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FRACTIONATION OF CONDENSED TANNINS BY COUNTER-CURRENT CHROMATOGRAPHY

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SUMMARY

Counter-current chromatography (CCC) was used to separate condensed tannin extracted from sorghum grain. Tannin already purified by another method was separated by CCC into nine fractions. Crude tannin extracts of sorghum were fractionated, showing different profiles for different sorghum cultivars. The amount of crude sample applied to the CCC column was increased for a large scale separation to obtain fractions of purified tannin large enough to be used for further characterization. Separation of tannins by CCC is partially due to differences in the length of the polymeric tannin molecules.

INTRODUCTION

Many foods including fruits, cereals, forages and beverages contain polyphenolic materials known as tannins. Condensed tannin in sorghum is associated with a diverse group of biological effects. In the field, effects of tannin in sorghum are generally beneficial; they include bird resistance[1], reduced pre-harvest germination[2] and disease resistance[3]. In the diet, sorghum tannin is usually harmful, being associated with decreased weight gain and lower feed efficiency in chicks and rats[4,5]. It is unlikely that a single tannin component or a single mechanism is responsible for such different effects. An understanding of the biological activities of tannins requires fractionation and purification of these complex polyphenols.

Elution from Sephadex LH-20 is the separation technique most often employed in condensed tannin purification[6]. Sephadex G-50 has also been used to estimate the molecular weights of tannins[7]. Further fractionation has been attempted with high-performance liquid chromatography, using Fractogel TSK HW-4[8], LiChrosorb Si 60[9] and μBondapak C_{18} (ref. 10) as packing materials. While separation of small chains was achieved, polymeric flavanols were often less resolved, giving broad peaks and complex elution patterns.

Counter-current chromatography[11] (CCC) eliminates the need for a solid support because it partitions tannin between two liquid phases. This method provides good resolution and no loss of sample by adsorption. Furthermore, extraneous material present in crude extracts which may interfere by clogging columns is not a

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problem in CCC. Crude extracts are therefore suitable samples and can be separated efficiently. Large scale separations are also possible because a large volume of the stationary phase is retained in CCC.

EXPERIMENTAL

Apparatus

Countercurrent chromatography was performed using a horizontal flow-through coil planet centrifuge[12,13] with an Ito Multi-layer coil[14] (P.C. Inc., Potomac, MD, U.S.A.). The coil consisted of approximately 130 m of PTFE tubing (1.6 mm I.D.) holding 315 ml. The tubing was wound around a spindle leaving an end at the center of the coil called the head and one at the outside of the coil called the tail.

The coil rotates around its own axis as it simultaneously revolves around a central axis causing a complex hydrodynamic motion of the two liquid phases[13]. The stationary phase is held in the column by this planetary motion, while the mobile phase is pumped through the column. The rotational speed is 800 rpm. A solute introduced at one end of the column is partitioned between the two phases as the mobile phase carries it through the column. When the upper phase of the solvent pair is the stationary phase, as it was here, the sample is introduced at the head and elution proceeds from head to tail.

An LDC/Milton Roy Minipump (Riviera Beach, FL, U.S.A.) was used to pump the solvents through the column and an Isco UA-5 absorbance monitor with a Type 6 optical unit (Lincoln, NE, U.S.A.) monitored the absorbance at 280 nm. Fractions (5 ml) were collected with a Pharmacia Frac 100 fraction collector (Uppsala, Sweden).

Materials

1-Butanol was obtained from Fischer Scientific. Sephadex LH-20, (+)-catechin and phytic acid were purchased from Sigma and 2,4-dimethoxybenzaldehyde was from Aldrich. Sorghum grain grown at the Purdue University Agronomy Farm (W. Lafayette, IN, U.S.A.) in 1983 was provided by Dr. John Axtell.

Selection of the solvent system

The solvent system was chosen on the basis of the partition coefficient of tannin in two liquid phases. Partition coefficients were determined in a test tube using equal volumes of two solvents and a small amount of sample. The absorbance at 280 nm of each phase was used to determine the relative concentration of tannin in the lower phase compared to the upper phase. Acceptable ratios are from 0.3 to 3.

A 1-butanol–aqueous phase solvent system was modified to obtain a suitable distribution of water-soluble tannin. The pH of the aqueous phase was varied from 3 to 7 with no significant change in partition coefficient. Adding different salts and varying salt concentration showed that the partition coefficient was dependent on the activity coefficient of the salt. Increasing the activity coefficient shifted the equilibrium to the organic phase, reducing the partition coefficient to an acceptable value, slightly less than 3. Sodium chloride (0.1 *M*) was chosen as the lower phase because of its sufficiently high activity coefficient and its insolubility in methanol. The latter property is important in recovery of tannin from the column. After the fractions were

dried, tannin was dissolved in methanol and separated from the insoluble salt. For larger scale separations the solvent system included 1 m*M* phytic acid in addition to the 0.1 *M* sodium chloride to protect tannin from oxidation[15] and preserve the fractions for further analysis[16].

Separation procedure

The two-phase solvent system was prepared by equilibrating equal volumes of 1-butanol and the aqueous component in a separatory funnel. The aqueous component was 0.1 *M* sodium chloride or 0.1 *M* sodium chloride with 1 m*M* phytic acid, pH 4.0. The solvents were degassed in the separatory funnel, then the phases were separated. The upper phase, used as the stationary phase, was pumped into the column and the sample, dissolved in equal parts of each phase, was injected at the head of the column. As rotation began, the mobile phase was pumped from head to tail at 38 ml/h.

At the completion of a separation, nitrogen gas was blown through the column displacing its contents into a 500-ml graduated cylinder. The percentage of stationary phase retained in the column was determined from the volume of upper phase divided by the total volume.

Preparation of samples

Purified tannin (8 mg) prepared from sorghum grain BR64 by the method of Hagerman and Butler[6] was dissolved in 1 ml of each of the two CCC phases for application to the column.

Crude tannin extracts were prepared from sorghum grain IS8768, a Group II type in which the tannin is extracted with acidic methanol but not with methanol as it is in group III sorghums. Ground grain was extracted twice with methanol (3 ml per gram of grain) by shaking for 0.5 h at room temperature. The extract was discarded and the grain residue was extracted as before but with 1% HCl in methanol. The acidic methanol extracts were combined and dried by rotary evaporation, redissolved in 1 ml of each of the two CCC phases, and filtered with a Buchner funnel.

Assays

Fractions from CCC to be assayed were dried by rotary evaporation and redissolved in methanol. Insoluble sodium chloride from the eluting solvent was removed by centrifugation.

The anthocyanidin formation assay in HCl–butanol was performed as described by Butler[17]. The "vanillin" assay carried out in glacial acetic acid[18] was further modified to increase its sensitivity by replacing vanillin with 2,4-dimethoxybenzaldehyde[18]. It is referred to as the DMBA assay. Concentrations, wavelengths of measurement, and other conditions were as previously described[18].

The degree of polymerization was estimated by a modification of the method of Butler[17] with the DMBA assay substituting for the vanillin assay. This method is based on the principle that the anthocyanidin formation assay detects all of the polymer's flavan-3-ol units except terminal units. A known amount of (+)-catechin was used as a standard in the DMBA assay to determine the absorbance (at 510 nm) per mol of terminal units. The absorbance (at 550 nm) per mol of non-terminal units detected in the anthocyanidin formation assay was determined by running a sample

of purified tannin in both the DMBA and the anthocyanidin formation assays. The DMBA assay was used to calculate the number of non-terminal units (in mol) present in the purified tannin while the anthocyanidin formation assay was used to determine the absorbance at 550 nm for this quantity of non-terminal units.

Knowing absorbance at 510 nm per mol of terminal units and absorbance at 550 nm per mol of non-terminal units for the DMBA and anthocyanidin formation assays respectively, the number of terminal and non-terminal units (in mol) were calculated for a particular sample. The chain length or degree of polymerization was determined by dividing the number of non-terminal units by the number of terminal units (both in mol) and adding 1.

RESULTS AND DISCUSSION

CCC of purified tannin

In our standard method of purifying tannin[6] the final step is elution from a Sephadex LH-20 column with water–acetone (50:50). Fig. 1 shows the elution of BR64 sorghum tannin from the column by measuring the absorbasnce at 540 nm (a tannin-associated pigment). Acetone in the eluting solvent forbids detection at the preferred wavelength, 280 nm, which detects the phenolic chromophores of tannin.

Purified tannin from Sephadex LH-20 was divided into three fractions, as shown in Fig. 1, which were lyophilized to dryness. Fraction C, the most heterogeneous, was separated by CCC into three major peaks plus a group of unresolved components (Fig. 2). According to the principles of CCC[11], those components which are most soluble in the mobile phase relative to the stationary phase will elute first. Thus, the order of elution follows the decreasing solubility in 0.1 *M* sodium chloride relative to 1-butanol. The anthocyanidin formation assay and the DMBA assay showed that the major peaks contained tannin.

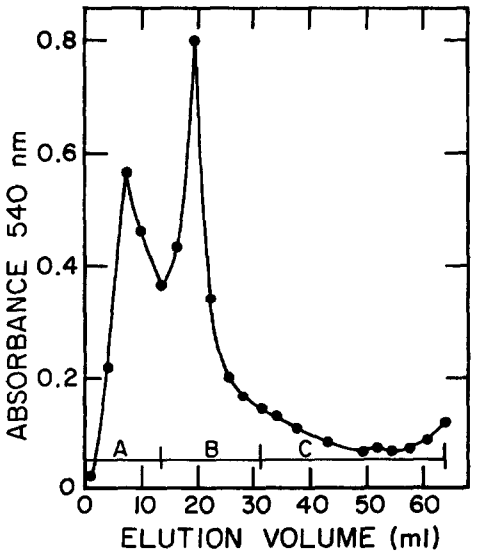

Fig. 1. Purification of BR64 sorghum tannin on Sephadex LH-20 with water–acetone (50:50). Column, 30 × 1.5 cm I.D. Fraction C was further separated by CCC.

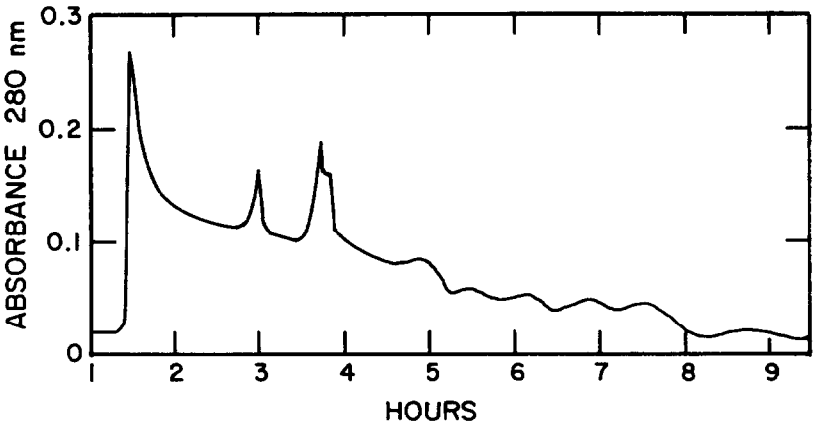

Fig. 2. CCC of purified BR64 sorghum tannin from fraction C in Fig. 1. Sample size: 8 mg dissolved in 1 ml of each phase; solvent system: 1-butanol–0.1 *M* sodium chloride (1:1); stationary phase: upper phase (1-butanol); flow-rate: 38 ml/h; retention of stationary phase: 84%.

CCC of crude extracts of tannin

Because there is no solid support in CCC which could become clogged, crude samples may be applied directly to the column. An acidic methanol extract of a group II sorghum (IS8768) was separated by CCC (Fig. 3). Although a single component predominates, many components are readily detected. This is in contrast to a crude

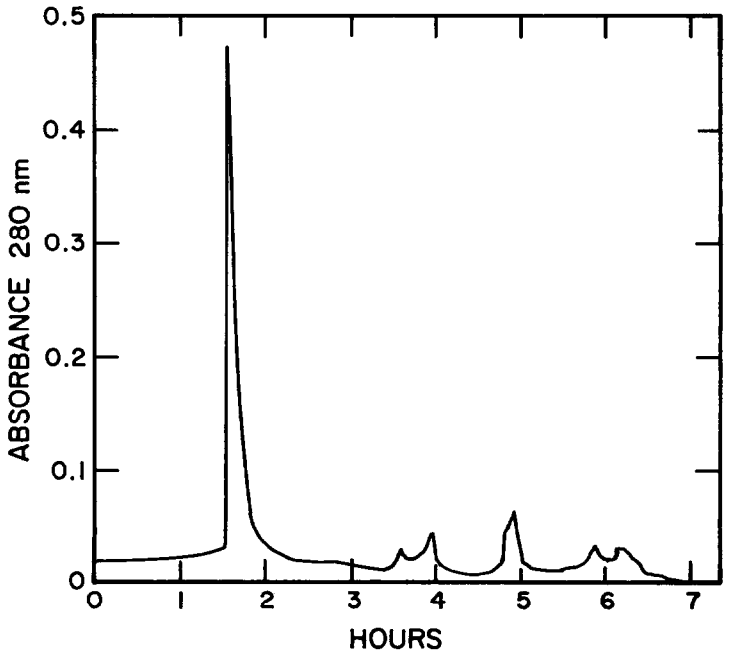

Fig. 3. CCC of the acidic methanol extract from 0.5 g IS8768 sorghum. Sample volume: 2 ml (1 ml of each phase); solvent system: 1-butanol–0.1 *M* sodium chloride (1:1); stationary phase: upper phase (1-butanol); flow-rate: 38 ml/h; retention of stationary phase: 83%.

methanol extract of RS610, a group I (low tannin) sorghum, where peaks are barely detectable. A crude methanol extract of a group III sorghum, BR64, has many more peaks which are less resolved than those from IS8768.

The capacity for obtaining a profile of tannin in sorghum from a crude extract is useful in tannin research. While many biological effects are associated with tannin they may not directly correspond to the quantity of tannin present. Chemical assays generally lack the specificity to measure particular components. CCC provides a means of comparing the tannin in different sorghum lines qualitatively. This can provide insight into tannin's association with various biological effects.

CCC on a large scale

With the advantage of having no solid support and retaining a large volume of stationary phase, CCC has potential for preparative scale separation[19]. In attempting to obtain purified tannin components in quantities suitable for further chemical and biological analysis, increasing quantities of crude IS8768 sorghum tannin were separated by CCC. Crude tannin extracts from 10, 20 and 50 g of seed were separated by CCC. The extracts contained a large amount of non-tannin, low-molecular-weight material, soluble in 1-butanol. This material, which might otherwise saturate the stationary phase, was removed by an ethyl acetate extraction.

Fig. 4 shows the separation of the extract from 20 g of seed. Fractions of tannin obtained were large enough to be analyzed by chemical methods. There was no loss in resolution by increasing the sample size from 10 to 20 g of seed extracted. However, separation of crude tannin from 50 g of sorghum (Fig. 5) showed a decrease in resolution and in retention of stationary phase. In addition to the large sample size the phytic acid in the solvent system contributed to this decrease.

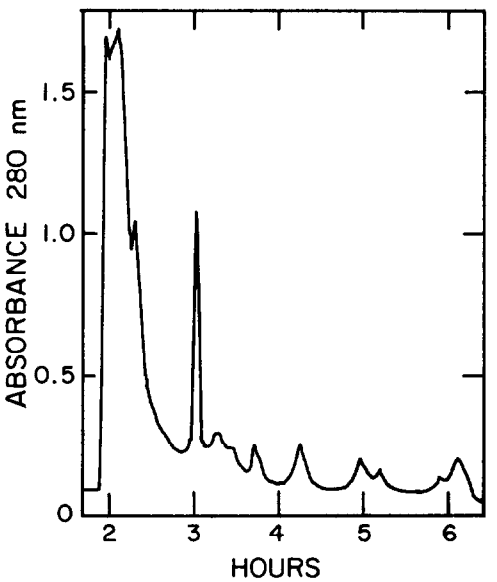

Fig. 4. CCC of crude tannin extracted from 20 g of IS8768 sorghum. Sample volume: 8 ml (4 ml of each phase); solvent system: 1-butanol–0.1 *M* sodium chloride (1:1); stationary phase: upper phase (1-butanol); flow-rate: 38 ml/h; retention of stationary phase: 78%.

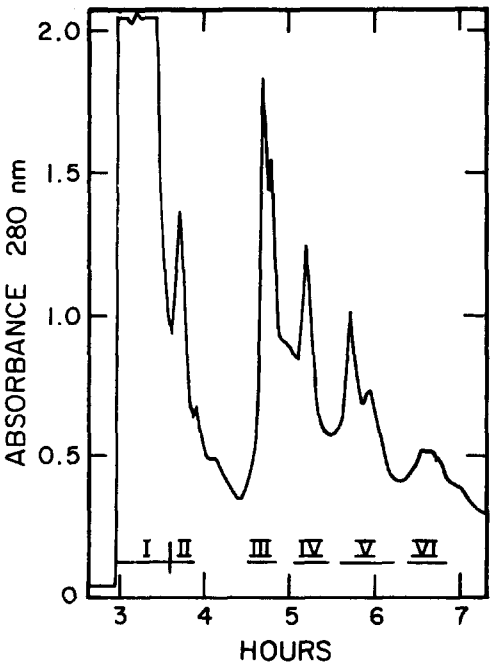

Fig. 5. CCC of crude tannin extracted from 50 g of IS8768 sorghum. Sample volume: 8 ml (4 ml of each phase); solvent system: 1-butanol–1 m*M* phytic acid in 0.1 *M* sodium chloride, pH 4.0 (1:1); stationary phase: upper phase (1-butanol); flow-rate: 38 ml/h; retention of stationary phase: 67%.

The fractions under each peak of Fig. 5 were combined and assayed by the anthocyanidin formation and DMBA assays to determine relative degree of polymerization. Table I shows that the first two peaks contain tannin with a higher degree of polymerization than the remaining four peaks. Longer chains are thus more soluble in the aqueous phase, possibly due to the secondary structure of tannin. The catechin and epicatechin monomeric units of tannin are linked through the 4,8-positions as shown in Fig. 6, or through the 4,6-positions[20]. In a long-chain molecule there can be stacking of monomer units to form a helical structure[20] in which hydrophobic groups are internal, making the molecule more water-soluble. As the chain length decreases, extensive intramolecular hydrophobic interactions are less probable, decreasing the molecule's affinity for water.

TABLE I

DEGREE OF POLYMERIZATION OF PEAKS FROM FIG. 5

Peak	*Degree of polymerization*
I	7.0
II	8.5
III	5.1
IV	5.0
V	4.6
VI	4.9

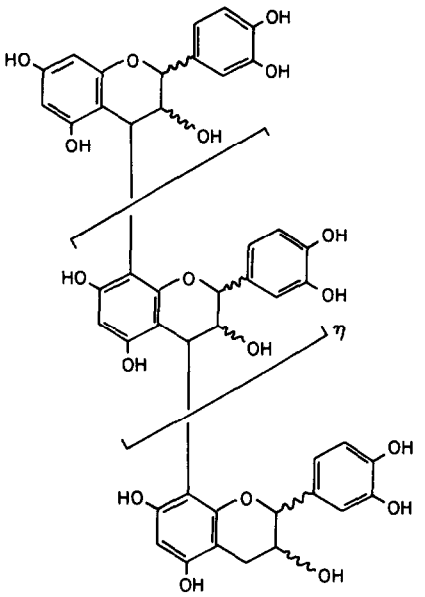

Fig. 6. Basic structure of a tannin molecule. Most common monomeric units are (+)-catechin (2,3-*trans*) or (−)-epicatechin (2,3-*cis*).

Peaks III–VI do not differ significantly in their degree of polymerization. Therefore, in addition to chain length, differences in linkage of the monomers, in the stereochemistry or in the number of hydroxyl groups on the monomeric units may also affect separation of tannin.

CONCLUSION

Tannin is a complex group of molecules with intricate interactions. Countercurrent chromatography can separate tannin into several fractions based on relative solubility in an aqueous salt solution *versus* 1-butanol. This is useful in two aspects of tannin research. First, breaking tannin down into subgroups facilitates study and characterization of its structure and chemical properties. These subgroups of tannin can be obtained in a relatively short time (two days) in quantities sufficient for most analytical techniques. Secondly, CCC can be used to study the relationship of tannin to biological effects by comparing the subgroups present with the biological characteristics of a particular sorghum cultivar.

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