The effects of environment on raspberry metabolites

Sean Conner

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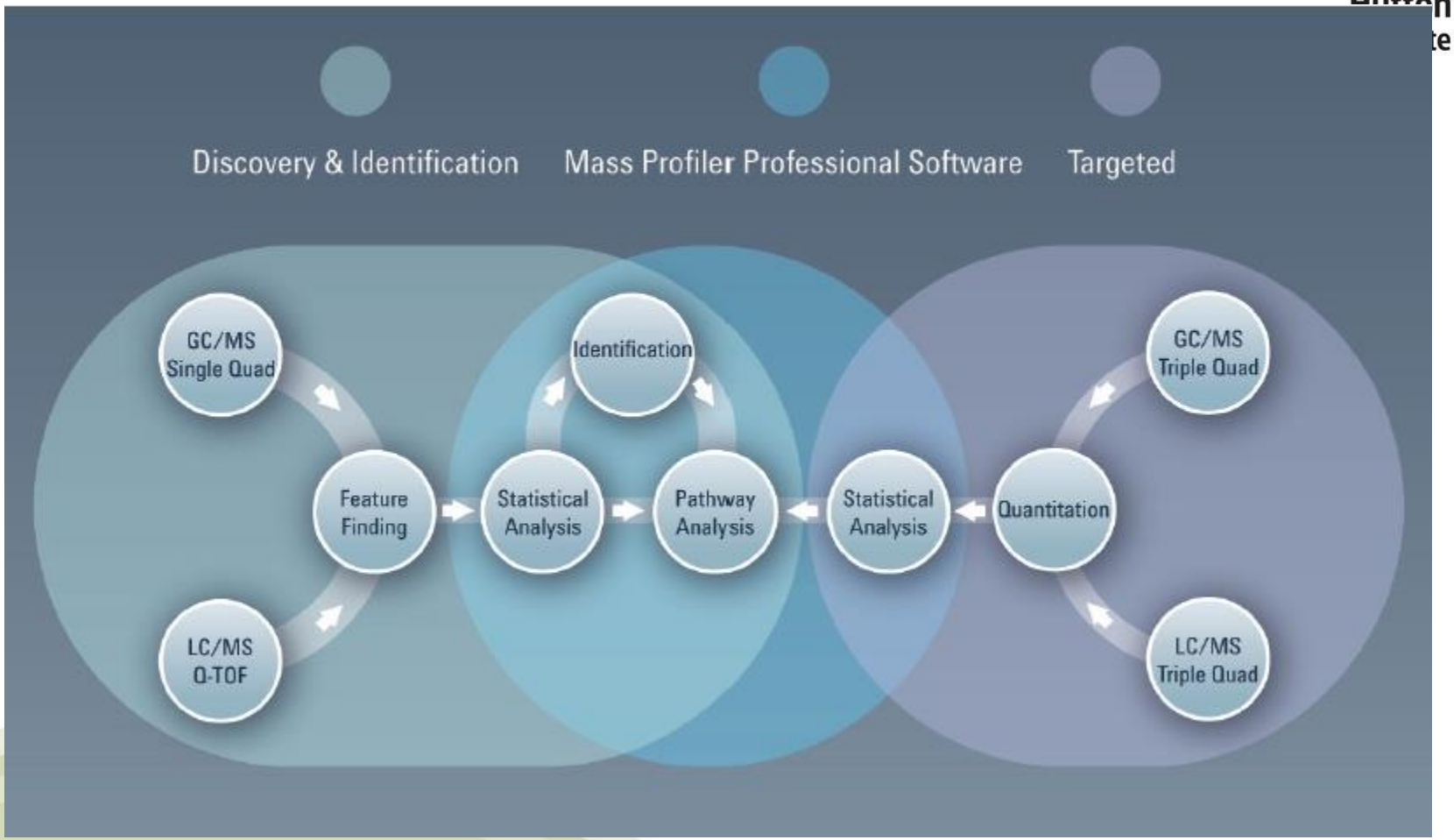
Future-proofing berryfruit CLIMAFRUIT

The Interreg IVB North Sea Region Programme

Chemometric Workflow:



Mass Spectrometry Based Chemometric Workflow

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GCMS

LCMS

Separate & Detect

GC/MSD
GC-QQQ

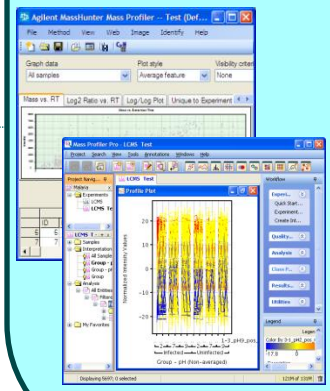
CE or LC-TOF/QTOF
CE or LC-QQQ

Feature Finding (Quantitate)

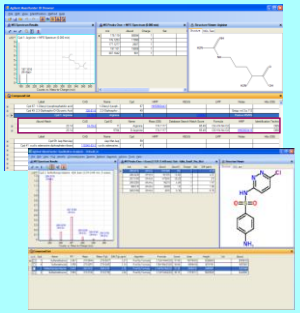
AMDIS* or *Find by chromatographic deconvolution

Molecular Feature Extraction*, *Find by Formula*, *Find by Ion

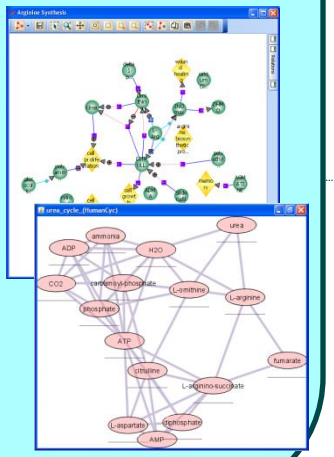
Alignment & Statistics



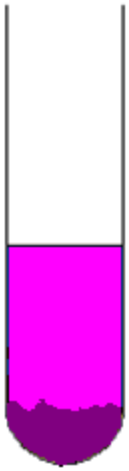
Identify



Pathways

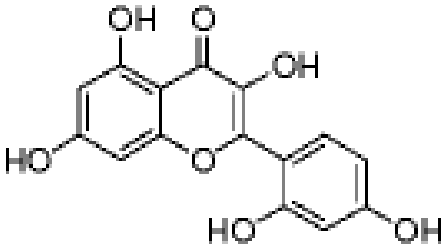


Extraction Protocol



3 ml 60:40 water/acetonitrile 1% acetic acid 0.1 mg/ml morin (internal standard)
100 mg freeze dried (FD) powder

Shaken for 60 minutes at 30 °C



Morin: Internal Standard

Experimental design

- Cultivars
- Countries
- Years 2011, 2012
- Organic/Conventional
- Randomised block design

Countries		Variety		Treatment	
Norway	NO	Autumn Treasure	AT	Conventional	Conv
Germany	DE	Glen Ample	GA	Organic	Org
Sweden	SE	Karaka Black	KB		
UK	UK	Erika	Er		
		Fall Gold	FG		
		Sugana	Sug		
		Glen Doll	GD		
		Cascade Delight	CD		
		Cowichane	Cow		
		Malling Hestia	MH		
		Glen Magna	GM		
		Tulameen	Tul		
		Polka	Pol		
		Glen Rosa	GR		

Experimental design

LC Parameters

Mobile Phase:	A: H2O 0.1% Formic Acid	
	B: Acetonitrile	
Flow:	0.3mls/min	
Column:	2.1mm x 100mm, Eclipse Plus C18 1.8um	
Injection:	0.5ul	
Temp:	30 °C	
Gradient:	**time**	**%B**
	0.0	5
	4.0	5
	32.0	100
	34.0	100

MS parameters:

ESI Positive:	
Scan Range:	100-2000 m/z
Scan Rate: MS	1 Hz
Reference ions POS:	121.05087 m/z, 922.009798 m/z
Reference ions NEG:	112.9856 m/z, 980.0164 m/z

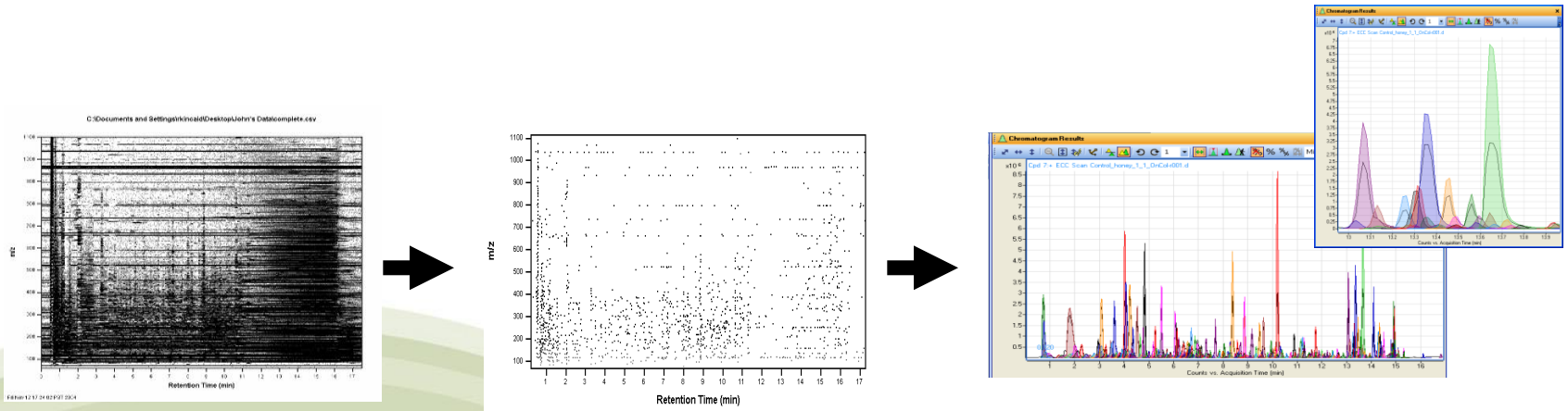
Source parameters :

Drying Gas Temp:	325°C
Drying Gas Flow:	10l/min
Nebuliser:	50p.s.i.
VCcap:	3500V
Fragmentor:	175V

Investigational Workflow – Feature Finding

Molecular Feature Extraction

- Works on the 3-dimensional data set
- Remove noise
- Group covariant mass signals
- Combine signals with chemical relations (isotopes, adduct ion clusters, multiply charged ions, dimers) into molecular features (= compounds)
  - Include any adducts, such as Na+ or K+
  - Include isotopes ([M+H]+, [M+H+1]+,…
  - Include different charge states
  - Check for dimers
- Create extracted compound chromatograms and compound spectra
- Create Datafile for export (*.cef)



Results: MFE of Complex Sample (Urine)

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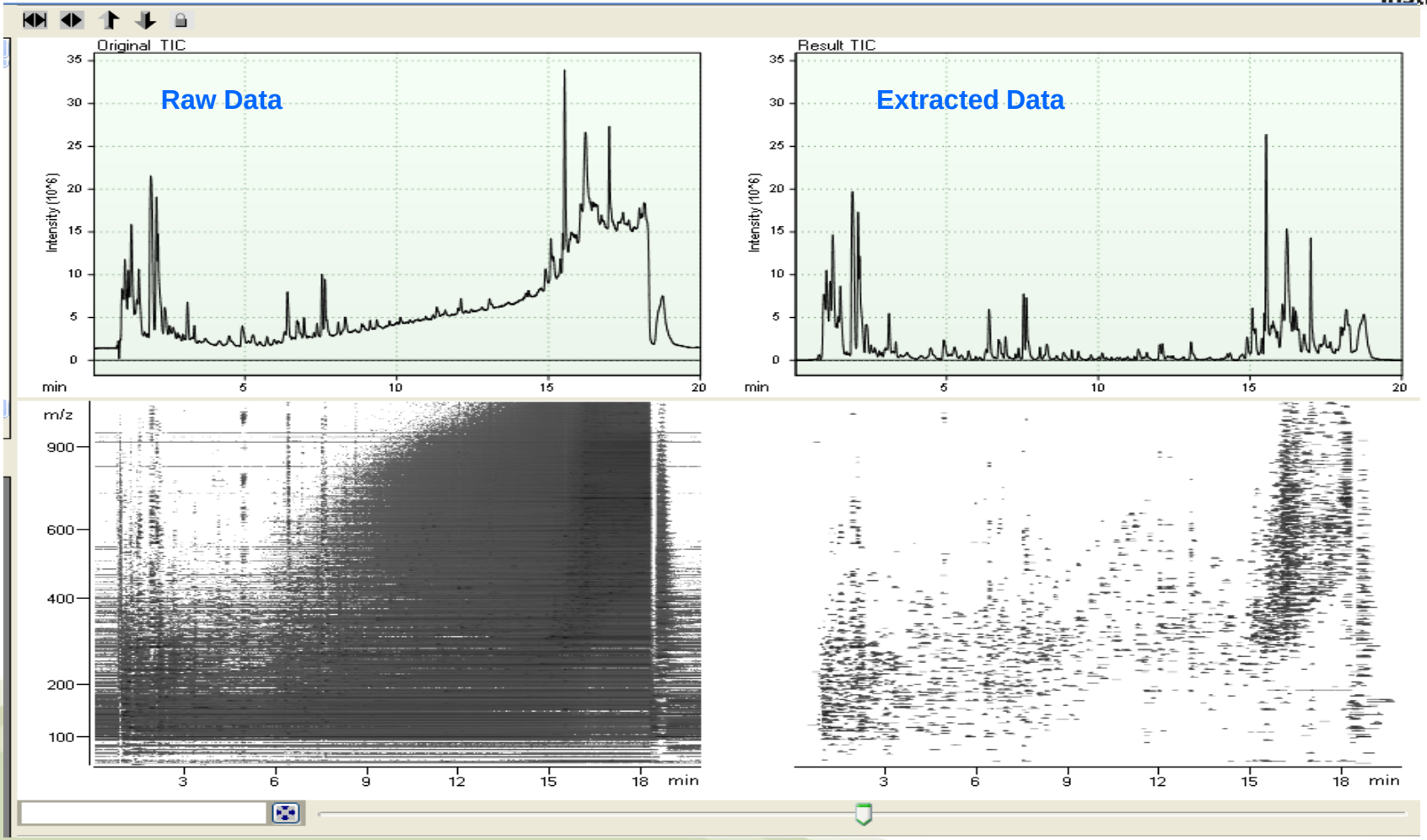
Normal Urine – >2200 Compounds found



Results: MFE of Autumn Treasure

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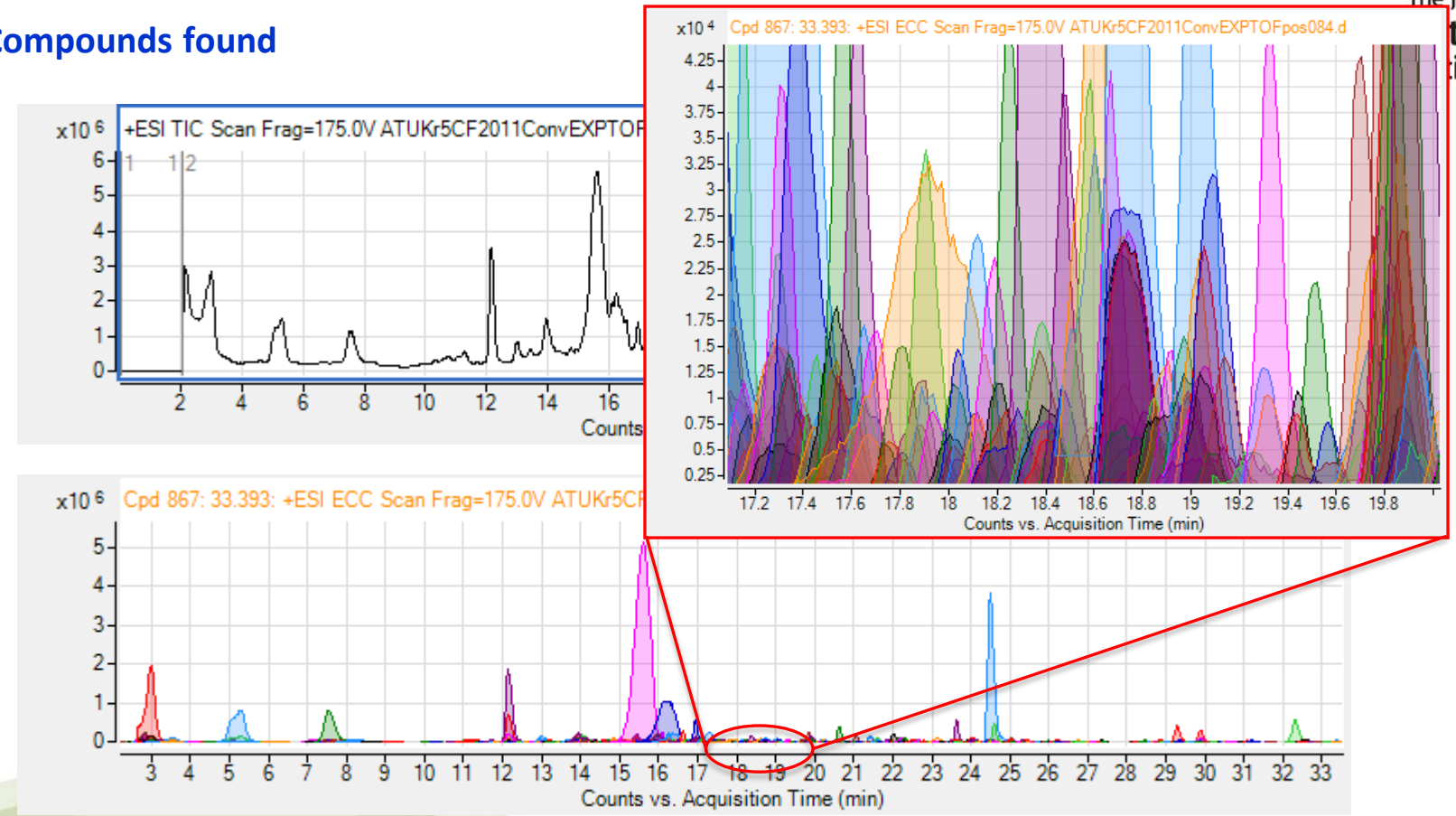
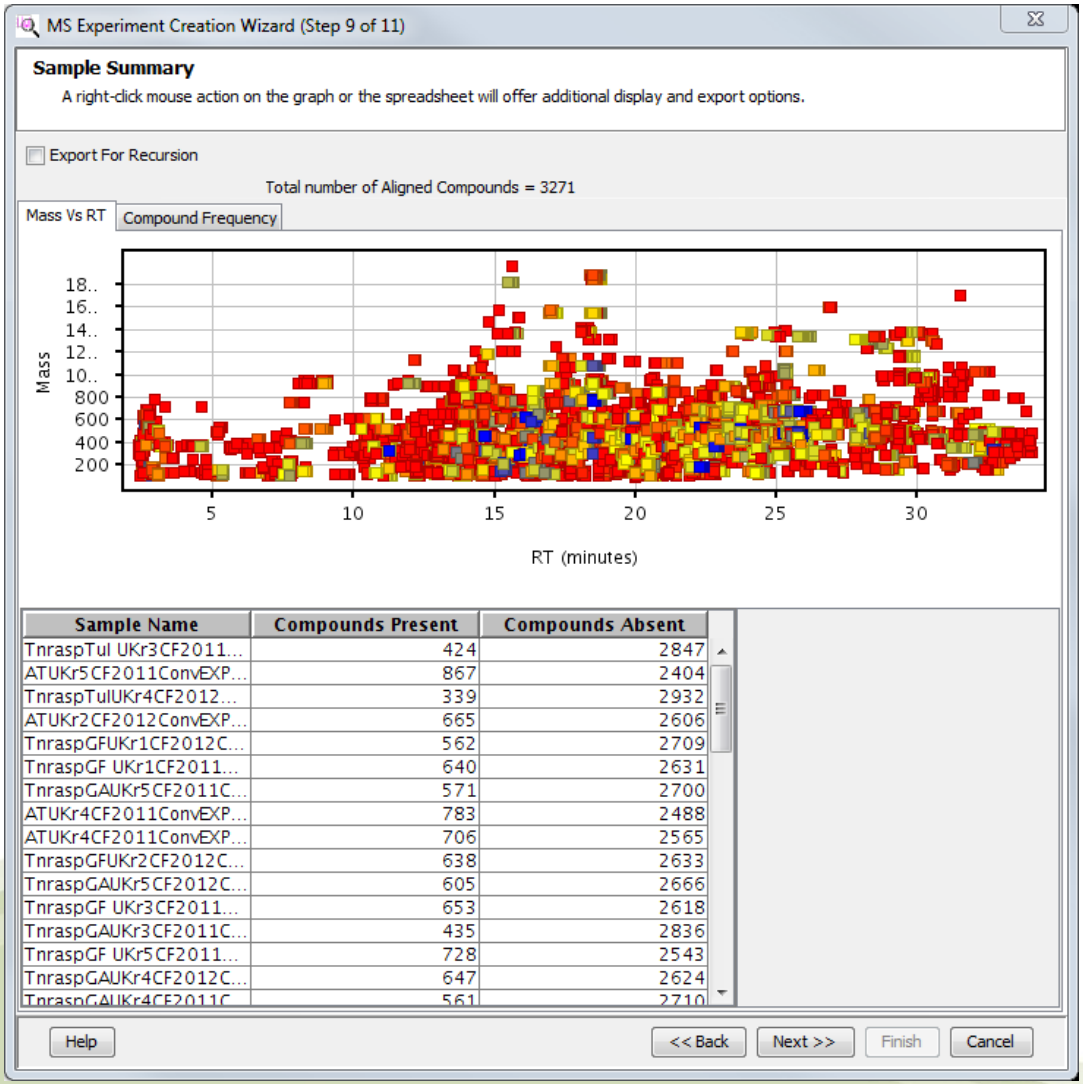
897 Compounds found



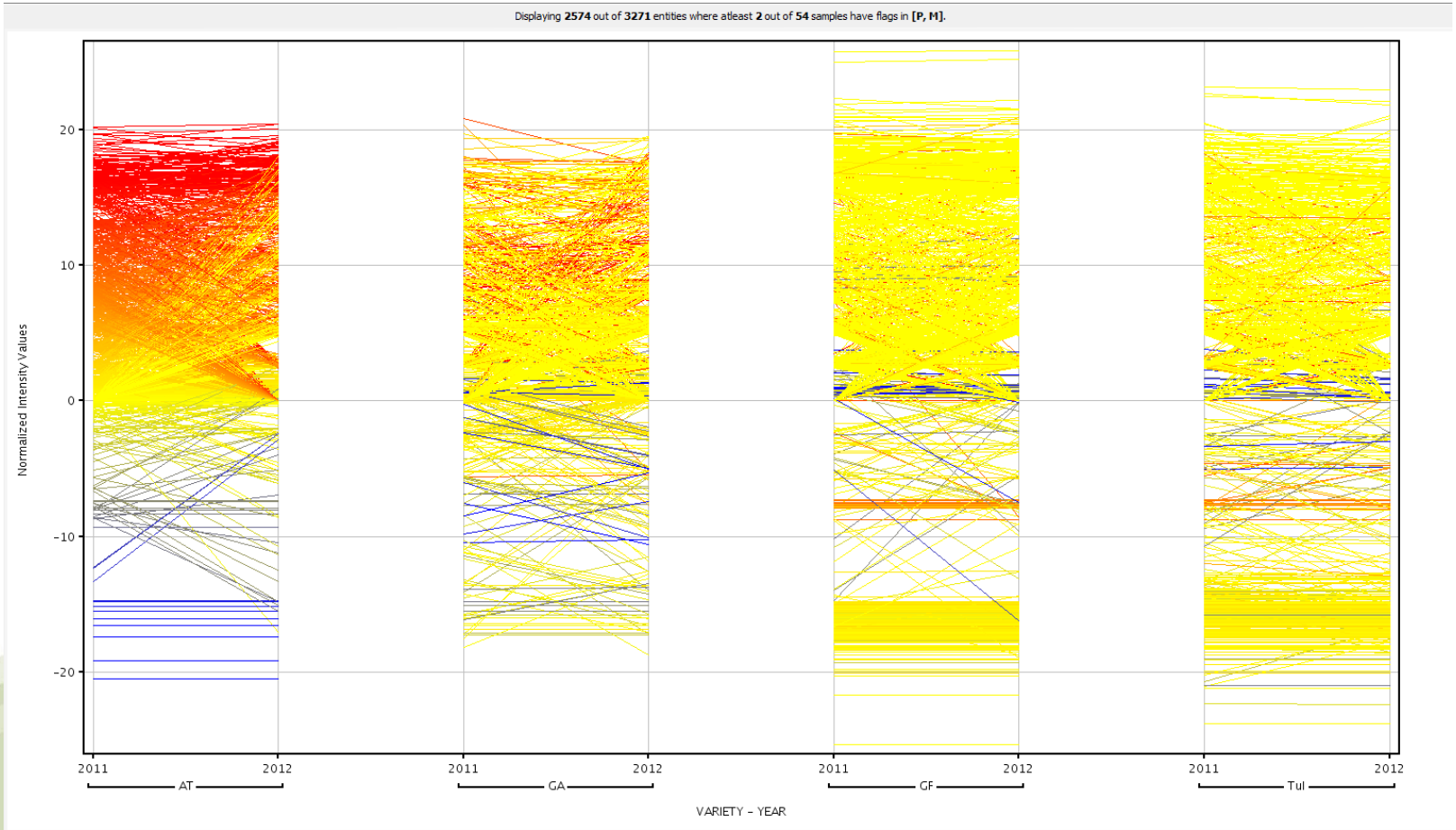
Sample Summary



A total of 3271 entities were found across the UK samples set of four different varieties

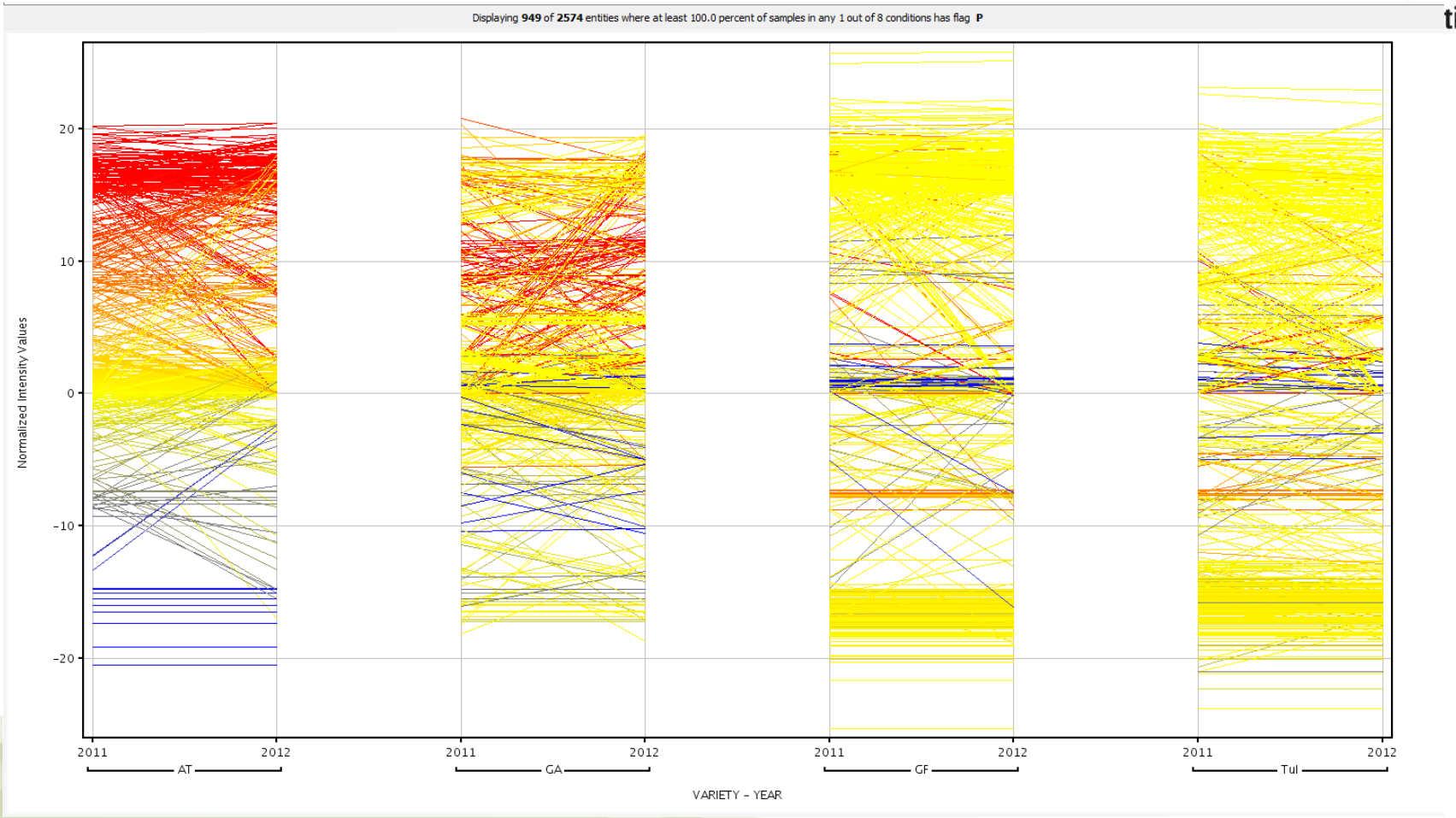
Flag Filter – remove ‘one hit wonders’

A total of 3271 entities was reduced to 2574 upon removing one-hit wonders. The profile plot here shows entities for each harvest year across four varieties of raspberry.



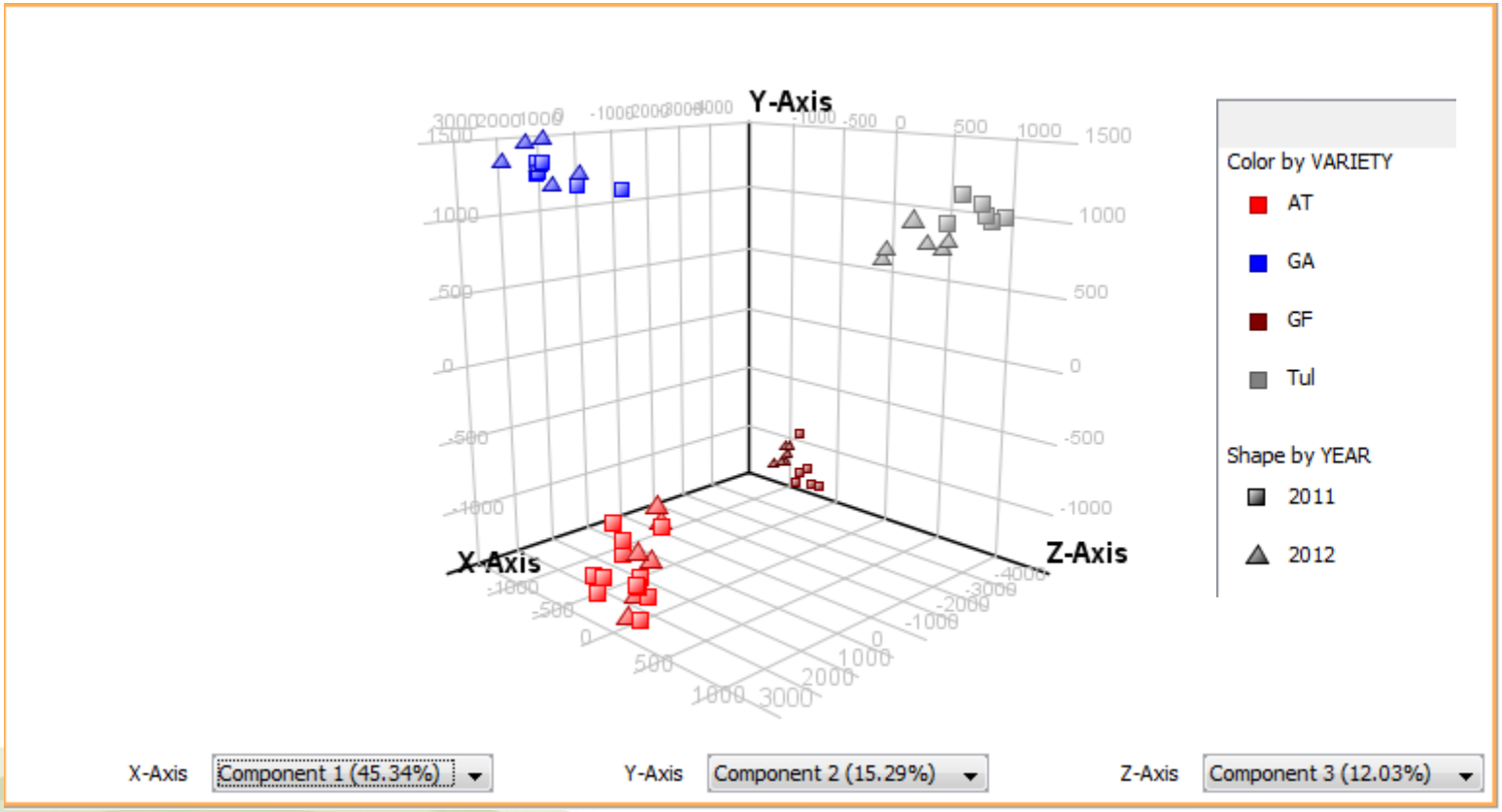
Frequency Filter – Entity is present in 100% of samples in one condition

The number of entities was further reduced by demanding that any entity is present in all samples within one condition. The profile plot here shows entities for each harvest year across four varieties of raspberry.



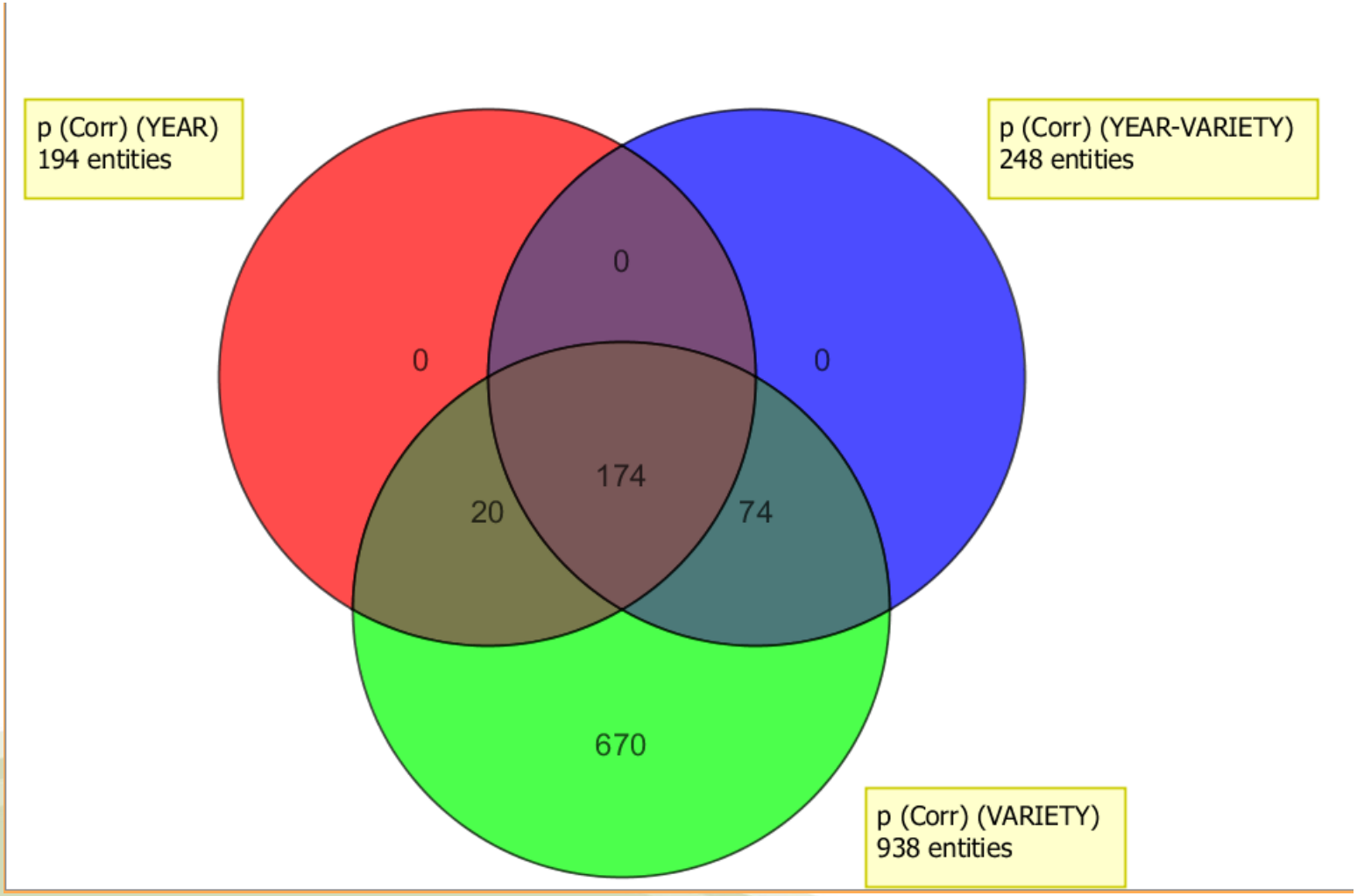
PCA Analysis showing variability across raspberry varieties

PCA analysis indicates that there is more difference in surviving entities between varieties than there is between years. Therefore, within the UK, harvest year is not significant for these compounds.



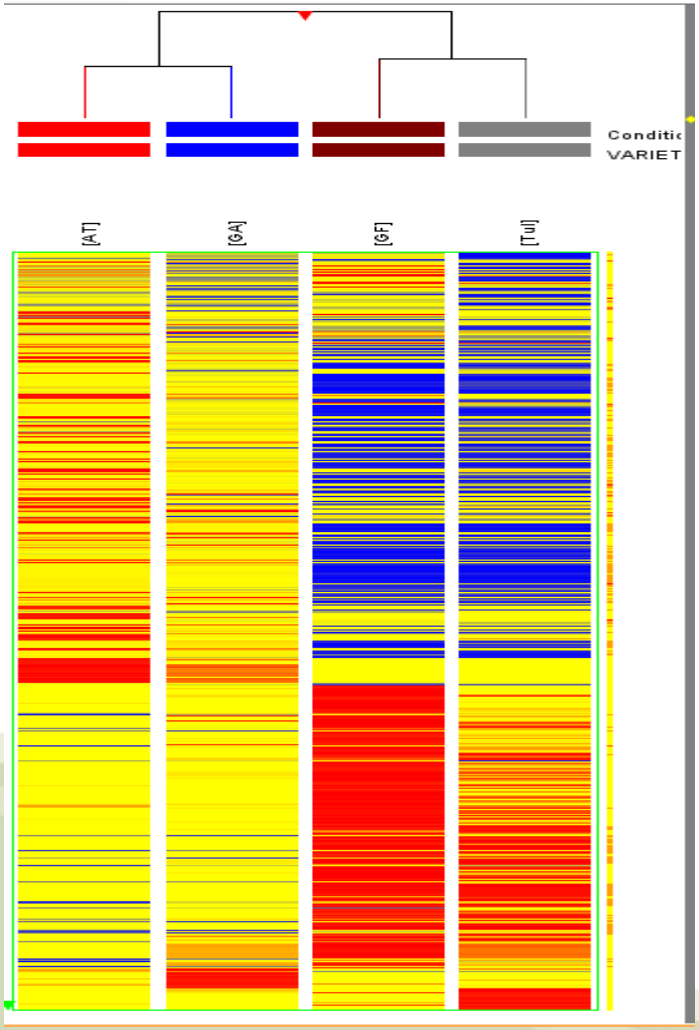
Significance Analysis – 2-way ANOVA (p <0.05)

This Venn Diagram supports the PCA in that differences between the samples were due to the raspberry variety and not the year of harvest.



Hierarchical Clustering

In this heat-map each line represents an entitiy whuile the colour indicates abundance of that compound. This dendrogram indicates similarities between AT and GA and GF and Tul while highlighting that AT anf GF are more different from GF and Tul than each pair is from each other.



Volcano Plot

Within the more similar pairs a volcano plot can be used to show those entities (red) which vary by a specified fold-change (5 x here) and a specified statistical significance ($p<0.001$ here). Therefore the red compounds are 5x more or less abundant in one variety vs the other with a significant of $p<0.001$.

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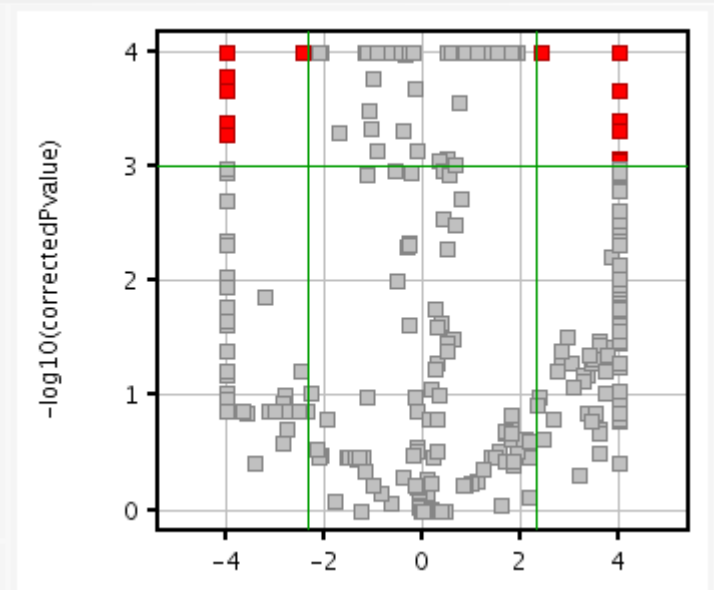
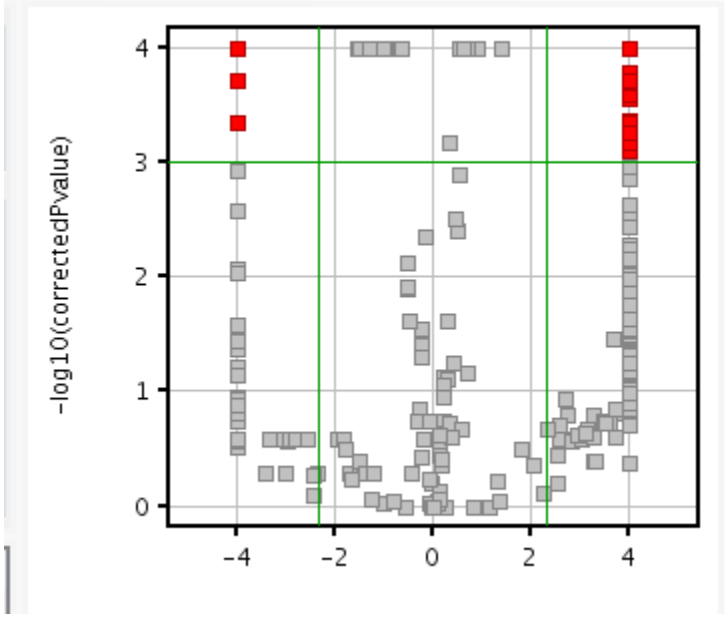
Volcano Plot AT vs GA



Volcano Plot GF vs Tul



Venn Diagram – Comparison of entities from Volcano plot (t-test) of two pairs of varieties.

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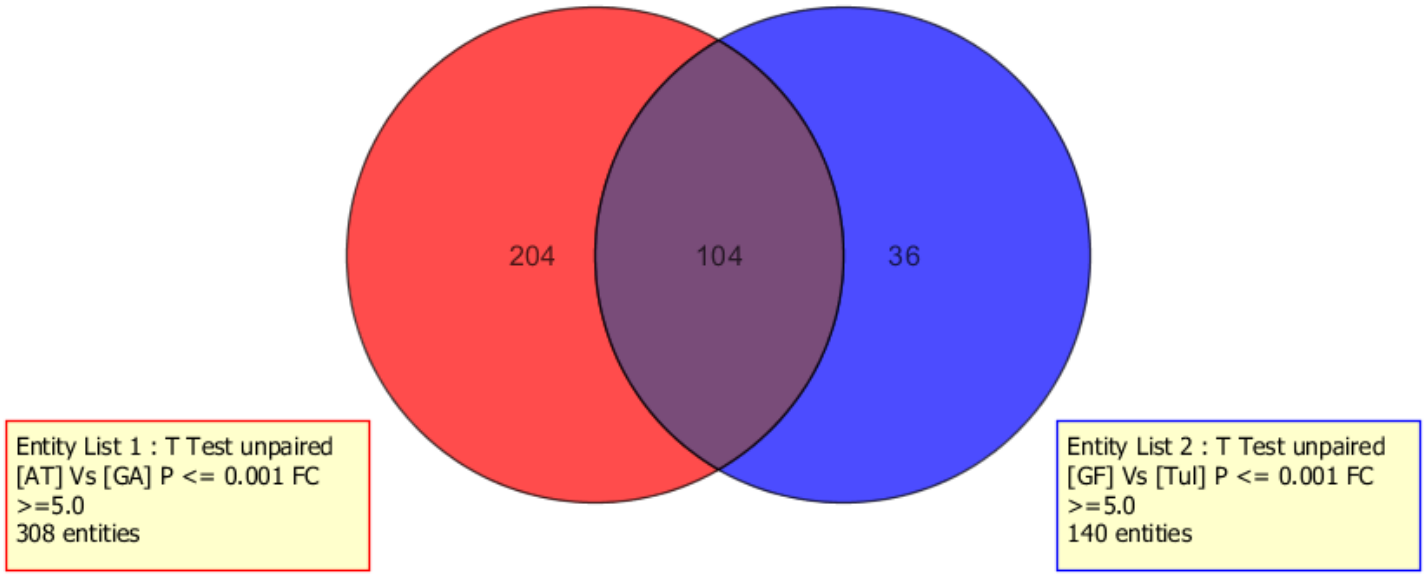
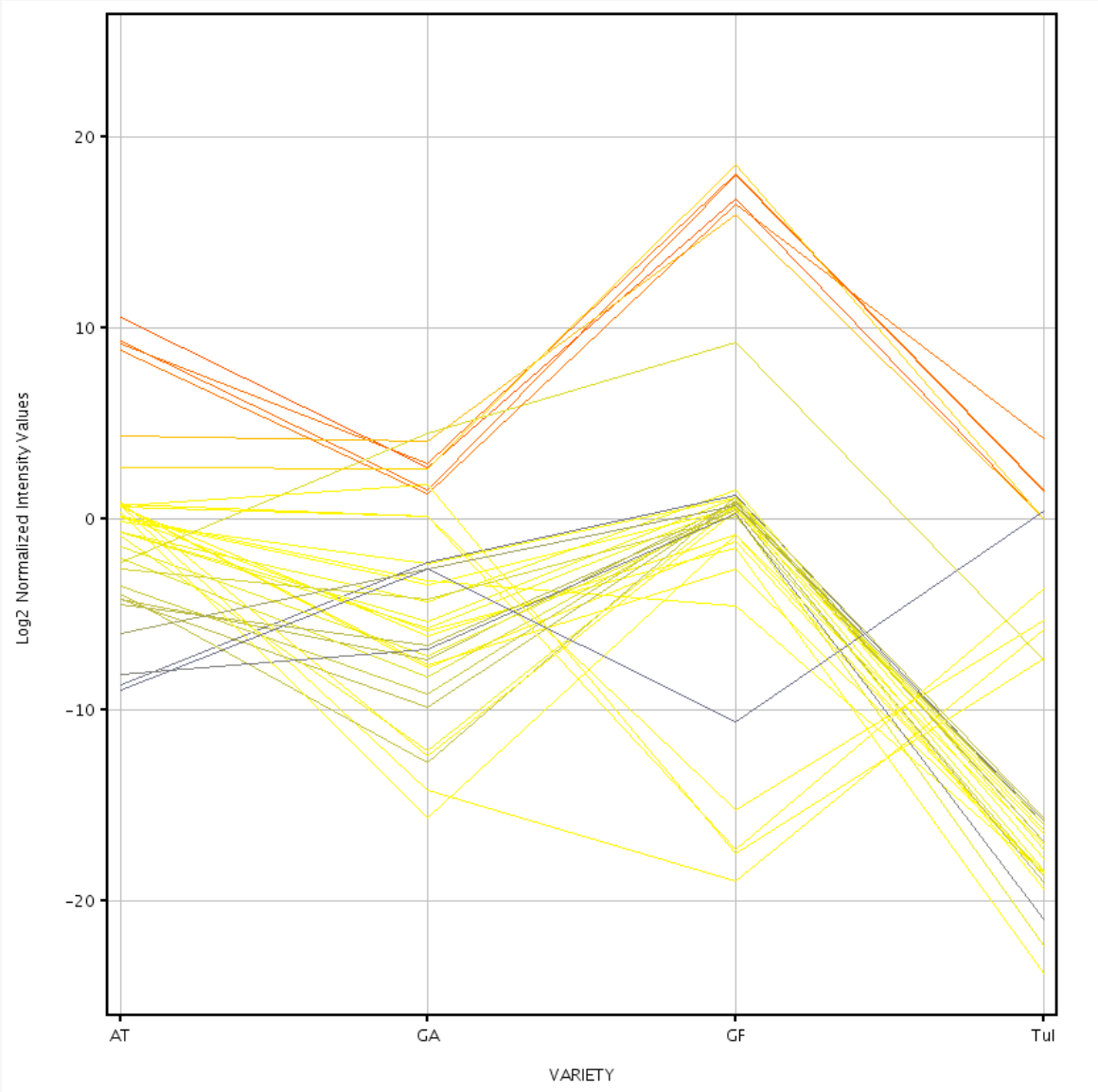
The entities identified from the volcano plots of t-testing of two pairs of varieties are exmanied using a Venn Diagram. This indicates that the same 104 compounds are 5x different in each pair of varieties. 36 compounds are 5x different only in the GF and Tul varieties.



Profile Plot of Entities

The 36 entities are shown here on a profile plot across all four investigated varieties. .



Identification

The entity list can be searched against a database (METLIN) and also used for formula generation

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Compound List

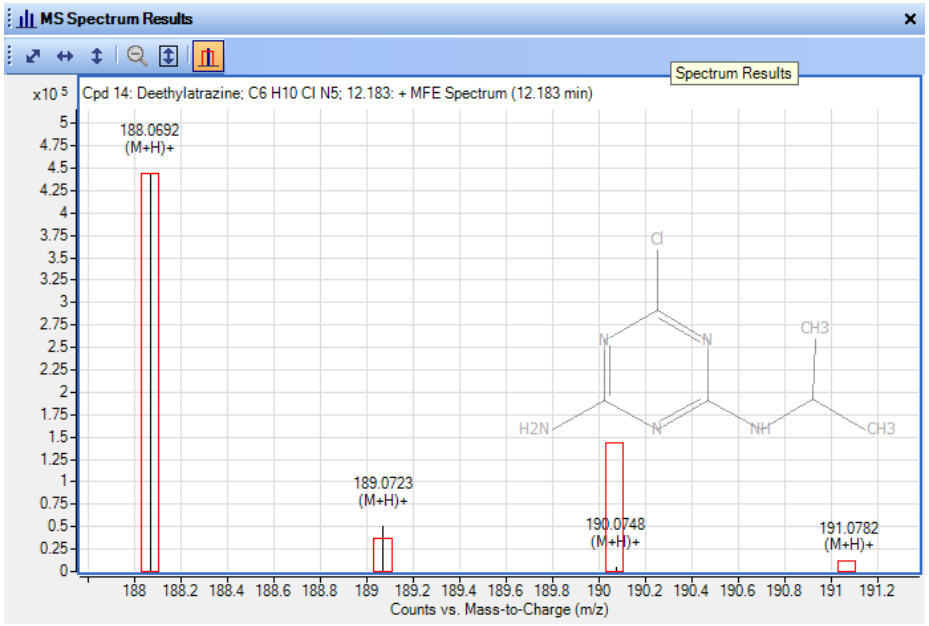
Cpd	Name	Notes	Diff (DB, ppm)	Hits (DB)	LMP	Formula	Score	Mass	RT	Mass (DB)	Mass (MFG)
1	Vitexin 2"-p-hydroxybenzoate		-3.13	3	LMPK12110225	C28 H24 O12	60.91	552.1285	2.675	552.1268	
25	Luteolin 7-galactoside-4'-glucoside		1.04	5	LMPK12110670	C27 H30 O16	98.92	610.1528	15.564	610.1534	
28	Iridodial glucoside tetraacetate		1.76	2		C24 H34 O11	75.61	498.2092	16.793	498.2101	
17	Indole	Group A 07/27/04	-3.07	3		C8 H7 N	82.49	117.0582	12.165	117.0578	
36	His His Arg		-1.31	3		C18 H28 N10 O4	84.55	448.2301	25.195	448.2295	
34	Glucosylgalactosyl hydroxylysine		0.64	2		C18 H34 N2 O13	86.09	486.2058	18.492	486.2061	
23	Disperse Blue 1		2.26	2		C14 H12 N4 O2	85.14	268.0954	14.976	268.096	
14	Deethylatrazine		1.65	1		C6 H10 Cl N5	68.39	187.0622	12.183	187.0625	
26	Asn-Trp-OH		0.51	5		C20 H18 N4 O7	98.46	426.1173	16.162	426.1175	
33	Asiatic acid		1.47	5		C30 H48 O5	97.12	488.3495	28.203	488.3502	
32	Argiotoxin 659		-0.42	3		C31 H53 N11 O5	68.59	659.4234	25.291	659.4231	
27	6-Chloropurine riboside		0.99	1		C10 H11 Cl N4...	65.32	286.0466	16.17	286.0469	
29	4-Hydroxybenzaldehyde		0.22	5		C7 H6 O2	87.34	122.0368	16.987	122.0368	
21	2-Hydroxy-7,8-dehydrograndiflorone		4.81	5	LMPK12120415	C19 H20 O5	85.67	328.1295	14.063	328.1311	
13	1-Benzylimidazole		12.86	3		C10 H10 N2	58.92	158.0824	12.183	158.0844	

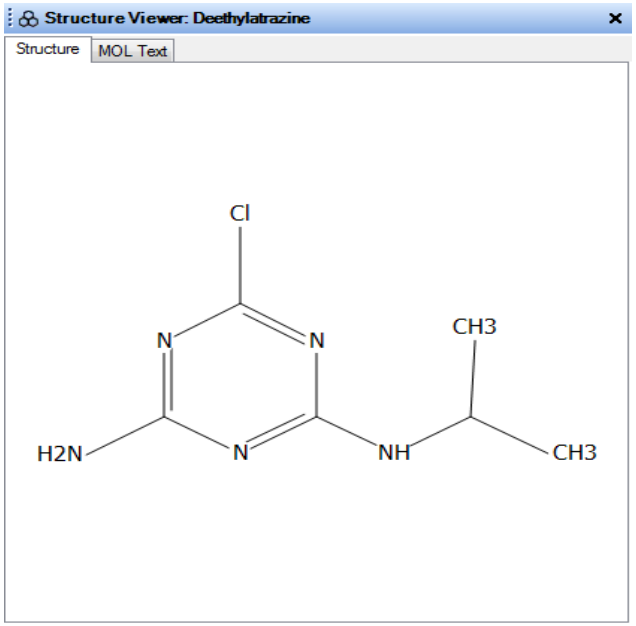
Compound List

Cpd	Label	Formula	Score	Diff (MFG, ppm	Mass	RT	Mass (MFG)	Diff (MFG, mDa)
31	Cpd 31: C11 H6 N6 O4; 19.700	C11 H6 N6 O4	83.29	1.85	286.0445	19.7	286.0451	0.53
24	Cpd 24: C13 H20 N2 O11; 15.018	C13 H20 N2 O11	92.45	1.2	380.1063	15.018	380.1067	0.46
11	Cpd 11: C9 H10 N5 O; 12.179	C9 H10 N5 O	99.06	1.01	204.0883	12.179	204.0885	0.21
8	Cpd 8: C24 H37 N O7 S; 3.035	C24 H37 N O7 S	90.19	0.9	483.2286	3.035	483.2291	0.44
20	Cpd 20: C32 H40 N O15; 14.068	C32 H40 N O15	92.76	0.71	678.2393	14.068	678.2398	0.48
30	Cpd 30: C12 H4 N3 O7; 19.713	C12 H4 N3 O7	84.98	0.57	302.0048	19.713	302.0049	0.17
10	Cpd 10: C14 H13 N7 O7; 8.221	C14 H13 N7 O7	93.97	0.55	391.0874	8.221	391.0876	0.22
9	Cpd 9: C20 H29 N4 O4; 3.019	C20 H29 N4 O4	83.84	0.45	389.2187	3.019	389.2189	0.18
2	Cpd 2: C3 H11 N5 O4; 2.816	C3 H11 N5 O4	87	-0.28	181.0812	2.816	181.0811	-0.05
18	Cpd 18: C7 H5 N4; 12.167	C7 H5 N4	95.86	-0.55	145.0515	12.167	145.0514	-0.08
35	Cpd 35: C11 H16 N3; 21.017	C11 H16 N3	84.77	-2.49	190.1349	21.017	190.1344	-0.47

Identification – Deethylatrazine ??

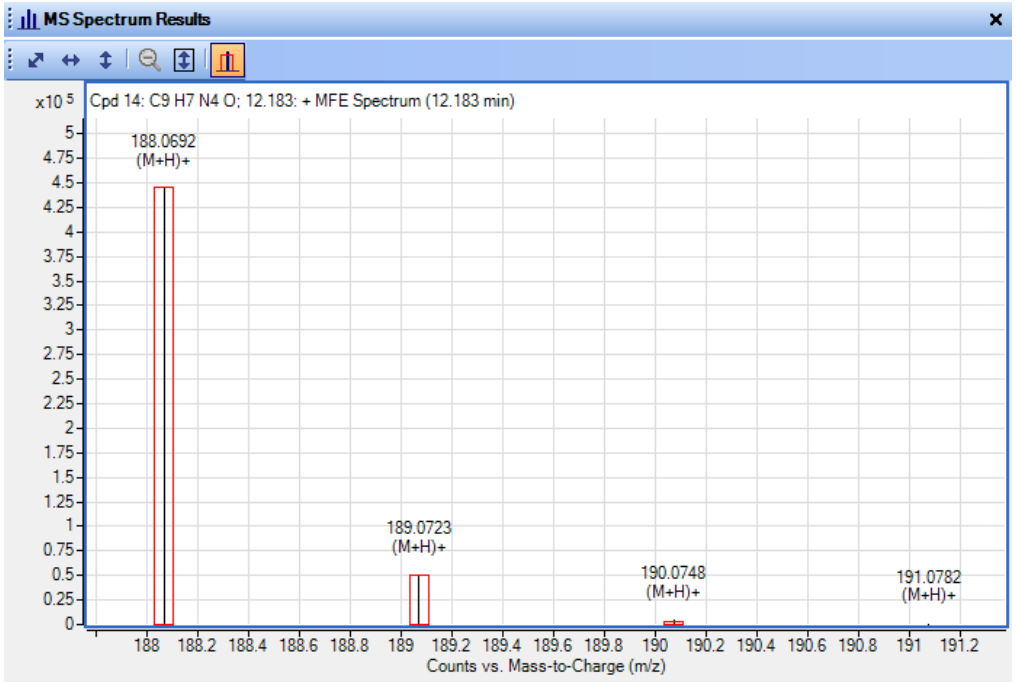
Database proposal of Deethylatrazine for compound 14 is not supported by the MS isotope pattern.





Identification – Formula Generation

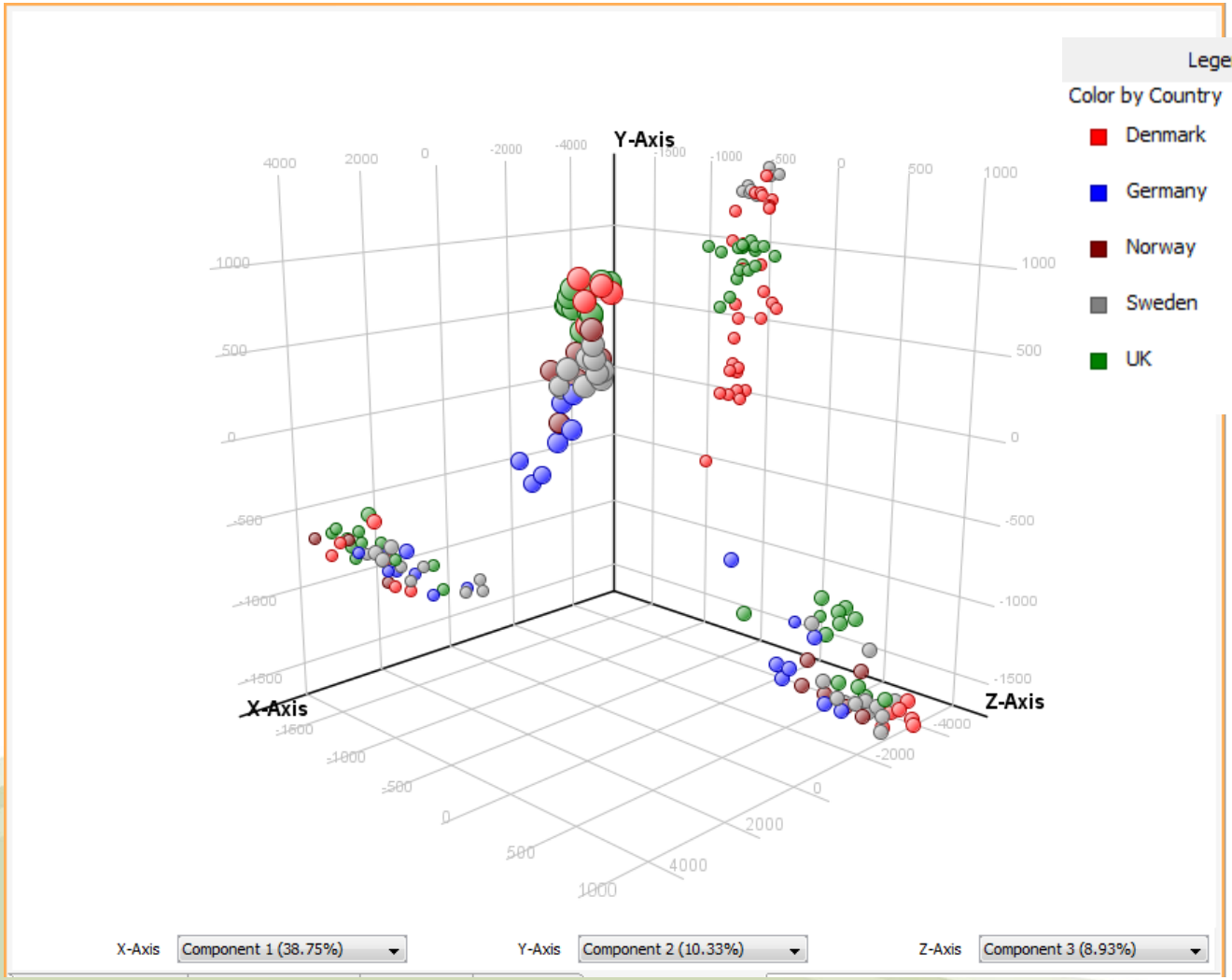
Formula generation proposes a formula of C9H7N4O which has a much larger score and better isotope pattern fit.



14	Cpd 14: C9 H7 N4 O; 12.183				1		C9 H7 N4 O	49.75	-0.19	187.062		187.062	-0.04	1	
Best	Name	Formula	Score	Mass	Mass (DB)	Mass (MFG)	Diff (ppm)	Diff (abs. ppm)	Diff (mDa)	RT	ID Source	Score (DB)	Score (MFG)	DBE	
◉		C9 H7 N4 O	49.75	187.062		187.062	-0.19	0.19	-0.04	12.183	MFG		99.51	8.5	
○		C9 H15 S2	40.52	187.0621		187.0615	-3.05	3.05	-0.57	12.183	MFG		81.05	2.5	
○	Deethylatrazine	C6 H10 Cl N5	34.2	187.0622	187.0625		1.65	1.65	0.31	12.183	DBSearch	68.39			

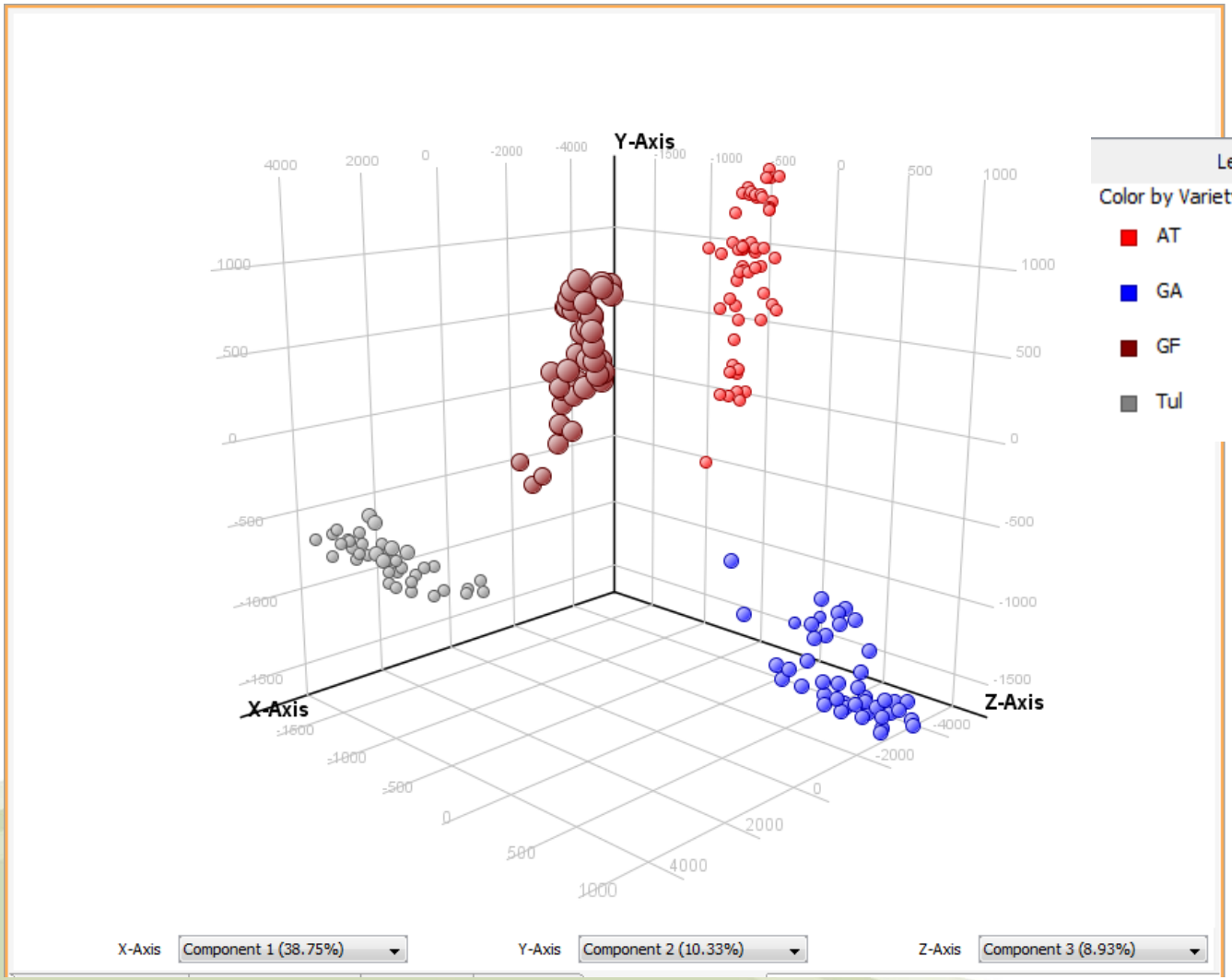
Country and Variety PCA

Four varieties across five countries were examined for variation.



Country and Variety PCA

Four varieties across five countries were examined for variation. This indicates that there is more variation between varieties than there is between countries.



Does Organic Cultivation Make A Difference ??

A single variety (AT) was grown under organic and conventional cultivation conditions.

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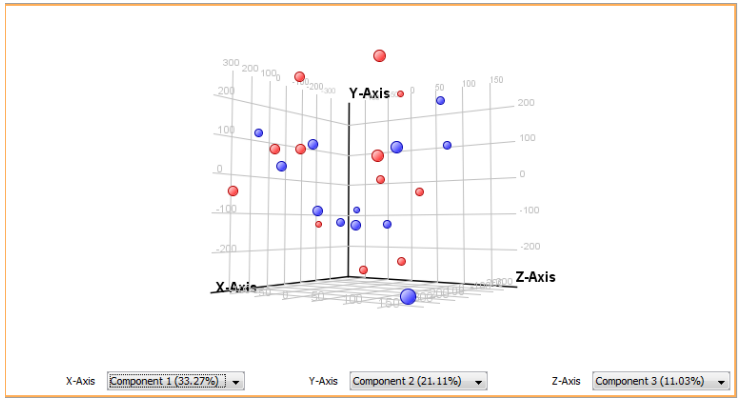

Result Summary

	P all	P < 0.05	P < 0.02	P < 0.01	P < 0.0050	P < 0.0010
FC all	151	0	0	0	0	0
FC > 1.1	81	0	0	0	0	0
FC > 1.5	50	0	0	0	0	0
FC > 2.0	50	0	0	0	0	0
FC > 3.0	21	0	0	0	0	0
Expected by chance		0	0	0	0	0

Summary

- LC/MS using retention time and accurate mass allows non-targeted investigation of multiple compounds
- Differences in different raspberry varieties grown in UK could be visualised using MPP
- The year of growth did not appear to be significant
- More details statistical processing provides smaller numbers of significantly differentiating m features
- These features can be putatively identified using databases and formula generation
- Variation between raspberry varieties appears greater than the variation in the country of growth.
- Organic cultivation made no difference to the entities found in ESI Positive RPLC/MS.