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Research article

Development of HPLC methods for determination of citrulline malate impurities

Tran Thu Hang[a], Hoang Thi Loan[a], Nguyen Tuyet Mai[a], Nguyen Thi Thuan[a*]
[a] *Hanoi University of Pharmacy, 13-15 Le Thanh Tong, Cua Nam, Hanoi, Vietnam*
** Corresponding author: thuannt@hup.edu.vn*

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ABSTRACT

Citrulline malate (CM) is a widely sought-after ingredient in the dietary supplement industry due to its ability to enhance exercise performance and promote muscle recovery. With the rising demand for CM, stringent quality control measures are essential, particularly in monitoring residual impurities in raw materials. An HPLC method was developed for the determination of fumaric acid (FA) and maleic acid (MA) impurities in CM raw material using a Shim-pack GIST C18 column (250 × 4.6 mm, 5 µm), detection at 210 nm, injection volume of 10 µL, and flow rate of 0.8 mL/min with a mobile phase consisting of acetonitrile and 3.3 mM potassium dihydrogen phosphate buffer (pH 2.0) (gradient). Another HPLC method was developed for the determination of *N*-acetylornithine (AO) impurity using the same C18 column, but with a detection wavelength of 200 nm, injection volume of 20 µL, flow rate of 1.0 mL/min, and a mobile phase consisting of 10 mM octane sulfonic acid sodium salt (pH 2.5) and acetonitrile under gradient condition. The methods were validated following AOAC International, confirming their accuracy and reliability (recovery not over than 100.0 ± 5.0% for all; RSD ≤ 1.1, 2.6, 3.0 % for FA, MA, AO respectively) in the range of concentration from 0.1 to 70 µg/mL (FA); 0.1 to 3.5 µg/mL (MA); 0.7 to 5.0 µg/mL (AO) with the coefficients very close of 1 (0.9999 for FA and MA, 0.9994 for AO). LOD, LOQ were established at 0.03, 0.1 for both FA, MA, and 0.5, 0.7 for AO.

INTRODUCTION

L-citrulline is an amino acid known for its role in supporting nitric oxide production, which promotes vasodilation, increases blood flow, regulates blood pressure, and enhances oxygen and nutrient delivery during exercise [1]. Additionally, *L*-citrulline participates in the urea cycle, facilitating the removal of ammonia, a metabolic byproduct associated with fatigue [2]. Beyond its standalone form,

*Corresponding author: thuannt@hup.edu.vn;
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L-citrulline is often combined with malic acid (malate), yielding citrulline malate (CM) in 1:1 or 2:1 ratio, with malate proposed to support energy metabolism through its role in the Krebs [2].

CM is synthesized through the combination of *L*-citrulline and malic acid, which may introduce impurities originating from these raw materials. According to the United States Pharmacopeia 2023 (USP 46), *N*-acetylornithine (AO) is a recognized impurity in *L*-citrulline, whereas fumaric acid (FA) and maleic acid (MA) are specified impurities that require monitoring in malic acid [3, 4]. The impurity limits for FA and MA are specified in the malic acid monograph as NMT 1.0% and NMT 0.05%, while the citrulline monograph sets a limit of NMT 0.5% for AO. Considering these established limits and the proportion of citrulline and malic acid in CM material, the acceptable thresholds for FA, MA, and AO in CM (1:1) material were determined to be 0.5%, 0.025%, and 0.25%, respectively.

Although there are both citrulline and malic acid monographs in most of pharmacopeia, including USP, BP, EP, but there isn't any pharmacopoeia monograph, or scientific study has addressed CM and its impurities. To facilitate the quality control of CM raw materials, this study developed a method for the simultaneous quantification of FA and MA in CM raw material based on reviewed studies on acid impurity determination, where chromatographic columns L17 (strong cation-exchange column) and L1 (C18 reversed-phase column) were commonly employed [4-7]. Due to the unavailability of the L17 column and the need to maintain consistency with the AO impurity analysis, this study utilized the L1 column with a suitable phase mobile. The analytical conditions for AO impurity determination were adapted from the USP 2023 monograph for citrulline [3].

MATERIALS AND METHODS

Materials

The PA substances used as reference standards include maleic acid (Bidepharmatech, Lot # DLX760, 99.0%), fumaric acid (Shanghai Zhanyun Chemical Co., Ltd, Lot # 231210, 99.5%), and *N*-acetylornithine (Adamas-beta, Lot # P2559006, 95%). HPLC-grade acetonitrile (Fisher) and other PA grade substances such as phosphoric acid, potassium dihydrogen phosphate (Xilong Scientific Co.), sodium octane sulfonate (Prolabo) was purchased and used directly. CM (1:1) material was obtained from Jing Jing Pharmaceutical Co., Ltd (Lot # C562305009).

The HPLC analysis was carried out on a Shimadzu Corporation (Japan) equipped with a low-pressure gradient pump (LC-30AD), a DAD detector (SPD-M20A), an autosampler (SIL-20A), and a column oven (CTO-10AS). All data acquired were proceeded by LabSolutions software (Shimadzu Japan).

Methods

Sample preparation

Eight mix standard solutions contained 0.1 to 70 µg/mL of FA and 0.1 to 3.5 µg/mL of MA were prepared in an acetonitrile (ACN)–phosphate buffer mixture (2:98) (solvent A). One other standard solution containing 50 µg/mL FA and 2.5 µg/mL MA was prepared by the same method to form *the solution for system suitability.*

Sevent AO standard solutions were prepared in water to obtain a range of final concentration from 0.7 to 5.0 µg/mL. The solution 4 – contained of 2.5 µg/mL was also used as *the solution for system suitability.*

A sample solution for determination of FA and MA at a concentration of approximatly10 mg/mL of CM in solvent A was prepared by dissolving of 251.3 mg CM in 25.0 mL solvent A, using 45 min of sonication.

The sample solution for determination of AO impurity at 1 mg/mL was prepared by dissolving 25.2 mg of CM raw material in 25.0 mL of water, following the same ultrasonic procedure as the previous analysis.

The spiked test solutions were prepared by adding a suitable amount of standard(s) into a 25.0 mL volumetric flask containing approximately 250 mg CM (accurately weighted), then continuing with the preparation of the sample solution.

All solutions were filtered through a 0.45 µm membrane prior to chromatographic analysis.

HPLC condition

Chromatographic separation was performed using a Shim-pack GIST C18 (250 × 4 mm, 5 µm) column. For the simultaneous determination of FA and MA impurities, the analysis was conducted at a flow rate of 0.8 mL/min, DAD detector of 210 nm, and an injection volume of 10 µL. A gradient elution system (Table 1) was employed using 3.3 mM potassium dihydrogen phosphate buffer (pH adjusted to 2.0 with phosphoric acid) and acetonitrile (ACN).

The determination of AO impurity was carried out according to the citrulline monograph of USP 2023 [3] with a flow rate of 1.0 mL/min and an injection volume of 20 µL. The mobile phase consisted of a gradient system (Table 1) using ACN and the buffer of sodium octane sulfonate in water (10 mM), adjusted to pH 2.5 with diluted phosphoric acid. The detection wavelength was set at 200 nm.

Table 1. Gradient conditions for the determination of impurities in CM material

FA and MA impurities			**AO impurity**		
Time (min)	*ACN (%)*	*Phosphate buffer (%)*	*Time (min)*	*ACN (%)*	*Sodium octane sulfonate buffer (%)*
0	1	99	0	15	85
10	1	99	4	15	85
15	25	75	15	30	70
22	25	75	20	15	85
			25	15	85

Method validation

According to AOAC International, the methods were validated for system suitability, specificity, limit of detection (LOD), limit of quantification (LOQ), linearity, calibration curve, accuracy, and precision (repeatability and intermediate precision) [8]. These validation parameters are fundamentally consistent with those required by pharmacopeial standards like USP <1225>, ensuring a robust assessment of method performance [9].

System suitability was assessed by injecting six successive injections o*f the solution for system suitability.* In general, system suitability was evaluated based on retention time (t_R), peak area (S_{pic}), theoretical plates (N), resolution (Rs), and tailing factor (T_f). The RSD of t_R and Spic of 6 injections should be not over than 2.0 %, Rs should be not less than 1.5, and T_f should be between 0.8 and 1.8.

Specificity of the methods was evaluated base on the chromatographs of solvents, standard solutions, and spiked sample solutions.

To estimate *Limit of detection (LOD) and Limit of quantification (LOQ),* standard solutions were diluted in suitable solvent and injected into the system. Chromatograms were recorded, then instrumental and method LOD/LOQ were determined based on signal-to-noise ratios, with S/N ≥ 3 commonly used for LOD estimation and S/N ≥ 10 for LOQ.

To evaluate the *linearity and calibration curve,* all standard solutions of each method were analyzed separately. Linear regression equations were established, with correlation coefficients (R) determined for each analytes.

The method *accuracy* was realized based on three spiked test solutions, corresponding to LQC, MQC, and HQC levels. Spiked test solutions of FA and MA were prepared at 30%, 100%, and 110% of their specified limits, while AO was assessed separately at 80%, 100%, and 120%. Each concentration level was analyzed in triplicate to evaluate recovery.

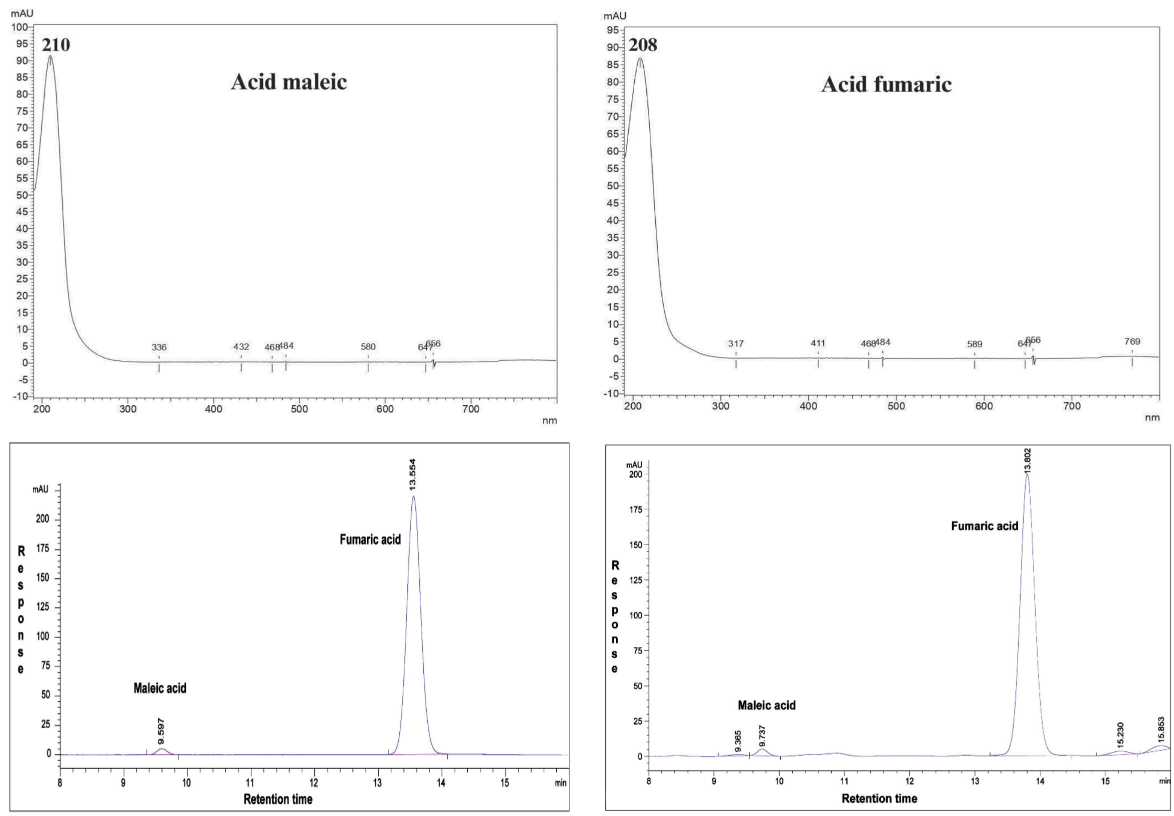
The method *precision (repeatability and intermediate precision)* were estimated with the analyses of six independent spiked test solutions contained 100% specified limits of each substance under their specific chromatographic conditions. Repeatability was evaluated based on the relative standard deviation (RSD) between analyses, and intermediate precision was assessed across different days and analysts under the same laboratory conditions. In general, RSD should not exceed 2%.

RESULTS AND DISCUSSIONS

RESULTS

Method development

To determinate the suitable condition for simultaneous analyzing of FA and MA, the



a) Mixed standard solution

b) Sample solution

Figure 1. Chromatograms of the mixed standard solution (a) and sample solution CM (b)

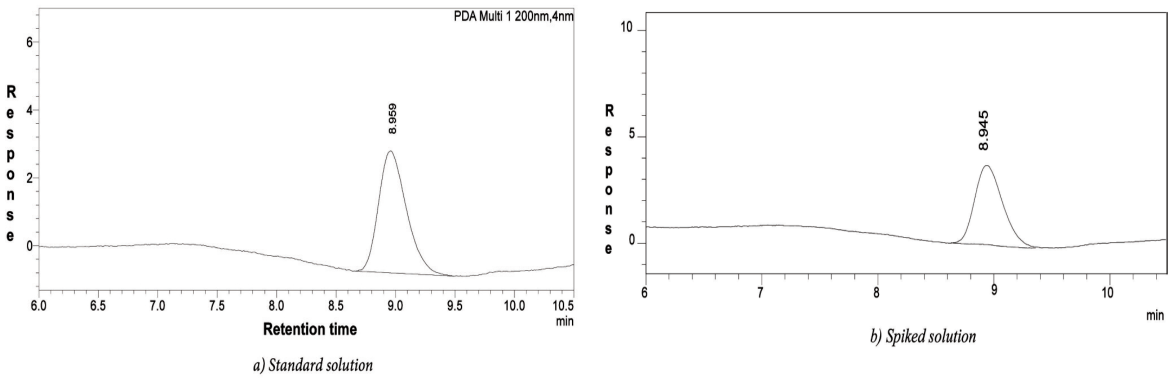


Figure 2. Chromatograms of the standard solution (a) and spiked sample (b) for the determination of AO impurity

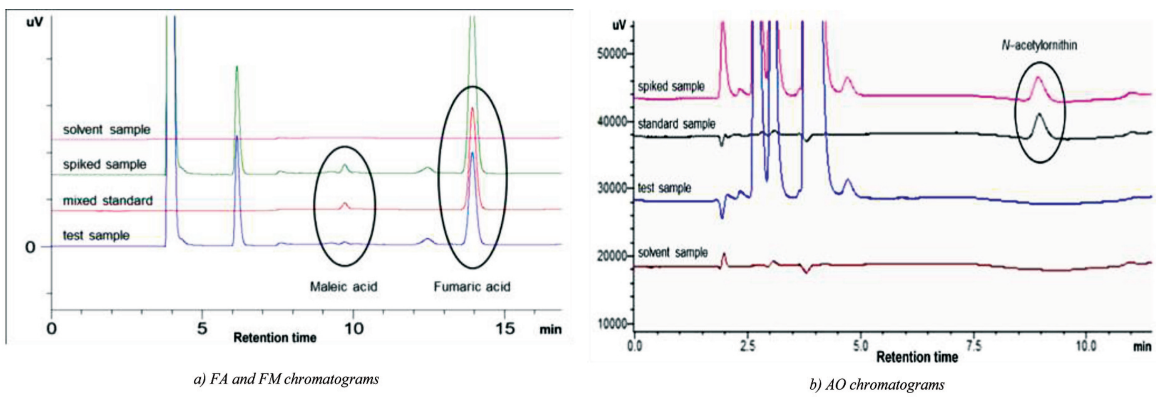


Figure 3. Chromatograms of solvent, standards, and spiked sample solutions

UV absorption spectra of individual FA (50 µg/mL) and MA (2.5 µg/mL) solutions were recorded (Figure 1) and showed maximum at 208 nm and 210 nm, respectively.

Considering MA's lower permissible limit and the risk of interference at lower wavelengths, 210 nm was selected as the detection wavelength for impurity analysis.

Several solvent programs had been tested. The best program (showed in table 1) achieved a good separation for both two impurities from other impurities (R_s of FA was 3.2, of MA was 1.6) with symmetric peaks ($T_f \approx 1.1$ for both peaks) (Figure 2).

To determine AO in CM raw material, HPLC condition of citrulline monograph of USP 2023 was used. The solvent program detailed in Table 1 showed effectively separation AO from solvent impurities and matrix impurities in the spiked sample (Figure 3). Therefore, this mobile phase programm was used for the determination of AO impurity in the CM sample.

Method validations

All validation results were summarized in figure 4, 5 and table 2.

The FA, MA method specificity was confirmed by the chromatograms (Figure 4a) of solvent, standard, spiked test samples. No interference was observed at their retention times in the blank solvent. In the spiked sample chromatogram, peaks appear at retention times with UV spectra matching those of FA and MA in the mixed standard solution. The spectral match factors for both FA and MA were > 0.99. Similarly, for AO determination, the chromatograms (Figure 4b) showed no interference at the AO retention time in the blank solvent. In the spiked sample, the AO peak matched the retention time and UV spectrum of the standard, with a spectral match factor of 0.9980, confirming method specificity.

A good system suitability was showed by results in the Table 2 with RSD values for retention time (t_R) and peak area (S_{pic}) below 2.0%. The average theoretical plate number (N) of each impurity exceeded 6000, the resolution (R_s) between peaks was greater than 1.5, and tailing factors (1.1 for FA, MA, 1.2 for AO) closed to 1 indicating symmetric peaks.

LOD of FA and MA were determined at 0.03 µg/mL for both with the S/N ratios of 2.6 and 2.9, respectively. Similarly, LOQ of FA and MA was estimated at 0.1 µg/mL with the S/N ratios of 9.6 and 11.6, respectively. For AO, at 0.5 µg/mL, the S/N ratio was 3.51, confirming the LOD, and the LOQ established at 0.7 µg/mL with the S/N ratio of 10.26. These results demonstrate the sensitivity of these two methods.

The linearity and calibration curve were obtained by analyzing standard solutions under the corresponding conditions. Within the investigated concentration range (0.1-70.0, 0.1-3.5, 0.7-5.0 for FA, MA and AO respectively) a linear correlation was observed between concentration and peak areas for each substance (Figure 5). The calibration curves exhibited correlation coefficients within 0.995 ≤ R ≤ 1, with bias at all points not exceeding ±15%. These results meet the requirements of AOAC International.

Table 2. Summary of methods validation results

Criteria	Parameter	FA	MA	AO
System suitability	RSD (%) of t_R (n = 6)	0.5	1.2	0.1
	RSD (%) of S_{pic} (n = 6)	0.1	0.9	0.9
	N (n=6)	17391.67	17403.83	6781.00
	R_s	11.64	11.64	13.76
	T_f	1.08	1.11	1.24
LOD	Concentration (µg/mL)	0.03	0.03	0.5
LOQ	Concentration (µg/mL)	0.1	0.1	0.7
Linearity	Range of concentrations (µg/mL)	0.1 – 70.0	0.1 – 3.5	0.7 – 5.0
	Coefficient of determination (R^2)	0.9999	0.9999	0.9994
Accuracy		(n = 3)	(n = 3)	(n = 6)
	Recovery (%) at low level	102.2 – 104.2	97.5 – 102.5	97.2 – 105.0
	RSD (%) at low level	1.1	2.6	3.0
		(n = 3)	(n = 3)	(n = 6)
	Recovery (%) at medium level	100.5 – 101.5	101.2 – 102.2	97.5 – 99.2
	RSD (%) at medium level	0.4	0.5	0.7
		(n = 3)	(n = 3)	(n = 6)
	Recovery (%) at high level	100.6 – 100.8	99.5 – 101.2	97.9 – 101.4
	RSD (%) at high level	0.1	0.9	1.2
Precision	RSD (%) for repeatability (n = 6, same day, same analyst)	0.7	1.4	1.1
	RSD (%) for intermediate precision (2 days, 2 analysts)	(n = 18) 0.8	(n = 18) 0.9	(n = 12) 2.6

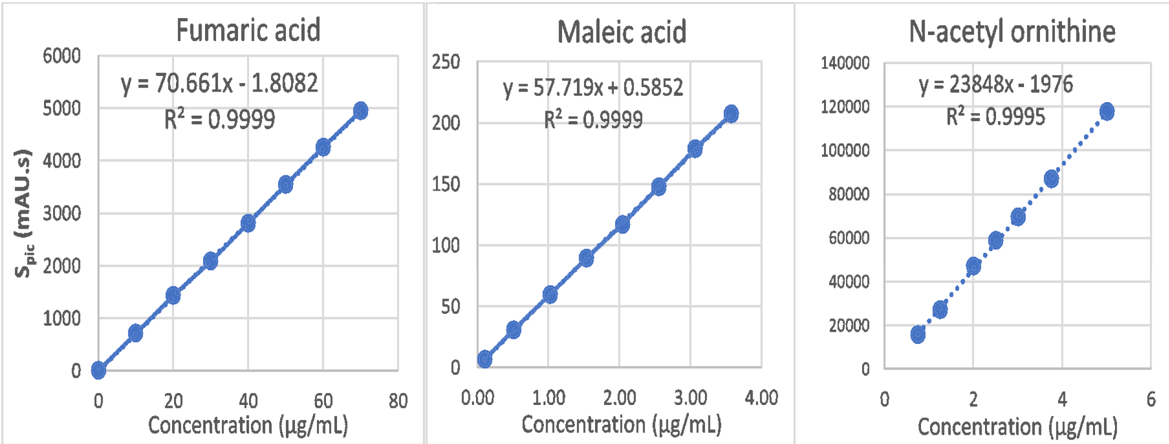


Figure 5. Chromatograms of solvent, standards, and spiked sample solutions

Table 3. Analytical results for the CM raw material batch

	FA and MA impurities			AO impurity	
Sample	Weight of sample (g)	FA conc. (%)	MA conc. (%)	Weight of sample (g)	AO conc. (%)
1	0.2502	0.4585	0.0099	0.0252	not detected
2	0.2503	0.4647	0.0100	0.0250	not detected
3	0.2499	0.4675	0.0101	0.0249	not detected
Average (%)		0.4636	0.0100		not detected

The next results presented in Table 2 demonstrated a high accuracy for all components in both methods, with the recovery ranging from 100.5% to 104.2%, RSD ≤ 1.1% for FA, from 97.5% to 102.5%, RSD ≤ 2.6% for MA, and from 97.2% to 105.0%, RSD ≤ 3.0% for AO (Table 2).

The precision was evaluated based on the relative standard deviation (RSD) between analyses of spiked testing samples contained 100% specified limit of each substance, which were carried out on one day by the same analyst and on two different days, by two different analysts. The method meets the precision requirements of AOAC with the precision criteria satisfied: RSD of FA ≤ 0.8%, of MA ≤ 1.4%, and of AO ≤ 2.6% across intra-day, inter-day, intra-analyst and inter-analyst variations.

Thus, the developed methods have been fully validated according to AOAC International.

Application of the methods

The validated methods were applied to determine the impurities FA, MA and AO in the CM raw material batch (No. C562305009) from Jing Jing Pharmaceutical Co., Ltd.

One standard solution at the concentration corresponding to 100% limit of each impurity and three independent test samples were prepared as indicated in Sample preparation for each method. The analysis was carried out under appropriate valuated conditions. The results were calculated based on the area of peak on standard chromatograms and of corresponding peak on sample chromatograms.

The results (Table 3) confirmed the presence of FA and MA impurities, with their levels within the acceptable limits (FA ≤ 0.5%, MA ≤ 0.025%). However, AO impurity was not detected in the sample of CM raw material from Jing Jing Pharmaceutical Co., Ltd.

DISCUSSIONS

Two HPLC methods were successfully established for the quantification of FA, MA and AO impurities in CM raw material with validated selectivity, accuracy, and repeatability according to AOAC guidelines. The use of a C18 column, rather than an ion-exchange column (L17), simplified the chromatographic system and aligned with commonly available laboratory setups while maintaining sufficient resolution between impurities and the main component. In the simultaneous determination of FA and MA, both compounds exhibit similar UV absorbance maxima at approximately 208 - 210 nm; however, FA is present at significantly higher levels (20-fold) compared to MA. Considering the lower specification limit for MA (0.025%) versus FA (0.5%), the detection wavelength was optimized at 210 nm to enhance MA quantification while minimizing chromatographic noise that is more pronounced at lower wavelengths. Adjusting the mobile phase pH to 2.0 was critical for improving FA and MA retention on the reversed-phase column by promoting their neutral molecular forms, thus increasing hydrophobic interactions and resolution from matrix interferences. For AO, optimal retention was achieved at pH 2.5 using sodium octane sulfonate as an ion-pairing agent, enabling the positively charged analyte to interact effectively with the hydrophobic stationary phase.

Validation data confirmed accurate quantification of all three impurities, with recoveries falling within acceptable limits and RSD values indicating good repeatability and intermediate precision. Application of the methods to a commercial batch showed that FA and MA were present at acceptable levels, while AO was not detected, supporting the material's compliance with impurity specifications.

These HPLC procedures provide a practical and reliable approach for impurity profiling in CM raw material, contributing to improved quality control, particularly considering increasingly stringent global regulations for nutraceutical products. Further validation across multiple production batches and laboratory settings is recommended to reinforce the methods' applicability.

CONCLUSION

Two high-performance liquid chromatography (HPLC) methods were established to quantify fumaric acid, maleic acid and *N*-acetylornithine impurities in citrulline malate raw material. The analysis utilized a Shim-pack GIST C18 column (250 × 4.6 mm, 5 µm). FA and MA were detected at 210 nm, AO at 200 nm. The methods underwent validation following AOAC International, ensuring its reliability and applicability. The system met suitability criteria with RSD < 2.0%, resolution > 1.5, and >2000 theoretical plates. Linearity was confirmed over 0.1–70.0 µg/mL (FA), 0.1–3.5 µg/mL (MA), and 0.7–5.0 µg/mL (AO).

LODs were 0.03 µg/mL for FA and MA, 0.5 µg/mL for AO; corresponding LOQs were 0.1 µg/mL and 0.7 µg/mL. Recoveries ranged from 100.5-104.2% (FA), 97.5-102.5% (MA), and 97.5-105.0% (AO) with repeatability and intermediate precision within acceptable RSD limits (≤ 1.1%; ≤ 2.6; and ≤ 3.0% respectively) showed a good reliability of these methods. These results provided an effective means for managing the quality of input CM material for food manufacturers and the controllers.

CONFLICTS OF INTEREST

None.

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