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GROWTH SUBSTANCES ISOLATED FROM WOODY CUTTINGS OF *CASTANEA SATIVA* MILL.*

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Abstract—A study of substances in the cuttings of *Castanea sativa* Mill. was made by extraction with methanol, paper chromatography and *Avena* coleoptile straight-growth test. Vanillic, *p*-hydroxybenzoic, salicylic and syringic acids were isolated and identified. A hydroxyaliphatic acid with interesting growth-inhibiting properties, which has not yet been identified, and an ester of ferulic acid were also found. Indol-3-acetic acid was not detected. The growth-stimulating zones in the biohistograms may be explained by their small content of vanillic and *p*-hydroxybenzoic acids. The growth-inhibiting zones must be due to the presence of the hydroxyaliphatic acid, mixed with salicylic and other hydroxybenzoic acids, which at a high level are toxic or inhibitors. Moreover, vanillic, *p*-hydroxybenzoic, *m*-hydroxybenzoic and salicylic acids were obtained from alkaline hydrolysis of the plant extract, insoluble in ether and soluble in water. Most of these probably come from glycosides cleavage.

INTRODUCTION

ISOLATION of growth regulators and inhibitors from woody cuttings of plants which are easy and difficult to root throw light on the chemical factors involved in rooting. We are studying both groups of plants, and although definitive conclusions can only be drawn from the study of many species of both groups we think it worth while to report on the substances which may be related to rooting isolated from *Castanea sativa*, a plant which is difficult to root.

There is no literature about growth regulators or inhibitors in *C. sativa* cuttings. Catechin (0·6 per cent) has been reported in the fruit bark of *C. sativa*,[1] and Bate-Smith[2] showed the presence of quercetin, kaempferol and caffeic, ellagic and *p*-coumaric acids in the leaves. It has also been shown that *C. sativa* trees growing in southern Europe contain 10–11% tannin,[3] and hexen-2-al-1 and dehidrodigallic acid have also been found in the leaves of this plant.[4]

Results of the Fractions of the Unhydrolysed Extract

Acidic fraction: biohistogram and compounds. Biohistogram of this fraction shows three different zones: (1) R_f 0·05–0·30; (2) R_f 0·30–0·55; (3) R_f 0·55–0·90 (Fig. 1). Zones 1 and 2 seemed to be growth stimulating and zone 3 growth inhibiting. However, as they contain a mixture of compounds, their eluate was rechromatographed and a mixture of vanillic and syringic acid was isolated at R_f 0·11–0·17 and *p*-hydroxybenzoic acid (PHB) at R_f 0·17–0·25.

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1 O. TH. SCHMIDT and G. HULL, *Chem. Ber.* **80**, 509 (1947).
2 E. C. BATE-SMITH, *J. Linn. Soc.* (*Botany*) **58**, 95 (1962).
3 W. E. HILLIS (Editor), *Wood Extractives*, p. 17. Academic Press, New York (1966).
4 W. KARRER, *Konstitution und Vorkommen der organischen Pflanzenstoffe*, No. 360 and 908. Birkhäuser, Basle (1958).

Every fraction which showed some growth stimulation was examined for IAA by chemical[5] and biological tests with negative results. Compounds responsible for growth inhibition of zone 3 (R_f 0·55–0·90) were salicylic acid, isolated by rechromatography (R 0·55–0·65), an unidentified aliphatic acid (R_f 0·70–0·80), an ester of ferulic acid and a complex phenol.

Identification of vanillic acid. This acid is very abundant reaching a concentration of at least 200 μg/kg plant, and was identified as follows: the eluate gave an u.v. spectrum with a maximum at 255 nm in neutral methanol, shifting to 285 nm by addition of alkali (vanillic acid, under the same conditions, gave λ_{max} 257 and 282 nm respectively); the eluate and pure vanillic acid both had in isopropanol–ammonia–water (IAW, 10:1:1) R_f 0·13; the eluate and vanillic acid produced the same ester with diazomethane. Chromatography of the reaction

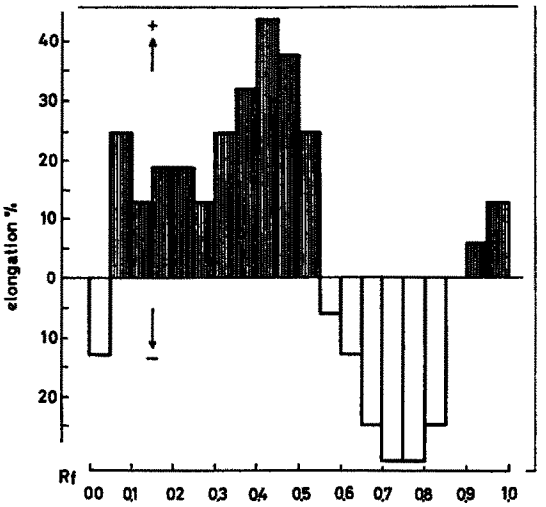

FIG. 1. BIOHISTOGRAMS (*Avena* COLEOPTILE SECTION TEST) OF THE ACIDIC FRACTION OF UNHYDROLYSED EXTRACTS OF *C. Sativa*. ORDINATE: EXCESS OF ELONGATION OF TREATED SECTIONS AS PERCENTAGE OF FINAL LENGTH EXCESS OF CONTROLS. ABSCISSA: R_f VALUES. LEAST SIGNIFICANT DIFFERENCE L.s.d. 16 PER CENT.

products, detected by successive sprays of diazotizated sulfanilic acid and 2,6-dichloroquinonechlorimide (DQC) reagent, gave yellow–red spots at R_f 0·60 in IAW; the eluate and vanillic acid gave the same reduction product (vanillylalcohol, R_f 0·71 in IAW) with $LiAlH_4$; fluorescence spectra of the eluate and vanillic acid were almost identical (Table 1).

Identification of syringic acid. This could not be isolated in a pure state because only a small amount was present but was identified by TLC using DQC as a detecting reagent in butanol saturated with water (R_f 0·33); chloroform:acetic acid (95:5) (R_f 0·65); and benzene:methanol:acetic acid (45:8:4) (R_f 0·75).

Identification of p-*hydroxybenzoic acid (PHB).* This was isolated in an amount of approximately 7 μg/kg and identified by its R_f (0·19) in IAW; that of its $LiAlH_4$ reduction product (R_f 0·72) and ester (R_f 0·69). It had an almost identical fluorescence spectrum to PHB (Table 1) and gave similar R_f values to the latter on TLC using butanol saturated with water (R_f 0·53) and chloroform:acetic acid (95:5) (R_f 0·44).

[5] E. SEOANE, A. CARNICER and E. VIEITEZ, *Microchem. J.* **9**, 432 (1965).

TABLE 1. MAXIMA OF THE ACTIVATION AND FLUORESCENCE SPECTRA OF COMPOUNDS ISOLATED FROM *C. sativa* AND OF PURE COMPARISON SPECIMENS, RUN IN NEUTRAL AND ALKALINE METHANOL

Substance	Neutral pH		Alkaline pH	
	λ Activation (max nm)	λ Fluorescence (max nm)	λ Activation (max nm)	λ Fluorescence (max nm)
Vanillic acid	297	345	307	355
Unknown	297	355; 430 (i)	307	365
p-Hydroxybenzoic acid	298	337		
Unknown	298	350		
m-Hydroxybenzoic acid	305	360	320	425
Unknown	305	370; 412 (i)	320	435
Salicylic acid	305	415	305	410
Unknown	305	425	305	420
Orcinol	282	305	297	350
Unknown	320	375; 395	345	435

(i) = impurities.

Identification of salicylic acid. In order to isolate and purify this acid zone 3 eluate was dissolved in ether and extracted with a solution of $NaHCO_3$ and the latter extract chromatographed on paper in butanol saturated with 1% ammonia. The salicylic band (R_f 0·35–0·45) was eluted and identified as above.

It had R_f 0·60 in IAW and its reduction product had R_f 0·77, identical with authentic samples. It gave an identical fluorescence spectra to salicylic acid (Table 1).

Aliphatic acid with growth-inhibiting properties. The zone 3 eluate $NaHCO_3$ extract was extracted with ether and this latter solution chromatographed on paper, using butanol saturated with 1% ammonia as solvent. The growth-inhibiting acid at R_f 0·45–0·60 was detected by biological assay. This acid has the following properties: it did not react with the specific phenol reagents; after removal of solvent an oil was obtained (about 2 mg/kg plant) which was insoluble in light petroleum, sparingly soluble in benzene, slightly soluble in ether and very soluble in methanol. By reaction with diazomethane a methyl ester was obtained, and an i.r. spectrum was run in compensated CCl_4. This spectrum showed bands at 3630 cm^{-1} (HO group), 1735 cm^{-1} (CO of an ester), 1460 and 1370 (CH_3 group), 1010 and 1151 cm^{-1}. No bands of an aromatic character were found.

On the basis of these results, the acid would appear to be an aliphatic hydroxyacid. However, attempts to detect it by reaction with bromocresol green and similar reagents failed.

An ester of ferulic acid. This was separated in zone 3 of the direct acidic fraction. TLC of this eluate using chloroform: acetic acid (95:5) as a solvent gave, with DQC, a blue–grey spot at R_f 0·21; if the eluate had been hydrolysed previously by alcoholic 5% NaOH the spot disappeared. In the study of this hydrolysis its acids were first isolated with sodium bicarbonate and then purified by chromatography. A highly fluorescent acid under u.v. was located at R_f 0·11–0·20, the eluate of this was identified as ferulic acid using the following tests: u.v. spectrum was similar to ferulic acid (methanol, 291 and 314 nm; with KOH these changed into 306 and 347–349 nm, respectively); R_f values (0·20) in IAW (authentic ferulic acid had R_f 0·24); TLC, using butanol saturated with water gave R_f 0·35 (ferulic acid, R_f 0·30); treatment of the eluate with diazomethane, followed by chromatography, afforded

two fluorescent spots under u.v. at R_f 0·59 (ferulic acid methyl ester) and R_f 0·79 (methoxiferulic acid methyl ester). Ferulic acid gave identical spots.

A complex phenol of R_f 0·75. When salicylic acid was separated with ether from the zone 3, and the organic layer was shaken with $NaHCO_3$, a complex phenol remained in the ether, this was shown by: paper chromatography which gave a blue spot with DQC at R_f 0·75 in IAW; the eluate phenol was recovered unchanged after alkaline hydrolysis with 5% alcoholic NaOH solution. Alkaline fusion gave vanillic acid and (tentatively) phloroglucinol identified chromatographically.

Neutral fraction: biohistograms and compounds. Biohistogram of this fraction showed growth stimulation (Fig. 2) at R_f 0·50. However, the zone eluate gave no chromogenic reaction with Ehrlich or DQC reagents. No peaks were found in the u.v. spectrum. Reduction with $LiAlH_4$ gave no detectable compound. Alkaline hydrolysis with 5% alcoholic NaOH, followed by a chromatography of the reaction products, gave a blue spot with DQC

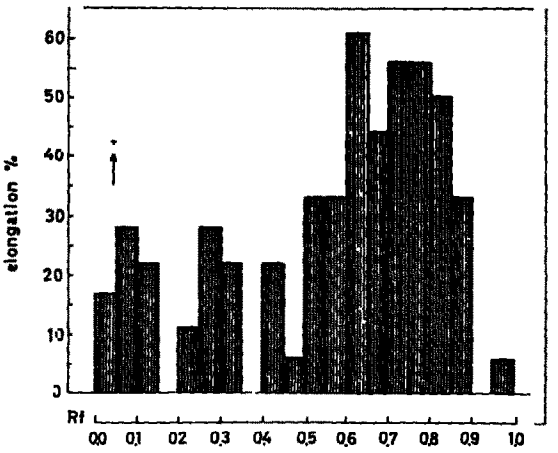

FIG. 2. BIOHISTOGRAM (*Avena* COLEOPTILE SECTION TEST) OF THE NEUTRAL FRACTION OF UNHYDROLYSED EXTRACTS OF *C. sativa*. ORDINATE: EXCESS OF ELONGATION OF TREATED SECTIONS AS PERCENTAGES OF FINAL LENGTH OF CONTROLS. ABSCISSA: R_f VALUES. L.s.d. 19 PER CENT.

at R_f 0·82 in IAW, which proved to be a phenol different from catechol, orcinol, cresols or resorcinol.

Phenolic fraction: biohistogram and compounds. This biohistogram was not significant in growth activity. A complex phenol at R_f 0·76 was separated, which by alkaline fusion yielded vanillic acid.

Results of Hydrolysate Fractions

Acidic fraction of hydrolysate. Although the related chromatogram was growth inhibiting along its full length, three different zones were cut according to the colour of a narrow strip sprayed with DQC. The related eluates were rechromatographed until the best possible resolution was obtained. In this way vanillic acid was isolated at R_f 0·03–0·15 (8 μg/kg plant); PHB (mixed with small amount of vanillic acid) at R_f 0·12–0·20; *m*-hydroxybenzoic acid (MHB) at 0·26–0·31; salicylic and inhibitory aliphatic acids at 0·55–0·65 and 0·70–0·80 respectively.

Identifications of salicylic, PHB, vanillic and aliphatic acids where made as described above.

Identification of m-*hydroxybenzoic acid.* The amount isolated was about 48 μg/kg plant. Because of impurities, its absorption and fluorescence spectra were not completely identical to those of MHB. However, its identification is beyond any doubt on the following evidence: fluorescence spectra (Table 1); paper chromatography of the acid (R_f 0·34) and its ester (R_f 0·79) in IAW were identical with authentic samples, as was that of its reduction product with $LiAlH_4$ (R_f 0·77).

Phenolic fraction: biohistogram and compounds. The biohistogram shows a zone of growth inhibition at R_f 0·65–0·95, which was confirmed by bioassay of its eluate. Chemically, a phenol was isolated which appeared as a brown spot at R_f 0·78. This spot was very close to that given by orcinol at R_f 0·80 (brown spot), but was not orcinol on the following evidence. Ultraviolet spectrum: orcinol in neutral methanol has a peak at 276 nm, which with alkali changes to 286 nm.; this eluate had peaks at 284 nm, and 280 nm respectively. Fluorescence spectrum (Table 1). It did not react with diazomethane and was recovered unchanged after alkaline hydrolysis.

Growth Activity of the Isolated Compounds

Growth stimulation and inhibition of the isolated substances was studied using either their eluate from *C. sativa* or authentic compounds. The results obtained from both sources were in good agreement. The limits of concentration, between which either growth stimulation or inhibition occurs, were studied using authentic compounds only.

p-*Hydroxybenzoic acid.* As has been shown elsewhere[6] PHB shows a significant growth promoting activity in the range 20–100 μg/ml with a maximum at 50 μg/ml. Between these limits PHB also shows a synergistic action when mixed with IAA. But PHB gives a strong growth inhibition at concentrations higher than 200 μg/ml.

Vanillic acid. Concentrations of vanillic acid lower than 10 μg/ml are ineffective on *Avena* coleoptiles. Betweeen 10 μg/ml and 150 μg/ml vanillic acid showed a noticeable growth stimulation, reaching a maximum at 70 μg/ml where it is equivalent to a solution of IAA of 0·1 μg/ml (Fig. 3).

Mixture of IAA and vanillic acid. Solutions containing IAA 0·1 μg/ml and 1 μg/ml together with increasing amounts of vanillic acid showed the following behaviour: growth activity of the solution is equal to that of IAA alone with concentrations of vanillic acid lower than 10 μg/ml; with concentrations of vanillic acid between 20–130 μg/ml a noticeable synergistic action was found, which reaches a maximum at 70 μg/ml of vanillic acid.

In a mixture of 20 μg/ml of IAA, vanillic acid neither enhances nor inhibits the IAA stimulation in the range 10–70 μg/ml, but the IAA growth stimulation was progressively diminished at concentrations higher than this.

m-*Hydroxybenzoic acid.* This acid in concentrations lower than 120 μg/ml showed some growth-promoting activity, which is especially noticeable between 40–100 μg/ml. An elongation maximum was obtained between 50–80 μg/ml being then almost the same growth stimulation as that brought about by 0·1 μg/ml of IAA. Concentrations higher than 150 μg/ml caused growth inhibition. No plasmolysis was observed.

Mixture of IAA and MHB. Solutions containing IAA 0·1 and 1 μg/ml with increasing amounts of MHB showed the following behaviour: with concentrations of MHB lower than 20 μg/ml the mixture had the same activity of the IAA alone; from 40 to 100 μg/ml of MHB the mixture had a growth stimulation, which reached a maximum equal to IAA alone at

[6] E. Vieitez, E. Seoane, D. V. Gesto, C. Mato, A. Vázquez and A. Carnicer, *Physiol. Plantarum* **19**, 294 (1966).

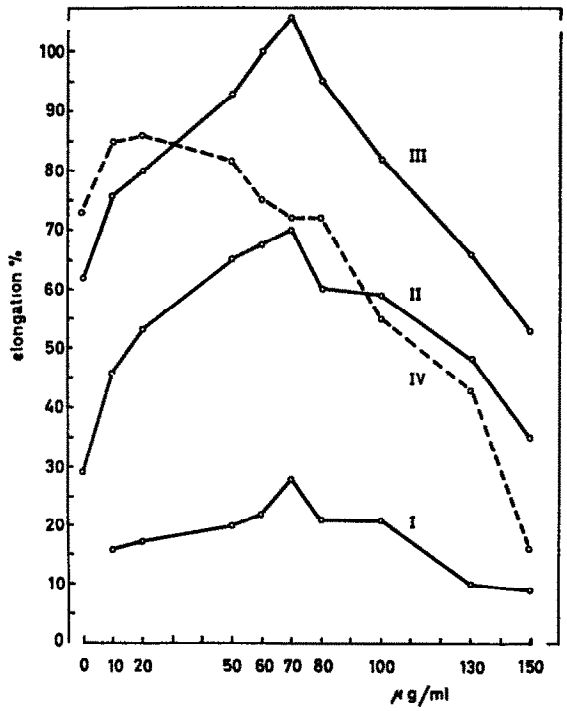

FIG. 3. BIOLOGICAL ACTIVITY (*Avena* COLEOPTILE SECTION TEST) OF PURE VANILLIC ACID (10–150 μG/ML) WITH AND WITHOUT IAA ADDED AT THREE CONCENTRATIONS. CURVES: I, NO IAA; II, 0·1 μG/ML IAA; III, 1 μG/ML IAA; IV, 20 μG/ML IAA. L.s.d. 15 PER CENT.

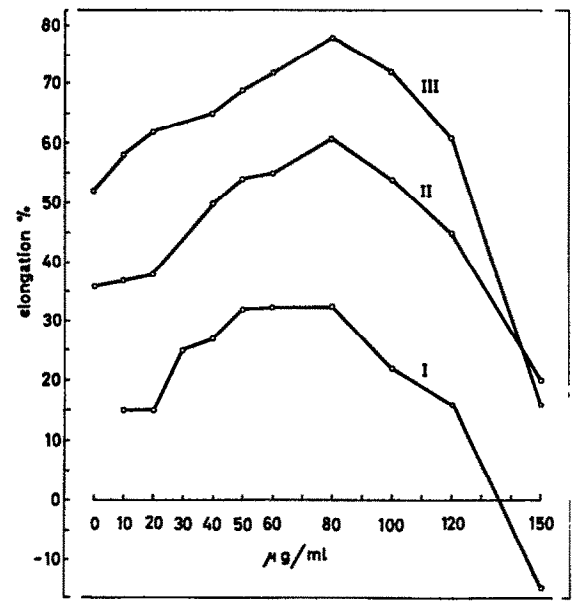

FIG. 4. BIOLOGICAL ACTIVITY (*Avena* COLEOPTILE SECTION TEST) OF PURE *m*-HYDROXYBENZOIC ACID (1–150 μG/ML) WITH AND WITHOUT IAA ADDED AT TWO CONCENTRATIONS. CURVES: I, NO IAA; II, 0·1 μG/ML IAA; III, 1 μG/ML IAA. L.s.d. 15 PER CENT.

80 μg/ml; the growth inhibition produced by 150 μg/ml of MHB alone, was neutralized by 0·1 and 1 μg/ml of IAA (Fig. 4). Behaviour of MHB alone is similar to that of PHB, but both acids show different behaviour in the mixtures with IAA: a synergistic growth stimulation was found with PHB; a simple activity enhancement with MHB.

Salicylic acid. Salicylic acid was ineffective in concentrations lower than 40 μg/ml. It brought about a growth inhibition at 50 μg/ml. For higher concentrations growth inhibition became more intense and it was accompanied by cell plasmolysis. The inhibition was complete at 80 μg/ml and the coleoptiles were dead.

Mixtures of IAA and salicylic acid. Salicylic acid was also ineffective in mixtures of 0·1 and 1 μg/ml IAA, which gave the same growth stimulation as IAA alone; but in concentrations higher than 40 μg/ml salicylic acid neutralized the IAA action and was growth inhibiting, producing the same cell plasmolysis as when it was used alone (Table 2).

TABLE 2. THE EFFECT OF SALICYLIC ACID WITH AND WITHOUT IAA ON THE PERCENTAGE INCREASE IN LENGTH OF *Avena* COLEOPTILE SECTIONS

	Salicylic acid (μg/ml)									
IAA (μg/ml)	0	0·1	0·5	5	20	40	50	60	80	100
0	0	8	7	6	7	1	−27	−53	−78	−98
0·1	35	42	43	42	45	31	1	−64	−96	
1·0	60	64	64	64	63	35	5	−57	−97	

DISCUSSION

Biohistogram of the acidic fraction of unhydrolysed extract showed an interesting zone of growth activity which might be due to its IAA content. However no IAA could be detected by chemical or biological tests and the growth-promoting activity must be due to the presence of other substances. PHB has been shown to be growth stimulating in the range of 20–100 μg/ml with an activity maximum at 50 μg/ml.[5] Between these limits PHB also shows a synergistic action when it is mixed with IAA.

Vanillic acid shows a growth stimulation in the range of 10–100 μg/ml, with activity maximum at 70 μg/ml. Vanillic acid also shows a synergistic action in mixtures with IAA. MHB has similar growth properties to PHB. Moreover, these acids in concentrations higher than 150 μg/ml are growth inhibitors or even toxic. Tests on the growth stimulation and synergistic action of other phenolic acids are still in progress. Accordingly, the presence of the above-mentioned acids in the acidic fractions may explain the different activity of their zones. Growth activity of a given zone may be stimulating, inhibiting or have no effect, according to its content of these acids.

Growth inhibitors seem to have a greater relevance than stimulators to the rooting of cuttings of *Castanea sativa.* Substantial amounts of vanillic and salicylic acids were found, which at levels higher than 150 μg/ml are growth inhibitors or toxic for coleoptiles. Furthermore an unidentified hydroxyaliphatic acid was found and it seems to be the most important growth inhibitor for this plant. One of us (E. V.) has found that *C. sativa* cuttings give some rooting after a long immersion in running water which, perhaps elutes the aliphatic acid after a long period of time. If this is a rooting inhibitor, in the same way that it inhibits coleoptile growth, then its elimination would be expected to restore the rooting ability of the cuttings.

EXPERIMENTAL

Extraction, separation and chromatographic analysis. 11·5 kg of fresh *Castanea sativa* Mill. woody cuttings, previously frozen at −45°, were sliced and extracted with methanol at 0–2° for 24 hr. Extraction, concentration, fractionation and chromatographic analysis were performed as previously reported.[6]

Acidic, phenolic and neutral substances were separated from ether solution by successive extraction with aqueous solutions of $NaCO_3$ and NaOH. Alkaline substances contained in the early methanolic aqueous extract, acidified with HCl, were separated by ether extraction, after basification with an excess of NaOH.

Hydrolysis of the aqueous residue. The aqueous residue after removal of ether promoted the growth of *Avena* coleoptiles and was hydrolysed with 0·5 N $Ba(OH)_2$ and the hydrolysate was fractionated as above.[6]

Bioassay. The straight growth of *Avena* coleoptile sections was used either with pieces of chromatograms or with the substances eluted from a chromatogram zone. Results were plotted as a biohistogram.[6,7]

Paper chromatography. Unless otherwise specified, isopropanol:ammonia:water (10:1:1) on Whatman paper No. 1 was used. The phenol reagent most commonly used was a 0·1% ethanolic 2,6-dichloroquinone-chlorimide solution with aqueous saturated borax solution overspray.[8] Substances isolated by chromatography were analysed as described elsewhere.[5,7]

Fluorescence spectra. A Zeiss spectrophotofluorometre ZFM 4C with two monochromators and a Xenon arc-lamp was used to run the activation and fluorescence spectra. Standard compounds were used in the concentrations of 5 μg/ml, or 1 μg/ml in neutral methanol or after addition of KOH.

Alkaline fusion. Extract concentrates were subjected to fusion by KOH for two successive 2-min periods at 240° and 280° in the presence of air. The ether extracts were analysed by paper chromatography.

[7] P. E. PILET and J. DUBOUCHET, *Rev. Gen. Botan.* **69**, 545 (1962).
[8] H. F. LINSKENS, *Papierchromatographie in der Botanik*, pp. 254, 333. Springer-Verlag, Berlin (1959).