Analysis of sugars and myo-inositol in tobacco with a new LC/MS/MS procedure

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Background

- The levels of sugars (non-polymeric carbohydrates) in tobacco are important for leaf characterization.
- Analyses of sugars such as glucose, fructose, and sucrose is typically performed using HPLC.
- Sugar molecules do not have chromophors and the detection is performed with a refractive index detector or a pulsed amperometric detector (PAD), isocratic separation, and identification based only on retention time [1-3].

1. W. Ellefson, *Curr. Protocols. Food Anal. Chem.,* 2002, E1.2.1-E1.2.9.2
2. M. Salman, M. T. Alghamdi, S. A. Bazaid, E. S. Abdel-Hameed, *Arch. Appl. Sci. Res.,* 3 (6) (2011) 488-496.
3. Thermo Scientific, *Dionex Application Brief* 127.

- A more recent LC/MS measurement in APCI mode of sugars requires a post column solvent addition.

4. H. Kumaguai, Agilent Application note 5988-4236EN, 2001.

Problems and solutions regarding MS analysis of carbohydrates

- Carbohydrates have low propensity to form ions in either positive or negative mode.
- Carbohydrates have the tendency to form clusters with alkaline ions such as Na^+, K^+, and with NH_4^+.
- Detection of small molecular ions such as Na^+ (atomic weight 23) or K^+ (atomic weight 39) is difficult using LC/MS/MS instruments.
- Cs^+ also forms clusters with carbohydrates and has the atomic weight 133.

Method description (sample extraction)

- A ground tobacco sample of 100 mg is weighed with precision of 0.1 mg and placed in a 50 mL vial.
- To this sample, 25 mL of water is added and the sample is extracted for 30 min on a wrist action shaker.
- A portion of the resulting extract is filtered through a 0.45 μm PVDF filter and 1 mL placed in a 2 mL vial for analysis. The samples are stable for at least 48 hours at 15 ^{o}C.
- To this solution are added 25 μL of a solution containing 2 mg/mL rhamnose (rhamnose is not present, or is at low trace level in tobacco).

Method description (HPLC separation)

- The column was a HILIC type YMC-Pack Polyamine II (YMC America, Inc., Allentown, PA, USA).
- 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 isocratic conditions and the mobile phase was 67% CH_3CN and 33% H_2O that has 80 mM $Cs(CH_3COO)$.
- The flow rate through the column was 1 mL/min. However, this flow is too high for the MS/MS detection and a split flow was used, such as only 250 μL/min flow was sent to the MS/MS detector, while 750 μL/min flow went to waste.
- The injection volume for the analysis was 5 μL.

Method description (MS/MS parameters)

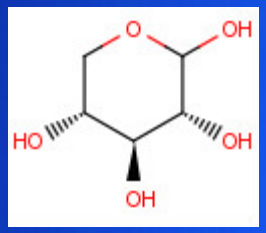
- API-5000 triple quadrupole mass spectrometer (AB Sciex, Framingham, MA, USA), working in multiple reaction monitoring (MRM) mode with the following parameters:

Parameter	Value
Polarity	Positive
Collision gas (CAD)	10 mL/min
Curtain gas (CUR)	40 mL/min
Ion source gas 1 (GS1)	45 mL/min
Ion source gas 2 (GS2)	45 mL/min
Ion spray voltage (IS)	5000 V
Temperature (TEM)	95 °C
Interface heater (Ihe)	on
Declustering potential (DP)	27.0 V
Entrance potential (EP)	10.0 V
Collision energy (CE)	15.0 V
Collision cell exit potential (CXP)	11.0 V
Acquisition time per ion	100 ms
Total acquisition time	20 min

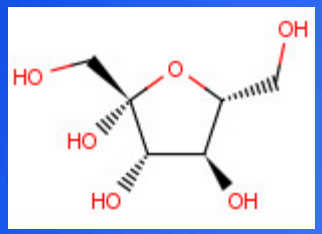
Method description (ions used for measurement)

No.	Compound	Precursor ion	Product ion
1	C5 sugars	283.0	133.0
2	Rhamnose	297.0	133.0
3	C6 sugars and inositol	313.2	133.0
4	C6 sugar alcohols	315.2	133.0
5	Disaccaharides (2 x C6)	475.2	133.0
6	Sucralose	529.0	133.0

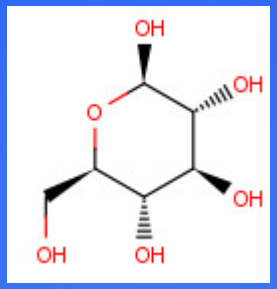
Compounds subject to quantitative analysis



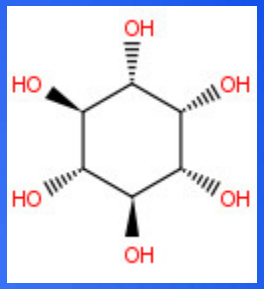
Xylose



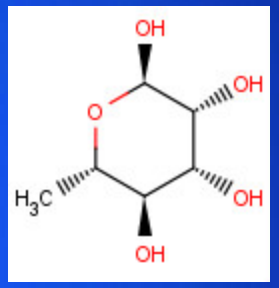
Fructose



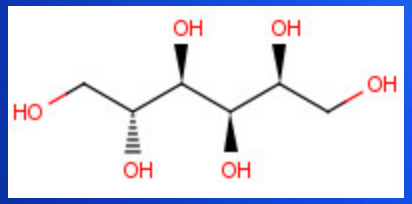
Glucose



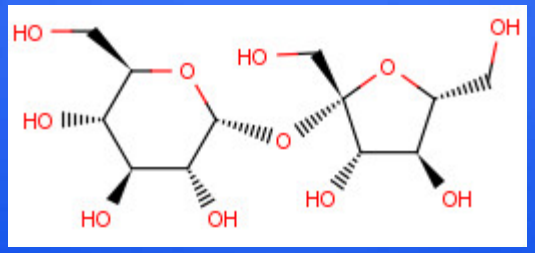
Myo-inositol



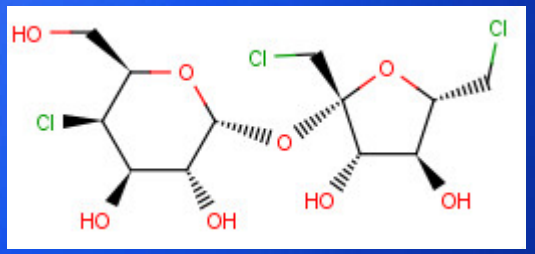
Rhamnose (I.S.)



Sorbitol



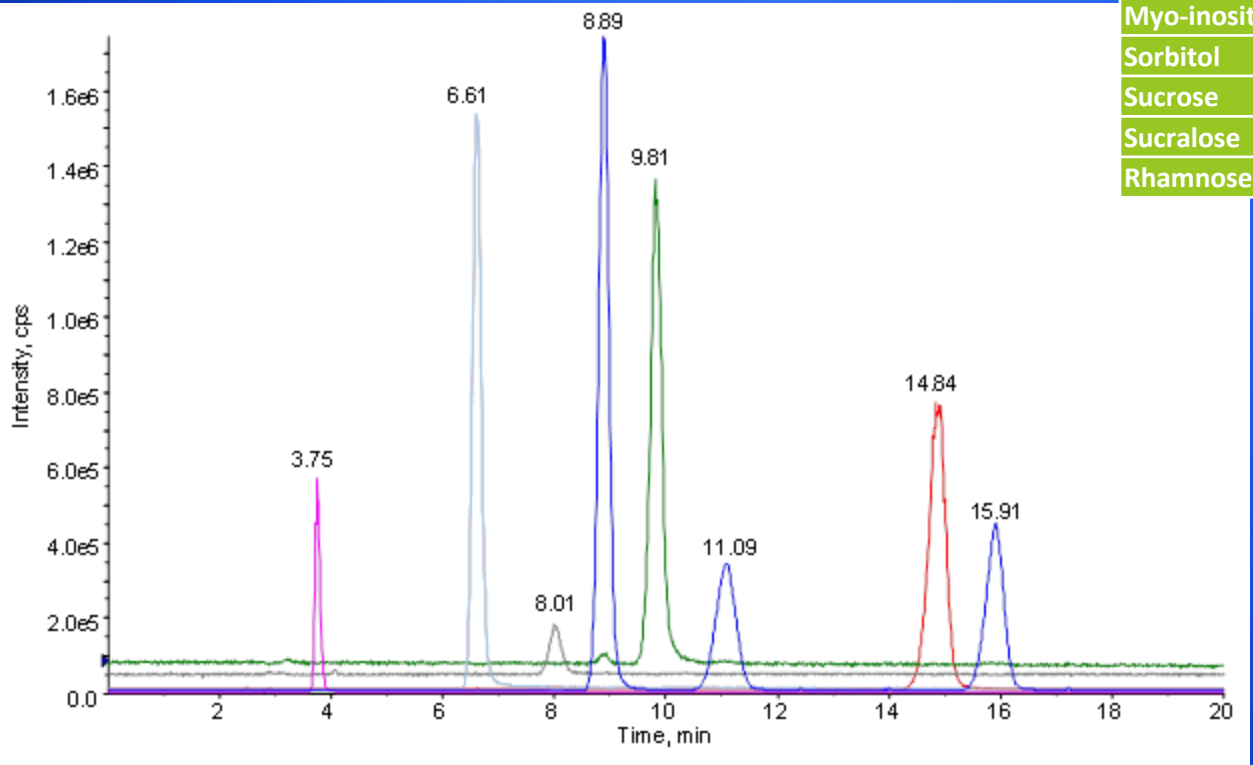
Sucrose



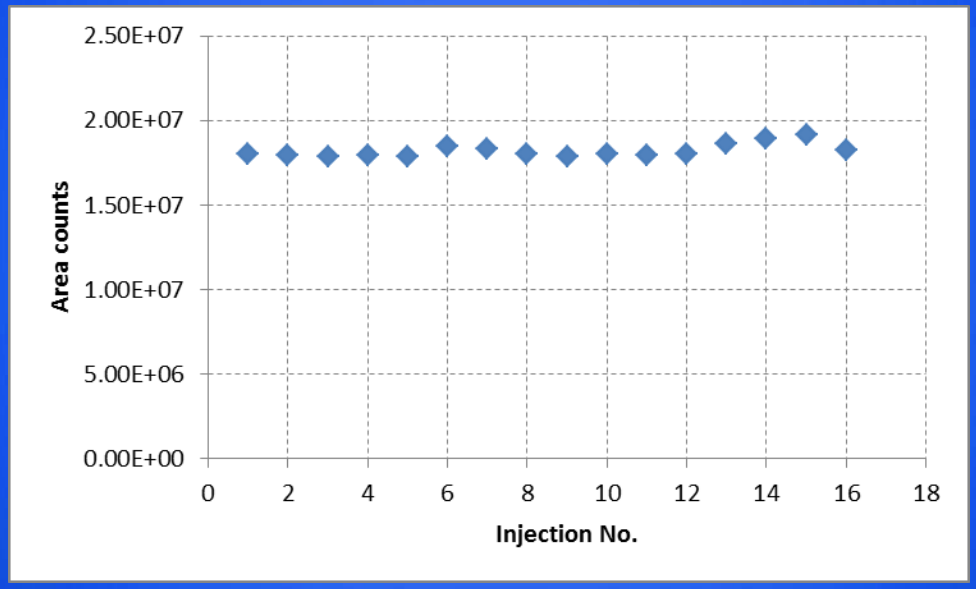
Sucralose

Example chromatogram for a set of standards

Compound	Ret. time min	Conc. µg/mL
Xylose	8.01	12.5
Fructose	8.89	75.0
Glucose	11.09	37.5
Myo-inositol	15.91	37.5
Sorbitol	9.81	37.5
Sucrose	14.84	37.5
Sucralose	3.75	12.5
Rhamnose (I.S.)	6.61	50

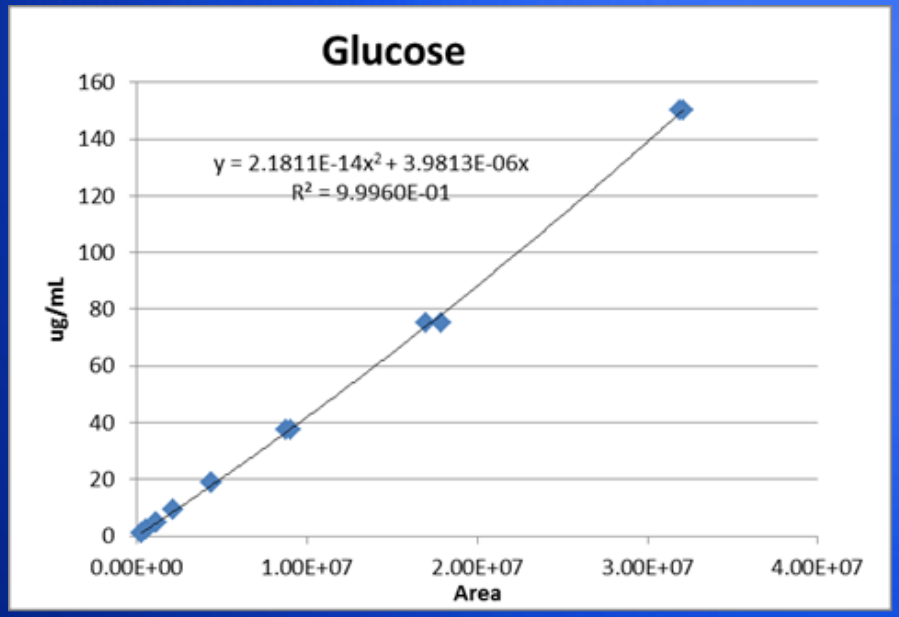


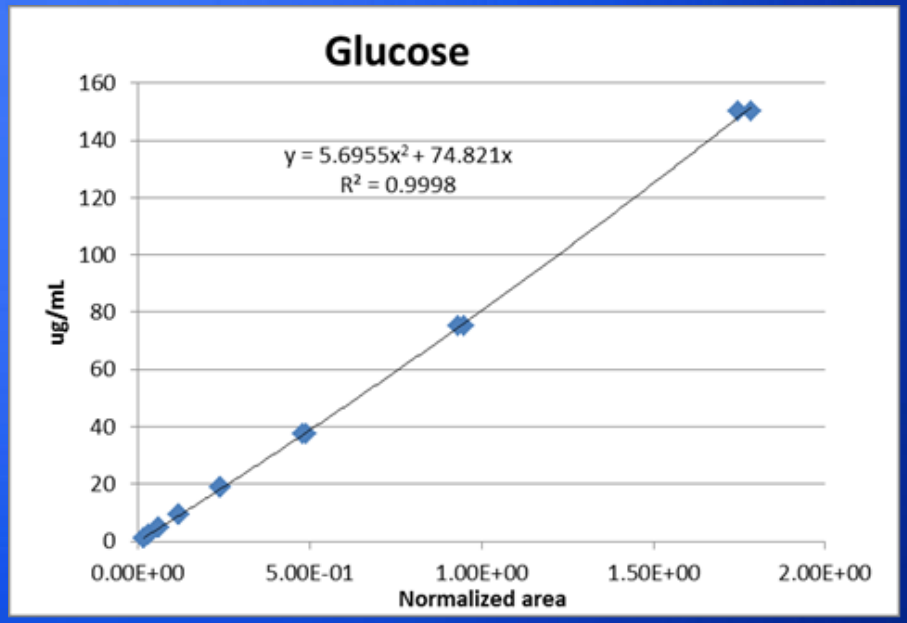
Variation in time of the peak area counts for the internal standard (rhamnose)



Quantitation of sugars

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

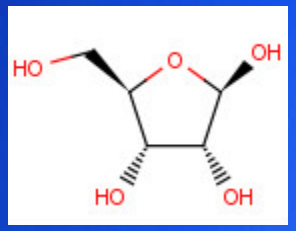




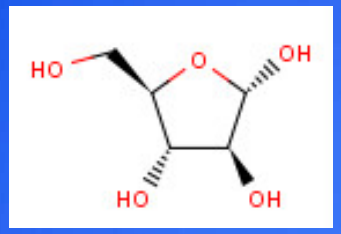
Equations for the quantitation of several analyzed compounds.

Compound	Conc. range	Eq. conc. vs. area	R^2	Eq. conc. vs. norm. area	R^2
Fructose	2.34 - 300 mg/mL	$y = 3.1851e\text{-}14x^2 + 2.2889e\text{-}6x$	0.9985	$y = 9.8229x^2 + 44.062x$	0.9993
Glucose	1.17 - 150 mg/mL	$y = 2.1811e\text{-}14x^2 + 3.9813e\text{-}6x$	0.9996	$y = 5.6955x^2 + 74.821x$	0.9998
Myo-inositol	1.17 - 150 mg/mL	$y = 2.8117e\text{-}14x^2 + 3.8556e\text{-}6x$	0.9995	$y = 8.0148x^2 + 63.753x$	0.9995
Sorbitol	1.17 - 150 mg/mL	$y = 2.0797e\text{-}14x^2 + 1.5151e\text{-}6x$	0.9988	$y = 6.4201x^2 + 28.882x$	0.9999
Sucrose	1.17 - 150 mg/mL	$y = 7.1834e\text{-}15x^2 + 2.1440e\text{-}6x$	1.0000	$y = 1.9364x^2 + 40.245x$	0.9995
Sucralose	0.39 - 50 mg/mL	$y = 3.5585e\text{-}14x^2 + 3.4924e\text{-}6x$	0.9997	$y = 8.9022x^2 + 65.332x$	0.9998
Xylose	0.39 - 50 mg/mL	$y = 1.1207e\text{-}13x^2 + 5.7044e\text{-}6x$	0.9975	$y = 29.48x^2 + 106.81x$	0.9992

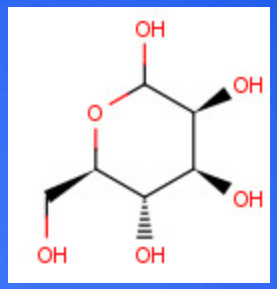
Other compounds that can be analyzed by this LC/MS/MS method



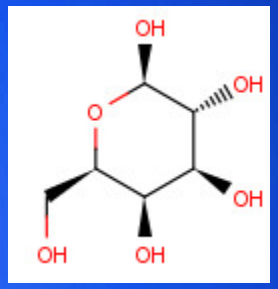
ribose



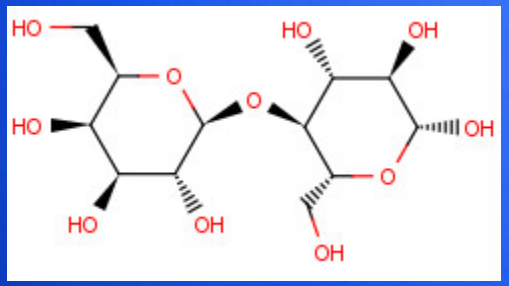
arabinose



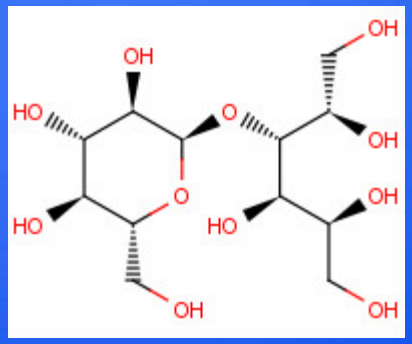
mannose



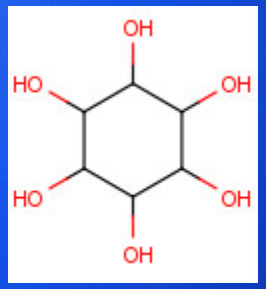
galactose



lactose

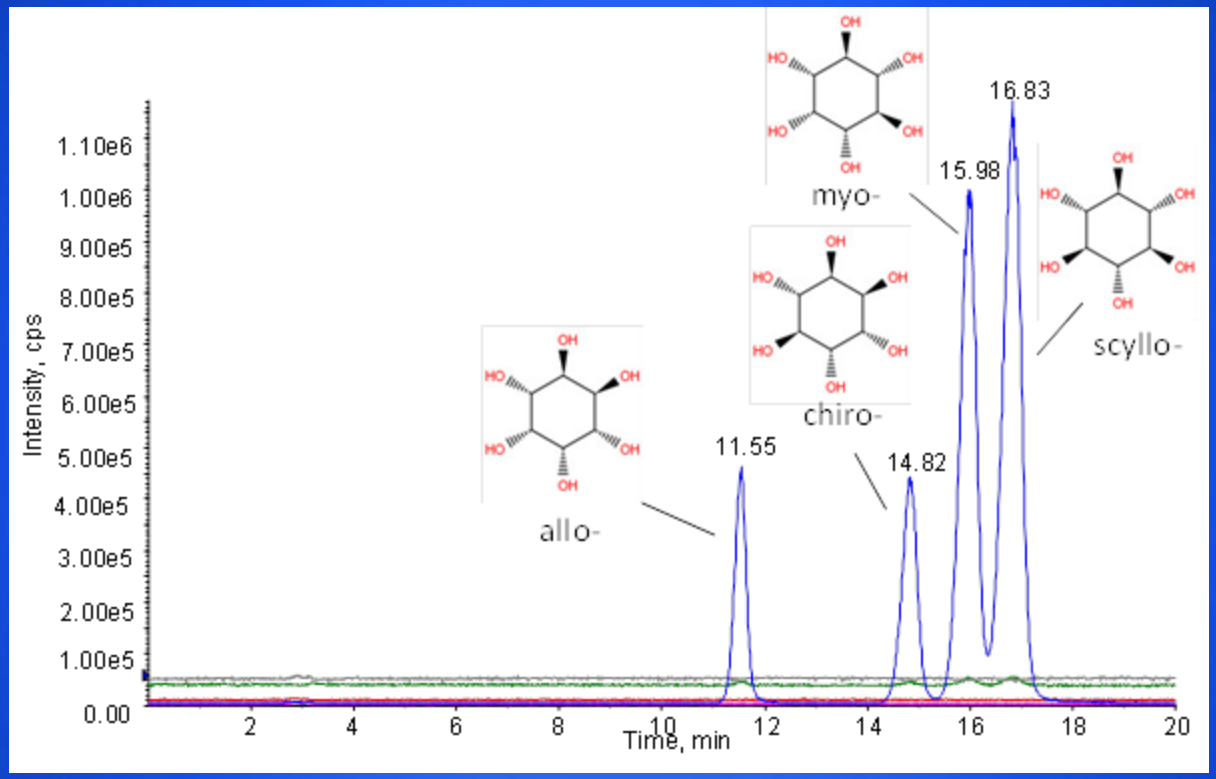


maltitol



various inositols

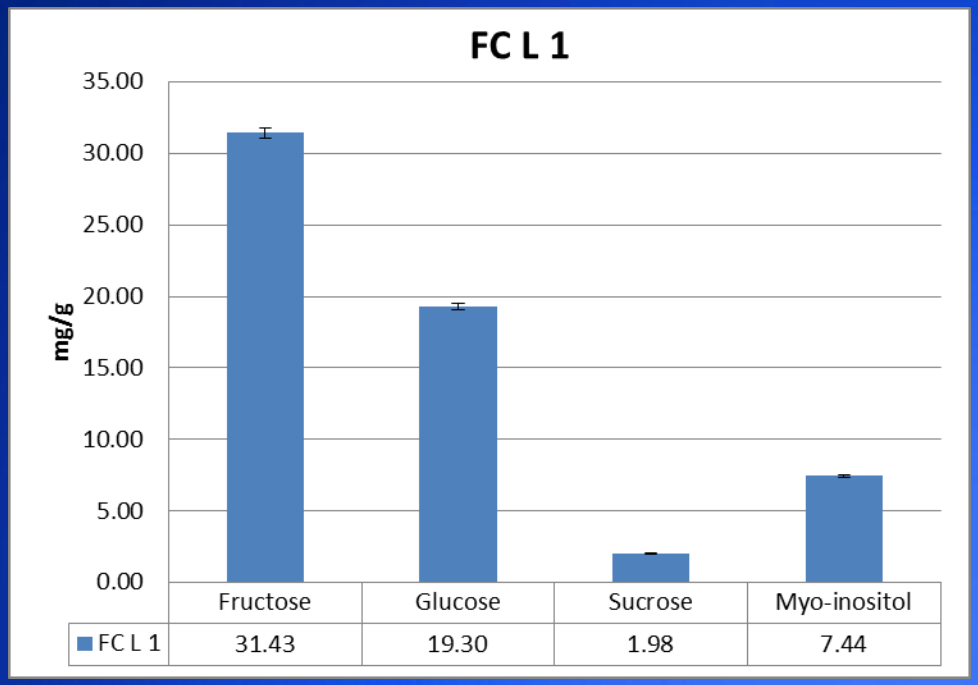
Separation of four inositols

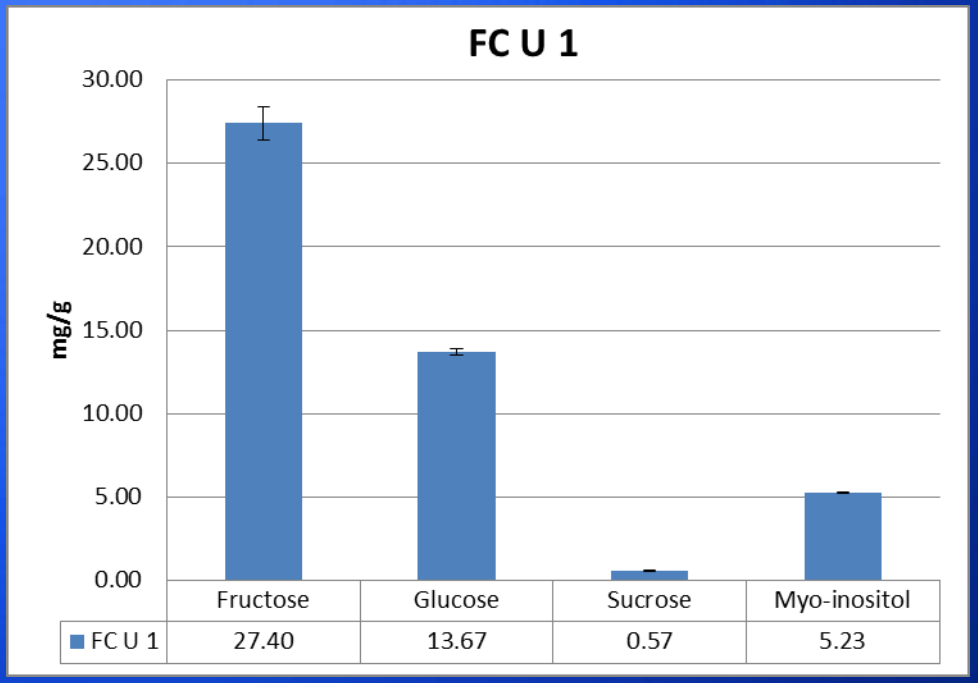


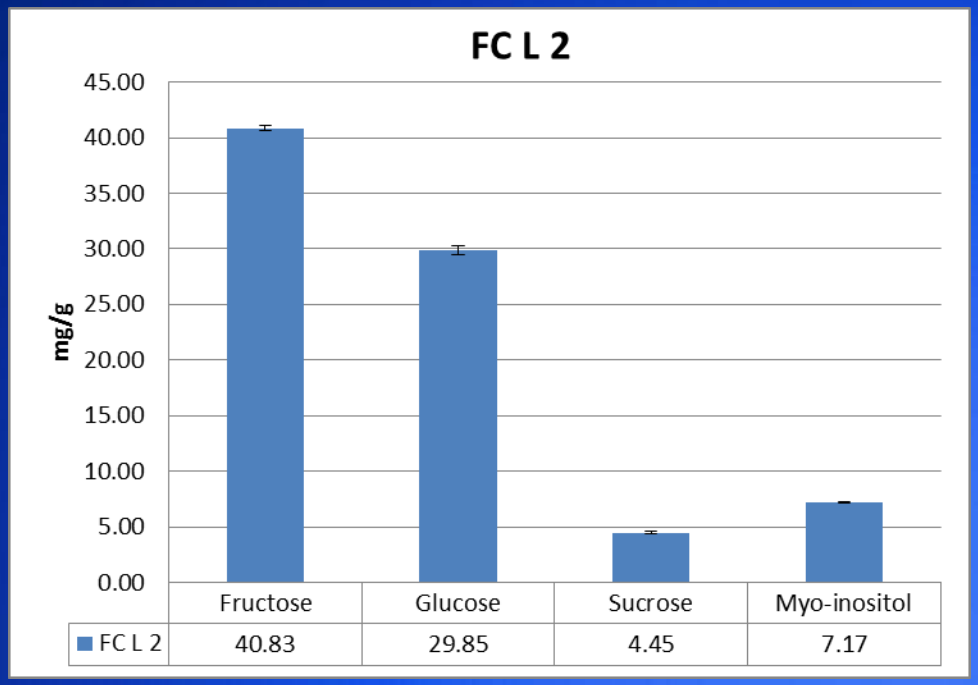
(Standards at 75 μg/mL).

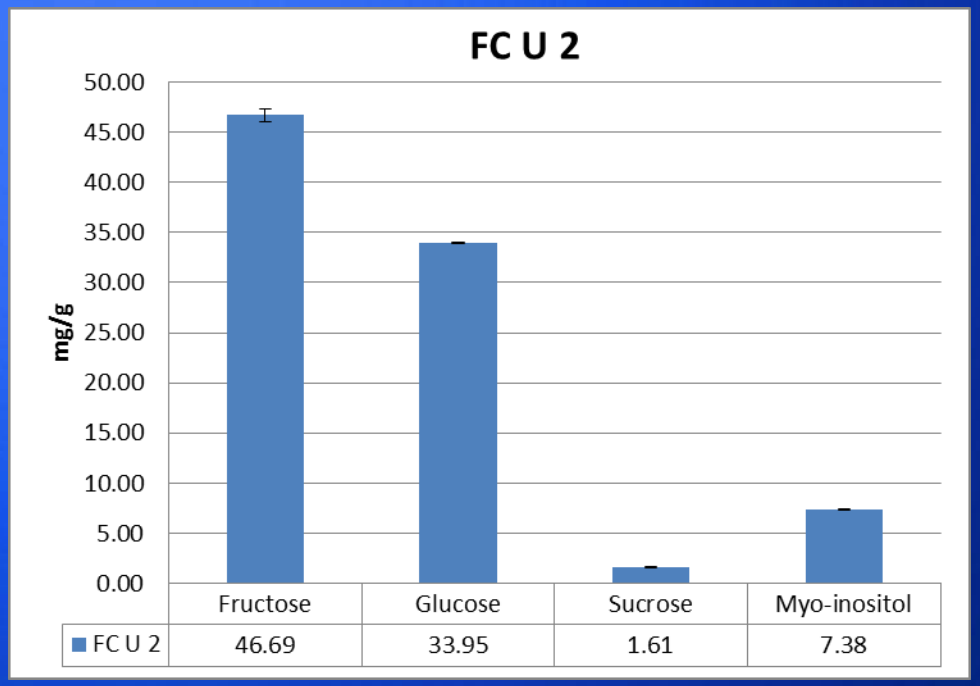
Tobacco analyzed by the LC/MS/MS method

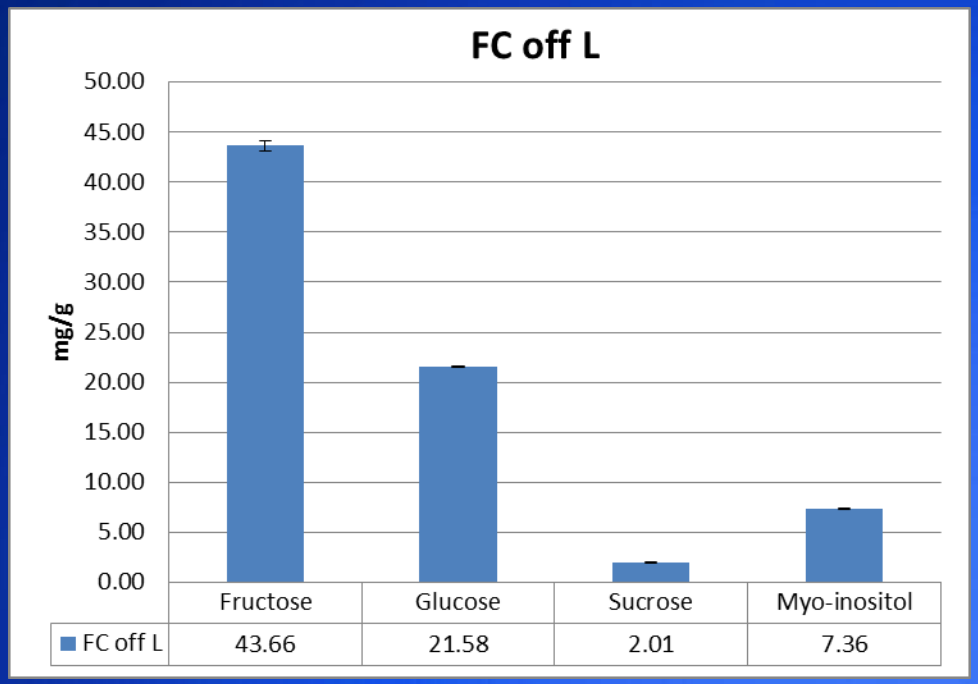
No	Tobacco type	Year	Description	Curing
Tob.1	FC L c (1)	2008	Eastern NC belt, lower stalk (lug) flue-cured	cured
Tob.2	FC U c (1)	2008	Eastern NC belt, upper stalk (leaf & some tips) flue-cured	cured
Tob.3	FC L c (2)	2009	South Carolina belt, lower stalk (lug) flue-cured	cured
Tob.4	FC U c (2)	2009	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

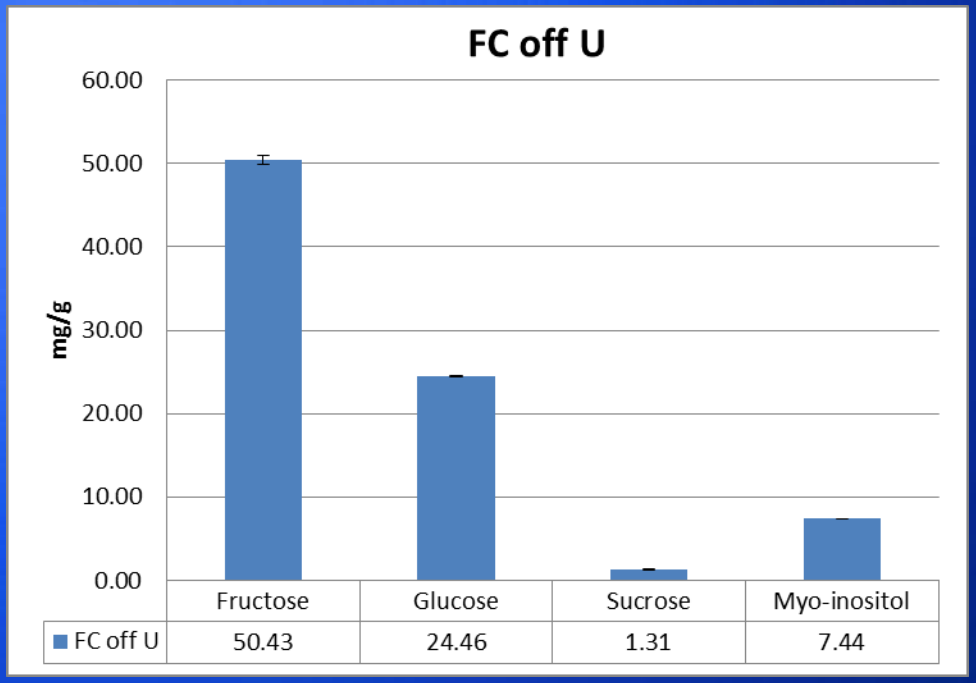


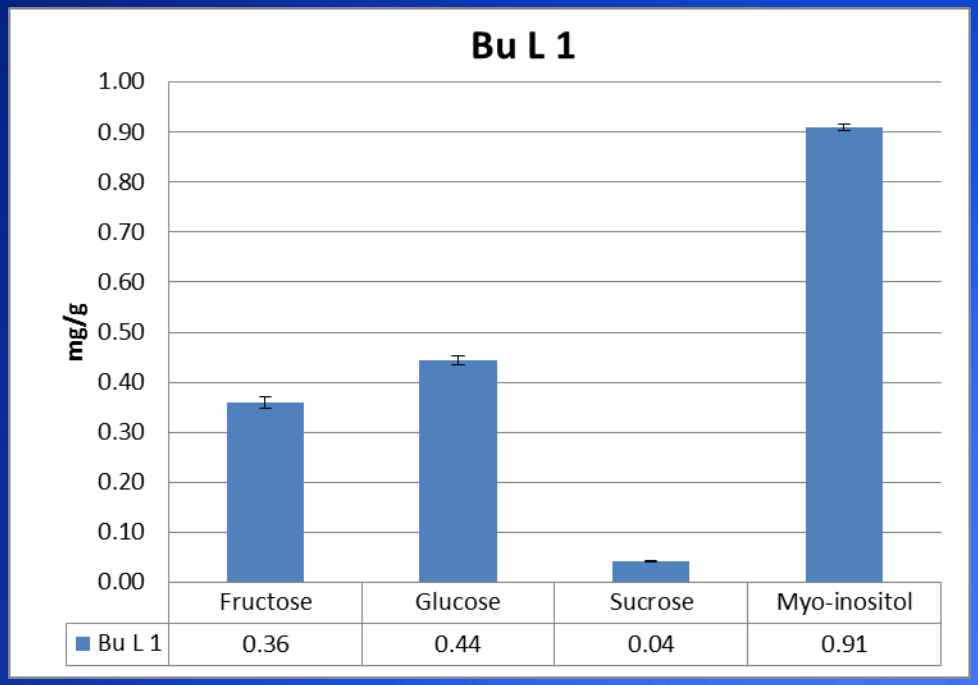


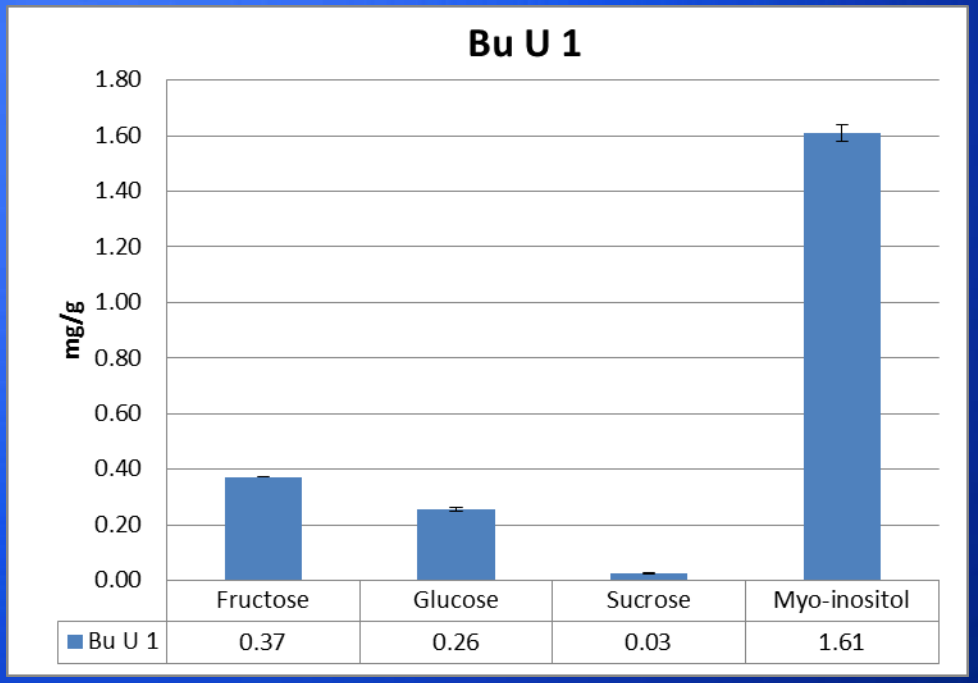


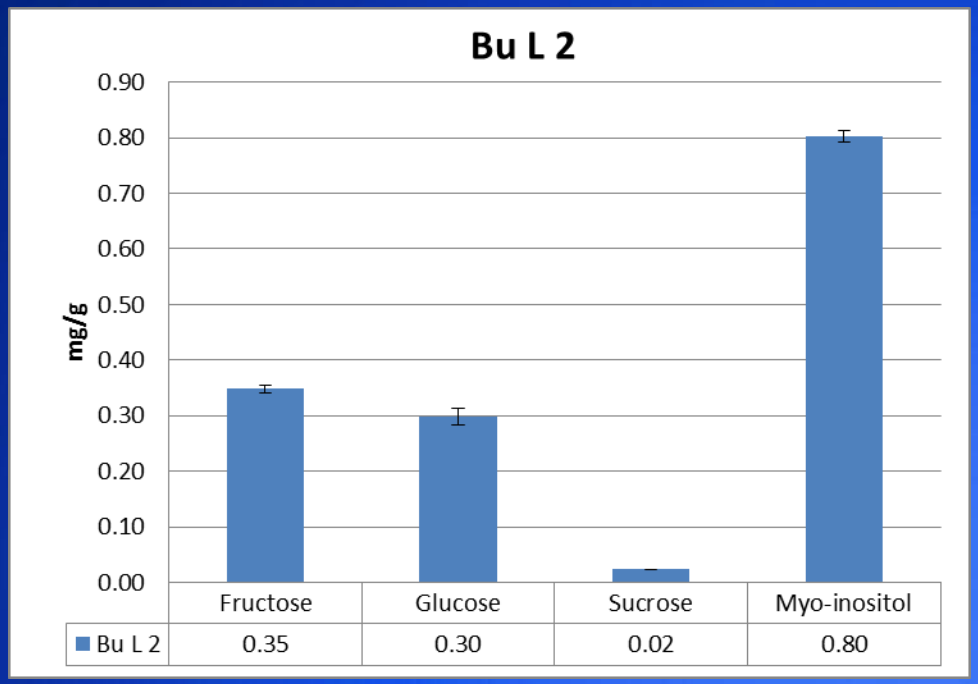


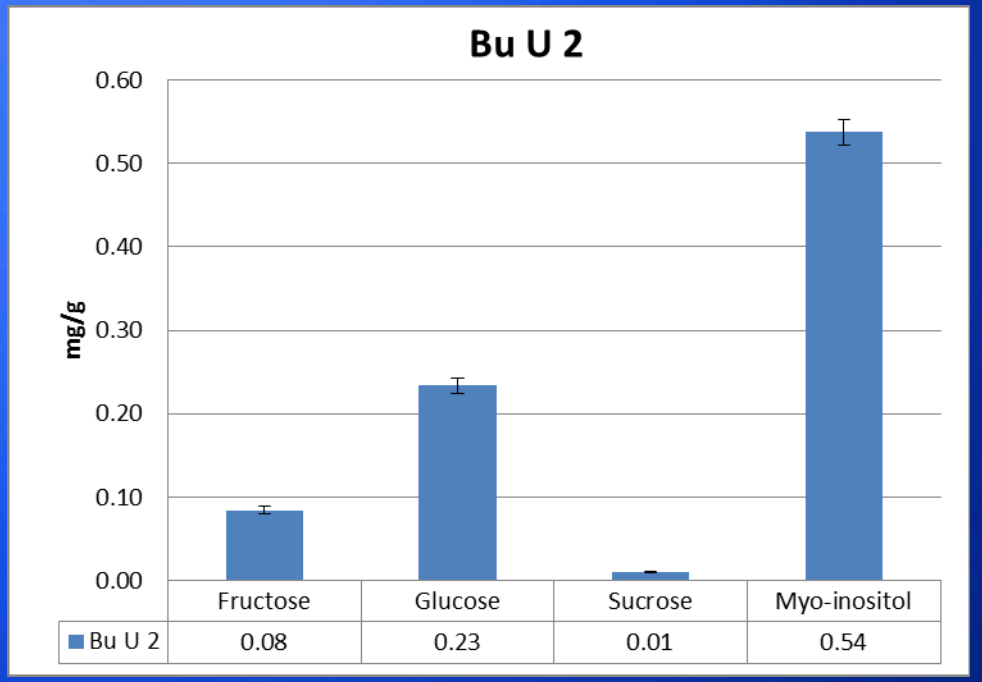


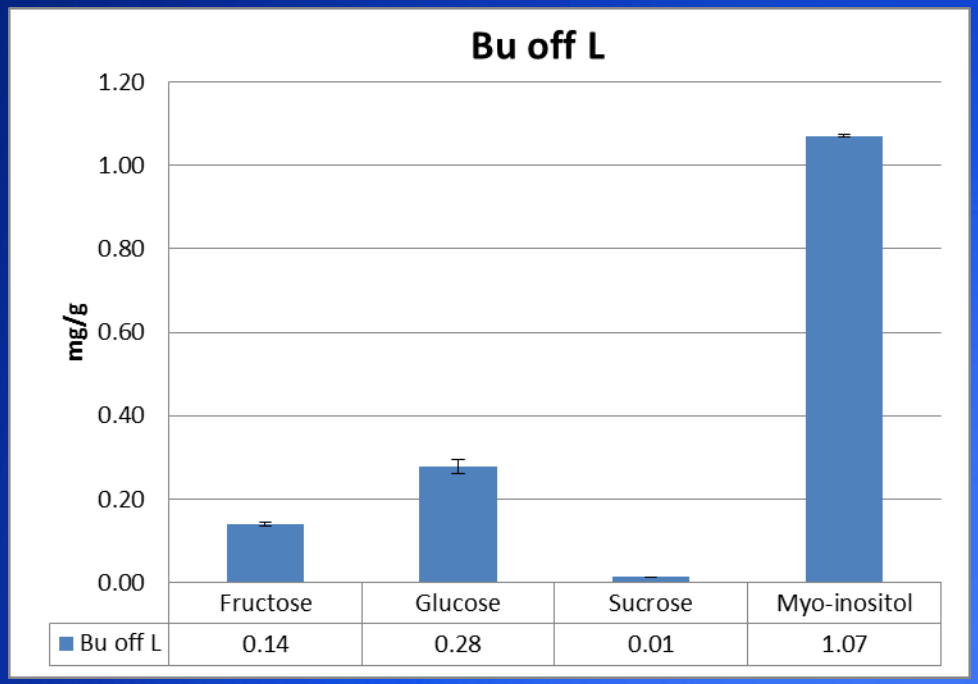


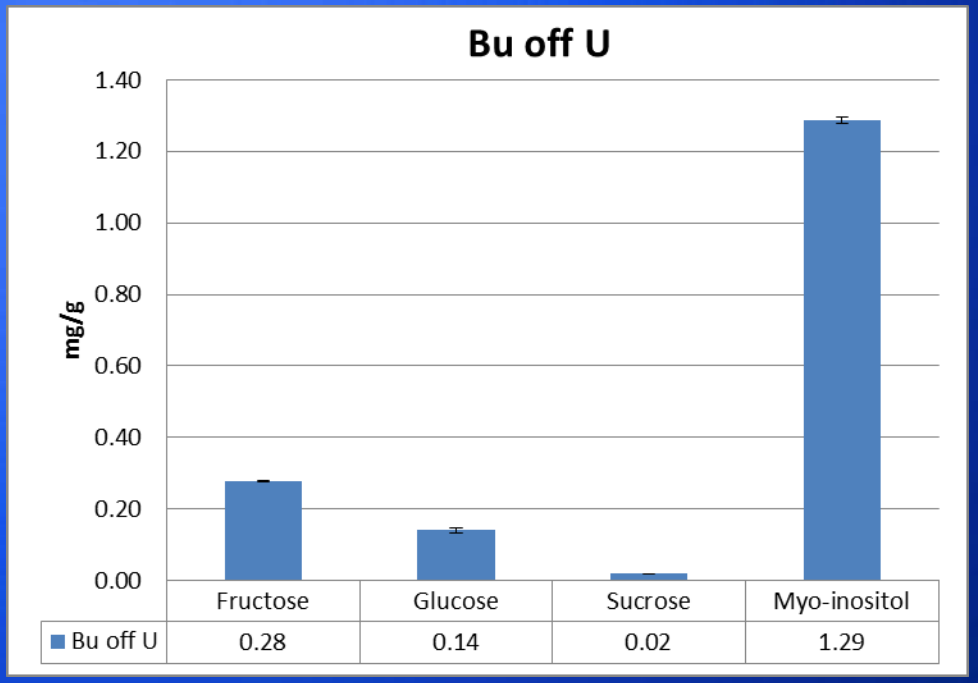


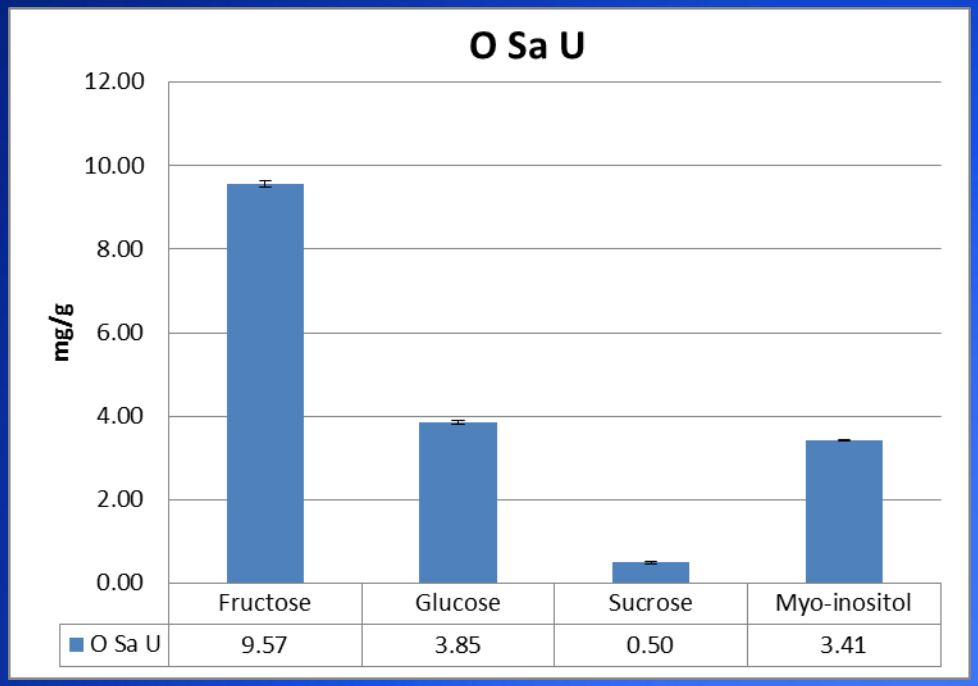


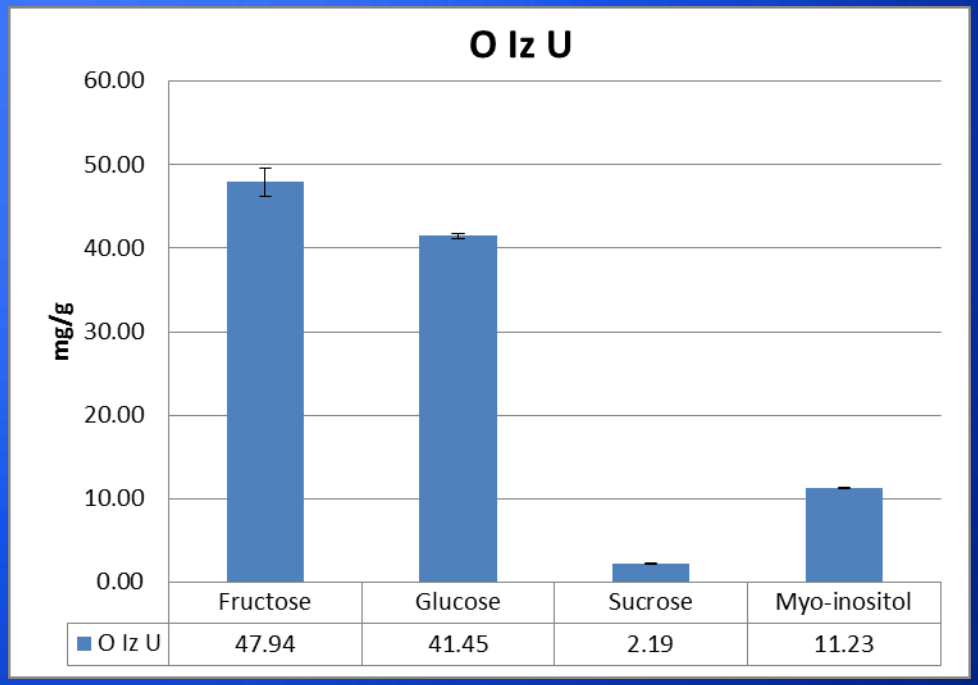


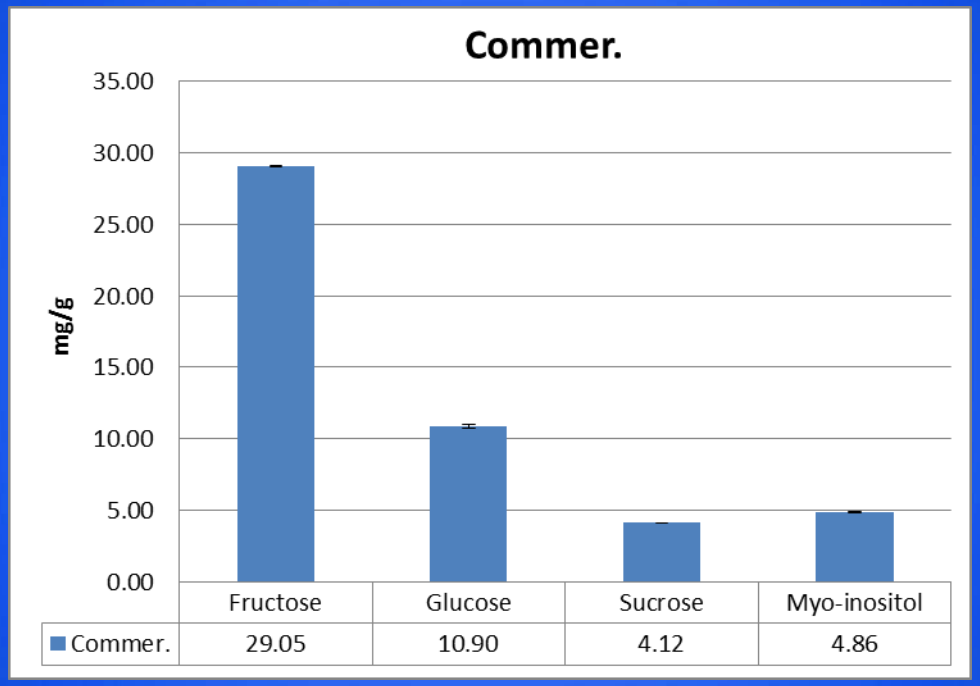












Conclusions (on the method)

- A new method for the analysis of several mono-sachharides, myo-inositol, sorbitol, sucrose, and sucralose using MS/MS detection has been developed.
- The method can be extended for the analysis of other carbohydrates.
- The separation is performed on a HILIC type column, YMC-Pack Polyamine II (YMC America, Inc., Allentown, PA, USA) with isocratic separation.
- The method has very good precision, sensitivity, and accuracy, and allows positive identification.
- The method can be successfully used for the analysis of tobacco samples.

Conclusions (on the tobaccos)

- The “profile” of sugars and even their actual levels do not differ too much from the lower stalk (lug) and upper stalk (leaf & some tips) of the plant.
- The burley tobaccos, as expected, are significantly lower in sugars and myo-inositol compared to the flue cured tobaccos.
- Differences that can be considered significant can be seen among the tobaccos of the same type but cultivated in different regions.
- The two Oriental tobaccos were very different regarding the level of sugars and myo-inositol.