Saudi Journal of Biological Sciences (2016) 23, S64–S68

King Saud University

Saudi Journal of Biological Sciences

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ORIGINAL ARTICLE

Determination of free amino acids in burley tobacco by high performance liquid chromatography

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Received 30 June 2015; revised 13 August 2015; accepted 16 August 2015
Available online 25 September 2015

KEYWORDS

Different ratio;
Cake fertilizer;
Inorganic fertilizer;
Free amino acids;
Burley leaves

Abstract A reversed-phase high performance liquid chromatographic method was developed for determining free amino acids in burley tobacco. The test was done by OPA/3-mercaptopropionic acid as the pre-column derivatizing reagent. Chromatographic column was Elitte C^{18} column (4.6 mm × 250 mm i.d., 5 μm). Mobile phase A was 18 mol/l NaAc (pH7.2) including 0.002%(v/v) triethylamine and 0.3%(v/v) furanidine. Mobile phase B was 100 mol/l NaAc (pH7.2)–acetoni trile–methanol (v/v = 1:2:2). The column temperature was 40 °C and the flow rate was 1.0 ml/min. The fluorescence detector was used with 350 nm excitation wave length and 450 nm emission wave length. The average recoveries of the method ranged from 95.3–100.7% with the relative standard deviation of 2.32–9.24%. The method is simple, accurate and has good repeatability. The results of the determination of seventeen kinds of free amino acids in burley leaves were produced by the way of different ratios of cake fertilizer and inorganic fertilizer. The results show that Aspartic acid has the highest content however ratio of cake fertilizer and inorganic fertilizer. The contents of most of the free amino acids are increased and then gradually decreased with the increase in organic manure. The contents of most of the free amino acids are very close at 15:85% ratio and 30:70% ratio of cake fertilizer and inorganic fertilizer. The total amount of free amino acids is the highest at 30:70% ratio of cake fertilizer and inorganic fertilizer. Considering comprehensively, the quality of burley leaves is the best at 30:70% ratio of cake fertilizer and inorganic fertilizer.

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1. Introduction

Amino acids are kinds of important nitrogenous compounds, and primary ingredients of Protein, also the precursors of Nicotine, Polyphenol and relative matters. Amino acids play

http://dx.doi.org/10.1016/j.sjbs.2015.08.008
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a vital role in the metabolism of nitrogen in tobacco plant and leaf quality and the formation of aroma compounds, so the analysis of amino acids is significant. Since the 1960s, extensive researches have been done on amino acids in tobacco domestic and overseas (Surhio et al., 2014; Batool et al., 2015; Thoronton et al., 1997). However most of these researches are centered on hydrolysis of amino acids in flue-cured tobacco. With the increasing importance of smoking and health and entering WTO, the development of the low tar blended cigarette is imperative under the situation. Researches on burley tobacco that is one of important raw tobaccos are vital, but less, especially about free amino acids least (Alinsinml and Zapico, 1994; Antoine and Wei, 1999; Jiarong and Hongguang, 2003). Research on the effect of different ratios of cake fertilizer and inorganic fertilizer on free amino acids has not been reported. The purpose of the current work was to qualitatively and quantitatively determine amino acids in burley tobacco and explore changing rule of different ratios of cake fertilizer and amino acids in burley tobacco to scientifically instruct fertilizing as well as provide reference for nitrogen metabolism in burley tobacco. At present, methods for researching amino acids include colorimetry, paper chromatography, gas chromatography (Ashraf et al., 2013a, b; Baizhan et al., 1999), liquid chromatography and capillary electrophoresis (Fatariah et al., 2014; Khaskheli et al., 2015; Naureen et al., 2014). This experiment was done by the improved high performance liquid chromatographic method. The process of treating is easy and rapid.

2. Material and methods

2.1. Instruments and reagents

Instruments: HPLC systems including Waters 515 pump, Waters 2475 fluorescence detector, Empower chromatography chemical station, Rheodyne 7725i local inlet valve, Sigma 3K18 high-speed centrifuge, Ultrasonic oscillator (KQ-250E, Kunshan), Mixing apparatus (CAT, 14-31, Japan).

Reagents: Standard sample: Aspartic acid(Asp), Serine(Ser), Glutamic acid(Glu), Glycine(Gly), Histidine(His), Arginine (Arg), Threonine(Thr), Alanine(Ala), Proline(Pro), Cystine (Cys), Tyrosine(Tyr), Valine(Val), Methionine(Met), Lysine (Lys), Isoleucine(Ile), Leucine(Leu), Phenylalanine(Phe), all are chromatographic pure, SIGMA company US, L-norvaline (chromatographic pure, Shanghai), 3-mercaptopropionic acid (chromatographic pure, chemical reagent company Shanghai), hydrochloric acid, sodium acetate, sodium borate, sodium hydroxide and triethylamine are chromatographic pure, methanol, tetrahydrofuran, acetonitrile, acetic acid are chromatographic pure, water is super-pure water.

2.2. Materials

Burley tobacco breed is 9803. The experimental field is in Enshi Hubei province; it is divided into five field experimental treatments: (1) No using A + B, (2) 15%A + 85%, (3) 30%A + 70B%, (4) 45%A + 55%B, (5) 100%A + No using B.

A – cake fertilizer (colza cake, N:6.07%; P:0.99%; K:1.42%), B – inorganic fertilizer (ammonium nitrate).

Fertilization level: $N:P_2O_5:K_2O$ ($kg/667m^2$) = 15 kg:15 kg: 30 kg.

Row spacing: 1.2 m.

Planting distance: 0.45 m.

Classifying them after air-cured burley tobacco, which have been treated in five steps the second-class tobacco leaves of which the stems have been moved out was chosen. Then they were dried at a temperature of 40 °C. They were crushed and sieved with 60 eyes sieves. Then it was sealed in a bottle for later use (Hamilton, 1991; Jomita et al., 1965).

2.3. Derivations reagents confecting

After weighing 5 mg OPA, it was put in a small bottle so that the volume was 2 ml, then 0.1 ml methanol was added to the bottle, and then 1 ml 0.1 mol buffer solution was added to the bottle. After shaking it for dissolving uniformly, 20 μl 3-mercaptopropionic acid was added to the bottle. After shaking it again for dissolving uniformly, put the bottle in the refrigerator where the temperature is 2 °C (Kato and Fujimaki, 1970).

2.4. Mobile phase formulating

Mobile phase A: Sodium acetate weighing (1.247 ± 0.025) g was dissolved with 200 ml pure water, then 100 μL triethylamine was added to the bottle and the solution was adjusted to pH7.20 ± 0.05 with 1% acetic acid. Then 1.5 mL tetrahydrofuran was added to the bottle. After mixing it uniformly, the volume was fixed to 500 mL then it was filtrated with 0.45 μm filtration membrane for later use.

Mobile phase B: Sodium acetate weighing (1.247 ± 0.025) g was dissolved with 200 ml pure water and the solution was adjusted to pH7.20 ± 0.05 with 1% acetic acid. Then the mixture of 400 ml acetonitrile and 400 ml methanol was added to the solution. After mixing it uniformly and fixing the volume to 500 mL, then it was filtrated with 0.45 μm filtration membrane for later use.

2.5. Chromatography conditions

Chromatographic column was Elitte C^{18} column (4.6 mm i.d. × 250 mm, 5 μm); Mobile phase A was 18 mol/l NaAc (pH7.2) including 0.002%(v/v) triethylamine and 0.3%(v/v) furanidine; Mobile phase B was methanol:water (40:60, v/v); The column temperature was 40 °C and the flow rate was 1.0 ml/min. The fluorescence detector was used with 350 nm excitation wave length and 450 nm emission wave length. The amount of sample injection is 20 μl. Table 1 shows the gradient time of mobile phase.

2.6. Pre-column derivation

After sucking up 25 μl buffer boric acid with a minute absorber, it was put into 1 mL derivatization tube. Again 5 μ1 0PA was sucked up with minute absorber and it was added to 1 mL derivatization tube. Then 10 μl sample was sucked up and was mixed uniformly with admixer, then sucking up 5 μl 3-MPA, again it was mixed uniformly with admixer. Then after setting it for ten minutes it was injected.

Table 1 The gradient time of mobile phase.

Time (min)	Flow rate (ml/min)	Mobile phase A (%)	Mobile phase B (%)	Curve
起始0	1.0	100	0	*
0.5	1.0	99	1	6
17	1.0	93	7	6
21	1.0	90	10	6
30	1.0	67	33	6
33	1.0	67	33	6
34	1.0	0	100	6
37	1.0	0	100	6
38	1.0	100	0	6
50	1.0	100	0	6

2.7. Samples pretreatment

Tobacco smalls weighing 2.0 g, was put in a 50 ml volumetric flask and 5 ml 0.1 mol/l hydrochloric acid was added and 0.2 ml 1.016 mg/mL L-norvaline (IS) to the flask. After fixing the volume, it was extracted for 20 min with ultra-sonic wave, then it was centrifuged for 20 min with centrifugal machine and pure solution was sucked up then it was filtrated with 0.45 μm filtration membrane for later injection.

2.8. Amino acid quantitative analysis

By means of standard chromatogram, references and standard samples injection, the order of peaks was confirmed by contrasting retention time to quality.

2.9. Quantifying with internal standard method

First, working curve of each amino acid with the ratio of the peak area of the amino acid standard sample to the peak area of internal standard and the concentration of respective amino acid was drawn to determine the respective linear equation, then the amount of corresponding amino acid was determined according to the ratio of the peak area of each amino acid in the sample measured to the peak area of internal standard (Haochun and Peizhang, 1993).

3. Results and discussion

3.1. Selection of method for tobacco sample pretreatment

3.1.1. Comparison of extractive solvent

Because amino acid can dissolve in water, ethanol, methanol and dilute acid and so on, these solvents or their mixture can extract free amino acids in tobacco. Traditional method uses 75% ethanol extractive. Another usual method uses 0.1 mol/l hydrochloric acid extractive. This experiment makes a contrast of the two methods.0.1 mol/l hydrochloric acid extractive method compared with 75% ethanol extractive method, the measured value of Glu, Pro and Lys slightly decreased, the amount of Val, Ser, Met, Phe and Tyr is similar. The amount of Gly, Asp and Cys slightly increased but the total amount relatively increased by 4%. Though hydrochloric acid extractive method is slow and it takes about half an hour, from the viewpoint of economy, this experiment adopted hydrochloric acid extractive method.

3.1.2. Comparison of ultra-sonic extraction time

Making the contrast of ultra-sonic extraction time e.g. 10 min, 20 min, 30 min, the amount of some amino acids increased with extraction time prolonging, but eleven in seventeen amino acids are the highest in 20 min, thus 20 min as ultra-sonic extraction time was selected (Kato, 1967; Jiarong and Zhiqiang, 2004).

3.2. Amino acid regression curve

1.000 g of each amino acid solid standard sample that was accurately weighed dissolved in 50 ml 0.1 mol/l hydrochloric acid as each amino acid single standard solution. The mixed standard mother solution was taken with 2 ml of each amino acid single mixed standard solution and the volume with super-pure water was fixed to 50 ml. Mixed standard solution takes 0.5, 1.0, 2.0, 5.0, 10 ml respectively. In the mixed standard mother solution 1.016 mg/ml L-norvaline 0.2 ml was added as an internal standard and the volume was fixed with super-pure water to 50 ml. 20 μl mixed standard solution was taken to make the chromatographic analysis. Table 2 shows regression curve equation and correlation coefficient (Qureshi et al., 2015; Husek, 1994).

According to Table 2, correlation coefficient is 0.9989–0.9999, indicating linear relation is all right.

3.3. Precisions and recovery

One of the samples makes the HPLC analysis in choosing chromatography conditions according to Section 2.6 methods and repeats six times ($n = 6$). Table 3 shows the results.

It is the recovery of process. The determining process is as follows: First, the amino acid standard blending solution was quantitatively added to a certain sample, then the following process was similar to Section 2.6 methods. Recovery = the determining result amount/the added amount. The average recovery of six times is shown in Table 4.

3.4. Samples determining

Tobacco smalls samples were respectively taken and determined according to Section 2.6 methods, then taking 20 μl solution to make the HPLC analysis. Table 5 shows the determining results.

It can be observed from Table 5: ① the amount of seventeen amino acids is distinctly different, the amount of Asp is the highest, and the amount of Ser and Glu is less. ② after air-cured, the amount of seventeen free amino acids has a distinct change with the different ratio of cake fertilizer to inorganic fertilizer. In total content, when the ratio of cake fertilizer to ammonium nitrate is 15%/85%, the total content of free amino acids is 3.027 mg/g, which is the highest; when the ratio of cake fertilizer to ammonium nitrate is 0/100, the total content of free amino acids is 0.185 mg/g, which is the lowest. The total content of free amino acids only using cake fertilizer is higher than that of only using inorganic fertilizer.

Table 2 Regression curve equation and correlation coefficient.

No.	Amino acids	Abridged eng.	Curve equation	Correlation coefficient
1	Aspartic acid	Asp	$Y = 0.7892X + 0.0045$	0.9996
2	Serine	Ser	$Y = 1.0215X + 0.0128$	0.9993
3	Glutamic acid	Glu	$Y = 0.7283X + 0.0321$	0.9994
4	Glycine	Gly	$Y = 1.4236X + 0.0413$	0.9992
5	Histidine	His	$Y = 0.3215X + 0.0214$	0.9998
6	Arginine	Arg	$Y = 0.7285X + 0.0124$	0.9991
7	Threonine	Thr	$Y = 0.8562X + 0.01657$	0.9992
8	Alanine	Ala	$Y = 1.1675X + 0.02869$	0.9997
9	Proline	Pro	$Y = 0.4658X + 0.04563$	0.9999
10	Cystine	Cys2	$Y = 0.9648X + 0.03654$	0.9997
11	Tyrosine	Tyr	$Y = 0.6375X + 0.00943$	0.9995
12	Valine	Val	$Y = 1.0456X + 0.02568$	0.9989
13	Methionine	Met	$Y = 0.8734X + 0.0086$	0.9990
14	Lysine	Lys	$Y = 0.3347X + 0.0062$	0.9993
15	Isoleucine	Ile	$Y = 0.9806X + 0.01789$	0.9997
16	Leucine	Leu	$Y = 0.09689X + 0.0189$	0.9993
17	Phenylalanine	Phe	$Y = 0.7652X + 0.0198$	0.9997

In curve equation, Y = the ratio of the peak area of substance measured to the peak area of internal standard. X = the ratio of the concentration of substance measured to the concentration of internal standard.

Table 3 Repeatability ($n = 6$).

No.	Amino acids	RSD (%, $n = 6$)	No.	Amino acids	RSD (%, $n = 6$)
1	Aspartic acid	3.46	10	Cystine	6.65
2	Serine	4.43	11	Tyrosine	8.36
3	Glutamic acid	2.32	12	Valine	4.75
4	Glycine	3.51	13	Methionine	9.24
5	Histidine	6.94	14	Lysine	4.24
6	Arginine	7.62	15	Isoleucine	3.45
7	Threonine	5.34	16	Leucine	4.36
8	Alanine	4.61	17	Phenylalanine	7.27
9	Proline	8.58			

Table 4 Recovery.

No.	Amino acids	Recovery (%)	No.	Amino acids	Recovery (%)
1	Aspartic acid	96.4	10	Cystine	95.8
2	Serine	100.3	11	Tyrosine	96.3
3	Glutamic acid	99.3	12	Valine	98.7
4	Glycine	99.4	13	Methionine	97.8
5	Histidine	100.7	14	Lysine	99.8
6	Arginine	96.2	15	Isoleucine	99.4
7	Threonine	98.6	16	Leucine	95.3
8	Alanine	97.2	17	Phenylalanine	99.6
9	Proline	96.5			

③ when the ratio of cake fertilizer to ammonium nitrate is 15%/85%, the content of ten amino acids in seventeen is the highest; when the ratio of cake fertilizer to ammonium nitrate is 30%/70%, the content of seven amino acids in seventeen is the highest. That is to say, the content of free amino acids in tobacco that produced only using inorganic fertilizer is low. The contents of most of the free amino acids are increased and then gradually decreased with the increase in organic manure. The reason for it is that using organic manure can increase the activity of soil microorganisms, improve soil physical and chemical characteristics and increase the capabilities of nitrogen metabolism. But because the nitrogen demand of burley tobacco is higher than that of flue-cured tobacco, and it will make an effect on the synthesis of free amino acids when the usage is to certain proportion (Xiezhong, 1997; Yusoff et al., 2013).

Table 5 The contents of free amino acids in burley leaves that were produced with different ratios of cake fertilizer (mg/g).

Amino acids	Samples				
	(1)	(2)	(3)	(4)	(5)
Aspartic acid	0.130	2.262	1.807	0.844	0.856
Serine	0.016	0.234	0.254	0.061	0.073
Glutamic acid	0.009	0.144	0.100	0.098	0.113
Glycine	0.001	0.012	0.013	0.008	0.009
Histidine	0.005	0.061	0.063	0.019	0.019
Arginine	0.002	0.035	0.031	0.029	0.030
Threonine	0.001	0.024	0.025	0.006	0.007
Alanine	0.003	0.042	0.043	0.032	0.032
Proline	0.002	0.011	0.011	0.01	0.009
Cystine	0.001	0.003	0.003	0.002	0.002
Tyrosine	0.001	0.009	0.008	0.008	0.007
Valine	0.001	0.017	0.016	0.018	0.017
Methionine	0.002	0.017	0.017	0.019	0.021
Lysine	0.004	0.064	0.054	0.041	0.049
Isoleucine	0.001	0.015	0.014	0.015	0.015
Leucine	0.002	0.025	0.024	0.023	0.024
Phenylalanine	0.004	0.052	0.049	0.021	0.022
Total content	0.185	3.027	2.532	1.254	1.305

4. Conclusions

Correlation coefficient of determining seventeen amino acids in tobacco is 2.32–9.24% and recovery is 95.3–100.7% which indicates that the precision and accuracy of this method is high to meet analysis demand. In addition, this method has the advantage of rapidness, accuracy, good separation, few samples, and easy sample preparation, little human influence and is suitable to be widely applied. The method was used to determine free amino acids in burley tobacco which were treated with different ratios of cake fertilizer and inorganic fertilizer. The results were satisfactory. It was concluded that most of amino acids which were useful for tobacco quality and total amount of amino acids were highest ratio of cake fertilizer and inorganic fertilizer was 30:70%. In other words, the quality of burley tobacco is the best at 30:70% ratio of cake fertilizer and inorganic fertilizer when fertilizer blended with cake fertilizer and inorganic fertilizer in burley tobacco.

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