Detector response and intensity cross-contribution as contributing factors to the observed non-linear calibration curves in mass spectrometric analysis

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It is a common knowledge that *detector fatigue* causes a calibration curve to deviate from the preferred linear relationship at the higher concentration end. With the adaptation of an isotopically labeled analog of the analyte as the internal standard (IS), *cross-contribution* (CC) of the intensities monitored for the ions designating the analyte and the IS can also result in a non-linear relationship at both ends. A novel approach developed to assess 'the extent and the effect of [CC]… in quantitative GC-MS analysis' can be extended (a) to examine whether a specific set of CC values is accurate; and (b) to differentiate whether the observed non-linear calibration curve is caused by detector fatigue or the CC phenomenon. Data derived from the exemplar secobarbital (SB)/SB-d_5 system (as di-butyl-derivatives) are used to illustrate this novel approach. Comparing the non-linear nature of calibration data that are empirically observed to that derived from theoretical calculation (with the incorporation of adjustment resulting from the ion CC phenomenon), supports the conclusions that (a) both CC and detector fatigue contribute significantly to the observed non-linear nature of the calibration curve based on ion-pair *m*/*z* 207/212; and (b) detector fatigue is the dominating contributor when the calibration curve is based on ion-pair *m*/*z* 263/268.

Introduction

It is common knowledge that *detector fatigue* causes a calibration curve for a GC-MS (gas chromatography-mass spectrometry)-based methodology to deviate from the desired linear dose–response relationship at the higher concentration end. On the other hand, we have demonstrated that a non-linear relationship may also occur,[1,2] at both the lower and the higher concentration ends, in the highly effective and now widely utilized 'stable isotope dilution mass spectrometry' methodology.‡

Therefore, choosing an ion-pair with no (or insignificant) CC to designate the IS and the analyte becomes an important task in any quantitative MS-based analytical protocol. For this reason, we have (a) conducted a comparative study on methods that can be used to measure the CC values between a specific pair of ions;[11] (b) developed a novel approach to assess 'the extent and the effect of [CC]… in quantitative GC-MS analysis';[12] and (c) compiled a comprehensive list of CC data for ion-pairs that may be adopted for designating the drug analytes and their ISs in MS-based methodologies.[10]

In this current study, we have carried out this line of research one step further. Specifically, the novel approach[12] reported earlier will be extended (a) to examine whether a specific set of CC values is accurate; and (b) to differentiate whether the observed non-linear calibration curve is caused by detector fatigue or the CC phenomenon. Secobarbital (SB)/SB-d_5 has been selected as the exemplar 'analyte/IS' system in this current study to illustrate the validity of this approach.

Experimental

Standards and reagents

The following analytes and deuterated ISs (in 1 or 0.1 mg/mL methanol solution) were purchased from Cerilliant Corp. (Austin, TX): secobarbital (SB) and SB-d_5; pentobarbital (PB). Derivatization reagents, iodobutane, were purchased from Pierce Chemical Co. (Rockford, IL). All other common chemicals and solvents were of HPLC grade.

Sample preparation and derivatization procedure

For SIM (selected ion monitoring) data collection, stock standard solutions for the *analyte*, secobarbital (SB), *IS* (SB-d_5) and the *reference* compound (PB) were first prepared. For the determination of CC values including only SB, 5 μL of the SB standard (1 mg/mL in methanol) was transferred into a 16 × 100 mm glass tube. For the run including only SB-d_5, 50 μL of the SB-d_5 standard (0.1 mg/mL methanol solution) was used. 5 μL of the PB standard (1 mg/mL in methanol) was also included in both tubes when the CC values were determined by the *internal standard* method. Thus, an equal amount of the SB and SB-d_5 was used in these two parallel experiments with the same amount of PB serving as the IS for ion intensity measurements.

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‡ In a 'stable isotope dilution mass spectrometry' assay protocol,[3–8] a pair of ions is selected to designate the analyte and its isotopic analog – the internal standard (IS). Observed intensities of these two ions are then used as the basis for quantification. However, the ion fragmentation processes of these two molecules may result in the contribution of the IS to the intensity of the ion designating the analyte, and *vice versa*, a phenomenon referred to as 'cross-contribution' (CC).[9,10]

For the study on a calibration curve, a series of standard solutions containing 100–40 000 ng/mL of the SB were prepared by using appropriate amounts of the stock standard solution of the analyte. Each of these standards included 500 ng/mL SB-d_5 (as the IS).

The following procedures were adopted to form the iodobutane derivatives of the analyte and the IS.[9] The 16 × 100 mm glass tube containing the analyte or IS (with PB serving as the IS for ion intensity measurements) as prepared in the last paragraph was evaporated to dryness under a stream of nitrogen at 50 °C. 100 μL freshly prepared TMAH/DMSO (1 : 20, v/v) solution was added to the dried residue, and 2 min later, 100 μL iodobutane was added. The tube was capped and briefly vortex-mixed, then incubated for 10 min at 40 °C in a heating block.[6] The mixture was cooled before adding 1 mL 0.1 N NaOH and 3 mL *n*-hexane, then followed by thorough mixing and centrifugation at 1500 rpm. The organic phase was isolated by decanting after freezing the lower aqueous layer. Final products were reconstituted with ethyl acetate for GC-MS analysis (see the next section). The structures of the resulting products are shown in respective mass spectrum figures (Fig. 1).

Instrumentation, analytical parameters, data collection and derivation

GC-MS analysis was performed on an Agilent 6890 GC interfaced to an Agilent 5975 MSD (Palo Alto, CA). A 30 m HP-ULTRA-1 cross-linked 100% methyl siloxane capillary column (0.20 mm ID, 0.33 μm film thickness) from Agilent (Wilmington, DE) was used for this study. Helium carrier gas flow rate was 1.0 mL/min. The injector and GC-MS interface temperatures were maintained at 260 °C and 280 °C, respectively. The GC oven temperature was initiated at 75 °C (held for 0.5 min), raised to 200 °C at 20 °C/min (held for 1 min), then to 275 °C at 40 °C/min (held for 1 min).

Full-scan mass spectrum data of SB and SB-d_5 were plotted (as shown in Fig. 1) and listed to examine the extents of CC for ion-pairs that may potentially be used to designate the analyte and the IS. For example, for the ion-pair *m*/*z* 207/212, the mass spectrum of SB (Fig. 1A) shows the presence (in low intensity) of the *m*/*z* 212 ion; similarly, the mass spectrum of SB-d_5 (Fig. 1B) shows the presence of the *m*/*z* 207 ion. Since ion intensity data collected by full-scan mode are not accurate enough for quantitative calculation, the intensities of all selected ions were collected again by re-injecting the same samples with the mass spectrometer operated under the SIM mode. Typical SIM operations monitor five ions, with 30 μs dwell time and 4.89 cycles/s, while the peak widths were typically tuned to 0.60 or 0.59. Details of the methodology have been described in our earlier publications[11] and briefly illustrated in the next section.

Normalization of SIM data derived from the analyte and the IS and the calculation of CC data

The CC measurement methods described in our earlier study[11] were used to obtain the CC data by two sets of experiments. Data derived from the first set of experiments were used to calculate CC data referred to as *direct measurement*, *normalized direct measurement*, *and internal standard* methods (data on the left-hand section of Table 1). The CC data derived from the direct measurement method were obtained based on raw ion intensity data; the CC data derived from the normalized direct

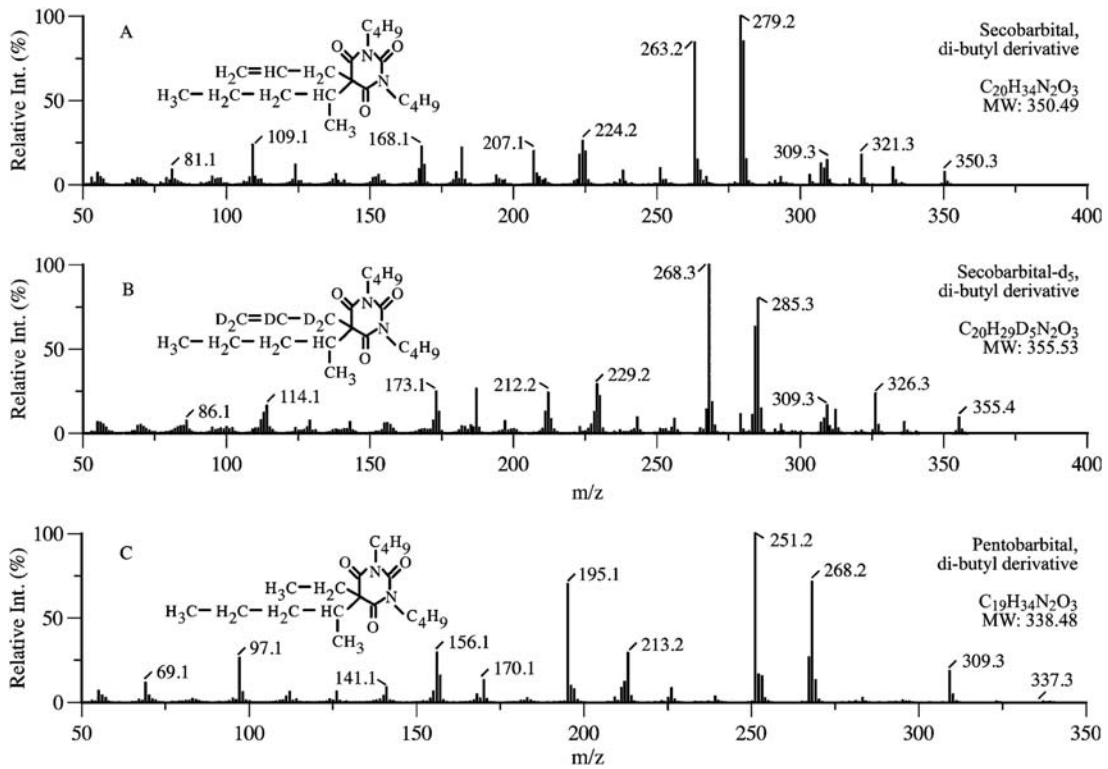

Fig. 1 Mass spectra and structures of secobarbital (A); secobarbital-d_5 (B); and pentobarbital (C) – all as di-butyl-derivatives.

Table 1 Cross-contribution (in %) to intensities of ions designating the analyte and its isotopic analog

					Standard addition[a] (M4)			
Ion (*m/z*)	Direct (M1)	Normalized direct (M2)	Internal standard (M3)	Mean	A	B	C	Mean
Ions designating secobarbital, but contributed by secobarbital-d_5								
207	4.14	4.61	4.44	4.40	0.85	1.07	0.89	0.94
224	3.02	3.37	3.24	3.21	2.70	2.64	2.72	2.69
263	0.24	0.27	0.26	0.26	0.072	0.046	0.049	0.056
280	2.14	2.38	2.29	2.27	1.55	1.57	1.49	1.54
321	7.02	7.84	7.54	7.47	—[b]	—	—	—
350	0.00	0.00	0.00	0.00	—	—	—	—
Ions designating secobarbital-d_5 , but contributed by secobarbital								
212	5.17	4.63	4.81	4.87	3.47	3.19	3.17	3.28
229	0.15	0.13	0.14	0.14	0.068	0.049	0.070	0.062
268	1.05	0.94	0.97	0.99	0.13	0.15	0.16	0.15
285	0.062	0.056	0.058	0.059	0.030	0.094	0.086	0.070
326	0.080	0.072	0.075	0.087	—	—	—	—
355	1.42	1.27	1.32	1.34	—	—	—	—

[a] Ion intensity used to derive the cross-contribution data were the observed value (for Method A), normalized to the base ion intensity of the analog receiving contribution (for Method B), and normalized to a selected ion (*m/z* 251) from a reference compound (pentobarbital) (for Method C). [b] Not determined.

measurement method and the internal standard method were calculated using the intensities of selected ions (*m/z* 350/355 from SB/SB-d_5 and *m/z* 251 from PB, respectively) to normalize the ion intensities for the analyte and the IS observed in different runs.

The three sets of CC data shown in the right-hand side of Table 1 were derived from the 'standard addition' approach following the same procedure described in our earlier study.[11] Using the contribution of SB to the intensity of *m/z* 212 (the ion selected to designate SB-d_5) as an example, the intensities of *m/z* 212 observed in 5 μg of SB were measured with the additions of 0, 0.10, 0.20, 0.30, and 0.40 μg of SB-d_5. These 5 intensity data were then used to derive a regression line, from which the intensity of *m/z* 212 in 5 μg SB (with the addition of 0 μg SB-d_5) was calculated. The resulting intensity data were divided by the intensity of this same ion observed when 5 μg of SB-d_5 was present by itself. The three sets of data (A, B, and C) shown in the right-hand side of Table 1 were derived using ion intensity data came from *direct measurement*, *normalized direct measurement*, *and internal standard* methods, respectively, as described in the last paragraph.

Results and discussion

Issues related to quantitative analysis using an isotopic analog of the analyte as the IS

When utilizing the highly effective stable isotope dilution mass spectrometry method, the concentration of the analyte in the test specimen is determined by: (a) monitoring the intensities of an ion derived from the analyte and an analogous ion derived from the IS; then (b) fitting the intensity ratio of these two ions in the test specimen to the ratio of this same pair of ions in a calibration curve. The calibration curve is established using a series of standard solutions containing the same concentration of the IS, but each with a different concentration of the analyte.

It is desirable to have a calibration curve that is linear over a wide concentration range if the concentrations of the analyte in test specimens vary significantly. However, the linear range of a calibration curve is often limited, because the detecting device may not respond linearly when the analyte's concentration exceeds a certain value – a phenomenon referred to as detector fatigue.

When an isotopic analog of the analyte is used as the IS, as commonly practised in GC-MS or LC-MS methodologies, the fragmentation of the IS and the occurrence of natural abundant isotopes may result in the presence of an ion having the same mass (low-resolution instrumentation) as the ion selected to designate the analyte, and *vice versa*.[1,2] Sine the retention times of the analyte and the IS are typically not adequately resolved, the measured intensities of the ions designating the analyte and the IS may contain interference originating from their respective isotopic analogs (the CC phenomenon). With less fragmentation resulting from the adopted ionization methods, LC-MS methodologies would expect to experience less interference of this nature.

We have studied various aspects of the CC phenomenon, including detailing the procedure for the measurement of CC in a book chapter.[10] We have also developed a novel approach[12] to assess 'the extent and the effect of [CC]… in quantitative GC-MS analysis', based on observing the characteristics of calibration curves resulting from the use of different ion-pairs. In this current study, we will demonstrate that this novel approach[12] can be extended to: (a) examine whether a specific set of CC values is accurate; and (b) differentiate whether the observed non-linear calibration curve is caused by *detector fatigue* or the *CC phenomenon*.

This current study was carried out in 4 steps as outlined below. The validity of the method described in Steps 1, 2,[9,11] 3a, and 3b[12] have been reported in our earlier studies and the procedures have also been briefly described in the Experimental section. Data thereby derived are used in the following sections to demonstrate the points advocated in Steps 3c and 4 – the subject matter of this current study.

(1) Ion-pairs that may potentially be used for designating SB and SB-d_5 are selected from the full-scan mass spectra (Fig. 1) of these two compounds.

(2) The CC values (Table 1) of these potentially useful ion-pairs are obtained by two categories of methods (last sub-section of the Experimental section) using data collected under SIM mode.

(3) Using data derived from a set of standard solutions (for calibration purposes) to determine whether a set of CC values evaluated is accurate.

(a) A set of standard solutions with the analyte's concentrations ranging from 100 to 40 000 ng/mL are prepared and analyzed to obtain a set of empirically observed concentrations. Deviations of the empirically derived concentration from the prepared (expected) concentration for each of these standard solutions are calculated (referred to as '*Deviation Data Set A*' hereafter).

(b) The CC values derived from the two sets of experiments mentioned in Step 2 are used as correction factors to derive two sets of concentrations (for the set of standard solutions) that would theoretically be expected when the CC phenomenon is considered.[12] Deviations of these two sets of theoretically derived concentrations from their prepared (expected) concentrations are calculated (referred to as '*Deviation Data Set B*' and '*Deviation Data Set C*' hereafter).

(c) Deviation Data Set B and Deviation Data Set C are sequentially compared to Deviation Data Set A. The set of CC values, responsible for generating the Deviation Data Set (B or C) that is consistent with Deviation Data Set A, is considered accurate.

(4) The last step determines whether the observed non-linear dose–response relationship (based on empirically data derived from the series of standard solutions described in Step 3) is caused by detector fatigue or the CC phenomenon. Details will be described in the sub-section entitled, 'Differentiating whether the observed non-linear calibration curve is caused by detector fatigue or the CC phenomenon'.

Selecting CC values and ion-pairs for study

The six sets of CC data shown in Table 1 came from two sets of experiments. Specifically, data shown as M1, M2, and M3 (data on the left-hand side of the table) were obtained from one set of experiments, while those shown under M4 (right-hand side of the table) were obtained from another set of experiments. Since the three sets of CC values obtained from each set of experiments are similar, these three CC values derived from each set of

Table 2 Empirically determined and theoretically calculated data derived from ion-pairs with different levels of cross-contribution – secobarbital/secobarbital-d_5 (as di-butyl-derivatives) example (reconstitution solvent volume: 50 μL)

			Theoretically calculated with CC derived from the mean of			
	Empirically observed		Standard addition methods[a]		Direct and normalized methods[b]	
Theor. conc.	Ion int. ratio	Observed conc. (% deviation)	Ion int. ratio	Calculated conc. (% deviation)	Ion int. ratio	Calculated conc. (% deviation)
m/z 207/212			0.94%/3.28%[a]		4.40%/4.87%[b]	
100	0.1642	111.9 (+11.9)	0.1563	106.6 (+6.56)	0.1795	122.4 (+22.4)
200	0.3346	228.1 (+14.1)	0.3035	206.9 (+3.45)	0.3221	219.6 (+9.81)
500	0.7334	500.0 (+0.00)	0.7334	500.0 (+0.00)	0.7334	500.0 (+0.00)
1000	1.559	1062 (+6.29)	1.414	963.7 (−3.63)	1.368	932.6 (−6.74)
2000	2.309	1574 (−21.3)	2.652	1808 (−9.60)	2.475	1687 (−15.6)
5000	5.974	4072 (−18.5)	5.615	3827 (−23.5)	4.897	3339 (−33.2)
8000	7.431	5066 (−36.7)	7.796	5314 (−33.6)	6.501	4432 (−44.6)
10 000	8.819	6013 (+39.9)	8.956	6105 (−38.9)	7.300	4977 (−50.2)
15 000	11.28	7693 (−48.7)	11.17	7617 (−49.3)	8.733	5954 (−60.3)
20 000	13.80	9408 (−52.9)	12.75	8694 (−56.5)	9.684	6602 (−66.9)
30 000	11.74	8006 (−73.3)	14.85	10 126 (−66.2)	10.87	7410 (−75.3)
40 000	14.45	9849 (−75.4)	16.19	11 035 (−72.4)	11.58	7893 (−80.3)
m/z 263/268			0.056%/0.15%[a]		0.26%/0.99%[b]	
100	0.1831	114.1 (+14.1)	0.1611	100.3 (+0.34)	0.1636	101.8 (+1.85)
200	0.3498	217.9 (+8.90)	0.3217	200.3 (+0.17)	0.3244	201.9 (+0.99)
500	0.8028	500.0 (+40.00)	0.8030	500.0 (+0.00)	0.8030	500.0 (+0.00)
1000	1.687	1050 (+5.03)	1.603	998.2 (−0.17)	1.588	988.9 (−1.11)
2000	3.015	1878 (−6.12)	3.196	1990 (−0.49)	3.113	1938 (−3.07)
5000	7.241	4509 (−9.83)	7.919	4930 (−1.38)	7.356	4580 (−8.39)
8000	10.12	6305 (−21.2)	12.56	7819 (−2.25)	11.16	6949 (−13.1)
10 000	13.02	8106 (−18.9)	15.51	9718 (−2.82)	13.48	8396 (−16.0)
15 000	17.13	10 667 (−28.9)	23.07	14 366 (−4.22)	18.67	11 625 (−22.5)
20 000	23.28	14 497 (−27.5)	30.33	18 884 (−5.58)	23.11	14 392 (−28.0)
30 000	24.79	14 444 (−48.5)	44.24	27 546 (−8.18)	30.34	18 889 (−37.0)
40 000	32.72	20 376 (−49.1)	57.40	35 742 (−10.6)	35.95	22 389 (−44.0)

[a] Ion cross-contribution data used for theoretically calculation are means of the value derived from direct measurement, normalized direct measurement, and internal standard methods. The contribution of secobarbital-d_5 to the intensities of ion designating secobarbital were 0.94% for *m/z* 207 and 0.056% for *m/z* 263; the contribution of secobarbital to the intensities of ions designating secobarbital-d_5 were 3.28% for *m/z* 212 and 0.15% for *m/z* 268. [b] Ion cross-contribution data used for theoretically calculation are means of the value derived from standard addition methods. The contribution of secobarbital-d_5 to the intensities of ion designating secobarbital were 4.40% for *m/z* 207 and 0.26% for *m/z* 263; the contribution of secobarbital to the intensities of ions designating secobarbital-d_5 were 4.87% for *m/z* 212 and 0.99% for *m/z* 268.

experiments were averaged to come up with two sets of CC values as shown in columns 5 and 9 in Table 1.

Data shown in Table 1 indicate that ion-pair *m/z* 263/268 exhibits excellent CC characteristics with high intensity; therefore, this ion-pair is the best choice for designating SB/SB-d_5 for the quantitation of the analyte. On the other hand, ion-pair *m/z* 207/212 exhibits significant CC values; adopting this ion-pair will cause a non-linear dose–response relationship at both ends of the calibration curve.[2] These two sets of ion-pairs are adapted as the *control* and the *test* sets, respectively, to illustrate two themes of this study as mentioned earlier. The other two ion-pairs, *m/z* 224/229, 280/285, will not be discussed further.

Determining the accuracy of CC value derived from two different sets of experiments

Mean CC values for ion-pair *m/z* 207/212 derived from two sets of experiments (as shown in columns 5 and 9 of Table 1) are significantly different, indicating that at least one set of these CC data is inaccurate. Thus, the first theme of this study is to determine which set (or any set) of these values is correct, using the approaches outlined in Steps 3 and 4 outlined at the beginning part of the 'Results and discussion' section.

Using the procedure described in our earlier study,[12] the resulting concentration data (adopting ion-pair *m/z* 207/212 for quantitation) for the set of standard solutions are shown in the upper section of Table 2. Data shown in columns 2 and 3 are the empirically observed intensity ratios for the ions designating SB and SB-d_5 and thereby derived concentrations (and deviations from the expected concentrations in percentages). Corresponding data derived from theoretical calculation[12] using the sets of CC values (shown in columns 5 and 9 in Table 1) are shown in columns 4 and 5 and columns 6 and 7, respectively.

Data shown in columns 3, 5, and 7 are Deviation Data Sets A, B, and C mentioned earlier. These data can be better interpreted by plotting these deviation values (data shown inside parentheses in percentage) as shown in Fig. 2A. Curve 'a' is the percent deviation of the empirically observed values from their

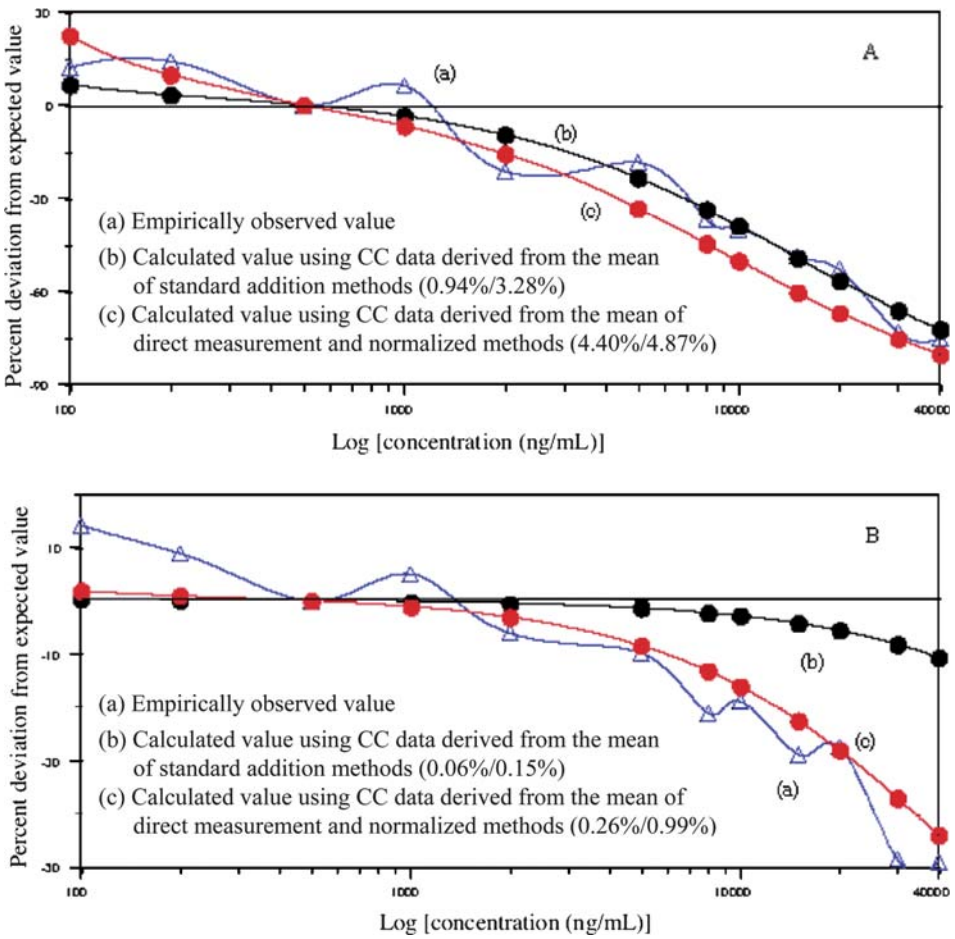

Fig. 2 Deviations (in %) of secobarbital concentrations from the expected values in a set of standard solutions ranging from 100 to 40 000 ng/mL (volume of ethyl acetate used for reconstituting the final derivation product: 50 μL): empirically observed (curve 'a'); theoretically calculated based on the mean of the cross-contribution data derived from three versions of the *standard addition* method (curve 'b'); and theoretically calculated based on the mean of the cross-contribution data derived from *direct measurement*, *normalized direct measurement*, *and internal standard* methods (curve 'c'). Part A (upper): ions *m/z* 207 and 212 are adopted for designating secobarbital and secobarbital-d_5, respectively. For curve 'b', the cross-contributions of the IS to the analyte and the analyte to the IS are 0.94% and 3.28%, respectively. For curve 'c', the corresponding cross-contribution data are 4.40% and 4.87%, respectively. Part B (lower): same as for Part A, except that the ions adopted for designating the analyte and the IS are *m/z* 263 and 268, respectively; the corresponding cross-contribution data are 0.056% and 0.15% for curve 'b' and 0.26% and 0.99% for curve 'c'.

respectively expected concentrations for this set of standard solutions, ranging from 100 to 40 000 ng/mL. Curves 'b' and 'c' are the corresponding data derived from theoretical calculation embedding two different sets of CC values.

It is noted that the experimentally derived data, curve 'a' in Fig. 2A, contain random errors (as indicated by the 'up-and-down' points), while the theoretically derived data, curves 'b' and 'c', follow definite trends characterized by their 'smoothness' nature. If the 'up-and-down' are considered acceptable experimental errors, then curves 'a' and 'b' overlap well throughout the entire concentration range of this set of standard solutions. This is to say that theoretical concentrations for these standard solutions, generated by embedding the set of CC values (0.94%/3.28% as shown in column 9 in Table 1), are consistent with what have been empirically observed.

On the other hand, that curve 'c' does not overlap well with curve 'a' is an indication that embedding the other set of CC values (4.40%/4.87% as shown in column 5 in Table 1) generated a set of theoretical concentrations with substantial differences from what have been empirically observed. The differences are particularly profound at the lower and the higher concentration ends. With these stimulations, it is concluded that for the ion-pair *m/z* 207/212, the correct set of CC values is 0.94%/3.28%, *i.e.*, 0.94% of the intensity measured for ion *m/z* 207 (designating SB) is actually contributed by SB-d_5, 3.28% of the intensity measured for ion *m/z* 212 (designating SB-d_5) is actually contributed by SB.

It is interesting to note that the CC data for the first three sets (Table 1: M1/M2/M3) and the second three sets (Table 1: M4A/M4B/M4C) are very similar within each group, but are significantly different between groups. This is an indication that both groups of experiments (based on different methods as described in the last sub-section of the Experimental section) generated data with good precision, but the data resulting from the first group of experiments (that have been proven less accurate) might have embedded systematic errors.

Differentiating whether the observed non-linear calibration curve is caused by detector fatigue or the CC phenomenon

Data shown in the lower section of Table 2 and the curves shown in Fig. 2B were derived from the ion-pair *m/z* 263/268, the same way as what have been documented in the upper section of Table 2 and Fig. 2A using *m/z* 207/212 as the quantitation ion-pair. As shown in columns 5 and 9 (Table 1), the corresponding CC

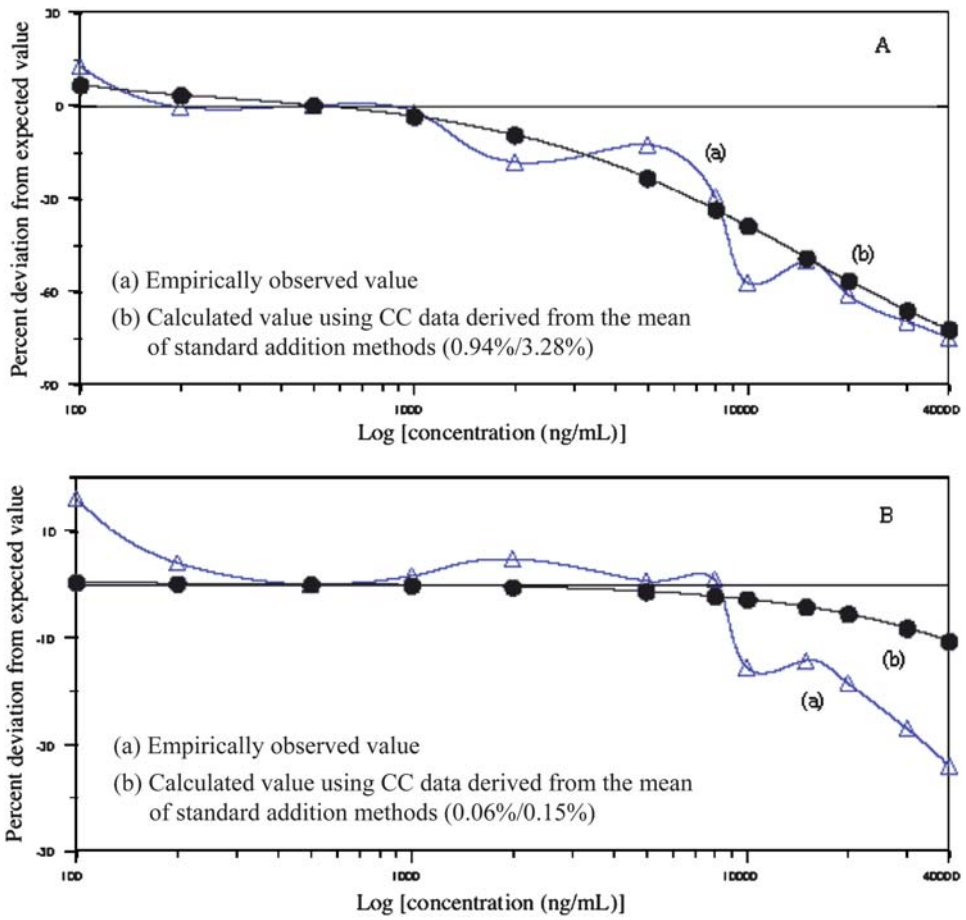

Fig. 3 Deviations (in %) of secobarbital concentrations from the expected values in a set of standard solutions ranging from 100 to 40 000 ng/mL (volume of ethyl acetate used for reconstituting the final derivation product: 100 μL): empirically observed (curve 'a'); and theoretically calculated based on the mean of the cross-contribution data derived from three versions of the *standard addition* method (curve 'b'). Part A (upper): ions *m/z* 207 and 212 are adopted for designating secobarbital and secobarbital-d_5, respectively. Part B (lower): same as for Part A, except that the ions adopted for designating the analyte and the IS are *m/z* 263 and 268, respectively. See Fig. 2 for the cross-contribution data for these ions.

values for ion-pair *m/z* 263/268 are 0.056%/0.15%, much lower than those derived from ion-pair *m/z* 207/212. With this set of lower CC values, the deviation data derived from the theoretically calculated concentrations, plotted as curve 'b' and in Fig. 2B, are expected to be significantly lower (especially at the lower and the higher concentration ends) than the corresponding data for *m/z* 207/212 shown in Fig. 2A. However, curves 'a' and 'b' in Fig. 2B do not overlap well as shown in Fig. 2A. It is thus suspected that the experimentally observed data may contain contributing factors other than the CC phenomenon.

Since the intensities of ion-pair *m/z* 263/268 are significantly higher than ion-pair *m/z* 207/212 (see Fig. 1A and 1B), we thought that the reason why curves 'a' and 'b' overlap well in Fig. 2A, but not in Fig. 2B, could have been caused by the contribution of the detector fatigue phenomenon to the data used to plot curve 'a' in Fig. 2B. This hypothesis was tested by re-injecting the set of fully prepared (derivatized and reconstituted) standard solutions at two additional dilution levels, *i.e.*, by adding additional 100 and 200 μL of ethyl acetate before the repeated injections.

The resulting data are plotted as curves 'a' and 'b' in Fig. 3 and 4. Curves in Fig. 3 and 4 were plotted in exactly the same way as the corresponding curves shown in Fig. 2, except that the reconstitution volumes of ethyl acetate were now 100 and 200 μL respectively, instead of 50 μL. Since curve 'c' in Fig. 2 was plotted based on an inaccurate set of CC values, this curve is not included in Fig. 3 and 4 where the detector fatigue phenomenon is the focus of discussion.

Curves 'a' and 'b' in Fig. 3A and 4A were plotted using data resulting from the ion-pair *m/z* 207/212. These two curves overlap well the same way as they do in Fig. 2A. On the other hand, the overlapping of these two curves improves from Fig. 2B to 3B to 4B as the amount of SB injected into the GC-MS was reduced (the injection volume was the same, while the volume of ethyl acetate used for reconstitution was increased from 50 to 100 to 200 μL).

In conclusion, the non-linear calibration curve observed in Fig. 2A (using *m/z* 207/212 as the quantitation ion-pair) can be fully explained by the CC phenomenon. On the other hand, the non-linear calibration curve observed in Fig. 2B (using *m/z* 263/268 as the quantitation ion-pair) is caused by the combination of the CC and the detector fatigue phenomena. Whether a specific ion-pair with CC is suitable for quantitation depends on the CC value, the desired linear calibration range, and the acceptable accuracy level. Ion-pair *m/z* 207/212 can be used in the 100–5000 ng/mL calibration range if a ±20% deviation is acceptable. Data shown in Table 2 indicate +11.9% and –18.5% deviations,

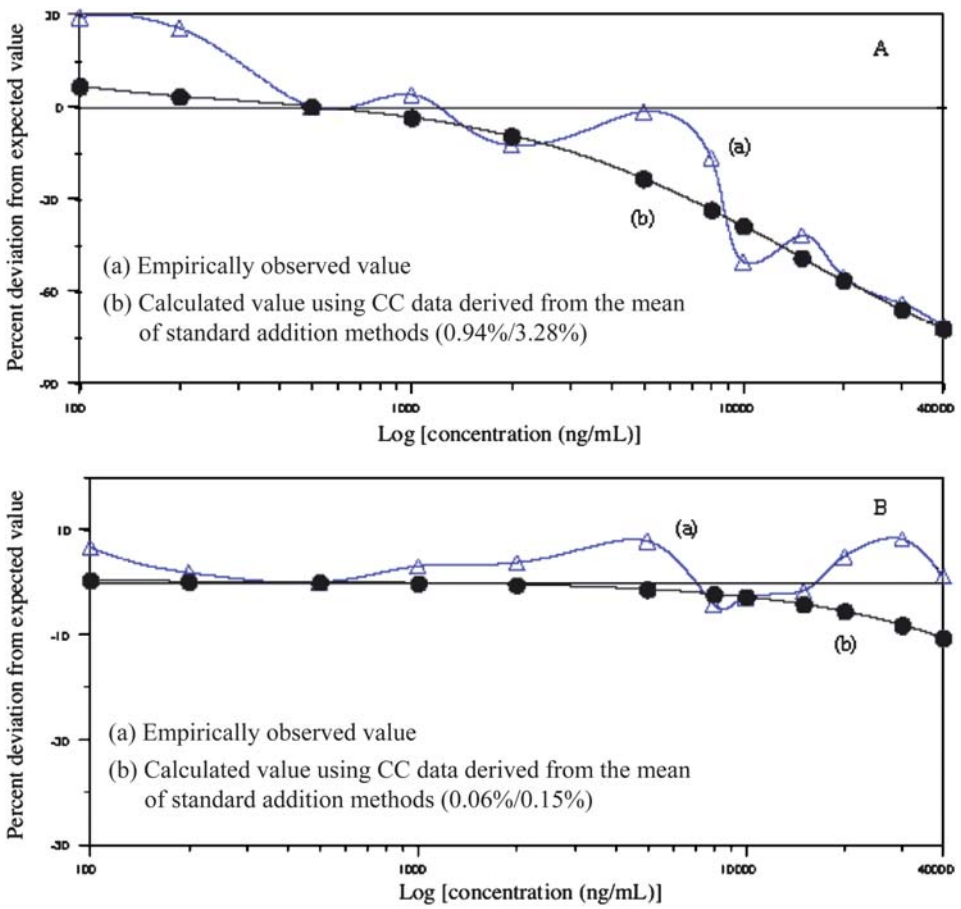

Fig. 4 Deviations (in %) of secobarbital concentrations from the expected values in a set of standard solutions ranging from 100 to 40 000 ng/mL (volume of ethyl acetate used for reconstituting the final derivation product: 200 μL): empirically observed (curve 'a'); and theoretically calculated based on the mean of the cross-contribution data derived from three version of the *standard addition* method (curve 'b'). Part A (upper): ions *m/z* 207 and 212 are adopted for designating secobarbital and secobarbital-d_5, respectively. Part B (lower): same as for Part A, except that the ions adopted for designating the analyte and the IS are *m/z* 263 and 268, respectively. See Fig. 2 for the cross-contribution data for these ions.

respectively, at the 100 ng/mL and the 5000 ng/mL concentration levels.

Conclusion

This study has made a very significant step toward understanding the CC phenomenon and its impacts on quantitation by the 'stable isotope dilution mass spectrometry' methodology. Specifically, it has been demonstrated, for the first time, that it is possible to determine whether a set of empirically determined CC values for a specific ion-pair is accurate. This study has further demonstrated that it is possible to differentiate whether an observed non-linear calibration curve is caused by the CC or the well known detector fatigue phenomenon.

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