CODEN [USA]: IAJPBB ISSN: 2349-7750

INDO AMERICAN JOURNAL OF
PHARMACEUTICAL SCIENCES

http://doi.org/10.5281/zenodo.1048813

Available online at: http://www.iajps.com Research Article

FLOW INJECTION DETERMINATION OF ASPARAGINE AND HISTIDINE USING DIPERIODATOCUPERATE BASED ON SPECTROPHOTOMETRIC INHIBITION DETECTION IN AMINO ACID SUPPLEMENTS

Zahoor Ahmad[1*], Muhammad Asghar[1], Samar Ali[1], Nusrat Munawar[2], Abdul Nabi[1], Mohammad Yaqoob[1]

[1]Department of Chemistry, University of Balochistan, Quetta–87300, Pakistan.
[2]Department of Chemistry, Sardar Bahadur Khan Women's University, Quetta, Pakistan

Abstract:
A rapid and sensitive spectrophotometric method is developed for the determination of asparagine (Asn) and histidine (His) based on their inhibition effect on diperiodatocuperate (DPC) absorption intensity at 415 nm in micellar alkaline medium using a double beam spectrophotometer. The effect of different experimental parameters such as concentration of DPC, potassium hydroxide and cetyl trimethyl ammonium bromide, flow rate, sample loop volume and reaction coil length was examined. The method presented a linear range of 0.2 – 10 μg mL^{-1} for Asn and 0.35 – 10 μg mL^{-1} for His under the optimized conditions. The limits of detection (LODs) were 0.06 μg mL^{-1} for Asn and 0.1 μg mL^{-1} for His with injection throughput of 120 h^{-1}. The limits of quantification (LOQs) were 0.2 μg mL^{-1} for Asn and 0.33 μg mL^{-1} for His. The relative standard deviations (RSDs, n = 10) of the method were 2.9 % and 3.4% for Asn and His at 0.1 μg mL^{-1} concentrations level. The method was applied for the determination of Asn and His amino acid supplements.

Keywords: *Flow injection analysis, UV-Vis spectrophotometry, Diperiodatocuperate, Asparagine, Histidine, Amino acid supplements*

***Corresponding author:**
Zahoor Ahmad,
Department of Chemistry,
University of Balochistan,
Quetta–87300, Pakistan.
E-mail address: zahoorahmad548@yahoo.com

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Please cite this article in press as Zahoor Ahmad et al, ***Flow Injection Determination of Asparagine and Histidine Using Diperiodatocuperate Based on Spectrophotometric Inhibition Detection in Amino Acid Supplements****, Indo Am. J. P. Sci, 2017; 4(11).*

INTRODUCTION:

Amino acids are fundamental building units of proteins and considered essential for numerous processes such as growth, formation of new tissues, generation of histamine, adrenaline, insulin and urea [1]. Imidazole side chain of His is considered to be the catalytic sites in many enzymes. It has the ability to participate in acid-base catalysis due to protonated and un-protonated states [2]. Histidine acts as a precursor for carnosine biosynthesis and can be converted into antioxidant and also found in skeletal muscles [3, 4]. Histidine possesses protective role against oxidative damage by 1 mM H2O2 solution in cataract model [5]. Amino acids are determined by various methods such as titrimetric [6], chromatographic [7] electroanalytical [8], electrophoresis [9], fluorescence [10], high performance liquid chromatography (HPLC) [11], chemiluminescence [12] and UV–Vis spectrophotometry [13, 14]. However, various analytical techniques have been reported such as using an HPLC or a specific amino acid analyzer [15], complex formation of vanadium(III) with L-histidine [16], identification of histidine by gas chromatography in food supplements [17] and development of a histidine-targeted spectrophotometric sensor [18].

Aspartic acid increases the mineral supplements absorption, promoting wound healing, reduces the fatigue, lowers the blood pressure, protects the liver by removing excess ammonia and other toxins from the bloodstream and is important for brain, function of RNA, DNA and production of immunoglobulin and antibodies [19]. The asparagine (Asn) is biosynthesized, enters the citric acid cycle as oxaloacetate in humans. Usually Asn plays essential role for the function and growth of the brain beside synthesis of ammonia [20]. The adding of acetyl-glucosamine to Asn can be achieved by oligo-saccharyl-transferase enzymes in the endoplasmic reticulum [21]. Such glycosylation is significant for both protein function and protein structure [22, 23]. Asn has been examined by column chromatography i.e., HPLC method [24] and a new approach HPLC-FTIR Malliard system [25]. The chemical structures of Asn and Histidine are shown in Figure 1.

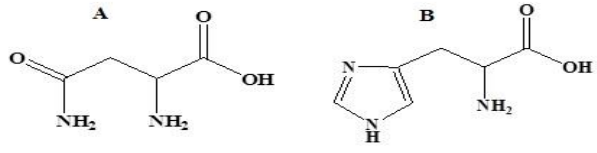

Fig 1: Chemical structures of A) Asparagine ($C_4H_8N_2O_3$, MW = 132.12) and B) Histidine ($C_6H_9N_3O_2$, MW = 155.16).

In this manuscript, a flow injection-spectrophotometric (FI-spec) method was developed for the determination of Asn and His using the quenching effect of these amino acids on the absorbance of diperiodatocuperate (DPC) in an alkaline medium. The effect of reagents concentration such as DPC, potassium hydroxide (KOH), cetyltrimethylammonium bromide (CTAB) and physical parameters such as sample loop volume and reaction coil length variables were investigated. The limits of detection (LODs) were 0.06 µg mL^{-1} and 0.1 µg mL^{-1} and the limits of quantification (LOQs) were 0.2 µg mL^{-1} and 0.33 µg mL^{-1} for Asn and His respectively. The relative standard deviations (RSDs) for Asn and His were 2.9 % (n = 10) and 3.4% (n = 10) at 0.1 µg mL^{-1} concentration level respectively and the injection throughput was 120 h^{-1}. The method was applied for the determination of Asn and His in amino acid supplements.

EXPERIMENTAL:

Instrumentation

The proposed two channel FI-spec manifold for the determination of Asn and His is shown in Figure 2. A four channel peristaltic pump (Ismatec: Reglo, Switzerland) was used to propel all the streams at a flow rate of 1.0 mL min^{-1}. The Asn/His standards were injected (120 µL) through rotary injection valve (Rheodyne 5020, Anachem, Luton, UK) into the carrier stream (0.01% CTAB w/v) and mixed with DPC (0.1 mM in 0.01 M KOH) stream. After passing the merged stream through reaction coil (100 cm), the absorbance was measured at 415 nm using a glass flow through cell (path length 1 cm, volume 80 µL, Hellma Analytics, Germany) fixed in a double-beam UV/Vis spectrophotometer (model UV-1700, Shimadzu, Japan). All the connections of FIA manifold and the reaction coil were made using PTFE tubes (0.8 mm i.d., Fischer scientific, Loughborough, UK). The output was recorded on a chart recorder (BD40, Kipp & Zonen, Delft, The Netherlands).

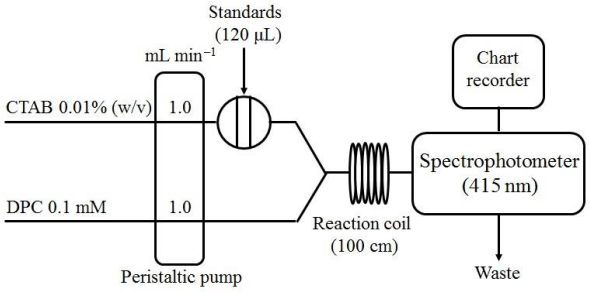

Fig. 2: FI-spectrophotometric manifold for the determination of Asn and His.

Reagents and chemicals

The reagents and chemicals used were of analytical reagent grade and all the solutions were prepared in ultra-high-purity (UHP) deionized water (Egla, Pure-lab Option, and UK). All the plastic and glassware were washed in detergent overnight followed by soaking in hydrochloric acid (HCL; 6% v/v) for a week and rising with deionized water for several times.

The DPC was prepared according to the previously reported procedures [26, 27]. For this purpose, potassium periodate (0.23 g), copper sulfate pentahydrate (0.125 g), sodium persulfate (0.14 g) and potassium hydroxide (0.8 g) were added to deionized water and the mixture was heated with constant stirring for about 20 min on a hot plate. The resulted red mixture was boiled for another 20 minutes for completion of the reaction. The mixture was cooled to room temperature and filtered through a sintered crucible. Using an ice bath, the filtrate was further cooled and then filtered. For the separation of synthesized complex from solution and crystallization, the mixture was left at room temperature for some time and was added 50% sodium nitrate in excess. The crystals were washed several times with deionized water after filtration and then dried. The synthesized DPC in KOH solution exhibited two absorption maxima at 263 and 415 nm. Fresh solutions of DPC were prepared in 0.01 M KOH when required and its concentration was calculated by measuring its absorbance at 415 nm spectrophotometrically (molar absorptivity ε = 6230 mol^{-1} L cm^{-1}).

Stock solutions (1000 µg mL^{-1}) of different amino acids such as histidine, asparagine, phenyl-alanine, tryptophan, arginine, cysteine, methionine, valine, aspartic acid, glutathione, glycine, acetyl cysteine, tyrosine, leucine, proline, glutamic acid and serine (Sigma Aldrich Chemical Company, USA) were prepared by dissolving 10 mg of each amino acid in 10 mL deionized water, sonicated for 5 min and stored at 4 ºC. Working standard solutions of these amino acids were prepared by serial dilution of the stock solutions with deionized water containing CTAB (0.01% w/v).

Stock solutions (500 µg mL^{-1}) of various organic compounds i.e., polyethylglycol, folic acid, tartaric acid, lactose, fructose, pantothenic acid and riboflavin were prepared by dissolving in deionized water and working standards were prepared by diluting the required aliquot of their stocks in deionized water CTAB (0.01% w/v).

Stock solutions (5% w/v) of various surfactants such as CTAB, Tween-20, Brij-35, SDS and Triton-X-100 were prepared by mixing 0.5 g of each surfactant in 10 mL of deionized water and stored in brown glass bottles. Working standards of all the surfactants were prepared by serial dilution of their stocks solution with deionized water.

Stock solutions (100 µg mL^{-1}) of different cations (Na^+, K^+, Mg^{2+}, Fe^{2+}, Fe^{3+}, Mn^{2+} and Sn^{2+}) and anions (NO_3^-, Cl^-, HCO_3^-, SO_4^{2-}, and PO_4^{3-}) were prepared by dissolving the required quantity from their respective salts and working standards were prepared from these stocks in deionized water containing CTAB (0.01% w/v).

JoMar's L-Asparagine and Lamberts® L-Histidine amino acid supplements were obtained from local market for analysis.

Sample preparation

The powdered content of 2 capsules (500 mg) of each of Asn and His samples were mixed thoroughly and 500 mg of each amino acid sample was dissolved in 100 mL deionized water. After sonication for almost 5 min, the solutions were filtered to remove any insoluble material. Appropriate aliquots were taken from these solutions and diluted with deionized water. For recovery experiments a series of standard solutions over the range of 0.5 – 1.5 µg mL^{-1} in deionized water containing CTAB (0.01% w/v) from these samples were spiked with Asn and His standards (0.5 – 1.5 µg mL^{-1}). The un-spiked and spiked samples were injected into the proposed FI-spec manifold to evaluate the applicability of the method.

RESULTS AND DISCUSSION:

Kinetic studies

The kinetics of Asn or His and DPC reaction in alkaline medium were studied by using a batch method. In this method, UV-Vis spectrum of DPC was taken from 250 – 600 nm and the DPC exhibited two wavelength maxima i.e. 263 and 415 nm (curve a) as shown in Figure 3 and 4. The DPC in quartz cuvette was then added Asn or His and quenching of DPC absorbance was observed after each 5 seconds as shown in Figure 3 (curve b and c) and Figure 4 (curve b – f) respectively. Almost 97.5% absorbance of DPC was quenched by Asn after 10 second while 96% was quenched by His in 25 seconds. These studies show that the redox reaction between DPC and Asn or His is fast enough and a FI-spec method with quenching effect can be established for the determination of such amino acids by selecting wavelength maximum for absorbance measurement at 415 nm.

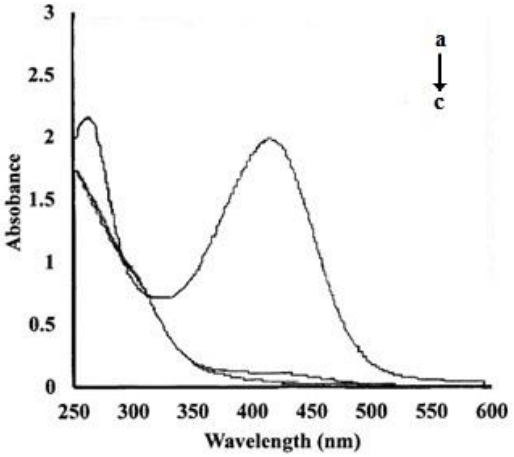

Fig 3: Batch kinetic UV-Vis spectrum between DPC–Asn in KOH 0.01 M: a = DPC (0.32 mM), b – c = DPC (0.32 mM) – Asn (1 µg mL^{-1}) after 5 and 10 seconds respectively.

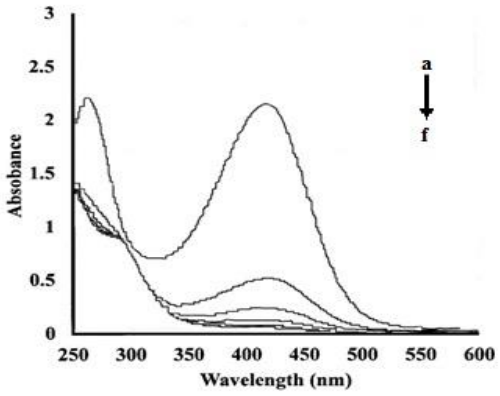

Fig 4: Batch kinetic spectrum between DPC–His in KOH 0.01 M: a = DPC (0.32 mM), b – f = DPC (0.32 mM) – His (1 µg mL^{-1}) after 5, 10, 15, 20 and 25 seconds respectively.

Optimization Studies of chemical and physical variables

The effect of various chemical and physical parameters was investigated for maximum sensitivity, selectivity, sample throughput and dynamic linear range. The effect of chemical variables includes the optimization of concentration of DPC, KOH and CTAB and physical variables include the effect of sample volume, flow rate and reaction coil length. The concentration of Asn and His selected for optimization studies was 2 µg mL^{-1}.

Effect of DPC concentration

The effect of DPC concentration was optimized over the range of 0.01 – 0.3 mM. As the concentration of DPC increased, the increase in % inhibition of absorbance intensity was observed up to 0.1 mM. Further increase in DPC concentration resulted in decrease of % inhibition of absorbance intensity as shown in Figure 5. Therefore, DPC of 0.10 mM was selected for subsequent experiments both for Asn and His.

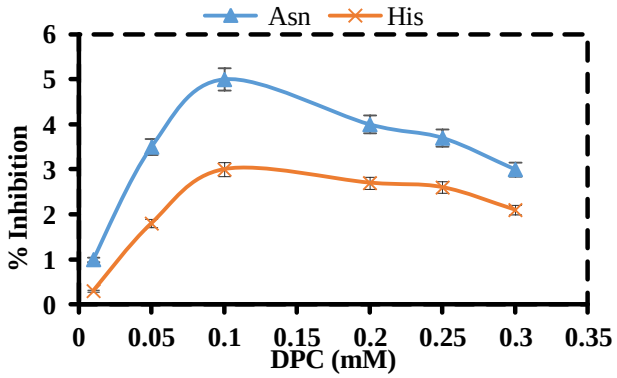

Fig 5: Effect of DPC concentration: KOH 0.1 M, Asn and His 2.0 µg mL^{-1} each; flow rate 1.5 mL min^{-1}, sample loop volume 60 µL and reaction coil length 100 cm.

Effect of KOH concentration

It is well known that DPC solution is stable in an alkaline medium. The influence of potassium hydroxide (KOH) concentration was examined over the range of 1 – 25 mM. The % inhibition of absorbance increased with the increase in KOH concentration up to 10 mM and further increase in KOH concentration drastically decreased the % inhibition as shown in Figure 6. Therefore, KOH of 10 mM was selected as an optimum and used in further experiments.

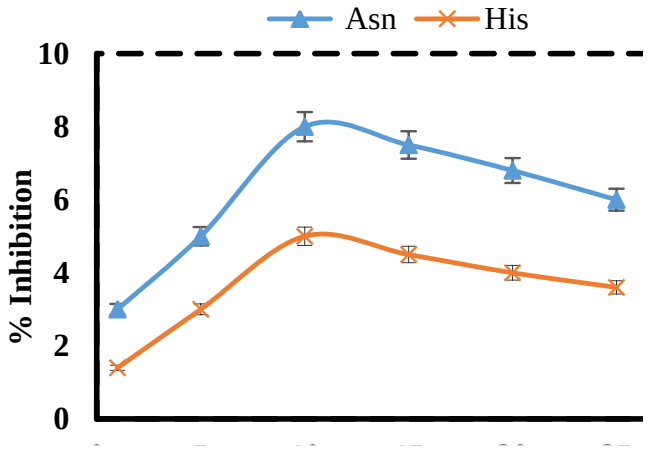

Fig 6: Effect of KOH concentration: DPC, 0.1 mM; Asn and His, 2.0 µg mL^{-1} each; flow rate, 1.5 mL min^{-1}; sample loop volume 60 µL and reaction coil length 100 cm.

Effect of surfactants

Comparison studies of various surfactants including Brij-35, SDS, Tween 20, CTAB and Triton–X 100 (0.015% w/v each) were performed, when propelled

as carrier stream in place of deionized water. The highest % inhibition both for Asn and His was obtained when CTAB solution was employed as carrier stream as shown in Figure 7. The increase in % inhibition of both the amino acid can be ascribed to increase in solubility and micelle formation in the presence of CTAB.

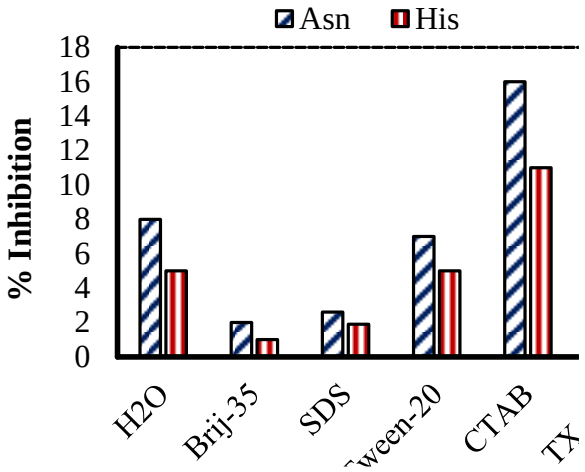

Fig 7: Effect of various surfactants: Asn and His, 2.0 µg mL^{-1} each; DPC, 0.1 mM; KOH, 10 mM; flow rate, 1.5 mL min^{-1}; sample loop volume 60 µL and reaction coil length 100 cm.

As a result, CTAB was selected as a carrier stream and its concentration was optimized over the range of 0.001 – 0.02% (w/v). As the concentration of CTAB is increased, the % inhibition was also increase up to 0.01% (w/v). Further increase in CTAB concentration resulted in decline of % inhibition of absorbance intensity as shown in Figure 8. Therefore, 0.01% of CTAB was selected as an optimum and used in subsequent experiments.

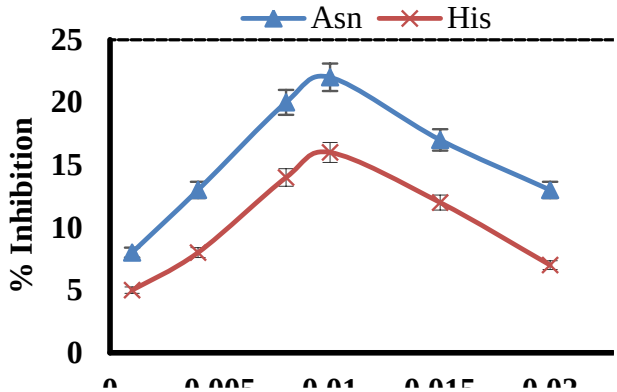

Fig 8: Effect of CTAB concentration: DPC, 0.10 mM; Asn and His, 2.0 µg mL^{-1} each; flow rate, 1.5 mL min^{-1}; sample loop volume 60 µL and reaction coil length 100 cm.

Effect of physical parameters

The effect of different physical variables such as sample loop volume, flow rate and reaction coil length was investigated as shown in Table 1. The effect of flow rate was checked over the range of 0.5 – 3.0 mL min^{-1}. The % inhibition of absorbance intensity was increased with the increase in flow rate up to 1.5 mL min^{-1} and therefore, flow rate of 1.5 mL min^{-1} was chosen as an optimum. The effect of sample loop volume was also studied over the range of 60 – 300 µL. As the sample volume increased, the % inhibition absorbance intensity was also increased up to 120 µL and further increase in sample loop volume did not result in an appreciable increase in % inhibition. Therefore, sample loop volume of 120 µL was selected as an optimum. The effect of reaction coil length was checked over the range of 50 – 120 cm and 100 cm was chosen as an optimum due to increased % inhibition of absorbance intensity.

Table 1: Effect of different physical parameters on the % inhibition of absorbance intensity

Parameter	Range studied	Optimum
Sample loop volume (µL)	60 – 300	120
Flow rate (mL min^{-1})	0.5 – 3.0	1.0
Reaction coil length (cm)	50 – 300	100

Table 2: Maximum tolerable concentration for the determination of Asn and His (*n* = 3).

Species analyzed	Tolerance (fold)	
	Asn	His
Na^+, K^+, Mg^{2+}, PO_4^{3-}	1000	500
Cr^{3+}, Cl^-, SO_4^{2-}, HCO_3^-	800	400
Stearate, starch, sucrose, polyethylglycol	300	150
Tartaric acid, lactose, fructose	140	70
Tryptophan, folic acid and Mn^{2+}	32	16
Methionine, phenyl-alanine, tryptophan, aspartic acid, serine, arginine, glycine and valine	20	10
Cholesterol and iodine	10	5

Study of Interferences

The influence of various cations, anions and organic compounds which could be used as excipients in various pharmaceutical formulations of amino acids was checked on the blank and on the determination of Asn (0.5 µg mL^{-1}) and His (1.0 µg mL^{-1}) to evaluate the selectivity of the proposed method. The tolerance limit was selected as a concentration which can produce ±5% error in the % inhibition of absorbance intensity. Tolerable concentrations of 1000 and 500 fold for Na^+, K^+, Mg^{2+}, PO_4^{3-}, 800 and 400 fold for Cr^{3+}, Cl^-, SO_4^{2-}, HCO_3^-, 300 and 150 fold for stearate, starch, sucrose, polyethylglycol, 140 and 70 fold for tartaric acid, lactose, fructose, 32 and 16 fold for tryptophan, folic acid and Mn^{2+}, 20 and 10 fold of methionine, phenyl-alanine, tryptophan, aspartic acid, serine, arginine, glycine, valine and 10 and 5 fold for cholesterol and iodine were found for the determination of Asn and His respectively as shown in Table 2.

Analytical figures of merit

Under optimum physical and chemical parameters, linear calibration graphs were obtained between the concentrations of Asn and His and % inhibition of absorbance intensity as shown in Figure 9. The linear range for Asn was obtained over the range of 0.2 – 10 µg mL^{-1} with LOD, LOQ, coefficient of determination (R^2) and regression equation of 0.06 µg mL^{-1}, 0.2 µg mL^{-1}, 0.9968 and $y = 9.588x + 3.9485$ (y = % inhibition of absorbance intensity and x = concentration of Asn (µg mL^{-1})) respectively. The linear range for His was achieved over the range of 0.35 – 10 µg mL^{-1} with LOD, LOQ, coefficient of determination (R^2) and regression equation of 0.1 µg mL^{-1}, 0.33 µg mL^{-1}, 0.9989 and $y = 8.635x + 2.2797$ (y = % inhibition of absorbance intensity and x = concentration of Asn (µg mL^{-1})) respectively. The relative standard deviations (RSDs, n = 10) of the method were 2.9 % and 3.4% for Asn and His at concentration level of 0.1 µg mL^{-1} for each respectively. The injection throughput was 120 per hour. The Table 3 shows a comparison between the proposed method and other methods reported in the literature. Most of the reported methods such as HPLC and GC have the advantages but require expensive instruments, high expertise, toxic solvents and long operational time. The proposed method is better than the methods reported in the table in term of injection throughput.

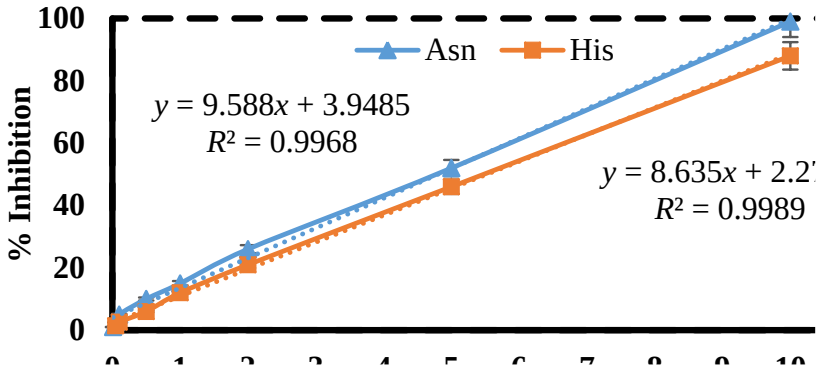

Fig 9: Calibration graphs for Asn and His.

Table 3: Comparison of the proposed FI-spec method with other reported methods for the determination of Asn and His.

Technique	Analyte	Sample matrix	Linear range (µg mL^{-1})	LOD (µg mL^{-1})	LOQ (µg mL^{-1})	Sample throughput h^{-1}	Reference
FI-spec	Asn	NR	8–1982	1.2	NR	30	[28]
Spec	His	NR	7.8–77.6	NR	NT	NR	[29]
CL	Asn	Biological	0.13–8	NR	NR	NR	[30]
	His	sample	0.16–23.3				
FI-spec	Asn	Food sample	26.4– 304	NR	NR	35	[31]
FI- pot	Asn	Food sample	13–264	NR	NR	30	[31]
CSV	His	Amino plasma Serum	0.016–0.19	NR	NR	NR	[32]
HPLC-PDA	Asn	Wine and beer	2.01−80.20	>0.4	NR	NR	[33]
	His		1.73−69.20	>0.4			
GC–MS	His	Human urine	1–300	0.5	NR	NR	[34]
HPLC-PDA	His	Drug	0.14–0.2	0.042	0.14	NR	[35]
FI-spec	Asn	Amino acid	0.2–10	0.06	0.2	120	This method
	His	supplements	0.35–10	0.1	0.33		

FI = Flow injection, spec = spectrophotometry, CL = chemiluminescence, pot = potentiometric, CSV = cathodic stripping voltammetry, HPLC-PDA = high performance liquid chromatography-photodiode array detector, GC–MS = gas chromatography–mass spectrometry, NR = not reported

Applications and validation

Asn and His belong to non-essential class of amino acids. Asn plays a vital role in the function of nervous system and brain, and transformation of amino acid from one form to other in liver. The role of His is in the utilization and regulation of many important trace minerals in body such as copper, iron, zinc and manganese and also in the formation of red and white blood cells**.** The proposed FI-spec method was applied for the determination of Asn and His in amino acid supplements. Recoveries for the analysis of Asn and His obtained over the range of 93 ± 2.4 – 112 ± 2.9 and 91 ± 4.4 – 108 ± 5.6 respectively as shown in Table 4.

Table 4: Recoveries of Asn and His from amino acid supplements (*n* = 3).

Sample	Asn and His (mg L^{-1})			Recovery ±RSD (%)
	Actual	Added	Found ±SD	
Asn	0.5	0.00	0.56 ± 0.016	112 ± 2.9
		0.5	0.93 ± 0.022	93 ± 2.4
		1	1.49 ± 0.05	99 ± 3.4
	1	0.00	1.08 ± 0.02	108 ± 1.9
		0.5	1.42± 0.05	95 ± 3.5
		1	1.91 ± 0.08	96 ± 4.2
	1.5	0.00	1.41 ± 0.05	94 ± 3.5
		0.5	1.91 ± 0.09	96 ± 4.7
		1	2.42 ± 0.11	97 ± 4.6
His	0.5	0.00	0.54 ± 0.03	108 ± 5.6
		0.5	0.94 ± 0.05	94 ± 5.3
		1.0	1.44 ± 0.07	96 ± 4.9
		1.5	1.93 ± 0.06	97 ± 3.1
	1	0.00	0.91 ± 0.04	91 ± 4.4
		0.5	1.52± 0.05	101 ± 3.3
		1.0	1.92 ± 0.09	96 ± 4.7
		1.5	2.55 ± 0.10	102 ± 3.9
	1.5	0.00	1.51 ± 0.06	101 ± 4.0
		0.5	2.08 ± 0.04	104 ± 1.9
		1.0	2.44 ± 0.07	98 ± 2.9
		1.5	3.10 ± 0.09	103 ± 2.9

Table 5: Determination of Asn and His in amino acid supplements with the proposed FI-spectroscopy method.

Sample	Labeled quantity	Proposed FI-spec method Found
Asn capsule	500 mg capsule^{-1}	494 mg
His capsule	500 mg capsule^{-1}	503 mg

The validation of the proposed method was performed by analyzing two amino acid supplements for Asn and His and the quantity found was almost similar to the labeled quantity which means the method is accurate for the determination of Asn and His in amino acid supplements as shown in Table 5.

CONCLUSION:

A rapid and sensitive FI-spec inhibition method was developed for the determination of Asn and His in the amino acid supplements. The absorbance intensity of DPC at 415 nm was quenched in the presence of Asn and His in micellar alkaline medium. The linear range for Asn was obtained over the range of 0.2 – 10 µg mL^{-1} with LOD and LOQ of 0.06 µg mL^{-1} and 0.2 µg mL^{-1} respectively and linear range for His was achieved over the range of 0.35 – 10 µg mL^{-1} with LOD and LOQ of 0.1 µg mL^{-1} and 0.33 µg mL^{-1} respectively. The relative standard deviations (RSDs, n = 10) of the method were 2.9% and 3.4% for Asn and His at concentration level of 0.1 µg mL^{-1} for each respectively. The injection throughput was 120 per hour. Recoveries for the analysis of Asn and His obtained over the range of 93 ± 2.4 – 112 ± 2.9 and 91 ± 4.4 – 108 ± 5.6 respectively. The method was sensitive and rapid for the determination of Asn and His in amino acid supplements.

ACKNOWLEDGEMENT:

The authors would like to acknowledge the Department of Chemistry, University of Balochistan, Quetta for providing the laboratory facilities.

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