

Hydroxycinnamoyl Esters of Malic Acid in Small Radish (*Raphanus sativus* L. var. *sativus*)

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Hydroxycinnamoylmalic Acids, Isolation, Identification, Quantitative Determination,
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The esters of *p*-coumaric, ferulic and caffeic acid with malic acid were isolated from leaves, the ester of sinapic acid with malic acid from cotyledons of *Raphanus sativus* plants via preparative HPLC. The esters were identified by hydrolyses and following capillary GC, by H-NMR and FAB-MS. Their concentrations in leaves and tubers were determined by gradient HPLC on reversed phase material.

Introduction

Hydroxycinnamic acid derivatives are present ubiquitously in the plant kingdom [1]. They have been detected in small radish by paper and thin layer chromatographic techniques [2]. Some of these compounds, especially those in small radish containing sinapic acid, could be analyzed by the investigations of Strack *et al.* [3, 4]. Two hydroxycinnamoylmalic acids are known up to now. Germs and leaves of beans (*Phaseolus vulgaris*) and red clover (*Trifolium pratense*) are containing caffeoylmalic acid (phaseolic acid) [5, 6] and sinapoylmalic acid has been determined in small radish seedlings.

In this present study, we report the occurrence of four hydroxycinnamoylmalic acids in small radish, the known esters of caffeic and sinapic acid and also the hitherto unknown esters of *p*-coumaric and ferulic acid.

Materials and Methods

Plant material

Leaves of the small radish varieties Hilmar, Riesenbutter and Cherry Bell were used for the isolation and determination of *p*-coumaroyl, caffeoyl and feruloylmalic acid. To obtain sinapoylmalic acid, seed of the variety Rota was sown on cotton

in the laboratory. The cotyledons were harvested and worked up 11 days after sowing [4].

Isolation of hydroxycinnamoylmalic acids

Sinapoylmalic acid: The cotyledons were comminuted in methanol and homogenized using a mixer and an Ultra Turrax. After filtering the plant pulp through a G4 glass frit and washing the residue with hot methanol the extract was evaporated at 40 °C to about 30 ml *in vacuo*. Sinapoylmalic acid was separated from the extract by preparative HPLC [7].

Apparatus: preparative HPLC system LCX (Philips), injection valve: Rheodyne 7125 with 2 ml sample loop, detection: UV 320 nm, solvent: 17% acetonitrile in 1% acetic acid, flow: 11.2 ml/min, column: 250 × 16 mm, LiChrosorb RP 18, 10 µm (Chrompack).

The fractions containing sinapoylmalic acid ($t_R = 5.4$ min) were collected and lyophilized. The purity of the compound was checked by analytical HPLC.

p-Coumaroyl, caffeoyl and feruloylmalic acid

A 300 g amount of small radish leaves was worked up to obtain these esters. They were isolated by preparative HPLC using the solvent 13% acetonitrile in 1% acetic acid. Retention times: caffeoylmalic acid: $t_R = 15.6$ min, *p*-coumaroylmalic acid: $t_R = 27.3$ min, feruloylmalic acid: $t_R = 32.7$ min. The

fractions collected were lyophilised and the purity of the obtained compounds was controlled by analytical HPLC. The fractions containing *p*-coumaroylmalic acid and feruloylmalic acid were cleaned up once more by preparative HPLC (solvent: 15% acetonitrile in 1% acetic acid).

Identification of the hydroxycinnamoylmalic acid components by GC

For the identification of the components of hydroxycinnamoylmalic acids about 1 mg of each compound was hydrolyzed by adding 0.5 ml of 0.5 N HCl and refluxing for 7 minutes in boiling water. The hydrolysate was evaporated to dryness *in vacuo* and trimethylsilylated by adding 0.5 ml BSA/5% TMCS and heating at 70 °C for 60 minutes. *p*-Coumaric, caffeic, ferulic, sinapic, malic and pimelic acid (internal standard) were treated in the same way for reference.

Apparatus: capillary GC (Carlo Erba), capillary: SE 30, 30 m (self made), carrier: N₂, detector: FID, temperatur gradient: 120 °C–8 °C/min–220 °C–15 °C/min–270 °C.

The hydroxycinnamic acid and malic acid could be detected in about equimolar ratio in each case.

H-NMR

H-NMR were recorded on a Bruker WH 270 spectrometer with puls fourier transformation techniques. DMSO d₆ (solvent) was assigned 2.5 ppm as secondary standard.

FAB-MS

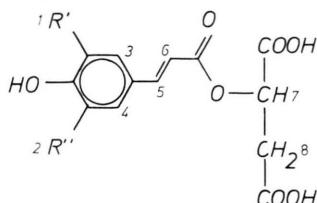
Negativion mass spectra were recorded on a Kratos MS 50 S spectrometer with a Kratos FAB source. A Xenon atom gun was used at 9 KV.

Quantitative determinations

The quantitative determinations were performed with: HPLC system LCX (Philips), injection valve: Rheodyne 7125 with 20 µl sample loop, column: 250 × 4.6 mm, LiChrosorb RP 18, 5 µm (Merck), detector: UV 320 nm, integrator: DP 101 Computing Integrator (Pye Unicam), solvent: A = 2% acetic acid, B = methanol, gradient: 5% B in A – 45% B in A in 45 minutes, flow: 1 ml/min.

Results and Discussion

The isolated compounds were identified as *trans*-*p*-coumaroylmalic acid, *trans*-caffeoylemalic acid, *trans*-feruloylmalic acid and *trans*-sinapoylmalic acid by acid hydrolysis and following identification of the components via GC, by H-NMR and negativion FAB-MS.



Characteristic proton signals of the hydroxycinnamic acids and malic acid could be detected by NMR (Table I). The coupling of the doublets (16 Hz) of the olefinic protons makes possible the determination of the *trans* compounds. The shift of about 5.2 ppm of the CH-proton of malic acid allows the identification of the ester bond with malic acid as alcoholic component (malic acid: δ (CH) = 4.27 ppm).

Negativion FAB mass spectroscopy affords a very good method for the mass spectrometric characterisation of conjugates, produced by plant cells, when they are bonded by oxygen. As well as intense deprotonated molecular ions characteristic sequence ions are also found. In the present compounds, conjugates of malic acid with *p*-coumaric acid, or ferulic acid, or caffeic acid, or sinapic acid show intense molecular ions at 279, 295, 309 and 339 d respectively. Cleavage of the bond between the ester oxygen of malic acid and the carbonyl of the aromatic acid gives the malic acid sequence ion at 133 d, while the characteristic aromatic acid sequence ions for cleavage between the carbon of the malic acid and the ester oxygen affords ions at 163 d for *p*-coumaric acid, 179 d for caffeic acid, 193 d for ferulic acid and 223 d for sinapic acid. Glycerol was used as matrix for the measurements and this gives rise to ions at 91, 183, 275 and 367 d (Figs. 1–4).

For the first time every possible ester of hydroxycinnamic acids with malic acid has been characterized and identified side by side in one plant. The thin layer characteristics (hR_f) of these esters are differing significantly from other hydroxycinnamic acid esters (Table II), facilitating their qualitative determination beside the frequently present esters with quinic acid or glucose.

Table I. H-NMR of hydroxycinnamoylmalic acids in DMSO d₆.

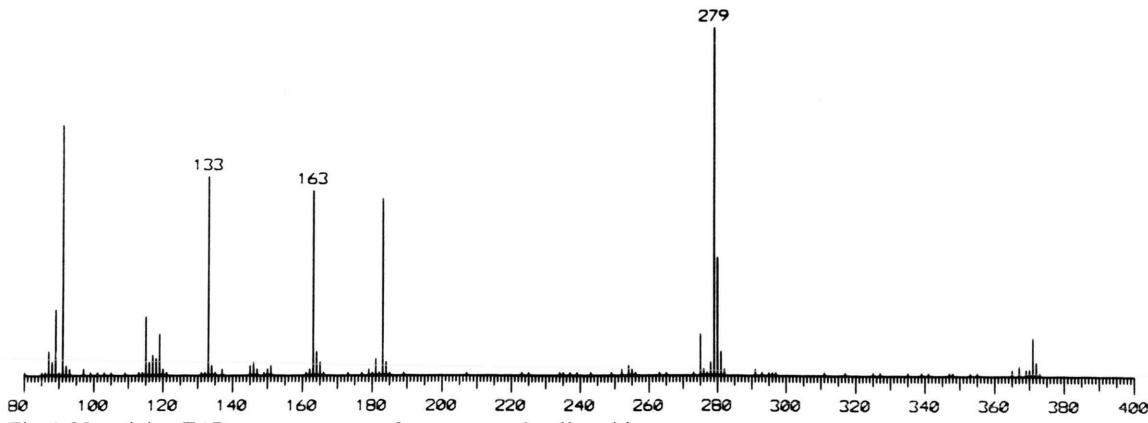
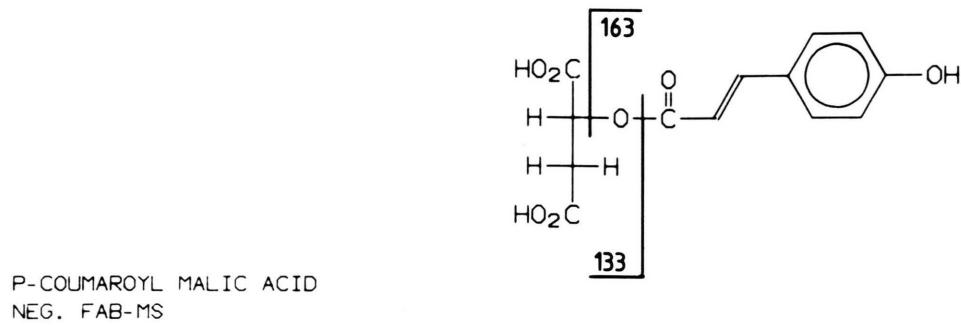
p-CM	ppm	CM	ppm	FM	ppm	SM	ppm
1 Ar.-H	7.59 d 15 Hz	OH		OCH ₃	3.82 s	OCH ₃	3.81 s
2 Ar.-H	7.59 d 15 Hz	Ar.-H	6.77 d 8 Hz	Ar.-H	7.14 d 8 Hz	OCH ₃	3.81 s
3 Ar.-H	6.80 d 15 Hz	Ar.-H	7.03 m	Ar.-H	6.80 d 8 Hz	Ar.-H	7.04 s
4 Ar.-H	6.80 d 15 Hz	Ar.-H	7.03 m	Ar.-H	7.35 s	Ar.-H	7.04 s
5 olef.-H	7.58 unres.	olef.-H	7.46 d 17 Hz	olef.-H	7.60 unres.	olef.-H	7.54 d 16 Hz
6 olef.-H	6.46 d 14 Hz	olef.-H	6.27 d 17 Hz	olef.-H	6.55 unres.	olef.-H	6.54 d 16 Hz
7 alk.-H	5.24 unres.	alk.-H	5.15 dd 8 Hz	alk.-H	5.23 unres.	alk.-H	5.08 dd 2 Hz
8 alk.-H	3.27 s	alk.-H	3.10 s	alk.-H	3.26 s	alk.-H	3.11 s

p-CM, *p*-coumaroylmalic acid; CM, caffeoylmalic acid; FM, feruloylmalic acid; SM, sinapoylmalic acid; d, doublet; dd, double doublet; s, singlet; unres., unresolved.

Table II. Thin layer chromatography (h R_f) of hydroxycinnamoylmalic acids on cellulose plates.

	BAW	BPW	KFW	EMW	UV	UV + NH ₃	UV + Ben.
<i>p</i> -coumaroylmalic acid	92	17	97	96	nv	blue	blue
caffeooylmalic acid	79	11	89	96	blue	green	brown
feruloylmalic acid	90	15	96	96	blue	green	blue
sinapoylmalic acid	81	8	93	95	blue	green	green

BAW, butanol/acetic acid/water = 40/10/50 (upper layer); BPW, butanol/pyridine/water = 14/3/3; KFW, methylisobutylketone/formic acid/water = 30/10/20 (upper layer); EMW, ethylacetate/methanol/water = 200/35/27; Ben., Benedict's reagents; nv, not visible.

Fig. 1. Negativion FAB mass spectrum of *p*-coumaroylmalic acid.

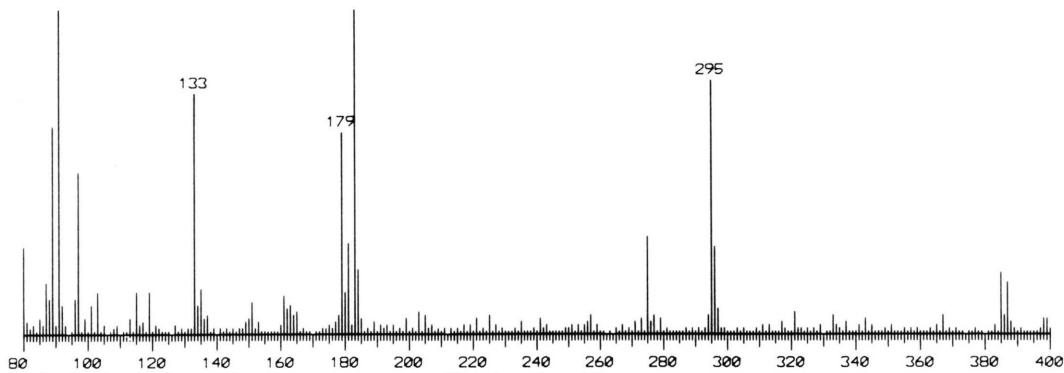
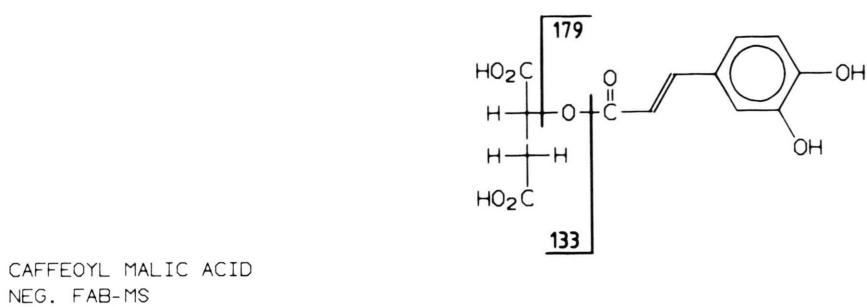


Fig. 2. Negativion FAB mass spectrum of caffeoylmalic acid.

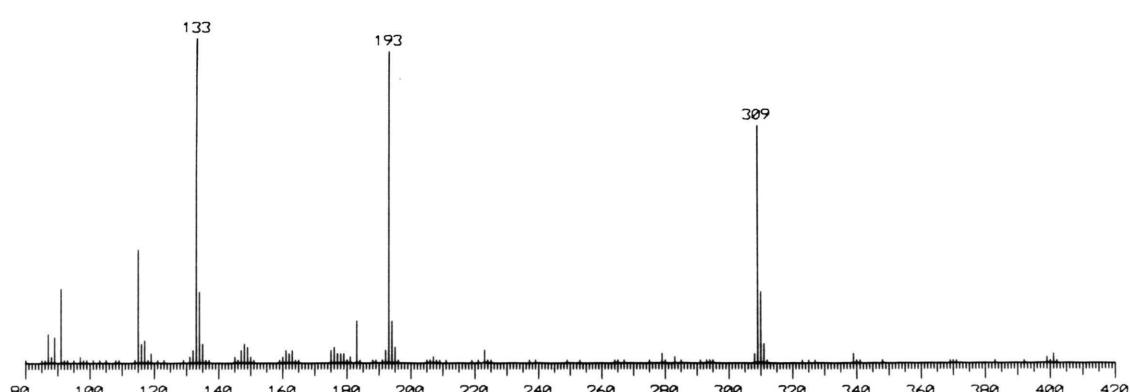
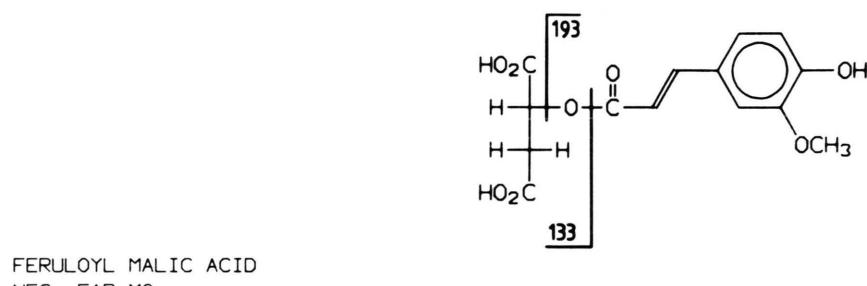


Fig. 3. Negativion FAB mass spectrum of feruloylmalic acid.

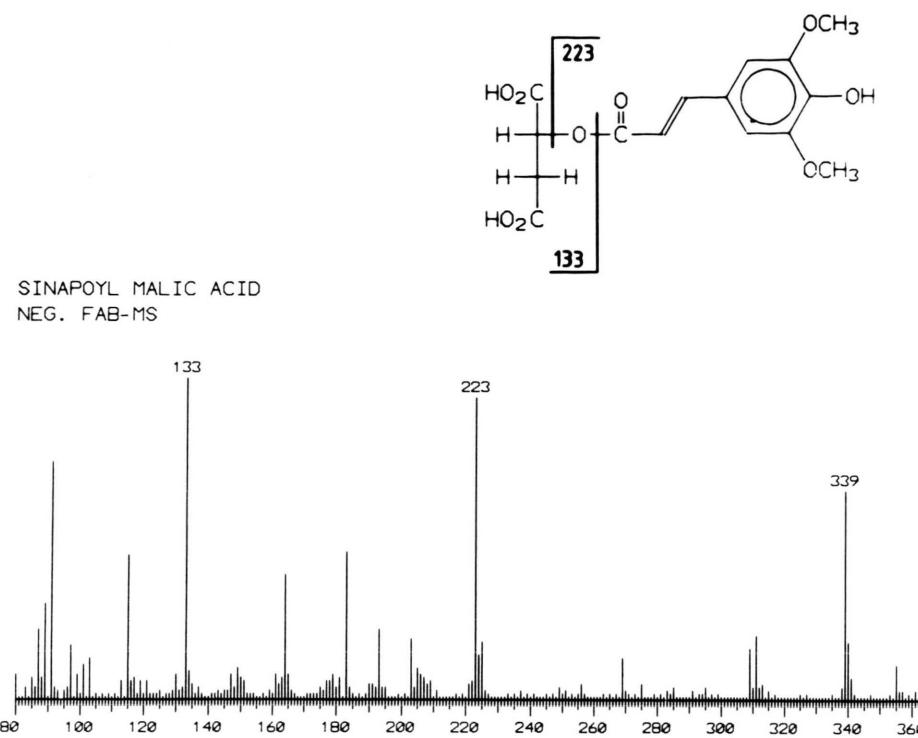


Fig. 4. Negativion FAB mass spectrum of sinapoylmalic acid.

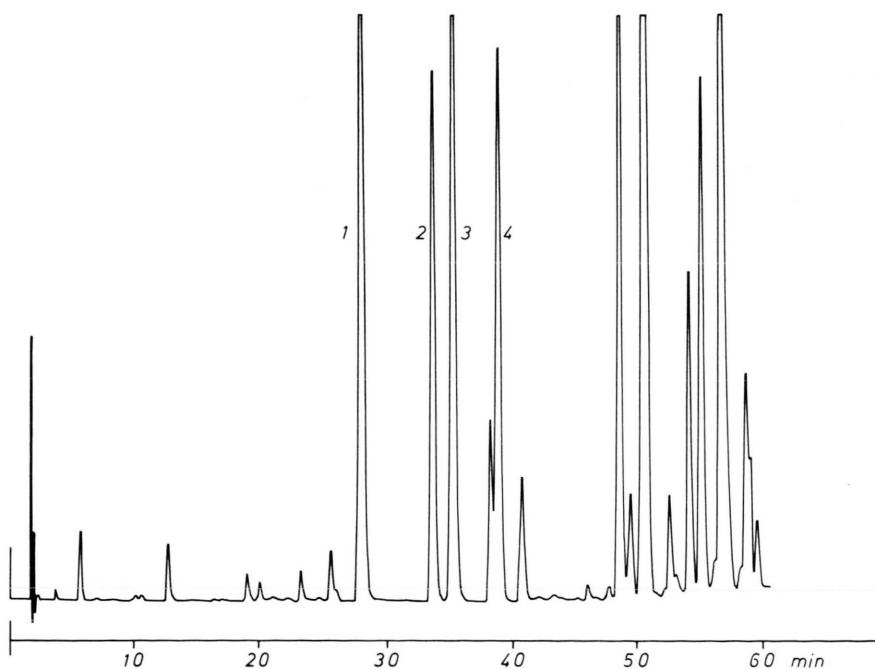
Fig. 5. HPLC chromatogram of *Raphanus sativus* leaves ("Hilmar"). 1, caffeoymalic acid; 2, *p*-coumaroylmalic acid; 3, feruloylmalic acid; 4, sinapoylmalic acid.

Table III. Concentrations of hydroxycinnamoylmalic acids in *Raphanus sativus* in ppm.

	<i>p</i> -CM	CM	FM	SM	3CQ	5CQ	CG	<i>p</i> -CG	FG
"Hilmar"									
leaves	43	80	81	56	1	+	6	3	3
tuber peeling	4	19	10	8	2	+	2	+	2
tuber peeled	nd	2	2	2	nd	nd	+	nd	nd
"Riesenbutter"									
leaves	44	137	88	62	2	2	6	2	3
tuber peeling	3	53	33	30	nd	1	2	nd	1
tuber peeled	nd	4	13	6	nd	nd	nd	nd	+
"Cherry Bell"									
leaves	79	255	156	60	2	1	7	2	3
tuber peeling	10	11	7	14	nd	1	1	nd	1
tuber peeled	nd	1	1	nd	nd	nd	+	nd	+

p-CM, *p*-coumaroylmalic acid; CM, caffeoylmalic acid; FM, feruloylmalic acid; SM, sinapoylmalic acid; 3CQ, 3-caffeoylequinic acid; 5CQ, 5-caffeoylequinic acid; CG, caffeoylglucose; *p*-CG, *p*-coumaroylglucose; FG, feruloylglucose; nd, not detected; +, traces.

The hydroxycinnamoylmalic acids are present in relative high concentrations in the leaves of small radish (Table III, Fig. 5). Lower but still considerable amounts are located in the red coloured peeling. In peeled small radish the concentrations are substantially lower, similar to observations at other cultivated plants like potatoes [8]. As in other cultivated plants the concentrations of these hydroxycinnamoyl derivatives might be depending on vari-

ety. Hydroxycinnamoylquinic acids and glucose were analyzed in addition to the hydroxycinnamoylmalic acids only in very low concentrations (Table III).

Acknowledgements

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