



Routine Analytical Chemistry Sub-Group

Technical Report

2015 Collaborative Study Developing a CRM for the Determination of Total Sugars in Tobacco by Continuous Flow Analysis

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1. Introduction

The CORESTA Recommended Methods, CRM N° 37 - DETERMINATION OF REDUCING SUBSTANCES IN TOBACCO BY CONTINUOUS FLOW ANALYSIS and CRM N° 38 - DETERMINATION OF REDUCING CARBOHYDRATES IN TOBACCO BY CONTINUOUS FLOW ANALYSIS are available for the analysis of the respective tobacco parameters. There is no harmonised method available for the analysis of 'Total Sugars' in tobacco.

A survey within the Routine Analytical Chemistry Sub-Group (RAC) revealed several methods for carbohydrates and their equivalents.

Therefore, the CORESTA Scientific Commission approved a study project for the development of a harmonised CRM for the determination of 'Total Sugars' by continuous flow analyses (CFA) in tobacco and tobacco products coordinated within the RAC Sub-Group.

A wide range of laboratories are using the above-mentioned CRMs for reducing substances or reducing carbohydrates in parallel to the determination of 'Total Sugars' by in-house methods. The new harmonised CRM for 'Total Sugars' should preferably be set up so that it can be used in parallel to the analysis of reducing carbohydrates utilising the advantage of the efficient automated CFA. Therefore, the existing CRM N° 38, which determines reducing carbohydrates, was chosen as basis for the development of a method for 'Total Sugars' measurements. 'Total sugars' are defined as total carbohydrates determined in dependence on the reaction of p-hydroxybenzoic acid hydrazide (PAHBAH), in which sucrose, the predominant sugar beside fructose and glucose with an up-streamed hydrolysis step transferred into these two monosaccharides, which reacts with PAHBAH to produce a coloured complex, described as a hydrazine^[1,2].

2. Organisation

The RAC Sub-Group agreed on an inter-laboratory study to be carried out as a first step. The employed (currently used) in-house methods of the participating laboratories were identified for comparison and further evaluation against first reasonable set-ups of the method to be developed. Therefore, the study was based on CRM N° 38, where reducing carbohydrates are measured by the reaction with p-hydroxybenzoic acid hydrazide (PAHBAH). The necessary up-streamed hydrolysis step was evaluated by using two different concentrations of hydrochloric acid (0.1 Mol/L and 0.5 Mol/L HCl). Additionally, the extraction solution (water or acetic acid) was examined, because of a note given in the CRM N° 38 regarding hydrolysis of sucrose may occur for some tobaccos if extracted with distilled water. With the experiences and results out of this inter-laboratory study (Appendix B) a final study protocol (Appendix A) with the suggested harmonized method was prepared by the RAC Sub-Group.

2.1 Participants

In total eight laboratories participated in the final study of 2015 using the study protocol and the suggested method. The list of laboratories and the continuous-flow equipment used are shown below in Table 1.

Table 1: Participating laboratories and equipment

Laboratory name	Equipment
BAT, Porto Alegre, BRAZIL	SEAL Analytical, AA3
China Tobacco Test Centre, Henan, CHINA	SEAL Analytical, AA3
BAT, Bayreuth, GERMANY	SEAL Analytical, AA3
SEAL Analytical, GERMANY	SEAL Analytical, AA3
SEITA ITG, FRANCE	Skalar, SA 5000
KT&G, Taejeon, KOREA	SEAL Analytical, AA3
BAT, Kuala Lumpur, MALAYSIA	SEAL Analytical, AA3
BAT, Stellenbosch, SOUTH AFRICA	Skalar, San++ plus System

2.2 Protocol

Eight samples were included into the study protocol, in which five tobacco samples were prepared and distributed by BAT Brazil. The CORESTA reference product CRP4, tobacco from the Kentucky Reference cigarette 3R4F and the CORESTA Monitor Test Piece CM8 were supplied by the participating laboratories from their own supply. Participants were asked to follow the supplied protocol (Appendix A). Sample descriptions and nominal levels of the analyte in question are given in Table 2.

Table 2: Sample description

Sample Name	Nominal levels (Total Sugars [%])
Burley (LLBY)	0.6
Virginia (low level) (LLVA)	3.5
Virginia (high level) (HLVA)	14
Cut Rag / Cig Blend (CIGB)	10
Cut Rag / RYO (RYOB)	8
CRP4	29*
3R4F	9
CM7	12

* CORESTA Guide N°15

2.3 Data Sets

The laboratories provided 130 data sets for the allocated tobacco, smokeless tobacco samples and an additional high sugar level flue-cured tobacco sample. While all 8 laboratories could provide 80 data sets (full data set) of the five tobacco samples, only four laboratories provided the full data set for the smokeless tobacco product. Laboratories not experienced in analysing smokeless tobacco noted difficulties in handling this tobacco material. With no explanation, even less results were provided for the two monitor test pieces. One laboratory substituted results for a high-level flue-cured tobacco sample results analysed by using 1.0 Molar HCL for the hydrolysis. Data packages overview as shown in Table 3.

Table 3: Number of laboratories providing full data sets by tobacco sample

Sample	Water Extracts	5 % Acetic Acid Extracts
Virginia (low level)	8	8
Virginia (high level)	8	8
Burley	8	8
Cut Rag / Cig Blend	8	8
Cut Rag / ROY	8	8
CRP4	4	4
3R4F	6	6
CM7	4	4
1R5F (not requested)	1	1
Flue-cured (high level) (not requested)	1	1

3. Raw data

The original data of the 8 laboratories and the statistical raw data values of the study results can be found in Appendix C. The data provided by one lab in addition to the protocol can also be found in Appendix C.

4. Statistical Analysis

The final statistical evaluation was carried out only for the five different levels of tobacco samples (120 data sets) and for the two different extraction versions (water / 5 % acetic acid; 5 % hydrogen acetate (HAC)), because no full data sets were available for the monitor test pieces and the smokeless tobacco product.

The statistical evaluation of data for this collaborative study followed the methods provided by ISO 5725-2^[3].

Estimation of h and k index to verify possible outlier values

$$h = \frac{d}{s_{\bar{x}}}$$

d : cell deviation $\bar{x} - \bar{\bar{x}}$

$s_{\bar{x}}$: standard deviation of cell averages $\sqrt{\sum_{i=1}^p d_i^2 / (p - 1)}$

$$k = \frac{s}{s_r}$$

s : cell standard deviation $\sqrt{\sum_{i=1}^n (x_i - \bar{x})^2 / (n - 1)}$

s_r : repeatability standard deviation $\sqrt{\sum_{i=1}^p s_i^2 / p}$

Estimation of repeatability (r) and reproducibility (R)

$$r = 2.8 * s_r$$

s_r : repeatability standard deviation

$$R = 2.8 * s_R$$

s_R : reproducibility standard deviation $\sqrt{(s_{\bar{x}})^2 + (s_r)^2(n - 1)/n}$

4.1 Mandel's h and k

Mandel's h and k statistics were calculated on the data of the final study of 2015. Only one outlier for Mandel's h (lab code 4) and two outliers for Mandel's k (lab code 6) for the extraction with acetic acid and three outliers for Mandel's k for the extraction with water were confirmed for the data. Group Mandel's h and k statistics were calculated to estimate h and k index to verify possible outlier values. h index checks the between laboratory data consistency, large h values (either positive or negative) indicate less agreement of mean values in comparison with other laboratories. k index checks the within laboratory data consistency, large k values indicate poorer repeatability in comparison with the other laboratories.

4.2 Mandel's h and k Critical Values and Detected Outliers

Table 4: Results of Mandel's h and k critical values

Laboratories (n)	Index	Critical Value
8	<i>h</i>	2.15
	<i>k</i>	2.06
9*	<i>h</i>	2.23
	<i>k</i>	2.09

* (one laboratory reported 2 trials for the cut rag/cig blend)

Table 5: Mandel's h and k outliers for the determination with 5 % acetic acid extraction

Sample	h Laboratory code	k Laboratory code
Virginia (low level)	/	/
Virginia (high level)	/	/
Burley	4	6
Cut Rag / Cig Blend	/	/
Cut Rag / ROY	/	6

Table 6: Mandel's h and k outliers for the determination with water extraction

Sample	h Laboratory code	k Laboratory code
Virginia (low level)	/	/
Virginia (high level)	/	4
Burley	/	/
Cut Rag / Cig Blend	/	4
Cut Rag / ROY	/	4

In Appendix C the Mandel's h and k data can be found. The charts can be found in Appendix D.

4.3 Repeatability and Reproducibility Estimation

The R & r values were calculated on the whole data sets (no outlier removal), because of the low number of final participating laboratories (n=8).

Table 7 and 8 show the r and R figures calculated for both extraction methods, 5 % HAC (Table 7) and water (Table 8).

Table 7: r & R results for the extraction with 5 % HAC

Sample	Mean [%]	r	R
Virginia (Low Level)	2.50	0.21	0.63
Virginia (High Level)	12.49	0.89	3.28
Burley	0.20	0.10	0.24
Cut Rag / Cig Blend	7.48	0.31	1.81
Cut Rag / ROY	5.33	0.29	1.65

Table 8: r & R Results for the extraction with water

Sample	Mean [%]	r	R
Virginia (Low Level)	2.64	0.26	1.00
Virginia (High Level)	12.65	0.85	3.37
Burley	0.25	0.14	0.31
Cut Rag / Cig Blend	7.68	0.47	2.38
Cut Rag / ROY	5.49	0.39	2.06

4.4 Comparison of 2015 Study to CRM N° 38 r & R Data

To further evaluate the data acquired in this study, r and R were also compared to CRM N° 38. The corresponding international study involved 11 laboratories and 4 samples and was conducted during 2006. Tables 9 and 10 list the r and R values of CRM Changed to CRM N° 38 and the 2015 study. The following graphs illustrate the comparison of repeatability (r), which refers to the variability within a laboratory and reproducibility (R), which refers to the variability of results between laboratories, found in this study against those in the published CRM N° 38.

Table 9: r and R of reducing carbohydrates from CRM N° 38, Collaborative Study 2006

Tobacco Type	Mean [% dwb]**	r	rCV*	R	RCV*
Flue-Cured A	5	0.3	6.0	2.1	42.0
Flue-Cured B	9.9	0.4	4.0	2.2	22.2
Flue-Cured C	12	1.2	9.8	3.5	28.7
Flue-Cured D	16	0.8	5.0	2.7	16.9

* rCV is $r/\text{mean} \times 100 \%$ and RCV is $R/\text{mean} \times 100 \%$

** [% dwb] unit of the result in % on dry weight basis

Table 10: r and R of ‘Total Sugars’ from Collaborative Study 2015

Extraction Method	Tobacco Type	Mean [%]	r	rCV*	R	RCV*
5 % Acetic Acid	Burley	0.20	0.10	50.0	0,24	120.0
	Virginia (Low Level)	2.50	0.21	8.4	0.63	25.2
	Cut Rag / RYO	5.33	0.29	5.4	1.65	31.0
	Cut Rag / Cig Blend	7.48	0.31	4.1	1.81	24.2
	Virginia (High Level)	12.49	0.89	7.1	3.28	26.3
Water	Burley	0.25	0.14	56.0	0.31	124.0
	Virginia (Low Level)	2.64	0.26	9.8	1.00	37.9
	Cut Rag / RYO	5.49	0.39	7.1	2.06	37.5
	Cut Rag / Cig Blend	7.68	0.47	6.1	2.38	31.0
	Virginia (High Level)	12.65	0.85	6.7	3.37	26.6

*rCV is $r/\text{mean} \times 100 \%$ and RCV is $R/\text{mean} \times 100 \%$

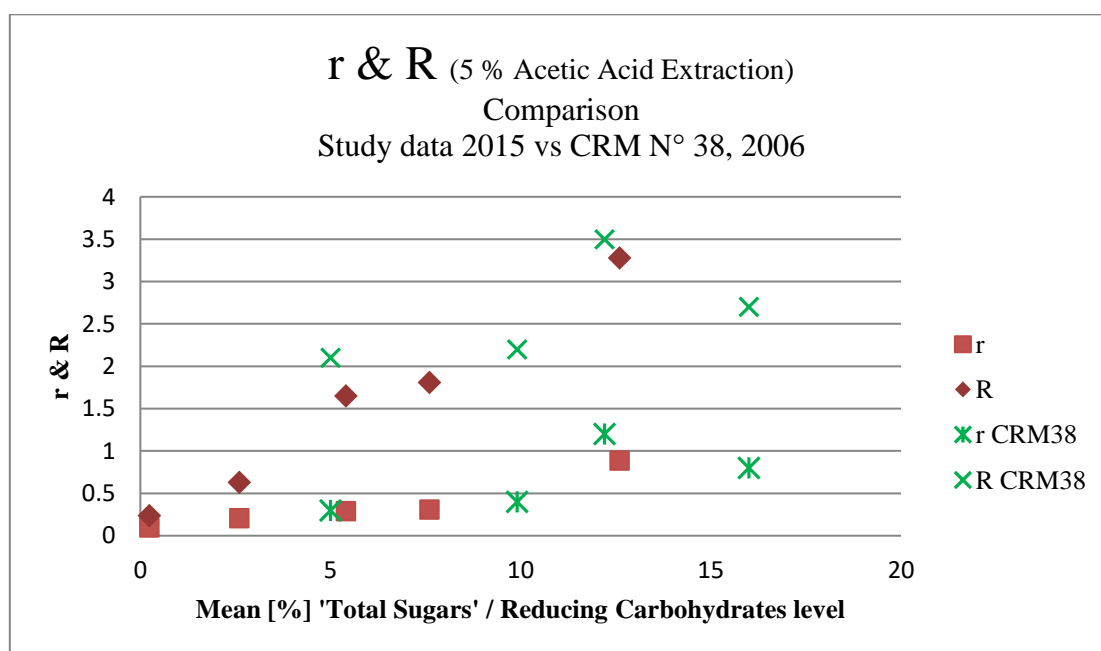


Figure 1: The r and R of ‘Total Sugars’ determined by the developed method (extraction with 5 % acetic acid) in 2015 study and the reducing carbohydrates of CRM N° 38, 2006 study.

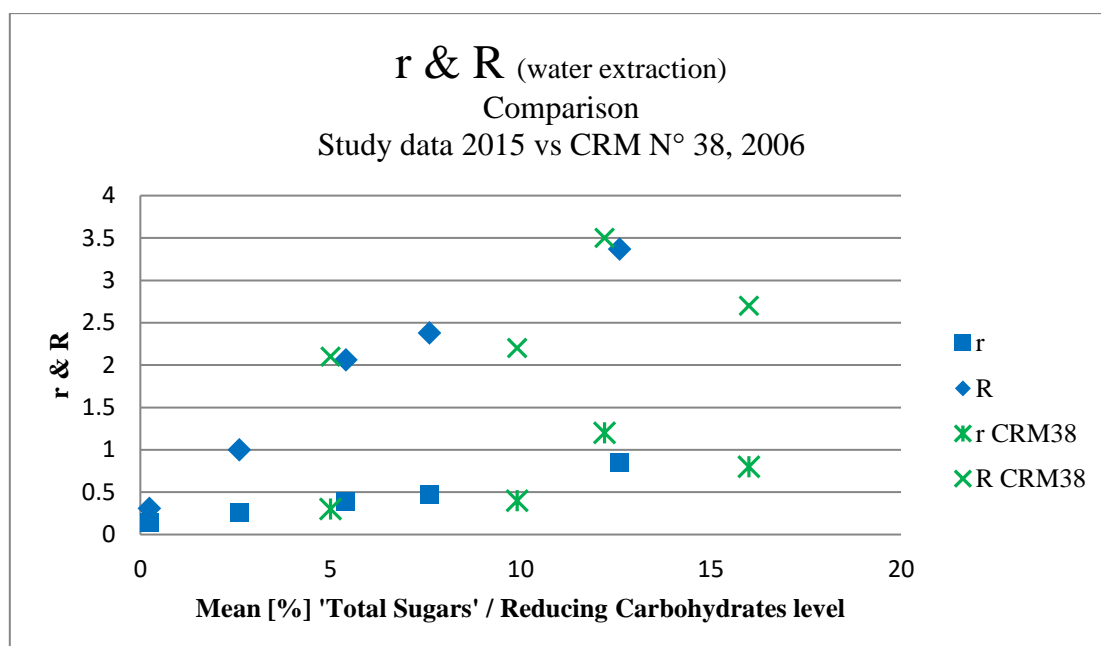


Figure 2: The r and R of ‘Total Sugars’ determined by the developed method (extraction with Water) in 2015 study and the Reducing Carbohydrates of CRM N° 38, 2006 study.

Comparing the data above for the same level of detection the r and R values of CRM N° 38 and for the collaborative studies of the method development are close to each other. Although at the very low level of ‘Total Sugars’ content relatively high values for rCV and RCV were calculated. This might be due to the fact that these results were analysed below the lowest calibration standard.

It can be observed from above tables and figures that the r and R data for the 5 % acetic acid extraction for all samples of the 2015 collaborative study are slightly lower than for the extraction with water. However, for both extracts, the r and R values of the developed method are lower than the observed comparable levels for CRM N° 38 and therefore acceptable for the new method.

5. Method Validation

5.1 Linearity in Solvent

Definition:

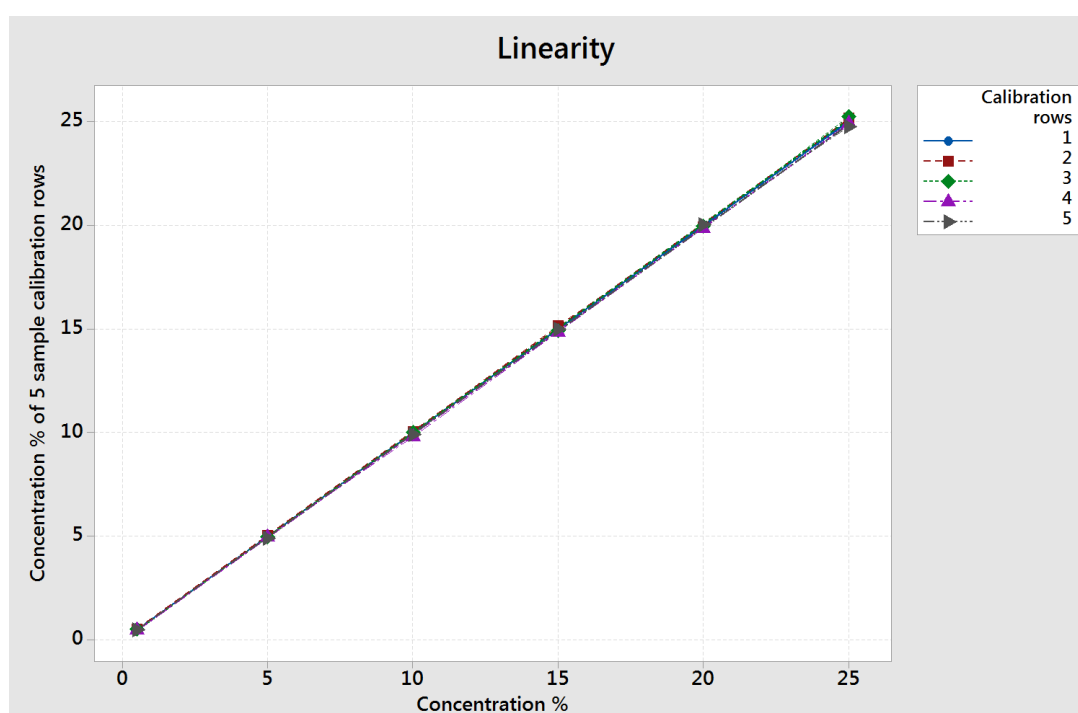
The purpose of this validation is to verify that the CFA method generates linear and reproducible calibration lines. The calibration was carried out based upon the measurement of 6 standard solutions (Table 11). The percentage of glucose is plotted versus the concentration of ‘Total Sugars’ in %.

Table 11: Concentration of the analyte in the calibration standards

Standards	'Total Sugars' concentration [mg/mL]	'Total Sugars' concentration (% w/w) if a sample dilution of 1:100 (0.25 g in 25 ml) is included into the calculation
1	0.05	0.5
2	0.50	5.0
3	1.00	10.0
4	1.50	15.0
5	2.00	20.0
6	2.50	25.0

Results:

The linear regression of calibration standards was calculated as $y_R = 1.0001 x - 0.0162$.

**Figure 3: Calibration Linearity**

The method delivered a linear calibration for the range of 'Total Sugars' concentration of 0.05 – 2.5 mg/mL (measured as glucose).

5.2 Accuracy and Precision of the Calibration Standards in Solvent**Definition:**

The purpose of this validation is to verify the accuracy of the calibration standard measurement by calculating the deviation of the actual values from the target values for the calibration standards. The relative standard deviation accounts for the required precision of the calibration standard measurement.

Results:

Table 12: Results for the evaluation of accuracy and precision

Calibration standard	1	2	3	4	5	6
Target concentration [%]	0.5	5	10	15	20	25
Measured concentration, mean values [%]	0.51	4.99	9.96	14.99	19.94	25.03
Deviation from target value [%]	1.60	-0.28	-0.42	-0.09	-0.29	0.13
SD [%]	0.008	0.047	0.100	0.112	0.064	0.201
RSD [%]	1.65	0.95	1.01	0.75	0.32	0.80

Accuracy:

The calibration standards are accurate since the calculated deviations of the actual standard concentrations from the target values are less than 5 % absolute, which was defined as acceptance criteria.

Precision:

The calibration standards are precise since the relative standard deviations of the calibration standard measurements are less than 5 % absolute, which was defined as acceptance criteria.

5.3 Recovery Rates

Recovery rates after adding analyte directly to a low TS level (LL) sample

Definition:

These tests (recovery rates after adding analyte directly to a low TS level (LL) sample) were carried out to determine the recovery rate (RR) of glucose and sucrose and to document and verify sample preparation accuracy as well as the hydrolysis step of the method.

Note: For the hydrolysis of one molecule Sucrose one molecule Water is added and yields to one molecule Fructose and one molecule Glucose (measured as two molecules Glucose), therefore the measured value of Sucrose as 'Glucose' is higher (342.3 g/Mol + 18.015 g/Mol → 180.16 g/Mol + 180.16 g/Mol (360.32 g/Mol); 100 mg/mL Sucrose = 105.26 mg/mL 'Glucose').

Table 13: Target concentrations of sucrose / glucose solutions

Target value sucrose / glucose solutions 1, 2 and 3			
	1	2	3
Sucrose: target value [mg/mL]	0.25	1.25	2.20
Sucrose: target value [%]	2.5	12.5	22.0
<i>Sucrose as glucose: target value [%]</i>	2.63	13.16	23.16
Glucose: target value [mg/mL]	0.25	1.25	2.20
<i>Glucose: target value [%]</i>	2.5	12.5	22.0

Table 14: Amount of sucrose respectively glucose in tobacco (LL) sample (matrix)

Applied amount and target concentrations	
Applied ~ amount [mg/mL] sucrose per tobacco sample	0.625
~ Concentration of sucrose in tobacco sample (matrix) [%]	6.25
~ Target concentration <u>as glucose</u> [%]	6.58
Applied ~ amount [mg/mL] glucose per tobacco sample	0.625
~ Target concentration glucose [%]	6.25

Results:

Table 15: Results of recovery rates and RSD of sucrose / glucose solutions (compare to Table 13)

Glucose / Sucrose solution			
	RR low	RR middle	RR high
Concentration sucrose (<u>as glucose</u>): Mean value of 5 reps [%]	2.70	13.38	23.62
RR sucrose: Mean value of 5 reps [%]	102.59	101.64	102.0
RSD [%]	0.550	0.606	1.053
Concentration glucose: Mean value of 5 reps [%]	2.51	12.43	22.14
RR glucose: Mean value of 5 reps [%]	100.48	99.42	100.63
RSD [%]	1.897	0.716	0.593

Table 16: Results of recovery rates and RSD of sucrose / glucose solutions (compare to Table 14)

Glucose / sucrose in tobacco (LL) sample	
Concentration of tobacco (LL) sample Mean value of 5 reps [%]	0.698
<i>Concentration sucrose (as glucose):</i> <i>Mean value of 5 reps [%]</i>	7.24 – 0.689
RR sucrose: Mean value of 5 reps [%]	99.42
RSD [%]	0.932
<i>Concentration glucose:</i> <i>Mean value of 5 reps [%]</i>	6.77 – 0.698
RR glucose: Mean value of 5 reps [%]	97.18
RSD [%]	1.092

The recovery rates of glucose and sucrose are satisfactory for the analyses in solution as well as in the tobacco matrix. The acceptance criteria of 100 % \pm 5 % for the recovery rate and a relative standard deviation of \leq 5 % were fulfilled.

6. Conclusions and Recommendations

6.1 Conclusions

- According to the study mean values, slightly higher results for the determination with the extraction in water could be recognized for some samples. This effect was seen as no appreciable difference in the results between the two extraction media.
- Moreover, the intra- as well as the inter-laboratory variation of the mean results given with 5 % acetic acid extraction were lower than for the water extraction.
- Therefore, one extraction solution is not preferred over the other and either may be used for this method. Nevertheless, if this method is used in combination with CRM N° 38 for reducing carbohydrates it might be considered to use the 5 % HAC extraction as recommended in CRM N° 38.
- Relatively high values for rCV and RCV are calculated for the very low ‘Total Sugars’ level of the Burley sample. This might be due to the fact, that these results are below the lowest calibration standard (above LOD of 0.03 mg/mL) and below the calculated LOQ of 0.1 mg/mL for tobacco matrix. However, linearity in solvent for the tested concentration range of 0.05 mg/mL – 2.5 mg/mL was given.
- Recovery rates for glucose and sucrose in solution as well as in matrix of 100 % \pm 5 % were demonstrated.
- The results of this collaborative study showed comparable or lower r and R data to those obtained by the CORESTA RAC Reproducibility and Repeatability 2006 International Collaborative Study given for CRM N° 38.

6.2 Recommendations

The RAC Sub-Group recommends that this new harmonised method is published as a CORESTA Recommended Method for the determination of ‘Total Sugars’ in Tobacco by Continuous flow analyses.

7. References

- [1] J. Agric. Food Chem. 2006, 54, 1975-1981. Determination of Carbohydrates in tobacco Products by Liquid Chromatography-Mass Spectrometry/Mass Spectrometry: A Comparion with Ion Chromatography and Application to Product Discrimination. Michelle B. Clarke, Dawit Z. Bezabeh, and Caitlin T. Howard
- [2] J. Agric. Food Chem. 2012, 60, 11714–11722. Comparison of Segmented Flow Analysis and Ion Chromatography for the Quantitative Characterization of Carbohydrates in Tobacco Products. John R. Shifflett, Lindsey A. Jones, Edward R. Limowski, and Dawit Z. Bezabeh
- [3] ISO 5725-2:1994: Accuracy (trueness and precision) of measurement methods and results - Part 2: Basic method for the determination of repeatability and reproducibility of a standard measurement method.
- [4] CORESTA Recommended Method CRM N° 38. Determination of Reducing Carbohydrates in Tobacco by Continuous Flow Analysis (August 2010).

APPENDIX A – Final Study Protocol

Protocol of Collaborative Test for Total Sugars in Tobacco 2015

1. Introduction

This protocol describes the suggested method procedure for the determination of Total Sugars in tobacco by CFA based on the results of the previous studies. According to the statistical evaluations it turned out, that the following settings showed the best conformity of the results from various laboratories. To be clear on the influence of the extraction solution conditions, this parameter will be included again in the study performance. It should support those laboratories, which perform the determination of Total Sugars and Reducing Sugar in accordance with CRM N° 37, CRM N° 38/ISO15153, ISO15154 (extraction with acetic acid required) in parallel.

It is requested that participating laboratories attempt to follow the method set-up with both extraction variations.

Any issues regarding the suggested options should be indicated on the respective result sheets.

Results are reported as % Total Sugars on an as-is-basis. The moisture content and the method used shall also be reported.

The study is designed for samples from 5 batches of materials, representing 5 different levels of Total Sugars, one sample with considerable difference to Reducing Sugar (smokeless reference product CRP4) and monitor test pieces such as CM7 and KR 3R4F should be included into the study.

The aim of the study is to show a robust hydrolysis step with 0.5 molar HCl for the determination of Total Sugars based on CRM N° 38 and getting clarity if an influence is given by the extraction solvent (water or acetic acid). In the case of enough statistical evaluable data sets a r&R study shall be performed and be included into the new CORESTA Standard Method for Total Sugars.

2. Scope

This document describes how the study procedure shall be conducted by the participating laboratories. In APPENDIX 1 a common way for the sample preparation and the result calculation is provided. In APPENDIX 2 the method set up for the determination of Total Sugars with the hydrolysis by 0.5 molar hydrochloric acid and a colour reaction with p-hydroxybenzoic acid hydrazide is given. The description of the two different extractions and combined different standard preparation is provided. Attention should be given to any issues in performing the test and should be recorded and submitted with the results.

Statistical analysis of the data will be conducted as reasonable with the received amount of data.

3. Procedure

Table AA1: Test samples

Estimated Total Sugars [%] level of the study samples:							
LLVAxx	HLVAxx	LLBYxx	CIGBxx	RYOBxx	CRP4	3R4F	CM7
3	14	0.6	10	8	25	9	11

- Five pouches of homogenised tobacco, at different Total Sugars levels, have been prepared and distributed by BAT Brazil to the participating laboratories. The Total Sugars (expressed as %TS) levels of the study samples incl. CRP4 and the monitor test pieces tobaccos are in a range of approximately 0.6 - 25 %.
- Each laboratory shall analyse 3 test portions, from each of the 5 pouches, the smokeless product CRP4 (see Appendix 1 for sample preparation), the monitor test pieces 3R4F and CM7 under repeatability conditions with the suggested method set-up (see Appendix 2).
- For quality control QC samples, should be included into the test runs (see Appendix 2).

Table AA2: Test protocol

	First set-up (according to Appendix 2)	Second set-up (according to Appendix 2)
Hydrolysis	HCl 0.5 mol/l	HCl 0.5 mol/l
Colour reaction	PAHBAH	PAHBAH
Extraction solvent	Water	5 % Acetic acid

This will result in 6 measurements (3 test portions for the suggested method with two different extractions) for each of the test samples being reported in the spreadsheet.

- The measurements should be conducted in March/beginning of April 2015 (results are requested by April 10th at the latest). If samples were kept in the freezer, leave the pouches at least 24 h at room temperature before using for analyses.

4. Reporting of Results

Using the spreadsheet provided.

The laboratory supervisor should report the following information in the relevant sections of the provided spreadsheet:

- The individual test results which should not be rounded (3 decimal places would be the preferred number).
- The moisture values for each of the samples.
- Method details for Total Sugars and Moisture should be given as requested in the columns of the spreadsheet.
- Information regarding any irregularities or disturbances during the measurement or issues during preparing the reagents.
- The date when the samples were received.
- The date when the samples were measured.
- Information regarding the equipment used.
- Any other relevant information (see 'Analyses parameters' in the spreadsheet)

The completed spreadsheet shall be returned not later than 10th of April 2015 to:

Ute Ernst, British American Tobacco Germany GmbH

e-mail address: [REDACTED]

telephone: [REDACTED]

Any questions or concerns regarding this protocol should also be addressed to Ute Ernst.

5. Definitions

a. Test result:

A test result is the value obtained by carrying out the complete test method once.

6. Appendix 1 – Sample Handling and Calculation

6.1 Sample preparation

- a. Mill the tobacco sample to a mesh size < 1 mm. If the tobacco is too moist for grinding, it should be dried down for about 3 hours at a temperature not exceeding 40 °C (for CPR4 see paragraph 2).

BAT samples for the study are already ground and ready to be used for the measurements!

- b. Determine the moisture content of the ground tobacco and provide the details for the method used (e.g. in accordance with ISO 6488 or 100 °C / 3 h)
- c. Weigh 0.250 g ± 0.001 g of the ground tobacco into a 50 ml Erlenmeyer flask and add 25 mL of the extraction solution.
- d. Place the Erlenmeyer on a shaker for 30 minutes and ~90 rpm.
- e. Filter the extract through a quantitative filter paper with medium speed and retention (~8 µm)

Note: The extracts should be analysed as soon as possible (if samples have to be stored longer than ½ a day before analysing, place them into the refrigerator. Note any time delay, if applicable in the result sheet)

6.2 Smokeless tobacco sample sourcing, handling, preparation

See CORESTA Guides N° 11 - Technical Guideline for Sample Handling of Smokeless Tobacco and Smokeless Tobacco Products

https://www.coresta.org/sites/default/files/technical_documents/main/Guide-No11-Smokeless-Sample-Handling_July11.pdf

and N° 15 - CORESTA Reference Products - Production and Evaluation

https://www.coresta.org/sites/default/files/technical_documents/main/Guide-No15_CRP-ProductionEvaluationRequirements_July14.pdf

- a. North Carolina State University (NCSU) Tobacco Analytical Services Laboratory (TASL) (<http://www.tobacco.ncsu.edu/strp.html>) provides the smokeless product. CRP4 is available in pouches of 85 grams and should be purchased shortly before usage. Store the samples at approximately 4 °C upon receipt if the analyses would be conducted within one week or store the samples at approximately -20 °C if the analyses would be delayed.

- b. Cryo-grinding is preferred. Add an entire pouch to a stainless still dish, add liquid nitrogen to the dish and then use a hard tool to fracture the frozen product after most of the nitrogen has evaporated. The tobacco should also be mixed after fracturing.
The frozen product could also be added to a mill (knife mill) for grinding.
- c. Second technique: take an entire pouch and chop or cut with a razor blade ensuring that all of the tobacco is mixed as effectively as possible.

The chosen procedure shall be noted as remark.

It is NOT acceptable to take small sample-size aliquots (0.25g – 1g) from a CRP4 pouch for preparation and analysis due to the inherent heterogeneity of the product.

- d. Take out of the pre-mixed tobacco the sample replicates for the analyses. Weigh for each replicate 0.250 g ± 0.001 g of the tobacco into a 50 ml Erlenmeyer flask and add 25 mL of the extraction solution.

6.3 Analysis and calculations

- a. In the determination of Total Sugars content, a tobacco extract in water and secondly in 5 % acetic acid is prepared. The sample must first undergo the inversion of the saccharose in a hot acid medium, forming glucose and fructose. The sample then goes through a dialyser and the Total Sugars content is determined as ‘Total Reducing Sugar’ by reaction with p-hydroxybenzoic acid hydrazide in alkaline medium at 85 °C. A yellow osazone is formed having an absorption maximum at 420 nm.
- b. Quantitation is obtained from a six-point external standard calibration of glucose. The amount of Total Sugars (in %) is determined by the following calculation

$$\%_{TS} = \frac{c * v * 100}{m}$$

where:

c = Glucose concentration (in mg/mL) obtained from the calibration curve

v = extraction volume (in mL)

m = mass of the sample (in mg)

7. Appendix 2 - Method for the Determination of Total Sugars in Tobacco by CFA

Method set-up based on the colour reaction of CRM N° 38 and a hydrolysis by 0.5 molar acetic acid.

7.1 Scope

This method is intended for use in the quantitative determination of Total Sugars in aqueous extracts or in acidic extract (to be evaluated in the study) of tobacco matrices by Continuous Flow Analyses (CFA) with a photometric detection.

7.2 Principle

A tobacco aqueous/acidic extract is prepared followed by a hydrolysis stage where sucrose and other disaccharides are hydrolysed to Reducing Sugars such as glucose and fructose to measure Total Sugars. The sample is first heated with HCl at 90°C and then passed through a dialyzer to eliminate interference from coloured compounds in the sample. The Total Sugars is then

determined as Reducing Carbohydrates in the extract by reaction with p-hydroxy benzoic acid hydrazide (PAHBAH). In alkaline medium at 85 °C a yellow osazone is formed whose absorbance is measured at 420 nm.

7.3 Apparatus and equipment

- a. Analytical balance
- b. CFA system with 90 °C heating bath, 85 °C heating bath, 6" dialyzer, sampler, pump, colorimeter with a filter for 420 nm, div. glass coils, tubing, etc.
- c. Standard laboratory glass ware e.g. volumetric flasks, pipettes

7.4 Reagents

Note: All reagents shall be of analytical grade quality.

Appropriate safety and health practices shall be established according to local EHS requirements.

- a. BRIJ-35 (Polyoxyethylene Lauryl Ether), 30 % SOLUTION

The amount of 0.5 ml 30 % solution per liter reagent (see below) is a recommendation but it depends on each individual system and could be suitable up to 1 ml per liter.

Brij-35 should not be more than a year old.

Other wetting agents may also be suitable¹

- b. BENZOIC ACID SOLUTION 0.1 % (w/v):

Dissolve 2.0 g of benzoic acid in 2 liters of distilled water

- c. ACETIC ACID SOLUTION 5 % (v/v):

Acetic acid, glacial 50 mL

DI water to 1000 mL

Add 50 mL of acetic acid (glacial) to about 500 mL of DI water. Dilute to 1000 mL with DI water and mix thoroughly.

- d. SODIUM HYDROXIDE SOLUTION, 0.5 M

Sodium hydroxide 20 g

DI water to 1000 mL

Brij-35, 30 % solution (a) 0.5 mL

Dissolve 20 g of sodium hydroxide in about 700 mL of DI water. Dilute to 1000 mL, add 0.5 ml Brij solution and mix thoroughly. Stable for as long as the solution remains clear.

- e. CALCIUM CHLORIDE SOLUTION, 0.008 M

Calcium chloride hexahydrate 1.75 g

DI water to 1000 mL

Brij-35, 30 % solution (a) 0.5 mL

¹ Wetting agent F has been found to be a good alternative to Brij-35 in heated acidic reagents.

Dissolve 1.75 g of calcium chloride hexahydrate in about 700 mL of DI water. Dilute to 1000 mL, add 0.5 ml Brij solution and mix thoroughly. If a precipitate occurs when dissolving the calcium chloride hexahydrate: filter the solution. Stable for as long as the solution remains clear.

f. HYDROCHLORIC ACID SOLUTION “A”, 0.5 M (HYDROLYSIS REAGENT)

Hydrochloric acid, 37 % 42 mL

DI water to 1000 mL

Brij-35, 30 % solution 0.5 mL

Slowly add 42 mL of hydrochloric acid (37 %) to about 500 mL of DI water. Dilute to 1000 mL with DI water, add 0.5 mL of Brij-35, 30 % solution and mix thoroughly. Stable for as long as the solution remains clear.

g. HYDROCHLORIC ACID SOLUTION “B”, 0.5 M

Hydrochloric acid, 37 % 42 mL

DI water to 1000 mL

Slowly add 42 mL of hydrochloric acid (37 %) to about 500 mL of DI water. Dilute to 1000 mL with DI water and mix thoroughly. Stable for as long as the solution remains clear.

h. SAMPLER WASH SOLUTION

DI water

i. PAHBAH (P-HYDROXY BENZOIC ACID HYDRAZIDE) SOLUTION

p-hydroxy benzoic acid hydrazide 25 g

Citric acid monohydrate 10.5 g

Hydrochloric acid “B”, 0.5 M to 500 mL

Place 400 mL of HCl solution (4.g) in a beaker, warm it to 45°C and under constant stirring add the PAHBAH and the citric acid monohydrate to the HCl solution. Let the solution cool down, transfer it to a volumetric flask and dilute to volume with the HCl solution (4.g).

7.5 Preparation of standards

a. D-GLUCOSE (C₆H₁₂O₆) STOCK SOLUTION:

Weigh, to the nearest 0.0001 g, 10.0 g of glucose, dissolve in about 800 mL of 0.1 % benzoic acid (4.b) respectively 5 % acetic acid (4.c), if used for sample extraction and dilute to volume. This solution contains 10 mg of glucose per liter. Store in a refrigerator.

b. WORKING STANDARDS:

From the stock glucose solution, prepare a series of at least six calibration solutions according to the Total Sugars concentration which is expected to be found in the test samples (e.g. 0.5 % - 25 % (w/w))

Table 3: Example of Working Standards

Accurately pipette volumes according to the table below into 200 ml volumetric flasks and make up to volume with DI water respectively 5 % acetic acid (4.c). Working standard	Volume of Stock solution (mL)	Total Volume (mL)	TS (mg/ml)	TS (% w/w)	TS (% w/w) if a sample dilution of 1:100 (0,25 g in 25 ml) is included into the calculation already
1	1	200	0.05	0.005	0.5
2	10	200	0.50	0.05	5
3	20	200	1.00	0.10	10.0
4	30	200	1.50	0.15	15.0
5	40	200	2.00	0.20	20.0
6	50	200	2.50	0.25	25.0

Store in a refrigerator.

c. QUALITY CONTROL STANDARDS:

Prepare as described in ‘5.b’ an independent (from the calibration row) standard solution of intermediate glucose concentration. This standard shall be used for the quality control samples in the run, e.g. every 10 injections. Calculate the concentration for these QCs and report the results as requested in the result sheet.

7.6 Calculation

Quantitation is obtained from a six-point external standard calibration of glucose. The amount of Total Sugars (in %) is determined by the following calculation

$$\%_{TS} = \frac{c * v * 100}{m}$$

where:

c = Glucose concentration (in mg/mL) obtained from the calibration curve

v = extraction volume (in mL)

m = mass of the sample (in mg)

7.7 Instrument set-up and operation notes

Configure the auto analyser according to the schematic found in Figure 1/2.

The following additional performance data can be given as an example for an AA3 colorimeter:

Carryover 0.2 %

A typical lag time is 14 min; it is related to the heating bath for the hydrolysis.

A typical reagent absorbance is ~0.02.

The position of the de-bubbler should be as near as possible to the pump (see picture).

Picture 1: De-bubbler position



For good reproducible results a well-shaped flow bubble pattern is necessary.

Note 1: If your system is not flowing correctly, please contact Ute Ernst (BAT Germany) or Lalia Potter (SEAL ANALYTICAL US) for help and advice.

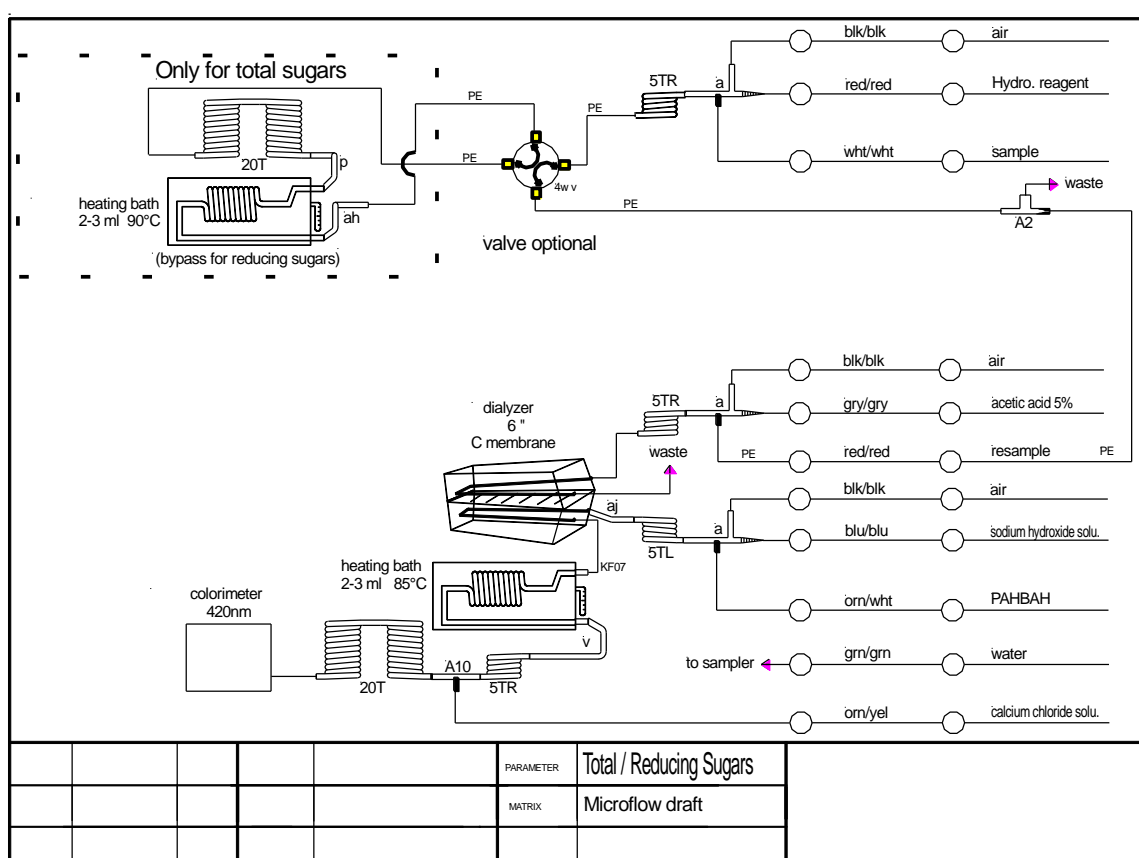


Figure 1: Example of a CFA flow chart (microflow)

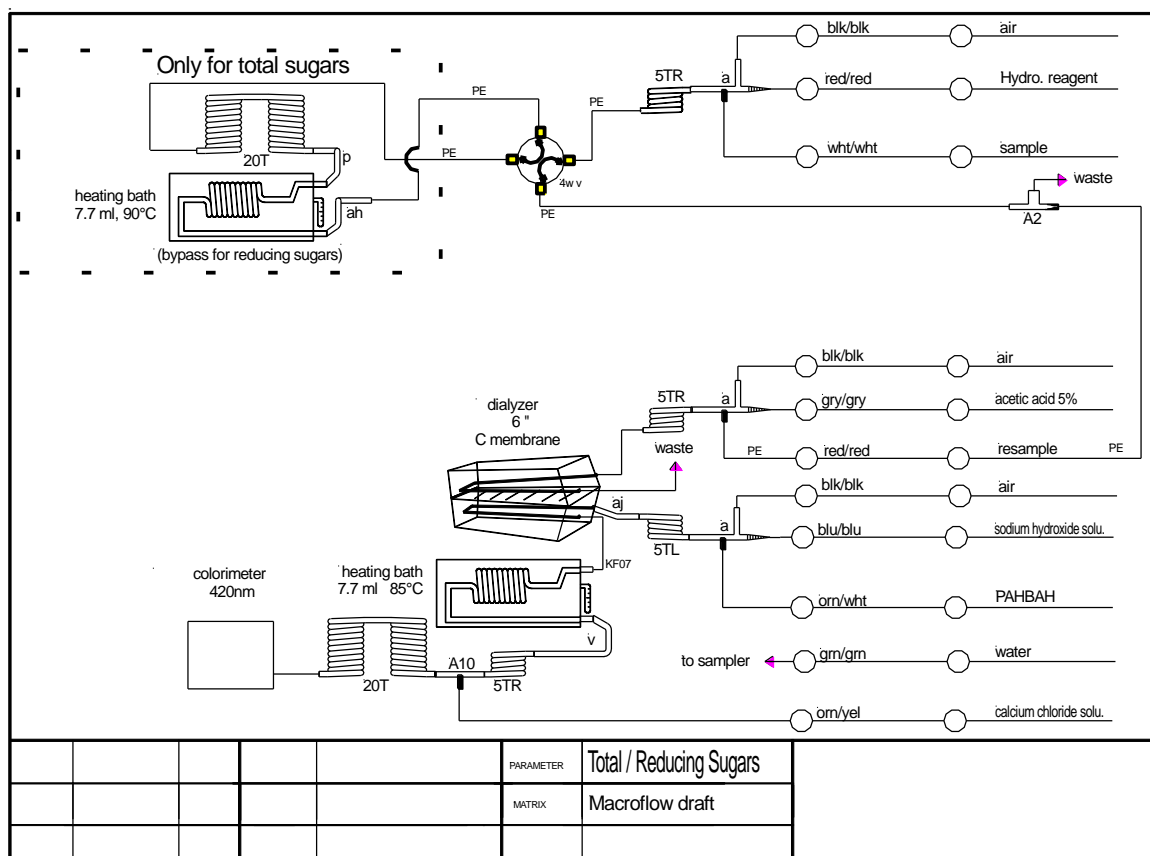


Figure 2: Example of a CFA flow chart (macroflow)

Note 2: The by-pass function of the first heating bath allows determining only Reducing Sugar without the hydrolysis step.

Note 3: See also Table 4 for the corresponding flow rates of the tubing.

Table 4: Flow rates of the tubing

PUMP TUBE	FLOW RATE (mL/min)	
	1 mm manifold (microflow)	2 mm manifold (macroflow)
orn/grn	0.05	0.10
orn/yel	0.08	0.16
orn/wht	0.11	0.23
blk/blk	0.15	0.32
orn/orn	0.19	0.42
wht/wht	0.26	0.60
red/red	0.32	0.80
gry/gry	0.38	1.00
yel/yel	0.43	1.20
yel/blu	0.48	1.40
blu/blu	0.54	1.60
grn/grn	0.64	2.00

APPENDIX B – Inter-Laboratory Study

1. Overview

- Response variable: Total Sugars (%)
- Laboratories: 9
- Methods: 5
 - Own Method
 - 0.1 M HCl / Water extract
 - 0.1 M HCl / Acetic Acid extract
 - 0.5 M HCl / Water extract
 - 0.5 M HCl / Acetic Acid extract
- Replicates by method: 3
- Samples: 5
 - Virginia Low Level (LLVA)
 - Virginia High Level (HLVA)
 - Burley Low Level (LLBY)
 - Cigarette Blend (CIGB)
 - Roll Your Own (RYOB)

2. Statistical Analyses

- To evaluate the results of Total Sugars, in average terms, we used the Kruskal Wallis multiple comparison test.
- h and k index were estimated in order to evaluate between and within laboratory data consistency.
- h checks the between lab data consistency, large h values (either positive or negative) indicate less agreement of mean values in comparison with other laboratories.
- k checks the within lab data consistency, large k values indicate poorer repeatability in comparison with the other laboratories.
- h and k were further analysed through the graphs that, with the critical values obtained in Table 5 of ASTM E691 -99 “Standard Practice for Conducting an Interlaboratory Study to Determine the Precision of a Test Method” allows to identify outlying observations.
- To compare ‘own methods’ between the laboratories and estimate h and k index all 9 laboratories results were used. In other comparisons, it was not possible to use results from all labs because they were not reported.

Averages with different letters (a - g), as shown in the following tables indicate significant differences between the results by Kruskal Wallis test with 5 % confidence level.

Comparison (between laboratories) of laboratories' own methods:

Table AB 1: Own methods – mean results [%] for Total Sugars

Lab ID	Average of own method				
	LLVA	HLVA	LLBY	CIGB	ROYB
1	4.83 b	13.69 b	1.49 b	8.81 c	6.80 a
2	5.78 ab	20.37 a	0.76 c	13.52 a	9.44 a
3	3.06 c	12.76 c	0.72 c	8.04 d	5.50 b
4	2.86 d	11.99 d	0.55 e	7.23 f	5.53 b
5	3.07 c	12.07 d	0.63 d	7.53 e	5.45 b
6	2.87 d	10.90 e	0.60 d	7.10 g	5.20 c
7	3.03 c	12.23 d	0.57 e	7.40 e	5.49 b
8	2.81 d	13.52 b	0.23 f	8.02 d	5.53 b
9	6.50 a	14.81 a	3.69 a	10.50 b	8.52 a

Comparison of the different extraction media for a defined hydrolysis step:

Table AB 2: Water against acetic acid extraction – hydrolysis with 0.1 molar HCl – mean results [%] for Total Sugars

Lab ID	Extract	Average of own method				
		LLVA	HLVA	LLBY	CIGB	ROYB
1	Acetic acid	2.44 b	11.89 b	0.12	7.39 b	5.03 b
	Water	3.02 a	16.59 a	0.13	9.35 a	5.45 a
5	Acetic acid	2.41	12.60	0.10	8.05	5.61
	Water	2.45	12.17	0.15	7.55	5.58
6	Acetic acid	2.79	13.76	0.17	8.84	6.22
	Water	2.87	13.61	0.17	8.70	6.22
7	Acetic acid	2.58	12.75	0.12 b	8.03	5.57 b
	Water	2.64	12.94	0.18 a	8.17	5.98 a

Table AB 3: Water against acetic acid extraction – hydrolysis with 0.5 molar HCl – mean results [%] for Total Sugars

Lab ID	Extract	Average of own method				
		LLVA	HLVA	LLBY	CIGB	ROYB
1	Acetic acid	2.43 a	11.75	0.12 a	7.14	5.16 a
	Water	2.27 b	11.67	0.09 b	7.41	4.83 b
5	Acetic acid	2.63	12.17	0.24	7.84	5.66
	Water	2.68	12.70	0.23	7.72	5.65
6	Acetic acid	2.88	13.86	0.23 b	8.79	6.34
	Water	2.88	13.77	0.25 a	8.66	6.32
7	Acetic acid	2.76	13.48	0.25 b	8.24	6.04
	Water	2.93	13.43	0.28 a	8.31	6.15

Comparison of the hydrolysis step with different acid concentration for the two extraction media:

Table AB 4: 0.1 molar HCl against 0.5 molar HCl hydrolysis – extraction with water – mean results [%] for Total Sugars

Lab ID	Extract	Average of own method				
		LLVA	HLVA	LLBY	CIGB	ROYB
1	0.1 M HCL	3.02 a	16.59 a	0.13	9.35 a	5.45 a
	0.5 M HCL	2.27 b	11.67 b	0.09	7.41 b	4.83 b
2	0.1 M HCL	4.91	18.82 a	0.91 b	11.07 a	8.20
	0.5 M HCL	4.91	17.36 b	1.19 a	10.48 b	8.05
5	0.1 M HCL	2.45 b	12.17	0.15	7.55	5.58
	0.5 M HCL	2.68 a	12.70	0.23	7.72	5.65
6	0.1 M HCL	2.87	13.61 b	0.17 b	8.70	6.22 b
	0.5 M HCL	2.88	13.77 a	0.25 a	8.66	6.32 b
7	0.1 M HCL	2.64 b	12.94 b	0.18 b	8.17 b	5.98 b
	0.5 M HCL	2.93 a	13.43 a	0.28 a	8.31 a	6.15 a

Table AB 5: 0.1 molar HCl against 0.5 molar HCl hydrolysis – extraction with acetic acid – mean results [%] for Total Sugars

Lab ID	Extract	Average of own method				
		LLVA	HLVA	LLBY	CIGB	ROYB
1	0.1 M HCL	2.44	11.89	0.12	7.39 a	5.03
	0.5 M HCL	2.43	11.75	0.12	7.14 b	5.16
5	0.1 M HCL	2.41	12.60	0.10 b	8.05	5.61
	0.5 M HCL	2.63	12.17	0.24 a	7.84	5.66
6	0.1 M HCL	2.79	13.76	0.17 b	8.84	6.22 b
	0.5 M HCL	2.88	13.86	0.23 a	8.79	6.34 a
7	0.1 M HCL	2.58	12.75 b	0.12 b	8.03	5.57 b
	0.5 M HCL	2.76	13.48 a	0.25 a	8.24	6.04 a

h and k indices of laboratories' own methods for all tobacco samples:

Table AB 6: h and k index for the tobacco sample LLVA

Lab ID	Test result			\bar{x}	s	d	h	k
	1	2	3					
1	4.82	4.82	4.84	4.8254	0.0117	0.9581	0.66	0.12
2	5.56	5.89	5.89	5.7793	0.1928	1.9120	1.33	2.01
3	3.11	3.00	3.08	3.0633	0.0569	-0.8039	-0.56	0.59
4	2.88	2.92	2.80	2.8640	0.0623	-1.0032	-0.70	0.65
5	3.10	3.10	3.00	3.0667	0.0577	-0.8006	-0.55	0.60
6	2.90	2.90	2.80	2.8667	0.0577	-1.0006	-0.69	0.60
7	3.01	3.10	2.98	3.0287	0.0624	-0.8385	-0.58	0.65
8	2.91	2.68	2.84	2.8070	0.1192	-1.0603	-0.73	1.24
9	6.45	6.43	6.64	6.5043	0.1171	2.6371	1.83	1.22

Table AB 7: h and k index for the tobacco sample HLVA

Lab ID	Test result			\bar{x}	s	d	h	k
	1	2	3					
1	13.71	13.75	13.59	13.6852	0.0830	0.0926	0.03	0.31
2	20.96	20.23	19.92	20.3685	0.5301	6.7760	2.43	1.96
3	12.65	12.79	12.85	12.7600	0.1048	-0.8325	-0.30	0.39
4	12.18	12.05	11.75	11.9942	0.2225	-1.5984	-0.57	0.82
5	11.90	12.00	12.30	12.0667	0.2082	-1.5259	-0.55	0.77
6	10.80	11.00	10.90	10.9000	0.1000	-2.6925	-0.97	0.37
7	12.22	12.01	12.48	12.2334	0.2330	-1.3592	-0.49	0.86
8	13.64	13.85	13.07	13.5177	0.4029	-0.0749	-0.03	1.49
9	14.85	14.98	14.59	14.8073	0.1952	1.2148	0.44	0.72

Table AB 8: h and k index for the tobacco sample LLBY

Lab ID	Test result			\bar{x}	s	d	h	k
	1	2	3					
1	1.49	1.45	1.51	1.4877	0.0311	0.4612	0.44	0.99
2	0.72	0.76	0.81	0.7612	0.0423	-0.2653	-0.25	1.35
3	0.75	0.73	0.68	0.7200	0.0361	-0.3065	-0.29	1.15
4	0.56	0.55	0.54	0.5495	0.0094	-0.4770	-0.45	0.30
5	0.60	0.60	0.70	0.6333	0.0577	-0.3932	-0.37	1.84
6	0.60	0.60	0.60	0.6000	0.0000	-0.4265	-0.41	0.00
7	0.59	0.55	0.57	0.5698	0.0199	-0.4567	-0.43	0.63
8	0.20	0.23	0.25	0.2291	0.0258	-0.7974	-0.76	0.82
9	3.69	3.67	3.70	3.6880	0.0190	2.6615	2.53	0.60

Table AB 9: h and k index for the tobacco sample CIGB

Lab ID	Test result			\bar{x}	s	d	h	k
	1	2	3					
1	8.75	8.83	8.87	8.8147	0.0576	0.1311	0.06	0.36
2	13.42	13.38	13.75	13.5161	0.2002	4.8324	2.31	1.26
3	8.11	8.08	7.94	8.0417	0.0936	-0.6420	-0.31	0.59
4	7.15	7.31	7.23	7.2301	0.0836	-1.4535	-0.69	0.53
5	7.60	7.40	7.60	7.5333	0.1155	-1.1503	-0.55	0.73
6	7.10	7.10	7.10	7.1000	0.0000	-1.5837	-0.76	0.00
7	7.45	7.32	7.43	7.3980	0.0707	-1.2857	-0.61	0.45
8	8.07	7.62	8.38	8.0221	0.3827	-0.6615	-0.32	2.42
9	10.45	10.53	10.51	10.4970	0.0394	1.8133	0.87	0.25

Table AB 10: h and k index for the tobacco sample RYOB

Lab ID	Test result			\bar{x}	s	d	h	k
	1	2	3					
1	6.78	6.74	6.90	68.042	0.0845	0.4204	0.27	0.72
2	9.39	9.44	9.51	9.4445	0.0578	3.0608	1.96	0.49
3	5.58	5.40	5.51	5.4950	0.0885	-0.8888	-0.57	0.75
4	5.58	5.60	5.40	5.5261	0.1059	-0.8576	-0.55	0.90
5	5.60	5.30	5.45	5.4500	0.1500	-0.9338	-0.60	1.27
6	5.20	5.20	5.20	5.2000	0.0000	-1.1838	-0.76	0.00
7	5.51	5.50	5.45	5.4859	0.0313	-0.8979	-0.58	0.27
8	5.43	5.33	5.82	5.5275	0.2589	-0.8562	-0.55	2.20
9	8.57	8.56	8.44	8.5207	0.0700	2.1369	1.37	0.59

APPENDIX C – Final Study - Raw Data and Statistical Data

1. Raw Data

Table AC 1: 0.5 molar HCl hydrolysis – extraction with acetic acid – raw data [%] for Total Sugars

Lab code	Method	0.5 M HCL _ acetic acid extract / PAHBAH								
	Sample Replicate	LLVAxx	HLVAxx	LLBYxx	CIGBxx	RYOBxx	3R4F	1R5F	CM7	CRP4
1	1	2.610	13.340	0.250	7.900	5.810	8.170	6.480	10.210	31.880
1	2	2.510	13.040	0.240	7.950	5.670	8.660	7.260	10.450	30.460
1	3	2.700	12.220	0.240	8.030	5.720	8.620	7.270	10.330	30.740
2	1	2.827	14.029	0.275	8.167	6.187	/	/	/	34.093
2	2	2.788	14.223	0.255	8.190	6.098	/	/	/	34.150
2	3	2.791	14.254	0.224	8.198	6.138	/	/	/	34.340
3	1	2.400	11.530	0.180	6.560	4.740	8.920	/	/	/
3	2	2.290	11.310	0.130	6.740	4.720	8.610	/	/	/
3	3	2.370	11.270	0.220	6.660	4.680	8.700	/	/	/
4	1	2.542	12.561	0.000	7.609	5.696	9.001	/	11.753	26.675
4	2	2.702	12.312	0.000	7.798	5.718	9.002	/	12.161	32.004
4	3	2.654	13.458	0.044	7.795	5.455	8.532	/	11.687	33.761
5	1	2.530	12.775	0.220	8.050	5.280	/	/	/	/
5	2	2.690	12.720	0.220	7.837	5.238	/	/	/	/
5	3	2.450	12.630	0.210	7.930	5.390	/	/	/	/
6	1	2.404	13.381	0.182	7.722	5.252	9.762	/	12.655	/
6	2	2.489	13.223	0.333	7.735	5.595	9.639	/	12.809	/
6	3	2.407	13.505	0.306	7.825	5.666	9.896	/	13.076	/
7/1	1	2.474	11.899	0.203	7.380	5.420	9.393	/	10.903	/
7/1	2	2.513	12.058	0.215	7.264	5.323	9.402	/	11.176	/
7/1	3	2.509	12.270	0.243	7.638	5.365	9.234	/	11.271	/
7/2	1	/	/	/	7.376	/	/	/	/	/
7/2	2	/	/	/	7.305	/	/	/	/	/
7/2	3	/	/	/	7.621	/	/	/	/	/
8	1	2.169	10.418	0.233	6.145	4.336	8.377	/	/	30.582
8	2	2.017	10.569	0.221	6.298	4.270	8.432	/	/	30.550
8	3	2.057	10.602	0.220	6.323	4.281	7.786	/	/	30.499

Table AC 2: 0.5 molar HCl hydrolysis – extraction with water – raw data [%] for Total Sugars

Lab code	Method	0.5 M HCL _ water extract / PAHBAH								
	Sample Replicate	LLVAxx	HLVAxx	LLBYxx	CIGBxx	RYOBxx	3R4F	1R5F	CM7	CRP4
1	1	2.960	12.600	0.310	8.060	5.880	8.680	7.200	10.540	30.800
1	2	2.720	12.430	0.320	8.440	5.950	8.580	6.040	10.520	32.140
1	3	2.840	12.490	0.300	8.270	5.850	8.720	6.570	10.830	31.880
2	1	3.185	14.417	0.198	8.897	6.736	/	/	/	35.463
2	2	3.176	14.443	0.161	8.954	6.581	/	/	/	35.586
2	3	3.198	14.433	0.143	8.956	6.587	/	/	/	35.675
3	1	2.210	11.840	0.140	6.850	4.720	8.700	/	/	/
3	2	2.290	11.690	0.190	6.860	4.750	8.720	/	/	/
3	3	2.350	11.950	0.160	6.990	4.790	8.920	/	/	/
4	1	2.661	13.123	0.455	7.879	5.725	8.919	/	12.092	30.369
4	2	2.980	12.837	0.357	7.823	6.321	9.412	/	12.293	31.641
4	3	2.738	14.153	0.363	8.538	5.921	9.171	/	12.501	32.495
5	1	2.949	13.330	0.290	8.127	5.598	/	/	/	/
5	2	2.900	13.145	0.290	8.280	5.520	/	/	/	/
5	3	2.829	12.835	0.280	8.217	5.560	/	/	/	/
6	1	2.426	13.000	0.033	7.534	5.286	9.886	/	12.854	/
6	2	2.432	13.546	0.122	7.792	5.627	9.898	/	12.977	/
6	3	2.299	13.158	0.107	7.752	5.542	9.997	/	12.966	/
7/1	1	2.637	12.485	0.278	7.549	5.291	9.143	/	10.980	/
7/1	2	2.550	11.913	0.427	7.293	5.170	8.836	/	10.709	/
7/1	3	2.534	12.279	0.291	7.498	5.422	9.322	/	11.372	/
7/2	1	/	/	/	7.509	/	/	/	/	/
7/2	2	/	/	/	7.391	/	/	/	/	/
7/2	3	/	/	/	7.526	/	/	/	/	/
8	1	2.233	10.406	0.192	6.035	4.214	8.125	/	/	30.039
8	2	2.073	10.533	0.351	6.101	4.353	8.116	/	/	31.059
8	3	2.189	10.583	0.265	6.085	4.347	8.031	/	/	30.627

Table AC 3: 0.5 molar HCl hydrolysis – acetic acid extraction – raw mean [%] and SD for Total Sugars

	Method	0.5 M HCL _ acetic acid extract / PAHBAH								
Lab code	Mean sd	LLVAxx	HLVAxx	LLBYxx	CIGBxx	RYOBxx	3R4F	1R5F	CM7	CRP4
1	mean	2.61	12.87	0.24	7.96	5.73	8.48	7.003	10.33	31.03
1	sd	0.095	0.580	0.006	0.066	0.071	0.272	0.453	0.120	0.752
2	mean	2.80	14.17	0.25	8.19	6.14	/	/	/	34.19
2	sd	0.022	0.122	0.026	0.016	0.045	/	/	/	0.129
3	mean	2.35	11.37	0.18	6.65	4.71	8.74	/	/	/
3	sd	0.057	0.140	0.045	0.090	0.031	0.159	/	/	/
4	mean	2.63	12.78	0.01	7.73	5.62	8.85	/	11.87	30.81
4	sd	0.082	0.603	0.025	0.108	0.146	0.271	/	0.257	3.690
5	mean	2.56	12.71	0.22	7.94	5.30	/	/	/	/
5	sd	0.122	0.073	0.006	0.107	0.079	/	/	/	/
6	mean	2.43	13.37	0.27	7.76	5.50	9.77	/	12.85	/
6	sd	0.048	0.141	0.081	0.056	0.221	0.129	/	0.213	/
7/1	mean	2.50	12.08	0.22	7.43	5.37	9.34	/	11.12	/
7/1	sd	0.021	0.186	0.021	0.191	0.049	0.095	/	0.191	/
7/2	mean	/	/	/	7.43	/	/	/	/	/
7/2	sd	/	/	/	0.166	/	/	/	/	/
8	mean	2.08	10.53	0.22	6.26	4.30	8.20	/	/	30.54
8	sd	0.079	0.098	0.007	0.096	0.035	0.358	/	/	0.042

Table AC 4: 0.5 molar HCl hydrolysis – water extraction – raw mean [%] and SD for Total Sugars

	Method	0.5 M HCL _ water extract / PAHBAH								
Lab code	Mean sd	LLVAxx	HLVAxx	LLBYxx	CIGBxx	RYOBxx	3R4F	1R5F	CM7	CRP4
1	mean	2.84	12.51	0.31	8.26	5.89	8.66	6.603	10.63	31.61
1	sd	0.120	0.086	0.010	0.190	0.051	0.072	0.581	0.173	0.711
2	mean	3.19	14.43	0.17	8.94	6.63	/	/	/	35.57
2	sd	0.011	0.013	0.028	0.034	0.088	/	/	/	0.106
3	mean	2.28	11.83	0.16	6.90	4.75	8.78	/	/	/
3	sd	0.070	0.131	0.025	0.078	0.035	0.122	/	/	/
4	mean	2.79	13.37	0.39	8.08	5.99	9.17	/	12.30	31.50
4	sd	0.166	0.692	0.055	0.398	0.304	0.247	/	0.205	1.070
5	mean	2.89	13.10	0.29	8.21	5.56	/	/	/	/
5	sd	0.060	0.250	0.006	0.077	0.039	/	/	/	/
6	mean	2.39	13.23	0.09	7.69	5.48	9.93	/	12.93	/
6	sd	0.075	0.281	0.048	0.139	0.178	0.061	/	0.068	/
7/1	mean	2.57	12.23	0.33	7.45	5.29	9.10	/	11.02	/
7/1	sd	0.055	0.290	0.083	0.136	0.126	0.246	/	0.333	/
7/2	mean	/	/	/	7.48	/	/	/	/	/
7/2	sd	/	/	/	0.074	/	/	/	/	/
8	mean	2.17	10.51	0.27	6.07	4.30	8.09	/	/	30.58
8	sd	0.083	0.091	0.080	0.034	0.079	0.052	/	/	0.512

Additional data as requested according to the study protocol from one participating laboratory – raw data [%] for Total Sugars (not included in statistical evaluations):

Table AC 5:

Method	0.5 M HCL _ acetic acid extract / PAHBAH	Method	0.5 M HCL _ water extract / PAHBAH
Sample Replicate / Mean sd	Flue-cured Tobacco	Sample Replicate/ Mean sd	Flue-cured Tobacco
1	29.775	1	30.271
2	29.685	2	30.024
3	30.062	3	31.042
mean	29.841	mean	30.446
sd	0.197	sd	0.531

Table AC 6:

Method	1 M HCL _ acetic acid extract / PAHBAH							
Sample Replicate	LLVAxx	HLVAxx	LLBYxx	CIGBxx	RYOBxx	3R4F	CM7	Flue-cured Tobacco
1	2.498	13.718	0.227	7.959	5.405	10.091	12.997	30.819
2	2.581	13.441	0.317	7.903	5.674	10.011	13.312	30.540
3	2.543	13.674	0.294	8.044	5.731	10.128	13.519	30.440
mean	2.541	13.611	0.279	7.969	5.603	10.076	13.276	30.600
sd	0.042	0.149	0.046	0.071	0.174	0.060	0.263	0.197
Method	1 M HCL _ water extract / PAHBAH							
Sample Replicate	LLVAxx	HLVAxx	LLBYxx	CIGBxx	RYOBxx	3R4F	CM7	Flue-cured Tobacco
1	3.007	13.423	0.541	7.933	5.882	10.168	13.000	30.861
2	3.029	13.973	0.605	8.116	6.130	10.095	13.359	31.174
3	2.876	13.490	0.617	8.116	6.085	10.291	13.302	30.676
mean	2.971	13.629	0.587	8.055	6.032	10.185	13.220	30.903
sd	0.083	0.300	0.041	0.106	0.132	0.099	0.193	0.252

2. Statistical Data

Table AC 7: h and k index of LLVA - 0.5 molar HCl hydrolysis – extraction with acetic acid

Lab ID	Test result			\bar{x}	s	h	k
	1	2	3				
1	2.610	2.510	2.700	2.61	0.095	0.52	1.29
2	2.827	2.788	2.791	2.8	0.022	1.42	0.29
3	2.400	2.290	2.370	2.35	0.057	-0.66	0.77
4	2.542	2.702	2.654	2.63	0.082	0.64	1.11
5	2.530	2.690	2.450	2.56	0.122	0.28	1.66
6	2.404	2.489	2.407	2.43	0.048	-0.29	0.66
7	2.474	2.513	2.509	2.5	0.021	0.01	0.29
8	2.169	2.017	2.057	2.08	0.079	-1.92	1.07

Table AC 8: h and k index of HLVA - 0.5 molar HCl hydrolysis – extraction with acetic acid

Lab ID	Test result			\bar{x}	s	h	k
	1	2	3				
1	13.340	13.040	12.220	12.87	0.58	0.34	1.83
2	14.029	14.223	14.254	14.17	0.122	1.48	0.38
3	11.530	11.310	11.270	11.37	0.14	-0.97	0.44
4	12.561	12.312	13.458	12.78	0.603	0.26	1.90
5	12.775	12.720	12.630	12.71	0.073	0.20	0.23
6	13.381	13.223	13.505	13.37	0.141	0.78	0.45
7	11.899	12.058	12.270	12.08	0.186	-0.36	0.59
8	10.418	10.569	10.602	10.53	0.098	-1.71	0.31

Table AC 9: h and k index of LLBY - 0.5 molar HCl hydrolysis – extraction with acetic acid

Lab ID	Test result			\bar{x}	s	h	k
	1	2	3				
1	0.250	0.240	0.240	0.24	0.006	0.50	0.16
2	0.275	0.255	0.224	0.25	0.026	0.60	0.71
3	0.180	0.130	0.220	0.18	0.045	-0.32	1.25
4	0.000	0.000	0.044	0.01	0.025	-2.32	0.70
5	0.220	0.220	0.210	0.22	0.006	0.17	0.16
6	0.182	0.333	0.306	0.27	0.081	0.88	2.24
7	0.203	0.215	0.243	0.22	0.021	0.22	0.57
8	0.233	0.221	0.220	0.22	0.007	0.27	0.20

Table AC 10: h and k index of CIGB - 0.5 molar HCl hydrolysis – extraction with acetic acid

Lab ID	Test result			\bar{x}	s	h	k
	1	2	3				
1	7.900	7.950	8.030	7.96	0.066	0.75	0.59
2	8.167	8.190	8.198	8.19	0.016	1.10	0.14
3	6.560	6.740	6.660	6.65	0.09	-1.30	0.81
4	7.609	7.798	7.795	7.73	0.108	0.39	0.97
5	8.050	7.837	7.930	7.94	0.107	0.71	0.96
6	7.722	7.735	7.825	7.76	0.056	0.43	0.50
7/1	7.380	7.264	7.638	7.43	0.191	-0.09	1.71
7/2	7.376	7.305	7.621	7.43	0.166	-0.08	1.48
8	6.145	6.298	6.323	6.26	0.096	-1.92	0.86

Table AC 11: h and k index of RYOB - 0.5 molar HCl hydrolysis – extraction with acetic acid

Lab ID	Test result			\bar{x}	s	h	k
	1	2	3				
1	5.810	5.670	5.720	5.73	0.071	0.68	0.68
2	6.187	6.098	6.138	6.14	0.045	1.38	0.42
3	4.740	4.720	4.680	4.71	0.031	-1.06	0.29
4	5.696	5.718	5.455	5.62	0.146	0.49	1.39
5	5.280	5.238	5.390	5.3	0.079	-0.06	0.75
6	5.252	5.595	5.666	5.5	0.221	0.29	2.11
7	5.420	5.323	5.365	5.37	0.049	0.06	0.46
8	4.336	4.270	4.281	4.3	0.035	-1.78	0.34

Table AC 12: h and k index of LLVA - 0.5 molar HCl hydrolysis – extraction with water

Lab ID	Test result			\bar{x}	s	h	k
	1	2	3				
1	2.960	2.720	2.840	2.840	0.120	0.57	1.32
2	3.185	3.176	3.198	3.186	0.011	1.57	0.12
3	2.210	2.290	2.350	2.283	0.070	-1.02	0.77
4	2.661	2.980	2.738	2.793	0.166	0.44	1.83
5	2.949	2.900	2.829	2.893	0.060	0.73	0.66
6	2.426	2.432	2.299	2.385	0.075	-0.73	0.83
7	2.637	2.550	2.534	2.574	0.055	-0.19	0.61
8	2.233	2.073	2.189	2.165	0.083	-1.36	0.91

Table AC 13: h and k index of HLVA - 0.5 molar HCl hydrolysis – extraction with water

Lab ID	Test result			\bar{x}	s	h	k
	1	2	3				
1	12.600	12.430	12.490	12.507	0.086	-0.12	0.28
2	14.417	14.443	14.433	14.431	0.013	1.51	0.04
3	11.840	11.690	11.950	11.827	0.131	-0.70	0.43
4	13.123	12.837	14.153	13.371	0.692	0.61	2.28
5	13.330	13.145	12.835	13.103	0.250	0.38	0.82
6	13.000	13.546	13.158	13.234	0.281	0.50	0.93
7	12.485	11.913	12.279	12.226	0.290	-0.36	0.95
8	10.406	10.533	10.583	10.507	0.091	-1.82	0.30

Table AC 14: h and k index of LLBY - 0.5 molar HCl hydrolysis – extraction with water

Lab ID	Test result			\bar{x}	s	h	k
	1	2	3				
1	0.310	0.320	0.300	0.310	0.010	0.58	0.20
2	0.198	0.161	0.143	0.167	0.028	-0.82	0.56
3	0.140	0.190	0.160	0.163	0.025	-0.86	0.50
4	0.455	0.357	0.363	0.392	0.055	1.38	1.10
5	0.290	0.290	0.280	0.287	0.006	0.35	0.11
6	0.033	0.122	0.107	0.087	0.048	-1.60	0.96
7	0.278	0.427	0.291	0.332	0.083	0.79	1.65
8	0.192	0.351	0.265	0.269	0.080	0.18	1.59

Table AC 15: h and k index of CIGB - 0.5 molar HCl hydrolysis – extraction with water

Lab ID	Test result			\bar{x}	s	h	k
	1	2	3				
1	8.060	8.440	8.270	8.257	0.190	0.69	1.14
2	8.897	8.954	8.956	8.936	0.034	1.50	0.20
3	6.850	6.860	6.990	6.900	0.078	-0.92	0.47
4	7.879	7.823	8.538	8.080	0.398	0.48	2.38
5	8.127	8.280	8.217	8.208	0.077	0.64	0.46
6	7.534	7.792	7.752	7.693	0.139	0.02	0.83
7/1	7.549	7.293	7.498	7.447	0.136	-0.27	0.81
7/2	7.509	7.391	7.526	7.475	0.074	-0.24	0.44
8	6.035	6.101	6.085	6.074	0.034	-1.91	0.21

Table AC 16: h and k index of RYOB - 0.5 molar HCl hydrolysis – extraction with water

Lab ID	Test result			\bar{x}	s	h	k
	1	2	3				
1	5.880	5.950	5.850	5.893	0.051	0.55	0.36
2	6.736	6.581	6.587	6.635	0.088	1.57	0.62
3	4.720	4.750	4.790	4.753	0.035	-1.01	0.25
4	5.725	6.321	5.921	5.989	0.304	0.69	2.15
5	5.598	5.520	5.560	5.559	0.039	0.10	0.28
6	5.286	5.627	5.542	5.485	0.178	-0.01	1.26
7	5.291	5.170	5.422	5.294	0.126	-0.27	0.89
8	4.214	4.353	4.347	4.305	0.079	-1.63	0.56

APPENDIX D – Final Study - Data Charts by Sample

Individual Value Plots by Sample for 0.5 molar HCl Hydrolysis – Extraction with Acetic Acid

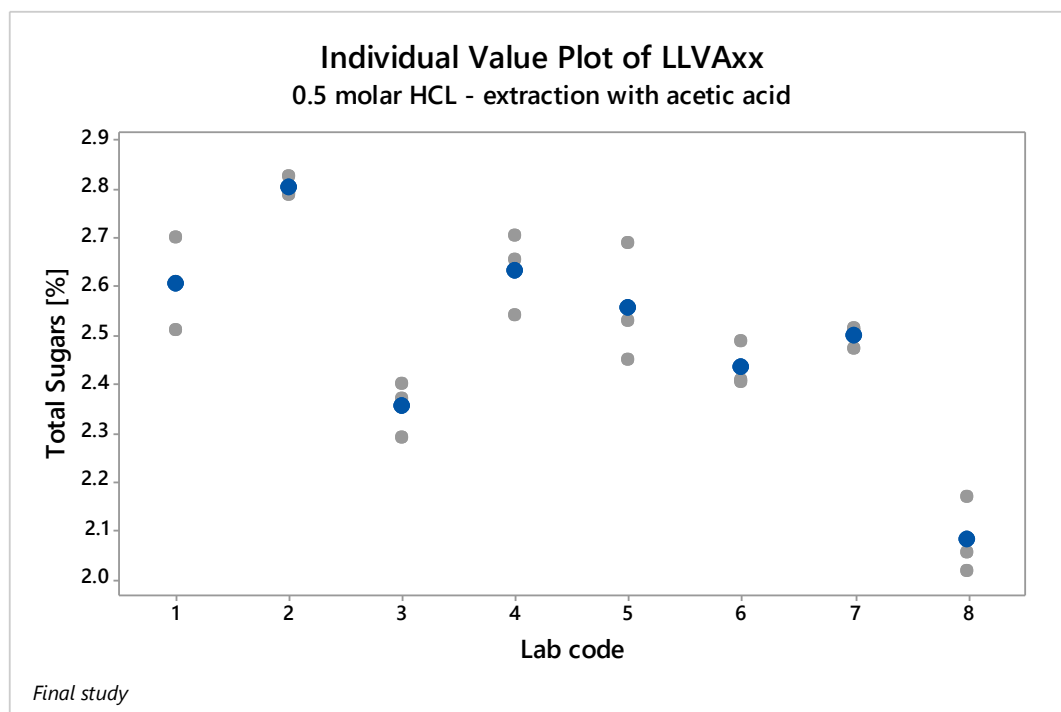


Figure AD 1

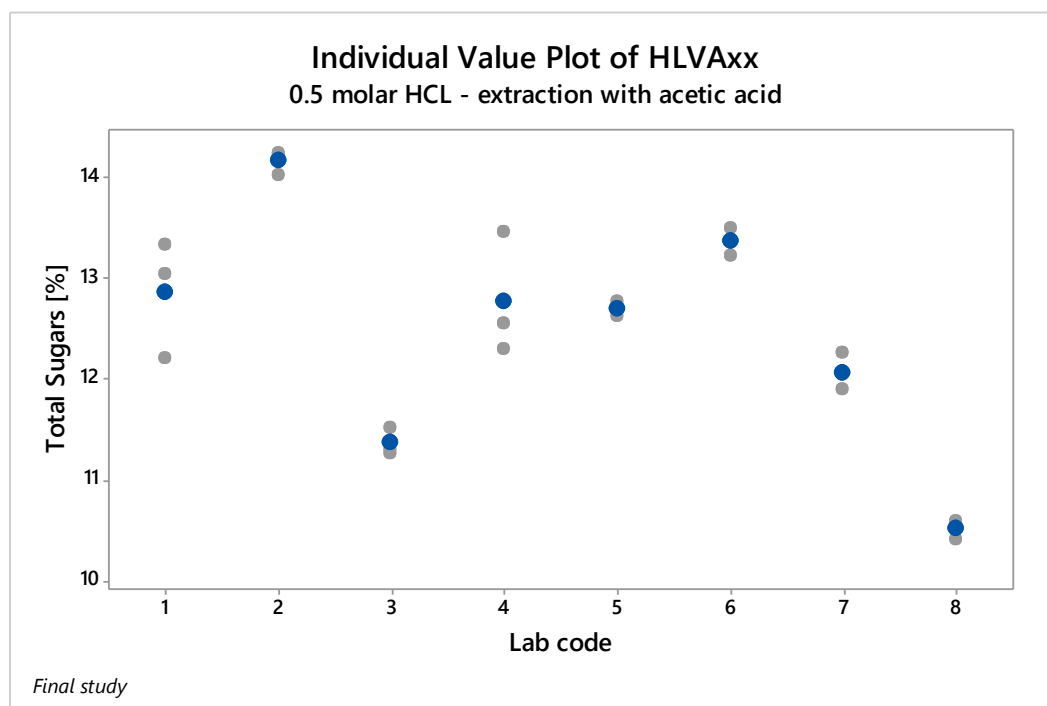


Figure AD 2

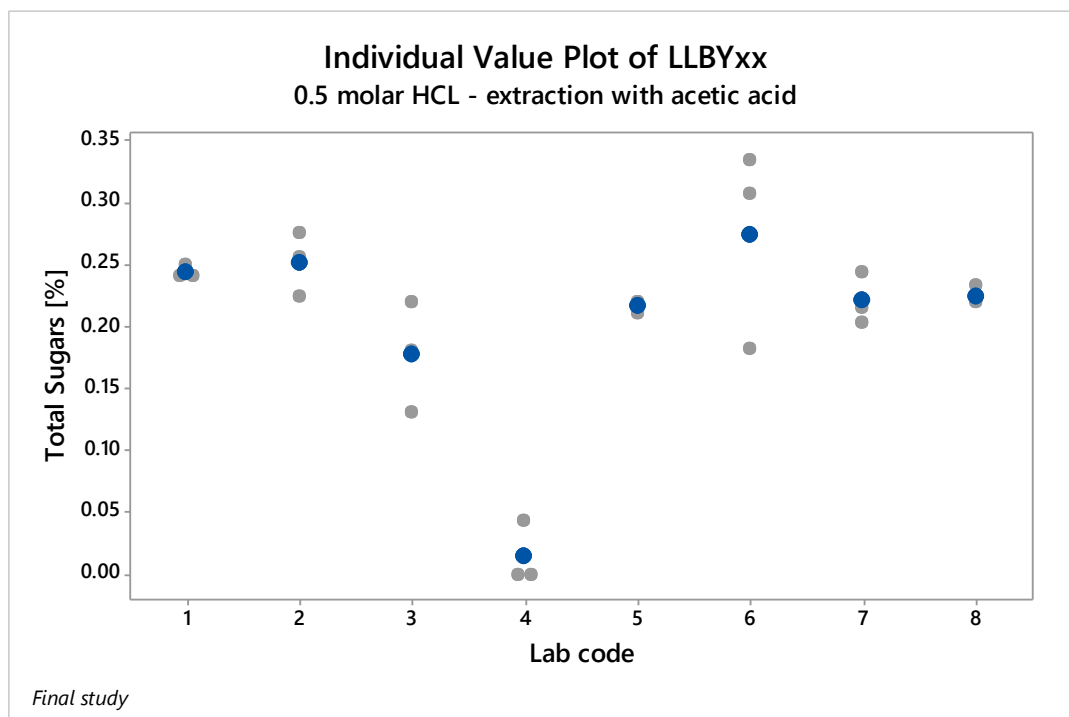


Figure AD 3

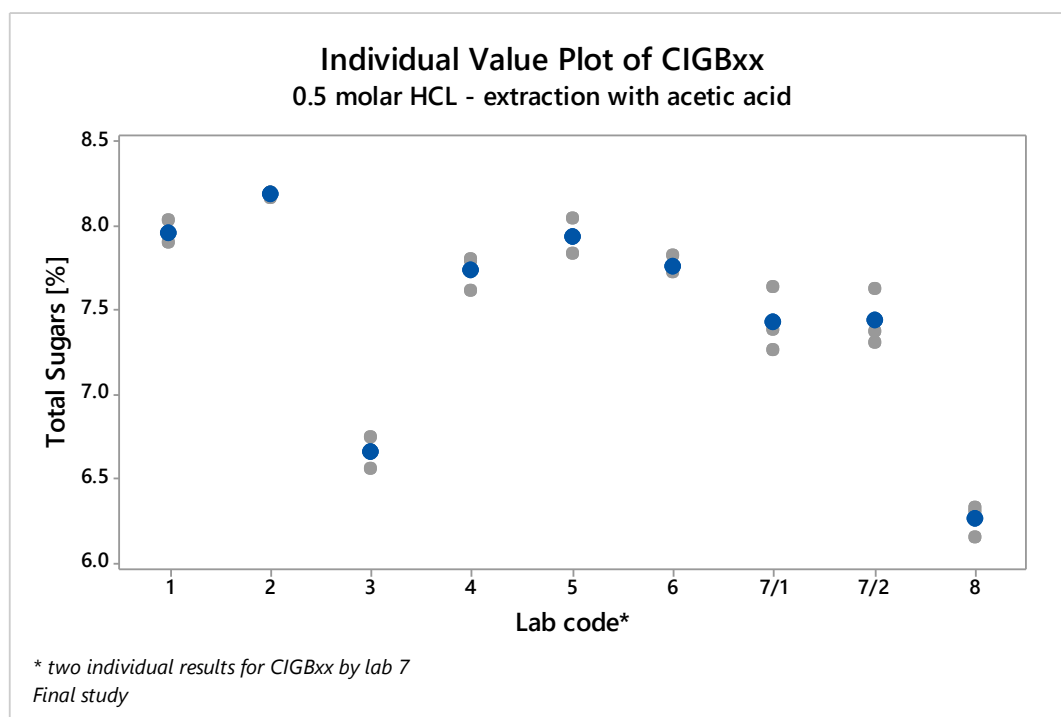


Figure AD 4

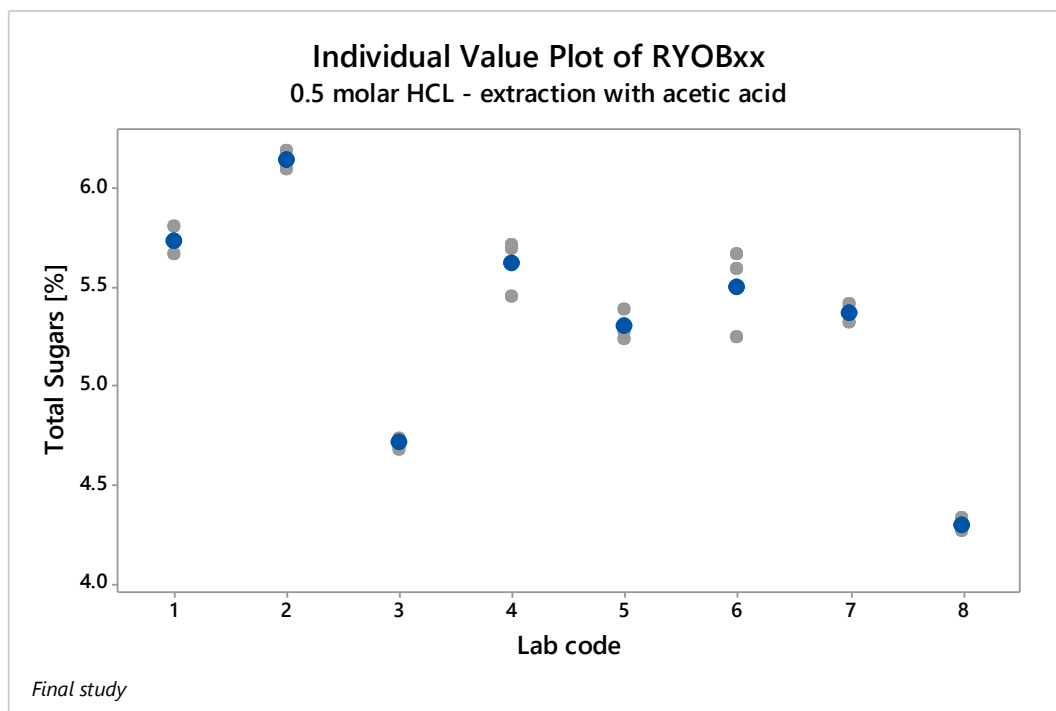


Figure AD 5

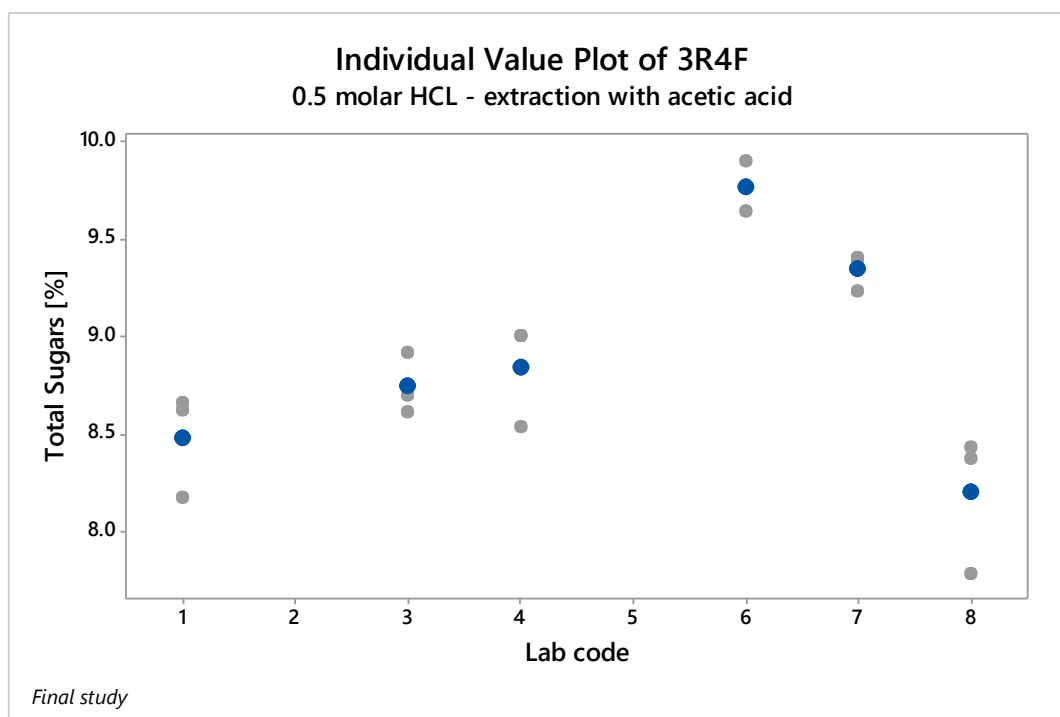


Figure AD 6

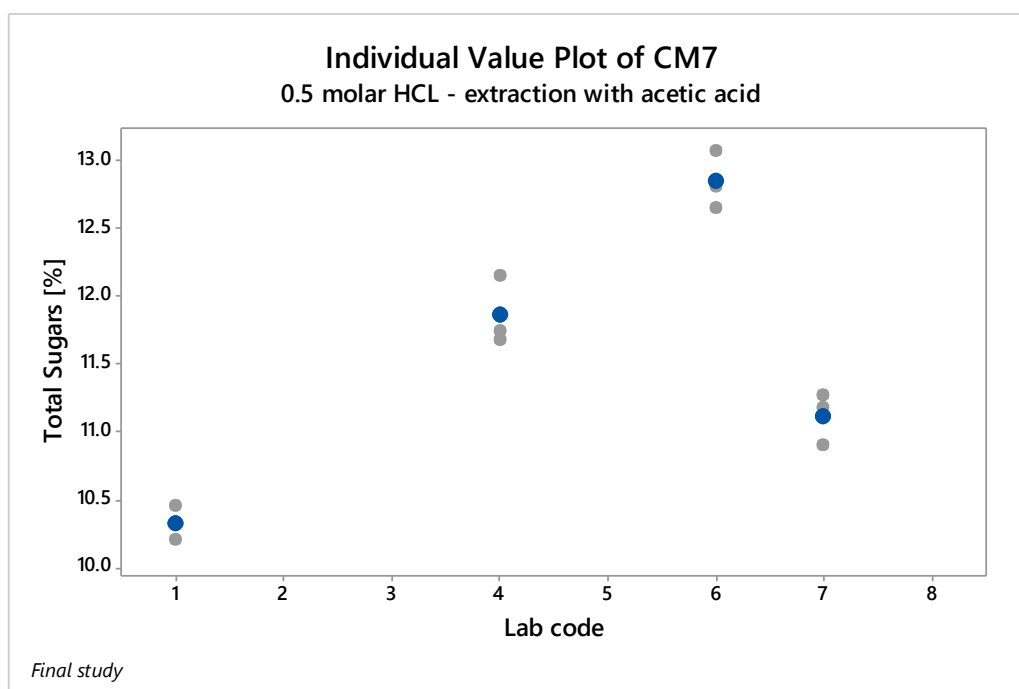


Figure AD 7

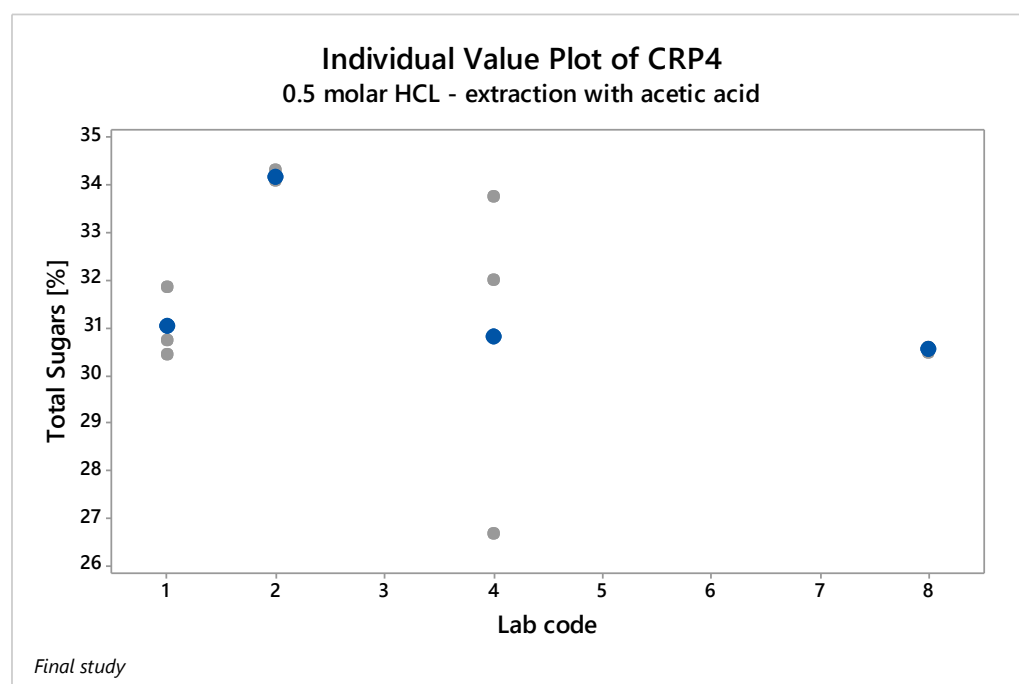


Figure AD 8

Individual Value Plots by Sample for 0.5 Molar HCl Hydrolysis – Extraction with Water

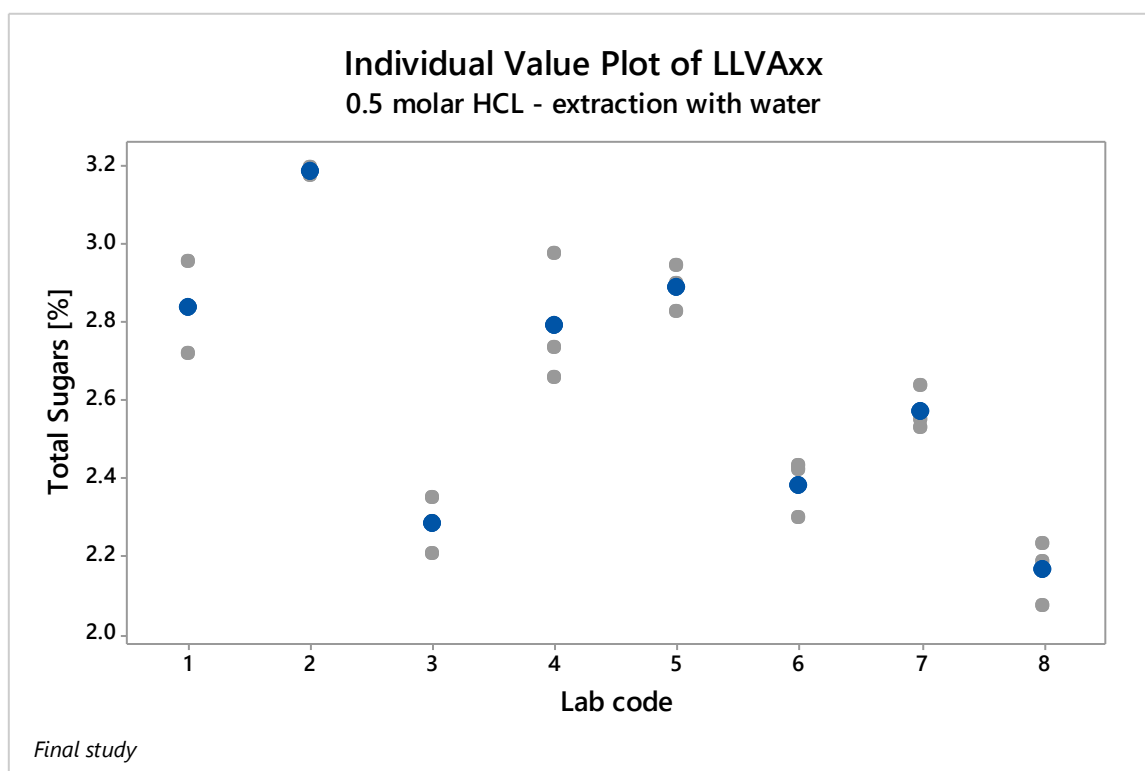


Figure AD 9

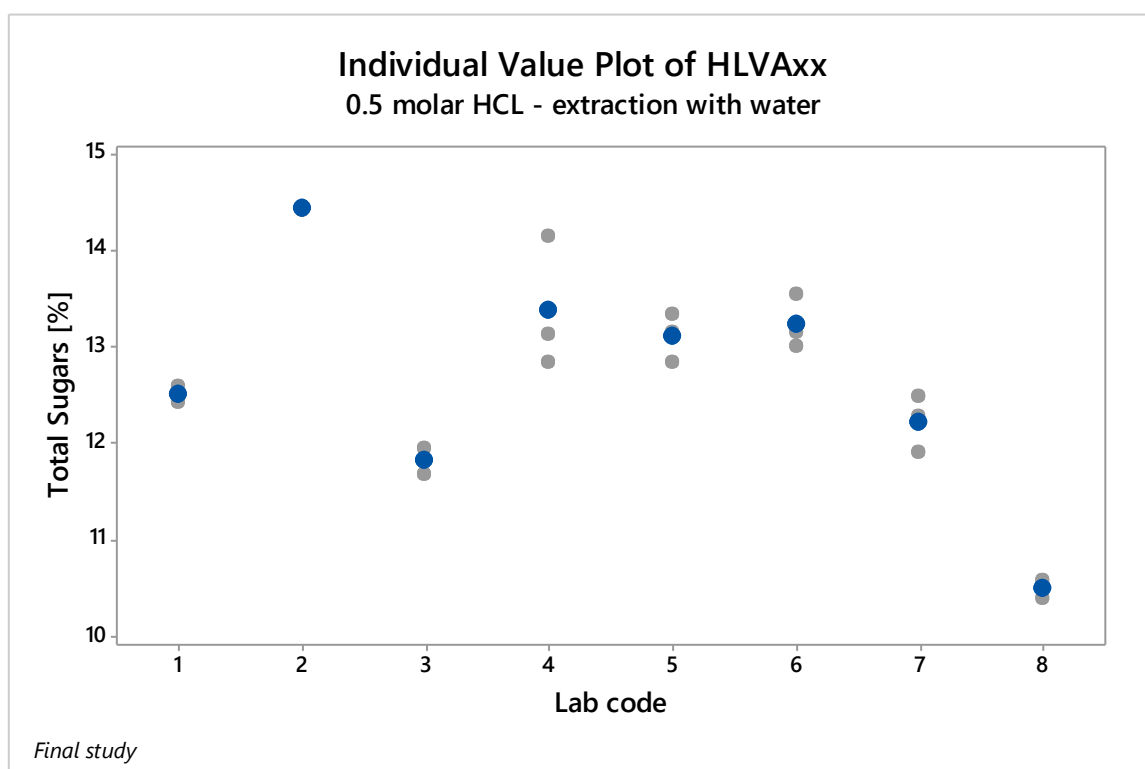


Figure AD 10

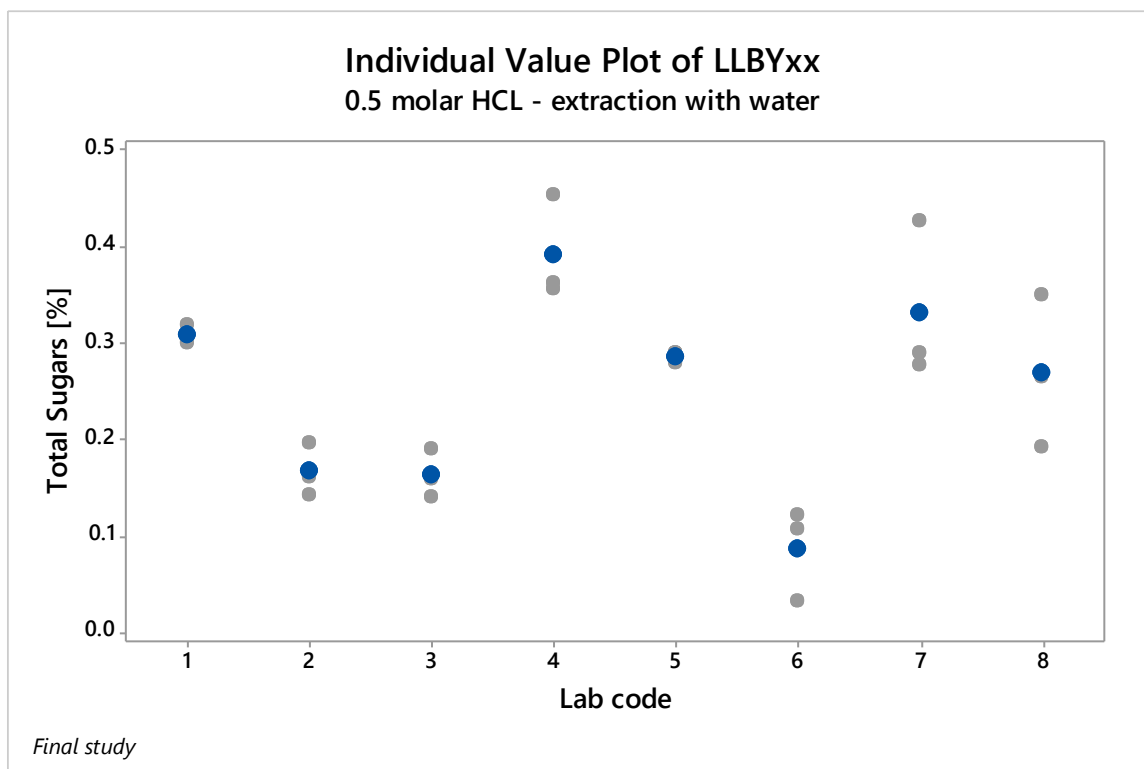


Figure AD 11

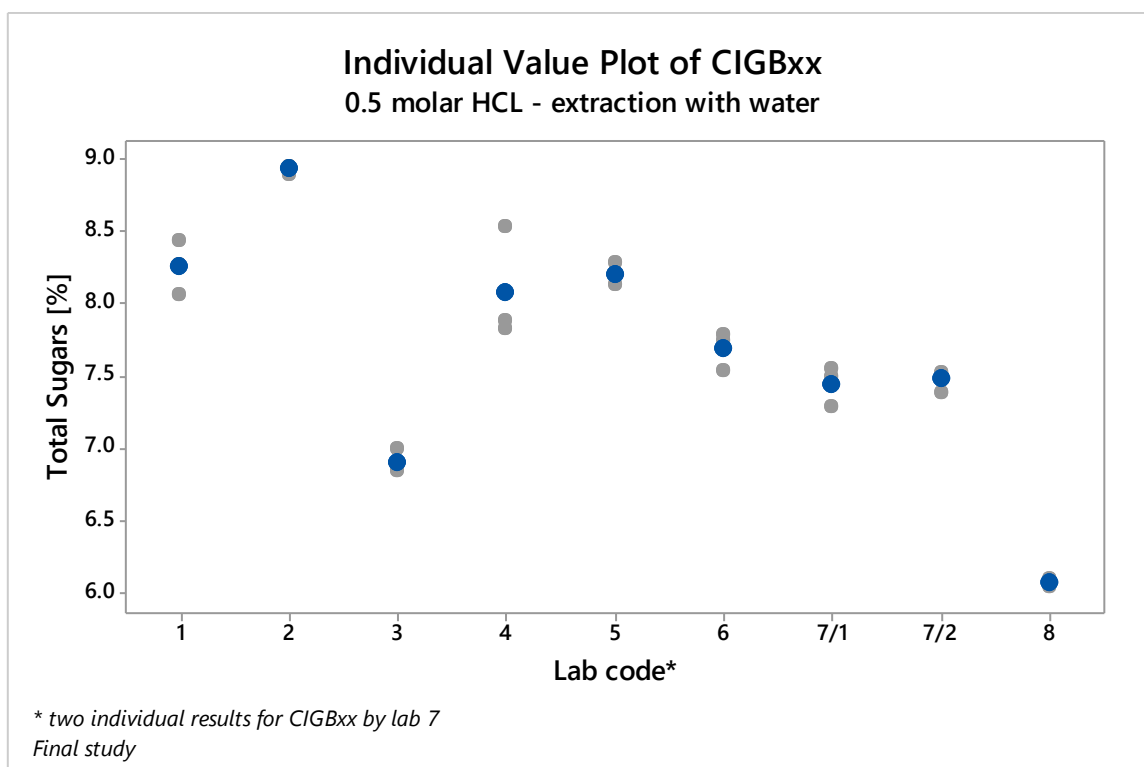


Figure AD 12

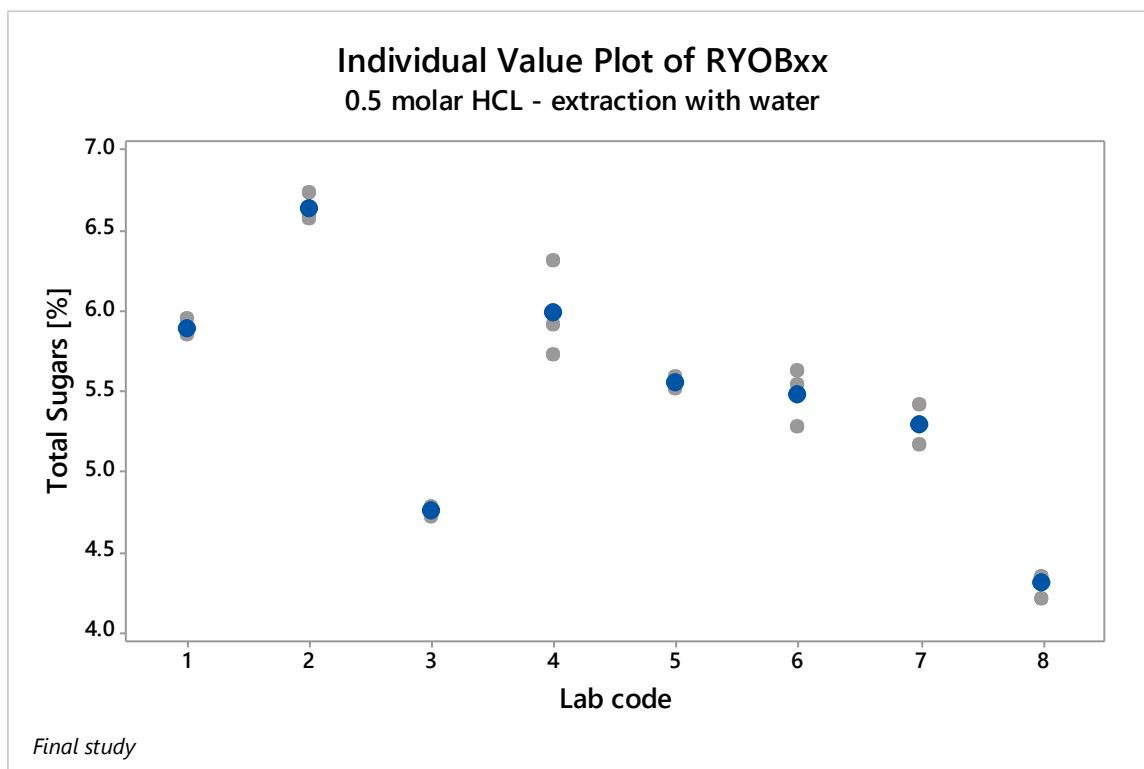


Figure AD 13

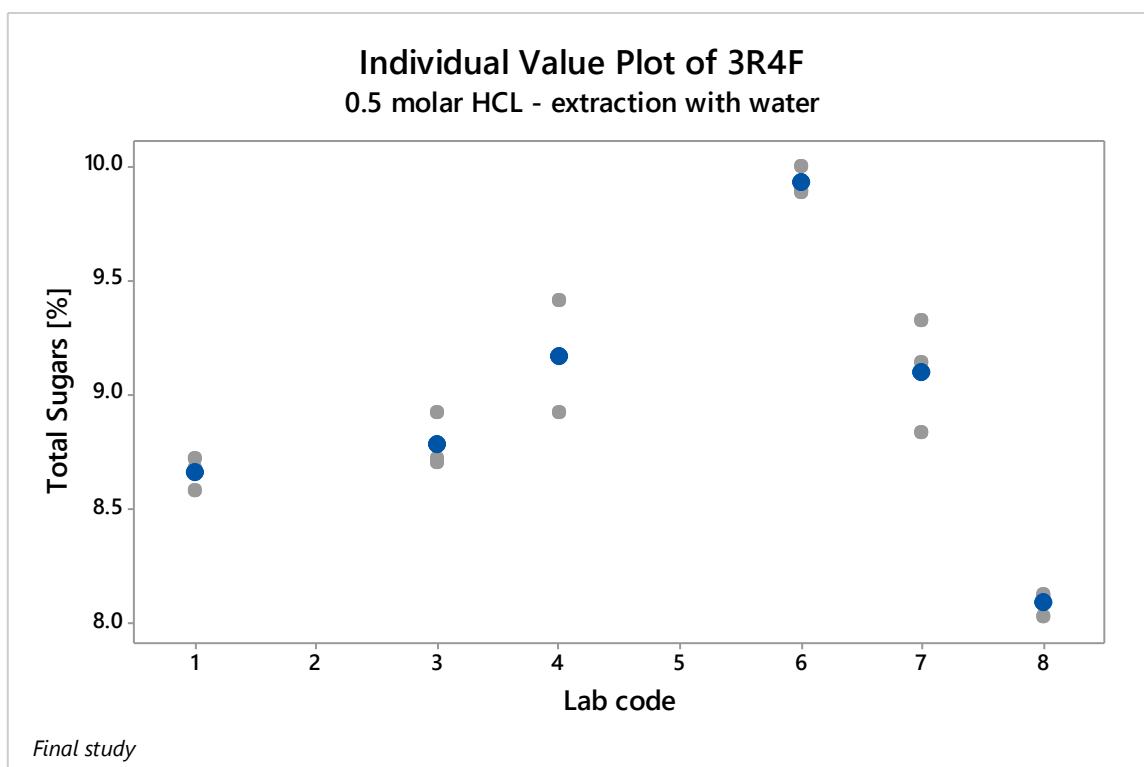


Figure AD 14

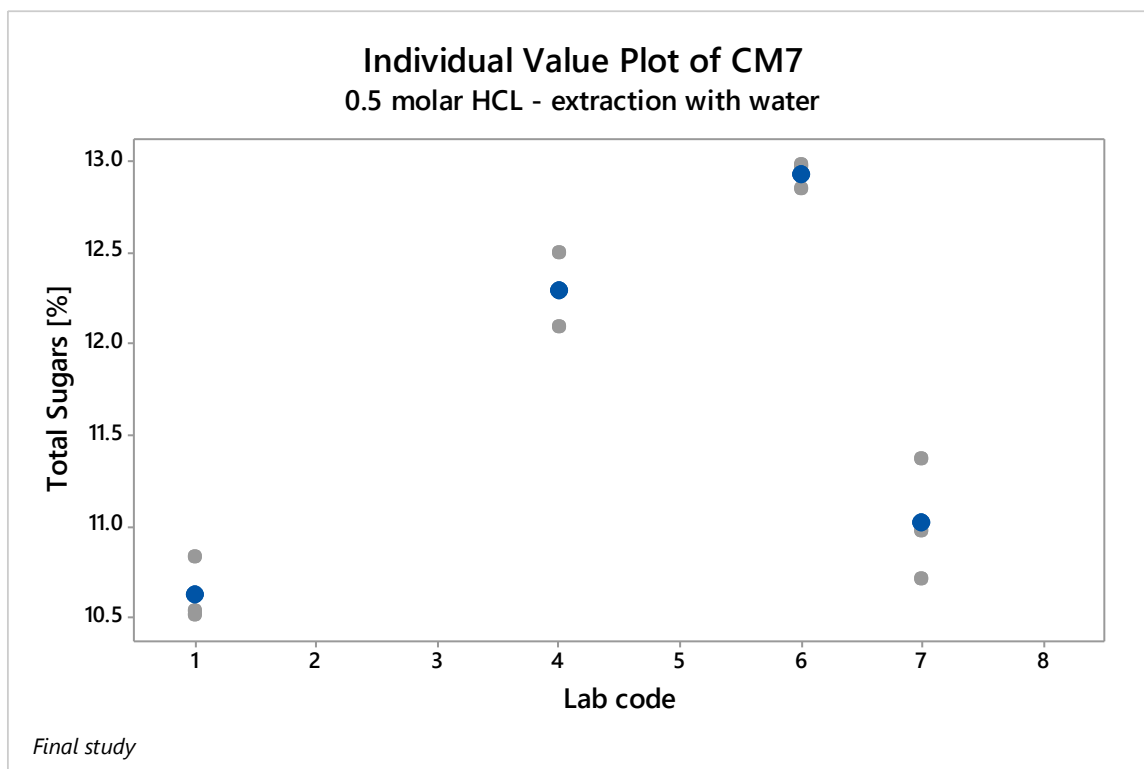


Figure AD 15

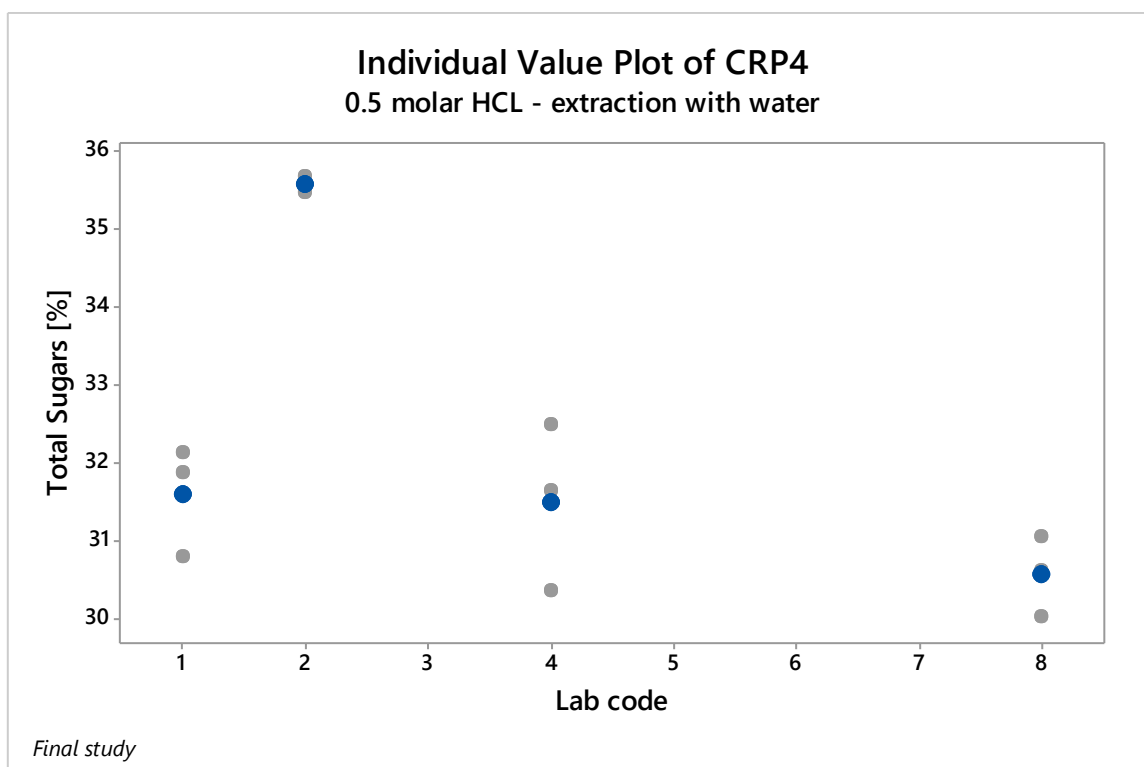


Figure AD 16

h and k Index by Sample for 0.5 Molar HCl Hydrolysis – Extraction with Acetic Acid

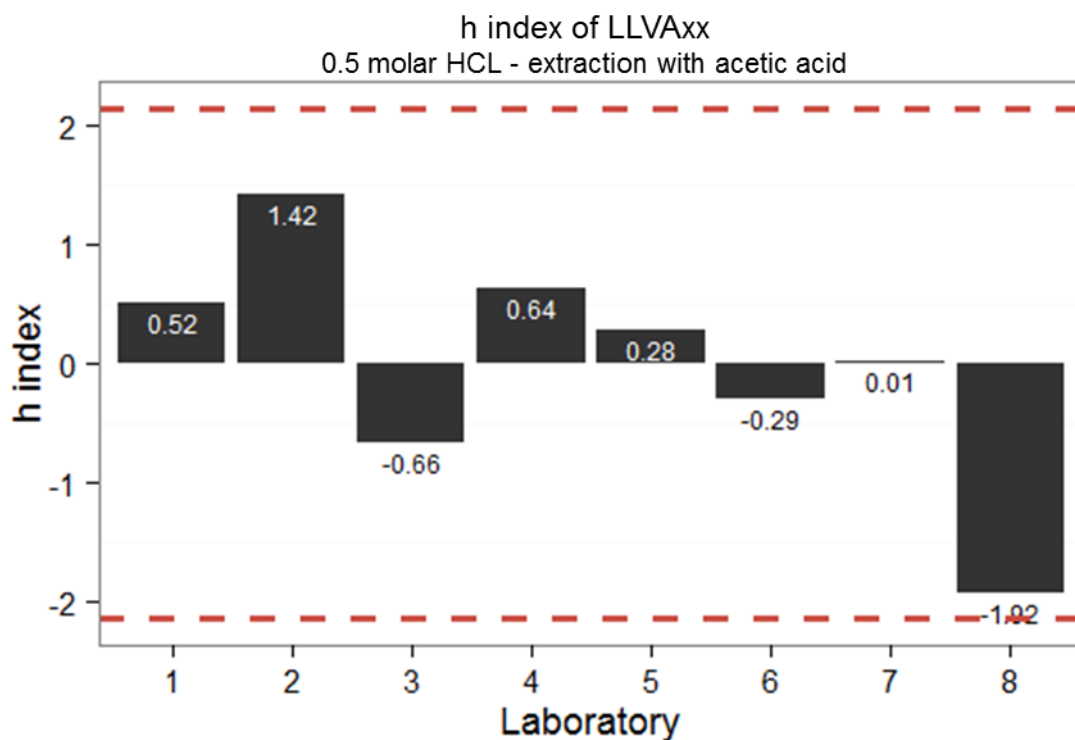


Figure AD 17

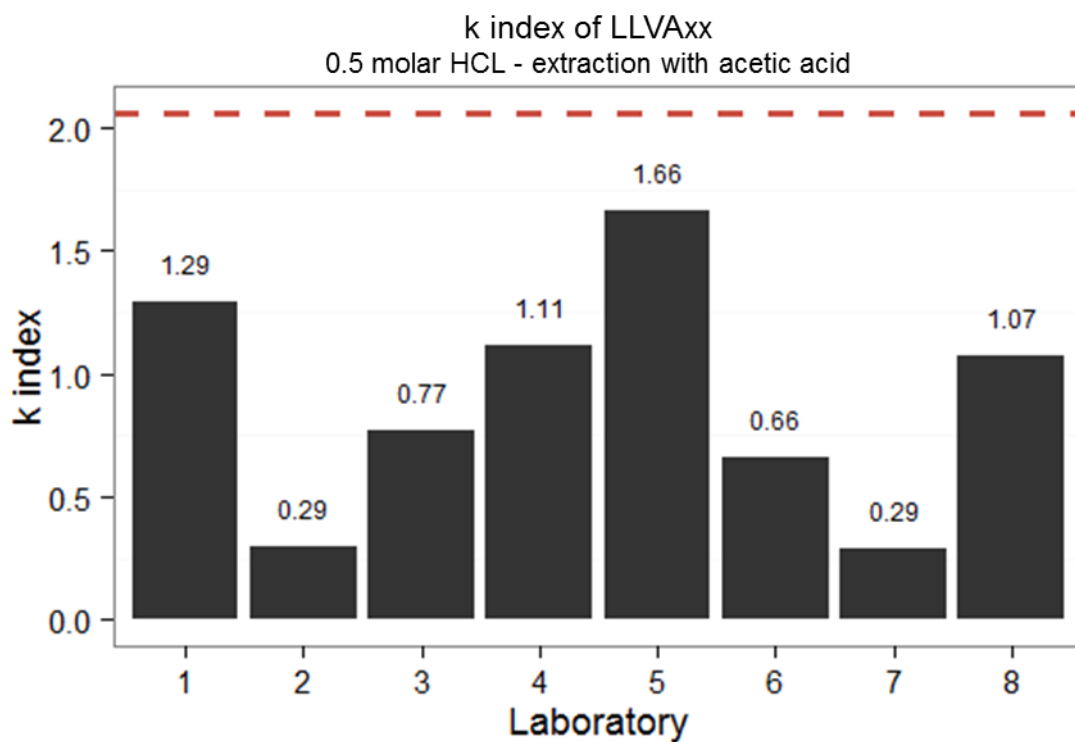


Figure AD 18

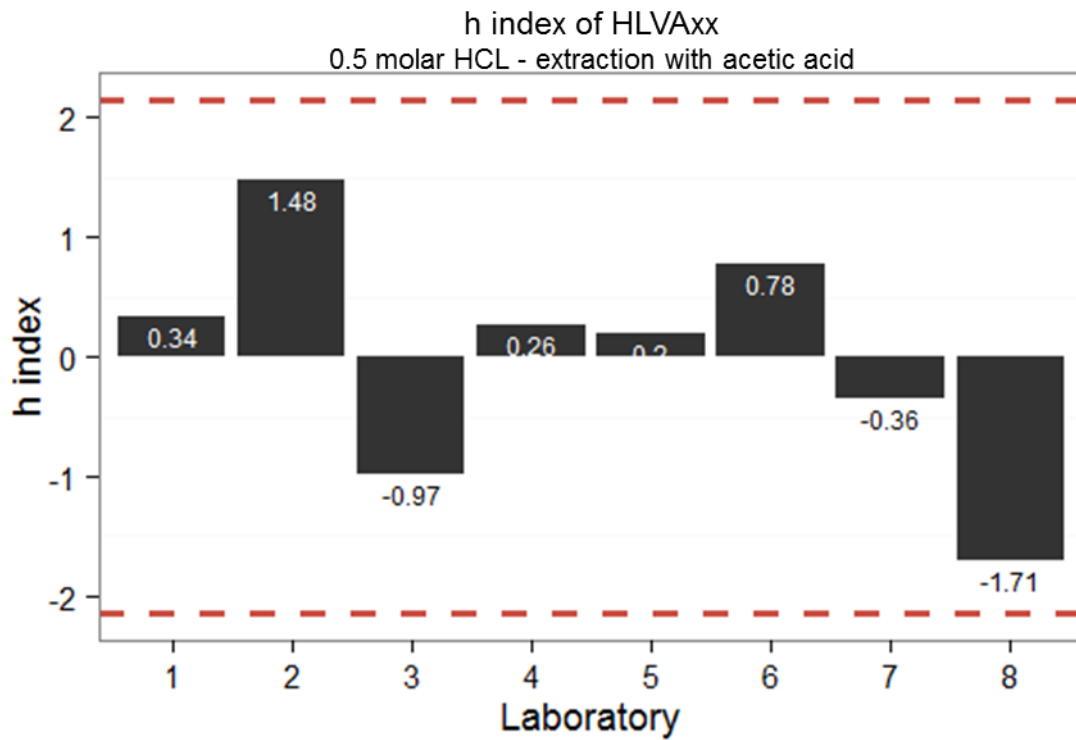


Figure AD 19

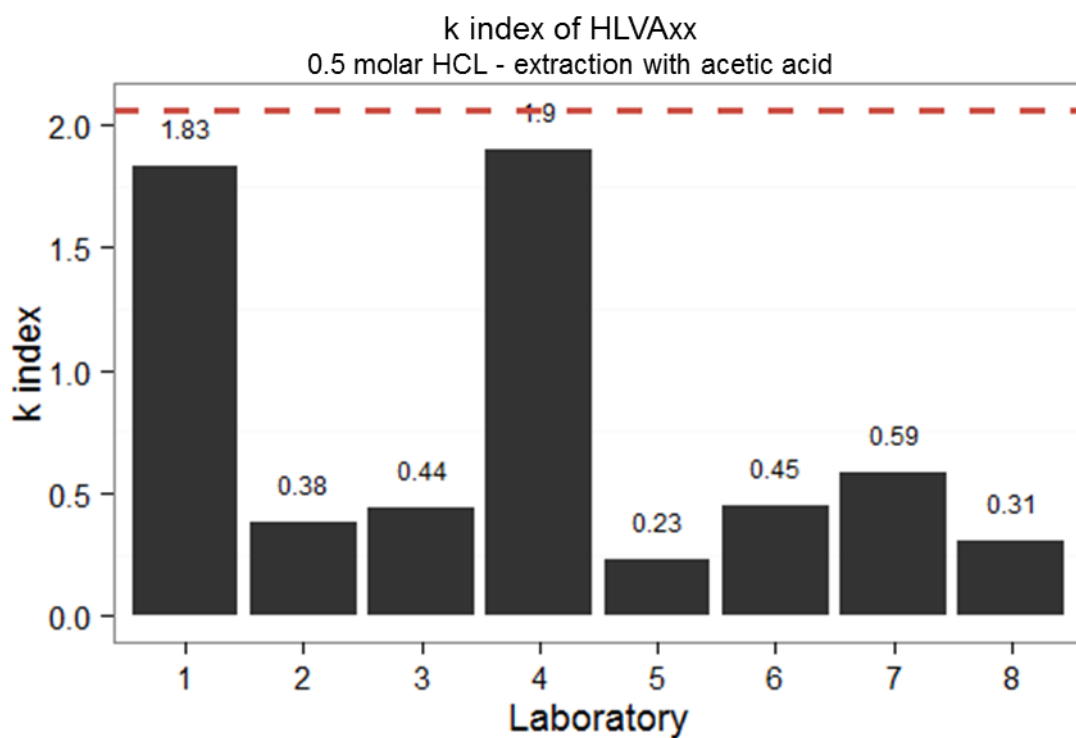


Figure AD 20

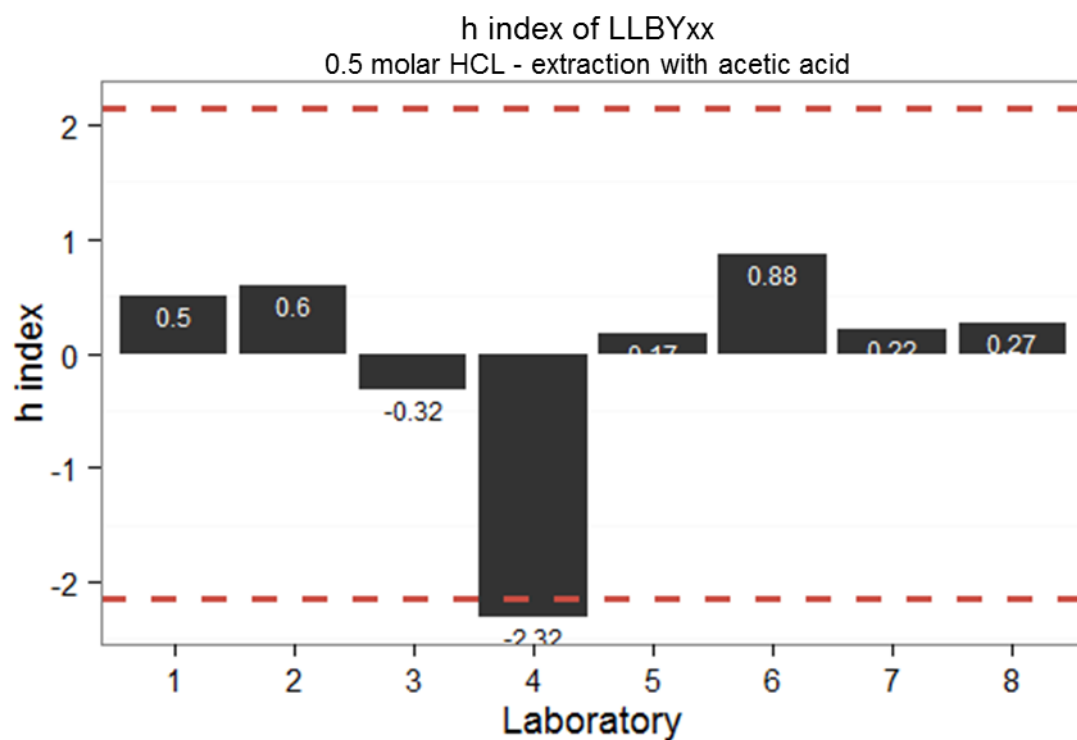


Figure AD 21

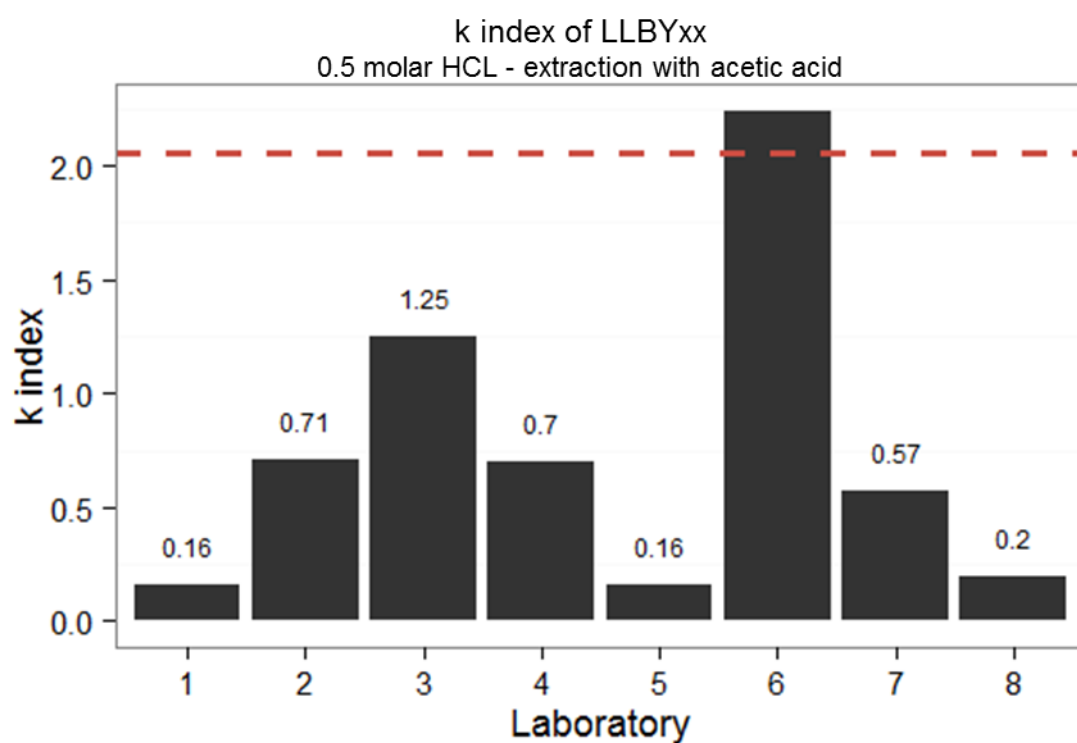


Figure AD 22

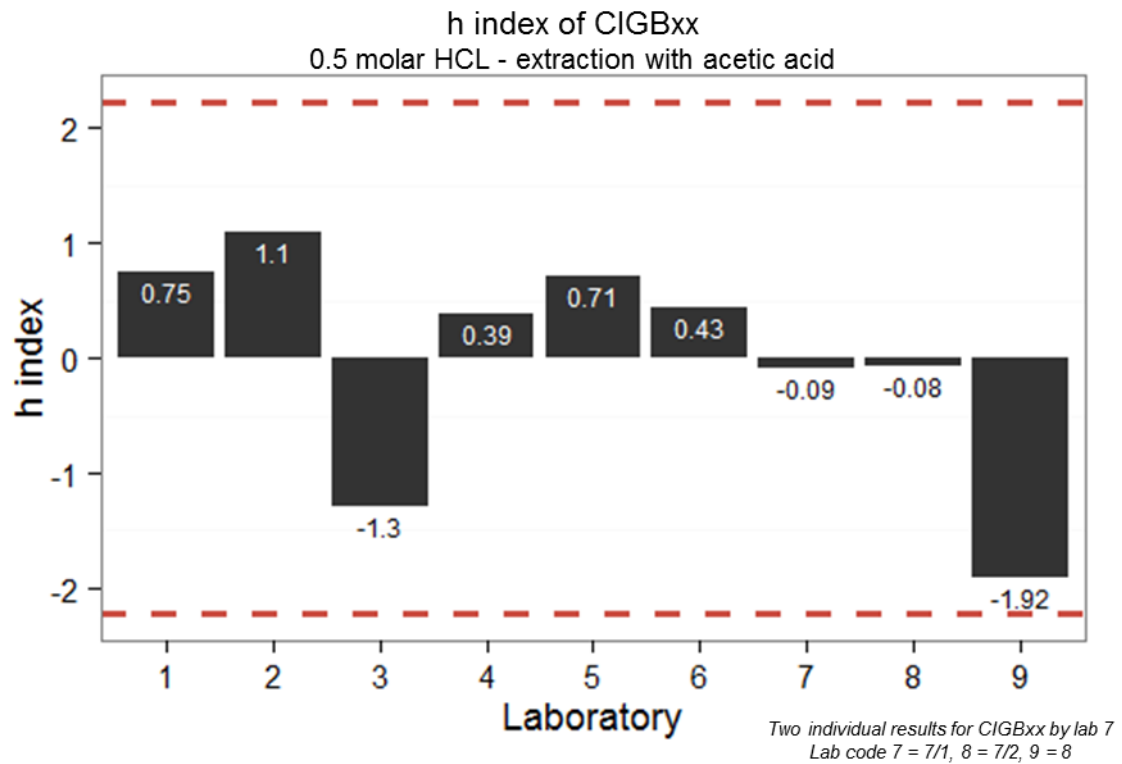


Figure AD 23

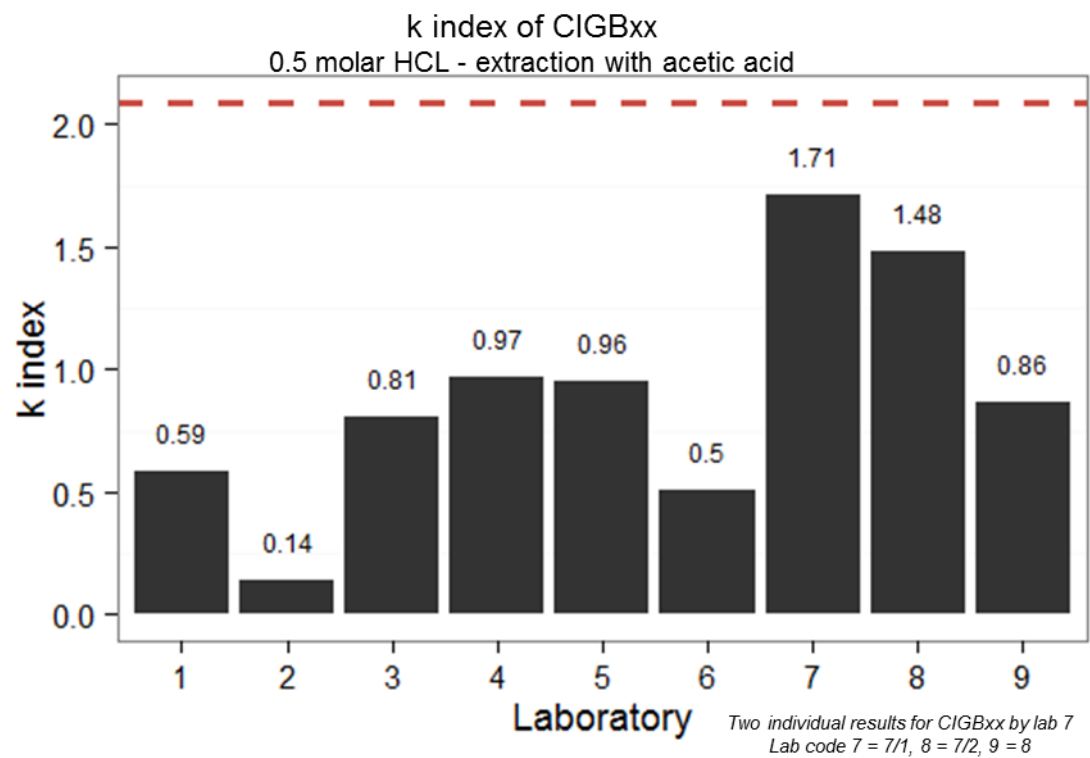


Figure AD 24

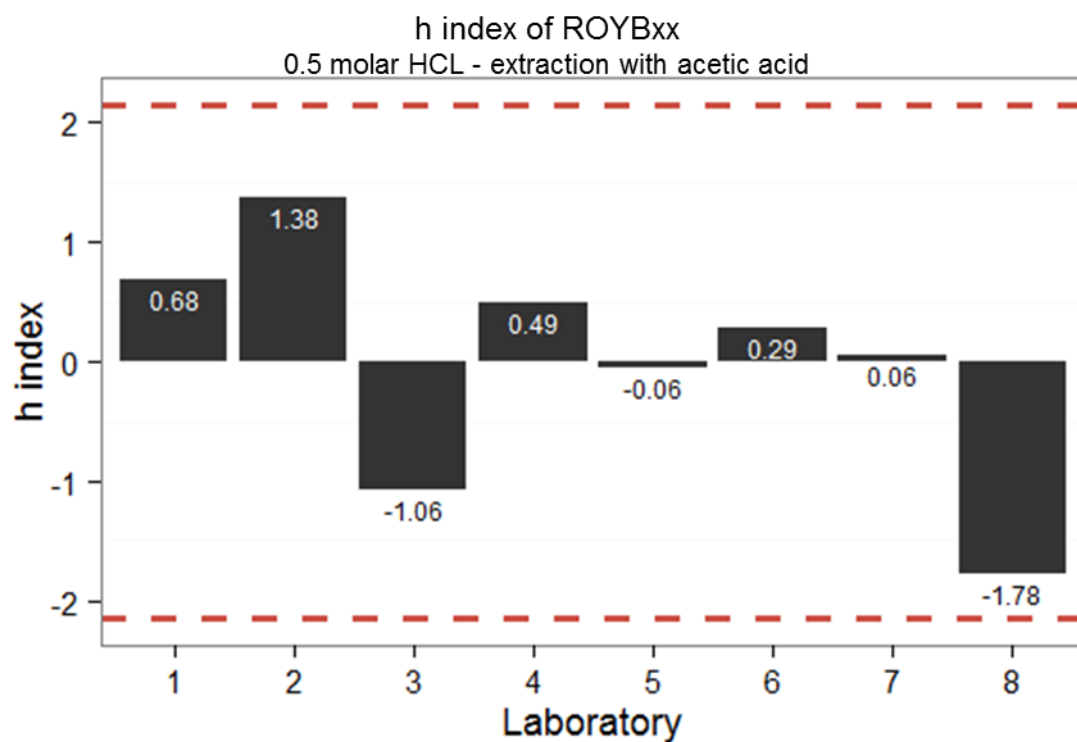


Figure AD 25

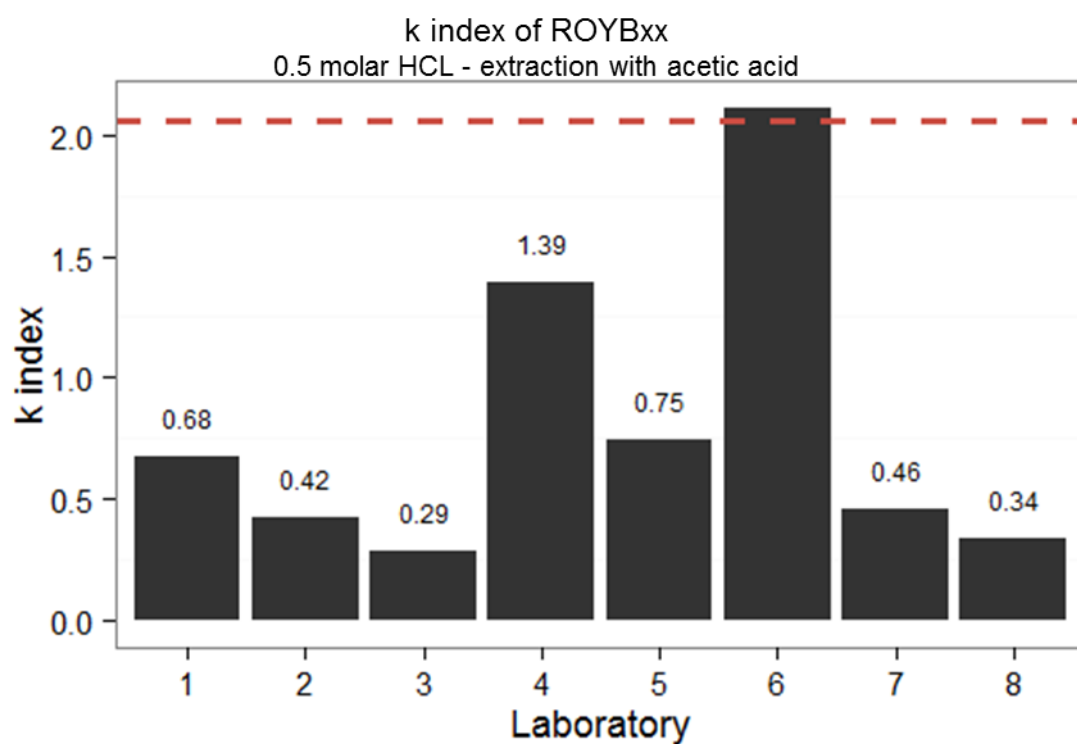


Figure AD 26

h and k Index by Sample for 0.5 Molar Hcl Hydrolysis – Extraction With Water

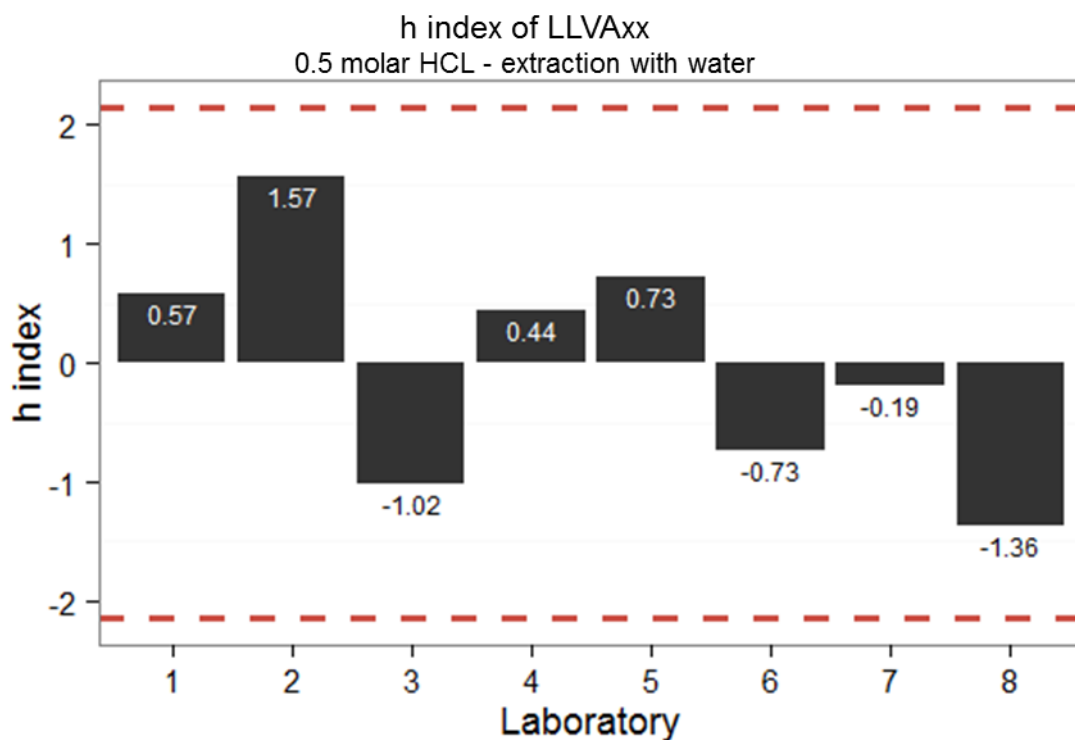


Figure AD 27

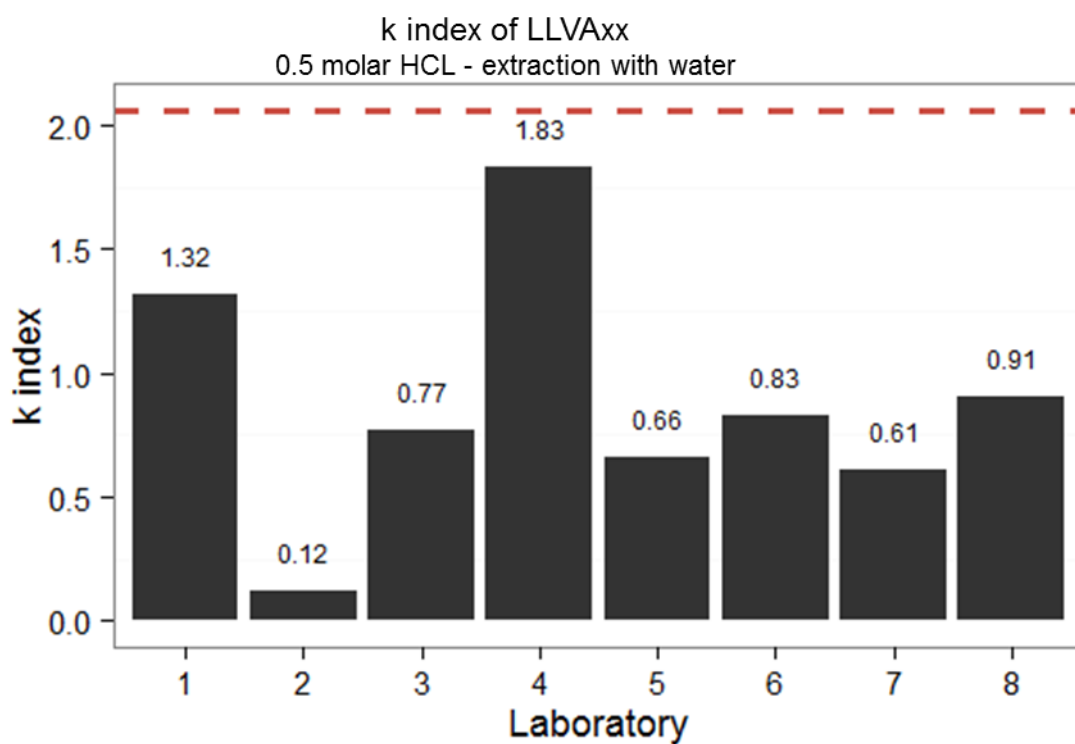


Figure AD 28

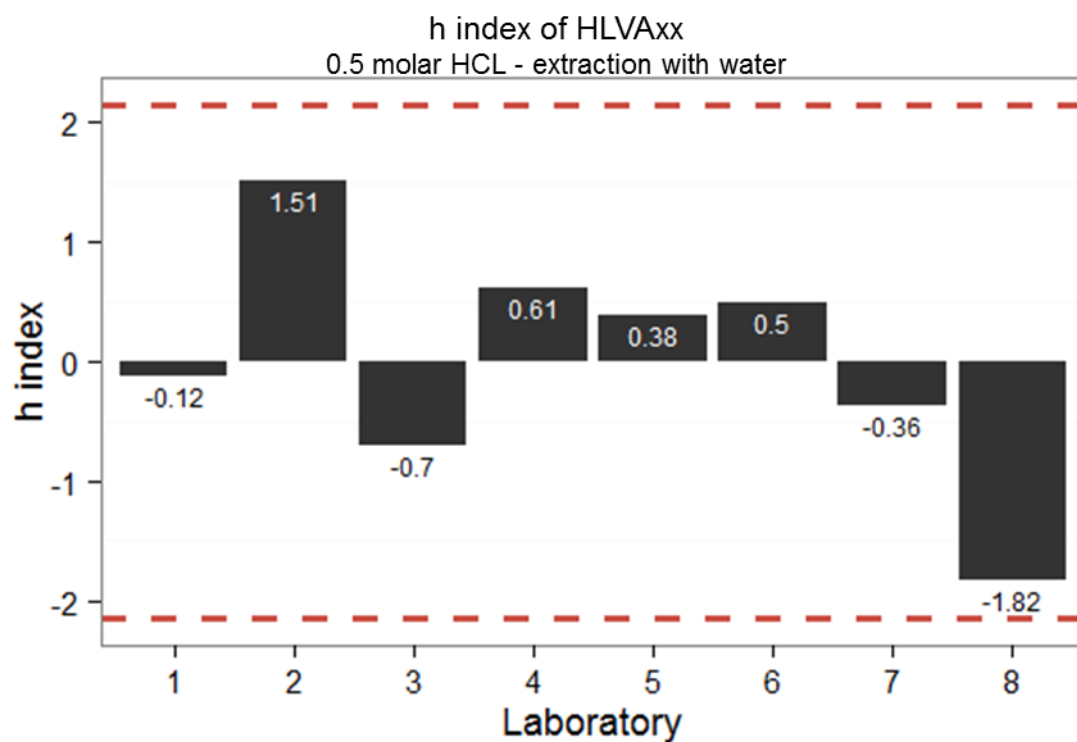


Figure AD 29

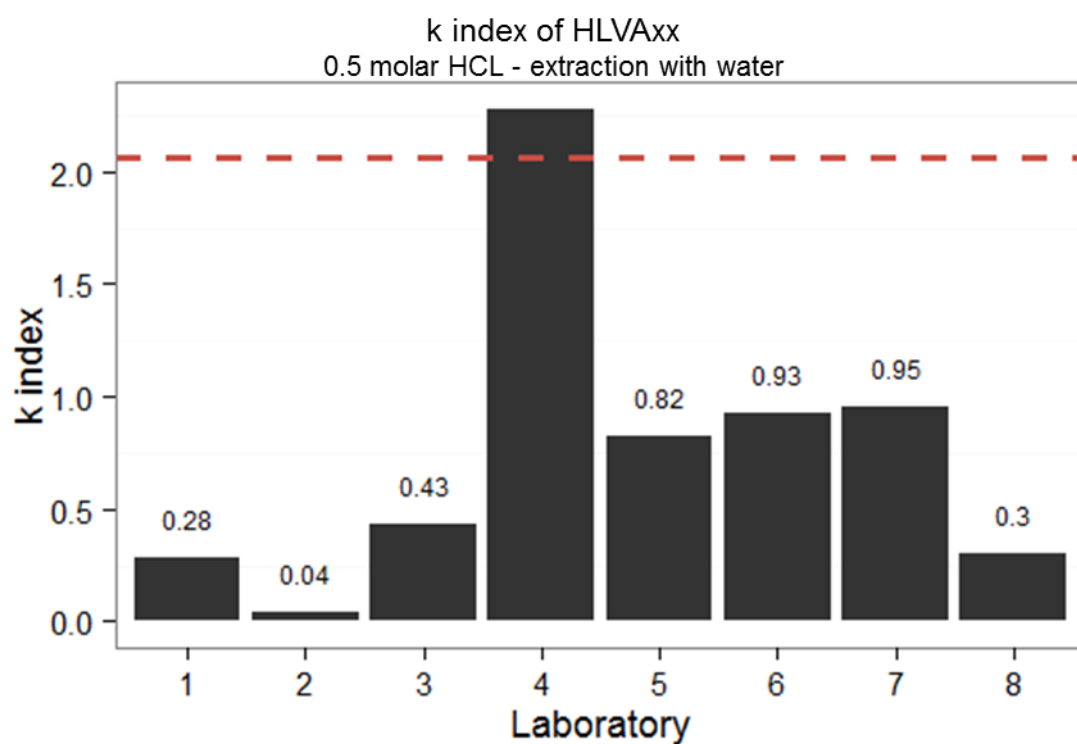


Figure AD 30

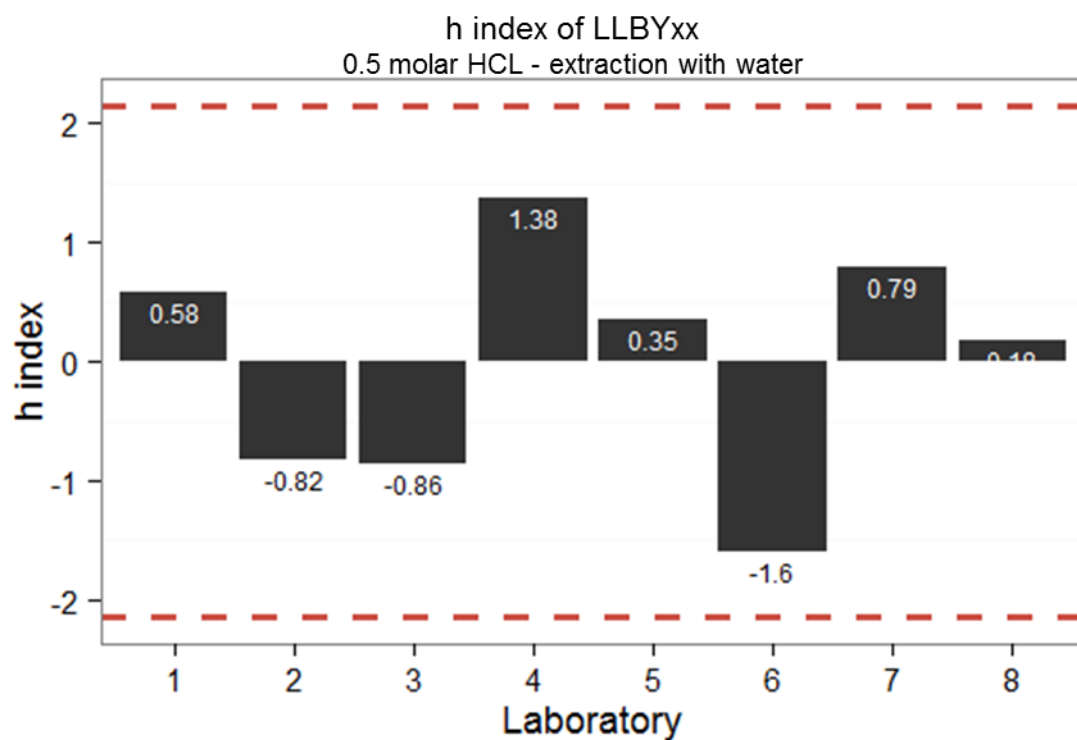


Figure AD 31

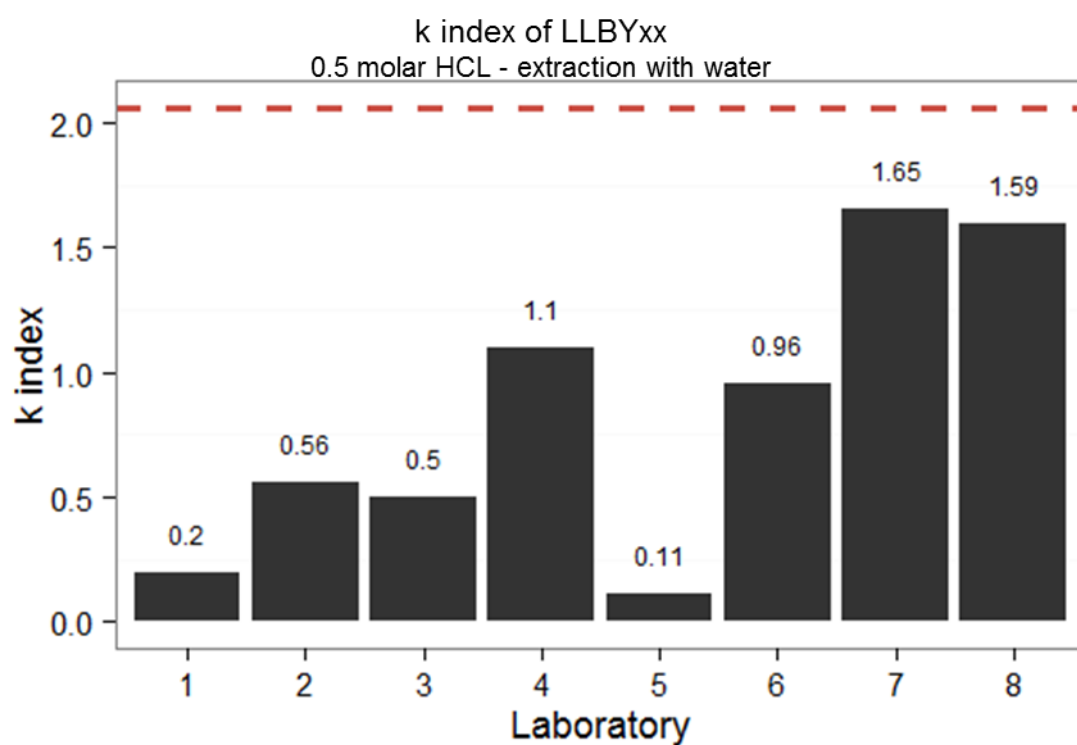


Figure AD 32

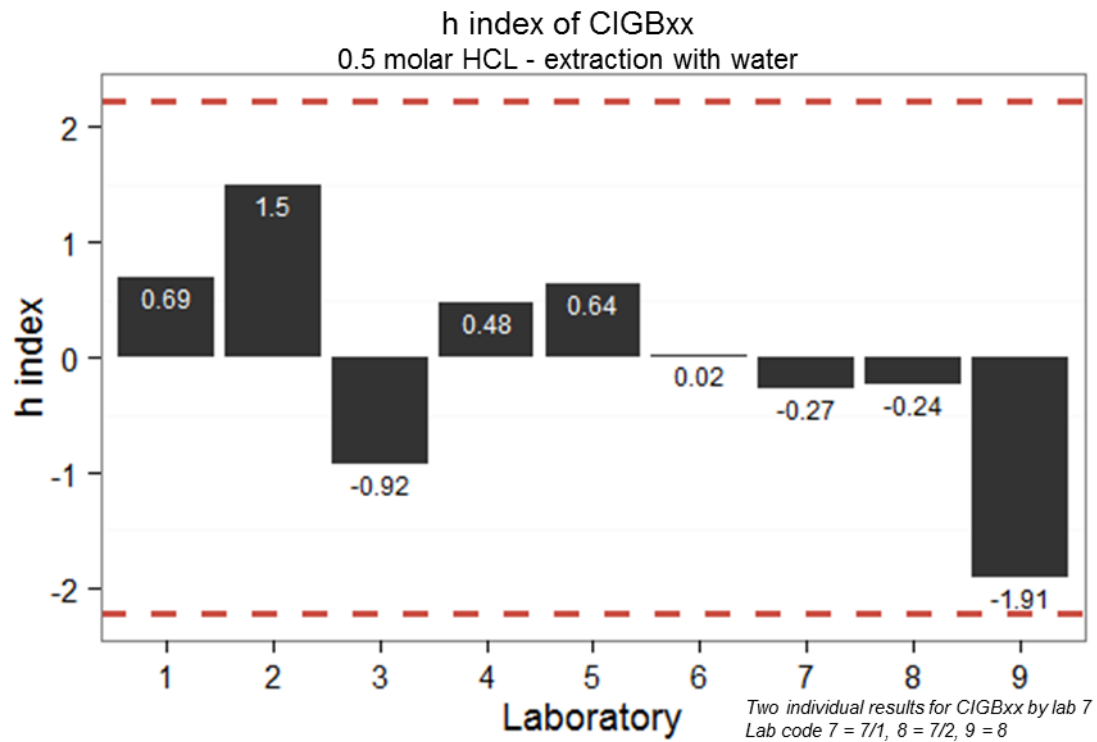


Figure AD 33

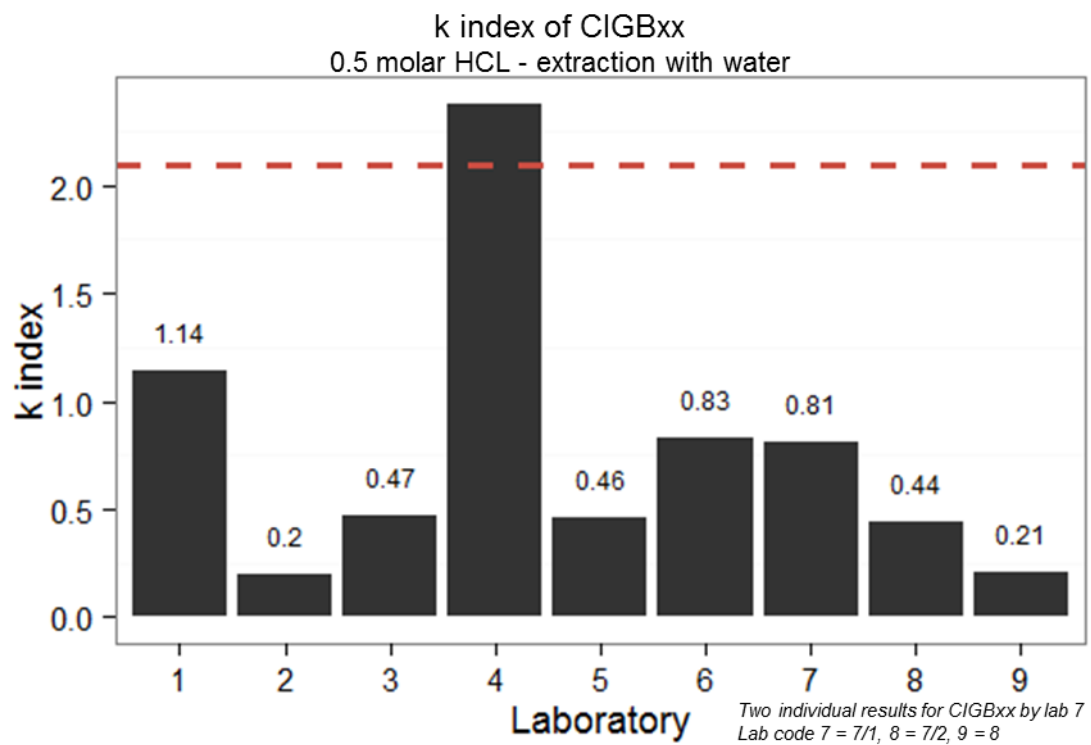


Figure AD 34

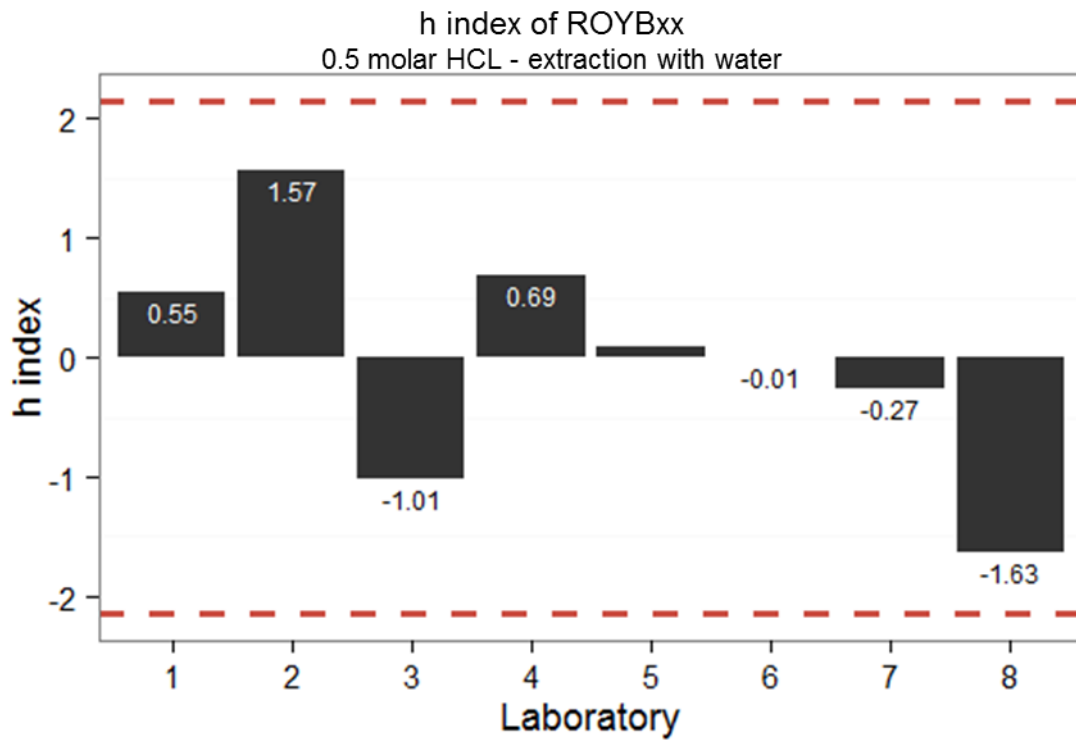


Figure AD 35

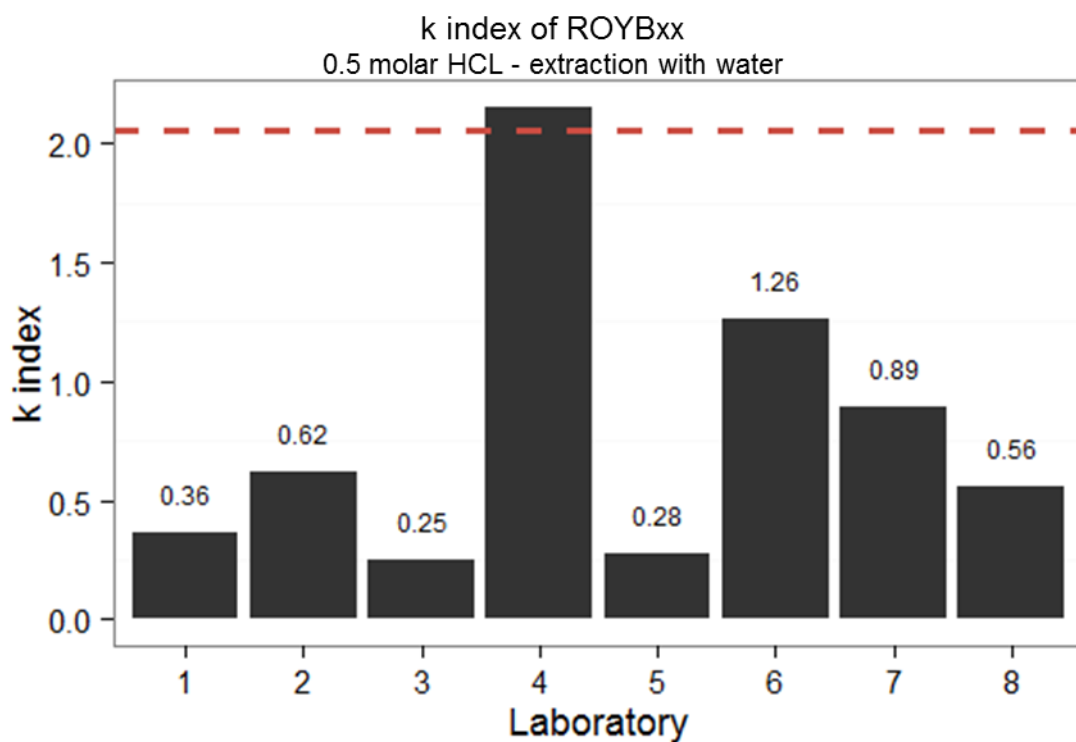


Figure AD 36