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JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

HPLC-DAD-MS/MS-ESI Screening of Phenolic Compounds in *Pieris brassicae* L. Reared on *Brassica rapa* var. *rapa* L

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The phenolic profiles of cabbage white butterfly (*Pieris brassicae* L.; Lepidoptera: Pieridae) at different development stages (larvae, exuviae, and butterfly), its excrements, and its host plant *Brassica rapa* var. *rapa* L. were determined by high performance liquid chromatography− diode-array detector−mass spectrometry/mass spectrometry−electrospray ionization (HPLC-DAD-MS/MS-ESI). Twenty-five acylated and nonacylated flavonoid glycosides and ferulic and sinapic acids were identified in host plant, from which only 12 compounds were found in the excrements. In addition, the excrements showed the presence of sulfate flavonoids and other flavonoid glycosides that were not detected in the leaves. In the larvae kept without food for 12 h, only 3 compounds common to the plant material and 2 others, also present in the excrements, were characterized. The results indicate that deacylation, deglycosylation, and sulfating steps are involved in the metabolic process of *P. brassicae* and that its excrements may constitute a promising source of bioactive compounds, which could be used to take profit of this common pest of *Brassica* cultures.

KEYWORDS: *Pieris brassicae* L.; *Brassica rapa* var. *rapa* L.; turnip; phenolic compounds; HPLC-DAD-MS/MS-ESI

INTRODUCTION

The role of plant chemistry in shaping plant−insect relationships is well recognized, with a close association of certain oligophagous insects with specific chemicals of their host plants (*1*). Phenolic compounds, namely flavonoids, are ubiquitous secondary metabolites in vascular plants with a prominent structural diversity and exhibit a variety of functions in plant herbivore interactions (*2*). They are important factors in pollination and oviposition, in allowing phytophagous insects to recognize their host plants, as feeding deterrents, and in insect pest management (*2*). Herbivores encounter these secondary metabolites during their feeding stages. Because insects are unable to synthesize flavonoids or their precursors de novo, the phenolic profile of the host plant has a determinant role in their flavonoid uptake and metabolism (*3*).

Large white butterfly *Pieris brassicae* L. (Lepidoptera: Pieridae), an insect whose larvae constitutes a frequent pest of some *Brassica* species, has a life cycle that lasts about 45 days from egg to adult. The larvae feed exclusively on crucifers, whereas adults feed on the nectar of several plants (*4*). Regarding *P. brassicae* relation with phenolic compounds from host plant, as far as we know there is one study, developed by our group, about the sequestration of flavonoids by the larvae fed with tronchuda cabbage leaves (*Brassica oleracea* L. var. *costata* DC) and kept without food for one hour (*5*). In this study it was observed that *P. brassicae* larvae was able to sequester and metabolize the flavonoids present in tronchuda cabbage, presenting only three compounds in common with the host plant.

Turnip (*Brassica rapa* var. *rapa* L.) is one of the oldest cultivated vegetables, being used for human consumption all over the world (*6*). The leaves are characterized by a peculiar bitter and pungent taste, distinguishing them from other *Brassica* vegetables, that is associated with the presence of glucosinolate degradation products (*7*). In what concerns phenolic compounds, several phenolic acids and flavonoids were previously determined by high performance liquid chromatography−diode-array detector (HPLC-DAD), namely hydroxycinnamic acids, kaempferol, and isorhamnetin derivatives (*8*). In addition, phenylpro-

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Table 1. R_t, UV and MS ($[M-H]^-$, $-MS^2[M-H]^-$ and $-MS^3[(M-H) \rightarrow Y^7_0]^-$) Data of Glycosilated Flavonoids from Saponificated Hydromethanolic Extract of *Brasica rapa* var. *rapa* Leaves: Flavonoid-3-*O*-Sophoroside/Sophorotrioside-7-*O*-Glucoside, −3,7-di-*O*-Glucoside, −3-*O*-Sophoroside, and −3/7-Glucoside[a]

Flavonoid-3-*O*-trihexoside-7-*O*-hexoside							
				$-MS^2[M-H]^-$ (*m/z*) (%)	$-MS^3[(M-H) \rightarrow Y^7_0]^-$ (*m/z*) (%)		
compounds[b]	R_t (min)	UV (nm)	$[M-H]^-$ (*m/z*)	$Y^7_0{}^-$ (−162)	$Y^7_0Y^3_2{}^-$ (−162)	$Y^7_0Z^3_1{}^-$ (−342)	$Y^7_0Y^3_0{}^-$ (−486)
2 Kaempf-3-*O*-Sophtr-7-*O*-Glc	11.2	266, 316sh, 348	933	771 (100)	609 (45)	429 (26)	284 (100)[c]

Flavonoid-3-*O*-dihexoside-7-*O*-hexoside						
				$-MS^2[M-H]^-$ (*m/z*) (%)	$-MS^3[(M-H) \rightarrow Y^7_0]^-$ (*m/z*) (%)	
compounds[b]	R_t (min)	UV (nm)	$[M-H]^-$ (*m/z*)	$Y^7_0{}^-$ (−162)	$Y^7_0Z^3_1{}^-$ (−180)	$Y^7_0Y^3_0{}^-$ (−324)
1 Querc-3-*O*-Soph-7-*O*-Glc	9.9		787	625 (100)	445 (60)	301 (100)
3 Kaempf-3-*O*-Soph-7-*O*-Glc	11.9	266, 317sh, 347	771	609 (100)	429 (100)	284 (50)[c]

Flavonoid-3,7-di-*O*-hexoside					
				$-MS^2[M-H]^-$ (*m/z*) (%)	
compounds[b]	R_t (min)	UV (nm)	$[M-H]^-$ (*m/z*)	$Y^7_0{}^-/Y^3_0{}^-$ (−162)	$[Agl-H]^-$ (−324)
4 Querc-3,7-di-*O*-Glc	15.6	255, 267sh, 294sh, 349	625	463 (100)	301 (50)
6 Kaempf-3,7-di-*O*-Glc	19.0	265, 317sh, 349	609	447 (100)	285 (30)
7 Isorhmnt-3,7-di-*O*-Glc	20.3	255, 267sh, 250	639	477 (100)	315 (19)

Flavonoid-3-*O*-dihexoside					
				$-MS^2[M-H]^-$ (*m/z*) (%)	
compounds[b]	R_t (min)	UV (nm)	$[M-H]^-$ (*m/z*)	$Z^3_1{}^-$ (−180)	$Y^3_0{}^-$ (−324)
10 Kaempf-3-*O*-Soph	27.4	266, 297sh, 347	609	429 (65)	285 (100)

Flavonoid-*O*-hexoside				
				$-MS^2[M-H]^-$ (*m/z*) (%)
compounds[b]	R_t (min)	UV (nm)	$[M-H]^-$ (*m/z*)	$Y^3_0{}^-$ (−162)
11 Kaempf-7-*O*-Glc	31.7	266, 318sh, 367	447	285 (100)
12 Kaempf-3-*O*-Glc	34.9	266, 299sh, 349	447	285 (100)
13 Isorhmnt-3-*O*-Glc	35.4	255, 265sh, 299sh, 350	477	314 (100)[c]

[a] Main observed fragments. Other ions were found but they have not been included. [b] Kaempf: Kaempferol. Querc: Quercetin. Isorhmnt: Isorhamnetin. Soph: Sophoroside. Sophtr: Sophorotrioside. Glc: Glucoside. [c] Fragments from homolytic cleavage of the glycosidic bond ($[Aglycon-2H]^{-\bullet}$) (*22*).

panoids were also described to occur in methyl jasmonate treated leaves, which are thought to play a role in plant defense (*6*).

The aim of the present study was to characterize the phenolic compounds of *B. rapa* var. *rapa* leaves and to establish possible relations with their ingestion, metabolism, and accumulation by *P. brassicae* in the different stages of its life cycle. For this purpose, turnip leaves, *P. brassicae* larvae reared on these leaves and deprived of food for 12 h, their excrements, exuviae, and butterflies were analyzed by HPLC-DAD-MS/MS-ESI, which constitutes a highly advanced and valuable technique for the characterization of complex phenolic molecules.

MATERIALS AND METHODS

Standards and Reagents. Methanol, sodium hydroxide, and hydrochloric and acetic acids were purchased from Merck (Darmstadt, Germany). The water was treated in a Milli-Q water purification system (Millipore, Bedford, Massachusetts).

Samples. Wild *P. brassicae* larvae were captured in Bragança (northeastern Portugal) and taken to the laboratory to complete their life cycle and for oviposition in turnip (*B. rapa* var. *rapa* L.) leaves. New larvae were developed having only this species as host plant. Fresh food was supplied every day ad libitum. New larvae at the fourth instar and correspondent excrements were picked for analysis. Some of them were collected and kept without food for twelve hours before freezing. Other larvae were left to develop until the butterfly stage, and 24 h maximum after eclosion (without food) they were also collected, together with the exuviae. *P. brassicae* (larvae, excrements, exuviae, and butterflies) and plant materials were freeze-dried and kept in a desiccator until analysis. Voucher specimens are deposited at Serviço de Farmacognosia from Faculdade de Farmácia, Universidade do Porto.

Extract Preparation. For the identification of the phenolic compounds present in turnip leaves and in *P. brassicae* larvae, excrements, exuviae, and butterflies, each lyophilised material (ca. 0.2 g) was thoroughly mixed with 1.5 mL of methanol/water (1:1), ultrasonicated (1 h), centrifuged (12 000 rpm, 5 min), and filtered through a 0.45 μm size pore membrane.

Alkaline Hydrolysis. Sodium hydroxide 2 N (1 mL) was added to 1 mL of the hydroalcoholic phenolic extract, obtained as described above, and the mixture was kept 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 hydrochloric acid (up to pH 1–2) and directly analyzed by HPLC/UV-DAD/ESI-MS^n.

Table 2. R_t, UV, and −MS ([M − H]$^-$, −MS^2[M − H]$^-$ and −MS^3[(M − H) → (M − H − 162)]$^-$) Data of Acylated Derivatives of Glycosilated Flavonoids from Native Hydromethanolic Extract of *Brasica rapa* var. *rapa* leaves[a]

				−MS^2[M − H]$^-$ (*m*/*z*) (%)										−MS^3[(M − H) → (M − H − 162)]$^-$ (*m*/*z*) (%)					
compounds[b]	R_t (min)	UV (nm)	[M − H]$^-$ (*m*/*z*)	−146 −*p*.Coum	−162 −Glc	−176 −Fer	−192 −MeOCf	−206 −Sinp	−308 −G−*p*C	−324 −G−C	−338 −G−F	−354 −G−MC	−368 −G−S	−146 −*p*.Coum	−162 −Caf	−176 −Fer	−192 −MeOCf	−206 −Sinp	[Agl − H]$^-$
Acylated Derivatives from **1**: Quercetin-3-*O*-Sophoroside-7-*O*-Glucoside																			
14 **1**-MeOCaf	9.4	255sh, 269sh, 333	979		817 (100)		787 (80)					625 (59)					625 (100)		
15 **1**-Caf	10.1	255, 269sh, 299sh, 343	949		787 (100)					625 (40)					625 (100)				301 (10)
18 **1**-Sinp	13.0	270, 337	993		831 (100)			787 (100)					625 (60)					625 (100)	301 (3)
19 **1**-Fer	13.4	255, 269sh, 299sh, 348	963		801 (100)	787 (45)					625 (40)					625 (100)			301 (10)
20 **1**-*p*.Coum	14.0	297sh, 327	933	787 (10)	771 (100)				625 (35)					625 (100)					301 (5)
24 **1**-Caf (**15** isomer)	17.1		949		787 (100)					625 (50)					625 (100)				300 (7)[c]
Acylated Derivatives from **3**: Kaempferol-3-*O*-Sophoroside-7-*O*-Glucoside																			
16 **3**-MeOCaf	11.3	269, 331	963		801 (100)							609 (5)					609 (100)		284 (30)[c]
17 **3**-Caf	12.2	269, 331	933		771 (100)					609 (6)					609 (100)				285 (4)
21 **3**-Sinp	15.0	269, 331	977		815 (100)								609 (3)					609 (100)[d]	284 (10)[c]
22 **3**-Fer	15.8	269, 331	947		785 (100)						609 (4)					609 (60)[d]			
23 **3**-*p*.Coum	16.6	269, 317	917		755 (100)				609 (5)										
25 **3**-Fer (22 isomer)	22.9	coeluting with **26**	947		785 (100)						609 (5)					609 (100)[d]			
27 **3**-*p*.Coum (**23** isomer)	23.3	268, 319	917		755 (100)				609 (3)					609 (100)					285 (5)

				−MS^2[M − H]$^-$ (*m*/*z*) (%)							−MS^3[(M − H) → (M − H − Acyl)]$^-$ (*m*/*z*) (%)	
compounds[b]	R_t (min)	UV (nm)	[M − H]$^-$ (*m*/*z*)	−146 −*p*.Coum	−162 −Caf	−176 −Fer	−192 −MeOCf	−206 −Sinp	−Acyl − 180	[Agl − H]$^-$	−180	[Agl − H]$^-$
Acylated Derivatives from **10**: Kaempferol-3-*O*-Sophoroside												
26 **10**-MeOCaf	22.9	coeluting with **25**	801				609 (100)		429 (5)	285 (12)	429 (30)	285 (100)
28 **10**-Caf	24.9	269, 299sh, 330	771		609 (100)				429 (5)	285 (8)	429 (35)	285 (100)
29 **10**-Sinp	25.5	269, 330	815					609 (100)[d]	429 (4)	285 (10)	429 (60)	285 (100)
30 **10**-Fer	27.4	coeluting with **10**	785			609 (100)[d]			429 (2)	284 (25)[c]	429 (65)	285 (100)
31 **10**-*p*.Coum	29.0	269, 317	755	609 (100)					429 (4)	285 (10)	429 (30)	285 (100)

[a] Main observed fragments. Other ions were found but they have not been included. [b] G (Glc), glucosyl; *p*C (*p*.Coum), *p*-coumaroyl; Caf, caffeoyl; F (Fer), feruloyl; S (Sinp), sinapoyl; MC (MeOCf), methoxycaffeoyl; **1**, quercetin-3-*O*-sophoroside-7-*O*-glucoside; **3**, kaempferol-3-*O*-sophoroside-7-*O*-glucoside; **10**, kaempferol-3-*O*-sophoroside. [c] Fragments from homolytic cleavage of the glycosidic bond ([Aglycon − 2H]$^{-\bullet}$) (*22*). [d] Compounds **21** and **29** (sinapoyl derivatives) and **22**, **25,** and **30** (feruloyl derivatives) showed an abundant ion at *m*/*z* 623, resultant from the loss of 192 u (206 – 14) and 162 u (176 – 14), respectively.

Table 3. R_t, and −MS: $[M - H]^-$, $-MS^2[M - H]^-$ and $-MS^3[(M - H) \rightarrow (M - H - 80)]^-$ Data of Glycosilated Flavonoid Sulfates from Hydromethanolic Extract of *Pieris brassicae* Excrements[a]

			$-MS^2[M - H]^-$ (*m*/*z*) (%)			$-MS^3[(M - H) \rightarrow (M - H - 80)]^-$ (*m*/*z*) (%)	
compounds[b]	R_t (min)	$[M - H]^-$ (*m*/*z*)	−80	−80 − 162	−80 − 2 × 162	−162	−2 × 162
				diglycosyl sulfates			
32 7-sulfate	11.3	719	639 (100)	477 (30)	315 (20)	477 (100)	315 (60)

			$-MS^2[M - H]^-$ (*m*/*z*) (%)		$-MS^3[(M - H) \rightarrow (M - H - 80)]^-$ (*m*/*z*) (%)
compounds[b]	R_t (min)	$[M - H]^-$ (*m*/*z*)	−80	−80 − 162	−162
			monoglycosyl sulfates		
33 12-sulfate	16.6	527	447 (100)	285 (50)	285 (100)
34 13-sulfate	17.1	557	477 (100)	315 (53)	315 (100)
35 13-sulfate (isomer of **34**)	18.5	557	477 (100)	315 (50)	315 (100)
36 13-sulfate (isomer of **34**)	19.1	557	477 (100)	315 (60)	315 (100)
37 12-sulfate (isomer of **33**)	19.4	527	447 (100)	285 (45)	285 (100)
38 13-sulfate (isomer of **34**)	20.2	557	477 (100)	315 (67)	315 (100)

[a] Main observed fragments. Other ions were found but they have not been included. [b] **7**, isorhamnetin-3,7-di-*O*-glucoside; **12**, kaempferol-3-*O*-glucoside; **13**, isorhamnetin-3-*O*-glucoside.

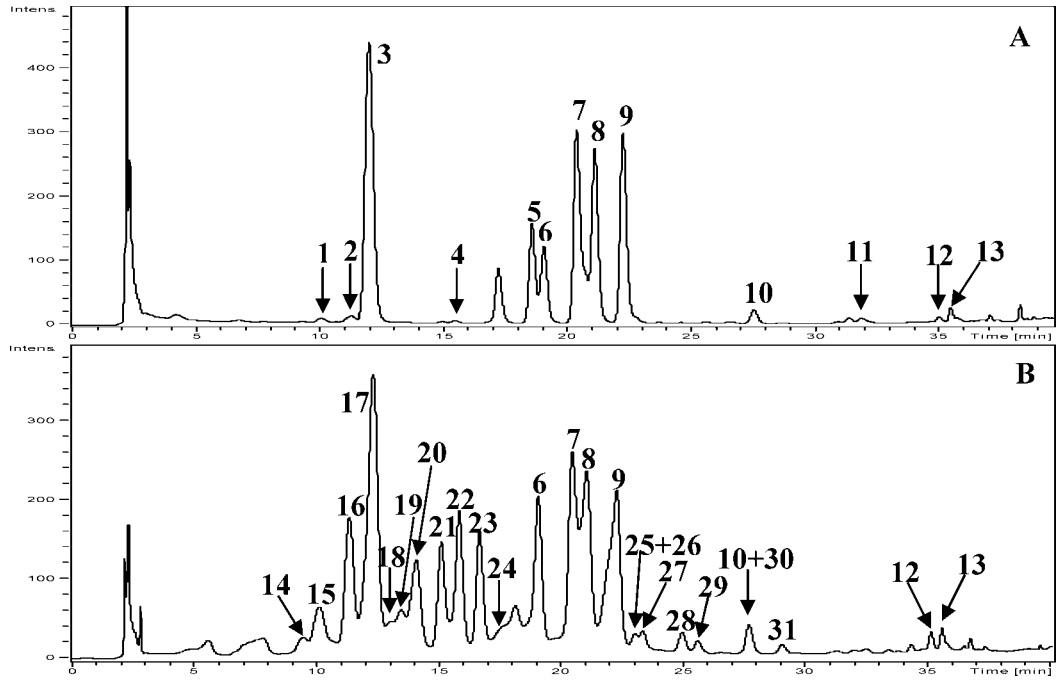


Figure 1. HPLC-DAD phenolic profile of *Brasica rapa* var. *rapa* leaves. (**A**) saponified hydromethanolic extract and (**B**) native hydromethanolic extract. Detection at 330 nm. Peaks: (**1**) quercetin-3-*O*-sophoroside-7-*O*-glucoside; (**2**) kaempferol-3-*O*-sophorotrioside-7-*O*-glucoside; (**3**) kaempferol-3-*O*-sophoroside-7-*O*-glucoside; (**4**) quercetin-3,7-di-*O*-glucoside; (**5**) *p*-coumaric acid; (**6**) kaempferol-3,7-di-*O*-glucoside; (**7**) isorhamnetin-3,7-di-*O*-glucoside; (**8**) ferulic acid; (**9**) sinapic acid; (**10**) kaempferol-3-*O*-sophoroside; (**11**) kaempferol-7-*O*-glucoside; (**12**) kaempferol-3-*O*-glucoside; (**13**) isorhamnetin-3-*O*-glucoside; (**14**) quercetin-3-*O*-(methoxycaffeoyl)sophoroside-7-*O*-glucoside; (**15**) quercetin-3-*O*-(caffeoyl)sophoroside-7-*O*-glucoside; (**16**) kaempferol-3-*O*-(methoxycaffeoyl)sophoroside-7-*O*-glucoside; (**17**) kaempferol-3-*O*-(caffeoyl)sophoroside-7-*O*-glucoside; (**18**) quercetin-3-*O*-(sinapoyl)sophoroside-7-*O*-glucoside; (**19**) quercetin-3-*O*-(feruloyl)sophoroside-7-*O*-glucoside; (**20**) quercetin-3-*O*-(*p*-coumaroyl)sophoroside-7-*O*-glucoside; (**21**) kaempferol-3-*O*-(sinapoyl)sophoroside-7-*O*-glucoside; (**22**) kaempferol-3-*O*-(feruloyl)sophoroside-7-*O*-glucoside; (**23**) kaempferol-3-*O*-(*p*-coumaroyl)sophoroside-7-*O*-glucoside; (**24**) quercetin-3-*O*-(caffeoyl)sophoroside-7-*O*-glucoside (isomer); (**25**) kaempferol-3-*O*-(feruloyl)sophoroside-7-*O*-glucoside (isomer); (**26**) Kaempferol-3-*O*-(methoxycaffeoyl)sophoroside; (**27**) kaempferol-3-*O*-(*p*-coumaroyl)sophoroside-7-*O*-glucoside (isomer); (**28**) kaempferol-3-*O*-(caffeoyl)sophoroside; (**29**) Kaempferol-3-*O*-(sinapoyl)sophoroside; (**30**) kaempferol-3-*O*-(feruloyl)sophoroside; (**31**) kaempferol-3-*O*-(*p*-coumaroyl)sophoroside.

HPLC/UV-DAD/ESI-MS^n analyses. Chromatographic analyses were carried out on a LiChroCART column (250 × 4 mm, RP-18, 5 μm particle size, LiChrospher 100 stationary phase, Merck, Darmstadt, Germany) protected with a LiChroCART guard column (4 × 4 mm, RP-18, 5 μm particle size, Merck, Darmstadt, Germany). The mobile phase consisted of a mixture of two solvents: water/acetic acid (1%) (A) and methanol (B). For studying both the free flavonol glycosides and the corresponding acylated derivatives, a linear gradient, starting with 15% B, was installed to reach 40% B at 30 min, 60% B at 35 min, 80% B at 37 min, and 80% B at 40 min. The flow rate was 1 mL min^{-1}, and the injection volume varied between 20 and 70 μL, depending on the compound and extract assayed. Spectral data from all peaks were accumulated in the range 240–400 nm, and chromatograms were recorded at 330 nm for glycosides and acylated derivatives. The HPLC/UV-DAD/ESI-MS^n analyses were carried out in an Agilent HPLC 1100 series equipped with a UV diode-array detector and mass detector in series (Agilent Technologies, Waldbronn, Germany). The HPLC consisted of a binary pump (model G1312A), an autosampler (model G1313A), a degasser (model G1322A), and a photodiode-array detector (model G1315B). The system was controlled by a ChemStation

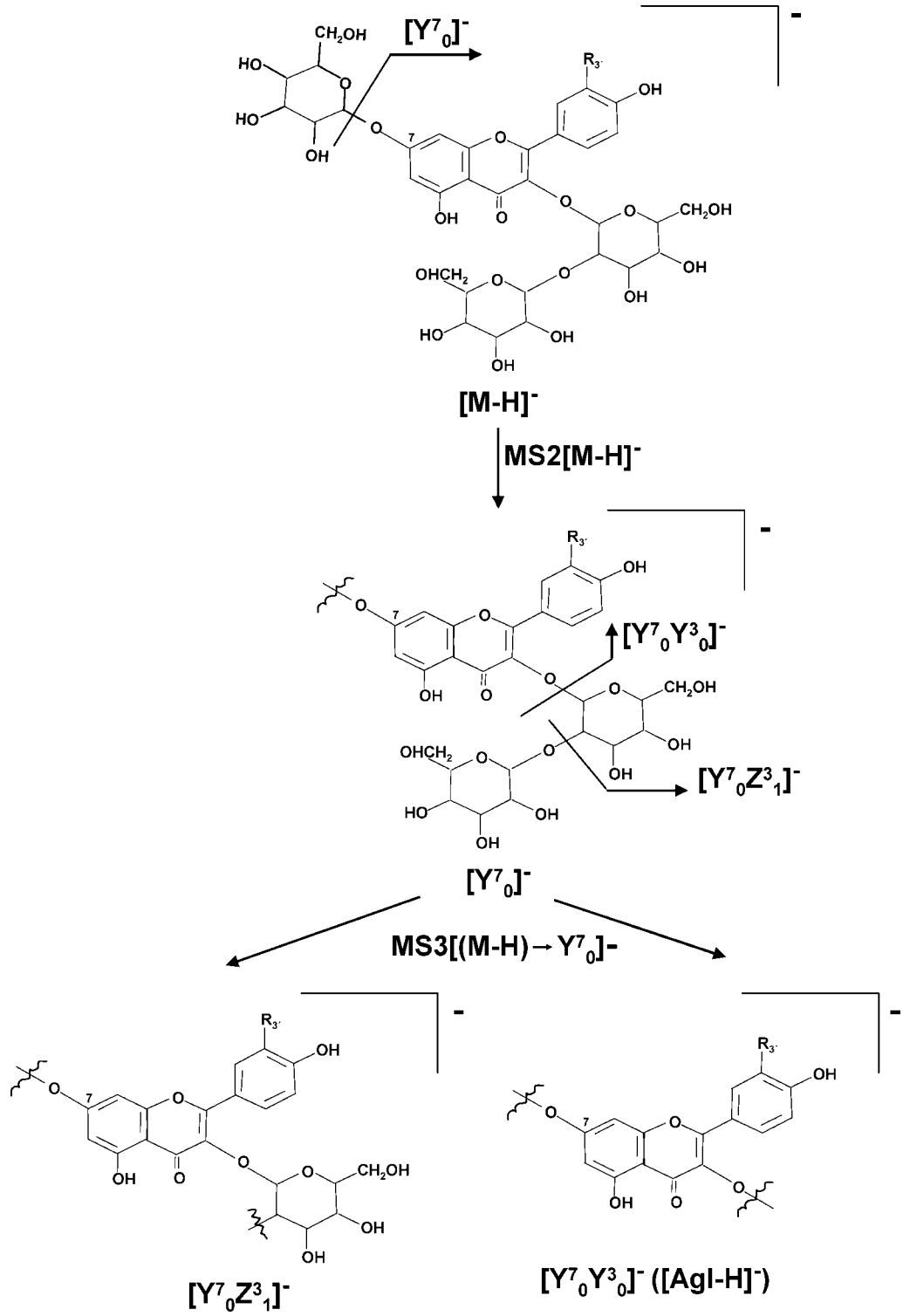


Figure 2. Fragmentation pathway of flavonol-3-*O*-sophoroside-7-*O*-glucoside. Compounds: **1** ($R_{3'}$: OH) and **3** ($R_{3'}$: H).

software (Agilent, v. 08.03). The mass detector was an ion trap spectrometer (model G2445A) equipped with an electrospray ionization interface and was controlled by LCMSD software (Agilent, v. 4.1). The ionization conditions were adjusted to 350 °C and 4 kV for capillary temperature and voltage, respectively. The nebulizer pressure and flow rate of nitrogen were 65.0 psi and 11 L min^{-1}, respectively. The full scan mass covered the range from *m/z* 100 to *m/z* 2000. Collision-induced fragmentation experiments were performed in the ion trap using helium as the collision gas, with voltage ramping cycles from 0.3 to 2 V. Mass spectrometry data were acquired in the negative ionization mode. MS^n was carried out in the automatic mode on the more abundant fragment ion in $MS^{(n-1)}$.

Tables 1–3 show the most frequent ions that characterize the fragmentation of the flavonoid *O*-glycosides. Other ions were found, but they have not been included because of their low significance on the MS behavior ions. The classical nomenclature (*9*) for glycoconjugates was adopted to designate the fragment ions. Ions ${}^{k,l}X_j$, Y^n_j, Z^n_j represent those fragments still containing the flavonoid aglycone, where *j* is the number of the interglycosidic bond broken, counted from the aglycone, *n* represents the position of the phenolic hydroxyl, where the oligosaccharide is attached, and *k* and *l* denote the cleavage within the carbohydrate rings.

The ions obtained as consequence of a second oligosaccharide fragmentation have been labeled according to a previous report (*10*).

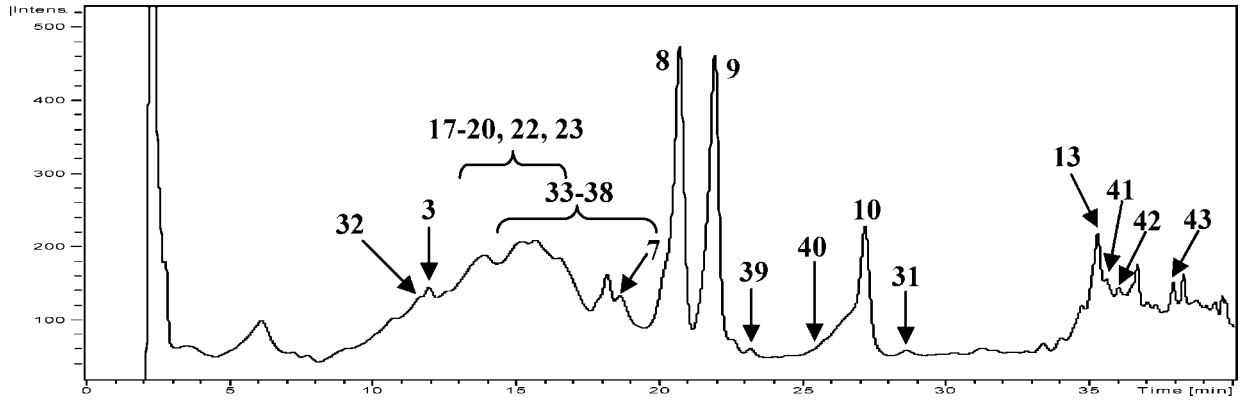


Figure 3. HPLC-DAD phenolic profile of *Pieris brassicae* excrements hydromethanolic extract. Detection at 330 nm. Peaks: **3**, **7–10**, **13**, **17–20**, **22**, **23**, and **31**, see **Figure 1**; (**32**) isorhamnetin-3,7-di-*O*-glucoside sulfate; (**33**) kaempferol-3-*O*-glucoside sulfate; (**34**) isorhamnetin-3-*O*-glucoside sulfate; (**35**) isorhamnetin-3-*O*-glucoside sulfate (isomer); (**36**) isorhamnetin-3-*O*-glucoside sulfate (isomer); (**37**) kaempferol-3-*O*-glucoside sulfate (isomer); (**38**) isorhamnetin-3-*O*-glucoside sulfate (isomer); (**39**) quercetin-3-*O*-sophoroside; (**40**) kaempferol-3-*O*-sophorotrioside; (**41**) kaempferol-3-*O*-(*p*-coumaroyl)-sophoroside (isomer); (**42**) kaempferol-3-*O*-(*p*-coumaroyl)sophoroside (isomer); and (**43**) kaempferol-3-*O*-(*p*-coumaroyl)sophoroside (isomer).

Thus, ions obtained from the ion $Y^7{}_0{}^-$ ($-MS^3[(M - H) \rightarrow Y^7{}_0]^-$) have been labeled starting with the ion $Y^7{}_0{}^-$ and followed by the resultant MS^3 ion, for example, the ion $Y^7{}_0Z^3{}_1{}^-$ (MS^3 of compound **1**, **Table 1** and **Figure 2**) denotes the loss of terminal glycosyl + 18 of the diglycoside in the 3 position ($Z^3{}_1{}^-$) from the fragmentation of ion $Y^7{}_0{}^-$ (total loss of a glycosylation in the 7 position). The losses indicated in the MS^3 scan show that the fragment came from the trapped and fragmented ion ($Y^7{}_0{}^-$) and not from the deprotonated molecular ion.

RESULTS AND DISCUSSION

***B. rapa* var. *rapa* Leaves.** The HPLC-DAD-MS screening of the *B. rapa* var. *rapa* leaves hydromethanolic extract revealed the existence of several flavonoids, the majority of them acylated with hydroxycinnamic acids. For their characterization, a saponification of the extract followed by the analysis of the deacylated derivatives were performed prior to the study of the native compounds.

Saponificated Hydromethanolic Extract. The HPLC-DAD-MS study of the saponificated extract of the leaves showed the presence of several kaempferol, quercetin, and isorhamnetin derivatives (**Figure 1A**). Their UV spectra indicated a substitution in the 3 and/or 7 position (**Table 1**) (*11*). Compounds **1–3** showed a MS fragmentation characteristic of flavonoids bearing a hexoside in the 7 position and a di- or trihexoside in the 3 position (*10*). In their $MS^2[M - H]^-$ spectra it was possible to observe the $Y^7{}_0{}^-$ ion as base peak ($[M - H - 162]^-$, loss of the sugar in 7 position), which was practically the only ion present. The $MS^3[(M - H) \rightarrow Y^7{}_0]^-$ event revealed the ions resultant from the fragmentation of the glycoside in 3 position (**Table 1**, **Figure 2**), whose high relative abundance is related with an 1 → 2 interglycosidic bond (*10*). Usually, for this kind of compound, the base peak in MS^3 corresponds to the aglycon. The presence of the ion at *m/z* 429 as base peak for compound **3** could give rise to some doubts regarding the nature of this fragment as *O*-diglycoside or di-*O*-glycoside. However, its provenance from the loss of 180 u (−162 – 18) indicates that it is an *O*-diglycoside (*12, 13*). So, these compounds were tentatively characterized as quercetin-3-*O*-sophoroside-7-*O*-glucoside (**1**), kaempferol-3-*O*-sophorotrioside-7-*O*-glucoside (**2**), and kaempferol-3-*O*-sophoroside-7-*O*-glucoside (**3**).

The MS fragmentation of compounds **4**, **6** and **7**, in which it was noticed that the ion $[(M - H) - 162]^-$ was the base peak, indicated a di-*O*-glycosilation. On the contrary, for compound **10** it was observed that the ion $Z^3{}_1{}^-$ ($[(M-H)-180]^-$) and the base peak belonged to the aglycon, indicating its *O*-diglycosidic nature. Thus, they were identified as quercetin-3,7-di-*O*-glucoside (**4**), kaempferol-3,7-di-*O*-glucoside (**6**), isorhamnetin-3,7-di-*O*-glucoside (**7**), and kaempferol-3-*O*-sophoroside (**10**).

Other detected compounds (**11**-**13**) are monoglycosides: kaempferol-7-*O*-glucoside (**11**), kaempferol-3-*O*-glucoside (**12**), and isorhamnetin-3-*O*-glucoside (**13**).

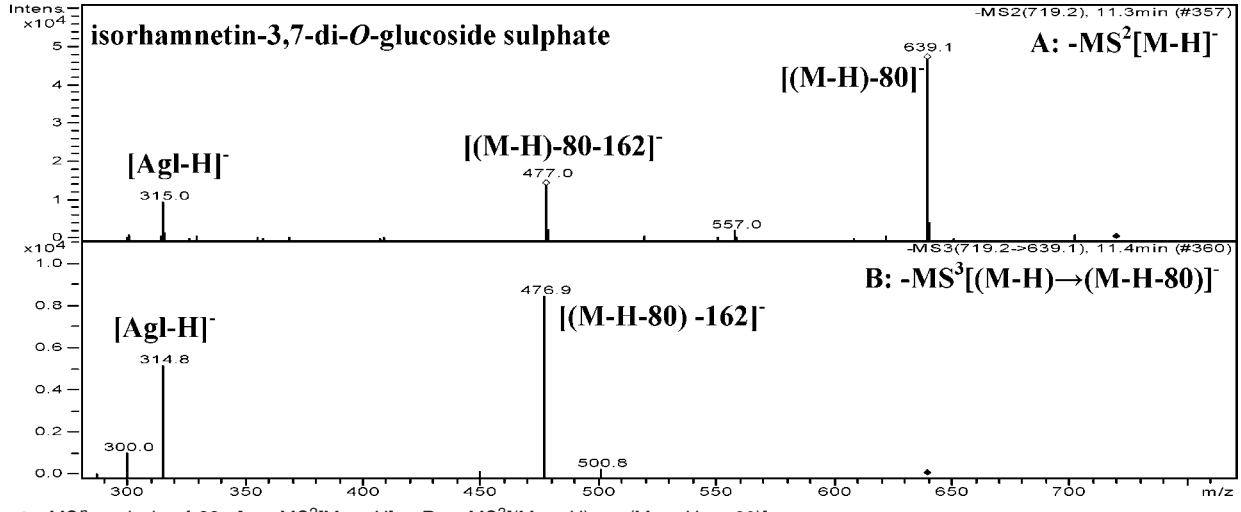


Figure 4. MS^n analysis of **32**. **A**: $-MS^2[M - H]^-$. **B**: $-MS^3[(M - H) \rightarrow (M - H - 80)]^-$.

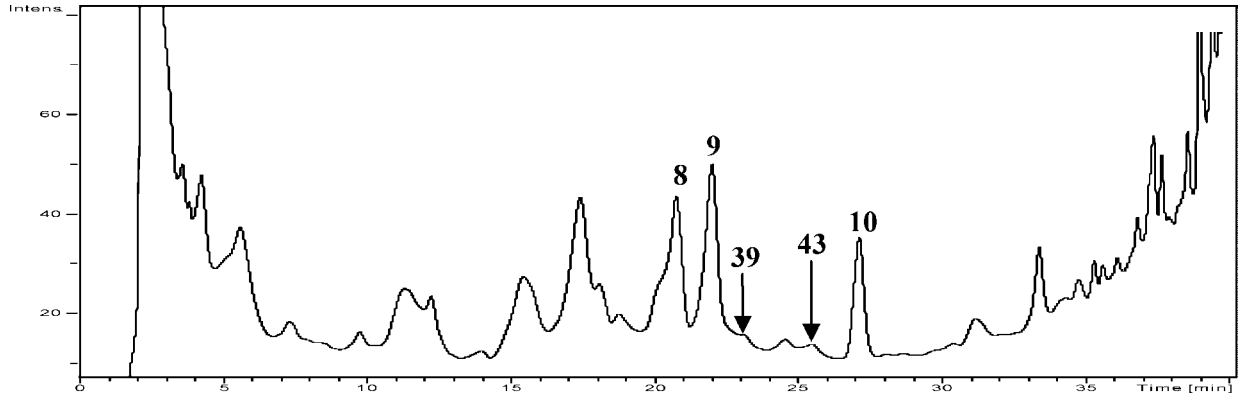
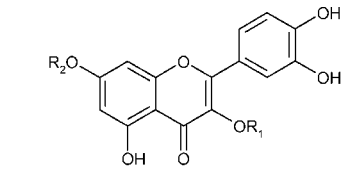
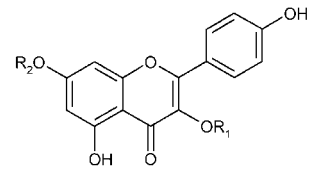


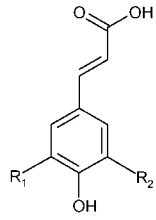
Figure 5. HPLC-DAD phenolic profile of *Pieris brassicae* larvae hydromethanolic extract. Detection at 330 nm. Peaks: **8**, **9**, and **10**; see **Figure 1**; **39** see **Figure 2**; (**43**) kaempferol-3-*O*-sophorotrioside.



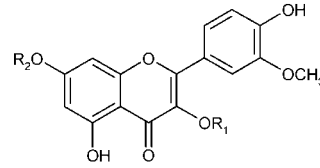
1 R_1 = sophorose; R_2 = glucose
4 $R_1 = R_2$ = glucose
14 R_1 = (methoxycaffeoyl)sophorose; R_2 = glucose
15, 24 R_1 = (caffeoyl)sophorose; R_2 = glucose
18 R_1 = (sinapoyl)sophorose; R_2 = glucose
19 R_1 = (feruloyl)sophorose; R_2 = glucose
20 R_1 = (*p*-coumaroyl)sophorose; R_2 = glucose
39 R_1 = sophorose; R_2 = H



2 R_1 = sophorotriose; R_2 = glucose
3 R_1 = sophorose; R_2 = glucose
6 $R_1 = R_2$ = glucose
10 R_1 = sophorose; R_2 = H
11 R_1 = H; R_2 = glucose
12 R_1 = glucose; R_2 = H
16 R_1 = (methoxycaffeoyl)sophorose; R_2 = glucose
17 R_1 = (caffeoyl)sophorose; R_2 = glucose
21 R_1 = (sinapoyl)sophorose; R_2 = glucose
22, 25 R_1 = (feruloyl)sophorose; R_2 = glucose
23, 27 R_1 = (*p*-coumaroyl)sophorose; R_2 = glucose
26 R_1 = (methoxycaffeoyl)sophorose; R_2 = H
28 R_1 = (caffeoyl)sophorose; R_2 = H
29 R_1 = (sinapoyl)sophorose; R_2 = H
30 R_1 = (feruloyl)sophorose; R_2 = H
31, 41, 42, 43 R_1 = (*p*-coumaroyl)sophorose; R_2 = H
40 R_1 = sophorotriose; R_2 = H



5 $R_1 = R_2$ = H
8 R_1 = OCH_3; R_2 = H
9 $R_1 = R_2$ = OCH_3



7 $R_1 = R_2$ = glucose
13 R_1 = glucose; R_2 = H

Figure 6. Chemical structures of several phenolic compounds identified in *Pieris brassicae* material and in the host *Brasica rapa* var. *rapa* leaves.

Apart from flavonoids, *p*-coumaric (**5**) (R_t 18.5 min; UV, 290sh and 310 nm; MS, $[M - H]^-$: 163, $MS^2[M - H]^-$: 119), ferulic (**8**) (R_t 21.0 min; UV, 295sh and 325 nm; MS, $[M - H]^-$: 193, $MS^2[M - H]^-$: 178, 149, 134) and sinapic (**9**) (R_t 22.2 min; UV, 325 nm; MS, $[M - H]^-$: 223, $MS^2[M - H]^-$: 208, 179, 164, and 149) acids have also been identified.

These flavonoid glycosides are common in *Brassica* species, either in the free form or as acylated derivatives. The most abundant, **3**, is the most extensively reported in the genus, being described in cauliflower (*B. oleracea* L. *var. botrytis*) (*14*), broccoli (*B. oleracea* L. var. *italica*) (*15*), tronchuda cabbage (*B. oleracea* L. var. *costata* DC) (*16–18*), and pak choi (*B. rapa* L. ssp. *chinensis* L. (Hanelt.)) (*19*). On the other hand, flavonol-3,7-di-*O*-glucosides are characteristics of *B. rapa*. In particular, **7** has been found in the free form in the nectar of ssp. *pekinensis* (*20*), as in ssp. *chinensis* L (*19*) and turnip tops of ssp. *sylvestris* (*11*).

Native Hydromethanolic Extract. The HPLC-DAD-MS study of the native leaves extract revealed the existence of several hydroxycinnamic acid derivatives of **1** (compounds **14**, **15**, **18**−**20**, and **24**), **3** (compounds **16**, **17**, **21**−**23**, **25**, and **27**), and **10** (compounds **26** and **28**−**31**) (**Table 2**, **Figure 1B**). In addition, ferulic (**8**) and sinapic (**9**) acids and the nonacylated flavonoid glycosides **6**, **7**, **10**, **12**, and **13**, detected in the saponificated extract, were also present. Compounds **6–10**, **12**, and **13** were already described in *B. rapa* var. *rapa* (*8*).

The fragmentation of the acylated derivatives of compounds **1** and **3** (**Table 2**) showed, in MS^2, a base peak resultant from the loss of the sugar in the 7 position ($[(M - H) - 162]^-$). The fragment correspondent to the loss of the acyl radical (an abundant peak for the derivatives of compound **1**, but absent in those of compound **3**) could also be observed, as well as the fragment obtained by the simultaneous loss of the sugar in the 7

position and of the acyl group (also more abundant in the derivatives of compound **1** than in those of compound **3**). The $MS^3[(M - H) \rightarrow (M - H - 162)]^-$ event revealed the loss of the acyl radical, giving the aglycon fragment linked to the glycosidic fraction at the 3 position. Despite having little abundance, the ion of the aglycon could also be noticed (*15*). Regarding the fragmentation of compound **10** derivatives (flavonoids without glycosilation in 7 position) in MS^2, the loss of the acyl radical was noted to give rise to the deprotonated ion of compound **10** (base peak). The $MS^3[(M - H) \rightarrow (M - H - Acyl)]^-$ event led to the typical fragmentation of a sophoroside (*10*) (**Table 2**).

The chromatographic behavior of compounds **26** and **28**−**30** (acyl derivatives of **10**), for which the retention times are equal or less than that of the deacylated compound (**10**), has been observed in previous studies (*14, 17*), indicating that the acylated derivatives at the 3 position of the sugar and without glycosilation in the 7 position exhibit an apparent irregular retention time. In the same way, the order of elution in reversed-phase HPLC of the acylated derivatives (caffeoyl, sinapoyl, feruloyl, and *p*-coumaroyl, respectively) is distinct from that of the free acids (caffeic, *p*-coumaric, ferulic, and sinapic, respectively), as previously reported (*14, 15*). Another apparently anomalous data is related with the UV spectra of compounds **15** and **19**, which are similar to those of the deacylated compounds.

To summarize, all acylated derivatives (methoxycaffeic, caffeic, sinapic, ferulic, and *p*-coumaric acids) of **1**, **3**, and **10** were characterized. The derivatives of **3** are the most abundant ones (**Figure 1B**), whereas those of **10** are present in trace amounts. Quercetin-3-*O*-sophoroside-7-*O*-glucoside derivatives are found in considerable amounts (**Figure 1B**). However, the deacylated glycoside (**1**) is detected only in vestigial amounts in the saponificated hydromethanolic extract (**Figure 1A**). This can be due to the alkaline decomposition, during the saponification process, of phenolic compounds with an *o*-dihydroxy group, resulting in the presence of quercetin derivatives in trace amounts while caffeic acid is not observed.

In general, this kind of acylated derivatives are very common in Brassicacea (*14–18*), and particularly in distinct *B. rapa* subspecies (*11, 19*). On the other hand, the presence of flavonol-3,7-di-*O*-glucosides, namely of nonacylated isorhamnetin-3,7-di-*O*-glucoside characterizes *B. rapa* (*8, 11, 19*) relatively to other *Brassica* species. Despite contributing to the organoleptic characteristics of the plant, these compounds may participate in the defense against external aggressions.

***P. brassicae* Material.** The HPLC-DAD-MS screening of the hydromethanolic extracts of *P. brassicae* larvae, excrements, exuviae, and butterflies led to chromatograms in which the compounds are present in very low or trace amounts. Besides, the first part of the excrements' chromatogram exhibited a bad resolution (**Figure 3**), making UV spectral analysis of the peaks impossible. Thus, for the study of their flavonoids, we had extracted from the HPLC-MS the MS^n ions at *m*/*z* 285, 301, and 315 ("extracted ion chromatogram") coming from kaempferol, quercetin, and isorhamnetin derivatives, respectively. Even so, for the detection of acylated derivatives that might still exist, we extracted those ions exhibiting the loss of the acyl radical (−146, −162, −176, −192, and −206 u) ("constant neutral loss chromatogram"), and compared these MS spectra and retention times with those of both native and saponificated extracts of *B. rapa* var. *rapa* leaves. Additionally, during the above-mentioned ions extraction process some fragmentation compounds were detected that revealed losses of 80 u (98 – 18), characteristic of sulfated derivatives. So, extraction of ions presenting this loss was also performed (constant neutral loss chromatogram).

Excrement. In the HPLC-DAD chromatogram of excrement hydromethanolic extract (**Figure 3**) the two main peaks corresponded to **8** and **9**. Other compounds present in considerable amounts, and that have been found before in the native hydromethanolic extract of *B. rapa* var. *rapa* leaves, were **10**, **7**, and **13**. Some of the acylated derivatives already described were also detected, in low or trace amounts: kaempferol-3-*O*-(caffeoyl)sophoroside-7-*O*-glucoside (**17**), quercetin-3-*O*-(sinapoyl)sophoroside-7-*O*-glucoside (**18**), quercetin-3-*O*-(feruloyl)sophoroside-7-*O*-glucoside (**19**), quercetin-3-*O*-(*p*-coumaroyl)sophoroside-7-*O*-glucoside (**20**), kaempferol-3-*O*-(feruloyl)sophoroside-7-*O*-glucoside (**22**), kaempferol-3-*O*-(*p*-coumaroyl)sophoroside-7-*O*-glucoside (**23**), and kaempferol-3-*O*-(*p*-coumaroyl)sophoroside (**31**). Other compounds that were not found in the native extract of the leaves were **3**, described in the saponificated extract, quercetin-3-*O*-sophoroside (**39**) (R_t 23 min; UV 255, 265sh, 300sh, and 350 nm; −MS: 625 $[M - H]^-$, $-MS^2[M - H]^-$: 463 (10%, $[(M - H) - 162]^-$), 445 (25%, $[(M - H) - 180]^-$), 301 (100%, $[Agl - H]^-$)), kaempferol-3-*O*-sophorotrioside (**40**) (R_t 25.4 min; −MS: 771 $[M - H]^-$, $-MS^2[M - H]^-$: 609 (58%, $[(M - H) - 162]^-$), 429 (50%, $[(M - H) - 342]^-$), 285 (100%, $[Agl - H]^-$)), and three isomers of **31** (compounds **41** (R_t 35.4 min), **42** (R_t 35.9 min), and **43** (R_t 37.8 min)), which have MS similar to that of compound **31**. In the first part of the chromatogram (**Figure 3**) several flavonoid derivatives were detected (**32**−**38**), for which $-MS^2[M - H]^-$ showed the loss of 80 u to give origin to the base peak that characterizes as sulfate flavonoids (**Table 3**). Other observed ions corresponded to the deprotonated aglycon and the one resulting from the simultaneous loss of the sulfate radical and the partial fragmentation of the glycosidic fraction in compound **32** (**Figure 4**). In the MS^3 fragmentation of compound **32** (**Table 3** and **Figure 4**) a base peak at *m*/*z* 477 was noticed, indicating its di-*O*-glucosidic nature: isorhamnetin-3,7-di-*O*-glucoside sulfate. The other sulfate derivatives were monoglucosides; **33** and **37** were kaempferol-3-*O*-glucoside sulfate isomers, and **34**−**36** and **38** corresponded to isorhamnetin-3-*O*-glucoside sulfate isomers. These kinds of compounds are very usual in animals' metabolic process, and their reversed phase HPLC chromatograms are badly defined. Considering the obtained results it can be inferred that during the metabolic process of *P. brassicae* the deacylation of flavonoids occurs, leading to the disappearance or decrease of acylated derivatives. Besides this, the absence of glycosilation in the 7 position in the majority of flavonoid sulfates, as well as in the remaining compounds (**10**, **39**, **40** and **13**), indicates the loss of the sugar in this position, together with the above-mentioned deacylation, in the derivatives of **3** and **1**, and in **2** and **7**, to originate **10**, **39**, **40**, and **13**, respectively. On the other hand, the monoglycosilation of the greater part of flavonoid sulfates (compounds **33**−**38**) points to a new deglycosilation process.

Larvae, Exuviae, and Butterflies. The hydromethanolic extract of *P. brassicae* larvae presented compounds found in the excrements: **8**, **9**, and **10** (**Figure 5**). Vestigial amounts of **39** and **40**, also detected in the excrements, were also noticed. These compounds may contribute to protect the larvae from external aggressions, such as light, undesirable environmental conditions, oxidative phenomena, or microbial agents.

The HPLC analysis of hydromethanolic extracts of exuviae and butterflies revealed peaks in trace amounts, none of them corresponding to the studied phenolic compounds or possibly

related with them. The chemical structures of the phenolics identified in the work herein are displayed in **Figure 6**.

Excrements are produced only at the larval stage. Although no quantification was performed, it seems clear that the excrements are the material containing higher phenolic content, which can be inferred not only by the diversity of compounds detected, but also by the intensity of the peaks in the chromatograms of the larvae and its excrements (**Figures 3** and **5**). This can be accomplished once the extracts of all matrices were prepared using exactly the same quantity of material and solvent volume. These results are not surprising, considering that phenolic compounds are sequestered and undergo metabolization, regarding their detoxification and excretion. If the compounds are excreted, then they will not be present in the subsequent stages, and this maybe the reason for not finding them in the exuviae and butterflies.

As far as we know, it is the first time that the phenolic compounds in *P. brassicae* larvae reared on B. rapa var. *rapa* leaves have been determined. In a previous work using *B. oleracea* var. *costata* as host plant (*5*), it was possible to see that the larva can sequester and metabolize this type of compounds. The phenolic composition of these two *Brassica* species is different, so the phenolic profile found for the larvae is also distinct from that observed before (*5*). This fact confirms the strong dependency on the phenolic pattern of the host plant. In addition, this is the first study involving *P. brassicae* excrements, exuviae, and butterflies, which allowed accomplishing that, besides deacylation and deglycosylation already reported (*5*), sulfating reactions also occur in the metabolic process of the larvae and that phenolic compounds are mainly excreted and not transferred into the wings.

In conclusion, as insects are unable to synthesize phenolic compounds or their precursors, their presence in the different stages of *P. brassicae* life cycle can only arise from the food it has ingested, that is, from the complex flavonoid derivatives and free phenolic acids of *B. rapa* var. *rapa* leaves. So, the detection of this kind of compounds in the larvae indicates that it has the ability to sequester them. Additionaly, the fact that both larvae and excrements exhibit phenolic compounds distinct from those of the host plant evidence that the larvae has the capacity to metabolize these phytochemicals and to excrete them by the faeces, which included sulfate derivatives, reported for the first time. As these kinds of flavonoids are known for their antioxidant potential (*16, 17, 21*), in what concerns the obtainment of potential health promoting compounds, unusual in nature and hard to be synthesized in the laboratory, *P. brassicae* excrements may constitute a promising source. Extracts of *P. brassicae* excrements fed with *B. rapa* var. *rapa* could be prepared to be used as antioxidants by, for example, food or pharmaceutical industries, allowing for some profit from this frequent pest in *Brassica* cabbage productions.

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