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ORIGINAL

Development and Validation of a Simple, Selective, and Accurate Reversed-Phase Liquid Chromatographic Method with Diode Array Detection (RP-HPLC/DAD) for the Simultaneous Analysis of 18 Free Amino Acids in Topical Formulations

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Abstract
Even though there are reported methods for the quantification of free amino acids (FAAs) in biological products, no work has been done on the analysis of these substances in formulations. Moreover, further research is required as the reported methods do not fulfill analytical method requirements. The objective of this study was, therefore, to develop and validate a rapid, reliable, and appropriate RP-HPLC/DAD method for the simultaneous determination of 18 FAAs (L-Ala, L-Arg, L-Asn, L-Asp, L-Gln, L-Glu, L-Gly, L-His, L-Ile, L-Lue, L-Lys, L-Met, L-Orn, L-Phe, L-Pro, L-Ser, L-Thr, and L-Val) in topical formulations. After appropriate method development, the technique was validated for selectivity, linearity and range, limit of detection, limit of quantification, precision, and accuracy. The samples were derivatized with 9-fluorenylmethyl chloroformate (Fmoc-Cl). Chromatographic separation was performed on InfinityLab Poroshell 120 E.C 18 (3 × 50) mm, 2.7 µm column at 25 °C. The mobile phase consisting of water and acetonitrile adjusted to appropriate pH was pumped in gradient mode at a flow rate of 0.7 mL/min. Ten microliters were injected and analyte detection was conducted using a DAD. The results indicate that the method was selective for these FAAs. It was linear over the concentration range of 5–80 µM with a correlation coefficient greater than 0.995. Moreover, it was sensitive, precise, accurate, and robust. All the reported drawbacks of RP-HPLC-based analysis of FAAs were resolved, and hence, this new method can be considered appropriate for the analysis of these FAAs in topical formulations.

Keywords FAAs · RP-HPLC/DAD · Fmoc-Cl · Formulations · Method validation

Introduction

Free amino acids (FAAs) are indispensable for healthy skin. They constitute the largest component (~40%) of the so-called natural moisturizing factor (NMF) [1] and are very important in maintaining the moisture balance of the skin. Among the different FAAs, the most abundant ones within the NMF are L-serine (L-Ser) (~36%), L-glycine (L-Gly) (~22%), and L-alanine (L-Ala) (~13%) [2]. The FAAs citrulline (Cit), ornithine (Orn), L-histidine (L-His), and L-arginine (L-Arg) account for 6–8%. As reported by Burke et al. [3], L-Ser, L-Gly, Cit, L-Ala, L-His, and L-threonine (L-Thr), in that order, are the dominant FAAs in the stratum corneum of human skin and account for as much as 80%. L-Proline (L-Pro) is also among the dominant FAAs; however, its presence is obscured, because it is masked by the large amount of Cit. The level of NMFs including FAAs can decline in

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dry skin due to disease conditions, such as atopic dermatitis, ichthyosis vulgaris, psoriasis, and age in addition to environmental conditions [4, 5]. One way to overcome such disease conditions is to formulate and deliver the major components of the NMF, i.e., FAAs, to the human skin in the form of semisolid or colloidal formulations [6]. The active pharmaceutical ingredients for such formulations (FAAs) can be sourced from different plants and mushrooms as reported in our previous work [7]. As with any other pharmaceutical or cosmeceutical products, the quality, safety, and efficacy of products loaded with FAAs should be ensured. In line with this, a rapid and efficient method for the simultaneous assay determination of the different FAAs in different formulations is of broad interest in the pharmaceutical and cosmeceutical industries.

Several methods have been described for the determination of FAAs, including automatic amino acid analyzers (cation-exchange chromatography), capillary electrophoresis, gas chromatography (GC), high-performance liquid chromatography (HPLC), and liquid chromatography–mass spectrometry (LC–MS/MS) [8–12]. Among the different methods, the combination of pre-column derivatization and separation by HPLC is the most convenient approach [13, 14]. To determine the concentration of compounds by this technique, it is necessary to chemically modify (derivatize) them into derivatives that absorb or fluoresce in the ultraviolet–visible (UV–Vis) wavelength range. Several reagents including but not limited to phenylisothiocyanate (PITC) [15], o-phthaldialdehyde (OPA) [16], 9-fluorenylmethyl chloroformate (Fmoc-Cl) [17, 18], naphthalene dicarboxaldehyde (NDA) [19], 5-dimethylamino-1-naphthalenesulfonyl chloride (dansyl-Cl) [20], 4-dimethylaminoazobenzene-4-sulfonyl chloride (dabsyl chloride) [21], 2,4-dinitrofluorobenzene (DNFB) [22], and 6-aminoquinolyl-*N*-hydroxysuccinimidylcarbamate (AQC) [23] have been used for this purpose.

Each of the derivatizing reagents that have been used in precolumn derivatization in HPLC analysis has its limitations. AQC amino acid derivatives require high solvent consumption and a long analysis time [24]. OPA and NDA do not react with secondary amino acids such as Pro and hydroxyproline and some of the amino acid derivatives are unstable, especially with OPA [16, 25]. Dansyl chloride lacks selectivity, and it reacts with both OH and NH_2 groups [20]. Moreover, the derivatization with dansyl-chloride is slow and the derivatives have poor stability. PITC derivatization results in hydrolysis and the production of by-products that interfere with the analysis [13]. Moreover, PITC derivatization methods have poor sensitivity, and the derivatization and excess solvent removal processes are time-consuming.

On the other hand, derivatization using Fmoc-Cl has been commonly used due to its distinct advantages. The derivatization is rapid and is done at ambient temperature, along with the resultant derivatives being very stable [26], Fmoc-Cl can react rapidly and quantitatively with both primary and secondary amino compounds in mild alkaline buffers [27]; it has also high sensitivity in the ultraviolet region [28]. Hence, the use of this reagent for the assay analysis of selected FAAs in cosmeceutical preparations can be considered one strategy for ensuring the quality of preparations containing these compounds.

However, the reported methods have a lot of limitations with respect to fulfilling method requirements and their applicability in sectors such as pharmaceutical and/or cosmeceutical industries where there is a strict regulatory requirement. The analytical methods mentioned in the literature involve the determination of FAAs mostly in biological matrixes (e.g., plant extracts), and the procedures are time-consuming and require excessive resources. Moreover, the common limitation of the reversed-phase HPLC (RP-HPLC) method for the analysis of FAAs, namely, poor retention of polar amino acids on the RP columns and difficulty of separation/resolution from the solvent peak and among the analyte peaks is still the major problem and needs further investigation. Due to this, there is an ongoing interest in the development of a reliable, rapid, and accurate method of analysis to assess the quality of FAAs, especially in the pharmaceutical and/or cosmeceutical industries. To our knowledge, there is no reported analytical method (that fulfills the pharmaceutical and/or cosmeceutical analytical method requirements) for the simultaneous analysis of the dominant FAAs of NMF in topical dosage forms. The objective of the present study was, therefore, to validate an RP-HPLC/DAD method for the simultaneous analysis of 18 selected FAAs, namely, L-Ala, L-Arg, L-Asn, L-Asp, L-Gln, L-Glu, L-Gly, L-His, L-Ilu, L-Lue, L-Lys, L-Met, L-Orn, L-Phe, L-Pro, L-Ser, L-Thr, and L-Val in colloidal formulations (microemulsions and microemulsion-based hydrogels) for dermal application. An attempt has been made to overcome the mentioned limitations of the RP-HPLC methods through appropriate method development and validation. The applicability of the method for the assay determination of the FAAs in other semi-solid preparations (microemulsion-based hydrogels) was also assessed.

Experimental

Reagents and Chemicals

The L-amino acids (L-Ala, L-Arg, L-Asn, L-Asp, L-Gln, L-Glu, L-Gly, L-His, L-Ilu, L-Lue, L-Lys, L-Met, L-Orn, L-Phe, L-Pro, L-Ser, L-Thr, and L-Val) were purchased from Sigma-Aldrich (St. Louis, USA). HPLC grade Fmoc-Cl, L-ornithine monohydrochloride, boric acid, sodium hydroxide, Brij O10, 2-phenoxyethanol, triethanolamine, and Transcutol®

P were also obtained from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). Carbopol® 934 was obtained from SERVA Electrophoresis GmbH (Heidelberg, Germany). 1-Adamantanamine (ADAM) was sourced from Thermo Fischer Scientific (Heysham, United Kingdom). HPLC grade water and acetonitrile were commercial products of Fischer Chemical (Loughborough, UK). Analytical grade glacial acetic acid and triethylamine were originated from Carl Roth GmbH + Co. KG (Karlsruhe, Germany). Isopropyl myristate and Poloxamer P407 were sourced from Caesar and Loretz GmbH (Hilden, Germany).

Six millimolar (6 mM) of Fmoc-Cl was prepared in acetonitrile for the derivatization and 12.5 mM of ADAM was prepared in a water: acetonitrile mixture (1:3). The 0.5 M borate buffer pH 8.6 was prepared by dissolving the required amount of boric acid in water and then adjusting the pH with 0.25 M sodium hydroxide.

Preparation of Microemulsions

In this study, Brij O10, Transcutol® P, and isopropyl myristate were used as a surfactant, co-surfactant, and oil phases, respectively; and a water solution containing a mixture of the 18 FAAs was used as the aqueous phase. An appropriate amount of Brij O10, Transcutol® P, and isopropyl myristate were weighed using an analytical balance (Type 870–13, KERN & SOHN GmbH, Germany), transferred to a glass vial, and mixed thoroughly using a magnetic stirrer (Model MR 3001, Heidolph Instruments GmbH & Co. KG Schwalbach, Germany). An aqueous solution containing a mixture of the 18 FAAs was prepared separately and this was slowly added to the surfactant/co-surfactant/oil mixture at room temperature with gentle stirring. The final mixture (that contains the selected FAAs) was gently shaken for complete mixing and stored in glass vials until analysis.

Method Validation

Preparation of Standard Solutions

Twenty millimolar (20 mM) stock solutions of each of the selected FAAs were prepared in 2-mL Eppendorf tubes using water as solvent. Then, 50 µL of each stock solution was transferred to a 1.5-mL Eppendorf tube and diluted to 1000 µL with the same solvent to obtain a stock solution of 1 mM. A series of six standard solutions having a concentration of 50, 100, 200, 400, 600, and 800 µM were then prepared by transferring the appropriate volume of the stock solution and diluting with water in separate 1.5 µL Eppendorf tubes. One hundred microliters (100 µL) of each of the standard solutions were transferred to 1.5 mL Eppendorf tubes. Two hundred microliters (200 µL) of 0.5 M sodium borate buffer pH 8.6 and 400 µL 6 mM Fmoc-Cl solution (in acetonitrile) were added to each solution. The resulting solutions were mixed very well and incubated for 10 min for complete derivatization. Three hundred microliters (300 µL) of 12.5 mM Adamantine HCl (ADAM) (in water: acetonitrile, 1:3 v/v) were added to each solution to terminate the reaction. The solutions were mixed again and incubated for another 2 min; centrifuged (Model Mega Star 3.0R, VWR International, LLC, Darmstadt, Germany) at $10{,}000 \times g$ for 5 min, and the supernatant was transferred to an HPLC autosampler vial. The concentrations of the final standard solutions were in the range of 5–80 µM.

Preparation of Sample and Placebo Solutions

A stock solution of microemulsions containing about 2.5 mg/mL of the respective FAA was extracted using methanol as solvent. The resulting solutions were then filtered through Whatman filter paper No. 42, and 40 µL of the filtrate was further diluted to 200 µL with bi-distilled water. The placebo solutions (formulations without FAAs) were prepared in the same manner. Then, 100 µL of each solution was derivatized as per the procedure mentioned in the standard preparation (starting from the addition of 200 µL of 0.5 M sodium borate buffer pH 8.6).

HPLC Chromatographic Conditions

The chromatographic apparatus consists of Shimadzu HPLC system (Shimadzu Corporation, Tokyo, Japan) with Solvent Delivery Module LC-40D, Auto-sampler Module SIL-40C, Diode Array Detector Module SPD-40 M, Column Oven Module CTO-40C, and System Controller Module CBM-40. Chromatographic separations were carried out on InfinityLab Poroshell 120 E.C 18 (3×50) mm, with a particle size of 2.7 µm (Agilent Technologies Germany GmbH & Co. KG, Waldbronn, Germany). Solvent A and solvent B consisted of water and acetonitrile, respectively, and each contained 0.2% glacial acetic acid and 0.1% trimethylamine as pH adjusters. The gradient system was adjusted as follows (time (min), %B): 0/15, 4/15, 7/23, 16/23, 18/38, 21/38, 27/60, 28/15, and 29/15. The auto-sampler temperature was maintained at 4 °C; 10 µL of each sample was injected. The column temperature was maintained at 25 °C and the detection of the derivatized samples was performed using a DAD. The total run time was set at 29 min.

Validation Parameters

Before performing the validation activities, method development was conducted to optimize the sample pretreatment, derivatization process, and chromatographic conditions. Robustness of the final experimental setup was also investigated using the "one-factor-a-time" method

("one-variable-at-a-time procedure") [29]. Analysis parameters such as pH, composition and flow rate of mobile phase, column temperature, column age, and solution stability were included in the robustness study. The effect of all these deliberate changes on the retention time, tailing factor, theoretical plate numbers (N), repeatability of peak areas, and resolution were studied.

After method development, the RP-HPLC/DAD method was validated in accordance with International Conference on Harmonization (ICH) guidelines on the validation of analytical procedures [30]. Validation parameters including a limit of detection (LOD), limit of quantification (LOQ), specificity/selectivity, linearity and range, accuracy, and precision were investigated. A system suitability test (SST) was also carried out throughout the validation work.

SST was conducted using standard solutions at the assay concentration of 20 µM after derivatization. The test was carried out by injecting standard solutions of all FAAs, each at a concentration of 20 µM, in six replicates. Chromatographic parameters such as retention time, peak area, tailing factor, number of theoretical plates (efficiency), height equivalent to the theoretical plate (HETP), tailing factor, and resolution were evaluated to assess the suitability of the HPLC system. The acceptance criteria for %RSD of retention time and peak area in replicate injections was less than 2 and that of tailing factor was less than 2. The limit for efficiency was not less than 2000 and that of HETP was not more than 2. A resolution value of 1.5 or greater between two peaks was taken aa s threshold value to ensure whether the sample components are well (baseline) separated to a degree at which the area or height of each peak may be accurately measured.

The specificity/selectivity of the analytical method was evaluated by analyzing the standard solution, FAA-loaded formulations, and the placebo samples (formulation without the FAAs) at the working concentration of 20 µM. The acceptance criterion for this test was % interference of less than 5 at the retention time of the respective analytics.

The linearity and range were evaluated using standard solutions of the FAAs at concentrations ranging from 5 to 100 µM. The concentration of each FAA was plotted against its corresponding peak area and linear regression equations were calculated. R^2 values of greater than 0.995 was taken as acceptance criteria for the linearity. The LOD and LOQ parameters were calculated by multiplying the S/Slope ratio by 3 and 10, respectively (where S is the standard deviation of the Y-intercepts of the five calibration curves and the slope is the mean slope of the five calibration curves), according to the ICH guidelines [30]. This estimate was further confirmed by the independent analysis of real samples prepared at the detection and quantification limits.

The accuracy/recovery of the method was determined by preparing three sample solutions at 50%, 100%, and 150% of the target concentration (20 µM) and calculating the recovery of each analyte as a percentage recovery The overall recovery of 90–110% was taken as a threshold value for this test.

The precision of the method was determined by measuring the repeatability (intra-day) and intermediate precision (inter-day) of the retention times and peak areas measured for each FAA. The intra-day variability was measured by the same analyst over one day, while inter-day precision tests were carried out by the same analyst on different days using different batches of reagents. The precision was determined by measuring the repeatability of the retention time and peak areas on replicate injections ($n=6$) at the sample solutions at the assay concentration (20 µM) and reported as a percentage of relative standard deviation (% RSD). The threshold value for precision was % RSD of less than 2% for both the retention time and peak area.

Application of the Method in a Routine Quality Control Tests

After performing the validation work, the applicability of the method was ensured by testing final topical preparations. In addition to the microemulsion stated in the above section, micro emulsion-based hydrogels were prepared and tested following the mentioned procedure. The microemulsion-based topical hydrogels were prepared using Carbopol® 934 and Poloxamer P407 as polymers. A 2% Carbopol® 934 dispersion was prepared in bi-distilled water, and this was mixed with the already prepared microemulsion (1:1 w/w). Then few drops of triethanolamine were added to neutralize the resulting solution and form the hydrogel. The Poloxamer P407 based hydrogel was prepared using the cold method. The prepared microemulsion was cooled to 4 °C. Then Poloxamer P407 was added under continuous mixing while maintaining the temperature at 4 °C. The final concentration of Poloxamer P407 in the hydrogel was 16% w/w. A few drops of 2-phenoxyethanol were added as a stabilizer in both preparations. The content of FAAs in the formulations was determined following the final experimental conditions of this method. Each experiment was done in triplicate.

Results and Discussion

Sample Pretreatment and Optimization of Derivatization Condition

Before performing the validation activities, a preliminary study (method development) was conducted to optimize the sample pretreatment, derivatization process, and chromatographic conditions. One common disadvantage of using Fmoc-Cl as a derivatizing reagent in the HPLC analysis of

FAAs is its reactivity towards water (after hydrolysis and decarboxylation, Fmoc-OH is formed and the peak due to this compound interferes with the peak area of some of the FAAs). Hence, an appropriate reagent should be selected to prepare the derivatizing reagent and remove the excess. Acetone is the most common reagent used for preparing the Fmoc-Cl solution. In the current study, we compared acetone and acetonitrile. Preparing the Fmoc-Cl in acetone resulted in a broad and big interfering peak as compared to that of acetonitrile. The common method of removing the excess Fmoc Cl reagent involves extraction with n-pentane [27]. The reaction of the unused Fmoc with excess ADAM [31] or heptylamine [32] is also reported in the literature. In the current study, both n-pentane and ADAM were checked and compared. The results obtained after extraction of the excess Fmoc-Cl with n-pentane were not consistent. Moreover, the concentrations obtained were lower than that obtained after reaction with ADAM, maybe due to considerable loss of the derivatized FAAs during the removal of excess n-pentane. Furthermore, a relatively longer extraction time is needed as it requires extracting the excess Fmoc-Cl solution at least three times with 500 µL of n-pentane. On the other hand, the reaction of the unused Fmoc with excess ADAM resulted in a spectacular decrease of the reagent peak, without any loss of the hydrophobic Fmoc derivatives. The extent of formation of Fmoc-OH was greatly decreased, and the excess Fmoc Cl was eliminated completely. The resulting Fmoc-OH peak was sufficiently resolved from the FAA derivatives and didn't interfere with the peaks of the analyte of interest. The response of the amino acid derivatives was independent of the ADAM concentration within the tested concentration range. Hence, acetonitrile and ADAM were used to prepare the Fmoc-Cl solution and to remove the excess Fmoc-Cl, respectively.

For Fmoc-Cl derivatization, the most commonly used buffer is borate buffer [18, 33–35], and therefore, this buffer was used in the present study. Different concentrations of borate buffer (20, 200, and 500 mM) were tested. Derivatization was effective at the highest concentration (500 mM) may be due to the promotion of reactivity of amine functional groups with the derivatization reagent at the highest concentration of borate buffer. Therefore, this concentration of buffer was selected and used. In the literature, it is reported that borate buffer is used in a wide concentration ranging, from 0.20 M [33], to 0.325 M [34], and 0.5 M [35].

The effect of the buffer pH on derivatization yields was investigated in the pH range of 7–10 using borate buffer solution (0.5 M). It was observed that the higher the pH, the higher the derivatization yield. The highest capacity of borate buffer was obtained at pH 10.0. However, performing derivatizations in reaction media at pH > 10.0 had several disadvantages including increased hydrolysis rate of the reagent, the need for high Fmoc concentration to ensure its excess, and the appearance of a huge Fmoc-OH peak. Hence, a lower pH of 8.6 was selected for the derivatization. In the literature, buffer-pH values in the range of 6 to 11.4 are reported, most of which are in the range of pH 8.0–9.0 [26, 34], and this is consistent with the present work. A reaction time of 10 min was sufficient for the complete reaction between the FAAs and Fmoc in borate buffer maintained at pH 8.6.

Optimization of Chromatographic Conditions

Of the two analytical columns (Lichrosphere C18, 150 mm × 4.6 mm, 5 µm particle size and Agilent InfinityLab Poroshell 120 E.C 18 (3 × 50) mm, 2.7 µm), better resolution, peak shape, and shorter end time were obtained with the latter one. Agilent InfinityLab Poroshell 120 columns are based on superficially porous particle technology, which features a solid silica core and a porous outer layer. Compared to traditional totally porous particles of the same (or similar) size, Poroshell particles deliver exceptional efficiency and reliability enabling fast and high-resolution separations. It provides superior peak shapes for faster, more accurate results due to high-purity silica and advanced bonding chemistries. This column is known for its lot-to-lot reproducibility giving confidence in chromatographic separations [36] and was used for the validation activity.

For mobile phase optimization, the effect of concentration of organic modifier, mobile phase pH, and flow rate were investigated. As the content of the organic modifier was decreased, the resolution was improved, and the end time was prolonged (a common feature for RP HPLC). The peak resolution and shape were improved when glacial acetic acid and trimethylamine were added. Among the different mobile phases tested, a gradient elution consisting of solvent A (water) and B (acetonitrile) each containing 0.2% glacial acetic acid and 0.1% trimethylamine showed a better resolution and peak shape. A decrease in pH increased the retention of all tested FAAs. The retention time of the Fmoc-OH was only slightly affected by the pH of the eluent. When the pH was increased, L-Arg, L-Asn, L-Gln, and L-Ser were not fully resolved. Mobile phase flow rates ranging from 0.5–1.0 mL/min were investigated. Optimum results (in terms of resolution and end time) were obtained at a flow rate of 0.70 mL/min ± 5%, which gave a total run time of about 30 min and resolution of > 2.0 for most of the investigated FAAs. With the longer column (Lichrosphere C18, 150 mm × 4.6 mm) the end time was about 50 min.

The effect of column temperature on resolution and peak shape was also investigated. As the temperature was increased, the resolution between successive peaks was poor. The best resolution was obtained at 25 °C followed by 30 °C and some of the peaks overlapped at 40 °C (for example the peaks due to L-Thr and L-Gly). Consistent results were

Table 1 Results of the system suitability test for the simultaneous analysis of 18 free amino acids by HPLC/DAD using Agilent InfinityLab Poroshell 120 E.C 18 (3 × 50 mm, 2.7 µm) column ($n = 6$)

FAA	Retention time (min)		Peak area (µV. min)		No. of theoretical plates		HETP		Tailing factor		Resolution	
	Average	% RSD	Average	% RSD	Average	% RSD	Average	% RSD	Average	% RSD	Average	% RSD
L-Arg	9.60	0.11	1084669.17	0.43	42067.50	0.47	1.19	0.47	1.02	1.32	–	–
L-Asn	10.54	0.12	624859.50	0.42	37194.83	0.42	1.34	0.42	1.04	0.542	4.00	0.82
L-Gln	10.95	0.13	1625946.50	0.86	29501.17	1.07	1.69	1.07	1.24	1.37	1.96	0.85
L-Ser	11.93	0.13	1614815.50	0.24	26605.50	1.28	1.88	1.29	1.16	0.29	3.21	0.60
L-Asp	12.93	0.14	585814.00	0.46	29619.67	0.95	1.69	0.94	1.232	0.94	2.84	0.77
L-Glu	14.27	0.16	754136.17	0.55	28415.00	0.96	1.76	0.96	1.10	0.80	3.64	0.54
L-Thr	15.57	0.18	1245178.67	1.12	29540.17	0.67	1.69	0.67	1.11	0.35	2.91	0.33
L-Gly	16.53	0.17	1638611.17	0.88	25670.33	1.32	1.95	1.34	1.00	0.23	2.89	1.17
L-Ala	18.81	0.04	1193973.81	4.20	194777.66	1.51	0.26	1.51	1.29	1.03	6.45	1.15
L-Pro	19.51	0.04	1138373.50	0.25	136283.50	1.64	0.37	1.65	1.15	1.28	3.66	1.27
L-Met	20.42	0.05	1253059.83	0.67	195740.16	0.89	0.25	0.89	1.22	0.74	4.60	1.09
L-Val	21.02	0.06	1168886.67	0.98	154665.17	1.26	0.32	1.25	1.17	1.32	2.98	1.47
L-Phe	22.75	0.07	932631.33	1.14	153655.50	1.15	0.32	1.16	1.20	1.48	17.19	1.23
L-Ile	23.27	0.05	1030549.67	0.31	196941.67	1.39	0.25	1.39	1.11	0.40	10.48	0.70
L-Leu	23.54	0.04	1133970.33	0.69	200119.00	1.56	0.25	1.55	1.07	0.44	2.01	0.22
L-His	26.38	0.02	1651545.17	1.23	356321.33	1.07	0.145	1.09	1.12	1.25	20.91	1.02
L-Orn	26.55	0.03	2649649.50	0.42	381551.33	1.62	0.13	1.62	1.21	0.16	1.64	0.72
L-Lys	27.12	0.03	2530910.17	1.11	334841.30	0.66	0.15	0.67	1.31	0.23	4.10	1.59

obtained at 25 °C ± 3 °C. The maximum detection for the derivatized compounds was at 263 nm and this was evidenced by the spectral analysis.

The effect of the presence of other FAAs such as L-cysteine HCl (L-Cys), L-tryptophan (L-Trp), and L-tyrosine (L-Tyr) was also investigated. Care should be taken as they could overlap with the other FAAs. Under the mentioned experimental conditions, L-Trp elutes just before L-Phe, L-Tyr elutes next to L-Ala, while L-Cys elutes at the end next to L-Lys. Solvent B should be reduced at the respective retention times to attain the required resolution.

Robustness

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage. Small changes in pH of mobile phase (solvent A), flow rate, and column temperature in the range 3.7 ± 0.2, 0.7 mL/min ± 5%, and 25 ± 3 °C, respectively, had no significant effect on the chromatographic performance. The repeatability of peak area and retention time, tailing factor, theoretical plate numbers, and resolution were not significantly affected under such small changes. Moreover, the percentage of solvent B can be varied within ± 2% in the gradient program. We used this method for more than a year and consistent results were obtained in both new and aged analytical columns. Solution stability was also investigated against freshly prepared standard solutions. The stock solutions were stable for 2 weeks at − 20 °C before derivatization and for 24 h after derivatization in HPLC auto-sampler at 4 °C. Hence, the method conditions can be considered robust.

System Suitability

System suitability testing is an integral part of many analytical procedures, and this test was considered as part of the validation work. It was studied by performing the experiment and looking for changes in retention times, peak area, efficiency, resolution, and asymmetry of the peaks. Different chromatographic parameters were recorded, and the results obtained are given in Table 1. The chromatograms of six replicates of the standard solution are shown in Fig. 1 and they were consistent in peak area, peak shape, and retention time.

The higher the plate number *N*, the greater the efficiency of the column. The lower the HETP, the better the resolution and the more efficient the separation. Efficiency is optimized when N is maximized and HETP is minimized. As can be seen from Table 1, the theoretical plate numbers for all the analytes were above 18,000 and the HETP was very low (not more than 2). The tailing factor was below 2 and this is within the pharmacopeial requirements (not more

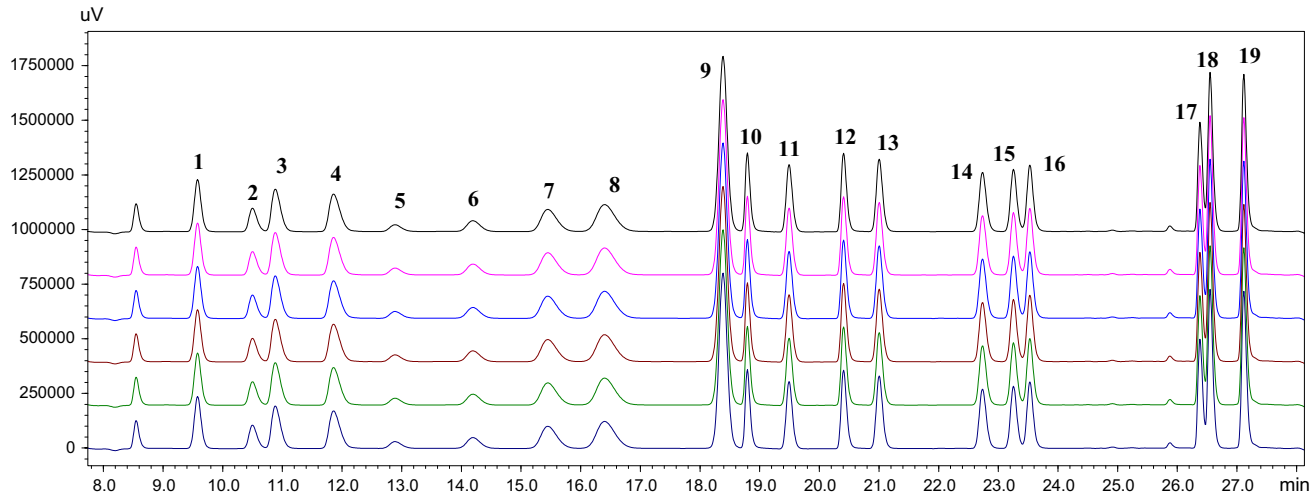

Fig. 1 Chromatograms of six replicates of the 20 µM standard solution (1: L-Arg, 2: L-Asn, 3: L-Gln, 4: L-Ser, 5: L-Asp, 6: L-Glu, 7: L-Thr, 8: L-Gly, 9: Fmoc-OH, 10: L-Ala, 11: L-Pro, 12: L-Met, 13: L-Val, 14: L-Phe, 15: L-Ile, 16: L-Leu, 17: L-His, 18: L-Orn, 19: L-Lys)

than 2). The resolutions among the different analytes were above 2 for most of the FAAs indicating sufficient resolution between successive peaks. Even though the resolution between L-Asn and L-Gln, and L-His L-Orn was below 2, the peaks were baseline separated (with a resolution of greater than 1.5) and there was no problem in the quantification. The peaks due to L-Ile and L-Leu were sufficiently resolved in the present work. The % RSD of each set of parameters (retention time, peak area, tailing factor, efficiency, and HETP) was less than 2% indicating the reproducibility of the RP-HPLC/DAD system for quantitative analysis of the 18 FAAs. Hence, the method fulfills the acceptance criteria for the mentioned SST parameters.

Method Validation

Specificity

Specificity is the ability to assess unequivocally the analyte in the presence of components that may be expected to be present. It was determined by comparing the chromatograms of the individual FAA working standards, FAA-loaded formulations, and placebo formulations. As can be seen in Fig. 2, there were no interfering peaks at the retention time of the respective analytes from the placebo solution. Hence, the validated method is specific for the selected FAAs. Moreover, the minimal differences between retention times and peak area (% difference of less than 0.5%) in the case of the standard and sample solutions allow confident and highly specific peak identification.

Linearity, Limit of Detection, and Limit of Quantification

The calibration curve was constructed within the linear concentration range of 5–80 µM. Each concentration was made in five independent replicates. Linearity was assessed using the correlation coefficient (R^2) of the regression line. The results in Table 2 show that R^2 was in the range of 0.9954–0.9991 indicating excellent linearity, which implies the reliable quantitation of FAAs. As estimated from the calibration curve, the LOD and LOQ values were in the range of 0.13–1.36 µM and 0.42–4.54 µM, respectively (Table 2). This estimate was further confirmed by the independent analysis of real samples prepared at the detection and quantification limit and the results were not significantly different from those reported in Table 2. In the literature, it is stated that Fmoc based derivatization of FAAs had better sensitivity than the other derivatizing reagents. López-Cervantes et al. [18] reported detection limits in the range of 23–72 ng/mL. LOD values in the range of 3 to 6 µM were obtained by the work of Fabiani et al. [31]. Garside et al. [37] also reported a detection limit of 0.5 µM with a fluorescence detection system and this was very close to the sensitivity of the current method. Hence, the developed method is sensitive enough to detect low concentrations of these FAAs.

Precision

The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions.

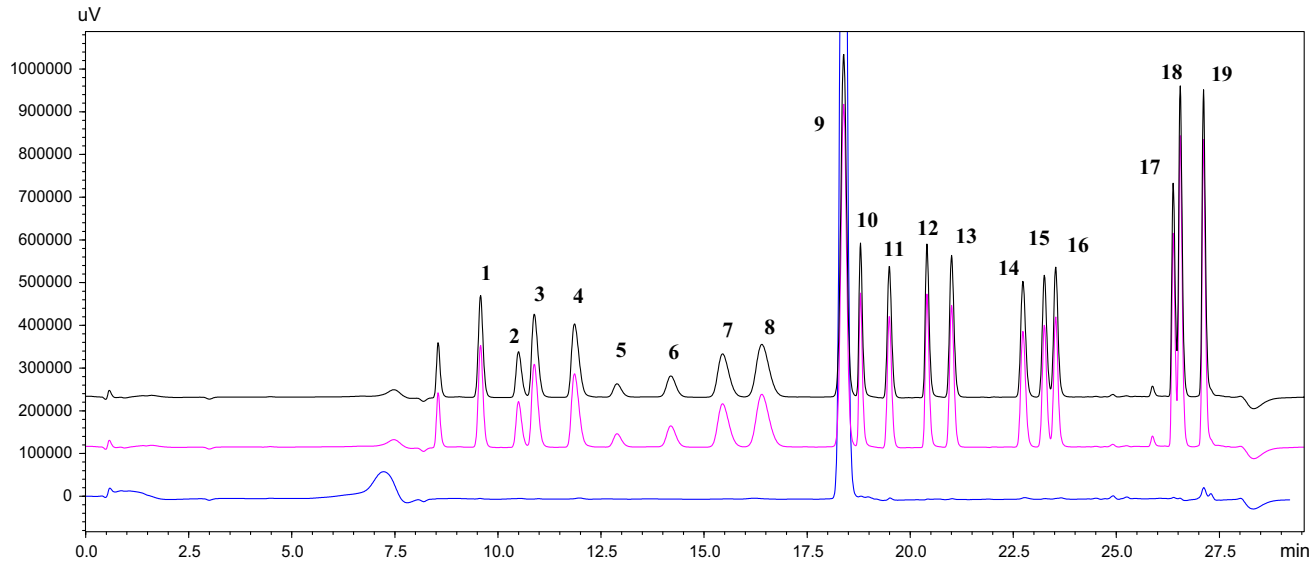

Fig. 2 Chromatograms of the placebo solution (blue), standard solution (violet), and sample solution (black) (1: L-Arg, 2: L-Asn, 3: L-Gln, 4: L-Ser, 5: L-Asp, 6: L-Glu, 7: L-Thr, 8: L-Gly, 9: Fmoc-OH, 10: L-Ala, 11: L-Pro, 12: L-Met, 13: L-Val, 14: L-Phe, 15: L-Ile, 16: L-Leu, 17: L-His, 18: L-Orn, 19: L-Lys)

Table 2 Results of linearity, LOD, LOQ ($n=5$) for the simultaneous analysis of 18 free amino acids by HPLC/DAD using Agilent InfinityLab Poroshell 120 E.C 18 (3 × 50 mm, 2.7 µm) column

Analyte	Linearity range	Linearity equation	R^2	S.D of Y-int	Mean Slope	LOD (µM)	LOQ (µM)
L-Arg	(5–80) µM	$Y=37077.90X+12437.73$	0.9975	11532.85	37077.90	0.93	3.11
L-Asn	(5–80) µM	$Y=20179.30X+6848.57$	0.9987	6156.32	20179.30	0.92	3.05
L-Gln	(5–80) µM	$Y=41283.27X+9426.13$	0.9991	4281.03	41283.27	0.31	1.04
L-Ser	(5–80) µM	$Y=50221.40X+26200.47$	0.9973	9292.66	50221.40	0.56	1.85
L-Asp	(5–80) µM	$Y=73157.17X+74215.60$	0.9986	13538.17	73157.17	0.56	1.85
L-Glu	(5–80) µM	$Y=59628.57X+51925.20$	0.9968	11124.60	59628.57	0.56	1.87
L-Thr	(5–80) µM	$Y=41665.73X+28593.10$	0.9978	17082.63	41665.73	1.23	4.11
L-Gly	(5–80) µM	$Y=51145:00X+21811.17$	0.9975	13134.49	51145.00	0.77	2.57
L-Ala	(5–80) µM	$Y=41042.80X+22072.10$	0.9973	6306.74	41042.80	0.46	1.54
L-Pro	(5–80) µM	$Y=40138.33X+567758.30$	0.9974	6306.74	40138.33	0.47	1.57
L-Met	(5–80) µM	$Y=47051.37X+11466.230$	0.9976	2296.22	47051.37	0.15	0.49
L-Val	(5–80) µM	$Y=43894.10X+13274.730$	0.9968	14242.17	43894.10	0.97	3.24
L-Phe	(5–80) µM	$Y=24227.33X+3694.03$	0.9986	1956.81	24227.33	0.24	0.81
L-Ile	(5–80) µM	$Y=42090.03X+15931.10$	0.9984	8597.02	42090.03	0.61	2.04
L-Leu	(5–80) µM	$Y=49001.73X+24230.40$	0.9972	15178.85	49001.73	0.93	3.10
L-His	(5–80) µM	$Y=21074.23X+26618.03$	0.9954	9560.45	21074.23	1.36	4.54
L-Orn	(5–80) µM	$Y=59201.90X+47779.87$	0.9981	3400.02	59201.90	0.17	0.57
L-Lys	(5–80) µM	$Y=76389.53X+69674.90$	0.9970	3213.82	76389.53	0.13	0.42

Precision/Repeatability

Repeatability, also called intra-day precision, expresses the precision under the same operating conditions over a short interval of time. It was determined on six determinations at 100% concentration of the test solution (20 µM). The results in Table 3 show that the validated method fulfills the requirements for precision (the % RSD was < 2%).

Intermediate Precision

The results of the intermediate precision are summarized in Table 4. As shown in the Table, the validated method fulfills the requirements for intermediate precision (the overall % RSD was < 2%).

Table 3 Results of precision in the simultaneous analysis of 18 free amino acids by HPLC/DAD using Agilent InfinityLab Poroshell 120 E.C 18 (3×50 mm, 2.7 µm) column

Sample	Concentration (µM)								
	1	2	3	4	5	6	Average	SD	%RSD
L-Arg	20.09	20.08	20.07	20.09	19.84	19.83	19.99	0.13	0.64
L-Asn	20.05	19.89	19.92	19.95	20.12	20.06	20.00	0.09	0.47
L-Gln	20.05	19.87	19.94	19.89	19.85	20.39	20.00	0.20	1.02
L-Ser	19.85	20.06	19.96	19.83	20.00	20.29	20.00	0.17	0.85
L-Asp	19.85	20.13	20.11	20.11	20.10	19.70	20.00	0.18	0.90
L-Glu	20.11	20.11	19.95	19.79	19.95	20.09	20.00	0.13	0.63
L-Thr	20.03	20.26	20.17	20.15	19.86	19.52	20.00	0.27	1.35
L-Gly	20.06	20.05	20.02	20.25	19.64	19.97	20.00	0.20	0.98
L-Ala	19.99	19.85	19.82	20.16	20.05	20.10	20.00	0.13	0.67
L-Pro	19.96	20.15	19.96	19.79	19.89	20.25	20.00	0.17	0.86
L-Met	20.08	19.61	20.04	20.19	19.92	20.15	20.00	0.21	1.06
L-Val	20.07	19.75	19.92	20.26	20.34	19.66	20.00	0.27	1.35
L-Phe	19.95	19.99	20.17	19.98	19.90	19.98	20.00	0.09	0.46
L-Ile	19.62	19.90	20.39	19.68	20.37	20.03	20.00	0.33	1.66
L-Leu	19.99	20.02	19.79	19.89	20.22	20.07	20.00	0.14	0.72
L-His	20.00	20.19	19.97	19.87	19.93	20.03	20.00	0.11	0.55
L-Orn	20.27	20.08	20.02	19.90	19.81	19.92	20.00	0.17	0.81
L-Lys	20.14	19.99	19.84	19.96	20.24	19.83	20.00	0.16	0.82

Table 4 Results of intermediate precision in the simultaneous analysis of 18 free amino acids by HPLC/DAD using Agilent InfinityLab Poroshell 120 E.C 18 (3×50 mm, 2.7 µm) column

Sample	Concentration (µM)														
	Day 1						Day 2						Overall		
	1	2	3	4	5	6	1	2	3	4	5	6	Mean	SD	%RSD
L-Arg	20.09	20.08	20.07	20.09	19.84	19.83	20.01	20.01	20.12	19.88	19.86	19.94	20.01	0.15	0.76
L-Asn	20.05	19.89	19.92	19.95	20.12	20.06	20.01	19.95	19.87	19.94	20.09	19.96	19.98	0.08	0.40
L-Gln	20.05	19.87	19.94	19.89	19.85	20.39	19.68	19.89	20.12	19.94	19.85	20.07	19.94	0.18	0.89
L-Ser	19.85	20.06	19.96	19.83	20.00	20.29	19.79	20.01	20.11	19.97	20.14	19.88	20.01	0.16	0.82
L-Asp	19.85	20.13	20.11	20.11	20.10	19.70	19.89	19.97	19.85	20.09	19.84	20.16	19.97	0.17	0.83
L-Glu	20.11	20.11	19.95	19.79	19.95	20.09	20.10	20.14	19.96	19.86	19.96	19.97	20.03	0.18	0.91
L-Thr	20.03	20.26	20.17	20.15	19.86	19.52	20.13	19.97	20.08	20.14	19.86	19.77	19.99	0.21	1.05
L-Gly	20.06	20.05	20.02	20.25	19.64	19.97	19.79	19.95	20.11	20.31	19.76	19.81	19.98	0.20	1.00
L-Ala	19.99	19.85	19.82	20.16	20.05	20.10	19.76	19.87	19.91	19.82	20.04	20.14	19.94	0.16	0.81
L-Pro	19.96	20.15	19.96	19.79	19.89	20.25	19.95	20.08	19.93	19.86	19.79	19.76	19.98	0.18	0.92
L-Met	20.08	19.61	20.04	20.19	19.92	20.15	19.87	19.95	20.02	20.17	19.97	19.49	19.95	0.21	1.08
L-Val	20.07	19.75	19.92	20.26	20.34	19.66	20.08	19.79	19.93	20.16	19.98	19.97	19.98	0.16	0.81
L-Phe	19.95	19.99	20.17	19.98	19.90	19.98	20.42	20.06	19.97	19.86	20.04	20.11	20.06	0.19	0.95
L-Ile	19.62	19.90	20.39	19.68	20.37	20.03	19.30	19.94	20.13	19.74	20.21	20.08	19.90	0.29	1.46
L-Leu	19.99	20.02	19.79	19.89	20.22	20.07	19.85	19.94	20.11	19.83	19.96	19.97	19.95	0.13	0.65
L-His	20.00	20.19	19.97	19.87	19.93	20.03	19.67	20.21	20.09	19.97	20.16	20.03	19.98	0.18	0.91
L-Orn	20.27	20.08	20.02	19.90	19.81	19.92	20.05	20.04	19.99	19.81	20.17	19.94	19.93	0.19	0.95
L-Lys	20.14	19.99	19.84	19.96	20.24	19.83	19.87	20.14	19.87	19.92	19.87	19.97	19.93	0.12	0.62

Table 5 Results of recovery in the analysis of the spiked free amino acid samples at three concentration levels by RP-HPLC/DAD using Agilent InfinityLab Poroshell 120 E.C 18 (3 × 50 mm, 2.7 µm) column ($n = 3$)

Analyte	Spiked free amino acid concentrations ($n = 3$)									Overall recovery (%)
	50% (10 µM)			100% (20 µM)			200% (40 µM)			
	Measured value	Recovery (%)	%RSD	Measured value	Recovery (%)	%RSD	Measured value	Recovery (%)	%RSD	
L-Arg	9.91	99.10	1.53	19.94	99.70	0.38	38.92	97.30	0.13	98.70
L-Asn	9.80	98.03	1.42	19.93	99.65	1.07	39.52	98.79	0.61	98.83
L-Gln	9.79	97.87	1.49	19.81	99.05	0.77	38.48	96.21	1.16	97.71
L-Ser	9.84	98.37	1.74	19.89	99.48	1.46	38.66	96.65	0.09	98.16
L-Asp	9.69	96.90	0.47	19.80	99.02	0.78	38.73	96.82	0.32	97.56
L-Glu	9.79	97.93	0.59	19.77	98.87	0.46	38.79	96.99	0.75	97.93
L-Thr	9.82	98.17	0.69	19.60	98.00	0.32	38.74	96.85	0.16	97.67
L-Gly	9.84	98.40	0.53	19.68	98.40	1.33	39.29	98.23	0.82	98.34
L-Ala	9.96	99.57	1.56	19.61	98.03	0.65	39.66	99.14	0.28	98.91
L-Pro	10.06	100.63	0.90	19.25	96.27	0.49	38.61	96.53	0.16	97.81
L-Met	9.92	99.23	1.57	19.57	97.87	0.53	38.82	97.04	0.47	98.05
L-Val	9.75	97.50	1.61	19.93	99.63	0.54	39.33	98.32	0.49	98.48
L-Phe	9.94	99.40	1.21	19.78	98.90	0.72	39.26	98.15	1.19	98.82
L-Ile	9.98	99.80	0.97	19.81	99.05	0.91	38.92	97.30	0.51	98.72
L-Leu	9.98	99.77	1.02	19.68	98.38	0.56	39.19	97.98	0.91	98.71
L-His	9.88	98.83	0.41	19.67	98.35	0.56	38.52	96.31	1.26	97.83
L-Orn	10.08	100.80	0.10	19.81	99.03	0.31	39.69	99.23	1.00	99.69
L-Lys	9.93	99.30	0.56	19.63	98.15	0.98	39.24	98.09	0.93	98.51

Accuracy

The accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found. The results of accuracy are shown in Table 5. As can be seen from the results, the values were within the acceptance criteria (90–110%) stated in the ICH guideline [30].

Application of the Method in Routine Quality Control Test

The applicability of the method in routine quality control testing was confirmed by conducting assay determinations on topical preparations. In addition to the microemulsion, the method was applied on microemulsion-based hydrogels (the additional ingredients included in the hydrogels did not interfere with the analyte peaks and the selectivity of the method was same as that of the microemulsion (Fig. 2). The content of the selected FAAs was determined by analyzing the assay content of the final finished products (microemulsions and microemulsion-based hydrogels). As can be seen in Table 6, the assay values were within the range of 95–105% which is within the pharmacopeoal assay requirements (90–110%). This confirms that the developed method

Table 6 Assay values of different topical preparations as determined by HPLC/DAD using Agilent InfinityLab Poroshell 120 E.C 18 (3 × 50 mm, 2.7 µm) column

Sample	Assay content (%) ($n = 3$)		
	Microemulsion	Carbopol® 934 based hydrogel	Poloxamer P407 based hydrogel
L-Arg	99.67 ± 0.68	98.73 ± 1.29	99.82 ± 1.77
L-Asn	100.08 ± 0.33	98.42 ± 0.38	98.80 ± 1.35
L-Gln	98.63 ± 1.17	99.11 ± 0.86	97.67 ± 1.50
L-Ser	99.18 ± 1.13	97.90 ± 1.12	98.82 ± 2.10
L-Asp	99.40 ± 0.98	97.65 ± 1.41	99.68 ± 1.19
L-Glu	97.90 ± 0.96	98.72 ± 0.67	97.10 ± 0.69
L-Thr	99.48 ± 1.65	97.66 ± 0.73	99.86 ± 1.98
L-Gly	101.56 ± 0.88	99.65 ± 2.33	98.22 ± 3.18
L-Ala	99.85 ± 0.71	99.23 ± 0.56	99.62 ± 0.76
L-Pro	97.92 ± 1.01	98.51 ± 2.08	98.76 ± 2.30
L-Met	100.67 ± 0.23	97.98 ± 1.15	99.88 ± 1.59
L-Val	99.81 ± 1.33	98.49 ± 1.10	99.28 ± 1.33
L-Phe	99.65 ± 0.31	99.05 ± 0.79	99.72 ± 0.36
L-Ile	98.98 ± 1.05	98.72 ± 1.31	99.45 ± 0.73
L-Leu	97.82 ± 0.61	98.71 ± 0.95	99.87 ± 0.63
L-His	99.00 ± 0.15	97.83 ± 1.37	97.32 ± 0.56
L-Orn	102.00 ± 1.20	99.69 ± 0.97	99.68 ± 0.67
L-Lys	99.82 ± 0.79	98.51 ± 0.69	99.56 ± 0.40

can be applied to any liquid and/or semisolid topical dosage forms without any further modification or after simple method verification.

In summary, the results indicate that this new method fulfills all the validation requirements of an analytical method as stated in the ICH Q2 (R1) guideline, and can be applied to liquid and semi-solid preparations.

Conclusions

A new, rapid, reliable, and accurate RP-HPLC/DAD method was developed and validated according to the ICH Q2 (R1) guideline for the simultaneous analysis of 18 FAAs in topical formulations for the first time. This method offers excellent sensitivity, selectivity, linearity, precision, as well as recovery. The most common drawback of the RP-HPLC method for the analysis of FAAs, namely, difficulty of separation/resolution from the solvent peak/Fmoc-OH, and among the analyte peaks are fully resolved. The limitations with Fmoc-Cl based derivatization and appropriate chromatographic conditions when analyzing these active components were also overcome during the method development phase. Unlike other reported methods, this method involves simple sample preparation and derivatization technique, a very short analysis time, and is economical with respect to reagent consumption. The FAAs are baseline separated and all components elute as narrow, well-defined, and nicely shaped peaks. Consistent results were obtained in different formulations indicating the appropriateness of the analytical method. Hence, this method can be adapted by official pharmacopeias and can be used in pharmaceutical and/or cosmeceutical quality control laboratories. We believe that our work is of scientific interest in these sectors and this method could have a significant contribution to ensuring the quality, safety, and efficacy of FAA-loaded topical formulations.

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Availability of Data and Materials All required data is included in the manuscript.

Code Availability Not applicable.

Declarations

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

Research Involving Human Participants and/or Animals This research did not involve human participants or animals.

Informed Consent Informed consent obtaining for this type of study is not required.

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