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Alkylresorcinols in rye (*Secale cereale* L.) grains*

I. Micromethod for determination of alkyl derivatives of resorcinol in rye grain

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Abstract

A pure preparation of alkylresorcinol from rye grains was obtained by preparative thin-layer chromatography, which gave a UV spectrum characteristic for 5-n-alkylresorcinols and orcinol. This preparation served as standard in the elaboration of a micro method for alkylresorcinols determination in acetone extract from rye grain. It was found that this method is suitable for selective determination of 5-n-alkylrezorcinols in acetone extracts from rye grains.

INTRODUCTIONS

A group of phenol compounds characteristic for cereal seeds namely 5-n-alkyl derivatives of resorcinol, not described so far in other plants — was isolated in 1964 by W e n c k e r t et all. (1964) from wheat and in 1967 from rye by W i e r i n g a (1967). The latter author demonstrated that these substances are localized in the outer layer (pericarp) of rye grains. These compounds can be extracted with the acetone and petroleum ether and they constitute a nonsaponifiable fraction of rye oil. The purified preparation is a mixture of alkyl derivatives of resorcinol substituted in position 5 with a straight, saturated chain of 15 to 25 C atoms. These substances inhibit growth in young animals and restrict, therefore, the possibility of using rye as fodder.

The fluorimetric method of alkylresorcinols determination in rye elaborated by W i e r i n g a (1967) is based on a Guareschi's test specific for

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1,3-dihydroxy-5-alkylbenzene. Musehold (1973) used for quantative determination of resorcinol derivatives in acetone extracts from whole, intact rye caryopses the reaction with diazotized sulphanilamide. Using the thin-layer chromatography the author demonstrated the specificity of this reaction for 5-n-alkylresorcinols. Bray (1952) for determination of phenol compounds in urine used p-nitroaniline. Modification of this method is applied for colorimetric determination of resorcinol derivatives in rye by Laboratory of Plant Breeding Station (Jakubowski 1974).

The present paper describes a simple method for extraction and partial purification of 5-n-alkylresorcinols from whole, intact rye grains. Obtained preparation was used as a reference substance to choose the optimal conditions for selective estimation of 5-n-alkylresorcinols in rye oil. For these purpose we adopted the p-nitroaniline method of Bray (1952). The conditions of the coupling of phenol derivatives with diazotized p-nitroaniline were adjusted so that 5-alkylresorcinols could be selectively determined in the presence of other phenol compounds.

MATERIAL AND METHODS

Material. Rye (*Secale cereale* L.), Variety Dańkowskie Złote from the 1973 harvest, Plant Breeding Station, Rogaczewo, Subunit Choryń. Purity 99.9, germinating power 90. Seed generation: Original. The phenol derivatives were extracted from whole, intact grain with acetone of analytical grade at 54° ± 1°C according to the procedure of Musehold (1973).

Methods

Thin-layer chromatography was done on aluminium foil sheets, 20 × × 20 cm, coated with a Silica Gel G layer at 0.2 mm thickness (Silufol-Kavalier, Czechoslovakia). For preparative purposes chromatography was performed on glass plates, 20 × 20 cm, coated with Silica Gel G layer at 1.5 mm thickness. The solvent systems used are as follows: 1. petroleum ether — ethyl ether — acetic acid (90 : 10 : 2) 2. petroleum ether — ethyl ether — acetic acid — methylisobutyl ketone (90 : 10 : 2 : 10); 3. petroleum ether — ethyl ether — acetic acid — methylisobutyl ketone (90 : 10 : 2 : 20); 4. n-hexane — ethyl acetate (95 : 5); 5. benzene; 6. chloroform — ethyl acetate (95 : 5) 7. chloroform : acetone (85 : 15); Partition was run on a 10 cm distance. After chromatography the plate was sprayed with a solution of 1 per cent vanilline in 50 per cent phosphoric acid or with the diazotized p-nitroaniline reagent used for quantitative determination of alkylresorcinols.

Column chromatography was carried out on Sephadex LH-20. The glass column of 1,2 cm × 49 cm was filled with swollen Sephadex LH-20 in methanol. The column was loaded with 20 mg of alkylresorcinol prepa-

ration dissolved in methanol and eluted with the same fluid at a flow rate of 15 ml/h. The amount of alkylresorcinol in the eluate was determined at 276 and 282 nm.

The p-nitroaniline reagent was prepared immediately before use by mixing 1 per cent aqueous $NaNO_2$ solution with 0.7 per cent p-nitroaniline solution in 1 N HCl. To 5 ml of cold sodium nitrite solution 4 ml of p-nitroaniline solution was added dropwise, with constant stirring, and the mixture was made up to 100 ml with cold water. The reagent stored at 0—4°C is stable for several hours.

Procedure. In calibrated test tubes with ground glass stoppers 1 ml of acet one or methanol solution, containing 10—100 ng alkyl resorcinols was placed and 1.5 ml of the p-nitroaniline reagent was added, the mixture was left to stand at room temperature for 60 min with intermited shaking. Extinction was read against a blank sample in a Specol (G. Dem. Rep.) with ZV attachment, spectrocolorimeter at 435 nm in 1 cm path-length.

Spectrophotometric measurements in the UV were performed on a Specord UV-VIS (Zeiss, Jena, G. Dem. Rep.) or Spectromnom 202 (MOM, Hungary) instruments in 1 cm path-length.

Reagents.

Silica gel G after Stahl-Merck, Darmstadt (Fed. Germ. Rep.) p-nitroaniline — Loba Chemie, Wien, Austria, orcinol-BDH Poole, England, 4-n-hexylresorcinol — Chemapol, Prague, Czechoslovakia, resorcinol — POCh, Gliwice, Poland, Sephadex LH-20 — Pharmacia, Uppsala Sweden; the remaining reagents were analytical grade chemicals purchased from POCh, Gliwice, Poland.

RESULTS

Table 1 shows the influence of the time of extraction of rye grains with acetone at 55°C on the phenol derivatives content determined by the dia-

Table 1

Influence of duration of extraction on the content of phenol derivatives in acetone extracts from rye grains

Duration of extraction hrs	E_{435}/gram of grains
1/2	5.5
1	7.5
2	9.0
3	9.5
5	9.5
24	9.5
Reextraction	0.2

Extraction at 54°. Phenol derivatives were determined with *p*-nitroaniline reagent.

zotized p-nitroaniline reagent. It was found that after 3 h of extraction the residue contained negligible amounts of resorcinol derivatives.

1. Thin layer chromatography of "rye oil"

Analysis of the acetone extracts from grain done on glass plate in the solvent system chloroform — acetone 85 : 15 demonstrated the presence of nine spots staining with vanilin-phosphate. The main fraction with Rf 0.55—0.57 stains red-brown. The remaining fractions occur in smaller quantities and stain purple-blue, purple-red or dark-blue (Fig 1). On the

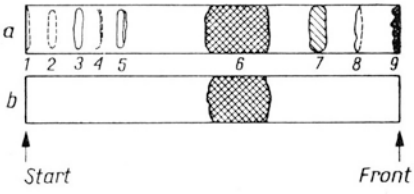

Fig. 1. Thin-layer chromatography on silica gel G of "acetone oil" from rye 'Dańkowskie Złote' in the system chloroform-acetone (85 : 15), 100-µg samples

a — chromatogram developed with vanillin solution in orthophosphoric acid; b — chromatogram developed with the p-nitroaniline reagent used for quantitative determinations

Colours: 1. start — grey; 2, 3, 4 and 5 — blue; 6 — red-brown; 7 — blue-violet; 8 — violet; 9 — red-violet

plate sprayed with the diazotized p-nitroaniline reagent used in the quantitive method appear only one yellow coloured fraction with Rf 0.55—0.57 which was eluted from plate with acetone. After evaporation of acetone the residue was dissolved in methanol and subjected to spectrophotometric analysis in the UV. Two peaks characteristic for 5-n-alkylresorcinol were found at 276 and 282 nm) (Fig. 4).

2. Preparation of alkylresorcinols from rye oil using methanol.

The following procedure, an example of wich is given below, was elaborated to obtain 5-n-alkylresorcinol preparations with omission of thin-layer chromatography.

One hundred gram of rye grains was treated with 250 ml of acetone and kept at 55°C for 3 h. The acetone extract was filtered off and the grains were once more extracted with 50 ml of acetone. The extracts were pooled and evaporated in a drier with air flow at 50°C. The "acetone oil" obtained was treated with 50 ml of methanol and warmed to 50° up to dissolution. The methanol solution was cooled to room temperature, filtered off and two volumes of water were added to filtrate. The whole was left to stand at 4°C for 2 h. The sediment was separated under suction by filtration on Büchner's funnel. The residue was transferred to an evaporating dish and dried with a warm air flux in a drier. The dry preparation was washed twice with petroleum ether, dried under reduced pressure and then in a vacuum exsiccator over P_2O_5 to constant weight. By this procedure a preparation of 5-n-alkylresorcinols was obtained in approxi-

mate 60 per cent yield. Fig. 2 shows chromatograms of the phenol derivatives obtained in different steps of preparation. The purity of the final preparation of 5-n-alkylresorcinols was tested in thin-layer chromato-

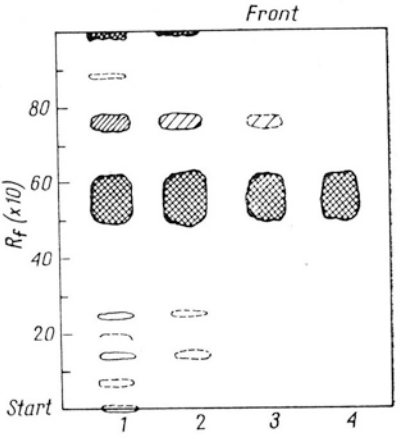

Fig. 2. Thin-layer chromatography on silica gel (Silufol foil) in the system chloroform-acetone (85 : 15); development with vanillin phosphate

1. "acetone oil" from rye — ca. 200-μg sample. 2. — methanol extract from "acetone oil" — ca. 200-μg sample, 3. — alkylresorcinol preparation after precipitation with water from methanol — 100-μg sample dissolved in acetone, 4 — alkylresorcinol preparation after washing with petroleum ether — 100-μg sample dissolved in acetone

graphy in seven different solvent systems (Table 2), and column chromatography on Sephadex LH-20 (Fig. 3). The elution profile shown one peak with slight asymetry.

Table 2

Thin-layer chromatography in different solvent systems of 5-n-alkylresorcinol preparation from rye grains obtained according to the methanol procedure

Systems solvents	R_f
petroleum-ether: ethyl-eter: acetic acid 90:10:2	0.18—0.20
petroleum-ether: ethyl ether: acetic acid: methyl-isobutylic ketone 90:10:2:10	0.34—0.36
petroleum-ether: ethyl ether: acetic acid: methyl-isobutylic ketone 90:10:2:20	0.45—0.47
n-hexane: ethyl acetate 95:5	0.01—0.03
Benzene	0.20—0.22
Chloroform: ethyl acetate 95:5	0.21—0.23
Chloroform: acetone 85:15	0.55—0.57

Chromatography was carried out on Silufol sheets. Distance of migration 10 cm. Chromatograms were stained with vanilline-phosphate. In all cases one spot with R_f given in the table was obtained.

The UV-spectra of resorcinol, 4-n-hexylresorcinol, orcinol (5-n-methylresorcinol) and the preparation of alkylresorcinols from rye are presented in fig. 4. The similar pattern of the spectra of orcinol and of the alkyl-

resorcinols preparation, and the different one for 4-n-hexylresorcinol provides further evidence that the alkyl derivatives of resorcinol from rye are substituted with hydrocarbon chain in position "5".

The alkylresorcinol preparation pure in thin-layer chromatography was used as standard for elaboration of the colorimetric micro method for determination of resorcinol derivatives in biological material. The phenol

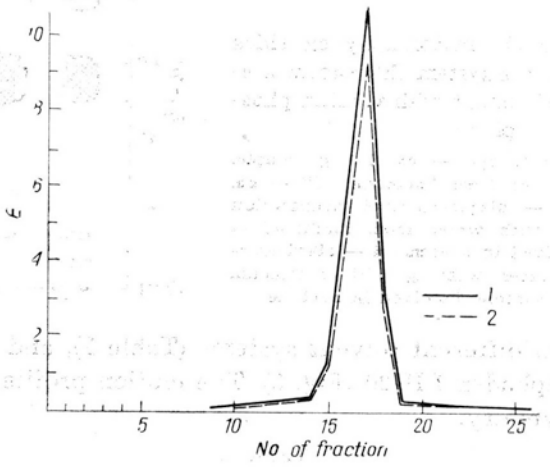

Fig. 3. Molecular filtration of methanol solution of 5-n-alkylresoricinols on Sephadex gel LH-20

Column 12 × 490 mm, elution with methanol (15 ml/h)
1 — 276 nm, 2 — 282 nm

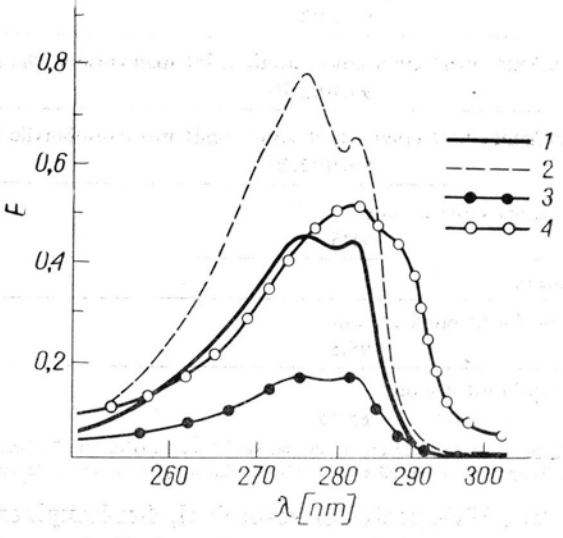

Fig. 4. UV-spectra of methanol solution: 1 — of resorcinol, 2 — of orcinol, 3 — of 5-alkylresorcinols, 4 — of 4-hexylresorcinol. Concentration of substance in methanol solutions 40 μg/ml.

derivatives coupled with diazotized p-nitroaniline are easier soluble in acetone than in ethanol which so far was used in quantitative methods.

Using acetone as a solvent make possible to extend the range of estimation of the reaction products, within which extinction value increases proportionally to alkylresorcinol concentration. Table 3 gives the mean extinction values for 10 to 100 μg of 5-n-alkylresorcinol per 1 ml of the standard solution. The method described fulfills the requirements of analytical methods since it gives results with a good reproducibility and precision.

Table 3

Dependence of extinction on concentration of standard solution of 5-alkylresorcinols determined by the p-nitroaniline method

Standard concentration in sample X (μg)	Mean extinction ± Y
10	0.099±0.0075
20	0.175±0.0095
40	0.362±0.0105
60	0.541±0.0081
80	0.704±0.0181
100	0,874±0.0163

Extinction measurements (n=60) were performed at 435 nm in Spekol spectrocolorimetr with 1 cm path length and ZV attachment.
From the data in the table the equation of the regression line was calculated: $y=ax+b$; $a=0.00868$, $b=0.00106$.

An essential parameter in analytical methods used for determination of given compounds in biological material is their selectivity. In order to establish the specificity of the method thin-layer chromatographic separation of the compounds contained in rye oil was performed. Then the plate was divided into 1-cm cuts and each was eluted with acetone. The eluates were divided in two samples, one of them use for colorimetric determination the other for spectrophotometric analysis. The spectrophotometric analysis was carried out after acetone evaporation and dissolution of the residue in methanol. Extinction was measured at 276 and 282 nm (fig. 5). It was found that the eluate from cuts with Rf 0.55—0.57 represented 97 and 60 per cent of the total extinction value in colorimetric and spectrophotometric estimation respectively. This indicates that the numerous phenol derivatives, detected with vanillin-phosphate, present in acetone extracts from rye with Rf different than that of 5-n-alkylresorcinols cannot be determined with the p-nitroaniline reagent. Extinction measurement at 276 and 282 nm is less specific. It may, therefore, be assumed that in rye acetone extracts mostly alkyl rezorcinols derivatives are determined by the above described colorimetric method.

This method was applied for determination of resorcinol, 5-methylresorcinol (orcinol) and 4-n-hexylresorcinol, with the purpose of using them as standards in determination of alkyl derivatives in biological material. Table 4 shows mean extinction values for solutions of the above named

standards. The data in this table give the converting factor for reference substances which allow the calculation of the content of alkylresorcinols in the sample. When 4-n-hexylresorcinol is used as standard, the read value should be multiplied by 0.89, that for resorcinol by 1,24 and for orcinol by 2.42.

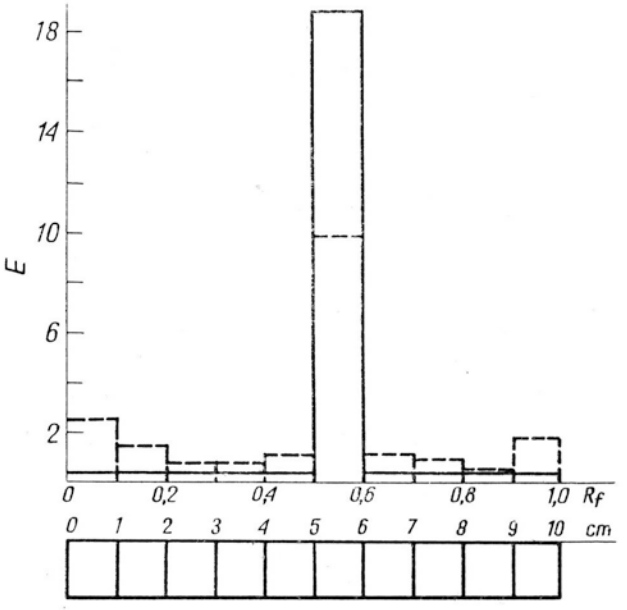

Fig. 5. Specificity of colorimetric p-nitroaniline micromethod in determination of 5-n-alkylresorcinols in acetone extracts from rye grain.

Thin-layer chromatography was run on 20 × 20 cm plates coated with a 1.5 -mm silica gel layer, solvent system chloroform-acetone (85 : 15); the 1 mg/0.2 ml sample was placed along the whole plate breadth. The developed chromatogram was divided into 1-cm fractions. The gel of the particular fractions was eluted with 5 ml of acetone. In the eluates the phenol derivatives content was determined by the p-nitroaniline method, and after exchange of acetone for methanol, extinction was read at 282 nm (— — —)

The described method was applied for determination of the 5-n-alkylresorcinol level in rye of the variety "Dańkowskie Złote". For this purpose 20 samples containing 30 grains each were weighed. They were placed in calibrated test tube with glass stoppers, treated with 3 ml acetone, placed in a water bath at 55°C for 3 h with intermited shaking. After cooling to room temperature the samples were made up with acetone to the initial volume of 3 ml, and 7 ml of acetone were added so that the final volume of the solvent was 10 ml. The test tubes were closed with the stoppers and mixed thoroughly. For colorimetric determinations 0.5 ml samples of the acetone extract were taken, made up to 1 ml with acetone and the reaction with diazotized p-nitroaniline reagent was run as described in Methods. The standard concentration value read from the calibration curve, multiplied by twenty gives the alkylresorcinols content in the rye sample (30 weighed grains). The determined mean alkylresorcinol con-

tent in one gram of rye of this veriety is 0.950 mg ± 6 per cent and is in agreement with the results of quantitative determinations reported by Wieringa in the fluorimetric method in reference to the pentadecylresorcinol standard (Wieringa, 1967).

It results from the data communicated to us by S. Jakubowski that the alkylresorcinols level determined in reference to orcinol as standard in various rye varieties varies within the limits of 0.270—0.550 mg per one g of rye. These valus multiplied by converting factor determined in the present study for orcinol — 2.4 (Table 4) give an alkylresorcinol level of 0.600—1.200 mg per 1 g of rye.

Table 4

Determination of resorcinol and its alkyl derivatives by the p-nitroaniline micromethod

Standard	Range of determinations µg/ml	E_{435}/mg	Conversion factor
Resorcinol (1,3-dihydroxybenzene) n=10	0—100	10.9±0.3	1.24
Orcinol (5-mehtyl-1,3-dihydroxybenzene) n=10	0—40	21.3±0.3	2.42
5-alkyloresorcinols (5-alkyl-1,3-dihydroxybenzene) n=60	0—100	8,8±0.1	1.00
4-hexylresorcinol (4-hexyl-1,3-dihydroxybenzene) n=10	0—100	7.8±0.4	0.89

Resorcinol, orcinol, 5-alkylresorcinols and 4-n-hexylresorcinol used as standards.

DISCUSSION

In view of the results of investigations of Wieringa (1967) and Musehold (1973) acetone was used for selective extraction of alkylresorcinols from grain. Musehold (1973) demonstrated that in acetone extracts from rye grain all alkylresorcinols are present. The extracts obtained by this author contained, however, a number of substances which in thin layer chromatography gave a colour reaction with vanilin-phosphate, but only one fraction reacted with Pauly's reagent (diazotized sulphanilamide).

The observations of Musehold (1973) were confirmed in the present work by the use of diazotized p-nitroaniline. It was demonstrated that both alkylresorcinol preparations obtained from acetone extracts from rye grains, either by preparative thin-layer chromatography, or with the use

of methanol precedure, are pure in thin-layer chromatography in seven different solvent systems and in column chromatography on sephadex-LH-20 and give a UV-spectrum characte ristic for 5-n-alkyl derivatives of resorcinol. This preparation served further as standard for elaboration of a colorimetric micromethod of alkylresorcinols determination by means of p-nitroaniline. The starting point was the method used in Plant Breeding Field Station with which we became acquainted owing to the courtesy of dr S. Jakubowski. In this method, like in all other modifications of Bray's original p-nitroaniline method, the coloured reaction products are dissolved in ethanol. It was found that 5-n-alkylresorcinols isolated from rye form with diazotized p-nitroaniline products difficulty soluble in ethanol, whereas these products are much more readily soluble in acetone. Use of acetone as solvent greatly improved the reproducibility and precision of determinations and increased the range of concentrations in which the colour reaction fulfills the Lambert-Beer's law.

The data presented here suggest that, from among the numerous compounds extractable with acetone from rye grains, the only substances which give a colour reaction with diazotized p-nitroaniline reagent are 5-n-alkylresorcinols. For calibration curve it is best to use a 5-n-alkylresorcinol preparation obtained from the same plant material. The application of other resorcinol derivatives as standards requires the use of appropriate conversion factor. The procedure here described allows selective determination of 5-n-alkylresorcinols with good reproducibility and a small error. Owing to the high sensitivity of the method it is possible to determined alkylresorcinols in single rye grains form one plant. These problems are the subject of the next paper.

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Alkilorezorcynole w ziarnach żyta (Secale cereale L.)

I. Mikrometoda oznaczania alkilowych pochodnych rezorcynolu w ziarnach żyta

Streszczenie

Opracowano warunki oznaczania i wydzielania alkilorezorcynoli z wyciągów acetonowych z ziaren żyta. Uzyskano homogenny w chromatografii cienkowarstwowej preparat alkilorezorcynoli, który dawał charakterystyczne dla 5-n-alkilorezorcynoli oraz orcynolu widmo w UV. Otrzymany preparat służył jako standard do opracowania mikrometody ilościowego oznaczania alkilorezorcynoli w wyciągach acetonowych z ziaren żyta. Wykazno, że atą metodą w ekstraktach acetonowych z ziaren żyta oznaczają się tylko 5-n-alkilorezorcynole.