Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy 173 (2017) 897–903

Contents lists available at ScienceDirect

Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy

journal homepage: www.elsevier.com/locate/saa

Ninhydrin-sodium molybdate chromogenic analytical probe for the assay of amino acids and proteins

Shivakumar Anantharaman [a,*], Nagaraja Padmarajaiah [b], Naef Ghllab Saeed Al-Tayar [c], Ashwinee Kumar Shrestha [d]

[a] PG Department of Chemistry, St. Philomena's College, Bannimantap, Mysore 570015, India
[b] DOS in Chemistry, Manasagangotri, Mysore 570 006, India
[c] Thamar University, Faculty of Applied Science, Department of Chemistry, Thamar, Yemen
[d] Department of Pharmacy, Kathmandu University, Nepal

ARTICLE INFO

Article history:
Received 19 December 2015
Received in revised form 20 October 2016
Accepted 20 October 2016
Available online 21 October 2016

Keywords:
Amino acid
Ninhydrin
Sodium molybdate
Hydrindantin
Ruhemann's purple
Leucine equivalents
Kjeldahl nitrogen

ABSTRACT

A sensitive method has been proposed for the quantification of amino acids and proteins using ninhydrin and sodium molybdate as chromogenic substrates in citrate buffer of pH 5.6. A weak molybdate-hydrindantin complex plays the role in the formation of Ruhemann's purple. The linear response for the amino acid, amino acid mixture and Bovine serum albumin is between 0.999 and 66.80 μM, 1.52 and 38 μM and 5 and 100 μg/L, respectively. The molar absorptivity of the individual amino acid by the proposed reaction extends from 0.58×10^4 to 2.86×10^4 M^{-1} cm^{-1}. The linearity equations for the proposed ninhydrin-molybdate for amino acid mixture is Abs = $0.021 \times$ Conc (μM) − 0.002. The applicability of the proposed method has been justified in food and biological samples in conjunction with Kjeldahl method.

1. Introduction

The notion that the nutritional value of foodstuffs depends on their chemical composition dates from the 19th century. Atwater, in 1894 states that "Chemical composition information is essential to improve family budget". At the same time this knowledge made the development of the first concept about the relation between diet and public health possible. McCance and Widdowson proposed in 1940 that "A knowledge of the chemical composition of food is the first essential in the dietary treatment of disease or any quantitative study of human nutrition" [1,2]. Currently, the importance of composition of food products information has been reassessed due to its wide range of application related to diet and nutritional programs, nutritional value labeling, nutritional education, international trade, promotion of new crops and transformation and consumption of new species, among others [3]. Availability of reliable data about the composition of food is essential. Therefore, exact analytical techniques, better still if they are fast and economical, are required.

The commonly available methods for the analysis of proteins and amino acids include biuret method [4], Lowry method [5], CB G-250 dye binding [6], bicinchoninic acid assay [7], Genipin [8], *o*-pthaldehyde [9], NBD-F [10], 3-(4-carboxybenzoyl)quinoline-2-carboxyaldehyde [11], Bromocresol green [12], Nile blue [13], Amido black [14], Ponceau [15], Erythrosin [16], thin layer chromatography [17], capillary electrophoresis-mass spectrometry [18], gas chromatography-flame ionization or mass spectrophotometric detection [19], and RP-HPLC [20]. Specific methods are also available for amino acids such as chemiluminescence-glutamine [21], and spectrophotometry-tryptophan [22]. Some of the methods that has been proposed includes differential scanning calorimetry-circular dichroism-UV spectroscopy [23], mass spectrophotometry [24], capillary electrophoresis-electrospray ionization-tandem mass spectrometry [25], HPLC with fluorescence detection [26], gas chromatography–mass spectrometry [27], micro-biomarker detection [28], ion-pairing liquid chromatography–high-resolution mass spectrometry [29], micellar electrokinetic chromatography with laser-induced fluorescence detection [30] and HPLC/electrospray negative ion tandem mass spectrometry [31].

Some of these methods, specifically colorimetric assay techniques have their own disadvantages; biuret protein determination interferes with constituents such as Tris buffer, sucrose, lactose, primary amines, glycerol, and dextran; the high dependence of the assay on protein composition presents a major problem to the broad use of CB binding as a quantitative protein assay and dye solubility in ethanol and acetic acid presents one of the major disadvantages; substituents such as EDTA, glucose, fructose, lactose, hydrogen peroxide, phospholipid, and Zwitter

* Corresponding author.
E-mail addresses: shivakem77@yahoo.co.in, shivakem77@gmail.com (S. Anantharaman).

http://dx.doi.org/10.1016/j.saa.2016.10.040
1386-1425/

ion buffers that have a tendency to bind to the cupric ions, dithiothrethiol, glutathione, and 2-mercaptoethanol. The fluorometric, HPLC, gas chromatography-mass spectrophotometry, micellar electrokinetic chromatography and tandem mass spectrophotometry methods involve high cost towards analysis as the instrument is not economical. Besides, the assay is so sensitive that it may require the dilution of the sample to be analyzed.

Several efforts have been made so far to find a generally applicable method for the analysis of amino acids. These include non-economical methods like gas chromatography and high performance liquid chromatography. Although these methods are highly sensitive in measuring picomolar levels of individual amino acids, they require time consuming pre or post-column derivatization and long chromatographic runs. It is often necessary to determine total free amino acids in very small amounts of biological tissues without chromatographic separation of individual amino acids and hence their routine determination requires a rapid and sensitive method.

Moore and Stein developed a reliable method for the determination of total amino acids using the ninhydrin reagent. Their method was further improved and utilized in the determination of amino acids in biological samples by automated amino acid analyzers. Doi et al. [32] modified Moore and Stein procedure using cadmium instead of tin in the preparation of ninhydrin reagent. As a result of this modification, many kinds of peptides were not detected. Therefore this latter method was proposed as appropriate in the determination of peptidase activity. Although the classical ninhydrin methods are precise and reliable for the determination of total amino acids they have limited sensitivity and are not suitable for the determination of the amino acid concentration in tissue samples which are available in very small quantities.

Determination of potency of amino acids may be done microbiologically since they are nutrients. Several classical methods have been developed. Some of them are modified so that they become specific to certain amino acids. The liberation of gaseous products like carbon dioxide, nitrogen, ammonia etc., by the reaction of primary amino acids with reagents has been developed. The volume of gas released is measured as an index of amino acid concentration. Some derivative methods involve the reaction of amino groups that include acid chloride, acid anhydrides, isocyanates, and dinitrofluorobenzene. The complexation reaction involving metals like copper, zinc, nickel, silver, and lead with amino acids has been accompanied by the analysis in the visible range. The important colorimetric method for the determination of amino acid involves the formation of purple colored hydrindantin product in weak acid solution with ninhydrin. This colorimetric technique presents some difficulties due to the instability of the color. Later stages of the research landed the method into modified procedures that included reagents such as sodium acetate [33], potassium cyanide [34], sodium borohydride [35], and ascorbic acid [36].

The objective of developing a sensitive method for amino acids and protein, the authors have attempted the use of ninhydrin and molybdate as reagents, which show a strong absorption at 570 nm. The narrow linearity range for the assay of amino acid and proteins makes the method more significant than the Ninhydrin alone. The assay has been adopted in the analysis of protein in various food and biological samples, concurrently using Kjeldahl procedure for the collection of ammonia. Effect of any foreign species on the reaction system has been analyzed while performing assay in biological and food samples. The proposed NSM is much more sensitive and superior compared to the classical ninhydrin method.

2. Experimental Details

2.1. Materials and Methods

A JASCO model UVIDEC-610 ultraviolet–visible (UV–vis) spectrophotometer with 1.0 cm matched cells was used for all absorbance measurements. A water bath shaker (NSW 133, New Delhi, India) was used to maintain a constant temperature for color development. All pH measurements and adjustments were done by a digital pH meter (model EQ-614, Equip-tronics, Mumbai, India).

2.2. Chemicals and Preparation of Reagents

All the amino acids and re-crystallized ninhydrin were purchased from BDH. Sodium molybdate and citric acid were purchased from SRL Chemicals, India. All other reagents used were of analytical grade and used without further purification. The standardization of sodium hydroxide for Kjeldahl method was carried out by preparing a standard solution of sodium carbonate (anhydrous) and sulphuric acid. Sufficient quantity of samples was used in the analysis for the determination of nitrogen content. Digestion of edible samples was carried out by a specially designed Kjeldahl flask for the collection of ammonia.

2.3. Preparation of Individual Amino Acids and Standard Mixture of Amino Acids

Each amino acid was prepared by dissolving 0.1 g in 100 mL of double distilled water and used as such or diluted appropriately in distilled water. A standard mixture of amino acids was prepared by dissolving L-serine, L-aspartic acid, L-methionine, L-glutamic acid, L-threonine, L-leucine, L-isoleucine, glycine, L-arginine, L-phenylalanine, L-histidine, L-alanine, L-tryptophan, L-valine, L-tyrosine, L-lysine, L-cysteine, and L-proline plus ammonium chloride such that each had the concentration of 0.4 mM (with a total concentration of 7.6 mM). The solutions of the non-targeted foreign species were prepared by using double distilled water.

2.4. Ninhydrin–Molybdate Reagent Preparation

This was prepared by dissolving 0.917 g of ninhydrin and 1.246 g of sodium molybdate in citrate buffer (pH 5.6) in a 25 mL standard flask. The final concentration of each ninhydrin and sodium molybdate was 0.206 M. The mixture prepared was used 6 h to get repeatable results.

2.5. Determination of Kjeldahl Nitrogen by Ninhydrin-Molybdate

Samples weighing ranging from 0.34 to 2.0 g placed in a Kjeldahl flask, 3.3 g of sodium sulphate and copper sulphate in the ratio of 10:1 and 10 mL of con. H_2SO_4 were added. Digestion was carried out at 430 °C until the solution was completely clear. Once the digestion was completed, the sample was distilled into a distillation flask and condensed into a highly alkaline solution by adding of 10 mL of 50% w/v NaOH. The released ammonia gets absorbed in 10 mL of 0.1 N H_2SO_4. The solution was made up to the mark in standard 50 mL flask. Then 10 μL volume was analyzed by the proposed method. The amount of total amino nitrogen in the sample was determined by standard addition method.

2.6. Procedure for the Quantification of Amino Acids by the Proposed Method

To each of the 10 mL standard flask, sufficient volume of the standard amino acid solution/amino acid mixture was transferred so that the final concentration was in the Beer's law range. Then, 0.5 mL of NSM reagent and 1.5 mL of citrate buffer (pH 5.6) were added. The mixture was boiled for about 10 min in a water bath and cooled under running tap water. The purple color developed on cooling was made up to the mark with distilled water, shaken well and the optical density of the colored solution was measured using 1.0 cm quartz cell at 570 nm against the reagent blank. The color developed is stable for >24 h which is normally sufficient to carry out the analytical procedures. The absorption spectrum of different combinations used in the reaction procedure is shown in Fig. 1.

A study related to the absorption visible spectra for the authentic RP in different 60:40 aqueous-nonaqueous solvent such as water with ethyl alcohol, DMF, DMSO revealed large differences in absorption at λ_{max}, and also ε values. The analysis of the above solvents was carried out by developing the RP in aqueous medium and the dilution was carried out by the respective 60:40 solvents such as ethyl alcohol, DMF and DMSO. A bathochromic shift has been observed in the change of the solvent from DMF to DMSO due to the stabilization of the RP. Finally, for the analysis of all amino acids, individually as well as in combined state, water was selected as a medium in justification of its economical and eco friendly property. The inset in Fig. 1 shows the effect of dilution in the respective 60:40 aqueous to non-aqueous solvents.

2.7. Evaluation of Optimized Conditions for the Assay Protocol

An attempt to add ninhydrin and molybdate separately to enhance the sensitivity of the colored reaction product was unsuccessful. Hence, equimolar concentration of about 0.206 M of ninhydrin and sodium molybdate was prepared by dissolving 0.917 g and 1.246 g respectively in 25 mL buffer solution of pH 5.6. The comparative graph to demonstrate the increase of sensitivity in the reaction that has been carried out for all the amino acids, shows increase in sensitivity limit by around 20 to 30 μM. The coefficient of regression for all the amino acids analyzed by this method has been around 0.999.

The effect of reaction conditions such as pH, volume of the buffer solution, reaction time, ninhydrin-molybdate reaction mixture, the sequential addition of reagents and temperature were studied.

2.7.1. Effect of pH

The buffer solutions that were studied for the color development were citrate buffer and acetate-phosphate buffer. The acetate-phosphate buffer was studied in the range 4.0 to 6.0 pH. As shown in Fig. 2, the experiment revealed that the optimal pH for maximum color development was 5.0–5.8. Citrate was comparatively more sensitive buffer solution system. Hence it was selected.

As the reaction was carried out in boiling water, the effective performance of the process at 100 °C was ascertained by optimization of temperature (Inset of Fig. 2). The optimization procedure has shown that relative color development against blank was maximum at its boiling temperature.

The results indicated that optical density is dependent on the order of addition of reagents. Hence, a sequence of addition was followed in the order amino acid, NSM mixture, and citrate buffer.

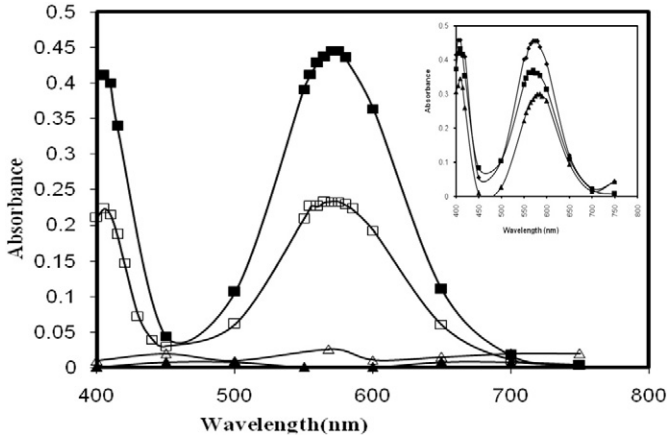


Fig. 1. The absorption spectrum of the RP color formation (■) Glycine + NSM mixture + citric acid-sodium hydroxide buffer (□) Glycine + ninhydrin + sodium molybdate + citric acid-sodium hydroxide buffer (Δ) Glycine + ninhydrin + citric acid-sodium hydroxide buffer (▲) Glycine + sodium molybdate + citric acid-sodium hydroxide buffer. The inset picture shows the effect of 60:40 ratio of aqueous:non-aqueous solvents on the RP color development.

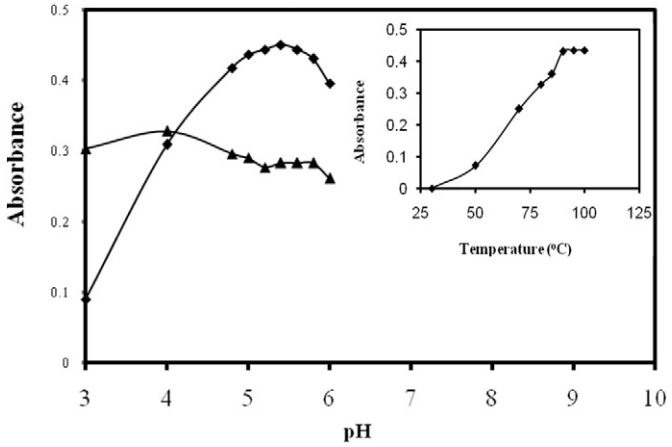


Fig. 2. Effect of buffer solutions (♦) citric acid-sodium hydroxide and (▲) citrate-phosphate on the reaction mixture. Inset picture shows the effect of temperature on the development of RP color.

3. Results and Discussion

3.1. Calibration Graph and Correlation

The calibration graph for individual amino acids/amino acids mixtures was constructed under optimized conditions. There was a good correlation between increase in absorbance and concentration of amino acid. The calibration graph for different amino acids and amino acid mixtures ranged from 0.999 to 66.80 μM and 1.52 to 38 μM. The lower and higher Beer's law limit for bovine serum albumin were 5 μg/L and 100 μg/L, respectively. The molar absorptivity of the individual amino acid by the proposed reaction extended from 0.58×10^4 to 2.86×10^4 M^{-1} cm^{-1}. Table 1 list out the optical parameters of the proposed NSM method for the amino acid mixture.

Table 1
Optical parameters for the determination of amino acid mixture by NSM method.

Parameters	Characteristics
Color	Ruhemann's purple
λ_{max}	570 nm
Stability	24 h
Beer's law range (μM)	1.52–38.0
Molar absorptivity (10^4 M^{-1} cm^{-1})	2.1
Detection limit	0.135 μM
Limit of quantification	0.447 μM
Regression equation[a]	
Correlation co-efficient	0.999
Slope (a)	0.021
Intercept (b)	0.002
Standard deviation[b]	0.006
Relative standard deviation[b] (%)	1.5
Reaction time	Instantaneous on heating at boiling temperature
Merits	• Avoids use of harmful chemicals like cadmium, stannous chloride, DMSO, DMF. • Reaction can be carried out in aqueous medium • The product is stable for 24 h • Minimum interference from glucose, sucrose and lactose • Helpful in nano levels detection of free amino acids especially in biological samples

[a] Y = ax + b where x is concentration in μM and Y is absorbance.
[b] Ten replicate measurements.

3.2. Determination of Leucine Equivalents for Fruits and Vegetables

The leucine equivalent for fruits and vegetables was carried out. Each sample was weighed in an analytical balance with four digit accuracy to get between 100 and 300 mg into test tubes. Citrate buffer (1.5 mL, pH 5.6) and NSM (0.5 mL) mixture were added to each tube. These tubes were covered with 10 mL beakers, placed in a circular rack in a boiling water bath, covered with aluminum foil and after boiling for 30 min, they were removed and placed in a water bath for 10 min. To each tube sufficient quantity of water was added. The tubes were vortexed, centrifuged and covered with parafilm for 5 min. The leucine equivalents were determined by the standard addition method using Beer's law. Leucine standards, samples and blanks were diluted with water to obtain in the absorbance range 0.4–0.7 at 570 nm. Leucine color equivalents were determined according to the relation optical density at 570 nm per mg of the sample/optical density of 0.01 mg of leucine. Table 2 gives results obtained by the proposed method for some selected vegetables, fruits and grams. Leucine has been selected as a standard since it produces stoichiometric amount of Ruhemann's purple in the ninhydrin reaction [37]. Table 3 depicts the molar absorptivity of selected amino acids.

3.3. Reaction Mechanism

Siegfried Ruhemann working at the University Chemical Laboratory in Cambridge, England made an accidental discovery in 1910 that revolutionized progress in chemistry and biochemistry of amino acids, peptides and proteins. The triketohydrindene hydrate led to results which appear to be of interest. It was found that a deep blue color was produced on warming a mixture of aqueous solution of this compound with aliphatic or an aliphatic aromatic amines which contained the amino group in the side chains. Later, this proposed reaction was explored by many researchers involving different amino acids to determine the extent to which the reaction is typical with different classes [37]. Of the 26 amino acids investigated 23 amino acids and 2 proteins produced typical purple-blue color, whereas the color was yellow for proline.

The most important discovery in the observation was that complexation of Ruhemann's purple with certain metal ions enhances the sensitivity of the analyses by allowing estimation of the resulting chromophore by fluorescent, luminescent, phosphorescent, and laser techniques. This modification now widely used in forensic sciences to determine faint finger prints, merits application to agricultural and biomedical sciences [37]. The authors on the same line made a successful attempt of replacing metal ions by molybdate.

The reaction mechanism for the formation of Ruhemann's purple can be explained based on the same route as that of the classical ninhydrin reaction, this involves the formation of hydrindantin, a dimer of the ninhydrin. The hydrindantin obtained follows a different route in the presence of molybdate. The sodium molybdate forms a weak π-complex with each aromatic ring of the ninhydrin component of the hydrindantin, which in turn undergoes reaction in a regular fashion as that of classical ninhydrin reaction. The quantitative 1:1 molar concentration of the sodium molybdate and ninhydrin substantiates the proposed mechanism of the reaction. The better molar absorptivity of the reaction involving sodium molybdate suggests that the sustained release of the hydrindantin reacts with ammonia giving Ruhemann's purple color with the formation of molybdic acid. The proposed reaction pathway for the formation of Ruhemann's purple is depicted in Scheme 1. The future research articles can debate the use of instrumental techniques to elaborately explain the mechanism of the reaction.

Table 2
Leucine equivalents and percentage nitrogen content of fruits, vegetables and some grams.

Common name	Binomial name	Nitrogen (%)	Leucine equivalents
Potato	*Solanum tuberosum*	0.11	56
Carrot	*Daucus carota*	0.01	29
Cucumber	*Cucumis sativus*	0.01	24
Onion	*Allium cepa*	0.01	30
Beans	*Phaseolus specica*	0.13	21
Mung bean	*Vigna radiata*	0.03	21
Chickpea	*Cicer arietinum*	0.13	32
Sesame	*Sesamum indica*	0.04	19
Papaya	*Carica papaya*	0.04	21
Banana	*Musa paradisica*	0.03	30
Grapes	*Vitis vivifera*	0.05	30
Pomegranate	*Punica granatum*	0.04	30
Apple	*Malus domestica*	0.01	23
Citrus	*Citrus limetta*	0.02	30

Table 3
Molar absorptivity of the proposed NSM for selected amino acids.

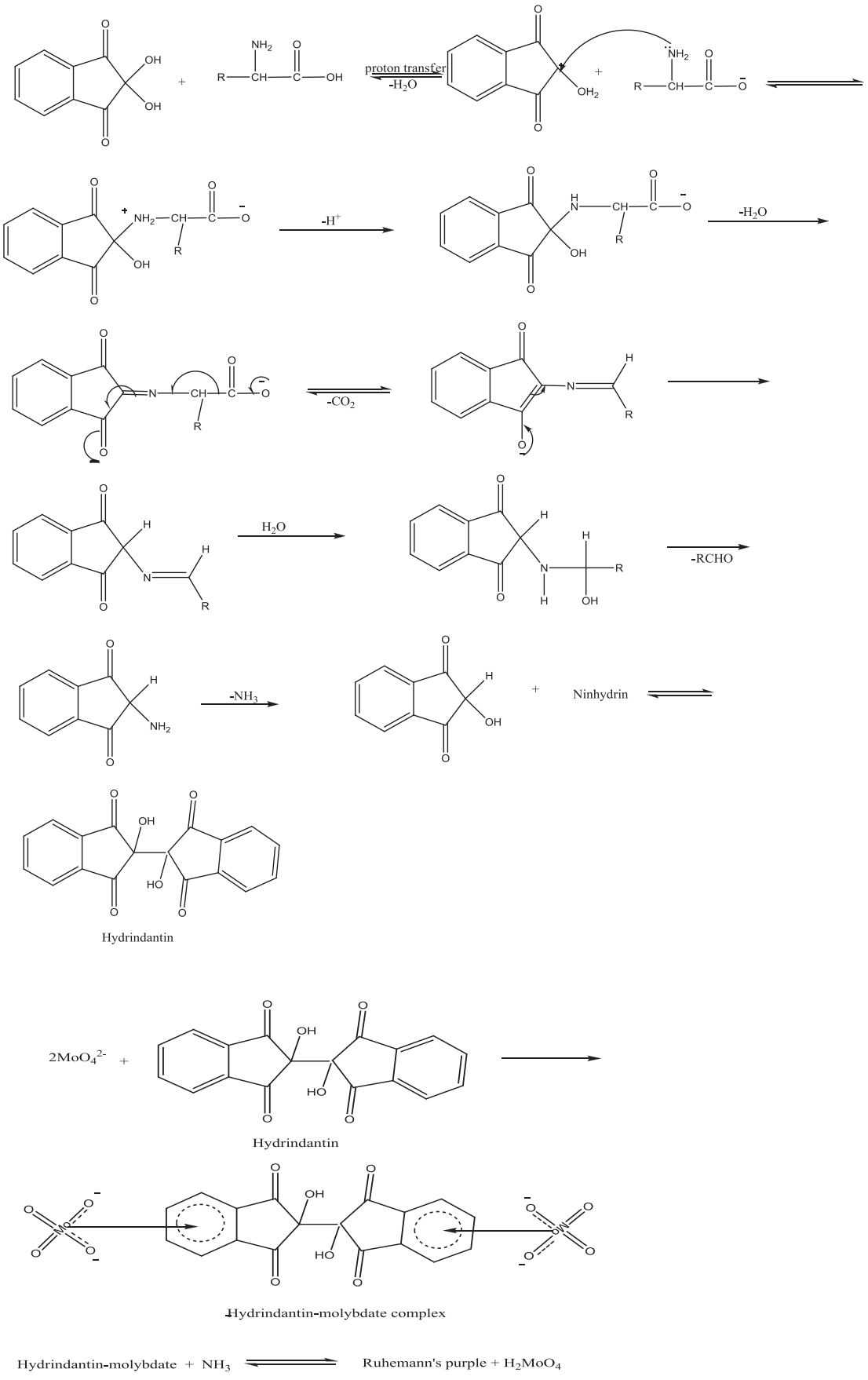
Amino acids/ammonia	Molar absorptivity, ε, 10^4 M^{-1} cm^{-1}
Cysteine	0.58
Dihydrophenylalanine	1.45
Serine	1.47
Glutathione	1.50
Arginine	1.61
Ornithine	1.72
Tyrosine	1.79
Norleucine	1.79
Threonine	1.8
Histidine	1.83
Ammonia	1.87
DL-2-Amino-*n*-butyric acid	1.92
Tryptophan	2.02
Isoleucine	2.03
Glycine	2.07
Alanine	2.08
Lysine	2.29
Leucine	2.32
Valine	2.34
Aspartic acid	2.36
Glutamic acid	2.42
Methionine	2.46
Phenylalanine	2.86

3.4. Stoichiometry of the Amino Acids with NSM Mixture

Ruhemann's purple color does not exactly correspond to the expected equivalent of amino group stoichiometry. The possible reasons that can be visualized for this non-ideal behavior include slow formation, side reaction, hydrolytic, oxidative and photolytic instability and interfering color. The following factor has contributed to the incompleteness of the formation of RP. Some amines react with ninhydrin considerably slower than others so that the low molar absorptivity of the reaction may be sometimes due to incompleteness of the reaction. As the formation of hydrindantin from the ninhydrin and amino acid involves complicated steps such as decarboxylation and concurrent aldehyde formation, which are essentially irreversible. So any equilibrium before the last irreversible step can relatively contribute to the slowness of the reaction which in turn influences the value of molar absorptivity.

3.5. Study of the Effect of Non-targeted Species in the Analysis of Amino Acids

Interference from foreign substances was tested by analyzing their effect in a fixed concentration of 3 mg/L of leucine. The results are summarized in the Table 4. It can be seen the method is highly sensitive and that some compounds, such as dextrose, sucrose, lactose, oxalate, phosphate, sodium tungstate, Na^+, acetate, brucine, nicotinamide, Ba^{2+}, Cd^{2+}, sulphate, sulphite, tartarate, Zn^{2+}, Co^{2+}, Ni^{2+}, Cl^-, Mn^{2+}, Cu^{2+}, F^-, Ca^{2+}, carbonate, EDTA, Mg^{2+}, NO_3^-, K^+, sulphamic acid, and Br^- do not interfere with the measurement in the given experimental conditions. Whereas, other species such as Fe^{3+}, Fe^{2+}, Pb^{2+}, I^-,



Scheme 1. Proposed reaction mechanism for the formation of Ruhemann's purple dye.

Table 4
Study of effect of non-targeted species in the amino nitrogen determination.

Foreign species	Tolerance limit[a] (μg/mL)
Dextrose, sucrose, lactose	≥25,000
Oxalate, phosphate, sodium tungstate	8000
Na^{+}	6000
Acetate	5000
Brucine	4000
Nicotinamide	2000
Ba^{2+}, Cd^{2+}	1200
Sulphate, sulphite, tartarate	900
Zn^{2+}	800
Co^{2+}	500
Ni^{2+}, Cl^{-}, Mn^{2+}, Cu^{2+}, F^{-}	400
Ca^{2+}, carbonate, EDTA	300
Mg^{2+}, NO_3^{-}, K^{+}	200
Sulphamic acid, Cd^{2+}, Br^{-}	120
Fe^{3+}, Fe^{2+}, Pb^{2+}, I^{-}, dichromate, Hg^{2+}, Ce^{4+}, Al^{3+} creatinine, nicotinic acid	50
Zr^{4+}	25
Sb^{3+}, urea	5
Tannic acid	4
Hydroxylamine hydrochloride, uric acid ascorbic acid	0.3

[a] Tolerance limit of interfering species was established at the concentration that do not cause error more than ±3% in absorbance values at 3 mg/L leucine concentration.

dichromate, Hg^{2+},Ce^{4+}, Al^{3+}, creatinine, nicotinic acid, Zr^{4+}, Sb^{3+}, urea, tannic acid, hydroxylamine hydrochloride, uric acid, and ascorbic acid showed a low tolerance level.

4. Conclusions

In conclusion, the proposed NSM spectrophotometric method for amino acids and amine related compounds can be used to an extent similar to that of the conventional ninhydrin techniques. Sodium molybdate, a substitute for cadmium and tin has greater chance of exploration for the qualitative and quantitative chemistry and biochemistry of amines, amino acids, peptides, proteins and related model compounds with ninhydrin. This reaction serves as a useful model for enzymatic and non-enzymatic decarboxylation, transamination, deamination and transpeptidation. NSM procedure has certain advantages particularly over conventional ninhydrin methods: it does not use toxic elements like cadmium, tin, aprotic solvents like DMSO, DMF and methyl cellosolve and above all it can be carried out in water as a medium of dilution, which is economical and eco friendly. Moreover, the proposed analytical probe has a minimum interference by glucose, sucrose and lactose, hence it can be adopted in the assay of glycoprotein. One of the major advantages of the proposed analytical technique is that the method is more sensitive than that of the conventional ninhydrin system, and thus enables the detection of nano mole levels of total free amino acids in small amounts of biological tissues that were otherwise not possible to detect by the conventional ninhydrin methods. Hence the authors strongly assert that the proposed NSM is widely tested for use as an alternative to the classical ninhydrin based reaction.

Abbreviations Used

CB	coomassie blue
DMF	dimethylformamide
DMSO	dimethylsulphoxide
EDTA	ethylenediaminetetraacetate
NSM	ninhydrin-sodium molybdate
RP	Ruhemann's purple

Conflict of Interest

We, the authors of the manuscript entitled "**Ninhydrin-Sodium Molybdate Chromogenic Analytical Probe for the Assay of Amino Acids and Proteins**" confirm that any aspect of the work covered in this manuscript has not involved either experimental animals or human patients.

We understand that the corresponding author is the sole contact for the editorial process (including editorial manager and direct communications with the office). He is responsible for communicating with the other authors about progress, submissions of revisions and final approval of proofs. We confirm that we have provided a current, correct email address which is accessible by the corresponding author and which has been configured to accept email from shivakem77@yahoo.co.in and shivakem77@gmail.com.

Acknowledgements

One of the author (A.S.K) expresses gratitude for the generous support by University Grants Commission, New Delhi, India (Fellowship Award 10-2(5)/2005(i)-E.U-II and 2166-MRP/15-16/KAMY006/UGC-SWRO). Also thank St. Philomena's College for providing facilities to carry out the research investigation.

References

[1] C.C. Hach, S.V. Brayton, A.B. Kopelove, A powerful Kjeldahl nitrogen method using peroxymonosulfuric acid, J. Agric. Food Chem. 33 (1985) 1117–1123.
[2] H. Greenfield, D.A. Southgate, Food Composition Data: Production, Management and Use, FAO, 2003.
[3] W.M. Rand, U.N. University, Food Composition Data: A User's Perspective: Report of a Conference Held in Logan, Utah, USA, 26–29 March 1985, United Nations University, 1987.
[4] X. Guobing, J. Lili, Z. Lihua, X. Tiean, Application of an improved biuret method to the determination of total protein in urine and cerebrospinal fluid without concentration step by use of Hitachi 7170 auto-analyzer, J. Clin. Lab. Anal. 15 (2001) 161–164.
[5] E.A. Ainsworth, K.M. Gillespie, Estimation of total phenolic content and other oxidation substrates in plant tissues using Folin-Ciocalteu reagent, Nat. Protoc. 2 (2007) 875–877.
[6] G. Candiano, M. Bruschi, L. Musante, L. Santucci, G.M. Ghiggeri, B. Carnemolla, P. Orecchia, L. Zardi, P.G. Righetti, Blue silver: a very sensitive colloidal Coomassie G-250 staining for proteome analysis, Electrophoresis 25 (2004) 1327–1333.
[7] J.M. Walker, The bicinchoninic acid (BCA) assay for protein quantitation, in: J.M. Walker (Ed.), The Protein Protocols Handbook, Humana Press, Totowa, NJ 2009, pp. 11–15.
[8] S.-W. Lee, J.-M. Lim, S.-H. Bhoo, Y.-S. Paik, T.-R. Hahn, Colorimetric determination of amino acids using genipin from *Gardenia jasminoides*, Anal. Chim. Acta 480 (2003) 267–274.
[9] Á. Kőrös, Z. Varga, I. Molnár-Perl, Simultaneous analysis of amino acids and amines as their o-phthalaldehyde-ethanethiol-9-fluorenylmethyl chloroformate derivatives in cheese by high-performance liquid chromatography, J. Chromatogr. A 1203 (2008) 146–152.
[10] C. Aoyama, T. Santa, M. Tsunoda, T. Fukushima, C. Kitada, K. Imai, A fully automated amino acid analyzer using NBD-F as a fluorescent derivatization reagent, Biomed. Chromatogr. 18 (2004) 630–636.
[11] E.A. Arriaga, Y. Zhang, N.J. Dovichi, Use of 3-(p-carboxybenzoyl)quinoline-2-carboxaldehyde to label amino acids for high-sensitivity fluorescence detection in capillary electrophoresis, Anal. Chim. Acta 299 (1995) 319–326.
[12] S. Yuji, Theoretical analysis concerning the characteristics of a dye-binding method for determining serum protein based on protein error of pH indicator: effect of buffer concentration of the color reagent on the color development, Anal. Sci. 21 (2005) 83–88.
[13] S.H. Lee, J.K. Suh, M. Li, Determination of bovine serum albumin by its enhancement effect of Nile blue fluorescence, Bull. Korean Chem. Soc. 24 (2003) 45–48.
[14] B. Okutucu, A. Dınçer, Ö. Habib, F. Zıhnıoglu, Comparison of five methods for determination of total plasma protein concentration, J. Biochem. Biophys. Methods 70 (2007) 709–711.
[15] H. Zhong, J.-J. Xu, H.-Y. Chen, A rapid and sensitive method for the determination of trace proteins based on the interaction between proteins and Ponceau 4R, Talanta 67 (2005) 749–754.
[16] X. Zhu, J. Sun, Y. Hu, Determination of protein by hydroxypropyl-β-cyclodextrin sensitized fluorescence quenching method with erythrosine sodium as a fluorescence probe, Anal. Chim. Acta 596 (2007) 298–302.
[17] Y. Nagata, T. Iida, M. Sakai, Enantiomeric resolution of amino acids by thin-layer chromatography, J. Mol. Catal. B Enzym. 12 (2001) 105–108.
[18] T. Soga, D.N. Heiger, Amino acid analysis by capillary electrophoresis electrospray ionization mass spectrometry, Anal. Chem. 72 (2000) 1236–1241.

[19] M.J. Nozal, J.L. Bernal, M.L. Toribio, J.C. Diego, A. Ruiz, Rapid and sensitive method for determining free amino acids in honey by gas chromatography with flame ionization or mass spectrometric detection, J. Chromatogr. A 1047 (2004) 137–146.
[20] L. Bosch, A. Alegría, R. Farré, Application of the 6-aminoquinolyl-N-hydroxysccinimidyl carbamate (AQC) reagent to the RP-HPLC determination of amino acids in infant foods, J. Chromatogr. B 831 (2006) 176–183.
[21] Y.-M. Liu, Z.-L. Liu, Y.-M. Shi, W. Tian, The determination of glutamine with flow-injection chemiluminescence detection and mechanism study, Luminescence 25 (2010) 50–54.
[22] P. Nagaraja, H.S. Yathirajan, R.A. Vasantha, Highly sensitive reaction of tryptophan with p-phenylenediamine, Anal. Biochem. 312 (2003) 157–161.
[23] M. Rezaei-Tavirani, S.H. Moghaddamnia, B. Ranjbar, M. Amani, S.-A. Marashi, Conformational study of human serum albumin in pre-denaturation temperatures by differential scanning calorimetry, circular dichroism and UV spectroscopy, BMB Rep. 39 (2006) 530–536.
[24] B. Domon, R. Aebersold, Mass spectrometry and protein analysis, Science 312 (2006) 212–217.
[25] T. soga, Y. kakazu, M. Robert, M. Tomita, T. Nishioka, Qualitative and quantitative analysis of amino acids by capillary electrophoresis-electrospray ionization-tandem mass spectrometry, Electrophoresis 25 (2004) 1964–1972.
[26] Z. Dai, Z. Wu, S. Jia, G. Wu, Analysis of amino acid composition in proteins of animal tissues and foods as pre-column o-phthaldialdehyde derivatives by HPLC with fluorescence detection, J. Chromatogr. B 964 (2014) 116–127.
[27] M.C.X. Pinto, M.J.N. de Paiva, O.C. Oliveira-Lima, H.C. Menezes, Z.d.L. Cardeal, M.V. Gomez, R.R. Resende, R.S. Gomez, Neurochemical study of amino acids in rodent brain structures using an improved gas chromatography–mass spectrometry method, J. Chem. Neuroanat. 55 (2014) 24–37.
[28] A.M. Skelley, J.R. Scherer, A.D. Aubrey, W.H. Grover, R.H.C. Ivester, P. Ehrenfreund, F.J. Grunthaner, J.L. Bada, R.A. Mathies, Development and evaluation of a microdevice for amino acid biomarker detection and analysis on Mars, Proc. Natl. Acad. Sci. U. S. A. 102 (2005) 1041–1046.
[29] A. Troise, A. Fiore, G. Roviello, S. Monti, V. Fogliano, Simultaneous quantification of amino acids and Amadori products in foods through ion-pairing liquid chromatography–high-resolution mass spectrometry, Amino Acids 47 (2015) 111–124.
[30] J. Yan, Y. Cai, Y. Wang, X. Lin, H. Li, Simultaneous determination of amino acids in tea leaves by micellar electrokinetic chromatography with laser-induced fluorescence detection, Food Chem. 143 (2014) 82–89.
[31] J. Ziegler, S. Abel, Analysis of amino acids by HPLC/electrospray negative ion tandem mass spectrometry using 9-fluorenylmethoxycarbonyl chloride (Fmoc-Cl) derivatization, Amino Acids 46 (2014) 2799–2808.
[32] E. Doi, D. Shibata, T. Matoba, Modified colorimetric ninhydrin methods for peptidase assay, Anal. Biochem. 118 (1981) 173–184.
[33] S.-W. Sun, Y.-C. Lin, Y.-M. Weng, M.-J. Chen, Efficiency improvements on ninhydrin method for amino acid quantification, J. Food Compos. Anal. 19 (2006) 112–117.
[34] D. Gabi, S. Ion, G. Robert, Z. Gheorghita, M. Ionel, Ninhydrin-based forensic investigations: II. Cyanide analytical toxicology, Int. J. Crim. Investig. 1 (2011) 213–226.
[35] K.M. Yiu, A. Valdeir, N.S. Jack, A $NaBH_4$ coupled ninhydrin-based assay for the quantification of protein/enzymes during the enzymatic hydrolysis of pretreated lignocellulosic biomass, Appl. Biochem. Biotechnol. 176 (2015) 1564–1580.
[36] S. Yokoyama, J.-I. Hiramatsu, A modified ninhydrin reagent using ascorbic acid instead of potassium cyanide, J. Biosci. Bioeng. 95 (2003) 204–205.
[37] M. Friedman, Applications of the ninhydrin reaction for analysis of amino acids, peptides, and proteins to agricultural and biomedical sciences, J. Agric. Food Chem. 52 (2004) 385–406.