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Methods of Analysis for Organic Contaminants

Methods

Module 8

Janie Dubois, Ph.D.

Food Safety Specialist

parera
Food Risk Analysis and Regulatory Excellence Platform

INAF
INSTITUTE OF NUTRITION AND FUNCTIONAL FOODS

UNIVERSITÉ LAVAL
Faculty of Agriculture and Food Sciences

Overview

Introduction

Module 8 of this training is centered on analytical methods for food contaminants using the technologies described in previous modules. A method is a procedure for the accomplishment of a specific objective or task. A number of different methods can be developed to address any given individual contaminant or group of contaminants, but each method is specifically aiming to provide a particular degree of certainty for the identification of the presence of a compound, and possibly quantification. In this regard, we present screening methods that aim to provide an indication of the presence of a contaminant, and confirmation methods that include additional information providing a higher degree of confidence that the contaminant is indeed present.

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

Learning Objectives

- Understand the process for developing a single-residue method
- Understand the reasons and challenges of developing multi-residue methods
- Understand the intricacies of a multi-residue method by LC-MS/MS
- Understand and practice an HPLC-FLD method
- Gain a better understanding of the sample preparation issues associated with pesticide residues in multi-residue methods

This document is part of a training that also includes video and in-person sections. As such, it should not be used as a stand-alone reference.

Lesson 1: Single Residue Methods

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 now 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 a multi residue method. The scope of any given laboratory helps define whether it should seek to implement largely multi residue methods or apply methods with a narrower scope of analytes and commodities.

The objective of the method has relevance for the selection of a number of parameters. 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 large 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 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 results, in this case a low level of false negatives. This topic will be revisited in module 9 on quality assurance systems.

Developing a single residue method

In this section, we use the example of AOAC Official Method 991.31 to walk through the process of method development for the determination of a small number of analytes, in this case, aflatoxins B1, B2, G1, and G2, which get reported as total aflatoxin. The objective of this section is to

understand official methods by going through the steps of method development which led to the decisions found in the method.

1. Purpose of the method

It is important to start method development by defining its objective. A method can aim to detect, quantitate or identify. Detecting means to determine if the compound is present. Quantitation goes one step further and measures how much of it is present. Identification is a more rigorous goal where we need to confirm that the analyte being measured is actually the analyte of interest with a certain degree of confidence.

With the objective of the method in mind, the focus moves to the analyte of interests. A suitable detection method must be selected, and it is strictly dependent on the properties of the analyte. For example, one would ask questions such as:

- Does the analyte absorb UV light;
- Is it fluorescent;
- Can it be ionized; and
- Is the analyte thermally stable?

When the scope of analysis includes quantitation, the concentration range expected from the type of samples targeted by the method must be considered. While the throughput, or the number of samples that must be analyzed per day, should be considered, the level of accuracy and precision required for the end-use of the method (regulatory action **versus** quality assurance in production for example) are the most important criteria for the selection of the best of the suitable techniques.

This is how the analytical technique is selected. In AOAC 991.31, The method is designed for the measurement of aflatoxins in corn, raw peanuts, and peanut butter. Section A indicates that this is a quantitation method for total aflatoxin and that the mode of measurement is fluorescence implemented with solution fluorometry for quantitation and liquid chromatography with fluorescence detection for identification and quantitation.

2. Sample preparation

Sample preparation is a critical step in food safety methods because foods are complex matrices that contain a very large number of components that can hinder the measurement of the analyte of interest. In most cases sample preparation can be divided in a few steps: Extraction, purification, and adjustment of the concentration and/or solvent to match their requirement of the analytical techniques selected.

Extraction is the series of steps taken to remove the majority of matrix components. Extraction usually includes homogenization of a relatively large sample achieved by physical means such as cutting or blending. The objective of the homogenization step is to obtain a sample where the concentration of the analyte of interest is representative of the concentration in a large amount of the food. The sample brought to the laboratory for homogenization should be very large when an analyte is expected to be heterogeneously distributed, such as is the case for aflatoxins.

The homogenized sample typically goes through a number of steps to remove solids and produce a solution containing the analytes of interest and a limited number of other compounds.

In the AOAC method, section E starts with a reference to sampling procedures that should be followed to ensure that the test portion sent to the laboratory is representative of the lot. The test portion is 25 grams. The method of homogenization is blending in an extraction solvent, and the initial cleaning is performed by filtration first through a filter with wider pores and secondly through a glass microfiber paper filter, which has much smaller pores. At this stage the sample no longer contains any solids and the sample components that are not soluble in the extraction solvent have been discarded.

The next step of sample preparation is the purification of the analytes of interest to a degree suitable for the analytical technique. The degree of purification can be very different for two analytical techniques. For example, high purity is required when there is no further separation in the sample and the detection mechanism is not selective. If chromatography is used and followed by a highly selective detector like mass spectrometry, the sample can proceed with in lesser degree of purity as long as the matrix components still present in the sample will not hinder the measurement or damage the instrument.

The purification step in the AOAC method consists in immunoaffinity chromatography. This type of antibody-based purification is very specific and consequently produces a sample of high purity. It also offers the advantage of enabling concentration of the sample to match the detection range of the analytical techniques selected.

3. Measurement

Once a sufficiently pure extract is available, the analytical technique selected is used to measure either the presence or the quantity of the analyte of interest, or both. The instrument must be properly calibrated both as part of the quality assurance program of the laboratory (instrument calibration) and calibrated for the specific purpose of the analysis (method calibration). The ladder calibration refers to the use of standards to determine the signal intensity and the retention time for example.

In our AOAC method, the quantitative method requires an additional step of sample preparation to produce a level of fluorescence compatible with the instrument. This is achieved through the addition of a bromine developer solution prepared in water. The fluorometer is calibrated using standards, in this case a surrogate for aflatoxin. A surrogate is a compound for which the fluorescence intensity can be correlated with the fluorescence intensity measured at one or more specific concentrations of the analyte of interest. In this case, a single concentration is used (20 ng/g equivalent), which is the MRL for aflatoxins in corn (in the United States). The sample is presented to the detector in a fixed pathlength cell, which is long enough to produce a signal intensity for which the signal to noise ratio enables quantitation at the target concentration, or the concentration range of interest.

The confirmation of identity is performed using LC with fluorescence detection using post column derivatization. In this case, a standard calibration curve is produced using certified aflatoxin standards. The LC fluorescence method requires the determination of a number of additional parameters during development. As discussed in previous modules the liquid chromatography method defines mobile phase, its flow rate, a column or stationary phase, a column temperature, and a detection mechanism. In the AOAC method the mobile phase is a degassed mixture of water, acetonitrile and isopropanol in proportions of (3+1+1) pumped at a flow rate of 1 ml/min. The column dimensions are 4.6 mm x 25 cm, the particle size is 5 µm, and the column chemistry is C18.

The post column derivatization is performed using an iodine solution and fluorescence is excited at 360 nm and emission measured with a >420 nm cutoff filter.

Point System for Confirmation

A point system has been developed to ensure agreement in the testing community on the validity of results. The point system is based on a number of characteristics of the analytical result of methods. It is important to remember that the analytical result is not a number or a concentration, but rather the chromatogram or the spectrum depending on the type of instrument used. The number (concentration) is an expression of the result. This is important because it means that a supervisor tasked with reviewing results should not be looking at the numbers, but rather at the chromatograms… Some examples of criteria assigned one point each include matching chromatographic retention time, selective detection with a matching retention time, quantitative agreement between alternate columns or detectors, and matching retention time of isomers.

When mass spectrometry is used in the method, the number of points assigned depends on whether the instrument operates at low or high resolution and if the ion detected is a precursor or a product ion. In low resolution mass spectrometry, the presence of an ion scores one point. In MSMS, a precursor ion scores one point while a product ion scores 1.5 points. In high resolution mass spectrometry, each ion is worth two or more points. A precursor ion is worth 2 points while a product ion scores 2.5. Codex has issued guidelines on the use of mass spectrometry for identification, confirmation and quantitative determination of residues[1] and using other techniques[2].

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[1] Guidelines on the use of mass spectrometry (MS) for identification, confirmation and quantitative determination of residues. CAC/GL 56-2005. Available at: http://www.fao.org/fao-who-codexalimentarius/sh-proxy/en/?lnk=1&url=https%253A%252F%252Fworkspace.fao.org%252Fsites%252Fcodex%252FStandards%252FCXG%2B56-2005%252FCXG_056e.pdf (accessed 11/11/20)

[2] Guidelines on performance criteria for methods of analysis for the determination of pesticide residues in food and feed, CXG 90-2017 Available at: 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 (Accessed 12/20/20)

It should be noted that any comparison for the allocation of points must be performed with the relevant standard analyzed under the same conditions and usually during the same sample run. Referencing parameters such as retention time or peak ratios to a spectral library or historical references is not acceptable in the regulatory environment.

As introduced earlier, the confirmation of identity of an analyte is achieved, traditionally, when two or more independent analyses yield the same result. When combining chromatography and a selective detection mechanism, the point system considers the matching retention time as one piece of information and the selective detection at that matching retention time as a second piece of information. When mass spectrometry is used the chromatography still earns one point for the matching retention time, the precursor ion scores one point, and product irons score 1.5 points each. Consequently, most MS methods acquire data about two product ions for each analyte of interest. The total score is therefore 1 point for the matching retention time, 1 for the precursor ion, and a total of three points for the two product ions. This result therefore meets the requirement for confirmation.

Acceptance Criteria

Tables of requirements from the European Union and the Food and Drug administration of the United States were included in the video portion of this training. They are examples of the acceptability criteria for mass spectrometry results. One of the most important decisions made during the development of a method using mass spectrometry is the selection of the diagnostic ions, or the product ions. The collision chamber located between the two quadrupoles causes a reproducible fragmentation that create a large number of fragments. These fragments may be very small and not chemically significant in the sense that they could have originated from the fragmentation of any number of precursor ions. The advantage of the tandem mass spectrometer is to be able to use the molecular ion as precursor and select large fragments that retain most of the chemically or functionally significant portion of the precursor. This is in contrast with the necessity to use adducts with single quadrupole mass spectrometry.

Besides the chemical relevance of the product ions, it is recommended to use an ion that results in a chromatographic peak with a signal to noise ratio of at least 10:1 as the primary ion for identification, while the second product ion should have a minimum S/N of 3:1. The abundance of the product ions can also be used as a guide in the selection of appropriate primary and secondary diagnostic ions to

take advantage of the fact that the most abundant product ions produce the highest peaks and therefore increase the sensitivity of the method compared to less abundant ions.

Lesson 2: Multi-Residue Methods

Single residue methods are by definition simpler to develop than multi residue methods because the chemical properties of a single or a small group of analytes must match the extraction, clean up and detection steps. Multiresidue analysis requires the consideration of a potentially large number of analytes and consequently must seek compromise between the parameters that may result in the best extraction of one component but not allow an acceptable level of recovery for another. The keyword in multi residue method development is compromise.

Developing a Multi-Residue Method

The steps of method development are essentially the same, except that each parameter must be contemplated and verified against all the different analytes. In addition, multi residue methods are often intended to be suitable for a large number of foods. While it is customary to seek a common sample preparation procedure for all samples, small variations may be needed to accommodate specific foods. In some cases, separate multi residue methods will be developed for different groups of commodities such as high fat, high sugar, or high water foods. In other cases, the method will be broadened and may include alternative steps for these different groups. Regardless of the approach, the sample extract must still meet the requirements for the analytical technique that follows it.

In this section, we walk through the process of methods development using AOAC Official Method 2007.01 for the determination of pesticide residues using GC-MS and LC-MS/MS.

1. Purpose of the method

The purpose of the multi residue method can be to detect, quantitate or identify. The scope, or the range of applicability of the method, is by definition much broader than that of a single residue method

as we try to use the same sample preparation, clean up and measurement for a large number of analytes. The analytes of interest must have common chemical properties to work relatively similarly through the process. Pesticide residues were among the first food contaminants targeted for the development of multi residue methods because they share many characteristics, such as their solubility in weak or strong organic solvents, their thermal stability range and the concentration range of interest. Many properties are also quite different for some pesticides. For example, some pesticides or metabolites of interest cannot be solubilized for liquid chromatography but can be volatilized for gas chromatography. Other distinctions include polarity, where some pesticide residues cannot be analyzed by reverse phase chromatography. The geometry of some pesticides also limits how they can be extracted from the matrix using steps that work very well for large groups of pesticides; planar pesticides are an example where the cleanup procedures that yield sufficiently purified samples in most cases may trap the planar pesticides and remove them from the sample prior to measurement.

In summary, the purpose of the method must include not only whether it is a detection, quantitation, or identification method, but also must include the scope of commodities or foods and analytes covered by the method.

Method AOA C 2007.01, the method is applicable for grapes, lettuces, an 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 this 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 except some compromises that are not necessary for laboratories with more 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 does not analyze as many commodities may be better served by a method with a limited scope. It is not always the best solution to use a method with a very broad scope just in case different types of samples may become of interest to the laboratory in the future. It can increase cost, time requirements and require analysts with training that is not available in the region among other disadvantages. Fit for purpose is the terminology used to describe how a method is suitable for the range of commodities, the range of analytes, the sample throughput and the equipment available in a particular laboratory.

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.

2. Sample Preparation

Since we have reviewed the steps of sample preparation in the previous lesson, we will focus here on how it translates in the AOAC method 2007.01. The sample preparation procedure is summarized in Figure 1 reproduced from the official method.

Step 0 specifies to combine a sample consisting of more than one kilogram of commodity. In the case of grapes, this corresponds to a number of individual grapes whereas for oranges, it is typical to quarter the oranges and use 1/4 of each orange until we reach one kilogram. This approach aims to obtain an analytical sample that is representative of the lot. This sample is chopped into small particles using a vertical cutter, or a knife, and a sub-sample of 200 grams is homogenized using and immersion blender.

It should be noted at this stage that Codex has published guidance on the parts of commodities that should be included in the testing[3]. In this case, oranges are analyzed with the peel.

Steps 1 and 2 specify the size of the test portion and where it should be transferred. Steps 3 to 5 describe the addition of solvents and salts whose function is to promote the solubilization of the analytes of interest in the organic phase while most commodity components (*i.e.* possible interferences) will rest in the aqueous phase. This salt-driven partitioning of analytes is at the heart of the QuEChERS methods. This sample preparation philosophy will be discussed during the hands-on portion of this training.

[3] Portion of commodities to which Codex maximum residue limits apply and which is analyzed. CAC/GL 41-1993. Available at: http://www.fao.org/input/download/standards/43/CXG_041e.pdf (accessed 11/12/20)

Step 6 and 7 describe the mixing and centrifugation that are used to first ensure good contact between the salts, solvents, and analytes, and then promote the separation of the two phases. Steps 8 to 10 consist in a second partitioning of analytes, following the removal of solids, promoted by the presence of salts that will help remove more water from the organic phase.

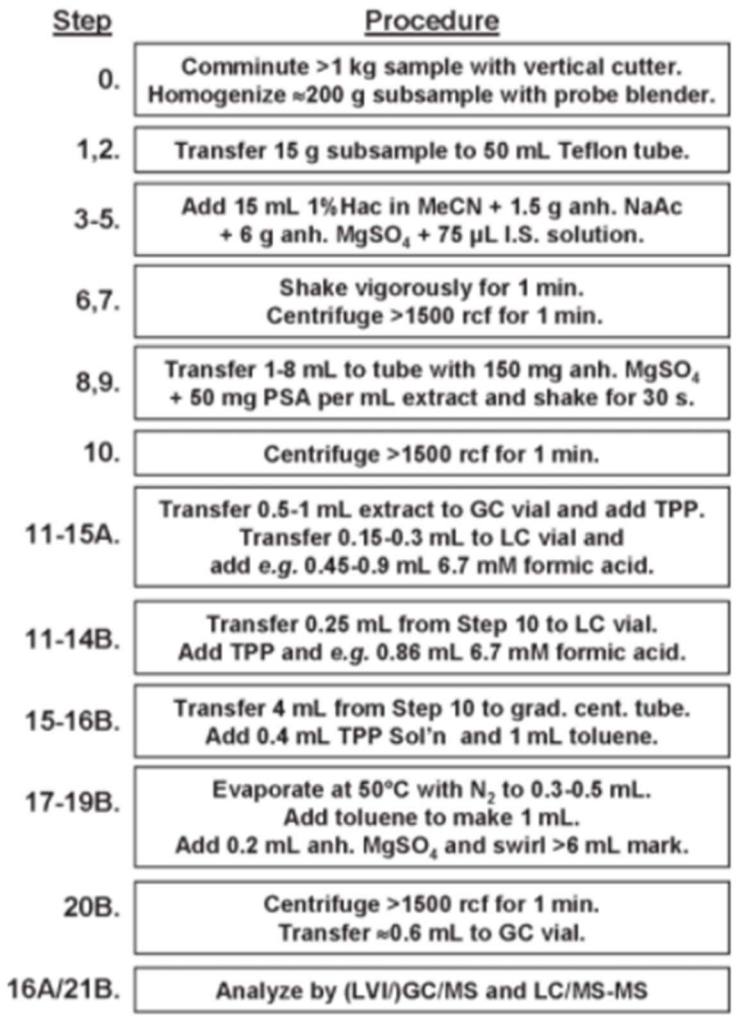

Figure 1: Sample preparation procedure for AOAC 2007.01.

At this point the method separates into an A and a B track. The A track is designed for the preparation of samples for LC analysis, while the B track includes additional steps required for the preparation of samples for GC analysis. While this is still considered a single method there are critical differences in the sample preparation for the two types of chromatography. Most pesticides are typically analyzed either by LC or by GC, but some are compatible with both instruments and could be included in the measurement on both. Generally, laboratories prefer to measure an analyte using a single method that provides confirmation of identity and quantitation rather than use two different methods to save time and money. The performance parameters of the method need to meet the requirements for regulatory action in either case.

The main difference between the A and B tracks are the requirement for solvent transfer for the sample going to the GC. Indeed, the acetonitrile used as the solvent for sample preparation is not

sufficiently volatile for GC analysis; it must be replaced with toluene. In addition, this method includes a step of concentration from 4.4 mL of sample to about 0.5 mL and adjustment of the volume to 1 mL. The concentration step aims to lower the limits of detection and quantitation.

3. Measurement

The measurement is performed using GC-MS and LC-MS/MS. The chromatography will provide one point to the score for the identification through matching the retention time with that of a reference standard measured in the same run, or at least on the same day. For the GC analysis, the molecular ion obtained from electron impact ionization will provide one point and a second ion is used to provide an additional point. For the LC analysis, the molecular ion formed in the electrospray ionization source scores one point and 2 product ions formed in the collision chamber score an additional 1.5 point each.

The effect of matrix components on measurements and mass spectrometry were discussed in Module 6. This method uses a matrix-matched calibration to account for matrix effects. Consequently, oranges, grapes and lettuces containing no pesticides are needed to process through the same sample preparation steps as the unknown samples and produce a clean extract that contains all the matrix components that could interfere in the measurement of the analytes of interest, but no pesticides. If such blank matrices are not available, some alternative techniques can be employed. We will discuss these techniques in the hands-on portion of this training. An internal standard is also used to calculate recovery along with a quality control spiked solution for quality control purposes. This will be discussed further in Module 9.

For both tracks, a calibration curve must be prepared. The calibration curve must span the range of concentration of interest expected in the unknowns, but must also fit within the linear response range of the detector. Consequently, it is customary to prepare a calibration curve that focuses on the level of contamination requiring regulatory action, in this case the maximum residue level (MRL). Many methods are simply developed with a calibration curve including a blank sample, a sample containing 1/4 of the MRL, 1/2 the MRL, 1X MRL, and 2X MRL. If any sample falls above the calibration range, the extract can be diluted until the intensity falls within the linear range of the detector and of the calibration. There is generally no real advantage in including concentrations much higher than the MRL in the regulatory laboratory, because the risk management actions are based solely on the exceedance of the MRL. Dilution of the sample can provide a final measurement of concentration that

could be used to implement additional risk management actions, such as educating farmers on how to apply the pesticides to ensure there is no excess residue at the time of harvest. For a food manufacturer, there may be other objectives to perform this type of analysis. For example, a laboratory could be testing raw materials to determine whether the lot needs to be processed to reduce the level of contamination prior to its use as an ingredient in production. This example emphasizes the importance of understanding the objective of the laboratory's work to determine if a method is well suited.

An official method, such as an AOAC method, must provide the data acquisition parameters for all the components of interests. In this particular method, the corresponding article published in the Journal of AOAC International is referenced; it contains the data acquisition parameters and conditions for the instruments. Figure 2 is a reproduction of a portion of the table where the chemical parameters necessary to determine the composition of the mobile phase, select the column, and decide whether a pesticide will be part of the GC or LC method are summarized.

Table 1. Information about the selected pesticides for the study

Pesticide	Use[a]	Class[b]	MW, g/mol[c]	Formula	Vp, mPa[d]	Solubility in water	pK_{ow}[e]	pK_a[f]	Analysis
Atrazine	H	Triazine	215.7	$C_8H_{14}ClN_5$	0.0385	33	2.5	1.7	GC and LC
Azoxystrobin	F	Strobilurin	403.4	$C_{22}H_{17}N_3O_5$	1.1×10^{-7}	6	2.5		LC[g] and GC
Bifenthrin	I	Pyrethroid	422.9	$C_{23}H_{22}ClF_3O_2$	0.024	<0.001	>6		GC
Carbaryl	I	Carbamate	201.2	$C_{12}H_{11}NO_2$	0.041	120	1.59		GC and LC[g]
Chlorothalonil	F	OC	265.9	$C_8Cl_4N_2$	0.076	0.81	2.92		GC
Chlorpyrifos	I	OP	350.6	$C_9H_{11}Cl_3NO_3PS$	2.7	1.4	4.7		GC[g] and LC
Chlorpyrifos-methyl	I	OP	322.5	$C_7H_7Cl_3NO_3PS$	3	2.6	4.24		GC[g] and LC

Figure 2: Information about the analytes included in the method, reproduced from JAOAC Int. (2007)90:485[4].

[4] S.J. Lehotay (2007) Determination of Pesticide Residues in Foods by Acetonitrole extraction and Partitioning using Magnesium Sulfate: Collaborative Study. Available at: https://www.researchgate.net/publication/233700986_Determination_of_Pesticide_Residues_in_Foods_by_Acetonitrile_Extraction_and_Partitioning_with_Magnesium_Sulfate_Collaborative_Study (accessed 11/12/20)

Similarly, the retention time, precursor ion and either other (precursor) ions in GC or product ions in LC-MS/MS are also identified in a second and third tables, for which a portion of each is reproduced in Figure 3.

Table 2. General GC/MS information for the pesticides[a]

Pesticide	M+	Base peak	Other ions	t_R, min
Atrazine	215	200	173, 217, 202	9.25
Azoxystrobin	403	344	372, 388	22.45
Bifenthrin	422	181	165, 166	17.77
Carbaryl	201	144	115, 116	11.14
Chlorothalonil	264	266	268	9.98
Chlorpyrifos	349	97	197, 199, 314, 316	12.03
Chlorpyrifos-methyl	321	286	288, 125, 197, 109	10.83

Table 3. General LC/MS/MS information for the pesticides[a]

Pesticides	$[M+H]^+$, *m/z*	MS/MS transition, *m/z*	t_r, min
Atrazine	216.0	216 → 174	16.46
Azoxystrobin	404.1	404 → 372	16.79
Carbaryl	201.8	202 → 145	15.46
Chlorpyrifos	349.9	350 → 200	20.20
Chlorpyrifos-methyl	321.8	322 → 125	19.37

Figure 3: Information for mass spectrometry, reproduced from JAOAC Int. (2007)90:485

While not unusual, using external publications to relay essential information for the application of a method adds one more level of complexity for the laboratory implementing the method. The preference is usually to include all the information in the official method, but the multi residue methods involve such large numbers of analytes and matrices that providing all the information necessary to convey the performance of the method would make the official document excessively long. Luckily, any reference cited in an official method is normally provided in the appendices or at least with appropriate links in the official method. The laboratory implementing the method should ensure to

have access to all this information and use the appropriate segments to develop its own standard operating procedure (SOP).

The equations necessary for the calculation of the results are given in the method. In a nutshell pesticide residue analysis for regulatory purposes requires a recovery between 70 and 120%, with some exceptions where a recovery of 50 to 150% is acceptable, reproducibility of less than 25% RSD and a repeatability of less than 15% RSD. RSD Is the relative standard deviation of reproducibility between laboratories or repeatability within a single laboratory.

Acceptance Criteria

The acceptance criteria for the different analytes included in a multi residue method can be different. In general, the acceptable range of recovery is the same for higher concentrations whereas lower recovery is acceptable at very low concentrations. These numbers may differ also between the GC and the LC methods, as can be expected because of the differences in the efficiency of separation, the concentration of the sample injected in the instrument and the efficiency of ionization in the source. The Codex guidelines on performance criteria for methods of analysis for the determination of pesticide residues in food and feed adopted in 2017 provides a detailed explanation of the acceptance criteria agreed upon in Codex, but each country's authority typically also publishes their own document listing these criteria [5, 6, 7, 8].

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[5] Guidelines on performance criteria for methods of analysis for the determination of pesticide residues in food and feed; Codex CXG 90-2017. Available at: 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 (Accessed 11/12/20)

[6] Guidelines for the validation of chemical methods in food feed cosmetics and veterinary products. US Food and Drug Administration. Available at: https://www.fda.gov/media/81810/download (Accessed 11/12/20)

[7] Analytical quality control an method validation procedures for pesticide residues analysis in food and feed. SANTE/2017/11813. Available at: https://ec.europa.eu/food/sites/food/files/plant/docs/pesticides_mrl_guidelines_wrkdoc_2019-12682.pdf (Accessed 11/12/20)

[8] Acceptance criteria for confirmation of identity of chemical residues using exact mass data for FDA FVM program. US Food and Drug Administration. Available at: https://www.fda.gov/media/96499/download (Accessed 11/12/20)

Conclusions

Multi residue methods involve a lot of compromise in order to obtain a sample preparation procedure that can be used for as many commodities and analytes as possible. The official method reviewed in this lesson provides a great example of both maximizing the scope of applicability of a certain number of steps, while not compromising on the recovery or sensitivity for other analytes by rather splitting into a separate measurement requiring its own additional steps of sample preparation. This method was the original multi residue method for pesticide residues and many methods have been developed since using it as a foundation. We chose to use the original method in this class to get a perspective on the evolution of the procedure as an increasing number of laboratories have adopted its basic principle. The method we use in the hands-on portion of this training is a variation of this original method.

Lesson 3: Advantages and Challenges of Multi-Residue Methods by LC-MS/MS

This third lesson of Module 8 focuses its attention on the sample preparation steps selected for AOAC Official Method AOAC 2007.01 and some of the critical parameters involved in the mass spectrometry. Many new analysts have never prepared pesticide residue samples using the more involving traditional methods and consequently don't appreciate the amount of simplification brought about by the QuEChERS methods.

Prior to the development and broad deployment of mass spectrometry, pesticide residue analysis required the use of a number of different GC columns in combination with element selective detectors. The resolution requirements for the GC separation were dictated by the availability of detectors and therefore the analyte peaks could not overlap. This is one of the greatest advantages of using mass spectrometry, which in itself is a filter, because the detector can sequentially measure multiple analytes eluting at the same retention time.

1. Sample preparation

However, multiresidue methods pose a number of challenges. For example, the sample preparation method must extract all compounds of interest. The cleanup steps must remove problematic co-extractives, but do so without losing any of the compounds of interest. Using a tandem quadrupole instrument reduces the level of clean up needed in a wide variety of samples, but there is still a need to remove any analytes that could negatively impact the ionization in the source, especially if it increases the limits of quantitation and detection to above the levels needed for the purpose of the method. The sample preparation with suitably broad retention must lead to an extraction efficiency meeting regulatory requirements, as discussed in lesson 2. It must not cause any degradation of any of the compounds of interest. When contaminants are present in vastly different concentrations or display very different coefficients of extinction, it may be necessary to include steps of concentration or dilution in order for the signal to fall within the linear range of the detector. Finally, it has become expected that multi residue methods will involve relatively easy and fast sample preparation; it is overall a tall order!

The graphical representation of the genealogy of sample preparation methods for pesticides prepared by colleagues at the US Food and Food Administration (Drs. Jon Wong, Alex Krynitsky and Kai Zhang) used in the video portion of this lesson is reproduced here in Figure 4. The steps of sample preparation described in lesson 2 can be compared with the procedure used in the 1970s at the US Food and Drug administration for the determination of halogenated pesticide residues. First, a test portion was extracted in 400 mL of hexane using a Soxhlet apparatus for 8 hours. The extract was then concentrated down to dryness and reconstituted in 20 mL of hexane. The next step was to run the sample through a florisil column to separate fats, using 200 mL of solvent, and a silica gel column to separate pesticides from PCBs, using another 500 mL of solvent. The quantitation was performed using gas chromatography with an ECD detector and confirmation was done by GC-MS. This method used over 1 L of organic solvent (hexane) per sample for the measurement of a few halogenated pesticides; in comparison, the QuEChERS method described in Lesson 2 used 15 mL of acetonitrile for LC and an additional 1.5 mL of toluene for GC per sample for the measurement of 20 pesticides. Modern variations on the QuEChERS method use the same volume of solvent for the measurement of upwards of 200 and even 300 pesticides. Besides being easier to perform, faster and less costly, these methods are better for the environment.

Most of the official methods adopted by regulatory laboratories for the trade of food use modifications of the original methods mentioned in Figure 4, with varying degrees of cleanup using salts. The preferences for one or another method are often justified by the regulatory requirements, such needing much lower limits of quantitation, or needing to test for many more pesticides, which would put more demand on the separation procedure to ensure that sufficient S/N can be obtained at the detector.

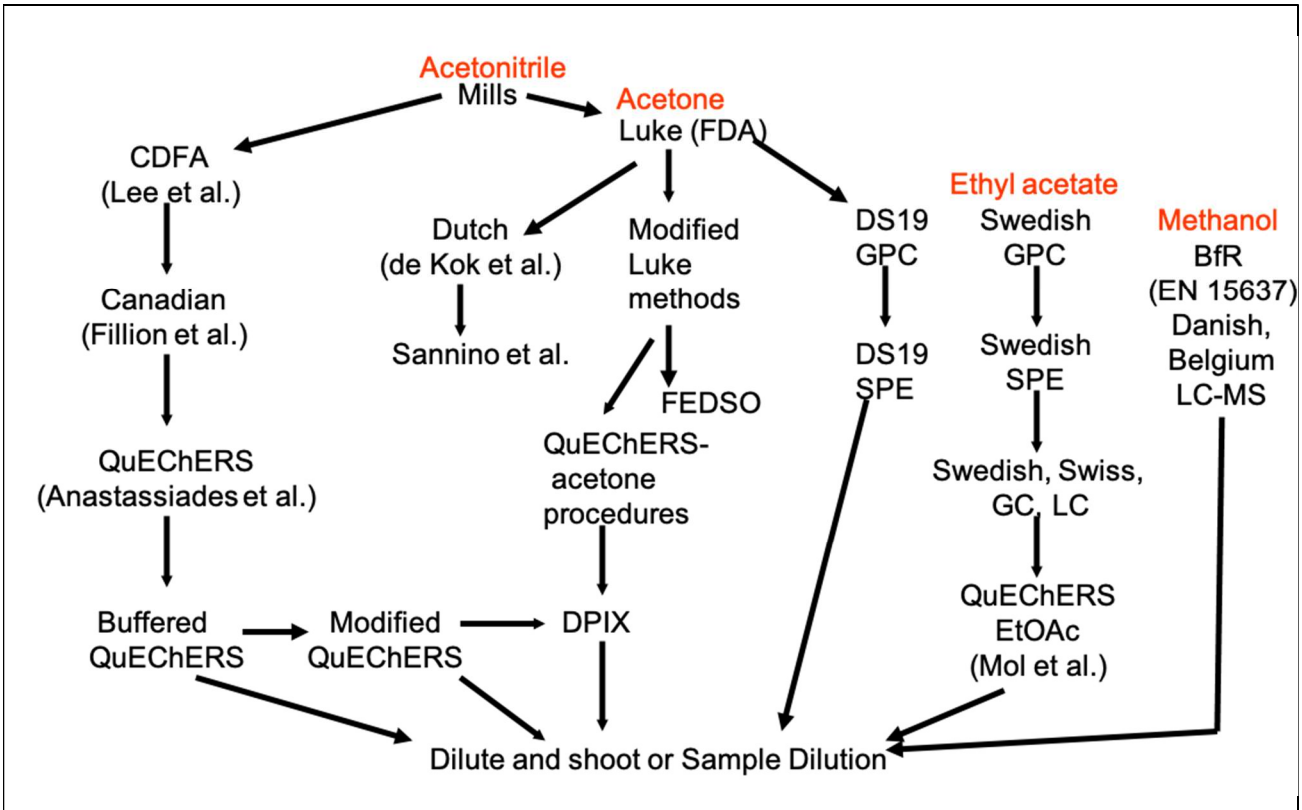

Figure 4: Genealogy of Sample Preparation (prepared by US FDA)

The modern sample preparation methods require fewer glassware needing washing and rely more heavily on consumables. This choice was made in part to lower the risk of cross contamination between samples associated with reusing glassware, and the cost of personnel to perform the task of cleaning. One of the challenges for developing countries that is directly related to this decision is that many laboratories have very small budgets for consumables and there is a temptation to use consumables and clean them for reuse. However, these consumables we're not meant to be used

repeatedly and they are often manufactured with soft materials such as different kinds of plastics, that can easily be scratched. These scratches make it increasingly hard to clean the vials and consequently increase the risk of cross contamination between samples. This is especially an issue when using highly sensitive detection techniques such as MS/MS. Sample extraction and cleanup procedures that use solely solvents and the matrix offer less risk of damaging the plasticware then procedures that involve shaking in the presence of glass beads for example. Nevertheless, in any case where material, whether it be glassware or plasticware, is reused for multiple analyses, the laboratory should have an SOP in place to describe the cleaning process to reduce the risk of cross contamination to an acceptably low level. The laboratory should check that the SOP achieves the objective through a check of the residues that are most likely to cause cross-contamination if not all analytes. If no attention is paid to the risk of cross-contamination, the laboratory could produce erroneous results. False-positive results could arise from contaminants trapped in the plastic that get released during sample preparation, while false negatives could happen if signal inhibitors gets released that are not compensated for by the matrix-matched calibration. While these should be caught by the quality assurance processes in place, it is always less costly (and damaging for one's reputation) to prevent a problem of cross-contamination than it is to fix it.

2. Measurement

The LC-MS/MS multiresidue method needs to be set up in order to separate the analytes sufficiently to enable MS analysis and produce peaks that are a compromise between sensitivity (*i.e*. narrow and high) and wide enough to allow enough time for the mass spectrometer to go through all the components of interest that elute in the same retention window. The effect of the selection of mobile phase on all analytes of the method must be understood. We discussed examples of the effect of using a gradient and different mixtures for isocratic runs in Module 4. The effect of pH is also important to produce symmetrical peaks and favor ionization. The column has to accommodate all analytes; luckily, food contaminants analysis has grown globally and now represents an interesting market for column manufacturers who now offer columns with optimal performance for these applications.

The real "new" topic in this section is the concept of running more than one analyte as a time in the mass spectrometer. It is not strictly what happens since only one m/z is allowed through MS1 in the MRM mode, but it describes the need for sharing of a time frame by two or more analytes.

Figure 5 shows a typical software window for a multi-reaction monitoring experiment in MS/MS. The green boxes span the portion of the time axis located at the top; data is to be acquired for each of the pesticides listed on the left (and including 2 product ions, identified here as mass pairs) during this time window. In this experiment, the period between 5.5 and 6.5 minutes shows 8 analytes. Consequently, MS1 will need to allow 8 different precursor ions through, the collision chamber will need to be energized at the best collision energy for each to produce 2 product ions. Finally, MS2 will need to filter 16 product ions onto the detector. This particular method includes over 150 pesticides, so there may be more than 16 overall. Nevertheless, if only these 8 pesticides elute within these 60 seconds, each product ion can receive a maximum of 60/16 = 3.75 seconds of attention. This has to include the time needed by the instrument to switch parameters, and ideally, we want to let the ions through MS1 as close as possible to the maximum intensity of the chromatographic peak.

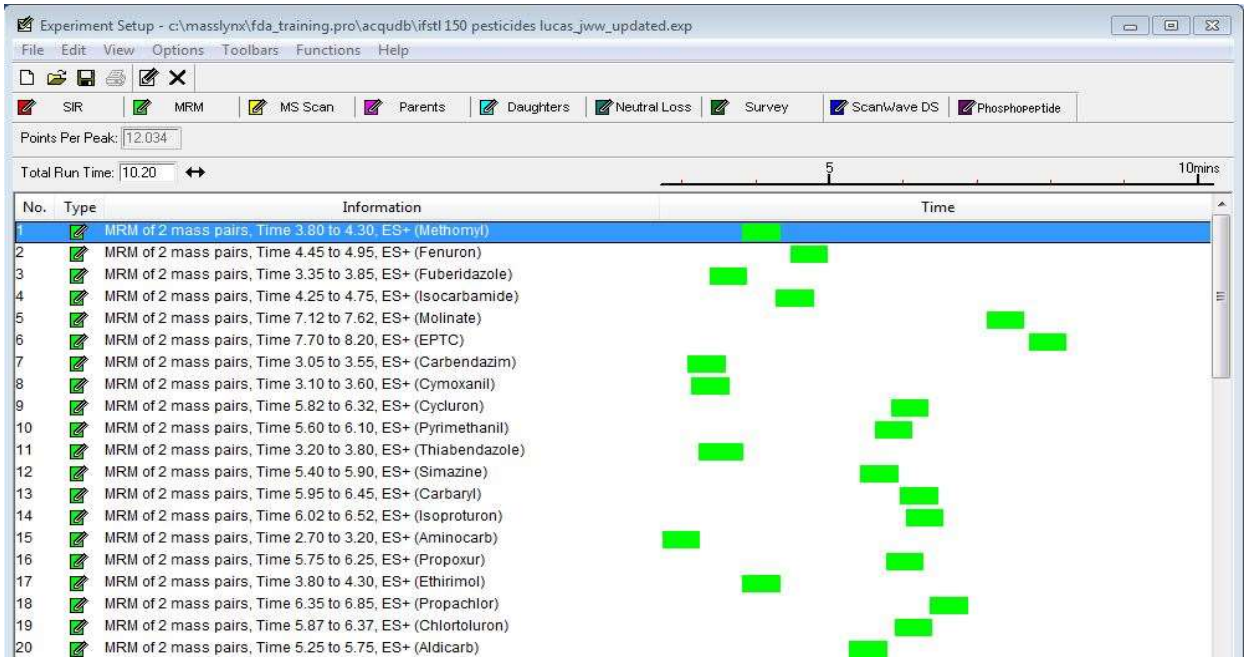

Figure 5: Waters' MassLynx window illustrating the period of time during which data acquisition is performed for a list of pesticides in an MRM method.

Some of the MS parameters are fixed during a run, such as the ionization flow rate at injection, desolvation gas and source temperature. Other parameters are tuned for each analyte, such as ionization current, positive/negative and the collision energy for fragmentation in the collision cell. In comes the concept of dwell time… Dwell time is the amount of time that the mass spectrometer spends accumulating data. We usually report dwell time and resulting points per peak, where a minimum of 10 to 15 points per peak is preferred. During the period of accumulation of the information (*i.e.* the dwell time), the signal is averaged, but so is the noise. This results in an increased S/N since the noise is random and will add/subtract, while the signal should always be about the same. Of course, a greater S/N translates in lower LOD and LOQ. In summary, we are working with very low concentrations in food safety, so we need to compromise in a way that provides a good-enough S/N ratio to meet the LOD required by the regulations, but allows us to analyze many components of interest in the same run.

Circling back to the dwell time, this is a value that can vary between instruments, but generally not by an order of magnitude. Using round numbers for illustrative purposes, an analyte may produce a S/N of 3:1 with 10 msec dwell time. This would be the absolute minimum dwell time to use to make a screening measurement (which needs S/N of 3 or more). In theory, the S/N improves according to the square root of the additional time. So, moving to a 100 msec dwell time should improve the S/N by 3.16X. Considering the margin of error, this is approximately the dwell time that would be needed to obtain a peak with a S/N of 10, the minimum requirement for a quantitative measurement.

An additional consideration is that we would like to acquire the data points as close as possible to the highest intensity of the peak, or the period of time where the signal is strongest, contributing further to a high signal to noise ratio. This means that even if we acquire data for a window of 20, 30, or 40 seconds for a particular analyte, the signal acquired at different time points within this window is not the same so the data acquisition is usually performed around the middle of the peak.

Putting all these considerations together clearly emphasizes why there is a limit to the number of analytes that can be included in a multiresidue method. It could be possible to increase the number of analytes included in the method by lengthening the experiment in a manner that would cause better separation of the analytes. A sufficiently long dwell time could then be allocated for each analyte in a very large method. At this time, methods including upwards of 300 pesticides are some of the largest methods for regulatory testing. As mentioned before, regulatory testing requires a high degree of

certainty because it will trigger regulatory action that is very costly. Screening methods comprising of around 1,000 pesticides are advertised, but they are generally not very popular because the preparation of the calibration curve for such a large method is both arduous and expensive. The sample preparation for such large methods would necessarily have to compromise in order to include more rather than less, which means that there are likely more matrix components also making their way into the column. These would contribute to gradually deteriorate the column at an accelerated pace compared to a cleaner sample. Matrix components can also negatively impact ionization as discussed in Module 6. These and other compromises such as the pH, buffers, column temperature, *etc*., reach a point where there is very little gain from trying to fit so many analytes in the same method. The trend is rather to invest in a high-resolution mass spectrometer to do a screening of unknowns rather than develop and use an unreasonably large MS/MS method.

In conclusion there is no such thing as a universal MRM. Analyte characteristics, such as whether they are volatile or not, polar or not, or sensitive to degradation at higher temperature fundamentally make the universal method impossible. More practically, there is generally not a need for exceedingly large methods because many laboratories have a restricted mandate that can limit the scope of commodities analyzed, the list of pesticides of interest, or both. When the concern is with completely unexpected analytes, high resolution mass spectrometry is a better fit for the purpose.

Lesson 4: Introduction to HRMS Methods

The original objective of high-resolution mass spectrometry was to be able to measure analytes having the same nominal mass by measuring the mass accurately to four decimal places. However, this was really never an issue in food safety, where the challenge is rather the number of different analytes that need to be analyzed. The expansion of the mass range compatible with the instrument compared to the tandem quadrupole provides significant advantages for large molecules, such as proteins, which is very useful for biomolecules in biopharmaceuticals. Still, there is a place for high resolution mass spectrometry in this field. Typically, it is the LC-time-of-flight and Q-trap

implementations that are seen in the food safety laboratory, but the GC-magnetic sector mass spectrometry is increasingly popular for dioxins analysis.

HRMS offers a good performance for quantitative analysis, in addition to the “number of points” that make it a confirmation method. These benefits make it compatible with many of the applications traditionally done on the LC-MS/MS, such as pesticide residues and intentional adulteration. As the technology comes down in price, some laboratories are going as far as replacing tandem quadrupole analyses with HRMS. Let’s review some of the features, advantages and disadvantages that drive such decisions.

HRMS for Pesticide Residues

This is the most obvious application because of the extremely large number of analytes of potential interest. For countries like the European Union, hazard-based regulation requires the determination of around 1,000 pesticide residues and metabolites and the duty is centralized in a relatively small number of laboratories that must test a very broad range of domestic and imported commodities. The flexibility and speed of data acquisition of HRMS can be a great advantage for them. In addition, these same laboratories are generously financed and can afford the technology itself, the highly educated and experienced staff, the infrastructure requirements and the maintenance. In the United States, this technology is not yet broadly deployed in routine regulatory laboratories, but it is available in the research laboratories of the regulatory agencies.

What makes HRMS attractive for pesticide residue analysis? The first advantage is its versatility. This instrumentation offers different modes of selectivity (discussed in Module 6), which in turn can help deal with matrix effects. This translates into the ability to increase the number of pesticides measured in a single injection and broaden the scope of the sample preparation to include more commodities. Such consolidation offers the additional advantage of maintaining laboratory accreditation for fewer methods while broadening the services offered by the laboratory.

The single method translates in timesavings for sample preparation as well. In addition, the duty cycle on the HRMS is greater than the quadrupole because the instrument can scan across the mass range constantly. While the scanning can be slower than in the tandem quadrupole, it proceeds while also

generating product ions in the collision cell in single-stage HRMS, which translates in the measurement of precursor and product ions for all ions present in the sample. Overall, this throughput enables the well-funded laboratory to analyze more samples for a broader range of potential hazards.

Finally, the technology may be adopted as a means of acquiring data for future reference. Indeed, the data set produced by the single stage HRMS, for example, contains more information than most laboratories need at this time. This information can be interrogated at a later time, for example if a new contaminant raises concerns in a particular commodity or from a particular geographical origin, it is possible to go back and examine data from samples where this contaminant was not a target and determine if, or even when, it started appearing.

One of the recent leaps forward in the analysis of pesticide residues happened with the replacement of HPLC with UHPLC. The chromatographic resolution gained finally provided a separation for analytes of similar mass that could not be easily resolved by MS or even MS/MS because of their similar nominal m/z and product ions. This improvement allows many more pesticides to be included in the same UHPLC/MS/MS method, without requiring HRMS, at a lower cost. HRMS however goes one step further in expanding the measurements to compounds we don't know yet are or may be significant.

HRMS for Adulterants

The reasons for using HRMS for food adulterants are probably the most obvious of any of the fields of application. In both intentional and unintentional adulteration, the laboratory doesn't know what might have been added. This is the true unknown-unknown. MS/MS is not well-suited for this category of unknowns because it requires that any analyte of interest be included in the instrument method in the form of the target mass to charge ratios of precursor and product ions, and supported by the analysis of reference standards. HRMS, with its identification of compounds based on the accurate mass, which can be kept calculated from the chemical structure and stored in large libraries, offers a clear advantage.

Concerns with the presence of pharmaceutical active substances in dietary supplements has created one of the most active areas of use for HRMS in adulteration. In most regulatory systems, traditional

medicines, also known as dietary supplements, must not contain synthetic pharmaceutical active ingredients. Dietary supplements typically do not require a prescription from a medical professional, so there is a high risk of negative interaction between prescribed medications and fraudulently added pharmaceutical ingredients. Active pharmaceutical ingredients are added to dietary supplements with the intention of causing a feeling of well-being that prompts consumers to continue to purchase the product. Dietary supplements do not go through the efficacy and safety data review imposed on drugs for registration and are therefore much less costly to bring to market. The absence of these studies however also means that there is no formal information about the effects of the product on any subpopulation, be it those with weakened immune system, pregnant, or taking pharmaceutical drugs. And there is generally also much less oversight on the market. The high level of health risk and the absence of logical reason for an active ingredient to be present, and therefore targeted for analysis, both highlight the value of high-resolution mass spectrometry data.

In research groups focusing on dietary supplements, there is also an interest in screening for plant toxins. While very few regulations exist in this area at this time, some regulatory agencies are contemplating regulations for secondary metabolites of health concern. In this application, certified reference materials and standards are generally not available, but the structure of the plant toxins is known, and their exact mass can be calculated. This is what makes HRMS an attractive tool for screening purposes. In addition, the post-acquisition data mining option mentioned above can be used to generate data from samples that were analyzed for a different purpose. For example, a lot of resources are allocated to testing for pesticide residues in edible plants (*i.e.* fruits, vegetables, herbs and spices); this data set could be mined for a survey of the presence of toxins.

HRMS and Omics

Last but not least, the field of “omics” sees great potential for HRMS data. In this broad range of applications in biology, the objective is to characterize and quantify large amounts of biological molecules that contribute directly or indirectly to the function, structure or dynamics of cells, tissues or whole organisms. This field is entirely based on data mining to identify relationships. Consequently, the availability of an extra-large amount of data is considered a benefit. Omics scientists are well acquainted with chemometrics, or the use of advanced mathematical processing for large datasets, something that is a little bit more challenging for routine food safety laboratories. Proteomics,

metabolomics and now foodomics aim to better understand the relationships between chemicals and health. We have also seen a new word, exposomics, that aims to look at exposure to pesticide metabolites in an untargeted profiling approach.

One of the main drivers for the use of HRMS in omics is the ability to measure large molecules such as proteins. Once it is possible to measure both the proteins in all their functional forms in living systems and the health promoting compounds, such as vitamins or amino acids, as well as known and unknown contaminants and their metabolites, there is great hope to learn about relationships that could lead to the development of new medicines or simply more specific dietary recommendations that promote the health of the general population or subpopulations affected by disease.

Use with Caution

One of the natural tendencies of researchers when a new tool becomes available is to use it for as many different applications as possible. HRMS is one of these tools that has a very broad scope of applicability and could help research analytes that are present at very small concentrations in food commodities either naturally or through addition. Intentional adulteration of food for economic gain is one of these applications where the compounds that are added can be completely unexpected. Most of the time, the adulteration aims to increase the quantity of products sold by weight. Any number of substances can be used for this purpose depending on the color, texture, aroma and flavor profile of the commodity. In other cases, water is added, and a variety of gums and texturing agents are used to reproduce the normal rheological properties of the food. Consequently, a high-resolution measurement supplemented by very large libraries can prove useful to identify these unknown unknowns.

However, one risk of this newfound ability to detect thousands of different compounds is that it could lead to a hunt for new food hazards that may not pose a significant health risk. While all significant risks for public health should be investigated for potential risk-reduction strategies, some of which may be regulated risk management strategies, scientific and economic considerations will need to be taken into account before long lists of naturally present food components that exist at very low

concentrations and have never shown health impacts become candidates for regulations. If and when some of these substances warrant investments in toxicological studies and prevalence evaluation over large territories, then HRMS may be the best tool for their detection and quantitation. Until then, the academic interest of finding substances with a potential health concern must be framed within the historical context that says that food is generally safe, and the modern risk based decision-making process that precedes the establishment of new rules or even the communication of concern with new hazards that is not supported by strong scientific evidence. As we have agreed in Codex, the mere presence of a hazard in food does not mean that is poses a risk to the population that warrants management actions.