Some Factors affecting the Extraction of Nitrogenous Materials from Leaves of Various Species

By E. M. CROOK* and M. HOLDEN, *Rothamsted Experimental Station, Harpenden, Herts*

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On the basis of experiments with tobacco, Crook (1946) has worked out a technique for the extraction of nitrogenous materials from leaves. Its usefulness has now been tested on leaves from other plants, although no attempt has been made to apply the technique to a comprehensive selection of plants. Those used had been brought to the laboratory for other studies or were conveniently available from local sources. At the same time it has seemed desirable to review and analyze the data which have accumulated over the last 5 years on the effect of certain factors such as the water and nitrogen contents of the leaves on the extraction of nitrogenous materials from them.

The literature relating to nitrogenous materials of leaves and methods of extracting them has been reviewed by Vickery (1945) and in the previous paper (Crook, 1946). More recently Wildman & Bonner (1947) have described some of the properties of proteins extracted from green leaves by a method differing somewhat from that used here.

EXPERIMENTAL

Leaves. Many species were available from the glasshouses. Fig and all cucurbits except marrow, cucumber and bryony were from the University Botanic Garden, Cambridge. The remainder were obtained from plants growing wild or in gardens in the vicinity of Rothamsted. The source is indicated for each species in the tables. No attempt was thus made to control the manurial treatment, age, etc., of the plants, but for certain experiments, advantage was taken of leaf material from fertilizer experiments with tobacco, potato and wheat in which combinations of N, P and K had been applied.

Extraction. The general procedure was that outlined by Crook (1946). The leaves were minced in a domestic meat mincer, squeezed through madapollam, re-minced and washed. In certain instances, e.g. strawberry and artichoke, very little or no sap was obtained after mincing. The mince was then washed three or four times with two or three times its weight of water. The washed fibres were then ground in the triple roller mill previously described (Bawden & Pirie, 1944) and again extracted with water. Neutralization of fibre to pH 8·0 with 0·2 N-NaOH was, in most instances, carried out before milling, but it was sometimes convenient to neutralize after milling. N and dry matter extracted during neutralization were included with the mill extract. The leaf fibre of most cucurbits and certain other species became alkaline on washing (Holden, 1948) and did not require neutralization. Results for these have been listed separately.

Analyses. Dry-matter determinations were made on portions of extracts by drying overnight at 95–100°. N was determined by a micro-Kjeldahl procedure (Crook, 1946).

* Now at University College, London.

RESULTS

Extraction of nitrogen and dry matter from various species. In Table 1 are summarized the data on the extraction of nitrogen and dry matter from 28 species distributed among 12 families. The disproportionate number of cucurbits (eight species) was included because these plants had been collected to study the 'alkaline drift' phenomenon (Holden, 1948). Species showing this effect are collected together in § (*b*) of the table, § (*a*) being for leaves whose fibre requires addition of sodium hydroxide to bring the pH to 8·0.

As was to be expected, the efficiency of the extraction process varies considerably for the different species. Extraction of nitrogen ranges from 93½ % with tobacco to 41½ % with nettle, and of dry matter from 78 % (bryony) to 38 % (horse tail). The differences are much more pronounced in mill than in mincer extracts. No consistent differences are noticeable between the two groups of plants. Indeed, § (*b*) of the table contains the two plants showing greatest and least extraction of nitrogen after milling (bryony and calabash). In general, more nitrogen and dry matter are found in mincer than in mill extracts. Strawberry, laurel, comfrey and bryony are the only species for which this is not true of nitrogen. The last two of these are also exceptional in having more dry matter in their mill extracts than is obtained after mincing.

The ratio of nitrogen in the soluble fraction to that in the material sedimentable from any extract at 3000 r.p.m. may vary widely, for the latter may form a small proportion of the extract as in fig (8½ %) or may constitute the major fraction (57 %) as in chrysanthemum. However, as there were no obvious regularities in the ratio and as figures for sedimentable material would have complicated the tables unduly, only total extractions have been shown.

The amount of sodium hydroxide required to neutralize the fibre of leaves quoted in § (*a*) of Table 1 varies considerably, and on this basis the plants can

Table 1. *Extraction of nitrogen and dry matter from leaves of various species*

Family	Latin name	Common name	Source*	Dry matter (% of wet wt.)	N (% of dry matter)	N (% of total)			Dry matter (% of total)		
						In extracts		In residue	In extracts		In residue
						Mince	Mill		Mince	Mill	
		(*a*) Plants whose fibres require neutralization									
Compositae	*Senecio vulgaris*	Groundsel	*W*	8·2	4·68	56	36	8	45	26½	28½
	Chrysanthemum hortorum	Chrysanthemum	*W*	18·6	2·06	31	31	38	41	20	39
Cruciferae	*Brassica oleracea*	Cabbage	*W*	9·7	4·47	55	30½	14½	52½	13½	34
Rosaceae	*Fragaria vesca*, var. Mayo	Strawberry	*W*	33·0	2·84	33	38	29	33	31	36
	var. Royal Sovereign	Strawberry	*W*	36·1	1·57	39½	—	(60½)	43½	—	(56½)
	Prunus laurocerasus	Laurel	*W*	28·8	1·75	21	31	48	30	16½	53½
Cucurbitaceae	*Ecballium elaterium*	Squirting cucumber	*C*	11·8	4·52	47½	42½	10	45½	25	29½
Leguminosae	*Phaseolus vulgaris*	Bean	*G*	14·1	2·80	54	19	27	49½	10	40½
Equisetaceae	*Equisetum* sp.	Horsetail	*W*	17·1	3·23	37	22	41	25	13	62
Gramineae	*Triticum vulgare*	Wheat	*W*	11·6	4·46	67	25	8	47½	17	35½
	Dactylis glomerata	Cocksfoot	*W*	27·0	2·49	35	34	31	28	15	57
Solanaceae	*Physalis alkekengi*	Winter cherry	*G*	23·7	4·03	47	17	36	30	17	53
	Nicotiana glutinosa	—	*G*	9·8	2·76	58	24	18	61½	12½	26
	Nicotiana tabacum	Tobacco	*G*	9·5	4·35	51	42½	6½	50	24	26
	Datura stramonium	Thorn apple	*G*	13·4	4·66	55	38	7	52	27½	20½
	Lycopersicum esculentum	Tomato	*W*	13·5	3·26	61	22	17	58	15½	26½
Buxaceae	*Buxus sempervirens*	Box	*W*	52·1	3·29	45	38	17	31	21	48
Moraceae	*Ficus carica*	Fig	*C*	20·2	4·52	50	37½	12½	38	19	43
		(*b*) Self-neutralizers									
Compositae	*Helianthus annuus*	Sunflower	*W*	17·6	3·24	46	10	44	36	19	45
	H. tuberosus	Artichoke	*W*	18·9	2·49	35	9½	55½	45	7½	47½
Boraginaceae	*Symphytum officinale*	Comfrey	*W*	13·2	4·14	24	28½	47½	21½	42	36½
Urticaceae	*Urtica dioica*	Nettle	*W*	21·8	3·75	29	12½	58½	22	8½	69½
Cucurbitaceae	*Cucurbita ovifera*	Marrow	*W*	14·7	4·66	53½	21½	25	38½	13	48½
	C. ovifera	Marrow	*W*	13·2	2·95	56½	12½	31	41	5	54
	Cucumis sativus	Cucumber	*W*	9·8	4·38	52½	20½	27	42½	17½	43
	Bryonia dioica	Bryony	*W*	11·1	4·60	36	52½	11½	34	—	—
	B. dioica	Bryony	*W*	11·5	6·21	40½	48½	11	37	41	22
	Lagenaria leucantha	Calabash	*C*	11·4	4·21	51½	3½	45	54	3½	42½
	Thladiantha sp.	—	*C*	13·9	3·17	72	5	23	45	5	50
	Cyclanthera explodens	—	*C*	12·0	4·85	58½	28½	13	45½	16	38½

* Source: *G* = glasshouse, *C* = Cambridge Botanic Garden, *W* = wild and garden.

be divided into two groups. In one are those species, among which is included tobacco, whose fibre takes up moderate amounts of alkali—30–60 ml. 0·2N-NaOH/100 g. In the other are those, such as strawberry and chrysanthemum, which take up very large amounts—200 ml./100 g. and more. The uptake of alkali in the former, which is due chiefly to the demethylation of pectin by pectase (Holden, 1945), proceeds rapidly at first and then more slowly, and is substantially complete in 24 hr. In the latter group, however, the uptake continues at a steady rate for as long as 6 days. Further, there is a simultaneous darkening in colour which does not occur with tobacco-like species and which suggests that oxidation of phenolic substances is concerned.

Relation between nitrogen and water contents of leaves. A review of the early data extending over the period 25 March 1942 to 26 September 1944 on the nitrogen and dry-matter contents of 20 batches of tobacco leaves showed that there was a linear relation between their nitrogen and water contents. In Table 2A are shown the constants for the best straight line through the experimental points calculated by the method of least squares. Application of Fisher's '*t* test' to this data gave a value of $t = 8{\cdot}86$. The '*t* test' measures the probability of a random set of data conforming to a hypothesis *by chance*, i.e. the probability that the data here presented should fall *by chance* on the calculated line with the present degree of exactitude. The value of *t* here obtained indicates that there is less than 1 chance in 10^6 that this is a random result. Subsequent data for tobacco from three manurial experiments (February and March 1946) treated with all combinations of N, P and K did not give results of such high significance, *t* being reduced from 8·86 to 2·62. No explanation for this difference can be given, but it should be noted that, throughout the early experiments, manurial treatment had been 'normal', whereas the later batches included many plants deficient in N or P. This did not appear to affect the other correlations noted below. Twenty batches of potato leaves from manurial experiments showed no relation between the nitrogen and water contents. However, when all species were considered together, a significant relation was again obtained, *t* being such that the probability of a chance result of this type was less than 0·001.

Effect of water and nitrogen content of the leaves on the extraction. Early experiments with tobacco suggested that more nitrogen was extracted from the wetter leaves. This was subsequently confirmed and was also found to hold for all species (Table 2B).

The factor of most significance for the extraction of nitrogenous materials was the initial nitrogen content of the leaves. No difference was detectable between the earlier and more recent data for tobacco, and the high positive correlation between nitrogen content and nitrogen extraction also held for all species together. The correlation was as good for extraction by mincing alone as for those by the whole procedure.

It has been found most convenient to express both extracted nitrogen and total nitrogen as a percentage of the total dry matter of the leaf. On this basis, Fig. 1 has been plotted from the combined data for the tobacco experiments referred to above and is included as an indication of the exactness of these relations. The lines shown are those calculated by the method of least squares.

Table 2. *Constants of the regression lines of the form $y = a + bx$ expressing the relations between extraction of nitrogenous materials from leaves and their nitrogen and water contents*

Material or fraction	No. of exp.	*a*	*b*		*t**
			Value	Standard error	
A. y = total N (as % dry matter) x = g. water/g. dry matter					
Early data for tobacco	20	1·39	0·16	±0·02	8·86
Later data for tobacco	24	−1·07	0·57	±0·22	2·62
Data for all species	31	2·33	0·24	±0·06	3·92
B. y = extractable N (as % dry matter) x = g. water/g. dry matter					
Tobacco—total extraction	24	−1·78	0·60	±0·20	2·94
All species—total extraction	30	1·20	0·28	±0·07	3·93
C. y = extractable N (as % dry matter) x = total N (as % dry matter)					
Tobacco: Mincer extraction	24	−0·28	0·69	±0·04	16·1
Total extraction	35	−0·34	0·95	±0·04	21·7
All species: Mincer extraction	22	−0·45	0·62	±0·08	7·6
Total extraction	31	−1·14	1·06	±0·10	10·1

* Calculated by Fisher's '*t* test'. From this value and the number of experiments can be obtained the probability of the experimental points falling on the calculated straight line by chance. For the number of experiments shown here, values of *t* greater than 2·5 indicate odds of more than 50 to 1 against it being a chance result, and for *t* greater than 3·5 the odds are more than 1000 to 1 against.

In Table 2C are collected the expressions relating extractable to total nitrogen, both for the tobacco data included in Fig. 1 and for all species listed in Table 1 together. As can be seen, all are highly significant, the values of t being such that the probability of obtaining any one by chance is much less than 1 in 10^6.

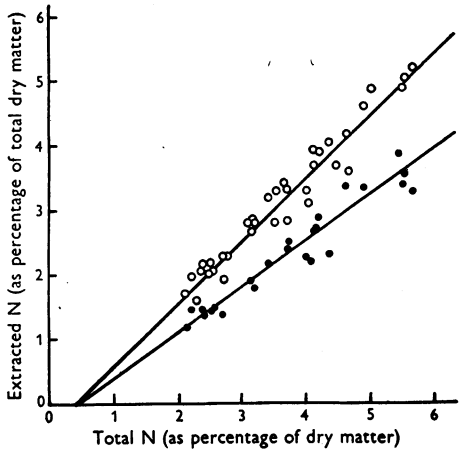

Fig. 1. The relationship between total and extractable nitrogen in tobacco leaves. ●——● extraction after mincing; ○——○ extraction after mincing and milling.

DISCUSSION

There are interesting differences in the ease of handling of various species of plants and in the character of the products. Few regularities of behaviour are observed among related families or even among the members of a single family. In general the wetter and less fibrous leaves are more easily worked although there are important exceptions, e.g. fig and bryony. We have also encountered certain unexplained differences within a single species, e.g. *Ecballium* is given among those plants requiring neutralization. Later samples of this species, however, showed an alkaline drift like other members of the same family.

As a rule, milling is not difficult with any species, provided the fibre is brought to a suitable moisture content. The leaves of some trees are exceptions to this, e.g. those of beech and horse chestnut are not only difficult to mince and give little or no sap on squeezing, but the fibre is so tough and stringy that it will not pass through the triple roller mill. Other plants, such as *Tilia*, contain substances that render their manipulation difficult. This species gives no sap, and, after the addition of water, becomes so slimy and viscous that it is impossible to squeeze extract through the cloths used.

The mincer extracts of several species, e.g. beech, dock and strawberry (var. Royal Sovereign), appear to be free from protein, i.e. give no precipitate with trichloroacetic acid. Bawden & Kleczkowski (1945) have observed the same phenomenon with this variety of strawberry and ascribed it to precipitation of protein by the large amounts of tannins in the extract. The difference in behaviour of even closely related plants is illustrated by a comparison of the two strawberry varieties. Although the amount of soluble nitrogen in mincer extracts of each is very similar, 53% is precipitable from Mayo extracts with trichloroacetic acid.

From the slope and position of the regression lines of Fig. 1 and Table 2C, several conclusions can be drawn. It can be calculated that the average total extraction of nitrogen was 86% from tobacco and 75% from all species. The figure for tobacco is lower than that reported by Crook (1946), but this was based on a procedure involving milling the fibre two or three times. In addition, the average nitrogen content of the leaves on that occasion was rather higher. Both these factors would tend to increase the extraction.

These regressions also make it possible to predict approximately, on the basis of the nitrogen content of the original leaf, the extraction likely to be reached, not only for tobacco, but for any species listed in Table 1. Thus, it can be calculated that, on the average, 78% of the nitrogen can be extracted from a tobacco leaf, 2% of whose dry matter is nitrogen, whereas the extraction rises to 90% for leaves with 6% nitrogen. The differences are greater when the data for all species are considered, the increase being from 48% extraction with leaves containing 2% nitrogen to 86% with those containing 6% nitrogen.

The lines are similar in slope and position both for the extensive data for a single species (tobacco) and for a random sample of plants of various species taken without regard to age, season, manurial treatment or botanical relationship. This perhaps suggests that the observed values are universally applicable and that, if separate regression lines were available for each species, they would all be very similar to that of tobacco.

When extrapolated back, the lines of Table 2C do not pass through the origin, but through points on the total nitrogen axis. This can be regarded as implying that, provided the equation still holds, leaves of this small nitrogen content would show zero extraction, or, what amounts to the same thing, it can be regarded as an illustration of the experimental fact that the last traces of nitrogen are extremely difficult to remove from fibre. As the slopes of the lines for total extraction do not differ significantly from unity, all the nitrogen over and above this limiting quantity is being extracted whatever the initial nitrogen content of the leaf. If the residue could be regarded as 'structural' and unaltered by

grinding and extraction, the picture would be simple —all nitrogen other than this would be extracted. However, the work of Bawden & Pirie (1944) and Crook (1946) suggests that the unextractable residue represents a cross-section of the nitrogenous material present in the cells fixed there by the extraction process itself. The plant cell contains a complex mixture of proteins of varying solubility and in various states of dispersion. These might be expected to be extracted at different rates and to different degrees of completeness. It is surprising therefore that extraction from all species of plants should proceed in such a manner that the residue is representative of all proteins and yet at the same time, the degree of extraction is so accurately expressed by a linear regression.

SUMMARY

1. The extraction of nitrogenous materials from the leaves of various plant species has been investigated by a procedure previously worked out.

2. The extraction varied from 41½ to 93½ % of the total leaf nitrogen, the average for all species being 75%.

3. The extraction was mainly influenced by the nitrogen content of the leaves, increasing with increasing nitrogen content.

4. The extraction was also influenced by the dry-matter content, as the wetter leaves have a higher nitrogen content.

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A Manometric Method for the Estimation of Milligram Quantities of Uronic Acids

By M. V. TRACEY, *Rothamsted Experimental Station, Harpenden, Herts*

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The most widely applicable method for the estimation of uronic acids is that based on the demonstration by Mann (1894) and Mann & Tollens (1896), that uronic acids are decomposed by heating with 12% (w/w) HCl giving a 25% yield of CO_2 (calculated to the anhydride). The method was further explored by Lefèvre (1907) and Lefèvre & Tollens (1907).

In the latter method, the sample was boiled with 12% HCl under reflux for 3·5 hr., and a stream of CO_2-free air passed through the apparatus. After washing and drying, the CO_2 carried from the flask was absorbed in a tube containing KOH. The CO_2 evolved was estimated by the increase in weight of the absorption tube. In subsequent years, many modifications of the original method were introduced, mainly concerned with alterations in the time of decomposition sometimes extended to 5 hr. and even to 8–10 hr. (Ehrlich & Schubert, 1929), and in the methods employed for washing, trapping and estimation of the CO_2. Barium hydroxide became a favoured absorption agent, and titrimetric methods were substituted for gravimetric (cf., for example, Dickson, Otterson & Link, 1930). More recently, attention has been directed to the use of stronger acid to shorten the time needed for decomposition (Colin & Lemoyne, 1938*b*, 18% HCl; McCready, Swenson & Maclay, 1946, 19% HCl), and to measurement of rates of CO_2 evolution in order to estimate traces of uronic acid derivatives in the presence of polysaccharides (Norman, 1939; Whistler, Martin & Harris, 1940).

The most radical departure in the determination of uronic acids by means of the CO_2 evolved on decomposition is that of Voss & Pfirschke (1937), who used an approximately 20 M-solution of $ZnCl_2$ at its b.p. of 146–147° instead of HCl. All these methods have in common the disadvantage of a complex apparatus and a long decomposition time, usually 5–8 hr.

The method to be described is based on the decomposition of not more than 50 mg. of the sample with 0·25 ml. of 12% HCl in a sealed tube at 111°. The CO_2 evolved is then measured by a modification of the method of Van Slyke & Folch (1940) for manometric carbon determination. The present method has the advantage that samples do not need attention during decomposition, and that, as a consequence, determinations may be made at the rate of 4/hr. for long periods.