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GAS CHROMATOGRAPHIC SEPARATION AND ANALYSIS OF TRIMETHYLSILYL DERIVATIVES OF SOME NATURALLY OCCURRING NON-VOLATILE PHENOLIC COMPOUNDS AND RELATED SUBSTANCES

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SUMMARY

The gas–liquid chromatographic (GLC) separation of a group of naturally occurring phenolic compounds embracing benzaldehyde, benzoic acid, cinnamaldehyde, cinnamic acid, coumarin, flavone and isoflavone and/or derivatives has been studied. By using N,N-bis(trimethylsilyl)trifluoroacetamide as a silylating agent, a glass column packed with Chromosorb W AW DMCS (80–100 mesh) coated with 1.5% SE-30 + 1.5% SE-52 and a temperature program about thirty-six of the above substances could be separated in a single run. Using preparative GLC, together with a new collecting device, it was found that the yield of trimethylsilyl (TMS) ^{14}C-labelled *p*-coumaric or ferulic acid was 81 to 85%. After hydrolysis of the collected radioactive TMS compounds it was further shown, via thin-layer chromatography and autoradiography, that only labelled *p*-coumaric or ferulic acid were present, indicating that little decomposition had occurred during GLC. In addition, ultraviolet, infrared, proton magnetic resonance and mass spectrometry were employed for studying the structures and conditions of the collected TMS derivatives from kaempferol, pyrocatechol and a series of phenolic acids.

All the above analyses confirmed that the compounds were not appreciably altered during GLC. It is further suggested that preliminary multiple elimination thin-layer chromatographic separation and purification of eventually unknown phenolic substances from plant extracts, in combination with the described GLC technique and the employed physical methods, will be of great value for quantitative analysis and structure determination.

INTRODUCTION

Phenolic compounds, which are widespread in nature, are frequently substances of physiological or economic interest[1–6]. Studies have therefore been made in order to improve the isolation, separation and determination of these compounds from plant and animal sources.

As indicated by Dallos and Koeppl[7], early attempts to determine phenolic compounds were limited to non-specific methods using Gibbs', nitrosophenol, 4-aminoantipyrine and other reagents[8]. Use of a combination of paper chromatography and some of the above quantitative techniques overcame, with certain phenolic compounds, the lack of specificity of the reagents[9,10]. However, the published methods are time consuming and are limited in separation power.

Thin-layer chromatographic (TLC) methods for the separation of phenolic compounds provide good resolution[3,11–16] and multiple elimination TLC (METLC)[5,17–20], in combination with ultraviolet (UV) or fluorimetric analysis, allows, theoretically, the separation and quantitative determination of almost every naturally occurring phenol[21]. However, the above methods, although usually faster than paper chromatography and far superior in separation power, are still time consuming and less satisfactory for phenols, which are either readily oxidized on the thin layers or not easily eluted from the plates. Gas chromatography does not suffer from these drawbacks. It has the required resolution and speed and, together with subsequent mass spectrometry or quantitative analysis, is therefore of great value in the study of naturally occurring phenols. However, as the use of gas chromatography is limited to volatile samples[22], it prohibits the direct analysis of most substituted, non-volatile and thermally unstable phenols[7,23].

Therefore, volatile methyl ethers[24,25] and trimethylsilyl (TMS) derivatives have been prepared in order to improve the gas–liquid chromatographic (GLC) analysis of phenolic compounds. For the preparation of the TMS derivatives reagents such as hexamethyldisilazane (HMDS), dimethylchlorosilane (DMCS) and trimethylchlorosilane (TMCS)[26–35] have been employed. Unfortunately, the above silylating agents suffer from multiple substitution and low efficiency[7]. Therefore, N,N-bis(trimethylsilyl)acetamide (BSA), a compound synthesized and applied by Klebe *et al.*[36], has also been used for phenols by several authors[7,37–39].

More recently, N,N-bis(trimethylsilyl)trifluoroacetamide (BSTFA) has been synthesized by Gehrke and Stalling[40] and employed as an active silylating agent for amino acids[41–43], purines and pyrimidines[44–46], some flavonoids[47] and the *cis* and *trans* isomers of *p*-coumaric, caffeic, ferulic and sinapic acids[48]. BSTFA shows a further increase in volatility (it appears with the solvent front) and has a lower detector response and greater solubility in some solvents than BSA[41]. According to Gehrke *et al.*[41] the fluorine in BSTFA results in fewer deposits of SiO_2 and thus decreases detector noise. Moreover, silylation at 125° for 10 min in a closed tube usually produces a single derivative of the investigated compounds.

We have accordingly carried out the GLC separation of a series of TMS derivatives of naturally occurring phenolic acids (from the benzoic and cinnamic acid series), coumarins, flavonoids and isoflavonoids, using BSTFA. In addition, the UV, infrared (IR) and proton magnetic resonance (PMR) spectra of certain TMS derivatives have been investigated, and the molecular weights, deduced from the mass spectra of these derivatives, isolated after preparative GLC, have been determined. Finally, the recovery and purity of derivatized [2-^{14}C]-*p*-coumaric acid or [2-^{14}C]-ferulic acid, as well as the hydrolysis of the derivatives to the labelled phenolic acids, have also been investigated.

EXPERIMENTAL

Materials

All the benzoic acid and cinnamic acid derivatives, as well as pyrocatechol, *p*-hydroxybenzaldehyde, vanillin, 2,4,6-trihydroxybenzaldehyde, flavone, galangin, catechin, morin, quercetin, naringin, coumarin, umbelliferone, esculetin, scopoletin and chlorogenic acid were purchased from Fluka (Buchs, Switzerland). Herniarin was obtained from K & K Laboratories (Plainview, N.Y., U.S.A.). The authors are further indebted to Dr. R. T. Williams, St. Mary's Hospital Medical School, University of London, Great Britain, for the gift of 3-hydroxy-, 6-hydroxy- and 8-hydroxy-coumarin; to Dr. J. C. Pew, Forest Products Laboratory, U.S. Department of Agriculture, Madison, Wisc., U.S.A., for the gift of 3,4-dimethoxycinnamaldehyde; to Prof. G. Hraszdina, Cornell University, New York State Agricultural Experiment Station, Geneva, N.Y., U.S.A., for the gift of chrysin, apigenin, myricetin and genistein; to Dr. J. B. Harborne, University of Reading, Great Britain, for the gift of rhamnetin; to Dr. J. Sachse, Eidgenössische Forschungsanstalt für Landwirtschaftlichen Pflanzenbau, Zürich, Switzerland, for the gift of daidzein, formononetin, genistein, biochanin A and pratensein.

Kaempferol was synthesized from naringin via kaempferol-7-glucorhamnoside according to the procedure of Pacheco and Grouiller[49]. The hydrolysis of kaempferol-7-glucorhamnoside was also performed as described by these authors[50]. Kaempferol was also obtained from Prof. H. Geiger, Organische Chemie der Universität (LH) Hohenheim, Stuttgart, G.F.R.). 3-O-Cinnamyl-D-(—)-quinic acid was prepared as described by De Pooter *et al.*[51].

Small samples of a mixture of TMS-*cis*- and *trans*-cinnamic acid derivatives were prepared by irradiating the TMS-*trans* compounds with UV light (366 nm) for 16 to 20 h. Thereafter the irradiated samples were subjected to GLC. In the case of ferulic acid the *cis* and *trans* derivatives were quantitatively prepared. For this purpose a concentrated solution of *trans*-ferulic acid (1.3 g in 14 ml of acetone plus 1 ml of water) was irradiated with UV light (366 nm) for 3 weeks. All further manipulations were performed in a dark room under a safelight (orange ICI Perspex filter No. 300). After irradiation the mixture of *trans*- and *cis*-ferulic acids was separated on a cellulose column (70 cm × 80 mm, Whatman Chromedia CF-11) with 2% acetic acid as solvent. Both stereoisomers were collected and the fraction was filtered through a 0.15 μm membrane filter.

After freeze-drying the residues were dissolved in acetone, filtered through Whatmann No. 1 filter paper, concentrated in a vacuum and dried over phosphorus pentoxide. [*cis*-Ferulic acid; yield 174 mg (13.4%); m.p. 112–112.5°; UV spectrum in ethanol: $\lambda_{max.}$ 317 nm, $\lambda_{min.}$ 259 nm, excitation max. (0.1 *N* NaOH) 357 nm, fluorescence max. (0.1 *N* NaOH) 470 nm; R_F (cellulose thin layers: solvent 2% acetic acid) 0.53. *trans*-Ferulic acid; yield 420 mg (32.3%); m.p. 169–170°; UV spectrum in ethanol: $\lambda_{max.}$ 236 and 322 nm, $\lambda_{min.}$ 230 and 262 nm, excitation max. (0.1 *N* NaOH) 357 nm, fluorescence max. (0.1 *N* NaOH) 470 nm; R_F (cellulose thin-layers: solvent 2% acetic acid) 0.25.]

[2-^{14}C]-Ferulic acid (specific activity 33.6 μCi/mmole) was prepared by the method of De Pooter *et al.*[52] and [2-^{14}C]-*p*-coumaric acid (specific activity 16.3 μCi/mmole) was synthesized in an analogous way. Chromosorb W AW DMCS (80–100

mesh), SE-30 and SE-52 were purchased from Varian Aerograph. BSTFA was obtained from Supelco (Bellefonte, Pa., U.S.A.) or E. Merck (Darmstadt, G.F.R.). Chloroform-d was obtained from Aldrich (Milwaukee, Wisc., U.S.A.).

Silica gel G for TLC and TLC aluminium sheets Polyamide 11 F_{254} (20 × 20 cm) layers (thickness 0.15 mm) were obtained from Merck.

MN cellulose powder 300, average particle size *ca.* 10 μm was purchased from Macherey, Nagel & Co. (Düren, G.F.R.). Soluene-100 sample solubilizer was purchased from Packard (Warrenville, Ill., U.S.A.).

METHODS

Trimethylsilylation

Each component (0.2–0.4 mg) was weighed for each mixture prepared. Subsequently, 400 μl of BSTFA were added (a maximum of 4 mg of a compound, or the mixture, could be silylated with 100 μl of the reagent) and the reaction mixture was maintained for 10 min at 125° in a sealed vial. Possible reactions of phenolic compounds and acids with BSTFA are as follows:

$$2\ R\text{–}OH + [(CH_3)_3\text{–}Si]_2 = N\text{–}\overset{\overset{O}{\|}}{C}\text{–}CF_3 \xrightarrow[10\ min]{125^\circ} 2\ R\text{–}O\text{–}Si\text{–}(CH_3)_3 + CF_3\text{–}\overset{\overset{O}{\|}}{C}\text{–}NH_2$$

$$2\ R\text{–}COOH + [(CH_3)_3\text{–}Si]_2 = N\text{–}\overset{\overset{O}{\|}}{C}\text{–}CF_3 \xrightarrow[10\ min]{125^\circ} 2\ R\text{–}\overset{\overset{O}{\|}}{C}O\text{–}Si\text{–}(CH_3)_3 + CF_3\text{–}\overset{\overset{O}{\|}}{C}\text{–}NH_2$$

After cooling, 7-μl aliquots of the reaction mixture were injected directly into the gas–liquid chromatograph.

Gas–liquid chromatography

Analyses were performed on a Varian-Aerograph HY FI Model 600 equipped with a glass column and flame ionization detector or on a Varian-Aerograph Autoprep Model A-700 equipped with the same column and a thermal conductivity detector (temperature 330°). The glass column (3.0 m × 2 mm I.D.) was packed with Chromosorb W AW DMCS (80–100 mesh) coated with 1.5% SE-30 plus 1.5% SE-52.

The carrier gas nitrogen (HY FI Model 600) or helium (Autoprep Model A-700), hydrogen and air flow-rates were 30, 30 and 275 ml/min, respectively.

The injection port temperature was 285°. The column temperatures were programmed as indicated in Fig. 1 (80–210°, programme power 55; 210–300°, programme power 80). The attenuator input, impedance and output sensitivity were 1, 10^7 and 1×, respectively. The detector temperature (Autoprep) was 330° and the filament current 150 mA.

The recorder (0–1.0 mV) chart speed was 1 cm/min. The analysis time amounted to 50 min.

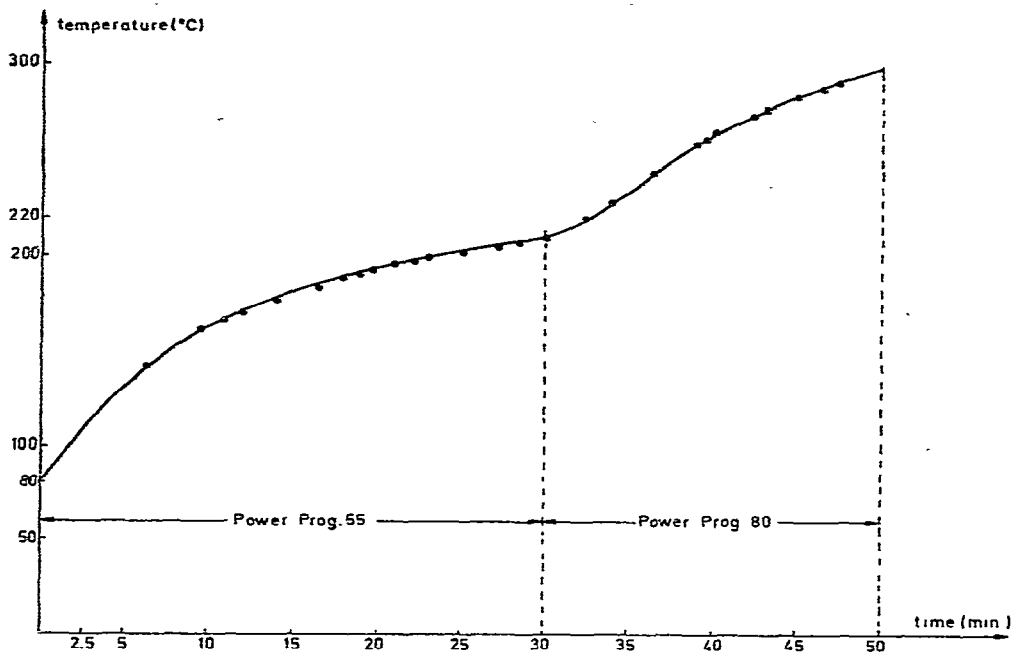


Fig. 1. Programming of the column oven temperature as a function of time.

Collection of TMS derivatives for mass spectrometry, UV, IR, PMR spectra determination and analysis of the radioactivity

For the collection of the TMS derivatives the Autoprep Model A-700 was modified. The auto-collector, chassis and panel sub-assembly were replaced by the collector device represented in Fig. 2. During the collection procedure, the temperature of the collector oven was maintained at least 20° above the column temperature required for the analysis of the compound(s) under investigation.

UV analysis

The collected TMS derivatives ($\pm$40 μg) were dissolved in dry dichloromethane and UV spectra were directly determined with a Shimadzu MPS-50L instrument.

IR analysis

The collected TMS derivatives (0.8 mg on the basis of the phenolic compounds) were again dissolved in dry dichloromethane. After addition of $\pm$125 mg of potassium bromide, the solvent was evaporated and the discs were then pressed in the usual way by application of a pressure of about 8000 kg. For the IR spectra of the TMS derivatives of pyrocatechol and cinnamic acid, an aliquot of the collected material was placed between the sodium chloride windows of a demountable cell. A Beckman IR-8 instrument was used for recording the spectra.

Mass spectrometry

An LKB 9000 gas chromatograph–mass spectrometer (LKB, Stockholm,

Sweden) was used to determine the mass of the parent ion of some of the collected TMS derivatives.

Preparation of samples for PMR analysis

The collected TMS derivatives (from 3 to 6 mg on the basis of the phenolic compounds) were dissolved in 0.25 ml of chloroform-d and the spectra were recorded with a Jeol JNM-PMX 60 instrument.

Radioactivity

The radioactivity was determined by means of liquid scintillation counting using an Automatic Tri-Carb, Model 3380, liquid scintillation spectrometer equipped with an Automatic Activity Analyzer (Packard). After collection, the labelled TMS derivatives were rinsed from the micro-collector glass tubes (Fig. 2a) into the counting vessels using scintillator liquid as a solvent. The soluene from the collector bottle (Fig. 2b) was first treated with 50 μl of hydrochloric acid and then scintillator liquid was added.

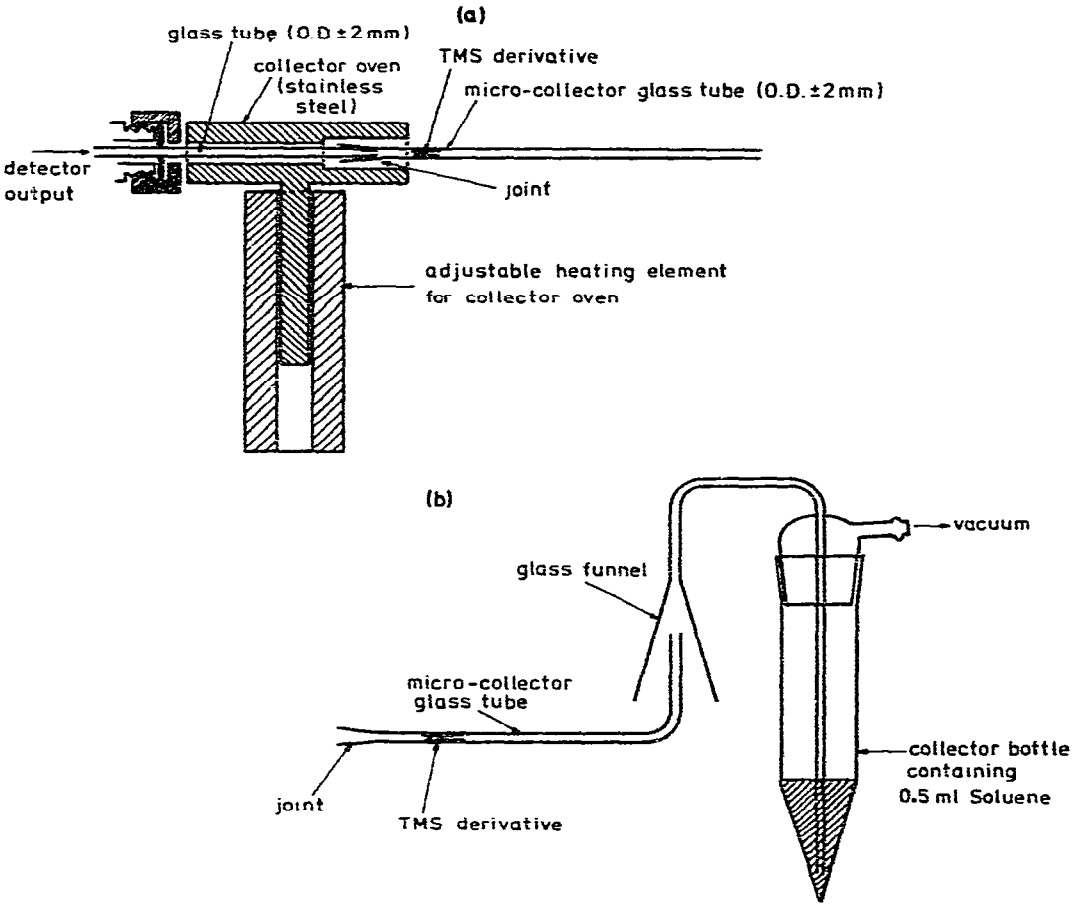


Fig. 2. Transformation of Varian Autoprep Model A-700 for the micro-collection of TMS derivatives (a) and labelled TMS derivatives (b).

Hydrolysis of bis-TMS-[2-^{14}C]-p-coumaric acid and bis-TMS-[2-^{14}C]-ferulic acid

To the bis-TMS derivatives collected in the micro-collector glass tubes (Fig. 2) a drop of water was added and the complete hydrolysis of the TMS derivatives was performed by maintaining the micro-collector glass tube for 48 h at room temperature.

Thin-layer chromatography and autoradiography

Thin-layer chromatography on silica gel–cellulose (1:1) layers was performed as described by Van Sumere and co-workers[3,11], using as solvent benzene–acetic acid (95:5). Before irrigation the stationary aqueous phase of the layers was increased by treatment with water vapour. The R_F values of [2-^{14}C]-*p*-coumaric acid and [2-^{14}C]-ferulic acid are shown in Table III.

Polyamide layers were used to separate esculetin, ferulic acid and isoferulic acid, which were collected as their TMS derivatives in one single peak (202°). Before spotting, the collected derivatives were first hydrolyzed and a good separation was obtained using the benzene–acetic acid solvent. R_F values obtained were: esculetin 0.00, isoferulic acid 0.17 and ferulic acid 0.23. Analogous separations of other compounds that show identical GLC retention volumes can be performed.

Detection of the phenolic compounds after TLC

The compounds were detected as described by Van Sumere and co-workers[3,11].

RESULTS AND DISCUSSION

The GLC separation of the TMS derivatives of a group of naturally occurring phenolic compounds and related substances is represented in Fig. 3. The compounds embrace benzaldehyde, benzoic acid, cinnamaldehyde, cinnamic acid, coumarin, flavone, isoflavone and/or their derivatives. The results show that the silylating agent BSTFA, in combination with a glass column, packed with Chromosorb W AW DMCS coated with 1.5% SE-30 plus 1.5% SE-52, an unsophisticated gas chromatograph and a temperature programme as represented in Fig. 1, gives a very good separation of most of the phenolic compounds.

When certain of the substances were found to have the same retention time, their TMS derivatives were collected and hydrolysed as described. Subsequently, the phenolic compounds were separated on thin-layers of silica gel–cellulose (1:1) or polyamide (see Experimental). When necessary METLC[3,17–20] in combination with chromogenic reagents, UV, fluorescence, IR, PMR and/or mass spectrometry can be employed for complementary identification. The TMS derivatives of *p*-hydroxybenzaldehyde showed further a shorter retention time than the silylated phenolic acids derived from benzoic acid, while the latter TMS derivatives were more rapidly eluted than the corresponding compounds from the cinnamic acid series. Coumarins proved to be more volatile than cinnamic acid derivatives with analogous substituents, and the derivatized flavonoids in turn to be less volatile than the cinnamic acid series.

In connection with the retention time of the TMS derivatives of the cinnamic acid group of compounds (each of them giving rise to the TMS ester produced by the –COOH function), the following conclusions with regard to an extra substituent or extra TMS-ether(s) (produced by one or more phenolic functions) can be formulated: $-CH_3 < -OCH_3 < -OTMS < 2\ -OCH_3 < -OCH_3 + -OTMS < 2\ -OTMS <$

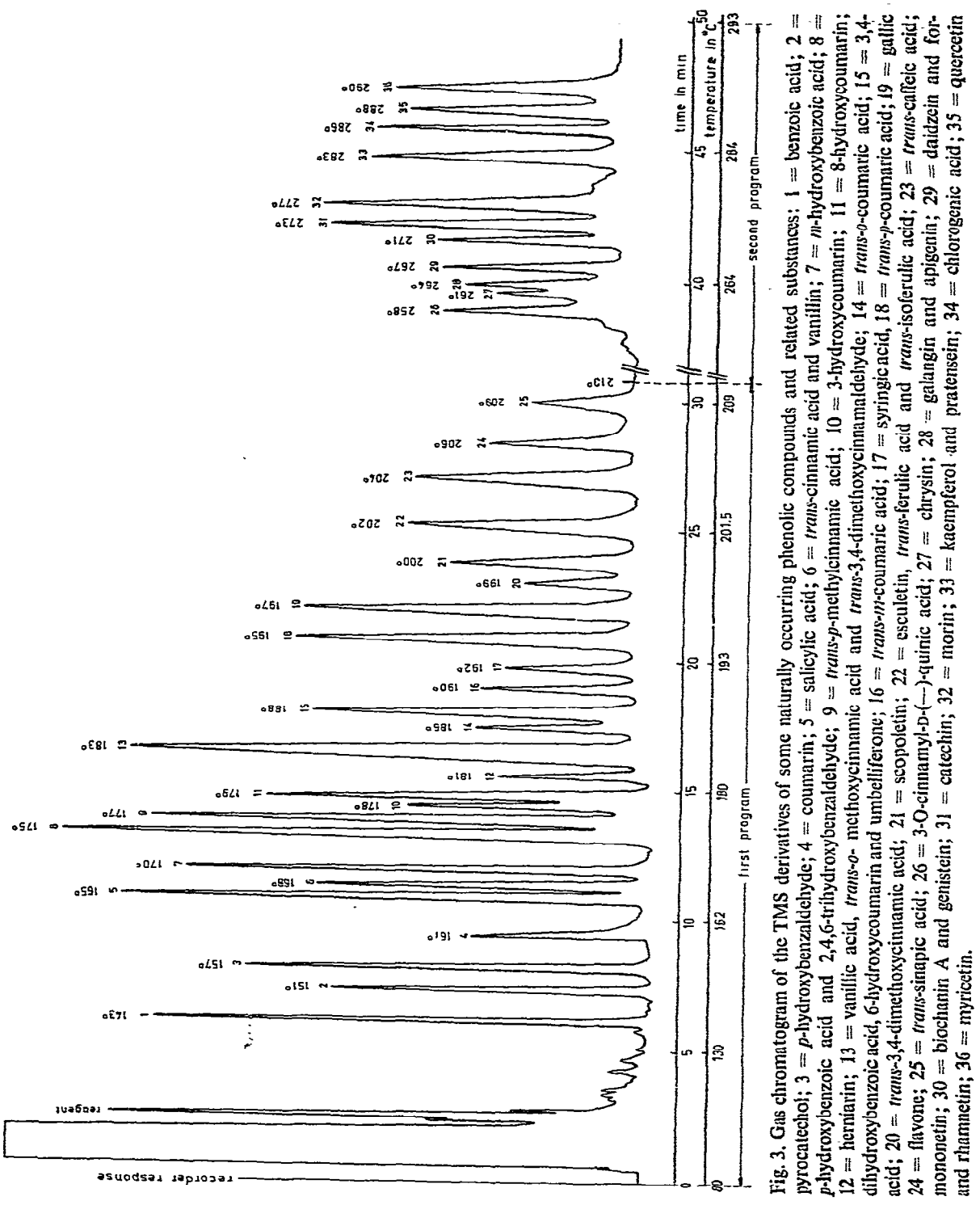


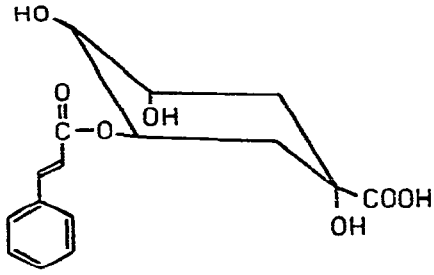
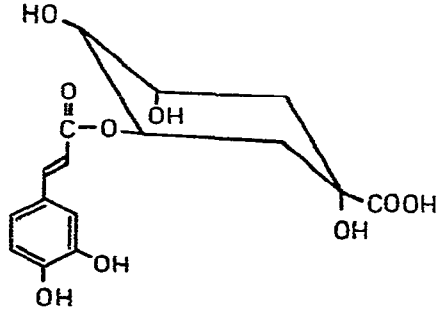
Fig. 3. Gas chromatogram of the TMS derivatives of some naturally occurring phenolic compounds and related substances: 1 = benzoic acid; 2 = pyrocatechol; 3 = *p*-hydroxybenzaldehyde; 4 = coumarin; 5 = salicylic acid; 6 = *trans*-cinnamic acid and vanillin; 7 = *m*-hydroxybenzoic acid; 8 = *p*-hydroxybenzoic acid and 2,4,6-trihydroxybenzaldehyde; 9 = *trans*-*p*-methylcinnamic acid; 10 = 3-hydroxycoumarin; 11 = 8-hydroxycoumarin; 12 = herniarin; 13 = vanillic acid, *trans*-*o*- methoxycinnamic acid and *trans*-3,4-dimethoxycinnamaldehyde; 14 = *trans*-*o*-coumaric acid; 15 = 3,4-dihydroxybenzoic acid, 6-hydroxycoumarin and umbelliferone; 16 = *trans*-*m*-coumaric acid; 17 = syringic acid, 18 = *trans*-*p*-coumaric acid; 19 = gallic acid; 20 = *trans*-3,4-dimethoxycinnamic acid; 21 = scopoletin; 22 = esculetin, *trans*-ferulic acid and *trans*-isoferulic acid; 23 = *trans*-caffeic acid; 24 = flavone; 25 = *trans*-sinapic acid; 26 = 3-O-cinnamyl-D-(—)-quinic acid; 27 = chrysin; 28 = galangin and apigenin; 29 = daidzein and formononetin; 30 = biochanin A and genistein; 31 = catechin; 32 = morin; 33 = kaempferol and pratensein; 34 = chlorogenic acid; 35 = quercetin and rhamnetin; 36 = myricetin.

2 –OCH_3 + –OTMS, etc. In addition, it can be seen from Fig. 3 that the retention time of the TMS derivative of *o*-hydroxycinnamic acid < that of *m*-hydroxycinnamic acid < that of *p*-hydroxycinnamic acid. Analogous statements can be made concerning the retention time of the benzoic acid series and the coumarins. For the retention time of flavone derivatives the following conclusions can be drawn: 2 –OTMS < 3 –OTMS < 4 –OTMS < 5 –OTMS = 4 –OTMS + –OCH_3 < 6 –OTMS.

However, exceptions to this rule exist as was shown for morin (for structure see Table I). Indeed, the retention time of the TMS derivative of this flavonol was unexpectedly found to be shorter than the retention time of the TMS derivative of kaempferol. In addition, the TMS derivatives of the isoflavones are, in general, less volatile than the flavone derivatives with the same number and type of substituents. For the compounds from the isoflavone group the following relationship between the TMS derivatives and, eventually, other substituents, and the retention time was found: 2 –OTMS = –OCH_3 + –OTMS < 3 –OTMS = –OCH_3 + 2 –OTMS < 3 –OTMS + –OCH_3.

TABLE I

STRUCTURE OF THE FLAVONOIDS AND 3-O-CINNAMYL-D-(−)-QUINIC ACID DERIVATIVES STUDIED

Compound	*Examples*	*Compound*	*Examples*
Flavones	Flavone Chrysin (5,7-di-OH) Apigenin (5,7,4′-tri-OH)	Quinic acid	3-O-Cinnamyl-D-(−)-quinic acid  3-O-Caffeyl-D-(−)-quinic acid (chlorogenic acid) 
Flavonols	Galangin (3,5,7-tri-OH) Kaempferol (3,5,7,4′-tetra-OH) Morin (3,5,7,2′,4′-penta-OH) Quercetin (3,5,7,3′,4′-penta-OH) Rhamnetin (3,5,3′,4′-tetra-OH, 7-OMe) Myricetin (3,5,7,3′,4′,5′-hexa-OH)		
Flavanol	Catechin (3,5,7,3′,4′-penta-OH)		
Isoflavones	Daidzein (7,4′-di-OH) Formononetin (7-OH, 4′-OMe) Genistein (5,7,4′-tri-OH) Biochanin A (5,7-di-OH, 4′-OMe) Pratensein (5,7,3′-tri-OH,4′-OMe)		

The TMS derivatives of the *cis* and *trans* stereoisomers of *p*-coumaric, caffeic, ferulic and sinapic acids have recently also been separated by GLC, using OV-25 as the stationary phase under isothermal conditions[48]. The temperature-programmed separation described above allows the simultaneous separation of the *cis* and *trans* isomers of all seven cinnamic acid derivatives tested (Table II). From a comparison of the retention times and the peak base width it can be seen that the method described in this paper gives better separations than that proposed by Hartley and Jones[48].

TABLE II

RELATIVE RETENTION TIMES AND COLUMN TEMPERATURE OF THE TMS DERIVATIVES OF THE *cis* AND *trans* STEREOISOMERS OF THE CINNAMIC ACID SERIES

Retention time of *trans-p*-methylcinnamic acid (13 min 54 sec) = 1.00.

Compound	*Relative retention time*	*Column temperature (°C)*
cis-Cinnamic acid	0.66	161
trans-Cinnamic acid	0.81	168
cis-o-Coumaric acid	1.03	177
trans-o-Coumaric acid	1.25	186
cis-m-Coumaric acid	1.12	180
trans-m-Coumaric acid	1.36	190
cis-p-Coumaric acid	1.21	184
trans-p-Coumaric acid	1.50	195
cis-Ferulic acid	1.49	194
trans-Ferulic acid	1.81	202
cis-Caffeic acid	1.63	199
trans-Caffeic acid	1.94	204
cis-Sinapic acid	1.76	201
trans-Sinapic acid	2.15	209

For quantitative work or additional analytical research on eventually unknown phenolic substances, it is also of great importance to investigate the yield and condition in which the derivatized compounds may be recovered after the described GLC.

For this purpose two types of additional experiments were performed. In the first experiment, the TMS derivatives of both [2-^{14}C]-*p*-coumaric acid and [2-^{14}C]-ferulic acid were gas chromatographed and collected. Comparison of the activity of the samples before and after GLC analysis (Table III) showed that the recovery of these compounds was 81–85%. The glass tube placed in the injector gave only 7 dpm, indicating that almost no decomposition occurs in the injector. After injection of water (steaming of the column) another 21 dpm could be accounted for. However, adsorption on to the stationary phase must be considered negligible, because the first, second and third 12.5 cm of the column material showed, after removal and elution, 46, 11 and 4 dpm, respectively. The catharometers gave 4 dpm and pyrolysis of the compounds is unlikely because only 4 dpm per analysis were found in the collector bottle (Fig. 2b). The loss of 12 to 15% of the material is therefore most probably due to leaks in the equipment (for instance, at the septa and joint of the micro-collector glass tube; Fig. 2a).

TABLE III

GLC OF DERIVATIZED [2-^{14}C]-*p*-COUMARIC ACID AND [2-^{14}C]-FERULIC ACID RECOVERY AND ANALYSIS OF THE COLLECTED MATERIALS

Compound	*Total activity of the sample in the syringe before injection (dpm)*	*Activity of the residual sample in the syringe after injection (dpm)*	*Activity of the injected sample (dpm)*	*Activity of the collected sample (dpm)*	*Recovery (%)*	*TLC of hydrolyzed sample (R_F)*	*TLC of reference (R_F)*
Bis-TMS-[2-^{14}C]-*p*-coumaric acid (specific activity 16.3 μCi/mmole)	3416	227	3189	2638	82	0.19	0.19
	3574	246	3328	2751	83		
	3436	282	3154	2679	85		
Bis-TMS-[2-^{14}C]-ferulic acid (specific activity 33.6 μCi/mmole)	4584	284	4300	3631	84	0.39	0.39
	4732	314	4418	3597	81		
	4629	333	4296	3518	82		

After hydrolysis of the collected TMS derivatives it was further established by TLC, followed by autoradiography, that the substances were identical with the starting materials. In a second set of experiments the TMS derivatives of pyrocatechol, vanillic, syringic, cinnamic, *p*-coumaric, and ferulic acids and kaempferol were separately chromatographed and collected on a preparative scale. Subsequently, the collected materials were analyzed by UV and IR spectrophotometry, PMR and, in part, mass spectrometry. The spectra, which were in accordance with the structures of the substances isolated (Tables IV and V), confirmed the previous finding that *p*-coumaric and ferulic acids are only slightly decomposed during GLC. In addition, the results presented in Tables IV and V indicate that analogous conclusions can be drawn for compounds ranging from pyrocatechol to kaempferol. Moreover, the mol.wt. deduced from the mass spectra showed that the peaks recorded correspond to the completely silylated substances. The IR spectra of the derivatized materials showed further peaks at $\pm$1680, $\pm$1250, $\pm$845 and $\pm$755 cm^{-1} corresponding to the –COOTMS and TMS groups[7,53]. In the spectra, two other bands occurred systematically at 1271–1285 cm^{-1} (m-vs) and 895–917 cm^{-1} (ms-vs). These bands are assigned to the O-Si- and the C_6H_5–O–Si– groups (Table IV), respectively. In the PMR spectra, the C_6H_5–O–TMS signal occurs between 0.23 and 0.30 δ and the –COOTMS signal between 0.33 and 0.38 δ. After preliminary METLC separation and purification of unknown phenolic substances from plant extracts, the proposed GLC technique, in combination with UV, IR, PMR and especially mass spectrometry, can be of great value to phytochemists.

TABLE IV

IR AND UV SPECTRA AND MOLECULAR WEIGHTS (DEDUCED FROM THE MASS SPECTRA) OF TMS DERIVATIVES ISOLATED AFTER PREPARATIVE GLC

vs = very strong; s = strong; ms = medium strong; m = medium; mw = medium weak; w = weak; m-sh = medium shoulder.

TMS derivative	*UV spectra (CH_2Cl_2)*		*IR spectra (cm^{-1})*				*Mass spectra*	
	$\lambda_{max.}$ *(nm)*	$\lambda_{min.}$ *(nm)*	*–COOTMS*	*O–Si*	*–Si(CH₃)₃*	*C_6H_5–O–Si*	*M^+/e*	*Mol. wt. calc.*
Pyrocatechol (1,2-Dihydroxybenzene)	278	250	—	1284 (ms)	1253 (m) 843 (vs) 750 (m)	906 (ms)	254	254
Vanillic acid (4-Hydroxy-3-methoxybenzoic acid)	260 294	237 279	1680 (m)	1285 (s)	1254 (w) 843 (vs) 759 (m)	895 (vs)	312	312
Cinnamic acid	278	—	1687 (vs)	1282 (ms)	1254 (m) 849 (vs) 772 (ms)	—	220	220
p-Coumaric acid (4-Hydroxycinnamic acid)	300 308	247 303	1673 (m)	1273 (m)	1255 (m) 845 (vs) 756 (ms)	917 (vs)	308	308
Caffeic acid (3,4-Dihydroxycinnamic acid)	241 299 321	261 303	1681 (mw)	1285 (vs)	1251 (s) 841 (vs) 753 (m)	910 (ms)	396	396
Ferulic acid (4-Hydroxy-3-methoxycinnamic acid)	242 298 324	263 305	1681 (m)	1271 (s)	1250 (s) 843 (vs) 758 (m)	904 (s)	338	338
Sinapic acid (4-Hydroxy-3,5-dimethoxycinnamic acid)	239 265	325	1683 (s)	1283 (s)	1250 (s) 844 (vs) 756 (m)	912 (s)	368	368
Kaempferol	262 310 345	244 285 316	—	1271 (m-sh)	1254 (s) 843 (vs) 752 (m)	913 (ms)	574	574

TABLE V

PMR SPECTRA OF TMS DERIVATIVES ISOLATED AFTER PREPARATIVE GLC

s = singlet; d = doublet; m = multiplet; br = broad.

TMS derivative	*Function*					
	C_6H_5–O–TMS	*–COOTMS*	*OCH_3*	*$\underline{CH}$ = CH–CO*	*Aromatic H*	*$\underline{CH}$ = CH–CO*
Pyrocatechol (1,2-Dihydroxybenzene)	0.25 δ (s)	—	—	—	6.83 δ (s)	—
Vanillic acid (4-Hydroxy-3-methoxybenzoic acid)	0.25 δ (s)	0.38 δ (s)	3.85 δ (s)	—	6.78 and 6.92 δ 7.47–7.67 δ (m)	—
Cinnamic acid	—	0.35 δ (s)	—	6.41 δ (d) J_{AB} = 16.0 cps	7.43 δ (m)	7.65 δ (d) J_{AB} = 15.8 cps
p-Coumaric acid (4-Hydroxycinnamic acid)	0.27 δ (s)	0.33 δ (s)	—	6.26 δ (d) J_{AB} = 16.0 cps	6.83 δ (d) J = 9 cps 7.41 δ (d) J = 9 cps	7.57 δ (d) J_{AB} = 16.0 cps
Caffeic acid (3,4-Dihydroxycinnamic acid)	0.26 δ (s)	0.33 δ (s)	—	6.23 δ (d) J_{AB} = 16.0 cps	6.88 δ (s) (1 H) 7.01 δ (s) (2 H)	7.53 δ (d) J_{AB} = 16.0 tps
Ferulic acid (4-Hydroxy-3-methoxycinnamic acid)	0.25 δ (s)	0.33 δ (s)	3.83 δ (s)	6.26 δ (d) J_{AB} = 16.0 cps	6.88 δ (s) (1 H) 6.93–7.08 δ (m) (2 H)	7.57 δ (d) J_{AB} = 16.0 cps
Sinapic acid (4-Hydroxy-3,5-dimethoxycinnamic acid)	0.23 δ (s)	0.35 δ (s)	3.85 δ (s)	6.28 δ (d) J_{AB} = 16.0 cps	6.77 δ (s)	7.55 δ (d) J_{AB} = 16.0 cps
Kaempferol	0.30 δ (s) (broad)	—	—	—	6.25 δ (s) (br) H-6 6.53 δ (s) (br) H-8 6.93 δ (d) (br) H-3′, H-5′ J = 9 cps 8.02 δ (d) (br) H-2′, H-6′ J = 9 cps	—

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