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Phenolics Metabolism in Insects: *Pieris brassicae*−*Brassica oleracea* var. *costata* Ecological Duo

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Changes in the phenolics composition of *Pieris brassicae* larvae fasted for distinct periods (1, 2, 4, 6, and 8 h) and their excrements and of *Brassica oleracea* L. var. *costata* DC leaves were determined by high-pressure liquid chromatography/UV-photo diode array detector/mass spectrometry−electrospray ionization. This is the first report following phenolics' metabolism by *P. brassicae* through time. The results evidence that *P. brassicae* sequesters and metabolizes the phenolic compounds from the host plant. In a general way, deacylation was the main metabolic reaction that took place, but deglycosylation and sulfate conjugation reactions also occur. Additionally, several kaempferol derivatives containing rhamnose, which is not common in *Brassica*, were found in the host plant. Attending to the bioactivities recognized for the type of identified compounds, the different materials may constitute an interesting source of bioactive compounds, namely, of highly glycosylated and acylated kaempferol and quercetin derivatives, constituting an economic advantage for producers who have great losses caused by this pest. In addition, a deeper understanding of phenolics metabolism in insects was pursued.

KEYWORDS: ***Pieris brassicae*** **L. larva;** ***Brassica oleracea*** **L. var.** ***costata*** **DC; phenolics; metabolism**

INTRODUCTION

Plants produce a great variety of secondary metabolites that almost all herbivores will encounter when feeding. Some plants contain compounds, like phenolics, that insects can sequester into their body cuticle for protection against pathogens and predators or into their wings to attract mates (*1*). The majority of phytophagous insects are monophagous or oligophagous, feeding on a limited range of plant species or families (*2*). The association between *Pieris* (Lepidoptera: Pieridae) and their Brassicaceae host plants was initially attributed to glucosinolates, but phenolic compounds, like quercetin and kaempferol derivatives, have also exhibited a role in the dynamic of the plant−insect biological system (*2*, *3*). Differently from other secondary metabolites accumulated by herbivores for defense or pheromone synthesis, phenolics are suggested to protect the insects against harmful radiation and to be involved in intra- or interspecific visual communication due to their UV-absorbing capacity (*4*, *5*).

The phenolic profiles of large white butterfly *Pieris brassicae* L. larvae reared on the leaves of three *Brassica* of which it is a frequent pest, namely, *Brassica oleracea* L. var. *costata* DC (*6*), *B. oleracea* L. var. *acephala* (*7*), and *Brassica rapa* var. *rapa* L. (*8*), have been determined before by our group. Several complex molecules, mainly flavonoid derivatives, were found before in extracts of the larvae kept without food for 1 and 12 h. Those studies provided evidence of the sequestration, metabolism, and excretion of the phenolic compounds from the host plant by *P. brassicae*. Also, the importance of the phenolic profile of the feeding materials in shaping the uptake and metabolic processes was demonstrated.

Furthermore, the distinct phenolic composition of the host plant was decisive for the bioactivity exhibited by several *P. brassicae* analyzed materials (*7*, *9*, *10*). However, the evolution of the metabolic process of *P. brassicae* was not assessed in any of those works. This information would contribute to the knowledge of the sequence of reactions occurring after phenolics uptake by the insect and could also allow the finding of interesting intermediary products.

As far as we are aware, there are no reported studies analyzing the evolution of phenolic compounds metabolism by this insect. Thus, the aim of the present work was to evaluate the overall phenolics profile evolution of *P. brassicae* reared on *B. oleracea* var. *costata* (tronchuda cabbage). On the other hand, attending to the bioactivities recognized for this type of compounds, their occurrence in insect materials may constitute an economic advantage for producers who have great losses due to the destruction of their cultures by this pest. For this purpose, extracts obtained from the larvae at different starvation periods,

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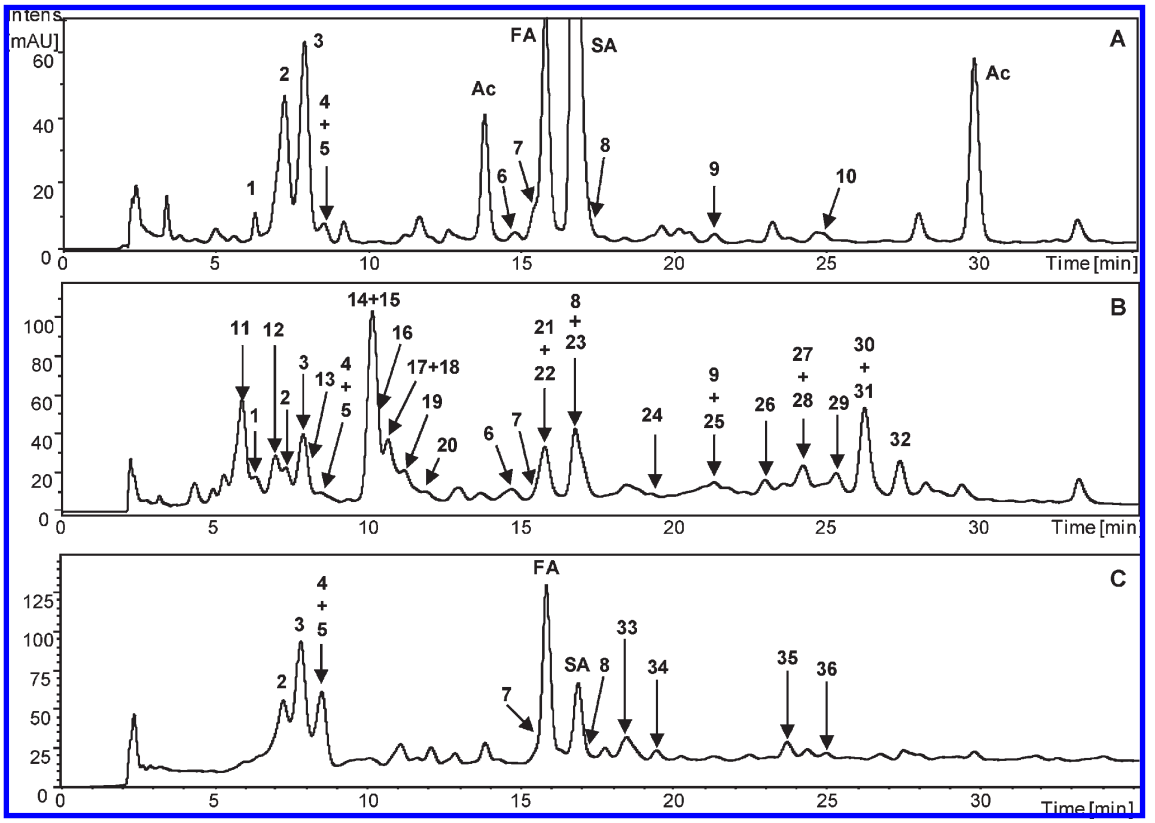

Figure 1. HPLC-PAD phenolic profile of (**A**) *B. oleracea* var. *costata* saponified extract, (**B**) *B. oleracea* var. *costata* native extract, and (**C**) *P. brassicae* excrement extract. Detection at 330 nm. Peaks: Ac, acylated derivatives not characterized; FA, ferulic acid; SA, sinapic acid; 1, quercetin-3-*O*-sophoroside-7-*O*-glucoside; 2, kaempferol-3-*O*-triglucoside-7-*O*-glucoside; 3, kaempferol-3-*O*-sophoroside-7-*O*-glucoside; 4, kaempferol-3-*O*-triglucoside-7-*O*-diglucoside; 5, kaempferol-3-*O*-sophoroside-7-*O*-diglucoside; 6, kaempferol-3-*O*-triglucoside-7-*O*-rhamnoglucoside; 7, kaempferol-3-*O*-triglucoside-7-*O*-rhamnoside; 8, kaempferol-3-*O*-sophoroside-7-*O*-rhamnoside; 9, kaempferol-3-*O*-sophoroside; 10, kaempferol-3-*O*-glucoside; 11, 3-*p*-coumaroylquinic acid; 12, 3-feruloylquinic acid; 13, kaempferol-3-*O*-(caffeoyl)sophoroside-7-*O*-glucoside; 14, kaempferol-3-*O*-(sinapoyl)triglucoside-7-*O*-glucoside; 15, kaempferol-3-*O*-(sinapoyl)-sophoroside-7-*O*-glucoside; 16, 4-*p*-coumaroylquinic acid; 17, kaempferol-3-*O*-(feruloyl)triglucoside-7-*O*-glucoside; 18, kaempferol-3-*O*-(feruloyl)sophoroside-7-*O*-glucoside; 19, 5-*p*-coumaroylquinic acid; 20, 4-feruloylquinic acid; 21, kaempferol-3-*O*-(sinapoyl)triglucoside-7-*O*-rhamnoside; 22, kaempferol-3-*O*-(sinapoyl)sophoroside-7-*O*-rhamnoside; 23, kaempferol-3-*O*-(feruloyl)sophoroside-7-*O*-rhamnoside; 24, kaempferol-3-*O*-(sinapoyl)sophoroside; 25, kaempferol-3-*O*-(feruloyl)sophoroside; 26, feruloylsinapoylgentiobioside; 27, disinapoylgentiobioside; 28, feruloylsinapoylgentiobioside isomers; 29, disinapoylgentiobioside isomers; 30, feruloylsinapoylgentiobioside isomers; 31, kaempferol-3-*O*-(disinapoyl)triglucoside-7-*O*-glucoside; 32, disinapoylgentiobioside isomer; 33, kaempferol-3-*O*-(feruloyl)sophoroside-7-*O*-diglucoside; 34, kaempferol-3-*O*-(feruloyl)sophoroside-7-*O*-glucoside isomer; 35, kaempferol-3-*O*-(feruloyl)triglucoside-7-*O*-diglucoside; and 36, kaempferol-3-*O*-(feruloyl)triglucoside-7-*O*-glucoside.

as well as of the excrements produced by the insect, were analyzed by high-pressure liquid chromatography/UV-photo diode array detector/mass spectrometry−electrospray ionization (HPLC/UV-PAD/MSn-ESI), an advanced and valuable tool in the elucidation of complex phenolic molecules.

MATERIALS AND METHODS

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

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 *B. oleracea* L. var. *costata* DC leaves. *B. oleracea* var. *costata* samples used to feed *P. brassicae* were specially produced for this work, in greenhouses of Escola Superior Agrária de Bragança. Samples were collected in November, 2008, which corresponds to higher insect activity, following the vegetative cycle of the plant (more developed vegetable).

New larvae were developed having only this plant as host, which was supplied every day *ad libitum*. New larvae at the fourth instar were collected for analysis. *P. brassicae* larvae were isolated and deprived of food for 8 h. After this starving time, individuals were placed in *B. oleracea* var. *costata* leaves for feeding. Afterward, they were divided in distinct groups, which were subjected to several starvation periods before being sacrificed (1, 2, 4, 6, and 8 h), together with their excrements. *P. brassicae* larvae at the distinct starvation periods, their excrements, and host *B. oleracea* var. *costata* leaves were freeze-dried. The dried material was powdered and kept in a desiccator in the dark until analysis. Voucher specimens (corresponding to aliquots of the samples that were subjected to extraction and phenolic compounds analysis) are deposited at the Department of Pharmacognosy, Faculty of Pharmacy, Porto University.

Phenolic Compounds Extraction. For the characterization of the phenolic compounds in *P. brassicae* larvae and excrements and in the host plant, ca. 1 g of dried material was boiled for 30 min in 800 mL of water. The resultant aqueous extracts were filtered over a Büchner funnel, frozen, and lyophilized. The lyophilized extracts were kept in a desiccator in the dark until analysis. For the identification of phenolic compounds, each lyophilized extract (0.05 g) was thoroughly mixed with 1 mL of methanol/water (1:1), ultrasonicated (60 min), centrifuged (12000 rpm, 5 min), and filtered through a 0.45 μm pore size membrane.

Alkaline Hydrolysis. For the study of the acyl flavonoids, alkaline hydrolysis was performed followed by mass spectrometric analysis of the deacylated derivatives. This hydrolysis procedure was necessary since losses of 146 mass units for *p*-coumaroyl moieties and of 162 mass units for caffeoyl residues coincide with the loss of rhamnosyl and hexosyl residues, respectively. Otherwise, a misassignment of the mass spectrometric data

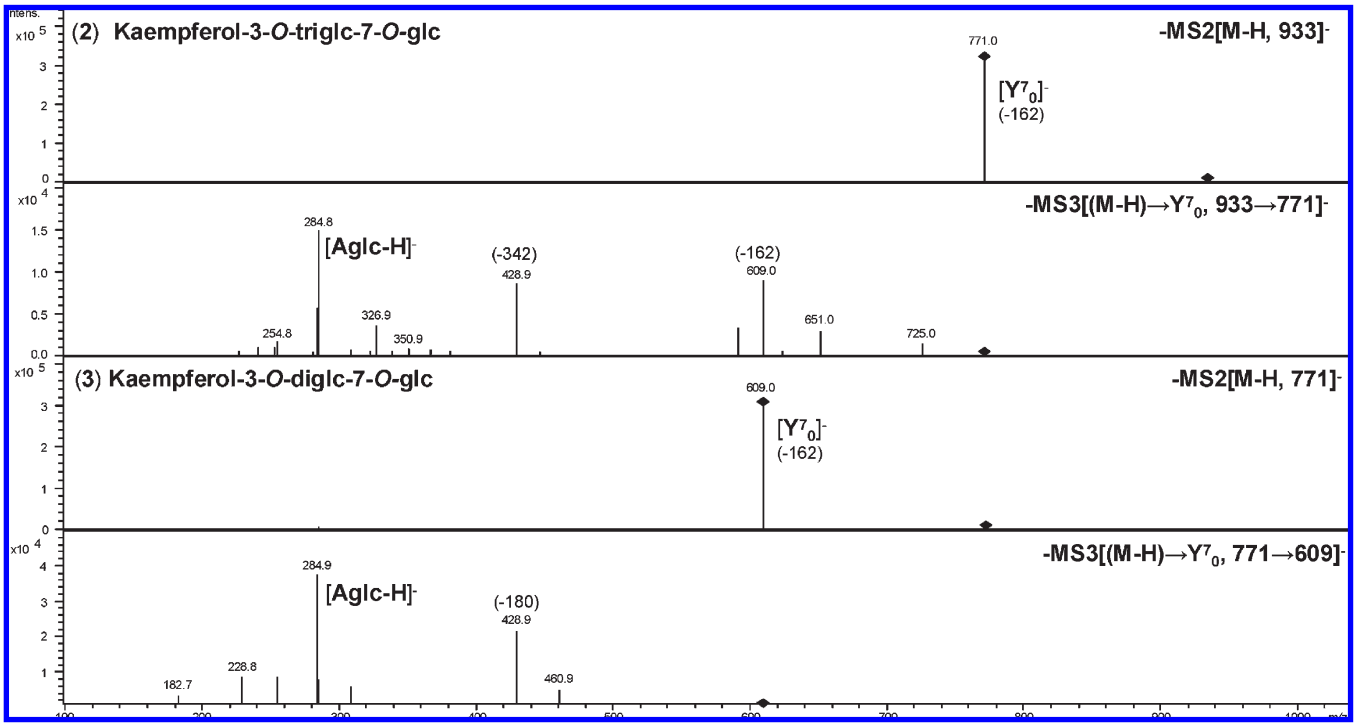

Figure 2. MS2 and MS3 spectra of the main flavonoids (**2** and **3**) in *B. oleracea* var. *costata* native extract. The identities of the compounds are as in **Figure 1**.

Table 1. R_t, UV, −MS[M − H]$^-$, −MS2[M − H]$^-$, and −MS3[(M − H)→Y_0^7]$^-$ Data of Nonacylated Glycosyl Flavonoids from Native Extract and from Saponified Extract of *B. oleracea* var. *costata* Leaves (**1**−**10**) and from *P. brassicae* Larvae (**39**)[a]

	compounds[b]	R_t (min)	UV (nm)	[M − H]$^-$ (*m*/*z*) (%)	−MS2[M − H]$^-$ (*m*/*z*) (%)	−MS3[(M − H)→Y_0^7]$^-$ (*m*/*z*) (%)			
						flavonol-3-*O*-tri/diglucoside-7-*O*-glucoside			
					Y_0^{7-} (−162)	(−162)	(−180)	(−342)	Aglc-H/2H
1	Q-3dG-7G	6.3	255, 266sh, 295sh, 352	787	625 (100)	463 (21)	445 (63)		300 (100)
2	K-3tG-7G	7.1	266, 320sh, 347	933	771 (100)	609 (60)		429 (57)	285 (100)
3	K-3dG-7G	7.9	266, 320sh, 347	771	609 (100)		429 (57)		285 (100)
						kaempferol-3-*O*-triglucoside-7-*O*-rhamnoglucoside			
					Y_0^{7-} (−308)				
6	K-3tG-7RG	14.8	266, 320sh, 348	1079	771 (100)	609 (100)		429 (25)	285 (65)
						kaempferol-3-*O*-tri/diglucoside-7-*O*-rhamnoside			
					Y_0^{7-} (−146)				
7	K-3tG-7R[c]	15.4		917	771 (100)	609 (90)		429 (40)	284 (100)
8	K-3dG-7R[c]	17.1		755	609 (100)		429 (68)		284 (100)
						kaempferol-3-*O*-tri/diglucoside-7-*O*-diglucoside			
					Y_0^{7-} (−324)				
4	K-3tG-7dG[c]	8.5		1095	771 (100)	609 (90)		429 (58);	285 (100)
5	K-3dG-7dG[c]	8.7		933	609 (100)		429 (40)		285 (100)
							flavonol-3-*O*-tri/diglucoside		
							−MS2[M − H]$^-$ (*m*/*z*) (%)		
39	Q-3dG	17.4	256, 266sh, 302sh, 353				445 (40)		300 (100)
9	K-3dG	21.3	266, 295sh, 348	609			429 (60)		285 (100)
10	K-3G[c]	24.7		447					285 (100)

[a] Main observed fragments. Other ions were found, but they have not been included. [b] Q, quercetin; K, kaempferol; G, glucose; R, rhamnose; and Q-3-tG-7-dG, quercetin-3-*O*-triglucoside-7-*O*-diglucoside. [c] Compounds hidden by others or in traces. Their UV spectra have not been properly observed.

might occur, due to the inability to distinguish between the presence of *p*-coumaroyl or ramhosyl moieties or the existence of caffeoyl or hexosyl groups

Sodium hydroxide (2 N; 0.5 mL) was added to 0.5 mL of the native methanol/water (1:1) solution from *B. oleracea* var. *costata* external leaves, 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-PAD/ESI-MSn.

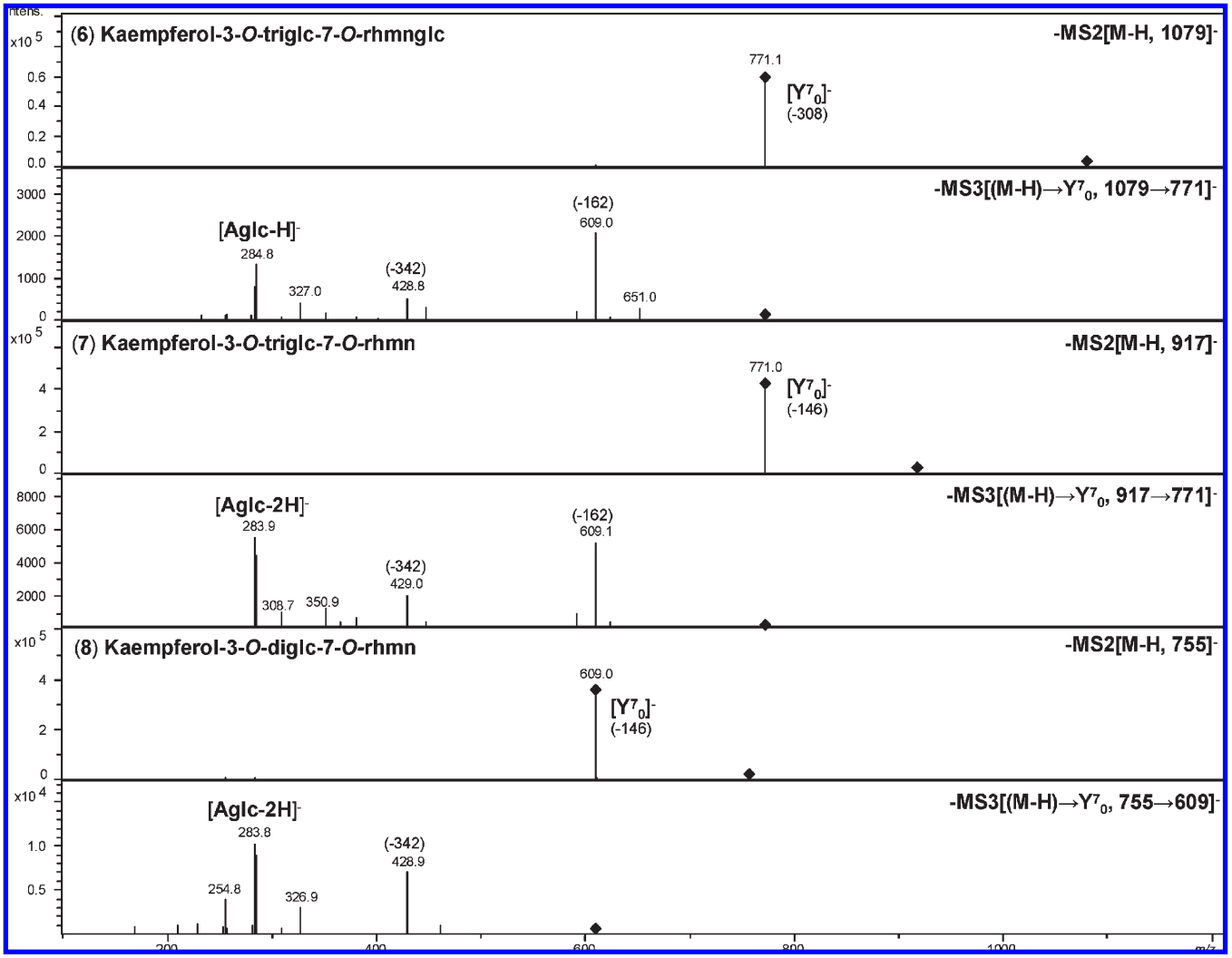

Figure 3. MS2 and MS3 spectra of some flavonoid derivatives with rhamnose, not common in *Brassica* (**6**−**8**). The identities of compounds are as in **Figure 1**.

HPLC/UV-PAD/ESI-MSn Analysis. Chromatographic analyses were carried out on a LiChroCART column (250 mm × 4 mm, RP-18, 5 μm particle size, LiChrospher100 stationary phase, Merck) protected with a LiChroCART guard column (4 mm × 4 mm, RP-18, 5 μm particle size, Merck). The mobile phase consisted of a mixture of two solvents: water−acetic acid (1%) (A) and methanol (B). For studying both free flavonol glycosides and the corresponding acylated derivatives, a linear gradient, starting with 20% B, was performed to reach 50% 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 was 20 μL. Spectral data from all peaks were accumulated in the range 240−400 nm, and chromatograms were recorded at 330 nm for the glycosides and their acylated derivatives. The HPLC/UV-PAD/ESI-MSn analyses were carried out in an Agilent HPLC 1100 series equipped with a 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 software (Agilent, v. 08.03). The mass detector was a linear 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 up 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 up to 2 V. Mass spectrometry data were acquired in the negative ionization mode. MSn was carried out in the automatic mode on the more abundant fragment ion in MS(n-1).

RESULTS AND DISCUSSION

Host Plant Analysis. When studying the metabolism of host plant's compounds by *P. brassicae*, a good knowledge of the phytochemistry of external leaves is required. The composition of *B. oleracea* var. *costata* has been previously reported (*11*), but as a living organism, variation in chemical composition may occur. So, the leaves of the host plant used to feed *P. brassicae* were analyzed.

In the present study and under the conditions described in the Materials and Methods, the HPLC-PAD-MSn screening of the deacylated glycosides resulting from the saponification of the native extract (**Figures 1A** and **2** and **Table 1**) shows as main compounds kaempferol-3-*O*-sophoroside-7-*O*-glucoside (**3**) and kaempferol-3-*O*-triglucoside-7-*O*-glucoside (**2**) (*12*, *13*), which was in line with the observations previously reported in external leaves (*11*). Other glycosides found before and equally present in this study were kaempferol derivatives (**4**, **5**, **9**, and **10**), although in very low or trace amounts. Quercetin-3-*O*-sophoroside-7-*O*-glucoside (**1**) has been described before, in both internal (*14*) and external (*6*) leaves of tronchuda cabbage. Apart from this, we now found three kaempferol derivatives, in low amounts, which contained rhamnose, which is not common in *Brassica*: **6**

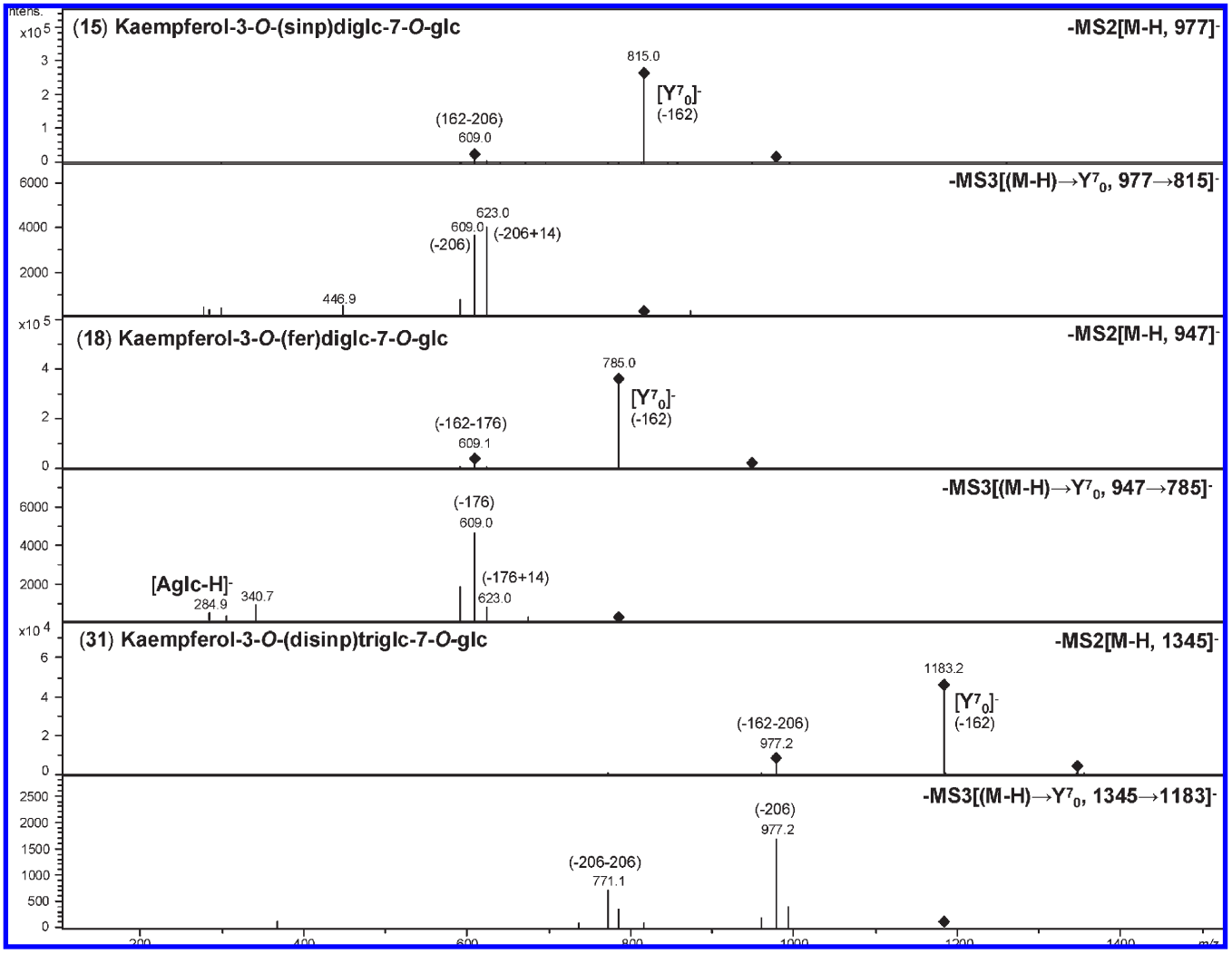

Figure 4. MS2 and MS3 spectra of some flavonoids acylated derivatives (**15**, **18**, and **31**). The identities of compounds are as in **Figure 1**.

(kaempferol-3-*O*-triglucoside-7-*O*-rhamnoglucoside), **7** (kaempferol-3-*O*-triglucoside-7-*O*-rhamnoside), and **8** (kaempferol-3-*O*-diglucoside-7-*O*-rhamnoside) (**Figure 3**). These kinds of compounds had been found before in watercress (*Nasturtium officinale* R. Br.) (*15*). In what concerns acylated flavonoid glycosides (**Figures 1B** and **4** and **Table 2**), the main compounds are related to the most abundant glycosides and are kaempferol-3-*O*-(sinapoyl)sophoroside-7-*O*-glucoside (**15**) and kaempferol-3-*O*-(feruloyl)sophoroside-7-*O*-glucoside (**18**). These compounds, which are important products observed in tronchuda cabbage internal leaves, coelute with kaempferol-3-*O*-(sinapoyl)triglucoside-7-*O*-glucoside (**14**) and kaempferol-3-*O*-(feruloyl)triglucoside-7-*O*-glucoside (**17**), respectively. These results, together with the presence of a diacylated derivative that elutes much later [R_t = 26.2, kaempferol-3-*O*-(disinapoyl)-triglucoside-7-*O*-glucoside (**31**)] suggest that the structures indicated for diacylated derivatives in external leaves were previously (*11*) wrongly attributed. In addition, these results allow us to establish that the composition of internal and external leaves is more similar among them than previously believed. Kaempferol-3-*O*-(caffeoyl)sophoroside-7-*O*-glucoside (**13**), kaempferol-3-O-(sinapoyl)sophoroside (**24**), and kaempferol-3-*O*-(feruloyl)-sophoroside (**25**) were detected and were reported before (*6*, *14*). Acylated glycoside derivatives with rhamnose in its structures were now identified for the first time in *B. oleracea* var. *costata*: kaempferol-3-*O*-(sinapoyl)triglucoside-7-*O*-rhamnoside (**21**), kaempferol-3-*O*-(sinapoyl)diglucoside-7-*O*-rhamnoside (**22**), and kaempferol-3-*O*-(feruloyl)diglucoside-7-*O*-rhamnoside (**23**). Other phenolics detected were hydroxycinnamic acids derivatives: 3-*p*-coumaroylquinic acid (**11**), 3-feruloylquinic acid (**12**), 4-*p*-coumaroylquinic acid (**16**), 5-*p*-coumaroylquinic acid (**19**), and 4-feruloylquinic acid (**20**) (*16*). Equally, three feruloylsinapoylgentiobioside isomer (**26**, **28**, and **30**) and three disinapoylgentiobioside isomers (**27**, **29**, and **32**) (*14*) were found.

Thus, as far as we know, from the 31 compounds noticed in *B. oleracea* var. *costata*, 12 compounds (**6**−**8**, **12**, **14**, **17**, **19**−**23**, and **31**) are reported for the first time in this matrix. The remaining ones have already been described (*6*, *8*, *11*, *14*).

***P. brassicae* Larvae and Excrements.** The HPLC-PAD chromatogram of the excrements' extract (**Figure 1C**) shows a set of peaks that are in line with the main deacylated glycosides observed in tronchuda cabbage saponified extract (**2**−**5**, **7**, and **8**), as well as ferulic and sinapic acids (**FA** and **SA**). On the other hand, it should be highlighted that the acyl derivatives of flavonols observed in the native extract were absent in excrements, whereas other acyl derivatives, which probably arose as a consequence of metabolism, were present (**33**−**36**) (**Figure 1C** and **Table 2**).

We previously established that sulfation is a metabolic process found in *P. brassicae* when fed with *B. rapa* var. *rapa* and *B. oleracea* var. *costata* (*6*, *8*). To confirm the presence of this kind of compounds in the excrements of the work herein, we extracted the ions that presented losses of 80 mass units

Table 2. R_t, −MS: $[M-H]^-$, −MS2$[M-H]^-$, and −MS3$[(M-H)\rightarrow Y_0^7)]^-$ Data of Acylated Glycosyl Flavonoids from Native Extract of *B. oleracea* var. *costata* Leaves (**13**−**15**, **17**, **18**, **21**−**25**, and **31**) from Excrements (**33**−**36**) and from *P. brassicae* Larvae (**37**)[a]

	compounds[b]	R_t (min)	$[M-H]^-$ (*m/z*)	−MS2$[M-H]^-$ (*m/z*) (%)			−MS3$[(M-H)\rightarrow Y_0^7]^-$ (*m/z*) (%)				
				kaempferol-3-*O*-(Acyl/diAcyl)-tri/diglucoside-7-*O*-glucoside							
				Y_0^{7-} (−162)	−162-Acyl+14	−162-Acyl	−Acyl+14	−Acyl	−diAcyl+14	−diAcyl	Aglc-2H/H
13	**3**-C	8.1	933	771 (100)		609 (55)		609 (100);			
14	**2**-S	10.2	1139	977 (100)		771 (6)		771 (100)			
15	**3**-S	10.2	977	815 (100)		609 (3)	623 (100)	609 (90)			
17	**2**-F	10.7	1109	947 (100)	785 (35)	771 (10)	785 (20)	771 (100)			
18	**3**-F	10.7	947	785 (100)		609 (5)	623 (16)	609 (100)			285 (10)
37	**3**-C isomer	13.7	933	771 (100)		609 (10)		609 (100)			285 (5)
34	**3**-F isomer	19.4	947	785 (100)		609 (3)	623 (90)	609 (100)			284 (7)
36	**2**-F isomer	24.9	1109	947 (100)			785 (100)	771 (30)			284 (17)
31	**2**-diS	26.3	1345	1183 (100)		977 (13)	991 (23)	977 (100)	785 (20)	771 (40)	
				kaempferol-3-*O*-(Acyl)-tri/diglucoside-7-*O*-diglucoside							
				Y_0^{7-} (−324)		−324-Acyl					
33	**5**-F	18.4	1109	785 (100)		609 (14)	623 (93)	609 (100)			285 (8)
35	**4**-F	23.7	1271	947 (100)			785 (100)	771 (23)			284 (8)
				kaempferol-3-*O*-(Acyl)-tri/diglucoside-7-*O*-rhamnoside							
				Y_0^{7-} (−146)		−146-Acyl	−Acyl+14	−Acyl			
21	**7**-S	15.6	1123	977 (100)		771 (11)	785 (20)	771 (100)			
22	**8**-S	16.0	961	815 (100)		609 (8)	623 (90)	609 (100)			285 (10)
23	**8**-F	17.1	931	785 (100)		609 (6)	623 (100)	609 (30)			285 (5)
				kaempferol-3-*O*-(Acyl)-tri/diglucoside							
							−MS2$[M-H]^-$ (*m/z*) (%)				
24	**9**-S	19.3	815				623 (95)	609 (100)			285 (10)
25	**9**-F	21.3	785				623 (100)	609 (85)			285 (6)

[a] Main observed fragments. Other ions were found, but they have not been included. [b] C, caffeoyl; S, sinapoyl; F, feruloyl; Aglc, aglycone; **2**, kaempferol-3-*O*-triglucoside-7-*O*-glucoside; **3**, kaempferol-3-*O*-sophoroside-7-*O*-glucoside; **4**, kaempferol-3-*O*-triglucoside-7-*O*-diglucoside; **5**, kaempferol-3-*O*-diglucoside-7-*O*-diglucoside; **7**, kaempferol-3-*O*-triglucoside-7-*O*-rhamnoside; **8**, kaempferol-3-*O*-sophoroside-7-*O*-rhamnoside; and **9**, kaempferol-3-*O*-sophoroside.

("constant neutral loss chromatogram"), but no sulfated compounds were found. The interest in sulfated conjugates arises from the biological activities observed before for this kind of compounds, like antioxidants or anticoagulants (*17*, *18*). Thus, their presence in excrements would contribute to increase the biological potential of this matrix.

In other works in which *P. brassicae* was fed with other *Brassica* species, such as *B. rapa* var. *rapa* (*8*, *10*) and *B. oleracea* var. *acephala* (*7*), the most abundant flavonoid in excrements was kaempferol-3-*O*-sophoroside, as a result of deglycosylation in the 7-position and deacylations (of sugars in the 3-position). However, in the study herein, this metabolic transformation was not as marked as in previous studies, as this compound was found in larvae but not in excrements, which indicates that it was converted into other compounds or accumulated in the insect's body. One possible explanation for the absence of this compound may be related to amounts of kaempferol-3-*O*-sophoroside or its derivatives in the leaves of the host plant. In the leaves of turnip (*10*) and kale (*7*), this compound and its acylated derivatives were found. Maybe in the amounts present in those plants, the mechanisms by which the insect accumulates these compounds were saturated and the compound "leaked" to the excrements. In *B. oleracea* var. *costata* leaves, however, perhaps a lower quantity of kaempferol-3-*O*-sophoroside, or of the acylated compounds that could originate it, were present; under those conditions, full accumulation takes place, and the compound cannot be found in excrements. Overall, in this study, it was observed that the flavonoids present in excrements are deacylation products and originate a chromatographic profile quite similar to the saponified extract of tronchuda cabbage external leaves (**Figure 1**).

The HPLC-PAD-MSn study of the phenolics present in larvae extracts at different time points (1, 2, 4, 6, and 8 h, **Figure 5**) revealed that at 1 h starvation, the major compounds were kaempferol-3-*O*-sophoroside-7-*O*-glucoside (**3**), FA and SA, and kaempferol-3-*O*-sophoroside (**9**), which results from deacylation and deglycosylation in position 7. Compound **3** was present in the native extract, in both free and acylated forms, and **9** was found in trace amounts. Other flavonoids that were also present in low or trace amounts, being equally found in the native extract, are the deacylated **1**, **2**, **5**, and **10**, and the acylated **14**, **15**, **17**, and **18**.

Compound **39** (quercetin-3-*O*-sophoroside) is the product of deglycosylation of **1** at position 7 (**Figure 6**). Other products of metabolism are **37**, kaempferol-3-*O*-(caffeoyl)sophoroside-7-*O*-glucoside (isomer of **13**), and **38**, quercetin-3-*O*-sophoroside sulfate (R_t = 13.8 min; −MS: 689 $[M-H]^-$, −MS2$[M-H]$: 609).

Phenolics Metabolism in *P. brassicae* Larva: Overview. In a general way, deacylation was the main metabolic reaction that took place. Seven compounds were present in the insect and absent in the leaves of *B. oleracea* var. *costata*: FA, SA, kaempferol-3-*O*-glucoside (**10**), kaempferol-3-*O*-(feruloyl)sophoroside-7-*O*-glucoside isomer (**34**), kaempferol-3-*O*-(caffeoyl)-sophoroside-7-*O*-glucoside (**37**), kaempferol-3-*O*-sophoroside sulfate (**38**), and quercetin-3-*O*-sophoroside (**39**). Five of these compounds are flavonoid derivatives, and because of the incapability of insects to synthesize this class of compounds (*19*), they must arise from the metabolism of compounds that do exist in the plant. **Figure 6** shows some reactions that could occur for these compounds. For instance, quercetin-3-*O*-sophoroside (**39**) is a compound that could be found in most of the analyzed starvation

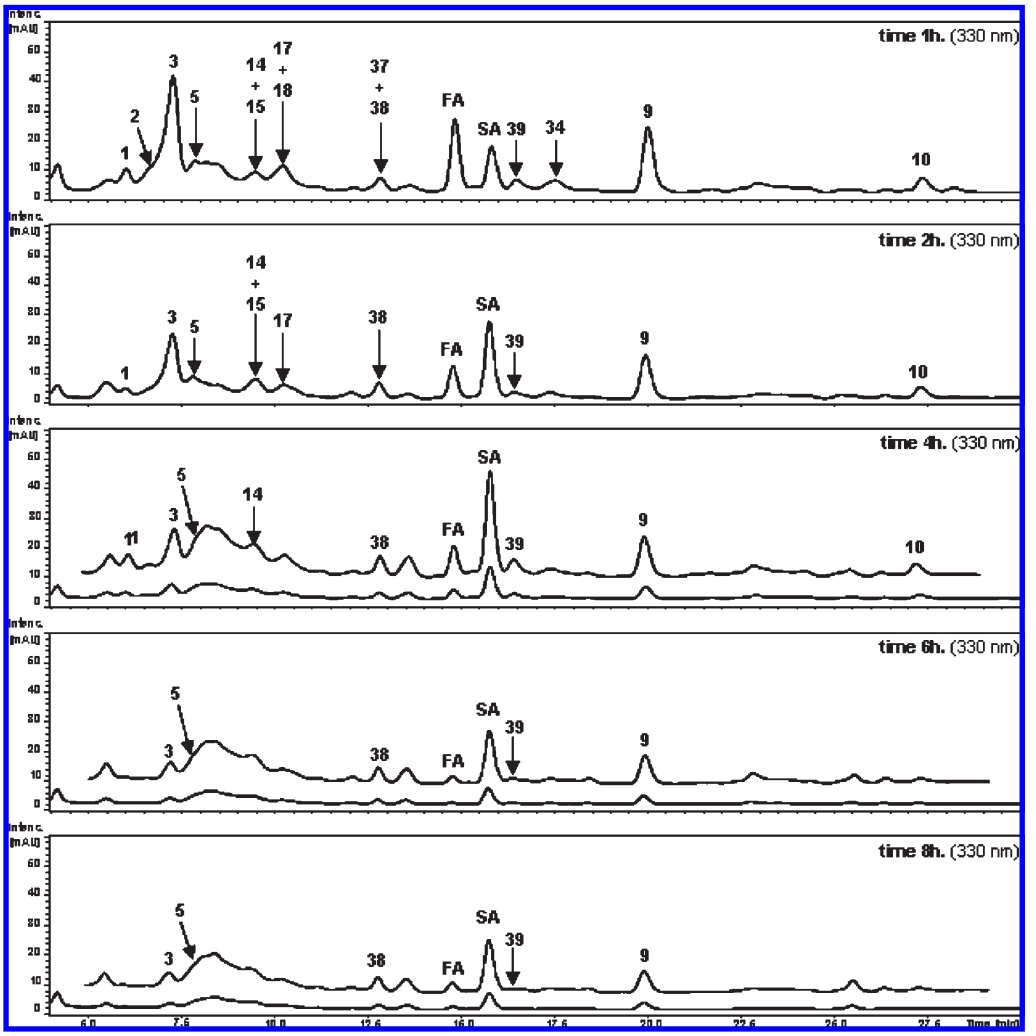

Figure 5. HPLC-PAD phenolic profile of *P. brassicae* larva. Detection at 330 nm. Peaks: 37, kaempferol-3-*O*-(caffeoyl)sophoroside-7-*O*-glucoside; 38, kaempferol-3-*O*-sophoroside sulfate; and 39, quercetin-3-*O*-sophoroside. For other peaks, see **Figure 1**.

periods, although absent in tronchuda cabbage. However, if we consider that quercetin-3-*O*-sophoroside-7-*O*-glucoside (**1**) was detected in the leaves, it is highly probable that a deglycosylation process occurred, which may also happen with kaempferol-3-*O*-sophoroside-7-*O*-glucoside (**3**), contributing to the presence of kaempferol-3-*O*-sophoroside (**9**) in the larvae. Likewise, ferulic and sinapic acids were among the major compounds in *P. brassicae*, although they were not found in the host plant. These compounds could have originated by deacylation of the acylated flavonols present in *B. oleracea* var. *costata*, as it is supported by the increasing amount of SA between 1 and 8 h after feeding, when digestion was taking place.

An interesting case is that of kaempferol-3-*O*-sophorose-sulfate (**38**), which was found in the insect at all times after feeding. As this compound was not found in tronchuda cabbage, the possibility of it coming from the diet is rejected. In this situation, kaempferol-3-*O*-sophoroside (**9**) present in the leaves of the plant or accumulated in the insect's body is the probable precursor (**Figure 6**). We have previously reported that sulfation is one of the metabolic processes that take place in *P. brassicae*. In a previous work by our group where *P. brassicae* was fed with *B. oleracea* var. *acephala* (kale) (*7*), of which *B. oleracea* var. *costata* is taxonomically related, three sulfated compounds had been found, kaempferol-3-*O*-sophoroside-sulfate, quercetin-3-*O*-glucoside-sulfate, and kaempferol-3-*O*-glucoside-sulfate. Thus, when insects fed upon these two *B. oleracea* varieties, at least one common sulfated metabolite was found. However, when *P. brassicae* was fed with *B. rapa* var. *rapa* (turnip) (*8*), the sulfated metabolite was isorhamnetin-3,7-di-*O*-glucoside. *B. oleracea* var. *costata* and *B. oleracea* var. *acephala* are, from a phytochemical point of view, much more similar between them than when compared with turnip. The results obtained reinforce the importance of the composition of the plant material in shaping the metabolic profile that arises upon feeding, being highly dependent on host plant.

From the 31 compounds identified in the host plant, 15 could not be detected in the insect at any time. Given the fact that they were, nevertheless, ingested, degradation or transformation during their metabolism is the only explanation for their absence in larvae. For instance, feruloylsinapoylgentiobioside (**26**), disinapoylgentiobiosides (**27**), and their isomers (**28**−**30**) were considerable peaks in the native extract of leaves but could not be found in the insect. As so, they had to be metabolized into other compounds. In addition, the feruloyl and sinapoyl moieties that are produced as a consequence of deacylation would contribute to the appearance of FA and SA, detected in the insect but not in the plant. A remarkable exception for the deacylation of acylated derivatives seems to be the case of *p*-coumaroyl derivatives. 3-*p*-Coumaroylquinc, 4-*p*-coumaroylquinic, and 5-*p*-coumaroylquinic acids were also present in the leaves of the host plant but were not detected in the insect or its excrements at any time. The hydrolysis of these compounds would originate *p*-coumaric acid

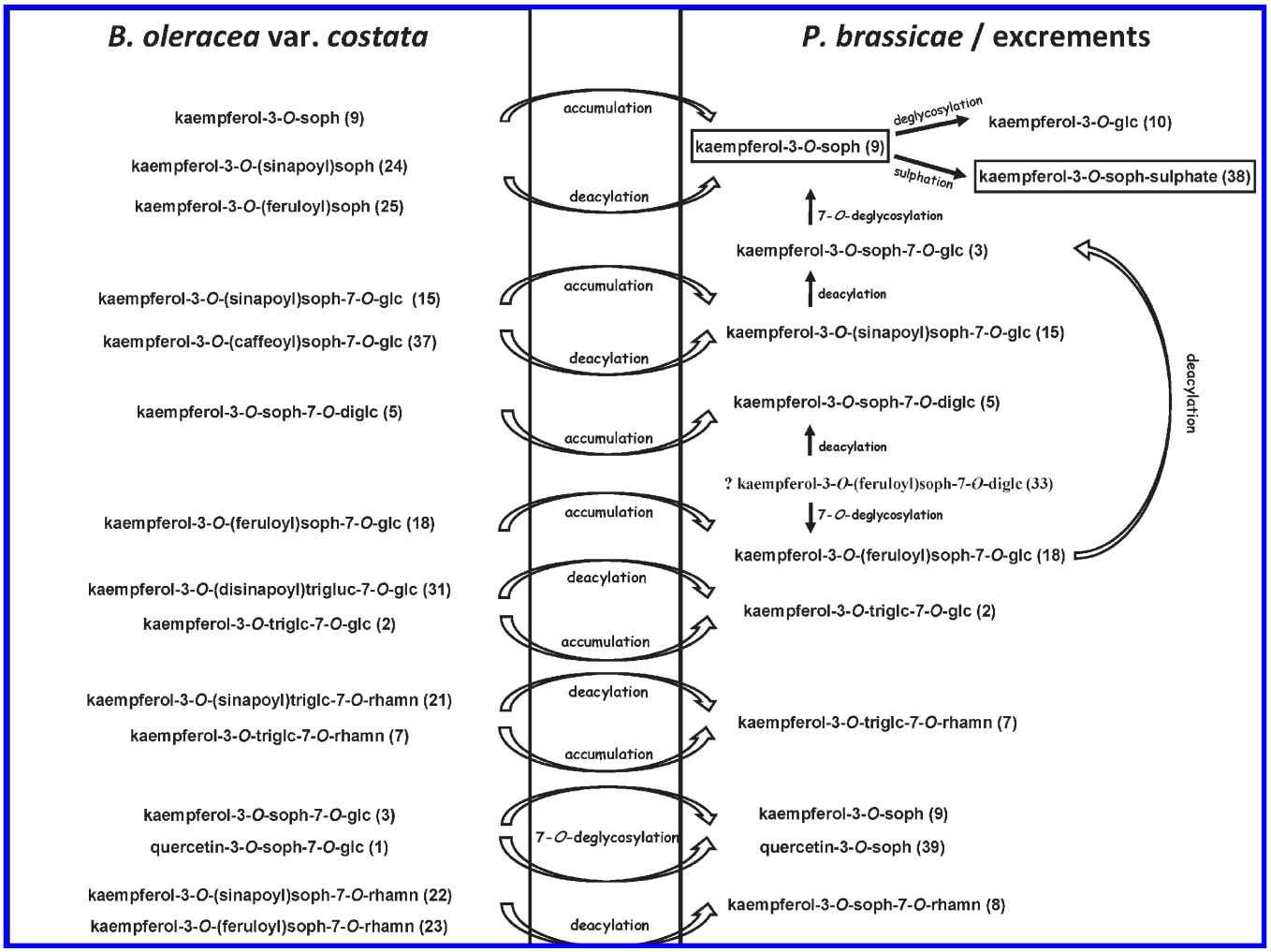

Figure 6. Possible metabolic reactions taking place in *P. brassicae*. "?" means that the origin of the compound is not known.

and quinic acid, in the same way that sinapoyl/feruloyl-gentiobiosides or sinapoyl/feruloyl acylated flavonoids originate free SA/FA and gentiobiosides/deacylated flavonoids; however, no *p*-coumaric acid was detected. This apparent dislike, by the insect, of *p*-coumaric acid was already described; when *P. brassicae* was fed with *B. rapa* var. *rapa*, which contained high *p*-coumaric acid levels (*10*), this compound was not found in either the insect's body or the excrements. Thus, *p*-coumaric acid metabolism must occur to a longer extension, being completely modified or destroyed. Another possibility was that *p*-coumaric acid was completely used to form quinones, as was described before in the autumnal moth *Epirrita autumnata* (*20*). We looked for the fragmentation patterns of quinones, and their absence was confirmed. With these results, the fate of *p*-coumaric acid in *P. brassicae* remains unknown, and further studies are required.

Among the 16 compounds found in the insect body (FA, **SA**, **1**−**3**, **5**, **9**, **10**, **14**, **15**, **17**, **18**, **34**, and **37**−**39**), six were equally found in the excrements: FA, SA, kaempferol-3-*O*-triglucoside-7-*O*-glucoside (**2**), kaempferol-3-*O*-sophoroside-7-*O*-glucoside (**3**), kaempferol-3-*O*-sophoroside-7-*O*-diglucoside (**5**), and kaempferol-3-*O*-(feruloyl)sophoroside-7-*O*-glucoside isomer (**34**). The 10 remaining compounds, absent in the excrements, were either accumulated or transformed into different molecules. In fact, the analysis of an insect starved for 8 h after feeding (**Figure 5**) revealed that the compounds are accumulated as follows: kaempferol-3-*O*-sophoroside-7-*O*-glucoside (**3**), kaempferol-3-*O*-sophoroside-7-*O*-diglucoside (**5**), FA, SA, kaempferol-3-*O*-sophoroside (**9**), kaempferol-3-*O*-sophoroside-sulfate (**38**), and quercetin-3-*O*-sophoroside (**39**).

If we take into account the changes registered through time, some patterns may be recognized. Kaempferol-3-*O*-sophoroside-7-*O*-glucoside (**3**) decreased between 1 and 8 h of starvation. Most probably, this compound underwent a deglycosylation at the 7-position, a process that is characteristic of *P. brassicae*, thus originating kaempferol-3-*O*-sophoroside (**9**). Contrary to what would be expected, the amounts of kaempferol-3-*O*-sophoroside did not increase accordingly. This may be explained, at least partially, by the fact that this compound was being consumed in the sulfation process that yields kaempferol-3-*O*-sophoroside-sulfate (**38**), which seems to slightly increase during this time range. Another compound that suffered an increase was FA. FA, which did not exist in *B. oleracea* var. *costata*, probably originated by the deacylation of the several acylated compounds that exist in the host plant. This compound was also present in larvae starved for 8 h, indicating that it is accumulated.

As this work constitutes the first insight on the time changes in phenolic contents in *P. brassicae* after feeding, no comparisons can be made regarding the qualitative and quantitative changes through time. However, some studies have analyzed the composition of *P. brassicae* after a 12 h starvation. In the insects fed with *B. rapa* var. *rapa* (*10*), the major compounds were ferulic and sinapic acids and the flavonoid in higher amounts was kaempferol-3-*O*-sophoroside. As for the excrements, ferulic and sinapic acids were, again, the main compounds, and the most expressive

flavonoid was isorhamnetin-3,7-di-*O*-sulfate. A major difference is that in the excrements of *P. brassicae* fed with turnip, only six compounds could be identified and quantified. On the other hand, in the study herein, 12 compounds were successfully identified in excrements. This difference can reflect the different composition of the host plants, as the analysis of *B. rapa* var. *rapa* led to the identification of 19 compounds, while in *B. oleracea* var. *costata*, 31 compounds were identified. A higher number of phytochemicals is thought to originate a higher number of metabolites. This is supported by the results obtained by feeding *P. brassicae* with kale (*7*). In kale, where 31 compounds were identified, the number of compounds in excrements, 25, was also higher than in the study with *B. rapa* var. *rapa*. In the study with kale, kaempferol-3-*O*-sophoroside was the major flavonoid in excrements, while in the work presented here, it was absent, although one of its derivatives, kaempferol-3-*O*-sophoroside-7-*O*-glucoside (**3**), was a major peak.

In conclusion, we proved a selective uptake of flavonoids from *P. brassicae* food source, as well as their bioconversion by the larvae. Additionally, as *P. brassicae* larvae act like a chemistry laboratory, it may provide compounds with different bioactivities from their host plant. The findings described herein may constitute an economic advantage for producers as the losses caused by this pest can be countered by the exploration of the insects' bioactive compounds.

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