Food Fraud Guide

Agilent
Trusted Answers

Methodologies for Food Fraud

Tips for robust experimental results

Executive summary

Knowing that food fraud scandals often drive public awareness and regulatory changes, the goal of this paper is to present analytical techniques and experimental methodologies, and introduce multivariate statistics and sample class prediction as it relates to food adulteration. Some approaches such as molecular spectroscopy tend to be less expensive, and a few of these instruments have been miniaturized to the point where they can be field-deployed. Spectroscopic instruments are useful in fingerprinting food because small changes in a sample's spectral profile can be detected with the latest technology, assuming appropriate data normalization techniques are applied. Similarly, the use of both unit- and high-resolution-based mass spectrometry (MS) can be important in food fraud testing because they can fingerprint food based on the pattern of discrete compounds they detect. While other techniques such as inductively coupled plasma mass spectrometry (ICP/MS) and inductively coupled plasma optical emission spectrometry (ICP/OES) have proven adept at identifying geographic origin based on trace element analysis. Genomic testing can accurately identify fish DNA, even from processed samples. From a methodological perspective, nontargeted approaches have proven effective in fingerprinting samples. The advent of inexpensive computer workstations and statistical software has made it possible to link nontargeted workflows with multivariate statistical analysis to extract useful information from analytical data. Until recently, these approaches have been too expensive or complex for researchers to perform by themselves; instead, the data had been handed over to dedicated statisticians or never fully investigated. But now, there are moderately priced tools that allow researchers to more easily use statistical approaches for determining attributes such as sample quality and building sample class prediction models.

Introduction

As background to why adulteration is an important issue, you may recall the melamine scandal that broke out in 2007 when dogs and cats were poisoned by their food.[1] Investigations discovered the dog and cat food was tainted with a mixture of melamine and its triazine analogs (ammelide, ammeline, and cyanuric acid). These inexpensive but highly nitrogenous industrial chemicals were being used to boost the nitrogen content in food to give the perception that the foods were rich in protein.[2]

As awareness grew, the adulteration of food with melamine quickly became an international problem. It was later detected in baby formula produced in the US, cookies distributed in Canada, chocolate sold in Asia and Australia, condensed milk in Thailand, and eggs in Hong Kong. During the scandal, international agencies such as the World Health Organization, the Food and Agriculture Organization, the European Food Safety Association, and the International Food Safety Authorities Network worked together to characterize and control the crisis. While 68 countries banned or recalled foods suspected of containing melamine,[3] many countries established allowable limits for melamine, with the FDA maximum residue limit (MRL) as 1 part per million (ppm) for infant baby formula, and 2.5 ppm for other products.[4] The FDA also set up an economically motivated adulteration (EMA) working group. In 2009, it defined EMA as the fraudulent, intentional substitution or addition of a substance in a product for the purpose of increasing the apparent value of that product or reducing the cost of its production (that is, for economic gain).[5]

Beyond fundamental safety concerns, food adulteration cheats consumers, as has been demonstrated with extra virgin olive oil (EVOO). A study from 2010 found that imported olive oil, which at that time accounted for 99% of the EVOO on the US market, often failed the sensory test for EVOO classification.[6] Experts now claim that up to 80% of EVOO is fraudulently labeled.[7] It is relatively straightforward to perform established tests for distinguishing EVOO and other grades of olive oils. Two popular examples are the HPLC measurement of diacylglycerol or pyropheophytin (a breakdown product of chlorophyll).[8] However, these tests can generate false positive and false negative results, and fraudsters can use an alternative adulterating oil to pass the tests.

Furthermore, there are flavor defects that can lead to a true EVOO not passing sensory testing. Mustiness, rancidity, and acidity can all be issues, especially with badly stored EVOO. Chemical analysis offers potential solutions to screen for these issues based on the concentration of specific markers such as acids, esters, and aldehydes being found at high ppb to low ppm concentrations.[9]

As chemists search for more reliable tests for EMA, new analytical instruments and methodologies are being explored. The key is finding the right technique for your application and budget.

MS-based food testing

Full spectrum (time of flight), scanning (quadrupole), and image building (Fourier transform) MS techniques are good tools for sample classification. They vary in price from inexpensive gas chromatography/mass spectrometry (GC/MS) instruments, more expensive Fourier transform mass spectrometers (FTMS), to high-price, high-value liquid phase ion mobility quadrupole time of flight instruments (IM/Q-TOF).

The Agilent 8890 GC system and Agilent 5977B GC/MSD.

Many volatile and semivolatile compounds are routinely analyzed by electron ionization (EI)-based GC/MS. This technique has the advantage of EI spectra that can easily be library searched against the large NIST 17/Wiley Registry 11 library that contains 597K compounds and over one million spectra. This gives an unprecedented ability to tentatively identify many volatile and semivolatile compounds. There are some limited structure-elucidation tools, such as the MS Interpreter and the substructure search feature provided by NIST, which are useful with unit mass data. Adding a full-spectrum accurate-mass instrument such as a GC/TOF or GC/Q-TOF provides structural information and allows identification of unknown compounds through the generation of fragmentation trees and thermodynamic-based structure elucidation tools such as Agilent Molecular Structure Correlator. The FT-traps have excellent mass resolution, but image building is not yet fully developed for EI analysis, and the match factors are lowered due to missing peaks. One solution to this problem is to develop a proprietary identification algorithm that places greater emphasis on mass accuracy, and less weight on missing ions.

Feature-finding analysis

There is also a mixture of commercial and academic feature-finding tools going back to AMDIS that was made public by NIST in the early 1990s.[10] A good academic solution for EI peak alignment and data extraction is the AMDIS/manual curation/SpectConnect. Unlike XCMS and MZmine, SpectConnect is specifically designed for EI data.

GC also lends itself to multidimensional chromatography with various commercially available solutions from companies such as Agilent, LECO, and ZOEX. An advantage for these techniques is that they typically have a higher signal-to-noise (S/N) than the unmodulated counterparts. Their disadvantage is that they require specialized hardware and peak deconvolution tools from companies such as GC Image. This software tool works well for analyzing samples sequentially, but has not been optimized for batch analysis, a cornerstone of nontargeted analysis.[11]

Nontargeted workflows depend on reproducible feature-finding software, ideally based on recursion. Recursive feature finding aims to minimize both false positives and false negatives. It does this by eliminating ions considered noise, aligning retention times, aligning masses, and ion binning to build a consensus library (consisting of a composite feature list and corresponding composite feature spectra). The consensus library is used by the algorithm to re-assign individual ions. This reduces the number of false positives. The remaining unassigned features are then used to perform a targeted search. This process reduces the number of false negatives due to missing values by re-examining the target list with less-stringent criteria. Recursive feature extraction is designed to increase the quality of the overall results. Since no algorithm is perfect, batch-wise manual editing of the results should be permitted following batch recursive feature extraction. XCMS is an online public resource for the nontargeted analysis of small molecules developed by The Scripps Research Institute that follows a similar workflow to fill in missing peak data.[12]

Once the feature-finding data have been reviewed and any integration errors addressed, the data can be exported as text files. If a peak is not found, it is considered missing. There may be an option in the statistical software to treat missing values either as zero abundance or as peaks that were not found. Selecting zero abundance strongly impacts the statistics. Choose this option only if the data have been carefully reviewed, and it has been confirmed that the peak is missing. Select missing values if the data are not carefully reviewed and the missing component might be a false negative.

Targeted and nontargeted methods

The combination of targeted and nontargeted chemometric approaches provides a good solution when evaluating samples that allow accurate identification of expected compounds, but also allow for the presence of unexpected components. A good example of this approach is the work done by Hjelmeland and colleagues evaluating the chemical and sensory profiles of Cabernet Sauvignon wines by HS-SPME. That study shows a targeted analysis using a nontargeted workflow with synchronous SIM/Scan.[13] Including a nontargeted component allows for retrospective analysis later.

Fragrant rice such as Basmati and Jasmine have higher concentrations of 2-acyl-1-pyrroline due to a loss of function mutation in the fgr gene product.[14] DNA testing could look for the mutated gene, but there are other key volatiles found in rice such as hexanal, which is associated with lipid oxidation. Volatile components such as these are relatively easy to separate chromatographically, and can be used to evaluate sensory attributes as well as characteristics associated with rice adulteration.[15] Subsequently, integrating headspace solid-phase microextraction into the methodology eliminates much of the sample preparation.[16]

The Agilent 1290 Infinity II LC system with an Agilent 6546 LC/Q-TOF.

To build reproducible prediction models, MS-based EMA methods should focus on finding robust identifiers instead of all identifiers. An example of this approach is when a LC/Q-TOF was used in full-spectrum mode to show the applicability of this instrument for differentiating mango variety. The approach relied on newer software tools to identify robust classifiers and constructing class prediction models. Even with the latest software tools, skill and expertise are required to develop a robust prediction method. However, the subsequent step of running the class prediction model should not require as much technical skill. Therefore, a software automation tool was created that contains the feature-finding method as well as the sample class prediction models. This allows production data files to be run in a streamlined manner against a previously developed MS-based model.[17]

Elemental fingerprinting techniques for geographic indication

Elemental fingerprinting can be used for many applications such as process quality control, identification of elemental contaminants, and speciating samples based on their trace elemental profiles. High-value foods and beverages that are characterized by origin and are produced in limited quantities are targets for EMA. To clarify the definitions of geographic indication (GI), the World Trade Organization Agreement on Trade-Related Aspects of Intellectual Property Rights (TRIPS) was agreed upon by all 164 member countries. The obligations are related to two articles, 22 in which each country has laws to prevent the use of marks that mislead the public as to the geographical origin of the goods. Article 23 says that governments may refuse to register or may invalidate a trademark that conflicts with a wine or spirits GI whether the trademark misleads or not.[18]

Food and beverages can be localized to country of origin by elemental content. The source of the trace and rare earth elements can originate from several sources such as the raw ingredients or from the local soil, environment, fertilizers, and agrochemicals. The processing steps such as $CuSO_4$ added to wine to remove thiols, or Zn and Fe leaching from steel fermentation equipment, or copper leaching from the distillation equipment can also contribute. Finally, bottling and storage can cause lead to leach from the metal capsule seal of a wine bottle.

Common analytical methods for the determination of trace metals in foods can be as simple as ion chromatography, electrochemical analysis, or atomic absorption spectrometry.

The electrochemical techniques are simple and inexpensive but are only useful for looking at a handful of specific metals. More expensive instruments such as atomic emission spectrometry (AES), ICP-OES, and ICP-MS have the ability to simultaneously detect trace and rare earth elements at low detection limits.[19]

Isotope-ratio mass spectrometry (IR-MS) has been shown to be useful in determining geographic indications using stable-isotope ratios. The samples are first converted into simple gasses such as hydrogen, carbon dioxide, carbon monoxide, nitrogen, oxygen, or sulfur dioxide using an elemental analyzer connected to a stable IR-MS. The isotope-pair ratios, such as $^{13}C/^{12}C$ and $^{15}N/^{14}N$ can be plotted together to help distinguish between geographic locations where the $^{13}C/^{12}C$ ratio depends mainly on the biochemical type of plant, while the nitrogen ratio reflects the local soil conditions. The nitrogen ratio can be greatly influenced by the type of fertilizer used, with mineral fertilizer fixed from atmospheric nitrogen having a ratio close to zero while organic fertilizer has a higher $^{15}N/^{14}N$ ratio.[20]

The Agilent 7900 ICP-MS system.

ICP-MS is a powerful tool for elemental analysis, providing part per billion (ppb) or trillion (ppt) limits of analytical detection for more than 70 elements. Trace-element availability depends on various geographic factors such as soil pH, humidity, clay, and humic acid complexes,[21] for example authenticity testing in Pu-erh tea from the Yunnan province in China. An ICP-MS was used to test 30 tea samples in triplicate for 29 elements. The results were analyzed by multivariate statistics using supervised learning. Canonical variate analysis showed two vectors. The first was based on macro elements such as Na, Mg, and Ca and micro elements such as Sr, Zr, Mo, and trace elements. This primary vector differed between the tea regions. The second vector separated the green teas from the black and fermented teas. This vector depended on the levels of Rb, Mn, W, Re, and Tl, all of which were higher in the black and fermented teas.[22]

Most ICP-MS interferences are polyatomic, and possess a greater ionic cross-section relative to mono-atomic ions. This characteristic can be exploited to remove background interferents through collisions with an inert gas. This process, called kinetic energy discrimination, causes all ions to lose energy in proportion to their collisional cross-section. The process reaches a point in which the polyatomic ions have lost enough energy and can be removed from the mass spectrum. With an inert collision gas such as helium, no side reactions or new product interferences are formed.[23] A triple quadrupole ICP-MS also benefits from kinetic energy discrimination. However, it also can eliminate mono-atomic interferences using reactive gases in the collision cell.[22] In both cases, modern ICP-MS instruments are significantly more selective and sensitive than their predecessors.

Spectroscopic techniques in authenticity testing

There are several types of EMA seen with wine and spirits. It can be as simple as sourcing an authentic bottle and either diluting the product with a cheaper alternative, counterfeiting a quality product with inexpensive alcohol and additives to simulate the original product, or it can be as bad as unscrupulously using diluted tax-free denatured alcohol to make a dangerous fake.[24]

Fourier transform infrared (FTIR), near infrared (NIR), and Raman spectroscopy

Milk is one of the seven most adulterated foods.[25] Water, whey, sodium hydroxide, urea, melamine, and hydrogen peroxide have been used to adulterate milk by increasing volume, obtaining higher protein content values, or sanitizing the product. Although direct measurement of milk samples is ideal, water and fat masks some of the spectral signals. A chloroform extraction was used to remove the fat, and an aliquot of the water fraction was dried on the attenuated total reflectance (ATR) diamond crystal. Regardless of which technique was used, there were prominent absorption bands. Midinfrared (MIR) showed major spectral differences between the control samples and the adulterated milk from 1,600 to 1,200 cm^{-1}. Spectra of milk adulterated with whey had prominent amide I stretching (C=O) at 1,635 cm^{-1} and amide II (N-H bending/C-H stretching) at 1,530 cm^{-1}. Samples adulterated with urea, synthetic milk, and urine showed a strong urea stretching (C=O) at 1,615 cm^{-1}, and NH_4^+ deformation at 1,454 cm^{-1}. There were also strong bands observed in the O-H stretching region 3,700 to 3,200 cm^{-1}, and additional absorption bands in the complex fingerprint region from 1,200 to 800 cm^{-1}.[26] The NIR region extends from 13,300 to 4,000 cm^{-1}. This energy range is not high enough for electron excitation but higher than necessary to promote molecules to their lowest vibrational states. NIR spectra are based on molecular overtones and combination bands, weak signals that are not allowed by quantum mechanics. One advantage of NIR's lower absorptivity is that it can penetrate further into a sample than MIR.[27] Specifically, the NIR milk adulteration samples showed two prominent absorption bands at 7,700 and 5,000 cm^{-1}.

While both MIR and NIR instruments are capable of adulteration testing, MIR tended to provide better classification models and quantification after principal component analysis (PCA)-based chemometrics.[26] Both UV-Vis and IR techniques are based on absorbance, and the amount of light absorbed is an absolute measurement. That is not to say that they are not independent of physical parameters, for example the granularity of a sample is very important, as shown in the following rice authenticity discussion where multiplicative scatter correction was required to correct for the granular nature of rice.

Rice is the staple food of more than half of the world's population, with approximately 480 million metric tons of milled rice produced annually, 85% of it for human consumption.[28] Unfortunately, EMA is a concern, as it is easy to dilute quality rice with substandard rice or add adulterants.[29] Field-based NIR techniques have been developed to identify rice by classifications such as geographical indication[30] or a measure of sample quality. In these NIR studies, normalization is an important component of the predictive quality of the NIR method. In the sample quality study, multiplicative scatter correction was able to distinguish sample classes of rice, while detrend and mean centering were not enough.[31] The use of multiplicative scatter correction is a common thread in NIR rice authenticity studies.[32]

Spatially offset Raman spectroscopy (SORS) is a technique used for security screening in European airports.[33] As a technology, it shows promise for EMA, as limitations such as fluorescence are being addressed through scientific advancement. A traditional Raman system uses a laser of approximately 785 nm, which is good for sensitivity but bad for interference from fluorescence. Newer Raman systems use longer wavelengths. The Agilent Resolve handheld SORS instrument has addressed fluorescence as a concern by increasing the wavelength to 830 nm, generating a smaller signal response, much less susceptible to fluorescence. The SORS method also reduces fluorescence from the packaging materials, such as colored plastics or glass. The operation of SORS includes a spectral normalization step. The instrument first collects zero mode spectra, which are like a traditional backscatter mode where the results are biased by the surface. The laser automatically shifts position before taking an offset measurement to correct for the container contribution to the spectrum.

EMA samples for SORS evaluation were provided by the Scotch Whiskey Research Institute at real-world levels of denaturants and adulterants. Six denaturants commonly used are: methyl ethyl ketone, isopropyl alcohol, methyl isopropyl ketone, ethyl *sec*-amyl ketone, methanol, and denatonium benzoate. These have been detected using a handheld SORS instrument down to ppm to sub-ppm levels. Typical flavorings used to adulterate whiskey are vanillin, sucrose, limonene, and trans-anethole. These have been detected in the low ppm range. All of

these were tested in closed glass threaded vials. To extend the usefulness of this technique to real-world conditions, additional samples were bought in stores to test the usefulness of SORS with the types of containers typically used for wines and spirits: clear flint, green, and brown glass. The dark glass reduced light transmission so that more measurements were required to achieve the highest S/N possible. With these stipulations, methanol was detected down to 250 ppm (well below the tolerance level of 20,000 ppm, or 2%), and all the other adulterants were detected down in the ppm range. The Resolve is sold as a field-based detection system for narcotics, explosives, and hazardous materials. The limits of detection (LODs) achieved in the above study used offline processing methods, not currently available on the Agilent Resolve handheld SORS instrument.

Genetic profiling for authenticity testing

The Agilent 2100 Bioanalyzer Instrument.

Traditional authenticity testing of fish has been based on visual inspection. Unfortunately, many fish are difficult to visually distinguish while alive, and the removal of fins and scales during processing makes visual identification impossible. An antibody-based solution could be used for field-based testing. However, this approach does not work after the meat has been cooked because proteins often denature though heating. Another approach, DNA testing, is relatively stable to food processing.[34] It can also be used to identify simple admixtures of meats and proteins until overlapping profiles complicate interpretation.[35] Despite the promise of DNA testing, there are several considerations that need to be considered. One drawback is that the nuclear genome is large and largely conserved. However, mitochondrial DNA (mtDNA) has a relatively fast mutation rate, comes from a single parent, and is 100,000 times smaller.[36] Initially, mtDNA approaches were developed on both mitochondrial cytochrome b and c regions. Eventually, the Barcode of Life Data System was adopted. The standard for animal identification became the 684 base pair region of the cytochrome C oxidase I (COI) gene.[37] The fish identification protocol was adapted to this standard.[38]

Other advancements in fish mtDNA analysis came from hardware improvements. The initial experiments were done using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) with capillary gel electrophoresis and staining for endpoint detection.[39] This approach was improved by replacing the gel electrophoresis step with a lab-on-a-chip capillary electrophoresis system.[40]

However, the Barcode of Life approach took longer to develop for land plants because of the high genetic diversity found in plant species. It was decided that targeting the conserved nuclear transcribed region would be reliable.[41] The CBOL Plant Working Group recommended using rbcL and matK genome sequences in 2009.[42] Prior to this standard approach being agreed on, various microsatellite markers were being used with PCR-RFLP. An example of this was the Basmati rice authenticity proof-of-concept project. In that study, it was shown that DNA testing could quickly determine admixtures of between 10 and 20% non-Basmati rice.[43]

PCR-RFLP approaches represent the standard authentication approach, and are good at generating a single sequence to compare with Barcode of Life-based public databases.[44] An approach is emerging designed to identify mixtures of unknown species, and is based on next-generation sequencing (NGS). NGS eliminates the need for cloning DNA fragments by bacteria. Instead, it is cell-free, can sequence multiple samples in a single run, and the results can be observed without gel electrophoresis.[45] Since NGS is based on fragmenting and processing DNA in parallel, it is suited to identify multispecies seafood products such as surimi. To distinguish between the fish and mollusk species, an experiment can use species-specific universal primers with a metabarcoding approach to classify genera and species of fish and mollusks. The Basic Local Alignment Tool (BLAST) is a specialized software for aligning and evaluating the results.[46]

Other techniques of note

Electronic Nose (e-Nose) detects volatiles using chemical (amperometric and conductometric), piezoelectric, and optical (smell-sensing) sensors, and those based on GC and MS.[47,48] The most frequently used e-Nose sensors are electrochemical. The number and type of sensors as well as their selectivity and sensitivity depend on the e-Nose application. E-Noses, due to their rapid screening capacity, can be an alternative to traditional GC. It has identified Emmentaler cheese from various geographic regions.[49] The drawback with this approach is that without chromatography or spectral data it is not possible to identify specific volatile compounds to monitor.

1D and 2D HR-NMR processed with multivariate statistics have detected fraud in the honey industry, and assisted

in identifying and quantifying several parameters such as sugars and amino acids. To ensure quality control while minimizing safety issues, the profiles of screened food products are compared to a larger database of genuine food samples.[50]

General nontargeted analysis workflow

For nonchromatographic techniques, such as a spectrometric analysis, we are not concerned with identifying individual components. In these experiments, we are simply looking for spectral features that differentiate between sample types. However, with MS-based techniques, we have the option of processing the samples through an identification protocol. This enriches the data, and allows us to compare results against known adulterants, sensory defects, and potential contaminants. Thus, adding a targeted component to a nontargeted approach can provide valuable insight.

It is important to recognize that an incorrect identification reduces the statistical significance of the experimental results. Therefore, leaving the results unidentified is preferential to including spurious identifications. Only include a targeted (known) component in your experiment if you are confident about your ability to consistently identify it. With chromatographically separated components, there are significant challenges to identification such as: poor retention time consistency, incomplete peak separation, isomeric and isobaric compounds, and unresolved peaks. These challenges lead to alignment problems, false peak detection (false positives and false negatives), and identification errors. Even in the best circumstances, data review is an important step in the workflow.

Most of the chemical tests used for adulteration only screen for a few well characterized attributes. Using EVOO as an example, known degradation products for sample quality are compounds such as pyropheophytin, a degradation product of chlorophyll, and 1-octen-3-ol, which contributes to a musty odor. Other known compounds have fermented, rancid, or musty attributes.

We can augment chemical tests with a sensory panel of experts. The biggest drawback is that this approach is labor intensive (expensive) and slow (experts only work for a short period of time before their ability to identify subtle characteristics wane). A combination of a sensory training set and multivariate statistics can offer a cheaper, more efficient, and reproducible approach for determining authenticity.

Key experimental factors for multivariate statistics

Ideally, adulteration would be tested in the raw materials, as opposed to downstream products, which can dilute and mask them. If possible, evaluate multiple analytical techniques to determine which one best meets your requirements. Spectroscopic, spectrometric, as well as nuclear magnetic resonance are all options for a specific food fraud test. Cost, complexity, sensitivity, and portability influence selection between competing methodologies.

Balance sample class prediction experiments with similar group sizes for authentic and adulterated samples, or unequal sampling can bias the statistical results because the heterogeneity of variance is an assumption in one-way analysis of variance (ANOVA) experiments. Modern statistical software tools can compensate for some of the bias, but it is better to avoid problems rather than having to try to minimize their effect after the data have been collected.

It is easy to set up a multivariate statistics sample class prediction method and get results that seem predictive based on the test data set. But having too many independent variables can lead to a class prediction method that fails to predict subsequent samples despite fitting the training data set. This situation is typically caused by overfitting the data. To reduce the likelihood of this scenario, see if simplifying to a two-dimensional PCA is effective at separating the sample classes. In general, prediction models that need fewer independent variables to predict class differences are stronger models, and are less prone to data overfitting.

Data quality is critical, so design the experiment around good analytical principals. Initially, minimize sample preparation for nontargeted workflows, as it biases what can be detected. When necessary, use dilution and simple extractions to deal with matrix effects. Considering that successful models use discreet class-specific characteristics for prediction, once these factors are confirmed, sample preparation and chromatography can be optimized to ensure that these variables are robustly measured in the final targeted prediction method.

Data normalization also needs to be carefully considered. With small sample class prediction methods, it is possible to pool samples for normalization controls. The advantage of this approach is that every component is present, and matrix and instrument effects can be reduced. However, it is not appropriate for the subsequent production sample class prediction methods in that the acts of pooling samples and running these pooled samples on a regular basis requires significantly more resources than adding appropriate internal standards. Instead, internal standards should be representative of the classes of adulterants being evaluated. Similarly, use proficiency

samples at the beginning and the end of each analytical batch to confirm that the instrument's performance is stable. The targeted sample class prediction method should be optimized for high-throughput production.

Sample class prediction is a form of supervised learning. It is a good practice to limit the number of dependent variables, ideally a single variable per predictive method. For example, sample type is the variable with vehicle blank, control, and known adulterants being instances. Using milk as an example, adulterants could be water, whey, sodium hydroxide, urea, melamine, and hydrogen peroxide. With obvious adulterants such as these, three technical replicates of every sample can confirm chromatographic reproducibility, if necessary, and five samples per condition should be enough for statistical significance. It may be that each target adulterant is distinguished enough to be characterized by its absorbance frequency in the sample spectra. In these cases, it is possible that a spectroscopic approach can be used to identify adulteration in the field using a handheld FTIR, potentially avoiding more involved lab-based approaches.

In other cases, as we see with the rampant miss-classification of EVOO, some substitutions are subtler and are therefore more difficult to expose. In these cases, chromatography and a sample class prediction workflow are necessary to detect fraud. Decide the scope of the method early. For example, fustiness, mustiness, rancidity, and vinegariness can all be issues, especially with badly stored EVOO, that lead to lower classifications such as virgin olive oil or worse. These four defects are well characterized, but there are many other factors that would lead to a failed EVOO classification that are dependent on the phenotype. Is it necessary to build a method robust enough to identify the major contributors to the most common flavor characteristics, and how many olive varieties are going to be included in the training set.

It is important to determine in subtle class prediction experiments if there is an appropriate amount of biological variation in the training data to cover the phenotypic differences and the various targeted characteristics. Are there enough samples to have statistical power? *Post-hoc* power analysis can determine the minimum required number of samples based on the statistical power of the pilot experiments.[51]

Chromatographic reproducibility is important, especially with mass spectrometry techniques. Even though there are chromatographic peak warping tools to help correct for chromatographic drift, peak alignment may not correctly identify structural isomers that nearly co-chromatograph if the retention times are drifting during the run. It is better to avoid chromatographic problems rather than deal with them after the fact. To ensure that any run order effects do not influence the overall outcome, analyze samples in random order. For example, matrix interferences can cause either signal suppression or enhancement. This often happens gradually throughout a batch.

After the data are collected, review them to look for chromatographic or spectral outliers that can bias the results. A simple approach is to visually review the data. If the problem seems to be a batch-based chromatographic effect, it may be possible to correct for it through location and scale normalization. The Combat algorithm, readily available online, is integrated in various multivariate statistical packages.[52] Also, to detect bad samples within a good batch, look for poor clustering on a PCA, poor correlation within a group, and check the box and whisker plot for nongaussian distribution. For a more systematic treatment, determine the Mahalanobis distance to show how many standard deviations away a value is from the mean value.[53]

Another useful approach is to use a recursive workflow to process the data when developing spectrometric-based sample class prediction methods. This helps the problem of false negatives and positives in the analytical data. Several feature finding and data alignment protocols have been optimized to deal with drifting chromatography and changes in instrument response. These tools perform alignment as part of feature extraction, and help address the sample variability that can come from chromatographic instability from various sources. If recursion is not an option, and there is too much data to manually check the results, consider treating missing values as missing as opposed to zero within the statistical software. This should be a data import feature of all multivariate statistical packages. A final recommendation is to keep track of all available metadata. It may be necessary to track down confounding factors that complicate data interpretation. A reference that goes into greater depth about spectrometric experimental design and quality control is written by Warwick Dunn *et al.*[54]

Multivariate statistics, significance testing, and confirmation

While not an instrument *per se*, multivariate statistics can play a pivotal role in identifying food fraud. This is the case for geographic authenticity, food quality evaluation, or adulteration. Whichever type of EMA is being evaluated, both biological and reference sample data need to be extracted to identify the characteristics (and underlying compounds) associated with acceptability, and which characteristics

lead to failure. This was typically done manually with a painstaking search for what is different between a failed sample and reference samples. As more failed samples were evaluated and more attributes discovered, a targeted list of components were added to a library. This is the state that many labs find themselves in today, combining tests for known degradation products along with a sensory evaluation. Neither chemical nor sensory tests are ideal on their own.

Agilent Profinder software does both mass and retention time alignment. Therefore, it can import the median retention and mass values, eliminating the need to perform a retention time alignment step in Agilent Mass Profiler Professional (MPP) software when the data are imported as a Profinder archive file. However, there is still a mass alignment option in MPP to allow people to import CEF files. When working with CEF files, because of the slight differences between how mass is defined in Profinder compared to MPP, it is best to increase the mass alignment window in MPP to accommodate the differences. To import data files from third-party instruments requires importing the file as a text file and using a targeted workflow.

There are also online statistical tools to perform feature extraction and simple chemometric analysis for pairwise experiments where there is only a single independent variable.[55,56] Fortunately, there are capable statistical tools available when an experiment contains more than one independent variable, and the researcher sees value in sophisticated techniques such as data clustering, class prediction, multiomic analysis, or pathway analysis. In these cases, other tools are available that can be used for differential analysis and visualization of complex data sets. Examples are MPP, SPSS from IBM, and Progenesis QI from Waters. Realizing there were many users interested in nontargeted analysis and sample profiling but less interested in learning how to use a sophisticated statistical package, Agilent focused on making MPP user friendly and instrument centric. As discussed earlier, scientific skill and analytical expertise are still needed to develop a robust prediction method. However, the subsequent step of running the class prediction model should not be complicated. Classifier was developed to automate LC/Q-TOF classification model analysis.

Finally, it is important to independently test the statistical power of your findings. There are standalone power analysis programs used to validate the statistical power of an experimental result.[51] Post-hoc power analysis calculates the power of the results given the sample size tested, where power is defined as the probability of rejecting the null hypothesis when the specific alternative hypothesis is true.

Class prediction/classifier

First, multivariate statistics provide separation of the sample classes, providing components (entities or features) that best discriminate between the classes. A sample class prediction (SCP) model is then built using these predictive components. Explained this way, it is understandable that another name for sample class prediction is supervised learning.

It is possible to have an entity list that seems to separate classes but is not capable of building a valid SCP model. The case where the model describes a random error in the training data instead of an underlying relationship in the samples is called overfitting the data.[57]

To generate the SCP model with the highest accuracy of prediction, the data quality is crucial. This facilitates construction of the right filtering and prediction model for the samples. SCP will provide the best results when the sample data are properly filtered. As mentioned in the multivariate statistics section, Agilent developed Profinder for recursive data extraction to reduce the number of false negatives and positives that a researcher needs to evaluate. This tool works with both scanning and full-spectrum mass spectral data. Similarly, with IR data generated from instruments from the 4300 Handheld FTIR to the 8700 LDIR for chemical imaging, Agilent provides optional chemometric and prediction modules in the MicroLab Expert software.

Multiple prediction models allow evaluation and customization since each prediction model has traits that make it applicable under certain situations:

- **Partial least squares discrimination** performs vector analysis to develop models that explain the difference between the classes. Outliers have significant influence on the results, and can cause this model to fail. It works well in situations where the data quality is consistent, and a simple model can distinguish between the classes.
- **Support vector machine** works with overlapping sample classes. The algorithm imagines each sample as a point in two- or three-dimensional space. When multiple planes exist that separate the classes, the algorithm maximizes the separation between the classes.
- **Naïve Bayesian** assumes that classes are independent from one another (called class conditional independence). This algorithm can work with small entity sets since only the variance within each class needs to be determined.

- **Decision tree** works well when the entities are present in all or nearly all the samples. It works through a series of if-then-else decisions to separate the classes.
- **Neural network** is suited for making classifications when there is a complex (or unknown) relationship between the classes. It is a good choice when there are two or more classes present in the data.

In general, there are two approaches to validate models once they are constructed. Leave one out is when a data file from each class is left out of the training set and is later classified with the prediction model. All the files go through this process to generate the confusion matrix, which shows the applicability of the training data with the model. The other approach is N-fold, in which the training data are randomly assigned to N groups. All but one of the groups are used to train the model, and the last group is used to validate the model. The process is repeated N times to generate the confusion matrix. Generally, an N value of three is enough. Once a model is constructed, trained, and validated, it should be verified using known samples since we need to be able to predict that they are completely independent of the training data.

There are numerous steps, and the whole process seems rather complex for researchers that do not have any experience in multivariate statistics. For people such as these, there are guided workflow options and tools that integrate feature finding, significance testing, model building, and validation into a single process in developing supervised learning models.[17]

Agilent's goal is to provide solutions for customers which includes sample preparation products, analytical instrumentation, software, workflows, and support. This guide is written to aid academic researchers evaluate the most commonly used techniques for fighting food fraud. It covers field-deployable spectrometric approaches using portable instruments like the Agilent Resolve Raman system and 4300 Handheld FTIR. It discusses the Agilent 2100 Bioanalyzer System as a lab based genomic test for accurately identifying fish, even from processed samples where the DNA has been degraded. The guide also shows that identifying geographic origin based on trace elemental analysis has never been easier with instruments such as the matrix-tolerant 7900 ICP-MS and the economical 5110 ICP-OES. Agilent has good solutions for both unit- and high-resolution MS-based food classification.

Agilent has good solutions for both unit- and high-resolution MS based food classification. An 8890 GC paired with the 5977B single quadrupole MS can be used to identify volatile and semivolatile adulterants with a spectral library. The Agilent 6546 LC/QTOF is a high-resolution, accurate-mass, research instrument for nonvolatile contaminants. Agilent provides a sample classification workflow solution that include MassHunter Profinder software for recursive mass spectrometric data analysis for both quadrupole and Time-of-Flight-based instruments. Curated accurate and unit-mass libraries are available for common food adulterants and allergens. Agilent offers instrument-centric statistical tools like Mass Profiler Professional and user friendly MassHunter Classifier to provide researchers with the ability build their own sample class prediction models and predict attributes such as sample quality.

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