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Determination of Seleno-Amino Acids in Enriched-Selenium Tobacco by Reversed-Phase High-Performance Liquid Chromatography with Pre-column Derivatization

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An analytical method has been developed for the simultaneous determination of selenocystine (SeCys), selenomethylcysteine (SeMeCys) and selenomethionine (SeMet). Three seleno-amino acids and 16 free amino acids were separated by high performance liquid chromatography on a reversed-phase column (Agilent Hypersil ODS column, 125 mm × 4.6 mm × 5 μm) with fluorometric detection (excitation wavelength at 340 nm and emission wavelength at 450 nm) by elution buffers based on sodium acetate with the flow rate 1 mL/min. The extraction methods of three seleno-amino acids and the chemical factors affecting the separation of the selenium species were optimized. All the three selenium compounds could be separated within 20 min. The method linearity, calculated for each seleno-amino acids, has a correlation coefficient higher than 0.990, in concentrations ranging from 0.1 to 10 mg/L (except for SeMeCys ranging from 0.1 to 100 mg/L). The stability of derivatives in acidified samples after ultrasonic was demonstrated. The limit of quantitation was estimated to be varying between 0.004 mg/L (SeMet) and 0.009 mg/L (SeCys) and recovery rates between 95.7 % (SeCys) and 112.9 % (SeMet). The repeatability of the method, expressed as R.S.D., ranged from 0.01 to 0.04 %. The presented method was applied for the quantitation of 16 amino acids and 3 seleno-amino acids contents in enriched-selenium Yunyan tobacco and ordinary Yunyan tobacco.

Key Words: Enriched-selenium tobacco, RP-HPLC, Seleno-amino acids, Amino acid, Pre-column derivatization.

INTRODUCTION

Selenium is an essential trace element for human, animals and some microorganisms, which is an important component of human and animal glutathione peroxidase (GSH-Px). This peroxidase can dioxide lipid hydroperoxide into harmless alcohol or water, so as to protect cells and membranes from oxidation damage. Therefore, adequate intake of selenium can help to enhance body immunity[1-5]. Selenium mainly exists in the organism by the form of selenium compounds and selenomethionine amino acid is the most important resource of getting selenium from people's daily dietary[6-10]. Common selenomethionine amino acid includes: selenocystine (SeCys), selenium methylcrysteine (SeMeCys) and selenomethionnie methionine (SeMet)

In recent years, as the increasing acute of the problem between smoking and health, the usefulness and safety of tobacco become an important research direction of tobacco science. Increasing the content of selenium can improve the safety of tobacco. The tobacco with selenium content higher than 0.14 μg/g is good for the health of smokers. The results show that the natural selenium content in cigarette is negatively correlated with tar content, specially when selenium content is between 0.1×10^{-6} to 1.0×10^{-6}, there is an obvious decrease of the tar content[11,12]. International cancer center found that selenium contents of tobacco of one country with low incidence of lung cancer triples one with high incidence. Increasing Se contents in cigarettes contributes to decrease some major diseases caused by smoking. Consumption of Se-rich vegetables and cereal grain has recently been associated with abnormal conditions and illness of human beings in China and India[13-15].

Selenium enriched tobacco is a hot research topic in agricultural products and tobacco fields. Studies show that tobacco has a strong ability of enriching selenium. Using selenium enriched fertilizer in soil or spraying sodium selenite can help to extract selenium enriched proteins from fresh leavers, especially soluble FI protein (bisphosphate carboxylase oxygenase, Rubisco). The mechanism of enriching selenium of protein is that, by metabolism process, inorganic selenium can be transferred into selenomethionine and selenocysteine, which can be further dehydrated into selenium enriched protein.

Similar as selenium enriched tobacco, research on easy and accurate qualitative and quantitative testing method for the functional selenium seleno-amino acids of selenium enriched tobacco is receiving increasing attention[11,16]. At this moment, considering that the mass spectrometric methods have not been fully universal, simple and common testing methods for seleno-amino acids mainly include capillary electrophoresis (CE) and chromatography, and high-performance liquid chromatography (HPLC) is the most common testing method. But for different samples, pretreatment methods, derivatization reagent, mobile phase and chromatographic conditions used by different researchers are different[17-22]. In this study, *o*-phthalaldehyde (OPA) pre-column automatic derivative method is used to testing seleno-amino acids in selenium enriched tobacco.

ortho-Phthalaldehyde reacts with the free amino groups in seleno-amino acids and free amino acids in the presents of a reducing reagent like β-mercaptoethanol to form their isoindole-derivatieves making them suitable for UV-and fluorescence detection (Fig. 1). This reaction is usually used in a pre-column derivatization step with an automated derivatizer set-up with on-line HPLC separation. UV detection is done at 338 nm. Fluorescence detection is done using excitation settings at 340 nm and emmission settings at 450 nm. In the meantime, optimize the extraction and testing method of selenocystine, selenium methyl cysteine and selenomethionine from selenium enriched tobacco and evaluate its accuracy, recovery rate and interference situation.

EXPERIMENTAL

Selenocystine (SeCys), selenomethylcysteine (SeMeCys), selenomethionine (SeMet) and *ortho*-phthalaldehyde (OPA) were purchased from Sigma-Aldrich (USA). Standard analytical kit, containing 16 L-amino acids were obtained from Agilent (USA), Super-gradient HPLC grade acetonitrile and methanol were obtained from Lab-Scan TEDIA (USA). Ultrapure water generated by the Milli-Q system Millipore (USA) was used. All other reagents were of the highest purity available. Four tobacco (including 2 enriched-selenium and 2 ordinary) samples were kindly supplied by Jiangnan University in China.

Standards: The stock standard solutions in 100 mg/L concentration of SeCys, SeMeCys and SeMet were prepared in 0.1 mol/L hydrochloric acid and kept at 4 °C. All calibrations and working solutions (0.01, 0.05, 0.25, 2.5, 5.0, 10.0, 15.0, 25.0 mg/L of SeCys, SeMeCys and SeMet) were prepared from stock standards by diluting with 0.1 mol/L hydrochloric acid.

Sample preparation: Seleno-amino acids and 16 free amino acids were extracted from enriched-selenium Yunyan and ordinary Yunyan tobacco samples. Weigh 1 g selenious samples into 15 mL centrifuge tubes. Ten millilitres of water (or 0.1 mol/L HCl) was added and kept at 50 °C for 2, 4, 8 or 16 h (with ultrasonic process or not). This extractant solution was left to cool to room temperature then centrifuged at 10000 × g for 10 min, the supernatants were used for HPLC analysis, after filtration through 0.22 μm membrane.

Derivatization buffer: Borate buffer was mixed from 0.2 mol/L boric acid (dissolved in 0.2 mol/L potassium chloride)-0.2 mol/L sodium hydroxide (pH 9.9 ± 0.05) (50:50, v/v). The buffer was filtered through 0.22 μm membrane, degassed in ultrasonic bath under vacuum and stored at 4 °C. During the monthly utilization no precipitation and pH changes were observed.

Derivatization: A 1 μL sample, 1 μL *o*-phthalaldehyde was added to 5 μL borate buffer in reaction vessels from Autosampler and mixed for 20 s. Then the 1 μL mixed solution were injected into ODS column for analysis.

HPLC equipment and chromatographic conditions: The analyses were carried out with Agilent 1100 (USA) chromatography system equipped with Quaternary Pump (G1311A), Autosampler (G1313A), Thermostatted Column Compartment (G1322A) and online Vacuum Degasser (G1322A). Detection of OPA derivatives of seleno-amino acids and amino acids was performed on a Mode G1321A fluorescent detector (FLD) operating at excitation and emission wavelengths of 340 nm and 450 nm, respectively. Data acquisition and processing were carried out with Agilent ChemStation B.04.01 chromatography software. The method was based on a reversed-phase column (Agilent Hypersil ODS column, 125 mm × 4.6 mm × 5 μm) and achieved by binary gradient with a flow-rate of 1.0 mL/min. Mobile phase A was 7.35 mmol/L sodium acetate-triethylamine-tetrahydrofuran (500:0.12:2.5, v/v/v), adjusted to pH 7.20 with acetic acid. Mobile phase B was 7.35 mmol/L sodium acetate-methanol-acetonitrile (1:2:2, v/v/v), the resulting pH was corrected to pH 7.20 with acetic acid. The utilized mobile phase elution gradient is as follows: 0 min, 8 % B; 17 min, 50 % B; 20.1 min, 100 % B; 24.0 min, 0 % B.

RESULTS AND DISCUSSION

Choice of extraction methods: By FLD scanning, detection wavelength of excitation and emission is decided to be 340 mm and 450 nm. When observing the reliability of testing the content of seleno-amino acid from selenium enriched tobacco by pre-column derivatization RP-HPLC method, we firstly optimize the extraction method of seleno-amino acid. By studying the linear between peak area and its concentration, the repeatability of RP-HPLC, the repeatability of pre-treatment process, the analysis about recovery rate and the interference of free amino acid on testing seleno-amino acid, we observe the reliability of the testing method.

Select the common extraction solves (purified water under 50 °C or 0.1 mol/L HCl) to extract seleno-amino acid and compare the effectiveness of two extraction solves and observe the extraction results under different extraction time. By analysis and comparison, the extraction result with 0.1 mol/ HCl is better than purified water, furthermore, ultrasonic treatment can improve extraction efficiency. Therefore, 0.1 mol/ HCl is used and ultrasonic treatment is taken to extract seleno-amino acid from samples. Comparing three different seleno-amino acids, ultrasonic treatment can maximum increase the extract efficiency of SeMet. The contents of SeMet are increased by 27.2, 26.0 and 25.9 % separately under extraction time of 4, 8 and 16 h. Besides that, the longer the extraction time, the higher the testing results of seleno-amino acid (except the extraction of SeCys with purified water or 0.1 mol/L HCl). But compared with 8 h's extraction time, the content of seleno-

TABLE-1
DETECTION VLAUES FOR SeCys, SeMeCys AND SeMet UNDER DIFFERENT EXTRACTION CONDITIONS (50 °C)

Time (h)	Extraction conditions	SeCys contents (mg/kg)	SeMeCys contents (mg/kg)	SeMet contents (mg/kg)
2	Pure water	0.11 ± 0.005	0.23 ± 0.012	0.27 ± 0.014
	0.1 mol/L HCl	0.29 ± 0.011	0.27 ± 0.008	0.47 ± 0.024
	Pure water + ultrasonic	0.12 ± 0.006	0.25 ± 0.010	0.25 ± 0.042
	0.1 mol/L HCl + ultrasonic	0.29 ± 0.007	0.33 ± 0.008	0.50 ± 0.041
4	Pure water	0.15 ± 0.008	0.21 ± 0.031	0.44 ± 0.017
	0.1 mol/L HCl	0.31 ± 0.003	0.34 ± 0.017	0.70 ± 0.024
	Pure water + ultrasonic	0.17 ± 0.001	0.26 ± 0.015	0.42 ± 0.030
	0.1 mol/L HCl + ultrasonic	0.31 ± 0.005	0.32 ± 0.021	0.89 ± 0.047
8	Pure water	0.14 ± 0.007	0.25 ± 0.014	0.60 ± 0.028
	0.1 mol/L HCl	0.32 ± 0.010	0.34 ± 0.018	0.74 ± 0.082
	Pure water + ultrasonic	0.18 ± 0.020	0.29 ± 0.017	0.55 ± 0.021
	0.1 mol/L HCl + ultrasonic	0.35 ± 0.010	0.33 ± 0.047	0.93 ± 0.068
16	Pure water	0.17 ± 0.008	0.26 ± 0.014	0.61 ± 0.012
	0.1 mol/L HCl	0.31 ± 0.008	0.34 ± 0.017	0.76 ± 0.016
	Pure water + ultrasonic	0.19 ± 0.007	0.29 ± 0.013	0.60 ± 0.013
	0.1 mol/L HCl + ultrasonic	0.36 ± 0.014	0.34 ± 0.007	0.96 ± 0.064

amino acid doesn't increase significantly after extracting for 16 h. Therefore, the extraction time is determined to be 8 h.

Standard curve and linear: Prepare standard solution of mixed SeCys, SeMeCys and SeMet with a certain concentration ranging between 0.01 and 100 mg/L with 0.1 mol/L HCl, and test respectively. Chromatographic analysis results are shown in Fig. 1. Repeat the test for three times to observe the lineal relationship between peak area and concentration, the results of which are shown in Table-2. It can be found in Fig. 1 that three different kinds of seleno-amino acids can be eluted within 20 min completely with good separation performance. To different seleno-amino acids, the linear ranges are different. The linear range for SeCys and SeMet is 0.01-10 mg/L, while the linear range for SeMeCys is 0.1-100 mg/L. There are good linear relationship (r > 0.999) between peak area and concentration for those three seleno-amino acids.

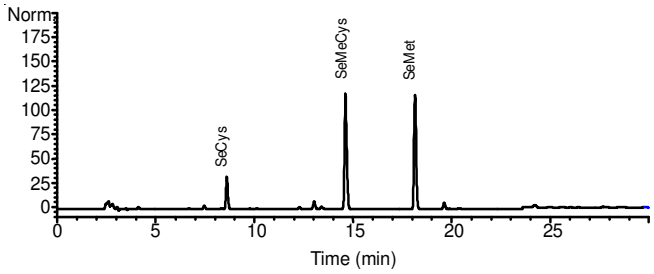

Fig. 1. Chromatogram of 3 seleno-amino acids

Taking signal-to-noise ratio (S/N) of 3 as a criterion, detection limit for different seleno-amino acids under acid condition or purified water condition are shown in Table-2. It is found out that the detection limit relates to the characteristics of the solvent itself, and there is a lower detection limit under acid condition, which is good for detection, especially when concentration is very low. The detection limits for SeMeCys and SeMet under acid condition is 1/30 and 1/25 of the detection limits under purified water condition. Under slightly acid condition, the extraction solutions for three seleno-amino acids with 0.1 mol/L HCl is very suitable for *ortho*-phthalaldehyde derivatization reaction. However, the sensitivities of each component under acid condition or purified water condition have significant difference, which mainly lies on two reasons: the selectivities of *ortho*-phthalaldehyde for different seleno-amino acids are different, and the maximum absorption wavelengths for different derivatives formed by different seleno-amino acids after *ortho*-phthalaldehyde are different. Chromatograms of seleno-amino acids from selenium enriched tobacco by retention qualitative detecting are shown in Fig. 2.

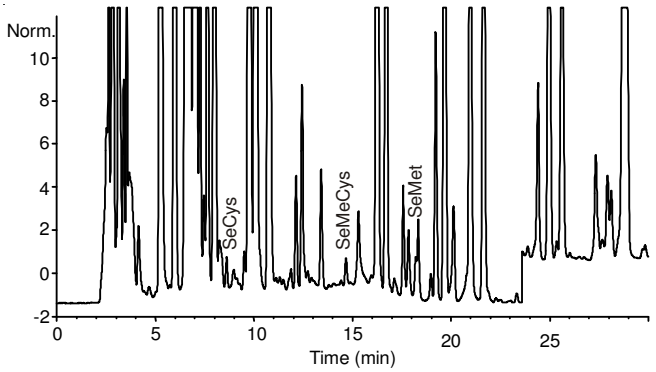

Fig.2. Chromatograms of 3 seleno-amino acids in enriched-selenium Yunyan 85 sample

Repeatability test: The repeatability tests of HPLC instrument and pre-treatment process for samples are performed

TABLE-2
REGRESSION EQUATION, R^2 AND DETECTION LIMITS AND REPEATABILITY OF 3 SELENO-AMINO ACIDS

Species	Retention time (min)	RSD (n = 3, %)	Linearity (mg/L)	Regression equation	r^2	Limit of quantitation (mg/L)	
						0.1 mol/L HCl	Pure water
SeCys	8.56 ± 0.01	0.12	0.01-10	Y = 0.5159X + 1.7825	0.9991	0.009	0.01
SeMeCys	14.73 ± 0.07	0.47	0.1-100	Y = 0.467X + 1.4031	0.9999	0.005	0.15
SeMet	18.10 ± 0.04	0.22	0.01-10	Y = 0.4545X + 1.5174	0.9997	0.004	0.01

TABLE-3
REPEATABILITY OF THE METHODS

	Repeatability of instrument			Repeatability of preparation		
	Peak area (μV s)	SD	RSD (n = 10, %)	Peak area (μV s)	SD	RSD (n = 10, %)
SeCys	31.99	1.30	0.04	1.59	0.07	0.04
SeMeCys	22.77	0.67	0.03	3.35	0.33	0.10
SeMet	46.17	0.59	0.01	25.52	1.22	0.05

respectively. In the repeatability test of HPLC instrument, test with 50 mg/L standard mixture for 10 times. Keep the same testing conditions for each test, average of peak area, standard deviation (SD) and related standard deviation (RSD) for each seleno-amino acid under this concentration are obtained (Table-3). Relative standard deviation of each seleno-amino acid are less than 5 %, indicating the good repeatability of HPLC equipment. It is because that by automatically derivatization procedure conditions, all seleno-amino acids can quickly response with derivative reagent, and can be quickly detected, therefore effectively reduce the deviations caused by the poor stability of seleno-amino acid after derivation[23].

In repeatability test of pre-treatment process, test the same sample with 0.1mol/L HCl, ultrasonic extraction and the pre-treatment process for 10 times described in above paragraph, and test by RP-HPLC under the same condition. Calculate the peak area, standard deviation (SD) and relative standard deviation (RSD), results of which are shown by Table-3. For trace analysis, test method can be accepted when RSD of repeatability test are no higher than 10 % (RSD = 10 %). In this method, RSD of all seleno-amino acids tested are no higher than 5 % (RSD = 5 %), indicating good repeatability of pre-treatment process.

Recovery test: Recovery tests are performed for three mixed seleno-amino acids under different concentration (2.5 mg/L, 5 mg/L and 10 mg/L). Parallel test the initial concentration and the added standards for 3 times. Quantitatively determine with external standard method, and calculate average recovery rate and relative standard deviation under with three different added standard samples (Table-4). The average recovery rate for three seleno-amino acid is between 95.7 % and 112.9 %, and relative standard deviation is between 2.96 % and 4.90 %, indicating the high accuracy, good repeatability and good reliability of this testing method.

Interference test: The most important interference substances in the extraction of selenium enriched tobacco are all kinds of free amino acids. They react with OPA, forming derivations which can be detected by VWD. Therefore, when investigating the influence of free amino acids from selenium enriched tobacco on the testing of seleno-amino acids, take mixture of aspartic acid, glutamic acid, serine, glutamine, histidine, glycine, threonine, arginine, alanine, tyrosine, cystine, valine, methionine, phenylalanine, isoleucine, leucine, lysine and three seleno-amino acids respectively to do pre-column derivatization reversed-phase high performance liquid chromatography analysis, results of which can be shown in Fig. 3. Fig. 3 showed that free amino acids (except SeMeCys) have less interference on the testing of seleno-amino acids, which will not influence quantitative determination, which further indicating that this method has advantages in testing seleno-amino acids in selenium enriched tobacco.

Application in enriched-selenium Yunyan and ordinary Yunyan tobacco samples: Four kinds of samples, enriched-selenium Yunyan 85, enriched-selenium Yunyan 87, ordinary Yunyan 85 and ordinary Yunyan 87, were analyzed. Judged from the retention time, no seleno-amino acids were detected in ordinary Yunyan 85 and ordinary Yunyan 87 samples and all SeCys, SeMeCys and SeMet were detected in enriched-selenium Yunyan 85 with the content of 0.35, 0.33 and 0.93 mg/kg, in Yunyan 87 with the content of 0.30, 0.26 and 0.84 mg/kg, respectively. It was further verified by spiking seleno-amino acids standard into the sample solution. The recovery of three seleno-amino acids spiked into the sample is between 93.7 % and 112.9 % (Table-5).

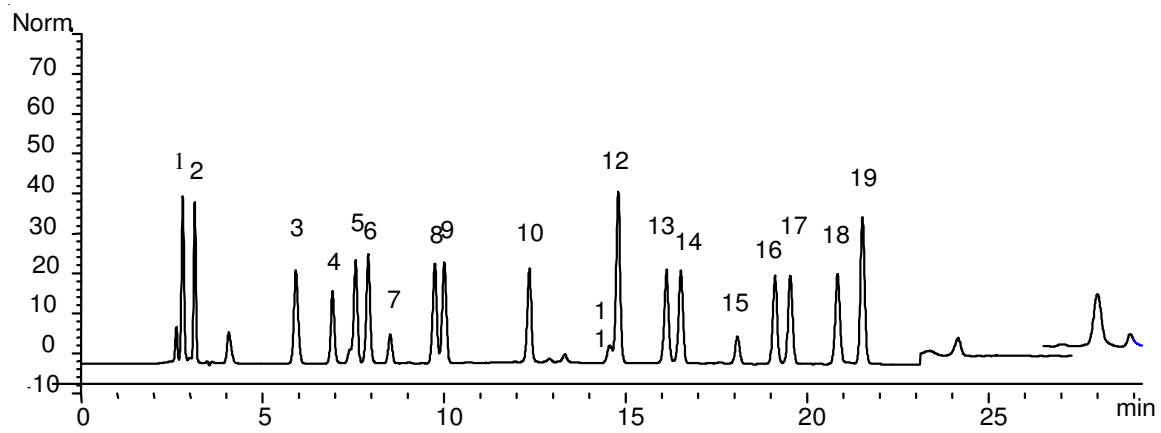

Fig.3. Chromatogram of 3 seleno-amino acids and 16 amino acids in standard solution: 1. aspartate (Asp); 2. glutamate (Glu); 3. serine (Ser); 4. histidine (His); 5. glycine (Gly); 6. threonine (Thr); 7. selenocystine (SeCys); 8. arginine (Arg); 9. alanine (Ala); 10. tyrosine (Tyr); 11. selenomethylcysteine (SeMeCys); 12. cysteine (Cys); 13. valine (Val); 14. methionine (Met); 15. selenomethionine (SeMet); 16. phenylalanine (Phe); 17. isoleucine (Ile); 18. leucine (Leu); 19. lysine (Lys)

TABLE-4
HPLC RECOVERY FOR DETERMINATION OF 3 SELENO-AMINO ACIDS IN ENRICHED-SELENIUM TOBACCO SAMPLE

Species	Sample analysis					RSD (n = 9, %)
	Initial concentration	Added standards (mg/kg) 2.5 (mg/kg)	Added standards (mg/kg) 5.0 (mg/kg)	Added standards (mg/kg) 10.0 (mg/kg)	Average recovery rate (n = 9, %)	
SeCys	3.61 ± 0.15	5.92 ± 0.26	8.37 ± 0.58	13.57 ± 0.29	95.7	4.49
SeMeCys	3.27 ± 0.44	6.05 ± 0.13	8.31 ± 0.67	13.54 ± 0.61	104.9	4.90
SeMet	13.98 ± 0.98	17.4 ± 0.78	19.10 ± 0.49	23.94 ± 0.44	112.9	2.96

TABLE-5
SELENO-AMINO ACIDS CONTENTS IN ENRICHED-SELENIUM YUNYAN AND ORDINARY YUNYAN SAMPLES

Samples	Species	Contents (mg/kg)	Spiked (mg/kg)	Recovery (%)
Enriched-selenium Yunyan 85	SeCys	0.35 ± 0.010	10	95.7
	SeMeCys	0.33 ± 0.047	10	104.9
	SeMet	0.93 ± 0.068	10	112.9
Enriched-selenium Yunyan 87	SeCys	0.30 ± 0.013	10	93.7
	SeMeCys	0.26 ± 0.052	10	101.6
	SeMet	0.84 ± 0.046	10	105.7
Ordinary Yunyan 85	SeCys	ND	10	98.5
	SeMeCys	ND	10	104.2
	SeMet	ND	10	105.6
Ordinary Yunyan 87	SeCys	ND	10	99.1
	SeMeCys	ND	10	103.2
	SeMet	ND	10	110.7

ND: not detected.

Conclusion

Derivatization with *ortho*-phthalaldehyde, subsequent liquid chromatographic separation of the derivatives on a reversed-phase column and detection by FLD is a suitable and sensitive method for the determination of SeCys, SeMeCys and SeMet. The fast reaction (within a few seconds at room temperature), the quantitative conversion and the excellent separation on conventional stationary phases are the advantages of this method. The derivatization of real samples needs no special attention, and a more elaborate sample preparation procedure may also reduce the matrix effects observed in reversed-phase chromatography.

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