Asian Journal of Chemistry Vol. 18, No. 4 (2006), 2573–2578

Reversed Phase HPLC Studies on the Change of the Free Amino Acid Content of Tobacco During Ageing

NING-NING MING, XIAO-YAN JIN, LIU QIANG†, SU YONG†
and JUN-CHENG GUO*
Research Center of Tobacco Science, University of Science and Technology of China
Meiling Road, 121, Hefei, Anhui 230052, China
Tel: (86)(551)3492064; E-mail: xyjin@mail.ustc.edu.cn, jcguo@ustc.edu.cn

Tobacco leaf samples from different leaf positions, different ageing times were extracted for the determination of free amino acid. The analysis methods involve cation exchange extraction and reversed phase HPLC using a pre-column derivatization with 6-aminoquinoyl-N-hydroxysuccinimidyl carbamate (AQC). The method is suited for the separation of most free amino acids. It allows high recovery and satisfies the necessary requirements with respect to accuracy, repeatability and sensitivity. Seventeen amino acids were unequivocally identified, including aspartate, serine, glutamate, etc. The results shows that free amino acid concentrations decrease with increasing ageing time and vary with leaf position.

Key Words: Amino acid, HPLC, 6-Aminoquinoyl-N-hydroxysuccinimidyl carbamate, Derivatization, Tobacco, Ageing.

INTRODUCTION

The free amino acid of tobacco plays an important role in tobacco quality[1, 2]. Only through ageing, tobacco leaf was processed into cigarette. Many chemical reactions of amino acid during ageing, especially browning reaction, occurred by the reaction of amino acid and glucose or other carbonyl compounds in tobacco, which produce a wide variety of flavour components, such as pyran, pyrrole, pyrazine and pyridine, enhances the flavour[3, 4]. Therefore, valid analytical methods of free amino acid and investigations on the change of amino acid content of tobacco during ageing are required.

Recently, the high-performance liquid chromatography technique was commonly used for determination of amino acids. This technique improved the sensitivity and resolution of amino acids determination and led to appearance of many derivatization reagents, such as *o*-phthaldialdehyde (OPA)[5, 6], 9-fluorenylmethyl chloroformate (FMOC-Cl)[7], phenyl isothiocyanate (PITC)[8–10], 1-dimethylaminonaphthalene-5-sulphonyl (Dansyl-Cl)[11] and 6-aminoquinoyl-N-hydroxysuccinimidyl carbamate (AQC)[12, 13]. In this study, we present a method of analysis

†Technical Center of Yuxi Hongta Group, Yuxi, Yunnan 653100, China.

allowing the separation of 17 amino acids in tobacco by cation exchange extraction to eliminate such interfering substances as pigment and soluble carbohydrate from ethanol extracts of tobacco leaf and RP-HPLC using pre-column derivatization with AQC. Meanwhile, using this method we acquire analytical data on the amino acid profiles in tobacco leaf from different leaf positions, different ageing times, which may be useful in establishing a relationship between the contents of amino acids and tobacco quality.

EXPERIMENTAL

All chemicals were of analytical reagent grade, except for acetonetrile, which was of HPLC grade (Yuwang Industrial & Commercial Co., Shandong, China). Sodium acetate, ethanol, hydrochloric acid, triethylamine, EDTA disodium salt and phosphoric acid were obtained from Chemical Reagent Co., Shanghai, China. 17 amino acid standards and AccQ·fluor reagent kit (including AQC, acetonitrile and sodium borate buffer) were obtained from Waters (Milford, MA, USA). Deionized water was obtained from a Millipore water purification system.

Plant materials: All flue-cured tobacco samples studied were collected from Yunnan, China, in 2001. To age the tobacco, leaves processed by modulation were placed in light-proof cartons. To avoid mildewing, relative humidity was set to 50–70% in every rainy season. Samples were taken for analysis in autumn every year.

Extraction of samples: Tobacco leaves were dried at 70°C to constant weight and then powdered. 1 g tobacco leaf powder was weighed exactly and extracted with 25 mL of 75% aqueous ethanol (v/v) for 30 min in an ultrasonic bath at room temperature. The mixture was filtered and the residue extracted twice more with ethanol. The filtrate mixed was purified by cation exchange extraction. Ion exchange was performed with an ion exchange column which was loaded with cation exchange resin. After the ethanol extracts combined were loaded, the column was washed with 50 mL of water to remove pigment and soluble carbohydrate and then eluted with 100 mL of ammonia (4 mol/L) to elute amino acids. The eluate was evaporated to dryness with a rotary evaporator. The residue was dissolved in 3 mL of hydrochloric acid (1 mol/L) and transferred quantitatively to a 25 mL volumetric flask and made up to 25.0 mL with deionized water.

Derivatization of standards and samples: 10 μL of standard or sample and 70 μL AccQ·fluor burate buffer were added into a clean sample tube (6 × 50 mm). The derivatization was carried out by adding 20 μL of AccQ·fluor reagent (10 mM AQC in acetonitrile) to the buffered mixture and immediately vortex mixing for several seconds. The reaction mixture was kept at room temperature for 1 min and then heated at 55°C for 10 min to complete the derivatization procedure. A 10 μL of aliquot was injected into the HPLC system.

HPLC analysis: Reversed-phase liquid chromatography was carried out using a Waters (Milford, MA, USA) HPLC system, which is equipped with a gradient controller (Waters 600) and a dual wavelength UV detector (Waters 2487). Data were recorded and analyzed using Waters 32 Millennium software. Chromatographic separation was carried out on a Tag CIS (Waters, Milford, MA)

column (150 mm × 30 mm i.d., 5 μm particle size) maintained at 36°C by a column oven. The ultraviolet detector was set to monitor the 248 nm wavelength. The flow rate was 1.0 mL/min and sample injection volume was 10 μL for each assay. Solvent A was made up of 0.23 M sodium acetate containing 77 mM triethylamine, 10 mM EDTA disodium salt, adjusted to pH 5.05 with phosphoric acid and solvent B was acetonetrile : water (3 : 2, v/v). The gradient elution was performed according to the program shown in Table-1. All standards and samples were filtered through 0.45 μm Millipore filter and the mobile phase solvents were degassed prior to use. The total analysis time was 46 min, including column stabilization.

TABLE-1
GRADIENT ELUTION SCHEME EMPLOYED FOR THE HPLC ANALYSIS OF AMINO ACIDS (Flow rate: 1 mL/min)

Time (min)	Solvent A	Solvent B
0	100	0
17	91	9
24	82	18
32	67	33
34	67	33
38	0	100
40	0	100
41	100	0
46	100	0

RESULTS AND DISCUSSION

HPLC chromatograms of the separation of 17 standard amino acids and of a leaf extract of tobacco are shown in Figs. 1 (A) and (B) respectively under the

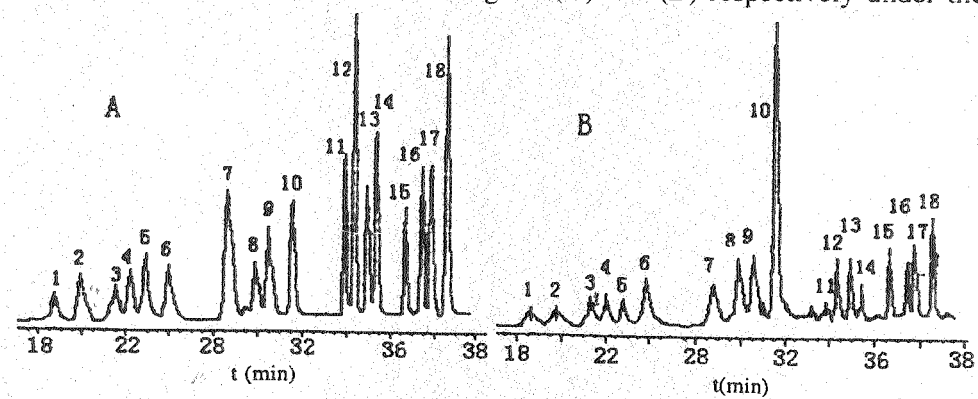

Fig. 1. HPLC chromatogram of (A) a standard mixture of amino acids, (B) a leaf extract of tobacco under the conditions described above. Key to peak identity: 1. aspartate (Asp); 2. serine (Ser); 3. glutamate (Glu); 4. glycyne (Gly); 5. histidine (His); 6. NH_3; 7. arginine (Arg); 8. threonine (Thr); 9. alanine (Ala); 10. proline (Pro); 11. cystine (Cys); 12. tyrosine (Tyr); 13. valine (Val); 14. methionine (Met); 15. lysine (Lys); 16. isoleucine (He); 17. leucine (Leu); 18. phenylalanine (Phe)

HPLC conditions described above. Most of the chosen standard compounds represent typical amino acids of tobacco influencing flavour and quality. As is shown in Fig. 1, a good resolution between these different peaks was obtained. A calibration curve for each standard amino acid was obtained by plotting the peak areas *vs.* the corresponding concentrations of standard solutions. All measurements were performed at least in triplicate (n = 3) and values were averaged and relative standard derivations were calculated (RSD). The correlation coefficient, limit of detection (LOD, at a signal-to-noise of 3), the regression equations and RSD are shown in Table-2.

TABLE-2
LINEAR REGRESSION EQUATION, CORRELATION COEFFICIENT, PRECISE AND DETECTION LIMIT OF AMINO ACIDS

Amino acid	Regression equation	Correlation coefficient	RSD (%) n = 3	Detection limit (S/N = 3)
Aspartate	A = 2.3019C + 3.9639	0.9927	1.41	19.97
Serine	A = 5.3765C + 18.6981	0.9995	1.20	15.76
Glutamate	A = 4.4018C – 6.6129	0.9966	0.60	22.07
Glycine	A = 12.7816C + 12.0139	0.9925	2.48	11.26
Histidine	A = 11.4487C – 7.5416	0.9918	1.87	23.57
Arginine	A = 11.5394C + 64.5625	0.9954	0.60	26.13
Threonine	A = 4.7689C + 12.9674	0.9946	1.40	17.87
Alanine	A = 8.8867C + 12.4650	0.9933	1.10	13.36
Proline	A = 8.3510C + 6.6863	0.9906	0.80	17.27
Cystine	A = 6.5141C + 19.9461	0.9938	2.41	36.04
Tyrosine	A = 19.3374C + 11.9487	0.9927	1.74	27.18
Valine	A = 6.5633C + 24.5972	0.9982	1.69	17.58
Methionine	A = 13.5398C + 4.6889	0.9906	0.80	22.38
Lysine	A = 6.1050C + 9.2827	0.9934	1.25	21.93
Isoleucine	A = 7.3019C + 28.6375	0.9983	1.44	19.68
Leucine	A = 10.4509C + 11.1878	0.9903	0.81	19.68
Phenylalanine	A = 18.4101C + 28.2924	0.9932	1.60	24.78

A: peak area; C: concentration of amino acid.

Correlation coefficients of over 0.99 indicated good linearity between concentration and peak area. In order to check the recovery capacity of the method, known quantities of amino acid standard mixture were added to known amounts of tobacco samples and the spiked samples were extracted and analyzed using the HPLC method described. Recoveries of 17 amino acids range from 86.7 to 112.2% and the average recovery value was found to be 99.15%, which showed the excellent reliability of the proposed method.

Cation exchange was performed to isolate the amino acid from interfering compounds in tobacco samples by a cation-exchange mechanism. In order to elute the amino acid completely, ninhydrin colorimetric method was utilized for evaluating eluate. EDTA disodium salt is commonly used as modifier in an HPLC mobile phase in order to remove the drift of base line. pH of mobile phase has enormous effect on chromatographic separation. The influence of pH was investigated in the pH interval of 4–6. Optimum separation was achieved at a pH value of 5.02. Below pH 4.9, Asp/Ser and Glu/Gly could not be resolved. At higher pH, Cys and Tyr comigrated. In addition, effect of the concentration of the extraction solvent on yields of amino acids was investigated and 75% of aqueous ethanol was employed on account of resulting in higher determined amino acid concentration.

The changes in the free amino acid content with leaf position and ageing time are summarized in Table-3.

TABLE-3
ANALYTICAL RESULTS OF AMINO ACIDS IN TOBACCO SAMPLES (mg/g)

Amino acids	Upper			Middle			Lower		
	2001	2002	2003	2001	2002	2003	2001	2002	2003
Aspartate	0.692	0.417	0.398	1.172	0.896	0.672	0.386	0.324	0.251
Serine	0.277	0.192	0.186	0.521	0.300	0.195	0.200	0.103	0.152
Glutamate	0.577	0.420	0.386	0.920	0.634	0.481	0.482	0.490	0.254
Glycine	0.384	0.293	0.181	0.323	0.215	0.171	0.170	0.111	0.108
Histidine	0.151	0.092	0.064	0.191	0.124	0.093	0.091	0.082	0.041
Arginine	0.366	0.371	0.191	0.392	0.235	0.195	0.260	0.171	0.140
Threonine	0.310	0.261	0.182	0.353	0.282	0.210	0.289	0.180	0.124
Alanine	0.612	0.498	0.391	0.600	0.418	0.361	0.477	0.403	0.201
Proline	2.916	1.724	1.401	3.911	2.078	1.653	1.222	0.861	0.651
Cystine	0.123	0.111	0.072	0.144	0.115	0.092	0.078	0.026	0.020
Tyrosine	0.126	0.120	0.114	0.210	0.174	0.131	0.062	0.059	0.033
Valine	0.298	0.318	0.210	0.310	0.251	0.250	0.181	0.171	0.131
Methionine	0.188	0.144	0.116	0.179	0.134	0.103	0.158	0.139	0.103
Lysine	0.374	0.276	0.204	0.424	0.221	0.214	0.190	0.155	0.921
Isoleucine	0.180	0.146	0.128	0.210	0.163	0.129	0.091	0.069	0.026
Leucine	0.225	0.240	0.203	0.402	0.247	0.207	0.253	0.194	0.124
Phenylalanine	0.468	0.332	0.151	0.459	0.314	0.121	0.299	0.246	0.143
Total	8.264	5.953	4.579	10.721	6.801	5.279	4.889	3.783	3.423

2001,2002 and 2003 denote analytical time respectively.

As is shown, proline was clearly the most predominant amino acid (which accounted for approximately 30% of the total content), whilst cystine and tyrosine were the constituents showing the lowest content, respectively. The total amounts of free amino acids varied significantly with leaf position. Amino acid concentration was highest in the middle leaves, due to high maturity level. The amino acids are considerably more abundant in upper leaves than in lower leaves, because nutrient in lower leaves was transferred to growing points above. Individual amino acids, such as glycine, alanine, methionine and phenylalanine, had higher concentration in upper leaves than in middle leaves due to more active metabolism.

In general, the total of free amino acids decreased sharply with progressive

ageing time. In the case of upper leaves, there is 31.11% decrease (from 8.264 to 5.693 mg/g) during the first year and 19.58% decrease (from 5.693 to 4.578% mg/g) during the following year. The decrease in amino acids with increasing ageing time is due to producing a series of chemical reactions as follows: (1) decarboxylation produced amines; (2) denitrification by oxidation of amino acid produced organic acids; (3) by browning reaction between amino group and reducing sugars, amino acids were degraded and such flavour compounds as pyrrole and pyrazine were formed. These chemical reaction productions have close relation to aroma and flavour of tobacco. In addition, the degree of reduction during the first year was higher, compared with the second year. Possible reason was that intensive chemical reactions occurred in tobacco leaf during the first year and the intensity was reduced with increasing ageing time. The order of the degree of reduction in different leaf positions was: middle > upper > lower. The principal reason for this order was that the high level of reducing sugars in middle leaf led to increase in consuming amino acids. However, there is an exception to the law. Arginine, valine and leucine in upper leaves increased slightly during the first year and decreased during the second year.

Conclusions

An analytical method for the determination of tobacco free amino acid has been developed, which is based primarily on cation exchange isolation of target compounds from tobacco extract followed by their HPLC analysis using a pre-column derivatization with AQC. From these analytical data, we conclude that free amino acid concentration was highest in middle leaves and higher in upper leaves than in lower leaves and amino acid level reduced when aging time was increased. These findings give useful information to tobacco processing and quality control.

ACKNOWLEDGEMENTS

The authors thank the State Tobacco Monopolization Agency (STMA) of China (No. 110200201035) for the financial support to carry out this work.

REFERENCES

1. S.S. Hecht, *Rec. Adv. Tob. Sci.*, **3**, 59 (1977).
2. H.R. Burton, L.P. Bush and J.L. Hamilton, *Rec. Adv. Tob. Sci.*, **9**, 91 (1983) and references therein.
3. H. Kato, *Agr. Biol. Chem.*, **31**, 1086 (1967).
4. H. Kato and M. Fujimaki, *Agr. Biol. Chem.*, **34**, 1071 (1970).
5. M. Roth, *Anal. Chem.*, **43**, 880 (1971).
6. D.W. Hill, F.H. Walters, T.D. Wilson and J.D. Stuart, *Anal. Chem.*, **51**, 1338 (1979).
7. S. Einarsson, B. Josefsson and S. Lagerkvist, *J. Chromatogr.*, **282**, 609 (1983).
8. D.R. Koop, E.T. Morgan, G.E. Tarr and M.J. Coon, *J. Biol. Chem.*, **257**, 8472 (1982).
9. R.L. Heinrikson and S.C. Meredith, *Anal. Biochem.*, **136**, 65 (1984).
10. P. Oravec and D.Podhradsky, *J. Biochem. Biophys. Methods*, **30**, 145 (1995).
11. J.M. Wilkinson, *J. Chromatogr. Sci.*, **16**, 547 (1978).
12. J. Diaz, J.L. Lliberia, L. Comellas and F. Broto-Puig, *J. Chromatogr. A*, **719**, 171 (1996).
13. H. Liu, M.C. Sanuda-Pena, J.D. Harvey-White, S. Kalra and S.A. Cohen, *J. Chromatogr. A*, **828**, 383 (1998).

(*Received*: 11 May 2005; *Accepted*: 10 April 2006) AJC-4766