Research Article
Effect of Different Ratio of Cake Fertilizer and Inorganic Fertilizer on Free-amino Acids in Burley Leaves

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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 derivatising reagent. Chromatographic column was Elitte C18 column (4.6 mm×250 mm i.d., 5 um). Mobile phase A was 18 mol/L NaAc (PH7.2) including 0.002% (v/v) trimethylamine and 0.3% (v/v) furanidine. Mobile phase B was 100 mol/L NaAc (PH7.2) -Acetonitrile-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 95.3 to 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 which were produced by way of different ratio 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 most of free Amino Acids are increased and then gradually decreased with the increase of organic manure. The contents most of 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.

Keywords: Burley leaves, different ratio of cake fertilizer and inorganic fertilizer, free amino acids

INTRODUCTION

Amino Acids are kinds of important nitrogenous Compound and primary ingredients of Protein, also the precursors of Nicotine, Polyphenol and relative matters. Amino Acids play 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 Xiao (1997), Kato (1967), Kato and Fujimaki (1970), Jomita *et al.* (1965) and Liu *et al.* (1999) have been done on Amino Acids in tobacco domestic and overseas. However most of these researches are centered on hydrolysis 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 (Hamilton, 1991; Chai *et al.*, 2003, 2004; Husek, 1994) on Burley tobacco that is one of important raw tobaccos are vital, but less, especially about free Amino Acids least. Research on the effect of different ratio 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 ratio 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 calorimetry, paper chromatography, gas chromatography (Li and Lu, 1993), liquid chromatography (Antoine *et al.*, 1999; Alonso *et al.*, 1994) and capillary electrophoresis

(Thornton *et al.*, 1997). This experiment was done by improved high performance liquid chromatographic method. The process of treating is easy and rapid.

EXPERIMENTS

Instruments and reagents: Instruments: HPLC systems including Waters 515 pump, Waters 2475 fluorescence detector, Empower chromatography chemical station, Rheodyne7725i local inlet valve; Sigma 3K18high-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 trimethylamine are chromatographic pure, methanol, tetrahydrofuran, acetonitrile, acetic acid are chromatographic pure, water is super-pure water.

Materials: Burley tobacco breed is 9803, the experimental field is in Enshi Hubei province; it is divided into five field experimental treatments:

- No using A+B
- 15%A+85%
- 30%A+70B%
- 45%A+55%B
- 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: P2O5:K2O (kg/667 m^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 with five steps. Choosing second-class tobacco leaves which the stems have been moved out. Then drying them with 40°C temperature. Crushing them and sieving them with 60 eyes sieves. Then sealing them with bottle for later using.

Derivations reagents confecting: Weighing 5 mg OPA, putting it in small bottle that the volume is 2 mL, adding 0.1 mL methanol to the bottle, then adding 1 mL 0.1 mol buffer solution to the bottle. After shaking it for

Table 1: The gradient time of mobile phase

Time (minute)	Flow rate (mL/min)	Mobile phase A %	Mobile phase B%	Curve
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

solving uniformly, adding 20 µL 3-mercaptopropionic acid to the bottle. After again shaking it for solving uniformly, put the bottle in the refrigerator where the temperature is 2°C.

Mobile phase formulating: Mobile phase A: Weighing (1.247±0.025) g sodium Acetate. Solving them with 200ml pure water, adding 100 µL Trimethylamine to the bottle and Adjusting solution to Ph7.20±0.05 with 1% acetic acid. Then adding 1.5 mL Tetrahydrofuran to the bottle. After mixing it uniformly, fixing the volume to 500 mL then filtrating it with 0.45 µm filtration membrane for later using.

Mobile phase B: Weighing (1.247±0.025) g sodium Acetate, solving it with 200 mL pure water and adjusting solution to pH7.20±0.05 with 1% acetic acid. Then adding the mixture of 400 mL Acetonitrile and 400 mL methanol to the solution. After mixing it uniformly and fixing the volume to 500 mL, then filtrating it with 0.45 µm filtration membrane for later using.

Chromatography conditions: Chromatographic column was Elitte C^{18} column (4.6 mm i.d.×250 mm, 5 µm); Mobile phase A was 18 mol/LNaAc (PH7.2) including 0.002% (v/v) trimethylamine 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.

Pre-column derivation: Sucking up 25 µL buffer boric acid with minute absorber, putting it to 1 mL derivatization tube. Again sucking up 5 µL 0PA with minute absorber and adding it to 1 mL derivatization tube. Then sucking up 10 µL sample and mixing them uniformly with admirer, then sucking up 5 µL 3-MPA, again mixing uniformly with admirer. Injecting after setting it for 10 min.

Samples pretreatment: Weighing 2.0 g tobacco smalls, then putting it in 50 mL volumetric flask and

adding 5 mL 0.1 mol/L hydrochloric acid and 0.2 mL 1.016 mg/mL L-nor valine (IS) to the flask. After fixing the volume, extracting it for 20 min with ultra-sonic wave, then centrifuging it for 20 min with centrifugal machine and sucking up pure solution then filtrating it with 0.45 μm filtration membrane for later injection.

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.

Quantifying with internal standard method: First, doing 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 to determine respective linear equation, then determining the amount of corresponding amino acid according to the ratio of the peak area of each amino acid in the sample measured to the peak area of internal standard.

RESULTS AND DISCUSSION

Selection of method for tobacco sample pretreatment: Comparison of extractive solvent, because amino acid can dissolve in water, ethanol and methanol and dilute acid and so on, these solvents or their mixture can extract free amino acids in tobacco. Traditional method is using 75% ethanol extracting. Another usual method is using 0.1 mol/L hydrochloric acid extracting. This experiment make 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 increasing 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.

Comparison of ultra-sonic extraction time: Making the contrast of ultra-sonic extraction time e.g., 10, 20, 30 min, respectively the amount of some amino acids increased with extraction time prolonging, but eleven in seventeen amino acids is highest in 20 min, thus selecting 20 min as ultra-sonic extraction time.

Amino acid regression curve: 1.000 g 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. Mixed standard mother solution is taking 2 mL each amino acid single standard solution mixed and fixes the volume with super-pure water to 50 mL. Mixed standard solution is respectively taking 0.5, 1, 2, 5, 10 mL Mixed standard mother solution and adding 1.016 mg/mL L-nor valine 0.2 mL as internal standard and fixing the volume with super-pure water to 50 mL. Taking 20 μL mixed standard solution to make the chromatographic analysis. Table 2 shows regression curve equation and correlation coefficient.

According to Table 2, Correlation coefficient is 0.9989-0.9999, indicating linear relation is all right. 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.

Precisions and recovery: One of samples makes the HPLC analysis in choosing chromatography conditions according to 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, I quantitatively added amino acid standard blending solution to a certain sample, then the following process is similar to 2.6 methods. Recovery = the determining result amount/the

Table 2: Regression curve equation and correlation coefficient

Amino acids	Abridged Eng.	Curve equation	Correlation coefficient
Aspartic acid	Asp	Y = 0.7892x+0.0045	0.9996
Serine	Ser	Y = 1.0215x+0.0128	0.9993
Glutamic acid	Glu	Y = 0.7283x+0.0321	0.9994
Glycine	Gly	Y = 1.4236x+0.0413	0.9992
Histidine	His	Y = 0.3215x+0.0214	0.9998
Arginine	Arg	Y = 0.7285x+0.0124	0.9991
Threonine	Thr	Y = 0.8562x+0.01657	0.9992
Alanine	Ala	Y = 1.1675x+0.02869	0.9997
Proline	Pro	Y = 0.4658x+0.04563	0.9999
Cystine	Cys2	Y = 0.9648x+0.03654	0.9997
Tyrosine	Tyr	Y = 0.6375x+0.00943	0.9995
Valine	Val	Y = 1.0456x+0.02568	0.9989
Methionine	Met	Y = 0.8734x+0.0086	0.9990
Lysine	Lys	Y = 0.3347x+0.0062	0.9993
Isoleucine	Ile	Y = 0.9806x+0.01789	0.9997
Leucine	Leu	Y = 0.09689x+0.0189	0.9993
Phenylalanine	Phe	Y = 0.7652x+0.0198	0.9997

Table 3: Repeatability (n = 6)

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

Table 4: Recovery

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

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

Samp/Ami acid	(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

added amount. The average recovery of six times shows in Table 4. Samples determining. Respectively taking tobacco smalls samples and determining them according to 2.6 methods, then taking 20 µL solution to make the HPLC analysis. Table 5 shows the determining results.

It will be observed from Table 5, the amount of seventeen amino acids is distinctly different, the amount of Asp is highest and the amount of Ser and Glu is less. 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 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

lowest. The total content of free amino acids only using cake fertilizer is higher than that of only using inorganic fertilizer. When the ratio of cake fertilizer to ammonium nitrate is 15%/85%, the content of ten amino acids in seventeen is highest; when the ratio of cake fertilizer to ammonium nitrate is 30%/70%, the content of seven amino acids in seventeen is highest. That is to say, the content of free amino acids in tobacco that produced only using inorganic fertilizer is low, The contents most of free Amino Acids are increased and then gradually decreased with the increase of organic manure. The reason for it is that using organic manure can increase the activity of soil microorganisms, improve soil Physical and chemical characters 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.

CONCLUSION

Correlation coefficient of determining seventeen amino acids in tobacco is 2.32-9.24% and recovery is 95.3-100.7% which indicating 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 separations, 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 different ratio of cake fertilizer and inorganic fertilizer. The results was satisfied. 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 fertilizing blended with cake fertilizer and inorganic fertilizer in burley tobacco.

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