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Fluorometric Assay of Free and Bound Coumarin rn Sweetclover

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Fluorometric Assay of Free and Bound Coumarin in Sweetclover[1]

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SYNOPSIS

Free and bound coumarin are extracted by autoclaving leaf tissue in water. Free coumarin is determined directly from an aliquot of the water extract diluted in alkali. Additional autoclaving of the extract in concentrated alkali is required for release of the coumarin from the bound form, permitting an assay for total coumarin. Bound coumarin values are then obtained by difference. Results of the assay as applied to four different phenotypes of sweetclover are given.

OF THE numerous methods which have been proposed for the assay of coumarin in sweetclover, those most widely used at the present time are based upon the conversion of coumarin to coumaric acid which, in basic solution, displays a characteristic yellowish-green fluorescence when exposed to ultraviolet light. Ufer (10) and Slatensek and Washburn (9) have stated that coumarin may be converted to coumaric acid by heating in concentrated alkali. Accordingly, published procedures for the fluorometric detection (3, 9, 10, 11) and assay (3, 8, 9) of coumarin in sweetclover all involve heating the plant tissue in alkali early in the procedure. One consequence of this treatment with hot alkali is the release of at least a part of the bound coumarin which has been extracted from the plant material (7, 9). (In this paper, bound coumarin is used as a general term to include the substance or substances present in sweetclover which produce the characteristic yellowish-green fluorescence only after autoclaving in alkali. Specific application of this term to the coumarin molecule is not intended.) The procedures cited do not permit distinction between the free and bound forms of coumarin. This aspect of the published methods is not particularly objectionable for certain breeding work in which both free and bound coumarin are considered undesirable. For genetic and physiological studies of coumarin production, however, it is essential that the two forms be distinguished. Conceivably, by combination of published fluorometric methods with the colorimetric method of Clayton and Larmour (2), one could assay for both forms. However, the desirability of measuring both forms in a single procedure is obvious.

The simple fluorometric method described in this paper provides for the assay of both free and total coumarin, and thus permits the calculation of bound coumarin content.

MATERIALS

Sweetclover plants of four different phenotypes were used in this study. The phenotypic classification was based upon the apparent fact that differences in coumarin production of the plant material used were chiefly attributable to the effects of two pairs of alleles which may be designated as *Cu/cu* and *B/b*.[3] When homozygous, the *cu* gene results in a greatly reduced level of total coumarin while the *b* gene prevents the formation or accumulation of free coumarin in the plant. Thus plants of the *Cu B* phenotype contain both free and bound coumarin, plants of the *Cu b* phenotype contain bound coumarin but are very low in free coumarin, and plants of the *cu B* and *cu b* phenotypes are low in both forms of coumarin. In these experiments, plants grown from certified seed of the varieties Spanish and Madrid were used as representatives of the *Cu B* phenotype. Plants representing the other three phenotypes were derived from breeding material obtained from Dr. W. K. Smith of the University of Wisconsin. All plants were grown in the greenhouse.

Sources of the coumarin, coumaric acid, and sodium hydroxide (reagent grade) were the Eastman Kodak Company, K and K Laboratories, and the J. T. Baker Chemical Co., respectively. A Gates M. R. 4 Laboratory Lamp with the T.F. 8 tube installed was used for ultraviolet treatment. According to the manufacturer, the energy peak of this tube is near 3600 Angstroms. For measurements of fluorescence, a Beckman DU Spectrophotometer equipped with a fluorescence attachment was used. Quinine sulfate (Merck, USP grade) was used as the fluorescence standard.

EXPERIMENTAL PROCEDURE AND RESULTS

The Method

In accordance with the recommendation of Brink,[4] testing was confined to material from terminal 3- to 4-inch portions from the upper branches of the plants. One such portion was cut from each plant to be tested, and the cut end was immediately submerged in water in a vial. The plant material was then taken to the laboratory where the mid-leaflet was removed from the youngest fully expanded leaf on each stem portion and immediately weighed to the nearest 0.1 mg. It was then transferred to a 12 by 75 mm. Pyrex test tube. A 2-ml. portion of water was added to each tube, followed by 2 drops of 95% ethyl alcohol applied directly to the leaflet. Gentle tapping of the tube following the alcohol addition usually resulted in submersion of the leaflet. The tubes and contents were then autoclaved for 15 minutes at 15 psi. Following removal from the autoclave, the tubes were cooled immediately in a cold water bath to reduce evaporation losses. Removal of the leaflet with a flamed platinum wire, to prevent obstruction of the pipette, facilitated later sampling of the extract. If necessary, extracts may be stored in the refrigerator at this stage.

Each leaflet extract was then assayed for free and total coumarin as follows: A 1-ml. aliquot of the well-mixed extract was added to 9 ml. of 2.5 *N* NaOH in a 20 by 150 mm. Pyrex test tube. Following mixing, a 2-ml. portion of the resulting solution was withdrawn and added to 8 ml. of water in a similar test tube. The remaining 8 ml. of solution was autoclaved for 30 minutes at 15 psi., and then cooled immediately in a cold water bath. A 1-ml. aliquot

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[3] Symbols as suggested by Dr. W. K. Smith of the University of Wisconsin in a personal communication.

[4] Brink, V. C. The content, distribution, and some metabolic aspects of coumarin in sweetclover (*Melilotus alba* Desr.). Unpublished Ph.D. thesis. University of Wisconsin. 1941.

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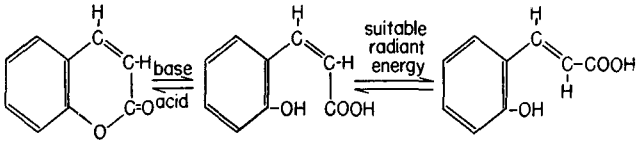
was drawn from the cooled solution and added to a mixture of 1 ml. of 2.5 *N* NaOH and 8 ml. of water in a test tube. The diluted samples withdrawn before and after autoclaving were then placed in a darkroom where they were subjected to 15 minutes of ultraviolet irradiation. The distance from the lamp to the solutions was approximately 15 cm. Following irradiation the tubes were immediately placed in a covered box in the darkroom. Tubes were removed from the box one at a time for fluorescence readings. A freshly prepared set of coumarin standards, including concentrations of 0.1, 0.25, 0.50, 0.75 and 1.00 γ of coumarin per ml., in 0.5 *N* NaOH, was irradiated and read with each set of samples. Before each sample or coumarin standard solution was read, the instrument was adjusted to read 100 with a fluorescence standard consisting of 0.77 γ of quinine sulfate per ml., in 0.1 *N* H_2SO_4. A curve was plotted from the readings of the coumarin standards, coumarin equivalence values for the samples were read from the curve, and coumarin contents of the leaflets were calculated. Readings made on samples withdrawn before autoclaving were used in calculating free coumarin levels, readings made from samples withdrawn after autoclaving were used in calculating levels of total coumarin, and bound coumarin values were obtained by difference.

In order that results might be expressed on the basis of dry weight, determinations of dry matter content were made. These determinations were based upon composite samples of approximately 15 leaflets similar in size and position on the plant to the leaflets used for extraction. Weights were taken before and after oven-drying at 95° C. for 3 to 4 hours, and the percentage dry matter was calculated. In an experiment designed to compare variations in dry matter content of leaflets between and within leaves, the within leaf variation was found to be the smaller. For the most precise work, therefore, the two side leaflets of a leaf could be used for dry matter determination and the mid-leaflet could be used for coumarin assay. Ordinarily, however, it is doubtful that the extra work required by this procedure would be justified.

Chemical Basis of the Method

As stated the fluorometric determination of coumarin is based upon the conversion of coumarin to the fluorescent compound, coumaric acid, and it has apparently been assumed that heating was required for this conversion. However, Harle and Lyons (4) and Patzak and Neugebauer (5) have shown conclusively that the following reactions proceed at room temperature.



From the standpoint of the fluorometric assay, it was of importance to know whether the conversion of the *cis* isomer to the *trans* form was complete under the conditions used. To obtain information of this point, 3.5×10^{-6} *M* solutions of coumarin and coumaric acid in 0.25 *N* NaOH were subjected to various durations of ultraviolet light, and fluorescence measurements were then made. The results, shown in figure 1, indicate that conversion was not complete, but rather that an equilibrium was established after approximately 8 minutes of irradiation. By taking values from a curve of fluorescence readings plotted against concentration of coumaric acid, it was found that at equilibrium the solutions were approximately 2.65×10^{-6} *M* with respect to coumaric acid. Thus, under the conditions used, it was possible to start with either pure coumarinic acid (coumarin in base) or coumaric acid in base, and after 8 minutes of ultraviolet treatment to have solutions in which approximately 76% of the o-hydroxycinnamic acid was in the fluorescent *trans* form and approximately 24% in the non-fluorescent *cis* form. Autoclaving coumarin or coumaric acid for 30 minutes in 2.5 *N* NaOH prior to ultraviolet treatment was without appreciable effect on the fluorescence of the solutions. Although conversion to the *trans* form was not complete, the fact that an equilibrium was established makes possible the use of fluorescence as an acceptable measure of coumarin concentration.

The importance of protecting the basic solutions from the usual light of the laboratory should be emphasized. The *cis* isomer, if exposed to such light, was partially converted to the *trans* form. More important is the apparent fact that the *cis* $\rightleftharpoons$ *trans* equilibrium established by ultraviolet irradiation was shifted in the direction of the *cis* isomer by the light normally present in the laboratory. Exposure to such light following ultraviolet irradiation resulted in a decrease in fluorescence. Reproducible readings were readily obtained, however, by keeping the solutions in the dark between ultraviolet treatment and measurement of fluorescence.

A typical curve of fluorescence reading plotted against coumarin concentration is shown in figure 2.

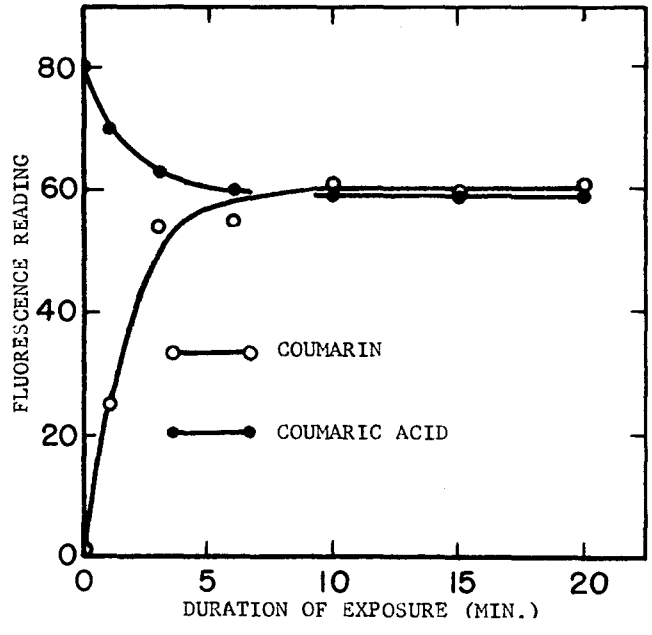


FIG. 1.—Fluorescence of solutions of coumarin and coumaric acid in 0.25 *N* NaOH with various durations of exposure to ultraviolet light. Concentration of solutions: 3.5×10^{-6} M. Both compounds were recrystallized from water prior to the experiment.

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Efficiency of Extraction

Individual extracts of 3 leaflets from each of 5 Spanish sweetclover plants were prepared as described above. Leaflets were then removed from the 15 extracts with a platinum wire, washed in distilled water, transferred to other tubes containing 2 ml. of water, and subjected to a second 15-minute autoclaving. First and second extracts were then assayed for free and total coumarin as previously described except that for the second extracts, the basic solutions were diluted 4 to 10 both before and after autoclaving, rather than 2 to 10 or 1 to 10. In this way measurement of lower levels of coumarin in the second extracts was possible. It is apparent from the results shown in table 1 that a single extraction with water in the autoclave was sufficient to remove a uniform and large fraction of the free and bound coumarin present in the leaflets. In another experiment of this type, a 10-minute extraction with 95% ethyl alcohol at 70° C. was used following the second water extraction. No coumarin was detected in the alcohol extract.

Duration of Ultraviolet Treatment

Five leaflets of Spanish sweetclover were autoclaved with 10 ml. of water, and 3 ml. of the extract thus obtained was added to 27 ml. of 2.5 *N* NaOH. A 16-ml. portion of the resulting basic solution was diluted to 80 ml. with water, the remaining basic solution was autoclaved for 30 minutes, and 8 ml. of the autoclaved solution was added to 8 ml. of 2.5 *N* NaOH and 64 ml. of water. Seven 10-ml. portions of each diluted solution were pipetted into test tubes for irradiation with ultraviolet light. Irradiation times of 0, 3, 7, 10½, 15, and 20 minutes were used, one portion of each diluted solution being irradiated for each of the periods. All operations subsequent to adding of the extracts to base were done in subdued light. From the results shown in figure 3, the 15-minute treatment was chosen for routine use in the assay. In a similar experiment, a leaf extract from a plant of the *Cu b* phenotype displayed essentially no fluorescence before autoclaving in base, and after autoclaving the fluorescence of the solution was very similar to that shown in the "after autoclaving" curve in figure 3.

Duration of Autoclaving in Base

Two extracts were prepared in the usual manner, one from Spanish, and one from a plant of the *Cu b* phenotype. Both extracts were diluted 1 to 10 with 2.5 *N* NaOH and aliquots were autoclaved at 15 psi. for these times: 0, 10, 30, 60, 120, and 180 minutes. Following autoclaving the aliquots were diluted 1 to 10 with water, the diluted

Table 1.—Efficiency of autoclaving in water as a means of extracting coumarin from Spanish sweetclover leaf tissue.

Assay for	Content of leaflet from two extractions * mean ± S.E.	Fraction of content obtained in first extract mean ± S. E.
	%	
Free coumarin	1.12 ± 0.09	0.97 ± 0.003
Total coumarin	4.43 ± 0.24	0.99 ± 0.001

*Dry weight basis.

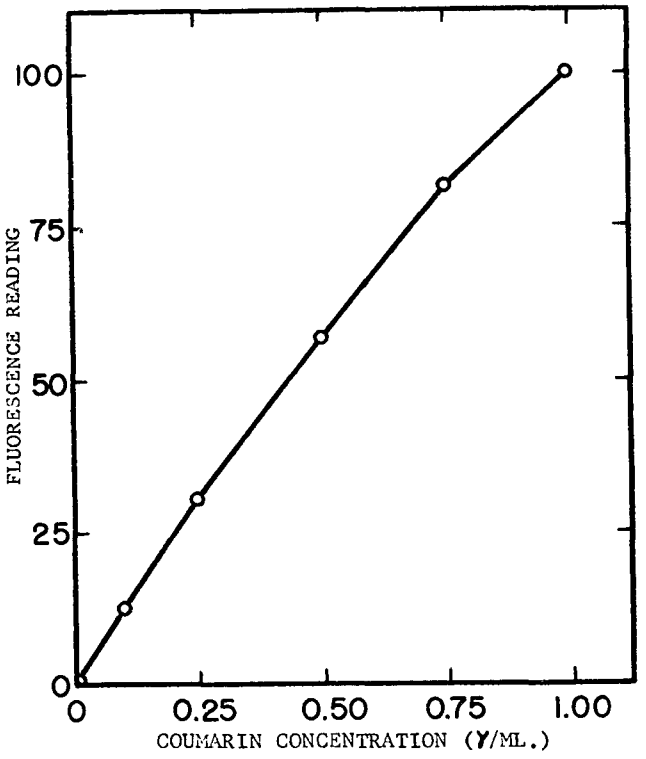

FIG. 2.—Relationship of fluorescence reading to coumarin concentration. Solutions were prepared in 0.5 *N* NaOH. Duration of ultraviolet treatment: 15 minutes.

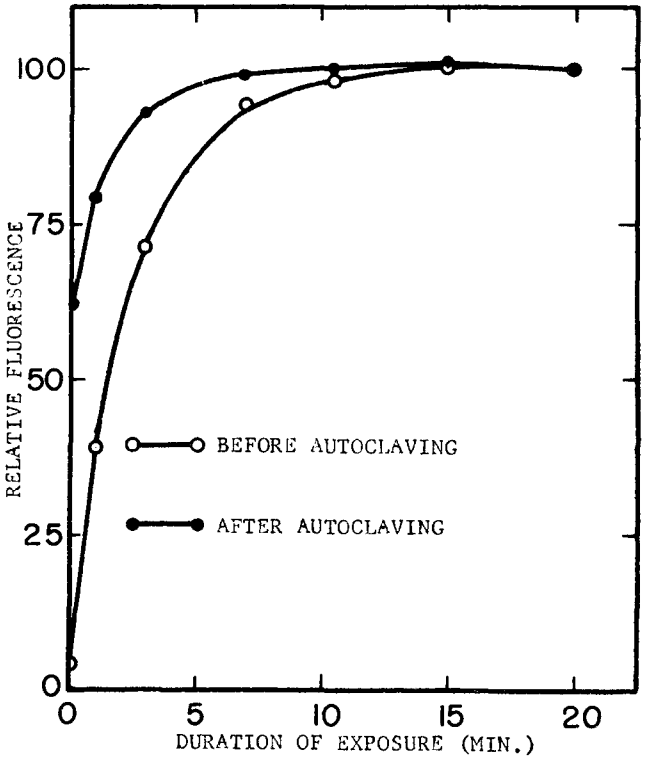

FIG. 3.—Fluorescence of a sweetclover leaf extract subjected to various durations of exposure to ultraviolet light before and after autoclaving in base. Calculated on the basis of a relative fluorescence of 100 for the 20-minute exposure in each set. Coumarin equivalence values for the plant extract were 23 γ per ml. before autoclaving and 86 γ per ml. after autoclaving.

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solutions were exposed to ultraviolet light for 15 minutes, and fluorescence readings were taken. Aliquots of a 2.5 *N* NaOH solution containing 10 γ of coumarin per ml. were autoclaved, diluted 1 to 10, irradiated, and subjected to fluorescence determination along with the plant extracts. As shown in figure 4, autoclaving the pure coumarin solution for periods longer than 30 minutes resulted in a small but definite loss in fluorescence. Failure of the plant extracts to show a similar loss may be the net result of continued liberation of fluorescent material from the bound form and destruction of the material by the longer periods of autoclaving. The 30-minute period was selected as satisfactory, both for its effectiveness and convenience.

Precision of the Method

Ten leaflets of the variety, Madrid, were extracted with 20 ml. of water in the usual manner. Fifteen 1-ml. aliquots of this extract were then separately assayed for free and total coumarin as described. Means and standard errors for free and total coumarin contents in the tissue (dry weight basis) were as follows:

Free coumarin—1.94 ± 0.008%
Total coumarin—3.27 ± 0.016%

The small standard errors obtained in this experiment, together with the data on efficiency of extraction given in table 1, indicate an excellent degree of precision for the laboratory procedures.

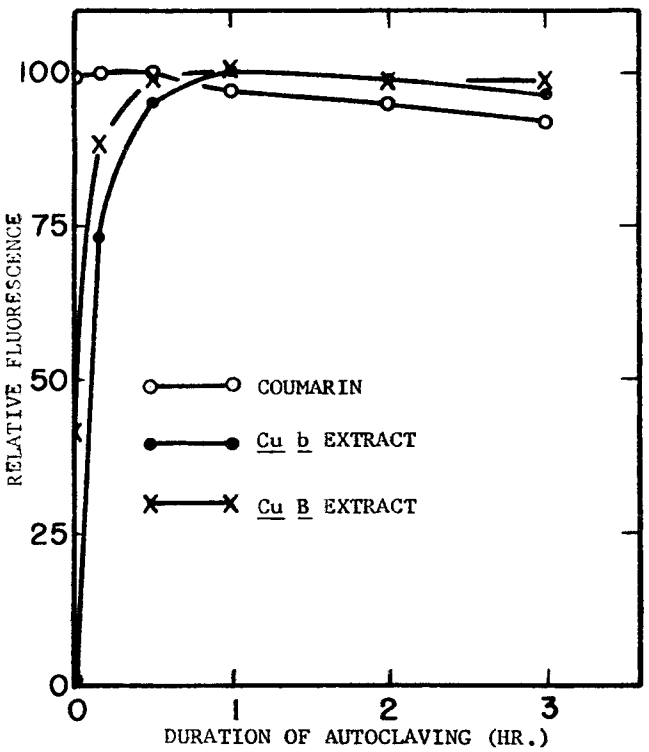

FIG. 4.—Fluorescence of leaf extracts from sweetclover plants of the *Cu B* and *Cu b* phenotypes and of coumarin subjected to various durations of autoclaving in base. Calculated on the basis of a relative fluorescence of 100 for the highest reading in each set. Coumarin equivalence values for the plant extracts were 61 γ per ml. for *Cu B* phenotype and 71 γ per ml. for *Cu b* phenotype.

Recovery of Added Coumarin

An extract of sweetclover leaves (Spanish) and a coumarin solution were mixed in various combinations in basic solution. In every case the final solution volume was 10 ml. and the final concentration of NaOH was 0.5 *N*. The solutions were assayed for content of free coumarin. As indicated in table 2, the fluorescence values ranged from 97 to 100% of values expected on the basis of the fluorescence of the plant extract and the quantities of coumarin added. These high recovery values lend support to the use of the proposed method as a measure of free coumarin in plant extracts. Further support was obtained in another experiment in which an acidified plant extract was subjected to ether extraction. At least 90% of the material responsible for fluorescence without autoclaving appeared in the ether phase. Pure coumarin added to a plant extract containing bound coumarin was also extractable with ether.

Assay of Four Phenotypes

Free and bound coumarin were determined in extracts of 15 plants of each of the 4 phenotypes listed previously. Results are presented in table 3. The magnitude of the standard errors indicates the variation among plants. This variation is much greater than that resulting from the laboratory procedures.

DISCUSSION

On the basis of the results obtained in these experiments, the following statements can be made regarding free and bound coumarin in sweetclover leaf tissue:

1. Both forms can be efficiently extracted by autoclaving the leaf tissue in water.
2. Basic solutions of free coumarin fluoresce in ultraviolet light after an initial exposure to light of suitable quality. Basic solutions of bound coumarin do not display appreciable fluorescence in ultraviolet light.
3. The fluorescence of a basic solution of free coumarin is changed very little by autoclaving the solution; however, autoclaving bound coumarin in base results in cleavage of

Table 2.—Recovery of coumarin added to Spanish sweetclover leaf extract.

Free coumarin equivalence of leaf extract	Coumarin added	Free coumarin equivalence of mixture		
		Expected	Observed	Observed / Expected
γ	γ	γ	γ	
2.3	2.5	4.8	4.8	1.00
2.3	5.0	7.3	7.1	0.97
2.3	7.5	9.8	9.5	0.97
4.5	2.5	7.0	7.0	1.00
4.5	5.0	9.5	9.2	0.97
6.6	2.5	9.1	9.0	0.99

Table 3.—Coumarin contents of leaves from sweetclover plants representing 4 phenotypes.

Phenotype	Coumarin equivalence (dry weight basis)	
	Free mean ± S. E.	Bound mean ± S. E.
	%	%
Cu B	1.28 ± 0.136	2.21 ± 0.107
Cu b	0.03 ± 0.001	5.02 ± 0.376
cu B	0.08 ± 0.018	0.08 ± 0.017
cu b	0.02 ± 0.002	0.16 ± 0.013

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the bound form and consequently in greatly increased fluorescence.

4. Pure coumarin added to an extract of sweetclover leaves is recoverable in excellent yield as free coumarin.

5. The method outlined provides a valid measure of free and bound coumarin contents.

An examination of the data (table 3) reveals that in plants of the *Cu B* phenotype, only 37% of the total coumarin content occurs as free coumarin. This figure varies considerably. In a number of other determinations on leaf tissue from plants of this phenotype, values between 25% and 60% have been obtained. Apparently, such plants contain considerably more bound coumarin than has been generally recognized.

Although the number of plants tested is too small to permit generalization, it appears that plants of the *Cu b* phenotype, in which nearly all of the coumarin present is in the bound form, can have even higher total coumarin values than *Cu B* plants. The identity of the bound coumarin which occurs in current varieties and selections of sweetclover has not been established, although Roberts and Link (6) and Slatensek[3] have suggested that it is glycosidic in nature. This suggestion appears reasonable on the basis of the behavior of the bound form in crude plant extracts. Also, the isolation of melilotoside, a crystalline glucoside of coumaric acid, from flowers of *Melilotus altissima* Thuil. and *M. arvensis* Wallr. was reported in 1925 by Charaux (1). If bound coumarin is indeed melilotoside or some substance(s) of similar molecular weight, the level of such material in the leaves must be extremely high, in some cases exceeding 10% of the dry weight.

Table 3 also illustrates clearly the effects of the *cu* and *b* genes on coumarin content. Thus, replacement of *Cu Cu* by *cu cu* results in a great reduction in the level of total coumarin, irrespective of *B* and *b*. Replacement of *BB* with *bb* results in the virtual disappearance of free coumarin, regardless of the level of total coumarin.

SUMMARY

Methods previously described for the assay of coumarin in sweetclover all involve heating the plant tissue in concentrated alkali early in the procedure. This permits the determination of total coumarin content but does not allow for the separate determination of the free and bound forms of coumarin. The method presented involves extraction of both forms of coumarin by autoclaving the leaf tissue in water, followed by dilution of the extract with alkali. Free coumarin content is determined directly from a sample of the alkaline solution, total coumarin content is determined from an autoclaved sample of the alkaline solution, and bound coumarin content is obtained by difference. Results are expressed on the basis of dry weight, as calculated from the green weights of the leaflets sampled, multiplied by the percentage dry weight of a composite sample of similar leaflets.

Experimental evidence pertaining to the reliability of the various steps in the procedure is presented. Results of the assay as applied to four different phenotypes of sweetclover are given.

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