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INFLUENCE OF THE EXTRACTION CONDITIONS ON THE RECOVERY OF FREE AMINO ACIDS IN PLANT MATERIAL

J. A. M. SCHRAUWEN and H. F. LINSKENS

Botanisch Laboratorium, Universiteit, Nijmegen

SUMMARY

From comparative investigations of five pretreatment-extraction conditions, using the composition of the pool of free amino acids as a parameter of disintegration during the extraction procedure, it was found that the optimal conditions are: disintegration of the fresh plant material in liquid nitrogen (–180 °C), lyophilization and homogenization of the dried powder in the extraction medium at 0 °C.

1. INTRODUCTION

Extraction of chemical compounds from plant material is the general way to get access to compounds which can then be qualitatively and quantitatively analyzed. Both the pre-extraction and extraction conditions are therefore crucial for the reliability of biochemical analyses of biological material.

The general procedures of disintegration and preparation of plant extracts have been treated by PAECH (1956) and PIRIE (1956). A comparative study of the drying conditions and the methods of extraction on samples of grass and clover was published by BARTHURST & ALLISON (1949). They concluded that extraction as well as procedure have a marked effect on the results of chemical analysis of the extracts, using nitrogen, ascorbic acid, saccharides, carotene, and peroxidase content as parameters for testing the efficiency of the various methods.

Amino acids too are an interesting parameter, because they are a good indicator of plasmatic integrity and can be extracted fairly easily (LINSKENS 1959). This paper deals with tissue preparation prior to extraction of free amino acids from full grown leaves of *Petunia* and stringbeans. The pool of free amino acids can give insight into the amount of hydrolysis of the peptide and protein fraction.

2. MATERIALS AND METHODS

Leaves from genetically homogeneous *Petunia hybrida* plants (clone W 166K with the self-incompatible alleles S_1S_2) were collected, immediately before the extraction, from cultures grown in the greenhouse with additional artificial light (HPL Philips). Fully expanded primary leaves from stringbeans (*Phaseolus vulgaris* cv. Widusa) were collected in the same way.

The freshly harvested leaves were dried superficially between filter paper and their fresh weight estimated.

The leaves were prehandled in five ways:

1. The leaves (about 200 mg) were cut into small pieces and homogenized in extraction medium (LINSKENS 1959) at 0 °C. An aliquot of the total extract was used for one analysis.
2. The intact leaves were placed in a vessel and dried in a lyophilization apparatus for 24 hours. About 25 mg were homogenized in extraction medium at 0 °C for one analysis.
3. Liquid nitrogen was poured over the intact leaves (about 200 mg) in a glass beaker and the brittle material pulverized. The powder, still frozen, was homogenized in extraction medium at 0 °C. A part of the total extract was used for one analysis.
4. Intact leaves were cooled with liquid nitrogen and pulverized in a glass beaker. The powder was lyophilized to complete dryness and 25 mg were weighed out and homogenized in extraction medium.
5. Intact leaves were placed in an oven at 105 °C and dried for 4 hours. About 25 mg were weighed out and homogenized in extraction medium at 0 °C.

Amino acids determinations were done as described earlier (LINSKENS & TUPÝ 1966; LINSKENS & SCHRAUWEN 1969).

3. RESULTS

Tables 1 and *2* show the effects of the different methods of treatment prior to extraction on the apparent concentration of the various amino acids in the extracts.

The total content of the free amino acids is almost the same for methods 1, 3 and 4, both with *Petunia* and bean leaves. Method 4 shows high values for the easily hydrolysable amino acids Asp, Glu, the amides $AspNH_2$, $GluNH_2$ and low contents of $EtNH_2$, GABA and NH_3. Nevertheless, the amounts of these particular compounds are not the same for both plant tissues, especially when comparing methods 1, 3 and 4.

Methods 2 and 5 resulted in a completely different amino acid distribution. The total content as well as the amino acid composition differed sharply from methods 1, 3 and 4. The greatest difference occurred with method 2. The amino acids GABA and Ala increased by 10–20 fold (*fig. 1*), while Pro, Ileu, Leu, Val and $EtNH_2$ also had higher values. This resulted also in a relatively high total amino acid content.

Method 5 gave the lowest amino acid content, especially for *Petunia*, the difference between methods 1, 3 and 4 is very large (*table 1*). Glu disappeared almost completely and the decrease in amounts of Asp, the amides + Thr + Ser is again large (*fig. 1*), while the increase in quantities of GABA, $EtNH_2$, Pro, Ileu, Leu and Val is not as high as obtained in method 2.

The variation from the mean value, for individual amino acids as well as for the total content, is lower in method 4 than in any other method, but generally below 5%.

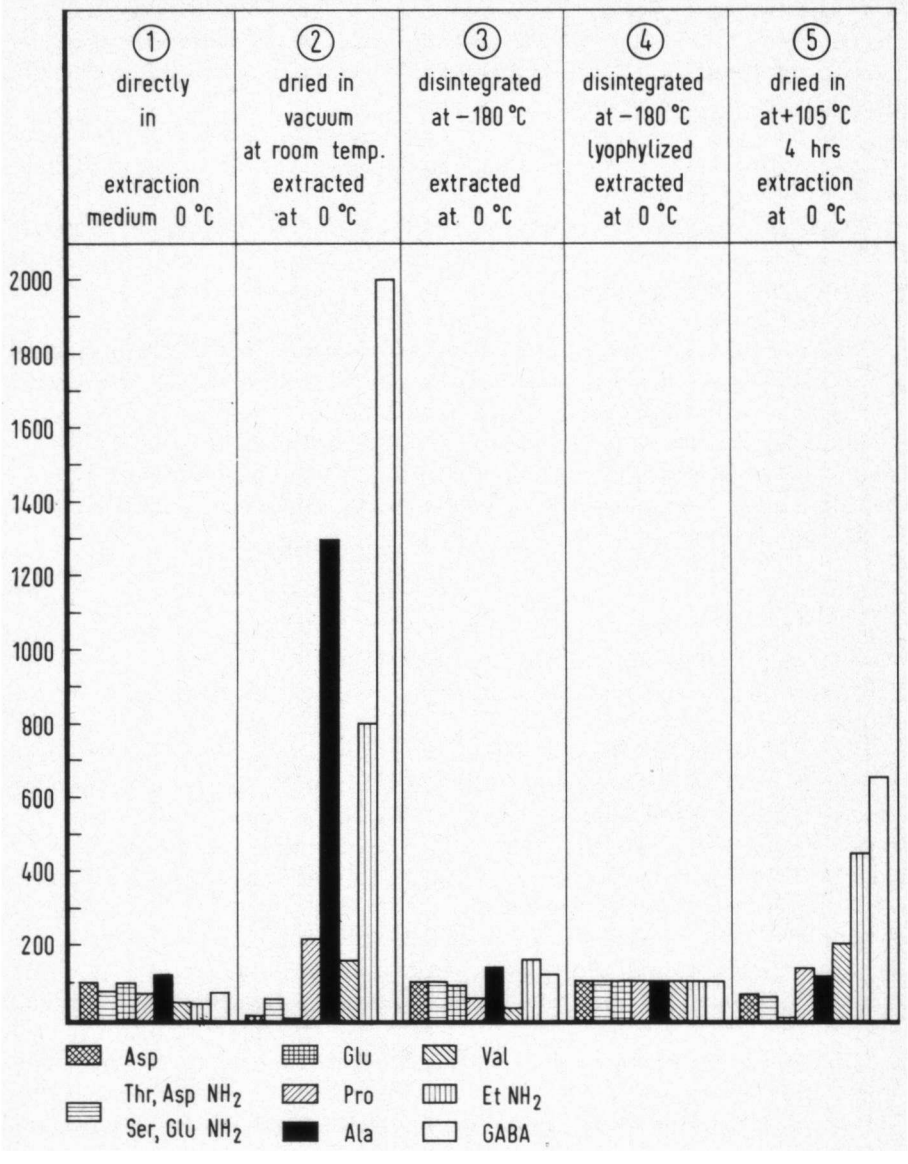

Fig. 1. The relative amino acids content of *Petunia* leaf extracts (method 4 = 100) after different treatments.

4. DISCUSSION

One of the main problems in extracting plant material is to isolate the compounds in the same proportion and composition as they are in situ (SYNGE 1968). The results presented in *tables 1* and *2* show that even common methods

Table 1. Free amino acid content of *Petunia* leaf extracts in nmol/mg dry weight after different treatments. (1) Directly in extraction medium 0 °C
(2) Dried in vacuum at room temperature, extracted at 0 °C
(3) Disintegrated at –180 °C, directly in extraction medium at 0 °C
(4) Disintegrated at –180 °C, lyophilized, extraction at 0 °C
(5) Dried at + 105 °C 4 hours, extraction at 0 °C

	(1)	(2)	(3)	(4)	(5)
Met SO_4	2.78	0.83	2.63	3.06	0.24
Asp	21.62	1.04	19.47	19.53	12.85
Thr, $AspNH_2$					
Ser, $GluNH_2$	12.50	9.01	15.42	15.82	9.53
Glu	35.35	1.07	31.60	34.77	2.31
Pro	0.35	1.10	0.25	0.50	0.70
Gly	0.82	1.02	1.01	0.86	0.44
Ala	3.97	42.06	4.50	3.25	3.69
Val	0.24	0.89	0.17	0.55	1.09
Met	0.03	0.15	0.02	0.05	0.13
Ileu	0.13	0.34	0.04	0.23	0.36
Leu	0.23	0.81	0.17	0.44	0.81
Tyr	0.01	0.28	+	0.10	0.18
Phe	0.20	0.46	0.15	0.29	0.32
$EtNH_2$	0.24	4.24	0.85	0.54	2.46
GABA	1.41	39.20	2.42	1.96	13.41
NH_3	11.88	26.50	12.75	6.16	5.70
Orn	0.27	0.30	0.21	0.48	0.18
Lys	0.23	0.71	0.13	0.41	0.71
Try	+	0.01		0.01	0.02
His	0.06	0.12	+	0.13	0.09
Arg	+	0.10	+	0.03	0.33
Cyst	+	0.08	0.04	0.09	
39?	1.02		1.37	1.05	
Pen		0.5?			
AABA + $GluNH_2$					0.72
Galact					6.27
total -NH_3	81.46	104.33	80.45	84.15	56.84

of preparation and extraction have a tremendous influence on the distribution of free amino acids isolated from leaves.

In method 4 the fresh leaves were kept frozen until completely dry and the dried material was extracted at 0 °C. The reproducible results obtained by this method suggest that neither chemical nor enzymatic reactions occur during the extraction procedure.

This view is strengthened by the low content of GABA, Ala, and $EtNH_2$, especially for bean leaves. The sums of Asp + Ala; Glu + GABA; the amides + Thre + Ser + Gly + NH_3 are almost constant for methods 1, 3 and 4 (*table 3*). This suggests that the increase of GABA, Ala and $EtNH_2$ are artefacts due to the decarboxylation of Asp, Glu, and Ser, respectively.

In methods 1 and 3 the plant material is still wet at the moment it contacts the extraction liquid and non-specific reactions probably cause the changes in

Table 2. Free amino acids of *Phaseolus* leaf extracts in nmol/mg dry weight after different treatments. Treatments as in *table 1*.

	(1)	(2)	(3)	(4)	(5)
Met SO_4	0.04	0.12	0.02	0.04	0.28
Asp	8.36	5.40	10.46	11.38	3.00
Thr, $AspNH_2$					
Ser, $GluNH_2$	9.33	8.40	11.15	11.67	7.45
Glu	11.36	5.00	13.87	16.98	0.18
Pro	0.62	2.10	0.66	0.61	0.93
Gly	0.93	0.80	0.64	0.27	0.33
Ala	6.00	22.20	3.85	3.24	6.79
Val	0.33	1.70	0.33	0.24	1.00
Met	0.01	0.49	—	0.03	0.04
Ileu	0.18	1.00	0.12	0.12	0.34
Leu	0.32	2.36	0.31	0.24	1.24
Tyr	0.21	0.80	0.12	0.13	0.34
Phe	0.63	1.40	0.65	0.63	0.79
$EtNH_2$	2.37	7.30	1.80	0.65	0.93
GABA	6.00	23.50	3.47	0.75	16.27
NH_3	4.77	10.80	4.42	4.56	6.95
Orn	0.35	0.27	0.28	0.25	0.25
Lys	0.37	2.00	0.33	0.40	0.52
Try	0.09	0.06	0.05	0.05	—
His	0.30	0.60	0.27	0.50	0.13
Arg	0.10	0.30	0.08	0.20	0.40
$GalactNH_2$					2.84
Total – NH_3	47.90	85.80	48.46	48.38	44.05

the content of Ala, Val, $EtNH_2$ and GABA (*tables 1* and *2*). The non-specificity of the reactions is borne out by the fact that the behaviour of both plants is not the same for methods 1, 3 and 4. The total amount of amino acids in leaves of either plant does not change significantly whether methods 1, 3, or 4 are used.

The results suggest that the decomposition of Glu, Asp, amides and Ser leads to the formation of GABA, $EtNH_2$, Ala, and Gly (FOWDEN 1967). The main reaction seems to be decarboxylation.

The situation is completely different with methods 2 and 5. The data obtained with method 2 show a large increase in the overall content of free amino acids (*tables 1* and *2*), caused by an increase in the sums of Asp + Ala; Glu + GABA; Thr + Ser + $GluNH_2$ + $AspNH_2$ + Gly + NH_3 (*table 3*) and an increase in the amino acids Pro, Val, Met, Ileu, Leu, Try, Lys in comparison with methods 1, 3 and 4. The content of almost all amino acids is increased, suggesting a breakdown of peptides by proteolytic enzymes.

The results of method 2 show that the use of this extraction procedure should be discouraged. Decomposition of the amino acids Asp, Glu, and the amides cause a 40% loss in total free amino acid content for *Petunia* in method 5 (*table 1*). The amino acids can be broken down either chemically or enzymati-

Table 3. Free amino acids combined in groups from leaf extracts after different preextraction conditions. Treatments as in *tables 1* and *2*. Amount in nmol/mg dry weight.

	(1)	(2)	(3)	(4)	(5)
Petunia					
Asp + Ala	25.60	43.10	23.97	22.78	16.54
Glu + GABA	36.76	40.27	34.02	36.73	15.73
Thr + Ser + Gly + amides + $EtNH_2$ + NH_3	25.44	40.77	30.03	23.38	18.13
Stringbean					
Asp + Ala	14.36	27.60	14.31	14.72	9.79
Glu + GABA	17.36	28.50	17.34	17.73	16.45
Thr + Ser + Gly + amides + $EtNH_2$ + NH_3	17.40	27.30	18.01	17.15	15.66

cally (KRETOVICH 1965; FOWDEN 1967). The contribution of the chemical decomposition must largely result in the decrease in the total amount of the amino acids. The time between plasmolysis and complete loss of enzyme activity must be very short, due to the rapid temperature change above enzyme activity level. So it is more likely that a chemical decomposition occurs in method 5.

Our conclusion is therefore that method 4 is the best for extracting free amino acids from leaves because of the low content of non-protein amino acids in comparison with the other methods, and the results are more reproducible, too.

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