Spectrophotometry Theory and Practice

Introduction

Spectrophotometry is an analytical technique used for measuring the concentration of an analyte dissolved in a solution. The measurement of the intensity of light transmitted by a solution may frequently be used for the quantitative determination of a substance dissolved in the solution, if the substance is itself colored (absorbs light) or can be converted to a colored compound. Such a method of analysis is known as a colorimetric method or a spectrophotometric method. The two terms are generally used interchangeably, although the earlier term colorimetry referred to methods using visible light and matching by eye. Spectrophotometry is now more generally used because analyses can be made using ultraviolet and infrared light as well as visible light and because measurements of intensity are now more commonly made using electronic detectors. Visible color is not essential for quantitative analysis, but the analyte must absorb some portion of the electromagnetic radiation passing through it, whether from the ultraviolet, visible, or infrared portion of the spectrum. Spectrophotometric methods of analysis are invariably comparison methods, where the unknown is compared to a standard of the same analyte under identical conditions of chemistry and lighting.

The physical laws describing the absorption of light on passage through a transparent material are relatively simple and may be applied to determining the amount of some absorbing analyte dissolved in a transparent solvent. The nature of light as electromagnetic radiation will be discussed as background for an understanding of how a spectrophotometer works and the principles of wavelength selection for chemical analysis.

Electromagnetic Radiation

Electromagnetic radiation (EMR) is a form of energy that may be treated as discrete particles known as photons, or in some instances it is more useful to use a wave theory of energy. The particle and wave descriptions are related to each other by the equation:

$$E = h\nu$$

where E is the energy of one photon of EMR energy, in joules; ν is the frequency of the wave, i.e., the number of repeating units (wavelengths) of the wave which pass a fixed point each second; and h is Planck's constant (6.625×10^{-34} joule-second). The energy can also be determined from the wavelength (λ) of the radiation. The wavelength of any wave is the length of one repeating unit of the wave. The wavelength of EMR is related to the frequency by the equation:

$$\lambda\nu = v$$

where **v** is the velocity of the EMR in a particular medium through which the radiation is traveling. The velocity in a particular medium can be calculated from the definition of the refractive index (η) of the medium.

$$\eta = c/v$$

where **c** is the velocity of EMR in a vacuum (3.0x108 meters/second). By combining equations, the relationship between frequency and wavelength is shown to be inversely proportional.

$$\lambda\nu = c/\eta$$

The energy of the EMR is related to the wavelength in the equation:

$$E = hc/(\lambda\eta)$$

In spectrophotometry it is common to use the wavelength to describe the radiation. The unit of measure for wavelength is nanometers (nm) which is $1x10^{-9}$ meters. In some literature the equivalent unit, millimicron (mμ) is used. See Figure 1.

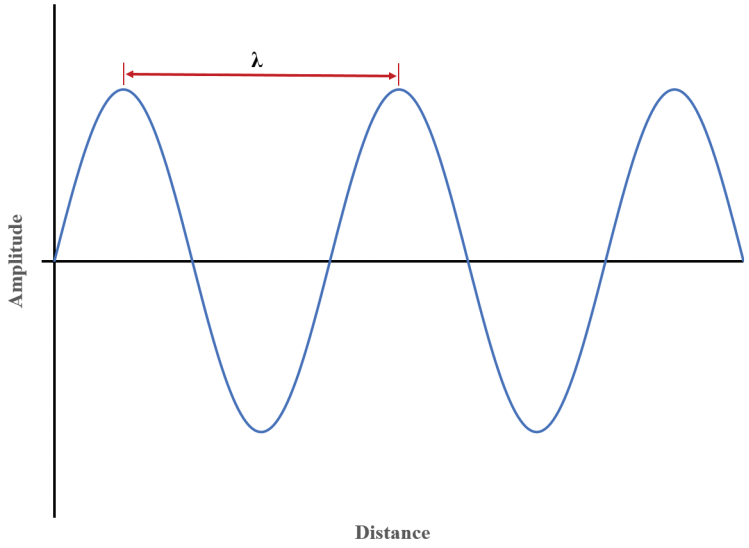


Figure 1 – EMR as a wave

The EMR spectrum is divided into several energy regions. The typical categories and associated wavelength ranges are shown in the following table.

EMR Region	Wavelength
X-Ray	0.01-10 nm
Vacuum Ultraviolet	10-180 nm
Near Ultraviolet	180-390 nm
Visible Figure 2	390-780 nm
Near Infrared	780-2500 nm
Fundamental Infrared	2.5-25 μm
Far Infrared	25-300 μm
Microwave	0.3 mm-500 mm
Radiowave	0.5-300 m

Although a large range of the spectrum is useful for chemical analysis, most quantitative analysis is performed by using radiation in the ultraviolet-visible region. In this region the radiation energy causes shifts in the electron energy levels of molecules and polyatomic ions. These shifts are called electronic transitions and result in absorption of radiation of a particular energy or wavelength. This absorption at specific wavelengths allows for quantitative analysis using ultraviolet or visible light in a spectrophotometer.

Absorption and Wavelength

Colored materials are colored because they absorb light in certain regions of the visible spectrum and transmit light in other regions. The regions of absorption are called absorption bands which may vary in width from just a few nanometers to over 300 nm. At the maximum point in the absorption band the material exhibits the largest change in absorbance with changes in concentration. This point is often chosen as the preferred wavelength for conducting the spectrophotometric analysis because it gives the maximum sensitivity, and most materials obey Beer's Law in this wavelength region.

The absorption bands are found by examining the light-absorbing profile of the material. A scan of absorbance versus wavelength for the material across the entire wavelength range of the spectrophotometer will reveal where absorption occurs and to what extent. Other factors may also be considered when selecting the wavelength, such as minimizing the absorption due to the reagent in the absence of the analyte or avoiding absorption from interferences that are likely to be present in the sample. The wavelength that is finally selected should be centered on the peak of the absorption band. See Figure 2.

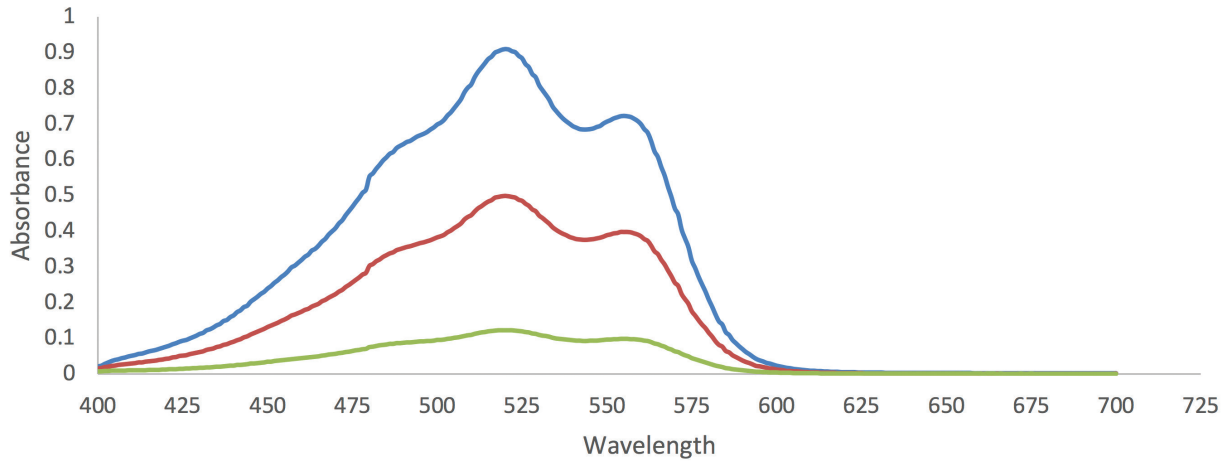


Figure 2 – Wavelength scans of increasing concentrations of a Würster dye

Beer-Lambert Law

The decrease in intensity of light on passage through a homogeneous material (solid, liquid, or gas) depends on the thickness of the material. For a transparent liquid containing a dissolved substance which absorbs light, that is a colored substance, the decrease in intensity depends also on the concentration of the absorbing substance. The absorption of light on passage through a transmitting material is exponential in character and is described by two laws: Lambert's Law dealing with the thickness, and Beers Law dealing with concentration. These laws are combined and often referred to as the Beer-Lambert Law Figure 3, which says:

$\log I/I_o = -\epsilon bc$

where I_o is the intensity of light entering the solution, the intensity of emerging light, **b** the length of the light path, **c** the concentration of the absorbing substance, and **ϵ** a proportionality constant. The constant, **ϵ**, is called the molar extinction coefficient when **b** is expressed in centimeters and **c** in moles per liter.

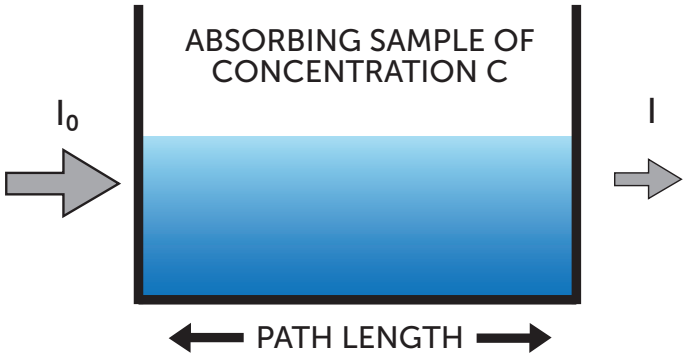


Figure 3 – Illustration of Beer-Lambert Law

In spectrophotometric analysis, the primary interest is in the concentration of some absorbing (colored) substance dissolved in a transparent solvent (usually water) when the length (pathlength) is held constant. When applying the Beer-Lambert Law to colored solutions the intensity of light emerging from the pure solvent is defined as ($\mathbf{I_o}$) and the intensity of light emerging from a solution of the absorbing material in the same solvent is defined as (**I**). The ratio of $\mathbf{I/I_o}$ is called the transmittance (**T**) and is usually expressed in percent, so:

$$\mathbf{T = (I/I_o) \times 100}$$

A related quantity is absorbance (A) which is defined by the equation:

$$\mathbf{A = \log I_o/I = \epsilon bc}$$

The relation between absorbance and percent transmittance is

$$\mathbf{A = \log (100/T)}$$

Although the spectrophotometer measures the relative amount of light transmitted, this value is usually converted to absorbance, which is directly proportional to concentration, and hence more useful to the chemist.

The Beer-Lambert Law and related equations only apply to true solutions. Particles in suspension (turbidity) will cause an error as they also absorb and scatter light but not in a manner consistent with the Beer-Lambert Law.

Spectrophotometers

A spectrophotometer is an instrument containing the components which do the following: generate light energy, select a specific wavelength of the light, pass the light beam through the sample, measure the change in intensity of the light on passage through the sample, and output the intensity signal on a display. See Figure 4.

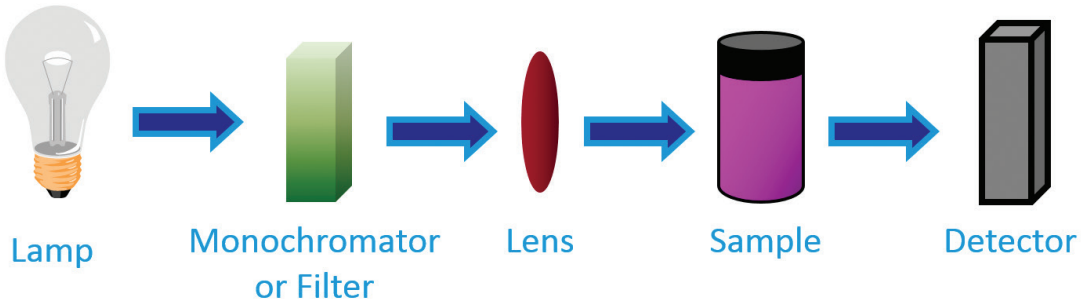


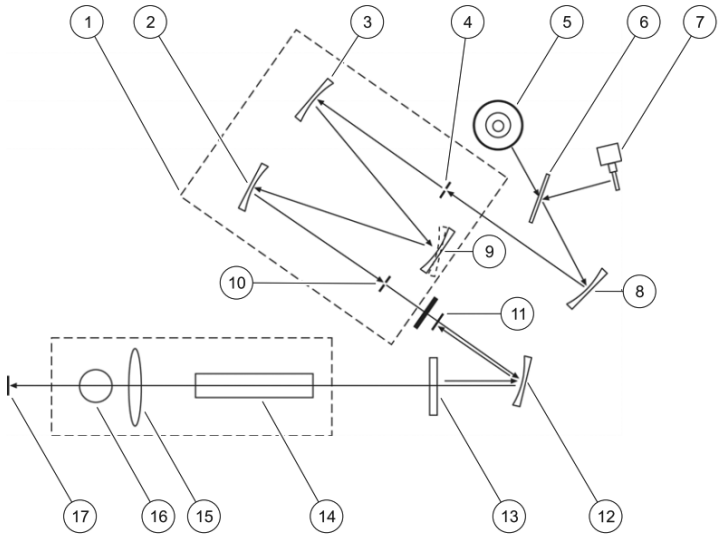
Figure 4 – Components of a spectrophotometer

Light Source

The source of visible light is usually a tungsten filament lamp which provides continuous radiation from about 350 to about 2500 nm. In the ultraviolet region the radiation source is often a hydrogen lamp, a deuterium lamp, or a xenon discharge lamp. These provide continuous radiation in the range of about 180-350 nm.

Wavelength Selection

Wavelength selection is accomplished in different ways, but the customary approach is through use of a monochromator. A monochromator is an optical component utilizing a prism or a diffraction grating to disperse the light into its wavelength continuum. Mirrors, lenses and slits are used to isolate a narrow band of wavelengths and direct the beam into the sample compartment. The wavelength range that exits the monochromator is referred to as the spectral bandwidth and ranges from as low as 0.1 nm to about 10 nm. See Figure 5.



1	Monochromator	**10**	Exit slit
2	Mirror	**11**	Lens reference element
3	Mirror	**12**	Mirror
4	Entrance slit	**13**	Splitter mirror
5	Deuterium lamp	**14**	Cell compartment (2) for universal cell adapter
6	Lamp selection mirror	**15**	Lens
7	Halogen lamp	**16**	Cell compartment (1) for round cells
8	Mirror	**17**	Reading element
9	Grating		

Figure 5 – Schematic of a DR6000 monochromator

Filters may be used instead of a monochromator to select the wavelength band. The bandwidth from color filters is on the order of 20-50 nm. Interference filters have considerably narrower bandpass. An instrument using filters is normally referred to as a colorimeter.

Some instruments achieve wavelength specificity from the light source itself without the need for filters or a monochromator. Light emitting diodes generate light in a narrow wavelength range suitable for spectrophotometric measurements.

Sample Containment

The instrument has a compartment for placement of a cell or cuvette which contains the sample to be analyzed. This cell compartment must allow uniform positioning of the cells so the light beam follows a consistent path. Borosilicate glass or certain plastics may be used for cell construction when measurements are made in the visible region. Quartz or fused silica cells must be used when working in the ultraviolet spectrum since glass absorbs ultraviolet light. The path length that the light beam travels through the sample must be consistent, so internal dimensions of cells are carefully controlled.

Detectors

The light beam finally strikes a detector which determines the energy level and converts it to an electrical signal. Different detectors may be used, but a typical example is a silicon photodiode. The voltage generated at the detector is used to drive a readout device, either an analog meter or a digital display. Microprocessors built into the instruments allow manipulation of the data in various ways.

Calibration Curves

While the concentration of an analyte in solution may in some cases be determined directly using Beer Lambert calculations, it is usually preferable to prepare a calibration curve. This is done by preparing standard solutions of the analyte to be determined and measuring the absorbance of these solutions under identical conditions to the unknown samples. The calibration curve is a plot of absorbance at a fixed wavelength as a function of concentration. The points, when plotted on linear graph paper, should define a straight line with absorbance and concentration increasing proportionally. The absorbance of the unknown sample may then be related to concentration. See Figures 6 and 7.

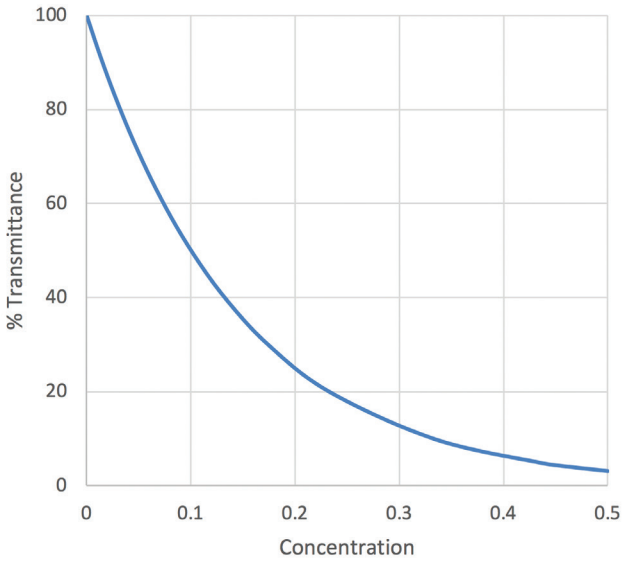


Figure 6 – Calibration curve, %T vs. Concentration

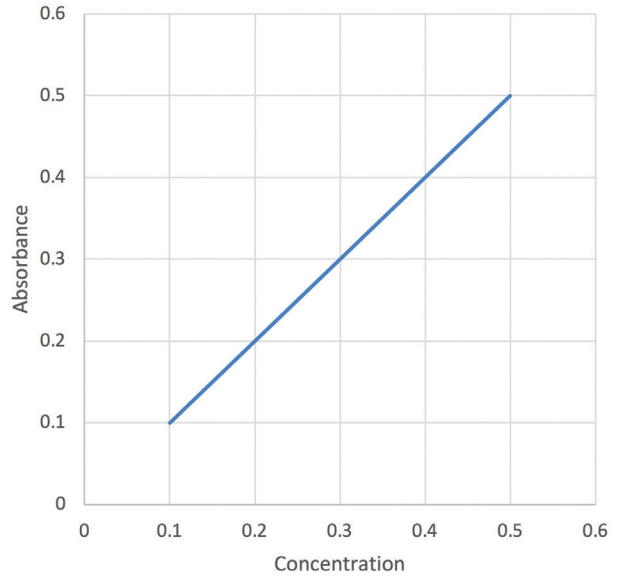


Figure 7 – Calibration curve, Abs. vs. Concentration

A calibration curve constructed from measurements in percent transmittance (%T) as a function of concentration yields a curved line on linear graph paper with %T decreasing as concentration increases. The same data produces a straight line calibration on semi-log graph paper when %T is plotted on the logarithmic axis and concentration is plotted on the linear axis. A calibration curve constructed from measurements in absorbance (A) as a function of concentration yields a straight line on linear graph paper when Beer's law applies.

Chemistry of the Spectrophotometric System

Color Development Factors

Quantitative analysis by spectrophotometry is strictly a comparison procedure. It is necessary that the unknown and the standards be prepared under identical chemical conditions. For chemical analysis, the colored system does not have to conform to Beer's Law perfectly since a series of standard points will be used to define the calibration curve. However, it is essential that a number of factors be considered, and controlled if they are important, to assure reproducible color development. The principal chemical factors which affect a colored system are:

- pH
- stability with respect to time
- ionic strength
- stability to the atmosphere
- temperature
- oxidation state of the element
- amount of reagent added
- nature of ions present
- specificity of the reagent

Sensitivity

The molar extinction coefficient of the complex formed between the colorimetric reagent and the target analyte is a good indication of the sensitivity of a method. The actual value of this coefficient may vary from one instrument to another but it provides a useful indicator for comparison of methods. Once the appropriate method has been selected the analyst must determine the optimum amount of colored material which should be present for the spectrophotometric measurement. This will dictate sample size, cell path length, and whether any dilutions are needed.

It is important to realize that spectrophotometric measurements are most precise in a certain range of absorbance (or %T) readings. The Twyman-Lothian curve relates percent error in concentration as a function of percent transmittance or absorbance. It shows that the optimum working range for colorimetric measurements is 10-80 %T or 0.1 - 1.0 A. On either end of this range the error associated with the reading increases dramatically. See Figure 8.

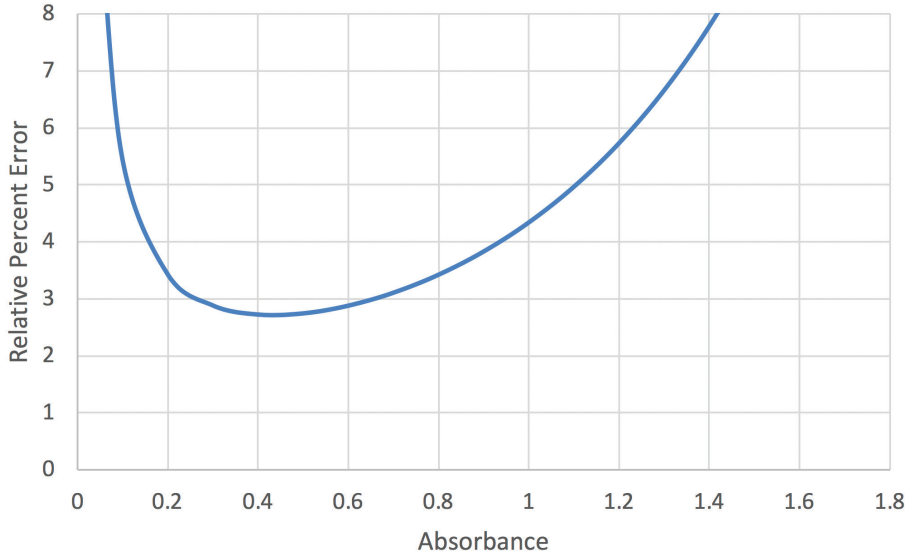


Figure 8 – Twyman-Lothian error curve

Interferences

Other substances which may be present in the unknown sample and cause errors in the result are classified as interferences. Control of these effects can often be achieved with masking agents that react with the offending substance. If this is not effective, preliminary separation of the analyte from the sample matrix or destruction of the matrix may be necessary. Several techniques are available, including extraction, distillation, absorption on ion exchange resin, precipitation and digestion.

Standards

Standards play a central role in spectrophotometry as they do in all methods used for quantitative analysis. Reliable standards are essential for preparing the standard solutions used for calibration. They are also indispensable for assuring accuracy in all steps associated with the analysis. Sample preservation and pretreatment operations such as digestion, extraction, distillation, etc. should all be performed on a standard to verify recovery. The technique known as standard additions or spiking, in which a standard is added to the sample, is useful to reveal interferences or other sources of error in the determination.

Laboratory Techniques and Manipulations

Blanks

Ideally, there is negligible color produced by the reagents in the absence of the analyte. Since spectrophotometry is based on comparisons, the sample must be referenced against a blank that is claimed to contain none of the analyte of interest. The instrument is "zeroed" to read zero absorbance or 100 %T on the blank. A sample blank is the sample without the addition of color forming reagents. A reagent blank is DI water which is treated with the same reagents as the sample.

Contamination

Due to the high sensitivity of most spectrophotometric reagents, the analyst must constantly guard against contaminating the developed colorimetric sample. Contamination is most likely from elements that are commonly found in nature: silica, aluminum, iron, sodium, potassium. calcium, magnesium and chloride. It may also come from other chemical reagents that were used in the same labware for a different determination. Sources of contamination are dust, labware, DI water and reagents.

To prevent contamination, use the following precautions:

- Acid rinse labware, especially glass.
- Expose labware to the same reagents used for analysis before use.
- Protect labware from atmosphere and dust.
- Use plasticware in certain metal tests.
- Carefully select cleaning agents and rinse thoroughly with DI water.

Turbidity

Accurate color measurements can't be made in the presence of suspended particles. Filter or centrifuge turbid samples or allow to settle prior to measurement. Be aware of sampling errors that may be introduced in the process.

Sample Cell Handling

Sample cells become part of the optical system and must not alter the beam of light passing through them. When using sample cells follow these guidelines:

- Clean thoroughly using acid or colorimetric reagent.
- Wipe with a lint free tissue to remove liquid droplets, fingerprints, or dust before inserting in the cell holder.
- Position in the instrument the same way every time.
- Be sure liquid level is high enough so all the light beam passes through the sample.
- Use matched cells for blanks and samples.
- Watch for scratches or scuffs on optical surfaces.

Aliquots and Dilutions

Often the analyte concentration in a sample is too concentrated for direct spectrophotometric determination. It becomes necessary to first make a more dilute solution for analysis. Taking an aliquot refers to removing an accurately measured portion of the sample. The aliquot of sample is then diluted to a specific final volume for analysis.

Illustrations and Figures

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HACH World Headquarters: Loveland, Colorado USA

United States:	800-227-4224 tel	970-669-2932 fax	orders@hach.com
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