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Plackett-Burman and Box-Behnken designs as chemometric tools for micro-determination of L-Ornithine

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ABSTRACT

Plackett-Burman (PB) and Box-Behnken (BB) screening and response surface factorial designs were used to evaluate spectrophotometric and spectrofluorimetric approaches for the determination of L-Ornithine (ORN) as per se and in dietary supplements. Both approaches were based on the derivatization of the primary amino group of ORN via Hantzsch condensation reaction producing yellow coloured adducts (dihydrolutidine derivative). The reaction product was determined spectrophotometrically (method A) at λ_{max} = 327 nm and spectrofluorimetrically (method B) at 480 nm (λ_{em}) after excitation at 325 nm (λ_{ex}). A multivariate scheme was tailored to investigate the process numerical variables; reaction temperature, heating time, reagent volume, and pH implementing PB as a screening design followed by BB as an optimization strategy. Categorical factors including diluting solvent and sequence of addition were kept invariable. Responses of the reaction systems were the maximum absorbance (Y1) and maximum fluorescence intensity (Y2), correspondingly. Quality tools as well as ANOVA testing, before and after response transformation were used to decide upon the substantial variables. Following the optimization of reaction variables using desirability plots, calibration graphs were found to be rectilinear in the range of 6–14 μg/mL and 0.4–1.2 μg/mL for methods A and B, respectively. Both methods proved to be sensitive with detection limits (DL) of 337 and 85 ng/mL, and quantitation limits (QL) of 1086 and 283 ng/mL, for methods A and B, respectively. An interference study was performed using potential foreign species. No significant interference effect was observed on any of the proposed procedures. System performance was addressed following ICH guidelines and considering parameters such as linearity, detection and quantification limits, accuracy and precision, robustness and specificity.

1. Introduction

Amino acids (AAs), generally viewed as the assembling blocks of proteins and the rudimentary metabolic intermediates, play a fundamental role in almost all biological processes. Having such a significance, AAs are important nutrients that are widely used in dietetic supplements, plant and soil composts, as well as food production biotechnology. Yet, not all AAs are "proteinogenic". Non-proteinogenic AAs, not only as their name implies do not exist in proteins, but also are not generated directly by the systematic cellular mechanisms.

L-Ornithine (ORN) is a non-proteinogenic AA that is un-coded for by DNA and accordingly not involved in protein synthesis. Being created, together with urea, by the deed of the enzyme arginase on the essential amino acid arginine, ORN is the centre of urea/ornithine cycle and its "rate limiting step" [1–4]. Produced ORN helps in converting ammonia into urea. Having such a fate, ORN administration would be helpful in treatment of diseases characterized by ammonia accumulation such as hepatic encephalopathy, and protracted cardiac exercise. Moreover, and by boosting ammonia excretion, ORN was found to help in extenuating lethargy [5,6].

The indirect role of ORN in protein synthesis, and hence on increasing the body mass, in addition to its role in improving fat metabolism, would explain marketing of ORN as a weightlifter for athletes. The effect of ORN on elevating serum human growth hormone was not confirmed at low dose [7]. Owing mainly to its nourishing and ergogenic effects, ORN is widely used in Qatar and the Middle East as an OTC dietary supplement. These facts about ORN as a non-proteinogenic AA make its analysis via lucrative, simple, sensitive, and time-saving procedure a challenging task.

L-Ornithine monohydrochloride; (*S*)-(+)-2,5-Diaminopentanoic acid hydrochloride, and as shown in Scheme 1, does not have a chromophoric functional group. Furthermore, ORN is present in biological fluids

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in the micro-concentration range, an issue that necessitates the development of an accurate, robust and highly sensitive procedure for its determination.

Few approaches have been reported in literature for micro-determination of ORN. Most of these procedures and to the best of our knowledge used sophisticated techniques such as chromatography as an analytical platform. Yet, with all the advantages such an approach offers, availability for routine analysis, cost-effectiveness, and laboriousness make their use in quality control laboratories questionable [8–11]. Nevertheless, none of the proposed procedures have touched the factorial design as an approach to assess the significant factors or their probable interactions. Moreover, most of these methods if not all, are based on the "outdated" approach of OVAT (one variable at time), where the impact of any of the reaction inputs is investigated one by one and without offering an assessment of the weight of their interactions. Chemometrics, on the other hand, propounds an invincible approach and a hypothetical basis that can account for all the previous obstacles. Engaging chemometrics for analytical method development would provide the 'comprehensive' knowledge of a phenomenon, and at the same time with less experimental effort [12,13].

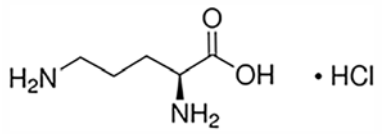
In the current investigation, two chemometrics-assisted approaches (spectrophotometry and spectrofluorimetry) were used to determine ORN both per se and in its alimentary supplement. Derivatization of ORN primary amine group via Hantzsch condensation reaction was employed as a good paradigm for testing the competency of the proposed factorial designs [14–17].

As a condensation reaction, Hantzsch pathway involves elimination of three water molecules following an interaction between an aldehyde, a β-diketone and an amino group [15–19]. Literature applications of Hantzsch reaction show that a set of known factors affects the fate of this reaction and the formation of a coloured dihydrolutidine derivative. Based on our previous knowledge, we restricted the initial investigation to only four 'continuous' factors: temperature, reaction time, reagent volume, and pH [15–18], while 'categorical' variables were run indiscriminately.

The current study implies an inclusive multivariate strategy for determination of ORN using spectrophotometry and spectrofluorimetry as a plan and formation of a coloured/ fluorescent ORN derivative via Hantzsch reaction as an itinerary. The proposed multivariate scheme combines Plackett-Burman (PBD) and Box-Behnken (BB) designs as screening and optimization designs, respectively [20–23].

The chief forte offered herein with lies in the expansive vision gained into the determination of ORN as per se and in supplements. A pool of factors with arbitrarily (or knowledge-based or in view of a preceding univariate testing) predefined upper and lower domains will be introduced into the screening phase exploiting PBD. The most influential factors will be determined by the end of this stage. Subsequent to this phase, and as a response surface methodology, BB design will serve to tune the factors and their levels to the optimum values. Maximum absorbance at the designated wavelength was the target.

Validation of any of the proposed designs will be done using analysis of variance (ANOVA) testing as well as the guidelines proposed by the ICH [24].



Scheme 1. L-Ornithine monohydrochloride.

2. Experimental

2.1. Materials and Reagents

All reagents used were of analytical grade. L-Ornithine monohydrochloride (ORN), acetylacetone, glacial acetic acid, and sodium acetate were obtained from Sigma-Aldrich (USA) and were used without further treatment. Formaldehyde (37% *w*/w) was procured from Merck (Darmstadt, Germany). Doubly distilled water (DDW) was the solvent of choice through the study. Now Food® Dietary Supplement Capsules (500 mg ORN/ capsule) were purchased from local pharmacy stores, Doha, Qatar.

2.2. Hardware and Software

For spectrophotometric measurements, an Agilent diode array UV–Vis Spectrometer with 10 mm quartz cell was used. PerkinElmer LS-45 spectrofluorimeter equipped with a high energy pulsed Xenon source for excitation, and an FL WinLab™ software was used. All measurements were done in a standard Hellma® flourescent 10 mm pathlength Suprasil® quartz cuvettes. Excitation and emission monochromators are scanned simultaneously with a constant difference $\Delta\lambda = 80$ nm and a response time of 8 s.

The pH of the working solutions was adjusted employing a Jenway pH meter equipped with a glass combination electrode (UK). A thermostatically controlled water bath (MLV, Salvis AG Emmenbruck, Germany) was exploited whenever heating is needed. A Minitab®17 software (Minitab Inc., State College, Pennsylvania, USA) was used for building and analyzing the factorial designs of choice. An OriginPro software (OriginLab, Northampton, MA) was utilized to execute line and curve fittings whenever needed.

2.3. Reagent and Stock Solution Preparation

Into a 50.0 mL volumetric flask, a volume of 20.0 mL acetate buffer (pH depends on optimization outcomes), 4.20 mL acetylacetone, and 10.0 mL formaldehyde were added. The volume was made to the mark with DDW. The reagent was prepared daily just before use and kept in a cool and dark place. ORN standard solution was prepared as 1 mg mL^{-1} using DDW. Further dilutions with DDW were prepared to get appropriate working solutions.

2.4. Optimized Reaction Procedure for Authentic ORN Samples

2.4.1. Method A; Spectrophotometry

Aliquots of standard drug solution containing 60.0–140.0 μg/mL ORN were transferred into 10.0 mL test tubes. A volume of 1.0 mL reagent prepared in acetate buffer of pH 3.6 was added. The mixture was heated in a boiling water bath (100 ± 2 °C) for 15 min. and then test tubes were left to cool down. Test tubes contents were transferred into 10.0 mL volumetric flasks and the volume was made up to the mark with DDW. A blank was similarly treated using water instead of ORN. Absorbance (Y1) of the reaction product was measured at 327 nm against the blank.

2.4.2. Method B; Spectrofluorimetry

To different aliquots of standard solutions containing 4–12 μg/mL ORN, 0.50 mL of the reagent solution prepared in acetate buffer pH 4.2. The prepared mixture was diluted to the mark (10.0 mL) using DDW. The flourescence intensity (Y2) of the reaction product was measured at an excitation wavelength of 325 nm and emission wavelength of 480 nm using a reagent blank in which ORN is omitted.