Analysis of Nutraceutical Soy Isoflavones Using LC/PDA

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Importance of Isoflavones

- ★ Potent natural antioxidants found in soy based food products
- ★ Implicated in reduction of LDL cholesterol
- ★ Genistein and Daidzein implicated in reducing cancer risk (breast, prostrate, & colon)
- ★ Weak estrogenic effect may relieve menopausal symptoms

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Importance of Isoflavones Nutraceutical

Federal Registar, Oct. 26, 1999,
the FDA allows a label claim that,

"…25 grams of soy protein a day,
as part of a diet low in saturated fat and cholesterol,
may reduce the risk of heart disease."

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Analysis of Isoflavones

- GC-MS method is sensitive and accurate, but laborious and expensive
  - several SPE steps required to isolate different forms
  - hydrolysis to aglucone
  - derivatization

- LC/PDA has an advantage because all isoflavone forms can be separated, and quantitated using UV Detection
  - Sensitivity
  - Coelution & Identification in complex matrices?

- LC/MS methods being developed

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Analysis of Isoflavones
Method Development Objectives

- Designed for UV and Mass Spec Detection
- Resolution of all 12 Soy Isoflavones
- Analysis time
  - less than 20 minutes for chromatography
  - less than 30 minutes injection to injection
- Confirmational Chemistries
- Application to Dietary Supplements and Food Matrices
- Use published references as foundation for
  - chromatography (Murphy, et.al., Franke,et.al., and Barnes, et.al)
  - sample prep (Murphy, et.al.)

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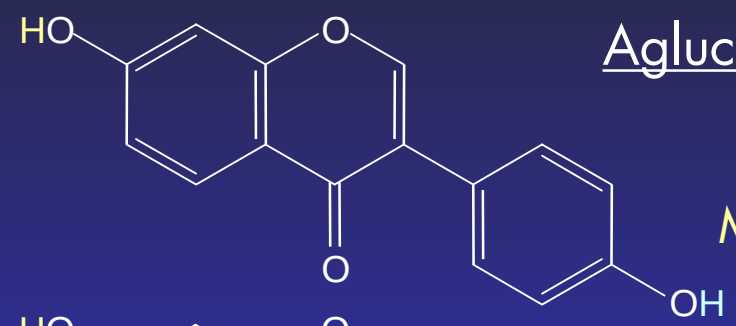
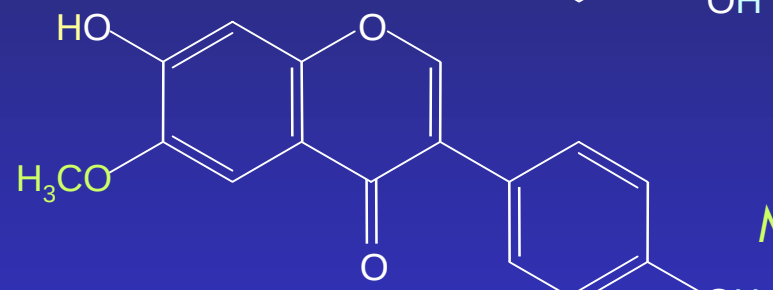
Common Soy IsoFlavone
Structure and pK_a

$pK_a \sim 7.6 \pm 0.1$



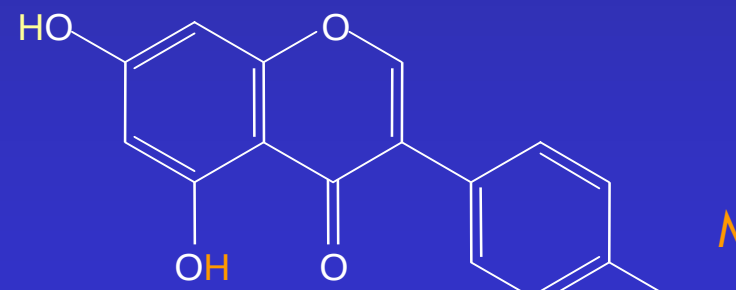
Aglucone Structure

Daidzein
MW = 254



Glycitein
MW = 284

$pK_a \sim 9.9 \pm 0.1$



Genistein
MW = 270

$pK_a \sim 9.2 \pm 0.1$

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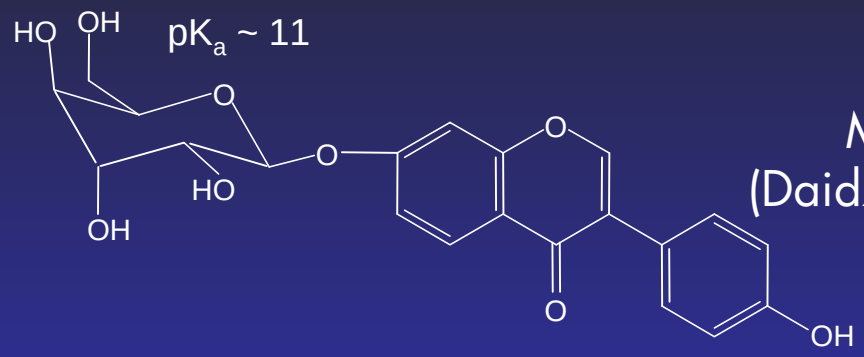
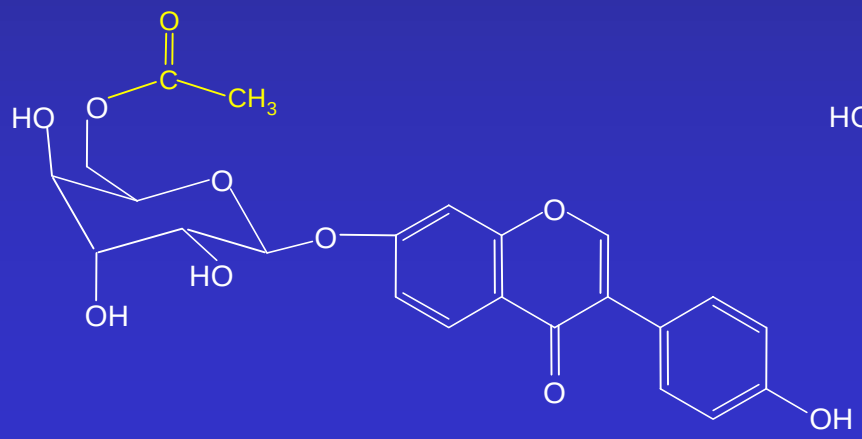
Common Soy IsoFlavone Glycoside

Structure and pK_a



Daidzin
MW = 416
(Daidzein Glucoside)

AcetylDaidzin
MW = 458
(Daidzin Acetyl Glucoside)



Malonyl Daidzin
MW = 502
(Daidzin Malonyl Glucoside)

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Price of Isoflavone Standards

Isoflavone Analytes	Qty (mg)	$$ Price
Daidzein	100	48
Daidzin	25	135
Acetyl Daidzin	5	165
Malonyl Daidzin*	5	165
Glycitein	25	338
Glycitin	25	235
Acetyl Glycitin	5	178
Malonyl Glycitin*	5	178
Genistein	100	45
Genistin	25	124
Acetyl Genistin	5	149
Malonyl Genistin*	5	149
Total price of Standards		$1909

* Malonyl Esters on backorder for several weeks

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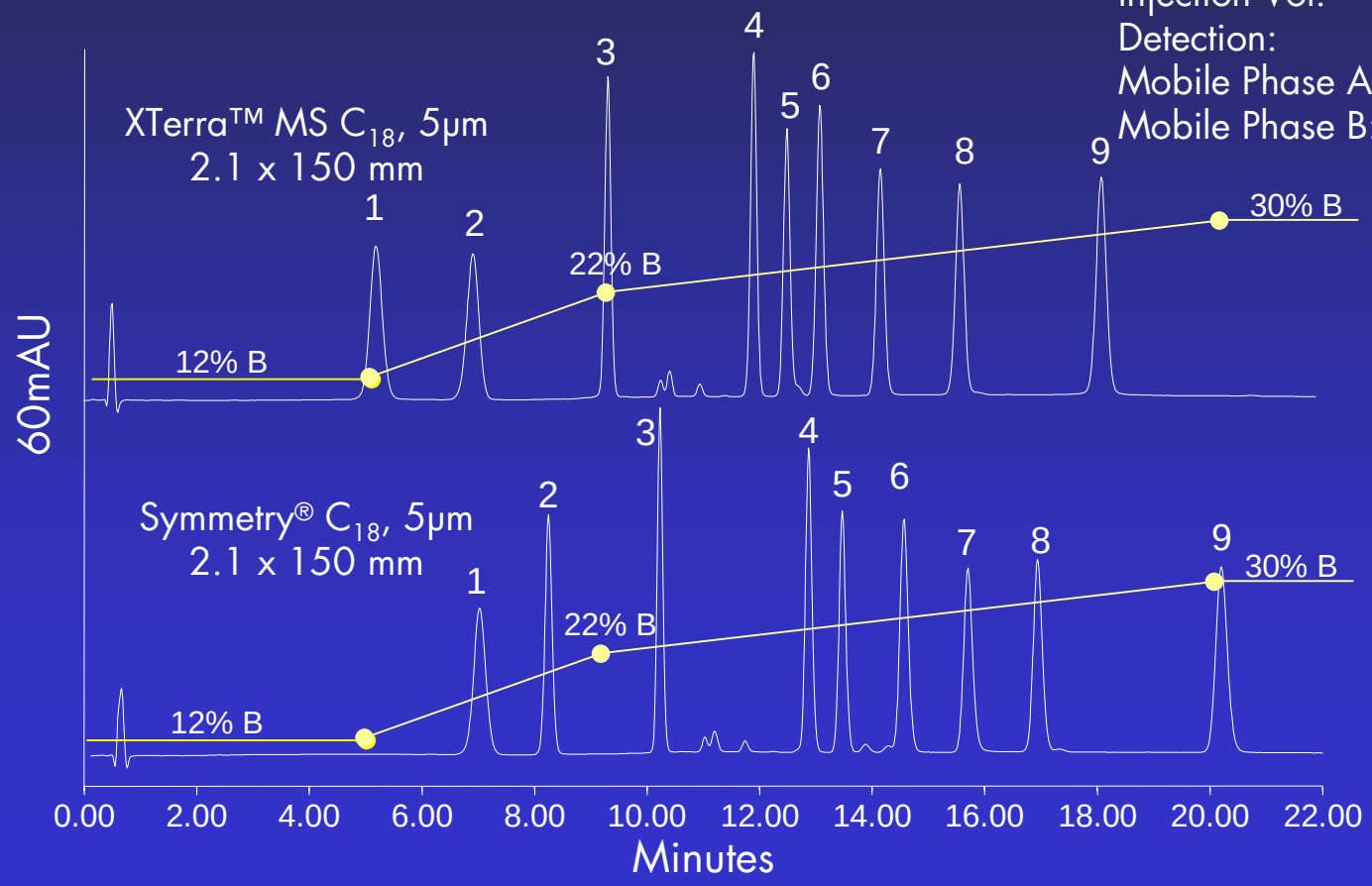
Chromatographic Character
Reverse Phase Columns are Not Alike

Soy Isoflavones

Flow Rate: 0.8 mL/min
Back Press: ~4200 psi
Injection Vol: 20 µL
Detection: 254 nm Extracted
Mobile Phase A: 0.1% HOAc, pH 3.4
Mobile Phase B: 80% AcCN/ 0.1% HOAc



10 µg/mL
1 Daidzin
2 Glycitin
3 Genistin
4 AcetylDaidzin
5 AcetylGlycitin
6 Daidzein
7 Glycitein
8 AcetylGenistin
9 Genistein

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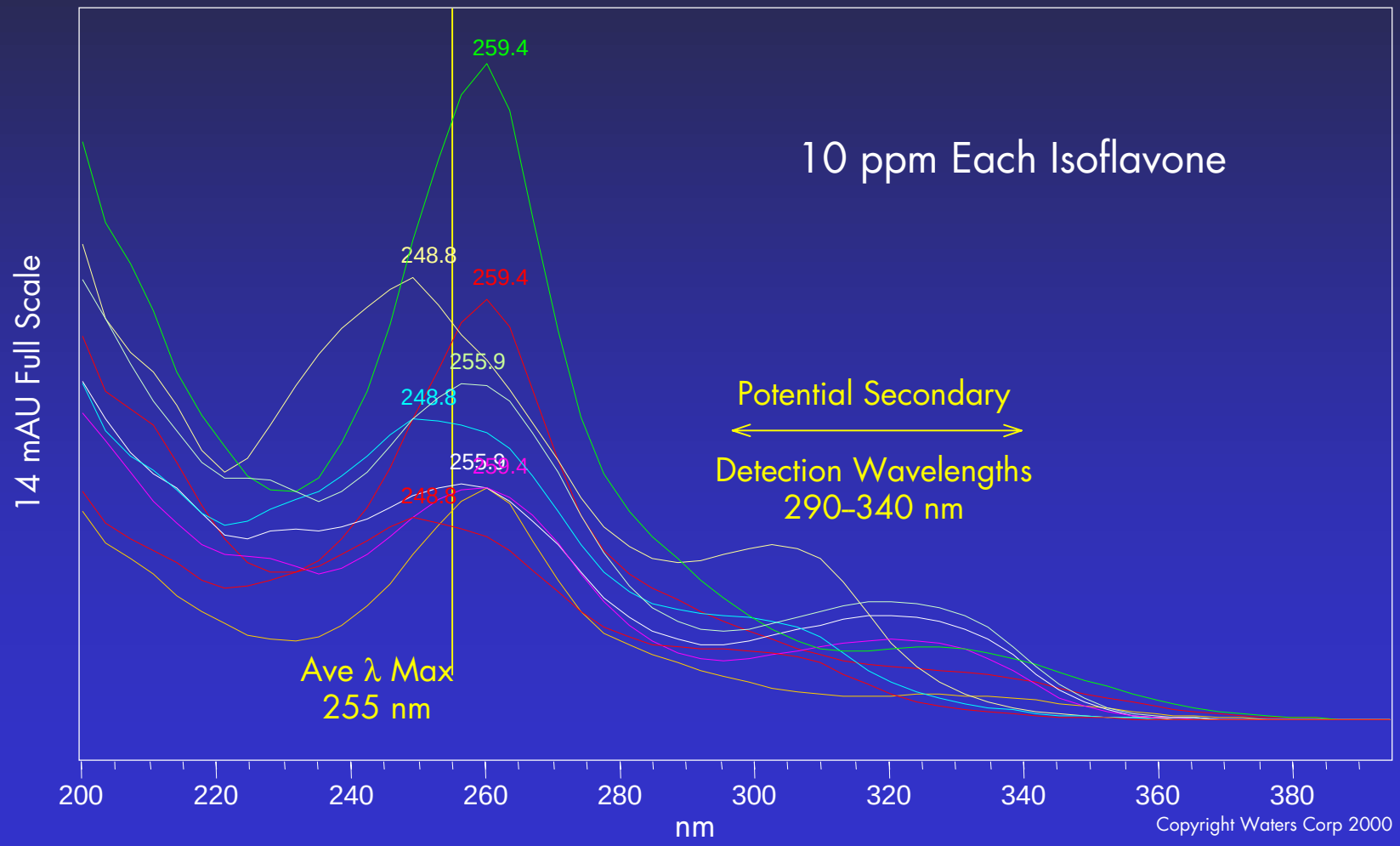
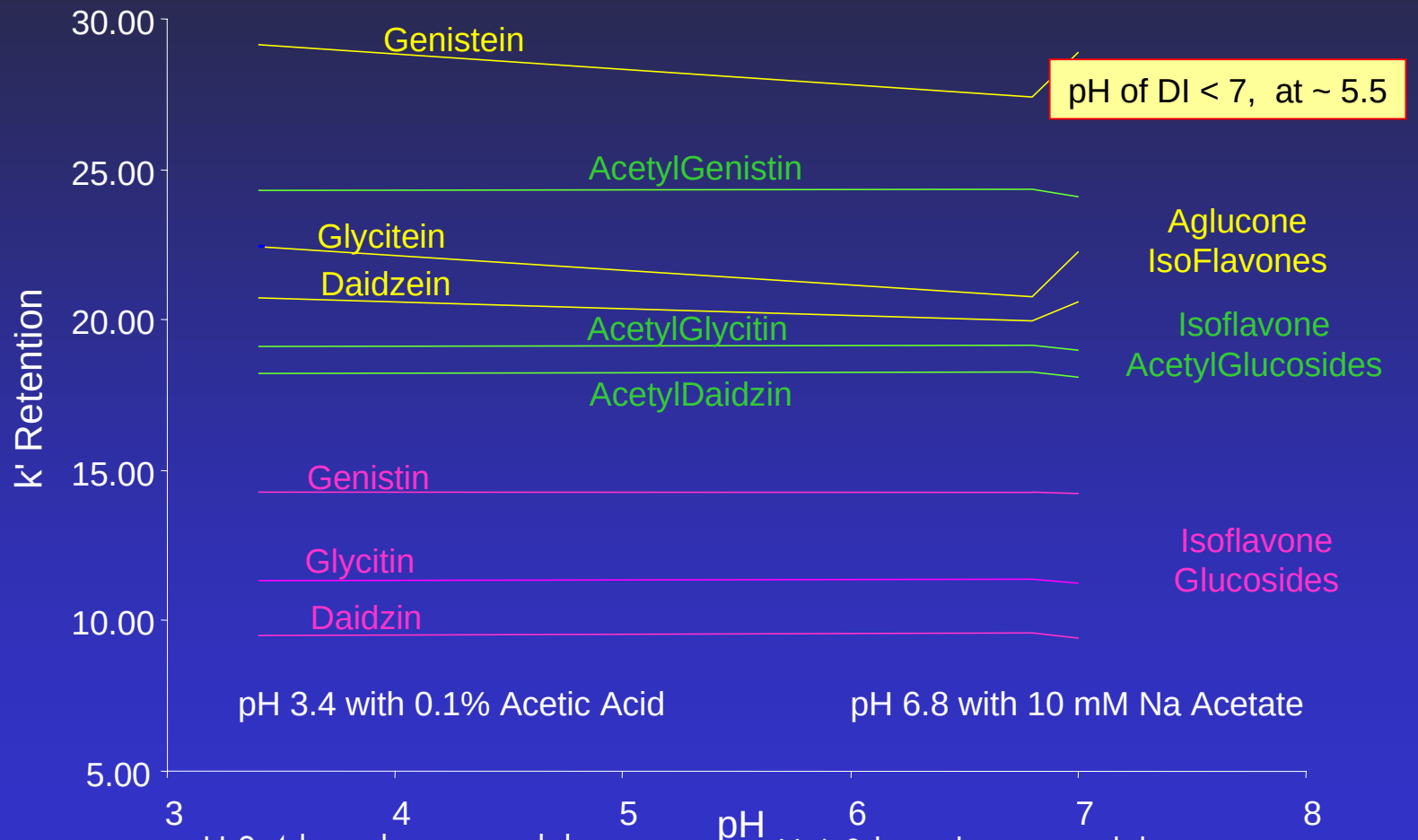
Isoflavone Spectra
in AcCN / 0.1% Acetic Acid / pH 3.4



Soy IsoFlavone Selectivity

Symmetry® C18, 5 µm
2.1 x 150 mm



pH 3.4 based upon work by
Pat Murphy, et.al., Iowa State Univ.

pH 6.8 based upon work by
Steve Barnes, et.al.Univ of Alabama

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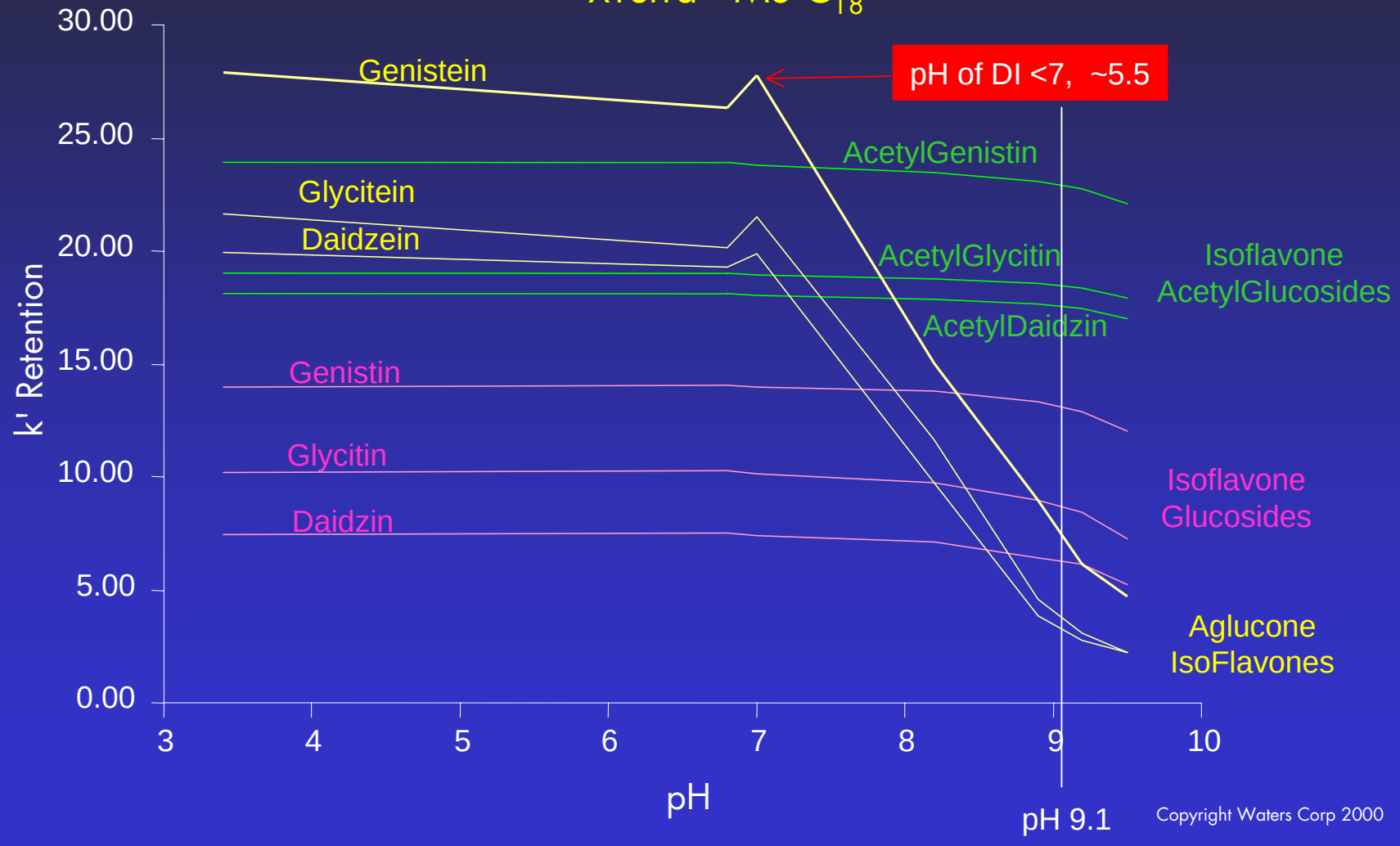
Soy IsoFlavone Selectivity

Alkaline pH with Ammonium BiCarbonate

XTerra™ MS C_{18}



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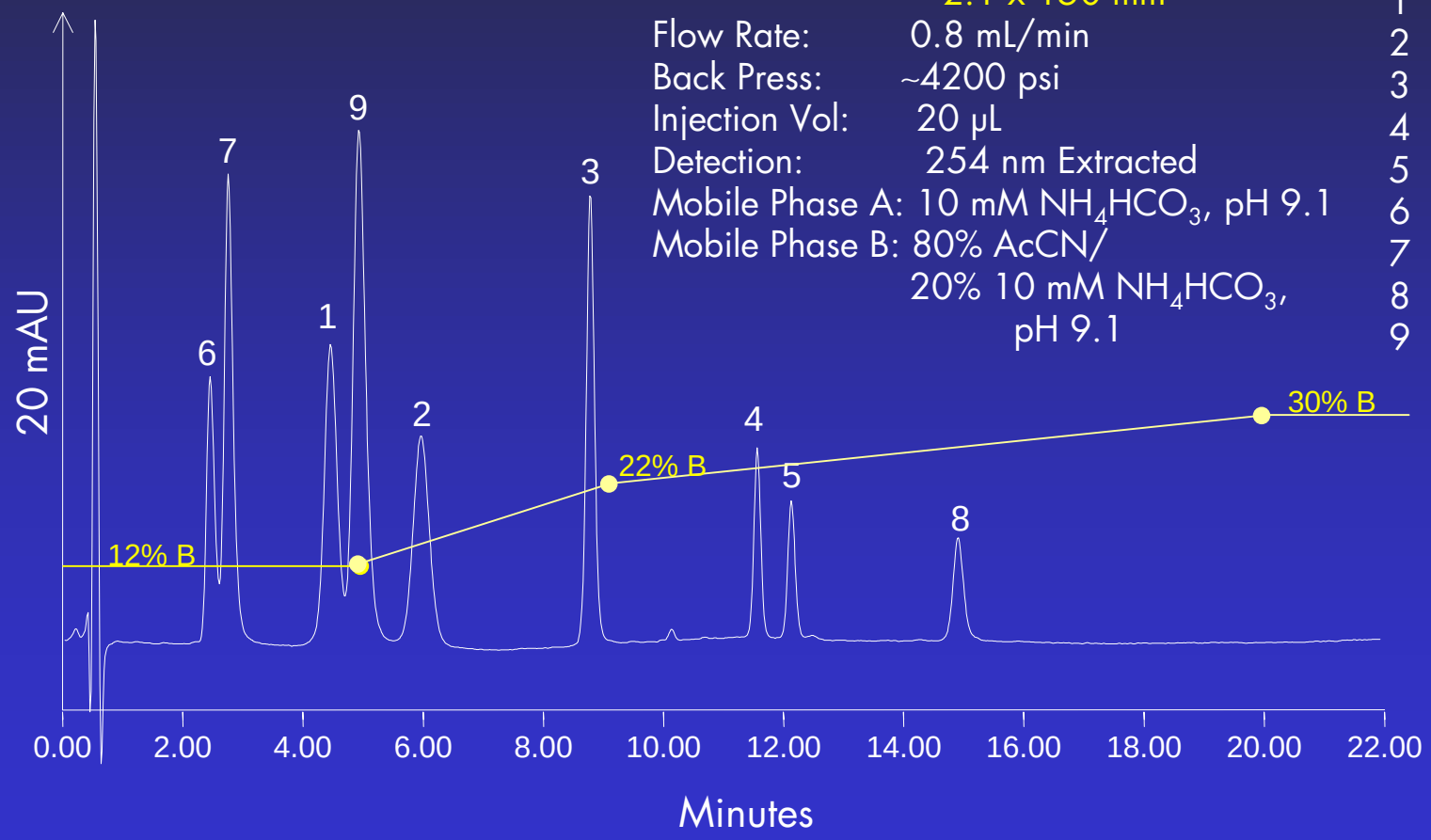
Soy IsoFlavones Resolution

Alkaline pH with Ammonium BiCarbonate Selectivity Change

Column: XTerra™ MS C_{18}, 5 µm 2.1 x 150 mm
Flow Rate: 0.8 mL/min
Back Press: ~4200 psi
Injection Vol: 20 µL
Detection: 254 nm Extracted
Mobile Phase A: 10 mM NH_4HCO_3, pH 9.1
Mobile Phase B: 80% AcCN/ 20% 10 mM NH_4HCO_3, pH 9.1

1 µg/mL
1 Daidzin
2 Glycitin
3 Genistin
4 AcetylDaidzin
5 AcetylGlycitin
6 Daidzein
7 Glycitein
8 AcetylGenistin
9 Genistein



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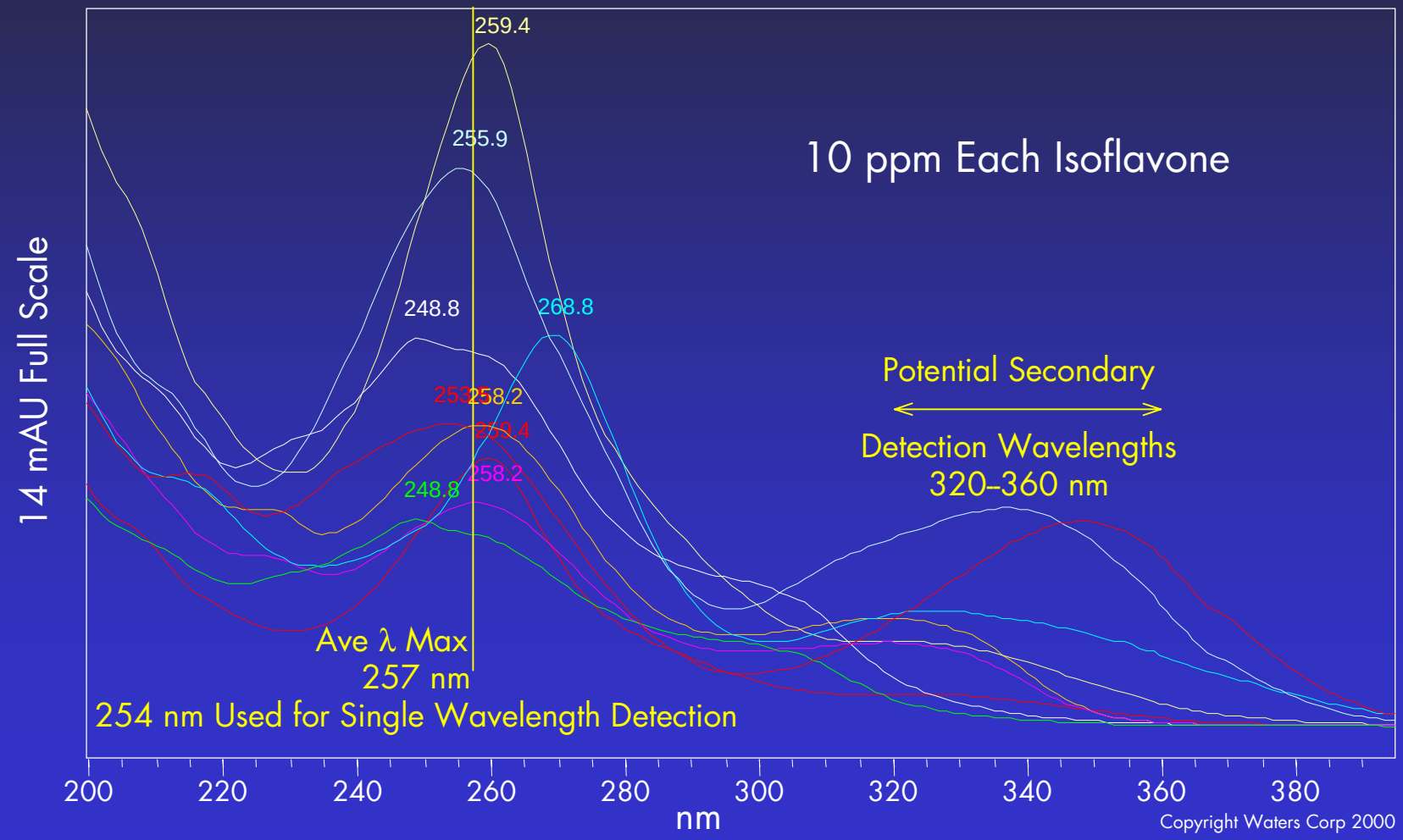
Isoflavone Spectra
in AcCN / NH_4HCO_3 / pH 9.1



Soy IsoFlavones Resolution

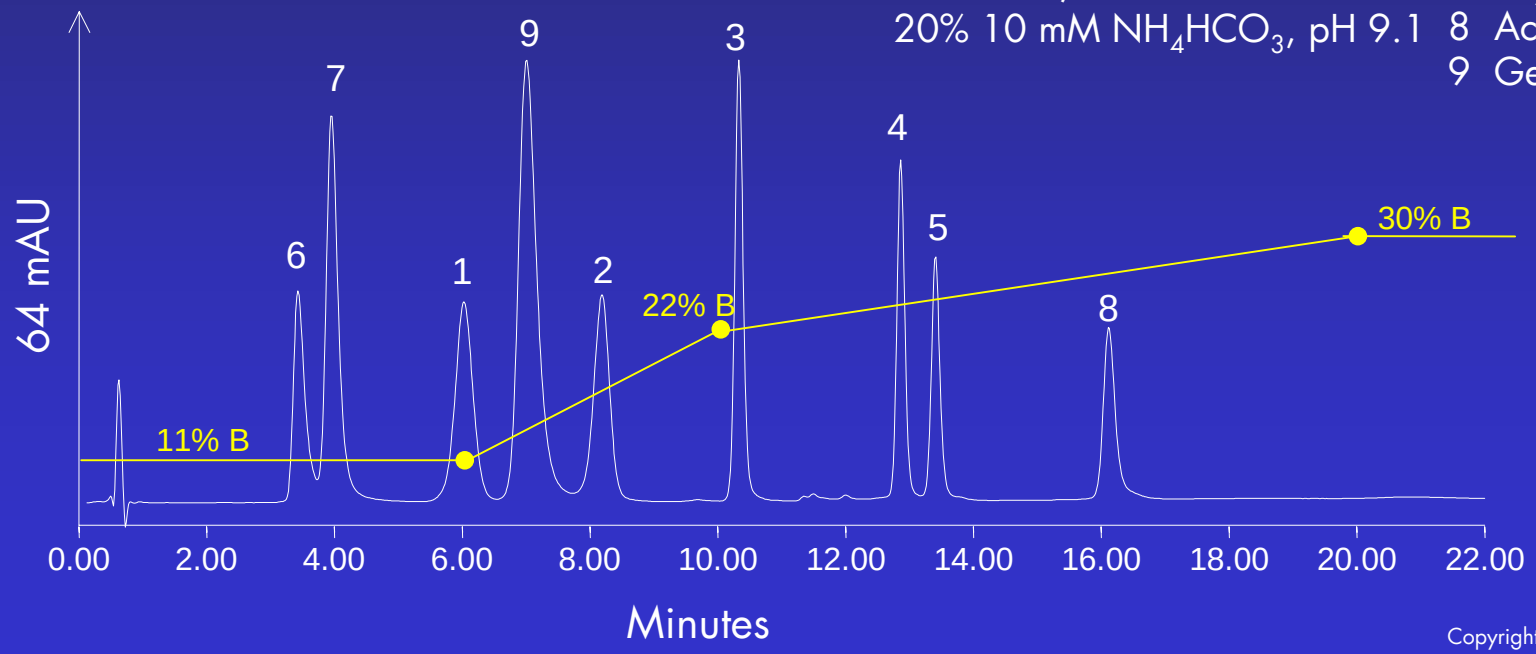
Alkaline pH with Ammonium BiCarbonate

Optimized Resolution

Column: XTerra™ MS C_{18}, 5 µm
2.1 x 150 mm
Flow Rate: 0.8 mL/min
Back Press: ~4200 psi
Injection Vol: 20 µL
Detection: 254 nm Extracted
Mobile Phase A: 10 mM NH_4HCO_3, pH 9.1
Mobile Phase B: 80% AcCN/
20% 10 mM NH_4HCO_3, pH 9.1

10 µg/mL
1 Daidzin
2 Glycitin
3 Genistin
4 AcetylDaidzin
5 AcetylGlycitin
6 Daidzein
7 Glycitein
8 AcetylGenistin
9 Genistein

Lower initial conditions by 1%
Extend time1 min for step 1 and 2



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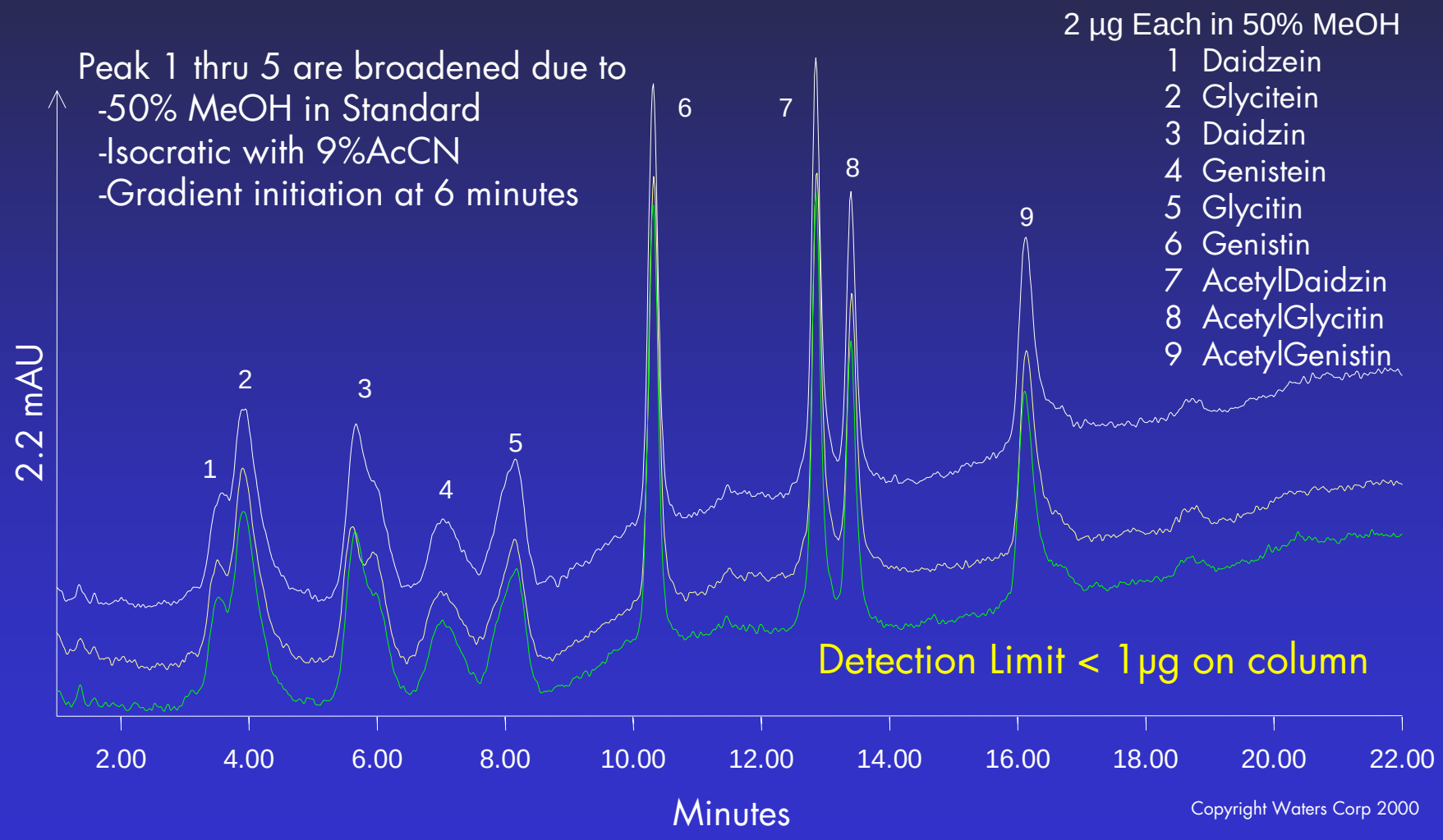
Soy IsoFlavones
Detection Estimate
Alkaline pH with Ammonium BiCarbonate



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Soy IsoFlavones
Sample Preparation

Based upon Murphy's procedure with soy based extracts and infant formula
J. Agric. Food Chem.,1997, 45, 4635- 4638

- Extract between 0.2 and 1 g of Sample with
  - 10 mL AcCN + 2 mL 100 mM HCl + 5 mL DI
  - stir overnight at ambient temperature
- Centrifuge extract to remove suspended solids
- Evaporate extract under N_2
- Resuspend in 100 mL of 50% MeOH
- Additional dilutions as appropriate with DI water

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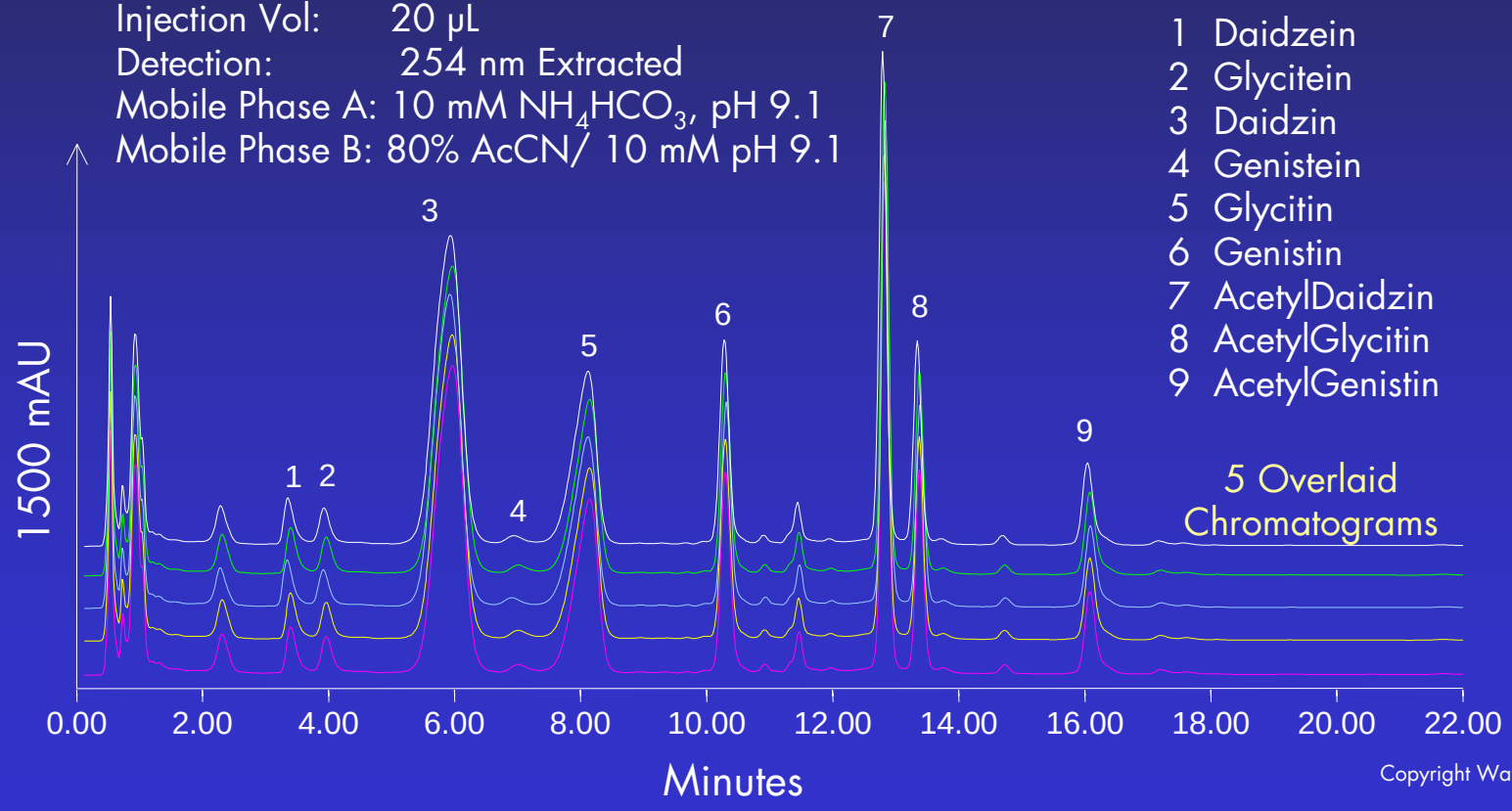
Soy Isoflavones
Concentrated Extract

Column: Xterra™ MS C_{18}, 5 µm
2.1 x 150 mm
Flow Rate: 0.8 mL/min
Back Press: ~4200 psi
Injection Vol: 20 µL
Detection: 254 nm Extracted
Mobile Phase A: 10 mM NH_4HCO_3, pH 9.1
Mobile Phase B: 80% AcCN/ 10 mM pH 9.1

Sample: SoyLife® 150, 15% Isoflavones, 98% as Glycosides
0.2 g / 10 mL (50% MeOH)



1 Daidzein
2 Glycitein
3 Daidzin
4 Genistein
5 Glycitin
6 Genistin
7 AcetylDaidzin
8 AcetylGlycitin
9 AcetylGenistin

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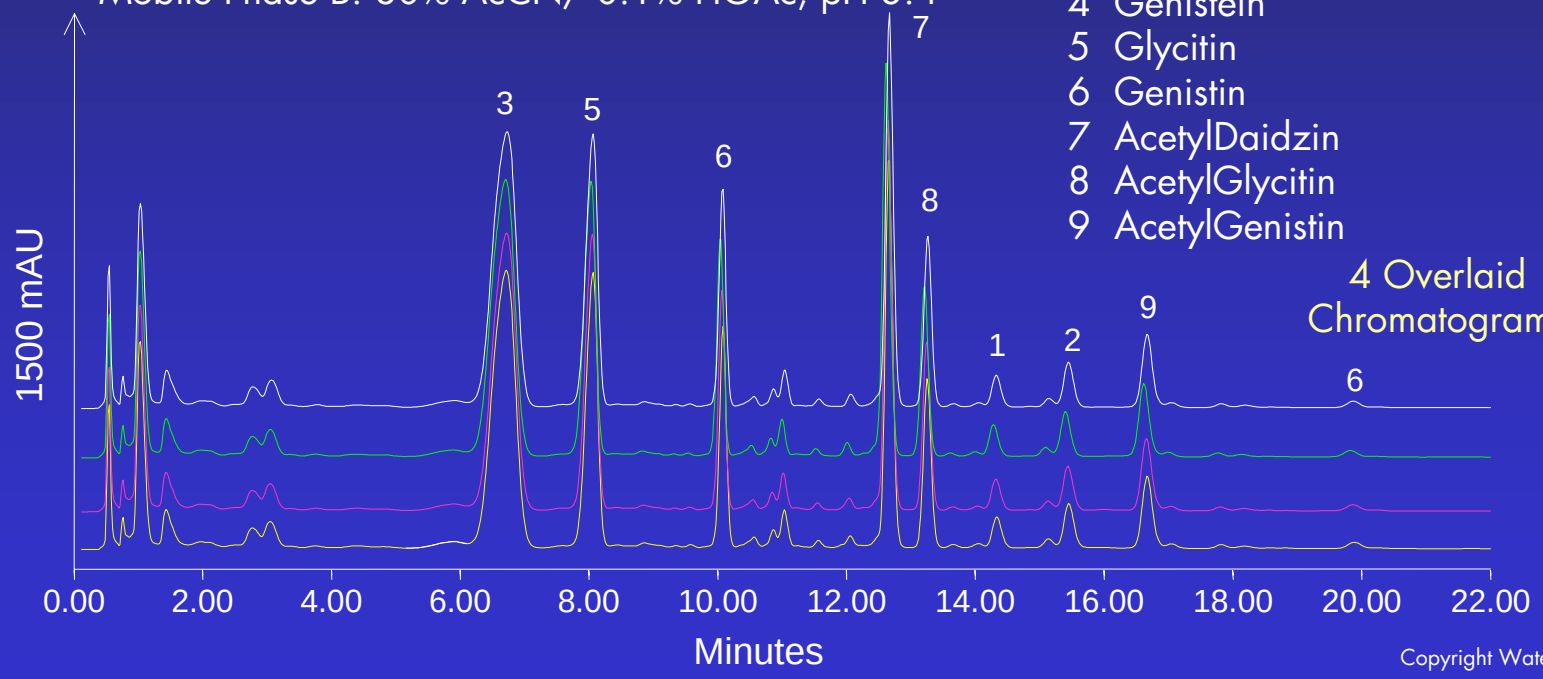
Soy Isoflavones Confirmation
Concentrated Extract

Column: Symmetry® C_{18}, 5 µm 2.1 x 150 mm
Flow Rate: 0.8 mL/min
Back Press: ~4200 psi
Injection Vol: 20 µL
Detection: 254 nm Extracted
Mobile Phase A: 0.1% HOAc, pH 3.4
Mobile Phase B: 80% AcCN/ 0.1% HOAc, pH 3.4

Sample: SoyLife® 150, 15% Isoflavones, 98% as Glycosides
0.2 g / 10 mL (50% MeOH)

1 Daidzein
2 Glycitein
3 Daidzin
4 Genistein
5 Glycitin
6 Genistin
7 AcetylDaidzin
8 AcetylGlycitin
9 AcetylGenistin



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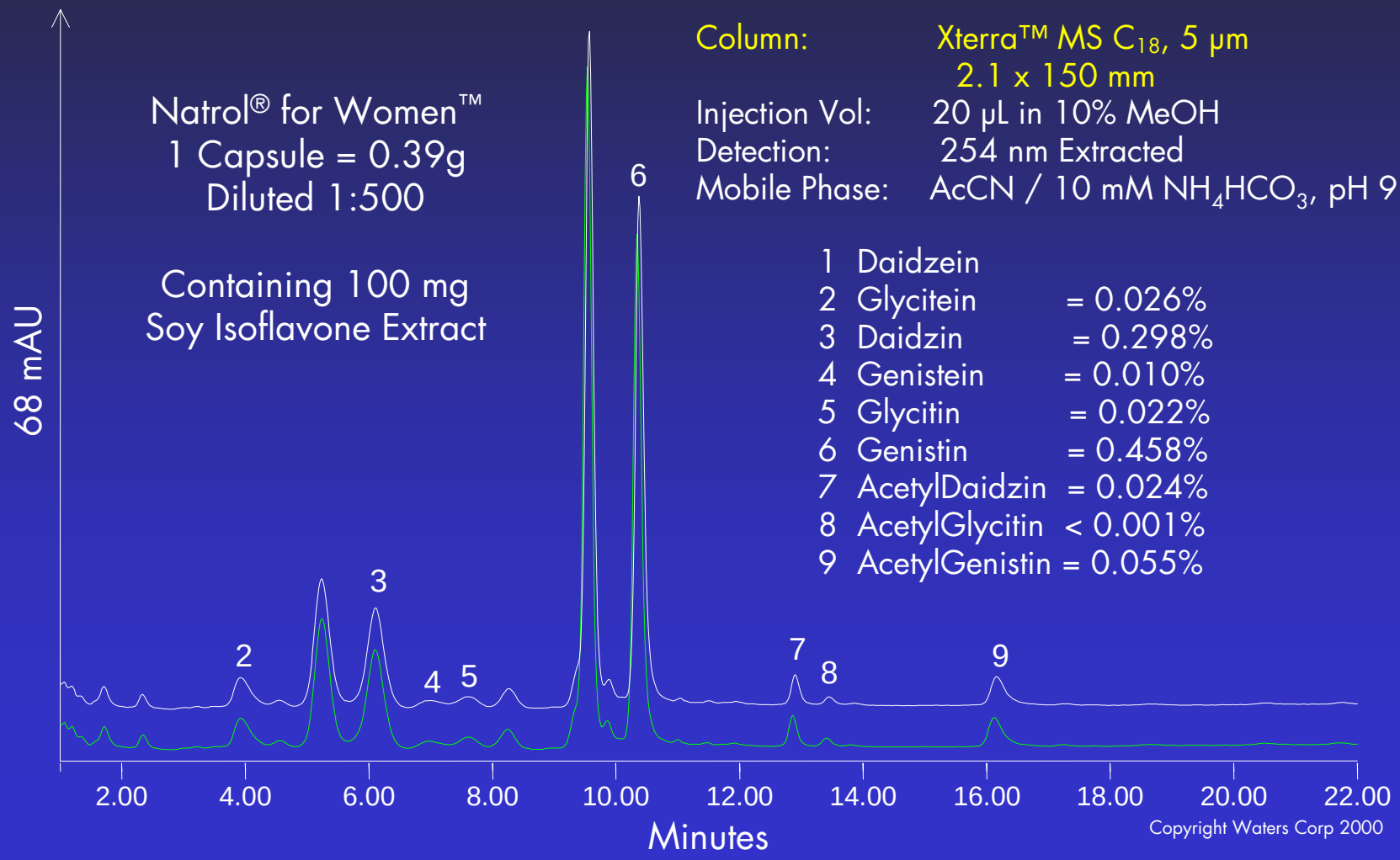
Soy Isoflavones
Dietary Supplement

Natrol® for Women™
1 Capsule = 0.39g
Diluted 1:500

Containing 100 mg
Soy Isoflavone Extract

Column: Xterra™ MS C_{18}, 5 µm
2.1 x 150 mm
Injection Vol: 20 µL in 10% MeOH
Detection: 254 nm Extracted
Mobile Phase: AcCN / 10 mM NH_4HCO_3, pH 9.1

1 Daidzein
2 Glycitein = 0.026%
3 Daidzin = 0.298%
4 Genistein = 0.010%
5 Glycitin = 0.022%
6 Genistin = 0.458%
7 AcetylDaidzin = 0.024%
8 AcetylGlycitin < 0.001%
9 AcetylGenistin = 0.055%



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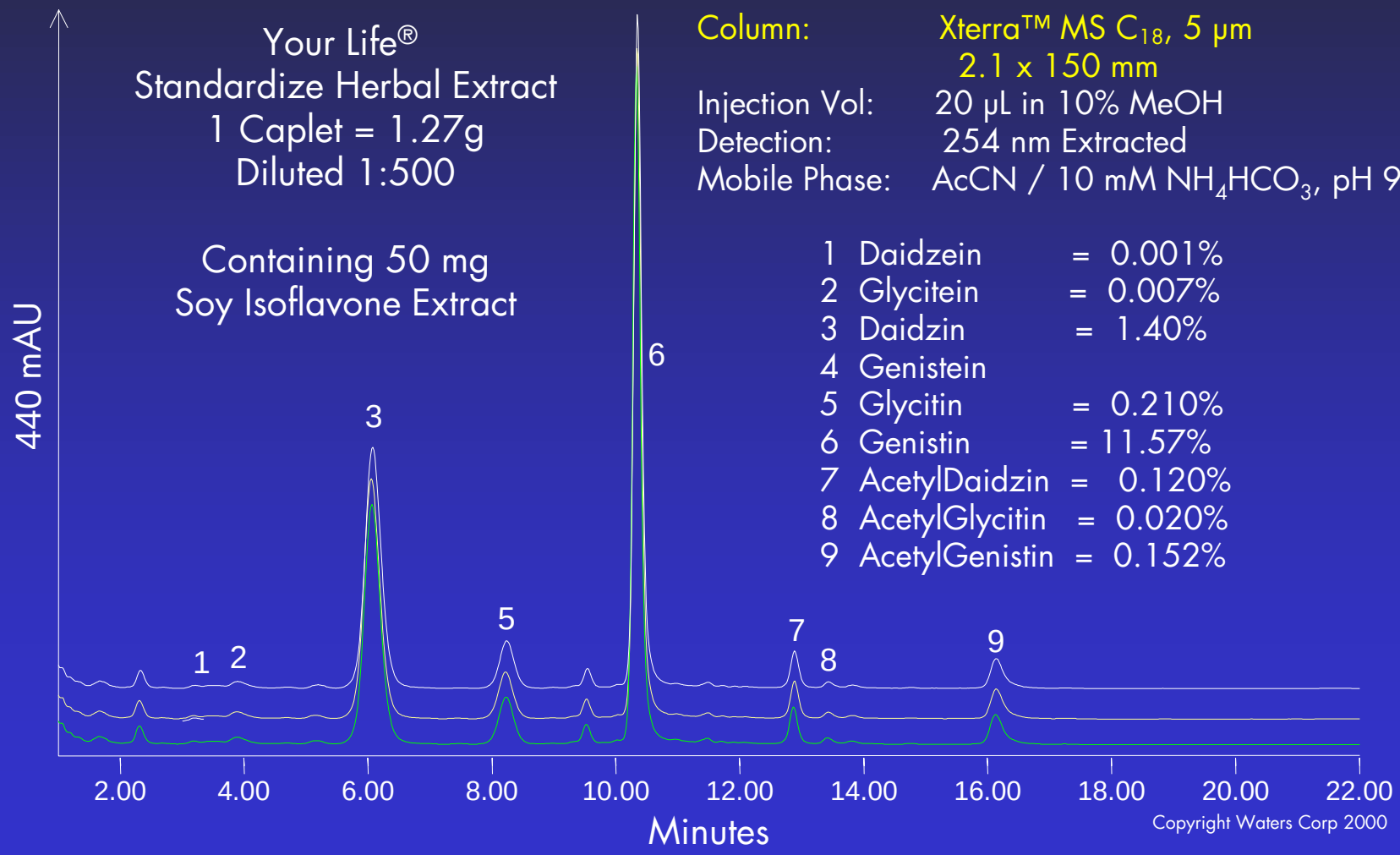
Soy Isoflavones
Dietary Supplement

Your Life®
Standardize Herbal Extract
1 Caplet = 1.27g
Diluted 1:500

Containing 50 mg
Soy Isoflavone Extract

Column: Xterra™ MS C_{18}, 5 µm
2.1 x 150 mm
Injection Vol: 20 µL in 10% MeOH
Detection: 254 nm Extracted
Mobile Phase: AcCN / 10 mM NH_4HCO_3, pH 9.1

1	Daidzein	= 0.001%
2	Glycitein	= 0.007%
3	Daidzin	= 1.40%
4	Genistein	
5	Glycitin	= 0.210%
6	Genistin	= 11.57%
7	AcetylDaidzin	= 0.120%
8	AcetylGlycitin	= 0.020%
9	AcetylGenistin	= 0.152%



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Method Development
Future Considerations

- Method needs to incorporate and resolve the malonyl glycoside esters from the 9 Isoflavones used in this study
  - Availability of standards
  - Expected to elute after the isoflavone glucosides and before the acetyl ester with acidic mobile phase
  - Expected to elute before isoflavone glucosides, in the region of aglucone elution with basic mobile phase
  - Use pH to optimize selectivity and resolution
  - Use Mass Spec to help look for analytes without the use of standards

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Method Development
Future Considerations

- Utility of the method for other isoflavones and flavone structures such as
  - Formononetin, Biochanin-A, Equol, Dihydrodaidzein, Luteolin, Quercetin, Apigenin, Kampferol, Myricetin, Naringenin, Hesperetin, Catechin
- Other polyphenolic structures

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Method Development
Future Considerations

Sample Preparation:

- Eliminate the need to evaporate the extract and resuspend in 50% to 80% MeOH
  - Improve early eluting peak shape using 5 to 10% MeOH
  - Prefer to extract in AcCN / NH_4HCO_3 mobile phase, then dilute with mobile phase

- Single sample prep for high lipid samples, such as dairy products
  - Potential for Solid Phase Extraction

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Method Development
Future Considerations

Chromatography:

- Optimize resolution and the gradient shape for malonyl glucosides with both acidic and basic mobile phases
- Develop a retention time index of known isoflavone analytes
- Optimize PDA identification in complex matrices
  - peak purity, and library spectrum matching
  - noise spectrum
- Use of Mass Spectrometry for identity confirmation
  - Molecular Weight aid to search for and identify unknowns

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Analysis of Soy Isoflavones
Using PDA and Mass Spec

- This work sets the foundation for 2 chromatographic conditions to function as complimentary and confirmational chemistries. Chromatographic selectivity
- In a complex matrix, such as food or natural products, there is always the risk of
  - quantitation bias due to analyte coelution with unknown components
  - false positive identification
- Use PDA <u>and</u> Mass Spec identification can eliminate this problem. Detection selectivity
- Amendable to QC and R&D

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