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ORIGINAL ARTICLE

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

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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 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

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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.