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REGULAR ARTICLE

Determination of mangiferin in *Mangifera indica* L. stem bark extract (Vimang®) and pharmaceuticals by liquid chromatography

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Abstract

A new liquid chromatography method for the analysis of mangiferin (I) from *Mangifera indica* L. stem bark extract (Vimang®), and pharmaceuticals is described. Screening experiments were performed by an experimental design to find the influence of some important chromatographic variables (methanol, column temperature and acetic acid) on the retention times and resolution between critical pairs in the separation. This design also permitted to estimate method robustness and optimal conditions to achieve the best resolution. The best separation was found using a LiChrospher RP 18, 5 µm, 250 x 4.6 mm I.D. column maintained at 25°C, a mobile phase comprising methanol-2.5% v/v aqueous solution of acetic acid (280:720, v/v) at a flow rate of 1.0 ml/min, and detection by UV at 254 nm. The method resolves I, the major component, from other components of the extract. The method showed good selectivity, repeatability (RSD < 2%) and linearity (r = 0.9998). The limits of detection and quantitation were 0.008% (0.9 ng) and 0.05% (6.2 ng), respectively, relative to a 0.6 mg/ml standard solution, injection volume 20 µl. This method was used to quantify I in some aqueous extracts from the natural product and pharmaceuticals.

Key words: *Mangifera indica*, Mangiferin, Vimang®, Pharmaceuticals, HPLC

Introduction

A new bioactive product of natural origin has been developed from the folk knowledge in Cuban ethnic medicine and it is used at present as antioxidant nutritional supplement (Guevara et al., 2004). The active ingredient of the developed pharmaceutical formulations (tablet, capsule, cream and syrup) is an extract of the *Mangifera índica* L. (mango) stem bark (Vimang®), obtained by decoction of some varieties grown in Cuba (Núñez Sellés et al., 2002; Acosta-Equijarosa et al., 2009). It is a fine brown powder, which has proven to be useful in the treatment of a large population sample presenting physical stress due to age or deteriorated physiological conditions caused by chronic lengthened diseases such as cancer, diabetes or cardiovascular disorders (Núñez-Sellés, 2005). Studies have shown that treatment with the extract provided significant protection against 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced oxidative damage, and the former lead to better protection when compared with other antioxidants (Vitamin C, E and beta–carotene) (Sánchez et al., 2000). Furthermore, the results indicate that Vimang® is bioavailable for some vital target organs, including liver and brain tissues, peritoneal cell exudates and serum. Therefore, it was concluded that it could be useful to prevent the production of reactive oxygen species (ROS) and the oxidative tissue damages in vivo (Sánchez et al., 2000). All these effects are likely due to the synergic action of several compounds such as polyphenols, terpenoids, steroids, fatty acids and microelements, which have been reported to be present in the extract (Núñez-Sellés et al., 2002a; Núñez-Sellés et al., 2007; Núñez-Sellés and Rastrelli, 2010).

Mangiferin (1,3,6,7-tetrahydroxyxanthone-2-C-ß-D-glucopyranoside), a C-glucosylxanthone, which was first isolated from the bark, branches and leaves of *Mangifera indica* L. (Bhatia, 1967; Nott, 1968),

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has been found to be the major component of this extract. It is known that mangiferin (**I**) (Figure 1) is potentially a naturally-occurring chemopreventive agent in rat colon carcinogenesis (Yoshimi et al., 2001), exerts antidiabetic activity by increasing insulin sensitivity (Ichiki et al., 1998; Sellamuthu et al., 2009), appears to act as a potential biological response modifier with antitumor, immune modulatory and anti-HIV effect (Guha et al., 1996), is useful as an analgesic without adverse effects (Makare et al., 2001), and inhibits the late event in Herpes Simplex/Virus-2 replication (Zhu et al., 1995).

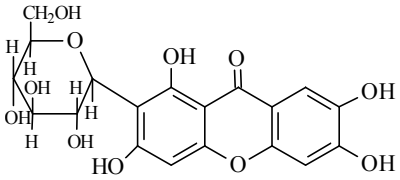

Figure 1. Structure of mangiferin (I).

A thin layer chromatography (TLC) densitometric method was developed for the quantitative determination of **I** in leaves of *Cratoxylum pruniflorum* (Nedialkov et al., 1998). On the other hand a high performance thin layer chromatographic (HPTLC) method was developed and validated as per ICH (International conferences on harmonization) guidelines for simultaneous quantification of mangiferin in *Salacia chinensis* roots (Nadagouda et al., 2010). Others liquid chromatography methods (LC) has been reported before for the quantitative determination of **I** and several other metabolites in *Mangifera indica* L. extracts or parts of the tree.

Several methods of LC for the determination of I simultaneous determination of several components including **I** in Chinese traditional pharmaceutical preparations (Ronghua et al., 2004) and determination of **I** in rat plasma and urine (Wang et al., 2006) are available. Recently report assessing the amount of mangiferin allowed into the eye (Hou et al., 2010). Therefore, a new LC method for the routine quantitative analysis of **I** in presence of other metabolites from *Mangifera indica* L. extracts and its pharmaceuticals had to be developed.

Materials and Methods

Reagents

Water was distilled twice from glass apparatus. Methanol, HPLC grade, and glacial acetic acid, analytical grade, were from Merck (Darmstadt, Germany).

Reference standards

The reference standard (RS) of mangiferin was a house standard with 94.8% of purity. The preparation chemical characterization of this reference standard was previously described (Sordo et al., 2010).

Natural product samples (Vimang®)

Natural product sample (Vimang®) was obtained by aqueous decoction of *Mangifera índica* L. stem bark grown in Cuba. The aqueous extract was dried by atomization in a spray dryer until a brown solid was achieved, which melts with decomposition between 215 and 218°C (Lot 601, water content (K. Fischer), 10.5% (RSD = 0.9%)) (Acosta Esquijarosa et al., 2009). Tablets and capsules conformed under wet granulation process (Vimang®, 300 mg/ units) were used for the applications.

Natural product sample preparation

Natural product samples of 50 mg were accurately weighed into 100 ml conical flasks. 25.0 mL of 85% v/v methanol were added, the flasks were sealed and shaken on a magnetic shaker for 20 min. Approximately 10 mL were centrifuged at 3000 rpm for 2 min. 5.0 ml of the clear supernatant were diluted to 10.0 mL with methanol 85%.

For pharmaceutical samples, 20 units were weighed and finely powdered. An accurately weighed amount of the analyte powder equivalent to the content of a unit was transferred into a 1000 mL conical flask. 250 mL of 85% v/v were added, the flask was sealed and shaken on a magnetic shaker for 20 min. Approximately 10 mL were centrifuged at 3000 rpm for 2 min. 5.0 mL of the clear supernatant were diluted to 10.0 mL with methanol 85%.

Recovery test

Four 50 mg amounts of natural product sample (one as a control) were weighed accurately and each portion (except the control) was spiked with known quantities of mangiferin house RS (1.26, 2.52, and 4.06 mg). All samples were extracted using the same procedure as indicated under 2.4 and the obtained solutions were injected for LC analysis to calculate the recovery. For pharmaceuticals, the same procedure was performed, but four amounts equivalent to a capsule and/or tablet were weighed accurately and each portion (except the control) was spiked with known quantities of mangiferin house RS (12.6, 25.2, and 40.6 mg). All samples were extracted using the same procedure as indicated under 2.4 and the obtained solutions were injected for LC analysis to calculate the recovery.

LC apparatus

The equipment consisted of an intelligent pump L-6200 (Merck-Hitachi, Darmstadt, Germany), a Model Rheodyne 7125 injector (Cotati, California, USA), with a 20 µL loop, a model L - 4250 UV detector (Merck-Hitachi). A PC was used to register the chromatograms using the software BioChrom 2.1 (CIGB, Havana, Cuba).

Experimental design

Screening and optimization of the selectivity were performed by experimental design and multivariate analysis. The screening experiment was carried out as a full-fraction factorial design at two levels. Response surface modeling (RSM) was used to optimize the significant variable factors. Three chromatographic variables that governed the separation most: quantity of methanol (mL) and acetic acid (%) in the mobile phase, and the column temperature were selected. This involves $2^3 = 8$ different experimental measurements. Considering the inclusion of the central point combination in the design (set on the preferred conditions found in the method development), as well as duplicate experiments, 18 measurements had to be performed. Robustness study for the optimized method conditions was performed using a Central Composite design Face Centered (CCF) for response surface modeling (RSM) investigations (17 measurements). The setup of the applied designs, the randomization of runs, the analysis of the measured responses, and the multivariate regression calculations, were performed using the statistical software Modde version 4.0 (Umetri, AB, Umeå, Sweden). One injection was done for each experiment and experiments were duplicated using the same mobile phase. The influence of the chromatographic variables and the interactions between two of them were considered in the analysis of response variables.

Results and Discussion

Method development

A 1 mg/ml reference standard solution of **I** in 85% methanol was initially chromatographed on a LiChrospher RP 18 column, 5 µm (250 x 4.6 mm I.D.) using water - methanol (70:30, v/v). To improve the symmetry factor of the observed peaks, a 3% v/v aqueous solution of acetic acid was used instead of water. Originally, a column temperature of 30°C was used but it was observed that by lowering the temperature to 25°C, the separation around the major peak was much improved, whereas the analysis time increased by only a few minutes.

Figure 2A shows a LC chromatogram of a 1 mg/mL preparation of a natural product sample from *Mangifera indica* L. In this chromatogram, peak **2** was assigned to **I**, and peak **3** to **II**. Peak **1** remained unidentified. UV spectra of peaks **2** and **3** were identical and showed four characteristic maximums of xanthones at 240, 258, 320 and 368 nm (Berardini et al.,2005; Barreto et al., 2008), whereas the one corresponding to peak **1** showed only two maxima at 224 and 274 nm. The three chromatographic peaks were homogeneous (UV spectra, which were recorded stopping the pump and using a facility of the UV detector, were the same at the beginning, in the maximum and at the end of each peak).

Optimization and Robustness

Optimization testing was performed using a full-fraction factorial experimental design including a low number of experiments, which allows estimating the effect of chromatographic parameters and their interactions. The estimated response surface plot for **R1** (resolution between 1 and 2) **R2** (resolution between 2 and 3) revealed that the best resolution within the studied range is achieved when the chromatographic variables were at their lower values (Figure 3). Due to the Cuban tropical climate it was preferred not to choose for temperatures below 25°C. Figure 2B shows a chromatogram obtained using the optimized conditions.

Robustness of the method was tested using a Central Composite design Face Centered (CCF). Table 1 shows the three chromatographic variables to be examined and the corresponding values for the design. As response variables in the factorial design, retention times of compounds were measured and selectivity between critical pairs was calculated.

The data collected were used to estimate the coefficients of the model, which represent the relationship between the response (Y) and the factors (X_n). Multiple linear regression (MLR) was used to estimate the coefficients of the terms in the model that are computed to minimize the sum of squares of the residuals, i.e. the sum of squared deviations between the observed and fitted values of each response. The least square regression method yields small variances for the coefficients and small prediction errors. It is important to note that MLR separately fits one response at a time and hence assumes them to be independent. One may review the fitted model by: examining the summary of the fit, R^2 and Q^2 for every response, examining the coefficients and their 95% confidence intervals, and examining the analysis of variance (ANOVA) table.

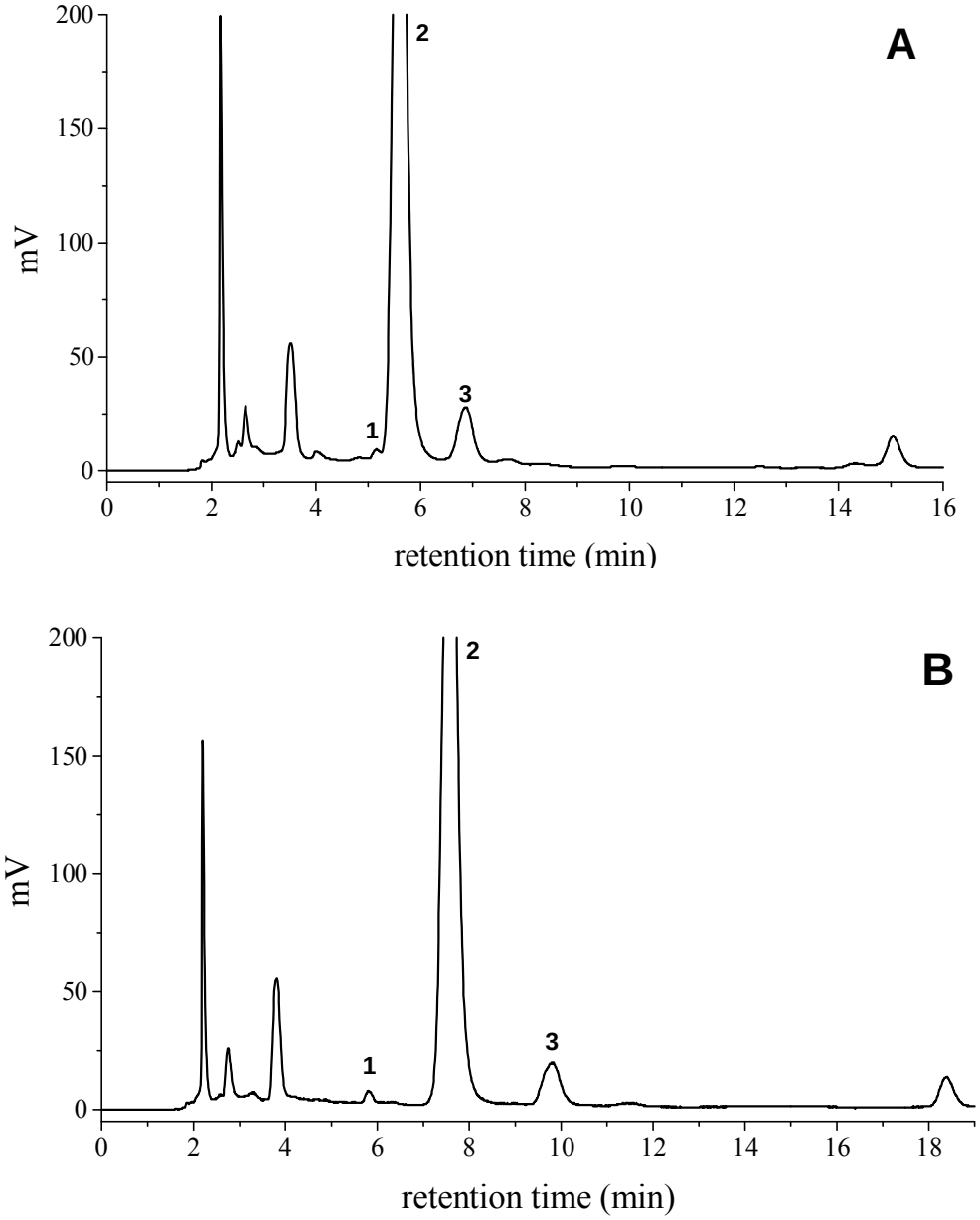

Figure 2. LC chromatogram of an extract of *Mangifera indica* L. (A) Stationary phase, LiChrospher RP 18, 5 µm (250 x 4.6 mm I.D.); column temperature, 30°C; mobile phase, 3% acetic acid aqueous solution – methanol (700:300, v/v); flow rate, 1.0 mL/min; sample concentration, 1 mg/mL; injection volume, 20 µl; detection, UV at 254 nm. Peaks: 1 = unknown, 2 = I, and 3 = compound structurally related to I. (B) as A, but column temperature, 25°C; mobile phase, 2.5% acetic acid aqueous solution – methanol (720:280, v/v).

Table 1. Values corresponding to –1, 0, and +1 levels.

Chromatographic variable	Low value (-1)	Central value (0)	High value (+1)
(A) methanol (mL)	260	280	300
(B) temperature (°C)	23	25	27
(C) acetic acid (%, v/v)	2.0	2.5	3.0

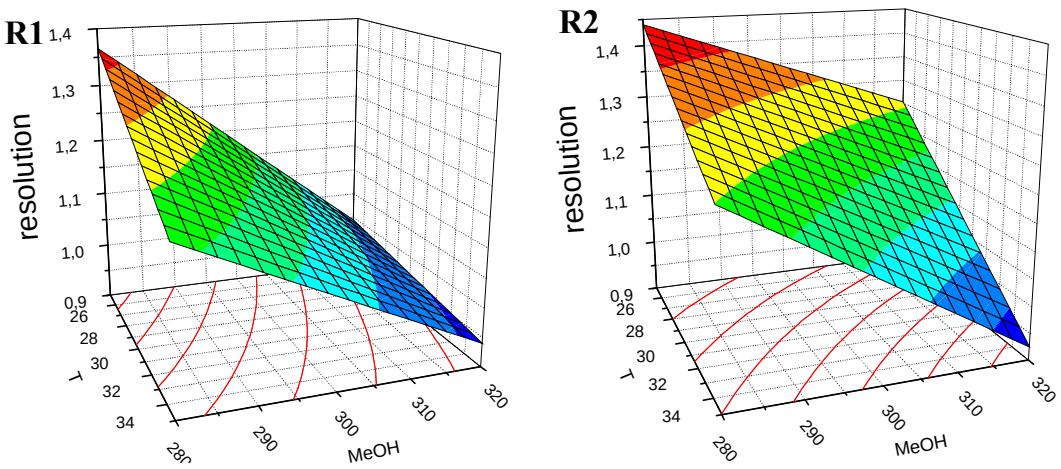

Figure 3. Response surface plot showing the resolution as a function of significant chromatographic variables (MeOH – methanol; T – column temperature). R1 – resolution between peaks 1-2; R2 – resolution between peaks 2-3. Percent of acetic acid was constant at 3 %.

The percent of the variation of the response explained by the model (R^2) and the predictive power of the model (Q^2) were over 0.97 and 0.7, respectively, implying that the data fitted well with the model. R^2 overestimates the good quality of fit, whereas Q^2 underestimates the veracity of fit. R^2 and Q^2 values close to 1 indicate an excellent model. A Q^2 larger than zero indicates that the dimension is significant (predictive). Large Q^2, 0.7 or larger, indicates that the model has good predictive ability and will have small prediction errors. The probability values for lack of fit in the ANOVA table were greater than 0.10 for every response variable. For this reason, the model appeared to be adequate for the observed data.

Evaluation of the coefficients calculated by the model allows obtaining the effect plot for each response variable. This plot displays the values of the effects (twice the coefficients) sorted (in absolute value) in descending order. The ± 95% confidence interval is drawn as an upper and lower line. For chromatographic variables the plot displays the predicted change in the response when the factor varies from its low to its high level, all other factors in the design being set on their average. When one selects a 2 factor interaction, the predicted change in the response when one factor varies from its low to its high level is plotted for both levels of the other factor, all remaining factors in the design being set on their average.

Peaks **1**, **2** and **3** were selected to review the effects of chromatographic variables and their interaction on the retention times and selectivity.

Figure 4 shows that two parameters, methanol and column temperature, have significant negative effects on the retention times of all compounds. This means that an increase of these variables provokes a decrease of the retention times. It can be noted that these effects are larger for peaks **2** and **3**. Interaction variables (column temperature and per cent of acetic acid) had only significant positive effect on the retention time for peak **1**. Within the range examined only methanol has significant negative effect on the selectivity between peaks **1-2** and **2-3**, denoting a decrease of the selectivity with an increase of methanol. Nevertheless, the peaks never overlapped, indicating the robustness of the method.

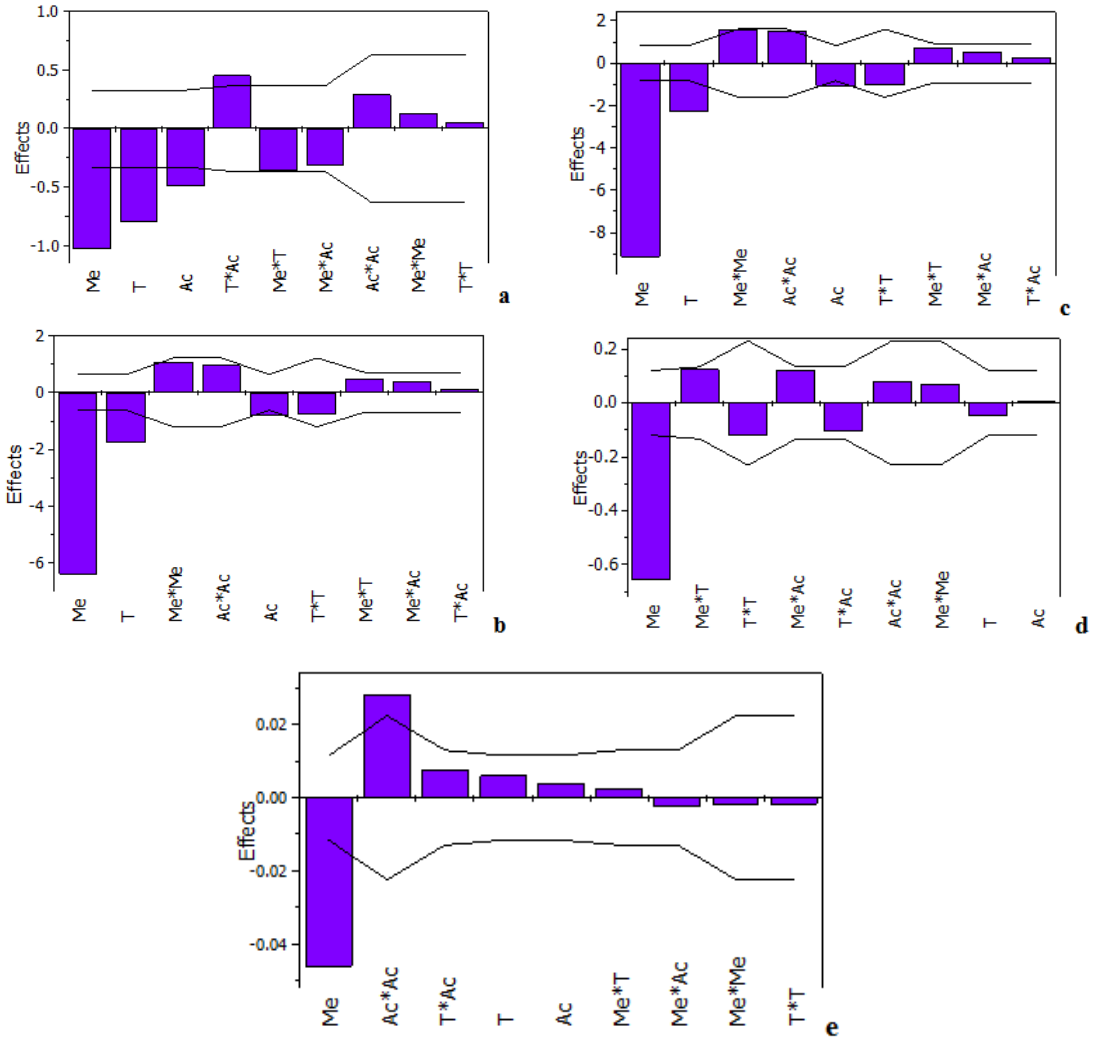

Figure 4. Effect plot for the response variables: a, b and c – retention times of peaks 1, 2 and 3, respectively; d and e – selectivity between peaks 1-2 and 2-3, respectively. Me – quantity of methanol (mL); Ac – percent of acetic acid; T – temperature.

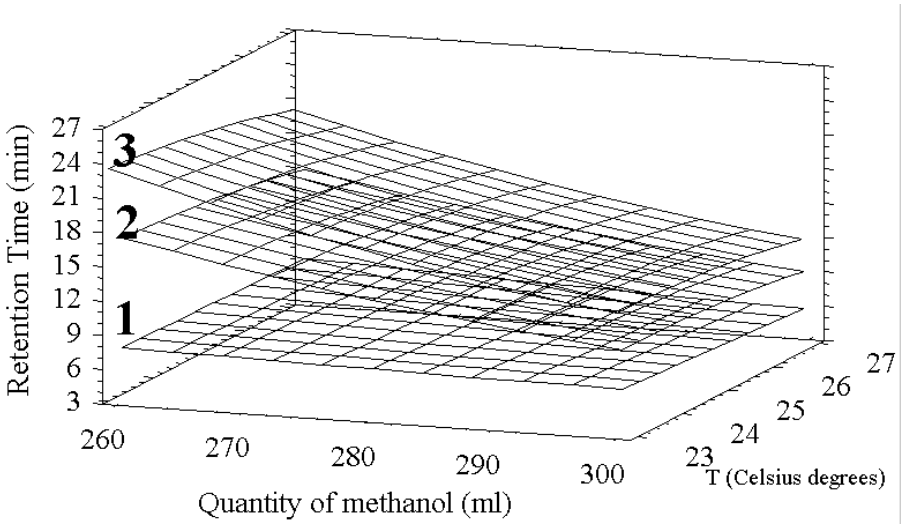

Figure 5. Estimated response surface plots constructed with retention times (tr) as a function of the quantity of methanol and the temperature of the column. The percent of acetic acid was kept at 3%.

Table 2. Relative recoveries of mangiferin in *Mangifera indica* L.

Added (mg)	Mean found (n = 3)	Relative Recovery (%) ± SD (n = 3)	Mean ± SD	RSD (%)
1.26	1.26	100.0 ± 3.2		
2.52	2.54	100.8 ± 2.2	100.9 ± 2.3	2.3
4.06	4.12	101.3 ± 1.8		

Response surface plots (Figure 5) constructed with retention time, as a function of the most significant chromatographic variables (methanol and column temperature) shows no overlapping, thus confirming the robustness of the method.

Quantitative analysis of mangiferin

Validation parameters such as precision, linearity, accuracy, selectivity and limits of detection (LOD) and quantitation (LOQ) were determined for mangiferin. Precision (repeatability) was checked with a solution of natural product sample having a 0.4 mg/mL concentration, equivalent to mangiferin. The relative standard deviation (RSD) was 1.6% (one analyst, n = 6) and 1.7% (two analysts, n = 12) for within-day and day-to-day repeatability, respectively. The calibration curve obtained by replicated analysis (n = 3) of a series of analyte concentrations corresponding to 0.1, 0.2, 0.3, 0.4, 0.5 and 0.6 mg/mL of mangiferin reference standard was subjected to linear regression analysis: y = -0.05 + 474.6 x, where x = mangiferin concentration in mg/ml, y = peak area; correlation coefficient r = 0.9998, standard error of the estimate $S_{y,x}$ = 1.8. The smallest difference of analyte concentration that can be recorded with a 95% probability is 0.006 mg/mL (1%).

The relative recoveries of **I** are shown in Table 2. 100.0% of **I** was recovered in presence of natural product sample with 85% methanol. The extraction recoveries of **I** from the related pharmaceuticals with three spiked levels were above 98% in all cases.

Homogeneous chromatographic peaks for **I** were obtained from 1 mg/ml solutions of natural product sample dissolved in acid (0.1 M HCl), aqueous and basic media (0.1 M NaOH), respectively, which were submitted to 1 h reflux, confirming the selectivity. The content of **I** was diminished only under basic media (51.0% of the original remained after degradation), whereas under other media no degradation was observed.

In the determination of LOD and LOQ, a solution of **I** reference standard was diluted gradually. The LOQ with signal-to-noise ratio of 10

was 0.05% of 0.6 mg/mL, i.e. 6.2 ng injected mass (n = 6; RSD = 7.6%). The LOD with signal-to-noise ratio of 3 was 0.016%, i.e. 1.98 ng injected mass.

Applications

The application of this method to the quality control of 16 batches of Vimang® active ingredient, obtained from different provinces of the country, and its pharmaceuticals were investigated and demonstrated in Table 3. Each sample was analyzed in triplicate and the average value was listed. All of the assay results fell between 100~300 µg of mangiferin per mg of Vimang ® powder, but samples No. 12 to No. 16 and they were rejected. The differences found are probably due to the fact that the mangiferin content in the plant varies with the season of the year and the zone in which it was grown. The claimed contents of this natural product required by ours producer are 85-115% for tablets and capsules. A typical chromatogram for the analysis of pharmaceuticals is shown in Figure 6. We established a simple and selective HPLC method for the assay of Vimang®.

Table 3. Content of mangiferin (µg/mg) in natural product samples from *Mangifera indica* L. and assay result of pharmaceuticals.

Sample	Content, µg/mg (RSD, %)	Sample	Content, µg/mg (RSD, %)
No.1 (batch 901)	254 (0.7)	No.9 (batch 0201)	125 (0.1)
No.2 (batch 903)	195 (2.0)	No.10 (batch 0202)	109 (1.5)
No.3 (batch E-923)	187 (1.6)	No.11 (batch 0203)	116 (1.2)
No.4 (batch E-924)	180 (1.4)	No.12 (batch 0204)	79 (2.4)*
No.5 (batch E-032)	206 (1.6)	No.13 (batch 0205)	56 (0.5)*
No.6 (batch 0103)	149 (5.7)	No.14 (batch 0206)	55 (2.3)*
No.7 (batch 0104)	162 (0.4)	No.15 (batch 0207)	66 (1.8)*
No.8 (batch 0112)	159 (0.3)	No.16 (batch 0208)	49 (0.2)*
Pharmaceuticals	Amount of Vimang® (mg)	Percentage of claimed content %, (RSD, %)	
Tablets (batch 1)	299.91	99.97 (5.47)	
Tablets (batch 2)	291.36	97.12 (3.14)	
Tablets (batch 3)	310.51	103.51 (1.42)	
Capsules (batch 1)	309.03	103.01 (1.66)	
Capsules (batch 2)	307.83	102.61(1.78)	
Capsules (batch 3)	313.89	104.63 (1.18)	

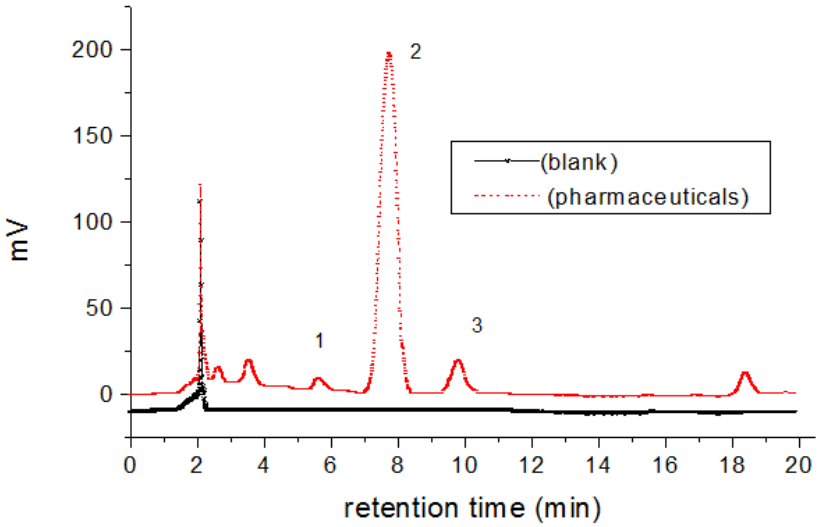

Figure 6. LC chromatograms of blank and Vimang® pharmaceuticals (tablets and capsules). Stationary phase, LiChrospher RP 18, 5 µm (250 x 4.6 mm I.D.); column temperature, 25°C; mobile phase, 2.5 % acetic acid aqueous solution – methanol (720:280, v/v); flow rate, 1.0 mL/min; injection volume, 20 µL; detection, UV at 254 nm. Peaks: 1 = unknown, 2 = I, and 3 = compound structurally related to I.

Conclusion

A LC method was developed for the quantitative determination of **I** as the major component of *Mangifera indica* L. extracts. A full-factorial design indicated that the three studied chromatographic variables (methanol, acetic acid and column temperature) have a significant effect on the retention times of all compounds, whereas the influence on the resolution among them is not significant, showing that the method was robust. It also pointed out the optimal conditions needed to achieve the best resolution. Validation of the method has shown its usefulness in the quantitative routine analysis of **I** in extracts and pharmaceuticals of the natural product under study.

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