Amino Acid Analysis of Spinach and Apple using a QuEChERS Sample Preparation Technique and Automated OPA/FMOC Derivatization LC Method

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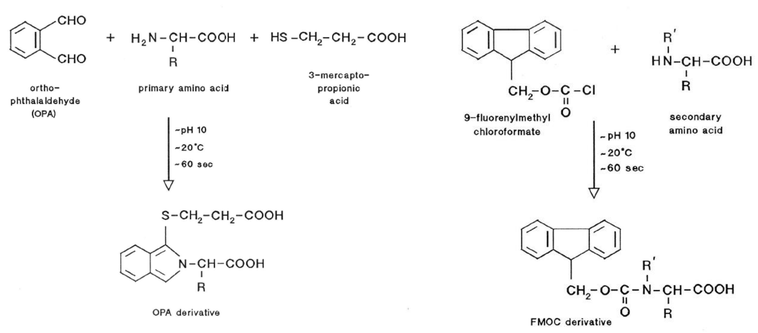
Introduction

An online automated OPA /FMOC derivatization method for amino acids will be used to analyze QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe) extracts of apple and spinach produce. Amino acid analysis of the food extracts will be compared. Scalability, batch-to-batch reproducibility, linearity, and longevity data of the amino acid method will be presented. Several column options will be shown, ranging from rapid nine minute analyses of 23 amino acids including re-equilibration using short (50 mm) Rapid Resolution High Throughput columns (1.8um), to 40 minute analyses using 250 mm traditional 5 um columns.

The LC Method

The Online Pre-Column Derivatizations

The primary amino groups react with ortho-phthalaldehyde (OPA) in the presence of 3-mercaptopropionic acid (3-MPA) at about pH 10 to form an isoindole derivative. Secondary amino groups do not react. The OPA derivatized amino acid is then detected by UV at 338 nm.



The secondary amino groups react with 9-fluorenylmethyl chloroformate (FMOC) at about pH 10 to form a secondary amide. Secondary amino groups do not react. The FMOC derivatized amino acid is then detected by UV at 262 nm.

The Automated Derivatization

G1376C well plate automatic liquid sampler (WPALS):

1) Draw 2.5 µL from Borate vial (Agilent PN 5061-3339)
2) Draw 1.0 µL from Sample vial
3) Mix 3.5 µL in washport 5X
4) Wait 0.2 min
5) Draw 0.5 µL from OPA vial (Agilent PN 5061-3335)
6) Mix 4.0 µL in washport 10X max speed
7) Draw 0.4 µL from FMOC vial (Agilent PN 5061-3337)
8) Mix 4.4 µL in washport 10X max speed
9) Draw 32 µL from Injection Diluent vial
10) Mix 20 µL in washport 8X
11) Inject
12) Wait 0.1 min
13) Valve bypass

The Mobile and Stationary Phase

Stationary Phase: ZORBAX Eclipse Plus C18
Column Temperature: 40 °C
Mobile Phase A: 10 mM Na_2HPO_4: 10 mM $Na_2B_4O_7$, pH 8.2: 5 mM NaN_3
Mobile Phase B: Acetonitrile: Methanol: Water (45:45:10, v: v: v)
Injection Diluent: (0.25 mL H_3PO_4 + 100 mL H_2O)

The Linear Gradients

The gradient profile (%B) is identical for all columns. The different gradient delay times are mitigated by reducing delay volume and the isocratic hold in the beginning of the gradient program.

Traditional high resolution method gradients

4.6 x 250, 5µm PN 959990-902	
time (min.)	%B
0	2
0.84	2
33.4	57
33.5	100
39.3	100
39.4	2
40	end
flow (mL/min.)	1.5

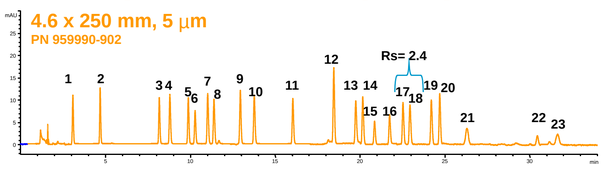
Rapid Resolution method gradients

	4.6 x 150, 3.5 µm PN959963-902	2.1x 150, 3.5 µm PN959763-902
time (min.)	%B	%B
0	2	2
0.5	2	2
20	57	57
20.1	100	100
23.5	100	100
23.6	2	2
25	end	end
flow (mL/min.)	1.5	0.42

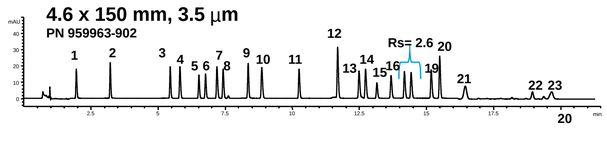
Rapid Resolution High Throughput method gradients

	4.6 x 100, 1.8 µm PN959964-902		4.6x50, 1.8 µm PN959941-902	
time (min.)	%B		time (min.)	%B
0	2		0	2
0.35	2		0.2	2
13.4	57		7.67	57
13.5	100		7.77	100
15.7	100		8.3	100
15.8	2		8.4	2
16	end		9	end
flow (mL/min.)	1.5		flow (mL/min.)	2.0

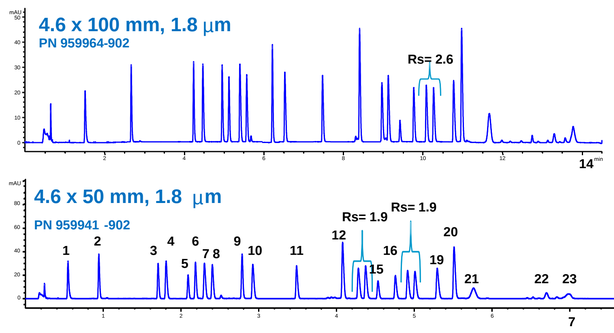
An Eclipse Plus C18 5 µm Option



A Rapid Resolution 3.5 µm Option



RRHT 1.8 µm Options



Amino Acid Identification and Detection

1. Aspartic acid
2. Glutamic acid
3. Asparagine
4. Serine
5. Glutamine
6. Histidine
7. Glycine
8. Threonine
9. Arginine
10. Alanine
11. Tyrosine
12. Cystine
13. Valine
14. Methionine
15. Norvaline
16. Tryptophan
17. Phenylalanine
18. Isoleucine
19. Leucine
20. Lysine
21. Hydroxyproline
22. Sarcosine
23. Proline

Primary Amino Acids 1-20 are detected at UV wavelength 338nm (DAD1, Sig=338,10 Ref=390,20 TT)

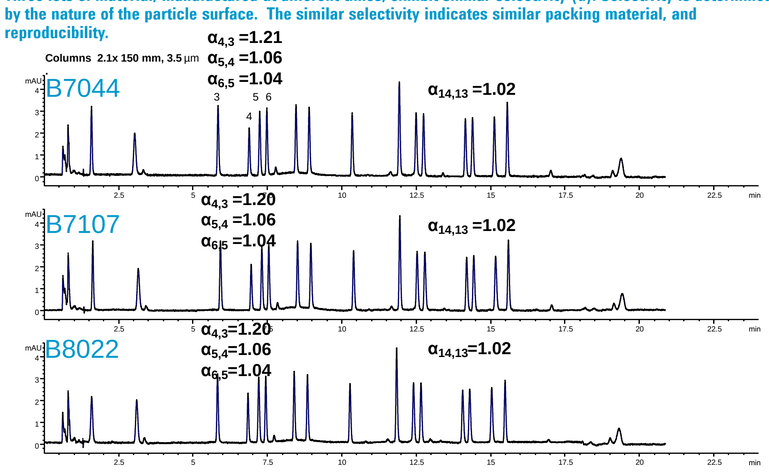
Secondary Amino Acids 21-23 are detected at UV wavelength 262nm (DAD1, Sig=262,4 Ref=390,20)

A programmed signal wavelength change from 338 nm to 262 nm is determined by choosing a switching time after lysine elutes and before hydroxyproline elutes

Method Ruggedness

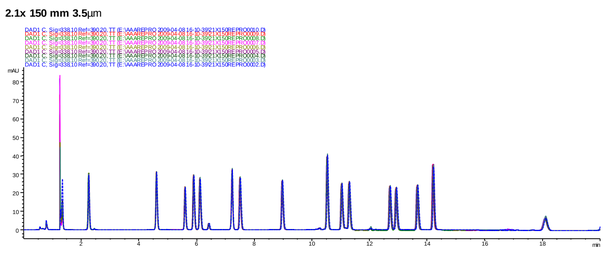
Lot-to-Lot Reproducibility

Three lots of material, manufactured at different times, exhibit similar selectivity (α). Selectivity is determined by the nature of the particle surface. The similar selectivity indicates similar packing material, and reproducibility.



Injection-to-Injection Reproducibility

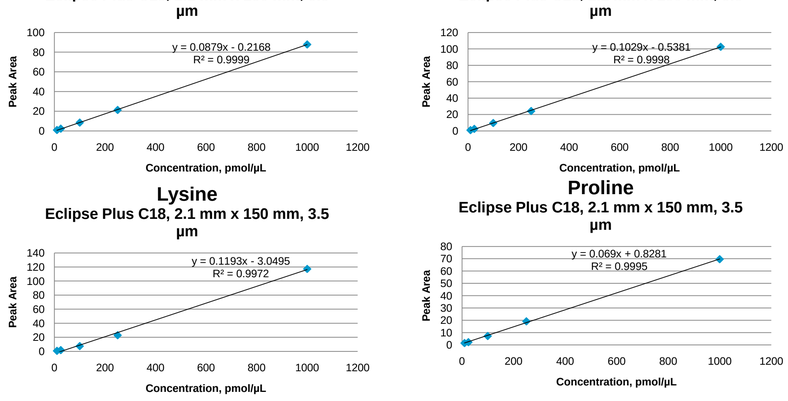
Overlay of eight sequential injections showing reproducibility of the online derivatization and gradient programs. Peak area of early, middle and late eluting amino acids are statistically tabulated below. The other amino acids had similar statistics.



amino acid	Peak Area Injection 1	Injection 2	Injection 3	Injection 4	Injection 5	Injection 6	Injection 7	Injection 8	Injection 9	StDEV	Mean	%RSD
glu	90.7	93.7	92.4	93.1	93.8	94.7	92.5	95.7	93.2	1.43	93.3	**1.5**
ala	109.9	113.2	111.8	112.1	113.3	114	111.6	116	113.3	1.72	112.8	**1.5**
cy2	153.2	157	155	157	157.8	159	154.4	162.6	158	2.79	157.1	1.8
lys	142.3	142.5	137.1	144.4	141.9	143.5	137.9	140.7	138.9	2.55	141.0	**1.8**
pro	60.8	62.9	61.8	65.8	60.9	63.2	61.2	70.2	61	3.11	63.1	**4.9**

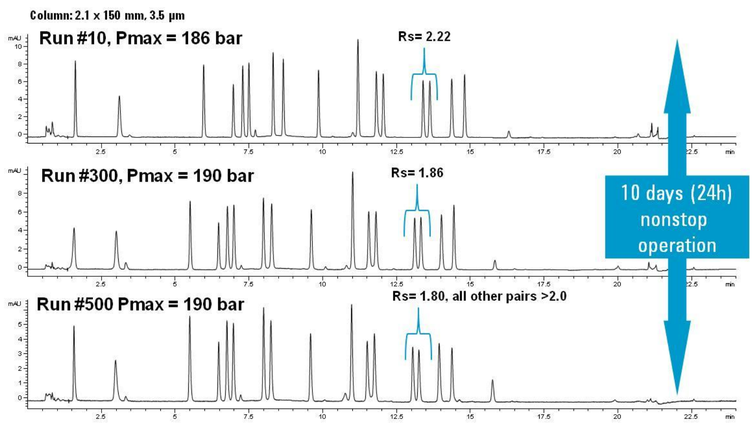
Linearity

Calibration curves of early, middle and late eluting amino acids show linearity over 1 to 1000 pmol/µL range using the Eclipse Plus C18, 2.1 x 150 mm, 3.5 µm method

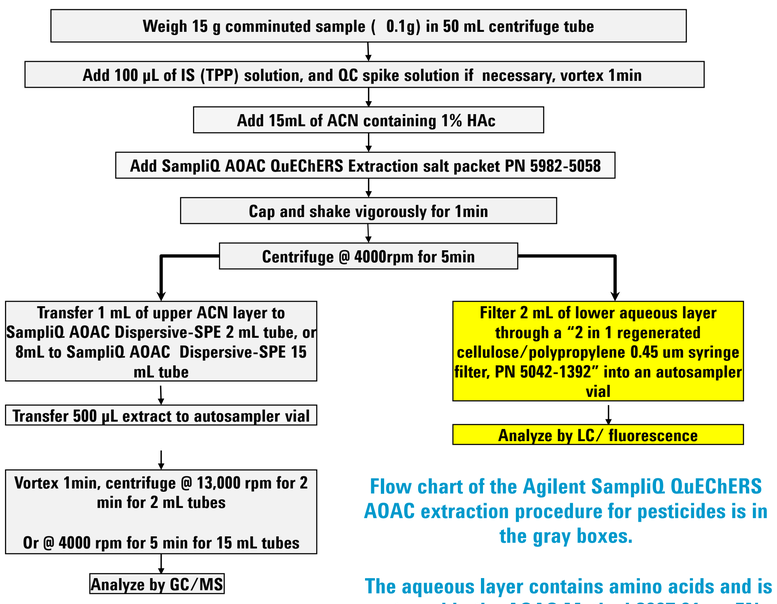


Lifetime

Overlay of early middle and late chromatograms of a 500 injection sequence



The QuEChERS Technique

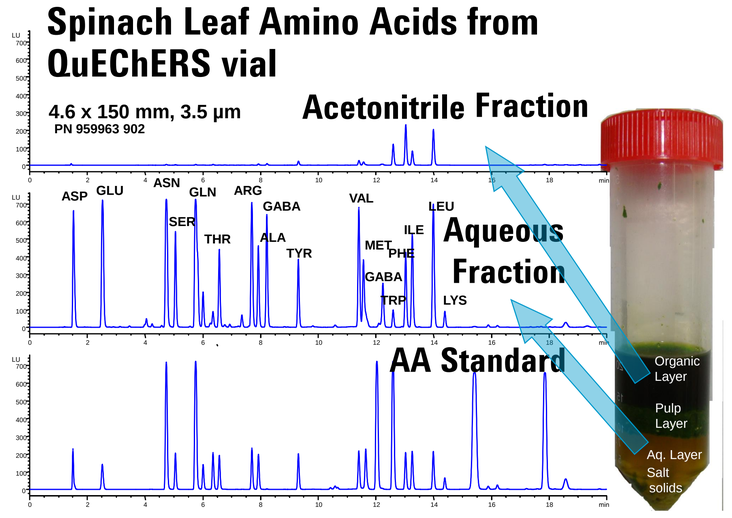


Flow chart of the Agilent SampliQ QuEChERS AOAC extraction procedure for pesticides is in the gray boxes.

The aqueous layer contains amino acids and is not used in the AOAC Method 2007.01 or EN Method 15662, but was analyzed with the Eclipse Plus C18 AAA LC method

Results

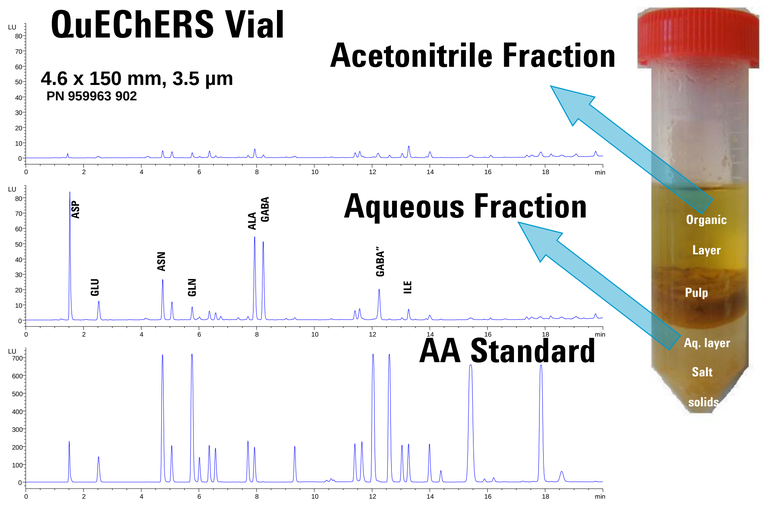
Spinach Leaf Amino Acids from QuEChERS vial



The $MgSO_4$ in the QuEChERS procedure partitions the Acetonitrile and water, concentrating the pesticides in the organic layer. The more polar amino acids concentrate in the aqueous layer.

Amino acid levels were under 100 pmol/uL therefore a G1321A fluorescence detector was used in place of UV for increased sensitivity, (λEx 340, λEm450, PMT gain 12 for OPA –AA, λEx 266, λEm305 for FMOC-AA (a programmed wavelength switch occurs after lysine elutes and before hydroxyproline elutes).

Apple Fruit Amino Acids from the QuEChERS Vial



Apples contained less amino acids compared to spinach. GABA (γ-aminobutyric acid) was also found in apple and spinach (GABA is not in standard but peak retention was confirmed, data not shown), and consists of two peaks. The major peak elutes about 8 minutes, and the minor elutes at about 12.2 minutes.

Besides amino acid content, the chromatographic "fingerprint" from the aqueous fraction QuEChERS protocol may be useful for determining ripeness, food quality, authenticity or adulteration, and variation of cultivars or origin.

Conclusions

- **An automated online derivatization method for amino acids using ZORBAX Eclipse Plus C18 was demonstrated as robust by longevity, lot-to-lot reproducibility, and linearity data.**
- **The Eclipse Plus C18 column choices offer the analyst high resolution, high speed, and reduced solvent consumption, in a combination that bests suits one's needs.**
- **QuEChERS extraction techniques may be a useful for analyzing fruit or vegetable for amino acids.**
- **Fluorescence detection can be substituted for UV detection for higher sensitivity.**

References

John W Henderson Jr, and Anne Mack "Improved Amino Acid Methods using Agilent ZORBAX Eclipse Plus C18 Columns for a Variety of Agilent LC Instrumentation and Separation Goals"
Agilent Pub.# 5990-4547EN (2009)

Cliff Woodward, John W Henderson Jr. and Todd Wielgos, "High-Speed Amino Acid Analysis (AAA) on Sub-Two Micron Reversed-phase (RP) Columns" Agilent Pub.# 5989-6297EN (2007)

Limian Zhao and Joan Stevens, "Analysis of Pesticide Residues in Spinach Using Agilent SampliQ QuEChERS AOAC Kits by GC/MS" Agilent Pub.# 5990-4305EN (2009)