Analysis of free amino acids in tobacco with a new LC/MS/MS procedure

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Eurofins

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Background

- A considerable number of analytical methods are reported in the literature for the analysis of free amino acids [1-3].
- 1. T.P. Mawhinney, R.S.R. Robinett, A. Atalay, M.A. Madson, *J. Chromatogr.* 358 (1986) 231–242.
- 2. J. W. Henderson, A. Brooks, *Agilent* Part No. 5990-4547EN.
- 3. H. Kaspar, K. Dettmer, Q. Chan, S. Daniels, S. Nimkar, M. L. Daviglus, J. Stamler, P. Elliott, P. J. Oefner, *J. Chromatogr. B*, 877 (2009) 1838-1846.
- Older procedures use HPLC separation on ion exchange type columns with post-column derivatization followed by UV detection.
- Other procedures use pre-column derivatization followed by HPLC analysis with fluorescence or UV detection or GC/MS analysis depending on the derivatization agent.
- More recent procedures use various techniques (with or without derivatization) HPLC separation and MS or MS/MS detection.

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Purpose of study

- Develop an HPLC method for free amino acid analysis without the use of derivatization.
- The separation should be on a reversed-phase type column, not on an ion exchange type or a HILIC type. This choice was made since reversed phase columns offer the best separation and chromatographic peak shapes.
- The detection procedure should be MS/MS working in MRM mode for providing positive identification for every peak in the chromatogram.
- The time length of the separation should be around 20 min or shorter.
- The method should be easily applicable to tobacco samples.

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Method description (sample extraction)

- A ground tobacco sample of 50 mg is weighed with precision of 0.1 mg and placed in a 20 mL vial.
- To this sample, 5 mL of extracting solution is added and the sample is extracted for 30 min on a wrist action shaker.
- The extracting solution contains 0.1 mol/L HCl, as well as 25 μmol/L (0.2286 μg/mL) d_3-alanine, and 25 μmol/L phenylglycine (0.3807 μg/mL) as internal standards (phenylglycine is not present in the tobacco leaf).
- A portion of the resulting extract is filtered through a 0.45 μm PVDF filter and placed in a 2 mL vial for analysis. The samples are stable for at least 48 hours at 15 ^{o}C.

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Method description (HPLC separation)

- The column was Acclaim™ RSLC Polar Advantage II, 2.1 x 250 mm with 2.2 μm particles from Dionex/Thermo Scientific.
- The HPLC system was a 1200 Ser. Agilent HPLC with degasser, dual pump (high pressure mixing), autosampler (samples kept at 15 ^{o}C), and column compartment (kept at 20 ^{o}C).
- The separation used gradient, solution A: aqueous with 1% acetonitrile, 0.5% heptafluorobutyric acid, 0.02% trifluoroacetic acid (by volume), and solution B: 0.1% trifluoroacetic acid in acetonitrile (by volume).
- The gradient started at 0% B to 60% B at 18 min, hold for 3 min and back to 0%.
- Flow rate was 0.2 mL/min and injection volume was 3 μL.

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Method description (MS/MS parameters)

- API-5000 triple quadrupole mass spectrometer (AB Sciex, Framingham, MA, USA), working in multiple reaction monitoring (MRM) mode with the following parameters:

Parameter	Value
Polarity	Positive
Collision gas (CAD)	5 mL/min
Curtain gas (CUR)	10 mL/min
Ion source gas 1 (GS1)	55 mL/min
Ion source gas 2 (GS2)	55 mL/min
Ion spray voltage (IS)	5500 V
Temperature (TEM)	450 ^{o}C
Interface heater (Ihe)	off
Declustering potential (DP)	60.0 V
Entrance potential (EP)	4.0 V
Collision energy (CE) for all amino acids	15.0 V
Collision energy (CE) for proline	5.0 V
Collision cell exit potential (CXP)	13.0 V
Acquisition time per ion	50 ms
Total acquisition time	25 min

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Method description (ions used for measurement)

No.	Amino Acid	Abbrev.	Precursor ion	Product ion	Ret. time min
1	Valine	Val	72	55	17.63
2	Glycine	Gly	76	30	4.58
3	Alanine	Ala	90	44	5.54
4	Sarcosine	Sar	90	30	5.97
5	d3-Alanine	d3-Ala	93	47	5.52
6	Serine	Ser	106	88	4.52
7	Proline	Pro	116	70	8.48
8	Threonine	Thr	120	56	5.18
9	Cysteine	Cys	122	87	12.01
10	Isoleucine	Ile	132	86	20.49
11	Leucine	Leu	132	86	20.17
12	Hydroxyproline	Hyp	132	86	4.59
13	Asparagine	Asn	133	74	4.43
14	Ornitine	Orn	133	70	20.16
15	Aspartic acid	Asa	134	88	4.88
16	Glutamine	Gln	147	101	4.79
17	Lysine	Lys	147	84	16.93
18	Glutamic acid	Glu	148	84	5.81
19	Methionine	Met	150	133	18.57
20	Phenylglycine	Phg	152	106	19.50
21	Histidine	His	156	110	18.05
22	Phenylalanine	Phe	166	120	21.29
23	Arginine	Arg	175	130	19.57
24	Citruline	Cit	176	70	9.61
25	Tyrosine	Tyr	182	165	19.77
26	Tryptophan	Trp	205	188	22.34

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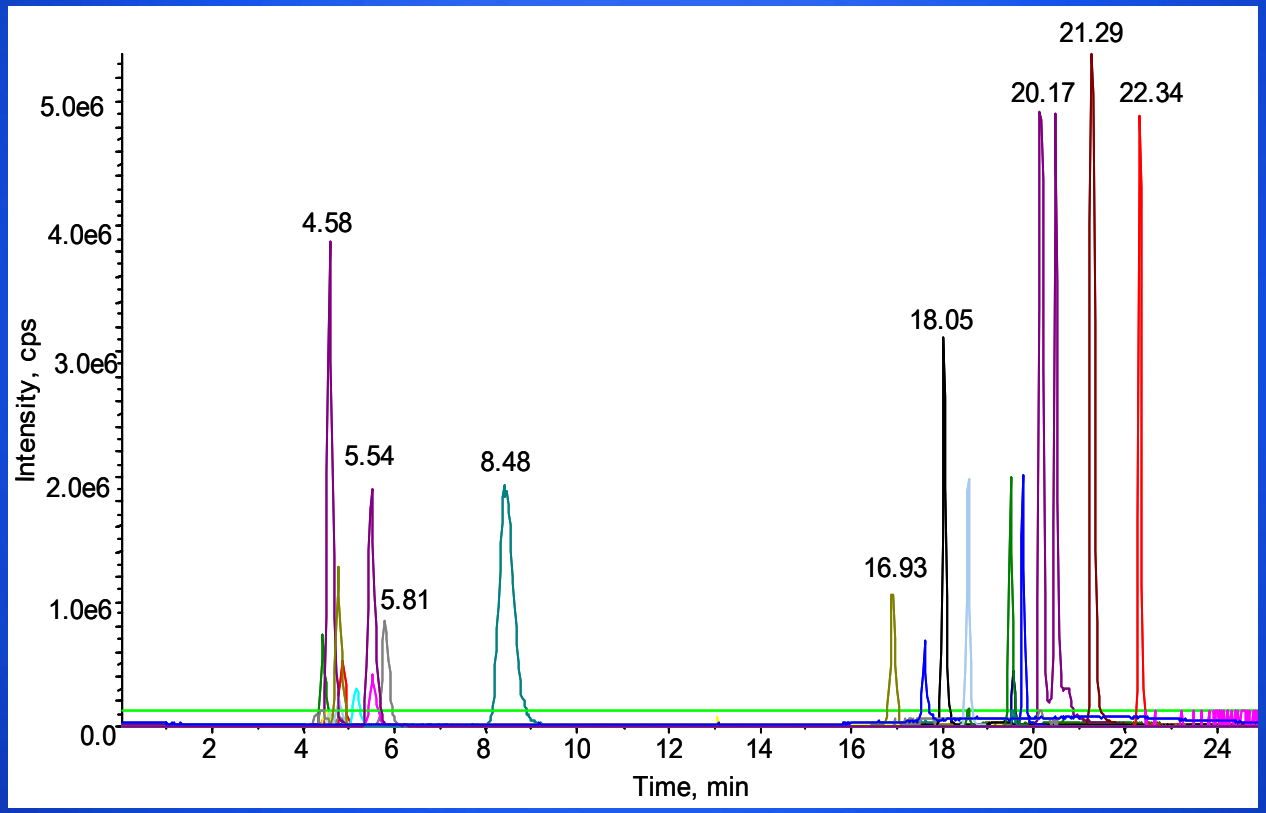
Example of chromatogram containing 25 μmol/L standards



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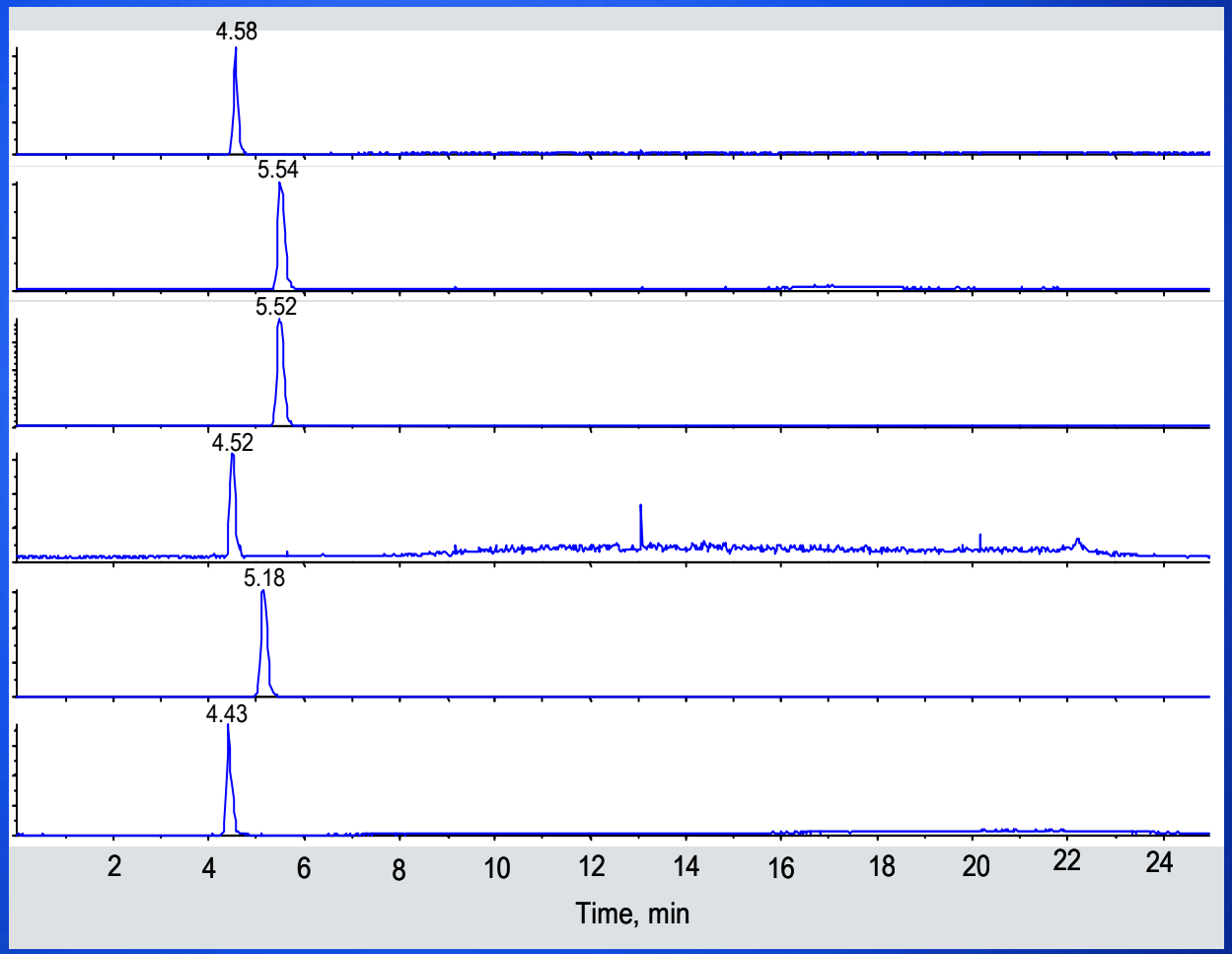
Extracted ions for six individual compounds

Compound	Ret. Time min
Glycine	4.58
Alanine	5.54
d3-Alanine	5.52
Serine	4.52
Threonine	5.18
Asparagine	4.43



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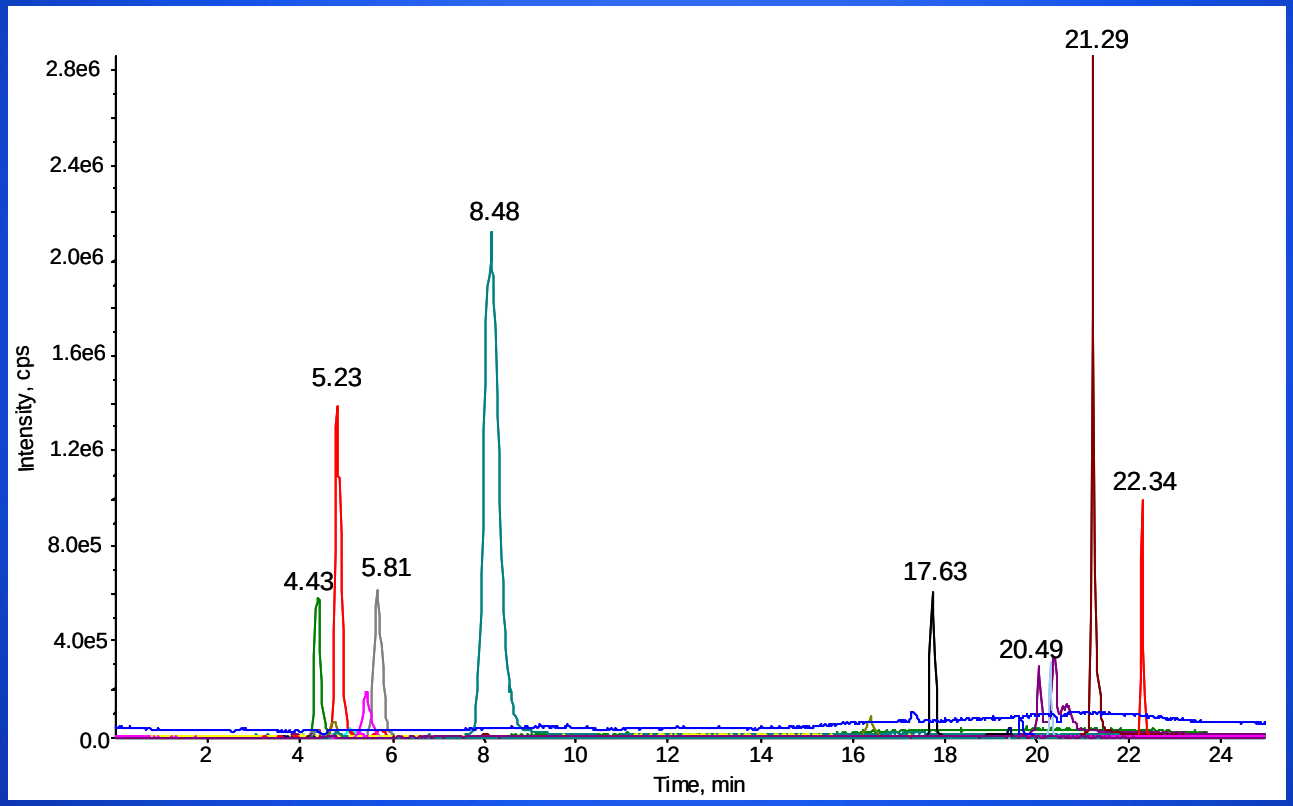
Example chromatogram of a flue cured tobacco extract



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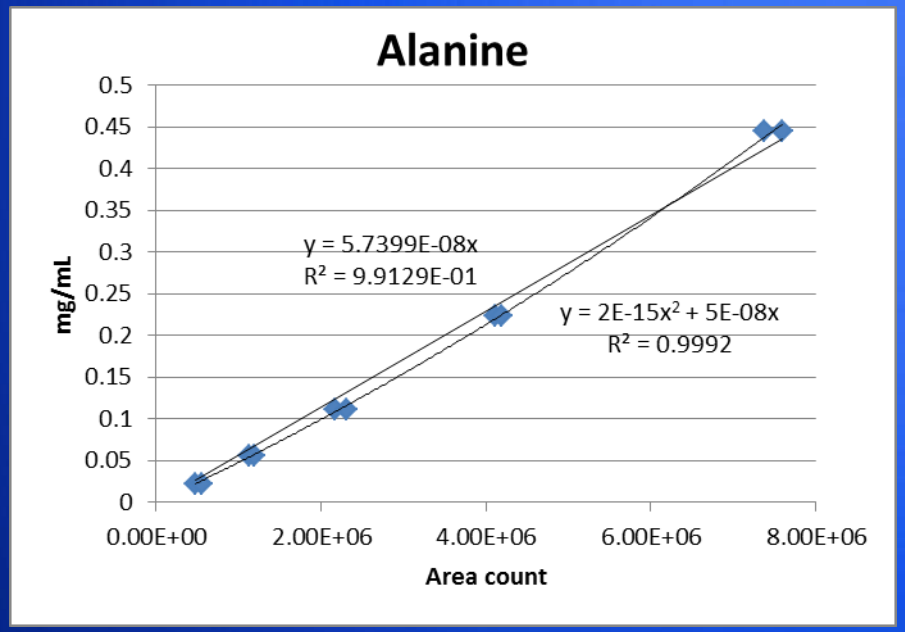
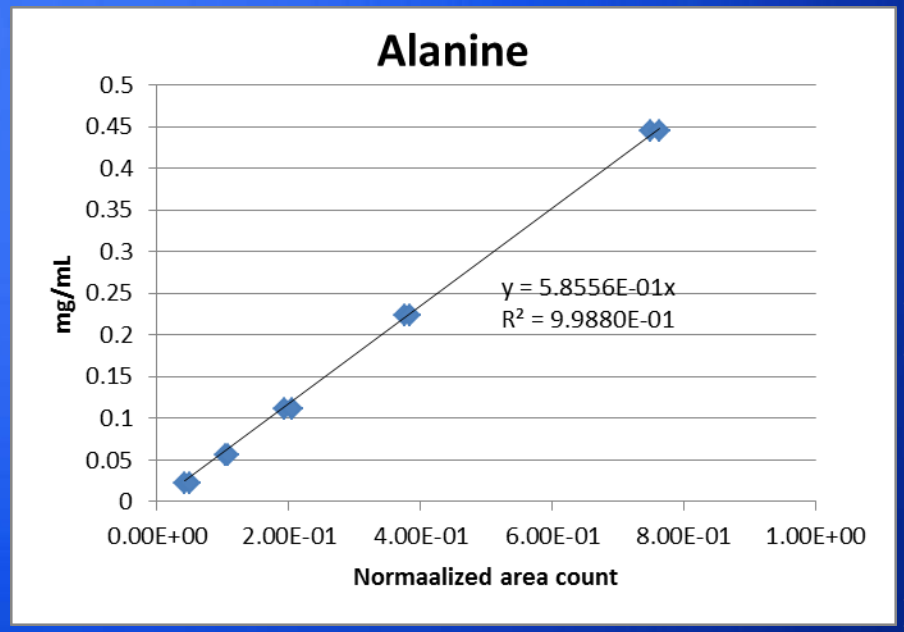
Quantitation of amino acids (AA)

- Quantitation is performed using calibration curves.
- The calibration curves can be either concentration of AA vs. area count, or concentration of AA vs. normalized area count (by I.S. area). Two examples are given below:





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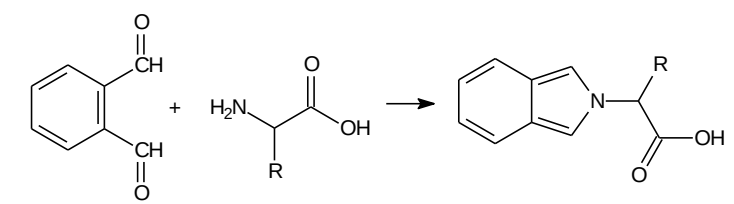
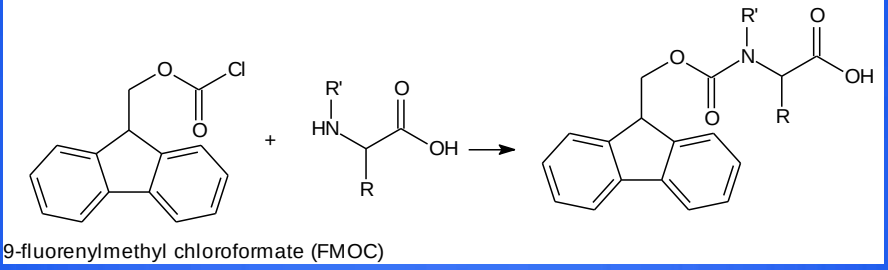
Standard HPLC method with OPA and FMOC derivatization and fluorescence detection used for comparison.



o-phthaldialdehyde (OPA)



9-fluorenylmethyl chloroformate (FMOC)

- Separation on a Zorbax Eclipse XDB-C18 column, 4.6 x 250 mm, 5 μm particles.
- Use of gradient with two solutions: (sol A) 10 mM Na_2HPO_4, 10 mM $Na_2B_4O_7$, 1% tetrahydrofuran in water at pH = 8.2, and (sol. B) methanol/acetonitrile/water 50/30/20 v/v/v.
- FLD detection (Ex = 340 nm, Em = 450 nm, and Ex = 266 nm, Em = 305 nm).

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Advantages of MS/MS technique versus OPA and FMOC analytical procedure.

- Positive identification of each amino acid by its specific precursor and product ions.
- Elimination of interferences using the specificity of detection and good LC separation.
- Excellent sensitivity, superior to fluorescent detection.
- Capability to adjust sensitivity from compound to compound by modifying the collision energy parameter, such that amino acids at low level are analyzed with higher sensitivity than those in large quantity.
- Relatively short chromatography run time, that can be made shorter when using higher flow rate for the mobile phase.
- Low solvent utilization.

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Tobacco analyzed by the LC/MS/MS method

No	Tobacco type	Year	Description	Curing
Tob.1	FC L c (1)	2008	Eastern NC belt, lower stalk (lug) flue-cured	cured
Tob.2	FC U c (1)	2008	Eastern NC belt, upper stalk (leaf & some tips) flue-cured	cured
Tob.3	FC L c (2)	2009	South Carolina belt, lower stalk (lug) flue-cured	cured
Tob.4	FC U c (2)	2009	South Carolina belt, upper stalk (leaf & some tips) flue-cured	cured
Tob.5	FC off L c	2006	Brazil, lower stalk (lugs & primings) flue-cured	cured
Tob.6	FC off U c	2006	Brazil, upper stalk (leaf & tips) flue-cured	cured
Tob.7	Bu L c (1)	2007	Kentucky & Tennessee, lower stalk (flyings & cutters) burley	cured
Tob.8	Bu U c (1)	2007	Kentucky & Tennessee, upper stalk (leaf) burley	cured
Tob.9	Bu L c (2)	2008	North Carolina & Virginia, lower stalk (flyings & cutters) burley	cured
Tob.10	Bu U c (2)	2008	North Carolina & Virginia, upper stalk (leaf) burley	cured
Tob.11	Bu off L c	2008	Malawi, lower stalk (flyings & cutters) burley	cured
Tob.12	Bu off U c	2008	Malawi, upper stalk (leaf) burley	cured
Tob.13	O Sa U c	2007	Turkey, good quality middle to upper stalk, Samsun oriental	cured
Tob.14	O Iz U c	2005	Turkey, good quality middle to upper stalk, Izmir oriental	cured
Tob.15	Commer. A	2010	Commercial cigarette A (“tar” 10.5)	cured

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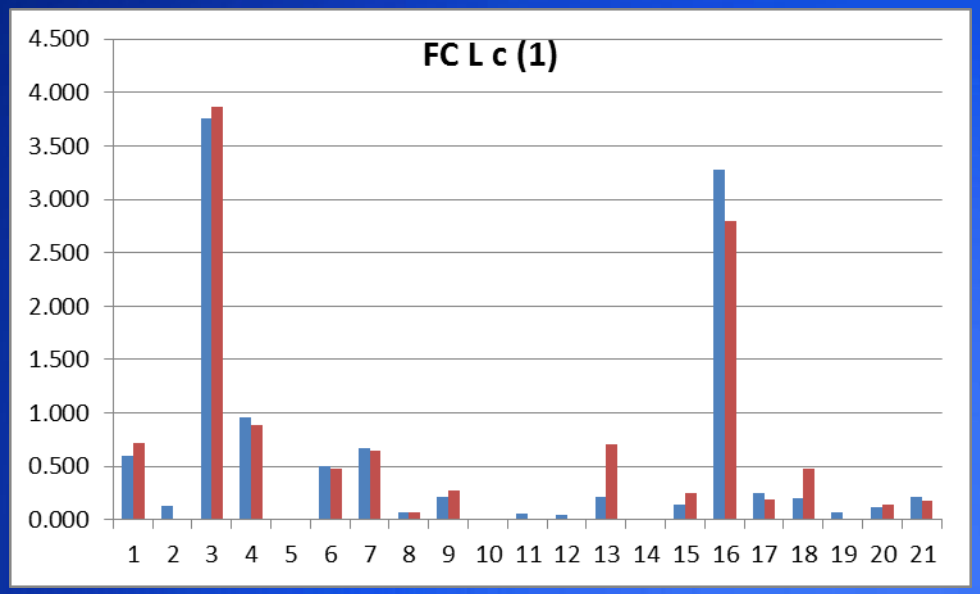

- X axis: amino acid No.
- Y axis: mg amino acid/g
- MS/MS method
- Standard method

No	Compound	No	Compound
1	Alanine	12	Leucine
2	Arginine	13	Lysine
3	Asparagine	14	Methionine
4	Aspartic acid	15	Phenylalanine
5	Cysteine	16	Proline
6	Glutamic acid	17	Serine
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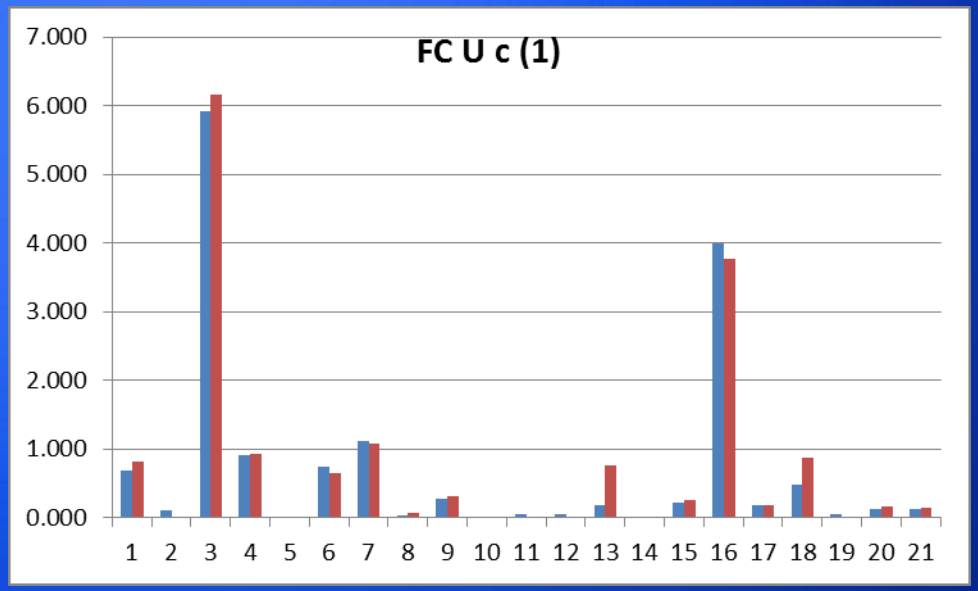

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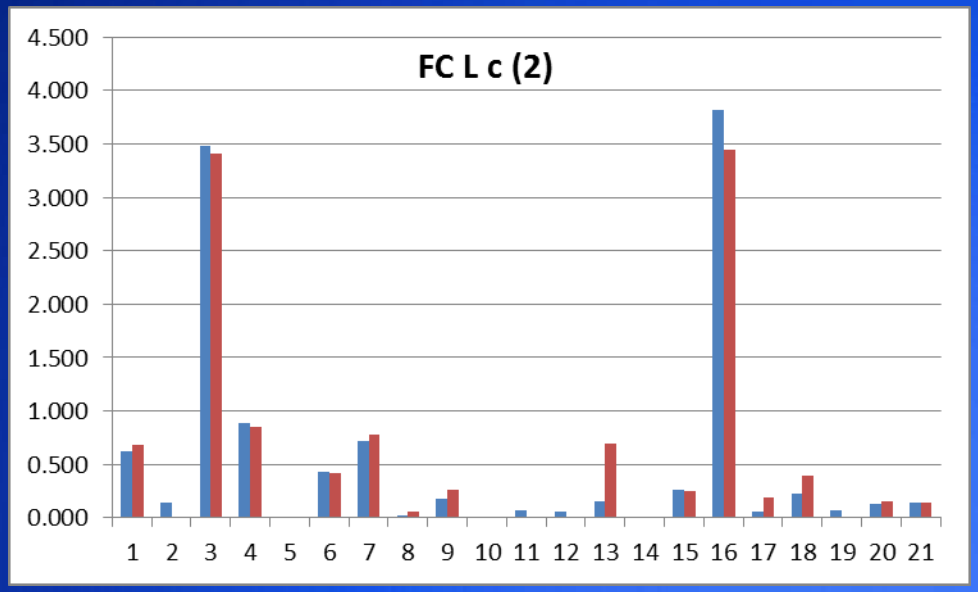

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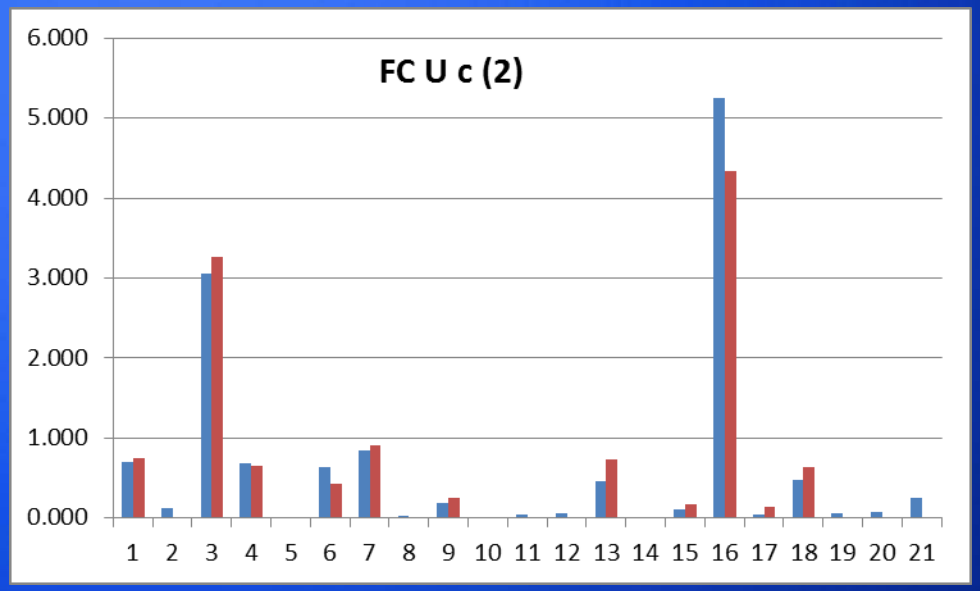

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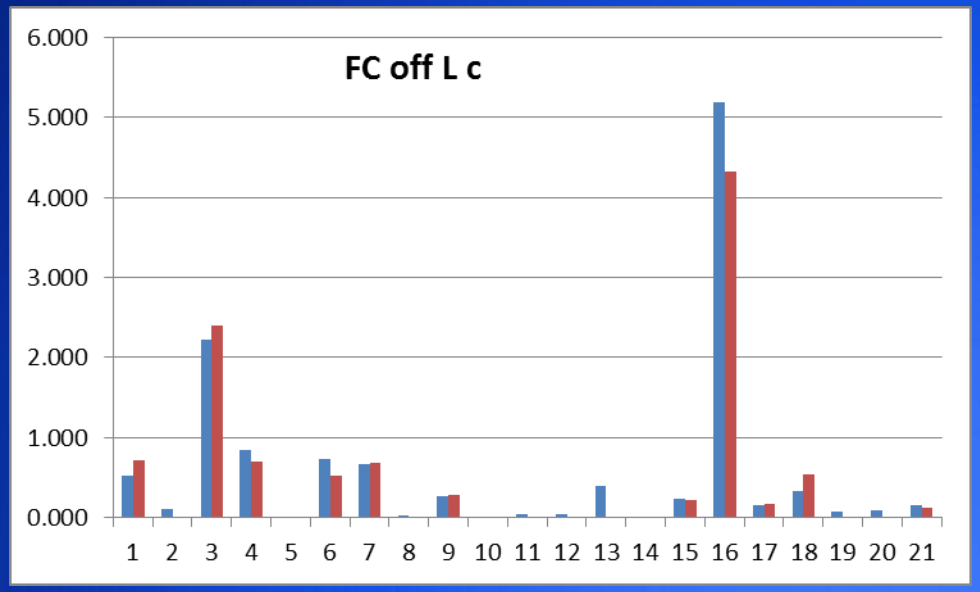

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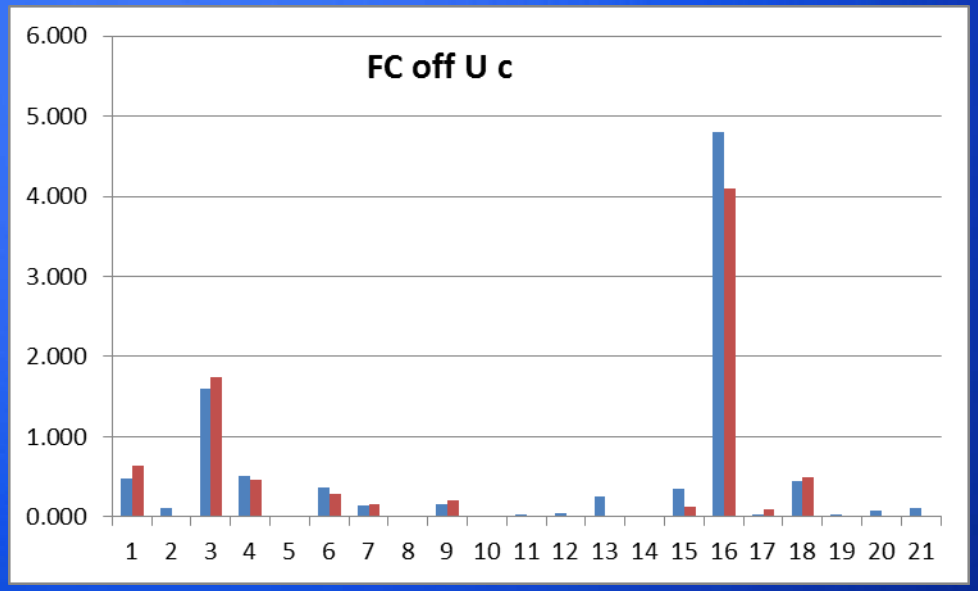

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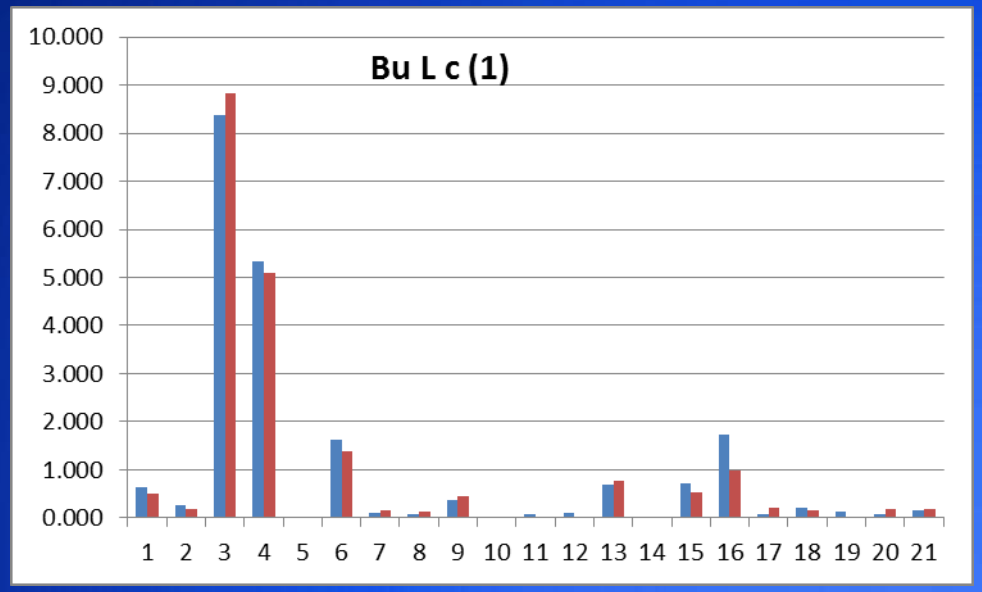

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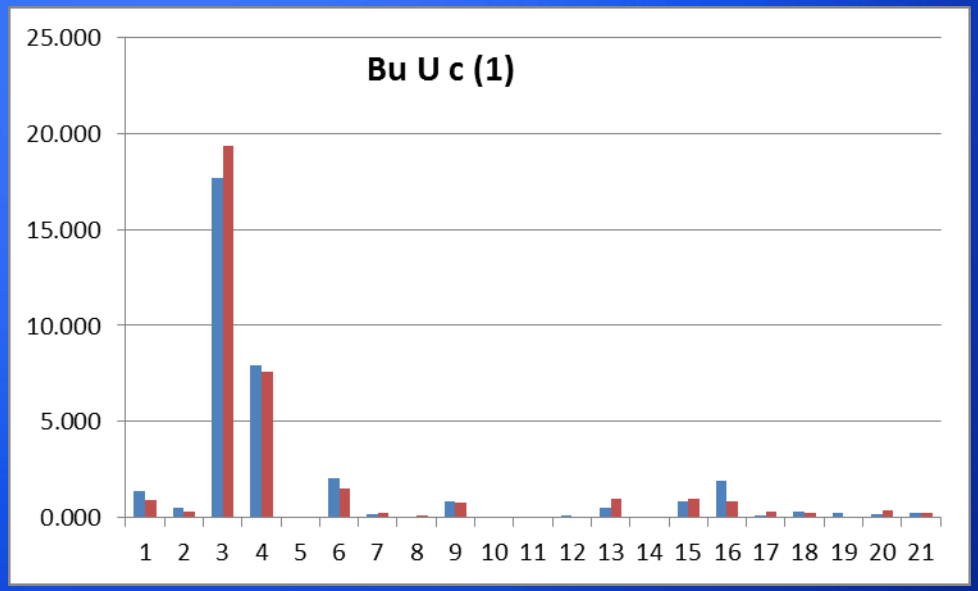

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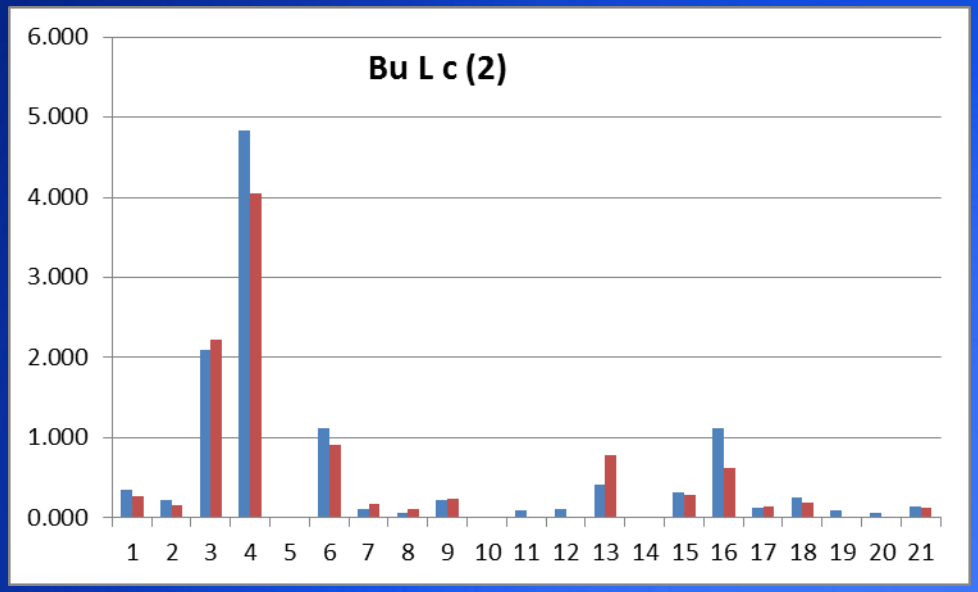

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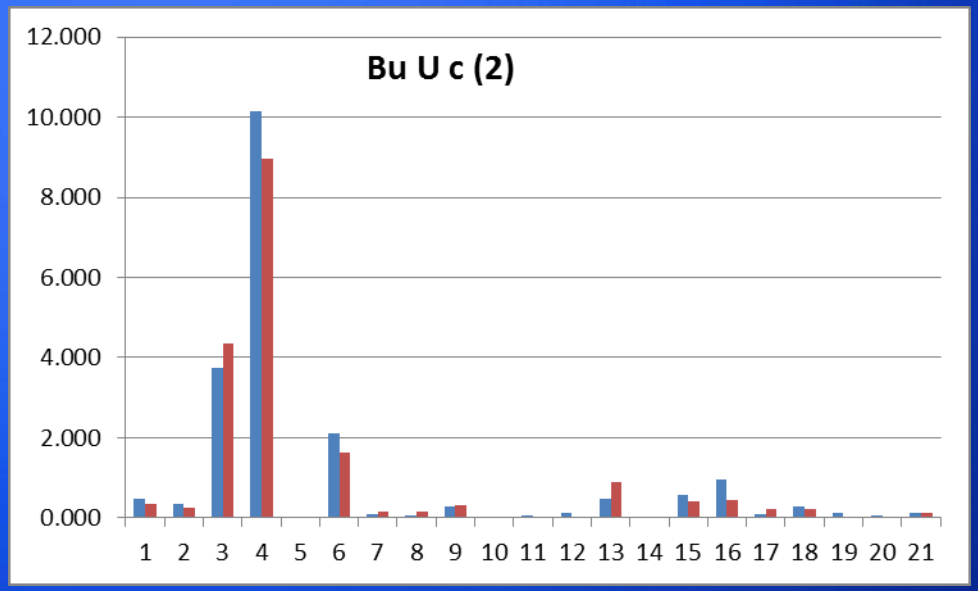

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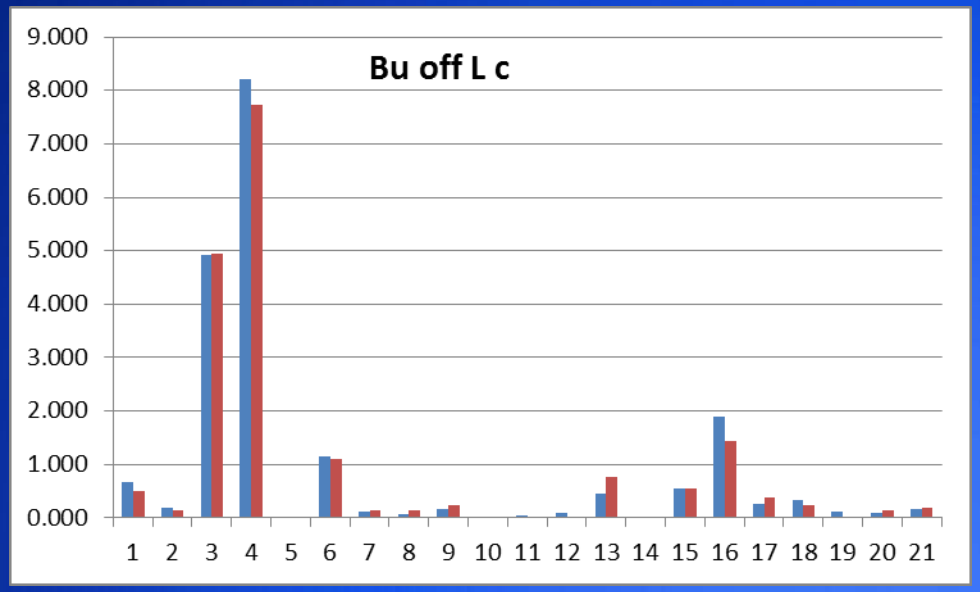

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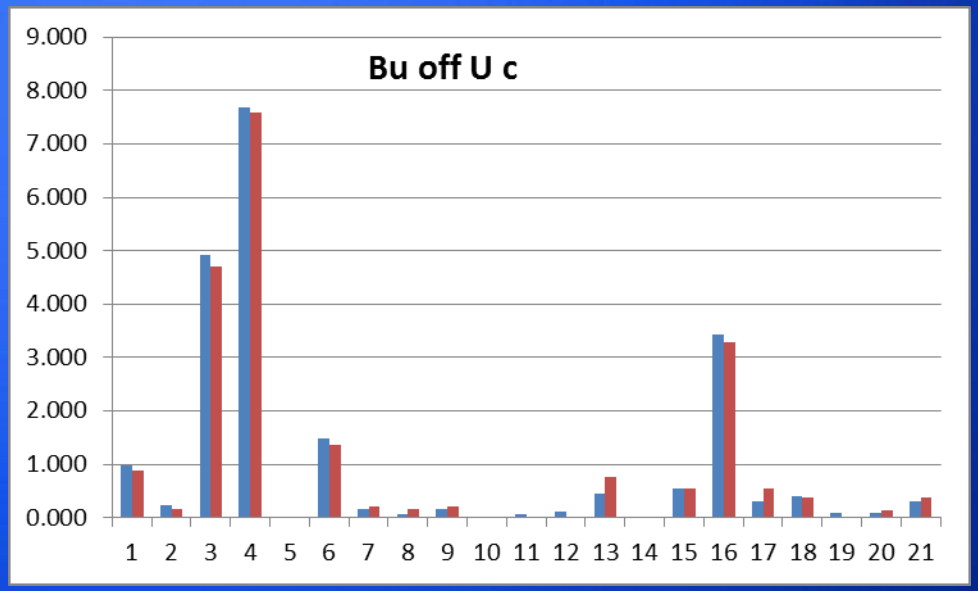

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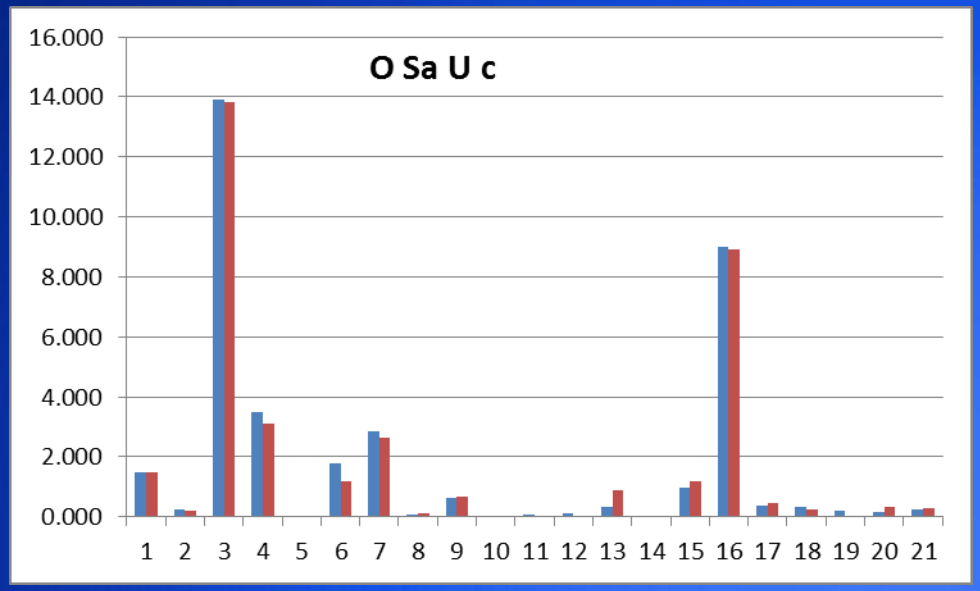

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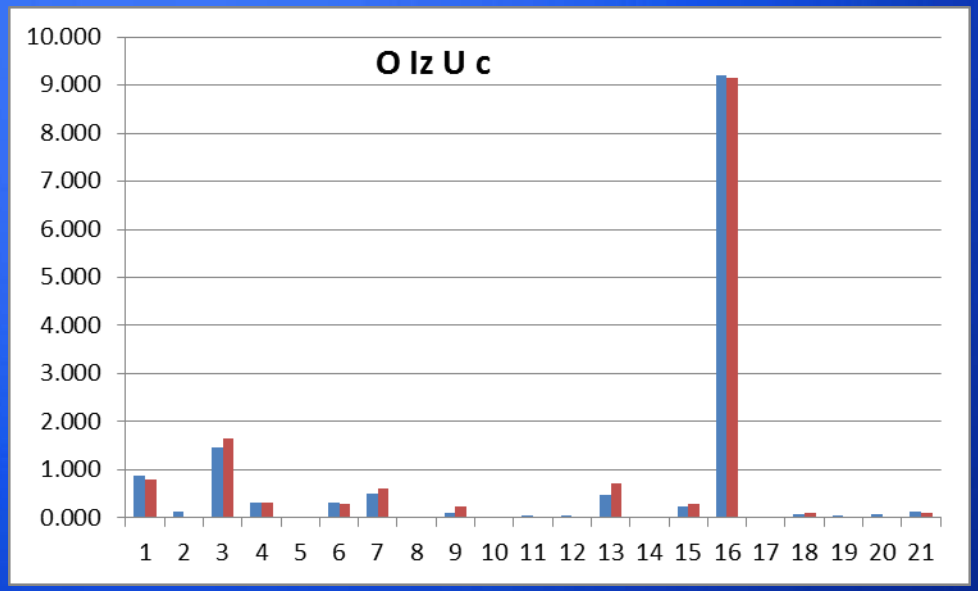

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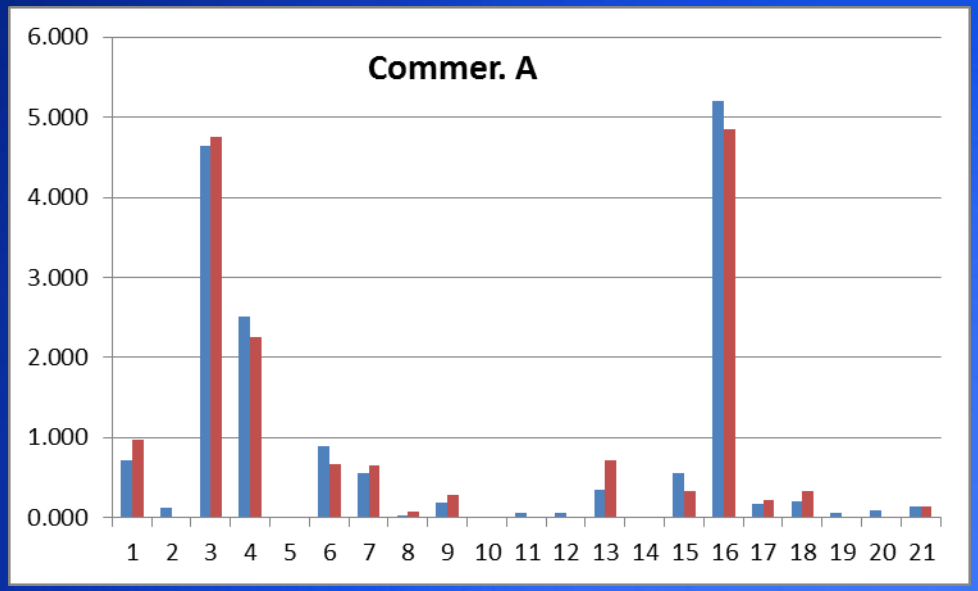

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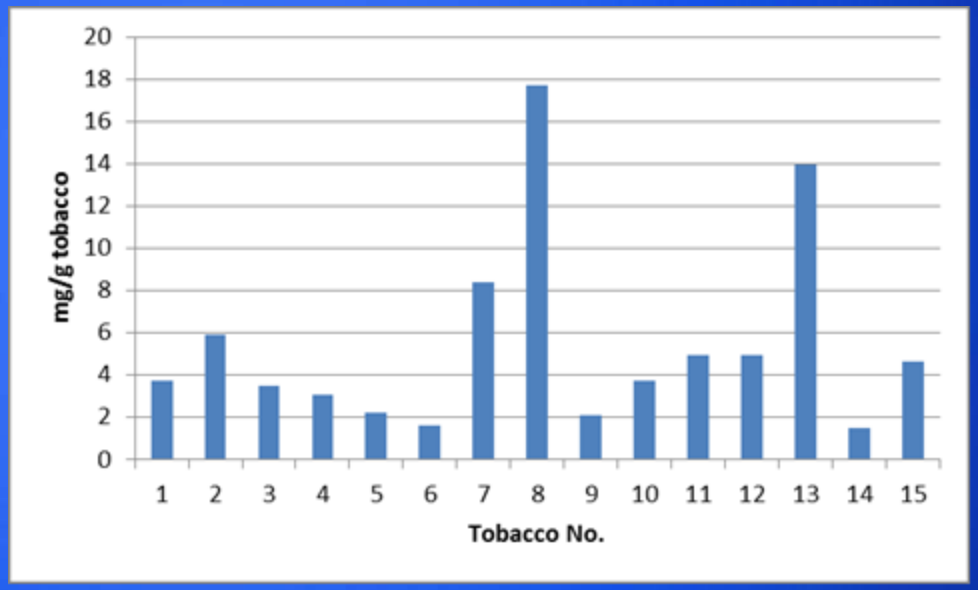
Variation in the level of asparagine (mg/g) in the analyzed tobacco types

No	Type
1	FC L c (1)
2	FC U c (1)
3	FC L c (2)
4	FC U c (1)
5	FC off L c
6	FC off U c
7	Bu L c (1)
8	Bu U c (1)
9	Bu L c (2)
10	Bu U c (2)
11	Bu off L c
12	Bu off U c
13	O Sa U c
14	O Is U c
15	Commer. A



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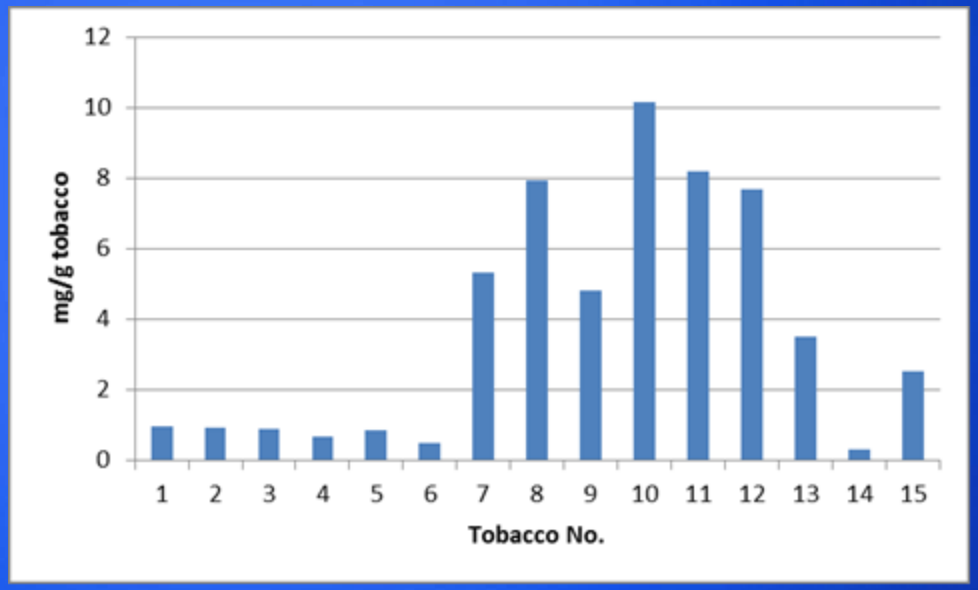
Variation in the level of aspartic acid (mg/g) in the analyzed tobacco types

No	Type
1	FC L c (1)
2	FC U c (1)
3	FC L c (2)
4	FC U c (1)
5	FC off L c
6	FC off U c
7	Bu L c (1)
8	Bu U c (1)
9	Bu L c (2)
10	Bu U c (2)
11	Bu off L c
12	Bu off U c
13	O Sa U c
14	O Is U c
15	Commer. A



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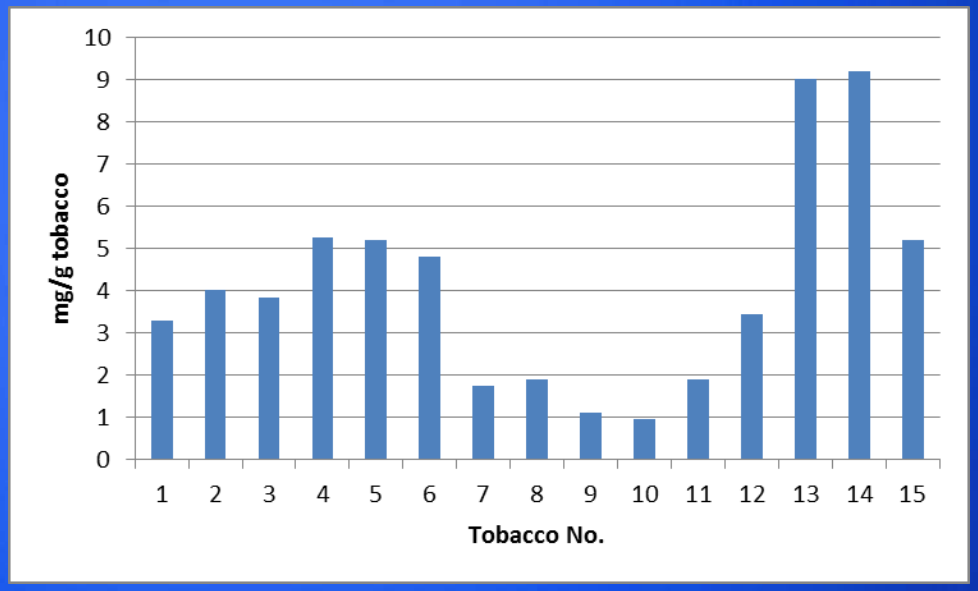
Variation in the level of proline (mg/g) in the analyzed tobacco types

No	Type
1	FC L c (1)
2	FC U c (1)
3	FC L c (2)
4	FC U c (1)
5	FC off L c
6	FC off U c
7	Bu L c (1)
8	Bu U c (1)
9	Bu L c (2)
10	Bu U c (2)
11	Bu off L c
12	Bu off U c
13	O Sa U c
14	O Is U c
15	Commer. A



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Conclusions (on the method)

- A new method for the analysis of free amino acids using MS/MS detection has been developed.
- The new method uses separation on an Acclaim™ RSLC Polar Advantage II column (C18), with gradient separation of amino acids and no derivatization.
- The method accuracy compared well with a HPLC-fluorescence with derivatization method, previously utilized for amino acids analysis.
- The method has very good precision, better sensitivity than the fluorescence detection method and allows positive identification of each amino acid.

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Conclusions (on the tobacco)

- The “profile” of amino acids and even the actual levels do not differ too much from the lower stalk (lug) and upper stalk (leaf & some tips) for the same tobacco material (some exceptions exist).
- Differences that can be considered significant can be seen among flue cured tobaccos from different regions. The same is true for burleys from different regions and for different Orientals.
- Significant differences (as expected) are seen between flue-cured, burley, and Oriental tobaccos regarding the content in amino acids.

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