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SIMULTANEOUS ESTIMATION OF AMINO ACID WITH PRE-COLUMN DERIVATIZATION USING HPLC METHOD

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Abstract:
In this review, a variety of techniques, such as HPLC and electrophoresis, are described for the estimation of individual amino acids as well as the simultaneous estimation of multiple amino acids. Pre-column or post-column derivatization may be utilised for amino acid determination using HPLC. In pre-column derivatization, the amino acid is first derivatized into a particular derivative and then analyzed in the column, whereas in post-column derivatization, the amino acid is first passed through the column for the purpose of separation, and then the separated amino acids are derivatized into their such derivatives that can be detected by fluorescence detector. Pre-column derivatization is employed more frequently than its post-column counterpart. A pre-column derivatization HPLC method was developed for the simultaneous determination of 17 types of amino acids in Asparagi Radix prior to and following heating. As a pre-column derivatization reagent, Phenyl isothiocynide[PITC] was utilised. On a C8 column (C8, 250 X 4.6 mm, 5.0 µm) using gradient elution with 0.1 mol/L pH6.5 acetate buffer solution-acetonitrile as mobile phase A and acetonitrile-water as mobile phase B,and methanol mobile phase C the amino acid derivatives were separated.
Keywords: Simultaneous, estimation, amino acid, pre-column derivatization and HPLC.

1 Introduction

In the study of amino acids, pre-column derivatization is an important step. So that the amino acids can move down the column, they are changed by chemicals called derivatizing agents. After this step, the products are put into an HPLC machine that is running in the opposite direction. This method is called pre-column derivatization in a more technical sense. Derivatizing phenyl thioisocynide and dansyl is needed to make the chemicals that can be made from these starting materials. The ingredient peaks may also be affected by derivatizing chemicals. Both 2-mercaptoethanol and o-phthalaldehyde are great molecules that can be derivatized before going into the column. One problem with the method is that none of the derivatizing chemicals can combine with secondary amines or amino acids. This is one thing the system doesn't do well. Both the UV/fluorometric detection method and the pre-column derivatization method use a variety of chemical parts and parts made from these parts. The following results came from a study that Chaitanya G. and his colleagues did in 2019 using the methods listed above. In the PITC 3 method, danskyl chloride and o-phthalaldehyde are used (1-3).

1.1 O-phthalaldehyde method

Analysis was performed using fluorescence instruments, however the final products needed to be made by hand. Any issues that 2-mercaptoethanol could have brought about were prevented by the experiment's use of 3-mercaptopropionic acid acetonitrile. You may be able to do the study using this approach in as little as 13 minutes. This technique may be used to count the amino acids present in the muscles, liver, and kidneys, among other systems (4, 5).

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1.2 Phynylisothiocynide[PITC] method

This material is sometimes referred to as Edman's reagent by scientists. If the right substances are utilised, it is possible to make derivatives of amino acids that are thiocarbonylated. After the creation of the items, they are the ones who put themselves in the analysis capsule. Thiocarbonyl compounds may be securely kept in the refrigerator for a few days, according to Moldoveanu SC (6-8).

1.3 Dansylchloride method

Estimation and models are two fields that use a lot of copies of primary and secondary amino acids made from dansyl chloride. Using devices that are sensitive to fluorescence, these leftovers can be found. Derivatization could take up to 35 minutes to finish in the dark. After that, an RP-HPLC test can be done on the products. Rainer Schuster's simple two-step method lets pre-column derivatization happen and lets primary and secondary amino acid analysis happen at the same time. During the study process, it may be harder to keep an eye on and measure the goal analytes if they have been derivatized. This pre-column derivatization method was made because the derivatizing agent was so hard to work with. At the beginning of the process, primary amino acids combine with o-phthalaldehyde and 3-mercaptopropionic acid. This makes a secondary amine. During the second step, secondary amino acids and FMOC (9-fluorenylmethylchloroformate) react with each other (9). Using a column with a reverse phase, you can separate the main amino acids from the secondary amino acids in about 15 to 20 minutes. With this equipment, derivatization can be done. Before mixing, all of the different chemicals and test results are put in a container (Figure a) (10).

2 Literature Review

Specialists must use pre-column derivatization because post-column derivatization wasn't used very often because of these things. Hong Ji Liu made 6-amino quinolyl-N-hydroxysuccinimidyl carbamate (AQC) in 1994 because no other derivatization reagent had all the right qualities. AQC became the most popular derivatizing agent because it worked well with all primary and secondary amino acids at an excitation wavelength of 250 nm and an emission wavelength of 390 nm using fluorescence detectors and UV detectors. In 2009, C. Bueneo et al. shared a second fact that made it possible to measure amino acids quickly and easily (11). They thought that since tryptophan, tyrosine, and phenylalanine all give off light on their own, other aromatic free amino acids could be found without having to change anything. Considering how important the three amino acids we just talked about are to the body's health as a whole, this is one of the most important things to know. Tryptophan is one of the most important amino acids because it is a building block for both niacin and serotonin, which are vitamins. The body makes the amino acid tyrosine, which is not necessary (12). Tyrosine helps control body temperature and gives energy to the central nervous system (CNS). The central nerve system is stimulated by the amino acid phenylalanine. There are a number of well-known ways to find these amino acids, such as gas chromatography, ion exchange chromatography, and HPLC study, which is the best and most commonly used method. In 1987, Mark C. Roach and Marlin D. Haromny used laser-generated fluorescence technology and a new substance called 2,3-Naphthalinedialdehyde to come up with a way to use HPLC to test amino acids. Visible light with a wavelength of 457.9 nm was used to find NDA, while ultraviolet light was used to find O-phthalaldehyde. Using the traditional derivatization method and the OPA reagent, scientists found up to 100 fmols of amino acids. To make it easier to find amino acids, a new chemical for derivatization and a new method called the laser fluorescence method were made. This meant that NDA amino acid molecules had to be lit up by a laser. Since this method was made, the

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amount of amino acids that can be found has gone down by up to 10 fmols (13-15).

3 Research Methodology

3.1 Trial 1

According to the study by Kakiyama G, Muto A, Takei H, Nittono H, Murai T, Kurosawa T, Hofmann AF, Pandak WM, and Bajaj JS. Aspartic acid, glutamic acid, serine, glycine, glutamine, threonine, alanine, histidine, tyrosine, valine, methionine, leucine, isoleucine, phenylalanine, tryptophan, lysine, and proline were the amino acids we chose to study in our other research on amino acids. Therefore, using the aforementioned study work as a reference, new technique development for Related Substances test was started (16). The results of the amino acid analysis of the sample using the old approach and the new methodology are compared. Results from amino acids are similar and meet the requirements for acceptance.

3.2 Trial 2

During analysis of Different amino acids by above reference method, only 13 peaks eluted.

3.2.1 Reagents, chemicals, filters and column:

Table (a) showing Reagents, chemicals, filters and column for of amino acid with precolumn derivatization Using HPLC.

3.2.2 Chromatographic system

Mode: LC
Column: Capcell Pak C8, 150*4.6mm, 5µm
Flow Rate: 1.7 mL/minutes
Run Time: 20 minutes
Injection Volume: 20µL
Column Oven Temperature: 40°C
Sampler Temperature: 25°C
Wavelength: 234 nm
Spike sample solution: spiked 2ml of each amino acid into the sample.

3.2.3 Preparation of solutions

Preparation of solutions and sample derivatization

Method A:

Standard stock solution preparation standard solution preparation Standard stock solutions were created by combining 10 mg of each of the designated amino acids with 6% HCl produced from HPLC-grade water. In addition, 60 ppm of each amino acid was present in the mixture of amino acids (17).

Method B:

with a phase of alkaline hydrolysis. After the cholylglycine hydrolase/sulfatase treatment (method A), 500 l of isopropanol and 100 µl of 1 N NaOH were added to the solution, which was then incubated at 60°C for 2.5 hours. Adding an IS, 50 nmol of norDCA, and 3 ml of 0.1 N NaOH, the amino acids were extracted in the same manner as described previously (18).

Sample derivatization process

Amino acids standards and sample derivatisation process are important to since the quantification of recovered AAs are affected by several reasons. A 1 ml of sample was pipetted into a 5 ml glass vial. It was then mixed with 1 ml of solution of methanol- water-triethylamine (2: 1: 1, v/v). The sample further derivatized using 20µl of a solution consisting ethanol, water, triethylamine, and phynylisothiocynide
[PITC] (7: l: l: 1, v/v). The vials were vortex-mixed and allowed to stand at room temperature for 4 hours and dried using the Speed Vat Concentrator. Furthermore, residue

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was reconstituted in ethanol and then it was injected into the HPLC system.

3.3 Trial 3

Mobile phase composition study to improve resolution between impurities peaks.

Mobile Phase Preparation:

Prepared a homogeneous mixture of 600 mL of buffer pH 3.00 and 400 mL of acetonitrile. Mixed well and sonicated to degas.

For other details refer above trial 1

3.4 Trial 4

Mobile phase, Gradient program composition study to improve resolution between impurities peaks.

3.4.1 Gradient program:

Table (b) showing Gradient Program for Trial 4 in amino acids.

3.5 Trial 5

3.5.1 Gradient program:

Table (c) showing Gradient Program for Trial 4 in amino acids.

3.6 Trial 6 (Final)

3.6.1 Chromatographic conditions

Instrument	:	HPLC
Column	:	C8, 250 X 4.6 mm, 5.0 µm (YMC Pack C8 is suitable)
Injection Volume	:	20µL
Wavelength	:	UV 234 nm
Column Ove Temperature	:	40ºC
Sample Temperature	:	25ºC
Flow rate	:	1.7mL /minute
Retention Time	:	About 15.3 minute for Fluvoxamine
Run Time	:	65 minute
Needle wash	:	Mixture of Acetonitrile and Water in the ratio of 40:60.
Seal wash	:	Mixture of Acetonitrile and Water in the ratio of 10:90.

3.6.2 Preparation of solutions:

Dilute Orthophosphoric acid:

Mix well after adding 5 mL of Orthophosphoric acid to a 50 mL volumetric flask that has around 10 mL of water in it. Mix after cooling and diluting with water to volume.

Buffer solution pH 3.0:

1 g of potassium dihydrogen phosphate and 8 g of sodium pentane-1-sulfonic acid salt should be precisely weighed before being added to 1000 mL of milli-Q water. Orthophosphoric acid should be diluted to a pH of 3.0 +/- 0.05. The solution should be filtered using a 0.45 µm PVDF membrane filter.

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Mobile phase
Mobile phase A:
Buffer Solution pH 3.0
Mobile phase B:
Acetonitrile
Mobile phase C:
Methanol
3.6.3 Gradient Program:
Table (d) showing Gradient Program for Trial 4 in amino acids.

4 Result And Discussion

4.1 Trail 1
4.1.1 Observation
- Only thirteen peaks, including aspartic acid, glutamic acid, serine, glycine, glutamine, threonine, alanine, histidine, tyrosine, valine, methionine, leucine, and isoleucine, were visible in the amino acid solution (Figure b).
- The other amino acids, such as phenylalanine, tryptophan, lysine, and proline, were not eluted (19).

4.1.2 Conclusion
- To enhance the morphologies of the eluted peaks, such as those for aspartic acid, glutamic acid, serine, glycine, glutamine, threonine, alanine, histidine, tyrosine, valine, methionine, Leucine, and isoleucine, gradient programmes need to be changed.
- Changes to the sample preparation, such as post-column derivitization, and the gradient program's evaluation of additional peaks, are required.

4.2 Trial 2
4.2.1 Observation
- Only 13 peaks, including aspartic acid, glutamic acid, serine, glycine, glutamine, threonine, alanine, histidine, tyrosine, valine, methionine, leucine, and isoleucine, were seen in the amino acid solution.
- Tyrosine and valine peak shapes are not properly separated after 15 minutes (Figure c).
- The last few amino acids, such as phenylalanine, tryptophan, lysine, and proline, were not eluted.

4.2.2 Conclusion
- To enhance the morphologies of the eluted peaks, such as those for aspartic acid, glutamic acid, serine, glycine, glutamine, threonine, alanine, histidine, tyrosine, valine, methionine, leucine, and isoleucine, the mobile phase's composition has to be changed.
- Changes to sample preparation, such as post-column derivitization, and the composition of the mobile phase are required to evaluate different peaks.

4.3 Trial 3
4.3.1 Observation
- Only 13 peaks, including aspartic acid, glutamic acid, serine, glycine, glutamine, threonine, alanine, histidine, tyrosine, valine, methionine, leucine, and isoleucine, were seen in the amino acid solution.
- Tyrosine and valine peaks did not separate as a result of this chromatographic modification (Figure d).
- The last few amino acids, such as phenylalanine, tryptophan, lysine, and proline, were not eluted.

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4.3.2 Conclusion

- To optimise the morphologies of the eluted peaks of aspartic acid, glutamic acid, serine, glycine, glutamine, threonine, alanine, histidine, tyrosine, valine, methionine, leucine, and isoleucine, it is necessary to alter the composition of the mobile phase as well as the gradient programme.
- Need to alter sample preparation, such as post-column derivitization, as well as the mobile phase's composition and gradient programme in order to evaluate different peaks.

4.4 Trail 4

4.4.1 Observation:

- All impurities, including phenylalanine, tryptophan, lysine, proline, aspartic acid, glutamic acid, serine, glycine, glutamine, threonine, alanine, histidine, tyrosine, valine, methionine, and leucine and isoleucine, are thoroughly separated (Figure e).
- No interference was seen using the placebo solution or the blank.

4.4.2 Conclusion:

- The experiment's methodology worked well. However, enough separation of all peaks was not observed, necessitating additional gradient analysis.

4.5 Trial 5

4.5.1 Observation:

Spike sample solution Separation is ok, but RT of lysine and proline is closer to each other i.e. Resolution between then is 1.36 (Figure f).

4.5.2 Conclusion:

In this experiment method workout satisfactorily. But proper separation of all peaks not found satisfactory hence further gradient study needs to be performed.

4.6 Trial 6

4.6.1 Observations

- All amino acids, including phenylalanine, tryptophan, lysine, proline, aspartic acid, glutamic acid, serine, glycine, glutamine, threonine, alanine, histidine, tyrosine, valine, methionine, and leucine and isoleucine, are resolved with excellent peak separation (Figure g).
- There was no placebo or blank interference when the contaminants' retention times peaked.

4.6.2 Conclusion

- From observation this methodology (Table e) can be finalized and Prevalidation study needs to be performed.

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5 Figures and Tables

5.1 Figures

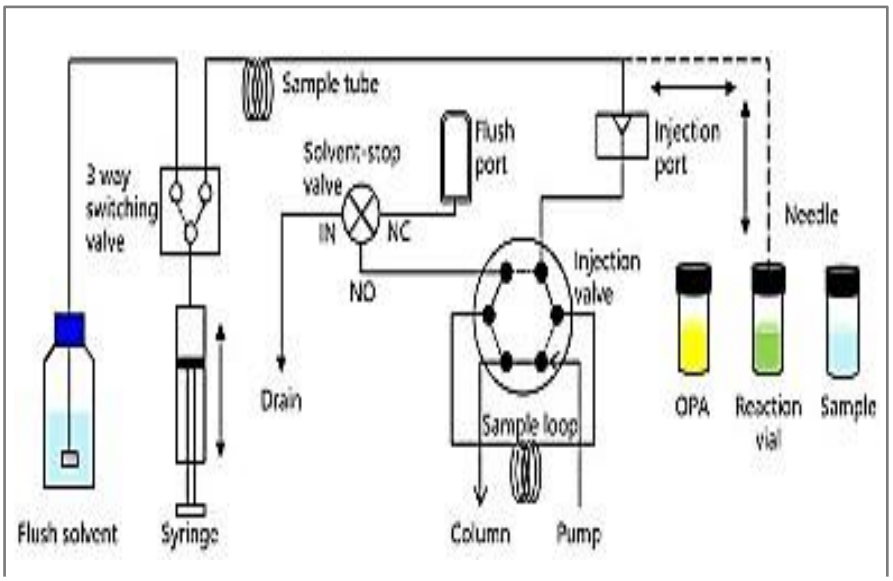

Figure (a) Mechanism of the pre-column derivatization technique.

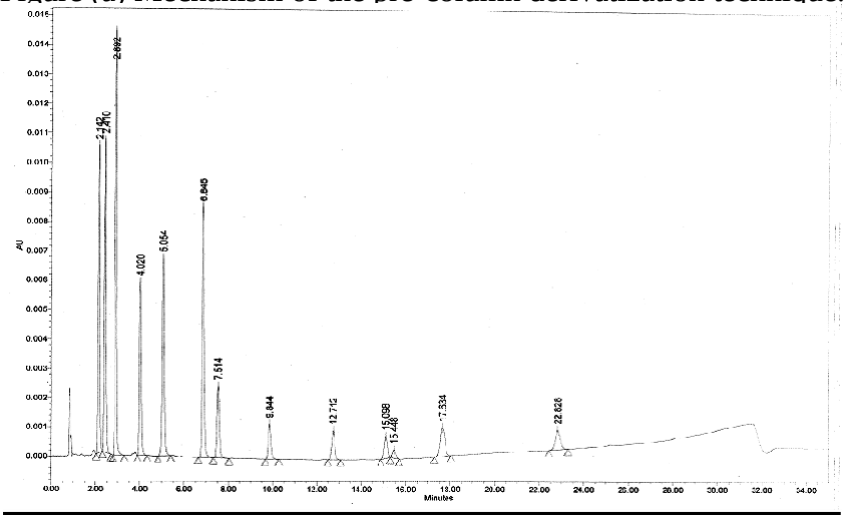

Figure (b) Chromatogram for trial 1 in Amino Acids (Precolumn Derivatization)

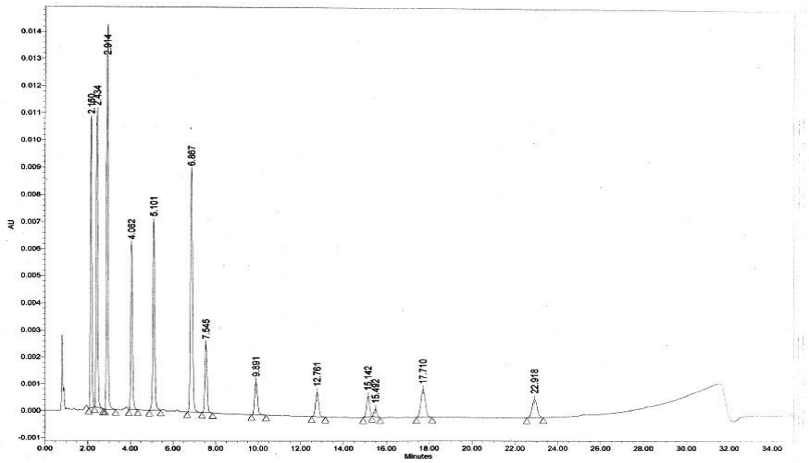

Figure (c) Chromatogram for trial 2 in Amino Acids (Precolumn Derivatization)

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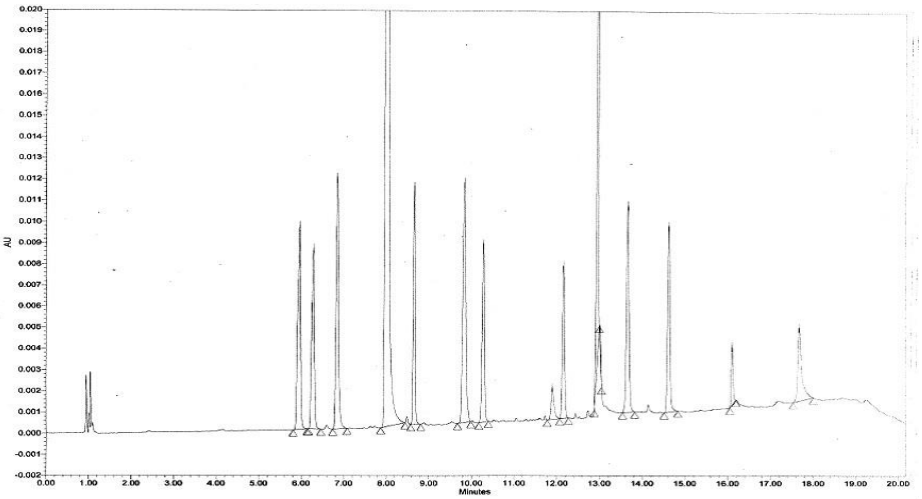

Figure (d) Chromatogram for trial 3 in Amino Acids (Precolumn Derivatization)

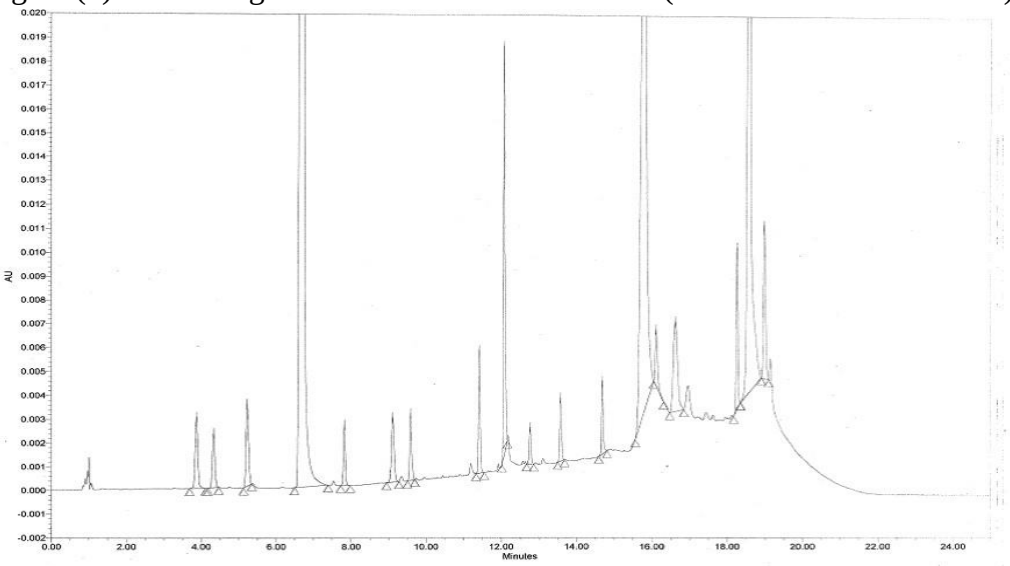

Figure (e) Chromatogram for trial 4 in Amino Acids (Precolumn Derivatization)

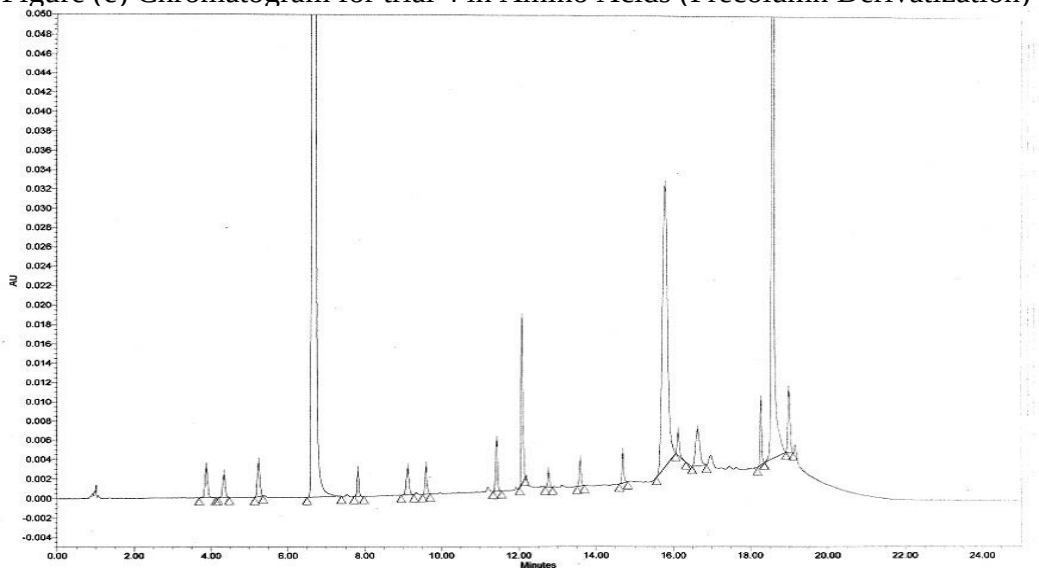

Figure (f) Chromatogram for trial 5 in Amino Acids (Precolumn Derivatization)

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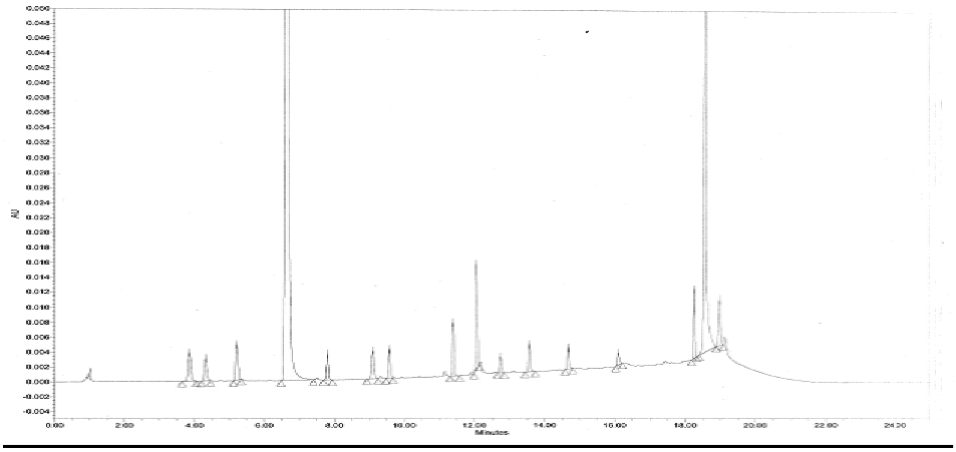

Figure (g) Chromatogram for trial 6 in Amino Acids (Precolumn Derivatization)

5.2 Tables

Table (a) Reagents, chemicals, filters and column for Trial 2 in amino acids

Sr. No.	Reagents, chemicals, filters and column	Grade	Make
1.	Ortho-phosphoric acid (88%)	HPLC	Honeywell
2.	Sodium dihydrogen phosphate monohydrate	EMPARTA®	Merck
3.	Acetonitrile	Supra-Gradient	Bisolve
4.	0.22 µm PVDF membrane filter (Item No.HVLP04700)	--	Millipore
5.	0.45µ Nylon Syringe filter (Cat No. SYNN0602MNXX104)	--	Mdi
6.	YMC Pack ODS-AM, 150 x 4.6 mm, 3µm(P/N –AM12S03-1546WT)	--	YMC Pack

Table (b) Gradient Program for Trial 4 in amino acids (Precolumn Derivatization)

Time (min.)	Mobile Phase A (%)	Mobile Phase B (%)	Mobile Phase C (%)
0	68	30	2
2	68	30	2
10	62	30	8
15	62	30	8
20	60	28	2
40	60	28	2
50	68	30	2
60	68	30	2

Table (c) Gradient Program for Trial 4 in amino acids (Precolumn Derivatization)

Time (min.)	Mobile Phase A (%)	Mobile Phase B (%)	Mobile Phase C (%)
0	68	30	2
2	68	30	2
10	62	30	8

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15	62	30	8
25	60	35	5
40	68	35	5
50	68	30	2
60	60	30	2

Table (d) Gradient Program for amino acids (Precolumn Derivatization)

Time (Minutes)	**Mobile Phase A (%)**	**Mobile Phase B (%)**	**Mobile Phase C (%)**
0	70	28	2
2	70	28	2
10	60	35	5
18	60	35	5
25	60	32	8
45	60	32	8
55	70	28	2
65	70	28	2

Table (e) Simultaneous estimation of amino acid with precolumn derivatization Using HPLC

Sr.no	**Peak Name**	**Retention Time in (min)**	**Area**	**% Area**	**Height**	**USP Resolution**	**USP Tailing**	**USP Plate count**
1	Aspartic acid	0.301	17311	2.65	5226	1.3	1.0	7449
2	Glutamic acid	4.111	12662	1.94	3910	2.7	1.0	9764
3	Serine	4.204	19939	3.06	6398	8.5	1.2	19537
4	Glycine	5.201	27517	4.22	5634	19.90	0.9	31192
5	Glutamine	6.201	27517	4.22	5634	19.90	0.9	31192
6	Threonine	7.502	2373	0.36	831	15.60	1.0	147385
7	Alanine	9.104	17676	2.71	8007	2.4	1.0	259777
8	Histidine	9.401	4390	0.67	3180	8.1	1.0	699783
9	Tyrosine	11.302	7161	1.10	3899	7.5	1.0	432548
10	Valine	12.012	9192	1.41	5628	6.9	1.1	606860
11	Methionine	12.302	3710	0.57	1305	3.3	1.1	240369
12	Leucine	13.102	13382	2.05	1989	2.5	1.1	37769
13	Isoleucine	14.502	3585	0.55	642	1.5	1.0	57452
14	Phenylalanine	16.0205	14221	2.18	6349	14.40	1.1	528543
15	Tryptophan	18.30	419157	64.28	89877	2.9	1.1	138935
16	Lysine	19.020	39443	6.05	9262	2.8	0.9	168927
17	Proline	19.102	11150	1.71	3368	1.1	1.1	266351

6 Conclusion

This article provides a variety of analytical methods that can be used to simultaneously estimate and evaluate amino acids. Using High Performance Liquid Chromatography

(HPLC) technology, numerous amino acid estimation techniques have been devised over time by various researchers. Pre-column derivatization has been shown to be more effective and efficient for the simultaneous estimation of amino acids than post-column derivatization (20). Pre-column derivatization requires less time and yields more precise and exact analytical results than post-column derivatization. In addition to HPLC, we discovered that there are additional methods for measuring amino acids. The capillary electrophoresis (CE) method could be considered for estimation purposes. Even without derivatization, the outcomes of this procedure are as anticipated. Nonetheless, as pre-column derivatization enhances analytical sensitivity and precision, it would eventually be favored (21).

7 Acknowldegements

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