Trend or necessity?

Chiara Dall'Asta, food chemistry professor at the University of Parma, debates the use of C-13 internal standards in mycotoxins analysis.

MYCOTOXINS ARE fungal secondary metabolites, occurring in a wide spectrum of food and feed commodities worldwide. They may exert acute and chronic adverse effects on humans and animals.

In consideration of the typical mycotoxin co-occurrence in crops and their extensive combination in food, co-exposure to multiple mycotoxins through one's diet should be considered for a proper risk assessment. A regulatory framework is currently enforced in many parts of the world, although still not harmonised at a global level. In the European Union, according to the farm to fork precautionary approach, the maximum permitted levels are particularly low, reaching sub-part per billion in the case of the most dangerous aflatoxins (eg, 0.05 µg/kg for aflatoxin M1).

"Over the last 20 years, mass spectrometry has become the benchmark in food analysis for a large range of contaminants"

Owing to the efforts of the agro-food system to meet regulatory compliance, the accuracy and reliability of analytical methods used in the industry and in official labs must be the highest possible, to avoid both false positive and false negative results.

Over the last 20 years, mass spectrometry has become the benchmark in food analysis for a large range of contaminants and, more recently, multi-analyte methods have been established as the golden standard for many classes of chemicals. However, analysts working with mass spectrometry are well aware of the possible matrix effect, due to ion suppression or ion enhancement, that may influence accurate quantification.

In the case of multiple analytes occurring at trace level in a complex food matrix, the choice of internal standard (ISTD) is crucial to minimise possible bias in quantification. The most effective strategy to ensure proper accuracy is Isotope Dilution Mass Spectrometry (IDMS). This approach is based on the use of stable isotope labelled standards, human-made compounds that cannot occur in naturally contaminated samples and are able to compensate for possible losses and matrix effects occurring during the analysis.[1,2]

Regarding mycotoxin analysis, all the regulated compounds are available on the market as C-13 standards, meaning that all the carbon atoms in the molecules are substituted by ^{13}C atoms.

The chemistry behind isotope dilution

Most elements occur in nature as a mixture of isotopes. Isotopes are atom species of the same chemical element that have different masses; eg, natural carbon consists of the stable isotopes ^{12}C (98.9 percent) and ^{13}C (1.1 percent) and the radioactive ^{14}C. For molecules of known empirical formula, the natural isotopic distribution of the entire compound is often available through online tools, such as Chemspider or Pubchem, or can be calculated from the relative frequency of the individual elements by the most common chemical software – ie, ChemDraw.

A compound can be enriched or depleted in a particular isotope. As a consequence, its natural isotopic distribution will vary accordingly giving rise to a so-called isotopologue compound. Stable isotopically-labelled compounds share the same physico-chemical properties with the analyte but have a different molecular mass. It means that they elute together with the target compound but are still distinguishable by MS. Once added to the sample in a suitable amount, losses of the analyte are completely compensated for by identical losses of the internal standard. As a consequence, IDMS can be regarded as an analytical method of the highest metrological standing.[3]

In IDMS, a given amount of the isotopically labelled form of the target analyte is added to the sample. Based on the known amount of the internal standard, the content of the analyte can be calculated.

"Over the past decade, labelled standards have become commercially available for all the regulated mycotoxins and are provided as uniformly ^{13}C-labelled ([U-13C]) isotopologues"

Uniform labelling with the stable isotopes has been used in MS for decades, in particular for (quantitative) proteomics studies. The first generation of stable isotope-labelled ISTD had the drawback of relying on deuterated compounds, which were relatively inexpensive but could lead to inaccuracy due to H/D exchange in the sample. Over the last decade, a second generation of labelled ISTD became available, characterised by ^{13}C and ^{15}N labelling or, in a few cases, deuterium placed into stable, non-exchangeable positions.[4]

C-13-labelled internal standard for mycotoxin analysis

The ideal compensation for losses makes C-13-labelled internal standard a perfect tool for contaminant analysis, which often requires tedious clean-up procedures due to matrix interferences. To ensure a proper quantification, the standard must be unequivocally distinguishable from the analyte. This has led to the production of isotope-labelled standards where all the carbon atoms in the molecules are replaced by the stable carbon isotope.

Fully labelled C-13 standards can be obtained in high isotopic purity (>99.5 percent) and are chemically stable with no $^{13}C/^{12}C$ exchange. In the LC-MS analysis, this ensures no co-elution problems between the labelled and unlabelled analytes, with a sharp and easily recognisable ISTD molecular ion.

Over the past decade, labelled standards have become commercially available for all the regulated mycotoxins and are provided as uniformly ^{13}C-labelled ([U-13C]) isotopologues. In addition to the major ones, some emerging or modified C-13-labelled mycotoxins are also available on the market. Since these compounds are usually not monitored in official controls, the labelled ISTD are mainly used for research purposes, ie, to study the effect of processing on modified mycotoxins.

As a consequence, a large number of methods have been published for mycotoxin analysis in almost all food and feed »

commodities. Results from interlaboratory validation studies have been reported, attesting the metrological advantage of the isotope dilution approach in the accurate mycotoxin quantification.[5] With the enlargement of the C-13-labelled standard portfolio, the first biomonitoring application has also been developed.[6]

Limitations and challenges of using C-13-labelled internal standards

The main limitation in the use of C-13 internal standards for mycotoxin analysis is the market cost. This can be particularly high, especially in routine controls when a huge number of samples are screened using a well-established official procedure.

To keep the costs under control, C-13-labelled ISTD is often added to the sample prior to injection into the MS system. It must be noted that in doing so, the internal standard may compensate for suppression/enhancement effects occurring in the MS instrument but does not account for analyte losses during sample preparation.

However, to efficiently counterbalance the analyte losses during the analytical procedure, the labelled standard should actually be added to the sample matrix at the beginning of the sample preparation. It must be said that 13-C-labelled internal standards may also account for possible degradation of mycotoxins during the analyses and differences between samples due to variations in sample processing by different technicians or protocols. Therefore, for newly developed methods, the higher cost of the internal standard is frequently offset by the reduced time spent on initial method development and qualifications. To make the most of the C-13-labelled internal standard, consider its use at the design stage of method development.

Based on my experience, the price of the C-13-labelled ISTD is negligible, if compared with personnel labour costs and general costs for consumables. According to the instrumental sensitivity, cost-effective clean up protocols, ie, dilute-and-shoot or QuEChERS, can be preferable to immunoaffinity column or SPEs.

"Costs are still the main barrier for the breakthrough of multiple isotope dilution required by extensive multi-analyte methods"

Nonetheless, costs are still the main barrier for the breakthrough of multiple isotope dilution required by extensive multi-analyte methods. When hundreds of mycotoxins and natural toxins are analysed simultaneously, the old strategy of adding a bunch of selected internal standards to correct distortions for groups of analytes is often the only way. In spite of the superior correction offered by the C-13 label, this approach still needs the determination of and correction for recovery to avoid possible bias in quantification.

Considering that the EU regulatory frame is moving from one-compound to multiple chemical risk assessment, this is – in my opinion – the major challenge in multi-analyte IDMS. The development of less extensive fit-for-purpose multi-toxin methods considering only those mycotoxins commonly co-occurring in a selected matrix, may be a reasonable choice while the costs of C-13-labelled standards will decrease on the market.

Chiara Dall'Asta

Chiara is a Professor in food chemistry at the University of Parma. Her interest lies in the occurrence and toxicological relevance of natural toxins, and in the application of advanced mass spectrometry for food safety and food fraud.

References

1. Rychlik M, Asam S. Stable isotope dilution assays in mycotoxin analysis. *Anal Bioanal Chem*. 2008 Jan;390(2):617-28. doi: 10.1007/s00216-007-1717-x
2. Asam S, Rychlik M. Recent developments in stable isotope dilution assays in mycotoxin analysis with special regard to Alternaria toxins. *Anal Bioanal Chem* 407, 7563–7577 (2015). doi: 10.1007/s00216-015-8904-y
3. Milton MJT, Wielgosz RI. (2000). "Uncertainty in SI-traceable measurements of amount of substance by isotope dilution mass spectrometry". *Metrologia*. 37 (3): 199–206. doi:10.1088/0026-1394/37/3/3.
4. Stokvis E, Rosing H, Beijnen JH. (2005) Stable isotopically labelled internal standards in quantitative bioanalysis using liquid chromatography/mass spectrometry: necessity or not? *Rapid Commun Mass Spectrom* 19:401–407
5. Zhang K,. Schaab MR, Southwood G, *et al.* A Collaborative Study: Determination of Mycotoxins in Corn, Peanut Butter, and Wheat Flour Using Stable Isotope Dilution Assay (SIDA) and Liquid Chromatography–Tandem Mass Spectrometry (LC-MS/MS). *Journal of Agricultural and Food Chemistry* 2017 65 (33), 7138-7152 DOI: 10.1021/acs.jafc.6b04872
6. Šarkanj B, Ezekiel CN, Turner PC, *et al.* Ultra-sensitive, stable isotope assisted quantification of multiple urinary mycotoxin exposure biomarkers. *Anal Chim Acta*. 2018 Aug 17;1019:84-92. doi: 10.1016/j.aca.2018.02.036.