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DERIVATIZATION OF PHENOLIC ACIDS FOR CAPILLARY GAS CHROMATOGRAPHY WITH HYDROGEN FLAME IONIZATION AND ELECTRON-CAPTURE DETECTION

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SUMMARY

Ten methods of derivatization of phenolic acids and the suitability of the derivatives so obtained for non-polar (SE-30, OV-1) capillary gas chromatographic (GC) quantitation with the flame ionization detection (FID) and electron-capture detection (ECD) have been studied. The phenolic acids included three benzoic and three *trans*-cinnamic acids, both series consisting of 4-hydroxy, 4-hydroxy-3-methoxy and 4-hydroxy-3,5-dimethoxy derivatives. The trimethylsilyl (TMS) ethers of the phenolic acid methyl esters, the TMS ethers of the phenolic acid TMS esters and the heptafluorobutyrates of the phenolic acid methyl esters were found to be suitable for quantitation with FID and the first mentioned derivatives are preferred. For quantitation with ECD, the perfluorobutyrates of the phenolic acid methyl esters were the best derivatives. The quantitation of phenolic acids by GC–FID is suitable for samples with concentrations in the range of 0.01–10 μg per injection. The concentrations applicable to GC–ECD quantitation are much lower, 0.05–1 ng per injection.

INTRODUCTION

Derivatization is a common praxis when analyzing relatively non-volatile compounds and polar molecules including various functional groups by gas and liquid chromatography. There are also many specific reasons for the use of derivatives, *e.g.*, (i) to improve the resolution of closely related compounds, (ii) to increase the detector response for trace components and (iii) to utilize selective detection such as electron-capture detection (ECD) and nitrogen-phosphorus detection (NPD), which allow the analysis of complex mixtures without prior separation from the matrix. Numerous examples of applications of various derivatization reactions have been described[1–3].

Extracts from soils contain small amounts of phenolic acids which occur together with humic substances[4]. Extractable matter from Finnish peat was found to contain phenolic acids although at a very low concentration[5,6]. Phenolic acids must be modified prior to their gas chromatography (GC) and numerous derivatization methods have been presented[1,3]. The present paper describes the derivatization of simple phenolic acids with various reagents to test their analytical usefulness for GC

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quantitation with flame ionization detection (FID) and ECD. The model compounds include various benzoic and cinnamic acids with one free phenolic hydroxyl group, one carboxyl group and 0, 1 or 2 methoxy groups.

EXPERIMENTAL

Reagents and solvents

The following phenolic acids (purities 97–99%) were obtained from EGA-Chemie (Steinheim, F.R.G.): 4-hydroxy-, 4-hydroxy-3-methoxy- and 4-hydroxy-3,5-dimethoxybenzoic acids, and *trans*-4-hydroxy-, *trans*-4-hydroxy-3-methoxy- and *trans*-4-hydroxy-3,5-dimethoxycinnamic acids. 3-Hydroxy-4-methoxybenzoic acid (99%, EGA-Chemie) was used as an internal standard.

The derivatization reagents were: N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane (TMCS) (purum; Fluka, Buchs, Switzerland), pentafluorobenzoyl chloride (PFBC) (98%, EGA-Chemie), bromoacetyl chloride (BAC) (Sigma, St. Louis, MO, U.S.A.), chloroacetic anhydride (CAA) (Sigma), iodoacetic anhydride (IAA) (Sigma), trifluoroacetic anhydride (TFAA) (99%, EGA-Chemie), heptafluorobutyric anhydride (HFBA) (EGA-Chemie), pyridine (silylation grade; Chrompack, Middelburg, The Netherlands), Diazald (N-methyl-N-nitroso-*p*-toluenesulphonamide) (99%, EGA-Chemie), potassium hydroxide (purum; EKA, Surte, Sweden). Diethyl ether, ethyl acetate, methanol (all of analytical grade) and toluene (LiChrosolv) were supplied by E. Merck (Darmstadt, F.R.G.). Ethanol (Aa, 99.5 wt. %) was obtained from Oy Alko Ab (Helsinki, Finland). All reagents and solvents were used as received.

Gas chromatography

All the products formed in the reactions between the reagents and phenolic acids in the model compound mixture were analyzed on a Micromat HRGC 412 gas chromatograph (Orion Analytica, Finland) equipped with both flame ionization and electron-capture detectors. The two-channel instrument was adapted with two similar wall-coated open tubular (WCOT) fused-silica capillary columns (25 m × 0.32 mm I.D.) coated with SE-30 silicone polymer at a film thickness of 0.25 μm (Oriola Prolab, Finland), or with one WCOT fused-silica capillary column (50 m × 0.20 mm I.D.) with chemically bonded OV-1 stationary phase at a film thickness of 0.10 μm (Oriola Prolab) (connected to the operating channel). Hydrogen was used as the carrier gas at flow-rates of 2.7 ml/min (0.50 bar) for the SE-30 columns and 0.6 ml/min (1.30 bar) for the OV-1 column (at 120°C). The make-up gases for FID were hydrogen (0.30 bar) and air (1.00 bar). The optimum standing current for ^{63}Ni-ECD was adjusted with the make-up gas (argon–methane, 95:5), 0.70–1.00 bar, and the compensation current, 180–255 units. The splitting ratio was adjusted to about 30:1, and the injection volume was 0.7 μl for the SE-30 columns and 0.2–0.3 μl for the OV-1 column. The temperature was raised from 120 to 270°C at 8°C/min (SE-30) or from 100 to 250°C at 4°C/min (OV-1). The injector and detector temperatures were maintained at 300°C. The peak areas were integrated with the built-in microcomputer and the chromatograms and data were printed with a Micromat printer-plotter.

Ultrasonication

An ordinary laboratory ultrasound cleaner with a capacity of 2.7 l was used (USF Finnsonic W 181, Oy Ultra sonic finland, Finland).

Derivatization procedures

Separate stock solutions were prepared for benzoic and cinnamic acids by dissolving them in ethyl acetate (Table I). The derivatization reactions were carried out in 3-ml test-tubes with PTFE cup liners. Samples for derivatization were made by mixing 50 μl of each stock solution (Table I) and adding 50 μl of the internal standard solution (17.22 mg of 3-hydroxy-4-methoxybenzoic acid in 50 ml of ethyl acetate). 3-Hydroxy-4-methoxybenzoic acid, a phenolic acid found naturally in negligible amounts if at all, was chosen as an internal standard to illustrate isomer resolution of the various derivatives produced (the samples contain 4-hydroxy-3-methoxybenzoic acid, 2, an isomer of the internal standard). Samples of the derivatization products were introduced into the gas chromatograph by a 10-μl Hamilton syringe.

TABLE I

STOCK SOLUTIONS OF PHENOLIC ACIDS IN ETHYL ACETATE

		Weighed amount (mg)	*Purity* (%)*	*Concentration (mmol/50 ml)*
Mixture of benzoic acids				
4-Hydroxy-	(1)	14.32	99	0.103
4-Hydroxy-3-methoxy-	(2)	18.32	97	0.106
4-Hydroxy-3,5-dimethoxy-	(3)	20.82	97	0.102
Mixture of cinnamic acids				
trans-4-Hydroxy-	(4)	17.11	98	0.102
trans-4-Hydroxy-3-methoxy-	(5)	20.10	98	0.101
trans-4-Hydroxy-3,5-dimethoxy-	(6)	24.90	98	0.109

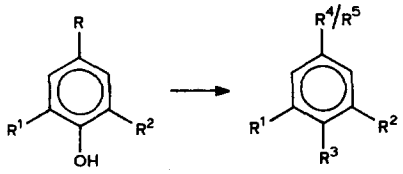
* According to the supplier.

All of the six phenolic acids (Table I) contain one free carboxyl group and one free phenolic hydroxyl group together with 0, 1 or 2 methoxy groups. In one of the ten derivatization procedures (A, Table II) the phenolic hydroxyl group was left free. All the other derivatization procedures (B to J, Table II) are capable of transforming both free carboxyl and phenolic hydroxyl groups into less polar groups. In all cases the aromatic carboxylic acids were esterified by diazomethane to methyl esters, except in procedure D which gave trimethylsilyl (TMS) esters. After esterification the free phenolic hydroxyl group was chemically modified in different ways. All ten procedures A to J are outlined in Table II and described below in more detail.

(A) *Methyl ester derivatives.* The phenolic acids were transformed into their methyl esters by diazomethane which was freshly prepared from Diazald and potassium hydroxide. After evaporating the solvent from the prepared model compound mixture under a stream of nitrogen, the residue was dissolved in 0.3 ml of diethyl

TABLE II

DERIVATIVES OF PHENOLIC ACIDS



	R	R^1	R^2
1	$-CO_2H$	H	H
2	$-CO_2H$	H	$-OCH_3$
3	$-CO_2H$	$-OCH_3$	$-OCH_3$
4	$-CH=CHCO_2H$	H	H
5	$-CH=CHCO_2H$	H	$-OCH_3$
6	$-CH=CHCO_2H$	$-OCH_3$	$-OCH_3$

Procedure	*Temp. (°C)*	*Time*	*Reagent*	*Derivatives**		
				R^3	*A* R^4	*B* R^5
A	−15	0.5 min	CH_2N_2	HO–	CH_3OOC-	$CH_3OOCCH=CH-$
B	55	24 h	CH_2N_2	CH_3O-	CH_3OOC-	$CH_3OOCCH=CH-$
C	−15	0.5 min	CH_2N_2 +	TMSO–	CH_3OOC-	$CH_3OOCCH=CH-$
	80	30 min	BSTFA/TMCS			
D	80	30 min	BSTFA/TMCS	TMSO–	TMSOOC–	TMSOOCCH=CH–
E	−15	0.5 min	CH_2N_2 +	C_6F_5COO	CH_3OOC-	$CH_3OOCCH=CH-$
	20	9 min	PFBC			
F	−15	0.5 min	CH_2N_2 +	$BrCH_2COO$	CH_3OOC-	$CH_3OOCCH=CH-$
	20	9 min	BAC			
G	90	72 h	CAA +	$ClCH_2COO$	CH_3OOC-	$CH_3OOCCH=CH-$
	−15	0.5 min	CH_2N_2			
H	90	24 h	IAA +	ICH_2COO	CH_3OOC-	$CH_3OOCCH=CH-$
	−15	0.5 min	CH_2N_2			
I	−15	0.5 min	CH_2N_2 +	CF_3COO	CH_3OOC-	$CH_3OOCCH=CH-$
	20	1 h	TFAA			
J	−15	0.5 min	CH_2N_2 +	$CF_3(CF_2)_2COO$	CH_3OOC-	$CH_3OOCCH=CH-$
	80	1 h	HFBA			

* R^1, R^2 as in the starting compounds. A = Benzoic acid esters; B = cinnamic acid esters.

ether–methanol (9:1, v/v). After cooling at −15°C for 5 min, dilute diazomethane in diethyl ether solution (−15°C) was added until a slight yellow colour persisted. In order to prevent the reaction of phenolic hydroxyl groups, the diazomethane was removed within 30 s at −15°C under a stream of nitrogen, whereafter the solvent was evaporated. The residue was dissolved in toluene (100 µl) and an aliquot was analyzed by GC.

(B) *Methyl ester–methyl ether derivatives.* After evaporating the solvent under nitrogen, the phenolic acid residue was dissolved in diethyl ether–methanol (9:1, v/v) and an excess of the ethereal diazomethane (0.5 ml) was added. After keeping the mixture at 55°C for 6 h, an additional amount (1 ml) of diazomethane solution was added and the mixture was kept at the same temperature for 18 h. After evaporating the excess of diazomethane and the solvent, the residue was dissolved in toluene (100 µl) prior to GC analysis.

(C) *Methyl ester–TMS ether derivatives.* The carboxyl groups were methylated by procedure A. After evaporating the solvent under nitrogen, the residue was dissolved in 70 µl of pyridine and 30 µl of BSTFA containing 1% of TMCS were added.

After heating at 80°C for 30 min, the mixture was analyzed by GC.

(D) *TMS ester–TMS ether derivatives.* To trimethylsilylate both the carboxyl and hydroxyl groups of phenolic acids simultaneously, method C was used except that the methyl esterification step was excluded. The derivatives obtained were found to decompose partly within a few hours.

(E) *Methyl ester–pentafluorobenzoate derivatives.* The carboxyl groups of the phenolic acids were carefully methylated by diazomethane as described in procedure A. After evaporating to dryness under nitrogen, the sample was dissolved in 0.2 ml of pyridine, and 10 μl (16 mg, 0.069 mmol) pentafluorobenzoyl chloride (PFBC) were added. The mixture was shaken occasionally for 5 min at room temperature and the reaction was completed by ultrasonication for 4 min. Then 2 ml of diethyl ether and 0.5 ml of 0.1 *M* hydrochloric acid were added and the mixture was shaken vigorously. After separation of two phases, the ether layer was isolated, taken up and washed three times with 0.5 ml of distilled water. The ether solution was dried over anhydrous sodium sulphate overnight. After evaporation of the solvent, the residue was dissolved in toluene (100 μl) and analyzed by GC.

(F) *Methyl ester–bromoacetate derivatives.* To prepare these derivatives the method was essentially the same as E but 10 μl (20 mg, 0.077 mmol) of bromoacetyl chloride (BAC) were used instead of PFBC.

(G) *Methyl ester–chloroacetate derivatives.* To a solution of the phenolic acid mixture in 0.2 ml of ethyl acetate were added 20 mg (0.117 mmol) of chloroacetic anhydride (CAA). Then the mixture was heated in a sealed test-tube at 90°C for 72 h. The solvent and the excess of reagent were evaporated under nitrogen at 60°C in a water-bath. The residue was dissolved in 0.3 ml of diethyl ether–methanol (9:1, v/v) and the carboxyl groups were esterified by diazomethane according to procedure A. The resulting methyl ester–chloroacetate derivatives were then dissolved in 100 μl of toluene prior to GC analysis.

(H) *Methyl ester–iodoacetate derivatives.* This procedure was similar to that of G with two exceptions: (i) 20 mg (0.057 mmol) of iodoacetic anhydride (IAA) were used instead of CAA, and (ii) the reaction time was 24 h at 90°C which, however, was found to be insufficient to complete the reaction.

(I) *Methyl ester–trifluoroacetate derivatives.* The carboxyl groups of the phenolic acids were esterified by diazomethane according to procedure A. After evaporating the solvent under nitrogen, the residue was dissolved in 50 μl of ethyl acetate and 50 μl (75 mg, 0.355 mmol) of trifluoroacetic anhydride (TFAA) were added. The mixture was then kept at room temperature. Within 1 h the reaction went to completion. Then the excess of reagent and the solvent were evaporated at room temperature and the residue was dissolved in 100 μl of toluene prior to GC analysis.

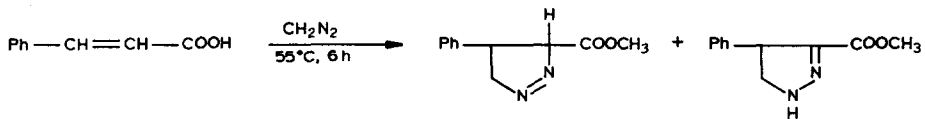
(J) *Methyl ester–heptafluorobutyrate derivatives.* This procedure was similar to I except that 50 μl (83.5 mg, 0.204 mmol) of heptafluorobutyric anhydride (HFBA) were used instead of TFAA and the reaction was carried out in a water-bath at 80°C for 1 h, which led to completion of the reaction. The reaction was quantitative but a longer reaction time (6 h) was found to result in a partial decomposition of the cinnamic acid derivatives.

RESULTS AND DISCUSSION

Methylation

Methylation of both carboxyl and phenolic hydroxyl groups occurs in diethyl ether–methanol solution (9:1, v/v) by ethereal diazomethane. In procedure A the esterification was carried out at −15°C with a dilute diazomethane-solution and the slight excess of diazomethane was removed immediately after reaction under stream of nitrogen to prevent ether formation. The methyl esters of the phenolic acids thus obtained contain a free phenolic hydroxyl group, which causes peak tailing on non-polar columns (and with slightly polar columns, too) and thus these derivatives are not very suitable for GC quantitation. The derivatization of the phenolic hydroxyl group with different reagents gives, however, as we shall see, very valuable derivatives and thus the carboxyl group methylation will be very meaningful.

For methyl ether formation, an elevated temperature (55°C), longer reaction time (24 h) and an excess of diazomethane were required (procedure B). The benzoic acids reacted quantitatively in 6 h, but no adequate reaction conditions were found for the cinnamic acids. Under the reaction conditions applied here some side products were formed from cinnamic acids when diazomethane reacted with the double bond activated by the adjacent aromatic ring (ref. 3, pp. 523–524):



Thus the simultaneous methylation of carboxyl and hydroxyl groups was found to be unsatisfactory for quantifying phenolic acids containing cinnamic acids or structurally related compounds. The procedure is unsuitable also for quantitation of hydroxybenzoic acids, if the sample contains corresponding methoxybenzoic acids.

Trimethylsilylation

The reagent BSTFA containing 1% TMCS is a very powerful silylating agent particularly for compounds with an acidic hydrogen atom, *e.g.*, carboxylic acids and phenols. The two-stage procedure C which includes methyl esterification of the carboxyl group and trimethylsilylation of the phenolic hydroxyl group is useful for quantitation of phenolic acids with FID. All phenolic acids in the model compound mixture were found to react quantitatively and the reaction products were stable for several days. Thus the trimethylsilyl ethers of the phenolic acid methyl esters are very suitable for the GC–FID analysis. Appropriate concentrations are 0.01–10 μg of phenolic acid per injection. When ECD was applied the responses for the cinnamic acid derivatives were much greater than those of the benzoic acid analogues. This is probably due to the conjugated electron-rich system comprised of a carboxyl group, a double bond in the side chain, a benzene ring and a phenolic hydroxyl group[7]. So these derivatives are useful also in cinnamic acid trace analysis when ECD is used. The gas chromatograms of the trimethylsilyl ethers of the phenolic acid methyl esters using FID and ECD are illustrated in Fig. 1.

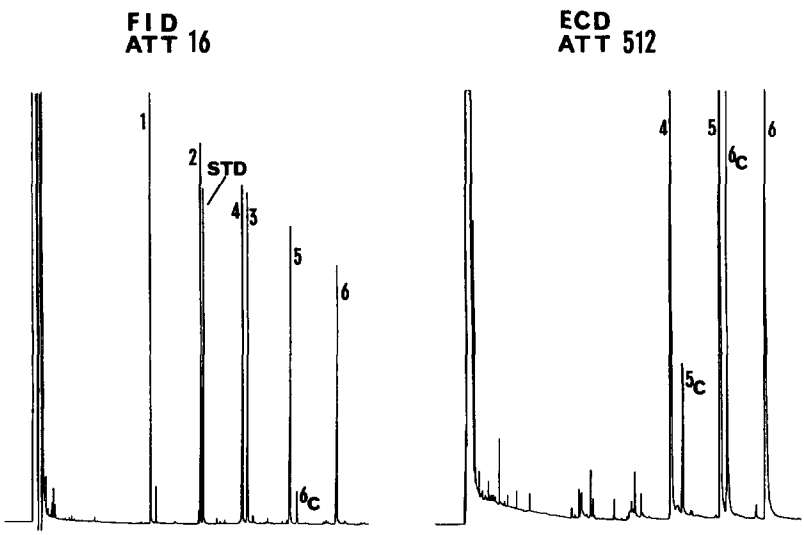


Fig. 1. Capillary gas chromatograms of the TMS ethers of phenolic acid methyl esters using FID and ECD. Peaks: 1 = 4-hydroxybenzoic acid; 2 = 4-hydroxy-3-methoxybenzoic acid; 3 = 4-hydroxy-3,5-dimethoxybenzoic acid; 4 = *trans*-4-hydroxycinnamic acid; 5 = *trans*-4-hydroxy-3-methoxycinnamic acid; 5C = *cis*-4-hydroxy-3-methoxycinnamic acid; 6 = *trans*-4-hydroxy-3,5-dimethoxycinnamic acid; 6C = *cis*-4-hydroxy-3,5-dimethoxycinnamic acid. STD = 3-hydroxy-4-methoxybenzoic acid as an internal standard. GC conditions: WCOT fused-silica capillary column (50 m × 0.20 mm I.D.) coated with chemically bonded OV-1, film thickness 0.10 μm; 1.3 bar hydrogen as the carrier gas; splitting ratio 30:1; attenuation (ATT) 16 for FID, 512 for ECD; column temperature raised from 100 to 250°C at 4°C/min.

The trimethylsilylation reactions both of carboxyl and hydroxyl groups were also found to proceed rapidly and quantitatively when procedure D was applied. These trimethylsilyl ethers of phenolic acid trimethylsilyl esters were also suitable for GC–FID analysis and cinnamic acid trace analysis when ECD was used. The resolution of the isomers 4-hydroxy-3-methoxybenzoic acid and 3-hydroxy-4-methoxybenzoic acid (internal standard) was, however, unsatisfactory when compared to the trimethylsilyl ether–methyl ester derivatives. The quantitation of these derivatives by GC should also be made immediately after derivatization because of the partial decomposition which was found to occur within a few hours.

Derivatization of phenolic hydroxyl group

The free hydroxyl groups in phenolic acid methyl esters were treated by six different derivatization reactions presented in Table II. Procedures E to J resulted in pentafluorobenzoates, various halogenoacetates and heptafluorobutyrates. Four procedures, namely E (reagent PFBC), G (CAA), I (TFAA) and J (HFBA) gave reaction products which were suitable for quantitation, but procedures F (BAC) and H (IAA) failed in this respect. In procedure F a decomposition of the reagent resulted in bromine which may react further with, *e.g.*, the double bonds of the cinnamic acid esters. In procedure H, 24 h at 90°C was insufficient for complete reaction. Some extraneous reaction products were also formed. Pentafluorobenzoates (procedure E) were formed quantitatively and they have high ECD responses, as do the other halo-

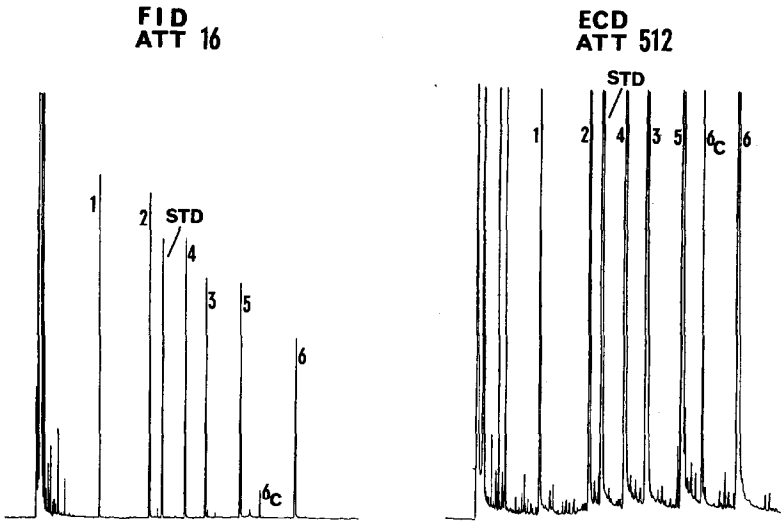


Fig. 2. Capillary gas chromatograms of the heptafluorobutyrates of phenolic acid methyl esters using FID and ECD. For peak identification and GC conditions, see Fig. 1.

genated derivatives. The derivatization was, however, complicated and troublesome. Chloroacetates which were obtained quantitatively by procedure G were useful derivatives for ECD. The drawback of this method is the long reaction time (72 h at 90°C). The trifluoroacetates (procedure I) and heptafluorobutyrates (procedure J) were also applicable to ECD and were prepared in 1 h which is short enough for practical work. The latter derivatives of cinnamic acid esters were found partly to decompose when longer reaction times and higher temperatures were applied. In the case of trifluoroacetates the acidification of the reaction mixture through the formation of trifluoroacetic acid may also promote decomposition of cinnamic acids. Newby *et al.*[8] have reported that hydroxycinnamic acids rapidly decompose in acidic and hot solution, whereas *p*-hydroxybenzoic acids are stable under the same conditions. The heptafluorobutyrates and the trifluoroacetates of phenolic acid methyl esters will, however, be the most useful derivatives in quantitation with ECD. The heptafluorobutyrated derivatives are preferred because of their higher stability and greater detector response. They are very suitable for quantiation by FID, too. The gas chromatograms of the heptafluorobutyrates of the phenolic acid methyl esters using both FID and ECD are illustrated in Fig. 2. In general, in our study a slight isomerization of cinnamic acids from *trans* to *cis* during their derivatization reactions could be observed.

CONCLUSIONS

The suitability of the different derivatives of phenolic acids prepared for GC quantitation with FID and ECD and the feasibility of various derivatization procedures can be summarized as follows:

1. The trimethylsilyl ethers of the phenolic acid methyl esters are the most

suitable derivatives for the GC quantitation with FID. In the first step of the derivatization procedure the carboxyl group is methylated with dilute ethereal diazomethane at −15°C and then in the second step the phenolic hydroxyl group is trimethylsilylated with BSTFA at 80°C for 30 min. The procedure is simple, rapid and quantitative. Resolution of isomers is adequate and the products are stable for several days. The GC quantitation is suitable for samples with relatively high concentrations of phenolic acids (0.01–10 μg per injection). Total trimethylsilylation of phenolic acids will also give suitable derivatives for GC–FID, but the resolution of isomers is not satisfactory. The reaction products are also unstable, partly decomposing within a few hours. The EC detection sensitive derivatives, heptafluorobutyrates of phenolic acid methyl esters will also be suitable for GC–FID quantitation, too, but the procedure J includes an additional step (evaporation–dissolution) and the detector responses are lower when compared to the trimethylsilylated phenolic acid methyl esters.

2. The best derivatives for quantitation with ECD are the heptafluorobutyrates of phenolic acid methyl esters. The derivatization (procedure J) is simple and quantitative. The possible decomposition of cinnamic acids can be prevented if the reaction time does not exceed 1 h at 80°C. The concentration for quantitation can vary in the range 0.05–1 ng of phenolic acid per injection.

ACKNOWLEDGEMENTS

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