Enzytec™ *Liquid* D-Glucose/D-Fructose – fully-automated application

Art. No. E8160

Verification report

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1. Automation on a Pictus 500 device

1.1 General

As many other spectrophotometric autoanalyzer, the Pictus 500 is only able to measure the absorbance after addition of one reagent and a second time after the next reagent(s). Therefore only the sum of D-glucose and D-fructose can be determined, because otherwise three time points have to be read. For differentiation between both sugars, the sample has to be analyzed additionally using the Enzytec™ *Liquid* D-Glucose test kit (E8140). Since both sugars have the same molecular weight, results obtained by using the D-Glucose test kit can be directly subtracted from the D-Glucose/D-Fructose test kit (E8160) results. This can be done automatically by the Pictus 500.

The re-usable cuvettes in the Pictus 500 device have a maximum volume of 350 µL with a light path of 0.6 cm which makes a calibration necessary. Therefore, reagent volumes for the manual application are divided by 10. To give a user the maximum flexibility, three different applications with different measurement ranges depending on the sample volume of 2, 10 and 100 µL are provided. Volumes of reagent 1, reagent 2 and reagent 3 were reduced to 200 µL, 50 µL and 50 µL respectively and are not changed for the three different applications. The Pictus 500 can automatically re-run samples and change between these three applications in case the concentration is below or above a measurement range.

1.2 Calibrators

High range application: two point linear calibration with 0 mg/L (water) and 7.5 g/L (use E8445 and dilute with water)

Basic range application: two point linear calibration with 0 mg/L (water) and 1500 mg/L (use E8445 and dilute with water)

Sensitive range application: four point linear calibration with 0 mg/L, 15 mg/L, 45 mg/L and 150 mg/L:

- 1500 mg/L: dilute E8445 with water
- 150 mg/L: dilute 1500 mg/L one + nine with water
- 45 mg/L: dilute 150 mg/L three + seven with water
- 15 mg/L: dilute 150 mg/L one + nine with water

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1.3 Analysis for the sum of D-glucose and D-fructose

- Add 200 µL reagent 1
- Add sample, control or calibrator/water; volumes depend on the application:
  a. 2 µL for High range application
  b. 10 µL for Basic range application
  c. 100 µL for Sensitive range application
- We recommended to pipette n=4 replicates for each calibrator
- Incubate for 2 min at 37 °C (99 °F)
- Read A1 at 340 nm
- Add 50 µL reagent 2
- Add 50 µL reagent 3
- Incubate for 10 min at 37 °C (99 °F)
- Read A2 at 340 nm

1.4 Calculations

The Pictus 500 device will calculate a linear calibration function from the single calibrators and use this function to calculate concentrations for unknown samples and control solutions. In case a differentiation between D-glucose and D-fructose is needed, the device will calculate the difference if samples were also analyzed using the Enzytec™ *Liquid* D-Glucose test kit before.

1.5 Recommendations for other automates

The ratio of 4:1 for reagent 1 and reagent 2 should not be changed and the sample volume should not be bigger than twice the volume of reagent 2. A calibration must be performed but is often stable for several days so that it must not be repeated on every day. Control solution(s) should be analyzed with every run to check the validity of the calibration. In case these control solutions are not within specifications, a re-calibration must be done.

2. Sample preparation

The sample preparation for the automated test method is identical to the sample preparation for manual testing.

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3. Criteria for acceptance

Recovery of aqueous standard solutions on automated devices is identical compared to the manual method and should be within 100 ± 5 %.

4. Automation on a Pictus 500 spectrophotometric analyzer

4.1 Comments on validation parameters independent on automation

Side-reactivity to other related sugars/sugar alcohols and interfering substances will not be characterized on the automated analyzer, because there is no know effect that an automated process will change the reactivities towards these substances.

The pipetting environment within a fully-automated device is much more regulated than the normal laboratory environment (including the analyst). For measurement, all reagents are cooled at 8 ± 2 °C (42 - 50 °F) while the reaction zone where the analysis takes place, is tempered to 37 °C (99 °F). This ensures a quick enzymatic reaction and highly reproducible results. Therefore, the characterization of incubation times and temperatures was not repeated. Incubation at 37 °C (99 °F) and the necessary incubation times are described for the 4 mL cuvette manual application (see validation report of the assay).

There is also no practical reason to analyze test kit components that were tested for their stabilities against transport and short-term storage at 37 °C (99 °F).

The Pictus 500 can automatically change between the three applications in case the concentration is below or above the Basic measurement range. Therefore, it was decided not to characterize each application for an LoD, but to characterize a proper LoQ.

4.2 LoQ (Limit of Quantification)

The lower end of the measurement range is the limit of quantification where acceptable recovery and precision are met. Our internal requirements are a recovery between 95 % and 105 %, and an RSD equal or lower than 10 %. For each of the three applications, aqueous solutions with different concentrations of D-glucose were analyzed at least five times. The concentration was calculated from the calibration of the system.

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Figure 1 shows the results for the Basic range application with a sample volume of 10 µL which is – despite a factor of 10 in volumes – the identical ratio of sample volume to reagents as the manual format with a sample volume of 100 µL, 2000 µL reagent 1 and 500 µL reagent 2. The automated analyzer has an LoQ of 12 mg/L using the criteria described before. The manual format also exerted a calculated LoQ of about 12 mg/L where the precision was still sufficient.

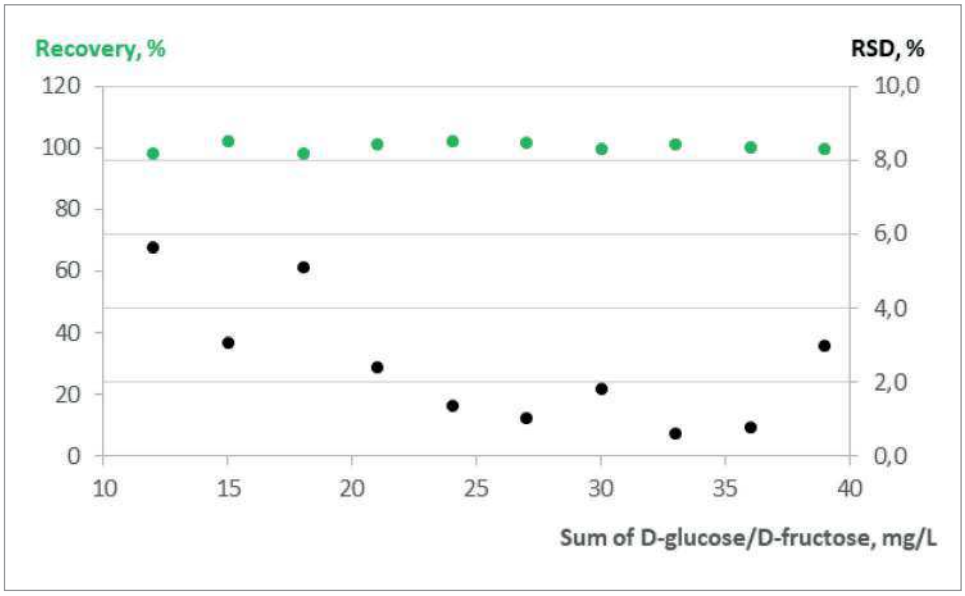

Figure 1: Confirmation of LoQ for the Basic range application with 10 µL sample volume.

Since D-glucose and D-fructose are often present at quite high concentrations in food such as fruit juices, the automated High range application with a low sample volume of 2 µL was introduced to analyze these matrices without dilution prior to measurement. As can be seen in figure 2, the LoQ for this application is 75 mg/L. This application was not investigated for the manual format, because this would require sample volumes of 20 µL which is challenging for untrained analysts. The Pictus 500 shows RSD values at or quite below 5 % for a sample volume of 2 µL.

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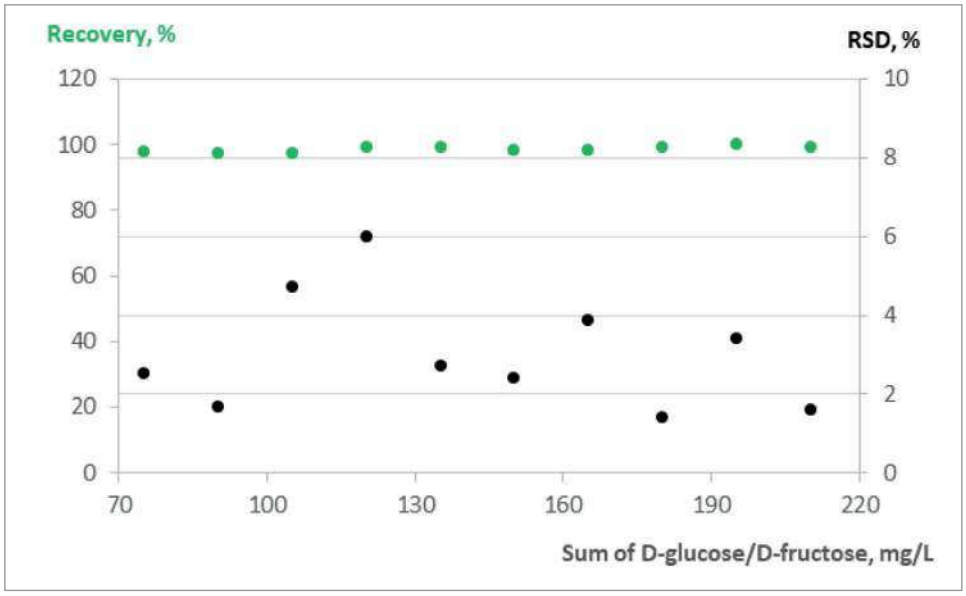

Figure 2: Confirmation of LoQ for the High range application with 2 µL sample volume.

In case trace analysis of D-glucose is necessary, the Sensitive range application with a sample volume of 100 µL was investigated for its LoQ, see figure 3. An LoQ of 5 mg/L can be claimed for this application where recovery and precision requirements were met.

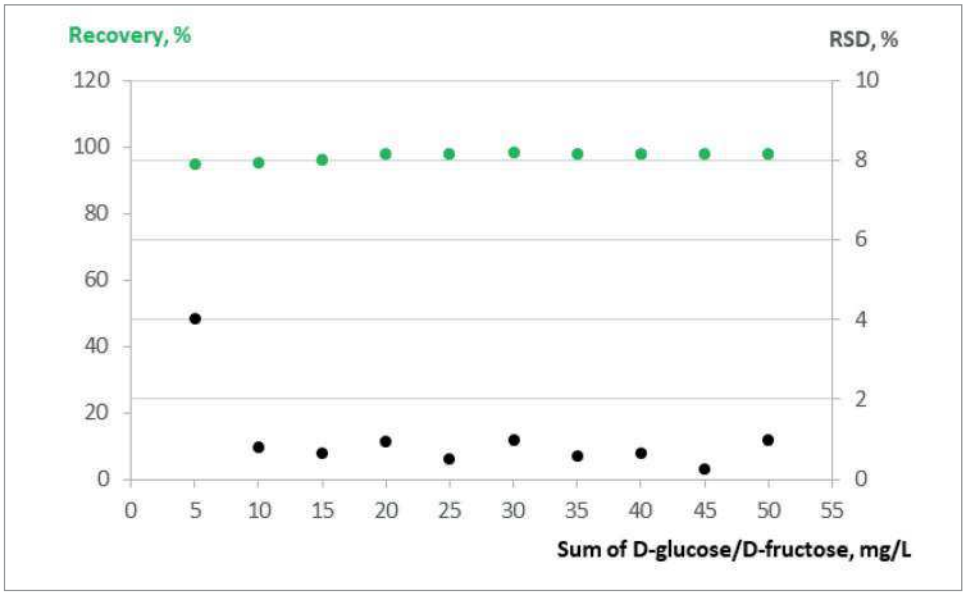

Figure 3: Confirmation of LoQ for the Sensitive range application with 100 µL sample volume.

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4.3 Linearity

The most important parameter for an automated application is the linear range, because in case of enzymatic analysis the analyte is often present in the sample and its proper quantification only depends on the proper choice of sample volume and calibration. For each of the three applications the optimal linear measurement range was characterized. Figure 4 shows that the upper measurement range is (at least) 1900 mg/L for a sample volume of 10 µL.

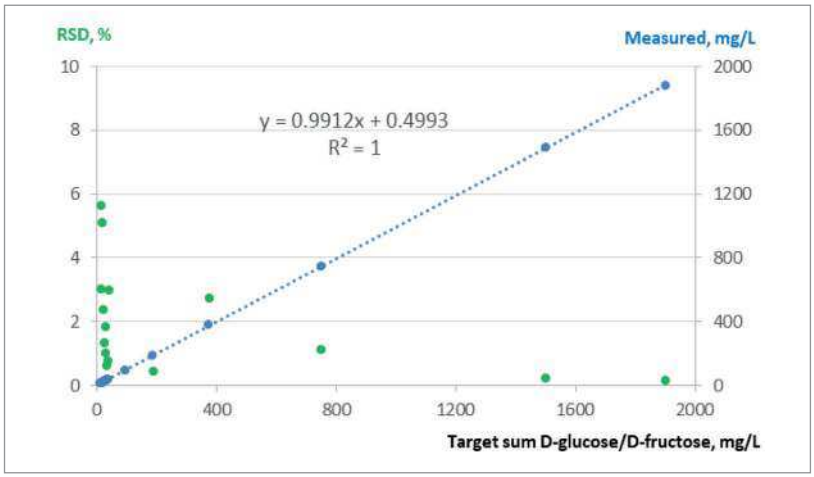

Figure 4: Characterization of linearity for the Basic range application with 10 µL sample volume.

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For the High range application with a sample volume of 2 µL, the upper measurement range is 10 g/L, see figure 5. This is nearly a factor of five compared to the Basic range application and perfectly fits to the increased sample volume of 10 µL.

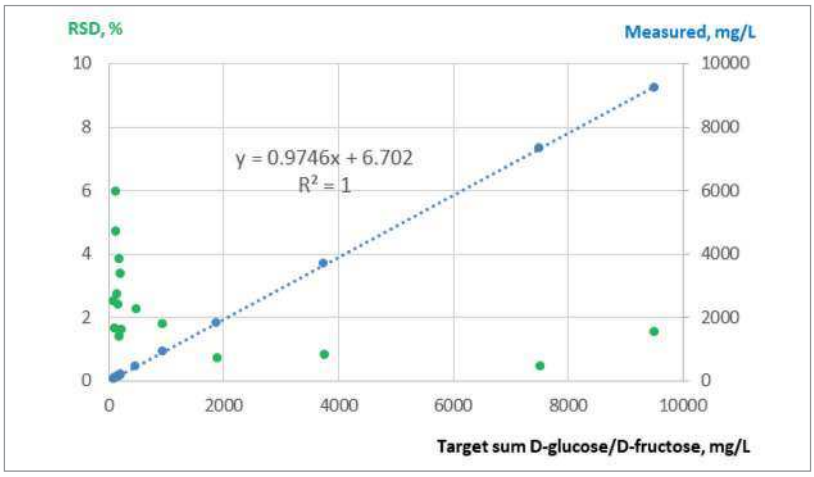

Figure 5: Characterization of linearity for the High range application with 2 µL sample volume.

For the Sensitive range application with a sample volume of 100 µL the upper measurement range is 190 mg/L, see figure 6. It is always recommended to include control samples at the upper range to check for linearity.

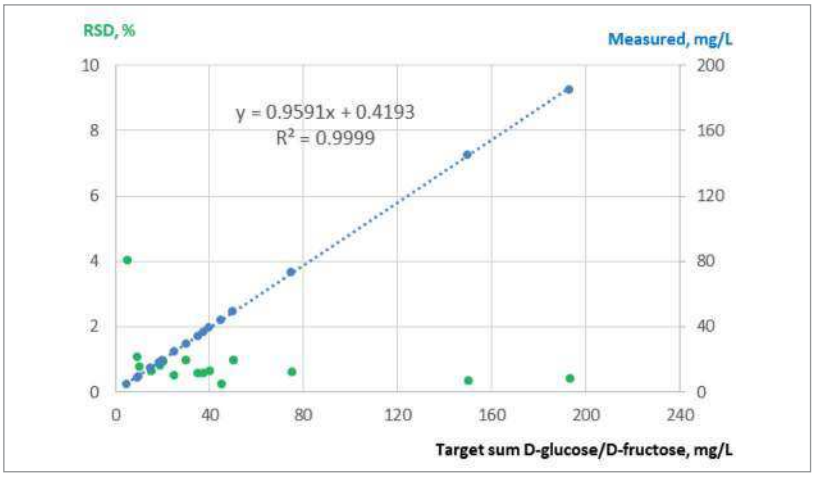

Figure 6: Characterization of linearity for the Sensitive range application with 100 µL sample volume.

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4.4 Precision and recovery

The precision of the automated pipetting was characterized for all three application. In most cases, aqueous solution were used because the characterization of different matrices was already done during the validation of the manual application. To check for trueness one standard wine was applied undiluted or diluted for all three applications.

Table 1 shows the results for the Basic range application with a sample volume of 10 µL. As expected for automated pipetting RSDs below 1 % were obtained for concentrations between 0.6 g/L and 1.4 g/L. The validity of the two-point calibration (0 g/L and 1.5 g/L) was also checked with these three solutions. Recoveries ranged between 99 % and 102 % and were thus clearly within specifications.

Table 1: Characterization of precision for the Basic range (10 µL sample volume) application using two aqueous control solutions and a standard wine.

	Aqueous solution		Standard wine
Replicate	Target: 1400 mg/L	Target: 1000 mg/L	Target: 591.2 mg/L
1	1394	1029	586.1
2	1409	1023	588.0
3	1404	1015	585.1
4	1400	1017	587.0
5	1405	1019	584.9
6	1404	1018	585.8
7	1411	1016	585.8
8	1404	1010	583.3
9	1402	1007	584.4
10	1387	1007	584.0
Mean, mg/L	1402	1016	585.4
SD, mg/L	7.08	6.93	1.41
RSD (%)	**0.50**	**0.68**	**0.24**
Recovery (%)	100	102	99

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Table 2 shows the results for the High range application with a sample volume of 2 µL. As expected for automated pipetting and the small volume, RSDs at or below 1.3 % were obtained for concentrations between 0.6 g/L and 1.4 g/L. The validity of the two-point calibration (0 g/L and 7.5 g/L) was also checked with these three solutions. Recoveries ranged between 99 % and 102 % and were thus clearly within specifications. The standard wine comes with a certificate so that trueness of the system was established for the application with the smallest volume of 2 µL.

Table 2: Characterization of precision for the High range (2 µL sample volume) application using two aqueous solutions and a standard wine.

	Aqueous solution		Standard wine
Replicate	Target: 1400 mg/L	Target: 1000 mg/L	Target: 591.2 mg/L
1	1391	1014	583.1
2	1412	1054	588.0
3	1410	1026	594.3
4	1394	1021	589.6
5	1402	1022	590.1
6	1410	1016	588.7
7	1404	1017	582.2
8	1406	1012	576.7
9	1405	1009	578.5
10	1388	1010	583.7
Mean, mg/L	1402	1020	585.5
SD, mg/L	8.49	13.11	5.58
RSD (%)	**0.61**	**1.28**	**0.95**
Recovery (%)	100	102	99

Table 3 shows the results for the Sensitive range application with a sample volume of 100 µL. For this application a four-point calibration (0, 15, 45 and 150 mg/L) was established and validated. RSD values were less than 1 % or lower for concentrations between 58.4 mg/L and 140 mg/L. The validity of the four-point calibration was also checked with these three solutions. Recoveries ranged between 99 % and 102 % and were thus within specifications.

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Table 3: Characterization of precision for the Sensitive range (100 µL sample volume) application using two aqueous control solutions and a red wine, which were diluted with water 1 + 9 before measurement.

	Aqueous solution		Standard wine
Replicate	Target: 140 mg/L	Target: 100 mg/L	Target: 59.12 mg/L
1	140	102	58.1
2	140	101	58.3
3	140	102	58.5
4	141	101	59.6
5	140	103	58.2
6	138	102	58.1
7	140	101	58.5
8	139	102	58.5
9	140	101	58.7
10	140	102	57.9
Mean, mg/L	140	102	58.4
SD, mg/L	0.63	0.75	0.48
RSD (%)	**0.45**	**0.74**	**0.83**
Recovery (%)	100	102	99

5. Conclusion

In summary, the data of the proof of concept show that the performance claims for food and beverages such as juices, chocolate, breakfast cereals, ice cream, sweetened condensed milk, jam, molasses, wine, beer and soft drinks are fulfilled. The method is robust and accurate for automated applications.