Isolation of formononetin and other phytoestrogens from red clover with the Agilent 1100 Series purification system

Application

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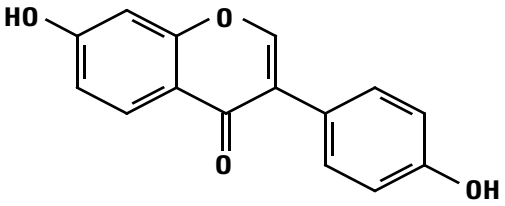

Abstract

Isolating active natural products from plant origin is frequent in the pharmaceutical industry when searching for new drugs. In this Application Note we show how this task can be accomplished using the Agilent 1100 Series purification systems AS (analytical scale) and PS (preparative scale)[1]. The isolation of formononetin and other phytoestrogens from red clover serves as an example to demonstrate the excellent performance of the Agilent purification solution at analytical and preparative scale flow rates.

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Introduction

Estrogens are used to treat menopause disorders and osteoporosis, because these disorders are caused by a low hormone level. Unfortunately, the steroids used have a high rate of undesired side effects, for example, thrombosis. Comparative studies between Asian and Western populations showed that these disorders and diseases are a lot less common in Asia. This is explained by the Asian soya-based diet, which contains high levels of phytoestrogens. Phytoestrogens[2] are currently under investigation especially because of their importance in hormone replacement therapy and cancer prevention without side effects.

The identification of phytoestrogenes with isoflavonoid-structure in red clover (*Trifolium pratense*, *L.*, *Leguminosae*) extract using fluorescence[3] and UV-visible detection is described in another Application Note[4]. In the Application Note here, we describe the separation and isolation of formononetin and other phytoestrogens from red clover in analytical and preparative scale using the Agilent 1100 Series purification systems AS and PS.

Equipment

The experiments were performed using the following systems:

Analytical scale system:
- Agilent 1100 Series vacuum degasser
- Agilent 1100 Series quaternary pump
- Agilent 1100 Series well-plate autosampler
- Agilent 1100 Series thermostatted column compartment
- Agilent 1100 Series diode array detector
- Agilent 1100 Series fraction collector AS

Preparative scale system:
- Two Agilent 1100 Series preparative pumps
- Agilent 1100 Series preparative autosampler
- Agilent 1100 Series column organizer
- Agilent 1100 Series diode-array detector
- Agilent 1100 Series preparative fraction collector PS

The systems were controlled using the Agilent ChemStation (rev. A.09.01) and the Purification/HighThruput software (rev. A.01.01).

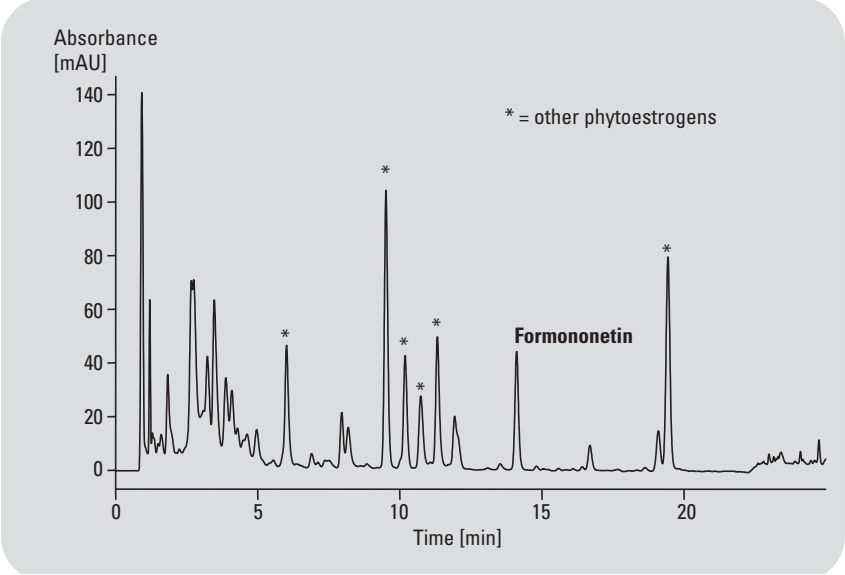
Results and Discussion

Extraction

25 g dried red clover was extracted ultrasonically with 250 ml methanol containing 25 ml 0.1 m H_2SO_4 for three hours. The mixture was removed from the ultrasonic bath and stirred overnight. After filtration the solution was evaporated at 40 °C to about 30 ml and filtered again.

Analytical method development

An analytical scale method was developed based on the method described previously[4] to replace the phosphoric acid by acetic acid. Figure 1 shows the resulting chromatogram. Formononetine and several other phytoestrogens[4] are marked in the chromatogram.



Columns	Zorbax SB-C18
	3 x 150 mm, 5 µm
Mobile phases:	0.1 % HOAc in water
	0.1 % HOAc in acetonitrile
Gradient:	20 % B to 45 % B in 20 min
	45 % B to 100 % B in 1 min
	100 % B for 4.5 min
	100 % B to 20 % B in 0.5 min
Stop time:	25 min
Post time:	5 min
Flow:	0.7 ml/min
Injection:	5 µl
Column temp.:	35 °C
UV detector:	DAD 260 nm/16
	(ref. 800 nm/100)
	standard flow cell
	(10 mm pathlength)

Figure 1
Chromatogram of crude red clover extract

Volume overloading experiment
Since concentration overloading was not possible due to the fixed concentration of the extract, volume overloading had to be done to isolate the compounds. Injecting up to 50 µl crude extract sample still lead to sufficient separation for analytical scale purification (figure 2).

Isolation of phytoestrogens in analytical scale.
A common method to isolate compounds from complex natural extracts is fractionation by time slices. Because of the good separation achieved in the overloading experiments, peak-based fractionation was used for the red clover extract. The chromatogram is shown in figure 3 – vertical lines indicate the collected fractions. The analytical method described in figure 1 was used with an injection volume of 50 µl. Fractions were collected between 6 and 20 minutes, based on threshold only (500 mAU).

Isolation of higher amounts
To gain more phytoestrogen material, pooling of fractions from several runs was carried out. This means repetitive injections were performed from one sample vial and the resulting fractions were collected in the same fraction vials. The pooling feature is described in detail in the User's Guide[5]. 450 µl of sample was

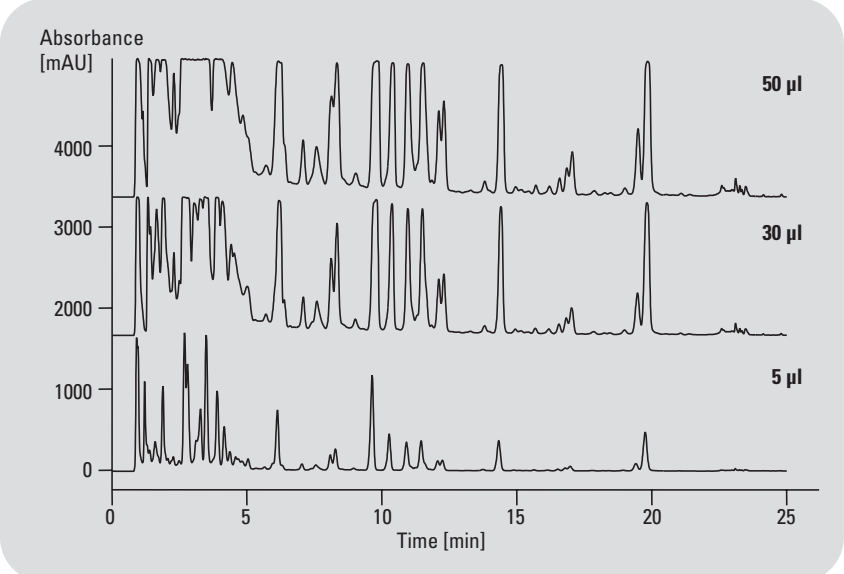


Figure 2
Volume overloading experiment

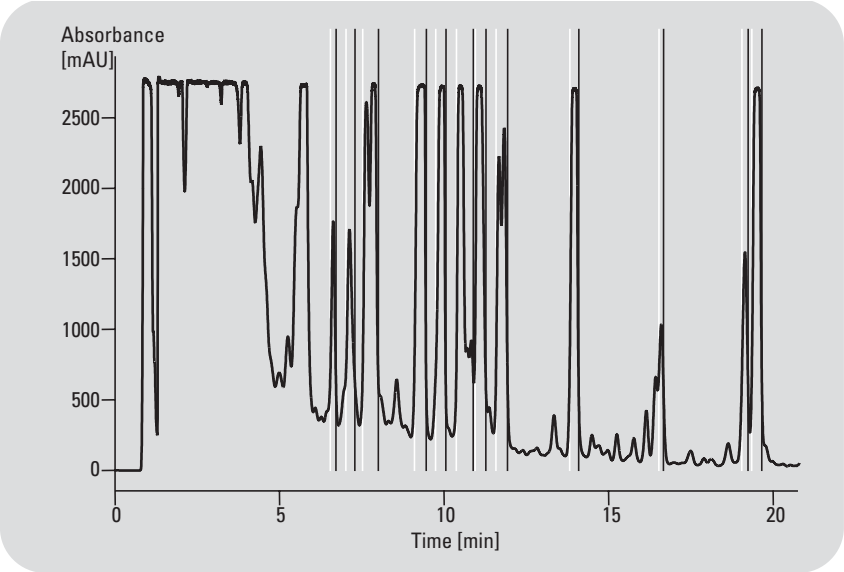


Figure 3
Analytical scale fractionation of red clover extract

injected in nine 50-µl injections and the resulting fractions were pooled automatically. Reanalysis of the fractions showed good results which demonstates the excellent performance of the instrument and software (figure 4).

Another possibility to purify more material is to scale-up to a larger column. Based on the analytical scale column overloading experiment (figure 2), scale-up calculations were done to inject 450 µl in one single injection. This was achieved on a 9.4 × 150 mm column at a flow rate of 7 ml/min. Since the Agilent 1100 Series wellplate autosampler AS can only be used up to a flow rate of 5 mL/min purification was transferred to a purification system PS. Figure 5 shows the chromatogram that was obtained. The lower peak heights

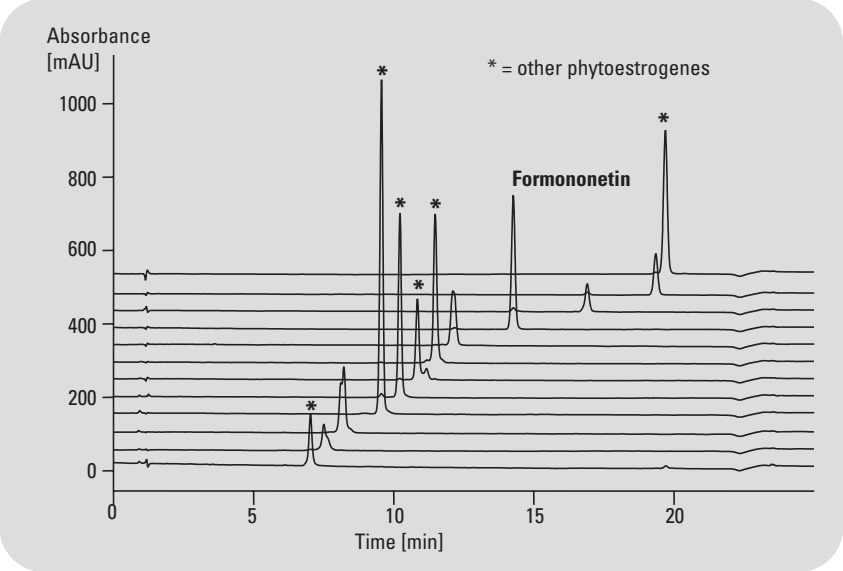


Figure 4
Re-analysis of fractions from pooling

Columns	Zorbax SB-C18 9.4 x 150 mm, 5 µm
Mobile phases:	0.1 % HOAc in water 0.1 % HOAc in acetonitrile
Gradient:	20 % B to 45 % B in 20 min 45 % B to 100 % B in 1 min 100 % B for 4.5 min 100 % B to 20 % B in 0.5 min
Stop time:	25 min
Post time:	5 min
Flow:	7 ml/min
Injection:	450 µl
Column temp.	ambient
UV detector:	DAD 260 nm/16 (ref. 800 nm/100) preparative flow cell (3 mm pathlength)
Fraction collection:	based on threshold only (100 mAU) between 5.5 and 21 minutes

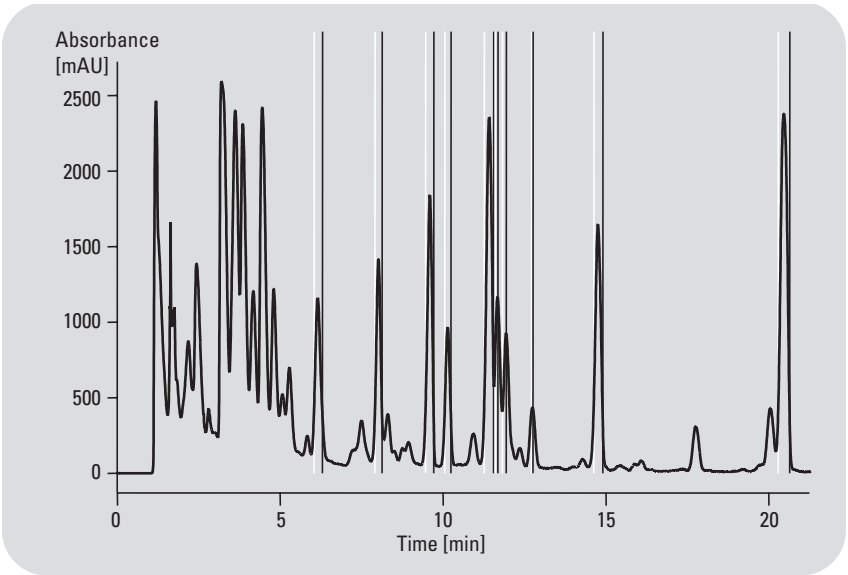
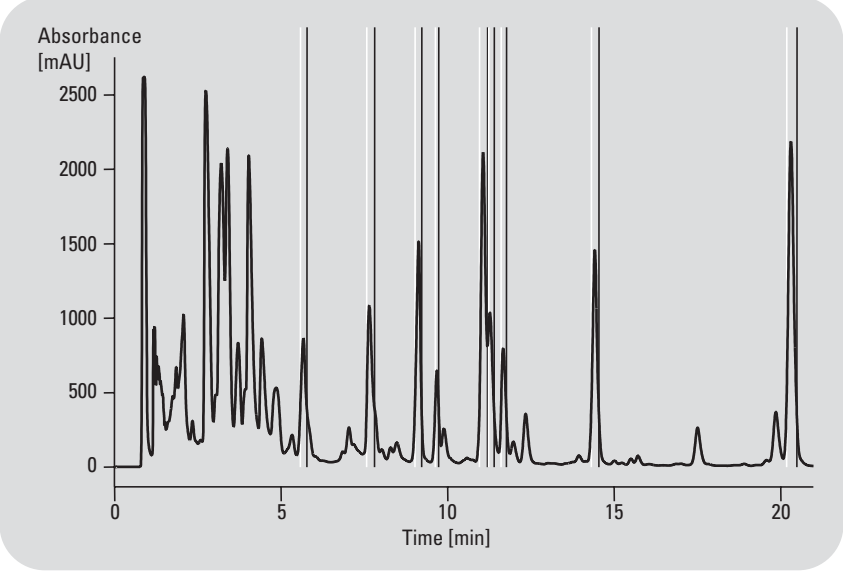


Figure 5
Preparative scale fractionation of red clover extract

and areas result from the shorter pathlength of the preparative flow cell (3 mm) compared to the standard flow cell (10 mm). Re-analysis of fractions showed comparable purities as for the pooling experiment. The injected sample volumes were the same (450 µl) for both the pooling experiments and when using the 9.4 mm id column. The solvent amounts used were also comparable (approximately 200 ml). However, the main advantage of purification on the 9.4-mm id column is the time that is saved in gaining the same amount of purified material.

Isolation of phytoestrogens in preparative scale

To purify even higher amounts of sample the method was further scaled up to a 21.2 × 150 mm column. At this scale it was possible to inject 2300 µl of sample in a single injection. The chromatogram and the method are shown in figure 6. Re-analysis of fractions showed that scale-up was possible without losing any performance with regard to the purity of the fractions.



Columns	Zorbax SB-C18 21.2 x 150 mm, 5 µm
Mobile phases:	0.1 % HOAc in water 0.1 % HOAc in acetonitrile
Gradient:	20 % B to 45 % B in 20 min 45 % B to 100 % B in 1 min 100 % B for 4.5 min 100 % B to 20 % B in 0.5 min
Stop time:	25 min
Post time:	5 min
Flow:	35 ml/min
Injection:	2300 µl
Column temp.	ambient
UV detector:	DAD 260 nm/16 (ref. 800 nm/100) Preparative flow cell (3 mm)
Fraction collection:	based on threshold only (100 mAU) between 5.5 and 21 minutes

Figure 6
Fractionation on a 21.2-mm preparative column

Conclusion

In this Application Note we showed the development of an analytical scale method to separate the compounds in a complex crude plant extract from red clover containing formononetin and several other phytoestrogen using the Agilent 1100 Series preparative system AS. Based on this method an analytical scale preparative separation with peak-based fraction collection was carried out. To obtain more phytoestrogen material the pooling feature of the Agilent 1100 Series purification system AS was used. The purity of the compounds gained was determined by the reanalysis of the fractions. Based on the analytical scale results the method was scaled up. The purification was repeated on two different columns on the Agilent 1100 Series purification system PS to gain higher amounts of the desired compounds in single runs. The results in preparative scale were comparable to the results achieved on the analytical scale system.

References

1.
"New perspectives in purification with HPLC and HPLC/MS" *Agilent Technologies Brochure*, **2001**, publication number 5988-3673EN.

2.
Julia Barrett, "Phytoestrogen: Friends or Foes", *Environmental Health Perspectives*, **1996** (104), 478.

3.
"Sensitive and Reliable Fluorescence Detection for HPLC", *Agilent Technologies Brochure*, **1999**, publication number 5968-9105E.

4.
"Separation of phytoestrogenes in red clover by reverse phase HPLC with UV-visible and fluorescence detection", *Agilent Technologies Application Note*, **2001**, publication number 5988-2399EN.

5.
"Agilent 1100 Series Purification System", *Agilent Technologies User's Guide*, **2001**, part number G2262-90001.

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www.agilent.com/chem/purification

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