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Sequestration and distribution of flavonoids in the common blue butterfly *Polyommatus icarus* reared on *Trifolium repens*

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Abstract

Larvae of *Polyommatus icarus* Rott. were reared on inflorescences of *Trifolium repens*. The three main flavonoid components of flower extracts, quercetin-3-*O*-galactoside, its 6″-*O*-acetyl derivative and myricetin-3-*O*-galactoside were isolated and identified by spectroscopic means. Individual extracts from larvae, pupae and imagines as well as extracts from exuviae, larval faeces and the host plant were analyzed by HPLC. Quercetin-3-*O*-galactoside was found to be the main component in *P. icarus*. Apart from this, only one additional plant flavonoid, tentatively identified as a kaempferol derivative on the basis of its online UV-spectrum, was also present in all developmental stages of *P. icarus*. Particularly in larvae the high percentage of flavonoids not detectable in extracts of *T. repens* suggested that *P. icarus* is able to metabolize dietary flavonoids. Dietary myricetin-3-*O*-galactoside was found to be selectively excreted. The flavonoid content of *P. icarus* correlated with the insects' dry weight and sex. Females contained about twice the flavonoid content of male imagines. This sex difference was significant both with regard to flavonoid amounts and concentrations and is considered to support the idea of flavonoids contributing to wing pigmentation and playing a role in intraspecific visual communication. Further support for this hypothesis comes from the analysis of dissected imagines revealing a distinct flavonoid distribution within the butterflies: 77% of the total flavonoid content of individuals analysed in this study were found to be located in the wings. Furthermore hindwings contained more flavonoids than forewings. Results are discussed in the context of earlier studies concerning *P. icarus* reared on three different fabaceous plants.

Keywords: *Polyommatus icarus*; Lycaenidae; Common blue butterfly; *Trifolium repens*; Fabaceae; White clover; Plant–insect interaction; Wing pigmentation; Flavonoids

1. Introduction

Herbivores continuously take up secondary plant metabolites with their food. A number of butterflies and some grasshopper species (Bernays, Howard, Champagne, & Estesen, 1991; Hopkins, & Ahmad, 1991) are known to sequester dietary flavonoids. Thomson (1926) was the first to describe the occurrence of flavonoids in butterflies after examining the wings of *Melanargia galathea*. Simple colour tests (Ford, 1941) and paper chromatograms (Wilson, 1985b, 1986b, 1987) revealed that species storing these UV-absorbing polyphenols are quite common among the diurnal Lepidoptera. Flavonoids are most frequently found in species belonging to the Papilionidae, Lycaenidae and Nymphalidae. Feeding experiments proved the dietary origin of the flavonoids sequestered (Wilson, 1985a; Wiesen, Krug, Fiedler, Wray, & Proksch, 1994; Burghardt, Fiedler, & Proksch, 1995). Ford (1941) suggested that flavonoids might contribute to the wing pigmentation of dayflying species. Analytical, physiological and behavioural experiments with different butterfly species confirmed the importance of UV-absorbing and UV-reflecting structures with regard to species recognition and mate selection

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(Bernard, & Remington, 1991; Meyer-Rochow, 1991; Brunton, & Majerus, 1995). However, the specific impact of flavonoids still has to be determined in this context.

Earlier studies (Feltwell, & Valadon, 1970; Wilson, 1987) on the occurrence of flavonoids in the common blue butterfly *P. icarus* were restricted to wild-caught butterflies with an unknown feeding history. As larvae of *P. icarus* are known to feed on a number of mostly herbaceous Fabaceae (Martin Cano, 1984) any comparison of plant and insect flavonoid patterns was difficult or impossible. More recent studies were conducted on individuals of *P. icarus* reared on inflorescences of five different natural host plants and on plants or plant parts not known to be used by *P. icarus* in the field (Wiesen et al., 1994; Burghardt, Fiedler, & Proksch, 1997a; Burghardt, Fiedler, & Proksch, 1997b). HPLC-analysis of these individuals showed that *P. icarus* accumulates varying amounts of dietary flavonoids depending on the ingested food plant and the sex of the butterflies.

This investigation of individuals reared on *Trifolium repens* further extends these studies on the interaction of the moderately polyphagous species *P. icarus* with its various fabaceous host plants. It contributes to the understanding of the accumulation process and the type and function of flavonoids sequestered by *P. icarus*.

2. Results and discussion

2.1. T. repens flavonoids

For a comparative analysis of insect and host plant flavonoid content it is necessary to know on which parts of the plant the larvae feed as the floral organs of *T. repens* were found to show distinct differences with regard to flavonoid quantities and identities (data not shown). Larvae were observed to feed only negligibly on sepals: the majority (64%) of 140 flowers examined had intact sepals. Sepals of the remaining flowers (36%) were only marginally damaged. Also petals were not ingested completely under laboratory conditions when food was present in excess. Unfortunately it was impossible to quantify the relative amounts of ingested petals, stamens and carpels. In the following the flavonoid pattern of sepal-free flower extracts is therefore considered to represent what larvae ingested and is referred to as the flower pattern (Fig. 1). The three main components in the flower pattern were identified as quercetin-3-*O*-galactoside (**3**), quercetin-3-*O*-(6″-*O*-acetyl)-galactoside (**5**) and myricetin-3-*O*-galactoside (**2**). The occurrence of the acetylated quercetin-3-*O*-galactoside has not been described for *T. repens* to date. Fourteen other HPLC-peaks showed UV-absorption patterns typical for flavonoid structures. Two of these minor flower components were tentatively identified as kaempferol derivatives (**4** and **6**) and two as isoflavonoids (**1** and **8**). By coelution (DC and HPLC) with the commercially available reference substance one peak was identified as the aglycon quercetin (**7**). Total flavonoid content of sepal-free flowers was 22.01 ± 0.26 μg/mg dry weight (three replicate injections of a pooled flower extract).

2.2. Accumulation of flavonoids in P. icarus

The flavonoid content of *P. icarus* reared on *T. repens* inflorescences correlated with the dry weight of the individuals analysed (larvae and pupae: $r_s = 0.76$, $p < 0.001$; male imagines: $r_s = 0.79$, $p < 0.0004$; female imagines: $r_s = 0.83$, $p < 0.003$). The average content of female imagines (59.04 μg, $n = 9$) was about twice that of male imagines (30.96 μg, $n = 14$). Apart from flavonoid amounts, flavonoid concentrations increased in the course of development which documents a process of enrichment in the insects' bodies (Table 1). Flavonoid concentrations in adult *P. icarus* butterflies are among the highest recorded so far and are only surpassed by conspecies reared on the related clover species *Trifolium pratense* (Burghardt et al., 1997b).

Only two plant flavonoids, quercetin-3-*O*-galactoside (**3**) and one of the kaempferol derivatives (**4**) were also detected in every insect extract (Fig. 1, Table 2). The kaempferol derivative (**4**) does not account for more than 5% of the total flavonoid content in any plant or insect sample. Quercetin-3-*O*-galactoside was the major flavonoid in insects and flowers. In *P. icarus* its percentage increased in the course of development from 43% in fourth instar larvae to 73% in pupae and 78% in imagines. Except for the presence of free quercetin (**7**) in pupal extracts, flavonoid patterns of pupae and imagines were very similar. The occurrence of a third kaempferol derivative (**9**) was restricted to pupae and imagines and accounted for about 5% of their total flavonoid content. The flavonoid pattern of fourth instar larvae differed qualitatively from those of pupae and imagines. The three flavonoids **10**, **11** and **12** were not found in *T. repens* but represented 11, 12 and 14% of the total larval flavonoid content, respectively. The occurrence of compound **12**, a quercetin derivative and five trace components was limited to larvae. Compound **11**, also a quercetin derivative, accounted for 10% of the total flavonoid content in pupae. The percentage in imagines was sex-dependent. In females it represented 9%, in males only 2% of the total flavonoid content (Table 2).

Flavonoid patterns of *P. icarus* and other lycaenid species are usually chemically more simple compared to those of the respective host plants. In adult butter-

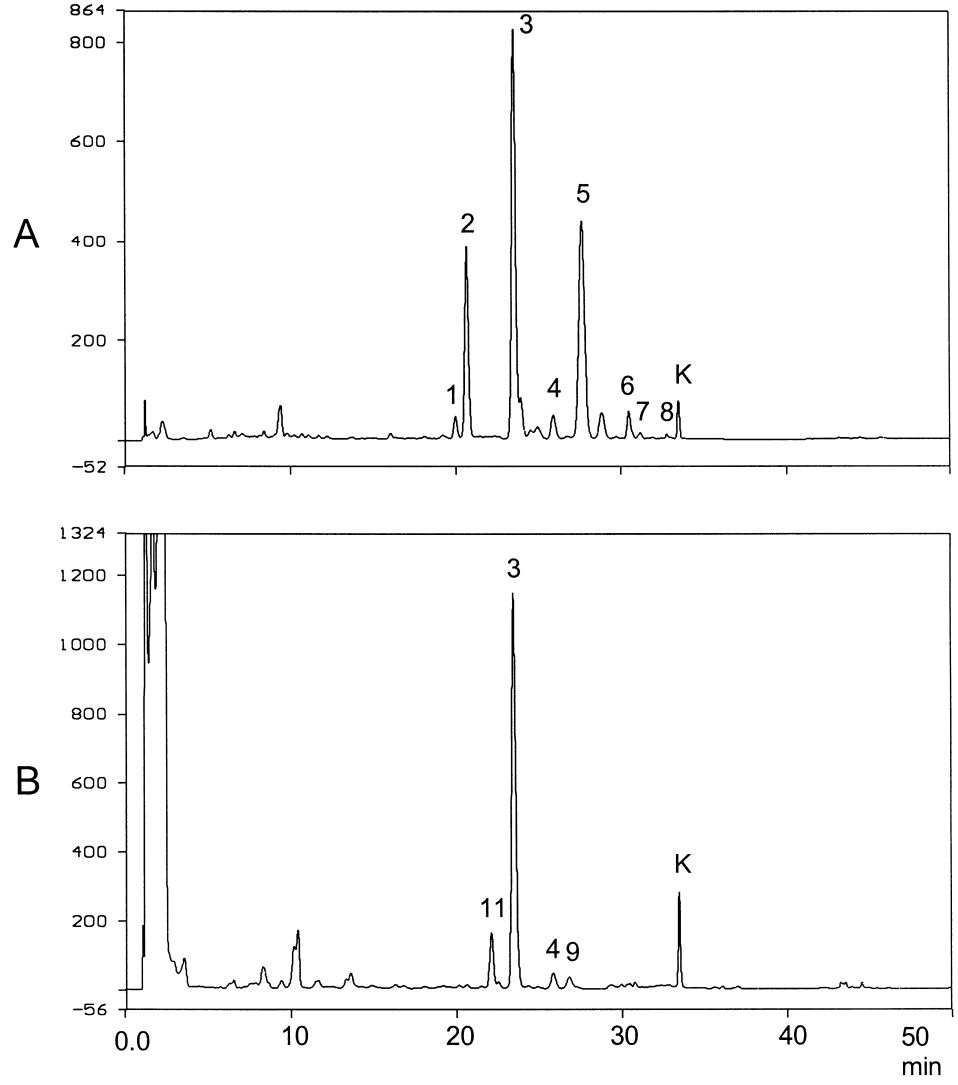

Fig. 1. Representative HPLC-chromatograms of methanolic extracts. Peaks were detected at 254 nm. (A) Sepal free flowers, (B) female butterfly of *P. icarus* reared on *Trifolium repens*. (K) Internal standard kaempferol, (1) isoflavonoid, (2) myricetin-3-*O*-galactoside, (3) quercetin-3-*O*-galactoside, (4) kaempferol-derivative, (5) quercetin-3-*O*-(6″-O-acetyl)-galactoside, (6) kaempferol derivative, (7) quercetin, (8) isoflavonoid, kaempferol derivative, (9) kaempferol derivative, (11) quercetin derivative.

Table 1
Weights, forewing lengths and flavonoid amounts of female and male imagines. Means are followed by the standard error. Statistics were performed using the two-sided *t*-test and two-sided Mann–Whitney *U*-test

Developmental stage	*n*	Dry weight (mg)	Forewing length (mm)	Flavonoid content (μg)	Flavonoid concentration (μg × mg^{-1} dry wt)
Third instar larvae	5	2.00 ± 0.13		0.99 ± 0.30	0.48 ± 0.12
Early fourth instar larvae	5	5.67 ± 0.34		12.52 ± 2.33	2.18 ± 0.32
Late fourth instar larvae	3	13.36 ± 1.29		44.42 ± 10.69	3.33 ± 0.82
Prepupae	3	13.32 ± 0.66		38.90 ± 9.43	3.01 ± 0.86
Pupae	5	12.50 ± 0.99		38.02 ± 5.44	3.15 ± 0.64
Female imagines	9	9.80 ± 0.78	14.53 ± 0.38	59.04 ± 7.43	5.85 ± 0.40
Male imagines	14	8.68 ± 0.25	15.18 ± 0.15	30.96 ± 3.20	3.49 ± 0.28
Significance of sex difference		$t = 0.817$, $p > 0.43$	$U = 38$, $Z = 1.576$, $p > 0.11$	$U = 17$, $Z = 2.898$, $p < 0.004$	$U = 9$, $Z = 3.403$, $p < 0.0007$

Table 2
Percentages of flavonoid compounds and total concentrations in plants, faeces and all developmental stages of *P. icarus*. The pattern of larvae represents the average of early fourth instar to prepupal stage caterpillars. Means are followed by the standard error

Component	Sepal free flowers, $n=3$	Faeces, $n=5$	Larvae, $n=9$	Pupae, $n=5$	Male imagines, $n=10$	Female imagines, $n=7$
(**2**) Myricetin-3-*O*-galactoside	17.1±0.4	23.6±2.0	minor component in 2 larvae			
(**3**) Quercetin-3-*O*-galactoside	38.1±0.4	52.1±1.6	42.6±3.9	73.1±5.4	79.3±0.4	76.0±1.9
(**4**) Kaempferol derivative	2.9±0.006	5.1±0.9	2.7±0.4	2.8±0.4	1.2±0.4	2.0±0.2
(**5**) Quercetin-3-*O*-(6″-*O*-acetyl)-galactoside	30.9±0.2	10.1±0.9	minor component in 5 larvae			
(**7**) Quercetin	minor component			5.5±2.1		
(**9**) Kaempferol derivative				3.0±0.6	6.6±0.7	4.2±0.3
(**10**) Flavonoid			11.1±2.2		minor component	minor component
(**11**) Quercetin derivative			12.2±2.6	10.0±4.8	2.1±0.6	9.3±1.6
(**12**) Quercetin derivative			14.1±2.4			
Minor components (n)	11.1±0.2 (13)	9.1±1.0 (8)	17.3±2.5 (7)	5.6±1.1 (3)	10.8±0.8	8.5±0.8
Total flavonoid concentration	22.01±0.15	9.08±0.99	2.84±0.4	3.15±0.49	3.80±0.33	6.26±0.38

flies one or two flavonoids usually represent at least 80% of the total flavonoid content whereas the flavonoid patterns of host plants are more diverse (Wilson, 1986a; Wiesen et al., 1994; Burghardt et al., 1997a; Geuder, Wray, Fiedler, & Proksch, 1997). Average quercetin-3-*O*-galactoside content of *P. icarus* imagines reared on *T. repens* accounted for 78% of their total flavonoid content, thus confirming the predominance of one major compound. In contrast to preceding studies, however, the number of minor components detectable in the butterfly extracts (12.4 ± 2.0 with a minimum of 5 minor components per butterfly) was about the same as in the larval food plant. This increase in the detection of minor components might reflect the improved sensitivity of HPLC techniques.

Flavonoids which do not occur in the larval food plants had been reported for *P. icarus* before. They had been detected in samples of individuals reared on inflorescences of *Medicago sativa*, *Coronilla varia* and *Vicia villosa* (Wiesen et al., 1994; Burghardt et al., 1997a) as well as in extracts of the related species *Polyommatus bellargus* reared on leaves of *Coronilla varia* (Geuder et al., 1997). The same is true for *Aricia agestis* feeding on *Geranium molle* leaves (Proksch et al., unpublished results) and *M. galathea* (Wilson, 1985b).

Differences between plant and insect flavonoid patterns and developmental changes in the flavonoid patterns of butterflies might be due to selective mechanisms (excretion and sequestration) as well as biotransformation reactions by the insects or their gut flora (Wilson, 1985b; Wiesen et al., 1994). Larvae of *P. icarus* feeding on an artificial diet were shown to be able to glycosylate flavonol aglycones (Wiesen et al., 1994). In *P. bellargus* flavonols are considered to enzymatically derive from flavones (Geuder et al., 1997) and in *M. galathea* a 4′-hydroxy-glycosyl-transferase is thought to be responsible for the occurrence of tricin 4′-glycoside, which could not be detected in the larval food plant (Harborne, 1991).

Apart from a few trace components, the flavonoid pattern of larval faeces and sepal-free flowers differed only with regard to relative flavonoid amounts (Fig. 1 and Table 2). In the faeces a lower percentage of quercetin-3-*O*-(6″-acetyl)-galactoside (**5**) was accompanied by a higher percentage of quercetin-3-*O*-galactoside (**3**) and myricetin-3-*O*-galactoside (**2**). Acylated flavonoids might easily be hydrolysed by esterases in the insect's gut (Ahmad, Brattsten, Mullin, & Yu, 1986). Floral myricetin-3-*O*-galactoside (**2**) was selectively excreted by the larvae of *P. icarus*. The same was true for myricetin derivatives ingested by individuals reared on other food plants (Wiesen et al., 1994; Burghardt et al., 1997a). The higher polarity and reactivity of myricetin compared to quercetin and kaempferol might be important in this context. Myricetin was shown to be

more toxic to *P. icarus* than quercetin and kaempferol (Otterbeck, unpublished data).

2.3. Flavonoid distribution in P. icarus and putative flavonoid functions

The difference between males and females was statistically significant with regard to both flavonoid amounts and flavonoid concentrations though sexes did not differ significantly with regard to weight and forewing size Table 1. Females contained 67% higher flavonoid concentrations than males. This difference is larger than the difference between males and females of *P. icarus* that were reared on any other host plant investigated so far (Wiesen et al., 1994; Burghardt et al., 1997a; Burghardt et al., 1997b). Up to now an even greater sex difference has only been reported for the related lycaenid butterfly species *P. bellargus* (Geuder et al., 1997). The marked difference between males and females supports the idea of flavonoids playing a role in intraspecific visual communication.

Flavonoids are predominantly accumulated in the wings of butterflies, where they might contribute to wing pigmentation. Generally 70 to 80% of the total flavonoid content seem to be located in the wings (Wilson, 1986a; Wiesen et al., 1994; Geuder et al., 1997). This distribution was confirmed by our investigation of *P. icarus*. In males reared on *T. repens* approximately 80% of all flavonoids were found in the wings whereas in females 70% of the total flavonoid was confined to the wings, although the wings accounted for only 26 and 20% of the total body dry weight, respectively.

In addition analysis of dissected male and female butterflies showed that the amount of flavonoids per area was larger in the hindwings than in the forewings. Interestingly this difference is more pronounced in males (3.4 times more flavonoids per area in the hindwings) than in females (1.7 times more). The sex difference was more pronounced in the forewings (2.7 times more flavonoids per area in females than in males) than in the hindwings (1.3 times more).

Sequestered flavonoids might also function as UV-protection pigments in insects as they do in plants (Lois, & Buchanan, 1994; Reuberr, Bornman, & Weissenboeck, 1996). This has been suggested for flavonoids present in the reproductive tissues of female *M. galathea* butterflies as well as for the flavonoids occurring in the eggs of this species (Wilson, 1985b, 1986a). Eggs of *P. icarus* have not been analysed for flavonoids yet. Flavonoid concentrations in the abdomina (1.88 μg/mg dry wt) of dissected female imagines reared on *T. repens* were 4.5 times higher than in the head–thorax complex of these females and twice as high as the concentrations in the bodies of males. The significance of this distribution pattern remains uncertain as ovaries are likely to be sufficiently protected within the females' bodies and oviposition sites are usually not exposed to UV-radiation but shaded by the host plant tissue.

Moreover, due to their antibiotic properties flavonoids were suggested to protect insects against infections Wilson, 1986a). Increased resistance of *P. icarus* on a quercetin-containing artificial diet compared to flavonoid-free diet has indeed been reported (Otterbeck, unpublished data), but exuviae of *P. icarus* examined in this study contained extremely small amounts of flavonoids (0.4–0.8 μg/mg dry wt). Retention of flavonoids through moulting supports a continuous flavonoid enrichment process until butterfly eclosion and underlines the significance of flavonoids present in imagines.

2.4. Variability of flavonoid patterns in P. icarus when grown on different host plants

The relative importance of flavonoid identity compared to flavonoid amounts found in plant and insect samples is hard to evaluate. Irrespective of the larval host plant all flavonoids identified in extracts of *P. icarus* so far are flavonols having similar UV-absorption spectra. Therefore visual pigmentation patterns probably differ mostly with regard to their intensity depending on the amounts of flavonoids sequestered. That is why flavonoid quantity is thought to be more important than flavonoid patterns with regard to flavonoid function (Burghardt et al., 1997a). This might not be true for the physiology of flavonoid uptake and sequestration by *P. icarus*, especially as the ability to metabolize dietary flavonoids seems to be limited: the flavonoid pattern of *P. icarus* is obviously dependent on the identity of the host plant's main compounds (this study, Wiesen et al., 1994; Burghardt et al., 1995). Feeding on a given host plant, larvae selectively sequester only some of the flavonoid compounds ingested. Physiological limitations might explain the fact that high flavonoid concentrations in the plant do not necessarily result in high flavonoid concentrations in *P. icarus* and vice versa (this study compared to Wiesen et al. (1994) and Burghardt et al. (1997a)). This might also be due to plant chemistry effects on the insect's feeding behaviour and therefore flavonoid uptake rate. For example, quercetin was reported to stimulate feeding of *P. icarus* larvae (Otterbeck, unpublished data).

This study extends our understanding of flavonoid variability in the herbivore *P. icarus*. Besides reporting an even larger sex difference than in any previous study on *P. icarus* we also found qualitative differences. For the first time a quercetin derivative is reported to represent the major flavonoid in *P. icarus*, even if quercetin derivatives have been described to

occur along with kaempferol derivatives earlier (Feltwell, & Valadon, 1970; Wilson, 1987; Wiesen et al., 1994; Burghardt et al., 1997a). Hence, quantitative as well as qualitative variation in the flavonoid load of adult butterflies must be taken into account when attempting to analyse the behavioural or ecological function of these phenolic pigments in lycaenid butterflies.

3. Experimental

3.1. T. repens flavonoids

Inflorescences for rearing *P. icarus* as well as for flavonoid isolation were harvested on Würzburg University campus in July and August 1996. Extraction was carried out with 90% aq. MeOH after grinding the freeze dried material. This extract was first partitioned against petroleum ether, then, after MeOH evaporation, between H_2O and EtOAc. Flavonoids accumulated in the EtOAc fraction. Isolation was achieved by column chromatography on Sephadex LH-20 in MeOH. The main components were identified based on their MS- and ^{1}H-NMR spectra. Positive-ion ESI MS was recorded in 50% aq. MeOH with 0.05% TFA. The solvent for NMR spectroscopy was DMSO-d_6. Minor components were tentatively identified based on their online UV-spectra and coelution with commercially available reference samples (HPLC and TLC).

3.2. P. icarus

For the rearing and sampling procedure see Burghardt et al. (1997a). Development of *P. icarus* took 29 ± 2 days (until hatching). Before freezing, all larvae had to starve for 5 to 6 h to make sure that their alimentary canal was empty. For analysis third instar larvae were not sorted according to their age. Fourth instar larvae were sampled either one day after moulting, three days after moulting or in the prepupal stage. Pupae were collected three days after pupation. Faeces were sampled from fourth instar larvae.

A number (too small for statistical analysis but comparable with regard to weight) of imagines was dissected to localize flavonoid occurrence in the insects. Forewings, hindwings, abdomina and the head–thorax complexes were separately analysed.

3.3. Comparative HPLC-analysis

For quantification, kaempferol was added as internal standard to freeze dried samples before grinding. Individual extraction was carried out in 90% aq. MeOH, three times for 24 h with stirring. In the case of larvae and faeces, extracts were partitioned between 90% aqueous MeOH and petroleum ether. Flavonoids accumulated in the MeOH layer. All samples were evaporated under reduced pressure and redissolved in 50% aq. MeOH and analysed by HPLC (Eurospher 100-C18 column, 125×4 mm) with gradient elution using aqueous H_3PO_4 (pH 2) containing increasing concentrations of MeOH (flow rate: 1 ml $\times$ min^{-1}). Flavonoids were detected at 254 nm. Identification of the flavonoids in the samples of *P. icarus* and the larval faeces was accomplished by comparing chromatograms and online UV-spectra with those of the plant samples.

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