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# Phenolic Compounds in External Leaves of Tronchuda Cabbage (*Brassica oleracea* L. var. *costata* DC)

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Glycosylated kaempferol derivatives from the external leaves of tronchuda cabbage (*Brassica oleracea* L. var. *costata* DC) characterized by reversed-phase HPLC-DAD-MS/MS-ESI were kaempferol 3-*O*-sophorotrioside-7-*O*-glucoside, kaempferol 3-*O*- (methoxycaffeoyl/caffeoyl)sophoroside-7-*O*-glucoside, kaempferol 3-*O*-sophoroside-7-*O*-glucoside, kaempferol 3-*O*-sophorotrioside-7-*O*-sophoroside, kaempferol 3-*O*-sophoroside-7-*O*-sophoroside, kaempferol 3-*O*-tetraglucoside-7-*O*-sophoroside, kaempferol 3-*O*-(sinapoyl/caffeoyl)sophoroside-7-*O*-glucoside, kaempferol 3-*O*-(feruloyl/caffeoyl)sophoroside-7-*O*-glucoside, kaempferol 3-*O*-sophorotrioside, kaempferol 3-*O*-(sinapoyl)sophoroside, kaempferol 3-*O*-(feruloyl)sophorotrioside, kaempferol 3-*O*-(feruloyl)sophoroside, kaempferol 3-*O*-sophoroside, and kaempferol 3-*O*-glucoside. These acylated derivatives are reported for the first time in nature, with the exception of kaempferol 3-*O*-(sinapoyl)sophoroside. Quantification of the identified compounds was achieved by HPLC-DAD and carried out in samples cultivated under conventional or organic practices and collected at different times. In general, samples from organic production exhibited higher total phenolics content than those from conventional practices collected in the same period.

**KEYWORDS:** ***Tronchuda cabbage*; *Brassica oleracea* L. var. *costata* DC; kaempferol derivatives; HPLC-DAD-MS/MS-ESI; HPLC-DAD**

## INTRODUCTION

*Brassica* vegetables are consumed in enormous quantities throughout the world and are important in human nutrition. *Brassica oleracea* is a native of the Mediterranean region and southwestern Europe, extending northward to southern England, growing on seaside cliffs. The wild species has evolved into a number of varieties in which different parts of the plant have become the edible constituents. Although essentially temperate, *Brassica oleracea* forms are now grown in other regions all over the world (*1*). Tronchuda cabbage (*Brassica oleracea* L. var. *costata* DC) is still considered to be a primitive cultivar, being 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 (*2*, *3*). It is a hardy crop offering the possibility of harvesting in the cold and frosty regions of northern Portugal and Spain, in which it constitutes a very important supply of vegetables during the winter (*2*, *3*). The tronchuda cabbage plant resembles a thick-stemmed collard with large floppy leaves. 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 preferences of consumers. Internal leaves are pale yellow and are tender and sweeter than external leaves, which present a dark green color. Previous research on tronchuda cabbage concerned its glucosinolate, protein, mineral, and free sugars composition (*2*−*4*), its resistance to crucifer downy mildew (*5*), the effect of silver nitrate on anther culture embryo production (*6*), and the effect of medium renovation and incubation temperature regimes on microspore culture embryogenesis (*7*).

In the past few years there has been growing interest in the chemopreventive and chemotherapeutic potential of naturally occurring compounds. *Brassica* species are reported to possess cancer preventive properties (*8*) that have been attributed to the glucosinolates and their derived products (*9*). Flavonoids and other phenolics also contribute to this capacity (*10*, *11*). Several studies have reported the presence of polyphenolic compounds in different *B. oleracea* varieties (*12*−*15*), but none involved tronchuda cabbage. Those polyphenols consisted of complex

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**Table 1.** Quantification of Tronchuda Cabbage Phenolic Compounds (Milligrams per Kilogram, Dry Basis)[a]

| sample | collection date | culti-vation[c] | compound[b] 1 + 2 | 3 | 4 | 5 + 6 | 7 | 8 | 9 + 10 | 11 | 12 + 13 | 14 | total |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | Oct 2002 | O | 556.9 (36.8) | 2.4 (0.1) | 5.8 (0.1) | 4.2 (0.0) | 91.6 (5.9) | 45.5 (2.6) | 157.6 (10.2) | 15.4 (0.4) | 87.8 (1.8) | nq[d] | 912.3 |
| 2 | Oct 2002 | C | 274.4 (11.2) | 112.6 (2.8) | 12.6 (0.5) | 10.5 (0.8) | 22.3 (1.6) | 95.4 (2.4) | 59.2 (1.9) | 6.7 (0.8) | 45.8 (1.8) | nq | 555.8 |
| 3 | Nov 2002 | O | 547.6 (46.0) | 750.2 (84.0) | 21.3 (2.5) | 59.1 (15.4) | 186.5 (10.7) | 429.7 (42.2) | 173.1 (6.7) | 16.2 (1.9) | 240.1 (29.6) | nq | 1952.0 |
| 4 | Nov 2002 | C | 191.0 (16.3) | 16.0 (9.3) | 8.1 (0.1) | 18.2 (0.3) | 1.3 (0.1) | 96.7 (14.3) | 97.1 (8.9) | 100.1 (1.5) | 38.7 (3.0) | nq | 561.4 |
| 5 | Dec 2002 | O | 9.2 (0.7) | 8.6 (0.1) | | 3.3 (0.1) | 1.3 (0.0) | 1.6 (0.2) | 2.0 (0.2) | 1.1 (0.1) | 7.5 (0.2) | nq | 27.4 |
| 6 | Dec 2002 | C | 18.4 (0.7) | 41.9 (0.6) | 3.3 (0.1) | 3.8 (0.2) | 7.3 (0.1) | 9.3 (3.4) | 60.5 (2.7) | 94.7 (4.6) | 23.0 (0.0) | nq | 252.5 |
| 7 | Jan 2003 | O | 245.7 (22.1) | 53.9 (2.9) | 2.5 (0.0) | 80.4 (1.9) | 5.9 (0.1) | 32.3 (2.5) | 115.7 (2.6) | 185.7 (3.4) | 3.2 (0.1) | nq | 712.6 |
| 8 | Jan 2003 | C | 62.5 (1.3) | 8.0 (0.6) | 1.4 (0.1) | 3.5 (0.5) | 34.2 (0.2) | 2.1 (0.0) | 34.5 (1.1) | | 33.6 (2.7) | nq | 254.1 |

[a] Results are expressed as mean (standard deviation) of two determinations. [b] Identity of compounds as in **Figure 1**. [c] O, organic; C, conventional [d] nq, not quantified.

flavonol glycosides, some of them being acylated derivatives, and hydrocycinnamic acid esters.

In a preliminary study we observed marked differences between the phenolic compositions of the two kinds of leaves. The work herein constitutes the first step for the characterization of phenolics in this cabbage. We aimed to identify and quantify the phenolics from tronchuda cabbage external leaves and to see if the phenolic composition is influenced by the agricultural procedure and the collection date. For this purpose, a methodology based on the solid-phase extraction (SPE) of phenolic compounds and reversed-phase HPLC-DAD-MS/MS-ESI and HPLC-DAD analysis of the methanolic extracts was applied to external leaf samples.

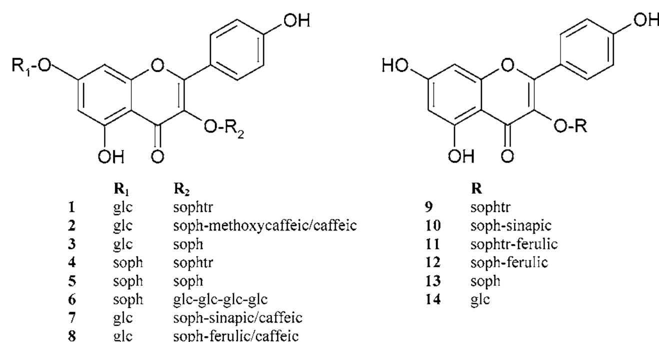


**Figure 1.** Chemical structures of phenolic compounds identified in tronchuda cabbage: glc, glucose; soph, sophorose; sophtr, sophorotriose.

## MATERIALS AND METHODS

**Standards and Reagents.** Kaempferol 3-*O*-rutinoside and kaempferol 3-*O*-glucoside were from Extrasynthése (Genay, France). Methanol, sodium hydroxide, and hydrochloric and formic acids were purchased from Merck (Darmstadt, Germany). The water was treated in a Milli-Q water purification system (Millipore, Bedford, MA).

**SPE Columns.** The Isolute C18 non-endcapped (NEC) columns (50 $\mu$m particle size, 60 Å porosity; 10 g of sorbent mass/70 mL of reservoir volume) were obtained from International Sorbent Technology Ltd. (Mid Glamorgan, U.K.).

**Plant Material and Sampling.** Tronchuda cabbages grown under different agronomic practices were studied. The experimental work was carried out in two fields located in Valbom dos Figos−Mirandela, northeastern Portugal (U.T. M. 29 PG5602). In one of the fields the production has organic status, certified by the national authority (Instituto de Desenvolvimento Rural e Hidráulica), following the guidelines of Council Regulation (EEC) 2092/91 of June 24, 1991 (organic production), and in the other field the production followed the standard cultural practices in the region (conventional production) (**Table 1**). The sowing date was in the end of June 2002, and the plant material was transplanted to the fields in the end of August. Water was provided by a local captation. In the organic field only organic fertilization was made with sheep manure, and no phytosanitary treatments were applied. In the conventional field, organic fertilization was made during the transplantation of the plants and, in the beginning of September, a mineral fertilization with 20.5% ammonium nitrate and 21.8% CaO (Nitrolusal 20.5%) (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 months (**Table 1**). On each harvesting date and in each field three plants were randomly selected and collected in the morning, approximately at the same hour. After harvesting, the plants were immediately transported to the laboratory and external and internal leaves were separated. Each sample corresponds to a mixture of the external leaves of the three plants, collected in the same field. Samples were stored in a freezer and freeze-dried before analysis. External leaves were subjected to phenolics extraction and HPLC analysis.

**Phenolic Compound 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 was concentrated to dryness under reduced pressure (30 °C) and redissolved in water acidified to pH 2 with HCl. The solution obtained was applied to an Isolute C18 (NEC) column, previously conditioned with 30 mL of methanol and 70 mL of acidified water. Polar compounds were removed with the aqueous solvent, and the retained phenolic compounds were then eluted with 50 mL of methanol. The extract was concentrated to dryness under reduced pressure (30 °C) and redissolved in methanol (1 mL).

**Alkaline and Acid Hydrolysis.** Hydrolysis was achieved according to previously reported methodology (*12*) by adding 0.5 mL of 4 N NaOH to the methanolic extract (0.5 mL) and keeping the mixture for 16 h at room temperature in a stoppered test tube under $N_2$ atmosphere. After this step, the alkaline hydrolysis products were acidified with concentrated HCl to pH 1−2 and directly analyzed by LC/UV-DAD/ESI-$MS^n$. This solution was used for partial (mild) acid hydrolysis and kept in a stoppered test tube during 30 min in an oven adjusted to 80 °C; then, it was directly analyzed by HPLC-DAD-MS/MS.

Total acid hydrolysis was carried out by adding 0.5 mL of 4 N HCl to 0.5 mL of the methanolic extract, and this solution was kept in a stoppered test tube, incubated for 30 min at 85 °C, and directly analyzed by LC/UV-DAD/ESI-$MS^n$.

**HPLC-DAD-MS/MS-ESI Qualitative Analysis.** Chromatographic separations were carried out on a 250 × 4 mm i.d., 5 $\mu$m, RP-18 LiChroCART column (Merck, Darmstadt, Germany) protected with a 4 × 4 mm LiChroCART guard column using formic acid 0.1% (A) and methanol (B) as solvents, starting with 20% B and using a gradient to obtain 50% B at 35 min and 80% B at 37 min. On the other hand, for the analysis of the acids and the aglycons obtained after hydrolysis, a linear gradient starting with 15% B and reaching 65% B at 50 min was used to reach 80% B at 52 min. The flow rate was 1 mL/min, and the injection volumes varied between 10 and 50 $\mu$L.

The HPLC system was equipped with an Agilent 1100 series diode array detector and a mass detector in series (Agilent Technologies, Waldbronn, Germany). It consisted of a G1312A binary pump, a G1313A autosampler, a G1322A degasser, and a G1315B photodiode array detector controlled by ChemStation software (v. 08.03). Spectroscopic data from all peaks were accumulated in the range 240−400

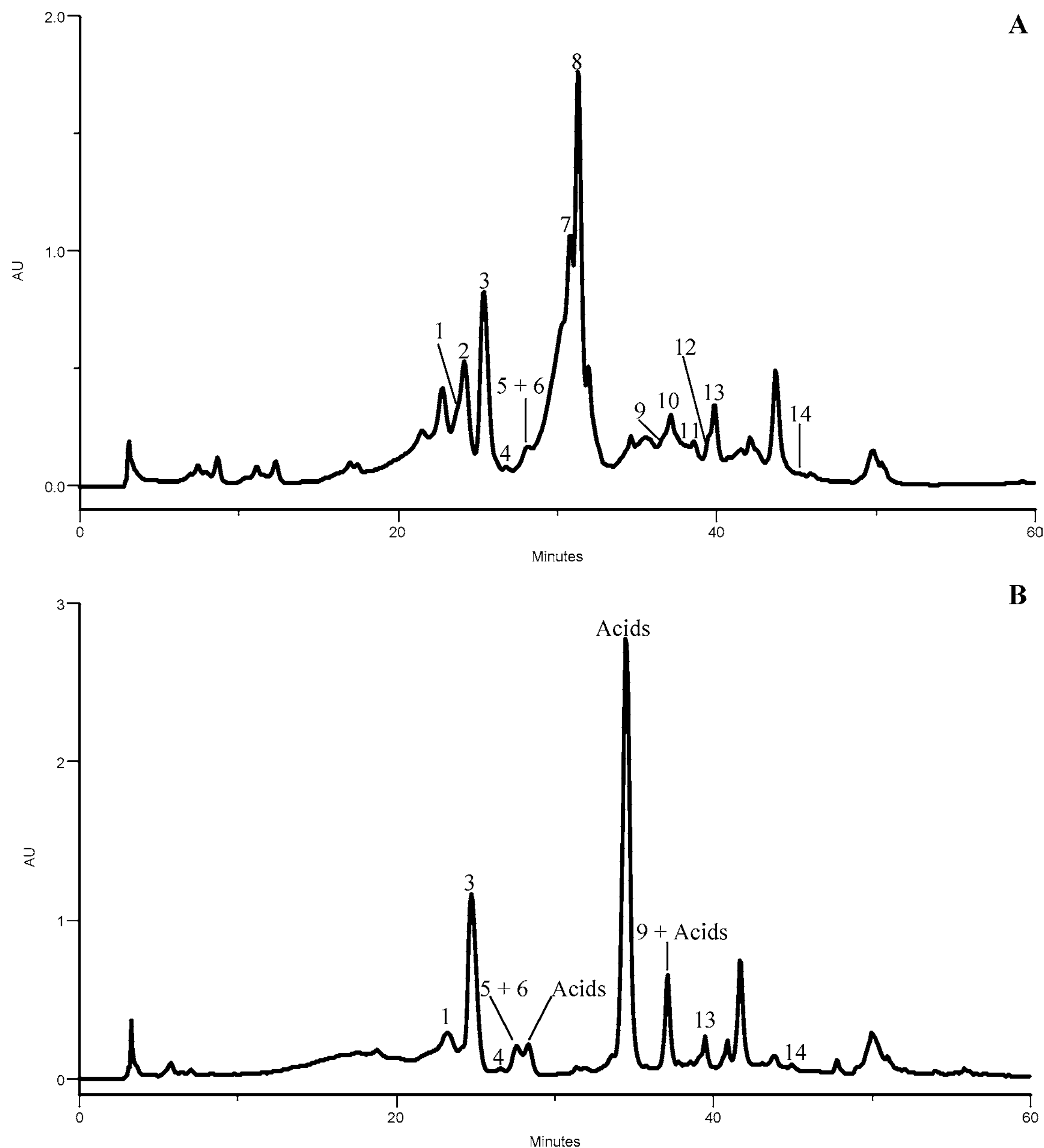


**Figure 2.** (**A**) HPLC-DAD chromatogram of tronchuda cabbage methanolic extract. (**B**) HPLC-DAD chromatogram of tronchuda cabbage methanolic extract after alkaline hydrolysis: (peak 1) kaempferol 3-*O*-sophorotrioside-7-*O*-glucoside; (peak 2) kaempferol 3-*O*-(methoxycaffeoyl/caffeoyl)sophoroside-7-*O*-glucoside; (peak 3) kaempferol 3-*O*-sophoroside-7-*O*-glucoside; (peak 4) kaempferol 3-*O*-sophorotrioside-7-*O*-sophoroside; (peak 5) kaempferol 3-*O*-sophoroside-7-*O*-sophoroside; (peak 6) kaempferol 3-*O*-tetraglucoside-7-*O*-sophoroside; (peak 7) kaempferol 3-*O*-(sinapoyl/caffeoyl)sophoroside-7-*O*-glucoside; (peak 8) kaempferol 3-*O*-(feruloyl/caffeoyl)sophoroside-7-*O*-glucoside; (peak 9) kaempferol 3-*O*-sophorotrioside; (peak 10) kaempferol 3-*O*-(sinapoyl)sophoroside; (peak 11) kaempferol 3-*O*-(feruloyl)sophorotrioside; (peak 12) kaempferol 3-*O*-(feruloyl)sophoroside; (peak 13) kaempferol 3-*O*-sophoroside; (peak 14) kaempferol 3-*O*-glucoside. Detection at 330 nm.

nm, and chromatograms were recorded at 330 nm for glycosides and acylated derivatives and at 330 and 360 nm for hydroxycinnamic acids and flavonoid aglycons, respectively. The mass detector was an Agilent G2445A ion trap mass spectrometer equipped with an electrospray ionization (ESI) system and controlled by LCMSD software (v. 4.1). Nitrogen was used as nebulizing gas at a pressure of 65 psi, and the flow was adjusted to 11 L/min. The heated capillary and voltage were maintained at 350 °C and 4 kV, respectively. The full scan mass covered the range from $m/z$ 200 to 2000 for free glycosides and acylated derivatives and from $m/z$ 90 to 400 for acids and aglycons. Collision-induced fragmentation experiments were performed in the ion trap using helium as collision gas, with voltage ramping cycles from 0.3 to 2 V. MS data were acquired in the negative ionization mode. $MS^n$ data were achieved in the automatic mode on the more abundant fragment ion in $MS^{n-1}$. **Tables 2** and **3** show the most frequent ions that characterize the fragmentation of the flavonoid *O*-glycosides. Other ions were found, but they have not been included due to their low significance on the MS behavior ions.

**HPLC-DAD Quantitative Analysis.** Twenty microliters of each extract was analyzed using a HPLC unit (Gilson) and a 250 × 4.6 mm i.d., 5 $\mu$m Spherisorb ODS2 column (Waters, Milford, USA). The solvent system was a mixture of formic acid 5% in water (A) and methanol (B), with a flow rate of 1 mL/min, and the gradient was as follows: 0 min, 10% B; 25 min, 20% B; 40 min, 50% B; 45 min, 50% B; 46 min, 90% B; 50 min, 90% B; 55 min, 100% B; 58 min, 100% B; and 60 min, 10% B. 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

**Table 2.** $t_R$, -MS $[M - H]^-$, -MS$^2$ $[M - H]^-$, and -MS$^3$ $[(M - H) \rightarrow Y^7_0]^-$ Data of Kaempferol Glycosides

| compound[a] | $t_R$ (min) | $[M - H]^-$ ($m/z$) | -MS$^2$ $[M - H]^-$ ($m/z$) (%) $Y^7_0{}^-$ | -MS$^3$[(M − H) → $Y^7_0$]$^-$ ($m/z$) (%) $Y^7_0{}^{0,2}X^-$ | $Y^7_0Y^3_3{}^-$ | $Y^7_0Y^3_2{}^-$ | $Y^7_0Y^3_1{}^-$ | $Y^7_0Z^3_1{}^-$ | $Y^7_0Y^3_0{}^-$ |
|---|---|---|---|---|---|---|---|---|---|
| | | | Kaempferol-3-*O*-tetraglcucoside-7-*O*-sophoroside | | | | | | |
| | | | (−324) | | (−162) | (−324) | | | (−648) |
| **6**, kaempf-3-TetraGlc-7-Soph | 10.8 | 1257 | 933 (100) | | 771 (12) | 609 (35) | | | 285 (100) |
| | | | Kaempferol-3-*O*-sophorotrioside-7-*O*-glucoside/sophoroside | | | | | | |
| | | | (−162/−324) | (−120) | | (−162) | | (−342) | (−486) |
| **1**, kaempf-3-Sophtr-7-Glc | 9.1 | 933 | 771 (100) | | | 609 (100) | | 429 (72) | 285 (39) |
| **4**, kaempf-3-Sophtr-7-Soph | 10.2 | 1095 | 771 (100) | 651 (6) | | 609 (100) | | 429 (35) | 285 (71) |
| | | | Kaempferol-3-*O*-sophoroside-7-*O*-glucoside/sophoroside | | | | | | |
| | | | (−162/−324) | (−120) | | | (−162) | (−180) | (−324) |
| **3**, kaempf-3-Soph-7-Glc | 10.0 | 771 | 609 (100) | | | | 447 (17) | 429 (45) | 285 (100) |
| **5**, kaempf-3-Soph-7-Soph | 10.5 | 933 | 609 (100) | 489 (11) | | | | 429 (92) | 284 (100)[b] |

Kaempferol-3-*O*-glycoside

| compound[a] | $t_R$ (min) | $[M - H]^-$ ($m/z$) | -MS$^2$ $[M - H]^-$ ($m/z$) (%) $^{0,2}X^-$ | $Y^3_2{}^-$ | $Z^3_2$ | $Y^3_1{}^-$ | $Z^3_1{}^-$ | $Y^3_0{}^-$ |
|---|---|---|---|---|---|---|---|---|
| | | | (−120) | (−162) | (−180) | (−324) | (−342) | (−486) |
| **9**, kaempf-3-Sophtr | 22.2 | 771 | | 609 (27) | 591 (29) | | 429 (63) | 285 (100) |
| | | | | | | (−162) | (−180) | (−324) |
| **13**, kaempf-3-Soph | 24.6 | 609 | 489 | | | 447 (7) | 429 (43) | 285 (100) |
| **14**, kaempf-3-Glc | 32.5 | 447 | | | | | | 285 (100) |

[a] Kaempf, kaempferol; Glc, glucose; Soph, sophorose; Sophtr, sophorotriose. [b] Fragments from homolytic cleavage of the glycosidic bond ($[Y^7_0Y^3_0{-}H]^{\bullet -}$) (*18*).

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. With the exception of kaempferol 3-*O*-glucoside, which was quantified as itself, the identified compounds were quantified as kaempferol 3-*O*-rutinoside, because none of them was commercially available. The average regression equations for kaempferol 3-*O*-rutinoside and kaempferol 3-*O*-glucoside were $y = 2.29 \times 10^7x$ and $y = 2.45 \times 10^7x$, respectively. The detection limits were 0.345 $\mu$g/mL for kaempferol 3-*O*-rutinoside and 0.322 for kaempferol 3-*O*-glucoside.

## RESULTS AND DISCUSSION

**Characterization of Phenolic Compounds.** The HPLC-DAD chromatogram of tronchuda cabbage methanolic extract revealed the existence of several compounds with UV spectra with two maxima at 267 and 349 nm and a shoulder at 300 nm, indicating the presence of kaempferol derivatives with substitution in position 3 (**Figures 1** and **2A**). In addition, acylated flavonoids were detected in the extract, and their UV spectra, characterized with a maximum with a high absorption at 330 nm and a little maximum between 255 and 268 nm, suggested that they were acylated with hydroxycinnamic acid derivatives. After alkaline hydrolysis, the chromatogram showed, apart from several hydroxycinnamic acids, various flavonoid glycosides and disappearance of the acylated derivatives (**Figure 2B**).

**Flavonoid Glycosides.** The MS study of free (nonacylated) glycosides was performed on the saponified extract so that the possible glucosyl radical losses (−162 amu) were not confused with those of the caffeoyl radical (−162 amu). The HPLC-DAD chromatogram of the saponified extract exhibited compound **3** as the main deacylated glycoside (**Figure 2B**).

The MS ion trap analysis of compound **3** and other minor flavonoids eluting in the first part of the chromatogram (**1**, **4**, **5**, and **6**), indicated that these compounds were kaempferol hexosides ($m/z$ of the aglycon at 285), most probably glucosides due to phylogenetic similarity with cauliflower (*12*) and broccoli (*15*). According to previous studies (*16*), from the study of their fragmentation in MS$^n$ ($n = 2-4$) (**Table 2**) it can be deduced that these derivatives are glycosylated in positions 3 and 7, with glucose, sophorose, or sophorotriose. The fragmentation pattern and the relative abundance of the obtained ions indicated the nature of the oligosaccharides linked to the hydroxyl groups in positions 3 and 7. The partial acid hydrolysis of the raw extract and its subsequent HPLC-DAD-MS$^n$ study gave rise to the characterization, besides kaempferol as the main flavonoid, of kaempferol 7-*O*-glucoside [UV, $\lambda$ 253sh, 266, 321sh, 367 nm; MS, 447 $(M - H)^-$; MS$^2$ $(M - H)^-$ 285], confirming the substitution of the hydroxyl at the 7-position of the most abundant glycoside (**3**). According to the above, the following kaempferol derivatives have been characterized both in the raw and in the saponified extracts: kaempferol 3-*O*-sophorotrioside-7-*O*-glucoside (**1**), kaempferol 3-*O*-sophoroside-7-*O*-glucoside (**3**), kaempferol 3-*O*-sophorotrioside-7-*O*-sophoroside (**4**), and kaempferol 3-*O*-sophoroside-7-*O*-sophoroside (**5**) (**Figure 1**).

Compounds **9**, **13**, and **14** exhibited UV spectra indicating a kaempferol derivative, with a substituent in position 3. Their fragmentation was characteristic of glycosides linked to only one hydroxyl group (*16*). They were identified as kaempferol 3-*O*-sophorotrioside (**9**), kaempferol 3-*O*-sophoroside (**13**), and kaempferol 3-*O*-glucoside (**14**), respectively. These glycosides have been recently characterized in cauliflower (*12*), some of them being detected for the first time in nature. It should be emphasized that flavonoids with more than three sugar residues are not usual and that pentaglycosides were observed for the first time in cauliflower (*12*) and broccoli (*15*).

The production of flavonoids with an unusually high degree of glycosylation by other *B. oleracea* varieties (*12*, *15*) and the useful application of HPLC-DAD-MS$^n$ ion trap in the identification of these kinds of complex flavonoids (*16*) led us to search for the possible existence of other kaempferol derivatives with six hexoses. Near compound **5** we detected in the extracted ion chromatogram (EIC) an ion at $m/z$ 1257 (compound **6**), present in trace amounts, which was in accordance with the deprotonated

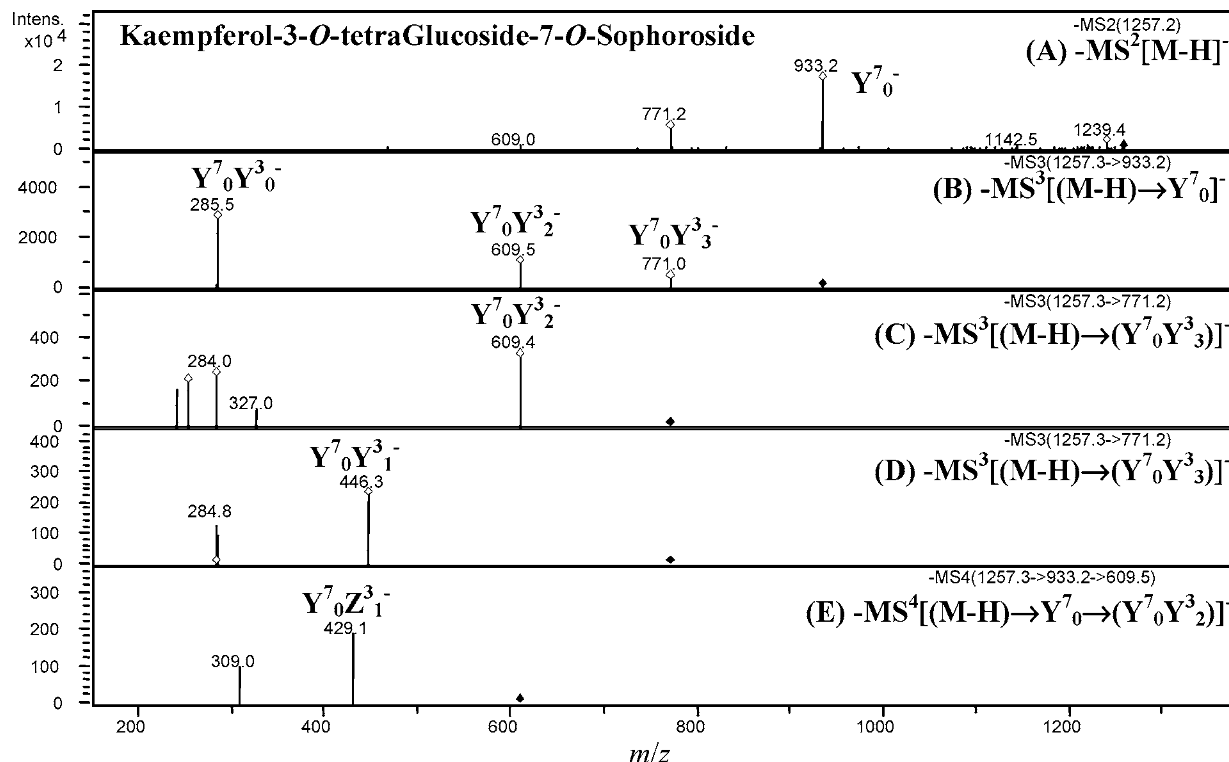


**Figure 3.** MS$^n$ analysis of kaempferol 3-*O*-tetraglucoside-7-*O*-sophoroside (**6**): (**A**) -MS$^2$ [M − H]$^-$; (**B**) -MS$^3$ [(M − H) → $Y^7{}_0$]$^-$; (**C**, **D**) -MS$^3$ [(M − H) → ($Y^7{}_0Y^3{}_3$)]$^-$; (**E**) -MS$^4$ [(M − H) → $Y^7{}_0$ → ($Y^7{}_0Y^3{}_2$)]$^-$.

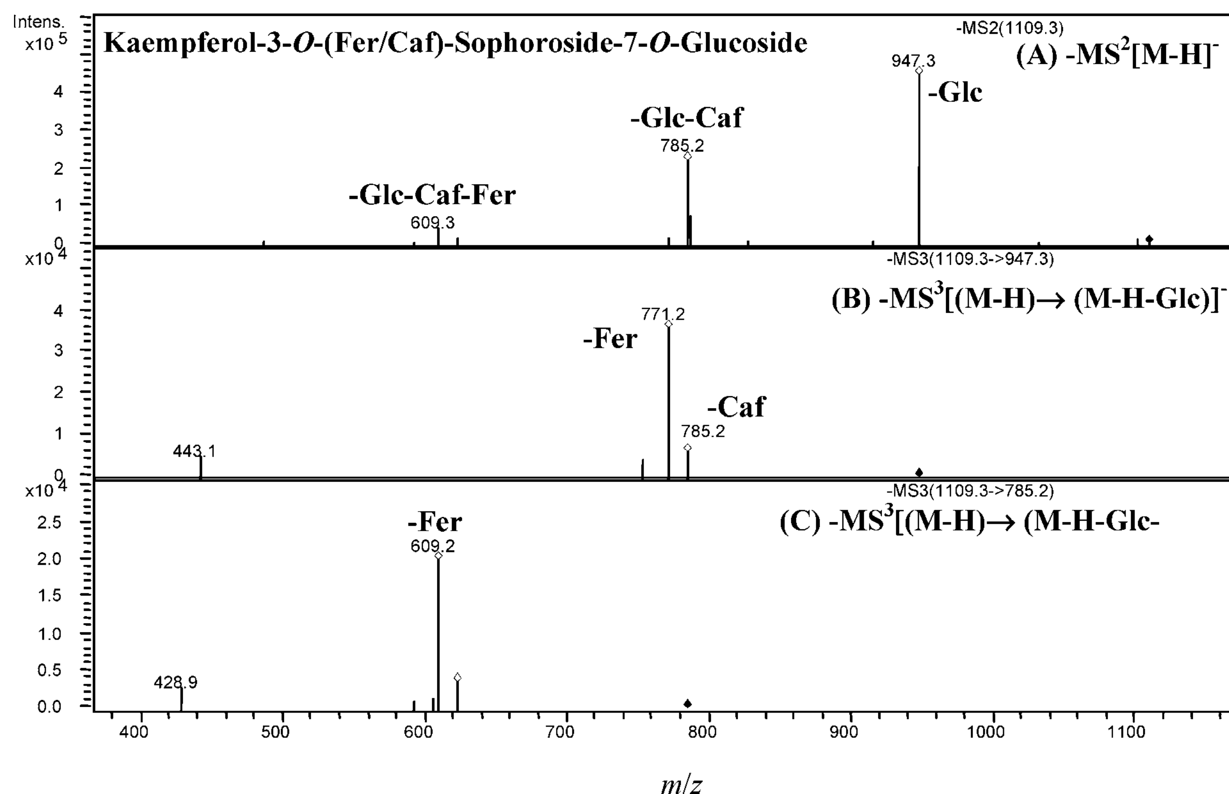


**Figure 4.** MS$^n$ analysis of kaempferol3-*O*-(feruloyl/caffeoyl)sophoroside-7-*O*-glucoside (**8**): (**A**) -MS$^2$ [M − H]$^-$; (**B**) -MS$^3$ [(M − H) → (M − H − Glc)]$^-$; (**C**) -MS$^3$ [(M − H) → (M − H − Glc − Caf)]$^-$.

molecular ion of an hexaglucosyl kaempferol. The loss of 324 amu, corresponding to two sugar moieties, in the MS$^2$ (M − H)$^-$ event to give an ion at *m/z* 933 (base peak), $Y^7{}_0{}^-$, indicated the presence of sophoroside linked to the hydroxyl group at the 7-position of kaempferol (**Table 2**; **Figure 3A**) and, therefore, the remaining four sugar residues should be tentatively considered as a tetrasaccharide linked to the hydroxyl group at the 3-position. In addition, the MS$^3$ [(M − H) → $Y^7{}_0$]$^-$ analysis allowed us to observe, apart from the ion of the aglycon (*m/z* 285), the fragmentation ions at *m/z* 771 and 609, corresponding to the losses of one and two glucoses from the ion at *m/z* 933 (**Figure 3B**), respectively, confirming the proposed substitution.

Other observed ions, which are not included in **Table 2**, were $Y^7{}_0Y^3{}_1{}^-$ (*m/z* 446) and $Y^7{}_0Z^3{}_1{}^-$ (*m/z* 429) (**Figure 3D,E**). The ions obtained have been labeled according to previous studies (*16*−*18*). According to the above-mentioned, compound **6** was identified as kaempferol 3-*O*-tetraglucoside-7-*O*-sophoroside.

**Acylated Derivatives.** The comparison of the HPLC-DAD chromatogram of the raw methanolic extract (**Figure 2A**) with that of the saponified extract (**Figure 2B**) indicated the existence of an acylated compound (compound **8**) present in high amounts. The HPLC-MS analysis showed the presence of other acylated compounds (compounds **2**, **7**, **10**−**12**) coeluting with other substances. From the MS study of these compounds two groups of acylated derivatives could be observed. For their retention times, and by comparison with broccoli compounds (*15*), one of them, grouping compounds **2**, **7**, and **8**, should be composed of monoacylated derivatives of kaempferol tetraglycoside, acylated with methoxycaffeic, sinapic, and ferulic acids, respectively. However, once the main product in the saponified extract is kaempferol 3-*O*-sophoroside-7-*O*-glucoside (**3**), they were tentatively considered as being diacylated derivatives of compound **3**, with caffeic acid as the second acylation acid, with a kind of link between the acyl groups that modifies their retention times, without discarding the possibility of being monocylated derivatives of compound **1** (kaempferol 3-*O*-sophorotrioside-7-*O*-glucoside). The other group of compounds (**10**−**12**) were monoacylated derivatives of kaempferol 3-*O*-glycosides, without glycosylation at the 7-position (**Table 3**).

As previously reported (*15*), in the $MS^2$ $(M - H)^-$ of compounds with glycosylation at both the 3- and 7-positions, a base peak corresponding to the loss of glycosylation at the 7-position was observed. Other important ions were also detected and were due to the simultaneous loss of the mentioned sugar and of one or two acids (**Table 3**; **Figure 4A**). After the base peak (M − H − Glc), the most abundant ion was (M − H − 324), resulting from the simultaneous loss of the glucose at the 7-position and of one caffeic acid (**Table 3**). In the fragmentation of the ion resulting from the loss of glycosylation at the 7-position ($MS^3$ $[(M - H) \rightarrow (M - H - Glc)]^-$) the loss of the two acids could be observed, confirming that the acylation was present on the sugars at the 3-position (**Table 3**; **Figure 4B**). The $MS^3$ $[(M - H) \rightarrow (M - H - Glc - Caf)]^-$ event gave rise to the ion of the aglycon glycosylated at the 3-position (kaempferol 3-*O*-sophorosyl) by loss of the other acid (**Table 3**; **Figure 4C**), the breakdown of which (data not shown in **Table 3**) was like the corresponding one for compound **3**. In the $MS^3$ $[(M - H) \rightarrow (M - H - Glc - Caf)]^-$ event of the derivatives with ferulic or sinapic acid from the first group and in the $MS^2$ $(M - H)^-$ event of the second group, an ion at *m/z* 623, corresponding to 609 + 14, could also be observed (data not shown in **Table 3**).

Therefore, the following diacyl derivatives from compound **3** have been characterized: kaempferol 3-*O*-(methoxycaffeoyl/caffeoyl)sophoroside-7-*O*-glucoside (**2**), kaempferol 3-*O*-(sinapoyl/caffeoyl)sophoroside-7-*O*-glucoside (**7**), and kaempferol 3-*O*-(feruloyl/caffeoyl)sophoroside-7-*O*-glucoside (**8**). In addition, the monoacylated derivatives kaempferol 3-*O*-(sinapoyl)sophoroside (**10**), kaempferol 3-*O*-(feruloyl)sophorotrioside (**11**), and kaempferol 3-*O*-(feruloyl)sophoroside (**12**) have also been identified. To the best of our knowledge, the characterization of these acylated derivatives has not been previously reported in nature, with the exception of compound **10**, recently described in cauliflower (*12*).

The comparative study of tronchuda cabbage flavonoids with those from cauliflower and broccoli indicates their resemblance

**Table 3.** $t_R$, -MS $[M - H]^-$, -$MS^2$ $[M - H]^-$, -$MS^3$ $[(M - H) \rightarrow (M - H - Glc)]^-$, and -$MS^3$ $[(M - H) \rightarrow (M - H - Glc - Caf)]^-$ Data of Acylated Derivatives from Kaempferol Glycosides

| | | | -$MS^2$$[M - H]^-$ (*m/z*) (%) | | | | | | | | | -$MS^3$ $[(M - H) \rightarrow (M - H - Glc)]^-$ (*m/z*) (%) | | | | -$MS^3$ $[(M - H) \rightarrow (M - H - Glc - Caf)]^-$ (*m/z*) (%) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| compound[a] | $t_R$ (min) | $[M - H]^-$ (*m/z*) | −162 −Glc | −176 −Fer | −206 −Sinp | −324 −G-C | −338 −G-F | −354 −G-MC | −516 −G-C-MC | −500 −G-C-F | −530 −G-C-S | −162 −Caf | −176 −Fer | −192 −MeOCaf | −206 −Sinp | −176 −Fer | −192 −MeOCaf | −206 −Sinp |
| diacylated derivatives from **3**: kaempferol-3-*O*-sophoroside-7-*O*-glucoside | | | | | | | | | | | | | | | | | | |
| **2**, 3-MeOCaf/Caf | 9.3 | 1125 | 963 (100) | | | 801 (67) | | 771 (16) | 609 (7) | | | 801 (6) | | 771 (100) | | | 609 (100) | |
| **7**, 3-Sinp/Caf | 12.5 | 1139 | 977 (100) | | | 815 (35) | | | | | 609 (5) | 815 (9) | | | 771 (100) | | | 609 (100) |
| **8**, 3-Fer/Caf | 13.2 | 1109 | 947 (100) | | | 785 (49) | 771 (4) | | | 609 (9) | | 785 (16) | 771 (100) | | | 609 (100) | | |
| monoacylated derivatives from kaempferol-3-*O*-glycosides | | | | | | | | | | | | | | | | | | |
| **10**, 13-Sinp | 22.6 | 815 | | | 609 (100) | | | | | | | | | | | | | |
| **11**, 9-Fer | 24.0 | 947 | | 771 (100) | | | | | | | | | | | | | | |
| **12**, 13-Fer | 24.6 | 785 | | 609 (100) | | | | | | | | | | | | | | |

[a] G (Glc), glucosyl; F (Fer), feruloyl; S (Sinp), sinapoyl; MC (MeOCaf), methoxycaffeoyl; **3**, kaempferol-3-*O*-sophoroside-7-*O*-glucoside; **9**, kaempferol-3-*O*-sophorotrioside; **13**, kaempferol-3-*O*-sophoroside.

to the presence of highly glycosylated cinnamoyl derivatives of flavonols.

**Quantitative Analysis.** The results obtained with the quantification of the identified phenolic compounds (**Table 1**) show that, in general, all 14 identified compounds were detected in the analyzed samples, with the exceptions of sample 5, which did not show kaempferol 3-*O*-sophorotrioside-7-*O*-sophoroside (**4**), and sample 8, which did not exhibit kaempferol 3-*O*-(feruloyl)sophorotrioside (**11**).

The highest amounts were found for the pair kaempferol 3-*O*-sophorotrioside-7-*O*-glucoside (**1**) plus kaempferol 3-*O*-(methoxycaffeoyl/caffeoyl)sophoroside-7-*O*-glucoside (**2**), except for sample 3, in which kaempferol 3-*O*-sophoroside-7-*O*-glucoside (**3**) was the major compound, and sample 6, which showed kaempferol 3-*O*-(feruloyl)sophorotrioside (**11**) as the major constituent. In general, kaempferol 3-*O*-sophorotrioside-7-*O*-sophoroside (**4**) and kaempferol 3-*O*-(feruloyl)-sophorotrioside (**11**) were minor compounds, although in sample 6, kaempferol 3-*O*-(feruloyl)sophorotrioside (**11**) is the compound present in highest amounts, as referred before. Kaempferol 3-*O*-glucoside (**14**) exists in trace amounts. Generally, samples from organic culture exhibited higher total phenolics content than those from conventional practices, collected in the same period, with the exception of the samples from December. This might be due to the interference of the mineral fertilizers and/or pesticides, used in conventional practices, in the biosinthetic pathway of flavonoids, decreasing phenolic amounts.

When considering the evolution during winter, we observed that between November and December there was a decrease of the total phenolics content, which is more evident in samples from organic culture. Additionally, a considerable increase of total phenolics in organic samples was noticed in January, mainly due to a higher content of the pairs kaempferol 3-*O*-sophorotrioside-7-*O*-glucoside (**1**) plus kaempferol 3-*O*-(methoxycaffeoyl/caffeoyl)-sophoroside-7-*O*-glucoside (**2**), kaempferol 3-*O*-sophoroside-7-*O*-sophoroside (**5**) plus kaempferol 3-*O*-tetraglucoside-7-*O*-sophoroside (**6**) and kaempferol 3-*O*-sophorotrioside (**9**) plus kaempferol 3-*O*-(sinapoyl)sophoroside (**10**) and of kaempferol 3-*O*-sophoroside-7-*O*-glucoside (**3**), kaempferol 3-*O*-(feruloyl/caffeoyl)sophoroside-7-*O*-glucoside (**8**), and kaempferol 3-*O*-(feruloyl)sophorotrioside (**11**). Geographical and climatic conditions cannot justify these observations, because all of the samples were collected in the same area. The lowest level of total phenolics observed in December in sample from organic production may be explained by the commitment of tronchuda cabbage cells to morphogenic developmental pathways, because those cabbages presented more developed leaves than those of conventional culture in the same period. Apparently that effect is less compatible with the biosynthesis and or turnover of phenolic secondary metabolites (*19*, *20*).

The results obtained in this study suggest that tronchuda cabbage external leaves may constitute a good source of health-promoting compounds, namely, flavonoids. However, more tronchuda cabbage external leaves, from other geographical origins, should be analyzed to establish the factors that affect its phenolic quantitative profile.

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