Trypsin Inhibitors and Hemagglutinins in Beans (*Phaseolus vulgaris*) and Their Relationship with the Content of Tannins and Associated Polyphenols

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For differentiation of true trypsin inhibitor (TI) from inhibition caused by polyphenolics (PP), water extracts at pH 7.6 from whole seeds, cotyledons, and seed coats of three varieties of common beans (*Phaseolus vulgaris*), black, white, and red, were treated as follows: (1) raw extract, untreated; (2) raw extract treated with poly(vinylpyrrolidone) (PVP); (3) cooked extract (115 °C and 15 psi, 20 min); and (4) cooked extract plus 1% PVP. Trypsin inhibitor was determined by the BAPA method. Group 1 would show total inhibition (TI + PP, "A"), group 2, inhibition due only to TI ("B"), group 3, PP inhibition plus possible remanent TI ("C"), and group 4, the inhibition due only to possible remanent TI ("D"). Their algebraic relationship is expressed by the equation $A = B + (C - D)$, where A = "calculated value" and $B + (C - D)$ = "analytical value". There were no differences between calculated and analytical values. A highly significant correlation ($r = 0.93$) indicated that with this methodology, trypsin inhibition due to TI and PP can be separated with a good degree of reliability.

Legume seeds contribute substantially to the protein content of the diets of a large part of the world's population, especially in those regions where animal protein consumption is relatively small due to its scarcity or to cultural taboos (Bressani and Elías, 1974; Fernández, 1975; Hulse et al., 1977; Jaffé, 1968, 1970; Liener, 1962; Ordóñez Gil, 1976). Unfortunately, consumption of legume seeds is not as high as it would be desirable, due to their relative scarcity and high market price—the result of their present low yields—and to certain drawbacks in their food and nutritional qualities. The drawbacks related to the nutritional and food qualities of legume seeds are well-known. One of the best documented is that related to the relative low nutritive value of the seeds' protein, which has been attributed to two main factors, the deficiency of sulfur-containing amino acids (Bressani and Elías, 1974; Bressani et al., 1961; Jaffé, 1949, 1950, 1970) and the presence of antiphysiological or toxic factors (Liener, 1962; Mendel, 1909).

Almost from their discovery, sulfur-containing amino acid deficiency and toxic factors have been overcome by supplementation in the first case and elimination in the second, with the aim of improving the nutritional quality of legumes. Generic and agronomic studies have also been involved in trying to improve legume seeds for human consumption. In spite of these measures, the incomplete knowledge subsisting to date on the antiphysiological factors has influenced negatively the magnitude and depth of the research on the improvement of the nutritional quality of these foods (Fernández et al., 1981). Despite this incomplete knowledge, there is a large number of publications on the subject, especially concerning those legume seeds more often consumed, such as soybeans and common beans. Of these factors, trypsin inhibitors (TI) and hemagglutinins (HA) have been considered as the main ones, although lately the importance of tannins and associated polyphenols has been recognized, since several authors have reported the ability of these components to inhibit the activity of a vast spectrum of enzymes, influencing the digestibility of the diets (Bressani and Elías, 1977; Elías et al., 1979; Glick and Joslyn, 1970a,b; Tamir and Alumot, 1969).

Recently, Elías et al. (1979) have brought to light a relationship between trypsin inhibitor activity and polyphenol (PP) content in common bean seeds. These authors studied beans of different seed coat color and found that trypsin inhibitor activity was influenced by a heat-labile factor (true trypsin inhibitor) and a heat-stable factor (PP). The former was concentrated in the cotyledons and the latter in the seed coat. Analysis of these data made it evident that the methodology available at present for the measurement of trypsin inhibitory activity is not suitable for differentiating between the inhibitory activity due to true TI and that due to PP. Furthermore, this activity has been usually attributed to the former. A methodology capable of distinguishing between the two types of inhibitory activity would be of great value in controlling the thermic destruction of true TI and the enzymatic inhibition caused by PP in diets containing cooked legume seeds and for correlating these data with those for digestibility.

The present study was carried out to determine the relationship between the main toxic factors (TI and HA) and PP in legume seeds and to develop a methodology capable of differentiating between true trypsin inhibitor activity and that due to PP.

EXPERIMENTAL SECTION

Relationship between TI Activity and PP Content. *Samples.* Samples of black, white, and red common beans (*Phaseolus vulgaris*) were used. For each bean color sample, besides the whole seed, the cotyledons and the seed coats were analyzed. The seed coats were removed with the aid of forceps and a scalpel from individual bean kernels. The following extracts were prepared from the whole bean, the cotyledons, and the seed coat from each bean color sample. (a) Raw: One gram of each ground (40-mesh) sample was suspended in 19 mL of 0.1 M, pH 7.6 phosphate buffer and mechanically shaken for 1 h. The suspension was then centrifuged and the supernatant withdrawn and frozen until analysis. (b) Cooked: Aliquots of the raw extract were cooked in an autoclave under controlled conditions (20 min, 115 °C, and 15 psi) and stored under the same conditions as the raw samples. (c) Cooked with poly(vinylpyrrolidone) (PVP) (Sigma Chemical Co., St. Louis, MO), which binds PP selectively, thus blocking their inhibiting action (Gustavson, 1954; Loomis and Battaile, 1966): The extracts were prepared as before, but previous to cooking PVP was added at a final con-

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Table I. Behavior of Antitrypsin Activity and Tannin Concentration in Bean Extracts under Several Treatments[a]

samples	raw		cooked		cooked + PVP	
	TUI[b]/mL of extract	tannins, mg/mL of extract	TUI/mL of extract	tannins, mg/mL of extract	TUI/mL of extract	tannins, mg/mL of extract
black, whole	12.46	0.449	4.05	0.733	0	0.800
black, cotyledons	14.98	0.316	1.04	0.456	0	0.587
black, seed coat	8.72	1.717	9.86	1.941	0.78	0.181
white, whole	11.77	0.355	3.47	0.456	0	0.512
white, cotyledons	5.22	0.305	0.81	0.364	0	0.619
white, seed coats	2.03	0.128	2.95	0.148	1.07	0.112
red, whole	13.92	0.463	2.43	0.651	0	0.536
red, cotyledons	13.07	0.355	3.95	0.429	0	0.736
red, seed coats	7.09	1.984	4.84	2.090	5.28	0.160

[a] Correlations: trypsin units inhibited per milliliter vs. tannins in raw, $r = -0.16$ (not significant), cooked, $r = 0.75$ ($P < 0.01$), and cooked plus PVP, $r = 0.79$ ($P < 0.01$). [b] Trypsin units inhibited.

centration of 50 mg/mL. For the actual determinations, a blank of phosphate buffer, pH 7.6, and the above concentration of PVP was used.

Trypsin inhibitors were determined by the method of Kakade et al. (1969) using *N*-benzoyl-DL-nitroanilide hydrochloride (BAPA) as the substrate for trypsin and tannins by the method described by Joslyn (1970), expressing the results as milligrams of tannic acid per milliliter of extract. It should be pointed out that this method uses a nonspecific reaction for tannins, since it is given by any compound containing a phenolic group.

Relationship between HA Activity and PP Content. *Samples.* The samples used in this section were the same extracts used in the previous section. In addition, other extracts from the same seeds but using a different technique were made as follows.

Preparation of the Extracts. (a) Raw: Ten grams of whole seeds was soaked in water for 18 h in 30 mL of water (1:3 sample:water ratio). After the seeds were soaked, water was added to restore the 1:3 ratio and the mixture was dried in an oven with maximum aeration at 60 °C. Once dried, the samples were treated in the same manner as described in the previous section. (b) Cooked: The treatment used was the same as above up to the replacing of the soaking water, after which the samples were cooked in an autoclave for 20 min at 115 °C and 15 psi and then dried with the cooking water as described above. (c) Cooked with PVP: Treatment was the same as above, with the difference that previous to cooking, PVP was added to reach a final concentration of 50 mg/mL. A blank of phosphate buffer and PVP was also run.

Hemagglutinating activity was determined by the method described by Jaffé and Brücher (1971) using trypsinized ox erythrocytes. This method used microtitration of the samples that are diluted in geometric proportion. The results are expressed as the highest dilution that results in agglutination of the erythrocytes in 1 h. Tannins were analyzed as described previously (Joslyn, 1970).

Development of a Methodology for Differentiating between Tryptic Inhibition Due to True TI and That Resulting from PP. *Samples.* Three replicates of the raw and cooked extracts used under Relationship between TI Activity and PP Content were used.

The methodology selected was based on the labile nature of TI to high temperatures. The method used is a modification of that described by Kakade et al. (1969), and it requires the determination of antitryptic activity in a raw and in a cooked extract.

(*a*) *Antitryptic Activity.* In a 50-mL volumetric flask 1 mL of the raw or cooked extract was diluted with 49 mL of distilled water. One milliliter of the same extract was placed in a similar flask and 5 mL of a 5% solution of PVP in distilled water was added; distilled water was added to volume. From this step onwards, measurement of the antitryptic activity was carried out.

(*b*) *Separation of Trypsin Inhibition.* For each sample, four determinations of antitryptic activity were performed: (1) in the crude extract; (2) in the crude extract plus PVP; (3) in the cooked extract; and (4) in the cooked extract plus PVP. In (1), that is, in the raw extract, the total trypsin inhibition ("A") was obtained by adding TI plus PP. In (2), the inhibition due to TI ("B") was obtained; in (3), upon heating, the inhibition by PP plus the possible remanent one from the undestroyed TI will be obtained ("C"); and (4) will provide the trypsin inhibition due to possible remanent undestroyed TI ("D"). Relating the different values algebraically, the following equation can be written:

$$A = B + (C - D)$$

The left side of the equation will be henceforth called the "calculated value" and the right side the "analytical value".

RESULTS

Relationship between TI Activity and PP Content. Table I presents the distribution of TI and PP in the several anatomic parts of the seeds and in relation to their seed coat color. In the case of raw whole beans, values for trypsin inhibitors were not significantly different in relation to the color of the seed. TI activity was not significantly different between cotyledons of black and red beans, but white beans showed a significantly lower activity than the other two varieties. The TI activity of the cotyledons of white beans was about 50% of the TI activity in the whole grain, in contrast with black- and red-colored samples. This was an unexpected finding and no explanation can be offered. It should be indicated, however, that trypsin inhibition on this sample by the Kunitz (1947) assay gave a lower value as compared to the whole grain. In contrast, the cotyledons of the colored beans gave inhibition values equal to those for the whole grain. Raw seed coats showed values of trypsin inhibitor lower than those for whole beans or their cotyledons. There was a significant difference between the TI activity of the seed coat of colored beans (black and red) and that of white beans.

In the case of tannins, similar values were obtained for colored whole beans and somewhat lower for white beans. Cotyledons showed values which were essentially similar for all varieties. Colored seed coats showed the highest PP values, while for white beans this value was even lower than that for cotyledons.

Table II. Hemagglutinin Titers in Bean Extracts

sample	titers for treatment[a]					
	A	B	C[b]	D	E	F
black, whole	4 (0.68)[c]	0 (0.77)	0	6 (0.35)	3 (0.30)	0
black, cotyledons	5 (0.29)	0 (0.57)	0	6 (0.30)	2 (0.10)	1
black, seed coats	3 (1.64)	3 (1.66)	0	4 (1.94)	5 (2.96)	3
white, whole	4 (0.46)	0 (0.61)	0	4 (0.35)	1 (0.13)	0
white, cotyledons	5 (0.60)	0 (0.69)	0	5 (0.30)	1 (0.14)	0
white, seed coats	2 (0.17)	1 (0.28)	0	3 (0.19)	1 (0.06)	0
red, whole	6 (0.76)	0 (0.94)	0	6 (0.45)	1 (0.26)	0
red, cotyledons	6 (0.54)	0 (0.67)	0	6 (0.34)	1 (0.07)	1
red, seed coats	3 (2.09)	3 (2.37)	0	4 (1.82)	4 (2.48)	2

[a] Treatment A, raw extract; treatment B, cooked extract; treatment C, cooked extract plus PVP; treatment D, extract from raw, soaked and dried beans; treatment E, extract from cooked and dried beans; treatment F, extract from beans cooked plus PVP and dried. Correlations: hemagglutinins—tannins in whole seeds: raw, $r = -0.35$ (not significant); cooked, $r = 0.72$ ($P < 0.01$); cooked plus PVP, $r = 0.93$ ($P < 0.01$). [b] Dilution number. [c] Values in parentheses are in milligrams of tannic acid per milliliter.

When the behavior of TI under the different treatments was studied, it was observed that cooking decreased antitrypsin activity to very low levels in the extracts from whole beans or from their cotyledons. On the other hand, in the case of seed coats, the TI values increased except in the case of red beans, in which these values decreased but not to the same degree as in whole beans or their cotyledons. Cooking resulted in an increase in all PP values, which was more marked in whole beans and their cotyledons, especially in the colored varieties.

Under the third treatment (cooking plus PVP), values of trypsin inhibitors close to zero were obtained both for whole beans and for cotyledons, regardless of seed color. Likewise, seed coats presented some remaining TI activity in exceedingly small amounts, except for the red variety that still contained considerable amounts of activity.

Regarding the relationship between TI activity and PP content, no significant correlation was found between these two components in the raw samples, while for the cooked samples the correlation was significant ($r = 0.75$; $P < 0.05$). Likewise, there was a significant correlation ($r = 0.79$; $P < 0.05$) between TI activity and PP content in the seed coats, which was not observed in the case of whole seeds or cotyledons.

Relationship between HA Activity and PP Content. Results discussed in this section are presented in Table II. The results paralleled those for TI, since no higher HA activity was detected in whole seeds and cotyledons than in seed coats. Regarding color, there was a higher activity in red, intermediate in black, and lower in white seeds, regardless of the anatomical part analyzed.

Comparing the results obtained with the first three treatments on the extracts (A, B, C) with the last three (D, E, F), it is evident that there is a higher HA activity in the latter. Regarding the behavior of HA on the different treatments, it can be observed that in treatment B (cooked extract) the titer of HA in whole seeds and cotyledons was zero, while in the seed coats of colored varieties the titer remained the same, except for the white variety, which showed a decreased HA activity, although never to zero levels.

In treatment E (cooked beans), the titers of HA were also decreased in whole seeds and cotyledons, although never to zero values. In colored seed coats, no reduction of HA activity was observed and even an increase of 1 unit was observed in seed coats of black beans.

When extracts were treated with heat and PVP (treatment C), not all the HA activity was eliminated in all samples. With treatment F (cooked beans plus PVP), similar results were obtained, except that HA activity was not reduced to zero in seed coats of colored varieties or their corresponding cotyledons, although in the latter the remaining activity was very close to zero.

Table III. Calculated and Determined Tryptic Inhibition[a]

sample	calculated $[B + (C - D)]$, TUI[b]/mL	analytical (A), TUI/mL
black, whole	9.59	11.34
black, cotyledon	8.65	8.96
black, seed coats	6.55	4.43
white, whole	7.49	6.81
white, cotyledon	9.29	8.57
white, seed coats	3.13	1.87
red, whole	11.93	12.63
red, cotyledon	9.93	12.24
red, seed coats	5.40	6.16
black, whole	12.51	13.36
black, cotyledon	13.03	13.62
white, whole	9.03	10.20
white, cotyledon	10.62	8.98
white, seed coats	4.18	3.52
red, whole	9.20	9.69
red, cotyledon	9.33	12.40
black, whole	8.82	8.91
black, cotyledon	8.18	9.67
black, seed coats	3.73	2.93
white, whole	9.00	10.70
white, cotyledon	9.72	9.97
white, seed coats	2.68	3.36
red, whole	13.56	15.42
red, cotyledon	12.67	13.54
red, seed coats	4.21	3.90

[a] Statistical analysis: $Y = 1.17X - 0.99$ (regression equation); $r = 0.96$ (correlation coefficient); $\chi^2 = 5.43$ ($P < 0.005$). [b] Trypsin units inhibited.

If HA activity is compared with PP content, a highly significant correlation is found in the case of seed coats ($r = 0.93$; $P < 0.01$), while no correlation was found between whole beans and their cotyledons. The latter results are important since, to our knowledge, the finding has not been reported in the literature.

Development of a Methodology for Differentiating between Trypsin Inhibition Due to True TI and That Resulting from PP Action. Table III presents results obtained by the methodology described in the previous section. In order to check the validity of the equation $A = B + (C - D)$ some statistical tests, including linear

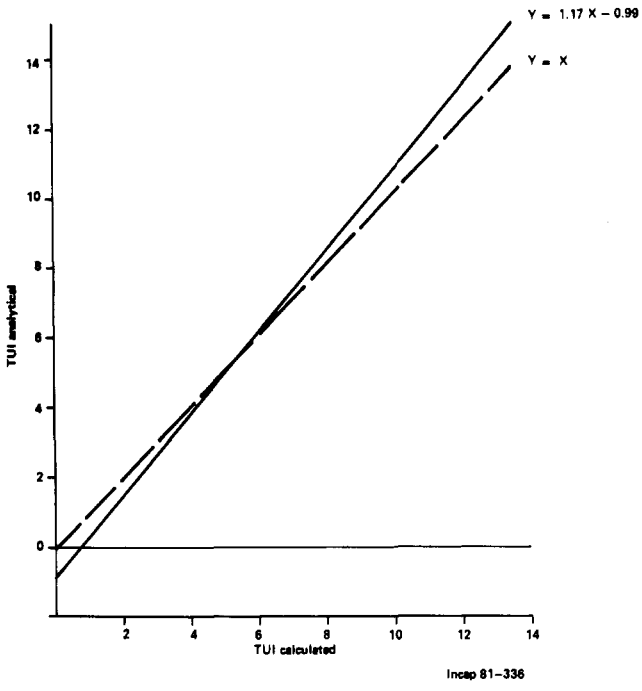

Figure 1. Comparison between the lines for the perfect equation ($Y = X$) and that obtained in experiment 3 ($Y = 1.17X - 0.99$).

correlation, regression and χ^2 were run on the results. A highly significant correlation ($r = 0.93$) between the two sides of the equation was found. Likewise, the χ^2 for expected and obtained data was highly significant ($P < 0.005$). The regression equation obtained was $Y = 1.15X - 0.99$. This equation is represented graphically in Figure 1, where it is compared with the ideal equation ($Y = X$).

The equation for the seeds of each color and for the different anatomical parts was also obtained. For the three colors, highly significant correlations were obtained ($r = 0.96$ for black, 0.94 for white, and 0.97 for red beans, respectively), with very small differences among them. Regarding the different anatomical parts, a highly significant correlation was obtained for whole seeds ($r = 0.96$), while for cotyledons and seed coats this relationship, although significant, was smaller ($r = 0.72$ and 0.70, respectively).

DISCUSSION

Relationship between TI Activity and PP Content. When the distribution of TI in the different anatomical parts of the seed was analyzed, the highest TI activity was found in whole seeds and in cotyledons, with little variation among beans of different colors. This is reasonable if one considers that true TI are proteins in nature, and since cotyledons are the seed structure with the highest protein content, it is logical to expect that they would contain the highest TI activity in the seed. Similarly, since the majority of the seed is cotyledon, the results on TI activity of the seed and the cotyledon would be very similar.

On the other hand, in the case of raw seed coats, the TI activity was lower than for the anatomical parts already mentioned, and values for TI in colored beans differed significantly from those of white beans. An explanation of this observation is the fact that the relative lowest protein content is found in the seed coat and, therefore, the concentration of true TI in them would be very low. Another important fact is that—as will be shown later—the highest concentration of tannins or PP is found in colored seed coats as compared to those found in white seed coats or in other anatomical parts of the seed. The latter would explain the difference in TI activity between colored and white seed coats since, as already discussed, PP are capable of exerting an inhibitory action on several enzymatic activities, among which is tryptic activity (Glick and Joslyn, 1970b; Goldstein and Swain, 1965; Tamir and Alumot, 1969).

This hypothesis is reinforced by the results of the comparison between the PP concentration in raw whole seeds and in cotyledons. Thus, while in the latter there was no significant difference between the PP concentrations regardless of color, this difference was evident in the case of whole white beans, which showed the lowest concentration. The highest level of PP in colored beans was due to the pigments present in their seed coats, which are, as previously stated, associated with tannins or PP (Elías et al., 1979; Fernández, 1975). The results from the determinations of PP in the seed coats corroborate this statement.

Several authors have reported that trypsin inhibition in raw beans is due to two factors: one, heat-labile factor and protein in nature which is present mainly in the cotyledons (true TI) and, the other, heat-resistant factor, located mainly in the seed coat and associated with tannins and PP (Elías et al., 1979; Fernández, 1975; Ordóñez Gil, 1976). Thus, when these conclusions are applied to the results obtained in the several anatomic parts of the seed, it can be said that in whole seeds and cotyledons true TI would be the main contributors to the total antitryptic activity, while in the seed coats, tannins and PP would be the main fractions responsible for this activity, especially in colored beans, since pigments are closely related to tannins and PP.

Cooking eliminated to a large extent the antitryptic activity of whole seeds and cotyledons, which is in line with the heat-labile nature of their main inhibitor. The remaining or heat-resistant activity could be attributed to the PP present in those anatomical parts, although an incomplete destruction of true TI should be taken into account and remanent inhibition would be, therefore, the result of the sum of both inhibitions.

TI activity increased in the case of cooked black and white seed coats. In red beans, on the other hand, activity decreased although to a lesser degree than in raw seeds and cotyledons. The increase in TI activity would imply a higher extraction through the action of heat. Previous studies have reached the same conclusion (Fernández, 1975). PP determination in the cooked extracts showed higher results for all anatomical parts when compared to the raw extracts, which corroborates the previous assertion.

The addition of PVP to the extracts and their consequent heating would result in the binding of tannins (heat-resistant factor) and destruction of true TI (heat-labile factor); therefore, one would expect no inhibition, which is what was obtained in the second treatment (cooking plus PVP), since all whole seeds and cotyledons subjected to this treatment showed zero TI activity. In black and white seed coats, although the result was not quite zero, it was low enough. Red seed coats were the exception, since some TI activity could be detected in them. The reason for these results is still under study.

The correlations calculated between PP content and TI activity demonstrated that for raw samples such correlation was not significant, a fact which agrees with previous observations, especially if one takes into account that in this type of samples the greatest part of trypsin inhibition is caused by heat-labile or protein factors. In the cooked samples, however, the correlation between the two parameters mentioned, although not very high, was statistically significant for the number of samples studied (P

< 0.05). This is logical, since trypsin inhibition will be conditioned mainly by the heat-resistant inhibitors or PP, because heat-labile factors would have been destroyed. The correlation could be higher; however, an explanation could be found in an incomplete destruction of true TI.

When the correlations were analyzed taking into account the different anatomical parts used, it was found that the only significant correlation between TI activity and PP content corresponded to the seed coats, which is understandable since they have the highest relative content of PP. These results are in agreement with findings by Singh and Jambunathan (1981), who reported that PP are found mainly in the seed coat of beans. Similar results were also reported by Elías et al. (1979). Cotyledons and whole seeds, on the contrary, do not have a relatively high content of PP and, therefore, would not be able to exhibit a high correlation between their content of these compounds and heat-resistant TI activity.

Relationship between HA Activity and PP Content. In the case of HA, results obtained regarding their localization in the raw seeds are similar to the previous findings with TI. Therefore, since HA are proteins, the highest HA titers were obtained in the raw beans and cotyledon extracts and the lowest values in the seed coats. Regarding color, the higher activity was found in red seeds and the lowest in white seeds; these variations, however, are due to cultivar differences. Elías et al. (1979) reached the conclusion that since HA are located mainly in the cotyledons, it would be very unlikely to find a direct relationship between HA activity and seed coat color.

Regarding the differences observed in HA titers in the first three treatments as compared to those obtained in the last three, the greatest activity in the latter could be explained by a higher extraction of HA due to the soaking step.

Since HA are heat labile, the cooked extracts (treatment B) should show activities close to zero; this, however, was true only for whole beans and their cotyledons, while in seed coats the activity remained unchanged in the case of colored varieties and decreased, although not to zero, in the white varieties. Again, it can be inferred that HA activity in colored seeds, and to a certain extent in white seeds, is the result of heat-stable factors, different from the classical HA. In this case, these factors could very well be tannins or PP.

Under treatment E (cooked beans) the HA titers decreased in whole seeds and cotyledons, although never reaching values as low as zero; the seed coats, again, showed an unchanged titer, as in the case of the previous treatment. It could be that HA in cotyledons are less susceptible to destruction by heat than they are in the extracts, and considering that this was the only difference between treatments E and B, that could be the reason why HA in whole seeds are not totally destroyed as they are in the extracts. These observations are very interesting because beans are always cooked before consumption, and it would be logical to assume that improperly cooked beans could contain still a certain amount of HA activity. This, of course, would be undesirable and even dangerous if one considers that HA are regarded as the most important antinutritional factor in legume seeds as well as the most toxic. The lack of effect of heat on seed coats' HA titers can be explained as was done for treatment B.

In treatment C, when heat and PVP action were used simultaneously, both the heat-labile and heat-stable HA fractions were totally destroyed even in the seed coats, whose HA activity was shown previously to be unaffected by heat. On the basis of these results, it is concluded that tannins and PP are responsible, in part, for the HA activity observed in seed coats. This fact has not been previously reported, on the one hand because other reports have focussed their attention on the HA content of whole seeds and on the other because HA activity has been determined in raw seed coat extracts but never in the cooked extracts.

The results of treatment F (cooked beans plus PVP) are similar in certain samples, although in others a total destruction was not obtained, which could be explained by an insufficient amount of PVP to bind all the tannins present.

The observation that a highly significant correlation was found between HA titers and PP content for seed coats, while for whole seeds or cotyledons, the correlation was not highly significant, is of value in supporting the previous contention that PP seed coats can contribute, at least partially, to HA activity in the same manner as they contribute to TI activity, although in the former case the activity was observed in seed coats and not in whole seeds or cotyledons.

Design of a Methodology for the Differentiation of Trypsin Inhibition Resulting from True TI and That Caused by PP. The methodology was based on the treatment with heat and with PVP to bean extracts. The first treatment would destroy the heat-labile TI and the second the activity due selectively to tannins (heat-stable factors). From the results of this methodology, the following equation was formulated:

$$A = B + (C - D)$$

where A stands for total trypsin inhibition (TI plus PP), B is the inhibition due to true TI, C stands for PP inhibition plus true TI activity not destroyed by heat, and D is the TI not destroyed by heat.

For evaluation of the methodology proposed, the results obtained for A and those due to $B + (C - D)$ were compared. Statistical analysis showed a highly significant correlation between them ($r = 0.93$; $P < 0.005$); besides, the regression equation line is very close to the ideal line resulting from the equation $X = Y$. From this it is concluded that the proposed equation and methodology can be used to separate trypsin inhibition due to heat-labile factors (true TI) and heat-stable ones (PP) with an acceptable degree of confidence. Due to the few data available, it would be convenient to test the methodology with a larger number of samples.

From the results of the statistical analysis using the data on seed color and anatomical part of the seed used, it can be concluded that the methodology can be used for any type of beans regardless of seed color. Nevertheless, red beans presented the highest correlation ($r = 0.96$), but the differences between black beans and red and white ones are minimal ($r = 0.96$ and 0.94, respectively).

When statistical analysis was run according to the anatomical structure of the seed, a highly significant correlation was found in the case of whole beans, while for cotyledons and seed coats this correlation, although significant, was smaller. Therefore, it could be concluded that the methodology may be more accurate for whole seeds than for cotyledons or seed coats.

It would be interesting to correlate the data on TI and HA with the content of the different PP instead of with their total content, since these activities are probably due to one or to very few groups of PP. The results would be not only of scientific interest but also of practical importance, since the greater the knowledge obtained on legume antinutritional factors, the sooner the attitude about these factors would be modified and the utilization and breeding programs on these foods improved.

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Volatile Components of Alfalfa Flowers and Pods

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The volatile components of alfalfa flowers and seed pods, isolated both by a Tenax trapping method and by vacuum steam distillation continuous extraction, were analyzed by the capillary gas–liquid chromatography–mass spectrometry combination. A total of 33 compounds was identified in the flowers and 31 compounds in the seed pods. The major component found associated with the flowers (Tenax trapping) was the previously identified (*E*)-β-ocimene. Other major flower volatiles identified included 2- and 3-methylbutanol, (*Z*)-3-hexenyl acetate, decanyl acetate, and dodecanyl acetate. Unusual flower components include neryl 2-methylbutyrate, α-copaene, and octan-3-one. Unusual pod components identified include γ-muurolene and an unidentified long-chain (ca. C_{16}–C_{18}) aliphatic methyl ketone. By use of the Tenax trapping method, the volatiles found associated with alfalfa flowers and pods were compared with those found associated with the leaves and stems.

Alfalfa is used as a food by a complex of insect pests and polinators. Individual parts of the plant are often selected to the exclusion of others. For example, the alfalfa seed chalcid (*Bruchophagus roddi* Guss.) oviposits only in the seed pods (Urbahns, 1920). The plant bug, *Lygus hesperius* Knight, sucks plant sap from the flowers and developing pods which prevents formation of seeds (Schull et al., 1934). Host plant volatiles often mediate the various behavior modes of the insects such as feeding, host finding, and oviposition (Dethier, 1953). We therefore decided to investigate whether alfalfa also contains volatile components that mediate the behavior of alfalfa pests.

Previously, we identified some volatiles from the alfalfa leaves and stems (Buttery and Kamm, 1980). In the present paper we set out to identify the volatiles specifically associated with the alfalfa flowers and seed pods and to compare these with those found in the leaves and stems.

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EXPERIMENTAL SECTION

Materials. Alfalfa (*Medicago sativa*) flowers, seed pods, and leaves plus stems were obtained from experimental fields in Albany, CA. Also, seed pods of known maturity (12 days) were obtained by pollination of alfalfa grown in a greenhouse at Corvallis, OR. Three different varieties, Lahontan, Narrangansett, and Caliverde, were examined. The mature flowers were picked fresh and used the same day. Green seed pods (exact maturity unknown) were picked from field plots and examined the same day. Samples of pods shipped by air from Oregon were kept cool by ice during shipping and overnight storage.

Most authentic chemical compounds were obtained from commercial sources (e.g., Aldrich Chemical Co. or synthesized by established methods and repurified by GLC separation, and their identity was checked by spectral means. Authentic sesquiterpenes (identity verified by