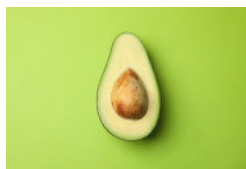




Improving Food Safety Analysis with LC/Q-TOF

FEBRUARY 2020

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Analysis of Pesticides in Complex Food Matrices

Christian Klein and Karen E. Yannell



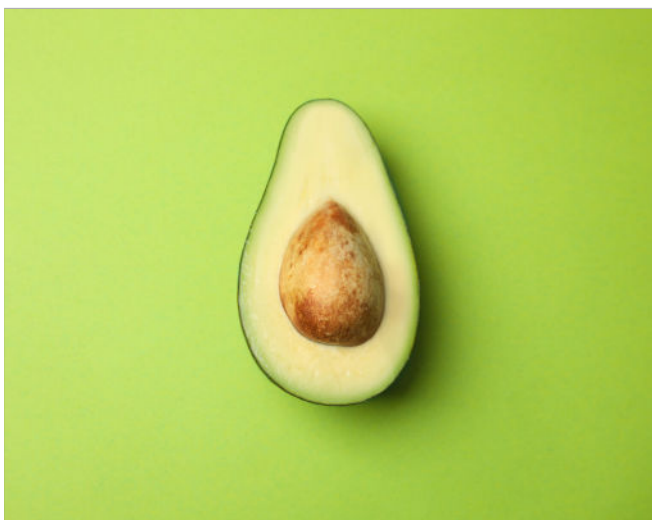
Nontargeted Screening Approaches for Potential Food Adulterants and Contaminants

Lewis Botcherby



Food Authenticity Testing with the Agilent 6546 LC/Q-TOF and MassHunter Classifier

Karen E. Yannell and Daniel Cuthbertson



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Analysis of Pesticides in Complex Food Matrices

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Increase your lab's productivity, decrease the complexity of your data analysis and review, and learn strategies for sample preparation.

Introduction

Many current analytical methods used to detect pesticides and other contaminants are falling short—the 2017 fipronil egg contamination scandal is a perfect example. As work is done to improve analytical techniques, this paper offers a workflow overview for pesticide analysis, addresses food safety guidelines accomplished with the Agilent 6546 liquid chromatography (LC) quadrupole time-of-flight (Q-TOF) system, and discusses food safety application using new screener software tools.

Mass Spectrometry

Mass spectrometry produces qualitative and quantitative results. Quantitative

is very directed—how much of a concentration of a compound can be detected. The qualitative aspect is what is in the sample. Can it be excluded from or included in the analysis? Both results are limited by the instrument's sensitivity and selectivity.

A comparison of full-spectrum to targeted analysis includes the following:

- **Full spectrum:** A spectrum is produced from the measurement. The resolution is a spectral width of each transmitted species (i.e., the peak width). Typically, this is done on high-resolution instruments such as a Q-TOF.
- **Targeted analysis:** Only a targeted window is transmitted. The resolution is the width of the transmission window. This is the typical workflow for triple-quadrupole instruments.



Triple quadrupole is the gold standard in quantitation with the highest sensitivity and an excellent dynamic range, but it has some limitations. The first is its unit mass resolution; it also has a very low mass accuracy and sample reanalysis for new compounds is not possible.

Q-TOF technology has high mass resolution, high mass accuracy, and is capable of sample reanalysis for nontarget compounds. Therefore, Q-TOF addresses the limitations of triple quadrupole, but there are sensitivity tradeoffs. Its sensitivity is comparable with mid-range triple quadrupoles, and it has only a slightly lower dynamic range.

Agilent is not only looking into the mass spectrometer, but it can also cover the entire workflow for pesticide analysis in food safety—from sample preparation and separation to detection and reporting, including added values for integrating technologies and services together.

Workflow Overview for Pesticide Analysis

The first step in the workflow is sample preparation; it is typically done with the QuEChERS extraction method. Agilent's Bond Elut reagents fulfill different requirements on the market such as different volumes in the sample extraction step or different methods (e.g., EN- or AOAC-based). After extraction, the next step is to select the dispersive solid-

phase extraction kit. It can, again, be dependent on the volume, the method, or the matrix dependencies.

Next is the separation step, which showcases the capabilities and impact of the Agilent 1290 Infinity II LC system. The 1290 Infinity II features:

- Touchscreen display with intuitive and adaptive control. Changes are made easily.
- Detector can achieve data rates appropriate for ultrahigh-pressure liquid chromatography peaks (240 Hertz VWD and DAD). It is suitable for fast chromatography.
- Column compartment with an innovative valve design for QuickChange and tool-free offers very low downtime and a flexible setup.
- Autosampler with a near-zero carryover with less than 9 ppm. It has the highest sample capacity (6,144 samples in 16x384 well plates), and it minimizes manual interaction during the sample analysis.
- 1,300 bar back pressure rating for high throughput analysis.

Focusing on the mass spectrometer, there are three different workflows:

1. **Target quantitation:** the quantitation of the compound with a limited list of targets (100). This workflow is highest throughput.



2. **Targeted screening:** confirms the presence of a compound. This is a medium-sized list of suspects (hundreds to a thousand), which is still a high-throughput routine analysis.
3. **Non-targeted screening:** sample is analyzed for significant compounds. This is a large size of analytes (thousands) with medium to low throughput.

Focusing on only two workflows, the Q-TOF workflow for target quantitation uses data independent acquisition (DIA), that are either All Ions or Q-RAI (Quadrupole Resolved All Ions), and a calibration curve generated to quantify compounds in unknown samples. This requires a reference standard for building the calibration curve, but the retention time is known and a concentration for the analyte is reported.

The workflow for targeted screening of suspects is also DIA and it confirms if a compound is detected with high confidence. A reference standard is optional here, therefore the analysis relies on the high mass accuracy, isotopic

“Analytes that are labeled as targets are quantitated and a larger set of suspects is screened in the same software at the same time.”

fidelity, and co-eluting fragments or spectral library match. The critical part in this workflow is the need for a fast review process to determine if an analyte is detected or undetected in a sample.

The workflows for both analyses are combined and can be performed simultaneously with the Agilent MassHunter Acquisition 10.1.

Analytes that are labeled as targets are quantitated and a larger set of suspects is screened in the same software at the same time. Additionally, the Screener Tool is embedded in this software to accelerate the analysis of the information-rich Q-TOF DIA data. After this process, a report is generated, which lists the detected or questionable compounds and targeted analytes that have a concentration reported.

Food Safety Guidelines within 6546 LC/Q-TOF

The 2017 SANTE Guidelines (11813) set identification requirements for pesticide residue analysis in Food and Feed for both unit mass resolution as well as accurate mass measurements. For the latter, the relevant mass spectrometers are Q-TOF, Orbitrap, FT-ICR, or a sector MS. The SANTE Guideline (SANTE/12682/2019) specifies a minimum number of ions required for identification; in this case, two ions with a mass accuracy of less than 5 ppm and at least one fragment ion are required. Another requirement



for identification is that the analyte chromatographic peaks for the precursor and/or product ion(s) must fully overlap. This implies screening cannot be performed in an MS-only mode.

Additionally, a single spectrum identification as typically used in auto MS/MS approaches will not work due to the chromatographic requirement of full peak overlap, and therefore DIA methods are the methods of choice. In a DIA mode called All Ions, one or more collision energies are applied to the whole mass spectra and all the ions present at that time are fragmented. Within this duty-cycle, an MS-only spectrum with zero collision energy is acquired, which allows one to have both molecular ions as well as fragment ions from non-zero collision energies. This method generates fully overlapping chromatographic peaks.

The difficulty is that, particularly in the low mass region, there are interfering ions because a lot of fragments exist between m/z 50 and 150. This causes the region to be noisy. Additionally, when fragmenting all the ions in the spectrum, it is hard to distinguish the origin of the fragments. To overcome this, the 6546 LC/Q-TOF has a new acquisition mode: Quadrupole Resolved All Ions (Q-RAI). With this technology, the quadrupole is used to filter a wide mass range before fragmentation. When the mass window filtering is done in sequence along the spectrum, less

complex fragment spectra is collected. Not only is the data less noisy, but more specificity is also provided to determine what molecular generated the fragment.

Duty Cycle Limitations

A typical chromatographic peak is about six seconds. For quantitation, at least 12 points over a chromatographic peak are needed, which means the duty cycle is 0.5 seconds, requiring 2 Hz as minimum acquisition rate. As a result, the more windows implemented in the experiment—for example, eight windows for Q-RAI in addition to the MS only spectrum—means the acquisition rate has to be $2 \times 8 + 1$, 17 Hz. And if there are multiple collision energies on it, it will go very fast into a fast acquisition rate. It is important to note that on a Q-TOF instrument, the acquisition rate is independent from the resolution. But this doesn't matter from the resolution side if you don't have the dynamic range. You can only resolve what you can see, and the dynamic range is one of the great features Q-TOF technology offers.

The dynamic range is achieved through two channels on the 6546 LC/Q-TOF: a low-gain channel that detects high-abundant signals and a high-gain channel that detects the low-abundance peaks. Both channels get summed together, yielding them in a dual-gain 10-GHz spectrum, which offers the advantage of having high resolution and extended



dynamic range together in order to detect compounds at high abundance next to compounds with very little detector response.

Technical Innovations in Quant 10.1: SureMass Data Conversion

Quant 10.1 allows one to process data to extract maximum information from the profile data for faster analysis. Profile data delivers the best sensitivity and mass accuracy, but historically is the slowest for processing. Agilent invented SureMass Data Conversion to move the profile data into SureMass features—it takes 3–5 minutes per sample, but it occurs after the acquisition with a post-acquisition script, so there is no delay time for data processing because the conversion is done while the next sample is already running. SureMass data improves the processing speed and shows mass accuracy at both low and high abundances.

Case Study: Food Safety Application with New Screener Software Tools

For food safety laboratories, it is advantageous to quantify commonly found pesticides in the first injection while also screening for hundreds more. As a proof of concept, 180 pesticides were quantified and over 200 were screened for at the same time. This was demonstrated in four different matrices: avocado, broccoli, black tea, and

strawberry. This analysis was done with the high-resolution 6546 LC/Q-TOF and the Quant 10.1 software. This software allows for simultaneous analysis of quantified and screened analytes. Additionally, it extracts and displays the LC/Q-TOF data in a way that makes the routine analysis possible.

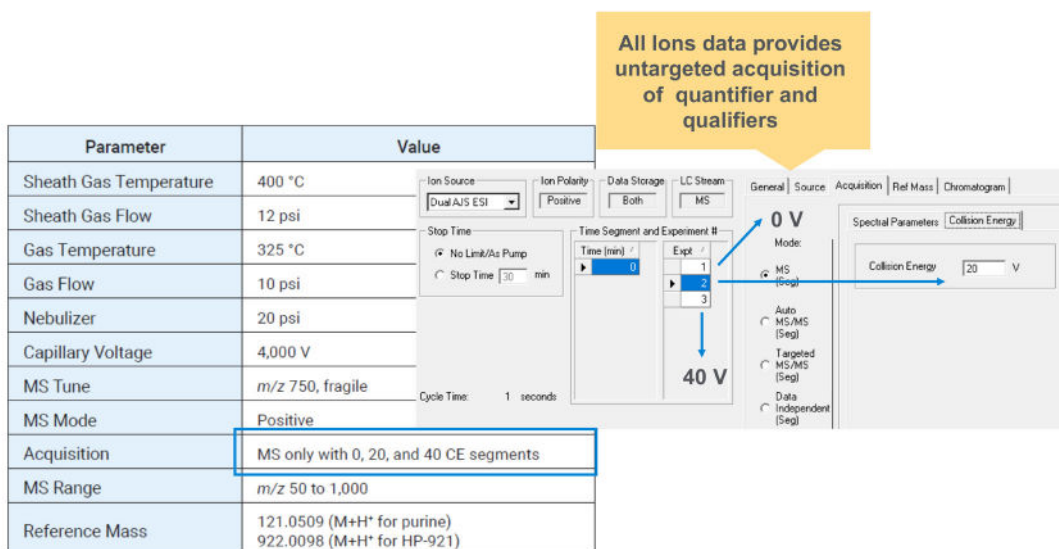
The method development was four steps:

1. **Sample Prep:** Prepares unknowns and calibrators for raw food products.
2. **Data Acquisition:** Both Q-RAI and All Ions are capable of high-acquisition rates that will also collect fragment data; they are both nontargeted acquisitions, so historical reanalysis can be done if needed.
3. **Build Method from PCDL:** Complete flexibility with method settings and set-up wizards to aid method development.
4. **Analyze Batch with LC/Q-TOF Screener Tool:** Fast analysis, confident results, and easy reporting.

Once the analysis method (Step 3) is built, it can be used again and again to analyze new batches of data successfully. Because of this, during routine analysis, there is no need to build it again—just take the developed analysis method and apply it to new data. In conjunction with the LC/Q-TOF Screener Tool, this is how routine



Figure 1: All Ions Q-TOF acquisition.



analysis is possible with Q-TOF data.

Sample preparation for the strawberry matrix required four steps:

1. **Blend:** Blended frozen strawberries until homogenized; 10 g were weighed into a conical tube.
2. **Extract:** 10 mL of acetonitrile was added; contents was shaken for 3 minutes.
3. **QuEChERS EN:** One EN packet was added to each tube; contents were shaken and centrifuged
4. **QuEChERS Dispersive SPE:** A Dispersive of SPE kit was added; shaken and centrifuged again.

The supernatant can be injected right away or stored. This process came directly

from the QuEChERS specification on Agilent's website. There is an extensive list of different matrices and clean-up requirements with the corresponding part numbers for products and procedures.

Data Acquisition

Next is data acquisition. A reverse-phase chromatography method was used with a 150 mm C18 column and a guard column, which helps extend the life of the chromatographic column. The gradient achieved excellent chromatographic separation of the analytes.

Figure 1 shows the All Ions data acquisition parameters. The data acquisition speed was 6 Hz, which allowed for the collection of 12 points across the chromatographic peak; this



“When an analyte is selected, its data is displayed in the Screener Tool and also in the Quant-My-Way UI.”

speed should be optimized for each chromatographic method. Remember, All Ions is an MS-only acquisition mode with different collision energies. The All Ions workflow enables collection of both molecular ion and fragment data, or quantifier or qualifier data, in a non-targeted manner. Although the data acquisition is untargeted, the data analysis is targeted.

Build Method from PCDL

When building the analysis method, you must know which pesticides you want to detect and have their molecular ion and fragment information. All this information is available in the Agilent Pesticide PCDL which is an expertly curated spectral library containing thousands of pesticides and their spectra collected at three different collision energies. Once analytes are imported into the Quant method from the PCDL, you can apply parameters to the compounds. These parameters, such as mass accuracy and signal-to-noise, are used in the Screener Tool to define outliers for rapid data analysis. The method can be saved and applied future batches of data.

Analyze with LC/Q-TOF Screener Tool

The LC/Q-TOF Screener Tool lists the method analytes in a sample and flags them as positively identified (green), needs review (orange), or not detected (red) based on the customizable analysis method settings. When an analyte is selected, its data is displayed in the Screener Tool and also in the Quant-My-Way UI. This gives a complete picture of the data and allows a reviewer to analyze a sample without digging deep in the software. For all analytes, the accurate mass data, overlapping fragments, and isotopic profile are displayed and easily understood so that reviewers have high confidence in the results. If the analyte is a target, a calibration curve is displayed and a concentration is generated. Screened analytes don't have a calibration curve, but they are analyzed in the same method—no need for an additional MassHunter Qual method.

This method gave good mass accuracy across the gradient and at different concentrations. Almost all the analytes had less than 2 ppm mass error at two different concentrations, and all of the analytes had less than 5 ppm error, which are the SANTE Guidelines.

Finally, 16 strawberry samples from different supermarkets across the United States were tested. The majority of the



“The samples were both organically and conventionally grown, and the number of detected pesticides followed the use trends with organic labeling.”

analytes were targets—this suggests the method would improve lab efficiency since you would quantify targets on the first injection. However, few analytes detected were suspects, emphasizing the importance of having a method with a broad enough scope for food safety. The samples were both organically and conventionally grown, and the number of detected pesticides followed the use trends with organic labeling.

Conclusion

Agilent offers reproducible sample preparation kits for your matrices, has trusted LC systems for your chromatography, and a high-resolution 6546 LC/Q-TOF, which meets regulatory requirements and has high-mass accuracy while collecting non-targeted data for screening and reanalysis purposes. This LC/Q-TOF system has fast acquisition rates and a large dynamic range which proves excellent for detecting pesticides in complex matrices. Finally, using MassHunter

Quantitative 10.1 software and LC/Q-TOF Screener Tool increases your lab’s productivity by combining the quantitative analysis and the suspect screening in one software, with the ability to analyze a batch of data of hundreds of analytes much quicker than traditional analysis workflows.



Christian Klein, PhD

LC/Q-TOF Product Manager
Agilent Technologies



Karen Yannell, PhD

Marketing Application Scientist
Agilent Technologies



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Nontargeted Screening Approaches for Potential Food Adulterants and Contaminants

Lewis Botcherby

Despite a wealth of analytical methods existing for food safety screening, the vast majority of these methods focus on specific compounds or a defined set of compounds, leaving unseen contaminants. Ann M. Knolhoff, a researcher for the U.S. Food and Drug Administration, spoke to LCGC Europe about the development of nontargeted screening approaches for potential contaminants and adulterants in food, and the considerations around sample preparation, chromatography, mass spectrometry, and data processing workflows.

Q. Why is the development of nontargeted screening approaches for food adulterants and contaminants important?

A: Many analytical methods used for food safety monitoring are designed to identify a specific compound or compound class, such as pesticides. While these targeted screening methods are important, compounds that are not contained in this defined method will not be found. A noteworthy example is melamine, which is a nitrogen-rich compound; melamine was being used as an economically motivated adulterant in pet food and milk products to increase the measured signal for total protein content. This adulteration resulted in illnesses and deaths among infants and pets (1). However, melamine and related compounds were not previously monitored. Nontargeted screening methods can aid in identifying the presence of adulterants and potentially hazardous compounds that may be present.

Approximately 11% of the total US food supply is imported; specific examples include 51% of fresh fruit,



28% of vegetables, and 95% of seafood by volume (2). This globalization of the food supply results in increased complexity. For example, each country has its own regulations for what is or is not acceptable. There are also more complicated supply chains, processing can vary between countries, and more diverse sample types are widely accessible. This necessitates the development of analytical methods that can identify potential health hazards that may arise. Nontargeted screening methods will ideally be able to identify potential issues with the food supply and aid in quickly identifying responsible compounds if a negative health effect is observed. These methods need to be accurate and able to quickly identify problematic samples and compounds.

Q. Sample preparation is an important part of most analytical methodologies, however, what are the issues surrounding the development of effective sample preparation for nontargeted methods?

A: Different analytical techniques are used for nontargeted screening, such as spectroscopy and mass spectrometry, which are often complementary to one another. Each of these methodologies have different requirements for sample preparation and it would be incredibly difficult to have one sample preparation workflow that could be used with all of these instrument platforms. Mass

spectrometry has the advantage of being able to detect many different compound classes—thousands of compounds can be detected in a single sample—and a large dynamic range can be measured which is useful for detecting both low- and high-levels of hazardous compounds. Because of these advantages, the discussed sample preparation challenges will be specific to mass spectrometry.

An optimal sample preparation method would extract compounds of interest, reduce potential interferences, and could be applied to different sample matrices without removing compounds of interest. Furthermore, compounds that differ in size, charge, acidity and alkalinity, and polarity would be reproducibly extracted (3). However, developing one method that fits all of these requirements is challenging, especially with sample matrices as complicated and diverse as food. To obtain the best compromise for different

“Nontargeted screening methods will ideally be able to identify potential issues with the food supply and aid in quickly identifying responsible compounds if a negative health effect is observed.”



compound classes, implemented methods strike a balance between sufficient sample clean-up to prevent instrument contamination and extracting as much as possible from a single sample. It is also likely that nontargeted methods will not exhibit the same recoveries as targeted methods because they may not be as selective. Food samples are also diverse, where different sample preparation strategies may need to be implemented depending on the sample type. The diversity of food matrices and compound classes also makes developing a universal sample preparation approach that will be successful for all cases unlikely for nontargeted screening. However, by using traditional approaches, such as preparing sufficient sample replicates, extraction blanks, and matrix spikes that contain diverse analytical standards, methods can be examined to determine if they are fit-for-purpose and reproducible for the needed application.

Q. Why is a chromatographic step important in a nontargeted method with regards to data quality?

A: One of the major advantages of using mass spectrometry is that thousands of compounds can be detected in a single food sample. This is especially true when combined with good chromatography because it can reduce the measured sample complexity, resulting in a greater number of compound identifications

“Nontargeted methods will not exhibit the same recoveries as targeted methods because they may not be as selective.”

(4). High-resolution mass spectrometry (HRMS) offers high mass accuracy and results in better separation of compounds that are similar in their mass-to-charge ratios (m/z). However, this resolution may not be sufficient in a complicated sample without a chromatographic step. We have observed matrix interferences at the 140,000 resolving power setting on an orbital trap instrument, despite using a long chromatographic gradient (50 min) (5). Using chromatography decreases the probability that these issues will occur. Increased mass accuracy errors can also be observed in orbital trap instruments when coeluting compounds of similar m/z are present, which can result in impaired molecular formula generation (4). Another consideration is that a significant number of food constituents may be measured in high abundance, which can result in ion suppression. Compounds with insufficient abundance will have higher isotopic ratio errors that can result in incorrect molecular formula generation (5). Likewise, quadrupole time-of-flight (Q-TOF) mass analyzers can be susceptible to higher



mass accuracy errors at low and high abundance (5). These matrix effects can be reduced if eluting compounds are chromatographically resolved. Achieving optimal data quality is vital to ensuring high-throughput, automated data processing workflows can be successful and reproducible.

Q. Are there any downsides to the inclusion of a chromatographic step?

A: Some may argue that a benefit to not using chromatography can be time. It can be faster to screen samples without chromatography, where analysis times can be 30–60 min per sample depending on the gradient length. However, because the rate-limiting step in nontargeted screening workflows is analyzing these information-rich data sets, I would argue that by using chromatography the data quality increases, which leads to faster data processing and more reproducible and accurate results. This is especially true when analyzing chemically complex sample matrices and data sets.

Q. What are the key considerations and potential pitfalls when generating the chemical formulae for unknown compounds?

A: Most available data analysis software programs that process HRMS data have functionality to generate molecular formulae. There are typically different settings that you can choose that will

influence the output, such as minimum and maximum numbers of elements that can be used. As the molecular weight increases, so does the number of possible chemical formulas. Seven golden rules were established for increasing the probability of generating a correct molecular formula (6). Among these rules are thresholds that should be used for mass accuracy (<3 ppm) and relative isotopic ratio error (<5%). This is another reason why ensuring high quality data is important.

Q. Chemical database can be incredibly useful when identifying unknown compounds as well as in certain data analysis approaches such as those in food “omics”. However, incomplete databases can lead to issues. How would complete databases change the prospect of untargeted screening methods, and can they ever truly be complete?

A: Compounds in chemical databases can be referred to as “known unknowns”. Suspect screening using liquid chromatography (LC)–HRMS uses a large database of specified compounds using the m/z and isotopic pattern to determine the presence of a compound. This strategy is different than nontargeted screening because the data are still being screened against a targeted compound list. There is a lot of merit to this workflow; it can be useful in ruling out the presence of known



“From a food safety standpoint, all of these compounds do not require identification.”

adulterants in these information-rich data sets and can complement nontargeted screening strategies.

The majority of compounds in foods are safe. From a food safety standpoint, all of these compounds do not require identification. If we could assume that databases are “complete,” known food compounds could be removed from the data and the remaining features could be identified. It would be useful if molecular databases would characterize compounds by being safe or hazardous; this information can be difficult to find but would be useful for high-throughput screening purposes to quickly highlight compounds of concern. A “complete” database in terms of the compounds that are present would also need to contain MS/MS spectra to identify and confirm generated molecular formulae. However, I don’t think that a database would ever be complete—I also don’t know what metrics could be used to define it as such. One of the ways, we’re trying to be less reliant on available molecular databases is to develop chemometric data analysis workflows to determine what compounds warrant identification or what sample requires further analysis (7).

Q. Automation is crucial in this type of screening to reduce costs and make the method viable for widespread use. Are there any special considerations that need to be taken to ensure a method can be automated?

A: The data analysis process is the most challenging part of a nontargeted LC–HRMS workflow to automate, especially if chemometrics methods are applied. Analyzing extraction blanks, a quality control mixture, matrix spikes, and sample replicates can help streamline this process (7). Analyzing extraction blanks enables the removal of features from a data set that are not inherent to the sample matrix. A quality control standard mixture can be used to monitor instrument performance by ensuring stable retention times, sufficient signal abundance, and measured mass accuracy errors are less than 3 ppm to promote correct molecular formula generation. Matrix spikes can indicate whether molecular features are being accurately extracted from the data set, if small chemical differences can be determined between sample groupings, and if the data processing workflow is effective. They can also be used to determine data quality. Sample replicates yield information regarding sample variability and should especially be used if applying chemometric methods for distinguishing sample groupings. However, processing workflows need to



incorporate mechanisms to automatically process and report findings.

Randomization of acquired samples can also limit the effects of instrumental differences on data output.

Q. Reproducibility has been a major issue across analytical chemistry. What steps would you recommend to ensure any developed nontargeted method can be reproduced?

A: In order for nontargeted screening methods to be reproduced, each part of the method will need to be examined, including the sample preparation, data acquisition, and data processing methods. This can be challenging if the available instrumentation and data analysis processing software differ from reported methods. However, the development of a standardized quality control standard mixture would help ensure that developed workflows are sufficient and that the same result could be obtained at different sites, on different instruments, and using different data processing software. This will also enable researchers to optimize each step of their own workflows. Additionally, incorporating the factors discussed with regard to automation in the previous question will also help establish workflows that will be easier to reproduce.

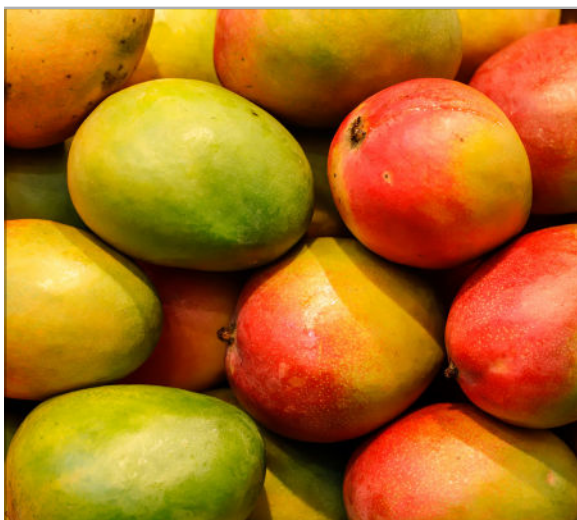
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Ann M. Knolhoff obtained her Ph.D. in chemistry at the University of Illinois at Urbana-Champaign, USA, working under the direction of Jonathan Sweedler. During her Ph.D., her research focused on analyzing different cell types within the brain using mass spectrometry, which included implementing metabolomic workflows. In 2011, she began working at the U.S. Food and Drug Administration in the Center for Food Safety and Applied Nutrition. As a research chemist, she develops nontargeted screening approaches using liquid chromatography and high-resolution mass spectrometry for food safety applications.

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False labeling and adulteration are growing problems in food manufacturing, underlining the need for easy-to-use tools for quality control analysis in this industry. This article describes a novel method enabling routine testing of food authenticity. The workflow consisted of an Agilent 6546 LC/Q-TOF used with MassHunter Profinder 10.0, Mass Profiler Professional 15.0, and Classifier 1.0 software. The method rapidly produced reliable results as insights into food quality.

Introduction

The food manufacturing industry is increasingly interested in food authenticity testing, as adulteration and fraudulent labeling becomes more common across a complex food supply chain. With the costs of premium

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Food Authenticity Testing with the Agilent 6546 LC/Q-TOF and MassHunter Classifier

Karen E. Yannell and Daniel Cuthbertson

ingredients and products rising, the incidence of adulterated or imitation products will continue to increase. Currently, the development and deployment of methods and procedures for testing for such activity is limited by the lack of user-friendly tools. Advancing such tools and workflows can improve quality control procedures of ingredients in the supply chain or final manufactured products in the consumer market place. This advance allows food manufacturers to consistently use authentic materials.

Mass spectrometry (MS) allows for the measurement and profiling of molecular components of food stuffs. These profiles can be used to classify a sample and determine if it is authentic or adulterated with high precision and accuracy. The Agilent 6546 LC/Q-TOF mass spectrometer has significantly improved low mass resolution with simultaneous broad dynamic range. These features allow more features to



be found and measured in a complex sample. Analysis software and workflows for development and use of food authenticity tests have required highly trained specialists, making it challenging for food labs to get involved.

This article introduces software for the development and implementation of a complete authentication workflow, from sample preparation to data analysis (**Figure 1**). Fast sample preparation is performed with a QuEChERS kit, while analyte separation and detection are accomplished with an Agilent 1290 Infinity II LC and 6546 LC/Q-TOF. An authenticity model can be built from a scientist's data processing, which can be automated with MassHunter Profinder 10.0 and Mass Profiler Professional (MPP) 15.0. Agilent MassHunter Classifier 1.0 performs automated authenticity analysis of samples to streamline results. With these improved tools, routine food authentication analyses can widely and easily be implemented.

Experimental

Sample set, preparation, and data acquisition

Certified authentic samples are needed for building a model. For this study, three different mango varieties were analyzed: Ataulfo, Keitt, and Tommy Atkins. Although these were not certified authentic, they have unique phenotypes that were used to correctly identify them,

Figure 1: A complete authentication workflow for the method developer and the routine analyst. The process begins with QuEChERS sample extraction and cleanup. In the method development procedure (top), data for the samples are acquired using a 6546 LC/Q-TOF. These data are analyzed using much improved Profinder 10.0 for feature extraction, and MPP 15.0 for building the model. The developer can test quality control samples and unknowns using their classification model in Classifier 1.0. In routine use, the Classifier software is simple to use, allowing an analyst to run and review samples productively (bottom). Result review includes a plot (center) to easily see if a sample is pure (colored circles) or adulterated (shown here, black diamond).

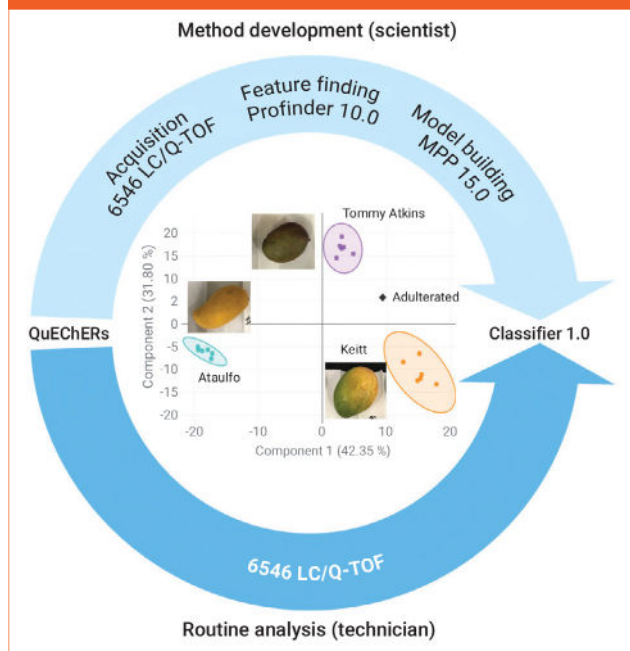
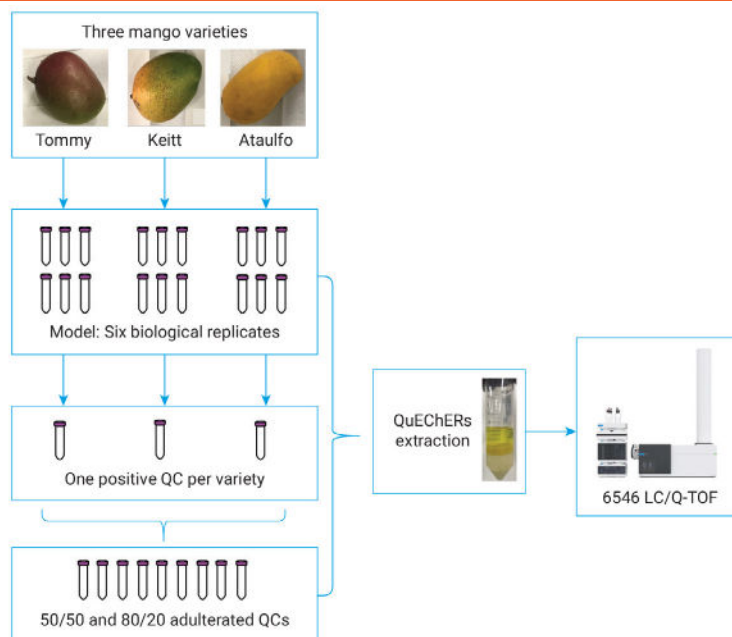


Figure 2: Sample and laboratory workflow for building a mango authenticity model. Six replicates of three mango classes were used to make the positive QC samples. Adulterated mixtures were made by mixing the pure QCs. Each sample was processed with the QuEChERS kit, and data were acquired using a 6546 LC/Q-TOF.



which is acceptable for this proof-of-concept study. Six biological replicates, or individual mangos per variety, were collected from local markets in the California region. The mangos were peeled, and the meat was homogenized. The homogenate was then processed with a QuEChERS EN protocol (1). In a 50-mL conical tube, 10 g of mango homogenate was mixed for two minutes with 10 mL of acetonitrile. An EN salt pouch was added and shaken for two minutes. Then, the sample was centrifuged for six minutes at 3,500 rpm. The upper layer was recovered and stored

at 7 °C in a glass HPLC vial until analysis.

A positive quality control (QC), or pure sample, for each variety was made by mixing the homogenate from the six biological replicates (**Figure 2**). Adulterated samples, or negative controls, were prepared by mixing the positive QC samples at known ratios, for example, 20:80 and 50:50. The QC samples were prepared with QuEChERS EN, and stored in the same way as the individual samples.

Samples were analyzed with a 1290 Infinity II LC coupled to a 6546 LC/Q-TOF. Using an injector program, 2 μ L

**Table 1:** Acquisition details for the 6546 LC/Q-TOF analysis.

Acquisition Parameters	
Column	Agilent ZORBAX SB-Aq, 3.0 × 150 mm, 3.5 μm
Mobile Phase A	Water + 0.1 % formic acid, 5 mM ammonium formate, 0.5 mM ammonium fluoride
Mobile Phase B	Acetonitrile + 0.1 % formic acid, 5 mM ammonium formate, 0.5 mM ammonium fluoride
Sheath Gas Temperature	400 °C
Sheath Gas Flow	12 psi
DryGas Temperature	325 °C
DryGas Flow	10 L/min
Nebulizer	20 psi
Capillary Voltage	4,000 V
MS Tune	<i>m/z</i> 1,700
MS Mode	Positive
Acquisition	MS only
MS Range	<i>m/z</i> 50 to 1,000

of sample was aspirated followed by a needle wash. Next, 1 μL of internal standard (IS), 100 ppb deuterated pesticide mix, was aspirated, the needle was washed, then all the sample and IS was injected. This internal standard allowed for the data quality to be monitored over the entire experiment. The Q-TOF was tuned and calibrated in positive mode (*m/z* 1,700 range).

Over five days of continuous acquisition, excellent mass accuracy was achieved with two internal reference

masses, purine and HP 921 (¹H, ¹H, ³H-tetrafluoropropoxy) phosphazine). MS data acquisition rates were set to maintain a minimum of 8 to 12 data points across chromatographic peaks. **Table 1** shows additional method details.

The model samples (six biological replicates • three mango varieties) were randomized in the worklist, followed by the injection of randomized adulterated samples. This was intended to mimic a typical laboratory workflow where model data are collected followed by the



Figure 3: Dimethoate-d6 (m/z 236.0446, RT 10.4 minutes) results during the entire analysis. The mass error for 100+ injections had less than 2 ppm error, and the area was stable with 6 % relative standard deviation (RSD). This is a representative result of six internal standards.

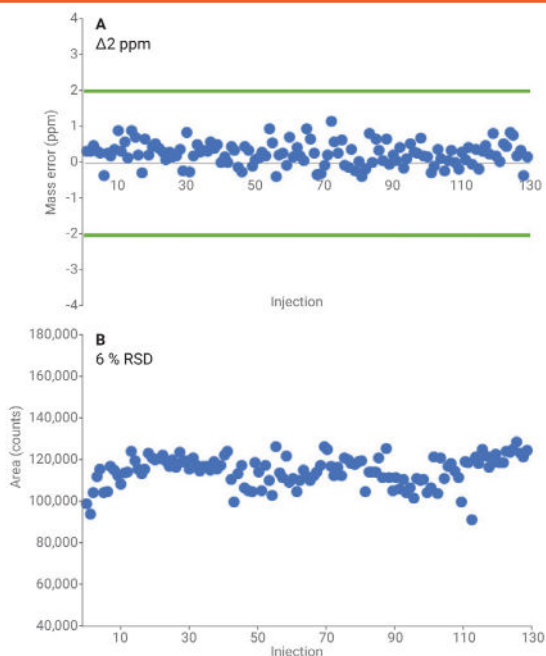
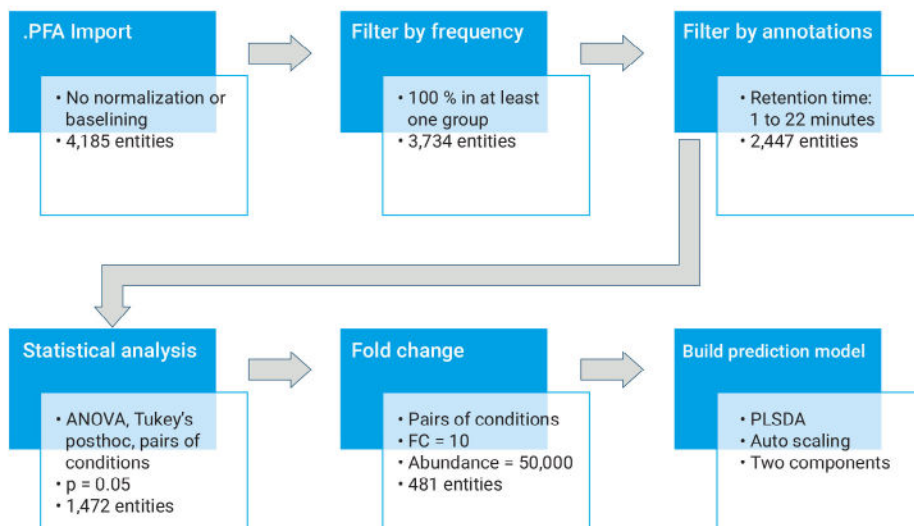


Figure 4: MPP analysis workflow and key parameters. At each filtering step, the number of entities remaining is noted.





unknown samples. In the worklist, after every 10 sample injections, the three positive QC samples were injected in a random order. To assess method and model longevity, 14 days after the data collection, freshly prepared positive QCs and adulterated samples were analyzed using the same method and model.

Results and discussion

Data quality

The mass accuracy and area of the deuterated internal standards are plotted in **Figure 3**. These results serve as a quality control check for not only every injection, but also the entire dataset. These data showed that throughout the entire experiment, mass error on the 6546 LC/Q-TOF was low (<2 ppm), and had a stable signal (<10 % RSD). The retention time drifted only 0.1 minutes throughout the whole worklist. This data reproducibility gives confidence in the instrument performance of each run.

Method development workflow: Profinder 10.0 and MPP 15.0

For food authenticity, the goal of the analysis is to find robust identifiers, not every differentiator. This strategy should allow for an analysis model to be used for an extended period without the need of a model update. There are many key differences between this Profinder and MPP workflow compared to other types of analysis (for example, metabolomics).

The 18 model samples were loaded into Profinder 10.0, and grouped by their mango variety. Because of the minimal retention time drift in this dataset, no retention time correction was needed for this analysis. The batch recursive feature extraction (small molecules/peptides) wizard was selected to detect features in an untargeted manner. A few changes were made to the default method. The protonated ion species was selected with the *common organic molecules (no halogens)* isotope model and a charge state limit of 1. A height filter of 3,000 was used whenever requested by the wizard. Finally, the molecular feature extractor (MFE) algorithm and the target score for feature quality was increased to 80.

The method was saved with a unique name to be used later in the Classifier 1.0 software. The untargeted analysis found over 4,000 features (entities), and these results were exported as a Profinder Archive (.PFA) file for import into MPP. Once imported into MPP, the data were filtered by frequency, retention time, ANOVA statistical analysis statistics, and fold change (**Figure 4**). Once the analysis was complete, a partial least square discriminant analysis (PLS-DA) model was created. The PLS-DA plot and groupings were inspected for goodness of fit (R^2), predictive power (Q^2), then exported as an MPP model.

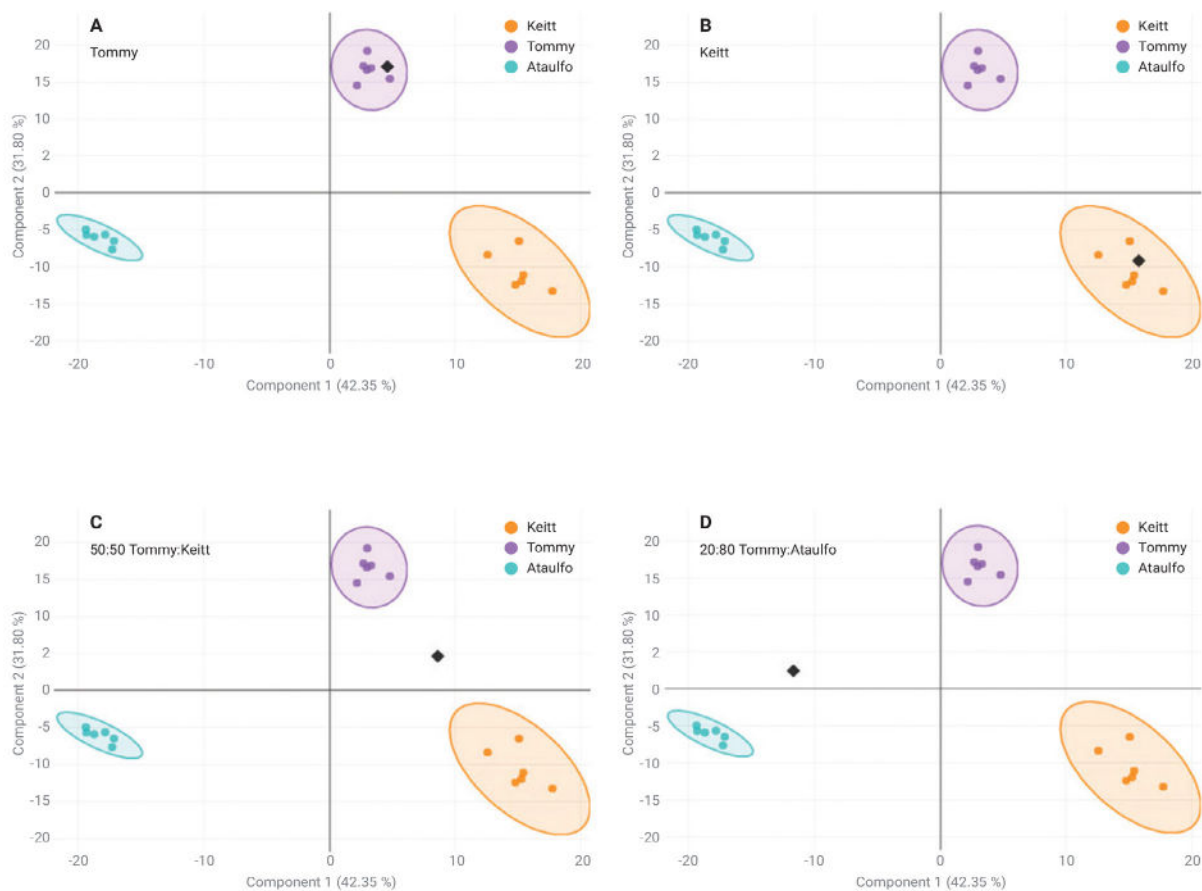
Figure 5: The Classifier 1.0 new project user interface is simple and easy to use. The Profinder method and MPP model, which are created during method development stage, are selected. One or more samples can be added.

Figure 6: Classifier 1.0 results are displayed in the Sample Table (A) and unanalyzed samples are queued (B). The individual sample details can be viewed by PCA plots (C) or feature details in the Compound Table (D).





Figure 7: Classifier 1.0 PCA plot results. The Keitt class is orange, the Tommy Atkins class is purple, and the Ataulfo class is teal. The black diamond represents the selected sample. The pure samples, (A) Tommy and (B) Keitt, have the sample fall directly within their group. The adulterated sample, (C) 50 % Tommy: 50 % Keitt and (D) 20 % Tommy: 80 % Ataulfo, are not near the model groupings.



Routine analysis workflow: Classifier 1.0

The development process ensures that the routine lab has an effective way of extracting features from samples (Profinder), and processing them to get a model (MPP). For routine classification of newly acquired samples, Profinder and MPP are not amenable for quick

and easy analysis because they require a high level of expertise. Classifier 1.0 is innovative software that allows an analyst to take saved Profinder and MPP methods and apply them to new samples and generate a fast result. This software does not require the analyst to use Profinder and MPP.



Classifier has a simple interface to input the method and sample information (**Figure 5**). Once samples are submitted, additional samples can be added to a project at any time during the Classifier analysis. Each sample takes a few minutes to analyze, and data can be reviewed as the processing moves down the sample queue. A project, containing one or more sample results, can be saved, re-opened, or exported as a report.

For this study, the MPP model, Profinder method, and all the adulterated samples were added to a Classifier 1.0 project.

Results were populated in the Sample Table (**Figure 6A**) where each row contains the sample name, predicted class, and confidence value associated with the classification. Pending samples that are not yet analyzed remain queued in the Sample Table until results are available (**Figure 6B**). To review data, a sample can be selected from the Sample Table. This selection updates the interface to show the reviewed sample's location in the model's principal component analysis (PCA) plot as a black diamond. This plot contains the model samples within Hotelling ellipses, or 95% confidence ellipses (**Figure 6C**). For every sample being analyzed, the individual features that belong to the model can also be reviewed in the Compound Table (**Figure 6D**).

In this analysis, the mango QC data were not included in the model building,

but rather were analyzed in Classifier 1.0 as a QC check for authentic samples. Each QC sample was classified correctly, and the PCA plots matched the correct variety for the QC. The six biological replicates that created the model are tightly contained in Hotelling ellipses (**Figure 7**). The review sample is shown as a black diamond in the PCA plot. The sample's position relative to the Hotelling's are indicative of their purity, as is the confidence value listed in the Sample Table (Figure 6A). When the sample is a pure QC, the black diamond is in or very near the mango grouping to which it belongs (**Figures 7A and 7B**). When the sample is adulterated, the black diamond is plotted further away from the grouping (**Figures 7C and 7D**).

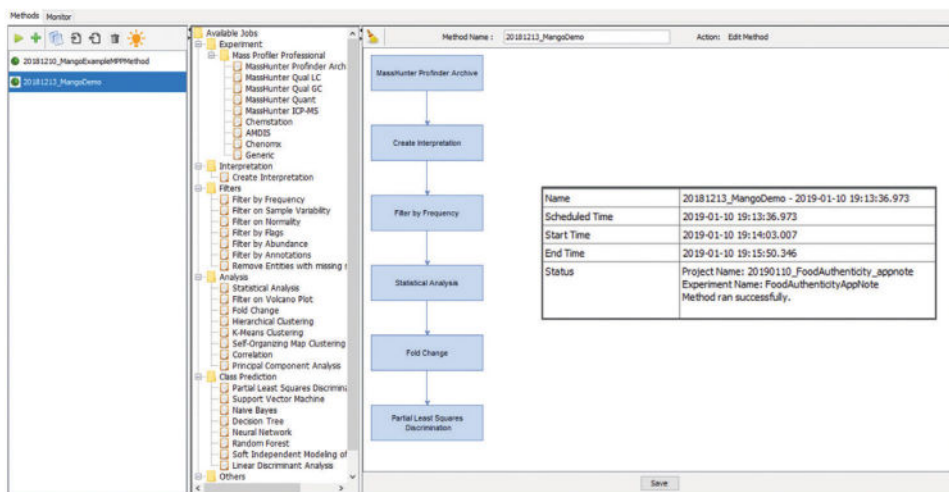
Figure 8 summarizes the confidence results for this study. Pure QC samples have high confidence values, whereas adulterated samples and negative controls have lower confidence values. In this case, a cutoff of 0.8 would have 100% accuracy for correctly identifying a sample as pure or adulterated based on the confidence value alone. Furthermore, the method holds good results for an extended series of analyses because the same results were achieved for samples collected on day 1 and day 14. The precision of the method is also very high, with an RSD <5 % for the confidence value (n = 10).



Figure 8: Summary plot of mango results from Classifier 1.0. The color represents the mango variety classification for each sample. Data from day 1 and day 14 experiments are plotted.



Figure 9: MPP 15.0 method automation user interface with new features. The left panel is a list of methods with a play button to initiate the desired method. New methods can be built on the right side by selecting steps for the middle panel to be added to the method. Using method automation makes the analysis faster and provides a report when complete (inset).





New workflow improvements for the method development scientist

A method automation tool in MPP 15.0 allows a method development scientist to efficiently create methods, and pass them off for routine analysis. The tool, shown in **Figure 9**, has easy drag and drop selection for building the method from a list of analysis options. Once the method is built, it can be saved and used on any new .PFA, eliminating the need to click through all the steps in the Workflow menu. This software improvement also makes it easier to add new authentic samples to a model since the saved analysis method can easily be re-applied. A statistical analysis method, such as the one in Figure 4, can be reprocessed with additional authentic samples to generate an updated model in just a mouse click with less time and human error.

Conclusion

Food authenticity workflows are needed to make food testing more routine as adulterated and mislabeled products and ingredients become more common. This desired workflow is realized with the 6546 LC/Q-TOF, Profinder 10.0, MPP 15.0, and Classifier 1.0. A method development scientist has a faster, automated workflow to help build a model. For routine analysis, an analyst only needs to use Classifier 1.0 for fast classification from new data. The

workflow produces fast, clear, and reliable results for labs to explore the quality of their ingredients and products.

References

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Daniel Cuthbertson,

Applications Scientist
Agilent Technologies



Karen Yannell, PhD

Marketing Application Scientist
Agilent Technologies