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Fluorometric Assay of Free and Bound, *cis*- and *trans*-*o*-Hydroxycinnamic Acid in a Single Plant Extract

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FLUOROMETRIC ASSAY OF FREE AND BOUND, *cis-* AND *trans-o*-HYDROXYCINNAMIC ACID IN A SINGLE PLANT EXTRACT[1]

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ABSTRACT

Fluorescence is measured on unhydrolyzed and hydrolyzed plant extracts, with and without prior ultraviolet treatment. Readings permit direct determination of free *o*-hydroxycinnamic acid (*o*-HCA), free *trans-o*-HCA, total *o*-HCA, and total *trans-o*-HCA. Levels of bound *trans-o*-HCA and free and bound *cis-o*-HCA are calculated by appropriate subtraction.

Additional index words: **Coumarin, Sweetclover, *Melilotus alba.***

SEVERAL procedures for the fluorometric determination of *o*-hydroxycinnamic acid (*o*-HCA) in sweetclover (*Melilotus* sp.) extracts are summarized in the review of Smith and Gorz (12). The earlier procedures (1, 11, 13) permitted determination of total *o*-HCA (commonly referred to as total coumarin). Subsequent modifications of these procedures provided for the fluorometric measurement of free as well as total *o*-HCA, and permitted the calculation of bound *o*-HCA levels by difference (4, 10). A later change in the procedure (7) made possible the assay of *trans* and total *o*-HCA, and from these values, levels of *cis-o*-HCA (the lactone of which is coumarin) were calculated by difference; however, the procedure did not provide estimates of the free and bound compounds. Determination of the various components contributing to total *o*-HCA may not always be required, but for some studies it is essential to know the amounts of both the *cis* and *trans* isomers which are present in the free and the glucosidically bound forms. The procedure described in this report combines and improves upon two earlier methods (4, 7) to permit the determination of the free and bound contents of both isomers of *o*-HCA in extracts of sweetclover and other *o*-HCA-containing plants. The method depends upon the fact that basic solutions of *cis-o*-HCA and of the glucosides of *cis-* and *trans-o*-HCA do not fluoresce under the conditions of the assay, while basic solutions of *trans-o*-HCA are highly fluorescent. Furthermore, ultraviolet irradiation of either isomer of *o*-HCA in basic solution brings about an equilibrium in which, under the conditions used in this work, approximately 72% of the compound is in the *trans* form and 28% is *cis*.

Tissue samples (10 to 100 mg fresh weight) are weighed to the nearest 0.1 mg, dropped into hot water (10 ml water in a 20 × 150 mm test tube in a boiling water bath), immediately submerged by shaking, and autoclaved for 20 min at 120 C. This treatment rapidly inactivates endogenous β-glucosidase which might be present (6), and effectively extracts both free and glucosidically bound *o*-HCA (4). Autoclaved extracts are cooled in a water bath, the contents of each tube are mixed, and the tissue residue is removed and discarded. These extracts may be frozen for later assay. An additional tissue sample of similar size is weighed and oven dried for determination of dry matter percentage. In sweetclover work, the mid-leaflet of a single leaf is often used as the moisture sample, and the two side leaflets are extracted for *o*-HCA assay.

Extracts are protected from light throughout all steps following submersion of the tissue in hot water. All subsequent operations are carried out in a darkroom under a Kodak Safelight[3] (Series OA Wratten filter), or in a very dimly lighted laboratory. This precaution is necessary to prevent spurious interconversion of the two isomers (5, 9).

Extracts are assayed fluorometrically by using a Turner Model 110 Fluorometer, with filters 7-60 and 8 as the primary and secondary filters, respectively, and with the smallest possible aperture between light source and sample. For typical sweetclover leaf extracts, the following sequence is used (all dilutions are made in 20 × 150 mm test tubes):

(a) Extracts are diluted 1 to 10 with water. This dilution may be omitted if the tissue is very low in *o*-HCA, or may be greater than 1 to 10 if the tissue is extremely high in *o*-HCA content.

(b) Solutions from step (a) are diluted 1 to 10 with 2.5*N* NaOH.

(c) Two 1-ml samples of the basic solution from step (b) are withdrawn, and each is diluted to 10 ml with water. One of the diluted samples is exposed to ultraviolet light for 15 min at a distance of approximately 15 cm from the T.F.8 tube of a Gates M.R.4 laboratory lamp (peak near 360 nm); the other is held in the dark prior to reading. The fluorescence reading of the ultraviolet-treated sample provides a measure of the total amount of free *o*-HCA present in the extract, the dark sample is used as a measure of free *trans-o*-HCA, and the content of free *cis-o*-HCA is calculated by difference (total free − free *trans* = free *cis*).

(d) The remaining 8 ml of basic solution from step (b) is autoclaved at 120 C for 45 min to hydrolyze the glucosidically bound *o*-HCA. The autoclaved solution is cooled, and two 1-ml samples are diluted to 10 ml with water. As in step (c), one of the samples is irradiated with ultraviolet light for 15 min, and the other is held in the dark. The dark and ultraviolet-treated samples from the hydrolyzed extract provide measures of the total (free + bound) *trans-o*-HCA, and total (free and bound *cis* + free and bound *trans*) *o*-HCA, respectively. Total *cis-o*-HCA values are obtained by difference (total *o*-HCA − total *trans*), and values for bound *trans-* and *cis-o*-HCA also are obtained by difference (total *trans* − free *trans* = bound *trans*, total *cis* − free *cis* = bound *cis*).

Fluorescence readings are converted to concentrations of *o*-HCA by reference to a conversion table, which is based on readings of a series of standards containing known concentrations of *o*-HCA. *o*-Coumaric acid (*trans-o*-HCA) is used in preparing the standards. Two series are prepared; one is exposed to ultraviolet

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[3] Mention of specific products is for identification only and does not imply endorsement by the USDA.

light for 15 min, while the other is held in the dark prior to reading. The relationship between fluorescence readings and concentration is linear. At the instrument settings used, a reading of 100 on a dark sample indicates a concentration of 3.64×10^{-2} micrograms *trans-o*-HCA/ml in the solution contained in the fluorometer cuvette. A reading of 100 on an ultraviolet-treated sample indicates a concentration of 5.07×10^{-2} micrograms *o*-HCA/ml.

Following adjustment for dilution of the plant extract, *o*-HCA levels are expressed as percentage of the oven-dry weight of the plant sample from which the extract came. Values may be expressed in terms of coumarin equivalence, if desired, by multiplying the *o*-HCA values by 0.890, the ratio of the molecular weight of coumarin to that of *o*-HCA.

Hot water extracts of sweetclover leaves contain numerous fluorescent substances other than *o*-HCA. However, for most purposes these substances do not interfere with the fluorometric assay. Evidence for this lack of interference is found in the fact that fluorescence readings on extracts of low-*o*-HCA sweetclover plants typically amount to only 1 or 2% of the readings on extracts of closely related high-*o*-HCA plants (3). The two kinds of extract would be expected to be similar with respect to fluorescence caused by compounds other than *o*-HCA. Thus, the extent of such fluorescence appears to be very small at the 1,000-fold dilution of plant extracts normally used. Furthermore, the fluorescence spectrum of alkaline sweetclover leaf extracts, as determined with an Aminco Bowman Spectrophotofluorometer, is almost identical to that of a basic solution of *trans-o*-HCA.

In this laboratory, the methods described have been used extensively in sweetclover research. They also have been applied successfully, although to a limited extent, in the measurement of *o*-HCA in various *Trigonella* species (2), and in tonka bean (*Dipteryx odorata* Willd.) (8), sweet vernal grass (*Anthoxanthum odoratum* L.), sweet grass (*Hierochloe odorata* (L.) Beauv.) and deer's tongue (*Trilisa odoratissima* (J. F. Gmel.) Cass.).

Literature Cited

1. Goplen, B. P., J. E. R. Greenshields, and W. J. White. 1956. Selection techniques in screening for coumarin-deficient sweet clover plants. Can. J. Bot. 34:711-719.
2. Gorz, H. J., and F. A. Haskins. 1964. Occurrence of *o*-hydroxycinnamic acid in species of *Melilotus* and *Trigonella*. Crop Sci. 4:193-196.
3. ——— and ———. 1969. Absence of dominance of the *Cu* gene in influencing *o*-hydroxycinnamic acid content in *Melilotus alba*. Crop Sci. 9:79-81.
4. Haskins, F. A., and H. J. Gorz. 1957. Fluorometric assay of free and bound coumarin in sweetclover. Agron. J. 49:493-497.
5. ——— and ———. 1959. Influence of spectral composition of light on *cis-trans* interconversion of *o*-hydroxycinnamic acid. Archiv. Biochem. Biophys. 81:204-210.
6. ——— and ———. 1961. A reappraisal of the relationship between free and bound coumarin in *Melilotus*. Crop Sci. 1:320-323.
7. ——— and ———. 1961. Assay of *cis*- and *trans-o*-hydroxycinnamic acids in sweetclover extracts. Biochem. and Biophys. Res. Comm. 6:298-303.
8. ——— and ———. 1963. Glucosides of coumarinic and *o*-coumaric acids in the tonka bean. Science 139:496-497.
9. ———, L. G. Williams, and H. J. Gorz. 1964. Light-induced *trans* to *cis* conversion of β-D-glucosyl *o*-hydroxycinnamic acid in *Melilotus alba* leaves. Plant Physiol. 39:777-781.
10. Kahnt, G., and W. J. Schön. 1962. Zur quantitativen Analyse des Cumarins und des Glucosids der Cumarinsäure in Blättern von *Melilotus albus*. Angewandte Botanik 36:33-49.
11. Slatensek, J. M., and E. R. Washburn. 1944. A rapid fluorometric method for the determination of coumarin and related compounds in sweet clover. J. Am. Soc. Agron. 36: 704-708.
12. Smith, W. K., and H. J. Gorz. 1965. Sweetclover improvement. Advance Agron. 17:163-231.
13. White, W. J., R. G. Savage, and F. B. Johnston. 1952. A slightly modified fluorometric method of testing for coumarin content in sweet clover. Sci. Agr. 32:278-280.