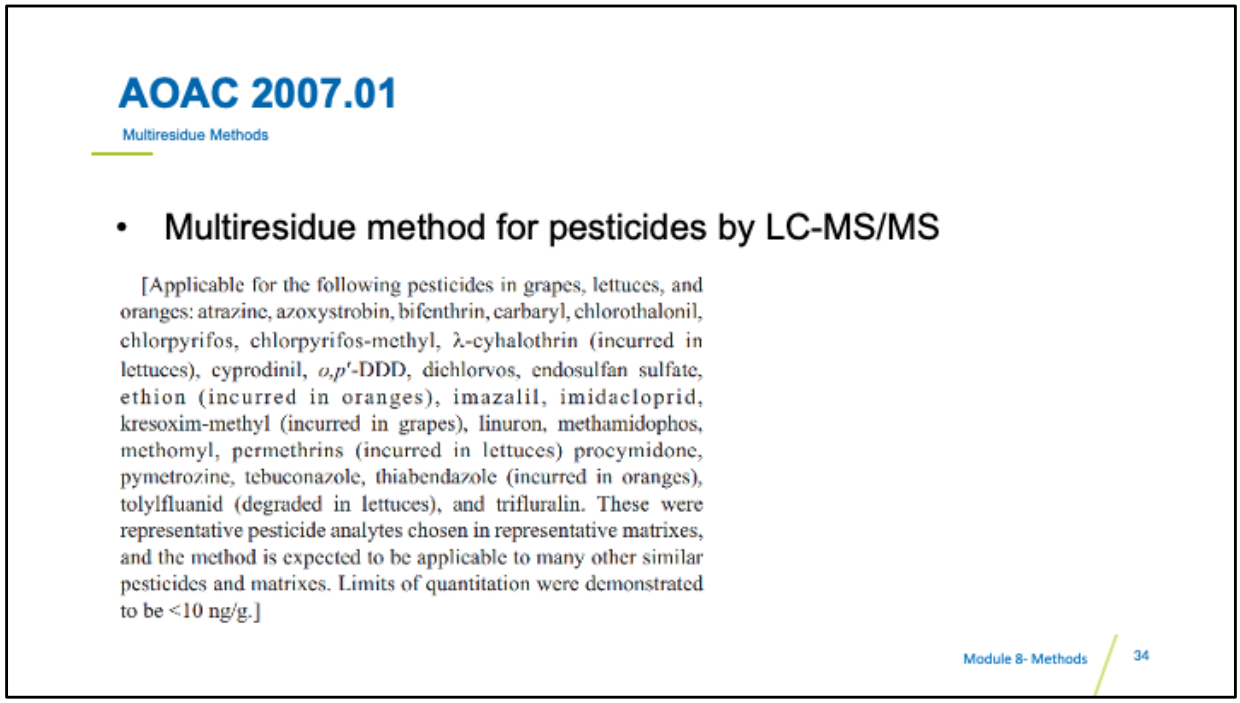
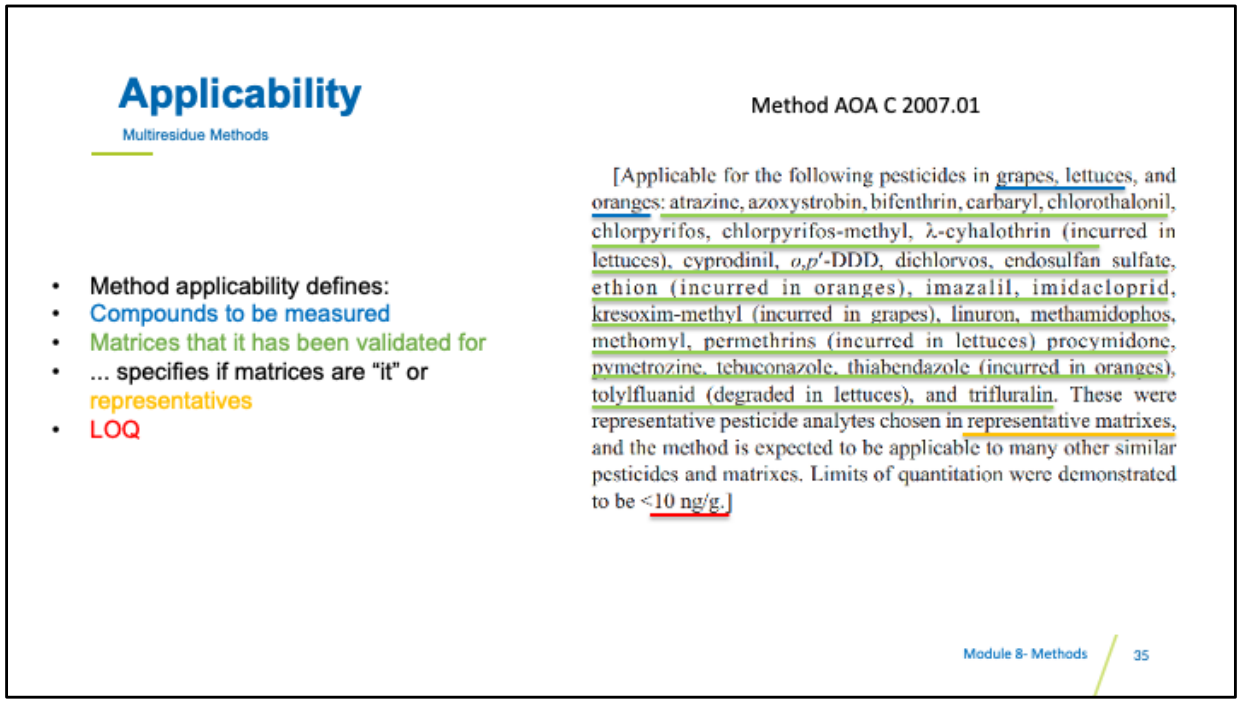
Welcome to lesson 2 of this module of method development. In this lesson, we look at important considerations in the development of multi-residue methods.

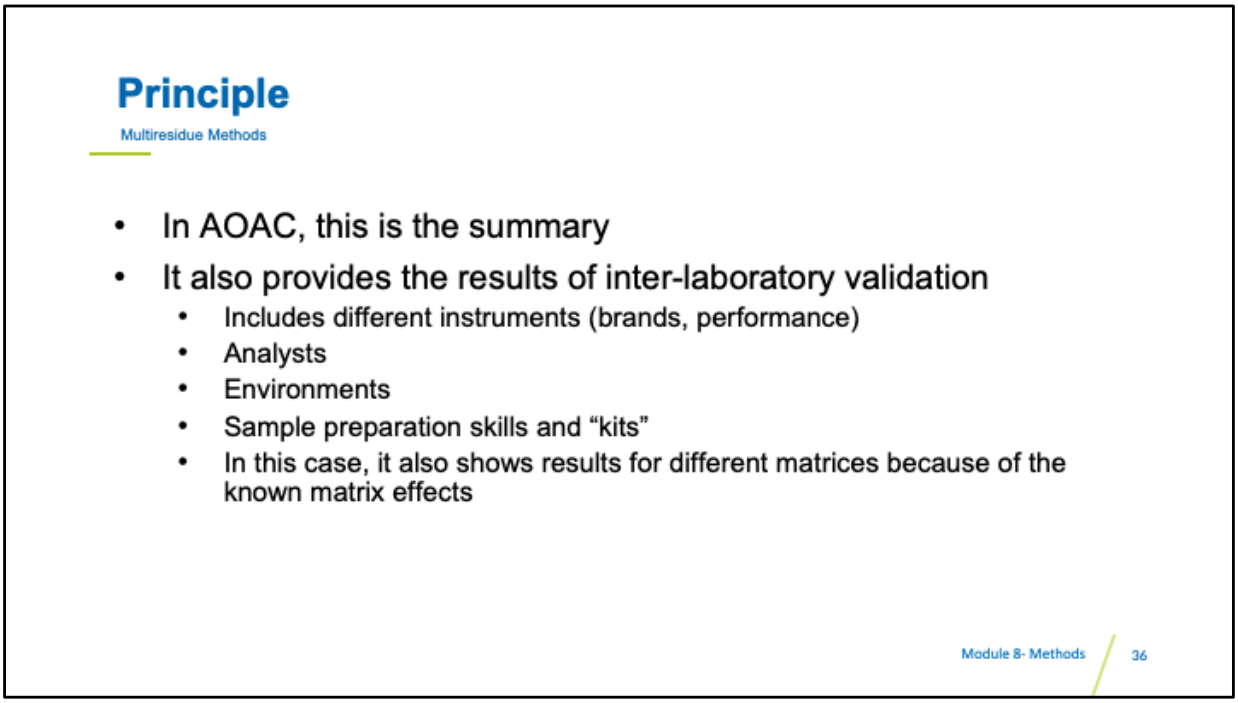


We took the time in lesson 1 to review how we decide which technique we want to use according to our purpose of either identification or confirmation. Now, let's look at it from a different angle. Let's look at an official method and dissect it to understand the important parameters. For this example, I chose AOAC official method 2007.01, a multi-residue method for pesticides residues using LC/MS/MS.

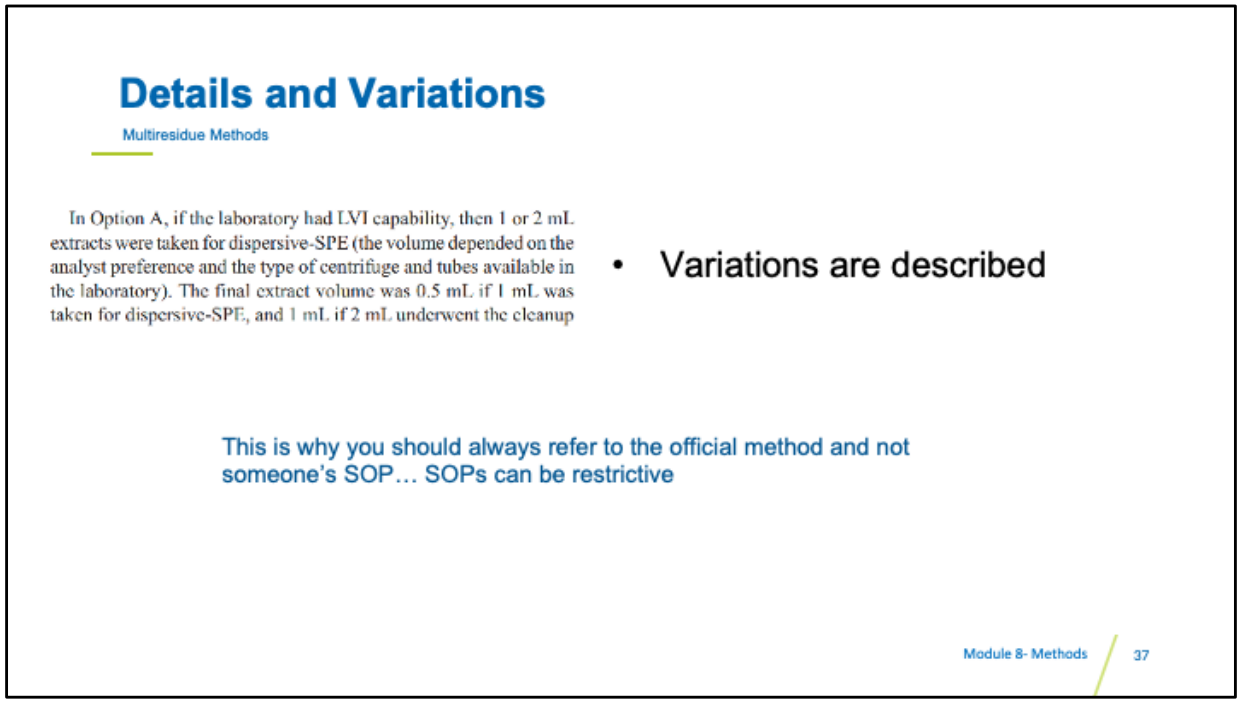


First, we look at the applicability. The method is applicable for grapes, lettuces and oranges, and for a relatively short list of pesticides. An important point to recognize is that methods are usually developed to fit the needs of one or more laboratories. In situations where the laboratory has regional responsibilities, it is quite common to see the method cover only commodities that are grown in the region and pesticides that are registered or expected to be used for these crops. Methods with extremely large scopes of application are much more difficult to develop and may require some compromises that are not necessary for laboratories with limited objectives. For example, sample preparation may require additional steps or more expensive consumables to be adapted for a broader range of commodities. A laboratory that doesn't have a broad range of commodities shouldn't add these steps and spend money on the consumables.

- Sometimes it will specify if matrices are "it" or representatives, in this case representativve, and finally, the LOQ is given.



In AOAC, this is the summary. It also provides the results of inter-laboratory validation and includes different instruments (brands, and performance profiles), analysts, environments, sample preparation, skills and "kits". In this case, it also shows results for different matrices because of the known matrix effects.



In this method, variations were used in laboratories with different equipment. So laboratories with large volume injection capability for their GC had an option shown here.

This is why you should always refer to the official method and not someone's SOP… SOPs can be restrictive

Apparatus and Conditions

Multiresidue Methods

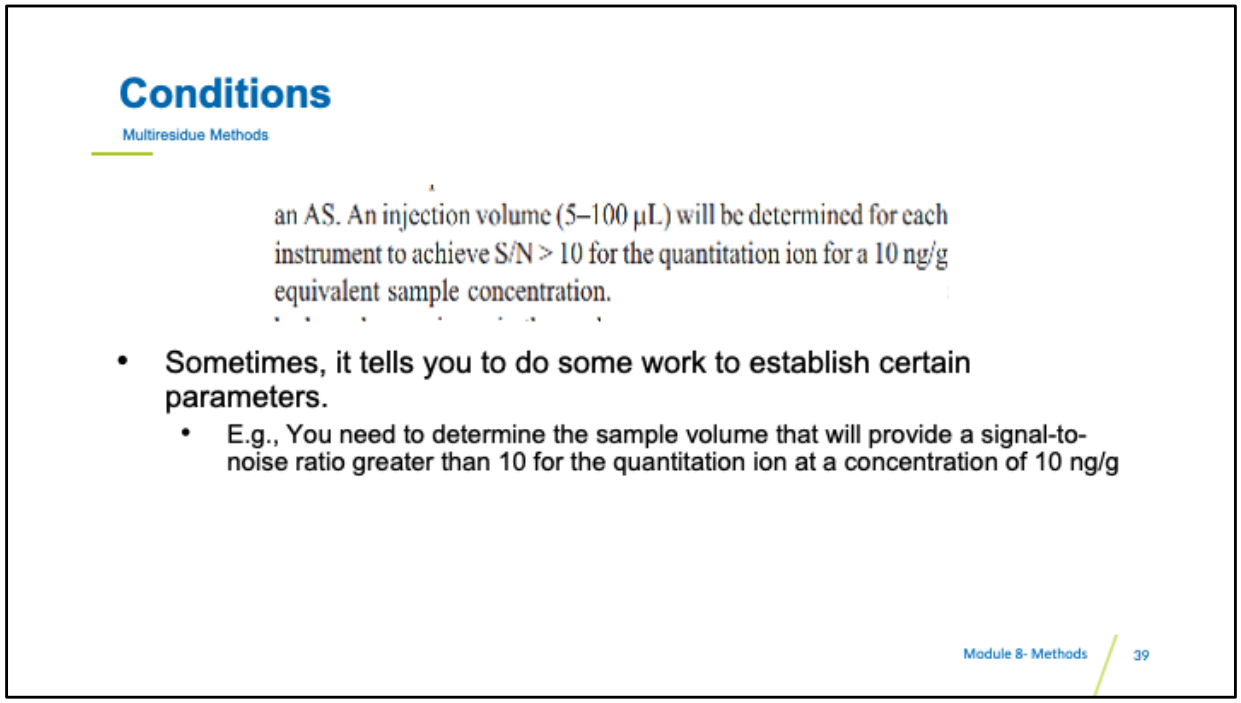
Defines the technologies applicable to the method

- Mass analyzer: Ion trap, TOF, MS/MS
- Ionization: EI
- Type of injection: Splitless (with volume)
- Final solvent(s) of extract
- Column

(a) *Gas chromatograph/mass spectrometer.*—An ion trap, quadrupole, time-of-flight (TOF), or other GC/MS instrument may be used with electron impact (EI) ionization, an autosampler (AS), and computerized instrument control/data collection. Either LVI of 8 µL for a 1 g/mL MeCN extract (e.g., 75°C ramped to 275°C at 200°C/min) or 2 µL splitless injection of 4 g/mL extracts in toluene at 250°C may be used. A 3–5 m, 0.25 mm id, phenylmethyl-deactivated guard column must be used as a retention gap in either case. The analytical column is a 30 m, 0.25 mm id, 0.25 µm film thickness (5%phenyl)-methylpolysiloxane (low bleed) analytical column (DB-5ms or equivalent). Set He head

The method also defines the technologies applicable to the method.
In method 2007.01, the pesticides of interest justify the requirement for two measurement techniques, namely GC-MS and LC/MS/MS. These different techniques in turn impose a number of different steps in the sample preparation.

The ionization is EI in GC, the type of injection is Splitless with volume, the final solvent of extract is toluene and the column is specified.



Sometimes, it tells you to do some work to establish certain parameters. In this example, you need to determine the sample volume that will provide a signal-to-noise ratio greater than 10 for the quantitation ion at a concentration of 10 ng/g.

Procedure

Multiresidue Methods

- Steps are clear
  - But you may need to adapt (e.g., Teflon tube)
  - Or get some help from references or vendors

(k) *Methanol (MeOH).*—Quality of sufficient purity that is free of interfering compounds in LC/MS/MS prepared in mobile phase solution.

(l) *Water.*—Quality of sufficient purity that is free of interfering compounds in LC/MS/MS.

Step	Procedure
0.	Comminute >1 kg sample with vertical cutter. Homogenize ≈200 g subsample with probe blender.
1,2.	Transfer 15 g subsample to 50 mL Teflon tube.
3-5.	Add 15 mL 1% Hac in MeCN + 1.5 g anh. NaAc + 6 g anh. $MgSO_4$ + 75 µL I.S. solution.
6,7.	Shake vigorously for 1 min. Centrifuge >1500 rcf for 1 min.
8,9.	Transfer 1-8 mL to tube with 150 mg anh. $MgSO_4$ + 50 mg PSA per mL extract and shake for 30 s.
10.	Centrifuge >1500 rcf for 1 min.
11-15A.	Transfer 0.5-1 mL extract to GC vial and add TPP. Transfer 0.15-0.3 mL to LC vial and add e.g. 0.45-0.9 mL 6.7 mM formic acid.
11-14B.	Transfer 0.25 mL from Step 10 to LC vial. Add TPP and e.g. 0.86 mL 6.7 mM formic acid.
15-16B.	Transfer 4 mL from Step 10 to grad. cent. tube. Add 0.4 mL TPP Sol'n and 1 mL toluene.
17-19B.	Evaporate at 50°C with N_2 to 0.3-0.5 mL. Add toluene to make 1 mL. Add 0.2 mL anh. $MgSO_4$ and swirl >6 mL mark.
20B.	Centrifuge >1500 rcf for 1 min. Transfer ≈0.6 mL to GC vial.
16A/21B.	Analyze by (LVI)GC/MS and LC/MS-MS

Figure 2007.01. Outline of the QuEChERS protocol used in the collaborative study.

The procedure is a list of steps to follow from commodities to final measurement. The procedure has enough specifications to enable the reproduction of the results, but also enough flexibility to be adapted to the conditions of different laboratories. For example here, paragraphs k and i mention ”sufficient purity”. What is sufficient depends on your instrument. It is quite different from GC-FPD to GC MS and MS/MS uses the highest purity.

Procedure

Multiresidue Methods

- The preparation of the mobile phase and standard mixes is described

(*1*) *Test mix in MeCN + 0.1% HOAc.*—4 ng/µL in 10 mL of all 30 compounds to be analyzed. Add 1 mL each of QC-spike solution + IS solution + TPP test solution + 1% HOAc in MeCN and fill to 10 mL with MeCN. Calibration spike standards in MeCN for 27 pesticide analytes (make 10 mL each in volumetric flasks, then transfer to 15 mL dark glass vials and store in freezer).

(*2*) *Cal-standard-1000.*—20 ng/µL of each pesticide + 4 ng/µL IS in MeCN + 0.1% HOAc. Add 5 mL QC-spike solution + 1 mL IS solution + 1 mL 1% HOAc in MeCN and fill to the mark with MeCN.

The preparation of all reagents and standards as well as the mobile phases is described in the procedure.

Data Analysis

Multiresidue Methods

- Data analysis is described
- The published study paper also shows the spreadsheet provided to collaborators …

I. Data Analysis

Quantitation is based on linear least squares calibration of analyte peak areas plotted versus analyte concentration. The *y*-intercept should be near zero and correlation coefficient (r^2) of the line should be >0.995. The integrated peak area (or the analyte peak area/IS peak area ratio if the IS is used) becomes the signal, S. Peak heights may be evaluated if peak areas are shown to give a problem. The

And the data analysis steps are listed. In this case, we are drawing a straight line through our calibration points and we don't force it to go through zero. The correlation coefficient of the regression should be greater than 0.995. Finally, this method suggested the optional use of an internal standard, so the the peak area that we correlate with the concentration would then actually be the area of the peak for the compound divided by the area of the peak from the standard.

In this case, the full multi-laboratory validation study was published as a paper and the paper contains the spreadsheet that was provided to the collaborators.

Quality of Results

Multiresidue Methods

The acceptability of the results was judged predominantly with respect to recoveries, intralaboratory repeatability, interlaboratory reproducibility, and the Horwitz ratio (HorRat), which is calculated from the equation:

$$HorRat = RSD_R / 2C^{-0.1505}$$

- When selecting a method for regulatory work, the acceptability criteria must match or be better than the performance criteria required…

Analyte	Avg. C, ng/g	s_r, ng/g	RSD_r, %	s_R, ng/g	Rec., %	RSD_R, %	HorRat	No. of labs	Outlier labs[a]
Atrazine	9.3	0.6	6.9	2.0	93	21	0.65	13	
	45	3.2	7.1	5.7	90	13	0.49	13	

s_r = Standard deviation for repeatability (within laboratory).

RSD_r = Relative standard deviation for repeatability.

s_R = Standard deviation for reproducibility (among laboratories).
RSD_R = Relative standard deviation for reproducibility.
C = Cochran outlier; SG = single Grubbs outlier.
RSD_r >15%; 120% < Rec. < 70%; RSD_R >25%; HorRat >1.2; and fewer than 8 laboratories in an assessment.
NA = Not applicable.

The last step is to check that the results meet the requirements for our purpose. If you are testing for commerce, there are national and international requirements that must be met.

Impact of Multi-Residue

Multiresidue Methods

- The method is comparable to single-residue, but:
  - All criteria must be met by each residue individually
  - Recovery can vary significantly, and steps may need to be added
    - Check the recovery of each residue individually with each change
    - Sometimes, split the sample preparation into two streams

Table 2007.01B. Interlaboratory study results for fortified pesticides in grapes

Analyte	Avg. C, ng/g	s_r, ng/g	RSD_r, %	s_R, ng/g	Rec., %	RSD_R, %	HorRat	No. of labs	Outlier labs[a]
Atrazine	9.3	0.6	6.9	2.0	99	21	0.65	13	
	45	3.2	7.1	5.7	90	13	0.49	13	
	365	23	6.2	71	91	19	1.04	13	
Azoxystrobin	9.4	0.6	6.6	2.0	94	21	0.64	13	
	82	8.7	9.4	11	92	12	0.51	12	8-SG
	182	17	9.2	26	91	14	0.70	12	8-SG
Bifenthrin	7.8	0.8	11	2.3	78	30[b]	0.89	11	2-C, 10-C
	88	5.9	6.9	14	88	17	0.73	12	6-C
	923	71	7.7	136	92	15	0.91	13	
Carbaryl	12	1.2	11	2.8	104	27[b]	0.85	12	5-SG
	50	6.4	13	11	100	22	0.87	13	
	1003	70	7.0	189	100	19	1.16	12	5-C
Chlorothalonil	8.3	0.9	14	2.1	63[b]	33[b]	0.97	8	10-C
	59	8.3	14	13	79	23	0.93	10	
	140	19	13	38	70	27[b]	1.27[b]	10	
Chlorpyrifos	8.1	1.5	18[b]	3.0	81	37[b]	1.12	12	
	68	8.3	12	14	84	20	0.84	13	
	395	25	6.4	50	79	12	0.68	12	11-SG

The method is comparable to single-residue method, but all criteria must be met by each individually residue. Recovery can vary significantly, and steps may need to be added. Check the recovery of each residue individually with each change. Sometimes, split the sample preparation into two streams

Conclusions

Multiresidue Methods

- Same criteria for acceptability as single-residue methods
- Each residue must meet the requirements for recovery, residual standard deviation, etc.
- If a method needs adaptation to accommodate more residues, ALL residues must be verified
  - One common option is to split sample preparation to meet completely different extraction conditions

In conclusion, multi-residue method impose the same criteria for acceptability as single-residue methods. Each residue must meet the requirements for recovery, residual standard deviation, and others. If a method needs adaptation to accommodate more residues, ALL residues must be verified. One common option is to split sample preparation to meet completely different extraction conditions.

You have reached the end of Section 2. In section 3, we will discuss the advantages and challenges of multi-residue methods.