

QUANTITATIVE ESTIMATIONS OF POLYPHENOLIC COMPOUNDS IN *VICIA FABA L.* (LEGUMINOSAE) SEEDS

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Abstract

Polyphenolic compounds and tannins are natural substances of secondary plant metabolism. They are well known for their antinutritional effects. These properties are due to their ability to interact and precipitate protein of the diet. They are responsible for the inhibition of digestion and the decrease of nutritive value of some legumes and cereals grains. Owing to their ability to interact strongly with protein of the ratio, they could be responsible to the inhibition activity of several enzymes [1,2]. So an important particularity must be reserved to these compounds when they are integrated into the diet. Five methods for the measurement of total phenolics, condensed tannins, and tanning capacity of *Vicia faba L.* were tested for their reliability; some of them were improved for their sensitivity. The results were interpreted so as to draw the phenolic pattern of each variety of *Vicia faba L.*, and they revealed different cases.

Key words: *Vicia faba L.*, genotype, polyphenols, condensed tannins, Antinutritional factors.

Résumé

Les polyphénols et les tannins sont des substances naturelles du métabolisme secondaire des végétaux supérieurs. Les tannins condensés sont connus pour leurs effets antinutritionnels aussi bien pour les animaux que pour l'homme. Cette propriété est due à leur capacité de se combiner et de précipiter les protéines, ce qui entraîne une diminution de la digestibilité et de la valeur nutritive de certaines graines de légumineuses et céréales qui en sont bien pourvues. En raison de leur capacité d'interagir étroitement avec les protéines, ils seraient responsables de l'inhibition de l'activité de plusieurs enzymes du tractus digestif [1,2]. Une importance particulière doit être réservée à ces composés quand ils sont présents dans la ration alimentaire. Cinq méthodes sont mises au point pour évaluer la teneur en phénols totaux, tannins condensés et pouvoir tannant des graines de *Vicia*. Ces méthodes sont testées pour leur fiabilité et leur sensibilité. Elles sont appliquées pour dévoiler la richesse polyphénolique de graines de plusieurs génotypes de *Vicia faba*. Les génotypes étudiés sont classés par famille biochimique selon les chémotypes déterminés par les cinq dosages.

Mots clés: *Vicia faba L.*, génotype, composés phénoliques, tannins condensés, facteurs antinutritionnels.

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ملخص

المركبات الفينولية و التانينات (Tannins condensés) مواد طبيعية للأبيض التانين للنباتات الزهرية. ومن بين المركبات الفينولية تعرف التانينات المكتفة بتفاعلاتها اللاغذائية سواء عند الحيوان أو الإنسان. وترجع هذه الخاصية إلى قدرة إرتباطها بالبروتينات، مما يؤدي إلى انخفاض الهضم وضعف القيمة الغذائية لبعض البقوليات الجافة والحبوب بصفة عامة. ولسبب قدرة تفاعلها الشديد مع البروتينات تكون هذه المركبات مسؤولة على تثبيط نشاط عدة أنزيمات هضمية. تعطى أهمية بالغة لهذه المركبات عندما تكون موجودة في الوجبة الغذائية، لكن جداول تركيب الأغذية لم تأخذ بعين الإعتبار هذه المغذيات الدقيقة ونشاطها الحيوي، وضعت خمسة طرق لتقييم المركبات الفينولية و التانينات المعقدة والقدرة التانينية لحبوب الفول (*Vicia faba L.*). أختبرت هذه الطرق لدقتها وحساسيتها، وطبقت لغرض الكشف عن عدة أصناف من الفول بالفينولات. وصنفت هذه المواد حسب العائلات البيوكيميائية والمجموعات الكيميائية المحددة بالطرق المدروسة.

الكلمات المفتاحية: *Vicia faba L.*, فينولات, تانينات معقدة, مواد لاغذائية.

The polyphenolic compounds such as tannins are well known for their antinutritional effects. These properties are due to their ability to interact with and precipitate proteins. They seem to be responsible for the inhibition of digestion enzymes and the decrease of nutritive value of some legumes and cereals. Several methods have been proposed to quantify these polyphenolic compounds. However most authors limit their quantitative approach to only one assay that is generally the vanillin test to Brodhurst *et al.* [8] and its numerous variants. Some works associate this test to the prussian blue assay [3] used for the detection of total phenolics. Other scientifics limit their approach to the anthocyanidin production method through acid treatment [4,7] or a protein precipitation technique such as BSA complexation [2]. Thus in most reports is presented a partial view of the phenolic pattern of plants studied. We propose a global procedure for polyphenol analysis of some varieties of *Vicia faba*, based on different chemical and biochemical assays.

EXPERIMENTAL SECTION

Materials

Vicia faba L. cultivars were provided by G. Duc (INRA, France). The varieties were chosen to represent the diversity of results obtained with different assays, called respectively n°949 T (red-brown testa), Minica (brown testa), Alferd (fawn testa), genotype n° 893

(green testa) and genotype n° 245 (beige-green testa). Catechin, tannic acid and vanillin were obtained from Sigma Chemical Co., gallic acid from Fluka AG. The anthocyanidin production and the vanillin assays were improved from Mimosa tannin, a condensed tannin source.

Methods

Folin-ciocalteu assay for total phenolics

1 ml of Folin-ciocalteu reagent was added to 7 ml of diluted aqueous tannin solution. 2 ml of 20% sodium carbonate was added 3 minutes later and this mixture was vortexed. After 20 minutes in a water bath at 40°C, the absorbance was read at 805 nm against a reagent blank.

Prussian blue assay for total phenolics

10 ml of 0,1 M FeCl_3 in 0,1 N HCl was added to this mixture, immediately followed by 1 ml of 0.008 M $\text{K}_3\text{Fe}(\text{CN})_6$. After 10 minutes at room temperature, the absorbance was measured at 725 nm against a reagent blank. For both assays, results were expressed as gallic acid, catechin, or tannic acid equivalents. As they are susceptible to interfere with non phenolics, it was necessary to associate them to PVP adsorption.

PVP adsorption

The PVP (polyvinylpyrrolidone) from Sigma Chemical Co. was conditioned during 10 minutes in boiling 10% HCl. After rinsing with distilled water, to recover a neutral pH, and then with acetone, it was dried at 70°C in drying oven. 25 ml of diluted aqueous tannin solution were acidified to pH 3.5 and added to 2g of PVP. The mixture was stirred gently for 10 minutes and centrifuged 20 minutes at 4500T/mn. The supernatant was assayed with the Folin ciocalteu or with the Prussian blue method. The PVP adsorbing all phenols, the difference between the assays realized before and after adsorption gave the exact phenolic content of the solution.

Anthocyanidin production through acid treatment for condensed tannins

The reagent was prepared just before use with 5% conc. HCl in n-BuOH. Four volumes reagent (3, 6, 9, 12 ml) were tested to hydrolyze 0.5 ml of aqueous solutions containing 0.25 to 1.5 mg.l^{-1} of mimosa tannins. The effect of addition of a ferric solution after Porter *et al.*, [10] on the yield of anthocyanidin productions was also studied: 0.2 ml of 2% (v/w) $\text{NH}_4(\text{FeSO}_4)_2 \cdot 12\text{H}_2\text{O}$ in 2N HCl was added in each tube for a reagent volume of 6 ml. The tube were stopped and placed in a boiling water bath for 2 hours. The absorbance of the cooled solution was read at 550 nm against a reagent blank. The results were expressed in mg/g from the $E_{1\%}^{1\text{cm}} = 150$, given by Bathe-Smith.

Vanillin assay for condensed tannins and catechins

To test the influence of vanillin and HCl concentrations of the reagent on the method sensitivity to Mimosa tannins, we employed the following combinations:

- Firstly, 8% HCl in MeOH was mixed v/v to successively: 1% [6], and 2.5 %, 4%, 5.8 % vanillin in MeOH.
- Secondly, 1 % Vanillin in MeOH was mixed v/v to successively : 8% [6], 24%, 48%, 72 % HCl in MeOH.

The reagents were prepared and mixed just before use.

To 1 ml of methanolic tannin solution were added 5ml of vanillin-HCl reagent. The absorbance was read at 500 nm after exactly 20 minutes in a water-bath at 30°C.

Haemanalysis

2ml of aqueous tannin solution were added to 3 ml of a sheep blood solution (1.5 ml in 100 ml of distilled water). After 10 minutes at room temperature, the mixture was centrifuged 10 mn at 4500 g and the absorbance of the supernatant was read at 578 nm.

Gelatin precipitation assay

The protein solution was prepared by adding 250 mg of bovine skin gelatin (Sigma chemical Co.) to 25 ml of distilled water. The gelatin was dissolved by stirring and gently heating the solution; then, 2 g of NaCl were dissolved in 20 ml of this gelatin solution. 10 ml of diluted aqueous tannin solution were acidified to pH 4.5. The tannins were precipitated by 0.2 ml of the NaCl-Gelatin mixture. After centrifugation, the supernatant was diluted and assayed by the Prussian blue method. The results were subtracted to those obtained with the total phenolic Prussian blue assay before PVP adsorption. For both biochemical assays, results were expressed as tannic acid equivalents.

Extraction of *Vicia faba* L testa

The testa were ground in a mortar and 0.5 g of the powder obtained was extracted twice for 20 minutes by 100 ml of hot acetone-water (7/3) containing 0.1 ml of 1% (w/v) sodium metabisulfite in distilled water, as an antioxidant. The extracts were filtered and concentrated under reduced pressure to a volume of 30 ml. A 5 ml fraction was evaporated to dryness and the residues was dissolved in warm methanol to be assayed by the vanillin test.

RESULTS

Determination of total phenolic content

Comparison between the Folin-ciocalteu and the Prussian blue assays is given in figure 1. The Prussian blue assay is about twice more sensitive to gallic acid than the Folin-ciocalteu assay. Moreover, the former presents an excellent reproducibility whereas the different standard curves obtained with the latter can't be considered as identical, as proved by the statistical analyses.

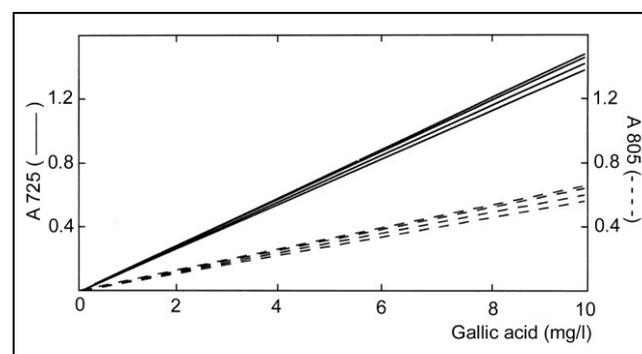


Figure 1: Standard curves obtained from gallic acid with Folin-ciocalteu (-----) and Prussian blue (—) assays.

Determination of condensed tannins content

The anthocyanidin production assay present an optimum yield of oxidation for a reagent volume twelve folds higher than the tannin solution volume, whatever the tannin content of the latter (Table 1). The absorbance can be also enhanced by addition of ferric ions brought by a ferric ammonium sulfate solution. In these conditions this method shows a good reproducibility, proved by the mathematical analysis.

Mimosa tannins mg/ml	Reagent volume				
	3 ml	6ml	9ml	12ml	6ml + (Fe ³⁺)
0.5	0.15	0.17	0.15	0.15	0.21
1	0.33	0.33	0.31	0.29	0.41
1.5	0.39	0.49	0.44	0.41	0.57
2	0.64	0.65	0.59	0.53	0.74
3	0.92	0.99	0.88	0.82	1.01

Table 1: Effect of reagent volume on the yield of anthocyanidins produced by butanol-chlorhydric acid treatment.

The most currently used variant of the vanillin test is that of Price *et al.*[6], which employs reagents containing respectively 1% vanillin and 8% HCl in MeOH, with a reaction temperature at 30°C. This method is perfectly reproducible; however, as several authors work with higher percentages of HCl and vanillin, we have tested the influence of higher percentages on the vanillin assay response, in hopes of improving its sensitivity. As shown in Figure 2, the increase of vanillin proportion has a positive effect on the test sensitivity, but it seems useless to exceed 5.8% of vanillin, as the improvement realized from 4 to 5.8% is rather weak. Likewise, a rising percentage of HCl induces a rehaussed response of the assay (Fig.3). However, for 72% HCl, the curve takes a hyperbolic shape and linear correlation gets weaker. Moreover, the performances obtained by this way (72% HCl, 1% vanillin) can be reproduced with reagents containing 24% HCl and 5.8% vanillin, while keeping a perfect linearity.

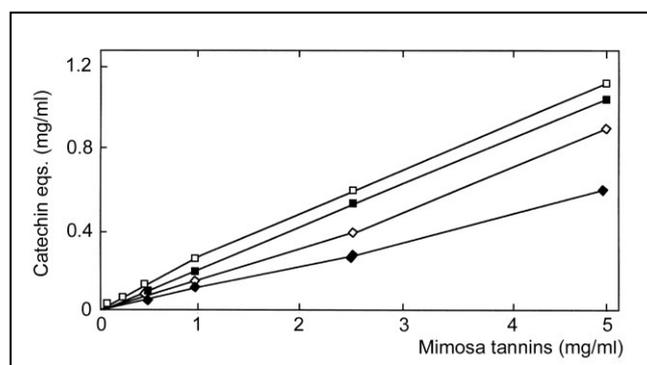


Figure 2: Effect of different concentrations of vanillin 1% (◆), 2,5%, (◇), 4% (■), 5,8 % (□) on the vanillin test sensitivity to mimosa tannin, for a reagent containing 8% of HCl.

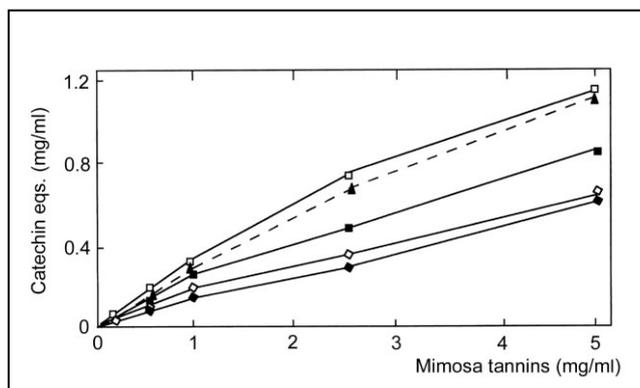


Figure 3: Effect of different concentration of HCl (8% (◆), 24% (◇), 48% (▲), 72% (□) on the vanillin test sensitivity to mimosa tannins, for reagents containing 1% (—) or 5.8% (---) of vanillin.

Determination of tanning capacity

Haemanalysis is a method easy and fast to realize. It is also very precise provided that a standard curve is remade for each new hemoglobin solution. These curves present a linear zone limited by two thresholds (Fig.4), corresponding respectively to the minimal content of tannic acid able to precipitate hemoglobin and to the complete precipitation of the bood solution. Of course, tannin solutions must be diluted in order that complexation occurs in the linear part of standard curve.

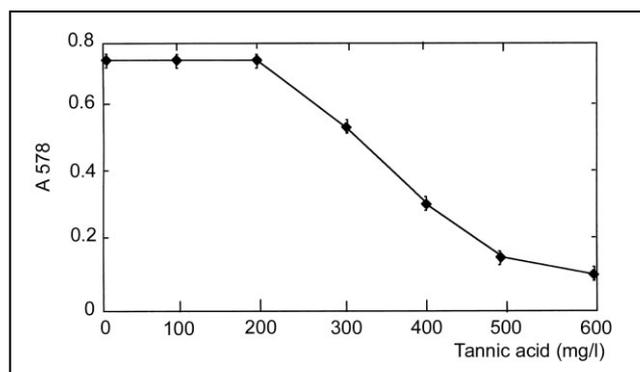


Figure 4: Standard curves obtained from tannic acid by hemoglobin precipitation.

Gelatin complexation method

Standard curves realized with tannic acid present the same aspect than the hemoglobin'ones (Fig.5). They were obtained adding a fixed volume of gelatin solution, contrary to Marigo [5], who advised to add gelatin until absence of flocculation of the solution. In our case, this volume was determined to allow the optimum complexation of *Vicia faba* extracts diluted from five ten folds.

Analysis of *Vicia faba* L. cultivars

All the exposed methods, except the Folin-ciocalteu assay, were applied to the analysis of the cultivars. The results are presented in table 2. On account of the good

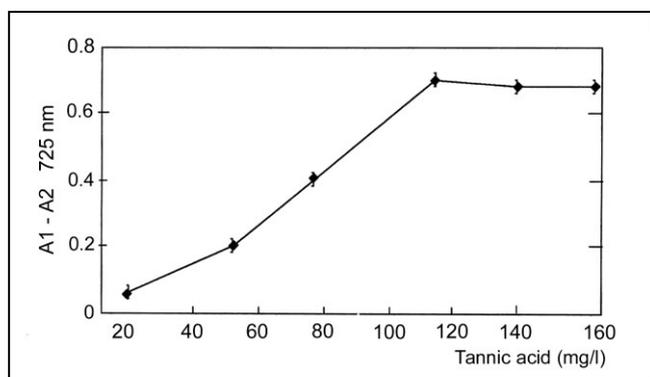


Figure 5: Standard curves obtained from tannic acid by gelatin precipitation.

reproducibility of the extraction and assay methods, with a standard deviation inferior to ten percents, only four extracts per variety were analysed. The results are expressed as catechin equivalent for tanning capacity and in $\text{mg}\cdot\text{g}^{-1}$ of dry matter for the anthocyanidin products.

Genotypes	N° 949T	Minica	Alfred	N° 893	N° 245
Total phenolics ^a	132.6±7.5	97.7±5.2	87.0±1.8	45.0±2.1	25.5±0.7
Total phenolics ^b	128.6±3.3	92.4±2.7	85.3±2.2	44.2±0.7	22.1±1.1
Condensed tannins:					
-A.P ^c	191.5±2.2	100.8±3.5	138.1±3.7	70.2±3.3	38.9±2.1
-Vanillin ^a	102.2±5.5	29.5±4.5	64.8±0.4	40.7±1.9	13.3±0.7
Tanning capacity:					
-Hemoglobin ^b	153.3±1.3	119.5±7.1	120.0±3.5	53.5±3.5	38.7±1.6
-Gelatin ^b	110.0±5.7	56.8±4.7	72.7±2.4	32.6±1.8	17.6±0.9
<i>AP/Ph.T</i>	1.4	1.0	1.6	1.6	1.5
<i>Hem/Ph.t</i>	1.2	1.3	1.4	1.2	1.6
<i>Hem/Van</i>	1.5	4.0	1.9	1.3	2.9
<i>AP/Van</i>	1.9	3.4	2.1	1.7	2.9

Table 2: Total phenolics, condensed tannins and tanning power of the testa of *Vicia faba* L. cultivars, expressed in $\text{mg}\cdot\text{g}^{-1}$ of dry matter. ^a Expressed as catechin equivalents. ^b Expressed as tannic acid equivalents. ^c Expressed in $\text{mg}\cdot\text{g}^{-1}$ from the $E1\% = 150$ given by Bate-smith.

The figure 6 summarise the specificity of the analytical methods employed to quantify the different chemical groups of polyphenolic compounds.

DISCUSSION

We dispose of a series of five methods to quantify the polyphenolic content. We have tried to demonstrate their reliability which was proved to be good. But, for all the assays, some other parameters must be taken into account. As matter of fact, not all tannins are extracted and there is certainly a differential extractibility of these molecules, according to their nature and molecular weight. Moreover, though phenolics are protected by an antioxidant, they can evolve and particularly polymerize themselves during extraction and concentration of extracts. At last the redissolution of products in methanol, for the vanillin test, it not complete, and the data obtained by this test are certainly undervalued.

However, all these parameters are supposed to interact in the same way for all extracts : the comparisons that can

be made between cultivars or among the different data obtained for one cultivar remain valuable. In the latter case, the ratios calculated can be considered as structural indicators of the cultivars phenolic pattern [4,6].

- Comparison between anthocyanidin production (AP) and Prussian blue assays gives the proportion of condensed tannins among other phenolics (*AP/Ph.T*).

- Comparison of biochemical methods against Prussian blue assay gives indication on the tanning capacity of all phenolics (*Hem/Ph.T*); while the ratio between the protein tests and assay gives the proportion of tanning proanthocyanidins.

- The ratio of hemoglobin and vanillin (*Hem/Van*) gives indication on the tanning efficiency of the true proanthocyanidins.

- The ratio between gelatin assay and haemanalysis represents the differential affinity of phenols towards two kinds of proteins. Gelatin is known to present a high affinity for many kinds of tannins : thus we are assured to complex the majority of astringent tannins with this protein.

On contrary, hemoglobin binds less easily to proanthocyanidins and so will select only molecules presenting a high tanning capacity. Nevertheless, this protein is precipitated by other phenolics or even non phenolics molecules, such as polysaccharids or lectins. These substances are able to complex hemoglobin and participate to the tanning activity of the plant extracts.

- At last, the ratio between Anthocyanidin products and Vanillin assays (*AP/Van*) indicates the approximate degree of polymerization of proanthocyanidins; when this ratio is high, it corresponds to the presence of high polymers; when it is low, it corresponds either to small polymers, or to high polymers with a high content in catechins [8]. These two case can be differentiated by their tanning capacity, as catechins do not react with proteins.

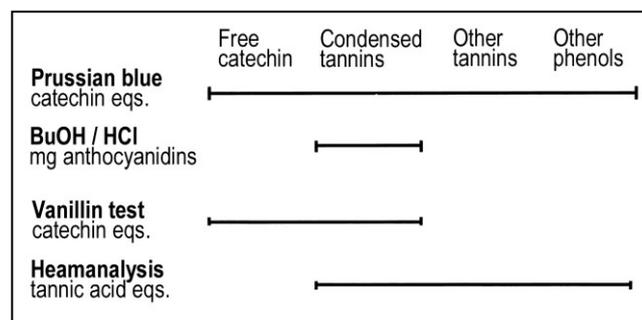


Figure 6: Specificity of analytical methods versus the different chemical groups of polyphenolic compounds.

The results obtained for the five cultivars of *Vicia faba* are representative of the three different cases that can be encountered with this set of methods:

- In all cases the yield in condensed tannins determined by Anthocyanidin Product assay (AP assay) is proportional to the total phenolic content determined by the Prussian blue assay ;
- The tanning efficiency of phenols, expressed by the ratio hemoglobin/vanillin varies more significantly : it is equal to 1.2 for cultivars n° 949 T and n° 893, up to 1.3 for the other cultivars
- The ratio AP/Vanillin distinguishes the cultivars n° 893 and n° 949 T (low ratio) from Alfred and genotype n° 245 (ratio = 2) and finally Minica with high ratio.

If we consider that the tanning capacity of proanthocyanidins increases with polymer length, at least for low degrees of polymerisation [10], these results can be explained as follows :

- The cultivar "Minica" and genotype n° 245 presents a high tanning efficiency and high ration *AP/Van*, i.e a high degree of polymerisation, they are probably rich in oligomers or polymers ;
- The cultivar "Alfred" present the high tanning efficiency but a low ratio *AP/Van* ; we can suppose either that we are in the same case than previously but a high content in catechin raises the vanillin value, or that the cultivar contains shorter polymers in a larger proportion.
- The cultivars n°s 949 T and n° 896 present a low tanning efficiency and low ratio *AP/Van* ; their composition should be rich in catechin and low oligomers. The results obtained with the gelatin test confirm the tendencies showed by the hemoglobin assay in a less marked way : gelatin seems to be more sensitive for the cultivars with high total phenolics.

CONCLUSION

We dispose actually of five methods for the measurement of the polyphenolic content of *Vicia faba* seeds. Results of the assays show that in the genus *Vicia* it exist a larger diversity of genotypes with several level of polyphenolic compounds.

All these methods will be applied to quantify the polyphenolic content to a larger collection of *Vicia faba* L. cultivars, and we hope to confirm the tendencies encountered here at a statistical scale.

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