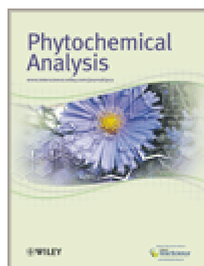

Phytochemical fingerprinting of vegetable Brassica oleracea and Brassica napus by simultaneous identification of glucosinolates and phenolics

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1 **Phytochemical fingerprinting of vegetable *Brassica oleracea* and *Brassica napus* by**
2 **simultaneous identification of glucosinolates and phenolics**
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17 **Abstract**

18 For the first time, intact glucosinolates and phenolic compounds were simultaneously
19 identified in kale, cabbage and leaf rape by LC-UV (PAD)-(ESI) MSn. This study led to the
20 identification of 12 glucosinolates and 32 phenolic compounds which included quinic acid
21 esters of hydroxycinnamic acids, hydroxycinnamic acyl gentobiosides and flavonoids. This
22 study provided a deeper and complete identification of health-promoting compounds in kale
23 and cabbage than previously reported and the new identification of the major phenolic
24 compounds in leaf rape.

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25 **KEYWORDS**: *Brassica oleracea*, *Brassica napus*, glucosinolates, phenolic compounds,

26 flavonol glycosides, hydroxycinnamic acids, LC/UV-PAD/ESI-MSn

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INTRODUCTION

Brassica vegetables have been related with the prevention of degenerative diseases and different types of cancer as well as cardiovascular health-promotion (Cartea and Velasco, 2008; Traka and Mithen, 2009). Compounds that appear to contribute to these health-related properties of brassicas and other food plants include isothiocyanates and their cognate glucosinolates, phenolics, including flavonoids, and other non-nutrients (Jahangir et al., 2009; Jeffery and Araya, 2009) inducing a variety of physiological functions as direct or indirect antioxidants, regulation of enzyme proteins and activities and controlling apoptosis and cell cycle (Duthie et al., 2000).

Glucosinolates are a large group of sulphur-containing secondary plant metabolites, which occur in all Brassica crops. A wide variety of glucosinolates exists but all share a common structure comprises a β-thioglucoside N-hydroxysulfates, a β-D glucopyranosyl moiety and a variable side-chain derived from methionine, tryptophan or phenylalanine. Upon cellular disruption, glucosinolates are hydrolyzed to various bioactive breakdown products by the endogenous enzyme myrosinase. Isothiocyanates and indole glucosinolate metabolites (in particular indol-3-carbinol) are two major groups of autolytic breakdown products of glucosinolates. Both of them exhibit protective activities against many types of cancer. *In vitro* and *in vivo* studies have reported that these compounds may affect many stages of cancer development, including the induction of detoxification enzymes (Phase II enzymes) and the inhibition of activation enzymes (Phase I enzymes) (Mithen et al., 2003) but also antiproliferative mechanisms like cell cycle arrest or apoptosis (Clarke et al., 2008).

Phenolic compounds are a large group of phytochemicals widespread in plant kingdom. Depending on their structure they can be classified into simple phenols, phenolic acids, hydroxycinnamic acid derivatives and flavonoids. The most common are the flavonoids which are built upon C_6–C_3–C_6 flavone skeleton. Flavonoids and hydroxycinnamic acid

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3 53 derivatives are widely distributed in plants and are important biologically active constituents
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6 54 of the human diet. In *Brassica* foods the flavonoids are complex, with up to five sugar
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8 55 residues present, and these may be further substituted with hydroxycinnamic residues (Vallejo
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11 56 et al., 2004). The bioavailability and activity of different glycosides depends on their
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13 57 substituents (Cermak et al., 2003). For this reason, it is important to characterize and quantify
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16 58 the different derivatives of phenolic compounds. These compounds have direct antioxidant
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18 59 and free radical-scavenging activities but can also induce expression of various genes
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20 60 encoding metabolic enzymes thought to decrease the risk of various diseases and disorders
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23 61 (Bennett et al., 2006).
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25 62 The Brassicaceae family has been widely investigated for glucosinolate (Kushad et al., 1999;
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27 63 Padilla et al., 2007c; Cartea et al., 2008a; 2008b) and for phenolic composition (Llorach et al.,
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30 64 2003a; Vallejo et al., 2004; Ferreres et al., 2005; 2006; Romani et al., 2006; Sousa et al.,
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32 65 2008). Nowadays, the profile of different *Brassica* species is well established. The analysis of
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35 66 these compounds by different methods is laborious and time consuming. Both phenolic
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37 67 compounds and glucosinolates have beneficial properties on human health and synergic
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39 68 effects could appear between both classes of metabolites. For this reason, a method to extract
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42 69 and analyze these compounds at the same time would be very useful. Bennet et al (2003;
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44 70 2006) used a method for analyzing both kinds of compounds on different species. Later, this
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46
47 71 method was applied to a set of turnip green and turnip top local populations (*B. rapa* var.
48
49 72 *rapa*) (Francisco et al., 2009) but, as far as we know, the method has not been used for other
50
51 73 *Brassica* crops, like *B. oleracea acephala* and *capitata* groups, and *B. napus*.
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53 74 In Galicia (northwestern Spain), different *Brassi*ca species are used as leaf vegetable products
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55 75 for human and also for animal consumption. Kales (*Brassica oleracea acephala* group),
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58 76 cabbages (*B. oleracea capitata* group), leaf rape (*B. napus pabularia* group), and turnip tops
59
60 77 and turnip greens (*B. rapa rapa* group) are the most important Brassica crops in this region.

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3 78 At the Misión Biológica de Galicia (CSIC, Spain), a collection of local varieties of *B.*
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6 79 *oleracea* and *B. napus* is kept as part of the *Brassica* genus germplasm bank. In previous
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8 80 reports, this collection was characterized based on morphological and agronomical traits
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10 81 (Picoaga et al., 2003; Rodriguez et al., 2005; Padilla et al., 2007a; Soengas et al., 2008; Vilar
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12 82 et al., 2008) and the profile of desulphoglucosinolates in leaves was studied (Cartea et al.,
13
14 83 2008a; 2008b; Velasco et al., 2008). To date, no information is available on content of intact
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17 84 glucosinolates and phenolic compounds in these species. Therefore, for a more
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19 85 comprehensive assessment that allows the nutritional study, the objective of this work was the
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22 86 identification of glucosinolates, flavonoids and hydroxycinnamic acids in a representative
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24 87 variety of kale, cabbage and leaf rape. Identification was carried out by LC-UV photodiode
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27 88 array detection (PAD)-electrospray ionization (ESI) MSn.
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3 89 **EXPERIMENTAL**
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6 90 **Plant material.** One local variety of each crop was evaluated in this study: a kale variety
7
8 91 named as 'MBG-BRS0468' (*B. oleracea acephala*), a white cabbage variety named as 'MBG-
9
10 92 BRS0057' (*B. oleracea capitata*), and a leaf rape variety named as 'MBG-BRS0063' (*B.*
11
12 93 *napus pabularia*). These varieties are in the germplasm collection at the Misión Biológica de
13
14 94 Galicia (CSIC) and were selected based on previous agronomic and nutritional evaluations
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16 95 (Rodriguez et al., 2005; Cartea et al., 2008a; 2008b; Soengas et al., 2008; Vilar et al., 2008).
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18 96 The populations were planted in multipot-trays and seedlings were transplanted into the field
19
20 97 at the five or six leaves stage to collect leaves in well-developed plants. After harvesting on
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22 98 dry ice, the material was immediately transferred to the laboratory and frozen at -80°C, prior
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24 99 to their lyophilisation. The dried material was powdered using an IKA-A10 (IKA-Werke
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26 100 GmbH & Co.KG) mill and the powder was used for analysis.
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30 102 **Sample preparation.** Extraction and the LC gradient for glucosinolate and phenolic analyses
31
32 103 is a multi-purpose chromatographic method that simultaneously separates glucosinolates and
33
34 104 phenolics (Bennett et al., 2003; 2006). Fifty mg of each sample were extracted in 1.5 mL 70%
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36 105 MeOH at 70°C for 30 min with vortex mixing every 5min to facilitate the extraction. The
37
38 106 samples were centrifuged (13000g, 15min, 4°C). The supernatants were collected and
39
40 107 methanol was completely removed using a rotary evaporator under vacuum at 37°C. The dry
41
42 108 material obtained was redissolved in 1mL of ultrapure water and filtered through a 0.20 µm
43
44 109 syringe PTFE filters (Anotop™, Whatman International Ltd, UK).
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46 110
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48 111 **Alkaline hydrolysis.** For the study of acyl flavonoid derivatives, an alkaline hydrolysis was
49
50 112 carried out to eliminate acid moieties like p-coumaroyl (*m/z* 146) and caffeoyl (*m/z* 162),
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52 113 which coincide with those of rhamnosyl and hexosyl residues respectively and, therefore, a
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3 114 miss-assignation can occur in MS analysis. Saponification was performed as follows: 1 mL of
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6 115 the extract plus 1 mL of 2M NaOH (up to pH 9–10) for 12 h at room temperature in a
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8 116 stoppered test tube under N_2 atmosphere. The alkaline hydrolysis products were acidified with
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10 117 concentrated HCl (up to pH 1–2) and directly analysed by LC/UV- PAD/ESI-MSn.
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15 119 **LC/UV- PAD/ESI-MSn analyses.** Chromatographic analyses were carried out on a Luna
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17 120 C18 column (250mm × 4.6mm, 5μm particle size; Phenomenex, Macclesfield, UK). The
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19
20 121 mobile phase was a mixture of (A) Trifluoro acetic acid (TFA) 0.1% and (B) acetonitrile/TFA
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22 122 (99.9:0.1). The flow rate was 1mL min^{-1} in a linear gradient starting with 0% B at 0–5 min,
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25 123 reaching 17% B at 15–17 min, 25% B at 22min, 35% B at 30min, 50% B at 35 min, 99% B at
26
27 124 50min and at 55-65min 0% B. The flow rate was 1mL min^{-1}, and the injection volume 20μL.
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30 125 Chromatograms were recorded at 330 nm for flavonoid glycosides and acylated derivatives
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32 126 and 227 nm for glucosinolates. The LC/UV- PAD/ESI-MSn analyses were carried out in an
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34
35 127 Agilent HPLC 1100 series equipped with a photodiode array detector and mass detector in
36
37 128 series (Agilent Technologies, Waldbronn, Germany). The HPLC consisted of a binary pump
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39 129 (model G1312A), an autosampler (model G1313A), a degasser (model G1322A), and a
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41 130 photodiode array detector (model G1315B). The HPLC system was controlled by a
42
43
44 131 ChemStation software (Agilent, v. 08.03). The mass detector was an ion trap spectrometer
45
46 132 (model G2445A) equipped with an electrospray ionisation interface and was controlled by
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48 133 LCMSD software (Agilent, v. 4.1). The ionisation conditions were adjusted at 350°C and 4kV
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50
51 134 for capillary temperature and voltage, respectively. The nebulizer pressure and flow rate of
52
53 135 nitrogen were 65.0 psi and 11 L min^{-1}, respectively. The full scan mass covered the range
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56 136 from *m/z* 100 up to *m/z* 1500. Collision-induced fragmentation experiments were performed
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58 137 in the ion trap using helium as the collision gas, with voltage ramping cycles from 0.3 up to 2
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138 V. Mass spectrometry data were acquired in the negative ionisation mode. MSn is carried out

139 in the automatic mode on the more abundant fragment ion in MS(n-1).

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141 **RESULTS AND DISCUSSION**

142 **Glucosinolate identification.**

143 The MS product ions obtained from glucosinolates were dominated by intense $[M–H]^-$ions
144 and other fragments of the composition of the side chain. Glucosinolates fragmentation
145 revealed two groups of typical fragments, one associated with the common moiety of the
146 glucosinolates (aglycone) and the other providing useful diagnostic ions for the identification
147 of the variable side chain. As it has been described by other authors (Fabre et al., 2007;
148 Rochfort et al., 2008) , the MS^2 fragmentation of the aglycone side chain produces specific
149 ions at *m/z* 195, 241, 259 and 275. The fragmentation of the ion *m/z* 259 (MS^3 $[(M-H)\rightarrow 259]^-$
150) gave rise to the fragments at *m/z* 139, 97 (corresponding to the sulphate group ($[SO_4H]^-$)),
151 and *m/z* 81 ($[SO_3H]^-$). The *m/z* 97 fragment ion is formed with high abundance in negative
152 ion ESI method (Mellon et al., 2002). Therefore fragments at *m/z* 259 and *m/z* 97 were used as
153 diagnostic ions of glucosinolates. These fragment ions were a very useful preliminary
154 screening method for determining the presence of glucosinolates in plant extracts.
155 The other group of fragments are compound-specific products ions that allowed structure
156 elucidation. All glucosinolates provided of constant neutral loss under the fragmentation
157 conditions. The most intense and consistent was the combined loss of sulphur trioxide and
158 anhydroglucose $[M-H-242]^-$. Other MS fragmentation pathways were to losses of glucose
159 radical $[M-H-162]^-$ and/or thioglucose moiety $[M-H-196]^-$ after the H-rearrangement of the
160 side chain to the sulphur atom in thioglucose moiety (Kokkonen et al., 1991). The indolic
161 glucosinolates were characterized also by the examination of characteristic product ions from
162 their specific R. This group contain two nitrogen atoms and the *m/z* values of their
163 deprotonated molecules were thus at odd mass numbers. Neoglucobrassicin and 4-
164 methoxyglucobrassicin exhibit identical molecular masses and fragmentation ions, they were

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3 165 differentiated by comparison with reported elution sequence during reversed phase HPLC
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6 166 (Kushad et al., 1999).
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8 167 Therefore the molecular ion $[M–H]^-$ of glucosinolates, their fragment ion pattern and the
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10 168 retention times allowed the identification of eight glucosinolates in kale and cabbage which
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13 169 exhibited the same glucosinolate profile and eleven glucosinolates in *B. napus* (Figure 1). In
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15 170 total were identified the following intact glucosinolates: *m/z* 422 glucoiberin, *m/z* 388
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18 171 progoitrin, *m/z* 358 sinigrin, *m/z* 438 glucoraphanin, *m/z* 402 gluconapoleiferin, *m/z* 372
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20 172 gluconapin, *m/z* 463 4-hydroxyglucobrassicin, *m/z* 386 glucobrassicanapin, *m/z* 447
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22 173 glucobrassicin, *m/z* 422 gluconasturtiin, *m/z* 477 4-methoxyglucobrassicin and *m/z* 477
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24
25 174 neoglucobrassicin. The mass spectral information of the glucosinolates identified is
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27 175 summarized in Table 1. The GS profile found in these three crops was similar to those
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30 176 reported by other authors in *B. oleracea* and *B. napus* leaves (Cartea et al., 2008a; 2008b).
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32 177 Cartea *et al.* (Cartea et al., 2008a; 2008b) studied the glucosinolate content of the collection of
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35 178 kale, cabbage and leaf rape kept at the MBG by HPLC-DAD and some differences were
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37 179 found regarding the current work. Eight glucosinolates were identified in the *B. napus*
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39 180 collection by HPLC-DAD. In the present work, 4-methoxyglucobrassicin was not detected
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42 181 and four other glucosinolates (4-hydroxyglucobrassicin, glucoiberin, sinigrin and
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44 182 glucoraphanin) were also identified although all of them in trace quantities (Table 1, Fig.1).
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46 183 With regard to kales and cabbages, the same authors found and quantified 10 and 15
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49 184 glucosinolates, respectively (Cartea et al., 2008a; 2008b). In this work, the most abundant
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51 185 glucosinolates (i.e. glucoiberin, sinigrin, or glucobrassicin) were identified in both crops but
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54 186 other minor glucosinolates like progoitrin, glucoiberverin, glucoalyssin, and
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56 187 glucobrassicanapin, were not found in kale and cabbage (Table 1, Fig.1).
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60 189 **Flavonoids identification.**

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3 190 The HPLC-DAD chromatogram of Brassica vegetable extracts revealed the existence of
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6 191 glycosylated derivatives of three flavonoids with substitution in position 3, that is, kaempferol
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8 192 (267, 300sh and 349 nm), quercetin (255, 267sh, and 355 nm) and isorhamnetin (255, 268sh,
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10 193 294sh, and 354 nm). In addition, acylated flavonoids were detected in the extract, and their
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13 194 UV spectra, characterized with a maximum with a high absorption at 330 nm and a little
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15 195 maximum between 255 and 268 nm, suggested that the flavonoid-glycoside molecules were
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17 196 linked with hydroxycinnamic acid derivatives, in which sinapic, ferulic, caffeic and *p*-
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19
20 197 coumaric acids were the most abundant. The results are summarized in Table 2. The
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22 198 chromatographic profiles, recorded at 330 nm, of the naturally occurring phenolic compounds
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24 199 in leaf rape, kale and cabbage extracts and the deacylated phenolic compounds, obtained after
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27 200 alkaline hydrolysis, are shown in (Fig. 2).
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29 201 Alkaline hydrolysis was performed to reduce the complexity of the naturally occurring
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32 202 compounds present in the plant extracts due to the release of the hydroxycinnamic acids by
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34 203 cleavage of the ester linkage between the acids and the glycosides (Martens, 2002). After
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36 204 alkaline hydrolysis, the chromatogram showed, apart from several hydroxycinnamic acids,
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39 205 various flavonoid glycosides and disappearance of the acylated derivatives (Fig. 2).
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41 206 *Deacylated flavonoids.*
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44 207 The MS ion trap analysis of the saponified extracts of the leaves showed mainly the presence
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46 208 of several kaempferol derivatives but quercetin and isorhamnetin were also determined. The
47
48 209 presence of the ion at *m/z* 285/284 $[Agl\text{-}H/2H]^-$ as base peak for compounds **2**, **3**, **4**, **7**, **8** and **9**
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51 210 show them as kaempferol derivatives (3,5,7,4´-tetrahydroxyflavone), while on compounds **1**
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53 211 and **6** this ion was *m/z* 301/300 indicative of quercetin derivatives ((3,5,7,3´,4´-
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55
56 212 pentahydroxyflavone pentahydroxyflavone) and *m/z* 315 for **5** isorhamnetin derivatives
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58 213 (3,5,7,4´-tetrahydroxy-3´-methoxyflavone) (Table 2). The $MS^2[M\text{-}H]^-$ fragmentation analysis
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60 214 of compounds **1-5** showed ions $[M\text{-}H\text{-}162]^-$ as base peak, indicated a loss of glycosidic

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215 residue at 7 position (Ferreres et al., 2004). According to previous studies (Llorach et al., 216 2003b; Ferreres et al., 2004; Vallejo et al., 2004) the fragmentation pattern and the relative 217 abundance of the obtained ions indicated that compounds **1-3** are glycosylated with a 218 hexoside in position 7 and a di- or trihexoside in the 3 position. The first fragmentation of the 219 deprotonated molecular ion $[M - H]^-$ in this compounds is expected to always be due to the 220 breakdown of the O-glycosidic bond at the 7-position (Ferreres et al., 2004). The remaining 221 glycosyl moieties of the flavonoid molecule are expected to be linked to the hydroxyl at the 3-222 position on the flavonol aglycone. The fragmentation $MS^3[(M\text{-}H)\rightarrow(M\text{-}H\text{-}162)]^-$ of **1-3** 223 showed losses to come from interglycosidic fragmentations at position 3 of the ring which in 224 agree with previous Brassica works, mentioned above, suggest the (1→2) interglycosidic 225 linkage between the disaccharide moieties of the flavonoids (mainly sophorosides). So, these 226 compounds were tentatively characterized as (**1**) quercetin-3-*O*-sophoroside-7-*O*-glucoside; 227 (**2**) kaempferol-3-*O*-sophorotrioside-7-*O*-glucoside and (**3**) kaempferol-3-*O*-sophoroside-7-*O*-228 glucoside. Compounds **4** and **5** were characterized as flavonoids with two sugar moieties 229 linked to different phenolic hydroxyl (di-*O*-glycosidics). According to Ferreres et al. (2004), 230 in these 3,7-di-*O*-glucosides, a base peak ion at $[M\text{–}H\text{-}162]^-$ in the $MS^2[M\text{–}H]^-$ mode is 231 always observed (Table 2). On the other hand, for compounds **6**, **7** and **8** it was observed the 232 fragment ion $[M\text{-}H\text{-}180]^-$ and the appearance of $[Agl\text{-}H]^-$ as base peak in the $MS^2[M\text{-}H]^-$, 233 together with the observed fragmentations, indicating them as flavonol-*O*-diglycosidics. The 234 UV spectra and MS fragmentation for compounds **7** and **8** show that they are kaempferol-3-235 *O*-dihexosides isomers. The $[M\text{-}H\text{-}180]^-$ ion was not observed in the fragmentation of **7;** 236 while this ion is very important for the compound **8**, indicating a interglycosidic linkage 237 (1→2) for this compound. Thus, they were identified as (**4**) kaempferol-3,7-di-*O*-glucoside; 238 (**5**) isorhamnetin-3,7-di-*O*-glucoside; (**6**) quercetin-3-*O*-sophoroside; (**7**) kaempferol-3-*O*-239 diglucoside; and (**8**) kaempferol-3-*O*-sophoroside**.** Compound **9** was identified as a

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3 240 monoglycoside kaempferol derivative with hydroxyl at 3 position free: (**9**) kaempferol-7-*O*-
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6 241 glucoside.
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8 242 In native extracts of kale, cabbage and leaf rape we observed compounds previously described
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11 243 by other authors in *Brassica* spp.(Llorach et al., 2003a; 2003b; Ferreres et al., 2004; Vallejo et
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13 244 al., 2004; Ferreres et al., 2005; 2006). The compound **5** was found only in *B. napus* extracts in
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15 245 trace quantities.
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17 246 *Acylated flavonoids.*
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20 247 Several of the flavonoids in the Brassica sample extracts had UV spectra with a broad
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22 248 maximum absorbance around 330-340 nm (Table 2), suggesting they were acylated with
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25 249 hydroxycinnamic acids (Fig. 2). The comparison of the HPLC-DAD chromatogram of the
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27 250 extracts with that of the saponified extract (Fig.2) indicated the existence of acylated
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29 251 compounds in high amounts. The MS study of these compounds allowed the detection of a
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32 252 total of 13 acylated flavonol glycosides (compounds **10-22**). These compounds were acyl
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34 253 derivatives from compounds **1** (**10, 13**), **3** (**11, 12**, **14**, **15, 16, 17**), **6** (**21**) and **8** (**18, 19, 20,**
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37 254 **22**). The fragmentation of some of these acylated derivatives showed in MS^2 a base peak
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39 255 resultant from the loss of the sugar in the 7 position $[(M-H)-162)]^-$. This fragmentation is
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42 256 typical from flavonid-3-*O*-(acyl)glycoside-7-*O*-hexoside and has been widely described in
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44 257 different *Brassicas* (Llorach et al., 2003a; Ferreres et al., 2005; 2006; 2008). Other important
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47 258 ion also detected was due to the loss of the acyl radical and/or the sugar and acids from the
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49 259 $[M–H]^-$. The resulting fragmentation after the loss of the sugar residues at 7 position (-
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51 260 $MS3[(M-H)\rightarrow(M-H-162)]^-$), showed that the acid loss is easily detected and that the acylation
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54 261 is always present on the sugars at 3 position in these compounds. Losses of 162, 206, 176,
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56 262 146 and 192 *m/z* has been identified as caffeic acid, sinapic acid, ferulic acid, *p*-coumaric
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59 263 acid and methoxycaffeic, respectively. The ion corresponding to the flavonoid 3-O-glycoside
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264 was always the base peak in MS^3 of these compounds. Thus, they had been characterized as

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265 acylated derivatives of quercetin-3-*O*-sophoroside-7-*O*-glucoside with caffeoyl (**10**) and
266 sinapoyl (**13**), and kaempferol-3-*O*-sophoroside-7-*O*-glucoside with methoxycaffeoyl (**11**),
267 caffeoyl (**12** and **17**), sinapoyl (**14**), feruloyl (**15**) and p-coumparoyl (**16**). Compounds **18-22**
268 presented a fragmentation MS^2 $[M-H]^-$ similar to the MS^3 of previous compounds (Table 2),
269 what is expected of flavonoids with glycosilation on a single phenolic hydroxyl. In addition
270 an ion resulting from loss of the acyl radical and fragment *m/z* 180 (162 +18) [(M-H)-acyl-
271 $180]^-$ that comes from the interglycosidic breakdown was observed in the fragmentation of
272 **19-22 ,** confirming the structure of flavonoid-*O*-diglycosides. These compounds have been
273 characterized as acyl derivatives of kaempferol-3-*O*-sophoroside with *p*-coumaroyl (**18**),
274 methoxycaffeoyl (**19**), caffeoyl (**20**), sinapoyl (**22**) and quercetin-3-*O*-(feruloyl) sophoroside
275 (**21**).
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277 ***Hydroxycinnamic acids identification.***

278 Ten hydroxycinnamic acids and derivatives (**3CQAc**, **3*p*CoQAc, SG, 4CQAc, 4FQAc, SA,**
279 **A, B. C, and D)** were detected in leaves of leaf rape, kale and cabbage (Table 3, Fig. 2). The
280 most abundant in the three crops were 3-caffeoyl quinic acid (**3CQAc**) (*R*t 17.3 min; UV
281 295sh, 325 nm; MS: 353, MS2(353): 191 (100%), 179 (62%)), 3-*p*-coumaroyl quinic acid
282 (**3*p*CoQAc**) (*R*t 19.1 min; UV 311 nm; MS: 337, MS2(337): 191 (7%), 179 (100%)) [30], and
283 sinapoylglucoside (**SG**) (*R*t 20.5 min; UV 329 nm; MS: 285, MS2(285): 291 (100%), 223
284 (85%)). 4-caffeoyl quinic (**4CQAc**) (*R*t 19.6 min; UV 295sh, 326 nm; MS: 353, MS2(353):
285 191 (16%), 179 (53), 173 (100%), 191 (16), 135 (12%)) and 4-feruloyl quinic (**4FQAc**) (*R*t
286 22.9 min; UV 325 nm; MS: 367, MS2(367): 191 (5%), 173 (3%), 163 (100%), 119 (5%))
287 were also identified in kale and cabbage but not in leaf rape. Derivatives formed from the
288 interaction of hydroxycinnamic acids with quinic acid and glucose were previously reported
289 in kale, pak choi, Chinese leaf mustard, turnip greens and turnip tops (Rochfort et al., 2006;

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3 290 Ayaz et al., 2008; Ferreres et al., 2008; Francisco et al., 2009; Olsen et al., 2009). Sinapic acid
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6 291 (**SA**) (*R*t 27.3 min; UV 329 nm; MS: 223, MS2(223): 208 (35%), 179(30%), 164(100%)) was
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8 292 a compound present in high quantities in *B. napus* and detected in trace amounts in the two *B.*
9
10 293 *oleracea* crops.
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13 294 Other identified hydroxycinnamic acid derivatives were sinapic and ferulic acids esterified
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15 295 carrying more than one hexose moiety (compounds **A, B, C and D**). It could be observed in
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17 296 all cases, the loss of 224 *m/z* from the deprotonated molecular ion, corresponding to sinapic
18
19 297 acid (Table 3). Compound **A** and **D** also presented ferulic acid and displayed the loss of this
20
21 298 acid (194 *m/z*). By comparison with data reported earlier in other *Brassicas* (Llorach et al.,
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23 299 2003b; Ferreres et al., 2006) these compounds were tentatively identified as: 1,2-
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25 300 disinapoylgentiobioside (**A**), 1-sinapoyl-2-feruloylgentiobioside (**B**), 1, 2, 2´-
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27 301 trisinapoylgentiobioside (**C**), and 1,2´-disinapoyl-2-feruloylgentiobioside (**D**). These results
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29 302 are in accordance with compounds detected in other *Brassica* species, like turnip tops
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31 303 (Romani et al., 2006; Francisco et al., 2009), tronchuda cabbage (Ferreres et al., 2006)
32
33 304 broccoli (Vallejo et al., 2004) and now for first time in kale and leaf rape.
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38 306 **CONCLUCIONS**
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41 307 A method to simultaneously extract and identify glucosinolates and phenolic compounds
42
43 308 (Bennett et al., 2003; 2006) is used for first time in *B. oleracea acephala* and *capitata* groups
44
45 309 and *B. napus* crops. Twelve intact glucosinolates and 32 phenolic compounds were
46
47 310 simultaneously characterized by LC/UV-PAD/ESI-MSn. The major flavonoid kaempferol
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49 311 was found as mono-, di-, and triglycosides acylated with different hydroxycinnamic acids.
50
51 312 The major hydroxycinnamic acid derivatives were present as esters of sinapic acid, ferulic
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53 313 acid, and glycosides. The highest difference between species is the number of sugar residues
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present on the flavonol core. It may also be expected that the different glycosides possess different biological activities for human health.

This study shows that kale, cabbage and leaf rape are a good source of phenolic antioxidants. The main naturally occurring phenolic compounds identified were flavonols and hydroxycinnamic acids. The majority of the flavonoids found in these varieties are kaempferol glycosylated and acylated with different hydroxycinnamic acids. Quercetin and isorhamnetin derivatives were also found. Kaempferol and quercetin are the most prevalent flavonoids in the *Brassicaceae* family. Kaempferol is known to be a strong antioxidant and quercetin also a potent free radical scavenger and is considered to be a protective against cardiovascular disease. Cabbage is a well established crop at world level as cauliflower or broccoli. Kale and leaf rape are minority crops in many parts of the world but they are a good source of nutritive compounds and due to their rusticity (Rodriguez et al., 2005; Padilla et al., 2007b) could be a good substitute of different *Brassica* species under hard climatic conditions.

Acknowledgements

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Table 1.List of identified glucosinolates with the corresponding retention times and MS data in extracts of leaf rape (*B. napus pabularia* group), cabbage (*B. oleracea capitata* group), and kale crops (*B. oleracea acephala* group)..

Code	Compound	Rt (min)	m/z $[M-H]^-$	$MS^2[M-H]^-$ *m/z* (%)	Leaf rape	Kale	Cabbage
GIB	Glucoiberin	4.8	422	358(100), 342(1), 291(2), 275(3), 259(5), 195(2), 180(6)	X	X	X
PRO	Progoitrin	5.9	388	332(29), 308(12), 301(17), 275(40), 259(100), 240(18) 210(78), 195(14), 192(24), 154(16),136(42), 130(30)	X	-	-
SIN	Sinigrin	6.6	358	278(2), 275(7), 259(100), 241(28), 195(4), 180(3), 162(8), 135(2), 116(5)	X	X	X
GRA	Glucoraphanin	7.4	436	420(6), 372(100) , 356(1), 291(2),275(1), 259(3), 194(2), 162(4)	X	X	X
GNL	Gluconapoleiferin	12.3	402	306(53), 275(20), 259(100), 240(7), 225(10), 215(24), 195(10), 163(13), 160(9), 145(18), 140(23), 120(9)	X	-	-
GNA	Gluconapin	12.5	472	292(4), 275(30), 259(100), 241(37), 227 (9), 195(25),176(8), 139 (11),130(10)	X	-	-
4-OHGBS	4-Hydroxiglucobrassicin	13.1	463	403(2), 383(10), 365(6), 300(6), 285(73), 267(100), 259(17), 240(25), 220(17), 169(30), 160(23), 132(5)	X	X	X
GBN	Glucobrassicanapin	15.7	386	306(4), 275(21), 259(100), 241(33), 208(12), 195(6), 163(4), 144(18) 139(8)	X	-	-
GBS	Glucobrassicin	16.8	447	367(22), 291(6), 275(34), 259(100), 251(17), 241(11), 224(3), 205(28), 195(11),144(9)	X	X	X
GST	Gluconasturtiin	19.5	422	342(9), 275(24), 259(100), 244(7), 229(8), 195(13), 180(24), 169(1), 163(5), 145(4), 140(6), 119(4)	X	X	X
4-MGBS	4-Methoxyglucobrassicin	20.4	477	397(25), 299(14), 291(69), 275(98), 259(62), 241(80), 235(100), 198(80), 195 (16), 144(70)	-	X	X
NGBS	Neoglucobrassicin	23.5	477	447(68), 446(100), 429(16), 385(5), 273(12), 259(16), 241(26), 224(4), 205(5)	X	X	X

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Table 2. Data of glycosilated flavonoids from saponificated extract (compounds 1-9) and acylated flavonoids from native extracts of leaf rape (*B. napus pabularia* group), cabbage (*B. oleracea capitata* group), and kale crops (*B. oleracea acephala* group). (compounds 10-22).

Peak	Compound	Rt (min)	UV (nm)	$[M-H]^-$ (*m/z*)	$MS^2[M-H]^-$ *m/z* (%)	MS^3	Leaf rape	Kale	Cabbage
1	Querc-3-*O*-shop-7-*O*-glc	16.9	255, 267sh, 351	787	625(100), 463(5), 301(3)	463(23), 445(21), 300(100)	-	X	X
2	Kaempf-3-*O*-triglc-7-*O*-glc	17.0	266, 316sh, 348	933	771(100)	489(15), 429(19), 285(100)	X	X	X
3	Kaempf-3-*O*-soph-7-*O*-glc	17.4	266.317sh, 347	771	609(100)	489(25), 429(21), 285(100)	X	X	X
4	Kaempf-3,7-di-*O*-glc	19.6	265, 317sh, 347	609	488(8), 447(100), 285(34)	285(100)	X	X	X
5	Isorhmnt-3,7-di-*O*-glc	19.9	255, 267sh, 352	639	519(12), 477(100), 315(19)	314(100)	X	-	-
6	Querc-3-*O*-soph	20.5	-	625	463(10), 445(51), 300(100)	-	-	X	X
7	Kaempf-3-*O*-diglc	22.8	266, 297sh, 347	609	447(100), 285(80)	285(100)	-	X	X
8	Kaempf-3-*O*-soph	27.4	266, 297sh, 347	609	447(21), 429(65), 285(100)	-	-	X	X
9	Kaempf-7-*O*-glc	28.1	266, 318sh, 367	447	285(100)	-	X	X	X
10	Querc-3-*O*(caffeoyl)-shop-7-*O*-glc	17.8	253, 269sh, 299sh, 337	949	787(100), 625(50)	625(100)	X	X	X
11	Kaempf-3-*O*-(methoxycaffeoyl)-soph-7-*O*-glc	18.4	269, 331	963	801(100), 609(7)	609(100), 285(12)	X	X	X
12	Kaempf-3-*O*-(caffeoyl)-soph-7-*O*-glc	18.6	269, 331	933	771(100), 609(7)	609(100), 285(10)	X	X	X
13	Querc-3-*O*-(sinapyl)-shop-7-*O*-glc	18.8	270, 337	993	831(100), 787(60), 625(65)	625(100)	X	X	X
14	Kaempf-3-*O*-(sinapoyl)-soph-7-*O*-glc	19.6	269, 331	977	815(100), 609(3)	609(100), 285(10)	X	X	X
15	Kaempf-3-*O*-(feruloyl)-soph-7-*O*-glc	20.0	Coeluting with 16	947	785(100), 609(6)	609(5)	X	X	X
16	Kaempf-3-*O*-(p-coumaroyl)-soph-7-*O*-glc	20.1	Coeluting with 15	917	755(100), 609(5),	-	X	X	X
17	Kaempf-3-*O*-(caffeoyl)-soph-7-*O*-glc (isomer)	20.8	269, 331	933	771(100), 609(9)	609(100)	X	-	-
18	Kaempf-3-*O*-(p-coumaroyl)-soph	23.4	269 , 317	755	609(100), 429(1), 284(15)	429(35), 285(100)	-	X	X
19	Kaempf-3-*O*-(methoxycaffeoyl)-soph	24.3	268, 330	801	609(100), 429(5), 285(10)	429(25), 285(100)	X	X	X
20	Kaempf-3-*O*-(caffeoyl)-soph	24.7	269, 330	771	609(100), 429(4), 285(9)	428(34), 285(100)	X	X	X
21	Querc-3-*O*-(feruloyl)-soph	25.1	-	801	625(100), 445(5), 301(4)	445(15), 301(100)	X	X	X
22	Kaempf-3-*O*-(sinapoyl)-soph	25.6	269, 330	815	609(100), 429(3), 285(9)	429(30), 285(100)	X	X	X

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1 Table 3. Data of hydroxycinnamic acids from extracts of leaf rape (*B. napus pabularia* group), cabbage (*B. oleracea capitata* group), and kale crops
2 (*B. oleracea acephala* group).
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Peak	Compound	Rt (min)	UV (nm)	$[M-H]^-$ (*m/z*)	$MS^2[M-H]^-$ *m/z* (%)	$MS^3[(M-H)\rightarrow(M-H-224)]^-$	Leaf rape	Kale	Cabbage
3CQAc	3-Caffeoyl quinic acid	17.3	295sh, 325	353	191 (100), 179 (62)	-	X	X	X
3*p*CoQAc	3-*p*-Coumaroyl quinic acid	19.1	311	337	191 (7), 179 (100)	-	X	X	X
4CQAc	4-Caffeoyl quinic acid	19.6	295sh, 326	353	191 (16), 179 (53), 173 (100), 135 (12)	-	-	X	X
SG	Sinapylglucoside	20.5	329	285	291(100%), 223 (85%)	-	X	X	X
4FQAc	4-Feruloyl quinic acid	22.9	325	367	191 (5), 173 (3), 163 (100), 119 (5)	-	-	X	X
SA	Sinapic acid	27.3	329	223	208 (35), 179 (30), 164 (100)	-	X	*In traces*	*In traces*
A	1,2-Disinapoylgentiobioside	28.1	330	753	529 (100), 223 (1)	223 (100), 205 (59)	X	X	X
B	1-Sinapoyl-2-feruloylgentiobioside	28.7	330	723	529 (11), 499 (100), 259 (4)	259 (28), 246 (20), 217 (53)	X	X	X
C	1,2,2'-Trisinapoylgentiobioside	30.5	330	959	735 (100)	717 (15), 529 (100)	X	X	X
D	1,2'-Disinapoyl-2-feruloylgentiobioside	31.2	330	929	735 (4), 705 (100)	499 (100)	X	X	X

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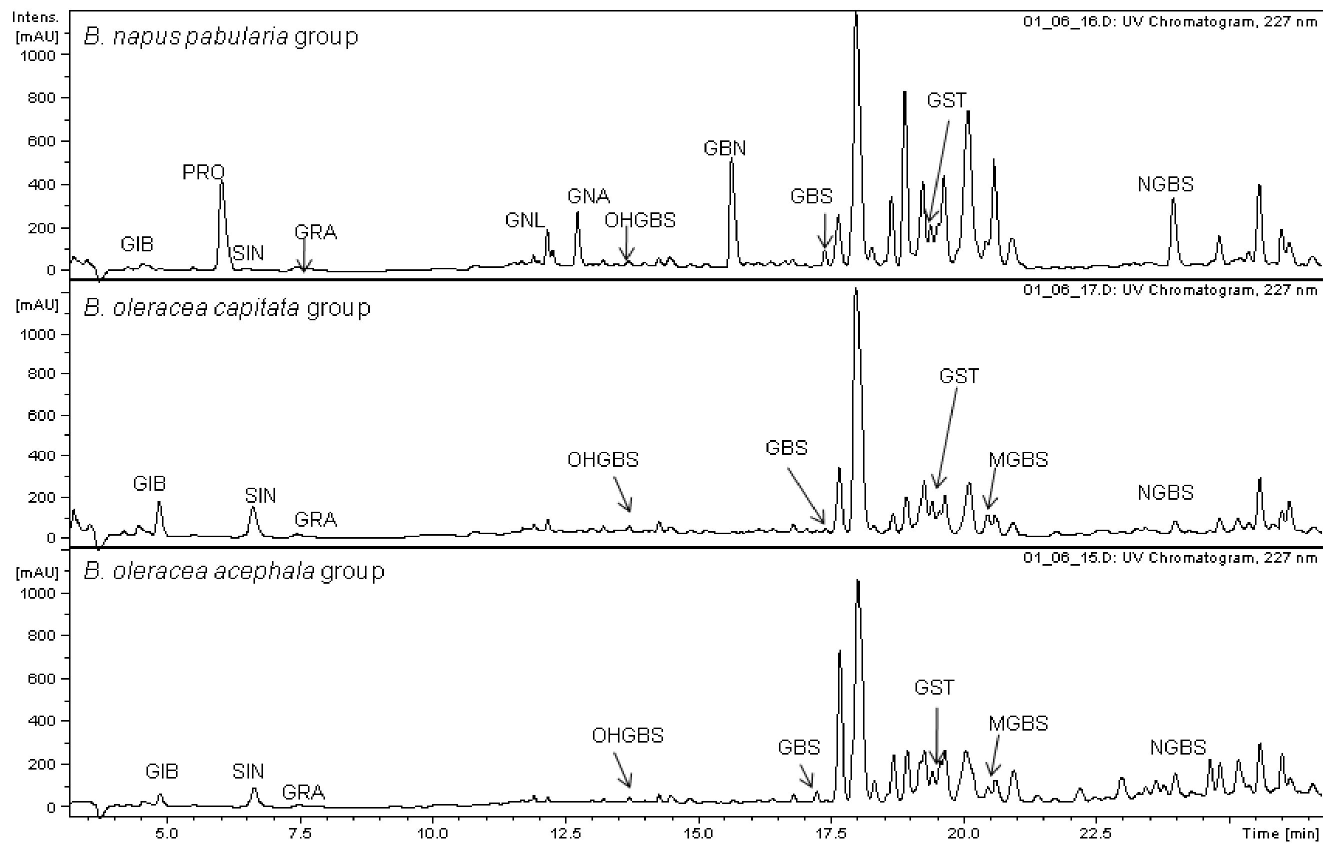
Figure 1. Glucosinolate profile of leaf rape (*B. napus pabularia* group), cabbage (*B. oleracea capitata* group), and kale crops (*B. oleracea acephala* group).



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GIB= Glucoiberin. **PRO=** Progoitrin. **SIN=** Sinigrin. **GRA=**Glucoraphanin. **GNL**=Gluconapoleiferin. **GNA**=Gluconapin. **4-OHGBS**=4-Hydroxiglucobrassicin. **GBN**=Glucobrassicanapin. **GBS**=Glucobrassicin. **GST**=Gluconasturtiin. **4-MGBS**=4-Methoxyglucobrassicin. **NGBS**=Neoglucobrassicin.

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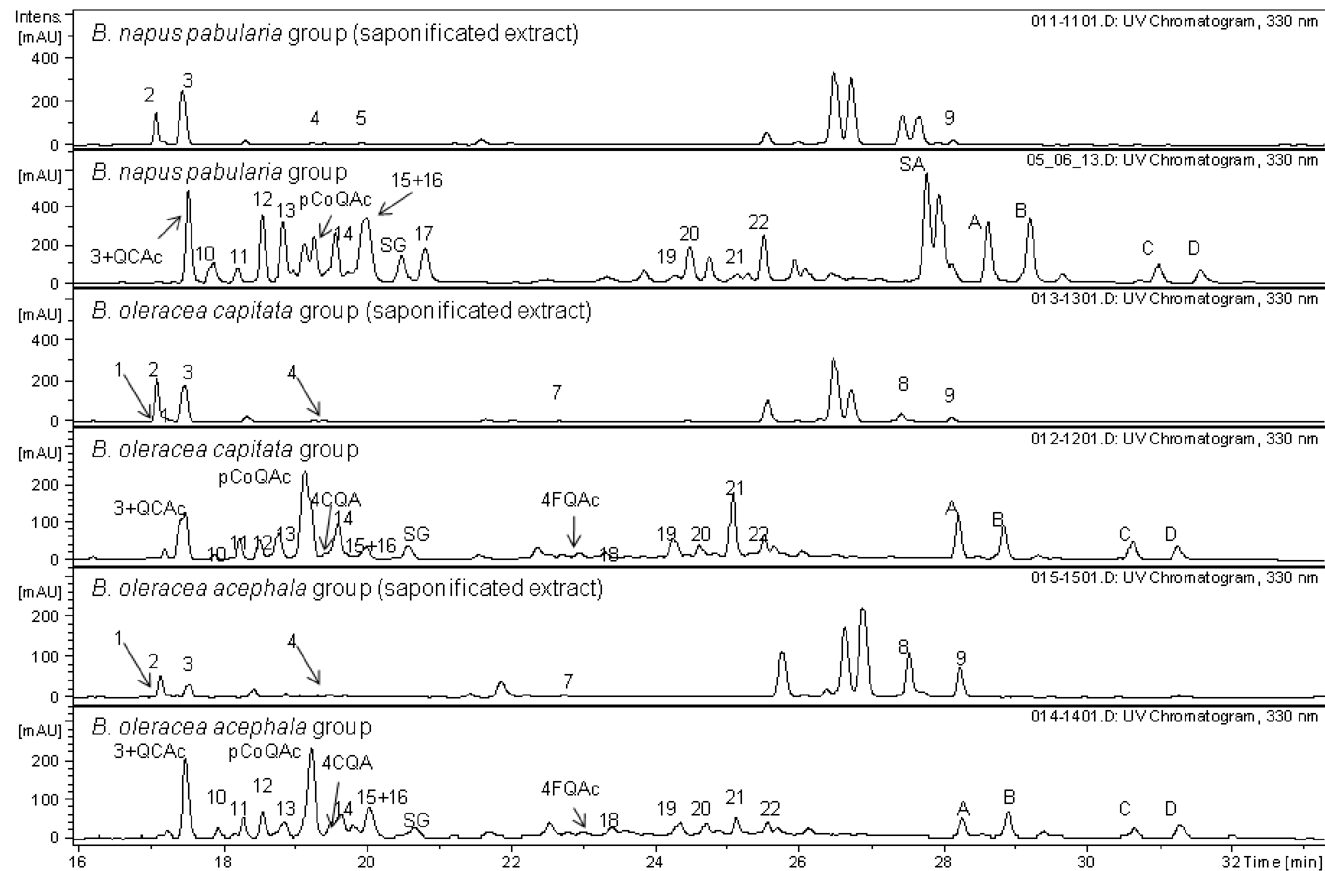
Figure 2. Phenolic profiles of leaf phenolics in rape (*B. napus pabularia* group), cabbage (*B. oleracea capitata* group), and kale (*B. oleracea acephala* group).



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1=Querc-3-*O*-shop-7-*O*-glc. **2=**Kaempf-3-*O*-triglc-7-*O*-glc. **3=**Kaempf-3-*O*-soph-7-*O*-glc. **4=**Kaempf-3,7-di-*O*-glc. **5=**Isorhmnt-3,7-di-*O*-glc. **6=**Querc-3-*O*-soph. **7=**Kaempf-3-*O*-diglc. **8=**Kaempf-3-*O*-soph. **9=**Kaempf-7-*O*-glc. **10=**Querc-3-*O*(caffeoyl)-shop-7-*O*-glc. **11=**Kaempf-3-*O*-(methoxycaffeoyl)-soph-7-*O*-glc. **12=**Kaempf-3-*O*-(caffeoyl)-soph-7-*O*-glc. **13=**Querc-3-*O*-(sinapyl)-shop-7-*O*-glc. **14=**Kaempf-3-*O*-(sinapoyl)-soph-7-*O*-glc. **15=**Kaempf-3-*O*-(feruloyl)-soph-7-*O*-glc. **16=**Kaempf-3-*O*-(p-coumaroyl)-soph-7-*O*-glc. **17=**Kaempf-3-*O*-(caffeoyl)-soph-7-*O*-glc (isomer). **18=**Kaempf-3-*O*-(p-coumaroyl)-soph. **19=**Kaempf-3-*O*-(methoxycaffeoyl)-soph. **20=**Kaempf-3-*O*-(caffeoyl)-soph. **21=**Querc-3-*O*-(feruloyl)-soph. **22=**Kaempf-3-*O*-(sinapoyl)-soph. **3CQAc=**3-Caffeoyl quinic acid. **3*p*CoQAc=**3-*p*-Coumaroyl quinic acid. **4CQAc=**4-Caffeoyl quinic acid. **SG=**Sinapylglucoside. **4FQAc=**4-Feruloyl quinic acid. **SA=**Sinapic acid. **A=**1,2-Disinapoylgentiobioside. **B=**1-Sinapoyl-2-feruloylgentiobioside. **C=**1,2,2'-Trisinapoylgentiobioside. **D=**1,2'-Disinapoyl-2-feruloylgentiobioside

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