DR EHRENSTORFER™

USE OF STABLE ISOTOPE INTERNAL STANDARDS FOR TRACE ORGANIC ANALYSIS

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INTRODUCTION

This paper explains why stable isotope internal standards are essential, particularly for test methods using electrospray mass spectrometry, to obtaining accurate results. It describes critical factors in the selection and use of isotopic internal standards, such as isotopic purity, molecular sites of the isotopic labels, optimising the mass difference between the internal standard and the analyte, and maintaining molar parity between the internal standard and the analyte.

All analytical measurements have an inherent uncertainty. Particularly in the case of chromatographic methods for trace organic analytes, this can be significant. It is not unknown for a reported result to have an associated uncertainty of over 100%. The overall measurement uncertainty is a combination of factors from each individual stage of the method; extraction, clean-up step(s), chromatography and detection. Even if the sample is calibrated against "fortified" matrix standards (a control sample, with a known amount of analyte added, taken through the entire procedure to mimic the losses suffered by the sample), full mitigation is rarely possible. There is too much unknown variation sample-to-sample, or tube-to-tube.

The traditional way to mitigate measurement uncertainty is to use an internal standard. Conventionally, this is a molecule structurally similar to the analyte(s) but unlikely to be present in the sample. It is added at the start of the procedure, in equal quantities to all samples, matrix standards, blanks and other control samples.

The assumption is that the internal standard within each sample will mirror everything that happens to the analyte within that same sample. Rather than the detector measuring the absolute response of the analyte, it measures the relative response of the analyte to the internal standard.

In practice, however, this assumption does not hold. The internal standard is chemically different to the analyte, and so behaves differently. Measurement uncertainty can be mitigated, but not fully compensated.

The use of mass spectrometry as a detector, however, allows for a significant refinement to this approach. If an isotopic analogue of the analyte can be used as an internal standard, then (in principle) it is chemically identical. It should exactly mirror the analyte at each stage of the process. But, because it has a different mass, it can be distinguished from the analyte by the detector. This provides, in concept, a complete compensation for uncertainty at every stage.

STABLE ISOTOPE INTERNAL STANDARDS

Isotopes are forms of a chemical element that differ only by the number of neutrons in the nucleus. They have the same chemical properties, but different mass. Isotopes can either be radio-isotopes (decay by emitting radiation) or stable (do not decay to any appreciable extent).

Most elements are a mix of isotopes. Natural carbon, for example, consists of approximately 99% stable ^{12}C, 1% stable ^{13}C, and a small amount of radioactive ^{14}C. These isotopes can be artificially enriched and purified.

Stable isotope internal standards are synthesised by using precursors where one or more atom has been replaced with a purified isotopic analogue. For example, a carbon atom (naturally predominantly ^{12}C) could be replaced by one that is predominantly ^{13}C. Thus an analogue of the analytical reference standard can be synthesised that is "labelled" with isotopes at different sites within the molecule. This analogue is the same chemical as the analyte, but has a different mass.

A large relative difference in mass between the analyte and its isotopic internal standard can cause them to behave differently; different energy levels lead to "Isotope Effects" which can affect chromatographic retention. In practical terms, however, they behave identically if the relative mass difference is small.

THE NEED TO MINIMISE MEASUREMENT UNCERTAINTY IN MASS SPECTROMETRY

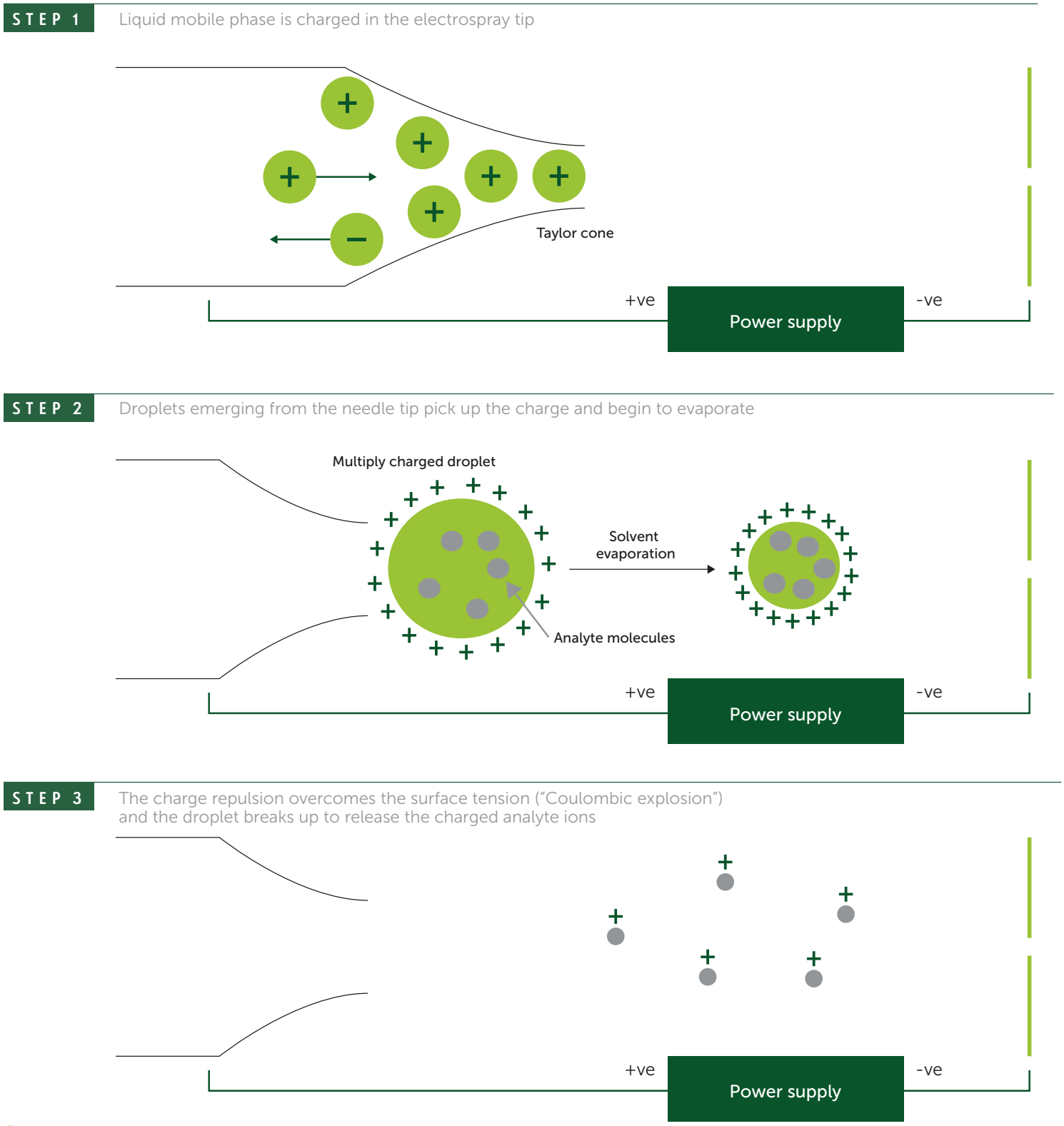
Mass spectrometry (MS) tends to have inherently worse precision than traditional detectors (LC-UV or GC-FID). For applications such as pharma ceutical purity measurement, where there is no need for sensitivity and where quantitation is vital, traditional detectors are preferred. MS, particularly liquid chromatography (LC-)MS, comes into its own when measuring multiple analytes of different chemistries in applications where a high degree of selectivity and sensitivity are required. There is an acceptance that quantitative precision may be sacrificed in these circumstances. It is the industry-standard technique for applications such as trace level impurities, residues and contaminants in food and the environment, traces of drugs of abuse and sports anti-doping.

For a few of these applications, it is true that accurate quantification is unimportant. For a banned substance in sports anti-doping, for example, it is only critical to prove the presence and identity of the substance. But for other applications accurate quantification is still important. An example is residues of the banned antibiotic, chloramphenicol, in shrimp. A concentration of 0.3 ug/kg is accepted as a trading limit – a result of 0.4 µg/kg could cause rejection of an international consignment, whilst 0.2 µg/kg would be accepted. Another example is surveys of toxic contaminants which are then fed into exposure assessments; a 200% measurement uncertainty on the result, which is not unusual for a trace-level LC-MSMS method, could change the conclusion of the risk assessment.

THE OVER-RIDING CASE FOR ISOTOPIC INTERNAL STANDARDS: 'MATRIX EFFECTS'

LC-MS suffers from inherent and large quantitative uncertainty when applied to trace level organics. To understand the reason and depth of the problem it is necessary to understand the principles of electrospray ionisation (Figure 1). When it emerges from the LC, the analyte is dissolved within the mobile phase. It is converted to free ions, for introduction into the mass spectrometer, in three steps.

Figure 1[1]: Electrospray ionisation principle.



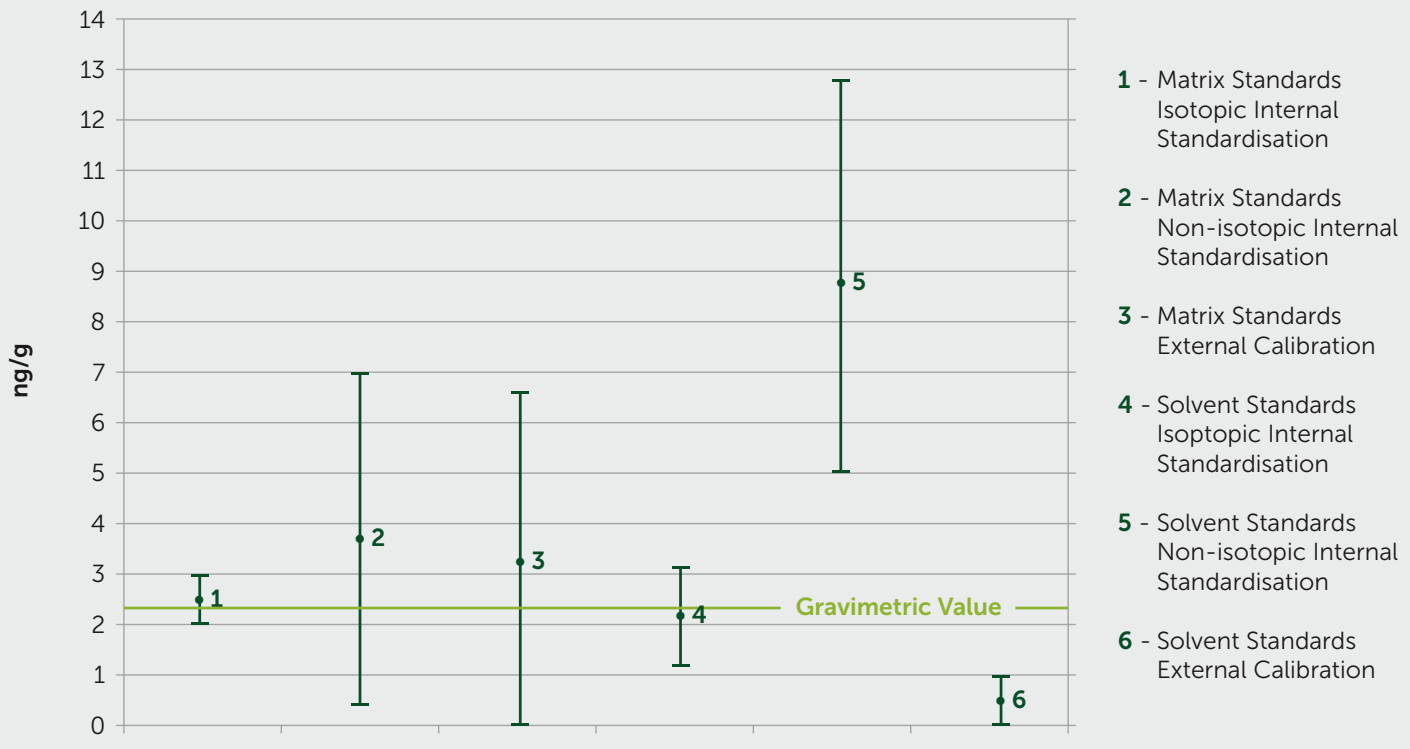
It is apparent that anything that co-elutes with the analyte and affects the dynamics of the Coulombic explosion in any manner – for example, anything that affects the charge distribution or affects the surface tension of the droplet – will affect the detector response. These are collectively known as "matrix effects". Co-eluents will vary from sample to sample. Salts, fats, surfactants and other polar molecules are all perennial offenders. In LC-MSMS the instrument is tuned to only detect the analyte. Any co-eluting substance remains undetected, meaning that there is no warning that the signal in an individual sample may have suffered from a matrix effect and is any different than would otherwise be expected.

Matrix effects cannot be compensated by using conventional internal standards. In fact, the use of a conventional internal standard will often increase the measurement uncertainty.

This is because both the analyte and the internal standard may suffer from independent unrelated matrix effects; the signal for one may be enhanced whilst the other is decreased, or vice-versa (Figure 2).

Matrix effects can be reduced by diluting the sample extract. But the only way to compensate for them (other than guaranteeing that all potential co-eluents are eliminated by sample preparation and clean-up) is to use stable isotope internal standards. These will be subject to identical matrix effects provided that they co-elute exactly with the analyte molecule. This means that a matching stable isotope internal standard is needed for accurate quantification of each and every analyte molecule in a multi-analyte method.

Figure 2[1] : LC-MSMS Measurement of Testosterone in Blood Serum Using Six Calibration Methods: No internal standard (external calibration); non-isotopic internal standard (nandrolone), isotopic internal standard (d3-testosterone). Each with and without matrix matching (preparing the standard in an extract of serum, rather than pure solvent).



CHOOSING THE RIGHT STABLE ISOTOPE INTERNAL STANDARD

There are a wide variety of internal standards available from different commercial suppliers. Most are based on substitutions of hydrogen atoms with ^{2}H (deuterium), carbon with ^{13}C or nitrogen with ^{15}N.

There are four key factors to consider when selecting a suitable internal standard.

1. Low residual contamination with the unlabelled molecule, caused by incomplete isotopic labelling of one of the precursors during synthesis of the internal standard. Ideally, the proportion of unlabelled molecule should be < 2%, to avoid having to make complex correction calculations.

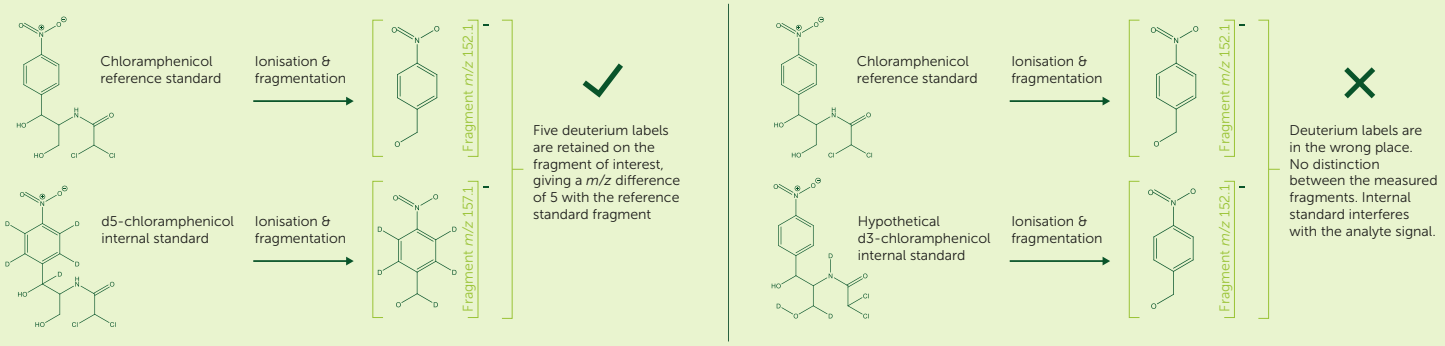
2. Optimal mass difference between the analyte and its isotope. Choose an isotope-labelled internal standard with sufficient mass difference to avoid overlap with the minor analyte spectral lines at M+1, M+2, M+3 etc. caused by the natural presence of stable isotopes in the analyte molecule (e.g. the 30% natural proportion of ^{37}Cl vs ^{35}Cl atoms, or the 1% natural proportion of ^{13}C vs ^{12}C atoms). The general rule for small organic molecules (e.g. 50 – 800 mass units) is that isotopic internal standards should be at least 3 mass units different to the analyte, or a greater mass difference for molecules with multiple chlorines which have particularly strong natural isotope ratios at M+2 and M+4. But if the total relative mass difference between the analyte and the isotopic standard is too great then "isotope effects" (caused by differing energy contents) can lead to unwanted chromatographic separation.

   The relative mass differences between ^{13}C and ^{12}C or between ^{15}N and ^{14}N are much lower than between ^{2}H and ^{1}H, making ^{13}C and ^{15}N popular choices to minimise isotope effects. If using deuterium, then the number of ^{2}H labels should be kept to the minimum needed to attain good mass resolution.

3. Depending on your choice of conditions, in many cases the labelled site(s) should be on the fragments of interest after fragmentation when using your specific test method (see Figure 3).

4. Good stability during the extraction method, e.g. avoid 2H labels sited adjacent to carbonyl groups that may be prone to proton-deuterium exchange under some conditions. ^{13}C and ^{15}N labels tend to be preferred to ^{2}H because they are less easily lost from the molecular structure. ^{18}O labels are seldom used because oxygen atoms are generally in labile positions within the molecule.

Figure 3: Example of right and wrong positions of deuterium labels for a given set of conditions.



CORRECT USE OF STABLE ISOTOPE INTERNAL STANDARDS

As with any conventional internal standard, stable isotopes should be added to the sample at the start of the procedure (prior to extraction) and left to equilibrate. If not, then there is increased risk of a difference in extraction efficiency of the internal standard compared to the analyte.

Stable isotope internal standards must be added at approximately the same concentration as the analyte calibration range. If the molar ratio between the internal standard and the analyte is significantly biased then the relatively small natural proportion of stable isotope in the analyte (or residual non-isotopic analyte in the stable isotope reference standard) become significant, and the calibration is no longer linear.

Stable isotope internal standards are uniquely matched to their analogous analytes. Therefore an individual internal standard is needed for the quantification of each and every analyte in a multi-analyte electrospray LC-MS method. Using d3-testosterone, for example, to also ratio the other androgens in a mixed steroid reference standard risks being worse than using no internal standard at all; the same problem as illustrated in Figure 2.

CONCLUSIONS

Stable isotope internal standards are a valuable resource for minimising measurement uncertainty. For accurate quantification using electrospray LC-MS, this verges towards an essential resource. They should only be used to standardise their own matching analytes, with an individual internal standard needed for each analyte to be quantified. Their correct selection and optimisation for any given application requires a degree of technical knowledge and scientific assessment.

ABOUT DR. EHRENSTORFER

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ABOUT THE AUTHOR

John Points is a UK-based interdependent consultant, offering advice on chemical risks and testing strategies to industry, regulators and laboratories in the food, environment and pharmaceuticals sectors.

REFERENCE

[1] Figures 1 & 2 from C Mussell, C Hopley and J Points, Matrix suppression profiling: a useful tool for LC-MS method optimisation, presented at Saskval I, Saskatoon, Canada, June 2007.

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