Optimization of a Reverse-Phase High Performance Liquid Chromatography (RP-HPLC) Method for Simultaneous Separation of Aloe-Emodin, Rhein, Emodin, Chrysophanol and Physcion

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Abstract

In this study, a reverse-phase high performance liquid chromatography (RP-HPLC) method with high separation efficiency, high detection sensitivity and excellent selectivity was successfully developed for the simultaneous determination of the aloe-emodin, rhein, emodin, chrysophanol, and physcion compounds. These optimum method conditions were determined by studying various columns, mobile phases and compositions, flow rates and column temperatures. The successful separation was carried out with a Supelcosil LC-18 column (250 x 4.6 mm, 5 μm) and a gradient program. The procedure was carried out at 20 °C with the flow rate of 1.0 mL/min. and the injection volume of 20 μL utilizing an RP-HPLC method with DAD detector at 225 nm. Deionized water containing 0.5% (v/v) orthophosphoric acid and methanol was used as the mobile phases A and B. This study showed an optimized analytical method can be effectively utilized to the qualitative determination of anthraquinone compounds.

Keywords: Reverse Phase High Performance Liquid Chromatography, Anthraquinon, Secondary Metabolites, Method Optimization.

Aloe-Emodin, Rhein, Emodin, Chrysophanol ve Physcion’un Eş Zamanlı Ayrımı için Ters Fazlı Yüksek Performanslı Sıvı Kromatografisi (RP-HPLC) Yönteminin Optimizasyonu

Öz

Bu çalışmada, aloe-emodin, rhein, emodin, chrysophanol ve physcion bileşiklerinin eş zamanlı tayini için yüksek ayırmaya verimliliği, yüksek saptama hassasiyetine ve üstün seçiciliğe sahip bir ters fazlı yüksek performanslı sıvı kromatografi (RP-HPLC) yöntemi başarıyla geliştirilmiştir. Optimum yöntem koşulları, çeşitli kolonlar, mobil fazlar ve bileşikleri, akış hızları ve kolon sıcaklıkları çalışma olarak belirlenmiştir. Ayırma, Supelcosil LC-18 kolonu (250 x 4.6 mm, 5 um) ile gradient programında gerçekleştilmiştir. Prosedür 20 °Cde, 1,0 mL/dk akış hızı ve 20 μL enjeksiyon hacmi ile 225 nm'de DAD dedektörü bir RP-HPLC yöntemi kullanılarak gerçekleştirilmiştir. Mobil faz A ve B olarak %0,5 (v/v) ortofosforik asit içeren deionize su ve metanol kullanılmıştır. Bu çalışma, antrakinon bileşiklerinin kalitativ tayini için optimize edilmiş bir analitik yöntemi etkin bir şekilde kullanılabileceğini göstermiştir.

Anahtar Kelimeler: Yüksek Performanslı Sıvı Kromatografi, Antrakinon, Sekonder Metabolitler, Metot Optimizasyonu.

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1. Introduction

The secondary metabolites are distributed in the plant family in various ways, and their biological functions are specific to the generating plants in which they are ascertained (Wolfender et al., 2015). The natural anthraquinones are the valuable part of the secondary metabolites naturally found in Polygonaceae, Leguminosae, Rubiaceae, Liliaceae and Rhamnaceae (Yao et al., 2004). The natural anthraquinones are interesting due to wide-ranging applications, and bioactive properties like anticancer (Koyama et al., 2002; Su et al., 2005; Chen et al., 2007), anti-inflammatory, antifungal (Agarwal et al., 2000), antimicrobial (Yanwen et al., 2005), diuretic, cathartic, laxative, vasorelaxing, antioxidant (Yen et al., 2000; Iizuka et al., 2004) and phytoestrogen activities (Chien et al., 2015; Locatelli, 2011; Reynolds, 2004). For the typical example; aloe-emodin and chrysophanol were found to possess anti-microbial bacterial activities (Smolarz et al., 2013). It has also been discovered that emodin, aloe-emodin and rhein naturally possess antitumor activities, anti-inflammatory and antiviral effects (Li-Weber, 2013). The rhein exhibit many biological properties, especially immuno-suppressive, anti-inflammatory, antitumor, anticancer (You et al., 2013). Additionally, the physician remains a potential candidate in the field of anticancer drug discovery against human cervical cancer (Wijesekara et al., 2014).

There are various analytical methods used for the qualitative or the quantitative analysis of the anthraquinones like the High Performance Liquid Chromatography (HPLC) (Zhang & Shi, 2010; Koyama et al., 2007; Zhang et al., 2013; Tian et al., 2012; Mandrioli et al., 2011) the Ultra-Performance Liquid Chromatography (UPLC) (Wang & Shi, 2014; Hu et al., 2014), the Capillary Cone Electrophoresis (CZE) (Tian et al., 2007; Wang et al., 2004), the Micellar Electrokinetic Chromatography (MEC) (Kuo & Su, 2003; Shang & Yuan, 2003; Sun & Yeh, 2205), the Capillary ElectroChromatography (CEC) (Li et al., 2007), the Thin Layer Chromatography (TLC) (Singh et al., 2005), the High-Speed Counter Current Chromatography (HSCCC) (Guo et al., 2011), the Cyclic Voltammetry (Wang et al., 2010) and the Supercritical Fluid Chromatography (Aichner & Ganzera, 2015) but are not sensitive enough. There are also extremely sensitive and reliable methods utilized with the HPLC-MS (Xu et al., 2008) and the GC-MS (EISohly et al., 2004; Zuo et al., 2008) available but these sophisticated devices are expensive, which limits their usability. Among all these methods, an HPLC with various detection systems offers high selectivity, sensitivity and separation efficiency with short analysis time.

The aim of this study was to develop a straightforward, rapid and highly sensitive method for the simultaneous separation of aloe-emodin, rhein, emodin, chrysophanol and physcion using diode array detector RP-HPLC. An effective separation of the five compounds of the similar chemical structure in only fifteen minutes was achieved with the developed method which also facilities elucidation and quantification of the structure of natural anthraquinones in plants.

2. Material and Method

2.1. Chemicals

All solvents (methanol, orthophosphoric acid), diclofenac (internal standard), uracil (t0) and standard compounds (aloe-emodin, rhein, emodin, chrysophanol and physcion) were obtained from Sigma-Aldrich. All chemicals used were HPLC grade and water was purified in a Milli-Q water purification system (Millipore, Bedford, MA, USA).

2.2. Instruments and Conditions

A Shimadzu HPLC system composed of a LC-10AD VP pump, SIL-30AC autosampler, a CTO-10AS column oven, a DGU-20A degasser and a SPD-M20A diode array detector (DAD) was utilized for chromatographic studies. The Supelcosil LC-18, the Mediterranea Sea 18 and the Cogent Phenyl Hydride columns used had the dimensions of a 25 cm × 4.6 mm, 5 µm, a 15 cm × 4.6 mm, 5 µm and a 25 cm × 4.6 mm, 4 µm, respectively. The gradient program was utilized for the chromatographic separation. The deionized water containing 0.5% (v/v) orthophosphoric acid was used as the mobile phase A; the methanol was used as the mobile phase B. The time-volume changes in the gradient program were as follows: 0-2 min., 0-70% B; 2-4 min., 70-75% B; 4-6 min., 75-80% B; 6-8 min., 80-85% B; 8-10 min., 85-90% B; 10-12 min., 90% B; 12-14 min., 90-85% B; 14-16 min., 85-80% B; and16-18 min., 80-70% B. The total injection time was 20 minutes with the injection volume of 20 µL. The optimum column temperature was at the 20 °C, the flow rate was at a 1.0 mL/min and the DAD detector was set at 225 nm.

2.3. Preparation of Stock Solution

The stock solutions of 250 µg/mL were traditionally prepared by dissolving the standard compounds (aloe-emodin, emodin, rhein, chrysophanol and physcion) in the methanol and properly storing at 4 °C. The dilutions were carefully made in methanol at the effective concentration determined for each compound from the stock solutions and required injections were made.

Internal standard was intentionally employed in the method optimization. This standard method adequately compensates the possible errors for pipetting and injection volumes, and also likely changes in the physical parameters during pretreatment or preparation steps. If the internal standard is determined appropriately, both the systematic and the random errors can be eliminated. However, the chemical and physical properties of the internal standard should be similar to those under the study. The diclofenac was intentionally chosen as the internal standard in our study. Even though the chemical structure of the diclofenac is not similar to the compounds studied in terms of the selectivity, peak acuity, resolution and analysis time, it was decided that the diclofenac would be suitable as an internal standard. The diclofenac solution (I.S) was dissolved in methanol to carefully prepare 20 µg/mL solution.

The uracil solution of 20 µg/mL prepared in methanol was chosen as the dead time (t0) to determine the capacity factors. The average retention time of the uracil was precisely determined with three injections without holding in the HPLC column. The standards, diclofenac, uracil solutions and mobile phases were filtered by a 0.45 µm pour size membrane filter.
3. Results and Discussion
3.1. Method Optimization

The most essential criteria for method development in chromatography systems can be listed as the analysis of the compounds in the shortest time possible, ensuring a good separation and obtaining symmetrical peaks. In providing these criteria; the actors like the chemical structure, the diameter and the length of the columns, the wavelength of compounds, the injection volume, the mobile phase composition, the flow rate and the column temperature have to be studied carefully. These criteria were taken into consideration in method optimization during the method development process to simultaneously determine the aloe-emodin, rhein, emodin, chrysophanol and physcion compounds.

The most properly used test for the optimization of RP chromatography is the resolution between peak pairs. Achieving a good resolution between all of the compounds under study is the main goal of chromatographic separation. On account of fundamental chromatographic parameters, the resolution, Rs., is affected by three independent variables:

$$R_s = (1/4) \sqrt{N \left[ (\alpha - 1)/\alpha \right] \left[ k_2/(k_2 - 1) \right]}$$

Eq. (1)

In general, for the separation to take place; the capacity factor (k) must be greater than 1, the selectivity factor (\( \alpha \)) greater than 1.15, and the resolution (Rs.) values must be greater than 1.5. For all optimization parameters Rs. values were calculated for all peak pairs according to Eq. (1).

**Wavelength**

The wavelengths at which the compounds in this study have the highest absorbance were determined properly using a DAD detector. The spectral scan graphs obtained are given in the Figure 1. It is clearly seen from the Figure 1 that all the compounds studied had a maximum absorbance at 225 nm. For the diclofenac (I.S) used as internal standard, the maximum absorbance wavelength of 205 nm was chosen (Figure 1b).

**Injection volume**

Injection of all six compounds with five different injection volumes (10 μL, 20 μL, 30 μL, 40 μL and 50 μL) were conducted for the determination of the optimum injection volume. The

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**Figure 1. λmax values of the compounds obtained by using DAD detector (a - Aloe-emodin, b - I.S c - Rhein, d - Emodin, e - Chrysophanol, f - Physcion)**

In the literature, the following wavelength results in the quantitative determination studies of aloe-emodin, rhein, emodin, chrysophanol and physcion were reported: Uzun and Demirezer (Uzun & Demirezer, 2019) at 440 nm (DAD detector), Liu et al., (Liu et al., 1997) at 280 and 450 nm (DAD detector), Gautam et al. (Gautam et al., 2011), Farooq et al., (Farooq et al., 2013) and Mehta (Mehta, 2012) at 254 nm (DAD and UV detector) with a HPLC.
chromatograms in the Figure 2 were obtained which shows that the peak shapes do not change with the different injection volumes. Therefore, a 20 µL injection volume was chosen as the optimum value to minimize material losses.

![Figure 2](image)

**Figure 2.** The standard mixture chromatograms obtained from different injection volumes of the compounds (1-Aloe-emodin, 2-I.S Rhein, 4-Emodin, 5-Chrysophanol, 6-Physcion)

**Column selection**

The column length and the column filler particle sizes are also important parameters for effective separation in the chromatography. Therefore, three different columns with different dimensions and particle sizes (Mediterranea Sea 18 (150 mm × 4.6mm, 5µm), Cogent Phenyl Hydride (250 mm × 4.6mm, 4µ) and Supelcosil LC-18 (250 mm × 4.6mm, 5µm)) were studied. While dimensions of the Cogent Phenyl Hydride and Supelcosil LC-18 columns are the same, the dimensions of the Mediterranea Sea 18 is different. In addition, the particle sizes of the the Cogent Phenyl Hydride and Supelcosil LC-18 columns were chosen differently from each other. The results obtained with three different columns are given in the Table 1. The resolution (Rs) values below 1.5 were obtained for I.S-Aloe-emodin and Physcion-Chrysophanol peak pairs in the Cogent Phenyl Hydride column and as clearly seen from the Figure 3a, these peaks were not separated effectively. The Rs. values calculated in the Supelcosil LC-18 column were higher than those in the Mediterranea Sea 18 column. In addition, high peaks were obtained with the the Mediterranea Sea 18 column, but the shapes of these peaks are not very good (Figure 3b). As seen in Figure 3c, the best separation and peak shapes were obtained with Supelcosil LC-18 column and thus, it was decided to conduct the study with this column.
Figure 3. The standard mixture chromatograms of the compounds obtained (1-Aloe-emodin, 2-I.S 3-Rhein, 4-Emodin, 5-Chrysophanol, 6-Physicon) in different columns a) Cogent Phenyl Hydride, b) Mediterranea Sea 18 c) Supelcosil LC-18

| Column                  | Compounds                  | k₂   | α     | k₂/(k₂+1) | (α-1)/α | (1/4)√N | Rₛ  |
|-------------------------|----------------------------|------|-------|-----------|---------|---------|-----|
| Cogent Phenyl Hydride   | I.S/Aloe-emodin            | 2.142| 1.065 | 0.682     | 0.061   | 30.656  | 1.275|
|                         | Rhein/I.S                  | 2.371| 1.107 | 0.703     | 0.097   | 35.221  | 2.402|
|                         | Emodin/ Rhein              | 3.246| 1.369 | 0.764     | 0.270   | 43.575  | 8.989|
|                         | Chrysophanol/Emodin        | 3.501| 1.079 | 0.778     | 0.073   | 45.326  | 2.574|
|                         | Physcion/Chrysophanol      | 3.734| 1.067 | 0.789     | 0.063   | 18.062  | 0.898|
| Mediterranea Sea 18     | I.S/Aloe-emodin            | 2.633| 1.221 | 0.725     | 0.181   | 30.214  | 3.965|
|                         | Rhein/I.S                  | 2.900| 1.101 | 0.744     | 0.092   | 30.649  | 2.098|
|                         | Emodin/ Rhein              | 3.761| 1.297 | 0.790     | 0.229   | 38.024  | 6.879|
|                         | Chrysophanol/Emodin        | 4.140| 1.101 | 0.805     | 0.092   | 41.650  | 3.085|
|                         | Physcion/Chrysophanol      | 4.854| 1.172 | 0.829     | 0.147   | 34.529  | 4.208|
| Supelcosil LC–18        | I.S/Aloe-emodin            | 1.916| 1.137 | 0.657     | 0.120   | 46.727  | 3.684|
|                         | Rhein/I.S                  | 2.076| 1.083 | 0.675     | 0.077   | 42.567  | 2.212|
|                         | Emodin/ Rhein              | 2.743| 1.321 | 0.733     | 0.243   | 55.438  | 9.875|
|                         | Chrysophanol/Emodin        | 3.093| 1.128 | 0.756     | 0.113   | 60.449  | 5.164|
|                         | Physcion/Chrysophanol      | 3.621| 1.171 | 0.784     | 0.146   | 64.903  | 7.429|

In a study by VanMen et al., (VanMen et al., 2012), a 25 cm long Optimapak C18 column was used where the retention times of approximately 29 and 31 minutes were determined for the emodin and the chrysophanol, respectively. Another study by Mehta (Mehta, 2012) with a 25 cm long C18 column reported the retention times of 10.75 for the rhein and 16.31 minutes for the emodin. Similar studies were carried out by Zou et al., (Zou et al., 2008) in a 15 cm long Phenyl-Hexyl column and Shi et al., (Shi et al., 2014) in a 25 cm long Diamonsil C18 column and the same retention times for aloe-emodin, rhein, emodin, chrysophanol and physcion were found with the results obtained in our study with 25 cm long Supelcosil C18 column.

**Figure 4.** Standard mixture chromatograms of the different compounds (1-Aloe-emodin, 2-I.S 3-Rhein, 4-Emodin, 5-Chrysophanol, 6-Physicon) obtained in a) Acetonitrile b) Methanol

**Mobile phase**

The methanol and the acetonitrile were studied to determine the mobile phase B composition and the data obtained were compared. As seen in the Figure 4a, the retention times of the compounds are shorter in the acetonitrile phase than in the methanol phase, but the separation could not be achieved since the aloe-emodin and the emodin compounds arrived at the same retention time. However, the separation of all compounds in the methanol was achieved (Figure 4b) and symmetrical peaks were obtained. As a result, the B mobile phase was determined to be methanol and the data obtained are given in Table 2.
Table 2. Values obtained for the methanol as the mobile phase B

| Mobile Phase B | Compounds          | \(k_2\) | \(\alpha\) | \(k_2/(k_2+1)\) | \((\alpha-1)/\alpha\) | \((1/4)\sqrt{N}\) | \(R_s\) |
|----------------|--------------------|---------|------------|-----------------|----------------------|-------------------|--------|
| Methanol       | I.S/Aloe-epidin    | 1.901   | 1.134      | 0.655           | 0.118                | 46.152            | 3.567  |
|                | Rhein/I.S          | 2.076   | 1.092      | 0.675           | 0.084                | 41.954            | 2.379  |
|                | Emodin/Rhein       | 2.747   | 1.323      | 0.733           | 0.244                | 55.008            | 9.838  |
|                | Chrysophanol/Emodin| 3.091   | 1.125      | 0.756           | 0.111                | 60.071            | 5.041  |
|                | Physcion/Chrysophanol| 3.626 | 1.173      | 0.784           | 0.147                | 62.699            | 7.226  |

In the gradient studies conducted in the literature, acetonitrile-water (Wang et al., 2013; Gao et al., 2009; Rafaelly et al., 2008) and the methanol-water (Uzun & Demirezer, 2019; Gautam et al., 2011; Wang et al., 2016) mobile phases are used. In this study, both mobile phases were studied, but the separation of the compounds could not be achieved in the acetonitrile mobile phase. In the experiments, five different ratios of the orthophosphoric acid-deionized water mixture (0.0% - 0.01% - 0.1% - 0.5% - 1.0%) were studied as the mobile phase A composition and the chromatograms obtained are given in Figure 5.

Figure 5. Standard mixture chromatograms of the compounds obtained in the 0.0% - 0.01% - 0.1% - 0.5% - 1.0% orthophosphoric acid-water

The data obtained as a result of the optimization of the mobile phase A composition are given in the Table 3. The \(R_s\) value for Rhein-IS in the 0.0% orthophosphoric acid-water mixture is 0.848 and these two peaks are not separated (Figure 5). However, the data show that the \(R_s\) values of the Rhein-IS peak pair increased with increasing orthophosphoric acid-water ratio and reached a maximum value of 2.227 for 0.5% orthophosphoric acid-water and remained constant at 1% orthophosphoric acid-water.

Table 3. Comparison of values obtained for mobile phase A composition optimization

| Mobile Phase A | Compounds                          | \(k_2\) | \(\alpha\) | \(k_2/(k_2+1)\) | \((\alpha-1)/\alpha\) | \((1/4)\sqrt{N}\) | \(R_s\) |
|----------------|------------------------------------|---------|------------|-----------------|----------------------|-------------------|--------|
| 0.0%           | I.S/Aloe-epidin                    | 1.714   | 2.002      | 0.632           | 0.500                | 10.560            | 3.337  |
|                | Rhein/I.S                          | 1.776   | 1.036      | 0.640           | 0.035                | 37.856            | 0.848  |
|                | Emodin/Rhein                       | 2.855   | 1.603      | 0.741           | 0.376                | 52.337            | 14.582 |
|                | Chrysophanol/Emodin                | 3.243   | 1.137      | 0.764           | 0.120                | 53.878            | 4.940  |
|                | Physcion/Chrysophanol              | 3.802   | 1.171      | 0.792           | 0.146                | 57.527            | 6.652  |
| 0.01%          | I.S/Aloe-epidin                    | 1.910   | 1.134      | 0.656           | 0.118                | 42.071            | 3.257  |
In earlier studies, separation of the compounds was carried out using 0.01% phosphoric acid-water (Zou et al., 2008), 0.1% phosphoric acid-water (Wei et al., 2013) and 0.05% phosphoric acid-water (Uzun & Demirezer, 2019) as the mobile phase composition. According to the data obtained in our study, 0.5% phosphoric acid-water was chosen to use as the mobile phase A composition.

**Flow rate**

In HPLC studies, a fast separation of the compounds is preferred so it is undesirable to have the flow rate to be below a certain value. Therefore, five different flow rates (1.5 mL/min, 1.2 mL/min, 1.0 mL/min, 0.8 mL/min and 0.5 mL/min) tested for the optimum flow rate value. The data obtained data are given in Table 4 and the chromatograms in Figure 6. The Table 4 clearly shows that the lower the flow rate, the slower the compounds leave the column and consequently the retention times in the column increase. Figure 6 shows that the retention times of the compounds increase at a flow rate of 0.5 mL/min and the physcion peak does not leave the column during the method period. Therefore, the flow rate of 0.5 mL/min was not included in the calculations.

**Table 4. Comparison of values obtained for flow rate optimization**

|                | Rhein/I.S | Emodin/ Rhein | Chrysophanol/Emodin | Physcion/Chrysophanol |
|----------------|-----------|---------------|---------------------|-----------------------|
| 0.1% Rhein/I.S | 1.929     | 2.112         | 3.132               | 3.676                 |
| 0.5% Rhein/I.S | 2.086     | 2.734         | 3.070               | 3.604                 |
| 1.0% Rhein/I.S | 2.135     | 2.786         | 3.126               | 3.669                 |

**Figure 6.** Standard mixture chromatograms of compounds obtained at flow rates of 1.5 mL/min, 1.2 mL/min, 1.0 mL/min, 0.8 mL/min and 0.5 mL/min
The Rs values of the Rhein-I.S peak pair were calculated as 1.824 and 1.920 at flow rates of 0.8 mL/min and 1.0 mL/min, respectively; it gradually decreased after flow rate of 1.0 mL/min. According to these results, the optimum flow rate in the study was determined as 1.0 mL/min.

In the literature, it had been reported that 1.0 mL/min flow rate was generally used in the analysis of aloe-emodin, rhein, emodin, chrysophanol and physcion by HPLC with different mobile phases (Uzun & Demirezer, 2019; Gautam et al., 2011; VanMen et al., 2012; Zou et al., 2008; He et al., 2009). Shi et al. (Shi et al., 2014) used as 0.8 mL/min and Wang et al. (Wang et al., 2013) used 0.5 mL/min flow rate in their studies. Thus, the flow rate of 1.0 mL/min determined in this study is compatible with the general results obtained in the literature.

**Column temperature**

In order to determine the optimum column temperature, five different column temperatures were studied (20 °C, 25 °C, 30 °C, 35 °C and 40 °C) and resulting chromatograms are given in the Figure 7. It is seen from the chromatograms that the retention times of the compounds decrease with the increasing temperature, but the peaks become closer to each other. In chromatographic studies, it is desired a short retention time and also high resolution for the studied substances.
Figure 7. Standard mixture chromatograms of the compounds obtained at column temperatures of 20 °C, 25 °C, 30 °C, 35 °C and 40 °C. The data obtained in the column temperature optimization study are given in Table 5. It was found that as the column temperature increased, the resolution value of the Rhein-I.S peak pair decreased and this value was less than 1.5 after 30 °C. The Figure 7 shows that as the temperature increases, the peaks get close to each other, and the Rhein-I.S peaks do not separate at 35 °C and 40 °C. Therefore, 20 °C column temperature was chosen since the resolution of Rhein and I.S peaks was the highest.

Table 5. Comparison of values obtained for column temperature optimization

| Column Temperature | Compounds       | k2   | α    | k2/(k2+1) | (α-1)/α | (1/4)√N | Rs  |
|--------------------|-----------------|------|------|-----------|----------|---------|-----|
| 20 °C              | I.S/Aloe-eminod | 1.685| 1.129| 0.628     | 0.114    | 31.428  | 2.250|
|                    | Rhein/I.S       | 1.906| 1.131| 0.656     | 0.116    | 26.682  | 2.030|
|                    | Emodin/Rhein    | 2.514| 1.319| 0.715     | 0.242    | 35.135  | 6.079|
|                    | Chrysophanol/Emodin | 2.806 | 1.116 | 0.737  | 0.104  | 36.819  | 2.822|
|                    | Physcion/Chrysophanol | 3.212 | 1.145 | 0.763 | 0.127 | 37.114 | 3.596 |
| 25 °C              | I.S/Aloe-eminod | 1.879| 1.135| 0.653     | 0.119    | 32.092  | 2.494|
|                    | Rhein/I.S       | 2.074| 1.104| 0.675     | 0.094    | 27.217  | 1.727|
|                    | Emodin/Rhein    | 2.719| 1.311| 0.731     | 0.237    | 35.491  | 6.079|
|                    | Chrysophanol/Emodin | 3.051 | 1.122 | 0.753 | 0.109 | 37.072 | 3.043 |
|                    | Physcion/Chrysophanol | 3.584 | 1.175 | 0.782 | 0.149 | 39.347 | 4.585 |
| 30 °C              | I.S/Aloe-eminod | 1.834| 1.145| 0.647     | 0.127    | 31.999  | 2.629|
|                    | Rhein/I.S       | 2.000| 1.091| 0.667     | 0.083    | 27.692  | 1.533|
|                    | Emodin/Rhein    | 2.626| 1.313| 0.724     | 0.277    | 35.218  | 7.063|
|                    | Chrysophanol/Emodin | 2.967 | 1.130 | 0.748 | 0.115 | 36.791 | 3.165 |
|                    | Physcion/Chrysophanol | 3.484 | 1.174 | 0.777 | 0.148 | 38.715 | 4.452 |
| 35 °C              | I.S/Aloe-eminod | 1.786| 1.155| 0.641     | 0.134    | 32.221  | 2.768|
|                    | Rhein/I.S       | 1.918| 1.073| 0.657     | 0.068    | 28.147  | 1.257|
|                    | Emodin/Rhein    | 2.526| 1.316| 0.716     | 0.240    | 35.368  | 6.078|
|                    | Chrysophanol/Emodin | 2.877 | 1.138 | 0.742 | 0.121 | 34.852 | 3.129 |
|                    | Physcion/Chrysophanol | 3.379 | 1.174 | 0.772 | 0.148 | 40.117 | 4.584 |
| 40 °C              | I.S/Aloe-eminod | 1.791| 1.162| 0.642     | 0.139    | 32.445  | 2.895|
|                    | Rhein/I.S       | 1.892| 1.056| 0.654     | 0.053    | 28.350  | 0.983|
|                    | Emodin/Rhein    | 2.488| 1.315| 0.713     | 0.240    | 35.516  | 6.077|
|                    | Chrysophanol/Emodin | 2.857 | 1.148 | 0.741 | 0.129 | 35.152 | 3.360 |
|                    | Physcion/Chrysophanol | 3.350 | 1.172 | 0.770 | 0.147 | 37.565 | 4.252 |

As a result of the literature review, it is seen that the column temperature of 25 °C was usually determined in HPLC methods (Zou et al., 2008; Wei et al., 2013; He et al., 2009; Ahmad et al., 2014). In the studies conducted by Feng et al., (Feng et al., 2017) and Sharma et al.(Sharma et al., 2012), 30 °C was used as the column temperature. Wang et al. (Wang et al., 2013) determined the column temperature as 35 °C; Gao et al. (Gao et al., 2009) and Shi et al. (Shi et al., 2014) determined it as 40 °C.

**Optimum conditions**

For the method to be used in the separation of compounds, three different columns, two different mobile phases B, five different mobile phase A compositions, five different flow rates and five different column temperatures were evaluated and the optimum separation conditions were determined (Table 6).

Table 6. The Optimum Chromatographic Conditions

| Column         | Supelcosil LC-18 (250 mm × 4,6mm, |
|----------------|----------------------------------|
| Wavelength     | 225 nm                           |
| Injection volume | 20 µL                            |
| Mobile Phase A | dH2O + 0.5% orthophosphoric acid |
| Mobile Phase B | Methanol                         |
Flow rate | 1.0 mL/min
---|---
Column | 20 °C
Injection time | 20 min

Separation of compounds at optimum conditions

The standard mixture chromatogram obtained under the optimized condition is given in the Figure 8. The retention times were found to be 8.413, 9.806, 11.858, 12.840 and 14.579 minutes for aloe-emodin, rhein, emodin, chrysophanol and physcion, respectively. This study on the simultaneous separation and determination of the anthraquinones was compared with the results from previous studies. In the studies of Chen et al., 2020; Rong et al., 2011 and Zhan et al., 2017; they separated these five anthraquinones in 22.5, 24.44 and 40.0 minutes, respectively. In this study separated five anthraquinones only 15 minutes. It observed that the developed method is sensitive, effective and selective and the retention time is shorter (Wang et al., 2016; Wei et al., 2013; Chen et al., 2020; Rong et al., 2011; Zhan et al., 2017).

Figure 8. Chromatogram of standard mixture (1-Aloe-emodin, 2-I.S, 3-Rhein, 4-Emodin, 5-Chrysophanol, 6-Physcion)

The chromatographic data obtained under optimum conditions are shown in Table 7. Under the proposed optimization conditions, good baseline resolution was obtained for the aloe-emodin, the rhein, the emodin, the chrysophanol and the physcion.

Table 7. The capacity factors, selectivity, and resolution factor values for the compounds studied

| Compounds                 | k2   | α      | (k2+1) | (α-1)/α | 1/(4√N) | Rr   |
|---------------------------|------|--------|--------|---------|---------|------|
| I.S/Aloe-emodin           | 1.685| 1.129  | 0.628  | 0.114   | 31.428  | 2.250|
| Rhein/1S                  | 1.906| 1.131  | 0.656  | 0.116   | 26.682  | 2.030|
| Emodin/Rhein              | 2.514| 1.319  | 0.715  | 0.242   | 35.135  | 6.079|
| Chrysophanol/Emodin       | 2.806| 1.116  | 0.737  | 0.104   | 36.819  | 2.822|
| Physcion/Chrysophanol     | 3.212| 1.145  | 0.763  | 0.127   | 37.114  | 3.596|

4. Conclusions and Recommendations

In this study, an RP-HPLC method was successfully developed for the simultaneous separation of the five anthraquinones compounds (aloe-emodin, rhein, emodin, chrysophanol and physcion). The developed method was optimized properly utilizing a Supercosil LC-18 column at the 20 °C with a 0.5% orthophosphoric acid-water mixture as the mobile phase A and methanol as the mobile phase B. The injection volume of 20 µL and the flow rate of 1.0 mL/min were studied with a DAD detector at 225 nm. In determination of the optimum separation conditions the capacity factor, the selectivity and the resolution parameters were all found to be above the reference values and five different standards were separated efficiently in 15 minutes. Therefore, this efficient RP-HPLC method can be recommended as the preferred separation method for the anthraquinones as both determination and routine analysis.

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