

EXTRACTION AND PURIFICATION OF PHENOLIC ACIDS FROM CORN STALK TISSUE

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ABSTRACT. — Published methods for extraction of fluorescent compounds were examined qualitatively. None gave complete extraction of fluorescent compounds from corn stalk pith tissue nor satisfactory separation from other substances which interfere with their chromatographic resolution. Evidence is presented that most fluorescent compounds are not present *in vivo* as free phenolic acids but as esters or glycosides. Only ferulic acid was found consistently free in ethanol extracts.

Recently, Guenzi and McCalla (1966) quantitatively determined five phenolic acids (ferulic, *p*-coumaric, syringic, vanillic, and *p*-hydroxybenzoic) in mature oats, wheat, sorghum, and corn residues. They reported that *p*-coumaric acid was present in greater concentration than the other acids and that higher concentrations of phenolic acids were found in alkaline hydrolyzates as compared to acid hydrolyzates.

We have qualitatively investigated the presence of phenolic acids in corn (*Zea mays* L.) stalks in an investigation of the resistance of corn to stalk rot since a great number of phenolic compounds have been implicated in the resistance responses of plants to infection. Only limited and unsystematic investigations have been made of the phenolic acids and other compounds which may be of significance in disease resistance in field corn. Coumarin, vanillic acid, fer-

ulic acid, and *p*-coumaric acid have been found in corn root tissue (Van Sumere, 1960). Reznik and Urban (1957, 1957a) presented chromatographic evidence that scopoletin 7-glucoside (scopolin) and chlorogenic acid are produced in corn leaf tissue from ferulic acid and they may be presumed to occur naturally. They also reported the presence of *p*-coumaric acid in corn leaf tissue. Several workers have identified 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DMBO) glucoside in corn stalk tissue, and it has been implicated in resistance of corn to *Diplodia zeae* (Schw.) Lev., a stalk-rot causing organism [For a literature review see BeMiller and Pappelis (1965)]. Genetic studies on corn pigmentation suggested that cyanidin, pelargonidin and quercetin 3-glucosides, leucocyanidin, and leucopelargonidin are present in the kernels (Coe, 1955; Straus, 1959).

The purpose of this study was to determine which method of extraction gives the best yields of UV fluorescent compounds to be phenolic by various chromogenic spray reactions.

MATERIALS AND METHODS

Five stalk rot resistant inbred lines of corn (B2, B14, C103, C190A, and Tx

173D) were planted on the Southern Illinois University Agronomy Farm on 21 May 1962, and stalks were harvested 11 weeks later (6 Aug. 1962). Internode pith cores of parenchyma tissue were obtained from the first five internodes of non-diseased plants. These cores contained both live and dead tissue (Pappelis and Smith, 1963). Fresh or frozen tissue was extracted with various solvents by heating on a steam bath for 5-10 minutes to inactivate enzymes and then finely divided in a Waring Blendor. The mixtures were filtered through glass wool. Extracts were then neutralized, if necessary.

Paper chromatographic comparison of the extracts was effected on Whatman No. 1 paper with a variety of irrigants, primarily 10% acetic acid, 1-butanol-acetic acid-water (4:1:5 v/v, upper layer), and water-saturated 1-butanol. Location of components was done by long wave (ca. 366 m μ) and short wave (ca. 254 m μ) ultraviolet radiation and suitable chromogenic sprays.

RESULTS

Alkaline extracts of the tissue, made for example with 1% NaOH in water or ethanol, were deeply colored and exhibited intense fluorescence. The brown color may have been due to oxidative degradation or polymerization of the extracted substances, inasmuch as the use of a Waring Blendor thoroughly aerated the mixture. To investigate this possibility, caffeic acid was treated with 1N NaOH under conditions simulating those of extraction (a) with no additional treatment, (b) under nitrogen, (c) under nitrogen with the addition of potassium metabisulfite ($K_2S_2O_5$) and sodium hyposulfite ($Na_2S_2O_4$) as antioxidants, and (d) under nitrogen with borate. All treatments destroyed caffeic acid to some extent; only borate gave any observable protection. This confirms the report of Rauscher et al. (1958) that many of the naturally occurring compounds

are esters which are hydrolyzed by alkaline solutions. Hence, alkaline extractions are undesirable for both quantitative and qualitative work.

Extractions of tissue with 95% and 90% ethanol and 90% ethanol with approximately 0.05% potassium metabisulfite or sodium hyposulfite (Vuataz et al., 1959) were about equally successful. The antioxidants partially prevented darkening of the solutions on standing.

Extractions of tissue were also effected with aqueous solutions of SO_2 , H_2S , NH_4SH and CN^- ; the former three are reducing compounds, and the third is basic in addition. Acidified sodium cyanide solutions contained the most fluorescent compounds. Apparently destructive enzymes are inhibited by cyanide ion. However, chromatographic comparison with the ethanol extracts showed no clear advantage for the use of cyanide over that of ethanol with antioxidant, and the latter method was easier and safer. Later, it was found that cyanide ion on cyanide-treated carbon reacts with DMBO glucoside in aqueous extracts of corn stalk parenchyma tissue to form an artifact (R. P. Hanzlik and J. N. BeMiller, unpublished data, 1966).

Neither should it be assumed that the alcohol extraction method is exhaustive or devoid of artifact formation. In fact, when the residue remaining from the ethanol extractions was thoroughly washed with hot water and petroleum ether, extracted with 95% ethanol in a Soxhlet extractor for 72 hr and then treated with a base, a number of fluorescent compounds, one of which was ferulic acid, were released. Also, it was shown that the original ethanol ex-

tracts contained large amounts of reducing sugars and amino acids; and, since heating these two together in the presence of ions such as metabisulfite and hyposulfite effects Mailard-type reactions (Ellis, 1959) to form compounds, some of which are fluorescent (Patton and Chism, 1951; Overby and Frost, 1952; BeMiller, 1966), artifacts may be formed in this way. The carbohydrates and amino acids also interfere greatly with chromatographic separations and identification of the substances by their R_f values.

To separate the fluorescent compounds from the water-soluble carbohydrates and amino acids, the ethanol extracts were evaporated under reduced pressure to sirups which were redissolved in water. Extractions of these aqueous solutions at various pH's with petroleum ether, benzene, chloroform, ethyl acetate, and ethyl ether were effected. Although the pH optimum was found to be about 6 and extraction with ethyl ether in a liquid-liquid extractor for 48 to 78 hours was found to be best, even these conditions did not give complete removal of the fluorescent compounds. The water-soluble, unextracted substances are probably esters and glycosides. Neither did a saturated lead acetate solution give complete precipitation of the phenolic compounds. Fermentation of the aqueous solutions with yeast removes many of the fluorescent compounds in addition to the carbohydrates and amino acids.

Adsorbents were compared for their ability to bind fluorescent compounds from the water solutions and for the ease with which these compounds can be eluted from them. Of

alumina, kaolin, silica gel, florisil, infusorial earth, bone carbon, Norit, Darco G-60 Carbon (untreated and treated with cone. HCl or stearic acid), polyamide, and Polyclar AT (a polyvinylpyrrolidone of Antara Chemicals, a division of General Aniline and Film Corporation), only the latter two showed promise. Polyamide powder has previously reported to be useful for the separations of phenols in columns (Seikel, 1962) and in thin-layer chromatography (Davidek and Prochazka, 1961; Davidek, 1962). The use of polyvinylpyrrolidone for the purification and separation of fluorescent compounds will be the subject of a subsequent report.

Qualitative identification of the components showed that, of the common phenolic acids, only ferulic acid was found free in the ethanol extracts of stalk parenchyma tissue of all five varieties. After treatment with bases, much more ferulic acid and *p*-coumaric and vanillic acids were found but no attempt was made to identify all the compounds so produced.

DISCUSSION

This study revealed that none of the extraction methods used, which were for the most part methods reported in the literature, gave complete extraction of the fluorescent substances or adequate separation of the fluorescent substances from carbohydrates, amino acids, etc. which interfere with their chromatographic resolution and identification, or protection of the extracted substances from degradation and/or polymerization. Methods now available for purification and separation of pheno-

lic compounds work well only with the free phenolic acids. Evidence obtained in our laboratories indicates that the phenolic acids of corn are not free but in the form of esters or glycosides; but identification of the phenolic acids released from these compound by treatment with acids or bases, while it does not identify the naturally occurring substances, may be of some use in determining the products of enzymic action such as might be found in areas of pathogen growth in a tissue or in decomposition of plant residues. However, caution must be used in relating the phenolic acids to biological effects until it has been established that they are indeed produced by such actions.

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