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Simultaneous analysis of AY and amino acids in corn oligopeptides by HPLC-fluorescence detector with OPA/FMOC-Cl pre-column derivatization

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Abstract

A high performance liquid chromatographic (HPLC) method for rapid simultaneous determination of alanine-tyrosine (AY) and 20 amino acids in corn oligopeptides was performed automatically by the autosampler, based on the online derivatization of amino groups with o-phthaldialdehyde (OPA) and 9-fluorenylmethyl chloroformate (FMOC-Cl) and the detection was carried out with fluorometric detection combined a C-18 column using gradient elution with external standard. The separation of 20 amino acids and AY was achieved within 30 min 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 1.0 mL/min. The method linearity, calculated for each amino acid and AY, has a correlation coefficient higher than 0.990, in concentrations ranging from 0.1 to 1000.0 mg/L. The stability of derivatives in acidified samples at 4°C and room temperature was demonstrated. The limit of quantitation was estimated to be varying between 0.005 mg/L (AY) and 0.02 mg/L(Asp) and recovery rates between 96.3% (Cys) and 102.8% (AY). The repeatability of the method, expressed as R.S.D., ranged from 1.06 to 4.51%. The method employs protein precipitation with trichloroacetic acid (TCA) and selected the reaction coil as derivatization. TCA clean-up showed a sufficient recovery for peptides as direct injection of the supernatant without evaporation or dilution. The presented method was applied for the quantitation of amino acid and AY contents in corn oligopeptides producing from microbial fermentation, complex enzyme hydrolysis and single enzyme hydrolysis methods.

Key words: Alanine-tyrosine(AY), corn oligopeptides, high performance liquid chromatography, pre-column derivatization.

Introduction

Alanine-tyrosine(AY) and amino acids co-exist in corn oligopeptides. Amino acids play a central role as building blocks of proteins and as intermediates in the metabolism. Bioactive peptide can exhibit antihypertensive, antioxidative, antimicrobial, immunomodulatory and anticancer activities, as a bioactive peptide, AY is a small protein fragment that is beneficial to body health [1-8]. AY could be isolated from the zein hydrolysate due to its high proportions of hydrophobic amino acids and the IC_{50} value (14.2 µM) of ACE-inhibitors [9-11].

Diverse analytical methods have been proposed for the analysis of amino acids or dipeptide including gas chromatography (GC), high-performance liquid chromatography (HPLC) and capillary electrophoresis (EC) [12]. More recently, liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS), micellar electro kinetic capillary chromatography (MECC) and micellar liquid chromatography (MLC) have been shown to be very sensitive and specific technique for the determination of underivatized amino acids [13-16], but these techniques are expensive and not available in many research laboratories.

This study indented to develop a simple RP-HPLC methodology for the simultaneous identification and quantification of AY and 20 amino acids in corn oligopeptides, based on a pre-column OPA/FMOC-Cl derivatization carried out in the chromatograph injection loop. OPA in the presence of borate buffer reacts with all 19 amino acids (except proline) and AY before FMOC-Cl synthesizing with proline, and proceeds to isoindolic derivatives at room temperature, in a quick and simple reaction. This methodology was applied to three different corn oligopeptide samples obtained from different production processes.

Materials and Methods

Materials: Standard analytical kit, containing 17 of L-amino acids + Asparagine + Glutamine + Tryptophan were obtained from Agilent (USA), AY (alanine-tyrosine), OPA (ortho-phthalperdialdehyde) and FMOC-Cl (9-fluorenylmethyl chloroformate) were purchased from Sigma-Aldrich (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.

Three oligopeptides were kindly supplied by 3 different manufactories in China, produced from corn protein with single enzyme hydrolysis, complex enzyme hydrolysis and microbial fermentation, respectively.

Standards: The stock standard solutions in 1.0 mM concentration of AY were prepared in 0.1M hydrochloric acid and kept at 4°C. All

calibrations and working solutions were prepared from stock standards by diluting with 0.1M hydrochloric acid.

Sample preparation: AY and amino acids were extracted from corn oligopeptide with pure water, 50 g/L TCA and 100 g/L TCA separately. Then they were centrifuged at 10,000×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 M boric acid (dissolved in 0.2 M potassium chloride) - 0.2 M 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 OPA and 1µL FMOC-CL were added to 5 µL borate buffer in reaction vessels from Autosampler and mixed for 20 s. Then 1 µL of mixed solution was 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) , Thermostated Column Compartment (G1322A) and online Vacuum Degasser (G1322A). Detection of OPA/FMOC-Cl derivatives of AY and amino acids was performed on a Mode G1321A fluorescent detector operating at excitation and emission wavelengths of 340 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 mM 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 mM 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 shown in Table 1.

Table 1. Chromatographic gradient conditions for simultaneous analysis of AY and amino acids.

Time(min)	0	20	21	21.5	24.5	25
Mobile phase A (%)	80	20	0	0	0	100
Mobile phase B (%)	20	80	100	100	100	0

Results and Discussion

Extraction of AY and 20 amino acids: The organic solvent or acid precipitation and solid-phase extractions or ultra filtration are often the methods used for sample preparation and cleaning up procedure in order to obtain complete extraction of all analyzed components and removal of proteins and other sample components [17, 18]. The acidic precipitation/extraction procedure seems to be the most promising method for complete recovery of low molecule weight compounds from complex matrices. Trichloroacetic acid (TCA) has strong characteristics of precipitating protein with MW limit, so a certain concentration of TCA is normally added in analysis of free amino acids. In this study, TCA was used as precipitating agent, and protein in corn oligopeptide could be obviously precipitated, besides that, AY can also be partly precipitated, so that the analyzed result of AY content becomes lower and so as the recovery rate [19, 20].

Series of experiments were carried out with different TCA concentrations (0 (pure water), 50 and 100 g/L), time for incubation (0.5, 1, 2 and 4 h) and ultrasound treatment to find the optimal sample preparation conditions. The comparison and the influence of the TCA to the precipitation performance of AY is shown in Table 2 and Fig. 1. When using pure water after ultrasound treatment, under the extraction time of 0.5, 1.0, 2.0 and 4.0, the extract efficiency increased by 5.8%, 6.4%, 4.8% and 3.6%. Normally, the longer the extraction time, the higher of AY value, which can be confirmed by the results of using pure water as extract solvent, but the results using TCA as extract solvent is different. Compared with the different extraction results by using TCA (50 and 100 g/L), under different extraction time the highest AY content was found when extracting for 0.5 h, which confirms the conclusion that AY can be partly precipitated during the extraction process by TCA. When using pure water as extract solvent, the extraction efficiency has no big difference when extraction time is longer than 2.0 h. So the following conditions were selected as apparently the best: pure water (0 g/L TCA) coupled with ultrasound treatment for 2.0 h.

Derivatization: The derivatization was carried out according to previously reported procedure with some modifications [21]. OPA/FMOC-Cl is known to have good activity and selectivity for amino compounds and employed as an excellent active group [22]. It can react with amines in low concentration to form stable derivatives under alkaline conditions in the presence of a thiol, the derivatization was achieved in an online flow-through derivatization coil, by mixing the scrubbing sample solution with OPA/FMOC-Cl reagents.

Chromatographic analysis: In order to make sure that there is good separation performance on ODS column among AY, free amino acids and other small peptides, it's very important to control the elution program and pH of mobile phase. Experiments were carried out using: (i) elution program (Table 1); (ii) mobile phase A, pH 7.2; (iii) controlling chromatographic conditions and column equilibration time.

The simultaneous analysis of AY and 20 amino acids in samples

Table 2. AY values at different extraction conditions.

Extraction method	AY content (g/100g)			
	0.5h	1.0h	2.0h	4.0h
Pure water	0.587± 0.013	0.591± 0.029	0.598± 0.018	0.604± 0.016
50g/L TCA	0.567± 0.018	0.473± 0.012	0.476± 0.006	0.477± 0.008
100g/L TCA	0.455± 0.011	0.416± 0.006	0.421± 0.009	0.405± 0.007
Pure water after ultrasound treatment	0.621± 0.008	0.629± 0.007	0.627± 0.011	0.626± 0.020
50g/L TCA after ultrasound treatment	0.558± 0.015	0.510± 0.014	0.491± 0.006	0.484± 0.012
100g/L TCA after ultrasound treatment	0.468± 0.012	0.427± 0.011	0.427± 0.008	0.421± 0.004

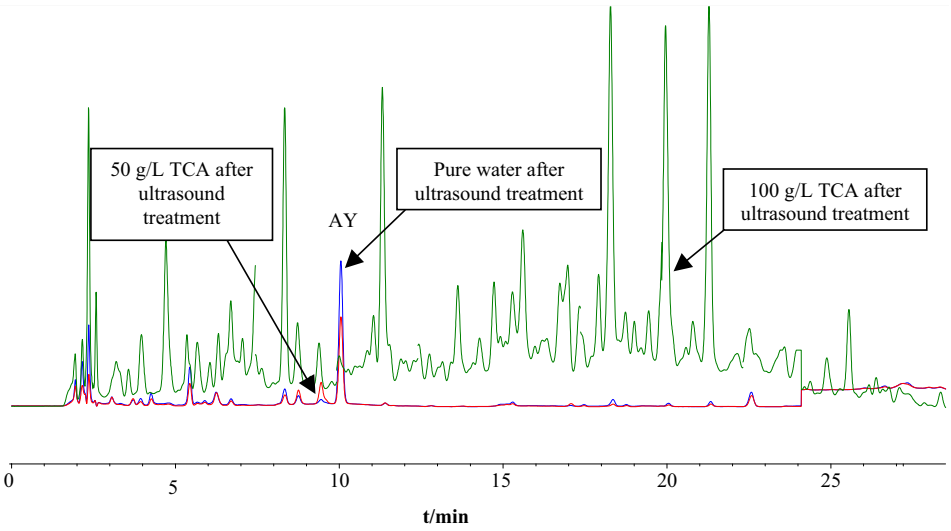

Figure 1. Chromatograms of AY in corn oligopeptide with different extraction.

of different production methods was achieved in only 30 min. AY separates well from amino acids and other peptides on chromatograms, thus allowing an easy switch to different modes of analysis-bioactive peptides or amino acids. The sample analytes were identified by comparison with the retention time of amino acid standard solutions. Quantification was performed by the external standard method based on peak areas of the eluted amino acids and AY derivatives.

Linearity, sensitivity and repeatability: The linearity was evaluated by the construction of calibration curves, using the chromatographic peak areas of the fluorescence response from triplicate injections of standards, at eight increasing concentrations in the 0.1-1000.0 mg/L range (0.1, 0.5, 1.0, 10.0, 100.0, 200.0, 500.0, 1000.0 mg/L) for AY and 20 amino acids (Table 3). In all cases, the relationship between concentrations and peak areas was linear over the tested range, with coefficients of determination greater than 0.990.

The repeatability of the method was evaluated by nine consecutive injections of the same sample during a working day. The detection limits (defined as three times the signal-to-noise ratio) for the amino acids were below 0.01 mg/L, with the exception of aspartic acid and proline, for which the limits were 0.02 mg/L. In the case of the AY analyzed, the detection limit was 0.005 mg/L.

The test of repeatability of the method consists of consecutive analysis of 10 replicates of a standard mixture and 10 replicates of a corn oligopeptide sample. All the resulting variation coefficients were below 5% (i.e. within normally acceptable limits), indicating the quick reaction among AY, 20 amino acids and derivatization reagent under automatic derivatization procedure conditions, so as to reduce the deviation caused by the instability of the AY and amino acids derivatives.

Recovery: Recovery in the analytical method was also studied by adding three increasing amounts of standards to a corn oligopeptide to cover the expected range of concentrations and analyzing each one in triplicate. The results are shown in Table 4; the mean recoveries were between 96.3% and 102.8%.

Analysis of corn oligopeptide samples: As a specific application of the proposed method, 3 oligopeptide samples produced with corn were analyzed simultaneously for AY and 20 amino acids (Fig. 2, Table 4). The first noteworthy aspect was the enormous variation among the different corn oligopeptides. The total amino acids in corn oligopeptides in order of abundance were microbial fermentation production > complex enzyme hydrolysis > single enzyme hydrolysis. Also, concentrations of AY were highest in oligopeptides from microbial fermentation production, this difference was probably due to the different process of enzyme hydrolysis and technical conditions. In the case of AY, from the standpoint of request in the New Resource Food List promulgated by China govenment, the mean value of the corn oligopeptides produced with microbial fermentation is above 6000.0 mg/kg and can be considered qualified.

Table 3. Regression equation, r^2, detection limits and repeatability of amino acids and AY.

Component	Regression equation	r^2	Detection limit(mg/L)	Repeatability (RSD(n=10, %))	
				Standard	Oligopeptide sample
aspartic acid	Y=0.358X+0.135	0.9997	0.02	0.65	2.68
glutamic acid	Y=0.372X+0.157	0.9988	0.01	0.75	4.51
asparagine	Y=0.374X+0.125	0.9980	0.005	0.98	3.26
serine	Y=0.243X+0.137	0.9990	0.005	3.14	1.59
glutamine	Y=0.400X+0.105	0.9991	0.01	0.24	1.85
histidine	Y=0.687X+0.103	0.9985	0.01	2.13	1.63
glycine	Y=0.191X+0.146	0.9993	0.01	1.15	2.58
threonine	Y=0.246X+0.114	0.9995	0.005	0.55	2.64
arginine	Y=0.405X+0.125	0.9993	0.01	0.67	2.45
alanine	Y=0.201X+0.127	0.9994	0.01	0.68	3.10
tyrosine	Y=0.445X+0.131	0.9997	0.01	0.59	3.05
AY	Y=0.958X+0.113	0.9997	0.005	0.45	1.79
cystenine	Y=0.333X+0.109	0.9996	0.01	1.10	1.65
valine	Y=0.276X+0.154	0.9987	0.01	0.68	3.28
methionine	Y=0.349X+0.164	0.9989	0.005	1.20	1.09
tryptophan	Y=0.642X+0.172	0.9994	0.005	1.48	1.06
phenylalanine	Y=0.412X+0.123	0.9999	0.01	1.36	3.25
isoleucine	Y=0.318X+0.130	0.9998	0.005	1.52	2.16
leucine	Y=0.302X+0.138	0.9996	0.005	1.12	1.58
lysine	Y=0.235X+0.156	0.9994	0.005	2.34	1.93
proline	Y=0.275X+0.113	0.9981	0.02	4.25	2.08

Table 4. HPLC recovery for determination of amino acids and AY in corn oligopeptides and concentrations in corn oligopeptides.

Component	Initial concentration (mg/kg)	Added standards (mg/kg) 250.0(mg/kg)	Added standards (mg/kg) 500.0(mg/kg)	Added standards (mg/kg) 1000.0(mg/kg)	Average recovery rate (n=9, %)	RSD (n=9, %)	Corn oligopeptides (mg/kg)		
							Single enzyme hydrolysis	Complex enzyme hydrolysis	Microbial fermentation pruduction
aspartic acid	601.4±26.8	853.8±28.4	1106.7±21.3	1642.3±26.2	102.0	2.28	20.5	3054.8	601.4
glutamic acid	3446.7±71.9	3685.3±70.1	3942.1±110.7	4436.8±21.4	97.8	1.73	1831.5	5279.3	3446.7
asparagine	1122.4±26.9	1362.6±28.9	1635.4±56.8	2129.7±46.9	99.8	2.60	89.5	730.0	1122.4
serine	1910.5±21.5	2166.8±48.7	2421.6±10.3	2889.4±69.8	100.9	1.70	94.6	909.2	1910.5
glutamine	972.5±9.2	1226.4±29.7	1461.2±33.8	1961.2±94.2	99.4	3.18	71.2	420.0	972.5
histidine	1598.3±15.7	1847.9±42.8	2085.2±51.5	2585.7±40.2	98.7	2.11	2491.4	1215.3	1598.3
glycine	347.2±6.6	594.5±18.5	849.7±11.5	1399.8±26.8	101.6	2.13	336.5	16152.9	347.2
threonine	848.7±5.7	1086.1±27.5	1356.5±45.9	1853.7±34.9	99.0	2.60	605.8	190.7	848.7
arginine	16862.8±210.8	17119.5±333.5	17390.0±555.6	17856.8±333.8	102.5	2.34	1052.1	616.2	16862.8
alanine	2319.9±42.5	2558.6±45.7	2811.0±81.6	3331.4±64.7	98.3	2.21	3426.4	1016.1	2319.9
tyrosine	9134.4±95.8	9386.8±184.4	9645.2±25.9	10124.9±65.8	100.7	0.96	694.8	687.5	9134.4
AY	6270.5±110.8	6515.8±62.2	6757.7±70.3	7398.7±69.8	102.8	0.98	3462.2	4070.0	6270.5
cystenine	936.6±15.4	1173.1±26.8	1421.1±48.6	1910.4±20.5	96.3	2.26	2023.1	46.6	936.6
valine	567.8±9.0	818.4±18.9	1061.5±36.7	1597.5±13.5	100.7	2.20	458.7	667.0	567.8
methionine	1996.0±21.0	2245.4±51.4	2486.0±88.9	2964.4±104.3	98.2	3.13	4553.6	23.5	1996.0
tryptophan	2933.4±37.4	3184.3±72.9	3438.9±111.5	3939.8±112.5	100.7	2.80	524.5	10.0	2933.4
phenylalanine	11881.1±214.5	12125.0±258.7	12399.5±376.8	12872.9±263.5	100.1	2.41	1658.4	24.6	11881.1
isoleucine	796.3±12.8	1045.2±24.7	1281.2±29.8	1759.6±16.7	97.6	1.88	825.4	386.4	796.3
leucine	14735.5± 11.4	14990.7±305.8	15222.5±445.7	15732.4±65.8	99.7	1.80	864.6	728.8	14735.5
lysine	10123.9±114.5	10367.2±211.6	10620.7±332.9	11165.8±28.9	100.3	1.81	161.0	2.9	10123.9
proline	73.0±3.0	334.9±12.2	556.4±16.9	1019.3±34.5	98.7	3.35	412.5	1134.3	73.0
					Total amino acids		25658.3	37366.3	89478.9

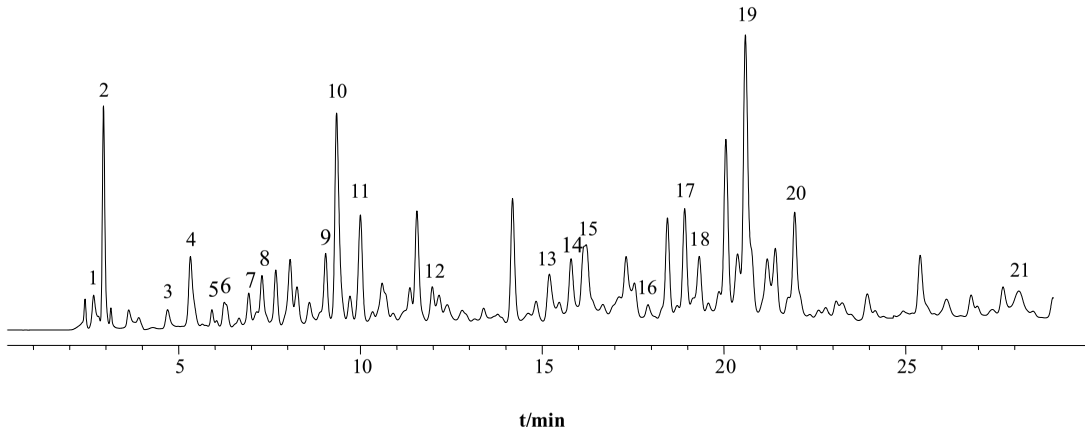

Figure 2. Chromatogram of AY and 20 amino acids in corn oligopeptide.

(1) aspartic acid, (2) glutamic acid, (3) asparagine, (4) serine, (5) glutamine, (6) histidine, (7) glycine, (8) threonine, (9) arginine, (10) alanine, (11) AY, (12) tyrosine, (13) cystine , (14) valine, (15) methionine, (16) tryptophan, (17) phenylalanine, (18) isoleucine, (19) leucine, (20) lysine, (21)proline.

Conclusions

A new chromatographic method for simultaneous determination of bioactively important groups of compounds - AY, amino acids in corn oligopeptides was proposed based on a pre-column derivatization with OPA/FMOC-Cl, performed in the sample injection loop and fluorescence detection. The separation and quantification of AY and 20 amino acids was carried out in a single run as their OPA/FMOC-Cl derivatives elute within 30 min, ensuring a reproducible quantification. The practical utility of the proposed chromatographic procedure was shown by the analysis of the AY and amino acids content in corn oligopeptide samples without any preliminary separation or clean-up steps. The method showed high sensitivity and response to minor compounds. The concentration range applicable for the new method coincides well with the requirements of food quality surveillance posted by legal authorities.

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