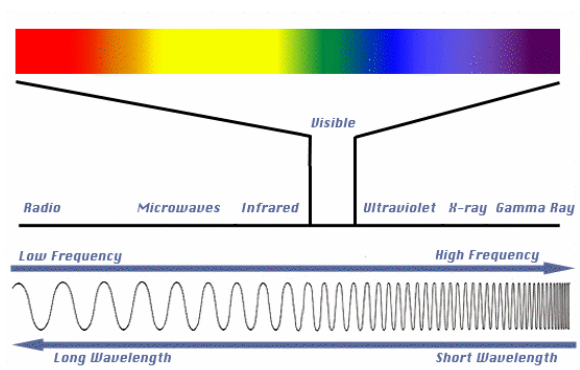
Chapter 11 Spectrophotometry

Spectrophotometry is a process using electromagnetic radiation (light) to measure an unknown analyte concentration. The spectrophotometry described in this section is commonly called UV-Vis Spectrophotometry since wavelengths of light in the visible spectrum are used to determine concentration. Light is described as a photon of energy. The amount of energy in each photon is determined by its wavelength. The shorter the wavelength the more energy the photon has. In UV-Vis Spectrophotometry, wavelength between 400-700 nm are generally used to determine the concentration of samples. Wavelengths shorter than 400 nm fall into the ultraviolet and x-ray range, while wavelengths longer than 700 nm fall into the infrared region.

Visible Spectrum

The visible spectrum is composed of the colors we see (the rainbow) ranging from red to violet. A typical incandescent light bulb puts out white light which is the result of all the visible colors being combined. By using a prism, this white light from the light bulb can be separated into individual wavelengths.



In spectrophotometry, chemical reactions are created with the analyte of interest. These reactions create a product that has a color. For example:

Iron + phenanthroline → orange color

The orange color is the result of the molecule **absorbing** photons of energy. The more molecules present, the darker the orange color. A spectrophotometer can be used to measure the intensity of the color. The spectrophotometer operates off the Beer-Lambert Law.

Beer-Lambert Law

The Beer-Lambert Law states that the absorbance of light is proportional to the color intensity. It also states that the intensity of the color is proportional to its concentration. In other words, the darker the color, the more concentrated the solution.

Absorbance = a b Concentration

Figure: This solution contains a small amount of iron

Figure: This solution contains a large amount of iron

The intensity of the orange color must be measured at a specific wavelength. This wavelength is determined by the method used. In this example, the wavelength selected is 510 nm. This wavelength was selected because it

had the highest sensitivity for iron. Each color will have its own special wavelength. For example, COD uses 620 nm, nitrate uses 400 nm, and phosphate uses 880 nm.

Spectrophotometer Components

The spectrophotometer is a light detecting instrument. It is composed of 6 major components.

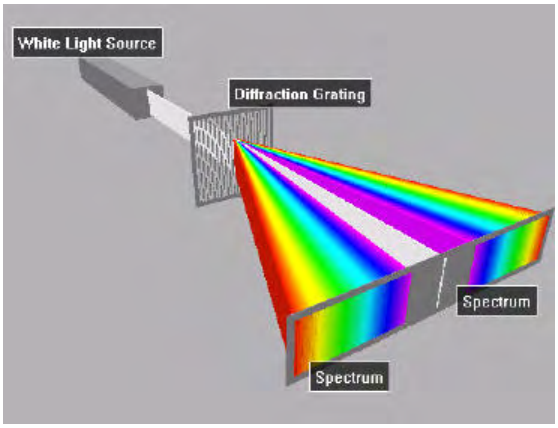
1. White light source
2. Diffraction Grating
3. Monochrometer
4. Exit slit
5. Sample cell holder
6. Light detector

White Light Source

This is usually a tungsten lamp that produces a constant white light output. The bulb is fairly cheap and can be run continuously. Remember that white light represents all the different colors.

Diffraction Grating

The diffraction grating is a special plate with hundreds of parallel grooved lines. The grooved lines act to separate the white light into the visible light spectrum (rainbow). The more lines the smaller the wavelength resolution. The more lines the more it costs.



Monochrometer

Once the light has been diffracted, a digital or mechanical knob is used to turn the diffraction grating and aim the desired wavelength at the exit slit.

Exit slit

The exit slit is a small hole that allows only a small amount of the diffracted light to leave the instrument. The smaller the exit slit, the more expensive the spectrophotometer. Common exit slits might be 2 - 20 nm. The exit slit dimension is often called the band pass. If the band pass is 20 nm, 20 nm of light is passing through the slit. If the spectrophotometer diffraction grating is set at 510 nm, then a spectrophotometer with a bandpass of 20 would be allowing wavelengths of light between 500 - 520 nm through the exit slit.

Sample Cell Holder

The 510 nm light passing through the exit slit now passes into the sample compartment. The sample compartment holds the sample cell or cuvette.

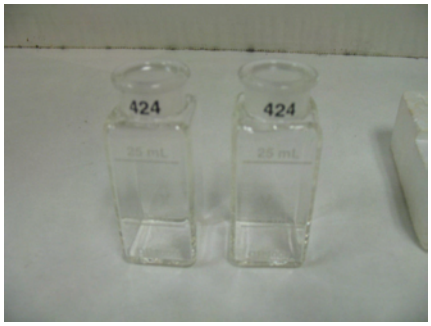


Figure: Matched Sample Cells

The sample cell holds the colored solution. The light is directed through the cuvette. The light energy either reacts with the colored molecules and is absorbed or it passes through the solution and is transmitted. The transmitted light is now sent to the detector. Sample cells should be "matched". Matched sample cells have the same optical properties and produce the same absorbance answer when a cobalt chloride solution is added to the

sample cell. The lab should have a minimum of 2 matched sample cells. One cell is for the reagent blank and the second is for all standards and samples.

Light Detector

The transmitted light hits a photomultiplier tube which converts the light energy into electrical energy. The electrical energy causes the electronics within the spectrophotometer to display an answer on the instrument. Unfortunately, the instrument does not know the correct answer because like the pH meter, it has not been standardized. Newer spectrophotometers may have computer programs built in which allow for immediate answer determination. These machines require the computer program to be validated.

Stock Preparation

Standards are used to calibrate the spectrophotometer. Standards are usually prepared from a stock solution purchased from reputable chemical supply houses. They commonly come as 1000 mg/L or are sometimes labeled in titer form as 1 ml = 1 mg. If the bottle only indicates the titer concentration, convert to mg/L by multiplying by 1000. For example, if the titer is 1 ml = 0.2 mg, then the concentration is 200 mg/L

$$\frac{0.2 \text{ mg}}{1 \text{ ml}} \times \frac{1000 \text{ ml}}{1 \text{ liter}} = 200 \text{ mg/L}$$

The stock solution is usually stable for a year. Discard any stock solution that changes color, shows growth or precipitation.

Intermediate Standard Preparation

The stock solution is normally too strong for most spectrophotometric analyses and must be diluted. This diluted stock solution is called an intermediate standard. The intermediate standard has a limited shelf life, usually no longer than 1 month. The intermediate standard is diluted to make working standards which are then used to calibrate the spectrophotometer. It is common practice to make at least 8 working standards to calibrate the spectrophotometer. For example, lets assume the phosphate procedure calls for working standards between 0 - 20 mg/L. The stock phosphate solution is 1000 mg/L. All standards should be prepared using volumetric pipets and volumetric flasks. Since the smallest volumetric pipet is 1.0 ml, pipetting 1.0 ml into a 100 ml volumetric flask will give a working standard of 10 mg/L.

$$1000 \text{ mg/L stock} \times \frac{1.0 \text{ ml}}{100 \text{ ml}} = 10 \text{ mg/L WS}$$

There is a dilemma here since working standards below 10 mg/L are needed but there are no volumetric pipets smaller than 1.0 ml. To avoid this dilemma, the intermediate standard is prepared. The concentration of the intermediate standard can be prepared using the following formula.

$$C_{stock} \times V_{stock} = C_{int.\ std} \times V_{int\ std.}$$

Where:

C_{stock} = stock concentration in mg/L
V_{stock} = volume of stock used
$C_{int.\ std}$ = concentration of intermediate standard
$V_{int\ std.}$ = volume of intermediate standard needed

In this example, lets prepare 250 ml of a 100 mg/L intermediate standard. Calculate how many milliliters of the stock solution will be needed.

$$C_{stock} \times V_{stock} = C_{int.\ std} \times V_{int\ std.}$$

$$1000 \text{ mg/L} \times V_{stock} = 100 \text{ mg/L} \times 250 \text{ ml}$$

$$V_{stock} = \frac{100 \text{ mg/L} \times 250 \text{ ml}}{1000 \text{ mg/L}} = 25.0 \text{ ml}$$

Prepare the intermediate standard by using a 25.0 ml volumetric pipet and a 250 ml volumetric flask. Pipet the stock solution into the flask, then fill to the mark with DI water and mix by inverting several times.

Working Standard Preparation

Working standards are the standards that will be used to create the colors needed to calibrate the spectrophotometer. Since 8 working standards are needed to produce a standard curve, the same formula can be used to calculate the volume of intermediate standard needed to prepare the working standards.

$C_{int.\ std} \times V_{int\ std} =. C_{work\ std} \times V_{work\ std}$

$C_{int.\ std}$ = intermediate standard concentration in mg/L

$V_{int\ std}$ = volume of intermediate standard used

$C_{work\ std}$ = concentration of working standard to be prepared

$V_{work\ std.}$ = volume of working standard needed

In this example, lets prepare 100 ml of a 1.0 mg/L phosphate working standard.

$C_{int.\ std} \times V_{int\ std} =. C_{work\ std} \times V_{work\ std}$

100 mg/L x X ml = 1.0 mg/L x 100 ml

$$X\ ml = \frac{1.0\ mg/L \times 100\ ml}{100\ mg/L}$$

X ml = 1.0 ml

Pipetting 1.0 ml using a volumetric pipet into a 100 ml volumetric flask will produce a 1.0 mg/L working standard which can now be used to calibrate the spectrophotometer.

Volume Inter. Std	Conc.	Absorbance
0 ml	0	
1.0 ml	1.0 mg/L	
3.0 ml	3.0 mg/L	
5.0 ml	5.0 mg/L	
8.0 ml	8.0 mg/L	
10.0 ml	10.0 mg/L	
15.0 ml	15.0 mg/L	
20.0 ml	20.0 mg/L	

The remainder of the chart can be filled in using volumetric pipets available in the lab. .

Reagent Blank

Notice the table has a "0" standard. This standard is call a reagent blank and will be used to zero the absorbance on the spectrophotometer. The reagent blank is prepared by taking deionized water through the same chemical reactions as the rest of the standards. A reagent blank has the advantage over a deionized water blank because any contaminants in the chemical reagents are ignored. For instance, if the deionized water has some phosphate contaminant, a background color will be produced in the reagent blank because the phosphate reagents will react to produce a color. This background color would be present in all the standards because they were all prepared using the same deionized water. If the reagent blank is used to zero the spectrophotometer, the background color will be subtracted out automatically from all the remaining standards.

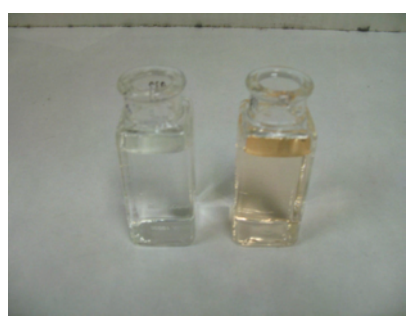


Figure: DI water blank vs. Reagent blank

Matched cuvettes are sample cells that have the same optical properties. Absorbance is a logarithmic function and small errors are magnified tremendously on the absorbance scale. It is important to handle cuvettes carefully to avoid errors which interfere with the absorbance reading. At low absorbance, contaminants such as dust, dirt, fingerprints, scratches, and bubbles interfere with the passage of light to the detector and will potentially cause significant false positive errors in the absorbance reading. The cuvette glass properties and cell positioning can also affect absorbance readings. Differences in glass such as scratches, thickness, and composition, also interfere with light passing through the same point on the cuvette. It is very important that the cuvette be lined up facing the same direction each time. Cleaning with detergent and deionized water should be completed as soon as possible to avoid having solids dry on the sides of the cuvette. Be careful using old brushes with wire to clean the sample cells. As the bristles from the brush wear, the exposed metal will scratch the sides of the sample cell.

Standard Curve Preparation

To prepare the calibration curve, the working standards are all taken through the chemical procedure and a colored solution is formed. The colored solution should increase in intensity as the concentration increases.

Figure: Cap and fill the volumetric flask, mix by inverting several times.

Figure: Be sure to label each flask with its concentration.

Now that the standards have been prepared following the test procedure, the lab technician should notice an increasing color intensity as the concentration of the analyte increases. This color needs to be measured by the spectrophotometer.

Step 1: Warm up the Lamp
Allow the spectrophotometer lamp to warm up for a sufficient time to eliminate electronic drift. This usually takes 10-20 minutes.

Step 2: Set the wavelength
Select and adjust the spectrophotometer to the desired wavelength. Older spectrophotometers have a knob to manually adjust the wavelength to the correct wavelength. The knob on the right in the picture below is the wavelength adjustment knob. Turn the knob slowly to avoid damaging the diffraction grating mechanism.

The wavelength on newer spectrophotometers is digitally selected.

Step 3: Zero the Spectrophotometer
Transfer the reagent blank to a matched sample cell.

Wipe the sample cell with Kimwipes® (non-scratching) to remove any water spots or fingerprints. It's important that sample cells be clean.

Place the reagent blank in the sample cell holder. Sample cells can be handled at the top without creating an error. The light generally passes through the lower half of the sample cell.

Close the cover to prevent outside light from entering. Press the absorbance button to select the absorbance mode, and then press the zero button to zero the absorbance. At this point the spectrophotometer is seeing the highest amount of light reaching its detector. In other words, no light is being absorbed because there is no color in the reagent blank.

Record the absorbance on the data sheet.

Step 4: Standard Curve Preparation
Using the second matched sample cell, transfer the lowest standard into the sample cell and wipe with a Kimwipe® to remove fingerprints.

Place the sample cell into the sample cell holder as before, close the door and read the absorbance displayed on the spectrophotometer. Record the absorbance on the data sheet.

Continue the same procedure using the same sample cell for the remaining working standards. The sample cell should be rinsed 3x using small volumes of each successive standard. Record the absorbance for each standard.

At this point, the absorbance of the 8 working standards should be recorded on the data sheet.

Volume Inter. Std	Conc.	Absorbance
0 ml	0	0.000
1.0 ml	1.0 mg/L	0.053
3.0 ml	3.0 mg/L	0.112
5.0 ml	5.0 mg/L	0.295
8.0 ml	8.0 mg/L	0.423
10.0 ml	10.0 mg/L	0.582
15.0 ml	15.0 mg/L	0.854
20.0 ml	20.0 mg/L	1.122

Step 5: Calibration curve
Now that the absorbencies for the working standards have been determined, a calibration curve must be prepared. Most calibration curves can be plotted on regular Cartesian graph paper. Beer's Law states that absorbance is proportional to concentration, so the y-axis will be labeled **"absorbance"** and the x-axis is labeled "**Concentration**". The scales should be divided up so as to include the highest absorbance and the highest concentration. The units of each scale do not need to be the same but they do need to be proportional. For instance if 1 block is 0.5 mg/L then the second block must be 0.5 mg/L higher (1.0 mg/L). Use as much of the graph paper as possible. Once all the points are plotted, the graph should fall in a reasonably straight line. Use a ruler to draw a line of best fit through the points. If the points do not fall on the line, an acceptable result is a line with an equal number of points above and below the line. Do NOT connect the dots. It is not permissible to extrapolate past the last point 20.0 mg/L (highest working standard) since you have no guarantee that the absorbance continues to be linear.

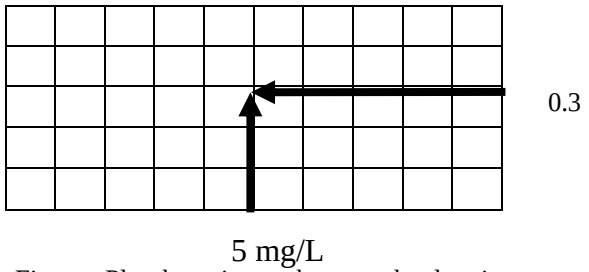
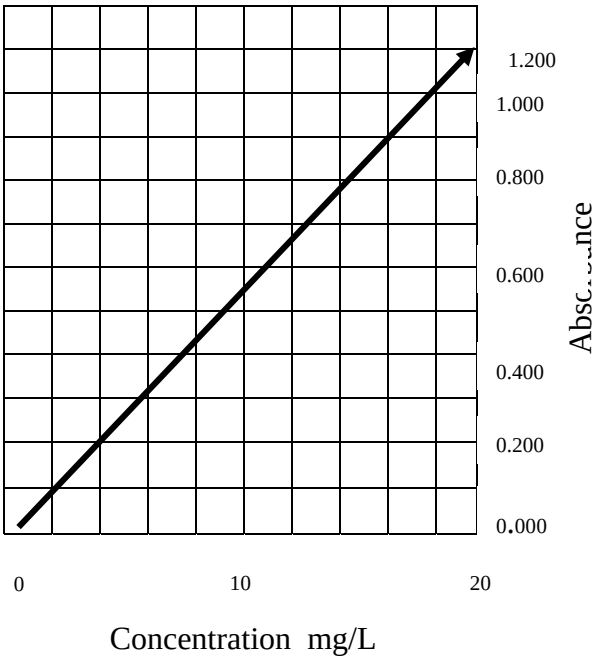


Figure: Plot the point on the curve by drawing a vertical line using the concentration data. Draw a horizontal line using the absorbance data. Make a point where the lines intersect. Repeat for all the remaining standards.

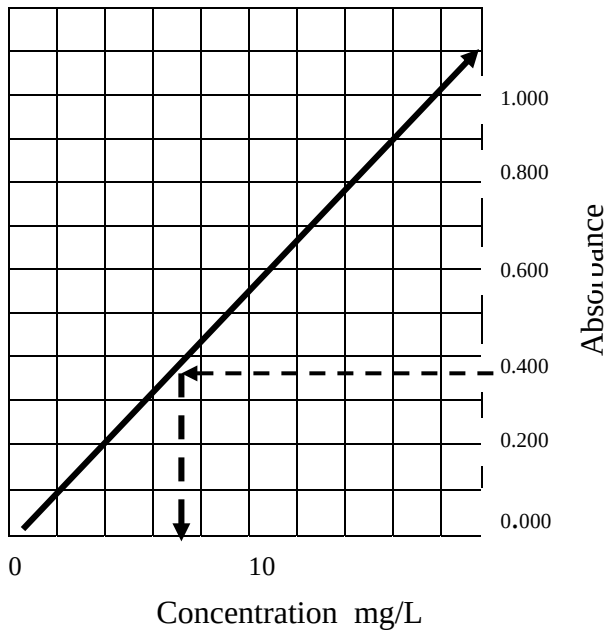


Draw the best fit straight line through all the points. This calibration curve will remain valid as long as the reagents that prepared it are not out of date. A high and low working standard should be run periodically to verify the curve has not changed.

Sample Measurement

Now that the calibration curve is prepared, numerous samples can be determined over several months. In this example, if the phosphate level in the effluent is to be measured, the sample will be taken through the test procedure to produce a color. The sample will be poured into the sample cell and the absorbance measured. The sample absorbance must fall within the calibration curve range to be able to determine the sample concentration.

Example 1:
The sample has an absorbance of 0.363. To determine the sample concentration, find 0.363 on the absorbance scale and draw a horizontal line until the line intersects the calibration curve. Drop a vertical line and read the concentration directly off the scale.

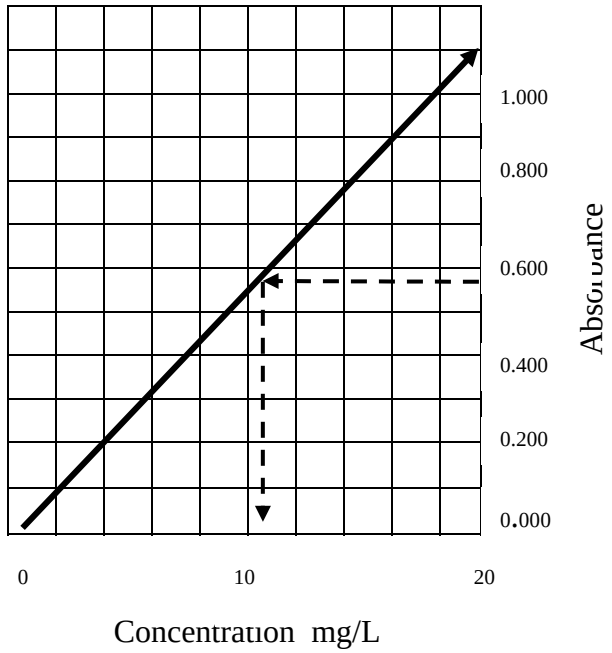


The sample concentration is 7.1 mg/L.

Example 2:
An influent sample is tested for phosphate. The absorbance is 1.334. Using the calibration curve, determine the sample concentration.

Notice the sample absorbance goes beyond the 20 mg/L standard absorbance. The sample is invalid and must be rerun using a diluted sample. In this example, the sample absorbance is just out of range, so a 1:2 dilution will most likely give a sample absorbance that will "hit" the middle of the calibration curve.

To prepare the 1:2 dilution, 50 ml of sample can be mixed with 50 ml of deionized water, then the diluted sample is tested for phosphate. The diluted sample absorbance is now 0.565. What is the phosphate concentration of the sample?



The diluted sample concentration is about 10.8 mg/L. Because the sample was diluted by 2, the sample concentration must be multiplied by 2 to get the sample concentration of 21.6 mg/L

Example 3
The sample absorbance is 0.011. The absorbance is so low, it is difficult to read the answer off the standard curve. Because you can't measure something that isn't there, the answer is reported as "less than" the detection limit. The detection limit can be the limit the manufacturer suggests or the limit the technician is confident of. In this case, the detection limit might be 0.2 mg/L, so the answer would be <0.2 mg/L or <mdl (minimum detection limit.)

Computer Generated Calibration Curves

Newer spectrophotometers have computer software which has the standard calibration curves built in for many procedures. A reagent blank still must be prepared to zero the spectrophotometer.

Figure: Place the reagent blank in the sample holder

Figure: Close the lid to keep out background light.

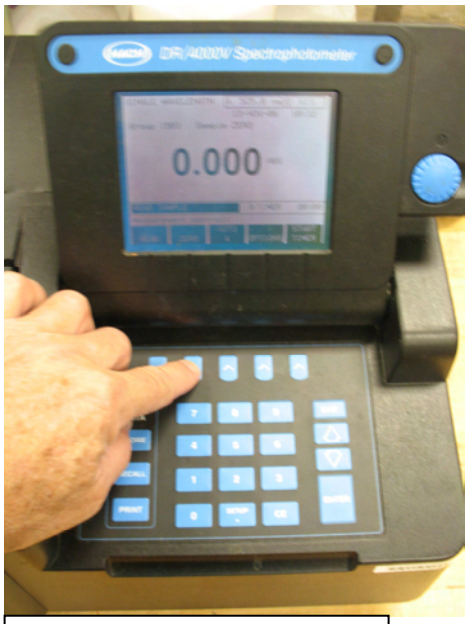


Figure: Press the zero button

The spectrophotometer is now calibrated using the manufacturer's calibration curve. The manufacturer's curve should be validated by running a technician prepared standard to verify the concentration. It is best to use a high and low standard to confirm that the technician's methodology will produce the same answer as the manufacturer's methodology.

The sample concentration can be determined directly from the spectrophotometer if using the manufacturer's calibration curve.

Figure: Place the sample into the sample cell holder, close the lid.

Figure: Press the read button to measure the concentration directly from the spectrophotometer.

Troubleshooting the Spectrophotometer

There are few problems with the spectrophotometer. The most common errors are usually due to sloppy technique. Failure to wipe sample cells, scratched sample cells, and improperly rinsed sample cells are common mistakes.

The other error associated with spectrophotometry revolves around interfering ions. Other compounds that will react with the reagents to produce an additional color are called interferences. A compound that makes the absorbance higher is called a false positive or positive interference while a compound that makes the absorbance lower is called a false negative or a negative interference.

Spectrophotometry Quality Control

- ☐ Method used is documented
- ☐ Make and model of spectrophotometer is documented.
- ☐ Reagent blank is used to zero the spectrophotometer.
- ☐ Standard calibration curve is prepared using a minimum of 8 working standards. Absorbances are documented.
- ☐ Record the purchase date, lot number, expiration date of stock solution.
- ☐ Wavelength used is documented.
- ☐ Calibration curve is periodically validated using a high and low working standard. Ideally, this should be performed with each run.
- ☐ Sample cells are free of dirt, scratches, fingerprints. Discard sample cells that are scratched, chipped, cracked.