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UV Visible Spectroscopy Method Development and Validation

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ABSTRACT

UV-Visible spectroscopy is a widely used analytical technique in various fields such as pharmaceuticals, environmental science, food industry, and materials science. This review explores the methodologies employed in the development and validation of UV-Visible spectroscopic methods. The focus is on discussing the key steps involved in method development, including selection of appropriate solvent, determination of suitable wavelength, optimization of instrumental parameters, and validation of the developed method according to regulatory guidelines.

A significant portion of the review is dedicated to method validation, focusing on parameters such as accuracy, precision, linearity, robustness, etc. In addition to method development and validation, the review explores various estimation techniques utilizing UV-Visible spectroscopy. These include Simultaneous Estimation, Q Absorbance Ratio, Derivative Spectroscopy, and Difference Spectroscopy. Overall, this review serves as a comprehensive resource for researchers and practitioners involved in UV-Visible spectroscopy, providing valuable insights into method development, validation, and estimation techniques

Introduction to Spectroscopy [1, 10]

Spectroscopy involves observing and understanding the electromagnetic radiation absorbed or emitted by molecules, atoms, or ions as they transition between different energy levels. These transitions occur from ground state to excited state or vice versa. The energy of a molecule in its ground state comprises rotational, vibrational, and electronic energies. Therefore, spectroscopy examines the alterations in these energy levels, providing valuable insights into the properties and behaviour of the studied substances.

Electromagnetic Radiation

Electromagnetic Radiation consists of distinct units known as photons. It exhibits both wave-like and particle-like properties, enabling it to propagate even through vacuum. It encompasses various forms such as visible light, ultraviolet (UV) radiation, infrared (IR) radiation, microwaves, radio waves, X-rays, gamma-rays, and cosmic rays. These classifications are based on differences in wavelength, frequency, or energy, providing convenient labels for each type of radiation.

Electromagnetic Spectrum

The complete electromagnetic spectrum is the arrangement of all forms of electromagnetic radiation in ascending order of wavelength or descending order of frequency. The visible spectrum (from violet to red) represents only a small portion of the electromagnetic spectrum.

If we arrange all types of electromagnetic radiations in order of their increasing wavelengths, then portion above the visible region is called infrared while that below it is the ultra –violet region. Infrared radiation possesses longer wavelengths, making it less energetic. Conversely, cosmic rays carry high energy levels, while radio waves exhibit energetic properties. Microwaves, characterized by their longer wavelengths, find utility in telephone transmission.

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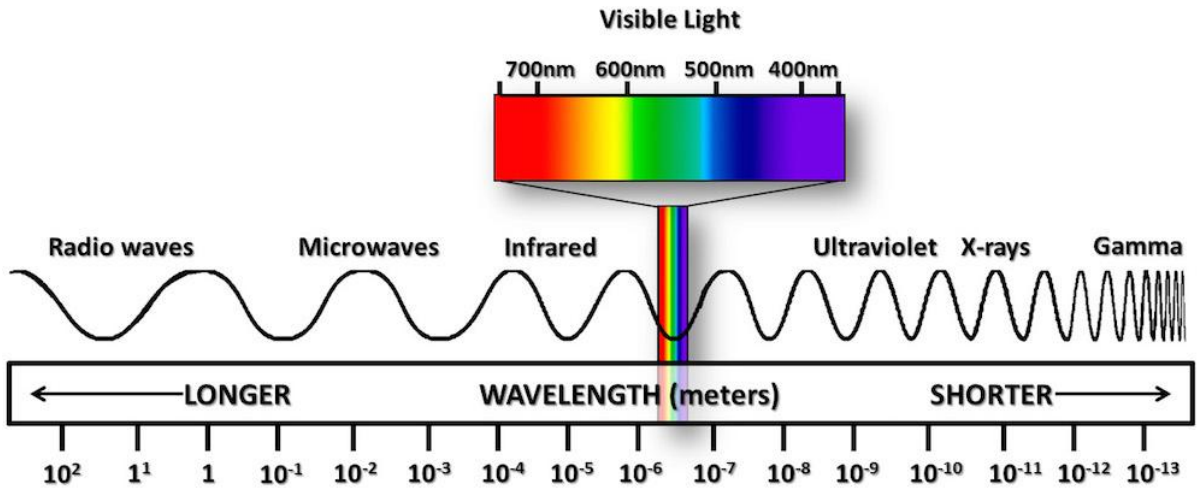

Figure 1 Electromagnetic Spectrum [4]

PRINCIPLE [2, 11]

The principle of UV visible spectroscopy is based on absorption of ultraviolet light or visible light by a chemical compounds, which gives spectra.

Beer Lambert law states that, "The absorbance (A) of monochromatic beam is directly proportional to concentration (C) & Path length (l).

A = abc

Where, A = Absorption

a = absorptivity

b = path length

c = concentration [2]

Electronic Transition [3, 15]

When radiation induces an electronic transition within a molecule or ion, the molecule or ion will show absorption, the molecule or ion will show absorption in the visible or ultraviolet area. As a result, when a sample absorb light in the ultraviolet or visible range, the molecules inside the sample experience a change in their electronic state. Electrons will be promoted from their ground state orbitals to higher energy excited state orbitals or antibonding orbitals by the energy provided by light. Ultraviolet and visible light can cause the following electronic transitions through absorption.

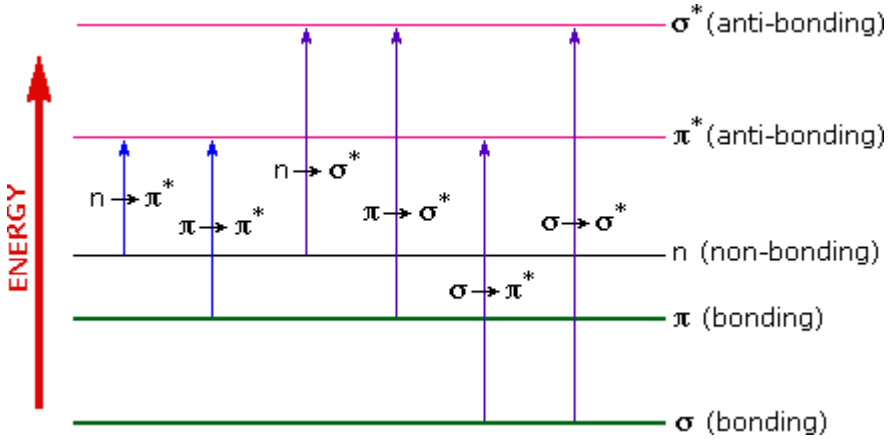

Figure 2 Electronic Transition of α, π, n electrons [5]

1) σ to σ* Transitions
2) n to σ* Transitions
3) n to π* and π to π* Transitions

Concept of Chromophore & Auxochrome [1]

Absorption of light in UV-visible region gives rise to absorption spectra. This spectrum arises due to transitions among electronic energy levels (Eo to E1) of certain groups present in the molecule. This group of atoms which is responsible for the absorption in UV-visible region is known as the **Chromophore**. Mostly the unsaturated groups, heteroatoms or heteroatoms containing lone pair of electrons are the possible chromophores. These chromophores show absorption in certain region of spectrum (Say from 400 to 200 am), however at particular wavelength it shows maximum absorption (say at 254 nm) and that wavelength is called as "Wavelength of maximum absorption." It is denoted by Λmax.

Auxochromes are groups that do not absorb radiation within the UV-visible region on their own. However, when they are connected to a chromophore, they can cause a shift in the absorption bands, either towards longer wavelengths (higher wavelengths) or shorter wavelengths (lower wavelengths) means they bring out the changes in absorption section of chromophore when attached to it. Typically, these auxochromes possess valence electrons that are not involved in bonding. Eg. –OH, - NH2, -Cl etc. It must be remembered that auxochrome shows intense absorption in far UV region i.e.at wavelengths greater than 780 nm. In short it can be stated as auxochromes itself do not absorb the radiation in UV region but when attached to chromophore causes changes in its absorption spectrum. Thus, in UV-visible region only chromophore absorbs the radiation.

Spectral shift:

Bathochromic shift (Red shift):

When an auxochrome is attached to the chromophore, sometimes the absorption band is shifted to longer wavelength is called as Bathochromic Shift. Bathochromic shift or red shift. The term red is used as it has the higher wavelength.

Hypsochromic shift (Blue shift):

When an auxochrome is attached to the chromophore, sometimes the absorption band is shifted towards shorter wavelength, this is called as. Hypsochromic shift or blue shift. The term blue is used as it has the shorter wavelength.

Hyperchromic shift: When an auxochrome is attached to the chromophore, sometimes the intensity of absorption band is increased; this is called as hyperchromic shift. • This in hyperchromic shift the absorptivity of molecule increases.

Hypochromic shift: When an auxochrome bonds with the chromophore, there are instances where the intensity of the absorption band decreases; this phenomenon is termed a hypochromic shift. In a hypochromic shift, the molecule's absorptivity decreases.

In summary, bathochromic and hypsochromic shifts involve changes in the wavelength (maximum) of absorption bands, while Hyperchromic shift and Hypochromic shift deals with intensity of absorbed radiation i.e. (Emax) or relative power of radiation.

Instrumentation [3]

The essential components of UV-Vis spectrophotometer are as follows:
1. Sources (UV- Visible)
2. Monochromator
3. Sample containers (cuvette)
4. Detector
5. Amplifier and recor

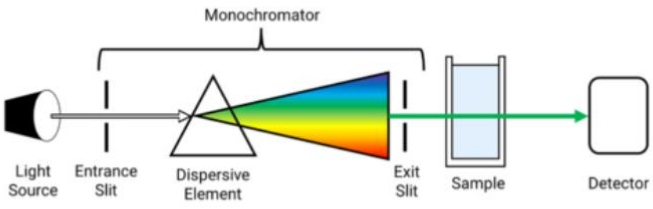

Figure 3 Schematic Diagram of UV Visible Spectrophotometer [6]

Sources:
A continuous source, or one that emits radiation at a variety of wavelengths, is necessary for UV- Vis spectroscopy. The following are many sources of uv radiation:
a) Hydrogen lamp
b) Deuterium lamp
c) Tungsten lamp
d) Xenon discharge lamp

Monochromator:
A Monochromator subtracts undesirable wavelengths from the radiation source light to produce Monochromator light. Through the entrance slit, multi-wave length polychromatic light enters Monochromator. After collimation, the beam is angled towards the dispersion component. The grating or prism then separates the beam into its constituent wavelengths. When the dispersing element or exit slit is adjusted, only radiation of a particular wavelength passes through the Monochromator via the exit slit.

The Monochromator (wavelength selector)
All monochromators contain the following component parts.
- An entrance slit
- A collimating lens
- A dispersing device (usually a prism or grating)
- A focusing lens
- An exit slit

Sample Cell:
To allow radiation to travel through them, the containers holding the sample and reference solution must be transparent. For UV spectroscopy, quartz or fused silica cuvettes are necessary.

Detector:
A semiconductor device called a photo detector transforms light energy into electrical energy. It is made up of a straightforward P-N junction diode and intended to function when reverse biased. As photons approach the diode, the photodiode absorbs them and produces electricity

Types of detectors
a) Barrier layer cell/photovoltaic cell
b) Phototubes/photo emissive tube
c) Photomultiplier tube

Recorder: [2]
The recorder detect & record the data of the experiment. It also stores the data in computer when it connected to computer.

ADVANTAGES & DISADVANTAGES

ADVANTAGES	DISADVANTAGES
UV visible spectroscopy gives accurate results	The results can be affected by Temperature, PH, impurities etc
Easy to handle	Only liquid samples are possible to analyse
Cost effective instrument.	Require proper handling of cuvette [2]

Applications [2, 12]
1. It is useful in quantitative analysis.
2. It is used in drug identification
3. It is used for determination of different species
4. It is used for beverage analysis
5. It is used in DNA & RNA analysis.
6. It is used to check nucleic acid purity
7. It is used in detection of impurities.
8. Structural elucidation of organic compounds.

Estimation Methods Used in Uv Spectroscopy [7, 13]
1) Simultaneous Equation method

2) Derivative Spectrophotometry
3) Q-Absorbance Ratio Method
4) Difference Spectroscopy

Simultaneous Equation Method:
If a sample contains two absorbing substances, X and Y, each of which absorbs at the maximum absorption wavelength (λ max) of the other, it is feasible to ascertain the concentrations of both substances under the following conditions:
• The absorptivities (molar absorptivities or extinction coefficients) of X at λ1 and λ2, denoted as ax1 and ax2, respectively.
• The absorptivities of substance Y at wavelengths λ1 and λ2, represented as ay1 and ay2, respectively.
• The absorbance of the diluted sample at wavelengths λ1 and λ2, denoted as A1 and A2, respectively.

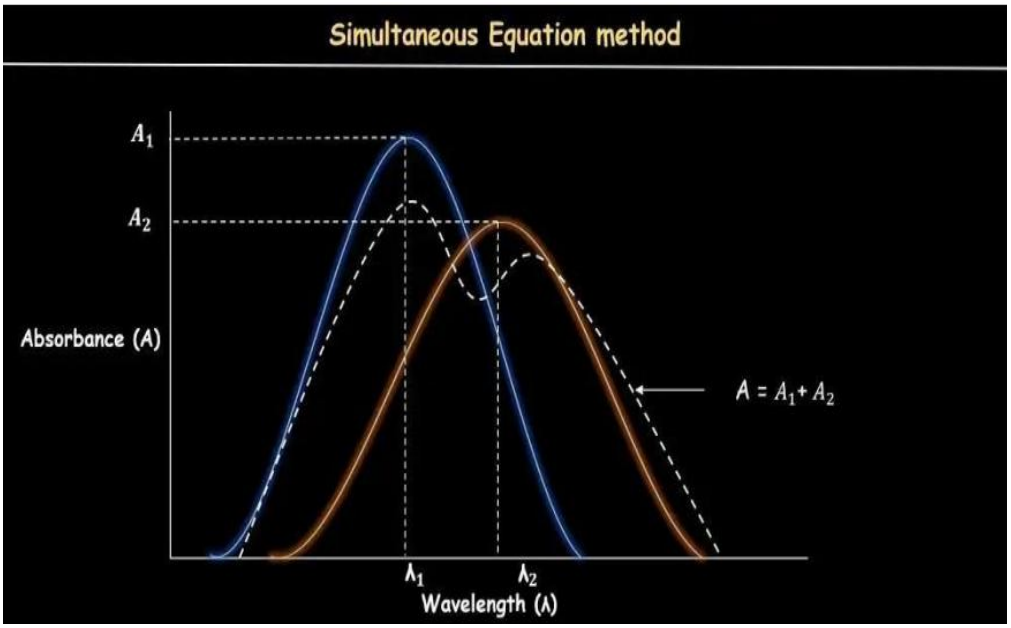

Figure 4 Simultaneous Estimation Method

Two equations are constructed based on the fact that at λ1, the absorbance of the mixture is the sum of the individual absorbances of X and Y:
Equation 1: A1 = (ax1 * Cx) + (ay1 * Cy)
Equation 2: A2 = (ax2 * Cx) + (ay2 * Cy)
Where: • A1 and A2 are the absorbances at λ1 and λ2, respectively.
• Cx and Cy are the concentrations of X and Y, respectively.
• ax1, ax2, ay1, and ay2 are the absorptivities of X and Y at the respective wavelengths. **Derivation:** For measurements in a 1 cm cell (b = 1 cm), Eq. (2) can be rearranged as follows: **Equation 3:** Cy = (A2 - ax2 * Cx) / ay2 Substituting the expression for Cy from Eq. (3) into Eq. (1) and rearranging gives:

Equation 4: A1 = (ax1 * Cx) + (ay1 * [(A2 - ax2 * Cx) / ay2]) Solving Eq. (4) for Cx yields: **Equation 5:** Cx = (A1 * ay2 - A2 * ay1) / (ax1 * ay2 - ax2 * ay1)

Q-Absorbance Ratio Method:
The Q-absorbance ratio method, also known as the absorption ratio method, is a variation of the simultaneous equation method used for quantitative analysis. This method relies on the principle that for a substance obeying Beer's law, the ratio of absorbance at two wavelengths is a constant value regardless of concentration and path length. This constant is referred to as "Hufner's Quotient" or Q-value. The technique involves measuring absorbance at two specific wavelengths: one corresponds to the λ max of one of the components (λ2), and the other wavelength where both components have equal absorptivity, known as the iso-absorptive point (λ1)

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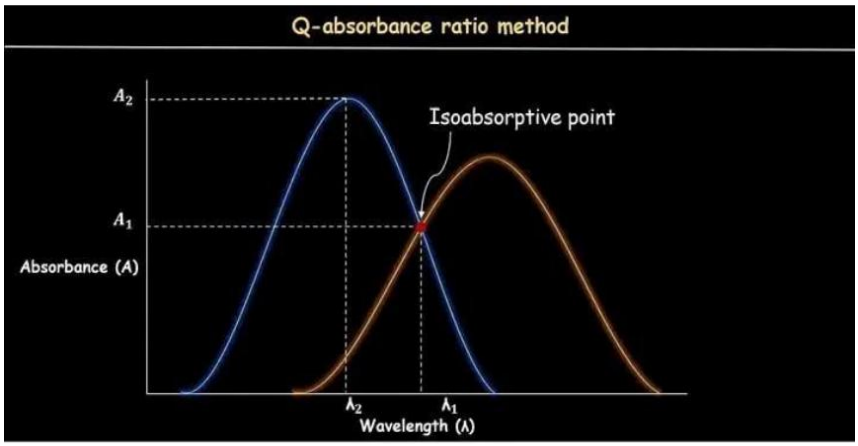

Figure 5 Q-Absorbance Ratio Method

To determine the concentrations of drugs X and Y using the Q-absorbance ratio method, the following equations are applied:
For drug X: Cx = ((QM - Qy) / (Qx - Qy)) × (A1 / ax1)
For drug Y: Cy = ((QM - Qx) / (Qy - Qx)) × (A1 / ay1)
In these equations:
Cx and Cy represent the concentrations of drugs X and Y, respectively. –
QX, QY, and QM are computed based on the average absorptivity values of the drugs at specific wavelengths. -
QX = ax2 / ax1 –
QY = ay2 / ay1 –
QM = A2 / A1
Where, ax1 and ax2 are the Absorptivities of drug X at wavelengths λ1 and λ2. ay1 and ay2 are the Absorptivities of drug Y at wavelengths λ1 and λ2. A1 and A2 indicate the absorbances of the sample solution at wavelengths λ1 and λ2.

Derivative Spectroscopy:
This straightforward spectrophotometric method is based on generating ratio spectra to resolve binary mixtures. It enables the utilization of the wavelength with the highest analytical signal, which exhibits multiple maxima and minima. This feature provides an opportunity to determine active compounds even when other substances and excipients are present, potentially causing interference in the assay.

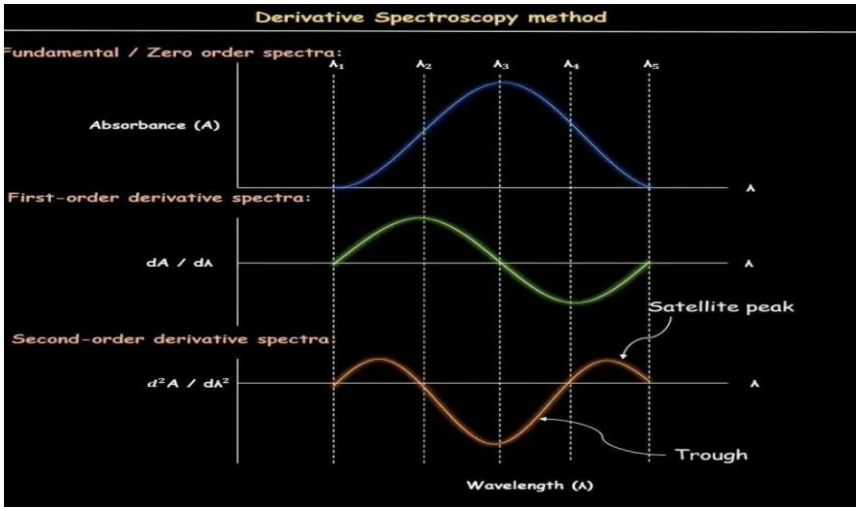

Figure 6 Derivative Spectroscopy Method

To facilitate the determination of the concentration of compound y without interference from drug x, one can calculate the first derivative. This operation effectively removes the constant value associated with compound x.

The observed difference between the two spectra arises from the constant interference value attributed to compound x. To eliminate this interference, you can either measure the difference in ratio spectra between two wavelengths or

compute the derivative of the ratio spectra.Additionally, employing the second derivative of the ratio spectra can enhance linearity, mean recoveries, and decrease relative standard deviation. The derivative ratio spectra technique has been adapted for the determination of ternary mixtures using the derivative ratio spectra zero-crossing method. In this approach, amplitudes are measured at the zero-crossing points in the derivative ratio spectra.

Difference Spectroscopy:
Difference spectrophotometry is a valuable technique in spectrophotometric analysis when dealing with samples containing interfering substances. It can significantly enhance selectivity and accuracy by measuring the absorbance difference (ΔA) between two equimolar solutions of the analyte in different chemical forms that exhibit distinct spectral characteristics..

To apply difference spectrophotometry effectively in the presence of interfering substances, the following criteria should be met:

1. Reproducible Changes in Analyte Spectrum: It should be possible to induce consistent and reproducible changes in the spectrum of the analyte by adding one or more reagents. This is crucial for ensuring that the observed differences are due to the analyte and not random variations.

2. No Alteration in Interfering Substance Absorbance: The addition of reagents should not affect the absorbance of interfering substances. This is important because you want to isolate the changes in the analyte's spectrum without causing unwanted shifts in the interfering substance signals.

One commonly employed technique to alter the spectral properties of the analyte is adjusting the pH using aqueous solutions of acids, alkalies, or buffers. This pH adjustment can lead to distinct chemical forms of the analyte with varying spectral characteristics, allowing for the measurement of ΔA

Method Development Steps For Uv Spectroscopy [8, 14]
1) Selection of Solvent System
2) Selection of Analytical Wavelength
3) Study of Beer-Lambert's Law
4) To perform Analysis of Standard Laboratory Mixture and Tablet Formulation by Proposed Method
5) To validate the developed method by using different validation parameters.

Validation For Uv Spectroscopy [9]
Validation is a procedure involving laboratory testing to confirm or determine that a method, system or assay provides correct and reproducible results within the intended and defined range for the intended analytical application.
Validation Parameters are as follows:

Precision:
Precision refers to the degree to which an analytical technique can be repeated. In fact, under normal conditions, it is usually distributed as the standard deviation value of a given sample in centimeters. Since an operationally sufficient number of samples is a statistically significant precision, it should be done at three levels according to ICH.
a) Repeatability (n=6)
b) Intraday Precision (n=3)
c) Interday Precision (n=3)

Accuracy:
Accuracy is a measure of the accuracy or uniformity of an analytical method. between values accepted as normal value, actual value or accepted control value and values accepted as standard value. , the real value or the generally accepted set point It is calculated as a percentage of the analytes obtained in the test when the samples are included in the blind study. The set of components, the accuracy of the quantification of impurities is determined by examining the samples (drugs).

Specificity:
The ability to correctly and precisely quantify the analyzes of interest in the laboratory is called specificity. The presence of other anticipated elements in the sample matrix. It is a measure used to determine the size of a group of people. Confounding factors such as excipients, contaminants and degradation are some of the other active ingredients. The products guarantee that only a single component elicits the highest response.

Linearity and Rage:
The ability of a method to produce test results that are directly related to the method inversely proportionate to the analyses linearity of dosage within a certain range is reported. The method's ability to produce test findings that are proportionate analysis. The capacity of the method to provide concentration within a particular range is known as linearity.

LOD:
The lowest detectable concentration of a material is called the detection limit. As concentration range (LOD). An analyte that is detectable but not quantifiable in a sample. According to the formula LOD = 3.3σ /S

LOQ:
The limit of measurement refers to the lowest concentration of the analytical method that can be determined with sufficient sensitivity and efficiency under laboratory-confirmed conditions of the product. According to the formula LOQ = 10 σ /S

Robustness:
It pertains to the capacity of a process to stay consistent despite minor alterations. Strength robustness denotes the capability to effectively manage deliberate modifications in input. Method parameters Variable Organisms, pH values and other method characteristics are used to measure the robustness of the technique. Determining the effect of ionic strength, temperature, and other variables (if any) on method robustness should be considered early in the development of a method that meets ICH standards.

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