Separation of phytoestrogens in red clover by reverse phase HPLC with UV-visible and fluorescence detection

Application

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Abstract

Phytoestrogen research has gained popularity due to the effect these plant estrogens have in cancer prevention, especially breast and prostate cancer. They are non-steroidal compounds with estrogenic effect. In this Application Note we describe the separation and identification of phytoestrogens in red clover using UV-visible and fluorescence detection.

Introduction

Phytoestrogens[1] have been investigated as possible cancer preventatives and for treating menopause and osteoporosis. Laboratory animal experiments and comparisons of Asian and Western human populations suggest that diet plays a large role in these types of health problems. One study found that Asian populations that eat large amounts of soy products - which contain high levels of phytoestrogens - have lower rates of hormone-dependent cancers and a lower incidence of menopausal symptoms and osteoporsis. Isoflavonoids, coumestans, and lignans are all types of phytoestrogens that are currently being studied for their potential health benefits.

In this Application Note we describe the separation and identification of seven phytoestrogens and their glucosides with isoflavonoid structure, genistin, ononin, daidzein, sissotrin, genistein, formononetin and biochanin A (figure 1) from red clover extract.

Identification of the isoflavonoids was based on retention times and by fluorescence[2,3] or UV-visible detection. To confirm the identification, the UV-visible spectra of some compounds were compared to standards.

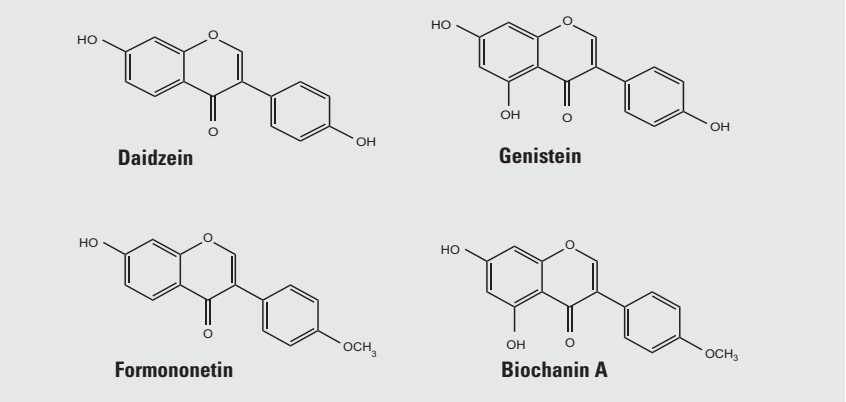

Figure 1
Structures of some phytoestrogens

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Equipment

The system included:

- Agilent 1100 Series degasser
- Agilent 1100 Series quaternary pump
- Agilent 1100 Series autosampler
- Agilent 1100 Series thermostatted column compartment
- Agilent 1100 Series diode array detector
- Agilent 1100 Series fluores cence detector

The system was controlled using the Agilent ChemStation.

Results and Discussion

Extraction

Extraction was done ultrasonically using methanol because previous extractions using methanol/2N HCl 80:20 v/v led to hydrolyzation of some isoflavone glycosides to aglycones and some unknown compounds.

Measurement of standards

A HPLC method was developed for eight phytoestrogens (genistin, ononin, daidzein, sissotrin, genistein, coumestrol, formononetin biochanin A) to achieve baseline separation using standards. Since four of the compounds showed good fluorescence, an Agilent 1100 Series fluorescence detector (FLD) was used to make identification of the compounds in the complex natural product extract easier. Using the FLD was also the only possibility to identify coumestrol, which co-elutes with genistein, because only coumestrol shows fluorescence. Chromatograms of the standards using diode array and fluorescence detection are shown in figures 2 and 3.

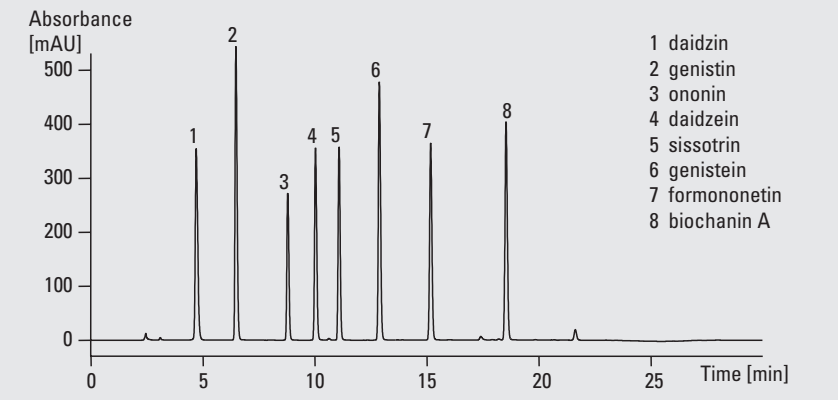

Figure 2
Chromatogram of standards using UV-visible detection at 260 nm

Column:	Zorbax SB-C18 4.6 x 250 mm, 5 µm
Mobile phases:	0.1 % H_3PO_4 in water = A 0.1 % H_3PO_4 in acetonitrile = B
Gradient:	20 % B to 80 % B in 25 min 80 % B to 20 % B in 5 min
Stop time:	30 min
Post time:	2 min
Flow:	1.0 ml/min
Injection:	10 µl
Column temp.:	35 °C
UV detector:	DAD 260 nm/40, (ref. 380 nm/30) standard flow cell (10 mm)
FLD:	excitation 249 nm emission 419 nm standard flow cell (8 µl)

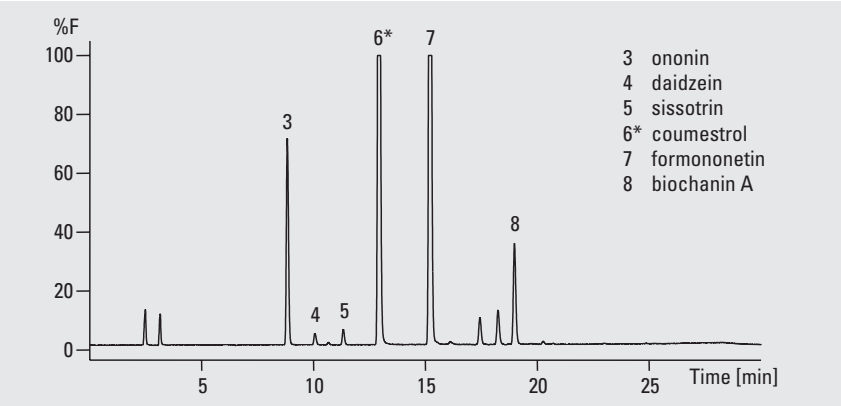

Figure 3
Chromatogram of standards using fluorescence detection at Ex = 249 nm, Em = 419 nm

Analysis of red clover extract

Identification of phytoestrogens in red clover was done using retention times, UV-visible spectra and fluorescence chromatograms. Since fluorescence detection is more selective than UV-visible detection, the Agilent 1100 Series FLD is a versatile tool for identification of compounds in complex natural product extracts. As shown in figure 4, three phytoestrogens, ononin, daidzein and fomononetin, could be identified by fluorescence detection in the red clover extract. Figure 5 shows the UV-visible chromatogram.

Seven phytoestrogens could be identified in the UV-visible chromatogram by their retention time. To confirm the identity of the compounds an additional comparison of the UV-visible spectra with standards was done. Since ononin, daidzein and formononetin were already identified by their fluorescence chromatogram, this was only done for the other compounds genistin, daidzein, sissotrin, genistein and biochanin A (figure 6).

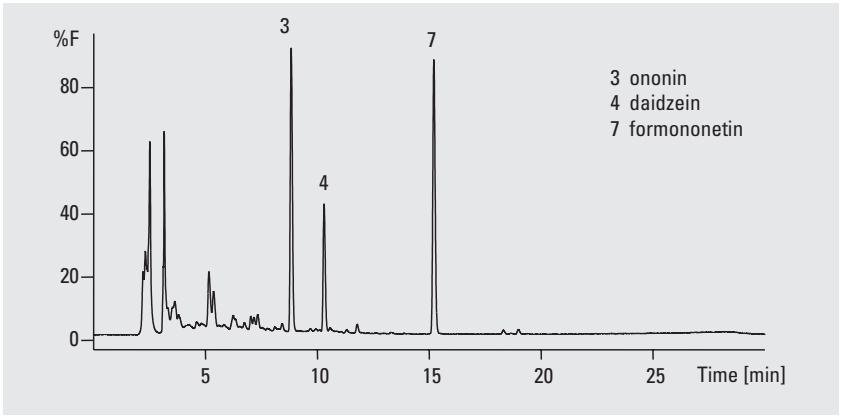

Figure 4
Fluorescence chromatogram of red clover extract, Ex = 249 nm, Em = 419 nm

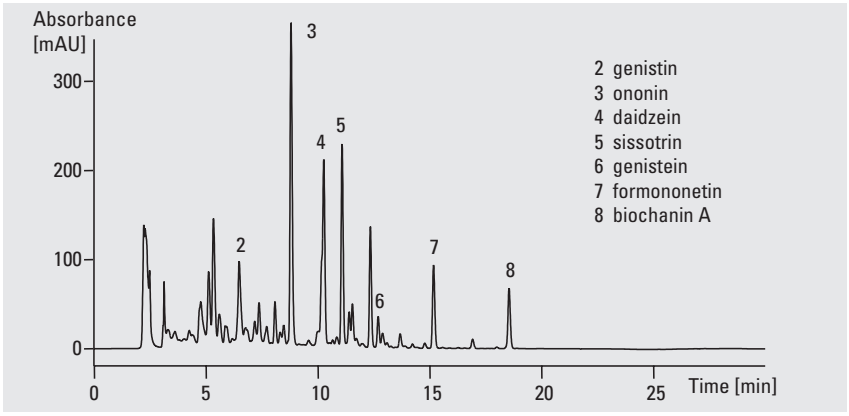

Figure 5
UV-visible chromatogram of red clover extract at 260 nm

Conclusion

In this Application Note we demonstrate the separation and identification of eight phytoestrogens from red clover extract using HPLC with UV-visible and fluorescence detection. The isoflavones were identified by their retention time in the UV-visible and fluorescence chromatogram as well as by their UV-visible spectra. Since phytoestrogens with isoflavone structure show promising activity against breast and endometrial cancer, and can be used in menopause and osteoporosis treatment, this method is a versatile tool, for quality control of natural phytoestrogen sources.

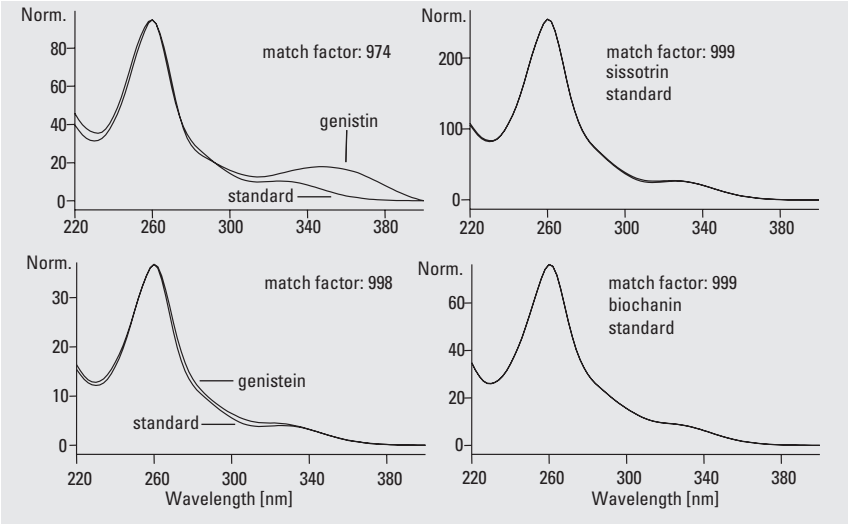

Figure 6
Spectra of phytoestrogens

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