modifications to the protein caused by processing conditions used, for example, to cleave the target protein from a larger fusion construct; or from the heterogeneity conferred on a glycoprotein by the covalently linked carbohydrate structures. Other peptides occurring at low levels in the tryptic map may arise as artifacts of the digestion process, resulting either from autoproteolysis of trypsin[38] or from nonspecific cleavages of the protein that are observed even in highly purified preparations of proteolytic enzymes. Finally, host cell proteins may also contribute to the low-level contaminants found in the peptide mixture, although in a pharmaceutical-grade protein these contaminants are expected to be present at undetectable levels.[40] Hence, the information content of the minor components of a mixture can be sufficient to warrant the development of methods, such as HPDC, for closer examination of the trace-level components of the mixture.

[40] S. E. Builder and W. S. Hancock, *Chem. Eng. Prog.* August 42 (1988).

[21] Measuring DNA Adducts by Gas Chromatography–Electron Capture–Mass Spectrometry: Trace Organic Analysis

By ROGER W. GIESE, MANASI SAHA, SAMY ABDEL-BAKY, and KARIMAN ALLAM

Introduction

This chapter presents our practical experience in method development for the determination of trace amounts of DNA adducts by gas chromatography–electron capture–mass spectrometry (GC–EC–MS). We have detected femtomole amounts of such analytes by optimizing sample preparation (involving extraction, chemical reaction, and purification steps starting with a biological sample) and low-attomole amounts of pure, derivatized standards by GC–EC–MS. Although such methodology is already useful, the concepts and techniques described should extend sample preparation to the attomole level.

In this chapter our work on chemical transformation is emphasized as part of sample preparation. This is a means to broaden the range of compounds that can be detected by GC–EC–MS. Also, our experience in operating a GC–EC–MS to achieve attomole detection limits routinely (for standards) is presented.

New ionization techniques for MS, such as electrospray (see [21] in this

volume[1]) and matrix-assisted laser desorption (see [22] in this volume[1a]), are increasing the ability of MS to analyze "nonvolatile" substances present even in aqueous samples. Less new but of continuing importance as a desorption/ionization technique in this respect is fast atom bombardment. In contrast, we are focusing on procedures in which significant chemical treatment of the sample precedes the "old technique" of GC to deliver the analyte into the MS. The desorption approaches are attractive because they can minimize sample preparation. They are also unique in their ability to achieve the direct detection of medium to high molecular weight biopolymers by MS. For trace organic analysis, however, the use of chemical steps to aid in the characterization and purification (including recovery) of smaller analytes by changing their physicochemical properties, coupled with the additional purification provided by GC (including the high purity of GC carrier gases), may be important.

DNA Adducts

DNA *adducts* result from covalent damage incurred *in vivo* by endogenous and exogenous agents. Measuring DNA adducts is of interest largely because carcinogens and mutagens (or their metabolites) tend to react covalently with DNA. Depending on many factors (e.g., adduct structure, location on the DNA, cell type, species) DNA adducts persist, are correctly repaired, or lead to mutations to different degrees. Potentially, the measurement of DNA adducts in human samples can help to assess how much of the human burden of cancer and genetic disease arises from exposure of people to DNA-reactive chemical and physical agents, or at least improve our measurement of individual exposure. High sensitivity is required, because cells contain little DNA (about 1 mg of DNA/g of wet tissue; 45 μg/ml of blood), and DNA contains relatively few adducts (e.g., 1 adduct in 10^7 or more nucleotides), which may nonetheless be biologically significant. Books and reviews are available on the meaning and measurement of DNA adducts, including the use of techniques other than GC–EC–MS for this purpose.[1b–4] These same sources also discuss other biomarkers of

[1] J. F. Banks, Jr., and C. M. Whitehouse, *Methods Enzymol.* **270,** Chap. 21, 1996.
[1a] R. C. Beavis and B. T. Chait, *Methods Enzymol.* **270,** Chap. 22, 1996.
[1b] R. C. Garner, P. B. Farmer, G. T. Steel, and A. S. Wright (eds.), "Human Carcinogen Exposure." IRL Press at Oxford University Press, Oxford, 1991.
[2] H. Bartsch, K. Hemmimki, and J. K. O'Neill (eds.), "Methods for Detecting DNA Damaging Agents in Humans: Applications in Cancer Epidemiology and Prevention." IARC, Lyon, France, 1988.
[3] M. C. Poirier and F. A. Beland, *Chem. Res. Toxicol.* **5,** 749 (1992).
[4] J. W. Groopman and P. W. Skipper, "Molecular Dosimetry and Human Cancer: Analytical, Epidemiological, and Social Considerations." CRC Press, Boca Raton, FL, 1991.

human exposure to chemicals, such as protein adducts and urinary metabolites.

Gas Chromatography–Electron Capture–Mass Spectrometry

Gas chromatography–electron capture–mass spectrometry (GC–EC–MS) is useful for the determination of electrophoric derivatives of DNA adducts. In this sensitive and specific technique, the derivatized sample is dissolved in an organic solvent and injected into a gas chromatograph, where the analyte is separated in the gas phase from many of the impurities that are present. Once the analyte elutes from the GC column, it encounters a cloud of low-energy electrons (and other reactive species including positive ions such as CH_5^+ when CH_4 is the reagent gas) in the ion source of the MS. If the analyte has an ability to capture an electron efficiently under such conditions (few compounds do), it will ionize by electron capture, forming an anionic product. The final, anionic product that is detected may be the initial one that forms (nondissociative electron capture), or a fragment that forms subsequently (dissociative electron capture). Multiple anionic fragments can also arise, depending on the structure of the analyte.[5,6]

A fused silica, bonded phase capillary GC column is employed because of its high performance in terms of chromatographic efficiency (sharp peaks), good analyte recovery (low level of analyte-destroying active sites), low bleed (the stationary phase is both cross-linked and bonded to the capillary wall), and durability. For less polar analytes, columns from different manufacturers give similar results, but a higher quality (usually more expensive) column is a wise choice for more polar analytes. This is because its level of performance will be higher when new and will degrade more slowly with use.

A moderately priced GC–EC–MS instrument can be used. Individual instruments from a given manufacturer may vary in their sensitivity, therefore the manufacturer should be requested to supply appropriate data for the particular instrument before it is shipped, e.g., whether the instrument can detect 50 amol of a representative compound. On the MS instrument in our laboratory [a Hewlett-Packard (HP, Palo Alto, CA) HP 5890A GC coupled to an HP 5988A MS], a reduced-volume, tighter source (narrow inlet hole for the GC column) was selected to enhance the sensitivity,[7] on the basis of a recommendation from the manufacturer.

[5] E. A. Stemmler and R. A. Hites, *Biomed. Environ. Mass Spectrom.* **17,** 311 (1988).

[6] A. G. Harrison, "Chemical Ionization Mass Spectrometry," 2nd Ed. CRC Press, Boca Raton, FL, 1992.

[7] S. Abdel-Baky and R. W. Giese, *Anal. Chem.* **63**(24), 2986 (1991).

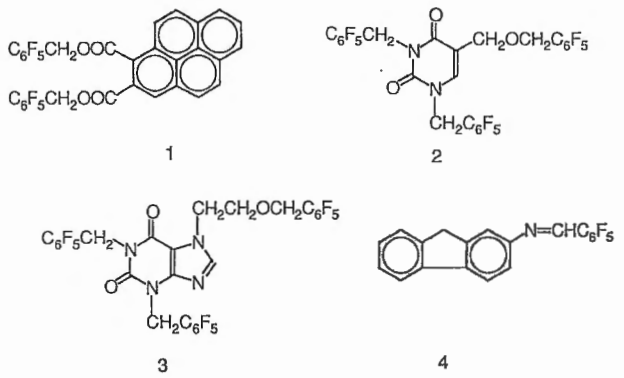


FIG. 1. Structures (**1–4**) of some high-response, single-ion electrophores related to DNA adducts. (Reprinted with permission from S. Abdel-Baky and R. W. Giese, *Anal. Chem.* **63**(24), 2986. Copyright 1991 American Chemical Society.)

High-Response, Single-Ion Electrophores

Although a variety of compounds can be detected by GC–EC–MS, the lowest detection limits result when high-response, single-ion electrophores (compounds that ionize by EC to give a single ion, or nearly so, aside from the isotope peak) are determined with selected ion monitoring. It is important for the ion to be structurally characteristic. The first examples of such compounds were demonstrated in 1978.[8] Some examples related to DNA adducts are shown in Fig. 1 (**1–4**); they all have been detected at the low-attomole level by GC–EC–MS.[7]

Not all high-response, single-ion electrophores have exactly the same sensitivity, for reasons that are not always completely clear. Two- to three-fold differences in response even of analogs are not uncommon. Differences in their susceptibility to losses at active sites in the overall GC–EC–MS system, ease of electron capture, participation in other reactions in the ion source, and inherent stability of the anion of interest (during its brief transit from the ion source to the electron multiplier detector) probably are the main reasons. Thus, one should select a single-ion electrophoric derivative for a given DNA adduct carefully, including testing at the attomole level on an aged GC column. For example, two such compounds may perform equivalently when tested as standards by GC–EC–MS down to the femtomole level, but only one may consistently give a high response at the attomole level, especially as the active sites increase on an aging GC column.

[8] D. F. Hunt and F. W. Crow, *Anal. Chem.* **50**, 1781 (1978).

Method Development

For a given adduct, one should first obtain 10 mg or more as a standard to develop a chemical procedure that converts the substance efficiently (yield ≥50%) into a high-response, single-ion electrophore. Once this is achieved, and a stable isotope internal standard is prepared, the procedure next is extended to a trace amount of the adduct as a standard. Down to about the 10-ng level, it is useful to monitor the procedure (yield, formation of side products) by high-performance liquid chromatography (HPLC) with ultraviolet (UV) detection. This reduces the workload on the GC–EC–MS; is rapid and convenient for optimizing the procedure; can reveal nonvolatile starting material, intermediates, and side products; and avoids the introduction of impure samples (early method development) into the GC–EC–MS.

Below the 10-ng level, one switches to GC–EC–MS (GC with electron capture detection can also be used conveniently down to roughly the midpicogram level, but with less convenience due to interference). Near the 10-ng level the reaction conditions (e.g., quantities of reagents and solvents employed in the procedure) are determined, and only dilutions of the analyte are required in proceeding to lower analyte levels. Further reduction in the amounts of the reagents yields concentrations that afford unacceptably long reaction times, or fail to form product efficiently for other reasons. The amount of each reagent and solvent should be minimized because every chemical contains impurities. Impurities increase in chemical reactions; and greater impurity early in a method generally leads to more extraneous peaks later in the chromatogram produced by the GC–EC–MS. Finally, in the method the sequential chemical steps should be compatible, e.g., the sample can simply be evaporated between any two chemical reactions, allowing the reactions to be conducted tandemly in the same vial.

As the amount of standard analyte is progressively reduced to the levels of interest anticipated in real samples, interference and losses will be encountered. Making a dilution of the analyte by only a factor of 10 at lower levels can increase these problems significantly. To address these difficulties, first test the last steps in the procedure, even the final evaporation step, with standards. Conduct each chemical reaction step as a blank (no analyte), and then spike in a known amount of authentic product at the end of the reaction. This approach determines whether it is the yield (from a chemical reaction) or recovery of analyte from a given reaction that is causing a loss. Unfortunately, the "tuning" largely needs to be done at the lower analyte level, because at higher analyte levels "everything works."

The later transition in method development to analyte-spiked, standard DNA, and then to real samples, means that the analyte will no longer be pure, and the impurities may consume some of the reagents being employed.

This can lead to a demand for higher amounts of the reagents than were effective for a pure standard of the analyte. Thus, one may elect to delay completed tuning of the procedure, especially the amounts of the reagents and column capacities, until real samples are tested.

DNA and Adduct Isolation

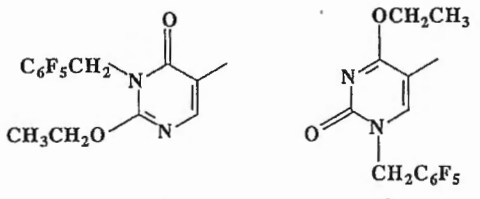
Genomic DNA is a relatively unique substance (e.g., large size, high negative charge and associated water solubility, resistance to enzymes other than deoxynucleases), so it is relatively easy to isolate from a biological sample, at least in moderate yield and purity. The common procedure involving protein and ribonucleic acid digestion/phenol–chloroform extraction/alcohol precipitation[9] is frequently selected. Many subtle variations of this method can be found in the literature. An automated instrument for this purpose is available from Applied Biosystems Inc. (Foster City, CA). Additional purification by ultracentrifugation in a cesium chloride gradient or by other methods is sometimes also performed.

Commercially available kits relying on extraction of the DNA onto a solid-phase packing (e.g., anion exchanger) are available, but the incomplete disclosure of the kit components by a manufacturer can discourage its use, because troubleshooting and tuning are then more difficult. A general problem with many procedures for DNA extraction is that the recovery of DNA declines with smaller samples.

Both chemical and enzymatic methods have been employed, in conjunction with liquid chromatography, to isolate adducts from the purified DNA. The choice is dictated largely by the special physicochemical properties of the adduct of interest, and the techniques most accessible to the laboratory. Strong acid hydrolysis degrades DNA to nucleobases. Moderate acid hydrolysis primarily depurinates the DNA. The latter depends on protonation at the N-7 position of guanine and adenine, which imposes a positive charge on the purine that makes the associated glycosidic bond susceptible to hydrolysis. Mild acid hydrolysis has been used to liberate benzo[*a*]pyrene-7,8,9,10-tetrahydrotetrol from its attachment at least to the N-2 position on guanine (see below). So-called "neutral thermal hydrolysis" (heating in buffer near pH 7) releases N^7-guanine and N^7-adenine alkyl adducts (because of the positive charge at the N-7 position) along with a small fraction of the normal purines.[10] Enymatic hydrolysis can be used to convert the DNA to nucleotides or nucleosides.

It is attractive to isolate the adduct from the bulk of normal DNA

[9] D. M. Wallace, *Methods Enzymol.* **152,** 33 (1987).
[10] D. N. Mhaskar, J. M. Raber, and S. M. D'Ambrosio, *Anal. Biochem.* **125,** 74 (1982).



5 6

FIG. 2. Structures of pentafluorobenzyl derivatives of O^2-ethylthymine (**5**) and O^4-ethylthymine (**6**).

monomers (derived from the hydrolyzed DNA sample) by solid-phase extraction on a short column, assuming that a column that more or less discriminates the adduct can be set up. This isolation procedure is convenient, can keep related adducts together (they can be resolved later by GC–EC–MS), and avoids two potential problems, which may be interrelated when HPLC instead is used at this stage: trace adduct retention times can change with column use, and contaminants can accumulate (including carryover of analyte) in the HPLC system. In one case it was found that 99.9% of the carryover in the HPLC system took place in the injector.[11] These two problems can be tedious to control because the amounts of the adducts tend to be too small for on-line detection. Nevertheless, in spite of these concerns about using HPLC when it has been used early in the overall procedure, it may be necessary to select this approach whenever solid-phase extraction falls short in purifying the adduct. It can be especially attractive to subject the hydrolyzed DNA sample to an antibody affinity column to extract the tiny amount of adduct away from the large excess of normal DNA monomers (nucleotides, nucleosides, or nucleobases). This method will be used increasingly as antibodies become more readily available, and adducts emerge that deserve extensive monitoring.

Chemical Transformation/Derivatization

Overview

The semipurified adduct is next converted into a single-ion electrophore. For some DNA adducts, such as certain alkyl derivatives of the nucleobases, electrophoric derivatization alone can accomplish this. Figure 2 shows such derivatives for O^2-ethylthymine and O^4-ethylthymine (**5** and **6**, respec-

[11] M. Saha and R. W. Giese, *J. Chromatogr.* **631,** 161 (1993).

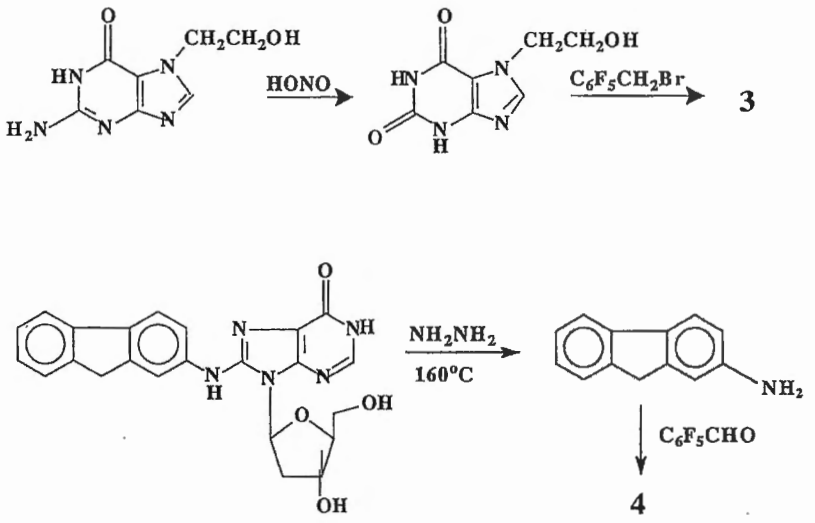


FIG. 3. Chemical transformation and electrophoric derivatization reactions. (Adapted from Allam *et al.*[13] and Bakthavachalam *et al.*[14])

tively).[12] These derivatives, possessing a pentafluorobenzyl moiety on a nucleobase nitrogen, tend to undergo efficient, dissociative electron capture, forming a pentafluorobenzyl radical and a nucleobase anion. Conveniently, the adducting alkyl moiety (the damage of interest on the DNA) reduces the degree of derivatization required for such adducts by inherently masking one of the other ionizable sites on the nucleobase. This type of derivative is obtained by reacting the alkyl nucleobase adduct with pentafluorobenzyl bromide in the presence of a base such as potassium carbonate (e.g., two-phase reaction with solid potassium carbonate in acetonitrile), triethylamine, or potassium hydroxide (e.g., phase transfer reaction involving a lower phase of dichloromethane and an upper phase of 1 *N* aqueous potassium hydroxide; see below).

When derivatization alone cannot convert a DNA adduct into a single-ion electrophore, chemical transformation is employed prior to derivatization. Two examples are shown in Fig. 3[13,14]: nitrous acid hydrolysis of an N^7-guanine adduct of ethylene oxide to a corresponding xanthine, and hydrazinolysis of an N^7-guanine adduct of 2-aminofluorene to liberate the

[12] M. Saha, G. M. Kresbach, R. W. Giese, R. S. Annan, and P. Vouros, *Biomed. Environ. Mass Spectrom.* **18,** 958 (1989).
[13] K. Allam, M. Saha, and R. W. Giese, *J. Chromatogr.* **499,** 571 (1990).
[14] J. Bakthavachalam, S. Abdel-Baky, and R. W. Giese, *J. Chromatogr.* **538,** 447 (1991).

2-aminofluorene moiety. A third example, mild acid hydrolysis/superoxide oxidation of an N^2-guanine benzo[*a*]pyrenediol epoxide adduct to liberate 2,3-pyrenedicarboxylic acid, is shown as part of an analytical scheme in Fig. 4. Also indicated in Fig. 3 is the subsequent electrophoric derivatization of the chemical transformation products to single-ion electrophores **3** and **4**. Not all single-ion electrophores are easy to obtain and purify in high yield, especially at a trace level, thus alternative chemical transformation/derivatization procedures sometimes should be explored.

Practical Considerations for Sample Preparation

We have chosen to employ standard, commercially available laboratory ware wherever possible. Conical vials are employed for the reactions, and subjected to extensive cleaning between procedures, including the vigorous use of a test tube brush, at least initially, to dislodge any microscopic particles that may be present on the vial walls. These particles are visible with a handheld microscope and can be present in the vials as received from the manufacturer.[15]

Plastics including polytetrafluoroethylene (PTFE) may need to be avoided. Not only can plastics emit volatile contaminants (e.g., residual plasticizers), but they can cause losses. We have observed analyte losses on PTFE-coated stirring bars. The use of a glass-stoppered vs plastic-capped vial for a trace chemical reaction can reduce the level of extraneous peaks observed by GC–EC–MS.[15]

Dilutions are performed with disposable glass capillary micropipettes (10- to 100-μl size) using a Drummond Captrol III (Drummond Scientific Co., Broomall, PA) to load and dispense aqueous samples, and gravity for samples in organic solvents. For both kinds of sample solutions, it is generally necessary to dispel the residual volume from the micropepette with a pulse of air. For this purpose, we either attach the plastic mouthpiece that comes with the micropipette to the dispensing end of a Pasteur pipette fitted with a rubber bulb, or use residual air in the Captrol III.

Reaction volumes in the 30- to 100-μl range are convenient, permit some evaporation during the reaction, and are adequate to dissolve prior evaporation residues. Reactions that involve mild conditions (no heating and no strong nucleophiles or electrophiles including pH extremes) should be selected, whenever possible, in order to minimize degradation of the reaction components, and thereby reduce interferencing peaks later in the GC–EC–MS chromatogram. Even selection of a more chemically inert solvent such as toluene relative to acetonitrile can reduce interference

[15] S. Abdel-Baky, K. Allam, and R. W. Giese, *Anal. Chem.* **64**, 2882 (1992).

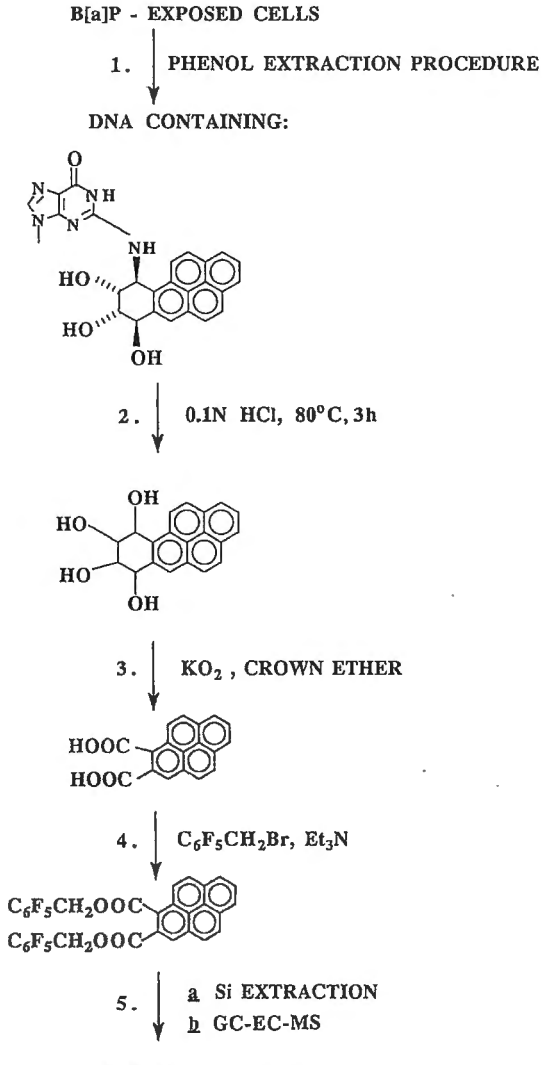


FIG. 4. Scheme for the detection of an acid-labile benzo[*a*]pyrenediol epoxide DNA adduct in cultured lymphocytes exposed to benzo[*a*]pyrene (B[*a*]P).

significantly.[16] A wider range of solvents can be used for trace vs conventional reactions because the low concentrations of the analyte and reagents reduce the need to select a solvent with good solubility properties toward the reaction components. Reactions should be selected that, at least on workup, yield no solid residues, to minimize adduct losses.

Continuous vortexing is attractive for agitating a trace chemical reaction (thereby avoiding a problematic PTFE or glass-coated stirring bar). The continuous vortexing unit that we employ (Multi-Mixer model 4600; Lab-Line Instruments, Melrose Park, IL) can be operated in an oven up to a temperature of 50°.

For evaporation of the solvent on completion of a chemical reaction, a stream of high-quality nitrogen may be preferred (with mild heating in a heating block, as necessary) rather than a centrifugal vacuum technique. The latter procedure may contaminate the sample (including analyte carryover; we encountered this problem with compound **1**) from exposure to plastic, paint, rubber tubing, and vacuum pump oil. However, the vacuum technique is more convenient and thus it may be selected if its performance is acceptable for a given analyte.

Whether it is useful to employ trimethylsilylated vs nontreated glassware for the dilutions, reactions, and evaporations depends on the adduct, reagents, and solvents involved. We resort to trimethylsilylation only when necessary. At the conclusion of an evaporation, it has proven to be better to redissolve the trace analyte immediately rather than store it dry until the GC–EC–MS is available for injection. We have observed that a dry, trace analyte stored in the refrigerator for 1 week can largely disappear relative to an equivalent, evaporated sample that was redissolved and stored as a solution.

Attomole Gas Chromatography–Electron Capture–Mass Spectrometry

Single-ion electrophores can be detected routinely at the low-attomole level on a GC–EC–MS that is dedicated to the determination of relatively pure, low-concentration compounds. We recommend injecting into the GC–EC–MS only samples that have been highly purified (e.g., by HPLC), form invisible residues on evaporation, and contain <10 pg/μl of analyte when derived from a low-level reaction, or <5 ng/μl of pure compound in the case of scanning detection.

Ultrahigh-purity gases (99.999%; total hydrocarbons < 0.5 ppm, O_2 < 3 ppm; H_2 < 3 ppm) are used: helium carrier gas and methane reagent

[16] K. Allam, S. Abdel-Baky, and R. W. Giese, *Anal. Chem.* **64**, 238 (1992).

gas. Each gas is filtered through an Oxysorb 1 in-line system (Med-Tech Gases, Medford, MA) to remove oxygen. The black adsorbent in this glass cartridge turns brown with use, with a lifetime of about 1 year for such ultrahigh-purity gases.

A 10-μl syringe (e.g., Hamilton 710) fitted with a stainless steel plunger (not PTFE tipped at the sample–contact end), and a 7-cm polyimide-coated fused silica needle, are used for 1-μl on-column injections. We have not found it necessary to remove the polyimide coating at the sample–contact end of the needle. Sequentially, 2 μl of air, 1 μl of sample (dissolved in toluene, hexane, or acetonitrile) and 2 μl of air are drawn into the syringe, the needle is blotted between a fold of paper tissue, and a fast (1 sec) on-column injection is made. (Nitrogen instead of air in the syringe improved the response for octafluoronaphthalene, an abnormal model test compound, when a small amount of this compound was injected.[7]) The syringe is then washed by pulling up 10 μl of warm (60°) 10× toluene (from a vial sitting on the top of the warm GC), and placed in a covered box until the next injection. Whenever a more contaminated or higher concentration (≥10-fold) analyte is injected (based on the resulting GC–EC–MS chromatogram), pure solvent is injected prior to the next sample to check for sample carryover. This overall approach has avoided a carryover problem in the GC–EC–MS. About twice a year, the duckbill and spring on the on-column injector (model 114; Hewlett-Packard) needs to be replaced, when needle insertion becomes difficult.

At the end of the day, 1-μl volumes of pure solvent (e.g., toluene) are injected until the baseline reequilibrates to its prior value at the start of the day (e.g., peak height abundance in the range of 1000–2000 units). Typically, a single injection is adequate after a full day of determining femtomole-level samples, whereas about five injections may be necessary when picomole-level samples have been analyzed (scanning conditions). The methane makeup gas then is turned off. (In fact, this should be done whenever a ≥1-hr delay is anticipated for the next injection, to minimize buildup of contamination in the system.) The carrier gas head pressure is reduced from 20 to 5 psi, and the column oven temperature is left at 220° until the following morning.

After about 2 weeks, we remove 10 cm from the injection end of the 15- to 30-m (when new) capillary GC column, because at this point the peaks have increased in width about 1.2-fold. This procedure improves the column performance (less so with time) until about 4 months (400 injections) has elapsed, at which point a new column is installed. On an older column, 20 cm is removed every 2 weeks. The peak width for the adduct after 400 injections is about 3 times its initial value on a new column, and no longer improves when an initial segment of the column is removed.

While some manufacturers have claimed that bonded-phase, fused silica capillaries can be restored by solvent washing after their performance degrades, this has not been successful in our work.

Tuning the instrument is accomplished generally every 2 weeks. Friday is a good day, so that the instrument can then "bake out" over the weekend. Tuning is accomplished in four stages, the first three of which [EI (electron impact), PCI (positive chemical ionization), and NCI (negative chemical ionization)] are performed as directed by the manufacturer (except that the source temperature is maintained at 250°), selecting recommended settings for high sensitivity. We then add an additional stage, in which the settings listed in Table I are selected (including, as seen, an elevated head pressure for the GC column). With these settings, the response of single-ion electrophores (at least **1–4**) is increased about 10-fold.[7] Routinely, we keep the emission current at 300 μA, however. Additional sensitivity

TABLE I
INCREASE IN SIGNAL-TO-NOISE RATIO[a]

Type	Conditions: Conventional setting	Conditions: Higher setting	Increase in S/N[b]
Head pressure (top of column), psi	5[c]	20[d]	6.5 ± 0.50 4.8 ± 0.40 (**2**)
Ion source pressure (methane), Torr	1.0[e]	2.0	2.8 ± 0.15 4.5 ± 0.32 (**3**)
Ion source T, °C	150[f]	250	2.1 ± 0.19
Electron energy, eV (ion source)	150	240	2.1 ± 0.15
Emission current, μA	300	450	2.0 ± 0.13

[a] Values of selected conditions in GC/EC–MS are increased one at a time (compound **1** tested except where indicated). (Reprinted with permission from S. Abdel-Baky and R. W. Giese, *Anal. Chem.* **63**(24), 2987. Copyright 1991 American Chemical Society.)

[b] Selected ion monitoring measurements were made. For each measurement, the other settings were conventional, as defined. Attomole amounts injected into 1 μl of toluene as separate solutions were 781 (**1**), 1123 (**2**), and 135 (**3**). The S/N was calculated by the computer for peak height. For each pair of settings (conventional and higher), the measurements were made on the same day. The precision shown is the range for triplicate measurements.

[c] Conventional range is 5–8 psi for a 12 m × 0.2 mm (length × i.d.) column. Linear velocity for the column was 25 cm sec^{-1} at 5.0 psi (air injection).

[d] Linear velocity was 35 cm sec^{-1}.

[e] Conventional range is 0.5–1.0 torr.

[f] Conventional range is 120–180° for EC.

sometimes can be achieved by raising the voltage on the electron multiplier by 600 V (the manufacturer recommends 400 V) above the value set in the EI tuning stage.

After about 6–9 months (when 3000 V is reached for the electron multiplier in the EI tuning stage), the ion source is cleaned and the electron multiplier replaced. (At times the ion source must be cleaned more frequently, e.g., after 4 months.) Individual electron multipliers can vary in sensitivity, so the response of a standard single-ion electrophore should be tested in the GC–EC–MS (with the column and ion source in good condition) immediately on installation of a new electron multiplier. Periodically, a new electron multiplier gives a 10-fold or lower response and needs to be exchanged for another one from the manufacturer. Selecting a sensitive electron multiplier currently is a matter of trial and error. Aside from unusual problems, the sensitivity of the instrument (for a given analyte) ordinarily depends on the condition of the column, ion source, and electron multiplier.

Example I: N^2 Guanine Benzo[a]pyrenediol Epoxide Adduct

Using the scheme shown in Fig. 4, we have detected the acid-labile N^2-guanine adduct of benzo[*a*]pyrenediol epoxide in cultured human lymphocytes exposed to benzo[*a*]pyrene (B[*a*]P). As seen, the method relies on the chemical transformation of the acid-released tetrahydrotetrol of B[*a*]P with potassium superoxide (step 3). It is attractive, as presented below, that steps 2–4 take place sequentially in the same vial, helping to make the procedure convenient.

After the DNA is isolated from the cell pellet by a conventional phenol extraction procedure, it is redissolved in water in an all-glass vial,[15] (300 μg of DNA in 500 μl of water). Extraction, once each, with 500 μl of water-saturated isoamyl alcohol and ethyl acetate (the intent being to remove nonadducted B[*a*]P metabolites) is followed by evaporation to 100 μl, dilution to 450 μl with water, addition of 50 μl of 1.2 *N* HCl, and heating at 90° for 3 hr with occasional vortexing. The sample is evaporated to dryness at 60° under nitrogen, followed by addition of 100 μl of acetonitrile and reevaporation. An internal standard is added (20 μl of methanol containing 106 pg of [1,2,3,4,5,6,11,12-$^2H_8$]9,10-dihydrobenzo[*a*]pyren-7(8*H*)-one, synthesized as described[17]) followed by evaporation. Addition of 25 μl of dimethylformamide containing 260 μg each of potassium superoxide and 18-crown-6 (freshly prepared mixture) is performed, followed by continuous vortexing at room temperature for 18 hr. After adding 500 μl of

[17] W. Li, C. Sotiriou-Leventis, S. Abdel-Baky, D. Fisher, and R. W. Giese, *J. Chromatogr.* **588,** 273 (1991).

1% acetic acid, followed by evaporation, 500 μl of 10% triethylamine in toluene is added. The sample is evaporated, 50 μl of toluene containing 2 μl of pentafluorobenzyl bromide and 2.5 μl of triethylamine is added, and the vial is vortexed at 50° for 5 hr and then overnight at room temperature. After evaporation, the sample is treated with 100 μl of hexane, followed by vortexing, evaporation, and addition of 500 μl of hexane. The sample is vortexed and then loaded with a Pasteur pipette onto a silica cartridge column: 100 mg of 40-μm silica gel particles from J. T. Baker (Phillipsburg, NJ) is sandwiched in a Pasteur pipette between two 10-mg plugs of silanized glass wool, followed by gravity washing with 3 ml each of ethyl acetate and hexane before the sample is applied. The column is washed twice with 2 ml of hexane taken through the vial, and the diester product is eluted with two 1-ml washes of ethyl acetate, followed by evaporation under N_2 at 60°. To focus the product at the bottom of the vial, the sample is treated with 100 μl of acetonitrile (the sample can be stored at this point) followed by vortexing and evaporation. Just before injection of 1 μl into the GC–EC–MS, 20 μl of acetonitrile is added and the sample is vortexed.

Representative, selective ion mass chromatograms from B[*a*]P-exposed and nonexposed cells are shown in Fig. 5.[18] Peak **1** in chromatogram A ("exposed cells" in Fig. 5) corresponds to the injection of 2.5 fmol of diester product. Corresponding peak **1′** in chromatogram A ("nonexposed cells" in Fig. 5) is an interference, as discussed in more detail elsewhere.[15] Detection of the internal standard (peak **2**) in the two kinds of samples is shown in the B chromatograms. On the basis of the analysis of a control sample consisting of calf thymus DNA spiked with a known amount of authentic 7,8,9,10-benzo[*a*]pyrenetetrahydrotetrol, the overall absolute yield of adduct in the procedure, at least throughout steps 3–5 (Fig. 4), is 22%.

Example II: N^7-(2′-Hydroxyethyl)xanthine

The electrophoric derivatization and detection of N^7-(2′-hydroxyethyl)-xanthine has been achieved. This compound is a chemical transformation product of the N^7-guanine adduct of ethylene oxide, as shown previously in Fig. 3. The final product that is formed and detected, after a two-step derivatization with pentafluorobenzyl bromide, is N^1, N^3-bis(pentafluorobenzyl)-N^7-[2′-(pentafluorobenzyloxy)ethyl]xanthine (compound **3**).

N^7-(2′-Hydroxyethyl)xanthine (95 pg, 480 fmol) in 10 μl of 1 *N* HCl is evaporated in a vial under N_2. From a stock solution of tetrabutylammonium hydrogen sulfate (5 mg in 5 ml of 1*N* KOH), 50 μl (0.15 μmol) is added to the vial, with subsequent addition of 150 μl of CH_2Cl_2 and 10 μl

[18] K. Allam, S. Abdel-Baky, and R. W. Giese, *Anal. Chem.* **65,** 1723 (1993).

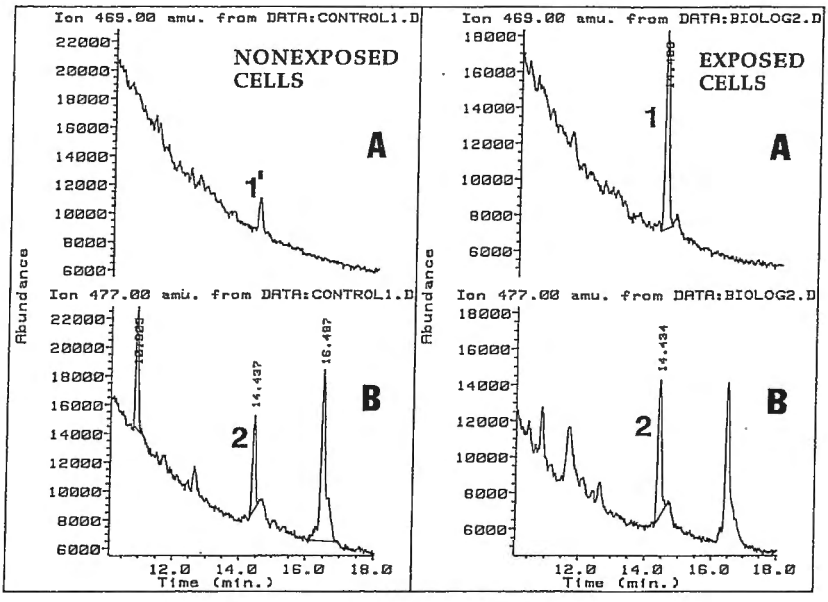


FIG. 5. Selected ion GC–EC–MS chromatograms from nonexposed and B[*a*]P-exposed cells. (A) *m*/*z* 469 to detect a diester product (**1**, which is the final product in Fig. 4) as peak **1**, and an interference (peak **1'**). (B) *m*/*z* 477 to detect a corresponding d_8-diester derived from the internal standard. GC–EC–MS: model 5988A mass spectrometer from Hewlett-Packard (Palo Alto, CA) fitted with a chemical ionization detector and connected to a Hewlett-Packard 5890 Series II gas chromatograph via a capillary interface kept at 290°. The capillary GC column (25-m length, 0.22-mm i.d., 0.1-μm film thickness, Ultra 1 from Hewlett-Packard) was temperature programmed from 140° immediately after on-column injection up to 290° at 70° min^{-1} and held for 13 min. Carrier gas, helium (20 psi); CI gas, methane (2 Torr). (Reprinted with permission from K. Allam *et al. Anal. Chem.* **65**, 1723. Copyright 1993 American Chemical Society.)

(0.065 μmol) of pentafluorobenzyl bromide. The reaction mixture is stirred for 20 hr at room temperature and the residual CH_2Cl_2 is slowly evaporated under N_2. Fifty microliters of H_2O and 150 μl of ethyl acetate are added, and the organic layer, after vortexing and centrifugation, is collected. Three more 150-μl ethyl acetate extractions are performed, and the combined organic layer is evaporated under N_2, redissolved in 50 μl of hexane : ethyl acetate, 1 : 1 (v/v), and applied to a Pasteur pipette column containing 200 mg of silica gel retained by silanized glass wool. This column had been prewashed (gravity flow) with 1 ml each of ethyl acetate and hexane. After washing with 4 ml of hexane and 8 ml of hexane : ethyl acetate, 90 : 10 (v/v), the product (**3**) is eluted with 2 ml of ethyl acetate, redissolved in 50

μl of toluene, and 1 μl is injected into the GC–EC–MS. The representative chromatogram shown in Fig. 6b[19] is obtained, corresponding to a yield of 56 ± 15% (n = 4). In Fig. 6a is shown a chromatogram from a blank reaction (no analyte).

Future

One of the attractive features of chemical transformation prior to GC–EC–MS for the detection of DNA adducts is that a given method, in principle, can be applied to an entire class of adducts. For example, the method presented above for the N^2-guanine adduct of benzo[*a*]pyrenediol epoxide is anticipated to detect diol epoxide polyaromatic hydrocarbon (PAH) DNA adducts in general,[20] including unknowns. Toward this goal, the described method for the tetrahydrotrol of B[*a*]P was found to detect the model compound chrysene-1,4-quinone.[20] Because the ions from the chemical transformation products of unknown diol epoxide PAH DNA adducts initially would be unknowns (making it impractical to use selected ion monitoring for their first-time detection), it would be attractive to use an MS instrument that simultaneously (essentially) detects many ions with high sensitivity, such as one equipped with an array detector.[21] Fourier transform ion cyclotron resonance and ion trap MS also are of interest in this regard. Once an ion (as a certain m/z value) is revealed with such equipment for an unknown, the compound then can be detected subsequently with high sensitivity by relying on selected ion monitoring on less expensive equipment. The ability to detect the adduct specifically, in turn, makes it possible to begin exploring the biological significance of the adduct even as an unknown, which in turn might trigger an interest in scaling it up for structural characterization.

We believe that GC–EC–MS methodology will be used increasingly in the trace detection of DNA adducts, and also for trace analytes in other areas of biomedical science. Toward this goal, it is important to continue making advances in the understanding and control over losses and interference during trace sample preparation. These problems can be best overcome once their mechanisms are revealed. Also, additional chemical transformation procedures are needed to bring a broader variety of trace analytes into the range of GC–EC–MS. With such advances, the high sensitivity and specificity of GC–EC–MS can be utilized more easily and generally in trace organic analysis.

[19] M. Saha and R. W. Giese, *J. Chromatogr.* **629,** 35 (1993).
[20] C. Sotiriou-Leventis, W. Li, and R. W. Giese, *J. Org. Chem.* **55,** 2159 (1990).
[21] J. A. Hill, J. E. Biller, S. A. Martin, K. Biemann, K. Yoshidome, and K. Sato, *Int. J. Mass Spectrom. Ion Process.* **92,** 211 (1989).

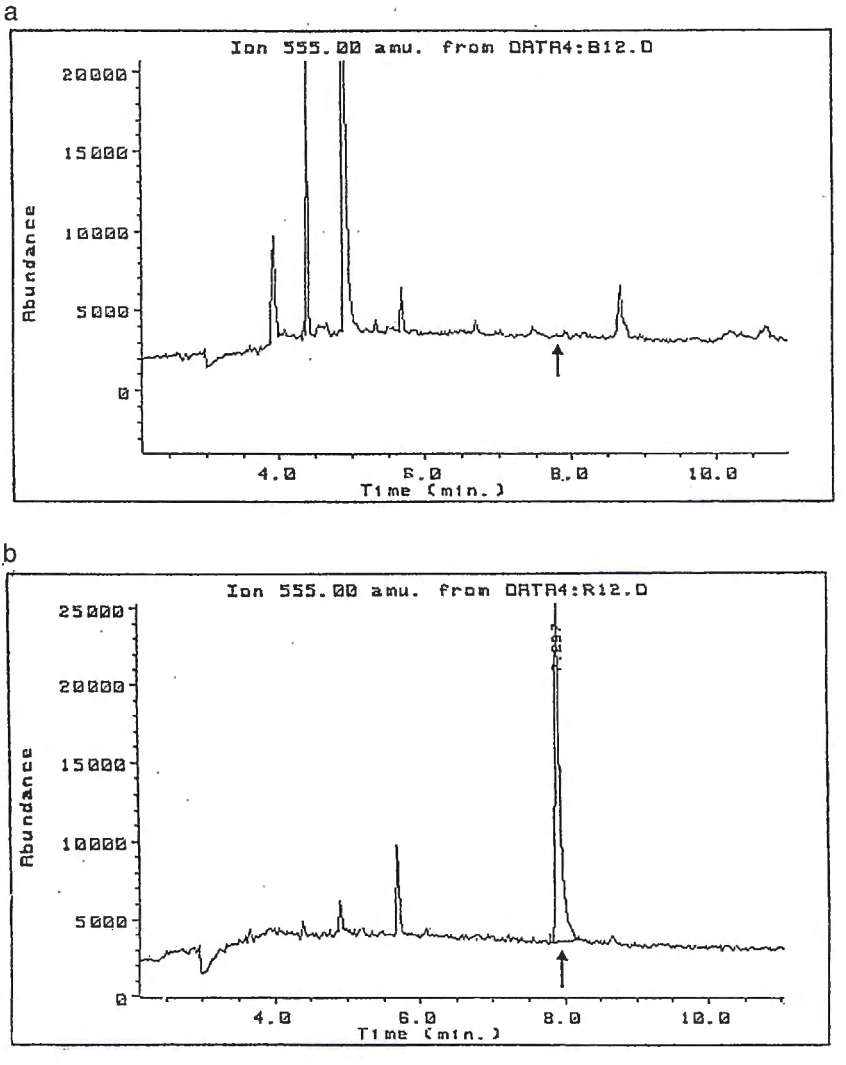


FIG. 6. Selected ion chromatograms obtained by derivatizing 0 pg (a) and 95 pg (b) of N^7-(2-hydroxyethyl)xanthine with pentafluorobenzyl bromide, followed by solid-phase extraction on a short silica column and injection of 1/50 of the final sample volume of 50 μl into a GC–EC–MS. Equipment and conditions were the same as in Fig. 5 except for the following: column, fused silica capillary, Ultra-1, 12 m, 0.2-mm i.d., 0.11-μm film thickness (Hewlett-Packard); GC oven temperatures, 120° start, then 70°/min immediately after injection up to 290° and hold for 6 min. [Reprinted with permission from M. Saha and R. W. Giese, *J. Chromatogr.* **629,** 40 (1993).]

Trace organic analysis by any technique is difficult. The tiny amount of analyte can easily fail to be detected because it is lost or inadequately purified during sample preparation. Additional purification steps tend to cause more losses. The losses and interference sometimes are no reproducible, increasing the difficulty of pinpointing their origin or learning their mechanisms. Highly purified reagents, exacting techniques, and instrumentation in top condition are often essential for success. Fortunately, each success makes it easier to achieve others, speeding up method development for related analytes. The talents and skills of a variety of scientists need to be combined to conquer the challenging frontier of trace organic analysis.

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