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Special Issue
ANALYTICAL ASPECTS OF
FORENSIC SCIENCE

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An international journal devoted to all branches of analytical chemistry

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ANALYTICA CHIMICA ACTA

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ANALYTICA CHIMICA ACTA

Preface

This special issue is devoted to Forensic Chemistry, an applied discipline directed towards the analysis and interpretation of physical evidence. Forensic Science includes the application of science to all matters of law — both criminal and civil. A necessary aspect of forensic science is the utilization of expert witnesses. An expert witness is charged to inform the trier of fact (judge or jury) in matters that require special knowledge.

Three things, taken collectively, set forensic science practice apart from many other disciplines. First, and most obviously, is the legal setting itself. But although this places special requirements on the scientific practice, it alone is a superficial distinction. A second aspect that sets forensic science apart are the types of materials that are considered. Many disciplines, for example, involve examinations of liquid blood, drugs in their legally manufactured form or drug metabolites in body fluids. But forensic scientists encounter dried blood that has been exposed to uncertain environmental insults, adultered drugs that have an uncertain and unreliable manufacturing history, and drugs in body fluids, taken as post-mortem samples, where the time and circumstances of death are in dispute. The uncertainties in sample quality and sample history are an inherent part of forensic science work. The third aspect setting forensic science practice apart is the types of problems that occur. An overview of these will be helpful to put the articles that follow in perspective.

There are three general types of problems in forensic science casework: identification, association and reconstruction. The first type of problem, identification, is common to all analytical work. In forensic chemistry the problem might be confirming the presence of an illicit drug, identifying traces of a flammable liquid in arson debris, or determining the amount of alcohol in a blood specimen. More often forensic science analyses involve development of associative evidence. Two samples are to be tested for the possibility of a common origin. Typically there is one larger sample from a known source and there is a smaller (often microscopic) sample that is of questioned origin. A comparison begins with characterization of the samples and their identification. The samples must first share the same *class characteristics*. These are more general properties that define a class of materials and are often those that are specified through manufacture or by species identifications. The comparison then extends, insofar as possible, towards *individualization*. Characteristics of the sample that might make the association more specific are sought. Thus, for example, trace elemental profiles, ratios of hydrocarbon mixtures, or distributions of particles are considered and evaluated for the degree of individuality that they contain. The specific form, size and appearance of a sample is just as critical as its chemical character and so there is a strong emphasis on chemical microscopy — where critical observations of physical and optical properties contribute to characterization and comparison. Instrumental analytical methods must often be designed or adapted to include a very critical analysis of instrumental precision in relationship to variation within and among samples — all with

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a sensitivity to uncertain sample history, limited sample quantity and, often, poor sample quality.

The third type of problem in forensic science is the reconstruction of specific past events. At issue can be associations between people or the nature of specific actions that may have taken place. We may be interested in whether a particular person had contact with a particular object, what the force of that contact was, whether there would have been transfers of any particles or patterns, or when the contact occurred.

Although collectively, the legal setting, types of materials and types of problems set forensic science practice apart, the scientific problems are shared individually with other disciplines. In epidemiology and industrial hygiene, for example, it is essential to track down a specific source, and in archeology and astronomy one must infer aspects of specific past events. In many aspects of analytical chemistry, samples can be limited and of uncertain past history. What we see emphasized in forensic science, however, is the specificity of characterization and a particular interest in judging which characteristics of a substance go beyond the step of classification and approach individualization. Combined with this is the necessity that methods be robust to all conceivable (unknown) environmental insults and the certainty that this aspect of the methods will receive careful scrutiny by the legal profession.

In the professional practice of forensic science, the potential value of samples as legal evidence must first be recognized. Examiners must then locate and collect these samples. Sampling must include methods that will preserve the physical state, and often the distribution of the sample on a substrate. Control samples must be taken to estimate, as well as possible, background levels, contamination, and sample condition. The samples must then be evaluated and appropriate tests must be chosen. Often these will be adapted to specific case circumstances. After these analyses are conducted, interpretation of the results often depends on the examiner's past experience with the specific type of material at issue. Directed background investigations may also be necessary. Lastly, and of critical importance, is the necessary communication of results to the non-scientific audience: police investigators, attorneys, and ultimately, to a judge or jury.

The articles in this special issue include many aspects of analytical chemistry where sample size or condition makes the method of particular interest to a forensic chemist. In most of the contributions you will see an explicit effort to separate the identification of a material in a class from the further characterization of a material that tends toward individualization. Throughout, you will see a sensitivity to the uncertain sample condition that dictates the choice of methodology.

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Studies on DNA polymorphisms in human bone and soft tissues

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Abstract

The determination of genetic characteristics in human bone and soft tissues is of interest both to forensic scientists and to physical anthropologists, although for different purposes. The application of genetic typing from human remains to anthropological and forensic identification problems is briefly reviewed. DNA analyses of ancient and modern human remains by Southern blot (RFLP) and amplification (PCR) techniques are reviewed. The results of studies on the effects of exposing bone and soft tissues to different environmental conditions for defined time periods on subsequent DNA typing are presented. Bone specimens were exposed to three temperatures and soil under dry and humid conditions, and a complete series of soft tissues were exposed to dry and moist air and to salt water, for periods of 1–9 months.

Keywords: Bone DNA; DNA; Deoxyribonucleic acid; Forensic DNA analysis; Forensic science; Human Identification; Molecular archaeology; Tissue DNA

Interest in determining genetic characteristics in human tissues other than blood is almost as old as ABO blood groups, the first genetic marker system, discovered at the turn of the century [1]. Development of methods for ABO typing in tissues has been the subject of continuing investigations by physical anthropologists and by medicolegal specialists for many years [2–8], and continues to the present day [9,10].

Use of bone or soft tissue ABO typing results to help answer anthropological questions has been called "paleoserology," and this literature was thoroughly reviewed by Borgonini Tarli [11,12]. We recently reviewed both anthropological and medicolegal work on bone and soft tissue genetic markers [13].

The development of DNA analysis techniques and their application to the forensic analysis of biological evidence have brought a new dimension to the use of genetic marker analysis in bone and soft tissues for human identification: the possibility of using DNA types as a means of identifying human remains that cannot be identified in some other way.

Positive identification of human remains has long been an important goal in civilized societies for both personal and legal reasons. Notification of next of kin allows them to provide the deceased an appropriate interment. Moreover, there

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are various legal matters that can be settled only after a legally valid certification of death. In criminal cases, positive identification is required in order to help establish corpus delicti, and aid in the investigation.

Methods used to identify human remains depend on the circumstances and the condition of the remains; they include direct facial identification, fingerprint matching, and dental matching, anthropological analysis, identification of clothing and belongings, and medical findings at autopsy. In many situations, however, the positive identification methods are not useful because of extensive putrefaction or destruction of the remains, because appropriate pre-mortem records are unavailable or cannot be located, or because the remains lack identifying features or markings due to natural or intentional destruction. Under these circumstances, less direct methods must be used to assist in the identification. In some situations, genetic marker analysis can be the only method available for assisting identification (or excluding possible misidentities).

Jeffreys' description of hypervariable regions of human DNA detected by two major multilocus probes [14], and the further showing that these probes were capable of virtually individualizing human DNA [15], marked the beginning of widespread interest in DNA analysis by forensic scientists. The potential applications of DNA typing to the resolution of disputed parentage and other affiliation problems [16,17] and to the individualization of blood and body fluids were immediately recognized [16,18]. This kind of DNA analysis detects variable numbers of tandem repeats (VNTR) polymorphism within sequences of repetitive, noncoding regions in the human genome [19–21]. Usually called "restriction fragment length polymorphism" (RFLP) analysis, it uses specific, labeled DNA probes to detect VNTRs in restriction endonuclease-digested human DNA that has been separated and immobilized on nylon membranes. Exploitation of the RFLP technique for forensic identification, particularly using single locus probes, has proceeded quickly [22–29].

The polymerase chain reaction (PCR) (sometimes called "amplification") is another method of DNA analysis that enables the primer-directed replication of millions of copies of a specific DNA sequence in vitro [30,31]. The specific sequence to be amplified is defined by the oligonucleotide primers that have sequences complementary to sequences flanking the specific region of interest. PCR design thus requires information about the base sequences flanking the specific region that is to be amplified. PCR takes advantage of thermostable DNA polymerases. The DNA polymerase from *Thermophilus aquaticus* [32,33], commonly called "Taq," is most widely used, but others, such as the enzyme from *Thermus flavis* [34,35] (commonly called "Replinase"), have been used. PCR techniques have been developed for a variety of genetic and clinical diagnostic applications [36–47].

Many specimens submitted for forensic DNA analysis contain limited quantities of DNA, and/or the DNA in the specimens may be degraded.

It is thus not always possible to obtain RFLP results, because the recovery of a certain minimal quantity of relatively undegraded DNA is required. These limitations have prompted the interest of forensic scientists in the applicability of PCR typing procedures [48]. DNA analysis based on the PCR may involve the amplification of regions exhibiting either sequence or length polymorphisms.

The most refined PCR procedure applicable to forensic identification thus far involves a region of sequence polymorphism. It utilizes specific primers and allele-specific oligonucleotide (ASO) probes to detect the types at the HLA-DQα locus [49,50]. HLA-DQα typing has been applied to blood and seminal stains [51–53] and to hair roots [54], and has been subject to extensive validation studies for forensic applications [55,56]. Other coding loci that have been studied and typed using PCR techniques include those for GC (vitamin D binding protein) [57] and ABO blood groups [58,59]. Here, the subtle differences between allele sequences were detected using differential restriction enzyme digestion.

The PCR has also been used to type several VNTR loci by amplifying the repeat region sequences, then characterizing the PCR products

by size following electrophoresis on agarose or polyacrylamide gels and visualization with ethidium bromide or silver staining [60]. Three loci exhibiting length polymorphism have been particularly studied in this respect: 3'APOB [61–64], D17S30 (sometimes referred to as D17S5) [65–67] and D1S80 (sometimes referred to as D1S58) [68–71].

In addition to its application to typing polymorphic DNA loci, PCR can be used to amplify specific sex chromosomes sequences to gain information about the sex of origin of a specimen. The centromeric regions of most human chromosomes are characterized by tandemly repeated arrays of satellite DNA [72], some of which are chromosome-specific. Some of these so-called alphoid repeats have been characterized and sequenced on the X and Y chromosomes [73–77], providing the information necessary to design PCR primers.

Witt and Erickson used these sequences to design primers for the specific amplification of X and Y alphoid repeat sequences [78,79], and showed that amplification of DNA from blood and bloodstains and detection of the specific PCR products on gels can be used to determine sex of origin. We have extended these X and Y chromosome amplification studies to bone and soft tissues [80], and found additionally that the sequences were primate-specific. Thus, species as well as sex of origin information can be obtained from PCR test results.

Many of the studies applying DNA analysis techniques to bone or soft tissue were done to explore the possibility of answering anthropological or phylogenetic questions. This type of inquiry has been called "molecular archaeology" [81]. Analysis has generally been more successful with mitochondrial DNA (mtDNA) [82] than with nuclear DNA in this material, and PCR has generally been a more successful approach than molecular cloning. A 3.4 kb fragment from a 2400 year old Egyptian mummy has been cloned [83], and mtDNA from the preserved tissues of an extinct member of the horse family was cloned and sequenced [84]. However, there are many problems associated with cloning DNA from old tissues [85], including the facts that most of the DNA isolated from old tissue samples is of low average length, and that it is extensively damaged [81,86, 87]. Because of the nature of the damage, there is a risk that cloned sequences could differ from the originals. This problem, and the extensive degradation of DNA from old sources, tend to make PCR a preferable method. There are a number of reports on the amplification of particular sequences of old to ancient DNA. Mitochondrial sequences from a 7000 year old human brain recovered in Florida [88], from human bones 300 to 5500 years old recovered in England [89], from human teeth and bones 150 to 6000 years old recovered in France [90] and in Japan [91], and various other human and animal sources [86,92], have been amplified and sometimes sequenced.

MtDNA was chosen for these studies partly because of its straightforward mother-to-child inheritance pattern, and partly because it is present in high copy number compared with most nuclear sequences of interest. Successful amplification of nuclear sequences from human bone DNA around a century old has also been reported [93,94]. The nuclear sequences that were amplified in these cases are present in multiple copies in the genome. These studies show that DNA is very stable, at least under some conditions. Preserved older or ancient tissue or bone specimens have been an important source of phylogenetic and anthropological information [95]. However, not all human remains can be expected to yield DNA that can be analyzed [96].

The stability and integrity of bone and soft tissue DNA demonstrated by the investigation of old specimens is highly encouraging for the potential medicolegal identification applications of DNA analysis. Bone (and teeth) are among the best sources of DNA from decomposed human remains [97–99]. Studies have been done on the stability, yield, and quality of DNA from postmortem tissues [100]. RFLP patterns were then generated with minisatellite probe 33.15 [14,15]. It was found that the larger restriction fragments (15–23 kb) gradually disappeared as autolysis progressed. No spurious bands were seen in any specimens, and, to the extent that bands were still present, the RFLP patterns matched in all tissues studied.

In cases where DNA typing is being used to identify human remains, there is generally no "known" DNA with which to compare the DNA isolated from bone or tissue. It is also unlikely that any pre-mortem DNA typing patterns would be available (although this situation may change as DNA type data bases are developed). Thus, at the present time, the application of DNA typing to human remains identification matters is limited to: (1) matching parts of human remains suspected of having a common origin; or (2) use in a parentage testing context to help establish identity, assuming parents or children of a missing person are available for testing.

In a recently reported case, single locus DNA probes were used to assist in identifying the decomposed body of a female homicide victim [101] using DNA isolated from the psoas muscle.

We first isolated DNA from human bone tissue, and studied the yield variation from spongy and compact forms of bone [102]. The bone DNA was suitable for RFLP as well as PCR analysis. The DNA from bone and blood of the same individuals showed the same RFLP patterns when analyzed with the single locus probe CRI-PAT-pL1077-1 (D6S22) [103,104] following HaeIII digestion, or with a cocktail of the single locus probes MS1 (D1S7), MS31 (D7S21), MS43 (D12S11) and g5 (D7S22) [105] following HinfI digestion. These studies helped establish that bone is a valuable source of DNA suitable for RFLP analysis. Likewise, PCR analyses of DNA from corresponding bone and blood using HLA-DQα and 3'APOB typing gave identical phenotypes. Other recent studies confirm that DNA isolated from bone can be analyzed by RFLP or PCR methods, and establish the value of a parentage testing approach to identification using these techniques [106,107]. The reported recovery of DNA from older pathology specimens [108] suggests that it is quite stable under the conditions of specimen fixation that were studied. These specimens may thus provide sources of known DNA from individuals who have died, and whose bodies are no longer available.

In this paper, we report the results of studies on DNA isolated from two series of specimens: bone tissues, after exposure of the specimens to dry and humid conditions at room temperature, 37°C, 56°C, and in soil for periods of up to 9 months; and bone and soft tissues, after exposure to dry and moist air at room temperature, and to salt water immersion. The results include data on total and human DNA yields, and on typing using both RFLP and PCR methods.

EXPERIMENTAL

DNA was isolated from bone and soft tissues using previously described organic solvent extraction methods [80,102], and recovered by ethanol precipitation or dialyzed through Centricon 100 miniconcentrators in TE (10 mM Tris–HCl, 1 mM Na_2EDTA, pH 7.8) or neutral distilled water. DNA recovered by ethanol precipitation and Centricon dialysis in TE buffer was used for RFLP analysis. DNA recovered by Centricon dialysis in neutral distilled water was used for PCR reactions. Several bone specimens were prepared with and without decalcification [106] prior to DNA extraction. Bone decalcification before DNA extraction has been used in the analysis of both old [89] and modern specimens [106]. The quality and quantity of DNA were assessed by UV visualization of ethidium bromide stained 1% agarose evaluation gels following electrophoresis in 1× TBE (89 mM Tris–HCl, 89 mM boric acid, 2 mM EDTA, pH 8). DNA quantity was also estimated by a spectrofluorimetric procedure [109]. To estimate the quality and quantity of human DNA in specimens, the evaluation gels were transferred [110] onto Zetaprobe nylon membranes using 0.4 M NaOH as transfer buffer, or onto Biodyne A nylon membranes using 10× SSC [111] as transfer buffer. Transferred DNA was fixed to Biodyne A by exposure to 0.2 J/cm^2 254 nm UV light. The membranes were then hybridized in 7% SDS, 4.5× SSPE, 10% PEG at 65°C with a human Alu sequence probe (Oncor), labeled with ^{32}P-dCTP (DuPont/NEN) using a random primer labeling kit (Promega) and Klenow DNA polymerase (Boehringer Mannheim). Labeled probes were recovered from Sephadex G-50 columns in 10 mM Tris–HCl, 1 mM Na_2EDTA, pH 7.8, 0.55 M NaCl using Blue Dextran as a

marker, and used at concentrations of 1.0–1.5 × 10^6 dpm/ml hybridization solution. Membranes were washed twice for 15 min in 2× SSC, 0.1% SDS at room temperature, then for 30 min in 0.1× SSC, 0.1% SDS at 65°C. Autoradiography was on Kodak XAR film for several hours to overnight at room temperature.

The quantity of human DNA in specimens was also estimated using a previously described dot blot procedure [112], except that the Alu probe (Oncor) was used instead of the p17H8 (D17Z1) probe. For dot blots, DNA was denatured in 0.3 M NaOH at 65°C for 1 h, then applied by vacuum to nylon membranes in 6.5× SSC, 0.325 M NaOH. Hybridization, washing and exposure conditions were essentially as described above for evaluation gel blots.

DNA for RFLP analysis was restricted with HaeIII (Boehringer Mannheim) following the supplier's protocol except that 0.045 μg/μl BSA and 2.2 mM spermidine were added to the digestion reactions. Restricted DNA fragments were separated electrophoretically on 1% agarose gels in 1× TBE using a 1 V/cm gradient for 24 h, then transferred to nylon membranes, hybridized and washed essentially as described above, except that labeled probes were added to a final concentration of 0.5 × 10^6 dpm/ml hybridization solution and stringent washes were for 15 min. Probes YNH24 and TBQ7 (Promega) detecting the VNTR loci D2S44 and D10S28, respectively, were used. Both DNA Analysis Marker (Bethesda Research Labs.) 30 fragment and "23 kb combination standard" (Lifecodes) sizing ladders were used. BRL ladder fragments were detected by following the supplier's protocol, and Lifecodes ladder fragments were detected using a ladder probe from the supplier.

PCR reactions for HLA DQα amplification, and the typing itself, were done with the Perkin Elmer/Roche kit following supplier's instructions, except that BSA was added to a final concentration of 160 μg/ml in PCR reactions and we used 20 ng template human DNA. Several bone DNA specimens were amplified in both the presence and absence of 2.8% formamide. Formamide at concentrations varying from 1.25% to 10% has been employed to enhance PCR specificity, particularly in GC-rich sequences [113], and 5% formamide has been reported to improve amplification of HLA-DQα type 1 alleles, thereby avoiding "allele dropout" (failure to amplify certain sequences that are actually present) [114].

PCR amplification of X, Y and VNTR sequences were carried out in 50 μl or in 100 μl final volumes. The 3'APOB reaction mixture contained 50 mM KCl, 200 μM dNTPs (Pharmacia), 10 mM Tris–HCl, pH 8.4, 2 mM $MgCl_2$, 0.01% (w/v) gelatin, 0.1 μM primers, 1 U Amplitaq (Perkin Elmer/Cetus), and 30–50 ng target DNA. Primers, modified from [61] were: APOB1: 5'-ATGGAAACGGAGAAATTATGGA, and APOB2: 5'-TTTTTTCCTTCTCACTTGGCAA. Amplifications were carried out in a Perkin Elmer Model 1000 thermal cycler following the profile: 3 cycles of 94°C, 4 min, 55°C, 2 min, 72°C, 2 min, followed by 27 cycles of 94°C, 2 min, 55°C, 2 min, 72°C, 2 min. D1S80 system PCR was according to the protocol of Kasai et al. [70] using 30 ng template human DNA. D17S30 locus [115] PCR was essentially according to the protocol described for 3'APOB above, except that 50 mM NaCl replaced 50 mM KCl in the buffer [116], the primers, modified from [66] were D17S30–1: 5'-TCGAAGAGTGAAGTGCACAG and D17S30–2: 5'-CCGCCTCCCAAGTTAACTTA, and the primer annealing temperature was 58°C. PCR products were detected by ethidium bromide staining of 1% agarose, 1% NuSieve agarose gels in 1× TBE and visualization by UV transillumination. Amplification of X and Y chromosome alphoid centromeric repeat sequences was carried out as previously described [80]; briefly, the reaction mixture was 10 mM Tris–HCl, pH 8.4, 50 mM KCl, 200 μM each dNTP, 2.5 mM $MgCl_2$, 0.01% gelatin, 2 U Amplitaq (Perkin Elmer/Roche, Norwalk, CT), 3.5% formamide and 0.1 μM primers as follows: X1: 5'-TATTTGGACTCTCTCTGAGGA; X2: 5'-TTCTACTACAAGGGTGTTGCA; or Y1: 5'-GTGTATTCACCTCCGGGAG; Y2: 5'-ACAAAAGGTTCAATTCTGTGAG. The thermal cycle profile was 3 cycles of 94°C for 4 min, 60°C for 2 min, 72°C for 2 min, followed by 30 cycles of 94°C for 2 min, 60°C for 2 min, 72°C for 2 min. XY PCR products

were detected on ethidium bromide stained 2.5% agarose, 1.5% NuSieve agarose gels.

Two series of specimens were studied. One series consisted of a set of 18 tissues (including bone and blood) from each of two undecomposed bodies, one male and one female.

Examples of each tissue were exposed to dry and humid conditions at room temperature (RT) and to salt water immersion for 30 days before DNA isolation and analysis. Some RFLP and PCR typing data on tissues from this series is presented below. The other series consisted of post-mortem bone specimens from 11 different individuals, each divided into 45 subspecimens. The subspecimens were kept under dry and humid conditions at room temperature (RT), 37°C, and 56°C, placed in dry and moist soil in the lab, and buried in the ground outdoors, for periods of 1, 2, 4, 6 and 9 months. Including the control specimens (those not exposed to any environmental condition), there were 506 specimens in the second series. DNA was isolated from all of them, and evaluated by yield gel, spectrofluorometry, and dot blot procedures to assess the quantity and quality of total and human DNA present. Selected examples of the specimens in the second series, as indicated below, were tested by PCR typing methods.

RESULTS AND DISCUSSION

Fig. 1a and b shows RFLP patterns obtained with blood and bone DNAs from the same individual using probes TBQ7 and YNH24, respectively. Fig. 1c, lanes 7 and 8, shows an additional RFLP pattern from the blood and bone of the same person using probe YNH24. These results confirm previous findings [102] that there are no discrepancies in patterns. Fig. 1c also shows the results of RFLP analysis of a bone and an ovarian tissue DNA, from different individuals, using

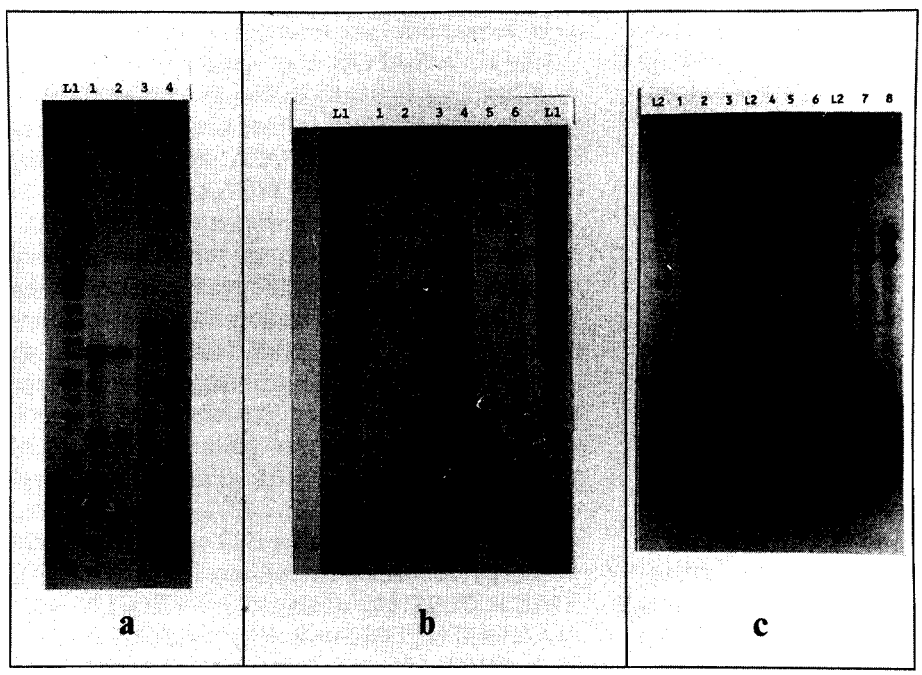

Fig. 1. RFLP analyses of blood, bone and soft tissue DNA (photo composites). (a) Blood–bone pair DNAs, lanes 1–2 and 3–4, hybridized with probe TBQ7. Lane L1, BRL 30 fragment sizing ladder. (b) Blood–bone pair DNAs, lanes 1–2, 3–4 and 5–6, hybridized with probe YNH24. Lane L1, BRL 30 fragment sizing ladder. (c) Lanes 1 and 2, known control DNAs; lane 3, bone tissue DNA; lane 4, bone tissue DNA "plug"; lane 5, ovarian tissue DNA; lane 6, ovarian tissue DNA "plug"; lanes 7–8, blood–bone pair DNA; lanes L2, lifecodes 23 kb combination standard ladder.

YNH24 probe. The "plug" lanes were obtained by excising the higher MW region of an evaluation gel lane containing the specimen, and performing the restriction and subsequent steps of the procedure directly on the DNA in the melted agarose. Although the signals are weak on this autoradiogram, this strategy provides a method for producing clearer single locus probe band patterns from degraded DNA specimens, which are common from bone and soft tissues, especially after any significant environmental exposure.

Figure 2 shows typical results obtained with DNA specimens from bone subjected to a variety of environmental conditions. Fig. 2a is an evaluation gel, Fig. 2b is an autoradiogram of an Alu-probed evaluation gel Southern blot, and Fig. 2c is an autoradiogram of an Alu-probed dot blot. Evaluation gels like that shown in Fig. 2a provide an estimate of the total DNA quantity in the specimen and of the quality of the DNA. The amount of sample needed for subsequent RFLP or PCR testing protocols, however, depends on estimating the quantity and degree of degradation of the human DNA in the specimen. The quantity of human DNA is readily assessed by an autoradiogram after hybridization of a radioactively labeled human probe (like Alu or p17H8) to dot blotted specimens, as in Fig. 2c. However, the dot blot format does not provide information

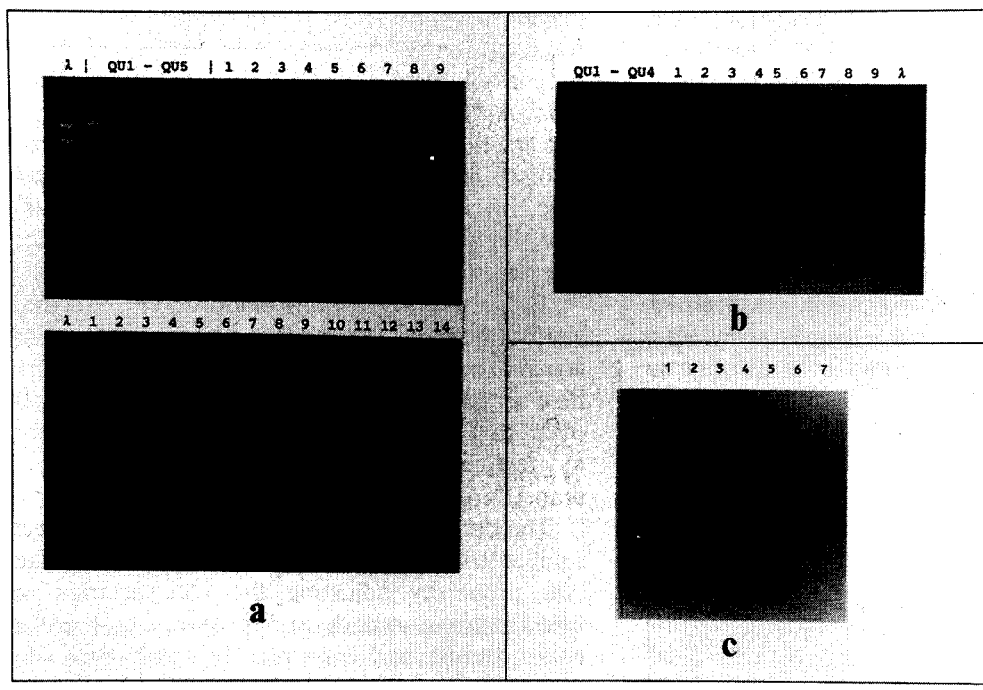

Fig. 2. Evaluation gel and autoradiograms of Alu probed membranes. (a) Evaluation gel. λ, λ-HindIII/EcoR1 ladder; QU1–QU5, 50, 25, 10, 5 and 1 ng λ DNA quantitation standards. Upper lanes: 1–2, control bone DNAs from same specimen decalcified and not decalcified prior to extraction; lanes 3–5, control bone, and one and four-month ground exposed bone DNAs from same specimen; lanes 6–8, control bone and six-month humid and dry 56°C exposed bone DNAs from same specimen; lane 9, control bone DNA from same specimen used for various environmental exposures to obtain results shown in lower lanes. Lower lanes: lanes 1–2, RT dry and humid for 4 months; lanes 3–4, RT dry and humid for six months; lanes 5–6, 37°C dry for one and two months; lanes 7–8, 37°C humid for one and two months; lanes 9–10, 56°C dry and humid for four months; lanes 11–12, dry and wet soil for four months; lanes 13–14, dry and wet soil for six months. (b) Autoradiogram of Southern blot of evaluation gel probed with Alu. λ, λ-HindIII/EcoR1 ladder; QU1–QU4, 50, 25, 10 and 5 ng human DNA quantitation standards; lanes 1–9, specimens correspond to those in upper lanes 1–9 of (a). (c) Autoradiogram of dot blotted Alu probed membrane. Top row, positions 1–7, 125, 100, 75, 50, 25, 10 and 1 ng human DNA quantitation standards; other rows, representative dot blot results from DNAs from various bone control specimens and those exposed to different environmental conditions.

about the extent of degradation of the human DNA present. For that purpose, autoradiograms of Southern blotted evaluation gels probed with human probes, as in Fig. 2b, are needed. Altogether, over 500 specimens were tested for total DNA, evaluation gels, Alu-probed evaluation gel blots, and Alu dot blots in these studies.

Initially, we thought that Alu autoradiograms of evaluation gels would enable predictions about the prospects for success in subsequent RFLP tests. Absence of higher MW human DNA would tend to suggest insufficient sample for an RFLP test. However, our expectations were not entirely borne out. In extreme cases, where there was no detectable human DNA, or where the human DNA was extremely degraded (< 1000 bp as judged by the λ-Hind III/EcoR1 ladder), RFLP testing was unsuccessful. But there were some specimens containing DNA that was quite degraded, and where there was no signal at the top of the lane (> 2000 bp as judged by the λ-Hind III/EcoR1 ladder) that nevertheless yielded satisfactory RFLP results, at least with YNH24.

The human DNA content of bone and soft tissue specimens, as assessed by the dot blot procedure, generally declined as a function of environmental exposure, especially in specimens exposed to soil, humid conditions or directly to salt water.

There was, however, significant variation in different specimens even under the same exposure conditions. With "control" bone and soft tissue specimens (no environmental exposure), human DNA as estimated by the standard dot blot assay typically comprised about 20% of total DNA, but varied in particular samples from undetectable (less than 0.2 ng/μl) to 80% of total DNA. With bone or tissue specimens exposed to soil or to humid conditions at any of the temperatures, human DNA typically comprised well under 1% of the total DNA.

Adams et al. [29] reported that no high molecular weight DNA could be extracted from soil that had been spiked with human blood (and, because no suitable DNA could be obtained, no RFLP typing results could be obtained). An additional experiment using sterile soil showed that the problem was not DNA degradation by soil constituents, but interference with the extraction of the human blood DNA by something in the soil. We examined a total of 133 bone specimens that had been exposed to a soil environment for 1–9 months. Of the 133, 45 specimens were in dry soil and 44 in wet soil at room temperature, and 44 specimens were buried in the ground outdoors. About 33% of the dry soil specimens, 20% of the wet soil specimens and 40% of the ground specimens yielded any human DNA, as judged by the dot blot and the Alu-probed evaluation gel blot assays. The remainder of the specimens yielded no detectable human DNA, e.g., lanes 4 and 5 of Fig. 2b. Our results on the bone fragments are not necessarily at odds with the results of Adams et al. [29]. Our experiments involved placing into and later recovering a physical bone fragment from the soil, and subjecting it to preparatory washing and cleaning steps prior to crushing it for DNA extraction. All the soil material itself is thus removed from the specimen prior to extraction. In the experiments involving blood in soil, there is obviously no way to separate the blood materials from the soil itself prior to extraction.

There were no significant differences in the yield of human DNA from the limited number of control (no environmental exposure) bones examined in parallel with and without decalcification prior to DNA extraction. We did not carry out any testing to determine the nature of the nonhuman DNA extracted from the specimens.

The dot blot assay can be constructed to detect smaller concentrations of human DNA with the Alu probe by adjusting the concentration of quantitation standards, and increasing both probe concentration and exposure time. We have run this assay successfully over a range of 5 ng to 1 pg DNA.

Figure 3 shows the results of RFLP analysis with YNH24 of DNA from heart and bone (from the same individual) that had been exposed to dry air, humid air, and salt water for 30 days. As indicated in the figure, air-exposed specimens yielded results but the salt water exposed ones did not. These results are typical of a series of tissues tested (see below). However, in a number of cases, DNA from specimens that failed to yield

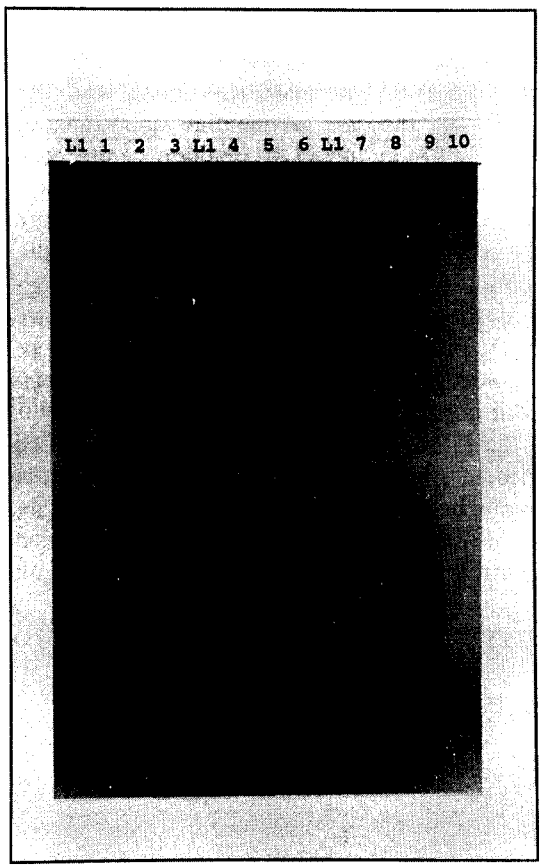

Fig. 3. Autoradiogram of RFLP analysis of kidney and heart tissue DNAs after exposure of tissues to environmental conditions. Lane L1, BRL 30 fragment sizing ladder; lanes 1 and 2, known control DNAs; lanes 3–6, DNA from control, RT dry, RT humid and salt water exposed kidney tissue, respectively; lanes 7–10, DNA from heart tissue in same format as lanes 3–6.

RFLP results could be successfully amplified and typed using PCR. The salt water exposed heart tissue DNA (lane 6, Fig. 3), for example, gave completely satisfactory 3'APOB results following PCR amplification (Fig. 4).

A total of 124 bone DNA specimens were tested using one or more of the PCR systems. A minimum quantity of human DNA is obviously required for testing, and only specimens that yielded sufficient human DNA could be considered for further testing. Insofar as possible, specimens were selected in an effort to gather data on bone from the same source that had been exposed to the gamut of environmental conditions and times. Further, more than one PCR system was used to test a given series of specimens to the extent possible. In all, 42 specimens were tested for DQα, 44 for D1S80, 21 for D17S30 and 17 for XY.

Overall, 35% of specimens tested gave results with one or more of the PCR systems tested. 55% of specimens gave D1S80 results, 36% gave DQα results, and 13% gave results with D17S30 and XY.

However, fewer total specimens were tested for D17S30 and XY than for DQα and D1S80.

Comparing the group of specimens aged under dry conditions (whether at RT, 37°C or 56°C) with the group aged under humid conditions, the percentage of positive results was higher with the former (48% overall) than with the latter (30% overall). The soil and ground group of specimens yielded 13% positive results overall.

Within the group of specimens aged under dry conditions, there were no major differences in percentage yielding positive results among those kept at RT, 37°C and 56°C. Within the group aged under humid conditions, however, the percentage of positive results was significantly better with RT specimens than with those kept at 37°C or at 56°C.

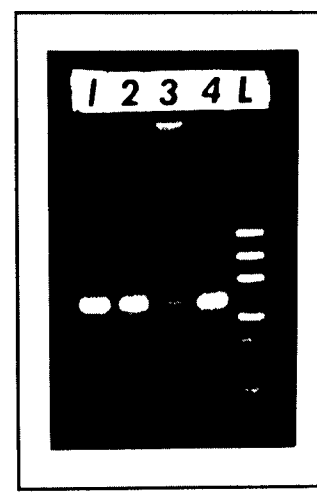

Fig. 4. 3'APOB PCR typing of heart tissue DNA. Lanes 1–4 contain same DNA and in same format as lanes 3–6 in Fig. 3; lane L, ϕX-174/HaeIII sizing ladder.

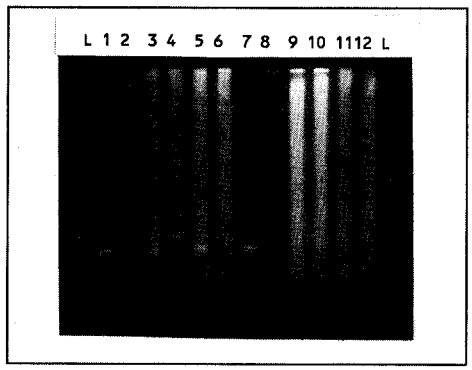

Fig. 5. Some representative XY amplification results from environmentally exposed bone specimen DNAs. Lane L, ϕX-174/HaeIII sizing ladder; odd lanes, amplification with X primers; even lanes, amplification with Y primers; lanes 1–2, control female bone DNA for lanes 3–6; lanes 3–4, one month RT dry; lanes 5–6, six month RT dry; lanes 7–8, control female bone DNA for lanes 9–12; lanes 9–10, one month RT dry; lanes 11–12, four month RT dry.

While the general trends noted just above were definite, and in general accord with what one would predict, individual samples did not always behave in accordance with predictions. For example, it could reasonably be expected that different specimens maintained under a particular environmental condition for comparable amounts of time would behave similarly. However, results in Fig. 5, which illustrates some results of XY amplification, show that different specimens aged dry at RT for 1 and 4 or 6 months did not yield comparable results. One specimen yielded results while the other did not. Similarly, specimens of the same bone sample might be expected to become untypable as the time of exposure to a particular condition increased. Figure 6, which illustrates some results of D1S80 amplification, shows that the specimen aged dry at RT behaves exactly as expected. However, the same specimen aged under humid conditions at RT yielded results for the six month sample but failed to give results at one and two months. Figure 6 also shows the failure of several soil and ground specimens to give specific PCR product. A number of similar observations were made in the DQα typing results. With one specimen, a four month ground sample yielded results, while a six month RT humid and a nine month 56°C dry sample did not.

It is possible that some of the bone DNA specimens contained an inhibitor of the PCR that co-extracted from the specimen along with the DNA. If so, the use of smaller quantities of template DNA in the PCR reaction would help to overcome the problem.

Our data thus indicate that there are definite trends in PCR typability vs. environmental exposure conditions and time, if specimens are considered in the aggregate. Further, the trends are generally in accord with expectation. Occasionally, however, the behavior of an individual specimen did not follow the predicted course.

In the DQα typing experiments, we amplified a series of four different specimen DNAs from the same bone sample in the presence and absence of 2.8% formamide. The series included the control, a six month RT humid, a nine month

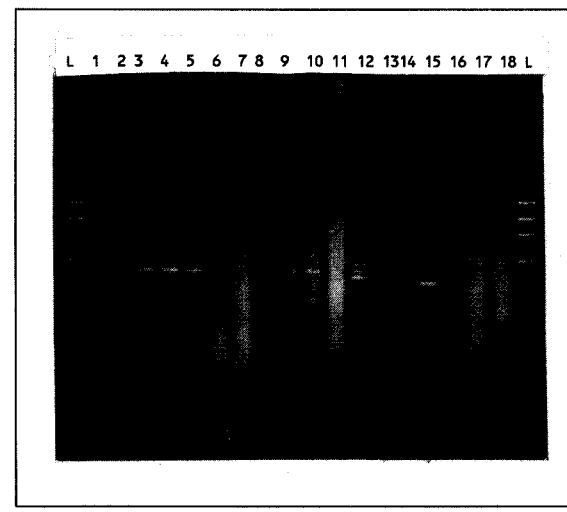

Fig. 6. Some representative D1S80 amplification results from environmentally exposed bone specimen DNAs. Lane L, ϕX-174/HaeIII sizing ladder; lane 1, control for lanes 2–11; lanes 2–6, RT dry specimens, 1, 2, 4, 6 and 9 months; lanes 7–9, RT humid specimens, 1, 2 and 6 months; lanes 10–11, two month specimens, 37°C dry and 37°C humid; lane 12, control for lanes 13–14; lanes 13–14, two month specimens, 56°C dry and 56°C humid; lane 15, control for lanes 16–18; lanes 16–18, six month dry soil and wet soil, and four month ground specimens. The three banded pattern seen in lanes 12 and 13 is a PCR artifact, probably due to the formation of heteroduplexes during amplification of repeat sequence regions in heterozygotes with different size alleles on the two chromosomes. The largest fragment is the artifact. On other gels with these specimens, the band was not seen.

TABLE 1

PCR amplification results with bone and soft tissues exposed to various environmental conditions before extraction of DNA and testing [a]

Tissue	Condition [b]	DQα	APOB	XY
Liver	C	+	−/+ [c]	± [d]
	A	+	±	+
	M	+	±	+
	S	−	−	−
Pancreas	C	+	+	+
	A	+	+	+
	M	+	+	+
	S	−	−	+
Kidney	C	+	+	+
	A	+	+	+
	M	+	+	+
	S	+	+	+
Fat	C	+	+	+
	A	+	±	+
	M	+	−	+
	S	−	−	−
Heart	C	+	+	+
	A	+	+	+
	M	+	+	+
	S	+	−	+
Brain	C	+	+	+
	A	+	±	+
	M	−	−	+
	S	+	+	+
Thyroid	C	+	+	+
	A	+	+	+
	M	+	±	+
	S	−	+	+
Lung	C	+	+	+
	A	+	+	+
	M	+	−	+
	S	+	−	+
Thymus	C	+	+	+
	A	+	+	+
	M	+	±	+
	S	−	−	−
Small Intestine	C	+	+	+
	A	+	+	+
	M	+	−	+
	S	−	−	−
Skeletal Muscle	C	+	+	+
	A	+	+	+
	M	+	−	+
	S	−	−	−
Spleen	C	+	+	+
	A	+	+	+
	M	+	+	+
	S	+	−	+
Bone	C	+	+	+
	A	+	+	+
	M	+	+	+
	S	+	+	+
Testis/ Ovary	C	+	+	+
	A	+	+	+
	M	+	± [e]	+
	S	−	−	−
Tendon	C	+	+	±
	A	+	+	+
	M	+	+	+
	S	+	−	+
Prostate/ Uterus	C	+	+	+
	A	+	+	+
	M	+	+	+
	S	−	−	+
Skin	C	+	+	+
	A	+	+	+
	M	+	−	+
	S	−	−	−
Blood	C	+	+	+
	A	+	+	+
	M	−	+	+
	S	nt [f]	nt	nt

[a] Tissues from two different individuals were exposed for one week to the designated condition before DNA extraction. + Means interpretable PCR results were obtained; − means no results. [b] Conditions of exposure: unexposed controls (C); dry air (A), humid air (M) or immersed in salt water (S). [c] −/+ Means negative in first PCR test, positive on repeat testing. [d] ± Means the specimen from one individual was positive; specimen from the other was negative. [e] Ovary was negative. [f] nt Means not tested.

56°C dry and a four month ground specimen. No differences were noted. Those that typed satisfactorily did so with or without the formamide. And those that were negative without the formamide were still negative when it was included. In the PCR, formamide acts primarily by effectively lowering the template melting temperature, and can thus be expected to have its most noticeable effects if the denaturation temperature selected for PCR cycling is too low, or the cycler fails to reach the nominal temperature.

Results of the experiments in which the series of eighteen tissues from each of two individuals were tested after a week's exposure to dry air at

RT, moist air at RT and salt water immersion are given in Table 1. Bone tissue was found to be the most robust in terms of yielding positive results with PCR testing of HLA DQα, 3'APOB and XY. Of the three PCR systems used, the 3'APOB results were most negatively affected by environmental exposure of the tissues. This effect was especially apparent in the RT moist air and in the salt water immersed specimens. In addition, XY amplification gave a higher percentage of positive results in the RT moist air and salt water immersed specimens than did DQα. Of the three environmental conditions tested, salt water immersion had the greatest negative effect on all the systems' results, as we expected.

In summary, these studies show that the exposure of bone or soft tissues to environmental conditions has variable effects on the ability to type the DNA that is subsequently isolated. In general, room temperature exposure of tissues under dry conditions is the least deleterious, while exposure to moist soil or immersion in salt water are the most deleterious. The general trends in DNA typability as a function of exposure conditions and time are in accord with expectations when all the data are considered, but a given occasional individual specimen may not always behave in accordance with the trends.

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Elemental analysis of small glass fragments in forensic science

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Abstract

The evidential value of small glass particles has been recognized for quite some time. While physical properties such as refractive index and density offer some discrimination of glass samples, they offer little in the way of classification of glass product type. Further, with improved glass manufacturing processes, the range of physical properties seen in modern glass samples is continually decreasing. Many have advocated the use of elemental analysis for the purposes of forensic glass analysis. Various instrumental techniques have been employed to this end, including flameless atomic absorption spectrometry, inductively coupled plasma atomic emission spectrometry, and energy dispersive X-ray fluorescence spectrometry. These more commonly employed methods are discussed with regard to the application to forensic glass analysis. Advantages and disadvantages of each technique are highlighted.

Keywords: Atomic absorption spectroscopy; Atomic emission spectrometry; Inductively coupled plasma; X-ray fluorescence spectrometry; Forensic analysis; Glass

The evidential value of glass fragments has been recognized for quite some time [1]. Although glass is most commonly associated as evidence in property offenses, it may be encountered in almost every type of criminal investigation. In fact, glass fragments have been reported as being among the various types of physical evidence most frequently encountered at crime scenes [2].

The large frequency of occurrence of glass as forensic evidence is attributable to many factors. Glass is ubiquitous, produced in large quantities and for a wide variety of applications, and due to the way in which it fractures, small particles of glass are common to the environment. These tiny glass fragments, either generated during the commission of a crime or pre-existing at the scene, can be carried away from the crime scene unwittingly by the perpetrator by adhering to clothing or shoes. Further, glass is chemically stable and resistant to environmental factors and therefore can be analyzed by the forensic laboratory with meaningful results even after a considerable amount of time has lapsed between the occurrence of the crime and the collection of questioned glass samples associated with the suspect.

Ideally, the analytical methods chosen for forensic comparisons should be capable of distinguishing all sources, as the ultimate goal of the criminalist is individualization; that is, proof that a questioned evidence sample originated from the known source to the exclusion of all other sources. Unfortunately, it is more the rarity than the norm that individualization is possible with many items of trace evidence. As with most forensic comparisons, if the known and questioned glass samples are indistinguishable by the various analytical methods employed, the analyst is forced to con-

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clude that the two samples "could have" shared a common origin. In most circumstances, the analyst must perform a battery of tests in an attempt to prove that the two samples are different. Clearly, the forensic scientist should be concerned with minimizing the number of false positives, which may tend to convict an innocent, while limiting the number of false negatives, which may exculpate a guilty individual. Therefore, the methods of analysis chosen should be highly accurate and precise and capable of distinguishing between as many sources as possible.

Historically, forensic comparisons of glass samples have consisted of comparisons between the physical properties of known and questioned samples, principally by refractive index and density measurements. Glass samples from different sources exhibit a range of refractive indices and densities, a fact which has been utilized in the forensic discrimination of glass samples. However, since these two physical properties are highly correlated, the value of performing both tests for discrimination purposes is questionable [3–6]. If, for example, refractive index determinations were performed on known and questioned glass samples and the samples could not be discriminated, it is expected that density measurements would yield similar results. Since these tests are not independent, in most cases the probability of common origin between known and questioned glass samples is not significantly increased by performing the second analysis. For this reason, and considering that refractive index measurements are generally simpler and less time-consuming to perform, many laboratories have opted to exclude density measurements from their routine analytical procedure for glass.

In recent years, there has been some concern within the forensic science community regarding the sole use of refractive index measurements for comparisons between known and questioned sources. This concern is rooted in the fact that, due to advances in glass manufacturing technology, the range of the refractive indices of modern glass is narrowing, thereby resulting in the potential for an increase in the number of false positives. It is imperative, therefore, that the refractive index measurements be performed with the highest possible precision and accuracy, and that the interpretation be tempered by the knowledge of these recent trends, particularly when the comparison results in a "match".

In light of the current situation with which the forensic glass examiner is faced, some have advocated the use of elemental analysis of these fragments as a complimentary technique to aid in the discrimination of glass samples. Although it has been explored for some time, the elemental analysis of glass for forensic purposes has only recently been actively pursued. One reason for this in the past may have been the inapplicability of various methods of elemental analysis to the small, irregularly shaped glass fragments which are common in forensic casework.

The qualitative elemental analysis of glass particles for the presence of elements which are intentionally added in small amounts to molten glass, or those which may be present as incidental trace contaminants in the raw materials may be quite significant in a forensic comparison. Major elements such as silicon, calcium, sodium, and aluminum are generally present in all soda-lime silica glasses; therefore, the evidential value associated with the mere presence of these elements in a sample is rather small.

Accurate quantitative elemental analysis of major, minor and trace components present in glass samples should provide a much more meaningful comparison. The mere presence of common minor and trace elements in two pieces of glass may indicate that the samples "could have" shared a common origin; however, a quantitative match of these elements present in an unknown sample with those of a known sample is more likely to increase the probability of common origin.

Quantitative elemental analysis can be a valuable technique for the discrimination between known and questioned samples, as well as for the classification of glass by end-product use. While "matching" an unknown glass sample to a particular known source is most often the concern of the criminalist, there are times when it may be important to determine the type of glass product from which the particle originated. Classification of glass fragments by product-use type can be

important in corroborating or disproving an alibi and in product tampering cases, and has been given much attention in recent years. It is similarly useful to identify the type of glass when evaluating the probability of finding such glass in the population. Regardless of the reasons for performing the elemental analysis, the methods utilized for such generally share similar concerns since forensic glass samples are generally small and irregularly shaped. However, prior to performing the analysis, the criminalist must bear in mind the purpose of the test, as the elements chosen and the level of precision and accuracy differ depending on whether one is interested in classification or discrimination of samples.

Various methods have been utilized for both of these purposes, including inductively coupled plasma atomic emission spectrometry (ICP-AES) [7–12], spark source mass spectrometry [13–16], neutron activation analysis [10], emission spectroscopy [10,16], flameless atomic absorption spectroscopy (FAAS) [10,23], and energy dispersive x-ray fluorescence spectrometry [17–22]. This paper will review several of these techniques with respect to their application and contribution to forensic glass analysis.

METHODS OF ELEMENTAL ANALYSIS

Since a number of methods for elemental determinations exist, the analyst may be faced with the decision of choosing the analytical technique(s) which will be employed for glass analysis in the forensic laboratory. Table 1 lists some of the important factors which should be considered when choosing an analytical technique. While they are not listed in specific order in Table 1, some of these parameters are of greater concern to the criminalist than to the general analytical chemist.

Three of the more common techniques, namely FAAS, ICP-AES and energy dispersive x-ray fluorescence spectrometry, will be discussed. These methods are being emphasized for several reasons. These techniques are well-established and sources of errors are understood and handled fairly easily. A considerable amount of research in glass analysis has already been performed with these techniques and information regarding reproducibility and detection limits is available. Further, many forensic laboratories may already have such instrumentation for other analytical purposes or the purchase of such instrumentation can be justified as any one of these need not be a dedicated instrument.

A comparison of some of the important features of these three instrumental methods may be found in Table 2. These considerations will be discussed with respect to the various techniques, as applied to forensic glass comparisons.

FAAS

Although flameless atomic absorption spectroscopy is an extremely sensitive technique, and therefore, ideal for trace element analysis, it has not been afforded much attention in the forensic analysis of glass samples. The sensitivity of this technique is down to the ppb range, with good precision and accuracy. This results in a minimal sample size required for analysis, generally 100 μg or less. Further, the equipment is available in many forensic science laboratories which perform gunshot residue analysis. Why, then, is this technique not widely used for elemental analysis of glass?

The fact is that FAAS has many disadvantages which render it an undesirable technique for daily, routine casework. Firstly, the technique is destructive, so if sample preservation is a concern, this is not the method of choice. Sample prepara-

TABLE 1

Some important factors to consider when choosing an analytical method

Destruction of sample
Level of reliable detection
Accuracy
Precision
Total analysis time
Ease of sample preparation
Sample size requirements
Cost, instrument and operation
Operator expertise required
Linear concentration range
Matrix effects and interferences easily controlled
Sources of error understood and controlled

tion can be tedious, requiring special digestion vessels for work with hydrofluoric acid. Procedures to avoid contamination are particularly important, as it is a trace element technique. Further, matrix effects and interferences exist, however, these can generally be handled by matrix matching samples and standards.

Perhaps the biggest drawback of this technique for routine analysis is that it is a single element technique. This requires multiple calibration curves to be performed, one for each element analyzed, and sample injections must be repeated for each element, all of which drastically increase analysis time. Fortunately, FAAS is currently automatable, and recent advances in graphite furnace technology allow for faster analyses.

Howden et al. [23] used FAAS to classify glass types by measuring iron and magnesium levels. Very small (100 μg) fragments of window glass were successfully analyzed with this technique, with good precision and accuracy. Precision for the iron measurement results of container glass samples was less, however, this is to be expected because of the much lower iron content in this type of glass. Matrix effects were noted, as silica in standard solutions yielded a suppression of the iron signal. No matrix effects were seen with magnesium. This problem can be overcome by matrix matching or by arithmetic correction of the absorbance readings by assuming an average suppression for iron in all samples, as was done in the reported research.

FAAS, while plagued by lengthy sample preparation and analysis, can be quite useful in forensic glass analyses, particularly when a limited number of elements are to be examined. Much of the research using furnace AAS was performed approximately fifteen years ago, when furnace performance was not as stable and reproducible as it is today. Detection limits of furnace AAS are about two orders of magnitude greater than ICP-AES or FAAS, and errors from interferences can be controlled. While the emphasis of the previously published research in FAAS [23]

TABLE 2

Comparison of some important parameters of three common techniques for elemental analysis of forensic glass samples

Parameter	Flameless AAS	ICP-AES	EDX/XRF
Destructive	Yes	Yes	No
Level of detection	Extremely sensitive	Good	Good, but varies with element, excitation and matrix.
Multi-element technique	No	Yes	Yes
Sample preparation	Intensive	Intensive	Virtually none
Automated	Yes	Yes	Possible
Cost	Moderate	High	Moderate (XRF) High (SEM/EDX)
Small sample size	Extremely small	Small to moderate	SEM, very small XRF, small but precision decreases with sample size
Speed of analysis	Very Slow	Rapid	Rapid
Interelement effects and interferences	Exist but easily handled	Virtually None	Exist but well understood and handled
Linear dynamic range	Short	Long	Moderate, varies with matrix
Precision	Good	High	Good, but not at low concentrations
Accuracy	High	High	Good
Operator skills	Moderate	High	Moderate
Equipment available in forensic labs	Possible	Highly unlikely	Possible

was the classification of glass type, the technique is also well suited for the discrimination between glass samples because of its low detection limits.

ICP-AES

Inductively coupled plasma atomic emission spectrometry has recently been investigated with regard to forensic glass analysis. Research in the areas of glass type classification and discrimination of samples has been reported. While not as sensitive as FAAS, ICP-AES is a fairly sensitive technique, with high precision and accuracy. The multi-element capability of simultaneous ICP-AES greatly reduces sample analysis time. ICP-AES has a large linear dynamic range, so major, minor and trace components can be analyzed without additional dilutions or recalibrations at different concentration ranges. Furthermore, this instrumental method is virtually free from interferences and interelement effects.

On the other hand, ICP-AES possesses many of the drawbacks of FAAS. Not only is ICP-AES destructive in nature, but it can be sample size limited. Sample sizes down to 200 μg have been reported, but in order to achieve good precision for discrimination sizes greater than 500 μg are recommended [12]. Sample preparation is similar to that of FAAS, and the related disadvantages of the preparation also apply here. The largest obstacles for the utilization of this technique in routine casework in crime laboratories, however, are cost and operator skills. ICP-AES instrumentation is quite expensive and is not likely to be currently operational in most forensic science laboratories in the United States. Further, considerable experience is needed to operate this instrument, which would likely require extensive training for a forensic chemist.

Catterick and Hickman [7] advocated the use of a five element survey using ICP-AES for the classification of glass samples. These authors proposed the use of manganese, iron, magnesium, aluminum, and barium for glass classification purposes. Details of the classification scheme derived from this work are presented by Hickman [8]. This five element method appears to be successful at classifying glass samples, and offers a small degree of discrimination within a single class. Hickman suggests that the addition of a number of other elements to the classification procedure, such as rubidium, strontium, cesium, antimony, and perhaps aluminum, should be more suitable for discrimination purposes. In more recent years, Hickman [11] modified this procedure to incorporate strontium, resulting in a six element survey protocol. This modification has resulted in the ability to relate certain glass samples to manufacturers' products.

An ICP-AES procedure for classification and discrimination of glass samples was also proposed by Koons et al. [12] which utilized six elements, namely, strontium, barium, magnesium, iron, manganese, and aluminum. While the classification scheme of Koons et al. differed from that of Hickman, both agreed that ICP-AES yielded highly accurate concentration determinations, which are essential for classification. Koons et al. reported good precision, suitable for discrimination purposes, could be obtained with this procedure on glass fragments larger than 500 μg. In most instances, Koons' method could distinguish individual container glass manufacturing plants.

ICP-AES has great potential in the area of forensic glass analysis. Prior to its widespread application to the comparison of two glass samples of similar source, Koons suggests that the homogeneity of glass evidence samples demands further investigation.

Energy dispersive x-ray fluorescence spectrometry

Energy dispersive x-ray fluorescence spectrometry provides a rapid and fairly sensitive means by which glass particles may be examined elementally. Perhaps the most significant advantage of this technique to the forensic examiner is that it is non-destructive in nature. Small samples may be analyzed with the x-ray fluorescence spectrometer utilizing a beam collimator and/or with a scanning electron microscope equipped with an EDX detector. Both x-ray-excited EDX spectrometry (XRF) and electron-excited EDX [as obtained by scanning electron microscopy (SEM)] have been utilized for forensic glass analysis purposes.

Electron excitation which is achieved in the SEM is complimentary to XRF in that each has a greater sensitivity in a different atomic number range. SEM/EDX is more sensitive to lower atomic number elements, which in glass samples are the major and only some minor components. However, since XRF is more sensitive to higher atomic number elements, it can be utilized to quantitate the major, minor, and some trace components of the glass sample. The combination of the two techniques offsets some of the disadvantages associated with each. While the SEM/EDX method requires some sample preparation, it can still be quicker than XRF and can compensate for the lower precision of XRF with respect to low atomic number elements in very small sample sizes.

Quantitative analysis of the spectra generated by these methods may be accomplished in a number of different ways using a computer system. As with any analytical method, the sources of error, particularly those affecting reproducibility, must be identified and properly controlled if meaningful analytical results are to be obtained. Interelement effects and matrix considerations are often important in EDX and XRF analyses. Peak sizes and ratios will vary with the method used, as well as with sample composition, irregular sample shapes, sample geometry, sample size and thickness. A brief discussion of these concerns as applied to glass analysis can be found in papers by Howden et al. [18] and Keely and Christofides [19]. For a comprehensive treatment of x-ray beam and sample interactions, the reader is directed to the literature [24].

Energy dispersive x-ray fluorescence spectrometry has been applied to the elemental analysis of various types of glass products. Authors have noted its utility in classification of glass into various product-use types [18–22], and, to a lesser extent, its ability to discriminate among glass samples of the same type [17–18,20]. EDX spectrometry is well suited for forensic glass analyses since it is a non-destructive, multi-element technique which can be performed on very small samples in a short period of time, with virtually no sample preparation.

Reeve et al. [20] established the significance of elemental analysis by energy dispersive x-ray fluorescence spectrometry in the forensic examination of glass using an XRF system. Of the 82 samples analyzed in their study, only two were indistinguishable by EDX alone, and when physical properties were considered along with the EDX results, all were distinguishable from each other. However, it is unclear how many of these samples could be distinguished by refractive index (and/or density) determinations alone. The authors suggested that the elemental analysis of glass samples prior to measurements of the physical properties in case situations would save a considerable amount of time.

Howden et al. [18] found similar results and indicated the potential of a collimated XRF beam to analyze small fragments (approximately 200 μg) of glass in forensic casework. These authors were able to successfully use this technique to distinguish glass samples of different product-use type whose refractive indices "matched". They also indicated the improved discrimination within a group of window glasses by this method.

The use of EDX in combination with a scanning electron microscope (SEM) for the analysis of small glass fragments was performed by Keely and Christofides [19], with XRF as a complimentary technique, and by Terry et al. [22]. Both studies were aimed at classification of glass samples and showed fairly reproducible results on small glass samples. The quantitative analysis was accomplished by Keely and Christofides by ratios of peak intensities obtained for major and minor elements within the glass samples, whereas Terry et al. reported the results of their study in percent concentrations of the oxides present.

Keely and Christofides found magnesium and iron levels to be significant in distinguishing window and non-window sources. Problems arose with sheet glass samples from older buildings since they contained low magnesium concentrations, which were on the order of those concentrations in container glasses. However, these glasses were finally classified correctly as sheet glass by noting their high iron content. The low magnesium concentrations which have been found in pre-1930 window glass samples were previously encountered by Howden et al. [18]. This author

has also encountered such window glass samples in working with XRF for discrimination between glass samples with indistinguishable refractive indices [25] and findings corroborate the low magnesium/high iron content previously reported.

Conversely, in a study of the comparison of physical methods versus elemental analysis, Andrasko and Maehly [17] reported that EDX analysis in a scanning electron microscope failed to distinguish between the glass samples, including samples which were different in their refractive indices. When emission spectrography was performed as a complimentary technique, all but two samples were distinguished. The discrimination by emission spectrography in this study hinged on the analysis of higher atomic number elements, such as manganese, chromium, and arsenic to name a few, for which the SEM/EDX method is insensitive.

Similar results could have been achieved with XRF as the complimentary technique, as the XRF method is more sensitive to the higher atomic number elements. Analysts are cautioned against using SEM/EDX as the sole method for elemental analysis for the discrimination of glass samples, as it will likely yield a high false positive rate.

A more recent study involving quantitative EDX analysis of glass samples was performed by Ryland [21]. The emphasis of this work was the end-use classification of glass by this technique. Ryland employed a combination of SEM/EDX and XRF methods and utilized peak intensity ratios for quantitative purposes. He determined that the Ca/Mg ratio, as determined by SEM/EDX, and the Ca/Fe ratio by XRF are useful parameters in the classification of glass products as originating from either a sheet or container source.

Ryland and Koons [26] recently compared SEM/EDX/XRF with ICP-AES for the classification of glass fragments as sheet or non-sheet. Both approaches were found to be reliable in classifying glass fragments into sheet or container groups. For discrimination purposes, however, ICP-AES offers an advantage over SEM/EDX/XRF in that geometric effects which will reduce the precision of trace element quantitation in SEM/EDX/XRF are not a concern in quantitation by ICP-AES.

A preliminary study [25] of the use of XRF in combination with refractive index for window glass discrimination purposes suggests that the XRF method is sensitive for discriminating between some window glasses with indistinguishable refractive indices. Qualitative differences were noted between window glass samples which "matched" in refractive index. While fully automated quantitation with a computer system was attempted, problems arose when unknown samples contained elements which were absent from the standard glass samples available, or when the concentration of an element differed grossly from that in the standards. Therefore, quantitative comparisons should be performed by a peak ratio method.

Data for elements ranging from sodium through zirconium have been collected for window glass samples by an XRF method. It is expected that poor precision will be seen in the Na and Mg results, yet Mg should be reliably detected if it is present in concentrations of greater than approximately 2%. This factor alone may aid in discrimination, particularly in identifying "old" (pre-1930) window glass samples with low magnesium levels as compared with modern window glass samples. Ryland's SEM/EDX procedure [21] should offer increased precision for Mg measurements, which may prove Mg to be a useful element for discrimination purposes.

Elements which are expected to show good precision and which are being investigated for discrimination with this method are Si, Ca, K, As, Sr, and possibly Mn, Fe, and Zr, though the precision for these later elements is less satisfactory. While barium and titanium could be useful for good discrimination, the Ti *K* lines and the Ba *L* lines cannot be resolved by XRF at the levels present in window glass samples.

Conclusions

Currently, there are a number of satisfactory techniques available for the elemental analysis of glass samples for forensic purposes. More research is needed in the area of discrimination among samples of the same glass type. Further,

survey information regarding the ranges of elemental compositions encountered in casework situations is necessary for a more meaningful interpretation of the elemental data.

Each of the various instrumental techniques has particular strengths and weaknesses, and not every technique is applicable to all casework situations. A laboratory interested in developing a routine analytical protocol for elemental analysis of glass must consider many factors when selecting an instrumental method. Some of these include cost, availability of instrumentation in the laboratory, space limitations, purpose of the analysis, sample destruction, application to other areas of evidence examination, and the levels of detection, precision and accuracy desired.

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Spectromicrography and colorimetry: sample and instrumental effects

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Abstract

Fiber comparison in forensic casework touches on many technical and legal areas including fiber manufacturing, thread and yarn production, knitting and weaving, dyeing, fabric ageing, fiber transfer, transferred fiber collection, analysis, evidence preservation, establishment of the limits and conclusions of testimony, and finally, maintaining meaningful and current reference materials, test equipment and proficiency studies. This paper will focus on the area of dye analysis in fiber comparisons and the specific instrumental analysis techniques of visible spectromicrography and colorimetry. The discussion will concentrate on instrumental effects that can damage or skew data.

Keywords: Spectrophotometry; Colorimetry; Dyes; Fibers; Forensic analysis; Microscopy; Microspectroscopy; Spectromicrography

Colorimetry utilizing visible light spectroscopy, whether by transmittance, diffuse reflectance or fluorescence techniques, is hardly a new or lightly explored topic. This familiarity and the lack of caution it can breed in forensic laboratory operations have potentially serious consequences when they and a spectrophotometer system are coupled to a microscope.

The current sophistication of digital technology, user friendly software and instrumental sensitivity not only make spectromicrography possible but also possibly misleading to the reassured analytical scientist. This reassurance is fortified by work such as that done by Workman and Mark [1] that in part states, "In general, it seems safe to say that error variance is more the result of the sample than the instrument/calibration combination". I have no doubt that this is true for bulk analysis routines, but it may not be the case for microscopical analyses. Spectromicrography is the core of microscopical colorimetry and an essential tool in forensic fiber analysis and dye or pigment comparisons.

Dye analysis has long been noted as an important feature of forensic fiber comparisons due to the diversity of visually and microscopically indistinguishable dye materials. Schemes for dye analysis and discrimination have taken several forms. A number of researchers have developed methods for isolating dyestuffs from fibers [2–4] and also for comparing dyes and dye mixtures with thin layer chromatography (TLC) techniques [5–7]. Some of these methods were compiled and presented as an analytical protocol in 1983 by the Trace Evidence Study Group of the California Association of Criminalists [8]. This work has been recently extended to cotton and rayon fibers by Laing et al. [9].

Spectrophotometry of fiber dyes was discussed, along with other methods, in 1979 [10] and the

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proposed analysis made extensive use of industrial methods developed and in use for the previous two decades. The forensic application of fiber dye spectrophotometry was improved somewhat by the application of existing Chromaticity Color Coordinate analysis techniques in 1980 [11]. At the same time, numerous difficulties became apparent with all the existing approaches to fiber dye comparisons which involved dye extractions as one of their first steps. These difficulties centered around the fact that each loose fiber in a forensic investigation represents, and must be handled as, a unique item of evidence in the case.

Extensive work in 1974 [12] had shown that the average length fiber transferred between clothing materials was about 5 mm. Subsequent work by Lowrie and Jackson [13] has reemphasized the limited length and size distribution of retained transferred fibers in typical casework, with the result that the average recovered transferred fiber had a length of less than 2 mm, when removed eight hours after the transfers took place. This is significant because all of the mentioned dye analysis protocols required 5 to 20 mm of a single fiber for analysis and they all involved dye extraction. Extraction solvents were not universally applicable, dyes were destroyed by the methods, extractions were not complete for all dye components, evidence fibers were rendered useless for future comparisons or reanalysis and not all commonly encountered dyes could be characterized. Typically, the protocol described by Wiersema [14] involved three major steps.

(1) Identification of the fiber type (usually involving microscopy).
(2) Selection and sequential application of increasingly powerful extracting solvents to identify dye types.
(3) Selection of appropriate TLC systems to discriminate extracted dyes.

Although these procedures and their limitations pose few if any problems when large samples of fiber or fabric are available for comparison, they represent substantial difficulties in typical forensic fiber comparisons. Many of these difficulties were noted by Paterson and Cook [15], and these authors proposed the in situ (in fiber) spectromicrographic comparison of dyes and the use of the International Commission on Illumination (CIE) Chromaticity Coordinate System established in 1931. The system is used throughout the dye and pigments industries. Later versions of the CIE system are extent but offer little advantage over the 1931 coordinate system for this type of instrumental work. Several sources are available that provide simple to detailed explanations of the CIE system [16–19]. In general terms, it is a method for graphically describing and distinguishing the multitude of color combinations, intensities and illuminants that produce visual impressions of color in a normal or "standard observer's" eye.

In 1985 Laing et al. [20] published a procedure using this system of colorimetry on normally mounted, single fiber samples. The procedure eliminated virtually all of the limitations previously noted for dye extraction methods. It should be added that a substantial degree of discriminating power was achieved with spectromicrography, by the simple comparison of raw visible spectrum absorption curves [21]. Normalization routines and standard deviation analysis offer additional discriminating power, but they were not directly pursued in the noted work. The procedure, some of its errors, background absorbance sources, and sample effects were discussed in 1986 [22]. The use of this or a similar system would seem to be unavoidable if colored fiber comparisons are to withstand the challenges of sample preservation, reanalysis before trial and cross-examination during testimony.

The balance of this paper will dwell on microscopical and spectrographic considerations necessary for producing useful colorimetric data from short lengths of single fibers. The goal is to yield data that is accurate and reproducible throughout the workday, as well as over a period of years, free of optical aberrations and suitable for comparison between laboratories. These considerations are critical in protracted or serial crime investigations that can sometimes take years to even recognize and often span numerous geographic and jurisdictional boundries before they are resolved. The considerations are also essential to addressing and withstanding challenges through opposing party reanalysis and testimony.

EQUIPMENT

The instrument used in this study is shown diagramatically in Fig. 1. It is a Zeiss UMSP-50 microscope spectrophotometer with a regulated 12 VDC, 100 W tungsten halogen, dual mirror illuminator. Optics are Neofluor with a 16×, 0.40 N.A. objective utilized as a condenser and a 25×, 0.60 N.A. objective. Sampled areas of slide mounted specimens are masked by user selectable, circular luminous field stops below the condenser and circular measuring apertures between the objective and monochromator.

The grating monochromator in the system has a mechanically ruled grating of 610 grooves/mm and blaze at 450 nm. Adjustable slit widths yield a selectable 1 to 28 nm bandwidth that passes the scanned image beam to a photomultiplier detector and an amplifier with software driven, three order of magnitude, gain switching during the scan.

Unless otherwise noted, fibers were mounted on 1 mm thick slides under No. 1–1/2 cover slips with Norland-NOA 65 optical adhesive (Norland, New Brunswick, NJ). This UV cured optical adhesive has a $\eta_D = 1.524$ and a $\eta_F - \eta_C \approx 0.018$.

Calibration scans were run with 1 nm bandwidths in 0.5 nm steps and sample scans were run with 5 nm bandwidths in 2.0 nm steps. The intensity at each monochromator step was measured ten times and the mean value was stored as a function of wavelength. The data was digitized through a Zeiss MPC 64 A–D/D–A converter and processed by Zeiss LAMBDA-SCAN software on an IBM-XT computer. Microscope shutters, monochrometer scan motor and filters are computer driven through the converter.

The wavelength calibration standard used is a holmium oxide glass plate versus a "blank" or "clear" reference standard consisting of an equal thickness of borosilicate glass, both mounted on a single glass slide with Norland-NOA 65 adhesive. Absorbance band positions were evaluated against reference wavelengths reported by McNeirney and Slavin [23] and used in ASTM Standard E275-83 [24]. This standard also includes a useful

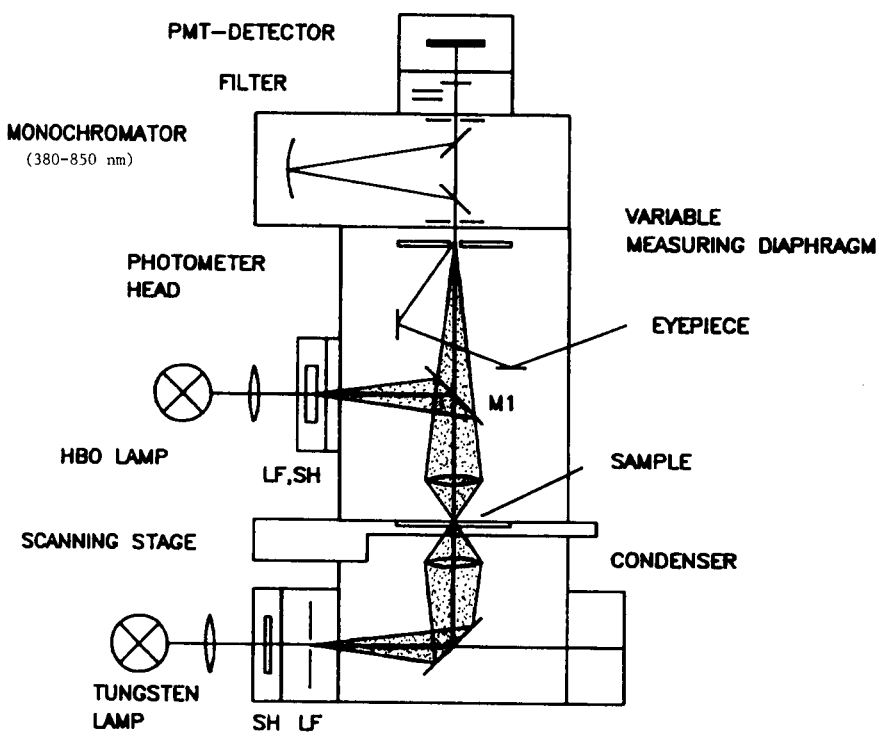

Fig. 1. Zeiss UMSP-50 microscope system (with permission).

form for documenting routine calibration data. Monochrometer errors were corrected by grating position offset during instrument software setup at the beginning of each work session. Sampling steps greater than 0.5 nm during calibration scans are not useful due to the nonsymmetry of most absorption bands in the holmium oxide glass standard. Monochrometer calibration offset over a 380 to 780 nm scanwidth has not exceeded 1.5 nm with the subject system over a two year period.

In normal casework, sampling steps and/or bandwidths of more than 5 nm have not been used because they lead to a rapid loss of inflection point discrimination that is vital to the critical evaluation of absorbances in metameric materials.

System responses including the blank luminance scan (S), object luminance scan (O) for holmium oxide glass and background corrected object or "quotient" spectrum ($Q = O/S$) are shown in Fig. 2. The benefits of automatic gain switching are exhibited at the spectrum's short wavelength extreme.

INSTRUMENTAL EFFECTS

Illuminator stability

Illumination stability and the reproducibility of luminance and color temperature are subject to several long and short term effects. An obvious first concern is power supply stability.

Standard microscope illuminator systems are not stabilized or closely regulated but are quite adequate for normal observation and photomicrography. The lamp used in this system exhibits an over 99% or 2 absorbance units (2 A.U.) change in luminance, and a luminance maxima peak shift of ≈ 50 nm between 4 VDC and 12 VDC (Fig. 3), or more usefully, an approximately 0.013 A.U. luminance change per 0.1 VDC and

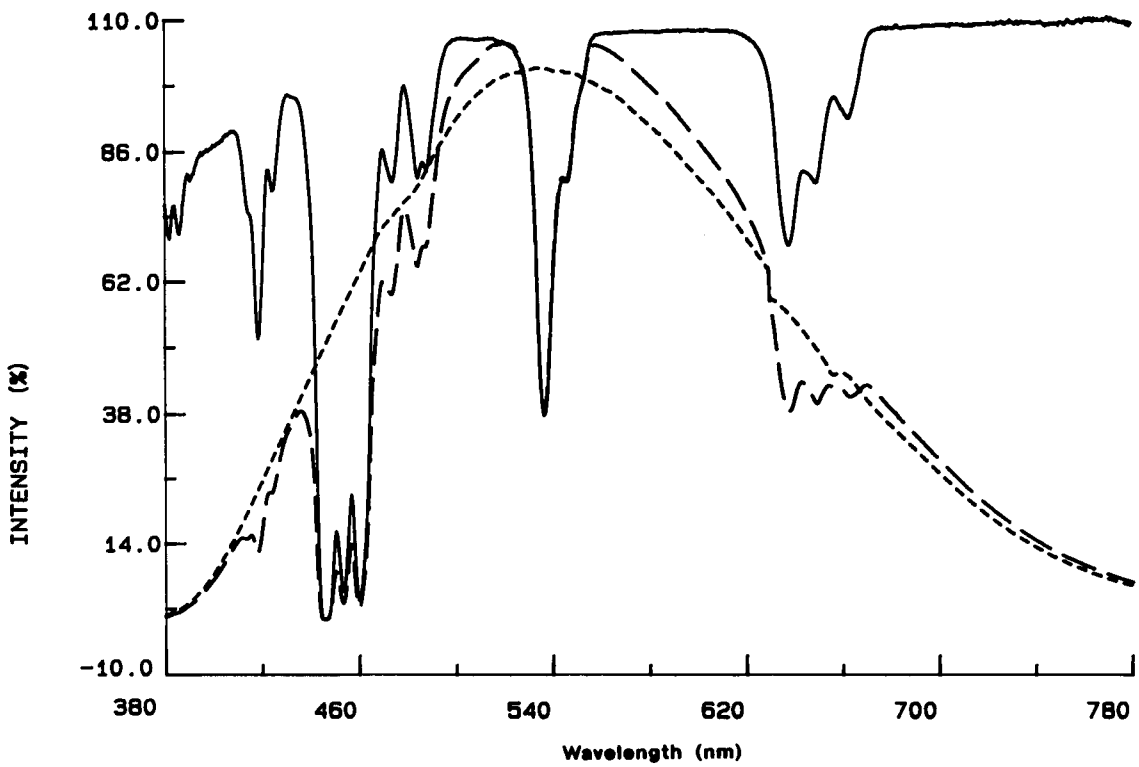

Fig. 2. Clear field (blank) system response, relative to a holmium oxide glass absorbance standard reference sample. Standard BSiO glass on slide, - - - - - -. Standard HoO glass on slide, — — —. Corrected HoO spectrum, ———.

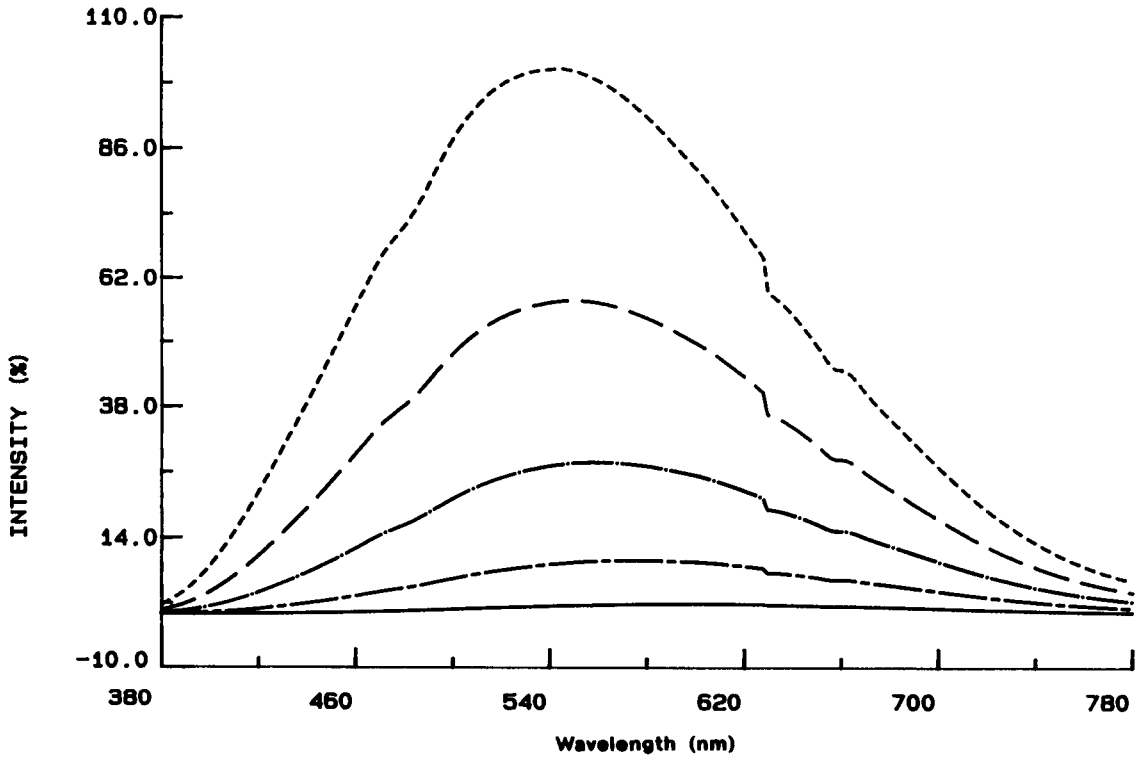

Fig. 3. System luminance response to varied illuminator power supply voltages. 12.00 VDC, - - - - - -; 10.00 VDC, — — —; 8.00 VDC, ·–·–·; 6.00 VDC, - — - — -; 4.00 VDC, ———.

0.7 nm luminance peak shift per 0.1 VDC. Common research grade microscopes in the author's possession show short term line voltage generated fluctuations of 1.0 VDC and 4 h voltage drifts of 0.5 VDC. The system used in this work is powered by a stabilized and regulated power supply with an integral digital voltmeter to ensure luminance reproducibility. The system is typically stabilized after 15–30 min from a cold startup (22°C) with a < 0.1 VDC voltage rise between startup and stabilization.

Illuminator voltage sensitive luminance and wave length shifts seem slight until they are applied to the 12.00 VDC system reference luminance spectrum (S) with slopes between 1.5 and 2 A.U. per 150 nm. The resulting quotients ($Q = O/S$) or reference corrected intensity spectra show significant changes. These clear field (slide, mountant, cover slip) spectra at 12.00, 11.90 and 11.50 VDC are shown in Fig. 4. Changes in chromaticity data between the reference voltage and two lower lamp voltages are shown in Table 1. Although the resulting chromaticity value changes might be considered insignificant for deeply dyed fibers, the shifts are dramatic when applied to pastel and "earth tone" colored fibers in popular fabrics today. Such fibers are almost visually colorless under the microscope and present a situation where spectromicrography is essential to their discrimination and comparison.

Köhler illumination

Spectromicrography systems and resulting chromaticity coordinate calculations are profoundly affected by alignment and focusing errors. Effects include signal to noise ratio (S/N) reduction and sensitivity loss, peak shifts and resulting chromaticity coordinate shifts.

Köhler illumination requires that the luminous field diaphragm, object image and measuring field aperture all be in coincident focus, and in optical alignment with the microscope lamp filament and

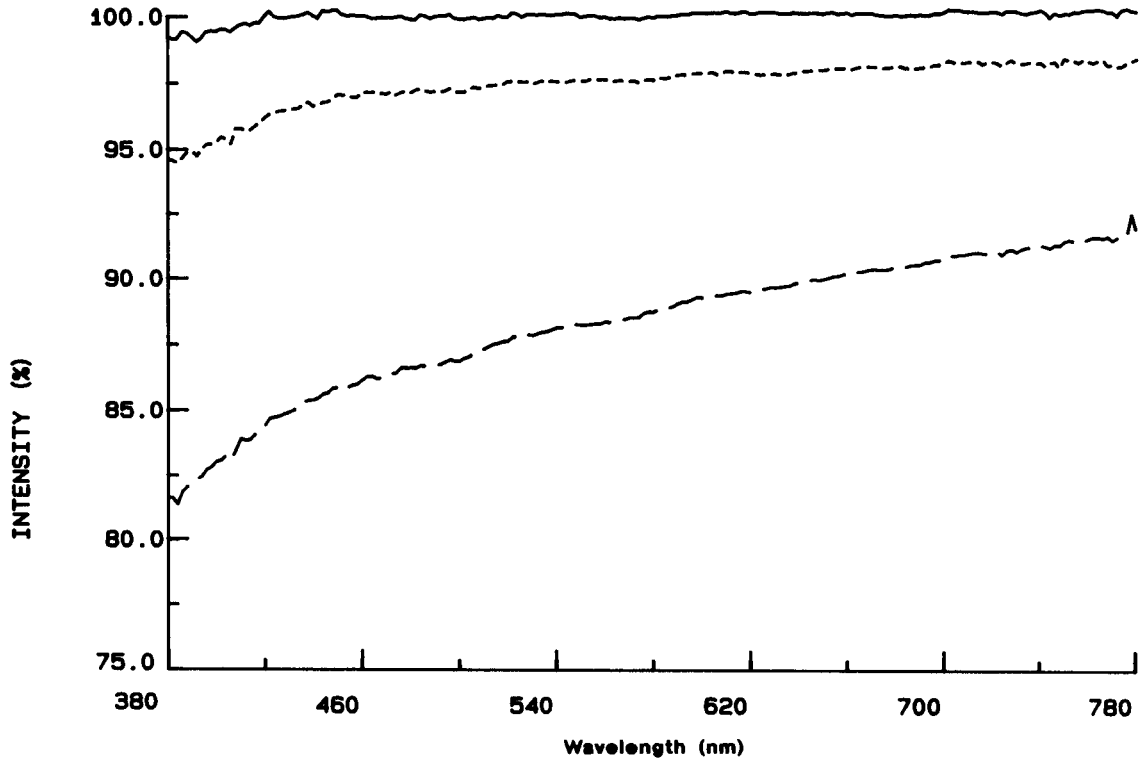

Fig. 4. Background corrected system response to illuminator power supply voltage changes of up to 0.5 VDC. 12.00 VDC, ———; 11.90 VDC, - - - - -; 11.50 VDC, — — —.

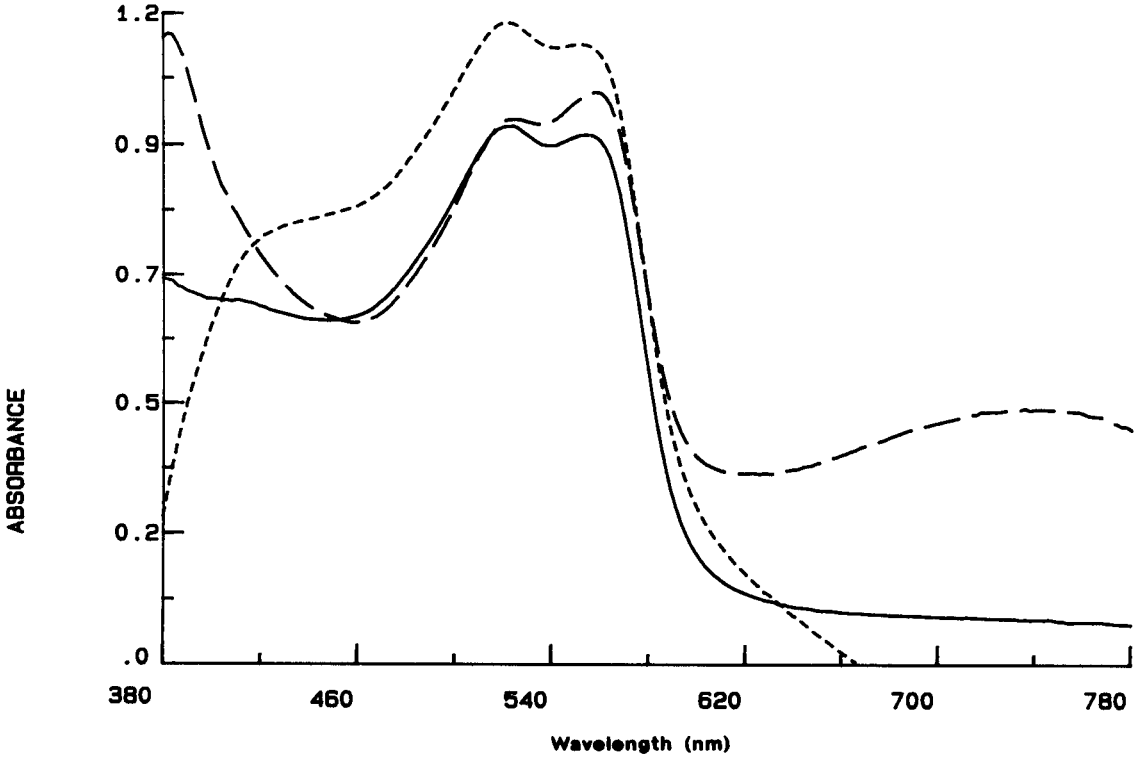

Fig. 5. Background corrected absorbance spectrum variations produced by condenser focusing errors with luminous field stop in place. Condenser in focal plane, ———; condenser below focal plane, - - - - - -; condenser above focal plane, — — —.

the photomultiplier detector. The lamp filament must also be focused at the back focal plane of the objective. These represent the minimum illumination conditions necessary for optimum and, most importantly, reproducible analyses.

Characteristically, a luminous field stop or field diaphragm will exhibit a magenta diffraction fringe at its edge when the stop or diaphragm is in focus with the objective. This fringe will shift to blue as the condenser is lowered below the focal point and to red as it is raised above the focal point. The fringe will also shift off the field stop centerline as the condenser is moved if the lamp assembly, condenser and/or objective are not centered, or if the stage, slide and coverslip are not normal to the system's optical centerline.

The color fringe seen at the edge of the luminous field stop represents scattered light diffracted or extracted from the analytical beam. Simply moving from one point to another on a single fiber or from one fiber to another on a single slide preparation, with normal focal plane changes of 5 to 10 μm, is sufficient to shift fringe colors from magenta to red or blue. Small condenser defocusing effects are dramatic (Fig. 5).

A spectroscopist might attempt to circumvent this effect by simply removing any and all luminous field stops. This does remove spectral shift effects but light intensity variations remain and S/N is reduced (Fig. 6). Making separate background or blank scans for each individual fiber focal point presents an almost unmanageable data "bookkeeping" problem, not to mention doubling analysis time, that can be simply avoided by refocusing the condenser just before each spectral scan. In addition, the deleterious effects of focusing errors can be minimized by using measuring apertures that are 2–3 times the size of their associated luminous field stop, and field stops that are smaller than the object of interest.

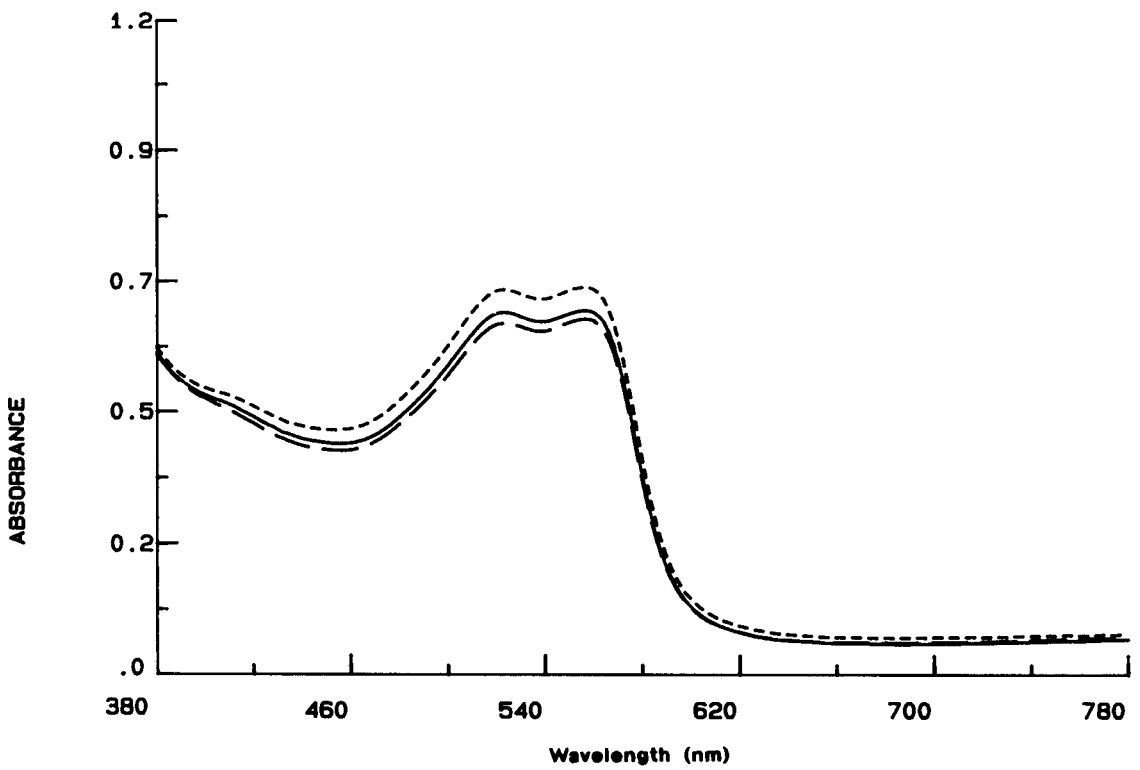

Fig. 6. Background corrected absorbance spectrum variations and S/N loss produced by condenser focusing errors in the absence of luminous field stops. Condenser in focal plane, ———; condenser below focal plane, - - - - -; condenser above focal plane, — — —.

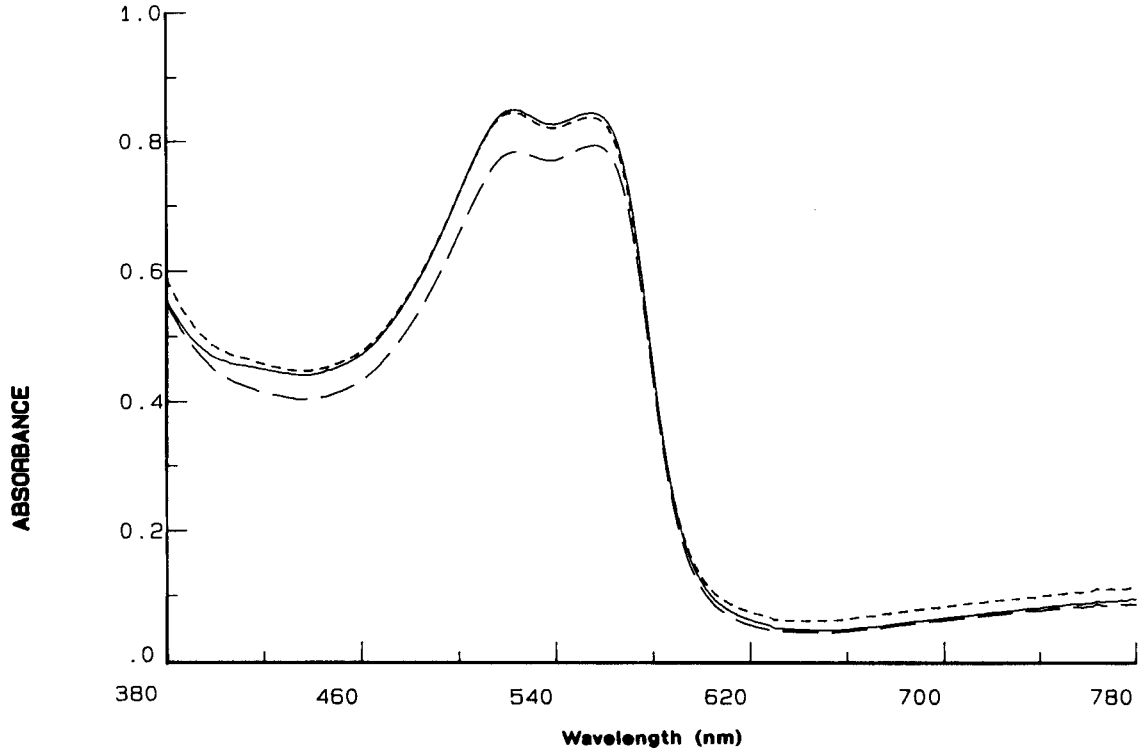

Fig. 7. Background corrected absorbance spectrum variations produced by object focusing errors and Bécke line interference. Object in focus, ———; object below focus, - - - - -; object above focus, — — —.

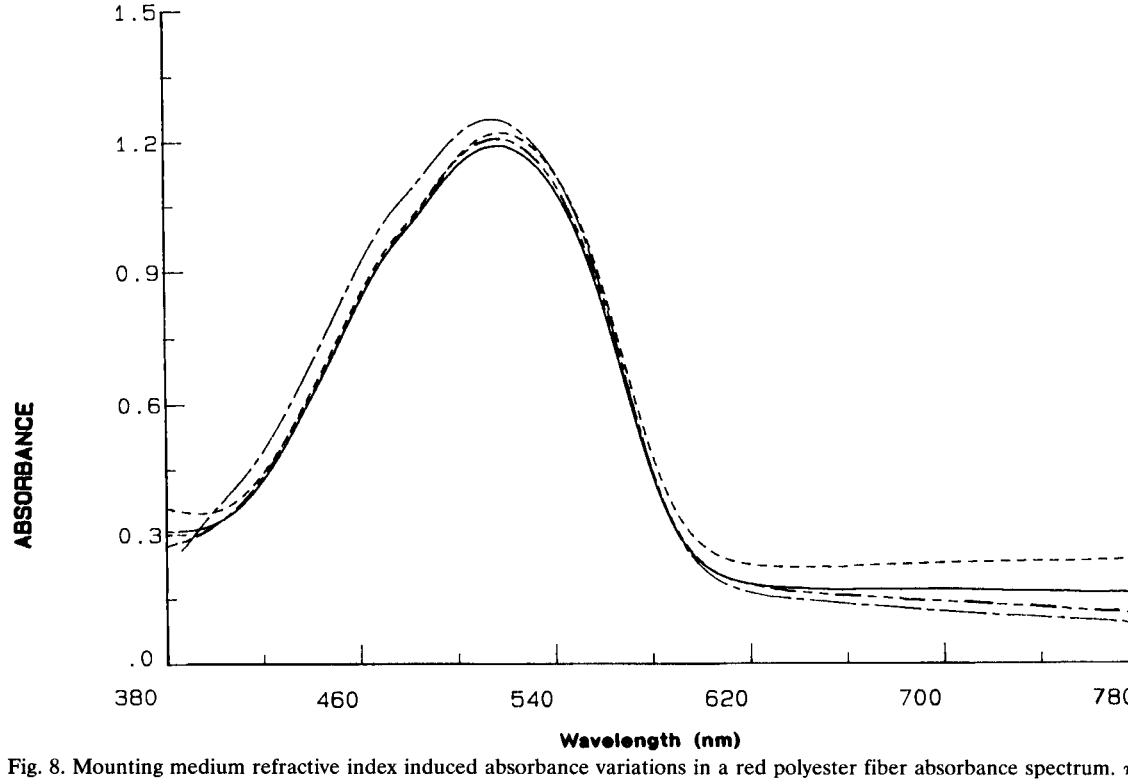

Fig. 8. Mounting medium refractive index induced absorbance variations in a red polyester fiber absorbance spectrum. $\eta_D = 1.524$ ———; $\eta_D = 1.424$ - - - - -; $\eta_D = 1.624$ — - - —; $\eta_D = 1.725$ - — - — -.

TABLE 1

Effects of illuminator voltages on chromaticity values (see Fig. 4) vs. 12.00 VDC standard

Illuminant:		A	C	D65	E
12.00	X	109.96	98.15	95.12	100.09
12.00	Y	100.06	100.05	100.05	100.05
	Z	35.60	118.28	108.94	100.04
	x	0.4477	0.3101	0.3128	0.3334
	y	0.4074	0.3161	0.3290	0.3333
	z	0.1449	0.3737	0.3582	0.3333
	dE *	0.07	0.06	0.06	0.06
11.90	X	107.42	95.72	92.78	97.64
12.00	Y	97.69	97.62	97.62	97.64
	Z	34.51	114.57	105.54	96.89
	x	0.4483	0.3109	0.3135	0.3342
	y	0.4077	0.3170	0.3299	0.3342
	z	0.1440	0.3721	0.3566	0.3316
	dE *	1.03	1.05	1.04	1.05
11.50	X	97.84	86.70	84.05	88.54
12.00	Y	88.65	88.35	88.34	88.41
	Z	30.62	101.51	93.51	85.82
	x	0.4506	0.3135	0.3161	0.3370
	y	0.4083	0.3195	0.3322	0.3364
	z	0.1411	0.3670	0.3517	0.3266
	dE *	5.00	5.03	5.03	5.04

Object focus

Accurate and reproducible object focusing is also an essential part of microscopical colorimetry. The culprit here is a Bécke line. The Bécke line will move into the analytical beam from the edge of the object fiber as the object focal plane is raised, when the object has a higher refractive index than the mountant. The result is to lower the S/N and the apparent absorbance of the object as shown in Fig. 7 where red cotton ($\eta_{\parallel} \approx 1.58$, $\eta_{\perp} \approx 1.53$) is mounted in low dispersion media with $\eta_D = 1.524$. If the relative refractive indices of object and mountant are reversed, this effect appears while lowering the focal plane below the object. Again, focal plane changes of only 5 to 10 μm are sufficient to cause the effects.

Mounting media

Increasing the mismatch between object and mountant refractive indices makes focusing effects more pronounced and more difficult to avoid. This is exhibited in Fig. 8, where a polyester fiber, of a nominal $\eta_{iso} = (2\eta_{\perp} + \eta_{\parallel})/3 = 1.60$ for ($\eta_{\parallel} \approx 1.70$, $\eta_{\perp} \approx 1.55$), is mounted in four different liquids of similar dispersion but varied refractive indices. The fiber in this case was immobilized on a slide and a single spot on the fiber measured repeatedly under different mounting liquids with the aid of a digitally controlled specimen stage and the fortuitous placement of a few delusterant particles. The close similarity of the curves is somewhat misleading as both object focus and luminous field stop focus became more difficult to achieve as mountant η_D values departed from 1.624.

Similar difficulties arise as the dispersion ($\eta_F - \eta_C$) of the mountant increases and tends to intensify the dispersive effects at the specimen edges. Reproducible results and improved operational ease require the use of a stable, low dispersion mountant with a refractive index close to that of the specimen.

Polarized illumination

Virtually all dyed fibers exhibit some degree of pleochromism or a color variation dependent on the fiber's orientation in a field of plane polarized light. This is a valuable point of fiber comparison with a polarized light microscope but is obviously not desired in the spectromicrography of randomly oriented fibers. Microscopes with any plane polarizing optics or dielectric mirror surfaces will introduce varying amounts of plane polarization to the illuminating beam. Metallic mirror surfaces without dielectric scratch resistant coatings are essential to colorimetric work. In short, the microscope needs to have been selected with spectroscopy in mind.

None of the forgoing effects need prove detrimental to a fiber comparison and are easily avoided once their symptoms are recognized. Avoiding them can easily become second nature.

ADDITIONAL INFORMATION

A rather dated (1949) but still valuable source of spectrophotometry considerations, errors and a discussion of standard materials is given by Gibson [25]. Although now out of print, it is still available through most technical libraries and is quite rigorous and informative.

The optimization of microscopical illumination and resolution can be confusing for spectroscopists who are working with a microscope for the first time. McCrone et al. [26] present a concise description of Köhler and other illumination modes with clear directions for adjusting a microscope to achieve each mode. Smith [27] also describes microscope adjustment with an eye toward photomicrography but many of his examples and explanations are equally useful for the spectromicrographer.

Currently used definitions of colorimetric terms are reviewed and explained by Hunt [28] while microscopical definitions are detailed by Aschoff [29].

Conclusions

The purpose of this paper is to present some of the reasons for, and significant considerations behind, the use of spectromicrography in the colorimetry of short single fibers. The items discussed are essential to yielding the reproducible results necessary for comparative analysis. The technique is distinguished from other applications of spectroscopy and colorimetry by the refractive and dispersive effects indigenous to microscopical analysis. Although these effects are well known to microscopists, their impact on spectroscopy is not generally self-evident. It is my hope that this discussion will prove useful to forensic fiber and paint analysis experts.

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A new approach to forensic analysis with infrared microscopy: internal reflection spectroscopy

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Abstract

A preliminary study has been conducted on the application of microscopical infrared internal reflection spectroscopy for the analysis of forensic evidence. Internal reflection spectra have been obtained from single fibers, hairs, paint chips, vehicle rubber bumpers, photocopy toners, carbon copies, writing ink on paper, lipstick on tissue and black electrical tapes. The technique is convenient, non-destructive and often permits spectra of smears to be obtained in situ. The technique shows great potential for analysis and comparison of forensic evidence.

Keywords: Infrared spectrometry; Forensic analysis; Microscopy, IR

Microscopical analysis with infrared (IR) spectrometry has greatly advanced trace analysis in forensic science. A wide variety of samples can be analyzed. Two common examples are single fibers [1–3] obtained as evidence at violent crime scenes and paint chips [4,5] found in hit and run cases. Infrared data are collected most frequently as transmission spectra, because (1) transmission is the traditional method, (2) reference spectra are usually in transmittance, and (3) reflectance and transmittance yield different spectral responses. Reflectance spectroscopy, however, requires less sample preparation, and is more convenient for certain specimens. Reflection–absorption [6,7], specular [6,8], and diffuse reflection [6–9] have been reported as IR microscopical techniques in the literature. Internal reflection spectroscopy, while commonly used on a macroscopic scale, has only recently become available as a microscopical technique [10,11].

Internal reflection spectroscopy (IRS) [a] has historically been used for surface analysis of samples which are too thick for transmission measurements [12–14]. IRS requires little or no sample preparation and has been very useful for

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[a] Internal reflection spectroscopy is the accepted nomenclature of the American Society for Testing and Materials (ASTM) for this technique [12]. The term attenuated total reflectance (ATR), from Fahrenfort [13], is also widely used. The manufacturer of the microscope objective described in this article refers to the device as an ATR objective.

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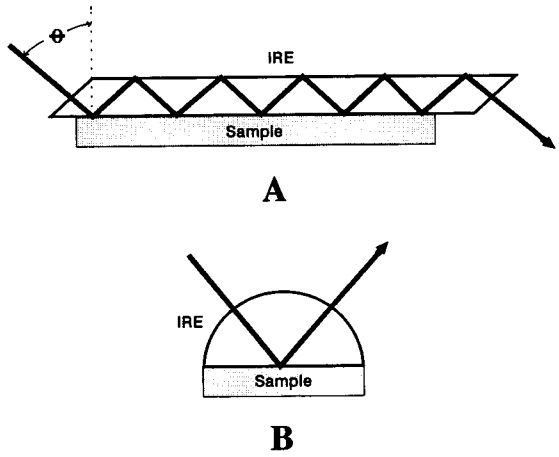

Fig. 1. (Upper) Multiple internal reflection in a parallelogram internal reflection element (IRE). (Lower) Single internal reflection in a hemispherical IRE.

surface analysis of bulk polymers, adhesive surfaces, and surface layers of laminates. An in-compartment accessory for IRS microsampling has recently been described [15], but its visual microscope optics are not coaxial with its infrared optics. Therefore, the ability to isolate small areas for analysis on the specimens is hindered. The recently introduced ATR microscope objective has the significant advantage of allowing the analyst to view the specimen directly where the measurements are taken. The method is nondestructive and generally allows the analysis of specimens in situ on evidence such as garments or documents. The work presented in this report is a preliminary study of the application of microscopical infrared IRS for the analysis of physical evidence.

INTERNAL REFLECTION SPECTROSCOPY

A typical experimental arrangement for obtaining internal reflection spectra of macro-sized samples is shown in Fig. 1A. A high refractive index, infrared transparent material and lower refractive index sample are interfaced in close contact with one another. When the IR beam's angle of incidence to the interface exceeds the critical angle, the beam undergoes multiple internal reflections in the IR transparent material. This material is called the internal reflection element (IRE) and is frequently referred to as an ATR crystal. Although the beam is totally reflected at the internal interface, the radiation's electrical field penetrates a small distance into the sample. This penetrating field is called the evanescent wave. If the sample absorbs the IR radiation at a particular wavelength (or frequency), the reflected beam is attenuated at that characteristic wavelength corresponding to an absorption band. This phenomenon is known as attenuated total reflectance (ATR) and the mea-

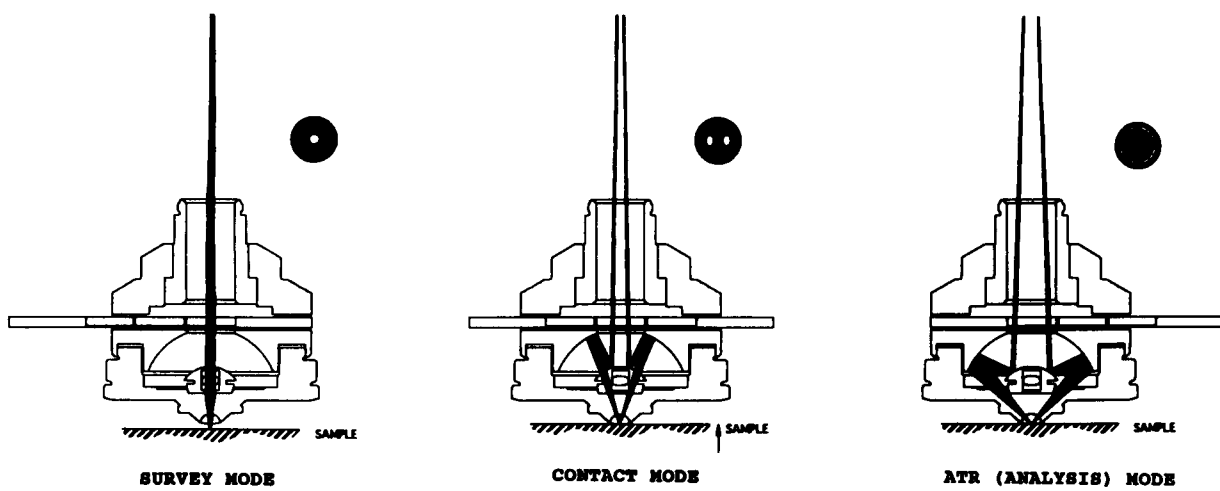

Fig. 2. Cross sections of the ATR objective and top view of the Fourier plane masks for the three modes of operation.

surement provides ATR spectra. Attenuation due to absorption can occur at each reflection and the sample may be placed on one or both sides of the IRE.

The depth of penetration, defined as the distance required for the electric field amplitude of the evanescent wave to fall to 1/e of its value at the surface, is given by:

$$d_p = \frac{\lambda}{2\pi\eta_e\left[\sin^2\theta - (\eta_s/\eta_e)^2\right]^{1/2}}$$

where λ is the wavelength in air, θ is the angle of incidence and η_s and η_e are the refractive indexes of the sample and IRE, respectively. Note that d_p is directly proportional to wavelength, thus ATR spectra display bands of increased absorption at longer wavelengths compared to transmission spectra. This wavelength dependence can be corrected with the appropriate computer software to make the spectra appear more like transmission spectra. In addition, peak shapes will differ between the two types of spectra because in ATR the peak shape is not only a function of absorption coefficient, but also a function of refractive index. This can introduce slight peak shifts and asymmetries in the band profile.

OBJECTIVE DESIGN

In contrast to the parallelogram design IRE shown for the macro configuration, the IRE used in the ATR objective is a single-reflection, hemispherical design as shown in Fig. 1B. The details of the ATR objective design are shown in Fig. 2. The ATR objective combines a Schwarzchild reflecting lens and an IRE for spectral measurement with a refractive lens for viewing through the IRE for visual microscopy.

The objective provides three operational modes: (1) the survey mode, (2) the contact mode and (3) the ATR (analysis) mode. These modes are produced by combining two coaxial optical systems with Fourier plane masks to separate each mode. Masks placed in the objective's Fourier plane define the angular aperture ranges needed to separate the three operational modes. The Fourier plane masks are on a slide, simplifying the change-over between modes. The survey mode allows visible light viewing to locate the microscopic area for analysis. In the survey mode visible light strikes the IRE at near-normal incidence allowing the analyst to view the sample. The refracting lens system used for near-normal survey viewing provides a free working distance of 1.5 mm. The sample must be in contact with the crystal for IRS. Because sample contact with the IRE restricts sample movement, the survey process must precede sample contact. Once an area of analysis is selected, the contact mode is used. In the contact mode, visible light strikes the IRE at less than the critical angle. This allows the analyst to view the crystal's surface as the sample is raised to make contact. After contact is made, the ATR mode is selected for data acquisition. In this mode, infrared radiation strikes the IRE at an angle greater than the critical angle and the internal reflection spectrum is acquired.

In general, the objective is convenient to use and produces high quality infrared spectra. The viewing mode is very useful when the samples are transparent or have a high specular reflectivity. For low or diffusely reflecting samples, it is sometimes necessary to use another objective lens for initial viewing. When contact between the sample and the IRE is observed in the contact mode, a significant reduction of the reflected light intensity occurs where contact takes place. While there is generally a clear indication of contact, in some instances contact cannot be seen. In such cases, while in the ATR mode, the observation of the interferogram as contact is made permits one to determine the contact position by a reduction of signal intensity.

For a ZnSe crystal, the ATR measurements require the radiant energy be incident at an angle greater than 45° to the crystal–sample interface. This high aperture is achieved with the reflective lens and hemispherical crystal. The hemispherical crystal also functions as an immersion lens, reducing the visible area of the analysis by the index of refraction of the IRE (2.4 times). Hence, when observing an apparent 100 μm diameter area, the radiation is only falling upon a 42-μm diameter spot of the sample. Because only a

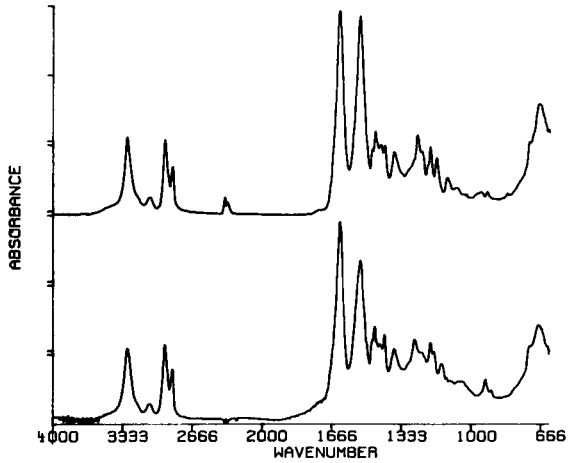

Fig. 3. ATR spectra of: (Upper) Nylon-6 truck carpet fiber. (Lower) Nylon-6,6 commercial building carpet fiber.

small area of the IRE makes contact with the sample, the contact efficiency is very high and produces high quality, single-reflection spectra.

EXPERIMENTAL

Spectra shown in Figs. 3–6 were obtained using a Nicolet 20SXC FT-IR spectrometer (Madison, WI) and Spectra-Tech research grade IR Plan microscope with Spectra-Tech ATR microscope objective (Stamford, CT). Spectra were acquired from 4000 to 650 cm^{-1} using a 0.25-mm, medium band, mercury cadmium telluride (MCT) detector. The spectra were obtained at 4 cm^{-1} resolution. The number of scans co-added for each spectrum varied with the sample type. Spectra shown in Figs. 7 and 8 were obtained using a Spectra-Tech IRμS^{TM} microscope/spectrometer system also equipped with a Spectra-Tech ATR microscope objective. Spectra were acquired at 8 cm^{-1} resolution and 250 scans (2 min) were co-added per spectrum. The majority of the spectra presented were baseline adjusted for flatness.

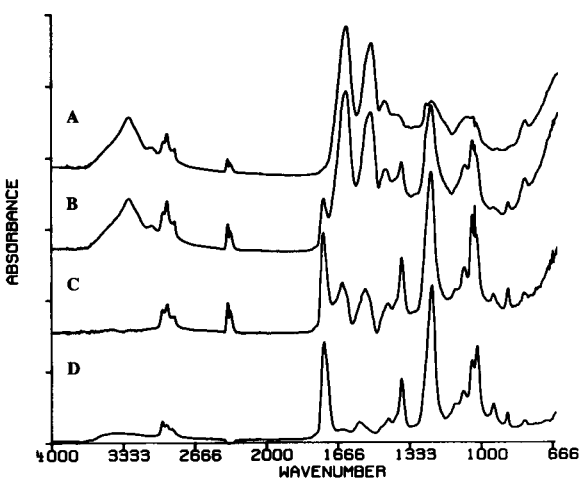

Fig. 4. ATR spectra of: (A) hair, (B) hair with hair spray, (C) difference spectrum obtained by subtracting spectrum A from spectrum B, (D) hair spray reference.

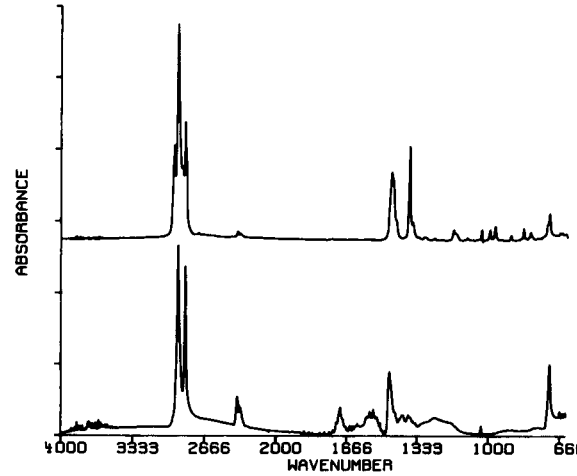

Fig. 5. ATR spectra of rubber chips from truck bumpers.

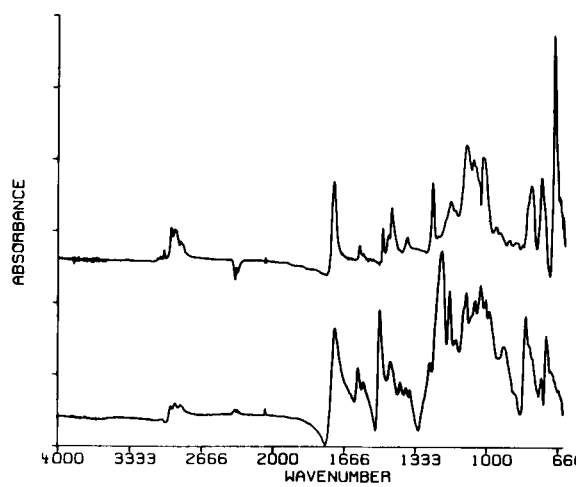

Fig. 6. ATR photocopy toner spectra.

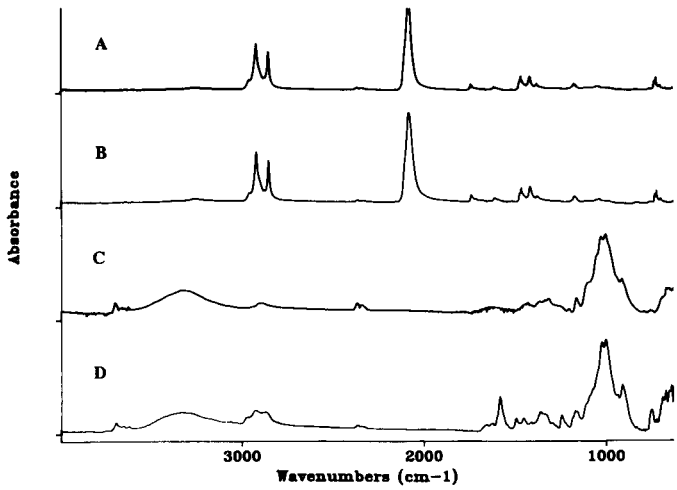

Fig. 7. ATR spectra of suspected carbon copy alteration: (A) carbon paper surface, (B) transferred carbon, (C) blank paper, (D) altered area.

The samples were placed on glass microscope slides for analysis. Other sample supports could have been used, however glass flexes slightly, thus reducing the chance of damaging the ZnSe crystal if too much pressure is applied. Tape was applied to the underside of the slide to prevent shattering if the glass broke.

RESULTS AND DISCUSSION

Many types of evidence lend themselves to infrared analysis using microscopical IRS. Fibers are frequently encountered as evidence in violent crimes. Single fibers are typically flattened and transmission spectra obtained [1–3]. Nylon carpet fibers are usually thicker than those originating from garments, and range on the order of 60 to 80 μm in diameter. The thickness and hardness of nylon make these fibers very difficult to flatten sufficiently for IR transmission analysis. Microscopical IRS is a very convenient technique for acquiring infrared spectra of these and other fibers. Figure 3 shows the ATR spectra of a nylon-6,6 fiber from a commercial building carpet (lower spectrum) and a nylon-6 fiber from a truck

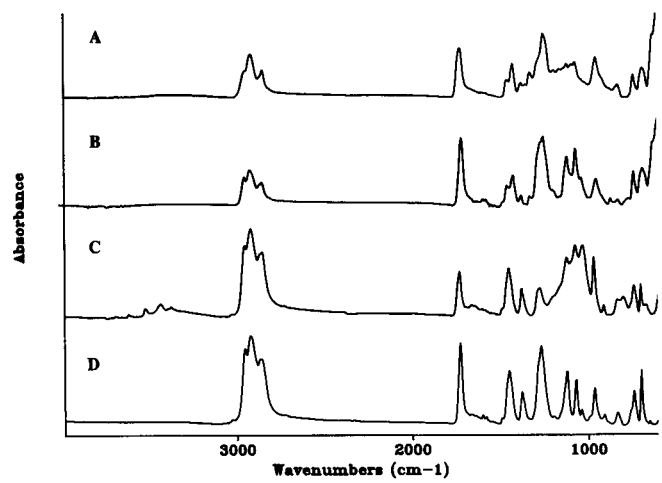

Fig. 8. ATR spectra of two different brands of black electrical tapes: (A and B) backing sides; (C and D) adhesive sides.

carpet (upper spectrum). Most pronounced in these spectra are the bands common to poly(amides): the N–H stretching band near 3300 cm^{-1} and the amide I and II bands near 1640 and 1540 cm^{-1}, respectively. The distinguishing features which differentiate the two types are the small, sharp 935 cm^{-1} crystalline band of nylon-6,6 and other small differences in the crystalline region from 1330 to 1000 cm^{-1}.

Hairs are also frequently found at crime scenes. The IR spectra of hairs are not readily distinguishable between individuals. The presence of hair spray or other coatings on hair, however, can significantly increase the value of the evidence. Fig. 4 shows ATR spectra of clean hair (spectrum A) and the same hair coated with hair spray (spectrum B). The difference spectrum obtained by subtracting spectrum A from spectrum B is shown in spectrum C, while a reference spectrum of the hair spray is shown in spectrum D. The difference spectrum closely resembles that of the hair spray reference except for the presence of residual amide bands near 1650 and 1530 cm^{-1} which could not be completely subtracted. Additional work is required to determine the compositional variation of composition of hair sprays and thus their spectra to establish the capability to discriminate between these products.

Hit-and-run accidents result in the transfer of material from the automobile to the victim [4]. These materials include chips and smears of automobile paint and rubber bumpers. Figure 5 shows ATR spectra of rubber bumper chips from two different vehicles. The analysis time for each spectrum was 4 min. The upper spectrum is indicative of poly(ethylene–propylene). The lower spectrum is that of poly(ethylene) with additives indicated by bands near 1670, 1500 and 1200 cm^{-1}. Spectra of bumpers from other autos have revealed poly(vinyl chloride) (PVC) based material. This variation of materials can sometimes permit discrimination between vehicle types for investigative purposes. Spectra of paint chips have also been obtained by this method.

This method demonstrates the potential for obtaining spectra of transferred smears of various materials while still resident on victims' clothing or other substrates. This capability would be non-destructive and require no sample preparation. Spectra have been obtained from lipstick smears on tissue paper. However, the ability to obtain spectra from auto paint and rubber bumper smears has not been extensively investigated as yet. Work is planned in this area.

The identification of photocopy toner manufacturers can play an important role in document cases. Lennard and co-workers [16,17] have reported a solvent extraction method with subsequent IR analysis by diffuse reflectance. While this is a viable method, it is both time consuming and destructive. With the application of microscopical IRS, however, spectra may be obtained in a few minutes without removing the copy toner from the document. The ATR spectra shown in Fig. 6 were obtained directly from photocopies made on different brand copiers. Total analysis time for each sample was 2 min. Spectral differences which indicate different polymer binders can readily be observed. The upper spectrum displays small bands near 3050, 670 and 700 cm^{-1} indicating a mono-substituted aromatic component, possibly from poly(styrene–butadiene), a common binder. Both spectra display carbonyl bands near 1730 cm^{-1} and C–O stretching bands in the region between 1200 and 1000 cm^{-1}, which are indicative of ester plasticizers in the binders. Different esters are apparent based on the band positions. The bottom photocopy toner spectrum displays a downward slope above 1700 cm^{-1}. This is due to dispersion from the carbon black filler which has a large refractive index change in that frequency region. The dispersion does not interfere with the interpretation of the spectrum.

Forensic document examinations also often involve the analysis of writing inks. Ink analysis has historically included comparison by thin-layer chromatography (TLC). More recently, an IR spectrometry method has been developed [18]. As with the photocopy toner method cited previously, this method consists of an extraction procedure with subsequent analysis by diffuse reflectance. If viable, the non-destructive microscopical IRS method could greatly facilitate ink examination. This approach has been demonstrated for a document case where a carbon copy was suspected to have been altered. Figure 7

shows a series of ATR spectra which confirms the alteration of the document. Spectrum A was obtained from the carbon paper and spectrum B was obtained from the carbon transfer where the writing was duplicated. The spectrum from the transfer matches that of the carbon paper as expected. Spectrum C was obtained from blank paper and is typical of cellulose. Spectrum D was acquired from a carbon copy area that was suspected to have been altered. The absence of the carbon copy spectral bands, along with the presence of additional bands superimposed on the paper bands, indicate that the document had indeed been altered. The additional bands appear to be those of a writing ink.

Pressure sensitive adhesive tapes are encountered frequently in forensic examinations. Tapes are used in the construction of bombs, for binding and gagging victims, and in the packaging of drugs. Several authors have reported the application of IRS as a fast means of obtaining infrared spectra of both tape backings and adhesives [19–21]. A study in our laboratory [21] using a standard-sized ATR accessory has shown the comparison of tape backing and adhesive spectra to be of great value and has proven very useful in case work. A considerable amount of tape is typically found in forensic cases, however, it is often difficult to find a clean area large enough to analyze with a standard-sized IRS accessory. Again, microscopical IRS is useful for this type of evidence. The spectra of black electrical tape adhesives and backings are shown in Fig. 8. Spectra A and B compare two common supplier's tape backings. The backings are both PVC films but differ in the plasticizer used. Spectra C and D were obtained from the adhesive sides of these two tapes. Adhesives differ extensively between different types of tapes with variations in rubber bases, oils, and tackifiers. Thus, adhesives provide much more discriminating value as evidence than do backings.

Conclusion

The application of microscopical infrared internal reflection spectroscopy has been demonstrated as a fast, simple and nondestructive method of analysis. This preliminary study has indicated that its application to forensic analysis should be an extremely useful and important approach to the examination of physical evidence. Certain products found as evidence can be readily discriminated from one another by the spectra obtained by this technique. However, additional work is required to determine the evidential value of spectra obtained from some materials.

This is publication number 93–06 of the Laboratory Division of the Federal Bureau of Investigation. Names of commercial manufacturers are provided for identification only and inclusion does not imply endorsement by the Federal Bureau of Investigation.

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Forensic analysis of explosives using ion chromatographic methods

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Abstract

Applications of ion chromatographic analysis techniques including capillary electrophoresis and inverse photometric detection are explored in the analysis of low explosives and their residues. Past procedures for this analysis are reviewed and new techniques are introduced. A scheme for the investigation of trace evidence from bombing crime scenes using ion chromatographic techniques is developed. This scheme includes two sets of anionic and cationic analytical methods to allow identification and confirmation of the presence of inorganic explosive residue. Several different types of explosive devices are examined using this scheme, and the results are shown.

Keywords: Electrophoresis; Ion chromatography; Explosives; Forensic analysis

Ion analysis is an important tool in the arsenal of the forensic explosives investigator. A large proportion of improvised explosive devices used in bombing incidents in the United States is produced using low explosives such as black powder, flash powder, or homemade mixtures. These bombs leave a significant amount of inorganic residue upon deflagration. While the complex chemical reactions that take place during the blast cannot be completely characterized, enough is known about the products and reactants left behind to allow a trained investigator to determine the nature of the explosive used in the device.

While microscopy, spot tests, thin-layer chromatography, and other techniques such as x-ray powder diffraction and infrared analysis can be used to obtain information on the nature of the residue, the most powerful tool in these investigations has been ion chromatography (IC) [1–4]. This technique, developed in the early 1970s, has the ability to detect μg per ml levels of the anions and cations left behind in the residue [5,6].

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For example, black powder which consists of charcoal, sulfur, and potassium nitrate, will show nitrite, nitrate, sulfate, sulfide, thiocyanate, and carbonate anions on analysis of an aqueous extract of its residue. Cation analysis of the same residue will yield primarily potassium ions along with residual amounts of sodium and ammonium. By noting the presence of each of these anions and cations, the forensic investigator can conclude that the explosive contained a potassium nitrate oxidizer with a carbon and sulfur fuel – strong evidence for the presence of black powder in the device. With experience, an investigator can also use the relative amounts of the ions in the residue to establish more conclusive proof of the identity and purity of the material used, helping to answer the question of whether a commercial or homemade composition was used.

Early instrumentation for IC consisted solely of suppressed or dual-column chromatographic methods [7]. These procedures used a strong ion exchanger followed by a second column or membrane which neutralized the ionic eluent allowing just the conductivity of the sample ions to be detected. These types of analysis were based on proprietary technology developed by Dionex (Sunnyvale, CA). However, there were problems with these methods due to the wide variety of charge densities present in the ions found in explosive residues [8]. As a result, no single combination of column and eluent could achieve separation of all relevant ions. In addition, the eluent used for the separation of anionic species was bicarbonate, making detection of this particular ion impossible.

To help alleviate this problem, gradient techniques and electronically compensated detectors have been designed [9,10]. A simpler solution to the above problems was developed through the use of inverse photometric analysis, a technique in which an absorbent eluent is used [11]. When the sample ions reach the vicinity of the detector cell, the eluent ions are displaced in order to maintain solution neutrality. The result is a decrease in absorbance at the detector in proportion to the amount of eluent displaced by the particular sample ion. An advantage of using absorbent ions as eluents is that weaker eluents can be used with low-capacity ion-exchange resins, allowing a wider range of sample ions to be analyzed on the same column [12,13]. The result is that the forensic chemist has for the first time the ability to analyze a wide range of anions and cations using simple liquid chromatographic equipment. Only two systems are required, one for cations and another for anions.

Unfortunately, the IC analyses used in the forensic laboratory suffer from the lack of good complementary techniques for peak confirmation. While x-ray and elemental analysis techniques can be used for residue analysis, these techniques lack the sensitivity and specificity required to verify chromatographic peaks. An additional problem with elemental analysis of cations is that important ions such as ammonium and monomethylamine cannot be detected. Thus the best way to confirm the identity of these IC peaks is to use a second chromatographic detection system. Two sets of two different column systems are used; one set for cations and another for anions. Each set might consist of (1) a traditional dual-column ion analyzer with suppressed conductivity detection and (2) a single-column ion analyzer with inverse photometric detection.

This solution to the problem of confirmation of peak identity is problematic both because of the above-mentioned problems with suppressed IC and also because peak elution order in both suppressed and non-suppressed IC is very similar. Thus it is possible to have an interfering peak show up at the same relative position in both systems.

The development of capillary electrophoresis (CE) for anion analysis has provided an opportunity to alleviate some of these problems [14,15]. The separation mechanism for ions in CE relies mainly on differences in the limiting equivalent conductances of the ions involved, unlike IC where separation results from complex interactions between a mobile and stationary phase [16,17]. As a result, peak elution order is completely different in the two techniques, and comparison of the same sample analyzed using both CE and IC is an excellent way to confirm peak identity [18].

Another recent development is the availability

of commercial cation columns capable of analyzing both singly and doubly charged ions using a single isocratic eluent. These columns rely on technology developed by Shomberg and utilize both ion exchange and coordination equilibria to produce their separation [19,20]. Due to the relatively low capacity of these columns, weak eluents can be used in combination with non-suppressed conductivity detection to achieve excellent results. This type of column can be used in combination with a column whose separation relies solely on ion exchange to provide confirmation of cation results [18].

By combining these assorted techniques, a system for the analysis of inorganic explosive residue can be proposed. In this paper, we will describe the methodology used to analyze inorganic explosive residue and give a number of examples of its application to real samples.

EXPERIMENTAL [a]

Ion chromatography

The ion chromatograph used for anion analysis was a Waters (Milford, MA) 600E multisolvent delivery system attached to a Kratos Spectra flow 783 variable-wavelength UV detector set at 280 nm and a Waters WISP 710B autosampler. The column used was a Vydac 302IC4.6 (Hesperia, CA) with a flow-rate of 2.5 ml/min and an injection volume of 25 μl [13]. Detector signals were analyzed using a Waters 840 data system. Isophthalic acid (Aldrich) was used as received. To prepare the eluent, 0.75 g of the isophthalic acid were added to 3 l of boiling water along with approximately 2 ml of 2 M KOH. Following dissolution of the acid, the solution was cooled and the pH adjusted to 4.6 using additional 2 M KOH.

For cations, two analytical systems were used. The first system used the same instrumentation mentioned above with an Interaction Ion 210 column and 0.10 mM cerium(III) sulfate as the eluent. The flow-rate was 1.0 ml/min and the detector was set to 254 nm. The second analytical system utilized a Waters IC-Pak M/D column with a 0.1 mM EDTA–3.0 mM nitric acid eluent. The flow rate was 1.0 ml/min and a Waters 431 conductivity detector was used.

Capillary electrophoresis

The capillary electrophoresis system used was a Spectra Physics 1000 CE (San Jose, CA) equipped with a 75 μm i.d. fused-silica column and a scanning ultraviolet detector. The detector was positioned at the positive end of the capillary (reversed polarity), and was operated in the dual-wavelength UV mode at wavelengths of 280 nm and 205 nm. The operating voltage was −20 000 V. Potassium dichromate, sodium tetraborate, boric acid, diethylenetriamine (DETA), and sodium hydroxide were used as received. The buffer system was prepared by adding 0.53 g of potassium dichromate, 0.76 g of sodium tetraborate, and 2.47 g of boric acid to 1 l of deionized water [21,32]. The pH was adjusted to 7.65 with DETA, and the solution was filtered through a 0.45 μm nylon 66 filter. The resultant buffer solution was 2 mM in borate, 40 mM in boric acid, and 1.8 mM in dichromate. The 75-μm fused-silica column was prepared for use by flushing for 2 min with 100 mM NaOH. Sample injection was performed using a hydrodynamic injection for 5 s.

Sample preparation. All solutions and extracts were prepared using 18 megohm deionized water. Pipe bombs containing a variety of explosive materials were deflagrated by the FBI Explosives Unit. Special care was taken in the treatment of these pipes in order to avoid sources of contamination. The pipes were rinsed with distilled water, air-dried, and carefully filled with the explosive preparation. To aid in this operation pipe threads were coated with petroleum jelly, and plastic bags were used in the filling operation, keeping the powder from contacting any surface near where the end cap screws back on. The pipe bombs were next rolled up in approximately 15 m of chain-link fence and set off while supported in the air between two posts. The fragments caught in the wire mesh of the fencing were then col-

[a] Names of commercial manufacturers are provided for identification purposes only, and inclusion does not imply endorsement by the Federal Bureau of Investigation.

lected and taken back to the laboratory for processing. The residue from the blast was collected by washing the fragments with deionized water and filtering through a prerinsed 0.2-μm syringe filter. These solutions were then left to dry overnight on watch glasses, and the dried crystalline material was collected for storage and later analysis.

RESULTS AND DISCUSSION

Anion analysis by ion chromatography

The ion chromatography system in use at the FBI laboratory is the Vydac 302IC 4.6 column with isophthalic acid at pH 4.6 as the eluent system. This methodology was developed specifically for the analysis of explosive residues [13]. The column uses a silica substrate which is bonded to a low-capacity ion-exchange media [22]. As a result, this column allows ions which would be too strongly retained on other ion-exchange columns to elute. Figure 1 shows the separation obtained using a standard containing ions of interest in the analysis of explosives. The perchlorate and carbonate peaks are difficult to analyze on standard IC systems, but generally show up in under 30 min with the Vydac column. While gradient techniques have been developed which improve peak shapes and separation efficiency, these procedures come at a cost of increased analysis complexity [9,10]. The Vydac system offers a number of significant advantages, especially for the average forensic laboratory. Because the method utilizes inverse photometric detection at 280 nm, no specialized equipment such as conductivity detectors or gradient pumps are required. Any laboratory with access to a standard liquid chromatograph with a fixed-wavelength detector can run the analysis. This is particularly important for laboratories with limited budgets or areas where such cases occur infrequently. Since anions such as phosphate, azide, iodide and fluoride can also be determined, this technique can also be used for other types of forensic trace analyses such as product tampering and soils analysis.

Anion analysis by capillary electrophoresis

While ion chromatography has been used for many years as a technique for the analysis of low explosive residue, CE is a relatively recent methodology for this application [18]. This technique achieves GC-like separation efficiencies of 70 000 or greater theoretical plates on liquid samples. This separation can be compared to that from a typical IC separation which has only about 3000 theoretical plates. Capillary electrophoresis

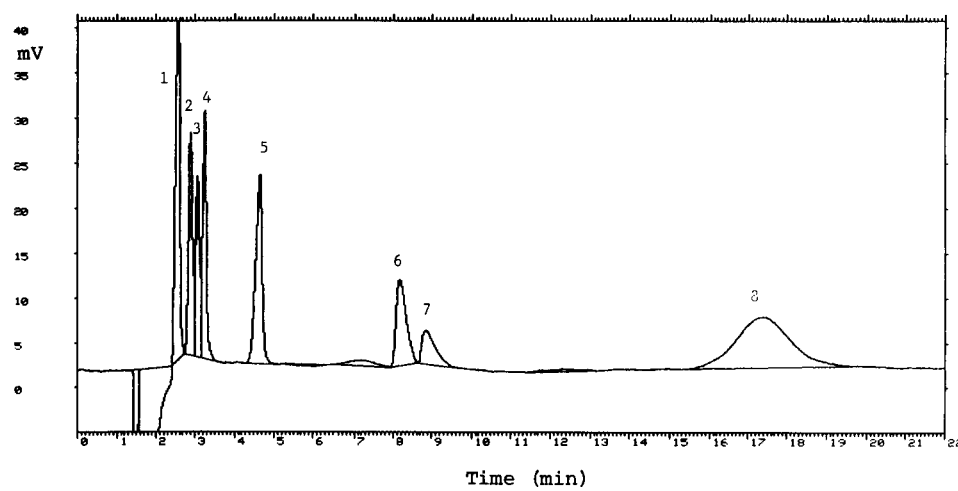

Fig. 1. Ion chromatogram of standard containing anions present in explosive residue. Vydac 302IC4.6 column with isophthalic acid eluent. Peak identification: (1) Cl^-; (2) NO_2^-; (3) ClO_3^-; (4) NO_3^-; (5) SO_4^{2-}; (6) SCN^-; (7) ClO_4^-; (8) HCO_3^-.

has been used in the FBI laboratory for over a year to confirm the results of the IC analyses with excellent results. Minimum detectable quantities are on the order of 0.5 μg/ml and a wide variety of ions can be separated and detected. Figure 2 shows an example of the separation obtained with an anion standard containing ions of interest in explosives residue analysis.

The capillary electrophoresis system accomplishes its separation by applying a high-voltage potential across a 70 cm × 75 μm i.d. fused-silica column. Injection occurs by introducing a small amount of sample into the column and then replacing the column into the buffer solution. High voltage is then applied and the samples are swept past an ultraviolet detector. Since most inorganic ions do not absorb ultraviolet light, the buffer contains absorbent dichromate ions, and samples are detected by absorbance loss as they displace the buffer in the detector's light path. In order to obtain reliable results with this technique, it is important to maintain proper control of the variables that affect the separation. Among the most important of these variables are electroosmotic flow-rate, pH, and the age of the buffer solution.

In capillary electrophoresis of anions, separation occurs due to the difference in charge to mass ratio of each solvated ion. The mass of the ion is also influenced by the size of its solvation shell. Thus heavily solvated ions like fluorine occur much later in the electropherogram than more massive ions like chloride, which are less well solvated. The ions are swept past the detector by means of the induced or electroosmotic flow. For proper anion analysis this flow must be oriented in the direction of the positive electrode where the detector is located [16]. Normally electroosmotic flow is induced by a layer of solution cations attracted to the negatively charged capillary wall. This results in flow towards the negative electrode. By the addition of the electroosmotic flow modifier, DETA, the capillary walls are coated with positively charged ions, effectively reversing the flow and allowing the anion separation to be performed quickly and efficiently.

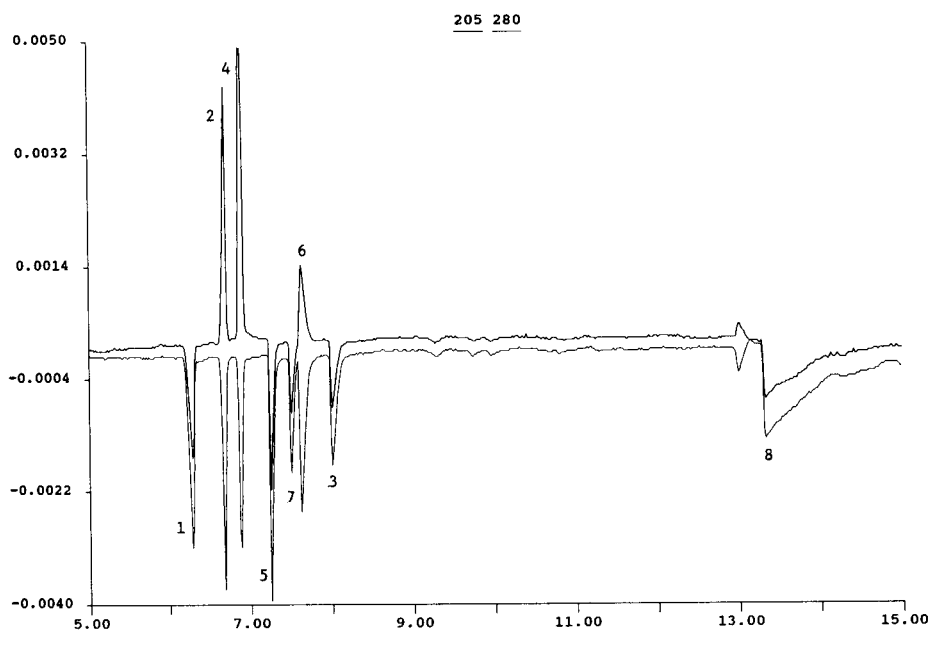

Fig. 2. Anion standard containing ions present in explosive residue. Analysis performed at 205 and 280 nm using capillary electrophoresis. Peak identification: (1) Cl^-; (2) NO_2^-, (3) ClO_3^-; (4) NO_3^-; (5) SO_4^{2-}; (6) SCN^-; (7) ClO_4^-; (8) HCO_3^-.

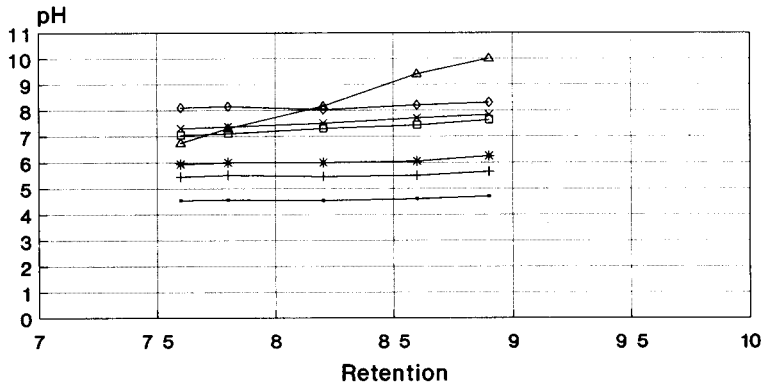

Fig. 3. Effect of pH adjustment with DETA on retention time in capillary electrophoresis. (■) Cl^-; (×) SCN^-; (+) NO_2^-; (◇) ClO_3^-; (*) NO_3^-; (△) SO_4^{2-}; (□) ClO_4^-.

In the methodology described above, the DETA electroosmotic-flow modifier is added to the buffer solution to adjust the pH of the eluent. This final step has important consequences for the quality of the separation. Since the concentration of DETA affects both the pH and the electroosmotic flow-rate, addition of this material has the potential to profoundly affect the separation. This effect is demonstrated in Fig. 3. Note the sensitivity of the sulfate ion to changes in pH produced by the addition of DETA. In this pH range sulfate is completely ionized, and thus this sensitivity must be related to the concentration of DETA. The ion pairing that reversed the electroosmotic flow in the capillary here is changing the relative migration rates of the anions. Sulfate, being doubly charged, ion pairs more extensively than the other anions and thus elutes more slowly as the DETA concentration increases. Phosphate, which is doubly charged at this pH, appears to behave similarly. Therefore, since sulfate is an important ion in the analysis of inorganic explosives, and is often present at the highest concentration, proper pH adjustment with DETA must be made for a successful separation. Often in preparation the pH is left slightly low and then adjusted based on the quality of the separation of the standard.

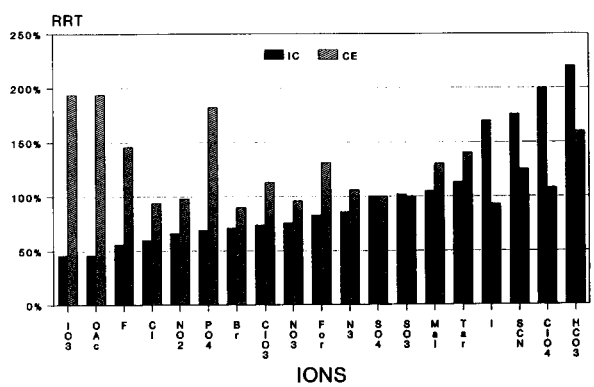

Fig. 4. Comparison of elution order in (hatched) capillary electrophoresis and (black) ion chromatography.

One of the reasons CE is so valuable for forensic explosive residue analysis is the fact that the separation mechanism is so very different from that of IC. This can be seen clearly in the results of Fig. 4 which shows a nearly orthogonal relationship in the relative retention times of the two techniques [18]. Peak interferences are easy to detect for if an interference occurs in one analysis technique, it is unlikely to appear in the other. An example of this situation appears in the analysis of explosive residue containing cyanate. This particular ion, OCN^-, interferes with the analysis of chlorate, ClO_3^-. Potassium chlorate is an oxidizer commonly present in illicit firecrackers and homemade explosives. It had also been suspected of being present in residue resulting from the deflagration of explosives containing potassium perchlorate oxidizers based on results obtained using IC. Figure 5 shows an example of the ion chromatogram of a sample of burned black powder. Peak number 3 appears at the

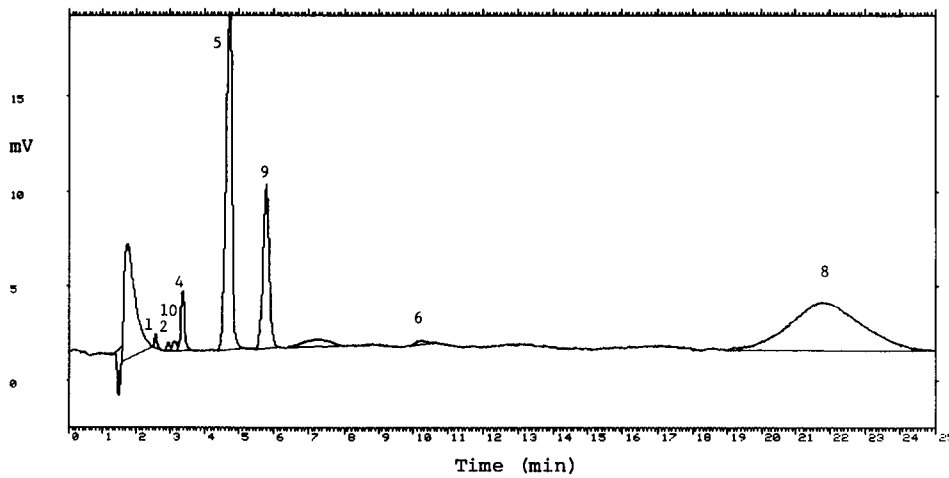

Fig. 5. Ion chromatogram of black powder pipe bomb residue. Peak identification: (1) Cl^-; (2) NO_2^-; (4) NO_3^-; (5) SO_4^{2-}; (6) SCN^-; (8) HCO_3^-; (9) HS^-; (10) OCN^-.

position at which the chlorate ion occurs. This oxidizer is not present in black powder. Subsequent analysis of this sample by CE, Fig. 6, revealed the presence of cyanate but not chlorate. The presence of cyanate was further confirmed by infrared analysis. Capillary electrophoresis has also shown value in the confirmation of IC peaks from fluoride to phosphate. In a similar way, we

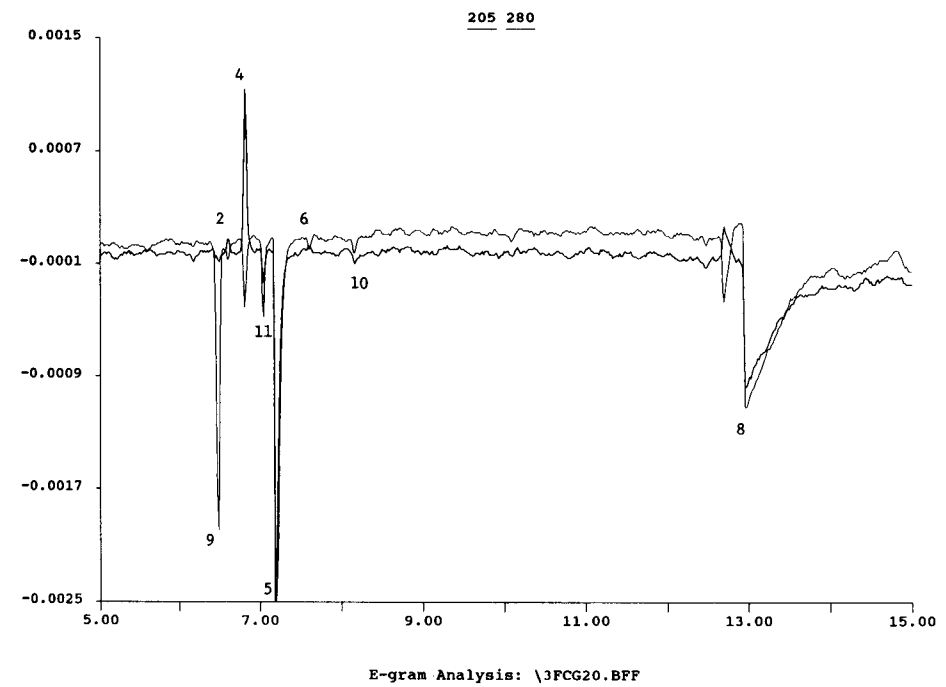

Fig. 6. Capillary electrophoresis of black powder pipe bomb residue. Peak identification: (2) NO_2^-; (4) NO_3^-; (5) SO_4^{2-}; (6) SCN^-; (8) HCO_3^-; (9) HS^-; (10) OCN^-.

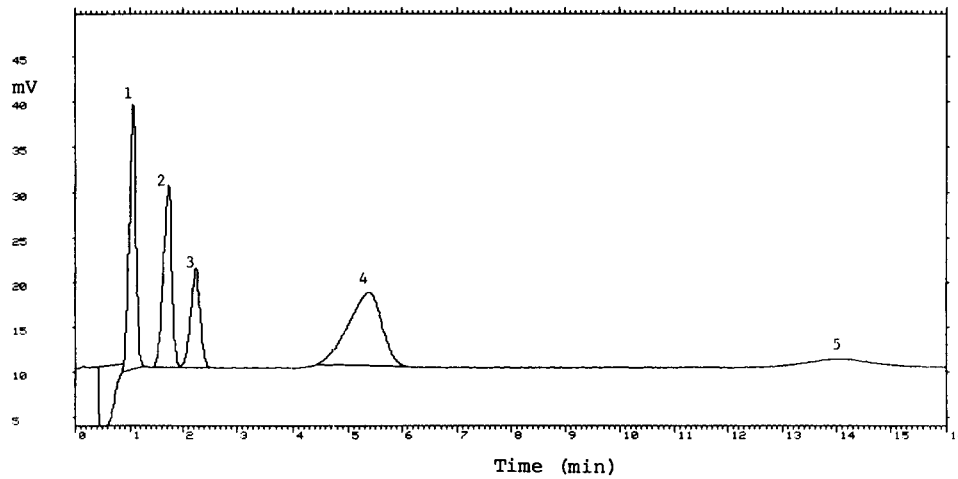

Fig. 7. Cation standard analyzed using the interaction ion 210 column with 0.10 mM cerium(III) sulfate eluent. Peak identification: (1) Na^+; (2) NH_4^+; (3) K^+; (4) Mg^{2+}; (5) Ca^{2+}.

have used ion chromatography to help elucidate problematic CE results.

Cation analysis by ion chromatography

As mentioned in the introduction, the primary reason for performing cation analysis of explosive residues is the fact that other methods of analysis such as atomic spectroscopy are incapable of determining organic cations such as ammonium and monomethylamine. These ions are important in the analysis of explosives used in the mining industry which are based on ammonium nitrate [8,23,24]. Another application of cation analysis is in the determination of the ratio of sodium to potassium in samples where a dry powder explosive is suspected. Commercial preparations of powdered explosives are unlikely to contain sodium-based oxidizers as sodium salts are more

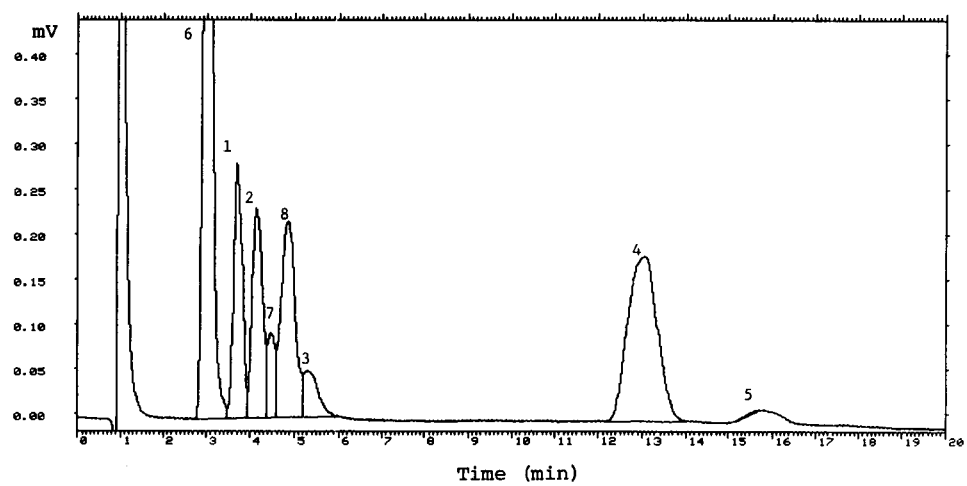

Fig. 8. Cation standard analyzed using the Waters M/D column with a 3 mM nitric acid–0.1 mM EDTA eluent. Peak identification: (1) Na^+; (2) NH_4^+; (3) K^+; (4) Mg^{2+}; (5) Ca^{2+}; (6) Li; (7) ethanolamine; (8) monomethylamine.

hygroscopic than potassium salts. The presence of high concentrations of sodium in the residue may be an indication of a homemade explosive mixture. Alkaline earth metals are also important in residue determinations. Magnesium metal is used in certain types of flares and flash powders, and calcium nitrate is an important component of certain commercial mining explosives. Thus the ideal analytical method for the analysis of cationic residue should be capable of determining group I and II metals as well as amines in a single run.

Initial methods for the determination of these ions either ignored the group II cations or utilized two different eluents; a weaker eluent for the group I cations and amines, and a second, stronger eluent for the group II cations [25,26]. More recently, a number of techniques have appeared in the literature which allow the analysis of mono- and divalent cations in a single isocratic run [12,27,28]. Among these are the two methods described previously; (1) a method using inverse photometric detection at 254 nm with a cerium sulfate eluent and a second procedure utilizing conductivity detection and (2) a nitric acid–EDTA eluent. The first procedure uses the Interaction Ion 210 column, Fig. 7, which while simpler and more robust, is incapable of distinguishing aliphatic amines as they coelute with ammonium and potassium. The second procedure, Fig. 8, has the capability to distinguish nine different ions, including lithium, sodium, ammonium, monomethylamine, ethanolamine, potassium, magnesium, calcium, and strontium. This method, which uses the Waters M/D column, has greater resolving power and higher sensitivity but is more vulnerable to contamination problems and subsequent loss of efficiency than the first method. The two procedures complement each other. The inverse photometric system is used for an initial screen, and the more sensitive and discriminating conductivity detection is applied later if ammonium is found to be present or if aliphatic amines are suspected in the residue.

Another possible methodology for the analysis of cationic explosive residues is CE. Applications are just beginning to appear in the literature for the analysis of cations using this technique [29,30]. While separations of ammonium and the aliphatic amines have yet to be shown, the wide range of metal ions separated demonstrate the potential power of the technique.

Analysis of pipe bombs

To test the capabilities of this analysis scheme, a series of experiments were conducted by initiating pipe bombs containing various explosive mixtures and carrying the fragments back to the laboratory. The explosives were set off on an explosives ordnance demolition range and prepared in the manner described above. Extreme care was taken in the preparation and manipulation of the unexploded devices.

Analysis of the explosive residues was conducted in an effort to document the anionic and cationic chemical profile of the residue. For the explosives examined in this trial, five replicates of pipes using the same type of powder were analyzed to determine what range of results could be expected. The fragments of each pipe were also sampled in two different locations to check for variations in the amounts of ions present and to look for the presence of pockets of unexploded material.

One persistent problem for the forensic chemist in analyzing evidence from bomb residues is the lack of an extensive knowledge base on what may be present in the residue. Typically, the development of new analysis techniques proceeds piecemeal, with conclusions based on very small sets of data. This is because experiments of this nature can only be performed in isolated and remote locations, and because these devices are extremely dangerous. Thus knowledge of the chemical makeup of explosive residues often comes from actual criminal casework; data in which little is known of the conditions used in preparation of the device or in the collection of the evidence. Indeed, in a certain percentage of cases such knowledge is lost due to the fact that the maker and his device are commingled in the residue.

Thus the goal of this study has been to examine a number of different types of explosives in order to obtain a semiquantitative analysis of the range and relative amounts of ions produced. While this work is still in progress, we have begun

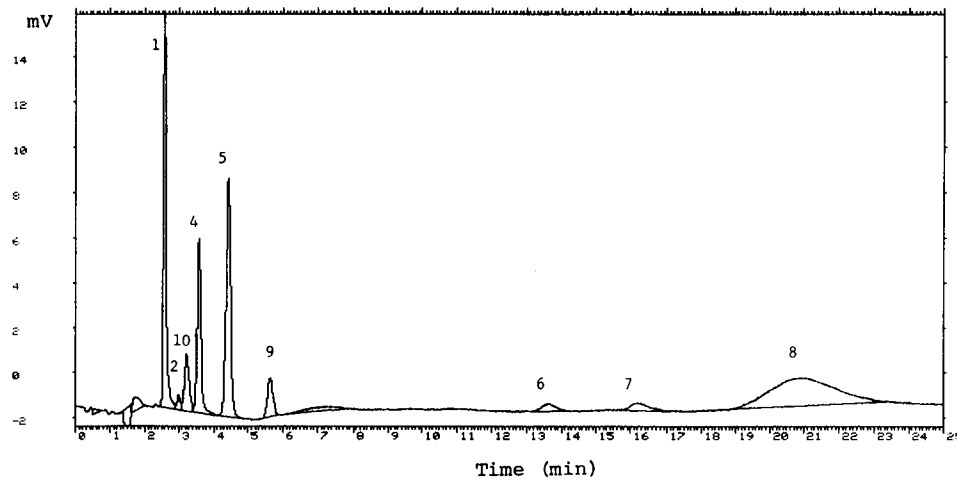

Fig. 9. Ion chromatogram of Pyrodex pipe bomb residue anions. Peak identification: (1) Cl^-; (2) NO_2^-; (4) NO_3^-; (5) SO_4^{2-}; (6) SCN^-; (7) ClO_4^-; (8) HCO_3^-; (9) HS^-; (10) OCN^-.

to acquire results on a number of common low explosives used to produce pipe bombs. For example, Figs. 9–12 show the results from the IC analysis of a pipe bomb filled with Pyrodex RS. This explosive is a black-powder substitute that contains potassium nitrate, sulfur, charcoal, potassium perchlorate, sodium benzoate, and dicyandiamide. The results show a wide variety of

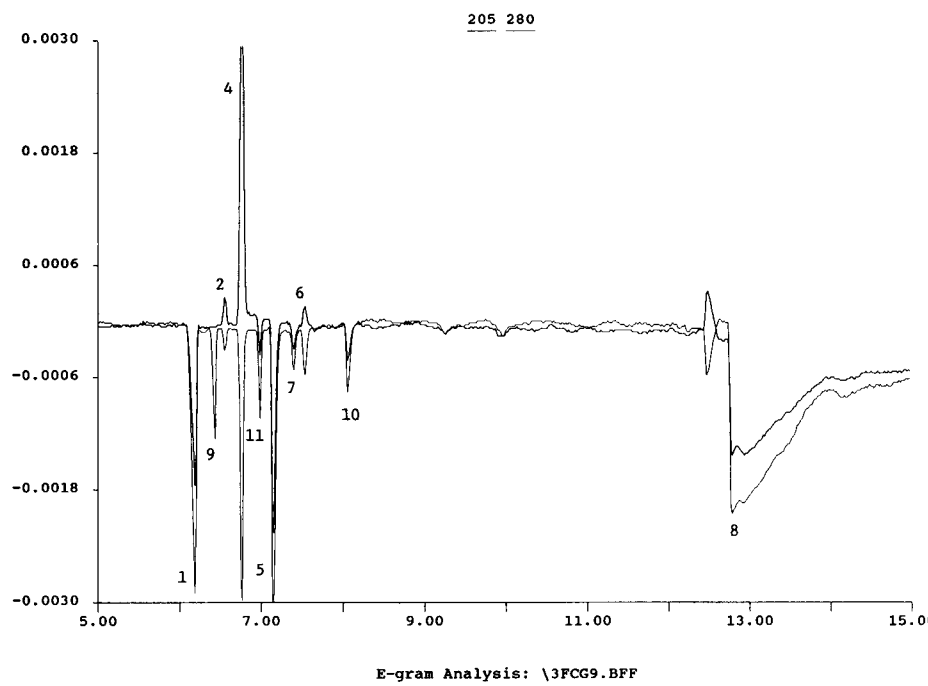

Fig. 10. Capillary electropherogram of Pyrodex pipe bomb residue anions. Peak identification: (1) Cl^-; (2) NO_2^-; (4) NO_3^-; (5) SO_4^{2-}; (6) SCN^-; (7) ClO_4^-; (8) HCO_3^-, (9) HS^-; (10) OCN^-; (11) unknown.

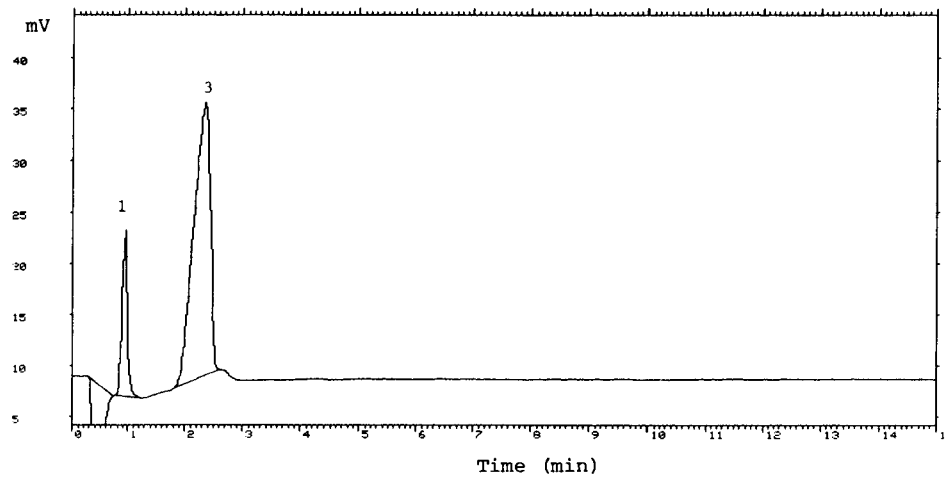

Fig. 11. Ion chromatogram of Pyrodex pipe bomb residue cations. Interaction ion 210 column. Peak identification: (1) Na^+; (3) K^+.

ions present in the anionic residue. These ions are the products and reactants of the following equation:

$$KNO_3 + KClO_4 + S + C + C_6H_5CO_2Na + NH_2C(NH)NHCN \rightarrow NO_2^- + Cl^- + SO_4^{2-} + HS^- + SCN^- + OCN^- + HCO_3^- + K^+ + Na^+$$

Note the difference in efficiency between the IC and CE analyses. The peak broadening that occurs with the late eluting ions such as SCN^- and ClO_4^- in the IC analysis limits the sensitivity for these ions. While LC or GC–MS can be used to identify the organic constituents of Pyrodex, the perchlorate ion is a particularly important clue for identifying this sample. The CE analysis is very helpful in confirming this ion. The ClO_4^- peak occurs relatively early and is sharp, allowing good sensitivity and a positive confirmation of its presence in the IC result. Note also the cyanate ion. Earlier predictions about the perchlorate oxi-

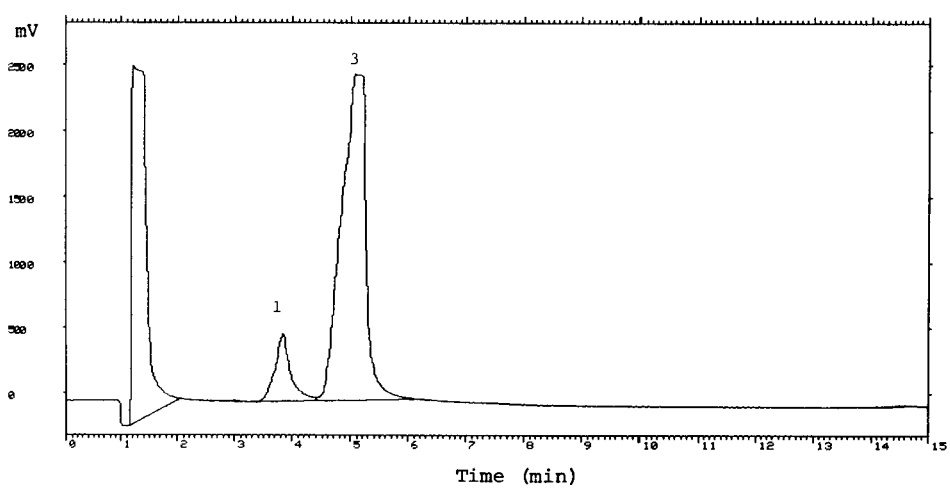

Fig. 12. Ion chromatogram of Pyrodex pipe bomb residue cations. Waters M/D column. Peak identification: (1) Na^+; (3) K^+.

dizer gave chlorate as an intermediate in the chemical reduction from perchlorate to chloride [13].

$$ClO_4^- \rightarrow ClO_3^- \rightarrow Cl^-$$

As mentioned previously, cyanate interferes with chlorate in the IC analysis. However, they are well separated in the capillary electropherogram, and in this example, cyanate is clearly present while chlorate is not. If only the IC analysis were performed, it is likely that this distinction would be lost and the peak mislabeled as chlorate. Thus at least in this instance, the perchlorate was reduced completely to chloride and no chlorate was observed. Further studies with perchlorate are needed in order to determine under what conditions if any, the chlorate ion does appear in the residue.

The cation analysis of this sample is not quite as circumstantial as the anion analysis. However, the fact that Pyrodex residues contain more sodium ions than might be expected if the sample were black powder can be of value to the forensic chemist in his evaluation of the results. Typically the sodium ions are the result of the presence of sodium benzoate in the sample, but in actual casework, such conclusions should be guarded because of the ubiquitous nature of sodium in the environment.

Contamination of samples as a result of the force of the blast is an ever-present concern,

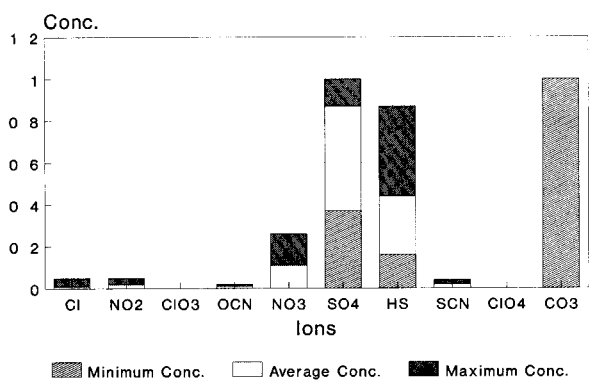

Fig. 13. Range of concentration of anions present in black powder pipe bomb residue. Determined by ion chromatography of 5 individual pipe bombs sampled in duplicate. (Carbonate not to scale.)

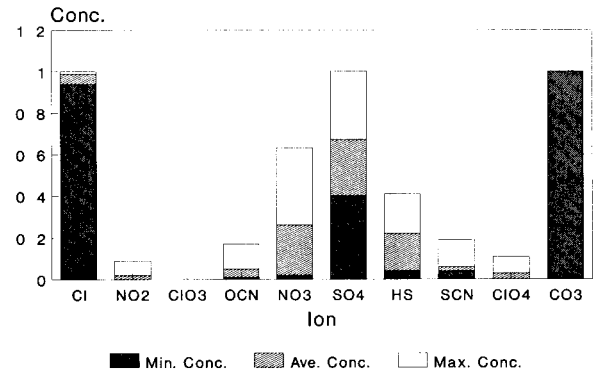

Fig. 14. Range of concentration of ions present in Pyrodex pipe bomb residue. Determined by ion chromatography of five individual pipe bombs sampled in duplicate. (Carbonate not to scale.)

since it is seldom that adequate control samples are taken at the crime scene, and most of these ions, positively and negatively charged, are present in some concentration in the environment. For this reason, the forensic investigator cannot rely on a single peak, such as nitrate, as an indication of the presence of a particular explosive. Instead, it is the overall chromatographic profile that is used to determine the identity of the bomb that produced the residue. Fortunately, the fragments resulting from the blast of a low explosive generally contains copious amounts of residue. For example, following the deflagration of black powder, fully 50 percent of the original mass is left behind as residue [31]. Under these circumstances, environmental contaminants are not so important, due to the sheer bulk of the residue ions present.

While the chromatographic profile can be used to determine the type of explosive utilized, it must also be stated that the absolute quantities of the ions cannot be used as an identifying feature. Variations in the type of containment device, in the heat of the blast, and in the amount of unburned material extracted can greatly affect the relative amounts of ions found in a residue extract. Figures 13 and 14 reveal the range of relative concentration of ions present in black powder and Pyrodex residue. This determination was made by analyzing the residue produced by five pipe bombs for each explosive. Each of the

bombs were prepared in exactly the same fashion on the same day. The results show that there is a large variation in the concentration of ions such as nitrate, which are present in the starting mixture. This is mainly due to the variability in the amounts of unburned material left on different areas of the pipes.

Among the threads and end caps of the pipes, relatively more of the starting mixture was left unburned, while fragments from the pipe center had correspondingly less unburned material. Other ions such as nitrite are intermediates, and their amounts vary depending on factors such as burn rates and the heat of the blast. The components that varied the least were the final products, such as chloride, sulfate, and sulfide. These materials were the most prevalent final products and the least subject to changes in reaction conditions. However, even the sulfate ion showed relative standard deviations approaching 50 percent. Carbonate ions were also present in both of these samples, however they were not quantified due to the large quantity of material and poor peak shapes produced with this ion.

From an analysis of the results displayed in Figures 13 and 14, it is clear that an investigator who bases his analysis on the presence of one particular ion could reach a faulty conclusion. Oxidizer ions from the original powder such as nitrate and perchlorate were sometimes found at very low levels or not detected at all. In such instances, the high sensitivity that ion analysis techniques can achieve can make the difference between a properly analyzed sample and a indeterminate result. Methods such as microscopy and chemical spot tests may not have the requisite sensitivity for this type of determination. It is also clear that the overall pattern of ions found, while variable in quantity, can be used in making an identification. These two samples, a black powder and a black powder substitute, have many of the same components, yet most of the ions appear at different average levels in the chromatographic analysis. Visual examination of the actual chromatogram give a clear picture of the distinctions between the two (Figs. 5 and 9).

Another benefit we have realized from this study is the importance of performing analyses of a number of the multiple fragments present in bomb residue. Small amounts of unburned powder are sometimes trapped in certain sections of the evidence, giving rise to the variability discussed above. By performing analyses of different sections of the residue, the examiner can be certain to have the greatest possible chance to have an accurate determination of the type of residue present.

Conclusions

Ion chromatography has been shown to be a useful technique in the analysis of low explosive residues since its development. The improved IC procedures described in this paper give the explosives investigator the best chance of properly and unequivocally determining the ions present in his residue sample. Inverse photometric detection techniques allow simple LC technology to be used in the initial screen of the sample. The columns and eluents described also permit the widest possible range of ions to be determined in a single isocratic run. A second set of techniques using CE for anions and conductivity detection for the cations permits confirmation of the ions determined in the first set of runs. The importance of careful analysis of the residue is underscored by analysis of the variation in a series of experiments on recovery of ions in black powder and Pyrodex pipe bomb residue. In the future, these experiments will be continued on a variety of commercial and homemade explosive mixtures.

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Characterization of smokeless gunpowder by means of diphenylamine stabilizer and its nitrated derivatives

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Abstract

Smokeless gunpowder frequently contains diphenylamine as a stabilizer. The diphenylamine acts as a nitrate scavenger and in turn is nitrated by complex processes. The various nitrated congeners of diphenylamine, which are numerous, may serve to characterize a sample of gunpowder because these derivatives reflect not only the production of the gunpowder, but also its storage career and thermal history following manufacture. These derivatives of diphenylamine may be isolated and identified by means of thin-layer chromatography and liquid chromatography.

Keywords: Chromatography; Diphenylamine; Forensic analysis; Gunpowder

Modern gunpowder is of the so-called "smokeless" type, consisting primarily of nitrated cellulose, but frequently with nitroglycerine as well. Gunpowder consisting of nitrocellulose alone is termed "single-base" gunpowder, and those powders that contain nitroglycerine besides nitrocellulose are called "double-base" gunpowders.

Upon standing, and particularly under hot conditions, the nitrocellulose and nitroglycerine will deteriorate with the release of nitrate. Free nitrate will in turn cause nitrous and nitric acid to be formed, which will further degrade the gunpowder. Consequently, a stabilizer of some sort is required. In the United States and in the United Kingdom, diphenylamine (DPA) is the most commonly used stabilizer, although resorcinol and ethyl centralite are also occasionally used.

A pristine gunpowder may contain diphenylamine alone, but soon the diphenylamine, acting as a nitrate scavenger, becomes nitrated itself in one of many ways. An aged cartridge (or a new one that has been exposed to a hot environment) may contain gunpowder with a witch's brew of nitrated diphenylamine compounds. The end member of this series of nitrated compounds is hexanitrodiphenylamine, in which all available sites have been nitrated.

The diversity of nitrated diphenylamine species provides an opportunity to characterize a particular gunpowder, because the nitrated diphenylamine species reflect not only the original production but also the subsequent thermal history and storage career of the gunpowder. And in

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fact, many color tests have been suggested for the characterization of gunpowders; these color tests rely in major part on the reaction of diphenylamine and resorcinol congeners [1,2].

Although DPA clearly acts as a nitrate scavenger and is increasingly nitrated as gunpowder ages, the available knowledge concerning the reactions of DPA nitration has been either fragmented or poorly understood. Although the sequences of events during diphenylamine nitration have been postulated (not always altogether plausibly [3,4]), these mechanisms have not been previously evaluated for forensic purposes. In the chemistry of gunpowder and propellants, and especially with respect to nitrocellulose containing powders, stabilizers are compounds that prevent the acid catalyzed decomposition of nitrocellulose or nitroglycerin. Stabilizers exert their effect by binding decomposition products such as free acid and nitrous gases; the stabilizers themselves are converted into relatively stable compounds in this process. Neither stabilizers nor their secondary products react with the parent nitroglycerin or nitrocellulose.

In recent years there has been renewed interest in DPA as an organic indicator of gunshot discharge residue [5–9]. Although the ubiquity of diphenylamine is such that the presence of DPA alone might not unequivocally suggest that a gunshot discharge has taken place, the presence of an entire group of nitrated derivatives of DPA is highly significant. Industrial and environmental uses of DPA is not normally associated with nitrating agents.

The intent of the present research was to investigate the mechanisms of nitration of DPA and the occurrence of nitrated derivatives of diphenylamine, and to evaluate the suitability of these derivatives as a test for characterizing gunpowders for determining commonality of source. Following a discussion of the possible mechanisms of diphenylamine nitration, the experimental work conducted in the present study was divided into three avenues of inquiry, namely, thermal stability of DPA, DPA nitration mechanisms, and presence and occurrence of DPA in gunpowders by thin-layer chromatography and high-pressure liquid chromatography analysis.

General reaction mechanisms

The classical explanation of the conversion of DPA into its various derivatives is the rearrangement of *N*-nitrosoamines to the *C*-nitroso compound (The Fisher–Hepp reaction) and its subsequent oxidation. Another theoretically plausible reaction would be the oxidation of a *N*-nitrosamine to the *N*-nitramine with subsequent rearrangement, although no direct evidence for this reaction has been reported. It seems unlikely that multiple simultaneous nitration, denitration, or rearrangement of a nitro group from one carbon atom to another would occur. Based on these types of reactions, several nitration schemes seem possible. The nitration scheme depicted in Fig. 1 is based upon a series of nitrosations, denitrosations and nitrations, but does not involve rearrangements. In this scheme the only nitrosation that occurs is the original *N*-nitrosation of DPA. Schroeder et al. [10] believed this to be the principal reaction of DPA, but that some direct nitration to the mononitro derivatives also occurs. According to this view, *N*-nitroso-DPA then is supposedly nitrated largely to *N*-nitroso-4-nitro-DPA, which in turn is further nitrated, although some denitrosation results at this stage. Since the accumulation of nitro groups would produce an increasing instability of the *N*-nitroso bond, Schroeder predicted that after formation of the *N*-nitroso-dinitro-DPA, essentially complete denitrosation occurs and that further reaction is by direct nitration.

To corroborate this nitration mechanism, Schroeder conducted further experiments [11–13] with a series of gunpowders into which he incorporated various derivatives of DPA and then heated the mixtures to accelerate the release of nitrate. Each of these propellants was doped with one (but only one) of the following compounds: *N*-nitroso-DPA, 4-nitro-DPA, 2-nitro-DPA, 3-nitro-DPA, 4,4′-dinitro-DPA, 2,4,4′-trinitro-DPA and hexanitro-DPA. The results showed that, except 4-nitroso-DPA, the heated powders were always found to contain the derivatives to be expected based on Fig. 1. Only traces of *N*-nitroso-2-nitro-DPA were present in that powder which originally contained 2-nitro-DPA, and yet *N*-nitroso-4-nitro-DPA was an important product

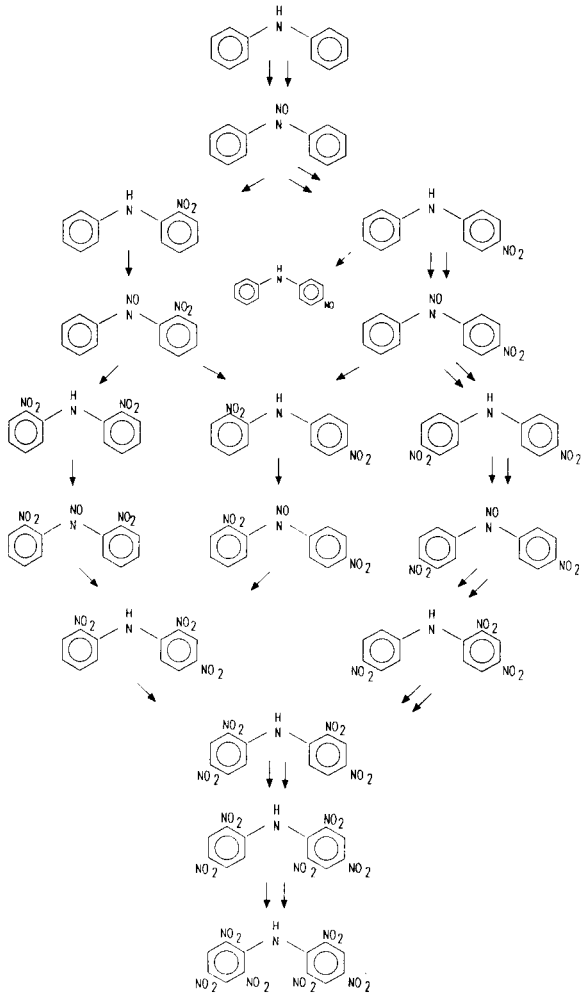

Fig. 1. Scheme of plausible nitration and nitrosation mechanisms of diphenylamine.

from 4-nitro-DPA. Denitrosation was demonstrated by the isolation of 4-nitro-DPA from the powder which originally contained *N*-nitroso-4-nitro-DPA. 4-Nitroso-DPA was found to be a very reactive compound which was depleted rapidly. Small amounts of 4-nitroso-DPA were isolated, but the reactivity of this derivative is such that it probably would not accumulate in gunpowder under any conditions.

On the basis of this it was concluded that the first reaction of DPA in smokeless powder is the nitrosation to *N*-nitroso-DPA, and that further reaction probably involves direct nitration to form *N*-nitroso-4-nitro-DPA. The probability of rearrangement of nitrated derivatives decreases as the DPA compounds become increasingly nitrated; the formation of the trinitro-DPAs and more highly nitrated compounds would probably involve direct introduction of the nitro group into the molecule.

Nitration mechanisms

The nitration of DPA in gunpowder has long been surrounded by a paradox, however. Nitration, in the classical sense, requires the presence of a strong acid to promote the formation of the nitronium cation. Although sulfuric acid is used in the manufacture of nitrocellulose, the trace amounts (typically ca. 0.5% sulfuric acid or less) found in nitrocellulose are insufficient to explain the nitration of DPA that accompanies the aging of gunpowder. On the other hand, nitrocellulose is an unstable compound in which thermal decomposition dissociates the ester bond [14–16]. This can produce nitrogen dioxide, a major decomposition product [17], which in turn can initiate a nitration reaction. This aspect of the chemistry of gunpowder may have been short shrift in previous work.

Explanations appearing in the literature concerning the nature of the nitration processes in DPA have ranged from classical to radical (the latter in the sense of electron transfer and radical pair collapse). The following discussion offers a review and summary explanation of both the classical and radical kind.

ELECTROPHILIC SUBSTITUTION: A CLASSICAL THEORY

Classically, nitration can be accomplished under the following conditions: (a) with nitric acid alone or with inorganic acids such as sulfuric acid; (b) with nitric acid in organic solvents such as nitromethane, acetic acid or acetic anhydride; (c) with dinitrogen pentaoxide in organic solvents; (d) with nitronium salts in organic solvents; (e) via nitrosation followed by oxidation. Although these conditions differ markedly from each other and the nitration rate of a given compound depends

on extant conditions, in all of the above the mechanism of nitration is essentially the same. The attacking entity is the nitronium ion, NO_2^+. Evidence of the existence of nitronium ion in nitrating media comes from Raman spectrum studies. A solution of nitric acid in sulfuric acid contains bands at 1400 and 1050 cm^{-1} that are not attributable to molecular nitric acid. Similar lines are also observed in other nitrating systems, namely dinitrogen pentaoxide in nitric acid (1400 and 1050 cm^{-1}), nitric acid in perchloric or selenic acid (1400 cm^{-1}) and crystalline nitronium perchlorate (1400 cm^{-1}). This last compound is completely ionized into nitronium ions and perchlorate ions [18], so that the 1400 cm^{-1} line is attributed to the nitronium ion. The 1050 cm^{-1} line is interpreted as the hydrogen sulphate ion in nitric acid–sulphuric acid, and to the nitrate ion in dinitrogen pentaoxide [19]. Raman spectroscopic measurements and other data are consistent with the establishment of the following equilibria:

$$H_2SO_4 + HNO_3 \rightleftharpoons HSO_4^- + [H_2O^+ - NO_2]^+ \quad (1)$$

$$[H_2O^+ - NO_2]^+ \rightleftharpoons H_2O + NO_2^+ \quad (2)$$

$$H_2SO_4 + H_2O \rightleftharpoons HSO_4^- + H_3O \quad (3)$$

The overall equilibrium may be expressed as:

$$2H_2SO_4 + HNO_3 \rightleftharpoons NO_2^+ + HSO_4^- + H_3O^+ \quad (4)$$

Evidence that nitronium ion is an active entity in diphenylamine nitration from thermally degraded nitrocellulose was shown by Yasuda [20], who effectively nitrated DPA in acetone by using dinitrogen pentaoxide. Nitration by dinitrogen pentaoxide is attributed to the ion pair $[NO_2^+NO_3^-]$, or by free nitronium ions by dissociation of this ion pair.

The nitrated derivatives of DPA in the experiments of Yasuda are consistent and similar to the nitrated derivatives of DPA found in gunpowder ageing studies by Schroeder et al. [10], Yasuda [21], and Volk [22] and consistent with the thermal studies of nitroglycerine conducted in the present study. Seven of the derivatives identified are nitroso compounds. Consequently, in the nitration of DPA there are either two sources of nitration (i.e., the nitrosonium ion, NO^+, and the nitronium ion, NO_2^+), or all nitration is via nitrosation with subsequent oxidation.

Nitration via nitrosation occurs in highly activated nuclei of amine derivatives, provided nitrous acid is present. The reaction appears to be in accordance, generally, with the following:

$$ArH + \underset{[\text{slow}]}{HNO_2} \rightarrow ArNO + H_2O \quad (5)$$

$$ArNO + \underset{[\text{fast}]}{HNO_3} \rightarrow ArNO_2 + HNO_2 \quad (6)$$

Since nitrous acid is continually reformed, its concentration remains constant throughout the reaction. Additionally, since the species NO^+, $H_2NO_2^+$, N_2O_4, N_2O_3 and HNO_2 are determined as nitrous acid by most conventional methods, any one of them could be acting as an effective catalyzing species to increase the rate of nitration.

In thermal degradation studies of cellulose nitrate [14], the products that could be identified by IR consisted of H_2O, HCHO, NO, CO_2, and NO_2. If nitrosation is the mechanism of DPA nitration, the following equilibrium reaction would agree with data from Yasuda [20]:

$$N_2O_4 \rightleftharpoons NO^+ + NO_3^+ \quad (7)$$

Rate limiting step in the aniline moiety of diphenylamine

Aniline is susceptible to nitronium attack because of the exposed π electrons. In the first step NO_2^+ takes two electrons of the π system to form a σ bond between it and one carbon of the phenol ring. This interrupts the cyclic system of π electrons, because in the formation of the sigma complex one carbon becomes sp^3 hybridized and no longer has an available p orbital. The four remaining π electrons of the σ complex (arenium ion) are delocalized over the remaining sp^2 carbons. In the second step the arenium ion loses a proton from the carbon that bears the nitronium. The two electrons that bonded this proton to carbon become a part of the π system. The

carbon that bears the electrophile becomes sp^2 hybridized again and an aniline derivative with fully delocalized π electrons is formed.

There exists experimental evidence that the arenium ion is a true intermediate in electrophilic substitution reactions involving benzene and its derivatives; the literature concerning DPA, however, is silent. Based on the benzene data the energy of reaction leading from DPA and the electrophile to the arenium ion is much greater than the energy of activation leading from the arenium ion to the final product. The reaction leading to the formation of the arenium ion is highly endothermic because the phenol ring loses its stabilization energy; conversely, the reaction leading from the arenium ion to the DPA derivative is highly exothermic because the phenol ring has regained its stabilization energy. The formation of the σ complex (arenium ion) is the rate limiting step in this reaction. The loss of a proton occurs rapidly compared with the arenium ion and does not affect the overall rate of reaction [19,23–25].

N-nitrosation, on the other hand, is the first nitration reaction found in ageing gunpowder studies. In the cases where *N*-nitrosation is the product of nitration, the rate limiting reaction is in the formation of the nitrosonium as shown:

$$C_6H_5NH_2 + HNO_2 \xrightarrow{[\text{slow}]} [C_6H_5NH - NO]^+ + H_2O \quad (8)$$

$$[C_6H_5NH - NO]^+ + HNO_3 \xrightarrow{[\text{fast}]} ArNO_2 + HNO_2 \quad (9)$$

Substituent effect of the amino group in the aniline moiety

The *ortho–para* directive effect of the amino group with an unshared electron pair is predominantly caused by resonance effect. This resonance operates primarily in the arenium ion and in the transition leading to it. The inductive effect of the amino group makes it slightly electron withdrawing. The difference between the electronegativities of nitrogen and carbon in aniline is not too large, however, since the carbon of the benzene ring is sp^2 hybridized it is somewhat more electronegative than it would be if it were sp^3 hybridized. Nevertheless, the resonance effect of the amino group in aniline is far more important than its inductive effect in electrophilic aromatic substitution and this resonance effect makes the amino group electron releasing and therefore *ortho–para* directing. Since the *ortho–para* directive effect is also observed in diphenylamine similar mechanisms of resonance and inductive effects are expected to be in accordance with present chemical thought. The mute literature does not support or discredit that the nitration of diphenylamine is similar to that of aniline. Since *N*-nitrosation is the first nitration reaction found in all ageing gunpowder studies, it seems plausible that in diphenylamine the inductive effect could be more important as the first step in nitrating this molecule. On the other hand, *N*-nitrosation can also be explained by the fact that secondary amines form *N*-nitroso compounds when the nitrating agent is nitrous acid.

ELECTRON TRANSFER AND RADICAL PAIR COLLAPSE, AN ALTERNATE NITRATION SCHEME

Perrin [27] proposed an alternate mechanism of electrophilic substitution reactions in aromatic molecules that are more reactive than toluene. Principally, the proposed mechanism involves an electron transfer from the aromatic to the nitronium ion followed by radical pair collapse to the σ complex intermediate:

$$NO_2^+ + ArH \rightarrow NO_2^{\cdot} + ArH^{+\cdot} \rightarrow HArNO_2^+ \quad (10)$$

For aromatics more reactive than toluene, the reaction with NO_2^+ is encounter-limited, so that all such aromatics react at the same rate. Yet although there is no intermolecular selectivity, there is intramolecular selectivity. If NO_2^+ is so reactive that it reacts at every encounter with a π system, it is reasonable to ask what distinguishes the *ortho* and *para* positions, which have only a slightly greater π electron density. It would be expected that NO_2^+ would not exhibit intramolecular selectivity, yet the selectivity is typical of

electrophilic substitution. In support of Perrin's mechanism is the fact that electron transfer to NO_2^+ is exothermic for all aromatics more reactive than toluene; additionally the estimated lifetime of an encounter pair (1×10^{-10} s) is too short to accommodate intramolecular selectivities. Therefore electron transfer mechanism provides an explanation for the encounter-limited nitration of aromatics more reactive than toluene. Electron transfer between aromatics and their radical cations is encounter-limited whenever the electron transfer is exothermic [27].

The electron transfer mechanism resolves the paradox of intramolecular selectivity without intermolecular selectivity, since the attacking species exhibiting the intramolecular selectivity (NO_2), is different from the one exhibiting no intramolecular selectivity (NO_2^+). Radical pair collapse, on the other hand, is quite likely to exhibit selectivity, due to the nonuniform spin density in the aromatic radical cation. There is a force of attraction between the NO_2 and each carbon of the radical cation. At long distances this force is strongest toward those positions of greatest spin density. At short distances this force is strongest toward those positions where bond formation will produce the most stable σ complex. Therefore, radical pair collapse is determined by a composite of spin density and σ complex stability. An effective test for the existence of this mechanism in diphenylamine was done by Lindblom [26], who performed a controlled-potential electrolysis of a mixture of diphenylamine and NO_2. The applied half-wave potential was incapable of oxidizing NO_2, but sufficed to generate the radical cation of diphenylamine. When the two radicals diffuse together, they combine to form the σ complex. The nitrated compound 4,4′-dinitrodiphenylamine was produced without HNO_3 and H_2SO_4. The observation that the same nitrated diphenylamine product is formed both electrochemically and via NO_2^+ is strong evidence that the radical pair is involved not only in the electrochemical synthesis but also in aromatic nitration.

Electron transfer and radical pair collapse also explains the paradox of nitration in gun powders without HNO_3 and H_2SO_4, which has defied classical nitration mechanisms. Additionally, the first nitration step of DPA in gun powders is *N*-nitrosation. Electron transfer from very reactive aromatics to NO^+ has been observed [27]. These data are consistent in that if nitration in gun powders is through an electron transfer and radical pair collapse, diphenylamine has such reactivity as to support this mechanism.

EXPERIMENTAL

Materials

Reagent grade 2,4-dinitrodiphenylamine, 2,5-dinitrophenol, 4-nitrophenol, *para*-diethylaminobenzaldehyde and *N*-nitrosodiphenylamine were purchased from Aldrich; 2-nitrodiphenylamine and 2-nitroresorcinol were purchased from Eastman; 4-nitrodiphenylamine, 4-nitrosodiphenylamine, *N*-nitrosodiphenylamine and 2,4-dinitrophenol were purchased from Pfaltz and Bauer; diphenylamine and triethylamine were purchased from Baker; methanol and tetrahydrofuran were purchased from Fisher; pharmaceutical-grade nitroglycerin was obtained from Marion Merrel Dow.

Pyrolytic flask experiments

A 1000-ml Kimax round bottom flask was modified to act as an electro-pyrolytic chamber. The sample tub that closed the electrical circuit was constructed of platinum and the electrical wires were of tungsten to support high temperatures. The stoppered end of the flask was modified to receive a Kontes thin-layer chromatography (TLC) tank inlet/outlet purge valve to allow the flask to be filled with nitrogen. A variable AC autotransformer was then connected to the tungsten terminals. The incandescence of the samples tub, and therefore indirectly the temperature, was measured with an optical pyrometer. The pyrolytic derivatives were identified by TLC.

Ageing studies of DPA nitration

Pharmaceutical grade nitroglycerin was obtained from Marion Merrel Dow (Nitro-Bid, 9 mg tablets), which yielded a total of 1620 mg of nitroglycerin. This was separated into three tubes

containing 540 mg of nitroglycerin each. Each aliquot was diluted to 6 ml with methylene chloride and 3 mg of diphenylamine in methylene chloride was added (0.6% w/v, DPA in methylene chloride). The tubes were incubated at (1) room temperature, (2) 37°C, and (3) 65°C. Nitration at the three different temperatures was stopped after 27 days and the 3 aliquots were nitrated at 120°C for 70 h. The nitrated derivatives were then identified by TLC.

TLC of gunpowders and nitrated derivatives of diphenylamine

Two-dimensional chromatography was done by the method of Volk, described in Refs. 21 and 22, on 254-nm UV fluorescence 20 × 20 silica gel plates (0.25 mm) (Brinkman Instruments), and developed with benzene–carbon tetrachloride–1,2-dichloroethane (50:30:25). The plates were dried and developed in the second dimension with petroleum ether–ethyl acetate (80:20). The plates were then dried, visualized with long-wave ultraviolet radiation (356 nm) and then sprayed with *p*-diethylaminobenzaldehyde (1 g in 75 ml methanol + 25 ml concentrated sulfuric acid). One-dimensional silica paper chromatography was done on Toxi-Lab TLC Systems purchased from Baxter. Toxi-grams were developed with benzene–carbon tetrachloride–1,2-dichloroethane (50:30:25) and visualized with potassium

TABLE 1

Possible derivatives from diphenylamine nitration

Compound	Abbreviation
Diphenylamine	DPA
N-Nitrosodiphenylamine	*N*-NO-DPA
2-Nitrosodiphenylamine	2-NO-DPA
4-Nitrosodiphenylamine	4-NO-DPA
2-Nitrodiphenylamine	2-*N*-DPA
4-Nitrodiphenylamine	4-*N*-DPA
N-4-Dinitrosodiphenylamine	*N*-4-NO-DPA
2,4-Dinitrodiphenylamine	2,4-di-DPA
2,6-Dinitrodiphenylamine	2,6-di-DPA
N-Nitroso-2-nitrodiphenylamine	*N*-NO-2-*N*-DPA
N-Nitroso-4-nitrodiphenylamine	*N*-NO-4-*N*-DPA
2,2′-Dinitrodiphenylamine	2,2′-di-DPA
2,4′-Dinitrodiphenylamine	2,5′-di-DPA
4,4′-Dinitrodiphenylamine	4,4′-di-DPA
4-Nitroso-2-nitrodiphenylamine	4-NO-2-*N*-DPA
N-Nitroso-2,4-dinitrodiphenylamine	*N*-NO-2,4-di-DPA
N-Nitroso-2,2′-dinitrodiphenylamine	*N*-NO-2,2′-di-DPA
N-Nitroso-2,4′-dinitrodiphenylamine	*N*-NO-2,4′-di-DPA
N-Nitroso-4,4-dinitrodiphenylamine	*N*-NO-4-4′-di-DPA
2,4,6-Trinitrodiphenylamine	2,4,6-tri-DPA
2,2′-4-Trinitrodiphenylamine	2,2′,4-tri-DPA
2,4,4′-Trinitrodiphenylamine	2,4,4′-tri-DPA
2,2′,6-Trinitrodiphenylamine	2,2′,6-tri-DPA
N-Nitroso-2,2′,4-trinitrodiphenylamine	*N*-NO-2,2′,4-DPA
N-Nitroso-2,4,4′-trinitrodiphenylamine	*N*-NO-2,4,4′-DPA
2,2′,4,4′-Tetranitrodiphenylamine	2,2′,4,4′-tetra-DPA
2,2′,4′,6-Tetranitrodiphenylamine	2,2′,4′6-tetra-DPA
2,2′,6,6′-Tetranitrodiphenylamine	2,2′,6,6′-tetra-DPA
2,4,4′,6-Tetranitrodiphenylamine	2,4,4′,6-tetra-DPA
2,2′,4,6-Tetranitrodiphenylamine	2,2′,4,6-tetra-DPA
2,2′,4,4′,6-Pentanitrodiphenylamine	2,2′,4,4′,6-penta-DPA
2,2′,4,6′,6-Pentanitrodiphenylamine	2,2′,4,6′,6-penta-DPA
2,2′,4,4′,6,6′-Hexanitrodiphenylamine	2,2′,4,4′,6,6′-hexa-DPA

dichromate (0.8 g in 100 ml 60% sulfuric acid). For the gunpowder experiments, 50 mg of gunpowder was extracted with 1 ml of methylene chloride in a screw top tube at room temperature for 12 h. One and three drops, respectively, of the extract were loaded onto a Toxi-gram strip. Following development, the diameter of each analyte was measured and the area calculated. Standard regression analysis confirmed a high correlation between spot area and spot amount, and TLC spot diameter was used in subsequent experimentation. To obtain a summary statistic that could characterize individual gunpowders, the areas of the analytes were summed; the resulting area reflects the total area of DPA and its derivatives detected in the sample.

LC analysis of gunpowders

A Spectra Physics 8700 high-pressure liquid chromatograph with a Beckman 254-nm UV detector was used for analysis. The analytical column was a Phenomenex Ultramex 5C18 (25 cm × 4.6 mm i.d.) reversed-phase C_{18} column. Samples were injected with an Altex 210 injector with a 20-μl loop. The Zeiss recorder was set at 1 mV and operated at 0.5 cm min^{-1}.

Separations were obtained isocratically with methanol–water–triethylamine (74:25:1). Flow-rate was maintained at 1 ml min^{-1} and column temperature was kept at 25°C.

Stock standards at a concentration of 200 μg ml^{-1} were made for diphenylamine, *N*-nitrosodiphenylamine, 2-nitrodiphenylamine and 4-nitrodiphenylamine. 4-Nitrosodiphenylamine (100 μg ml^{-1}) and 4-nitroaniline (200 μg ml^{-1}) were prepared for internal standards. All solutions were made by dissolving the solute in 20 ml of tetrahydrofuran and adding methanol to 100 ml in a volumetric flask. Working standards and internal standards were diluted to 40 μg ml^{-1}.

Sample preparation for LC was as follows: 50 mg of gunpowder was weighed and transferred to a screw top tube. 1 ml of methylene chloride was added and allowed to extract for 12 h after which the extract solution was filtered through an Acro LC13 0.2-μm filter (Gelman Sciences 4450). 50 μl of the filtered extract was then transferred to an evaporation tube and 50 μl of the internal stan-

TABLE 2

TLC R_F values for DPA and nitrated derivatives

Compound	Silica gel		Visual	*p*-DEAB	Paper silica ToxiGram
	vertical R_F	Horizontal R_F			
DPA	0.94	0.96	N/S [a]	Blue	0.93
2-*N*-DPA	0.86	0.96	Orange/yellow	Pink	0.90
N-NO-DPA	0.72	0.96	N/S	Blue	0.78
2,4-di-*N*-DPA	0.70	0.76	Yellow	Lemon yellow	0.72
N-NO-4-*N*-DPA	0.69	0.82	Orange	Purple	0.72
2,2′-di-*N*-DPA	0.64	0.53	Yellow	Yellow	0.70
2,4′-di-*N*-DPA	0.58	0.69	N/S	Yellow/orange	0.65
N-NO-4,4′-di-DPA	0.52	0.76	Yellow	Yellow	?
N-NO-2,4′-di-DPA	0.47	0.35	Yellow	Yellow	?
4-*N*-DPA	0.45	0.62	Yellow	Purple violet	0.59
N-NO-2,2′-di-DPA	0.45	0.24	Yellow	Yellow	?
2,4,4′-tri-*N*-DPA	0.31	0.60	Yellow	Yellow	0.42
2,2′-4,4′-tetra-*N*-DPA	0.20	0.45	Yellow	Yellow	0.32
4,4′-di-*N*-DPA	0.12	0.29	Yellow	Yellow	0.24
2,2′,4,4′,6,6′-hexa-*N*-DPA	0.03	0	N/S	Fluorescence	0.03

[a] N/S = not seen.

TABLE 3

Nitration derivatives from room temperature reactions

Day	0	1	3	6	8	10	13	15	17	20	22	24	27
DPA	+	+	+	+	+	+	+	+	+	+	+	+	+
N-NO-DPA	–	–	–	–	–	–	–	–	+	+	–	–	+

dard was added to each sample and allowed to evaporate to dryness at room temperature. The sediment was reconstituted with 50 μl of tetrahydrofuran, swirled, and 200 μl of methanol was added. The samples were then analyzed by LC.

RESULTS AND DISCUSSION

A list of the possible nitrated derivatives of DPA is shown in Table 1, and TLC reference values are shown in Table 2.

Thermal stability of diphenylamine

To test the hypothesis that DPA might undergo pyrolytic degradation to certain products (e.g., phenol and aniline), the sample holder in the electro-pyrolytic chamber was loaded with 50 mg of DPA and heated to incandescence, i.e., 700–800°C, by applying an AC potential of 14 V to the filament. Oxidation within the chamber was inhibited by purging with nitrogen gas. After pyrolysis the condensates of the fumes that were generated during the experiment were analyzed by TLC for diphenylamine derivatives, aniline and phenols. Only DPA was recovered. The greatest quantity of DPA was recovered from the pyrolytic condensate and not the solid residue remaining on the sample holder (an observable ten-fold difference of sample area on TLC), implying that a large portion of the sample reached the vapor state. Similar results were noted when the sample holder was loaded with 4-nitro-DPA.

Pyrolysis of *N*-nitroso-DPA was performed until fumes were no longer generated and again the chamber and sample holder were analyzed. An unknown compound was formed during this experiment that was not identified by spectrophotometric or TLC analysis. Assays for phenol and aniline were negative. The temperature reached by the electro-pyrolytic chamber was between 700 and 800°C. This was determined with an optical pyrometer and by melting point correlations. This suggests that DPA and some of its derivatives may survive a limited exposure to high temperatures in a nitrogen atmosphere, and equally important, that DPA is not thermally degraded to aniline or phenol in these experiments. These data support the empirical findings of Dahl et al. [5–7] that DPA is relatively stable in the shooting environment.

The electro-pyrolytic chamber could not be tested with air for fear of a filament failure, but it seems certain that oxidation derivatives would form under such conditions; these compounds would not resemble diphenylamine or its nitrated derivatives.

Ageing studies of diphenylamine nitration

The nitration/nitrosation of DPA by nitroglycerine at room temperature yielded the products listed in Table 3.

TABLE 4

Nitration derivatives from 37°C reactions

Day	0	1	3	6	8	10	13	15	17	20	22	24	27
DPA	+	+	+	+	+	+	+	+	+	+	+	+	+
N-NO-DPA	–	–	–	–	–	–	–	+	+	+	+	+	+

TABLE 5

Nitration derivatives from 65°C reactions

Day	0	1	3	6	8	10	13	15	17	20	22	24	27
DPA	+	+	+	+	+	+	+	+	+	+	+	+	+
N-NO-DPA	–	–	+	+	+	+	+	+	+	+	+	+	+
4-*N*-DPA	–	–	–	–	+	+	+	+	+	+	+	+	+
2-*N*-DPA	–	–	–	–	–	–	–	+	+	+	+	+	+
4-NO-DPA	–	–	–	–	–	–	–	–	+	–	+	+	+

When the nitration was carried out at 37°C, the products consisted of the compounds listed in Table 4.

Nitration/nitrosation of DPA at 65°C yielded the products shown in Table 5.

Nitration for the three different temperatures was stopped after 27 days and all three samples were nitrated at 120°C for 70 h. The nitrated compounds yielded are listed in Table 6.

A summary of the nitrated derivatives of diphenylamine encountered in this research, as well as those described in the relevant work of others, are listed in Table 7.

The presence of nitroso derivatives in addition to nitro derivatives appears to be related to temperature. This was shown in the present work and in that of others [10,21,22]. As the temperature of incubation increases, the formation of the species of the diphenylamine derivatives varies, especially in the case of nitroso derivatives.

In the ageing of gunpowders, where the temperature is dependent upon the ambient environment, the nitration pathways are straighforward, since environmental temperatures rarely exceed 45°C. Therefore, the first step in the nitration of DPA is *N*-nitrosation, followed by simultaneous *C*-nitration and *N*-denitrosation. This is most likely due to the electron withdrawing effect of the nitro group, a process that would destabilize the *N*-nitroso bond. The *C*-nitration occurs preferentially at the para position; nitration at the ortho position was noted 7 days after nitration occurred at the para position in the 65°C incuba-

TABLE 6

Nitration derivatives of all samples after 70 h at 120°C

Compound	Room temp.	37°C	65°C
DPA	–	–	–
N-NO-DPA	–	–	–
2-*N*-DPA	–	–	–
4-*N*-DPA	–	–	+
N-NO-2-*N*-DPA	–	–	–
N-NO-4-*N*-DPA	–	–	+
2,2′-di-DPA	+	–	+
2,4′-di-DPA	+	–	+
4,4′-di-DPA	+	–	+
N-NO-2,2′-di-DPA	+	–	+
N-NO-2,4′-di-DPA	–	–	–
N-NO-4,4′-di-DPA	+	–	+
N-NO-2,2′,4-tri-DPA	+	–	+
2,4,′-tri-DPA	+	+	–
2,2′-4,4′-tetra-DPA	–	–	–
2,2′-4,4′,6-penta-DPA	–	+	–
2,2′,4,4′,6,6′-hexa-DPA	–	–	+

TABLE 7

Comparison of derivatives produced from diphenylamine nitration

Compound	Schroeder et al. [10]	Yasuda [20]	Volk [22]	Current work
DPA	+	+	+	+
N-NO-DPA	+	+	+	+
2-*N*-DPA	+	+	+	+
4-*N*-DPA	+	+	+	+
N-NO-2-*N*-DPA	–	–	–	–
N-NO-4-*N*-DPA	–	+	+	+
2,2′-di-DPA	+	+	+	+
2,4-di-DPA	–	+	+	–
2,4′-di-DPA	+	+	+	+
4,4′-di-DPA	+	+	+	+
N-NO-2,2′-di-DPA	–	+	–	+
N-NO-4,4′-di-DPA	–	+	+	+
2,4,4′-tri-DPA	+	+	+	+
2,2′,4-tri-DPA	+	+	+	–
2,2′,4,4′-tetra-DPA	+	+	+	–
2,2′-4,4′,6-penta-DPA	+	+	+	+
2,2′-4,4′,6,6′-hexane-DPA	+	+	+	+

tion experiment. Further nitration at the 4'-position on the other phenyl ring occurred more often than the 2,2'-dinitro-DPA. It therefore seems reasonable to postulate that the preferential nitration pathway would be 4-nitro-DPA, followed by 4,4'-dinitro-DPA.

Schroeder et al. [10] studied DPA by heating small samples of powder at 71°C in separate tin containers for periods up to 258 days. Rough quantitative determination of the predominant nitroso and nitro derivatives of DPA was identified by column chromatography and colorimetric techniques on eleven separate samples of the propellant. These nitrated derivatives showed an increase and decrease of each compound when plotted in terms of the quantity of DPA derivatives produced.

Nitrates may decompose into such nitration agents as nitrous acid, nitric acid, nitrogen dioxide or nitrogen tetraoxide. These agents may react independently, or may be in an essential equilibrium with each other so that there is effectively a "single" nitrating agent that is responsible for the reactions.

Based on the current research, as well as the data of Schroeder et al. [10], Yasuda [21] and Volk [22], it is apparent that direct *N*-nitrosation occurs, since no other process could be expected to yield *N*-nitroso-DPA. It is also certain that denitrosation occurs. Direct nitrations of the phenol rings will involve (a) direct nitration of amines (including partially nitrated derivatives), and (b) direct nitration of nitrosamines (which may be accompanied or followed by denitrosation).

Presence and occurrence of DPA in gunpowders by TLC and LC analysis

The sensitivity of the Toxi-Lab TLC plates is unquestionably less than that of conventional silica gel TLC plates; extracts analyzed on both systems showed that two dimensional chromatography resolved compounds not adequately resolved or separated by one dimensional chromatography or by the Toxi-Lab system. However, because of the convenience of the Toxi-Lab system, this system was used for the narrow purpose of characterizing a large number of gunpowders and cartridges.

It was found that by using TLC, gunpowders may be successfully characterized and compared in a semi-quantitative manner to establish their provenance. It is accepted, nevertheless, that this comparison is at least in part a feature of time and that the nitration derivatives found at any moment are a reflection of the history and environment to which the propellants have been submitted. The characterization of 118 gunpowders based on the presence of DPA and its nitrated derivatives showed this approach to be straightforward and unequivocal with respect to interpretation. As in the work of Archer [28], the most frequently detected stabilizers in gunpowders was DPA and three of its derivatives: *N*-nitroso-DPA, 2-nitro-DPA and 4-nitro-DPA. In the older manufactured gunpowders, polynitrodiphenylamines were detected, inferring advanced stages of nitrocellulose degradation.

When TLC techniques were used, only 5 propellants from 118 samples could not be differentiated. The similarities in these gunpowders could be attributed to samples having experienced similar environmental conditions, or alternatively, they could be propellants that originated from a common source and were distributed to various cartridge loader manufacturers.

Assay time by TLC is about 45 min, and because the equipment needs are minimal, these analyses can be accomplished in virtually any laboratory. Increased sensitivity and quantitation could be obtained by using a scanning densitometer.

	Dupont Hi-Skor	Hercules Herco 110	Hercules Red Dot 10	DuPont 18
(1) DPA	31.7	31.7	40.0	1.9
(2) N-NO-DPA	49.5	27.8	44.6	54.5
(3) 2-N-DPA	15.0	15.0	17.8	17.8
(4) 4-N-DPA	17.8	2.0	6.0	17.8
(5) 2,2'-di-DPA				1.9
(6) 4,4'-di-DPA				10.0

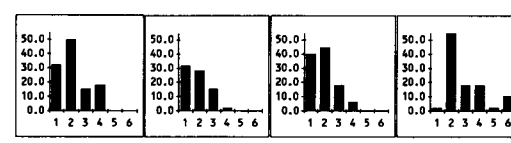

Fig. 2. Bar graph of selected gunpowders analyzed by TLC to illustrate the relative content of diphenylamine and its nitrated derivatives. Values represent area of TLC spots.

TABLE 8

Chemicals and samples tested for assay interference

p-Nitroaniline	3-Nitrophenol
m-Nitroaniline	4-Nitrophenol
Matches	*p*-Phenylphenol
Urine	Nitrobenzene
K_2NO_2	1,2-Dinitrobenzene
Phenol	1,3-Dinitrobenzene
2-Nitrophenol	2,4-Dinitrophenol

Figure 2 exhibits bar graphs of selected gunpowders and their relative content of DPA and its nitrated derivatives.

To evaluate the possibility of false positive reactions, the chemicals listed in Table 8 were tested by TLC and it was established that differentiation was possible based on color and/or R_F. The chemicals listed in Table 8 were chosen because of their chemistry, however implausible, or because of their historical significance in connection with the testing of gunshot discharge residues.

A typical chromatogram of DPA and nitrated derivatives reference compounds is shown in Fig. 3; the peaks are well-resolved, symmetrical, and the retention times are reproducible.

Gunpowders and cartridges were analyzed for DPA, *N*-nitroso-DPA, 2-nitro-DPA and 4-nitro-DPA. Gunpowder characterization using LC techniques produced a highly stylized profile. Figure 4 illustrates the DPA derivatives from the

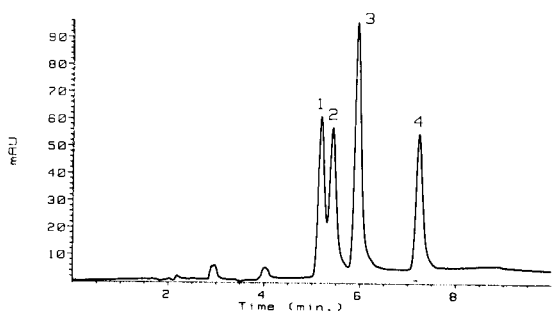

Fig. 3. Standard chromatogram of diphenylamine and its nitrated derivatives by LC. Column: Phenomenex RP C18; isocratic mobile phase of methanol–water–triethylamine (74:25:1); flow-rate 1.0 ml min^{-1}; injection volume 20 μl. Peaks: (1) *N*-nitrosodiphenylamine, (2) 4-nitrodiphenylamine, (3) diphenylamine, and (4) 2-nitrodiphenylamine.

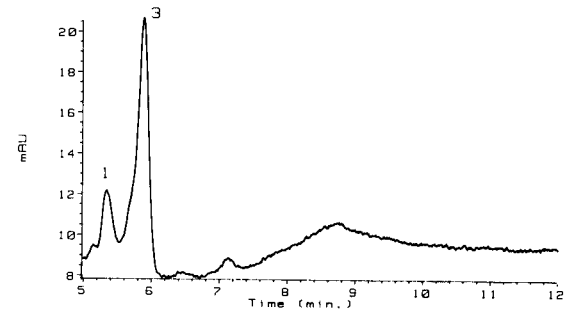

Fig. 4. LC of diphenylamine and its nitrated derivatives of gunpowder from a 7-mm Remington Magnum cartridge. Peaks: (1) *N*-nitrosodiphenylamine, and (3) diphenylamine.

gunpowder of a 7-mm Remington Magnum cartridge. Those gunpowders that contained ethyl centralite as the main source of stabilizer could easily be distinguished, since the elution of ethyl centralite did not interfere with any of the DPA derivatives of interest. Assay time by LC is about 45 min and produces a quantitative number that revealed some information regarding the current extent of nitration for a given sample.

The sensitivity of the LC assay was 0.25 μg ml^{-1} or 5 ng total detected when a 20-μl sample loop was used. The LC response for DPA, *N*-NO-DPA, 2-nitro-DPA and 4-nitro-DPA was linear from 50 to 4000 ng.

It is of interest to note that none of the Federal brand cartridges and some other cartridges analyzed had DPA or its derivatives as their main source of stabilizer.

A profile of DPA and its various derivatives found in 4 representative gunpowders is shown in Fig. 5. An experiment to determine the feasibility of recovery of DPA and its derivatives on the

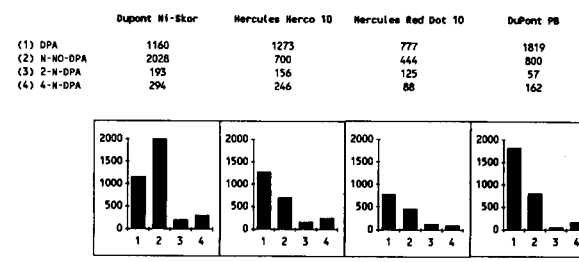

Fig. 5. Bar graph of representative gunpowders analyzed by LC showing total content of diphenylamine and its nitrated derivatives (μg ml^{-1}).

hands of a person after firing a handgun was conducted. As performed in the manner discussed above, the LC or TLC techniques were inadequate with respect to sensitivity to conclude that this type of analysis would be applicable to all shooting incidents and to all types of ammunition or firearms. Attempts to recover DPA and its derivatives from the hands of a shooter were unsuccessful in 0.22 and 0.25 caliber handguns. Successful recovery of DPA derivatives was ordinarily observed, however, with calibers equal to or greater than 0.38 Special. Increased sensitivity could be obtained, however, by measuring DPA derivatives by coupling liquid chromatography with an electrochemical detector, or the use of liquid chromatography–mass spectrometry (LC–MS); this would achieve an order of magnitude greater sensitivity and would permit the LC technique to be applied to calibers less than 0.38. In conclusion, the TLC approach to characterizing gunpowders was found useful for the purpose of distinguishing between samples of different provenance and different age. LC was found to provide a more suitable means for the quantitation of minor diphenylamine / derivatives. The scheme in Fig. 1 illustrates the most likely nitration events; a large group of derivative compounds may be formed, which in turn will give gunpowders a highly stylized profile of nitrated derivatives. The mechanism of DPA nitration is most likely that of electron transfer followed by radical pair collapse. This mechanism explains the paradox of nitration in gunpowders without HNO_3 and H_2SO_4, an aspect of the chemistry of diphenylamine that has defied classical notions of nitration mechanisms.

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The chemistry of death by gunshot

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Abstract

When a firearm is discharged, a complex series of events takes place within an interval of approximately 1 millisecond. Many of these events are associated with chemical as opposed to mechanical processes, and an understanding of these processes, and of the materials involved in these processes, may be of value in the reconstruction of the factual circumstances at the instant of discharge. These chemical processes include the formation of uprange and downrange gunshot residues of several diverse sorts. Proper analysis and interpretation of gunshot residues and other components of ammunition may provide useful information in the investigation of shooting incidents.

Keywords: Explosives; Forensic analysis; Gunshot residue analysis

Modern firearms are discharged by depressing the trigger. This action typically requires 2 to 6 kg of force, but the mechanical advantage supplied by the trigger lever is such that under ordinary circumstances even a child is able to discharge the firearm. Pressure on the trigger causes a part called the sear to become disengaged from the hammer, which travels forward under spring tension to strike the firing pin. The firing pin in turn strikes the primer of the cartridge to initiate the discharge. From the instant the firing pin strikes the cartridge to the time the bullet emerges from the barrel is typically about a millisecond; the process is irreversible, and, depending on the caliber, powder type and charge, the bullet is sent on its way by a force of 1 to 50 billion ergs of energy.

This energy is all too frequently irresponsible. The majority of homicides committed in the United States are by gunshot. Homicide is the leading cause of death in American males aged 15 to 23, and the fourth leading cause of death in all Americans under the age of 65. Unless something occurs to cause the homicide rate to level off, let alone decline, two million Americans who are currently alive will be murdered before the end of their natural lifetime.

The firearm is but the applicator, and the bullet which causes death is but the vehicle. Fundamentally, the bullet is given its impulse by chemistry, and it is chemical principles which govern these processes. An understanding of these processes may be of value in the reconstruction of the factual circumstances at the instant of discharge.

The chemistry of ammunition components and the residues of firearms discharge is a vast landscape, and none too tidy, either. Many aspects of this broad subject will call out for some specific detail, which in turn will cry out for the specific exception. This review is intended to paint the landscape with broad strokes, but is not intended

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to exhaustively treat each theme in this complex subject. As appropriate, the specific details are provided; in most instances, the specific exceptions are not.

Cartridge discharge events

When the firing pin strikes the primer of a cartridge, the soft brass metal of the primer is crushed. The metal deforms under the force of the firing pin and is pushed toward the interior of the cartridge. When this occurs, a small amount of a sensitive explosive compound is pinched between the crushed metal and (in centerfire cartridges) a hard, immobile part of the primer called the anvil. The explosive detonates, and the temperature and pressure go from ambient to about 10^4 kPa and 1500–2000°C in a tenth of a millisecond.

Over the years, manufacturers of ammunition have used a number of initiating primer materials; Table 1 lists explosives that have been used. The heat from this sudden explosion causes the main charge of smokeless gunpowder, a mixture of nitrocellulose and (generally) nitroglycerine, to ignite. In addition to nitrocellulose and nitroglycerine, the propellant often contains nitrate esters (such as ethylene glycol dinitrate), stabilizers (such as diphenylamine), burn modifiers (such as 2,4-dinitrotoluene), plasticizers (such as dibutylphthalate), and various other materials in slight concentration. Table 2 lists materials which may be encountered in smokeless gunpowder and primer mixtures. (It should be understood that this table lists organic compounds which *may* have been included in gunpowder or primer mixtures at some time, and is not a list of compounds which will be found in any given sample.) The smokeless gunpowder does not detonate, or explode, but deflagrates. That is, it burns fast, very fast indeed, but not fast enough to qualify as a true detonation. (Most onlookers would be unaware of the distinction, since the entire process is over in about a millisecond.) The gunpowder will begin to ignite about 0.5 ms after the crushing of the primer, with an increase in temperature and pressure to about 3600°C and 3×10^5 kPa. The pressure and temperature reach peak values between 0.5 and 0.75 ms, and then begin to subside. When the bullet leaves the muzzle of the firearm, the pressure is vented to the atmosphere. This typically occurs before all of the gunpowder is consumed, causing fragments of unburned gunpowder to follow the bullet downrange. Other particulates, apart from the fragments of unburned gunpowder, are also formed during the detona-

TABLE 1

Primer explosives

Lead styphnate	Diazodinitrophenol
Lead azide	Lead thiocyanate/chlorate
Silver azide	2,6-Dinitroquinone-2-diazide
Tetracene	Diazoaminotetrazole
Mercuric fulminate	Diazodinitrophenol (DDNP)
Lead ethylenedinitramine	Dinitrodihydroxydiazobenzene
Lead 2,4-dinitrobenzoate	Lead dinitrophenylazide
Lead 3,5-dinitrobenzoate	Lead 2,4-dinitroresorcinate
Lead 3-nitrophthalate	Lead nitroamino guanidine
Lead dinitrosalicylate	Lead trinitrophloroglucinol
Lead hypophosphite	Ferric styphnate/ hypophosphite
Red phosphorus/K_2ClO_4	Lead styphnate/ nitroaminotetrazole
Lead nitroaminotetrazole/ styphnate	

TABLE 2

Miscellaneous organic compounds that may be found in smokeless gunpowder and primer mixtures

Nitrocellulose	Nitroglycerine
Diphenylamine	Trinitrotoluene (TNT)
Cresol	Pentaerythritol tetranitrate (PETN)
Resorcinol	Diethyl phthalate
Carbazole	Dimethyl phthalate
Triacetin	Dimethyl sebacate
Nitrotoluene	*N,N*-Diethylcarbanilide
Carbanilide	(Ethylcentralite)
Dinitrocresol	*N,N*-Dimethylcarbanilide
Cyclonite (RDX)	(Methylcentralite)
2,5-Dinitrotoluene	1,2,4-Butanetriol trinitrate
2,6-Dinitrotoluene	*N,N*-Dibutylcarbanilide
2,4-Dinitrotoluene	(Butylcentralite)
Dibutyl sebacate	Diethylene glycol dinitrate
Dibutyl phthalate	Di-*n*-propyl adipate (DNPA)
Graphite	*N*-Methyl-*p*-nitroaniline
	Trimethylolethane trinitrate
	Triethylene glycol dinitrate (TFGDN)
	Nitroguanidine

tion of the primer and the ignition of the main propellant.

The bullet is driven out of the cartridge case by the pressure of the burning gunpowder. It is propelled through the barrel of the firearm, and thence to its ultimate destination, which alas may be a human being. Bullets are typically formed of lead, some of lead alone and some with a jacket of copper around the lead. Even if the bullet has a copper jacket, it is likely to have an area of lead at the base which is exposed to the high heat of the discharge environment.

Some firearms, for example, revolvers and bolt-action rifles, do not eject an expended cartridge case unless the shooter does so deliberately. Consequently, when firearms of this type are used, a cartridge case may not be encountered at the crime scene because it is still in the firearm, and is removed as the assailant leaves the scene. Other firearms are of the so-called 'automatic' type, in which the cartridge case is normally ejected from the firearm. With weapons of this type, the cartridge case will be found at the crime scene unless the assailant notices it and deliberately picks it up and leaves with it. The point here is that although bullets are invariably deposited at the crime scene, cartridge cases may or may not be.

When bullets and cartridge cases are found at the scene, their greatest value as evidence is in the comparison of markings on their surfaces with test firings from a suspect firearm; in this manner the forensic scientist may be able to state that the bullet or cartridge case had been fired in a particular firearm, to the total exclusion of all other firearms.

But if the firearm is subsequently lost, hidden, or destroyed, it will not be available for purposes of test firing. In those instances, it may be possible to compare the evidence bullet or cartridge case with another lot of fired or unfired ammunition to determine if the composition of the evidence is consistent with the ammunition found in the possession of the suspect.

Bullets are frequently encountered which contain lead or copper as major components. The minor and trace elements might be almost anything, but it is common to encounter lead bullets with 0.05–2.5% antimony intentionally added as a hardening agent, and with copper at a concentration of 7–50 ppm and zinc at a concentration of 1 ppm. Copper-jacketed bullets may contain 5–10% zinc, antimony at a level of 2–5 ppm, and silver at 30–90 ppm. Tin and bismuth may be encountered, although infrequently, and aluminum and nickel are occasionally represented.

Cartridge cases are primarily copper and zinc, but may also contain iron, nickel, and lead. As cartridge cases are increasingly manufactured from scrap and recycled brass, virtually any element with which copper is alloyed may creep into the cartridge case brass. The more complex the composition of the cartridge case, the more unique is the 'signature' of the brass of the evidence found at the crime scene.

In practice, the chemistry of gunpowder and ammunition components is considerably more complex than would be suggested by the preceding discussion. It is possible to focus on any one aspect of the chemistry of gunpowder and expand a discussion of the subject to some length. For example, the explosive mixture used in the primer may contain, in addition to the primary explosive, an oxidizer (e.g., barium nitrate, potassium chlorate, lead nitrate, or lead dioxide), a burn rate modifier (e.g., K_2SO_4, Na_2SO_4, SnO_2), a fuel (e.g., antimony sulphide, gum arabic, calcium silicide, nitrocellulose, carbon black, lead thiocyanate, and powdered zirconium, aluminium, or magnesium), a frictionator (e.g., aluminum powder, ground glass), a sensitizer (e.g., TNT, tetracene, PETN), and a binder (e.g., gum arabic, tragacanth gum, dextrin, sodium alginate, rubber cement, karaya gum).

Composition of the projectile

Many bullets, and particularly revolver bullets, are made of lead. But lead is such a ductile metal that bullets comprised solely of lead are generally considered to be too soft for optimum use; consequently, antimony at a level of 0.05–2.5% is often intentionally introduced to harden the lead. In addition to antimony, there may be trace concentrations of virtually any element associated with either lead or antimony when the metals are extracted from ore. Arsenic is one such example.

Guinn and co-workers [1–3] have made extensive studies of the composition of bullet lead. Using neutron activation analysis (NAA) to measure 5 elements, viz., ^{124}Sb, ^{125}Sn, ^{108}Ag, ^{76}As, and ^{66}Cu, most samples of bullet lead may be distinguished [4]. The selection of these five elements represents a compromise between the optimum detection of short-lived and longer-lived isotopes.

Many other bullets are formed with a copper jacket surrounding a lead core. Ammunition intended for use in autoloading firearms is generally of this type, as is most hunting ammunition. Other bullets may be formed of aluminum, or of an aluminum–nickel alloy, while some specialized bullets, e.g., armor-piercing ammunition, may have a core of tungsten, steel or iron, beryllium copper, bronze, or even depleted uranium.

Composition of the cartridge case

The cartridge case is typically brass, with a basic composition of 70% copper and 30% zinc, although aluminum and nickel-plated brass cases are not uncommon, and even steel cases have been used. In addition to copper and zinc, it is common to encounter traces of nickel, lead, and iron. It is not beyond the bounds of credulity to occasionally find traces of bismuth, silver, tin, silicon, cadmium, phosphorus, cobalt, or manganese. As with copper-jacketed projectiles, the suite of minor and trace elements in cartridge case brass may be enlarged by the use of recycled or scrap brass, a practice that is increasingly prevalent because of the rising cost of copper.

Composition of unburned gunpowder

Smokeless gunpowder from a cartridge or recovered in an unburned state from a downrange target may be characterized by testing the suspected particle with color reagents [5–8]; those reagents based on concentrated sulfuric acid appear to be the most efficacious [8]. Table 3 lists the reactivity of a few selected gunpowders with a number of sulfuric acid reagents.

Unburned gunpowder may also be characterized by means of thin-layer chromatography (TLC) of the numerous gunpowder additives [9–11]. A fragment of the gunpowder may be dissolved in acetone and subjected to one-dimensional thin-layer chromatography, or alternatively by two-dimensional TLC if the ultimate in discrimination is desired. Numerous solvent systems have been described for the separation of minor components of smokeless gunpowder; the TLC requirements are not taxing. For two-dimensional TLC, the present author prefers a Silica Gel G system with benzene in the first dimension and with benzene–ethyl acetate (85 : 15) in the second dimension, and visualizes the components of the gunpowder by spraying the plate with a solution of 0.5% vanillin in glacial acetic acid to which is added 5 ml of 85% phosphoric acid. This system permits most smokeless powders to be identified and distinguished from one another. An alternative spray reagent is 1 g potassium dichromate in 80 ml water to which is added 20 ml of concentrated H_2SO_4. This latter spray reagent is reported [12] to detect 1 μg of diphenylamine and *N*-nitrosodiphenylamine, and 0.3 μg of the 1,3-dialkyl-1,3-diphenylureas.

Many smokeless gunpowders will show diphenylamine, along with the nitrated products *N*-nitrosodiphenylamine, and 2- and 4-nitrodiphenylamine. Polynitrodiphenylamine derivatives may be present, but these appear to be primarily associated with aged samples. Thin-layer chromatography will often show the presence of glyceryltrinitrate, 2,4-dinitrotoluene, and 1,3-dialkyl-1,3-diphenylurea in smokeless gunpowder [10]. Smokeless powder may also be characterized by means of gas chromatography (GC) [13,14], gas chromatography–mass spectrometry (GC–MS)

TABLE 3

Reactivity of representative gunpowders with acidic color reagents [8] (Marquis Reagent: 3 drops of 40% formaldehyde in 3 ml of conc. H_2SO_4; Mecke Reagent: 5 mg of selenious acid (H_2SeO_3) in 1 ml of conc. H_2SO_4)

	Concentrated H_2SO_4	Marquis	Mecke
Alcan 101	Light brown	Orange yellow	Orange yellow
DuPont 4227	Bright yellow	Dark olive	Grayish olive
Hercules 210	Reddish brown	Deep orange	Brownish orange
Hogdon 375	Medium olive	Deep blue	Deep blue
Olin 450LS	Greenish yellow	Dark olive	Light olive

[15,16], infrared (IR) spectrometry [17], differential thermal analysis [18], pyrolysis–gas chromatography [19,20], liquid chromatography (LC) [21–28], or capillary electrophoresis [29]. A very powerful technique for this purpose is Fourier transform IR (FTIR) combined with GC–MS [30], or capillary supercritical fluid chromatography with FTIR [31]. Both GC and LC may be capable of distinguishing between different production lots of the same ammunition; GC would seem to have a slight edge over LC in distinguishing between various manufacturers of gunpowder [32].

Gunshot residues, downrange

The combustion processes related to the discharge of a firearm are not totally efficient, and as a consequence some unburned gunpowder is invariably blown down the barrel and ejected from the muzzle of the firearm. Ejection of unburned gunpowder is more pronounced with shorter-barreled firearms, but is never totally absent, even with long-barreled firearms which provide a better opportunity for the powder to burn completely.

The significance of this to the forensic scientist is that the distance from the muzzle of the firearm to the target may be determined by an inspection of the size and density of the pattern of downrange residue. If the gunpowder residues are distributed downrange in the shape of a cone with the muzzle at the vertex, then the conic section corresponding to a close distance would be a circle of small diameter; a circle of larger diameter would suggest a greater distance between the firearm and the target. For these types of interpretation to be valid, however, the forensic scientist must be assured that the pattern is in fact due to gunpowder and not some other artifact unrelated to the shooting incident.

The distance these fragments of gunpowder will travel downrange depends on the burning rate of the powder, the total powder charge, the barrel length, and the geometry of the unburned powder. A powder cut into millimeter sized square flakes, for example, will not be as aerodynamically stable in flight as a sphere of the same size, and consequently will not travel as far downrange. It is unusual to observe fragments of unburned gunpowder on downrange targets at distances greater than 70 cm, although in some extreme instances with rifle ammunition the fragments may travel in excess of 2 m.

In addition to (millimeter sized) fragments of unburned powder, which are still more or less recognizable as fragments of gunpowder, sub-micron sized particles of smoke are blown down the barrel and ejected from the muzzle. By virtue of their exceedingly small mass, these particles do not extend far downrange. It is unusual to visually observe smoke darkening on downrange targets at distances greater than 50 cm, and in many instances the pattern of smoke darkening will be scarcely perceptible at 20 cm. In other instances it will be quite conspicuous at 30 cm and moderately conspicuous at 40 cm.

How does one verify, however, that the residues of the gunshot discharge that one sees on downrange targets are in fact fragments of unburned gunpowder or sub-micron sized products of gunpowder deflagration?

With unburned gunpowder, a suspected particle may be examined under a stereoscopic microscope. In some instances, the morphology of the particle permits an unambiguous identification, but in others the fragments may be heat-deformed to such an extent that the fragment is unrecognizable. In the latter case, the fragment may be tested in a white porcelain spot plate with 0.2% diphenylamine in concentrated sulfuric acid. In the presence of nitrate, primarily from the nitrocellulose, the test solution will turn deep blue. The diphenylamine test is sensitive, but has its shortcomings with respect to how the test results should be interpreted. This issue is considered in connection with the dermal nitrate test in the discussion of uprange gunshot residues.

The particle of suspected gunpowder may also be tested with color tests, TLC, GC with or without mass spectrometry, or by pyrolysis–gas chromatography; these methods are discussed in other sections. In some instances, soft x-ray photography or autoradiography may be of value.

Gunshot residues, uprange

No other area dealing with the chemistry of gunshot evidence is so complex, and controver-

sial, as the identification of uprange discharge residues. (Uprange residues are those which are deposited on surfaces in close proximity to the firearm, as contrasted with downrange residues which are propelled along the trajectory of the bullet.) When a firearm is discharged, the majority of the residues related to the discharge are ejected downrange from the muzzle. A small amount, however, is deposited on the hands of the person firing a handgun, or onto the face of a person firing a rifle. If these residues can be properly identified and interpreted, it will provide enormous assistance to the police investigator in a reconstruction of the factual events at the time the shooting took place. For example, in a suspected suicide case, does the deceased have gunshot residues on his or her hand which would indicate that the firearm was held by that person at the instant of discharge? In a case where a suspect denies firing or even holding a gun, are there discharge residues which would support or contradict his or her statement? These issues have historically caused problems for the forensic chemist. The chemistry is not trivial, and, broadly speaking, the interpretation of data is difficult.

Nitrate

The initial attempt to determine by chemical means if a person had fired a weapon was a disaster. In 1933, Teodoro Gonzales of the Criminal Identification Laboratory of Mexico City introduced a test to detect nitrates on the hands of a suspect to the Milwaukee, Wisconsin police department [33]. The test came to be known as the "dermal nitrate test", the "paraffin test", the "Gonzales test", or the "diphenylamine test", and with the benefit of hindsight perhaps represents the nadir of forensic science in the 20th Century. Molten paraffin at just above the melting point was brushed onto the hands of a person suspected of having discharged a firearm. When the paraffin froze, it was pulled off intact and the paraffin sprayed with a 0.2% solution of diphenylamine in concentrated (or, alternatively, 70% v/v) sulfuric acid. If the person had recently fired a gun, the paraffin would, ostensibly, turn blue.

The test is not fundamentally a test for a firearm discharge event, of course, but a test for oxidizers. While the test had enormous intrinsic appeal to police investigators, the test was fatally flawed. Nitrates and chlorates are so ubiquitous in nature that, if the test was used as a test for shooting, false positive reactions abound. Persons with urine on their hands will test positively, as will people who have recently struck a match. Oxidizers causing a positive reaction are also found in tobacco ash, fingernail polish, numerous pharmaceuticals, fertilizers, and in leguminous plants. On the other hand, false negative tests were common; in some instances the hands of police officers at a pistol range would fail to give a positive reaction. The test was soon criticized [34–36], and abandoned by most forensic laboratories. Turkel and Lipman [37] reported that based on their studies, there was a 75% chance of a wrong conclusion when relating the results of the test to whether a person had in fact discharged a firearm. Many forensic chemists now hold that to run the test would not only be unwise and unprofessional, but downright unethical. *Nevertheless, fifty years after the test was discredited in the scientific literature, the test is still utilized in a number of places in the world!* One may argue that those jurisdictions that still use this test are in fact aware of its failings, but don't really care.

Nitrite

Although nitrates are exceedingly common in our environment, nitrites are less so. Nitrites associated with the discharge of a firearm may be demonstrated by electrochemical methods [38], or by some modification [39–41] of the classical Greiss test [42]. The Greiss test is a diazo coupling of an aminobenzene sulfonate with a naphthylamine; modifications of the test are necessary to avoid the use of potentially carcinogenic materials. The testing of downrange targets for nitrite to determine shooting distance was first suggested in 1937 by Walker [43]. Walker used 'C' acid (2-naphthylamine-4,8-disulfonic acid) or 'H' acid (1-amino-8-naphthol-3,6-disulfonic acid) to form a colored complex with nitrite. Many

modifications have been suggested, mostly variations of the diazo dye used, and a review exists of 52 different nitrite determinations [44].

Barium, antimony, and lead

The majority, but not all, of centerfire cartridge case primers contain barium, antimony, and lead. And even if the primer does not contain antimony or lead, the high temperature of combustion of the gunpowder may cause some lead and antimony to sputter off of the exposed lead base of the projectile during discharge. If one can successfully demonstrate the presence of these residues on the hands of a person, it may suggest that a person had discharged a firearm, or had handled one without having discharged it. The secondary transfer of material from other surfaces on which gunshot residues have been deposited may be a factor in some instances.

The average amount of barium, antimony, and lead on the hands of persons who have not discharged or handled a firearm, (and for a moment eschewing certain elevated occupational levels), is 0.8 μg for barium, 0.08 μg for antimony, and 20 μg for lead [45]. Current thought on the interpretation of residue levels on the hands of persons who have discharged or handled a firearm is not in all respects crystallized, but tends to center around three standard deviations above the background levels. Hence, the threshold values which are indicative of a person having discharged or handled a firearm are 1.8 μg for barium, 0.22 μg for antimony, and 49 μg for lead [45]. These levels are low, but within the capability of a number of analytical techniques to deliver accurate results. The values given here are only one criterion; others are widely used, and, additionally, the use of predetermined threshold levels of barium and antimony to indicate the presence of gunshot residues has been challenged on the basis of the variability of barium and antimony composition [46].

These residues do not persist very long on the hands of a person who is living, but remain longer on the hands of a deceased person. Although residues of barium, antimony, and lead have been found on the hands of a person who has discharged a firearm several hours before the sampling is conducted, it is common to fail to find the residues after 1 to 2 h have elapsed since the shooting incident. In the context of a criminal investigation, this requires an aggressive concern on the part of the investigator to have the hands of the suspect sampled as soon after the incident as possible. If the person has washed his or her hands, or if more than a few hours have elapsed since the shooting occurred, attempts to demonstrate gunshot residues will very likely be unsuccessful.

The hands of persons in a few specific occupations may show elevated levels of barium, antimony, or lead. Lead is found in plumbing materials, in storage batteries, and (increasingly rarely) in paint. Antimony is found in some paint and in the Babbitt metal used in motor bearings. Barium is found in paint and in automobile grease. It is uncommon, however, that the levels of all three elements will be elevated, or that the levels will adhere to the ratio of the three elements encountered in gunshot residues. If, for example, an analyst encountered a test subject with an antimony level greatly in excess of the barium level, the analyst would suspect environmental contamination rather than association with firearm discharge, and would consequently hesitate to express an opinion. A more extensive list of possible sources of these three elements is given in Table 4.

Sampling for gunshot residue determination

Sampling of the hands for gunshot residue determination may be conducted in a number of ways. The sampling for scanning electron microscopy (SEM) is unique; an adhesive (e.g., rubber cement diluted 5:1 with toluene) is applied to a carbon or metal SEM sample stub and the solvent allowed to evaporate. The stub is pressed repeatedly with moderate pressure against the area of the skin to be tested. The stub is then mounted in the SEM sample holder and examined.

For chemical testing for Ba, Sb, or Pb residues, the skin is swabbed with cotton swabs [47] or filter paper [48] moistened with dilute nitric acid, sloshing the hands around in a plastic bag containing a few ml of dilute nitric acid [49], using

TABLE 4
Sources of barium, antimony and lead in the general environment

Barium	Antimony	Lead
Paint	Paint	Paint
Grease	Flame retardants	Putty
Rubber	Lacquers	Glass
Radio-opaque x-ray media	Enamels	Gasoline
	Storage batteries	Batteries
Paper	Pewterware	Pewterware
Printing fabric mordants	Tin alloys	Hair dye
	Copper alloys	Candle wicks
Rat poison	Lead alloys	Printer's ink
Photocells	Printer's type	Glazed ceramics
Sunscreen	Matches	Solder
Wood	Bearings	Plumbing
Fireworks	Pyrotechnic mixtures	Auto body filter
Lubricating oil	Eye makeup	Wheel balancing weights
Printer's ink		
Glass		

molten paraffin in much the same fashion as the discredited "dermal nitrate" test, or by pressing transparent tape against the skin and ashing the tape prior to analysis [50].

The subject of GSR sampling has been reviewed by Goleb and Midkiff [51]. No one technique stands out as being applicable to all case situations, and some latitude in the selection of the sampling method would seem to be a desirable professional practice.

Wet chemical tests

A significant advance in the area of gunshot residue testing occurred in 1959 when Harrison and Gilroy [52] described the use of sodium rhodizonate to detect the barium, antimony, and lead of primer residues. The rhodizonate test is a rather good test for these three elements at the levels encountered in downrange residue tests, but has shortcomings as a test for uprange residues. The test has, at best, only marginal sensitivity for the levels of barium, antimony, and lead on uprange targets.

A more serious concern, however, is that the three elements associated with firearm primer residue are occasionally found on the hands of persons who have not discharged a firearm, i.e., the levels of these elements may not be nil. A qualitative test, such as suggested by Harrison and Gilroy, is not what is needed. What is needed is a quantitative test to distinguish normal background levels of these elements from the levels associated with actual discharge of a firearm.

Apart from the value of the rhodizonate test for downrange residue determination, the greatest contribution of the work of Harrison and Gilroy is that the forensic science community was alerted to the possibility of using primer residues, viz., barium, antimony, and lead, as an indicator of whether a person had actually fired or handled a weapon. It was only a matter of time until some sort of a 'messiah' technique emerged with the requisite sensitivity to properly analyze for these elements in a quantitative sense.

Neutron activation analysis

The messiah technique materialized in the early 1960s in the form of neutron activation analysis (NAA). Neutron activation analysis is well suited for the determination of barium and antimony in gunshot residues, but is unsuited for lead determination. This instrumental approach is limited in its practical application by the requirement of a reactor installation; a thermal neutron flux of approximately 10^{12} neutrons/cm/s and the necessity of working with radioisotopes. The radioactivity typically is measured with a high resolution lithium-drifted germanium detector. ^{139}Ba and ^{122}Sb are quantitatively determined by comparison with simultaneously-irradiated standards. Radiochemical separation is generally necessary to eliminate the interference that otherwise would occur as the result of sodium. NAA enjoyed a fairly brief period of popularity in the 1960s for the analysis of gunshot residues, and much of the information necessary for the interpretation of gunshot residues was developed by means of this technique [45,53–57]. An extensive study of more than 1500 test firings over a period of eight years was published by Krishnan [58], and Rudzitis reported results from 1250 tests from a six year period [59]. Although the consensus of opinion within the forensic science community is that NAA represents a legitimate means of

determining gunshot residues, it has been eclipsed by atomic absorption spectrophotometry (AAS) because of the widespread availability of AAS, and by scanning electron microscopy because of the ability to actually visualize the particles with SEM.

Atomic absorption spectrophotometry

The inability of NAA to detect lead at very low levels served as an impetus to the application of atomic absorption spectrophotometry to the study of gunshot residues, but flame atomic absorption methods suffered from a lack of sensitivity as well. With the advent of flameless AAS, and particularly the graphite furnace method, atomic absorption spectrophotometry was able to achieve the sensitivity necessary to determine the very low levels of barium, antimony, and lead encountered in gunshot residues [60–63]. Initial efforts at flameless AAS with the carbon rod atomizer and graphite tube atomizer were successful in the case of antimony, problems were experienced with barium because barium reacts with carbon or graphite to form refractory carbides. The tantalum strip atomizer, on the other hand, was found to efficiently atomize barium as well as lead and antimony [64].

Anodic stripping voltammetry

Lead and antimony in the levels encountered in gunshot residues may be successfully determined by anodic stripping voltammetry [65,66]. In a system where a platinum wire acts as an anode and a hanging mercury drop electrode acts as a cathode, lead and antimony (and copper, should this element be of concern to the forensic chemist as well) will be deposited by electrolysis on the mercury drop. Slowly decreasing the negative potential of the cathode will induce the dissolution of the metal, viz.: Pb, −0.47 V; Sb, −0.16 V; Cu, −0.26 V. The sensitivity of this method can be greatly enhanced by applying the negative potential in the dissolution step in small pulses. This modification is called differential pulse anodic stripping voltammetry.

Barium is unsuited for this instrumental technique. This is a major limitation to this approach, because in the interpretation of gunshot residues, the combination of Ba and Sb is often more reliable as an indicator of gunshot residue than Pb and Sb alone.

Scanning electron microscopy

The intrinsic appeal of the use of scanning electron microscopy for GSR detection is that the gunshot residue particles may be visualized directly, and when a candidate particle is located, the elemental profile of the particle may be analyzed by energy-dispersive x-ray analysis (EDX). This approach provides an opportunity, where the morphology of the particle permits, to exclude other possible sources, (i.e., non-gunshot sources), of barium, antimony, and lead. Fine grained spheroidal particles containing an abundance of barium, antimony and lead are strongly suggestive of a shooting environment.

In 1971, Boehm observed, by means of scanning electron microscopy, micron sized particles of a distinctive nature which appeared to be associated with the discharge of a firearm [67]. His work was expanded upon by a research group at the Aerospace Corporation in El Segundo, California [68–70], and by Andrasko and Maehly in Sweden [71]. In a later work [72], Wolten and Nesbitt of the Aerospace group addressed the issue of the origin of these GSR particles, and concluded that while certain of the particles were due to the primer and the bullet, other particles were due to the bullet alone. The expanded gases cause the bullet to be compressed axially. The bullet then expands radially with very strong frictional heating taking place as the bullet travels down the barrel. Fragments of lead are torn off the bullet and partly vaporized. The lead may then condense on its own, or may condense around other particles.

In 1982, Basu [73] further described the formation of gunshot residue particles as a function of their exposure to heat and pressure. When the primer mixture explodes, the temperature rises to above 1500–2000°C within 0.2 ms. The temperature now exceeds the vaporization temperature of lead (1620°C), barium (1140°C), and antimony (1380°C). Although driven into the vapor state, they condense into the liquid state as well because of supersaturation.

Unless these particles are subsequently modified, they appear as spheroids with diameters in the range of 2 to 10 μm, although some may be as small as 0.1 μm. These spheroids are often fairly homogeneous with respect to lead, barium, and antimony [73].

Heat from the primer explosion, as well as impinging hot spheroidal primer residue particles, causes the smokeless gunpowder to ignite. By 0.5 ms the pressure and temperature has risen to $2–3 \times 10^5$ kPa (ca. 40 000 p.s.i.) and approximately 3600°C. The prototypical spheroidal particles may survive this sharp increase in temperature and pressure without a change in form, or they may be modified by a combination of one or more of the processes of boiling, etching, fragmenting, or coalescence. At this stage, extending approximately from 0.5 to 0.8 ms, hollow spheroids are formed with holes and cavities resulting from gas pockets. These particles range between 15 and 55 μm [73].

The cooling phase now commences, and is virtually complete by 1.0 ms. Barium will freeze at 725°C, antimony at 631°C, and lead at 327°C. The gunshot residue particles will freeze in whatever form they were in, but since barium and antimony are frozen at a temperature more than 300°C over the solidification temperature of lead, spheroids of barium and antimony may acquire an accretion layer of lead to form a particle having the appearance of a partially-peeled orange. These particles range between 10 and 35 μm [73].

This discussion must be extended, however, to include the contribution of the bullet to the total array of elements in the particulate residue. Lead, which may contain on the order of 0.05–2.5% antimony as a hardening agent, will boil off the exposed base of the bullet under the conditions of high temperature. Lead from this source will also assume a spheroidal shape. If the bullet was of the type that is lead with a very thin coating of copper, most of the bullet lead particles will also show copper by EDX. If the bullet was copper jacketed, however, many fewer particles of lead are produced, and only a small fraction of them contain copper.

The manual examination of a SEM sample stub for gunshot residues is both time-consuming and tedious. A thorough examination may require 8 h; it helps to be a Zen master. Consequently, the residue may be subjected to an initial concentration step [74], or the entire scanning may be automated [75–77]. An automated system will scan the sample in a spot mode, stop at a particle with a promising backscatter electron (BSE) image as determined by a preset BSE video threshold, analyze the elemental composition of the particle, and then, having recorded the coordinates of the particle, continue the scan for other particles. The human operator may return later to the coordinates for an inspection of the particle. While well-formed particles with a distinctive morphology are certainly welcome, they are not absolutely necessary in the interpretation of gunshot residues.

But in the forensic sciences, where every fact seems to have three sides and every thought seems to have six, nothing is simple or easy. Although the SEM and EDX together represent a rigorous means of identification, it is not absolute. It is reported that cigarette lighter flint particles mimic GSR residues, at least with respect to morphology, and it is noted that the morphology of the gunshot residue particles as seen under the SEM is influenced to some extent by the surface of the target. In this area of the chemistry of death, there are still questions to be answered.

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Firearm cartridge case comparison by graphite furnace atomic absorption spectrochemical determination of nickel, iron and lead

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Abstract

Graphite furnace atomic absorption spectrometry was used to determine the concentrations of nickel, iron, and lead in 26 different production lots of Winchester-Western cartridge case brass. In most cases, the measured elemental concentrations were found to be normally distributed within a lot, and the variables lacked any strong association. It was found that the concentrations of these three elements could be used as a basis for the comparison of individual cartridge cases to determine their provenance. Using the *T*-test with a significance level of 0.01, the frequency of obtaining a 'true match' and a 'false match' were found to be 98.1% and 0.43%, respectively. Additionally, a likelihood ratio was used to evaluate the extent to which provenance, or "common origin" among brass cartridge cases can be established.

Keywords: Atomic absorption spectrometry; Iron; Lead; Nickel; Firearms; Forensic analysis

In recent years, the use of firearms during the commission of crimes has increased enormously. In 1989, firearms are estimated to have been used in 11832 murders, 204617 aggravated assaults, and 192004 robberies in the United States [1]. This results in an annual health care cost of approximately 1×10^9 US dollars for acute hospitalization [2], and a measureless cost in human anguish in cases where death ensues.

In light of these statistics, numerous attempts to regulate the sale and ownership of firearms have been promoted, with some success. However, given that there are approximately 130 million firearms in America, with another 5.5 million being produced and 750000 being imported each year [3], it is unlikely that the number of firearms in existence will decrease dramatically in the near future. Likewise, it is also unlikely that there will be a significant decrease in the availability of firearms to individuals intent on their felonious use.

Because of the prevalence of firearms, numerous techniques have been developed to analyze the evidence associated with them, e.g., bullets and cartridge cases comparisons, gunshot residues, fingerprints or blood on the firearm, and trajectory reconstruction. Typically, the most definitive evidence obtained from material associated with firearms is not intrinsic to the firearm per se. It arises rather from the interaction of the firearm with components of the cartridge; a comparison under the microscope of evidence bullets or cartridge cases with test firings from a suspect's firearm may often enable a forensic scientist to

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conclude that the evidence bullet or cartridge was in fact fired through the suspect's weapon, to the total exclusion of all other firearms in the world.

With regard to a cartridge case retrieved from a crime scene and a firearm retrieved from a suspect, one may ask whether the cartridge was discharged in this particular weapon. Initially the forensic scientist will determine whether the cartridge case is of the appropriate size and form to be fired in the weapon, and then will determine whether the class characteristics, i.e., the position of extractor and ejector markings, the form of the firing pin impression, etc., imparted to the case by the weapon correspond. If the class characteristics are consistent, then it is possible that the cartridge was discharged in that firearm, but it does not establish that it was in fact discharged in that firearm. In order to draw such a conclusion, the number of firearms which could have fired the cartridge must be reduced to one. This reduction is usually accomplished by comparing striations left on the evidence cartridge case with the striations on a test cartridge case fired in the suspect weapon. The pattern of these unique striations is not shared by other firearms, not even successively manufactured specimens from the same manufacturer. By utilizing these individual characteristics, "causal identity" is established, that is, the markings on both evidence and test case were caused by the same event, namely, the interaction of that firearm with the cartridge case [4].

But to do all of this, however, requires that the suspect firearm must be available for test firing. This is, unfortunately, not always the case; subsequent to the shooting event, the firearm may be destroyed, hidden, lost or modified. When the firearm is not available for purposes of test firing, the forensic scientist is often limited to the information revealed by the gross morphology of the cartridge case and the class characteristics imparted to the cartridge case by the firearm.

Alternatively, those properties intrinsic to the cartridge case could be investigated. For example, the trace elemental composition of the brass (the material out of which most cartridge cases are made), could be determined to ascertain whether a compositional 'match' between evidence samples exist. If a 'match' is established, the next issue to be confronted is whether the samples have a "common origin", that is, whether they were produced from the same melt of brass. Given sufficient agreement, a second form of identity may be tentatively assumed, namely, "identity of source" [5]. However, a 'match' between the measured trace elemental concentrations of two samples does not necessarily imply that the samples have a common origin, because it is possible that the two samples may coincidentally have similar elemental compositions. Therefore, it becomes necessary to evaluate the extent of this coincidental occurrence in order to evaluate the notion of "common origin." The present work is directed toward this latter issue.

The present work addresses two questions: First, can trace elemental composition of cartridge brass be used as a basis for comparing one melt of brass with another melt of brass, and more specifically, can trace elemental composition be used as a basis for comparing one cartridge case from a production lot with another cartridge case from another production lot? Second, if a compositional 'match' is established, what is the extent of a coincidental 'match', given that the two samples come from different production lots of cartridge cases? To answer these questions, the T-test and likelihood ratios were used to determine if a particular cartridge case originated from the same production lot as a group of cartridge cases.

Cartridge case brass

Originally, the function of the firearm cartridge case was to facilitate loading of the projectile (bullet or shot) and the propellant powder charge as a single unit into the firearm. Initially, these components were contained within a paper housing [6]. However, as firearm and ammunition development progressed, more durable materials were used, such as linen, steel, and copper [7,8].

In the mid-1800s, with the development of a functional breech loading firearm, the cartridge acquired an additional function, that of sealing the breech against the rearward leakage of combustion gases. In order to accomplish this and still allow the weapon to operate properly, the

case material had to possess special properties. For example, if the material was too hard, it would be brittle and vulnerable to cracking during discharge of the cartridge. If, however, the material was too soft, the case would not return to its original dimensions after the pressure produced by the combustion gases decreased. This would not only make it difficult to extract the case from the firing chamber but would lengthen the time required for reloading, cause excessive wear on the inside of the chamber walls, shorten the life of the firearm, and make the cartridge case unsuitable for any subsequent reloading. After considerable "trial-and-error" experimentation, an alloy consisting of approximately 30% zinc and 70% copper was found to be best suited for the fabrication of cartridge cases. Not only did this alloy have the ductility needed, but it was also resistant to corrosion. Consequently, 30:70 brass became known as cartridge brass.

To ensure that the cartridge case fulfills its function, it was necessary to stringently control and monitor the production of cartridge cases, both in terms of their case dimensions and the quality of the material from which the cases are produced. Certain standards have been established by the U.S. Department of Defense and agreed upon by cartridge manufacturers as set forth by the American Society for Testing and Materials (ASTM). For example, with regard to the chemical composition of the cartridge brass, the material shall conform to the following chemical requirements: copper, 68.5% to 72.5%; lead, 0.07% maximum; iron, 0.05% maximum; zinc, the remainder. If bismuth is found in excess of 0.006%, the lot shall be rejected. Furthermore, when all of the major elements are analyzed, their sum shall be 99.7% minimum [9].

In addition to the concentration limits regarding *specifically named* minor (0.01% to 1%) and trace (1 ppm to 100 ppm) elements, other elements may be present. All other minor and trace elements may not exceed 0.3%. These may include tin, silicon, aluminum, nickel, phosphorus, manganese, cobalt, cadmium and silver [10,11]. Other elements may be present at levels below 1 ppm [12]. The presence and concentration of elements other than the major constituents depend on a number of factors, such as the mineral source from which the copper and zinc are extracted, the mineral processing procedure employed during the extraction of virgin copper and zinc metal, and the use of recycled or scrap brass in the production of cartridge brass. The manufacturer of cartridge cases is likely to be oblivious to the presence or concentration of trace elements, and ordinarily would be in no position to do anything about it in any event.

In addition to the specified maximum concentration limits on specifically named elements, the limits for any ASTM specified or unspecified elements may be established by agreement between the supplier and the purchaser as long as the specifications comply with industry standards. Consequently, it is not uncommon to find specific composition requirements for cartridge brass to vary from purchaser to purchaser while still remaining within industry standards. The prevailing attitude within the ammunition industry is that if the brass meets ASTM standards and functions properly, the trace element composition is of no particular concern. This situation provides a provident opportunity, for forensic purposes, to characterize cartridge brass as having originated from a particular production run.

EXPERIMENTAL

Three criteria were used in selecting the elements Ni, Fe, and Pb for determination. First, the elements must be present at relatively high concentrations, so that a minimal amount of sample would be consumed during the analysis. (This consideration, although not unique to forensic work, is a salient aspect of any forensic analysis; courts of law view with askance on any analytical technique that consumes the totality of the evidence with no possibility of an independent re-examination.) Second, the concentration of each must vary significantly from melt to melt, so that it will be possible to distinguish various melts of brass. Third, the elements should have different electron configuration in their outermost principal energy level in order to minimize any similarity in chemical properties and to avoid any poten-

tially strong statistical association between the variables.

Graphite furnace atomic absorption spectrometry and accessory equipment

The sample analysis was conducted on a Perkin-Elmer (P-E) Model 380 atomic absorption spectrometer controlled by a HGA-400 programmer. The characteristic radiation was produced with the following P-E hollow cathode lamps: Ni (P-E No. 0303-6047), Fe (P-E No. 0303-6037), and Pb (P-E No. 0303-6039). All of the lamps had less than 42 h of use at the beginning of this research. The graphite tubes (P-E No. 290-1766) were pyrolytically coated, and the purge gas was argon of greater than or equal to 99.996% purity. A 1-mV OmniScribe strip chart recorder, Model 35247R-5, was used. The potentiometer in the temperature control assembly of the furnace was calibrated with a Model 22-211 Migronta multimeter so as to fall within the designated range specified by the manufacturer.

Sample selection and collection

Winchester-Western (W-W) cartridge cases of 0.308 NATO caliber were selected for three reasons: (1) sufficient brass would be available with each case to allow for multiple determinations and confirmatory testing by another analytical method if necessary; (2) Winchester-Western is one of the largest manufacturer of cartridge cases in the world, and is a subsidiary of the Olin Corporation, one of the largest produces of cartridge brass, and (3) the methods of brass production and cartridge case manufacturing utilized by Olin typifies industry methods.

The number of cartridge cases to be analyzed from each lot was based on the assumption that the concentrations of Fe, Pb, and Ni in brass lack any strong association and that, as a rule of thumb, a minimum of approximately 15 samples should be analyzed for each variable studied. Ideally, approximately 45 cases from each lot number should therefore have been analyzed, but because the cases are typically sold as a unit of twenty per box, only two boxes or 40 cases were actually analyzed from each lot number. This number of cases not only fulfills the predicted sample requirements cited above, but also enabled the determination of the intra-lot number variability of the analyte concentrations as well as the degree of inter-box variability within a specific lot number.

Using the same line of reasoning, it was decided that cartridge cases from a minimum of 15 different lot numbers should be analyzed in order to determine the inter-lot number variability of the analyte concentrations.

Table 1 lists the lot numbers of the W-W cartridge cases analyzed, the location where the cases were acquired, and the dates of purchase. A total of 26 lot numbers are represented in the table, all of which were collected over a two year period from the western United States. Furthermore, it is worth noting that samples from every lot number encountered during phone inquiries

TABLE 1

Cartridge case samples

Lot number	Quantity	Purchase location	Purchase date
030YD1	40	Oakland, CA	06/07/88
030YD41	40	Lacey, WA	06/17/88
03VA3	40	Lewiston, ID	06/20/89
009RG32	40	Santa Ana, CA	07/07/89
022RG32	40	Santa Ana, CA	07/07/89
030YD51	40	Oakland, CA	01/13/90
007YD41	40	Modesto, CA	03/24/90
030CC12	40	Sacramento, CA	03/29/90
007YD9	40	Oakland, CA	04/13/90
022RE72	40	Oakland, CA	04/13/90
007YD51	40	San Leandro, CA	04/13/90
006XM42	40	El Cerrito, CA	05/24/90
007YC13	20	El Cerrito, CA	05/24/90
030YC13	20	Sacramento, CA	05/30/90
002XL01	20	Sacramento, CA	05/30/90
030CD11	20	Oakland, CA	06/02/90
007YD9	40	Oakland, CA	06/02/90
030CD11	20	San Francisco, CA	06/02/90
006XL7	20	Castro Valley, CA	06/02/90
006XL7	20	San Jose, CA	06/02/90
030CH13	40	San Carlos, CA	06/02/90
002XM62	20	Pleasant Hill, CA	06/15/90
006XM62	20	Pleasant Hill, CA	06/15/90
030CD12	40	Edmonds, WA	07/18/90
030CD52	40	Lynnwood, WA	07/18/90
030CD42	40	Lynnwood, WA	07/18/90
024NH03	40	Portland, OR	09/14/90
030CH9	20	Portland, OR	09/14/90
005WG52	40	Redding, OR	09/20/90

or visits to retailers were purchased, and that there are six lot numbers for which only 20 cases could be located. In short, the sampling was fairly exhaustive as to what was available to the public for this two year period.

For purposes of brevity in the discussion following, the various lots of cartridge cases were assigned a letter prefix as follows: A002XL01, B002XM62, C003VA3, D005WG52, E006XL7, F006XM42, G006XM62, H007YC13, I007YD41, J007YD51, K007YD9, L009RG32, M022RE72, N022RG32, O024NH03, P030CC12, Q030CD11, R030CD12, S030CD42, T030CD52, U030CH13, V030CH9, W030YD1, X030YD13, Y030VD41, Z030YD51.

Sample preparation

Each lot of cases was soaked in acetone for one hour to remove any organic material, e.g., oil, remaining on the cases from production processes. After air drying, the cases were transversely cut with a circular saw and an aluminum oxide cut-off wheel at the junction of the case body and the initial tapering point leading to the case neck. By selecting this point for cutting, a piece weighing slightly more than 0.5 g was obtained. The cut end of the tapering piece was trimmed to remove any contaminated portions due to cutting, and weighed on a Type 21 Magni-Grad analytical balance with a precision of 0.1 mg. The samples were then dissolved in 5 ml of 1:1 'Baker Instra-Analyzed' (BIA) analytical reagent grade nitric acid in 100 ml Nalgene high density polyethylene (HDPE) beakers and diluted to 50 ml in volumetric flasks with water having a resistivity of 12 megohms produced from a Milli-Q reagent grade water system. The dissolved sample remained in the volumetric flasks no longer than 20 min before being transferred to 125-ml Nalgene HDPE bottles, and all of the samples were analyzed within 13 h of preparation.

TABLE 2

Drying time parameters

Volume of sample (μl)	Ramp time (s)	Hold time (s)
10	10	15
15	15	15
20	15	15
30	20	20
40	25	20

TABLE 3

Ashing and atomization temperature and time parameters

Element	Cycle	Temp. (°C)	Ramp (s)	Hold (s)	Integration (s)
Fe	Ash	900	15	10	–
	Atomize	2400	–	10	10
Pb	Ash	–	–	–	–
	Atomize	2050	–	10	10
Ni	Ash	1000	15	10	–
	Atomize	2500	–	10	10

Standard preparation

Standards containing 10.00, 5.00, 2.50, 1.00, 0.50, and 0.25 μg/ml of Fe, Pb, and Ni were prepared daily by serial dilutions of stock standard solutions containing 1003 μg/ml Fe, 1000 μg/ml Pb, and 1006 μg/ml Ni. Sufficient volumes of stock standard solutions containing 0.1003 g/ml Zn and 0.1003 g/ml Cu were added to each of the described standards to match the 30:70 Zn–Cu matrix typically found in cartridge brass. All of the stock standard solutions, except that of Pb, were prepared by dissolving the respective metal ($\geq$ 99.9996% purity) in a minimum volume of 1:1 BIA nitric acid and diluting to volume. These standards were stored in Nalgene HDPE bottles and were discarded after six months.

Graphite furnace atomic absorption spectrometry

The experimental parameters used in this study are provided in Tables 2–4.

TABLE 4

Spectral parameters for cartridge brass analysis

Element	Wavelength (nm)	Slit width (nm)	Lamp current (mA)
Fe	272.0	0.2	25
Pb	283.3	0.2	8
Ni	232.0	0.2	25

RESULTS AND DISCUSSION

Data and summary statistics

Provided in Tables 5–7 are the summary statistics for the analyte concentration in each lot of brass tested, and Figs. 1–3 are scatterplots of the measured elemental concentrations.

Qualitatively the plots show a close grouping of points within lots in comparison to the overall variation. The data do not distinguish between variation due to measurement error and variation of true concentration across cartridge cases within lots, as there is only one measurement for each element on each cartridge case. To gauge the order of the magnitude of the measurement error, additional data were collected as follows: Two cartridge cases from each of two boxes with lot number 030YD1 were arbitrarily selected. The [Ni], [Fe], and [Pb] of each case were repetitively determined five times using the analytical procedure previously described. Table 8 lists the measured concentrations determined in the four cartridge cases. In addition, the correlation coefficients for the variables within each cartridge case are provided in Table 9.

As can be seen from Table 8, the relative standard deviation in measurement precision is approximately 4.64%, 4.44%, and 4.83%, respectively, for the variables [Ni], [Fe], and [Pb]. Upon comparison of these replicate measurement errors with the observed within-lot variations of the variables provided in Tables 5–7, it appears that most of the within-lot variation is attributable to imprecision of the measuring technique rather

TABLE 5

Summary statistics by lot for nickel

Lot number	Average ($\%\times10^{-4}$)	Std. Dev.$_{(n-1)}$ ($\%\times10^{-4}$)	Low ($\%\times10^{-4}$)	High ($\%\times10^{-4}$)
002XL01 [a]	285	3.85	279	292
002XM62 [a]	261	5.62	250	270
003VA3	175	5.40	163	184
005WG52	224	2.56	220	229
006XL7	272	3.47	261	277
006XM42	384	8.92	363	397
006XM62 [a]	264	3.00	258	269
007YC13 [a]	238	4.99	228	248
007YD41	294	7.67	271	302
007YD51	278	4.95	269	289
007YD9	247	3.45	241	253
009RG32	218	2.99	212	224
022RE72	312	12.5	289	341
022RG32	226	2.88	221	232
024NH03	411	6.21	398	422
030CC12	266	3.78	260	278
030CD11	175	7.39	157	201
030CD12	392	5.74	379	407
030CD42	199	4.41	191	209
030CD52	179	4.18	171	187
030CH13	233	3.27	225	240
030CH9 [a]	238	3.58	232	247
030YD1	196	7.83	173	215
030YD13 [a]	165	2.86	160	170
030YD41	195	2.77	190	200
030YD51	284	3.99	277	296

[a] Only 20 cartridge cases from these lot numbers were analyzed.

TABLE 6

Summary statistics by lot for iron

Lot number	Average ($\%\times10^{-4}$)	Std. Dev.$_{(n-1)}$ ($\%\times10^{-4}$)	Low ($\%\times10^{-4}$)	High ($\%\times10^{-4}$)
002XL01 [a]	274	4.27	263	281
002XM62 [a]	276	9.43	253	288
003VA3	247	3.22	241	253
005WG52	239	2.62	235	244
006XL7	371	4.46	361	381
006XM42	268	6.51	254	280
006XM62 [a]	359	3.96	351	365
007YC13 [a]	262	6.15	253	273
007YD41	276	3.60	266	284
007YD51	224	4.43	216	236
007YD9	403	4.18	393	417
009RG32	224	3.27	214	229
022RE72	244	6.01	229	261
022RG32	220	3.93	214	233
024NH03	249	5.20	240	259
030CC12	270	6.10	258	289
030CD11	322	8.98	306	343
030CD12	334	7.43	319	347
030CD42	250	4.89	239	262
030CD52	210	3.52	204	217
030CH13	213	5.67	197	226
030CH9 [a]	298	5.95	288	310
030YD1	391	9.98	372	409
030YD13 [a]	240	3.30	233	244
030YD41	225	2.86	221	232
030YD51	512	10.4	489	530

[a] Only 20 cartridge cases from these lot numbers were analyzed.

TABLE 7

Summary statistics by lot for lead

Lot number	Average ($\%\times10^{-4}$)	Std. $Dev._{(n-1)}$ ($\%\times10^{-4}$)	Low ($\%\times10^{-4}$)	High ($\%\times10^{-4}$)
002XL01 [a]	128	2.40	123	132
002XM62 [a]	143	2.87	138	149
003VA3	52.7	0.834	49.4	53.7
005WG52	163	3.29	156	169
006XL7	161	2.82	157	166
006XM42	159	5.10	144	169
006XM62 [a]	159	2.56	154	164
007YC13 [a]	132	5.33	116	139
007YD41	123	2.82	119	128
007YD51	125	3.20	120	133
007YD9	250	4.42	238	263
009RG32	150	3.00	144	155
022RE72	129	4.13	121	138
022RG32	110	3.25	101	117
024NH03	197	6.00	185	209
030CC12	186	7.44	167	208
030CD11	193	4.74	180	203
030CD12	206	5.64	190	214
030CD42	155	5.00	142	164
030CD52	173	5.75	156	188
030CH13	120	3.37	112	127
030CH9 [a]	190	2.56	185	194
030YD1	163	5.11	151	180
030YD13 [a]	132	3.28	125	137
030YD41	145	3.05	140	151
030YD51	215	2.71	210	220

[a] Only 20 cartridge cases from these lot numbers were analyzed.

than a result of actual differences in elemental concentrations.

However, the heterogeneity of the within-lot variances is statistically significant with some within-lot variances being ten times greater than other within-lot variances. This heterogeneity may be due to the manner in which cartridge cases are packaged into boxes. In particular, it is possible for one box to contain cartridge cases produced from two different melts of brass. Generally this occurs when the end of one cartridge case production run is mixed with a subsequent production run of other cartridge cases.

The overall correlation coefficients between variable combinations Ni–Fe, Ni–Pb, and Fe–Pb were found to be 0.1436, 0.2768, and 0.5916, respectively. These mainly reflect correlation at

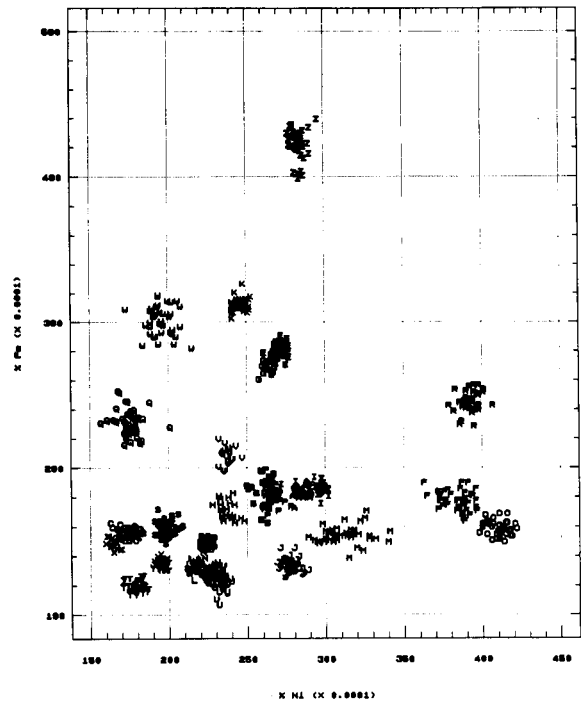

Fig. 1. Fe concentration vs. Ni concentration.

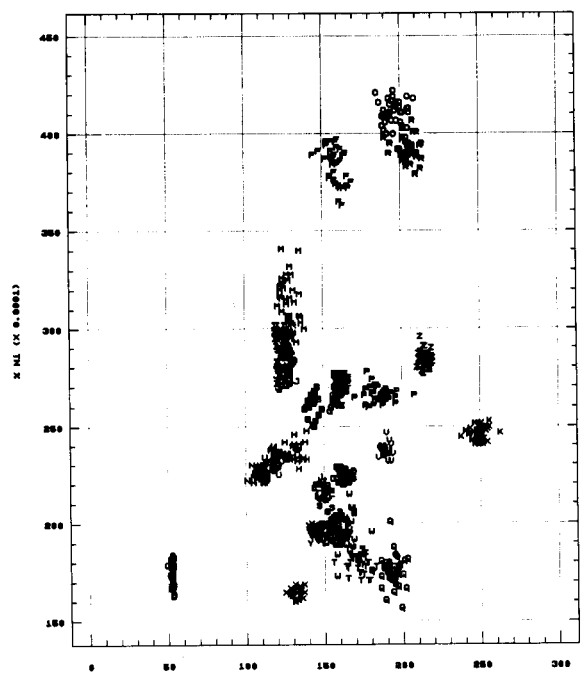

Fig. 2. Ni concentration vs. Pb concentration.

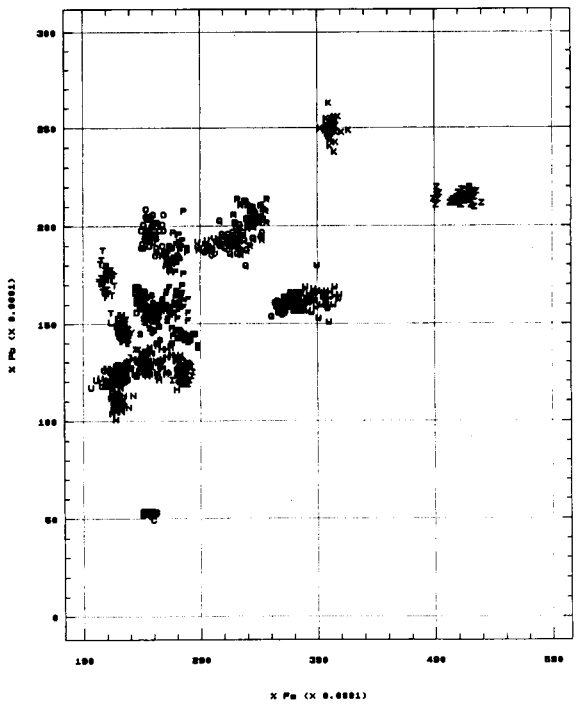

Fig. 3. Pb concentration vs. Fe concentration.

TABLE 8

Replicate determinations of [Ni], [Fe], and [Pb] on selected cartridge cases

Element	Sample No.	% Concentration ($\times 10^{-4}$)					
		Replicate No.					Mean, S.D.
		1	2	3	4	5	
Ni	3A	209	201	184	196	190	196, 9.66
	9A	207	204	192	188	187	196, 9.29
	22B	194	199	211	189	186	196, 9.83
	36B	210	193	202	185	186	195, 10.7
	Group						196, 9.08
Fe	3A	413	391	377	366	404	390, 19.2
	9A	363	409	394	410	383	392, 19.6
	22B	372	399	409	412	374	393, 19.1
	36B	392	371	379	416	399	391, 17.6
	Group						392, 17.4
Pb	3A	175	157	163	166	157	163, 8.21
	9A	155	169	151	162	177	163, 10.5
	22B	149	170	160	158	166	162, 10.7
	36B	167	158	171	169	154	164, 7.40
	Group						163, 7.86

TABLE 9

Correlation coefficients of replicate measurements on selected cartridge cases

Variable combination	Cartridge case number			
	3A	9A	22B	36B
[Ni] and [Fe]	+0.5070	−0.3490	−0.0971	−0.4672
[Ni] and [Pb]	−0.0570	−0.2830	+0.0208	−0.0659
[Fe] and [Pb]	+0.1979	−0.2013	+0.2254	+0.1625

the between-lot level and suggest that weak associations exist between the variables, with the exception of a stronger association between Fe and Pb.

In addition, the within-lot correlation coefficients are listed in Table 10. With few exceptions, these associations are weak. This is consistent with the notion that the measurement errors for the three analytes are independent (as the above correlation of replicate measurements suggest), and the variation within lots is mainly attributable to measurement imprecision.

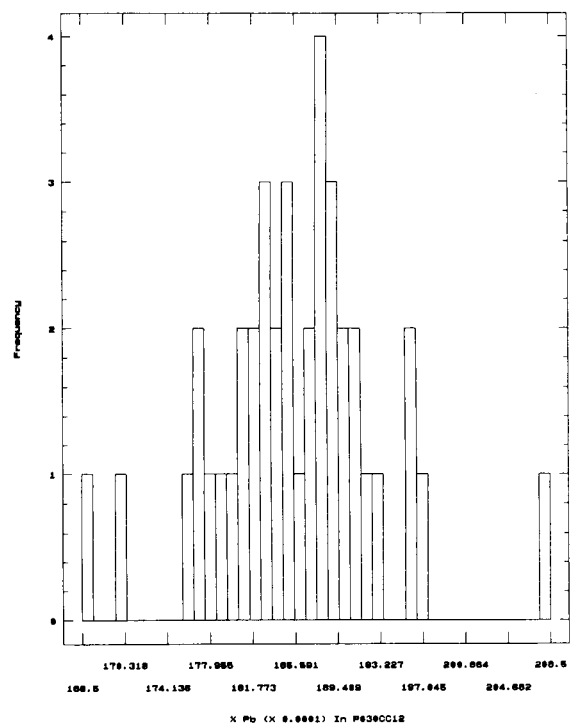

Fig. 4. Frequency histogram for Pb in P030CC12.

Normality of the data at the intra-lot level

Each variable within each of the 26 lots was investigated by visual inspection of its frequency histogram and normal probability plot to determine if its distribution was reasonably normal. Rather than to provide 156 frequency histograms and normal probability plots, the distribution of Pb in lot P030CC12 has been selected as being representative for illustration; its frequency histogram is shown in Fig. 4. Qualitatively, only five of the 78 empirical distributions are less suggestive of normality. It would seem reasonable to conclude that each variable is approximately normally distributed within lots.

Determination of a 'match'

The goal of trace elemental analysis of cartridge case brass would be to establish whether two groups of one or more cartridge cases came from the same source. Source, in the sense of having been all together at some stage in their history, perhaps in some larger collection, and separated more or less randomly into the two groups as well as from any additional cartridge cases in the collection. The question would be whether the two groups 'match' with respect to [Ni], [Fe], and [Pb], or whatever other variables are used. If so, the groups of cases under investigation could have come from the same source.

The "one dissimilarity principle", commonly invoked in forensic science, demands that all of the variables 'match' individually. This doctrine asserts that a single *unexplained* difference between two samples is sufficient justification to conclude that the samples have different origins. It is generally believed that in a situation of conflicting or uncertain data, this principle defaults in favor of the accused defendant. Hypothesis tests, such as the *T*-test, are commonly used in forensic science, at least to the extent that statistics are used at all. Such a test can be used to determine whether or not two groups of car-

TABLE 10

Within-lot correlation coefficients by lot number

Lot number	Elements		
	[Ni] and [Fe]	[Ni] and [Pb]	[Fe] and [Pb]
A002XL01	0.2948	−0.1257	0.0444
B002XM62	0.0586	0.1156	−0.2920
C003VA3	−0.2867	−0.2409	−0.2101
D005WG52	0.1210	0.0175	−0.3033
E006XL7	0.1405	−0.1137	−0.0585
F006XM42	−0.7754	0.4821	−0.4617
G006XM62	0.2525	0.7146	0.2730
H007YC13	−0.3904	0.0578	−0.0151
I007YD41	−0.0515	−0.0911	0.2271
J007YD51	−0.2789	−0.0457	0.0483
K007YD9	0.1832	0.1590	−0.0030
L009RG32	0.3301	−0.0942	−0.0873
M022RE72	0.0976	−0.2999	−0.0958
N022RG32	0.0045	0.2555	0.0830
O024NH03	−0.1485	−0.0196	−0.0294
P030CC12	0.0264	−0.1582	0.2632
Q030CD11	−0.1926	−0.0445	0.0763
R030CD12	0.2278	−0.1124	0.0307
S030CD42	0.0039	−0.0160	0.1003
T030CD52	0.0063	−0.1721	−0.1600
U030CH13	0.0309	0.0663	−0.0996
V030CH9	−0.0012	−0.0387	0.1261
W030YD1	−0.1133	0.3152	0.0141
X030YD13	−0.3255	0.0904	−0.0228
Y030YD41	0.0127	−0.0502	−0.2644
Z030YD51	−0.0507	−0.0992	0.0529

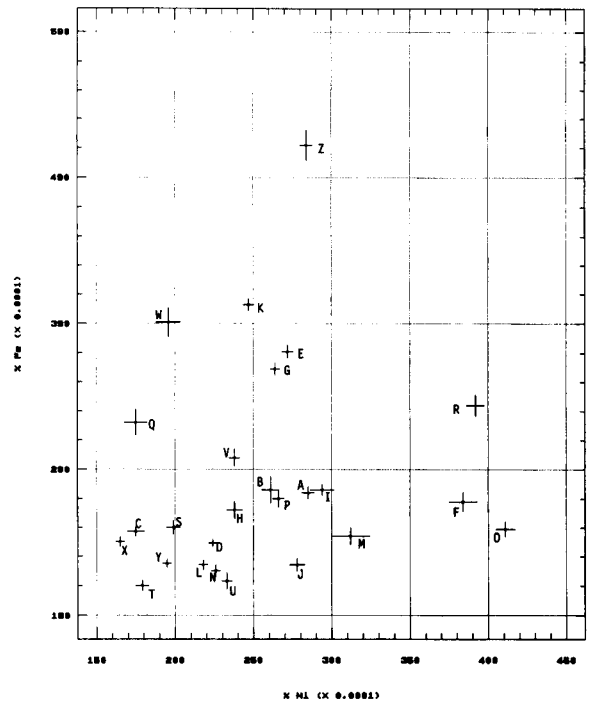

Fig. 5. Lot mean Fe concentration vs. lot mean Ni concentration.

tridge cases 'match' with respect to a given variable.

Presupposing each group comes from a single source, the null and alternative hypotheses are:

H_0: from the same source (1)

H_1: from different sources (2)

Of the four possible inferential outcomes from such a test, three would be of interest to the judicial system in the United States, where courts have a history of being skeptical concerning the introduction of statistical methods. The first outcome is to obtain a 'match' when in fact H_0 is true. This will be referred to here as a 'true match.' The second outcome is to obtain a 'non-match' when H_0 is true. This outcome may be considered beneficial to a guilty party because the association between the guilty party and the crime is not made, i.e., the error will favor the defendant. Such an occurrence corresponds to a Type I error and will be called a 'false non-match.' An error of this type will not deliver justice, but would not be viewed by the courts as egregious since it would favor the interests of the accused; this error will falsely exonerate a person who is truly guilty. The third outcome is to obtain a 'match' when in fact H_0 is false, i.e., when H_1 is true. This type of mis-identification is of greatest concern because of its potential of adverse consequences for an innocent individual, that is, the establishment of a false association between the defendant and the crime. This occurrence corresponds to a Type II error and will be referred to here as a 'false match.'

Unfortunately, yet unavoidably, most tabulated critical values for hypothesis testing concern the level of significance (α), the probability of a Type I error, whereas the judicial system is more interested in the probability of a Type II error. This latter probability is designated (β), and is influenced by a change in α. For example, when α is increased from 0.01 to 0.05, the criterion for a 'match' is restricted. The probability of a false non-match increases, but the probability of a false match decreases. Conversely, if α is reduced, the probability of a false non-match decreases, but the probability of a false match increases.

Consider single measurements of a single variable [Ni], [Fe], or [Pb] on two groups of two or more cartridge cases. A working definition of a *match* will now be constructed. Define a statistic T by

$$T = |x_1 - x_2| / \sqrt{[(\text{within SS})/(n_1 + n_2 - 2)] \times [(1/n_1) + (1/n_2)]} \quad (3)$$

where n_1 and n_2 are the number of cartridge cases in groups 1 and 2, respectively, x_1 and x_2 are the means within the groups, and the within SS is the pooled within group sum of squares. If the absolute value of T is found to be less than a critical value, it may be said that there is a *match*. The critical value will be the $100(1 - \alpha/2)$ percentile of the standard normal distribution with a specific significance level α and $n_1 + n_2 - 2$ degrees of freedom.

Inter-lot number comparison

The indistinguishability of lots based on the determination of one element is apparent in Table 11, which lists those lots whose measured elemental concentrations match (in the sense discussed above) at the 0.01, 0.05, and 0.10 significance levels. Although there is a seemingly high rate of indistinguishability, it is not unexpected given the narrow range over which the [Ni], [Fe], and [Pb] are distributed. This narrow range is the result of the artificial maximum impurity concentration limits established by industrial specifications and the practical lower impurity concentration limits resulting from geological, extractive metallurgical, and economic factors.

However, as becomes evident upon further review of Table 11, none of the lot number combinations whose mean concentrations match for any one element also match when a second element is considered, regardless of which α level is chosen. Even with the lowest α level at 0.01, and whichever pair of elements is used, each lot may be distinguished from every other lot. This is illustrated in Figs. 5–7, which are plots of the mean elemental concentrations for two elements with their respective one standard deviation bars.

Although the determination of two elemental concentrations is sufficient to differentiate two of

the lots at the 0.01 significance level, there is some inter-lot overlap for individual cartridge cases. For example, in Fig. 5, lots A and I, B and P, and N and U overlap. However, when all three variables are used as seen in Fig. 8, the degree of overlap substantially decreases.

True match and false match frequencies between one cartridge case and a group of cases

In a typical investigation, the forensic scientist may be presented with a single cartridge case and asked to compare it with a group of cartridge cases. As before, the frequencies of false matches is of enormous importance. To investigate this, every cartridge case was compared with the lot to which it belonged, and with the other 25 lots. The results of these comparisons, based on considera-

TABLE 11

Lot number combinations which can be falsely matched

Lot number combinations	$\alpha = 0.10$	$\alpha = 0.05$	$\alpha = 0.01$
Nickel			
A002XL01 and Z030YD51	Yes	Yes	Yes
A002XL01 and G006XM62	No	No	Yes
C003VA3 and Q030CD11	Yes	Yes	Yes
G006XM62 and P030CC12	No	No	Yes
H007YC13 and V030CH9	Yes	Yes	Yes
S030CD42 and W030YD13	No	No	Yes
W030YD13 and Y030YD41	Yes	Yes	Yes
Iron			
A002XL01 and B002XM62	Yes	Yes	Yes
A002XL01 and I007YD41	No	Yes	Yes
B002XM62 and I007YD41	Yes	Yes	Yes
C003VA3 and O024NH03	No	No	Yes
D005WG52 and X030YD13	Yes	Yes	Yes
F006XM42 and P030CC12	Yes	Yes	Yes
J007YD51 and L009RG32	Yes	Yes	Yes
J007YD51 and Y030YD41	Yes	Yes	Yes
L009RG32 and Y030YD41	No	Yes	Yes
O024NH03 and S030CD42	Yes	Yes	Yes
Lead			
A002XL01 and M022RE72	Yes	Yes	Yes
B002XM62 and Y030YD41	No	No	Yes
D005WG52 and W030YD13	Yes	Yes	Yes
E006XL7 and F006XM42	No	No	Yes
E006XL7 and W030YD13	No	No	Yes
F006XM42 and G006XM62	Yes	Yes	Yes
H007YC13 and M022RE72	No	No	Yes
H007YC13 and X030YD13	Yes	Yes	Yes

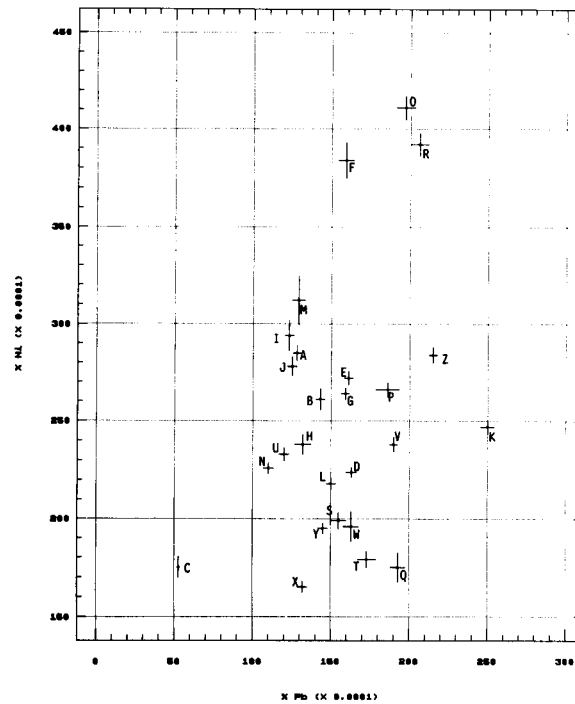

Fig. 6. Lot mean Ni concentration vs. lot mean Pb concentration.

tion of one, two and three variables and an α of 0.01, are provided in Table 11.

Table 12 illustrates the particular true match and false match frequencies. The frequency of true matches are ~ 99.3% to 99.6%, ~ 98.8% to 99.6%, and ~ 98.1%, respectively when one, two, and three elements are considered. Similarly, the frequency with which false matches can be expected are ~ 11.0% to 16.8%, and ~ 1.22% to 2.89%, respectively, when one and two variables are considered. When three variables are considered, the observed frequency of a false match is ~ 0.43%. Of the 902 cartridges analyzed in this study, only 0.43% of the cartridge cases could be false matched with non-progenitorial lots when three variables were considered and α was set at 0.01.

The likelihood ratio

As discussed previously, the purpose of comparative trace element analysis would be the determination of common origin, that is, whether two groups of cartridge cases originated from the same lot. Thus far, this work has focused on the

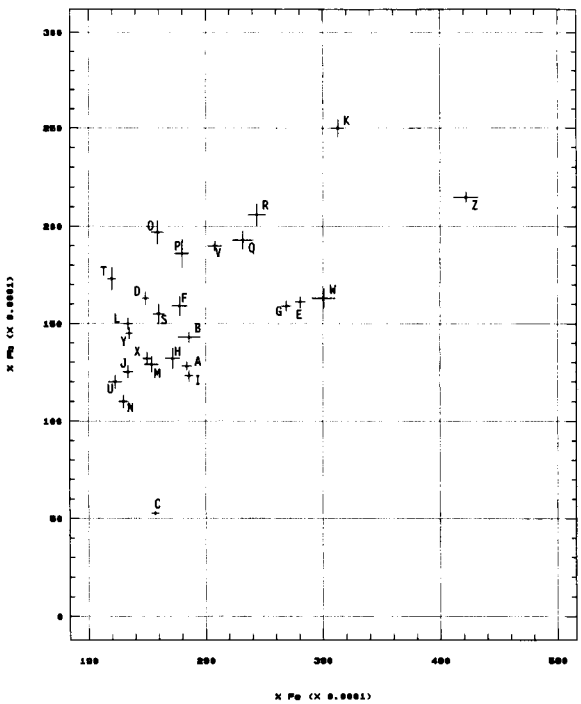

Fig. 7. Lot mean Pb concentration vs. lot mean Fe concentration.

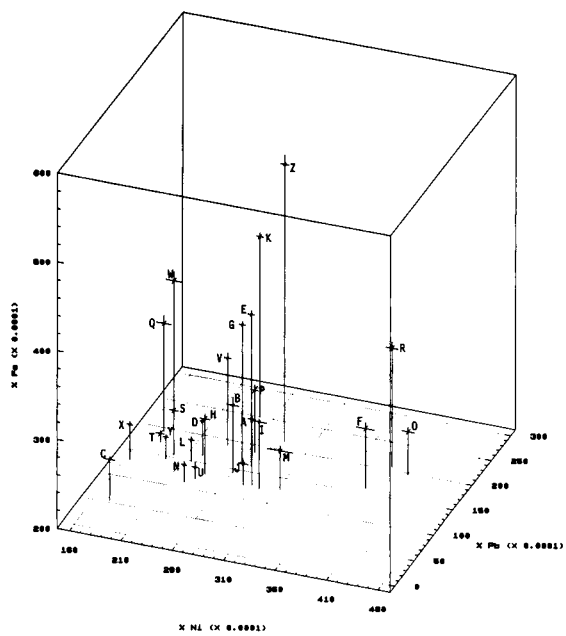

Fig. 8. Mean Fe concentration vs. mean Ni concentration and mean Pb concentration for each lot analyzed.

TABLE 12

True match and false match frequencies when a single cartridge case is compared with a group of cartridge cases and one, two or three variables are considered

Variable(s) considered	True match Freq. (%)	False match Freq. (%)	
Ni [a]	907/912 (99.4)	2498/22775	(11.0)
Fe	910/914 (99.6)	3133/22825	(13.7)
Pb	909/915 (99.3)	3837/22859	(16.8)
Ni–Fe	896/900 (99.6)	276/22625	(1.22)
Ni–Pb	895/906 (98.8)	654/22650	(2.89)
Fe–Pb	899/909 (98.9)	517/22725	(2.28)
Ni–Fe–Pb	885/902 (98.1)	97/22525	(0.431)

[a] The denominator is the number of true matches and the number of false matches possible respectively.

determination of a match utilizing standard hypothesis testing. Unfortunately, these methods do not address the question of whether two groups of cartridge cases *did* have a common origin, but rather whether two groups *could* have a common origin. Regrettably, many juries are provided with only this limited contextual information and are asked to infer whether a common origin exists without knowing the frequency with which a true match, a false non-match, and a false match are likely to occur. Furthermore, even if the jury is provided with this information, it usually lacks the understanding or means necessary to assess the significance of a match. Consequently, the jury must rely on the expert witness to clarify and ascribe meaning to a match. One method of clarifying the significance of a match, and which is slowly gaining acceptance in the forensic sciences, is the application of the likelihood ratio [13–21].

If A denotes the event that the two groups of cartridge cases originated from the same source, and if $\bar{A}$ ('not A') denotes its negation, i.e., the event that the two groups of cartridge cases came from different sources, then prior to the determination of whether a match occurs the event A has some probability $P(A)$, and $\bar{A}$ has the probability $P(A) = 1 - P(A)$. The odds on A is correspondingly

$$\text{Odds on } A = P(A)/P(\bar{A}) \qquad (4)$$

When scientific evidence is introduced in a trial, it is the responsibility of the expert witness to present the information in such a manner that the court can incorporate it into the context of the trial. If there is a match between the two groups of cartridge cases the jury will be interested in the probability of A given that a match exists, $P(A \mid B)$, where B denotes the event that there is a match, or equivalently, the odds on A given B

$$\text{Odds on } A \mid B = P(A \mid B)/P(\bar{A} \mid B) \quad (5)$$

According to the Bayes theorem

$$P(B \mid A) = [P(A \mid B)P(B)]/P(A) \quad (6)$$

$$P(A) = [P(A \mid B)P(B)]/P(B \mid A) \quad (7)$$

$$P(\bar{A}) = [P(\bar{A} \mid B)P(B)]/P(B \mid \bar{A}) \quad (8)$$

Hence the odds on A given B, i.e., given a match, is related to the odds on A (prior to the determination of whether there is a match) via the formula

$$P(A \mid B)/P(\bar{A} \mid B) = [P(A)/P(\bar{A})][P(B \mid A)/P(B \mid \bar{A})] \quad (9)$$

The odds on A is referred to as the "prior odds"; the odds on A given B is called the "posterior odds"; the factor $P(B \mid A)/P(B \mid \bar{A})$ is termed the "likelihood ratio" (LR).

It must be emphasized here that the event B, existence of a match, is a reduction of the raw evidence obtained from the two groups of cartridge cases. In a more comprehensive approach, B (or $\bar{B}$) would be replaced with $B^{\ddagger}$, an event specifying the observed values of every variable measured on every cartridge case in both groups.

Determination of the likelihood ratio

The numerical value of the likelihood ratio can be established by determining the probability of obtaining a match when a cartridge case is compared with its progenitorial lot, and by determining the probability of obtaining a match when the cartridge case is compared with a non-progenitorial lot. This last probability corresponds to the probability of a false match occurring, which has already been approximated at 0.43% when three variables are considered and α is set at 0.01. In addition, the former probability has been approximated at 98.1% when the same three variables are considered and α equals 0.01. The resulting likelihood ratio is equal to (885/902)/(97/22 525) or 227.

Utilizing Evett's and Buckleton's verbal equivalent to a likelihood ratio [22], this value of 227 would "strongly support" the inference that a specific cartridge case did come from a specific lot of cartridge cases given a match between the variables [Ni], [Fe], and [Pb] exist, that is, the cartridge case and the lot of cartridge cases have a common origin. However, it is worth remembering that while there is extensive clustering of the measured concentrations within most lots, and notable inter-lot differences in measured elemental concentrations exist, there is also significant variation between measured intra-lot elemental concentration variances which warrants caution when applying the likelihood ratio value.

It should be recognized, however, that the likelihood ratio is not without its shortcomings. It is extremely difficult to determine $P(B \mid A)$ in many practical situations, including this one. The within-lot, and more importantly, the between-lot, distributions of [Ni], [Fe], and [Pb] is unestablished. A distribution model for the variables in the "non-progenitorial" lots posited in event A must be assumed, and consequently, the likelihood ratio calculated here should be viewed as a plausible approximation rather than a precise value.

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Target-compound method for the analysis of accelerant residues in fire debris

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Abstract

A primary task of arson analysis is to identify residues of commonly targeted accelerants (gasoline, petroleum naphthas, kerosine, and diesel fuel) when present in samples of fire debris. The commonly employed method separates volatile debris components by flame-ionization gas chromatography (GC-FID) and compares the resulting profile with profiles for neat and partially evaporated accelerant exemplars. This method fails, that is, neither establishes nor disproves the presence of an accelerant, when GC peaks of non-accelerant origin (usually pyrolysis products) are sufficient in number and size to conceal an otherwise diagnostic accelerant pattern. In such cases the GC effluent can be analyzed by mass spectrometry (MS) to distinguish accelerant-related species from co-eluting, but non-identical, substrate-related species. Recent work by others has shown that the GC–MS data extraction and patterning technique known as mass (or extracted ion) chromatography contributes substantially to accelerant recognition in high-background samples by limiting interferences to only a few classes of non-accelerant species. A different GC–MS approach, developed in this laboratory and described in this paper, employs target compound analysis to exclude interference by all such species.

Keywords: Gas chromatography–mass spectrometry; Accelerant; Arson; Pattern recognition; Pyrolysis products; Target compound analysis

For over 30 years, pattern recognition based on gas chromatography (GC) has been commonly used to identify residues of petroleum-derived accelerants (flammable/combustible liquids used to "accelerate" the initiation of a fire) in debris samples removed from scenes of suspected arson [1–5]. Gasoline, petroleum naphthas, kerosine, and diesel fuel are the accelerants routinely targeted for detection. Except napthas, each of these products, irrespective of brand or grade, produces a characteristic gas chromatographic pattern. Naphthas (also known as middle-range petroleum distillates or MPD's) comprise a less homogeneous accelerant class and, as a result, are usually represented by many chromatograms in reference pattern collections. The standard detection procedure entails isolating volatile components from the debris by any of a number of extraction and concentration techniques, chromatographing the isolate, and interpreting the chromatographic pattern by visual comparison with patterns obtained for accelerant exemplars. The problem with this method is its vulnerability to interferences by contaminant peaks. Contaminants generally consist of volatile organic components produced by the pyrolysis of substrate materials at the scene of the fire. All debris isolates contain contaminant species in very wide ranging number, chemical variety, and relative abun-

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dance. When accelerant species are also present, they compete with contaminants for space on the chromatogram. If accelerant levels are high enough, relative to contaminant levels, the accelerant pattern prevails and will be recognized. In the opposite case, contaminants prevail and the accelerant escapes detection. Since the nature of the fire scene generally favors weak accelerant residues over strong ones, the average accelerant-containing isolate can be expected to fall closer to the second of these two extremes. This view is supported by the frequency with which hard-to-interpret or uninterpretable gas chromatography–flame-ionization detection (GC–FID) patterns are encountered in arson casework.

A rapid and broadly effective method of sample clean-up prior to analysis would be very useful in the arson laboratory. Selective-reaction and selective-adsorption methods have been applied successfully in special arson-related situations [6,7]. However, the sources and chemical compositions of contaminants suggest that such techniques are unlikely to be effective for most high-background samples. Accelerant residues are most likely to survive building fires at or near floor level. Consequently, incoming samples of debris usually contain scorched or charred wood flooring, plywood subflooring, carpet, carpet padding, plastic tile, and various adhesives. In their pre-fire condition, all of these materials consist largely of organic polymers which, when exposed to fire, decompose into hundreds of different volatile species. Many of these pyrolysis products remain entrapped where formed; others may be adsorbed from the fire atmosphere. A number of arson analysts have used GC–FID to display the compositional complexity of volatiles

TABLE 1

GC–MS operating parameters

Column	DB-1 Non-polar capillary, 60 m × 0.32 mm i.d., 0.25-μm methylsilicone bonded phase (J&W Scientific, Folsom, CA)	
Carrier gas	Helium at 20 p.s.i.g. (138 KPa), 2.0 ml min^{-1} at 70°C, 42 cm s^{-1} linear velocity	
Injector	Split/splitless in splitless mode, 0.6 min splitless time, temperature 260°C	
Transfer line	250°C	
Temperature programs:	Method 1 (gasoline/MPD)	Method 2 (HPD)
Initial temperature	70°C	70°C
Initial hold	None	None
First ramp rate	2° min^{-1}	2° min^{-1}
Intermediate temperature	130°C	130°C
Intermediate hold	0.2 min	0.2 min
Second ramp rate	30° min^{-1}	10° min^{-1}
Final temperature	260°C	260°C
Final hold	3 min	10 min
Run time	38 min	54 min
Mass spectrometer:		
Scan range	50–200 a.m.u.	
Start time	4.00 min	
Scan cycle	1.25 s (16 A/D samples)	
Source temperature	200°C	
Ionization	Electron impact	
Electron energy	70 eV	
Emission current	300 μA	
Threshold	20 counts	

TABLE 2

ID file for gasoline target compounds

Target compound	Retention time (min)	Ion (m/z)	Relative abundance (%)
1,3,5-Trimethyl-benzene	6.8	105	100
		120	50
1,2,4-Trimethyl-benzene	7.6	105	100
		120	45
1,2,3-Trimethyl-benzene	8.4	105	100
		120	45
Indane	8.8	117	100
		118	55
		115	35
1,2,4,5-Tetra-methylbenzene	12.3	119	100
		134	50
1,2,3,5-Tetra-methylbenzene	12.5	119	100
		134	50
5-Methylindane	13.2	117	100
		132	40
4-Methylindane	13.7	117	100
		132	40
Dodecane	17.5	57	100
		71	65
		85	50
4,7-Dimethyl-indane	19.1	131	100
		146	40
2-Methylnaph-thalene	21.2	142	100
		141	80
1-Methylnaph-thalene	22.0	142	100
		141	85
Ethylnaphthalenes (mixed)	27.7	141	100
1,3-Dimethyl-naphthalene	28.5	156	100
		141	90
2,3-Dimethyl-naphthalene	29.6	156	100
		141	90

isolated from pyrolyzates produced in the laboratory by heating assorted woods and floor coverings [7–10]. In this laboratory, gas chromatography–mass spectrometry (GC–MS) has been used to identify major volatile components of a dozen such pyrolyzates and many isolates (accelerant-free) obtained in casework. Nearly 40% of the pyrolyzate peaks examined were identified as hydrocarbons of assorted homologous series and structural types, including saturated and unsaturated aliphatic, naphthenic, and aromatic compounds. A simple and effective pre-analysis clean-up procedure for removing these hydrocarbons from target petroleum-based hydrocarbons has yet to be discovered.

Replacement of a flame-ionization detector, which responds non-selectively, by a mass spectrometer, which can be operated in a compound-specific manner, affords a means of eliminating interference by many species of contaminants [5,9–13]. A useful GC–MS analysis of a high-background isolate may be performed as follows.

TABLE 3

ID file for MPD target compounds

Target compound	Retention time (min)	Ion (m/z)	Relative abundance (%)
Nonane	5.4	57	100
		85	50
		71	35
Propylcyclo-hexane	6.1	83	100
1,3,5-Trimethyl-benzene	6.8	105	100
		120	50
1,2,4-Trimethyl-benzene	7.6	105	100
		120	45
Decane	8.2	57	100
		71	40
		85	30
1,2,3-Trimethyl-benzene	8.4	105	100
		120	45
n-Butylcyclo-hexane	9.3	83	100
		82	70
trans-Decalin	10.0	138	100
		96	65
		81	55
Undecane	12.4	57	100
		71	55
		85	35
1,2,3,5-Tetra-methylbenzene	12.5	119	100
		134	50
n-Pentylcyclo-hexane	13.8	83	100
		82	80
		55	70
C_{12} alkane	15.5	57	100
		71	40
Dodecane	17.6	57	100
		71	65
		85	50
C_{13} alkane	18.4	57	100
		71	40
n-Hexylcyclo-hexane	19.5	83	100
		82	80
		55	60

TABLE 4

ID file for HPD target compounds

Target compound	Retention time (min)	Ion (m/z)	Relative abundance (%)
Decane	8.2	57	100
		71	40
		85	30
n-Butylcyclo-hexane	9.3	83	100
		82	70
trans-Decalin	10.0	138	100
		96	65
		81	55
Undecane	12.4	57	100
		71	55
		85	35
1,2,3,5-Tetra-methylbenzene	12.5	119	100
		134	50
n-Pentylcyclo-hexane	13.8	83	100
		82	80
		55	70
Dodecane	17.6	57	100
		71	65
		85	50
n-Hexylcyclo-hexane	19.5	83	100
		82	80
		55	60
2-Methylnaph-thalene	21.2	142	100
		141	80
1-Methylnaph-thalene	22.0	142	100
		141	85
Tridecane	23.6	57	100
		71	65
		85	40
n-Heptylcyclo-hexane	25.6	83	100
		82	85
1,3-Dimethylnaph-thalene	28.5	156	100
		141	90
Tetradecane	29.7	57	100
		71	65
		85	50
n-Octylcyclo-hexane	31.7	83	100
		82	90
2,3,5-Trimethyl-naphthalene	33.8	170	100
Pentadecane	34.0	57	100
		71	70
		85	50
n-Nonylcyclo-hexane	35.1	83	100
		82	80
Hexadecane	36.5	57	100
		71	70
		85	50
Heptadecane	38.4	57	100
		71	80
		85	55

TABLE 4 (continued)

Target compound	Retention time (min)	Ion (m/z)	Relative abundance (%)
Pristane	38.6	57	100
		71	80
		85	40
Octadecane	39.8	57	100
		71	80
		85	55
Phytane	40.0	57	100
		71	75
		85	60
Nonadecane	41.1	57	100
		71	75
		85	60
Eicosane	42.1	57	100
		71	80
		85	55
Heneicosane	43.2	57	100
		71	80
		85	55

The sample is separated by capillary GC and the effluent is linearly scanned to acquire mass spectra and retention times for all components eluting within the accelerant-characteristic time range. The system computer is then used to extract accelerant-related data from the acquired data file and to present the extracted data in a graphical form suitable for comparison with graphs obtained for accelerant exemplars. In work of this kind to date, two different procedures have been used for the data extraction step. One procedure, extracted ion profiling (or "mass chromatography") [14], has been discussed at length in other papers [5,9,10,12,13]. Although not hydrocarbon specific, this technique is selective enough to reduce substantially the number of non-accelerant species capable of interfering with accelerant pattern recognition. The other procedure, developed in this laboratory, eliminates that kind of interference by targeting individual hydrocarbons for identification and quantitation [15]. Potential interference is thereby restricted to a single category of contaminant, namely, those hydrocarbons of pyrolytic origin that are structurally identical to the target compounds.

This paper describes the target-compound technique and results of performance tests which demonstrate its ability to detect accelerants in high-background samples.

EXPERIMENTAL

The GC–MS system consists of a Hewlett-Packard Model 5890 gas chromatograph, a Hewlett-Packard Model 5988A mass spectrometer, and an RTE-A operating system running on an HP A400 MICRO 24 computer with 160-MB hard drive (Hewlett-Packard, Avondale, PA). Operating parameters are listed in Table 1. Reagent-grade carbon disulfide (J.T. Baker, Phillipsburg, NJ) was used as the diluent for accelerant exemplars and as the extracting solvent for charcoal tubes employed to isolate volatiles from pyrolyzates. The charcoal tubes contained 50–200 mesh activated carbon (Fisher Scientific, Pittsburgh, PA) that was heated at 275°C for one hour before use. Gasoline, kerosine, and diesel fuel were obtained from local service stations. Paint thinner, charcoal lighter fluid, and mineral spirits were obtained at local hardware stores. Fir plywood (interior grade), vinyl floor tile, cut-pile nylon carpeting with jute backing, and polyurethane foam carpet padding were used to generate pyrolysis products.

Weathered samples of gasoline, kerosine, diesel fuel, and mineral spirits were produced by warming the fresh liquids in shallow containers until the desired degree of evaporation (by volume) had been attained. Pyrolysis products were produced by placing plywood chips, floor tile fragments, and carpet swatches with padding in separate 1-gal metal paint cans, attaching perforated lids, and heating the cans strongly over a Meker burner until heavy smoke was observed at

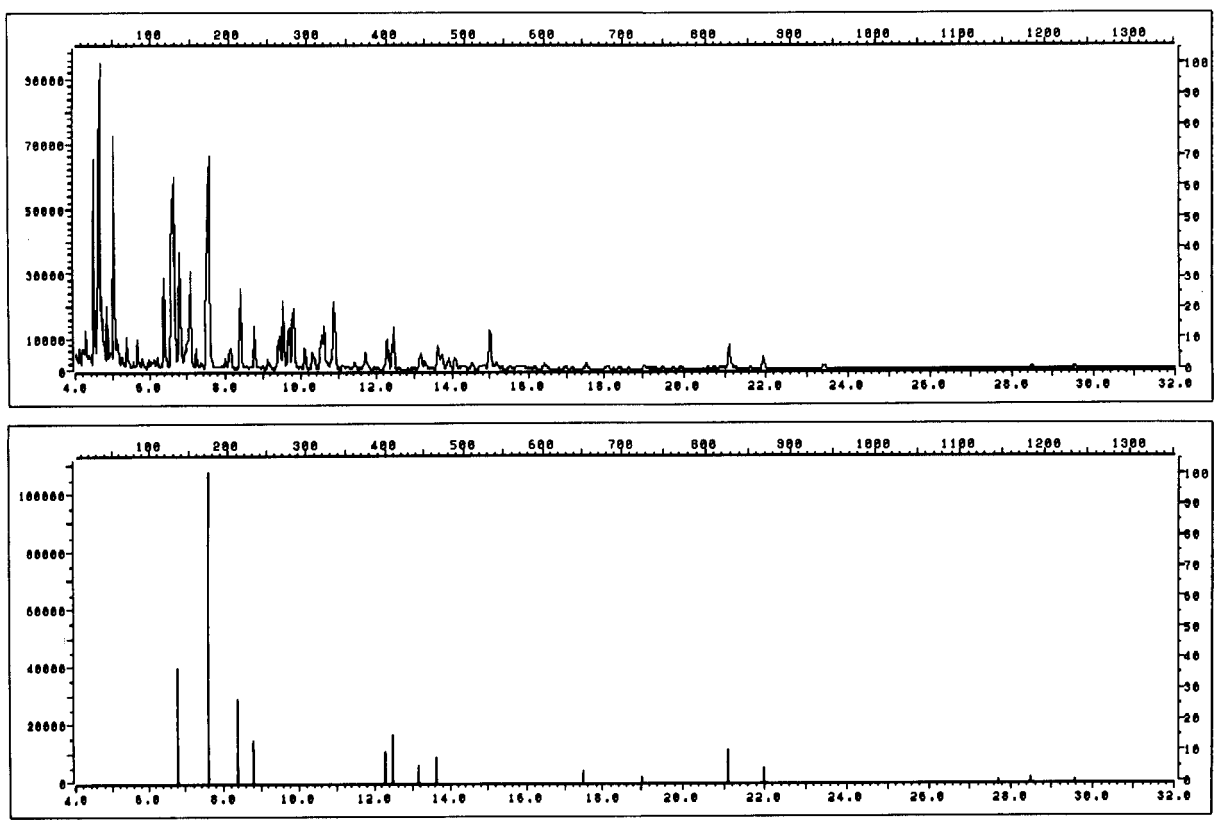

Fig. 1. Total ion chromatogram (TIC), and target compound chromatogram (TCC) for fresh unleaded gasoline.

the vents. After cooling, the vapors within each can were collected by charcoal tube adsorption [16,17], and the tubes were eluted with carbon disulfide.

Liquid petroleum products were diluted with carbon disulfide to approximately 1% (v/v) prior to injection for GC–MS patterning. For the performance tests, the extracted pyrolyzate solutions were spiked with approximately 0.1% (v/v) of weathered petroleum product.

The evaporation of petroleum samples, preparation of pyrolysis products, and dilution of samples were carried out in a fume hood to prevent respiratory exposure to smoke and hazardous chemical vapors.

Splitless injections were used to avoid the discrimination effects commonly experienced with split injections of mixed solutes of wide boiling range [18]. The GC parameters listed in Table 1 provided near-baseline resolution of all target compounds. The specified start time (4.00 min) assured complete elution of solvent and served to exclude the acquisition of data for sample components deemed to have little or no diagnostic value, namely, hydrocarbons of carbon number 8 or less. The selected scan range (50–200 a.m.u.) accommodated all of the ions necessary for the identification and quantitation of the 40 different target compounds employed in this procedure. A wider scan would have reduced sensitivity while adding little of importance to selectivity. Of the two GC–MS methods listed in Table 1, the longer was used if the GC–FID trace supported the possible existence of heavy petroleum distillate

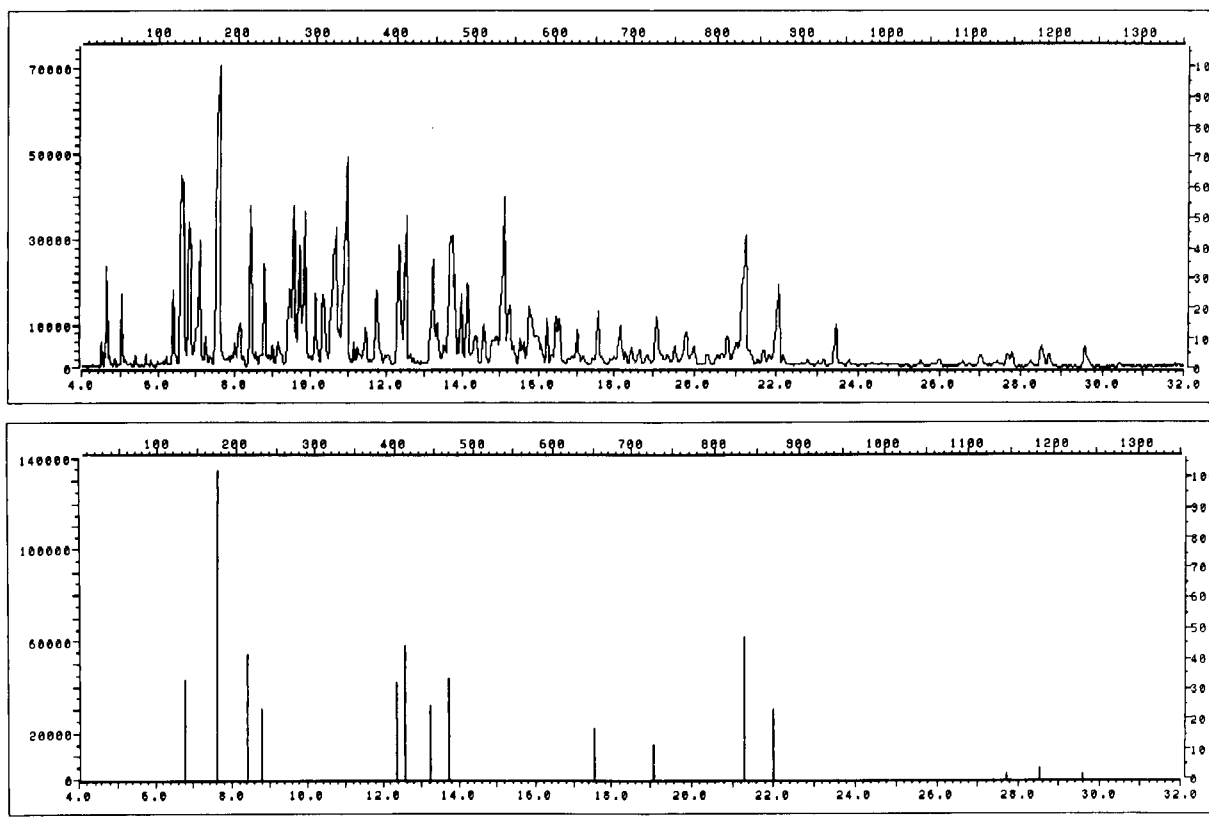

Fig. 2. TIC and TCC for 80% evaporated gasoline.

(HPD), or mixtures of HPD with gasoline and/or MPD. The shorter method was chosen if only gasoline or MPD were sought.

Accelerant identification files (ID files)

Separate ID files have been created for gasoline, MPDs, and HPDs. MPDs contain hydrocarbons in the *n*-octane through dodecane boiling range [19] and include paint/varnish thinners, mineral spirits, degreasing solvents, and several brands of charcoal lighter fluid. HPDs contain hydrocarbons in the *n*-nonane through tricosane boiling range [19] and include kerosine, jet fuel, diesel fuel, and heating oil. Tables 2, 3, and 4 show the pertinent contents of these files. Peak retention time windows were set at 0.3 min for all target compounds except for the closely eluting tetramethylbenzene isomers, for which a setting of 0.15 min was used. A tolerance limit of ±25% was applied to relative ion intensities when searching a sample's data file for targeted species. The most intense ion (base ion) was used for quantitation of each target compound.

A homologous series of normal alkanes was the starting point for MPD and HPD identification, and provided the "bell"-shaped peak envelope characteristic of petroleum distillate. Naphthenic hydrocarbons (alkylcyclohexanes) were included to insure against false positives from polyolefin pyrolyzates, which also contain homologous series alkanes [20]. The isoprenoids pristane and phytane provided additional insurance for HPD identification because of their recognition as biological markers in crude oil [21]. Unidentified branched C_{12} and C_{13} alkanes were added to the MPD file to fill relatively long gaps in the short (14 min) pattern. The homologous series alkanes (nonane and higher) do not make good patterns

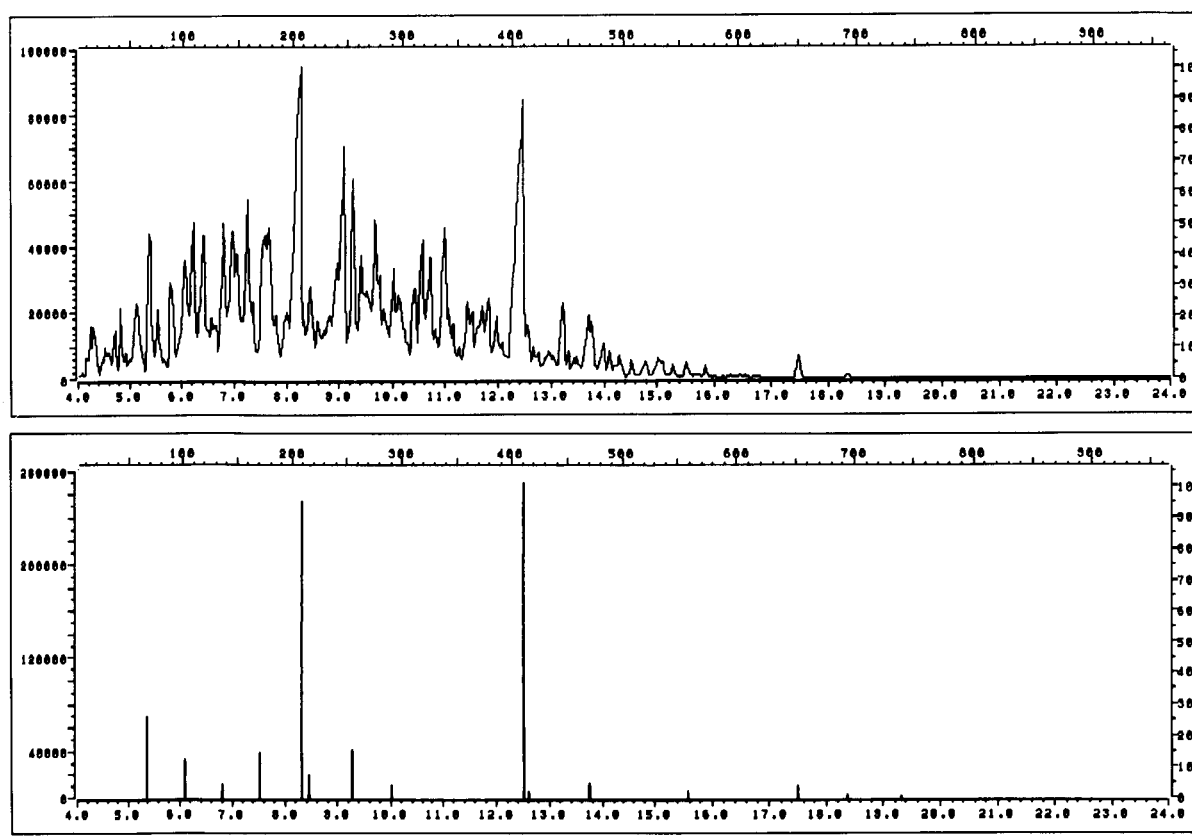

Fig. 3. TIC and TCC for fresh mineral spirits.

with gasoline due to low concentrations and co-elution with gasoline aromatics; however, dodecane was included in the gasoline ID file as an indicator for the presence of heavier petroleum fractions. Isomer pairs for aromatic compounds were included, where practicable, in all three accelerant files because experience has shown that the abundance ratios of these compounds to their respective isomers are fairly consistent in petroleum distillates [11]. Naphthalene, an obvious target for petroleum identification, was found to be too common in pyrolysis products to be useful.

The ions used for identification purposes consisted of the base ion for each target and one or two other strong ions where their inclusion significantly increased selectivity without seriously affecting sensitivity. Accordingly, ions having intensities less than 30% of the base ion were not included. The ion intensities reflect the actual performance of the instrument during a GC–MS run.

Data processing

Processing of the raw data acquired during a GC–MS run consisted of the following steps: (1) generation of a total ion chromatogram (TIC); (2) generation of a "Quant Report"; and (3) generation of a target compound chromatogram (TCC).

The TIC is a plot of the total ion current recorded during each scan versus GC retention time. Its only role in the present scheme was to assist selection of an ID file for "first trial" use in the next step. During the GC–MS run, the effluent from the GC column is scanned by the mass spectrometer, from 50 to 200 daltons, every 1.25 s. The resulting data are stored on the data system's hard drive as a series of mass spectra

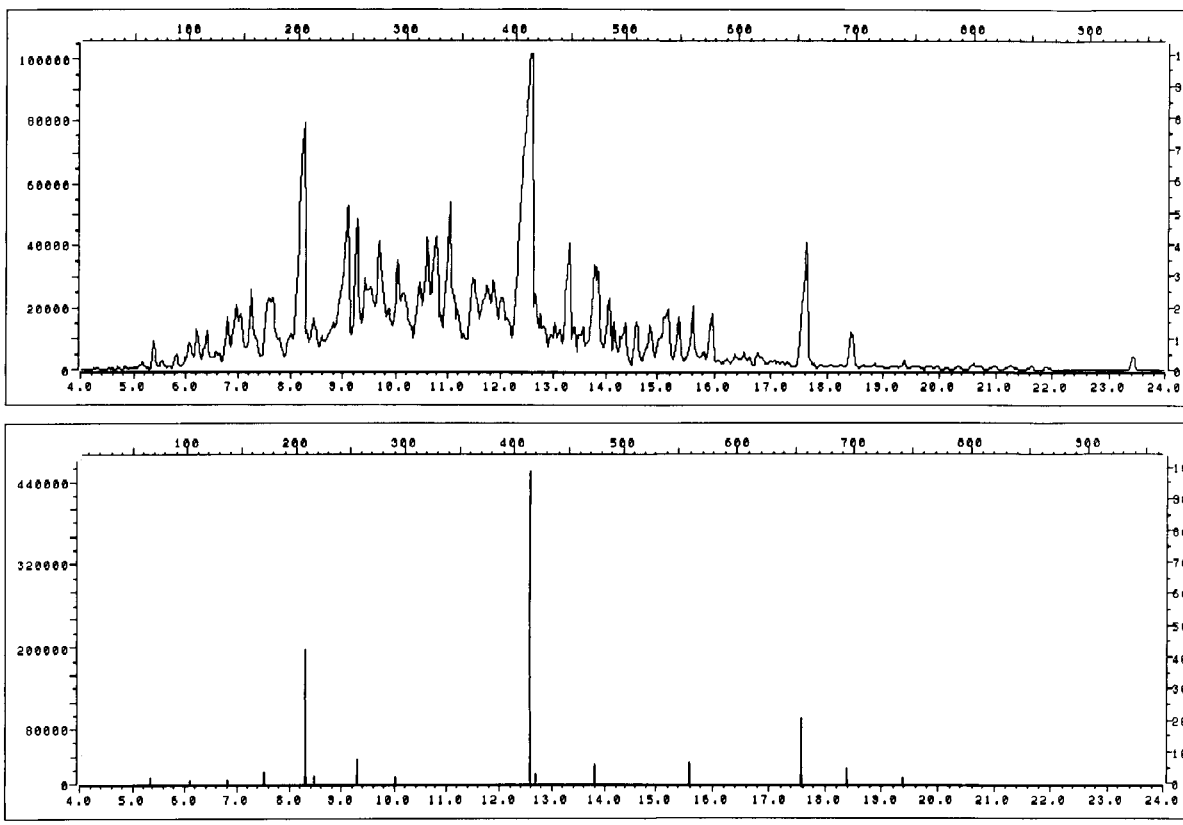

Fig. 4. TIC and TCC for 80% evaporated mineral spirits.

totalling approximately 1600 for Method 1 and 2400 for Method 2. The sum of intensities for all detected masses within a single mass spectral scan is the total ion current for that point on the chromatogram.

Programmed comparison of the raw data file with an ID file produces a Quant Report (step 2). For each target compound sought, the computer searches for the required ions within the specified retention time window. If the ions exist with the correct relative intensities and maximize in the same scan, the compound is identified and its base ion quantitated. The Quant Report that is output from the data system consists of a listing of the identified target compounds and their respective peak areas.

The last step reproduces the Quant Report data as a bar graph (histogram) in which the target-compound pattern is plotted, with the *y*-coordinate being peak area and the *x*-coordinate being retention time. These patterns were produced through use of a user-generated program (procedure file), and have been coined by us as target compound chromatograms (TCCs). TCCs for questioned samples were interpreted by visual comparison with a library of TCCs for fresh and partially evaporated accelerant exemplars.

Retention time and relative abundance are the two parameters used in accelerant pattern recognition. The ratio of abundances for two consecutive target compounds is relatively insensitive to changes caused by partial evaporation or incomplete sample recovery. Target compounds that elute at wider intervals have greater vapor pressure (boiling point) differences than neighboring compounds, and are subject to greater abundance ratio mismatch when compared against library TCCs. Because of this, long-term variations are

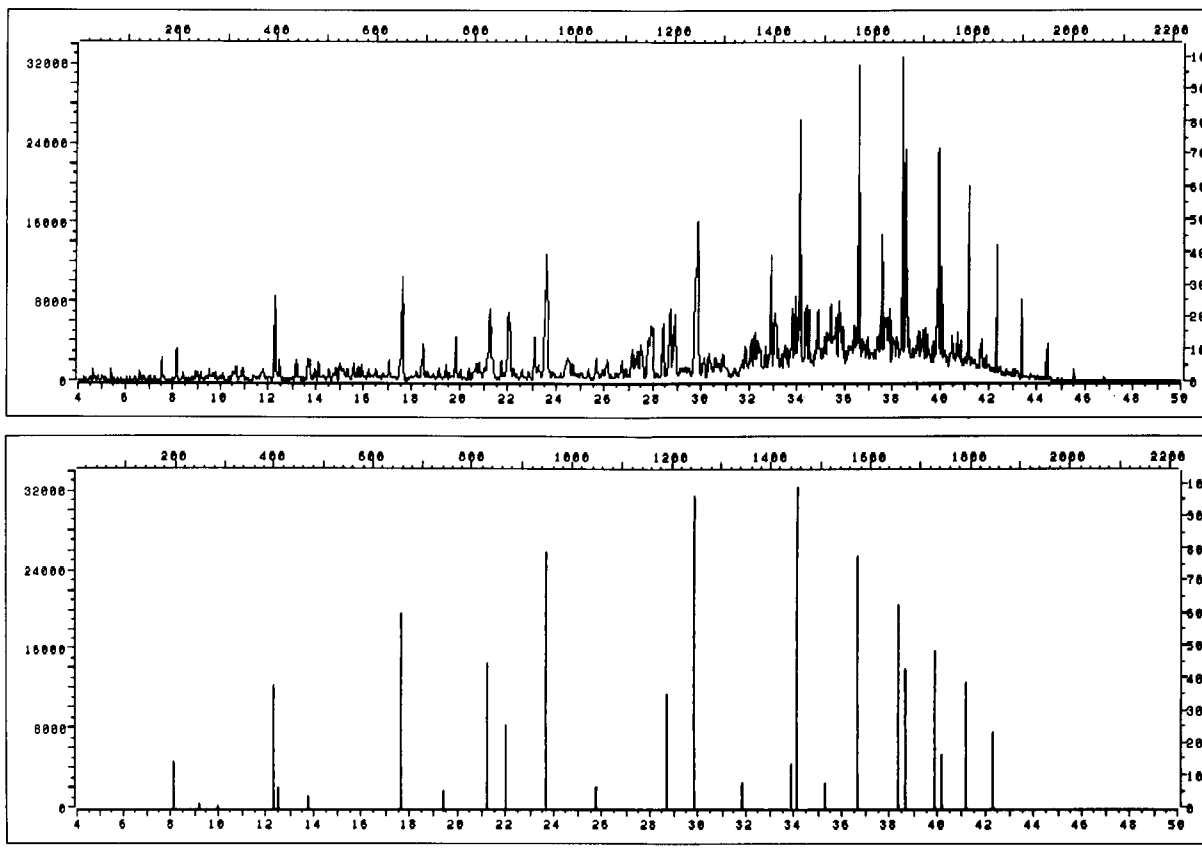

Fig. 5. TIC and TCC for fresh diesel fuel (fuel oil No. 2).

more acceptable in visual pattern matching than are short-term variations, which should be treated as questionable.

The ordinate on a TCC, being in terms of peak area for each compound's base ion, is not directly comparable to the ordinate on a TIC, which is in terms of total detector response for all ions. This should be kept in mind when comparing the two different graphical representations of the same data.

RESULTS AND DISCUSSION

Accelerant profiles

TICs and TCCs for fresh and weathered accelerants are shown in Figs. 1 through 6. Figure 1 was obtained on a blend of 18 fresh gasolines, and Fig. 2 represents the same gasoline evaporated to 20% of its original volume. Figures 3 and 4 show the TICs and TCCs for a representative MPD (mineral spirits) before and after evaporation to 20% of its original volume. Results of similar treatment to an HPD (diesel fuel) are shown in Figs. 5 and 6. Patterns of partially evaporated petroleum products are needed to represent the large effects of accelerant exposure to weathering, that is, evaporation and/or partial combustion at the fire scene. Either kind of exposure results in progressive loss of light-end components and consequent enhancement of the relative abundances of less-volatile species. The effect is especially large for gasoline, which, when fresh, contains a high weight fraction of low-boiling species. To ensure the proper identification of samples with unknown histories, it is essential to have a reference library of TCCs for accelerants in various stages of evaporation.

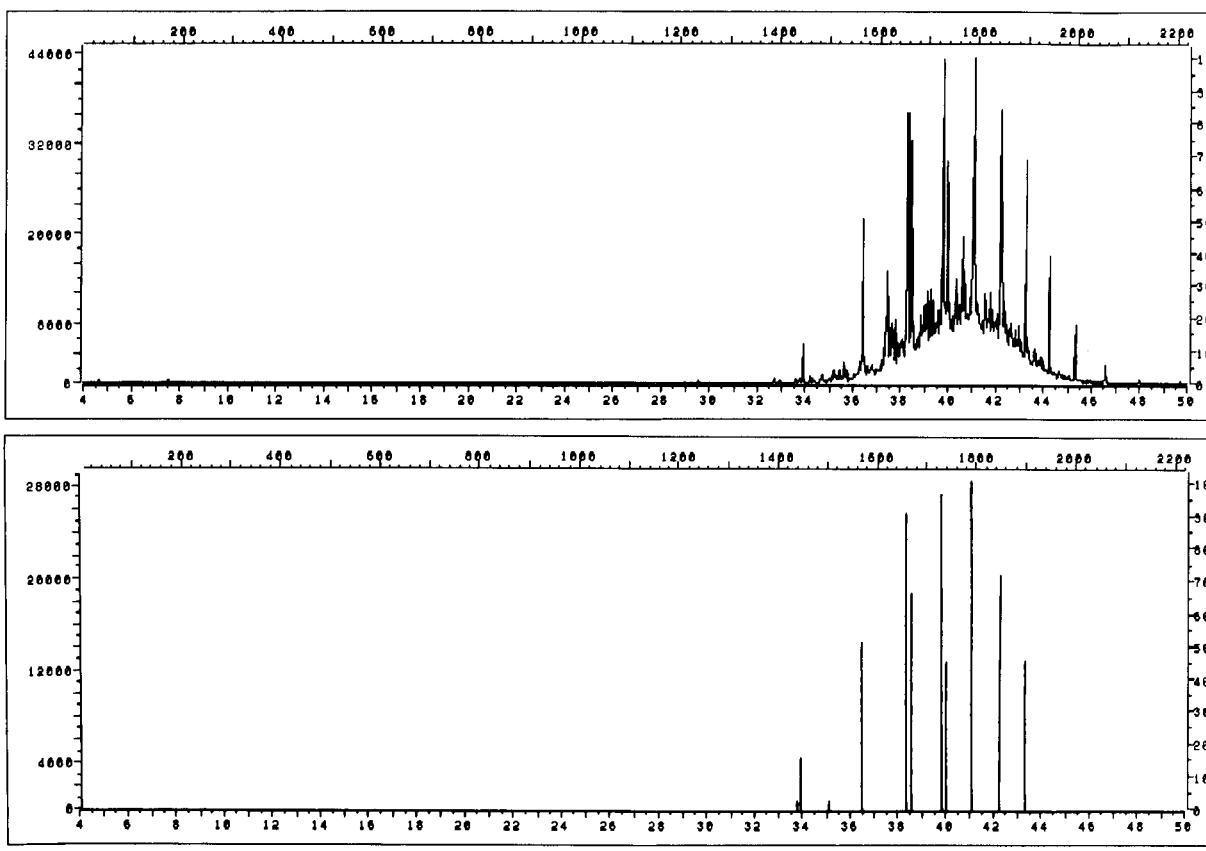

Fig. 6. TIC and TCC for 80% evaporated diesel fuel (fuel oil No. 2).

Performance tests

The ability of this procedure to extract accelerant-diagnostic patterns from GC–MS data for highly contaminated accelerants was tested by mixing partially evaporated exemplars of gasoline, mineral spirits, and diesel fuel with pyrolyzates of plywood, floor tile, and carpet/padding. The level of added accelerant was adjusted so that the accelerant's chromatographic pattern would be obscured. The resulting total-ion and target-compound chromatograms appear in Figs. 7–9, respectively. Comparison of these patterns with corresponding patterns obtained from the same petroleum products without contaminants (Figs. 2, 4, and 6, respectively) shows that the target-compound patterns, unlike the total ion patterns, provide conclusive evidence as to the presence and type of accelerant in each test sample.

Interferences

The target-compound approach to accelerant recognition precludes interference by all sample components except those which are target compounds of substrate (i.e. pyrolytic) origin. It is apparent that pyrolysis products will interfere seriously only when present in amounts sufficient to produce excessive distortion of an otherwise diagnostic accelerant profile. An indication of the number of target compounds that may be encountered solely as a result of substrate pyrolysis is provided in Fig. 10. These patterns were obtained by extracting targeted components of gasoline, MPD, and HPD from raw GC–MS data for each of the pyrolyzates previously described. Two inferences can be drawn from these results. First, by showing that many of the more abundant components of accelerants can be produced by pyrolyzing materials commonly present at fire

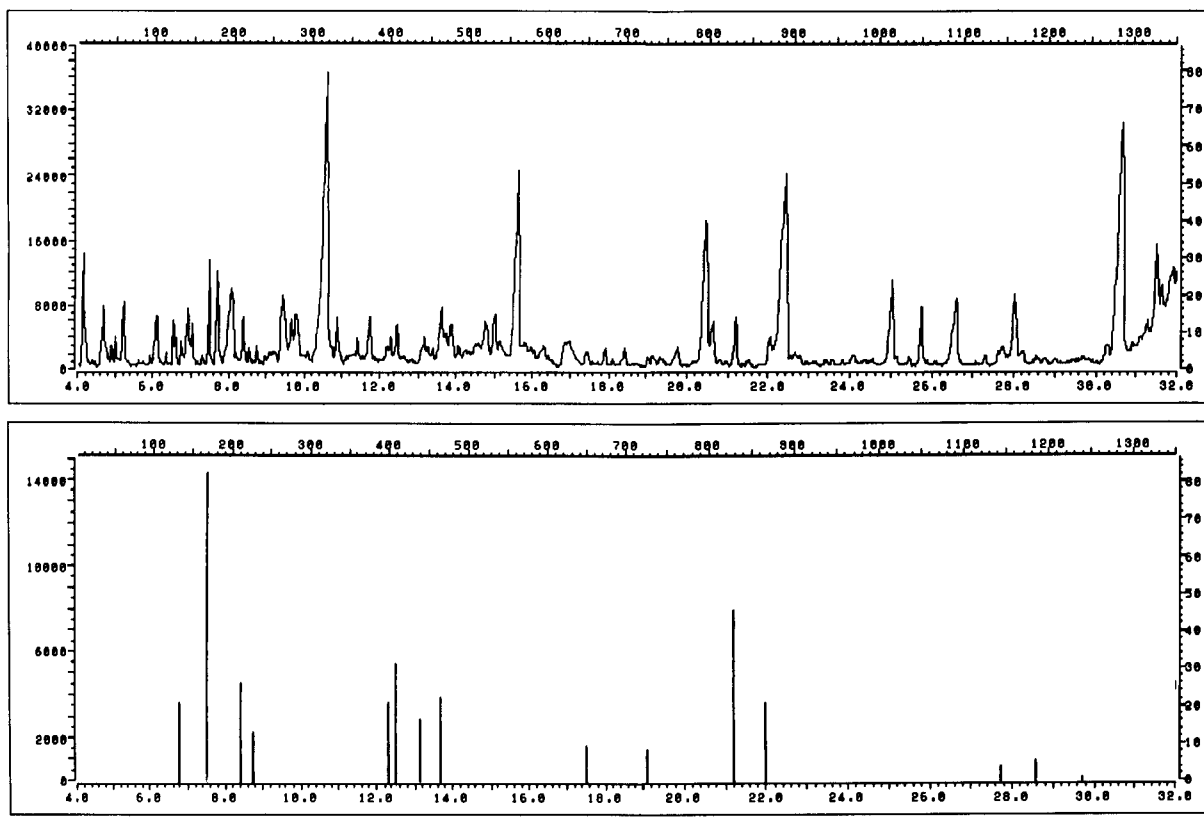

Fig. 7. TIC and TCC for plywood pyrolyzate spiked with 80% evaporated gasoline (compare Fig. 2).

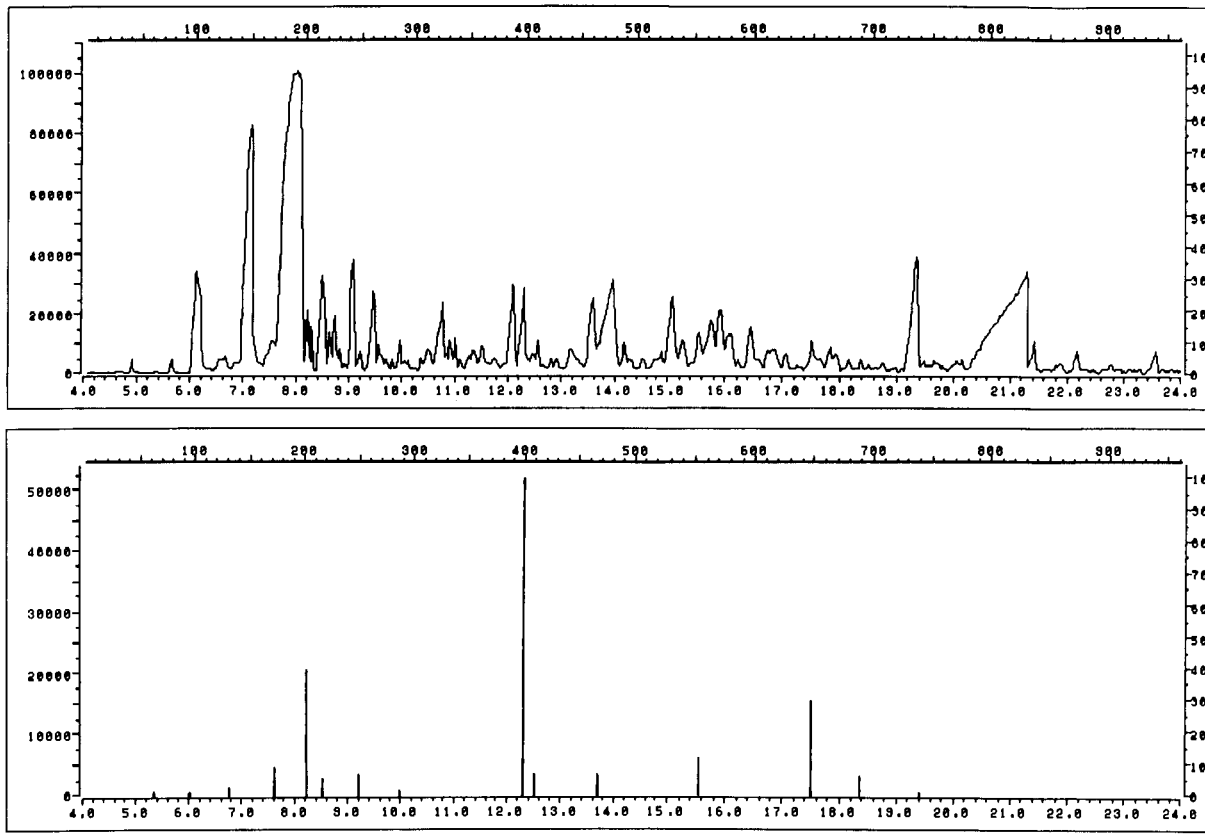

Fig. 8. TIC and TCC for floor tile pyrolyzate spiked with 80% evaporated mineral spirits (compare Fig. 4).

scenes, these data suggest the impossibility of conceiving a totally interference-free procedure for accelerant detection in arson samples, that is, a procedure incapable of yielding an occasional false negative result. Secondly, the absence of a significant degree of similarity between TCCs of these pyrolyzates and those of accelerant exemplars implies that the present procedure entails practically no risk of mistaking a pyrolyzate for an accelerant, that is, a false positive result.

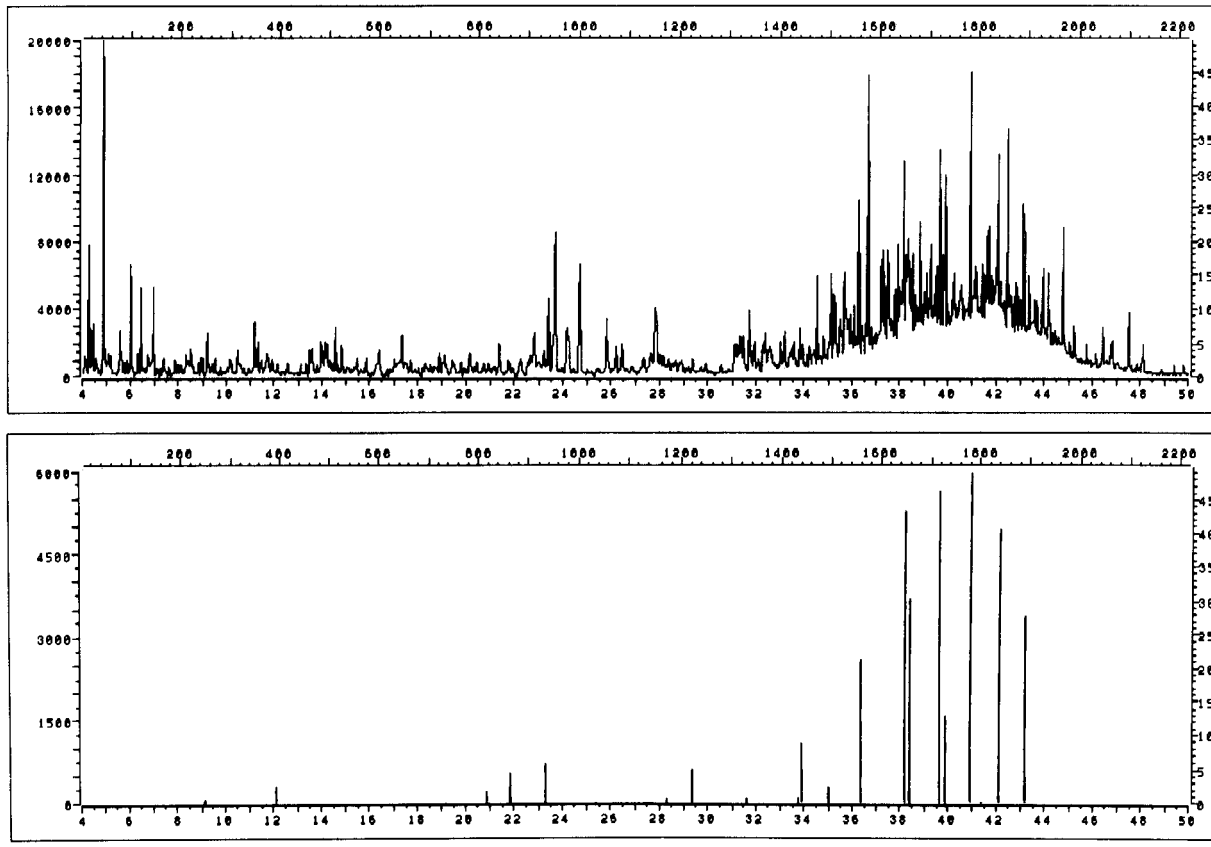

Fig. 9. TIC and TCC for carpet/padding pyrolyzate spiked with 80% evaporated diesel fuel (compare Fig. 6).

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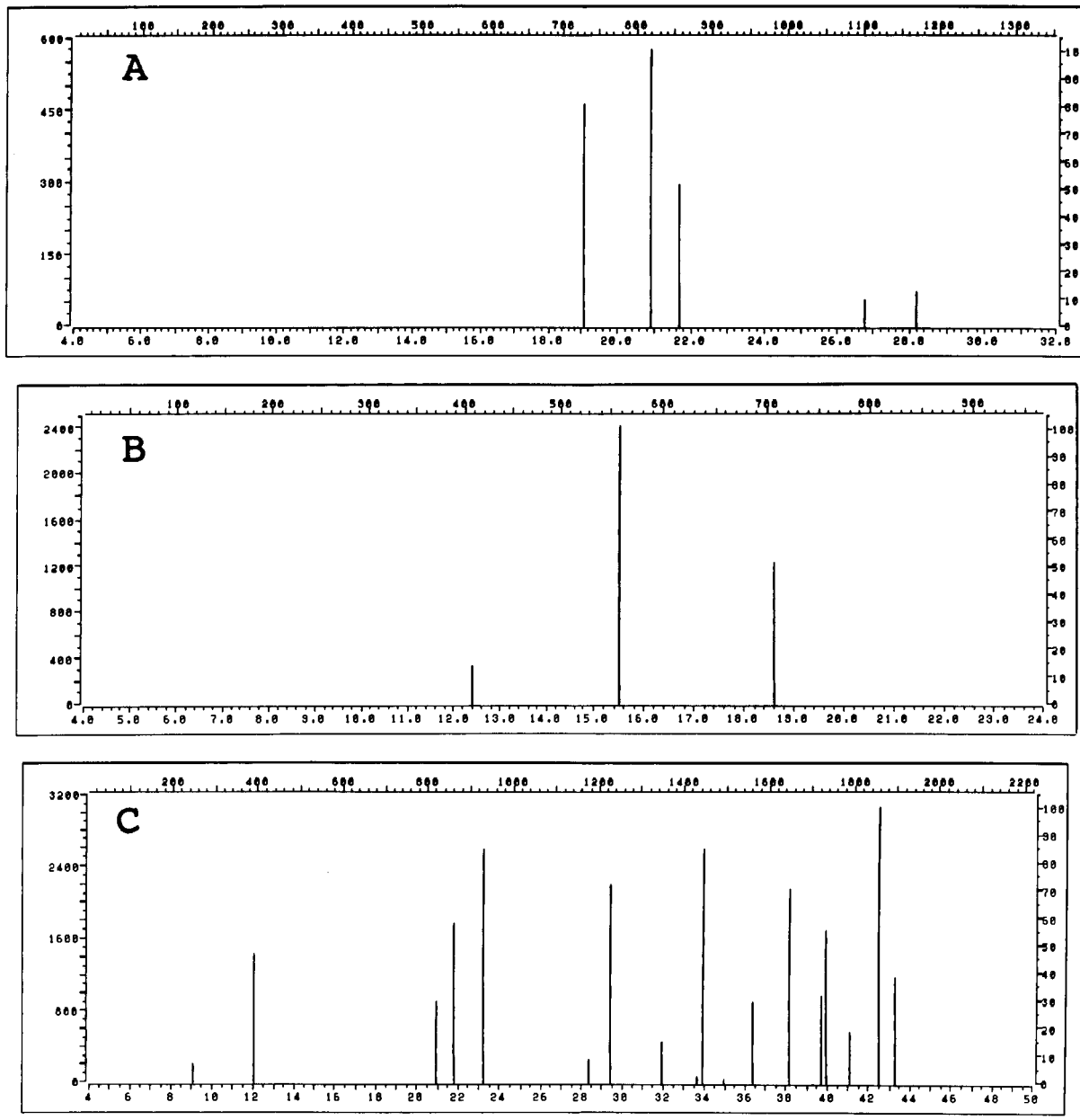

Fig. 10. (A) Gasoline target compounds present in plywood pyrolyzate; (B) MPD target compounds present in floor tile pyrolyzate; (C) HPD target compounds present in carpet/padding pyrolyzate.

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Liquid chromatography with photodiode array spectrophotometric detection in the forensic sciences

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Abstract

Liquid chromatography (LC) is used in forensic laboratories for numerous applications including examination of drugs and poisons, plastics and polymers, oils, hydrocarbons, cosmetics, inks, dyes and pigments. LC with photodiode array detection (PDA) is a hybrid technique which can provide complete UV–visible spectral information on a given peak in a chromatogram, enabling determinations of peak purity to be made, and identification of unknown peaks to be assigned by library searches of spectral information in combination with retention behavior. These are valuable features normally associated with gas chromatography–mass spectrometry. The additional information available on each peak makes LC-PDA a particularly attractive technique for the forensic laboratory where higher levels of certainty are often demanded in test results. This paper reviews some of those applications for LC-PDA in the forensic sciences, including drug screening, drug and pharmaceutical analysis, differentiation of inks, identification of pesticides, fungi, quality control testing and profiling of cosmetics, street drugs and profiling of other complex mixtures. The practical and technical limitations of the technique are explored and its place in the hierarchy of methods available in forensic laboratories is evaluated.

Keywords: Liquid chromatography; UV–Visible spectrophotometry; Forensic analysis; Photodiode array detection; Street drug analysis; Toxicology

Liquid chromatography (LC) has been used extensively in forensic laboratories for a number of years, with applications in drug testing, toxicology, and trace evidence examination including dyes and intermediates, explosives, hydrocarbons, lipids, optical brighteners, plastics and polymers, plasticizers and other additives [1]. Until recently the detectors available for LC have given only limited information about a chromatographic peak; namely its retention time, and its response to a single measuring probe (e.g., absorbance at a single wavelength, electrochemical activity at a given potential, etc.). This has restricted the role of LC mostly to quantitative analysis of compounds whose identity was already known [2], and to two-dimensional profile comparisons for complex mixtures [3,4].

The availability of low-cost diode array UV–visible spectrophotometric detection since the late 1980s [5,6], now allows the collection of multiple data points on any chromatographic peak, and subsequent manipulation of the data, markedly improving the discriminating power of LC for qualitative as well as quantitative applications.

A central tenet of chemical analysis in the forensic sciences is the confirmation of an analytical result by an independent chemical means.

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For example, drug testing laboratories certified by the National Institute of Drug Abuse (NIDA), typically rely on an initial drug screen using an immunoassay method, followed by a confirmatory quantitative analysis by gas chromatography–mass spectrometry (GC–MS) [7]. The initial identification is based on biochemical recognition of part of the compound's molecular structure, while the confirmatory test is based on the volatility, polarity, and predictable fragmentation behavior of the compound in the source of the mass spectrometer. The advent of photodiode array (PDA) spectrophotometric detectors for LC provides the chromatographer with the means of identifying by retention time the nature of a peak in a chromatogram, and confirming this identification by compiling a ratiogram at two or more selective wavelengths, or by examining the complete UV–visible spectrum of a given peak. The success of this technique depends on the compound in question having a spectrum with distinctive features which, while typically less specific than mass spectrometry [8], is often sufficient to provide confirmation with a high degree of confidence, particularly when considered along with retention time information, the compounds behavior during a selective extraction, and any ancillary information from screening tests or other sources.

The growing body of literature describing photodiode array detection in LC analysis of forensic samples has borne this out, particularly where well-characterized compounds are being tested, such as in tests for cocaine, heroin, PCP, diazepam, marijuana, etc., but also in identification of components in unknown mixtures such as illicit drugs and pharmaceuticals, extracts from biological fluids, inks and dyes. This paper reviews some of the applications in which LC-PDA has been used routinely as both a complement to, and an alternative to GC–MS in the forensic sciences.

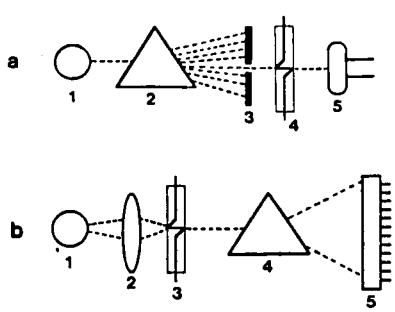

Fig. 1. Optics of conventional and photodiode array detectors. (a) Conventional variable-wavelength UV–visible detector for LC. (1) Light source, (2) grating, (3) monochromator, (4) flow cell, (5) single photodiode. (b) Reversed optical bench for photodiode array (PDA) detection for LC. (1) Light source, (2) lens system, (3) flow cell, (4) grating, (5) photodiode array.

Instrument considerations

Measuring UV–visible absorbance at a single wavelength has been the favored detection method for LC for many years, because of its sensitivity, general applicability and relatively low cost [1,9]. PDA detectors provide one means of recording in real-time, constant flow mode, a full UV–visible spectrum of the contents of the detector flow cell. The optics design for the PDA detector is the reverse of traditional LC UV–visible detector as shown in Fig. 1. In a PDA detector, a collimator lens focuses white light through the flow cell, where absorbance across the complete spectrum takes place. The original light signal minus the absorbed energy is then refocused and split by a holographic grating. The resulting spectrum impinges on an array of photodiodes, whose signals are amplified and fed to the data system. Each photodiode therefore continually monitors a discrete section of the spectrum. The size of this section and the degree of dispersion by the grating, determines the instrument's spectral resolution. Figure 2 shows an example of the three dimensional representation of the spectral/temporal data set known as a spectrochromatogram. This particular example shows a series of benzodiazepines, separated chromatographically and illustrates how their identity can be further ascertained by comparison of characteristic spectral features.

The issue of resolution is crucial in the selection of a PDA instrument for forensic applications. Resolution is the instrument's ability to discriminate between two closely adjacent wavelengths. Two wavelengths are said to be resolved if the trough between the two peaks is lower than 80% of the maximum. The ratio of the spectral bandwidth, i.e. the bandwidth of the source light at half the intensity, to the natural bandwidth, i.e.

the width of the absorption bandwidth at half the maximum absorbance, must be 0.1 or less otherwise inaccuracies in absorbance measurement for that band will result [5,10]. Most organic compounds in an aqueous environment have absorption bands with natural bandwidths greater than 20 nm, and therefore adequate resolution can be achieved on an instrument with a spectral bandwidth of 2 nm [10,11]. However, for some applications e.g., the fine absorbance detail of the five 'fingers' of benzene, 2 nm resolution would be insufficient. In practical terms, resolution is primarily a function of the number of diodes on the array, and increased resolution is achieved only at the cost of sensitivity, or spectral range. In considering the use of LC-PDA, for most applications including forensic science, an instrument with 2 nm resolution is optimum, greater resolution is usually unnecessary and poorer resolution compromises the value of the spectroscopic data.

A competing technology to PDA which also allows the collection of full UV–visible spectra is the rapid mechanical scanning UV–visible detector. In this instrument, the light source is split prior to passing through the flow cell, as with the standard optical bench. In rapid mechanical scanning instruments however, the grating is mounted on a transducer platform, the frequency of which is set at several cycles per second to ensure that, for typical chromatographic peaks, several full spectral scans take place during the passage of each component through the flow cell. This counters risks of obtaining skewed spectral data on peak upslope or downslope. This approach may impart improved sensitivity and can offer resolution down to 1 nm, allowing the measurement of

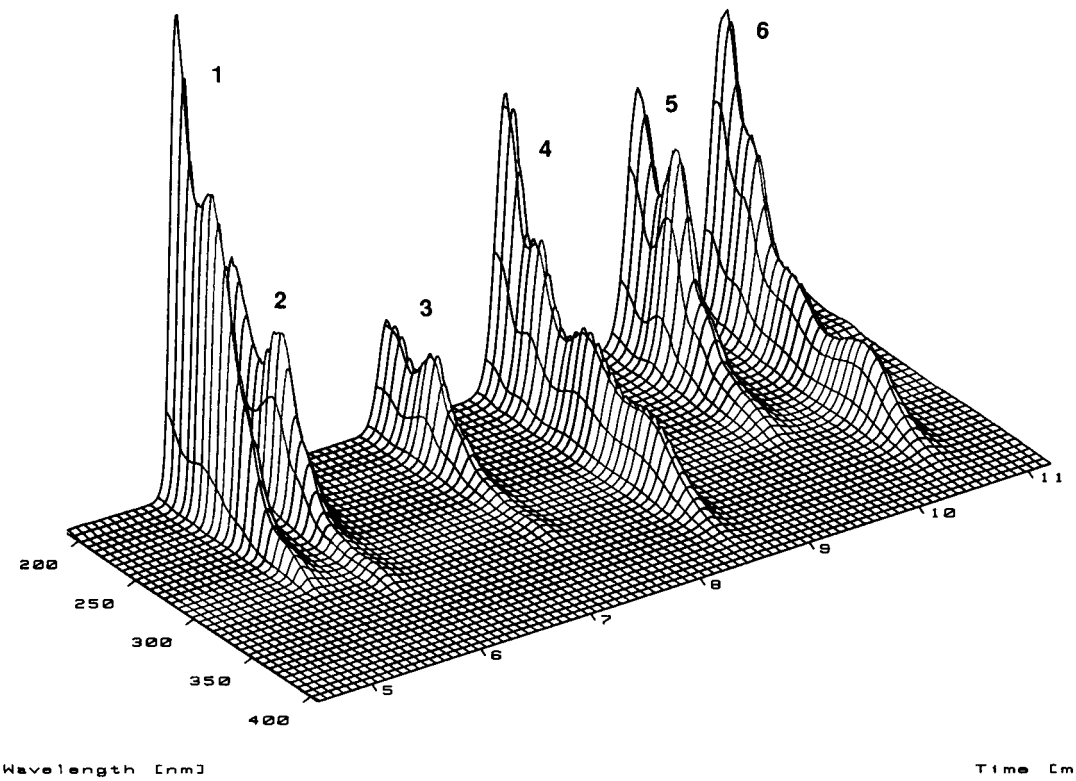

Fig. 2. Spectrochromatogram showing spectral and chromatographic data for the separation of six benzodiazepines. (1) Midazolam, (2) flurazepam, (3) oxazepam, (4) nitrazepam, (5) alprazolam, (6) clonazepam. Separation was accomplished on a 25-cm Lichrosorb RP-8 column (Merck), using 35% acetonitrile in 0.05 M KH_2PO_4–H_3PO_4 buffer (pH 3). Flow-rate was 1.5 ml min^{-1} (Gilson 307) and all spectra were collected between 190 and 400 nm (Hewlett Packard, 1040 M).

spectroscopic features with natural bandwidths of 10 nm [10,12]. Both approaches achieve the goal of obtaining full spectral information of chromatographic peaks, and additional features such as library matching, peak purity determinations, and derivative spectra are functions of the available software. The relative advantages of each configuration can be debated, but the capabilities of the software system, the required sensitivity for a given assay, and the choice of chromatographic conditions are likely to be more important considerations for the typical user [9].

Method development applications

The method development stage of any LC assay is as critical in forensic science as in any other field and is made considerably more straightforward when a PDA detector is available. It aids in selecting the optimum wavelength for maximum sensitivity and eliminating interference from other components in the sample or in biological extracts—a frequent problem in dealing with forensic samples.

In an LC-PDA method for colchicine in postmortem bile [9], a wavelength of 390 nm was selected after examining the spectrochromatogram for extracts from drug-free bile samples, resulting in an interference-free chromatogram and a sensitive assay with minimal sample preparation. Other peaks with similar spectral features to colchicine were noted and assumed to be metabolites or conjugates.

During method development, knowing the UV–visible absorption characteristics of the components of a mixture allows their relative elution order to be determined through a single injection of the mixture, providing they have sufficiently different spectra.

Once peaks have been identified, elution conditions can be changed on successive runs, and a mixture of all components can be injected simultaneously. Any changes in peak elution order which might otherwise have gone undetected, can be determined spectrophotometrically, as can the identity of any other drug or artifact which could potentially interfere with the analyte of interest. In traditional method development, compounds must be injected individually under each set of elution conditions to determine peak elution order. In cases where peaks co-elute under one set of chromatographic conditions, having spectral information about each component in the mixture can indicate co-elution [13,14]. Where manipulation of chromatographic conditions will not allow the separation of two or more compounds, spectral deconvolution and spectral suppression software is available which allows independent quantitative measurement of both components in that peak, if their spectra are sufficiently distinct [15].

The reference or library spectra should be acquired in the mobile phase to be used for the particular application. When using gradient elution LC methods, the spectrum may change depending on the content of the mobile phase at the time of elution from the column, particularly if a pH or ionic strength gradient is being run. Because UV–visible spectra are sensitive to the conditions of pH, ionic environment, and solvent system, generic UV–visible spectral libraries can be of limited use, and comparative spectra should always be acquired under local conditions. As proper identification should be based on the behavior of the compound in the time domain as well as the spectral domain, the generally variable interlaboratory reproducibility of LC assays lends further arguments for users to construct their own spectral/chromatographic libraries.

In toxicological applications, when method development reaches the stage of testing biological extracts, PDA detection provides the ability to examine through software, ratiograms at two or more wavelengths to ensure separation of the analyte of interest from any co-extracted material —a frequent problem when dealing with putrified post mortem material routinely encountered in post mortem forensic toxicology. In a homogeneous peak, the ratio at two carefully chosen characteristic wavelengths, should be constant throughout the peak, independent of concentration, and thus identical from run to run [16]. Performing a plot of chromatograms at two wavelengths results in a ratiogram which will show the presence of non-homogeneous peaks. These may appear with non-linear apices (Fig. 3) if the peaks

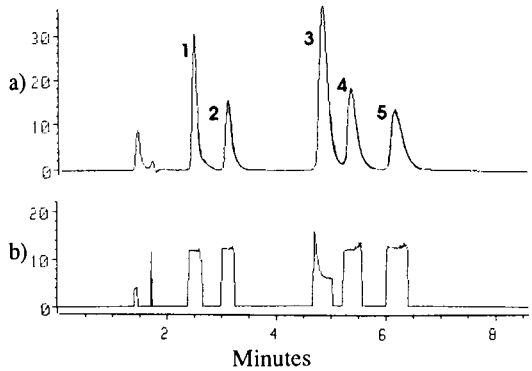

Fig. 3. Use of wavelength ratioing to indicate peak impurity. Analysis of tricyclic antidepressants and metabolites by LC-PDA on a 250-mm Lichrospher RP-8 (CH100) column. Mobile phase: acetonitrile–0.05 M phosphate buffer (pH 3) (40:60) at 2.0 ml min^{-1}. (a) Chromatogram at 252 nm: (1) 10-hydroxy nortriptyline, (2) 10-hydroxy amitriptyline, (3) protryptyline/imipramine (co-eluting peak), (4) nortriptyline, (5) amitriptyline. (b) Ratiogram 252 nm/230 nm. Asymmetric ratio of peak 3 indicates inhomogeneity and co-elution.

are not exactly coincident. Even exactly coincident peaks may appear to have a different ratio relative to an internal standard than that obtained for the known pure component.

As with selected ion monitoring in mass spectrometry, where the degree of certainty of any identification increases with the number of channels of data collected, so the certainty of peak identity increases with the number of wavelengths monitored, and with increased spectral resolution as discussed above. Similarly, the certainty of peak purity increases with the number of ratiograms constructed. It should be noted that because of the relatively broad spectral bandwidths typical in UV–visible spectra for organic compounds, monitoring wavelengths for ratiograms should be selected at well spaced but distinctive points on the spectrum, since adjacent points are usually close in relative absorbance. Ratios of immediately adjacent points on the spectrum are relatively non-specific and therefore of limited value. This is in contrast to the use of ion ratios in mass spectrometry, where the ratios of consecutive ion m/e values are not necessarily related, and ratios of immediately adjacent ion fragments can be very compound specific.

Spectral libraries and searching

Another feature common to most PDA instruments is the availability of spectral libraries and software to compare spectra generated from an unknown sample with spectra acquired under local conditions and stored in a library on disk. Various approaches for making this comparison have been used, and have been reviewed elsewhere [17,18]. Methods include generation of tables for normalized absorbance at a given wavelength for both the unknown and standard spectra, and calculation of coefficient of correlation for the two data sets (Hewlett Packard, Palo Alto, CA) [5]. Software available through another vendor (Varian, Walnut Creek, CA) adopts a different approach, calculating a constant value (purity parameter) as shown in Eqn. 1, for the spectrum in the library and comparing the constant value with that generated for an unknown spectrum.

$$\text{Purity parameter} = \frac{\Sigma A^2}{\Sigma A^2} \qquad (1)$$

The same product also determines similarity and dissimilarity of the unknown spectrum with library spectra from a comparison of the sine and cosine vectors respectively of the spectra. In practical applications, these approaches proved comparable in their ability to identify unknown spectra, although with the latter method, the precalculation of the comparison factors for the library spectra made the search routine more rapid. Other approaches to archive retrieval include generation of inverse files of key spectral features for area normalized spectra, and comparison with an unknown spectrum [17].

Regardless of which method is used for spectral comparisons however, we strongly recommend that the spectra of the unknown and the library matches be overlayed and examined for the presence of any gross inconsistencies which may not be apparent to the search/retrieval algorithm used. Several structurally related compounds may have very similar spectra, resulting in almost identical match values. It is also worth noting that spectral noise in either the unknown or reference spectrum, can lead to poor matches. Furthermore, as spectral noise can influence the degree of match, it is advisable to evaluate not

only the best match, but the top three or four matches. Library comparison is undoubtedly one of the most useful and commonly used features of PDA detectors in the forensic sciences, for the purposes of suggesting possible identities of unknown peaks, for checking peak purity, and for confirming the identities of compounds identified by other methods.

Automated method development

As noted above, determining optimum chromatographic conditions for a new assay can be a time consuming and subjective process. A variety of method development and optimization software packages are available commercially which exploit the multiwavelength data set generated by the PDA. Several of these systems are entirely automated, selecting the chromatographic conditions, performing the analysis and collecting and evaluating the data before selecting the solvent conditions for a subsequent run. These programs make use of mixture design statistical techniques such as overlapping resolution mapping and simplex search algorithms to produce a user-weighted optimized separation [19]. Most of these systems use PDA data to construct ratiograms or use full spectral comparisons to detect peak crossover or coelution during the optimization procedure. Undetected peak crossover between iterations can invalidate the optimization.

Practical considerations

As a result of the amount of data generated, PDA instruments require a computer with a disk drive. Disk space can be used most economically by collecting full spectral data only when a peak elutes (threshold values can be set on most instruments, as with computing integrators). For peak purity determinations, complete chromatograms at several wavelengths must also be collected throughout the run.

Photodiode array detection has many of the same limitations as conventional UV–visible spectroscopy, specifically, its less specific nature compared to mass spectrometry, and particulary for compounds which share major structural features. While this can be a limitation, it can be exploited in the identification of unknown spectra. Inspection of any collection of UV–visible spectra will show that many compounds within a class will share the same spectral features, for example [20] benzodiazepines, opiates and phenothiazines, and some of their metabolites each possess features which would indicate to which class they belong. In contrast to fragmentation patterns in mass spectrometry which can change appreciably following relatively minor structural modifications, UV–visible spectra display greater similarity within a class. PDA detection can therefore be a useful tool in identifying, for example, an unknown drug according to its class even when its absolute identity cannot be immediately determined. There are significant exceptions to this general rule however, since some metabolic modifications, particularly those involving changes in saturation, conjugation, and hydroxylation of delocalized systems, and major conformational changes such as ring opening, can also cause considerable changes in absorbance behavior [21].

A further limitation which applies to LC-PDA is a result of the UV–visible absorbance properties of many of the solvents typically used in normal phase LC, such as chlorinated hydrocarbons, *n*-alkanes, unsaturated hydrocarbons, and cyclic hydrocarbons, ethers, amides and esters. As these compounds can have very high absorbances in the low UV (190–250 nm), and many analytes have their only significant absorbance in this region, signal-to-noise ratios tend to be very poor and spectral comparisons can be compromised. This also prevents the use of some mobile phase additives and LC-PDA is often limited to use with reversed phase LC using solvent systems composed of buffer, water, short chain alcohols, nitriles and organic acids. Constraints on design forced by the optical layout, together with the electronics noise limitations of the instruments, typically result in a sensitivity of 2–5 times less than state-of-the-art single variable wavelength UV–visible detectors for LC [8–10]. The greater potential for stray light in reversed optics systems may also limit the linear range of the instrument, although in practice this is not a widely reported problem.

Forensic science applications

LC with PDA detection has two major strengths which make it an indispensable technique for forensic laboratories. The first is the ability to provide reliable confirmation of peak identity and purity, equivalent to selected ion monitoring in mass spectrometry. The second valuable feature is in the wealth of spectral information which is available for each peak in a chromatogram. This makes LC-PDA suitable for constructing three dimensional spectrochromatograms for profiling of complex mixtures, such as in the comparison of inks, dyes, copier toners, cosmetics, explosives, oils, hydrocarbons and pigments, all of which have been shown to have applications in the forensic sciences [1]. To date this feature has been exploited only to a limited extent [9,12].

Drug testing in biological fluids and tissues

The major forensic application for LC-PDA is in drug analysis—both of street drugs and in detecting and identifying drugs and poisons in extracts from biological fluids and tissues. Because of the increased certainty of a peak identification many LC-PDA methods have been reported which attempt to provide a systematic toxicological screen and confirmation for drugs in serum or urine samples. This application has previously been the sole domain of GC–MS.

A method for the extraction of basic drugs from urine using a cation-exchange solid phase extraction (SPE) procedure followed by LC with PDA detection [20] used a gradient elution reversed-phase system with a C-8 column (Lichrospher 100 CH-8, Merck, Darmstadt) to screen for over 100 drugs and metabolites in a 1-ml urine sample. The authors recommended that although retention times were reproducible within-day and over extended periods of time, it was important that standards be run frequently, to prevent peaks falling outside the time domain identification window of the library search routine. This could lead to misidentified peaks when the spectrum was actually available. Our subsequent experience with this method has been that the setting of a wide window (20% or 3–5 min absolute), is the optimum for a screening method, as this permits the computer library match system to tentatively identify unknown peaks according to chromophore class, which can aid with identification of compounds unknown to the library. In addition, the authors urge that laboratories adopting this method should acquire their own spectral library to ensure accurate criteria for identification under local conditions. The mobile phase composition which ran from 10 to 50% acetonitrile in 0.05 M phosphate buffer (pH 3), allowed elution of compounds of extremely disparate polarity in a single run, for example cocaine, cocaethylene and benzoylecgonine were all detected, and morphine and thioridazine have been detected in the same extract. Classes of compounds were found to elute within discrete blocks with respect to retention time, as compounds within a class are typically close in polarity. This extraction procedure has provided extracts of excellent quality from urine samples and has been used successfully with cerebrospinal fluid and vitreous humor samples. Untreated postmortem blood samples however were not compatible with the SPE procedure [22], and samples with high salt concentration can reduce recoveries for very polar compounds. The LC-PDA method was validated by comparison against an enzyme multiplied immunoassay technique (EMIT)-GC/GC–MS procedure with excellent agreement.

A standardized system for drug screening of 225 street drug and pharmaceutical samples [23] enhances identification based on retention behavior by relating it to the 1-nitroalkane scale [24], and includes a daily analysis of acid/neutral and basic drug standards to provide correction factors. This approach effectively compensates for changes in retention time with column deterioration and may improve interlaboratory comparisons, provided that identical chromatographic conditions are used. The system has also been used with simple ethyl acetate extracts from post mortem blood, although the extract quality appears poor and unsuitable for routine screening purposes with typical forensic specimens.

Microbore LC has achieved popularity with some users of LC, and has several advantages over normal-bore LC, particularly when PDA de-

tection is being used. Along with the usual reduction in mobile phase volumes, the elution volume for the peak is lower, therefore the concentration of the eluted drugs is consequently higher, improving sensitivity by a factor of 2–3. This offsets to some extent the lower sensitivity typically available from diode array detectors [25]. Similar effects can be obtained through the use of low dispersion chromatographic techniques [26]. In a method for drug screening in serum samples, microbore chromatography has been used effectively in this way [21]. The authors describe retention time information for over 350 drugs, metabolites and artifacts. Also noted is the utility of using UV–visible spectral information to classify drugs according to their chromophore class which, with some notable exceptions, can be very helpful in identifying unknown peaks. This method was used successfully with a simple dichloromethane extraction procedure from clinical serum samples for the diagnosis of drug poisoning, but was not used with autopsy material.

An isocratic LC method with PDA detection [27] was characterized for over 30 drugs and metabolites including antidepressants, antihistamines, phenothiazines and analgesics in serum. The authors make the point that the two technique approach—identification and quantitation chromatographically, and confirmation spectrophotometrically—can be sufficient to ensure absolute identification to the degree of certainty required for most clinical purposes. This makes LC-PDA an indispensable technique for clinical laboratories. The procedure described was particularly successful in identifying the class of tricyclic antidepressants, and discusses the common problems of interference and false positives with traditional single wavelength instrumentation. A reversed phase ion-pair LC-DA procedure has also been described for drug screening of serum samples in an emergency toxicology setting [18]. This report also evaluated the discriminatory efficiency of five different similarity tests in library searches and concluded that a multicomponent analysis provided the most reliable results. The application of LC-PDA to the identification of drugs in post mortem blood samples has been described, using an integrated LC-PDA system [28]. This procedure uses a dual isocratic chromatographic system approach with two different columns, and a multiple step fractionated manual liquid–liquid extraction. The authors also address the issue of co-elution of peaks and the use of peak purity determinations to flag this.

In consideration of the above methods, it should be noted that while urine is certainly a relevant forensic specimen, serum is considerably less so and some of these methods which specifically discuss serum would require considerable changes in extraction procedures to make them suitable for post mortem whole blood, tissue homogenates or other typical forensic specimens. In-line solid phase extraction by on-column injection with column switching has been described for post mortem vitreous humor and cerebrospinal fluid sample, specifically for the identification of barbiturates [29] and cocaine and benzoylecgonine [30]. Diode array detection was used to confirm the identity and purity of the drug peaks in the samples, and aided in the identification of other peaks as possible metabolites. A rapid scanning UV detector has also been used for the measurement of cocaine and metabolites in post mortem brain samples after enzymatic digestion and solid phase extraction [31], again providing the added assurance of spectral information in determining peak identity and peak purity prior to quantitation.

By making the chromatographic conditions more selective for a given drug or group of structurally related compounds, the specificity of an LC-PDA method can be further enhanced. A number of methods developed for drug assays in clinical samples have been described. The experience of these workers validates the use of LC-PDA, and is certainly relevant to forensic drug testing. Among the most common applications is the analysis of benzodiazepines and their metabolites in both serum and urine. One method [32] allows for the specific identification of more than 11 benzodiazepines in serum, and utilized library comparison and peak purity features of the available software. Another report validated the LC-PDA method against an EMIT benzodiazepine assay [33]. The sample preparation includes sample hydrolysis resulting in conversion of the ben-

zodiazepines and their metabolites to the corresponding benzophenone (13 in all are characterized here, corresponding to 15 of the most commonly encountered benzodiazepines and their metabolites). This conversion limits identification of the specific benzodiazepine present, but improves the sensitivity of the assay. These workers reported concordant results between LC-PDA and EMIT in greater than 80% of cases. LC-PDA was also used effectively for the detection, identification and quantitation of chlordiazepoxide and its metabolites in serum [34], while another method reported a sensitive and specific gradient elution LC method for the separation and identification of 19 benzodiazepines in biological fluids using a rapid scanning UV–visible detector [35]. Reported detection limits were in the 3–5 ng ml^{-1} range. The method uses an efficient solid phase extraction procedure, which appears to work well for clinical specimens, but would probably make the method unsuitable for untreated post mortem blood.

Analysis of street drug material

An LC-PDA method for the detection of manufacturing by-products and impurities in illicitly produced cocaine has been described in which dual UV detection at 215 and 277 nm was used [36]. The procedure identified benzoic acid, cinnamic acid (*cis* and *trans*) and several isomers of truxillic and truxinic acid, benzoylecgonine and cinnamoylcocaine (*cis* and *trans*). The use of complete spectral information greatly facilitated compound identification. Δ^9-tetrahydrocannabinol (THC), the active component of marijuana was identified in extracts from THC-containing gelatin capsules, using LC-PDA to evaluate peak homogeneity during the chromatographic optimization process [37]. Sub- and supercritical fluid chromatography were also successfully attempted with a PDA detector, using carbon dioxide as the primary mobile phase component. This was then applied to the separation, identification and purity determination of opiate alkaloids from a poppy straw extract [38]. LC-PDA has also been used for the identification of components, cutting agents, impurities and reaction by-products in illicit heroin samples [39]. LC-PDA methods are eminently suitable for this application, as the number of likely analytes is relatively low and they are well characterized and easily recognizable in terms of their spectral properties.

Drug testing in sports

Drug testing in sports is another area of forensic science where LC-PDA is being used extensively. Diuretics have been used by athletes to reduce body weight prior to weigh-in to allow them to qualify in lower weight categories. They have also been used to enhance the excretion of other banned performance enhancing drugs. The identification of diuretics in the urine of athletes has therefore been an important determination in sports toxicology since the International Olympic Committee (IOC) banned their use in 1988. LC-PDA has been described as a screening method for these compounds [40,41], and although the UV–visible spectra are probably sufficiently distinct on their own to allow confirmation of identity, the protocol for IOC drug testing currently requires GC–MS confirmation. The measurement of diuretics however illustrates the advantage that LC has over GC for screening for certain compounds. The polar structural features of the drugs in this class make them unsuitable for GC without prior derivatization, making screening for their presence by GC or GC–MS a more tedious procedure. The xanthine stimulants such as caffeine, theophylline and theobromine are banned in certain sports also. An LC-PDA method for the gradient elution separation and identification of eight of these compounds and some of their metabolites has been described, where identification is based on retention time and peak identity confirmed by UV–visible data [42].

Also of concern in athletic drug testing is the use of corticosteroids as performance enhancing drugs. LC-PDA has been shown to be capable of resolving and identifying spectrally 10 commonly used corticosteroids [43]. Because of the structural features shared by many of these compounds, first derivative spectroscopy was found to be more useful in discriminating between them than the spectra themselves. Methods have also been described using LC-PDA for the identifica-

tion of more than 60 anabolic and androgenic steroids in illicit drug preparations [44–46]. These compounds are used by athletes and also used illegally in animal racing and in cattle breeding and rearing, which may constitute a forensic setting. A method has been described for the analysis of these steroids in tissue from slaughtered cattle using LC-PDA [47].

The general experience of those using LC-PDA in drug testing applications which have relevance in forensic toxicology, represents an enthusiastic endorsement of the technique. Features most commonly used include multiple wavelength monitoring, library comparisons, first and second derivative spectra, peak purity determinations and peak integration features for quantitation.

Comparative analysis in the forensic sciences

The second application for LC-PDA in the forensic sciences is in profiling of complex mixtures and matching these with known standards. LC-PDA was first used in this way for the diagnosis of metabolic disorders. Urine samples were analyzed to determine their carboxylic acid profiles, and used to diagnose various inborn errors of metabolism [48–50]. The complementary relationship between LC-PDA and GC–MS in this regard has been addressed by some authors [51].

Applications relevant to forensic science include the ability to produce distinctive profiles which would allow the identification of hallucinogenic and toxic mushrooms and fungi [52–54]. The active components of *psilocybe* mushrooms, psilocybin and psilocin have been identified [9,55], and the tentative identification of the other tryptamine alkaloids made. While absolute identification of each peak has not been accomplished the pattern is representative of mushrooms of that particular family, and the additional dimension of complete UV–visible spectra for each peak allows confirmation of an identification made on the basis of a matching chromatographic pattern.

Constructing profiles from complex mixtures can assist in matching and identifying trace evidence. LC-PDA has been used to profile and differentiate between methylisothiazolone components from cosmetics [56], and plant extracts used in cosmetics [57]. A method using multiple wavelength detection has been described for the differentiation of over 100 non-ball pen inks, using a rapid scanning UV–visible detector [12]. This work illustrates the utility of three dimensional information sets in discriminating between complex mixtures of very similar composition. In addition, the use of first and second derivative spectra, was used effectively to distinguish between ink components with similar UV–visible spectra.

Identification of pesticides also occasionally forms part of forensic cases, and the chromatographic properties of 51 of the common pesticides have been compiled [58], including UV–visible absorption maxima, and retention times.

Discussion

As indicated in the work cited above, there are limitations on UV–visible spectra as generated by LC-PDA instruments, both in terms of specificity, sensitivity and inter-instrument differences. The principle of the technique however, namely the use of an independent chemical means of confirmation, places LC-PDA in a category of hybrid techniques along with GC–MS and gas chromatography with Fourier transform infrared spectroscopy (GC–FT-IR). The level of certainty required or perceived to be required by the analyst for a given application for a given analyte will dictate the acceptability of LC-PDA in place of GC–MS. LC-PDA is most likely to find its niche in circumstances where LC is currently used for screening or identification and GC–MS is not available or not considered essential, or where LC with a single wavelength UV–visible detection is currently being used as confirmation of another technique [EMIT, fluorescence polarization immunoassay (FPIA), TLC]. Quantitative chromatographic techniques are ranked in terms of their discriminating power in Table 1, although exceptions will exist on a compound by compound, and matrix type basis. Other factors in selecting a quantitative chromatographic technique include the purpose of the analysis, the cost of the instrumentation and how robust a given technique is. When these factors are taken into account, LC-PDA is an attractive option.

TABLE 1

Selectivity of quantitative chromatographic methods (decreasing) based on chromatographic resolving power and detector specificity

Gas chromatography–mass spectrometry (GC–MS)
LC–MS
GC–FT-IR
LC-PDA, specific chromatographic assay
LC-PDA, screening chromatographic assay
GC, dual column and selective detector
GC, dual column *or* selective detector
LC, UV dual wavelength
GC, flame ionization detection
LC, selective detector
LC, UV single wavelength

With diode array detection, since comprehensive UV absorbance information is available, compounds can be identified on the basis either of relative absorbance at discrete, selective wavelengths (wavelength ratioing) or by examination of the complete spectrum. The former approach requires that the analyte possesses distinctive absorbance maxima, which limits its use to a few compounds, but increases its specificity over a single wavelength determination. Full spectral comparison further allows differentiation even between similar compounds, by consideration of absorbances at all wavelengths. Minor differences between spectra can be enhanced by the comparison of 1st or 2nd derivative spectra.

UV spectra are however a function of the electronic properties of the atoms in the molecule and as such are subject to effects arising from the solvent environment of the compound. pH induced differences in UV spectra of barbiturates have long been fundamental to their identification and quantitation [59]. Similarly, the spectra of some compounds are affected by the presence of methanol or acetonitrile which are typical components of reversed phase mobile phases. The difficulty of reproducing retention times and chromatographic conditions from laboratory to laboratory has been a major factor in restricting the use of LC as a standardized method. This irreproducibility also limits the usefulness of collected spectral data bases, and the sharing of spectra between laboratories. Ideally, reference spectra should be collected under conditions identical to those under which the sample is being run.

In our experience sensitivity of spectral characteristics to pH and solvent effects does not apply to all compounds, and we have successfully identified a number of compounds (including verapamil, colchicine, temazepam, azaperone and cyclizine) for which we had no reference spectra, from tabulations in the literature. The most valuable sources for this spectral information include Clarke's Isolation and Identification of Drugs [60] and Instrumental Data for Drug Analysis [61]. It is emphasized however that literature values are no substitute for the acquisition of a spectral library under local conditions. This is not only to correct for minor variations in UV absorbance resulting from local water supply, instrument configuration, solvent grade, pH measurements etc., but also because literature tabulations are restricted by necessity to absorbance maxima, and do not generally give information regarding relative intensities of the maxima, or the presence of points of inflexion.

The introduction of diode array detection into LC has provided the ability to identify and confirm the identity of compounds eluting from the LC column, thus eliminating the doubts that often accompanied identification by retention time alone, a property which is subject to variations resulting from small changes in solvent composition, small temperature changes, injection vehicle effects and system idiosyncrasies.

As a complimentary technique to GC–MS, the most heavily exploited features of LC-PDA are undoubtedly peak purity, method development, spectral identification and spectrochromatogram construction for profiling complex mixtures. As an alternative to GC–MS, its most important application will be in the analysis of compounds with temperature labile or polar functional groups which are otherwise unsuitable for GC analysis.

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Supercritical fluid extraction of benzodiazepines in solid dosage forms

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Abstract

Several benzodiazepines were separated from the matrices of their standard dosage forms using supercritical fluid extraction. Extracts were further processed by gas chromatography–mass spectrometry and/or Fourier transform infrared spectrometry. Extracts of suitable purity and quantity for mass spectral analysis were easily obtainable from even the lowest concentration dosage forms of the drugs tested.

Keywords: Gas chromatography–mass spectrometry; Infrared spectrometry; Benzodiazepines; Supercritical fluid extraction

Supercritical fluid extraction (SFE) has been used in industry to separate oils from soybeans and various compounds from other plants [1–3]. SFE similarly has been used to extract drugs from plants [4,5]. While supercritical fluid chromatography (SFC) has been used in the preliminary identification of drugs of abuse [6–8] including several benzodiazepines [9], the field of forensic science has been slow to adapt SFE for use in extracting drugs from their matrices.

Due to the short time which SFE instrumentation has been available, there exist some problems which narrow the range of use. Because so few methods exist for SFE, method development is required. The field of forensic science, always inundated with actual case work, has little time to devote to the development of new techniques. This factor could explain the reason it is slow to adopt SFE for the extraction of drugs from their matrices, both pharmaceutical and biological.

SFE has several desirable traits when compared to solvent extractions [10,11]. It requires no more and often less time than conventional solvent extractions. After purchase of the apparatus, the costs per extraction are less in terms of commodities and labor expenses [12].

In a conventional extraction of a pharmaceutical from its solid dosage form, the sample must be dissolved, pH adjusted, and extracted with an organic solvent. Some methods require that the pH of the aqueous phase be readjusted and re-extracted with more solvent. This procedure requires approximately 15 min, with more needed if an emulsion forms. It will use at least 10 ml of solvent with some procedures using many times more. The eluting solvent must then be evaporated before any further analysis can take place. The solvents normally employed for drugs of abuse are diethyl ether, hexane, chloroform and

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methylene chloride. These are all flammable and/or potentially hazardous to the health of the scientist. Since SFE does not use chlorinated solvents, it can decrease the hazards posed to analysts and aid in the reduction of environmentally hazardous laboratory wastes.

SFE utilizes a compound maintained above its critical temperature (C_t), which is high above its boiling point, and critical pressure (C_p). Under these conditions, the fluid exists in a state neither liquid nor gas, but with some of the most chromatographically useful properties of both. The properties of a supercritical fluid are a combination of those of liquids and gases. They are less viscous than liquids, yet have a density of 100–1000 times that of a gas, being much closer to a liquid state. The solvating properties and solvent power of the fluid are greater than a gas. The diffusion coefficient of the supercritical fluid is nearly 1000 times less than that of a gas, but 10 times greater than that of a liquid, allowing for greater efficiency of extraction [13].

The most frequently used solvent in SFE is supercritical carbon dioxide. Figure 1, the phase diagram for carbon dioxide (CO_2), shows the relationship of the supercritical fluid to the solid,

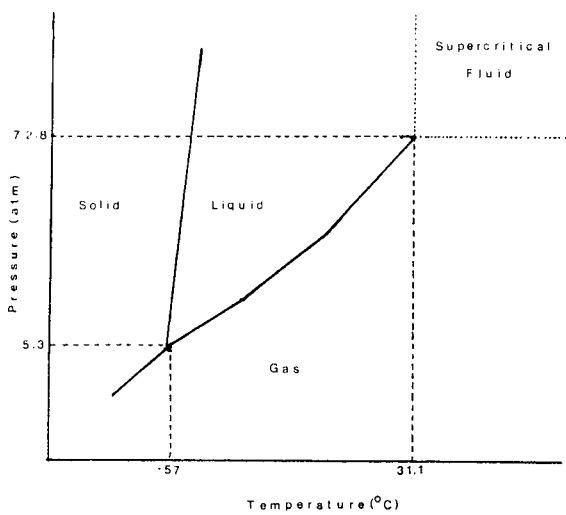

Fig. 1. Phase diagram of carbon dioxide including supercritical fluid region.

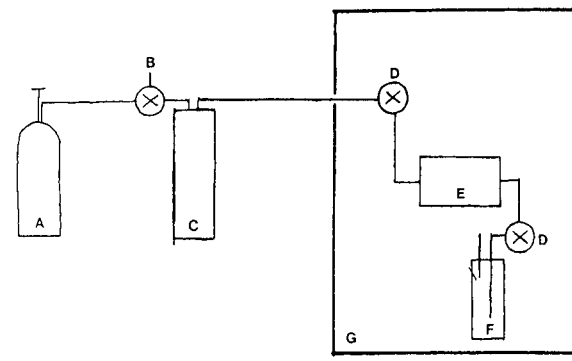

Fig. 2. Flow diagram of supercritical fluid extraction. A = Solvent (CO_2); B = solvent modifier valve; C = syringe pump; D = switching valve; E = extraction vessel; F = collection vessel; G = oven.

liquid and gas phases. The section in the upper right quadrant of the diagram is the supercritical region. Carbon dioxide has low C_t (31.1°C) and C_p (72.8 atm) values. It is not hazardous to the environment and is safer than the common solvents used by analysts in classical extraction procedures. Mixtures of solvents in the supercritical state allow the analyst greater selectivity than conventional extraction techniques. Although CO_2 is non-polar with a dipole moment of zero, when used in SFE, its polarity can be manipulated by adding a small amount of an entraining solvent, such as methanol [14].

The many substituted benzodiazepines are used as anxiolytics, sedative hypnotics, muscle relaxants and anticonvulsants. The newer drugs of this class are of increased potency resulting in a lower concentration of drug in their dosage forms. This has hampered the analysis of these often abused drugs. SFE using supercritical carbon dioxide as the solvent separates benzodiazepines from the matrix of their solid dosage forms, and concentrates the active component allowing maximum utilization of the limited amount of drug present. This method of extraction gives the forensic scientist the flexibility to use either gas chromatography–mass spectrometry (GC–MS) or Fourier transform infrared spectrometry (FT-IR) as a confirmatory test.

EXPERIMENTAL

Instrumentation

Supercritical fluid extractor. A Suprex SFE 50 supercritical fluid extractor (Suprex, Pittsburgh, PA) was used to extract the drugs from both tablets and capsules.

GC–MS. A Hewlett-Packard (Hewlett-Packard, Avondale, PA) Model 5890 Series II gas chromatograph coupled to a Model 5970 mass selective detector with a HP 59940 MS Chemstation as the data system was used for the mass spectral analysis. The gas chromatographic conditions were as follows. Column: 25 m × 0.2 mm i.d., HP-1 with a 0.33-μm film thickness. Injector temperature: 240°C. Oven temperature: 270°C for 1 min followed by a ramp of 30°C min^{-1} to a final temperature of 290°C, and held there for 15 min. Carrier gas: helium at 10 p.s.i. (70 kPa). Injection: split 10:1.

FI-IR

A Nicolet (Nicolet Instruments, Madison, WI) 5DX Fourier transform infrared spectrophotometer with a NEC PC-8853N data system was used for the infrared analysis. The sample was scanned at one scan per second for 10 s from 3800 to 350 cm^{-1}.

Reagents

The extraction solvent was supercritical fluid grade carbon dioxide (Scott Specialty Gases, Plumstead, PA). This was modified by the addition of 2% (v/v) HPLC grade methanol (Baxter

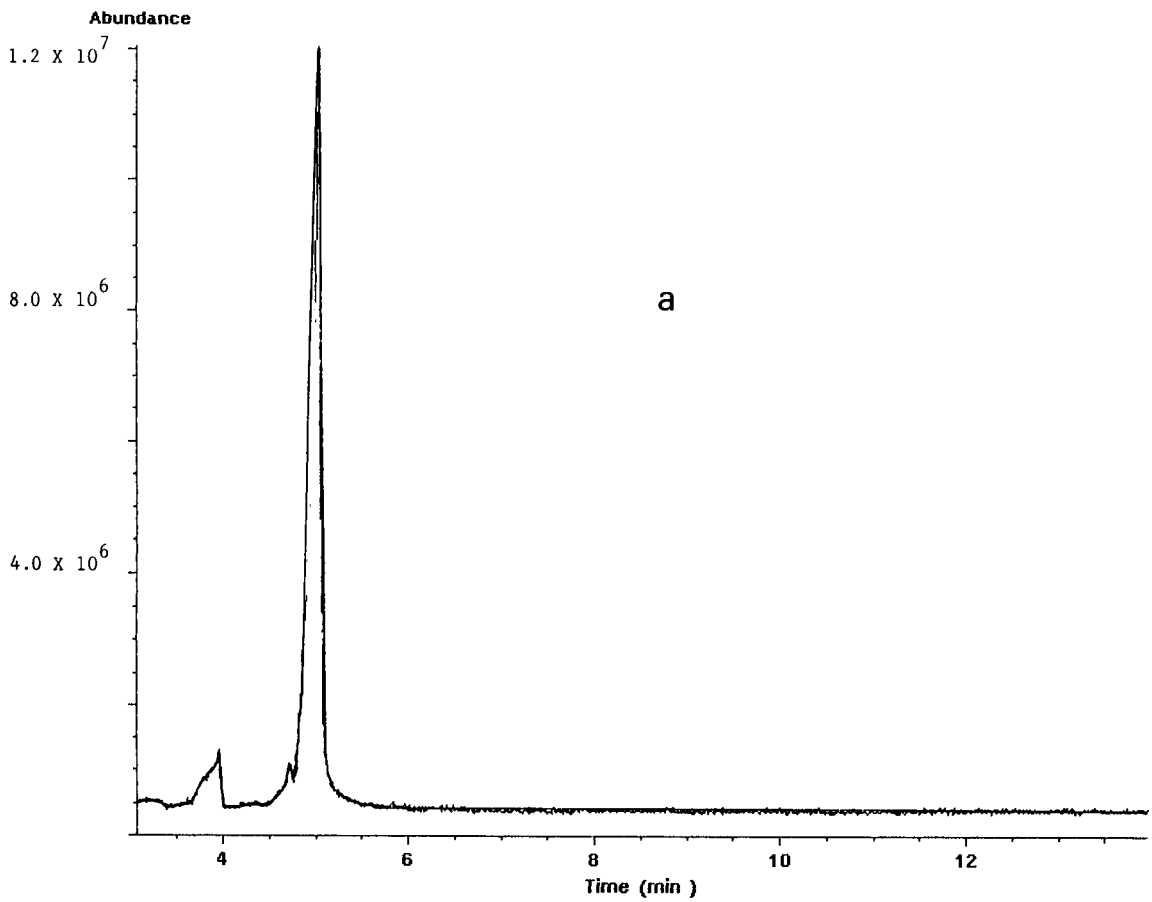

Fig. 3. (a) Total ion chromatogram of the SFE eluent of a Librium tablet.

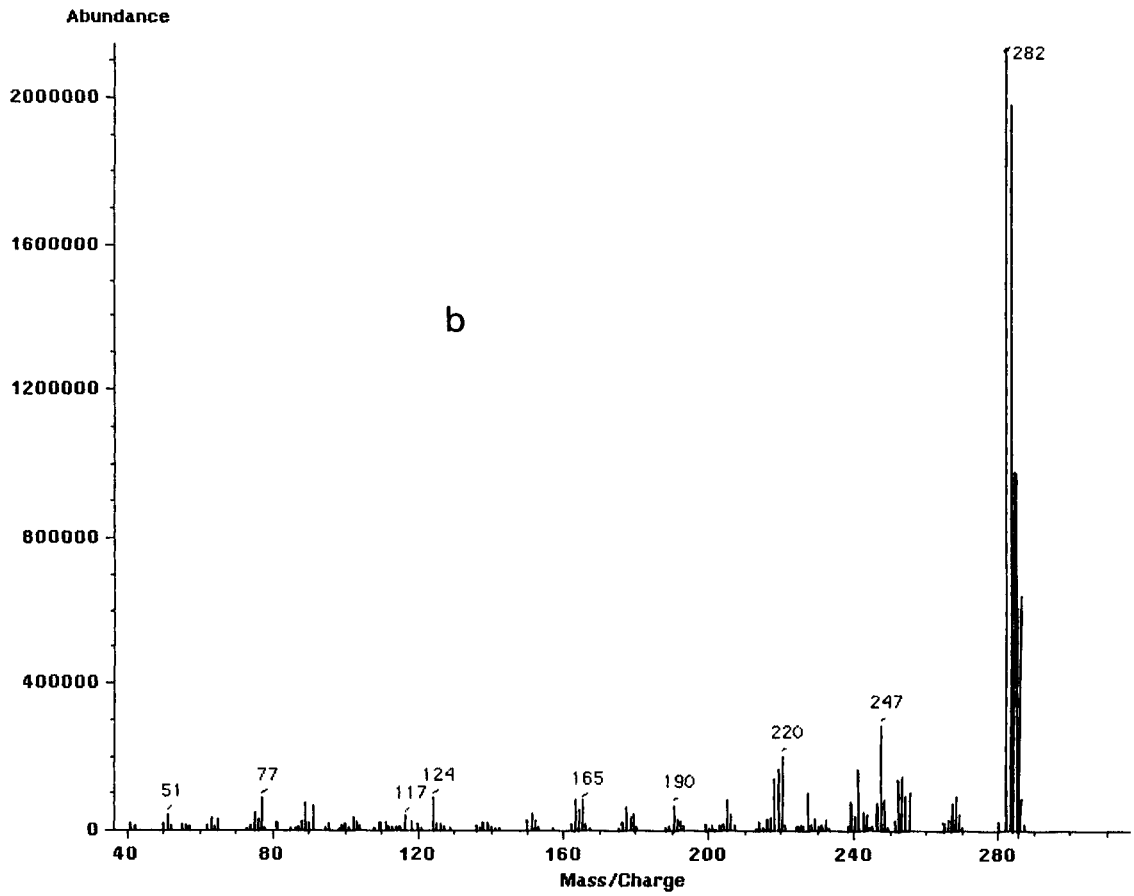

Fig. 3. (b) Mass spectrum of diazepam from extraction.

Healthcare, McGaw Park, IL). During mass spectral analysis, this same methanol was used to dissolve the extracted drug for injection into the GC–MS system. The extraction vessel or cell of the SFE system was filled with Hydromatrix, a solid support (Analytichem, Harbor City, CA). Infrared grade potassium bromide (Mallinckrodt, St. Louis, MO) was used as the support matrix during infrared analysis.

Method

The contents of each capsule or tablet were ground using a mortar and pestle. Approximately one quarter of each sample was used in an extraction. The portion used was carefully dispersed throughout a premeasured amount of the hydromatrix (450 mg). The extraction cell was placed in the Suprex SFE-50 supercritical fluid extractor. A diagrammatic representation of a typical SFE unit is shown in Fig. 2. The oven was used isothermally at 65°C. The extraction program was 15 min long. The pressure was 100 atm. The extractor was held 5 min in the static mode and switched for 10 min to the dynamic extraction mode. The eluent was collected in a 10×75 mm glass tube containing 500 μl of methanol. The methanol was allowed to evaporate before subsequent analysis. The dried sample was reconstituted with 50 μl of methanol. A 1-μl volume was injected onto the GC–MS system. Total ion

TABLE 1

Dosage forms, manufacturers and instrumental analysis performed on drugs tested

Benzodiazepine	Source	FT-IR	GC–MS
Alprazolam	Upjohn (Xanax, 0.5 mg)	No	Yes
Clorazepate	Abbott (Tranxene, 7.5 mg)	No	Yes
Chlordiazepoxide	Roche (Librium, 5 mg)	No	Yes
Diazepam	Roche (Valium, 5 mg)	Yes	Yes
Oxazepam	Wyeth (Serax, 30 mg)	Yes	Yes
Prazepam	Parke-Davis (Centrax, 30 mg)	No	Yes
Temazepam	Sandoz (Restoril, 30 mg)	Yes	Yes
Triazolam	Upjohn (Halcion, 0.125 mg)	No	Yes

chromatograms and mass spectra were evaluated for purity and apparent relative concentration of the drug. Those samples identified as pure enough and with sufficient amount available were analyzed by FT-IR. All spectral data, both MS and IR, were compared to laboratory-generated libraries of standards.

RESULTS AND DISCUSSION

Table 1 is a compilation of the results of the analyses. All dosage forms tested were suitable for analysis by GC–MS. Figures 3a (chlordiazepoxide) and 4a (diazepam) are total ion chromatograms in which can be observed only one peak for the drug of interest. The resulting mass spectra for these compounds can be seen in Figs. 3b and 4b, respectively. Infrared data were collected on only four of the drugs. A representative

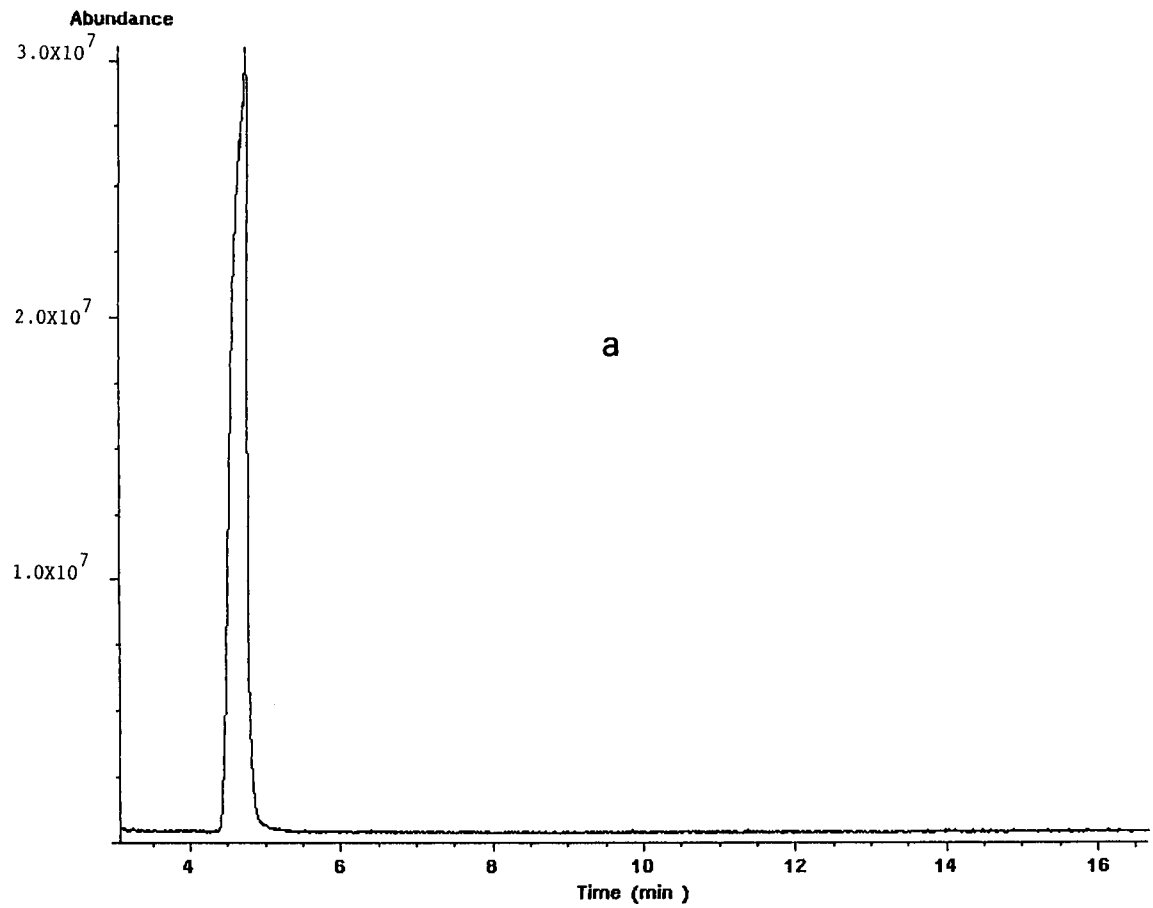

Fig. 4. (a) Total ion chromatogram of the SFE eluent of a Valium capsule.

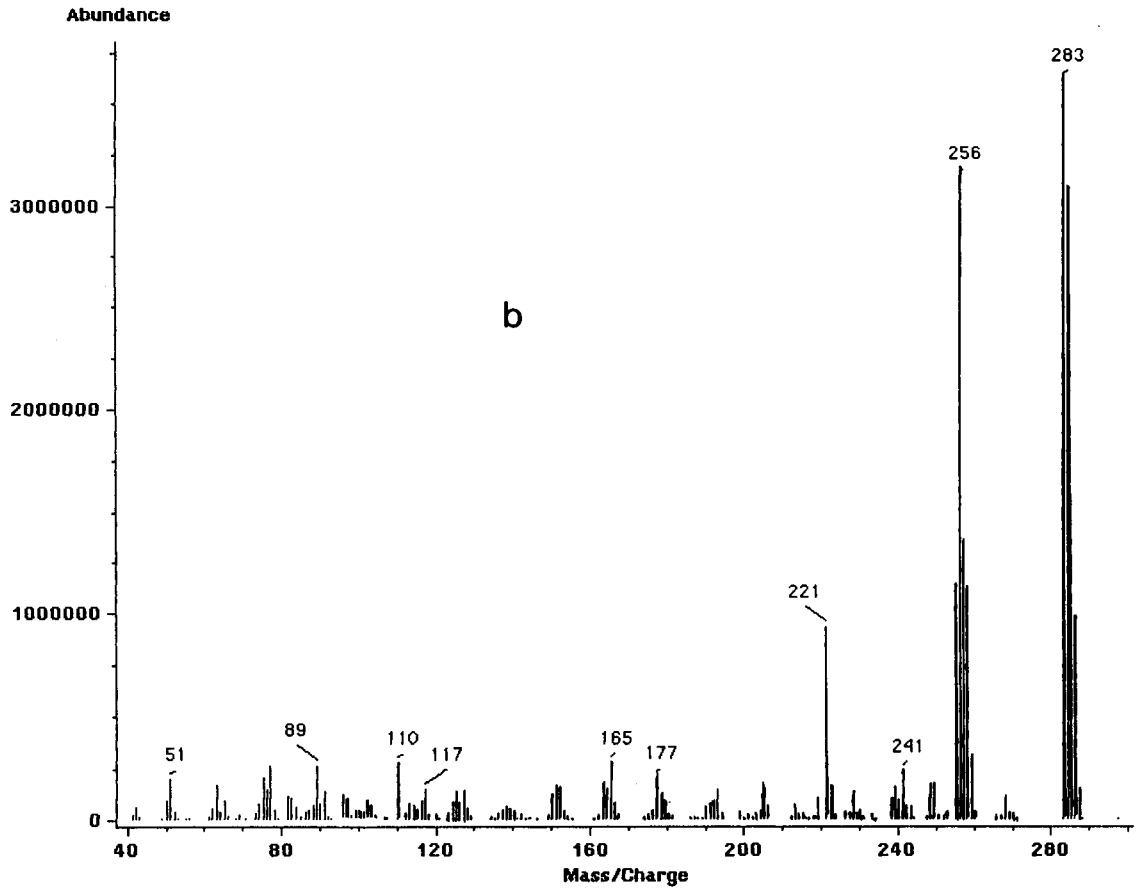

Fig. 4. (b) Mass spectrum of chlordiazepoxide from extraction.

spectrum obtained from diazepam is presented in Fig. 5. Only those drugs in dosage units over 1 mg per tablet produced enough purified drug for FT-IR analysis. Prazepam was not subjected to FT-IR since this compound is only used in this laboratory as an internal standard for quantitation. The last drug without an IR spectrum was clorazepate. This extract was analyzed by FT-IR, but the spectrum could not be matched to any available in house or published data. Although the GC–MS total ion chromatogram showed no other contaminants, and the mass spectrum matched the standard, we were unable to obtain a representative IR spectrum. Further work, including nuclear magnetic resonance (NMR) spectrometry, is planned to determine the cause of this discrepancy.

Care had to be taken during the extraction process that all tablet and capsule contents were finely ground and well dispersed throughout the matrix in the extraction vessel. If not well mixed with the matrix, the extraction procedure molded the powder into a hard mass and decreased the recovery of active component. The reason for this is yet unknown, and under further evaluation.

Supercritical fluid extractions of a wide range of benzodiazepines were successfully accomplished. The drugs which were analyzed represented parent drugs and metabolites. Oxazepam, a metabolite of several other compounds, was easily extracted. In order to determine how well the drugs were separated from their matrices by SFE, GC–MS analyses were performed. Utilizing the separation properties of GC–MS, the purity

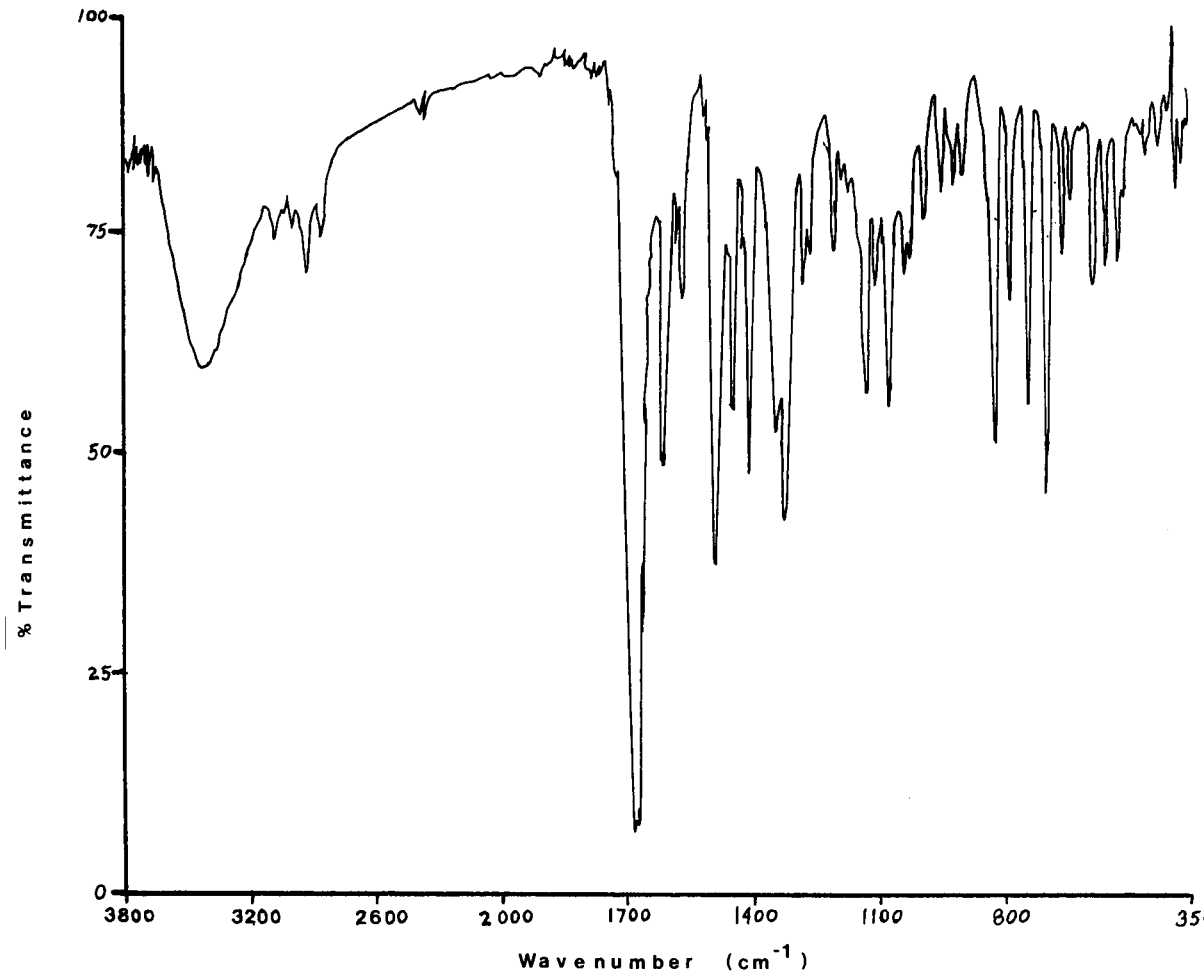

Fig. 5. FT-IR spectrum of diazepam from extract.

of each drug was determined. No matrix components or contaminants were identified in the mass spectral data. While FT-IR analysis was performed only on the more concentrated capsules or tablets, these analyses also confirmed the high purity of the drugs following SFE.

The use of SFE for extraction of benzodiazepines from their solid dosage forms has no advantage over the commonly used technique of methanol dissolution and direct injection on the GC–MS. However, the development of this novel extraction procedure for benzodiazepines from a simple matrix has led to further research testing the ability of SFE to remove these drugs and metabolites from biological matrices of forensic interest, such as blood and urine. The presentation of this preliminary work on SFE is intended to introduce this new extraction technique to the forensic science community and encourage further research into the uses of supercritical fluid extractions.

We would like to acknowledge the Illinois State Police, Bureau of Forensic Sciences, for their cooperation during this research.

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The Chemistry of Artificial Lighting Devices

Lamps, Phosphors and Cathode Ray Tubes

By **R.C. Ropp**

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Both the early use of artificial lighting and current manufacturing methods concerning incandescent and fluorescent lamps are covered in this book. The protocols for manufacture of fluorescent lamp phosphors and those used in cathode ray tubes are also treated in some detail. This text surveys the amazing, vast array of artificial lighting devices known to date in terms of how they arose and are, or have been used by mankind.

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