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ANALYSIS OF HYDROXYBENZOIC AND HYDROXYCINNAMIC ACIDS IN PLANT MATERIAL

II. DETERMINATION BY GAS–LIQUID CHROMATOGRAPHY

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SUMMARY

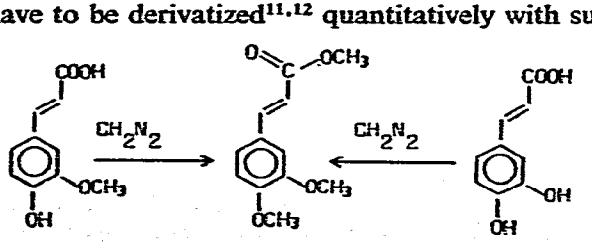
Factors affecting the quantitative determination of phenolic acids in plant material by gas–liquid chromatography of their trimethylsilyl derivatives, employing silicone stationary phases, have been investigated. The use of soft glass must be strictly avoided, while borosilicate (Pyrex), quartz and support surfaces must be deactivated by silanization. Both capillary and packed columns were used. The method developed was applied to the analysis of phenolic acids in spices, and by careful attention to the factors discussed, excellent results were achieved.

INTRODUCTION

Although previous workers[1–9] have employed gas–liquid chromatography (GLC) for the determination of phenolic acids (mostly in standard solutions, rarely in actual biological samples), no comprehensive study appears to have been undertaken of the various factors affecting the accuracy of the method.

A general method for preparing a quantitative extract of phenolic acids from plant material was described in Part I[10]. Although this method yields an extract suitable for the quantitative determination of phenolic acids in most cases, some adaptation to sample and circumstances may be necessary for best results in specific instances.

Normally phenolic acids are insufficiently volatile for direct analysis by GLC, and have to be derivatized[11,12] quantitatively with suitable reagents. The very



trans-ferulic acid trans-caffeic acid

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popular permethylation (with diazomethane) is simple and rapid, but unsuitable for phenolic acids because methylated hydroxy groups cannot be distinguished from those occurring naturally.

Perethylation[13] results in solid, separable and distinguishable compounds, but quantitative derivatization is not always readily achieved. Methylesterification with additional silylation results in compounds well separable by chromatography. Such derivatives are less sensitive to incomplete deactivation of GLC columns than per-silylated derivatives, but their formation presents problems in individual cases. Special silylations such as the formation of sulphonyltrimethylsilyl derivatives[14] generally require further expertise, so their use is restricted to difficult analytical problems. Specific derivatization methods for electron-capture detection[12,15–17] are described in the literature, but were not investigated in this study.

Trimethylsilylation was chosen for the present study because it is achieved readily and quantitatively although the derivatives, particulary trimethylsilyl (TMS) esters, are very sensitive to moisture and catalytic influences. Many silylation reagents are available commercially. The use of N,O-bis(trimethylsilyl)acetamide (BSA) in pyridine was chosen because the reaction is efficient, the reagent is comparatively inexpensive and a homogeneous reaction mixture is obtained. Various combinations of silylation reagents have been tested by Drawert and Leupold[7] for phenolic acids.

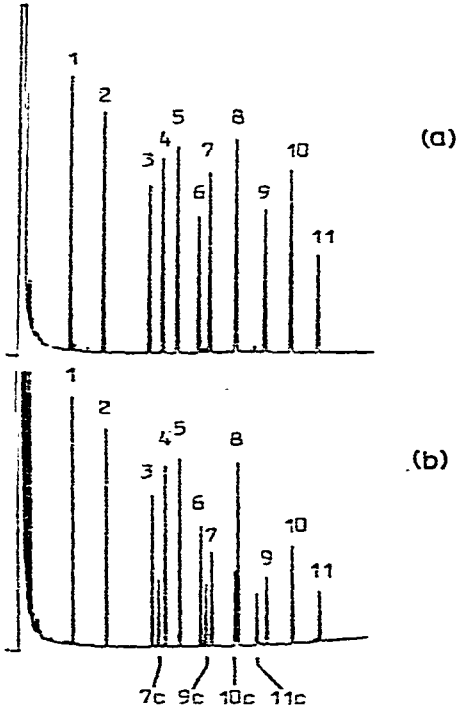

Fig. 1. Chromatograms of phenolic acid reference mixture: a, freshly silylated; b, after standing for 3 weeks in a closed flask in diffuse laboratory light. Conditions as in Fig. 6. Peaks: 7c = *cis*-*p*-coumaric acid; 9c = *cis*-ferulic acid; 10c = *cis*-caffeic acid; 11c = *cis*-sinapic acid; others as in Fig. 3.

A characteristic property of hydroxycinnamic acids is *trans–cis* isomerization which can occur during sample preparation, even on only slight exposure to UV light. Reference *cis*-isomers can readily be obtained from UV-irradiated solutions of authentic *trans*-isomers by preparative paper or thin-layer chromatography (*cf.* refs. 18 and 19).

EXPERIMENTAL

Evaporations were performed in a rotary vacuum evaporator at a temperature less than 40°C unless stated otherwise.

Derivatization and GLC

The preparation of the plant materials, to obtain a "parent solution", was as described in Part I[10]. Two samples of the parent solution (0.5–5.0 ml, generally 2.0 ml) were pipetted (1) into a 20-ml conical flask and 1 ml of a reference solution was added (*e.g.*, 50 mg of each phenolic acid per 100 ml of methanol), and (2) into a second flask, without reference solution. Samples 1 and 2 were evaporated carefully, residual water being removed azeotropically by several additions of methanol. After adding *ca.* 1 ml pyridine and *ca.* 0.5 ml BSA to samples 1 and 2, the flasks were sealed with polyethylene stoppers and heated in an oil-bath. Silylation is generally complete after 30 min at 80°C (for pure phenolic acids treatment of 15 min is adequate), but as a precautionary measure a reaction time of 2 h was allowed.

Exactly 1 ml of internal standard solution (*e.g.*, 50 mg of the *n*-alkanes C_{13} and C_{14} dissolved in 100 ml benzene) was added next to sample 2, and 1 ml of pure benzene to sample 1. *n*-Alkanes were selected as internal standards because they are unaffected by silylation and by column deactivation, and are readily obtainable.

Silylated hydroxycinnamic acids tend to undergo *trans–cis* isomerization, therefore solutions (especially of reference compounds) should be kept in the dark. Stability was maintained for several days simply by keeping the flasks in a small, closed cardboard box.

A reference solution of silylated phenolic acids (containing the same amount of standard as sample 2) and samples 1 and 2 were analyzed under the same GLC conditions, employing at least two different silicone phases (*e.g.*, OV-101 and OV-17).

Evaluation

Any irregularities in the gas chromatographic system became evident immediately from changes in the characteristic chromatograms of the standard reference solution.

By comparing the chromatograms of the reference solution with those of samples 1 and 2, the recovery for the phenolic acids added to sample 1 can be examined. This, in turn, serves as a check on the completeness of silylation and reveals also the degree of separation of the internal standards from other constituents. The comparison of the chromatograms permits a more reliable identification of the phenolic acids than would be possible from retention data[9] alone. Constituents of the complex plant extracts are able to affect each other's retention during the temperature-programmed GLC.

Remarks on silylation

Pure phenolic acids are soluble directly in BSA, but natural samples generally require a suitable solvent prior to reaction with BSA. Dioxane and pyridine are equally suitable for this purpose. Pyridine is a slightly better solvent, but dioxane shows less tailing on elution from the column. Silylation of phenolic acids proceeds equally effectively in dioxane –BSA (2:1) and pyridine–BSA (2:1).

Moisture must be rigorously excluded. A slight formation of white vapour during BSA addition to the sample indicates the presence of volatile acids or residues of methanol or water. In such cases completeness of silylation should be checked. Salicylic acid serves as a good indicator for this purpose as its silylation is particulary sensitive to the presence of moisture.

Remarks on GC columns

The support material must not be alkaline. Base-treated supports (often described as "high quality" supports) can cause decomposition of silylated phenolic acids. Deactivation of commercially available support materials is generally inadequate (Fig. 2), so the products should be resilanized before coating.

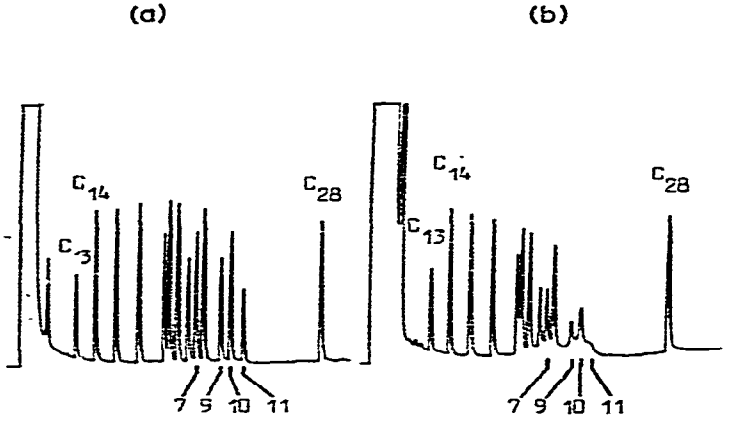

Fig. 2. Chromatograms of phenolic acid reference mixture (as in Fig. 3a including a qualitative mixture of *n*-alkanes C_{13}, C_{14}, C_{28}) under same conditions: a, using resilanized Chromosorb G AW DMCS; b, using Chromosorb G HP.

The support material (Chromosorb G AW) was dried for more than 10 h at *ca.* 150°C and refluxed with pure dichlorodimethylsilane (DMCS) for *ca.* 1 h. The mixture was cooled to room temperature and the DMCS was removed by decantation and repeated washing with toluene. The support material was then washed well with anhydrous methanol, allowed to stand for *ca.* 10 min under the solvent and rewashed. The methanol was removed by repeated washing with toluene, and finally with the solvent to be used for coating.

Metal columns may be used. The glass columns, glass wool and vaporization chamber must be made from borosilicate (Pyrex) glass and must be properly deactivated (silanized). Glass capillary columns must also be made of borosilicate glass if they are to be used for quantitative determination of phenolic acids as their tri-

methylsilyl derivatives. Leaching with hydrochloric acid and a careful silanization[20–22] were the only pretreatments of the inner glass surface necessary when apolar silicone phases (*e.g.*, OV-101) were used.

Silyl derivatives of hydroxycinnamic acids (especially sinapic acid) are very sensitive to contact with soft glass and incompletely deactivated surfaces. To be acceptable, columns must give the same TMS-sinapic acid to internal standard peak ratio after injecting different amounts of a test solution.

Silanized quartz wool in the lower part of the injection port retains non-volatile matter. It should be changed frequently. The final temperature of the heating program in the oven should be higher than the temperature of the injection port. If non-volatile matter contaminates the top of the column despite these precautions and leads to column deterioration (peak tailing and decreased resolution), the first 1–2 cm of packing should be renewed.

A further consideration is the area of the injection port and top part of the column which must not be obstructed to allow the vaporized sample to pass this hot area as rapidly as possible. TMS derivatives of *ortho*-hydroxybenzoic acids are particularly sensitive to hydrolysis when traces of water are present. On the column proper, however, water is separated rapidly from the sensitive TMS derivatives so that programming to high oven temperatures may be employed safely.

RESULTS

The retention properties of phenolic acids on silicone stationary phases of various polarities are shown in Figs. 3a–d. *cis*-Hydroxycinnamic acids always have shorter retention times than the corresponding *trans*-isomers.

The mass spectra of silylated phenolic acids[3,23–25] show molecular ion peaks of high relative intensity (Table I). The compounds would therefore be readily

TABLE I

MASS SPECTRA OF THE MOST FREQUENTLY OCCURRING HYDROXYBENZOIC AND HYDROXYCINNAMIC ACIDS AS TRIMETHYLSILYL DERIVATIVES

Data in *m/e*: in parentheses, relative intensity. M = Molecular ion; BP = base peak. Conditions: Finnigan 4023 gas chromatography–quadrupole mass spectrometry system with open-split interface as recommended by Stan and Abraham[26]; interface, 250°C; ion source, 220°C; electron beam energy 70 eV; ionizing current, 300 μA.

Acid	*M*	*BP*	*Other characteristic ions*
Salicylic	276 (51)	73 (100)	135 (11)
p-Hydroxybenzoic	276 (56)	73 (100)	223 (40), 193 (40)
Vanillic	312 (29)	73 (100)	297 (46), 267 (32), 223 (28)
Syringic	342 (25)	73 (100)	327 (30), 312 (24), 297 (18)
Gentisic	355 (38)	73 (100)	
Protocatechuic	370 (14)	73 (100)	193 (53), 355 (10)
Gallic	458 (11)	73 (100)	281 (22)
p-Coumaric	308 (18)	73 (100)	219 (31), 293 (25), 249 (15)
Ferulic	338 (44)	73 (100)	323 (25), 308 (23), 249 (20)
Sinapic	368 (43)	73 (100)	338 (37), 353 (15)
Caffeic	396 (28)	73 (100)	219 (43), 191 (10)

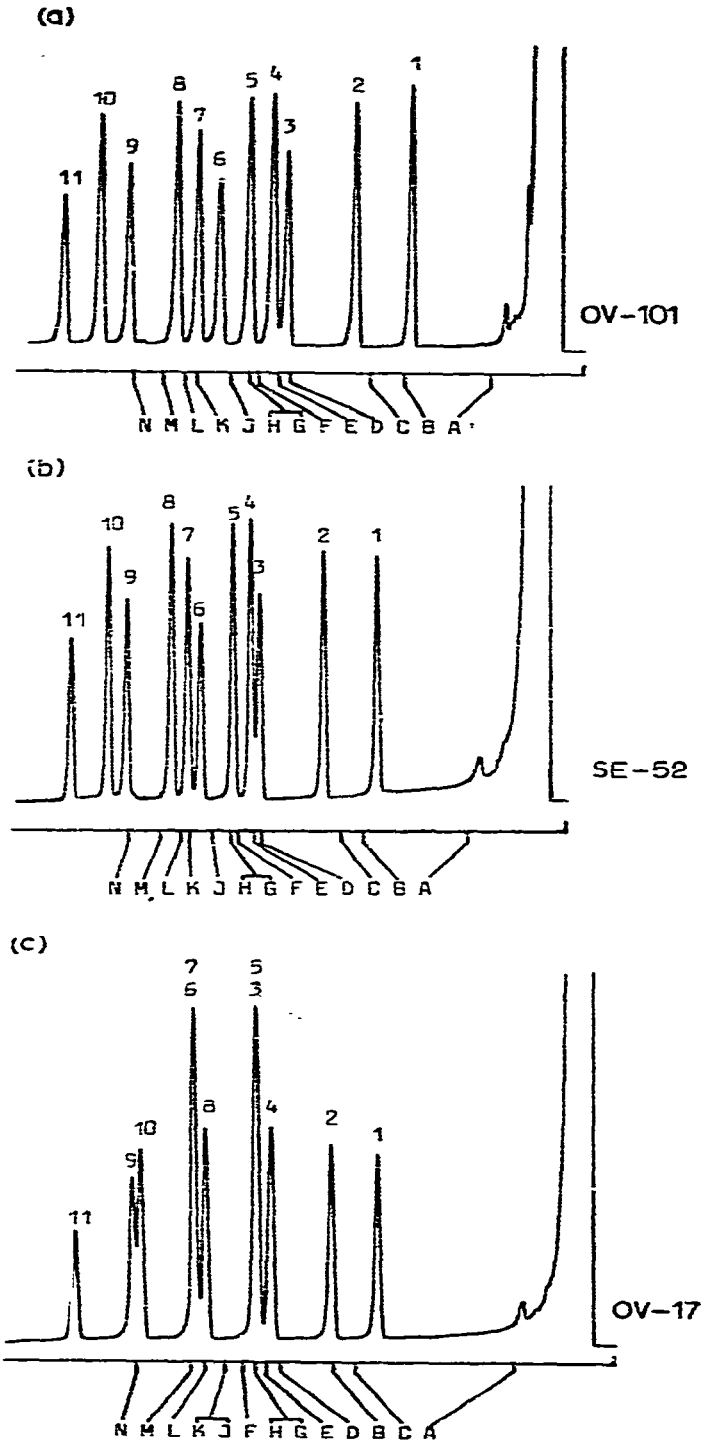
(a)
11
10
9
8
7
6
5
4
3
2
1
OV-101
N M L K J H G F E D C B A
(b)
11
10
9
8
7
6
5
4
3
2
1
SE-52
N M L K J H G F E D C B A
(c)
7
6
5
3
11
10
9
8
4
2
1
OV-17
N M L K J F H G E D B C A

Fig. 3.

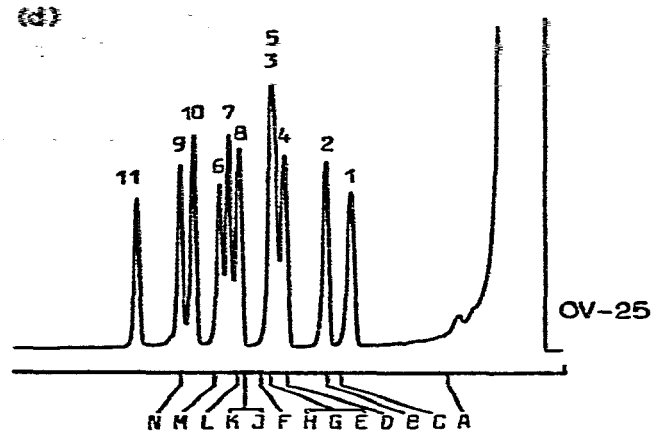

Fig. 3. Retention sequences. Chromatograms of a silylated reference solution containing equal quantities of the most frequently occurring natural hydroxybenzoic and hydroxycinnamic acids. Peaks: 1 = salicylic acid; 2 = *p*-hydroxybenzoic acid; 3 = vanillic acid; 4 = gentisic acid; 5 = protocatechuic acid; 6 = syringic acid; 7 = *trans*-*p*-coumaric acid; 8 = gallic acid; 9 = *trans*-ferulic acid; 10 = *trans*-caffeic acid; 11 = *trans*-sinapic acid. Positions at which rarely naturally occurring phenolic acids appear: A = benzoic acid; B = *trans*-cinnamic acid; C = *m*-hydroxybenzoic acid; D = 2,3-dihydroxybenzoic acid; E = 2,6-dihydroxybenzoic acid; F = *trans*-*o*-coumaric acid; G = 2,4-dihydroxybenzoic acid; H = 3,5-dihydroxybenzoic acid; J = *trans*-*m*-coumaric acid; K = 2,3,4-trihydroxybenzoic acid; L = dihydrocaffeic acid; M = 2,4,6-trihydroxybenzoic acid; N = *trans*-isoferulic acid. Conditions: a, glass column (2 m × 2 mm I.D.), 1% OV-101 on Chromosorb G AW (silanized) (80–100 mesh), Erba 2350, flame ionization detection, injector and detector 300°C, oven 2 min at 110°C then 8°/min to 320°C, N_2, flow-rate 15 ml/min; b, glass column (3 m × 4 mm I.D.), 3% SE-52 on Chromosorb W AW (silanized) (80–100 mesh), Erba 2150, flame ionization detection, injector and detector 300°C, oven 7°/min from 100°C to 300°C, 0.5 μar N_2; c, glass column (3 m × 2 mm I.D.), 1% OV-17 on Chromosorb G AW (silanized) (80–100 mesh), other conditions as in Fig. 3a; d, glass column (3 m × 3 mm I.D.), 2% OV-25 on Chromosorb W AW (silanized) (80–100 mesh), Siemens L 402, flame ionization detection, injector 290°C, detector 310°C, oven 3 min at 110°C then 8°/min to 300°C, N_2, flow-rate 30 ml/min.

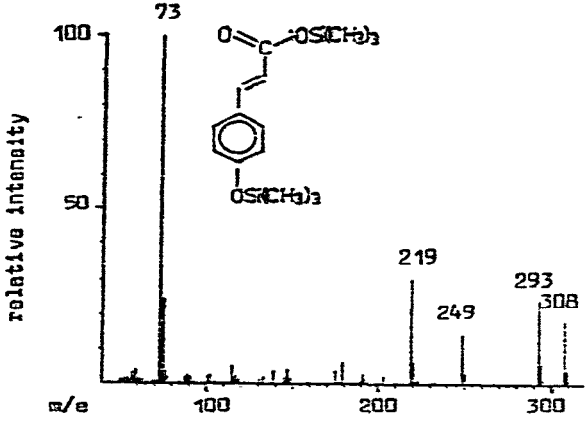

Fig. 4. Mass spectrum of *p*-coumaric acid TMS derivative with intense molecular ion peak, typical of the spectra of silylated phenolic acids.

amenable to selected ion monitoring (SIM). Usually the ion $[(CH_3)_3Si]^+$, *m/e* 73, constitutes the base peak. Fragmentation schemes are given by Horman and Viani[25] and Lhugenot *et al.*[27]. In the present study mass spectra of *cis*- and *trans*-isomers of silylated hydroxycinnamic acids were found to be identical.

A 1-mg amount of each phenolic acid and 1 mg tetradecane were derivatized in a 1-ml silylation mixture and analyzed by GLC [1 μl per injection; parameters in Fig. 3a and c; integrator, System I (Spectra-Physics, Santa Clara, CA, U.S.A.)] to determine the ratio of peak areas of acid to tetradecane (relative response factors): protocatechuic acid, 1.29; gallic acid, 1.28; *p*-hydroxybenzoic acid, 1.22; gentisic acid, 1.22; salicylic acid, 1.17; caffeic acid, 1.11; *p*-coumaric acid, 1.00; vanillic acid, 0.96; syringic acid, 0.85; ferulic acid, 0.84; sinapic acid, 0.72; (tetradecane, 1.00).

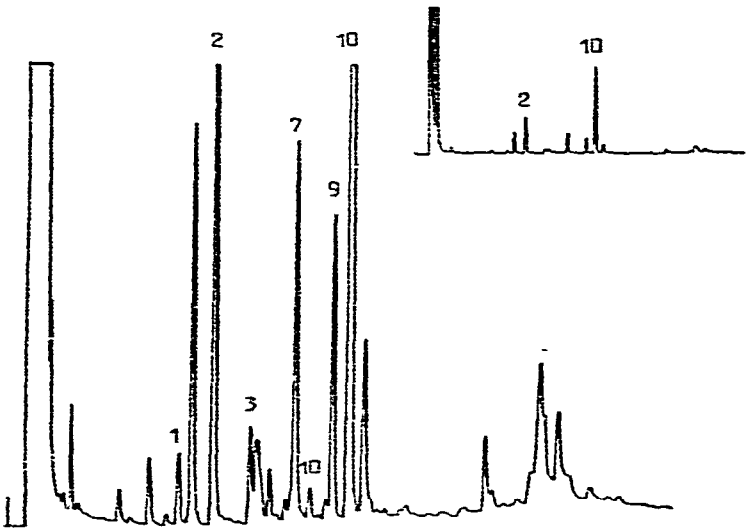

Fig. 5. Phenolic acids extracted from dried aniseed (*Pimpinella anisum* L.). Peaks: 1 = salicylic acid (63 ppm); 2 = *p*-hydroxybenzoic acid (1030 ppm); 3 = vanillic acid (133 ppm); 7 = *p*-coumaric acid (737 ppm); 9 = ferulic acid (642 ppm); 10 = caffeic acid (2060 ppm); 7, 9, 10 = *trans*-isomers. Conditions as in Fig. 3a.

In very low concentration ranges (*e.g.*, below 0.05 μg phenolic acid per injection) the peak areas obtained were relatively too small, especially from the sensitive sinapic acid. Thus we did not rely upon response factors only, but also calibrated GLC columns in the range 0.005–1.00 μg phenolic acid per injection before each series of analyses. This and the use of internal standards permitted reliable measurements.

The methods described in this paper were applied successfully to the quantitative analysis of phenolic acids in 33 spices. Figs. 5 and 6 illustrate the distribution of phenolic acids in dry aniseed and fresh thyme leaves. The results for other spices will be reported elsewhere[28].

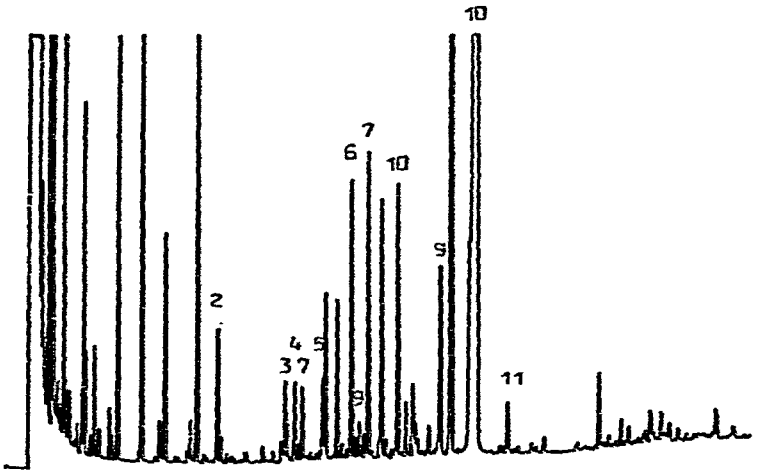

Fig. 6. Phenolic acids extracted from fresh leaves of thyme (*Thymus vulgaris* L.). Peaks: 2 = *p*-hydroxybenzoic acid (26 ppm); 3 = vanillic acid (10 ppm); 4 = gentisic acid (*ca.* 25 ppm); 5 = protocatechuic acid (5 ppm); 6 = syringic acid (62 ppm); 7 = *cis*- + *trans*-*p*-coumaric acid (80 ppm); 9 = *cis*- + *trans*-ferulic acid (40 ppm); 10 = *cis*- + *trans*-caffeic acid (3330 ppm); 11 = *cis*- + *trans*-sinapic acid (*ca.* 50 ppm). Conditions: 20-m glass capillary coated dynamically with 5% OV-101, Erba 2150, flame ionization detection, injector and detector 250°C, oven 110°C, then 5°/min to 250°C, 0.33 bar H_2, splitting ratio 1:10.

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