Basic Microbiological and Chemical Analyses for Wine

WASHINGTON STATE UNIVERSITY EXTENSION • EM047

Table of Contents

Preface ... iii
General Safety ... 1
Preparation of Solutions ... 2
Unit prefixes and symbols ... 2
Unit conversions ... 2
Molecular weight ... 2
Preparation of solutions ... 3
Reading a meniscus ... 4
Use of pipettes. ... 5
Storage and stability of chemical solutions ... 5
Other useful laboratory information ... 5
Selection and Validation of an Analytical Method ... 6
Uncertainty and error in scientific measurements ... 6
Verification and validation of testing methods ... 7
Must and Wine Analyses (Microbiology) ... 8
Method 1: Use and calibration of a phase contrast microscope ... 9
Method 2: Wet mount preparation ... 11
Method 3: Isolating microorganism ... 12
Method 4: Concentration of cells ... 15
Method 5: Enumeration/detection of microorganisms ... 17
Method 6: Fermentation tubes ... 19
Must and Wine Analyses (Chemistry) ... 20
Method 7: Standardization of NaOH ... 21
Method 8: pH ... 23
Method 9: Titratable acidity ... 26
Method 10: Volatile acidity ... 30
Method 11: Yeast assimilable nitrogen ... 34
Method 12: Ammonia ... 37
Method 13: Soluble solids (hydrometer) ... 39
Method 14: Soluble solids (refractometer) ... 41
Method 15: Free/total sulfur dioxide (Ripper) ... 43
Method 16: Free/total sulfur dioxide (aeration/oxidation) ... 48
Method 17: Alcohol (Ditta Ing C. Bulio ebulliometer) ... 53
Method 18: Alcohol (AllaFrance ebulliometer) ... 56
Method 19: Reducing sugars (Clinitest) ... 58
Method 20: Reducing sugars (modified Lane-Eynon) ... 60
Method 21: Malic acid ... 63
Method 22: Reduced sulfur compounds ... 65
Method 23: Wine color ... 68
Method 24: Protein removal (bentonite) ... 69
Method 25: Potassium hydrogen tartrate stability ... 72
Appendix I. Parts of a Microscope ... 75
Appendix II. Descriptions of Microorganisms ... 77
Appendix III. Table for Addition of Potassium Metabisulfite ... 80
Glossary ... 81
References ... 86
The Authors ... 88

Preface

This manual is intended for those wishing to conduct general laboratory analyses of grape musts and wines. Determinations of sugar, sulfur dioxide, titratable and volatile acidities, pH, and alcohol content are integral measurements performed by commercial and home winemakers. While these determinations allow chemical adjustments to musts and wines, they are also critical for compliance with state and federal laws regarding composition (e.g., alcohol, volatile acidity, and sulfur dioxide).

One area that the authors have tried to address throughout this manual is the question of quality assurance regarding laboratory results. Wine statistics are notoriously difficult to compare between wineries due to variability in methods and practices between laboratories. In fact, wineries occasionally encounter difficulty in obtaining reproducible results even within the same laboratory. In this manual, we have suggested some controls that may help wineries address these issues, but the opportunity exists for more work to be done in this area.

This work represents a follow-up to the *Laboratory Manual for Wineries* published in 1990 (MISC 0146). Rather than publish a second edition, the authors felt a new title was justified, given the extensive changes to format and expanded content. We hope that you find *Basic Microbiological and Chemical Analyses for Wine* useful in your situation (winery, laboratory, or classroom). Please feel free to write or email us if you have questions or comments.

The authors wish to thank Mr. Glenn O'Dell (Constellation Brands US, California) for his advice and assistance. Additional thanks are extended to Washington State University for support of this project.

—*Charles G. Edwards and Bruce A. Watson*
January 3, 2013

Basic Microbiological and Chemical Analyses for Wine

General Safety

Safety is very important when working in a laboratory or winery. To keep everyone safe, the following instructions must be obeyed while in the winery and laboratory:

1. All employees, visitors, and students MUST wear safety goggles/glasses at all times. Although eye protection equipment can be uncomfortable, it can save eyesight! Locate a good, comfortable pair of safety glasses or goggles with side shields for laboratory work.
2. Eating or drinking should not be allowed in the laboratory. Glassware used for analytical methods should be kept separate from that used for human consumption.
3. Avoid wearing open-toe shoes and loose-fitting clothing. Chemical spills do happen and it is important to avoid spills directly onto feet. Loose-fitting clothing can catch on equipment and result in hands or arms being pulled into turning/moving equipment such as a stemmer/crusher.
4. Lab coats are optional but may be desirable to protect clothing from wine stains or chemicals.
5. The laboratory or winery is required by law to have Material Safety Data Sheets (MSDS) for every chemical on the premises, on file in a readily accessible location. These sheets include all information about a chemical including flammability, toxicity, chemical properties, and disposal methods. Be sure to know the location, format, and contents of these highly useful pamphlets.
6. If a chemical is spilled, clean up using appropriate and approved methods of disposal. Be sure to consult the relevant MSDS for information regarding disposal.
7. Know the location of such safety items as (a) safety manual information, (b) eye wash units, (c) fire extinguishers, (d) first aid kits, (e) overhead showers, and (f) telephones with emergency numbers.
8. Mouth pipetting is not allowed for any reagent at any time.
9. In the winery, the floors may be wet and much of the equipment has moving parts. Use EXTREME caution at all times to avoid falls or being injured.

Preparation of Solutions

Unit prefixes and symbols

			Prefix	Symbol
1,000,000	=	10^{6}	mega	M
1000	=	10^{3}	kilo	k
100	=	10^{2}	hecto	h
0.1	=	10^{-1}	deci	d
0.01	=	10^{-2}	centi	c
0.001	=	10^{-3}	milli	m
0.000001	=	10^{-6}	micro	µ
0.000000001	=	10^{-9}	nano	n

Unit conversions

1. Weight
    - grams (g) = 0.035 oz = 0.0022 lb
    - pounds (lb) = 454 g
    - tons (short) = 2000 lb

2. Volume
    - liters (L) = 0.264 gal
    - gallon (gal) = 3.785 L = 128 fl oz
    - fluid ounce (fl oz) = 0.0297 L

3. Temperature
    - °C = $^{5}/_{9}$ (°F - 32)
    - °F = $^{9}/_{5}$ (°C) + 32

Molecular weight

Like the term "dozen" equals 12 units, one mole in chemical terms is equal to 6.02 x 10^{23} particles (atoms or molecules) which is known as Avogadro's number. The weight of a mole of atoms is termed the molecular weight (MW) or gram formula weight (GFW). The weight of a mole varies with the type of element or compound; 1 mole of hydrogen weighs 1 gram while sodium, a heavier molecule, has a molecular weight of 23 g/mole. Individual elements react together to form compounds, which, in turn, can further react to form new compounds. For instance, one oxygen atom (element) will combine with two hydrogen atoms to form one compound of water or H_2O. As individual atoms or molecules cannot be counted (only estimated using Avogadro's number), physical portions can be weighed. So, one mole of oxygen (16 g) will combine completely with two moles of hydrogen atoms (2 g) to form one mole of water (18 g).

The molecular weight of other elements is shown in Table 1.

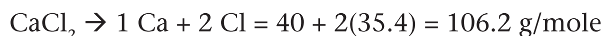
The molecular weight of some common compounds can be determined by using Table 1. For example, the molecular weight of sodium hydroxide (chemical formula NaOH) is 40 g/mole while calcium chloride

Table 1. Molecular weights of some common elements.

Symbol	Name	MW	Symbol	Name	MW
H	Hydrogen	1	Al	Aluminum	27
C	Carbon	12	Cl	Chlorine	35.4
N	Nitrogen	14	Ca	Calcium	40
O	Oxygen	16	Fe	Iron	55.8
Na	Sodium	23	Cu	Copper	63.5
Mg	Magnesium	24.3	Zn	Zinc	65.4

($CaCl_2$) has a molecular weight of 106.2 g/mole.

NaOH → 1 Na + 1 O + 1 H = 23 + 16 + 1 = 40 g/mole


$CaCl_2$ → 1 Ca + 2 Cl = 40 + 2(35.4) = 106.2 g/mole

Determination of the molecular weight of solutes is essential in the preparation of chemical solutions. Moles can be used to express solute concentration in two different ways:

> **Molarity** (M) is defined as the number of moles of solute per liter of solution. To calculate M, the weight of dissolved solute and its molecular weight need to be known.
>
> **Normality** (N) is defined as the number of equivalents of solute per liter of solution. To calculate N, the weight of dissolved solute and its equivalent weight are required.

One equivalent weight is the weight that contains 1 mole of replaceable hydrogen (acid) or hydroxyl (base). Molarity and normality are therefore related by N = nM where n equals the number of replaceable H^+ or OH^- per molecule (for acids and bases). Because sulfuric acid (H_2SO_4) has two H^+, a 0.01M solution is equivalent to 0.02 N.

The molarities and normalities of concentrated acids commonly used in the laboratory are shown in Table 2.

Table 2. Common reagents and their molarities and normalities.

Reagent	Symbol	Molarity	Normality
Acetic acid	$HC_2H_3O_2$	17.4	17.4
Formic acid	$HCHO_2$	23.6	23.6
Hydrochloric acid	HCl	12.1	12.1
Nitric acid	HNO_3	15.8	15.8
Sulfuric acid	H_2SO_4	18	36
Phosphoric acid	H_3PO_4	14.8	44.6

Preparation of solutions

When preparing solutions or reagents, be sure to use only distilled or deionized water as well as clean glassware. To adequately clean glassware, wash in hot tap water with residue-free soap (for instance, Alconox® or similar product). Rinse using hot tap water and then apply a final rinse of distilled water to remove any residual deposits (for example, minerals from hard water). Clean glassware should not have any water spots or other residue present.

As an example, how many grams of NaOH are needed to prepare one liter of 0.1 M solution?

MW of NaOH = 40 g/mole

$$\frac{0.1 \text{ mole}}{1 \text{ liter}} \times \frac{40 \text{ g NaOH}}{1 \text{ mole}} \times 1 \text{ liter} = 4 \text{ g NaOH}$$

Add 4 g NaOH to a 1 L volumetric flask. Add distilled water to dissolve solid NaOH and bring to 1 L volume.

Note that this calculation assumes 100% purity of the NaOH crystals. Like some other compounds, NaOH reacts with compounds present in the atmosphere such as CO_2 and H_2O, which decrease its purity and strength. Other examples are sugars (glucose, fructose, or sucrose) and $K_2S_2O_5$ (potassium metabisulfite) which react with atmospheric H_2O. In some situations, it is important to remove the absorbed water prior to preparation of solutions. For example, preparation of potassium hydrogen phthalate ($KHC_8H_4O_4$) may be used to standardize NaOH solutions (Method 7). However, other compounds do not have this requirement. Be sure to read the label on the chemical when determining an expiration date or if water must be chemically removed before use.

Reading a meniscus

An important technique to be able to use a volumetric flask, pipette, or graduated cylinder is to determine when the container holds the exact volume specified. The level (top surface) of liquid in a container does not form a straight edge; it is curved. To correctly measure the amount of liquid, scientists have used the bottom of the curved surface, that is, the **meniscus**, as the point at which volume is determined (Figure 1).

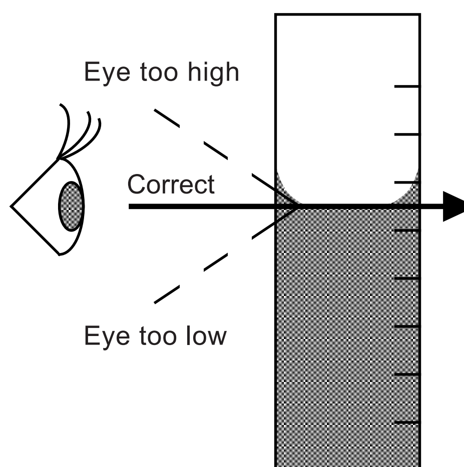


Figure 1. Proper viewing of a meniscus.

Use of pipettes

1. Pipetting techniques

   "To Contain" (T.C.). Pipettes must be rinsed out after all liquid has drained and the rinsing added to the sample.

   "To Deliver" (T.D.). Drain pipette vertically with the tip against the side of the receiving vessel.

   "To Deliver/Blow Out." Drain pipette vertically and blow out the last drop that remains in the tip. Some manufacturers place two bands near the mouthpiece to differentiate from T.D. pipettes.

2. On a graduated pipette, the numbering system "10 mL in 1/10" means that the pipette contains a total volume of 10 mL and has graduations of 0.1 mL.

Storage and stability of chemical solutions

Important considerations in the laboratory are the stability and storage of chemicals. Some dry chemicals can be stored in cupboards or on shelves in the lab and are stable for many years. Others may adsorb moisture or CO_2 from the atmosphere or otherwise lose strength or change composition.

Liquids may require special considerations including (a) low temperature, (b) minimal light exposure, (c) compatibility with other chemicals, and (d) flammability. Always consult the label or the materials data safety sheet (MSDS) for storage and shelf life.

WARNING! Under no circumstances should flammable solvents be stored in a regular refrigerator or freezer because organic vapors can create a very serious explosion hazard in a confined space.

In general, solutions have limited stability and require either frequent standardization or periodic replacement. Microbial contamination and degradation can be a problem with some solutions.

Other useful laboratory information

1. Always add *ACID TO WATER* when preparing mineral acid solutions (sulfuric, hydrochloric or nitric acids). Place most of the required water in the bottom of the volumetric flask and cool the flask in an ice bath as the acid is added *slowly*. The best way to add the acid is to allow it to slowly flow along the inside wall of the flask, to avoid splashing.
2. Make solutions in volumetric flasks by placing the solute in the flask before adding the solvent in small increments (EXCEPT FOR ACIDS).
3. Always have another person present in the laboratory for safety reasons and for assistance in checking calculations before preparation of solutions.
4. MSDS (materials safety data sheets) sheets explain cautions for chemicals and are available from chemical companies. These are

required by law to be in a safety file on site where the chemicals are used. The Merck Index is another source for chemical hazard information.

5. Always use appropriate safety equipment in the laboratory (protective eye goggles, etc.).
6. NEVER return a chemical to the original container if some is left-over from weighing operations.
7. Never add liquids to hot, dry glassware since glass breakage is probable.
8. Most of the chemical solutions described in this manual can be purchased from commercial chemical companies or wine analytical laboratories. While these prepared, sometimes standardized, solutions can be more expensive, they save in preparation time and reduce error.

Selection and Validation of an Analytical Method

In this manual, methods are described for a range of analyses but these may or may not be the best method for any given situation. In general, selection of a method depends on several variables including (a) dependability, (b) practicality (can you perform the method with available equipment and within the time allowed?), (c) specificity, (d) sensitivity, (e) the required accuracy and precision, and (f) expense.

Finally, some analyses are chosen based on legal considerations. For example, there are federal legal limits for acetic acid in wine but the analytical method must be one that is approved for that analysis. Be sure to consult either the Official Methods of Analysis or Code of Federal Regulations (Title 27, Part 240) for more information.

It is important to periodically analyze samples of the standards of known concentration of the analyte to validate the method and to be sure it is performing as expected. Standards can be either purchased from commercial chemical supply companies or easily prepared in the laboratory. Refer to the different methods for specific procedures of validation.

Uncertainty and error in scientific measurements

All measurements made in a laboratory have some inherent degree of uncertainty because a measurement is vastly different from counting. Imagine a box that contains 18 glass marbles. Although the number of marbles is known, the mass (weight) cannot be determined by counting; it must be measured. Depending upon available equipment, the 18 marbles may have a combined mass of 89 g, or, using an expensive scale (balance), 89.137 g. In either case, there is a degree of uncertainty implied, depending on the equipment. In this case, the first scale may provide an uncertainty of 89 ± 1 g while the second may yield 89.137 ± 0.005 g. Thus, it is useful to know the size of the uncertainty associated with any scientific measurement.

Degree of uncertainty can be described in terms of precision and accuracy (Figure 2). Precision is the degree of reproducibility in a measurement. In other words, if the same measurement is performed a dozen times, precision describes the variability of the values obtained. In

contrast, accuracy refers to how closely the results relate to the actual physical reality in question. In the example of the marbles, above, repeat measurement of the mass of the marbles may yield values ± 0.01 g but these values may all be incorrect if the actual weight was 92.56 g instead of 89.14 g. In that case, the measurements exhibited high precision but low accuracy, probably due to an incorrectly calibrated scale.

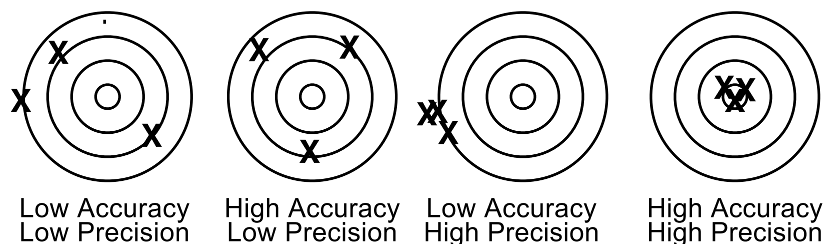


Figure 2. Illustration of accuracy versus precision.

When we express an uncertainty for a particular measurement, it assumes that the instruments involved were properly calibrated and that the operations were carried out correctly. Operator error (poor lab technique) will also contribute to both imprecision and inaccuracy.

When a measurement is made in the laboratory, the goal would be that the measurement be both reasonably precise and accurate. However, this can be difficult to achieve. For example, sending the same wine sample to ten different laboratories often yields different measurements. In fact, it is sometimes difficult to believe that the same wine was actually analyzed!

Verification and validation of testing methods

A regular program of method verification and validation is part of laboratory quality assurance. For many of the laboratory procedures in this manual, method verification procedures will be provided as a means to ensure that measurements are precise and accurate. In addition, acceptable levels of uncertainty will be presented (Table 3). For example, while a pH meter will probably not reliably distinguish between pH 3.56 and 3.57, the meter should reliably distinguish pH 3.56 from 3.66.

Table 3. Approximate expected uncertainties associated with specific wine analyses.

Method	Expected Uncertainty
pH (Method 8)	±0.04
Titratable acidity (Method 9)	±0.15 g/L
Volatile acidity (Method 10)	±0.05 g/L
Free SO_2 by Ripper (Method 15)	±3 mg/L (white), ±5 mg/L (red)
Total SO_2 by Ripper (Method 15)	±5 mg/L (white), ±10 mg/L (red)
Free/Total SO_2 by aeration/oxidation (Method 16)	±2 mg/L
Alcohol by ebulliometer (Methods 17 & 18)	±0.4% v/v
Reducing sugar by Clinitest® (Method 19)	±1 g/L (for wines <0.5% residual sugar)
Reducing sugar by modified Lane-Eynon (Method 20)	±2 g/L

Suggestions for method verification will be based on three types of procedures: (a) use of standards, (b) standard addition where an analyte is added to a wine, and (c) use of wine samples of known composition.

Many methods use standards to calibrate equipment or to verify procedures. For example, pH standards are solutions purchased to calibrate pH meters, while malic acid solutions can be used to verify enzymatic malate assays. If a pH 7.00 and a pH 4.00 standard are used to calibrate the pH meter, an additional check would be to another standard, say pH 3.00. A difficulty is that wine samples are not pure solutions of individual chemical components, which can be a problem due to chemicals that interfere with the analysis. As such, other constituents can interfere with measurements (e.g., CO_2 interferes when measuring titratable acidity) so special steps must be taken.

Another type of method verification relies on a standard addition of the analyte in question to a wine. Here, a wine is analyzed for some analyte (for example, titratable acidity) and then additional tartaric acid is added to the same wine before analysis. Obviously the second sample should have a higher titratable acidity than the first, by the amount of the addition. This provides confidence that the sample preparation procedures are good and that linearity is achievable in the assay response.

The final approach to verify a method that revolves around analysis of wines with known compositions. The difficulty with these types of methods is knowing the true composition of a test wine. One procedure is to send a wine to a commercial analytical laboratory of good reputation, and then to compare results. Alternatively, some suppliers have wines of known composition that can be used.

Each assay method described in this manual will provide (a) instructions for instrument calibration and/or assay calibration using purchased or prepared standards, (b) at least one method for assay verification, (c) the best estimate of the uncertainty of the result in the hands of a competent technician using good instruments and reagents and (d) a list of common pitfalls for each procedure. For some assays, some of the above objectives may not be relevant or may simply be beyond reach. However, the degree of uncertainty inherent in the measurement is a rather subjective value. This value will be based on personal experiences in winery and academic laboratories and on the advice of others in the industry with substantial experience (Table 3).

Must and Wine Analyses (Microbiology)

In addition to using chemical analyses, wineries utilize various microbiological methods to evaluate fermentations and finished wines. Commonly, phase-contrast microscopy is used as a means to quickly examine fermenting must and wines for specific microorganisms. In addition, potential causes of spoilage initially detected by smelling/tasting wines can also be confirmed using a microscope. At times, the winemaker will want to quickly examine a wine sample but may also want to try to isolate the microorganism observed. As light microscopes require use of stains or dyes in order to observe microorganisms, investment in a phase contrast microscope can be well worth the expense.

Method 1: Use and calibration of a phase-contrast microscope

A. Concepts

Unlike normal bright field microscopy, phase-contrast microscopy allows the microbiologist to view living objects (for example, cells) without the use of stains or dyes. This technique relies on the fact that light is retarded and diffracted when it passes through a medium that is denser (the cell) than the surrounding medium (water or wine). The degree to which light is diffracted is defined by the refractive index of the medium. Microscopes equipped with a phase-contrast optical system are designed to enhance the differences in refractive index between the microorganism and surrounding medium, thereby allowing the microorganism to be observed. Because an image viewed under phase-contrast is not colored, this technique should not be used for the examination of stained or dyed microorganisms.

At times, wine microbiologists want to determine the sizes of the microorganisms being viewed through a microscope. This information can help to determine the identity of the microbe in question. To do this, an ocular micrometer is placed into the eyepiece of a microscope, but it must be calibrated for each objective using the outlined procedure, below. Refer to Appendix I for identification of the common parts of a microscope.

B. Equipment/supplies

1. Phase-contrast microscope.
2. Clean microscope slides and coverslips.
3. Ocular and stage micrometers.
4. Immersion oil (100x oil immersion objective only).
5. Lens cleaning solution and lens paper (NOT Kimwipes®-type tissues).

C. Procedure

1. Once sample is prepared, place the microscopic slide onto the stage and turn on the light source.
2. Move the condenser all the way up to the stage.
3. Using the low magnification objective (10x or 20x), attempt to focus on the image on the slide using the coarse adjustment knob.

   NOTE: To help with adjustment, mark an "x" on a clean slide and place the slide onto the stage. As the image is viewed, the condenser and diaphragm can be adjusted to obtain optimal light and image. Alternatively, look for the edge between coverslip and microscope slide or an air bubble under the coverslip.

4. Once the object is in focus, higher magnifications can be achieved by rotating the nosepiece to the proper objective.

   NOTE: Oil immersion objectives require immersion oil to be placed between the objective and the slide. Only one drop is normally needed.

NOTE: Higher magnifications of 40x or 100x will require more light.

5. Once finished, be sure to use lens paper and lens cleaner to clean all objectives and the stage, especially excess immersion oil.
6. To calibrate the microscope, place the ocular micrometer in the eyepiece and place the stage micrometer on the center of the stage. Viewed through the eyepiece of the microscope, the micrometers appear as shown in Figure 3 with lines exactly 10 microns (μm) apart on stage micrometer.

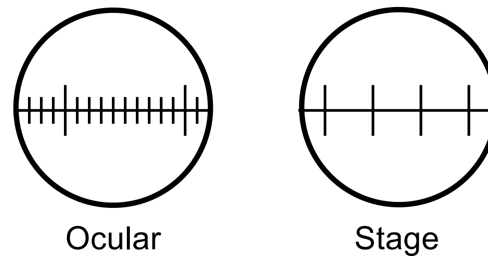


Figure 3. Comparison of markings for an ocular micrometer (left) and stage micrometer (right).

7. Use the low objective (10x) to focus on the scale of the stage micrometer.
8. Once the low objective is focused, rotate the objective to be calibrated into place.

   NOTE: Be sure to use oil with the oil immersion objective.
9. Use the control knobs to move the stage until the two micrometer scales are close to overlapping (Figure 4).

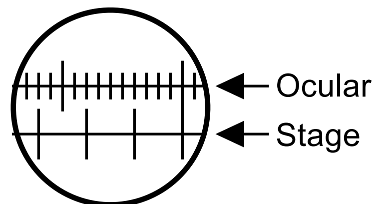


Figure 4. View of ocular and stage micrometer markings, as they move into alignment during calibration.

10. Count the number of ocular micrometer lines that exactly occupy the space between any two of the stage micrometer lines. With four ocular lines per space on the stage micrometer lines, one space of ocular micrometer would be equal to 10 μm/4 = 2.5 μm.
11. For this objective and microscope, the distance between two lines in the ocular micrometer are 2.5 μm. How large are the two microorganisms as viewed using the calibrated ocular micrometer shown in Figure 5?

Approximate size of the cocci-shaped microorganism:

Diameter = (3 spaces)(2.5) = 7.5 μm

Approximate size of the rod-shaped microorganism:

Length = (9.5 spaces)(2.5) = 24 μm
Width = (1.5 spaces)(2.5) = 4 μm

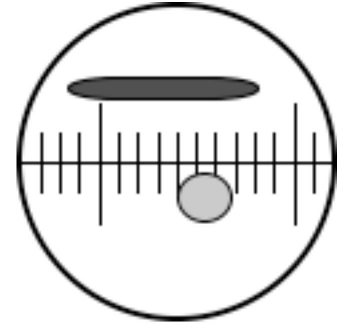


Figure 5. Example of two microorganisms that can be measured with the ocular micrometer.

D. Helpful hints/interpretations

1. General descriptions of microorganisms as viewed under a microscope are found in Appendix II.
2. Immersion oil left on an objective can severely damage the O-rings, so it is important to remove any residue. Clean objectives, stage, and eyepiece using an approved lens cleaning fluid and lens paper after each use.

   WARNING! Never use Kimwipes-type tissues as these will scratch the objectives.

Method 2: Wet mount preparation

A. Concepts

Wine microbiologists will commonly perform a microscopic evaluation by looking at a sample under a wet mount using a phase-contrast microscope. Normally, a minimal population of 10^4 cells per mL is necessary to observe cells microscopically, although some report being able to detect populations as low as 10^3 cells/mL. As such, several microscopic fields should be examined and/or samples should be centrifuged to concentrate microorganisms (Method 4) prior to microscopic evaluation (Method 1). General descriptions of microorganisms as viewed under a microscope are found in Appendix II.

Observing microorganisms under a microscope is enhanced by adding a dye or stain to the culture. Cells will take up the dye or stain, allowing the cell to be observed using bright field microscopy. One such stain, methylene blue, is also used to evaluate viability.

Methylene blue exists in two redox-dependent states: reduced and oxidized. The reduced state (*leuco* form) is colorless, while the oxidized form is blue. If a viable yeast cell takes up the dye, methylene blue is reduced to the colorless form so that the cell appears colorless (or white) against the blue (dyed) background. Thus, cells that reduce the dye to the colorless form are assumed to be viable (live), while dead yeast cells do not reduce the dye and appear blue or black. As methylene blue rapidly becomes toxic to microorganisms, preparations should be microscopically examined within 10 minutes (Fugelsang and Edwards, 2007).

B. Equipment/supplies

1. Phase-contrast microscope.
2. Microscope slides and coverslips.
3. Immersion oil (100x oil immersion objective only).

4. Lens cleaning solution and lens paper (NOT Kimwipes-type tissues).
5. Laboratory (transfer) loops and/or needles with wooden or metal handles (can also substitute toothpicks).
6. Methylene blue dye.
    a. Prepare a citrate buffer by dissolving 2.4 g disodium hydrogen citrate ($Na_2HC_6H_5O_7$) and 2.1 g sodium dihydrogen citrate ($NaH_2C_6H_5O_7$) in a minimal amount of distilled water and dilute to 100 mL. Adjust pH to 4.6 if necessary.
    b. Dissolve 0.3 g methylene blue chloride in 30 mL of 95% v/v ethanol.
    c. Add 100 mL of the citrate buffer to the methylene blue solution.

C. Procedure

1. Using either a laboratory loop or small pipette, place a drop of juice/wine on a clean microscope slide.

    NOTE: It is sometimes necessary to centrifuge samples to concentrate the microorganisms in the pellet, especially if the number of microorganisms in the juice/wine are low. Refer to Method 4 for more information on concentration of cells prior to examination.

2. If desired, add a drop of the methylene blue dye to the sample on the slide to distinguish live cells from dead cells. Skip this step if live cell/dead cell comparison is not needed.
3. Place a coverslip over the suspension.

    NOTE: For longer term storage, the coverslip/slide edge can be sealed with petroleum jelly in order to prevent drying of the mount.

4. Examine the slide using low and oil immersion powers.

D. Helpful hints/interpretations

1. General descriptions of microorganisms viewed under a microscope are found in Appendix II.
2. Immersion oil left on an objective can severely damage the O-rings, so it is important to remove any residual oil after use. Be sure to clean objectives, stage, and eyepiece using approved lens cleaning fluid and lens paper after each use. Never use Kimwipes-type tissues as these will scratch the objectives.

Method 3: Isolating microorganisms

A. Concepts

In order to physically separate microorganisms from a wine, a small aliquot of wine (normally 0.1 mL) is placed on solidified media and allowed to incubate. After incubation, individual colonies on these plates are removed and "streaked" on non-selective (general) and selective media. The objective of streaking is to inoculate fewer and fewer microbes on each of the three consecutive sectors, thereby increasing the chance of producing well separated, isolated

colonies. Various microorganisms present in a sample will appear as different colonies on a plate containing a suitable solidified medium (Table 4). Different microorganisms are evidenced by differences in colony size, color, and shape (Appendix II).

Named after the now defunct Wallerstein Laboratories, WL nutrient broths (liquid) and agars (solid) are general cultivation media for yeasts and molds including non–*Saccharomyces* yeasts, *Saccharomyces*, *Zygosaccharomyces*, and *Brettanomyces*. In fact, these media can be used to monitor yeast population diversity during fermentation because of the unique colony morphologies of various wine yeasts (Pallman et al., 2001). WL media contain a pH indicator, bromcresol green, which permits rapid screening of acid–producing colonies. A related medium known by two names, WL-Differential (WLD) or WL-Cycloheximide (WLC), contains the selective agent cycloheximide added at concentrations of approximately 50 to 100 mg/L, making the medium selective for *Brettanomyces* against *Saccharomyces.*

Table 4. Growth of specific yeast and bacteria in various culturing media.

		Medium			
	Microorganism	**WL**	**WLC**	**MR**	**MRC**
Yeasts	*Saccharomyces*	+	–	+	–
	Non-*Saccharomyces*	+	V	+	V
	Brettanomyces	+	+	+	+
	Zygosaccharomyces	+	V	+	V
Bacteria	Acetobacter	+	+	+	+
	Oenococcus	–	–	+	+
	Lactobacillus	V	V	+	+
	Pediococcus	–	–	+	+

(+) growth; (–) no growth; (V) growth depends on species/strain

WARNING! Cycloheximide is highly toxic and extreme care should be exerted using this chemical. All glassware exposed to the agent should be disposed of or decontaminated properly, which can include autoclaving. Consult the MSDS for more information on use and disposal.

Lactic acid bacteria are nutritionally fastidious, microaerophilic microorganisms, which grow best on media enriched with fruit or vegetable juices and under conditions of low oxygen tension. Many wineries have been successful cultivating lactic acid bacteria using large mouth glass jars with candles or using specific GasPak® systems. A "modified Rogosa" (MR) medium commonly used for growing these bacteria contains apple juice and can be made selective against *Saccharomyces* by the addition of cycloheximide (MRC).

Most yeasts exhibit colonies after 1 to 3 days incubation on WL. However, *Brettanomyces* grown on WLC requires at least 3 days (more commonly 5 to 7). *Zygosaccharomyces* is normally sensitive to cycloheximide so grows well

on WL but some strains may have higher tolerance than previously thought. *Acetobacter* will grow on most media, with colony appearance within 2 to 4 days. Being somewhat fastidious, lactic acid bacteria (*Oenococcus*, *Lactobacillus*, and *Pediococcus*) need between 5 and 10 days.

B. Equipment/supplies

1. WL, WLC, and/or MRC agar in Petri plates.
   a. Approximately 20 mL of a medium should be added to each 150 mm diameter Petri plate.
   b. Choice of specific medium to use depends on type of microbe to be cultivated.
2. Hockey sticks.
3. Sterile 1 mL pipettes with 0.1 graduations.
4. Phase-contrast microscope.
5. Microscope slides and cover-slips.
6. Immersion oil (100x oil immersion objective only).
7. Lens cleaning solution and lens paper (NOT Kimwipes-type tissues).
8. Laboratory (transfer) loops and/or needles with wooden or metal handles (can also substitute toothpicks).

C. Procedure

1. Write sample name, date, and media type on the bottoms of the Petri dishes.
2. Aseptically transfer 0.1 mL of the wine to the Petri plate.
3. Dip the hockey stick in 95% ethanol and ignite. Once ethanol is gone (flame is extinguished), spread the wine evenly over the agar surface, avoiding digging into the agar.
4. Incubate all media in a 22° to 25°C incubator with the side of the Petri plate containing the agar as the lid (agar side up, that is, the wine-cultured face down) to reduce airborne microbial contamination.

   NOTE: If a larger volume is added to a spread plate (>0.5 mL), the plates must first incubate with agar side down (cultured face up) to allow the large volume of wine to dry. Once dry, the plates are turned upside down (that is, the side containing the agar becomes the lid).

5. After a suitable incubation, examine each plate for growth of different colony types. Count each colony type and record a brief description of these observations (sizes, surfaces, edges, shapes, colors, and smells).
6. Individual colonies can be further isolated by streaking that colony onto fresh agar using the pattern in Figure 6 as a guide. Using a loop, remove the colony from the plate and starting at point #1, move the loop back and forth in a zigzag pattern. Once completed, flame the loop, turn the plate $^{1}/_{3}$ of a turn, and start at point #2 to streak over some of the pattern left previously. Re-flame loop again and finish streaking by starting at point #3 on the same plate.

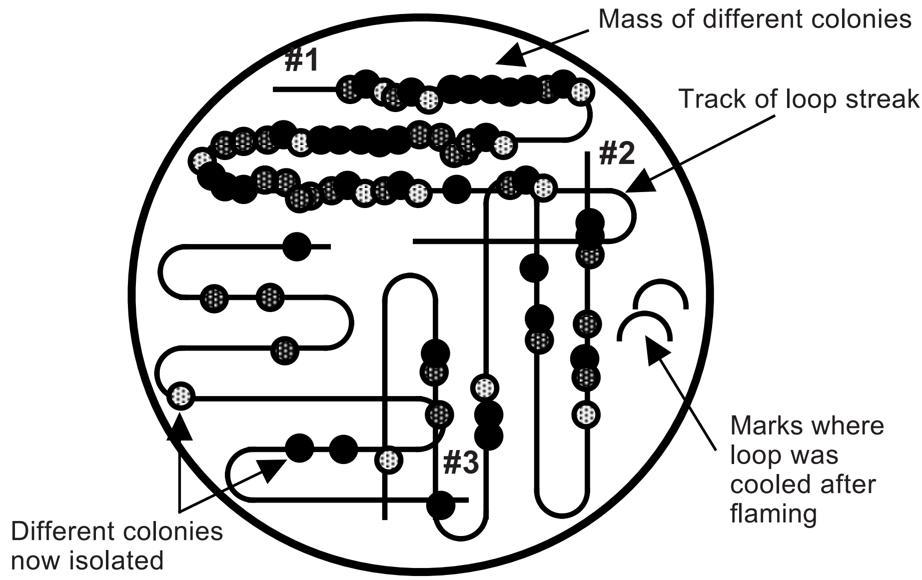


Figure 6. Three-step streak pattern for isolation of microorganisms.

7. After a suitable incubation, record descriptions of each colony type and examine each by preparing wet mounts (Method 2) for phase-contrast microscopically (Method 1). Record observations and compare to the descriptions of different microorganisms found in Appendix II.

Method 4: Concentrating microorganisms

A. Concepts

When sampling from the bottling line or other areas where microorganisms are either absent or present at low populations (<5 CFU/mL), microorganisms will not be easily seen under a microscope. In general, the minimum observable population is about 1 x 10^4 CFU/mL. In these cases, the wines are not cloudy and probably contain low concentrations of microorganisms. To increase the number of microbes in a sample to be examined, the microorganisms need to be concentrated into a pellet.

Two methods for concentration are (a) centrifugation and (b) membrane filtration. In centrifugation, a certain volume of wine is centrifuged at high speed (3000 x *g* for 15 to 30 minutes) and, after carefully decanting the wine, the pellet is mixed using a small glass rod or Pasteur pipette. A small drop of the pellet can be made into a wet mount (Method 2) in preparation for examination by phase-contrast microscopy (Method 1). With membrane filtration, a specific volume of wine is passed through a sterile filter membrane (Figure 7), often with an absolute pore size of 0.45 µm or less. This membrane, with the trapped microorganisms on the upper surface, is then aseptically transferred onto a Petri dish containing a solidified medium. Although useful for wines that have low viable populations of microorganisms (e.g., on a bottling line), these concentration methods are difficult if particulate matter is present. Particu-

late matter makes viewing samples microscopically challenging and will plug or clog membranes.

For the membrane method, many choices of filtration units are available. Some units can be reused after cleaning and sterilization (autoclaving) and use pre–sterilized membranes that are packaged individually. Alternatively, disposable units are easy to use and convenient with some even containing both the filtration apparatus and a Petri dish attached. With both systems, it is necessary to use aseptic techniques when transferring the membrane to the agar surface. Some membranes are available with grids which can be helpful with microscopic counting.

The volume of sample needed to provide statistically reliable results is also important. By experience, if recovery of >10 cells/L at bottling likely results in instability, detection limits can be calculated when only 100 mL of a 750 mL bottle is membrane filtered. However, the detection limit is better when the entire bottle contents (750 mL) are filtered. Although the theoretical ability to detect microorganisms has improved with an increase in sample volume, results can still be below the minimal CFU per plate requirement of 25 colonies.

B. Equipment/supplies

1. WL, WLC, and MRC agar in Petri plates.
    a. Approximately 20 mL of a medium should be added to each 150 mm diameter Petri plate.
2. Vacuum filtration system including vacuum tubing.
3. Sampling instrument (tanks or barrels).
4. Forceps.
5. 95% v/v ethanol.
6. Membrane filtration system.
    a. Disposable plastic-types equipped with 0.45 µm membranes work well (Figure 7).

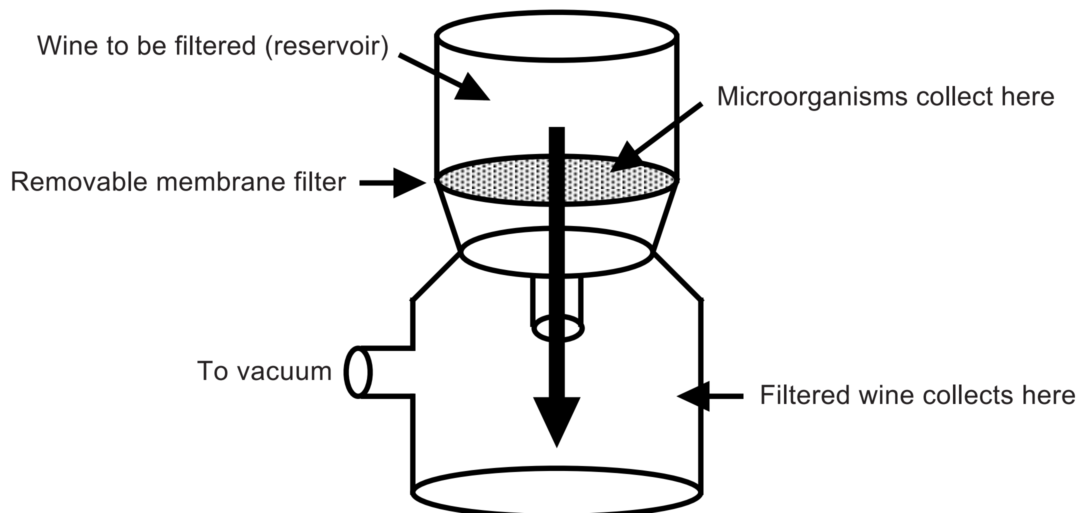


Figure 7. Pre-sterilized membrane filtration system.

C. Procedure

1. Before starting, clean and sanitize the laboratory work surface using 70% v/v ethanol.

   NOTE: To minimize fire danger, work on a fire retardant surface.

2. Sampling

   a. Bottle: Using 70% v/v ethanol, swab the neck of the bottle and dip the cork screw (not a two-pronged or "Ah-So" type). Ignite the ethanol ("flame") but keep the corkscrew and the bottle pointed DOWNWARD. If the cork begins to burn, do not blow to extinguish the flame (shake bottle rapidly).

   b. Tanks: Remember that yeast and bacteria will settle towards the bottom of a large container. Therefore, sample from the bottom of the tank or agitate the tank prior to sampling. Be sure to collect sample as aseptically as possible including flaming the sampling instrument before use and putting the sample into a sterile container.

3. Flame the open neck of the bottle for a couple of seconds and immediately pour at least 250 mL into the reservoir of a sterile filter unit (Figure 7).

4. Apply suction with a vacuum source until all sample has been collected in the lower chamber. Be sure to have a trap flask located between the vacuum source and the sterile filter unit to catch additional mL of sample.

5. Flame the forceps, then aseptically remove the membrane that now contains microbial cells.

6. Roll the membrane onto a sterile Petri dish containing suitable agar medium, making sure of contact between the agar and the membrane. Use the forceps to remove any air bubbles from underneath the membrane by pushing lightly on the membrane.

7. Incubate the filter pad and observe microbial growth indicated by colonies.

8. Record each colony type and prepare wet mounts (Method 2) for phase-contrast microscopy (Method 1).

D. Helpful hints/interpretations

1. Because microbes cannot grow unless the membrane is in contact with the agar, be sure to remove any air bubbles between the membrane and agar surface.

2. General descriptions of microorganisms as colonies and as viewed under a phase contrast microscope are found in Appendix II.

Method 5: Enumeration/detection of microorganisms

A. Concepts

In direct plating, a known volume of sample (0.1 to 1.0 mL) is placed into either a sterile Petri dish and mixed with a molten agar medium (pour plate method) or transferred onto a pre-poured agar medium in a sterile Petri dish

(spread plate method). In theory, each viable cell in the sample then grows into a visible colony. After incubation for a period of time, the growing colonies are counted and the number of viable organisms per milliliter or per gram of sample can be calculated. Microbiological kits can be purchased with pre-sterilized and pre-poured agar as well as other reagents and materials, giving even the smallest of wineries the ability to perform these tests.

Spread plates are used to enumerate aerobic or heat-sensitive microorganisms. These can be prepared a few days prior to use, but normally accept smaller sample volumes of closer to 0.1 mL. In some situations, volumes up to 1 mL are possible but the agar surface must dry before incubation. By contrast, pour plates can be used with either 0.1 or 1 mL sample volumes.

B. Equipment/supplies

1. Sterile Petri dishes and suitable sterilized medium in flask (pour plate method) or in dishes (spread plate method).
2. Sterile pipettes (0.1 mL and 1.0 mL).
3. Hockey sticks.
4. Sterile dilution blanks (9 mL 0.1% peptone water in test tubes).
5. Flame source (Bunsen burner or alcohol flame will work well).
6. 70% v/v Ethanol.

C. Procedure

1. Refer to Figure 8 for a scheme for setting up a dilution series using sterile peptone water dilution blanks for either pour plates or spread plates. Serially dilute the juice/wine, thoroughly mixing each dilution blank before transferring 1 mL into the next blank.

   Pour plate method:

   a. Place 1.0 mL from each dilution into a sterile Petri dish.
   b. Aseptically add ≈20 mL sterilized and molten agar to each plate. The agar should be liquid and warm to the touch but not so hot as to incur microbial injury. Carefully mix each plate. Allow the plates to cool and harden undisturbed, usually an hour.

   Spread plate method:

   a. Place 0.1 mL from each dilution onto a sterile Petri dish containing the appropriate sterile and hardened medium.
   b. Holding its handle, dip a glass hockey stick into ethanol, flame it, and allow the flame to extinguish. Spread the 0.1 mL around on the plate using the now-sterile hockey stick.

2. Invert the plates (agar side up) and incubate for the appropriate time.
3. After incubation, find the plate (dilution) that has between 30 and 300 visible colonies and count all colonies. For

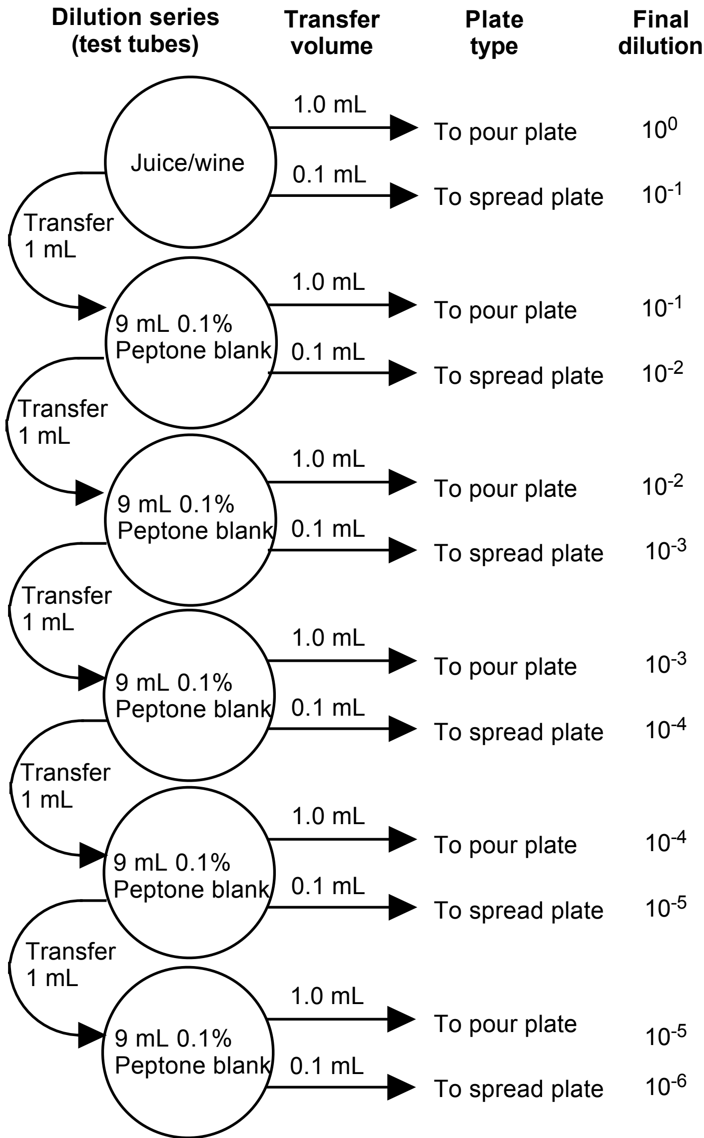


Figure 8. Dilution series scheme using pour or spread plates.

calculation, multiply the count by the reciprocal of the dilution.

D. Helpful hints/interpretations

1. If the 10^{-3} plate (dilution) had 122 colonies, the estimated count of viable microorganisms in the original juice or wine sample would be 122 x 10^3 or 1.22 x 10^5 colony forming units (CFU) per mL.

Method 6: Fermentation tubes

A. Concepts

Durham tubes are very small test tubes that can be placed, upside down, into a larger test tube that contains liquid media. These tubes trap any gas produced by any microorganisms growing in the media.

Normally, two tests can be run on purified cultures of unidentified yeasts: (a) fermentation test and (b) alcohol tolerance test.

B. Equipment/supplies

1. Durham tubes.
2. Large test tubes (18 x 150 mm) with screw caps.
3. Glucose fermentation broth (20 g/L glucose, 5 g/L yeast extract, adjusted to pH 3.3 to 3.5 with phosphoric acid).
4. Glucose fermentation broth with ethanol (20 g/L glucose, 5 g/L yeast extract, adjusted to pH 3.3 to 3.5 with phosphoric acid. Add 900 mL H_2O, autoclave (121°C for 15 minutes), and then add 100 mL absolute ethanol).

C. Procedure

1. Fermentation test.
   a. Fill a large text tube containing a Durham tube with glucose fermentation broth and inoculate with the unknown microorganism.
   b. Incubate at 20° to 25°C and watch for gas production and growth.
2. Alcohol tolerance test.
   a. Fill a large text tube containing a Durham tube with glucose fermentation broth supplemented with 10% ethanol and inoculate the unknown microorganism.
   b. Incubate at 20° to 25°C and watch for gas production and growth.

D. Helpful hints/interpretations

1. Refer to Table 5 for interpretations.

Table 5. Growth and appearance of various yeasts in glucose broth.

Microbe	Glucose broth	Glucose broth (+ alcohol)
Saccharomyces, Zygosaccharomyces	Very gassy; shell vial floats in 1 to 3 days	Very gassy; shell vial floats in 1 to 3 days
Most wine-surface film yeasts	Little or no gas; film on surface	Little or no gas; film on surface
Dekkera & *Brettanomyces*	Growth very slow; vial floats in 1 to 2 weeks	Growth very slow; vial floats in 1 to 2 weeks
Non-wine yeasts	Gas, film, or just growth	Little or no growth

Must and Wine Analyses (Chemistry)

Routine chemical analyses are performed in wineries for many reasons. Some constituents present in wine are regulated as to maximum allowable concentrations (e.g., volatile acidity and SO_2) or for taxation purposes (e.g., alcohol). In addition, chemical analyses can be indicators of potential or spoilage problems.

The basic analytical methods employed by most wineries are for pH, titratable acidity, volatile acidity, free/total SO_2, alcohol, and °Brix or residual sugar.

Method 7: Standardization of NaOH

A. Concepts

Once a NaOH solution used for titrating is prepared, it must be standardized to determine the exact concentration of base present. "Standardized NaOH" is a solution for which the exact concentration is known to three significant figures (e.g., 0.145 M). Standard solutions of NaOH are used in a number of wine laboratory analyses, most notably titratable and volatile acidities and SO_2 by aeration/oxidation. The following method can be found in Skoog and West (1980) and Zoecklein et al. (1995) for preparation and standardization of NaOH.

While preparation of standardized solutions of NaOH is possible, it can be easier and cheaper to obtain ready-made standards. Available from chemical supply companies, these solutions can be purchased at the desired concentration and are traceable to the National Institute of Standards designated as NIST on the label. Because degradation begins once the bottle is opened, careful measures are required to maintain the stated concentrations. When a container is left open or otherwise exposed to the atmosphere, NaOH solutions rapidly adsorb CO_2 which neutralizes NaOH and forms sodium carbonate (Na_2CO_3). This problem can be worse during grape harvest when concentrations of CO_2 within a winery are elevated because of CO_2 evolution from fermenting tanks. In addition, dilution of these standard solutions can also cause errors because of poor quality laboratory water or through pipetting mistakes.

If a standard is diluted or the container is accidentally left open, the solution must be discarded or re-standardized using the following protocol. Standardization will require the use of an analytical balance with precision of no less than 0.001 g, preferably 0.0001 g. Alternatively, some laboratories standardize against fresh standard solutions of hydrochloric or sulfuric acid also purchased from chemical supply houses. Any acid used for this purpose must itself be quite fresh as these solutions also degrade during storage.

B. Equipment/supplies

1. NaOH (solid).
2. Distilled water.
    a. Recently boiled and allowed to cool to room temperature while covered with a watch glass.
3. Volumetric flasks.
4. Potassium hydrogen phthalate ($KHC_8H_4O_4$, abbreviated as "KHP").
    a. Place a quantity of primary standard potassium hydrogen phthalate into a porcelain dish and dry in an oven for 2 hours at 110°C. Cool in a desiccator.

5. Erlenmeyer flasks.
    a. 125 or 250 mL.
6. Large storage vessel (e.g., carboy) set-up as shown in Figure 9.
    a. The absorbent is either soda lime or Ascarite II which removes any CO_2.
7. pH meter or indicator.
    a. To prepare the pH indicator, dissolve 0.5 to 1.0 g phenolphthalein in a solution of 80% v/v ethyl alcohol.
8. Burettes with ring stands.
    a. 25 or 50 mL.

C. Procedure

1. Weigh out an appropriate amount of NaOH to prepare a 50% w/v NaOH solution.
    a. To prepare 1 liter of a 0.1 M standardized solution, ≈4 g of NaOH dissolved in 8 mL of distilled water will be required.
2. Add the dry NaOH to small beaker and add a minimal amount of distilled water to dissolve.
3. Allow the Na_2CO_3 to settle out from the 50% w/v NaOH solution.
4. Carefully decant the supernatant into a volumetric flask.
    a. The amount of 50% w/v NaOH produced should contain more than the total NaOH needed for the diluted solution given losses during transfer.
5. Dilute to volume with cool distilled water and store in an appropriate container.

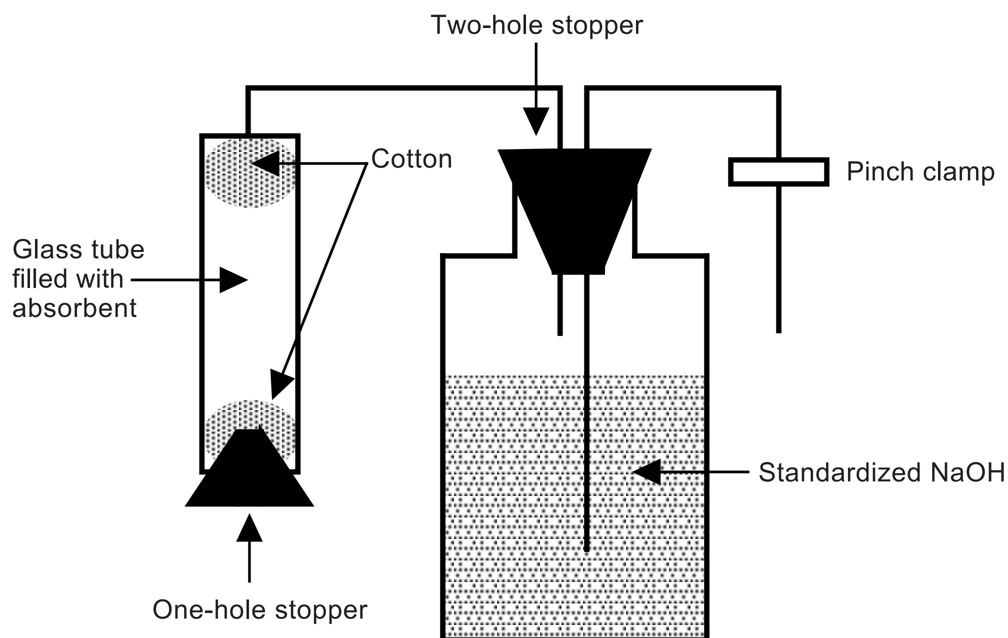


Figure 9. Apparatus for storage of standardized NaOH.

6. To standardize the NaOH solution, weigh 0.7 to 0.9 g KHP (to the nearest 0.0002 g) into 250 mL conical flasks using an analytical balance. Record weight to ± 0.0001 g.
7. Dissolve KHP in 50 to 75 mL of freshly boiled and cooled distilled water.
8. Add 2 drops phenolphthalein and titrate with base solution until pink color of the indicator persists for 30 seconds, OR titrate to pH 8.2 with a pH meter.
9. Record the volume of base required to complete the titration and calculate the concentration of NaOH to four significant figures (0.0001 M) as given below.

$$\text{NaOH (N)} = \frac{(\text{g KHP})(1000)}{(\text{mL NaOH})(204.229)}$$

D. Helpful hints/interpretations

1. Standardized base solutions are reasonably stable if protected from prolonged exposure to glass or air. Here, silicates are formed slowly from glass while carbonates are formed from atmospheric carbon dioxide.
2. For longer term storage, use a tightly sealed polyethylene bottle.

E. Validation

1. Given the problem of CO_2 adsorption by standardized NaOH, it is advisable to periodically re-standardize.

Method 8: pH

A. Concepts

The modern pH meter and electrode are probably the most essential pieces of laboratory equipment in any winery. The quality known as pH is a measurement of the concentration of hydrogen ion activity, [H+], in a solution. The pH scale is a logarithmic scale, so small numerical differences mark larger differences in $[H^+]$. In fact, pH is mathematically defined as:

$$pH = -\log[H^+]$$

While many pH measurement tools include an “automatic temperature compensation” probe, the heart of the system is the electrode. In a pH probe, the electrode measures a response in voltage where, as pH increases, the mV reading decreases. From pH 0 to 12, the relationship between voltage and pH is roughly linear, approximately 59 mV per pH unit or per log of $[H^+]$.

Modern pH electrodes are usually, in fact, two electrodes in one: a combination of the glass membrane electrode, which develops a cross-membrane voltage in response to external hydrogen ions, and a reference electrode to complete the circuit and provide a stable reference potential. The reference electrode must be connected to the solution through some sort of liquid junction—either a conventional ceramic

junction or some type of open junction as in the Ross Sure Flow design. Many pH measurement problems are a result of a clogged junction, and thought must be given to both the nature of the junction and the composition of the fill solution.

The differential between [H^+] inside and outside of the glass electrode causes a measurable electric potential (voltage) across the membrane. The electrode puts out a voltage signal which is converted by the pH meter (essentially just a voltmeter) to a pH readout. Thus, the meter must be standardized (calibrated) to determine what mV corresponds to what pH. Standardization, or calibration, is typically performed using at least two standard buffer solutions.

To standardize the system, standard pH buffers are used and can be purchased from scientific supply companies. Normally, two buffers are used for standardization but the selection of those buffers depends on the goal. For instance, measurement of wine pH (3 to 4) requires use of buffers at pH 4 and 7. It is desirable to check the standardization using pH 3.00 standard or saturated potassium bitartrate (pH = 3.59). In fact, some pH meters will accept standardization using up to five buffers. Titration of a wine where the endpoint is between pH 8 and 9 normally requires standardization with pH 7 and 10 buffers.

In addition to establishing a response curve with the standards, a pH meter will normally calculate the slope of the line. This should be 59 mV per pH unit and will normally be displayed as percent of that theoretical response. If in doubt, switch to mV readout mode and the difference between pH 7 and 4 should be roughly 3 x 59 mV, or 177 mV. In practice, the slope of the response will change over the life of the electrode. A large deviation from 100% slope usually indicates an electrode in need of maintenance or retirement. In addition, clogged junctions will exhibit a slow response, extensive drift, sensitivity to sample stirring, and a general lack of reproducibility.

Three types of electrodes are: (a) gel-filled, (b) those with AgCl or HgCl in the reference electrode, or (c) those designed without Ag^+ or Hg^+ such as Ross electrodes. Gel-filled electrodes are inexpensive and maintenance-free but have a short service life in a winery. One problem is that sulfide and other metal-reactive, reduced sulfur compounds present in wine react with the silver ions in an electrode to form insoluble silver sulfide (Ag_2S). The Ag_2S precipitates in the ceramic junction, and clogs the system.

Probes or meters with AgCl or HgCl in the reference electrode reservoir are more expensive than gel-filled electrodes but are just as rugged and have a longer service life. Their junctions can be cleaned using agents such as NH_4OH. (Drain the electrode, add the agent, rinse, and refill. Be sure to follow manufacturer's instructions.)

Although more expensive than gel filled electrodes and quite as rugged, they have a longer service life. Electrodes that do not contain Ag^+ or Hg^+ lack metal to react with

sulfides, so the junctions rarely plug. While electrodes with flushable junctions also tend to have much longer service lives, these types can be expensive.

B. Equipment/supplies

1. pH meter with electrode(s), preferably with automatic temperature correction probe.
2. pH buffers (pH 4, 7, and 10).
3. Beakers (150 and 250 mL).
4. Wash bottle.
5. Disposable wipers (Kimwipes-type tissues).

C. Procedure

1. Calibrate the pH meter using one, if not two, standard buffer(s), normally pH 7 and pH 4 but sometimes pH 10. Refer to the instrument's instructions manual for details of standardization.

    NOTE: As a rule of thumb, standardize the pH meter with standards on either side of the test material. For example, use pH 7 and 10 buffers when titrating a wine sample to an endpoint of pH 8.2.

2. Once standardized, place the electrode in juice or wine and measure the pH.

D. Helpful hints/interpretations

1. Always keep the electrode tip submerged in a solution except when moving it from one solution to another.
2. Store electrodes in the storage solution recommended by the manufacturer.
3. Before placing an electrode in another solution, rinse it with distilled water. Lightly wipe the electrode body dry with disposable wipes. The electrode can then be rinsed with the sample, if desired, prior to measuring the pH.
4. Check the junction point at the bottom of an electrode to be sure it is not obstructed. The junction is clogged if (a) stirring the sample changes the reading of the meter; or (b) meter response is sluggish, exceeding 10 to 15 seconds (depending on the probe). Clogged junctions will often appear dark gray or even black from the presence of precipitated AgCl. Gel-filled electrodes will generally need to be discarded when their junctions plug. If the junction is clogged or the electrode is otherwise responding erratically, either fix the problem or replace the electrode before proceeding. Immersing an electrode in 0.1M HCl for 10 seconds or less can sometimes regenerate electrodes. Consult the electrode manufacturer for more details regarding regeneration procedures.
5. Keep the electrode body filled with the proper solution, periodically changing that filling solution.
6. DO NOT put an electrode so deep into a solution that the level of its internal filling solution is below that of the external solution.

7. Make sure the electrode sleeve is open when in use and closed when not in use. Located towards top of the electrode body, this sleeve allows the internal liquid to be at atmospheric pressure.
8. Never return buffer solutions to the stock container. Properly dispose of extra buffer solution to avoid contamination of the stock buffer.
9. Always bring solutions to room temperature (20°C) because automatic temperature compensation (ATC) probes are not completely satisfactory for correcting for temperature variations. The electrode itself needs to be at the temperature of the solution for proper ATC function, and for solutions varying considerably from room temperature, this requires several minutes of equilibration. While electrode response depends on temperature, the pH of any buffered solution such as wine also changes with temperature.

E. Validation and uncertainty

1. Always validate and/or re-standardize your pH meter if the operation has not been carried out within the last hour using fresh, commercially prepared pH standards.
2. Expected uncertainty is ±0.04 units.

Method 9: Titratable acidity

A. Concepts

Titratable acidity (TA) differs from pH in that TA measures the amount of titratable acid present in a sample, whereas pH is a measure of the strength of the acidity. For example, a 1:1 dilution of a wine yields a TA value half the TA value of the original wine (e.g., the concentration of acids is reduced from, say, 4 g/L down to 2 g/L after dilution) while the pH would remain virtually unchanged due to the buffering capacity of the wine. That is, titratable acidity is not dependent on pH.

To measure TA, a sample of wine is titrated with a standardized base (Method 7) until the acids present are neutralized at approximately pH 8.2. At the neutralization point, the number of moles of acid equals the number of moles of base. The number of moles of base added to neutralize the acids is calculated from the amount and the molarity of base. Once the number of moles of acid is determined, the concentration of titratable acids present (usually calculated as tartaric acid) can be determined. However, the concentration of titratable acids in a wine are not equal to the total moles of the different organic acids present. At wine pH, all of the acids present are already partially titrated, and thus there is no clear correlation between titratable acidity and total acidity. In fact, TA is not the same as “total acidity,” where the latter implies that each individual acid is quantified, most frequently performed using chromatographic methods (e.g., high pressure liquid chromatography or HPLC). To make things more confusing, some government agencies use the term “total acidity” when the more correct term would be “titratable acidity.”

A pH meter and/or an indicator solution is used to determine when the reaction between acid and base is complete. Both devices work on the same principle: neutralization at pH 8.2 is either measured as a number (pH meter) or visually observed through a change in the color (indicator). The acids in grape musts and wines are weak acids and when titrated, the true endpoint will be greater than pH 7. By convention, the titration endpoint is pH 8.2, although in Europe the endpoint is sometimes accepted as being pH 7.

By convention in the USA, titratable acidities of wines are based on tartaric acid, expressed as g/100 mL or g/L. As cellar additions are normally written as g/L, many wineries express TA as g/L. Other conventions exist, particularly in Europe, both regarding the titration end point and the type of acid in which the TA is expressed. For example, TA is commonly expressed as sulfuric acid in France. In Germany, TA is sometimes calculated as the volume of 0.1N base needed to titrate 100 mL sample. To convert from one acid value to another, use the factors in Table 6 and multiply the given acid value by the factors listed for the desired acid.

For titration, the indicator used is phenolphthalein. This indicator is colorless in acid solutions and red in alkaline solutions. The color change may be difficult to see in red wines. In practice, small variations in end point will have minimal effect on the calculated value of TA as wine is poorly buffered at pH 7 to 9 and small additions of base will result in large changes to pH.

One of the largest sources of error in determining TA is the presence of CO_2. Whether dissolved in the wine, reagents, or water used to dilute samples, carbonic acid (H_2CO_3) is formed from CO_2 and this increases TA values. While carbonic acid contributes only one proton to a titration, a concentration of 500 mg/L CO_2 will increase the apparent TA by 0.85 g/L if it is not removed by degassing prior to analysis.

Wines are typically "degassed" of CO_2 by one of several methods. The most reliable method is vigorous stirring under vacuum. Typically, a volume of wine is placed in a large vacuum flask with a stir bar and agitated under low vacuum conditions, such as provided by a single stage vacuum pump or water aspirator. More commonly, laboratories rely upon heating the wine sample, either undiluted or after dilution with distilled water. It is generally necessary to heat the wine or wine/water mix to boiling to drive off the CO_2.

Table 6. Conversion factors for expressing titratable acidity in wine using different acids.

	Transform to:					
Expressed as:	**Tartaric**	**Malic**	**Citric**	**Lactic**	**Sulfuric**	**Acetic**
Tartaric	1.000	0.893	0.853	1.200	0.653	0.800
Malic	1.119	1.000	0.955	1.343	0.731	0.896
Citric	1.172	1.047	1.000	1.406	0.766	0.938
Lactic	0.833	0.744	0.711	1.000	0.544	0.667
Sulfuric	1.531	1.367	1.306	1.837	1.000	1.225
Acetic	1.250	1.117	1.067	1.500	0.817	1.000

Caution must be used in this method to not to boil too vigorously because that will also cause loss of acetic acid which should be included as part of the TA.

Dissolved CO_2 is also an issue in the water used for dilution of the wine sample. While some distilled or deionized water contains little dissolved CO_2, other sources will need to be boiled and then cooled (while protected from atmospheric CO_2) before use. One indicator of CO_2 in water is a slow downward drift in pH after apparently adding the final drop of NaOH during titration. This drift is due to the slow conversion of dissolved CO_2 to bicarbonate ion (HCO_3^-) at pH values close to endpoint (8.2). In addition, distilled or deionized water containing CO_2 will have noticeable buffering capacity at neutral pH. One drop of 0.1 N NaOH in 50 mL of "pure" water should result in a pH of about 9 or higher, while water with dissolved CO_2 will be much more resistant to pH change.

Titratable acidity is commonly determined on field samples, at harvest, both before and after fermentation, and in the cellar, especially if the acidity levels are altered by cold stabilization, malolactic fermentation, or other means. However, TA is not performed on samples with high amounts of CO_2 present. Unfermented juice from maturity samples and initial tank samples may be assumed to contain negligible CO_2, but all other wine samples must be assumed to contain large concentrations of CO_2. In general, newly fermented wines can be saturated (≈1500 mg/L) or even oversaturated in CO_2. During subsequent processing and transfers, CO_2 concentrations tend to decrease. At bottling, most red and dry white wines have CO_2 levels at or below the human sensory detection threshold of about 500 mg/L. Off-dry or sweeter white or rose wines are often intentionally bottled with higher dissolved CO_2 levels, in some cases approaching or exceeding 2000 mg/L.

B. Equipment/supplies

1. NaOH (0.1 N standardized).
    a. Refer to Method 7 for preparation of standardized NaOH solutions.
    b. Weekly, check the concentration of the standardized NaOH using KHP or 0.1 N H_2SO_4.
2. Burette (10 or 25 mL).

    NOTE: "Auto-zeroing" burettes that refill automatically are greatly preferred if large numbers of samples are to be processed.
3. Erlenmeyer flasks (125 or 250 mL).
4. pH meter or phenolphthalein.
    a. If using phenolphthalein, dissolve 0.5 to 1.0 g in 80% v/v ethyl alcohol/distilled water.
5. Beakers (250 or 400 mL).
6. Watch glasses.
7. Pipettes (5 mL; volumetric preferred).

C. Procedure

1. Add approximately 50 to 100 mL distilled water to a 250 mL beaker containing a Teflon®-coated magnet.
2. Pipette 5 mL of the sample into the beaker.
3. Heat the contents to a rolling boil, remove from heat, cover with a watch glass, and cool the beaker contents to room temperature.

   CAUTION! Boiling the sample too long will result in the loss of acetic acid, thereby lowering the titratable acidity and introducing error into the measurement.

   NOTE: The sample and the distilled water can be heated separately. Here, add the 5 mL of sample to a 250 mL beaker and place on a preheated hot plate or small flame. Bring the sample to an incipient boil (point at which bubbles are just beginning to break the surface but before a rolling/active boil occurs). Add approximately 50 mL CO_2-free water prior to titration.

   NOTE: An alternative method to boiling that also removes CO_2 is to use a vacuum system. Here, a sample of wine (20 to 50 mL) at 20° to 25°C is placed into a 1 L vacuum flask with a large stir bar and degassed under vacuum (Air Cadet pump or similar single stage vacuum pump yielding a vacuum of 20–28 inches of Hg) with constant stirring for at least 5 minutes. Each laboratory should confirm the period of time necessary for degassing using their particular apparatus by running a series of trials. Wines containing a large amount of CO_2 (e.g., sparkling wines) may require extra degassing. Must samples will not normally require degassing.
4. Once cool, place the beaker on a stir plate and start mixing.
5. Insert the pH electrode and titrate with standardized 0.1 N NaOH to pH 8.2.
6. Record the volume of 0.1 N NaOH used.
7. Alternatively, the titration can be conducted using the pH indicator phenolphthalein. Here, a few drops of 1% phenolphthalein are added to colorless wines and standardized NaOH added until a pink color persists for 20 seconds.

   NOTE: Phenolphthalein cannot be used in red wines and commonly results in over-estimation of titratable acidity due to by-passing the colored end point.
8. Calculations

$$\text{Tartaric acid (g/L)} = \frac{(\text{mL NaOH})\ (\text{N NaOH})\ (0.075)\ (1000)}{(\text{mL sample size})}$$

D. Helpful hints/interpretations

1. Suspended sediments such as grape pulp, yeast cells, or potassium bitartrate must be removed from the sample by centrifugation, settling, or filtration prior to titration.
2. Use only high quality deionized or distilled water for this analysis. Addition of one drop of 0.1 N NaOH to 100 ml of water should result in a pH value >9.0.
3. Sodium hydroxide solutions rapidly absorb CO_2 from the atmosphere, resulting in a decrease in concentration and an over-estimation of titratable acidity. A glass column containing soda lime can be used to prevent entry of CO_2 into the container storing standardized NaOH.

E. Validation/uncertainty

1. Send a duplicate wine sample to a commercial laboratory for analysis to compare results and ensure accuracy.
2. To evaluate precision, analyze the same wine sample repeatedly three or four times. Expected uncertainty is ±0.15 g/L.
3. Prepare a "spiked" sample by dissolving 0.200 g tartaric acid in 100 mL of a wine sample, making sure all tartaric acid dissolves. After determining the TA on both unspiked and spiked wine samples, subtract the values; the difference should equal 2.00 ± 0.1g/L.

Method 10: Volatile acidity

A. Concepts

Volatile acidity (VA) is used as an indicator of wine spoilage from various microorganisms during vinification. Therefore, many wineries regularly monitor VA to identify those processing points where microbial spoilage is occurring. Although VA is expressed as grams of acetic acid per 100 mL or g/L, the value will not be identical to the actual concentration present. The reason is that other acids also contribute to the VA value and some acetic acid will remain in the wine even after distillation.

Volatile acidity is most frequently determined on finished wines for regulatory purposes. In the USA, legal limits are 0.12 g/100 mL for white wines and 0.14 g/100 mL for red wines. Most consumers will notice elevated VA levels well below the legal limit, often starting at 0.8 g/L. Note that acetic acid is not primarily responsible for off-odor; excessive amounts of ethyl acetate can lead to this spoilage. The odor of ethyl acetate has been described as being similar to that of 'fingernail polish remover.' According to Ough and Amerine (1988), acetic acid and ethyl acetate are usually produced at the same time and in proportional amounts, so determination of acetic acid via Cash still (Figure 10) can provide a good indicator of spoilage.

The basic principle is to distill, collect, and titrate the components of the wine which are volatile. In theory, this should exclude the fixed acidity of dicarboxylic acids and, to a large degree, exclude the monocarboxylic lactic acid. In

practice, there are several possible interfering factors such as CO_2 dissolved in the distillate, wine, or employed for steam generation. Furthermore, steam distillation of SO_2 and/or lactic acid is also possible. Degassing the sample, preboiling the water, and heating the distillate prior to titration can minimize difficulties associated with CO_2. Any residual SO_2 present in the sample can be removed by addition of hydrogen peroxide (H_2O_2) which oxidizes sulfite (SO_3^{2-}) to sulfate (SO_4^{-2}).

Additional sources of error are (a) incomplete distillation of acetic acid; (b) inadequate standardization of the NaOH; (c) condenser water is not cold enough to condense all the distillate vapors; or (d) leaks in the Cash still apparatus, such as at the stopcock or ground glass unions. It has also been noted that the blank titration value is dependent upon the time interval elapsed between distillation and titration. Thus, it is important to perform each titration (including the blank titration) at the same intervals after distillation, i.e., one to three hours.

B. Equipment/supplies

1. Volumetric flasks (100 mL).
2. Erlenmeyer flasks (250 mL).
3. Volumetric pipettes (10 mL).
4. Graduated pipettes (1 mL graduated in 0.1 mL graduations).
5. Burette (50 mL).
6. NaOH (two solutions: standardized 0.025 N and approximately 2 N).
   a. Refer to Method 7 for preparation of standardized base solutions.
   b. Weekly checking of the concentration of the standardized NaOH using KHP or 0.1 N H_2SO_4 is advisable.
7. Phenolphthalein (1%) or pH meter.
   a. Dissolve 1 g of phenolphthalein in 70 mL of 95% ethanol and add 30 mL of distilled water. Mix well.
8. Hydrochloric acid-ferric chloride solution (HCl-$FeCl_3$•$6H_2O$).
   a. Dissolve 2.0 g $FeCl_3$•$6H_2O$ in 40 mL of concentrated HCl in a 100 mL volumetric flask. Dilute to volume with distilled water.
9. Cash still with rheostat (Figure 10).

C. Procedure

1. Refer to specific manufacturer's instructions, because each Cash still is a little different in its operation and design. Figure 10 roughly represents the R&D 80 Volatile Acid Still manufactured by Research & Development Glass (Berkeley, CA).

   NOTE: Be sure to connect all hoses to the condenser and the aspirator unit and make sure that the drain line is clear, per the instructions of the particular unit.

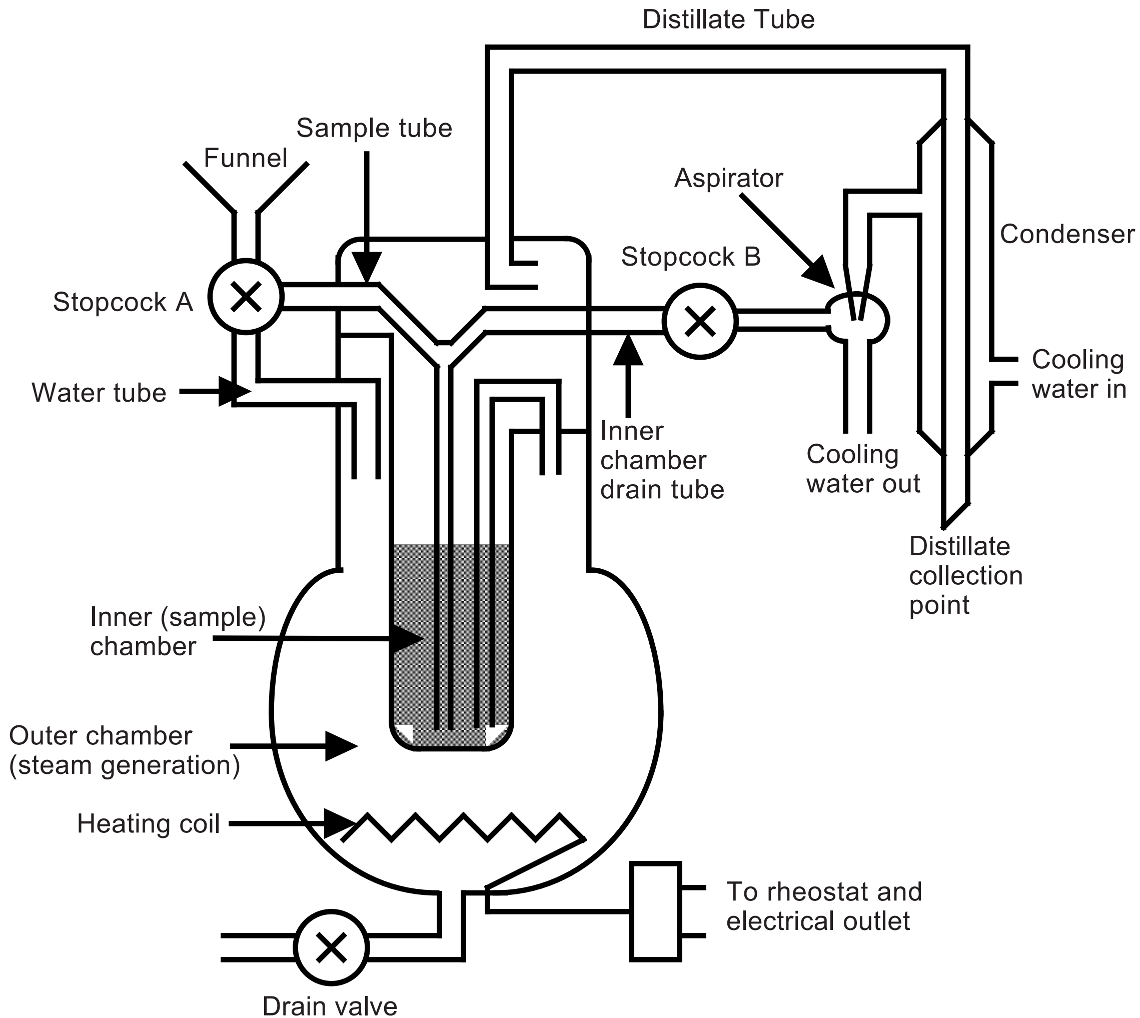


Figure 10. Schematic diagram of a Cash still.

2. Turn the green handle on Stopcock A up and fill the outer chamber with distilled water (≈500 mL or up to demarcation line) by pouring water into the funnel.

   NOTE: Be sure distilled water covers the heating coil in the outer chamber.

   WARNING! NEVER fill outer chamber with distilled water if the chamber is empty and WARM OR HOT. Heat shock will break the glass.

   WARNING! NEVER apply power to the coil unless it is submerged in water.

3. Turn on the cold tap water to the condenser with enough flow to create suction with the aspirator.

   NOTE: Check this by adding a little distilled water to the inner (sample) chamber through the funnel, with the green handle on Stopcock A in the up position. Now, slowly open Stopcock B which is attached to aspirator. If there is sufficient tap water flowing, the

water in the sample chamber will be sucked out. If not, increase the tap water flow slightly until there is enough suction to remove the distilled water.

4. Close Stopcock B. Place a 250 mL Erlenmeyer flask at the end of the condenser outlet to collect condensate.
5. Using a 10 mL volumetric pipette, add 10.00 mL distilled water or wine to a 20 mL glass vial.

   NOTE: At least one 10.00 mL distilled water blank should be carried through the following steps before distilling the wine. If titration of the blank requires >0.2 mL standardized 0.025 N NaOH, the Cash still must be cleaned.

6. With the red handle of Stopcock A in the up position, add distilled water or wine to inner chamber. Rinse the sample tube with additional distilled water (5 to 10 mL).
7. Turn the green handle on Stopcock A up. Turn on unit.

   NOTE: If the unit has a rheostat, set to ≈110.

8. After the water in the outer chamber begins to boil, wait 20 seconds and then close Stopcock A.

   NOTE: This allows any CO_2 to be vented.

9. When the distillation is complete by collection of 100 mL (≈5 minutes), open aspirator Stopcock B to remove the distilled sample in the inner chamber.

   NOTE: To rinse the inner chamber, close the aspirator Stopcock B, turn the red handle on Stopcock A up, and add distilled water through the funnel. Open the aspirator Stopcock B to flush and repeat these processes until the inner chamber is adequately clean.

   NOTE: The exact volume of distillate collected is not critical because most of the acetic acid distills early in the process. For consistency, it is best to collect 100 mL ± 5 mL but do not discard excess distillate! In these situations, simply titrate the entire amount of distillate.

   CAUTION! When the inner chamber is clean, check the water level in the outer flask. Add more distilled water as required. ALWAYS be sure there is adequate water in the outer flask!

   WARNING! If the heating coil is exposed or if the flask runs dry, there is RISK OF GLASS BREAKAGE if the outer chamber is still warm to hot. In this situation, do **not** add water. Turn off the power and let the still cool slowly before restarting the procedures.

10. To the Erlenmeyer flask containing the distillate, titrate with 0.025 N NaOH by either adding 3 drops of phenolphthalein (faint pink endpoint) or using a pH meter (to pH 8.2).
11. Calculation.

$$\text{Volatile acidity (g/L)} = \frac{(\text{mL NaOH})(\text{N NaOH})(60)}{(\text{mL sample size})}$$

D. Helpful hints/interpretations

1. Young wines may require the addition of a small amount of inert silicone antifoam solution prior to distillation.
2. If recently-boiled distilled water is used to fill the still, the boiling step of the distillate can be omitted. Boiled distilled water that is still warm can be added to the still to decrease the time before the next sample can be distilled.
3. Steam must not be allowed to leak from the condenser joints because this leads to the loss of acetic acid.
4. At the beginning and end of the day, distilled water should be boiled in the still to clean it.
5. Sorbates present in the wine can add error because this compound distills with acetic acid. Subtract 0.010 from the volatile acidity value for every 200 mg/L sorbate present.
6. Correction is not normally made for the amount of SO_2 present in the wine. However, correction may be necessary if the amount of SO_2 present is high or if the volatile acidity concentration is close to the legal limit. Ough and Amerine (1988) suggest adding 0.5 mL 2 N NaOH to the wine prior to distillation, incubating for five minutes, and then adding 0.5 mL of hydrochloric acid-ferric chloride solution. Another method is to add 1 mL of 1% H_2O_2 to 10 mL of wine. In either case, any SO_2 present will be oxidized and thereby any interference is removed.

E. Validation/uncertainty

1. Distill three samples of the same red wine. Expected uncertainty is ±0.05 g/L.
2. Prepare a 10% w/v acetic acid solution by adding 10.00 g glacial acetic acid to a 100 mL volumetric flask and dilute to volume with distilled water. Transfer 2.5 mL to a 250 mL volumetric flask and dilute to volume with the same wine before distillation. This should increase volatile acidity by 1.0 g/L with recoveries ranging between 92 and 102%.

Method 11: Yeast assimilable nitrogen

A. Concepts

The concentration and composition of nitrogen–containing compounds in grape musts play a crucial role in the nutrition of microorganisms involved in both fermentation and the potential spoilage after fermentation. The nitrogenous components that are metabolically available to *Saccharomyces* are present as ammonium salts (NH_4^+) and alpha-amino compounds (amino acids), collectively known as "yeast assimilable nitrogen" (YAN). Alpha-amino compounds are measured by the assay described here, while NH_4^+ is frequently determined by using an ammonium electrode (Method 12).

Many grape musts are considered to be deficient in nitrogen based on the estimated minimal requirement of 140 to 150 mg N/L. However, the needed amount of nitrogen may be dependent on other parameters including sugar concentration of the must (Table 7), as suggested by Bisson and

Butzke (2000). In the United States, the maximum level of $(NH_4)_2HPO_4$ legally permitted for correcting nutritional deficiencies is 960 mg/L (8 lb/1,000 gal), a concentration that provides 203 mg N/L assimilable nitrogen.

It is generally recommended to delay nitrogen addition for either 48 hours (for red fermentations) or 72 hours (for white fermentations) after yeast inoculation. Addition of ammonium late in fermentation should be avoided since this compound is not consumed by *Saccharomyces* at this stage of fermentation.

Table 7. Possible relationships between must sugar concentrations and required YAN.

Must Ripeness (°Brix)	Yeast Assimilable Nitrogen (mg N/L)
21	200
23	250
25	300
27	350

In this assay, developed by Dukes and Butzke (1998), alpha-amino compounds present in grape musts are measured through reaction with *o*-phthaldialdehyde (OPA). Having the formula of $C_6H_4(CHO)_2$, the molecule is a dialdehyde with two formyl (CHO) groups attached to a benzene ring. OPA is used as a very sensitive fluorescent reagent for assaying amines in solution, notably contained in proteins, peptides, and amino acids.

B. Equipment/supplies

1. NOPA reagent buffer (pH 9.5).
    a. Dissolve 0.168 g OPA (*o*-phthaldialdehyde, purity = 99%) in 25 mL of 95% v/v ethanol.
    b. To a 250 mL volumetric flask, add a minimal amount of distilled water (≈50 mL) and dissolve 0.960 g NaOH, 2.117 g *o*-boric acid, and 0.204 g N-acetyl-L-cysteine (NAC).
    c. Once the OPA solution prepared in step (a) is dissolved, add entire solution to the 250 mL volumetric flask and <u>then</u> dilute to volume.
    d. Store reagent at 4°C for up to 21 days. Be sure to warm reagent to room temperature prior to use.
2. Reagent buffer without OPA (pH 9.5).
    a. To a second 250 mL volumetric flask, add a minimal amount of distilled water (≈50 mL) and dissolve 0.960 g NaOH, 2.117 g *o*-boric acid, and 0.204 g N-acetyl-L-cysteine (NAC). Dilute to volume.
    b. Store reagent at 4°C for up to 21 days. Be sure to warm reagent to room temperature prior to use.
3. Isoleucine (ILE) stock solution.
    a. Dissolve 0.132 ± 0.002 g of l-isoleucine (purity = 98%) and dilute to volume in a 100 mL volumetric flask (approximately 10 mM). Be sure to record exact weight of

ILE to calculate exact concentration for the calibration standards.

4. UV-VIS Spectrophotometer (335 nm) with UV grade cuvettes.

   NOTE: Can use the methyl acrylate disposable cuvettes but NOT the polystyrene ones.

5. Test tubes (6 mL).
6. Micropipette with appropriate pipette tips (50 and 100 µL volumes).

C. Procedure

1. Prepare calibration standards as per Table 8 by addition of reagents to 6 mL test tubes.
2. Set spectrophotometer to 335 nm and allow a warm-up time of 30 minutes.
3. Add 50 µL of clarified juice sample to each of two 6 mL test tubes.

   NOTE: Centrifugation (3000 x *g* for 10 minutes) is sufficient to remove the solids from the juice.

4. To one of the test tubes containing 50 µL juice, add 3.0 mL of the NOPA buffer containing the OPA.
5. Repeat step above (4) using the prepared calibration standards.
6. To the other test tube containing 50 µL juice, add 3.0 mL the reagent buffer without OPA.

   NOTE: The net absorbance of the amino acid derivatives is calculated by subtracting the absorbance of the blank from the absorbance of the derivatized sample.

7. Vortex the test tubes and allow the reaction to occur for 10 minutes.
8. Measure the absorbance of the samples and the calibration standards.
9. Plot absorbance of each calibration standard vs. mg/L of nitrogen (Table 8). Determine the linear regression equation of the line (y = mx + b) and calculate the concentration of nitrogen in the sample.

Table 8. Calibration standards for measuring yeast assimilable nitrogen.

Test Tube	Isoleucine Stock Solution (µL)	dH_2O (µL)	Resultant Concentrations: Isoleucine (ILE) (approximate mM)	Resultant Concentrations: Nitrogen (approximate mg/L)
Blank 1*	0	50	0	0
Blank 2**	0	50	0	0
Standard 1	10	40	2.0	28
Standard 2	20	30	4.0	56
Standard 3	30	20	6.0	84
Standard 4	40	10	8.0	112
Standard 5	50	0	10.0	140

*Blank 1 contains NOPA reagent buffer. **Blank 2 contains reagent buffer without OPA.

Method 12: Ammonia

A. Concepts

Ammonia is an important form of nitrogen for yeast nutrition. Adequate nitrogen is essential to prevent sluggish fermentations and reduce the chances of undesirable sulfides. Monitoring ammonia levels during fermentation may help reduce the occurrence of "stuck" fermentations. Musts that are low in ammonia can be supplemented with diammonium phosphate (DAP) at the start of fermentation to provide adequate nitrogen levels. Note that a 1 lb/1000 gal addition of DAP (0.12 g/L) should result in a rise of 25 mg/L in the ammonia concentration.

There are two commonly employed methods for determination of ammonia in musts and wines: an enzymatic assay or ion-specific probes. Enzymatic methods are commercially available as kits while probes can be used with any pH meter capable of a millivolt readout. While there are two types of ion-specific probes available, the method below uses a gas-sensing ammonia probe.

The response of an electrode membrane changes as it ages, which implies the calibration procedure must be done daily. Samples and standards must be stirred at the same rate and must be at the same temperature (preferably 20°C). A slow response of the electrode indicates that the membrane is clogged. When in doubt, it is best to change the membrane to ensure proper performance. In general, change the membrane at the end of the day to let it "age" overnight prior use to yield a more stable response.

As the presented method comes from Zoecklein et al. (1995), be sure to review manufacturer instructions for specific information.

B. Equipment/supplies

1. Ammonia combination electrode with spare membranes.
   a. The electrode should be fitted into a rubber stopper that fits a 125 mL Erlenmeyer flask and suspends the electrode at a height above the bottom to avoid being hit by the stir bar. The stopper should not seal tightly in the flask during analysis to avoid drifting readings.

      NOTE: Prepare the electrode for service as described in the instruction manual. Pay careful attention to stretching the membrane across the sensor so that it leaves a smooth surface. Sluggish or suspect membranes should be replaced at the end of the day to give them ample time to equilibrate. Rinse the electrode with distilled water between samples.
   b. Store electrode between measurements and overnight in the 1000 mg/L standard without added NaOH.
2. Expanded scale pH meter (can substitute specific ion meter).
3. Magnetic stirrer and stir bars.
4. Volumetric flasks (500 mL and 1000 mL).
5. Erlenmeyer flasks (125 mL) with stopper bored to fit the probe.

6. NaOH solution (10 M).
    a. Dissolve 200 g NaOH pellets in a minimal amount of distilled water in a 500 mL volumetric flask. Once cool, dilute to volume.
    b. Some laboratories use 5 M NaOH containing 0.05 M EDTA (14.6 g per 1L).
7. HCl (1 N).
    a. Place 500 mL distilled water into a 1000 mL volumetric flask.
    b. While stirring, slowly pour 82.8 mL of 37.1% concentrated HCl into the flask. Bring to volume with distilled water and mix well.
8. Ammonia stock solution (1000 mg/L).
    a. Dry ammonium chloride (NH_4Cl) in an oven at 105°C overnight. Place in a desiccator and allow to completely cool to room temperature.
    b. To a 1000 mL volumetric flask, add 3.141 ± 0.002 g NH_4Cl and dissolve in a minimal amount of water.
    c. Add 0.1 mL of 1 N HCl and dilute to volume.
    d. Alternatively, ammonia standards are available for purchase from chemical supply companies.

C. Procedure
1. Prepare probe calibration standards by diluting the 1,000 mg/L ammonia stock solution according to Table 9.

Table 9. Ammonia calibration standards.

Ammonia Stock Solution (mL)	Volumetric Flask (mL)	Ammonia Standard (mg/L)	Log Ammonia Standard (mg/L)
1.0	1,000	1.0	0
1.0	1,000	10.0	1
100.0	1,000	100.0	2

    a. Transfer 100 mL of the 1 mg/L standard into a 125 mL Erlenmeyer flask fitted with a stopper bored out to fit the probe. Add 1 mL 10 M NaOH. Place the electrode into the beaker and begin stirring with the magnetic stirrer and stir bar.
    b. Allow the mV reading to stabilize and record the value.
    c. Repeat steps (a) and (b) using the 10.0 and 100.0 mg/L ammonia standards.

    NOTE: Rinse the electrode with distilled water and blot dry between readings.
    d. Construct a calibration curve of mV readings vs. log concentration.

    NOTE: Use semi-log graph paper to construct the curve.
2. Must/wine analysis.
    a. Add 100 mL of must or wine to a 125 Erlenmeyer fitted with a stopper bored out to fit the probe. Add 1 mL 10M

NaOH. Place the electrode into the flask and begin stirring with the magnetic stirrer and stir bar.

b. Allow the mV reading to stabilize and record the value.

NOTE: If the reading is off the scale (higher than the highest value), dilute the grape must or wine before repeating step (a). Be sure to record the dilution.

NOTE: A common method for diluting the sample is by adding a 20 mL sample to a 100 mL volumetric flask. Dilute to volume with distilled water.

c. Compare the mV value to the calibration curve to find the concentration of ammonia.

NOTE: Multiply the obtained concentration by the dilution factor if the sample was previously diluted.

D. Helpful hints/interpretations

1. The standard curve should be checked periodically by re-analyzing one or more of the standards.

Method 13: Soluble solids (hydrometer)

A. Concepts

The soluble solids on musts are primarily composed of two fermentable sugars, glucose and fructose, normally present in a 1:1 ratio. Commonly, soluble solids are used as a measure of the maturity of grapes and thus an index of the time to harvest. In addition, wineries can use hydrometers to monitor fermentations to determine how fast (or slow) sugar is being consumed by the wine yeast, *Saccharomyces*.

One method for determining soluble solids in musts is **hydrometry.** Hydrometry is based on a principle of buoyancy: a floating object will sink in a liquid to the point where the weight of the volume of liquid displaced by the submerged portion is equal to the weight of the object itself.

Hydrometers are glass tubes weighted on one end to be able to float upright. To the unweighted end is attached a narrow stem of a given length marked to represent small increases in the displaced volume of liquid. The stem portion is calibrated to read the relative density of a liquid. Because most hydrometers are calibrated at 20°C, the temperature of the must should be recorded and the observed reading corrected (Table 10).

When soluble solids such as sugar are added to water, the water density increases and the hydrometer rises. When ethanol is added to water, for instance, during alcoholic fermentation, the water density decreases and the hydrometer sinks. Because ethanol is a by-product of *Saccharomyces*, the °Brix value obtained at the end of fermentation will likely be less than 0°, often -2° to -0.5°, even though most of the fermentable sugar has been metabolized.

One of the biggest sources of error is CO_2 in fermenting wine. Any CO_2 present can adhere to the hydrometer, thereby increasing its buoyancy (making the hydrometer float higher). Stirring, agitation, or sonication can help to remove excess CO_2 from the sample prior to measurement. In the case of handheld meters, CO_2 can be removed by pressurizing the wine (hold finger over tube and apply pressure to the plunger).

Although hydrometers have the advantage of being cheap, newer instrumentation such as handheld and bench-style density meters, like those from Anton Paar, are increasingly being used by wineries. These are being used not only for monitoring fermentation but for wine density measurements for bottle fill volume calculations.

Table 10. Correction table for hydrometers (°Brix) calibrated at 20°C.

	Sucrose (%)						
°C	**0**	**5**	**10**	**15**	**20**	**25**	**30**
				Subtract from reading			
15	0.20	0.22	0.24	0.26	0.28	0.30	0.32
16	0.17	0.18	0.20	0.22	0.23	0.25	0.26
17	0.13	0.14	0.15	0.16	0.18	0.19	0.20
18	0.09	0.10	0.10	0.11	0.12	0.13	0.13
19	0.05	0.05	0.05	0.06	0.06	0.06	0.07
				Add to reading			
21	0.04	0.05	0.06	0.06	0.06	0.07	0.07
22	0.10	0.10	0.11	0.12	0.12	0.13	0.14
23	0.16	0.16	0.17	0.17	0.19	0.20	0.21
24	0.21	0.22	0.23	0.24	0.26	0.27	0.28
25	0.27	0.28	0.30	0.31	0.32	0.34	0.35
26	0.33	0.34	0.36	0.37	0.40	0.40	0.42
27	0.40	0.41	0.42	0.44	0.46	0.48	0.50
28	0.46	0.47	0.49	0.51	0.54	0.56	0.58
29	0.54	0.55	0.56	0.59	0.61	0.63	0.66
30	0.61	0.62	0.63	0.66	0.68	0.71	0.73

B. Equipment/supplies

1. Hydrometers (-5° to +30° Brix or Balling).
2. Hydrometer jar or graduated cylinder (250 mL).
3. Thermometer.
4. Disposable wipes.

C. Procedure

1. Cool approximately 250 mL of clear juice/wine to a few degrees below room temperature (20°C).
2. Carefully pour the juice/wine into a graduated cylinder/hydrometer jar, creating as few bubbles as possible.

3. Lower the hydrometer into the liquid and give it a slight spin to remove air bubbles attached to the outside of the hydrometer.

4. Record the reading when the temperature reaches 20°C am-ing sure to view the bottom of the meniscus.

   NOTE: If the temperature of the must or wine is different from 20°C, use the values in Table 10 to correct for the actual temperature.

D. Helpful hints/interpretations

1. Always use a CLEAN hydrometer. Watch for bubbles attached to the hydrometer as these will increase buoyancy and may indicate dirty glassware.

2. When viewing the hydrometer scale, numbers above the zero are read as negative and numbers below the zero are read as positive.

Method 14: Soluble solids (refractometer)

A. Concepts

Changes in refractive index (RI) can be used as a measure of total soluble solids since the most important components affecting the RI value in grape juices are sugars.

The principle of refractive index involves the bending of light as it passes through a liquid. Refractive indices of liquids are dependent on many variables including temperature and alcohol content. If the temperature of the juice being measured is other than 20°C, an RI reading must be corrected, using a corrections table. However, refractometers should never be used to monitor an alcoholic fermentation because alcohol greatly affects the reading.

The following procedure is for measuring soluble solids in a liquid, using an AO Abbé bench top refractometer. While the general procedure is similar for other refractometers, consult the manufacturer's directions before use. In general, desk-top refractometers are generally more accurate than hand-held ones. Addition of a thermometer to the refractometer is important for temperature compensation purposes. In recent years, digital refractometers have gained in popularity due to their increased speed and lower cost.

B. Equipment/supplies

1. AO Abbé refractometer.
2. Plastic disposable pipettes.
3. Methanol.
4. Disposable wipes.

C. Procedure

   CAUTION! When using a refractometer, it is important to avoid scratching the prisms. NEVER dry-wipe the prisms, and use only plastic pipettes when transferring liquid onto the prisms.

1. Calibration of an AO Abbé refractometer.

a. Open prism assembly and remove the disposable wipe that cushions the prisms.

   NOTE: Inspect the prisms for scratches and replace if needed.

b. Clean each prism surface with water and dry with methanol. Wipe with a disposable wipe.

c. Set mode selector switch (the on-off switch on the front panel of the machine) to Brix-T/C. In this mode, the °Brix value is automatically corrected to 20°C.

d. Position the illuminator arm and lens to illuminate the face of the upper prism.

e. Use triple distilled water, if available, to wet the refracting (lower or measuring) prism and close the prism assembly.

f. While looking through the eyepiece, turn the adjustment control knob on the side of the machine until the shadow line appears in the reticle field (the collection of fine lines that appears through the eyepiece). The adjustment should be counter-clockwise when the field of view appears to be dark and clockwise when bright.

g. To adjust the eyepiece, rotate the eyepiece counter-clockwise all the way and then slowly rotate in a clockwise direction until the reticle cross-hair is sharply focused.

h. Turn the dispersion correction wheel at the base of the eyepiece so the access hole points to the 6 o'clock position.

i. Readjust the lamp for maximum contrast. Rotate the dispersion correction wheel for minimum color.

j. Turn the adjustment control knob to adjust the shadow line so it precisely intersects with the cross-hairs (Figure 11). This is the most critical step in achieving accurate instrument readings.

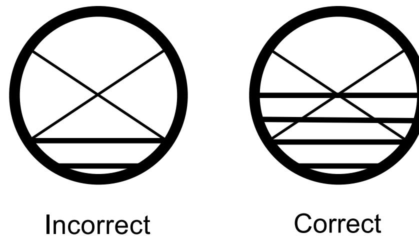


Figure 11. Comparison of reticle field adjustments. When properly adjusted, the top-most reticle just meets the intersection point of the cross-hairs (shown on right).

k. Press the Read display button below the eyepiece barrel. An acceptable reading should be 0.0 or 0.1 °Brix. Press the Temperature display button next to the Read

display button. If the temperature shown is over 20°C, the refractometer is in calibration and the temperature compensation is operating properly. If you get a reading other than 0.0 or 0.1 in the Brix-T/C mode, clean the prism and repeat procedure.

2. Measurement of refractive index (°Brix).
   a. Follow procedure outlined above for the calibration of the refractometer, substituting juice for distilled water.
   b. Make sure to clean prism surfaces immediately after use. The bulk of any sample should be wiped off first with a Kimwipe-type tissue, followed with a water cleaning. Use methanol to help dry the prisms.

D. Helpful hints/interpretations

1. Prism surfaces should be cleaned with distilled water to remove sugar or other residue, then dried with methanol.
2. When closing the prism assembly after it has been cleaned and dried, place only ONE layer of lens tissue or disposable wipe between prisms.
3. Refractometers cannot be used to monitor a fermentation because alcohol interferes with the reading.
4. °Brix can be converted from the Baume scale used in certain parts of Europe or the Oechsle scale used in Germany/Austria using tables found in Amerine and Ough (1988) or by the following approximations:

   °Brix = (1.8)(Baume)

   °Brix = (0.25)(Oechsle) – (2.5)

5. To prepare grape samples, place grapes into a sample bag (a thick, freezer-style bag with a press-and-seal closure works well) and carefully knead the bag with hands and fingers. Avoid using hard tools like hammers as these could break seeds. Once a uniform slurry is obtained, pour into a beaker for further analyses. Samples for titratable acidity and °Brix should first be clarified, either by centrifugation or settling by gravity.

E. Validation

1. Sugar standards can be prepared by accurately weighing sucrose and mixing with a known weight of distilled water. To prepare a 20°Brix solution, dissolve 20.00 g sucrose in 80.00 g water. It is important to weigh the water as it is not readily feasible to volumetrically measure the water with sufficient accuracy. Furthermore, °Brix is a weight/weight relationship, not weight/volume.

Method 15: Free/total sulfur dioxide (Ripper)

A. Concepts

Sulfur dioxide (SO_2) is commonly added to wines for its antioxidant and antimicrobial properties. SO_2 protects wine from some forms of microbial spoilage, binds to products of oxidation such as acetaldehyde to form odorless bisulfite compounds, binds to phenolic components to retard their

oxidation, scavenges peroxides, and reacts with molecular oxygen—albeit very slowly. Amounts of SO_2 in excess of the optimum can retard aging undesirably, reduce fruit aromas, and negatively impact the sensory impact of the wine. In addition, some individuals are allergic to SO_2, particularly in elevated amounts. The maximum amount of sulfur dioxide allowed in the production of wines is regulated and so must be measured.

In solution, SO_2 exists in equilibrium between three different forms: dissolved gas or molecular ($SO_2{\bullet}H_2O$), bisulfite (HSO_3^-), or sulfite (SO_3^{2-}). All three of these forms make up free SO_2. However, free SO_2 will react with and bind to other molecules such as aldehydes, phenols, and even glucose. Thus, the relationship between free, bound, and total SO_2 is defined as:

$$[\text{Free } SO_2] + [\text{Bound } SO_2] = [\text{Total } SO_2]$$

It is important to note that some forms of bound SO_2 are bound more tightly than others. Tight associations (e.g., SO_2-acetaldehyde) dissociate more slowly than others (e.g., SO_2-glucose). Because of this difference, free SO_2 commonly may also include weakly-bound SO_2. In the Ripper assay, the rate of titration during the determination of free SO_2 will affect the end point, which contributes to precision difficulties.

The Ripper method is based on the oxidation-reduction reaction (Amerine and Ough 1988). Formation of a blue starch-iodine complex due to excess iodine indicates the endpoint of the titration.

$$SO_2 + I_3^- + H_2O \text{ à } SO_3 + 3I^- + 2H^+$$

A number of the reagents used in this assay (e.g., 1+3 H_2SO_4, starch indicator, standardized thiosulfate) can also be purchased pre-made from commercial chemical supply companies.

Traditionally used to determine SO_2 levels in wines, the Ripper assay has several limitations, the largest of which is that (red) wine or must color interferes with the visual endpoint of the titration. Other limitations include (a) the reaction of iodine with other iodine-reducing substances like phenolics and ascorbic acid; (b) the easy dissociation of SO_2 in red wines can lead to falsely high values for free SO_2 (SO_2 complexes with anthocyanin or glucose); and (c) the instability of iodine solutions requires daily standardizing. Although fast and relatively easy, the Ripper method is limited by poor precision and large systematic error (Vahl and Converse 1980). Wines with added ascorbic acid cannot be tested for SO_2 by the Ripper method.

A number of the reagents used in this assay (e.g., 1+3 H_2SO_4, starch indicator, standardized thiosulfate) can be purchased pre-made from commercial chemical supply companies.

See Appendix III for determining amounts of potassium metabisulfite to yield various concentrations of total SO_2.

B. Equipment/supplies

1. Erlenmeyer flasks (250 mL).
2. Large Erlenmeyer flask or beaker (2000 mL).
3. Volumetric flasks (250, 500, and 1000 mL).
4. Volumetric pipettes (20 and 25 mL).
5. Burette with ring stand.
6. Lamp.

   NOTE: Yellow light is helpful for seeing the endpoint when titrating red wines.

7. Iodine (reagent grade crystals of I_2).
8. Sodium thiosulfate ($Na_2S_2O_3 \bullet 5H_2O$).
9. Anhydrous sodium carbonate (Na_2CO_3).
10. Sodium bicarbonate ($NaHCO_3$).
11. Potassium iodate (KIO_3).
12. Potassium iodide (KI).
13. NaOH (1 N).
14. HCl (6 M).
    a. Prepare by carefully pouring 10 mL of concentrated HCl into 10 mL of distilled water. Stir to dissolve.
15. H_2SO_4 (1+3).
    a. In a 1000 mL Erlenmeyer flask, add approximately 500 mL distilled water. Place in an ice bath under a fume hood. Carefully and slowly, add 250 mL of concentrated H_2SO_4. Allow solution to cool to room temperature and add distilled water to volume.

       CAUTION! Add the acid slowly to water over a period of several hours and allow it to cool. This reaction generates a great deal of heat. Use an ice water bath to assist in cooling the solution. Be sure to pre-cool the solution container in an ice bath. Once mixed, store in glass containers.

       NOTE: Pre-made solutions of 1+3 H_2SO_4 (25%) can also be purchased commercially.
16. Starch indicator.
    a. Prepare by weighing 10.0 g soluble potato starch into a beaker. Make into a paste with distilled water. Dribble paste into approximately 450 mL boiling distilled water. Boil and stir for several minutes. Cool and add distilled water to bring volume to 500 mL. Store in refrigerator to reduce potential microbiological spoilage.

       NOTE: Pre-made starch indicator solutions can also be purchased commercially.

C. Procedure

1. Preparation and standardization of thiosulfate.
   a. Gently boil 1.5 L of distilled water for 5 minutes in a 2000 mL beaker or Erlenmeyer flask and allow to cool.

b. Weigh out 18.8 g $Na_2S_2O_3$•$5H_2O$ and 0.1 g of Na_2CO_3 and add to the cooled water in step (a) above. Stir to mix. Transfer the 0.05 M $Na_2S_2O_3$ solution to clean, labeled bottles with screw caps.

c. Dry approximately 1.5 g KIO_3 at 170° to 180°C. After cooling in a desiccator, accurately weigh out between 0.5 and 0.8 g (to the nearest 0.0001 g) and transfer to a 250 mL volumetric flask. Dissolve the KIO_3 in approximately 150 mL distilled water, mix the solution, and dilute to the 250 mL mark.

d. Pipette 25.00 mL of standard KIO_3 solution into each of three 250 mL flasks.

e. Place 3 g KI into each of three 100 mL beakers and dissolve with 25 mL of distilled water.

f. Boil 2 mL of 6 M HCl in each of three 10 mL beakers to remove any chlorine and add each to one flask containing the KI solution.

NOTE: No iodine color should appear at this point since color will indicate the presence of iodate or of other oxidizing agents.

g. Add the acid iodide (KI) solution without delay to one of flasks containing the iodate (KIO_3) solution. Slowly swirl and titrate with thiosulfate.

NOTE: Vigorous shaking and undue delay must be avoided to minimize loss of iodine vapor.

h. When the iodine color has become a very pale yellow, but NOT before, add 5 mL of starch indicator and continue the titration with fractions of drops until the starch-iodine color has disappeared.

i. Repeat titration from step (g) one at a time for the remaining flasks containing iodate.

j. Calculations:

$$\underset{(N)}{Na_2S_2O_3} = \frac{(g\ KIO_3)\ (2.803)}{(mL\ Na_2S_2O_3)}$$

NOTE: Standardized thiosulfate must be frequently discarded and new solutions purchased because these standards rapidly degrade during storage.

2. Preparation and standardization of iodine.

a. Weigh 12.9 g I_2 and 25.0 g KI into 80 mL distilled water in a 500 mL volumetric flask and dissolve by stirring. Bring to volume with distilled water. The concentration of this solution of iodine is approximately 0.2 N.

NOTE: Store this stock solution in an airtight brown bottle in the refrigerator.

b. Dilute 50 mL of the 0.2 N iodine solution to 500 mL with distilled water to a concentration of approximately 0.02 N.

NOTE: Store this solution in an airtight brown bottle in the refrigerator.

c. To standardize the iodine solution, pipette 25.00 mL of the approximately 0.02 N iodine solution into an Erlenmeyer flask and titrate with standardized $Na_2S_2O_3$ solution to a light yellow color, adding 5 mL starch indicator, and continuing the titration until the blue color just disappears.

d. Calculations:

$$\text{Iodine (N)} = \frac{(\text{mL } Na_2S_2O_3)\ (\text{N } Na_2S_2O_3)}{(\text{mL iodine})}$$

NOTE: Because iodine solutions change titer rapidly, it is necessary to re-standardize daily, including any commercially supplied iodine (Step 2c).

NOTE: Purchase of 0.020 N iodine from a commercial supplier will eliminate preparation of the iodine reagent. This reagent should be standardized as described above (Step 2c). Rapidly titrate the free sulfurous acid using 0.020 N iodine solution. The endpoint is the first darkening of the solution to a blue or blue-black color which persists for 1 minute. Record volume of iodine used in titration.

3. Procedure for determining free sulfur dioxide.

a. Pipette 25.00 mL of wine into a 250 mL Erlenmeyer flask. Keep stoppered until ready to run.

b. Add 5 mL of starch indicator, 5 mL of (1+3) sulfuric acid and a pinch of sodium bicarbonate to expel air. For red wines, 50 mL of distilled water may be added to help visualize the endpoint.

c. Rapidly titrate the free sulfurous acid using 0.02 N iodine solution. The endpoint is the first darkening of the solution to a blue or blue-black color which persists for 20 seconds. Record volume of iodine used in titration.

NOTE: The temperature of the solution being titrated should not exceed 20°C/68°F.

NOTE: For red wines, a strong source of yellow light (such as a sodium "bug" light) can be placed so the light is transmitted through the solution from the side in order to better distinguish the endpoint.

NOTE: Some laboratories use a persistence of blue-black color for 60 seconds, rather than 20, as the endpoint.

d. Calculations:

$$\text{Free } SO_2 \text{ (mg/L)} = \frac{(\text{mL iodine})\ (\text{N iodine})\ (32)\ (1000)}{(\text{mL sample})}$$

4. Procedure for determining total sulfur dioxide.

a. Pipette 20.00 mL of wine and 25.00 mL of 1N NaOH into a 250 mL Erlenmeyer flask.

b. Mix, stopper the flask, and let stand for 10 min to permit hydrolysis of the acetaldehyde-sulfurous acid.

c. Add a "pinch" of $NaHCO_3$, 5 mL of starch indicator, and 10.00 mL of 1+3 H_2SO_4 and mix.

d. Titrate rapidly with 0.020 N iodine to a blue or blue-black color which persists for 30 seconds.

e. Calculations:

$$\text{Total } SO_2 \text{ (mg/L)} = \frac{\text{(mL iodine) (N iodine) (32) (1000)}}{\text{(mL sample)}}$$

D. Helpful hints/interpretations

1. The endpoint of a blue-black color can be very difficult to see in red wines. Some laboratories use a yellow lamp to help illuminate the end point. In addition, conducting the titration in a larger flask (1L) can also help as the solution is spread into a thinner layer and the color change is more easily seen.
2. Knowing sample pH and concentration of free SO_2, the amount of molecular SO_2 present can be calculated:

$$[\text{Molecular } SO_2] = \frac{[\text{Free } SO_2]}{[1 + 10^{pH - 1.8}]}$$

E. Validation/uncertainty

1. Expected uncertainty for free SO_2 is ±3 mg/L (white wines) and ±5 mg/L (red wines) while total SO_2 is ±5 mg/L (white) and ±10 mg/L (red).

Method 16: Free/total sulfur dioxide (aeration/oxidation)

A. Concepts

Sulfur dioxide (SO_2) is commonly added to wines for its antioxidant and antimicrobial properties. SO_2 protects wine from some forms of microbial spoilage, binds to products of oxidation such as acetaldehyde to form odorless, bisulfite adducts, binds to phenolic components to retard their oxidation, scavenges peroxides, and reacts with molecular oxygen, albeit very slowly. Amounts in excess of the optimum can retard aging undesirably, reduce fruit aromas and negatively impact the sensory impact of the wine. In addition, some individuals are allergic to SO_2, particularly in elevated amounts. The maximum amount of sulfur dioxide allowed in the production of wines is regulated and so must be measured.

In solution, SO_2 exists in equilibrium between three different forms; dissolved gas or molecular ($SO_2 \bullet H_2O$), bisulfite (HSO_3^-), or sulfite (SO_3^{2-}). All three of these forms make up free SO_2. However, free SO_2 will react with other molecules such as aldehydes, phenols, glucose, and other wine com-

ponents to become bound. Thus, the relationship between free, bound and total SO_2 is defined as:

$$[\text{Free } SO_2] + [\text{Bound } SO_2] = [\text{Total } SO_2]$$

It is important to note that some forms of bound SO_2 are bound more tightly than others. Tight associations (e.g., SO_2-acetaldehyde) dissociate more slowly than others (e.g., SO_2-glucose). Because of this difference, free SO_2 commonly may also include weakly-bound SO_2.

In the aeration-oxidation method (A/O) originally described by Buechsenstein and Ough (1978), the wine is acidified with phosphoric acid to put all free SO_2 in the form of dissolved SO_2 gas (the pH is reduced below 2). Free SO_2 is carried by suction or by pressurized air or gas into a bubbling tube where it combines with peroxide to form H_2SO_4 an acid which is then titrated with standardized NaOH. Air will not oxidize the SO_2 because sulfite ion is the only form that reacts with O_2. The overall chemical equation is a net gain of $2H^+$ for every SO_2 molecule carried over. One of the advantages of this method is that colored compounds in the wine do not interfere because the must/wine is not directly titrated.

Both free and total SO_2 can be measured depending on aspiration (suction) conditions but a new sample must be used each time. For the measurement of free SO_2, the extraction of SO_2 gas is carried out near 0°C to minimize the conversion of bound forms to free. For the determination of total SO_2, the extraction of SO_2 gas is carried out at 100°C under reflux conditions to cause all of the bound SO_2 to be converted to free during the course of the bubbling. There are many glassware designs for measuring SO_2 by the A/O method. The original reference Buechsenstein and Ough (1978) used Lieb-Zacherl design (Apparatus #1, Figure 12) while a modified system used by some wineries (Apparatus #2, Figure 13) involves flat-bottom impinger vials coupled to sidearm flasks.

B. Equipment/supplies

1. Vacuum source + tubing (can use a water aspirator).
2. Burette and ring stand.
3. NaOH (standardized 0.01 N).
   a. Refer to Method 7 for preparation of standardized base solutions.
   b. Weekly check the concentration of the standardized NaOH using KHP or 0.1 N H_2SO_4.
4. Hydrogen peroxide (0.3% v/v).
5. Indicator solution.
   a. Dissolve 0.100 g methyl red and 0.05 g methylene blue in 100 mL of a 50:50 v/v ethanol:water solution.
   b. Alternatively, this may be purchased from a scientific supply house.

6. Phosphoric acid (25%).
   a. Add 280 mL of 90% H_3PO_4 to ≈500 mL water in a 1L volumetric flask. Dilute with distilled water.

      CAUTION! Always add acid to some water and **never** add water to concentrated acid.
   b. Alternatively, using the approximate graduations on an Erlenmeyer flask can be used.
7. Apparatus #1: Lieb-Zacherl ($^{14}/_{20}$ ground glass joints) as shown in Figure 12.
   a. Connecting adapter (A).
   b. Liebig condenser (B).

      NOTE: Be sure to have water that is as cold as possible circulating through the condenser.

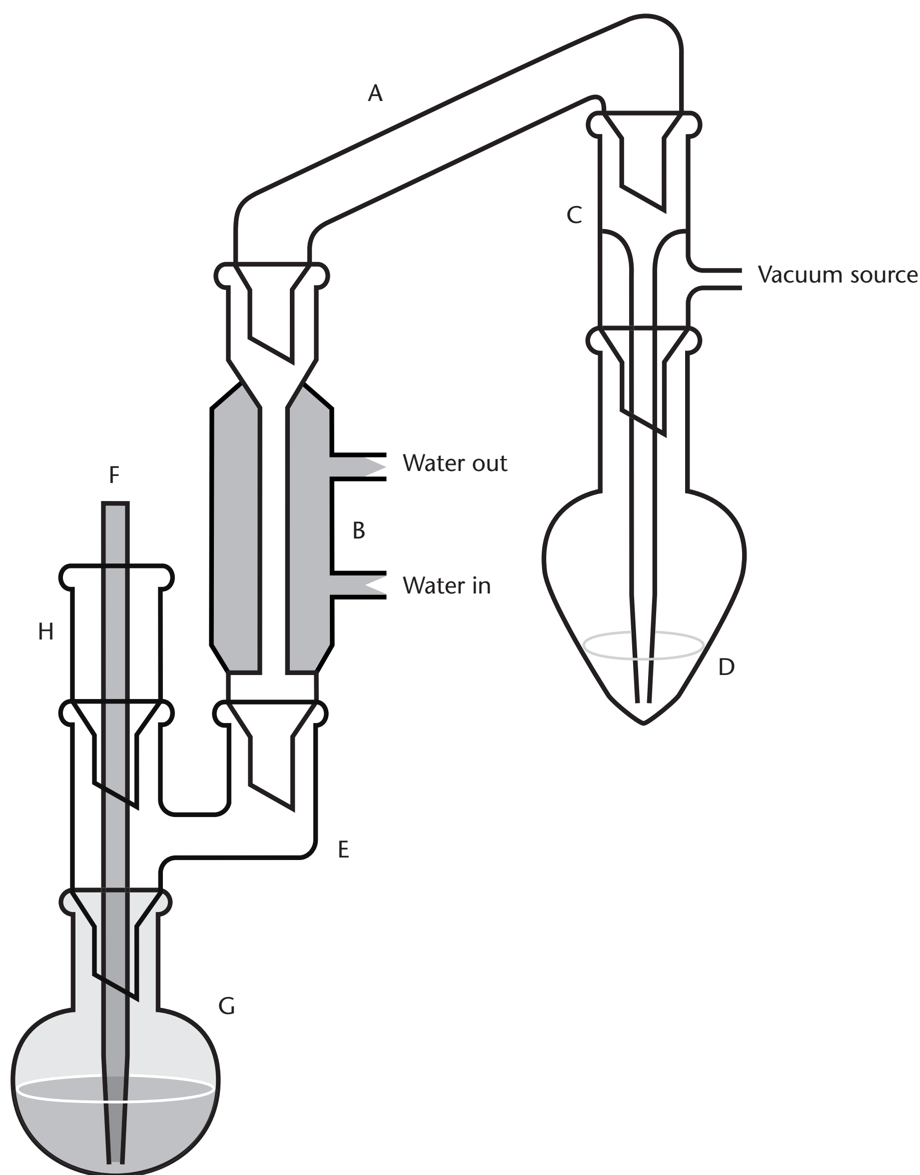


Figure 12. Lieb-Zacherl apparatus for determining free/total SO_2 ("Apparatus #1").

c. Vacuum adapter (C).

NOTE: Using a short piece of Tygon® tubing, connect a Pasteur pipette to the central glass tube of the vacuum adapter. Adjust the length of the pipette so that it will reach within 1 mm of the bottom of the pear-shaped receiver flask.

d. Pear-shaped flask, 50 mL (D).

e. Claissen adapter (E).

f. Pasteur pipette (F).

NOTE: Adjust the length of the pipette so that it will reach within 1 mm of the bottom of the round-bottom sample flask.

g. Round-bottom flask, 50 mL (G).

h. Universal adapter (H).

2. Apparatus #2: Flat-bottom impinger vials (Figure 13).

NOTE: Be sure to have water that is as cold as possible circulating through the condenser.

C. Procedure

NOTE: The procedure outlined refers to Apparatus #1 (Lieb-Zacherl) but the steps can be easily adapted to Apparatus #2 (flat-bottom impinger vials).

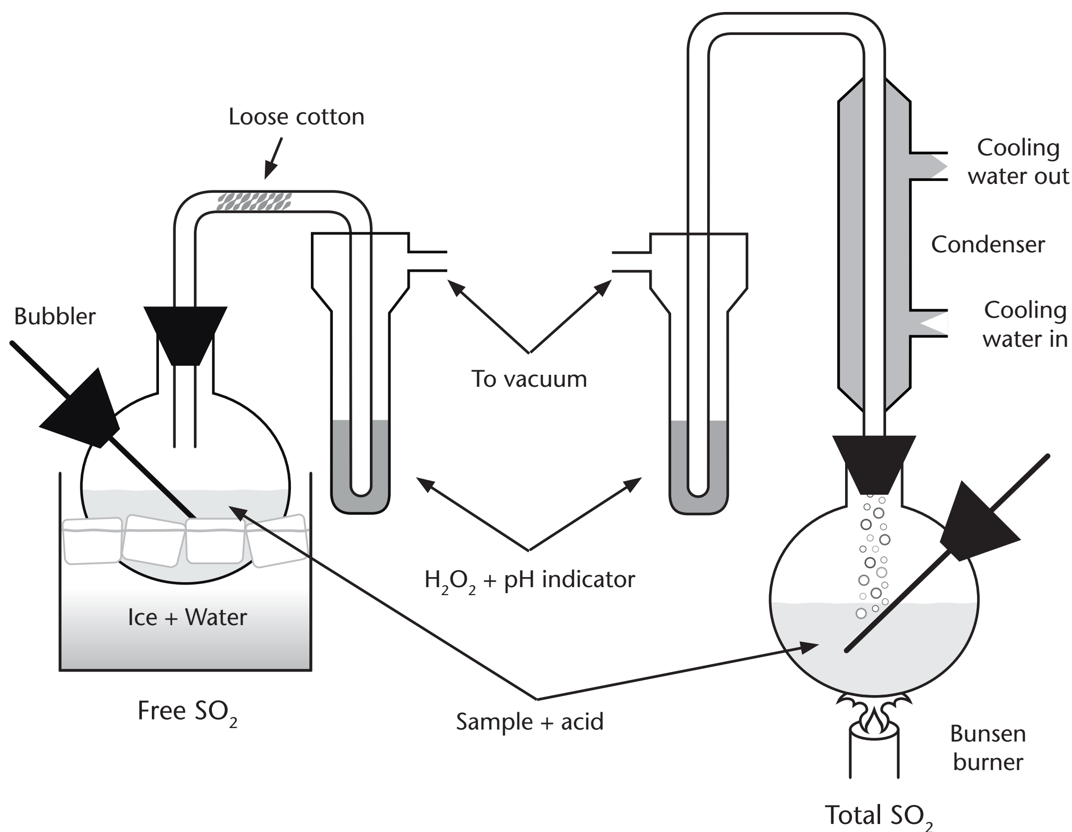


Figure 13. Alternative apparatus ("Apparatus #2") for measuring free SO_2 (left) and total SO_2 (right) using flat-bottom impinger vials.

1. Pipette 10 mL of 0.3% H_2O_2 into the pear-shaped flask (D).
2. Add 3 drops of indicator to turn the solution purple; then adjust the "purple" to "turquoise green" with 0.01 N NaOH (usually requires only 1 drop). Connect the flask to the vacuum adapter (C).

   NOTE: It is important to remember the shade of "turquoise green" observed since this same color will be the endpoint of the titration.

3. Pipette 20 mL sample (e.g., wine) into the round-bottom flask (G).
4. Pipette 10 mL 25% phosphoric acid into the round-bottom flask and connect flask to the apparatus.

   Free SO_2: Submerge flask (G) in a 0°C ice-water bath.

   Total SO_2: Gently boil contents of flask (G) with a micro-burner flame directly touching the bottom of the flask.

5. To begin, draw air rapidly through the system using a vacuum source or, alternatively, use N_2 gas through the Pasteur pipette.

   Free SO_2: Aspirate for 10 minutes.

   Total SO_2: Aspirate for 15 minutes. If foaming becomes a problem, add a few drops of inert silicone antifoam solution to the wine.

6. After aspiration, remove the trapping flask (D) and rinse the vacuum adapter tube (C) into the flask.
7. Titrate the contents of the flask (D) to the initial "turquoise green" color.

   NOTE: Before titration of the free SO_2 sample, set-up and beginning the aeration of the total SO_2 sample will reduce overall analysis time.

8. Calculation:

$$\text{Free or Total } SO_2 \text{ (mg/L)} = \frac{\text{(mL iodine) (N iodine) (32) (1000)}}{\text{(mL sample)}}$$

D. Helpful hints/interpretations

1. Foaming occurs occasionally and can be controlled by adding 95% v/v ethanol or inert silicone antifoam to the acidified wine.
2. A small bit of loose cotton in line between the free SO_2 wine flask and the impinger vial will help to eliminate carry-over of acidic aerosol from the flask. This cotton must not be packed so tightly as to impede the flow of gas.
3. Acetic acid concentrations below 1.2 g/L have a negligible effect but above this concentration, trace amounts are carried over during the distillation and can cause slightly higher results.
4. Aspiration for more than 20 minutes results in a lower SO_2 reading because of a slight release of combined sulfur dioxide.

5. If the flask for the total SO_2 determination is not actively refluxing (boiling) during the course of bubbling, the total SO_2 figure obtained will be low due to incomplete disassociation of the bound SO_2 complexes. Be certain that the heat source used provides for active boiling.
6. If total SO_2 is above 160 mg/L, the quantities of wine and phosphoric acid must be halved prior to assay.
7. Knowing sample pH and concentration of free SO_2, the amount of molecular SO_2 present can be calculated:

$$[\text{Molecular } SO_2] = \frac{[\text{Free } SO_2]}{[1 + 10^{\text{pH} - 1.8}]}$$

E. Validation/uncertainty

1. A time course of SO_2 accumulation in the impinger vial solution should be run periodically. Normally, an initial rapid release of SO_2 is observed for several minutes, resulting in a quick increase in the apparent SO_2 value. At some time point, this apparent value will plateau and rise only slightly thereafter or not at all. In the case of the free SO_2 assay, a slow release of bound SO_2 will normally be observed in the plateau phase. The time point at which the plateau begins may differ, dependent upon flow rate as determined by the efficiency of the vacuum pump or on other factors such as the size (fineness) of the bubbles in the wine chamber. As such, it is essential to confirm that one is working in the plateau region of the assay time course for each apparatus and to reconfirm this on a periodic basis.

   Six minutes may be enough to collect all free or total SO_2 but some equipment combinations may require as much as 15 to 20 minutes to reach the plateau area of the response. For example, lower gas flow will necessitate longer bubbling so a gas flow monitor or regulator may help to control the rate of bubbling.

   Once per month, the assay should be validated so that enough air flow is passing through the solutions for a sufficient length of time. This is done by conducting the aeration phase of the assay for both free and total SO_2 for intervals of 6, 10, 14, and 20 minutes. The titrated values for SO_2 at the longest time value will normally be within 5% of the chosen “normal” aspiration time. Obviously, if longer times are required to reach the plateau value, it will be necessary to either adjust the apparatus to obtain greater air flow, or to lengthen the “normal time” of bubbling.
2. Expected uncertainty is ±2 mg/L.

Method 17: Alcohol (Ditta Ing C. Bulio ebulliometer)

A. Concepts

Use of an ebulliometer relies on boiling point depression of water–alcohol solutions. Addition of alcohol (ethanol)

to water will reduce the boiling point of a water solution depending on the amount of alcohol present. However, the presence of other solutes (e.g., sugar and acids) also affect boiling points. Because of these interferences, alcohol concentrations determined by ebulliometry should never be used for labeling. For determination of alcohol content for label and tax purposes, it is recommended that wineries submit their wines to a TTB-certified commercial laboratory. There, alcohol can be more accurately measured using such methods as gas chromatography, near-IR spectroscopy, or Fourier Transform IR spectroscopy. Although highly accurate and precise, these methods require a considerable financial investment in equipment.

Barometric pressure has a large impact on the temperature at which liquids boil on any given day. It is therefore necessary to calibrate the ebulliometer before beginning measurement of wine alcohols and to check calibration after every tenth sample. If the weather (and hence the barometric pressure) is unstable, it may be best to wait for a more stable day to run these determinations. Because water boils at 100°C under 760 mm of Hg (about 29.9 inches), check the barometer reading to see if the observed boiling point of water is in agreement.

The procedure outlined is one for the Ditta Ing C. Bulio ebulliometer (metal boiler and condenser). Consult the instruction manual for the particular ebulliometer that will be used for analysis.

B. Equipment/supplies

1. Graduated cylinder (50 mL).
2. Volumetric flask (100 mL).
3. NaOH (2% w/v).
    a. Dissolve approximately 2 g NaOH in a minimal amount of distilled water in a 100 mL volumetric flask. Once cooled, dilute to volume.
4. Denatured alcohol for alcohol lamp.
5. Crushed ice for condenser.
6. Ebulliometer, complete with specialized thermometer and alcohol lamp (Figure 14).

C. Procedure

1. Calibrate the ebulliometer every few hours depending on the weather conditions.
    a. Rinse boiling chamber with 50 mL distilled water and drain.
    b. Screw condenser onto boiling chamber and fill condenser with ice-cold distilled water. Insert the special thermometer into the boiling chamber.
    c. Add 50 mL distilled water to the boiling chamber, then ignite the alcohol lamp and heat the chamber.
    d. When the mercury reaches a constant position on the thermometer, move wheel on the “Alcohol Percentage

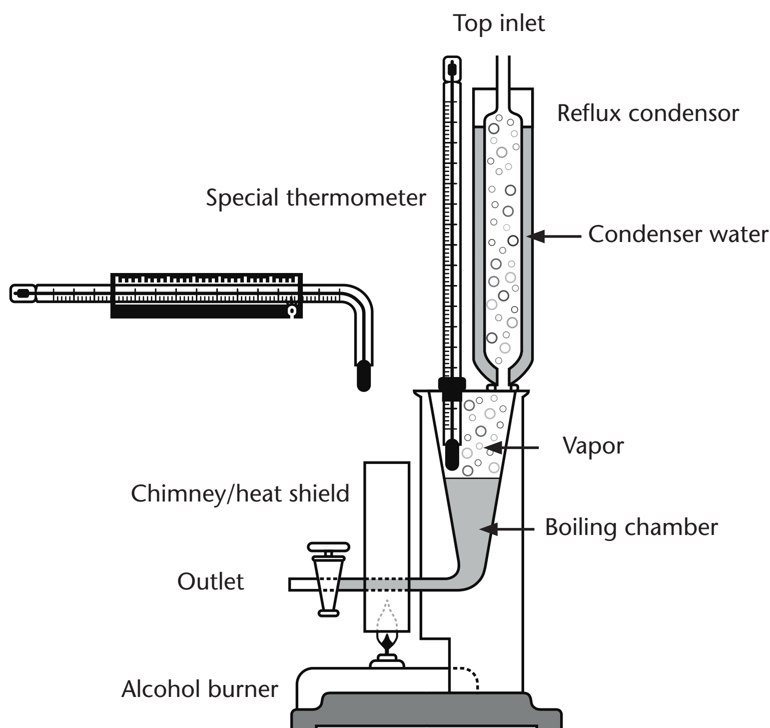


Figure 14. Diagram of an ebulliometer. Image courtesy of Monash Scientific Glass Blowing Services (Dandenong, Victoria, Australia).

Calculator" until "0" is aligned with the temperature indicated on the thermometer. Lock scale into position using the thumb screw in the center of the calculator.

2. Measurement of alcohol content in wine.
    a. Dilute wine sample to approximately 5% v/v alcohol for increased accuracy. A common dilution is to add 50 mL wine to a 100 mL volumetric flask and dilute to volume (1:2 dilution).
    b. Rinse the boiling chamber with diluted wine and fill with 50 mL of the diluted wine.
    c. Screw condenser onto boiling chamber "finger tight" (not too tight) and fill condenser with ice cold distilled water. Insert the special thermometer into the chamber and ignite alcohol lamp to heat the boiling chamber.
3. Calculation.
    a. Use the "Alcohol Percentage Calculator" to determine alcohol content knowing the temperature at which the diluted wine samples boils. Be sure to take into account any dilution factors in the calculation.
    b. The ebulliometer reading is influenced by the presence of sugar. Dilute the wine so that the sugar content is less than 2%. Tables are available for correcting the observed values (Love 1939).

D. Helpful hints/interpretations
1. To keep ebulliometer free of mineral scale deposits, boil 2%

w/v NaOH for a few minutes and rinse with distilled water. Boil distilled water in the chamber and again rinse. This procedure should be repeated every 50 alcohol determinations.

> **NOTE:** Do not attach condenser or thermometer when boiling the chamber with NaOH.

2. The amount of heat received from the alcohol lamp MUST be constant. During operation, protect the ebulliometer from air drafts in the room.

> **CAUTION!** Do not put a hot thermometer in contact with cold water or wine since breakage is probable.

3. Be wary of weather storms because these will change the barometric pressure and thus the boiling point of water. Ebulliometers should be calibrated at least twice a day.
4. Empty and refill the condenser with ice-cold distilled water for every alcohol determination.
5. Wines high in alcohol (over 14% v/v) or high in sugar (over 3% w/w) should be diluted 1:1 prior to alcohol determination. This is best done by pipetting 50 mL of wine and 50 mL of deionized water together into a container using exactly the same pipette for each measurement.

E. Validation/uncertainty

1. Prepare an ethanol standard by diluting 95% ethanol (Everclear®, not denatured alcohol) by one part in seven parts (10 mL Everclear + 60 mL distilled water). This should yield an alcohol concentration of 13.6%. To improve accuracy, the same volumetric pipette should be used for measuring all seven volumes of ethanol and water. If the ebulliometer does not yield a value of 13.5–13.7%, the apparatus is not functioning correctly. Before repeating the calibration step, check for mercury separations in the thermometer or use a different one.
2. Alternatively, wines of known alcoholic content can be periodically used to check the accuracy of the measurements.
3. Expected uncertainty is ±0.4% v/v.

Method 18: Alcohol (AllaFrance ebulliometer)

A. Concepts

Use of an ebulliometer relies on boiling point depression of water-alcohol solutions. Addition of alcohol (ethanol) to water will reduce the boiling point of a water solution. The degree of reduction is depends on the amount of alcohol present. However, the presence of other solutes (e.g., sugar and acids) also affect boiling points. Because of these interferences, alcohol concentrations determined by ebulliometry should never be used for labeling.

Barometric pressure has a large impact on the temperature at which liquids boil on any given day. It is therefore necessary to calibrate the ebulliometer before beginning measurements of wine alcohols and to check calibration after every tenth sample. If the weather (and hence the barometric

pressure) is unstable, it may be best to wait for a more stable day to run these determinations. As water boils at 100°C under 760 mm of Hg (about 29.9 inches), check the barometer reading to see if the observed boiling point of water is in agreement.

The procedure outlined here is one for the AllaFrance ebulliometer. Consult the instruction manual for the particular ebulliometer that will be used for analysis.

B. Equipment/supplies

1. Ebulliometer, complete with specialized thermometer.
2. Graduated cylinder (50 mL).

C. Procedure for the AllaFrance ebulliometer

1. Turn on cooling water.
2. Pour a small quantity of distilled water in the top funnel, rinsing the cylinder at the same time.
3. Empty the top funnel and fill it up to the inscribed mark.
4. Turn on the ebulliometer (switch is in the back).

   NOTE: An LED light in front should appear 'red' indicating the device is switched on.

5. Press ON/Off button on the front panel ONLY once.

   NOTE: An LED light in front should appear 'green' indicating the device is heating.

   WARNING! Do not push the ON/OFF button without having water or wine inside the glass cylinder.

6. Wait for the mercury column in the thermometer to stabilize for approximately 6 to 7 minutes.
7. Record the temperature of boiling and press ON/OFF button on the front panel once to stop the boiling process.

   NOTE: Set the circular slide rule to set "0% alcohol" to the temperature of boiling distilled water.

8. Carefully remove the thermometer and open the drainage valve located at the rear of the device.

   NOTE: Let the glass column cool to the touch. Rinse using at least 750 mL distilled water inside the glass cylinder.

9. Replace the thermometer and repeat steps for wine.

D. Helpful hints/interpretations

1. In the case of overheating, the green LED light will flash and the device will stop heating. In this situation, allow the device to cool until the green LED light stops flashing. Rinse with distilled water and restart the analyses.
2. To eliminate foaming when boiling, add a drop of antifoam (silicone).
3. The ebulliometer reading is influenced by the presence of sugar. Dilute the wine so that the sugar content is less than 2% w/v. Tables are available for correcting the observed values (Love 1939).

4. Be wary of weather storms because these will change the barometric pressure and thus the boiling point of water. Ebulliometers should be calibrated at least twice a day.

   CAUTION! Do not put a hot thermometer in contact with cold water or wine since breakage is probable.

5. Wines high in alcohol (over 14% v/v) or high in sugar (over 3% w/w) should be diluted 1:1 prior to alcohol determination. This is best done by pipetting 50 mL of wine and 50 mL of deionized water together into a container using exactly the same pipette for each measurement.

E. Validation/uncertainty

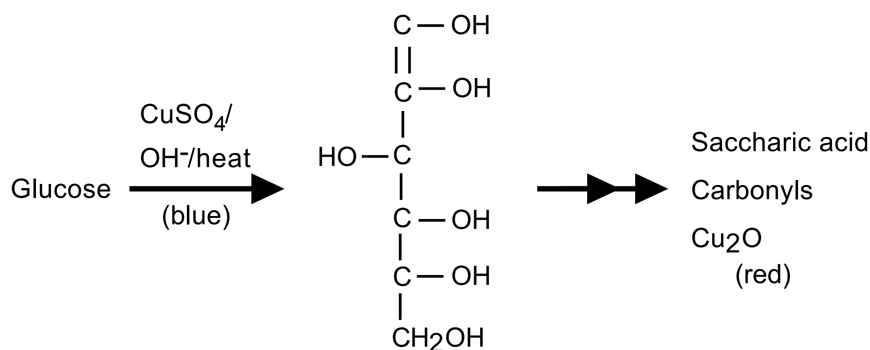
1. Prepare an ethanol standard by diluting 95% ethanol (Everclear, not denatured alcohol) by one part in seven parts (10 mL Everclear + 60 mL distilled water). This should yield an alcohol concentration of 13.6%. To improve accuracy, the same volumetric pipette should be used for measuring all seven volumes of ethanol and water. If the ebulliometer does not yield a value of 13.5 to 13.7%, the apparatus is not functioning correctly. Before repeating the calibration step, check for mercury separations in the thermometer or use a different one.
2. Alternatively, wines of known alcoholic content can be periodically used to check the accuracy of the measurements.
3. Expected uncertainty is ±0.4% v/v.

Method 19: Reducing sugars (Clinitest®)

A. Concepts

Reducing sugars are those sugars with a free carbonyl (C=O) group like glucose or fructose. Reducing sugars are analyzed towards the completion of fermentation and prior to bottling. Although not overly accurate, this method can provide winemakers with a quick check as to completion of their wines' of fermentations where 0.2% w/v reducing sugar is considered to be "dry" (fermentation is completed). This method is best restricted to wines of not more than 1% reducing sugar; i.e., wines that are not in need of dilution. It is strongly recommended that standard glucose solutions of 0, 0.25, 0.5, 0.75, and 1% be prepared and tested so that the color standards may be confirmed.

The reagent tablets made by Ames Laboratories are no longer marketed as Dextrocheck; they have been replaced by Clinitest which is used in medical laboratories for urine analysis. Although the basic procedure for urine analysis is similar to that for wine, differences exist between the package directions and wine analysis. It is important to follow the wine analysis procedure as outlined below. Furthermore,



it is important to *not* use a product that only detects glucose (tablets which contain glucose oxidase) since fructose will be present in the wine as well and would go undetected.

This method is based on the reaction of reducing sugars with an alkaline solution of cupric ions and either tartrate (Fehling's solution) or citrate (Benedict's solution) ions forming a colored complex. The concentration of reducing sugars are determined by matching colors using a key provided by the manufacturer. It is important to note that reducing sugars (i.e., those with free anomeric carbons) are not equivalent to residual sugar; the latter can contain both reducing and non-reducing sugars. Sucrose is a non-reducing sugar and cannot be detected using these types of methods.

B. Equipment/supplies

1. Test tubes and rack.
2. Pipettes (0.5 mL).
3. Long-stem glass funnels.
4. Filter paper.
5. Activated carbon.
6. Clinitest tablets (Miles Laboratories).

C. Procedure

1. Place 0.5 mL of wine in a test tube.
    a. Cloudy wines must be filtered.
    b. Red wines must be decolorized by treating a 50 mL aliquot with 1 g of activated carbon and filtering through Whatman #1 filter paper. For highly pigmented wines, additional charcoal may be required. Normally, 25 to 50 mg of carbon per mL should be sufficient to yield a sample of pink/red color.
2. Drop 1 tablet into the test tube.
3. Do not shake the tubes during boiling or for 15 seconds after the boiling has stopped.
4. Watch the reaction to see the color "pass through" the orange shade of 1% and turn brownish (refer to the color chart in Table 11). If this occurs, sample dilution may be required (see below).

5. Once the 15-second period after boiling has ended, shake the test tube gently and compare the color of the liquid fraction to the color chart provided by the manufacturer. Ignore sediment in the bottom of the tube or color changes after the 15-second period.

D. Helpful hints/interpretations

1. Using the color chart in Table 11, the amount of reducing sugar in the wine can be determined.

Table 11. Interpretation of color results from Clinitest tablets

		CIELAB Color Scale*		
Reducing Sugar (%)	**Descriptor Color**	**L**	**a**	**b**
Negligible	Dark blue	30.1	-11.0	-7.28
0.25%	Guacamole	45.4	-6.75	20.7
0.50%	Sage	49.1	-3.53	29.8
0.75%	Foothills	51.4	2.55	33.0
1%	Tan	57.4	8.71	41.0
>2%	Bright orange	61.8	32.0	50.8

*See http://dba.med.sc.edu/price/irf/Adobe_tg/models/cielab.html.

2. If a "pass through" is noted during boiling or during the 15-second waiting period, then the sample contains >1% reducing sugar and needs to be diluted prior to analysis.

   a. Dilute the wine 1:5 (0.1 mL wine + 0.4 mL water) if the wine contains 1 to 5% residual sugar. Multiply the result from the color chart by 5.

   b. Dilute the wine 1:50 (0.1 mL wine + 0.9 mL water and then 0.1 mL diluted wine + 0.4 mL water). If the sample contains >5% residual sugar, multiply result from the chart by 50.

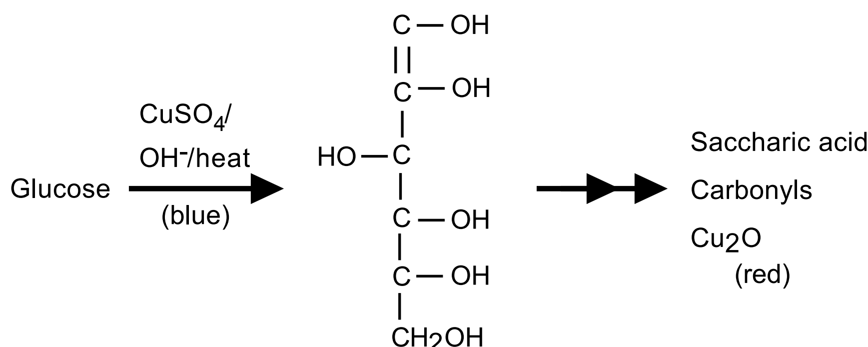
E. Validation/uncertainty

1. Expected uncertainty is ±1 g/L for wines with <0.5% residual sugar.

Method 20: Reducing sugars (modified Lane-Eynon)

A. Concepts

Residual sugar determinations employ a variety of means to determine the concentration of reducing sugars (sugars which undergo oxidation with concomitant reduction of cupric ion as shown below).



Glucose and fructose are both reducing sugars which are present in grapes along with smaller amounts of unfermentable pentoses, primarily arabinose. Thus, the presence of these unfermentable, reducing pentoses prevents the reducing sugar concentration from reaching zero in dry wines. If the amount of fermentable sugar present in a dry wine needs to be determined, it will be necessary to run an enzymatic assay for glucose and fructose.

Residual sugar contents above 0.5% can be measured by such assays as Gold Coast, Rebelein, or the modified Lane-Eynon as described here (Zoecklein et al., 1995). For measurement of concentrations below 0.5%, Clinitest (Method 19) is suggested. It is important to note that sucrose is a non-reducing sugar and will therefore not be detected by this method.

B. Equipment/supplies

1. Volumetric flasks (100 and 500 mL).
2. Erlenmeyer flasks (500 mL, narrow mouth).
3. Burette (25 mL with offset delivery) + ring stand.
4. Spin bar.
5. Stir/hot plate.
6. Fehling's A solution.
   a. Prepare by dissolving 34.65 g of hydrated copper sulfate ($CuSO_4 \cdot 5H_2O$) in water in a 500 mL volumetric flask and dilute to volume.
   b. Store at 0° to 4°C but discard if a precipitate forms.
7. Fehling's B solution.
   a. Prepare by dissolving 173 g of sodium potassium tartrate (Rochelle salt) and 50 g of NaOH in water in a 500 mL volumetric flask and dilute to volume.
   b. Store in a Pyrex® or alkali-resistant bottle at room temperature.
8. Soxhlet's reagent.
   a. Mix Fehling's A and B reagents in equal volumes just prior to analysis.
9. Standard glucose solution (0.50% w/v).
   a. Fructose can substitute for glucose.

      NOTE: Sucrose ("table sugar") is not a reducing sugar and should not be used for preparation of the standards.
   b. Use an analytical balance to prepare the standard.
10. Methylene blue solution (1% w/v).
   a. Dissolve 1 g methylene blue in 100 mL distilled water.

C. Procedure

1. Standardization.
   a. Add 70 mL of distilled water to a 500 mL Erlenmeyer flask containing a large stir bar.

b. Pipette 20 mL of Soxhlet's reagent to the flask and mix well.

c. Fill a 25 mL burette with 0.5% glucose.

d. Place the Erlenmeyer flask under the burette and add 18 mL of the 0.5% glucose solution to the flask.

e. Place flask onto a preheated stir/hot plate.

f. When the solution reaches a full boil, add 5 drops of methylene blue indicator and begin titrating (while stirring) with the 0.5% glucose.

   WARNING! Boiling the caustic solution can result in serious eye or other injury. Splash goggles (not safety glasses) are therefore mandatory for conducting this assay.

   NOTE: Endpoint is a color change from blue to red. The number of mL of 0.5% glucose used represents the blank titration value (should be close to 21 mL).

   NOTE: The titration must be completed within 3 minutes from the start of boiling.

g. Repeat the titration until the results do not differ by more than 0.1 mL.

2. Analysis of wine.

   a. Add 70 mL of distilled water to a 500 mL Erlenmeyer flask containing a large stir bar.

   b. Pipette 20 mL of Soxhlet's reagent to the flask and mix well.

   c. Add 1 mL of wine to the flask.

      NOTE: If residual sugar concentration of the wine is >5%, dilute to between 1 and 5%. Be sure to include any dilution factors in the final calculation.

   d. Fill a 25 mL burette with 0.5% glucose.

   e. Place the Erlenmeyer flask under the burette and add 18 mL of the 0.5% glucose solution to the flask.

   f. Place flask onto a preheated stir/hot plate.

   g. When the solution reaches a full boil, add 5 drops of methylene blue indicator and begin titrating (while stirring) with the 0.5% glucose.

      WARNING! Boiling the caustic solution can result in serious eye or other injury. Splash goggles (not safety glasses) are therefore mandatory for conducting this assay.

      NOTE: Endpoint is a color change from blue to red.

      NOTE: The titration must be completed within 3 minutes from the start of boiling.

   h. Repeat the titration until the results do not differ by more than 0.1 mL.

$$\text{Reducing sugar (g/100 mL)} = \frac{(A - B)(0.005)(100)}{V}$$

Where: A = Volume of standard sugar for Soxhlet's reagent (mL)
B = Volume of standard sugar for wine sample (mL)
V = Volume of wine in final aliquot

D. Helpful hints/interpretations

1. If the 20 mL aliquot of clarified wine contains too much sugar for the Soxhlet's reagent, use 20 mL of a wine diluted 1:2 with water. Note any dilution factors for calculation purposes.
2. All cloudy wines must be clarified prior to analysis.
3. Glucose is hydroscopic so must be dried (60°C for 3 or 4 hours) prior to use as a standard.
4. Check the blank titration frequently to be sure that this value is repeatable. A pair of blank determinations should be run before each set of assays (at least once per day or whenever new solutions are mixed) and if the two values differ by more than 0.1 mL, repeat until reproducibility is obtained. A lack of repeatability of the blank suggests inadequate boiling, inconsistent rate of titration, or differences in volumes of color indicator added.

E. Validation/uncertainty

1. Determine the residual sugar concentration in a wine, ideally 0.5 to 3% w/v. Carefully add 1 mL of 1.00% w/v glucose (called a "known addition") to an assay flask containing 1 mL of the wine. The additional sugar should increase the concentration by 1.0% based on using the original volume of undiluted wine present (1 mL). Perform a verification once for every 20 assays with any variance indicative of the need for a new reagent or changes to the protocol.
2. Glucose standards can be prepared and analyzed, but this sugar is hygroscopic. Prior to use, dry at 60°C for 3 or 4 hours and store in a desiccator.
3. Expected uncertainty is ±2 g/L.

Method 21: Malic acid

A. Concepts

Wine contains several organic acids with the most notable are malic, tartaric, succinic, lactic, and citric (if added). The presence or absence of these acids can be detected in wines using paper chromatography. Commonly, winemakers use some method to determine if malolactic fermentation is complete, as indicated by the disappearance of malic acid and the formation of lactic acid. Paper chromatography has the advantages of being relatively quick and inexpensive. However, the limit of detection is relatively high compared to other methods such as enzyme kits which require a spectrophotometer or high performance liquid chromatography (HPLC).

Either solvent 1 or solvent 2 can be used for paper chromatography.

B. Equipment/supplies

1. Glass tank or a one gallon wide-mouth jar with lids.
2. Whatman No. 1 chromatography paper and filter paper (11 cm diameter).
3. Separatory funnel (1000 mL) with support ring and stand.
4. Micropipettes (glass capillary 1.1 to 1.2 mm i.d. and 75 mm long).
5. Standard solutions (1% solutions of malic acid, lactic acid, or tartaric acid).
6. Solvent 1.

   Prepare by mixing 500 mL N-butanol (1-butanol), 53.5 mL reagent grade formic acid, 100 mL 1% bromcresol green $_{(aq)}$ and 500 mL distilled H_2O in a separatory funnel. Mix by inverting funnel and occasionally vent using the stopcock. Place upright and discard lower water layer. Filter solvent through filter paper to remove small residual water.

7. Solvent 2.

   Prepare by mixing 500 mL N-amyl alcohol (1-pentanol), 109 mL concentrated formic acid, 0.5 g bromophenol blue, and 391 mL distilled H_2O in a separatory funnel. Mix by inverting funnel and occasionally vent using the stopcock. Place upright and discard lower water layer. Filter solvent through filter paper to remove small residual water.

   CAUTION! Avoid breathing the solvents, and wear chemical-resistant gloves and goggles when handling the solvent. Work in a room with adequate ventilation and use a fume hood.

C. Procedure

1. Prepare either solvent 1 or 2 as described above and pour ½ inch of solvent into the glass tank/jar or reservoir. Replace lid.
2. Cut a 9 x 14 inch piece of chromatography paper (Figure 15). Wear disposable vinyl gloves while handling the paper. The size and shape of the paper will vary depending on whether the chromatography will be conducted in an ascending or descending direction. Cut the paper, noting the "machine direction" on the box (run the chromatogram in the same direction).
3. Draw a faint pencil line across the paper, $^3/_4$ to 1 inch from the bottom for ascending <u>or</u> 2 $^3/_4$ inch from the bottom for descending chromatography. Samples will be applied ("spotted") along this line (the origin).
4. Apply 10 µL of each of the standards on the same spot using the micropipettes. Spot 10 µL of each wine an inch apart along the origin, one wine sample per spot, and allow the chromatogram (paper) to dry before placing it into the tank or jar.

NOTE: Do not immerse the wine spots into the solvent since this will ruin the chromatogram and contaminate the solvent. For running chromatograms in a jar, staple the ends of the paper together widthwise to form a paper tube and place in the jar.

5. Develop the chromatogram for 4 to 12 hours, until the solvent moves close to the top of the paper.
6. Once developed (when the solvent line is near the top of the paper), remove the paper from the tank and hang it to dry under a fume hood. Be sure to wear chemical-resistant gloves to avoid contaminating the paper with your hands.

D. Helpful hints/interpretations

1. Do not spot all of the 10 µL at once. Put a little at a time on the chromatography paper and dry using a hair dryer before reapplication. If spots are too large and diffuse, reduce the volume of wine added.
2. If the chromatogram is poor in quality (in either color or resolution), filter the solvent again through Whatman #1 paper to remove small residual water.
3. Do not let the paper touch the sides of the tank/jar. This can result in "wicking" of the solvent, leading to a poorly developed chromatogram.

Method 22: Reduced sulfur compounds

A. Concepts

Hydrogen sulfite (H_2S) and other sulfur-containing compounds such as mercaptans (R-SH), monosulfides (R-S-R), and disulfides (R-S-S-R) give wines "reduced characters" which can be quite unpleasant. While H_2S

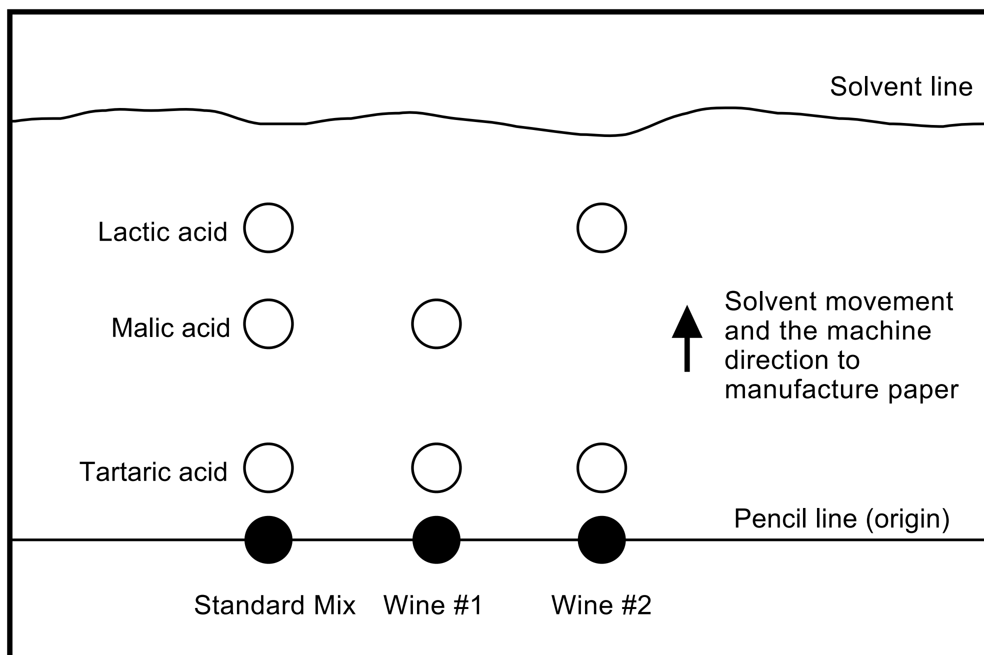
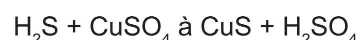


Figure 15. Typical results from paper chromatography showing acid separation.

smells of ‘rotten eggs,’, methyl mercaptan (CH_3-SH) has been described as ‘putrefaction,’ ‘cooked cabbage,’ ‘rotten cabbage,’ ‘burnt-rubber,’ or ‘burnt -tire’ (Park 2001), ethyl mercaptan (CH_3CH_2-SH) as ‘onion,’ ‘rubber,’ ‘burnt-match,’ ‘earthy,’ or ‘fecal’ (Mestres et al. 2000), and dimethyl sulfide (CH_3-S-CH_3) as ‘cooked cabbage,’ ‘asparagus,’ ‘canned corn’ (Park 2001). Failure to treat H_2S in its early stages will add to sensory problems through production of the other sulfur-containing compounds.

To remove H_2S and mercaptans from a wine, wineries and home winemakers add copper in some form. This can include including passing the wine through a simple copper pot scrubber (Molly Maid®) inserted into a small PVC pipe. Larger wineries will use a copper salt, most commonly $CuSO_4$, based on the following chemistry:



$$H_2S + CuSO_4 \text{ à } CuS + H_2SO_4$$

Being a precipitate, CuS will fall out of solution and is easily removed by racking or filtration.

Although the legal limit in the U.S. is 6 mg/L, the concentration of residual copper in wine cannot exceed 0.5 mg/L. As such, care must be taken as to how much copper is initially added. Addition of 2.98g $CuSO_4 \bullet 5H_2O$ to 1000 gallons of wine is a copper addition of 0.2 mg/L. Here, $CuSO_4 \bullet 5H_2O$ (MW = 249.7 g/mole) represents 25.4% copper (MW = 63.55 g/mole). As a concentration of 0.2 mg/L requires a total of 757 mg copper per 1000 gallons (3785 L) of wine, this would equal 2.98 g copper sulfate (0.757/0.254 = 2.98).

This addition may not increase the copper content of the wine, if most of the added copper is precipitated. Wines should be tested for copper levels by a commercial laboratory both before and after additions. In fact, it may be prudent to test wines from lab trials to make sure that excess copper is not being added. All samples should be filtered prior to testing to ensure removal of copper-containing sediments.

It is important to note that while H_2S and mercaptans will readily react with copper, disulfides do not. Therefore, addition of copper to a wine will not influence any odors exerted by disulfides. In addition, it is possible to confuse these off-odors with those of a *Brettanomyces* infection. The testing method described here can help determine if sulfur-containing compounds are the causative agents for the perceived off-odor.

One should be aware that a number of important varietal aroma compounds, particularly those found in Sauvignon Blanc, are mercaptans and will be therefore be removed or attenuated by copper. In these situations, judgement and finesse is required to determine appropriate strategies.

Sensorily testing for reduced sulfur compounds in wine and designing a treatment plan is carried out in two phases. First, the nature of the problem is characterized by additions

of copper and/or ascorbic acid using small, laboratory-scale samples prior to winery application. Once the problem is characterized, careful trials are set up to determine the treatment levels required.

B. Equipment/supplies

1. $CuSO_4 \bullet 5H_2O$ stock solution (0.05%).
   a. Weigh 0.500 g $CuSO_4$ into a 1000 mL volumetric flask.
   b. Add a minimal amount of distilled water to dissolve the salt, and dilute to volume.
2. Ascorbic acid stock solution (1%).
   a. Weigh 1.0 g ascorbic acid into a 100 mL volumetric flask.
   b. Add a minimal amount of distilled water to dissolve the salt, and dilute to volume.
3. Three wineglasses.

C. Procedure

1. Place approximately 50 mL of suspect wine into three wineglasses.
2. To wineglass #1, add nothing (control wine).
3. To wineglass #2, add five drops of $CuSO_4$ stock solution and mix well.
4. To wineglass #3, add five drops of ascorbic acid stock solution and mix well. After 5 to 10 minutes, add five drops of $CuSO_4$ stock solution and again stir well.
5. Interpret the results according to Table 12.

Table 12. Sensory comparisons of wines for diagnosing reduced sulfur compounds.

	Odor, compared to Wineglass #1		
	Wineglass #2	**Wineglass #3**	**Conclusion**
1st Possibility	No change	No change	Not a sulfide problem
2nd Possibility	No change	Reduction/Elimination	Disulfide
3rd Possibility	Reduction	Elimination	H_2s, mercaptan, & disulfide
4th Possibility	Elimination	Elimination	H_2s and/or mercaptan

D. Helpful hints/interpretations

1. For determination of proper addition levels for the cellar, it is recommended that a series of trials employing various levels of copper additions with or without ascorbic acid be prepared. Sensorily evaluate any wines from these trials after 24 hours to allow reactions to go to completion. Wine samples of 375 mL are employed as these can be stored easily in bottles of similar size and will provide plenty of wine for evaluation. After filtration, these samples may also be submitted to a commercial laboratory for residual copper determination.

Table 13. Amount of copper or ascorbic acid to add to wine (375 mL).

	$CuSO_4 \cdot 5H_2O$ (0.05%)		Ascorbic Acid (1%)	
	Volume Added (µL)	**Copper Concentration (mg/L)**	**Volume Added (mL)**	**Ascorbic Acid Concentration (mg/L)**
Bottle 1	0	0	0	0
Bottle 2	295	0.1	0	0
Bottle 3	590	0.2	0	0
Bottle 4	885	0.3	0	0
Bottle 5	0	0	0.75	20
Bottle 6	0	0	1.5	40
Bottle 7	0	0	2.25	60

Method 23: Wine color

A. Concepts

The methods involved in the analysis of wine color are based on treating wine with different reagents and measuring light absorbance at different wavelengths. The procedure outlined below is a modification of the method published by Somers and Evans (1977).

B. Equipment/supplies

1. UV-VIS spectrophotometer.
2. Filtration system (0.45 µm).
3. Cuvettes (1 and 10 mm path lengths).

   NOTE: A quartz insert may be used with the 10 mm cuvette to get 1 mm path length.

C. Procedure

1. White wine.
   a. Filter wine through 0.45 µm filter.
   b. Measure absorbance with 10 mm cuvette at 420 nm using a water blank to zero spectrophotometer.
   c. Calculation (A = absorbance):

$$\text{Color} = A_{420}$$

2. Red wine.
   a. Filter wine through 0.45 µm filter.
   b. For pure wine color, absorbance should be measured with a 1 mm cuvette at 420 and 520 nm using a water blank for the zero.

      NOTE: It is very important that the wine not be diluted, to avoid resultant changes in pH.

c. Calculation (A = absorbance):

$$\text{Color density} = A_{420} + A_{520}$$

$$\text{Color hue} = \frac{A_{420}}{A_{520}}$$

Method 24: Protein removal (bentonite)

A. Concepts

Proteins represent a diverse class of biopolymers which perform both enzymatic and structural roles in the grapes and in the yeast. Present in most wines after fermentation, these proteins react with tannins from red wines to yield insoluble complexes that precipitate. Because white wines usually lack sufficient tannins to remove the proteins, these proteins have the potential to form hazes and precipitates upon storage. The precipitation process may occur spontaneously during storage, but also may be accelerated by high ethanol concentrations and by higher temperatures. This is why the terms “protein stability” and “heat stability” are often used interchangeably. Once protein precipitation has occurred, the haze or precipitate will not redissolve and will render the wine unacceptable to the consumer.

Wines at risk for post-bottling precipitation commonly have causative proteins removed by reaction with a number of fining agents such as bentonite. Bentonite and other fining agents react with protein, making larger insoluble complexes that can be removed by racking or filtration. After fining, it is hoped that protein concentrations are low enough that precipitation is unlikely to occur under storage and shipping conditions routinely encountered by the wines. However, addition of excessive amounts of fining agents can remove important flavors and aromas from the product.

All white (and pink) wines must be tested for protein stability. While many tests have been used over the years, simple heating tests work well in terms of achieving acceptable stability. A variety of factors such as pH, ionic strength, and ethanol concentration all affect the stability of the proteins in solution, so it is essential to conduct stability testing and any subsequent fining operations after the final blend has been made and any additions of acid are completed.

If a wine is determined to be protein instable, a fining trial must be conducted using various levels of bentonite additions to establish the minimum amount of bentonite necessary to achieve stability. These additions are tested for effectiveness by the same heat stress test used to establish the stability or instability of the unfined wine. Note that the same bentonite used in the cellar must be used for the trials, and that preparation of the bentonite suspension in the laboratory should mimic the preparation used in the cellar to the greatest possible degree.

Evaluation of the stressed wines for turbidity may be carried out either by visual examination using a good light source such as a high intensity lamp, or by use of a turbidity meter to quantitate the turbidity in Nephelometric units. In all cases, the wine samples for testing must be scrupulously clear and free of haze and sediments prior to application of the heat stress, as it is impossible to evaluate haze formation if the heated sample was not originally clear. Centrifugation followed by filtration is normally used to clarify wine samples.

A turbidity meter may be used to confirm visual observation of the stable/unstable wines. It is important to use clean cuvettes to accurately determine turbidity expressed as NTU values. With increasing amounts of bentonite added, the NTU values should decline to a stable point which should remain relatively unchanged at higher bentonite additions.

B. Equipment/supplies

1. Bentonite suspension (3% w/v).
    a. Prepare according to the manufacturer's directions regarding water temperature and hydration time.
    b. Commonly, bentonite (3 g) is weighed into a 250 mL beaker and slowly sprinkled into water. Keep stirring to avoid clumping.
    c. When all bentonite is added, dilute to volume (100 mL).

       NOTE: It will also be necessary to stir the suspension continuously while pipetting the suspension into the tubes for trials. Bentonite will rapidly settle out of an unstirred suspension but may be easily resuspended by resuming stirring. This suspension may be used over a period of several days without risk of spoilage.
    d. Allow the suspension to swell before use following manufacturer's instructions.
2. Screw-capped test tubes (50 mL).
3. Variable volume pipettor (1000 µL).
4. Centrifuge (optional but recommended).
5. Oven (80°C).
    a. Can also substitute an incubator or water bath.
6. Filtration apparatus.
    a. 25 mm syringe-type filter holders with glass fiber depth filters (coarse) and 0.8 µm membranes.

C. Procedure

1. Pre-filter the wine to be tested, to remove obvious hazes or precipitates.
2. Incubate the wine at 80°C for 4 hours before transferring to 4°C for an additional 4-hour incubation.
3. Allow the sample to warm to room temperature for at least 2 hours to eliminate condensation and to promote additional coagulation of protein precipitates.

4. Evaluate for turbidity either by eye or by use of a turbidity meter.

   NOTE: Those wines that remain clear may be regarded as stable and do not require additional stabilizing against protein precipitations.

   NOTE: Wines that show obvious hazes or flocculant precipitations should be regarded as protein instable. Slight hazes indicate marginal stability and may require additional fining (step 5).

5. Set up eight screw-capped test tubes labeled as shown in Table 14. Add the appropriate volume of 3% bentonite and 50 mL wine to each tube.

Table 14. Set-up for a bentonite evaluation trial.

Test Tube	Volume of 3% Bentonite (µL)	Bentonite (g/L)
Tube 1	0	0
Tube 2	165	0.1
Tube 3	335	0.2
Tube 4	665	0.4
Tube 5	1000	0.6
Tube 6	1331	0.8
Tube 7	1665	1.0
Tube 8	2000	1.2

6. Invert all tubes several times to mix.
7. Clarify each tube by centrifugation or allow to settle at least overnight.
8. Filter each wine using a syringe-mounted 25 mm filter, first through a glass fiber filter and then through 0.8 µm filter membrane.
   a. Filtrate from one filter unit may be flowed directly into the second syringe containing the 0.8 µm membrane.
   b. If the same filter is to be used for all tubes of given wine, start with the highest bentonite addition to prevent contamination of a stable wine with unstable wine.
9. Incubate the filtered wines at 80°C for 4 hours before transferring to 4°C for an additional 4-hour incubation.
10. Allow the samples to warm to room temperature for at least 2 hours to eliminate condensation and to promote additional coagulation of protein precipitates.
11. Evaluate for turbidity either by eye or by use of a turbidity meter.

   NOTE: Those wines that remain clear may be regarded as stable and do not require additional stabilizing.

   NOTE: The test tube with the lowest bentonite addition that satisfactorily stabilizes the wine against protein precipitation will normally represent the level chosen for a cellar addition.

D. Helpful hints/interpretations

1. It is essential that the wine sample for testing is truly representative of the lot. Mix barrels or tanks prior to sampling, as required.
2. As soon as the bentonite has been well mixed into a tank of wine, a sample should be removed, clarified, and heat stressed to confirm protein stability.
3. Wines must not be excessively cold (≤4°C) during the bentonite addition process. Cold wines risk crystallization of potassium hydrogen tartrate on the bentonite platelets, reducing the bentonite's effectiveness for protein removal.
4. Bentonite requirements of wines change during aging. Recheck wines soon after fining or after preparing blends of supposedly heat-stable wines.
5. Heat stability of wines should be checked after further processing including acid additions, incorporation of other protein fining agents, etc., prior to bottling.

Method 25: Potassium hydrogen tartrate stability

A. Concepts

After fermentation, wines are super-saturated with potassium hydrogen tartrate or potassium bitartrate (KHT), a salt that is not overly soluble at cool to cold temperatures in alcoholic solutions. Precipitates of KHT crystals tend to form on nucleation surfaces including the bottom of a cork. While this precipitate does not normally affect wine flavor or aroma, consumers are often concerned because the precipitate can resemble broken glass. In general, cold instability tends to be more of an issue in white wines which may be stored at or near 0°C in a refrigerator or cold room for several weeks or months.

Large wineries almost universally strive to prevent such precipitation by cold stabilization, while smaller operations without adequate chilling and filtration capacity to accomplish effective stabilization may opt for other solutions, such as a back label explanation of the nature of the precipitate. Commercially, stabilization is accomplished by chilling the wine in-tank to below 0°C and encouraging KHT deposition by the addition of KHT seed crystals. Once precipitation has completed, it is then essential to remove KHT crystals from the wine by filtration before the wine is allowed to warm up as the precipitated KHT will easily redissolve upon warming of the wine. To ensure that this was correctly accomplished in the cellar, it is important to test cold stability after the separation of the KHT crystals from the wine is complete.

To determine if a wine is cold stable or not, small volumes of wines are chilled to various temperatures and examined for crystal formation over time. All of these tests tend to be dependent upon the presence or absence of nucleation surfaces for crystal formation and are highly

subjective in their interpretation. These methods are also time consuming and may not yield results for several days, which may hamper bottling decisions.

An alternative method gaining popularity is the Davis Conductivity test, a rapid and easy method that yields a consistent, reproducible, and objective assessment of cold stability. The Davis Conductivity test relies upon the fact that soluble KHT carries electric current and thus increases the conductivity of a liquid. As KHT precipitates, the conductivity of the liquid decreases (fewer ions are present). Testing for cold stability essentially involves measuring the conductivity of two samples: (a) tank sample and (b) stabilized sample. The latter is prepared by adding excess KHT to the tank sample kept at 0°C. If the conductivity values are close (<4% for white wine; <5% for red wine), the wine is considered to be cold stable to precipitation from KHT.

B. Equipment/supplies

1. Wide-mouth Erlenmeyer flasks (250 mL).
2. Conductivity probe.
3. Finely ground KHT.
    a. Use a mortar and pestle to finely grind the crystals, if necessary.
4. Ice bath.
    a. An ice-water bath is always at 0°C.
5. Stir plate and magnetic stir bar.

C. Procedure

1. Prepare a 0°C bath of water and ice cubes that can be placed onto a stir plate.
2. Place the probe in the bath to begin chilling it to 0°C.

    NOTE: The probe itself must reach 0°C.

3. Place 200 mL of a freshly drawn tank sample in a 250 mL wide-mouth flask and bring its temperature to 0°C in the ice bath.

    NOTE: The sample must be representative of the whole tank. Beware of temperature stratification in the tank!

4. Place the flask sitting in the ice bath on a stir plate and use a magnetic stir bar to stir the wine throughout the test.
5. Place the conductivity probe in the wine and allow the reading to stabilize.

    NOTE: This may take several minutes.

6. Record this “tank value” which represents the conductivity of the sample.
7. To the same sample, add 4 g of KHT and continue stirring until the reading again stabilizes, usually after 20 to 40 minutes.

NOTE: A reading is considered stable when no decrease in value is seen across three successive measurements made at intervals of at least two minutes.

8. Record this “stabilized value” which represents the conductivity of the KHT-seeded sample.
9. Calculation.
    a. Divide the “tank value” by the “stabilized value” to yield a calculated ratio.
    b. The decimal portion of the calculated ratio represents the percent decrease in conductance; i.e., a value of 1.048 equates to a 4.8% decrease in conductance.

D. Helpful hints/interpretations

1. Calculated ratios of less than 4% imply the white wine is cold stable, while red wines are considered stable with slightly higher values (Δ conductance of less than 5%).
2. Due to the increase in solubility of KHT with temperature, a difference in conductivity values of 15% at 0°C will correspond to a 5% decrease in conductivity if the test were performed at 2°C. Thus, if one wishes to stabilize to 2°C, a drop of conductivity at 0°C of 15% could, in theory, be tolerated. However, experience indicates that wines with a difference of 7% at 0°C will tend to precipitate crystals.
3. It is critical to perform the method at exactly 0°C and maintain constant stirring.

    NOTE: Cellar stabilization should also be performed at or preferably below 0°C.

4. The stability test should be carried out on wines in tank that are in the process of being KHT stabilized and then again on stabilized wines after separation of seed KHT. This will confirm that the separation of the KHT crystals from the wine was carried out correctly in the cellar.

Appendix I. Parts of a Microscope

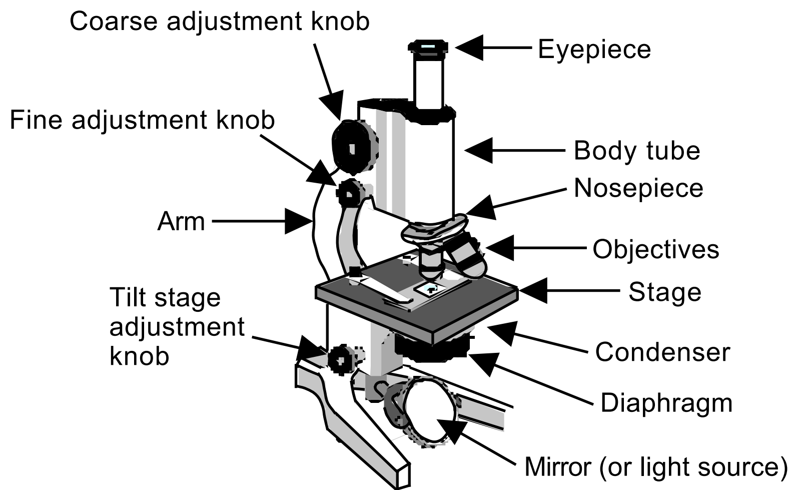


Figure A-1. Parts of a basic microscope.

Arm. This physically supports the microscope and is the part that is held when moving the microscope to different locations.

Body tube. The tube holds the eyepiece lenses and nosepiece in the correct position and orientation.

Condenser. This part condenses light rays into a "pencil-shaped" cone of light, thereby allowing more light to enter the microscope. The position of the condenser can be raised or lowered to change the amount of light entering the microscope.

Coarse adjustment knob. This knob moves the stage up and down in *large* increments to permit quick focusing on the sample of interest.

Diaphragm. Controlled by a lever, the diaphragm increases or decreases the amount of light passing through the slide containing a sample to be examined.

Eyepiece (ocular) lenses. These lenses are those that the microbiologist uses to view samples (normal magnification = 10X).

Fine adjustment knob. This knob moves the stage up and down in *small* increments to permit fine focusing on the sample of interest.

Magnification. The ratio of the apparent size of the sample as seen through the microscope to the actual size of the sample seen by the unassisted eye at a distance of 25 cm.

Total magnification = (ocular magnification) × (objective magnification)

Mirror. Used to direct light into the microscope from a separate, unattached light source.

Nosepiece. Objective lenses are screwed into the nosepiece, which allows changing objectives quickly by rotating its base.

Objective lenses. Most microscopes contain three objectives, which act to further magnify the image. These objectives can be low (10X), high (40X), and oil immersion (100X). Objectives can be switched by simply rotating the nosepiece.

Oil immersion objective. Commonly the highest magnified objective, its use requires a drop of immersion oil to be placed between the objective and the sample in order to provide clear images of the sample.

Parfocal. Microscopes are designed so that once an image is in focus using the low (10X) objective, other objectives can be used with a minimum of re-focusing.

Stage. The platform that holds the sample to be examined. Most microscopes have controls to move the stage so that more of the sample can be completely examined.

Tilt stage adjustment knob. Allows the microscope to be tilted toward/away from the microbiologist to assist viewing. Be careful of liquid samples spilling over the stage if the stage is tilted too far.

Appendix II. Colony and Microscopic Descriptions of Must/Wine Microorganisms

A. Descriptions of yeasts

1. *Saccharomyces*.

   Colony: Grows in 1 to 3 days with sizes that range 2 to 5 mm (up to 10 mm in 2 to 4 weeks); sensitive to cycloheximide (1 to 4 mg/L); rounded, later umbonate; smooth, satiny surface with smooth edges; white, cream, light or medium green on WL agar; does not grow on lysine agar.

   Microscopic: Round to ovoid, little clumping or chaining; 10 to 20 μm in diameter; multipolar budding; strong fermenter.

2. *Zygosaccharomyces*.

   Colony: Similar to *Saccharomyces* but sometimes darker green; may develop bumpy appearance; does not grow on lysine agar.

   Microscopic: Similar to *Saccharomyces* when young but more oval; may form some chains; conjugation in 1 to 2 weeks but only after growth on suitable media (bizarre conjoined structures that look like “dumb-bells” or cells with “protrusions”); strong fermenter; resistant to sorbate.

3. *Kloeckera/Hanseniaspora*.

   Colony: Growth of colonies 1 to 3 mm in diameter only needs 1 to 2 days; resistant to cycloheximide; rounded, smooth, and dark green on WL agar; acetone smell (not acetic); limited growth on lysine agar.

   Microscopic: Apiculate or ellipsoid; cells can resemble "lemons," "bowling pins," or "footballs;" can be mistaken for *Brettanomyces* due to similar size and shape; very often seen in juice and fermenting must.

4. *Dekkera/Brettanomyces*.

   Colony: Small (<1 to 3 mm) but visible in 3 to 7 days; Initially white and shiny but become olive or metallic green after a week; hemispherical and smooth; Resistant to >1000 ppm cycloheximide; acetic acid produced so colonies smell and agar color changes quickly to yellow; does not grow on lysine agar (usually).

   Microscopic: Polymorphic but can be ogive (olive-shaped), "bowling pin," or even almost ovoid; smaller than *Saccharomyces*; bud scars flatten one or both ends in older cells (cell looks like "rowboat," "barrel," or rectangle).

5. *Candida*, *Pichia* and other surface film yeasts.

   Colony: Grow in 1 to 3 days to a size of 2 to 5 mm; very few resistant to cycloheximide (>10 ppm); rounded or flat; varied surface that may resemble *Saccharomyces* or may be rough or wrinkly; colony edges may be smooth or irregular; white or light green on WL agar; grow well on lysine agar.

Microscopic: Shape is variable due to several genera/species in wine (polymorphic); range in sizes; round, ovoid, or elongate; often form clumps and branched chains.

B. Descriptions of bacteria

1. *Oenococcus*.

Colony: Pinpoint or small (1 to 2 mm) and never spreading; flat or slightly rounded; clear or white or tan on MR agar; clear or dark green on WL agar (if can grow); resistant to cycloheximide (>100 ppm); generally slow growing so require 5 to 10 days; no or limited odors.

Microscopic: Nearly round or lenticular but very small (0.5–0.7 x 0.7–1.2 μm); pairs and chains; occasional elongated cells within a chain; Gram positive; catalase-negative.

2. *Lactobacillus*.

Colony: Pinpoint, small, or larger colonies (1 to 5 mm) and never spreading; flat or slightly rounded; clear or white or tan on MR agar; clear or dark green on WL agar (if can grow); resistant to cycloheximide (>100 ppm); growth varies so may require 3 to 10 days; no or limited odors.

Microscopic: Rod-shaped (0.5 to 2 μm in width) but extremely variable in length with short rods (*L. brevis*) and very long rods (*L. fructivorans*) found in wines; single, in pairs, or chains; ends of cells are not tapered like *Acetobacter*; Gram positive; some species may be weakly catalase-positive.

3. *Pediococcus*.

Colony: Pinpoint or small (1 to 2 mm) and never spreading; flat or slightly rounded; clear or white or tan on MR agar; clear or dark green on WL agar (if can grow); can be a bit larger than *Oenococcus* and more pearly or shiny white; resistant to cycloheximide (>100 ppm); generally slow growing so require 5 to 10 days; no or limited odors.

Microscopic: Spherical and larger than *Oenococcus*; pairs, tetrads or even clumps (no single cells); cell diameters range 0.5 to 1 μm; Gram positive; generally catalase negative.

4. *Acetobacter*.

Colony: Colonies usually small (<1 to 2 mm) after 2 to 3 days growth; green or clear colonies on WL agar but agar can turn yellow due to acid production; tan or clear colonies on MR agar; may or may not produce acetic smell.

Microscopic: In culture, ellipsoidal to rod-shaped, straight or slightly curved; most cells very short (0.6–0.8 x 1–3 μm), some cells longer; swollen, snake-like or

club-shaped forms of variable width; Gram-negative when young but may be Gram variable in older cultures; catalase positive. In wine, cells are often quite short and resemble *Pediococcus* (do not normally see swollen, snake-like or club-shaped forms of variable width); may form chains that resemble *Oenococcus* but cells are larger; catalase positive. To perform a catalase test, add a few drops of 3% H_2O_2 to a culture growing on agar and watch for bubbles of O_2 (positive result).

Appendix III. Addition of Potassium Metabisulfite to Musts/Wines

Opened containers of potassium metabisulfite (KMS) should not be stored for lengthy periods of time (>1 year). If an older supply of the salt is being used, be sure to measure how much active SO_2 is actually added to the wine (measure 24 hours after addition).

Once the volume of must or wine is determined, carefully weigh out potassium metabisulfite, per the table below, and add. Be sure to mix the must/wine very well to equally disperse the salt.

Table for Addition of Potassium Metabisulfite

Desired Amount of Total SO_2 (mg/L)	Volume of Liquid (Wine or Must): 0.5 gal	1.0 gal	2.5 gal	3.0 gal	3.5 gal	4.0 gal	5.0 gal
	Quantity of Potassium Metabisulfite to add (g)						
5	0.02	0.04	0.09	0.11	0.12	0.14	0.18
10	0.04	0.07	0.18	0.21	0.25	0.28	0.35
20	0.07	0.14	0.35	0.42	0.49	0.56	0.70
30	0.11	0.21	0.53	0.63	0.74	0.84	1.05
40	0.14	0.28	0.70	0.84	0.98	1.12	1.40
50	0.18	0.35	0.88	1.05	1.23	1.40	1.75
60	0.21	0.42	1.05	1.26	1.47	1.68	2.10
70	0.25	0.49	1.23	1.47	1.72	1.96	2.45
80	0.28	0.56	1.41	1.68	1.97	2.24	2.80
90	0.32	0.63	1.59	1.89	2.23	2.52	3.15
100	0.35	0.70	1.77	2.10	2.48	2.80	3.50

Glossary

accuracy. For measurements, how closely the results relate to the actual physical reality in question.

aerobe. A microorganism whose growth requires the presence of air (oxygen). *Acetobacter* would be an example of an obligate ("must have") aerobe.

agar. This is a complex carbohydrate material refined from marine algae and used to produce semi-solid media (like Jello®). It solidifies at temperatures between 40° and 45°C and will not remelt until it is boiled. Any liquid medium can be solidified by the addition of 1 to 2% w/v agar.

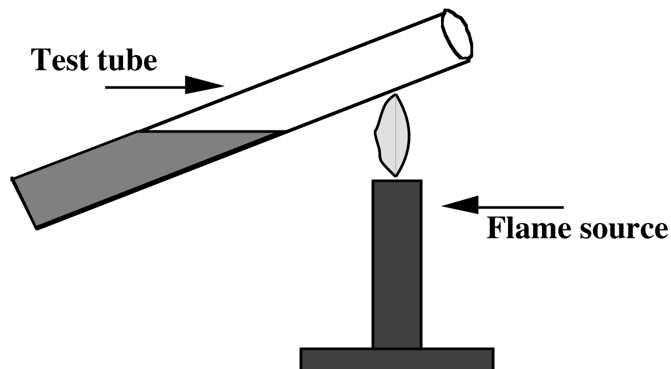
aliquot. A portion or sample taken for testing or analysis.

anaerobe. A microorganism that grows only or best in the absence of air (oxygen). Many lactic acid bacteria are considered to be facultative ("not required") anaerobes; these microorganisms grow well under anaerobic conditions but can also grow in the presence of some oxygen.

anaerobic jar. As most laboratories do not have anaerobic incubators (these are very expensive!), a good substitute is a large plastic jar that contains racks that hold disposable CO_2 generators. Alternatively, some winemakers rely on "candle jars" in which a candle is lit, and the jar sealed (the flame will remove the oxygen that is present before being extinguished).

aseptic technique. Any technique or procedure in which precautions against microbial contamination are taken. Once media or instruments are sterile, they are kept relatively free of microorganisms using this technique.

In the case of transferring into and out of flasks or test tubes containing sterile media, the neck of the flask or test tube is flamed (put into a flame). This creates a convection current of hot air, which forces air out of the flask/tube so that microorganisms in the surrounding air cannot enter. A Bunsen burner or an alcohol lamp is commonly used as a flame source.



Another means of minimizing contamination is through the use of laminar flow hoods. These special hoods maintain a sterile environ-

ment so that microbiological analyses can be easily performed, but they are expensive.

autoclave. This is a large pressure vessel capable of reaching temperatures in excess of 100°C (normally, 121°C) to sterilize media and instruments.

Brownian motion. Random movement and motion of microorganisms as viewed in a wet mount under a microscope.

colonies. When a viable cell is deposited on the surface of a solidified agar medium, it reproduces and forms a cluster (or colony) of cells, which are counted for enumeration. Thus, microbial populations in a sample are frequently reported as CFU/mL (colony-forming units per mL).

dilution blank. A sterilized solution consisting of 0.1% peptone water. These solutions are used to dilute samples that contain large numbers of viable microorganisms. Samples are normally serially diluted (1:10, 1:100, 1:1000, 1:10000, etc.) prior to plating using solidified agar.

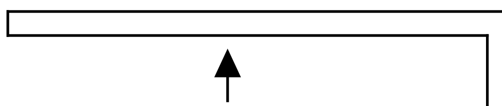
enzyme. A protein catalyst that causes changes in other molecules without undergoing any alterations itself. For instance, the enzyme catalase acts on hydrogen peroxide (H_2O_2) to break it down into water (H_2O) and oxygen (O_2).

equivalent weight (EW). The weight of a solution that contains 1 mole of replaceable hydrogen (acid) or hydroxyl (base).

gram formula weight (GFW). The weight of one mole of atoms or molecules. Also called the molecular weight (MW).

Gram stain. A differential staining procedure that classifies microorganisms as either Gram (+) or Gram (-) based on retention of a specific dye (crystal violet). Staining a microorganism on a microscope slide with a specific dye will allow for viewing the cells with a bright field microscope.

hockey stick. A glass rod bent into the shape of an "L" that is used to evenly spread samples onto solidified media.



Glass rod with "handle"

hydrometry. Measure of liquid densities, using the principles of buoyancy, through the use of a hydrometer.

inoculum. A small quantity of viable microorganisms introduced into a medium or juice with the goal of growing the microorganisms.

loop. A device, with a handle and a metal loop on the end, used to transfer microorganisms. It is also known as a "laboratory loop" or a "transfer loop" and is sterilized by holding the metal loop in an open flame.

medium. A material formulated using different ingredients to support the growth of different microorganisms. Such ingredients include glucose, peptone, yeast extract, liver extract, and others. A medium can be used as a liquid or solid, the latter requiring the addition of agar.

meniscus. The bottom of the curved upper surface of a liquid in a container.

methylene blue. A differential stain that can be added to a liquid suspension to determine which yeast cells are alive and which are dead. Live yeasts will reduce the dye to a colorless form, while dead cells appear blue/black, when viewed through a microscope.

molarity (M). The number of moles of solute per liter of solution.

molecular weight (MW). The weight of one mole of atoms; Also called the gram formula weight (GFW).

normality (N). The number of equivalents (see also **equivalent weight**) of solute per liter of solution. To calculate N, the weight of dissolved solute and its equivalent weight are required.

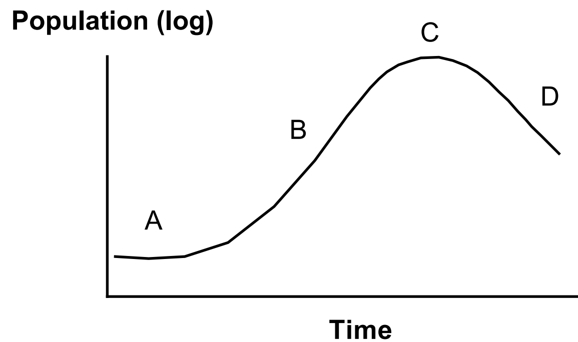
Petri dish. A sterilized glass or plastic dish with cover that is used to hold solidified microbial media.

pH. A measurement of hydrogen ion activity in a solution. A solution with a pH of 7 is neutral; solutions with pH lower than 7 are acidic; solutions with pH greater than 7 are basic (alkaline).

phase contrast microscope. Rather than staining a culture in order to visibly see the microorganisms using a bright field microscope, phase contrast microscopes allow direct viewing of microbes in liquid cultures. Here, light passing through a denser medium (a microbial cell) than another medium (the liquid) will be retarded. Phase contrast enhances differences in refractive index between these two media (a cell and the surrounding liquid).

pour plate. A plate (such as a Petri dish) into which a liquid sample to be microbiologically enumerated is aseptically transferred, with cooled (yet still liquid) medium then added. The medium and the liquid sample must be well mixed prior to allowing solidification of the medium (typically agar gel).

phase (growth). Microbial growth in juice, wine, or a medium has four distinctive phases: (A) lag, (B) logarithmic, (C) stationary, and (D) death.



During lag phase (A), cells are adjusting to the new environment and they increase in size (with no increase in cell numbers). Logarithmic phase (B) is the period when cell numbers rapidly increase. At some point, the growth rate decreases and the cells enter stationary phase (C) in which growth and death rates are approximately equal. The accumulation of toxic wastes and the decreasing availability of nutrients eventually result in death of the cells (D).

plates. Petri dishes used to contain solidified microbiological media for enumeration or isolation of microbes.

precision. The degree of reproducibility of a measurement.

reducing sugars. Sugars such as glucose and fructose that have a free carbonyl function (C=O) and will therefore react with Fehling's reagent.

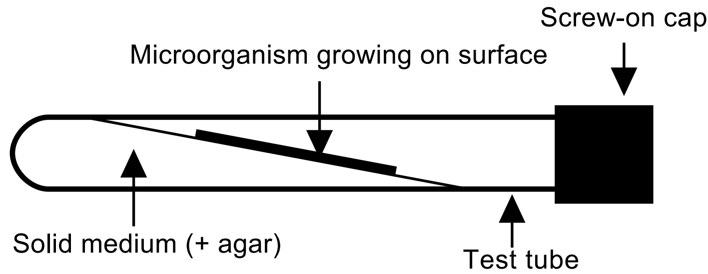
refractive index. Describes how light is bent (refracted) as it moves through a medium such as glass or a liquid.

reticle. A collection of fine lines that appears through the eyepiece of a microscope or other viewer.

sanitation. The term refers to reducing microbial populations in the winery using physical (e.g., heat) or chemical means. In contrast, "sterilization" implies destruction of *all* microorganisms.

selective agent. A chemical added to a medium so that undesirable microorganisms will not grow while desirable microbes will grow. Cycloheximide is one of the more important selective agents used in wine microbiology.

slant. Liquefied media containing agar is aseptically placed into a test tube. The test tube is placed at a slight angle on a tabletop while the agar media cools and sets. Slants are used to aseptically store yeast and bacteria cultures for longer periods of time than with Petri dishes.



spread plate. Culture dishes in which a microbiological sample is aseptically added onto the surface of media already solidified (gel formed) in Petri dishes. The sample, normally 0.1 mL, is evenly spread on the surface of the media by using a "hockey stick" glass rod.

supernatant. Solution above a precipitate or pellet at the bottom of a container.

titratable acidity. The quantity of all acids in a must or wine sample that can be titrated with a strong base (e.g., NaOH) to an end point of pH 8.2.

volatile acidity. The concentration of acetic acid in a wine as measured using a Cash steam distillation unit (still).

References

Amerine, M.A. and M.A. Joslyn. 1970. *Table Wines.* 2nd ed. Berkeley, CA: University of California Press. ISBN 0-520-01657-2.

Amerine, M.A., and E.B. Roessler. 1983. *Wines - Their Sensory Evaluation.* San Francisco: W. H. Freeman and Co.

Boulton, R.B., V.L. Singleton, L.F. Bisson, and R.E. Kunkee. 1996. *Principles and Practices of Winemaking*. New York: The Chapman and Hall Enology Library. ISBN 0-412-06411-1.

Buechsenstein, J.W. and C.S. Ough. 1978. SO_2 Determination by Aeration-Oxidation: A Comparison with Ripper. *Am. J. Enol. Vitic*. 29: 161–164.

Dharmadhikari, M.R. and K.L. Wilker. 2001. *Micro Vinification A Practical Guide to Small-Scale Wine Production*. Mountain Grove, MO: Missouri State Fruit Experiment Station. ISBN 0-9707971-0-9.

Difco Laboratories. 1984. *Difco Manual: Dehydrated Culture Media and Reagents for Microbiology.* 10th ed. Detroit, MI: Difco Laboratories.

Dukes, B.C. and C.E. Butzke. 1998. Rapid Determination of Primary Amino Acids in Grape Juice Using an o-phthaldialdehyde/N-acetyl-L-cysteine Spectrophotometric Assay. *Am. J. Enol. Vitic*. 49(2): 125–134.

Edwards, C.G. 2005. *Illustrated Guide to Microbes and Sediments in Wine, Beer, and Juice*. Pullman, WA: WineBugs LLC. ISBN 978-0-977-25220-6.

Fugelsang, K.C. and C.G. Edwards. 2007. *Wine Microbiology: Practical Applications and Procedures*. 2nd ed. New York: Springer Science and Business Media. ISBN 978-0-387-33349-6.

Iland, P., A. Ewart, and J. Sitters. 1993. *Techniques for Chemical Analysis and Stability Tests of Grape Juice and Wine*. Campbelltown, South Australia: P. Iland Wine Promotions. ISBN 0-646-13102-8.

King, S.W. and R.B. Beelman. 1986. Metabolic Interactions Between *Saccharomyces cerevisiae* and *Leuconostoc oenos* in a Model Grape Juice/Wine System. *Am. J. Enol. Vitic*. 37: 53–60.

Love, R.F. 1939. A Table for Ebulliometers for Use with Alcoholic Liquids Containing Solid Matter. *Ind. Eng. Chem. Anal. Ed*. 11: 548–550.

Ough, C.S. and M.A. Amerine. 1988. *Methods for Analysis of Musts and Wines*. 2nd ed. New York: John Wiley and Sons. ISBN 0-471-62757-7.

Pallman, C.L., J.A. Brown, T.L. Olineka, L. Cocolin, D.A. Mills, and L.F. Bisson. 2001. Use of WL Medium to Profile Native Flora Fermentations. *Am. J. Enol. Vitic*. 52: 198–203.

Sakata, K. 1986. Methods for the Detection of Protein Instability. In: *Proceedings of the 1986 Pacific Northwest Winemaking Seminar.* Edited by S.E. Spayd and J. Watson. Prosser, WA: Washington State University.

Skoog, D.A. and D.M. West. 1980. *Analytical Chemistry.* Philadelphia, PA: Saunders College Publishing.

Somers, T.C. and M.E. Evans. 1977. Spectral Evaluation of Young Red Wines: Anthocyanin Equilibria, Total Phenolics, Free and Molecular SO_2, "Chemical Age." *J. Sci. Food Agric.* 28: 279–287.

Vahl, J.M. and J.E. Converse. 1980. Ripper Procedure for Determining Sulfur Dioxide in Wine: Collaborative Study. *J. Assoc. Off. Anal. Chem.* 63: 194–199.

Vilas, M. 1993. Bottling Line Sampling and Diagnostic Techniques. *Vineyard and Winery Management.* Sept/Oct. 33–35.

Vine, R.P. 2002. *Winemaking: From Grape Growing to Marketplace.* Springer Science+Business Media, LLC, NY. ISBN 0-306-47272-4.

Zoecklein, B.W., K.C. Fugelsang, B.H. Gump, and F.S. Nury. 1995. *Wine Analysis and Production.* New York: Chapman and Hall. ISBN 0-412-989210-2.

The Authors

Charles G. Edwards was raised in the San Joaquin Valley of California where he worked on his family's vineyard near Acampo. He received his Bachelor of Science, Master of Science, and Doctor of Philosophy degrees in food science from Oregon State University (1982), Cornell University (1985), and The Pennsylvania State University (1989), respectively. Currently, he is a professor (enology) with the Washington State University/University of Idaho School of Food Science. His enological research focuses on problem alcoholic fermentations, the malolactic fermentation, and spoilage issues such as *Brettanomyces* and *Lactobacillus*. As part of the on-going research effort, his laboratory discovered two novel species of *Lactobacillus* implicated in wine spoilage, *Lactobacillus kunkeei* and *Lactobacillus nagelii*. Dr. Edwards is the sole author of *Illustrated Guide to Microbes and Sediments in Wine, Beer, and Juice* and co-author of *Wine Microbiology* (2nd edition), the latter of which won top honors from L'Organisation Internationale de la Vigne et du Vin (O.I.V.) for the best written work in enology in 2007. Besides research, Dr. Edwards has taught and continues to teach undergraduate and graduate courses in food chemistry, wine microbiology/processing, and winery internships as well as the WSU Extension enology certificate. He completed a year-long sabbatical leave in 2001–2002 working at Chr Hansen (Hørsholm, Denmark) and won the Excellence in Research award from the College of Agriculture, Human, and Natural Resources in 2006.

Bruce A. Watson grew up in Eugene, Oregon, and from a very early age knew that he wanted to become a chemist, but didn't discover wine until graduate school. He received a Bachelor of Science in chemistry from the University of Oregon and a Doctor of Philosophy in biochemistry from the University of Washington (1975). After working in retail wine sales for a short period, Dr. Watson returned to science as a postdoctoral fellow in the Department of Botany at the University of Washington, studying a variety of organisms and problems. In 1986 and 1987, he worked during the harvest period at Columbia Winery (Woodinville, WA) and left academic research in 1988 to head up the quality management program at the winery. In 2001, Constellation Brands acquired Columbia, Covey Run (Zillah, WA) and Ste. Chapelle (Caldwell, ID) wineries and he oversaw the quality management program for the northwest operations of the company. In that role, he traveled numerous times to Australia to select wine for the Alice White brand and worked with the extensive quality management group of Constellation Brands in the USA. Although retired in 2008, Dr. Watson continues to consult for Washington wineries while teaching wine chemistry and microbiology at South Seattle Community College (Seattle, WA).

Copyright 2013 Washington State University

WSU Extension bulletins contain material written and produced for public distribution. Alternate formats of our educational materials are available upon request for persons with disabilities. Please contact Washington State University Extension for more information.

You may download copies of this and other publications from WSU Extension at http://pubs.wsu.edu.

Issued by Washington State University Extension and the U.S. Department of Agriculture in furtherance of the Acts of May 8 and June 30, 1914. Extension programs and policies are consistent with federal and state laws and regulations on nondiscrimination regarding race, sex, religion, age, color, creed, and national or ethnic origin; physical, mental, or sensory disability; marital status or sexual orientation; and status as a Vietnam-era or disabled veteran. Evidence of noncompliance may be reported through your local WSU Extension office. Trade names have been used to simplify information; no endorsement is intended. Published January 2013. *(This publication replaces MISC0146.)*

EM047