Comparison of Carotenoid Storage Stability in Alfalfa Leaf Protein (Pro-Xan) and Dehydrated Meals

A. Lyle Livingston,* George O. Kohler, and Donald D. Kuzmicky

Both carotene and xanthophyll were found to be more stable in alfalfa leaf protein concentrates (Pro-Xan) than in dehydrated alfalfa during storage of 1- or 2-g samples in open vials in the dark. Elevated temperatures (38 °C) made the differences in storage stability more pronounced. Freeze-drying alfalfa yielded a product with improved storage stability as compared with heat dehydration, but no such differences occurred with Pro-Xan. The addition of an antioxidant such as ethoxyquin improved the storage stability of carotene and xanthophyll in all meals. Addition of the water-soluble fraction from alfalfa leaf protein concentrate preparation only slightly improved the stability of carotenoids in Pro-Xan.

Dehydrated alfalfa meal has long been one of the principal pigmentation sources in poultry rations and has been marketed on a guaranteed carotene–xanthophyll content. Recently alfalfa leaf protein concentrates (Pro-Xan) have become available from pilot plant (Edwards et al., 1975a,b; Knuckles et al., 1972; Kohler et al., 1968, 1978) and commercial production. Pro-Xan is currently being produced by the Valley Dehydrating Co., Sterling, CO, and France Luzerne, Chalons Sur Marne, France. Due to high carotene–xanthophyll as well as high protein contents and low fiber levels, Pro-Xan shows great promise for use in poultry (Kuzmicky et al., 1977; Kuzmicky and Kohler, 1977) and swine feeds (Cheeke et al. 1977).

The ultimate use of both Pro-Xan and dehydrated alfalfa as pigmentation sources depends on the availability and stability of the carotenoids in these plant products. Kuzmicky et al. (1977) found xanthophyll in Pro-Xan to be about 1.7 times as available as that of dehydrated alfalfa and about three times that of marigold meal. There is considerable information in the literature comparing the xanthophyll availability of dehydrated alfalfa with other pigmentation sources such as corn gluten meal and synthetic carotenoids such as β-apo-8-carotenal (Kuzmicky et al., 1968; Marusich, 1965, 1967, 1969). Equally important is the stability of the carotenoids during storage. Storage under an inert gas (Graham, 1944) or treatment with the antioxidant ethoxyquin (Thompson, 1951), alone or combined with an oil (Livingston et al., 1955; Bickoff et al., 1955; Mitchell et al., 1954), has been shown to be effective in minimizing oxidation losses during storage of dehydrated alfalfa or Pro-Xan (Witt et al., 1971). The present study was undertaken to compare the storage stability of the carotenoids in Pro-Xan and dehydrated alfalfa meals prepared from the same freshly harvested alfalfa.

EXPERIMENTAL SECTION

Preparation of Meals. Freshly harvested alfalfa was brought to the laboratory and chopped, and a portion was dehydrated in a pilot Arnold dehydrator as previously described (Livingston et al., 1968) to provide dehydrated (dehy) alfalfa meal. Other portions of the freshly chopped alfalfa were pressed with a twin screw press to provide a whole green juice which was adjusted to pH 8.5 with concentrated NH_4OH. After heating the whole green juice to 80–85 °C by direct steam injection, the whole coagulated green protein concentrate was collected by centrifugation as described by Edwards et al. (1975b). The concentrate was pressed through a die to give uniform noodles and either freeze-dried (Buffalo Vac drier) or dried in the pilot Arnold dehydrator or on a fluidized bed drier (Witte drier). The moisture of the final product was adjusted in the fluid bed drier by varying the number of passes over the drier. The moisture in the products dried in the pilot Arnold dehydrator were varied by adjusting the outlet temperature of the drier. These temperatures ranged from 82 to 121 °C.

For comparative purposes a sample of freshly dried Pro-Xan meal was collected at a commercial plant (Valley Dehydrating Company, Sterling, CO) which utilizes an Arnold dehydrator. A sample of a commercially dried turf grass meal dried in an Arnold dehydrator (Livingston et al., 1971b) was obtained from Warren Turf Nursery (Suisun, CA).

Selected levels of water solubles [brown juice; Witt et al. (1971)] were added back to the Pro-Xan prior to drying so as to give a final concentration of water solubles of 5.98, 12.21, and 18.91% on a dry weight basis. Prior to analyses and storage the samples were left in sealed bottles at −12 °C.

Preparation of Storage Samples. The dried samples were ground through a 40-mesh screen in a Wiley mill. Ethoxyquin, 0.0125–0.125% by weight, was added to certain of the dried samples following the procedure of Livingston et al. (1955). Moisture levels were determined by drying in a forced draft oven at 105 °C for 16 h.

Storage Tests. A series of 1- or 2-g samples of each Pro-Xan, alfalfa and turf grass meal was stored in open vials in the dark at 2, 20, and 38 °C. Samples were analyzed after 4, 8, and 12 weeks of storage.

Carotenoid Analyses. The dehydrated alfalfa and turf grass meals were analyzed for carotene and nonepoxide xanthophyll according to the method of Livingston et al. (1971a). Similar analyses were performed on the Pro-Xan meals according to the method of Knuckles et al. (1971). All analyses were in duplicate.

RESULTS AND DISCUSSION

As shown in Table I carotene was less stable during 12 weeks of storage at all temperatures than xanthophyll in both the Pro-Xan and dehy alfalfa meals. Knowles et al. (1968) and Witt et al. (1971) also found carotene to be less stable than xanthophyll during accelerated storage in alfalfa and Pro-Xan, respectively. The carotenoids in both the low moisture Pro-Xan and alfalfa meals were more stable than those in the high moisture meals. It has previously been demonstrated that during alfalfa dehydration, carotenoids were more stable during drying to high meal

Western Regional Research Center, Science and Education Administration, U.S. Department of Agriculture, Berkeley, California 94710.

Table I. Effect of Storage Temperature and Meal Moisture on Carotenoid Stability in Leaf Protein and Dehydrated Alfalfa Meals

sample	storage temp, °C	meal moisture, %	initial values, mg/kg		carotene retained, %		xanthophyll retained, %	
			carotene	xanthophyll	4 weeks	12 weeks	4 weeks	12 weeks
Pro-Xan	2	7.6	881.1	1191.6	82	55	81	67
	2	2.3	898.2	1201.8	84	53	85	71
	20	7.6			66	49	85	74
	20	2.3			74	55	88	76
	38	7.6			56	31	68	54
	38	2.3			62	36	75	56
dehy alfalfa	2	8.0	277.5	343.8	80	56	86	66
	2	2.8	308.4	341.4	92	84	97	94
	20	8.0			72	46	85	63
	20	2.8			82	61	95	91
	38	8.0			44	21	81	58
	38	2.8			48	25	88	69

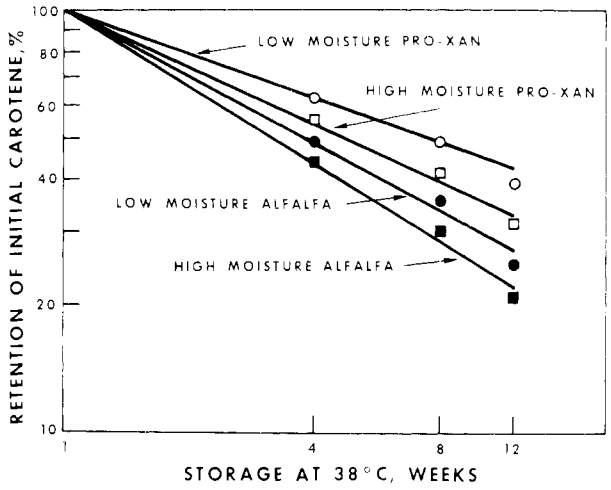

Figure 1. Effect of meal moisture upon stability of carotene during storage of Pro-Xan and alfalfa.

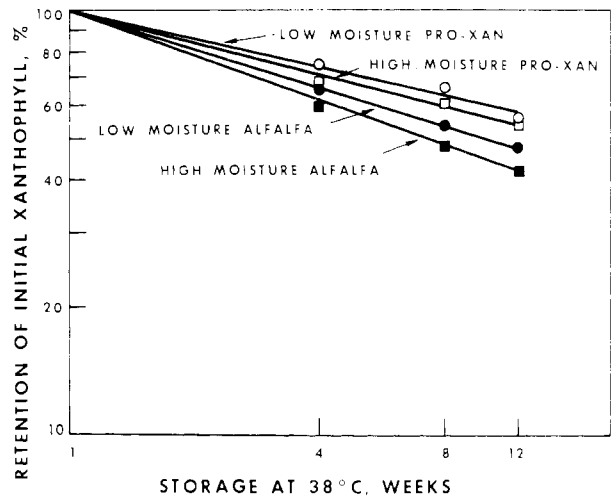

Figure 2. Effect of meal moisture upon stability of xanthophyll during storage of Pro-Xan and alfalfa.

moisture levels (Livingston et al., 1968) than when dried to low meal moisture levels. In the present study the meals were stored in open vials and, therefore, the moisture equilibrated to about 6–8% within 1 or 2 weeks. Accordingly, the moisture levels during storage were nearly the same for all meals. Differences in stability must, therefore, be due to effects occurring during the drying.

Carotene (Figure 1) and xanthophyll (Figure 2) were more stable in the Pro-Xan meals than in either the low or high moisture dehydrated alfalfa meals prepared from comparable fresh alfalfa.

Figure 3 compares the storage stability of carotene and xanthophyll in freeze-dried Pro-Xan and alfalfa prepared from the same fresh plant material. As shown in this figure the excellent storage stability of the carotenoids in freeze-dried alfalfa exceeded the storage stability of the carotenoids in the freeze-dried Pro-Xan. The lower stability of the freeze-dried Pro-Xan carotenoids may be due in part to the loss of the water-soluble antioxidants in the brown juice during the Pro-Xan preparation (Grossman et al., 1969). It has previously been shown that due to the excellent storage stability of the carotenoids in freeze-dried alfalfa there was only a slight enhancement by the addition of an antioxidant such as ethoxyquin (Knowles et al., 1968). In this present study several levels of the antioxidant ethoxyquin were added to Pro-Xan and alfalfa meals that had been dried to high and low moisture levels (Table II). Again the carotenoids in the low moisture Pro-Xan and dehydrated alfalfa meals were more stable than those in the high moisture meals. Although the carotenoids in the untreated Pro-Xan meals were more stable than those in

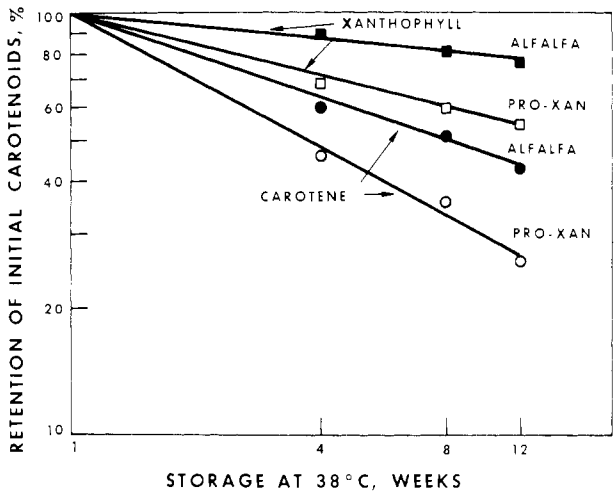

Figure 3. Retention of carotene and xanthophyll in freeze-dried Pro-Xan and alfalfa.

the untreated alfalfa meals, the addition of ethoxyquin made the carotenoid storage stability more nearly equivalent. There was further enhancement of carotenoid storage stability by increasing the antioxidant level from 0.0125 to 0.0500%, particularly for the Pro-Xan meals. Increasing the level of antioxidant to 0.125% resulted in only an additional slight increase in the storage stability of the carotene or xanthophyll.

Table III presents the results of the carotenoid storage stability in commercially prepared Pro-Xan and turf grass

Table II. Effect of Level of Ethoxyquin and Meal Moisture on Carotenoid Stability in Leaf Protein and Dehydrated Alfalfa Meals[a]

sample	moisture, %	ethoxyquin added, %	carotene retained, %		xanthophyll retained, %	
			4 weeks	12 weeks	4 weeks	12 weeks
Pro-Xan	7.6	0.000	56	31	68	54
	7.6	0.0125	60	43	69	66
	7.6	0.050	63	49	80	75
	7.6	0.125	64	53	83	77
	2.3	0.000	62	36	75	56
	2.3	0.0125	69	51	78	74
	2.3	0.050	72	57	85	80
	2.3	0.125	77	63	89	81
dehy alfalfa	8.0	0.000	44	21	60	42
	8.0	0.0125	62	38	72	62
	8.0	0.0500	67	50	80	70
	8.0	0.125	76	57	80	69
	2.8	0.000	48	25	66	48
	2.8	0.0125	68	48	72	67
	2.8	0.050	78	63	86	75
	2.8	0.125	85	74	86	76

[a] For initial analyses of these meals, see Table I.

Table III. Carotenoid Stability in Forage Products Stored at 38 °C

sample	ethoxquin added, 0.0125%	moisture, %	initial values, mg/kg		carotene retained, %		xanthophyll retained, %	
			carotene	xanthophyll	4 weeks	12 weeks	4 weeks	12 weeks
Pro-Xan[a]		7.3	911.9	1265.9	53	36	70	51
Pro Xan[a]	+	7.3	852.9	1196.0	65	54	77	70
turf grass[b]		7.9	558.4	805.6	55	48	81	65
turf grass[b]	+	7.9	552.9	780.1	75	73	87	84
Pro-Xan[c]		8.5	959.2	1056	46	27	85	63
Pro-Xan	+	8.5	865.3	1032.2	58	40	86	70
Pro-Xan		1.6	1032.5	1099.1	77	27	89	63
Pro-Xan	+	1.6	955.7	1096.3	90	70	96	82
dehy alfalfa[c]		6.0	366.7	440	41	16	56	37
dehy alfalfa	+	6.0	297.7	365.6	75	50	85	67
dehy alfalfa		2.5	308.0	356.2	49	25	66	48
dehy alfalfa	+	2.5	319.4	357.9	87	65	95	77
freeze-dried alfalfa		2.8	348.7	446.6	69	54	91	82
freeze-dried alfalfa	+	2.8	331.5	437.8	82	75	94	95

[a] Sample from Valley Dehydrating Co. [b] Sample from Warren Turf Nursery. [c] Samples from WRRC.

meals along with high and low moisture Pro-Xan and dehydrated alfalfa meals prepared at this laboratory. For this study both the Pro-Xan and dehydrated alfalfa meals were dried in the pilot Arnold dehydrator.

The commercial Pro-Xan meals initially contained the highest levels of xanthophyll of any meals used in these studies. The storage stability of the carotenoids in all of the meals was greatly improved by the addition of ethoxyquin, with the carotene in any particular meal being the most improved by the added antioxidant. The turf grass carotenoids were the most stable during storage of any of the meals included in this study. It has previously been found at this laboratory that during dehydration turf grass has a very short retention time in the drum, resulting in little loss of carotenoids during drying. Apparently, there is also little loss of the natural antioxidants in the turf grass during drying, thus accounting for the exceptional storage stability of the carotenoids. Witt et al. (1971) also reported that the carotenoids in a commercially prepared and drum dried alfalfa leaf protein concentrate to be exceptionally stable during storage tests. The larger scale equipment used in commercial drying may give a more rapid throughput time, resulting in a lower loss of natural antioxidants.

Figures 4 and 5 show the improved storage stability of carotene and xanthophyll in both Pro-Xan and dehydrated alfalfa by the addition of 0.0125% ethoxyquin. Although

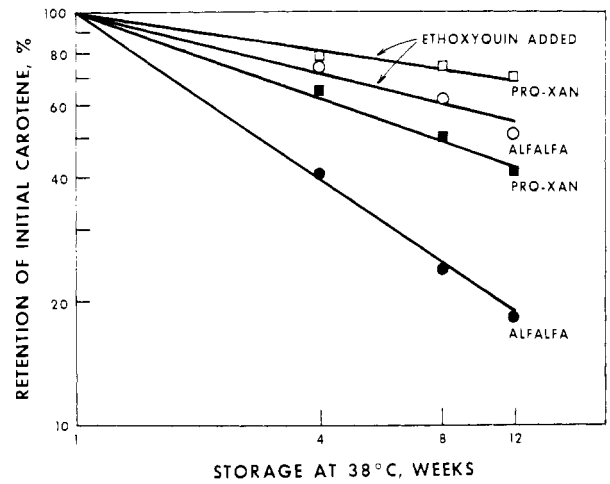

Figure 4. Effect of added ethoxyquin upon carotene stability in alfalfa and Pro-Xan.

the storage stability of the carotenoids in both meals was improved by the addition of ethoxyquin, Pro-Xan still contained the more stable carotenoids.

Since alfalfa contains both water-soluble (Grossman et al., 1969) and fat-soluble antioxidants (Livingston et al., 1968), the addition of portions of the pressed water solubles back to the leaf protein concentrate should enhance the

Table IV. Effect of Processing and Drying Procedures on Storage Stability of Carotenoids in Pro-Xan

sample	% water solubles added	drying method	initial values, mg/kg carotene	initial values, mg/kg xanthophyll	carotene retained, % 4 weeks	carotene retained, % 12 weeks	xanthophyll retained, % 4 weeks	xanthophyll retained, % 12 weeks
Pro-Xan	5.98[a]	fluid bed	829.8	949.7	57	33	73	59
Pro-Xan[b]	5.98[a]	fluid bed	829.8	949.7	54	38	83	70
Pro-Xan	5.98	freeze-dried	968.0	1184.5	56	28	76	56
Pro-Xan[b]	5.98	freeze-dried	968.0	1184.5	55	38	81	68
Pro-Xan	13.21	fluid bed	700.5	701.8	48	32	81	63
Pro-Xan[b]	13.21	fluid bed	700.5	701.8	56	42	89	79
Pro-Xan	13.21	freeze-dried	774.8	1022.3	56	43	70	46
Pro-Xan[b]	13.21	freeze-dried	774.8	1022.3	60	46	77	49
Pro-Xan	18.91	fluid bed	732.4	775.9	54	30	83	64
Pro-Xan[b]	18.91	fluid bed	732.4	775.9	57	47	90	77
Pro-Xan	18.91	freeze-dried	771.4	902.0	57	36	81	63
Pro-Xan[b]	18.91	freeze-dried	771.4	902.0	62	45	85	79
alfalfa meal		drum dried	366.7	440.0	41	16	60	37
alfalfa meal[b]		drum dried	366.7	440.0	76	50	80	67
alfalfa meal		freeze-dried	348.7	446.6	69	54	91	82
alfalfa meal[b]		freeze-dried	348.7	446.6	82	75	94	95

[a] Not added but present due to incomplete dewatering in the centrifuge. [b] Added 0.0125% ethoxyquin.

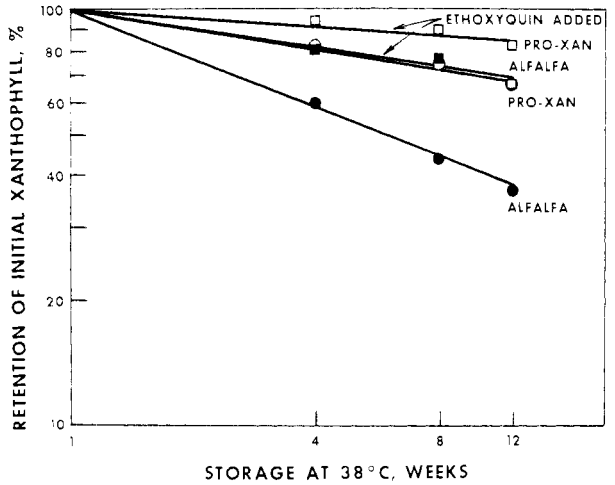

Figure 5. Effect of added ethoxyquin upon xanthophyll stability in alfalfa and Pro-Xan.

storage stability of the carotenoids. Table IV presents the results of adding one–three times the equivalent level of water solubles back to the Pro-Xan prior to drying. The Pro-Xan in this study was dried either on a fluidized bed drier or freeze-dried. For comparative purposes alfalfa meal samples, either drum dried or freeze-dried, are also presented in this study. Prior to storage, portions of each of the meals were treated with ethoxyquin.

The decrease in initial carotenoid values of the freeze-dried Pro-Xan meals with increasing levels of added water solubles is apparently a dilution effect. In this study there was only a slight increase in carotenoid storage stability with increasing levels of added water solubles. Furthermore, the carotenoids in Pro-Xan dried on the fluid bed drier were nearly as stable as those in the freeze-dried Pro-Xan. Again, the addition of ethoxyquin increased the storage stability of the carotenoids in all meals. Although the carotenoids in the dehydrated alfalfa meal were the least stable, the freeze-dried alfalfa with added ethoxyquin was the most stable of all of the meals studied. Only 5% of the xanthophyll was lost following 12 weeks of storage. There would seem to be an additive or, possibly, even a synergistic effect of the added and natural antioxidants in the Pro-Xan meals with the added water solubles.

Although the stabilizing value of more water solubles added to Pro-Xan containing about 6% water solubles is questionable, it is of obvious advantage to commercial plants producing alfalfa leaf protein, dehydrated alfalfa, or turf grass to add the antioxidant ethoxyquin in order to protect carotene and xanthophyll during storage.

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Transformations of Chemical Constituents during Flue-curing of *Nicotiana tabacum* L. 2. Metabolism of Nitrogenous and Related Constituents

Aumeed N. Amin, R. C. Long,* and W. W. Weeks

Changes in certain nitrogen fractions of tobacco leaf during flue-curing were studied by sampling leaves from two distinct primings in 1976 and 1977 at short time intervals during the yellowing and drying stages of flue-curing. Analytical data were corrected for dry weight loss. Percentage total nitrogen decreased during curing, while nitrate nitrogen showed no significant pattern of change. Total alkaloid concentrations exhibited stalk position and seasonal responses. Proteolysis extended throughout flue-curing, resulting in increased α-amino nitrogen during yellowing, but declined during drying. A net decrease in the concentration of total carbonyls (possible products of amino acid metabolism) occurred in both primings of one season, but not in the other. Miscellaneous nitrogen increased during yellowing and decreased during drying. The results suggest that flue-curing substantially alters the components of the various nitrogenous fractions of the harvested leaf.

Nitrogenous components of tobacco are important not only because of the presence of nicotine, but also for their contribution to flavor and aroma which Leffingwell (1976) reviewed in detail. Changes in some of these chemical constituents of tobacco leaf during flue-curing have been reviewed by Frankenburg (1946) and Johnson (1966, 1975). Early investigations of changes in the constituents during curing indicated the importance of curing in modifying qualitatively and quantitatively the chemistry of the green, mature leaf which, in turn, influences the quality of the cured leaf (Bacon, 1952; Chang, 1961; Sastry, 1960). Weybrew et al. (1966) have suggested that some carbonyls contributing to the aroma and flavor of tobacco may be generated through the decarboxylation and/or deamination of certain amino acids during curing.

By sampling at short time intervals during curing and determining the metabolic activities of leaf tissue and the constituent levels associated with them, one can gain a better understanding of how these factors influence the development of quality. The objective of this study was to investigate the transformations which occur in various nitrogen fractions during curing of tobacco from upper and lower stalk positions.

MATERIALS AND METHODS

As described previously (Amin et al., 1980), *Nicotiana tabacum* L. cv NC 2326 plants were grown and cultured normally in 1976 and 1977. The first and fourth primings (comprising approximately leaves 1–4 and 13–16, respectively) were harvested at maturity and flue-cured in conventional barns. Leaf samples were withdrawn at 6- and 12-h intervals during the yellowing and drying cycles of curing, respectively. After separating the midrib from the lamina, the lamina was quick-frozen, freeze-dried, ground, and stored for analysis.

Samples were analyzed for total alkaloids according to the method of Harvey et al. (1969). Soluble protein nitrogen [extracted according to the method of Gaines (1977)] and total nitrogen were determined colorimetrically as ammoniacal nitrogen/BD acid digest (Technicon Industrial Method No. 312-74A). Nitrate-nitrogen analysis was accomplished according to the method of Collins et al. (1967), while α-amino nitrogen was determined using a ninhydrin-positive procedure. Miscellaneous nitrogen was calculated from the equation:

$$\text{miscellaneous N} = \text{total N} - (\text{protein N} + \alpha\text{-amino N} + \text{nitrate N}) \quad (1)$$

Total carbonyls were determined by a modification of the procedure of Henick et al. (1954). Analytical grade CH_2Cl_2 was distilled with 2,4-dinitrophenylhydrazine (1.0 g/L) to eliminate carbonyls. Carbonyl-free ethanol was prepared by distilling 100% ethanol with 2,4-dinitrophenylhydrazine (0.5 g/L). One gram of ground tobacco was extracted with 100 mL of carbonyl-free ethanol for 24 h in the dark. The extract was filtered and made to 100-mL volume with carbonyl-free ethanol. Five milliliters was diluted to 100 mL. Five milliliters of diluted extract was pipetted into a 25-mL volumetric flask, and 3 mL of 4.3% trichloroacetic acid in carbonyl-free CH_2Cl_2 and 5 mL of 0.15% 2,4-dinitrophenylhydrazine in carbonyl-free ethanol were added. The flask was heated in a water bath at 40 °C for 30 min and cooled to room temperature. Color was developed by adding 10 mL of 4% KOH (prepared fresh in carbonyl-free ethanol). The flask was adjusted to volume with carbonyl-free CH_2Cl_2 and mixed. The absorption at 435 nm was determined spectrophotometrically after exactly 10 min against a blank prepared in the

Department of Crop Science, North Carolina State University, Raleigh, North Carolina 27650.

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