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Analysis of phenolic acids in hyssop (Hyssopus officinalis L.) by HPLC-DAD with monolithic column Chromolith™ RP-18e

Analiza zawartości kwasów fenolowych występujących w hyzopie lekarskim przy użyciu HPLC-DAD z kolumną monolityczną Chromolith™ RP-18e

INTRODUCTION

Phenolic acids are a group of second metabolites widely occurring in various plants. They play an important part in growth and reproduction processes. They are also produced in response to injuries in order to defend the plant from pathogens. Phenolic acids posses many different pharmacological properties and are widely used in traditional and modern medicine [10,14].

The anti-inflammatory properties of phenolic acids are attributable to (e.g. rosmarinic or caffeic acid) the inhibition of lipoxygenases and cyclooxygenases as well as interference with the complement cascade [7,9,12,15].

The important feature of phenolic acids is their immunomodulating activity (e.g. rosmarinic, gentisic, chlorogenic and caffeic acids) [16].

In the event of a complex plant matrix, selection of appropriate chromatographic conditions for HPLC is a matter of great importance as well as a potential analytical problem. For example, selection of optimal mobile phase composition or mobile phase gradient is often based on trial-and-error approach, which is indubitably extremely time consuming and expensive.

In the present study the ChromSword® software (Merck KGaA, Darmstadt, Germany) was employed in the separation of mixture of various phenolic acids in a plant extract sample. The main concept of computer-assisted method development is to receive information from the chromatographer about the mixture to be separated, and then to apply computer simulation to predict results for different chromatographic conditions, thus finding the ideal conditions for separating the mixture. It is well known that optimizing of a gradient profile by trial-and-error method is often very complicated, and moreover, it is costly and time-consuming. The more breakpoints on the gradient profile and the more complicated the sample – the more time needed for optimization [2].

The gradient profile was optimized on the basis of only two initial chromatographic runs (two linear gradients with different slopes).

Additionally, the software used in this study did not contain any database of the characteristic of the applied monolithic column, i.e., Chromolith™ RP-18e, 100 x 4.6 mm (Merck, Germany), but nevertheless the collected information about many different commercial reversed-phase columns together with various types of mobile phases, allowed us to predict the retention behavior of analytes.

Monolithic columns offer unique characteristics leading to rapid analysis times, and compared to conventional column technology, allow for a substantial reduction in the pressure requirements to drive the mobile phase through the chromatographic column [1,4-6,8,11,13].

EXPERIMENTAL

Apparatus and reagents. High Performance Liquid Chromatography was performed on a *LaChrom® Elite* HPLC System (Merck KGaA, Darmstadt, Germany) equipped with autosampler (L- 2200), 20 µl sample loop, HPLC Pump (L-2100) with on-line degassing system, Chromolith™ RP-18e column (100 x 4.6 mm, Merck), UV detector (L-2400) and Diode Array Detector (D-7455). The data obtained were analyzed with *EZChromelite* HPLC software, version 2.2 (Merck), together with ChromSword HPLC method optimization software (Merck KGaA, Darmstadt, Germany).

Reagents for HPLC analysis**,** acetonitrile (Merck, Germany), acetic acid (POCh, Poland) and water were all of chromatographic grade. Methanol, diethyl ether, sodium sulphate (POCh, Poland)**,** used for isolation of phenolic acids and other operations concerning sample preparation**,** were of analytical grade. Standards of phenolic acids were purchased from Roth (Germany)**.** Working standard solutions, 10-100 µg/mL, were prepared by dilution from stock standard solutions with acetonitrile.

Plant material. Hyssop (*Hyssopus officinalis* L.) was grown in the herb garden at Faculty of Pharmacy, Medical University in Lublin, Poland (N 51°16’ E 22°34’). Aerial parts of hyssop were harvested during flowering stage in August 2005. The taxonomic identification was confirmed by plant taxonomist, Stanislaw Kwiatkowski, at the Dept. of Pharmacognosy with the Medicinal Plant Unit, Medical University in Lublin, Poland. After identification, plant material was dried at 35ºC and ground. The voucher specimen was deposited at the Herbarium of the Dept. of Pharmacognosy, Medical University in Lublin, Poland (No. 0501).

Sample preparation. Dried and pulverized sample (10 g) of hyssop was extracted with methanol (50 mL) under reflux condenser in a water bath for 30 min. After the extraction process, the liquid was decanted and the plant material was re-extracted with another portion (50 mL) of methanol. The extraction procedure was then performed one more time. All supernatants were combined and the solvent was evaporated under reduced pressure (Vacuum Rotary Evaporator type 350P, Unipan, Poland). The dried residue was rinsed with 100 mL of hot distillated water and left for 12 hours in a refrigerator. Afterwards, the aqueous fraction was extracted (liquid-liquid extraction) with diethyl ether (10 x 50 mL and 20 x 30 mL). Ether fractions were combined, dried with anhydrous sodium sulphate and filtered. Subsequently, the ether was removed under reduced pressure and the residue containing phenolic acids was dissolved in 10 mL of a mixture containing acetonitrile and methanol.

RESULTS AND DISCUSSION

Optimization of RP-HPLC method.

Since the Chromolith™ RP-18e chromatographic column was not available in the software's database, RP-HPLC method was optimized using the empirical approach. At the beginning, the system started with chosen starting conditions (80% ACN: 20% H_2O). After data collection, the second chromatographic run was proposed by the system. On the basis of the second run, an empirical linear retention model was set up from the observed retention data. Thus, the application of the following conditions, and the possession of data of three different chromatographic conditions facilitated setting of the quadratic retention model (the system performs further optimization process cycles until the retention model is accurate enough to predict optimum separation conditions).

Applying the retention models established before, the ChromSword® generated gradient profiles and established the best gradient profile automatically using Monte-Carlo optimization method [2].

The use of ChromSword® software assisted in the development of the composition of the mobile phase suitable for qualitative and quantitative analysis of phenolic acids. Chromatographic conditions are listed in Table 1. The Chromolith™ RP-18e column was flushed for 5 min. with 100% ACN and later equilibrated in mobile phase before each injection.

Table 1. Conditions of chromatographic HPLC analysis

Temperature	26 ± 1°C		
Pressure	5.781 MPa		
Flow rate	2 ml/min		
Sample loop	20 µL		
Mobile phase composition	A – HPLC grade water + 1% acetic acid B – 100% acetonitrile		
	Time	Gradient composition	
	0 min	1% B	99 % A
	2.5 min	5% B	95% A
	9.2 min	16% B	84% A

Phenolic acid identification was performed by comparing their retention times (t_R) with t_R standards, and by determining their spectra in UV-light (220-400 nm). Contents of particular phenolic acids in analyzed fractions were calculated on a base of calibration curves plotted as dependence of area surface under peaks and concentration for diluted standard phenolic acids.

The data obtained during analysis of phenolic acids standards (dissolved in methanol), namely retention times, statistical evaluation, limits of detection (LOD) and linearity results, are shown in Table 2.

Table 2. Data of HPLC-DAD analysis of phenolic acids standards

Phenolic acid	Retention time t_R	Standard deviation (n=3)*	Coefficient of variation	LOD** (μg/L)	Linear correlation coefficient (n_p=6) *** (%)
gallic	1.65	0.24	14.54	7.9	99.90
protocatechuic	2.80	0.16	5.71	1.98	99.90
gentisic	3.41	0.16	4.69	2.52	99.87
chlorogenic	3.84	0.08	2.08	2.4	99.75
caffeic	4.13	0.19	4.60	2.6	99.10
ferulic	5.01	0.02	0.40	1.9	99.91
rosmarinic	5.57	0.02	0.35	3.2	99.51

* n - number of injections

**LOD – limit of detection

*** n_p - number of data points considered for the linear regression

The optimized conditions of analysis made it possible to obtain satisfactory separation of standard phenolic acid mixtures, as well as low detection limits – 1.9 to 7.9 μg/L. The linearity of the HPLC method was investigated for phenolic acids standards in the range of 0.1-100 μg/mL at six concentration levels. The obtained results of linearity ranging from 99.1 to 99.91% suggest high accuracy of quantitative analysis.

Application of optimized HPLC method to the analysis of phenolic acids in hyssop.

Table 3 presents retention time and content of phenolic acids present in aerial part of *Hyssopus officinalis* L. Fig. 1 presents chromatogram HPLC of investigated plant.

Table 3. Data of retention times and content of determined phenolic acids from aerial part of *Hyssopus officinalis* L.

Phenolic acid	Retention time t_R	Content of compounds (μg/g dry wt)
gallic	1.90	0.089
protocatechuic	2.90	254
Gentisic	3.60	496
chlorogenic	3.75	0.026
caffeic	4.30	1674
ferulic	5.20	432
rosmarinic	5.60	2590

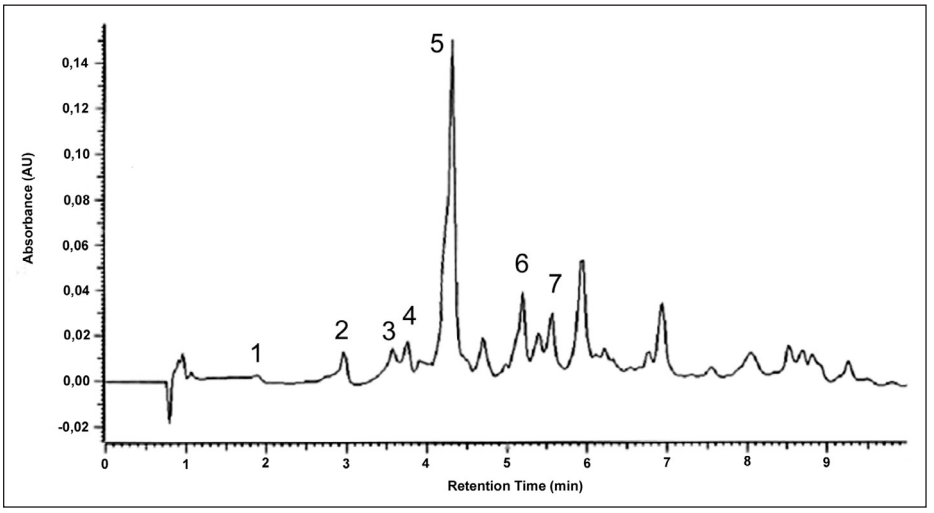

Figure 1. Chromatogram factions from aerial parts of *Hyssopus officinalis* L. Peak identification: 1. gallic acids, 2. protocatechuic acid, 3. gentisic acid, 4. chlorogenic acid, 5. caffeic acid, 6. ferulic acid, 7. rosmarinic acid. Chromatographic conditions see tab. 1.

In the analysis of free phenolic acids present in *Hyssopus officinalis* L., high amounts of rosmarinic (2590 μg/g) and caffeic acid (1674 μg/g) were noted. In comparison, the levels of gallic (0.089 μg/g) and chlorogenic acid (0.026μg/g) were very low (Tab. 3). HPLC-DAD analysis confirmed literature data [16] concerning occurrence of protocatechuic, gentic, chlorogenic, caffeic, ferulic and rosmarinic acid in the herb of hyssop. Furthermore, the combined use of the monolithic column and optimized mobile phase gradient made the identification and quantification of gallic acid in this plant possible for the first time.

CONCLUSIONS

The use of ChromSword® computer simulation program for optimization of phenolic acid separation on Chromolith™ RP-18e monolith column made very fast analysis possible. The automated method development system created a method with considerably shorter run times (6 min.).

Typically, the analysis of phenolic acids in real samples takes 21 min. (using RP-HPLC gradient) [3] and efficient separation of standard substances and constituents of the plant extract.

The application of optimized HPLC-DAD method to the analysis of free phenolic acids in *Hyssopus officinalis* L. confirmed the Chromolith™ RP-18e as a very efficient column in quantitative and qualitative analysis. In addition, the ChromSword® software turned out to be a useful tool in predicting the retention behavior of analytes, appropriate mobile phase gradient and separation conditions. Thanks to the optimized method presented in this work, both the time of analysis, as well as the use of solvents, were significantly reduced in comparison to costly and time-consuming trial-and-error approach.

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SUMMARY

In the present study the ChromSword® software was used in order to optimize the chromatographic separation of selected phenolic acids in the monolithic stationary phase. A mobile phase gradient composed of a mixture of water, acetonitrile and acidic acid was used. The computer simulation of HPLC separation made obtaining optimal conditions of separation possible, as well as high linearity (linear correlation coefficient ranging from 99.1 to 99.9%), suggesting high accuracy of quantitative analysis. By using monolithic columns, less than 6 minutes were required to analyze seven phenolic acids. Very low limit of detection (LOD) values of phenolic acids, ranging from 1.9 to 7.9 μg/L, were achieved. The application of optimized mobile phase gradient for the analysis of phenolic acids occurring in *Hyssopus officinalis* L. allowed for confirmation of the presence of protocatechuic, gentisinic, chlorogenic, caffeic, ferulic, gallic and rosmarinic acids in this plant.

Keywords: Chromolith, ChromSword, HPLC, hyssop, *Hyssopus officinalis* L., phenolic acids

STRESZCZENIE

W prezentowanej pracy przedstawiono optymalizację rozdziału chromatograficznego kwasów fenolowych przy zastosowaniu oprogramowania komputeriwego ChromSword®. Zastosowano gradient fazy mobilnej woda, acetonitryl, kwas octowy. Symulacja komputerowa rozdziału kwasów fenolowych wykazała wysoką korelacje liniową od 99,1 do 99,9%. Użycie kolumny monolitycznej pozwoliło na skrócenie czasu rozdziału kwasów fenolowych do 6 minut, przy niskim limicie detekcji 1,9 do 7,9 μg/L. W ekstrakcie z hyzopu lekarskiego zidentyfikowano następujące kwasy fenolowe: protokatechowy, gentyzynowy, chlorogenowy, ferulowy, galusowy i rozmarynowy.

Słowa kluczowe: Chromolith, ChromSword, HPLC, hyzop lekarski, *Hyssopus officinalis* L., kwasy fenolowe.