Journal of Chromatography, 627 (1992) 193–201
Elsevier Science Publishers B.V., Amsterdam

CHROM. 24 578

Quantitative comparisons of reaction products using gas chromatography–mass spectrometry and dual-isotope techniques

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(First received May 14th, 1992; revised manuscript received August 21st, 1992)

ABSTRACT

Special dual-isotope methods for GC–MS measurements show response relations necessary for quantitative comparisons of reaction product abundances, even for multiple-pathway reaction systems. The quantitative comparisons are compatible with the use of isotopically labeled reference substances generated by reference reactions, for which suites of reaction products may be compared via compounds common to both reference and sample reaction systems. The results of comparisons studied are fairly insensitive to large variations in relative concentrations and to high uncertainties in individual analyte measurements. Moreover, the approach may, for some circumstances, be used for analytes of unknown identities or with reference compounds of unknown concentrations. The dual-isotope GC–MS methods described are suitable for photolysis reactions and should be especially helpful in toxicologic metabolism comparisons, environmental degradation studies or other kinetic systems.

INTRODUCTION

GS–MS measurements are especially helpful in experiments involving many analytes, partly because GC–MS can provide excellent selectivity and high sensitivity. Resulting low limits of detection and freedom from many interferences can thus make GC–MS procedures powerful quantitative methods. The use of GC–MS for complicated analyses is well established, and a variety of versatile commercial instruments are available. However, several factors plague GC–MS procedures for analyte measurements from complex sample matrices, predominantly in pretreatment steps [1]. Variations in extraction efficiencies and variable losses during solvent volume reductions can be partially compensated by traditional recovery standard and internal standard techniques [2], sometimes using added compounds which are isotopically labeled but otherwise identical to target analytes [3,4]. Consequently, those techniques require both availability and careful characterization of appropriate reference materials for *every* measured component. However, for complex systems such as studies of metabolisms or environmental exposures several reaction product analytes may be measured for each sample, which exacerbates difficulties associated with use of traditional methods, especially if pure reference materials are not available for every analyte. Moreover, identities of the reaction product analytes are not always known, which precludes use of conventional internal or external standard methods.

Analyses using added isotopically labeled substances can be very powerful for quantitative measurements and comparisons [3–9]. Use of two or more radioactive isotopes is not uncommon and exploits the great selectivities and sensitivities of ra-

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0021-9673/92/$05.00

dioactivity measurements. However, potential health hazards and regulations make alternatives to use of radiolabeled materials attractive. Consequently, isotope selective methods which use non-radioactive substances, *e.g.* MS or atomic emission, are attractive options to radiometric procedures if appropriate isotope selectivities with sufficient corresponding sensitivities can be attained.

Dual-isotope methods with MS of equilibrated mixtures of target analytes with appropriate isotopically labeled compounds have been used for many years for accurate analyses [4]. Approaches which mimic isotope dilution and use GC–MS have gained general acceptance for use in important environmental analyses [3]. For those procedures an isotopically labeled form of each target analyte is added to samples before pretreatment and the two forms of each analyte thereby undergo essentially identical effects during sample preparation. Subsamples are then analyzed by GC–MS using accepted procedures, with each analyte and its isotopically labeled form being measured via their respective characteristic m/z values. However, the good selectivity and sensitivity of GC–MS is sometimes not sufficient to allow for reliable measurements via those approaches, partly due to variations in ionization efficiencies in the MS source, perhaps from variable source pressures or coeluting interfering compounds. Ensuring coelution of both forms of each analyte can partially remedy effects of varying ionization efficiencies [10], even those caused by interfering compounds or variable source pressures.

We have developed dual-label radiometric methods for measurements via chromatography, exploiting the good selectivity and high sensitivity fo radioactivity measurements [4–9]. In previous work, compounds labeled with two different radioactive isotopes were used with HPLC for special dual-label procedures which mimic multiple internal standard methods. In those studies biologically generated radiolabeled reference solutions were used and their components separated along with differently labeled respective coeluting reaction products from samples from reaction experiments. One method employs a homogeneous reference solution of multiple radiolabeled reaction products, generated by a reference reaction system, as a mixed internal standard reference solution [5,6,8]. A known amount of the reference solution is added to each experimental sample containing corresponding differently labeled reaction products before sample preparation, but after investigated reactions have taken place. This procedure may be used to quantitatively compare reaction product profiles and to test for differences between control *versus* test groups in reaction efficacy, *e.g.*, in metabolism or environmental degradation experiments.

The dual-label approach described above allows for compensation for variations in extraction efficiencies, variable losses during volume reduction of extracts, imprecisions of volume measurements and uncertainties in specific activities of reactant compounds and reaction products. The procedures may greatly obviate difficulties caused by unavailability of pure reference compounds. Also, the method yields great improvements in data quality for quantitative measurements [8]. Moreover, theoretically valid comparisons which may be tested statistically are allowed via these methods and show dramatic increases in quality of results *versus* conventional procedures [8,9]. The dual-label techniques are powerful for comparing reaction efficiencies for multiple-pathway reactions [6,8] and for assessing complications caused by impurities or isotope effects [7,9].

Dual-isotope GC–MS procedures reported in this study show advantages of the dual-label radiometric method described above, but avoid use of radioactive materials.

THEORY

A main advantage of the dual-isotope procedures for GC–MS measurements described below is that several special ratios may yield well defined, theoretically predictable results which could be tested statistically [6–9]. Herein we call normal-isotope-composition compounds "Y-labeled compounds".

Dual-isotope reaction product determination

In the absence of pure standards, a fixed known volume, V_a, of a homogeneous internal standard solution generated by a reference reaction system which contains several isotopically labeled reference compounds could be used in place of a conventional standard solution made by mixing known quantities of pure labeled substances [5,6,8]. One may add these X-labeled reference compounds to

subsamples of mixtures of normal isotopic composition, *i.e.* Y-labeled compounds, of unknown concentrations which have been generated from reactions being investigated. By judicious selection, some of the X-labeled components would be chemically identical to the Y-labeled components, except for their respective molecular weights. Hence, pretreatments of the mixtures should yield equivalent extraction/concentration/dilution efficiencies, E_i, for the two extractable forms of each common component; thus, if V_t is the volume of the prepared subsample, and V_{ss} is the reproducible volume of the prepared subsample used for chromatographic separation, then $E_{Y,i} = A_{X,i}V_t(A_{s,X,i}V_{ss})^{-1} = E_{X,i}$, where $A_{X,i}$ and $A_{s,X,i}$ are measured GC–MS areas for the X-labeled component i for samples from the volumes V_{ss} and V_a, respectively. This equivalence is a reasonable assumption when isotope exchange is negligible, and the two forms are chemically alike and are not entrapped or bound in tissue or precipitates.

This method is similar to use of several isotopically labeled internal standards using conventional internal standard calculations. Consequently, the amount of each normal-isotopic-composition component from the sample could be determined by using that subsample component's integrated MS ion current data from the dual-isotope chromatograms, $A_{X,i}$ and $A_{Y,i}$, and the X-label areas for a sample from volume V_a for the reference solution, $A_{s,X,i}$. These quantitative determinations may be useful but are subject to several uncertainties and restrictions which could be avoided by use of the powerful R and U ratio methods discussed below.

Single-component comparisons between experiments using the R-ratio

In many experminents the absolute amounts of analytes are often less important than their relative concentrations between experiments, *e.g.* in comparisons of metabolism in control *versus* test organisms [5,6]. Such comparisons may be suitable for use of multiple internal standards generated by a reference reaction system and the dual-isotope procedures described herein.

One can expose two sets of reactions, 1 *versus* 2, to the same homogeneous dosage of normal isotopic composition Y-labeled compound, add volume V_a of X-labeled reference solution to each resulting sample, and then pretreat and separate each, with GC–MS measurements of eluates. If $V_{a,1} = V_{a,2}$, then $A_{s,X,1} = A_{s,X,2}$. Moreover, if $V_{t,1} = V_{t,2}$ and $V_{ss,1} = V_{ss,2}$ by design, and M is the mass of selected analyte in the indicated subsample, then

$$R_{12} = (M_1/M_2) = (A_{Y,1}A_{X,2})\,(A_{Y,2}A_{X,1})^{-1} \quad (1)$$

for the component of interest, and this R ratio may be calculated from GC–MS area data only.

If reaction efficacy were hypothesized to be not different between two groups, *e.g.* control *versus* test groups, then $R_{12} = 1$ if this null hypothesis is valid and replicated R_{12} values could be tested statistically to ascertain if R_{12} were different from unity for the specified component of interest, *e.g.* if the compound reacts differently in the compared systems.

Multiple-component comparisons using the U ratio

If the procedure used for the R ratio above is extended to several reaction products, then the resulting multi-parametric method could be used to characterize several reaction pathways. Thus, relative magnitudes of several intra-sample parameters may be tested and yield results which are more important than their absolute magnitudes or individual single-component comparisons between groups. For those comparisons, an extension of the R ratio method can be formulated for groups 1 *versus* 2 and components i *versus* j such that

$$U_{12} = (R_{12,i}/R_{12,j}) = (A_{Y,i,1}A_{X,j,1}A_{Y,j,2}A_{X,i,2}) \cdot (A_{Y,j,1}A_{X,i,1}A_{Y,i,2}A_{X,j,2})^{-1} \quad (2)$$

where X and Y represent the measured forms, subscripts i and j indicate the two components of interest, the subscripts 1 and 2 indicate samples from which the components were isolated and A indicates measured GC–MS areas. If the reaction product profile for both groups were the same, then $U_{12} = 1$. This U ratio may be tested statistically, and the null hypothesis of identical relative reaction rates for those components could be assumed unless U_{12} is shown to be significantly different from unity.

Of course, by comparing several components, i, j, k, l..., one might evaluate reaction product profiles representing several modes, *e.g.* several metabolism pathways.

EXPERIMENTAL

Reagents

All organic solvents used were Mallinkrodt ChomAR grade. Anthracene and decadeuteroanthracene were purchased from Aldrich, both at > 99% purity. Helium carrier gas was > 99.9999% 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 carrier gas was used with a pressure of 15 kPa in the split–splitless inlet, yielding a carrier gas flow-rate of 1.0 ml/min at 25°C. Splitless mode was used for all injections and elutions. Injections of 1.0 μl were used unless otherwise stated.

The GC column used was a 12 m × 0.2 mm I.D.

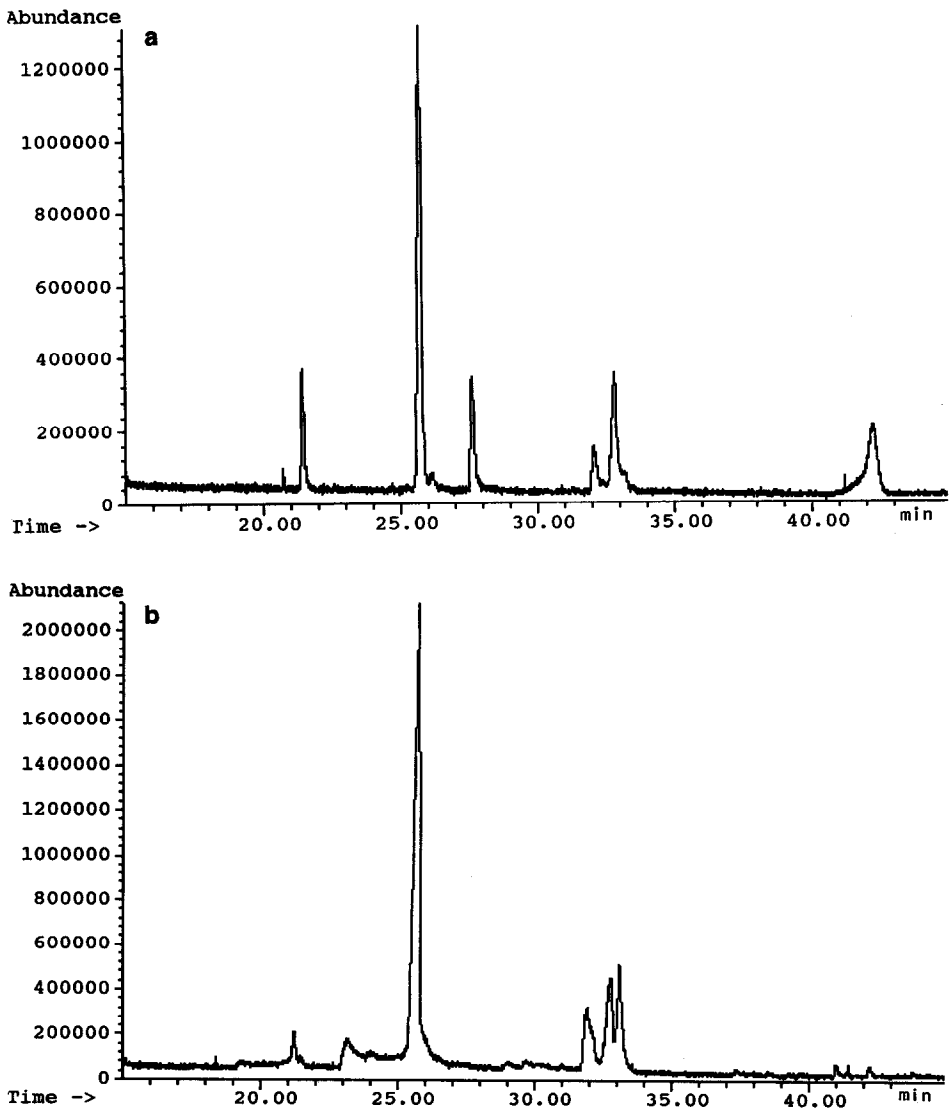

Fig. 1. Total ion chromatograms for 1-μl subsamples of (a) photolyzed methanolic anthracene and (b) photolyzed methanolic [$^2H_{10}$]anthracene.

fused-silica capillary column with a 0.33-μm-thick cross-linked methyl silicone stationary phase.

Procedure

Separate saturated mixtures, with excess solid substance, were made by mixing 10.0 ml of methanol and 10^{-4} mol of either anthracene or [$^2H_{10}$]anthracene. Both of these mixtures were exposed to sunlight, through window glass, for 35 days and then maintained at 4°C in darkness until use. The methanolic solutions above the remaining crystals were mixed, 100 μl of [$^2H_{10}$]anthracenic solution

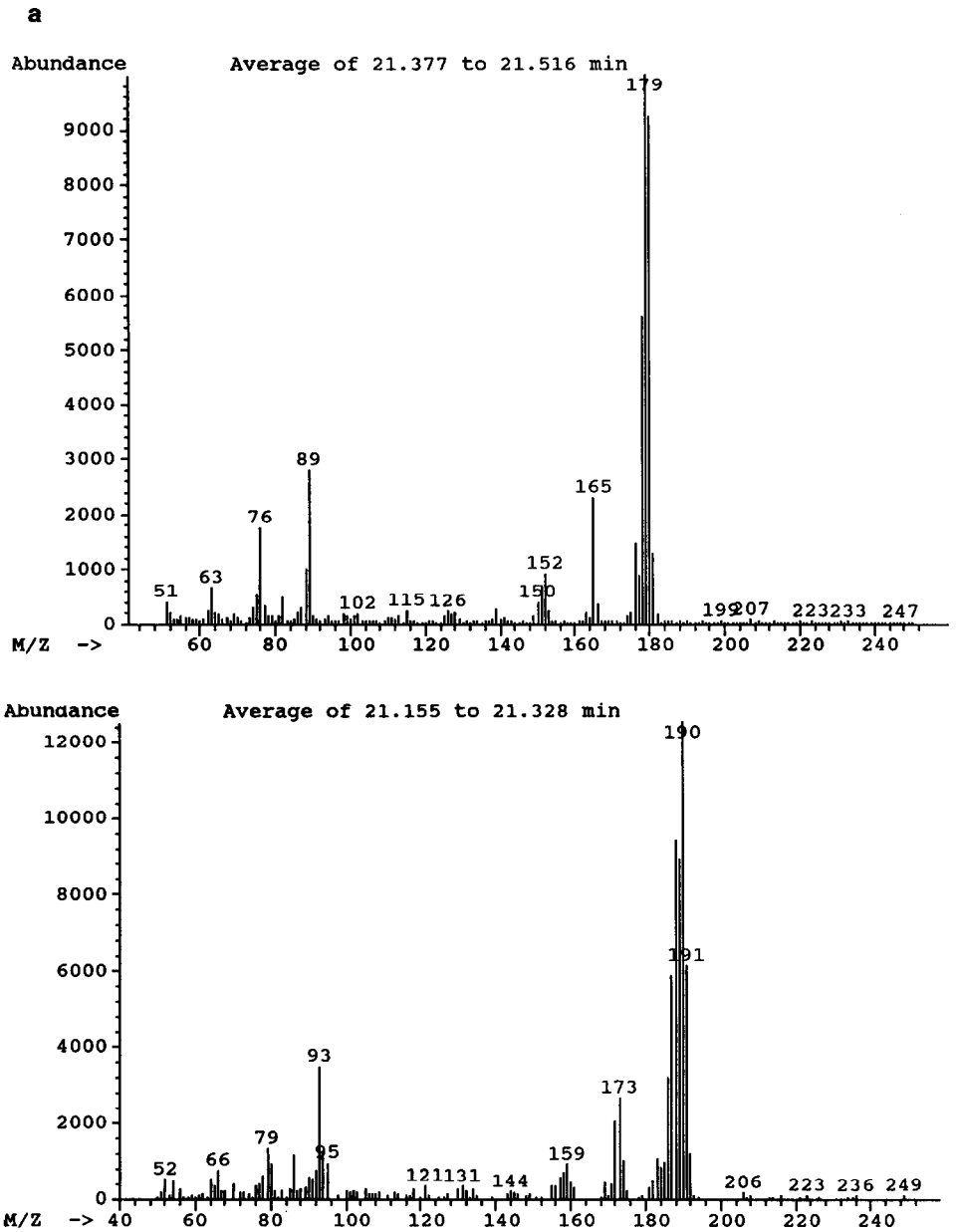

Fig. 2.

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plus 5–1000 μl of anthracenic solution and diluted to 1.50 ml with methanol. Actual concentrations of constituents were thus unknown, other than the saturation of the anthracenes. Triplicate dilutions were made for all relative concentrations.

Triplicate subsamples for all dilutions of the methanolic reactants and their photolysis products were analyzed by GC–MS. Subsamples of 1 μl were injected into the 325°C injector, in the splitless mode, with the oven temperature at 60°C. The 60°C initial temperature was maintained for 5 min, then raised to 120°C at 10°C/min, held at 120°C for 2 min, raised to 160°C at 2°C/min, held at 160°C for 2 min, raised to 250°C at 5°C/min and maintained at

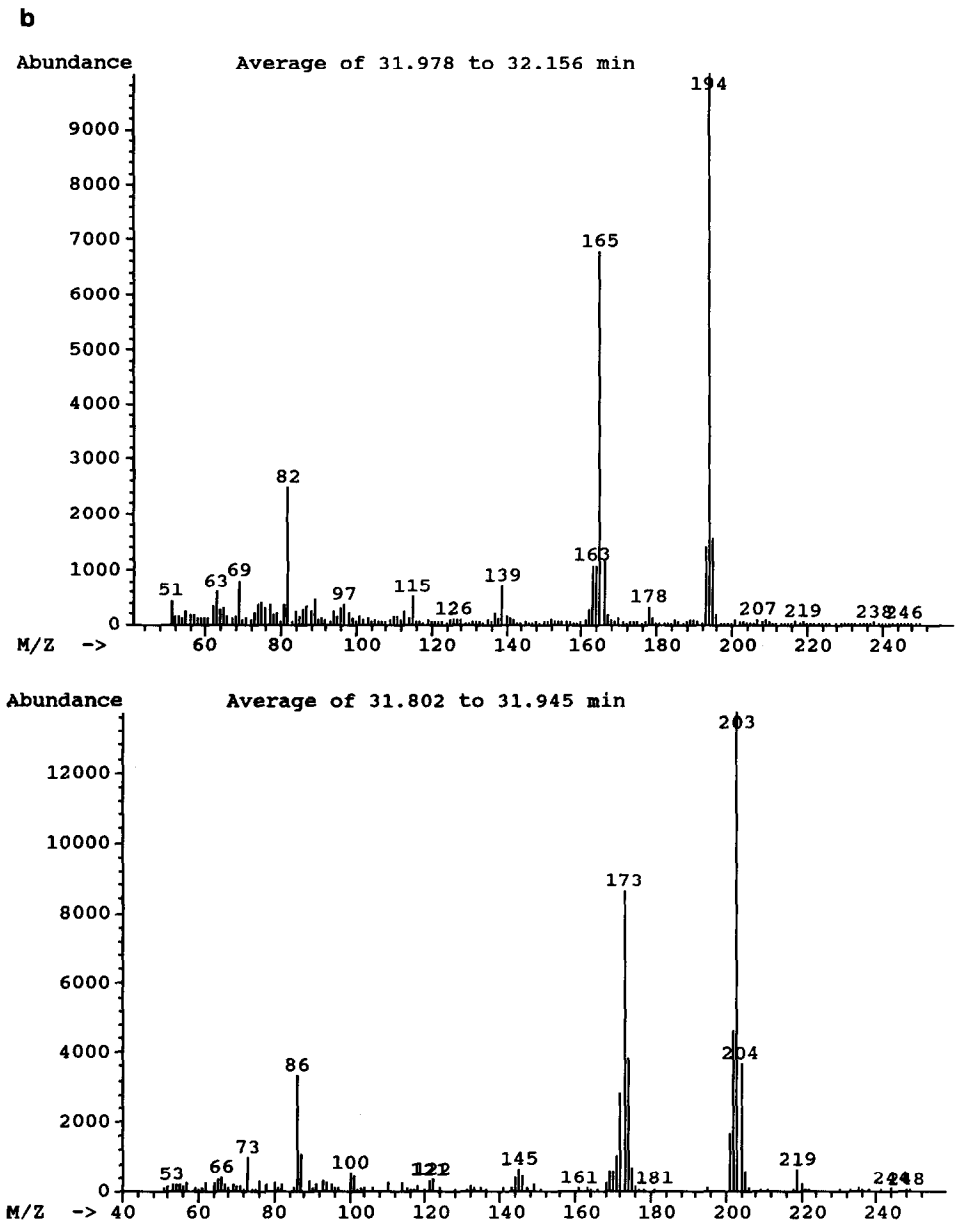

Fig. 2.

250°C for 2 min. The GC–MS transfer line was isothermal at 285°C.

Eluates were measured by selected ion detection (SID) unless stated otherwise. Between retention times of 15 and 30 min, ions at m/z 178, 180, 188, 190, 208 and 216 were monitored every 0.81 s with 100-ms measurement times for each. Between retention times of 30 min and 45 min, ions at m/z 194, 202, 203, 208, 216 and 217 were monitored every 0.69 s with 100-ms measurement times for each. For

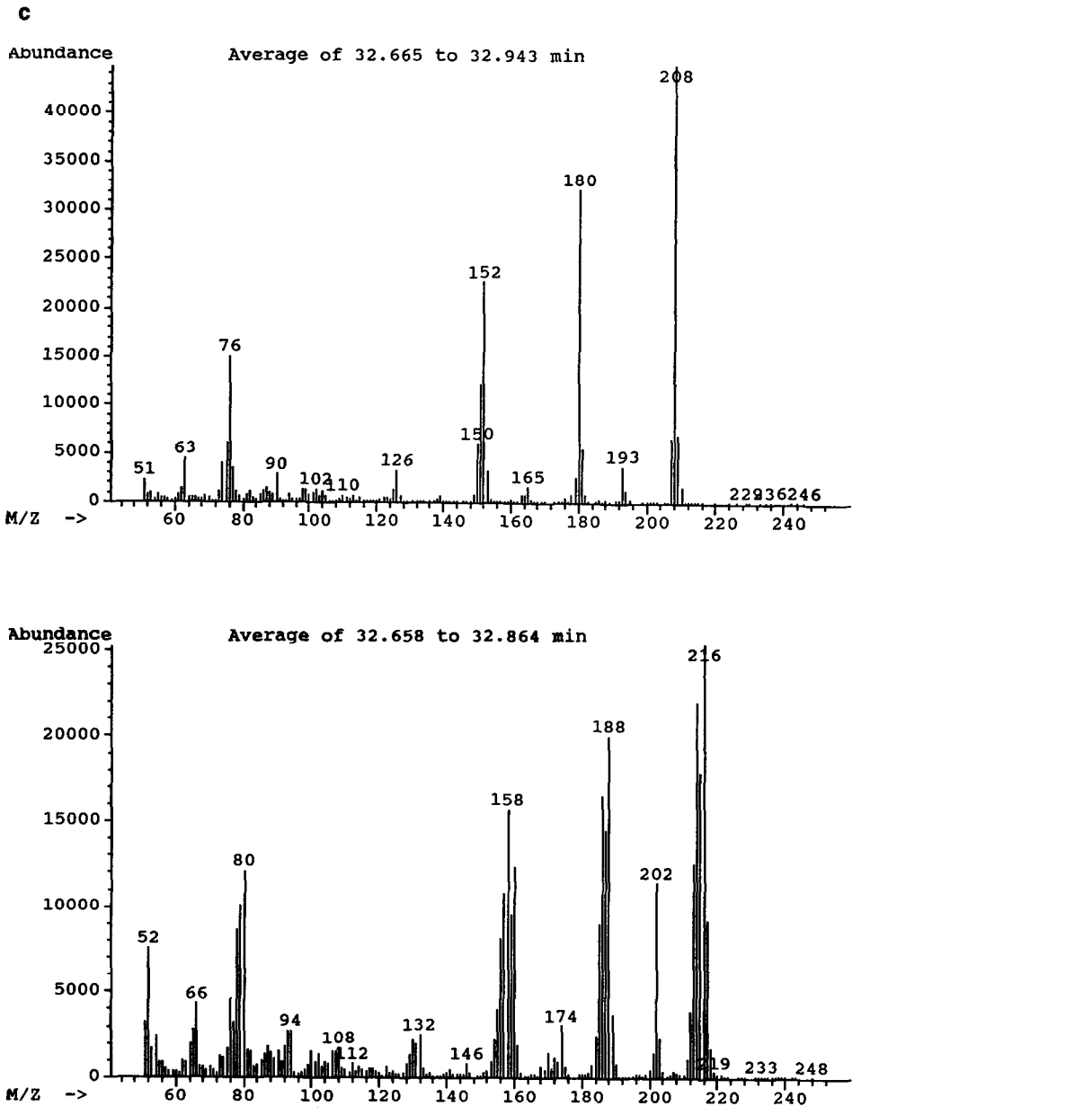

Fig. 2. Mass spectra for tentatively identified anthracene products and corresponding [$^2H_{10}$]anthracene products, derived from GC–MS measurement of mixed solutions of anthracene, decadeuteroanthracene and their photolysis products in methanolic mixtures: (a) likely 9,10-dihydroanthracenes, (b) possibly 9-anthracenones, and (c) likely anthraquinones.

eluate measurements using m/z scanning, detector currents for m/z values between 50 and 250 were measured at a rate of 2.4 scans/s between retention times of 15 and 45 min. Total ion chromatograms (TIC) and selected ion chromatograms were produced and integrated via provided algorithms, and statistical calculations were made by conventional techniques.

RESULTS AND DISCUSSION

Subsamples of the separate methanolic anthracene or [$^2H_{10}$]anthracene mixtures and their respective photolysis products were separated via GC–MS with m/z scanning (Fig. 1) The anthracene and its photolysis products showed several TIC peaks similar to peaks corresponding to [$^2H_{10}$]anthracene and its products. Mass spectra for anthracene (M.W. = 178 u) eluting at 25.7 min and for decadeuteroanthracene (M.W. = 188 u) eluting at 25.7 min were adequate to identify those eluates.

Mass spectra (Fig. 2) for the anthracene product eluting at 21.4 min were like reference spectra for 9,10-dihydroanthracene (M.W. = 180 u), and the [$^2H_{10}$]anthracene product eluting at 21.3 min showed mass spectra like that for 9,10-dihydroanthracene but displaced 10 m/z units, consistent with its being 9H,9^{2}H,10H,10^{2}H-dihydrodecadeute-

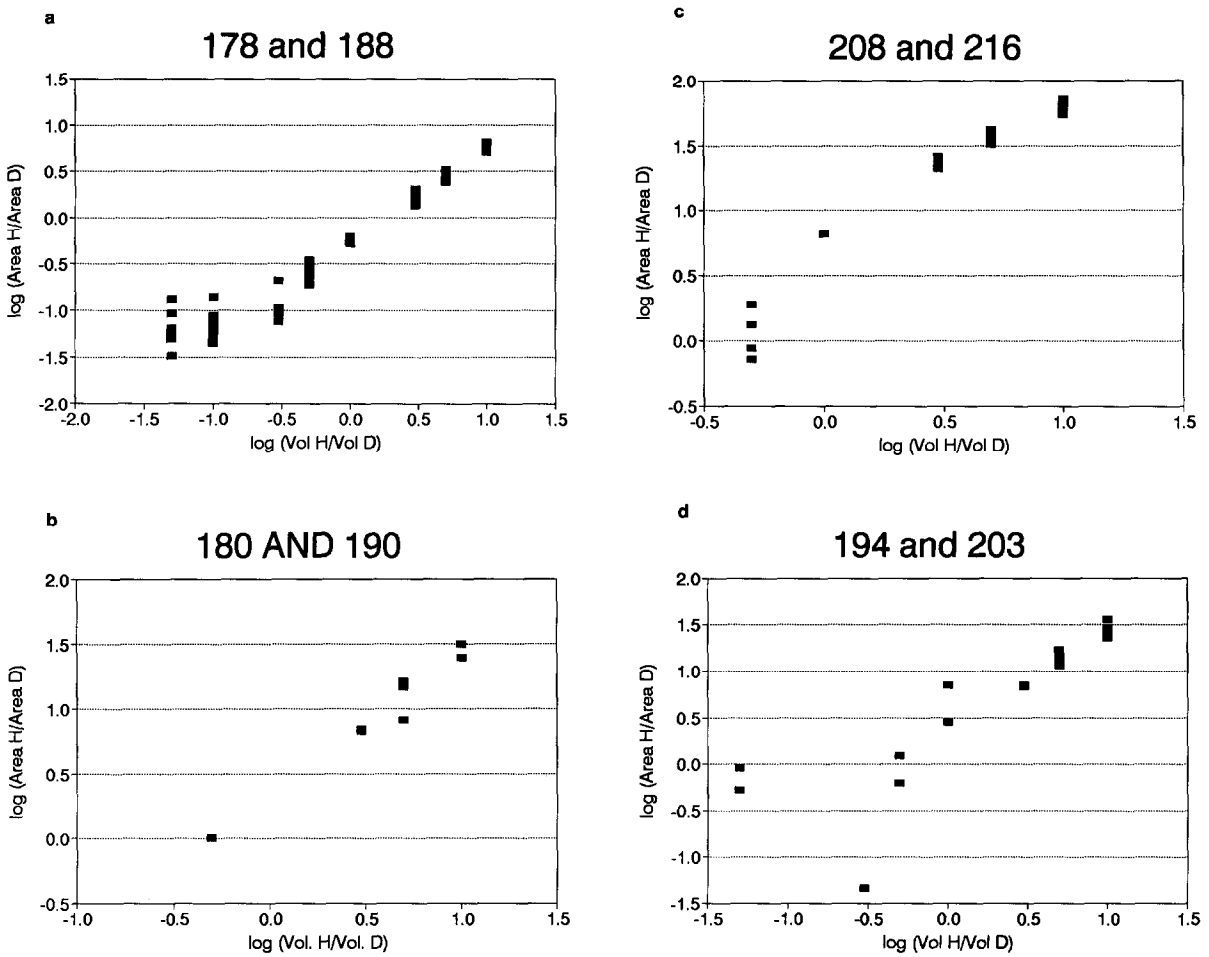

Fig. 3. Relative GC–MS response relations, log(area H/area D) *versus* log(volume H/volume D), with concentration of deuterated species constant for selected eluates including (a) anthracenes, (b) likely 9,10-dihydroanthracenes, (c) likely anthraquinones and (d) possibly 9-anthracenones.

roanthracene (M.W. = 190 u). Similarly, an eluate at 32.8 min showed mass spectra like 9,10-anthracenedione (M.W. = 208 u) (anthraquinone) with the corresponding octadeuteroanthraquinone (M.W. = 216 u) eluting at 32.6 min. Another eluate, at 31.9 min, was tentatively identified as 9-anthracenone (M.W. = 194 u) with its corresponding 9-[$^2H_9$]anthracenone (M.W. = 203 u) eluting at 31.8 min. Derivations could be done for improved identifications but may not be required for dual-isotope comparisons discussed herein. Other photolysis product eluates were present, but significant peak overlaps and low ion currents precluded confident accumulations of mass spectra for them, and corresponding SID measurements for them were inconclusive.

All four selected eluate pairs showed constant relative sensitivities, *i.e.* linear relative response *versus* relative concentration relations: linear log/log relations with slope = 1, over nearly three orders of magnitude of relative concentrations (see Fig. 3). All were calculated relative to responses from eluates from the reproduced 100 μl of [$^2H_{10}$]anthracenic photolysis product solution diluted to 1.50 ml with methanol and anthracene product solution, with none of the concentrations known. Consequently, the respective measured deuterated eluates derived from the [$^2H_{10}$]anthracene reference reaction system may serve as appropriate internal standards for calculations of relative concentrations, as described above for R and U ratios.

Uncertainties in easily measured relative responses for specified relative concentrations typically varied between 6% (R.S.D.) and 12% (R.S.D.), but became greater at low concentrations of anthracenic substances near the limits of reliable measurement, as expected. Corresponding expected uncertainties for R ratios would approximate 10–15% (R.S.D.) and for U ratios approximately 15–25% (R.S.D.). Consequently, for relative responses which can be measured with typical reasonable precisions, *e.g.* ± 5–10% R.S.D., products formed in reactions may be compared with good reliability without determining their actual concentrations, sometimes without their identifications, via use of appropriate isotopically labeled substances generated by a reference reaction.

Use of the dual-isotope GC–MS procedures discussed above have potential for dramatically improving statistical comparisons between reaction product profiles in experiments. This is mainly due to the theoretically valid predictability for the R and U ratios, *i.e.* $R = 1$ or $U = 1$ for the null hypothesis. Also, because the reproducibly added reference substances may be used as internal standards, contributions due to uncertainties in recoveries, volume measurements, etc. can be compensated, resulting in the small standard deviations for corresponding R and U ratios, as indicated above.

ACKNOWLEDGEMENT

We thank the Petroleum Research Foundation for support of this work via Grant PRF 25307-B5.

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