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Amino acid analysis by high-performance liquid chromatography with methanesulfonic acid hydrolysis and 9-fluorenylmethylchloroformate derivatization

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ABSTRACT

Experiments were undertaken to verify a method for complete amino acid analysis of plant and animal tissues and waste products from a single hydrolysis and high-performance liquid chromatographic run. Using methanesulfonic acid, hydrolysis of cytochrome *c* at 115°C for 22 h yielded recoveries equal to or higher than hydrolysis at 115°C for 70 h or at 150°C for 22 h. Triple evacuation of the hydrolysis tube alternated with nitrogen flush gave recovery improvements over single evacuation. Refrigerated storage of samples under vacuum for up to 4 days between hydrolysis and further analysis was not different from immediate analysis. However, recoveries of several amino acids were reduced by refrigerated storage in air. Recoveries of individual amino acids were determined by hydrolysis of biological samples with and without added cytochrome *c*. Although recoveries from biological samples were lower for several amino acids, precision was sufficient to allow quantitation after correction for incomplete recoveries.

Derivatization with 9-fluorenylmethylchloroformate (FMOC) was chosen because derivatives are formed with both primary and secondary amino acids, derivatives are quite stable, and detection may be either UV absorbance or fluorescence. Derivative yield is sensitive to the pH of the reaction mixture. A pH of 8.0 gave reproducible derivative yield for all physiological amino acids. Solvent extraction of excess FMOC, when compared to addition of amantadine to react with excess FMOC, gave both higher recoveries and greater precision. Following derivatization, samples could be kept at 4°C for at least 24 h before high-performance liquid chromatographic analysis without loss of response. Derivative yield and detector response were constant across a wide range of molar ratio of FMOC to total amino acids.

Gradient elution was required to separate FMOC derivatives on a reversed-phase column. The capability of the pumping system to produce exponential gradients permitted rapid and easy fine-tuning of the gradient.

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INTRODUCTION

Research into methods for amino acid determination has been an active area of investigation during recent years. The Biosis data base lists more than 9000 English papers published on this subject during the 1980s. The focus for much of this research has been on precise determination of amino acid composition from limited quantities of relatively pure proteins. The information obtained is often used in conjunction with sequence analysis to elucidate protein structure. Relatively little attention has been given to the very different problems involved in amino acid analysis on mixed tissue samples for ecological or nutritional purposes[1]. Mixed tissue samples contain, in addition to proteins and free amino acids, varying amounts of carbohydrates and lipids which may interfere with hydrolysis and subsequent analysis. These samples are necessarily heterogeneous in nature, making larger sample sizes an advantage. Some decrease in effciency of recovery and precision of determination is tolerable in exchange for speed and ease in sample preparation.

The present set of experiments was undertaken with the purpose of validating a method whereby complete amino acid composition could be determined for plant and animal tissues and waste products using a single hydrolysis and high-performance liquid chromatographic (HPLC) run. Methanesulfonic acid hydrolysis was chosen because of its ability to preserve tryptophan in the presence of moderate amounts of carbohydrate[2,3] and because derivatization may be carried out on the hydrolysis mixture after only pH adjustment. Since the exclusion of oxygen during hydrolysis is important for the complete recovery of several amino acids, the effect of flushing the sample with nitrogen during evacuation, prior to hydrolysis, was studied. The effects of hydrolysis time and temperature and of sample storage on recoveries were determined by hydrolysis and analysis of cytochrome *c*. Recovery efficiency for each amino acid from cytochrome *c* added to biological samples was determined by hydrolysis of biological samples alone and with added cytochrome *c*.

9-Fluorenylmethylchloroformate (FMOC) was chosen as a pre-column derivatization reagent based on the method of Cunico *et al.*[4]. Other derivatization methods were rejected because of considerations such as long analysis time and necessity for a post-column derivatization system (ninhydrin)[5], inability to detect secondary amino acids (*o*-phthalaldehyde)[6], instability of derivatives (dansyl chlorides)[7,8], or the requirement for evaporation prior to HPLC injection (phenylisothiocyanate)[9]. FMOC reacts with amino acids in dilute, basic solution to form UV absorbing and fluorescent derivatives with 88–98% yield[10]. This derivatization avoids the problems mentioned above and gives a choice of detection by fluorescence in the low pmole range or by UV absorbance at higher concentrations. Also, it is less sensitive to interferences from extraneous sample components than the other methods[4,10].

The effects of conditions during and after derivatization were studied to maximize the yield of amino acid derivatives. Optimum pH and reaction time for derivative formation were determined. Comparison was made between the use of amantadine to scavenge excess FMOC following reaction and the use of pentane to extract the alcoholic by-product. The effect on derivative formation of the molar ratio of FMOC to total amino acids and the stability of FMOC derivatives at both 4°C and at room temperature were determined. The mobile phase gradient was optimized to allow complete separation of all essential amino acids.

MATERIALS AND METHODS

Hydrolysis

Samples of black cherry leaves (*Prunus serotina*), fifth instar cecropia larvae (*Hyalophora cecropia*), and feces produced by the larvae when fed black cherry leaves were lyophilized and ground, using a Wiley Mill, to pass a 40-mesh screen. Samples were weighed on a Cahn Model 49 electrobalance to contain 0.2 to 2 mg of protein and introduced below the neck restriction of a reusable hydrolysis tube with PTFE stopper (Pierce)[11]. Hydrolysis reagent was freshly prepared by diluting methanesulfonic acid (Kodak) to 4 *M* with HPLC-grade water and adding approximately 0.2% 3-(2-aminoethyl)indole (Sigma). A 2-ml volume of reagent was added to each sample and the tube was closed to the first stop. Samples were frozen in dry ice–acetone and evacuated to 50 μTorr. The efficacy of nitrogen flush compared with simple evacuation was tested by flushing the sample tube twice with nitrogen and re-evacuating. Samples were hydrolyzed in an oil bath at 115°C (±2°C) for 22 or 70 h and at 150°C for 22 h.

Except when testing for sample deterioration with storage between hydrolysis and derivatization, samples were either derivatized and analyzed immediately or were kept sealed at 4°C. Cytochrome *c* (1.0 mg) and samples with added cytochrome *c* (about 1 mg total protein) were treated in a similar manner. Equine cytochrome *c* used for standardization has no serine residues[12], therefore serine does not appear in the tables for recovery of amino acids.

Hydrolysis results in conversion of glutamine and asparagine to glutamate and aspartate, respectively. The HPLC conditions used did not resolve glutamine from asparagine. Therefore, these four amino acids are grouped as diamino plus diacid (DAA) in the tables.

Derivatization

Standard amino acids, cytochrome *c* and FMOC were purchased from Sigma. c-Allylglycine (Sigma) was added to each hydrolyzed sample as an internal standard. Samples were transferred quantitatively to 15-ml centrifuge tubes, placed in an ice bath and either neutralized with NaOH and buffered with 0.1 *M* $NaHCO_3$, pH 8.0, or buffered with 0.1 *M* $Na_2Br_1O_4$, pH 8.0, and adjusted to pH 8.0 with NaOH. All samples were brought to a final volume of 12 ml. Following centrifugation, 1.0 ml of sample was mixed with 1.0 ml of $6.0 \cdot 10^{-3}$ *M* FMOC in acetone. After completion of the reaction at room temperature, excess FMOC was destroyed by addition of approximately 6 μmol of amantadine or removed by double extraction with 2.0-ml portions of *n*-pentane. The samples were filtered through a 0.45-μm-pore syringe filter. The first few drops were discarded.

Chromatography

The HPLC system (Waters Division of Millipore) consisted of a Model 600 pump with exponential quaternary gradient capability and column heater, a refrigerated WISP autosampler, a 990 photodiode array detector and a NEC APC IV computer with Waters 990+ software. The column was a 150 mm × 4.6 mm I.D. end-capped ODS-80TM Aminotag column supplied by Varian Instruments and was maintained at 30°C.

Solvents used were Baker, HPLC grade. Tetrahydrofuran was distilled under

TABLE I

MOBILE PHASE COMPOSITION

Components were measured separately by volume and mixed without correction to final volume. Buffer composition was $15 \cdot 10^{-3}$ *M* citric acid, $10 \cdot 10^{-3}$ *M* tetramethylammonium chloride, pH adjusted with NaOH.

Mobile phase	*Buffer pH*	*Buffer (%)*	*Aceto-nitrile (%)*	*Tetrahydro-furan (%)*	*Final pH*
A	1.85	73	27	0	3.25
B	4.50	60	35	5	5.30
C	4.50	25	62	13	6.20

nitrogen within 72 h before use. Water was freshly purified by passage through a Continental Modulab, Type 1, HPLC-grade system. All mobile phases were prepared and filtered fresh daily. The mobile phase buffer was $15 \cdot 10^{-3}$ *M* citric acid with $10 \cdot 10^{-3}$ *M* tetramethylammonium chloride, pH adjusted with NaOH. Mobile phase composition and gradient profile are given in Tables I and II, respectively. These are modified from a method developed in the Varian LC Applications Laboratory[13].

Statistical analyses were done using SPSS/PC+ on an IBM/AT computer[14].

RESULTS AND DISCUSSION

Hydrolysis

The Student's *t*-test showed significantly higher mean recoveries from cytochrome *c* hydrolysate for cysteine ($P < 0.01$), methionine ($P < 0.01$) and tyrosine ($P < 0.05$) when the hydrolysis tube was flushed twice with nitrogen during

TABLE II

GRADIENT PROFILE

Composition of buffers A, B and C are given in Table I. Flow-rate, 1.4 ml/min.

Time (min)	*A (%)*	*B (%)*	*C (%)*	*Curve No.*[a]
0	100	0	0	
3	50	50	0	6
17	0	100	0	7
23	0	96	4	7
27	0	85	15	6
31	0	50	50	6
35	0	35	65	6
40	0	0	100	6

[a] Curve No. refers to the exponential gradient designation given in the Waters operating manual for the 600 pump. Curve 6 is a linear gradient, curve 7 is the shallowest of the concave gradients described by the equation: $A = A_s + (A_e - A_s)\,[(t - t_0)/(t - t_1)]^{(n-5)}$, where A = % of A at time t; A_s = % of A at the beginning of the segment; A_e = % of A at the end of the segment; t = elapsed time; t_0 = time at the beginning of the segment; t_1 = time at the end of the segment; n = curve number.

TABLE III

RECOVERY OF AMINO ACIDS FROM HYDROLYZED CYTOCHROME *c* — EFFECT OF NITROGEN FLUSH DURING EVACUATION PRIOR TO HYDROLYSIS

Samples of 1.0 mg cytochrome *c* were hydrolyzed at 115°C for 22 h. Evacuation was to 50 μTorr. For double nitrogen flush, after evacuation, vacuum was turned off, tube was flushed with nitrogen and re-evacuated, then nitrogen flush and evacuation were repeated.

Amino acid	*Evacuation only*		*Double nitrogen flush*	
	Mean recovery (%)	*C.V. (n = 3)*	*Mean recovery (%)*	*C.V. (n = 3)*
Ala	91.8	2.5	91.7	3.1
Arg	90.7	5.8	92.1	4.1
Cys	42.5	5.3	68.4	8.8[b]
DAA[a]	91.2	2.7	92.2	4.5
Gly	96.8	6.1	95.0	5.1
His	89.9	7.5	94.7	5.4
Ile	86.1	2.8	90.2	2.7
Leu	88.0	1.8	90.2	2.7
Lys	88.4	6.8	96.1	3.8
Met	63.1	6.2	92.7	3.1[b]
Phe	88.6	10.3	92.2	4.4
Pro	90.4	4.9	97.8	5.6
Thr	82.6	4.9	85.4	2.4
Trp	78.4	2.6	90.0	6.7
Tyr	73.4	4.3	82.0	6.5[c]
Val	87.6	4.2	89.1	2.5

[a] Sum of Asp, Glu, Asn, Gln.
[b] $P < 0.01$.
[c] $P < 0.05$.

evacuation prior to hydrolysis (Table III). Although individual recoveries of other amino acids were not improved by nitrogen flush, overall recovery was tested for significance of improvement by data transformation. Excluding the three amino acids for which individual recoveries were significantly higher, individual recoveries for nitrogen flush were divided by the corresponding recoveries for evacuation only. The mean of the results was tested against the hypothesis that it was not different from one. It was found to be greater than one by a Student's *t*-test at $P < 0.01$.

Neither longer (115°C for 70 h) nor higher-temperature (150°C for 22 h) hydrolysis yielded any significant improvements for individual or overall recoveries when compared to hydrolysis at 115°C for 22 h (Table IV, one-way ANOVA, Scheffe multiple range test, $P > 0.05$). At 150°C, there was significant loss of cysteine ($P < 0.05$), methionine ($P < 0.05$), threonine ($P < 0.01$) and tyrosine ($P < 0.05$) (one-way ANOVA, Scheffe multiple range test). Since peptide linkages involving branched chain amino acids are particularly difficult to hydrolyze[15], their recovery may be taken as a measure of hydrolysis efficiency. The 115°C, 22 h hydrolysis yielded 86% recovery of isoleucine and 89% recovery of leucine and valine (Table IV).

When replicate hydrolyzed samples ($n = 3$) were derivatized and analyzed immediately, stored at 4°C under vacuum or stored at 4°C after releasing the vacuum

TABLE IV

EFFECT OF HYDROLYSIS CONDITIONS ON RECOVERY OF CYTOCHROME *c*

Samples of 1.0 mg cytochrome *c* were hydrolyzed as indicated.

Amino acid	*22 h, 115°C*		*70 h, 115°C*		*22 h, 150°C*	
	Mean recovery (%)	*C.V. (n = 3)*	*Mean recovery (%)*	*C.V. (n = 3)*	*Mean recovery (%)*	*C.V. (n = 3)*
Ala	96.6	3.0	97.8	7.3	102.3	2.9
Arg	94.2	4.8	100.2	2.9	96.4	3.4
Cys	69.8	3.4	62.2	5.0	53.0	9.4[c]
DAA[a]	94.4	3.0	95.2	4.5	90.3	3.5
Gly	98.3	4.4	99.7	2.6	106.5	8.7
His	92.4	3.1	94.1	5.2	89.2	4.9
Ile	85.5	2.9	86.8	2.9	90.2	3.4
Leu	89.3	2.9	88.6	3.0	91.4	2.8
Lys	93.8	4.3	93.1	4.0	88.1	4.0
Met	90.7	5.6	87.6	10.0	85.7	1.8[c]
Phe	89.7	5.5	88.6	10.4	90.6	5.2
Pro	94.4	3.9	94.5	5.0	95.6	10.5
Thr	84.0	5.5	79.2	5.5	67.5	6.1[b]
Trp	89.5	4.2	85.5	2.3	87.2	3.0
Tyr	84.4	6.1	81.4	5.3	73.2	7.5[c]
Val	88.7	3.1	89.3	3.7	92.6	3.3

[a] Sum of Asp, Glu, Asn, Gln.
[b] $P < 0.01$.
[c] $P < 0.05$.

from the hydrolysis tube, there was found to be no difference between storage under vacuum for up to 4 days and immediate derivatization (Table V). However, there was a significant loss of cysteine, histidine, lysine and tryptophan when samples were stored in air at 4°C for 4 days (Table V, one-way ANOVA, Scheffe multiple range test, $P < 0.05$).

Individual amino acid recoveries from leaves, larvae and feces were tested separately against recoveries from cytochrome *c* using a Student's *t*-test. Most amino acids were recovered from the cytochrome *c* spike in leaves, larvae and feces with efficiencies similar to those from pure protein (Table VI). Exceptions were methionine from all three samples and tryptophan from leaves and feces. However, the coefficient of variation (C.V.) is low enough to allow estimation of total amino acid content to within *ca.* 12% for tryptophan and to less than *ca.* 10% for all others.

Derivatization

An advantage of sulfonic acid hydrolysis over HCl hydrolysis is that sulfonic acid need not be evaporated prior to derivatization. It is sufficient to adjust the pH and buffer the sample prior to addition of the derivatization reagent. No one pH is optimum for derivatization of all amino acids. Many amino acids show maximum derivative formation at a pH of 7.0; however, efficiency falls off quickly at slightly higher or lower pH. At pH 8.0, derivative formation is slightly less than maximum, but

TABLE V

EFFECT OF STORAGE OF HYDROLYZED SAMPLES ON RECOVERY OF AMINO ACIDS FROM CYTOCHROME *c*

Replicate 1.0 mg samples of cytochrome *c* were hydrolyzed at 115°C for 22 h. Three were refrigerated for 4 days without opening the hydrolysis tube. Three were refrigerated for 4 days after opening the hydrolysis tube. Three were derivatized and analyzed immediately.

Amino acid	*Immediate deriv.*		*4°C, Vacuum*		*4°C, Air*	
	Mean recovery (%)	*C.V. (n = 3)*	*Mean recovery (%)*	*C.V. (n = 3)*	*Mean recovery (%)*	*C.V. (n = 3)*
Ala	97.3	2.5	96.2	4.8	96.1	3.6
Arg	92.9	4.5	94.1	4.5	97.7	5.3
Cys	69.8	4.0	70.4	4.0	57.9	4.9[b]
DAA[a]	92.8	2.7	94.3	4.2	93.9	4.8
Gly	95.5	4.9	97.4	3.4	96.5	5.3
His	96.3	3.5	92.4	4.0	78.5	8.8[b]
Ile	85.3	2.6	87.2	3.6	86.8	3.9
Leu	88.7	3.3	88.9	3.9	87.7	3.7
Lys	93.5	5.4	94.3	4.5	78.6	6.2[b]
Met	92.2	5.4	90.9	4.8	92.8	3.3
Phe	89.7	5.7	90.0	6.4	89.3	5.4
Pro	95.2	3.2	94.6	3.8	91.9	5.6
Thr	83.5	2.9	81.4	5.0	82.8	3.5
Trp	89.8	3.3	88.6	6.6	75.0	6.6[b]
Tyr	84.5	6.4	84.5	5.5	85.4	7.9
Val	88.4	4.4	88.8	4.7	89.5	3.0

[a] Sum of Asp, Glu, Asn, Gln.
[b] $P < 0.05$.

is less affected by small variations in pH[16]. A pH of 8.0 gave reproducible results for all amino acids. Mean C.V. was less than 5.0 for all biological samples (Table VII). Sodium borate buffer was easier to use and gave greater precision of recovery than sodium bicarbonate buffer. With sodium bicarbonate it is necessary to approximately neutralize the sample prior to adding the buffer. Otherwise carbon dioxide is evolved reducing the buffering strength of the mixture. With borate buffer, it is possible to add the buffer to the acidic hydrolysis mixture and then adjust pH with a few drops of NaOH. Whichever procedure is used, pH of each individual sample should be verified before proceeding with derivatization because derivative yield is strongly dependent on pH.

Einarsson *et al.*[17] found that the FMOC derivatization reaction is essentially complete in less than 1 min at room temperature. FMOC also reacts with water to produce FMOC-alcohol. The alcohol elutes from the HPLC column at mid-run and has fluorescence and UV absorbance spectra similar to the derivatized amino acids. Large FMOC-alcohol peaks interfere with quantitation of smaller amino acid peaks. Unless derivatization is done on line immediately prior to HPLC injection, excess FMOC must be removed following derivatization. Amantadine, added after amino acid derivatives have formed, reacts with excess FMOC, preventing further formation

TABLE VI

RECOVERY OF AMINO ACIDS FROM HYDROLYZED CYTOCHROME *c* ADDED TO BIOLOGICAL SAMPLES

Three samples each of leaves, larvae and feces containing approximately 1.0 mg of protein were hydrolyzed. Three samples of each containing approximately 0.5 mg of sample protein plus 0.5 mg of cytochrome *c* were hydrolyzed. Amino acids recovered from cytochrome *c* in mixed samples were calculated by subtraction of amino acids from corresponding single component samples.

Amino acid	*Leaves*		*Larvae*		*Feces*	
	Mean recovery (%)	*C.V. (n = 3)*	*Mean recovery (%)*	*C.V. (n = 3)*	*Mean recovery (%)*	*C.V. (n = 3)*
Ala	92.6	3.8	90.5	6.6	90.4	5.5
Arg	96.0	7.1	93.0	6.5	98.0	6.5
Cys	67.3	7.3	72.4	5.5	76.6	6.1
DAA[a]	96.7	1.2	100.7	3.7	92.7	4.4
Gly	95.1	2.4	93.9	1.1	93.6	2.9
His	95.2	4.6	90.1	2.3	92.5	4.5
Ile	86.1	5.6	88.6	4.8	85.7	3.9
Leu	86.2	3.4	90.8	4.5	88.8	2.0
Lys	97.1	4.3	96.4	8.5	91.1	6.4
Met	77.6	3.9[b]	71.4	4.4[b]	75.3	2.2[b]
Phe	89.5	4.2	92.1	5.0	89.3	6.3
Pro	93.8	4.8	101.0	3.5	92.1	6.4
Thr	73.4	4.5	75.4	3.2	71.7	6.1
Trp	64.4	12.1[b]	87.0	1.4	54.0	12.9[b]
Tyr	80.9	4.3	90.5	4.5	82.8	4.9
Val	85.0	3.2	96.6	3.0	90.5	4.4

[a] Sum of Asp, Glu, Asn, Gln.
[b] $P < 0.01$.

of alcohol[6,18]. It does not, however, remove alcohol formed prior to addition. If amantadine is used to scavenge excess FMOC, it must be added at accurately timed intervals within five minutes of reaction time to minimize alcohol formation. Amantadine–FMOC has a reversed-phase retention time longer than any of the physiological amino acids and the HPLC run time must be extended by as much as 10 min to completely remove it from the column. Alternatively, an organic solvent may be used to remove both excess FMOC and previously formed FMOC-alcohol. Since alcohol is extracted along with excess FMOC, it is not necessary to minimize alcohol formation. The derivatization reaction may be carried out for a longer time and exact timing is not so critical. The HPLC run may be terminated shortly after the last amino acid derivative is eluted.

The average specific response (integrated area per pmoles injected) for most amino acids was less with amantadine addition at 5 min than with pentane extraction at 15 min (Tables VIII) (Student's *t*-test). If amantadine addition was delayed to 15 min, the FMOC-alcohol peak completely masked the alanine peak and made quantitation of tyrosine and proline difficult. Although it has been found that pentane extraction may remove some of the more hydrophobic amino acids[18], the data in Table

TABLE VII

MEAN COEFFICIENTS OF VARIATION OF AMINO ACIDS IN BIOLOGICAL MATERIALS

Hydrolysis, derivatization and analysis were performed in duplicate or triplicate for the indicated number of samples. Values given are the mean of the CV's (%) calculated separately for those samples.

Amino acid	*Leaves (n = 15) (%)*	*Larvae (n = 11) (%)*	*Feces (n = 8) (%)*
Ala	2.8	5.0	4.5
Arg	4.7	4.9	5.1
Cys	9.0	8.1	9.3
DAA[a]	3.4	4.5	4.2
Gly	3.8	3.9	4.2
His	3.6	4.4	4.3
Ile	4.7	5.0	4.4
Leu	4.0	3.4	4.0
Lys	4.8	4.6	4.1
Met	4.9	3.7	4.6
Phe	4.7	4.1	4.7
Pro	4.3	4.7	4.1
Thr	2.6	4.7	4.5
Trp	6.2	5.5	9.4
Tyr	7.6	3.9	4.1
Val	3.9	3.5	4.7

[a] Sum of Asn, Gln, Asp and Glu.

VIII indicate that specific response and thereby sensitivity are enhanced by pentane extraction following an extended reaction time.

To study the effect of the molar ratio of FMOC to total amino acids on derivative formation, equimolar standard amino acid mixtures were prepared to contain total amino acids from 30 to 4000 nmol in 1.0 ml. These were derivatized with 6000 nmol of FMOC in 1.0 ml of acetone, giving a molar ratio of FMOC to total amino acids ranging from 200 to 1.5. For each amino acid, a regression equation was calculated for specific response against concentration. Correlation was found to be significant (Student's t-test, $P < 0.05$) only for glycine and the correction was less than 2% over the entire concentration range. Although several researchers have stressed the importance of maintaining the molar ratio of FMOC to amino acids within a certain range[4,15,16], these data do not show any effect due to a 133-fold change in that ratio.

To test for derivative deterioration over time, standard mixtures were derivatized and injected immediately, then reinjected after 1, 2, 6, 12, 18 and 24 h either at 4°C or at room temperature. Regression equations and correlation coefficients were calculated for specific response against time for each amino acid at each temperature. At 4°C, aspartate and glutamate both showed a significant (Student's t-test, $P < 0.05$) positive correlation with storage time following derivatization. In both cases, the correction was less than 2% over a 24-h period. At room temperature only tryptophan was significantly correlated with storage time (Student's t-test, $P < 0.05$). Again the correction was less than 2%. Derivatives were, therefore, sufficiently stable to allow simultaneous preparation of enough samples to load the autosampler for a 24-h run.

TABLE VIII

SPECIFIC RESPONSE USING AMANTADINE OR PENTANE TO REMOVE EXCESS FMOC

Mixtures containing 10 nmoles each of 20 standard amino acids in 1.0 ml buffer were derivatized with 6 μmoles of FMOC in 1.0 ml acetone. Where amantadine was used, 6 μmoles were added after 5 min. If pentane was used, 2.0 ml was added after 15 min. After shaking and centrifugation, pentane was removed and extraction was repeated. Injection size was 20 μl.

Amino acid	*Amantadine*		*Pentane*	
	Response (area/pmol)	*C.V.* (n = 3)	*Response (area/pmol)*	*C.V. (n = 3)*
Ala	16.1	3.2	15.7	2.7
Arg	9.4	2.5	12.1	2.8[b]
Cys	23.8	8.1	22.8	2.8
DAA[a]	15.1	2.8	20.4	3.2[b]
Gly	12.6	6.0	16.8	3.1[b]
His	15.9	2.5	18.5	2.2[b]
Ile	13.6	2.6	18.5	2.1[b]
Leu	15.4	3.2	20.6	1.8[b]
Lys	26.6	2.4	34.3	1.5[b]
Met	12.6	3.4	17.9	1.6[b]
Phe	11.1	3.8	14.6	2.0[b]
Pro	11.6	3.5	13.5	1.8[c]
Thr	10.2	3.0	13.1	2.0[b]
Trp	15.8	8.9	21.4	1.6[b]
Tyr	11.4	2.6	15.2	3.6[b]
Val	13.1	2.4	17.8	2.0[b]

[a] Sum of Asn, Gln, Asp plus Glu.
[b] $P < 0.01$.
[c] $P < 0.05$.

Chromatography

A binary, three-point optimization technique[19] was used in a stepwise manner to effect separation among groups of amino acids which proved difficult to separate. The final gradient (Table II) was a combination of linear and exponential segments. Separation was near baseline for all amino acids with the exception of asparagine and glutamine (Fig. 1). These are non-essential for the larvae studied. Also, hydrolysis converts asparagine and glutamine to aspartate and glutamate, respectively. Therefore separation of this pair was not considered essential to our goals. The injection volune was 20 μl. If this is changed it is necessary to reoptimize the gradient, because the sample buffer disturbs the pH profile of the gradient near the beginning of the run. While much of the separation is insensitive to slight changes in pH or organic content of the elution gradient, the separation among phenylalanine, tryptophan, isoleucine and leucine remained extremely sensitive to such changes as well as to the effects of column ageing. The capability of the pumping system to produce exponential gradients made tuning this section of the chromatogram quite easy using only a change in the curve number.

Histidine and tyrosine form both mon- and di-FMOC derivatives. Quantitation was achieved by summing weighted peak areas. Also, no attempt was made to prevent

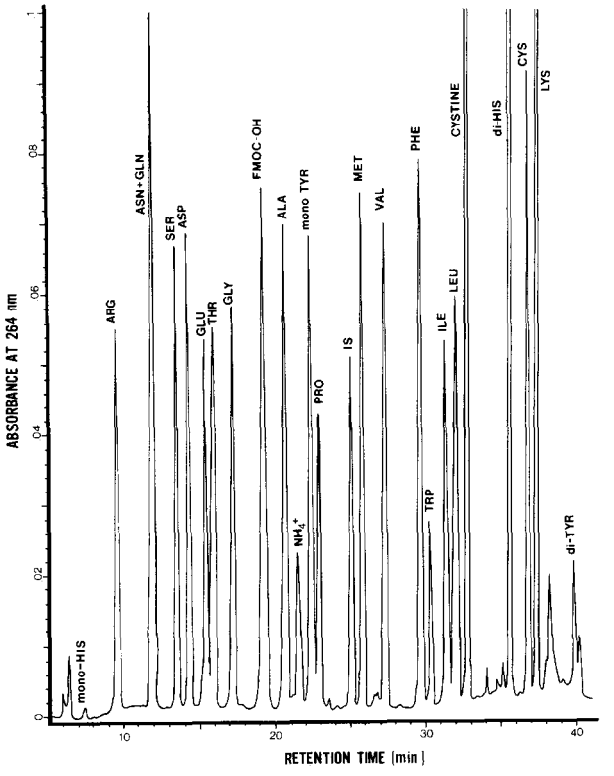

Fig. 1. Separation of FMOC derivatives of standard amino acids. Approximately 1.0 nmole of each amino acid was injected onto a 150 mm × 4.6 mm I.D. ODS-80TM Aminotag column. Internal standard (IS) was c-allylglycine. Gradient elution conditions are given in Tables I and II.

interconversion of cysteine and cystine during hydrolysis. These were quantitated from the weighted-area sum of the two peaks.

Biological samples

Fig. 2 is a typical chromatogram obtained from a sample of larval tissue hydrolyzed, derivatized and chromatographed under the conditions described. All physiological amino acids, with the exceptions noted above, are easily separated and peak areas may be accurately determined.

Biological samples analyzed under the conditions described above generally had C.V. values under 5%. Cysteine and tryptophan were somewhat higher, but under 10%. Our mean C.V. for all amino acids for leaves and larvae was 4.6% and for feces 4.9% (Table VII). These compare favorably with published values of precision for other methods used to analyze mixed biological samples. A recent multi-laboratory study used HCl hydrolysates of fish meal to compare ion-exchange HPLC with gas chromatography (GC) for amino acid analysis[20]. The mean within-laboratory C.V. for all amino acids was 6.4% for ion-exchange HPLC and 5.9% for GC. Adeola *et al.*[21] analyzed soybean meal, corn and triticale using HCl hydrolysis and

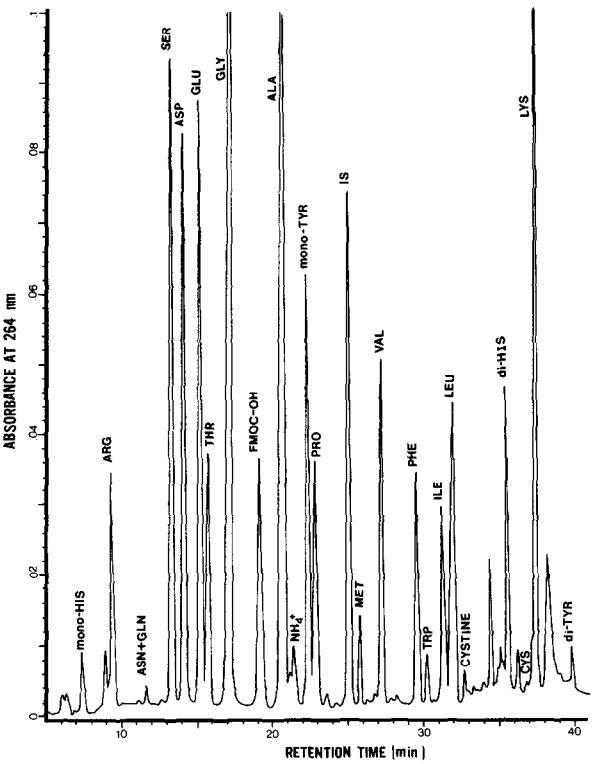

Fig. 2. Separation of FMOC derivatives of amino acids from hydrolyzed cecropia larvae. Approximately 5 mg of sample was hydrolyzed. Final injection represented approximately 4 μg of tissue injected onto a 150 mm × 4.6 mm I.D. ODS-80TM Aminotag column. Internal standard (IS) was c-allylglycine. Gradient elution conditions are given in Tables I and II.

reversed-phase HPLC of phenylthiocarbamyl derivatives or GC of N-heptafluorobutyryl derivatives. Using HPLC, the mean C.V. for soybean meal was 6.7%. Using GC, the mean C.V. for soybean meal was 3.6%, but neither arginine nor histidine, both essential amino acids, were determined. Corresponding mean C.V. values for corn were 6.1% using HPLC and 5.1% using GC and for triticale 5.3% using HPLC and 6.2% using GC.

CONCLUSIONS

Nitrogen flush used in preparation for methanesulfonic acid hydrolysis significantly increased the recovery of cysteine, methionine and tyrosine. Hydrolysis for 22 h at 115°C gave less than complete recoveries of amino acids, but with sufficient precision to allow accurate quantitation after correction for recoveries. Longer (72 h) or higher-temperature (150°C) hydrolysis did not significantly enhance efficiency of recovery and the higher temperature caused partial loss of cysteine, methionine, threonine and tyrosine. Hydrolyzed samples were stored for up to 4 days at 4°C under vacuum without significant loss of amino acids. Storage for 4 days in air resulted in loss of cysteine, histidine, lysine and tryptophan. For some amino acids, recovery efficiency

was lower from biological samples than from pure protein. However, precision was high enough to achieve reliable quantitation of amino acid content when correction was made for recoveries of individual amino acids.

With FMOC derivatization, pH control is important to obtain repeatable derivative yield. Although pH 8.0 did not give maximum yield for all amino acids, yield was less dependent on small changes at pH 8.0 than at lower pH. Borate buffer was judged to be easier to use than bicarbonate. For removal of excess FMOC, pentane extraction, compared with amantadine addition, gave a higher derivative yield for most amino acids. Derivative yield and specific response for UV absorbance was constant for molar ratios of FMOC to total amino acids ranging from 1.5 to 200. Derivatized samples were kept for at least 24 h without significant change in amino acid derivatives.

Complete HPLC separation of FMOC amino acids was achieved during a 40-min run time. Small differences in both pH and organic content of gradients are important for separation among phenylalanine, tryptophan, isoleucine and leucine.

Biological samples were analyzed with a mean C.V. for all amino acids in all samples of under 5%. This could be achieved on a routine basis using an autosampler for 24 or more samples daily.

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