Estimation of the degree of polymerization of condensed tannins of some wild fruits of Zimbabwe (*Uapaca kirkiana* and *Ziziphus mauritiana*) using the modified vanillin-HCl method

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Abstract: Methanol and glacial acetic acid were compared for their effect in the vanillin assay for tannins. The time taken for the development of colour was followed using a spectrophotometer. By reacting vanillin and condensed tannins in the presence of acetic acid, the time for the development of the chromogenic substance was reduced approximately threefold compared with the time taken in the presence of methanol. The degree of polymerization was followed by reacting a catechin standard, the tannin of the *Uapaca kirkiana* and *Ziziphus mauritiana*, with vanillin in acetic acid medium at 30 °C and measuring absorbance at intervals. The values obtained suggested between 4 and 10 monomer units of catechin per polymer of tannins for the different portions of the two fruits.
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Keywords: polymerisation; *Uapaca kirkiana*; *Ziziphus mauritiana*; chromogenic; vanillin

INTRODUCTION

The vanillin-HCl method has been widely used for the quantitative determination of condensed tannins in plant materials.[1] The assay is sensitive, relatively simple and is specific for flavan-3-ols, dihydrochalcones and complex condensed tannins. Flavanols, unlike the majority of natural phenolics, react with vanillin in acidic medium to yield a coloured product with absorbance maximum at 500 nm. The vanillin method for estimating tannin is most attractive in that it is specific, but the drawback is that it suffers from a reported lack of repeatability between samples, days and laboratory.[1–3]

Tannins are phenolic plant secondary compounds and are widely distributed in the plant kingdom.[4–6] Methanol is the usual solvent for the extraction of tannins from many plant materials but acetic acid can also be used. By reacting tannins with vanillin in glacial acetic acid, the degree of polymerization of purified tannins can be determined. Butler *et al* reported that, in glacial acetic acid, the reactions of tannins and catechin with vanillin are kinetically similar.[4,7–11]

Generally the concentration of phenolic compounds decreases with decrease in flesh firmness. Bashir *et al*[12] reported a decrease in total phenolics of white and pink guavas as the fruit ripens. The decrease in phenolics as the fruit ripens was also reported in bananas and mango. The decrease in total phenolics as fruit ripens results in a better taste than the astringent taste of unripe fruits. This decrease was associated with the increase in polymerization of condensed tannins and hydrolysis of the astringent sugars, such as arabinose ester of hexahydrodiphenic acid.[12]

Goldstein and Swain,[13] also suggested that the decline in tannin content and the associated loss in astringency of fruits on ripening is due to an increase in the degree of polymerization. The tannin content reaches a maximum early in the maturation process and declines to levels that are different for different plants.[13,14]

The *Uapaca kirkiana* fruit is a fleshy round berry, up to 40 mm across, with a tough reddish brown skin surrounding juicy yellow-brown pulp, in which several hard white-ridged seeds are embedded. The ripe fruit is edible during the period November to February. The unripe *Uapaca kirkiana* fruit has an astringent taste and this may probably be due to the presence of tannins in this fruit, which will depolymerise on ripening. The pulp is honey-sweet with a slight flavour or oranges. It is eaten fresh, the tough seeds and skin being discarded.[15]

Zizphus mauritiana fruits are yellow, 10 mm in diameter, ripening to brown and progressing to black as they dry. They are pleasant tasting and thirst

quenching, eaten fresh, or are sometimes sun-dried as a preserve. The ripe fruits, crushed and soaked for some hours in water make a refreshing drink. If allowed to ferment the liquid may be distilled to make a potent spirit called *kachaso*. The roots have a very commonly known medicinal use. They can be soaked in water for several hours and used to cure convulsions in children.[15]

Our aim of this study was to compare the effects of methanol and acetic acid on the reaction of condensed tannins with vanillin and use the reactions to estimate the degree of polymerisation of the condensed tannins of *Uapaca kirkiana* and *Ziziphus mauritiana* fruits of Zimbabwe.

MATERIALS AND METHODS
Reagents
All reagents used were of analytical grade and were obtained locally except for vanillin and catechin which were obtained from Sigma-Aldrich Chemie (Steinheim, Germany). Standard catechin ($4\,mg\,ml^{-1}$), methanol:HCl (5:1 v/v), glacial acetic acid:HCl (5:1 v/v), vanillin reagent ($1\,g\,100\,ml^{-1}$), 50% methanol in distilled water.

Fruit sampling and storage
The fresh ripe fruits were collected in November at random from fruit trees in Greendale about 10 km from Harare. The fruits were cleaned, packaged in polyethylene containers, transported to our laboratory and stored at $4\,°C$ until further use. The ripe fruits are edible during the period November to late February.

Extraction of total tannin
The peel, pulp, seed coat and seed embryo of the fruits were completely separated and exposed to sunlight until they were completely dry. The dried samples were ground into a very fine powder. The powdered sample (2 g) was extracted twice with cold 50% methanol (10 ml) by suspending a tube containing the mixture for 60 s on a shaker. The two volumes were combined and centrifuged at $3000 \times g$ for 10 min and then the supernatant was filtered into small sample bottles ready for analysis. In another set of investigations the powdered sample (2 g) was extracted twice with glacial acetic acid (10 ml) by suspending a tube containing the mixture for a minute on a shaker. The two volumes were combined and centrifuged at $3000 \times g$ for 10 min and then the supernatant was filtered into small sample bottles ready for analysis.

Colorimetric methods
The sample (5 µl) was placed in a test-tube, 2.5 ml of methanol-HCl added and then 2.5 ml of the vanillin reagent. The mixture was shaken and initial absorbance values were read, using a spectronic 20® Genesys™ (USA) spectrophotometer at 500 nm, followed by incubation at $30\,°C$ in a water-bath, taking absorbance values at 5-min intervals until

no absorbance changes were noted. This point was termed the absorbance maximum (A_{max}).

In another test-tube, the sample (5 µl) was mixed with 2.5 ml of acetic acid-HCl and then 2.5 ml of the vanillin reagent was added. The mixture was shaken and initial absorbance values were read using a spectronic 20® genesys™ spectrophotometer at 500 nm, followed by incubation at $30\,°C$ in a water-bath, taking absorbance values at 5-min intervals until no absorbance changes were noted. This point was termed the absorbance maximum (A_{max}).

In a separate experiment, catechin (5 µl) was placed in a test tube with 2.5 ml of methanol-HCl and then 2.5 ml of the vanillin reagent was added. The mixture was shaken on a Vo-tex and initial absorbance values were read using a spectronic 20® genesys™ spectrophotometer at 500 nm, followed by incubation at $30\,°C$ in a water-bath, taking absorbance values at 5-min intervals. After 35 min, methanol (0.5 µl) was added and absorbance readings were taken for a further 30 min at 5-min intervals.

RESULTS AND DISCUSSION
The rate of chromophore development in the reaction of catechin and vanillin was seen to be dependent on solvents as shown in Figs 1 and 2. The change
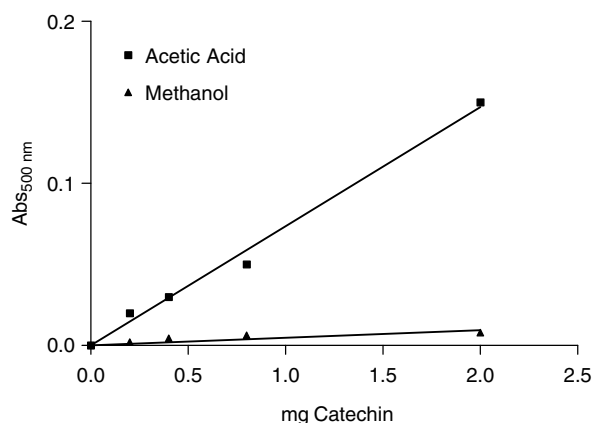

Figure 1. Standard curve for the reaction of catechin and vanillin in acetic acid and methanol.
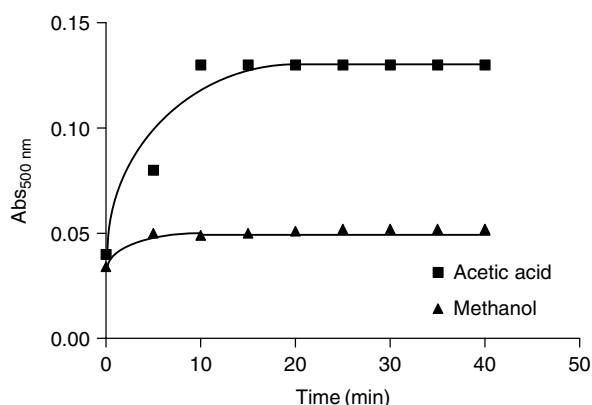

Figure 2. Reaction of catechin with vanillin in acetic acid and in methanol.
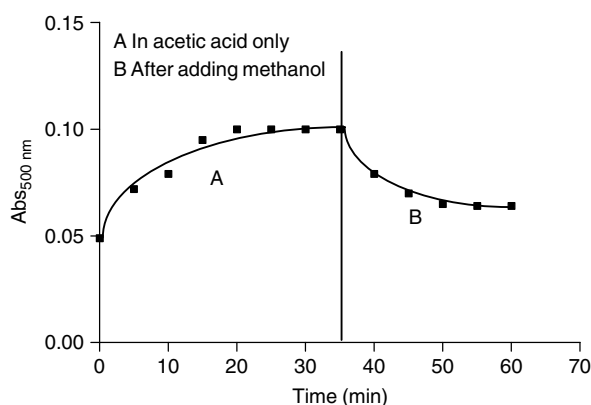
Figure 3. The reaction of catechin with vanillin in acetic acid; after 35 min, methanol was added.

in absorbance over time for the reaction in acetic acid is shown in Fig 2 and the values are almost threefold compared with values obtained when the same reaction was carried out in methanol. This was also true for the standard curve that is illustrated in Fig 1. This suggests that, in methanol, the reaction is slower and the mechanism is not the same since the curve for acetic acid in Fig 2 does not intercept with the methanol curve. Methanol may also have some interfering effects on the reaction.

Introducing methanol into a reaction that initially contained acetic acid was used to test this result, as shown in Fig 3. Addition of methanol to a reaction that initially contained acetic acid decreased the absorbance, as shown in curves A and B in Fig 3. Butler *et al* suggested that the chromophore development in a reaction in methanol is dependent upon several factors, including the degree of polymerization, the types of linkages between the monomer units and their nature.[1] They also suggested that the reaction in acetic acid occurred only at the end-groups. Methanol inhibits chromophore development in this reaction, so that addition of small amounts of methanol resulted in colour loss, as shown in Fig 3. It has also been reported that methanol affects the reaction of tannin monomers and oligomers differently in a complex kinetic pattern that makes the standardisation using catechin difficult. The reaction in both solvents involves condensation of vanillin with the condensed tannins without depolymerisation of the tannins.[16]

The reaction kinetics of Figs 4 and 5 provided the maximum absorbancies (A_{max}), as shown in Table 1, which were used to estimate the degree of polymerization as the ratio of extinction coefficient of monomer to polymer. The extinction coefficient (E_{500}, m^2 mol^{-1}) of catechin calculated from the standard curve (Fig 1), the data in Table 1 and the formula $E_{500} = A_{max}/lc$ derived from the Beer–Lambert Law (where l is the path length and c is the amount of substance concentration), gave the value 20.8 m^2 mol^{-1}. This being the case, the degree of polymerization of tannins of *Uapaca kirkiana* and *Ziziphus mauritiana* in the different portions was calculated using Butler's method of dividing the polymer extinction coefficient by the
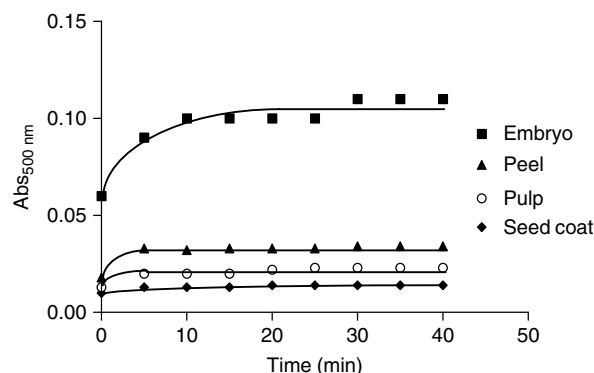

Figure 4. The reaction of condensed tannins of various portions of *Uapaca kirkiana* fruit with vanillin in acetic acid. The reactions were used to estimate the degree of polymerization of the tannins in the fruit.
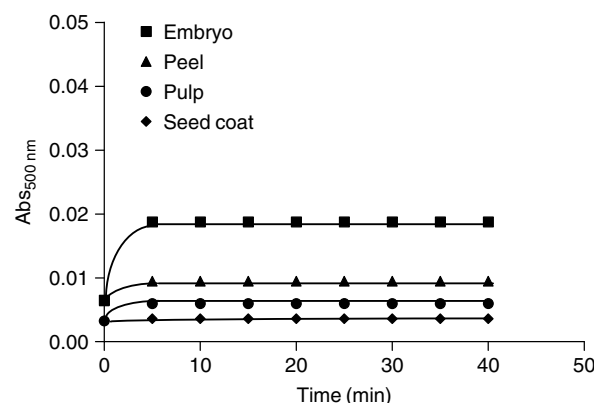

Figure 5. The reaction of portions of *Ziziphus mauritianus* tannins with vanillin in acetic acid. The reactions were used to estimate the degree of polymerization of the tannins in the fruit.

Table 1. Extinction coefficient of the reactions of catechin, *Uapaca kirkiana* and *Ziziphus mauritiana* fruit portions with vanillin in acetic acid

Sample		A_{max}	Concentration (mol l^{-1})	E_{500} in acetic acid (m^2 mol^{-1})
Catechin		0.052	0.0025	20.8
U kirkiana	Peel	0.340	0.0016	212.5
	Pulp	0.230	0.0011	209.1
	Seed coat	0.140	0.0007	200.0
	Embryo	0.410	0.0053	77.4
Z mauritiana	Peel	0.750	0.0040	187.5
	Pulp	0.600	0.0030	200.0
	Seed coat	0.360	0.0020	180.0
	Embryo	0.980	0.0090	108.9

monomer coefficient for catechin, 20.8 referred above, so obtaining the estimates presented in Table 2. The results obtained suggest a range of between 4 and 10 monomer units of catechin per polymer of tannins in the different portions of the two fruits, shown in Table 2. The fruit peel, pulp and seed coat of *Uapaca kirkiana* have each 10 monomer units of catechin whilst the embryo only has 4 monomer units. A more or less similar trend was observed in *Ziziphus mauritiana* fruit peel and seed coat having 9 catechin

Table 2. Estimated degree of polymerization (DP) of *Uapaca kirkiana* and *Ziziphus mauritiana* fruit portions

Sample		Estimated DP
U kirkiana	Peel	10.2
	Pulp	10
	Seed coat	9.6
	Embryo	3.7
Z mauritiana	Peel	9
	Pulp	9.6
	Seed coat	8.7
	Embryo	5.2

monomer units per polymer and pulp having 10 with the embryo having only 5 units. This is, however, comparable with the findings of Butler et al,[1] where they estimated 5 monomer units per polymer of sorghum tannins, and of Gupta and Haslam[17] who suggested 6–7 monomer units per polymer in an independent research on the sample from the same source. The peel had the highest degree of polymerization, followed by the pulp, with the embryo having the least degree of polymerization in both fruits. Bashir et al[12] reported higher degrees of polymerization in the peels of white and pink guavas than the pulp. This is because changes in the tannin constituent occur more in the peel during fruit ripening. This is true of *Uapaca kirkiana*, which loses its astringent taste as the fruit ripens.[1,12,17]

The reaction in acetic acid is very fast and produces more colour but, for the estimation of total tannins, the methanol reaction is more useful, because the insensitivity of the reaction in methanol towards monomers is an advantage since the absorption mostly due to polymers is maximised while the absorption due to monomers is minimised. From this result we concluded that glacial acetic acid should not replace methanol as the solvent for the vanillin assay of tannins. The reaction in methanol is much less sensitive to monomer units such as catechin than it is to the polymeric tannins.

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