



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

Gizem Yıldırım Baştemur<sup>1,2,\*</sup>, Reyhan Akpınar<sup>3</sup>, Sabriye Perçin Özkorucuklu<sup>4</sup>, Esengül Kır<sup>5</sup>

<sup>1\*</sup> Süleyman Demirel University, Institute of Graduate Studies in Sciences, Programme of Chemistry, Isparta, Turkey, (ORCID: 0000-0002-4634-4525), gizemyildirimbastemur@istanbul.edu.tr

<sup>2</sup> Istanbul University, Faculty of Science, Department of Molecular Biology and Genetics, İstanbul, Turkey, (ORCID: 0000-0002-4634-4525), gizemyildirimbastemur@istanbul.edu.tr

<sup>3</sup> Istanbul University, Institute of Graduate Studies in Sciences, Programme of Molecular Biotechnology and Genetics, İstanbul, Turkey, (ORCID: 0000-0001-5910-4160), reyhanakpnr1@gmail.com

<sup>4</sup> Istanbul University, Faculty of Science, Department of Molecular Biology and Genetics, İstanbul, Turkey, (ORCID: 0000-0001-9778-2034), sabriyeo@istanbul.edu.tr

<sup>5</sup> Süleyman Demirel University, Faculty of Art and Science, Department of Chemistry, Isparta, Turkey, (ORCID: 0000-0002-6641-3444), esengulkir@sdu.edu.tr

(First received 26 August 2021 and in final form 28 November 2021)

(DOI: 10.31590/ejosat.987628)

**ATIF/REFERENCE:** Yıldırım Baştemur, G., Akpınar, R., Perçin Özkorucuklu, S. & Kır, Esengül. (2021). Optimization of a Reverse-Phase High Performance Liquid Chromatography (RP-HPLC) Method for Simultaneous Separation of Aloe-Emodin, Rhein, Emodin, Chrysophanol and Physcion. *European Journal of Science and Technology*, (27), 972-984.

## 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 × 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 ayırma verimliliğine, yüksek saptama hassasiyetine ve üstün seçiciliğe sahip bir ters fazlı yüksek performanslı sıvı kromatografisi (RP-HPLC) yöntemi başarıyla geliştirilmiştir. Optimum yöntem koşulları, çeşitli kolonlar, mobil fazlar ve bileşimleri, akış hızları ve kolon sıcaklıkları çalışılarak belirlenmiştir. Ayırma, Supelcosil LC-18 kolonu (250 x 4.6 mm, 5 µm) ile gradient programda gerçekleştirilmiştir. Prosedür 20 °C'de, 1,0 mL/dk akış hızı ve 20 µL enjeksiyon hacmi ile 225 nm'de DAD dedektörlü bir RP-HPLC yöntemi kullanılarak gerçekleştirilmiştir. Mobil faz A ve B olarak %0,5 (v/v) ortofosforik asit içeren deiyonize su ve metanol kullanılmıştır. Bu çalışma, antrakinon bileşiklerinin kalitatif tayini için optimize edilmiş bir analitik yöntemin etkin bir şekilde kullanılabileceğini göstermiştir.

**Anahtar Kelimeler:** Yüksek Performans Sıvı Kromatografisi, Antrakinon, Sekonder Metabolitler, Metot Optimizasyonu.

\* Corresponding Author: gizemyildirimbastemur@istanbul.edu.tr

## 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-micro 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 immunosuppressive, antiinflammatory, antitumor, anticancer (You et al., 2013). Additionally, the physcion 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, 2005), the Capillary ElectroChromatography (CEC) (Lü 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&Ganzer, 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 (ElSohly 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 facilitates 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 ( $t_0$ ) 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 Mediterranean 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; and 16-18 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 ( $t_0$ ) 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 aloec-emodin, rhein, emodin, chrysophanol and physicon 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

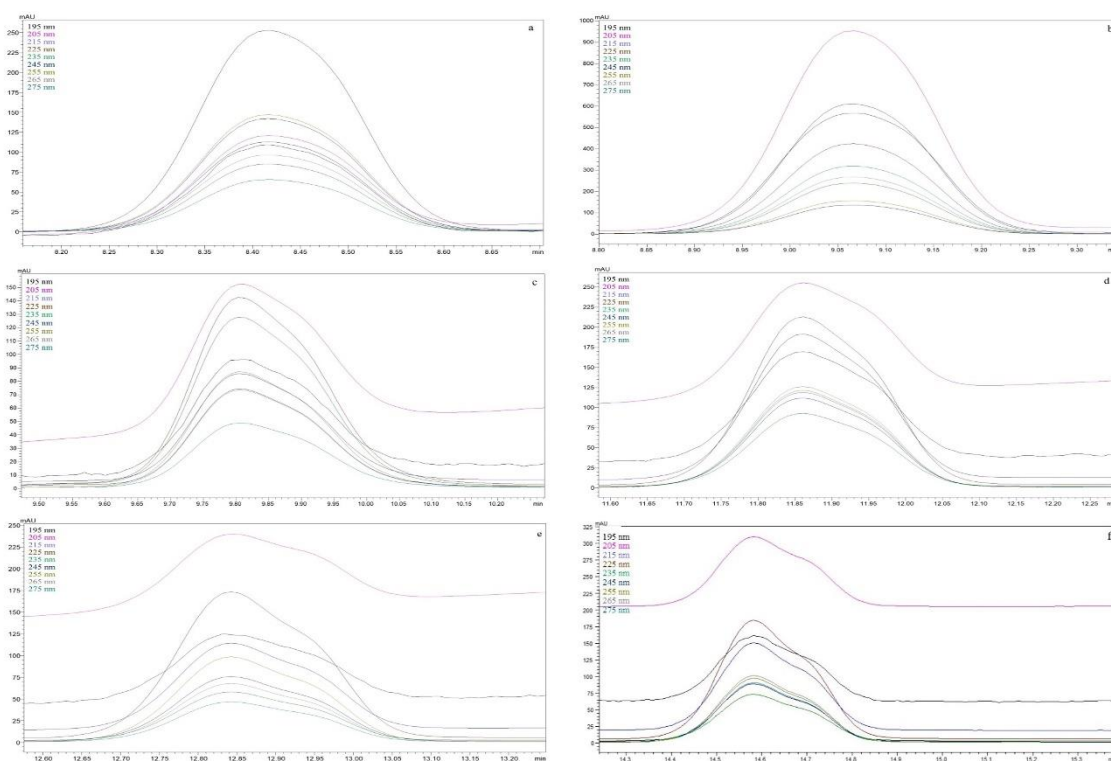
fundamental chromatographic parameters, the resolution,  $R_s$ , is affected by three independent variables:

$$R_s = (1/4) \sqrt{N} [(\alpha-1)/\alpha] [k_2/(k_2-1)] \quad \text{Eq. (1)}$$

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 ( $R_s$ ) values must be greater than 1.5. For all optimization parameters  $R_s$  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).



**Figure 1.**  $\lambda_{max}$  values of the compounds obtained by using DAD detector (a-Aloe-emodin, b-I.S c-Rhein, d-Emodin, e-Chrysophanol, f-Physicon)

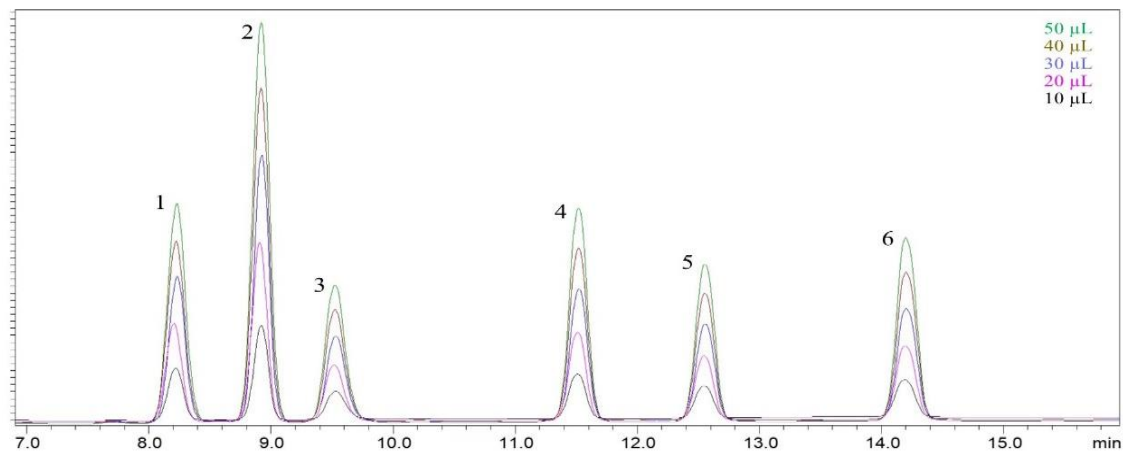
In the literature, the following wavelength results in the quantitative determination studies of aloec-emodin, rhein, emodin, chrysophanol and physicon were reported: Uzun and Demirezer (Uzun&Demirezer, 2019) at 440 nm (DAD detector), Liu et al., (Liu et al., 1997) at 280 and 450 nm (DAD detector), Gautam 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.

#### Injection volume

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

chromatograms in the Figure 2 were obtained which shows that the peak shapes do not change with the different injection volumes. Therefore, a 20  $\mu\text{L}$  injection volume was chosen as the optimum value to minimize material losses.

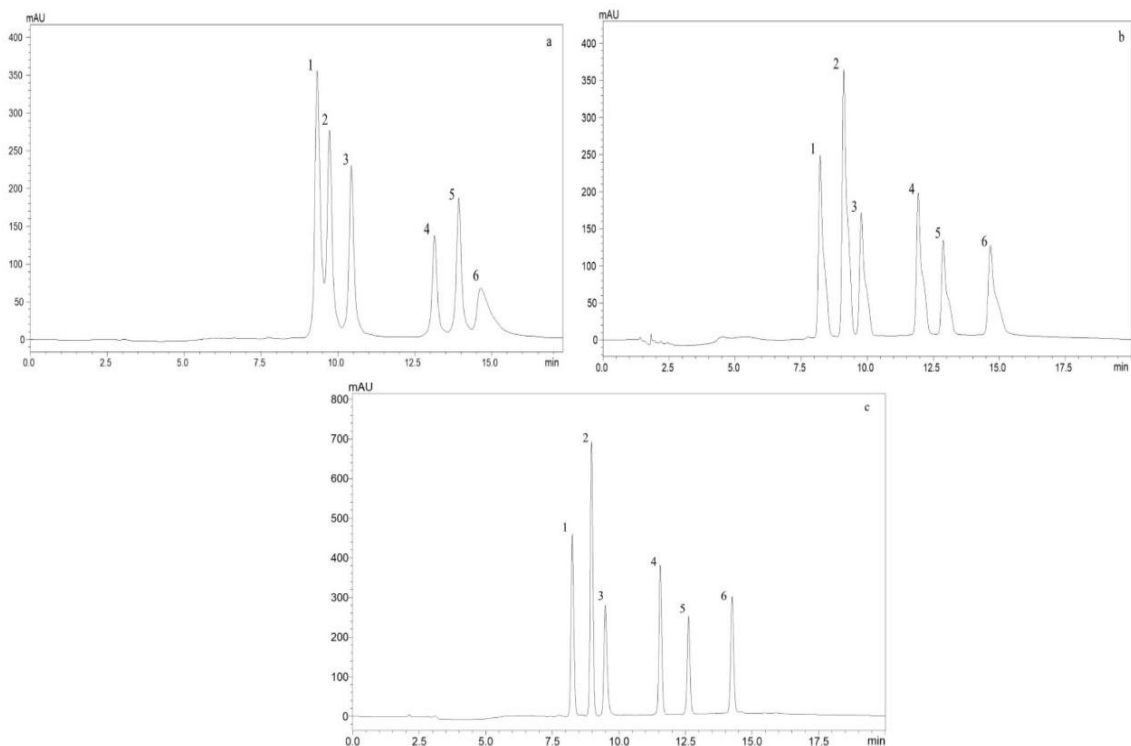


**Figure 2.** The standard mixture chromatograms obtained from different injection volumes of the compounds (1-Aloe-emodin, 2-I.S 3-Rhein, 4-Emodin, 5-Chrysophanol, 6-Physicon)

### 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  $\times$  4.6mm, 5 $\mu\text{m}$ ), Cogent Phenyl Hydride (250 mm  $\times$  4.6mm, 4 $\mu\text{m}$ ) and Supelcosil LC-18 (250 mm  $\times$  4.6mm, 5 $\mu\text{m}$ )) 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 ( $R_s$ ) values below 1.5 were obtained for I.S-Aloe-emodin and Physicon-Chrysophanol peak pairs in the Cogent Phenyl Hydride column and as clearly seen from the Figure 3a, these peaks were not separated effectively. The  $R_s$  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-Physcion) in different columns a) Cogent Phenyl Hydride, b) Mediterranean Sea 18 c) Supelcosil LC-18

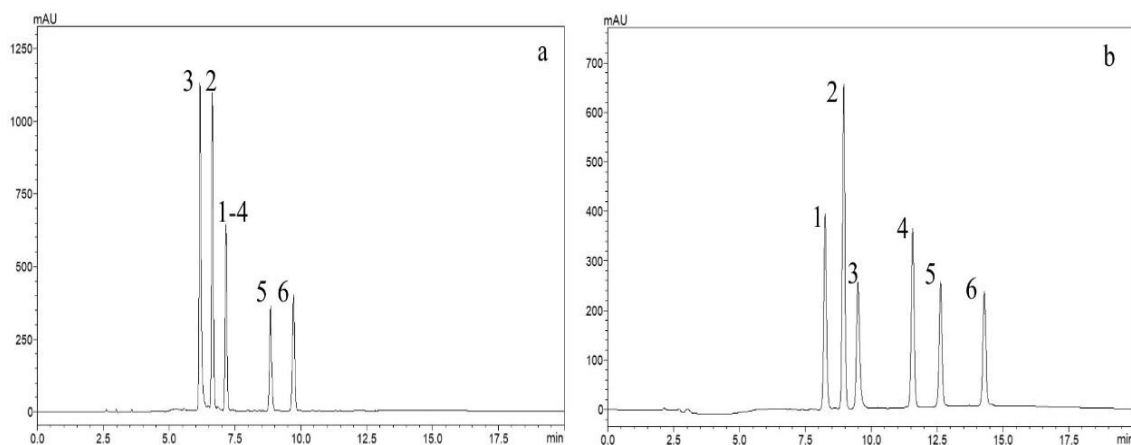
**Table 1.** Comparison of the values obtained for all the compounds in the different columns

Column	Compounds	$k_2$	$\alpha$	$k_2/(k_2+1)$	$(\alpha-1)/\alpha$	$(1/4)\sqrt{N}$	$R_s$
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.

**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.



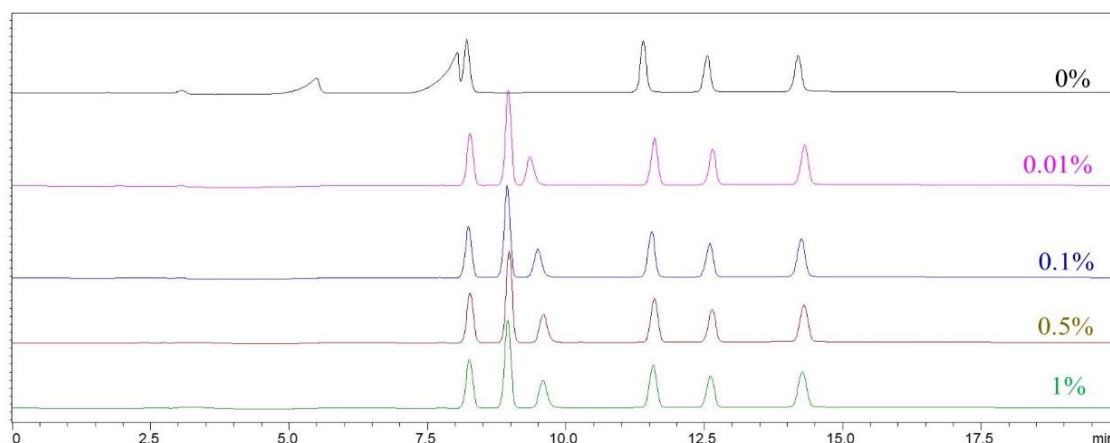
**Figure 4.** Standard mixture chromatograms of the different compounds (1-Aloe-emodin, 2-I.S 3-Rhein, 4-Emodin, 5-Chrysophanol, 6-Physcion) obtained in a) Acetonitrile b) Methanol

**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-emodin	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; Rafaely 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 the 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-emodin	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-emodin	1.910	1.134	0.656	0.118	42.071	3.257

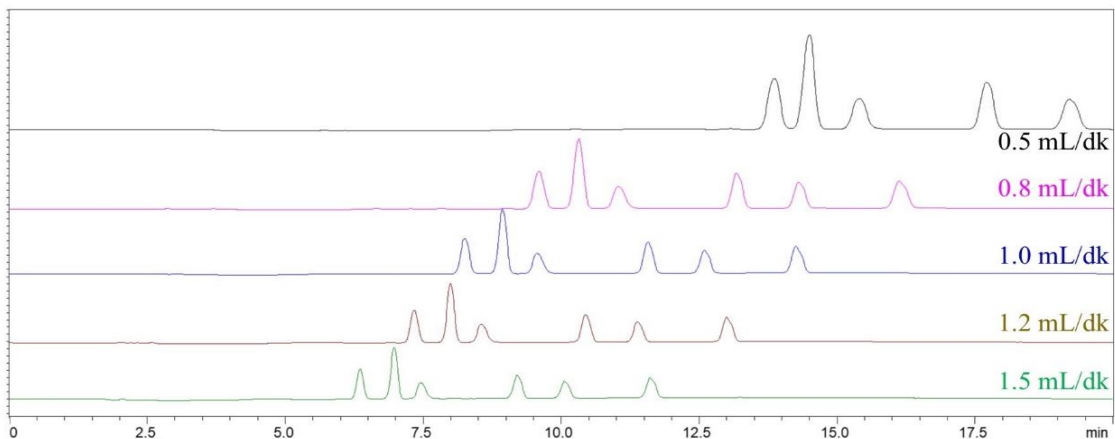
	Rhein/I.S	2.039	1.068	0.671	0.064	35.969	1.545
	Emodin/ Rhein	2.769	1.358	0.735	0.264	49.089	9.525
	Chrysophanol/Emodin	3.111	1.124	0.757	0.110	52.524	4.374
	Physcion/Chrysophanol	3.652	1.174	0.785	0.148	54.959	6.385
<b>0.1%</b>	I.S/Aloe-emodin	1.929	1.135	0.659	0.119	40.547	3.180
	Rhein/I.S	2.112	1.095	0.679	0.087	35.051	2.071
	Emodin/ Rhein	2.789	1.321	0.736	0.243	46.793	8.369
	Chrysophanol/Emodin	3.132	1.123	0.758	0.110	49.586	4.134
	Physcion/Chrysophanol	3.676	1.174	0.786	0.148	52.479	6.105
<b>0.5%</b>	I.S/Aloe-emodin	1.888	1.137	0.654	0.120	40.086	3.146
	Rhein/I.S	2.086	1.105	0.676	0.095	34.681	2.227
	Emodin/ Rhein	2.734	1.311	0.732	0.237	45.557	7.903
	Chrysophanol/Emodin	3.070	1.123	0.754	0.110	48.225	4.000
	Physcion/Chrysophanol	3.604	1.174	0.783	0.148	50.603	5.864
<b>1.0%</b>	I.S/Aloe-emodin	1.927	1.134	0.658	0.118	38.738	3.008
	Rhein/I.S	2.135	1.108	0.681	0.097	33.722	2.227
	Emodin/ Rhein	2.786	1.305	0.736	0.234	44.035	7.584
	Chrysophanol/Emodin	3.126	1.122	0.758	0.109	46.448	3.838
	Physcion/Chrysophanol	3.669	1.174	0.786	0.148	48.941	5.593

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.



**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

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

Flow rate (mL/min)	Compounds	$k_2$	$\alpha$	$k_2/(k_2+1)$	$(\alpha-1)/\alpha$	$(1/4)\sqrt{N}$	$R_s$
0.8 mL/min	I.S/Aloe-emodin	1.678	1.124	0.627	0.110	32.576	2.247
	Rhein/I.S	1.864	1.110	0.651	0.099	28.303	1.824
	Emodin/ Rhein	2.419	1.297	0.708	0.229	37.775	6.125
	Chrysophanol/Emodin	2.710	1.120	0.730	0.107	39.745	3.104
	Physcion/Chrysophanol	3.183	1.175	0.761	0.149	37.524	4.255
1.0 mL/min	I.S/Aloe-emodin	1.883	1.133	0.653	0.117	32.632	2.493
	Rhein/I.S	2.086	1.108	0.676	0.097	29.275	1.920
	Emodin/ Rhein	2.734	1.311	0.732	0.237	36.529	6.337
	Chrysophanol/Emodin	3.064	1.121	0.754	0.108	38.213	3.112
	Physcion/Chrysophanol	3.599	1.175	0.783	0.149	40.324	4.704
1.2 mL/min	I.S/Aloe-emodin	2.100	1.133	0.677	0.117	31.409	2.488
	Rhein/I.S	2.317	1.103	0.699	0.093	26.581	1.728
	Emodin/ Rhein	3.050	1.316	0.753	0.240	34.641	6.260
	Chrysophanol/Emodin	3.415	1.120	0.773	0.107	36.375	3.009
	Physcion/Chrysophanol	4.042	1.184	0.802	0.155	40.384	5.020
1.5 mL/min	I.S/Aloe-emodin	2.416	1.144	0.707	0.126	29.956	2.669
	Rhein/I.S	2.652	1.098	0.726	0.089	25.583	1.653
	Emodin/ Rhein	3.510	1.324	0.778	0.245	32.153	6.129
	Chrysophanol/Emodin	3.930	1.120	0.797	0.107	33.930	2.894
	Physcion/Chrysophanol	4.695	1.195	0.824	0.163	37.541	5.042

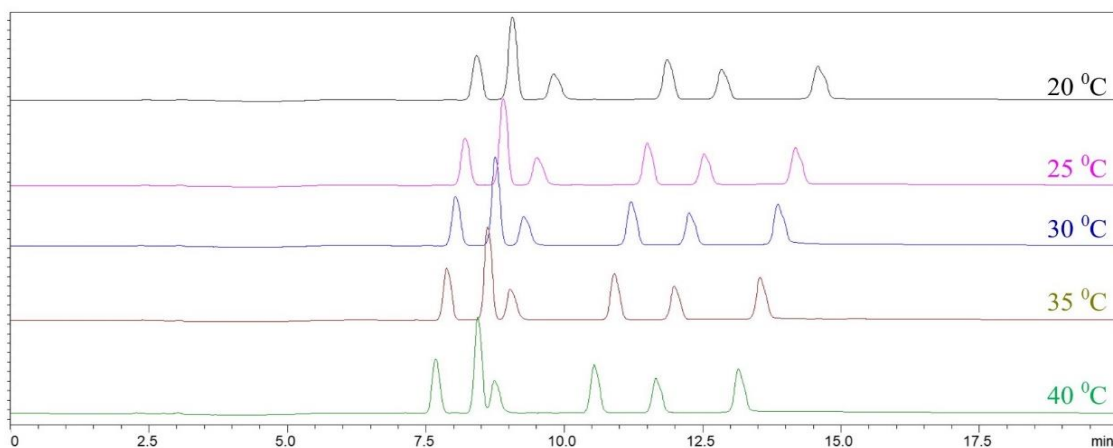
The  $R_s$  values of the Rhein-I.S peak pair were calculated as 1.824 and 1.920 at flow rates of 0.8 mL/min and 1.0 mL/min, respectively; it gradually decreased after flow rate of 1.0 mL/min. According to these results, the optimum flow rate in the study was determined as 1.0 mL/min.

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	$k_2$	$\alpha$	$k_2/(k_2+1)$	$(\alpha-1)/\alpha$	$(1/4)\sqrt{N}$	$R_s$
20 °C	I.S/Aloe-emodin	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-emodin	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	6.149
	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-emodin	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-emodin	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-emodin	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).

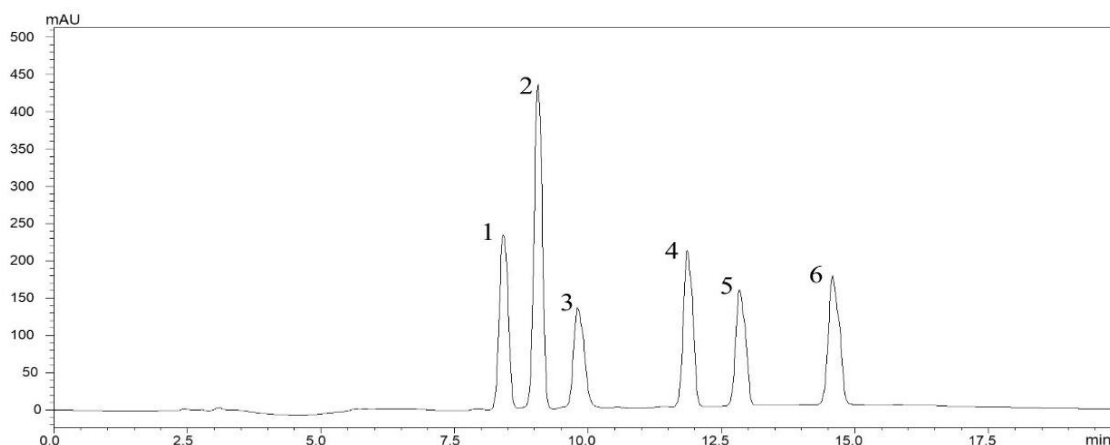
**Table 6.** The Optimum Chromatographic Conditions

Column	Supelcosil LC-18 (250 mm × 4,6mm,
Wavelength	225 nm
Injection volume	20 µL
Mobile Phase A	dH <sub>2</sub> O + 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



**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,

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

Compounds	$k_2$	$\alpha$	$k_2/(k_2+1)$	$(\alpha-1)/\alpha$	$(1/4)\sqrt{N}$	$R_s$
I.S/Aloe-emodin	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

## 4. Conclusions and Recommendations

In this study, an RP-HPLC method was successfully developed for the simultaneous separation of the five anthraquinone 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  $\mu$ L 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

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).

good baseline resolution was obtained for the aloe-emodin, the rhein, the emodin, the chrysophanol and the physcion.

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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