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ANALYSIS OF METHYL AND ETHYL ESTERS OF HYDROXYBENZOIC AND HYDROXYCINNAMIC ACIDS IN PLANT MATERIAL

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SUMMARY

A method is described for the extraction and purification of methyl and ethyl esters of hydroxybenzoic and hydroxycinnamic acids from plant material. The esters were analyzed as trimethylsilyl derivatives by glass capillary gas chromatography (OV-1, OV-73, Dexsil 300) and gas chromatography–mass spectrometry. The method has been applied to analysis of methyl and ethyl esters of hydroxybenzoic and hydroxycinnamic acids in vegetables and potato peels.

INTRODUCTION

In plants, phenolic acids occur ubiquitously and in various forms. Fruits and vegetables chiefly contain esters of hydroxycinnamic acids with quinic acid or D-glucose besides smaller concentrations of hydroxybenzoic acid compounds. In contrast, methyl and ethyl esters of hydroxybenzoic and hydroxycinnamic acids have been discovered in nature only in a few cases, with the exception of methyl salicylate and methyl gallate[1]. Bohlmann and co-workers[2–6] isolated, amongst other natural substances, methyl and ethyl caffeate, methyl 4-coumarate and methyl ferulate from some plants.

Phenolic acids are important constituents of plants. The antioxidative and antimicrobial effect of hydroxycinnamic and hydroxybenzoic acids is well known[7]. Gallic acid esters are used in the food industry as antioxidants and methyl, ethyl and propyl 4-hydroxybenzoates (PHB esters) as preservatives. Hydroxycinnamic and hydroxybenzoic acids are further known as growth regulators[8,9] and in the resistance of cultivated plants against pathogenic microorganisms[10,11], especially chlorogenic acid. Chlorogenic acid is easily oxidized enzymatically by phenol oxidases, which for example causes brown discoloration of light fruits, fruit juices and wines.

In this laboratory Sontag *et al.*[12] separated the hydroxycinnamic acid esters by high-performance liquid chromatography (HPLC). It seemed important to develop a method for qualitative and quantitative analysis of the esters of the frequently occurring phenolic acids including the hydroxybenzoic acid esters. Schulz and Herrmann[13,14] employed gas–liquid chromatography for the determination of phenolic

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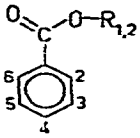
TABLE I

HYDROXYBENZOIC ACID ESTERS



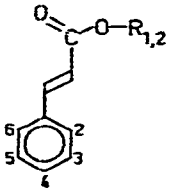
R_1= $-CH_3$
R_2= $-CH_2-CH_3$

Substituents	*Trivial name*	*Source*
2-OH	Methyl salicylate	E. Merck (Darmstadt, G.F.R.)
	Ethyl salicylate	Aldrich-Europe (Nettetal, G.F.R.)
4-OH	Methyl 4-hydroxybenzoate	Riedel de Haen (Seelze, G.F.R.)
	Ethyl 4-hydroxybenzoate	Roth (Karlsruhe, G.F.R.)
2,5-di-OH	Methyl gentisate	—
	Ethyl gentisate	
3,4-di-OH	Methyl protocatechuate	—
	Ethyl protocatechuate	Nipa Ltd. (Pontypridd, Great Britain)
3,4,5-tri-OH	Methyl gallate	Roth
	Ethyl gallate	E. Merck
3-OCH_3, 4-OH	Methyl vanillate	Aldrich-Europe
	Ethyl vanillate	Nipa Ltd.
3,5-di-OCH_3, 4-OH	Methyl syringate	Nipa Ltd.
	Ethyl syringate	

acids, after hydrolysis, as trimethylsilyl (TMS) derivatives. Methyl and ethyl esters of TMS-hydroxybenzoic and -hydroxycinnamic acids are suitable for GLC because they are more volatile than the free acids (TMS derivatives) and because they allow one to distinguish naturally occurring hydroxyl groups from methoxy groups. So we chose capillary GLC in addition to HPLC because of its high performance and to develop

TABLE II

HYDROXYCINNAMIC ACID ESTERS



R_1= $-CH_3$
R_2= $-CH_2-CH_3$

Substituents	*Trivial name*
4-OH	Methyl 4-coumarate
	Ethyl 4-coumarate
3,4-di-OH	Methyl caffeate
	Ethyl caffeate
3-OCH_3, 4-OH	Methyl ferulate
	Ethyl ferulate
3,5-di-OCH_3, 4-OH	Methyl sinapate
	Ethyl sinapate

another chromatographic system for the alkyl esters of the frequently occurring phenolic acids. Another advantage of glass capillary GLC is the possibility to use a mass spectrometer, a highly specific detector.

EXPERIMENTAL

Some of the esters are not commercially available. These esters were synthesized by esterification of the acid with the corresponding alcohol and characterized by melting point and GLC and mass spectrometric (MS) data. MS data of some hydroxycinnamic acid methyl esters (TMS derivatives) have been reported by Horman and Viani[15].

Evaporations were performed in a rotary vacuum evaporator at a temperature not higher than 40°C.

Sample extraction

Fresh plant material (10 g) was homogenized with 25 ml acetonitrile for about 5 min in a centrifuge tube using an Ultra-Turrax (Janke & Kunkel, Staufen i. Br., G.F.R.). The Ultra-Turrax was washed with 25 ml acetonitrile. The two acetonitrile fractions were combined and centrifuged for about 5 min at 4300 *g*. The supernatant was filtered through glass wool into a 250-ml round-bottomed flask. The residue was re-extracted once by homogenizing with 25 ml acetonitrile, washing the Ultra-Turrax and centrifuging. The acetonitrile was evaporated, leaving an aqueous solution.

Preparation of polyamide columns

Polycaprolactam powder (MN-Polyamid SC-6, 0.05–0.16 mm; Macherey, Nagel & Co., Düren, G.F.R.) was suspended in water–methanol (1:1). After about 3 h the suspension was poured into a tube (250 × 14 mm I.D., with a G2-frit and a stopcock) to a height of 150 mm. To remove soluble polyamide components, the column was washed with 50 ml methanol–25% aqueous ammonia (990:10), 50 ml water–acetic acid (998:2) and 50 ml water.

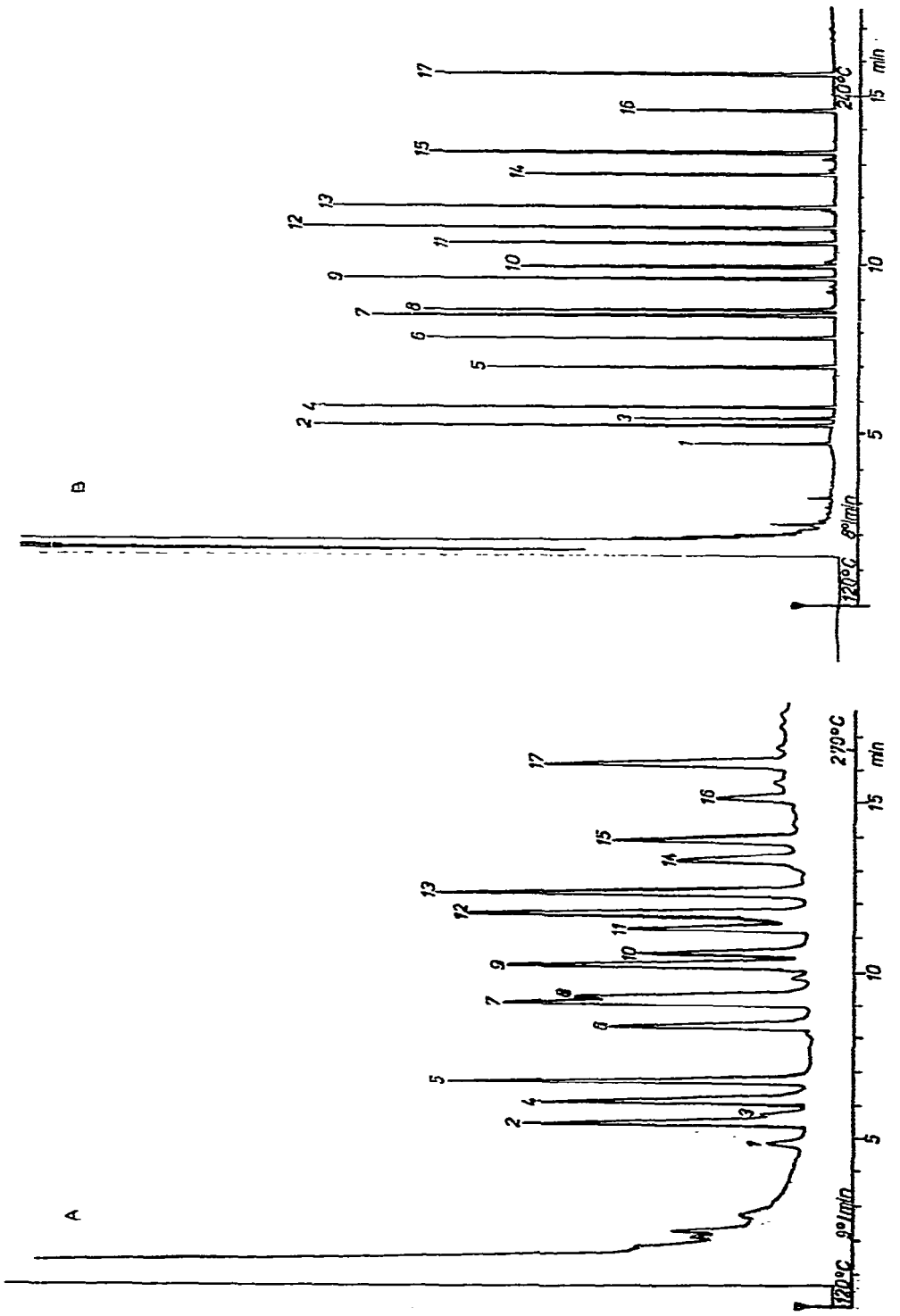
The aqueous plant extract was placed on top of the polyamide column by rinsing the flask three times with 5 ml hot water. The column was washed with 100 ml water to remove carbohydrates, salts and other undesired compounds. Elution was performed with 30 ml methanol. The eluate was collected when methanol first reached the end of the column, producing visible streaks.

Liquid–liquid extraction

For further clean-up, extraction with diethyl ether was useful. The water-containing eluate was adjusted to pH 5 and extracted twice by shaking with 20 ml diethyl ether. The ether solutions were combined, dried over sodium sulphate and filtered into a conical flask.

Derivatization and quantification

After adding 500 μl internal standard solution [tricosane and 3-*tert*.-butyl-4-hydroxyanisole (BHA), each 20 mg per 100 ml hexane], the sample was evaporated to dryness. About 0.4 ml N,O-bis(trimethylsilyl)acetamide (BSA) were added and the


A
B
120°C
9°/min
270°C
min
5
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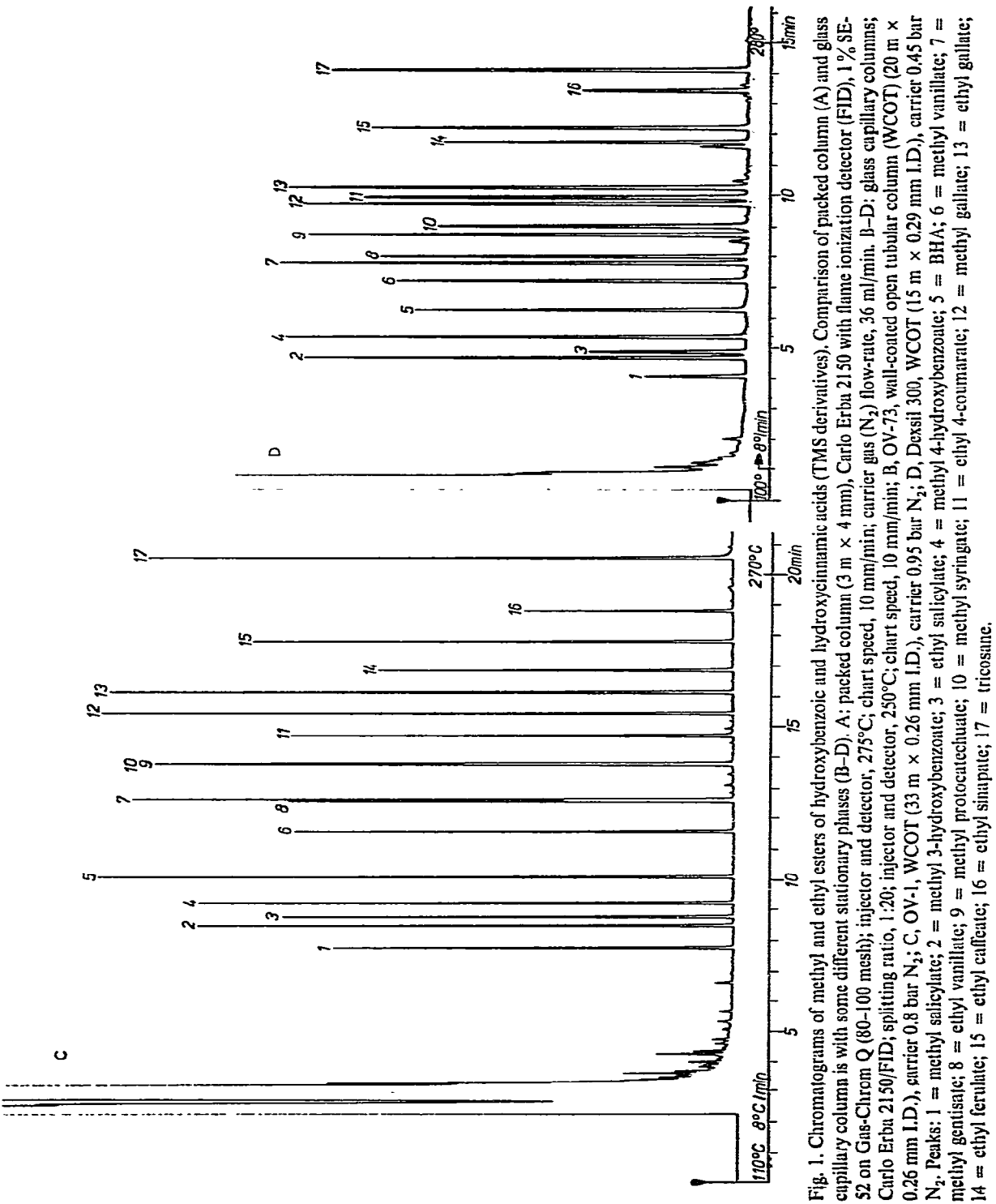


Fig. 1. Chromatograms of methyl and ethyl esters of hydroxybenzoic and hydroxycinnamic acids (TMS derivatives). Comparison of packed column (A) and glass capillary column is with some different stationary phases (B–D). A: packed column (3 m × 4 mm), Carlo Erba 2150 with flame ionization detector (FID), 1% SE-52 on Gas-Chrom Q (80–100 mesh); injector and detector, 275°C; chart speed, 10 mm/min; carrier gas (N_2) flow-rate, 36 ml/min. B–D: glass capillary columns; Carlo Erba 2150/FID; splitting ratio, 1:20; injector and detector, 250°C; chart speed, 10 mm/min; B, OV-73, wall-coated open tubular column (WCOT) (20 m × 0.26 mm I.D.), carrier 0.8 bar N_2; C, OV-1, WCOT (33 m × 0.26 mm I.D.), carrier 0.95 bar N_2; D, Dexsil 300, WCOT (15 m × 0.29 mm I.D.), carrier 0.45 bar N_2. Peaks: 1 = methyl salicylate; 2 = methyl 3-hydroxybenzoate; 3 = ethyl salicylate; 4 = methyl 4-hydroxybenzoate; 5 = BHA; 6 = methyl vanillate; 7 = methyl gentisate; 8 = ethyl vanillate; 9 = methyl protocatechuate; 10 = methyl syringate; 11 = ethyl 4-coumarate; 12 = methyl gallate; 13 = ethyl gallate; 14 = ethyl ferulate; 15 = ethyl caffeate; 16 = ethyl sinapate; 17 = tricosane.

flask was sealed with a polyethylene stopper followed by heating on an oil-bath. Silylation was complete after 60 min at 70°C. Tricosane (C_{23}) was selected as internal standard, because it is unaffected by silylation and column deactivation. The ratio BHA/C_{23} was used to check on the efficiency of silylation.

A reference solution of phenolic acid ester TMS derivatives, containing 100 μg per 0.4 ml of each ester and the same amount of internal standard, was analysed under the same GLC conditions. A computing integrator SP4100 (Spectra-Physics, Santa Clara, CA, U.S.A.) was used for calculations.

Gas–liquid chromatography

For separation, glass capillary GLC was performed because of its high resolution (Fig. 1). Fig. 1A shows the chromatogram of a standard solution of the esters

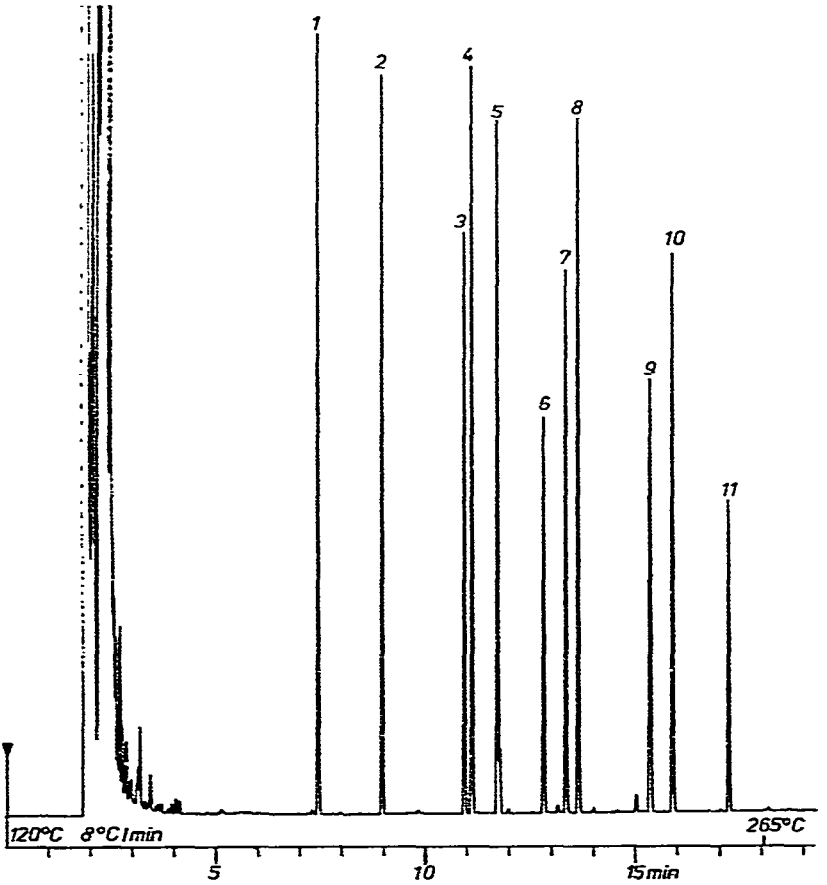


Fig. 2. Chromatogram of phenolic acids (TMS derivatives) on OV-73 (WCOT, 28 m × 0.26 mm I.D.). Conditions: Carlo Erba 2150 with FID; carrier, 0.95 bar N_2; injector and detector, 250°C; chart speed, 10 mm/min. Peaks: 1 = salicylic acid; 2 = 4-hydroxybenzoic acid; 3 = vanillic acid; 4 = gentisic acid; 5 = protocatechuic acid; 6 = syringic acid; 7 = 4-coumaric acid; 8 = gallic acid; 9 = ferulic acid; 10 = caffeic acid; 11 = sinapic acid.

separated on a packed column and Fig. 1B–D the same mixture separated on glass capillaries with different stationary phases.

The sensitivity of TMS-phenolic acids to alkaline supports and glasses has been reported by Schulz and Herrmann[14]. This sensitivity to alkaline and insufficiently deactivated glass capillaries may be the reason for the statement of Verzele and Sandra[16] that some separations succeed better on packed columns than on capillaries. As an example they mentioned the separation of persilylated plant phenolic acids. We succeeded in separating TMS-phenolic acids on a glass capillary without any loss (Fig. 2).

The sensitivity to insufficiently deactivated glass capillaries caused problems initially as it was not possible to buy a column suitable for analyzing TMS-phenolic acid esters. Therefore, we prepared glass capillary columns using exclusively borosilicate glass. A slightly modified procedure, based on that described by Grob[17], involving acidic leaching, flushing, dehydration, persilylation with tetraphenyldimethyldisilazane (TPDMDS)[18] and static coating[19–21], gave excellently deactivated capillary columns for use up to 340°C. Column evaluation was performed according to Donike[22] because this is a temperature-programmed test at elevated temperatures using fatty acid TMS esters. Fig. 1B shows the chromatogram of fifteen methyl and ethyl esters of hydroxybenzoic and hydroxycinnamic acids separated on a glass capillary column produced as described.

TABLE III

RELATIVE RETENTION TIMES OF METHYL AND ETHYL ESTERS OF HYDROXYBENZOIC AND HYDROXYCINNAMIC ACIDS ON THREE STATIONARY PHASES RELATED TO TRICOSANE (C_{23}) AND 3-*tert.*-BUTYL-4-HYDROXYANISOLE (BHA)

Compound	*OV-1*		*OV-73*		*Dexsil 300*	
	BHA	C_{23}	*BHA*	C_{23}	*BHA*	C_{23}
Methyl salicylate	0.686	0.214	0.737	0.286	0.643	0.209
Ethyl salicylate	0.809	0.254	0.852	0.331	0.814	0.266
Methyl 4-hydroxybenzoate	0.872	0.272	0.902	0.350	0.929	0.301
BHA	1.000	0.313	1.000	0.388	1.000	0.324
Ethyl 4-hydroxybenzoate	1.050	0.329	1.047	0.407	1.146	0.374
Methyl vanillate	1.270	0.396	1.235	0.479	1.372	0.445
Ethyl vanillate	1.467	0.460	1.354	0.526	1.523	0.494
Methyl gentisate	1.493	0.465	1.381	0.536	1.569	0.512
Methyl protocatechuate	1.536	0.479	1.394	0.541	1.569	0.509
Ethyl gentisate	1.660	0.521	1.475	0.573	1.675	0.546
Methyl syringate	1.664	0.519	1.530	0.594	1.745	0.569
Ethyl protocatechuate	1.725	0.541	1.532	0.595	1.815	0.589
Methyl 4-coumarate	1.732	0.540	1.589	0.617	1.824	0.591
Ethyl syringate	1.916	0.601	1.512	0.665	1.983	0.647
Ethyl 4-coumarate	1.916	0.601	1.722	0.668	2.004	0.649
Methyl gallate	2.104	0.656	1.784	0.693	2.089	0.665
Methyl ferulate	2.142	0.667	1.892	0.735	2.131	0.695
Ethyl gallate	2.244	0.704	1.894	0.735	2.275	0.738
Ethyl ferulate	2.364	0.741	2.019	0.784	2.404	0.779
Methyl caffeate	2.374	0.740	2.061	0.800	2.478	0.809
Ethyl caffeate	2.579	0.808	2.176	0.845	2.587	0.844
Methyl sinapate	2.592	0.809	2.237	0.868	2.709	0.879
Ethyl sinapate	2.785	0.873	2.388	0.927	2.880	0.939
C_{23}	3.208	1.000	2.534	1.000	3.073	1.000

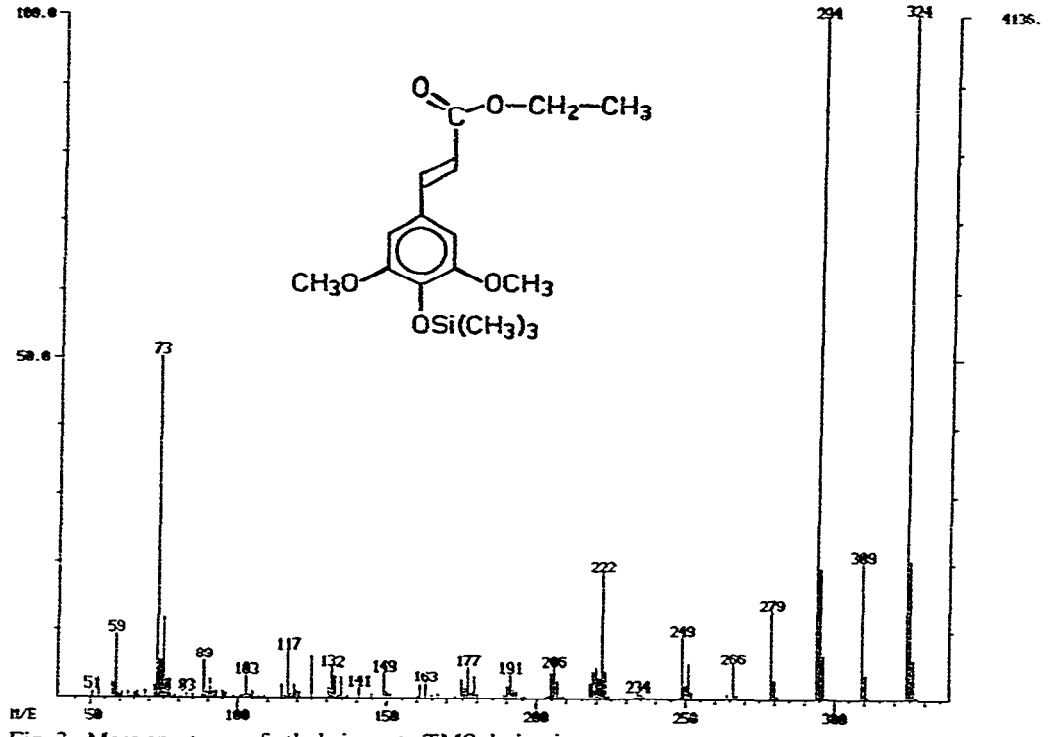


Fig. 3. Mass spectrum of ethyl sinapate TMS derivative.

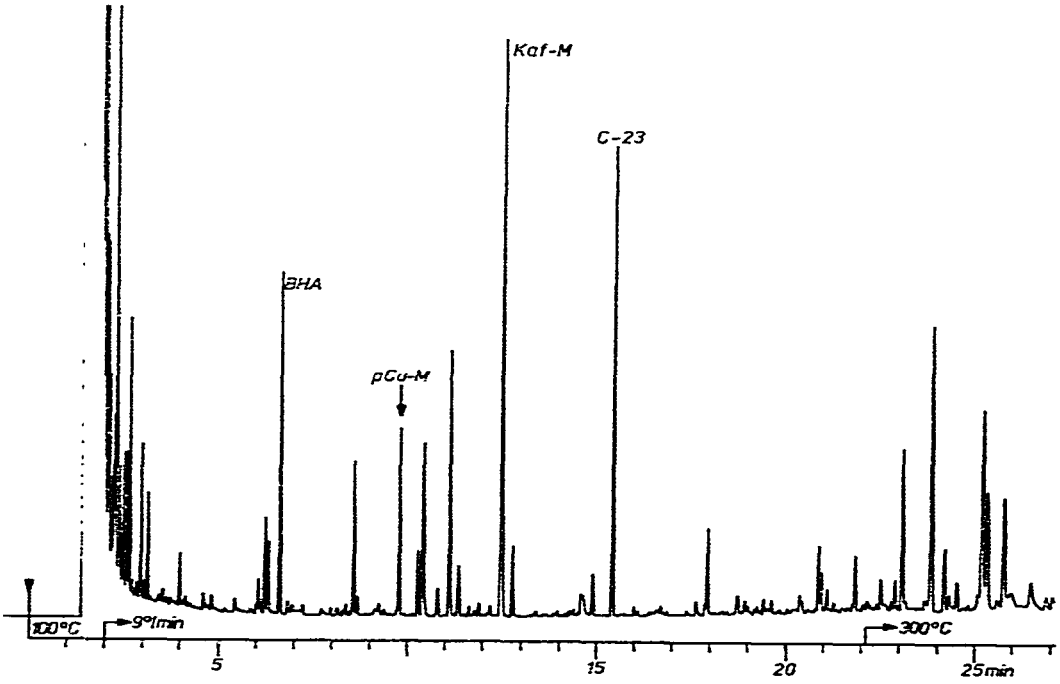


Fig. 4. Chromatogram of extract from fresh potato peelings. Peaks: pCu-M = methyl-4-coumarate; Kaf-M = methyl caffeate.

RESULTS AND DISCUSSION

Gas–liquid chromatography

In Table III the relative retention time data for 22 methyl and ethyl esters of phenolic acids (TMS derivatives) are listed. Relative retention time was related to both tricosane and BHA for three stationary phases, OV-1, OV-73 and Dexsil 300. The retention behaviour on different stationary phases is shown in Fig. 1B–D. Methyl and ethyl esters of salicylic acid cannot be determined with the described extraction method because of their great volatility and these esters are not retained on the polyamide column during washing with water. Methods for determination of methyl and ethyl salicylate have been described for example in refs. 23–25.

Mass spectrometry

The mass spectra of silylated methyl and ethyl esters of hydroxybenzoic and hydroxycinnamic acids show molecular ion peaks of high relative intensity (Fig. 3) if the ion source temperature is not too high (130°C). The compounds would therefore be readily amenable to single-ion monitoring (SIM). Often the ion $(CH_3)_3Si^+$, *m/e* 73, constitutes the base peak, especially when the ion source temperature exceeds 130°C.

The fragmentation pattern of the methyl and ethyl esters of the hydroxybenzoic acids is similar to that of the methyl esters of TMS-cinnamic acids discussed by Horman and Viani[15]. The esters with two neighbouring hydroxyl groups and TMS substituents on the aromatic ring show simple spectra. Besides the molecular ion peak

TABLE IV

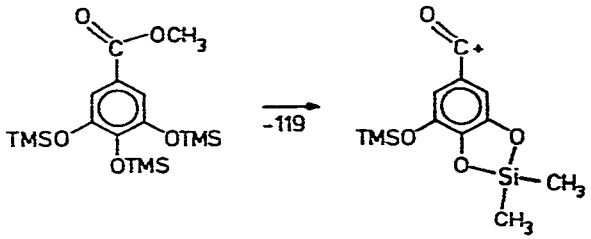
MASS SPECTRAL DATA FOR THE METHYL AND ETHYL ESTERS OF HYDROXYBENZOIC AND HYDROXYCINNAMIC ACIDS (TMS DERIVATIVES)

Data in *m/e*, relative intensities in parentheses. M = Molecular ion; BP = base peak. Conditions: Finigan 4023 gas chromatography–quadrupole mass spectrometry system with open-split interface[26]; interface, 250°C; ion source, 130°C; electron beam energy, 70 eV; ionizing current, 300 μA.

Compound	*M*	*BP*	*Other characteristic ions*
Methyl 4-coumarate	250(100)	250(100)	235(66),219(40),203(39)
Ethyl 4-coumarate	264(100)	264(100)	219(53),249(35),203(30),192(37)
Methyl ferulate	280(72)	72(100)	250(97),217(85)
Ethyl ferulate	294(100)	294(100)	264(92)
Methyl caffeate	338(50)	219(100)	
Ethyl caffeate*	352(22)	73(100)	219(66)
Methyl sinapate	310(78)	280(100)	
Ethyl sinapate	324(100)	324(100)	294(97)
Methyl salicylate*	224	59(100)	209(88)
Ethyl salicylate*	238	195(100)	223(44)
Methyl 4-hydroxybenzoate*	224(62)	209(100)	193(28)
Ethyl 4-hydroxybenzoate*	238(18)	73(100)	193(32)
Methyl vanillate*	254(32)	224(100)	239(56),193(62)
Ethyl vanillate*	268(26)	73(100)	253(48),193(56)
Methyl gentisate*	312	297(100)	267(24)
Ethyl gentisate*	326	73(100)	311(17),239(45)
Methyl protocatechuate*	312(18)	193(100)	
Ethyl protocatechuate*	326(12)	193(100)	
Methyl syringate*	284(24)	254(100)	269(50),223(39)
Ethyl syringate*	298(12)	73(100)	283(18),268(43)
Methyl gallate*	400(6)	73(100)	281(15)
Ethyl gallate*	414(7)	73(100)	281(34)

* Varian/MAT 44S gas chromatography–quadrupole mass spectrometry system modified with a Carlo-Erba split 76; interface, 220°C; ion source, 220°C; electron beam energy, 70 eV; ionizing current, 300 μA.

there are only two other peaks in the spectrum of methyl gallate for example, *m/e* 73 and *m/e* M − 119. M − 119 is of high intensity and seems to be formed by cleavage of the methyl ester group, one TMS group and another methyl group, forming a ring of high stability resulting in an intensive peak at *m/e* 281.



M = 400 281

Application

The method described in this paper was applied successfully to the qualitative and quantitative analysis of methyl and ethyl esters of phenolic acids in vegetables, for example, and potato peels (Fig. 4). The results will be reported elsewhere[27].

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