# Enzym Methods in Wine Analysis

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***Key words:*** enzyme-test measurements, statistical comparison of enzymatically and instrumentally measured results

***Summary:*** In our laboratory for special determinations BOERINGER-MANNHEIM's enzyme-test combinations have been used for 10 years. Our present work deals with the practical aspects of the enzymatic determination of so important wine components like L-malic, L-lactic and citric acid, glycerol, D-gluconic acid, D-sorbitol, acetic aldehyde and D-glucose to D-fructose (G/F) ratio. Whenever possible, the results are compared to the results of other methods (spectrophotometry, gas chromatography, polarimetry) used at our department.

## Introduction

Wine could presumably be considered one of the most complex systems among food products. The growth of quality demands, that is equal to the criteria of marketability and exportability, makes necessary the measurement of more and more wine constituents. The determination of certain compounds is a separation task. For economic reasons analytical equipment like GC, HPLC...*etc.* are often not available either in production quality control or in research. In these cases microbiological *(Horváth J.* 1974.) and enzyme-test *(Handbuch Boehringer,* 1987.) methods may give a serious help in measuring different compounds.

## Material and methods

The description of the determinations in details are omitted because they are included in every package of the enzyme-tests sent by the producer. The mere basics of the methods used will be discussed.

A common feature of the test-combinations determining the compounds above, except D-sorbitol is, that all of them is based on the measurement of the NADH's or NADPH's UV absorbance changes occuring in the enzyme-catalyzed or connected reaction. The amount of the NADH reacted or the quantity of the formed NADPH are proportional to the concentration of the chemical substansce being analysed. Absorbances of NADH and NADPH were measured at the wavelengths of 334, 340 or 365 nm. When determining the D-sorbitol, the absorbtion of formazane equivalent to the component itself was measured at 492 nm.

Our experiments were performed as follows:

- measuring the discrepancies between the concentrations of the model solutions set by analytical balance (nominal concentration) and the real one determined by the enzyme-test, means accuracy control,
- determination of the accuracy of the enzyme methods in preservative ($SO_2$ , potassium sorbate) free wines by standard addition,
- investigation of the effect of preservatives on the enzyme-reactions and as a consequence, on the accuracy of the methods at two concentration-level combinations;
  - a.) normal concentrations:
    total $SO_2$ = 300 mg/dm$^3$
    potassium sorbate = 250 mg/dm$^3$
  - b.) extreme high concentrations:
    total $SO_2$ = 600 mg/dm$^3$
    potassium sorbate = 1000 mg/dm$^3$-

examination of the effect of dilution (10- and 100-fold) on the accuracy in model solutions,

- comparison of the results of the enzymatic glycerol and G/F ratio measurements to the result of the other methods (spectrophotometry, gas chromatography, polarimetry) used in our lab.

## Results and discussion

Based on the results of the investigations the conclusions could be drawn, as follows:

- To control the accuracy of the enzym methods, solutions of chomatography-grade standards with several concentrations were prepared by analytical-balance mass measurements and were determined in 5 parallels enzymatically. According to the model solution measurements (*see Table 1.*) it could be stated, that the results of the enzymatic concentration determinations were lower than the nominal ones by 1.0–5.0%. It means that 95.0–99.0% efficiency could be achieved by the enzymatic methods. The highest difference occurred in the case of acetic aldehyde, that could be explained by the troubles of preparing an accurate nominal concentration model solution as well. Due to the t-probe results (degree of freedom= 4, probability level = 95 %) the difference was significant.
- In the case of wine measurements free of preservatives standard addition proved (*see Table 2.*), that concentrations determined enzymatically were lower by 3.4–12.5% than the nominal values set by analytical-balance. The smallest discrepancy occurred in the citric acid and the highest in the acetic aldehyde determinations. Discrepancies have been analysed by mathematical-statistical methods. Based on the results of t-tests it has been stated that the differences of the two extreme values are significant both in the citric acid and in the acetic aldehyde measurements.
- In the experiments of the wines containing two level preservative concentrations the discrepancies were similar (*see Tables 3* and *4).* It is of great importance however, that the preservatives in the concentrations applied do not disturb the determinations, they do not inhibit the perfection of the enzyme reactions.
- The effect of dilution on the accuracy is demonstrated in one characteristic case picked out of L-lactic acid determination in model solution (*see Tables 5* and *6).* The data of the tables prove the importance of the right choice of dilution from the point of view of accuracy. The discrepancies between the tenfold and hundredfold diluted samples are already significant. The mistake of the undiluted sample determination is unacceptably great compared to the tenfold (optimally) diluted one.
- In the comparison of the enzyme-test results to the results of other instrumental analytical determinations the next measurements have been performed:

***Table 1*** Statistical probe of an experimental average measured in acetic aldehyde model solution

| Number of measurement | Nominal | Enzymatic | Discrepancy |
|---|---|---|---|
| 1. | 0.120 | 0.114 | 0.006 |
| 2. | 0.120 | 0.118 | 0.002 |
| 3. | 0.120 | 0.112 | 0.008 |
| 4 | 0.120 | 0.116 | 0.004 |
| 5. | 0.120 | 0.109 | 0.011 |

(X) average of discrepancies = 0.0062
(S) deviation of discrepancies = 0.0035
$t_{critical}$ = 2.776 (95% probability, degr. of freedom = 4)

$$t_{calculated} = \frac{X\,(n)^{1/2}}{S} = 3.961$$

Being $t_{critical} < t_{calculated}$, ***the difference*** between the nominal (set by analytical-balance) and the enzymatically measured concentration ***is significant!***

***Table 2*** Statistical probe of an experimental average measured in preservative free wine for citric acid

| Number of measurement | Nominal | Enzymatic | Discrepancy |
|---|---|---|---|
| 1. | 1.000 | 0.964 | 0.036 |
| 2. | 1.000 | 0.984 | 0.016 |
| 3. | 1.000 | 0.942 | 0.058 |
| 4. | 1.000 | 0.999 | 0.001 |
| 5. | 1.000 | 0.941 | 0.059 |

(X) average of discrepancies = 0.0340
(S) deviation of discrepancies = 0.0256
$t_{critical}$ = 2.776 (95% probability, degr. of freedom = 4)

$$t_{calculated} = \frac{X\,(n)^{1/2}}{S} = 2.970$$

Being $t_{critical} < t_{calculated}$, ***the difference*** between the nominal (set by analytical-balance) and the enzymatically measured concentration ***is significant!***

***Table 3*** Accuracy test in model solutions in five parallels

| Compound | Addition. (g/dm$^3$) | Concentration measured enzymatically (g/dm$^3$) | | Diff. of nom.& enz. conc. (%) |
|---|---|---|---|---|
| | | average | variance | |
| Acetic aldehyde | 0.120 | 0.114 | 0.003 | 5.0 |
| D-Sorbitol | 0.200 | 0.195 | 0.002 | 2.5 |
| D-Gluconic acid | 1.000 | 0.967 | 0.017 | 3.3 |
| G/F | 22.500/27.500 | 21.833/26.377 | 0.197/0.235 | 3.0/4.1 |
| Glycerol | 0.389 | 0.379 | 0.008 | 2.5 |
| L-Malic acid | 0.196 | 0.198 | 0.003 | 1.0 |
| L-Lactic acid | 2.000 | 1.946 | 0.023 | 2.7 |
| Citric acid | 0.400 | 0.385 | 0.008 | 3.8 |

*Table 4* Standard addition in preservative free wines in five parallels

| Compound | Additional conc. ($g/dm^3$) | Enzymatically meas.conc. in the base wine ($g/dm^3$) | | Enzymatically meas.conc. in the additioned wine ($g/dm^3$) | | Var.of add.& enz. detn.conc. (%) |
|---|---|---|---|---|---|---|
| | | verage | variance | average | variance | |
| Acetic aldehyde | 0.080 | 0.041 | 0.003 | 0.111 | 0.005 | 12.5 |
| D-Sorbitol | 0.105 | 0.173 | 0.006 | 0.269 | 0.011 | 9.0 |
| D-Gluconic acid | 1.020 | 0.142 | 0.010 | 1.090 | 0.039 | 7.1 |
| G/F | 12.500/12.500 | 10.900/11.290 | 0.300/0.300 | 22.61/23.32 | 0.46/0.39 | 4.8/3.8 |
| Glycerol | 0.970 | 6.669 | 0.096 | 7.582 | 0.110 | 5.9 |
| L-Malic acid | 1.005 | 1.191 | 0.026 | 2.126 | 0.088 | 7.0 |
| L-Lactic acid | 1.030 | 2.460 | 0.139 | 3.389 | 0.118 | 9.8 |
| Citric acid | 1.000 | 0.253 | 0.016 | 1.219 | 0.011 | 3.4 |

*Table 5* Effect of the dilution on the accuracy of the determination of model solution concentrations in five parallel measurements

| Compound | Added std. conc. | Concentration determined enzymatically ($g/dm^3$) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Undiluted | | | 10-fold diluted | | | 100-fold diluted | | |
| | (g/dm3) | avg | deviation | var.% | avg | deviation | var.% | avg | deviation | var.% |
| Acetic aldehyde | 0.120 | 0.077 | 0.005 | 35.8 | 0.112 | 0.004 | 6.7 | Ø | Ø | 100 |
| D-Sorbitol | 0.200 | 0.191 | 0.003 | 4.5 | 0.192 | 0.004 | 4.0 | 0.083 | 0.009 | 58.5 |
| D-Gluconic acid | 1.000 | 0.748 | 0.033 | 74.8 | 0.958 | 0.014 | 4.2 | 0.057 | 0.085 | 94.3 |
| G/F | 22.5/27.5 | 0.39/0.47 | 0.04/0.03 | 98.3/98.3 | 3.36/3.64 | 0.44/0.28 | 85.1/86.8 | 21.4/26.5 | 0.48/0.34 | 4.9/3.5 |
| Glycerol | 0.389 | 0.378 | 0.006 | 2.8 | 0.357 | 0.011 | 8.2 | 0.078 | 0.035 | 79.9 |
| L-Malic acid | 0.196 | 0.192 | 0.004 | 2.0 | 0.185 | 0.009 | 5.6 | 0.038 | 0.039 | 80.6 |
| L-Lactic acid | 2.000 | 0.319 | 0.011 | 84.1 | 1.944 | 0.027 | 2.8 | 1.840 | 0.067 | 8.0 |
| Citric acid | 0.400 | 0.387 | 0.005 | 3.3 | 0.382 | 0.009 | 4.5 | 0.028 | 0.041 | 93.0 |

*Table 6* Standard addition in wines with preservatives ($SO_2$ :300 p.p.m., K-Sorbate: 250 p.p.m.) in five parallels

| Compound | Additional conc. ($g/dm^3$) | Enzymatically meas.conc. in the base wine ($g/dm^3$) | | Enzymatically meas.conc. in the additioned wine ($g/dm^3$) | | Var.of add.& enz. detn.conc. (%) |
|---|---|---|---|---|---|---|
| | | average | variance | average | variance | |
| Acetic aldehyde | 0.080 | 0.040 | 0.006 | 0.112 | 0.006 | 10.0 |
| D-Sorbitol | 0.102 | 0.172 | 0.008 | 0.268 | 0.006 | 5.9 |
| D-Gluconic acid | 1.025 | 0.139 | 0.012 | 1.089 | 0.040 | 7.3 |
| G/F | 12.500/12.500 | 10.740/11.180 | 0.340/0.470 | 22.51/23.14 | 0.25/0.48 | 5.8/4.3 |
| Glycerol | 0.965 | 6.542 | 0.126 | 7.442 | 0.091 | 6.7 |
| L-Malic acid | 1.005 | 1.186 | 0.027 | 2.088 | 0.122 | 10.2 |
| L-Lactic acid | 1.030 | 2.460 | 0.166 | 3.389 | 0.131 | 9.8 |
| Citric acid | 1.000 | 0.241 | 0.014 | 1.191 | 0.011 | 5.0 |

1. comparing the enzymatic glycerol determination to gas-chromatography,
2. comparing the enzymatic G/F ratio measurement to polarimetry,
3. comparing the enzymatic acetic aldehyde determination to spectrophotometry.

Our experiments aimed at the elucidation of the discrepancies between the enzymatic and analitical methods.

1. When the comparison of the enzymatic to gas-chromatographic glycerol measuring method was made, the glycerol content of 41 wine samples was also determined. The mathematical-statistical parameters of the regression analysis are summarized in *Table 7*.
   According to the results of the statistical evaluation the differences between the two methods are not significant. The equation of the linear regression ($y = 1.0134x + 0.0418$) shows that concentrations determined by the ezymatic method are a bit lower than the gas-chromatographic ones. Data obtained by any of the methods can be converted into each other by the regression equation.
2. The results of G/F ratio measurements of 22 wine samples prove, that the enzymatic and polarimetric methods are practically equal. Based on the standard deviation quotients, on the ratios calculated from the slope of the regression equation and on the correlation coefficient it has been stated, that the enzymatic method is a bit more accurate, but the difference between the two methods is not significant.
3. The comparison of enzymatic to spectrophotometric acetic aldehyde determinations show a close linear relationship between the results of the two methods. The values can be converted into each other by the equation of the regression straight. Spectrophotometric acetic aldehyde concentrations of wines and wine distillates are significantly lower than the enzymatic ones.

*Table 7* Standard addition in wines with preservatives ($SO_2$ :600 p.p.m., K-Sorbate: 1000 p.p.m.) in five parallels

| Compound | Additional conc. ($g/dm^3$) | Enzymatically meas.conc. in the base wine ($g/dm^3$) | | Enzymatically meas.conc. in the additioned wine ($g/dm^3$) | | Var.of add.& enz. detn.conc. (%) |
|---|---|---|---|---|---|---|
| | | average | variance | average | variance | |
| Acetic aldehyde | 0.080 | 0.038 | 0.005 | 0.113 | 0.006 | 6.3 |
| D-Sorbitol | 0.103 | 0.172 | 0.172 | 0.265 | 0.009 | 9.7 |
| D-Gluconic acid | 1.020 | 0.140 | 0.011 | 1.103 | 0.056 | 5.6 |
| G/F | 12.500/12.500 | 10.050/11.250 | 0.290/0.470 | 22.83/23.37 | 0.38/0.51 | 5.8/3.0 |
| Glycerol | 1.060 | 6.436 | 0.114 | 7.425 | 0.122 | 6.7 |
| L-Malic acid | 1.003 | 1.185 | 0.038 | 2.079 | 0.089 | 10.9 |
| L-Lactic acid | 1.030 | 2.428 | 0.190 | 3.376 | 0.198 | 8.0 |
| Citric acid | 1.000 | 0.231 | 0.015 | 1.174 | 0.018 | 5.7 |

Finally summarizing the above results it can be stated, that enzyme-test methods applied in wine analysis can be considered standard methods. They are really capable of determining such components when the measurement is too expensive and time consuming and the results of what are not reliable enough. Regarding the expense and the need of highly qualified operators of HPLC and GC systems, the national production of enzyme-test preparations should be reconsidered.

*Table 8* Optimal dilution rates in case of enzymatic measurements determined by model solutions

| Compound | Dilution rate |
|---|---|
| Acetic aldehyde | 10 |
| D-Sorbitol | 10 |
| D-Gluconic acid | 10 |
| G/F | 100 |
| Glycerol | 1 |
| L-Malic acid | 1 |
| L-Lactic acid | 10 |
| Citric acid | 1 |

*Table 9* Statistical parameters of the measurements

| Parameter | Enzym. and GC Glycerol detn. | G/F determination | | Aceticaldehyde detn. |
|---|---|---|---|---|
| | | Enzymatic and Polarimetric | Enzyme and s.photometric | |
| | | Glucose | Fructose | |
| Number of measurements(N) | 41 | 22 | 22 | 15 |
| Slope of regression straight(a) | 1.0134 | 1.065 | 0.977 | 0.557 |
| Intercept of regression straight(b) | 0.0418 | –0.946 | –0.182 | 1.319 |
| Correlation coefficient(r) | 0.997 | 1.000 | 0.999 | 0.997 |
| Standard deviation of X-values ($S_x$) | 2.6825 | 20.457 | 20.753 | 135.919 |
| Standard deviation of Y-values ($S_y$) | 2.7268 | 20.214 | 20.302 | 75.833 |

Note: X values are the results of the enzymatic determinations

## References

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