Ultra-fast LC analysis of soy isoflavones in foods and dietary supplements using newly developed 2 μm RP-column designed for polar compounds

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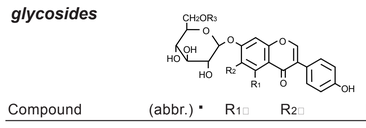
1. Introduction

Soybeans are known to contain 9 kinds of isoflavone glycosides and their corresponding aglycones (Fig.1). Soy-containing foods and dietary supplements are widely consumed for their putative health benefits (e.g., reduction of osteoporosis, relief of menopausal symptoms, breast cancer chemoprevention). However, there is also some concern that excessive intake of soy isoflavones can stimulate tumor growth in women with estrogen-related cancers. Recently, increasing attention has been focused on determination of the levels of isoflavones in soy-derived foods and the recommended daily intake.

Reversed-phase (RP) HPLC has been widely applied to analysis of soy isoflavones. However, because of the poor selectivity to highly polar compounds of similar structure like soy isoflavones, acceptable separation has not been often achieved without long column length and long analysis time. For these types of challenging separations, we have already developed a silica-based C18 column named Hydrosphere C18, which provides strong retention and superior selectivity of polar compounds.

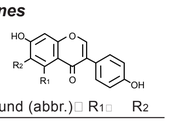
This study developed high-throughput methods of both conventional LC and ultra-fast LC for quantifying isoflavones in soybeans, soy foods and dietary supplements, using Hydrosphere C18 with short column length. Twelve isoflavones were separated completely within 10 min in conventional LC method with 3 μm particles and a 50 x 4.6 mm column. Moreover, the conventional LC method can be transferred easily to ultra-fast LC method with 2 μm particles and a 50 x 2.0 mm column; its analysis time can be reduced to about 3 min without losing resolution.

Figure 1: Structures of 12 isoflavones in soybeans



glycosides

Compound	(abbr.) *	R_1	R_2	R_3
Daidzin	(D)	H	H	H
Glycitin	(Gl)	H	OCH_3	H
Genistin	(G)	OH	H	H
6"-*O*-Acetyldaidzin	(AD)	H	H	$COCH_3$
6"-*O*-Acetylglycitin	(AGl)	H	OCH_3	$COCH_3$
6"-*O*-Acetylgenistin	(AG)	OH	H	$COCH_3$
6"-*O*-Malonyldaidzin	(MD)	H	H	$COCH_2COOH$
6"-*O*-Malonylglycitin	(MGl)	H	OCH_3	$COCH_2COOH$
6"-*O*-Malonylgenistin	(MG)	OH	H	$COCH_2COOH$



aglycones

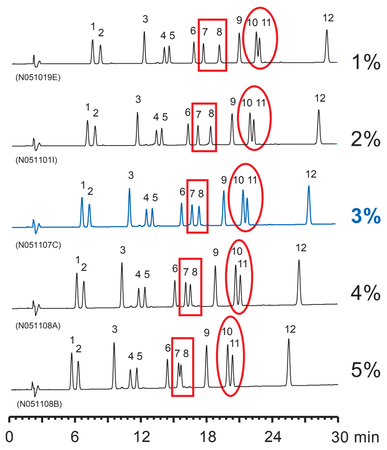
Compound	(abbr.)	R_1	R_2
Daidzein	(De)	H	H
Glycitein	(Gle)	H	OCH_3
Genistein	(Ge)	OH	H

2. Experiments

The gradient elution of water and acetonitrile containing acetic acid has been used commonly in RP-HPLC separation of soy isoflavones.

In Figure 2, the influence of acetic acid concentration of soy isoflavone separation is evaluated under equivalent gradient condition. With increasing acetic acid concentration, the resolution of peak 10 and 11 is improved, while the resolution of peak 7 and peak 8 is worse than initial. Considering both resolutions, the mobile phase containing 3% acetic acid would be suitable for this separation.

Figure 2: Influence of acetic acid concentration on soy isoflavone separation

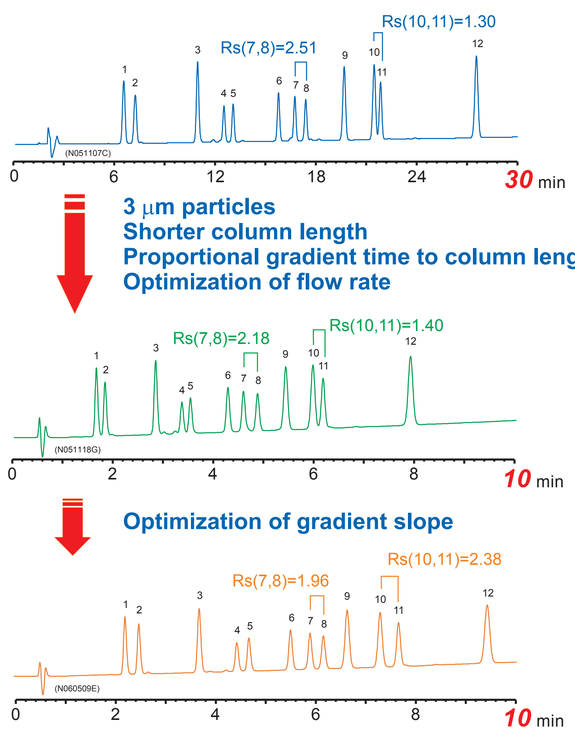


acetic acid concentration	Resolution (Rs) peak 7, 8	peak 10, 11
1%	5.82	1.04
2%	4.55	1.22
3%	**2.51**	**1.30**
4%	1.67	1.47
5%	*n.c.*	1.51

1. D 7. AGl
2. Gl 8. MG
3. G 9. De
4. MD 10. Gle
5. MGl 11. AG
6. AD 12. Ge

Column : Hydrosphere C18
150 x 4.6 mm i.d., 5 μm
Flow rate : 1.0 ml/min
Temperature : 35℃
Detection : UV at 254 nm
Injection : 10 μl (0.01 mg/ml)
Eluent : A) water / acetic acid
B) acetonitrile / acetic acid
Gradient : 15-35% acetonitrile (0-30 min)

Figure 3: Optimization of conventional LC conditions with 3 μm particles



a) 150 x 4.6 mm i.d., 5 μm
15-35% ACN (0-30 min)
1.0 ml/min
10 μl Inj.

b) **50** x 4.6 mm i.d., **3** μm
15-35% ACN (0-**10** min)
1.5 ml/min
3 μl Inj.

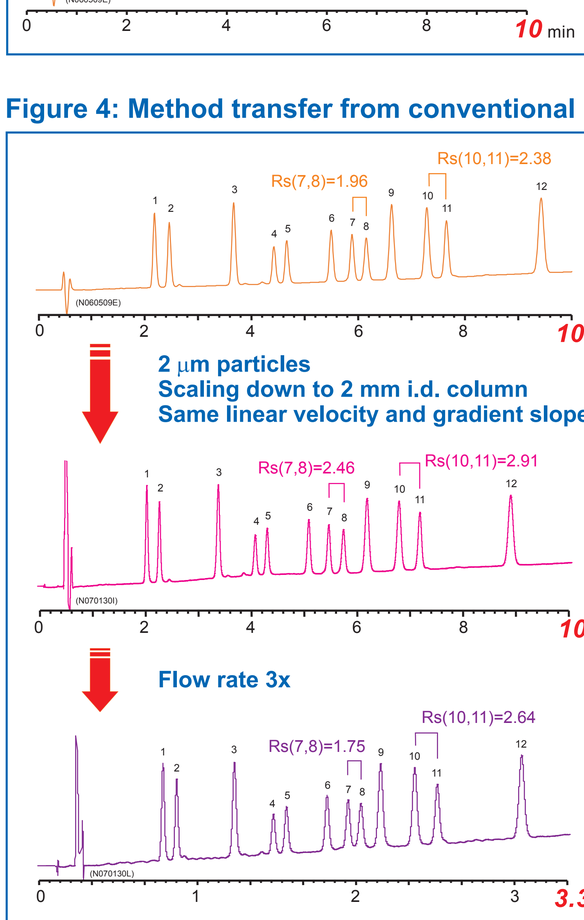
Column : Hydrosphere C18
Temperature : 35℃
Detection : UV at 254 nm
Eluent : A) water / acetic acid (100/3)
B) acetonitrile / acetic acid (100/3)

Optimized method for conventional LC

c) 50 x 4.6 mm i.d., 3 μm
12.5-30% ACN (0-10 min)
1.5 ml/min
3 μl Inj.

1. D 7. AGl
2. Gl 8. MG
3. G 9. De
4. MD 10. Gle
5. MGl 11. AG
6. AD 12. Ge

Figure 4: Method transfer from conventional LC with 3 μm to ultra-fast LC with 2 μm



Optimized method for conventional LC

a) 50 x 4.6 mm i.d., 3 μm
12.5-30% ACN (0-10 min)
1.5 ml/min
3 μl Inj.

b) 50 x **2.0** mm i.d., **2** μm
12.5-30% ACN (0-10 min)
0.3 ml/min
0.6 μl Inj.

Column : Hydrosphere C18
Temperature : 35℃
Detection : UV at 254 nm
Eluent : A) water / acetic acid (100/3)
B) acetonitrile / acetic acid (100/3)

Optimized method for ultra-fast LC

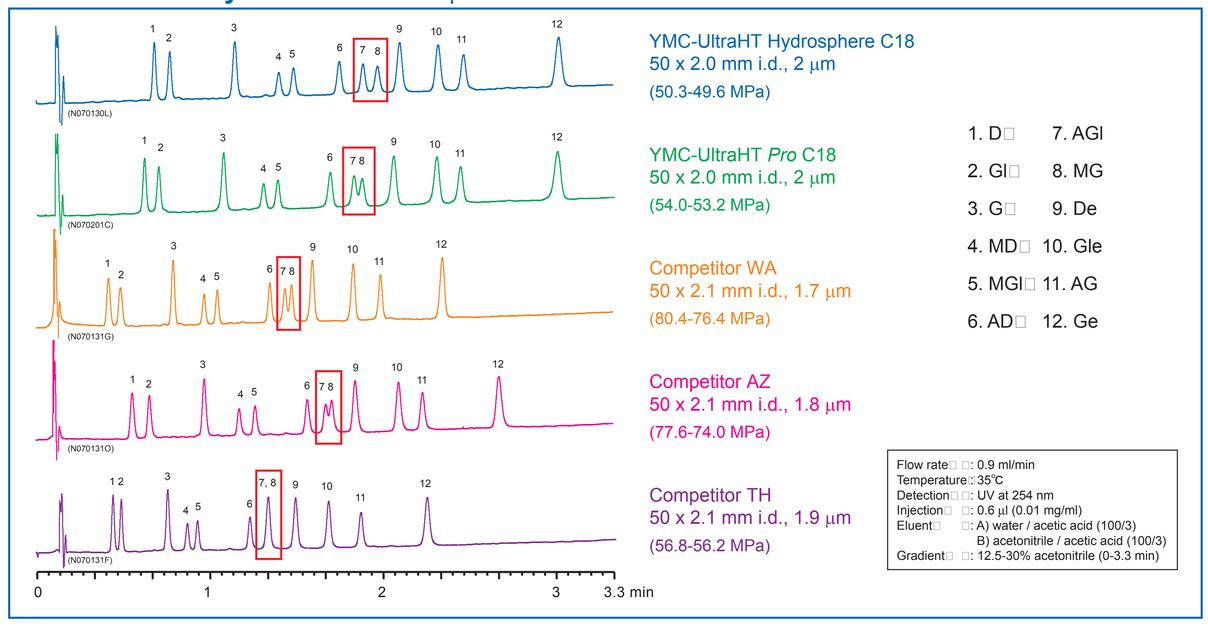
c) 50 x 2.0 mm i.d., 2 μm
12.5-30% ACN (0-**3.3** min)
0.9 ml/min
0.6 μl Inj.

1. D 7. AGl
2. Gl 8. MG
3. G 9. De
4. MD 10. Gle
5. MGl 11. AG
6. AD 12. Ge

Figure 3 shows the optimization process of soy isoflavone separation for conventional LC using Hydrosphere C18 column. As shown in Chromatograms a and b, changing column length from 150 mm to 50 mm and changing flow rate from 1.0 ml/min to 1.5 ml/min with 3 μm particles reduce the analysis time without losing resolution. Furthermore, the resolution of a critical pair (peak 10 and 11) is improved by optimization of gradient slope and 12 isoflavones are resolved completely within 10 min as shown in Chromatogram c.

Figure 4 shows the method transfer from conventional LC to ultra-fast LC using the newly developed 2 μm particles YMC-UltraHT Hydrosphere C18. The 2 μm Hydrosphere C18 has the equivalent separation selectivity to that of 3 μm and 5 μm particles, so it can be easily scaled down without changing eluent conditions, as shown in Chromatograms a and b. Furthermore, the column packed with 2 μm particles maintains the high efficiency and resolution even at a 3 times higher linear velocity than that optimized for the column packed with 3 μm particles. Ultimately, the analysis time can be reduced in 3.3 min.

Figure 5: Comparison of soybean isoflavone separation among 2 μm Hydrosphere C18 and commercially available sub-2 μm



1. D 7. AGl
2. Gl 8. MG
3. G 9. De
4. MD 10. Gle
5. MGl 11. AG
6. AD 12. Ge

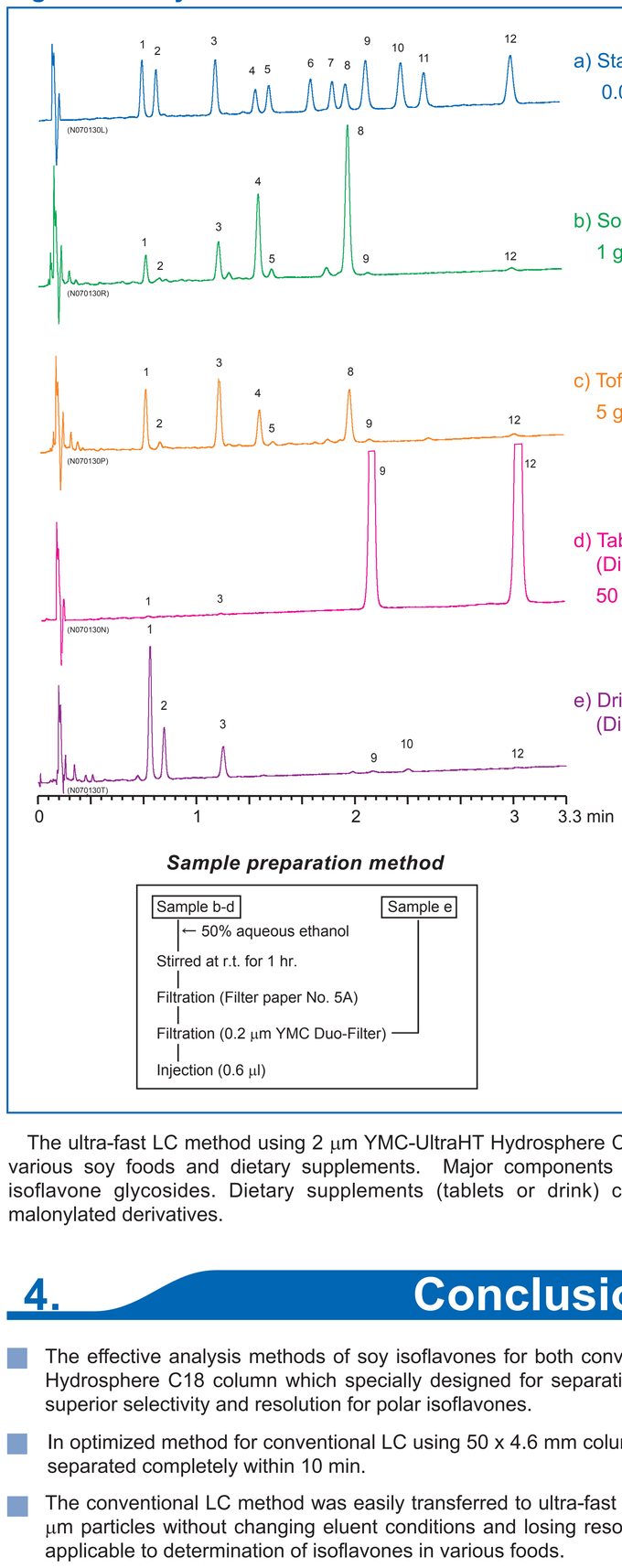
Flow rate : 0.9 ml/min
Temperature : 35℃
Detection : UV at 254 nm
Injection : 0.6 μl (0.01 mg/ml)
Eluent : A) water / acetic acid (100/3)
B) acetonitrile / acetic acid (100/3)
Gradient : 12.5-30% acetonitrile (0-3.3 min)

Figure 5 compares the chromatographic performances of Hydrosphere C18 and four commercially available C18 columns packed with 2 μm or sub-2 μm particles with the ultra-fast LC method which has been developed in Figure 4c. Although efficiencies of these 2 μm or sub-2 μm columns are almost same, any columns except Hydrosphere C18 can not achieve favorable resolution of peak 7 and peak 8. Hydrosphere C18 provides enhanced retention and superior resolution for polar compounds such as isoflavone glycosides.

The combination of excellent efficiency and unique selectivity, 2 μm YMC-UltraHT Hydrosphere C18, enables the development of robust and high-throughput analysis methods for polar compounds in short time.

3. Application

Figure 6: Analyses of extracts obtained from various soy foods and dietary supplements

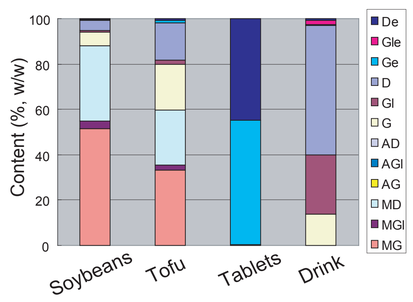


1. D 7. AGl
2. Gl 8. MG
3. G 9. De
4. MD 10. Gle
5. MGl 11. AG
6. AD 12. Ge

Column : YMC-UltraHT Hydrosphere C18
50 x 2.0 mm i.d., 2 μm
Flow rate : 0.9 ml/min
Temperature : 35℃
Detection : UV at 254 nm
Injection : 0.6 μl
Eluent : A) water / acetic acid (100/3)
B) acetonitrile / acetic acid (100/3)
Gradient : 12.5-30% acetonitrile (0-3.3 min)

Sample preparation method

Content of isoflavones in soy foods and dietary supplements



The ultra-fast LC method using 2 μm YMC-UltraHT Hydrosphere C18 was applied to the determination of isoflavones in various soy foods and dietary supplements. Major components in soy foods (soybeans or tofu) were malonylated isoflavone glycosides. Dietary supplements (tablets or drink) contained isoflavone glycosides or aglycones, not malonylated derivatives.

4. Conclusions

- The effective analysis methods of soy isoflavones for both conventional LC and ultra-fast LC were developed using Hydrosphere C18 column which specially designed for separation of polar compounds. Hydrosphere C18 showed superior selectivity and resolution for polar isoflavones.
- In optimized method for conventional LC using 50 x 4.6 mm column packed with 3 μm particles, 12 isoflavones were separated completely within 10 min.
- The conventional LC method was easily transferred to ultra-fast LC method using 50 x 2.0 mm column packed with 2 μm particles without changing eluent conditions and losing resolution. This ultra-fast LC method within 3.3 min was applicable to determination of isoflavones in various foods.