allied to G_1- or G_2-glutelins and coextracted with zein. It is the reason why in an elaborate study on zein (Landry and Sallantin, 1978; Landry, 1979) the whole maize grain, rather than the endosperm, was used as the starting material, thus eliminating the disadvantages inherent to hand dissection of grain.

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Separation of Phenolics of *Sorghum bicolor* (L.) Moench Grain

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Two column supports, useful in the resolution of sorghum grain phenolics, are introduced. The first, Sepharose CL-6B, divides the phenolics into three main groups, F_1, F_2, and F_3, while the second, Sephacryl S-200, subdivides the three fractions obtained from the Sepharose column. A continuous automated monitoring system is described for polyphenol detection. This system can utilize either Folin–Ciocalteu reagent or a solution of $FeCl_3$ in Rochelle salt. On addition of alkali, the Folin detection system monitors total phenolic hydroxyls present, while the $FeCl_3$ method monitors only those hydroxyl phenolics which are spatially acceptable for complexing with the Fe^{3+}. The F_3 group phenolics are the largest in size, consisting of two polymeric forms, one of molecular weight approximately 10 000 and the other of molecular weight greater than 80 000.

Work has been done on the separation and nature of the phenolics of sorghum. Blessin et al. (1963) isolated a material after extracting grain with water, by diphasic solvent distribution and separation on a Dowex 1-X10 column. They tentatively noted that cold acid treatment yielded fisetinidin, while hot acid caused oxidation to a flavonol. Later, Yasumatsu et al. (1965) extracted sorghum with methanol and using a crystalline cellulose column separated the extract into three leucoanthocyanidins. Boiling acid hydrolysis yielded eriodictyol and pelargonidin, though it is probable that the eriodictyol arose by oxidation from luteoferol (leucoluteolinidin).

Bate-Smith (1969) prepared luteoferol from eriodictyol by $NaBH_4$ reduction and showed its presence in both birdproof (high tannin content) and nonbirdproof sorghum cultivars. However, he did not attempt any separatory work and based his conclusions on color changes peculiar to luteoferol.

Nip and Burns (1969, 1971) studied both red and white cultivars of sorghum (colors may have nothing to do with tannin content). They extracted grain with acidified methanol and purified the extract by paper chromatography. Identification by comparison with two known compounds (not authenticated samples) suggested the

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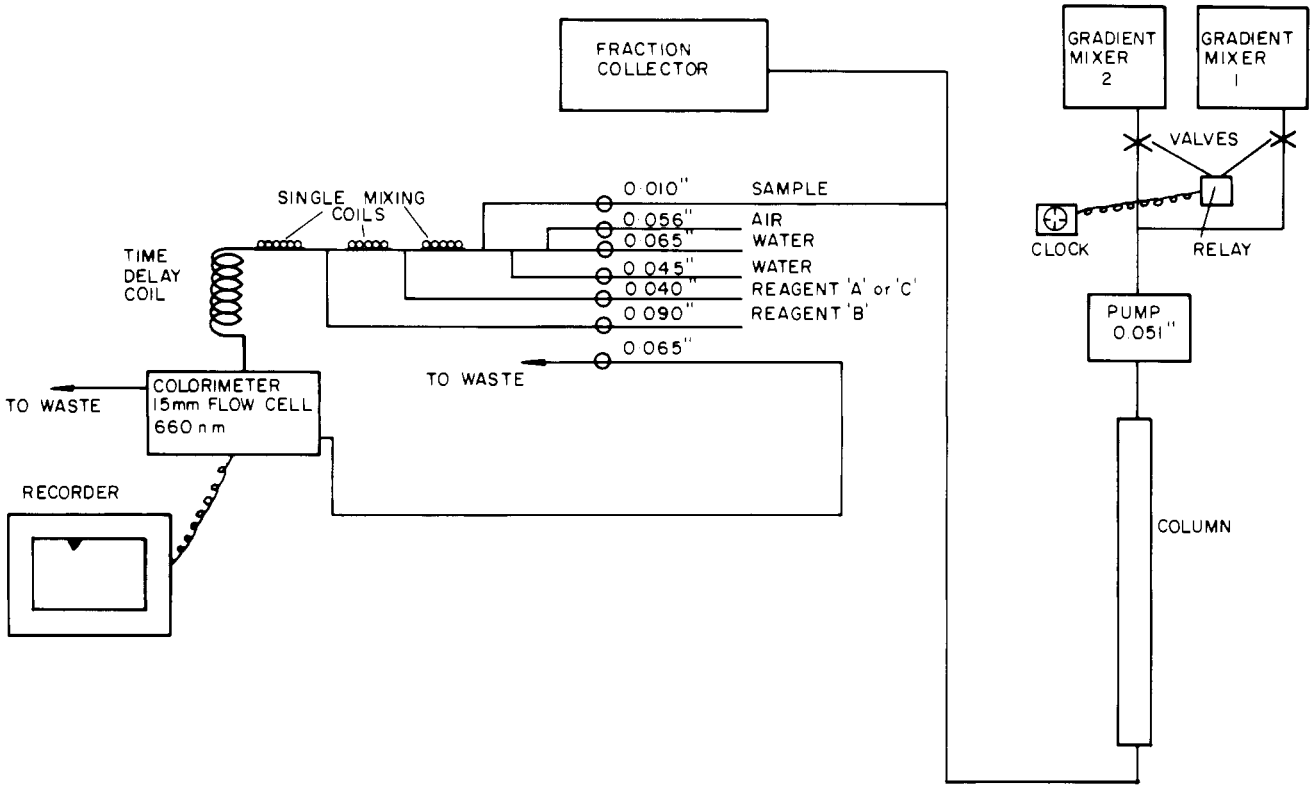

Figure 1. Scheme showing the system used in the continuous and automated detection of sorghum phenolics. Reagent A: A 1:4 dilution of Folin–Ciocalteu reagent supplied by Merck, Darmstadt, W. Germany; resultant color blue (660 nm). Reagent B: 0.5 M ethanolamine. Reagent C: 0.025% (w/v) $FeCl_3$ in a 0.3% (w/v) solution of sodium potassium tartrate; resultant color violet (505 nm). The valves shown are solvent resistant (supplied by the Automatic Switch Co., NJ) and were used only to avoid manual switchover during the night. The equipment used was the Technicon AutoAnalyser system.

presence of apigenidin 5-kaempferol 3-rutinoside 7-glucuronide. In both cultivars they found different glycosides of apigenidin and luteolinidin. Strumeyer and Malin (1975) developed new procedures for the isolation of phenolics from sorghum. They extracted grain with 95% ethanol and separated the tannin from nontannin polyphenols on Sephadex LH-20. The tannins were further purified on LH-20, using 50% aqueous acetone, into five different components. These tannins yielded only cyanidin after hot acid hydrolysis.

In these laboratories it was found that neither polyamide nor poly(vinylpyrrolidone) columns could be used for the separation of tannins from birdproof cultivars of sorghum because of a loss of more than 70% of the applied material. Furthermore, two-dimensional chromatography on paper was too complex to be useful. We therefore decided to utilize the principle of Strumeyer and Malin's method, i.e., hydrogen bonding to a polyol column support. Our choice was Sepharose CL-6B for two reasons: it does not swell or shrink; it has a very large pore and will, therefore, accept a greater load of polymerized polyphenol than LH-20. Further we investigated Sephacryl S-200 because of the amide bond, which would bind polyphenols with a different binding constant, and because of its uniform small particle size and stability to solvent changes.

EXPERIMENTAL PROCEDURES

Materials. All the work described here was performed on a birdproof cultivar of sorghum, known as SSK2, grown by the Plant and Seed Control Division, Roodeplaat Experimental Farm, South African Department of Agricultural Technical Services. It was harvested and worked on fresh, or stored over a few months at 5 °C in the dark. It was chosen because it was the grain available with the most complex phenolic pattern.

Table I. Polyphenol Extraction from Milled Sorghum[a]

extractant	OD 525	OD as % of OD for 75% acetone
acetone	0.985	87.5
aqueous acetone (80% v/v)	1.108	98.2
aqueous acetone (75% v/v)	1.125	100
aqueous acetone (60% v/v)	1.098	97.6
aqueous acetone (50% v/v)	1.066	94.7
aqueous acetone (25% v/v)	0.845	75.2
water, distilled	0.127	11.3
aqueous dimethylformamide (75% v/v)	1.027	91.3
	1.027	91.3
1% HCl in ethanol (95%)	0.126	11.2
1% HCl in methanol (A.R.)	0.215	19.1
acetone–methanol (1:1 v/v)	0.819	72.8
ethanol (absolute)	0.605	53.8
aqueous ethanol (80% v/v)	0.656	58.3
ethanol (95% v/v)	0.635	56.4

[a] The phenolics were detected by the Jerumanis method.

Extraction. In a preliminary study we found that around 75% aqueous acetone gave the best extraction of phenolics. Table I illustrates the superiority of aqueous acetone as compared to methanolic and ethanolic extractants used by most workers. In fact, percolation on a Büchner funnel using 500 mL of 80% (v/v) acetone/100 g of ground grain completely exhausted the marc of phenolics. The sorghum was milled and extracted by percolation as follows (for 100-g sample): (1) with 300 mL of $CHCl_3$ to remove lipids; (2) with 400 mL of $CHCl_3$–MeOH (3:1 v/v) to remove more polar lipids; and (3) with 500 mL of 80% (v/v) aqueous acetone to extract all of the phenolic material.

Detection of Phenolics. Two continuous methods using the same system were devised, shown diagrammat-

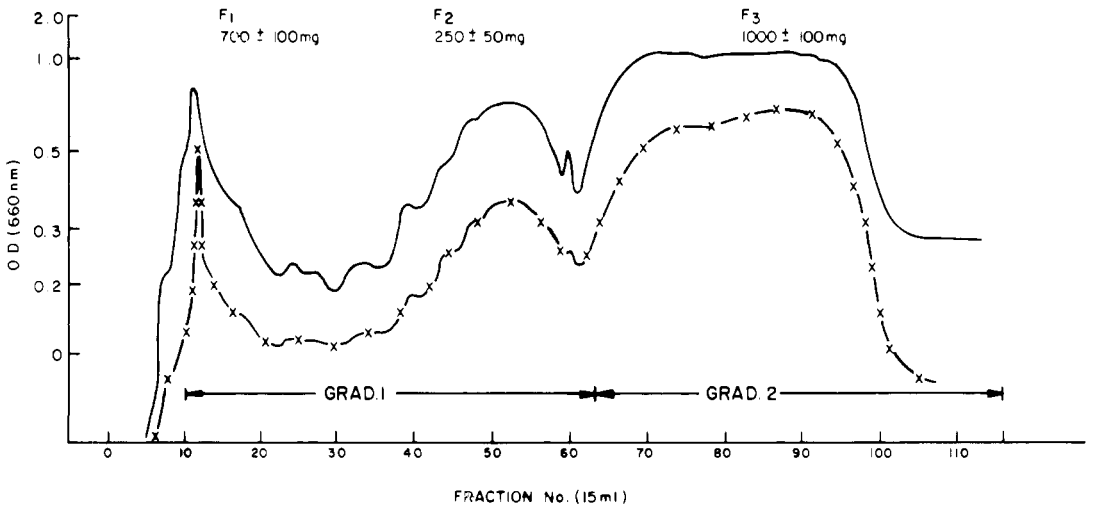

Figure 2. The fractionation of an 80% (v/v) aqueous acetone extract of a SSK 2 sorghum variety on Sepharose CL-6B (2.5 × 33.0 cm). The column was pumped at 1.0 mL/min and 15-mL fractions were collected. Samples were drawn off continuously at 0.05 and 0.23 mL/min, respectively, for the Folin–Ciocalteu (solid line) and the Fe^{3+} chelation (×) determinations. The weights quoted represent an average of six different experiments. The chart speed was 2 cm/h.

ically in Figure 1. One is based on the reductive power of aromatic hyroxyls with the phosphomolybdate complex of the Folin–Ciocalteu reagent. The other depends on the steric configuration of the hydroxyls that, when correct, can complex Fe^{3+} to give a red-blue color adsorbing at 505 nm (a modification of a method introduced by Jerumanis, 1972). The former method measures total hydroxyl groups and is about ten times more sensitive than the latter.

The two main eluting solvents may be either DMF or acetone, both noted hydrogen-bond-breaking solvents. However, acetone acts as a weak reductant for the Folin reagent and so, with gradients of acetone, this reagent cannot be used. In this case the Fe^{3+} chelation technique can be used. However, should the sensitivity of the Folin method be required, then DMF may be used in place of acetone. This may be acceptable if only the recording of the elution profile is important; if material is being collected for further study, then acetone is preferable because of its low boiling point.

Separation. The gradients used in the following work were set up using two three-chambered gradient mixers. The nine-chambered gradient mixer as described by Peterson and Sober (1959) may also be used. The latter has the advantages of not requiring either switchover or pumping. Any other solvent-resistant gradient device can be used.

(*i*) *Using Sepharose CL-6B.* The acetone solution containing the phenolics had 50 g (wet weight) of water-washed Sepharose added to it, and the acetone was then removed under reduced pressure at room temperature. This load was placed on top of a Sepharose column (2.5 × 25 cm) which was previously equilibrated with water and eluted with the following gradients from two three-chambered gradient mixers: (1) H_2O–H_2O–80% (v/v) aqueous MeOH (250:250:300 (mL)); (2) 80% (v/v) aqueous MeOH–25% (v/v) aqueous Me_2CO–50% (v/v) aqueous Me_2CO (282:250:264 (mL)). The gradients were pumped at 1 mL/min by using a peristaltic pump and solvent resistant tubing (Solvaflex, Technicon Instruments).

(*ii*) *Using Sephacryl S-200.* The three fractions (F_1, F_2, and F_3) obtained from the Sepharose column (see Figure 2) were loaded separately onto a Sephacryl S-200 column (2.5 × 33 cm). For F_1 and F_2 the columns were equilibrated with H_2O and for F_3 15% (v/v) aqueous DMF containing 1 mM HCl was used. All solvents referred to as acidic contained 10 mM HCl.

For fraction 1 (F_1) the following gradients were applied: (1) H_2O–10 mM HCl (250:250 (mL)); (2) 10 mM HCl–60% (v/v) aqueous acidic MeOH (250:285 (mL)); (3) 60% (v/v) aqueous acidic MeOH–25% (v/v) acidic dimethylformamide (DMF)–50% (v/v) aqueous acidic DMF (285:253:256 (mL)). For fraction 2 (F_2) the following gradients were applied: (1) H_2O–60% (v/v) aqueous MeOH (250:285 (mL)); (2) 60% (v/v) aqueous MeOH–60% (v/v) aqueous acidic MeOH (285:285 (mL)); (3) 60% (v/v) acidic MeOH–25% (v/v) aqueous acidic DMF–50% (v/v) aqueous acidic DMF (285:253:256 (mL)). To partition fraction 3 (F_3) the gradient was applied with six chambers of the commercial glass nine-chambered mixer (Technicon Instruments). The gradient was made up with the following solutions containing 1 mM HCl and 15%, 25%, 25%, 25%, 30%, and 50% (v/v) aqueous DMF.

Figures 2, 3, 5, and 6 exemplify the separation achieved by using the gradients described here.

Regeneration of Column Material. Both Sepharose CL-6B and Sephacryl S-200 can be regenerated, but the methods differ. Sepharose was washed with 0.5 M NaOH by stirring gently for about 1 h. Three such washings were sufficient to remove the small amount of phenolics adhering. Before reusing, it was imperative to wash well with 0.5 M acetic acid. Washing with water alone was not sufficient to remove the base, and phenolics are readily oxidized under basic conditions.

Base did not remove the phenolics from Sephacryl S-200 and, in fact, made their removal imposssible. Two or three washings with 50% (v/v) aqueous DMF containing 1 mM HCl was found sufficient to remove most of the phenolics; however, after a time, the Sephacryl became permanently stained and was discarded. If the column was not broken down it was sufficient to occasionally remove and discard the upper cm or so.

RESULTS AND DISCUSSION

Separation. Figure 2 shows the preliminary separation of sorghum phenolics into three main groups by using Sepharose CL-6B. If a polyamide column (Strack and Mansell, 1975) was used instead of a Sepharose column, then about 70% of the phenolics were lost. Even fraction 1 (Figure 2), the less tightly bound phenolics, showed 15 to 30% material loss on polyamide. Use of poly(vinylpyrrolidone) resulted in even greater losses. However, the loss with Sepharose was no more than 5%. From Figure

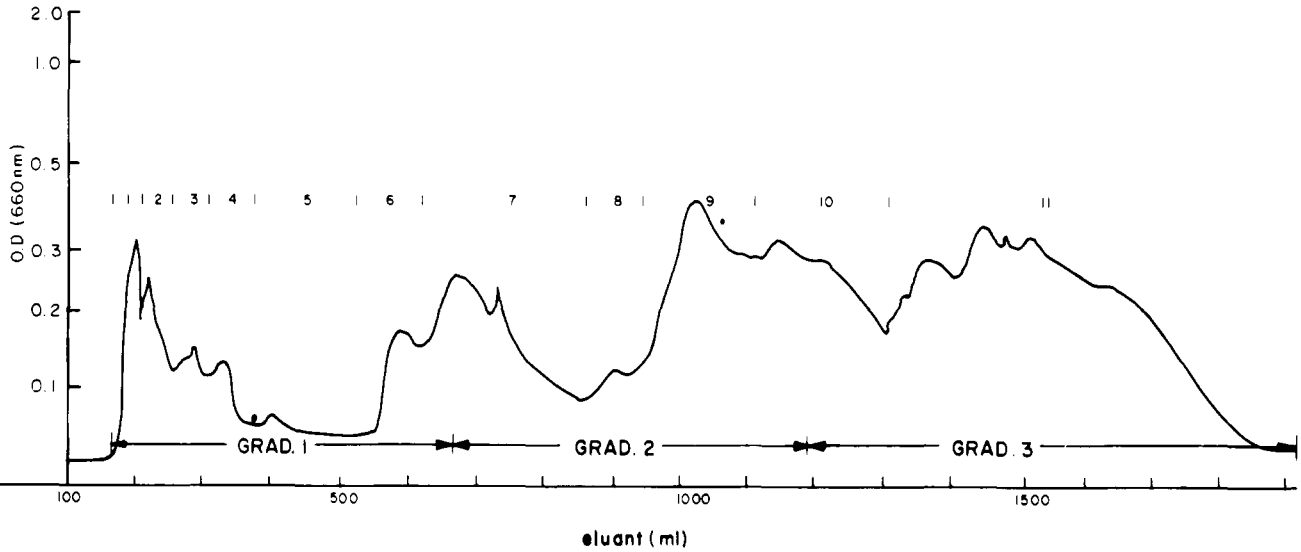

Figure 3. The separation of F_1 (250 mg) from Figure 2 on a Sephacryl S-200 column (1.6 × 75 cm). The pump rate was 0.75 mL/min. Samples were drawn off continuously at 0.32 mL/min and detected with Folin–Ciocalteu reagent. A column (2.5 × 35 cm) pumped at 1.3 mL/min gave a similar pattern. The chart speed was 2 cm/h.

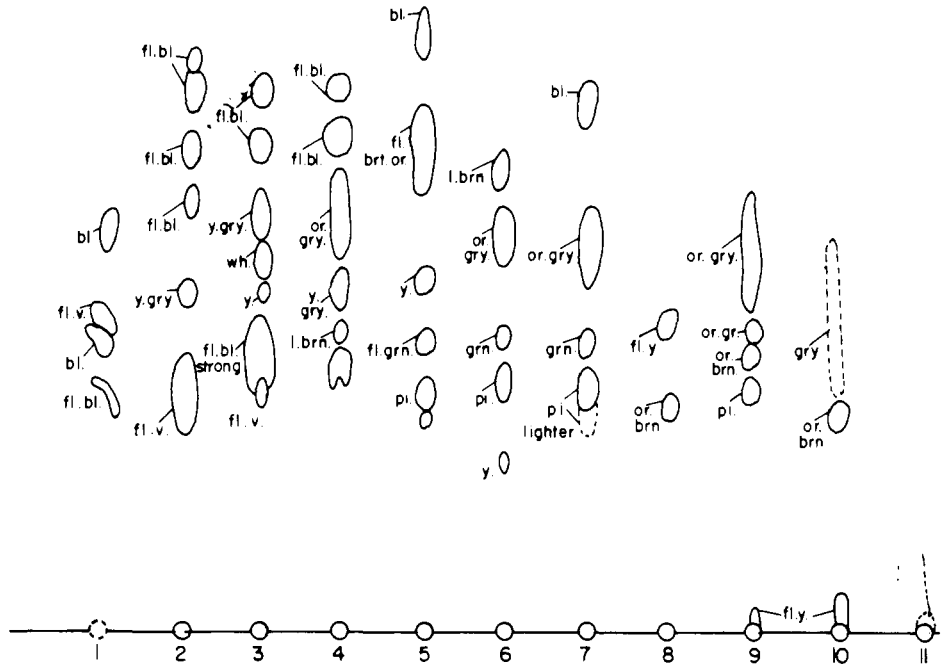

Figure 4. Thin-layer chromatography of the fractions marked 1 to 11 in Figure 3 on a polyamide plate with MeOH–HOAc–H_2O (90:5:5 (mL)) as solvent. After spraying with (2-aminoethyl)diphenylboric acid, the plates were examined under UV light (366 nm). Abbreviations used: bl, blue; brn, brown; gry, grey; grn, green; y, yellow; or, orange; v, violet, wh, white; pi, pink, brt, bright; fl, fluorescent.

2 it can be seen that the main difference between the two detection systems is one of sensitivity. The correlation shown here between the two widely different systems supports the use of the sensitive Folin reagent, as described here, in the detection of phenols.

The Sepharose column was developed to make paper chromatography of F_1 more useful; the phenolic pattern of the crude acetone extract is so complex that no meaningful separation on paper could be obtained by us. However, F_1 (Figure 2) was still very complex, and at least 14 different phenolics could be detected by two-dimensional chromatography on paper. We therefore attempted further column chromatography of F_1 and selected Sephacryl S-200 because of its novel polydextran and polyacrylamide structure. We assumed that the introduction of the amide structure allied to the polydextran would be worthy of investigation because the amide groups would be more widely dispersed than that of the pure polyamide matrixes normally used. Figure 3 shows that the separation of phenolics was good. The separation is independent of length diameter ratio to quite a large extent; two columns of 2.5 × 33 cm and 1.6 × 75 cm gave identical results. The broader column is to be preferred for quantitative work.

The column subfractionation of F_1 is shown from 1 to 11 in Figure 3. These fractions were dried, then dissolved in MeOH, applied to a polyamide plate, and developed with MeOH–HOAc–H_2O (90:5:5 (mL)). Figure 4 shows the various phenolics revealed after spraying with (2-aminoethyl)diphenylboric acid ester (Somaroo et al., 1973). The complexity of F_1 and the usefulness of Sephacryl S-200 are borne out.

Further fractionation of F_2 (from Figure 2) on Sephacryl S-200, shown in Figure 5, suggests a less complex system in comparison with F_1, but it is still quite complex. However, F_3 appears more simple, with only two major

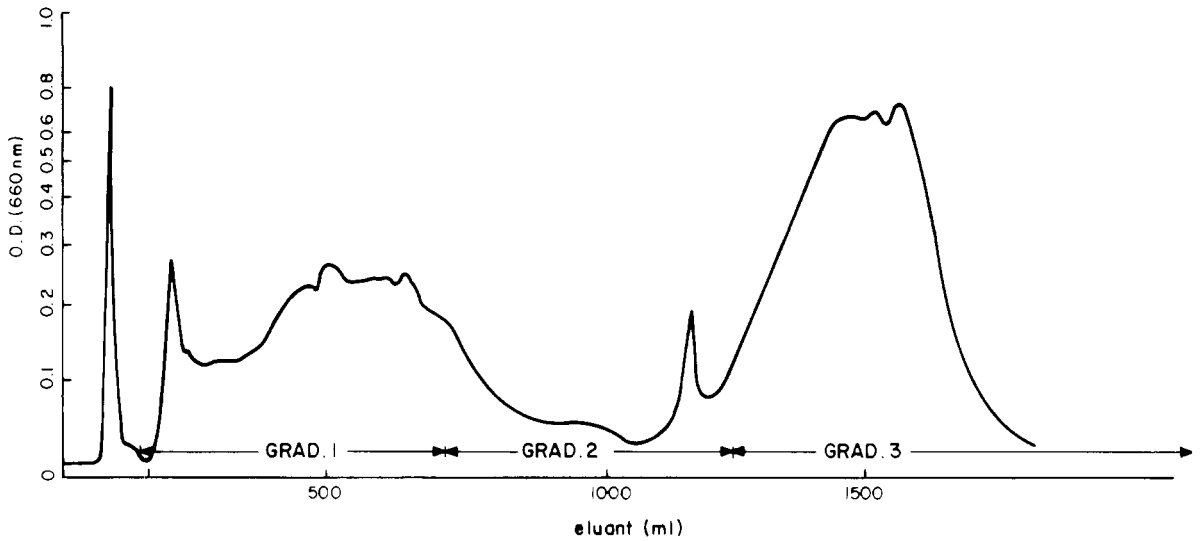

Figure 5. The separation of F_2 (250 mg) from Figure 2 on a Sephacryl S-200 column (2.5 × 33 cm). The pump rate was 1.06 mL/min. Samples were drawn off continuously at 0.1 mL/min and detected with Folin–Ciocalteu reagent. The chart speed was 2 cm/h.

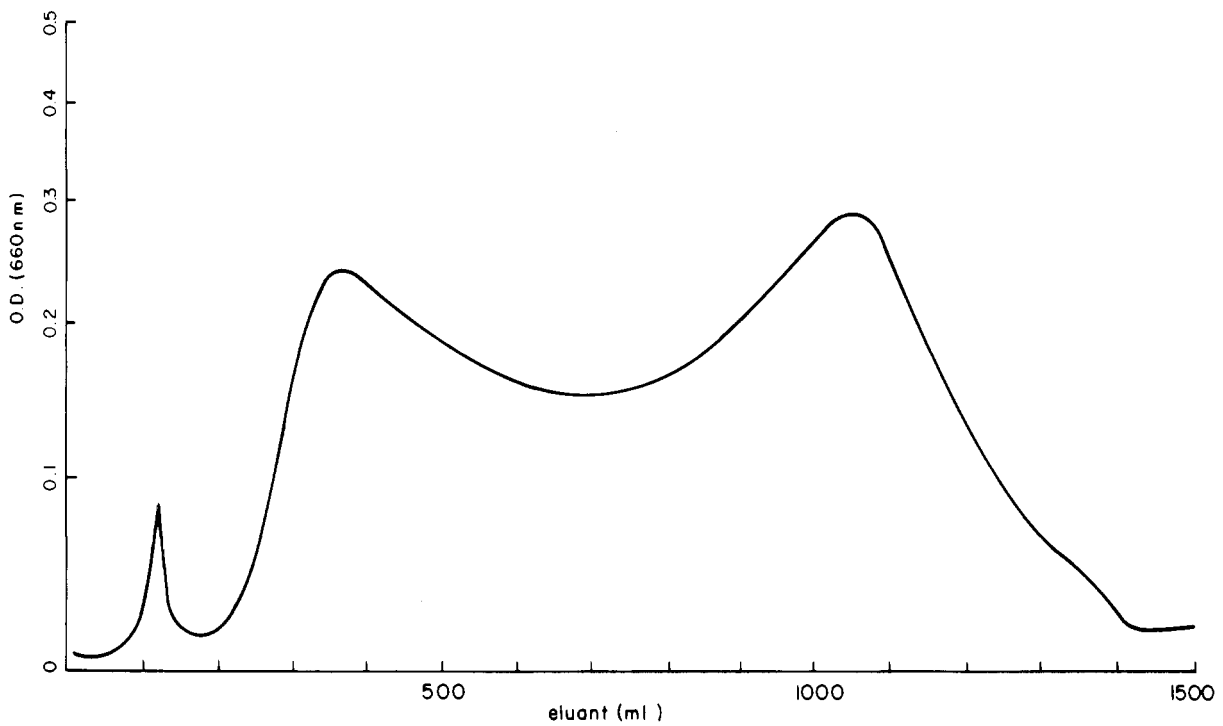

Figure 6. The separation of F_3 (100 mg) from Figure 2 on a Sephacryl S-200 column (2.5 × 33 cm). The flow rate was 3 mL/min under gravity and a six-chambered gradient mixer used, each chamber containing: 15, 25, 25, 25, 30, and 50% DMF all with 0.001 M HCl. Samples were drawn off continuously at 0.1 mL/min and detected with Folin–Ciocalteu reagent. The chart speed was 2.5 cm/hr.

peaks (Figure 6). The two materials appeared on the column as yellow and red bands and were eluted in that order.

Looking at the gradients required for elution from Sephacryl S-200 as compared to Sepharose CL-6B, one can say that binding is much stronger to the former support. Furthermore, the three fractions obtained from Sepharose show a general order of binding $F_1 < F_2 < F_3$ on Sephacryl with an increasing demand for methanol, then dimethylformamide, for elution. This tendency suggests that the main force responsible for the binding is hydrogen bonding, rather than ionic bonding.

Because hydrogen bonding is a result of hydroxyl groups both in the support and in the phenol, and since the support is constant, we postulated that the main difference between F_1, F_2, and F_3 could be the number of hydroxyl groups, which might in turn be dependent on the components being either small molecule(s) containing more hydroxyls or a polymer. For the former concept there might be a small difference in size while the latter might exhibit quite a large change. To examine these possibilities, we subjected F_1, F_2, and F_3 to gel permeation chromatography on Sephacryl S-200 after first showing that the eluant [50% (v/v) aqueous DMF containing 0.01 M HCl] exhibited no partitioning effects; it failed to separate benzoquinone from $CuSO_4$. This solvent also prevents binding of the phenols to the column support. Figure 7 shows that the size order is $F_3 > F_2 > F_1$, but it is not at all a simple relationship; e.g., F_3, F_2, and F_1 all possess material of the same order of size and yet all exhibit vastly different binding constants to Sepharose CL-6B. It is, therefore, possible that size and number of hydroxyls are

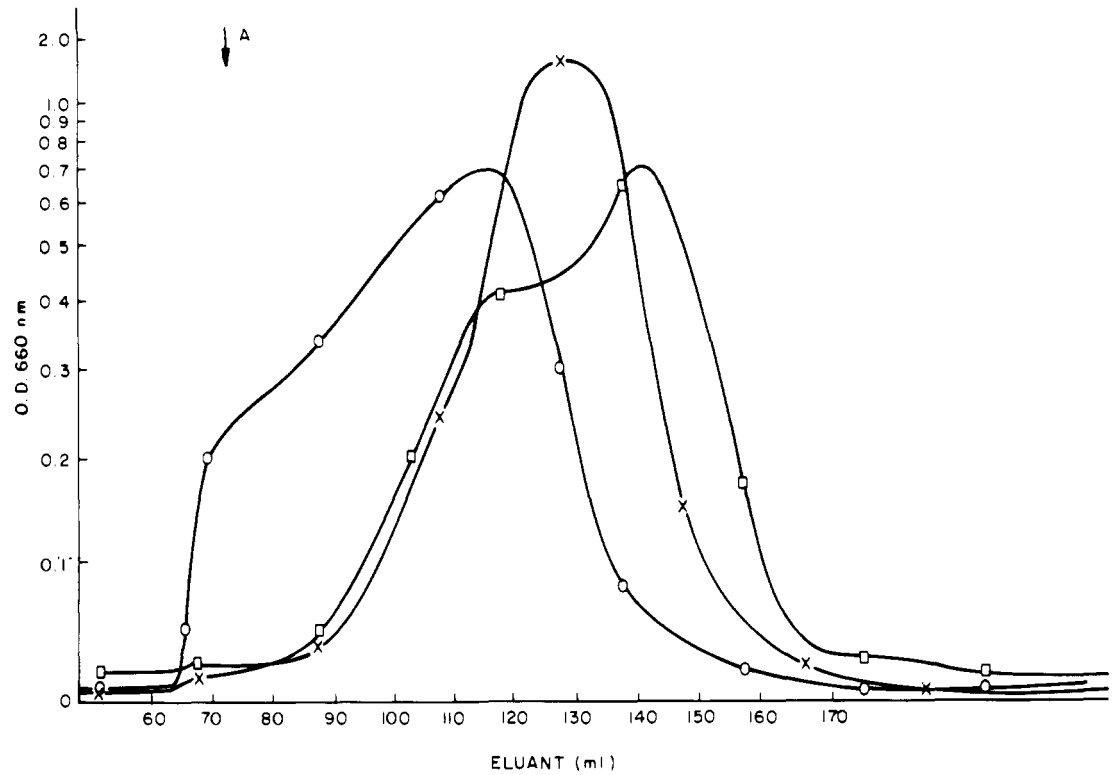

Figure 7. The gel permeation of F_1 (□), F_2 (×) and F_3 (○) on a column of Sephacryl S-200 (91.5 × 1.6 cm) with 50% (v/v) aqueous DMF containing 0.01 M HCl as eluant. The arrow marks the void volume.

both involved in the binding to Sepharose CL-6B rather than one to the exclusion of the order. Further, the position of the hydroxyls on the flavonoid skeleton may also be important.

These concepts are not at all novel and were surveyed as long ago as 1958 by Roux and Evelyn who, in fact, showed a relationship between the molecular weight of wattle tannin and their R_f's on paper chromatography using 1-BuOH–HOAc–H_2O upper phase as solvent. The higher molecular weight material showed a lower mobility, which was the same order as we have found.

The largest molecular weight shown in Figure 6 must be greater than 80 000 because this is the exclusion size of Sephacryl S-200 for linear polydextrans, and the smaller F_3 material must have a molecular weight of 10 000.

In Figure 6 it was noted that the two peaks eluted from Sepharose CL-6B were colored, the first one being yellow and the second red. Interestingly, the elution pattern was similar in Figure 7. In other words, the large phenolic compound was bound less tightly than the smaller ones. Another explanation might be that both separations were due only to gel permeation, i.e., size, and that they have similar binding constants.

CONCLUSIONS

While we developed these techniques to investigate the phenolics of sorghum, we believe that the methods may be more generally useful to people who find it necessary to resolve complex mixtures of natural phenolics. These techniques have subsequently been used to separate phenolics isolated from other plant materials (Glennie).

The main advantage of these procedures probably lies in the preliminary separation of large samples of phenolics prior to their ultimate purification by other well-established techniques such as paper and thin-layer chromatography. A more meaningful discussion of the forces involved in the binding awaits a more detailed examination of the binding itself and the structures of the phenolics involved.

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