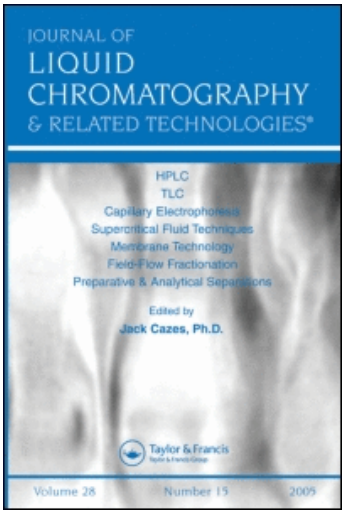

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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To cite this Article Schmidt, Alexander H.(2005) 'Development of an HPLC Method for the Determination of Hydroxycinnamic Acid Derivatives in *Cimicifuga racemosa* (Black Cohosh) Extracts Using an Automated Method Development System', Journal of Liquid Chromatography & Related Technologies, 28: 6, 871 — 881
To link to this Article: DOI: 10.1081/JLC-200051475
URL: http://dx.doi.org/10.1081/JLC-200051475

Journal of Liquid Chromatography & Related Technologies®, 28: 871–881, 2005

ISSN 1082-6076 print/1520-572X online
DOI: 10.1081/JLC-200051475

Development of an HPLC Method for the Determination of Hydroxycinnamic Acid Derivatives in *Cimicifuga racemosa* (Black Cohosh) Extracts Using an Automated Method Development System

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Abstract: The separation of a complex mixture, such as the ingredients in medicinal plants, is typically difficult and the development of a HPLC method is a labor-intensive and time-consuming process if carried out manually. Automation of this process can increase productivity of a pharmaceutical R&D department substantially.

This paper describes the development of a high performance liquid chromatographic method for the determination of hydroxycinnamic acid derivatives in *Cimicifuga racemosa* extracts and its preparations by using a fully automated method development system (Waters AMDS).

The developed method is based on the baseline chromatographic separation of six hydroxycinnamic acid derivatives (caffeic acid, ferulic acid, isoferulic acid, fukinolic acid, cimicifuga acid A, and cimicifuga acid B), the major constituents in *Cimicifuga racemosa* (Black Cohosh), on a XTerra MS C_{18} column with a water-methanol gradient and photodiode array detection.

Keywords: HPLC, Automated method development, *Cimicifuga racemosa* (black cohosh)

INTRODUCTION

Although high performance liquid chromatography (HPLC) is the most widespread tool for the analysis of complex mixtures like medicinal plant

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extracts, the method development process is very time-consuming and usually requires much expertise. Despite the advances made with chromatographic columns, the HPLC method development is still based mainly on trial-and-error experiments, and small changes in the experimental conditions often result in confusing peak movements.

In a previous paper, the method development using DryLab, a chromatography simulation software, was reported for the separation of kava pyrones in *Piper methysticum* extracts.[1,2] By using this software, it was possible to develop a HPLC method with baseline separation of all major constituents, with an analysis run-time of only 15 min.

DryLab has long had the capability of identifying the optimum gradient condition and the column temperature on the basis of retention data from four experimental initial runs.[3–6]

The recently introduced fully automated method development software package AMDS, is an interface between DryLab and Empower, the CDS software for system control, data acquisition, and data processing. DryLab systematically explores a range of options like stationary and organic mobile phase type, gradient time, temperature, and pH. It allows each variable to be explored on the basis of a limited number of experimental runs and uses the results from these runs to refine the method. AMDS automatically exchanges all result and peak information between DryLab and Empower.

In this present study, the AMDS software is used for the separation of hydroxycinnamic acid derivatives in *Cimicifuga racemosa.* Extracts and its phytopharmaceutical preparations of *Cimicifuga racemosa*, popularly known as Black Cohosh, have traditionally been used as a safe alternative to hormone-replacement therapy for the treatment of hot flashes and some other unpleasant symptoms of menopause.[7]

Because of the widespread interest in Black Cohosh, analytical methods are needed to identify and determine the constituents, which are responsible for the pharmacological properties.

A large number of compounds have been isolated from the plant including triterpene glycosides and hydroxycinnamic acid derivatives (Figure 1); most of them are esters of caffeic acid, ferulic acid, or isoferulic acid with fukiic acid or piscidic acid. The compound or combination of compounds responsible for the pharmacological activity of *Cimicifuga* are yet unknown, but the hydroxycinnamic acid derivatives have been reported to show numerous biological activities such as antioxidant activity, suppression of interleukin-8 production, and anti-inflammatory activity.[8,9]

Although a lot of analytical methods for the analysis of the triterpene glycosides have been published in recent years,[10–12] relatively few reports are available for the analysis of the hydroxycinnamic acid derivatives[13,14] and there is still a need for developing an easy and rapid method, which could be used for quality control of phytopharmaceutical preparations.

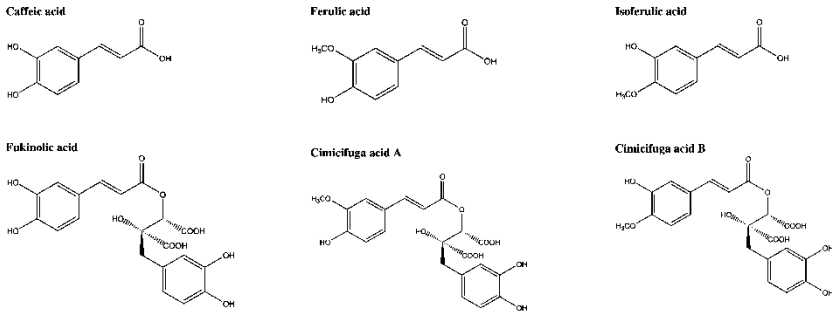

Figure 1. Chemical structures of the phenolic compounds from *Cimicifuga racemosa.*

EXPERIMENTAL

Chemicals

Solvent A was water pH = 2.0, prepared by adding o-phosphoric acid (Suprapur, Merck, Darmstadt, Germany) to water (Milli Q water purification system, Millipore, Eschborn, Germany) up to the desired pH. Solvent B was acetonitrile and solvent C was methanol and both were of HPLC-grade (LiChrosolv, Merck).

The columns used were a XTerra RP_{18}, 100 × 3.9 mm, 3.5 μm and a XTerra MS C_{18}, 100 × 3.9 mm, 3.5 μm (Waters, Eschborn, Germany).

Samples and Standards

For the method development, a dried hydroalcoholic extract of *Cimicifuga racemosa* (Steiner and Co., Berlin, Germany) with a weight ratio drug/extract of 4.5–8.5 : 1 was used.

The reference standards of caffeic acid and ferulic acid were purchased from Sigma (Taufkirchen, Germany), isoferulic acid was from ChromaDex (via Promochem, Wesel, Germany), and cimicifuga acid B was from Dr. Kolkmann & Partner (Oldenburg, Germany).

HPLC Equipment

Chromatographic analysis was performed using a Waters Alliance HPLC system (Eschborn, Germany) equipped with a 2695XE separation module with degasser, temperature-controlled sample compartment and column

heater, and a 2996 photodiode-array detector. The dwell volume of the chromatographic system used was 0.8 mL. Chromatograms were monitored at a wavelength of 325 nm. UV spectra were taken in the range of 210–440 nm.

For system control, data acquisition, and data processing the Empower software (Waters, Eschborn, Germany) was used. For the automated method development, we used the AMDS software (Waters, Milford, MA) and DryLab 2000plus for AMDS software (LC Resources, now: Rheodyne LLC, Walnut Creek, CA, USA).

Sample Preparation Procedure

Cimicifuga racemosa extract (160 mg) were exactly weighed into a 25-mL volumetric flask and ultrasonically extracted (Sonorex RK1029, Bandelin, Berlin, Germany) with 20 mL methanol 80% for 15 min. The mixture was made up to volume with methanol 80%. An aliquot of the preparation was filtered through a 0.45 μm-PTFE membrane filtration cartridge (Gelman Sciences, Dreieich, Germany) directly into a vial, and transferred into the HPLC autosampler.

Powdered samples of a commercial product (Sinei capsules, Steiner and Co., Berlin, Germany), equivalent to about 160 mg of *Cimicifuga racemosa* extract, were treated in the same way.

Method Development Process

The first step is to provide the AMDS software with information about the separation, such as sample classes (acids, bases, neutrals), stationary and mobile phases, instruments dwell volume, and method goals (maximum resolution, minimum run-time). Four initial experiments are carried out fully automated. They consist of a short and a long gradient, each at a low and a higher temperature (for an illustration of the experimental design for optimizing gradient time and temperature see Figure 2).

After the initial runs are made, an automated peak tracking process by the AMDS software is necessary due to a change in the sequence of the constituents, which would complicate the optimization process if made manually. The peak tracking process is based on UV spectra taken from the PDA detector and the peak area. The AMDS software transferred the peak data (retention time and peak area) automatically into the DryLab 2000plus software, which calculates a two-dimensional resolution map (gradient time versus column temperature) to find the optimum chromatographic conditions.[5,6]

A verification run is performed to confirm the optimal chromatographic conditions. User intervention for interpretation could be necessary and is possible for fine-tuning if ghost peaks, baseline fluctuation, or blank peaks appear.

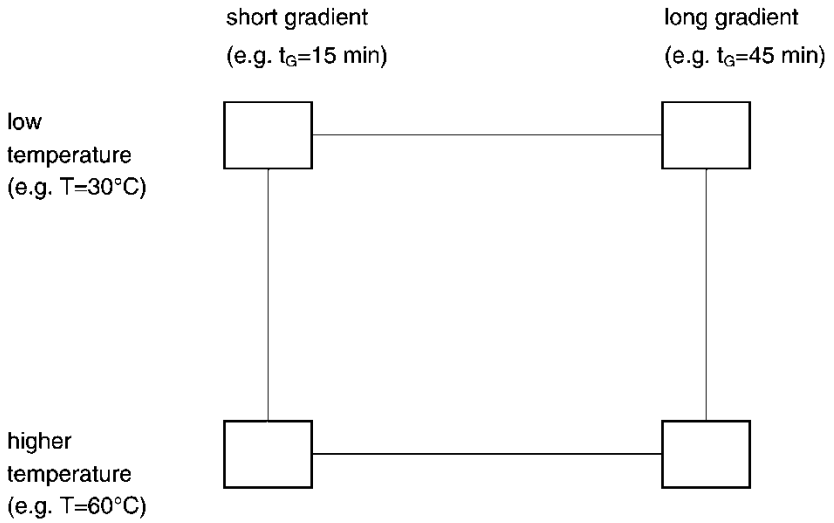

Figure 2. Illustration of an experimental design for simultaneous optimizing gradient time (t_G) and column temperature (T).

Peak Identification

The peaks of interest in the final chromatogram were identified by comparing retention times and UV-spectra (210–440 nm) of the peaks with those from the reference standards. Because of the lack of fukinol acid and cimicifuga acid A, these hydroxycinnamic acid derivatives were identified by comparison of the UV-Vis spectra with those of literature data.[15]

RESULTS AND DISCUSSION

The first step of the automated method development process for the separation of the hydroxycinnamic acid derivatives started with the water–acetonitrile system on the XTerra RP_{18} column. The XTerra column is based on a complex three-dimensional network of polymeric material incorporated into silica. The RP_{18} type is a monofunctional silane with an embedded polar group reversed-phase ligand. The four initial runs were made at 30 and 60°C, respectively, and with a linear gradient from 10 to 85% solvent B (acetonitrile) in $t_G = 10$ and 30 min, at a flow rate of 1.4 mL/min. The resulting chromatograms at 325 nm are given in Figure 3. The software indicated that the relevant peaks couldn't be separated from each other. Therefore, acetonitrile is not useful as an organic solvent and any further optimization with acetonitrile would be meaningless.

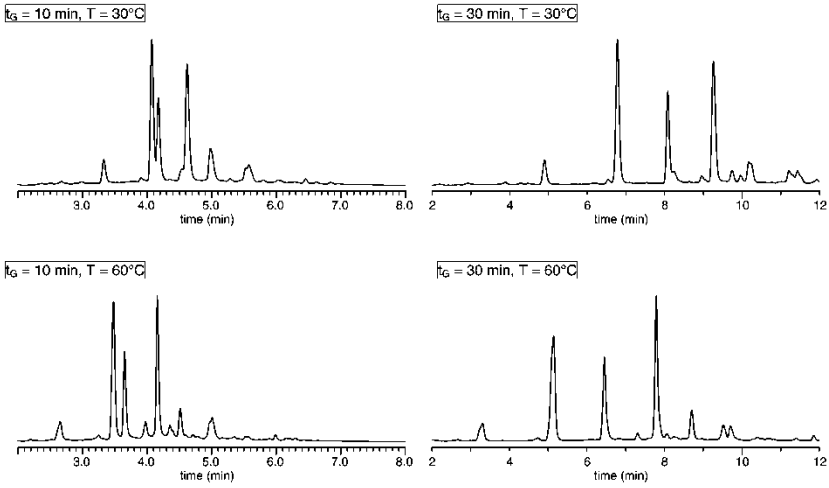

Figure 3. Four chromatograms of *Cimicifuga* extract at 325 nm with different t_G/T values using acetonitrile as the organic solvent on XTerra RP_{18}. For other conditions see text.

In the following experiments, the software automatically changed the organic solvent of the mobile phase to methanol. The optimization procedure of the water-methanol system was applied in a similar way as the water-acetonitrile system, but with a linear gradient from 10 to 90% solvent C (methanol) in $t_G = 14$ and 42 min, respectively, at a flow rate of 0.7 mL/min. As indicated in the resulting chromatograms at 325 nm, which are given in Figure 4, this change didn't improve the quality of the separation.

In the second development step a XTerra MS C_{18} column was used, which is based on a trifunctional silane with a classical reversed-phase ligand. The chromatograms of the water-acetonitrile system (not shown here) and the water-methanol system (see Figure 5) showed that the XTerra MS C_{18} column is a good choice, because all peaks are very well separated from each other and it provided excellent peak symmetry. The four initial runs indicate that good separation for most of the peaks can be reached by a longer gradient time at the higher temperature.

As a result of that, the software recommended an optimum separation with a linear gradient from 5.0 to 43.3% methanol within 6.7 min, followed by an isocratic elution for 12 min, at a flow rate of 0.7 mL/min and a column temperature of 38°C.

A verification run was performed to confirm the optimal chromatographic conditions. The resulting chromatogram is presented in Figure 6. It shows excellent separation of all peaks.

However, for batch control of phytopharmaceutical preparations, in which speed of analysis is crucial, a shorter run-time than 23 min would be

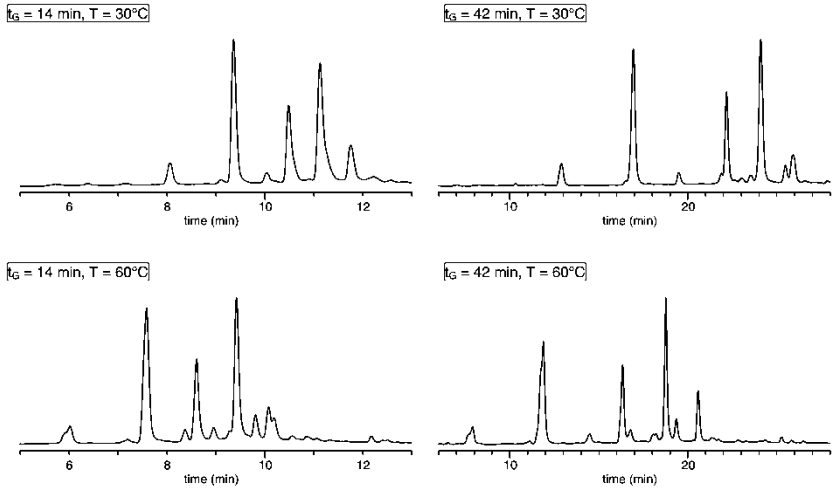

Figure 4. Four chromatograms of *Cimicifuga* extract at 325 nm with different t_G/T values using methanol as the organic solvent on XTerra RP_{18}. For other conditions, see text.

preferred. For fine-tuning and further optimization of the run-time, the entry data of the system after peak tracking was stored by the software and available for reprocessing. By using DryLab's two-dimensional critical resolution map (gradient time versus column temperature), it was possible

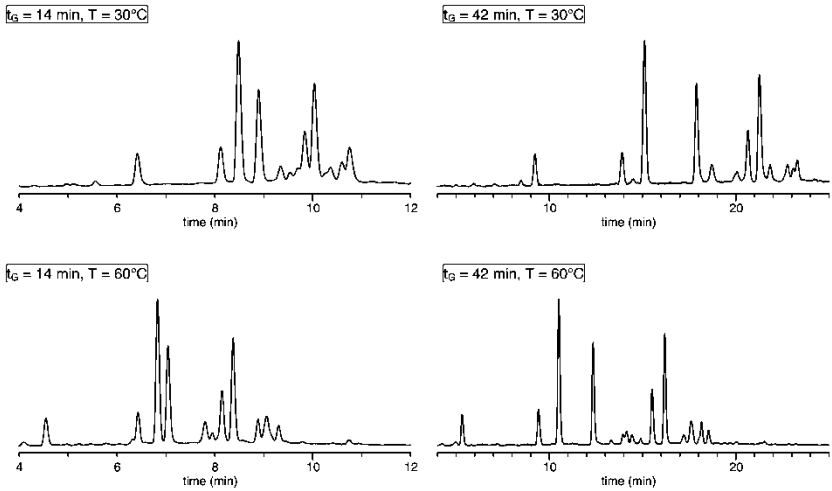

Figure 5. Four chromatograms of *Cimicifuga* extract at 325 nm with different t_G/T values using methanol as the organic solvent on XTerra MS C_{18}. For other conditions, see text.

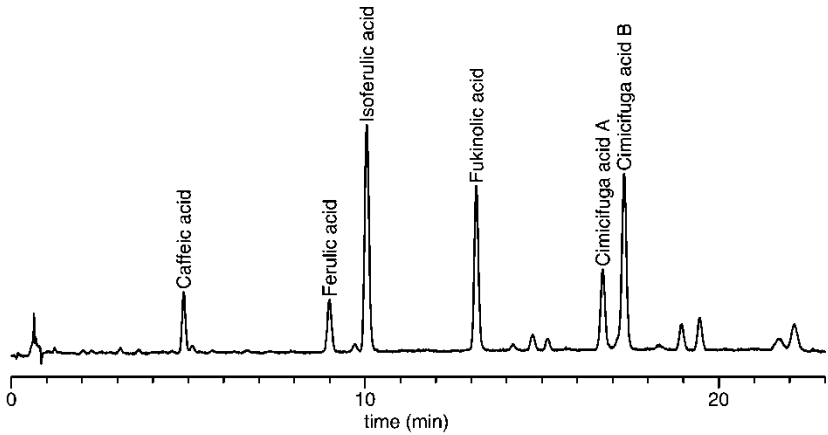

Figure 6. Automatically generated verification run of *Cimicifuga* extract on a XTerra MS C_{18} column with linear gradient from 5.0 to 43.3% MeOH within 6.7 min, followed by an isocratic elution for 12 min at 38°C and a flow rate of 0.7 mL/min. Peaks of interest are separated very well and could be identified as described.

to find baseline separation for the six peaks of interest (hydroxycinnamic acid derivatives) with a linear gradient from 20 to 50% methanol in $t_G = 12$ min and $T = 50°C$ ($R_S > 1.5$). The final chromatogram is shown in Figure 7.

As shown in Table 1, several samples were analyzed according to the method described above. The average content of total hydroxycinnamic acid derivatives, calculated as caffeic acid, in *Cimicifuga racemosa* extract

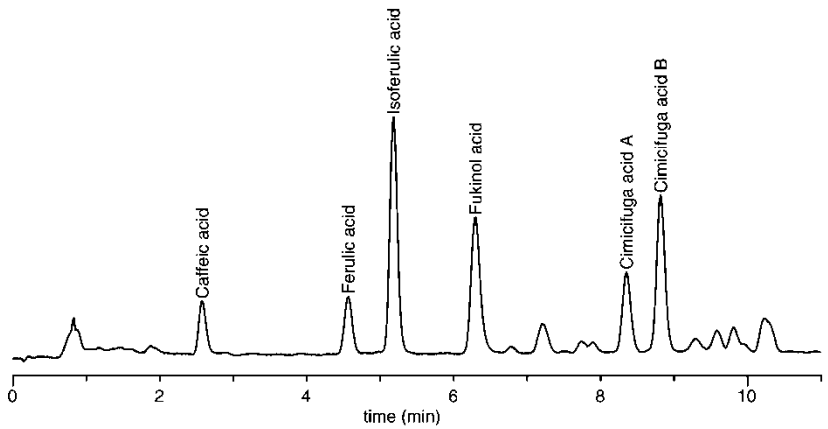

Figure 7. Further optimized and final chromatogram of *Cimicifuga* extract at 325 nm on a XTerra MS C_{18} column with linear gradient from 20 to 50% MeOH within 12 min at 50°C, and a flow rate of 1 mL/min. Peaks of interest could be identified as described.

Table 1. Results for *Cimicifuga racemosa* extracts and a commercial product. The amounts of the hydroxycinnamic acid derivatives were calculated as caffeic acid

	Amount (mg/100 mg)						
Cimicifuga racemosa extract	Caffeic acid	Ferulic acid	Isoferulic acid	Fukinolic acid	Cimicifuga acid A	Cimicifuga acid B	Total
Batch WB07812	0.024	0.026	0.091	0.088	0.044	0.078	0.352
Batch WB26542	0.029	0.034	0.134	0.095	0.05	0.098	0.439
Batch WB31013	0.026	0.03	0.128	0.086	0.047	0.091	0.407
	Amount (mg/capsule)						
Commercial product (Sinei capsules)	Caffeic acid	Ferulic acid	Isoferulic acid	Fukinolic acid	Cimicifuga acid A	Cimicifuga acid B	Total
Batch 7300122	0.008	0.009	0.032	0.03	0.015	0.028	0.122
Batch 7300813	0.008	0.009	0.037	0.028	0.015	0.028	0.125
Batch 7301433	0.009	0.011	0.044	0.03	0.016	0.032	0.142

and a commercial product was found to be 0.4% (w/w) for the extract and 0.13 mg for the capsule, respectively.

CONCLUSION

The main problem in separating complex mixtures, such as plant extracts by high performance liquid chromatography, is in finding a system, which has the specific selectivity. With the fully automated method development process provided by the AMDS software and the implemented chromatography simulation software DryLab 2000plus, the baseline separation of the hydroxycinnamic acid derivatives in *Cimicifuga* extracts is possible with a linear gradient of water pH = 2.0 and methanol on a XTerra MS C_{18} column.

The time saved, by using this software, is the most important aspect of the method development procedure. Only the necessary minimum numbers of chromatographic runs are performed by the system. Most of it can be achieved in over-night runs. The AMDS software optimizes the maximum peak resolution in minimum analysis run-time. By using solvent and column switching, the most suitable separation of samples can be established very rapidly.

The developed HPLC method was successfully applied to the separation of hydroxycinnamic acid derivatives for the quality control of *Cimicifuga racemosa* extracts and its phytopharmaceutical preparations.

ACKNOWLEDGMENTS

The author is very thankful to Dr Hans-Ulrich Kuffner (Waters Corp., Vienna, Austria) for providing the AMDS software and for many helpful discussions, and to Kerstin Stransky and Katrin Pöchmann for their technical assistance during the method development studies. Last but not least, the author would like to thank Eugene Bonk for reviewing the manuscript.

Parts of this work has been presented at the HPLC Symposium in Philadelphia, PA in June 2004.

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Received October 31, 2004
Accepted December 03, 2004
Manuscript 6544