Effect of Storage Method on Content of Chlorogenic Acid and Rutin in Tobacco

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Green leaves harvested from tobacco plants were processed and stored by various methods prior to determination of chlorogenic acid and rutin. Samples lyophilized immediately after harvest, with or without prior quick-freeze, retained levels of chlorogenic acid and rutin comparable with those of fresh tissue. Samples stored at −85° C. showed a moderate loss in chlorogenic acid content, but no loss of rutin. Samples stored in a freezer at −15° C. for 3 weeks prior to extraction and analysis showed large reduction in amounts of both chlorogenic acid and rutin. Similar losses of these phenolic compounds were observed in leaf samples which were oven-dried at 60° or 70° C. immediately after harvest. When reduction in levels of polyphenols occurred, the chlorogenic acid content was always more severely affected than the rutin content. Thus, total chlorogenic acid and rutin levels as well as the relative proportions of these two polyphenols may be altered by methods of processing and storage prior to chemical analysis.

It is often necessary to store green-harvested plant samples prior to chemical analysis. This is critical when a large number of samples must be collected at a particular stage of growth, and the analytical procedure employed is so time-consuming that few samples can be processed at one time. These problems confront many researchers involved in studies of natural products of tobacco. Methods of sample storage must be selected with caution because chemical changes which occur in stored tissue may invalidate the results of chemical analysis.

Polyphenols are readily hydrolyzed and oxidized, and form complexes with proteins. Thus, changes may occur in stored tissue such that data derived after storage do not reflect the condition of the tissue at the time of sampling.

The objective of this research was to determine the relative merits of various methods of processing and storage of tobacco leaf samples as related to their content of chlorogenic acid (3-caffeoylquinic acid) and rutin (quercetin 3-rhamnoglucoside), the principal soluble polyphenols of tobacco.

MATERIALS AND METHODS

Experiment I. Burley tobacco (*Nicotiana tabacum* L. var. Burley-21) plants were cultured outdoors in large pots of soil that were irrigated, as needed, with half-strength Hoagland's Solution No. 1 (Hoagland and Arnon, 1938). The plants were cultured outdoors because preliminary experiments showed that the polyphenol content of tobacco was low in plants grown inside a glasshouse.

When the plants attained an average height of about 90 cm., they were transferred, still in their respective containers, to an analytical laboratory where samples were harvested and processed. The two uppermost, fully expanded leaves per plant were harvested. Midveins were removed, lamina weights were recorded, and extraction or storage procedures were started within seconds after the leaves were removed from the plants. Seven replicates were collected for each of the eight storage treatments.

Processing and Storage. Immediately after fresh weights were obtained, the samples were analyzed or processed and stored (as described below) until 21 days after the harvest, when extractions were conducted. Processing and storage procedures were as follows:

FRESH (control). Samples were extracted, concentrated, and immediately chromatographed.

LYOPHILIZED (followed by storage in an evacuated desiccator).

Without Prefreeze. Fresh samples were placed directly in glass flasks and dried in glass vessels on the side arms of the lyophilizer.

After Quick-Freeze in Liquid Nitrogen. Fresh samples were placed in Soxhlet extraction thimbles and immersed in the liquid nitrogen. The frozen samples, still in the thimbles, were placed in glass flasks and lyophilized on the side arms of the lyophilizer.

After Quick-Freeze in Dry Ice. Fresh samples were layered between paper toweling and packed in powdered dry ice for 1 to 2 hours. Subsequently, the frozen samples were transferred to glass flasks and lyophilized on the side arms of the lyophilizer.

STORED IN FREEZER AT −15° C. Without Prefreeze. Fresh samples were enclosed in plastic bags and placed in the freezer compartment of an ordinary laboratory refrigerator.

After Quick-Freeze in Liquid Nitrogen. Fresh samples were placed in extraction thimbles and immersed in liquid nitrogen. The frozen samples were placed in plastic bags and transferred to the freezer.

After Quick-Freeze in Dry Ice. Fresh samples were placed in bags and packed in powdered dry ice for 1 to 2 hours, after which they were transferred to the freezer.

OVEN-DRIED. Samples were placed in paper bags and dried to constant weight at 60° C. in a forced-draft oven. The dried samples were stored in an evacuated desiccator until final processing.

Additional Experiments. Additional experiments were carried out during fall and winter months using

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glasshouse-grown dark tobacco, Ky 151. For comparison of fresh and stored tissue, leaves were halved along the midrib. One half of a fully expanded leaf served as a fresh tissue sample and was immediately processed. The remaining half was processed as described below:

OVEN-DRIED AT 70° C. Samples were placed in paper bags and dried to constant weight at 70° C. in a forced-draft oven. Dried samples were extracted without further storage.

STORED AT −85° C. Samples were placed in plastic bags and stored for 3 weeks in a Revco ultra-low-temperature freezer at −85° C.

Determination of Chlorogenic Acid and Rutin. Each sample was exhaustively extracted in boiling absolute methanol and concentrated to 5 ml. One-hundred-microliter portions of the concentrate were then streaked in a 2.5-cm. band on 46 × 57 cm. sheets of Whatman No. 1 paper and developed in two dimensions by descending chromatography. Solvent systems employed were 1-butanol–acetic acid–water (6:1:2) (BAW) and 2-propanol–water–formic acid (5:95:0.1) (IWF), respectively. Zones containing chlorogenic acid (R_fBAW, 0.53; R_fIWF, 0.58) and rutin (R_fBAW, 0.34; R_fIWF, 0.32) were previously determined by comparison of R_f values and spectral curves with standard compounds recovered from the same chromatography system. These zones were cut out of the chromatograms and the chlorogenic acid was eluted with 30% methanol prior to assay by addition of Arnow's reagent (Arnow, 1937). Absorbance of the solution was measured at 510 mμ on a Bausch & Lomb Spectronic 20 colorimeter. Rutin was assayed by eluting its zone in a 1% methanolic aluminum chloride solution. Absorbance of the solution at 432 mμ was measured on a Beckman DBG recording spectrophotometer.

Amounts of chlorogenic acid and rutin present in the extracts were determined by means of internal standard curves prepared by recovery of known amounts of both compounds from the paper chromatography system described above. Statistical analysis by the method of least significant difference was performed on data obtained in the first experiment. Analysis of paired variates was used in experiments in which the half-leaf technique was employed.

RESULTS AND DISCUSSION

The results of comparison of seven methods of tissue storage against a fresh tissue control are shown in Table I. Samples that were lyophilized and stored for 3 weeks after harvest retained chlorogenic acid and rutin levels comparable with those of fresh tissue. Levels of the two compounds did not differ significantly between samples that were or were not prefrozen prior to placement on the lyophilizer. This suggests that a prior prefreeze treatment may not be necessary. However, this result should be interpreted with caution, since a blue fluorescing spot appeared on chromatograms of tissues freeze-dried without prior quick-freeze. The spot, which has an R_f of 0.79 in BAW and an R_f of 0.21 in IWF, did not appear on chromatograms of fresh tissue or of samples that were quick-frozen prior to lyophilization. Since this spot was also found on samples that were oven-dried at 60° C., it may have been a degradation product of one of the phenolic constituents, and, therefore, may indicate that some breakdown of phenols occurred in samples placed in the lyophilizer without a prior quick-freeze.

Samples that were oven-dried at 60° C., stored at −15° C. after a prefreeze in liquid nitrogen, or stored at −15° C. without a prefreeze treatment incurred large losses of both chlorogenic acid and rutin. Following these treatments, only about 25 to 28% of the rutin and approximately 2 to 5% of the chlorogenic acid were recovered as compared with levels of these compounds found in fresh tissue. Slightly more chlorogenic acid (8%) and a significantly greater amount of rutin (75%) were obtained from tissue that was prefrozen and held in dry ice for 1 to 2 hours prior to storage at −15° C. Since the improved recovery of rutin appeared to be inconsistent, this portion of the experiment was repeated using the half-leaf technique described above. Rutin values were low in all but the first experiment, because the plants for subsequent experiments were grown under glass. Results of the second experiment (Table II) agree

Table I. Effects of Storage Method on Levels of Chlorogenic Acid and Rutin in Tobacco Lamina

Treatment	Chlorogenic Acid (CA)[a]		Rutin (R)[a]		Ratio (CA/R)
	μg./g. fresh lamina	Recovery, % of control	μg./g. fresh lamina	Recovery, % of control	
Fresh (control)	2054		627		3.2
Oven-dried at 60° C.	92	4.5	159	25.3	0.6
Lyophilized					
Without prefreeze	1865	90.8	659	105.0	2.8
After liquid N	2072	100.9	690	110.0	3.0
After dry ice	1879	91.5	625	99.6	3.0
Stored at −15° C.					
Without prefreeze	36	1.7	178	28.3	0.2
After liquid N	43	2.1	170	27.1	0.3
After dry ice	158	7.7	443	70.6	0.4
LSD 0.05	248		140		
LSD 0.01	331		187		

[a] Values are means of 7 replicates.

Table II. Recovery[a] of Chlorogenic Acid and Rutin after Freezing in Dry Ice Followed by Storage for 3 Weeks at −15° C.
(μg./g. fresh wt.)

	Chlorogenic Acid	Rutin
Fresh (control)	836	80
Dry ice and storage at −15° C.	119[b]	42[c]
Recovery, %	14	53

[a] Values are means of 4 replicates.
[b] Difference from control significant at 1% level.
[c] Difference from control significant at 5% level.

Table IV. Recovery[a] of Chlorogenic Acid and Rutin after Storage for 3 Weeks at −85° C.
(μg./g. fresh wt.)

	Chlorogenic Acid	Rutin
Fresh (control)	1298	51
Stored at −85° C.	968[b]	54[c]
Recovery, %	74	106

[a] Values are means of 6 replicates.
[b] Difference from control significant at 1% level.
[c] No significant difference from control.

with those previously obtained. Slightly more than half the amount of rutin found in fresh tissue was recovered, although treated tissue contained only 14% as much chlorogenic acid as did fresh lamina. We do not have an explanation for the apparent improved recovery of rutin in comparison with other freezer storage methods.

The data shown in Table I indicate that lamina dried in an oven at 60° C. lost large amounts of chlorogenic acid and rutin. Since polyphenol oxidase in tobacco has been shown to be less stable at 70° C. than at 60° C. (Sheen, 1968), it was of interest to see if raising the oven temperature to 70° C. would decrease enzymatic degradation and provide a stored sample more comparable to fresh tissue. Temperatures exceeding 70° C. were not desirable because a number of other compounds are lost either through breakdown or because of volatility at such temperatures. The results obtained from drying half-leaf samples at 70° C. are presented in Table III. These samples were analyzed within 3 days after drying. Although recovery of chlorogenic acid (41%) and rutin (56%) was improved over those of the stored sample that was oven-dried at 60° C. (4 and 25%, respectively), losses of the desired constituents were still large enough to indicate that oven-drying of tobacco leaves at either temperature preparatory to storage is undesirable.

Samples stored in a freezer at −15° C. incurred large losses of both principal polyphenols (Table I). The apparent explanation is that the tobacco leaves were not completely frozen and chemical changes continued to occur. Leaves stored at −15° C. differ markedly in appearance from those stored at −85° C. in an ultra-low-temperature freezer. The former had soft, spongy areas scattered over the surface of the lamina. In contrast, tobacco leaves stored at −85° C. were uniformly green in color and completely brittle to the touch. To investigate the possibility that the content of chlorogenic acid and rutin in tobacco tissue stored at an ultra-low temperature would approximate that of fresh tissue, a comparison of half leaves under these two conditions was made (Table IV). While there was approximately a 30% decrease in the chlorogenic acid content of the stored tissue, no significant difference was found in rutin content between the two treatments. On the basis of this relatively small loss in only one of the two compounds of interest, together with the fact that there were no qualitative differences in chromatogram patterns of fresh tissue and that stored at −85° C., maintaining green-harvested leaf samples for short periods at ultra-low temperatures seems to be a satisfactory method of tissue storage.

In each of the four treatments (Table I) in which decreases in the levels of the two polyphenols occurred, chlorogenic acid content was reduced in much greater amounts than was content of rutin. Whereas in fresh and lyophilized tissue, the ratio of chlorogenic acid to rutin on a microgram per gram fresh weight basis was approximately 3 to 1, this ratio fell to values ranging from 0.6:1 to 0.2:1 for other treatments tested. This finding differs from an earlier report that chlorogenic acid content remains relatively constant under conditions resulting in the decomposition of rutin in tobacco leaves during flue-curing (Penn and Weybrew, 1958). Our results indicate that rutin may be the more stable of the two compounds in green-harvested tobacco leaves.

The data obtained in these experiments underscore the need for critical selection of a method of storage for tissue samples when it is not feasible to use fresh material. Not only were losses in total amounts of chlorogenic acid and rutin found in several of our treatments, but also large changes in the relative proportions of these two polyphenols occurred during oven-drying and storage of samples at −15° C. Lyophilization is a reliable method of processing tissue for storage and is recommended where such equipment is available. If lyophilization is not possible, storage at ultra-low temperatures may be advantageous. When samples must be brought from field plots to the laboratory, it may be advisable to quick-freeze the tissue immediately and maintain it in dry ice until processing, lyophilization, or transfer to an ultra-low-temperature freezer.

Table III. Recovery[a] of Chlorogenic Acid and Rutin after Oven-Drying at 70° C.
(μg./g. fresh wt.)

	Chlorogenic Acid	Rutin
Fresh (control)	1894	182
Oven-dried	779[b]	101[b]
Recovery, %	41	56

[a] Values are means of 5 replicates.
[b] Difference from control significant at 1% level.

Polyphenols as a group are reactive chemical substances. The degradation products of polyphenols will react with a number of other constituents in plant tissue —for example, protein (Loomis and Battaile, 1966)— thereby altering the original condition of the sample with regard to other compounds of interest. In addition, it is probable that conditions causing breakdown of polyphenols result in breakdown of other constituents. Our study has pointed to the need for careful selection of storage methods of fresh tobacco tissue destined for chemical analysis.

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