STABLE ISOTOPE DILUTION ASSAY MASS SPECTROMETRY IN FLAVOUR RESEARCH: INTERNAL STANDARD AND CALIBRATION ISSUES

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Abstract

Mass spectrometry is widely used in flavour research to quantify compounds by using isotopically labelled internal standards. However, equimolar amounts of different molecules do not give equal instrumental responses. Therefore, calibration of the mass spectrometer is needed prior to any sample quantification. If the mass of the internal standard differs from that of the analyte by more than 3 Da, the calibration curve is linear. If this mass difference is lower, a second-order curve is obtained that can be linearised by applying various mathematical treatments. We tested a number of linearisation techniques and recommend the method that uses isotopic enrichment calculation to control the quality of the mass spectrometer's calibration.

Introduction

Quantitative analysis is a major growth area in mass spectrometry. Determination of the quantity of a molecule by mass spectrometry is not straightforward because mass spectrometric measurements are not exactly reproducible and ionisation of the compounds depends on several instrumental parameters that are difficult to precisely control (e.g. pressure at the ion source, conditions of the detector). Measurements are generally made relative to an internal standard, thus achieving results with a high level of precision and accuracy. To assure a reliable ratio measurement, the internal standard must be a chemical and physical mimic of the analyte. Therefore, isotopically labelled analogues of the analyte are considered to be the best choice.

This technique, called stable isotope dilution-mass spectrometry (SID-MS), has a long history starting from the dawn of mass spectrometers. Isotope dilution analysis of complex organic compounds was first described in 1940 by Rittenberg and Foster (1). It is still being used in an ever widening range of quantitative applications (e.g. clinical, pharmacological, nuclear chemistry, etc.). Reference methodologies needed to prepare certified material samples are based on SID-MS (2). In aroma research, SID-MS was applied for the first time in 1987 (3). Since then, it has been successfully used to quantify more than 100 potent aroma compounds (4).

SID-MS is based on the fact that the molar ratio of a compound to its stable isotope analogue is directly related to the ratio of the ion intensities of each compound. However, equimolar amounts of different compounds do not give equal instrumental responses because the ionisation efficiency depends partly on the molecular structure. Therefore, the mass spectrometer must be calibrated.

The mathematical background of calibration in SID-MS is well documented (5, 6) and has been recently reviewed by De Bièvre (7) and Sabot (8). However,

considerable differences exist in the analytical approaches taken to determine unknown mole ratios from ion intensity data. These approaches are the result of the complexities introduced into the analysis by the overlapping signals of the unlabelled analyte and its labelled analogue used as internal standard. This overlap is due to the natural occurrence of isotopes in the analyte and to incomplete labelling of the internal standard. This overlap leads to non-linear calibration curves complicating calculation and making necessary the use of linearising methods. This paper will focus on the problem of non-linear calibration curves. Different linearising methods will be compared.

Experimental

Materials

Unlabelled benzaldehyde (purity 99.8 %) was purchased from Fluka (Buchs, Switzerland). [$^2H_1$]-benzaldehyde and [$^2H_6$]-benzaldehyde (98 % enrichment) were obtained from Numelec (Geneva, Switzerland). All solvents were from Merck (Darmstadt, Germany).

Calibration curves were prepared using mixtures of defined amounts of analyte and labelled internal standard making variable either the analyte and keeping the internal standard constant (experiment A) or varying each molecule and keeping the total amount of the two compounds constant (experiment B).

GC-MS analysis

GC-MS analyses were performed on a Finnigan SSQ 7000 mass spectrometer (Bremen, Germany) working in electron ionisation mode at 70 eV. Samples were introduced via a Hewlett-Packard HP-5890 gas chromatograph (Geneva, Switzerland) equipped with an HP-7673 autosampler using the following conditions: splitless injection at 250°C, fused silica capillary column DB-5 (J&W Sci, MSP Friedli, Koeniz, Switzerland) 30 m, 0.32 mm I.D., film thickness 0.25 µm. The carrier gas was helium (90 kPa). The temperature program was 35°C (1 min), then 20°C/min up to 150°C and 30°C/min up to 250°C (1 min). The measurements were carried out at unit resolution measuring molecular ions of the analyte (m/z 106) and the internal standards (m/z 107 and 112 for [$^2H_1$]-benzaldehyde and [$^2H_6$]-benzaldehyde, respectively) with a dwell time of 100 ms. Each sample was analysed in triplicate.

Results and discussion

The determination of the level of an analyte in any sample by SID-MS requires the establishment of a calibration curve. Flavour compounds are present in food samples over a wide range of concentrations. The calibration curve should be constructed to cover this wide amplitude and to assure that no sample falls outside the calibrated range. To study the influence of the overlap due to the natural occurrence of isotopes in the analyte and to incomplete labelling of the internal standard, we have used benzaldehyde as analyte and either [$^2H_1$]-benzaldehyde or [$^2H_6$]-benzaldehyde as internal standard. Figure 1 presents the molecular ion region after electron impact ionisation of the three compounds. It clearly shows that in the case of the analyte and the monolabelled internal standard, the ion at m/z 106 will arise from the $M^{+\cdot}$ of benzaldehyde and from unlabelled material contained in the internal standard.

Similarly the ion at m/z 107 will be found in the spectrum of $[^2H_1]$-benzaldehyde as molecular ion but also in the spectrum of unlabelled benzaldehyde mainly as natural ^{13}C-benzaldehyde. When benzaldehyde was used as analyte and $[^2H_6]$-benzaldehyde as internal standard, the two molecular ions (at m/z 106 and 112) did not overlap.

The calibration curves were established following two different ways. In the first series (experiment A), the internal standard was kept constant (1 ng per sample) and the analyte was added in increasing amounts (from 0 to 50 ng per sample). This procedure is classically used for drug quantification or biochemical analyses (9).

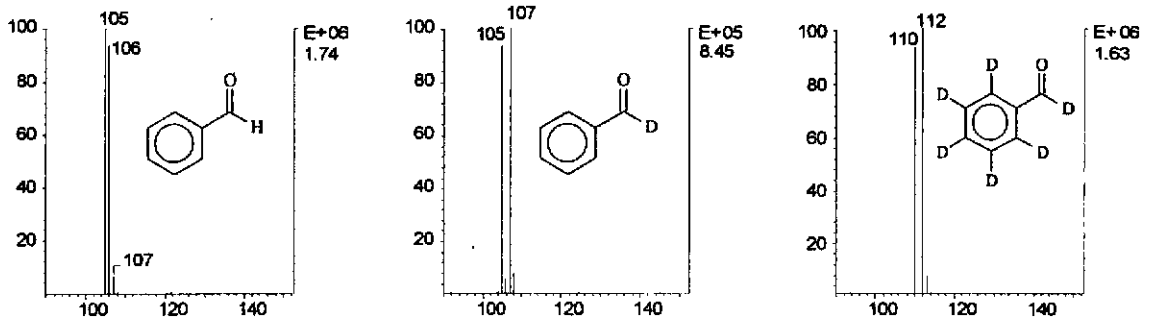

Figure 1. *Molecular ion region of benzaldehyde, $[^2H_1]$-benzaldehyde and $[^2H_6]$-benzaldehyde. The spectra were obtained after electron impact at 70 eV.*

In the second series (experiment B), both the analyte and the internal standard amounts were varied (from 0 to 50 ng per sample for the analyte and from 50 to 0 ng per sample for the internal standard) keeping constant the sum of the two compounds. These two sets of experiments A and B were performed using either $[^2H_1]$-benzaldehyde or $[^2H_6]$-benzaldehyde as internal standard.

The four calibration curves obtained are presented in Figure 2. It shows that the use of $[^2H_1]$-benzaldehyde as internal standard leads to second order curves independently of the way that has been used to build the calibration (curves A-1 and B-1). The use of $[^2H_6]$-benzaldehyde leads to better results. In experiment A (curve A-2), the calibration curve is linear. Experiment B leads, however, to a non-linear curve (curve B-2) showing that elevated concentrations of $[^2H_6]$-benzaldehyde can overlap low concentrations of unlabelled benzaldehyde.

To linearise the second order curves A-1 and B-1 obtained when $[^2H_1]$-benzaldehyde was used as internal standard (where maximum ion overlap is encountered), three methods were employed and compared.

The first method uses a calculation procedure published by Colby and McCaman (10). It transforms the ion ratio (y value) into a value calculated according to the following formula:

$$y = \frac{[(Ry - Rm)(Rx + 1)]}{[(Rm - Rx)(Ry + 1)]}$$

Where *Rx* is the ion ratio of labelled to unlabelled isotope determined with pure analyte, *Ry* is the same ratio determined with pure internal standard, and *Rm* the same ratio calculated with the mixtures of analyte and internal standard for each calibration point.

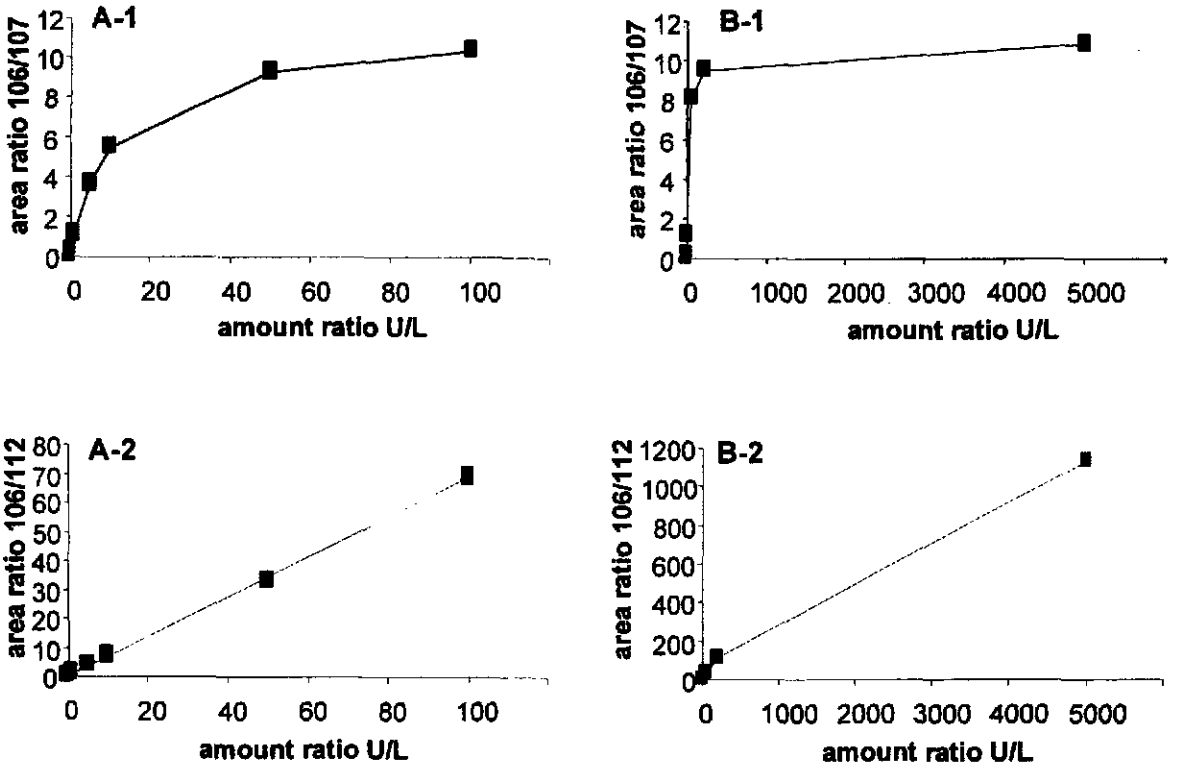

Figure 2. *Calibration curves of benzaldehyde using either [$^2H_1$]-benzaldehyde or [$^2H_6$]-benzaldehyde as internal standards. The curves A-1 and A-2 were obtained with constant amount of the internal standard ([$^2H_1$]-benzaldehyde or [$^2H_6$]-benzaldehyde, respectively). In the case of curves B-1 and B-2, both the amounts of the analyte and the internal standards ([$^2H_1$]-benzaldehyde or [$^2H_6$]-benzaldehyde respectively) were varied*

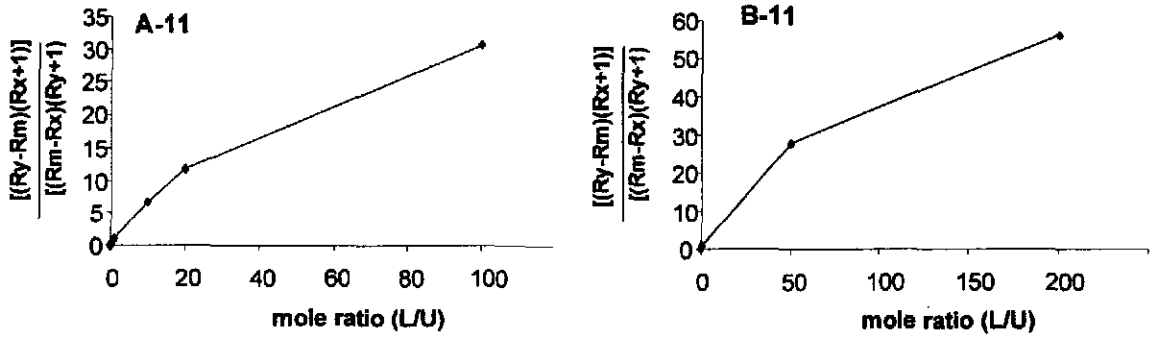

Figure 3. *Linearisation of the calibration curves of benzaldehyde using [$^2H_1$]-benzaldehyde as internal standards according to the method of Colby and McCaman (10). The curve A-11 was obtained with constant amount of the internal standard and the curve B-11 with variable amounts of the analyte and the internal standard*

The application of this method to the calibration curves obtained when unlabelled benzaldehyde is quantified using [$^2H_1$]-benzaldehyde as internal standard gives the results presented in Figure 3 (curves A-11 and B-11). The method is clearly not

powerful enough to linearise the curves built up over the wide range of concentrations used.

The second linearisation method, developed by Bush and Trager (11), generates calibration curves where the y values are calculated as follows:

$$y = Ql(Qu + tQl)$$

The value *Qu* is the amount of unlabelled analyte, *Ql* the amount of labelled internal standard and t corresponds to the fixed fraction of the internal standard which is identical to the unlabelled analyte.

The application of this technique leads to the curves presented in Figure 4. It shows that the method is valid when the amount of internal standard is kept constant (experiment A, curve A-12), but does not work properly when both compounds are varied over a wide range of concentrations (experiment B, curve B-12).

The third linearisation method transforms the amount ratio and ion ratio data into theoretical and measured isotopic enrichments (12-14).

The theoretical enrichments, expressed in mole per cent excess (MPE_{Theo}), were calculated according to the following formula:

$$MPE_{Theo} = \left(\frac{Ql}{(Ql + Qu)}\right) x100$$

where *Ql* is the amount of labelled internal standard and *Qu* the amount of unlabelled analyte.

The measured enrichments (MPE_{Mes}) were calculated according to the following formula :

$$MPE_{Mes} = \left(\frac{(Rs - Ro)}{(1 + (Rs - Ro))}\right) x100$$

where *Rs* is the ion ratio (labelled to unlabelled) in each calibration point and *Ro* the same ratio measured with pure unlabelled analyte.

Figure 5 presents the linear calibration curves obtained by this method. The relationship between the theoretical enrichment and the measured enrichment is linear in both cases (experiment A (curve A-13) where the internal standard [$^2H_1$]-benzaldehyde was kept constant and the analyte was added in increasing amounts and experiment B (curve B-13) where both the analyte and the internal standard were varied).

Moreover the accuracy of the calibration can be checked by observing the slopes of the curves. Indeed, if no bias are introduced into the measurement the slope should be close to the unity, i.e. 1.0000. Using [$^2H_1$]-benzaldehyde as internal standard, values of 0.8872 and 0.8947 (n = 8) were obtained for experiment A (curve A-13) and B (curve B-13), respectively. The deviation of these values from the theoretical value of 1.0000 is due to an overlap of the ions measured for the analyte and the internal standard. If [$^2H_6$]-benzaldehyde is used as internal standard, the slopes have values of 0.9656 and 0.9912, as shown with the curves A-14 and B-14, respectively. These numbers are slightly different from the purity values given by the manufacturer of the two compounds (see Experimental). These results highlight the importance of the labelling degree of the internal standard. When possible, the

internal standard should be chosen according to the rule that the monitored ions for the analyte and the internal standard have a difference of at least 3 mass units.

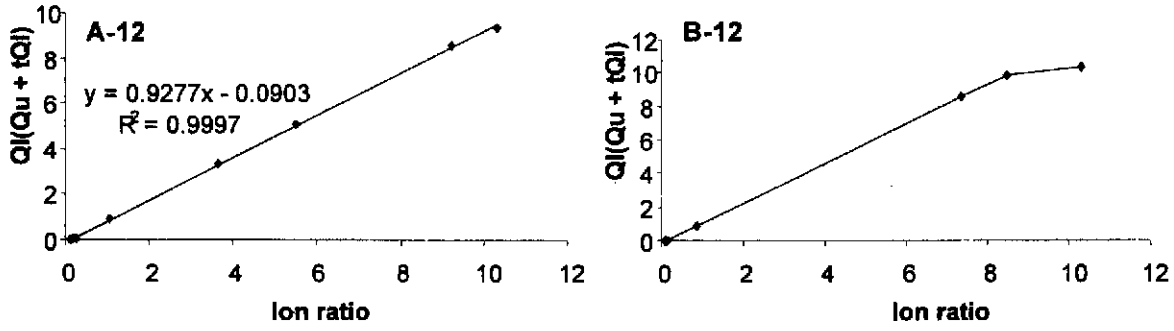

Figure 4. *Linearisation of the calibration curves of benzaldehyde using [$^2H_1$]-benzaldehyde as internal standard according to the method of Bush and Trager (11). The curve A-12 was obtained with constant amount of the internal standard and the curve B-12 with variable amounts of the analyte and the internal standard*

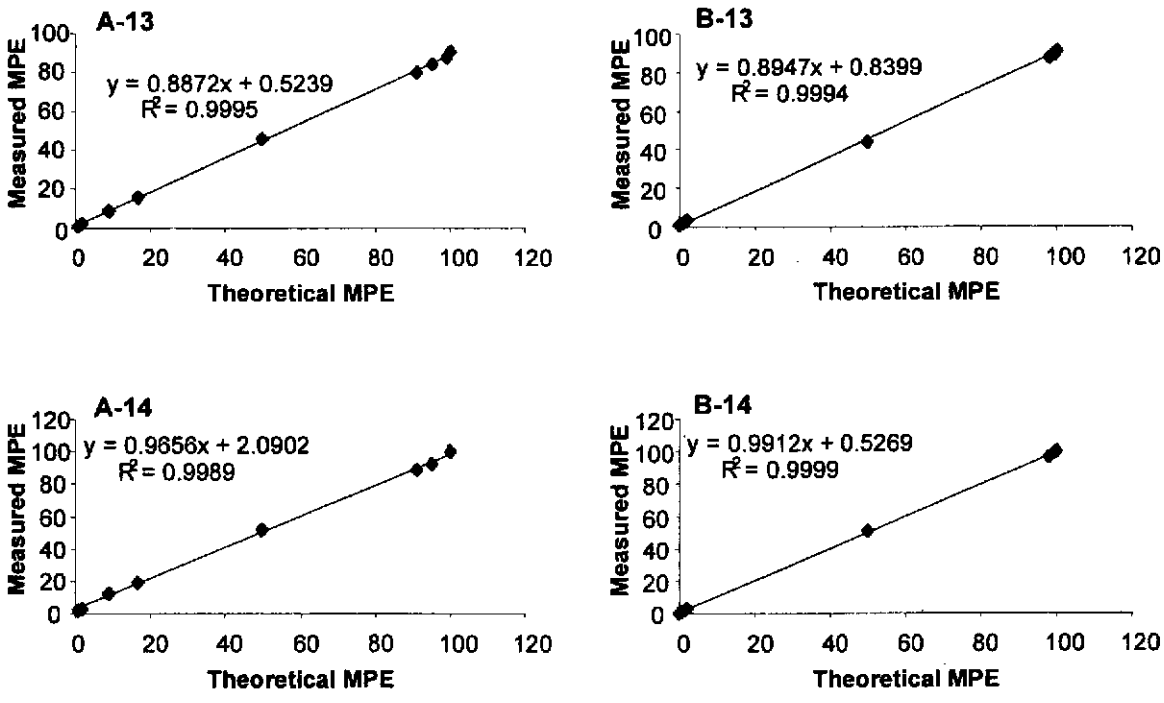

Figure 5. *Linearisation of the calibration curves of benzaldehyde using the isotopic enrichment calculations. The curve A-13 was obtained with constant amount of [$^2H_1$]-benzaldehyde and the curve B-13 with variable amounts of the analyte and the monodeuteriated internal standard. Similar experiments with [$^2H_6$]-benzaldehyde led to the curves A-14 and B-14*

Conclusions

Isotopic dilution is one of the most precise and accurate methods available today to quantify flavour compounds. To avoid overlap between internal standard ions and analyte ions, the mass difference between the two compounds should be higher than 3. When this condition cannot be fulfilled (e.g. in the case of monodeuteriated compounds), and when calibration is needed over a large amplitude, the calibration curve can be linearised using the calculation of the theoretical and measured isotopic enrichments. This method can also be used to control the accuracy of the calibration and we recommend its use as a quality control of the calibration curve.

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