Journal of Chromatography, 639 (1993) 215–220
Elsevier Science Publishers B.V., Amsterdam

CHROM. 24 986

Quantitative comparisons using gas chromatography–mass spectrometry and dual-isotope techniques for detection of isotope and impurity interferences

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(First received September 11th, 1992; revised manuscript received February 17th, 1993)

ABSTRACT

Dual-isotope methods for special quantitative comparisons using GC–MS are evaluated for assessing interferences in reaction experiments. The procedure is suitable for detecting interferences from impurities in reactants, errors due to isotope exchange and effects of kinetic differences between labeled and non-labeled forms. Combined reaction of both isotopic forms of test compounds in the same reaction system, followed by concurrent pretreatment and GC–MS measurements, allow for special ratios to be tested statistically to determine if the interferences are significant.

The comparisons may be relatively unaffected by large uncertainties in pretreatment and measurement steps and thus may yield greatly improved results relative to corresponding conventional assessments. Moreover, concentrations of the individual eluates need not be known and eluate identifications may not be required for proper use of this approach.

INTRODUCTION

Dual-isotope methods can be used for quantitative analyses or comparisons [1–4]. Radioactive isotopes are used in dual-label studies to take advantage of the good selectivity and high sensitivity of radiometric measurements, but are typically restricted to only special procedures, partly due to potential health hazards or regulations. Alternatively, non-radioactive isotopic labels may be preferred, and mass spectrometry (MS) measurements may be useful with them if sufficient mass discrimination and sensitivities may be attained.

Dual-isotope MS methods which mimic isotope dilution, using equilibrated mixtures of isotopically labeled target compounds have been used for years [5] and have been adapted to important environmental determinations [6]. A known amount of that analyte's selected isotopically labeled form is added to samples before pretreatment and measurement and the two analyte forms thereby undergo identical treatment. An appropriate subsample is then analyzed by GC–MS with each analyte and its corresponding isotopically labeled form measured via characteristic m/z values.

GC–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 [7]. Variations in extraction efficiencies, variable losses during solvent volume

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0021-9673/93/$06.00

reductions and other factors can be partially compensated by traditional recovery standard and internal standard techniques [8], sometimes using added compounds which are isotopically labeled but otherwise identical to target analytes [5,6]. Consequently, those techniques require both availability and careful characterization of appropriate reference materials for *every* measured component. Unfortunately, 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 internal standard 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 [2–4].

In previous work, compounds labeled with two different radioactive isotopes were used with high performance liquid chromatography (HPLC) for special dual-label procedures which mimic multiple internal standard methods [1–4,9,10]. The dual-label approaches allow for compensation for: (a) variations in extraction efficiencies, (b) variable losses during concentration of extracts, (c) imprecisions of volume measurements, and (d) uncertainties in specific activities of reactant compounds and reaction products. Moreover, the procedures may greatly obviate difficulties caused by unavailability of pure reference compounds. For special cases [3,10] dual-isotope GC–MS procedures may be used for quantitative comparisons to evaluate: (a) effects of impurities on reaction product formation experiments, (b) effects of isotope exchange upon those experiments, and (c) effects of kinetic differences between the forms which may be caused by their different respective masses. Moreover, theoretically valid comparisons which may be tested statistically are allowed by those methods and show dramatic increases in quality of results when compared to conventional procedures [4,10]. The dual-label techniques are powerful for comparing reaction efficiencies for multiple pathway reactions [2,4] and for assessing complications caused by impurities or isotope effects [3,10].

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

THEORY

A main advantage of the dual-isotope procedures for GC–MS measurements for assessing isotope and impurity effects is that special ratios may yield well-defined, theoretically predictable results which can be tested statistically [2–4,10]: one may concurrently react in the same solution both an isotopically labeled (X-labeled) test compound and the same compound which is unlabeled, (herein called Y-labeled) with perhaps one or both being of unknown concentration. If the two differently labeled reactant compounds are chemically identical, their respective reaction products also typically will be identical, except for their respective molecular masses; this equivalence is a reasonable assumption unless isotope exchange occurs during reaction, or if kinetics are affected as a result of differences in isotopic masses. Moreover, if the reaction products are pretreated together in the same solution prior to measurement, the pretreatments will typically have equivalent extraction/concentration/dilution efficiencies, E, for the two forms of each reaction product i, *i.e.*, $E_{X,i} = E_{Y,i}$. Thus, if V_a is the volume of the pretreated sample solution derived from the reaction mixture, V_{ss} is the volume of the prepared subsample used for chromatographic separation, and M is the mass of specified analyte in the original sample, then $A_{X,i} = (V_{ss}/V_a)M_{X,i}E_{X,i}S_{X,i}$ and $A_{Y,i} = (V_{ss}/V_a)M_{Y,i}E_{Y,i}S_{Y,i}$, where $A_{X,i}$ and $A_{Y,i}$ are measured GC–MS areas, respectively, for the X-labeled and unlabeled forms of eluate component i from volume V_{ss}, and $S_{X,i}$ and $S_{Y,i}$ are the integrated response factors for GC–MS measurement of the m/z values characteristic for the respective measured species [11].

Intrasample comparisons

To evaluate isotope or impurity effects for reaction systems, special intrasample ratios can be defined for X *vs.* unlabeled reaction products.

For this, V_a and V_{ss} are respectively identical for both labeled and unlabeled forms of each reaction product taken together from the same sample solution. Accordingly, as $E_{X,i} = E_{Y,i}$, a ratio of respective masses can be calculated and $M_{X,i}M_{Y,i}^{-1} = (A_{X,i}S_{Y,i})(A_{Y,i}S_{X,i})^{-1}$ for any component i. Furthermore, separated reaction products, i *vs.* j, derived from the same reaction experiment can then be compared.

$$Q_{ij} = (M_{X,i}M_{Y,i}^{-1})(M_{X,j}M_{Y,j}^{-1})^{-1}$$
$$= (A_{X,i}A_{Y,j})(A_{Y,i}A_{X,j})^{-1}(S_{Y,i}/S_{X,i})$$
$$\times (S_{Y,j}/S_{X,j})^{-1}$$

If relative integrated response factors for components i and j are known, then Q_{ij} may be calculated from GC–MS area data in combination with those appropriate $S_{Y,i}/S_{Y,j}$ and $S_{X,i}/S_{X,j}$ ratios. Moreover, if the relative integrated response factors for both i and j are directly proportional to their relative injected amounts [11], *i.e.*, fulfill a basic assumption for common internal standard procedures, then $Q_{ij} = (A_{X,i}A_{Y,i})(A_{Y,i}A_{X,j})^{-1}$. Thus, Q_{ij} may be computed from measured GC–MS area data of the dual-isotope chromatograms only.

If there has been neither isotope exchange nor differences in kinetics, nor contributions to the specific components due to impurities and the data used are sufficiently precise, then $Q_{ij} = 1$; that is, the relative isotopic composition for the components is the same. If isotope effects or impurities cause interferences, then Q_{ij} may be significantly different than unity, and one may test Q_{ij} statistically for each i,j pair through replications and a null hypothesis that the measured reaction products are unaffected by impurities or isotope effects: If, statistically, $Q_{ij} \neq 1$ one may not accept the null hypothesis.

Intersample comparisons

Similarly, one may compare intersample Q-ratios to ascertain intersample differences due to impurities or isotope effects; for compound i, $Q_{12,i} = (M_{X,i,1}M_{Y,i,1}^{-1})(M_{X,i,2}M_{Y,i,2}^{-1})^{-1}$ for comparisons for samples 1 *vs.* 2, both resulting from reactions of identical aliquots of the same test solution containing both X- and Y-labeled reactant. Conveniently, if the relative integrated response factors do not vary, *i.e.*, $(S_{X,i,1}/S_{Y,i,1}) = (S_{X,i,2}/S_{Y,i,2})$, for those intersample comparisons, then $Q_{12,i} = (A_{X,i,1}A_{Y,i,2})(A_{X,i,2}A_{Y,i,1})^{-1}$, which can be readily calculated from GC–MS area data only and can be tested statistically for $Q_{12,i} = 1$, as above.

Likewise, intrasample comparisons can be extended to comparisons of two or more component concentration ratios relative to the same components in intersample sets of measurements, *e.g.*, $Q_{ij,1}/Q_{ij,2} = Q_{ij,12}$. This might correspond to comparing products of two reaction pathways and $Q_{ij,12} = 1$ if the reaction product profiles are unaffected by impurities or isotope effects. Again, the Q-ratio can be calculated from GC–MS area data only and replications can be tested statistically.

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. Automated MS optimizations were performed daily using provided algorithms. 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

Solutions of anthracene and [$^2H_{10}$]anthracene were prepared in methanol, $4.1 \cdot 10^{-4}$ M and $3.8 \cdot 10^{-4}$ M, respectively. Measured volumes of

the two solutions were mixed to 10 ml total volume, yielding relative concentrations, [anthracene]/[[$^2H_{10}$]anthracene], varying between 10^{-3} and 10^3. The mixed solutions were exposed to sunlight through window glass for 35 days and then stored at 0°C in darkness until used.

Four subsamples for each dilution of the methanolic reactants and their photolysis products were measured via GC–MS. Over several days' duration, 1-μl subsamples were injected into the 325°C injector, in splitless mode, with oven temperature at 60°C. The 60°C initial temperature was maintained for 5 min, then raised to 120°C at 10°C min^{-1}, held at 120°C for 2 min, raised to 160°C at 2°C min^{-1}, held at 160°C for 2 min, raised to 250°C at 5°C min^{-1} and maintained at 250°C for 2 min. The GC–MS transfer line was isothermal at 285°C.

Eluates were measured by selected-ion monitoring (SIM) unless stated otherwise. Between retention times of 15 min 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. Selected ion chromatograms were produced and integrated via provided algorithms, and statistical calculations were made by conventional techniques. For tentative eluate identifications using full m/z scanning detector currents for m/z values between 50 and 250 were measured at a rate of 2.4 scans s^{-1}, between retention times of 15 min and 45 min.

RESULTS AND DISCUSSION

Several subsamples of anthracene–[$^2H_{10}$]anthracene mixtures and their respective photolysis products were separated by GC–MS with m/z scanning. Anthracene (parent ion = 178 m/z) and [$^2H_{10}$]anthracene (parent ion = 188 m/z) were identified by their mass spectra at elution times of 27.2 min and 27.1 min, respectively. Similarly, 9,10-anthracenedione (anthraquinone, parent ion = 208 m/z) and its corresponding octadeuterated species (parent ion = 216 m/z) were tentatively identified at elution times of 35.9 min and 35.7 min, respectively. None of the identifications were confirmed by supplemental methods, as true identities need not be known for Q-ratio evaluations. Other eluates were present but showed significant peak overlaps or insufficient ion currents for reliable measurements.

The selected eluate pairs measured by SIM (see Fig. 1) fulfilled the basic relative sensitivity assumption for use with Q-ratios. They showed linear relative response *vs.* relative initial reactant concentration relations, *i.e.*, linear log/log relations with slope = 1, over approximately three orders of magnitude (see Fig. 2). These individual GC–MS measurements had good relative precisions of approximately ±10% with nonlinearities at extremes due to one of the amounts being near its limit of reliable measurement and hence showing Poisson data distributions for non-zero entries. Although the initial reactant concentrations were controlled, none of the

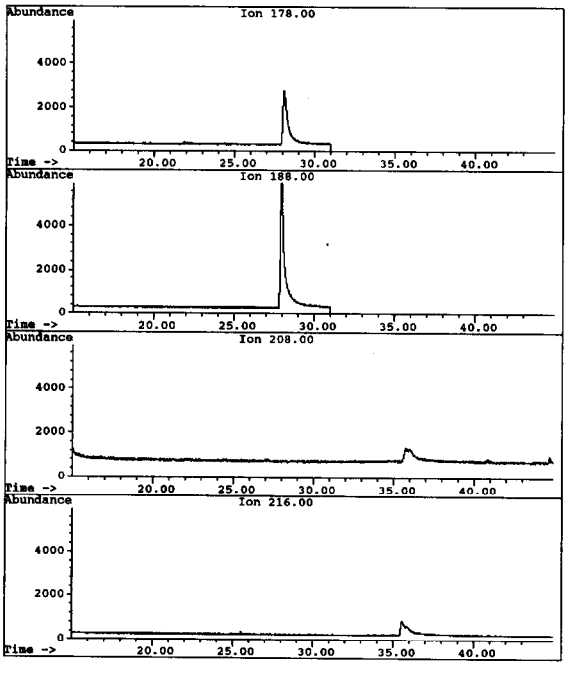

Fig. 1. Selected-ion (SIM) chromatograms for m/z values 178, 188, 208, 216 for 1-μl subsample of a photolyzed solution with [[$^2H_{10}$]anthracene]/[anthracene] = 2.0. Time in min.

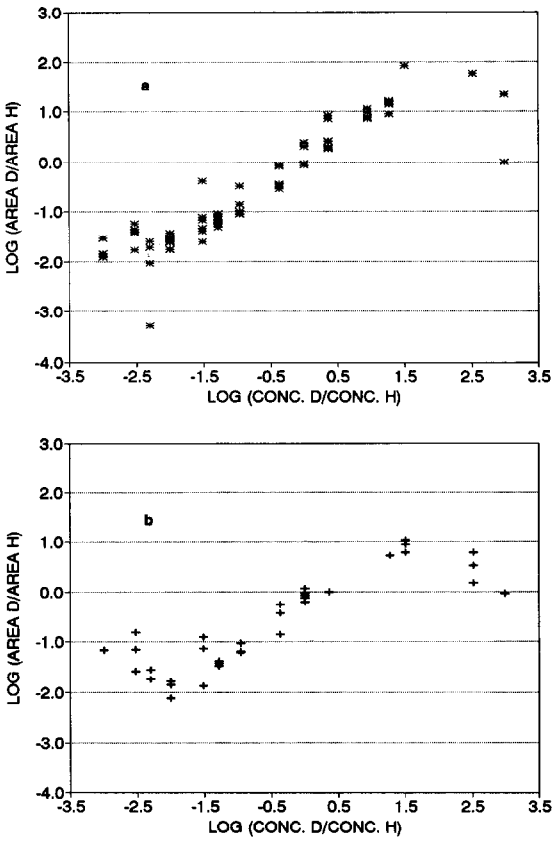

Fig. 2. Relative GC–MS areas log ($Area_{[^2H_{10}]anthracene\ product}$/ $Area_{anthracene\ product}$) *vs.* log of relative concentrations of reactants prior to reaction, pretreatment and measurements: (a) [$^2H_{10}$]anthracene/anthracene, (b) probable [$^2H_8$]anthraquinone/anthraquinone.

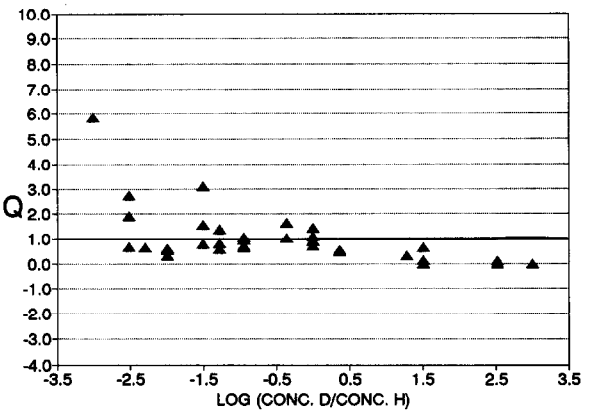

Fig. 3. *Q*-ratios *vs.* log of relative concentrations of reactants prior to reaction, pretreatment and measurements. *Q*-ratio here is $(A_{[^2H_8]anthraquinone}/A_{anthraquinone})/(A_{[^2H_{10}]anthracene}/A_{anthracene})$.

postreaction concentrations were known. However, the relative integrated responses for corresponding labeled and non-labeled species varied directly (see Fig. 2) with initial reactant concentrations, which indicates compatibility with the use of the *Q*-ratios described above.

Q-ratios for anthracenes *vs.* tentatively identified anthraquinones were not statistically different than unity (Fig. 3) over the 2 1/2 orders of magnitude, $10^{-2.0}$ to $10^{0.5}$, spanning reliable measurements for all included components. Results in that range were consistent with the theoretical prediction, *i.e.*, acceptance of the null hypothesis, for reactions in the absence of impurity interferences or isotope effects.

The *Q*-ratio methods for GC–MS described herein are compatible with eluates of unknown concentrations and perhaps of unknown identities and therefore may be used when conventional techniques such as standard additions and isotope dilution are not feasible. They use well-accepted statistical tests and are useful for comparing relative concentrations, especially for evaluating interferences in dual-isotope reaction systems. The required GC–MS assumptions are similar to those accepted for dual-isotope GC–MS procedures which mimic isotope dilution. However, use with eluates of unknown identities has the risk of improper pairing of eluates and improper selection of appropriate m/z values for measurements.

ACKNOWLEDGEMENT

We thank the Petroleum Research Foundation for support of this work with grant PRF No. 25307-B5.

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