



Development of a validated RP-HPLC/DAD method for the quantitative determination of methyl jasmonate in an insect repellent semi-solid formulation



Johirul Islam^{a,*}, Saurav Phukan^b, Pronobesh Chattopadhyay^a

^a Division of Pharmaceutical Technology, Defence Research Laboratory, Tezpur, 784001, Assam, India

^b Department of Molecular Biology and Biotechnology, Tezpur University, Tezpur, 784 028, Assam, India

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ABSTRACT

A simple and efficient reversed-phase high-performance liquid chromatographic (RP-HPLC) method has been developed for the first time for the estimation of a mosquito repellent, methyl jasmonate in a cream formulation, and validated as per the International Conference on Harmonization guidelines. Acetonitrile and water (75:25 v/v) were used as the mobile phase and the flow-rate of the mobile phase was kept constant at 1.0 mL/min. The analysis was performed isocratically on a C₁₈ analytical column (250 × 4.4 mm, 5 μm) using a Diode Array Detector for the detection of methyl jasmonate at 214 nm. The presence of excipients did not interfere with the quantification of methyl jasmonate. The calibration curve was linear in the concentration range of 25–300 μg/mL. The relative standard deviations for intra-day and inter-day precision, and repeatability were less than 2%. The recovery ranged from 88.5% to 90.7% with relative standard deviations not higher than 2%. The limit of detection and quantification were 9.4 μg/mL and 28.5 μg/mL, respectively. System suitability parameters were within the accepted range. The proposed method was also robust. Thus, the present report puts forward a novel analytical method for the estimation of an emerging mosquito repellent, methyl jasmonate by using the RP-HPLC technique.

1. Introduction

Methyl jasmonate (MeJA) is an important phytohormone involved in the regulation of diverse developmental processes of plants, such as seed germination, root growth, tuber formation, fertility, fruit ripening, leaf senescence and so on [1, 2, 3]. It also triggers unique plant defense mechanisms against biotic and abiotic stresses, such as insect-driven wounding, a range of pathogens, and ecological stresses, such as drought, low temperature and salinity [4, 5]. MeJA has also been proven to induce suppression of cellular proliferation and necrosis, and this expanded its potential application to cancer therapy [6, 7, 8]. Furthermore, MeJA has been found to cause aversion in number of ticks such as nymphal *Ixodes ricinis*, *Hyalomma marginatum rufipes* Koch, etc. [9]. Most interestingly, MeJA has recently been implicated in repelling *Culex quinquefasciatus* mosquitoes [10]. *Culex quinquefasciatus* act as a vector for numerous deadly pathogens including avian malaria parasite, St. Louis encephalitis virus, Western equine encephalitis virus, West Nile virus and Japanese encephalitis virus. These mosquito-vector viruses

are responsible for causing many severe human tropical diseases that incapacitate and seriously debilitate millions of people and unfortunately, decimate countless lives annually [11]. In addition, absence of vaccines and the lack of effective medical treatments for some of the diseases further alleviate the sufferings and economic consequences among populations [12]. Overwhelmingly, recent evidences on the involvement of *Culex quinquefasciatus* mosquitoes to vector zika virus (which is generally considered an *Aedes*-transmitted pathogen) has raised grave concern [13, 14]. Thus, these scientific findings advocate urgent strategies to control this mosquito vector. Despite numerous anti-mosquito formulations (of both synthetic and natural origins) are currently available on the market, the menace posed by this mosquito vector remains. It is important to note that applicability of many commercially available anti-mosquito formulations has lessened noticeably, and this can be attributed either to their ineffectiveness, toxic effects or the development of resistance in mosquitoes to these anti-mosquito formulations [12]. Therefore, there is a need to explore new and more efficient preventive measures against the *Culex*

* Corresponding author.

E-mail address: johirulmon@gmail.com (J. Islam).

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quinquefasciatus mosquitoes. To the best of our knowledge, insect repellent formulation containing MeJA has not been attempted to date in any form. Hence, there is a vast opportunity in formulation development.

In view of the above, a semi-solid formulation, MeJA-loaded mosquito repellent cream (MeJA-MRC) was developed in our laboratory and its efficacy was investigated against laboratory-reared *Culex quinquefasciatus* mosquitoes. Although, MeJA-MRC has exhibited promising activity against *Culex quinquefasciatus* (data not shown), for practical applicability of MeJA-MRC, a suitable analytical technique is must for the quantification of MeJA adhering to the international standards. Available literatures have presented a number of analytical methods utilizing sophisticated LC-MS-MS [15], LC-ESI-MS/MS [16], SPME-GC-MS [17], RPLC-GC [18], PMME-HPLC [19], SPE-HPLC [20], SPME-HPLC [21] and HPLC-UV [22] based techniques for the quantitative analysis of MeJA. Although the effectiveness of these reported methods undoubtedly contribute to efficient separation of MeJA, they have nonetheless several disadvantages, which limit their application. For instances, LC-MS-MS, LC-ESI-MS/MS and SPME-GC-MS may not be available in all laboratories owing to the high capital cost of these equipments. Furthermore, complex instrumentation, extensive sample preparation schemes and requirements for a skilled labor force present major constraints that may impede the application of these highly sophisticated analytical instruments [23]. Similarly, PMME-HPLC, SPE-HPLC and SPME-HPLC require complex procedure of sample preparation, and involves relatively high consumption of reagents and longer run time [24]. Whilst a HPLC-UV method is available, this method necessitates the synthesis of fluorescent hydrazones of jasmonic acid by chemical derivatization to render the analyte quantifiable by HPLC. In addition, complex chromatographic conditions and long runs (~ 36 min) discourages the application of this method [22]. Thus, growing concern on the raised transmission of *Culex quinquefasciatus*-vectored diseases, absence of appropriate preventive measures and lack of simple and efficient analytical techniques prompted us to undertake this investigation. Hence, the present investigation focused on to the development of a reproducible, simple, fast and sensitive RP-HPLC method for the quantification of MeJA in MeJA-MRC and validate it conforming to the guidelines of the International Conference on Harmonization (ICH) [25].

2. Materials and methods

2.1. Reagents and chemicals

Methyl jasmonate, and HPLC grade solvents such as acetonitrile, methanol and water were purchased from Sigma Aldrich (Sigma Aldrich Chemical, Co., St. Luis, MO, USA).

2.2. Instrumentation

All experiments were performed on a fully automatic HPLC system (Agilent Technology, 1260 Infinity, USA), equipped with an online degasser, a quaternary pump, an auto-sampler, a column compartment with oven and a diode array detector (DAD) system. The analysis was performed isocratically on a C₁₈ analytical column (ZORBAX Eclipse plus, 250 × 4.4 mm, 5 µm). The Open LAB CDS Chemstation chromatographic software was used for HPLC data processing (version-C.01.04).

2.3. Standard solution preparation

Standard stock solution of MeJA (1000 µg/mL) was prepared by dissolving an appropriate amount of MeJA in ethanol. The solution of primary standard stock was stored at 4 ± 0.5 °C until further use.

2.4. Selection of the detection wavelength

To determine the detection wavelength for HPLC analysis, 10 µg/mL

solution of MeJA was prepared by diluting the primary standard stock solution with mobile phase [acetonitrile and water at the ratio of 75:25 (v/v)] and scanned in the range of 190–400 nm using the mobile phase as blank.

2.5. Preparation of sample solutions of MeJA-MRC.

One gram of MeJA-MRC was transferred to a 100 mL volumetric flask and extracted with 10 mL ethanol. The flask was then shaken mechanically for about 2 h for complete extraction of MeJA. The resultant mixture was then centrifuged at 6000 × g for 20 min and the supernatant (1 mL) was collected. The supernatant was further diluted with mobile phase to obtain 300 µg/mL of MeJA, approximately. The aliquots were filtered through syringe filter (HiMedia Laboratories Pvt. Ltd., Maharashtra, India) and injected subsequently for HPLC analysis.

2.6. Method development

Preliminary method was developed and optimized as per the method described elsewhere with slight modifications [24]. Briefly, various HPLC parameters including columns, detection wavelengths, solvent systems, mobile phase compositions and flow rates were altered to obtain a set of suitable chromatographic conditions. However, detector, injection volume, oven temperature (25 ± 2 °C) and mode of elution remained unaltered during the process of method development and validation. A fixed concentration (100 µg/mL) of sample solution of MeJA-MRC was injected, and a spectrum at the desired wavelength was recorded. Finally, peak purity of the resultant chromatogram was evaluated to finalize the suitable detection wavelength. Other aspects that were typically considered in the preliminary method development include resolution, separation time, quantitation, column pressure, peak height and solvent consumption per run.

2.7. Validation

After obtaining the suitable chromatographic conditions, the method was validated as per the ICH Q2B guidelines [25] by following the method as described elsewhere [24]. The method was validated in terms of linearity, precision, accuracy, specificity, limit of detection (LOD) and limit of quantification (LOQ), system suitability and robustness. Further, stability of reagents and solvents were investigated as described elsewhere [26].

2.8. Application of the developed method

The validated HPLC method was used for the determination and quantification of MeJA in MeJA-MRC. The preparation of MeJA-MRC solutions followed the method as described in section 2.5. Triplicate injections were done for each sample.

3. Results and discussion

3.1. Method development and optimization

In the present contribution, we attempted to develop and validate a RP-HPLC/DAD method for the quantification of MeJA in a cream formulation. In this investigation, theoretical and empirical approaches were combined for successful method development and validation. The theoretical approach included the understanding of the physico-chemical characteristics of MeJA that allows the selection of appropriate chromatographic conditions from the available vast literatures. The development of the preliminary method was therefore based on the compilation of comprehensive information of MeJA including molecular mass, solubility, partition coefficient, chemical structure and functionality and UV absorption spectrum. Solubility is an important aspect since it helps to select the suitable mobile phase compositions and column to

be used in HPLC method development. MeJA is very slightly soluble in water (340 mg/L at 25 °C) but freely soluble in alcohol. Therefore, ethanol was used for preparing the stock solution of MeJA. Chemically, MeJA (molar mass: 224.3 g/mol; logP: 2.76) is methyl (1R,2R)-3-Oxo-2-(2Z)-2-pentenyl cyclopentaneacetate that contains an ester and a ketone group, and a cyclopentane parent ring (Fig. 1a). Both the ester and the ketone groups exhibited strong UV absorbance at 214 and 293 nm in acetonitrile: water (75:25 v/v) (Fig. 1b). Interestingly, each of these two wavelengths was suitable for the quantification of MeJA by HPLC using the DAD detector (Fig. 2). After obtaining this preliminary information, the method was developed and optimized by altering various HPLC parameters as described in method section.

Taking into account the physico-chemical characteristics of MeJA, both C₈ and C₁₈ analytical columns were used for the analysis of MeJA. C₁₈ column displayed better results than the C₈ in terms of optimum plate number (*N*), tailing factor (*T_f*) and resolution (*R_s*). To finalize the optimum absorbance, the instrument's LOD was obtained for 214 and 293 nm by DAD detector. Ideally, a good method should exhibit minimum LOD while deciding a detection wavelength. In addition, the peak area should be maximum at the selected wavelength [24]. It is worthy to note that, the minimum LOD was obtained at 214 nm with suitable precision and accuracy. Moreover, the corresponding peak area of MeJA was maximum at this wavelength. Importantly, co-eluting interferences that may likely to appear due the presence of excipients were also absent for this wavelength at the retention time of MeJA (4.5 min). Sensitivity obtained for 293 nm was in-adequate with the proposed analytical method. The selection of the optimum wavelength was further confirmed by peak purity of the resultant peak. At 214 nm, spectra of MeJA exhibited a threshold and similarity curve (Fig. 3a), where later was found below the threshold curve. Moreover, all diamonds (each diamond represents the scan path) were found at the green zone. As per the manufacturer instructions, the above conditions persist when a single compound is present in a sample solution (Agilent technologies). Additionally, Fig. 3b presented a superimposable peak. It is worth to note that if a single component is present in a peak, the spectra obtained across peak should be superimposable [27]. Thus, these findings indicated the presence of only MeJA in the sample solutions. Results obtained in this investigation are not at variance with our previous publication. In that particular contribution, we reported the peak purity while developing a method for the estimation of a mosquito repellent, ethyl anthranilate in a polymeric patch [24]. Thus, all requisite criteria were met at 214 nm and hence it was selected as an ideal detection wavelength with respect to sensitivity and conditions of the proposed analytical method.

MeJA is an oily liquid, and remained as a neutral molecule during the separation. Thus, the mobile phase may be a simple combination of

organic solvent and water, and a buffer solution is not required. Two different solvent systems, acetonitrile and methanol were tested for continuous measurement of MeJA (Supplementary Figure 1). It was observed that the acetonitrile-water system gave a well-resolved peak and a better resolution than the methanol-water system, providing suitable retention time for MeJA. Further, separation parameters such as retention time, peak area and plates count obtained with the acetonitrile-water system were better than the methanol-water system (Supplementary Table 1). In addition, an unknown peak was observed with methanol-water system at 5.8 min that may directly influence the accurate quantitation of MeJA. Results obtained in this experiment are similar to other previous findings. For example, Xia et al., 2010 [28] developed and validated a method for the quantification of linalool using acetonitrile and water as a mobile phase. In their experiments, this mobile phase composition also generated similar well-resolved peaks as obtained in this investigation. Compositions of the mobile phase (60:40, 65:35, 70:30, 75:25, 80:20 and 85:15) have also exerted similar influences on the separation especially peak spacing and resolution. Significant changes in the band spacing with improved peak resolution were evident with the increased concentration of organic solvent i.e. acetonitrile. However, a decrease in the concentration of acetonitrile resulted in increased retention with varied selectivity, band spacing and resolution. Thus, careful selection of both solvent type and composition is essential for successful method development. Finally, acetonitrile and water: (75:25 v/v) was found to be the ideal mobile phase composition for the quantification of MeJA. Similarly, the influence of flow rates (0.5, 1.0 and 1.5 mL/min) was also found to affect the separation. As the flow rate varied, the retention time of MeJA also varied. The optimum flow rate was found to be 1.0 mL/min. At this flow rate, the *N* was maximum and exhibited the best *R_s* between all components. However, the quantification of MeJA was performed at ambient temperature, i.e. 25 ± 2 °C owing to its neutral characteristics. Generally, reversed phase chromatographic separation of neutral compounds at higher temperatures results in decrease selectivity [27]. Given the MeJA's neutral characteristics and to obtain improved separation, temperature was therefore kept constant throughout the experiment. Finally, a C₁₈ column, a DAD detector, a solvent system comprising of acetonitrile:water (75:25 v/v) with flow rate 1 mL/min and 214 nm detection wavelength was chosen as the most suitable conditions for the proposed analytical method. Symmetric and well defined peak of MeJA with an average retention of 4.5 min was observed with the above conditions. The percent relative standard deviation (%RSD) was less than 2% and column pressure was found well below the defined limit. Ideally, column pressure <150 bar is desirable; and <200 bar is usually essential [27].

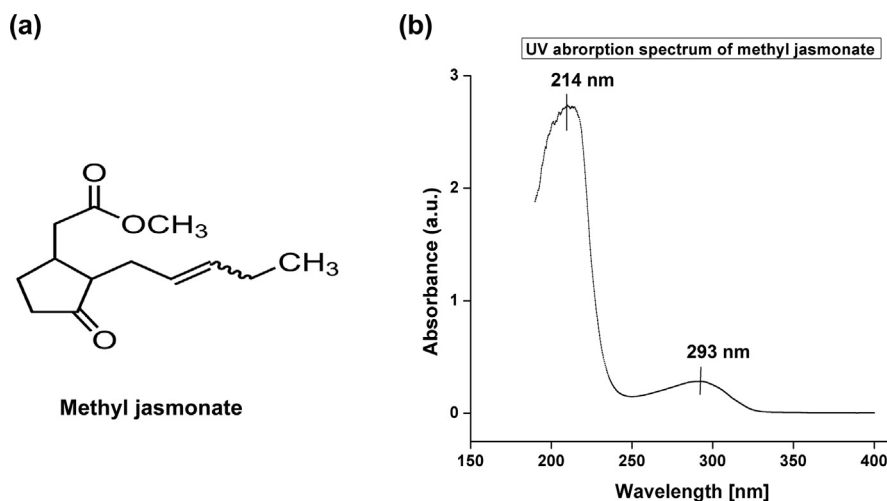


Fig. 1. (a) Chemical structure of MeJA; and (b) UV absorption spectrum of MeJA.

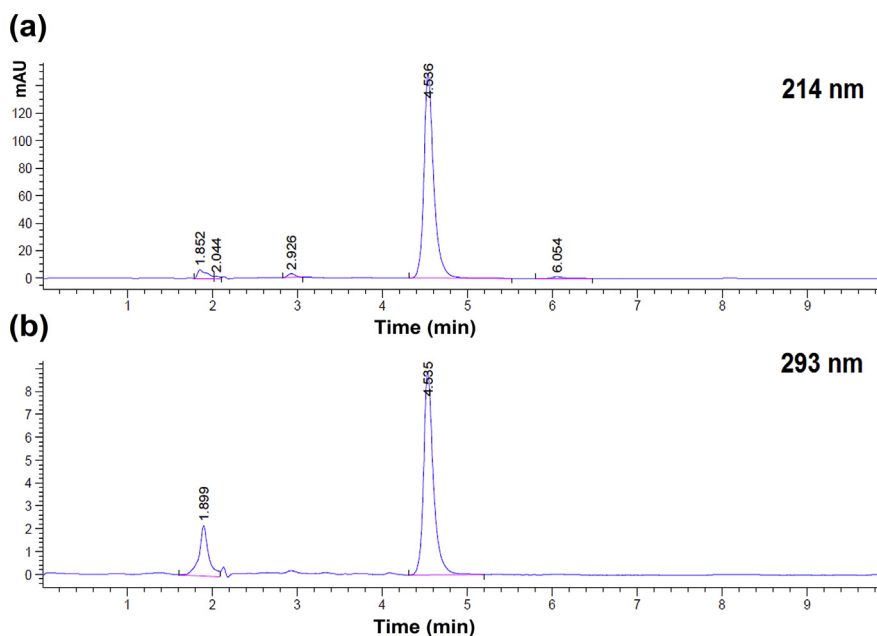


Fig. 2. Chromatogram of MeJA obtained at (a) 214 nm; and (b) 293 nm with a DAD detector.

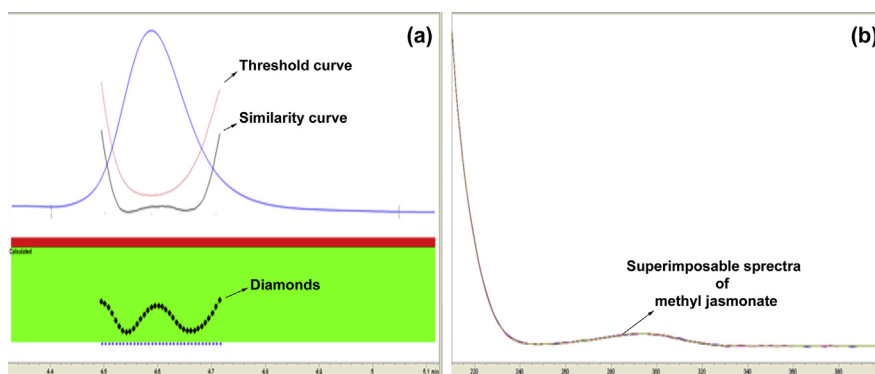


Fig. 3. (a) Peak purity curve of MeJA in MeJA-MRC at 4.5 min; (b) superimposable spectra of MeJA.

3.2. Method validation

3.2.1. Linearity

The linearity of a method is used to determine how well the data fits to the linear equation. In other words it is the measure of how well a calibration plot of the response vs. analyte concentration approximates a straight line [27]. For system linearity, five analyte concentrations were used to obtain a calibration curve. The stock sample solution of MeJA-MRC was diluted with mobile phase to prepare solutions of MeJA containing 25, 50, 100, 200, and 300 $\mu\text{g/mL}$. A fixed volume (20 μL) of each solution was injected in triplicate into the HPLC column and their corresponding responses were recorded. Analysis of variance (ANOVA) was used to validate the linearity of the developed method (GraphPad Instat. version-3.05).

The calibration curve was linear over the range of 25–300 $\mu\text{g/mL}$. The equation of the calibration curve based on the peak response was $y = 3.987x + 2.491$ with a correlation coefficient (R^2) of 0.999. The results obtained from linear regression analysis indicated the significance of the proposed method with a p value < 0.005 . Ideally, a calibration curve should be linear with a R^2 value of 0.999 and have a zero intercept [27]. The intercept obtained in this experiment was not zero though; it was found quite low as 2.491 with the R^2 value approaching unity, thus suggesting the linearity of the method.

3.2.2. Precision

The precision of the method was established by determining the system precision including intra-day and inter-day precision, and repeatability and was expressed as %RSD. Ideally, the %RSD value should be less than 2% [27]. For intra-day and inter-day precision evaluations, three different concentrations (50, 100 and 200 $\mu\text{g/mL}$ of MeJA) were prepared by diluting stock sample solution of MeJA-MRC and each solution was injected in triplicate in the same day and different days, respectively and their %RSD values were calculated. While, repeatability of the method was determined by injecting six replicate injections of the sample (100 $\mu\text{g/mL}$) and %RSD was calculated.

The results of precision experiments are presented in Table 1a. The % RSDs for intra-day and inter-day precision, and repeatability were less than 2%.

3.2.3. Accuracy

The accuracy of an analytical method is used to determine the closeness of the measured value to the true value. Accuracy of a method is generally determined by recovery of the analyte spiked into a placebo matrix. In general, the recovery at each level is determined by comparison to the known amount added. For a major component assay, spiked levels typically should be at 50%, 100% and 150% of the level expected for the analyte in a normal assay [27]. For accuracy analysis, three

Table 1a

Precision and values of the proposed method.

Analyte	Concentration (µg/mL)	Inter-day			Intra-day			Concentration (µg/mL)	Repeatability		
		Area (mAU)	Mean ± SD	% RSD	Area (mAU)	Mean ± SD	% RSD		Area (mAU)	Mean ± SD	% RSD
MeJA	50	179.81	178.8 ± 1.21	0.67	177.73	177.86 ± 0.37	0.21	100	388.26	383.99 ± 8.01	2.08
		177.46			177.56				374.24		
		179.13			178.28				373.13		
	100	384.44	383.45 ± 0.86	0.22	372.62	372.97 ± 0.34	389.94				
		383.04			373.32		389.20				
		382.86			372.98						
	200	733.79	739.20 ± 9.1	1.23	722.71	723.00 ± 0.55	0.07				
		734.08			723.65						
		749.74			722.64						

different concentrations (50, 100 and 150 µg/mL corresponding to 50%, 100% and 150%, respectively) of standard MeJA were prepared and spiked with placebo samples. Each sample was injected in triplicate and recovery was determined by comparing the obtained concentration with the nominal concentration.

The recovery of the proposed analytical method ranged from 88.5% to 90.7% with %RSD less than 2% (Table 1b). The closeness of values between found analyte and claimed theoretical concentrations at different levels indicated the trueness/accuracy of the proposed method.

3.2.4. Specificity

The specificity of a method is a measure of how well a method can quantify the concentration of the analyte in the presence of all other sample materials [24, 27]. The potential effects from matrix components used in MeJA-MRC was investigated, where placebo matrix was composed of silicon oil (Sigma Aldrich); cetostearyl alcohol, glycerine, glycerine monostearate, light liquid paraffin, methyl paraben, propyl paraben, stearic acid and tocopherol (HiMedia); and Aquagel 35S® (Tinci Materials Technology Co. Ltd., Guangzhou, China). The specificity of the proposed method for determination of MeJA was established by injecting mobile phase, placebo matrix extracted solution, pure MeJA (100 µg/mL) and MeJA-MRC (100 µg/mL) solution into the HPLC system with a run time of 10 min. The resultant chromatograms were then examined for the presence of any interfering peaks at the retention time of MeJA. The specificity of the method was further validated by injecting a fixed concentration (100 µg/mL) of MeJA-MRC and determination of peak purity factor of the resultant peak.

The representative chromatograms of mobile phase, placebo, pure MeJA and MeJA in MeJA-MRC are presented in Fig. 4. The solution of MeJA-MRC showed a distinct, well-resolved and symmetric peak at the retention time of 4.5 min (Fig. 4d). It is worthwhile to note that interfering peaks of placebo matrix were absent at the retention time of MeJA, indicating the specificity of the proposed method.

Table 1b

Accuracy of the method determined as per the ICH Q2B guidelines.

Analyte	Concentration (µg/mL)	Area (mAU)	Obtained concentration (µg/mL)	% Recovery	Mean ± SD	% RSD
MeJA	50	179.08	44.29	88.58	88.5 ± 0.22	0.25
		178.3	44.09	88.19		
		179.1	44.29	88.59		
	100	361.43	90.02	90.02	89.9 ± 0.11	0.12
		360.67	89.83	89.83		
		361.45	90.03	90.03		
	150	544.74	136	90.66	90.7 ± 0.08	0.09
		545.5	136.19	90.79		
		544.49	135.94	90.62		

3.2.5. LOD and LOQ

To determine LOD and LOQ, five different concentrations (25, 50, 100, 200, and 300 µg/mL of MeJA) were prepared by diluting the sample solution of MeJA-MRC with mobile phase and subsequently injected into the HPLC system. The LOD and LOQ were then calculated by the following formulas as described elsewhere [24].

$$\text{LOD} = 3.3 \sigma / S \quad (1)$$

$$\text{LOQ} = 10 \sigma / S \quad (2)$$

where, σ and S are the intercept and slope of the calibration curve, respectively.

The LOD and LOQ of MeJA were 9.4 and 28.5 µg/mL, respectively.

3.2.6. System suitability

System suitability parameters such as N , T_f , capacity factor (k), R_s and %RSD of the peak area were assessed by injecting six replicate injections of sample solution of MeJA-MRC containing 100 µg/mL of MeJA.

The results of system suitability parameters are presented in Table 2a and they were within the accepted range. MeJA was repeatedly retained and well separated at 4.5 min with %RSD less than 1% (as per the USP [29], %RSD should be <1) indicating good repeatability of replicate injections on the integral HPLC system used. In addition, it was established that $k = 0.86$. In general, k affects peak resolution. The resolution usually worsens or improves depending on whether k becomes smaller or increases. Better k values are achieved by changing either the mobile phase composition or the stationary phase [27]. In contrast, changes in the column conditions such as flow rate, column length and particle size do not affect k . Ideally, a precise and rugged method should have R_s greater than 1.5 [27]. The present method exhibited R_s of 5.82 thus indicating an optimum k value. The acceptance limit of $T_f < 1.5$ was achieved with this method and T_f never exceeded 1.26; indicating excellent peak symmetry. Finally, N always exceeded 2000 in all chromatographic runs, which ensured good column efficacy throughout the

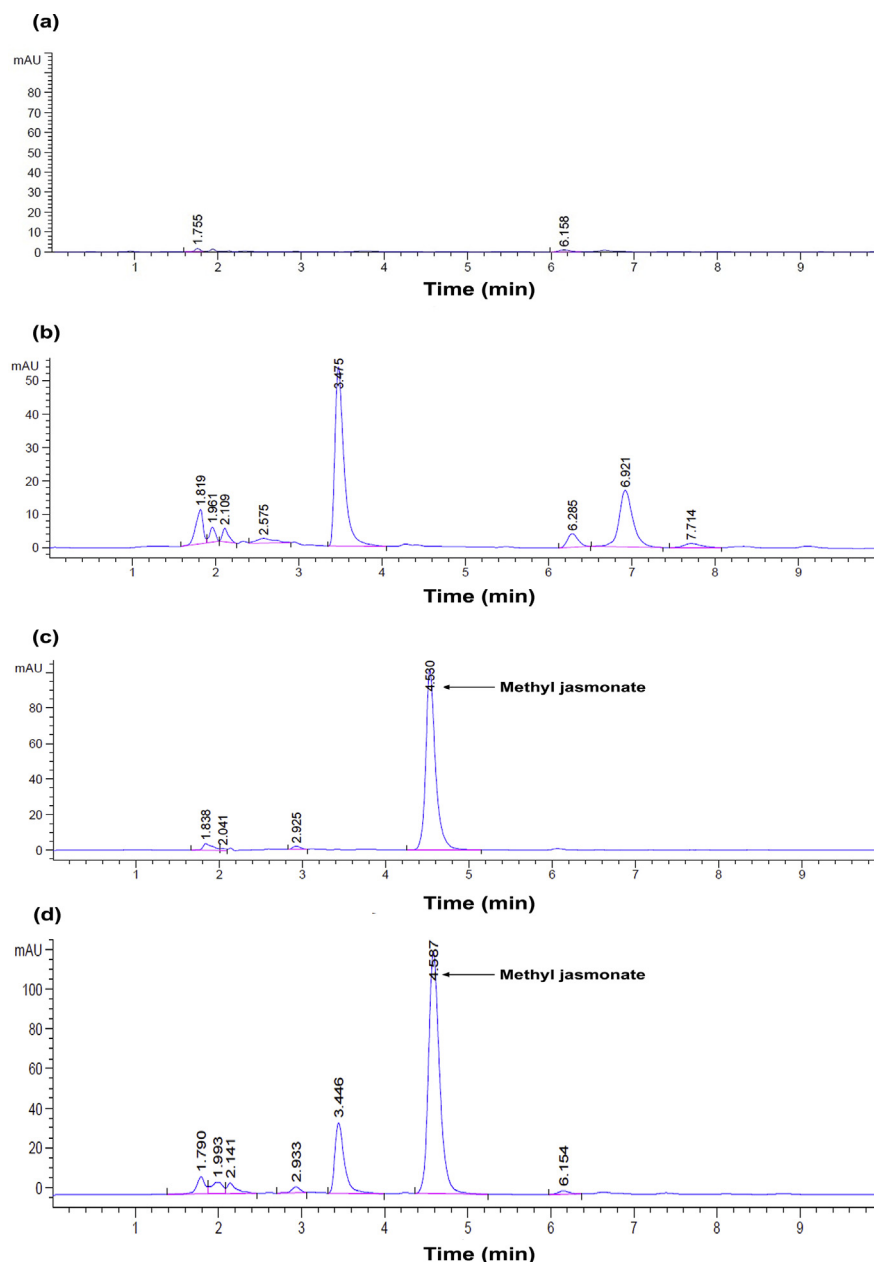


Fig. 4. Chromatograms of (a) mobile phase; (b) placebo matrix; (c) pure MeJA; and (d) MeJA-MRC containing MeJA.

Table 2a

System suitability parameters.

Parameters	Retention time (R_t)	Capacity factor (k)	Area (mAU)	Tailing factor (T_f)	Plates count (N)	Resolution (R_s)
Mean ($n = 6$)	4.54	0.86	371.3	1.26	7266.16	5.82
SD	0.006	0.005	1.59	0.01	241.24	0.08
% RSD	0.15	0.59	0.42	1.43	3.32	1.4
Required limits	<15 min		% RSD < 1	Tf < 1.5	$N > 2000$	

developed separation process.

3.2.7. Robustness

The ICH defines the concept of robustness of an analytical procedure as “a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters” [27]. Robustness of the proposed method was investigated by altering the flow rates and compositions of the mobile phase, and thereby estimating their influences on different HPLC parameters including N , T_f , k and R_s .

No significant changes in N , T_f , k or R_s were noted upon applying small variations to the chromatographic conditions indicating that the method was robust to small deliberate changes. The robustness of the method was further demonstrated by the presence of well-resolved peak of MeJA following the changes in the chromatographic conditions. Results of the robustness investigation are presented in Table 2b.

3.2.8. Stability of samples and reagents

To generate reproducible and reliable results, the samples, standards,

Table 2b
Robustness study.

Parameters	Values	Retention time (R_t)	Area (mAU)	Tailing factor (T_f)	Plates count (N)	Resolution (R_s)
Flow rate (mL/min)	0.5	9.29 $\pm$ 0.03	785.87 $\pm$ 1.67	1.19 $\pm$ 0.01	8654.33 $\pm$ 155.13	6.27 $\pm$ 0.04
	1	4.58 $\pm$ 0.0008	384.00 $\pm$ 8.01	1.2 $\pm$ 0.08	6171 $\pm$ 46.66	5.31 $\pm$ 0.01
	1.5	3.04 $\pm$ 0.003	268.74 $\pm$ 0.44	1.27 $\pm$ 0.02	5735.66 $\pm$ 38.28	5.008 $\pm$ 0.02
Mobile phase composition (acetonitrile: water)	70:30	5.26 $\pm$ 0.01	371.66 $\pm$ 1.0	1.18 $\pm$ 0.01	6046.33 $\pm$ 62.98	6.16 $\pm$ 0.02
	75:25	4.58 $\pm$ 0.0008	384.00 $\pm$ 8.01	1.2 $\pm$ 0.08	6171 $\pm$ 46.66	5.31 $\pm$ 0.01
	80:20	4.12 $\pm$ 0.01	416.39 $\pm$ 29.53	1.28 $\pm$ 0.10	6703.33 $\pm$ 571.11	4.65 $\pm$ 0.16

and reagents used for the HPLC analysis must remain stable for a reasonable time. To investigate the stability, sample solution of MeJA-MRC containing 100 μ g/mL of MeJA was prepared and assessed after 24 h in auto-sampler, after 24 h at room temperature and after a week in the refrigerator (2–8 $^{\circ}$ C). Further, the sample solution was injected into the HPLC system at day 0, 15, 30 and 60 and their corresponding areas were compared to determine the content of MeJA. Typically, stable solutions result in uniformity in content with least standard deviations.

No significant changes were observed in the content of both samples and standard thus ensuring their stability during the specified period (data not shown). Further, acetonitrile, water and alcohol remained stable during the period of observation.

3.2.9. Application of the developed method

The developed method was used successfully for the analysis of MeJA in MeJA-MRC. The chromatogram of the corresponding MeJA in MeJA-MRC is presented in Fig. 4 (d). The assay of MeJA in MeJA-MRC was found to be 94.6 $\pm$ 0.2% (Table 3). Ideally, higher percentage of assay of a compound is desired while developing a method as it enables other investigator in the routine analysis of same compound as such, or in various other dosage forms.

4. Conclusion

The present investigation resulted in the development of an RP-HPLC/DAD analysis method for MeJA that was validated in terms of linearity, precision, accuracy, specificity, system suitability and robustness. The method is rapid, precise, accurate, sensitive and easy to apply, making it suitable for the quantification of MeJA in MeJA-MRC and any other formulations. The method involves a single step procedure for the preparation of samples and direct injections. Moreover, the run time was also short (4.5 min). Therefore, the proposed analytical method is recommended for the routine analysis of MeJA as such, or in various dosage forms. In addition, the method can be applied in many developing countries or field stations where advanced analytical equipments such as LC/MS or GC/MS are not available. However, it may be necessary to verify the method if other polymers/excipients are used instead of polymers/excipients employed in this study. We speculate a two way outcome when using different polymers. The first one is the interference of polymers, or their monomer units with MeJA. The second one is the poor recovery due to the strong interaction between MeJA and polymers so that MeJA is encapsulated and cannot be extracted with the method used in the present study. This is why a release profile is usually needed when polymers and their rates are determined during encapsulation development.

Table 3
Assay (%) of MeJA by the proposed method.

Formulation code	% Assay of MeJA
F1	94.36
F2	94.76
F3	94.63
Mean	94.6
SD	0.2

Declarations

Author contribution statement

Johirul Islam: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Pronobesh Chattopadhyay: Conceived and designed the experiments; Analyzed and interpreted the data. Contributed reagents, materials, analysis tools or data.

Saurav Phukan: Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

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