

## OIV-MA-AS311-10 Determination of D-glucose and D-fructose in wines by automated enzymatic method

Type III method

### 1. Scope of application

This method makes it possible to determine the sum of D-glucose and D-fructose in wine by specific enzyme analysis using an automatic sequential analyser.

In this document a collaborative study is reported which demonstrates application of the method for measurement of D-glucose and D-fructose from 0.1 to 96.31 g/L, taking into account the introduction of a dilution of the sample above 5 g/L.

Note: Where necessary, each laboratory using this method may refine, and potentially widen, this range through a validation study.

### 2. Standard references

OIV *Compendium of International Methods of Analysis*: Glucose and fructose – enzymatic method, OIV-MA-AS311-02,

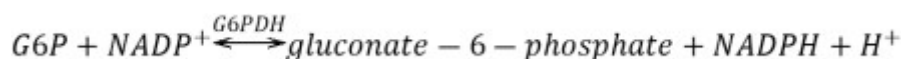
ISO 78-2: Chemistry – Layouts for standards.

### 3. Reaction principles

D-glucose and D-fructose are phosphorylated by adenosine triphosphate (ATP) during an enzymatic reaction catalysed by hexokinase (HK) to produce glucose-6-phosphate (G6P) and fructose-6-phosphate (F6P).



Glucose-6-phosphate is first oxidised to gluconate-6-phosphate by nicotinamide adenine dinucleotide phosphate (NADP) in the presence of the enzyme glucose-6-phosphate dehydrogenase (G6PDH). The quantity of reduced nicotinamide adenine dinucleotide phosphate (NADPH) is directly correlated with that of glucose-6-phosphate and thus with that of D-glucose.



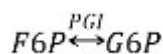
Fructose-6-phosphate (F6P) is converted into glucose-6-phosphate (G6P) in the

# COMPENDIUM OF INTERNATIONAL METHODS OF WINE AND MUST ANALYSIS

## Determination of D-glucose and D-fructose in wines by automated enzymatic method (Type-III)

---

presence of phosphoglucose isomerase (PGI):



The glucose-6-phosphate thus formed reacts as shown in the above formula.

The reduced nicotinamide adenine dinucleotide phosphate (NADPH) produced is measured based on its absorption at 340 nm.

### 4. Reagents and working solutions

During the analysis – unless stated otherwise – only use reagents of recognised analytical grade and water that is distilled, demineralised or of equivalent purity.

#### 4.1. Reagents

- 4.1.1. Quality I or II water for analytical usage (ISO 3696 standard)
- 4.1.2. Triethanolamine hydrochloride (CAS no. 637-39-8)
- 4.1.3. NADP (nicotinamide adenine dinucleotide phosphate) (CAS no. 24292-60-2)
- 4.1.4. ATP (adenosine-5'-triphosphate) (CAS no. 34369-07-8)
- 4.1.5. MgSO<sub>4</sub> (anhydrous magnesium sulphate) (CAS no. 7487-88-9)
- 4.1.6. Sodium hydroxide (CAS no. 1310-73-2)
- 4.1.7. Hexokinase (HK) (CAS no. 9001-51-8)
- 4.1.8. Glucose-6-phosphate dehydrogenase (G6PDH) (CAS no. 9001-40-5)
- 4.1.9. Phosphoglucose isomerase (PGI): lyophilised powder, 400-600 units/mg protein (CAS no. 9001-41-6)

*Note: One unit ensures the conversion of 1.0 μmole of D-fructose-6-phosphate into D-glucose-6-phosphate per minute at pH 7.4 and 25 °C*

- 4.1.10. Polyvinylpyrrolidone (PVP) (CAS no. 9003-39-8)
- 4.1.11. D-glucose: purity ≥ 99.5% (CAS no. 50-99-7)
- 4.1.12. D-fructose: purity ≥ 99% (CAS no. 57-48-7)

**Note 1:** There are commercial kits for the determination of D-glucose and D-fructose. The user needs to check the composition to ensure it contains the above-indicated reagents.

**Note 2:** The use of PVP is recommended to eliminate any possible negative effect of tannins in wine on the enzyme protein molecules. This is the case particularly in red wines. Should the use of PVP not prove effective, the laboratory should ensure that the wine tannins do not interfere with the enzymes.

#### 4.2. Working solutions

- 4.2.1. Triethanolamine hydrochloride buffer and magnesium sulphate adjusted to pH

# COMPENDIUM OF INTERNATIONAL METHODS OF WINE AND MUST ANALYSIS

## Determination of D-glucose and D-fructose in wines by automated enzymatic method (Type-III)

---

7.6. The preparation may be as follows:

- triethanolamine hydrochloride (4.1.2): 11.2 g,
- magnesium sulphate (4.1.5): 0.2 g,
- PVP (4.1.10): 2 g,
- water for analytical usage (4.1.1): 150 mL.

The mixture is adjusted to pH 7.6 using a 5 M sodium hydroxide solution, then made up to 200 mL with water for analytical usage. The solution is stable for at least 4 weeks at 2-8 °C.

4.2.2. R1 working solution (example):

- triethanolamine buffer (4.2.1): 50 mL,
- NADP (4.1.3): 117 mg,
- ATP (4.1.4): 150 mg.

3. R2 working solution (example):

- triethanolamine buffer (4.2.1): 2 mL,
- HK (4.1.7): 270 U,
- G6PDH (4.1.8): 340 U,
- PGI (4.1.9): 640 U.

**Note:** Commercial preparations of a HK/6GPDH mixture may be used.

**Note:** When preparing these solutions, they should be mixed gently to prevent foam from forming. The life cycle of the working solutions is limited and should be evaluated and respected by the laboratory.

Calibration solutions

To ensure the closest possible connection to the International System of Units (SI), the calibration range should be created using pure solutions of D-glucose and D-fructose prepared by weighing and covering the measurement range.

## 5. Apparatus

5.1. Analyser

5.1.1. Equipment type

**Automatic** sequential analyser equipped with a spectrophotometer with UV detector.

# COMPENDIUM OF INTERNATIONAL METHODS OF WINE AND MUST ANALYSIS

## Determination of D-glucose and D-fructose in wines by automated enzymatic method (Type-III)

---

The reaction temperature should be stable (around 37 °C). The reaction cuvettes are glass, methacrylate or quartz. The equipment is controlled by software ensuring its operation, data acquisition and useful calculations.

### 5.1.2. Absorbance reading

The concentration of the analytes directly relates to the absorbance difference read by the spectrophotometer. The precision of the absorbance reading should be a minimum of 0.1 absorbance unit (AU). It is preferable not to use absorbance values higher than 2.0.

### 5.1.3. Precision of volumes collected

The precision of the volumes of reagents and samples collected by the pipettes of the analyser influences the measurement result. Quality control of the results using appropriate strategies (e.g. according to the guides published by the OIV) is recommended.

### 5.1.4. Reaction duration and temperature

In general, the reaction time is 10 minutes and the temperature is 37 °C. Certain pieces of apparatus may use slightly different values.

### 5.1.5. Wavelength

The wavelength of maximum absorption of the NADPH formed by the reaction is 340 nm. This wavelength will be selected for the spectrophotometers commonly used. Some analysers are equipped with photometers that use a mercury-vapor lamp. In this case, a wavelength with a reading of 365 or 334 nm is to be selected.

## 5.2. Balance

This should be calibrated to the International System of Units and have 1 mg precision.

## 5.3. pH meter

## 5.4. Measuring glassware

The measuring glassware for the preparation of reagents and calibration solutions is class A.

## 6. Sampling

### 6.1. Preparation of samples of musts and wines

The majority of wine and must samples may be analysed without preparation. In some cases, a preparation may be introduced:

filtration should be used for highly turbid samples,

sample dilution (manual or automatic) with water for analytical usage (4.1.1) should be used for values exceeding the measurement range. By way of example, factors of 10x, 20x or 40x are used for musts. Given their impact on the uncertainty budget, these

dilutions should be conducted with the utmost care.

### 6.2. Preparation of samples of wines containing CO<sub>2</sub>

Wine samples containing CO<sub>2</sub> may produce bubbling effects. They must be degassed beforehand by stirring under vacuum, ultrasonic processing or any method enabling the required degassing.

## 7. Procedure

Given that different analysers may be used, it is recommended that the conditions of use provided by the manufacturer are strictly observed. This also applies to the different enzymatic kits available on the market.

The procedure takes place as follows:

1. The sample (S) is placed in a reaction cuvette.
2. Working solution R1 (4.2.2) is then added to the cuvette.
3. The two are mixed together. Time is then allowed for a lag period, in order to guarantee absorbance stability. This lag period may last from 1-5 min, and is defined by the laboratory, according to the characteristics of the equipment used.

Working solution R2 (4.2.3) is added and the reaction takes place.

By way of example, the quantities of different elements may be as follows:

- sample: 2.0 µL,
- R1: 40 µL,
- R2: 40 µL.

The equipment takes regular measurements (every 12 seconds, for example) that make it possible to obtain a reaction curve, an example of which is given in Figure 1.

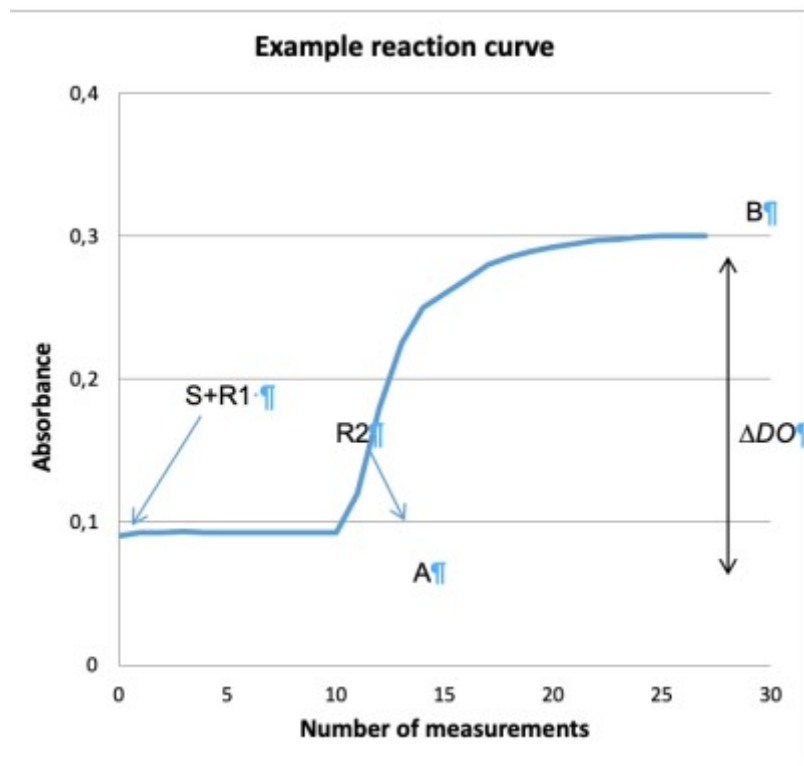


Figure 1

The equipment makes it possible to choose the reading points for the difference in absorbance sought, for example A and B in Figure 1.

#### 8. Calculation of results

The measurement used for the determination of the result is as follows:

$$\Delta DO = (\text{Absorbance } B - \text{Absorbance } A)$$

In order to correlate this  $\Delta DO$  value with the desired concentration of D-glucose and D-fructose, calibration of the equipment is carried out using the calibration solutions at a minimum of 3 points (§4.3) covering the measurement range. In addition, a reagent blank is used comprising all of the reagents but no sample (point 0 of the calibration).

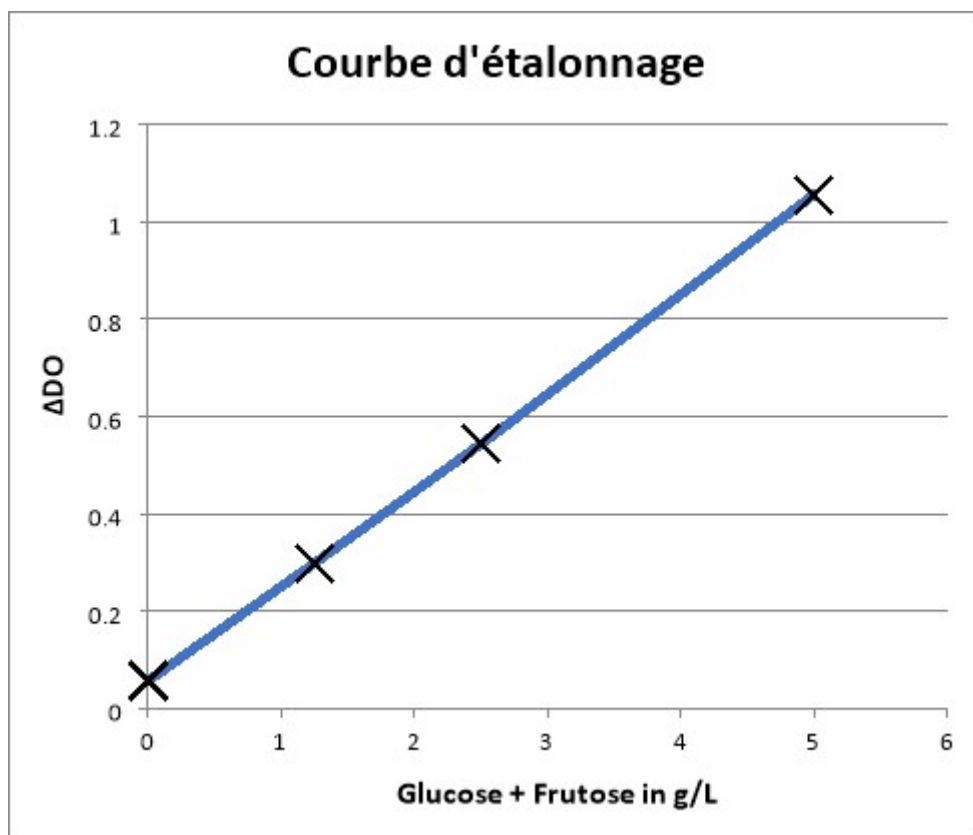


Figure 2: Calibration curve

The calibration curve can be order 1 ( $Concentration = a \cdot \Delta DO + b$ ) or even order 2 ( $Concentration = a \cdot \Delta DO^2 + b \cdot \Delta DO + c$ ). If using a calibration curve of order 2, the laboratory should take care to limit the calibration domain in order to maintain sufficient sensitivity of the method (risk of crushing the curve).

The final value obtained should be multiplied by any coefficient of dilution used.

### 9. Expression of results

The D-glucose + D-fructose results are expressed in g/L to 2 d.p.

### 10 Precision

#### Interlaboratory reproducibility

$RSD_R = 5\%$  (from 1 g/L)

$CV_R\% (k=2) = 2 \cdot RSD_R = 10\%$ , (from 1 g/L)

#### Repeatability

# COMPENDIUM OF INTERNATIONAL METHODS OF WINE AND MUST ANALYSIS

## Determination of D-glucose and D-fructose in wines by automated enzymatic method (Type-III)

---

$RSD_r = 1.5\%$  (from 1 g/L)

$CV_r\% (k=2) = 2 RSD_r = 3\%$  (from 1 g/L)

### Limit of quantification

Validated LOQ = 0, 10 g/L

(Concentration where  $CV_R\% (k=2) = 60\%$ )

### Annex Results of the interlaboratory tests

#### Collaborative study

A total of 17 laboratories from different countries participated in the collaborative study, organised in 2016.

Labo	Country
Miguel Torres S.A.- Finca Mas La Plana	SPAIN
Estación Enológica de Castilla y León	SPAIN
INGACAL -Consellería do Medio Rural Estación de Viticultura e Enoloxía de Galicia	SPAIN
Estación Enológica de Haro	SPAIN
Instituto dos Vinhos do Douro e do Porto, IP	PORTUGAL
Comissão de Viticultura da Região dos Vinhos Verdes	PORTUGAL
Laboratoires Dubernet	FRANCE
Laboratoire Diœnos Rhône	FRANCE
Laboratoire Natoli	FRANCE
SCL Montpellier	FRANCE
Agricultural institute of Slovenia	SLOVENIA



# COMPENDIUM OF INTERNATIONAL METHODS OF WINE AND MUST ANALYSIS

## Determination of D-glucose and D-fructose in wines by automated enzymatic method (Type-III)

Fachbereich: Wein, Weinüberwachung - Chemisches und Veterinäruntersuchungsamt Karlsruhe	GERMANY
HBLAuBA Wein - und Obstbau	AUSTRIA
Landesuntersuchungsamt Mainz	GERMANY
Hochschule GEISENHEIM University Institut Weinanalytik und Getränkeforschung	GERMANY
Unità Chimica Vitienologica e Agroalimentare - Centro Trasferimento Tecnologico - Fondazione Edmund Mach	ITALY
Unione Italiana Vini soc. Coop.	ITALY

For analysis, 2 x 10 blind duplicate samples were used, with 1 repetition. The wines analysed are wines originating from France and Portugal, dry wines and liqueur

Sample	A		B		C		D		E		F		G		H		I		J		
Position	1	9	2	13	3	4	5	15	6	10	16	20	7	11	12	17	8	19	14	18	
Labo3	rep#1	94.00	96.00	3.40	3.50	<b>0.40</b>	<b>0.40</b>	0.90	1.10	<b>2.10</b>	<b>2.50</b>	0.10	0.10	1.40	1.40	5.60	5.90	<b>4.70</b>	<b>4.20</b>	17.50	17.00
	rep#2	96.00	98.00	3.50	3.60	<b>0.40</b>	<b>0.30</b>	1.00	1.10	<b>2.20</b>	<b>2.40</b>	0.10	0.10	1.40	1.40	5.70	6.00	<b>4.30</b>	<b>4.50</b>	17.50	17.00
Labo6	rep#1	97.50	95.00	3.42	3.25	<b>0.35</b>	<b>0.48</b>	1.05	0.98	<b>3.24</b>	<b>2.65</b>	0.08	0.05	1.42	1.40	5.49	5.57	4.04	4.11	13.63	19.00
	rep#2	97.00	94.50	3.39	3.29	<b>0.37</b>	<b>0.57</b>	1.08	1.01	<b>3.34</b>	<b>2.66</b>	0.08	0.08	1.52	1.45	5.42	5.52	3.95	4.13	13.70	20.50
Labo7	rep#1	99.22	99.53	3.46	3.56	0.31	0.34	1.00	0.98	2.50	2.58	0.04	0.04	1.49	1.39	5.77	5.75	4.26	4.35	17.66	17.35
	rep#2	100.30	98.90	3.53	3.53	0.31	0.32	1.02	1.02	2.48	2.50	0.04	0.02	1.48	1.34	5.89	5.79	4.23	4.40	17.21	17.94
Labo9	rep#1	92.00	94.20	3.05	3.03	0.29	0.30	0.93	0.97	2.30	2.16	0.04	0.04	1.25	1.25	5.02	5.01	3.98	3.76	15.60	15.76
	rep#2	95.00	97.25	3.03	3.23	0.32	0.31	0.94	0.90	2.20	2.29	0.03	0.04	1.27	1.25	5.14	5.39	3.80	4.06	16.64	16.40
Labo10	rep#1	90.79	92.31	3.27	3.36	0.34	0.34	0.97	1.01	2.28	2.30	0.09	0.07	1.28	1.26	5.46	5.42	3.27	3.36	17.92	17.99
	rep#2	92.13	91.65	3.34	3.24	0.32	0.35	0.97	1.04	2.28	2.33	0.08	0.08	1.32	1.28	5.18	5.37	3.34	3.24	17.58	17.68
Labo11	rep#1	91.40	91.28	3.06	3.12	0.57	0.30	0.95	0.93	2.15	2.18	0.07	0.05	1.16	1.22	5.19	5.34	3.70	3.86	16.22	16.47
	rep#2	90.13	89.94	3.10	3.14	0.56	0.30	0.93	0.93	2.14	2.18	0.07	0.06	1.16	1.20	5.28	5.18	3.76	3.86	16.13	16.33
Labo12	rep#1	100.00	100.00	3.25	3.27	0.34	0.33	1.03	1.10	<b>2.35</b>	<b>2.75</b>	0.08	0.10	1.30	1.39	5.66	5.64	4.07	4.13	17.30	17.44

# COMPENDIUM OF INTERNATIONAL METHODS OF WINE AND MUST ANALYSIS

## Determination of D-glucose and D-fructose in wines by automated enzymatic method (Type-III)

	rep#2	101.00 97.00	3.22 3.25	0.34 0.33	1.03 1.11	<b>2.36 2.75</b>	0.08 0.10	1.30 1.39	5.62 5.68	4.07 4.15	17.50 17.80
Labo13	rep#1	96.60 96.00	3.04 3.07	0.34 0.31	0.97 0.94	<b>2.26 2.50</b>	0.05 0.04	1.25 1.25	5.21 5.29	3.84 3.99	16.08 16.03
	rep#2	96.00 95.10	3.07 3.12	0.32 0.32	0.97 1.04	<b>2.25 2.25</b>	0.04 0.04	1.25 1.28	5.24 5.31	3.90 3.97	15.95 16.18
Labo14	rep#1	104.00 98.00	3.19 3.16	0.33 0.33	0.97 0.96	<b>2.47 2.44</b>	0.05 0.05	1.34 1.32	5.77 5.81	4.20 4.21	17.76 17.04
	rep#2	103.00 96.00	3.18 3.17	0.33 0.33	0.97 0.97	<b>2.48 2.44</b>	0.05 0.05	1.34 1.32	5.77 5.78	4.20 4.14	17.44 17.24
Labo15	rep#1	110.03 99.25	3.63 3.60	0.20 0.19	0.94 0.97	<b>2.54 2.36</b>		1.30 1.20	5.65 6.14	4.56 4.43	17.16 19.33
	rep#2	104.39 99.34	3.59 3.72	0.20 0.20	0.94 0.95	<b>2.52 2.32</b>		1.32 1.20	5.62 6.19	4.39 4.54	17.41 19.29
Labo16	rep#1	95.20 94.08	3.20 3.22	0.32 0.32	0.96 0.96	<b>2.24 2.26</b>	0.06 0.06	1.23 1.23	5.19 5.19	3.89 3.84	17.82 17.38
	rep#2	96.00 94.41	3.17 3.18	0.31 0.33	0.95 0.94	<b>2.25 2.22</b>	0.06 0.06	1.24 1.22	5.13 5.15	3.85 3.86	17.84 17.24
Labo17	rep#1	96.68 97.10	3.28 3.38	0.47 0.43	1.03 1.03	<b>2.41 2.46</b>	0.10 0.20	1.36 1.36	5.52 5.53	4.09 4.00	16.42 17.30
	rep#2	97.08 99.40	3.24 3.33	0.39 0.38	0.95 0.96	<b>2.30 2.36</b>	0.20 0.15	1.32 1.24	5.38 5.40	3.95 4.10	16.50 16.60
Labo18	rep#1	90.23 91.39	3.14 3.26	0.46 0.47	1.12 1.10	<b>2.30 2.44</b>	0.23 0.24	1.38 1.30	5.19 5.49	3.91 4.10	14.83 14.89
	rep#2	90.02 91.74	3.18 3.31	0.47 0.47	1.07 1.07	<b>2.31 2.40</b>	0.23 0.24	1.38 1.32	5.23 5.45	3.94 4.04	14.82 14.85
Labo19	rep#1	99.63 103.55	3.34 3.41	0.32 0.32	0.98 0.97	<b>2.38 2.41</b>	0.04 0.05	1.29 1.30	5.68 5.56	4.10 4.11	17.61 17.49
	rep#2	100.57 103.28	3.36 3.42	0.32 0.32	0.98 0.97	<b>2.36 2.42</b>	0.05 0.05	1.29 1.31	5.61 5.59	4.10 4.11	17.53 17.51
Labo20	rep#1	96.41 96.18	3.20 3.23	0.32 0.32	0.96 0.95	<b>2.26 2.32</b>	0.07 0.08	1.24 1.24	5.35 5.40	3.92 4.03	16.36 16.51
	rep#2	96.32 95.89	3.18 3.23	0.32 0.32	0.96 0.95	2.26 2.32	0.07 0.08	1.24 1.24	5.35 5.38	3.92 4.03	16.38 16.49
Labo21	rep#1	103.60 102.02	3.37 3.60	0.23 0.25	0.95 0.98	2.41 2.49	0.05 0.05	1.27 1.33	5.95 6.12	<b>4.02 4.53</b>	<b>18.41 19.70</b>
	rep#2	102.50 103.02	3.34 3.51	0.23 0.26	0.92 0.98	2.45 2.45	0.03 0.05	1.26 1.27	6.02 5.99	<b>4.09 4.42</b>	<b>18.96 19.90</b>
Labo22	rep#1	96.73 96.59	3.25 3.28	0.28 0.28	0.92 0.93	2.25 2.31	0.06 0.05	1.23 1.28	5.51 5.47	4.02 3.98	17.09 17.10
	rep#2	97.06 96.34	3.24 3.21	0.30 0.30	0.93 0.93	2.26 2.30	0.04 0.05	1.21 1.24	5.40 5.39	4.03 4.04	17.05 17.01

**Table of the data obtained.** The values in bold correspond with the values rejected in accordance with the Cochran (variance outliers) test with a 2.5% significance level (one-tailed test), and the Grubbs (outliers from the mean) test with significance levels of 2.5% (two-tailed test).

*Note: The absent values have not been provided by the laboratory in question.*

Sample	A	B	C	D	E	F	G	H	I	J
--------	---	---	---	---	---	---	---	---	---	---

# COMPENDIUM OF INTERNATIONAL METHODS OF WINE AND MUST ANALYSIS

## Determination of D-glucose and D-fructose in wines by automated enzymatic method (Type-III)

No. of laboratories selected	15	17	14	17	14	14	17	16	15	14
No. of repetitions	4	4	4	4	4	4	4	4	4	4
Min.	90.69	3.08	0.20	0.93	2.16	0.04	1.19	5.14	3.80	14.85
Max.	102.79	3.64	0.47	1.09	2.52	0.10	1.45	6.02	4.48	17.79
Overall average	96.31	3.29	0.32	0.98	2.34	0.06	1.30	5.50	4.05	16.86
Repeatability variance	1.449	0.004	0.000	0.001	0.004	0.000	0.001	0.009	0.005	0.065
Inter-laboratory stand. dev.	3.60	0.16	0.06	0.05	0.10	0.02	0.07	0.26	0.17	0.83
Reproducibility variance	14.037	0.029	0.004	0.003	0.013	0.000	0.006	0.073	0.034	0.739
Repeatability variance	1.20	0.06	0.01	0.04	0.06	0.01	0.04	0.09	0.07	0.26
r limit	3.40	0.17	0.04	0.10	0.17	0.02	0.11	0.26	0.21	0.72
Repeatability RSD <sub>r</sub>	1.2%	1.8%	4.4%	3.6%	2.5%	13.2%	2.9%	1.7%	1.8%	1.5%
Reproducibility stand. dev.	3.75	0.17	0.07	0.06	0.11	0.02	0.08	0.27	0.19	0.86
R limit	10.60	0.48	0.19	0.16	0.32	0.06	0.22	0.76	0.52	2.43
Reproducibility RSD <sub>R</sub>	3.9%	5.1%	20.4%	5.7%	4.8%	35.3%	6.1%	4.9%	4.6%	5.1%
Horwitz RSD <sub>r</sub>	1.877	3.120	4.425	3.742	3.284	5.694	3.588	2.889	3.025	2.440
Horrat <sub>r</sub>	0.666	0.587	1.001	0.952	0.773	2.315	0.804	0.585	0.593	0.621
Horwitz RSD <sub>R</sub>	2.84	4.73	6.70	5.67	4.98	8.63	5.44	4.38	4.58	3.70
Horrat <sub>R</sub>	1.368	1.086	3.036	0.997	0.965	4.087	1.123	1.122	1.000	1.378

### Table of the results obtained

*Note: The results from sample F should be taken with caution due to the very low concentration levels, which are below to the laboratories' limit of quantification.*

# COMPENDIUM OF INTERNATIONAL METHODS OF WINE AND MUST ANALYSIS

## Determination of D-glucose and D-fructose in wines by automated enzymatic method (Type-III)

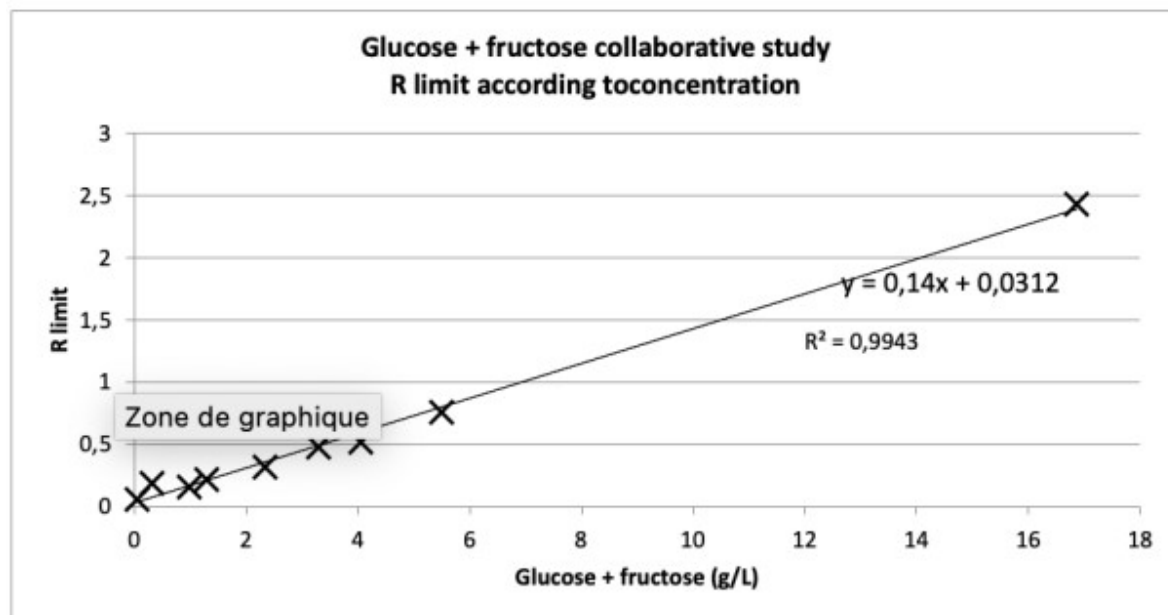


Figure 3: R limit according to concentration

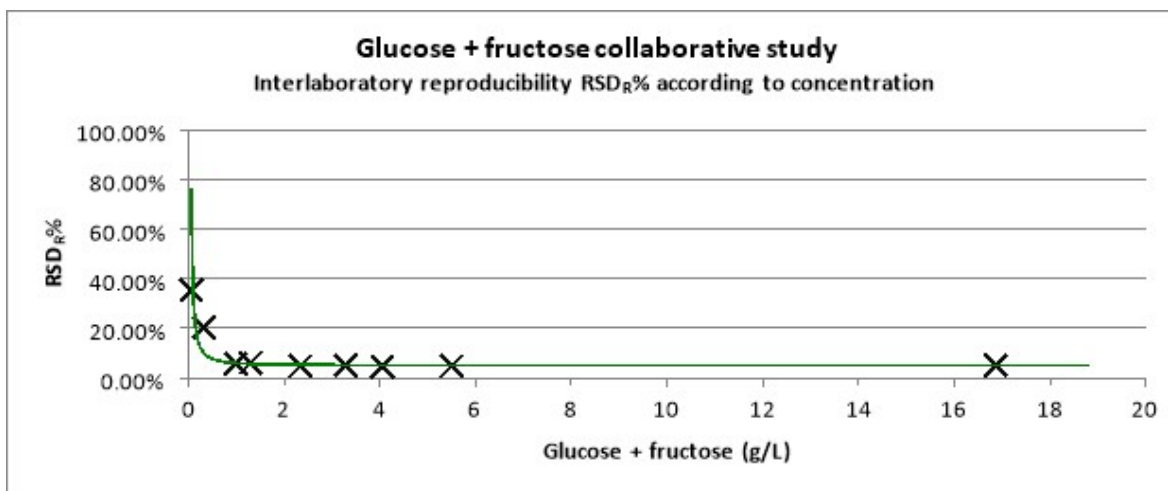


Figure 4: Interlaboratory RSDR % according to concentration.

Modelling:  $RSD_R\% = 1 \cdot C^{(-1,424)} + 5$