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Influence of Tissue Drying on *o*-Hydroxycinnamic Acid Content in Sweetclover

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ABSTRACT

A fluorometric procedure was used to study changes in levels of the free and bound forms of the *cis* and *trans* isomers of *o*-hydroxycinnamic acid (*o*-HCA) associated with drying sweetclover (*Melilotus*) tissues under various conditions of time and temperature. Leaves, or leaves and stems, of three sweetclover varieties, 'Spanish,' 'Evergreen,' and 'Goldtop,' and two closely related, highly inbred lines (*CuCuBB* and *CuCubb* genotypes) were used. Drying temperatures of 30 and 40 C were used most extensively. When dried at 30 C, leaves lost more than half of their bound *cis-o*-HCA, and a small amount of free *cis-o*-HCA (coumarin) was produced. In leaves dried at 40 C, up to 90% or more of the bound *cis-o*-HCA was converted to the free form by the enzyme, β-glucosidase, and little loss of total *o*-HCA occurred. Thus, levels of free and bound *cis-o*-HCA were appreciably modified by the drying treatments imposed. Content of *trans-o*-HCA, on the other hand, was not greatly changed by drying. The results obtained may have application in the drying of leaves of deer's tongue [*Trilisa odoratissima* (Walt. ex J. F. Gmel.) Cassini], an *o*-HCA-containing plant used in the tobacco (*Nicotiana tabacum* L.) industry.

Additional index words: Coumarin, *Melilotus*, *cis* and *trans* isomers, β-Glucosidase.

COUMARIN and the related compounds *cis*- and *trans-o*-hydroxycinnamic acid (*cis*- and *trans-o*-HCA) are regarded as undesirable constituents of sweetclover (*Melilotus*) forage because they adversely affect forage palatability (coumarin, in particular, has a bitter taste), and they appear to be metabolically related to the anticoagulant dicoumarol (14). Therefore, it is important to know whether levels of these compounds may be reduced substantially by a simple treatment such as air- or oven-drying of the forage. Furthermore, a question exists as to whether assays of dried samples provide a reliable measure of the amount of coumarin and related compounds present in the green plant. Several investigations have touched

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on this problem in sweetclover (4, 6, 15), sweet vernalgrass (*Anthoxanthum odoratum* L.) (3), and sweet woodruff (*Asperula odorata* L.) (4), and in some instances, conflicting results were reported. Stevenson and Clayton (15) and Goplen, Greenshields, and White (6) reported a lowering of coumarin content upon drying, and Behr, Hülsmann, and Thilo (4) noted an increase. Behr et al. made the reasonable suggestion that the discrepancy probably resulted from the different assay methods used by the various workers.

The studies just cited were published before a simple fluorometric assay for the free and bound forms of *cis*- and *trans-o*-HCA was available. Such a procedure has now been developed (7, 9) and has been used to investigate again the question of "coumarin loss" during drying of sweetclover tissue. The results are the subject of this report.

MATERIALS AND METHODS

Plant materials. White-flowered biennial sweetclover (*M. alba* Desr.), varieties 'Spanish' and 'Evergreen;' yellow-flowered biennial sweetclover [*M. officinalis* (L.) Lam.], var. 'Goldtop;' and two closely related, highly inbred *M. alba* lines of the *CuCuBB* and *CuCubb* genotypes were used in this study. Derivation of the *CuCuBB* and *CuCubb* lines paralleled the derivation of the *cucuBB* and *cucubb* lines described by Gilchrist, Haskins, and Gorz (5). Extracts of *CuCuBB* plants are high in content of glucosidically bound *cis*- and *trans-o*-HCA and in β-glucosidase activity (8). With respect to the *Cu/cu* and *B/b* alleles, Spanish, Evergreen, and Goldtop are of the *CuCuBB* genotype.

Plants were grown in the field, greenhouse, or growth chamber. Harvested stems were processed in the laboratory where, in most instances, only the youngest fully expanded leaf was removed from each main or side branch. Leaves were dissected into the three constituent leaflets, and individual leaflets or bulked corresponding leaflets were weighed. One leaflet or one group of bulked corresponding leaflets was used for initial extraction; the other two were subjected to various drying treatments, either two times at one temperature or two temperatures for one time. In a single experiment with both stem and leaf tissue, terminal portions (stems and attached leaves) 30 cm long from Goldtop plants about 120 cm in height were harvested, weighed, and subjected to drying and extraction treatments.

Drying treatments. Samples were dried in laboratory ovens of the gravity convection type or were air-dried at room temperature (ca 25 C). In the ovens, samples were placed as centrally as possible to facilitate air circulation. Oven temperatures were generally maintained within ± 1 C of the indicated values.

Extraction and assay. Individual or bulked leaflets were immersed in hot water (10 ml/leaflet) and immediately autoclaved for 20 min at ca 120 C. Aliquots of the resulting extracts were held in a freezer until assayed. In the experiment on Goldtop, each stem portion with attached leaves was immersed in 400 ml of hot water and autoclaved for 30 min. Samples of the extracts were frozen for later assay.

All extracts were assayed fluorometrically for free and bound *cis*- and *trans-o*-HCA, as previously described (9).

RESULTS AND DISCUSSION

Losses in total *o*-HCA from *CuCuBB* and *CuCubb* leaflets were most pronounced when the samples were dried at room temperature (Table 1). Changes in level of *trans-o*-HCA during drying were neither large nor consistent, and values for free *trans-o*-HCA (not shown in the table) were extremely low in all experiments. For practical purposes, the *trans-o*-HCA values in the tables and figures represent the amount of this compound present in the glucosidically bound form. Levels of the *trans* compound accounted for slightly more than one-tenth of the total *o*-HCA in the initial (zero time) samples. This value agrees well with fractions of the *trans* isomer previously observed in field-grown *CuCu* leaves (1).

Inasmuch as *trans-o*-HCA levels were relatively unaffected by drying, it is apparent that the observed losses in total *o*-HCA resulted from decreased levels of the *cis* isomer. The lactone form of *cis-o*-HCA is coumarin, and "bound coumarin" has been identified as the β-D-glucoside of *cis-o*-HCA (12). In chopped or homogenized tissue of the *CuCuBB* genotype, endogenous β-glucosidase hydrolyzes *cis-o*-HCA glucoside, and the liberated *cis-o*-HCA lactonizes spontaneously to produce coumarin (7). A measure of free coumarin content is provided by the free *cis-o*-HCA values in Tables 1 and 2. To convert percentage of free *cis-o*-HCA to free coumarin percentage, the free *cis-o*-HCA values should be multiplied by 0.89, the ratio of the molecular weight of coumarin to that of *o*-HCA.

Table 1. Relative weight and *o*-hydroxycinnamic acid (*o*-HCA) content of leaflets from field-grown *CuCuBB* and *CuCubb* sweetclover plants. At each temperature, midleaflets from 10 leaves were used for initial (0 time) extraction, and the two groups of 10 side leaflets were dried for two different times as indicated. Leaflets were individually weighed, extracted, and assayed.

Genotype	Drying temp. (C)	Drying time (hour)	Relative weight (initial=100)	*o*-HCA percentage*			
				trans	cis		
					Bound	Free	Total
CuCuBB	Room temp. (ca25)	0	100	0.51 ± 0.04	4.19 ± 0.32	0.02 ± 0.006	4.72 ± 0.36
		16	50.8 ± 3.1	0.52 ± 0.05	3.36 ± 0.43	0.08 ± 0.02	3.97 ± 0.48
		64	18.3 ± 0.5	0.47 ± 0.04	1.02 ± 0.24	0.56 ± 0.10	2.05 ± 0.35
	40	0	100	0.56 ± 0.07	4.68 ± 0.35	0.02 ± 0.006	5.26 ± 0.41
		16	18.0 ± 0.4	0.60 ± 0.08	3.37 ± 0.34	1.15 ± 0.16	5.11 ± 0.38
		64	18.7 ± 0.6	0.59 ± 0.08	3.44 ± 0.32	0.79 ± 0.15	4.83 ± 0.38
	60	0	100	0.57 ± 0.04	4.56 ± 0.21	0.14 ± 0.02	5.26 ± 0.25
		4	17.7 ± 0.5	0.44 ± 0.05	0.37 ± 0.08	2.38 ± 0.11	3.19 ± 0.19
		16	18.0 ± 0.5	0.41 ± 0.03	0.31 ± 0.05	2.25 ± 0.12	2.95 ± 0.14
	100	0	100	0.62 ± 0.05	4.45 ± 0.30	0.15 ± 0.02	5.23 ± 0.35
		1	18.8 ± 0.8	0.50 ± 0.03	1.96 ± 0.24	2.06 ± 0.12	4.52 ± 0.28
		4	18.5 ± 0.6	0.50 ± 0.04	1.87 ± 0.21	1.84 ± 0.14	4.20 ± 0.26
CuCubb	Room temp. (ca25)	0	100	0.42 ± 0.04	3.71 ± 0.24	0.02 ± 0.008	4.14 ± 0.27
		16	42.7 ± 3.2	0.37 ± 0.03	1.56 ± 0.14	0.04 ± 0.012	1.98 ± 0.16
		64	19.2 ± 1.1	0.42 ± 0.03	1.31 ± 0.20	0.06 ± 0.007	1.78 ± 0.21
	40	0	100	0.34 ± 0.03	3.13 ± 0.22	<0.01	3.47 ± 0.25
		16	19.1 ± 0.9	0.37 ± 0.05	3.02 ± 0.34	0.05 ± 0.012	3.43 ± 0.39
		64	18.7 ± 0.8	0.36 ± 0.05	3.14 ± 0.25	0.03 ± 0.008	3.53 ± 0.28
	60	0	100	0.45 ± 0.04	3.68 ± 0.23	0.03 ± 0.006	4.16 ± 0.27
		4	18.9 ± 0.6	0.31 ± 0.03	3.58 ± 0.19	<0.01	3.88 ± 0.21
		16	19.1 ± 1.0	0.32 ± 0.03	3.60 ± 0.23	<0.01	3.91 ± 0.25
	100	0	100	0.37 ± 0.06	3.02 ± 0.38	0.03 ± 0.012	3.43 ± 0.44
		1	17.4 ± 0.6	0.27 ± 0.05	3.01 ± 0.34	0.03 ± 0.011	3.31 ± 0.37
		4	17.3 ± 0.5	0.31 ± 0.06	2.82 ± 0.38	0.04 ± 0.009	3.16 ± 0.44

* Dry weight basis, mean of 10 determinations ± SE.

Table 2. **Relative weight and *o*-hydroxycinnamic acid content of terminal 30-cm portions of field-grown Goldtop sweetclover plants. Five portions were used as the initial sample, and five were subjected to each of the indicated drying treatments.**

Drying temp. (C)	Drying time (hour)	Relative weight (initial=100)	*o*-HCA percentage*			
			trans	cis Bound	cis Free	Total
Initial		100	0.27 ± 0.01	1.91 ± 0.04	0.02 ± 0.005	2.20 ± 0.05
30	118	25.3 ± 0.7	0.27 ± 0.01	0.96 ± 0.05	0.31 ± 0.04	1.54 ± 0.09
40	94	20.6 ± 0.5	0.27 ± 0.01	0.97 ± 0.07	0.57 ± 0.05	1.81 ± 0.08
75	28	20.2 ± 0.5	0.27 ± 0.01	0.23 ± 0.06	1.22 ± 0.08	1.71 ± 0.08

* Dry weight basis, mean of five determinations ± SE.

In leaflets of the *CuCuBB* genotype, all drying treatments effected decreases in level of bound *cis-o*-HCA (Table 1). At room temperature, this loss was offset to a small extent by a gain in free *cis-o*-HCA at 64 hr, but the decrease in total *o*-HCA was still pronounced. The decrease in bound *cis-o*-HCA was smaller at 40 C than at room temperature, but, as evidenced by the larger contents of free *cis-o*-HCA, enzymatic hydrolysis of the bound form was considerably greater at 40 C. The gain in the free form accounted almost completely for the loss in the bound form. Thus, no significant reduction in level of total *o*-HCA was seen at 40 C. At 60 C, the decrease in bound *cis-o*-HCA amounted to more than 90%, and levels of free *cis-o*-HCA were higher than at 40 C. Apparently the 60 C temperature provided a degree of tissue disruption that permitted extensive contact of β-glucosidase with bound *cis-o*-HCA, and at the same time allowed the enzyme to remain active long enough to effect almost complete hydrolysis of this substrate. Some loss of a compound as volatile as coumarin would be expected during prolonged heating at 60 C; thus, volatilization may account for the observed net loss in total *o*-HCA at this temperature. Loss in bound *cis-o*-HCA was less extensive at 100 C than at 60 C, probably because of the greater lability of β-glucosidase at the higher temperature.

The loss in bound *cis-o*-HCA from *CuCubb* leaflets dried at room temperature was similar to that observed for the *CuCuBB* genotype (Table 1). In the *bb* leaflets, which lack β-glucosidase activity, the formation of free *cis-o*-HCA was negligible. The similarity of *CuCuBB* and *CuCubb* leaflets, with respect both to loss of bound *cis-o*-HCA and limited production of free *cis-o*-HCA, leads to the conclusion that the decrease in bound *cis-o*-HCA associated with drying at room temperature involved metabolic conversions other than β-glucosidase-mediated hydrolysis. In marked contrast to results obtained with *CuCuBB* leaflets, no appreciable loss of *o*-HCA and no conversion of bound *cis-o*-HCA to the free form were noted in *CuCubb* leaflets dried at 40, 60, or 100 C. These observations strengthen the conclusion, suggested in the preceding paragraph, that β-glucosidase played a significant role in the conversions and losses noted in *CuCuBB* leaflets dried at these three temperatures.

Results with leaves of field-grown Spanish sweetclover were generally similar to those obtained with *CuCuBB* leaves. These and other experiments indicated that field-grown leaves dried at 25 to 30 C lost much of their bound *cis-o*-HCA without extensive production of free *cis-o*-HCA, whereas at 40 C extensive

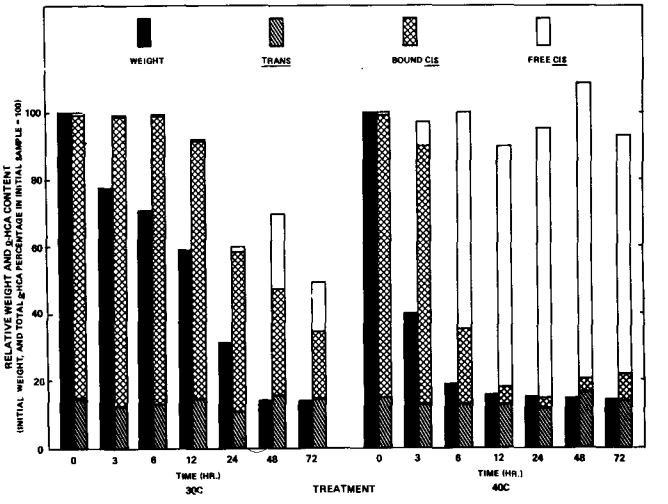

Fig. 1. Relative weights and *o*-hydroxycinnamic acid (*o*-HCA) contents of field-grown Evergreen leaflets dried for various times at 30 and 40 C. Ten leaves were used for each drying time. Within each set of 10 leaves, bulked midleaflets were used for the initial sample, one group of side leaflets (bulked) was dried at 30 C and the other group at 40 C. The zero-time values in the graph represent the means of the six initial extracts (one for each drying time); other values are based on assays of single extracts.

free *cis-o*-HCA was produced with little loss of total *o*-HCA.

In a further investigation of the rather striking differences produced by drying at 30 as opposed to 40 C, observations were made over a longer time span. Field-grown plants of the variety Evergreen were used. Magnitudes of the standard errors in earlier experiments (Table 1) indicated that variation among similarly treated leaflets was not excessive; accordingly, bulked 10-leaflet samples were used in this experiment. Results (Fig. 1) indicate that at 30 C, relatively little *o*-HCA was lost until leaflets had been dried for more than 12 hours. Between 12 and 24 hours, an appreciable drop in content of bound *cis-o*-HCA occurred, and only a small part of this loss was offset by a gain in free *cis-o*-HCA. Between 24 and 48 hours, bound *cis-o*-HCA continued to decline, but levels of the free *cis* compound increased, indicating that β-glucosidase was still active and that sufficient water was still present in the tissues to permit some hydrolysis of the bound form to occur.

Drying of the Evergreen leaflets was much more rapid at 40 C than at 30 C, as expected. The extent of hydrolysis of bound *cis-o*-HCA was considerably greater than that indicated in Table 1 for *CuCuBB* leaflets dried at 40 C. Thus, within 12 hours at 40 C, more than 90% of the bound *cis* isomer in Evergreen leaflets had been hydrolyzed to the free form. Possibly this extensive hydrolysis was related to the fact that the Evergreen leaflets were more succulent (about 15% dry matter) than the *CuCuBB* leaflets (more than 18% dry matter). Losses of free *cis-o*-HCA (coumarin) through volatilization evidently were not great over the 72-hour duration of this experiment. Levels of *trans-o*-HCA remained relatively constant throughout the drying treatments.

The drying treatments discussed in the preceding paragraphs involved removal of the leaves from the

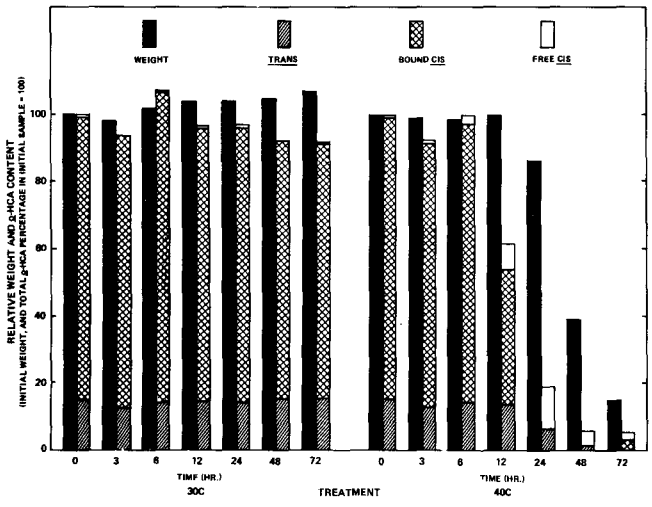

Fig. 2. Relative weights and *o*-hydroxycinnnamic acid (*o*-HCA) contents of field-grown Evergreen leaflets incubated under moist conditions for various times at 30 and 40 C. Except for the use of moist rather than dry conditions, the experimental procedure was identical to that described in the legend for Fig. 1.

plants. This situation gave rise to a question as to whether the observed changes in *o*-HCA content might have resulted from cutting off the supply of nutrients to the leaves rather than from the drying process. To obtain information on this point, detached Evergreen leaflets were placed on moist filter paper in Petri dishes and incubated over pans of water in 30 and 40 C ovens. Treatment durations were identical to those used in the experiment on drying Evergreen leaves. As shown in Fig. 2, leaflets held at 30 C under these moist conditions maintained their fresh weight and *o*-HCA content, and very little bound *o*-HCA was hydrolyzed during the 72-hour course of the experiment. At 40 C, fresh weight was maintained for about 12 hours and *o*-HCA content for about 6 hours; longer durations of incubation resulted in extensive tissue degradation and loss of both *cis*- and *trans-o*-HCA. These patterns differ drastically from those shown in Fig. 1. Inasmuch as detached leaflets were used in the experiments on which both Fig. 1 and Fig. 2 were based, the differences in patterns of change and loss shown in the two figures cannot be attributed to the separation of the leaflets from their source of metabolites, but must have resulted from the different conditions of incubation imposed.

The ratio of *cis*- to *trans-o*-HCA in sweetclover leaves depends to a great extent on the quality of the light to which the leaves are exposed (11). Almost 90% of the *o*-HCA present in the field-grown leaves used in the foregoing experiments occurred as the *cis* isomer. To investigate loss patterns in leaves having lower percentages of the *cis* form, *CuCuBB* and *CuCubb* leaves from greenhouse- and chamber-grown plants were dried at 30 and 40 C for various times. In the greenhouse-grown leaves of both genotypes, about 65% of the *o*-HCA was in the *cis* form; the corresponding percentage for chamber-grown leaves was slightly less than 10%. Losses from greenhouse-grown leaves were similar to those recorded in Table 1 for field-grown leaves of these genotypes. However, losses from and conversions in the chamber-grown leaves were slight in comparison with changes in leaves from the other sources. When chamber-grown leaves were exposed to sunlight for 2 hours before the initiation of drying, more than 60% of the *trans-o*-HCA was converted to the *cis* form, and losses from drying such leaves were generally intermediate between those observed for greenhouse-grown and nonexposed chamber-grown leaves. These observations confirm the conclusion that leaf drying has much less effect on the *trans* than on the *cis* isomer.

From a practical standpoint, it was desirable to know whether the type of changes in *o*-HCA content observed in relatively small samples of sweetclover leaves occurred also in sweetclover plants dried for hay. Unfortunately, available equipment did not permit the drying and processing of hay samples under closely controlled conditions. However, a small scale investigation was made using the terminal 30-cm portion of main stem and leaves from field-grown Goldtop sweetclover plants that were about 120 cm in height. These samples were extracted immediately or after drying at 30, 40, or 75 C. Based on preliminary experiments, respective drying times of 118, 94, and 28 hours were chosen for obtaining approximately equal dry weight percentages at the three temperatures. Results (Table 2) indicate that in these samples which included both leaf and stem tissue, (1) *trans-o*-HCA was not affected by drying; (2) bound *cis-o*-HCA was significantly reduced by each of the drying treatments; and (3) conversions of bound to free *cis-o*-HCA were in the order 75 C > 40 C > 30 C. Differences between the 30 and 40 C treatments, although less pronounced, were in the same direction as those noted in the leaf drying experiments. Based on these results, a considerable reduction in *o*-HCA level would be expected during the curing of sweetclover hay in the field. Assays based on oven- or air-dried samples certainly cannot be relied on to give an accurate indication of *o*-HCA level and form in the intact plant.

Information of the sort presented here for sweetclover is needed for other plants containing *o*-HCA. In particular, such information would be valuable for deer's tongue [*Trilisa odoratissima* (Walt. ex J. F. Gmel.) Cassini]. Because they are rich in coumarin, dried leaves of this plant are used in the tobacco (*Nicotiana tabacum* L.) industry (2, 13). Recent studies indicate that fresh leaves of deer's tongue, like fresh sweetclover leaves, contain relatively large quantities of the glucosides of *cis*- and *trans-o*-HCA, and no more than slight amounts of free coumarin (10). An investigation of optimal conditions for producing and curing deer's tongue leaves might well lead to significant improvement in yield of the apparently desired constituent, coumarin.

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