APPLICATION NOTE

Residual sugar measurement in wine using the SpectraMax Plus 384 Microplate Reader

Introduction

Analysis of malic acid, residual sugar, volatile acidity and ammonia is very important in quality control during wine production. Enzymatic assays carried out in microplate format are quantitative and help achieve high throughput with respect to time and labor. The residual sugar assay involves enzymatic conversion of the analytes to give NADPH as a by-product. Measuring NADPH production by measuring absorbance at 340 nm allows direct quantitation of the analytes in wine samples. Here we describe the use of Molecular Devices SpectraMax® Plus 384 Microplate Reader and SoftMax® Pro Software to efficiently collect and analyze the data for enzymatic determination of residual sugar in wine.

Some of the unique features of the SpectraMax Plus 384 Microplate Reader are:

- Wavelength Range: 190–1000 nm in 1-nm increments with no need for separate filters; covers UV range
- Read Speed: 96 wells: 9 Seconds
  384 wells: 29 Seconds
- Temperature: 4°C above ambient to 45°C
- Cuvette port: Holds standard cuvettes and 12 x 75 mm test tubes
- OD range: 0–4 OD

Enzymatic measurement of residual sugar (RS) in wine

Enzymatic Reactions:

(1) Glucose + ATP $\xleftrightarrow{(HK)}$ Glucose-6-Phosphate + ADP

(2) Fructose + ATP $\xleftrightarrow{(HK)}$ Fructose-6-Phosphate + ADP

(3) Fructose-6-phosphate $\xleftrightarrow{(PGI)}$ Glucose-6-Phosphate

(4) Glucose-6-phosphate + NADP $\xleftrightarrow{(G6P\text{-}DH)}$ Glucose acid-6-phosphate + NADPH + H^+

HK: Hexokinase

PGI: Phosphoglucose isomerase

G6P-DH: Glucose-6-phosphate dehydrogenase

Reaction dynamics

This assay measures the two major sugars in wine, glucose and fructose. Since the formation of NADPH is stoichiometrically related to the oxidation of glucose-6-phosphate, measurements for glucose + fructose can be determined by the absorbance of NADPH at 340 nm.

Benefits

- **Direct quantitation of analytes in wine samples**
- **Improved throughput relative to traditional single-tube methods**
- **Automated calculation of results with SoftMax Pro Software**

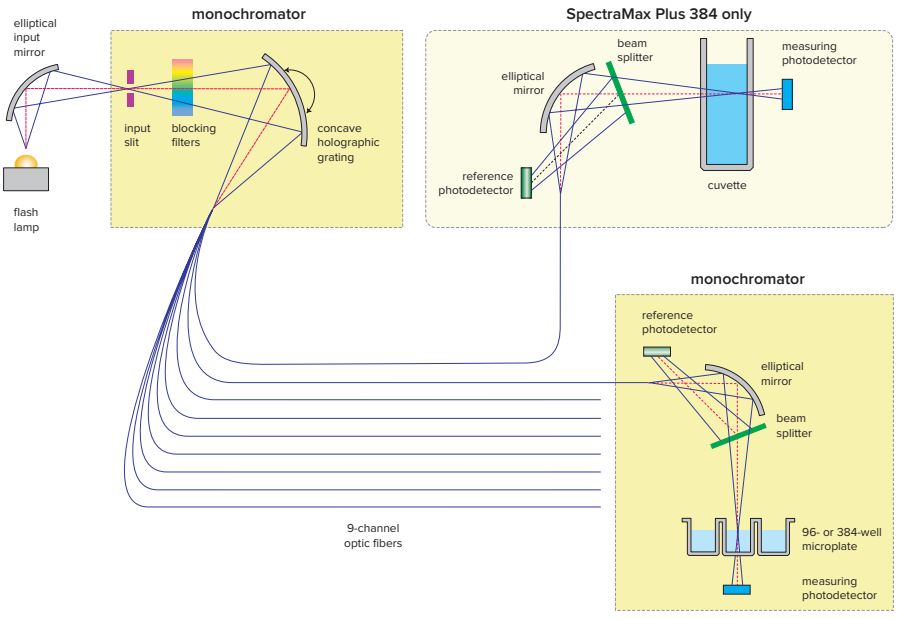


Figure 1. SpectraMax® Plus 384 optics.

PathCheck Sensor

The PathCheck® Sensor is a patented feature from Molecular Devices that measures the optical pathlength of samples in microplate wells. It is an innovative way of normalizing the absorbance reading in a microplate well to that of a 1-cm cuvette..

Beer-Lambert Law states that
Absorbance = E * C * L

where
E = absorptivity (extinction coefficient)
C = concentration
L = pathlength

In case of a cuvette, the optical path is horizontal, thus the pathlength is fixed and is equal to 1 cm. But in case of a microplate, the optical path is vertical. So, the pathlength depends on the volume of the sample, as illustrated in Figure 2.

SoftMax Pro Software

The software controls the instrument, collects the data, and provides complete data analysis. Customized protocols with appropriate instrument settings and calculations can be pre-written and saved. The end user can conveniently open a preconfigured protocol and obtain complete results and analysis with no protocol setup time.

Materials

- SpectraMax Plus 384 Microplate Reader (Molecular Devices cat. #PLUS 384)
- UV-transparent 96-well microplates (Corning cat. #3635)
- Transferpette micropipette (Drummond Scientific cat. #2704174, 2705402, 2705412, 2704180; Drummond Digital Microdispenser cat. #3-000-510)
- Microtips for the micropipettes (Eppendorf cat. # epT.I.P.S. Reloads 022491539, 022491512, 022491547)
- Centrifuge for microfuge tubes (Eppendorf cat. #5424)
- Microfuge tubes 1.5 mL capacity (Eppendorf cat. #022364111, 022363557, 022363514, 2236357-3)
- D-Fructose (Fisher Scientific cat. #L95-500)
- Hexokinase/Glucose-6-Phosphate Dehydrogenase 30 mg (10 mL, Roche cat. #10737275001)

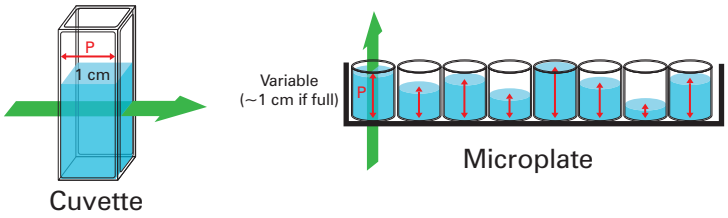


Figure 2. Pathlength in a cuvette and a microplate are different.

Concentration	0.1 g/ 100 mL	0.2 g/ 100 mL	0.4 g/ 100 mL
DI H_2O	95 mL	95 mL	95 mL
D-Fructose	0.1 g	0.2 g	0.4 g
Bring Volume To	100 mL	100 mL	100 mL

Table 1. Preparation of Fructose Standards.

Assay Limits	Dilution	Sample Volume
0.01-0.4 g/100 mL	No Dilution Required	5 µL
0.4-1.0 g/100 mL	No Dilution Required	5 µL
1.0-4.0 g/100 mL	1:10 Dilution	5 µL
4.0-40.0 g/100 mL	1:100 Dilution	5 µL

Table 2. Processing of wine samples for the RS assay.

Amount of Buffer Required	Amount of PGI Added
10 µL	14 µL
50 µL	28 µL
100 µL	56 µL
150 µL	84 µL
200 µL	112 µL

Table 3. Calculation of required amounts of phosphoglucose isomerase per volume of reaction buffer.

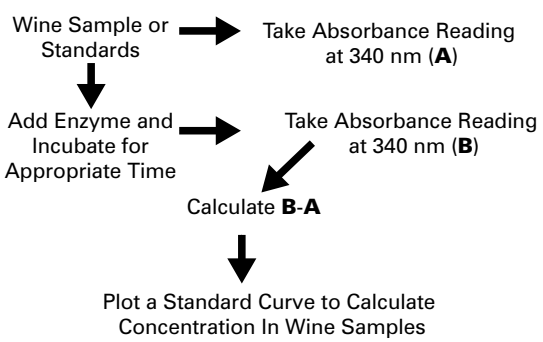


Figure 3. Measurement of residual sugar (RS) in wine.

- Phosphoglucose isomerase 10 mg/mL (Roche cat. #10128139001)
- Polyvinyl pyrrolidone (Fisher Scientific cat. #BP431-100)
- TRIS (Amresco cat. #0497-500G)
- MgSO4 • 7H2O (Fisher Scientific cat. #M63-500)
- ATP (Boehringer Mannheim cat. #10519987001)
- NADP (Boehringer Mannheim cat. #10240354001)
- 1.0 M HCl (Fisher Scientific cat. #SA431-500)

Preparation of fructose standards

The reagents were stirred until dissolved. Standards producing a standard curve with coefficient of determination (R)=1.000 were stored in 1.5 mL freeze tubes at -4°C. Reagents that are too old may not give an ideal standard curve.

Preparation of RS buffer solution

Polyvinyl pyrrolidone (PVP) 2.0 g, TRIS 3.0 g and $MgSO_4 \cdot 7H_2O$ 0.5 g were added to 350 mL deionized (DI) H_2O in a 400-mL beaker. The pH of this solution was adjusted to 7.6 with 1.0 M HCl (≈36 mL). Further, ATP 0.15 g and NADP 0.175 g were then added to this solution, and the volume was adjusted to 500 mL with a volumetric flask. The buffer was stored at 4°C.

Processing of wine samples for the RS assay

If necessary, wine samples were diluted in DI water according to Table 2. Turbid samples were centrifuged or filtered prior to analysis, either before or after dilution.

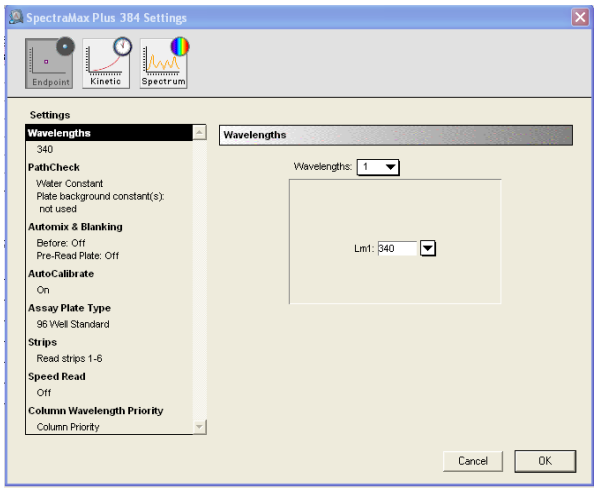
Samples diluted in DI water 1:10 were accompanied by 4.0 g/100 mL standard diluted 1:10. Samples diluted in DI water 1:100 were accompanied by 10.0 g/100 mL standard diluted 1:100.

Methods

Step 1: Blanks, standards and samples were run in duplicate.

Reagent Blank	Buffer 300 µL	DI Water 5 µL
Standards	Buffer 300 µL	Standards 5 µL
Samples	Buffer 300 µL	Samples 5 µL

A



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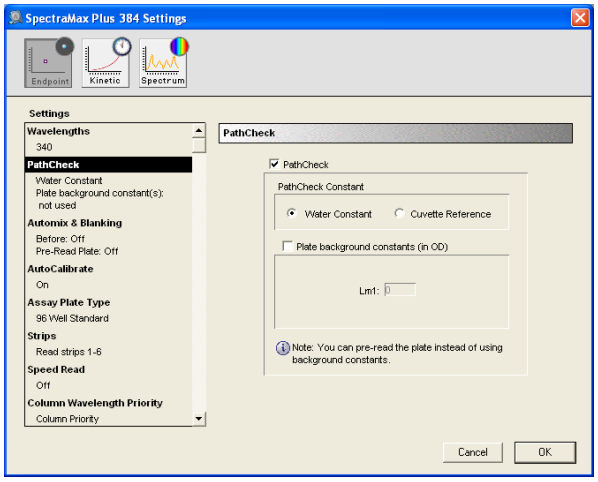

Figure 4. Instrument settings for RS assay. A: Overall settings, **B:** PathCheck Sensor settings.

Parameter	Setting
Read Type	Endpoint
Wavelength	340 nm
PathCheck	PathCheck selected, Water Constant (no plate background constant)
Automix & Blanking	Off
AutoCalibrate	On
Assay Plate Type	96-Well Standard
Strips	Selected as needed

Table 4. RS assay instrument settings.

Step 2: D-Fructose standards were removed from the freezer and allowed to warm to room temperature.

Step 3: The amount of buffer necessary to analyze standards and samples was measured out into a dedicated “RS” beaker and warmed to room temperature. This step is necessary for achieving an ideal coefficient of determination.

Step 4: Phosphoglucose isomerase (PGI) enzyme was added to the buffer according to the values in Table 3. PGI was mixed into buffer thoroughly, but gently to avoid denaturing the enzyme.

Step 5: 300 µL of buffer was dispensed into each well using the 300-µL multichannel pipette.

Step 6: 5 µL of DI H_2O, standard, or sample was dispensed into the appropriate wells.

Step 7: The reagents were mixed by pipetting up and down (trituration).

Step 8: The plate was mixed by vortexing for 30 seconds; an initial absorbance reading was then taken on the microplate reader.

Step 9: Immediately after the initial read, 5 µL of HK/G6P-DH was dispensed into each well using the 1–20 µL multichannel pipette.

Step 10: The plate was mixed by vortexing for 30 seconds and incubated at room temperature for 21 minutes.

Step 11: After 21 minutes of incubation a “Final” reading was taken with the same instrument settings on the microplate reader.

Instrument setup

The instrument was programmed through SoftMax Pro Software. The settings were adjusted in the plate section of the software (Figure 4). Table 4 shows the instrument settings used for the RS assays. Figure 4 shows screenshots for the wavelength settings and “PathCheck” selection.

Two identical plate sections were created. The initial plate section involved reading the plate with all the reagents but no enzyme. The second plate section was the actual reaction with enzyme. The optical density readings from the initial plate

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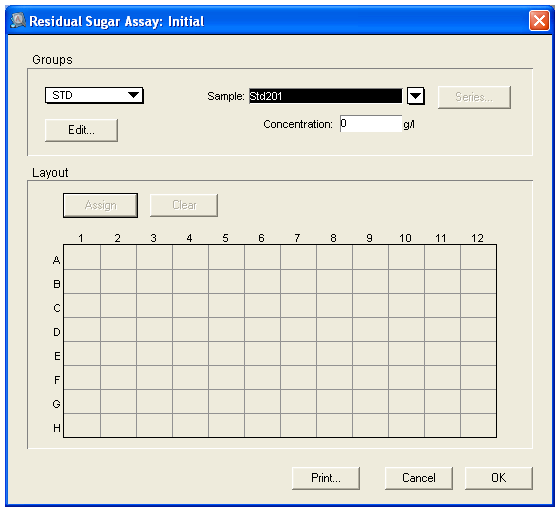

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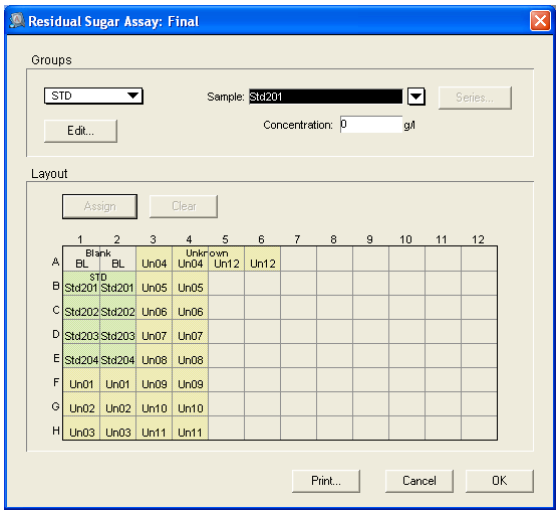


Figure 5. Template setup for RS assay. A: Initial plate—no template was set up. **B:** Final plate—template with plate, standards, and unknown samples.

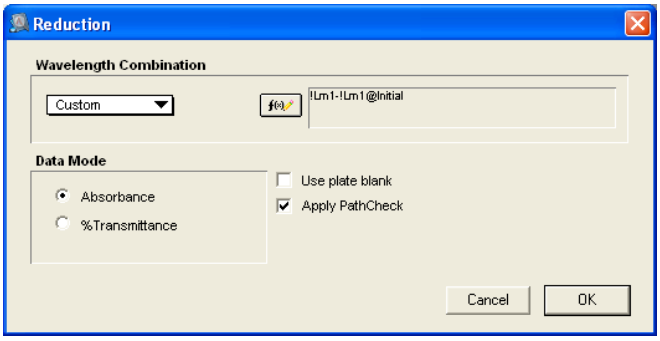


Figure 6. Reduction of data. Reduction setup showing custom formula being used for the subtracting optical density of each well in the initial plate from the optical density of the same well in the final plate. PathCheck Sensor values were applied for the calculation.

section were subtracted from the optical density readings of the second plate section through reduction settings.

Template setup for RS assay

A template was set up in the software to denote placement of the standards and samples in wells of the microplate. The flexible template layout allows easy addition of more samples and replicates. For the initial plate, no template was set up. For the final plate, standards and unknown samples were assigned. Concentration of each standard was assigned by clicking on the wells and typing the appropriate concentration in the box. The range was 0–0.4 g/100 mL (Figure 5).

Reduction setup

The final plate was the actual reaction with the enzyme. The optical density readings from the initial plate were subtracted from the optical density readings of the final plate through reduction settings (Figure 6). Hence the plate blank was not used. A plate blank was incorporated in the experiment for quality control.

Results

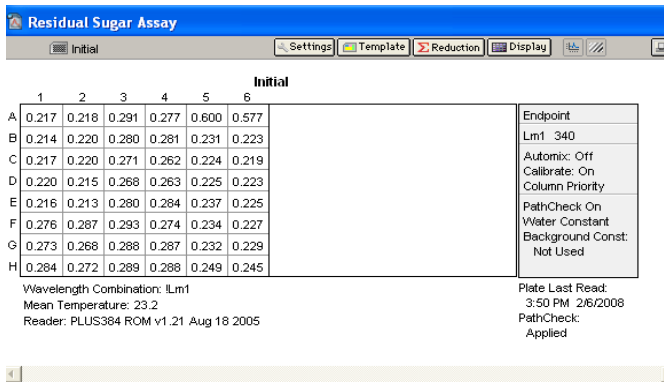
The experiment was performed as described in the Materials and Methods section. A baseline reading was taken with the plate containing all the components of the reaction mixture except the enzyme (Figure 7, Panel A). After adding the enzyme to the reaction mixture, the plate was incubated for 21 minutes at room temperature and a final reading was taken (Figure 7, Panel B).

SoftMax Pro Software automatically calculated mean, standard deviation, and %CV for the standard and tabulated the results in a group section (Figure 8).

Standard curve

A standard curve was created using information from group section “STD” with concentration on the x-axis and mean OD value of the replicates on the y-axis (Figure 9). Standard deviation was used to denote the error bars, while a linear curve fit was assigned to the plot. The standard curve was used in determining the residual sugar concentration in wine samples denoted as “Unknown.”

A



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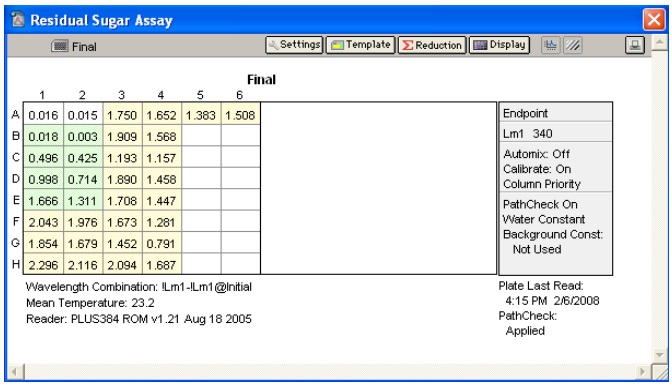


Figure 7. Results for RS assay. A: Plate with all the reagents and no enzyme. **B:** Plate read after addition of enzyme and incubation.

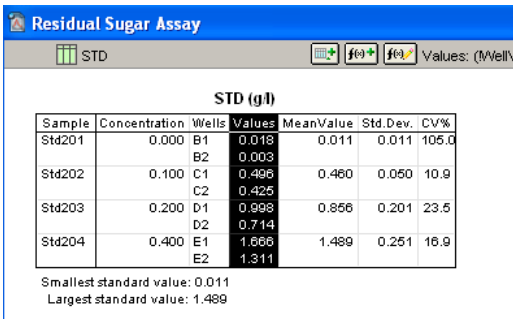


Figure 8. Calculations for standards. Group section standard with mean, standard deviation and %CV calculations.

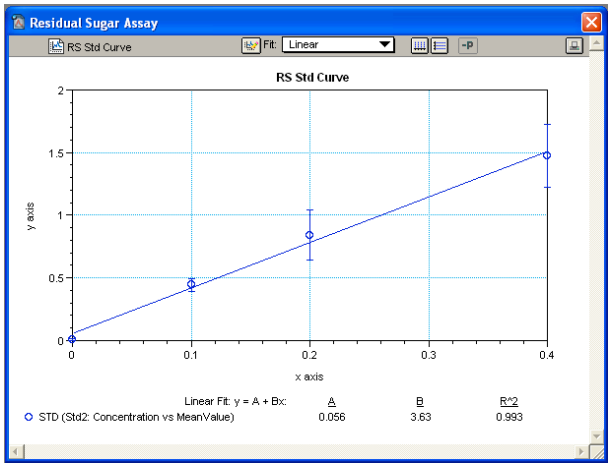


Figure 9. RS assay standard curve. A standard curve plotted and used for deriving the concentration of residual sugar in wine samples.

A group section designated as “Unknown” was populated automatically with data from samples designated as “Unknown” in the template.

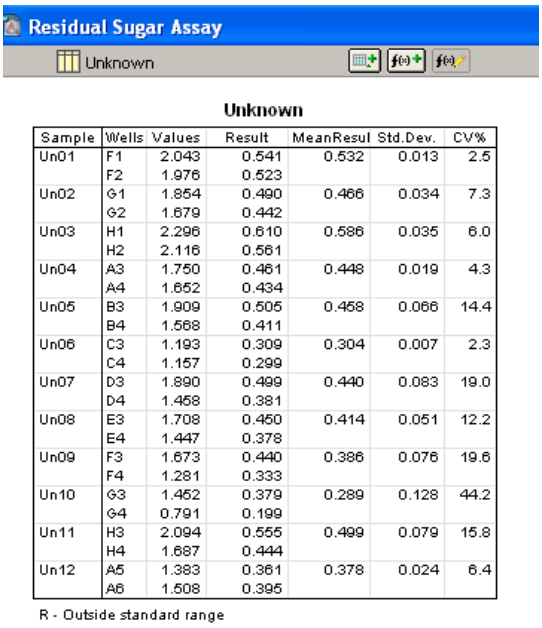
Residual sugar in each sample was calculated by the software using the standard curve for interpolation (Figures 10 and 11).

Conclusion

The SpectraMax Plus 384 Microplate Reader is a good choice for running enzymatic quantitative measurement of residual sugar (RS) and Malate (ML) in wines and can be extended to similar assays, including ammonia and volatile acidity measurement.

Advantages include the following:

- It is cost- and time-effective.
- Tunability and the PathCheck Sensor features of the SpectraMax Plus 384 reader aid in reaching higher precision and accuracy.
- SoftMax Pro Software is a convenient tool for analysis and calculation of complicated and large data sets. It offers pre-written, ready-to-use protocols, custom formulas and appropriate graphing options.
- Other Molecular Devices readers with absorbance detection mode, e.g. the SpectraMax i3x Multi-Mode Microplate Reader, may also be used for this application.
- For increased throughput requirements, Molecular Devices StakMax® microplate handling system integrates with SpectraMax readers to enable automated processing of batches of 20, 40, or 50 microplates.



Unknown

Sample	Wells	Values	Result	MeanResul	Std.Dev.	CV%
Un01	F1	2.043	0.541	0.532	0.013	2.5
	F2	1.976	0.523			
Un02	G1	1.854	0.490	0.466	0.034	7.3
	G2	1.679	0.442			
Un03	H1	2.296	0.610	0.586	0.035	6.0
	H2	2.116	0.561			
Un04	A3	1.750	0.461	0.448	0.019	4.3
	A4	1.652	0.434			
Un05	B3	1.909	0.505	0.458	0.066	14.4
	B4	1.568	0.411			
Un06	C3	1.193	0.309	0.304	0.007	2.3
	C4	1.157	0.299			
Un07	D3	1.890	0.499	0.440	0.083	19.0
	D4	1.458	0.381			
Un08	E3	1.708	0.450	0.414	0.051	12.2
	E4	1.447	0.378			
Un09	F3	1.673	0.440	0.386	0.076	19.6
	F4	1.281	0.333			
Un10	G3	1.452	0.379	0.289	0.128	44.2
	G4	0.791	0.199			
Un11	H3	2.094	0.555	0.499	0.079	15.8
	H4	1.687	0.444			
Un12	A5	1.383	0.361	0.378	0.024	6.4
	A6	1.508	0.395			

R - Outside standard range

Figure 10. Calculation of residual sugar concentration in wine samples. Residual sugar concentration in wine samples was calculated using the standard curve.

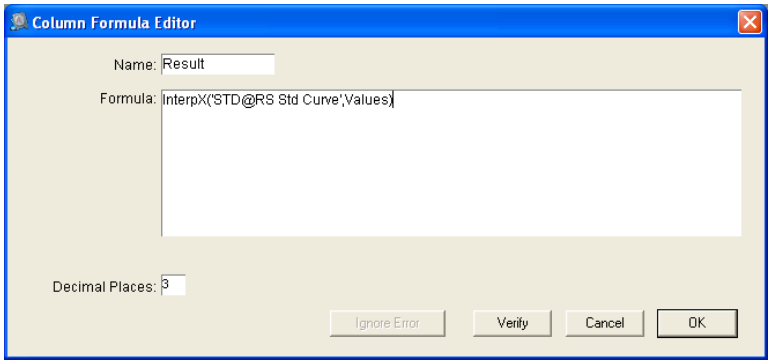


Figure 11. Custom formula for interpolation of standard curve. Formula for the column “Result” that automatically calculated residual sugar in wine samples by interpolation from the standard curve.

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