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COMMUNICATION

# A critical analysis of L-asparaginase activity quantification methods—colorimetric methods versus high-performance liquid chromatography

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## Abstract

L-asparaginase or ASNase (L-asparagine aminohydrolase, E.C.3.5.1.1) is an enzyme clinically accepted as an antitumor agent to treat acute lymphoblastic leukemia (ALL) and lymphosarcoma through the depletion of L-asparagine (L-Asn) resulting in cytotoxicity to leukemic cells. ASNase is also important in the food industry, preventing acrylamide formation in processed foods. Several quantification techniques have been developed and used for the measurement of the ASNase activity, but standard pharmaceutical quality control methods were hardly reported, and in general, no official quality control guidelines were defined. To overcome this lack of information and to demonstrate the advantages and limitations, this work properly compares the traditional colorimetric methods (Nessler; L-aspartic acid β-hydroxamate (AHA); and indooxine) and the high-performance liquid chromatography (HPLC) method. A comparison of the methods using pure ASNase shows that the colorimetric methods both overestimate (Nessler) and underestimate (AHA and indooxine) the ASNase activity when compared to the values obtained with HPLC, considered the most precise method as this method monitors both substrate consumption and product formation, allowing for overall mass-balance. Correlation and critical analysis of each method relative to the HPLC method were carried out, resulting in a demonstration that it is crucial to select a proper method for the quantification of ASNase activity, allowing bioequivalence studies and individualized monitoring of different ASNase preparations.

**Keywords** L-asparaginase · Enzymatic activity · L-aspartic acid · Nessler · L-aspartic acid β-hydroxamate · Indooxine

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## Introduction

L-asparaginase (ASNase) has been used as a well-established antitumor agent for acute lymphoblastic leukemia [1] and other related forms of cancer, and even recently against pathogenic bacterial infection [2]. ASNase is also successfully applied in the food industry for preventing acrylamide formation in processed foods with high starch content [3]. Considering its pharmacological and food significance and its diversity of applications [2], the demand for an efficient, sensitive, and reliable quantification method to determine ASNase activity has been increasing.

The main reaction of ASNase consists of the hydrolysis of L-Asn, resulting in the formation of L-aspartic acid (L-Asp) and ammonia ($NH_3$). The enzymatic activity is quantified by the measurement of the substrate conversion at the maximum rate, where one unit of ASNase (U) corresponds to the amount of enzyme required to convert 1 μmol of L-Asn into 1 μmol of

L-Asp and 1 μmol $NH_3$ per minute at 37 °C [4]. To measure the extension of the substrate conversion several quantification methods have been developed based on the determination of L-Asp or $NH_3$ produced. The methods for determining L-Asp include high-performance liquid chromatography (HPLC) [5], direct amino acid quantification by circular dichroism (CD) [6], electrophoresis assays [7], and determination by a colorimetric assay from complexation with hydroxylamine [8]. To measure the amount of $NH_3$ produced, methods typically involve a reaction with colorimetric reactants, such as the Nessler reagent [9] or indophenol [10], followed by a spectrophotometric determination. Additional assays include the measurement of alternative substrate degradation, such as indooxine, [4] L-aspartic acid β-(7-amido-4-methylcoumarin), [11] nicotinamide adenine dinucleotide (NADH) [12], and 5-diazo-4-oxo-L-norvaline [13]. Conductometry can also measure the ASNase activity, where the increase of conductivity corresponds to the simultaneous production of L-Asp and $NH_4^+$ [14].

Despite the quantity and breadth of ASNase activity quantification methods, only the Chinese Pharmacopoeia have adopted ASNase monographs [15], and for food applications, the Food and Drug Administration (FDA) indicates that the Nessler method can be used with pure ASNase [16]. With a scarcity of standardized protocols and pharmaceutical quality control guidelines, the standard protocols for measuring the activity of ASNase preparations are hardly reported [17]. Lack of defined guidelines results in wildly varying ASNase activities reported in the literature, and are thus difficult to compare effectively within the wide range of ASNase applications.

The most common method for ASNase quantification is the simple, colorimetric Nessler method. This method consists of the reaction between Nessler's reagent (dipotassium tetraiodomercurate (II)) and the ammonia released during the conversion of L-Asn into L-Asp, providing a characteristic yellow reaction mixture that can be quantified by spectrophotometry [18]. The Nessler method exhibits good reproducibility, but requires meticulous care [19], has a high level of detection (LOD ≥ 20 U $L^{-1}$), [18] and involves the use of highly toxic reagents [11]. Nessler's reagent is not very selective for ammonia, thus, when used in complex media, such as fermented broths or food preparations, the presence of contaminants, including polymers, esters, salts, surfactants, alcohols, amines, and aldehydes, interferes in the quantification, hindering the estimation of real enzymatic activity [20]. Additionally, reaction temperature, color equilibration time, and rate of mixing also impact the color development of the solution, and thus contribute to the variability of the results for this method [21].

To overcome some of the limitations of the Nessler method in fermented broth samples or in media with high concentrations of non-ASNase produced ammonia, alternative methods for the determination of ASNase activity have been proposed [22]. One approach based on L-aspartic acid β-hydroxamate (AHA), the AHA method, utilizes the determination of asparto- and glutamo-hydroxamic acids formed by the enzymatic conversion of L-Glu and L-Asn, respectively [8]. ASNase converts L-Asn into L-aspartic acid β-hydroxamate (AHA) in the presence of hydroxylamine, which is then reacted with ferric chloride ($FeCl_3$) developing a characteristic red color measurable by spectrophotometry [8]. Another common technique uses the hydrolysis of AHA (from the mentioned reaction above) to liberate hydroxylamine for reaction with 8-hydroxyquinoline at alkaline pH. This method, the indooxine method, results in an intensely green-colored, oxindole dye (indooxine) easily detectable between 705 and 710 nm [4, 23]. The indooxine method has a low detection limit ($> 2 \times 10^{-5}$ U $L^{-1}$), but has a limited working pH range due to the hydroxylamine instability above neutrality [23].

With continuing research into increasing sensitivity, accuracy, reproducibility, and the reliability of the existing protocols, the variability and lack of consistency in results still plagued these techniques [4, 17]. HPLC-based methods overcome the drawbacks of colorimetric methods and determine ASNase activity through quantification of L-Asp and/or L-Asn (depletion) [5, 24]. The method by Nath et al. [5] determined the ASNase activity by the quantification of L-Asp produced after incubation of plasma samples with L-Asn, using an isocratic HPLC fluorescence. The assay includes the derivatization of enzymatic products with *o*-pthaldialdehyde (OPA), followed by separation using reverse-phase chromatography and detection using fluorescence [5]. This method shows excellent reproducibility (< 10%), accuracy (< 13%), precision (< 13%), and linearity (correlation coefficients of 0.9992 ± 0.0007) when compared with colorimetric assays, being internally validated with the determination of the production of L-Asp or L-Glu, as well as the depletion of L-Asn and L-Gln [5].

With the variability, weaknesses, and strengths of several ASNase activity quantification methods and without "official" standardization by most of the regulatory agencies, our goal was to critically compare the three traditionally applied colorimetric assays (Nessler, AHA, and indooxine methods) and correlate the ASNase activity with HPLC-based method results. We sought to gain an important analysis of each technique and provide the community with correlation and correction factors to consider and use when choosing one technique over another.

## Material and methods

### Material

L-asparaginase (E.C.3.5.1.1) was purchased from ProSpec-Tany (ENZ-287, Rehovot, Israel). Standards for HPLC

L-Asn, L-Asp and L-Asp acid β-hydroxamate, *o*-phtaldialdehyde (OPA) reagent, Trizma-HCl buffer base, HPLC grade methanol, trichloroacetic acid (TCA), mercaptoethanol, sodium tetraborate, and 8-hydroxy quinoline were purchased from Sigma-Aldrich (St. Louis, MO, USA). Nessler reagent was provided by Merck with following composition: potassium hydroxide (≥ 10–< 20%) and dipotassium tetraiodomercurate (II) (≥ 1–< 2%) (Hohenbrunn, Germany). All other reagents are of analytical grade and were used as received. Ultrapure water double distilled, passed through a reserve-osmosis system and further treated by filtration through a Millipore Milli-Q ion-exchange system (18 MΩ cm) (Bedford, MA, USA), was used.

### Quantification of ASNase activity

Aqueous solutions of the ASNase commercial enzyme were prepared at concentrations of 0.01 to 0.4 mg $mL^{-1}$ by dilution in phosphate buffer (20 mM) at pH 7.4. For the determination of ASNase activity [see Fig. S1 (a) in Electronic Supplementary Material (ESM)], 50 μL of each diluted ASNase solution was added to a glass test tube containing 500 μL of Trizma-HCl (50 mM) pH 8.6 buffer, 50 μL of 189 mM L-Asn solution, and 450 μL of deionized water. After incubation at 37 °C for 10 min in a controlled temperature bath, the reaction was quenched by the addition of 250 μL of sulfosalicylic acid solution (4 wt%). The ASNase activity of each solution was then determined through three different approaches, one HPLC method and two colorimetric methods (Nessler and AHA), as detailed in the sections S1.1 to S1.3 in ESM, respectively. The quantification using indooxine method was based on a different ASNase hydrolysis protocol, in which a solution of AHA was used as the substrate, as detailed in the section S1.4 in ESM. All experiments were carried out in three independent assays, with the respective blank assays (using denatured ASNase) to subtract the interferences.

## Results and discussion

### Comparison of ASNase activity quantification methods

The enzymatic activity of pure L-asparaginase was measured by three independent colorimetric methods (Nessler, AHA, and indooxine) and was compared with the activity determined by HPLC, which quantifies the activity by direct determination of L-Asp (Fig. S1 in ESM). The comparison between all the methods was carried out using a range of ASNase aqueous solution concentrations (0 to 0.4 mg $mL^{-1}$), and the corresponding ASNase activities (U $mL^{-1}$) quantified through each method were correlated with the ASNase concentration (mg $mL^{-1}$) in Fig. 1 (experimental details and detailed values are provided in the ESM section).

As seen in Fig. 1a, each method resulted in a similar trend between ASNase activity and enzyme concentration, where a direct linear correlation was observed until a concentration of 0.2 mg $mL^{-1}$, wherein a saturation profile was then observed. These results suggest that at a high ASNase concentration (> 0.2 mg $mL^{-1}$), the substrate was fully converted into L-Asp (189 mM of L-Asn for HPLC, Nessler and AHA methods, and 10 mM of AHA for the indooxine method). This hypothesis was confirmed by the HPLC analysis, where L-Asn was not detected in the samples hydrolyzed with high ASNase concentration samples (> 0.3 mg $mL^{-1}$) (see Fig. S2 in ESM). Considering this threshold, Fig. 1b presents a method comparison analysis of the range up to 0.2 mg $mL^{-1}$ ASNase. The linear least-squares regression analysis shows good correlation for all the methods in this concentration, where linearity was maintained with $R^2 > 0.98$ for all methods.

Despite the similarity between the profiles of ASNase activity as a function of ASNase concentration and good correlation of each method, the methods result in statistically different enzymatic activities for the same ASNase concentration

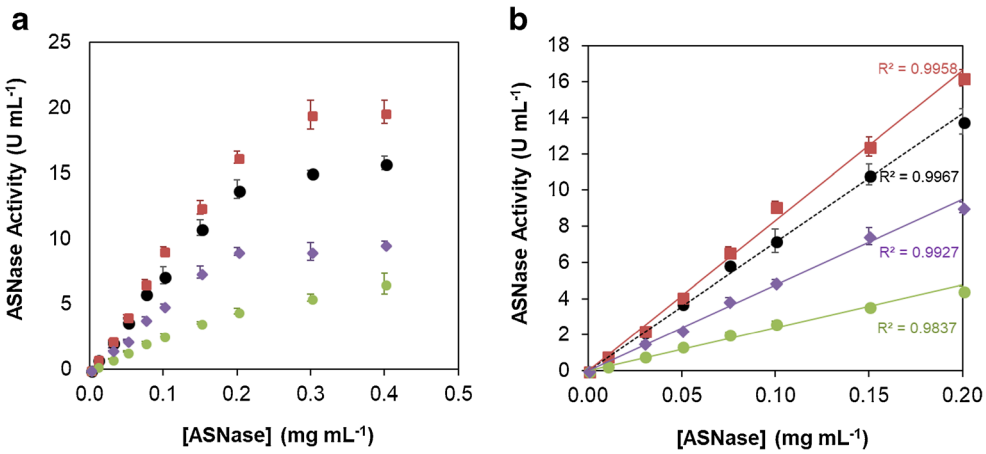


**Fig. 1** **a** ASNase activity (U $mL^{-1}$) at 37 °C as a function of the concentration of ASNase (mg $mL^{-1}$). **b** Linear regression and respective correlation coefficients ($R^2$) within the range 0 to 0.2 mg $mL^{-1}$, determined using the following methods: HPLC (●); Nessler (■); indooxine (♦); and AHA (●). Each value corresponds to the mean of at least three independent assays ± standard deviation

(see ESM, Table S1). Roughly, the Nessler method overestimates the ASNase activity, while indooxine and AHA methods underestimate the enzymatic activity in comparison with the direct quantification of L-Asp by HPLC. In detail, at a low ASNase concentration (< 0.1 mg $mL^{-1}$), both HPLC and Nessler methods result in statistically similar activities, but above this concentration, Nessler method overestimates the ASNase activity in comparison with the L-Asp produced. Both indooxine and AHA methods underestimate the activities at all concentrations of ASNase studied.

To understand and quantify the differences of the ASNase activity between the four methods, the linear least-squares regression of ASNase activity values obtained by each of three colorimetric methods were plotted against the direct enzymatic activity measured by HPLC (Fig. 2). The linear regression only considered the experimental values in the range of ASNase concentrations that exhibit linearity with ASNase activity (up to 0.2 mg $mL^{-1}$).

Linear least-squares regression analysis of each method indicates the following trend to quantify the ASNase activity (slope, $R^2$): AHA (0.3352, 0.9932) < indooxine (0.6663, 0.9975) < HPLC (1, 1) < Nessler (1.1682, 0.9965). All methods showed strong positive correlations ($R^2 > 0.95$). The slopes of the linear regressions confirm that the Nessler method overestimated the ASNase activity in approximately 17% higher than HPLC, while if determined through the indooxine or AHA protocols, the enzymatic activity values are, approximately, 33 and 67% lower than the values obtained through the determination of L-Asp by HPLC, respectively. Considering that the direct quantification of L-Asp by HPLC provides the most accurate value of ASNase activity (it measures the product directly), it is quite evident that of the colorimetric methods compared, the Nessler method provides the most accurate, followed by the indooxine method. The AHA method exhibits the lowest accuracy, since it quantifies

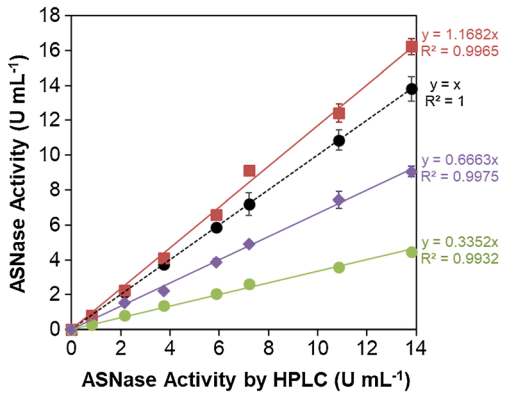


**Fig. 2** ASNase activity (U $mL^{-1}$) ratio of colorimetric methods activity, Nessler (■), indooxine (♦), and AHA (●) to HPLC quantification (●). The lines correspond to linear regression analysis based on the method of least squares of each method, according to the corresponding equations and $R^2$ values. Each value corresponds to the mean of at least three independent assays ± standard deviation

approximately threefold less enzymatic activity than the direct method. The ASNase activity is based on the measurement of substrate turnover at the maximum rate [4]. Thus, both Nessler and AHA method can be directly compared with HPLC, since 189 mM L-Asn was hydrolyzed by ASNase following the same reaction protocol; however, a more careful comparison with the indooxine method should be performed because this method uses a different type and concentration of substrate (10 mM of AHA), which can lead to a change in the substrate hydrolysis rate.

The Nessler method exhibited the lowest deviation from the HPLC method in measured activities, yet at concentrations greater than 0.1 mg $mL^{-1}$, it overestimates the enzymatic activity. Lanvers et al. [4] previously compared the Nessler and indooxine methods and found a similar overestimation profile for the Nessler method. The Nessler method quantifies the enzymatic activity indirectly by metal complexation of ammonia to form a colored-complex and compares its production with a product standard curve [$(NH_4)_2SO_4$ stock solution]. While considered an acceptably accurate and inexpensive method, several disadvantages and experimental parameters result in overestimation of the enzyme activity [25]. For example, the chromogen that results from the Nesslerization process is time-sensitive and must be carefully controlled to maintain consistent absorbance measurements [25]. Additionally, when substantial quantities of protein are present (particularly at high ASNase content), Nessler's reagent can produce flocculent precipitates interfering with the solution's absorbance, thus requiring an additional centrifugation step to avoid interferences [25]. Finally, and as mentioned previously, the determination of ASNase activity in complex mixtures also measures residual ammonium ions present, reducing the method's accuracy and precision if the system is complex. Evidence of this interference in the Nessler method was observed when higher ASNase activities were measured for the crude extract compared to purified ASNase [26–28].

The AHA and indooxine methods underestimate the ASNase activity in comparison with HPLC. From a practical point, both methods are quite useful for the determination of enzymatic activity in complex media (*i.e.*, fermented broths), particularly mixtures containing residual ammonium content, since these methods exhibit low interference with ammonium ions. AHA and indooxine protocols are based on the conversion of different substrates, and thus the underestimation is method-specific. The AHA method deviation results directly from the hydroxylaminolysis reaction, which does not occur at the same rate as that of the asparaginolytic reaction. Thus, the L-Asp is only quantified after reaction with hydroxylamine by a second reaction using ASNase as a catalyst yielding AHA, which is then complexed with Fe (III) under acidic conditions to produce the colored species. Then, the AHA protocol is effectively measuring the enzymatic activity for two reactions, and since ASNase activity is calculated by time

of the reaction rate the values returned from this method are much lower than the other methods. Additionally, ASNase can use AHA as a substrate potentially resulting in the reverse catalytic reaction where the product AHA is converted into L-Asp and hydroxylamine. This back-reaction would reduce the AHA concentration, effectively decreasing the values of enzymatic activity. Although the AHA method underestimates the catalytic activity and is relatively insensitive, the method is cheap and simple, [25], which supports its use in certain ASNase-based processes.

The other colorimetric method studied was the indooxine method, which is based on the catalytic conversion of AHA to L-Asp and hydroxylamine by ASNase. This method provides detection of resulting hydroxylamine through condensation with 8-hydroxyquinoline and oxidation to indooxine [4]. As highlighted above, this method returns enzymatic values lower than HPLC and Nessler methods and higher than AHA method. Considering the possible reverse catalytic reaction, similarly to the AHA method, low activity values are somewhat expected in contrast to the single-step reaction of HPLC and Nessler methods. Lanvers et al. [4] also measured lower catalytic activity using the indooxine method than in the Nessler method with serum samples. In addition to the difference in the experimental procedures (buffer, pH, and type and concentration of substrate), the authors noted that the determination of the calibration curve could also induce differences in the catalytic responses. Yao et al. [17] have also compared the theoretical and operational characteristics of indooxine and Nessler methods and found that the values of enzymatic activity provided by the Nessler method were higher than indooxine method. They demonstrated that the Nessler method was more sensitive, simpler, and less laborious than indooxine method [17].

In summary, all the methods have good linearity and precision, which make them useful for the determination of ASNase catalytic behavior. Naturally, each method comes with its advantages and disadvantages (some are more sensitive, while others are more simple and economical). Depending on the ASNase source (serum, fermented broth, commercial buffer solution, etc.), each researcher should make their own choice when deciding on the appropriate and adequate method for measuring their catalytic activities.

Finally, this work is also intended to provide a set of correction factors or deviations between each of the colorimetric methods and the HPLC method, allowing a quick and easy adjustment for accurate ASNase activities. Due to the high number of ASNase enzymatic protocols, we have specifically focused on the protocols used with ASNase obtained through microbial sources, rather than attempt to correlate all available ASNase sources across the literature. We have compared several published values of ASNase activity and shown their adjustment using the correlations determined (Table S3 in ESM). The table combines the previously reported ASNase activity values and the corresponding HPLC estimated values (which correspond to an estimation of would be obtained through the HPLC method, as: Nessler: $y = x*1.1682$; AHA: $y = x*0.6663$; indooxine: $y = x*0.3352$). Nessler and AHA methods are mainly used to quantify ASNase from the fermented broth by a microorganism, while the indooxine method is exclusively used for ASNase serum determination. Methods that use different substrates for the quantification of ASNase, such as AHA and indooxine, suffer less interference from the $NH_3$ present in the medium, since it does not quantify this molecule. However, the Nessler method is highly influenced by the fermented medium, since after the microorganism growth, the release of $NH_3$ occurs as a by-product of the metabolism of the nitrogen sources. Large amounts of $NH_3$ affect the color development of Nessler reagent, leading to a turbid solution [22]. After normalization, by previous equations (Fig. 2 and Table S3 footnote), some discrepant values became closer, but this condition does not apply to all values reported. For example, the value obtained by the AHA method [29] became greater than one obtained by Nessler method [26], such as, 7.1 U $mL^{-1}$ by AHA and 20.9 U $mL^{-1}$ by Nessler, when their activities are estimated these values become 21.2 and 17.9 U $mL^{-1}$, respectively.

## Conclusions

We compared three commonly used colorimetric methods for the determination of ASNase activity to an HPLC method. While several comparisons of these methods have been presented, here, we present for the first time a set of normalization factors for each method to provide more accurate results of the enzymatic activity and a protocol of comparing data between methods. The standardization of ASNase quantification by a simple, reliable, rapid, and robust method is a very advantageous tool to overcome the disadvantages in the great disparity of methods that exist to determine enzymatic activity in the literature. Additionally, adoption of these factors allows for the interpreting, converting, and comparison of results obtained from other methods. Furthermore, comparing all colorimetric methodologies evaluated in our work, the Nessler method is a simple methodology, with relatively inexpensive reagents and less time to analyze the results. Thus, we have tried to reinforce the importance of the standardization of enzymatic methodologies, proposing a correlation that provides a statistically significant comparison between the activity values obtained from three well-used methods (Nessler, AHA, and indooxine) and the direct measurement by HPLC, which we considered the most accurate measurement. It is evident that the fully understanding of the differences among the enzymatic methods and their limitations becomes extremely important within this context. Although HPLC is the most accurate, considering that the Nessler method has the shortest execution

time, the cheapest, and operationally easy, we believe that it will continue to be the most applied. Besides that, it is worth mentioning this method is more suitable, as recognized by the FDA, for pure ASNase formulations; if the purpose is to measure the ASNase activity in a more complex media, it is recommended that alternative methods or adequate blanks be used to decrease the interferences of the method. In order to properly compare new measurements to previously reported values, we recommend that the correction factors described here be considered to all activity values measured utilizing these colorimetric methods to ensure a comparison between the most accurate and representative activities of ASNase.

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## Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

## References

1. Hill JM, Roberts J, Loeb E, Khan A, MacLellan A, Hill RW. L-asparaginase therapy for leukemia and other malignant neoplasms. JAMA. 1967;202:882–8.
2. Vimal A, Kumar A. Biotechnological production and practical application of L-asparaginase enzyme. Biotechnol Genet Eng Rev. 2017;8725:1–22.
3. Aiswarya R, Baskar G. Enzymatic mitigation of acrylamide in fried potato chips using asparaginase from *Aspergillus terreus*. Int J Food Sci Technol. 2018;53:491–8.
4. Lanvers C, Paulo J, Pinheiro V, Hempel G, Wuerthwein G, Boos J. Analytical validation of a microplate reader-based method for the therapeutic drug monitoring of L-asparaginase in human serum. Anal Biochem. 2002;309:117–26.
5. Nath CE, Dallapozza L, Eslick AE, Misra A, Carr D, Earl JW. An isocratic fluorescence HPLC assay for the monitoring of L-asparaginase activity and L-asparagine depletion in children receiving *E. coli* L-asparaginase for the treatment of acute lymphoblastic leukaemia. Biomed Chromatogr. 2009;23:152–9.
6. Kudryashova EV, Sukhoverkov KV. "Reagent-free" L-asparaginase activity assay based on CD spectroscopy and conductometry. Anal Bioanal Chem. 2016;408:1183–9.
7. Broome JD. Factors which may influence the effectiveness of L-asparaginases as tumor inhibitors. Br J Cancer. 1968;22:595–602.
8. Grossowicz N, Wainfan E, Borek E, Waelsch H. The enzymatic formation of hydroxamic acids from glutamine and asparagine. J Biol Chem. 1950;187:111–25.
9. Shifrin S, Parrott CL, Luborsky SW. Substrate binding and intersubunit interactions in L-asparaginase. J Biol Chem. 1974;249:1335–40.
10. Scheiner D. Determination of ammonia and kjeldahl nitrogen by indophenol method. Water Res. 1976;10:31–6.
11. Ylikangas P, Mononen I. A fluorometric assay for L-asparaginase activity and monitoring of L-asparaginase therapy. Anal Biochem. 2000;280:42–5.
12. Cooney DA, Handschumacher RE. L-asparaginase and L-asparagine metabolism. Annu Rev Pharmacol. 1970;10:421–40.
13. Handschumacher RE, Bates CJ, Chang PK, Andrews AT, Fischer GA. 5-Diazo-4-Oxo-L-norvaline: reactive asparagine analog with biological specificity. Science. 1968;161:62–3.
14. Drainas D, Drainas C. A conductimetric method for assaying asparaginase activity in *Aspergillus nidulans*. Eur J Biochem. 1985;593:591–3.
15. Chinese Pharmacopoeia Commission. General monographs part 1. In: Pharmacopoeia of the People's Republic of China. 2010. pp. 35–38.
16. Food and Drug Administration Guidance for Industry Acrylamide in Foods. FDA Food Guid. 2018. http://www.fda.gov/FoodGuidances. Accessed 16 April 2018.
17. Yao H, Vancoillie J, D'Hondt M, Wynendaele E, Bracke N, Spiegeleer BD. An analytical quality by design (aQbD) approach for a L-asparaginase activity method. J Pharm Biomed Anal. 2016;117:232–9.
18. Meister A. Glutaminase, asparaginase, and α-keto acid-ω-amidase. Methods Enzymol. 1955;2:380–5.
19. Cooney DA, Capizzi RL, Handschumacher RE. Evaluation of L-asparagine metabolism in animals and man. Cancer Res. 1970;30:929–35.
20. Miller GL, Miller EE. Determination of nitrogen in biological materials. Anal Chem. 1948;20:481–8.
21. Thompson JF, Morrison GR. Determination of organic nitrogen control of variables in the use of nessler's reagent. Anal Chem. 1951;23:1153–7.
22. Morrison GR. Microchemical determination of organic nitrogen with Nessler reagent. Anal Biochem. 1971;43:527–32.
23. Wehner A, Harms E, Jennings MP, Beacham IR, Derst C, Bast P, et al. Site-specific mutagenesis of *Escherichia coli* asparaginase II: none of the three histidine residues is required for catalysis. Eur J Biochem. 1992;208:475–80.
24. Gentili D, Zucchetti M, Conter V, Masera G, D'Incalci M. Determination of L-asparagine in biological samples in the presence of L-asparaginase. J Chromatogr B Biomed Sci Appl. 1994;657:47–52.
25. Jayaram HN, Cooney DA, Jayaram S, Rosenblum L. A simple and rapid method for the estimation of l-asparaginase in chromatographic and electrophoretic effluents: comparison with other methods. Anal Biochem. 1974;59:327–46.
26. Khushoo A, Pal Y, Singh BN, Mukherjee KJ. Extracellular expression and single step purification of recombinant *Escherichia coli* L-asparaginase II. Protein Expr Purif. 2004;38:29–36.
27. Zhu J, Yan X, Chen H, Wang Z. In situ extraction of intracellular L-asparaginase using thermoseparating aqueous two-phase systems. J Chromatogr A. 2007;1147:127–34.
28. Amena S, Vishalakshi N, Prabhakar M, Dayanand A, Lingappa K. Production, purification and characterization of L-asparaginase from *Streptomyces gulbargensis*. Brazilian J Microbiol. 2010;41:173–8.
29. Kumar NSM, Ramasamy R, Manonmani HK. Production and optimization of L-asparaginase from *Cladosporium* sp. using agricultural residues in solid state fermentation. Ind Crop Prod. 2013;43:150–8.