RESEARCH ARTICLE

Development and Validation of an HPLC Method for the Quantification of Morin Flavonoid Encapsulated within PLGA Nanoparticles

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Abstract: ***Background***: Morin flavonoid exerts neuroprotective effects with potential interest in neurodegenerative disorders. For this, the use of surface-modified polymeric nanoparticles loaded with morin is an interesting approach.

Objective: To develop and validate an HPLC method for the quantification of morin released from a new delivery system consisting of poly lactic-co-glycolic (PLGA) nanoparticles functionalized with the dipeptide phe-phe (phenylalanine-phenylalanine) used to facilitate their access to the CNS.

Method: The HPLC procedure was developed and validated with morin hydrate dissolved either in methanol (method A) or in methanol: 0.1N HCl (50:50, v/v, method B). Two new nanoparticle formulations were developed and characterized: morin-loaded PLGA nanoparticles (formulation F1), and morin-loaded PLGA phe-phe nanoparticles (formulation F2).

Results: Method A was linear within the concentration range of 5-30 µg mL^{-1} and, 1-30 µg mL^{-1} for method B. LOD and LOQ with method A were 1.23 µg.mL^{-1} and 3.90 µg mL^{-1}, respectively, and 0.481 µg.mL^{-1} and 1.458 µg mL^{-1}, respectively for method B. The average amount of phe-phe bound to formulation F2 (50 mg of NPs) was 431.33 µg. The encapsulation efficiency of morin within PLGA nanoparticles was around 80%. After functionalization, this value decreased significantly.

Conclusion: Method B showed better sensitivity, accuracy, and precision for the quantification of morin. The procedure used to functionalize the nanoparticles was adequate for linking the dipeptide to their surfaces, but this procedure is not adequate when encapsulating water-soluble compounds.

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1. INTRODUCTION

Morin (3,5,7,2',4'pentahydrxyflavone) belongs to the flavonoid group of phytochemicals. Several studies postulate the potential of flavonoids in the treatment of neurological disorders such as Alzheimer´s or Parkinson´s diseases as a consequence of their neuroprotective effects [1-3]. Abnormal accumulation of proteins is a primary characteristic of these diseases [4] as well as oxidative stress and neuroinflammation [5]. In this regard, flavonoids have proven to be able to reduce the formation of reactive oxygen species (ROS) and decrease pro-inflammatory factors such as TNF-α [6]. Moreover, among flavonoids, morin can inhibit the glycogen synthase kinase 3-beta enzyme (GSK3β) involved in the hyperphosphorylation of tau protein in Alzheimer´s disease [1].

However, due to the lack of information regarding the ability of flavonoids to cross the blood-brain barrier (BBB), it is difficult to fully understand the mechanisms involved in their neuroprotective effects. There are very few studies in which the passage of flavonoids through the BBB has been addressed [7, 8], and in which only very small amounts of the compound were able to reach the brain [9]. To overcome this drawback and increase their passage through the BBB, the use of nanoparticles loaded with active ingredients, such as morin flavonoid, is a potentially valuable approach in the

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treatment of neurodegenerative disorders [10-12]. Poly lactic-co-glycolic acid (PLGA) is a biodegradable and biocompatible polymer, approved by the FDA and widely used for the preparation of both micro- and nano-particulate devices as delivery systems for drugs, proteins, and other macromolecules such as RNA, DNA or peptides [13-15]. Moreover, to improve the passage of nanoparticles across the BBB, different ligands such as transferrin, glucose or insulin can be attached to their surfaces [16-19]. Among them, phenylalanine (phe), an essential amino acid, exhibits a higher affinity to cerebral tissue than to other tissues [20, 21]. For instance, various nanosystems, including this amino acid as a surface modifier, have been developed when targeting the LAT1 receptor which is expressed in capillary endothelial cells forming the BBB [22-24].

In this regard, we have developed a new formulation consisting of morin hydrate-loaded PLGA nanoparticles (NPs) without and with functionalization of their surfaces with phe-phe dipeptide, in order to overcome their passage across the BBB and deliver the neuroprotective agent into the CNS.

However, for the detection and quantification of flavonoids, specifically morin, a few analytical methods have been reported. The HPLC method employed by Ikeuchi-Takahashi *et al.* [25] uses as mobile phase acetonitrile: 0.2% ortho-phosphoric acid in water (27:73, v/v) with detection wavelength set at 250 nm but with no indication of the calibration curve range used. In the method described by El-Haddad *et al.* [26], the concentration range used to prepare the calibration curve is 50-75 µg mL^{-1}, which is not suitable to detect low concentrations. Taking into consideration that detection of morin shows both low sensitivity and selectivity, none of the previous methods would be adequate for the quantification of the compound when encapsulated within polymeric nanoparticles.

For this, we have developed a new HPLC method suitable for the quantification of morin either in the form of hydrate (method A) or hydrochloride (method B). In method A, morin hydrate is dissolved in methanol, whereas in method B, the flavonoid is initially dissolved in a mixture of methanol:HCl. In both cases, the method is validated and applied to the quantification of morin flavonoid encapsulated within the PLGA nanoparticle formulations developed in this work.

2. MATERIALS AND METHODS

2.1. Chemicals, Reagents, Materials, and Apparatus

Morin hydrate was purchased from Sigma Aldrich (Madrid, Spain). PLGA (Resomer RG®502) with a ratio of 50:50 was purchased from Evonik (Essen, Germany). The dipeptide phenylalanine-phenylalanine (phe-phe) was obtained from Sigma-Aldrich (Madrid, Spain). Polyvinyl alcohol (PVA) Mw 72,000 Da was obtained from Merck (Madrid, Spain). HPLC grade acetonitrile, methanol, and other solvents were all of the analytical grade and purchased from Panreac (Madrid, Spain). Water was purified by the Milli-Q filtration system (Millipore, Madrid, Spain).

An HPLC chromatograph Jasco (Jasco International Co Ltd, Tokyo, Japan) equipped with an LG-2080-04 quaternary low-gradient unit, a PU-2080 pump, a DG-2080- 54 Degasser, an AS-2050-plus autosampler and a UV-2070 plus UV/Vis detector was used. The fluorescence detector was a Jasco FP-4025 (Jasco International Co Ltd, Tokyo, Japan) coupled to the HPLC system. The chromatographic column used was a Gemini® 5 µm NX-C18, 250 mmx4.6 mm (Phenomenex, Madrid, Spain)

2.2. Stability of Morin Hydrate

To establish the analytical conditions for the development of a simple and rapid HPLC method for the detection and quantification of morin either in the form of hydrate or hydrochloride, and encapsulated within PLGA nanoparticles, stability studies of morin in solution were initially performed to identify the analyte in the presence of components which could be present in the sample (impurities, degradation products). The procedure used involved the search for possible degradation products by means of stress stability studies. For this, stability of morin hydrate in methanol solution at a concentration of 30 µg mL^{-1} was analysed under the following stress conditions: 0.1N HCl at 60ºC for 24 h; 1N NaOH at 105ºC for 20 min; and oxidative conditions (3% H_2O_2) for 24 h. Moreover, the stability of morin hydrate in solution was tested after storage at room temperature (25ºC) and at 4ºC for 24 h [Table **1**]. All samples were prepared in triplicate.

Table 1. Stability results.

Conditions	Morin Recovery ± SD (%)
25ºC, 24 h	103.42±0.57
4ºC, 24 h	106.19±8.03
0.1N HCl, 60ºC, 24 h	214.05±8.14
1N NaOH, 105ºC, 20 min	2.85±0.86
3% H_2O_2, 24 h	73.77±7.29

2.3. Sample Preparation

Standard solutions of morin hydrate were prepared either in methanol (method (A) or in a mixture of methanol and 0.1 N HCl (method B). In method A, morin hydrate was dissolved in methanol at a concentration of 100 µg mL^{-1} and from this, the calibration curve was prepared. Method B was simultaneously developed to improve the sensitivity of the detection of the flavonoid. In this case, morin hydrate was dissolved in methanol: 0.1N HCl (50:50 v/v), to form the hydrochloride, at a concentration of 100 µg mL^{-1} under magnetic stirring for 2 h. Then, this solution was diluted with methanol to construct the calibration curve.

2.4. Chromatographic Conditions

For the selection of the most adequate chromatographic conditions, the following parameters were tested: the composition and pH of the mobile phase, flow rate, and effect of the column temperature [Table **2**].

Table 2. Chromatographic conditions assayed. Tr: retention time.

Parameter	Value	Recovery (%)	Tr (min)
pH	2.3	109.24	8.02
	2.5	100.56	6.92
	2.6	95.54	7.11
Flow rate (mL min^{-1})	1.2	102.56	8.14
	1.4	100.56	6.92
Mobile phase (% acetonitrile)	20	91.46	18.66
	27	100.56	6.92
	30	115.85	6.10
Column temperature (±0.5ºC)	30	90.77	7.29
	40	99.66	6.92

2.5. Method Validation

Method validation was carried out according to the International Conference on Harmonization (ICH) guidelines [27] with the preparation of morin samples being performed either in methanol (method A) or in methanol: 0.1N HCl (50:50, v/v, method B). In both cases, validation for linearity, precision, accuracy, specificity, detection limit (LOD) and quantification limit (LOQ) was carried out.

2.5.1. Linearity

Calibration curves were prepared for morin hydrate at concentrations of 5, 10, 15, 20, and 30 µg mL^{-1} for method A, and 1, 5, 10, 15, 20 and 30 µg mL^{-1} for method B. Each solution was prepared in triplicate and injected three times. Linearity was tested by linear regression using ANOVA.

2.5.2. Precision

Five replicate injections of three different concentrations of morin and corresponding to both, method A and B were used for evaluating the instrumental precision, repeatability (intra-day) and intermediate precision (inter-day), and expressed as percentage relative standard deviation (%RSD). Instrumental precision was evaluated after performing five replicate injections of a sample containing the flavonoid at a concentration of 15 µg mL^{-1}. Repeatability (intra-day) of methods A and B was evaluated by analyzing three different solutions of morin (5, 15 and 30 µg mL^{-1}) on the same day. Each solution was analyzed five times the same day under the same experimental conditions. The intermediate precision (inter-day) was tested by analyzing three independent solutions of the flavonoid (5, 15 and 30 µg mL^{-1}) on three different days, with five runs carried out for each sample.

2.5.3. Accuracy/Recovery

Accuracy was determined based on the recovery (percentage) of a known amount of morin. Different solutions of the flavonoid (5, 15 and 30 µg mL^{-1}) were prepared for method A and B and injected into the chromatographic system. All samples were prepared in triplicate. The accuracy of the method was determined by comparing the concentration found with the actual concentration and expressed in terms of percentage relative standard deviation (% RSD).

2.5.4. Sensitivity

Both the limit of detection (LOD) and the limit of quantification (LOQ) were calculated according to the ICH guidelines [27]. According to these guidelines, LOD and LOQ can be calculated based on the standard deviation of the response (standard error of the intercept) and the slope of the calibration curves. Therefore, for the calculation of LOD and LOQ, the results of the ANOVA obtained from the calibration curves were used. Calculations for LOD were then based on the standard deviation of the calibration curve (σ) and the slope of the curve (S), by means of the equation LOD= 3.3× σ/S. LOQ was calculated from the equation: LOQ=10 x σ/S. Once LOD and LOQ were estimated, samples with similar concentrations to their values were analyzed five times to check the values obtained.

2.6. Preparation of Morin Nanoparticles

2.6.1. Preparation of Morin-Loaded PLGA Nanoparticles

Morin-loaded PLGA nanoparticles (formulation 1, F1) were prepared by the solvent extraction-evaporation method. Briefly; an amount of 5 mg of morin hydrate was dissolved in 100 µL DMSO, and added to 2 mL CH_2Cl_2. Then, 50 mg of PLGA 502 were dissolved in the CH_2Cl_2 solution. This solution was added over 4 mL of 1% PVA and sonicated for 10 min. The organic solvent was removed under magnetic stirring for 4 h. Nanoparticles (NPs) were washed with water and centrifuged (Sorvall ST 8R centrifuge, Thermo Scientific, Waltham, USA) at 13.000 rpm for 30 min. Finally, the NPs were resuspended in 1 mL of 1% sucrose solution and freeze-dried for 24 h. Three batches of formulation F1 were prepared.

2.6.2. Preparation of Morin-Loaded PLGA-phe-phe Nanoparticles

Morin-loaded PLGA-phe-phe nanoparticles (formulation 2, F2) were prepared with the same solvent extraction-evaporation method, but with NPs functionalized with the dipeptide phe-phe. For this, and according to the method described by Constantino *et al.* [28], the union between the carboxylic group of the polymer and the terminal amine group of phe-phe was achieved by activating the carboxylic group of PLGA after the formation of an ester bond with N-hydroxysuccinimide (NHS) in the presence of N'-ethylcarbodiimide hydrochloride (EDC) in acidic medium. Three batches of formulation F2 were prepared.

2.6.3. Quantification of Bound Peptide

Indirect quantification of the amount of phe-phe bound to the NPs was performed. For this, the supernatant containing the unbound peptide was analyzed according to the method described by Neurauter *et al.* [29], with the fluorescence detector set at 210 nm excitation and 302 nm emission. The calibration curve used for this quantification (50, 100, 250, 300, 400 and 500 µg mL^{-1}) was $y = 35447+638.32x$ ($r^2 = 0.9991$).

2.6.4. Quantification of Morin Encapsulated within the Nanoparticles

Nanoparticles (formulation F1) and morin hydrate-loaded PLGA nanoparticles functionalized with phe-phe dipeptide (formulation F2). The dipeptide was used to improve their passage across the BBB and deliver the neuroprotective agent to the CNS.

To quantify morin encapsulated within the nanoparticle formulations developed, an adequate analytical method is required. For this, and in order to establish the best analytical conditions for the HPLC analysis of morin samples, a preliminary study was conducted under stress conditions to detect all possible degradation events occurring when a solution of morin hydrate is dissolved in methanol (concentration 30 µg mL^{-1}). From the results obtained it was found that under oxidative and basic conditions mean recovery of morin was 74±7% and 3±0.8%, respectively (Table **1**). The results obtained in basic conditions were as expected and in agreement with those described by other authors [30], which have demonstrated that morin is very unstable at high pH values, with decomposition occurring by a sequence of multistep reactions under basic conditions. However, under acidic conditions (0.1N HCl at 60ºC for 24 h), morin recovery was higher than expected (214 ± 8%), a result that led us to the development and validation of an analytical method adapted for the quantification of morin hydrate (method A) in which the flavonoid is dissolved in methanol, and another version of the latter, named as method B, in which morin hydrate is firstly dissolved in methanol:0.1N HCl (50:50, v/v), as an analytical approach to increase the sensitivity of morin detection by HPLC.

Both formulations of morin-loaded NPs (F1 and F2) were analysed by means of the HPLC method (method A and B) previously described. Tests were performed in triplicate.

The amount of morin encapsulated within the NPs was determined by weighing an exact amount (10 mg) of NPs which were dissolved in 1 mL CH_2Cl_2. PLGA was precipitated with methanol (4 mL) and separated by centrifugation at 13,000 rpm for 5 min. Then, 1 mL of the methanol solution was drawn from each sample and used for the HPLC analysis. Finally, the morin content of each formulation was quantified using methods A and B.

Encapsulation efficacy (EE%) of the flavonoid within formulations F1 and F2 was calculated as the ratio between the amount of morin content in the NPs and the amount of drug used for their preparation and expressed as percentage.

3. RESULTS AND DISCUSSION

Morin, a phytochemical flavonoid, exerts neuroprotective effects with potential interest when treating neurodegenerative disorders such as Alzheimer´s or Parkinson´s diseases.

For this, we have developed a new drug delivery system consisting of morin hydrate-loaded PLGA Chromatograms obtained after storage of morin solutions at 25ºC and 4ºC for 24 h (chromatographs not shown) were similar to those obtained right after preparation of a morin solution of the same concentration (30 µg mL-1) by methods A and B (Fig. **1**).

Under these conditions (25ºC for 24 h and 4ºC for and B, respectively. By means of Students t-test the 24 h), mean morin recovery was found to be 103±0.6% and 106±8%, respectively (Table **1**).

For the selection of the best chromatographic conditions, the following parameters were analyzed: composition and pH of the mobile phase, flow rate, and column temperature (Table **2**). In method A, when the effect of the pH was studied within the range of 2.3 and 2.6, the highest value resulted in a chromatographic peak with a hump, whereas the lowest pH value tested resulted in smaller and less symmetrical peaks (chromatographs not shown). From our results, the pH value selected was 2.5, for which a well-defined and symmetrical peak was obtained. The pH value was a key factor to obtain better peak resolution.

The effect of the mobile phase composition (acetonitrile: water) was also analyzed. With the lower proportion of acetonitrile assayed (20%), the peak of morin eluted at 18.66 min, whereas when acetonitrile was incorporated at 30%, the retention time was close to the solvent elution (chromatographs not shown). Finally, the composition selected was 27% acetonitrile:73% water, with the pH of water adjusted at pH 2.5±0.05 with phosphoric acid. With this, a retention time of 6.92 min was obtained for morin (Table **2**).

When two different flow rates of the mobile phase were analyzed (1.2 and 1.4 mL min^{-1}), the best resolution was achieved with a flow rate of 1.4 mL min^{-1}, resulting in retention time for the flavonoid of 6.92 min (Table **2**). The same retention time resulted when keeping the column temperature at 40ºC.

From the results obtained the final chromatographic conditions selected for methods A and B were as follows: the mobile phase was composed of acetonitrile:water (27:73, v/v) with the pH of water adjusted to 2.5±0.05 with phosphoric acid. Flow rate was 1.4 mL min^{-1} and the injection volume 50 µL. The mobile phase was vacuum- filtered through 0.45 µm nylon membranes (Millipore, Madrid, Spain) and degassed by ultrasonication for 20 min before use. The chromatographic column used was Gemini®

5 µm NX-C18, 250 mmx4.6 mm (Phenomenex, Madrid, Spain). The temperature of the column was fixed at 40ºC. The detection wavelength was set 256 nm and the total run time was 20 min.

Under these analytical conditions, a retention time of 6.79 min, a symmetry factor of 0.77 and a value of 2936 for the theoretical plates were obtained by method A [Table **3**].

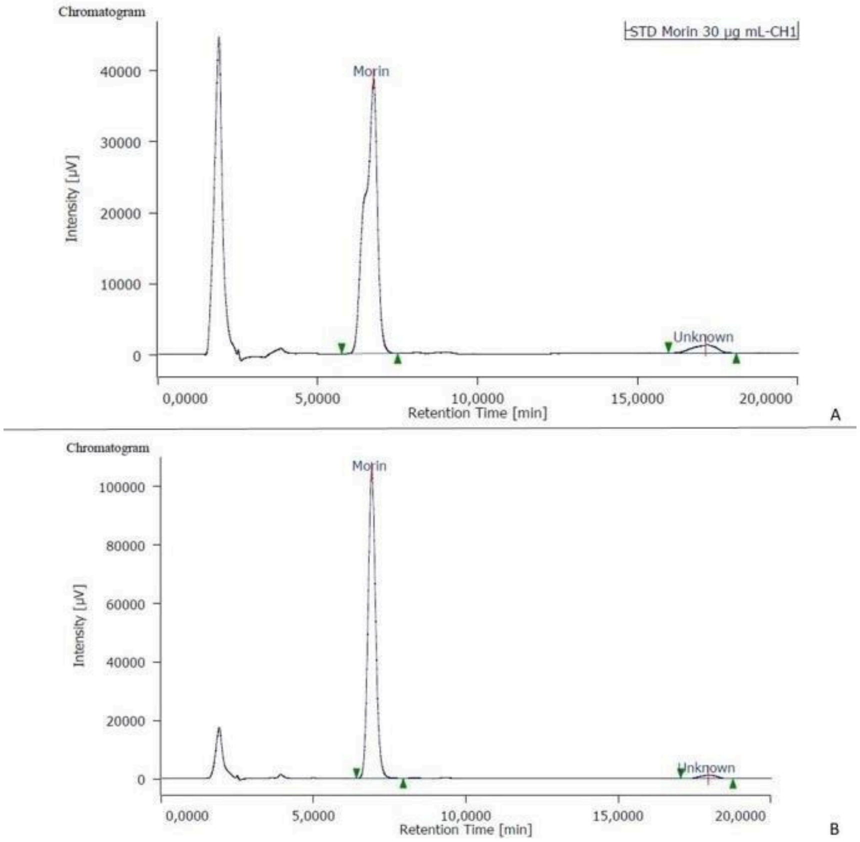

Fig. (1). Typical chromatograms corresponding to a standard solution of morin (30 µg mL^{-1}) and analyzed by methods A and B.

Table 3. Linearity results.

	Method A	Method B
Range (µl mL^{-1})	5-30	1-30
Slope	28026.95	61986.31
Standard error of the slope	602.689	544.691
Intercept	9515.80	-4625.74
Standard error of the intercept	10948.40	9035.41
Correlation coefficient (r)	0.9970	0.9993
Determination coefficient (r^2)	0.9940	0.9987
F-ANOVA test regression (p- value)	2162.54 (0.0000)	12950.63 (0.0000)
t-Stadistic intercept (p-value)	0.8691 (0.4005)	-0.5119 (0.6157)
t-Stadistic slope (p-value)	46.5031 (0.0000)	113.8008 (0.0000)
R (R>2)	7.71	13.91
Theorical plates (N>2,000)	2936	4032
Tailing factor (T<2)	0.77	1.09
Retention time (min)	6.79	6.92

When morin was prepared in methanol:0,1N HCl (50:50, v/v), which corresponds to method B, the same chromatographic conditions were analysed with similar results obtained. In this case, the peak of morin eluted at 6.92 min, with a symmetry factor of 1.09 and 4000 theoretical plates [Table **3**].

Once established the chromatographic conditions, the linearity of method A and B was checked within concentration ranges of 5-30 µg mL^{-1} for method A and 1-30 µg mL^{-1} for method B. Both standard calibration curves were linear over the concentration ranges studied (Table **3**) with correlation coefficients of 0.9970 and 0.9993 for method A proportionality ($p > 0.005$) and regression ($p < 0.005$) of the method A and B was determined. Therefore, according to statistical analysis by ANOVA, the curves were linear with $p<0.005$ however, the lower concentration range found linear in method B suits better our purposes of detecting and quantifying morin encapsulated within nanoparticulate delivery systems.

Measurement of intra-day and inter-day precision was expressed as a percentage relative standard deviation (% RSD). The results obtained are depicted in Table **4**. With method A, inter-day and intra-day precision ranged between 5.50-6.80% and 2.10-6.20%, respectively, whereas with method B, RSD values for inter-day and intra-day precision ranged between 2.20-3.40 and 0.90-3.50%. In both cases adequate values were obtained however, method B exhibited better performance in terms of precision.

Table 4. Precision results obtained for method A and B. RSD (relative standard deviation), RE (relative error).

Morin Concentration (μg.mL^{-1})	Intra-day			Inter-day		
	Mean recovery (%)	RSD (%)	RE (%)	Mean recovery (%)	RSD (%)	RE (%)
Method A						
5	99.71	6.20	-0.28	95.95	6.77	-4.09
15	104.20	3.66	4.20	102.55	5.50	2.55
30	104.60	2.10	4.60	105.49	5.63	5.48
Method B						
5	98.11	3.54	-1.89	97.12	3.13	-2.87
15	100.30	0.92	0.30	100.08	2.18	0.08
30	100.90	2.47	0.90	97.34	3.38	-2.65

Table 5. Accuracy results.

Method A				Method B		
Morin (μg mL^{-1})	Recovery (μg mL^{-1})	Recovery (%)	Mean ± SD %RSD	Recovery (μg mL^{-1})	Recovery (%)	Mean ± SD %RSD
1	-	-	-	1.03	103.28	100.3± 2.98 2.97%
1	-	-		1.00	100.30	
1	-	-		0.97	97.32	
5	5.34	106.91	102.44 ± 5.08 4.96%	4.86	97.26	99.94 ± 2.37 2.38%
5	5.17	103.48		5.04	100.8	
5	4.84	96.91		4.84	96.72	
10	10.28	102.84	92.90 ± 9.99 10.72%	10.22	102.21	101.46 ± 2.74 2.70%
10	8.28	82.85		9.84	98.42	
10	9.30	93.02		10.37	103.74	
15	14.57	97.13	97.40 ± 1.21 1.24%	15.50	103.37	100.83 ± 2.41 2.38%
15	14.45	96.36		14.79	98.57	
15	14.81	98.73		15.08	100.55	
20	20.38	101.92	95.58 ± 5.49 5.74%	20.61	103.04	100.67 ± 2.07 2.06%
20	18.50	92.50		19.85	99.24	
20	18.46	92.31		19.94	99.72	
30	29.32	97.73	97.02 ± 2.01 2.07%	29.51	98.37	99.47 ± 2.15 2.16%
30	28.42	94.75		29.43	98.10	
30	29.57	98.58		30.58	101.94	

Moreover, low RSD values were obtained with method B for all the concentrations assayed (1-30 μg.mL^{-1}), thereby indicating low variability of the analytical method and adequate agreement between experimental and theoretical values. Regarding accuracy, the results obtained (Table **5**) show that recovery ranged from 83% to 107% for method A, but with RSD values as high as 10.7%, whereas for method B, recovery ranged from 91% to 103% with RSD values lower than 3.6%, thereby indicating the suitable accuracy obtained with method B.

With respect to sensitivity, the values obtained for LOD and LOQ with method A were 1.2 μg.mL^{-1} and 3.90 μg mL^{-1}, and 0.5 μg.mL^{-1} and 1.5 μg mL^{-1}, for method B, respectively. For the determination of LOD and LOQ samples were analyzed five times with the results obtained being similar to the theoretical concentrations used in method A and B. Taking into consideration that our objective was to develop a new nanoparticulate delivery system for morin flavonoid, and in order to be able to detect and quantify the probable small amounts of flavonoid encapsulated within the NPs, method B exhibits better sensitivity than method A.

Once method validation was conducted, method A and method B were used for the quantification of morin encapsulated within the PLGA nanoparticle formulations developed: morin-loaded PLGA NPs (formulation F1) and morin-loaded PLGA-phe-phe NPs (formulation F2).

Figure **2** shows a chromatogram obtained after analyzing a sample containing phe-phe bound to PLGA nanoparticles, in which a well-defined peak for the dipeptide is observed.

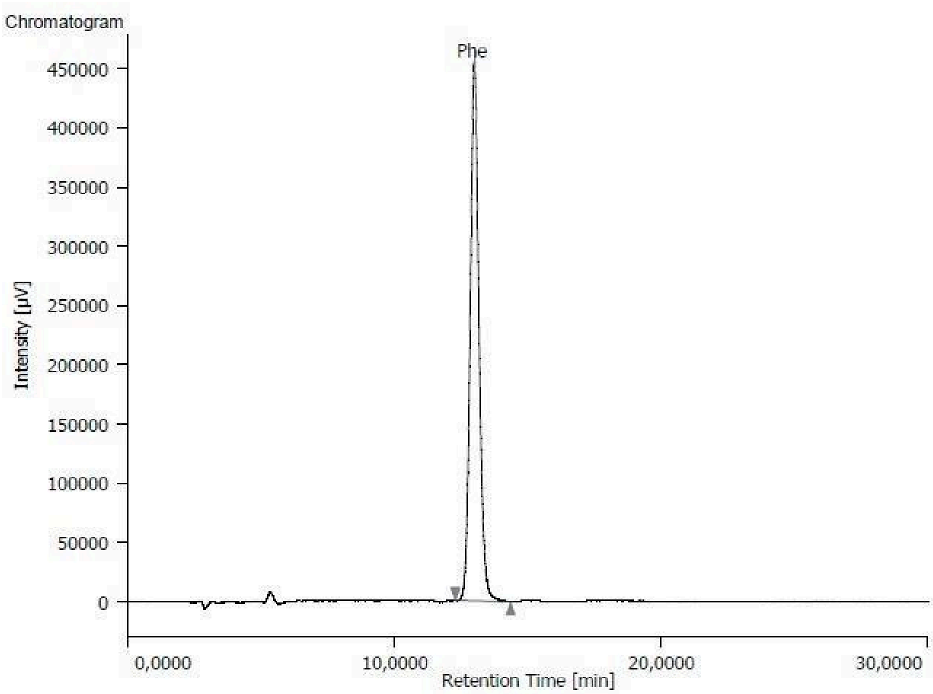

Fig. (2). Typical fluorescence chromatogram corresponding to a sample of phe-phe-linked nanoparticles.

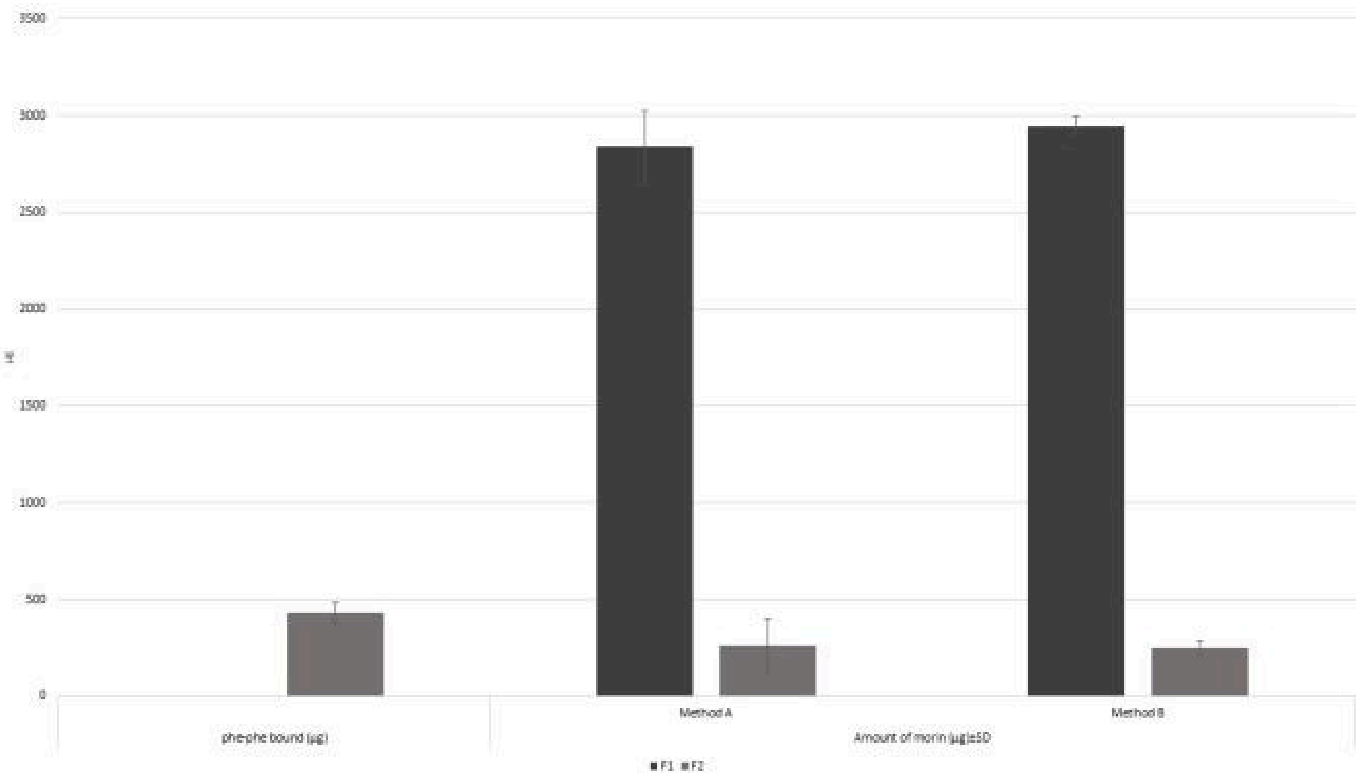

Fig. (3). Mean results (± SD) of the amount of phe-phe dipeptide bound and the amount of morin encapsulated within PLGA nanoparticles. F1: morin-loaded PLGA nanoparticles, F2: Morin- loaded PLGA-phe-phe nanoparticles. phe-phe: phenylalanine- phenylalanine.

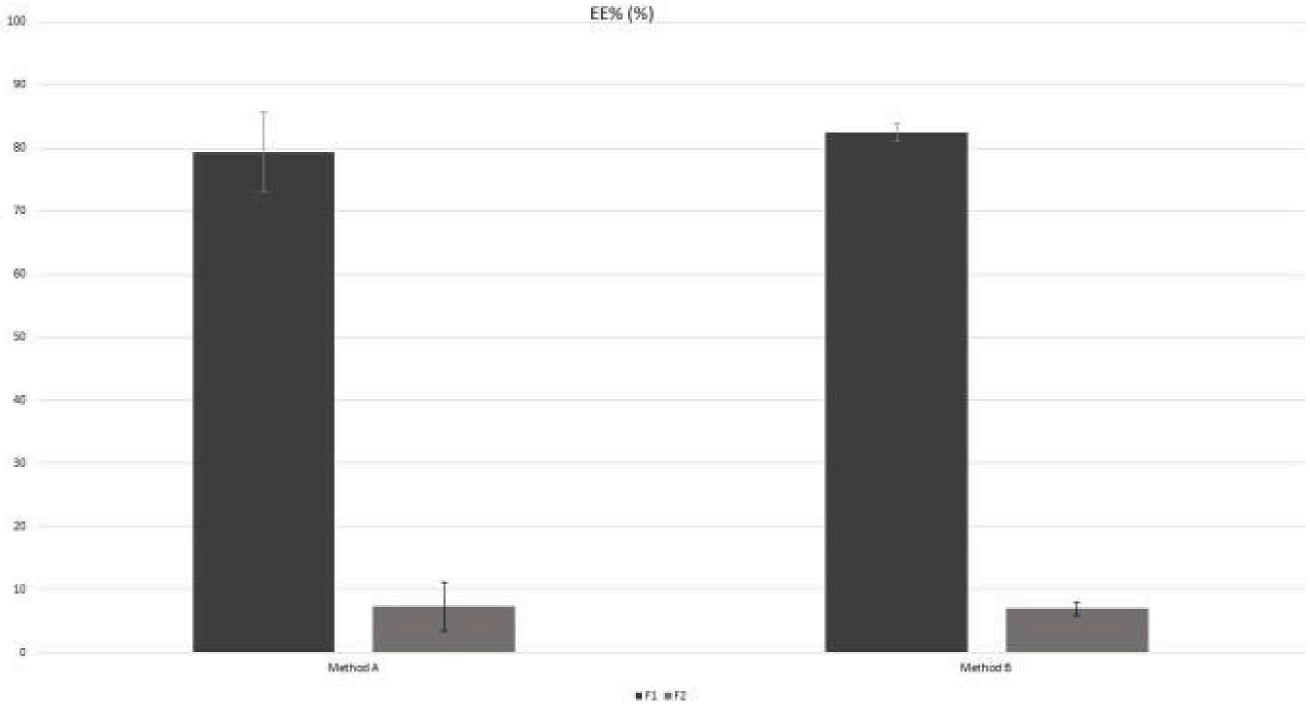

Fig. (4). Mean results (± SD) of the encapsulation efficiency (EE%) of morin within PLGA nanoparticles. F1: morin-loaded PLGA nanoparticles, F2: Morin-loaded PLGA-phe-phe nanoparticles. phe-phe: phenylalanine-phenylalanine.

Indirect quantification of the amount of phe-phe bound to the NPs was performed according to the method described by Neurauter *et al*. [29]. The average amount of phe-phe bound to formulation F2 (50 mg of NPs) was around 430±50 µg as calculated by HPLC with fluorescence detection (Fig. **3**).

Fig. (**4**). Shows the mean results (± SD) obtained for the encapsulation efficiency (EE%) of morin within PLGA nanoparticles and corresponding to formulations F1 (morin- loaded PLGA nanoparticles) and F2 (morin-loaded PLGA- phe-phe nanoparticles).

When morin-loaded NPs were prepared without phe-phe (formulation F1), the values obtained for the encapsulation efficiency were high and around 80% (79±6% and 82±1% from method A and B). However, when phe-phe was bound to the NPs (formulation F2) encapsulation efficiency was significantly reduced, with mean values of 7±4% and 7±1% when analyzed by methods A and B, respectively. This decrease in the encapsulation efficiency was probably caused by the ligand-binding process which is performed meanwhile the NPs are being loaded with the flavonoid under stirring in an acidic medium, in which morin is soluble. Moreover, this process also requires 24 h of keeping suspended in water the nanoparticles as well as 3 h for attaching the dipeptide to their surfaces. During this time, it is possible that part of the encapsulated morin is lost.

Our results have demonstrated that treating the samples with methanol and HCl 0.1N (50:50 v/v) increases the precision and sensitivity of the HPLC determination of morin, that when compared with other published analytical methods for the compound, is a clear advantage, which in turn becomes specially important when dealing with nanoparticulate systems in which the amounts of drugs encapsulated are very small.

CONCLUSION

The HPLC method developed, validated, and adapted for the quantification of morin flavonoid, either in the form of hydrate (method A) or hydrochloride (method B), is adequate for the quantification of the flavonoid encapsulated within PLGA nanoparticles. However, with method B, in which the flavonoid is initially dissolved in methanol:0.1N HCl (50:50, v/v) better sensitivity, accuracy, and precision are achieved, therefore; the use of HCl resulted to be a key point to improve the HPLC quantification of morin flavonoid in a complex formulation (polymeric nanoparticles).

A new formulation consisting of morin hydrate-loaded PLGA nanoparticles is developed with the method used to functionalize the nanoparticles proven to be suitable for linking the dipeptide phe-phe to their surfaces. However, this functionalization procedure is not adequate for nanoparticles containing water-soluble compounds, therefore; further research is needed. One approach could be to perform BBB tests to evaluate whether the formulation can access the CNS, or another approach could be to evaluate if binding the peptide to the polymer before the nanoparticles are prepared improves the efficiency of this process.

LIST OF ABBREVIATIONS

BBB = Blood-Brain Barrier
CNS = Central Nervous System
EDC = Ethylcarbodihimiide
EE% = Encapsulation Efficiency
F1 = Formulation1; morin-loaded PLGA nanoparticles
F2 = Formulation 2; morin-loaded PLGA-phe-phe NPs
HPLC = High Performance Liquid Chromatography
LOD = Limit of Detection
LOQ = Limit of Quantification
NHS = N-hidroxysucciniimide
NPs = Nanoparticles
phe-phe = phenylalanine-phenylalanine
PLGA = Polylactic-co-glycolic acid
PVA = Polyvinyl Alcohol
RSD = Relative Standard Deviation

ETHICAL APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No Animals/Humans were used for studies that are the basis of this research.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

Not applicable.

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CONFLICT OF INTEREST

The authors report no conflict of interest.

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