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Determination of $^{13}C/^{12}C$ ratios of saccharides in honey by liquid chromatography – isotope ratio mass spectrometry

Results of an interlaboratory comparison

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Contents

Abstract

An interlaboratory comparison (ILC) was organised to provide an opportunity for interested laboratories to assess and compare their competence in determining the $^{13}C/^{12}C$ ratios of fructose, glucose, di- and trisaccharides in honey by using liquid chromatography – isotope ratio mass spectrometry. Fourteen laboratories participated in the ILC and tested six honey samples. For proficiency assessment, the mean values reported back by the participating laboratory were transformed into z-scores by using the consensus value (grand mean) and the reproducibility standard deviation.

For fructose 3 out of 84 z-scores (3.6 %) were $|2| \leq z \leq |3|$, for glucose 2 out of 84 (2.4 %) were $|2| \leq z \leq |3|$ and 4 (4.8 %) were $\geq |3|$, for disaccharides 2 out of 78 (2.6 %) were $|2| \leq z \leq |3|$ and 2 (2.6 %) were $\geq |3|$, for trisaccharides 2 out of 69 (2.9 %) were $|2| \leq z \leq |3|$ and 3 (4.3 %) were $\geq |3|$.

The majority of the participating laboratories demonstrated the proficient use of the applied LC-IRMS for mono-, di- and trisaccharides in honey, which will allow them to apply the technique for detecting adulterated honey samples within the scope of the method. Further guidance on the proper detection and evaluation of the oligosaccharide fraction will be needed to provide proof that the method is fit for compliance assessment of honey with purity criteria.

In general, the results of the ILC demonstrate that LC-IRMS is a suitable technique for determining carbon isotope ratios of fructose, glucose, di- and trisaccharides in honey with sufficient precision and it is fit for assessing whether sugar syrups have been added to honey, within the limits of the method.

Introduction

Honey quality is regulated at international level by the FAO/WHO Codex Alimentarius (CODEX STAN 12-1981 [1], and accordingly at European level by Council Directive 2001/110/EC [2] establishing methods for their analysis.

The European Commission has regularly been informed of the presence on the market, in a potentially significant proportion, of honey that may not meet the composition criteria laid down by the Council Directive 2001/110/EC and/or that is not the result of the production process required by the legal definition of honey. Moreover, despite the progress realised so far, scientific knowledge concerning honey chemistry and technology lags behind the inventiveness of dishonest operators. Therefore, the development of efficient anti-fraud methods is necessary in order to avoid disturbance of the market and the deterioration of the image of honey.

Isotopes of the light elements can provide useful information regarding the authenticity of food products. Most agronomically important plants use the Calvin–Benson photosynthetic cycle (C3 plants), while certain plants originating from warmer climates use the Hatch–Slack pathway (C4 plants). Carbon isotopes ratios, determined by isotope ratio mass spectrometry (IRMS), can be employed to discriminate plants that use different biochemical pathways of CO_2 fixation.

Adulteration of honey with sugar syrups produced from maize starch or from sugar cane, which are C4 plants, can be detected by IRMS coupled to an elemental analyser (EA-IRMS) down to a level of 7 % addition [3]. Exogenous sugar syrups derived from C3 plants such as beet root, wheat, rice, potato cannot be detected by this method. A novel approach combining chromatographic separation of honey saccharides by liquid chromatography and IRMS (LC-IRMS) can detect syrups originating from C3 plants at levels in excess of 10 % and allows detection of maize starch syrups down to 1 % [4]. Although the LC-IRMS method is in routine use for detecting honey adulteration with exogenous sugar syrups, it has not been subjected to multi-laboratory validation and was not yet included in proficiency testing schemes. Therefore, an interlaboratory comparison was organised to explore how well laboratory results produced by LC-IRMS methods in routine usage agree and how well laboratories perform.

Purpose and scope

The interlaboratory comparison was organised to assess the agreement of testing results produced by applying LC-IRMS for determining the $^{13}C/^{12}C$ ratios of saccharides in honey with a view to providing evidence that the approach is fit for detecting adulterated honey. The generated data should also allow evaluating the performance of participating laboratories.

Organisation of the interlaboratory comparison

<u>Test items</u>

Bulk honey samples were warmed in a water bath to 40 °C, homogenised by stirring and aliquots of 2 g filled into screw-cap glass vials, coded and stored at room temperature in the dark until dispatch (Table 1).

Table 1. Characteristics of honey samples used in the interlaboratory comparison.

Sample code	Characteristic	Indicated by
HS-00045 *(*Monofloral - Lemon*)*	Adulterated	Presence of C4 sugars by EA/LC-IRMS
HS-00048 (Polyfloral)	Genuine	Conformity with purity criteria [5] and absence of oligosaccharides
HS-00329 (Honeydew)	Undecided	Presence of oligosaccharides by LC-IRMS but otherwise conforming with purity criteria [5]
HS-00378 (Honeydew)	Adulterated	Presence of oligosaccharides by LC-IRMS and UHPLC-MS
HS-00959 (Monofloral - Acacia)	Undecided	Different δ ^{13}C observed in the trisaccharide fraction
HS-01158 (Monofloral - Lavender)	Genuine	Conformity with purity criteria [5] and absence of oligosaccharides

<u>Homogeneity and stability</u>

Homogeneity of the test items was not studied as honey is a liquid, though viscous, but stirring at elevated temperature should ensure proper homogeneity. Stability of the isotope ratio values was assumed over the duration of the ILC.

<u>Distribution of test items and timing of the ILC</u>

Test items were dispatched by over-night courier on 06/12/2017 and the reporting deadline was 28/01/2018. However, the last report was received on 31/07/2018.

Instructions to partic0ipants

Participants were free to apply an LC-IRMS method of their choice and were requested to make duplicate analysis of all samples and report $\delta^{13}C$ (‰) values for fructose, glucose, di- and trisaccharides as well as the area-% for the oligosaccharide peak. They were provided with MS Excel files to report test conditions and test results to the ILC organiser and instructed to store the test items in a cool and dry place, warm the test items and stir them before taking test portions. $\delta^{13}C$ (‰) values had to be calculated as $[(^{13}C/^{12}C \text{ sample} - ^{13}C/^{12}C \text{ reference}) \times 1000] \div ^{13}C/^{12}C \text{ reference}$; the reference either being Vienna Pee Dee Belemnite (VPDB) or a secondary reference material traceable to VPDB.

<u>Confidentiality</u>

The ILC organiser committed to anonymise testing results reported by the participants and not share raw data with third parties.

Results

Data reported back by the participants were checked for completeness and validity and plotted (Figures A1 to A4 in the Annex).

Details of the analytical methods used by the participants are given in Table 2. All participants but one used polymeric styrene-divinylbenzene columns for sugar separation; one participant used a CarboPac anion-exchange column instead.

Table 2. Experimental conditions used by the interlaboratory comparison participants.

Lab	Column	Flow-rate (ml/min)
1 [1)]	Shodex SUGAR Series® 7 µm, 20 Å, 300 x 8 m)m (pre-column: Shodex Guard Column, 8 µm SP-G Spherical Polymer, 50 x 6 mm)	0.50
2	Agilent HiPlex Ca column, 7.7 x 300 mm (pre-column: Agilent HiPlex Ca column, 7.7 x 50 mm)	0.60
3	Phenomenex Rezex RCM - Monosaccharide Ca2+ (8%), 300 x 7.8 mm	0.45
4	Phenomenex Rezex Ca^{2+}, 300 x 8 mm	0.30
5	Phenomenex Rezex RCM-Monosaccharide Ca2+ (8%)	0.40
6 [2)]	repromer H+, Dr. Maisch (pre-column: repromer H+, Dr. Maisch)	0.25
7	ThermoFisher XP HyperREZ Carbohydrate H+, 8 µm, 300 x 8 mm	0.40
8	Alltech 700CH carbohydrate column	0.40
9	Ca^{2+} column, 8 µm, 300 x 7.8 mm (pre-column: Ca2+ column, 8 µm, 10 x 7.8 mm)	0.30
10	ThermoFisher CarboPac PA1	0.20
11	Phenomenex Rezex ECM-monosaccharide Ca2+, 300 x 7.8 mm	0.30
12	Waters Sugar-Pack I, 10 µm, 300 x 6.5 mm	0.50
13	no details reported	
14	Phenomenex Rezex RCM-monosacharide Ca^{2+}, 300 x 7.8 mm	0.30

[1)] Collection of sugar fractions, followed by EA-IRMS

[2)] Thermal conversion interface

Individual laboratory performance was assessed in terms of z-scores in accordance with ISO 13528:2005 [6] and the IUPAC International Harmonised Protocol [7].

$$\text{z-score} = (x_{lab} - X_{ref}) / \sigma_p$$

where:

x_{lab} is the measurement result reported by a participant
X_{ref} is the reference value (grand mean of participant results)
σ_p is the standard deviation for proficiency assessment (target standard deviation)

The grand mean of all valid results reported by the ILC participants was taken as the reference value X_{ref}. A standard deviation for proficiency assessment (target standard deviation) was not set by the ILC organiser as the Horwitz equation [8], which is frequently used for that purpose, cannot be applied to measurands expressed as isotope ratios instead of mass ratios. Therefore, the reproducibility standard deviation calculated according to ISO 5725:2005 [9] was used as the target standard deviation σ_p for deriving z-scores. Consensus means and standard deviations for the test items are given in Table 3.

Table 3. Consensus means and reproducibility standard deviations for $\delta^{13}C$ (‰) values of saccharides in the test items.

	Honey					
	HS-00045	HS-00048	HS-00329	HS-00378	HS-00959	HS-01158
Fructose						
Mean	-24.33	-25.07	-24.40	-24.26	-24.49	-26.41
Reproducibility standard deviation	0.38	0.28	0.19	0.27	0.38	0.37
Glucose						
Mean	-24.43	-24.47	-23.58	-23.71	-24.53	-26.03
Reproducibility standard deviation	0.31	0.43	0.34	0.33	0.29	0.42
Disaccharides						
Mean	-24.85	-26.27	-25.23	-25.13	-24.88	-27.01
Reproducibility standard deviation	0.35	0.34	0.36	0.24	0.36	0.31
Trisaccharides						
Mean	-24.05	-26.74	-24.69	-25.18	-22.73	-25.73
Reproducibility standard deviation	0.68	0.36	0.35	0.50	0.45	0.57

The z-scores for all test items and measurands are graphically summarised in Figures 1 to 4, and the respective numerical values are given in the Annex, Tables A1 to A4.

Figure 1. z-scores for $\delta^{13}C$ (‰) values for fructose in the ILC test items (blue: z<|2|; yellow: |2|<z<|3|, red: z>|3|).

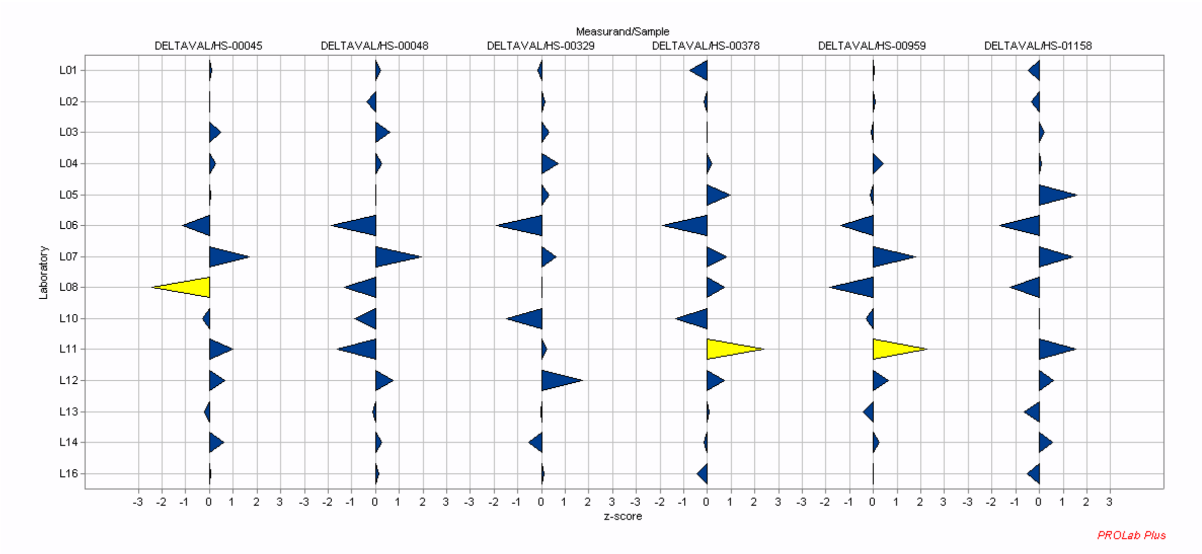


Figure 2. z-scores for $\delta^{13}C$ (‰) values for glucose in the ILC test items (blue: z<|2|; yellow: |2|<z<|3|, red: z>|3|).

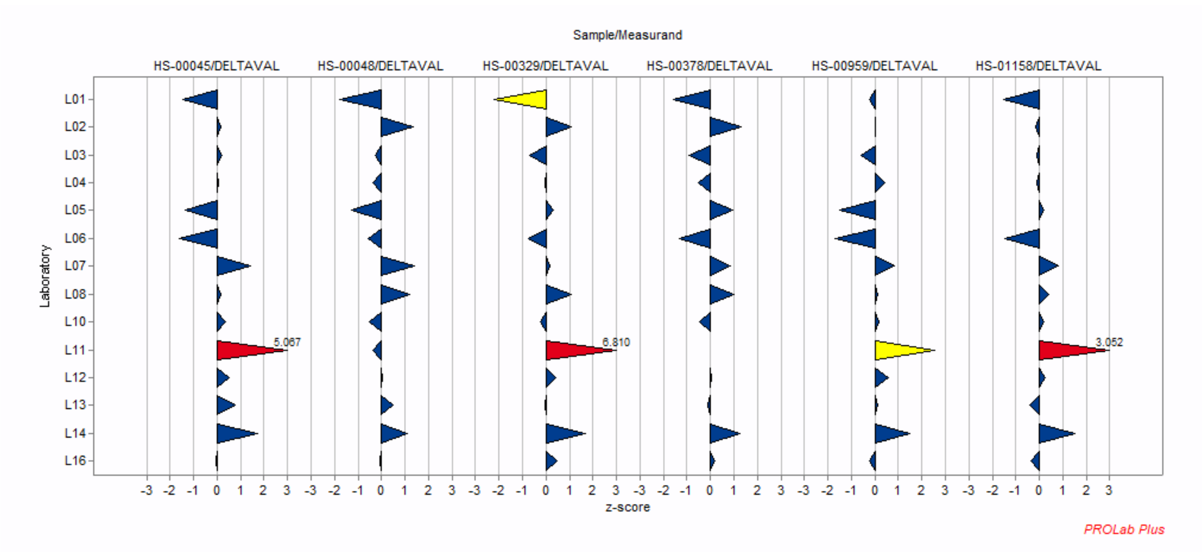


Figure 3. z-scores for $\delta^{13}C$ (‰) values for disaccharides in the ILC test items (blue: z<|2|; yellow: |2|<z<|3|, red: z>|3|).

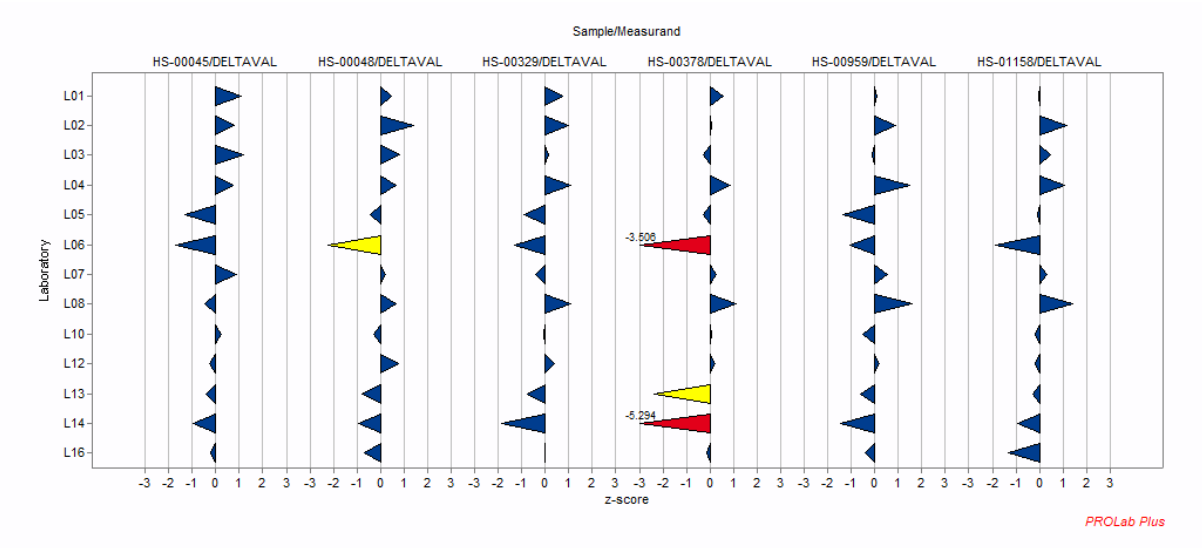
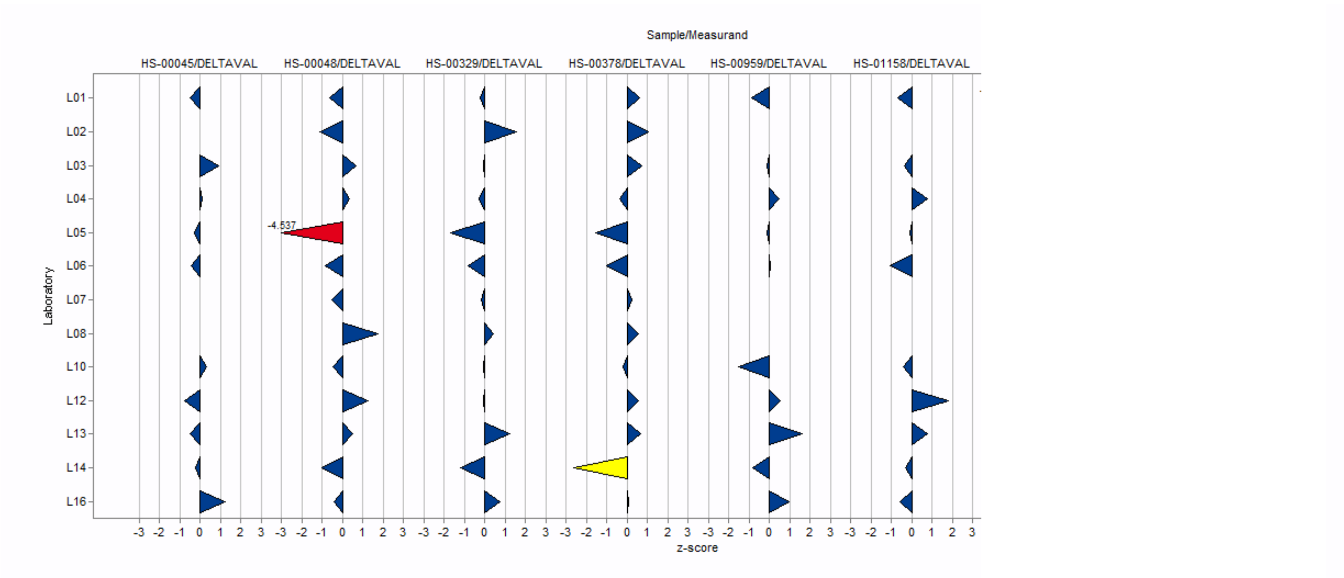


Figure 4. z-scores for $\delta^{13}C$ (‰) values for trisaccharides in the ILC test items (blue: z<|2|; yellow: |2|<z<|3|, red: z>|3|).



Discussion

Most participating laboratories obtained z-scores ≤ |2| for nearly all of the test items, which was expected, as the z-scores were computed on the basis of the grand mean and the standard deviation of the reported values. However, this does not lessen the validity of the ILC outcome as the reproducibility standard deviation obtained in this exercise was in good agreement with respective values reported by the Forensic Isotope Ratio Mass Spectrometry (FIRMS) network (median $\delta^{13}C$ of 0.20 ‰ for 6 ILCs) [10], and for hard cheeses (0. 2 ‰ for $\delta^{13}C$ values) [11]. The reproducibility standard deviation for trisaccharides was higher, which may be due to their lower amount in honey but also due to the inability of polymeric styrene-divinylbenzene columns to separate individual trisaccharides, resulting in overlapping peaks with broad widths and sometimes irregular peak shapes. This was even more pronounced for the oligosaccharide fraction; only few participants reported values for oligosaccharides (as area-%), when present (i.e. HS-00329 & HS-00378), and therefore, oligosaccharides were excluded from further evaluations.

The laboratory using a strong anion-exchange column for sugar separation reported only values for the monosaccharides and the obtained z-scores were for some of the test items $\geq |3|$.

For fructose 3 out of 84 z-scores (3.6 %) were $|2| \leq z \leq |3|$, for glucose 2 out of 84 (2.4 %) were $|2| \leq z \leq |3|$ and 4 (4.8 %) were $\geq |3|$, for disaccharides 2 out of 78 (2.6 %) were $|2| \leq z \leq |3|$ and 2 (2.6 %) were $\geq |3|$, for trisaccharides 2 out of 69 (2.9 %) were $|2| \leq z \leq |3|$ and 3 (4.3 %) were $\geq |3|$.

According to the results obtained in this ILC the majority of the participating laboratories had the applied LC-IRMS under control for mono-, di- and trisaccharides, which will allow them to make use of the technique for detecting adulterated honey samples within the scope of the method. For oligosaccharides only a few data sets were reported, which did not allow to make meaningful statistical analyses. Further guidance on the proper detection and evaluation of the oligosaccharide fraction will be needed to provide proof that the method is fit for compliance assessment of honey with purity criteria.

In general, the utility of LC-IRMS for determining carbon isotope ratios of fructose, glucose, di- and trisaccharides with sufficient precision was demonstrated by this ILC and that the measurement procedure is fit for assessing whether sugar syrups have been added to honey, within the limits of the method.

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- Elementar Analysensysteme GmbH, Langenselbold, Germany
- Eurofins Food Integrity Control Services GmbH, Ritterhude, Germany
- FERA Science Ltd, York, United Kingdom
- Floramo Corporation Srl, Rocca de'Baldi (CN), Italy
- Imprint Analytics GmbH, Neutal, Austria
- Institut des Sciences Analytiques (ISA), Villeurbanne, France
- Intertek Food Services GmbH, Bremen, Germany
- LAVES Lebensmittel- und Veterinärinstitut Oldenburg, Oldenburg, Germany
- MAPAMA - Laboratorio Arbitral Agroalimentario, Madrid, Spain
- QSI - Quality Services International GmbH, Bremen, Germany

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[1] FAO/WHO Codex Standard for Honey, CODEX STAN 12-1981, http://www.fao.org/3/w0076e/w0076e30.htm

[2] Council Directive 2001/110/EC of 20 December 2001 relating to honey, https://eur-lex.europa.eu/legal-content/EN/ALL/?uri=CELEX%3A32001L0110

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Annex

Figure A1a. Laboratory results for δ^{13}C (‰) values for fructose in sample HS-00045 (limit of tolerance gives the range for z-scores ≤ |2|).

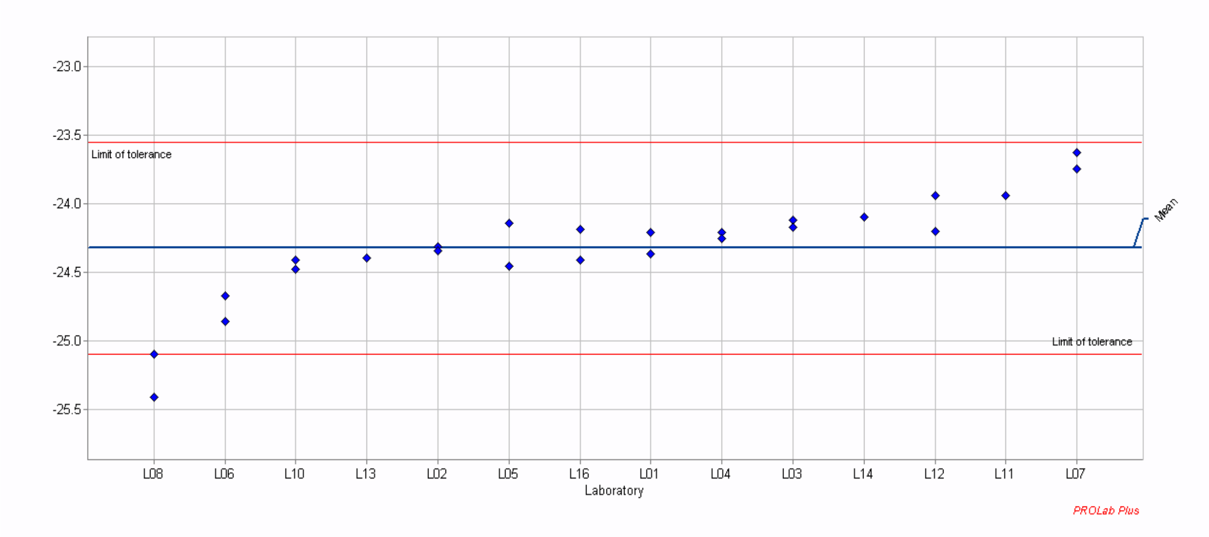

Figure A1b. Laboratory results for δ^{13}C (‰) values for fructose in sample HS-00048 (limit of tolerance gives the range for z-scores ≤ |2|).

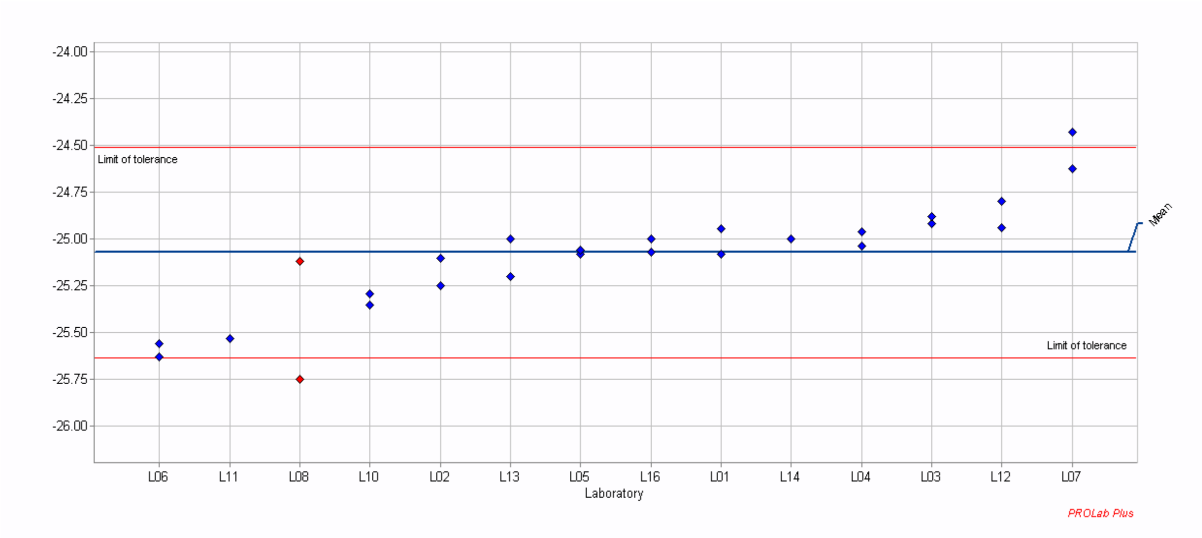

Figure A1c. Laboratory results for δ^{13}C (‰) values for fructose in sample HS-00329 (limit of tolerance gives the range for z-scores ≤ |2|).

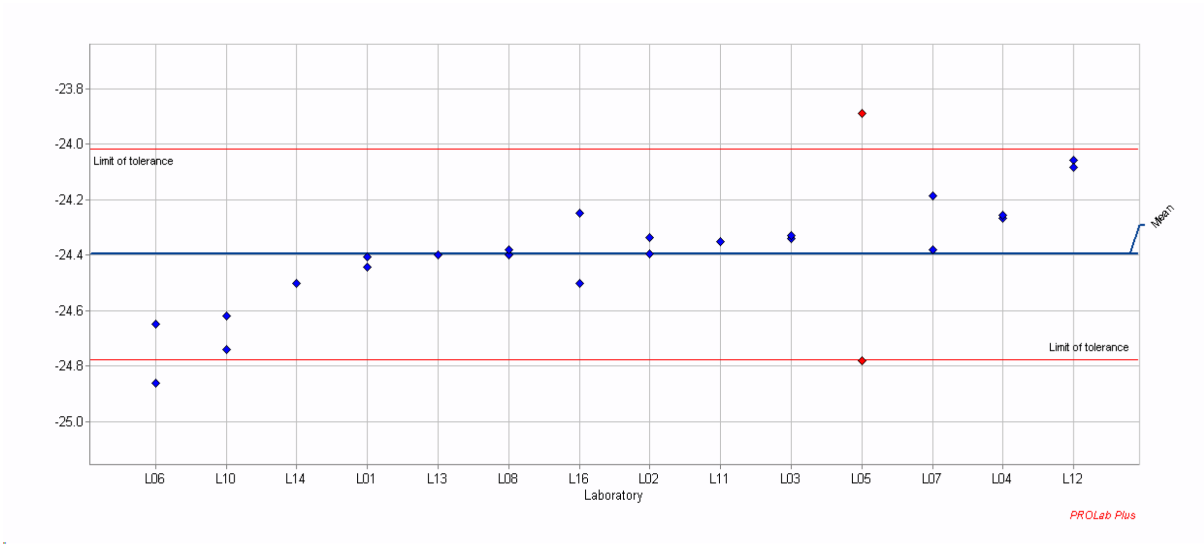

Figure A1d. Laboratory results for δ^{13}C (‰) values for fructose in sample HS-00378 (limit of tolerance gives the range for z-scores ≤ |2|).

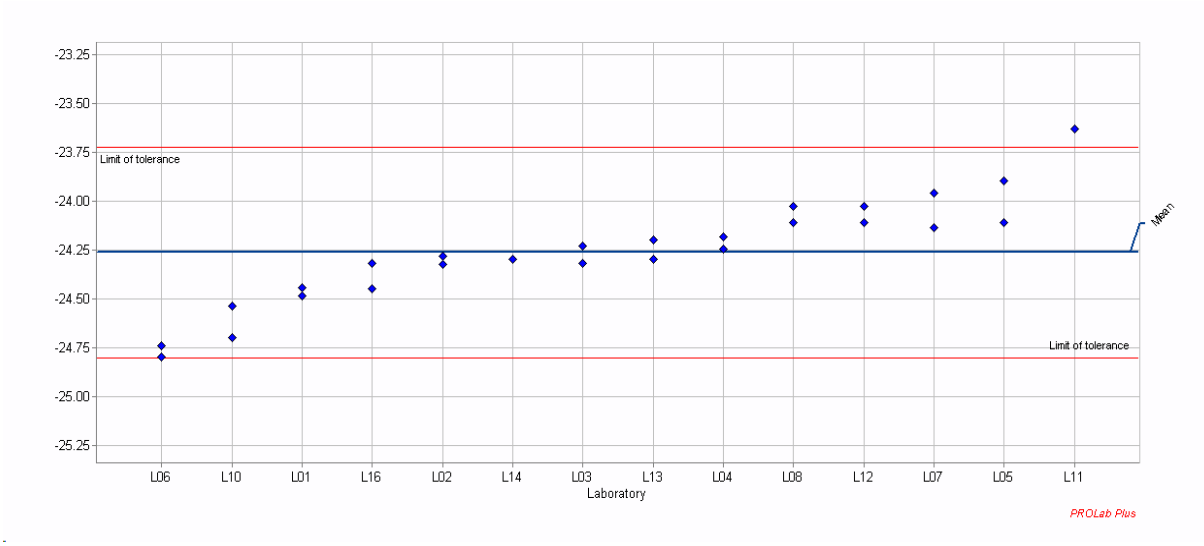

Figure A1e. Laboratory results for δ^{13}C (‰) values for fructose in sample HS-00959 (limit of tolerance gives the range for z-scores ≤ |2|).

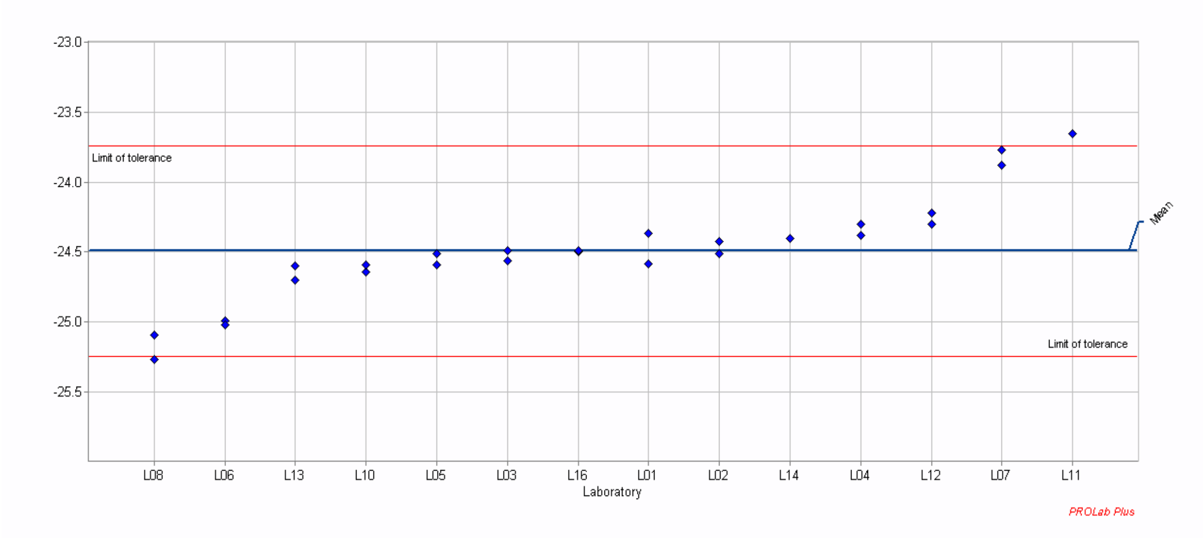

Figure A1f. Laboratory results for δ^{13}C (‰) values for fructose in sample HS-01158 (limit of tolerance gives the range for z-scores ≤ |2|).

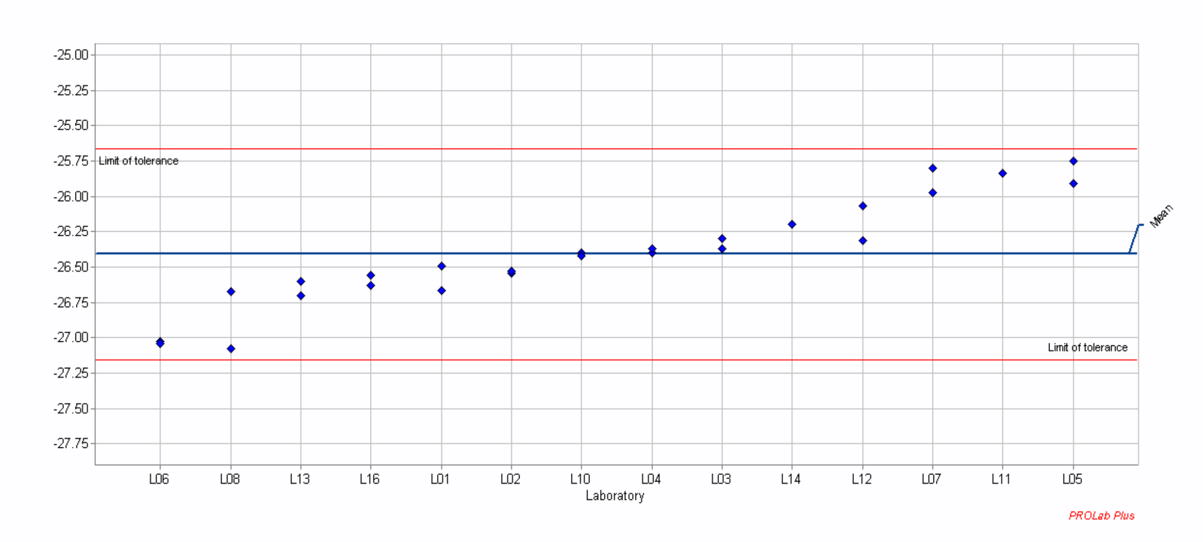

Table 1A. z-Scores for δ^{13}C (‰) values for fructose.

Laboratory	HS-00045	HS-00048	HS-00329	HS-00378	HS-00959	HS-01158
L01	0.10	0.21	-0.14	-0.75	0.05	-0.45
L02	-0.01	-0.36	0.16	-0.14	0.07	-0.34
L03	0.47	0.61	0.33	-0.04	-0.09	0.20
L04	0.25	0.26	0.72	0.18	0.41	0.06
L05	0.07	0.01	0.33	0.97	-0.15	1.56
L06	-1.14	-1.86	-1.89	-1.88	-1.36	-1.68
L07	1.66	1.95	0.60	0.81	1.79	1.40
L08	-2.41	-1.29	0.04	0.72	-1.83	-1.25
L10	-0.31	-0.88	-1.49	-1.32	-0.33	0.00
L11	1.00	-1.63	0.25	2.36	2.24	1.53
L12	0.65	0.72	1.72	0.73	0.62	0.59
L13	-0.19	-0.10	-0.02	0.05	-0.42	-0.64
L14	0.59	0.26	-0.54	-0.13	0.25	0.56
L16	0.07	0.13	0.12	-0.45	-0.01	-0.50

Figure A2a. Laboratory results for δ^{13}C (‰) values for glucose in sample HS-00045 (limit of tolerance gives the range for z-scores ≤ |2|).

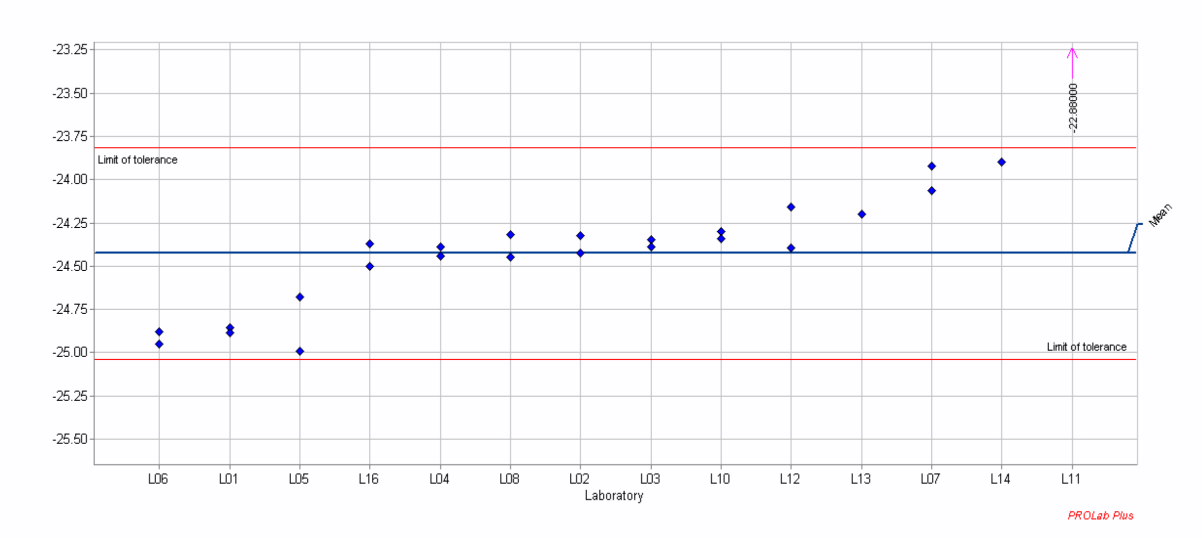

Figure A2b. Laboratory results for δ^{13}C (‰) values for glucose in sample HS-00048 (limit of tolerance gives the range for z-scores ≤ |2|).

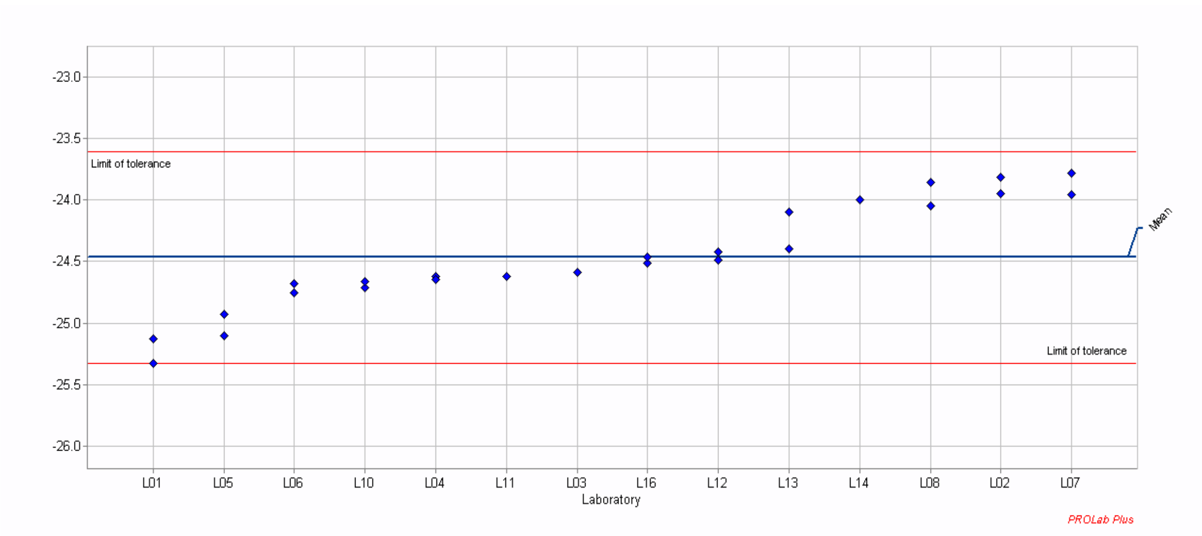

Figure A2c. Laboratory results for δ^{13}C (‰) values for glucose in sample HS-00329 (limit of tolerance gives the range for z-scores ≤ |2|).

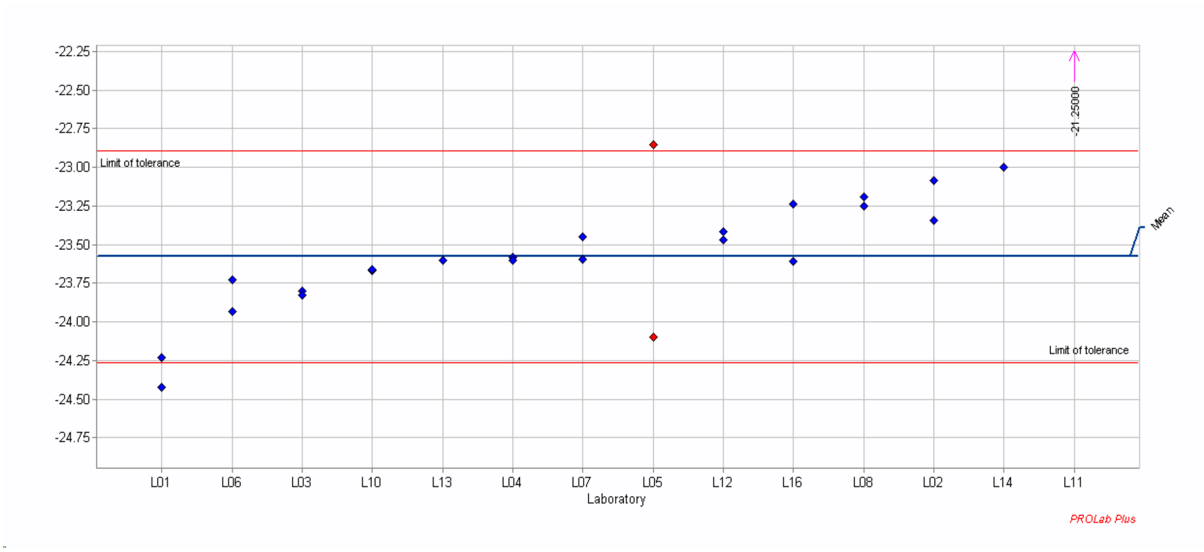

Figure A2d. Laboratory results for δ^{13}C (‰) values for glucose in sample HS-00378 (limit of tolerance gives the range for z-scores ≤ |2|).

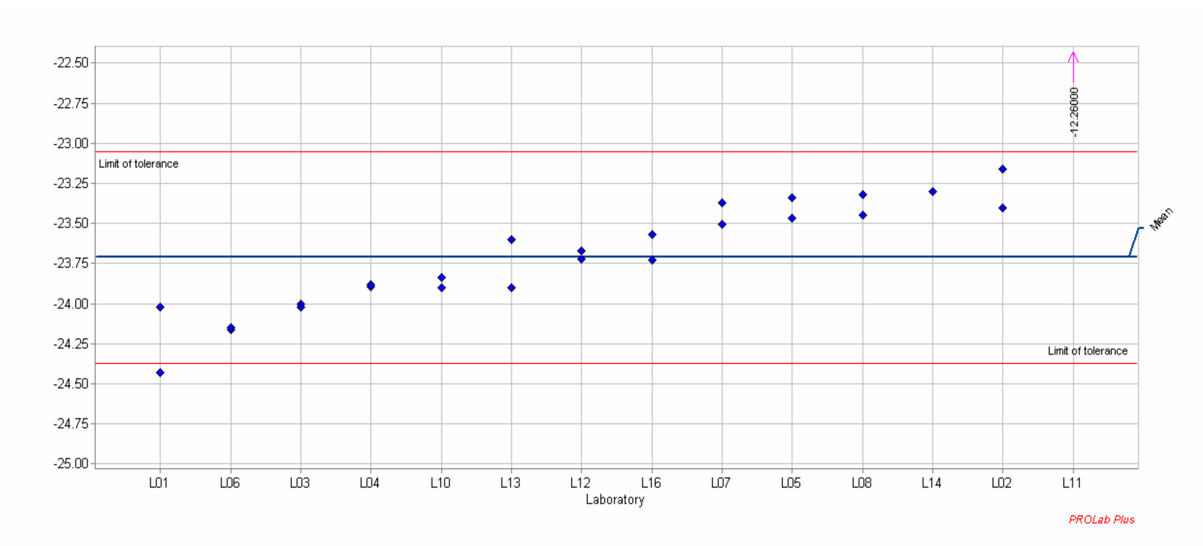

Figure A2e. Laboratory results for $\delta^{13}C$ (‰) values for glucose in sample HS-00959 (limit of tolerance gives the range for z-scores ≤ |2|).

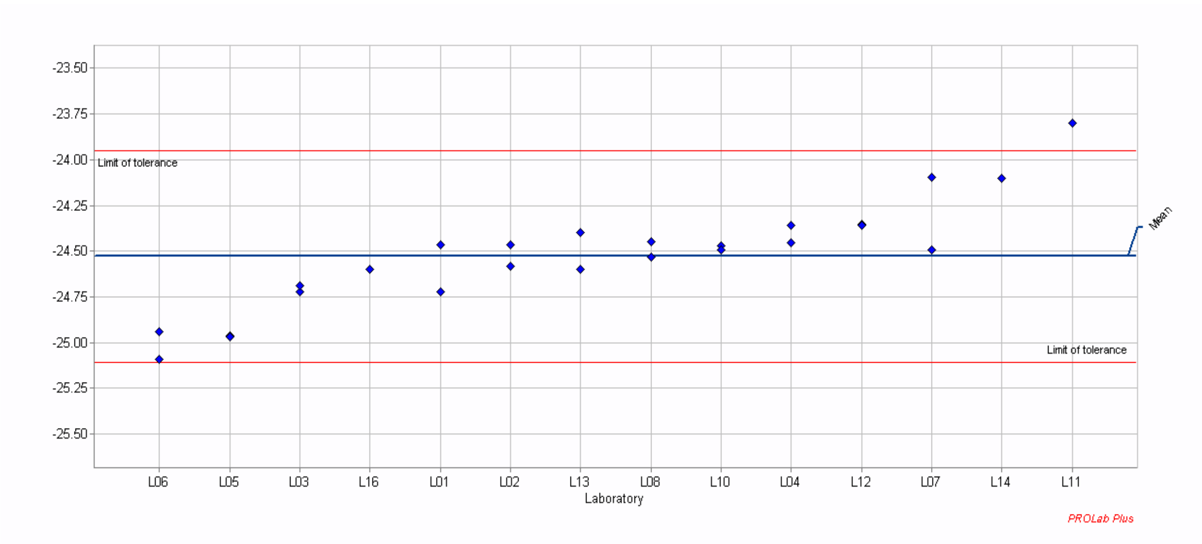

Figure A2f. Laboratory results for $\delta^{13}C$ (‰) values for glucose in sample HS-01158 (limit of tolerance gives the range for z-scores ≤ |2|).

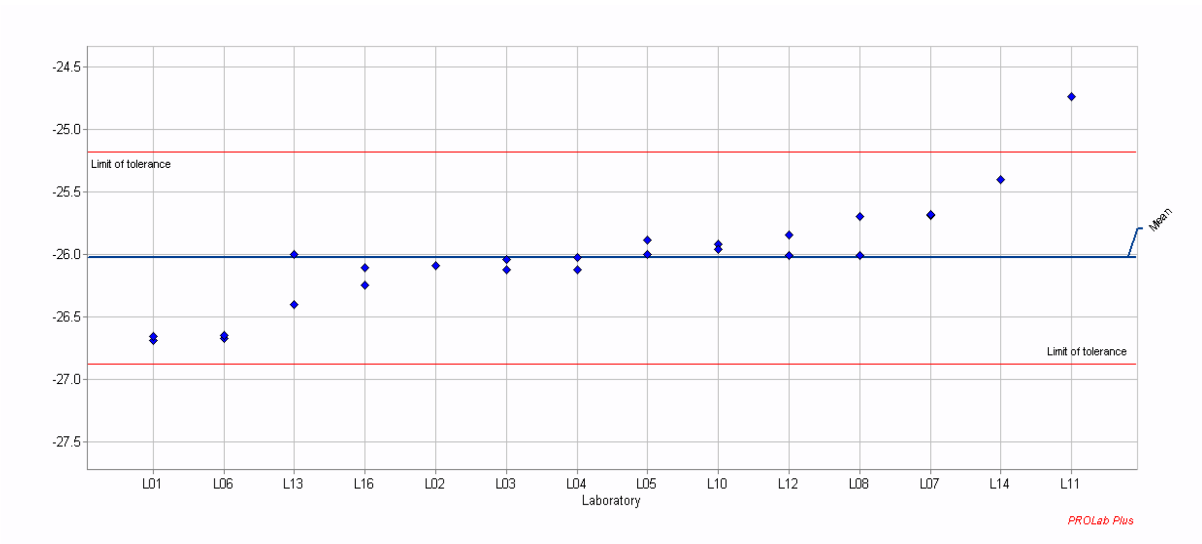

Table 2A. z-Scores for $\delta^{13}C$ (‰) values for glucose.

Laboratory	HS-00045	HS-00048	HS-00329	HS-00378	HS-00959	HS-01158
L01	-1.45	-1.77	-2.19	-1.56	-0.22	-1.52
L02	0.18	1.36	1.06	1.31	0.01	-0.14
L03	0.19	-0.29	-0.69	-0.90	-0.61	-0.12
L04	0.04	-0.39	-0.04	-0.53	0.43	-0.11
L05	-1.34	-1.28	0.30	0.94	-1.51	0.20
L06	-1.60	-0.58	-0.74	-1.34	-1.68	-1.49
L07	1.42	1.39	0.16	0.83	0.81	0.81
L08	0.14	1.19	1.05	1.00	0.14	0.41
L10	0.35	-0.51	-0.25	-0.48	0.17	0.21
L11	5.07	-0.36	6.81	34.79	2.53	3.05
L12	0.50	0.03	0.40	0.05	0.59	0.24
L13	0.74	0.51	-0.06	-0.11	0.10	-0.40
L14	1.73	1.09	1.69	1.25	1.49	1.49
L16	-0.03	-0.04	0.45	0.19	-0.25	-0.36

Figure A3a. Laboratory results for δ^{13}C (‰) values for disaccharides in sample HS-00045 (limit of tolerance gives the range for z-scores ≤ |2|).

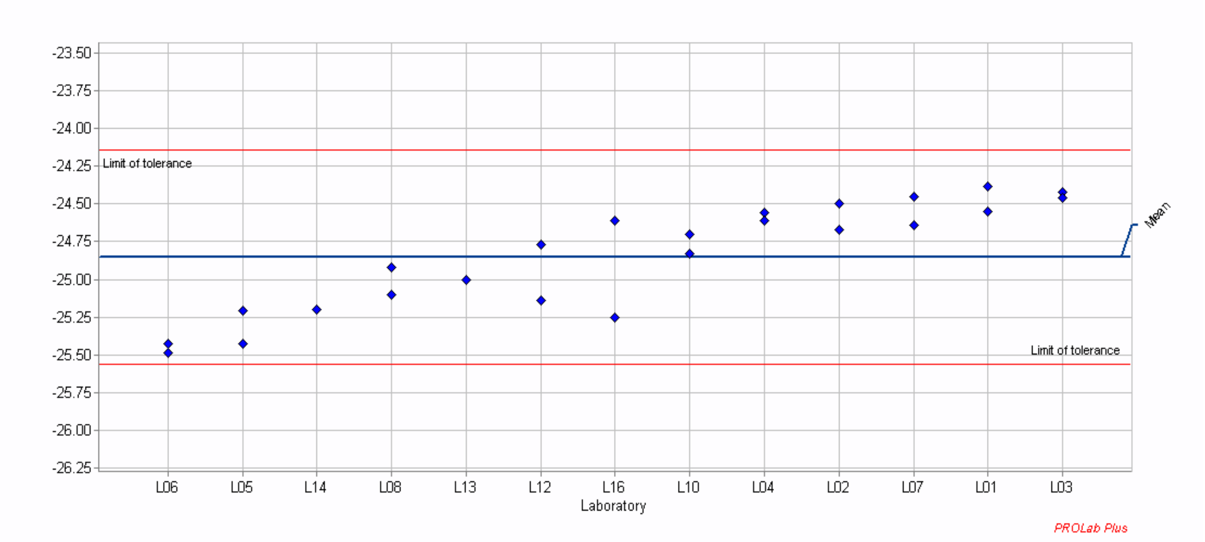

Figure A3b. Laboratory results for δ^{13}C (‰) values for disaccharides in sample HS-00048 (limit of tolerance gives the range for z-scores ≤ |2|).

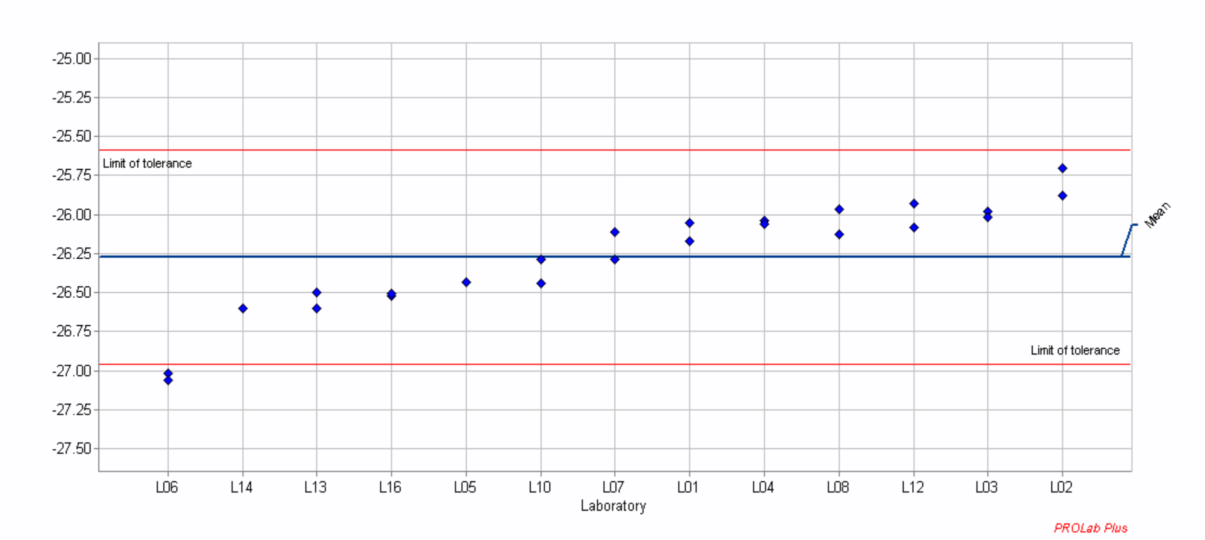

Figure A3c. Laboratory results for δ^{13}C (‰) values for disaccharides in sample HS-00329 (limit of tolerance gives the range for z-scores ≤ |2|).

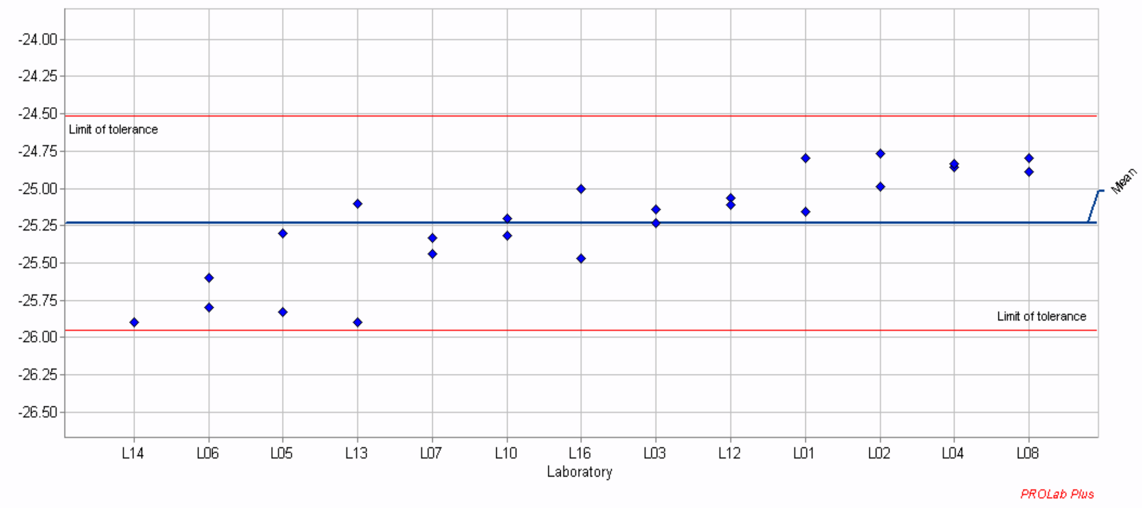

Figure A3d. Laboratory results for δ^{13}C (‰) values for disaccharides in sample HS-00378 (limit of tolerance gives the range for z-scores ≤ |2|).

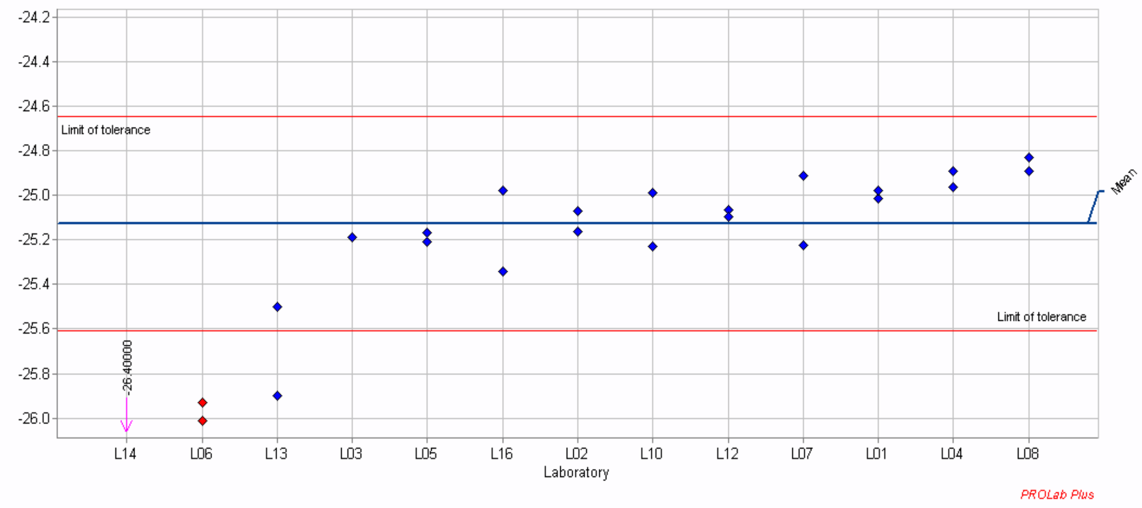

Figure A3e. Laboratory results for δ^{13}C (‰) values for disaccharides in sample HS-00959 (limit of tolerance gives the range for z-scores ≤ |2|).

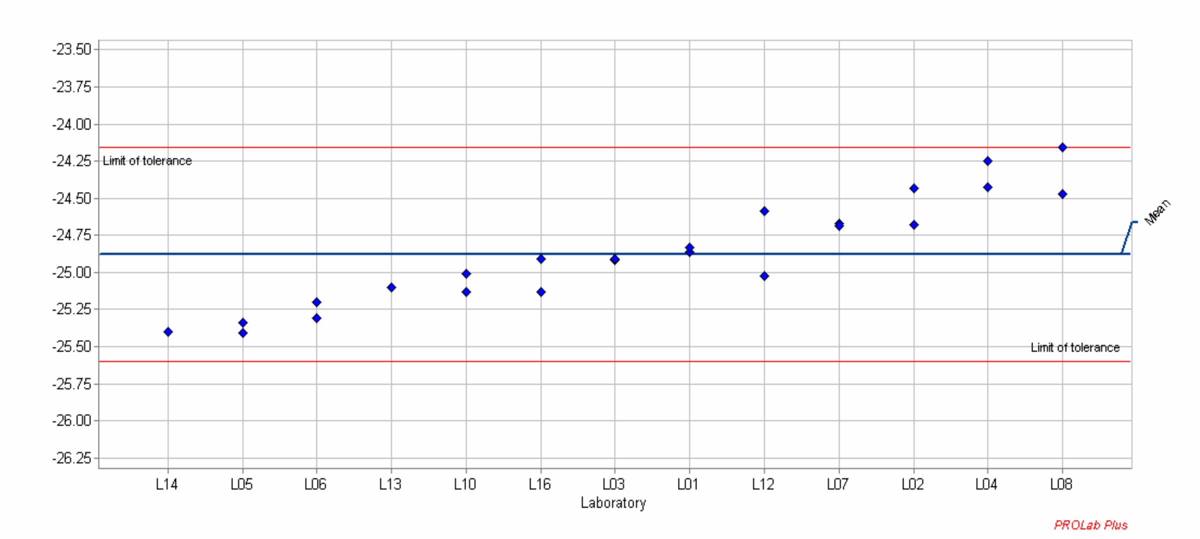

Figure A3f. Laboratory results for δ^{13}C (‰) values for disaccharides in sample HS-01158 (limit of tolerance gives the range for z-scores ≤ |2|).

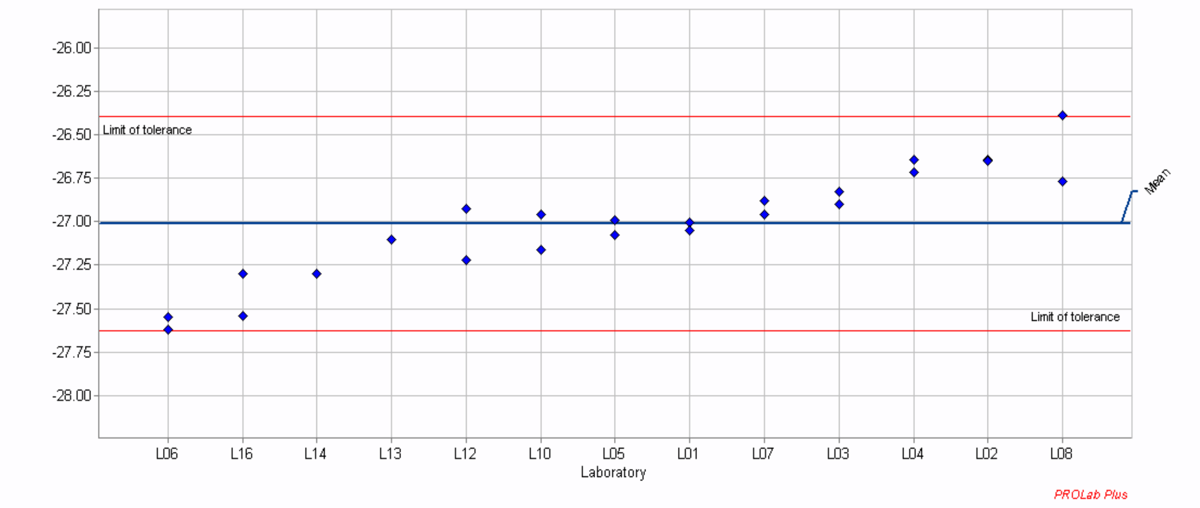

Table 3A. z-Scores for $\delta^{13}C$ (‰) values for disaccharides.

Laboratory	HS-00045	HS-00048	HS-00329	HS-00378	HS-00959	HS-01158
L01	1.09	0.47	0.72	0.55	0.08	-0.05
L02	0.77	1.41	0.99	0.04	0.89	1.18
L03	1.16	0.80	0.13	-0.26	-0.10	0.48
L04	0.75	0.64	1.08	0.84	1.50	1.07
L05	-1.32	-0.46	-0.93	-0.26	-1.38	-0.08
L06	-1.71	-2.24	-1.30	-3.51	-1.04	-1.86
L07	0.87	0.22	-0.42	0.25	0.55	0.30
L08	-0.44	0.65	1.08	1.11	1.56	1.40
L10	0.25	-0.27	-0.08	0.07	-0.53	-0.16
L12	-0.29	0.77	0.41	0.19	0.20	-0.20
L13	-0.42	-0.81	-0.74	-2.38	-0.61	-0.29
L14	-0.98	-0.95	-1.86	-5.29	-1.45	-0.94
L16	-0.22	-0.71	-0.01	-0.14	-0.39	-1.33

Figure A4a. Laboratory results for δ^{13}C (‰) values for trisaccharides in sample HS-00045 (limit of tolerance gives the range for z-scores ≤ |2|).

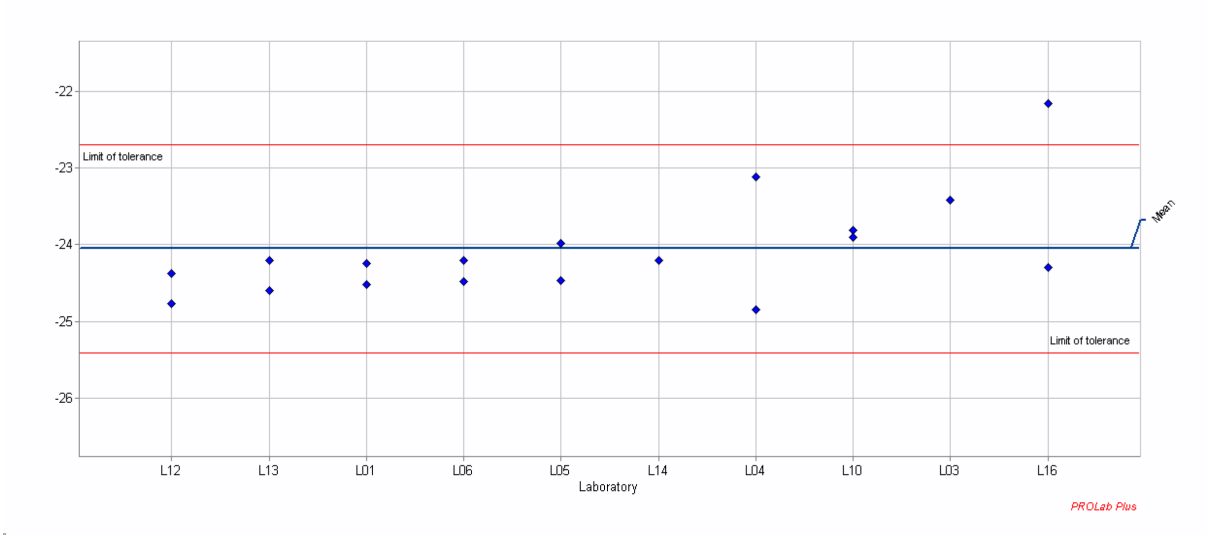

Figure A4b. Laboratory results for δ^{13}C (‰) values for trisaccharides in sample HS-00048 (limit of tolerance gives the range for z-scores ≤ |2|).

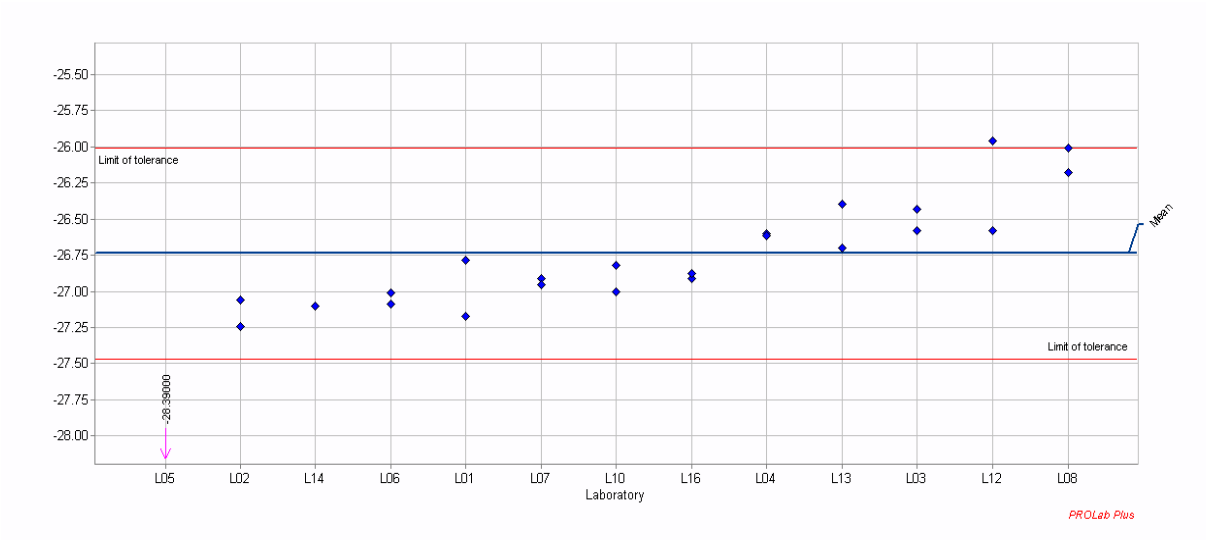

Figure A4c. Laboratory results for δ[13]C (‰) values for trisaccharides in sample HS-00329 (limit of tolerance gives the range for z-scores ≤ |2|).

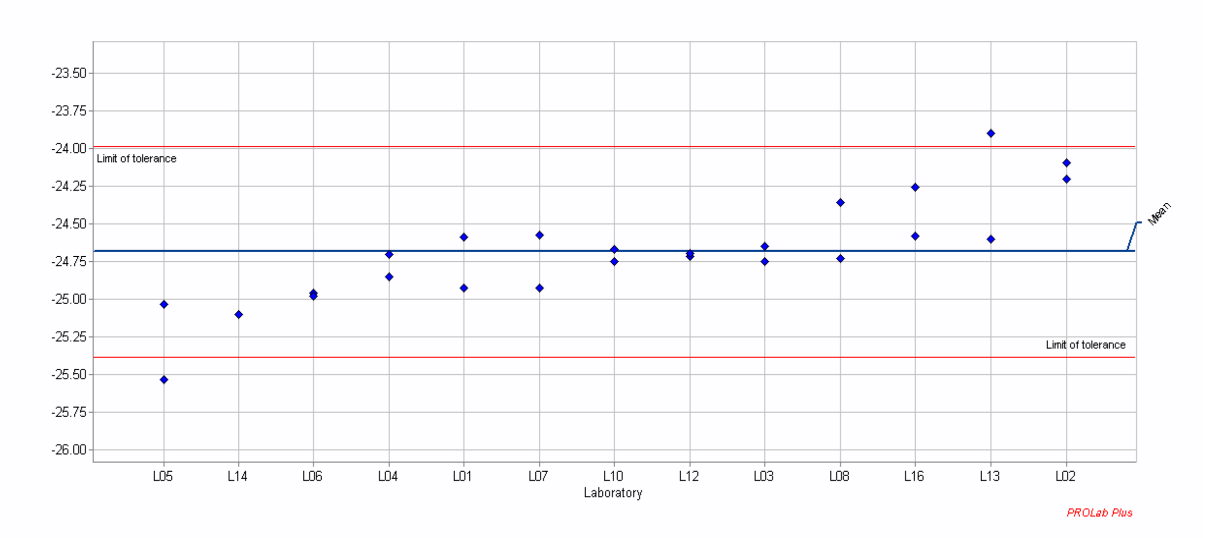

Figure A4d. Laboratory results for δ[13]C (‰) values for trisaccharides in sample HS-00378 (limit of tolerance gives the range for z-scores ≤ |2|).

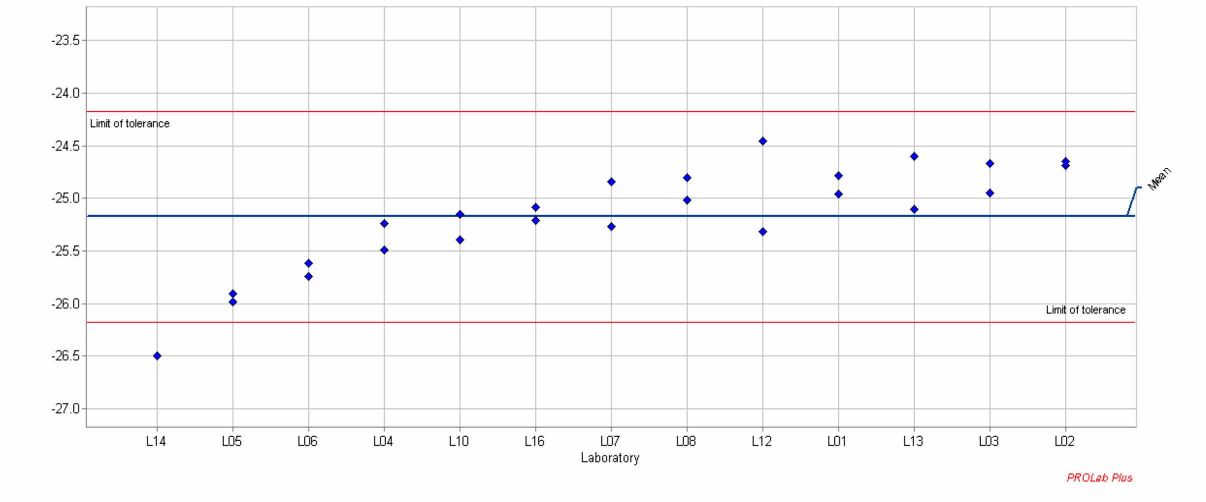

Figure A4e. Laboratory results for $\delta^{13}C$ (‰) values for trisaccharides in sample HS-00959 (limit of tolerance gives the range for z-scores ≤ |2|).

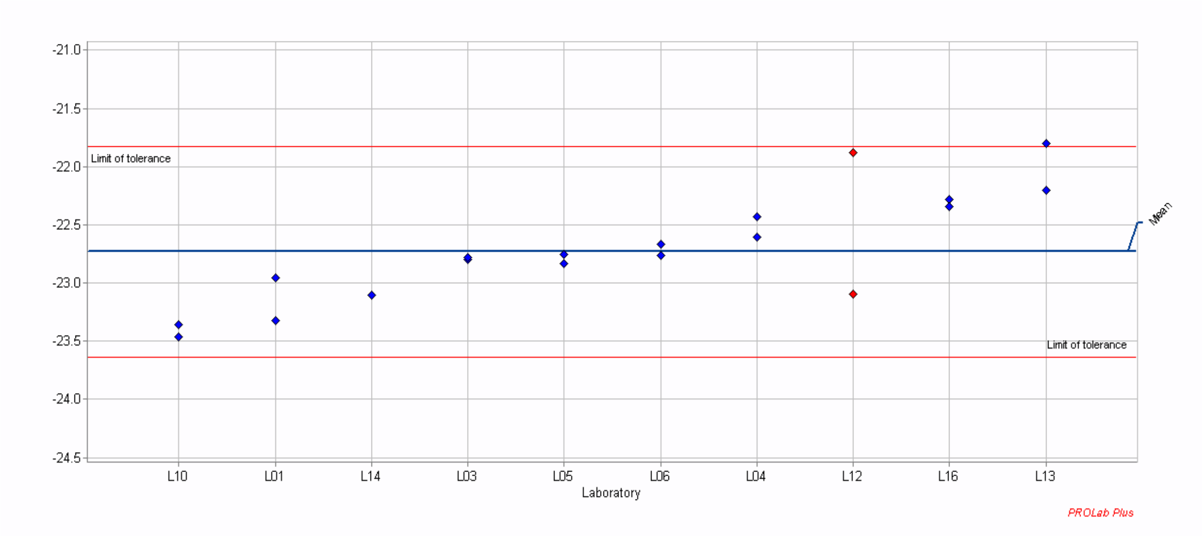

Figure A4f. Laboratory results for $\delta^{13}C$ (‰) values for trisaccharides in sample HS-01158 (limit of tolerance gives the range for z-scores ≤ |2|).

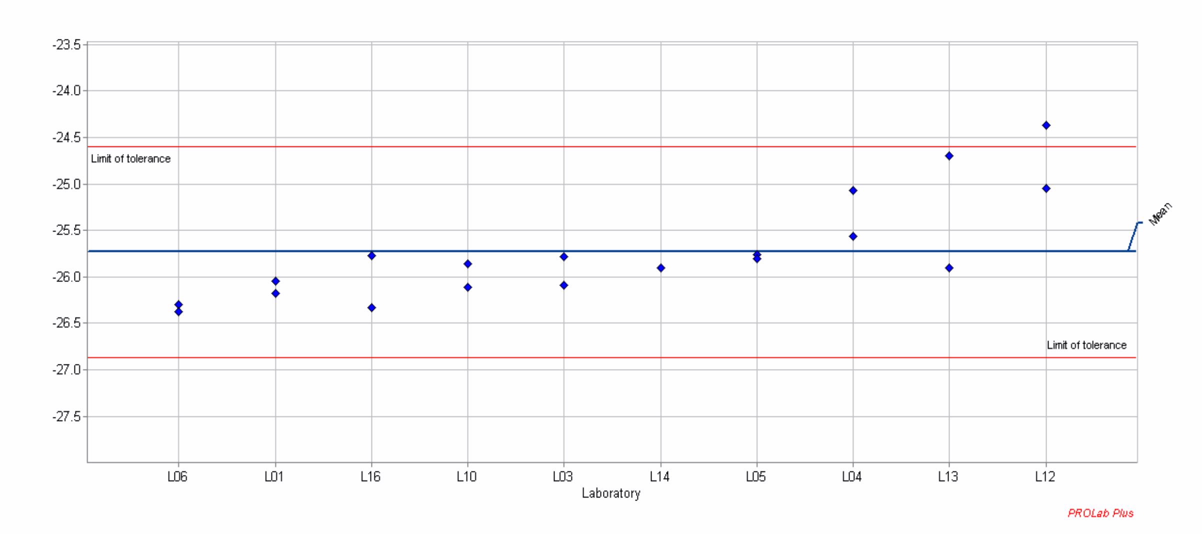

Table 4A. z-Scores for $\delta^{13}C$ (‰) values for trisaccharides (n.d., not determined)

Laboratory	HS-00045	HS-00048	HS-00329	HS-00378	HS-00959	HS-01158
L01	-0.48	-0.65	-0.20	0.61	-0.90	-0.66
L02	n.d	-1.13	1.54	1.02	n.d	n.d
L03	0.93	0.64	-0.04	0.73	-0.13	-0.36
L04	0.10	0.36	-0.26	-0.38	0.47	0.74
L05	-0.25	-4.54	-1.71	-1.54	-0.13	-0.08
L06	-0.42	-0.85	-0.82	-1.01	0.04	-1.07
L07	n.d	-0.54	-0.19	0.24	n.d	n.d
L08	n.d	1.77	0.40	0.53	n.d	n.d
L10	0.29	-0.47	-0.07	-0.19	-1.50	-0.44
L12	-0.77	1.28	-0.06	0.58	0.54	1.81
L13	-0.51	0.52	1.25	0.65	1.62	0.77
L14	-0.22	-0.99	-1.19	-2.65	-0.81	-0.29
L16	1.22	-0.43	0.76	0.06	0.93	-0.56

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