Analysis of several common organic acids in tobacco leaf, snus, and moist snuff

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Background

- Among the organic acids commonly present in tobacco, are the following: citric, fumaric, lactic, oxalic, maleic, malic, quinic, pyruvic, trihydroxybutanoic, and several sugar acids (e.g. gluconic).
- The content in these acids covers a wide range of values, some being present at % level, and other only in traces.
- The information about the level of these acids is important because of their contribution to the sensory properties of cigarette smoke and also to the taste of oral tobacco products.
- Analysis of these acids is not simple because they are not volatile to be amenable for gas chromatographic analysis without derivatization, they are very polar for good separation in reversed phase liquid chromatography, and they do not have chromophore groups for allowing sensitive ultra violet (UV) detection.

Background (cont.)

- The most common analytical technique used for analysis of these acids is ion exclusion chromatography (IEC).
- The common detection techniques used in IEC are based on conductometry, refraction index, or UV absorption at low wavelengths (210-215 nm).
- These techniques do not offer very good sensitivity, and also are non-selective such that the only procedure for the differentiation between the analytes is based on the retention time.
- A RP-HPLC method coupled with mass spectral detection has been developed for the quantitation of citric, fumaric, lactic, oxalic, maleic, malic, quinic, pyruvic, and trihydroxybutanoic acids.
- A qualitative GC-MS analysis of the acids in the plant material was performed before the LC-MS quantitation.

Qualitative GC-MS analysis

- The GC-MS analysis utilized direct derivatization of the plant material. The samples were silylated without using a preliminary extraction*.
- 50 mg plant material + 400 μL of DMF that contains 400 μg/mL *tert*-butylhydroquinone as internal standard + 800 μL BSTFA with 1% TMCS were added into 2 mL GC vials.
- The vials were kept at 73 °C (in a heating block) for 30 min, and subsequently allowed to cool at room temperature.
- The solution from each vial was filtered through 0.45 μm PVDF filters and analyzed on an Agilent 6890/5973 GC-MS instrument.
- Peak identification in the total ion chromatogram (TIC) was done using mass spectral library searches (NIST14 mass spectra library).

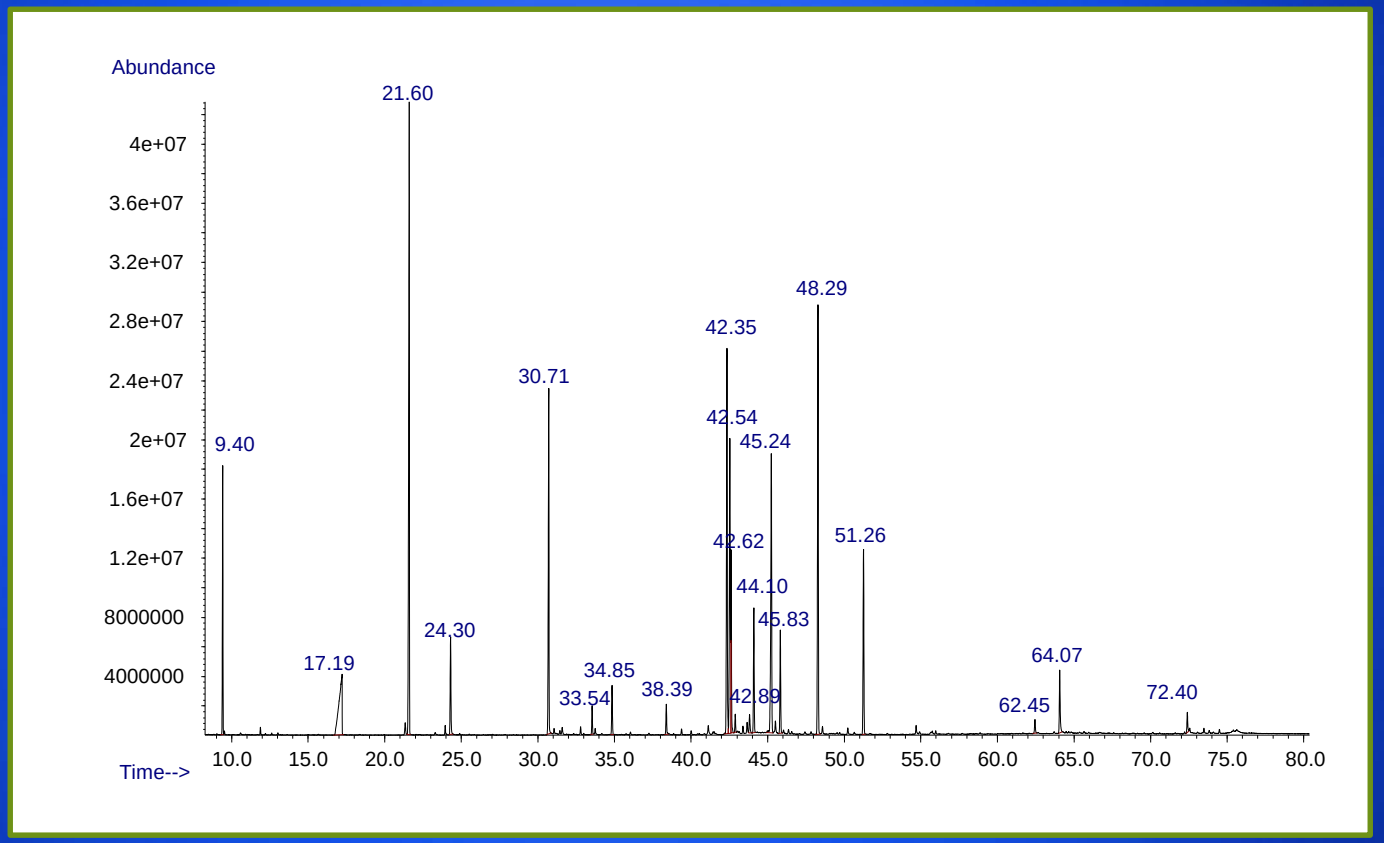
- * E. D. Alford, J. H. Lauterbach, 41st TCRC, abstract 56, Greensboro, NC, October 4-7, 1987

GC-MS operating parameters

(DB5-MS column, 30 m, 0.25 mm i.d., 0.25 μm film thickness)

Parameter	Description	Parameter	Description
Initial oven temp.	50°C	Flow mode	Constant flow
Initial time	0.5 min	Flow rate	1.0 mL/min
Oven ramp rate	3°C/min	Nominal initial pressure	7.65 psi
Oven final first ramp	200°C	Split ratio	30:1
Final time first ramp	0 min	Split flow	30 mL/min
Oven ramp rate	4°C/min	Outlet pressure	Vacuum
Oven final temp.	300°C	Transfer line heater	280°C
Final time	10 min	Ion source temp.	230°C
Total run time	85.5 min	Quadrupole temp.	150°C
Inlet temp.	300°C	Resulting EM Voltage	2000 V
Inlet mode	Split	MSD solvent delay	7.0 min
Injection volume	1.0 mL	MSD acquisition mode	TIC
Carrier gas	Helium	Mass range	33 to 1050 AMU

Total ion chromatogram of a tobacco sample (cigarette blend)



Acids detected in a tobacco sample (cigarette blend)

Peak #	Compound	Ret. Time	Peak #	Compound	Ret. Time
1	Lactic acid*	11.87	13	2-Keto-L-gluconic acid	40.87
2	Glycolic acid	12.61	14	Citric acid*	42.89
3	Pyruvic acid*	15.65	15	Quinic acid*	44.10
4	2-Butenedioic acid* (Z)	22.72	16	Gluconic acid	48.59
5	Succinic acid	23.28	17	Galactaric acid	48.83
6	Glyceric acid	23.94	18	Hexadecanoic acid	50.24
7	2-Butenedioic acid* (E)	24.90	19	Caffeic acid	52.81
8	Malic acid*	30.71	20	Linoleic acid	54.77
9	Pyroglutamic acid	31.59	21	Linolenic acid	54.91
10	Trihydroxybutanoic acid (1)*	32.80	22	Glucuronic acid	58.88
11	Trihydroxybutanoic acid (2)*	33.54	23	Chlorogenic acid (1)	72.40
12	Tetrahydroxypentanoic acid	40.45	24	Chlorogenic acid (2)	73.83

* NOTE 1: Acids targeted for quantitation.

NOTE 2: Acetic, and oxalic acids not seen by this analysis.

Quantitative LC-MS analysis

- 50 mg of plant material (weighed with 0.1 mg precision) extracted for 30 min (at room temperature on a wrist action shaker) with 15 mL water containing 0.1% formic acid.
- A portion of this solution was filtered through a 0.45 μm PVDF filter and 200 μL from this solution were placed in a 2 mL screw top cap vial.
- To the vial were added 800 μL of a solution containing 0.1% formic acid and two internal standards.
- The internal standard solution contained 6.156 μg/mL citric acid d4, and 6.813 μg/mL Na^+ salt of lactic acid d3.
- Injections of 1 μL sample were made into a Agilent 1200 HPLC system, coupled with a API 5000 triple quadrupole MS from AB Sciex.

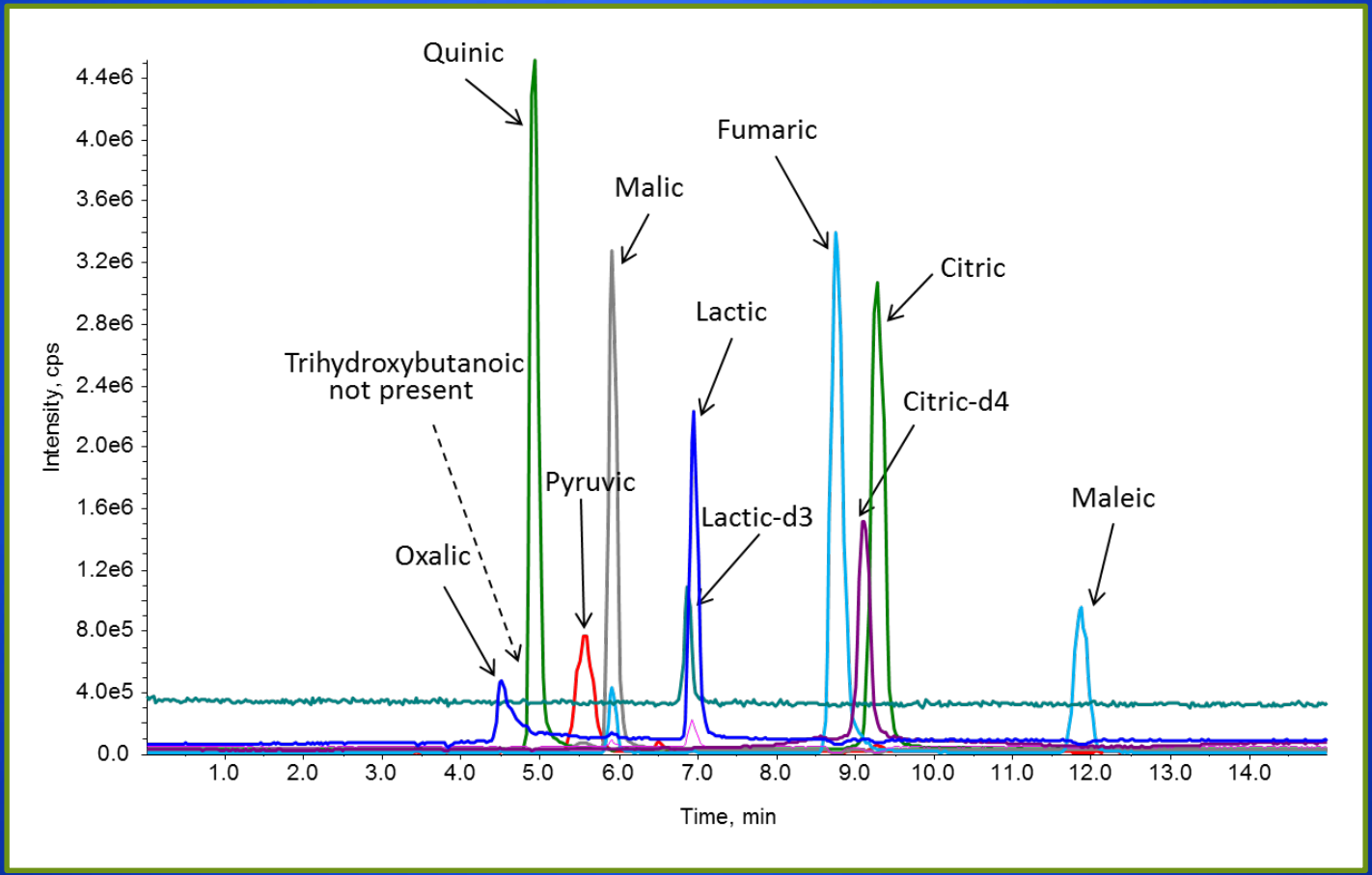
Quantitative LC-MS analysis (cont.)

- The separation was performed on a Synergy Hydro-RP column 250 x 4.6 mm with 4 um particles from Phenomenex in isocratic mode.
- The mobile phase contained 95% water and 5% methanol, to which was added 1.5 mL formic acid per 1L solution.
- The flow rate was 0.6 mL/min.
- The detection was performed in Q1 Multiple Ion mode using negative ionization.
- The optimized parameters for MS were: curtain gas CUR = 10 mL/min, ion spray voltage IS = - 4000 V, temperature TEM = 600 ^{o}C, ion source gas 1 GS1 = 45 mL/min, ion source gas 2 GS2 = 35 mL/min, declustering potential DE = -10V, entrance potential EP = -5V.

Ions used for detection and the elution retention time of each compound.

Compound	Ion for Q1	Retention time (min)
Citric acid	191.1	9.44
Citric acid-d4	195.1	9.26
Fumaric acid	115.1	8.88
Lactic acid	89.0	6.98
Lactic acid-d3	92.1	6.90
Maleic acid	115.1	12.03
Malic acid	133.1	5.96
Oxalic acid	89.0	4.51
Pyruvic acid	87.0	5.57
Quinic acid	191.1	4.93
Trihydroxybutanoic (1)	135.1	4.52
Trihydroxybutanoic (2)	135.1	4.62

Chromatogram for a standard mixture containing about 10 µg/mL from each analyte and about 5 µg/mL of deuterated standards*.



NOTE: Acetic acid cannot be measured by this method.

Calibration for quantitation

- The calibrations were done using seven standards. The targets for the standards were 40 μg/mL, 20 μg/mL, 10 μg/mL, 5 μg/mL, 2.5 μg/mL, 1.25 μg/mL, and 0.625 μg/mL of each analyte.
- The actual initial mixture contained levels close to target levels but slightly different.
- Each standard solution contained the same amount of internal standard with 5.45 mg/mL lactic acid-d3 sodium salt and 4.925 mg/mL citric acid-d4.
- Quadratic lines were fitting better than linear ones the calibration points.
- The equations of the form $Y = a X^2 + b X + c$ were utilized for the calibration, where X = (peak area of standard) / (peak area of internal standard) and Y is μg/mL analyte.

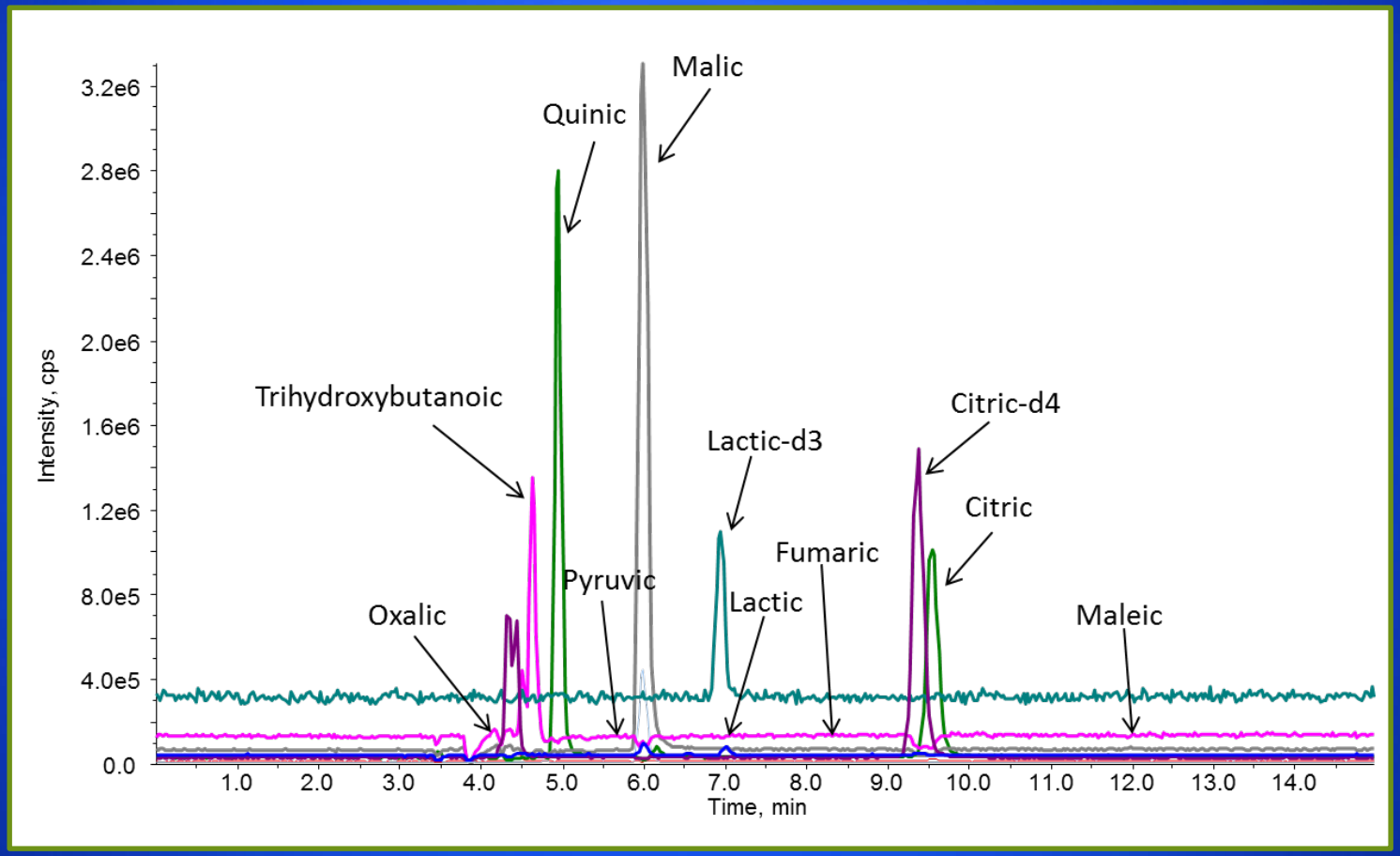
Coefficients for the equations $Y = a X^2 + b X + c$ used for the calibration of the analytes, the corresponding internal standard, and coefficient of determination R^2 for the analytes.

Analyte	I.S.	*a*	*b*	*c*	R^2
Citric acid	Citric acid-d4	2.04150	3.06250	0.68096	0.9953
Fumaric acid	Citric acid-d4	2.02200	3.19470	0.37591	0.9931
Lactic acid	Lactic salt-d3	0.34434	4.91290	-0.25554	0.9971
Maleic acid	Citric acid-d4	6.59860	22.03300	-0.22275	0.9985
Malic acid	Lactic salt-d3	0.43470	1.88910	0.13887	0.9916
Oxalic acid*	Lactic salt-d3	-2.00700	19.48300	-0.49165	0.9679
Pyruvic acid	Lactic salt-d3	0.12554	9.08260	0.01696	0.9951
Quinic acid	Lactic salt-d3	0.35164	-0.06276	0.91719	0.9886

The values for LOQ in μg/mL in the analyzed solution for various analytes.

Compound	LOQ μg/mL (solution)
Citric acid	0.15
Fumaric acid	0.16
Lactic acid	0.11
Maleic acid	0.02
Malic acid	0.04
Oxalic acid	3.45
Pyruvic acid	0.21
Quinic acid	0.01

Chromatogram of a tobacco sample also containing about 5 μg/mL of deuterated standards.



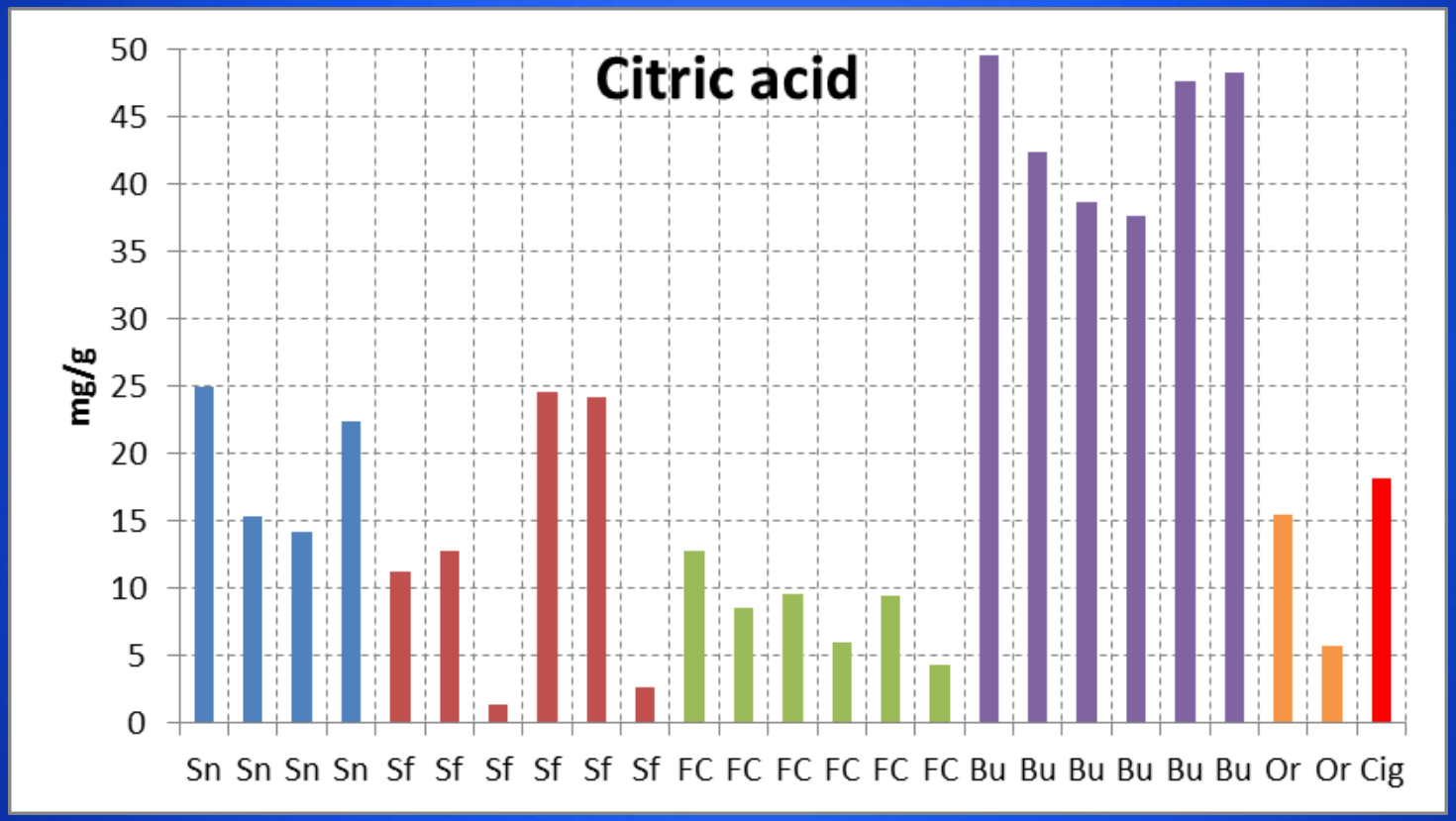
Description of the analyzed samples

No.	Sample	Description
1	Snus 1	Natural
2	Snus 2	Wintergreen
3	Snus 3	Natural
4	Snus 4	Wintergreen
5	Moist snuff 1	Natural
6	Moist snuff 2	Natural
7	Moist snuff 3	Natural
8	Moist snuff 4	Natural
9	Moist snuff 5	Wintergreen
10	Moist snuff 6	Wintergreen
11	FC L (1)	Eastern NC belt, lower stalk (lug) flue-cured
12	FC U (1)	Eastern NC belt, upper stalk (leaf & some tips) flue-cured
13	FC L (2)	South Carolina belt, lower stalk (lug) flue-cured
14	FC U (2)	South Carolina belt, upper stalk (leaf & some tips) flue-cured
15	FC off L	Brazil, lower stalk (lugs & primings) flue-cured
16	FC off U	Brazil, upper stalk (leaf & tips) flue-cured
17	Bu L (1)	Kentucky & Tennessee, lower stalk (flyings & cutters) burley
18	Bu U (1)	Kentucky & Tennessee, upper stalk (leaf) burley
19	Bu L (2)	North Carolina & Virginia, lower stalk (flyings & cutters) burley
20	Bu U (2)	North Carolina & Virginia, upper stalk (leaf) burley
21	Bu off L	Malawi, lower stalk (flyings & cutters) burley
22	Bu off U	Malawi, upper stalk (leaf) burley
23	O Sa U	Turkey, good quality middle to upper stalk, Samsun oriental
24	O Iz U	Turkey, good quality middle to upper stalk, Izmir oriental
25	Commercial cigarette	Tobacco blend

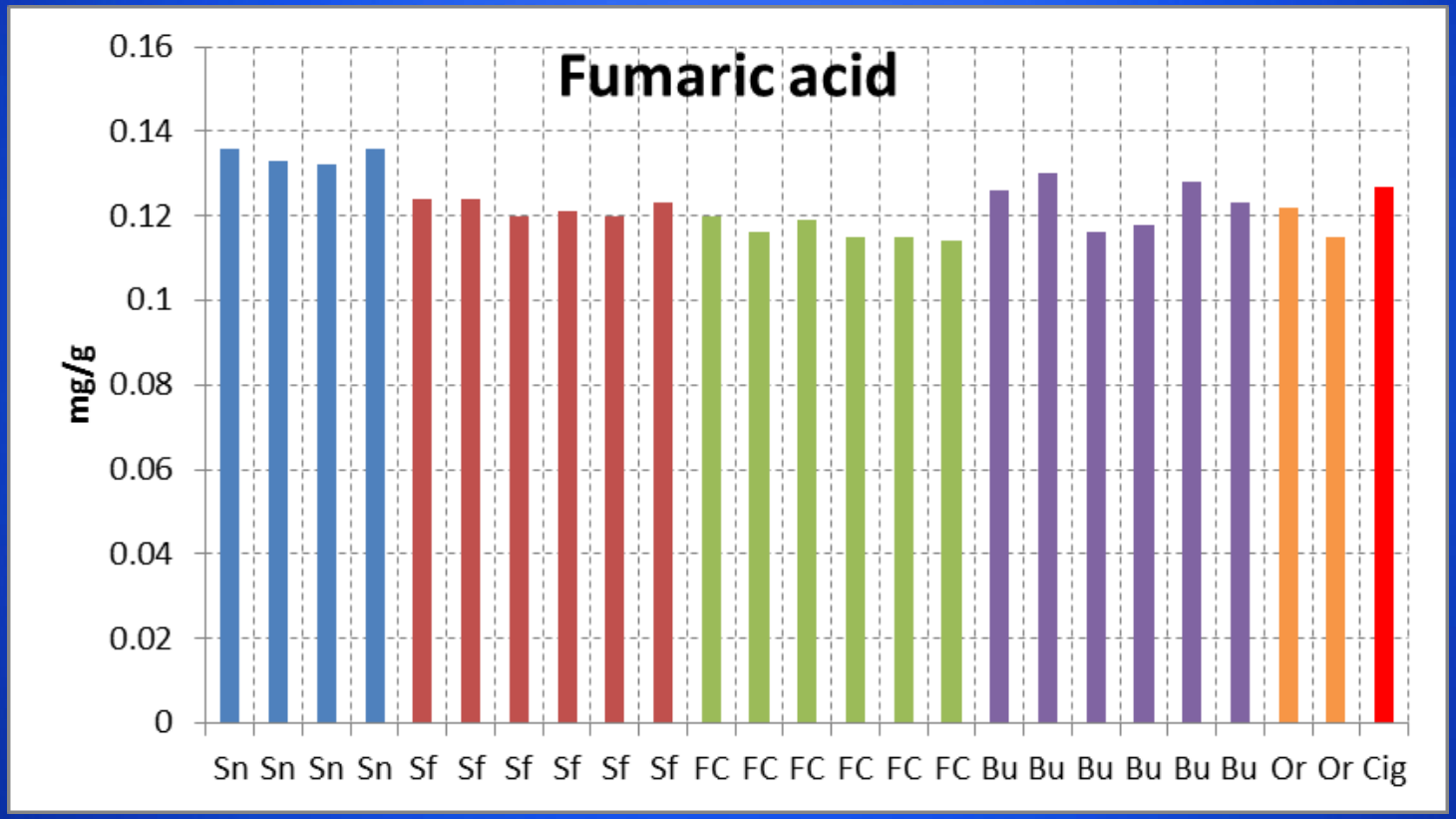
Results of levels of organic acids in mg/g

No.	Sample	Citric mg/g	RSD%	Fumaric mg/g	RSD%	Lactic mg/g	RSD%
1	Snus 1	24.971	0.35	0.136	1.04	0.652	0.05
2	Snus 2	15.356	2.95	0.133	1.50	0.527	1.08
3	Snus 3	14.144	3.07	0.132	10.76	0.532	6.94
4	Snus 4	22.347	5.22	0.136	3.28	0.622	10.40
5	Moist snuff 1	11.181	3.20	0.124	3.34	0.746	10.35
6	Moist snuff 2	12.799	4.21	0.124	5.53	0.370	0.68
7	Moist snuff 3	1.343	0.16	0.120	0.27	1.301	0.05
8	Moist snuff 4	24.573	1.47	0.121	5.46	0.031	3.49
9	Moist snuff 5	24.091	1.89	0.120	0.16	2.156	7.53
10	Moist snuff 6	2.588	1.10	0.123	2.56	0.379	1.78
11	FC L (1)	12.686	1.76	0.120	6.96	0.220	0.61
12	FC U (1)	8.543	4.05	0.116	12.04	0.200	10.40
13	FC L (2)	9.556	2.08	0.119	5.93	0.270	0.91
14	FC U (2)	5.889	0.19	0.115	0.38	0.198	0.44
15	FC off L	9.394	0.83	0.115	0.59	0.296	1.66
16	FC off U	4.236	1.38	0.114	2.07	0.380	3.82
17	Bu L (1)	49.492	4.45	0.126	5.09	0.140	6.92
18	Bu U (1)	42.354	4.58	0.130	8.57	0.137	1.44
19	Bu L (2)	38.608	2.89	0.116	1.93	0.128	2.84
20	Bu U (2)	37.562	1.01	0.118	3.17	0.155	3.56
21	Bu off L	47.529	2.17	0.128	0.42	0.124	7.25
22	Bu off U	48.228	4.28	0.123	15.81	0.181	12.18
23	O Sa U	15.438	2.41	0.122	9.42	0.179	3.32
24	O Iz U	5.736	1.39	0.115	1.40	0.233	4.03
25	Commercial cigarette	18.133	2.76	0.127	3.74	0.584	8.89

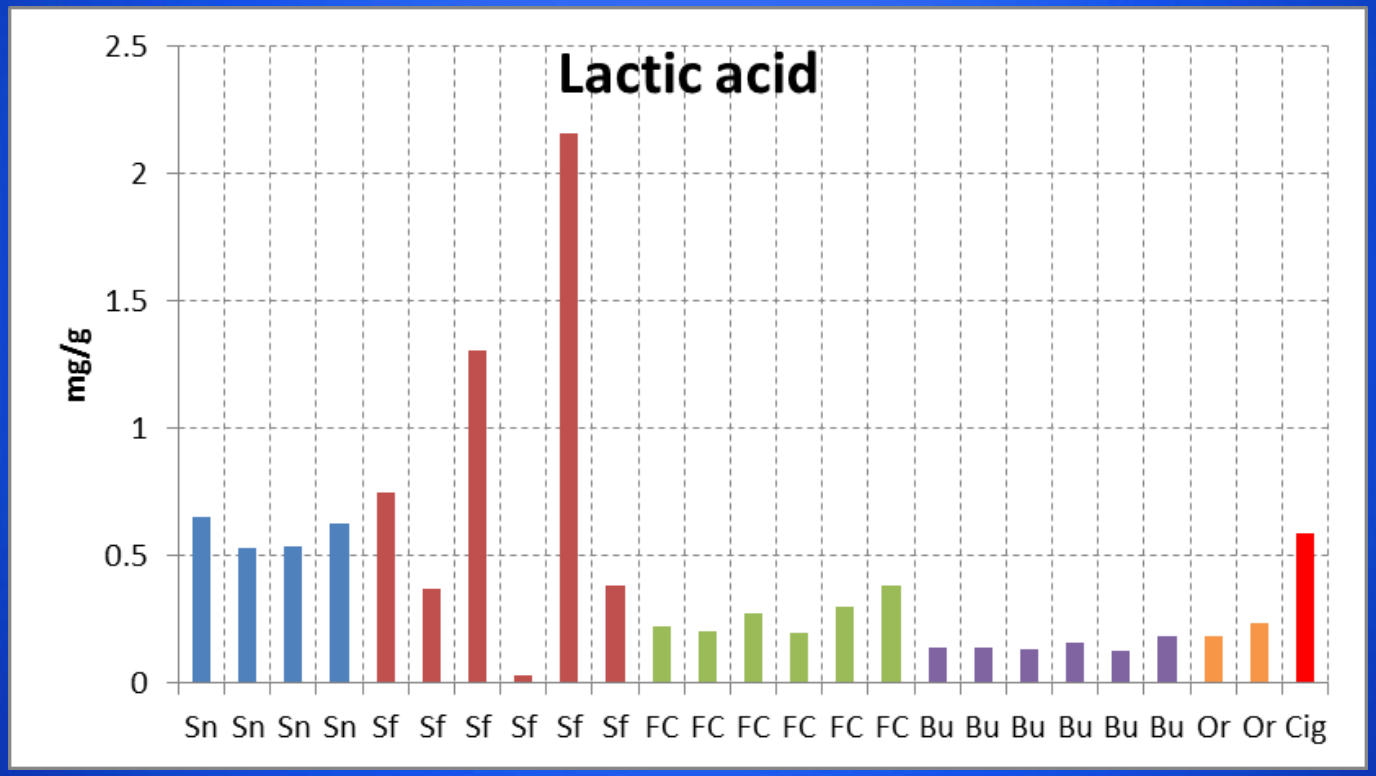
Results of levels of organic acids in mg/g



Results of levels of organic acids in mg/g



Results of levels of organic acids in mg/g

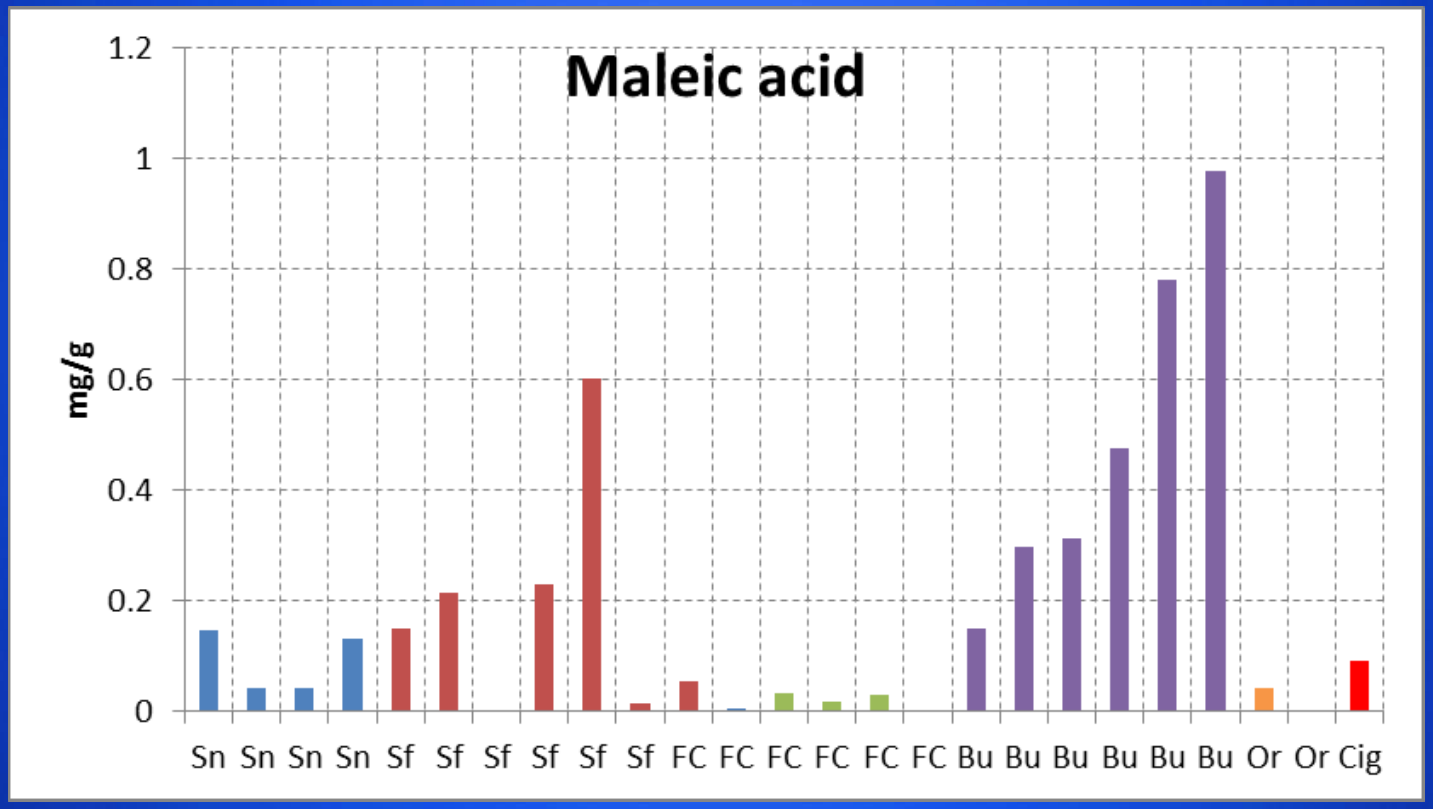


Results of levels of organic acids in mg/g

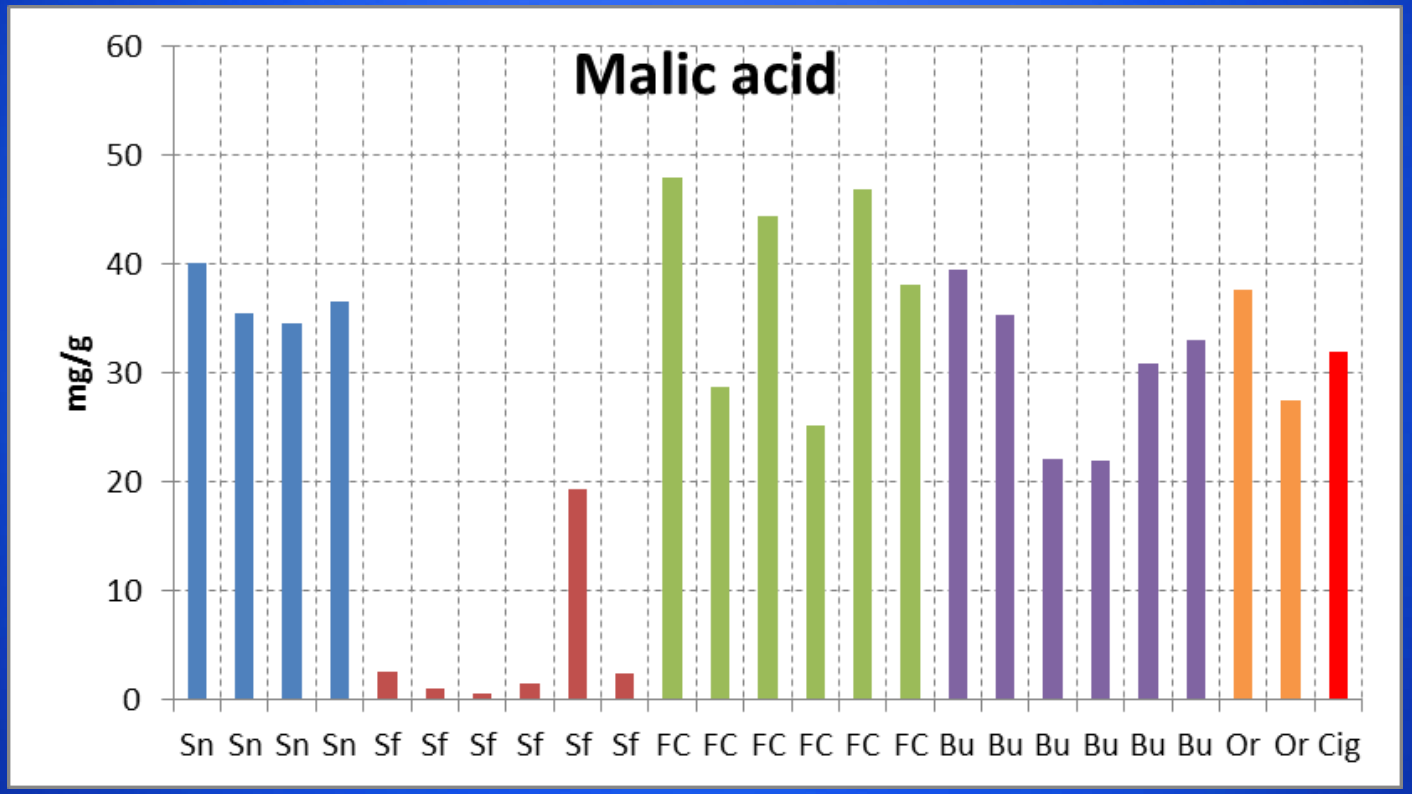
No.	Sample	Maleic mg/g	RSD%	Malic mg/g	RSD%	Oxalic mg/g	RSD%
1	Snus 1	0.146	1.18	40.109	1.45	0.023*	1.22
2	Snus 2	0.042	4.65	35.353	1.39	0.063	0.92
3	Snus 3	0.042	5.92	34.538	1.94	0.020*	2.81
4	Snus 4	0.129	10.76	36.524	6.13	0.130	12.82
5	Moist snuff 1	0.150	12.38	2.492	4.05	N.D.	-
6	Moist snuff 2	0.212	6.56	1.000	2.75	N.D.	-
7	Moist snuff 3	N.D.	-	0.480	1.81	N.D.	-
8	Moist snuff 4	0.229	2.28	1.415	3.46	N.D.	-
9	Moist snuff 5	0.600	3.37	19.351	2.36	0.058	3.86
10	Moist snuff 6	0.015*	4.34	2.387	0.72	0.075	2.82
11	FC L (1)	0.053	1.34	47.861	4.33	2.637	5.08
12	FC U (1)	0.002*	5.00	28.667	5.17	2.994	5.73
13	FC L (2)	0.031*	0.53	44.276	9.95	2.243	3.29
14	FC U (2)	0.016*	0.49	25.054	9.28	3.162	0.23
15	FC off L	0.029*	1.54	46.839	9.46	2.439	2.07
16	FC off U	N.D.	-	37.963	6.73	2.107	1.69
17	Bu L (1)	0.150	15.44	39.463	3.86	0.118	1.25
18	Bu U (1)	0.296	4.39	35.220	0.34	0.107	1.22
19	Bu L (2)	0.311	2.00	22.036	9.07	0.457	7.54
20	Bu U (2)	0.475	1.75	21.937	2.22	0.462	3.38
21	Bu off L	0.780	4.61	30.788	4.59	0.271	2.42
22	Bu off U	0.975	4.61	32.935	4.85	0.119	0.70
23	O Sa U	0.041	0.46	37.540	9.92	0.339	4.73
24	O Iz U	N.D.	-	27.411	9.02	1.383	3.99
25	Commercial cigarette	0.092	9.38	31.812	9.07	0.926	9.34

* NOTE: Below the lowest standard.

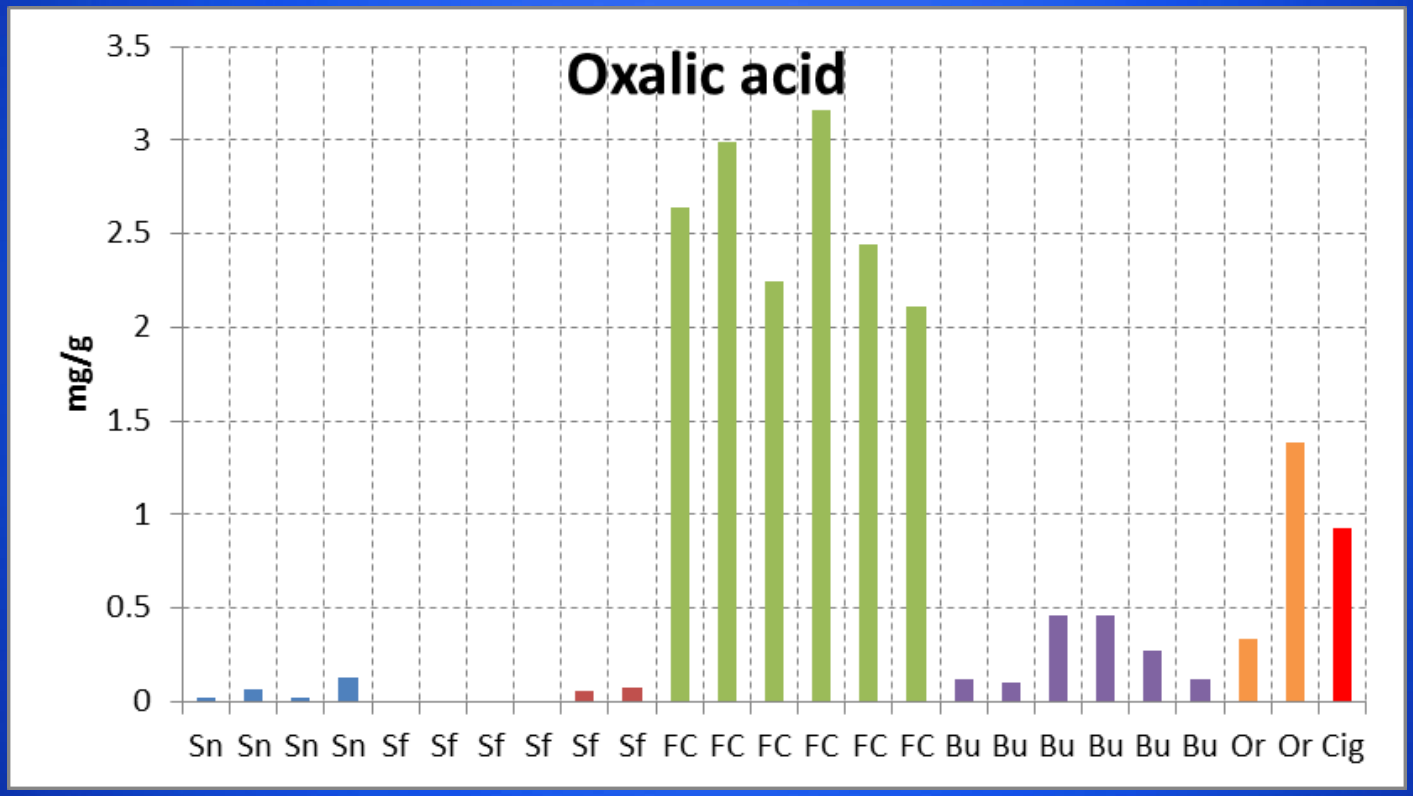
Results of levels of organic acids in mg/g



Results of levels of organic acids in mg/g



Results of levels of organic acids in mg/g

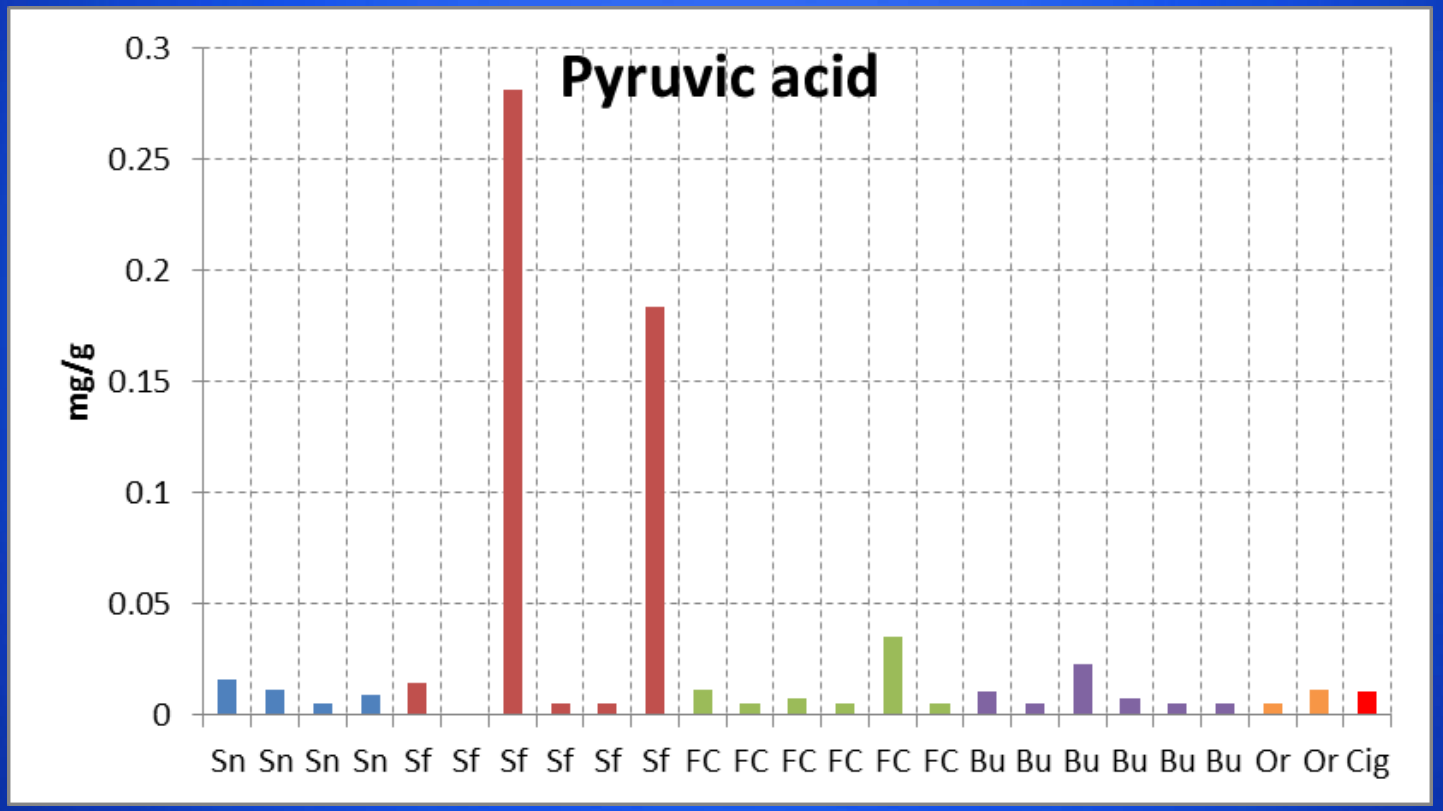


Results of levels of organic acids in mg/g

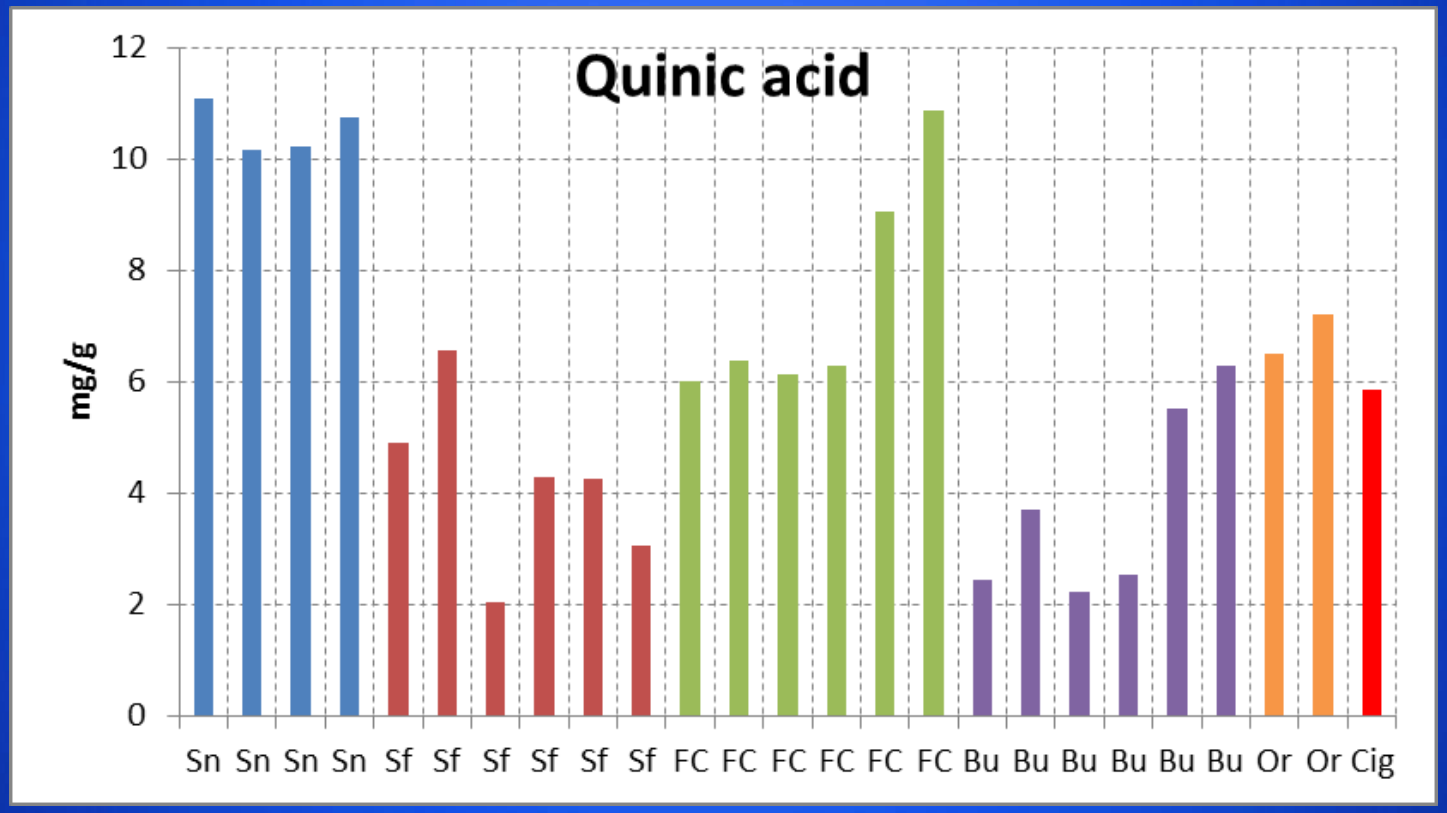
No.	Sample	*Pyruvic mg/g*	*RSD%*	*Quinic mg/g*	*RSD%*	*Trihydr. mg/g*	*RSD%*
1	Snus 1	0.016*	0.70	11.097	5.74	2.582	0.50
2	Snus 2	0.011*	5.50	10.168	5.04	3.042	11.09
3	Snus 3	0.005*	9.66	10.209	4.92	2.948	1.39
4	Snus 4	0.009*	14.46	10.754	1.07	2.349	12.62
5	Moist snuff 1	0.014*	5.22	4.911	5.12	0.274	6.41
6	Moist snuff 2	N.D.	-	6.573	2.00	N.D.	-
7	Moist snuff 3	0.281	0.43	2.028	4.85	0.275	0.63
8	Moist snuff 4	0.005*	3.89	4.302	2.33	0.274	1.89
9	Moist snuff 5	0.005*	2.99	4.246	4.09	0.645	7.11
10	Moist snuff 6	0.183	2.98	3.054	1.58	0.282	0.16
11	FC L (1)	0.011*	0.92	6.014	6.23	1.527	4.24
12	FC U (1)	0.005*	12.62	6.391	4.55	1.816	10.03
13	FC L (2)	0.007*	0.25	6.134	11.62	1.653	0.01
14	FC U (2)	0.005*	0.21	6.273	1.21	1.668	0.51
15	FC off L	0.035	1.30	9.057	12.05	3.049	1.76
16	FC off U	0.005*	2.36	10.858	13.16	2.536	4.56
17	Bu L (1)	0.010*	12.45	2.437	0.03	0.693	9.04
18	Bu U (1)	0.005*	17.03	3.695	1.59	0.890	1.08
19	Bu L (2)	0.023*	1.74	2.231	8.00	0.512	1.98
20	Bu U (2)	0.007*	3.89	2.546	10.19	0.521	3.38
21	Bu off L	0.005*	7.62	5.510	12.33	1.992	7.09
22	Bu off U	0.005*	12.72	6.284	12.01	1.935	9.07
23	O Sa U	0.005*	5.01	6.496	5.77	3.563	9.02
24	O Iz U	0.011*	1.43	7.207	7.44	3.109	2.69
25	Commercial cigarette	0.010*	0.25	5.870	3.39	2.342	6.04

* NOTE: Below the lowest standard.

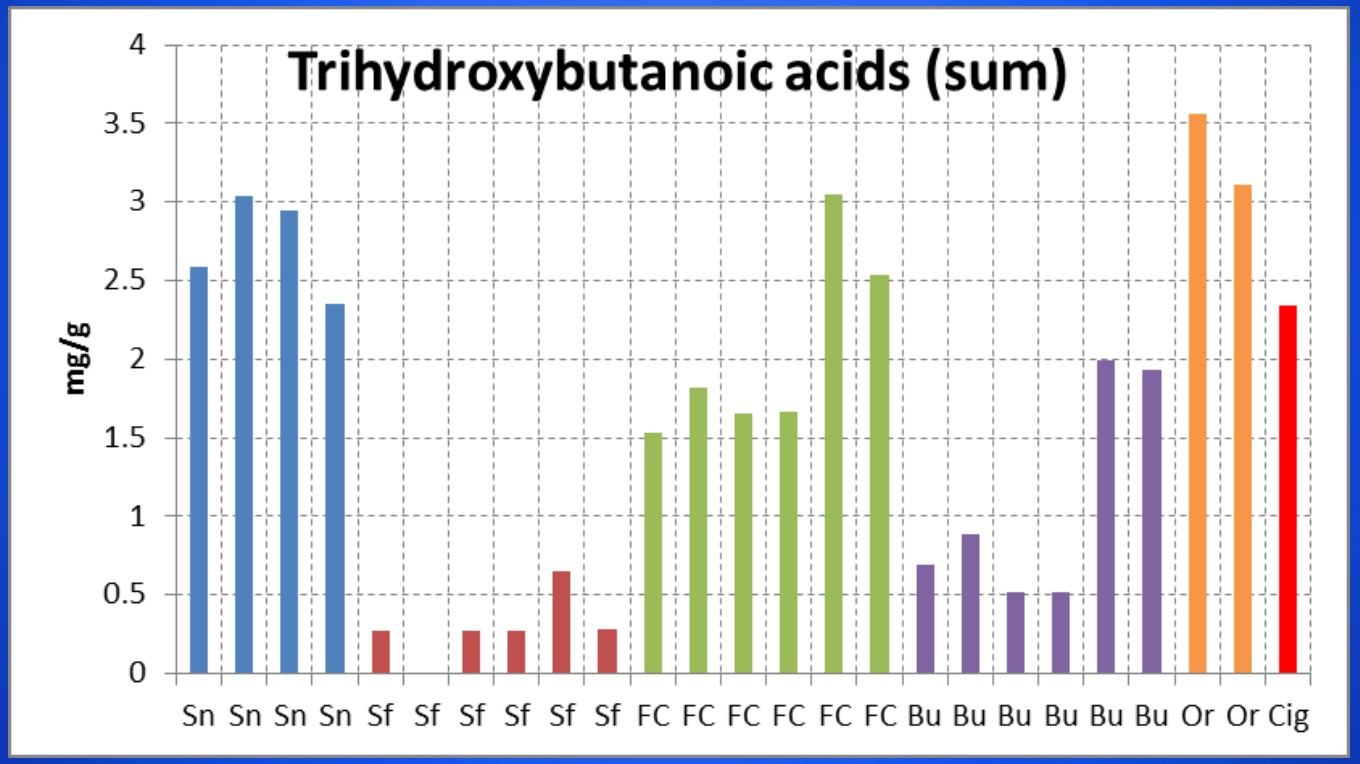
Results of levels of organic acids in mg/g



Results of levels of organic acids in mg/g



Results of levels of organic acids in mg/g



Conclusions

- A qualitative GC-MS and a quantitative LC-MS method have been utilized for the analysis of several common organic acids in tobacco, snus, and moist snuff.
- The GC-MS method, which has been successfully used for qualitative analysis of tobacco for almost 30 years, indicated the presence of about 24 acids in tobacco.
- Nine such acids that were at three different levels (%, mg, and μg) were selected for quantitation using a novel LC-MS method.
- The LC-MS method has been validated and provides reliable results for the quantitation.
- Specific patterns were seen for the variation of the analyzed organic acids in the different types of samples.