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Short communication

Matrix matching in liquid chromatography–mass spectrometry with stable isotope labelled internal standards—Is it necessary?

Amitha K. Hewavitharana*

School of Pharmacy, Pharmacy Australia Centre of Excellence, The University of Queensland, 20 Cornwall Street, Woolloongabba, QLD 4102, Australia

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ABSTRACT

Matrix matching is used in analysis to compensate for matrix effects that influence analytical response. It has been a widely discussed topic in electro-spray mass spectrometry where the ionization suppression is a major problem in accurate quantitative analysis. However, the unique strength of mass spectrometry to detect and quantify accurately a co-eluting stable isotope labelled internal standard offers an easy solution to the ionization suppression problem. Given the fact that it is impossible to match the matrix of the calibration standards with all samples, mass spectrometry allows accurate quantitation without the need for matrix matching, as long as the internal standard co-elutes with the analyte of interest. If the analyte and internal standard co-elute, the slope of the calibration curve analyte response/internal standard vs. analyte concentration is independent of the matrix composition, eliminating the need for matrix matching.

It is common practice in analysis to match the matrix of the calibration standards to that of the samples. The concept of matrix matching has been important in techniques such as atomic absorption spectrometry, in order to compensate for the matrix effects (ME). However, the impracticality of exact matching of the matrix of the calibration standards with all samples lead to the calibration by standard addition [1,2] the method of choice in atomic spectroscopy.

In the past, if the analysis was carried out using a technique other than atomic spectroscopy, the effect of the matrix was not significant enough to cause a major reduction in accuracy. However, in LC–MS, especially in liquid chromatography coupled to electrospray mass spectrometry (LC–ESI-MS) where the ion suppression/enhancement effects due to matrix can significantly reduce or enhance the analyte response [3–10], the matrix matching concept has re-surfaced. Ion suppression is more common of the two effects and it is of major concern in quantitative analysis when using LC–MS and LC–MS–MS because it affects accuracy, precision and the limit of detection [10]. When the analyte co-elutes with another compound, the MS detector signal of the analyte is suppressed due to competition between analyte and the co-eluting compound for either the total available charge or the available surface area of the droplet [10] in the interface of MS detector. The effect of the ion suppression alone has been shown to reduce the accuracy of the assay by 26% [8].

Let us look at the possible ways to eliminate and/or correct the ion suppression effects, in order to obtain accurate data with ESI-MS.

Operating LC–ESI-MS under "total solvent consumption" condition where heat and low flow rates to ESI can reduce or eliminate ME [11]. However, this is not a practical option for a routine analytical laboratory.

It is common practice to carry out extensive sample clean up to reduce ME. However, it is important to appreciate the fact that even the most extensive sample cleanup protocols such as solid phase extraction (SPE) will not remove the impurities that co-elute with the analyte, which are the compounds that cause ion suppression. The principal behind such clean up regimes is to remove compounds that have different polarities/hydrophobicities to those of the analyte of interest. Compounds co-elute with the analyte do so because they have very similar polarities/hydrophobicities to those of the analyte. Therefore, the analyte in a complex matrix is subject to ion-suppression regardless of the extent of cleanup. A recent study found that 134 analytes out of the total of 198 in biological samples exhibited signal suppression or enhancement with LC–MS followed by SPE clean up [12]. In fact, because SPE preconcentrates the analyte (along with interferents that have similar polarities) this clean up process can often magnify the ion suppression/enhancement effects [13].

Improvement and optimization of the chromatography can minimize co-elution of other compounds with the analyte of interest. However, if analyte specific detector parameters such as selected or multiple reaction monitoring (SRM or MRM) are used, the co-eluting compounds will not be detected. Unlike with modes of

* Tel.: +61 7 3346 1898; fax: +61 7 3346 1999.
E-mail address: a.hewavitharana@pharmacy.uq.edu.au

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detectors such as UV and fluorescence where the co-eluting compounds (if not detected) have no effect on the magnitude of the analyte response, the "unseen" co-eluting compounds in LC–ESI-MS can enhance or suppress the analyte response.

When the best possible chromatography is unable to separate all compounds in the matrix from the analyte, which is often the case with any biological or environmental sample, the best way to correct for the analyte signal suppression or enhancement is to co-elute a stable isotope labelled (SIL) analogue of the analyte. Since the SIL will normally elute exactly at the retention time of the analyte, it will experience the same suppression/enhancement effects as the analyte. This allows the analyst to treat the SIL as the internal standard and to correct for the suppression/enhancement effects caused by the matrix.

As each sample will contain different co-eluting compounds at different concentrations, the extent of suppression in each will be different. However, the co-eluting SIL internal standard (SIL-IS), added to each sample, can be used to fully correct for these effects. The question now is about the composition of the calibration standards. The common practice is to match the matrix of the calibration standards with that of the sample. Is it practical to do an exact matching of the matrix in calibration standards with the samples, when each sample contains different co-eluting compounds at different concentrations? Many protocols have been suggested to address the problem of exact matrix matching such as: using the actual sample (containing the analyte) as the matrix and preparing calibration standards by spiking it with SIL analogue [14] (here the SIL analogue is used as the calibration standard rather than the internal standard); preparation of calibration standards in many different lots of blank matrixes and comparison of slopes obtained for each matrix [15], and then establishing an acceptable coefficient of variation for the method to be reliable. However, some of these protocols are more diagnostic in nature rather than solutions to the problem and do not fully address the sample to sample variation in matrix.

In realising the impracticality of exact matrix matching, the analyst is forced to use the standard addition method of calibration to obtain accurate data so that the standard addition method has been resurfacing in recent times [13]. However, this too is certainly not an option that a routine laboratory, with a demand for high throughput, can afford.

Mass spectrometry offers an option that is not available in atomic spectrometry or any other chromatography detection method. In mass spectrometry, a co-eluting compound with a different mass can be determined accurately as long as the compound and its mass are known. This allows the use of a SIL-IS for calibration. Although the magnitudes of the individual responses for the analyte and the internal standard will differ in the presence and absence of ion suppression/enhancement, the ratio of responses will be unaffected [13]. This means that whether the calibration standards are prepared in the matrix or in the water or in the mobile phase solvent, the calibration curve (ratio of analyte response/internal standard response vs. ratio of analyte concentration/internal standard concentration or analyte concentration) obtained will be exactly the same. This means that the calibration standards prepared in water or the mobile phase solvent will give the same results as those prepared in the matrix. In fact, it was found that when SIL-IS's are used for analysis, the precision of the slopes of calibration standards prepared in five different lots of biofluid were within the range 0.19–2.4%, irrespective of the HPLC–MS interface utilized [15]. This shows that the use of SIL-IS compensates for not only the matrix variability but also for the other variabilities such as sample preparation, chromatography and mass spectrometry. This comprehensive study used five different lots of biofluid matrixes and the narrow precision obtained, 0.19–2.4%, was for 39 different analytes [15]. It also showed that when the structural analogues were used as internal standards instead of the SIL analogues, the precision of the five slopes varied up to 16%. This study clearly demonstrates the importance of SIL-IS in quantitative analysis by MS and the effective elimination of ME by the utilisation of SIL-IS.

The common approach used for calibration, in the analysis of complex samples such as plasma, is to prepare the calibration standards in blank matrix and to subject all calibration standards to the lengthy extraction procedure that is used for other samples. In fact, Food and Drug Administration (FDA) guidelines specifically state the requirement for preparing standards by spiking the matrix with known concentrations of analyte [16]. This requirement and the common approach to analysing biological and environmental samples do not take into account the unique advantage offered by the relatively new, increasingly popular, mass spectrometric detection: the ability to accurately quantitate the co-eluting SIL analogue of the analyte. Also, it must be mentioned here that there is no such "blank matrix" that resembles all samples in a batch that can be spiked to prepare "matrix matched" calibration standards. When added to the sample at the beginning of the extraction, the SIL-IS will experience all changes experienced by the analyte, including the ion suppression effects as described above. Therefore, if a SIL analogue is used as the internal standard, there is no need to prepare calibration standards in matrix and subsequent cumbersome extractions. The response ratio calibration using the standards prepared in water or mobile phase solvent will produce the same accurate results as that produced using standards prepared in matrix. This allows the analyst to cut down the preparatory work without sacrificing the accuracy of the data.

In choosing SIL-IS, labelling such as ^{13}C and ^{15}N are preferred to deuterium labelling because deuterium labelled internal standards may not sufficiently co-elute with the analyte causing differences in ion suppression effects in analyte and the internal standard [17]. The deuterium atoms were found to be less lipophilic causing a slightly earlier elution on a reversed phase column. This effect can be facilitated by the presence of high concentrations of co-eluting suppressing ions to cause a significant reduction of accuracy [18]. For the SIL-IS to fully correct for the ion suppression effects, the analyte must completely co-elute with it. Therefore, it is important to check whether they co-elute in each sample chromatogram. However, if the elution time is found to be slightly different, the gradient can be altered to force co-elution of the analyte and the internal standard. It was shown [19] that a linear calibration plot of peak area ratio vs. concentration ratio ensures that the mutual suppression effects of analyte at different concentrations and SIL-IS are equal and therefore the phenomenon does not affect the validity of quantitative analysis. When the mutual suppression effects cause non-linearity, it can be corrected by selecting appropriate concentration of internal standard [20].

A precaution that the analyst must take, regardless of the fact that the matrix of calibration standards is matched or not, is to check the linearity of the internal standard response within the range of expected concentrations. The commonly used response ratio method of calibration, described above, uses a single-point calibration for the internal standard. In other words, this method has a built-in assumption that the internal standard response is linear within the range of internal standard concentrations expected in all samples analysed [21]. In the case of ion suppression/enhancement in LC–ESI-MS, the range of responses obtained for the internal standard can be wide because of the differences in the co-eluting compounds in each sample. Therefore, ensuring the linearity over a wide range of concentrations above and below the added internal standard concentration is paramount for obtaining accurate data.

When more than one analyte is chromatographed and detected by LC–ESI-MS a SIL-IS must be included for each analyte of interest in order to correct for ion suppression effects. As pointed out

above, matrix matching of the calibration standards cannot compensate for ME's such as ion suppression because the matrix varies from sample to sample. In situations where a SIL-IS or a structural analogue of the analyte that will co-elute with the analyte is not available for each and every analyte in the sample, the standard addition method of calibration will be the only option that will guarantee accurate results with LC–ESI-MS.

In summary, to obtain accurate concentration data for an analyte in mass spectrometry the analyst must: optimise chromatography, use a SIL-IS, prepare calibration standards in water or mobile phase solvent and check the linearity of the internal standard response. Matching the matrix of the calibration standard with samples is not necessary as the SIL-IS can be used to efficiently correct for ME.

It is important that the guidelines used for analysis such as FDA are changed with the rapidly changing strengths of the analytical techniques. The approach, one guideline fits all analytical techniques deprives the analyst of the advantages offered by the ongoing advancements made in analytical technology. Revisiting and changing guidelines on a frequent basis, in keeping with the rapidly changing technologies, will enable analyst to take full advantage of the new developments and cut down the time and cost associated with routine analysis.

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