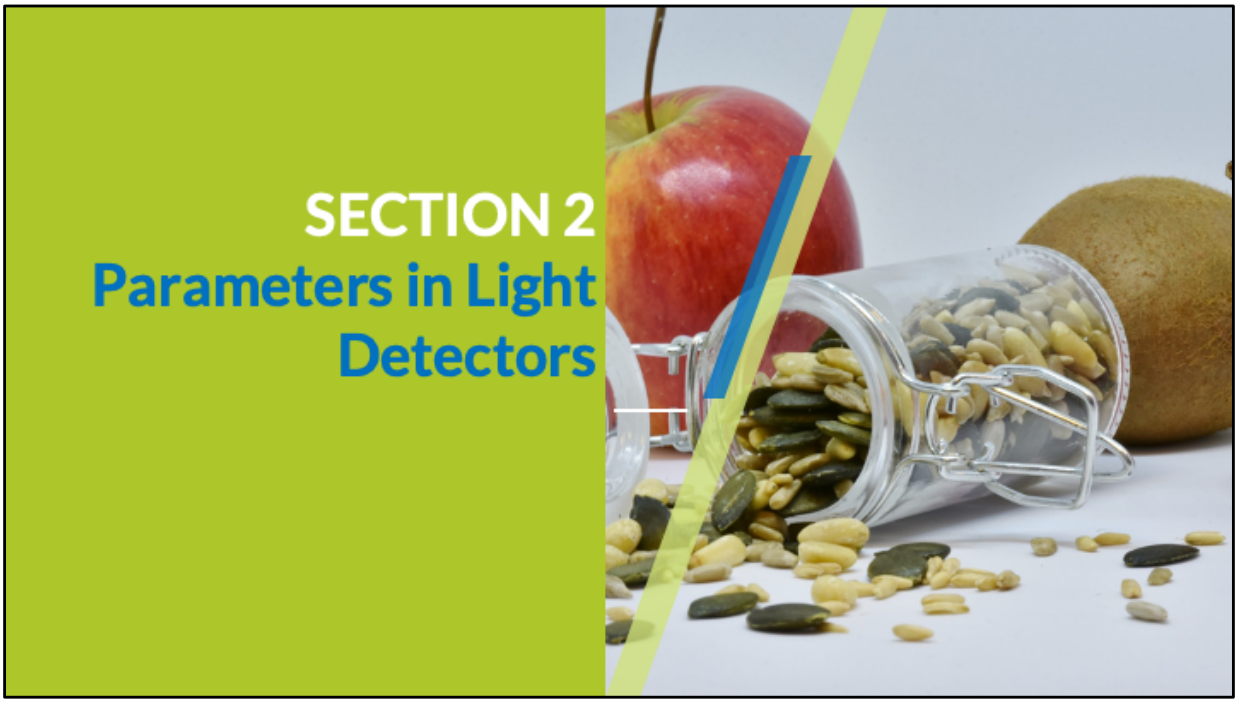
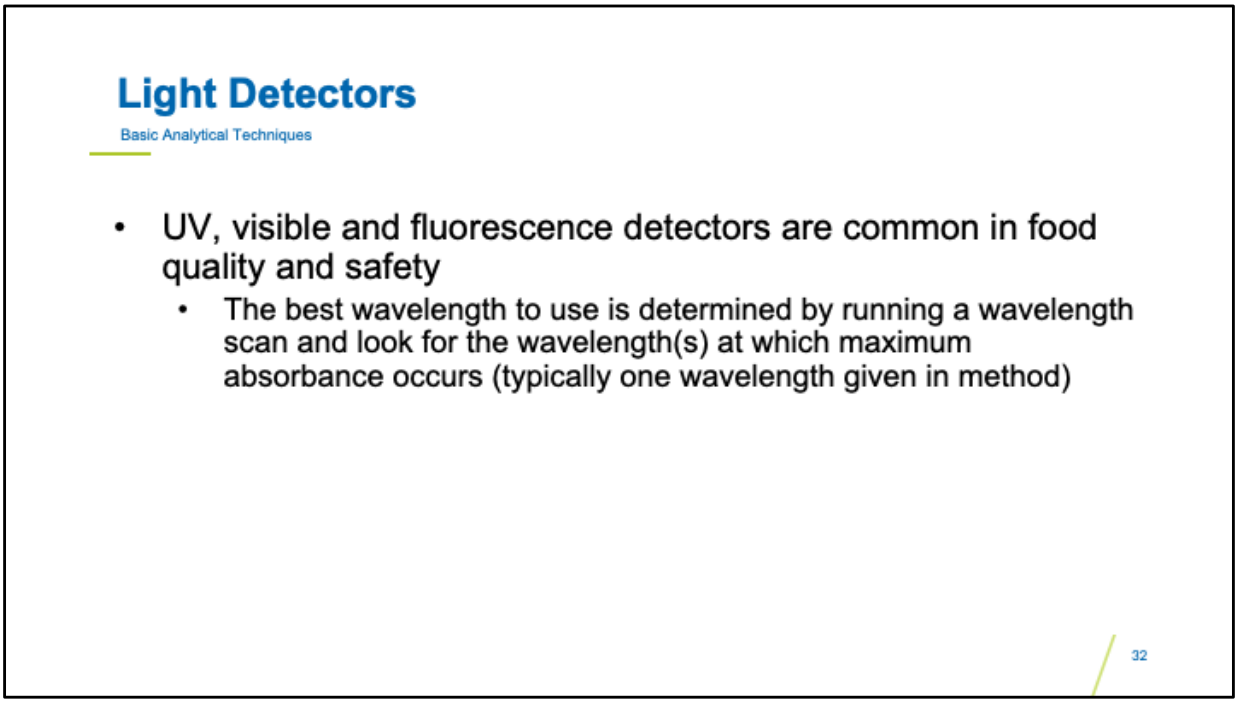
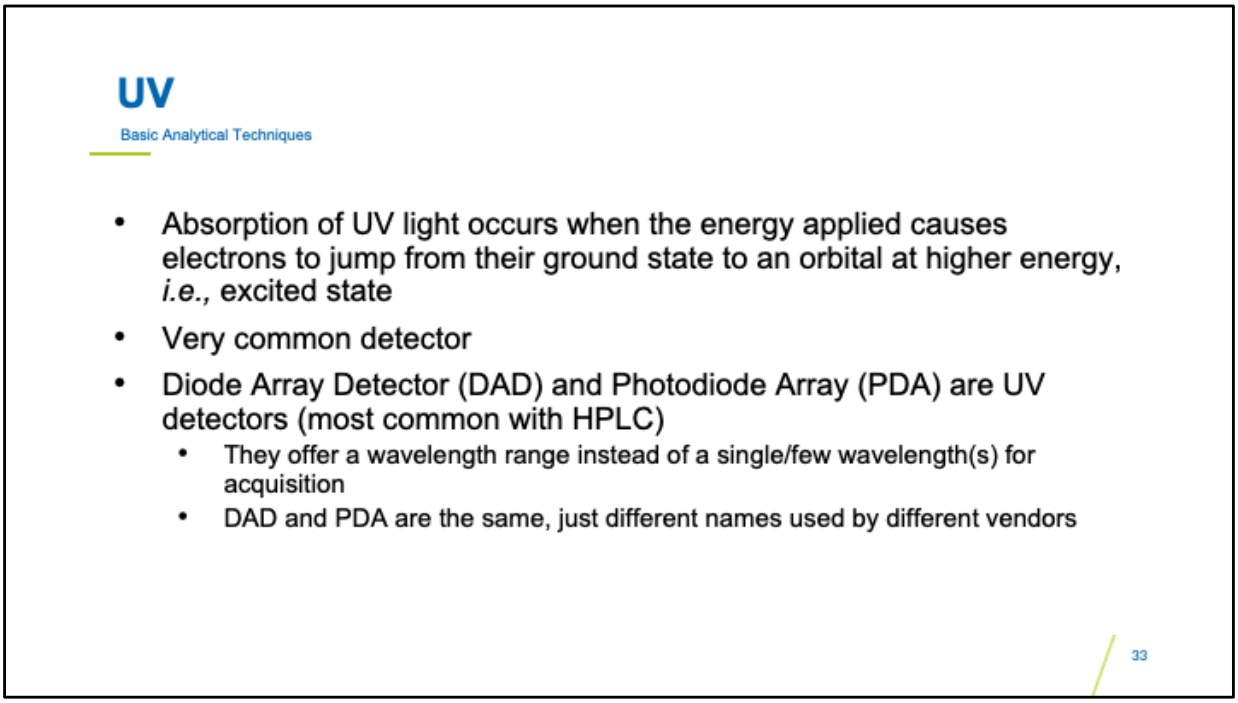
Welcome to Module 7 on instrument operation.



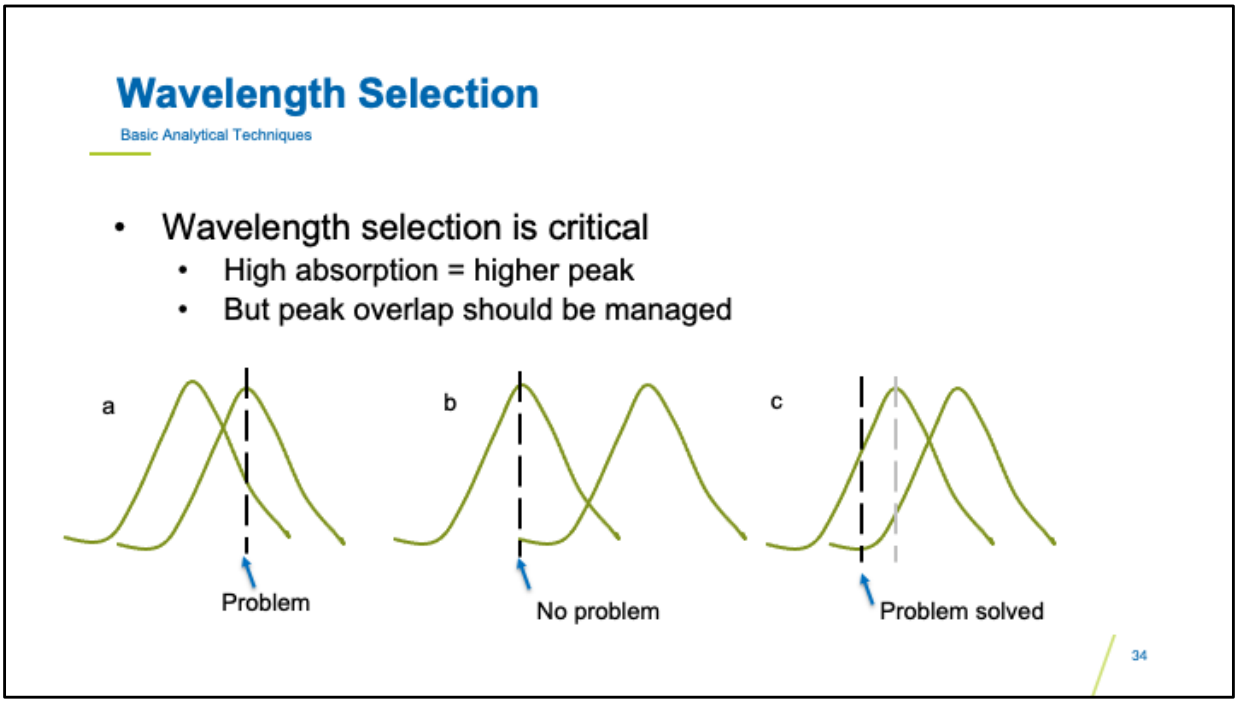
Module 7, Section 2: Parameters in light detectors



Light, or photometric, detectors are very popular in the food safety laboratory because of their relatively low cost and broad applicability. Fluorescence, UV and visible detectors are some of the default detectors associated with liquid chromatography instruments. Method development with these detectors is straightforward; the analyst determines the best wavelength to use by running a wavelength scan and identifying the wavelength or wavelengths at which maximum absorbance or fluorescence occur. A routine method would then use that single wavelength for the detection and quantitation of the target analytes. For most food safety laboratories, this development work has been done elsewhere and the official method states the wavelength to use. In this section, we very briefly review the principle of these techniques in order to understand the parameters that affect analytical results.



The absorption of ultraviolet light occurs when the energy applied to a molecule causes some of its electrons to jump from their ground state to an orbital at higher energy. This is called the excited state. The detector therefore measures the loss of UV light from the source as it went through a sample and presents it inverted as a peak. UV detectors are very common and can be in the form of a diode array detector called DAD or PDA, which stands for photodiode array. The advantage of the arrays is to offer a wavelength range instead of a single or selection of few wavelengths for data acquisition. While only a single or a few wavelengths are required for any given method, the array broadens the range of applicability of the instrument to more methods that aim to measure different analytes with different UV absorption characteristics.



Being a very simple detector, there are only a few parameters that affect the signal reported in the chromatogram. The selection of an appropriate wavelength is critical to obtain any signal at all, but the intuitive selection of the wavelength of maximum absorption, while usually optimal, may in certain cases not be. This would happen if two analytes absorbing UV light at nearby wavelengths are targeted by the analysis. It would be best for selectivity to use wavelengths at which there is no overlap in signal. The figure on this slide illustrates this example, where in a, we have a problem because the maximum of the second peak is affected by the presence of the first peak. In example b, there is no problem because the first and second peaks are not overlapping. If we go back to example a, we could measure the absorbance of the first peak by doing it off of the peak maximum, where there is no interference from the second peak.

A method developed to measure a larger number of analytes has an increased risk of seeing overlapping bands. Overlapping bands can be separated by changing the column chemistry or the mobile phase composition, or both. This essentially corresponds to going from the chromatography depicted a to the one depicted in b. If it is not possible to modify them, a measurement

could be made away from the top of the absorption peak, where the second component does not absorb. The disadvantage of this approach is that it reduces the sensitivity of the method and consequently increases the limit of detection and limit of quantitation. Flow rate and column length also impact overlap; a column that is too short may not allow sufficient interaction with the stationary phase to cause the separation of the two peaks. Similarly, a mobile phase moving at an excessively high flow rate will prevent these interactions or at least limit them. Slowing down the flow of the mobile phase may result in the separation of the peaks and using a longer column could lead to the same outcome. In both cases, we would be going from a to b. When it is not possible to change the column chemistry or the column length, the chemistry of the mobile phase and the flow rate should be considered first before settling on using a wavelength of lower sensitivity (which is Figure c). If using a wavelength of lower sensitivity is the only solution, it may be possible to increase the pathlength of the cell, which results in an increased signal intensity and lower limits of detection and quantitation. However, this is best done when it is possible to increase the pathlength without widening the diameter of the cell because this would cause peak broadening and possibly increased overlap of the bands.

The regulatory requirements for the confirmation of identity of a contaminant will be discussed further in module 9. For UV-visible detectors, a confirmation of identity would require the use of either two different chromatography columns or two chemically-independent wavelengths of absorption to deliver the level of certainty required for regulatory action.

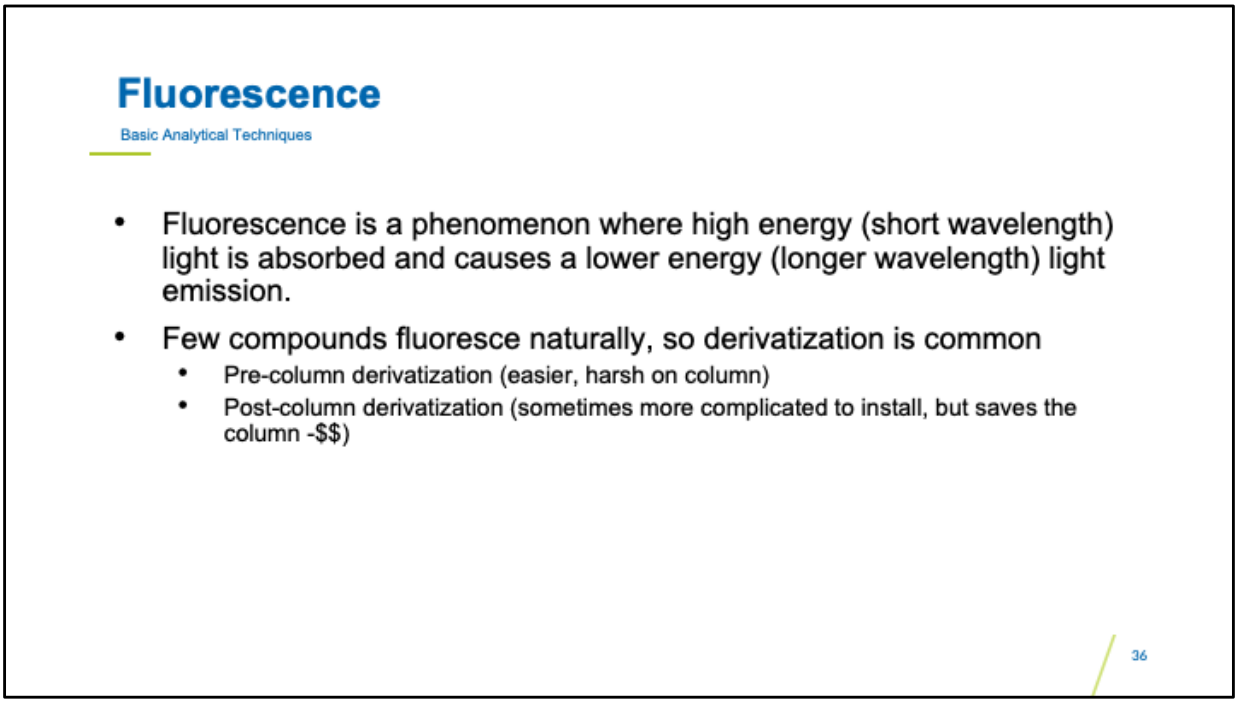
View the Inside

Basic Analytical Techniques

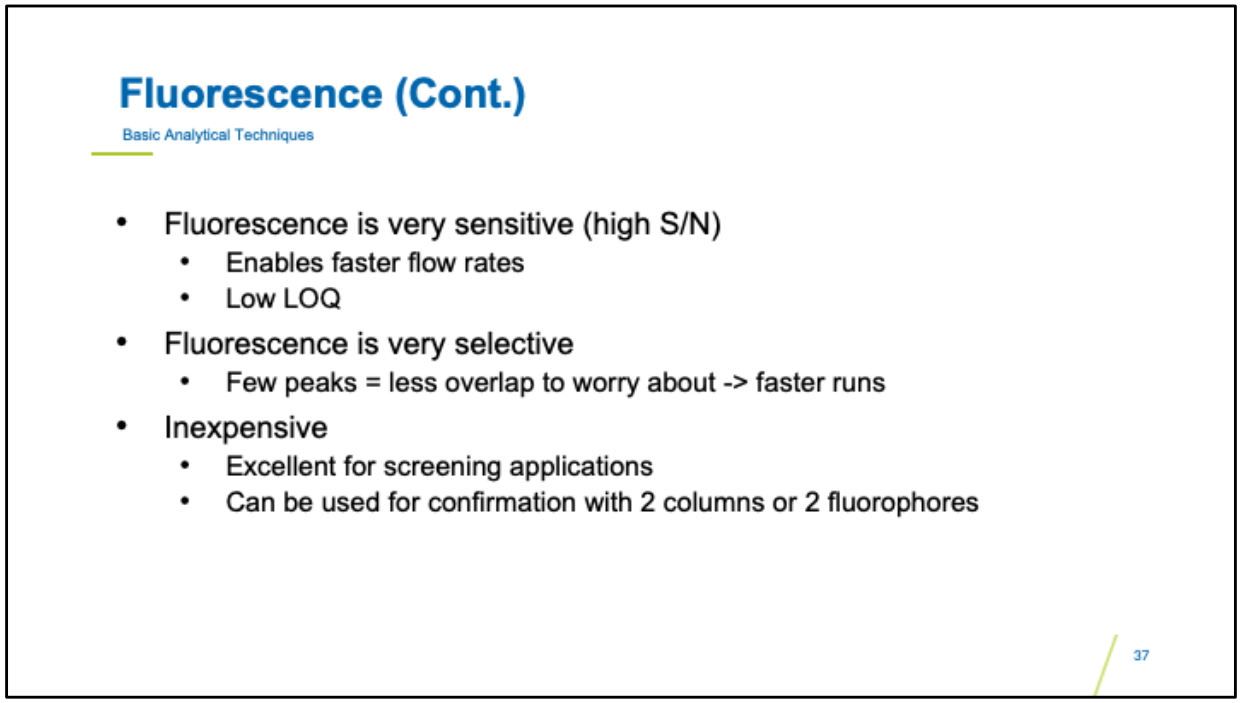
- When possible, watch maintenance videos to understand the inside of your instrument…

35

I always recommend to new analysts to learn as much as they can about their instrument. When your vendor makes then available, it is a good idea to watch maintenance videos to understand the insides of your instrument…



Fluorescence is emitted when a higher energy short wavelength of light is absorbed and as a result, a lower energy longer wavelength burst is emitted. Few compounds fluoresce naturally so derivatization is common in methods using fluorescence detectors. Generally, pre column derivatization is easier perform or even to install if it uses an accessory but can be damaging for the column. Post column derivatization can be a little more complicated to install between the column and the detector, but it is often preferred because it ensures a longer column lifespan. This is an important consideration because of the high cost of chromatography columns.



Fluorescence is very common for contaminant analysis because it is a very sensitive technique. It is characterized by a high signal to noise ratio which enables the use of faster flow rate and delivers suitably low limits of quantitation. Fluorescence is considered very selective because few components are naturally fluorescent. However, interferences from matrix components that may result in diminished fluorescence or fluoresce themselves impose a requirement for sample preparation steps that result in a very clean sample.

The complicated matrix characteristic of food safety applications often requires that we use sample preparation methods that are either time consuming or expensive or both. In the case of aflatoxins for example, the extract from corn must be purified through an immunoaffinity column or at least a highly specific chemical clean-up column to remove all matrix components that are naturally fluorescent. In general, fruits and vegetables must go through extensive cleanup before they can be measured using fluorescence detection because they contain chlorophyll which displays a really strong fluorescence that would overwhelm most of our peaks of interest.

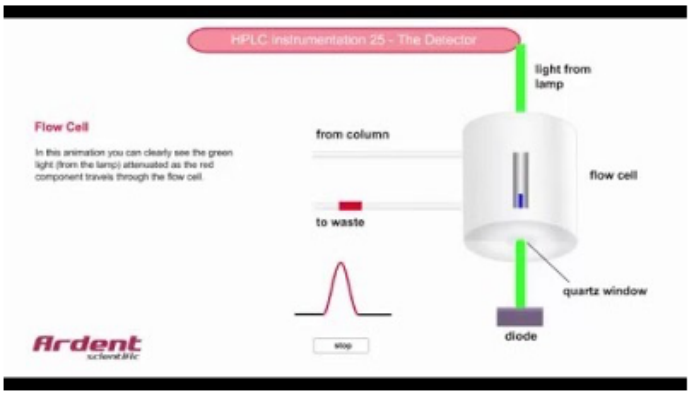
Fluorescence detectors are generally inexpensive,

excellent for screening applications and they can be used for confirmation with the combination of 2 columns or 2 fluorophores. T hese requirements will be discussed further in Module 9.

View the inside

Basic Analytical Techniques

- This video looks at the flow cell in the fluorescence detector…



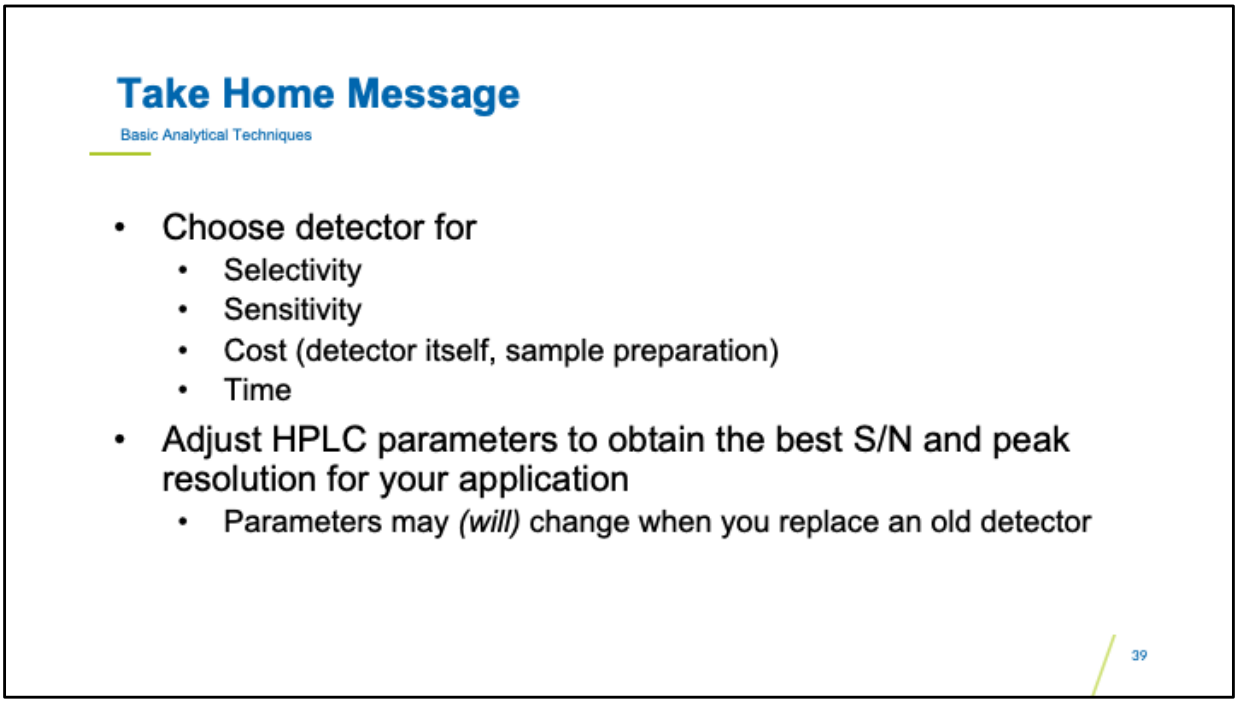
https://youtu.be/ixb7QTqZkaA

38

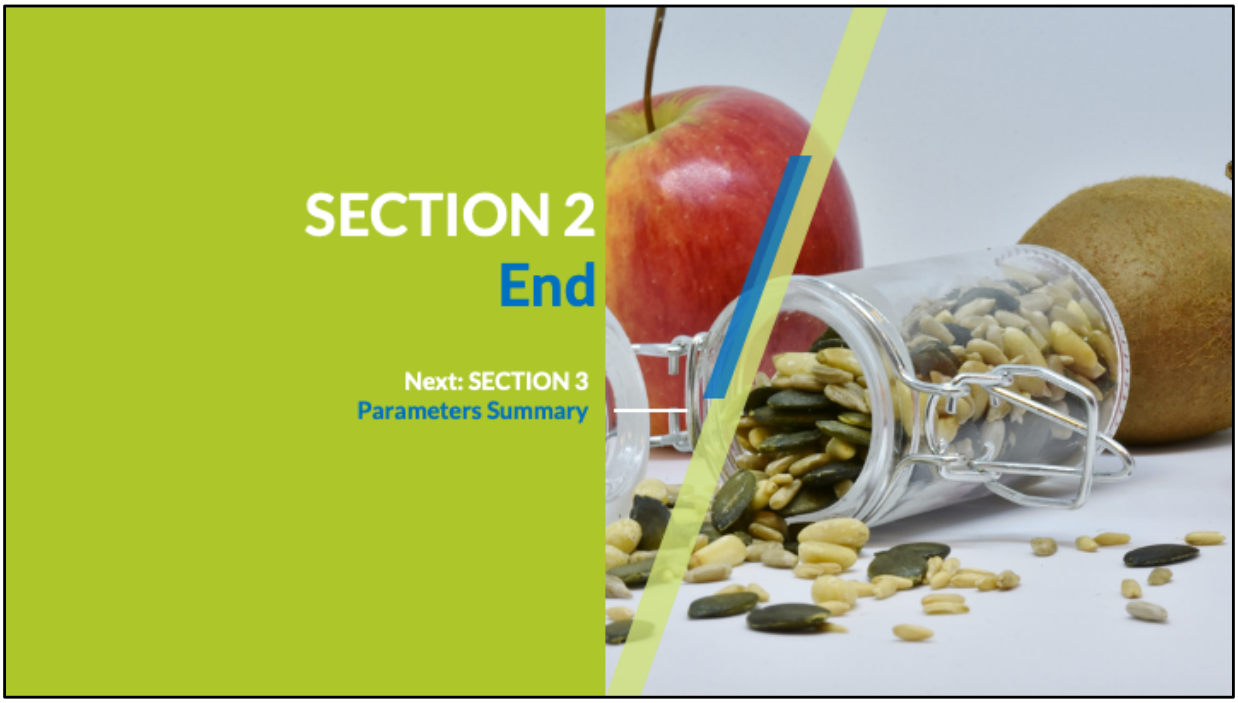
Let's talk about the inside of the fluorescence detector. This video looks at the flow cell

Video:

As we have a closer look at the flow cell after the separation, we can see the components flowing through the cell. As the red component flows through, some of the green light is absorbed, resulting in a reduced intensity of green light hitting the diode. This reduced transmission is inverted and then translated to the peak response. In a variable wavelength detector, a wavelength is chosen, which is specific for the components of interest in the mixture and also one that will give the maximum response.



In summary, we have a bit of a take home message. There is no “one-size-fits-all” solution that can satisfy the breadth of requirements of food safety contaminants analysis. A detector must be chosen on the basis of its selectivity, sensitivity, the cost of the detector and special sample preparation needed, and also the amount of time available for the analysis. The HPLC parameters must be adjusted to obtain the best signal to noise ratio, peak resolution and peak isolation. It is important to also monitor the deterioration of the signal as detectors age as they may require adjustments in the method parameters to compensate for peak distortion or reduced intensity. Similarly, method parameters are likely to need adjustment when a new detector is installed.



In section 3, we summarize all these parameters.