

Development and Validation of HPLC-PDA Assay method of *Frangula emodin*

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Abstract: *Frangula emodin*, (1,3,8-trihydroxy-6-methyl-anthraquinone), is one of the anthraquinone derivatives found abundantly in the roots and bark of a number of plant families traditionally used to treat constipation and haemorrhoids. The present study describes the development and subsequent validation of a specific assay HPLC method for emodin. The separation was achieved on a Waters Symmetry C18, 4.6 × 250 mm, 5 μm particle size, column at a temperature of 35 °C, with UV detection at 287 and 436 nm. An isocratic elution mode consisting of 0.1% formic acid and 0.01% trifluoroacetic acid as the aqueous mobile phase, and methanol was used. The method was successfully and statistically validated for linearity, range, precision, accuracy, specificity and solution stability.

Keywords: Emodin; anthraquinone; HPLC; assay; development; validation.

Introduction

A significant number of herbal medicinal products, despite their long tradition, do not fulfill the requirements imposed by European Union (EU) Directive 2004/24/EC on Traditional Herbal Medicinal Products, introduced in 2005¹, which states that companies manufacturing herbal medicinal drugs must show proof of the drug's efficacy and pharmaceutical quality. To maintain these products on the market, companies must supply scientific evidence that proves the quality, safety and efficacy of their products. *Frangula emodin*, (1,3,8-trihydroxy-6-methyl-anthraquinone) (Fig. 1), is one of the anthraquinone derivatives found abundantly in the roots and bark of Fabaceae (*Cassia* spp.), Polygonaceae (*Rheum*, *Rumex* and *Polygonum* spp.) and Rhamnaceae (*Rhamnus* and *Ventilago* spp.). These plants have been used traditionally in the Eastern world for their purgative effects to treat constipation and haemorrhoid^{2, 3}. The laxative properties have been attributed to the active constituents of these plants, one of which is emodin.

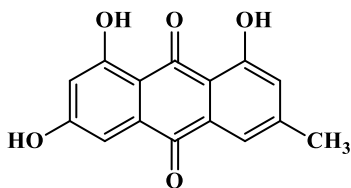


Figure 1. Emodin structure

Studies have shown emodin to display a number of biological activities such as anti-microbial⁴, immunosuppressive⁵, anti-diabetic⁶ and anti-inflammatory activities^{7, 8}. Moreover, emodin is thought to have anti-tumor properties. Studies have been performed both *in vitro* and *in vivo*, and results have shown emodin to have potential as a cytotoxic agent against a variety of human cancers including lung cancer⁹, liver cancer¹⁰, and gastric cancer¹¹.

Various methods have been reported in the literature for the analysis of emodin in a mixture of anthraquinones including thin layer chromatography¹², high performance liquid chromatography (HPLC) with ultraviolet-visible (UV-Vis)¹³, photodiode array (PDA)¹⁴, fluorescence¹⁵ and mass spectrometric (MS)¹⁶ detection. The disadvantage of running methods that were developed for the simultaneous determination of a number of anthraquinones is that in order to achieve sufficient separation between the peaks, they usually incorporate long run times. For routine analysis of emodin, a simple and rapid analytical method is preferred. Few reports of a specific validated UV spectrophotometric method for the assay quantification of *Frangula emodin* have been published to date^{17, 18}. This study focused on supplying chromatographic information on the emodin molecule to meet the requirements imposed by the aforementioned directive, and enable further research and product development. The first objective was to establish the physicochemical properties of emodin that were relevant to the development of a chromatographic method in terms

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of sample preparation and HPLC analysis. Secondly, an assay HPLC method capable of identifying and quantifying emodin in the presence of related substances was developed and subsequently validated in accordance with the International Conference on Harmonisation (ICH) guideline Q2(R1) relating to the validation of analytical procedures¹⁹.

Experimental Section

Instrumentation

A **Waters Alliance 2695 Separations Module**, consisting of an on-line degasser, quaternary pump, thermostatted sample compartment, auto-sampler, column heater/cooler and Waters 2998 PDA detector was employed. Data were acquired and processed using the Waters Empower 2.0 software. A C18 reversed phase column Waters Symmetry C18, 4.6 × 250 mm, 5 µm particle size, was used for the separation. A **Bruker AM250 Nuclear magnetic resonance (NMR) spectrophotometer** fitted with ¹H and ¹³C probe of frequency 250.1 MHz for ¹H and 62.9 MHz for the ¹³C probe with the corresponding software Bruker Aspect 3000 pc using 16K for ¹H NMR was used.

Software

ACD ChemsSketch software version 10.00 Calculator Plugins were used to predict pK_a values and generate pK_a plots, Marvin 6.1.6, 2014, ChemAxon (<http://www.chemaxon.com>). Statistical analysis was performed using Microsoft Excel and SPSS 20.0 for Windows.

Chemicals and Reagents

HPLC gradient grade methanol and trifluoroacetic acid (TFA) (> 99.0% purity) were purchased from Sigma Aldrich. Formic acid (98% purity) was obtained from Fischer Scientific (UK.). Deionized water was purified using the ELGA Pure Lab Classic System with 0.2 µm filter, TOC 1 to 3 ppb and resistivity ≥ 18.2 MΩ-cm. The **emodin working standard** utilized during this research project was obtained from Applichem, Germany with the item code A2056. It was certified as being of HPLC grade and extracted from *Rhamnus frangula*. This working standard was used as the sample throughout the project. The reference standard of emodin of item code E7881 was obtained from Sigma Aldrich. The standard was extracted from *Frangula* bark and its HPLC purity was stated as being 96.10%.

Standard Solution

Working and reference standard solutions of emodin were prepared by dissolving accurately weighed amounts of emodin standard in 100% methanol, followed by 15 minute sonication and

topping up to the mark to give a final concentration of 0.5 mg/mL. A similar standard concentration was selected in multiple studies^{20, 21, 22}. The solutions were stored at 4 °C.

Method Scouting and Development

Several HPLC methods have been developed and validated for the analysis of anthraquinones. These methods primarily differ in the type of column used, mobile phase, flow rate and their applications. A literature survey was first performed in search of HPLC methods that included emodin as one of the analytes. Most methods were developed for the separation and determination of mixtures of anthraquinones present in various herbal extracts. Additionally they incorporated rather long runtimes to allow for the separation of the various components.

Methods that included phosphate buffers^{23, 24} were excluded as phosphate buffers are incompatible with certain detectors, including mass spectrometry. Hence in order to allow the rapid transfer of methods from UV detection to MS detection, the chromatographic parameters were chosen to be both UV and MS compatible. Preliminary testing was performed to establish the pK_a of the emodin molecule and also to identify the UV wavelengths at which the emodin molecule absorbed most intensely.

The shortlisted methods (Systems 1, 2 and 3), which were selected based upon the results of the preliminary testing, were attempted on the Waters Alliance HPLC injecting only the emodin working standard. The resultant chromatograms were examined for sensitivity, efficiency and separation characteristics. The retention time, peak area, United States Pharmacopeia (USP) plate count and USP tailing were measured. The method that produced the best results for the emodin peak (System 3) was further optimized for column temperature, detection wavelength and mobile phase composition to obtain specificity of the emodin peak in the shortest run time possible (Systems 4, 5 and 6). A small quantity of TFA was incorporated into the mobile phase to sharpen peaks and as a result improve peak efficiency and resolution. In order to determine the optimal TFA concentration that was compatible with the emodin molecule without exceeding the pH limit recommended by the column, two concentrations of TFA were analyzed on the HPLC (Systems 7 and 8) and the resultant chromatograms were examined for efficiency of peaks. Specificity and the optimal detection wavelength were determined by performing peak purity testing using the PDA detector. The optimized method was then validated in accordance to the International Conference of Harmonization (ICH) guideline Q2(R1)¹⁹.

Table 1. Summary of HPLC methods investigated during method scouting and development.

System	Mobile Phase Constituents (A:B)	Isocratic/Gradient Profile	Flow Rate (mL/min)	Column Temp. (°C)	UV λ (nm)	Retention Time (mins)	Reference
1	Water : methanol	Gradient 0 mins: 55% A 0 to 6 min: 55% A 6 to 9 min: 25% A 9 to 18min: 0% A 18 to 25min: 0% A	1.0	25	436	21.090	18
2	0.1% (v/v) Formic acid : methanol	Gradient 0 mins: 28% A 0 to 5 min: 20% A 5 to 10 min: 15% A 10 to 14min: 10% A	1.0	50	N/A	21.871	25
3	0.1% (v/v) Formic acid : methanol	Isocratic 15% A : 85% B	1.0	25	254	9.629	26
4	0.1% (v/v) Formic Acid : methanol	Gradient 0 mins: 28% A 0 to 10 min: 28% A 10 to 21 min: 15% A 21 to 30 min: 10% A	1.0	35	254	19.408	Optimization of System 2
5	0.1% (v/v) Formic Acid : methanol	Gradient 0 mins: 55% A 0 to 6 min: 55% A 6 to 9 min: 25% A 9 to 18min: 0% A 18 to 25min: 0% A	1.0	35	254 436	17.051	Optimization of Systems 1 and 2
6	0.1% (v/v) Formic Acid : methanol	Isocratic 10% A : 90% B	1.0	35	254 436	6.425	Optimization of System 3
7	0.1% TFA : methanol	Isocratic 10% A : 90% B	1.0	35	254 436	5.985	Investigating the effect of TFA
8	0.01% TFA : methanol	Isocratic 10% A : 90% B	1.0	35	254 436	5.891	Investigating the effect of TFA
9	0.01% TFA 0.1% formic acid : methanol	Isocratic 10% A : 90% B	1.0	35	254 436	5.905	Chosen optimized chromatographic conditions

Results and Discussion

Preliminary study of the emodin molecule

The pK_a of emodin generated using ACD/ChemSketch software version 10.00, was found to be 6.39 with an error of 0.2. This value appears to be a combination of the dissociation constants pK_{a1} and pK_{a2} which were reported as being 5.70 and 7.94 respectively²⁷. From this information it was deduced that an acidic mobile phase was preferable with a pH lower than 4.70 in order to have the emodin in a single ionization form. Due to the molecule's acidic functionality, the emodin would be in its unionized form at pH's lower than 4.70 and moreover in a 'robust pH zone' which is vital for reproducible retention times.

The UV spectrum of the emodin working standard dissolved in methanol (Fig. 2) gave a preliminary idea of the wavelengths that showed the highest absorption for emodin which is an important factor to consider when deciding on the detection wavelength for the HPLC method. A spectrum of pure methanol was also included as a blank to ensure that no interferences from the diluent were present. The electronic absorption spectrum of emodin was measured in methanol at room temperature in the wavelength region 200 to 700 nm. Five prominent bands were identified with maxima at 221, 252, 265, 288 and 435 nm. The spectrum matched the spectrum reported by Marković et al.²⁸. The broad band at 435 nm was reported to have a dominant charge transfer character in the same research study.

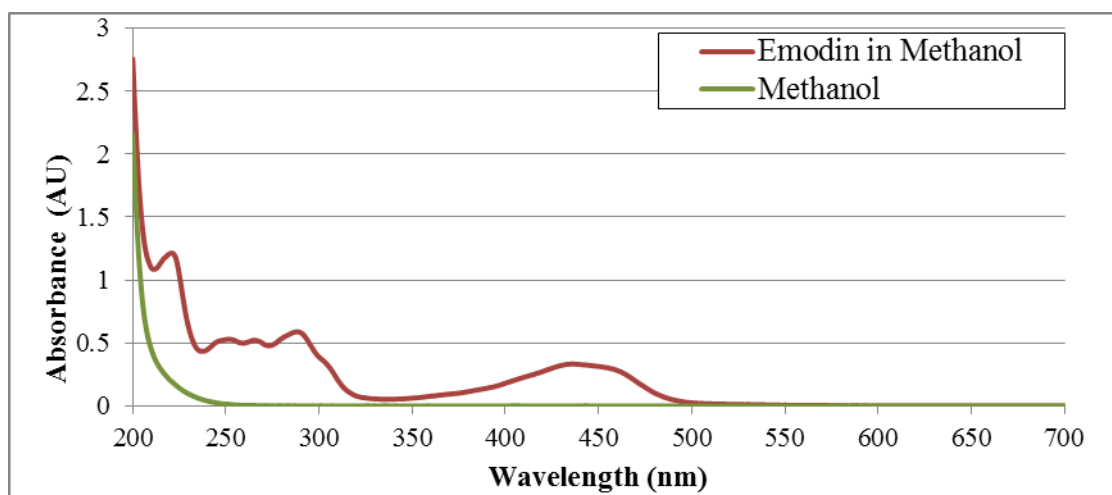


Figure 2. UV-VIS spectrum of the emodin working standard in methanol using the Shimadzu UV-Vis spectrophotometer

Method Development and Optimisation

The optimal chromatographic conditions for the HPLC assay method for emodin are listed in Table.

2, Full and Expanded integrated chromatograms are displayed in Fig. 3 (A) and 3 (B).

Table 2. HPLC method parameters for the assay determination of emodin (System 9).

Column	Waters Symmetry C18 250 x 4.6 mm, 5 μ m
Mobile Phase	Mobile Phase A: 0.01% (v/v) TFA 0.1% (v/v) formic acid Mobile Phase B: 100% methanol HPLC grade
Isocratic Profile	10% MP A : 90% MP B
Flow rate	1.00 mL/min
Run Time	15 minutes
Column Temperature	35 $^{\circ}$ C
Sample Temperature	4 $^{\circ}$ C
Injection volume	10 μ L
UV detection wavelength	287 nm, 436 nm and PDA Analysis (210-500 nm) Resolution: 1.2 nm
Diluent	100% HPLC grade methanol
Solution Filters	0.45 μ m Pall GHP filters of code PN-4562
Test concentration	0.5 mg/mL

On injecting the working standard, four peaks were eluted, one of which was more significant than the other three (Fig. 3). An injection of the reference standard of emodin indicated that the emodin peak was expected to elute at approximately 5.9 minutes. The peaks were labelled emodin, Unknown 1, 2 and 3, all of which had excellent USP Tailing and USP plate count. The total run time of the system was 15 minutes which was considerably short. The chromatograms at 287 nm and 436 nm were visually identical so only chromatograms for the 436 nm wavelength are reported in this paper.

The method was run at dual wavelengths, 287 nm and 436 nm, as they provided two distinctive insights into the performance of the analytical method. Investigation at 287 nm, albeit having slight interferences from the mobile phase additives which

may reduce the robustness of the method, offers a broader view of the components in the sample since most samples absorb at low wavelengths. On the other hand, selecting a longer wavelength is more selective towards the drug substance and picks up less system peaks and degradation products. The result of both wavelengths was reported to relate both perspectives (Table. 3).

The peaks were all baseline resolved from each other with a resolution larger than 3.0 for all the four peaks. Nevertheless peak purity testing was performed using the Waters Empower software to ensure that the peaks were also spectrally pure. A max plot for a standard injection analysed using system 9 (Tables. 1 and 2) was generated and the results are displayed in Table. 4. The results indicated the emodin peak was spectrally pure, with

purity angle values lower than the purity threshold. Thus, the method was specific for emodin as per ICH criteria for specificity, namely, “the ability of the method to unequivocally assess the analyte in the presence of other components that may be expected to be present for example, impurities, degradation products and matrix components”. The first two peaks in the chromatogram (Fig. 3) were spectrally impure (Table. 4) and were hence referred to as

Unknown 1 and Unknown 2. The second major peak in the system, at retention time 10.222 minutes (Table 4) was labelled Unknown 3, and with purity angle values below the threshold, was also found to be spectrally pure. All three unknown peaks were most likely impurities or matrix components. The next section describes tests that were performed in an attempt to identify the unknown peaks.

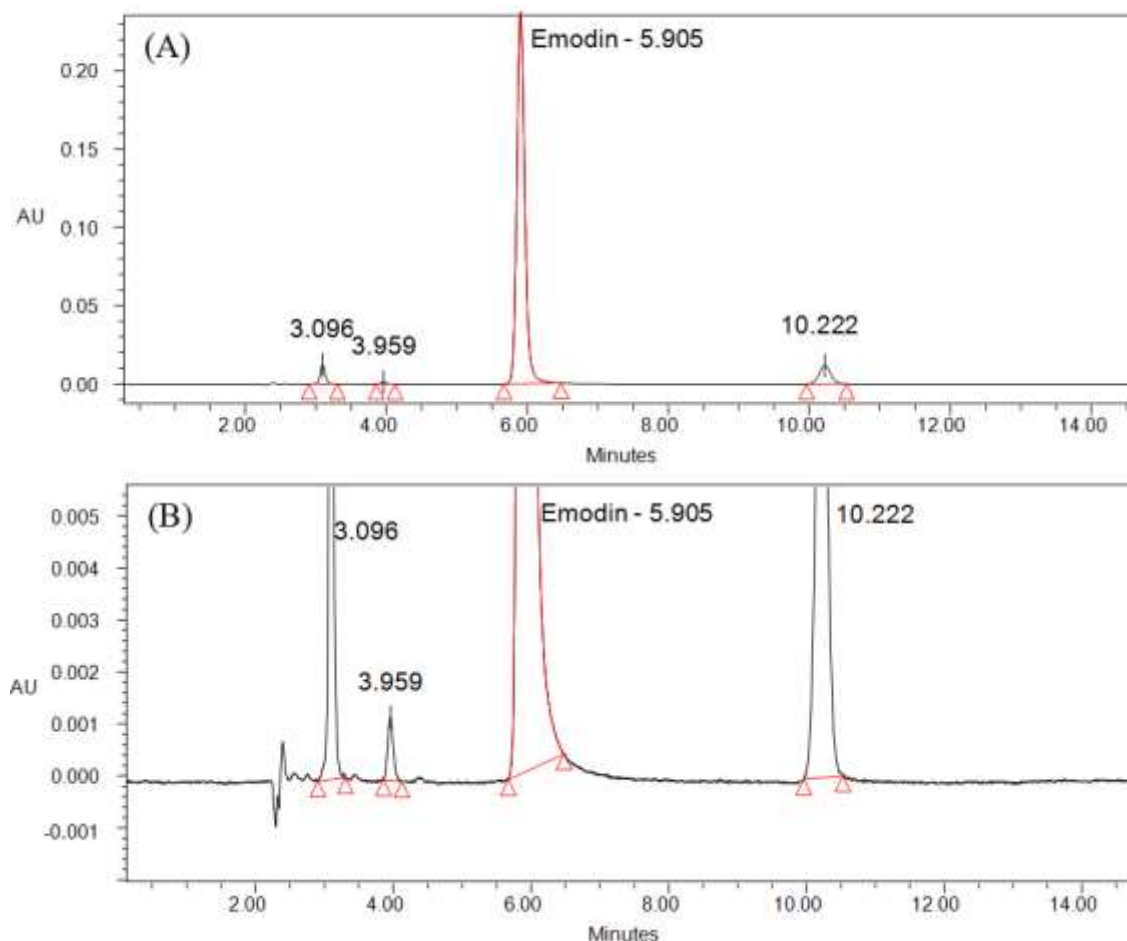


Figure 3. Full (A) and expanded (B) chromatograms obtained using chromatographic System 9 at 436 nm; Injection peak at 2.361 minutes.

Table 3. Chromatographic profile obtained for the optimised HPLC assay method for emodin at 254 nm and 436 nm.

At 287nm							
Peak number	Retention time (mins)	Peak name	Area ($\mu\text{v}\cdot\text{sec}$)	% Area	USP count	Plate	USP tailing
1	3.096	Unknown 1	101984	2.77	10111		1.160
2	3.959	Unknown 2	10295	0.28	10752		1.150
3	5.905	Emodin	17366864	91.36	15941		1.170
4	10.222	Unknown 3	205965	5.59	18908		1.021
At 436nm							
Peak number	Retention time (mins)	Peak name	Area ($\mu\text{v}\cdot\text{sec}$)	% Area	USP count	Plate	USP tailing
1	3.096	Unknown 1	59603	2.89	10115		1.150
2	3.959	Unknown 2	6959	0.34	11719		1.203
3	5.905	Emodin	1863743	90.34	15958		1.168
4	10.222	Unknown 3	132767	6.44	18937		1.020

Table 4. PDA Peak Purity Output obtained using chromatographic System 9

Peak number	Retention time (min)	Peak name	Purity angle	Purity threshold	Spectral status
1	3.096	Unknown 1	2.193	0.469	Impure
2	3.959	Unknown 2	15.182	2.417	Impure
3	5.905	Emodin	0.217	0.261	Pure
4	10.222	Unknown 3	0.225	0.402	Pure

Identification of impurities

Identification of the unknown peaks that had eluted in the optimised system was attempted. The UV absorption spectra for the four individual peaks (Fig. 4) were extracted using the Empower PDA software.

On comparing the four absorption spectra, several similarities were observed. The peaks and troughs of the spectra, as well as the λ_{\max} values, were similar to each other suggesting that all three unknown peaks were anthraquinones, albeit with different side groups.

A common observation made from most systems that were investigated is that one of the unknown peaks that eluted after emodin was always present in the approximate range of 6 to 7.5 % area of the total peak area. This was a good indication that the peak in these systems represented the same impurity or degradation product. Since this component was the second most abundant component in the sample it was important to attempt to identify its structure.

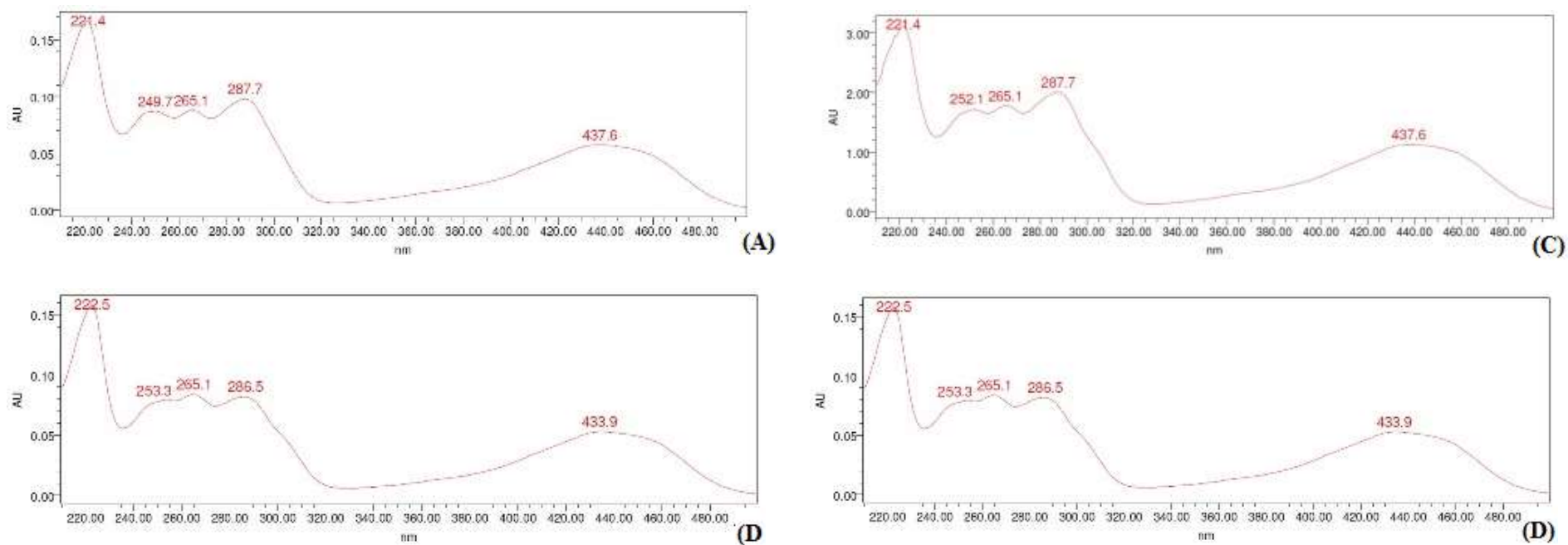


Figure 4. Absorption spectra generated using PDA software of (A) peak at retention time 3.097 minutes (Unknown 1); (B) peak at retention time 3.960 minutes (Unknown 2); (C) emodin peak at retention time 5.905 minutes; (D) peak at retention time 10.222 minutes (Unknown 3)

The ^1H NMR spectrum of the emodin working standard was acquired on a Bruker AM250NMR spectrophotometer. Analysis was carried out at room temperature using 5 mg of emodin in approximately 0.8 mL of deuterated dimethyl sulfoxide solvent. The result obtained is documented below:

^1H NMR (DMSO, 250.1MHz) δ : 12.12 (1H, s, -OH), 12.07 (1H, s, -OH), 12.04 (1H, s, -OH), 7.47 (1H, s, H-4), 7.16 (1H, s, H-2), 7.10 (1H, d, H-5), 6.57 (1H, d, H-7), 3.93 (3H, s, -OCH₃), 2.41 (3H, s, CH₃)

The NMR spectrum of emodin working standard matched the data reported by Chu et al.²⁹ and Guo et al.³⁰ with the exception of a signal at 3.93 ppm. The 3.93 ppm signal is typical of the three hydrogens of the methoxy group. The integration ratio of the two signals at 3.93 and 2.41 ppm was approximately 1 : 17.45, which corresponded to 5.73 % and 94.27 % respectively. This result coincided with the results extracted from the HPLC chromatogram, where the peak labelled as Unknown 3, which eluted at a retention time of approximately 12.222 minutes, was also present in approximately the same amount. Hence Unknown 3 peak was most likely an anthraquinone containing a methoxy side group and it was present in approximately 6% of the total mass of the emodin working standard. NMR results also confirmed that the impurity peaks were not formed *in situ* on dissolving the standard in methanol.

Physcion (Fig. 5), which is an anthraquinone with a methoxy group, might be the impurity present in the working standard at a 6% percentage ratio.

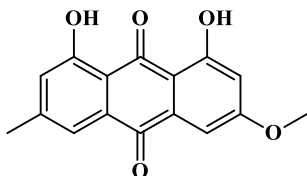


Figure 5. Structure of Physcion

On cross-referencing the UV spectrum of the peak (Fig. 4D), with a documented spectrum of physcion, there seemed to be a positive match. Marković et al.²⁸, reported the UV spectrum of physcion in methanol with the following λ_{max} : 225, 253, 265, 286 and 433 nm. Therefore this information was all pointing to the fact that the emodin working standard contained approximately 6% of physcion. The peaks referred to as Unknown 1 and 2 were present in too small a concentration to be picked up by NMR. Moreover it had already been established by the peak purity analysis that the peaks were not spectrally pure and hence could be a combination of a number of smaller peaks. It is most likely that these are process impurities that could not be separated during the extraction process.

Validation

The purpose of the purity assay HPLC method was to identify and quantify emodin in the presence of other impurities. In this regard, only the emodin peak was considered when evaluating validation parameters. The validation was conducted in accordance with ICH guideline Q2(R1) entitled 'Validation of analytical procedures'¹⁹. Six replicates were injected to the HPLC system. Validation of the HPLC assay method was performed at 287 nm and 436 nm for the emodin test concentration of 0.5 mg/mL. The proposed method was validated for specificity, linearity, range, accuracy, precision and solution stability.

- System Suitability

System suitability parameters were evaluated by injecting a standard solution of emodin of concentration 0.5 mg/mL in methanol for six consecutive times. The acceptance criteria were less than 2% relative standard deviation (RSD) for peak areas and peak retention times of the emodin peak, USP theoretical plates greater than 3000 and USP tailing factor smaller than 1.5.

- Specificity

Specificity was demonstrated by performing PDA peak purity analysis on the emodin peak using the Empower 2.0 software. The emodin reference and working standards were injected following a system suitability test, several blank injections and a bracketing standard at the end. Sections of the chromatograms that contained no noise were marked as 'baseline' noise to enable the software to measure the spectral baseline. Subsequently the software measured peak purity angles and threshold values. In cases where the peak purity angles were smaller than their respective threshold values, the peaks were considered to be spectrally pure. Spectral purity was confirmed for emodin and physcion but not for the first two unknown peaks. Nevertheless specificity was achieved.

- Linearity

The concentration range of the analytical procedure was identified through a linearity study. Linearity was evaluated for the emodin peak across a range of ten solutions of concentration ranging from 25 to 150% of the test concentration, namely 0.125 to 0.750 mg/mL. Each solution was prepared by dilution from a stock standard solution of concentration 1.25 mg/mL. Triplicate injections of each linearity solution were injected after the system suitability injections with adequate bracketing. The results were analysed at 287 and 436 nm. The results of the statistical tests performed for the linearity determination are illustrated in Table. 5. Linearity was confirmed at both wavelengths; between 25 and 125 % of the test concentration at 287nm and 25 to 150% of the test concentration at 436 nm.

Table 5. Linearity results and ICH compliant acceptance criteria.

Parameter	Results at 287 nm	Results at 436 nm	ICH compliant acceptance criteria
Number of linearity solutions	10	10	≥ 5
Range of area %RSD of triplicate injections	0.01 – 0.76%	0.03 – 0.79%	< 2.0%
%RSD of response factor	0.97%	0.55%	< 2.0%
Residual sum of squares	23680275298	3101722671	n/a
Range of % response residual	-1.56% to 0.64%	-0.58% to 0.83%	≤ 2.0%
Residual standard deviation	62822.9	19690.5	n/a
Relative residual standard deviation	0.54%	0.25%	≤ 2.0%
Slope ($\mu\text{v}\cdot\text{sec}\cdot\text{mg}^{-1}\cdot\text{mL}$)	30344150	17117041	n/a
y Intercept ($\mu\text{v}\cdot\text{sec}$)	170797	39451	n/a
% y Intercept relative to 0.5 mg/mL	1.11%	0.46%	≤ 2.0%
r^2	0.999892	0.999975	$r \geq 0.998$
Verified range of analytical method	25-125%	25-150%	80 – 120%

The limit of quantitation (LOQ) and limit of detection (LOD) concentration levels were not established since they are not required for assay methods as per the ICH Q2(R1) guideline¹⁹.

- Accuracy

The accuracy between the emodin reference standard, representing the accepted reference value, and the emodin working standard was measured across the range of the analytical procedure as determined through the linearity study. Accuracy was assessed using nine determinations over three concentration levels covering the specified range. The reference standard was prepared at 100% of the test concentration and the working standard was prepared in triplicate at 80, 100 and 120% of the test concentration, corresponding to 0.4 mg/mL, 0.5

mg/mL and 0.6 mg/mL respectively. The reference standard and the nine working standard solutions were injected in triplicate following the system suitability and were adequately bracketed by standard. Accuracy was reported as percentage recovery. A summary of the results is illustrated in Table. 6. At the two wavelengths the minimum and maximum recovery values were within the recommended 98.0 to 102.0% range. The calculated %RSD of the overall percent recovery also satisfied the stated criteria, which is less than 2.0%

Table 6. Results of the accuracy validation study for the emodin peak.

287 nm			436 nm		
Concentration of Standard (mg/mL)	Average Recovery of n=3 replicates (%)	Average RSD of n=3 replicates (%)	Concentration of Standard (mg/mL)	Average Recovery of n=3 replicates (%)	Average RSD of n=3 replicates (%)
0.406	99.38	0.80	0.407	98.90	0.81
0.509	100.10	0.60	0.509	99.94	0.60
0.612	98.48	0.37	0.612	98.77	0.38
Overall average	99.32		Overall average	99.20	
Overall RSD (%)	0.82		Overall RSD (%)	0.65	

- Precision

Repeatability

Both the system precision and the overall repeatability were examined. System precision was measured by injecting one of the repeatability standards for six times consecutively. The mean peak area, retention time, theoretical plates or plate count and tailing for the emodin peaks and their respective

% RSD were measured. System precision was confirmed as at both wavelengths, the percentage relative standard deviations were less than 2.0% for all the parameters that were investigated.

To assess the overall repeatability, six standard preparations at 100% of the test concentration were prepared and analysed on the same chromatographic

system. The six standard preparations were injected in triplicate following system suitability and adequate bracketing by a standard. The percentage recovery for each sample was calculated. The results of the six standards prepared at 100% of the test concentration are displayed in Table. 7. The % RSD at both wavelengths were below 2.0% indicating that

the technique applied for sample preparation entailed a very high degree of repeatability for the quantification of emodin. Moreover, an adequate recovery of the drug was observed with a 95% confidence interval (C.I.) that included the theoretical 100% recovery.

Table 7. Overall repeatability results for the emodin peak at 287 and 436 nm.

Solution	287 nm	436 nm
	Mean % Recovery of 3 injections	Mean % Recovery of 3 injections
Repeatability standard A	100.32	100.14
Repeatability standard B	100.56	100.42
Repeatability standard C	99.42	99.27
Repeatability standard D	99.90	99.76
Repeatability standard E	100.70	100.50
Repeatability standard F	99.89	99.73
Mean	100.13	99.97
%RSD	0.48	0.47
95% C.I.	99.62 to 100.64%	99.48 to 100.46%

Intermediate precision

The intermediate precision was tested by running six freshly prepared standard replicates of the 100% test concentration on a different chromatographic system and on a different day (Day 2), and comparing them to the repeatability standards (Day 1). Each standard was injected in triplicate following a system suitability and adequately bracketed by a

standard. The corresponding percentage recoveries were measured and the mean percentage recovery of the three replicate injections was measured. The % RSD values that were obtained for the individual data sets and for the pooled data set are shown in Tables. 8 and 9. The individual and overall %RSD values were less than 2.0% for both wavelengths indicating that the method showed good precision.

Table 8. Intermediate precision for the emodin peak at 287nm

DAY 1		DAY 2	
Solution	Mean % Recovery of 3 replicate injections	Solution	Mean % Recovery of 3 replicate injections
Repeatability standard A	100.32	Int. Precision Standard A	99.51
Repeatability standard B	100.56	Int. Precision Standard B	100.15
Repeatability standard C	99.42	Int. Precision Standard C	100.73
Repeatability standard D	99.90	Int. Precision Standard D	100.11
Repeatability standard E	100.70	Int. Precision Standard E	99.19
Repeatability standard F	99.89	Int. Precision Standard F	99.20
Mean	100.13	Mean	99.82
%RSD	0.48	%RSD	0.62
Variance	0.23	Variance	0.38
95% C.I.	99.63 to 100.64%	95% C.I.	99.17 to 100.46%
<i>Calculations on pooled data sets (Day 1 and 2):</i>			
Overall Mean		99.97	
Overall %RSD		0.59	
Overall variance		0.34	
Overall Confidence Interval at 95%		99.78 to 100.17%	

Table 9. Intermediate precision for the emodin peak at 436nm.

DAY 1		DAY 2	
Solution	Mean % Recovery of 3 replicate injections	Solution	Mean % Recovery of 3 replicate injections
Repeatability standard A	100.14	Int. Precision standard A	99.45
Repeatability standard B	100.42	Int. Precision standard B	100.04
Repeatability standard C	99.27	Int. Precision standard C	100.61
Repeatability standard D	99.76	Int. Precision standard D	100.10
Repeatability standard E	100.50	Int. Precision standard E	99.10
Repeatability standard F	99.73	Int. Precision standard F	99.08
Mean	99.97	Mean	99.73
%RSD	0.47	%RSD	0.62
Variance	0.22	Variance	0.38
95% C.I.	99.48 to 100.46%	95% C.I.	99.08 to 100.38%
<i>Calculations on pooled data sets (Day 1 and 2):</i>			
Overall Mean		99.85	
Overall %RSD		0.57	
Overall variance		0.33	
95% Overall Confidence Interval		99.66 to 100.04%	

Range

The minimum specified range by ICH guideline Q2(R1) of 80 to 120% of the emodin test concentration was demonstrated from the accuracy, linearity and precision data. The range of the assay method at 287 nm was demonstrated to be from 25 to 125% of the test concentration. The range of the assay method at 436 nm was found to be from 25 to 150% of the test concentration.

Stability of solution

Stability of solution was assessed for both the reference and working standards over a period of 24 hours. Storage temperature and light were examined for the purpose of this study. Stability was determined by comparing the response of the aged standards to the response obtained for the initial injection.

Part 1: Both standard and reference solutions were injected at the following time points: 0, 2, 4, 6, 8, 10, 12, and 24 hours. Duplicate injections were taken for each time point. The sequence was started off with a system suitability and was adequately bracketed. Both solutions showed no significant change in the total analyte composition, emodin retention time or emodin peak area. The % RSD measured for the peak areas of emodin for both standards at 287 and 436 nm were all below 2%.

Part 2: Aliquots of the standard and reference solutions were taken and stored for 24 hours in both clear and amber vials, both in a fridge (4-8 °C) and at room temperature (25 °C). At both wavelengths, the percentage difference in the peak area of emodin was lower than 2.0% for all the factors that were investigated. Emodin was shown to be insensitive to

light and stable at a temperature range of 4°C to 25 °C.

Comparison of methods

Only a few chromatographic methods have been developed for the exclusive determination of emodin in natural products. One such study was a HPLC method coupled to a diode array detector (DAD) to identify emodin in Tartary Buckwheat¹⁷. The method was optimized at a single detection wavelength of 436 nm, and the emodin peak eluted at approximately 11.7 minutes. It also incorporated phosphoric acid in the mobile phase which limits the applicability of the method as phosphoric acid is incompatible with a number of detectors, namely MS. A second study determined emodin from *Polygonum cuspidatum* by HPLC-DAD¹⁸. This method incorporated a gradient elution with a run time of 25 minutes.

The method developed in this study is more rapid than the previously reported studies as the emodin peak eluted at approximately 6 minutes with a total run time of 15 minutes. This is extremely important as it utilizes less solvent and is therefore both time and cost effective. It incorporates TFA and formic acid in its mobile phase which allows use and interfacing with a large number of detectors. The method has been validated at dual wavelengths which enables the detection of emodin related substances. It is also linear over a concentration range of 0.125 to 0.625 mg/mL, as well as highly accurate and precise. This method can also be applied in the determination and quantification of physcion as peak purity analysis showed that the physcion peak was spectrally pure.

Conclusion

A simple, rapid, accurate and precise assay method for the HPLC analysis of emodin has been developed and validated in accordance to ICH criteria. All the validation criteria have been satisfied at 287 nm and 436 nm. The method was also developed to be specific to emodin in the presence of related substances including process impurities such as physcion.

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