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Simultaneous Determination of 17 Amino Acids in Microbial Pigments from *Monascus spp* By UHPLC Amino Acid Analyser Using Pre-Column Derivatization

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A B S T R A C T

Microbial pigments were produced from *Monascus purpureus* (MTCC 410), *Monascus purpureus* (MTCC 369), *Monascus ruber* (MTCC 1880) and *Monascus ruber* (MTCC 2326) by submerged fermentation. The essential amino acids were determined in the pigment extract natural pigments by UHPLC method using precolumn derivatization method with **o**-phthalaldehyde-2-mercaptoethanol. The results shown in the *Monascus purpureus* (MTCC 410) produced the mamimum amount of amino acids, viz, Aspartic Acid (5.90 mg/L), Glutamic Acid (1.30 mg/L), Serine (1.80 mg/L),Histldine (0.60 mg/L), Tyrosine (5.70 mg/L), Cystine (0.80 mg/L), Valline (2.40 mg/L), Leucine (0.40 mg/L) and Proline (88.20 mg/L). Analysis of these amino acids may be of great importance to determine food safety and consumer safety.

Introduction

The increasing interest in the beneficial ingredients of *Monascus* derived products and the attempt of decreasing the toxic components for natural colorants in food make the progress of research in the exploitation of fungal biotechnology. There are two reasons for the determination of amino acids in foods: their potential toxicity and a possibility of using them as food quality indicators. Some of the major applications of amino acids analysis are; quality control of raw materials, intermediates and end products, monitoring fermentation processes, process control and research & development (Onal, 2007). UHPLC with precolumn derivatization is the mostly frequently reported technique for Amino acids separation and quantification (Bomke *et al*., 2009).This paper presents a method to determine the amino acids by UHPLC using precolum derivatization method with **o**-phthalaldehyde-2-

mercaptoethanol in pigments from *Monascus purpureus* (MTCC 410), *Monascus purpureus* (MTCC 369), *Monascus ruber* (MTCC 1880) and *Monascus ruber* (MTCC 2326).

Materials and Methods

Monascus Pigment Production Using Submerged Fermentation

Yeast phosphate soluble starch broth was prepared and sterilized at 121^{o}c at 15lbs for 15 minutes. After sterilization, the medium was inoculated with the *Monascus purpureus* (MTCC 410), *Monascus purpureus* (MTCC 369), *Monascus ruber* (MTCC 1880) and *Monascus ruber* (MTCC 2326) and incubated for 7-15 days at 25-30^{o}C. After incubation period the culture was sterilized at 121^{0}c at 15lbs pressure for 15mins. After sterilization, the broth was filtered through Whatmann No. 1 filter paper.

Determination of Amino Acid in Pigments by UHPLC Amino Acid Analyser

Standard solutions

A solution of Amino acids standards from Sigma (AAS-18) solution containing 2.5, 5.0 and 7.5 mg/L (Aspartic Acid, Glutamic Acid, Serine, Histldine, Glyeine, L-Threonine, Arginine, Alanine, Tyrosine, Cystine, Valline, Methionine, Phyenylalanine, Isoleucine Leucine, Lysine, Proline) was prepared for calibration curves.

Hydrolysis of samples

The method of Zhaolai Dai *et al.*, for amino acid extraction was followed with modification. The pigment samples were filtered through a 0.45 mm. The samples were then hydrolyzed with HCl 6 M at 150°C for 6 h. After hydrolysis, the acid was removed by rotary evaporation. Sample was resuspended in 2 mL of sodium citrate buffer at pH 2.2.

UHPLC Condition

The amino acid is determined by UHPLC method (Rouba and Ulrich, 2013), Instrument : Nexera UHPLC (Shimadzu) with SIL-30AC autosampler, Column : YMC-Triart C18, 1.9 µm (75 mmL. × 3.0 mm I.D., 1.9 µm, YMC Co., Ltd.), Mobile Phase : A : 20 mmol/L Phosphate Potassium Buffer (pH 6.9), B : 45/40/15 Acetonitrile/Methanol/Water, Time Program : B Conc.11 % → 13 % (0.00-3.00 min), → 31 % (5.00 min) → 37 % (7.5 min), → 70 % (10.00 min) → 100 % (10.50-13.50 min), → 11 % (14.00 min), Flow Rate : 0.8 mL/min, Column Temp. : 35 °C, Injection Volume: 1 µL, Detection : RF-20Axs Ex. at 350 nm, Em. at 450 nm, → Ex. at 266 nm, Em. at 305 nm (9.0 min), Cell Temp.: 20 °C. Flow Cell: Conventional cell.

Preparation of derivatization Reagents

Mercaptopropionic Acid- 3-Mercaptopropionic Acid 10 µL in 0.1 mol/L Borate Buffer (pH 9.2) 10 mL, 2. *o*-Phthalaldehyde SolutionL: *o*-Phthalaldehyde 10 mg in 0.1 mol/L Borate Buffer (pH 9.2) 5 mL.3. Fluorenyl Methyl Chloro Formate (FMOC) - Acetonitrile Solution 9-Fluorenyl Methyl Chloro Formate 4 mg in Acetonitrile 20 Ml.

Derivatization Procedure

Mercaptopropionic Acid ,OPA and sample was taken in (µL) ratio of 45:22:7.5 and thoroughly mixed and kept for 1 min. then added 10 µL of FMOC and sample was injected to the UHPLC using Autosampler.

Results and Discussion

Linearity

The linearity was established using Three concentrations of amino acid standards (Table1-2 & Figure 1-2). The data of peak area *vs.* amino acid concentration were treated by linear least squares regression analysis. The linearity data obtained should obey the equation $y = bx + a$, where a is zero within the 95 % confidence limits, and the coefficient of determination (R^2) is greater than 0.984 (Armagan onal *et al.*, 2013 & Perucho *et.al.*, 2015). The retention time of amino acid standards show in UHPLC, are L-Aspartic acid (Rt -0.453), L-Glutamic acid (Rt -0.667), L-Serine (Rt - 0.97) L-Histidine (Rt-1.418), Glycine (Rt -1.915), Threonine (Rt- 2.372), Arginine (Rt-2.772), Alanine (Rt-3.16) ,Tyrosine 3.693, Cystine 5.185, Valine (Rt-6.269), Methionine (Rt-7.847) ,Phenylalanine (Rt-8.579), Isoleucine (Rt-8.986), Leucine (Rt-9.848), Lysine (Rt-9.988) and Proline (Rt-11.198) (Table 3-6).

The results shown in the *Monascus purpureus* (MTCC 410) produced the maximum amount of amino acids, Aspartic Acid (5.90 mg/L), Glutamic Acid (1.30 mg/L), Serine (1.80 mg/L),Histidine (0.60 mg/L), Tyrosine (5.70 mg/L), Cystine (0.80 mg/L), Valline (2.40 mg/L), Leucine (0.40 mg/L) and Proline (88.20 mg/L) followed by the *Monascus ruber* (MTCC 2326) and given in table 1.The analytical column they used was a 3µm C18 column and the chromatogram run time is only 11 min. Although total run time was 17 min, this paper which describes a reliable HPLC method for the quantitation of plasma amino acids in such a short time. Although the simple sample preparation with OPA as derivatization reagent and the short analysis time, make this method very appropriate for fast usual analysis of microbial samples (Figure.1-5).

Table.1 Summary of Amino Acid Content in Monascus Pigments

Amino acids	Amino acids Conc. (mg/L)			
	Monascus purpureus (MTCC 410)	*Monascus purpureus* (MTCC 369)	*Monascus ruber* (MTCC 1880)	*Monascus ruber* (MTCC 2326)
Aspartic Acid	5.90	0.00	6.50	BDL
Glutamic Acid	1.30	1.40	2.20	1.90
Serine	1.80	1.40	1.40	1.70
Histldine	0.60	0.60	0.90	1.00
Glyeine	BDL*	2.40	2.80	2.50
L-Threonine	BDL*	BDL*	BDL*	BDL*
Arginine	BDL*	0.30	BDL*	BDL*
Alanine	BDL*	BDL*	BDL*	BDL*
Tyrosine	5.70	5.00	3.40	4.00
Cystine	0.80	0.30	0.20	0.30
Valline	2.40	2.00	2.50	4.30
Methionine	BDL*	0.00	9.70	20.80
Phyenylalanine	BDL*	BDL*	BDL*	BDL*
Isoleucine	BDL*	23.60	BDL*	22.60
Leucine	0.40	1.20	BDL*	0.50
Lysine	BDL*	0.30	BDL*	BDL*
Proline	88.20	BDL*	59.30	19.50

* Below Detection Limit

Table.2 UHPLC Validation Data for Amino Acid Standards

Peak#	Ret. Time	Area	Height	Area%	Name	Conc. (mg/L)
1.	0.453	16377	1670	0.253	L-Aspartic acid	2.5
2.	0.667	129126	31880	1.992	L-Glutamic acid	2.5
3.	0.97	238650	53580	3.682	L-Serine	2.5
4.	1.418	16918	3609	0.261	L-Histidine	2.5
5.	1.915	195540	38754	3.017	Glycine	2.5
6.	2.372	12009	1332	0.185	Threonine	2.5
7.	2.772	14353	1664	0.221	Arginine	2.5
8.	3.16	3286630	316310	50.702	Alanine	2.5
9.	3.693	429450	31822	6.276	Tyrosine	2.5
10.	5.185	444133	36688	6.852	Cystine	1.25
11.	6.269	144847	11371	2.235	Valine	2.5
12.	7.847	39286	3306	0.606	Methionine	2.5
13.	8.579	271763	49250	4.192	Phenylalanine	2.5
14.	8.986	795899	124754	12.278	Isoleucine	2.5
15.	9.848	125000	26467	1.928	Leucine	2.5
16.	9.988	105799	25163	1.632	Lysine	2.5
17.	11.198	239071	32888	3.688	Proline	2.5

Table.3 UHPLC Validation Data for Amino Acid Content in *Monascus purpureus* (MTCC 369)

Peak#	Ret. Time	Area	Height	Area%	Name	Conc. (mg/L)
1.	0.433	14355	4742	1.041	Aspartic Acid	5.90
2.	0.703	6505	1171	0.472	Glutamic Acid	1.30
3.	1.364	11190	2174	0.812	Serine	1.80
4.	2.414	30597	3592	2.22	Histldine	0.60
5.	No peak is Detected.	0	0	0	Glyeine	BDL
6.	No peak is Detected.	0	0	0	L-Threonine	BDL
7.	No peak is Detected.	0	0	0	Arginine	BDL
8.	No peak is Detected.	0	0	0	Alanine	BDL
9.	5.653	43681	4135	3.169	Tyrosine	5.70
10.	6.329	6703	1008	0.486	Cystine	0.80
11.	6.648	27494	3309	1.995	Valline	2.40
12.	7.104	4622	120	0.335	Methionine	BDL
13.	No peak is Detected.	0	0	0	Phyenylalanine	BDL
14.	No peak is Detected.	0	0	0	Isoleucine	BDL
15.	8.735	2223	392	0.161	Leucine	0.40
16.	No peak is Detected.	0	0	0	Lysine	BDL
17.	10.944	1232895	95424	89.439	Proline	88.20

*BDL- Below Detection Limit

Table.4 UHPLC Validation Data for Amino Acid Content in *Monascus purpureus* (MTCC 410)

Peak#	Ret. Time	Area	Height	Area%	Name	Conc. (mg/L)
1	No peak is detected.	0	0	0	Aspartic Acid	BDL
2	0.695	6851	1340	3.018	Glutamic Acid	1.40
3	1.338	8734	2047	3.848	Serine	1.40
4	2.371	33144	3765	14.601	Histldine	0.60
5	3.278	1755	140	0.773	Glyeine	2.40
6	No peak is detected.	0	0	0	L-Threonine	BDL
7	4.542	2585	316	1.139	Arginine	0.30
8	No peak is detected.	0	0	0	Alanine	BDL
9	5.633	38163	4728	16.812	Tyrosine	5.00
10	6.28	2896	474	1.276	Cystine	0.30
11	6.658	22158	2049	9.761	Valline	2.00
12	7.104	-2101	107	-0.925	Methionine	BDL
13	No peak is detected.	0	0	0	Phyenylalanine	BDL
14	7.912	94857	3115	41.788	Isoleucine	23.60
15	8.525	6019	692	2.652	Leucine	1.20
16	8.763	2275	384	1.002	Lysine	0.30
17	No peak is detected.	0	0	0	Proline	BDL

*BDL- Below Detection Limit

Table.5 UHPLC Validation Data for Amino Acid Content in *Monascus ruber* (MTCC 1880)

Peak#	Ret. Time	Area	Height	Area%	Name	Conc. (mg/L)
1	0.433	15787	5430	1.538	Aspartic Acid	6.50
2	0.703	10433	2131	1.016	Glutamic Acid	2.20
3	1.354	8475	1391	0.826	Serine	1.40
4	2.371	48047	3305	4.68	Histldine	0.90
5	3.165	2044	80	0.199	Glyeine	2.80
6	No peak is detected.	0	0	0	L-Threonine	BDL
7	No peak is detected.	0	0	0	Arginine	BDL
8	No peak is detected.	0	0	0	Alanine	BDL
9	5.628	26160	2428	2.548	Tyrosine	3.40
10	6.262	1488	287	0.145	Cystine	0.20
11	6.654	27709	2069	2.699	Valline	2.50
12	7.052	54345	3187	5.294	Methionine	9.70
13	No peak is detected.	0	0	0	Phyenylalanine	BDL
14	No peak is detected.	0	0	0	Isoleucine	BDL
15	No peak is detected.	0	0	0	Leucine	BDL
16	No peak is detected.	0	0	0	Lysine	BDL
17	11.024	828383	66195	80.693	Proline	59.30

*BDL- Below Detection Limit

Table.6 UHPLC Validation Data for Amino Acid Content in *Monascus ruber* (MTCC 2326)

Peak#	Ret. Time	Area	Height	Area%	Name	Conc. (mg/L)
1	No peak is detected.	0	0	0	Aspartic Acid	BDL
2	0.686	9000	1735	1.396	Glutamic Acid	1.90
3	1.311	10754	1979	1.668	Serine	1.70
4	2.315	53550	3355	8.306	Histldine	1.00
5	3.095	1835	138	0.285	Glyeine	2.50
6	No peak is detected.	0	0	0	L-Threonine	BDL
7	No peak is detected.	0	0	0	Arginine	BDL
8	No peak is detected.	0	0	0	Alanine	BDL
9	5.605	30813	3069	4.779	Tyrosine	4.00
10	6.258	2954	324	0.458	Cystine	0.30
11	6.654	48841	2929	7.576	Valline	4.30
12	7.019	116579	6159	18.083	Methionine	20.80
13	No peak is detected.	0	0	0	Phyenylalanine	0.00
14	7.946	90891	3078	14.098	Isoleucine	22.60
15	8.705	2597	322	0.403	Leucine	0.50
16	No peak is detected.	0	0	0	Lysine	BDL
17	11.056	272379	33757	42.248	Proline	19.50

*BDL- Below Detection Limit

Figure.1 UHPLC Chromatogram of Amino Acid Standards

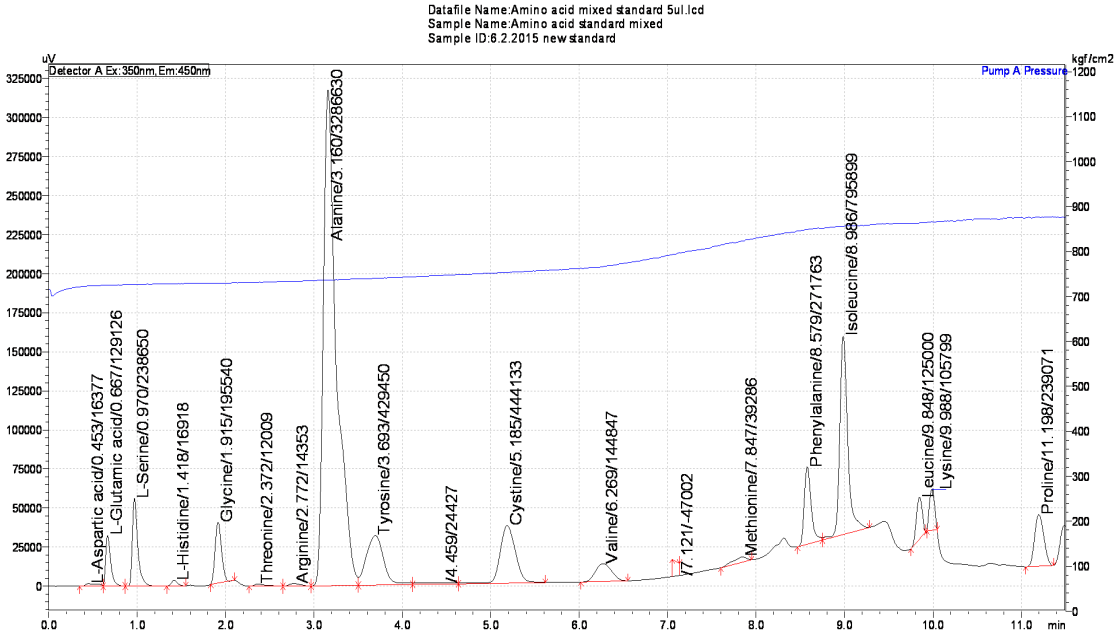


Figure.2 UHPLC Chromatogram of Comparative Amino acid Standards

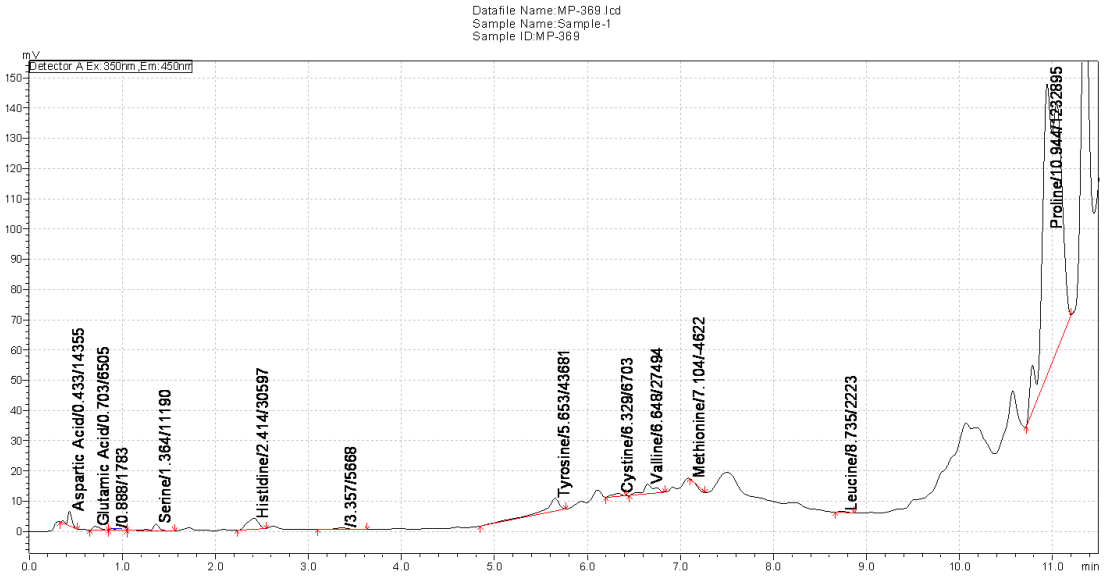


Figure.3 UHPLC Chromatogram of Amino Acid Content in *Monascus purpureus* (MTCC 410)

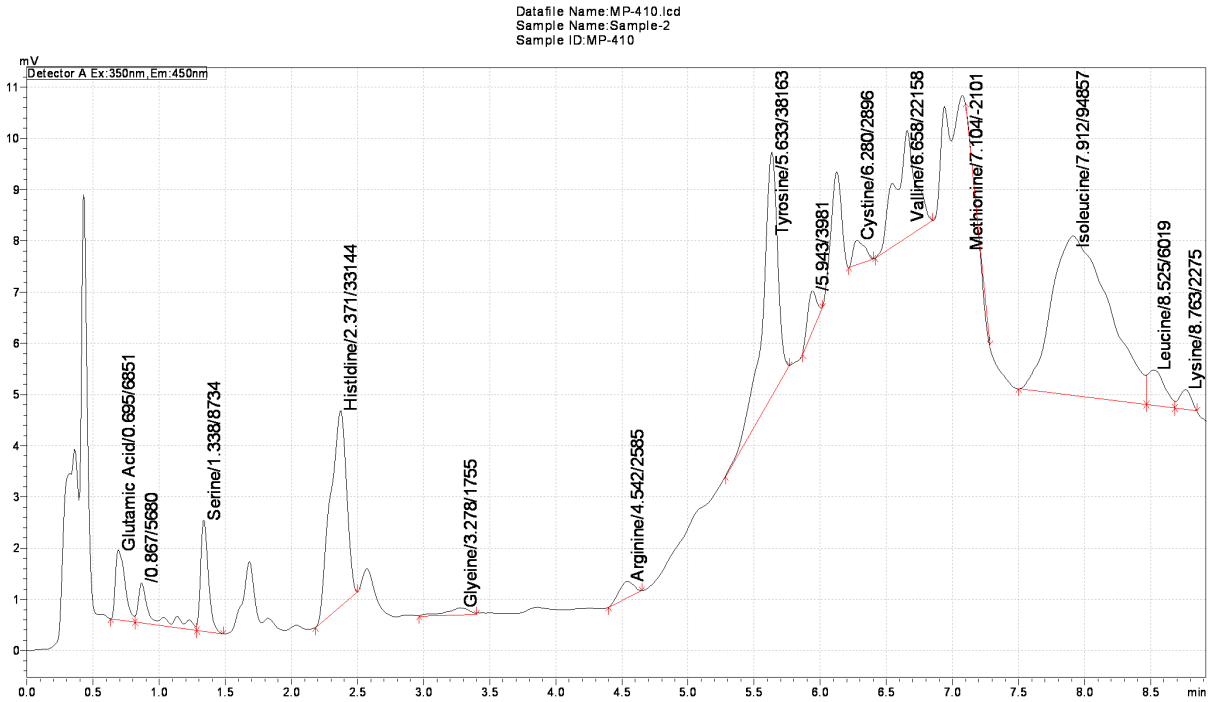


Figure.4 UHPLC Chromatogram of Amino acid Content in *Monascus ruber* (MTCC 1880)

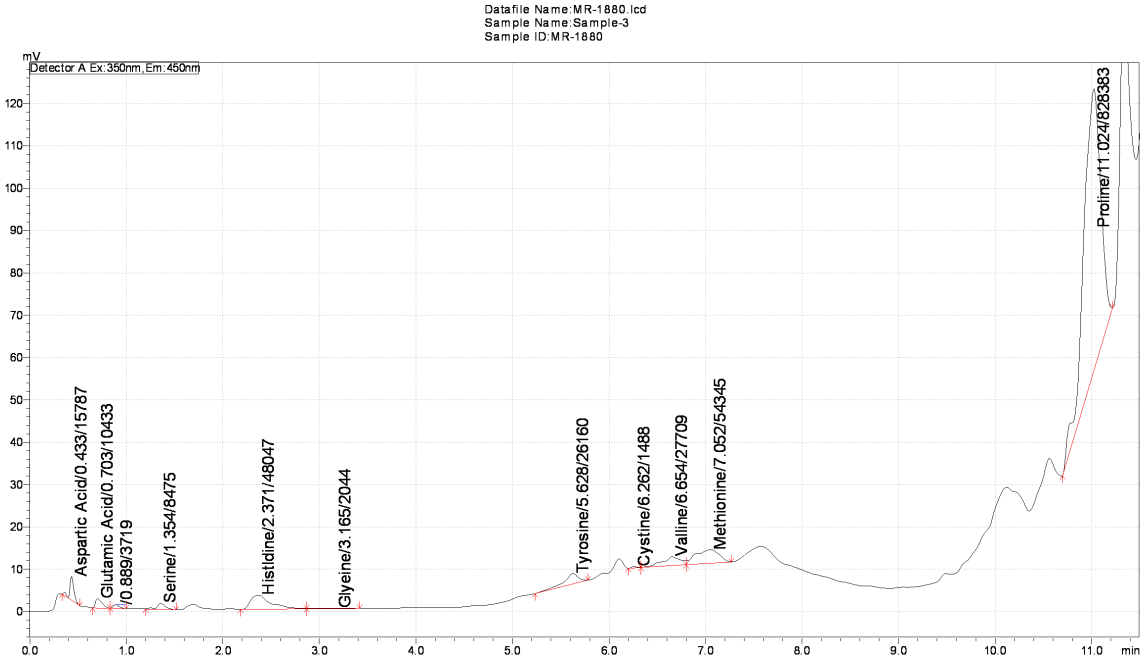
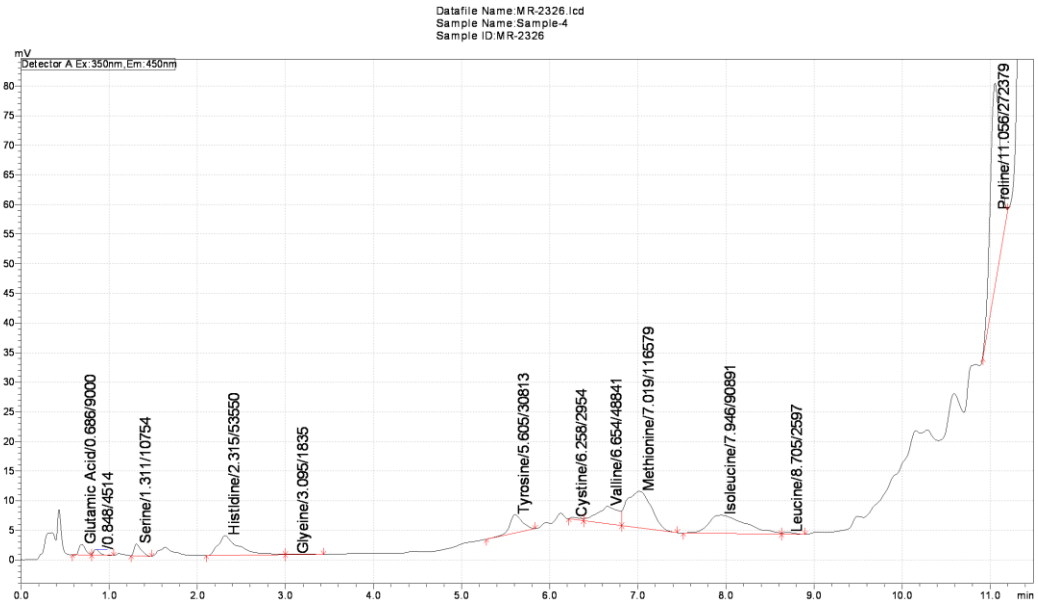


Figure.5 UHPLC Chromatogram of Amino Acid Content in *Monascus ruber* (MTCC 2326)



In conclusion, the attainment of this study is a reliable and high throughput method for the separation and quantification of amino acids in the monascus pigments from *Monascus purpureus* (MTCC 410), *Monascus purpureus* (MTCC 369), *Monascus ruber* (MTCC 1880) and *Monascus ruber* (MTCC 2326). The determination of amino acids using UHPLC in combination with OPA as a derivatization agent provides a very useful method.

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