A Comparison of Amino Acid Analysis (AAA) using UHPLC-ToF and CE-ToF

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Purpose of Work

- Detection and quantification of amino acids plays an important role in protein analysis, food analysis, and physiological fluids.
- Sample preparation is an important issue in amino acid analysis and the sample matrix.
- Samples are often complex and the separation can prove challenging.
- Amino acids are often difficult to detect so amino acid analysis (AAA) typically involves chemical derivatization to improve detection by UV or fluorescence.
- Time of Flight (ToF) Mass Spectrometry coupled with either Ultra-High Pressure Liquid Chromatography (UHPLC) or Capillary Electrophoresis (CE) provides a sensitive and discriminating detection technique which will preclude the need for derivatization and avoid some of the sample matrix interferences.

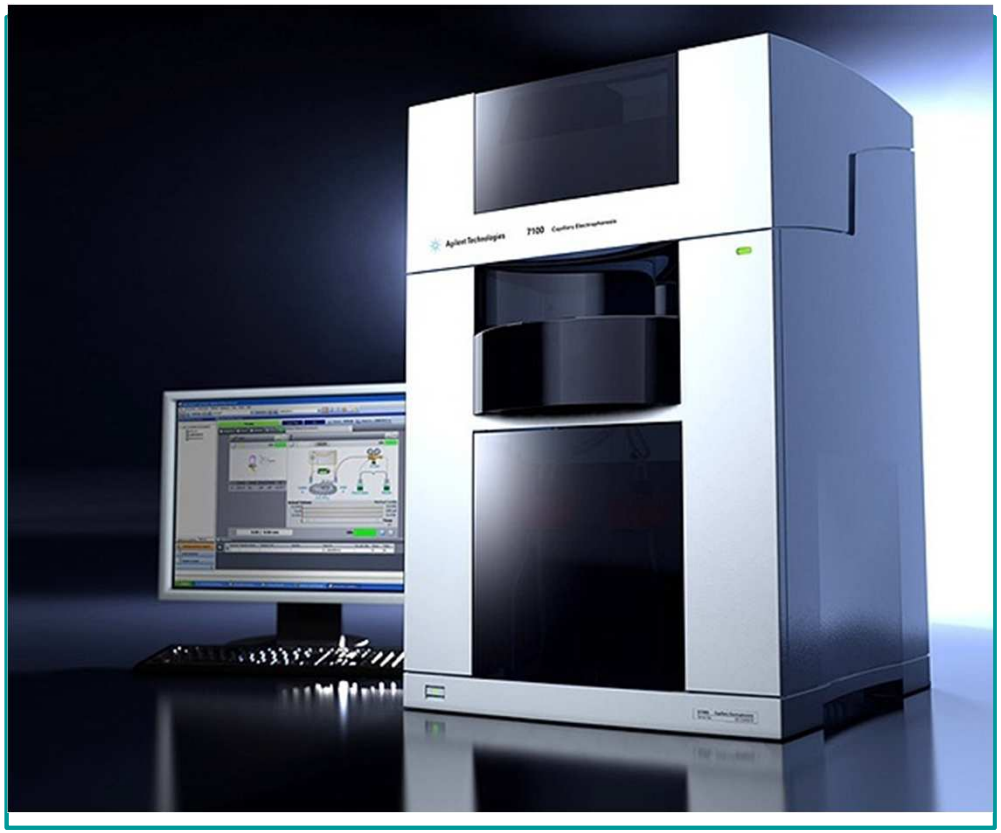
- **Goal:** To compare these two sample introduction systems using a time-of-flight mass spectrometer as the detection system.

Agilent 1290 Infinity LC

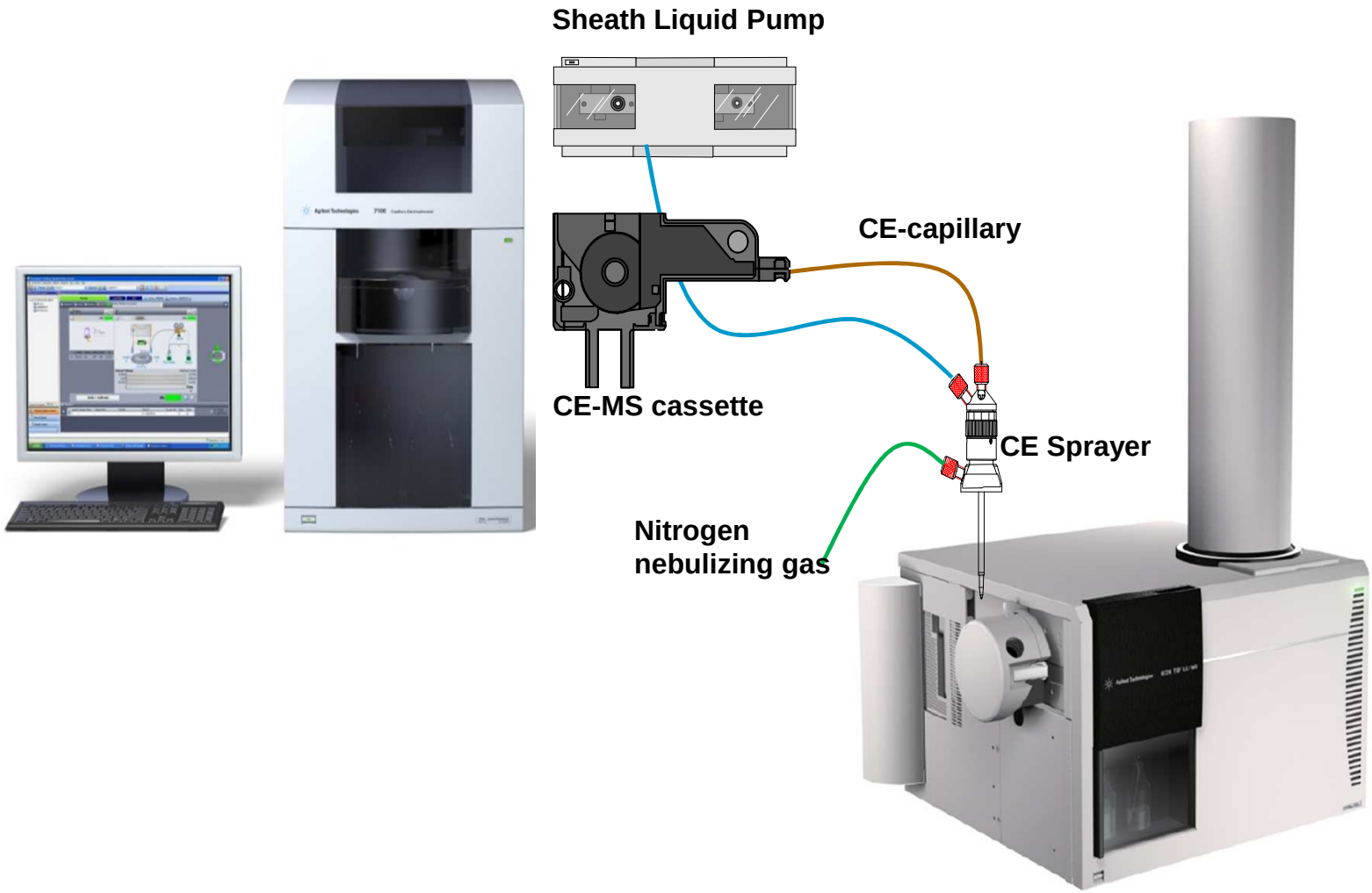
- Power range 1200 bar (2 ml/min);
  800 bar 5 ml/min)
- Delay volume (Pump (w/o) mixer: 10 µl)
- Pump: e.g RT stability < 0.02 % (1.5 min runs)
- ALS precision for small volumes
- Injection principles: flow-through
- TCC: column switching, easy valve exchange
- DAD: lowest detector noise < +/- 3 µAU/cm
  Max-Light High Sensitivity cell

Agilent 7100 Capillary Electrophoresis System Summary

- Updated UV detector with 40Hz acquisition rate
- Smaller footprint
- Flexible detection options
  - UV, MS, LIF, CCD
- Plug & play CE-MS
  - Grounded source allows easy interfacing

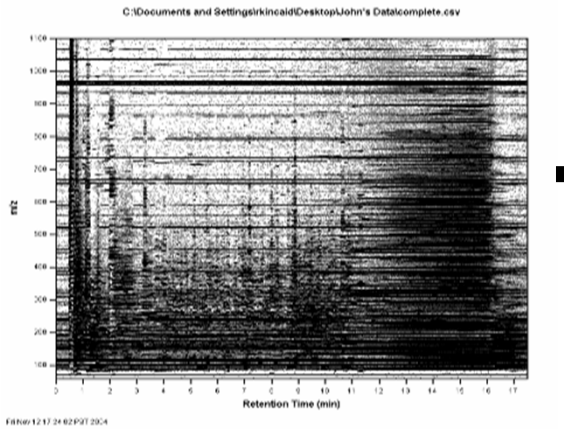


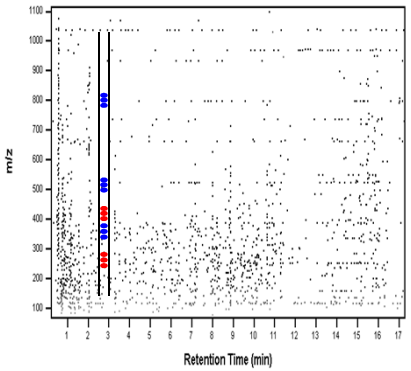
Agilent CE-ESI-MS Setup



Molecular Feature Extractor (MFE)

- How the MFE works
  - Map signals in the 3-dim. space in time and mass at the MS level.
    IMPORTANT: High resolution in mass and time!
  - Remove areas which only contain noise, and NO signals.
  - Identify all mass signals with a common RT (narrow time window)
  - Combine mass signals with common RT and chemical relation (isotope, adduct, dimer, higher charge state) => Molecular feature or Compound.
  - Create Extracted Compound Chromatograms (ECC) and Compound Mass Spectra from ions associated to a molecular feature (compound)
  - Feature consists of an accurate neutral mass, a retention time, and a peak volume





RT	m/z	Abund
...	...	...
...	...	...
2.11	195.1745	21000
2.11	257.2566	33550
2.34	224.2134	11784
...	...	...
...	...	...

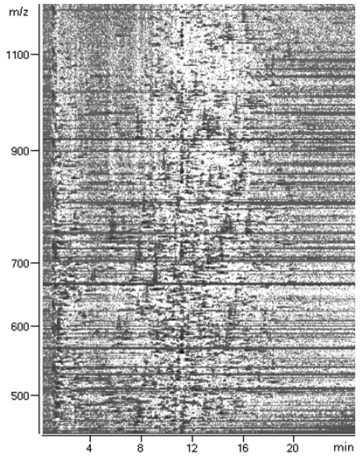
Compound list or Molecular feature list

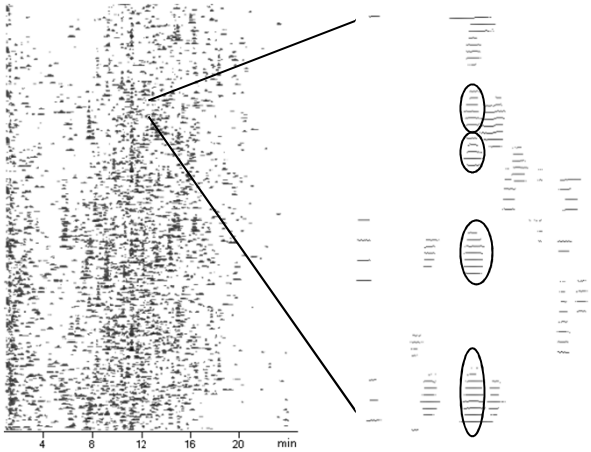
Export features:4664 / groups:1167

	RT	mass	abund.	height	#ions
463	7.715	1271.5948	11146	1329	3
464	7.716	1579.7000	1223	188	2
465	7.716	748.3860	18753	1216	4
466	7.718	1970.7935	2788	281	3
467	13.242	2826.4049	11853	662	4
468	13.244	2814.4034	60168	1698	8
469	13.245	2816.4119	8707	680	3
470	13.246	2331.1504	3191	291	3
471	13.247	1407.6968	4377	385	2
472	13.249	2761.4877	4778072	79795	78
473	13.250	2433.2736	3736	399	3
474	13.250	2806.4575	16286	650	7
475	13.252	1123.6411	4246	416	2
476	13.253	2068.0019	14029	665	7
477	13.253	2783.4528	27728	1212	8
478	9.159	677.2975	8801	714	2
479	9.159	2173.8394	3492	427	3
480	9.161	1310.6477	1838172	70500	23
481	9.161		12218	1585	1
482	9.162	1334.6137	75374	2869	10
483	10.385	1540.6853	2023	344	2

Isotope clusters grouped into molecular features

Data Analysis: Molecular Feature Extractor





species	RT	m/z	mass	abund.
M+3H	13.248	921.5025	2761.4856	356764
M+3H+1	13.247	921.8373	2761.4874	548320
M+3H+2	13.248	922.1722	2761.4897	476780
M+3H+3	13.249	922.5072	2761.4924	256680
M+3H+4	13.249	922.8416	2761.4934	117149
M+3H+5	13.251	923.1744	2761.4892	45705
M+3H+6	13.252	923.5058	2761.4810	15721
M+3H+7	13.246	923.8387	2761.4775	4492
M+3H+8	13.263	924.1649	2761.4535	677
M+2H+Na	13.248	928.8293	2761.4842	29521
M+2H+Na+1	13.249	929.1643	2761.4863	46140
M+2H+Na+2	13.247	929.4957	2761.4781	43276
M+2H+Na+3	13.247	929.8283	2761.4737	33217
M+2H+Na+4	13.258	930.1614	2761.4706	23203
M+2H+Na+5	13.285	930.5000	2761.4842	11741
M+2H+Na+6	13.277	930.8343	2761.4846	3970
M+2H+K	13.247	934.1483	2761.4673	8064
M+2H+K+1	13.241	934.4889	2761.4863	13793
M+2H+K+2	13.244	934.8196	2761.4759	11070
M+2H+K+3	13.250	935.1566	2761.4847	5695
M+2H+K+4	13.247	935.4933	2761.4926	3721
M+2H+K+5	13.280	935.8045	2761.4237	1182
M+H+2Na	13.238	936.1547	2761.4783	6951
M+H+2Na+1	13.241	936.4933	2761.4913	9925
M+H+2Na+2	13.244	936.8254	2761.4853	8407
M+H+2Na+3	13.251	937.1567	2761.4769	7074
M+H+2Na+4	13.256	937.4817	2761.4497	3386
M+H+2Na+5	13.242	937.8123	2761.4392	2586

Export features:4664 / groups:1167

	RT	mass	abund.	height	#ions
463	7.715	1271.5948	11146	1329	3
464	7.716	1579.7000	1223	188	2
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480	9.161	1310.6477	1838172	70500	23
481	9.161		12218	1585	1
482	9.162	1334.6137	75374	2869	10
483	10.385	1540.6853	2023	344	2

Raw data → **Background noise removed** → **Individual m/z peaks grouped into isotope clusters** → **Isotope clusters grouped into molecular features**

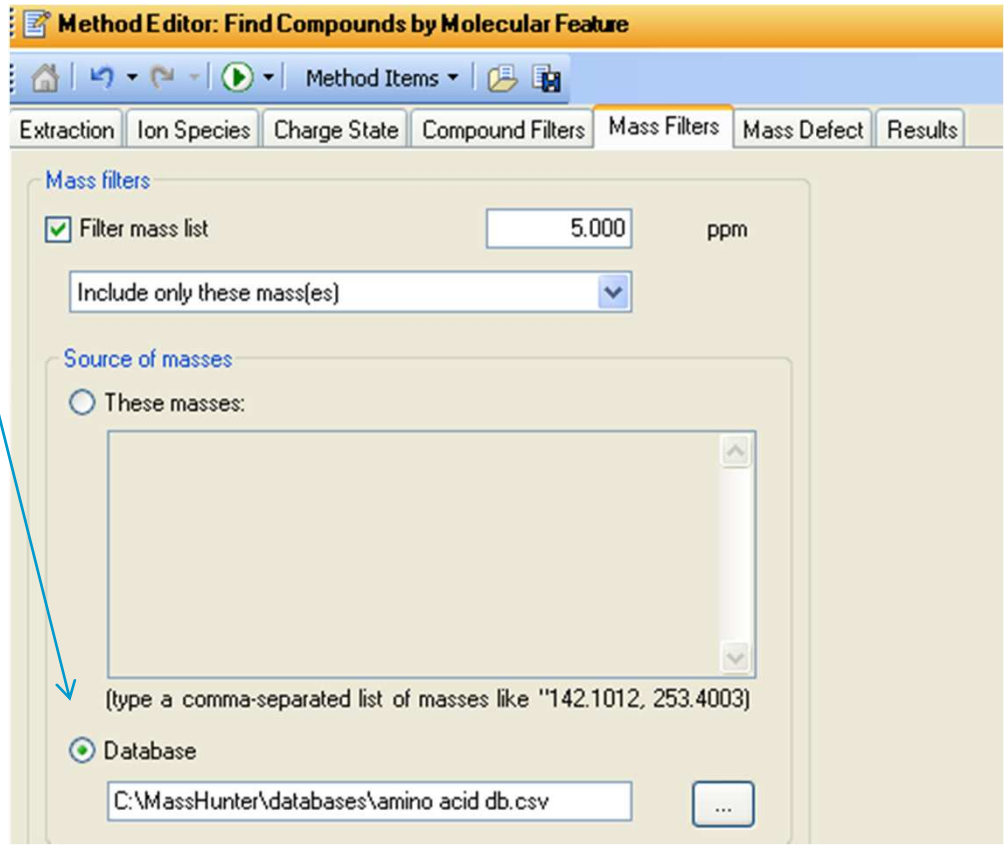
Identification, Quantification, Differential Analysis are performed on *chemically qualified compound data*

- Looks at Mass spectral data first → Groups co-eluting isotopes & converts to neutral mass
- Checks that there is a chromatographic response
- Groups all charge states, adducts, etc. associated with a given feature into a “peak volume”
- Feature consists of an accurate neutral mass, a retention time, and a peak volume

Data Analysis: Filter Molecular Feature Extractor Results by Amino Acid Database

Contents of Database (.csv file)

Formula	Mass	Compound name
C3H7NO2	89.0477	L-Alanine
C6H14N4O2	174.1117	L-Arginine
C4H7NO4	133.0375	L-Aspartic Acid
C6H12N2O4S2	240.0238	L-Cystine
C5H9NO4	147.0532	L-Glutamic Acid
C2H5NO2	75.032	Glycine
C6H9N3O2	155.0695	L-Histidine
C6H13NO2	131.0946	L-Isoleucine
C6H13NO2	131.0946	L-Leucine
C6H14N2O2	146.1055	L-Lysine
C5H11NO2S	149.051	L-Methionine
C9H11NO2	165.079	L-Phenylalanine
C5H9NO2	115.0633	L-Proline
C3H7NO3	105.0426	L-Serine
C4H9NO3	119.0582	L-Threonine
C9H11NO3	181.0739	L-Tyrosine
C5H11NO2	117.079	L-Valine



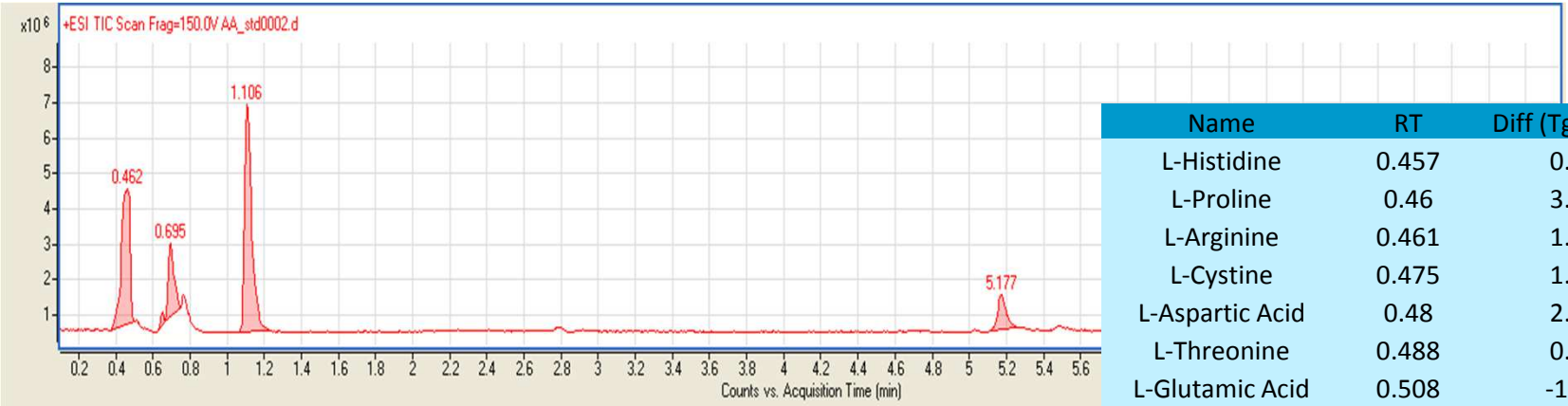
***Database contents only contain amino acids found in the standard mix**

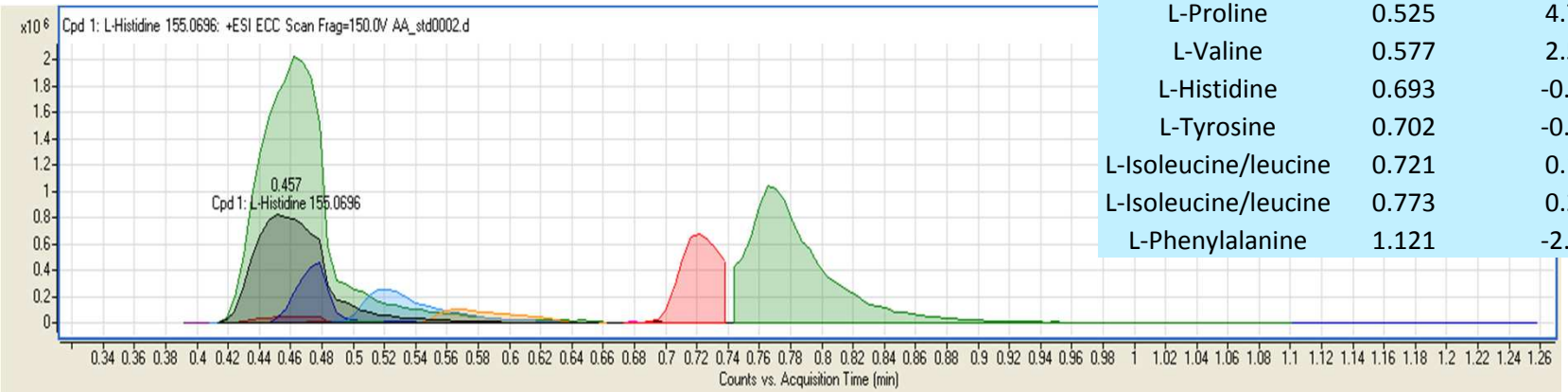
TOF Instrument Parameters for LC method:

Parameter	Setting
Source Type	Dual spray ESI in Positive mode
Gas temperature	325 ºC
Drying Gas Flow rate	12 L/min
Nebulizer Pressure	45 psi
Capillary Voltage	4000 V
Fragmentor Voltage	150 V
Acquisition rate	3 scans/sec
Reference correction	1 point @ 922.009798
Acquisition Mode	2 GHz, extended dynamic range 50 to 1000 M/Z

Initial LC Method Development (Amino Acid Standard)

Traditional chromatography using typical MS compatible mobile phases
- Zorbax HD SB C18 (1.8µm, 2.1x150mm) with 0.1% formic acid in water and 0.1% formic acid in ACN
- 95/5 (A/B) to 5/95 (A/B) over 10 minutes



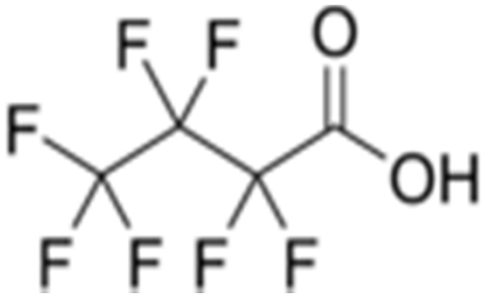


Name	RT	Diff (Tgt, ppm)	Vol %
L-Histidine	0.457	0.99	16.95
L-Proline	0.46	3.08	0.88
L-Arginine	0.461	1.14	39.12
L-Cystine	0.475	1.63	4.65
L-Aspartic Acid	0.48	2.82	0.17
L-Threonine	0.488	0.87	0.29
L-Glutamic Acid	0.508	-1.09	0.29
L-Proline	0.525	4.71	5.43
L-Valine	0.577	2.56	2.52
L-Histidine	0.693	-0.79	0.23
L-Tyrosine	0.702	-0.19	0.26
L-Isoleucine/leucine	0.721	0.19	7.94
L-Isoleucine/leucine	0.773	0.25	21.12
L-Phenylalanine	1.121	-2.28	0.17

-But underivatized amino acids are more polar than derivatized . . .
  - -minimal retention and separation
  - -Ion suppression (from co-elution) results in lack of sensitivity
  - -Possible co-elution with sample matrix (eluting near void volume)

Change Modifier from Formic Acid to Heptafluorobutyric acid

HFBA has a variety of niche applications in analytical and synthetic chemistry. It is an ion pair reagent for reverse phase HPLC. It is used in the sequencing, synthesis, and solubilizing of proteins and peptides.



Final 1290 Infinity UHPLC Parameters

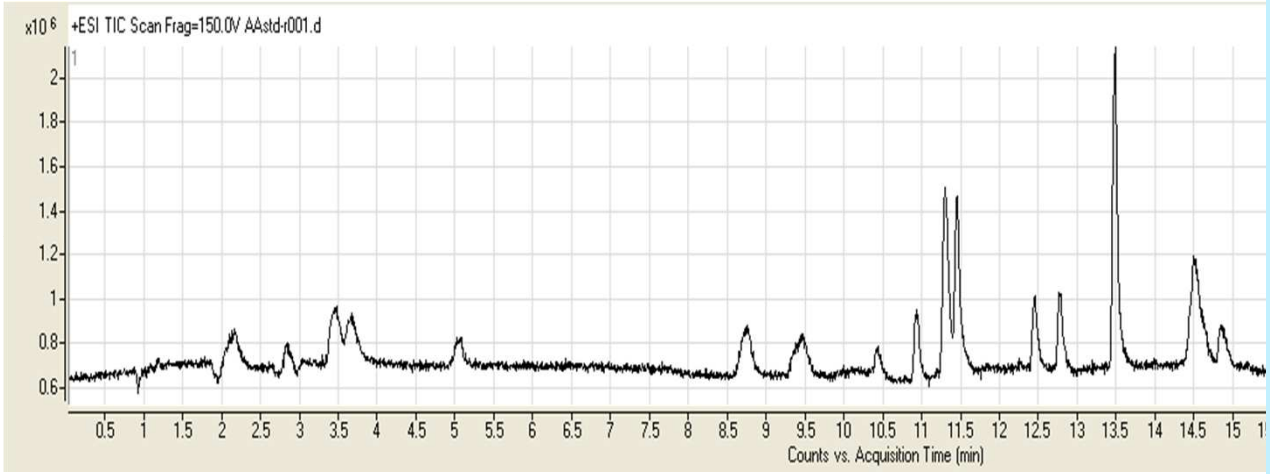
HFBA offers better retention/peak shape and is MS compatible

Parameter	Setting
Mixer	35 µL jet weaver
Column	Zorbax HD SB C18 (1.8µm, 2.1x150mm)
Column Temperature	45 ºC
Flow rate	0.3 mL/min
Mobile phase A	Water, 0.1 % HFBA
Mobile phase B	Acetonitrile, 0.1% HFBA
Injection Volume	3 µL

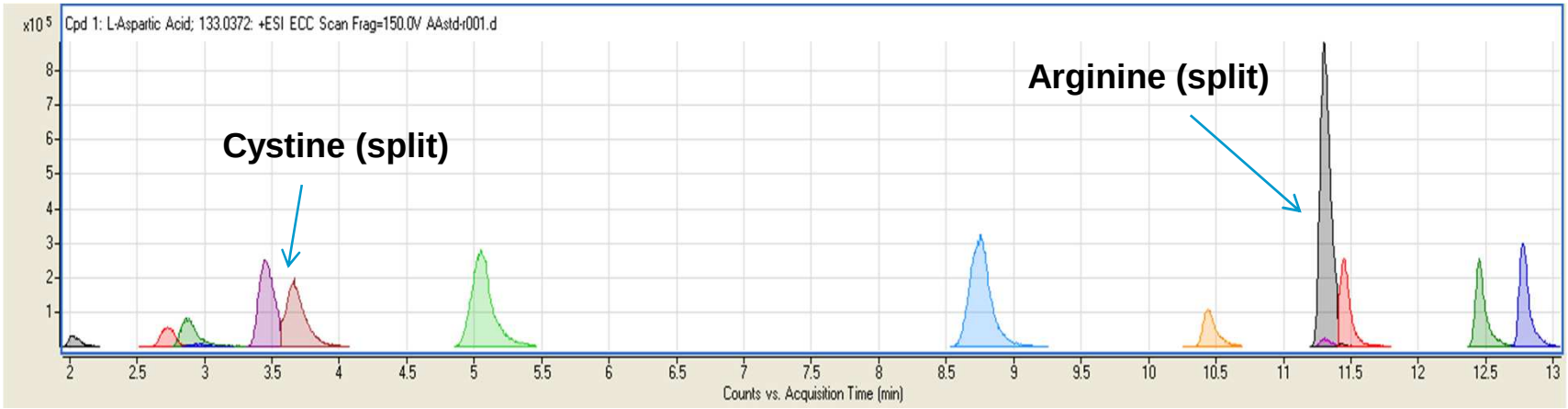
Gradient Program

Time (min)	%A	%B
0.0	100	0
5.0	100	0
15.0	70	30
15.1	40	60
18.0	40	60
18.1	100	0
21.0	100	0

Final LC Conditions (Amino Acid Standard)

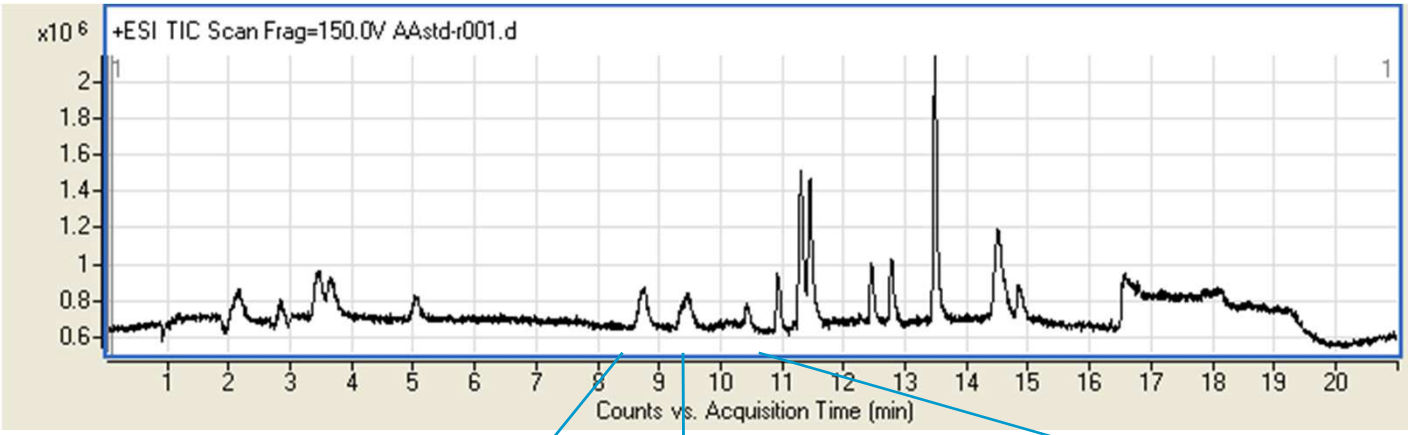


Name	RT	Diff (Tgt, ppm)	Vol %
L-Aspartic Acid	2.032	-2.48	0.86
L-Threonine	2.729	-3.13	1.92
L-Glutamic Acid	2.879	-1.97	3.26
L-Alanine	2.971	1.91	0.32
L-Cystine	3.456	0.83	9.14
L-Cystine	3.671	0.93	8.42
L-Proline	5.05	0.68	13.54
L-Histidine	8.747	-0.62	15.67
L-Valine	10.444	-1.15	3.42
L-Proline	11.306	-0.03	0.57
L-Arginine	11.307	-0.31	23.35
L-Arginine	11.455	0.4	6.27
L-Isoleucine/Leucine	12.455	-1.34	6.05
L-Isoleucine/Leucine	12.779	-1.28	7.21

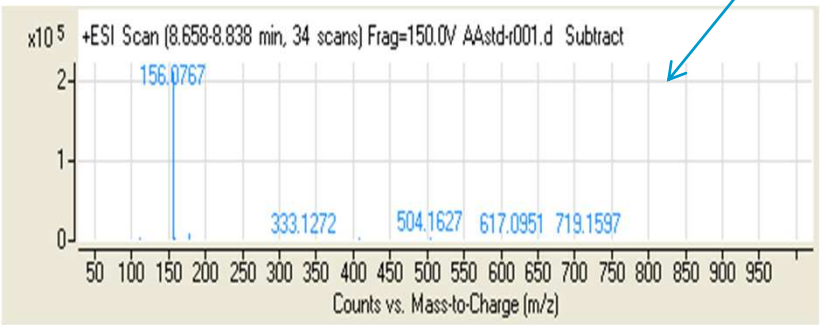


-Sufficient retention and separation
-Ion suppression from co-elution should be minimal
-Possible co-elution with sample matrix (near void volume) less likely

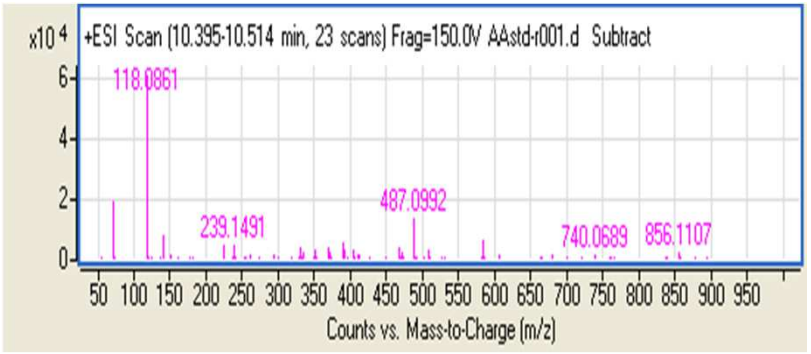
LC Method Example Spectra (Amino Acid Standard)



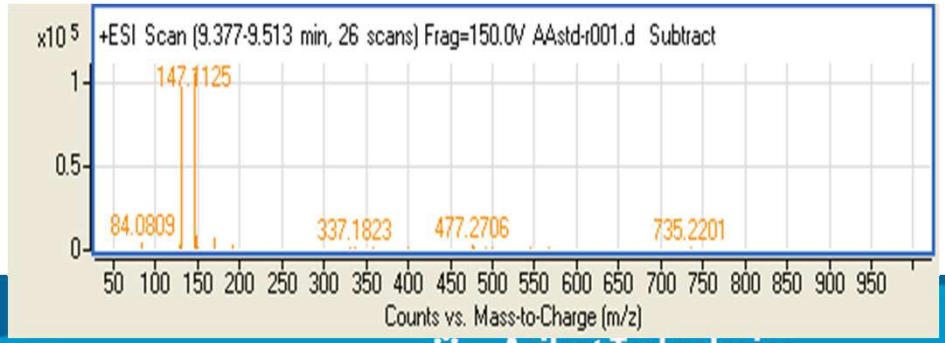
Histidine



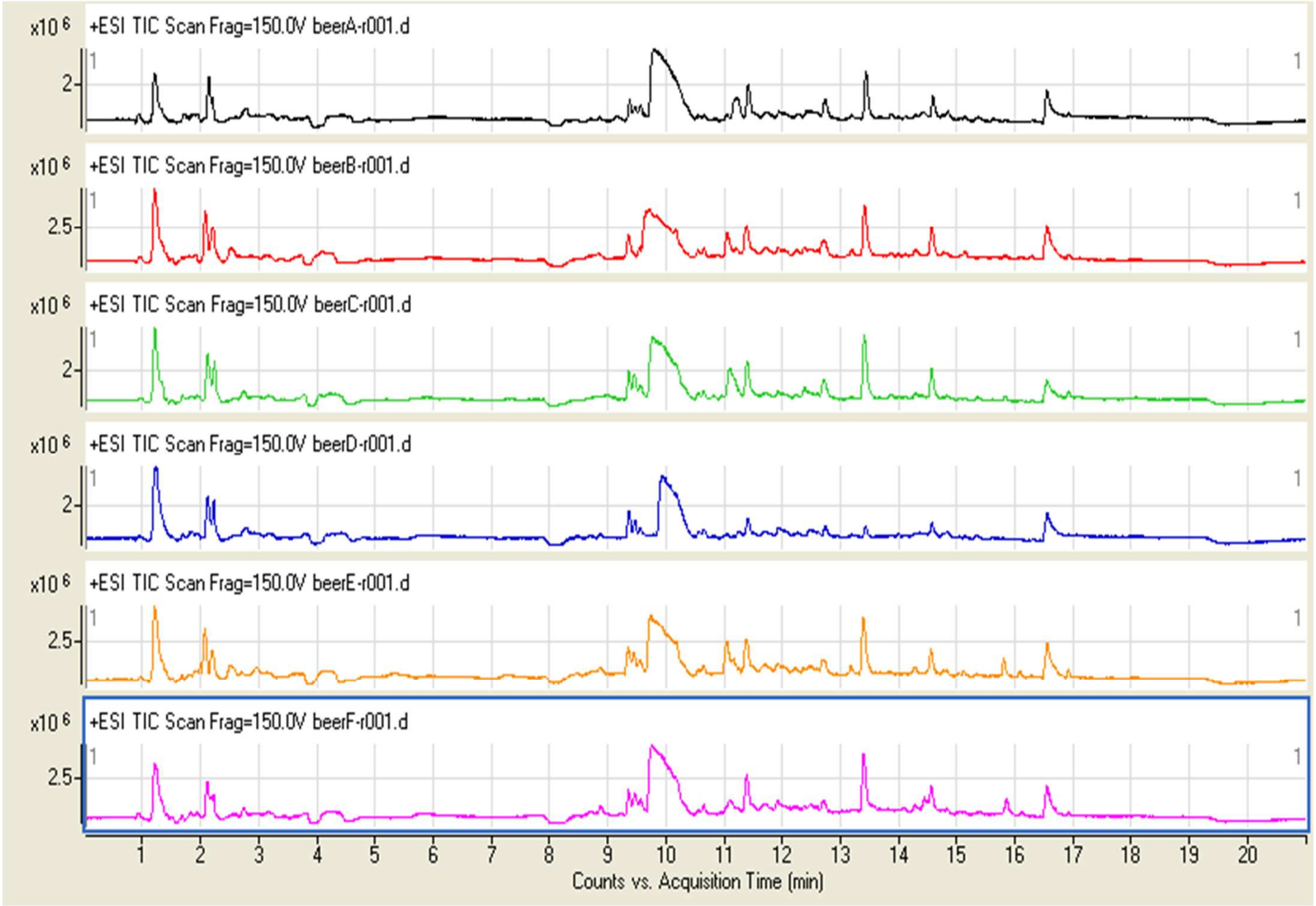
Valine



Lysine



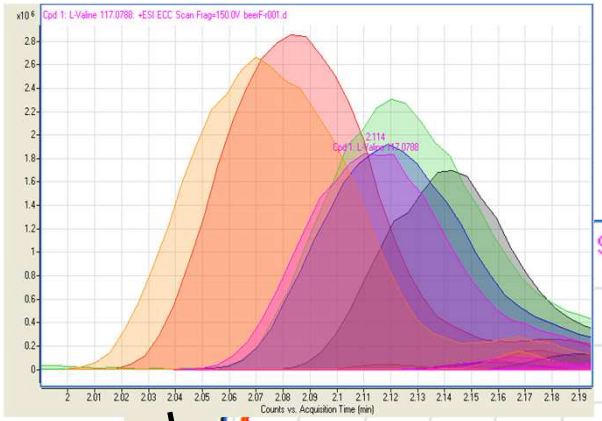
TICs of Different Beer Brands by LC



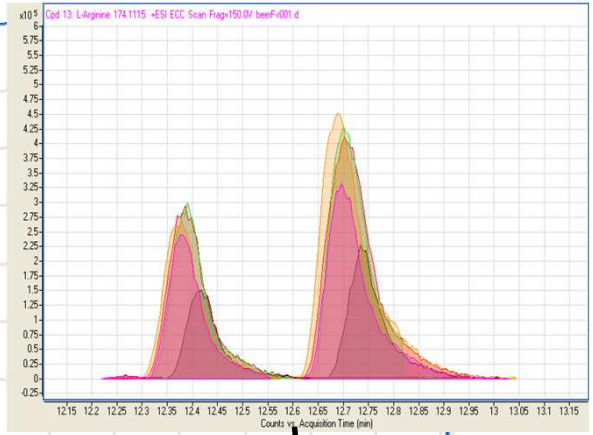
Overlay of Amino Acids In Different Beer Brands By LC

Present in all 6
Misidentified as Valine!
Valine is at 10.3min

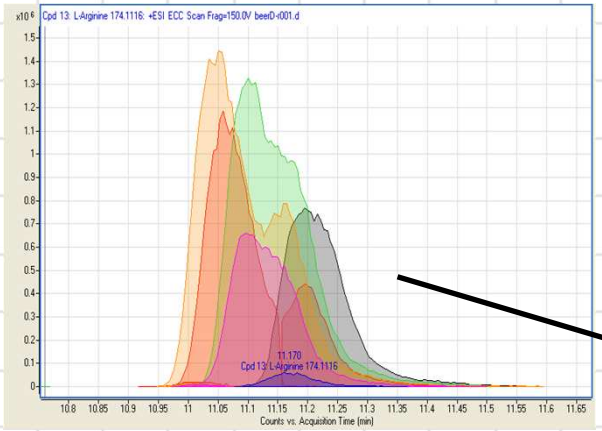
- Arginine has a bad peak shape
- Leucine and Isoleucine is baseline separated
- Overall peaks appeared tailed



Leucine and Isoleucine
present in 5 or 6 beers
Missing in Beer D



Arginine Present in all 6
Minimal in Beer D



7100 CE Parameters

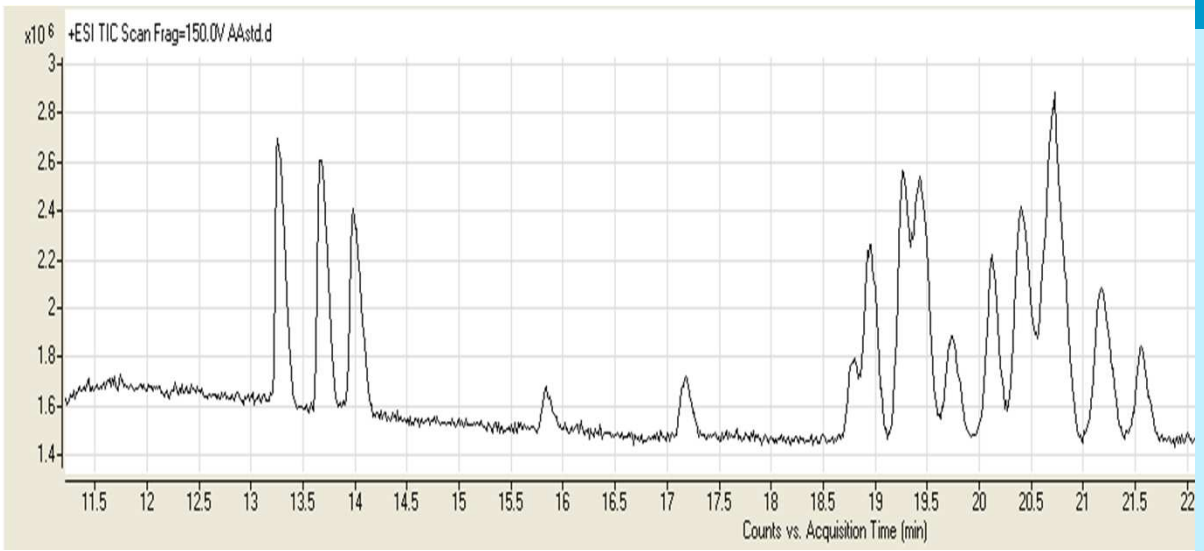
Parameter	Setting
Capillary Type	50 µm uncoated fused silica, 105 cm long
Cassette Temperature	20 ºC
Voltage	20 kV
Buffer	1N Formic Acid
Injection	50 mBar for 10 sec
Sheath Buffer	50:50 water:Methanol 0.1% Formic Acid
Sheath Flow rate	5 µL/min

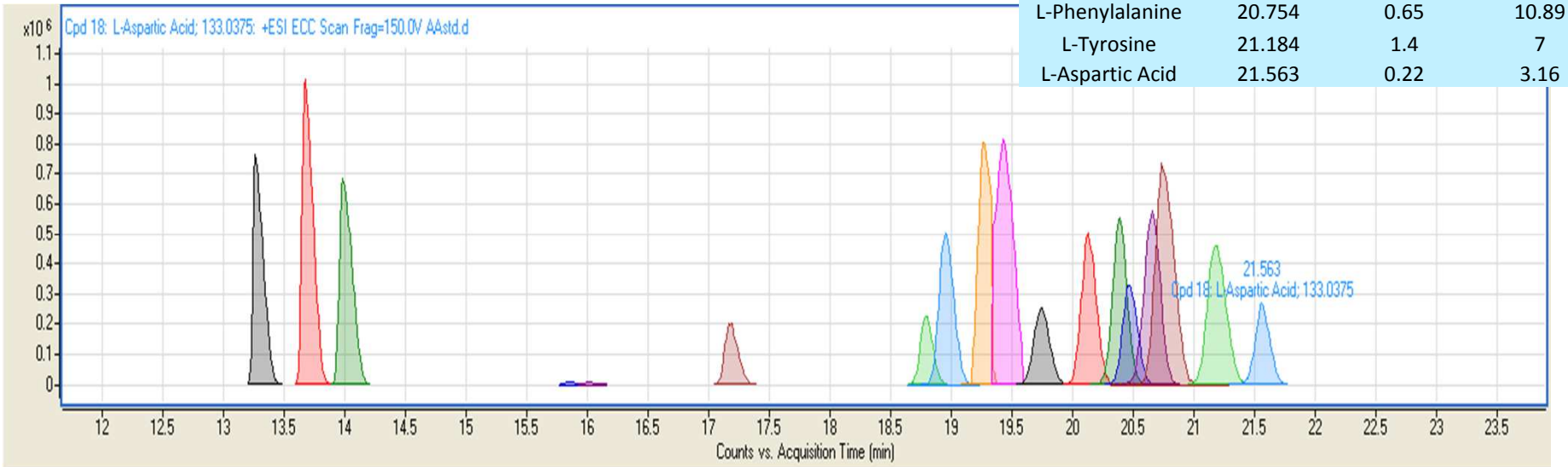
TOF Instrument Parameters for CE Method:

Parameter	Setting
Source Type	ESI in Positive mode
Gas temperature*	250 ºC
Drying Gas Flow rate*	5 L/min
Nebulizer Pressure*	5 psi
Capillary Voltage	4000 V
Fragmentor Voltage	150 V
Acquisition rate	3 scans/sec
Reference correction	1 point @ 922.009798
Acquisition Mode	2 GHz, extended dynamic range 50 to 1000 M/Z

***These parameters need to be adjusted lower compared to the source conditions used for the LC method due to the lower flow rate (5 µL/min from the sheath pump vs 0.3 mL/min for the LC method)**

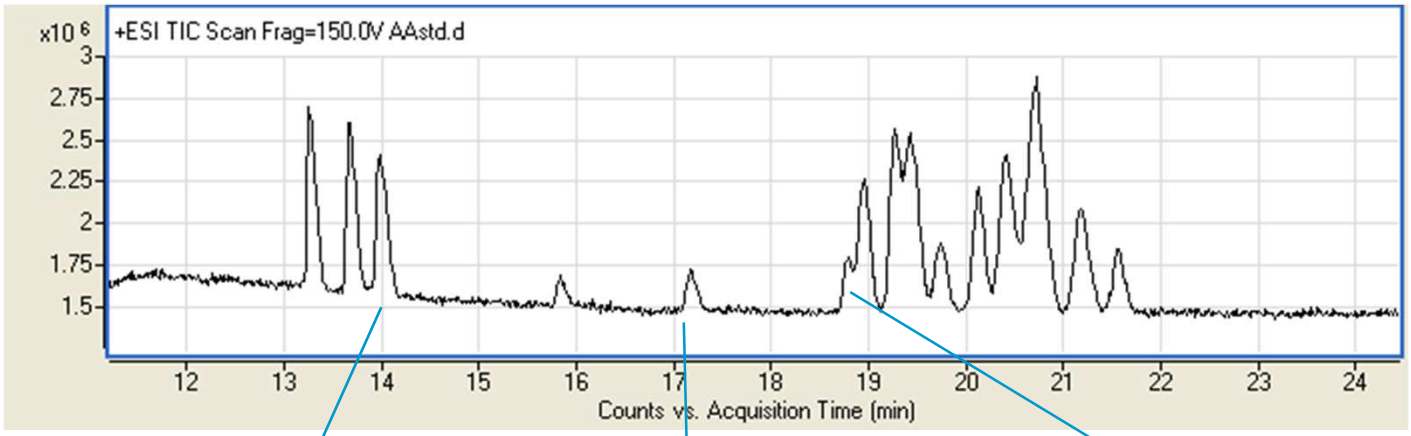
CE data: Amino Acid Standard



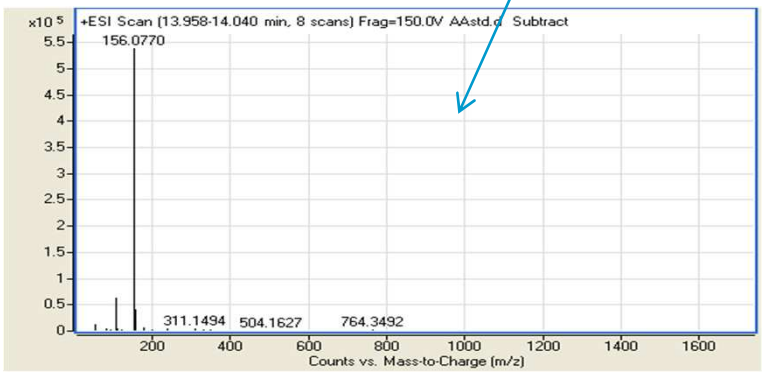


Name	RT	Diff (Tgt, ppm)	Vol %
L-Lysine	13.276	1.64	6.23
L-Arginine	13.684	2.08	8.88
L-Histidine	14	1.64	6.97
L-Alanine	17.18	4.85	2.11
L-Serine	18.792	1.32	2.28
L-Valine	18.955	1.4	5.92
L-Isoleucine/leucine	19.269	1.76	7.98
L-Isoleucine/leucine	19.436	1.82	11.33
L-Threonine	19.742	-2.87	3.21
L-Methionine	20.13	1.21	5.95
L-Proline	20.387	1.77	6.38
L-Glutamic Acid	20.47	0.86	3.98
L-Cystine	20.654	1.59	7.59
L-Phenylalanine	20.754	0.65	10.89
L-Tyrosine	21.184	1.4	7
L-Aspartic Acid	21.563	0.22	3.16

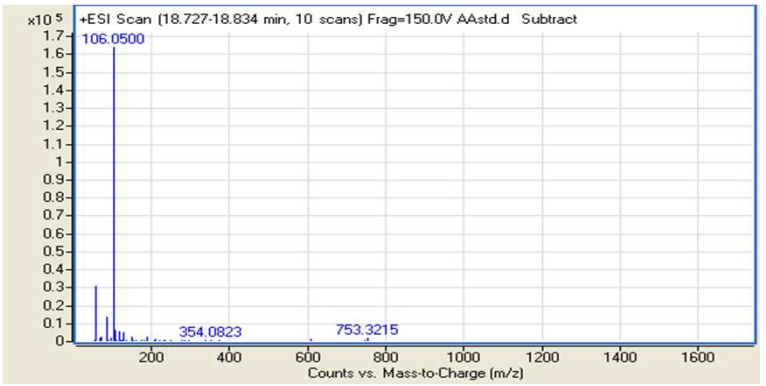
CE Method Example Spectra (Amino Acid Standard)



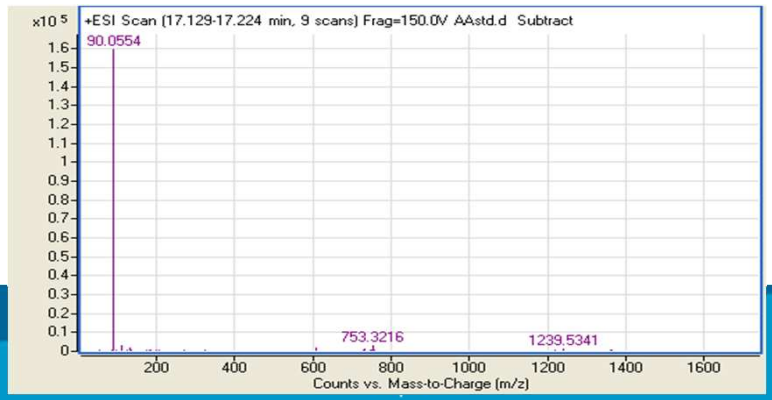
Histidine



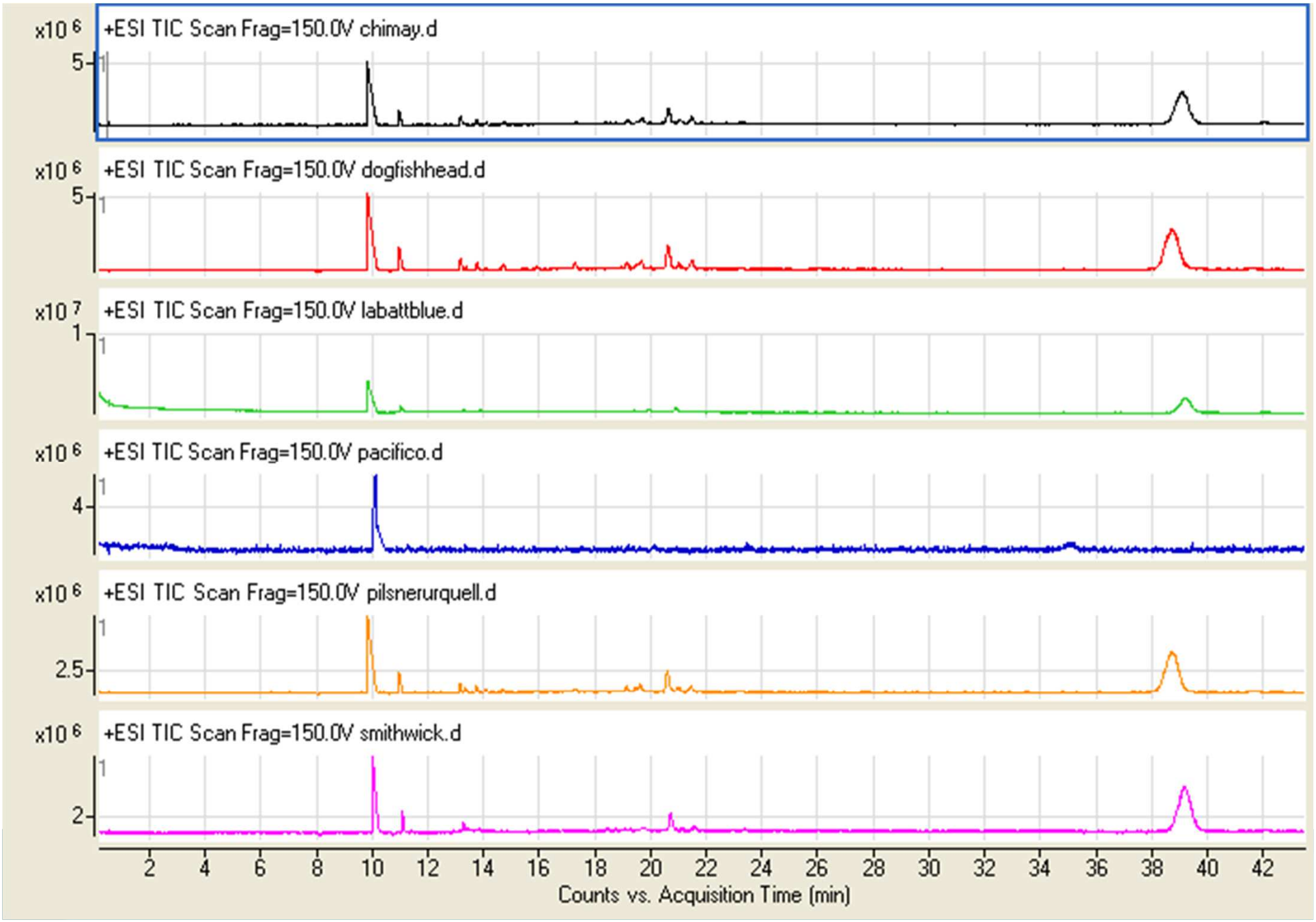
Serine



Alanine



TIC of Different Beer Brands by CE

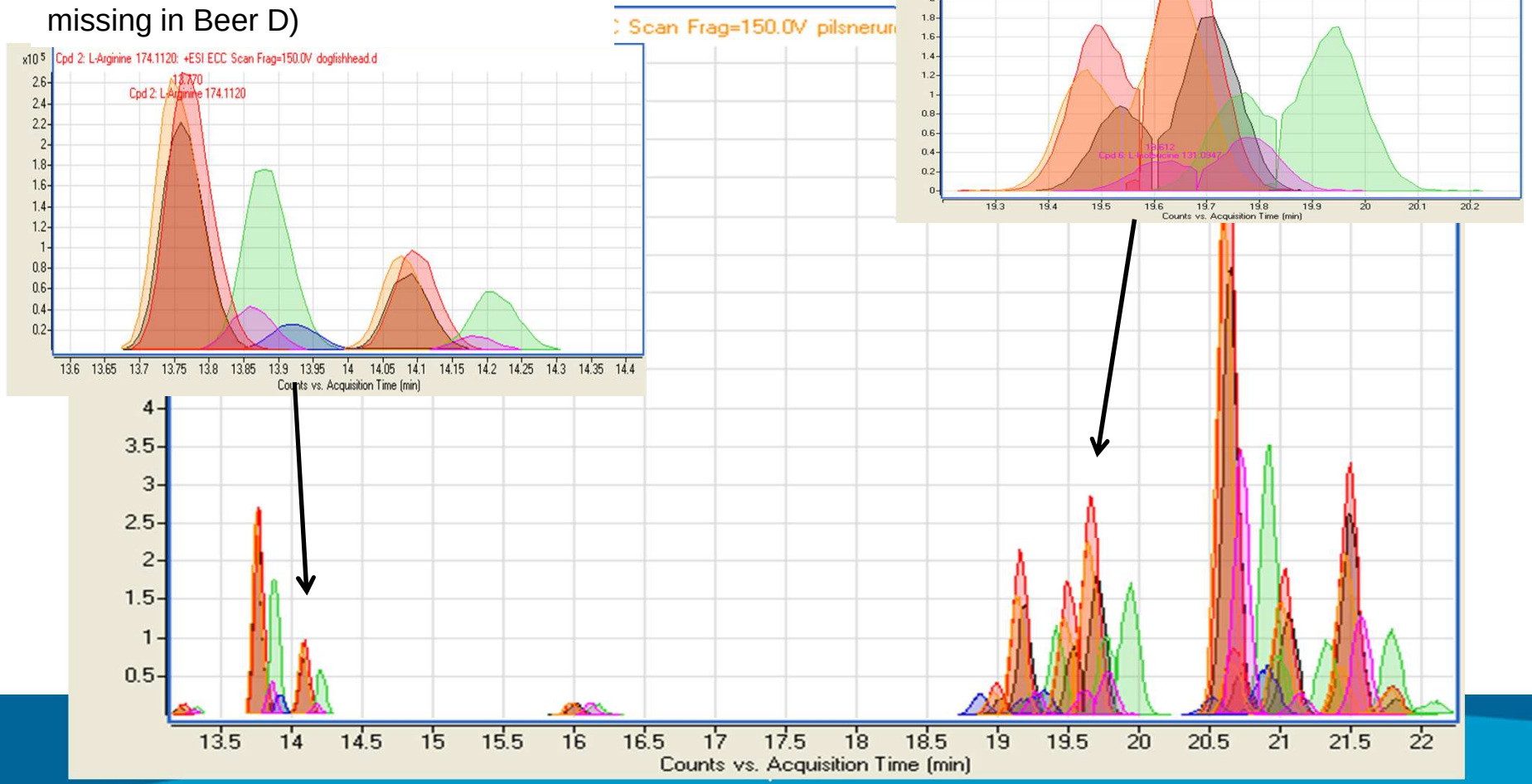


Overlay of Amino Acids In Different Beer Brands by CE

- Peak shapes are more symmetrical
- Leucine and Isoleucine not fully baseline separated
- Retention times have a higher %RSD

Leucine and Isoleucine present in 5 or 6 beers
Missing in Beer D

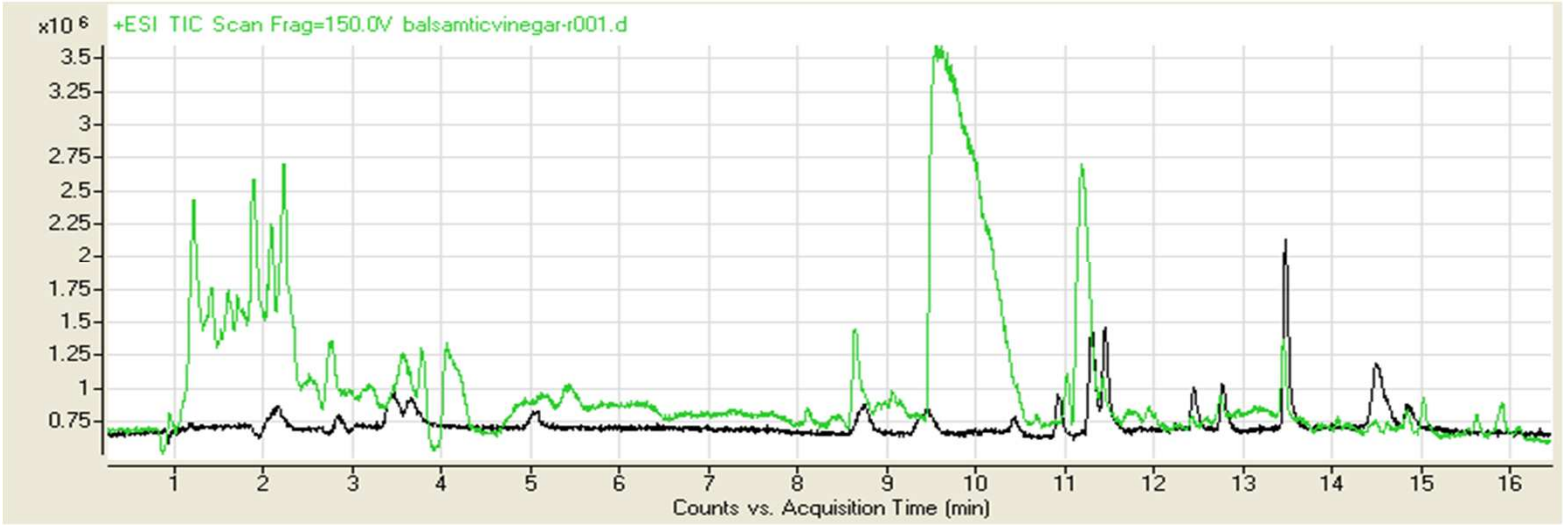
Arginine (present in all 6, minimal in D)
Histidine (present in 5 of 6 beers, missing in Beer D)



Comparison of TICs for Balsamic Vinegar

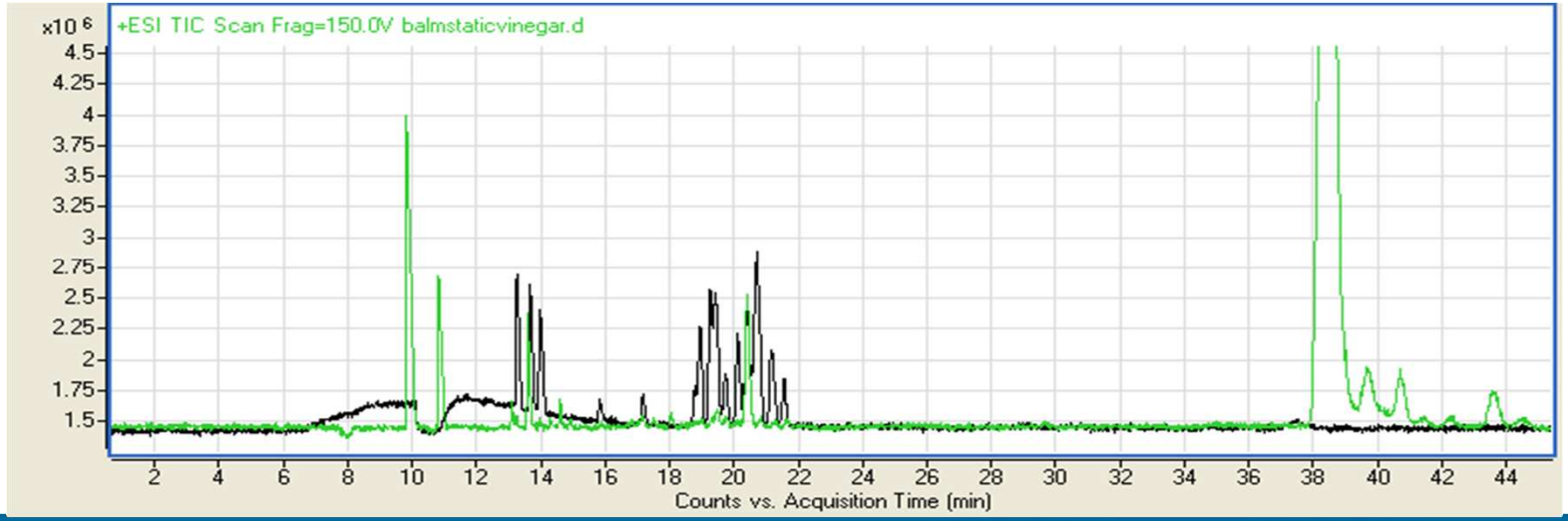
Overlay of Amino Acid Standard (Black) with Balsamic Vinegar (Green)

LC



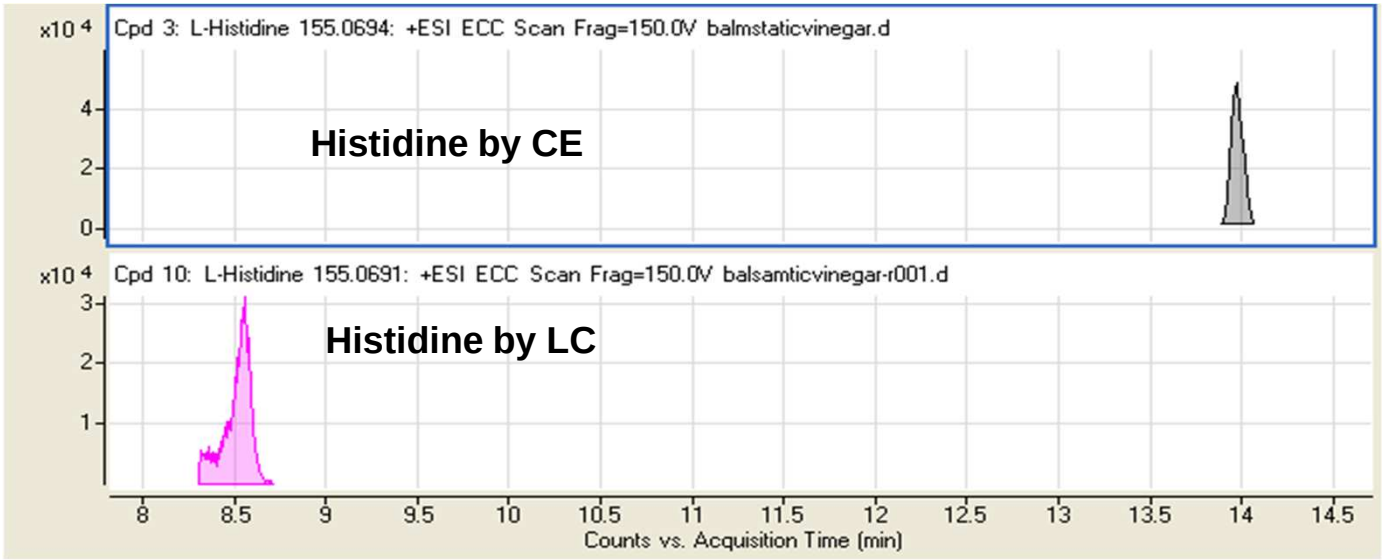
For LC the interferences are spread out across the run where amino acids are eluting
For CE there are less interferences from the sample matrix (comes early and late in the run)

CE

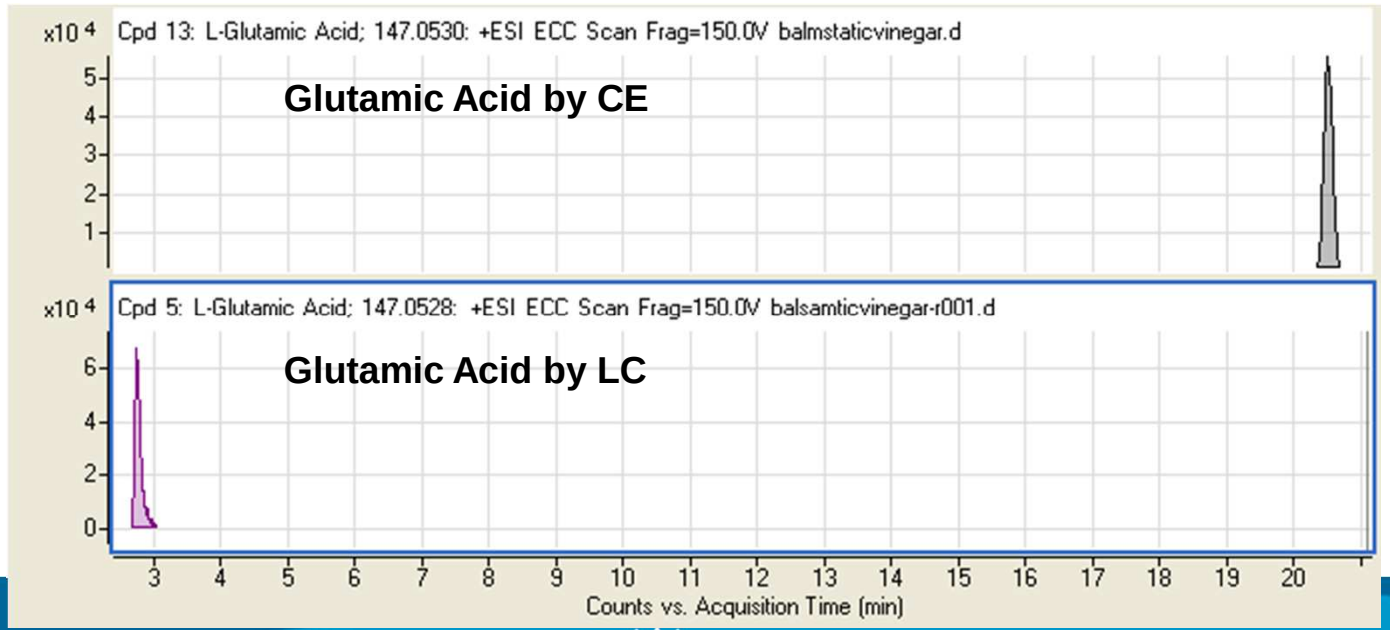


Comparison of Peak Shapes for Balsamic Vinegar

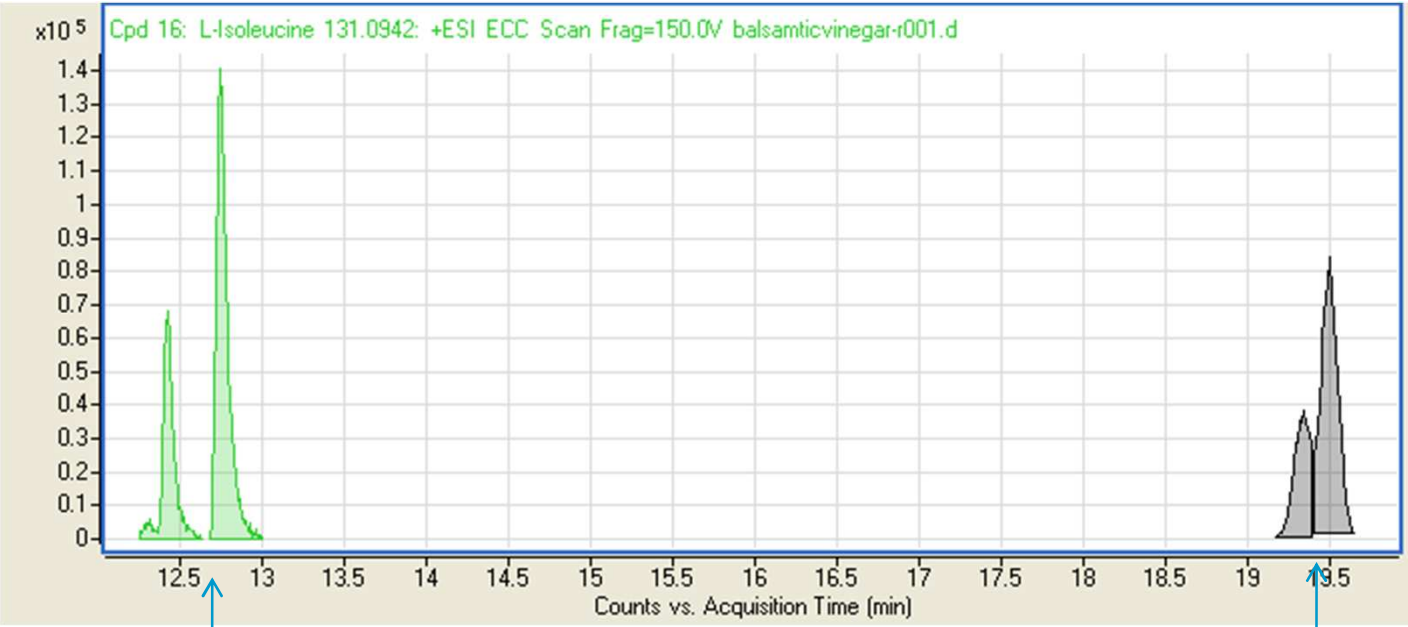
Example 1



Example 2



Comparison of Leucine/Isoleucine Separation

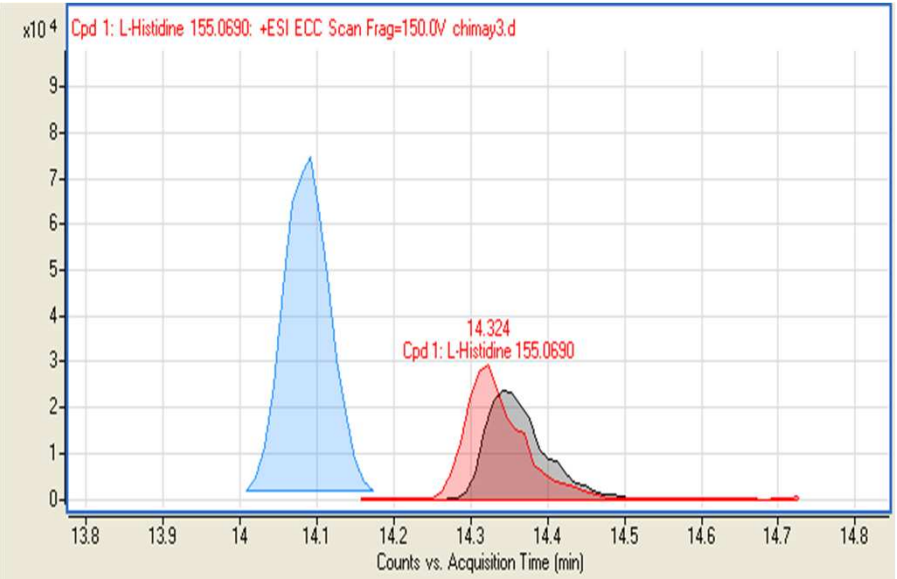


LC baseline separated

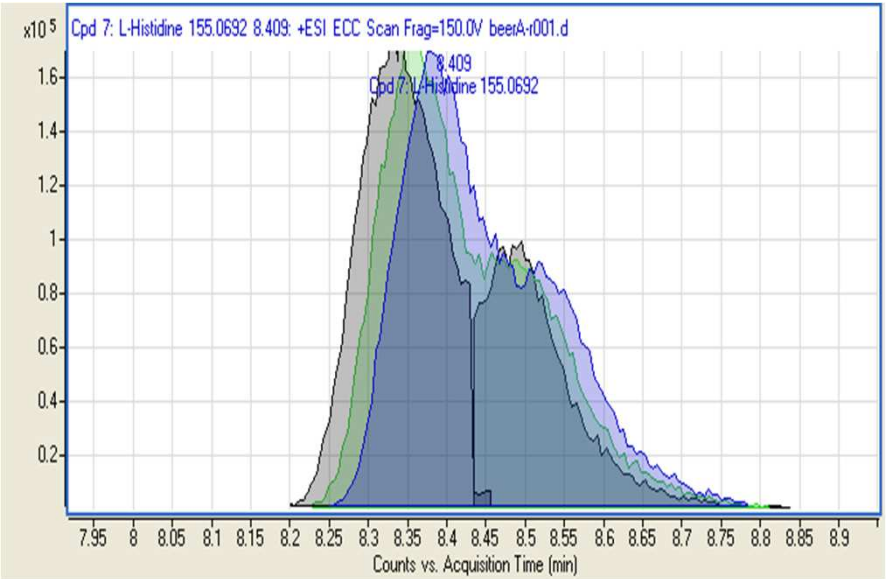
CE partially resolved

Comparison of Migration/Retention Time Precision

CE: Histidine for 3 replicate injections



LC: Histidine for 3 replicate injections



	CE RT (min)	LC RT (min)
Run 1	14.324	8.409
Run 2	14.321	8.382
Run 3	14.087	8.336
Average	14.244	8.375667
Std Dev	0.135974	0.03691
% RSD	0.954607	0.440679

Conclusion

LC method Advantages

-Easier to setup and implement
-User's have more familiarity with LC
-Faster run and cycle time
-Better retention time precision
-Leucine and Isoleucine are fully baseline separated

CE method Advantages

-Amino acids are better separated from matrix effects
-Less false positive identifications
-Sample cleanup/preparation is less involved
-Peak shapes are more symmetrical