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2-*O*-(*p*-COUMAROYL)-L-MALATE, 2-*O*-CAFFEOYL-L-MALATE AND 2-*O*-FERULOYL-L-MALATE IN *RAPHANUS SATIVUS*

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Key Word Index—*Raphanus sativus*; Cruciferae; 2-*O*-(*p*-coumaroyl)-L-malate; 2-*O*-caffeoyl-L-malate; 2-*O*-feruloyl-L-malate; 2-*O*-sinapoyl-L-malate; metabolism; insect feeding behaviour.

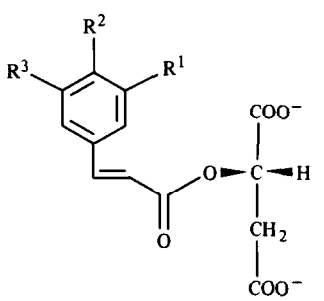
Abstract—The cotyledons, leaves and inflorescences of the radish, *Raphanus sativus*, have been examined for their content of hydroxylated cinnamic acid malate esters. 2-*O*-(*p*-coumaroyl)-L-malate, 2-*O*-caffeoyl-L-malate and 2-*O*-feruloyl-L-malate were found to be quantitatively predominating compounds in the fraction of carboxylic acids isolated from radish leaves and inflorescences. Identifications were based on PC, TLC, HVE, GC, HPLC, UV and NMR spectroscopy. The L- or (2*S*)-configuration of malic acid released by hydrolysis from the esters was determined by using L-malate dehydrogenase. A combined column chromatography technique applied prior to HVE, HPLC and GC was useful for separating the malate esters from most other low MW plant constituents. The significance of the present investigation is briefly discussed in relation to the metabolism of phenolic constituents of glucosinolate-containing plants, and the effects of these compounds in relation to insect feeding behaviour.

INTRODUCTION

Higher plants contain a large number of different benzoic and cinnamic acid derivatives including several compounds with appreciable physiological effects [1]. Within different plant families and/or genera, structural variation seems to be limited but depends on the plant parts examined, growth conditions and the developmental stage of the plant.

The group of naturally-occurring hydroxylated cinnamic acid esters with aliphatic hydroxycarboxylic acids as the alcohol moiety comprises some few esters of quinic acid, shikimic acid, tartaric acid and malic acid [1]. Only two hydroxylated cinnamic acid malate esters have been isolated from natural sources: 2-*O*-caffeoyl-L-malate (**2**) (Fig. 1) from *Phaseolus vulgaris* [2] and from *Trifolium pratense* [3], and 2-*O*-sinapoyl-L-malate (**4**) from *Raphanus sativus* [4]. Compound **4** is biosynthesized during germination of radish seeds; sinapoylcholine disappears simultaneously with the formation of 1-*O*-sinapoyl-β-D-glucose, and after a few days **4** is biosynthesized [5]. Other cruciferous plants contain choline esters of different aromatic acids as seed constituents [6–8].

The present work is a continuation of our previous investigations of phenolic plant constituents and their possible function in relation to host-plant selection by crucifer-feeding insects [9, 10]. We report here on the isolation of two new natural products, 2-*O*-(*p*-coumaroyl)-L-malate (**1**) and 2-*O*-feruloyl-L-malate (**3**), as well as the identification of **2** which is new among the known constituents of cruciferous plants. A simple method of semiquantitative analysis has been used to determine the concentration of **1**–**4** in different parts of *R. sativus*.



		R^1	R^2	R^3
2-*O*-(*p*-Coumaroyl)-L-malate	**1**	H	OH	H
2-*O*-Caffeoyl-L-malate	**2**	OH	OH	H
2-*O*-Feruloyl-L-malate	**3**	OMe	OH	H
2-*O*-Sinapoyl-L-malate	**4**	OMe	OH	OMe

Fig. 1. Structures of *E*(*trans*) isomers of hydroxylated cinnamic acid-L-malate esters occurring in *Raphanus sativus*.

RESULTS AND DISCUSSION

The total fraction of carboxylic acids and intact glucosinolates in leaves and inflorescences of *R. sativus* was obtained by a well-established extraction and isolation procedure [7, 11, 12]. The ion-exchange principles utilized resulted in a pyridine eluate from the Ecteola 23 cellulose column containing all glucosinolates and carboxylic acids present in the extract. The hydroxylated cinnamic acid malate esters were separated from each other and from the glucosinolates by column chromatography on Polyclar-AT, and, finally, they were purified by preparative HVE. Identification of **1**–**3** was performed by chromatographic and spectroscopic investigations of the intact esters and their hydrolysis products. The results obtained with the hydrolysis products were identical to those obtained with authentic reference compounds.

Table 1 shows the results obtained by PC, TLC, HVE and HPLC of the compounds and by GC of the per-

Table 1. R_f values from PC and TLC, ionic mobilities by HVE and k' values by HPLC of carboxylic acids occurring in *R. sativus*, and R_t values by GC of their pertrimethylsilyl derivatives

Compounds*	R_f values on PC in solvent systems†			R_f values on TLC in solvent systems†		HVE mobilities (cm) in buffer		GC R_t values‡ (min)	HPLC k' values§
	1	2	3	4	5	pH 3.6	pH 6.5		
2-*O*-(*p*-Coumaroyl)-L-malate (**1**)	0.86	0.31	0.08	0.14	0.23	16.0	21.8	40.5	7.57
2-*O*-Caffeoyl-L-malate (**2**)	0.77	0.20	0.04	0.04	0.11	14.5	20.5	42.9	4.21
2-*O*-Feruloyl-L-malate (**3**)	0.83	0.37	0.06	0.22	0.35	13.4	19.4	42.3	9.50
2-*O*-Sinapoyl-L-malate (**4**)	0.80	0.42	0.06	0.20	0.32	12.6	18.1	44.0	11.14
p-Coumaric acid	0.87	0.51	0.27	0.72	0.78	−1.0	15.6	28.1	5.21
Caffeic acid	0.78	0.26	0.19	0.39	0.53	−1.0	13.4	32.0	2.71
Ferulic acid	0.84	0.75	0.25	0.81	0.86	−1.0	11.2	30.9	6.71
Sinapic acid	0.80	0.85	0.19	0.85	0.95	−1.0	7.6	33.5	7.86
Malic acid	0.38	0.12	0.08	0.02	0.09	19.4	34.4	19.2	—

*For the *E*-(*trans*) isomers, see Fig. 1.

†For experimental conditions, see Experimental.

‡For GC conditions, see ref. [14].

§For HPLC conditions and column type, see Experimental. $k' = (t_R - t_0)/t_0$; $t_0 = 7.0$ mm.

trimethylsilylated compounds. HVE at pH 1.9 separates efficiently the glucosinolates [11] from the carboxylic acids. The mobilities of **1**–**4** in HVE at pH 6.5 compared with the mobilities of the monocarboxylic acids show that **1**–**4** are dicarboxylic acids. The results from HVE at pH 3.6 revealed the rather low pK_{a1} value of **1**–**4** and malic acid. The R_f values by PC and TLC are in agreement with the structures proposed, and the results in Table 1 reveal, furthermore, that the most efficient separations of **1**–**4** are obtained by HPLC and by GC of the TMSi derivatives.

The presence of the hydroxylated cinnamic acid malate esters on chromatograms and pherograms was easily revealed by their strong but different fluorescences in long-wave UV light and also in short-wave UV light for **1**. The dark blue colour of **1** and the light blue colours of **2**–**4** changed characteristically on exposure to ammonia vapour in accordance with their UV spectra as described for the corresponding choline esters [6, 8]. Like other carboxylic acids, **1**–**4** reacted with the aniline–xylose spray reagent.

The ^{1}H NMR chemical shifts and coupling patterns for **1**–**3** dissolved in D_2O were compared with the corresponding spectra of *p*-coumaric acid, caffeic acid, ferulic acid and malic acid. The results confirmed their structures and disclosed the presence of both the *Z*- (*cis*) and *E*-(*trans*) isomers in the isolated preparations. Mixtures of the *Z*- and *E*-isomers have also been recognized with other naturally-occurring cinnamic acid derivatives [4, 7].

The hydroxylated cinnamic acid malate esters **1**–**3** were easily hydrolysed in acidic and especially alkaline solutions, transforming the compounds into malate and *p*-coumaric acid from **1**; malate and caffeic acid from **2**; and malate and ferulic acid from **3**. The hydrolysis mixtures were analysed by HVE, PC, TLC and HPLC as well as by GC of the TMSi derivatives (Table 1). The identity of the carboxylic acids was further strengthened by cochromatography with authentic samples. The malate produced in the hydrolysis of **1**–**3** was shown to possess (2*S*)- or L-configuration by use of the coupled assay containing L-malate dehydrogenase (EC 1.1.1.37) [13].

Semiquantitative determinations of **1**–**4** in extracts from 1.0 g amounts of freeze-dried cotyledons, leaves and inflorescences were based on HPLC in a reversed-phase system after group separation of the low MW constituents according to a recently described method [12]. The results are shown in Table 2. Determination of hydroxylated cinnamic acid malate esters by HPLC is simple, sensitive and elegant [12]. The results revealed that **1**–**3** occur in

Table 2. The content of malate esters (μg/g freeze-dried plant material)* in different parts of *R. sativus*

Plant material	2-*O*-(*p*-Coumaroyl)-L-malate	2-*O*-Caffeoyl-L-malate	2-*O*-Feruloyl-L-malate	2-*O*-Sinapoyl-L-malate
Cotyledons of 14-day-old seedlings	1983	988	482	1410
Leaves of 28-day-old plants	1876	1980	1221	152
Leaves of flowering plants	2324	1760	1012	167
Inflorescences	507	71	553	15

*Determined by use of HPLC after isolation, as described in Experimental.

appreciable amounts in cotyledons, leaves and inflorescences of radish. The previously well-described radish constituent **4** [4] was only quantitatively predominating among the hydroxylated cinnamic acid malate esters in the cotyledons, which is in agreement with the previous investigation [5]. Free hydroxylated cinnamic acids were only present in small amounts in the extracts.

EXPERIMENTAL

Plant material. Seeds of *Raphanus sativus* L. cv Copenhagen Market were sown in field plots at the Agricultural Experimental Station, Taastrup. Cotyledons were harvested from seedlings on the 14th day and leaves from plants on the 28th day after sowing. Leaves and inflorescences were furthermore harvested from flowering plants. The plant materials were freeze-dried and stored at −20°, until extractions were carried out.

General methods and instrumentation. Methods and equipment used for PC, TLC, HVE, GC, HPLC and ^{1}H NMR have been described elsewhere [6, 11, 12, 14]. PC was performed on Whatman No. 1 paper in solvents: (1) *n*-BuOH–HOAc–H_2O (12:3:5); (2) PhOH–H_2O–13 M NH_4OH (120:30:1); (3) *i*-PrOH–H_2O–13M NH_4OH (8:1:1). HVE was carried out on Whatman No. 3 MM paper in buffer systems: (1) pH 1.9 HOAc–HCOOH–H_2O (4:1:45), 2 hr at 3.2 kV and 90 mA; (2) pH 3.6 pyridine–HOAc–H_2O (1:10:200), 2 hr at 3 kV and 90 mA; (3) pH 6.5 pyridine–HOAc–H_2O (25:1:500), 50 min at 5 kV and 90 mA. TLC was performed on silica gel plates (DC-Alufolien, Kieselgel 60, 20 × 20 cm, Merck) in solvents: (4) $CHCl_3$–*t*-amyl alcohol–HCOOH–H_2O (136:24:27:83); (5) C_6H_6–HOAc–H_2O (125:73:2). HPLC was performed on 250 × 4.6 mm i.d. columns (Knauer, Berlin, West Germany) packed by the dilute slurry technique with Nucleosil 5 C_{18} (5 μm) (Macherey, Nagel & Co., Düren, West Germany) in an isocratic system with 5% HCOOH in H_2O modified with 25% MeOH as the mobile phase. The detection wavelength was 313 nm.

Isolation. Homogenization and extractions were performed as previously described [11]. Fresh plant material (leaves and inflorescences) was freeze-dried and homogenized (300 g) with an Ultra-Turrax homogenizer in boiling MeOH–H_2O (7:3), cooled and filtered. The combined filtrates (3 × 4 l.) were evapd to dryness, redissolved in 200 ml H_2O and extracted with $CHCl_3$ (3 × 100 ml). The H_2O phase was concd to *ca* 50 ml and applied to a column of CM Sephadex C-25 (H^+, 2.5 × 100 cm) connected in series to a column of Amberlite IR 120 (H^+, 2.5 × 100 cm) which again was connected in series to a column of Ecteola 23 cellulose (AcO^-, 2.5 × 100 cm). The system was flushed with H_2O and the respective columns were then eluted according to the principles described previously [12]. The Ecteola 23 cellulose column was eluted with 1M pyridine and fractions (20 ml) were collected at 40 ml/hr. The glucosinolate and hydroxylated cinnamic acid malate ester containing fractions Nos. 10–100 were pooled and taken to dryness. Further purification and separation were obtained by chromatography on a Polyclar-AT column (2.5 × 45 cm). MeOH–H_2O (3:1) was used as eluant and fractions of 20 ml were collected at 40 ml/hr. The glucosinolates appeared in fractions 5–30; 2-*O*-feruloyl-L-malate (**3**) was the quantitatively predominating compound in fractions 51–70; 2-*O*-(*p*-coumaroyl)-L-malate (**1**) was the quantitatively predominating compound in fractions 71–114; 2-*O*-caffeoyl-L-malate (**2**) was the quantitatively predominating compound in fractions 115–170. Final purification of the three compounds was obtained by prep. HVE at pH 6.5 and prep. PC in solvent 1. For PC, TLC, HVE, HPLC of **1**, **2** and **3** and GC of their TMSi derivatives, see Table 1. For UV and ^{1}H NMR spectra, see Results and Discussion.

Hydrolysis. The hydroxylated cinnamic acid malate esters (5 mg) were dissolved in 2.5 ml 2 M HCl and hydrolysed for 4 hr at 100°. The reaction mixture was taken to dryness and stored under vacuum in a desiccator containing $CaCl_2$. Silylation and GC were performed as described previously [14]. The hydroxylated cinnamic acid malate esters were dissolved in 2 M NH_4OH and hydrolysed for 1 hr at 60°. The reaction mixture was taken to dryness and analysed. L-malate dehydrogenase (L-malate: NAD oxidoreductase, EC 1.1.1.37) was used in conjunction with glutamate-oxaloacetate transaminase (L-aspartate: 2-oxaloacetate transferase, EC 2.6.1.1.) for both quantitative and qualitative determinations of L-malate in an assay as described in the information sheet from Boehringer Mannheim GmBH [13].

Isolation and semiquantitative determination. Freshly harvested cotyledons, leaves and inflorescences were freeze-dried and homogenized (1 g) in boiling MeOH–H_2O (7:3) as described above. After filtration and lyophilization, the hydroxylated cinnamic acid malate esters were purified by ion-exchange chromatography as described above using small columns (Pasteur pipettes, 0.5 × 7 cm) [12]. The lyophilized 1 M pyridine eluate from the Ecteola 23 cellulose column was further purified by prep. HVE at pH 1.9 (the hydroxylated cinnamic acid malate esters do not migrate; glucosinolates migrate towards the anode) followed by prep. HVE at pH 6.5 which separates the hydroxylated cinnamic acid malate esters from monocarboxylic acids (Table 1). Compounds **1**–**4** were quantitatively determined by HPLC in the system described above using standard curves for *p*-coumaric acid, ferulic acid, caffeic acid and sinapic acid, respectively, run in the same chromatographic system.

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