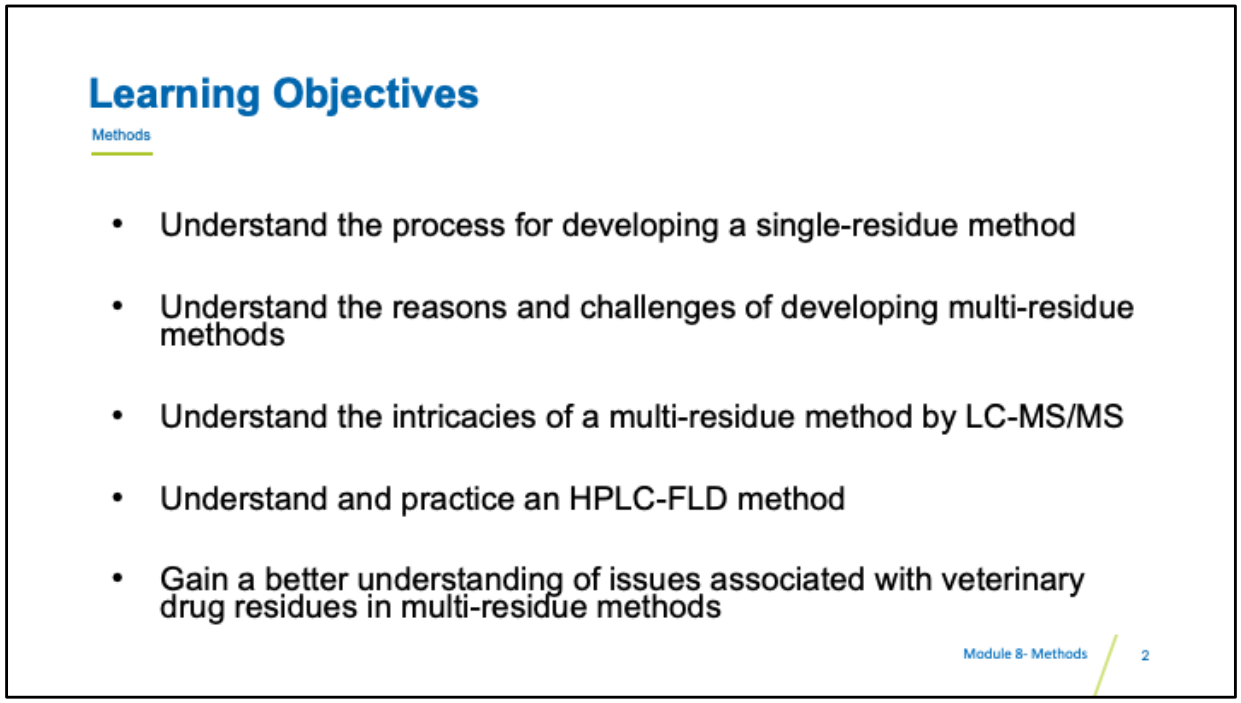
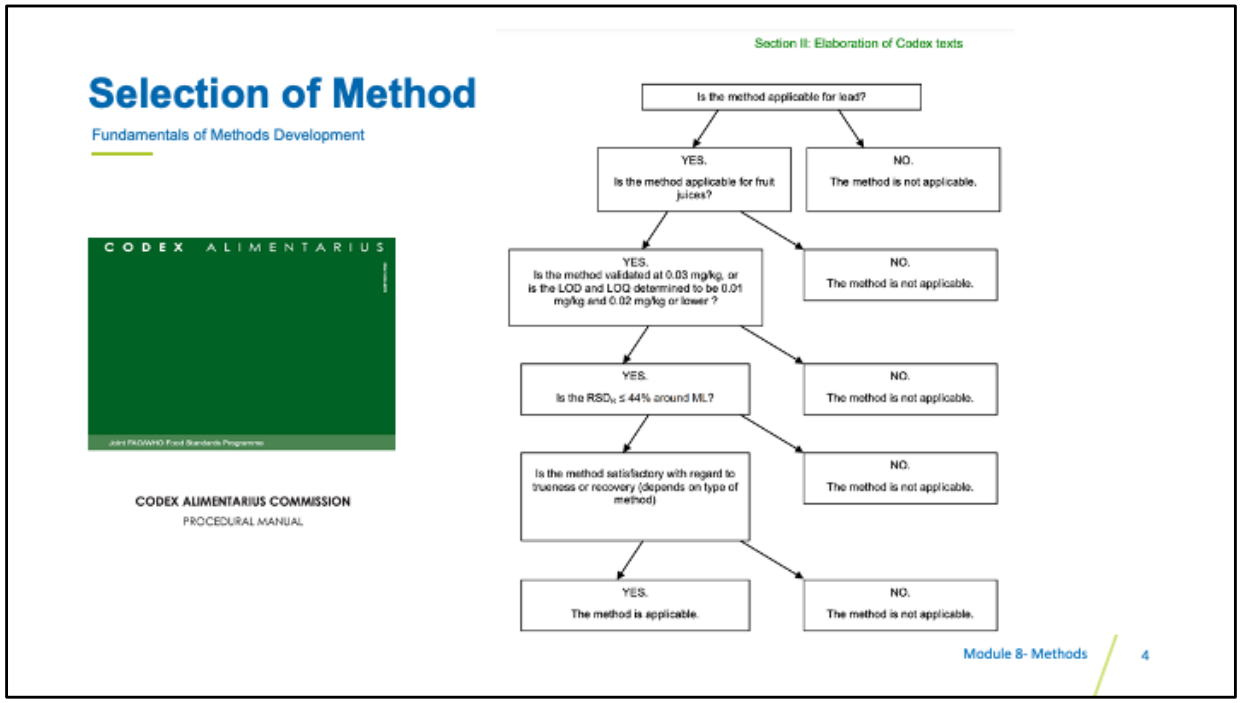
Welcome to Module 8 of the online portion of this training on confirmation methods for organic chemical contaminants in food.



The objectives of this module are to understand the process for developing a single-residue method; to understand the reasons and challenges of developing multi-residue methods; to understand the intricacies of a multi-residue method by LC-MS/MS; understand and practice an HPLC-Fluorescence method and finally, get a better understanding of issues associated with veterinary drug residues in multi-residue methods

In section 1, we look at single residue methods and the factors taken into account during method development.



Traditional analytical methods were developed for the determination of a single or a small number of contaminants at once. With the advent of LC-MS/MS, methods that determine the presence and quantity of large numbers of analytes are commonplace. While the multi residue methods provide speed and cost savings, some analytes are not compatible enough with large groups of other analytes to be included in a multi residue method. In addition, some analytes require special sample preparation steps that may not be optimal for multi residue methods.

A method needs to be selected with a though process of the sort shown here, from the Codex Procedural Manual. It must be able to measure the contaminant of interest, in this case lead. Then ,we ask if it is applicable for the matrix, in this case juice. Then, whether it can achieve the LOQ of interest and generally meets the performance requirements for our purpose.

Selection of Method

Fundamentals of Methods Development

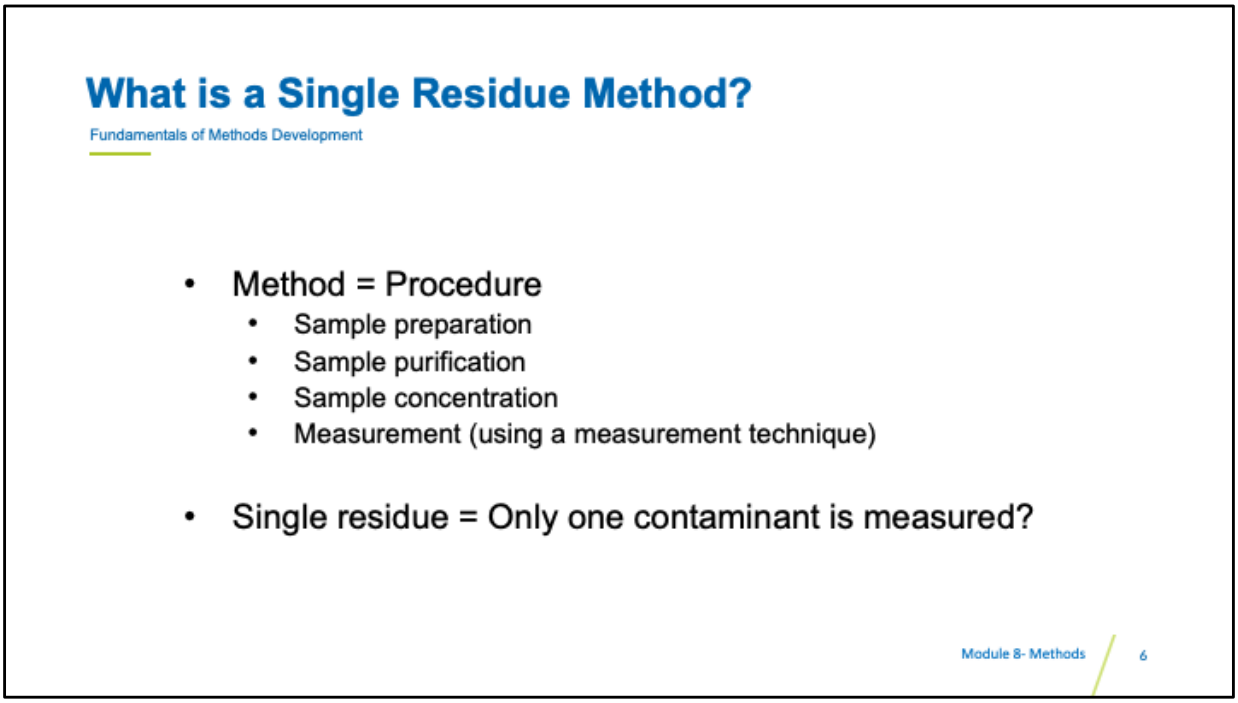
CODEX ALIMENTARIUS COMMISSION

PROCEDURAL MANUAL

Twenty-third edition

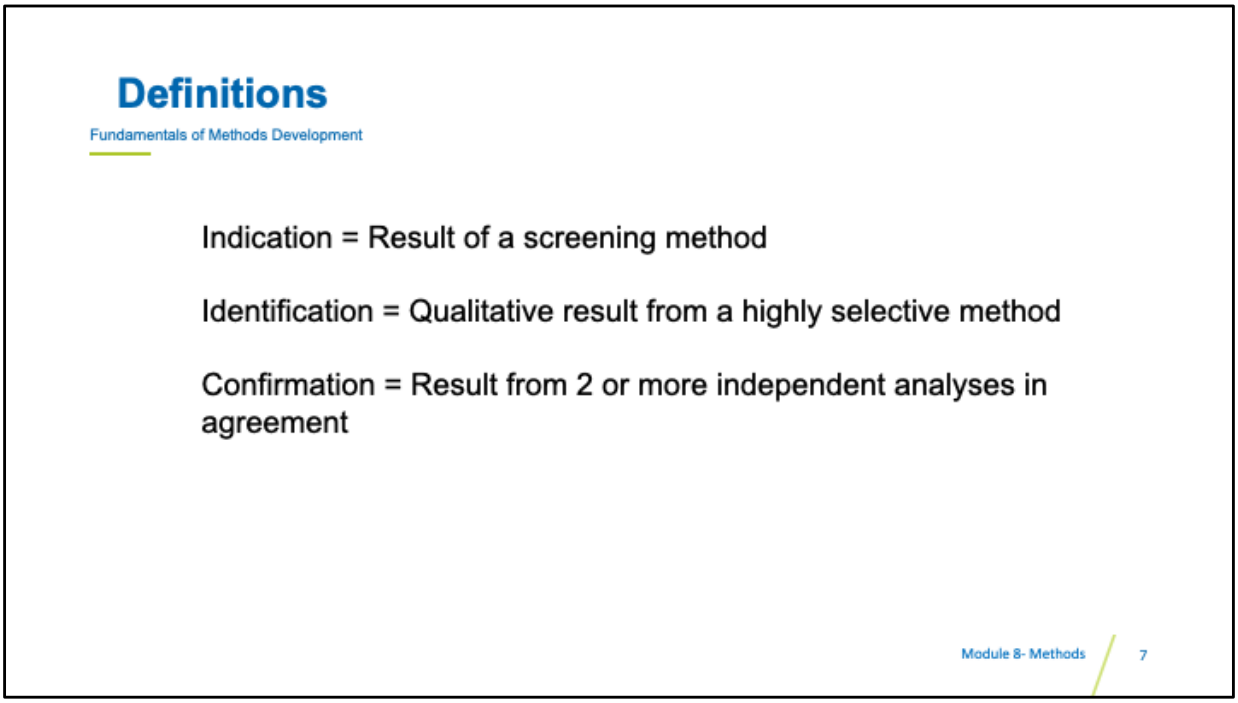
- trueness
- applicability (matrix, concentration range and preference given to 'general' methods)
- limit of detection
- limit of quantification
- precision; repeatability intra-laboratory (within laboratory), reproducibility inter-laboratory (within laboratory and between laboratories), but generated from collaborative trial data rather than measurement uncertainty considerations
- recovery
- selectivity
- sensitivity
- linearity

There are many performance requirements to evaluate. We will review this topic in Module 9. Briefly, we have to look at trueness, applicability, limit of detection, limit of quantitation, precision, repeatability intra-laboratory and inter-laboratory reproducibility, recovery, selectivity, sensitivity and linearity.

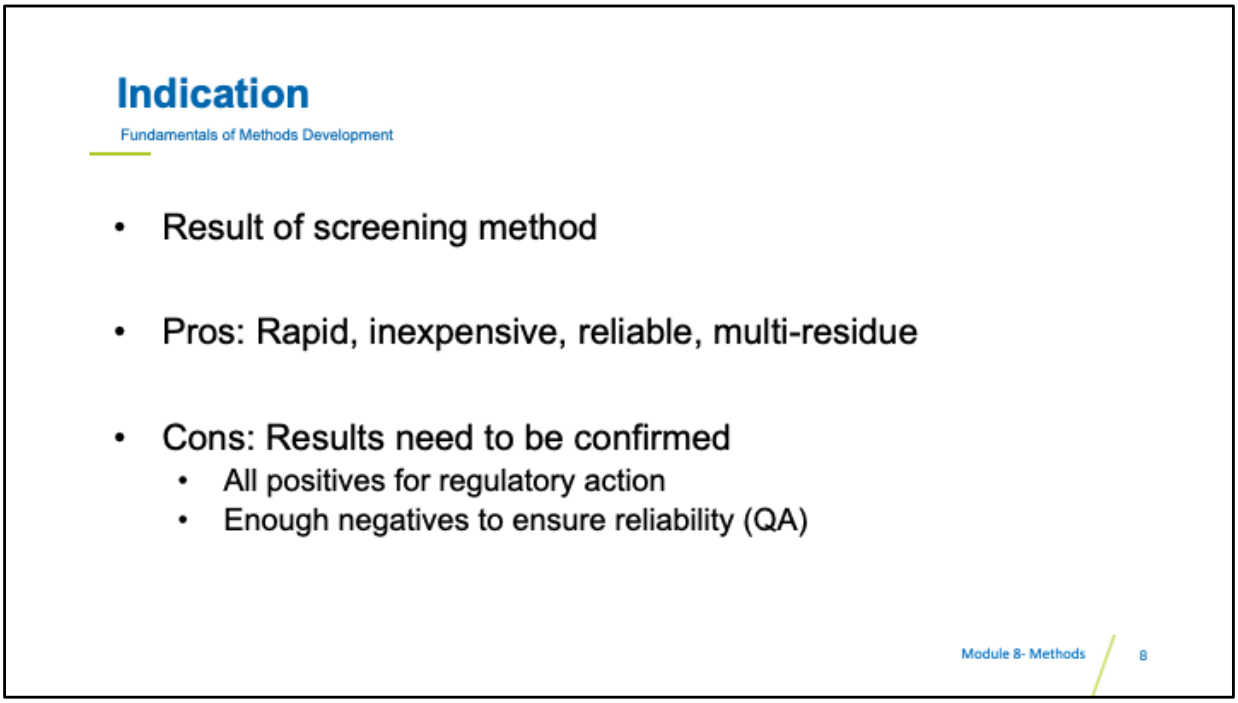


A method typically comprises steps for sample preparation, including homogenization and extraction, purification, adjustment of the concentration either through concentration or dilution, and measurement.

A single residue method is one that measures only one component, but sometimes a group is considered one, like aflatoxins for example. In the case of aflatoxins, there are 4 separate aflatoxins and we could measure them together as one, or individually depending on the measurement technique that we choose and the purpose.



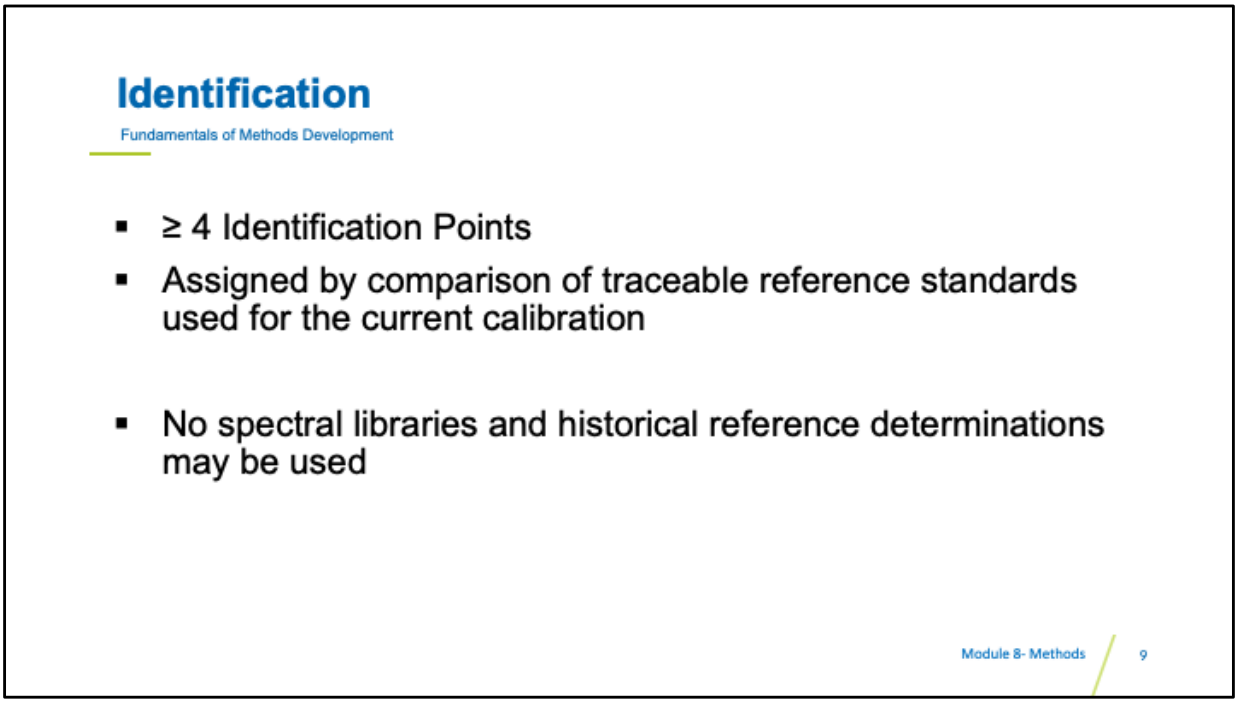
Some definitions are important at this stage. In general, indication is the result of a screening method. Identification is a qualitative result obtained from a highly selective method and confirmation is the agreement of results from two or more independent analyses.



Screening methods are very important in food safety because of the large number of samples and an increasingly larger number of contaminants covered by regulations.

Screening methods are typically rapid, inexpensive and reliable, and they can be single or multi residue methods.

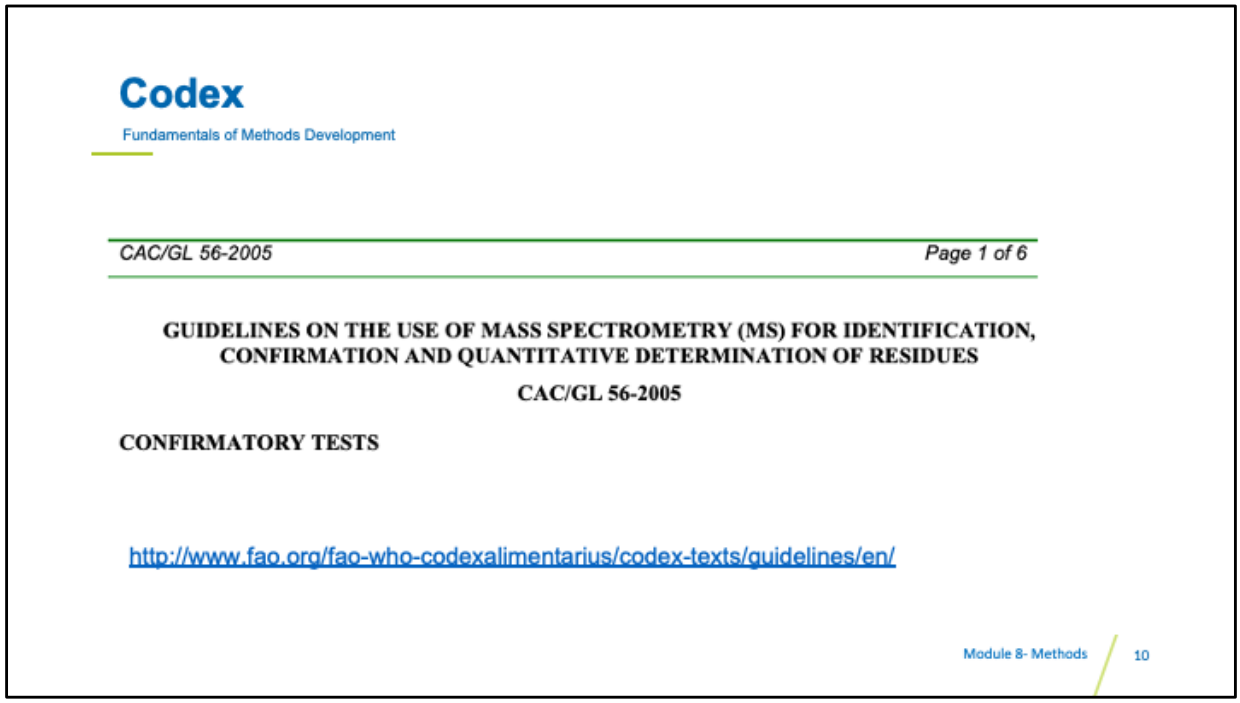
The main disadvantage is that the result must be confirmed. In most regulatory systems, all positive screening results must be confirmed through a second analysis before regulatory action can be taken. This is to avoid acting on a false positive result. The number of negative results that are confirmed is more variable. It is important that enough negative results are confirmed to ensure the reliability of the screening method, in this case a low level of false negatives. This topic will be revisited in module 9 on quality assurance systems.



A method used for the identification can be indicative or must confirm this identity. It is important to know what the terminology means in your regulations. We use a set of criteria that have been assigned point values to determine if a method is just for indication or for confirmation. An identification in the United States must score at least 4 points, while confirmation methods need to score at least 5 points.

First and foremost, the points are based on a comparison with a reference standard used in the same run as our samples.

We cannot use a library or historical data for comparison with a current chromatogram...



Codex issues guidelines on the use of mass spectrometry for the identification, confirmation and quantitative determination of residues, which is identified by this document number. The reference at the bottom of the page is to the library of guidelines issued by Codex, including this one.

Assigning Identification Points

Fundamentals of Methods Development

Criteria	Point Assignment
Matching chromatographic retention time (RT)	1 point per alternative systems
Selective detection with matching RT	1 point per detector
Quantitative agreement between alternate column/detectors	1 point per sample
Isomers with matching RT	1 point

These are criteria associated with the chromatography. In other words, one point is allocated if we have a peak at the same retention time as our reference standard. If we have a single detector and run the sample on two different columns, we can accumulate 2 points.

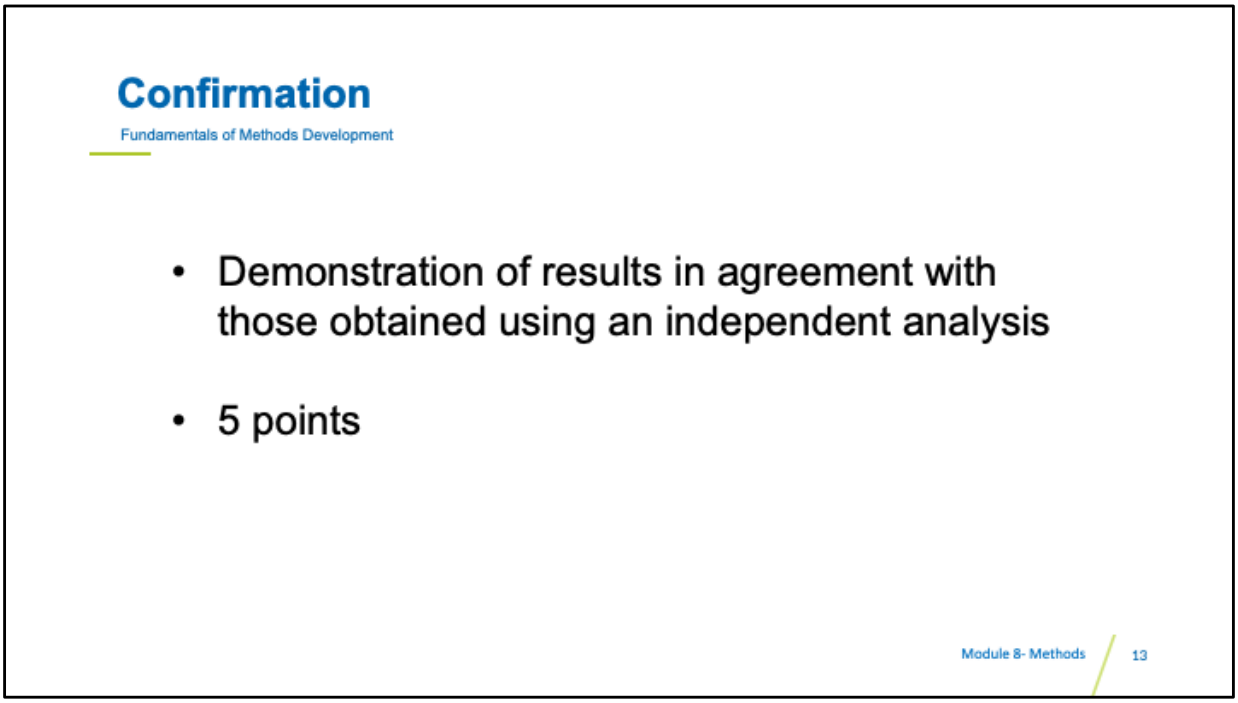
Assigning Identification Points

Fundamentals of Methods Development

Criteria	Point Assignment
Low resolution MS ion	1 point per ion
Low Resolution MS/MS precursor ion	1 point per precursor ion
Low resolution MS/MS product ion (transition)	1.5 points per ion
High resolution MS (HRMS) ion	2.0 points per ion
High resolution MS precursor ion	2.0 points per ion
High resolution MS product ion (transition)	2.5 points per ion

When using mass spectrometry as the detector, then there is a point for the precursor ion, or when using MS/MS, there is a 1.5 value for each transition. As we mentioned before, a transition is the combination of a precursor and a product ion. With high resolution mass spectrometry, there are 2 points per precursor ion, and 2.5 points per transition.

Consequently, a matching retention time and two transitions provide enough certainty for confirmation in LC-MS/MS.



A confirmation is a demonstration of results in agreement with those obtained using an independent analysis. In the points system we just saw, the chromatography and mass spectrometry are considered 2 independent analyses since they use completely different principles. Two columns are also considered two analyses.

In the point system, we want 5 points for confirmation.

Codex Criteria for Confirmation for non-MS methods

Fundamentals of Methods Development

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Table 2. Examples of detection methods suitable for the confirmatory analysis of substances

Detection method	Criterion
LC or GC and MS	If sufficient number of fragment ions are monitored
LC-DAD	If the UV spectrum is characteristic
LC – fluorescence	In combination with other techniques
2-D TLC – (spectrophotometry)	In combination with other techniques
GC-ECD, NPD, FPD	Only if combined with two or more separation techniques
LC-immunoaffinity	In combination with other techniques
LC-UV/VIS (single wavelength)	In combination with other techniques

http://www.fao.org/fao-who-codexalimentarius/shproxy/en/?lnk=1&url=https%253A%252F%252Fworkspace.fao.org%252Fsites%252Fcodex%252FStandards%252FCXG%2B90-2017%252FCXG_090e.pdf

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Codex has published a list of criteria for confirmation using different methods of analysis. The rationale is the same as in the example from the previous two slides, but it is less prescriptive as Codex lets each authority decide the specifics of how many points they want.

As a general rule, only the combination of chromatography and mass spectrometry provides enough evidence in a single analysis to be considered a confirmed result. LC-diode array can be enough if the whole spectrum is characteristic. The others all need a second analysis to confirm.

http://www.fao.org/fao-who-codexalimentarius/sh-proxy/en/?lnk=1&url=https%253A%252F%252Fworkspace.fao.org%252Fsites%252Fcodex%252FStandards%252FCXG%2B90-2017%252FCXG_090e.pdf

Criteria

Fundamentals of Methods Development

European Method		
Relative intensity (% of base peak)	EI-GC-MS (relative units)	CI-GC-MS, GC-MSn, LC-MS, LC-MSn (relative units)
>50 %	±10 %	± 2 %
>20-50 %	± 15 %	± 25 %
>10 – 20 %	± 20 %	± 30 %
≤ 10 %	± 25 %	± 50 %

FDA-ORA Lab 10 Document		
Relative intensity (% of base peak)	Tolerance Window EI-GC/MS	Tolerance Window LC/MS
>40 %	±10 % absolute units	± 20 % relative units
>10 – 40 %	± 25 % relative units	± 25 % relative units
≤ 10 %	± 50 % relative units	± 50 % relative units

In the criteria associated with the chromatogram, the peaks not only need to appear at the same retention time, but also need to have similar intensities for similar concentration of standards. These two tables compare criteria from the European methods and from the US FDA. We will talk about these in more details in the in-person section of this course.

Criteria

Fundamentals of Methods Development

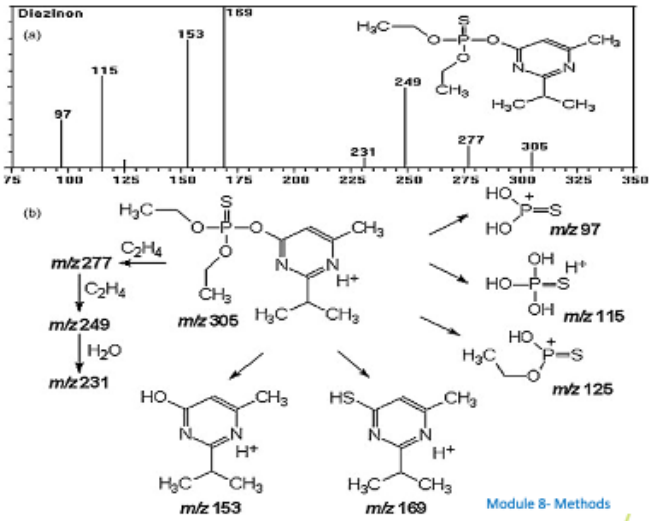
	LC-MS	GC-MS
Ion Ratio	50 %	40 - 60 % Calculated from ± 20 % relative units
Tolerance Window	50 %	40 – 60% Calculated from ± 10 % absolute units

There are also quantitative criteria associated with the ion ratios. If the ion ratio is not similar under the same ionization conditions, there is a good likelihood that the compounds are not the same even if they share a precursor and a product ion.

Selection of Diagnostic Ions in MS

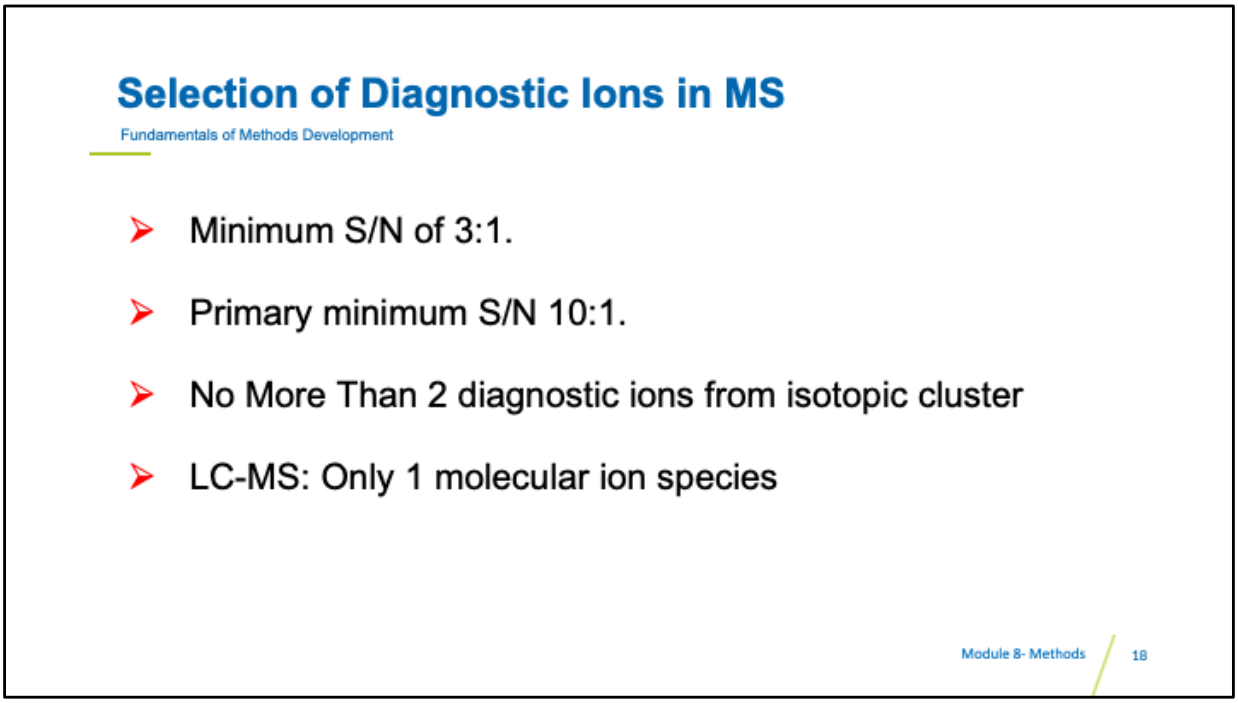
Fundamentals of Methods Development

Rational selection based on ion chemistry



Let's go back more specifically to the method development steps. We need to select diagnostic ions for our compound of interest. We know that the collision chamber will create many different product ions, so we need to choose wisely. A good ion is one that is specific to the fragmentation of the compound of interest. In other words, it contains important portions of the original molecule. It should not be common from the fragmentation of a lot of molecules.

In the example illustrated here, we have the precursor ion in the center, and a number of fragments with relevant chemistries. The mass spectrum shows the peak intensities for all of them. We would want to use ions with a high intensity, because the intensity affects our limit of quantitation. So we might choose the transition of 305 > 169.



There are minimum values of peak intensities to look for. This is in the chromatogram.

A good primary ion has a signal to noise ratio of of at least 10 to 1. This is the minimum for quantitation. The second ion should have a S/N of at least 3:1. If there is an isotopic cluster, we should not use more than 2 diagnostic ions from the cluster... More than that is just redundant information.

Finally, in LC/MS, we have only one molecular ion and we should always use it as our precursor. As we discussed in Module 5, we can have 2 in GC, so we could choose which one to use for MS/MS, or use both for GC/MS.

Practical Example

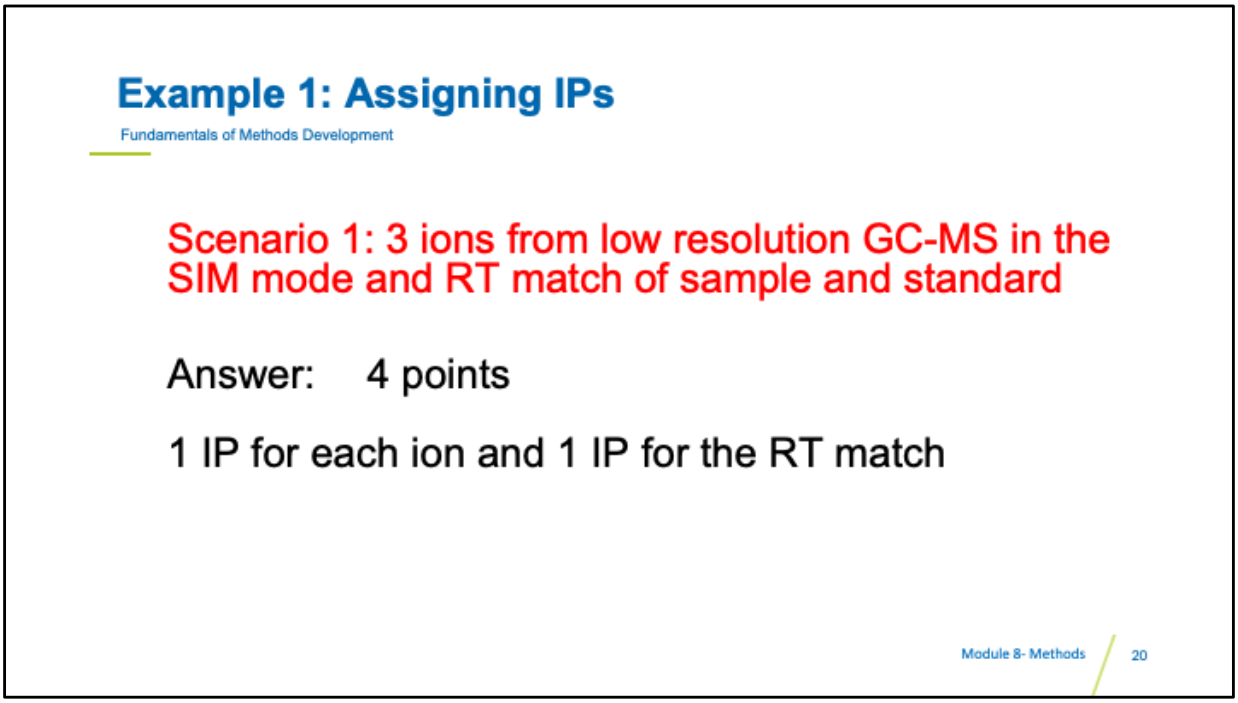
Fundamentals of Methods Development

Perchlorate MRM Ratios
(Secondary/Primary)

ng/g Found	Average Ratios in Std %	Ratios in Sample %	FDA-ORA Lab 10	EU Acceptable Range
			Acceptable Range ± 25% Relative	Acceptable Range ± 25% Relative
1.7	33.65	33.65	25.24-42.06	25.24-42.06
3.9	33.65	36.81	25.24-42.06	25.24-42.06
7.5	33.65	29.01	25.24-42.06	25.24-42.06
1.9	33.65	32.44	25.24-42.06	25.24-42.06

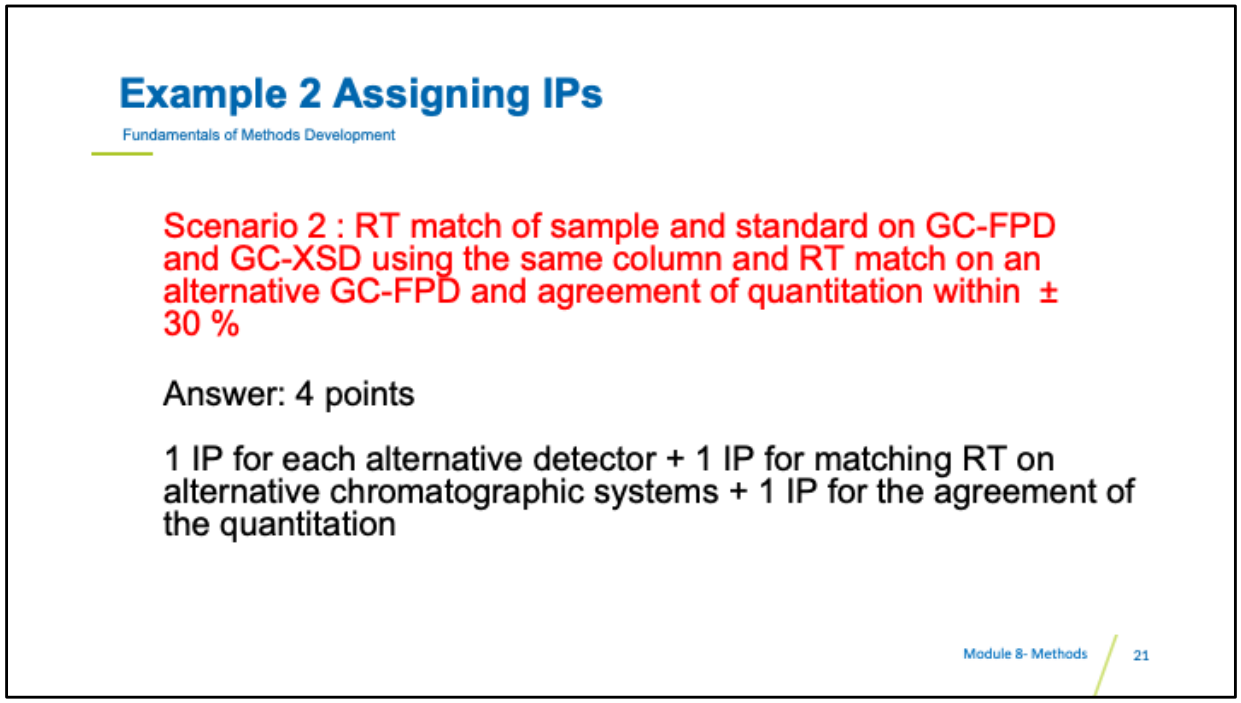
NOTE: LOQ is 1.0 ng/g

Let's look at an example here. This is perchlorate in melons. The ion ratio of the secondary over the primary diagnostic ions is just under 34% in the standard, and in the sample, it varies from 29 to 37%. The two columns on the right indicate that these ions would be acceptable both in the US and in the EU.



Let's review a few examples of how we calculate the score, or the number of identification points for methods to decide if we have enough for identification. In this first example, a result is submitted to us, which contains 3 ions from low resolution GC-MS in the SIM mode and RT match of the standard and sample. Is it an identification?

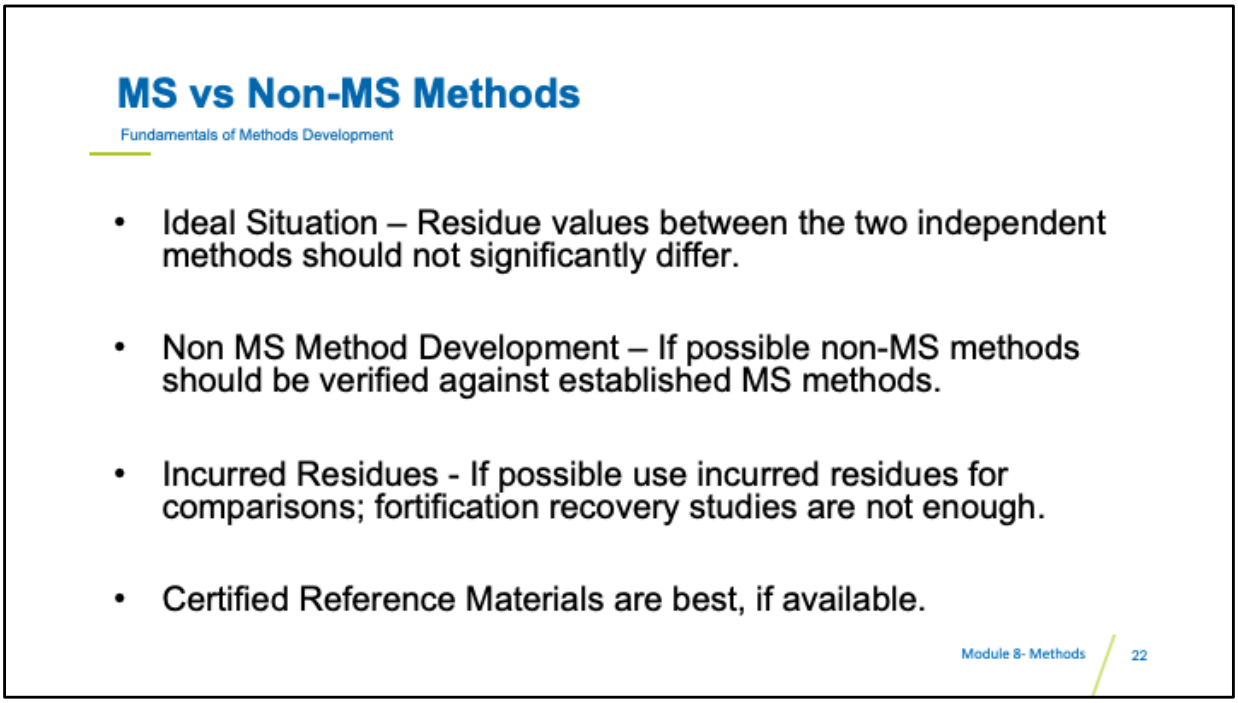
Yes, it scores 4 points. Here, it is a single MS, so precursor ions are all 1 point. 1 identification point for each ion and 1 identification point for the retention time match, for a total of 4.



In this 2nd example, we have RT match of sample and standard on GC-FPD and GC-XSD using the same column and RT match on an alternative GC-FPD and agreement of quantitation within ± 30 %. Do we have identification?

The answer is yes, this has scored 4 points.

1 IP is given for each alternative detector + 1 IP for matching RT on alternative chromatography systems + 1 IP for the agreement of quantitation by two independent methods, for a total of 4.

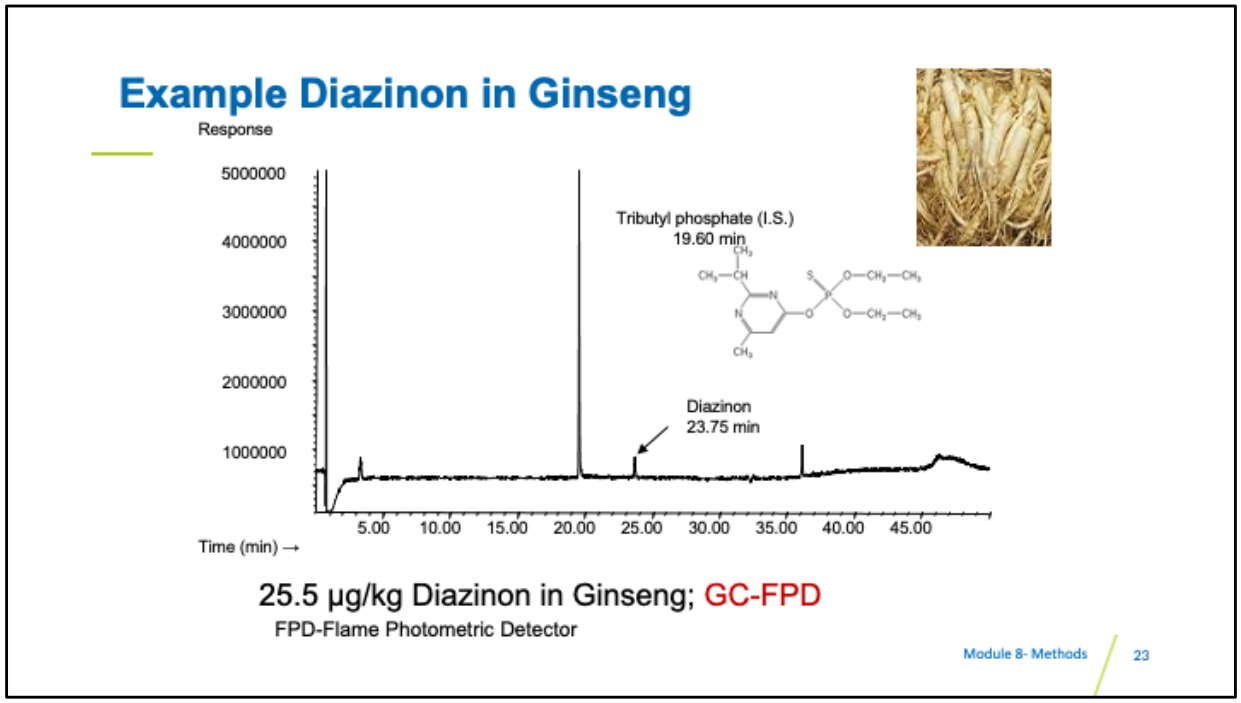


These two examples showed that MS is not the only instrument that can be used, especially since it can be difficult to deploy in laboratories where the electrical supply is not stable.

If we use two no-mass spectrometry methods, then residue values between the two independent methods should not be significantly different. In an ideal world, we would be able to verify our method with mass spectrometry for confirmation and see that we obtain the same results.

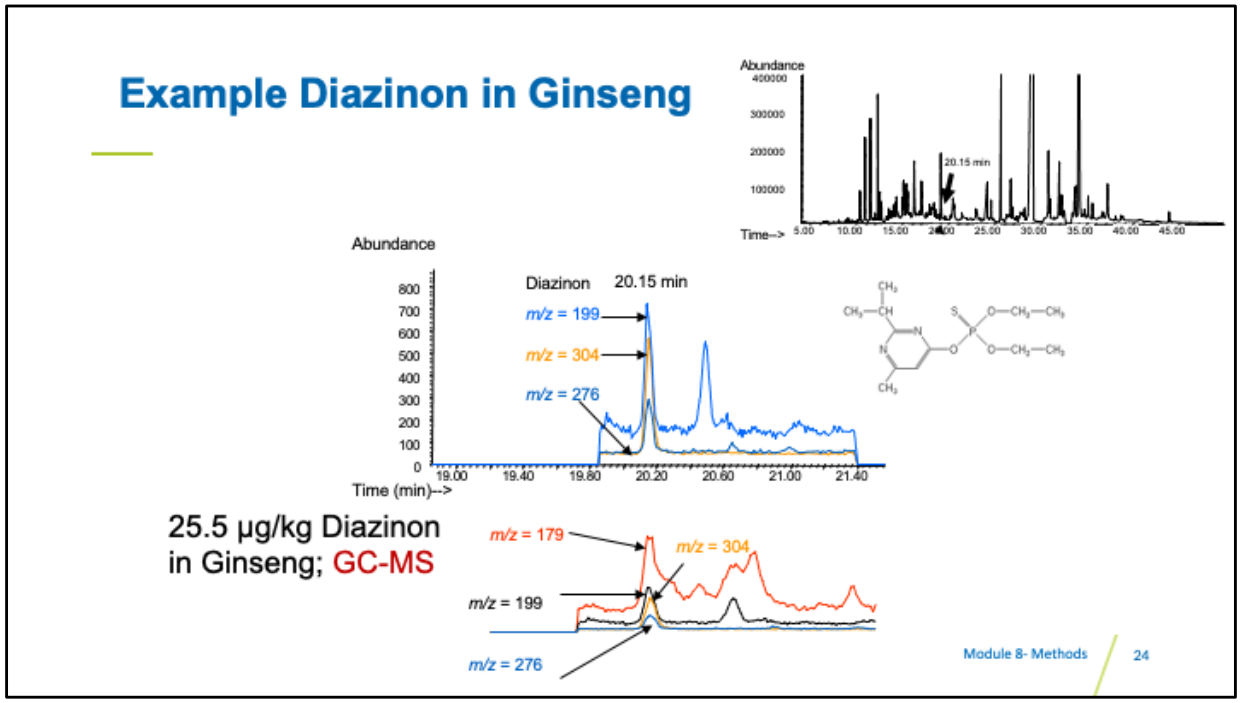
If possible, we also need to verify these parameters with incurred samples. An incurred sample is one that is naturally contaminated, as opposed to a laboratory spike. Recovery determination using fortified samples in not sufficient.

Finally, the reference standards should be certified if possible, or at least verified by an independent analysis.



Let's look at a real life example with the data now. This work was presented by Drs. Alex Krinitsky and Jon Wong of the US FDA. We are looking for diazinon in ginseng. Diazinon is an organophosphate insecticide. As a side note, ginseng is a difficult sample, that's why it is a good example for limitations of methods.

In GC with an FPD detector, we have a peak at 23.75 minutes and our reference standard has a peak at 19.6 minutes.



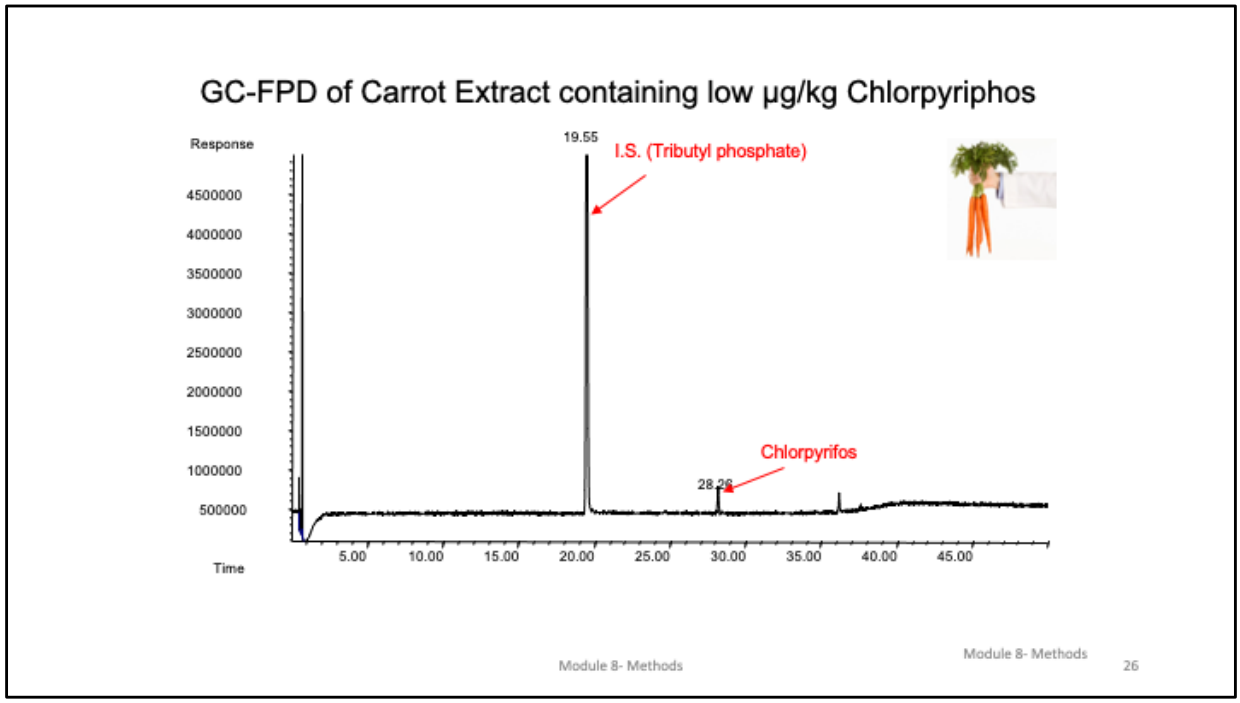
In GC-MS, we have a peak in the chromatogram on the top right at 20.15 min, and we see the overlapping peaks of our internal standard and ions for diazinon.

Findings with Diazinon in Ginseng

- GC-MS/SIM
  - satisfy identification criteria
  - RT match 4 ID Points
- GC-MS/SIM combined with FPD
  - satisfy identification & confirmation criteria
  - RT match with diazinon 5 ID Points

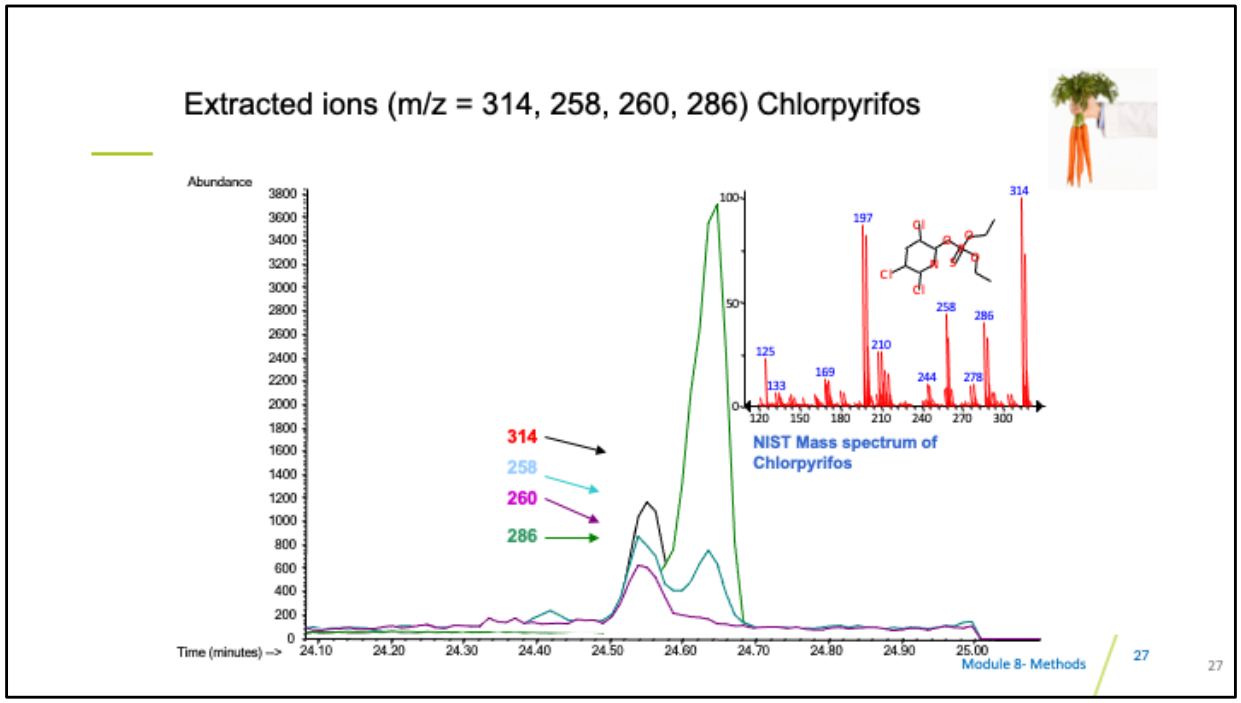
So, what can we conclude? The GC with two single ion monitoring by MS is enough for identification.

When combining the results of GC FPD and GC-MS with two single ions and observing the matching retention time, then we have confirmation.



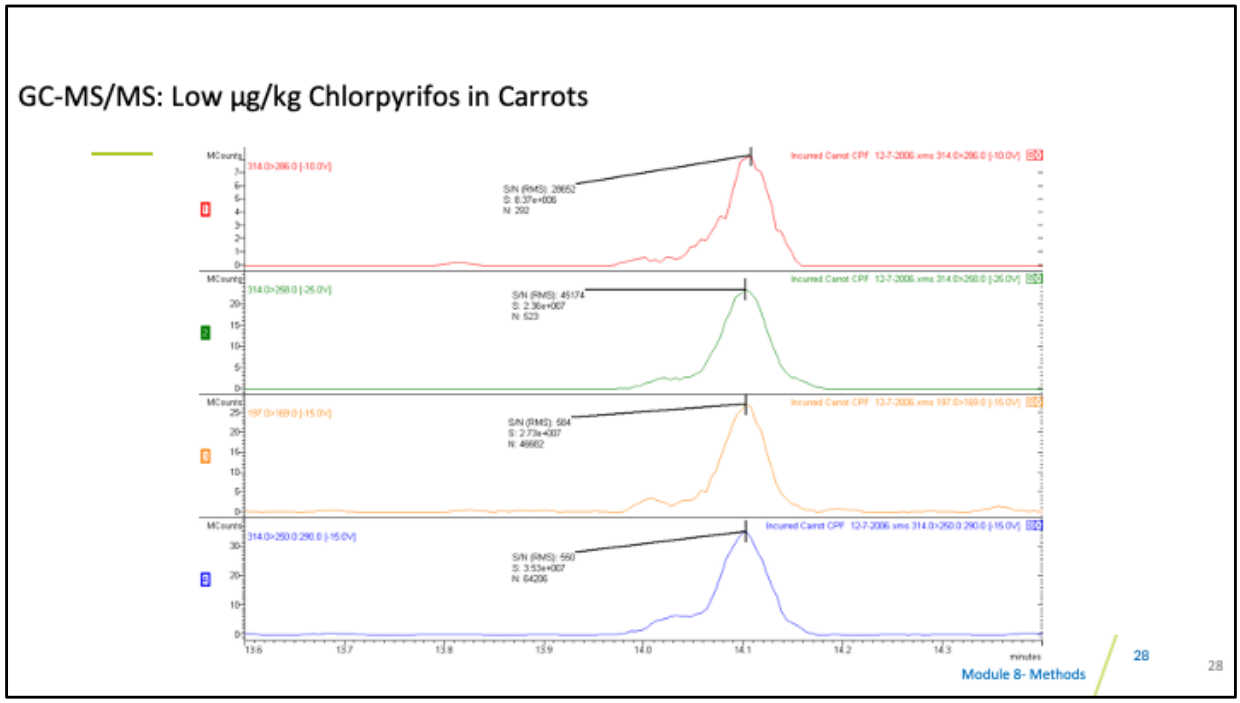
In this second example, we are looking at GC-FPD of Carrot Extract containing low concentration of Chlorpyrifos, another organophosphate pesticide.

The GC-FPD chromatogram shows a retention time of just over 28 minutes.

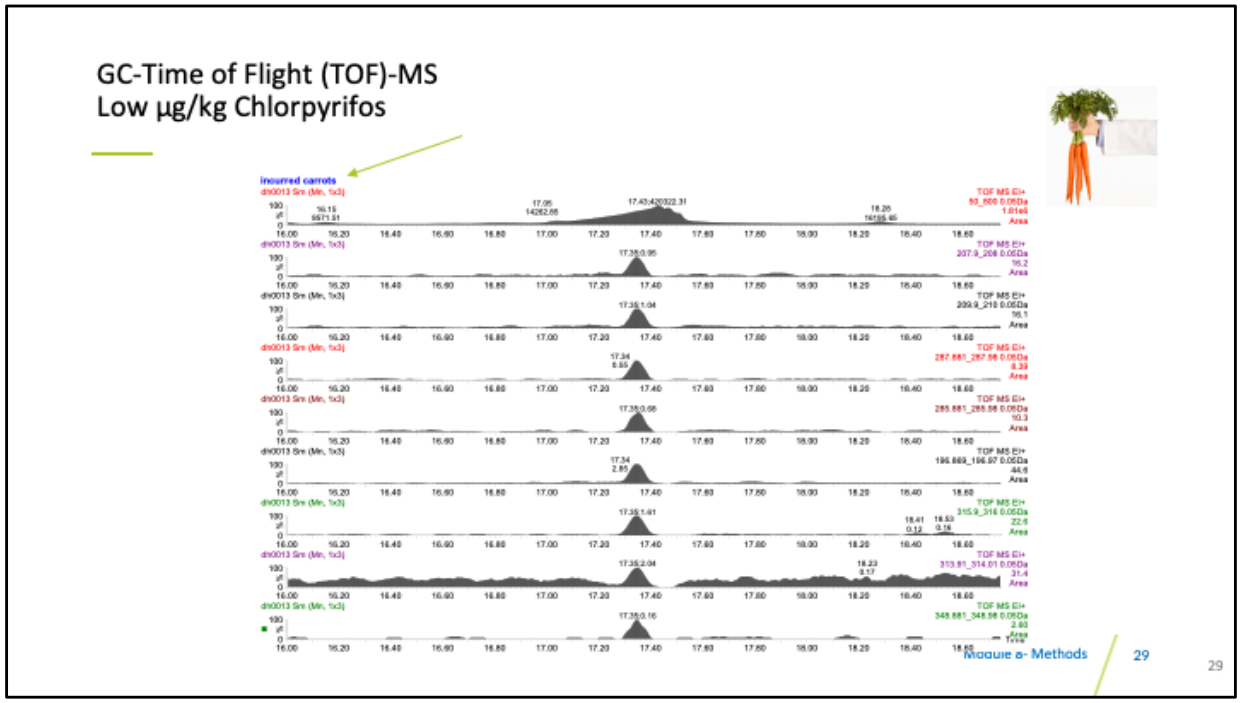


Now we have the mass spectrum on the right and the three chromatograms from the single reaction ion monitoring for m/z = 314, 258, 260, 286.

The problem we see here is an interference with two of the ions.



So we go to GC-MS/MS: for the same sample, where we are looking at the transitions: 314>286, 314>258, and 314>250, and 197>169. All of them have very high S/N ratio and no interference.



Next, we look at the GC-Time of Flight (TOF)-MS chromatograms.

Low µg/kg Chlorpyrifos in carrots is observed again. I should mention that all of these chromatograms were acquired from incurred samples.

Findings with Chlorpyrifos in Carrots

- GC-MS/SIM alone did not satisfy identification criteria
- GC-MS/SIM with FPD satisfied identification and confirmation
- GC-MS/MS and GC-TOF-MS each alone satisfied identification criteria

The findings in this case are that GC-MS/SIM alone did not satisfy identification criteria since only two diagnostic ions, with the proper ion ratios, were free from interferences.

GC-MS/SIM combined with FPD did satisfy identification and confirmation since they are two different detectors and the retention times matched with chlorpyrifos.

GC-MS/MS and GC-TOF-MS each alone satisfied identification criteria since diagnostic ions, with the proper ion ratios, were free from interferences. These also satisfy confirmation criteria.

LOD Calculation

Fundamentals of Method Development

Estimated LODs (S/N = 3) converted into picograms

Pesticide	3-Ion SIM	1-Ion SIM	GC-TOF	GCxGC TOF	HRMS w/TOF	Triple Quad. MS/MS
Endosulfan	24	15	12	4	0.8	0.5
Endosulfan sulfate	97	3	8	5	0.3	0.5
Heptachlor	24	2	5	0.6	0.2	0.2
Lindane	24	3	9	0.5	0.2	0.05

From **Alexander B. Fialkov et al., *International Journal of Mass Spectrometry* (2006), doi:10.1016/j.ijms.2006.07.002**

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Last but not least, the LOQ and LOD of a method need to be calculated. We will do this in our lab session, but here is a good reference. Briefly, we calculate the LOQ as the concentraton that provides a peak with a S/N of 10:1, and LOD at a S/N ratio of 3:1, and then we back calculate how much was in the original sample, before it went through sample preparation, which may include concentration of the residue.

The point of showing the results here is to compare the values of LOD for different techniques. If an MRL is really low, there may not be very many techniques to measure it...

You have reached the end of lesson 1 focusing on the thinking that goes into method development for a single residue method.

Next is lesson 2, where we expand to multi-residue methods.