

Effect of drying method and length of storage on tannin and total phenol concentrations in *Pigeon pea* seeds

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Abstract

Total phenols and condensed tannins in *Pigeon pea* seeds were determined by flow injection spectrophotometry. Folin-Denis and vanillin assays were used for total phenol and condensed tannin, respectively. Three *Pigeon pea* accessions were cultivated in a greenhouse. After harvesting, one access was separated into two aliquots. One aliquot was freeze-dried ($-196\text{ }^{\circ}\text{C}$), and the other was dried in a forced air oven at $50\text{ }^{\circ}\text{C}$, like the other access samples, to evaluate drying temperature interference. The seeds were initially analyzed and then stored at around $-10\text{ }^{\circ}\text{C}$, $16\text{ }^{\circ}\text{C}$ or at ambient temperature ($25 \pm 10\text{ }^{\circ}\text{C}$). The stored samples were analysed every 30 days, for a 90 day period. A significant difference ($P < 0.05$) occurred between the drying methods for condensed tannin as well as for total phenols, regardless of the storage conditions. The experiments for comparing total phenols, obtained at different storage times, showed significant differences ($P < 0.05$) for total phenols, indicating that tannin quantification has to be done immediately after harvesting to avoid formation of protein complexes or polymerisation, which lead to erroneous results. In accordance with results, freeze-drying proved to be best for preserving the characteristics of the sample. In order to compare the tannin and phenol contents within accessions, fresh seeds should be used.

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1. Introduction

Phenolics are secondary compounds widely distributed in the plant kingdom. Tannins are a special group of phenolics, with high molecular weight, that occur in higher plants. The chemical diversity of tannins confers many kinds of reactions. Thus, tannin present in some food can react with proteins or minerals in a biological medium, causing damage to the consumer's diet (Deshpande, Cheryan, & Salunkhe, 1986; Ikan, 1969).

Analytical methods used to quantify tannins are based on properties such as precipitation by inorganic or organic reagents, formation of coloured compounds and oxidation with acid permanganate or ferricyanide, on a volumetric basis. The most common methods for tannin determination in legumes and cereals are the

Prussian blue and the Folin-Denis tests. Based on the reducing power of phenolic hydroxyl groups, these methods are not specific, as they detect all phenols, phenolic acids, flavonoids, and tannins (Deshpande et al., 1986). Condensed tannins are normally determined using the vanillin method as chromogenic reagent in acid media (Price, Van Scoyoc, & Butler, 1978).

Tannin quantification methods have been automated by flow injection, due its intrinsic advantages, such as the kinetic characteristic. All determinations are performed with the help of a standard curve, before and after the samples, providing the same conditions for both, standards and samples, and allowing fast and reliable results (Ruzicka & Hansen, 1988). In contrast to batch methods, the physical conditions in a flow system are reproducible because it contains no components that create random turbulence and only reproducible convection is observed (Karlberg & Pacey, 1989). Moreover, this kind of system has advantages, such as reduction of manual steps,

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higher throughput and low reagent consumption, than the manual method.

Sample preparation for tannin and phenol quantification is an important task and it should be considered before analysis. The initial harvesting, drying, storage and extraction methods have significant effects on condensed tannin analysis (Schofield, Mbugua, & Pell, 2001). Atanasova, Fulcrand, Cheynier, and Moutounet (2002), in a study with wine, observed significant changes in pigments during storage. The wines without storage or one-month-stored showed higher levels of pigments including, in particular, genuine anthocyanins. With more than one month of storage time, they observed that anthocyanin concentrations were progressively diminished and more stable structures, such as pyranoanthocyanins, gradually accumulated.

Deshpande and Cheryan (1985), Deshpande et al. (1986), and Price et al. (1978) have also reported influences of time, temperature and humidity during storage of sample before analysis, with decrease of catechin equivalent values for the studied samples.

Drying methods also play an important role in sample preparation for tannin analysis. Palmer, Jones, Wina, and Tangendjaja (2000) studied the effects of temperature and drying method on condensed tannin content in leaf samples of *Calliandra calothyrsus*. At higher temperatures, under aerobic drying, there was more bound tannin (protein- and fibre-bound). The free tannin, under anaerobic conditions, increased slightly with increasing temperature, whereas there was a large linear decrease under aerobic drying. The authors also noticed that total condensed tannin increased slightly with temperature under anaerobic drying, but decreased by a similar magnitude (about 10%) with aerobic drying. Their results also showed that freeze-dried samples had values similar to samples dried at the lower temperatures (25 and 45 °C) for all measures.

The objective of this study was to determine the effects of temperature in sample preparation and length of storage on condensed tannin and total phenol levels in *Pigeon pea* seed samples (*Cajanus cajan* (L.) Millsp. sp.) originating from three different accessions. Folin-Denis and vanillin flow injection methods were used to determine phenols and condensed tannin, respectively.

2. Materials and methods

2.1. General

The amounts of total phenol and condensed tannin were determined in *Pigeon pea* seed samples, using a colorimetric approach, by flow injection systems. The results were statistically treated using the SAS statistical package.

2.2. Solutions

Extraction solution (ES), was prepared by dissolving 1.0 mol hydrochloric acid in absolute methanol (Deshpande et al., 1986).

Folin-Denis reagent, used for total phenol determination, was prepared by mixing 25.0 g of sodium tungstate dihydrate, 5.0 g of molybdato-phosphoric acid, 12.5 ml of concentrated phosphoric acid and 200 ml of water. The mixture was submitted to reflux during 3 h and the resulting solution was made up to 250.0 ml with water. 0.5 M NaOH was used to increase pH.

Catechol (Sigma, Deisenhofen, Germany), a simple phenol (Fig. 1(a)), was used as an analytical standard for total phenol quantification. Reference solutions were prepared in ES from stock solution (100.0 mg l⁻¹) to contain 0.00, 20.0, 40.0, 60.0, 80.0 and 100.0 mg l⁻¹ of catechol.

Vanillin (Sigma, Deisenhofen, Germany) was the chromogenic reagent for condensed tannin analysis. It was prepared by solubilization of 20.0 g vanillin in 250 ml of ethanol. 1.0 M HCl in ethanol was used to decrease the pH and 20 % (v v⁻¹) of the ES in ethanol solution was used as carrier stream.

The reagent, (-)-epicatechin (Sigma, Deisenhofen, Germany) (Fig. 1(b)), was used as analytical standard in the vanillin assay. The stock reference solution (1000.0 mg l⁻¹), was prepared by solubilization of 100 mg of (-)-epicatechin in 100 ml of ES (Ferreira & Nogueira, 2000). Considering the expected contents of condensed tannin, the reference solutions were prepared in ES to contain 0.00, 10.0, 30.0, 50.0, 70.0 and 100.0 mg l⁻¹ of (-)-epicatechin.

2.3. Instrumentation

The flow systems, described in the Figs. 2 and 3, comprised a model ISM 761 peristaltic pump (Ismatec, Switzerland) furnished with silicon pumping tubes, a lab-made injector-commutator (Bergamin, Zagatto, Krug, & Reis, 1985) (Fig. 2) and a 4-way injection valve (Gilson, USA) (Fig. 3), a model 432 spectrophotometer (FEMTO, S. Paulo, Brazil) containing a tubular flow cell (inner volume ca 80 µl, optical path 12 mm) and a model BD111 stripchart recorder (Kipp & Zonen, Delft Holland). The manifolds were built up with 0.8 mm i.d.

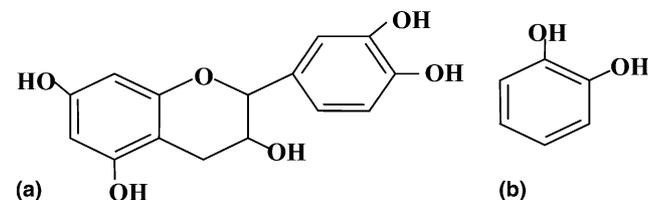


Fig. 1. Molecular structures: (a) catechol (Giner-Chavez, 1996) (b) (-)-epicatechin (Haslam, 1996).

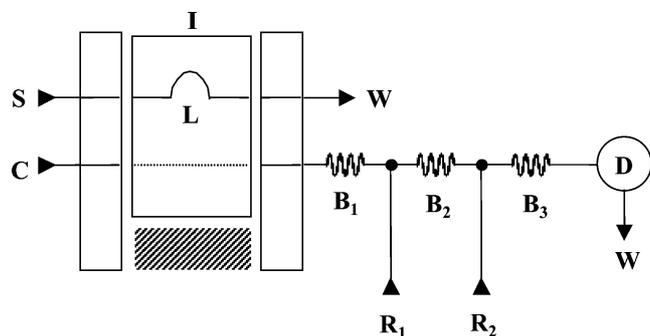


Fig. 2. Flow injection system used for total phenol determination: I, injector-commutator; S, sample; L, sample loop (5 cm, 25 μ l); C, carrier stream (desionized water, 4.5 ml min^{-1}); B₁, B₂, and B₃, reaction coils (50; 50 and 300 cm); R₁ NaOH solution (1.0 ml min^{-1}); R₂, Folin-Denis reagent (0.5 ml min^{-1}); D, detector ($\lambda = 760$ nm); W, waste. More details in the text.

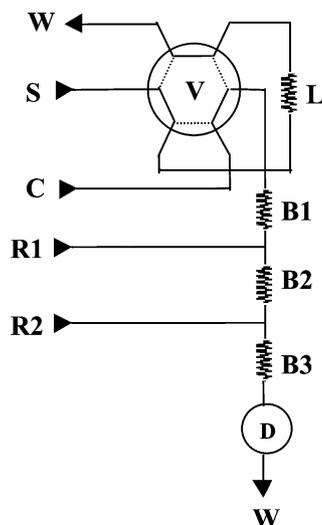


Fig. 3. Flow injection system used for condensed tannin determination: V, injection valve; S, sample; L, sample loop (100 cm); C, carrier stream (ES in ethanol, 2 ml min^{-1}); B₁, B₂ and B₃ reaction coils (50, 50 and 150 cm); R₁, HCl in ethanol (2 ml min^{-1}); R₂, vanillin in ethanol reagent (2.0 ml min^{-1}); D, detector ($\lambda = 500$ nm); W, waste. (Ferreira & Nogueira, 2000).

polyethylene tubing of a non-collapsible wall type and Perspex[®] Y-shaped connectors.

2.4. Sample preparation

Three accessions of *Pigeon pea*, randomly named G84, G146 and G155, were cultivated in a greenhouse. After harvest, samples were oven-dried at 50 °C for 48 h in a forced air oven and ground in a cutting mill fitted with a 20 mesh screen at the bottom of the cutting chamber. About 53 h from harvest, total phenols and condensed tannins were determined in seed samples of all accessions ground. After the first analysis, the samples were stored under three conditions: in a freezer (T around -10 °C), in a cold chamber (T around 16 °C) or

at ambient temperature ($T = 25 \pm 10$ °C). The analysis of condensed tannins and total phenols were accomplished after 30, 60 and 90 days from the first.

Effects of sample preparation were evaluated using the G84 accession. Seeds of this accession were collected and immediately frozen at -196 °C with liquid nitrogen, followed by grinding in a blender. Samples were freeze-dried (E-C Micromodulyo, New York, EUA) and stored under the same three conditions as described above.

Oven- or freeze-dried ground seeds (0.25 g) were mixed with 10.0 ml of extraction solution (ES). The mixture was shaken for 20 min in a mechanical shaker, followed by 8 min of centrifugation at 693 g.

2.5. Flow system

The analytical methodology used for total phenol determination is based on phosphomolybdic-phosphotungstic acid reduction by phenols in alkaline medium.

The original method (Deshpande et al., 1986) was adapted to a flow injection system, similar to that previously used for total phenol determination in water and soil leachates (Zhi, Ríos, & Valcárcel, 1996). The flow set up is outlined in Fig. 2. The length of the sample loop (L), which the sample was aspirated to, was 5 cm (ca. 25 μ l). By switching the injector-commutator, the sample aliquot was introduced in water, the carrier stream (C, 4.5 ml min^{-1}), and a sample zone is created. This sample zone passed through the mixing coil (B₁, 50 cm), received the 0.5 M NaOH reagent (R₁, 1.0 ml min^{-1}), passed through the B₂ mixing coil (50 cm), received the Folin-Denis reagent (R₂, 0.5 ml min^{-1}), passed through the B₃ reaction coil (300 cm), and reached the detection unit (D). The passage of the processed sample through the flow cell resulted in a transient signal, which was measured at 760 nm and was proportional to the total phenol content in the sample. After achieving the analytical signal, the processed sample was discarded (W) and the injector-commutator was switched back to the sampling position in Fig. 2 for the next sample aliquot introduction.

The vanillin flow system used for condensed tannin determination is outlined in Fig. 3. This methodology was previously described (Ferreira & Nogueira, 2000) and it is based on reaction between condensed tannin and vanillin reagent in acid medium. The system performance was similar to that described for total phenol. The injector-commutator was changed by a selector valve to avoid dead volume, which was harmful to the analytical signal. The sample loop was 100 cm (ca. 500 μ l) and the carrier stream was 20% v v⁻¹ of ES in ethanol (2.0 ml min^{-1}). The reaction mixing coils, B₁, B₂ and B₃ were 50, 50 and 150 cm, respectively and the maximum absorbance was measured at 500 nm. The solution of 1.0 M HCl in ethanol (R₁, 2.0 ml min^{-1}) and

the vanillin chromogenic reagent (R_2 , 2.0 ml min⁻¹) were the reagents used for condensed tannin determination.

2.6. Statistical procedures

The experimental design was the incomplete factorial, with six replicates, and the main average effects in the two experiments were appraised by the Tukey test at a probability of 5%. The programme SAS v. 8, procedure GLM III (SAS Institute, 1999) was applied to perform these analyses.

2.6.1. Experiment 1

In experiment 1, the effects evaluated were seed sample preparation (i.e. freeze- or oven-dried), sample storage conditions (i.e. freezer, cold chamber and ambient temperature) and time of storage (i.e. fresh sample, 30, 60 and 90 days after harvest). For this study, only G84 accession was used.

2.6.2. Experiment 2

In the second experiment, amounts of condensed tannins and total phenols of three accessions from *Pigeon pea* were evaluated and compared with time and storage conditions. So, the main effects considered were accesses (i.e. G146, G155 and G84), sample storage conditions (i.e. freezer, cold chamber and ambient temperature) and time of storage (i.e. fresh sample, 30, 60 and 90 days after harvest).

3. Results and discussion

3.1. Experimental procedures

Folin-Denis and vanillin colorimetric reactions, which are based on interactions between different functional groups, have broadly been used (Deshpande & Cheryan, 1985; Dressler, Machado, & Martins, 1995;

Price et al., 1978; Sarkar & Howarth, 1976; Swain & Hillis, 1959). They have also been considered a good alternative for phenolic compound determination (Dressler et al., 1995). Therefore, they were chosen for proposed analysis and were used in the automated flow injection mode.

As the Folin-Denis reagent, phosphomolybdic-phosphotungstic acid, oxidizes any phenol, it was used in the present work for total phenol determination. The flow system adapted for total phenol determination presented good sensitivity and an analytical frequency of 72 samples per hour.

The flow methodology for condensed tannin determination, based on the vanillin–tannin reaction, has been successfully used. This procedure focusses on line reaction between vanillin and flavonoids, with a simple link in the 2–3 position, and free hydroxyls that are meta-orientated in a ring called B, in their structures (Deshpande et al., 1986). The proposed flow system for condensed tannin analysis was stable for a 4 h work period (r.s.d. <1%) and handles about 60 samples per hour.

3.2. Statistical procedures

3.2.1. Experiment 1

Variance analysis results and average estimations for condensed tannins and total phenols and their relationships among seed sample preparations, sample storage conditions and times of storage, obtained by statistical analysis, are presented in Tables 1 and 2.

A significant statistical difference ($P < 0.05$) occurred among the drying methods for condensed tannin as well as for total phenol, regardless of the storage conditions (Table 1). Levels of condensed tannins and total phenols were lower for freeze-dried than for oven-dried samples (Table 2). This contrasts with Terril, Windham, Evans, and Hoveland (1994), who found higher amounts of condensed tannins in freeze- than oven-dried samples

Table 1
Experiment 1. Variance analyses summary

RV	DF	MS			Pr > F ^a		
		CT	TP	CT/TP	CT	TP	CT/TP
Replication	5	3531.91	7441.78	0.0199	0.6984	0.9154	0.0845
Seed sample preparation	1	599078.34	3169187.48	0.0273	0.0001	0.0001	0.0993
Sample storage conditions (C)	2	7623.78	1645.09	0.0197	0.2812	0.9376	0.1396
Time of storage (T)	3	158801.92	1588737.48	0.4300	0.0001	0.0001	0.0001
C ^a T	6	2353.61	8935.56	0.0072	0.8748	0.9065	0.6139
Experimental error	52	5864.29	25502.50	0.0096	–	–	–
Average		550.21	992.96	0.6043			
VC (%)		13.91	16.08	16.27			
R ₂		0.81	0.87	0.79			

Degrees of freedom (DF), medium-square (MS) and the significance of the Snedecor test (F) for the variables: condensed tannin (CT), total phenols (TP) and their relationships (CT/TP) in the random variances (RV).

^a F test.

Table 2

Average estimation in experiment 1 for the condensed tannin (CT) and total phenol (TP) determinations and their relationships in the different variation sources (seed sample preparations, sample storage conditions and times of storage)

Source	Average (mg kg ⁻¹) (N = 3)		
	CT	TP	CT/TP
<i>Seed sample preparation</i>			
Freeze-drying	466 B ^a	786 B	0.628 A
Oven-drying	640 A	1212 A	0.580 B
<i>Sample storage conditions^b</i>			
CC	589 A	1020 A	0.637 A
RT	554 A	1004 A	0.601 A
F	507 A	955 A	0.575 A
<i>Time of storage (days)</i>			
0	682 B	1016 C	0.678 A
30	823 A	1535 A	0.540 B
60	486 C	1258 B	0.401 C
90	533 C	724 D	0.743 A

^a Means followed by the same letter were not different from each other by the Tukey test ($P < 0.05$).

^b Sample storage conditions: CC, cold chamber; RT, ambient temperature; F, freezer.

from *Sericacea lespedeza*. However, these results are in accordance with those obtained by Palmer et al. (2000) who observed a reduction in condensed contents of samples oven-dried compared with freeze-dried, both in aerobic conditions. Hagerman (1988) also found differences among freeze-, air- and oven-dried procedures, depending on the period of harvest and the species used, and suggested that, if a sample must be stored, freeze-drying is recommended instead of air- or oven-drying, because it is better for preservation. In the present work, probably some non-phenolic compounds from *Pigeon pea* were modified, owing to the high temperatures used during sample preparation, and they reacted as a phenolic compound, interfering with assays.

Storage conditions (freezer, cold chamber and ambient temperature) did not interfere with either tannin or total phenol levels ($P > 0.05$). In spite of this, significant results ($P < 0.05$) were observed for total phenol at dif-

ferent storage times. The interaction was also positive ($P < 0.05$) for the condensed tannins in the first 60 days (Table 1). This confirms that phenolic compounds are better determined in fresh samples, since they could form complexes with protein or be polymerised (Hagerman, 1988). They could also become more susceptible to air and light exposure, reducing the intensity of oxidation reactions by the Folin-Denis and vanillin methods.

These results suggest that time of storage and drying procedures are the most important parameters to be considered in condensed tannin and total phenol determinations.

3.2.2. Experiment 2

Tables 3 and 4 show the summary of variance analysis and the average estimation of condensed tannins and total phenols and their relationships among accessions, sample storage conditions and times of storage.

Table 3

Experiment 2. Variance analyses summary

RV	DF	MS			Pr > F ^a		
		CT	TP	CT/TP	CT	TP	CT/TP
Replicate	5	5170.33	32618.29	0.0015	0.7761	0.0185	0.9041
Accession (A)	2	1144901.56	54460127.24	1.4187	0.0001	0.0001	0.0001
Sample storage condition (C)	2	52019.037	138637.41	0.0011	0.0001	0.0001	0.7969
Time of storage (T)	3	551064.67	3926785.17	0.4881	0.0001	0.0001	0.0001
A*C	4	42620.51	36216.03	0.0018	0.0011	0.0166	0.8231
A*T	6	128869.93	524579.37	0.1420	0.0001	0.0001	0.0001
C*T	4	17182.94	57633.58	0.0020	0.0942	0.0011	0.7986
A*C*T	8	24101.89	60173.87	0.0009	0.1913	0.0001	0.9921
Experimental error	67	2073.38	11099.57	0.0049	–	–	–
Average		537.00	2267.13	0.4454			
VC (%)		8.48	4.65	15.69			
R ₂		0.96	0.99	0.97			

Degrees of freedom (DF), medium square (MS) and the significance of the Snedecor test (F) for the variables: condensed tannins (CT), total phenols (TP) and their relationships (CT/TP) in the random variances (RV).

^a F test.

Table 4

Average estimation in experiment 2 for the condensed tannins (CT) and total phenols (TP) determination and their relationships in the different variation sources (accessions, sample storage conditions and times of storage)

Source	Average (mg kg ⁻¹) (N = 3)		
	CT	TP	CT/TP
<i>Accession</i>			
G146	348 B ^a	4578 A	0.0771 C
G155	622 A	1011 C	0.679 A
G84	641 A	1212 B	0.580 B
<i>Sample storage conditions^b</i>			
CC	568 A	2324 A	0.453 A
RT	558 A	2297 A	0.452 A
F	483 B	2177 B	0.431 A
<i>Time of storage (days)</i>			
0	836 A	1746 D	0.622 A
30	757 B	3010 A	0.391 B
60	494 C	2588 B	0.282 C
90	513 C	1958 C	0.554 A

^aMeans followed by the same letter were not different from each other by the Tukey test ($P < 0.05$).

^bSample storage conditions: CC, cold chamber; RT, ambient temperature; F, freezer.

A significant statistical difference ($P < 0.05$) occurred among accessions, sample storage conditions and times of storage for both, condensed tannins and total phenols (Table 3). The level of total phenols was higher than that of the condensed tannins, in all accessions, during the 90 days (total observation period) (Table 4). This was expected because the condensed tannins are a class of polyphenol, which reacts with the Folin-Denis reagent. The vanillin assay is a more specific test, and only the condensed tannins and some dihydrochalcones could be quantified (Deshpande et al., 1986). The content of total phenols presented increasing values for the three accessions, especially in the first 30 days, and the amount of the condensed tannin was approximately the same during this period. The increasing of phenolic levels could be due to interference from chemical variations, which reacted with Folin-Denis reagent, especially in the first 30 days.

The G146 accession presented the lowest levels of tannin and the greatest levels of phenols when compared with G155 and G84 levels (Table 4). This was expected, because G155 is an accession of collection which suffered agronomic changes regarding tannin amounts.

4. Conclusions

Time and storage conditions mainly affect the phenol analyses. The oven-drying procedure changes the phenolic compounds to greater or lesser degrees, depending on the species and the maturation stage of the plant, and some non-phenolic compounds could be modified owing

to the high temperatures, used during the sample preparation, interfering with the results. The freeze-drying procedure should be chosen if the samples need storage, because temperature, air and light exposure could reduce the intensity of oxidation reactions of the analytical methods. Otherwise, the best time for phenol or tannin determination is immediately, or until 30 days after harvest. The freeze-drying procedure and immediate determination of total phenols or condensed tannins are recommended for reliable results.

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