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Quantitative measurements via co-elution and dual-isotope detection by gas chromatography–mass spectrometry

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ABSTRACT

Dual-isotope measurements by gas chromatography–mass spectrometry (GC–MS) which mimic isotope dilution may suffer from irreproducibilities or unduly large uncertainties because of variations in ionization efficacies for the respective forms in the MS source. Such variations are sometimes avoided via extensive pretreatments and high-resolution GC separations. However, in some circumstances, an alternative approach is feasible which instead exploits the advantages of decreasing GC resolution. By forcing both forms of each analyte to co-elute, their ionization efficacies in the MS source will be virtually identical, thereby allowing for highly reproducible relative response ratios to be attained despite dramatically lowered GC resolution. The co-elution results described here are nearly as precise as results from moderate-resolution separations in the absence of interferents. Thus, dual-isotope GC–MS measurements with co-elution of the target analytes and their respective isotopically labeled internal standards offer a powerful alternative to the conventional approach of requiring expensive and labor-intensive additional pretreatments and separations; however, the effects of interferences may be exacerbated by the forced co-elution and must also be considered.

INTRODUCTION

Dual-label methods can be useful for measuring or comparing analytes [1–4]. Radioactive substances are often employed for dual-label measurements in order to exploit their good selectivities and high sensitivities. However, unless required because of their low limits of detection and freedom from interferences, radioactivity measurements are typically avoided because of potential health hazards or regulations. Consequently, methods utilizing non-radioactive isotopically labeled substances may be preferred, and GC–MS measurements can be used owing to the mass discrimination offered by mass spectrometry (MS). For example, MS with equilibrated mixtures of isotopically labeled compounds is a valuable approach for accurate analyses and has been used for many years [5].

Recently, dual-isotope techniques which mimic isotope dilution have become popular. Some gas chromatographic (GC)–MS approaches have been adopted for use in a variety of important environmental measurements, *e.g.*, via US Environmental Protection gency (EPA) Methods 1624 and 1625 [6]. Typically, for each analyte, a known amount of that analyte's selected isotopically labeled form is added to samples before pretreatment. The two analyte forms thereby undergo identical treatment because they exist together in the same conditions. A subsample of the prepared sample is then analyzed via GC–MS according to established protocols, with the analyte and its isotopically labeled form typically eluting separately and being measured via their respective characteristic m/z values.

Unfortunately, even those dual-isotope procedures are not always accurate, and it is recommended that other quantitative approaches be used if the two forms do not both show baseline separation from all potential interferents [6]. Such improved temporal resolution can sometimes be at-

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tained via modified pretreatment schemes and/or changes in instrumentation variables, but corresponding adaptations can be expensive and labor intensive. An alternative approach, detailed here, ensuring co-elution of the labeled and non-labeled forms, may offer sufficient data quality to avoid costly and time-consuming adaptations which are used to enforce baseline resolution of eluates.

THEORY: ASSURING CONSTANT RELATIVE SENSITIVITIES VIA CO-ELUTION

If an isotopically labeled, *e.g.*, deuterium-labeled, form of analyte i is added to sample n prior to pretreatment, then the peak area for its elution and measurement by GC–MS may be modeled as

$$A_{\text{ind}} = \frac{V_{sn} C_{ind} E_{ind} V_{\text{col},n} S_{ind} V_{\text{inj},n}}{V_{cn}(V_{\text{col}} + V_{\text{split}})_n V_{cn}} \int_{RT-bw}^{RT+bw} k_{ind}(I_{ind})_t \mathrm{d}t \quad (1)$$

with

$$(I_{ind})_t = (E_{\text{ion},ind})_t (E_{\text{ext},ind} E_{\text{sel},ind} P_{\text{mult},id} f_{\text{p}})_t \quad (2)$$

where A_{ind} is the peak area for analyte i from sample n and isotopic label d (which could be deuterium or ^{13}C or another isotope), V_{s} and V_{c} correspond to the original sample volume and the volume after pretreatment, C is the concentration of the specified analyte in the sample, E is the efficacy of the pretreatment as the fraction of the specified substance recovered, V_{col} and V_{split} correspond to carrier volumes which flow to the column and out of the split vent during the duration while the analyte resides in the injector, S is the fraction of the analyte which is in the carrier during the duration the analyte resides in the injector, V_{inj} is the volume of pretreated sample injected, RT is the retention time of the analyte, bw is some selected multiple of the analyte's peak width, k relates electrometer current to the monitored response, E_{ion} is the ionization efficiency in the source for the target analyte at the specified time t, E_{ext} and E_{sel} are efficiencies of extraction from the source and delivery to the first stage of the electron multiplier through the m/z selector and $P_{\text{mult}} f_{\text{p}}$ defines the response of the electron multiplier to incident selected ions.

Relative response factors (RRF) for naturally occurring analytes, indicated here as h, relative to the isotopically labeled form, d, can be defined for the overall pretreatment and measurement using known amounts of both forms in a reference sample, r:

$$RRF_{irhd} = (A_{irh}/C_{irh})(A_{ird}/C_{ird})^{-1} \quad (3)$$

In subsequent determination, a known amount of the isotopically labeled form is added to each sample before analysis, and the relative response factor may be assumed to be invariant; thus, for sample m, using a relative response factor determined from reference sample r:

$$C_{imh} = (C_{imd} A_{imh})(RRF_{imhd} A_{imd})^{-1} = (C_{imd} A_{imh})(RRF_{irhd} A_{imd})^{-1} \quad (4)$$

if both forms, h and d, are chemically identical through the pretreatment and delivery onto the separations column, and the measurements are not confounded by interferences. Thus, internal standard calculations are valid only if relative measurement sensitivities are stable.

More important for this discussion are special cases for which one ensures that the ionization efficiency of both forms in the source are effectively the same, *i.e.*, $E_{\text{ion},ih} = E_{\text{ion},id}$. For those situations, the response factor assumption is valid when

$$\frac{\int k_{imh}(E_{\text{ext},imh} E_{\text{sel},imh} P_{\text{mult},imh} f_{\text{p}})_t \mathrm{d}t}{\int k_{imd}(E_{\text{ext},imd} E_{\text{sel},imd} P_{\text{mult},imd} f_{\text{p}})_t \mathrm{d}t} \cdot \frac{\int k_{ird}(E_{\text{ext},ird} E_{\text{sel},ird} P_{\text{mult},ird} f_{\text{p}})_t \mathrm{d}t}{\int k_{irh}(E_{\text{ext},irh} E_{\text{sel},irh} P_{\text{mult},irh} f_{\text{p}})_t \mathrm{d}t} = 1 \quad (5)$$

That is, if ionization efficiencies are identical for the target analyte and its isotopomer standard, then internal standard quantitative calculations are appropriate if the relative instrumental responses are reproducible for both analyte and internal standard ions in the source.

However, the ionization efficiency for a specified species can vary dramatically with source pressure and concentrations of other species in the source. Consequently, if interferents co-elute with one of the analyte's forms but not the other, both source pressure and the presence of interferences may cause $E_{\text{ion},ih} \neq E_{\text{ion},id}$, and their relative response factors may therefore be irreproducible or unduly imprecise, perhaps precluding valid internal standard calculations if they elute separately.

The traditional approach to attempts for ensuring

$E_{ion,ih} = E_{ion,id}$ is to extensively pretreat samples and otherwise to separate measured species to yield baseline-resolved eluates, consistent with EPA recommendations [6], and to likewise ensure reproducible source pressures, gas-phase compositions in the source and measurement voltages. However, ensuring baseline resolution is not always feasible or economical, and thus an alternative approach might be used to ensure $E_{ion,ih} = E_{ion,id}$.

An alternative to assuring baseline separations is instead to force co-elution so that both forms of the target analyte are present together at the same time in the same ionizing environment. In such conditions their ionization efficiencies should be identical, neglecting tiny differences in ionization potentials due to differences in ground-state vibrational and rotational energies due to their different reduced masses. Of course, lowering GC resolution to assure co-elution may also cause interferences by other compounds which might contribute to the measured MS signal, and such interferences must be evaluated and compensated for the best results. Also, decreased GC resolution may sacrifice sensitivity and degrade limits of detection. However, because the internal standard and target analyte are chemically identical, fluctuations in ion source conditions may not affect their relative sensitivity, which is required for valid internal standard calculations for GC. Consequently, despite tradition, one might purposely decrease the GC resolution to cause co-elution of labeled and non-labeled forms of the target analytes in order to achieve valid quantitative analyses in dual-isotope procedures which mimic isotope dilution: effects of increased interferences and worse limits of detection due to lowered resolution must be considered and weighed against possible benefits of forced co-elution.

EXPERIMENTAL

Reagents

Anthracene and decadeuterated anthracene were purchased from Aldrich, both at >99% purity. All solvents used were of ChromAR grade from Mallinckrodt and helium carrier gas was >99.9995% pure.

Apparatus

A Hewlett-Packard Model 5971A mass-selective detector interfaced to a Hewlett-Packard Model 5890 Series II gas chromatograph was used, controlled and monitored by a Hewlett-Packard Model QS-20 Vectra computer. Helium was used as the carrier gas at 50 kPa pressure in the split–splitless inlet, yielding a 1.0 ml min^{-1} flow-rate out of the 12 m × 0.2 mm I.D. (0.33-μm film thickness) cross-linked methylsilicone fused-silica capillary column at 25°C. A splitting ratio of 60:1 was used, with a 1.0-min splitless period after each injection. Injection volumes of 1.0 μl were used, as indicated below.

Procedures

Separate solutions of natural isotopic abundance anthracene and decadeuterated anthracene were prepared, dissolving 1 mmol of each compound in 10 ml of methanol; other isotopes such as ^{13}C could likewise have been used, perhaps yielding improved results. These solutions were exposed to sunlight for 2 weeks, generating reaction products 9,10-dihydroanthracene [formula weight (FW) = 180 u], $9^1H,9^2H,10^1H,10^2H$-dihydrooctadeuteroanthracene (FW = 190 u), 9,10-anthracenedione (anthraquinone, FW = 208 u) and octadeuteroanthraquinone (FW = 216 u). The resulting solutions were mixed by volume, as indicated in Table I, and analyzed via two GC separation schemes: (a) isothermal at 160°C for 15 min after injection, then programmed from 160 to 250°C at 20°C min^{-1}, and (b) isothermal at 60°C for 5 min, then programmed to 120°C at 10°C min^{-1}, to 160°C at 2°C min^{-1} and to 250°C at 5°C min^{-1}. The former temperature programme caused co-elution of the deuterated and non-deuterated forms, and the latter allowed for their partial separations. Injections of 1 μl were used and ion currents monitored for selected m/z values over durations spanning the respective components' elutions: m/z = 180 and 190 for the dihydroanthracenes, m/z = 178 and 188 for the anthracenes and m/z = 208 and 216 for the anthraquinones. Five replicates were done for each solution tested, with respective integrated ion currents being calculated for each. For each replicate, ratios of the respective integrated ion currents for non-deuterated and deuterated components were found and corresponding average ratios and standard deviations were calculated.

RESULTS AND DISCUSSION

Deuterated d_{10}-anthracene, normal isotopic composition anthracene and their respective photolysis products, dihydroanthracene and anthraquinone, were separated and measured by GC–MS. Moderate-resolution GC allowed for the modest separation of the respective forms (see Figs. 1 and 2). Peak areas were calculated for each eluate via its parent m/z signal and within-run relative integrated responses compared for each eluate's natural and deuterated pair. For moderate-resolution separations, results within one set of replicates, mixed 1:1 (v/v), varied (n = 5): dihydroanthracene (m/z 180 : 190 = 1.436 ± 0.025), anthracene (m/z 178 : 188 = 1.155 ± 0.008) and anthraquinone (m/z 208 : 216 = 0.926 ± 0.011). These measurements were very reproducible, being made for pure solutions with no interferences detected other than the mutual overlap of the respective forms of the measured compounds. Baseline separations might produce better ratios, but these represent high-quality data.

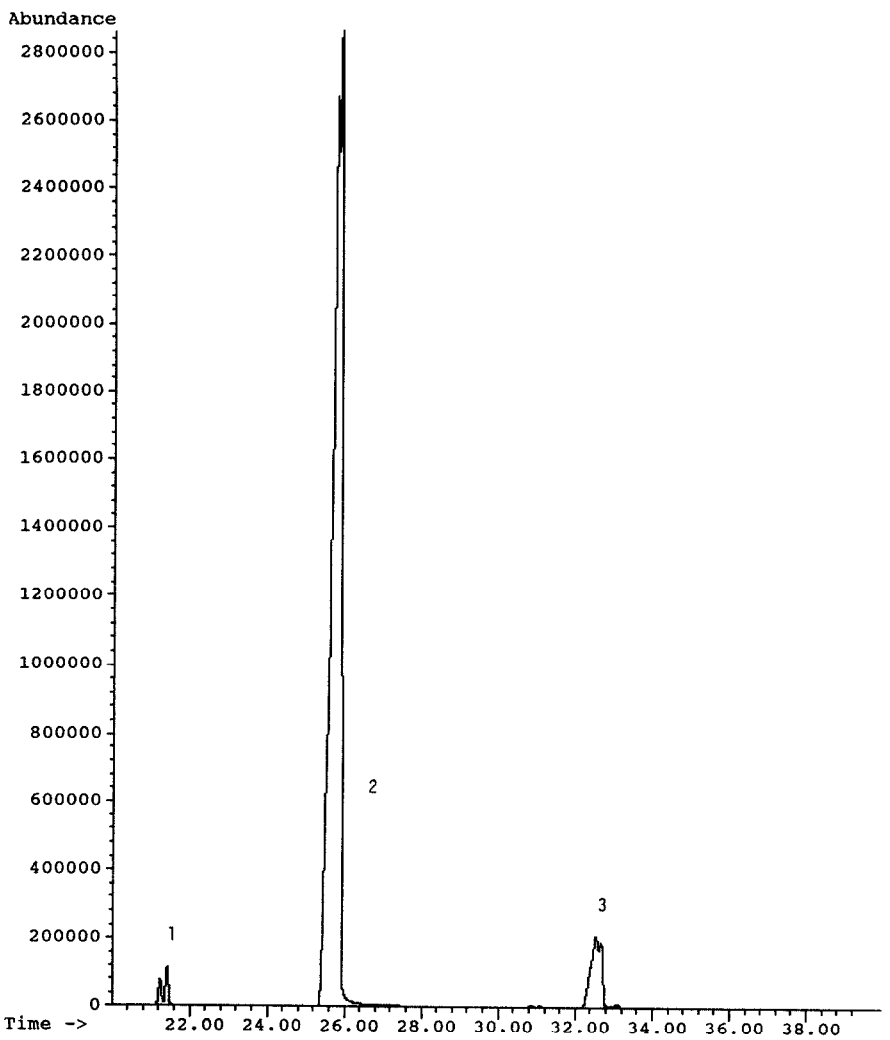

Fig. 1. Moderate-resolution GC–MS separation and measurements of non-deuterated and deuterated forms of (1) 9,10-dihydroanthracene, (2) anthracene and (3) 9,10-anthracenedione. This chromatogram is for the sum of all ion currents for m/z = 178, 180, 188, 190, 208 and 216 over the measurement duration. Time in min.

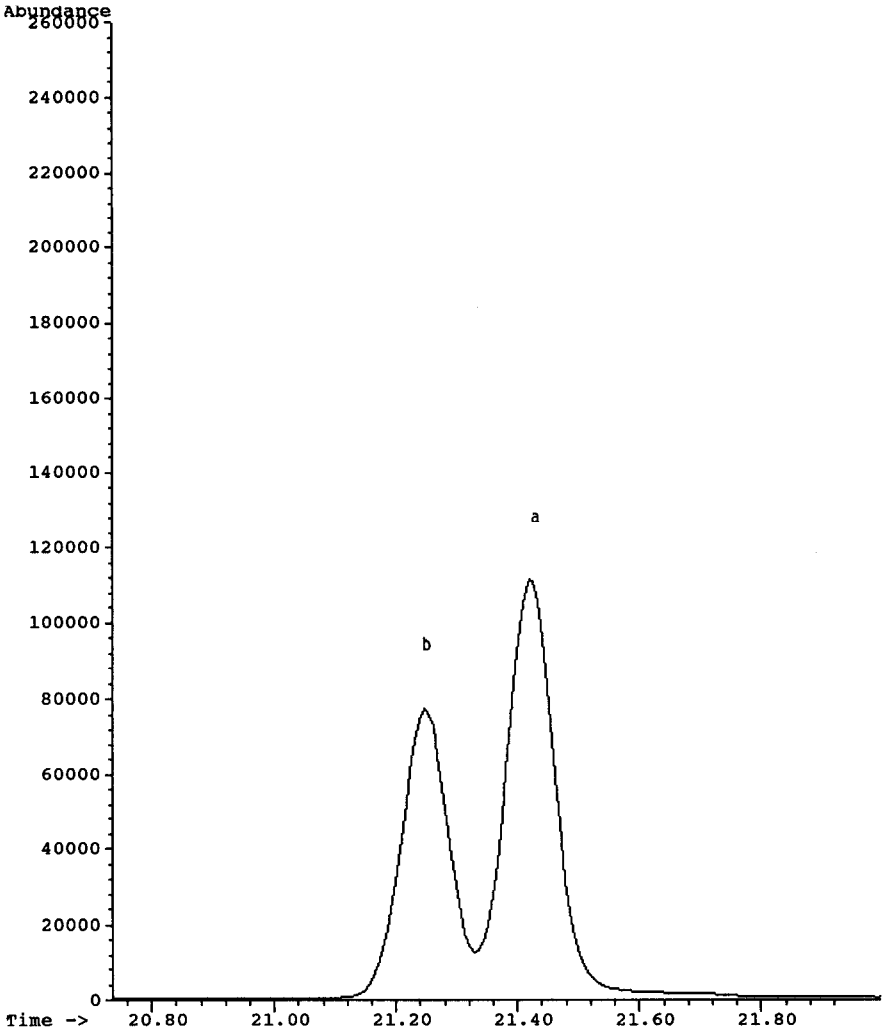

Fig. 2. Moderate-resolution GC–MS separation for selected ion measurements of (a) non-deuterated (m/z = 180) and (b) deuterated (m/z = 190) forms of 9,10-dihydroanthracene, showing nearly baseline resolution. Time in min.

The same solution, and others, were separated by low-resolution GC, yielding nearly complete co-elution of the respective deuterated *vs.* non-deuterated forms (see Figs. 3 and 4). The limits of detection were much worse for the lowered resolution separations and measurements, a disadvantage of the forced co-elution. However, the within-run relative integrated responses for sets of replicates (n = 5) varied by about the same amount as for moderate-resolution separations (see Table I). The relative responses were not as reproducible as those for moderate-resolution separations for pure solutions described above, partly owing to the much broader peak widths. However, the ratios for co-eluted forms were very reproducible and sufficient for good quantitative calculations, and might be superior to corresponding higher resolution measurements if significant interferences are present.

The dual-isotope example above indicates that good quantitative precision may be achieved despite the lowered resolution to achieve co-elution of target analytes and their isotopomer standards. Forcing

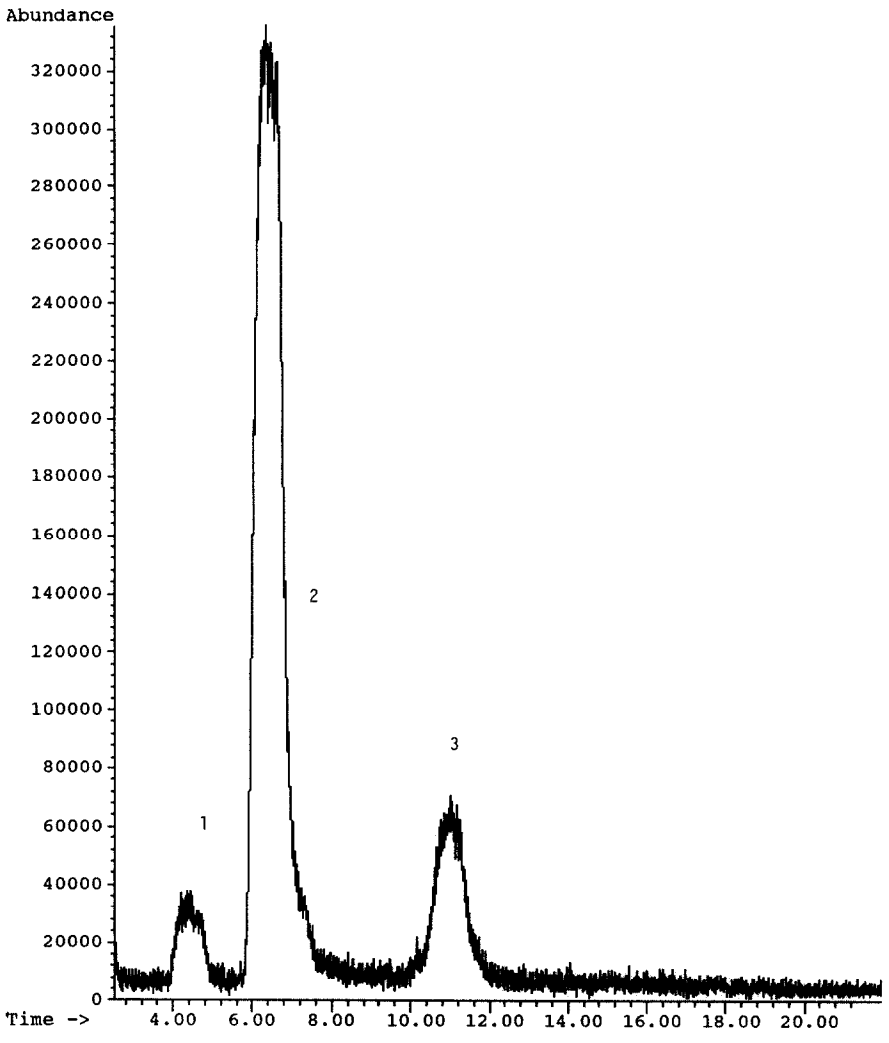

Fig. 3. Low-resolution GC–MS separation and measurements of both non-deuterated and deuterated forms of (1) 9,10-dihydroanthracene, (2) anthracene and (3) 9,10-anthracenedione. This chromatogram is for the sum of all ions for m/z = 178, 180, 188, 190, 208 and 216 over the measurement duration. Time in min.

identical ionization efficiencies via co-elution of the target analyte and its labeled isotopomer could thereby improve the precision and accuracy of ionization efficiencies which might otherwise vary dramatically. However, the feasible improvements are not without potential disadvantages, such as degradation of signal-to-noise ratios and correspondingly worse limits of detection, increased chances of errors in the selected m/z measurements owing to co-eluting interferents and possible isotope exchanges causing errors in selected m/z measurements and associated calculations. Compensation for isotopic abundances and assessment of interferences are typical problems in GC–MS, and using ^{13}C or other isotopes may be better than using deuterium, especially for avoiding isotope exchanges. These difficulties caused by co-elution are much like those encountered in direct-insertion MS, but the GC co-elution approach allows for the separation of analytes from solvents and other

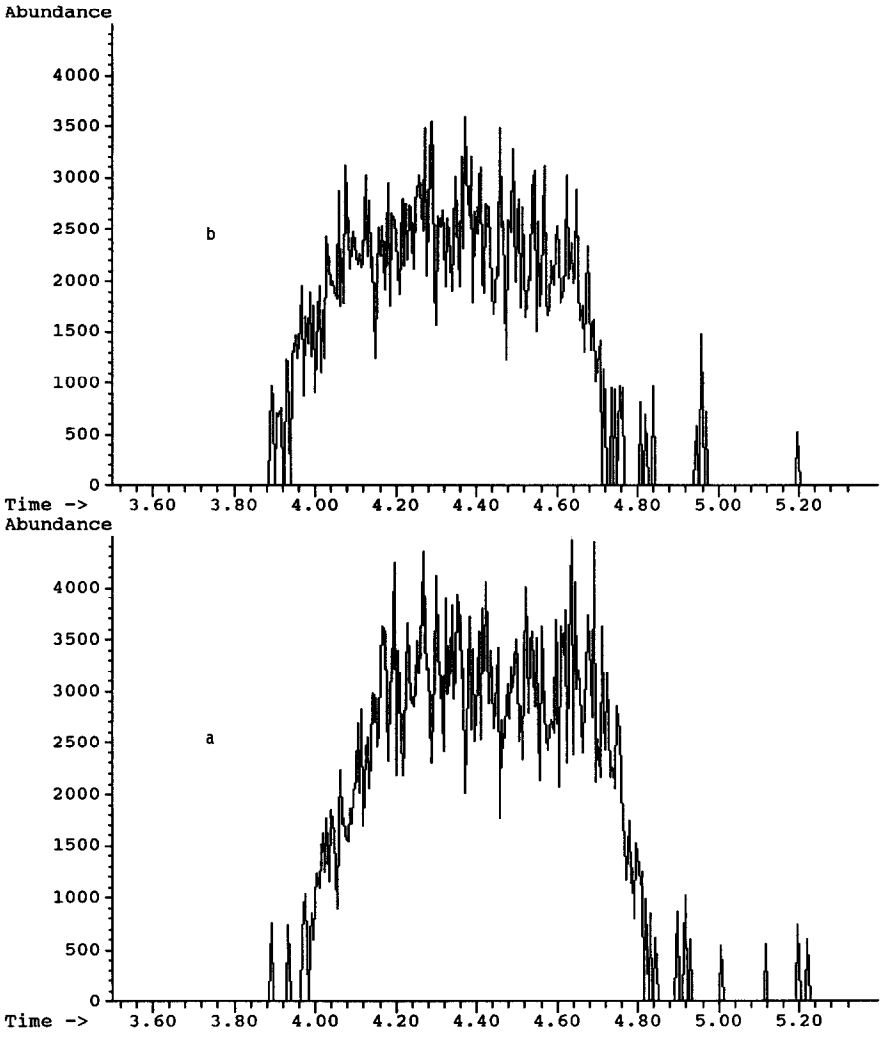

Fig. 4. Low-resolution GC–MS separation and selected ion measurements of (a) non-deuterated (m/z = 180) and (b) deuterated (m/z = 190) forms of 9,10-dihydroanthracene, showing nearly complete overlap of the eluates. Time in min.

major interferences which might preclude direct-injection or very low-resolution GC techniques.

The dual-isotope co-elution approach suggested here may require considerable compromise for its effective use. However, for situations in which direct-insertion MS is not feasible and for which higher resolution GC techniques yield intolerable fluctuations of source ionization efficiencies, forced co-elution of target analytes with their added isotopically labeled internal standards may in some instances allow for good quantitative GC–MS results.

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TABLE I

RELATIVE RESPONSES FOR DEUTERATED *VS.* NON-DEUTERATED ELUATES FOR COELUTION OF TARGET ANALYTES

Relative concentration	Relative integrated response[a]		
	Dihydroanthracene[b]	Anthracene[b]	Anthraquinone[b]
3:1	3.720 ± 0.179	3.609 ± 0.078	3.002 ± 0.134
2:1	2.485 ± 0.106	2.450 ± 0.038	1.988 ± 0.064
1:1	1.178 ± 0.014	1.130 ± 0.039	0.906 ± 0.028
2:3	0.841 ± 0.018	0.772 ± 0.013	0.610 ± 0.018
1:2	0.674 ± 0.012	0.606 ± 0.013	0.477 ± 0.008
1:3	0.401 ± 0.005	0.339 ± 0.010	0.276 ± 0.009

[a] Area for non-deuterated form/area for deuterated form, average ± S.D. (n = 5).

[b] For dihydroanthracene m/z = 190 *vs.* m/z = 180, for anthracene m/z = 188 *vs.* m/z 178 and for anthraquinone m/z = 216 *vs.* m/z = 208 were measured.

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