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RESEARCH

# Development and Validation of a Novel and Eco-Friendly RP-HPLC Method for the Simultaneous Analysis of Betaine, Citric Acid, L-Carnitine, and Niacinamide in Dozliv Forte-P Ultra

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## Abstract

**Purpose** A simple, novel, and eco-friendly analytical method was developed for the simultaneous analysis of Betaine, Citric acid, L-carnitine, and Niacinamide in Dozliv Forte-P Ultra liquid. The developed method was validated in accordance with ICH Q2 (R1) guidelines to ensure reliable and accurate quantitative analysis.

**Methods** Chromatographic separation was achieved using a C-18 column under the optimized isocratic elution conditions. The developed method was evaluated for various validation parameters, including system suitability, specificity, linearity, LOD, LOQ, accuracy, precision, robustness, and solution stability. Moreover, the environmental and analytical sustainability of the method was examined using the greenness (AGREE, MoGAPI), blueness (BAGI) and redness (RAPI) assessment tools.

**Results** The developed method complied with the ICH Q2 (R1) guidelines and all the validation parameters were found to be within acceptable limits. The method exhibited good suitability with tailing factor below 2.0, and theoretical plate counts greater than 2000. It also demonstrated excellent specificity along with good robustness and linearity ($R^2 > 0.990$), The obtained LOD, LOQ values were acceptable, while precision studies showed %RSD values below 2% and accuracy studies demonstrated recovery rates greater than 98%. Furthermore, the method achieved favorable AGREE (0.77), MoGAPI (85), BAGI (85) and acceptable RAPI scores indicating that the developed method is reliable, sustainable and environment friendly.

**Conclusion** The developed method proved to be novel, precise, reliable, robust and eco-friendly. Hence, the developed method can be effectively utilized for the simultaneous quantification of these compounds in pharmaceutical formulations, veterinary products, and feed supplements.

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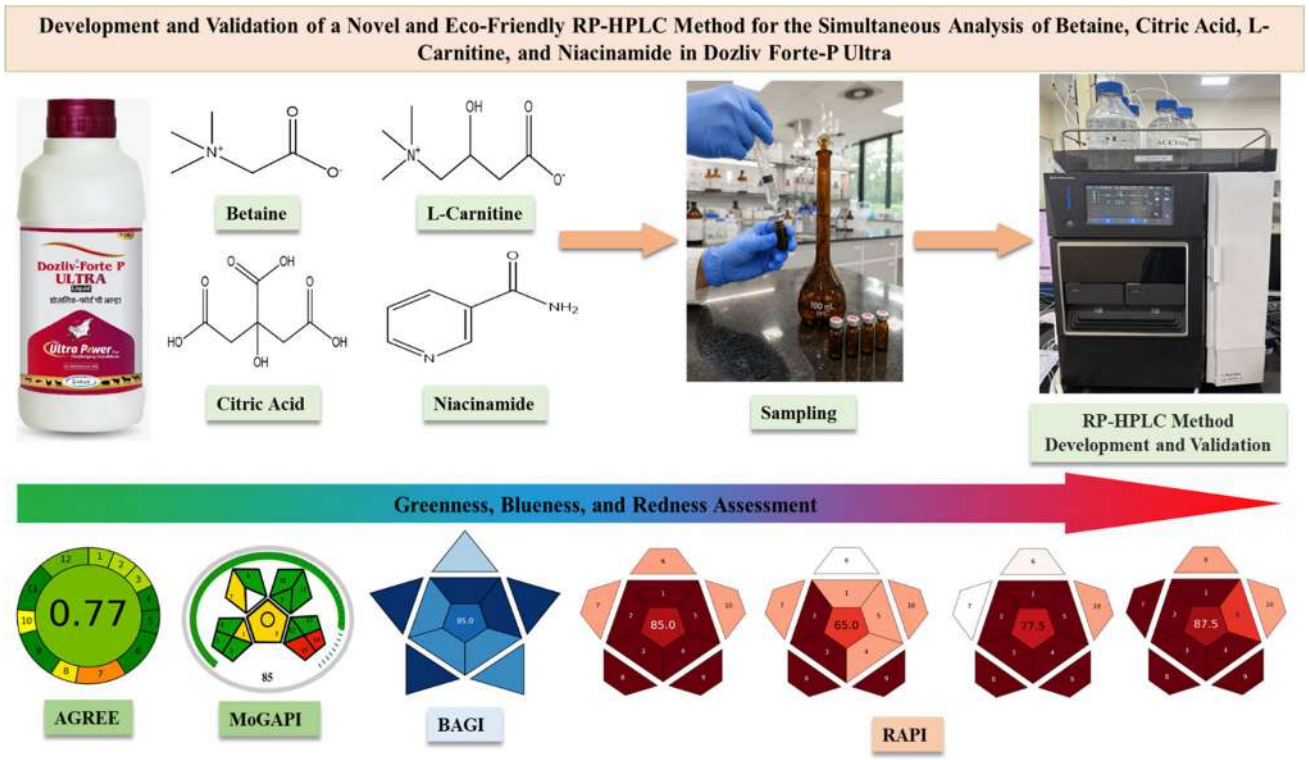
## Graphical Abstract




**Keywords** RP-HPLC · Betaine · L-Carnitine · Citric acid · Niacinamide · Validation · Green Analytical Chemistry

## Introduction

Dozliv Forte-P Ultra is a poultry feed supplement designed to support liver health and alleviate associated disorders. It contains key bioactive components, including Betaine, Citric acid, L-carnitine, Niacinamide, and other hepatoprotective agents, that support hepatic function and promote recovery [1]. Betaine hydrochloride (Fig. 1a, $C5H12NO_2$. Cl, M.W: 153.61 g/mol, M.P: 301 °C) known as the hydrochloride form of Betaine which is in the form of white crystalline powder with sweet taste and very soluble in water [PubChem CID: 11545, 247]. Betaine serves as a methyl donor in the folate–methionine cycle and modulates gene expression via epigenetic mechanisms [2, 3]. It has been reported that Betaine enhances growth and laying performance [4–6], reduces serum triglyceride levels, and promotes lipid metabolism and triglyceride regulation [7, 8]. Recent studies have shown that Betaine supplementation attenuates high-fat diet–induced hepatic lipid accumulation in rats by suppressing hepatic lipogenesis and promoting fatty acid oxidation [9]. Citric acid (Fig. 1b, C6H8O7, M.W: 192.12 g/mol, M.P: 153 °C) is a colourless crystals with an acid taste and very soluble in water [PubChem CID: 311]. It was reported in a previous study that the ability of Citric acid to enhance energy metabolism and inhibit lipid droplet accumulation may be associated with the activation of AMP-activated protein kinase (AMPK) in broilers [10–12]. L-Carnitine (Fig. 1c, $C7H15NO_3$, M.W: 161.20 g/mol, M.P: 197 °C) is a white crystalline powder and readily soluble in water [PubChem CID: 10917). It plays an essential role in energy metabolism by enabling the transport of long-chain fatty acids into mitochondria for β-oxidation, thereby promoting ATP production through fatty acid oxidation [13]. Studies have demonstrated that L-carnitine supplementation can significantly influence lipid profiles and reduce abdominal fat deposition in chickens [14]. Niacinamide (Fig. 1d, C6H6N2O, M.W: 122.12 g/mol, M.P: 130 °C) is a white crystalline powder with bitter taste and very soluble in water [PubChem CID: 936]. Niacinamide supplementation has been shown to increase hepatic $NAD^+$ levels, thereby enhancing the activity and expression of sirtuins and their associated signaling pathways [15]. Activation of these pathways suppresses lipogenesis while promoting fatty acid oxidation [16]. Additionally, Niacinamide has been reported to decrease vascular oxidative stress, prevent hepatic fat accumulation, and reduce pre-existing hepatic steatosis in a high-fat diet-induced NAFLD rat model [17]. Dietary Niacinamide supplementation has been shown to

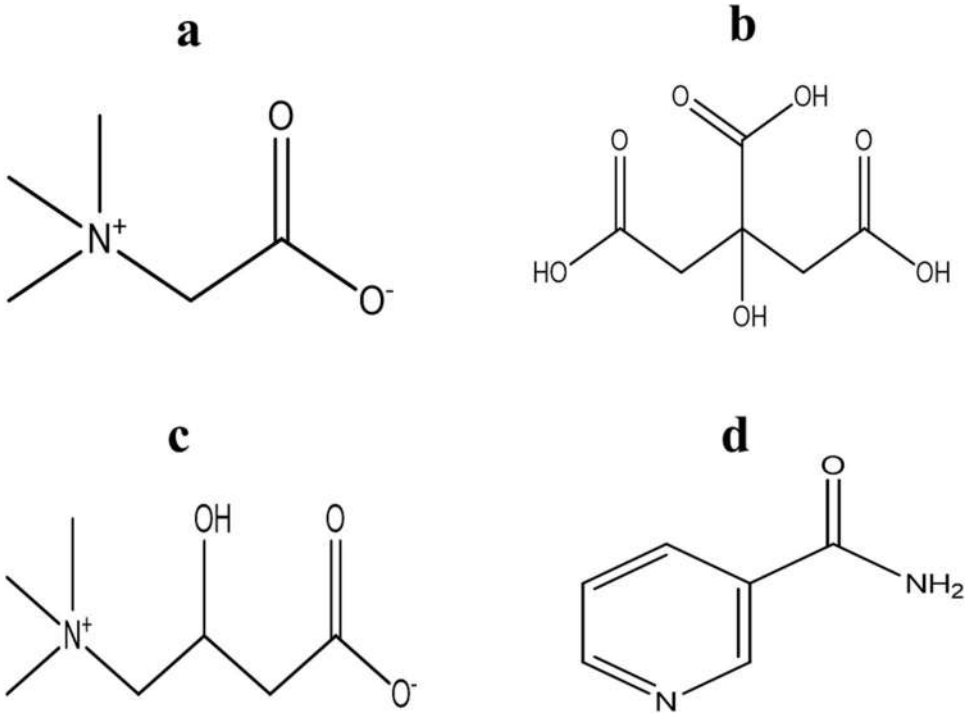



**Fig. 1** Chemical Structures (**a**) Betaine, (**b**) Citric acid, (**c**) L-Carnitine, (**d**) Niacinamide

improve growth performance and regulate lipid metabolism in poultry [18].

A variety of chromatographic and spectroscopic techniques have been reported in the literature for the analysis of Betaine, Citric acid, L-Carnitine, and Niacinamide, in different matrices. For Betaine analysis, techniques such as UHPLC-MS–MS, HILIC-LC–MS, LC-MRM-MS, MALDI-TOF MS, HILIC-ELSD, HPLC–ICPMS-ESMS, HPLC–UV, and Tandem mass spectrometry have been reported [19–26]. Similarly, for Citric acid analysis, analytical methods such as LC–MS/MS, HPLC–DAD, Ion Chromatography, Capillary electrophoresis have been reported [27–31]. Likewise, the determination of L-Carnitine has been carried out using UPLC-MS/MS, Nano LC/UV, HPLC-Tandem mass spectrometry, HPLC/FLD, and Chiral liquid chromatography-Tandem mass spectrometry [32–36]. For Niacinamide estimation, analytical approaches including UV spectrophotometry, RP-HPLC, HPLC–UV, and LC–MS/MS have been extensively utilized [37–45]. In addition, several studies have reported the simultaneous determination of Betaine and L-Carnitine using ion chromatography, UHPLC-MS/MS, and LC–MS/MS methods [46, 47].

However, no isocratic RP-HPLC method has yet been established for the simultaneous estimation and validation of Betaine, Citric Acid, L-Carnitine, and Niacinamide in a single chromatographic run using a C-18 column and a mobile phase containing sodium dihydrogen phosphate, orthophosphoric acid, and sodium 1-hexane sulphonic acid.

In the present research work, a novel RP-HPLC method was successfully developed and validated in accordance with ICH Q2 (R1) guidelines for the simultaneous estimation of all four analytes in a single chromatographic run. The developed method demonstrated high accuracy, precision, specificity, sensitivity, and excellent linearity. Additionally, the analytical performance and environmental sustainability of the method were evaluated using greenness (AGREE, MoGAPI), blueness (BAGI), and redness (RAPI) assessment tools.

## Materials and Methods

### Chemicals and Reagents

All the chemicals and reagents used in the analysis were of analytical grade. Methanol was purchased from Qualigens, 1-Hexane Sulphonic Acid Sodium Salt was obtained from Molychem, Orthophosphoric Acid, Sodium Hydroxide Pellets and sodium dihydrogen phosphate were obtained from SRL, HPLC water was purchased from Loba Chemie Pvt. Ltd., The working standard of Betaine, L-Carnitine, Niacinamide were procured from Tokyo Chemical Industry Co. Ltd., and Citric Acid was obtained from SRL.

### Instrumentation

A Shimadzu LC-2050C 3D i-series RP-HPLC system equipped with a Photodiode array (PDA) detector was utilized for the method development and validation studies.

## Analytical Method Development

### Chromatographic Conditions

The mobile phase for Betaine, Citric Acid, L-Carnitine, and Niacinamide was composed of Buffer solution and Methanol in a ratio of 98:02. The buffer solution was prepared by adding 0.065 M sodium dihydrogen phosphate, 0.1% (v/v) orthophosphoric acid, and 0.008 M sodium 1-hexane sulphonic acid in HPLC water. The pH was then adjusted to 2.50±0.05 using Sodium Hydroxide. The flow rate was maintained at 0.8 ml per minute. A stainless steel column 250 mm × 4.6 mm, packed with octadecylsilane bonded to porous silica (5µm) was utilized. A volume of 20 µl of the prepared solution was injected. The detection was carried out at a wavelength of 210 nm.

### Preparation of Standard Solution

Firstly, a stock solution A was prepared by accurately weighing 200.0 mg of L-carnitine into a 20 ml volumetric flask, diluted with HPLC water, followed by sonication for 2 min, and the volume was made up to the mark with HPLC water. Secondly, a stock solution B was prepared by transferring 20.0 mg of Citric acid and 30.0 mg of Niacinamide into a 100 ml volumetric flask. The contents were diluted with HPLC water, followed by sonication for 2 min, and the volume was made up to the mark with HPLC water. Lastly, the working standard solution was prepared by adding Betaine (18.0 mg) into a 100 ml volumetric flask, and dissolved in HPLC water, followed by sonication for 2 min. Now, from stock solution A, 5.0 ml solution was transferred, and 10.0 ml solution from stock solution B was transferred into a volumetric flask containing the standard solution, and the final volume was made up to 100 ml with HPLC water to obtain the final working standard solution. Before filling into the HPLC vials, the standard solution was filtered through a 0.20 µm nylon filter.

### Preparation of Sample Solution

For testing Betaine, Citric Acid, L-Carnitine, and Niacinamide simultaneously, 1.0 g of the sample was taken in a 100 ml volumetric flask, and diluted with HPLC water up to the mark. The solution was shaken for 2 min and then sonicated for 2 min. Before filling into the HPLC vials, the sample was filtered through a 0.20 µm nylon filter.

## Analytical Method Validation

### Specificity

The developed method should demonstrate excellent specificity by accurately detecting the analytes of interest without any interference from other components. To evaluate the specificity of the method, blank, placebo, standard, and test solutions containing Betaine, Citric Acid, L-Carnitine, and Niacinamide were injected into the chromatographic system, and the corresponding representative chromatograms were recorded [48–52].

### Linearity, LOD, and LOQ

The linearity of the developed method was assessed by evaluating the relationship between the concentration of the analytes and their respective peak responses. Calibration graphs were plotted to determine whether the method exhibited a linear response across the selected concentration range, and the obtained results were further confirmed using appropriate statistical analysis. In this study, solutions of Betaine, Citric Acid, L-Carnitine, and Niacinamide were prepared at five concentration levels ranging from 50 to 150% of the working concentration. The calibration curves generated from these concentration levels were utilized to evaluate the linearity of the method, while the corresponding peak area data were analyzed using linear regression analysis. The limit of detection (LOD) and limit of quantification (LOQ) were determined from the linear regression equation using the formulas 3.3 × SD/S and 10 × SD/S, respectively [49, 53].

### Accuracy

The accuracy of the developed method was evaluated by adding known amounts of Betaine, Citric Acid, L-Carnitine, and Niacinamide into a placebo preparation to determine the method's ability to accurately measure the analyte. The actual concentrations of Betaine, Citric Acid, L-Carnitine, and Niacinamide were compared with the measured concentrations, and their percentage recoveries were calculated to evaluate the accuracy of the method. The recovery study was performed at three distinct concentration levels corresponding to 50%, 100%, and 150% of the test preparation concentration. At each concentration level, triplicate preparations were prepared and analyzed to ensure the reliability and consistency of the recovery results [50, 54].

### Precision

The precision of an analytical method indicates the closeness of agreement among a series of measurements obtained from multiple sampling of the same homogeneous sample under specified conditions. In the present study, method precision was evaluated in terms of repeatability by performing six replicate assays of Betaine, Citric Acid, L-Carnitine, and Niacinamide within the same day. Intermediate precision was further assessed by repeating the analysis on a different day, by a different analyst, and in a different laboratory, under the same

analytical conditions. The method was considered precise when the %RSD was found to be not more than 2% [51, 55].

### Robustness

The robustness of an analytical method represents its ability to remain unaffected by small and deliberate variations in method parameters. It serves as an indicator of how consistent and dependable the method will be under typical operational conditions. In the present study, robustness was evaluated by introducing minor variations in analytical parameters, including wavelength variation (±2 nm) and flow rate variation (±10%). These variations were applied to assess their influence on the simultaneous analysis of Betaine, Citric Acid, L-Carnitine, and Niacinamide. The impact of these variations was examined by monitoring chromatographic parameters, such as retention time (RT), asymmetry factor, theoretical plate count, and the assay values [49, 50].

### System Suitability

System suitability analysis is an important step used to assess the adequacy and reliability of the developed chromatographic method for the intended analysis. In the present study, system performance was evaluated by examining several parameters %RSD, retention time, peak area, tailing factors, and the number of theoretical plates. For this purpose, six replicate injections containing Betaine, Citric Acid, L-Carnitine, and Niacinamide were analyzed using the newly developed RP-HPLC method. The obtained results were assessed to ensure that the chromatographic system fulfilled the required criteria for reliable analytical performance [48, 49, 56].

### Solution Stability

To assess solution stability, standard and sample solutions of Betaine, Citric Acid, L-Carnitine, and Niacinamide were analyzed by HPLC at initial, 12, and 24 h. The % assay difference and % RSD values were determined at each time point to monitor any changes over the study period. The solutions were considered stable if the % assay difference and % RSD values complied with the acceptance criteria of not more than 2.0% [57].

## Results and Discussion

### Optimization of Chromatographic Conditions

A C18 analytical column (250 mm × 4.6 mm, 5 µm) was selected for HPLC method development, with mobile phase optimization carried out to achieve sharp, well-resolved chromatographic peaks for Betaine, Citric Acid, L-Carnitine, and Niacinamide. Separation was performed under isocratic elution conditions at a flow rate of 0.8 mL/min, with the column and cooler temperature held at 30 °C and 10 °C. The injection volume was kept at 20 µL. Detection was performed using a Photodiode Array (PDA) detector operating at 210 nm wavelength, which demonstrated optimal sensitivity and peak response across all four compounds. Under the optimized chromatographic conditions, all four analytes were successfully separated within a single 30 min run. Retention times observed under the optimized conditions were approximately 4 min for Betaine, 5 min for Citric acid, 10 min for L-Carnitine, and 18 min for Niacinamide, respectively.

### Analytical Method Validation

#### Specificity

The developed method showed good specificity, without any significant interference from other substances. The specificity of Betaine, Citric Acid, L-Carnitine, and Niacinamide was evaluated by comparing the chromatograms of the sample solution with those of the standard solution, placebo solution, and blank solution. The peak observed in the chromatogram were well-defined and corresponded only to the target compound. The chromatograms obtained for blank, placebo, standard, and sample solutions are shown in Fig. 2 respectively.

#### Linearity, LOD, and LOQ

The linearity study showed regression coefficient ($R^2$) values of 0.9998 for Betaine, 0.9993 for Citric acid, 0.9993 for L-Carnitine, and 1.0000 for Niacinamide, respectively. All values were within the acceptable range of 0.990 to 1.000, indicating a good linear relationship between concentration and peak area. The linearity, LOD, and LOQ results are summarized in Table 1. These results confirmed that the method is suitable and reliable for the quantification of all four analytes in the sample. The representative calibration graphs for linearity are shown in Figures S1-S4.

#### Accuracy

The method showed good accuracy at three concentration levels (50%, 100%, and 150%). The recovery percentages for Betaine, Citric Acid, L-Carnitine, and Niacinamide at these levels are presented in Table 2. The %RSD values were found to be 0.17%, 0.19%, 0.24%, and 0.19% for Betaine, Citric Acid, L-Carnitine, and Niacinamide, respectively, all

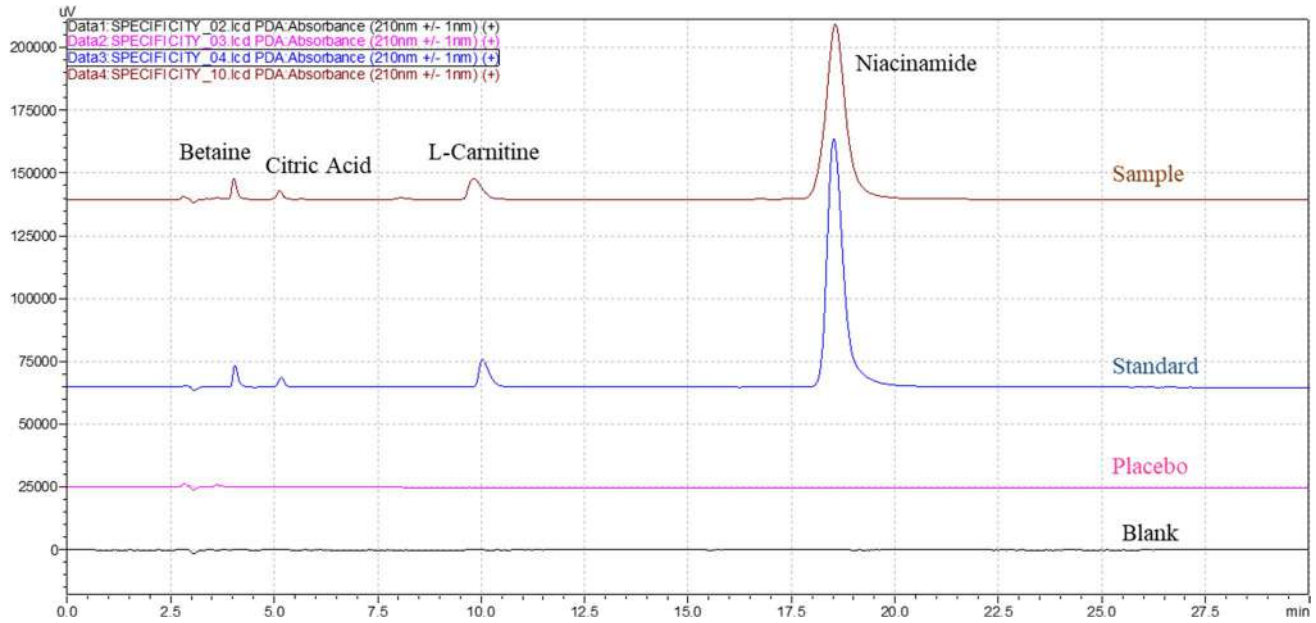



**Fig. 2** Representative specificity chromatograms of betaine (180 µg/ml), citric acid (20 µg/ml), L-Carnitine (500 µg/ml), and niacinamide (30 µg/ml)

**Table 1** Linear regression data

| Parameters | Betaine | Citric Acid | L-Carnitine | Niacinamide |
|---|---|---|---|---|
| Slope | 395.24 | 1790.00 | 369.60 | 91259.00 |
| Intercept | 586.57 | 137.50 | 538.60 | 8087.00 |
| Standard Error | 280.11 | 418.52 | 983.74 | 5506.85 |
| Regression Coefficient ($R^2$) | 0.9998 | 0.9993 | 0.9993 | 1.0000 |
| Range (µg/ml) | 90–270 | 10–30 | 250–750 | 15–45 |
| LOD (µg/ml) | 5.73 | 1.89 | 21.52 | 0.49 |
| LOQ (µg/ml) | 17.36 | 5.73 | 65.21 | 1.48 |

**Table 2** Accuracy and precision studies data

| Concentration Levels | Accuracy (Percentage of Recovery, %RSD) | | | |
|---|---|---|---|---|
| | Betaine | Citric Acid | L-Carnitine | Niacinamide |
| At 50% | 99.79%, 0.17% | 98.36%, 0.19% | 99.24%, 0.24% | 99.15%, 0.19% |
| At 100% | 99.71%, 0.17% | 98.73%, 0.19% | 99.70%, 0.24% | 99.26%, 0.19% |
| At 150% | 99.47%, 0.17% | 98.56%, 0.19% | 99.57%, 0.24% | 99.53%, 0.19% |
| Precision (%RSD) | | | | |
| Intra-day Precision Data | 0.28% | 0.76% | 0.28% | 0.16% |
| Inter-day Precision Data | 0.73% | 0.57% | 0.36% | 0.42% |

of which were within the acceptable limit of less than 2.0%. The accuracy chromatograms at 50%, 100%, and 150% for all four analytes are shown in Fig. 3 respectively.

## Precision

The relative standard deviation values for six preparations were calculated and found to be within the acceptance limit of less than 2.0%. For Intra-day precision, the % RSD values for Betaine, Citric Acid, L-Carnitine, and Niacinamide were found to be 0.28%, 0.76%, 0.28%, 0.16% respectively. Similarly, for inter-day precision, the % RSD values were found to be 0.73%, 0.57%, 0.36%, and 0.42%, respectively. Both intra-day and inter-day results confirm that the method is precise and meets the acceptance criteria of not more than 2.0%. The results of precision study are provided in Table 2, and the corresponding chromatograms are shown in Figure S5.

## Robustness

The robustness study showed that the method was not significantly affected by small changes in flow rate (± 10%) and wavelength (± 2 nm). The analytical parameters, including retention time, peak area, tailing factor, number of theoretical plates, and percentage assay, remained stable under these varied conditions. As shown in Table 3, the percentage relative difference between assay values was within the acceptable limit of 2.0%. This confirms that the method is

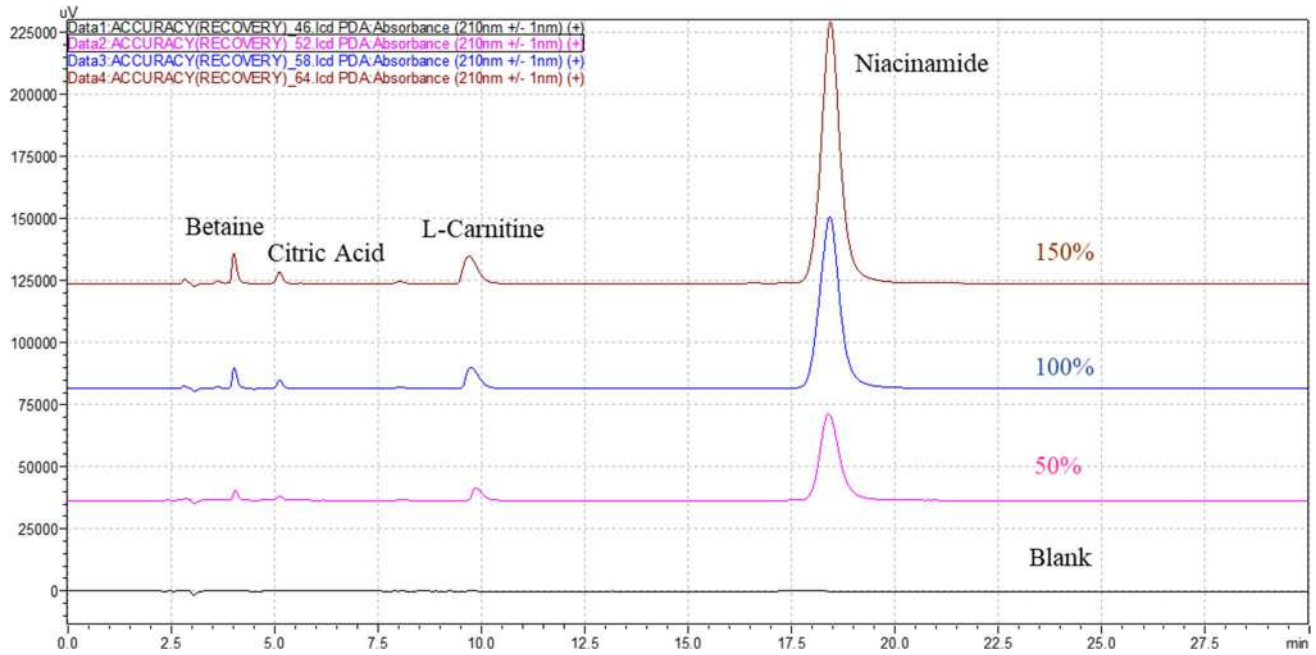



**Fig. 3** Representative accuracy chromatograms of betaine, citric acid, L-Carnitine, and niacinamide

**Table 3** Robustness study data based on flow rate and wavelength

| Sample | Parameters | | % Assay | % Assay Difference |
|---|---|---|---|---|
| Betaine | Flow Rate | 0.80 ml/min | 99.71 | - |
| | | 0.72 ml/min | 99.59 | 0.12 |
| | | 0.88 ml/min | 100.59 | 0.88 |
| | Wavelength | 210 nm | 99.71 | - |
| | | 208 nm | 99.86 | 0.15 |
| | | 212 nm | 100.22 | 0.51 |
| Citric Acid | Flow Rate | 0.80 ml/min | 99.47 | - |
| | | 0.72 ml/min | 101.18 | 1.71 |
| | | 0.88 ml/min | 99.37 | 0.10 |
| | Wavelength | 210 nm | 99.47 | - |
| | | 208 nm | 98.19 | 1.28 |
| | | 212 nm | 98.65 | 0.82 |
| L-Carnitine | Flow Rate | 0.80 ml/min | 105.41 | - |
| | | 0.72 ml/min | 105.03 | 0.38 |
| | | 0.88 ml/min | 104.26 | 1.15 |
| | Wavelength | 210 nm | 105.41 | - |
| | | 208 nm | 105.18 | 0.23 |
| | | 212 nm | 105.33 | 0.08 |
| Niacinamide | Flow Rate | 0.80 ml/min | 99.98 | - |
| | | 0.72 ml/min | 100.40 | 0.42 |
| | | 0.88 ml/min | 99.79 | 0.19 |
| | Wavelength | 210 nm | 99.98 | - |
| | | 208 nm | 99.82 | 0.16 |
| | | 212 nm | 100.15 | 0.17 |

reliable and robust, and unaffected by minor variations in conditions. The chromatograms obtained during the robustness study are shown in Figure S6 respectively.

## System Suitability

The chromatogram was analyzed, and peak areas were recorded for all analytes. The % RSD values of six replicate injections of Betaine, Citric Acid, L-Carnitine, and Niacinamide were found to be within the acceptance criteria of less than 2.0%. Moreover, the tailing factor was found less than 2.0, and theoretical plates were greater than 2000, which met the acceptance criteria. The data of system suitability for Betaine, Citric Acid, L-Carnitine, and Niacinamide are presented in Table 4, and the respective chromatogram is shown in Fig. 4.

**Table 4** Data of system suitability study

| Parameter | Betaine | Citric Acid | L-Carnitine | Niacinamide |
|---|---|---|---|---|
| Retention Time (Minutes) | 4.021 | 5.131 | 9.973 | 18.411 |
| Tailing Factor | 1.469 | 1.118 | 1.643 | 1.451 |
| Theoretical Plates | 31478 | 38906 | 51601 | 80034 |
| %RSD | 0.46 | 0.57 | 0.22 | 0.29 |

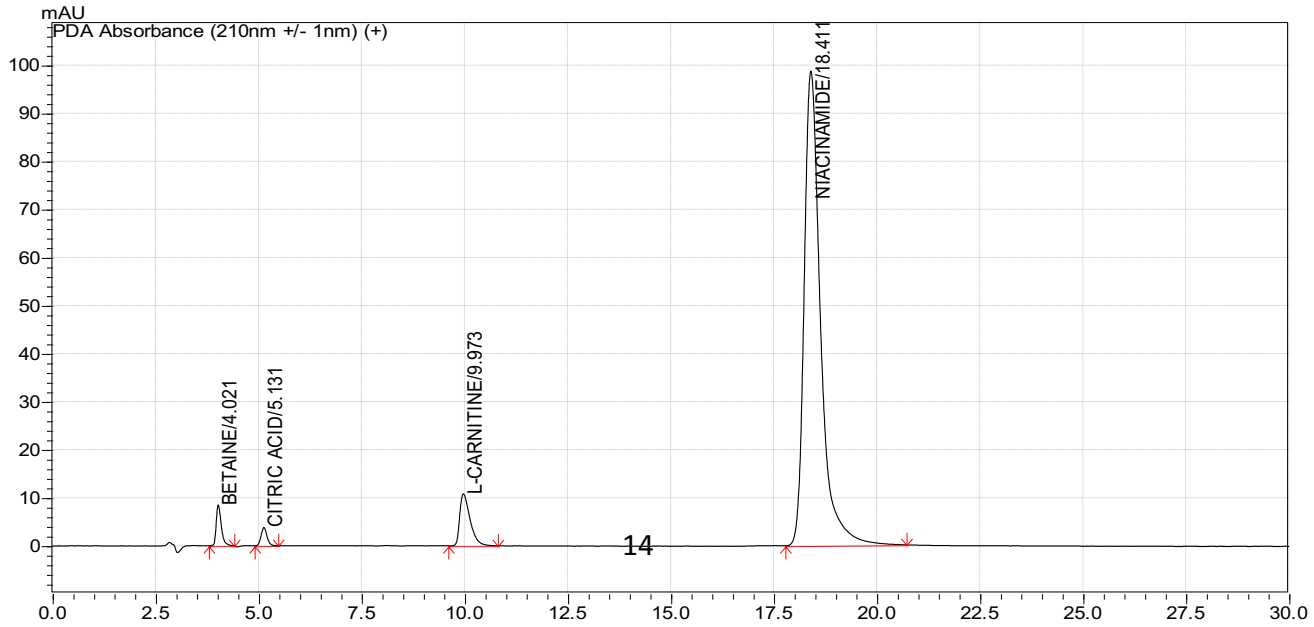



**Fig. 4** Representative chromatograms for system suitability of Betaine, Citric acid, L-Carnitine, and Niacinamide

### Solution Stability

The stability of standard and sample solutions was evaluated for Betaine, Citric Acid, L-Carnitine, and Niacinamide. All solutions were found to be stable after 24 h, with % assay differences and % RSD values falling within the acceptable limit of less than 2.0%. The results are summarized in Table 5, and the corresponding chromatograms are depicted in Figure S7.

### Greenness Assessment (AGREE and MoGAPI)

Green Analytical Chemistry (GAC) emphasizes the development of environment friendly analytical methods by encouraging sustainable practices that reduce the use of hazardous chemicals, waste generation, and energy consumption [58, 59]. The greenness of an analytical method is assessed using several criteria, including the toxicity and quantity of reagents, waste production, energy requirements, procedural complexity, miniaturization, and automation [60]. To evaluate the environmental sustainability and compliance of the developed RP-HPLC method with GAC principles, two multi-parameter assessment tools, namely Analytical GREEnness Metric (AGREE) and Modified Green Analytical Procedure Index (MoGAPI), were utilized [58].

The AGREE tool evaluates analytical methods based on all 12 principles of GAC using a segmented pictogram approach. Since certain criteria contribute differently to the overall greenness of a method, weighted scoring was assigned, giving higher importance to factors such as method simplicity, sample quantity, analytical throughput, and energy efficiency. According to the AGREE evaluation scale, scores $\geq 0.75$ represent excellent greenness, scores between 0.50–0.74 indicate acceptable greenness, and

**Table 5** Data of solution stability study

| Samples | Time Interval | %Assay | %Assay Difference | Overall Mean% | Overall SD% | Overall RSD% |
|---|---|---|---|---|---|---|
| Betaine | Initial | 99.09 | - | 98.90 | 0.43 | 0.44 |
| | After 12 h | 99.20 | 0.11 | | | |
| | After 24 h | 98.40 | 0.69 | | | |
| Citric Acid | Initial | 100.63 | - | 101.47 | 0.80 | 0.79 |
| | After 12 h | 101.53 | 0.90 | | | |
| | After 24 h | 102.23 | 1.60 | | | |
| L-Carnitine | Initial | 104.17 | - | 103.51 | 0.61 | 0.59 |
| | After 12 h | 102.98 | 1.19 | | | |
| | After 24 h | 103.36 | 0.81 | | | |
| Niacinamide | Initial | 98.15 | - | 98.34 | 0.18 | 0.18 |
| | After 12 h | 98.50 | 0.35 | | | |
| | After 24 h | 98.37 | 0.22 | | | |

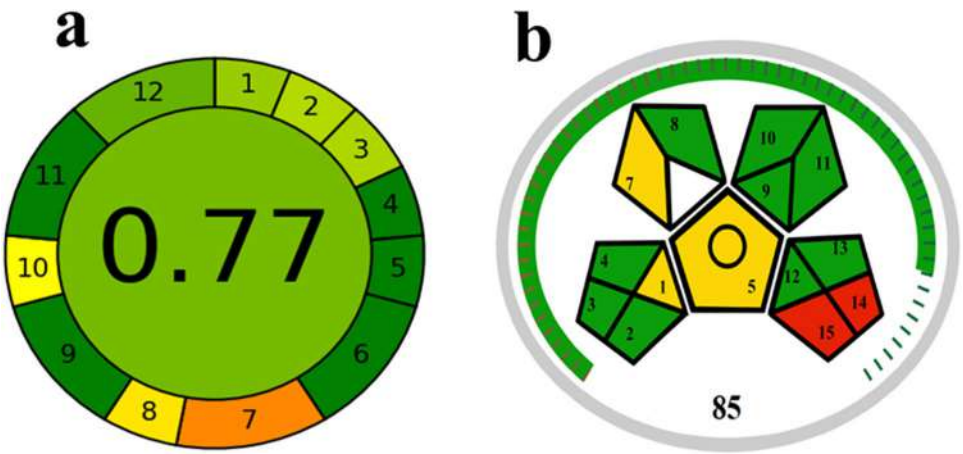



**Fig. 5** Greenness Assessment Pictograms (**a**) AGREE and (**b**) MoGAPI

scores < 0.50 reflect inadequate greenness [60]. The developed RP-HPLC method achieved an overall AGREE score of 0.77, as presented in Fig. 5a, indicating excellent environmental sustainability.

The Modified Green Analytical Procedure Index (MoGAPI) evaluates the environmental sustainability of analytical methods using 15 different assessment parameters by combining the visual GAPI pictogram with the quantitative analytical Eco-Scale scoring system [61]. According to the obtained overall score, analytical methods are classified as excellent green (≥75), acceptable green (50–74), or inadequate green (<50) [62]. The developed RP-HPLC method achieved a MoGAPI score of 85, as shown in Fig. 5b, indicating excellent greenness. Although the overall greenness score was slightly affected by waste generation and the limited use of renewable or sustainable materials, the method still demonstrated several environmentally favorable features, including low consumption of samples and reagents, reduced energy usage, and enhanced operator safety. Therefore, the AGREE and MoGAPI assessment tools demonstrated the excellent environmental sustainability of the developed method and its strong adherence to GAC principles.

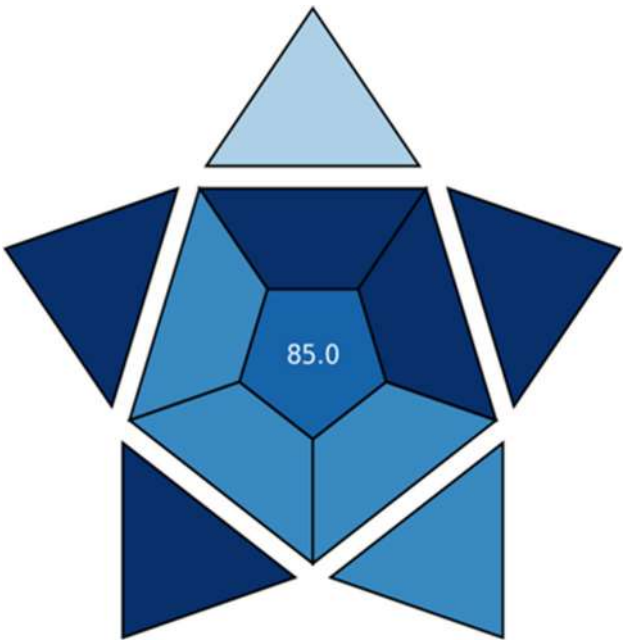



**Fig. 6** BAGI assessment pictogram

### Blueness Assessment (BAGI)

The Blue Applicability Grade Index (BAGI) system evaluates the applicability and environmental sustainability of analytical methods [63]. The final representation is shown as an asteroid-shaped pictogram with a numerical score at its center. The color of the pictogram reflects the level of compliance with the defined criteria, ranging from dark blue (high compliance), blue (moderate compliance), light blue (low compliance), to white (no compliance) [64]. The central value indicates the overall score of the method, which ranges from 25 to 100, where 25 corresponds to poor performance, and 100 signifies excellent performance [63, 65]. The developed RP-HPLC method achieved a score of 85, as presented in Fig. 6, indicating a high level of environmental sustainability.

### Redness Assessment (RAPI)

The Red Analytical Performance Index (RAPI) is a tool mainly used for the evaluation of quantitative analytical methods, with assessment criteria selected in accordance with ICH validation guidelines [66]. Each parameter is scored on a scale ranging from 0 to 10 with increments of 2.5, and the scores are represented visually using a color gradient from white (0) to dark red (10). The overall analytical performance is displayed at the center of the RAPI pictogram as a star-shaped diagram, providing an average score on a scale of 0–100.

In this study, the RAPI tool was applied to assess the RP-HPLC method developed for the simultaneous analysis of the selected analytes, where higher scores indicated better analytical performance [67, 68]. Separate RAPI scores were

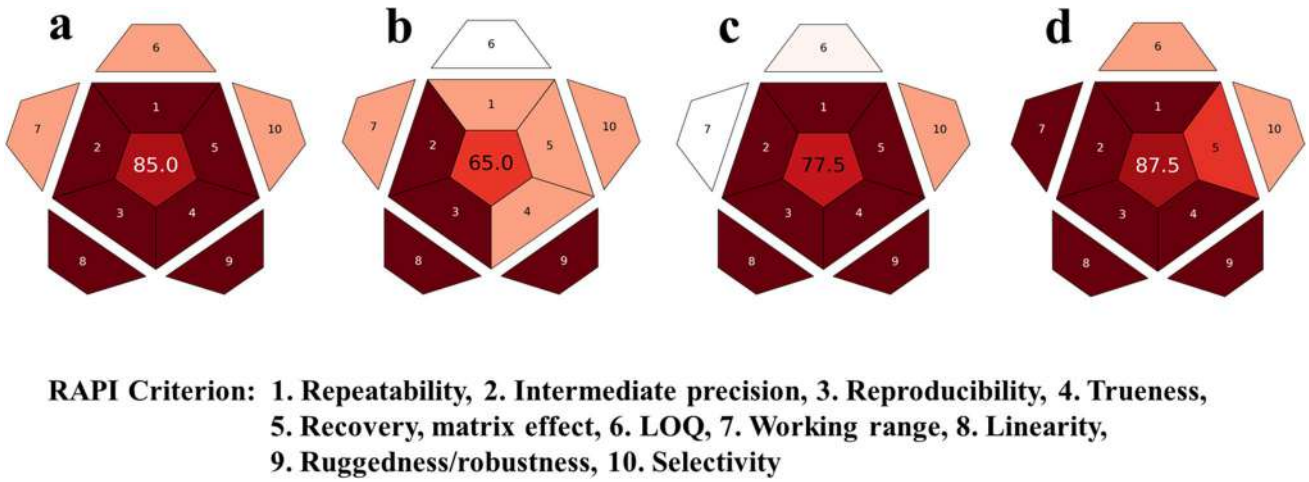



**Fig. 7** RAPI Assessment Pictograms (**a**) Betaine, (**b**) Citric Acid, (**c**) L-Carnitine, (**d**) Niacinamide

obtained for Betaine (85.0), Citric Acid (65.0), L-Carnitine (77.5), and Niacinamide (87.5), as the results of each criterion differed among all four analytes, as presented in Fig. 7a–d. The lower scores were mainly attributed to comparatively higher LOQ values and moderate working concentration ranges. In contrast, higher scores reflected excellent precision, linearity, robustness, reproducibility, and acceptable recovery values. The obtained RAPI results demonstrated that the developed RP-HPLC method is reliable, suitable, and effective for the simultaneous quantitative determination of Betaine, Citric Acid, L-Carnitine, and Niacinamide.

## Conclusion

A simple, reliable, and green RP-HPLC method was successfully developed and validated according to the current ICH guidelines for the simultaneous estimation of Betaine, Citric Acid, L-Carnitine, and Niacinamide in Dozliv Forte-P Ultra liquid. All validation parameters were found within the acceptable limits, exhibiting excellent specificity, linearity, precision, and recovery rates. The developed method also showed good compliance with GAC principles along with acceptable BAGI, and RAPI assessment results, demonstrating that the developed method is novel, reliable, and eco-friendly. Hence, the developed method can be effectively utilized for the simultaneous quantification of these compounds in pharmaceutical formulations, veterinary products, and feed supplements.

### Abbreviations

| | |
|---|---|
| RP-HPLC | Reverse Phase High-Performance Liquid Chromatography |
| M.W | Molecular Weight |
| M.P | Melting Point |
| ATP | Adenosine Triphosphate |
| $NAD^+$ | Nicotinamide Adenine Dinucleotide |
| NAFLD | Non-Alcoholic Fatty Liver Disease |
| UHPLC-MS-MS | Ultra-High Performance Liquid Chromatography-Tandem Mass Spectrometry |
| HILIC-LC-MS | Hydrophilic interaction Liquid Chromatography-Liquid Chromatography-Mass Spectrometry |
| LC-MRM-MS | Liquid Chromatography-Multiple Reaction Monitoring-Mass Spectrometry |
| MALDI-TOF MS | Matrix-Assisted Laser Desorption/ Ionization Time-of-Flight Mass Spectroscopy |
| HILIC-ELSD | Hydrophilic Interaction Liquid Chromatography-Evaporative Light Scattering Detector |
| HPLC–ICPMS-ESMS | High-Performance Liquid Chromatography-Inductively Coupled Plasma Tandem Mass Spectrometry |
| UV | Ultra-Violet |
| LC–MS/MS | Liquid Chromatography-Tandem Mass Spectrometry |
| DAD | Diode Array Detector |
| UPLC-MS/MS | Ultra Performance Liquid Chromatography-Tandem Mass Spectrometry |
| FLD | Fluorescence Detector |
| ICH | International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use |
| SD | Standard Deviation |
| RSD | Relative Standard Deviation |

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**Data Availability** No datasets were generated or analysed during the current study.

## Declarations

**Ethical Approval** Not applicable.

**Disclosure Statement** This manuscript was developed using established and widely accepted RP-HPLC method development and validation approaches, including standard ICH guideline-based validation parameters and conventional analytical workflows commonly employed in similar pharmaceutical analytical studies. The use of such standard methodologies may account for certain similarities between this submission and our previously published method development research. However, the current study is an independent and original investigation, and all experimental work, analytical methods, validation data, and results presented are novel and specific to this research. Furthermore, no generative or assistive artificial intelligence tools were used in the preparation of this manuscript.

**Competing interest** The authors declare no competing interests.

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