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Tronchuda cabbage flavonoids uptake by *Pieris brassicae*

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Abstract

The flavonoid pattern of larvae of cabbage white butterfly (*Pieris brassicae* L.; Lepidoptera: Pieridae) reared on the leaves of tronchuda cabbage was analysed by HPLC-DAD-MS/MS-ESI. Twenty flavonoids were identified or characterised, namely 16 kaempferol and four quercetin derivatives. Kaempferol 3-*O*-sophoroside, a minor component of tronchuda cabbage, was found to be the main component in *P. brassicae* (15.8%). Apart from this, only two other flavonoids present in significant amounts in tronchuda cabbage (kaempferol 3-*O*-sophoroside-7-*O*-glucoside and kaempferol 3-*O*-sophoroside-7-*O*-sophoroside) were found in the larvae. The larvae have high amounts of quercetin derivatives (18.5%), which were present only in trace amounts in tronchuda cabbage extracts, suggesting that *P. brassicae* is able to selectively sequester these flavonoids. The occurrence of a high content of flavonoids not detectable in tronchuda cabbage extracts indicates that *P. brassicae* larvae are able to metabolize dietary flavonoids.

Keywords: *Pieris brassicae* L. larvae; Tronchuda cabbage; *Brassica oleracea* L. var. *costata* DC; Flavonoids

1. Introduction

Larvae of *Pieris brassicae* L. (Lepidoptera: Pieridae) are specialists on crucifers, whereas adults feed of the nectar of a variety of plants. The larvae can feed on various species of Brassicaceae, namely, cauliflower, cabbage, turnip, nasturtium and, more rarely, on red cabbage and radish.

The close association between *Pieris* sp. butterflies and crucifers was linked to the presence of glucosinolates in these plants (Renwick, 2002). As well as glucosinolates, flavonoids can modulate the feeding behaviour of larvae and oviposition of adult insects (van Loon et al., 2002).

Flavonoid uptake is relatively widespread in the Lepidoptera, in particular in butterfly families like the Papilionidae, Nymphalidae, and Lycaenidae, where they form part of the wing pigmentation (Burghardt et al., 1997, 2001; Schittko et al., 1999). In fact, although most of pigments are synthesised de novo during scale development in the pupa, some are secondary plant metabolites taken up from the larval diet since insects are unable to synthesize flavonoids or their precursors de novo (Knüttel and Fiedler, 2001). Feeding experiments proved the dietary origin of the flavonoids (Burghardt et al., 1997; Schittko et al., 1999; Knüttel and Fiedler, 2001; Harborne and Grayer, 1994). Thus, flavonoid uptake and metabolism by insects is strongly dependent on the specific flavonoid pattern of their host plants (Burghardt et al., 1997, 2001; Schittko et al., 1999; Geuder et al., 1997).

The flavonoids sequestered by the larvae are subsequently metabolised, stored and transferred into the wings during the late pupal stage (Geuder et al., 1997). Flavonoids are known for their antioxidant potential (Ferreres et al., 2006; Vrchovská et al., 2006) and perhaps they act as antibiotic and antiviral agents in insects (Harborne and Grayer, 1994).

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In what concerns flavonoids patterns it was shown that (i) larvae sequester only specific fractions of the flavonoid load of the host plant, (ii) the sequestered flavonoids are subjected to various glycosydation processes, (iii) the flavonoid content of butterflies from the same species may drastically vary according to the host plant actually used during the larval stages, and (iv) female butterflies tend to be richer in flavonoids than males (Burghardt et al., 2001).

As referred above, flavonoids in the insects are positively associated with the amounts of flavonoids in the food that they had consumed (Burghardt et al., 2000). Larvae selectively sequester and metabolise quercetin and kaempferol derivatives, the predominant flavonoids in the analysed plants. Other flavonoids such as myricetin derivatives, flavones and isoflavonoids were mostly excreted (Burghardt et al., 2001).

As far as we know, there is no study concerning the sequestration of phenolic compounds by *P. brassicae* from tronchuda cabbage leaves (*Brassica oleracea* L. var. *costata* DC) or other cabbages. This study can be relevant from the nutritional point of view, considering that the larvae may accumulate or even metabolize trochuda cabbage constituents, namely complex flavonol glycosides (Ferreres et al., 2005, 2006), constituting a source of potential bioactive compounds not available in nature.

In this paper, the flavonoids present in larvae of *P. brassicae* were tentatively identified by HPLC-DAD-MS/MS-ESI and they were compared to the flavonoid pattern of the tronchuda cabbage external leaves, which were the only ones on which they have fed. Some considerations about ingestion, metabolism and accumulation of flavonoids in *P. brassicae* are made.

2. Results and discussion

2.1. Characterisation of *P. brassicae* phenolic compounds

The HPLC-DAD-MS/MS-ESI screening of the aqueous methanolic extract of *P. brassicae* larvae (Fig. 1) shows several peaks with UV spectra characteristic of flavonols, with two maxima at around 260 and 350 nm (Table 1), and their corresponding acylated derivatives (Table 2). The UV spectra shape of the acylated flavonols resembles the overlapping of a flavonol spectrum with a hydroxycinnamic acid one, with a broad maximum around 310–330 nm and a short maximum or shoulder around 265–271 nm (Table 2), which, therefore, can be misunderstood as a cinnamic acid derivative. Some spectra are poorly defined because the compounds are found in trace amounts and/or coelute with other ones. Thus, UV spectra of compounds **7**, **10** and **12** are not included in Table 2, and those of compounds **8**, **13** and **16** differ from the expected for isolated and purified compounds, probably due to possible co-elution with other products.

The ESI-MS/MS ion trap study confirms the above mentioned and shows several flavonoids which are structurally similar to those already described in various Brassicaceae species (Ferreres et al., 2005, 2006; Llorach et al., 2003; Vallejo et al., 2004). These compounds are characterised to be

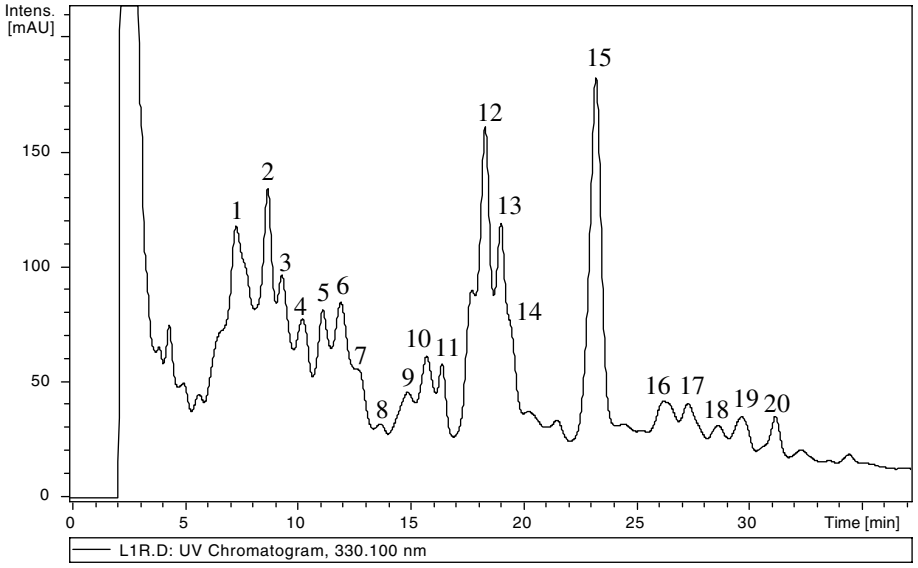

Fig. 1. HPLC-DAD phenolic profile of larvae hydromethanolic extract. Detection at 330 nm. Peaks: (**1**) quercetin 3-*O*-sophoroside-7-*O*-glucoside; (**2**) kaempferol 3-*O*-sophoroside-7-*O*-glucoside; (**3**) kaempferol 3-*O*-sophoroside-7-*O*-sophoroside; (**4**) quercetin 3-*O*-(feruloyl)-triglucoside-7-*O*-glucoside; (**5**) kaempferol 3-*O*-(sinapoyl)-triglucoside-7-*O*-glucoside; (**6**) kaempferol 3-*O*-(feruloyl)-triglucoside-7-*O*-glucoside; (**7**) kaempferol 3-*O*-(*p*-coumaroyl)-triglucoside-7-*O*-glucoside; (**8**) kaempferol 3-*O*-(methoxycaffeoyl)-sophoroside-7-*O*-glucoside; (**9**) kaempferol 3-*O*-(caffeoyl)-sophoroside-7-*O*-glucoside; (**10**) quercetin 3-*O*-(*p*-coumaroyl)-sophoroside; (**11**) kaempferol 3-*O*-(*p*-coumaroyl)-triglucoside; (**12**) kaempferol 3-*O*-(*p*-coumaroyl)-sophoroside; (**13**) kaempferol 3-*O*-(methoxycaffeoyl)-sophoroside; (**14**) quercetin 3-*O*-sophoroside; (**15**) kaempferol 3-*O*-sophoroside; (**16**) kaempferol 3-*O*-(*p*-coumaroyl)-sophoroside (isomer); (**17**) kaempferol 3-*O*-(disinapoyl)-triglucoside-7-*O*-glucoside; (**18**) kaempferol 3-*O*-(feruloyl/sinapoyl)-triglucoside-7-*O*-glucoside; (**19**) quercetin 3-*O*-(feruloyl)-triglucoside; (**20**) kaempferol 3-*O*-glucoside.

Table 1
R_t, UV, -MS: $[M-H]^-$, -$MS^2[M-H]^-$ and -$MS^3[(M-H) \rightarrow 625/609]^-$ data of flavonol glycosides without acylation found in *P. brassicae*[a]

Compounds[b]	R_t (min)	UV (nm)	$[M-H]^-$ (*m/z*)	-$MS^2[M-H]^-$ (*m/z*) (%)			-$MS^3[(M-H) \rightarrow 625/609]^-$ (*m/z*) (%)		
				−162	−180	−324	−162	−180	−324
1 Querc-3-Soph-7-Glc[c]	7.2		787	625 (100)			463 (25)	445 (50)	300 (100)
2 Kaempf-3-Soph-7-Glc	8.6	267, 347	771	609 (100)			447 (10)	429 (35)	285 (100)
3 Kaempf-3-Soph-7-Soph[c]	9.2		933	771 (25)		609 (100)		429 (45)	285 (100)
14 Querc-3-Soph	19.4	255, 267, 350	625	463 (15)	445 (25)	300 (100)			
15 Kaempf-3-Soph	23.1	265, 293sh, 347	609	447 (10)	529 (50)	285 (100)			
20 Kaempf-3-Glc	31.0	265, 295sh, 348	447	285 (100)					

[a] Main observed fragments. Other ions were found but they have not been included.
[b] Querc, quercetin; Kaempf, kaempferol; Soph, sophoroside; Glc, glucoside.
[c] Compounds in trace amounts and hidden by other. Their UV spectra have not been observed properly.

Table 2
R_t, UV, -MS: $[M-H]^-$, -$MS^2[M-H]^-$ and -$MS^3[(M-H) \rightarrow (M-H\text{-}162)]^-$ data of flavonol acyl-glycosides found in *P. brassicae*[a]

Compounds[b]	R_t (min)	UV (nm)	$[M-H]^-$ (*m/z*)	Flavonol-3(acyl)glycosyl-7-glucoside derivatives					
				$MS^2[M-H]^-$	$MS^3[(M-H) \rightarrow (M-H\text{-}162)]^-$				
				-Glc (162)	-Caf (162)	-MCaf (192)	-*p*Coum (146)	-Fer (176)	-Sinp (206)
4 Querc-3-(Fer)triGlc-7-Glc	10.1	265, 291sh, 321	1125	963				787	
5 Kaempf-3-(Sinp)triGlc-7-Glc	11.1	269, 290sh, 325	1139	977					771
6 Kaempf-3-(Fer)triGlc-7-Glc	11.8	270, 299sh, 323	1109	947				771	
7 Kaempf-3-(*p*-Coum)triGlc-7-Glc[c]	12.5		1079	917			771		
8 Kaempf-3-(MCaf)Soph-7-Glc	13.6	271, 325	963	801		609			
9 Kaempf-3-(Caf)Soph-7-Glc	14.8	269, 320	933	771	609				
17 Kaempf-3-(diSinp)triGlc-7-Glc[d]	27.2	270, 331	1345	1183					977[d]
18 Kaempf-3-(FerSinp)triGlc-7-Glc[e]	28.6	271, 331	1315	1153				977	947[e]
				Flavonol-3(acyl)glycoside derivatives					
				$MS^2[M-H]^-$					
				-Caf	-MCaf	-*p*Coum	-Fer	-Sinp	
10 Querc-3-(*p*-Coum)Soph[c]	15.8		771			625			
11 Kaempf-3-(*p*-Coum)triGlc	16.2	270sh, 293sh, 315	917			771			
12 Kaempf-3-(*p*-Coum)Soph[c]	18.2		755			609			
13 Kaempf-3-(MCaf)Soph	18.9	269, 325	801		609				
16 Kaempf-3-(*p*-Coum)Soph (isom)	26.1	267, 338	755			609			
19 Querc-3-(Fer)triGlc	29.5	269, 327	963				787		

[a] Main observed fragments. Other ions were found but they have not been included.
[b] Querc, quercetin; Kaempf, kaempferol; Soph, sophoroside; Glc, glucoside; Fer, feruloyl; Sinp, sinpapoyl; *p*-Coum, *p*-coumaroyl; Caf, caffeoyl; MCaf, methoxycaffeoyl.
[c] Compounds in trace amounts and hidden by other. Their UV spectra have not been observed properly.
[d] $MS^4(1345 \rightarrow 1183 \rightarrow 977)$: 771.
[e] $MS^4(1315 \rightarrow 1153 \rightarrow 947)$:771.

kaempferol and quercetin derivatives, the later in lesser amounts, with a high degree of glycosilation, as well as a possible acylation at the glycosidic fraction linked to the hydroxyl group at the 3 position of the flavonol.

From the MS study it is possible to distinguish a group of flavonol glycoside derivatives without acylation (Table 1), whose MS fragmentations (Ferreres et al., 2004) are characteristic of flavonol-3-*O*-sophoroside-7-*O*-glucoside (compounds **1** and **2**) (Fig. 2), flavonol-3-*O*-sophoroside-7-*O*-sophoroside (**3**), flavonol-3-*O*-sophoroside (**14** and **15**) and flavonol-3-*O*-glucoside (**20**).

On the other hand, the MS fragmentation pattern of the acylated flavonol derivatives (Vallejo et al., 2004) confirms the presence of a series of flavonol-3-*O*-(acyl)glycoside-7-*O*-glucoside (Table 2) (compounds **4**–**9**, **17** and **18**), in which we can observe a loss of 162 u in the $MS^2[M-H]^-$ due to the break of glucose at the 7-position. The $MS^3[(M-H) \rightarrow (M-H\text{-}162)]^-$ shows the loss of the acyl radical giving the aglycone fragment linked to the glycosidic fraction at the 3-position, triglucoside (**4**–**7**) or sophorose (**8** and **9**) (Table 2). For the diacylated derivatives, compounds **17** and **18**, a new MS event

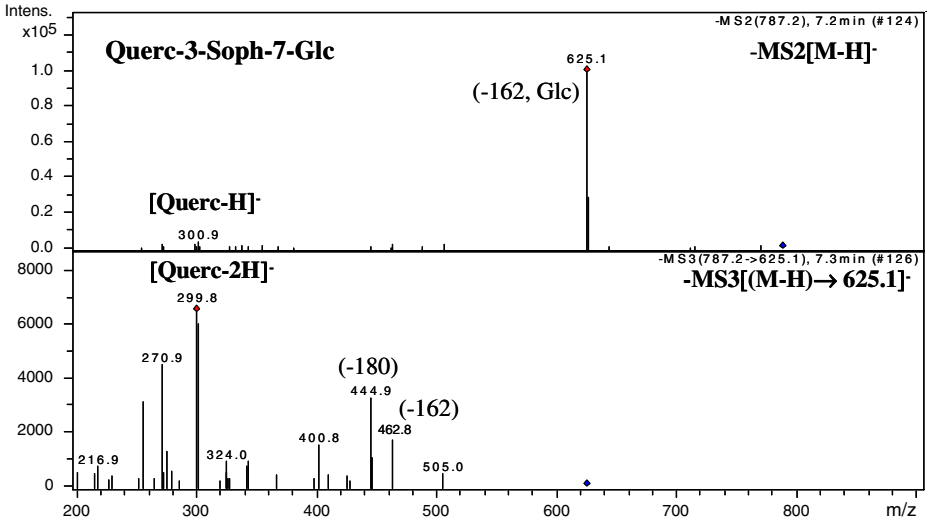

Fig. 2. MS^n analysis of quercetin 3-*O*-sophoroside-7-*O*-glucoside (**1**).

(MS^3[(M−H) → (M−H-162) → (M−H-162-acyl)]$^-$) was necessary to obtain the referred fragment.

Another set of complex flavonol derivatives (compounds **10**–**13**, **16** and **19**) shows a MS fragmentation (Table 2) in which MS^2[M−H]$^-$ event the loss could be interpreted as that of an acyl radical to give rise to the aglycone fragment linked to the glycosidic fraction at the 3-position (Figs. 3 and 4), confirming the lack of glycosilation at the 7-position (Vallejo et al., 2004). In this group of compounds it is observed an apparently anomalous chromatographic behaviour of compounds **10**, **12** and **13**, once they elute before the deacylated compounds from which they derive from. This behaviour has already been described for similar compounds found in cauliflower (Llorach et al., 2003), being noticed that the acylated derivatives at the 3-position of the sugar and without glycosilation in the 7-position exhibited an apparent irregular retention time. On the other hand, the presence of two isomers of kaempferol 3-*O*-(*p*-coumaroyl)-sophoroside with lower (compound **12**) and higher retention times (compound **16**) than kaempferol 3-*O*-sophoroside (**15**) indicates that the position of the acylation over the sugar also influences the order of elution.

In addition, the loss of 146 u in the MS fragmentation of compounds **7**, **10**, **11**, **12** and **16**, the majority of them with poor or badly defined UV spectrum as mentioned above, could be due to the presence of a rhamnosyl radical, instead of a *p*-coumaroyl one. However, in tronchuda cabbage leaves neither rhamnosyl nor *p*-coumaroyl derivatives were found (Ferreres et al., 2005, 2006). So, we consider that the demethoxylation of the sinapoyl and/or feruloyl derivatives originating *p*-coumaroyl derivatives is more likely to occur during the metabolism process in the larvae, as indicated below, rather than the demethylation of glucose to give rise to rhamnosyl derivatives.

Thus, in *P. brassicae* the following compounds were identified or tentatively identified: quercetin 3-*O*-sophoroside-7-*O*-glucoside (**1**), kaempferol 3-*O*-sophoroside-7-*O*-glucoside (**2**), kaempferol 3-*O*-sophoroside-7-*O*-sophoroside (**3**), querce-

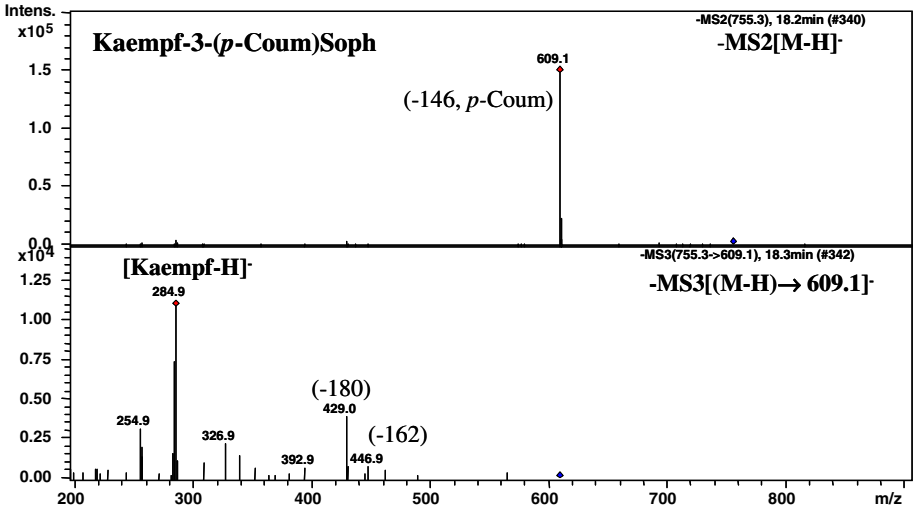

Fig. 3. MS^n analysis of kaempferol 3-*O*-(*p*-coumaroyl)-sophoroside (**12**).

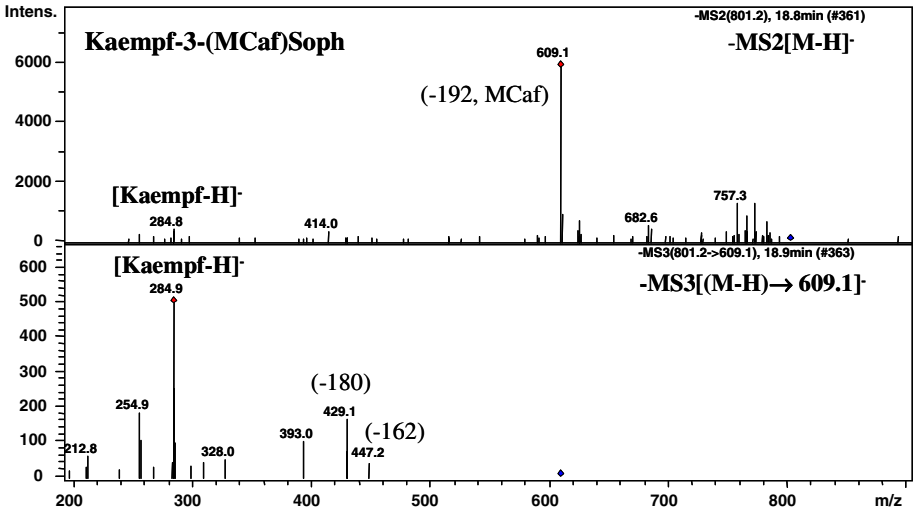

Fig. 4. MS^n analysis of kaempferol 3-*O*-(methoxycaffeoyl)-sophoroside (**13**).

tin 3-*O*-(feruloyl)-triglucoside-7-*O*-glucoside (**4**), kaempferol 3-*O*-(sinapoyl)-triglucoside-7-*O*-glucoside (**5**), kaempferol 3-*O*-(feruloyl)-triglucoside-7-*O*-glucoside (**6**), kaempferol 3-*O*-(*p*-coumaroyl)-triglucoside-7-*O*-glucoside (**7**) (tentatively), kaempferol 3-*O*-(methoxycaffeoyl)-sophoroside-7-*O*-glucoside (**8**) (tentatively), kaempferol 3-*O*-(caffeoyl)-sophoroside-7-*O*-glucoside (**9**) (tentatively), quercetin 3-*O*-(*p*-coumaroyl)-sophoroside (**10**) (tentatively), kaempferol 3-*O*-(*p*-coumaroyl)-triglucoside (**11**) (tentatively), two isomers of kaempferol 3-*O*-(*p*-coumaroyl)-sophoroside (**12** and **16**) (tentatively), kaempferol 3-*O*-(methoxycaffeoyl)-sophoroside (**13**) (tentatively), quercetin 3-*O*-sophoroside (**14**), kaempferol 3-*O*-sophoroside (**15**), kaempferol 3-*O*-(disinapoyl)-triglucoside-7-*O*-glucoside (**17**), kaempferol 3-*O*-(feruloyl/sinapoyl)-triglucoside-7-*O*-glucoside (**18**), quercetin 3-*O*-(feruloyl)-triglucoside (**19**) and kaempferol 3-*O*-glucoside (**20**).

2.2. Comparison between flavonoids in *P. brassicae* and tronchuda cabbage host

The phenolic composition of the tronchuda cabbage external leaves is well defined in a previous work, analysing several samples, from both organic and conventional agricultural practices (Ferreres et al., 2005). The composition of the host leaves, from which the larvae feed, revealed to be similar to that described before, being detected 13 kaempferol derivatives (Table 3). The flavonoid profile obtained with *P. brassicae* was then compared with that of the cabbage. Kaempferol 3-*O*-sophoroside-7-*O*-glucoside, kaempferol 3-*O*-sophoroside-7-*O*-sophoroside and kaempferol 3-*O*-sophoroside were the only compounds that the larvae and cabbage had in common.

Although the glycosylation pattern of the flavonols is the same in both extracts, it can be observed that the flavonol 3-*O*-glycosides represent more than ca. 50% in the larvae extract (Table 4), while they correspond to ca.12% of their food plant (Table 3). This can be ascribed to the

Table 3
Phenolic composition of tronchuda cabbage external leaves

Compound	%
21+ Kaempferol 3-*O*-sophorotrioside-7-*O*-glucoside	7.6
22 Kaempferol 3-*O*-(methoxycaffeoyl/caffeoyl)-sophoroside-7-*O*-glucoside	
2 Kaempferol 3-*O*-sophoroside-7-*O*-glucoside	22.9
23 Kaempferol 3-*O*-sophorotrioside-7-*O*-sophoroside	1.4
3+ Kaempferol 3-*O*-sophoroside-7-*O*-sophoroside	11.4
24 Kaempferol 3-*O*-tetraglucoside-7-*O*-sophoroside	
25 Kaempferol 3-*O*-(sinapoyl/caffeoyl)-sophoroside-7-*O*-glucoside	17.1
26 Kaempferol 3-*O*-(feruloyl/caffeoyl)-sophoroside-7-*O*-glucoside	27.8
27+ Kaempferol 3-*O*-sophorotrioside	5.1
28 Kaempferol 3-*O*-(sinapoyl)-sophoroside	
29 Kaempferol 3-*O*-(feruloyl)-sophorotrioside	0.4
30 Kaempferol 3-*O*-(feruloyl)-sophoroside	1.1
15 Kaempferol 3-*O*-sophoroside	5.2

Table 4
Phenolic composition of *P. brassicae*

Compound	%
1 Quercetin 3-*O*-sophoroside-7-*O*-glucoside	8.7
2 Kaempferol 3-*O*-sophoroside-7-*O*-glucoside	10.0
3 Kaempferol 3-*O*-sophoroside-7-*O*-sophoroside	6.6
4 Quercetin 3-*O*-(feruloyl)-triglucoside-7-*O*-glucoside	4.5
5 Kaempferol 3-*O*-(sinapoyl)-triglucoside-7-*O*-glucoside	5.0
6 Kaempferol 3-*O*-(feruloyl)-triglucoside-7-*O*-glucoside	5.6
7 Kaempferol 3-*O*-(*p*-coumaroyl)-triglucoside-7-*O*-glucoside	2.6
8 Kaempferol 3-*O*-(methoxycaffeoyl)-sophoroside-7-*O*-glucoside	0.5
9 Kaempferol 3-*O*-(caffeoyl)-sophoroside-7-*O*-glucoside	1.8
10 Quercetin 3-*O*-(*p*-coumaroyl)-sophoroside	3.4
11 Kaempferol 3-*O*-(*p*-coumaroyl)-triglucoside	3.3
12 Kaempferol 3-*O*-(*p*-coumaroyl)-sophoroside	13.4
13+ Kaempferol 3-*O*-(methoxycaffeoyl)-sophoroside	9.2
14 Quercetin 3-*O*-sophoroside	
15 Kaempferol 3-*O*-sophoroside	15.8
16 Kaempferol 3-*O*-(*p*-coumaroyl)-sophoroside (isomer)	2.4
17 Kaempferol 3-*O*-(disinapoyl)-triglucoside-7-*O*-glucoside	2.1
18 Kaempferol 3-*O*-(feruloyl/sinapoyl)-triglucoside-7-*O*-glucoside	1.3
19 Quercetin 3-*O*-(feruloyl)-triglucoside	1.9
20 Kaempferol 3-*O*-glucoside	1.9

metabolism of the flavonols glycosylated at 3 and 7 positions present on tronchuda cabbage, or to a higher efficiency of sequestration of flavonol 3-*O*-glycosides.

Among the flavonol glycoside derivatives without acylation, kaempferol 3-*O*-sophoroside is the most abundant, corresponding to ca. 16% of the total amount of phenolic compounds of the larvae (Table 4), while in tronchuda cabbage leaves it only represented ca. 5% (Table 3). This difference possibly results from the metabolism of kaempferol 3-*O*-sophoroside-7-*O*-glucoside and its acylated derivatives, which are the most abundant compounds of tronchuda cabbage external leaves.

In what concerns the presence of quercetin derivatives, the composition of the external leaves of tronchuda cabbage (Ferreres et al., 2005) was re-analysed and these were detected in vestigial amounts, which also happened with the tronchuda cabbage external leaves eaten by *P. brassicae*. The larvae contain high amounts of quercetin derivatives (ca. 18% of the total amount of phenolic compounds) (Table 4), while in tronchuda cabbage these compounds are present only in trace amounts, suggesting that *P. brassicae* selectively sequester these flavonoids or that the kaempferol glycosides are metabolised into quercetin glycosides by the larvae.

The presence of *p*-coumaroyl derivatives, which have not been found on either the internal or external leaves of tronchuda cabbage (Ferreres et al., 2005; Sousa et al., 2005) can be explained by the demethoxylation of the sinapoyl and/or feruloyl derivatives during the metabolism process in the larvae. On the other hand, the absorbance of the peaks observed in Fig. 1 for acylated flavonoid derivatives, cannot be taken as proportional to their abundance, as some of them co-eluted with other unidentified cinnamoyl acids derivatives, presenting a similar UV spectrum and contributing to the overall absorbance of those peaks. Another possible explanation is their existence in tronchuda cabbage leaves in concentrations below the detection limits, being, however, selectively uptaken and accumulated by *P. brassicae*.

The existence of two methoxylated flavonol derivatives in *P. brassicae* (compounds **8** and 1**3**) (Fig. 4) can be resultant from the metabolism of kaempferol 3-*O*-(methoxycaffeoyl/caffeoyl)-sophoroside-7-*O*-glucoside present on the external leaves of the tronchuda cabbage.

As far as we know, this is the first report about the uptake of flavonoids by *P. brassicae* and it was observed they sequestered flavonoids from tronchuda cabbage external leaves. In addition, some of them may undergo metabolism during ingestion. The results suggest that *P. brassicae* may have interest for the synthesis and/or accumulation of potential health promoting compounds, which are rather unusual in nature. Although the *P. brassicae* gut was not removed and longer starving periods should be tested, some conclusions could be obtained as larvae and feeding plant contain only three compounds in common, indicating that the 17 different phenolics found in larvae are directly related with the insect, being a consequence of its metabolism and/or selective sequestration. Further studies should be done, varying the starving periods before freezing and analysing gut and remaining body separately, in order to obtain more information about the metabolic process. Larvae in different instars should also be studied, to evaluate the effect of the developmental stage in sequestration rate of flavonoids. Also the study of the faeces of larvae can provide valuable data about the excretion of flavonoids.

3. Experimental

3.1. Standards and reagents

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

3.2. Larvae, plant material and sampling

Wild *P. brassicae* larvae (fourth instar) and respective tronchuda cabbage external leaves (of three individuals with 45 days-old) host plants were collected on fields located in Samil, Bragança, northeastern Portugal. Voucher specimens of tronchuda cabbage leaves are deposited at Serviço de Farmacognosia from Faculdade de Farmácia, Universidade do Porto. After collection the larvae were kept without food for 1 h before they were frozen. The frozen larvae and plant material were freeze-dried and kept in a dessicator until analysis.

3.3. Extraction of the phenolic compounds

The identification of the phenolic compounds was performed using a hydromethanolic extract of the lyophilised larvae: ca. 1.5 g powdered larvae was thoroughly mixed with 2.5 ml methanol–water (1:1), ultra-sonicated and filtered.

The same extraction methodology was used for quantification purposes of phenolic compounds in larvae and plant material.

3.4. HPLC-DAD-MS/MS-ESI qualitative analysis

Chromatographic separations were carried out on a 250 mm × 4 mm, 5-μm particle size, RP-18 LiChroCART (Merck, Darmstadt, Germany) column protected with a 4 mm × 4 mm LiChroCART guard column using acetic acid 1% (A) and methanol (B) as solvents, starting with 20% B and using a gradient to obtain 50% B at 35 min. The flow rate was 1 ml min^{-1} and the injection volume was 20 μl. The HPLC system was equipped with an Agilent 1100 Series diode array and a mass detector in series (Agilent Technologies, Waldbronn, Germany). It consisted of a G1312A binary pump, an G1313A autosampler, a G1322A

degasser and a G1315B photodiode array detector controlled by a ChemStation software (Agilent, v. 08.03). Spectroscopic data from all peaks were accumulated in the range 240–400 nm, and chromatograms were recorded at 330 nm. The mass detector was a G2445A Ion-Trap Mass Spectrometer equipped with an electrospray ionisation (ESI) system and controlled by LCMSD software (Agilent, v. 4.1.). Nitrogen was used as nebulising gas at a pressure of 65 psi and the flow was adjusted to 11 l min^{-1}. The heated capillary and voltage were maintained at 350 °C and 4 kV, respectively. The full-scan mass covered the range from *m/z* 90 up to *m/z* 2000. Collision-induced fragmentation experiments were performed in the ion trap using helium as collision gas, with voltage ramping cycles from 0.3 up to 2 V. MS data were acquired in the negative ionisation mode. MS^n data were achieved in the automatic mode on the more abundant fragment ion in MS^{n-1}. Tables 1 and 2 show the most frequent ions which characterise the fragmentation of the compounds. Other ions were found but they have not been included due to their low significance on the MS behaviour ions.

3.5. HPLC-DAD quantitative analysis

Twenty microliters of each extract were analysed using a HPLC unit (Gilson) and a 250 × 4.6 mm i.d., 5 μm 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^{-1}, 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 Medical Electronics, Villiers le Bel, France). Peak purity was checked by the software contrast facilities.

Phenolic compounds quantification was achieved by the peak areas recorded in the chromatograms relative to that registered for known concentrations of external standards. With the exception of kaempferol 3-*O*-glucoside, which was quantified as it, the other kaempferol and quercetin derivatives were quantified as kaempferol 3-*O*-rutinoside and quercetin 3-*O*-rutinoside, respectively, since none of the identified compounds was commercially available.

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