on tronchuda cabbage (*Brassica oleracea* L. var. *costata* DC) cultivated under conventional and organic practices and collected at different times. Six organic acids (aconitic, citric, ascorbic, malic, shikimic, and fumaric acids) were identified and quantified by HPLC-UV. Qualitative and quantitative differences were noted between internal and external leaves. Analysis of the phenolics of the internal leaves was achieved by HPLC-DAD, and the phenolic profile obtained was revealed to be distinct from that of the external leaves. By this means were identified and quantified 11 compounds: 3-*p*-coumaroylquinic acid, kaempferol 3-*O*-sophoroside-7-*O*-glucoside, kaempferol 3-*O*-(caffeoyl)sophoroside-7-*O*-glucoside, kaempferol 3-*O*-(sinapoyl)sophoroside-7-*O*-glucoside, kaempferol 3-*O*-(feruloyl)sophoroside-7-*O*-glucoside, kaempferol 3-*O*-sophoroside, two isomeric forms of 1,2-disinapoylgentiobiose, 1-sinapoyl-2-feruloylgentiobiose, 1,2′-trisinapoylgentiobiose, and 1,2′-disinapoyl-2-feruloylgentiobiose. In general, internal leaves exhibited more constant chemical profiles.

KEYWORDS: Tronchuda cabbage; *Brassica oleracea* L. var. *costata* DC; organic acids; phenolic compounds; organic and conventional production

INTRODUCTION

Nowadays consumers are aware of the need for a constant supply of phytochemical-containing plants to get optimal health benefits. Vegetables form an essential part of a well-balanced diet. *Brassica* vegetables, including all cabbage-like ones, are very popular, being consumed in enormous quantities all over the world. *Brassica* species are reported to possess cancer preventive activity, due to glucosinolates and their derived properties (*1*−*3*). Their leaves have little starch, sugar, or fat, their fiber is useful; they contain some vitamin E and a range of B vitamins, and they present significant carotenes and ascorbic acid contents. Within *Brassica* genus, the *B. oleracea* species has evolved into a number of varieties of which different parts of the plant have become the edible constituents (*1*). Although essentially temperate, *B. oleracea* forms are grown in other regions throughout the world (*1*). Tronchuda cabbage (*B. oleracea* L. var. *costata* DC) is known to be a cultivar well adapted to the soil and climate conditions and generally grown with little or no agrochemical input (*4*). It is very important in the Portuguese diet and agricultural systems. Tronchuda cabbage exhibits large floppy leaves, which are close together, round, smooth, and slightly notched at the margins. Its dark green external leaves are rather bitter and tough and are usually prepared by boiling. The internal leaves are pale yellow, tender, and sweeter than the external ones, being consumed raw or, most usually, cooked.

Organic acids and phenolic compounds are known to contribute to the organoleptic characteristics of fruits and vegetables (*5*). These compounds have been used for the quality control of several matrices (*6*−*10*). In addition, they may also exert a protective role against various diseases due to their antioxidant potential (*11*). The phenolic composition of tronchuda cabbage external leaves has already been characterized (*12*). The phenolic compounds of the internal leaves were identified by HPLC-DAD-MS/MS-ESI (*13*), but no study concerning their variation has been done. The organic acids present in the external and internal leaves have been characterized (*13*), but their variation was not analyzed.

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Table 1. Quantification of Tronchuda Cabbage Organic Acids (Milligrams per Kilogram, Dry Basis)[a]

				compound						
sample	collection date	leaves	cultivation[b]	aconitic acid (t_R 24.2 min)	citric acid (t_R 28.7 min)	ascorbic acid (t_R 29.8 min)	malic acid (t_R 34.3 min)	shikimic acid (t_R 46.5 min)	fumaric acid (t_R 57.7 min)	total
1	Oct 2002	external	O		7429.8 (1019.6)	9778.3 (358.0)	9133.5 (1087.1)	143.5 (7.5)	21.9 (0.7)	26507.0
2	Oct 2002	external	C	61.0 (0.9)	18980.4 (1112.2)	28843.7 (1344.4)	25904.3 (307.6)	171.3 (12.7)	34.6 (0.6)	73995.2
3	Nov 2002	external	O		9749.6 (576.2)	10978.7 (546.9)	11183.7 (164.8)	124.9 (5.7)	8.6 (1.2)	32045.6
4	Nov 2002	external	C	78.0 (22.4)	9362.6 (458.9)	28441.8 (2914.0)	22184.6 (1073.3)	226.1 (15.3)	27.8 (0.3)	60321.0
5	Dec 2002	external	O	64.8 (6.1)	5041.3 (946.8)	31179.1 (4054.5)	39459.0 (2357.6)	118.3 (16.9)	1.3 (0.1)	75863.8
6	Dec 2002	external	C	76.3 (16.4)	9962.8 (2164.2)	30634.3 (3468.0)	28385.9 (1391.4)	172.7 (2.2)	2.5 (0.2)	69234.5
7	Jan 2003	external	O		3017.6 (182.5)	15516.5 (3519.2)	12030.4 (583.1)	138.0 (12.5)	25.8 (1.2)	30728.3
8	Jan 2003	external	C	93.0 (16.1)	8282.4 (392.8)	30906.2 (7289.2)	32207.5 (2452.6)	232.1 (2.9)	7.9 (3.8)	71729.0
9	Oct 2002	internal	O	114.2 (10.1)	9681.7 (12.8)	31069.4 (632.4)	33449.7 (149.9)	269.2 (2.3)	41.6 (1.1)	74625.9
10	Oct 2002	internal	C	20.2 (1.1)	4689.9 (29.8)	nq[c]	32270.5 (193.6)	257.4 (3.4)	26.1 (16.3)	37264.1
11	Nov 2002	internal	O	176.4 (13.7)	6912.3 (21.0)	15852.7 (930.4)	17620.8 (688.8)	155.8 (1.6)	143.3 (2.8)	40861.4
12	Nov 2002	internal	C	59.7 (0.7)	5198.1 (305.0)	nq	32710.7 (924.3)	216.5 (0.8)	26.2 (1.9)	38211.2
13	Dec 2002	internal	O	135.4 (2.7)	8525.4 (328.0)	25043.9 (181.6)	52869.6 (582.8)	200.9 (3.9)	53.9 (3.2)	86829.1
14	Dec 2002	internal	C	83.7 (8.1)	4108.7 (76.7)	nq	6735.9 (657.0)	178.7 (4.6)	58.8 (1.0)	11165.8
15	Jan 2003	internal	O	113.8 (18.2)	5560.4 (2.4)	36225.6 (3005.6)	10080.3 (1069.9)	248.9 (16.1)	91.3 (5.2)	52320.2
16	Jan 2003	Internal	C	101.8 (42.0)	9448.6 (1701.5)	21030.6 (5206.2)	5907.2 (625.5)	303.6 (3.7)	62.6 (0.1)	36854.4

[a] Results are expressed as mean (standard deviation) of three determinations. [b] O, organic; C, conventional. [c] Not quantified.

Table 2. Quantification of Tronchuda Cabbage Internal Leaves Phenolic Compounds (Milligrams per Kilogram, Dry Basis)[a]

				compound[b]								
sample	collection date	leaves	cultivation[c]	**1** (t_R 14.1 min)	**2** (t_R 26.3 min)	**3** (t_R 27.3 min)	**4** (t_R 31.8 min)	**5** (t_R 32.6 min)	**6** (t_R 40.1 min)	**7** + **8** + **9** + **10** (t_R 43.6–45.0 min)	**11** (t_R 46.0 min)	total
9	Oct 2002	internal	O	69.7 (3.8)		7.4 (0.2)	3.5 (0.7)	0.9 (0.8)	2.9 (0.1)	85.0 (2.5)	3.1 (0.1)	172.5
10	Oct 2002	internal	C	61.0 (0.1)		14.1 (0.6)	1.5 (0.0)	14.5 (0.2)	1.4 (0.0)	54.1 (4.3)		146.6
11	Nov 2002	internal	O	23.2 (1.0)		2.7 (0.4)	nq[d]	nq		42.1 (0.3)	2.5 (0.2)	70.5
12	Nov 2002	internal	C	57.4 (8.7)		6.3 (1.1)			0.9 (0.0)	33.1 (6.1)	1.4 (0.2)	99.1
13	Dec 2002	internal	O	34.3 (0.6)		3.7 (0.1)			1.0 (0.0)	32.0 (0.1)	0.0 (0.0)	71.2
14	Dec 2002	internal	C	17.7 (2.2)		1.5 (0.0)			2.7 (0.1)	14.6 (2.7)	0.3 (0.1)	36.8
15	Jan 2003	internal	O	33.3 (2.2)	nq	5.2 (0.1)	2.0 (0.1)	3.8 (0.1)	4.0 (0.7)	73.3 (0.2)	0.7 (0.9)	122.3
16	Jan 2003	internal	C	16.7 (2.8)	12.2 (0.6)		28.0 (5.4)	17.9 (1.3)	13.8 (0.9)	9.0 (0.9)		97.6

[a] Results are expressed as mean (standard deviation) of three determinations. [b] Identified according to ref *13*: **1**, 3-*p*-coumaroylquinic acid; **2**, kaempferol 3-*O*-sophoroside-7-*O*-glucoside; **3**, kaempferol 3-*O*-(caffeoyl)sophoroside-7-*O*-glucoside; **4**, kaempferol 3-*O*-(sinapoyl)sophoroside-7-*O*-glucoside; **5**, kaempferol 3-*O*-(feruloyl)sophoroside-7-*O*-glucoside; **6**, kaempferol 3-*O*-sophoroside; 7, 1,2-disinapoylgentiobiose; **8**, 1-sinapoyl-2-feruloylgentiobiose; **9**, isomer of 1,2-disinapoylgentiobiose; **10**, 1,2,2′-trisinapoylgentiobiose; **11**, 1,2′-disinapoyl-2-feruloylgentiobiose. [c] O, organic; C, conventional. [d] Not quantified.

The combination of several environmental factors may lead either to irreversible injuries or to the induction of reactions resulting in the plant acclimation (*14*). Thus, the purpose of this work was to evaluate the influence of two fertilization regimens and collection date on the organic acids and phenolic compounds profiles of tronchuda cabbage leaves. To accomplish this, a methodology based on HPLC-UV of organic acids was applied to the internal and external leaves aqueous extracts. The internal leaves phenolic compounds were determined by reversed-phase HPLC-DAD analysis of their methanolic extracts.

MATERIALS AND METHODS

Standards and Reagents. Aconitic, citric, ascorbic, and sinapic acids and kaempferol 3-*O*-rutinoside were purchased from Extrasynthése (Genay, France). Malic, shikimic, fumaric, and *p*-coumaric acids were from Sigma (St. Louis, MO). Methanol, sodium hydroxide, and formic acid were purchased from Merck (Darmstadt, Germany), and sulfuric acid was from Pronalab (Lisboa, Portugal). The water was treated in a Milli-Q water purification system (Millipore, Bedford, MA).

Plant Material and Sampling. Tronchuda cabbages were grown in two fields located in Mirandela, northeastern Portugal (U.T.M. 29 PG5602), according to two different agronomic practices previously described (*12*). Briefly, in one of the fields the production followed the organic status, and in the other field the production was developed according to the standard cultural practices of the region (conventional production). Plant material was sowed by the end of June 2002 and transplanted to the fields at the end of August. In the organic field only organic fertilization was applied with sheep manure. In the conventional field, organic fertilization was made during the transplantation of the plants and, at the beginning of September, a mineral fertilization with ammonium nitrate and CaO (ADP Adubos de Portugal) was applied with a side dress rate of 50 kg of N/ha. In this field, at the end of September, one pesticide treatment with deltamethrin (Decis) (Bayer Crop Science) at a rate of 30 mL/hL was made.

Plant material was harvested for four consecutive months (**Tables 1** and **2**). At each harvesting date and in each field three plants were randomly selected and collected in the morning. After harvesting, the plants were immediately transported to the laboratory and the dark green external leaves were separated from the pale yellow internal ones. Samples were stored in a freezer at −20 °C and then lyophilized (Modulyo 4K Freeze-Dryer Edwards). The three lyophilized materials were powdered, mixed, and kept in an exsiccator, in the dark. Each sample corresponds to a mixture of the external or internal leaves of the three plants, collected in the same field and on the same date.

Organic Acids Extraction. Internal and external leaf extracts were prepared by putting 1.5 g of lyophilized plant material in 300 mL of boiling water. The mixture was boiled for 1 h and then filtered over a Büchner funnel. The resulting extracts were then lyophilized (Modulyo 4K Freeze-Dryer Edwards). The lyophilized extracts were kept in an exsiccator in the dark and redissolved in 0.01 N sulfuric acid prior to analysis by HPLC-UV.

Phenolic Compounds Extraction. Each sample (∼0.5 g) was thoroughly mixed with methanol until complete extraction of the phenolic compounds (negative reaction to 20% NaOH). The extract

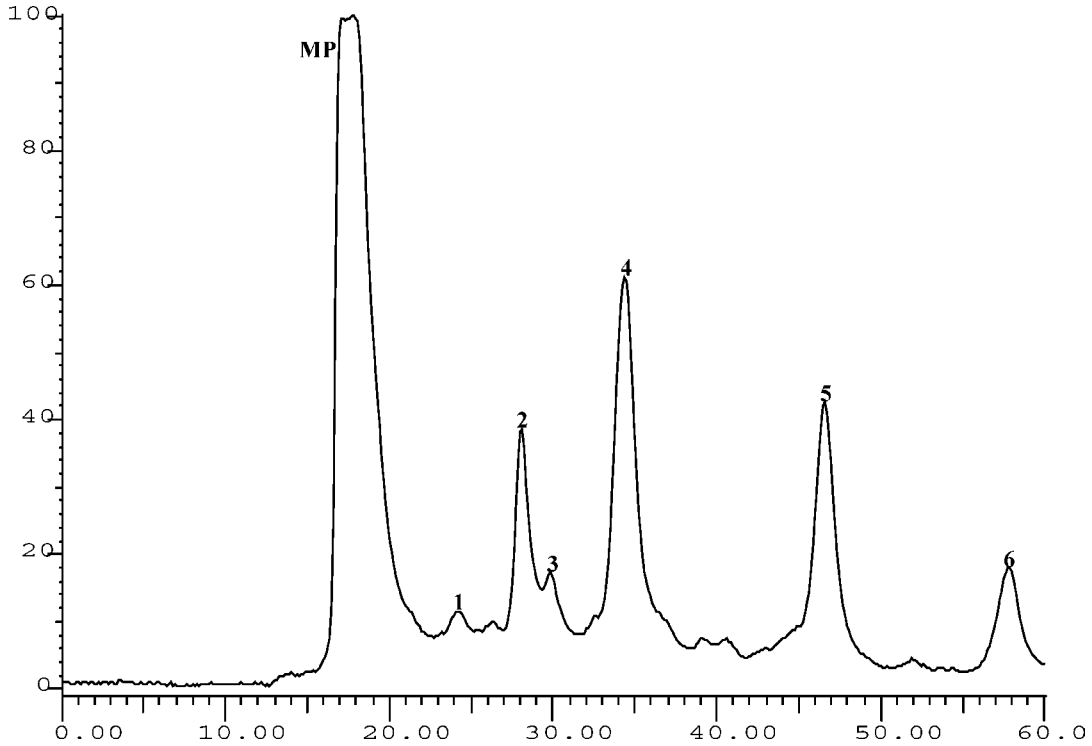

Figure 1. HPLC-UV organic acid profile of tronchuda cabbage internal leaves. Detection was at 214 nm. Peaks: (MP) mobile phase; (1) aconitic acid; (2) citric acid; (3) ascorbic acid; (4) malic acid; (5) shikimic acid; (6) fumaric acid.

was concentrated to dryness under reduced pressure (30 °C), redissolved in methanol (0.5 mL), and 20 μL was analyzed by HPLC-DAD.

HPLC Analysis of Organic Acids. Twenty microliters of each extract was analyzed as previously reported (*9*) with some modifications. The system consisted of an analytical HPLC unit (Gilson) with an ion exclusion column, Nucleogel Ion 300 OA (300 × 7.7 mm), in conjunction with a column-heating device set at 30 °C. Elution was carried out isocratically, at a solvent flow rate of 0.2 mL/min, with 0.01 N sulfuric acid. The injection volume was 20 μL. Detection was performed with a UV detector set at 214 nm.

Organic acids quantification was achieved by the absorbance recorded in the chromatograms relative to external standards. The peaks in the chromatograms were integrated using a default baseline construction technique.

HPLC Analysis of Phenolic Compounds. The separation was carried out with a HPLC unit (Gilson) and a 250 × 4.6 mm i.d., 5 μm Spherisorb ODS2 column (Waters, Milford, MA). The solvent system was a mixture of formic acid 5% in water (A) and methanol (B), at a flow rate of 1 mL/min. Elution started with 10% B and reached 20% B at 25 min, 50% B at 40 min, 50% B at 45 min, 90% B at 46 min, and 90% B at 48 min. Detection was achieved with a Gilson diode array detector. Spectroscopic data from all peaks were accumulated in the range of 200−400 nm, and chromatograms were recorded at 330 nm. The data were processed on Unipoint system software (Gilson Medical Electronics, Villiers le Bel, France). Peak purity was checked by the software contrast facilities.

Phenolic compounds quantification was achieved by the absorbance recorded in the chromatograms relative to external standards. The peaks in the chromatograms were integrated using a default baseline construction technique. Because standards of the compounds identified in the internal leaf methanolic extracts were not commercially available, 3-*p*-coumaroylquinic acid was quantified as *p*-coumaric acid, the kaempferol derivatives were quantified as kaempferol 3-*O*-rutinoside, and sinapic acid derivatives were quantified as sinapic acid.

RESULTS AND DISCUSSION

Organic Acids. Tronchuda cabbage internal and external leaves presented a chemical profile composed by six identified organic acids: aconitic, citric, ascorbic, malic, shikimic, and fumaric acids (**Figure 1**). The external leaves from organic culture exhibited aconitic acid only in December (**Table 1**).

The lyophilized extracts showed a high content of organic acids, ranging from ca. 11 to 87 g/kg (**Table 1**). Fumaric acid was the compound present in lower amounts, with the exception of sample 10, in which aconitic acid was the minor organic acid (**Table 1**). In the external leaves malic and ascorbic acids were the compounds present in highest amounts, representing from 69 to 93% of total acids, in samples from both organic and conventional culture (**Table 1**). The internal leaves exhibited more variety in the relative amounts of each organic acid. Anyway, in these samples malic acid was the major compound until December, accounting for 43−87% of total identified compounds, and in January ascorbic acid became the main compound, corresponding to 57−69% of total acids (**Table 1**).

With regard to the agronomic procedure both internal and external leaves from organic culture exhibit a similar behavior. The date of collection affects the organic acids profile in the same way, with an increase of ascorbic acid relative amount in January. The highest production of organic acids in the organic samples occurred in December (**Table 1**), following the development of the cabbage: organic tronchuda cabbage presented more developed leaves than those of conventional culture in the same period. This is in accordance with previous results (*12*), in which the commitment of organic tronchuda cabbage cells to morphogenic developmental pathways was accompanied by the lowest level of secondary metabolites (phenolics) in December. Apparently, the nutrients are mainly used for primary metabolites biosynthesis (*15*), which is more related with cabbage growth.

The conventional procedure seems to affect the organic acids profile of tronchuda cabbage, resulting in some discrepancies in the relative amounts of the compounds of external and internal leaves (**Table 1**). In the internal leaves ascorbic acid is a vestigial compound until December (**Table 1**), a fact that remains unexplained. October was the month in which the production

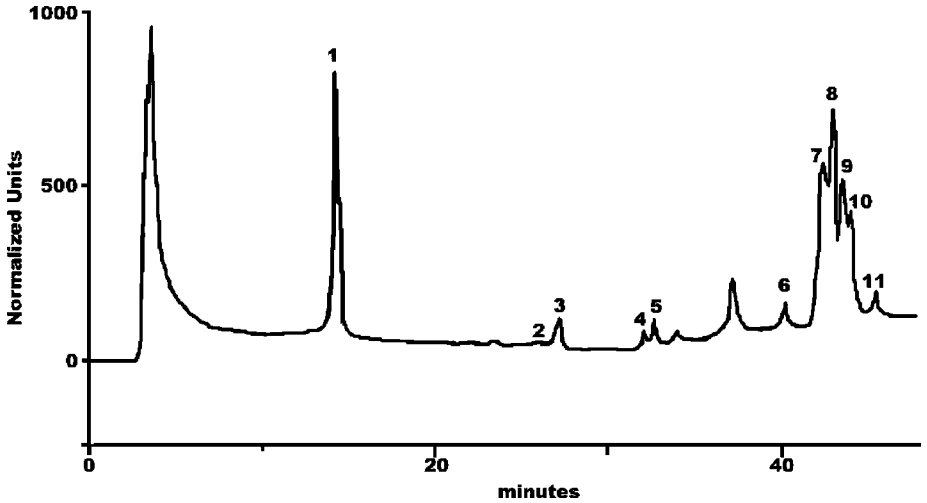

Figure 2. HPLC-DAD phenolic profile of tronchuda cabbage internal leaves. Detection was at 330 nm. Peaks: (1) 3-*p*-coumaroylquinic acid; (2) kaempferol 3-*O*-sophoroside-7-*O*-glucoside; (3) kaempferol 3-*O*-(caffeoyl)sophoroside-7-*O*-glucoside; (4) kaempferol 3-*O*-(sinapoyl)sophoroside-7-*O*-glucoside; (5) kaempferol 3-*O*-(feruloyl)sophoroside-7-*O*-glucoside; (6) kaempferol 3-*O*-sophoroside; (7) 1,2-disinapoylgentiobiose; (8) 1-sinapoyl-2-feruloylgentiobiose; (9) isomer of 1,2-disinapoylgentiobiose; (10) 1,2,2′-trisinapoylgentiobiose; (11) 1,2′-disinapoyl-2-feruloylgentiobiose.

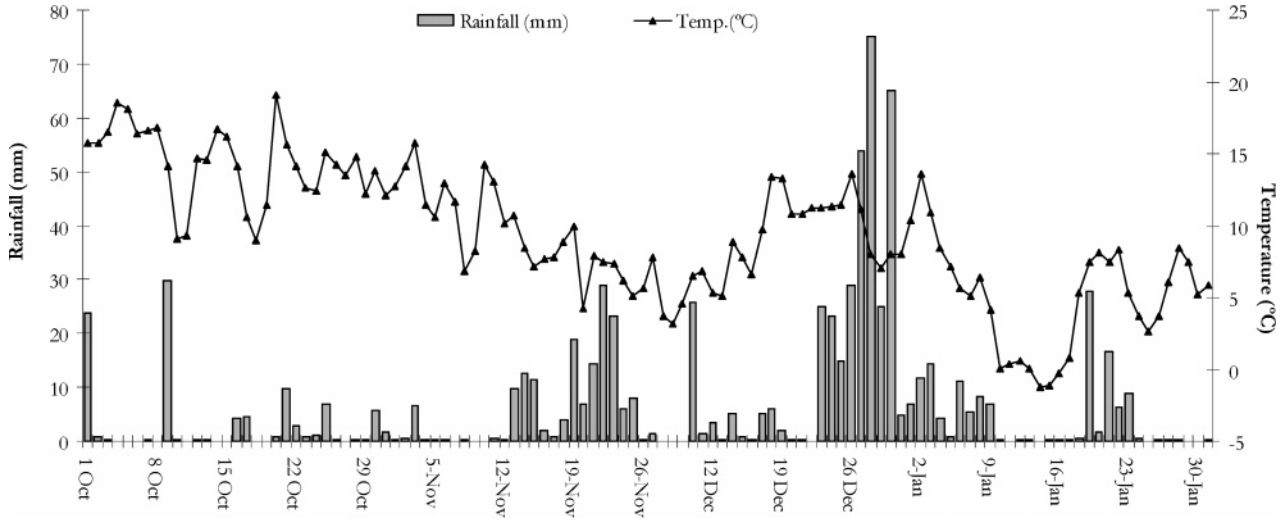

Figure 3. Climatic conditions observed in Mirandela from October 2002 to January 2003.

of citric and malic acids by the samples from conventional practice, subjected to mineral fertilization, was higher. This could be attributed to the existence of nitrate in the fertilizer, which is available in high quantity by that time, leading to higher citric and malic acids contents, as described before (*16*).

Phenolic Compounds. The analysis by HPLC-DAD of the methanolic extracts of tronchuda cabbage internal leaves revealed the presence of several hydroxycinnamic acids and kaempferol derivatives: 3-*p*-coumaroylquinic acid, kaempferol 3-*O*-(caffeoyl)sophoroside-7-*O*-glucoside, kaempferol 3-*O*-(sinapoyl)sophoroside-7-*O*-glucoside, kaempferol 3-*O*-(feruloyl)-sophoroside-7-*O*-glucoside, kaempferol 3-*O*-sophoroside, two isomers of 1,2-disinapoylgentiobiose, 1-sinapoyl-2-feruloylgentiobiose, 1,2,2′-trisinapoylgentiobiose, and 1,2′-disinapoyl-2-feruloylgentiobiose (**Figure 2**). Kaempferol 3-*O*-sophoroside-7-*O*-glucoside (**2**) was found only in the samples collected in January (samples 15 and 16). The above-mentioned compounds have already been characterized before in tronchuda cabbage internal leaves (*13*). The results obtained in this study indicate that the phenolic composition of tronchuda cabbage internal leaves is distinct from that of the external ones: the internal leaves present phenolic acid derivatives as the main compounds and small amounts of flavonol glycosides (**Figure 2**), whereas external leaves exhibit only flavonol derivatives (*12*). Besides, kaempferol 3-*O*-sophoroside-7-*O*-glucoside (**2**) and kaempferol 3-*O*-sophoroside (**6**) are the only phenolic compounds presented in both kinds of leaves.

Data from the quantification of the identified phenolic compounds (**Table 2**) showed that 3-*p*-coumaroylquinic acid (**1**) and the sinapic acid derivatives, namely, the two isomers of 1,2-disinapoylgentiobiose (**7** and **9**), 1-sinapoyl-2-feruloyl-gentiobiose (**8**) and 1,2,2′-trisinapoylgentiobiose (**10**), were the major compounds, representing >79% of total phenolics, with the exception of sample 16, collected in January from the conventional culture, in which kaempferol 3-*O*-(sinapoyl)-sophoroside-7-*O*-glucoside (**4**) was the compound present in highest amount (∼29% of total phenolics). Compound **11** (1,2′-disinapoyl-2-feruloylgentiobiose) was the minor compound (**Table 2**). The phenolic profile of tronchuda cabbage internal leaves was revealed to be more homogeneous than that of the external ones (*12*), which is not surprising considering that the internal leaves are less exposed to external factors and phenolic compounds are very susceptible to the external environment.

Generally, internal leaf samples from organic culture exhibited higher total phenolics content than those from conventional practice collected in the same period, as was observed with the

external leaves (*12*), with the exception of the samples from November. The interference of the mineral fertilizers and/or pesticides, used in conventional culture, in the biosynthetic pathway of phenolic compounds could explain the lower amounts presented by those samples.

When considering the changes in phenolic composition during winter, we observed a decrease of the total phenolics content until December, which was more evident in samples from conventional culture. A considerable increase of total phenolics in both organic and conventional samples was noticed in January, as was observed before with the external leaves (*12*) (**Table 2**). Additionally, the production of flavonoids was higher in January for the two agronomic practices, a fact that could be explained by the very low temperatures registered in Mirandela region during January (**Figure 3**). In fact, the existence of a positive correlation between high levels of flavonoid glycosides and increased frost resistance is well-known (*17*). As these compounds contain sugar residues, they can delay water crystallization by the formation of hydrogen bonds between their hydroxyl groups and water molecules. Also, the action of flavonoids as antioxidants can be invoked, as protectors of plant tissues against adverse effects of low-temperature oxidative stress (*17*). It seems that phenylalanine ammonia-lyase activity, a key enzyme of phenylpropanoid biosynthesis, is increased under low-temperature conditions (*14*), which may justify the production of flavonoids as defense agents.

The results obtained in this study indicate that, in a general way, tronchuda cabbages from organic culture present higher phenolics contents than those from the conventional one. Tronchuda cabbage may constitute a good source of health-promoting compounds, namely, organic acids and phenolic compounds. It should be emphasized that internal and external leaves supply distinct phenolics. This could be of great relevance when biological activities are considered and deserves further studies.

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