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IMPROVING THE ANALYSIS OF CHEMICAL RESIDUES IN FOOD

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485F US Highway One South, Suite 210,
Iselin, NJ 08830
(732) 596-0276

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Vice President/Group Publisher
Michael.Tessalone@ubm.com

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INTRODUCTION

With health and safety concerns linked to the presence of pesticides, pathogens, heavy metals, and chemical contaminants in food, scientists must continue to advance analytical methods for testing food safety. The North American Chemical Residues Workshop (NACRW) has long been an important meeting for scientists interested in sharing information about this important topic and learning about the latest developments in trace level analysis of food and agricultural samples. The NACRW's organizers have again collaborated with *LCGC* to bring readers an informative ebook highlighting some of the latest studies and findings from the 2017 meeting.

In *Improving the Analysis of Chemical Residues in Food* (sponsored by Thermo Fisher Scientific), hear first from Jo Marie Cook, the chief of the Division of Food Safety at the Florida Department of Agriculture and Consumer Services. She stresses the importance of proper sampling, and cautions readers that failing to ensure representative sampling and proper sample processing can affect a method's reproducibility and accuracy.

Next, Yelena Sapozhnikova, a research chemist at the US Department of Agriculture's Agricultural Research Service, talks about her recent research findings indicating an automated mini-solid-phase extraction cleanup coupled with low-pressure gas chromatography–mass spectrometry (MS)/MS increased the reliability and quality of a multiresidue method for the analysis of pesticides and environmental contaminants in meats.

Eric Verdon, the head of the European Union Reference Laboratory (EU-RL) for Antibiotic Veterinary and Dye Residues in Food from Animal Origin at the Anses-Fougeres Laboratory in France, describes some of the new methods developed by his Reference Laboratory that can measure more than 70 antimicrobial substances in a single run without losing critical sensitivity.

Last, hear from Paul Reibach, the technical director of chemistry at Smithers Viscient, about how the health and environmental risks of new pesticides and other chemical residues are assessed and how the allowable levels of residues are determined.

This timely information coming out of the NACRW meeting will surely be helpful in advancing the field of pesticides and chemical residues analysis in foods.

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WHY SAMPLING THEORY MATTERS IN PESTICIDE RESIDUE TESTING OF FOOD

An interview with Jo Marie Cook

Because it has become a standard practice in pesticide residue testing of food to use small (~100-mg) sample sizes, concern has risen about representative sampling and proper sample processing. This concern increases further when one considers the desire of contract laboratories and agricultural businesses to use even smaller sample sizes to enable high-throughput methods. Jo Marie Cook, the chief of the Bureau of Chemical Residue Laboratories of the Division of Food Safety at the Florida Department of Agriculture and Consumer Services is a passionate advocate of the importance of proper sampling. She recently spoke to *LCGC* about this topic.

You have raised concern about sampling practices in pesticide residue testing (1). How significant a problem is unrepresentative sampling today?

The practice of sampling from a single case or drum is very common. Raw agricultural samples are often taken at distribution sites far from the field where they were grown. A single box of seafood or a few grams of specialty cheese may be taken for laboratory analysis. Getting access to the most representative sample is impossible for some organizations. Paying for and processing larger sample sizes is expensive and time-consuming.

Is it problematic if sample reduction takes place in the field where food samples are collected, rather than in the laboratory?

I think it is problematic, because insufficient numbers of samplers are assigned to sampling. In addition, field sampling personnel seldom have the proper equipment and training to do it properly (2). It is far more difficult to avoid contamination and ensure sample integrity in a hot, muddy agricultural field or an extremely cold refrigerator storage unit. Growers and manufacturers are in the best position to develop representative sampling plans and provide the best environment and procedures for mass reduction.

Do changes need to be made in the guidelines for how many samples to take and what sizes those samples should be?

For pesticide residue analysis, there has been much work done by Codex Alimentarius to develop guidelines for

sample size, number of increments and estimated sampling uncertainty, both for registrants conducting field trials and for regulators enforcing maximum residue limits (MRLs) (3–6). More recently, some additional guidance documents on this subject have been published (7–10). However, these guidelines could do a better job of emphasizing sampling correctness (11). As more research is done, it reveals the tremendous variability that can exist in residues for different pesticides and crops and how difficult it is to know the uncertainty.

If we talk about sampling of all types of foods by all organizations, the answer to your question is yes. The number of increments taken and the total size of the sample are important, but the manner in which these increments are taken is also very important. These guidelines are frequently misused. If the increments taken do not represent the whole of the product, the result can be very biased. This concept of *sample correctness* is not well understood or practiced. Sampling is quite expensive but the analyses are even more expensive. How does it benefit management to make decisions on analyses that do not adequately represent the product tested? They need to know why they are sampling and how they are going to use the results. Rather than develop charts of sample size and number of increments, we need to emphasize a comprehensive understanding of sampling theory so that those who are developing sampling protocols and procedures, those who are conducting the sampling and analyses, and those who are using the data all understand the uncertainty in the results.

For spot contaminations such as in microbiological analysis, it is very difficult to obtain a representative sample that will detect isolated contamination before a product, such as baby leafy vegetables, is commingled in large batch processing before packaging. We need to develop strategies for detection of point source contamination that may involve moving the product through a wash cycle and continuously screening the process for contamination. This type of testing can be done at the harvest or manufacturing level to far greater effect than any sampling after the product is packaged and shipped to sites across the world.

Guidelines are often intended for a very specific process, so everyone using these guidelines needs to recognize that there are no general practices that fit all situations. Sampling protocols should be based on the sample quality criteria (SQC) and the global estimation error (GEE) that can be tolerated in the final results (2). Although it is easy for sampling experts to encourage customized sampling protocols based on SQC and GEE, the data to estimate the error are often not available, so producers and regulators alike have to begin somewhere. In our bureau, we have begun collecting larger samples of fresh produce from fields ready for harvest or at packing houses as the product arrives. In our laboratory, we have begun

conducting some replicate processing and analysis experiments to help us to estimate error in our processing (1). It would be so very wonderful if we could partner with growers and manufacturers to conduct the same types of replicate sampling and testing to better understand the true variability in sampling across the tremendous variety of products and analytes being tested.

What do you think analytical chemists need to understand about Gy's theory of sampling that they don't understand well?

As I describe in my presentations, it is very important to understand the relationship of mass and number of increments to error. In general, testing smaller masses leads to increased fundamental error and collecting too few increments leads to increased distributional error (2). The theory of sampling is so very much more complex that chemists need at least an introductory knowledge of Pierre Gy's theory of sampling and how it may contribute to errors in their measurements. Much more important is the concept of *sample correctness* and the many sources of hidden bias and their contribution to error (3).

What are the primary sources of sampling error?

There are both random errors such as fundamental sampling error caused by the variability in the concentration of the analyte of interest from element to element in a material and grouping and segregation errors caused by the variability in the location in time and/or space of the elements (fragments, particles) of a material in relation to each other. In addition, there are a number of systematic errors such as increment delimitation error, increment preparation error, increment extraction error, sample preparation error, sample processing error, and others (2,3).

How should differences in types of food and analytes be taken into account in sampling?

Once scientists study their types of samples and analytes of interest through the eyes of the theory of sampling, they will begin to identify the characteristics that are most important in developing their specific sampling protocols.

As sampling studies are conducted, it will reveal the most appropriate sample size and number of increments needed for a specific purpose. More importantly, it should also reveal those sampling practices needed to ensure representative samples and those procedures to be avoided because they might lead to sampling bias. One of the most important material selection errors occurs where some of the elements of the material are not sampled at all. For pesticide residues, the mass and number of increments has been standardized but not the implementation of good, representative sampling.

Modern testing approaches often use 10–15-g test portions. Is that test portion size adequate?

First, it has to be established that correct sampling procedures have been used at all sampling stages. The procedures to adequately describe and validate sampling and sample processing procedures are lacking. If we don't know how a sample was processed and how the test portions were selected, then we cannot assume, from repeatability studies alone, that we have identified all of the errors.

Second, there is far too little data using incurred residues. Most validations are conducted by spiking analytes into the test portion, which does not identify the possible sources of error from sample handing, sample processing, and insufficient analyte extractability, which can lead to loss or change in analyte.

Third, it is not only the test portion size, but also the size and uniformity of the elements in the processed fraction and how the test portion is selected that are important and should be studied and described in standard operating procedures. For example, many laboratories still use room-temperature processing, which is known to contribute to analyte losses for some pesticides. These may not be known or are ignored and not measured as part of the overall uncertainty. It is important to establish, as a part of analytical validation, that a test portion will meet the SQC requirements for the analytes of interest in the particular decision unit (DU) of interest. For example, it might be much more important to have a very precise analytical measurement of any contaminants in baby food than is needed for routine surveillance to enforce MRLs in fresh vegetables.

What factors should be considered in determining the minimum analytical sample size?

If all other sources of error are small, the compositional and distributional heterogeneity of the material being sampled is very important in determining the minimum analytical test portion mass. The particle size and distribution in the comminuted analytical test sample, from which the test portion is selected, is directly related to the minimum analytical sample size needed to achieve a given error rate. Many laboratories are turning to cryomilling to produce very small, submillimeter particle size test samples. However, cryomills will only process a few grams of material, so many procedures use a two-step process, which introduces its own errors. In addition, very small test portions must then be selected and weighed from these extremely cold test samples. I would suggest that incurred residues of known concentration need to be tested to validate these methods. Radiolabeled materials are sometimes used for this purpose and are very useful in identifying losses of analytes

throughout the process. They may often be analyzed with more accuracy, which allows a more precise estimate of errors throughout the process (12–15).

Can the use of microsamples of ~0.1 g, combined with automated methods, lead to accurate results?

Yes, it is possible for some analytes, in some types of materials to be tested using milligram sample sizes. What has not yet been shown is that the field-to-test-portion errors are sufficiently controlled. Replicating a test portion does not estimate the entire error. In the field of pesticide residue, so much work has been done on some aspects of sampling and sample processing errors that with some additional study, we may see some adequately characterized micro methods. There is just a lot of work to be done for over 1,000 pesticide chemicals and hundreds of human and animal food matrices. Registrants and regulators alike need to provide clearly described procedures and data to support the use of such micro methods. It would be very worthwhile if sampling validation data could be shared openly among industry and government scientists so that some of these questions could be addressed.

Is the traditional sample comminution method adequate?

Most traditional sample comminution methods are not adequate for sub-millimeter test portions. As explained above, cryomilling may provide better comminution for some but not all applications. It adds time and expense to processing, so it depends on how many samples must be analyzed and how much savings is realized by adopting micro methodologies.

Does cryomilling of samples lead to greater accuracy in testing?

Yes, it can, for some applications, because a finer particle size can be achieved and analytes may not degrade as easily. But only if correct sampling has been achieved at every preceding sampling stage and the appropriate test portion size chosen.

What recommendations do you have for laboratories that want to ensure their sampling and sample processing protocols do not contribute to inaccurate testing results?

I recommend that more scientists study and understand the theory of sampling and not simply the statistical approach to sampling that is often taught because the number of increments taken is just not the whole story. I would also really like to see more studies using well-described incurred residues and reference materials. Establish and monitor the error contributions for every sampling stage, validate laboratory-sampling procedures just as is done for analytical methods, incorporate quality control (QC) into sampling

stages, and evaluate the QC, as is done for analytical methods. Recently, I have been suggesting that laboratories can spike surrogates at earlier stages in their procedures, such as before comminution or to the comminuted analytical test sample before selection of the test portion, as a means of gathering more information about sampling errors. Very little has been done to routinely measure sampling uncertainty in laboratory processing, so it remains to be seen if this suggestion is truly useful.

What resources or guidelines on sampling practice should analysts refer to?

Initially, I would recommend that scientists start with *GOOD Samples* (2) as a very general introduction. It is available for free download. *GOOD Samples* lists additional references including a very good series of articles in the April/May 2015 issue of the *Journal of AOAC International*. Chapters 9 and 10 in *Food Safety Assessment of Pesticide Residues* provides more detailed sampling theory for the residue chemist (3). I personally like Gy's Sampling for Analytical Purposes (16) and Pitard's *Pierre Gy's Sampling Theory and Sampling Practice* (17).

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Jo Marie Cook is the chief of the Bureau of Chemical Residue Laboratories of the Division of Food Safety at the Florida Department of Agriculture and Consumer Services.

AUTOMATING SOLID-PHASE EXTRACTION CLEANUP TO SAVE TIME AND REDUCE COSTS IN FOOD SAFETY ANALYSIS

An interview with Yelena Sapozhnikova

Given increasing concerns about food safety worldwide, a research team from the U.S. Department of Agriculture developed and validated a fast and cost-efficient method for the analysis of pesticides and environmental contaminants in meats. Here, Yelena Sapozhnikova, PhD, a research chemist at the Eastern Regional Research Center of the U.S. Department of Agriculture's Agricultural Research Service, discusses how an automated mini-solid-phase extraction (SPE) cleanup approach coupled with low-pressure gas chromatography–mass spectrometry (MS)/MS can achieve reliable, high-quality results without the labor and costs required with other methods.

Why did your team decide to develop and validate a multiresidue method for analysis of pesticides and environmental contaminants in meats (1)?

For many food commodities, including meat, global food trade is on the rise. Fast, efficient, and inexpensive analytical techniques are needed for the analysis of contaminants in meat samples. We previously developed a high-throughput analytical method for the analysis of pesticides and environmental contaminants in fish and seafood samples, based on QuEChERS (quick, easy, cheap, efficient, rugged, and safe) extraction and filter-vial dispersive SPE (d-SPE) cleanup. We decided to optimize and validate this method for the analysis of poultry (specifically chicken), cattle, and pork muscle. To achieve a wide scope of analysis, we included both polar (liquid chromatography [LC]-amenable) and nonpolar (gas chromatography [GC]-

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amenable) pesticides, and environmental contaminants: polychlorinated biphenyls (PCBs), polyaromatic hydrocarbons (PAHs), polybrominated diphenyl ethers (PBDEs), and other flame retardants in our method.

Why did you use a low-pressure GC (LPGC)–mass spectrometry (MS)/MS method in your study? What were the advantages of this technique over conventional GC–MS?

Our goal was to match the speed of ultrahigh-pressure liquid chromatography (UHPLC) analysis, which is typically 10 min, and to conduct GC– and UHPLC–MS/MS analysis in parallel from the same sample extract in 10 min. The low-pressure (LP) vacuum outlet GC–MS/MS technique has been used in our laboratory for more than 10 years.

LPGC analysis has many advantages over conventional GC methods. First is its speed. LPGC analysis is at least two to three times faster than a conventional GC method; our total LPGC separation of >200 analytes takes only 10 min. This results in high sample throughput, increased productivity, and faster turnaround time because more samples can be analyzed during a work shift.

Other advantages of the LPGC technique include greater sensitivity, lower detection limits, increased sample loadability, and greater ruggedness than conventional GC methods (2). Using both LPGC– and UHPLC–MS/MS techniques in parallel enabled us to analyze a wide range of both polar (LC) and nonpolar (GC) pesticides from one sample, affording a wide scope of analysis. In addition, we had 55 mid-polarity pesticides, which are both LC and GC amenable. Consequently, they were analyzed by both instrumental methods, thus providing an additional degree of confirmation by using orthogonal techniques.

How did you validate your method, and what were the results?

We validated our method for poultry, cattle, and pork muscle tissue according to the U.S. Department of Agriculture (USDA) Food Safety and Inspection Service (FSIS) protocol, which requires 10 replicated samples of each matrix type at each spiking level. Spiking levels are usually selected to be below, at, and above established tolerance levels for pesticides.

We acquired cattle, pork, and poultry muscle tissues from different parts of the animal (raised organically) from local grocery stores. For example, chicken wings, breast, thigh, drumsticks, and whole Cornish hens were used as representative muscle for poultry samples.

We evaluated recoveries and relative standard deviations (RSDs) of 243 analytes from pork, beef, and chicken samples (n = 10 each) fortified at three spiking levels. In terms of the results, satisfactory method validation criteria, 70–120% recoveries and RSDs of ≤20% were achieved for 200 of 243 contaminants, which is 82% of all tested analytes.

When trying to cover hundreds of analytes in one multiclass, multiresidue method, there are always "problem analytes" that don't behave and fail the validation criteria. For example, some contaminants had recoveries below 70%, but they were consistent with low RSDs. For others, mostly brominated flame retardants, we were able to validate the method at higher spiking levels, but failed at the lowest level because of their low sensitivity with GC–MS/MS-electron ionization (EI) methods.

Did any of the results from the study surprise you? Why or why not?

When we used post-column infusion to estimate matrix effects for LC-amenable pesticides with and without d-SPE cleanup, we learned that there was only a 10% difference in matrix effects. Therefore, we decided not to use d-SPE cleanup before UHPLC analysis. It was particularly helpful because some polar LC-amenable pesticides were retained by d-SPE sorbents, which led to lower recoveries.

Analytical chemists tend to think the more cleanup, the better, but that is not necessarily true, and our findings supported that premise. In fact, more steps in the sample preparation process bring more opportunities for errors. Recent studies also showed that dilution is the solution, and diluting the final extracts can be more efficient than cleaning, provided we can still measure the desired concentrations after the dilution, which is achievable with modern analytical instrumentation.

The sample preparation and instrumental analysis you developed are rather fast. How about data analysis and reporting? How long does it take to process data for 243 contaminants in one sample? In many samples?

For identification with MS/MS analysis, retention time (t_R), two ion transitions and their ratios are required (3). This is four identification points per analyte, multiplied by 243 analytes, plus internal and quality control standards, resulting in over 1,000 data points to review from one sample! For validation experiments, we ran batches of 50–60 samples per day, totaling as many as 60,000 data points to review from only one batch. By the end of a day reviewing peaks, you can end up cross-eyed. With all the improvements in analytical instrumentation—new sensitive mass spectrometers, better analytical columns, and sample preparation (QuEChERS, and robotic cleanup)—data analysis remains a bottleneck in terms of the time spent by an analyst integrating and reviewing peaks.

From our experience, we found that no matter how well we set up the software parameters to integrate peaks, unanticipated mistakes always happen. For example, the software integrates a closely eluting peak instead of the one needed. Another problem concerns human error; when you have to stare at

the screen for hours and days integrating and reviewing data, sooner or later, you are going to make a mistake. It is unavoidable.

We recently discovered a better and faster way to accomplish this task using a summation integration function (4). This function is present in many software packages, and simply draws a line from point A (where the peak starts) to point B (where the peak ends). UHPLC instruments have rock-solid retention times, and we use analyte protectants in our LPGC approach, and as a result we see very few deviations in retention times and consistent peak shapes over many batches of samples. Although it takes some time to set up start and stop times for every compound, it pays off when you don't have to manually reintegrate or correct wrong integration by the software.

We also found that, understandably, the consistency of the summation integrations is superior to human capabilities. So, right now, we spend an hour or so setting up summation integration parameters in the quantitation–processing method, and it takes a few seconds (rather than hours and days) for consistent automated integration. You can find more information about summation integration in an article recently published in *LCGC* (5).

What are you currently working on?

We are currently validating the method for 270 pesticides and environmental contaminants in meat muscles, and plan to optimize and validate the methods for catfish and eggs in the future.

The list of pesticides we are currently working on is recommended for routine monitoring in meats by the U.S. Environmental Protection Agency (EPA). The selection is based on pesticides' occurrence in foods as reported by the National Pesticide Residue Program, as well as their assigned importance ranking derived from the information on their usage, properties and toxicological effects. Environmental contaminants include: PCBs, recommended for monitoring by the World Health Organization (WHO), including dioxin-like congeners; PAHs from the EPA priority list and the European Union list (EU 15+1); PBDEs; and other flame retardants.

Sample preparation is based on QuEChERS extraction, but we adopted an automated robotic mini-column SPE approach for cleanup, as recently reported (4), instead of the d-SPE cleanup we previously used. Automated cleanup is operated by a robotic liquid-handling system, where sample preparation steps are programmed through the software.

A simple cleanup procedure, as reported recently (4), entails passing 300 µL of the QuEChERS extract with a flow rate of 2 µL/s through a small 35-mm mini-column containing 45 mg of sorbents. The same sorbents as for d-SPE cleanup (that is, anhydrous $MgSO_4$, PSA, C18, and Carbon X) are used, but they are packed inside a small mini-column. No conditioning or elution of the sorbents

are required as in traditional SPE, and the resulting cleaned extract is collected in a mini-insert inside an autosampler vial.

After the addition of analyte protectants and acetonitrile (for samples) or calibration standards (for calibration samples), the extract is injected into an LPGC–MS/MS system. We evaluated high-throughput cleanup for 94 pesticides and environmental contaminants in 10 food commodities using this approach (4) with LPGC–MS/MS, and achieved efficient, robust cleanup with high-quality results. At that time, we used the robotic handler in a stand-alone fashion, but now it is installed on the top of our LPGC–MS/MS instrument, which allows consequent injection of the cleaned extracts as well as continuous operation of cleaning–injection cycle, thus saving time. No cleanup is performed for extracts subjected to UHPLC analysis; QuEChERS extracts are filtered and injected. After the method is validated, we plan to transfer it to the USDA FSIS laboratory for routine monitoring of contaminants in meats, catfish, and other commodities.

How does the method you are currently investigating improve upon the methods most labs are currently using?

In terms of sample preparation, we demonstrated that QuEChERS batch extraction with acetonitrile is very fast and efficient. In fact, as many as 50 samples can be extracted in one batch on a platform shaker. It takes one analyst in our laboratory approximately 3 h to prepare a batch of 40 pre-homogenized samples and submit the extracts to UHPLC–MS/MS analysis and robotic cleanup in parallel with LPGC–MS/MS analysis.

Most labs use accelerated solvent extraction (ASE), gel permeation chromatography (GPC), or column SPE cleanup for analysis of pesticides in meats. Those methods are time- and labor-consuming, and they utilize large amounts of solvent and materials. We calculated the cost for materials and supplies using our method at $8–9/sample, while the estimated cost for ASE, GPC, and SPE methods is at least two-fold greater.

The same is true with the amount of time needed for sample preparation; our method is significantly faster. Automated robotic cleanup is efficient and robust, which also helps to increase instrument ruggedness and decrease maintenance and down time.

Another difference is in using three ion transitions and their ratios instead of two. Meat samples are complex, and sometimes there are interferences leading to skewed ion ratios, and, consequently, false-negative results. With t_R, three ion transitions and the three resulting ion ratios, we have seven identification points instead of four using two ion transitions, resulting in more accurate identification. Finally, automated data analysis using summation integration saves much time (and eye exhaustion).

Might there be any barriers to the implementation of this technique?

Many laboratories use the QuEChERS extraction method now; however, there is some resistance to using acetonitrile as an extraction solvent. It is believed that nonpolar solvents such as hexane and ethyl acetate are better for extracting nonpolar lipophilic contaminants, but we have shown that they also extract up to 18 times more co-extractive materials from biological samples (6) compared to acetonitrile, which in turn, requires more extensive cleanup. We have demonstrated on incurred samples and NIST standard reference materials (SRMs) (7) that acetonitrile as an extraction solvent achieved efficient extraction of many contaminants with the use of isotopically labeled internal standards.

In terms of implementing our robotic automated cleanup procedure, the biggest barrier may be in acquiring the robotic handler. It costs $30,000–40,000, but the benefits are significant because it provides efficient, fast, and robust extract cleanup for many food matrices.

In summary, what do you feel is the top take-home message for analysts reading about this method?

The method we developed is simple, fast, efficient, and inexpensive, and it can be implemented in any laboratory. It can save significant amount of solvents and sorbents compared with other commonly used methods (using ASE, GPC, or SPE), thus leading to money, time, and labor savings.

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Yelena Sapozhnikova, PhD, is a research chemist at the Eastern Regional Research Center of the U.S. Department of Agriculture's Agricultural Research Service.

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DEVELOPING MULTIRESIDUE AND MULTICLASS METHODS FOR THE ANALYSIS OF VETERINARY MEDICINAL PRODUCTS FOR USE BY ROUTINE TESTING LABORATORIES

An interview with Eric Verdon

Reference Laboratories have an important role in food safety within the European Union, as they advance the science of analytical testing in a very practical way, by developing new methods that can be used effectively by EU Member State National Reference Laboratories and by official routine laboratories. As a result, the methods that Reference Laboratories develop must be rugged and as efficient as possible—ideally being capable of measuring many analytes in a single run—without losing critical sensitivity. Eric Verdon, the head of the European Union Reference Laboratory (EU-RL) for Antibiotic Veterinary and Dye Residues in Food from Animal Origin at the Anses-Fougeres Laboratory in France, spoke to *LCGC* about some of these methods and the process of developing them.

You recently developed and validated a fast and simple screening method for 75 antimicrobials in meat and aquaculture products using liquid chromatography–tandem mass spectrometry (LC–MS/MS) (1). Why did you undertake this work? How does this method fit into the larger trend of developing multiresidue food safety methods?

In the European Union and in many other parts of the world, public health

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concerns and food safety control have been changing a lot over the past 30 years. At the same time, many advances have been made to the technological tools available for monitoring chemical residues in food. The field of veterinary drug residue control in food undertook this very same evolution. Back in the 1980s, only wide-scope microbiological methods were employed for control of veterinary antimicrobial residues in food from animal origin. This methodology was the only one able at the time to rapidly and reliably detect, within a single sample and at parts-per-million levels, the antibacterial activity of a certain number of antimicrobials.

In the early 1990s the European Union (EU) regulations under the so-called "Food Law" strengthened food safety controls on veterinary medicinal products (VMP) in European Union countries. The presence of VMP traces in food had also to be fully controlled under application of enforcing regulations such as the Regulation No. 1990/2377/EEC. This piece of regulation was the first to introduce the concept of "maximum residue limits" (MRLs) to be assessed and established for each VMP residual substance in each animal product (muscle, liver, kidney, fat, milk, egg, honey) in each relevant species (bovine, porcine, ovine, caprine, equine, poultry, farmed game, farmed fish, and other aquaculture species). Annual National Residue Monitoring Programmes were therefore implemented over the same period (under Directive No. 1996/23/EC). And numerous analytical methods were developed by means of biological or physicochemical technologies, or both, to cover the need for monitoring. For example, in the case of antibacterial substances, microbiological inhibitory methods were challenged by new immunochemical and immuno-enzymological methods but also by analytical chemistry methods based on separation techniques such as high performance liquid chromatography (HPLC) and gas chromatography (GC). By the end of the 1990s, it appeared necessary to incorporate technological developments into monitoring programs to have better coverage of many chemical substances while avoiding having so many single-residue methods. One important step in this process was to develop multiresidue antimicrobial HPLC methods able to cover a whole family of very similar substances such as sulfonamides, tetracyclines, or even beta-lactams, at least in meat production at first. But the most important revolution in physicochemistry applied to the VMP residue control was engaged in the early 2000s with the regular use of reliable and sensitive MS detectors coupled to these HPLC systems and particularly with the use of the tandem MS detectors, which are also called *triple-quadrupole* detectors (LC–MS/MS).

More and more research and development was then conducted on these physicochemical systems and their electronic and informatics devices. It

quickly became possible to apply more speed and more sensitivity for numerous signals (analytes) to be detected in the same sample run and if possible after the same extraction procedure and within the same injection into the ionization source. Multiresidue methods were born and their monitoring capacities have not stopped improving. Today, with more and more robust and sensitive LC–MS/MS instruments, 50 to more than 150 veterinary analytes can be analyzed in a single method with high speed.

This method, therefore, represents another step forward in the development of multiresidue methods for food safety, by covering a large set of analytes of a type (antimicrobials) and in a food matrix (meat) that had previously not been covered by a single method.

Can you briefly describe the method? How did you optimize the method to be able to screen for such a large number of analytes?

This method is aimed at swiftly screening as many antimicrobial residual analytes as possible extracted from the same sample, with the same extraction procedure, and injected into the same chromatographic analytical column and using the same detection system. As a consequence, the extraction procedure is kept as technically simple as possible and with a wide scope for fishing as many antimicrobial veterinary substances as possible from the biological food matrix, regardless of what tissue-like matrix (such as bovine, porcine, and poultry muscle, or fish flesh) the sample is collected from. This is why we ultimately preferred an acetonitrile liquid–liquid extraction. The chromatographic system was also designed to maintain a simple, reliable, and robust separation of the analytes. The main issue was to get rid of the more polar substances extracted from the food matrix, which we accomplished by controlling the monitoring of the whole set of our antimicrobial substances within a short separation window and maintaining a reduced analysis run time. We also achieved a sufficient separation from the polar endogenously extracted molecules to avoid or at least minimize the matrix effects issues generally observed in the ionization source. Detection with a tandem mass spectrometer is today considered one of the best choices for reliable identification of each of the antimicrobial residual substances. It leads to ionized analytes that can be easily monitored by means of at least two of their significant product ions. Also it is the best choice for providing accurate enough quantification of each of the relevant substances, especially when appropriate internal standards can be used. In our method, we did use a single internal standard, however, because the method was first developed to be a fast screening and identification method. Our main objective was to transfer a reliable method for routine use to a large network of official French laboratories.

You originally started with 75 antibiotics, and successfully validated the method for 73. Why was the method not able to be validated for the other two antibiotics—bacitracin and virginiamycin?
The method has been developed to consider all the antimicrobial substances currently regulated in the EU with an MRL set and also in use in the veterinary sector in France. Bacitracin and virginiamycin were first considered as relevant compounds. However, it appeared quickly during our development process that these two polypeptidic antibiotics were not chemically behaving the same way as the other antimicrobials. Our wide-scope method could not be adjusted and sufficiently focused to cover all the characteristics (extraction, separation, ionization, detection) needed to track these two polypeptidic substances as well. In general, a multiresidue method will often be insufficient for a few interesting substances whose physicochemical characteristics are different from the other analytes in such a way not to be covered by the method.

This method is clearly intended to be easy to use. How did you ensure its ruggedness, particularly for use by a wide variety of laboratories?
The general process of method development in our Reference Laboratory is to consider first where the method will be used. Certain methods are kept at a reference status in our hands, but most methods are designed to be deployed in our network of routine laboratories. When we developed this method, we kept in mind that the transferred method might need to be adjusted to work for a large scope of routine laboratory facilities and for their LC–MS/MS instrumentation setups. Ruggedness is one parameter that is on the minds of all our developers at the Reference Laboratory from early development to the validation steps. We also continuously evaluate its ruggedness throughout the process of transferring a method to the network of laboratories. First, it is discussed and handled technically during the hands-on training sessions we invite our partners to participate in at our facilities. Second, during the process of setting up and tuning the method in their routine laboratories, they can request some support and assistance from the Reference Laboratory. And third, the level of performance and the reliability of the method are controlled within the network of field laboratories by means of an initial collaborative testing study, which is then followed by continuous organization of proficiency testing (PT) studies over appropriate periods proposed by our Reference Laboratory behaving as the PT provider under ISO 17043.

Overall, what results did you see in terms of specificity, limit of detection, sensitivity, and detection capability?
This method was developed to be employed in France under the EU Food

Law as an official qualitative screening method for antimicrobial residues in all meat tissues including the aquaculture products. And thus the analytical performance of the method has been validated in accordance with Decision (EC) No. 657/2002, which is the current regulation for the validation of analytical methods dedicated to VMP residue control in food. This decision was enforced in 2002 in line with two pieces of MRL regulations, (EU) No. 2009/270 and No. 2010/37, which replaced in 2009 the regulation (EEC) No. 90/2377 and was created to support the Directive (EC) No. 96/23 adopted in 1996 for the implementation of the annual national official monitoring plans of the EU member states. Practically speaking, the method is validated by comparing its analytical performance to the criteria of Decision (EC) No. 657/2002 for this kind of MRL-substance method. Table 9 of this Decision lists the appropriate performance criteria to be assessed and validated for each substance controlled under a qualitative screening: that is, the detection limit (CCbeta), the selectivity–specificity, and the applicability–ruggedness–stability. In this regard, the method was demonstrated to be fully validated for 73 antimicrobials against the 75 that were initially considered. Only bacitracin and the virginiamycin were not successfully validated for this method.

You recently conducted a study to compare the levels of contaminants—pesticides, antibiotics, persistent organic pollutants (POPs), metals, antimicrobials and more—in standard and organically raised beef, pork, and poultry (2). Why did you undertake that study?

In a report published in 2011 to define the main prospects of the organic food sector, the French Scientific Council for Organic Agriculture underlined that food safety and the absence of contaminant residues was the primary motivation driving 95% of organic food consumers. Yet according to the same report, very few scientific data were available to support the presumption of a health benefit associated with organic products. The issue is particularly critical for animal-derived food products due not only to recent safety crises, but also to the known tendency of these food matrices to bioaccumulate toxic contaminants such as environmental micropollutants, mycotoxins, and antibiotics. The project thus aimed at providing scientific data to fuel the debate on the presumed health benefit of organic meat products in regard to their possible chemical contaminants and the putative resulting toxicity potential for consumers. This multidisciplinary project covered both basic and industrial research and involved both French scientific institutes (INRA, ANSES, ONIRIS, and AgroParisTech) and the three main R&D organizations of the meat sector for beef, pork, and

poultry species (IDELE, IFIP, and ITAVI). With this large French consortium, novel research and development was undertaken in analytical chemistry, food chemistry, toxicology of contaminant mixtures, risk analysis, experimental economics, metabolomics, genomics, and chemometrics.

What methods did you use in that study?

Our focus at the Anses-Laboratory of Fougeres was the analytical chemistry control of the residues of antimicrobials in beef, pork, and poultry samples and also of anticoccidials in the poultry samples. Two first screening methods were carried out by LC–MS/MS instrumentation; positive findings in the initial screening were followed by analysis using several of our confirmatory quantitative methods. One first screening method was dedicated to 75 antimicrobial residues in muscle tissues including eight penicillins, 10 cephalosporins, 17 sulfonamides, four tetracyclines, 13 macrolides, 10 quinolones, and 13 other compounds. The second screening method was able to monitor 10 coccidiostatic substances in poultry meat including chemical coccidiostats and polyether ionophores. Coccidiostat residues were investigated only for the set of poultry samples, considering the exclusive usage of these feed additives in poultry farming. Internal standards and quality control samples suitable for each species matrix (beef, pork, and poultry) were used to ensure the reliability of the results. The required criteria of performance of the methods according to Decision (EC) No. 2002/657 were carefully examined and all quantitative results in terms of concentration were expressed in milligrams per kilogram. Limits of quantification were in the range 0.2–1.8 mg/kg for antimicrobial residues and between 0.02 and 8 mg/kg for coccidiostats. A number of other analytical methods were carried out by the two other reference laboratories, the Anses-Laboratory of Maisons-Alfort and the ONIRIS-Laberca of Nantes, for screening environmental contaminants such as inorganic trace elements, mycotoxins, and pesticides, and also POPs like PCDD/F congeners, PCB-DL congeners, PCB-NDL congeners, and HBCD, respectively.

What were your major findings?

As a first reference study to fuel the debate on the presumed health benefits of organic meat products in regard to their possible chemical contaminant contents, the major findings of this work highlighted that chemical residues arising from veterinary (antimicrobials and anticoccidials) or phytosanitary (pesticides) practices were generally not detected or were detected at levels far below their MRL or tolerance level. Although this result was expected for the organic products as a direct consequence of the implementation of organic specifications, our study

highlighted that conventional meat was also observed to be free of such residues. These can therefore not be considered as criteria to distinguish between organic and conventional breeding practices.

On the other hand, some environmental and inorganic contaminants were highlighted in both farming practices. Organic production, favoring older animals or outdoor access, or both, can also lead to environmental contamination when dealing with contaminants accumulating as a function of age or fat content. While in most cases the explanatory factors have been advanced or hypothesized, it is not possible to anticipate at this stage the consequences in terms of consumer exposure to these contaminants. This study is in this respect considered a pilot study where results have highlighted some consequences of contamination of meat from particular types of farming. Results are intended to serve as input to assess the corresponding chemical risk for conventional and organic meat consumers. The scope of this project could now be expanded from meat only to the organic food basket, while increasing the statistical power; a study like a total diet study (TDS) including a large sampling of organic products may for instance lead to a conclusion about the exposure of organic consumers, in a complete risk assessment study.

You have also developed a fast, multiclass method for the determination of antimicrobial residues in honey (3). How big a concern is the presence of antimicrobials in honey? What is their source?

In apiculture, beekeepers may treat their hives with antibacterial agents against bacterial diseases such as American foulbrood (AFB) and European foulbrood (EFB). However, in some countries, like the United Kingdom and New Zealand, when bee colonies are infected with AFB, the hives must be destroyed by burning because the spores are considered highly resistant and can remain infectious for more than 35 years. So far, there are no MRLs set for antimicrobial residues in honey within the European Union (Reg (EU) No 2010/37, 2009). Therefore, the presence of such VMPs in honey is not authorized. The European Union Reference Laboratories (EU-RLs) provided in 2007 recommended concentrations (RCs) for the control of nonauthorized substances included in honey for tetracyclines, sulfonamides, streptomycins, and macrolides (erythromycin and tylosin) to improve and harmonize the performance of monitoring analytical methods. Furthermore, the Codex Committee on Residues of Veterinary Drugs in Foods (CCRVDF) drafted guidance by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) (JECFA, 2013) for the establishment of MRLs in honey based on the acceptable

daily intake (ADI) of VMP residues and their depletion studies in honey.

Many of the residues you analyzed in this study—such as sulfonamides, tetracyclines, macrolides, and aminoglycosides—present specific challenges in terms of matrix effects. That meant there were many aspects of the sample preparation approach to optimize. What were the most important aspects to your optimization of the sample preparation steps?
The aim of this study was to develop and validate a simple multiclass method for identification and quantification by LC–electrospray ionization (ESI)-MS/MS of at least 21 antimicrobial VMPs reputed to be used in treatments for bee colonies. These drugs belong to several classes of antimicrobials that include sulfonamides, macrolides, tetracyclines, lincosamides, and aminoglycosides. Simple extraction and cleanup steps were investigated and optimized using ultrasonic-assisted extraction and dispersive solid-phase extraction (dSPE).

Honey is a complex biological matrix that contains a high concentration of several sugars and other substances like vitamins, proteins, minerals, organic acids, and enzymes. The composition of these substances can vary widely, depending on the nectar source and other external factors such as seasonal and environmental conditions. These variations pose analytical challenges regarding sample processing and analysis of trace contaminants in honey. One of these challenges is to remove interfering substances such as sugar, wax, and pigments from honey extracts before VMP residue analysis, to reduce matrix effects. There are several other challenges for the analyst to overcome during the development of a multiclass method for analysis of VMPs in honey. For example, sulfonamide residues in honey combine with reducing sugar to form *N*-glycoside bonds, which leads to poor recoveries for almost all sulfonamides that could be found in the sample. For that reason, it is necessary to include a pretreatment hydrolysis step to break the sugar–sulfonamide bond. Studies have demonstrated that methanol and hydrochloric acid were the main reagents used to hydrolyze *N*-glycoside bonds to give better recovery. However, macrolides are not stable at acidic conditions; they are usually extracted under basic conditions to avoid their degradation. Erythromycin A degrades rapidly to anhydro-erythromycin A in honey, which is known to be an acidic matrix (pH ranges from 3.4 to 6). Other studies of honey samples identified desmycosin (tylosin B), the degradation product of tylosin A. Therefore, it is important to include not only the parent VMPs but also their metabolites or other transformation products when monitoring the use of their residues in honey. A similar phenomenon is observed for tetracyclines; these compounds can undergo structural epimerization in acidic conditions

(pH 2–6). Furthermore, they have a strong affinity to form complexes with divalent metal cations, which leads to inadequate recoveries during the sample extraction processing. To improve recoveries, the interaction can be disrupted by adding EDTA to the extraction solvent because it has greater affinity to chelate cations than tetracyclines. Another issue associated to the development of multiclass analytical method is associated with aminoglycosidic antibiotics. These VMPs are highly polar organic basic compounds that show practically no retention in reversed-phase LC; unless an ion-pairing reagent such as a perfluorocarboxylic acid is added to the mobile phase, also taking into account the suitable concentration to minimize ionization suppression.

When you validated the method, what results did you see?

The multiantimicrobial method for control in honey was validated according to recommended criteria of Commission Decision (EC) No 2002/657, and satisfactory performance data were obtained for most of the studied analytes. The relevant criteria for evaluating the performance of this screening–confirmatory method were specificity, linearity, recovery, precision, limit of decision (CCα), and capacity of detection in screening (CCβ). They all have been tested and shown to be in compliance with the EU legislation requirements, thus demonstrating the fitness-for-purpose of this method for the selected antimicrobials. This demonstration by an in-house validation process was complemented in our development program by satisfactory participation in several external proficiency testing studies.

What is the next challenge in chemical residue analysis that you plan to take on?

Along with several other reference laboratories working in the control of chemical residues for food safety, our laboratory is aiming at developing a simple and rapid extraction method followed by a post-targeted trace-level identification of VMP residues in different complex biological matrices. In this program, as part of our Anses analytical research activity, we will focus on the identification of nontargeted veterinary drug metabolites and degradation products of biological origin. The methodology that we will consider here for the near future would allow specialized laboratories to control, via a high-throughput screening, a large number of regulated compounds through a quick one-day, one-shot analysis to build consumer confidence in the safety of the food on the market. Unambiguous identification of all nontargeted drug metabolites, except those of simple known drug modifications (such as oxidation, reduction, and acetylation), in a given complex biological matrix, is still a challenging task for food-testing laboratories.

However, some of these challenges can be addressed using the following four approaches: obtaining high-resolution mass measurement of VMP residue analytes in full-scan MS or MS/MS modes, or both; determining the elemental composition of $[M + H]^+$ or $[M - H]^-$ ions in full scan mode and product ions in MS/MS mode with mass errors < 2 ppm; data processing through specialized software to find biomarker fingerprints; and synthesizing analytical standards of the suspected compounds.

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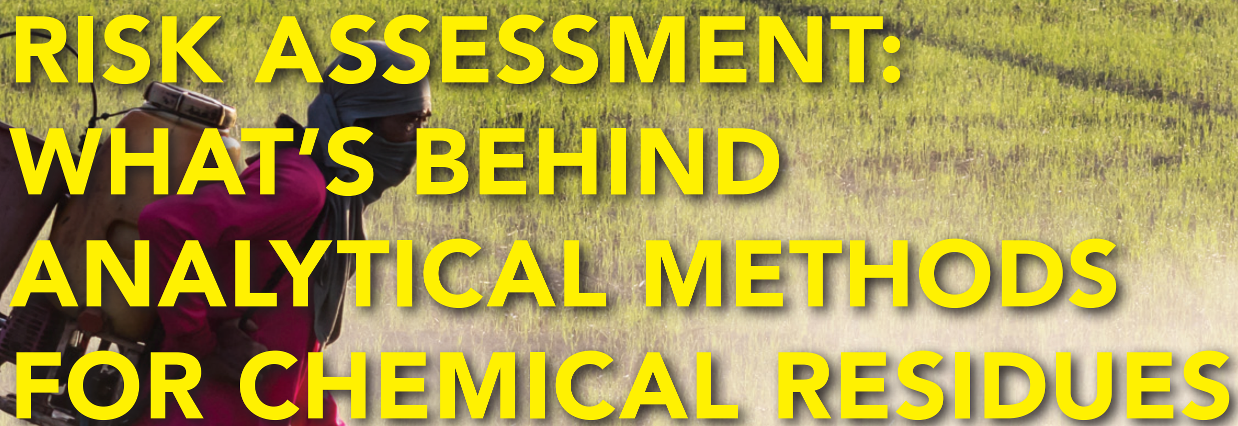
Eric Verdon, PhD, *is the head of the European Union Reference Laboratory (EU-RL) for Antibiotic Veterinary and Dye Residues in Food from Animal Origin at the Anses-Fougeres Laboratory in France.*



RISK ASSESSMENT: WHAT'S BEHIND ANALYTICAL METHODS FOR CHEMICAL RESIDUES

An interview with Paul Reibach

In the analytical community, many excellent methods are developed for analyzing pesticides and other chemical residues, and these methods are often used to determine whether the levels of residues in a food exceed allowed levels. But of course, there is an important process to determine what those allowed levels should be, and that process is based on assessing the risks to human health and the environment associated with those compounds. Paul Reibach, the technical director of chemistry at the contract research organization Smithers Viscient, talked to *LCGC* about how the risks of pesticides, and botanical insecticides in particular, are assessed.

What are the key components of assessing the risk of exposure to a given pesticide?

Risk assessments take into account the inherent toxicity of the molecule in question and the ultimate exposures through diet, dermal, and inhalation routes of exposure. Simplistically, risk = toxicity × exposure. The toxicity is determined from a wide variety of toxicology and ecotoxicology studies. The specific studies required are prescribed by various global regulatory authorities. In the United States, these studies are mandated and regulated by the U.S. Environmental Protection Agency (EPA) and the U.S. Food and Drug Administration (FDA). Examples of guidelines can be found at the EPA website under "Series 870—Health Effects Test Guidelines for Toxicology" and "Series 850—Ecological Effects Test Guidelines for Ecotoxicology." These studies assess a variety of potential acute, sub-chronic, and chronic concerns. Once toxicity endpoints are understood they are compared with potential exposures. Based on a variety of additional studies such as plant and animal metabolism, field magnitude of residue trials, animal

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feeding studies, food processing studies, and drinking water studies, a residue definition is determined and maximum permitted levels are defined. These levels, which are referred to as the *tolerance* in the United States and as the *maximum residue level* (MRL) in the EU, cover potential exposures from all that we eat and drink. Various computer models are then employed to estimate exposures based on potential food and drink consumption. Factors considered here are residue levels in treated food crops where the pesticide is in actual use, the percentage of those crops treated, any processing factors before getting the crop to market, composition of an average diet, and the population being evaluated. Additional safety factors or margins of error are also applied. Additional evaluations are made for worker exposures during application and harvesting.

You have done work analyzing the risks of botanical insecticides. How are such compounds defined?

The term *botanical insecticides* has both legal and practical applicability. One of the best examples of a botanical insecticide are the pyrethrins. Pyrethrins are insecticides derived from chrysanthemum flowers. These extracts are a complex mixture with many components. They are commonly found in chrysanthemum species from Australia and Africa. Pyrethrins work by altering insect nerve function, which causes paralysis in target insect pests, eventually resulting in death. There is evidence that these have been used as an insecticide in China for over a thousand years. Because there are similarities in the nervous systems of insects and mammals, there is some potential inherent neurotoxicity for humans; however, as discussed above the exposure levels are a key component of the risk equation. Based on mammalian toxicology studies, pyrethrins have low toxicity to humans. The EPA has concluded that the pyrethroids as a group (natural and synthetic) are at least 10 times less toxic to mammals than they are to insects. Also pyrethrins are rapidly degraded in the environment so exposures are minimal. Since they are derived from flowers in nature, have been in use for so long, and are considered nontoxic in mammals, they are considered safe.

Simply being derived from plant extracts is no guarantee of safety as there are many molecules extracted from plants that are known to have high toxicity. Remember Socrates died from drinking "tea" made from poison hemlock. So, all potential botanical insecticides need to be evaluated on a case by case basis.

Are these insecticides permitted to be used on food labeled as "organic"? And is their mechanism of action generally different from that of synthetic insecticides?

A discussion of organic versus nonorganic is a very complex topic and is outside

the scope of my presentation. There are numerous regulatory bodies involved and rigorous certification criteria needed to comply with the organic claims.

Is there anything fundamentally different about the way the safety of a botanical insecticide is tested compared with how the safety of a synthetic pesticide is tested?
Safety testing for all pesticides is a tiered approach with an initial set of studies needed for all potential products. Based on the results of initial testing, botanical insecticides have generally not triggered the higher tiers of testing. Should these tests be triggered, they would be conducted the same way that these tests are conducted for any synthetic insecticide.

How does the process for assessing the risk of new botanical insecticides depend on the compound's initial classification by the Directorate General for Health and Consumer Affairs of the European Community (SANCO)?
Depending upon previous regulatory classifications before pesticide uses (such as food additives, flavorings, and so on), some botanicals have received reduced registration requirements. I believe this route may no longer be acceptable to the authorities.

What are the steps in a risk assessment of a new botanical insecticide? What mammalian toxicology tests must be performed? What ecotoxicology tests are done?
For mammalian toxicology, the EPA requires an initial set of studies referred to as the "six-pack" tests. These are acute oral toxicity, acute dermal toxicity, acute inhalation toxicity, primary eye irritation, primary dermal irritation, and dermal sensitization. In some cases, an applicant may receive a waiver allowing them to skip even these studies.

What considerations are given for how the compound may break down in the environment? How are the transformation products tracked?
The more rapidly the material is degraded, the less the potential exposure. Metabolism and environmental fate studies are required to assess degradation. Significant degradation products and metabolites may be treated like the active component. The toxicity and levels of the metabolites and degradation products need to be assessed. Generally, they are less toxic and present at lower levels.

What are the "residue tests" that are performed on new insecticides?
Depending on the toxicological concern, the full scale and magnitude of the residue studies may be triggered. In this case, the pesticide material is applied under actual agricultural production use conditions and raw agricultural commodities are analyzed

at harvest. Studies such as these require the development and validation of rigorous analytical methods. These methods require a formal validation and subsequent independent lab validation by a second laboratory. A tolerance enforcement method is then generated which can be used by the EPA, FDA and others to evaluate residue levels entering the market as to assess misuse of the pesticide.

Paul Reibach, PhD, *is the technical director of chemistry at the contract research organization Smithers Viscient.*

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