Recent Applications of LC–MS in Forensic Science

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Introduction

The term "forensic science" covers those professions that are involved in the application of the social and physical sciences to the criminal justice system.

Forensic experts are obliged to explain the smallest details of the methods used, to substantiate the choice of the applied technique and to give their unbiased conclusions. The final result of the work of the forensic scientist, the expert evidence, exerts a direct influence on the fate of a given individual. This burden is a most important stimulus and one that determines the way of thinking and acting in forensic sciences. Consequently, the methods applied in forensic laboratories should assure a very high level of reliability and must be subjected to extensive quality assurance and rigid quality control programmes.[1]

Legal systems are based on the belief that the legal process results in justice — a belief that has come under some question in recent years. Of course, the forensic scientist cannot change scepticism and mistrust single-handedly. He or she can, however, contribute to restoring faith in the judicial processes by using science and technology in the search for facts in civil, criminal and regulatory matters.

The ability of mass spectrometry (MS) to extract chemical fingerprints from microscopic levels of analyte is invaluable in this quest, enabling the legally defensible identification and quantification of a wide range of compounds.

Recent years have seen the development of powerful technologies that have provided forensic scientists with new analytical capabilities, which were unimaginable only a few years ago. Gas chromatography (GC)–MS, liquid chromatography (LC)–MS, isotope ratio MS and inductively coupled plasma-MS have become routine tools to enable detection and characterization of minute quantities in what can often be very complex matrices.

In LC–MS, there has been an explosion in the range of new products available for solving many analytical problems, particularly those applications in which non-volatile, labile and/or high molecular weight compounds are being analysed. Many analysts and laboratories have reached the point at which they are considering the acquisition of LC–MS instrumentation.

According to Willoughby et al. LC–MS has progressed from the "innovators" stage through the "early adaptors" and on to the "early majority" stage, and is now open to specialists from a variety of disciplines. This has been as a direct result of the introduction of robust, user-friendly atmospheric pressure ionization (API)-MS instruments at an affordable price.[2]

Toxicology

Drugs of abuse: Traditionally, laboratories use GC–MS for the confirmation of illicit drug use. However, this is a time-consuming and labour-intensive procedure, particularly as sample preparation; that is, solid-phase extraction (SPE) and derivatization are usually unavoidable. LC–MS may well be the solution to the identification and quantification problems often encountered by analytical toxicologists because it permits the confirmation analysis of polar or non-volatile compounds without the need for derivatization.

In comparison to single quadrupole methods, tandem mass spectrometry (MS–MS) offers superior sensitivity and selectivity for the species of interest. These instruments, when operated in multiple reaction monitoring (MRM) mode, enable the quantification of low levels of compounds in biological matrices, often with much reduced sample preparation and analysis times. Nowadays, stable isotopically labelled analogues of many drugs of interest are readily available. These prove to be valuable tools for the purpose of internal standardization and quantification. Additionally, because these standards have almost identical physicochemical properties to the unlabelled drug, they will compensate for any effects of reduced sample preparation or ion suppression produced by the matrix.[3]

To date, LC–MS (and MS–MS) methods have been described for most of the main drug classes including opiates and synthetic opioids, amphetamines, cocaine and metabolites, cannabinoids, hallucinogens and benzodiazepine derivatives.[4]

Compounds from these classes can be determined using a simpler sample preparation (no hydrolysis, one-step extraction, no derivatization) and with a sensitivity, at least that of GC–MS.[5]

Furthermore, LC–MS now makes the analysis of polar metabolites of illicit compounds possible. What was previously a difficult exercise by GC–MS, this additional metabolite data now allows a more complete interpretation of the analytical results. Recently, Bogusz and co-workers described the simultaneous analysis of diacetylmorphine, morphine and 6-acetylcodeine and their respective metabolites (i.e., monoacetylmorphine, morphine glucuronides, codeine and codeine-6-glucuronide).[6] This method enabled the differentiation of prescribed and non-prescription heroin.

Moreover, in some situations the collection and subsequent analysis of biological specimens may take place several hours (or days) after drug administration. Often by this stage, only the metabolites are detectable.

For the detection of illicit drugs, plasma and urine are currently the most common matrices investigated. However, over the last few years there has been an increasing interest in the use of more convenient, less invasive alternative specimens such as oral fluid. Use of this sample, in particular, is an attractive possibility because collection is simple, rapid and requires no special equipment or facilities. Furthermore, the procedure can be supervised, thus reducing the opportunity for sample adulteration. However, since the volume of this biological specimen is limited, a highly sensitive analytical procedure is required. Indeed, analysis by GC–MS can be problematic for this very reason. In some instances, the volume of oral fluid may be even further reduced; for example, frequent users of amphetamines can suffer from a dry mouth because of the sympathomimetic effects of the drugs. In addition, the inherent viscosity of oral fluid can lead to problems during the unavoidable SPE procedure.

Wood et al. reported a validated LC–MS–MS method that enables the simultaneous quantification of MDMA (± 3,4-methylenedioxymethamphetamine, 'Ecstasy'), MDA, MDEA (3,4-methylenedioxethylamphetamine, EVE), amphetamine, methamphetamine and ephedrine in plasma or oral fluid. The procedure requires only 50 µL of plasma or oral fluid to achieve limits of detection of 2 µg/L or better, and comprises simple and rapid sample preparation (i.e., methanol clean-up) followed by LC–MS–MS analysis (Figure 1).[3]

It seems likely that, as a result of the high sensitivity afforded by some LC–MS–MS procedures, the routine use of more convenient, less-invasive, alternative specimens for the confirmation of drug abuse, will increase in the future.

Doping: At this particular moment in time, the International Olympic Committee (IOC) still relies on capillary GC–MS and/or high-resolution mass spectrometry for confirmation of positive findings. The IOC lists more than 150 illegal substances that chemists must try to identify from blood and urine, and perhaps, in the future, from alternative specimens. Chief among these are anabolic agents, such as steroids, which increase muscle mass and strength, and peptide hormones, such as erythropoietin (EPO), which raise the level of oxygen-carrying red blood cells. The IOC also bans the use of stimulants such as amphetamines, analgesics for pain relief, and urine-manipulating agents used to mask illegal substances.[7]

The use and abuse of anabolic steroids is a growing problem. Current detection techniques suffer from an extensive sample pretreatment and thus from low sample throughput.

Those fighting drug abuse are faced with a number of problems ranging from the simple lack of suitable reference materials to the need to improve the instrumentation used. Therefore, the European Commission started the *SGLC/MS Project*: (Steroid glucuronides; development of liquid chromatography–mass spectrometric analysis).[8] This project involves the development of new test methods that will allow modern drug testing techniques to be more widely and effectively used. The preparation of suitable reference compounds is likely to play a vital role in this process. Once perfected, these synthesis methods could be further scaled-up so that reference standards can be manufactured to meet the needs of modern forensic and clinical analysis methods.

LC–MS may well be the solution to the identification and quantification problems often encountered by analytical toxicologists because it permits the confirmation analysis of polar or non-volatile compounds without the need for derivatization.

The project participants should be in a good position to evaluate the suitability of their synthesis methods and LC–MS techniques when the next Olympic Games comes around in Athens in 2004.

In the near future it is expected that, for doping control, there is likely to be an increase in the development and validation of LC–MS methods.

Screening: Before establishing LC–MS screening procedures in routine work, several limitations should be kept in mind. The spectral data of electrospray ionization (ESI) and/or atmospheric pressure chemical ionization (APCI) are limited in contrast with GC–MS electron impact mass spectra, and the reproducibility depends on the apparatus. Another problem for ESI is the reduction in compound ionization (ion suppression) when other compounds (e.g., matrix) co-elute. In such instances a relevant toxicant could be overlooked. In the opinion and experience of Maurer et al., analytes that are volatile in GC should be screened for by GC–MS. However, they state that LC–MS is an excellent method for screening, library-assisted identification, and quantification of low-dosed and/or rather polar compounds, especially in plasma.[9]

Decaestecker et al. presented an LC–MS strategy for systematic toxicological analysis (STA). The investigators employed a quadrupole time-of-flight (Q-TOF) instrument and used the ability to automatically switch from MS to MS–MS whilst 'on the fly'. During the chromatographic run, the quadrupole is initially set to transmit all masses until an ion(s) reaches a certain set threshold. Thereupon, the instrument automatically switches to MS–MS mode, selecting the ion(s), which are subsequently fragmented in the high-efficiency hexapole collision cell, thus generating product ions that are further mass analysed by the TOF. By limiting the TOF spectral accumulation time in MS–MS mode to a statistically acceptable minimum, the quadrupole almost instantly switches back to MS mode. Qualitative information, comprising the complementary MS ($[M + H]^+$ (precursor ion)

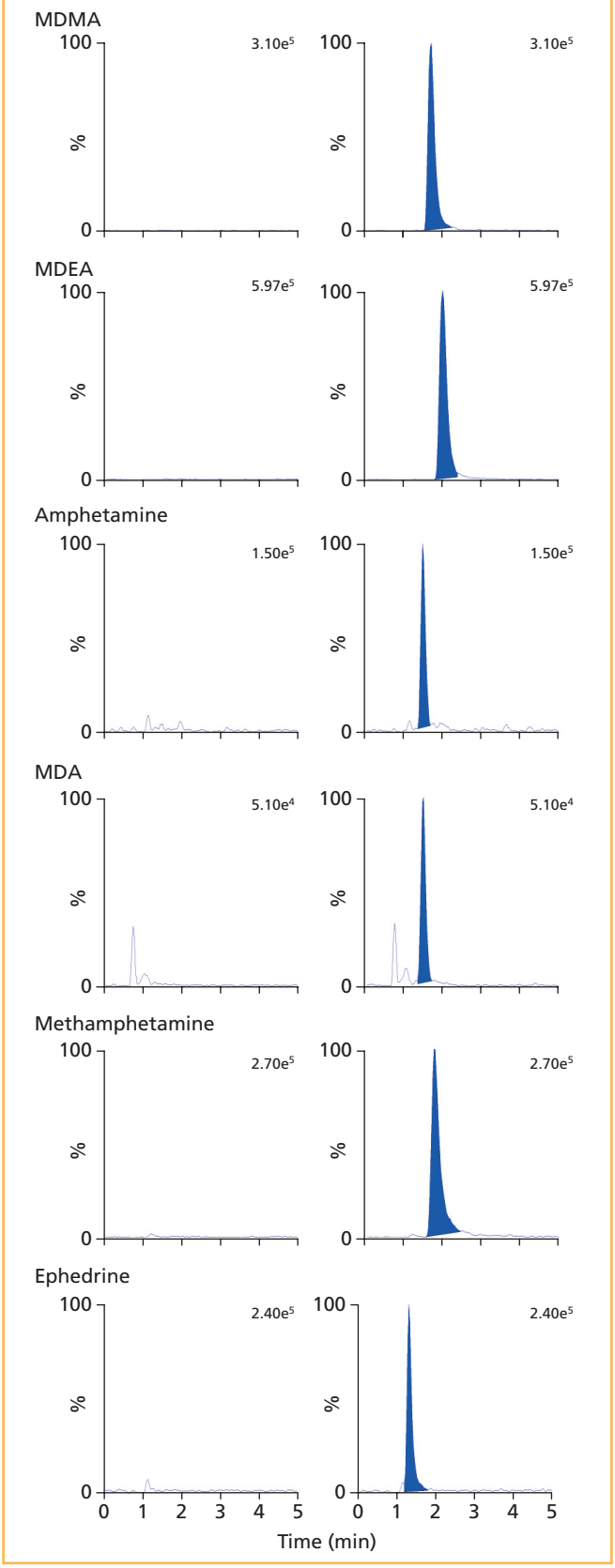


Figure 1: MRM chromatograms obtained with a single injection of blank oral fluid and amphetamine-enriched oral fluid with 5 µg/L of the various amphetamines. Peak intensity is shown in the top right-hand corner of each trace.

and MS–MS (informative product ion profile) data, as well as quantitative information obtained by integration of the MS extracted ion chromatogram(s), can be obtained in one single acquisition. The complete separation of drugs is not necessary because up to eight different ions can be 'simultaneously' selected for MS–MS if they reach the preset criteria. This innovative approach clearly has the potential for a substantial advance in the introduction of LC–MS in STA (Figure 2).[10]

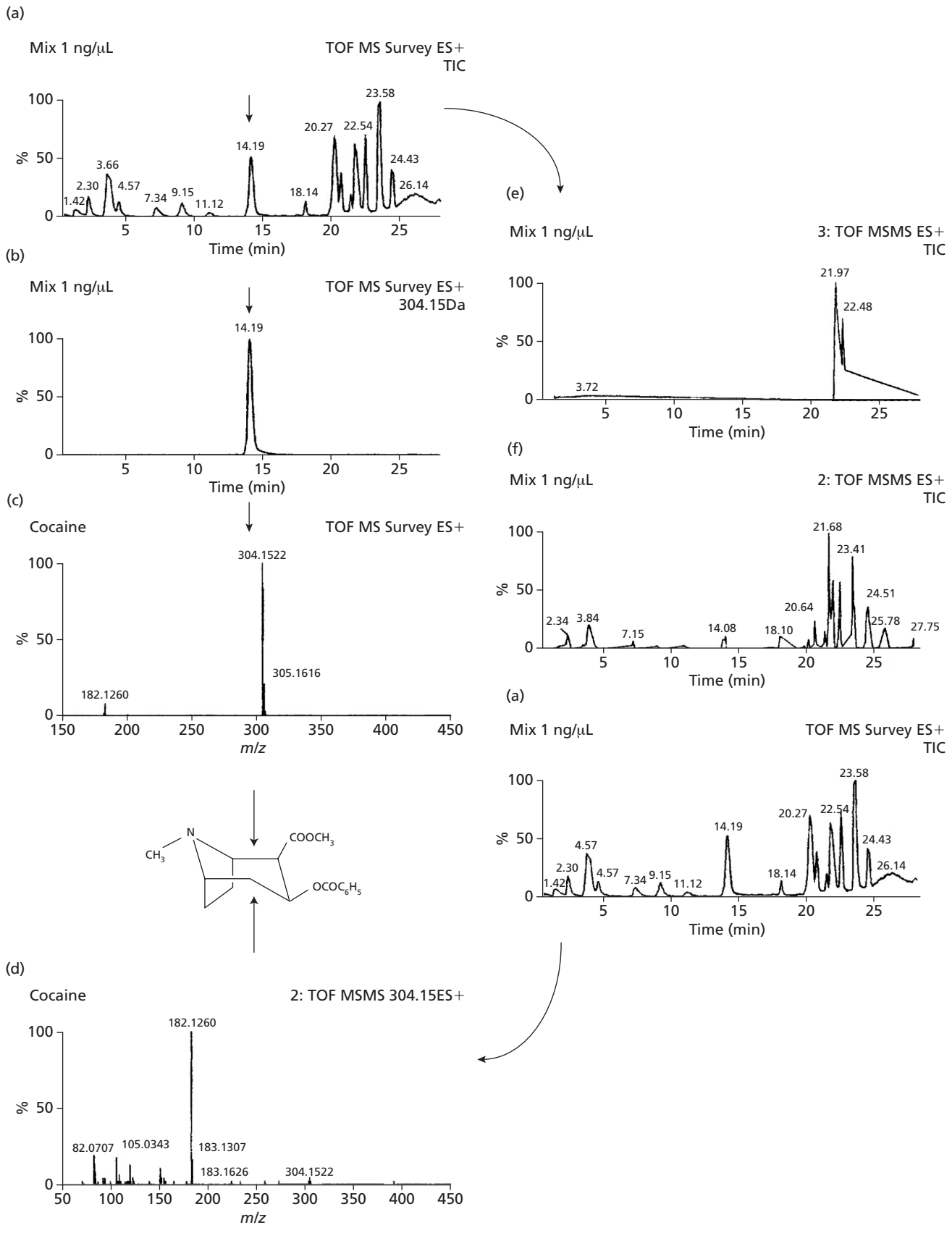
High throughput: Generally, it is the speed of the LC separation that is the main rate-limiting factor for high-throughput bioanalyses. However, the increased use of 96-well plates and robotic liquid handling systems, the availability of robust triple quadrupole mass spectrometers, and developments in chromatographic and sample preparation techniques, have all increased the rate at which this data can be generated.

The demand for ultrafast LC–MS–MS analysis might be met by using short columns packed with small particle sizes, which are stable over a wide range of pH values. Yung-Fong Cheng et al. have outlined a simple and comprehensive LC–MS–MS strategy for the rapid analysis of a wide range of pharmaceutical compound types. This approach uses a combination of chromatographic techniques plus detection by MS–MS to simultaneously obtain data on acidic, neutral and basic pharmaceutical compounds.[11]

Deng Y et al. describe a high-throughput, high-performance bioanalytical system capable of extracting and analysing 1152 plasma samples in 10 hours. A track robot system interfaced with a liquid handler was used for simultaneous solid-phase extraction of four 96-well plates in a fully automated fashion. The extracted plasma samples were injected onto four parallel monolithic columns for separation via a four-injector autosampler. The effluent from the four columns was directed to a triple quadrupole mass spectrometer equipped with an indexed four-probe ESI source (Micromass MUX™ interface). Hence, sample extraction, separation and detection were all performed in a four-channel parallel format that resulted in an overall throughput of approximately 30 s/sample from plasma.[12]

Entomotoxicology: Entomotoxicology is the study of drugs in insects and is a relatively new branch of forensic entomology.[13] The use of insect larvae to aid in the establishment of the post-mortem interval is a well-recognized procedure in the performance of medico-legal autopsies. In addition, insects may serve as reliable alternative specimens for toxicological analyses in the absence of sufficient tissues and fluids normally taken for such purpose. Currently, scientists from the departments of Anatomo-pathology from the Academic Hospital of the Free University of Brussels, Entomology of the Royal Belgian Institute of Natural Sciences and the Toxicology and Microtraces sections of the National Institute of Criminalistics in Belgium are working together in the field of forensic entomology. This is an excellent example of the multidisciplinary character of forensic sciences. In these investigations, LC–MS–MS has been used to detect Nordiazepam and its metabolite Oxazepam in the larvae of *Calliphora vicina* (Diptera, Calliphoridae). Flies and larvae were from a stock colony of *Calliphora vicina* maintained in an environmental chamber. They were reared on artificial foodstuff spiked with a range of concentrations of Nordiazepam. The concentrations were equivalent to those expected in skeletal muscle following fatal human overdoses. Only one larva was needed to prove positive drug

Figure 2: (a) TIC in the MS mode, (b) MS extracted ion chromatogram of cocaine, (c) MS and (d) MS–MS spectra of cocaine, (e,f) TICs of the first two channels in the MS–MS mode.



concentrations (Figure 3). Further investigations are required to determine the metabolism of other benzodiazepines (e.g.,Valium and Rohypnol) in the *Calliphora vicina* larvae and the effect of toxic compounds on larvae development.[14]

Biological and Chemical Agents

NBC (Nuclear, Biological and Chemical) agents can be obtained from many sources including home "breweries", laboratories or other commercial sources, or stolen from industrial sites.

Mass spectrometry cannot be used in cases of nuclear threats, but it can be used as a detection system when chemical and biological substances are involved.

On March 20, 1995, terrorism changed. For the first time, terrorists used a chemical warfare agent against a civilian population. The nerve agent sarin (GB) was released into the Tokyo subway system causing over 5500 people to seek medical attention. Nerve agents are extremely potent organophosphorus compounds that cause biological effects by inhibiting the enzyme acetylcholinesterase. The more widely known nerve agents are tabun (GA), sarin (GB), soman (GD), GF and VX.

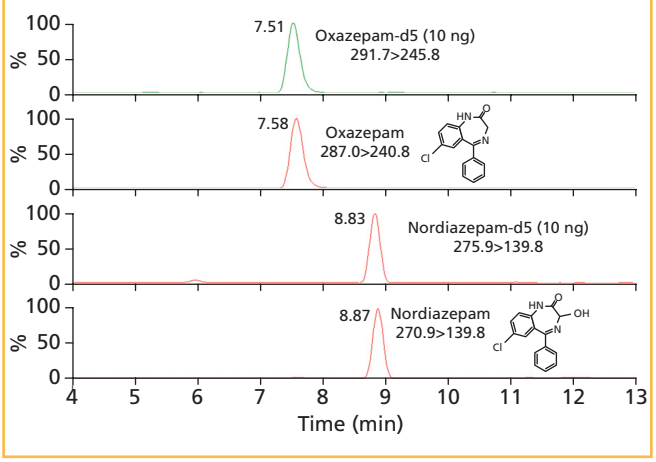
Organophosphorus chemical warfare agents have been studied extensively by electron impact and chemical ionization MS. The use of these complementary ionization techniques facilitates the acquisition of molecular and fragmentation ion information, that may be used for unambiguous identification. Increasingly, researchers have developed LC–MS separation methods to deal with the analysis of aqueous samples containing these non-volatile products. Benefits over GC analysis include reduced or no sample handling, and no requirement for derivatization to increase the volatility of the hydrolysis products. The first application of packed capillary column LC–ESI-MS for the characterization of organophosphorus chemical warfare agents was presented by D'Agostino et al.[15]

The same group has analysed snow contaminated with sarin (isopropyl methylphosphonofluoridate). ESI-MS data were acquired using a TOF mass spectrometer. A sample of the snow from near a split 105 mm chemical shell was collected for laboratory analysis to confirm the identity of the chemical warfare agent(s). Sarin, its hydrolysis products and several previously uncharacterized related compounds were identified on the basis of LC–ESI-MS data. Full mass spectra were acquired for 14 compounds detected during LC–ESI-MS analysis.[16]

Terrorist organizations have also shown a strong interest in the use of biological weapons because these are inexpensive to produce, difficult to monitor, and can produce illness and death in large numbers of people. Inhaling 40 000 spores of anthrax (enough to fit on the head of a pin) would result in a death rate of 95 per cent.

Besides the fast screening of bacteria and viruses, the identification of protein toxins has become of utmost importance. Using protein biomarkers all three classes of Agents of Biological Origin (ABO classes) can be detected, meanwhile DNA based techniques are limited to the detection of bacteria and viruses only. An LC–MS technique has been developed using a capillary/nano LC system that allows for the identification of all three types of ABO classes (bacteria, viruses and protein toxins) based on protein/peptide identification. The bacterial samples are initially lysed by ultrasonification, to release cell proteins, prior to filtration of the proteins and subsequent injection onto the multidimensional capillary LC system. The latter comprises an ion-exchange column (for concentration of proteins and removal of non-ionic detergents), an immobilized enzyme column for on-line digestion of proteins, a reversed-phase (RP) precolumn for concentration/desalting of peptides and a nano RP separation column for peptide mapping. The protein biomarkers present in the bacterial lysates are selectively detected by an ion-trap mass spectrometer. Identification of the bacteria is based on MS–MS sequence data from the proteolytic peptides. On-line digestion of the protein biomarkers has been optimized. At elevated temperatures digestion is accomplished within a few minutes (compared with hours in solution). A fast and sensitive analytical method is extremely important to measure pathogenic bacteria at lowest levels and to take protective measures thereafter. The total analysis time is ~25 min and the required amount of protein biomarkers are in the low fmol range. Other advantages resulting from the use of such protein-based identification techniques is the capability to differentiate between different bacteria strains, important for the investigation of the bacteria's origin.[17]

Figure 3: These MRM chromatograms are the result of the extraction of a single larva that had previously been reared on artificial foodstuff spiked with Nordiazepam. The concentration of the latter was equivalent to that typically observed in skeletal muscle following fatal overdoses of Nordiazepam in humans. During the extraction procedure, the larva was spiked with 10 ng Oxazepam-d5 and 10 ng Nordiazepam-d5. The larva is both positive for Nordiazepam and its main metabolite Oxazepam. A triple quadrupole mass spectrometer was used for the analysis. Ionization was achieved using electrospray in the positive ionization mode (ES+).



Dyes

Trace evidence abounds at most crime scenes. Fibres are transferred from the assailant to the victim when contact is made. Textile fibres found at a crime scene can be used as chemical evidence in a wide range of crimes. Fibre colour chemical composition, shape and size are used to characterize fibres. An important part of forensic fibre examination involves the characterization of textile dyestuffs. As many hundreds of dyes are used in the textile dying industry, spectroscopic

methods are not sufficient for unambiguous identification because similar dyes having the same colour might have different molecular structures. Projects are on-going to characterize and compare fibres according to molecular structure of the extracted dye. A reference database of LC–MS spectra of these dyes will be created.[18]

The problem of determining the relative age of ballpoint pen inks, written on the same paper with the same ink formula, has eluded forensic scientists worldwide. Each year, millions of dollars are lost because of fraud, forgery, tax evasion, falsified wills etc. Here, we report investigations of LC–MS and LC–MS–MS methods for characterizing the ageing of inks on paper. The use of mass spectrometry allows for the identification of the ink components chosen as indicators of ageing. Ink on paper was extracted into solution. Written paper samples from 1990 and 2000 made using BIC blue and black pens were analysed. The extent of extraction of the dyes from the ink into the solvent was much greater for the newer ink than for the older one.

The amount of dye extracted from the paper into a weak solvent has also been measured relative to the amount extracted into a strong solvent. The general trend shows that as the extraction time is increased, the percent extraction of the dye into the weak solvent also increases. The identification of degradation products of the dyes is also being examined as a potential new ageing parameter.[19]

Gunshot Residue

Gunshot residue (GSR) consists of a variety of organic and inorganic substances. GSR may be found on the skin or clothing of the person who fired the gun, on an entrance wound of a victim, or on other target materials at the scene.

Therefore, either the inorganic (primarily metallic) or organic residues can be tested.

The two common analytical methods of detection of the inorganic residues are atomic absorption (AA) and scanning electron microscopy with energy dispersive X-ray analysis (SEM/EDX). Of these, the more specific is SEM/EDX, but it is also more time-consuming and expensive.

Smokeless powders are a class of propellants that were developed in the late 19th century to replace black powder. The term smokeless refers to the minimal residue left in the gun barrel following the use of smokeless powder. In forensic analysis, smokeless powders are often encountered as organic gunshot residue or as the explosive charge in improvised explosive devices.

All smokeless powders can be placed into one of three different classes according to the chemical composition of their primary energetic ingredients. A single-base powder contains nitrocellulose, whereas a double-base powder contains nitrocellulose and nitroglycerine. The energetic ingredients in triple-base powders are nitrocellulose, nitroglycerine and nitroguanidine, but because triple-base powders are primarily used in large calibre munitions, they are difficult to obtain on the open market.[20]

Modern gunpowder, or "smokeless" powder, can contain up to 23 organic compounds (FBI study).

At present the chemical ballistics unit of the National Institute of Criminalistics and Criminology (NICC) is dealing with a research project sponsored by the Belgian Federal Office for Scientific, Technical and Cultural Affairs (OSTC). The aim of the project is the development of new methods for organic GSR collection on the skin and clothes and the qualitative and quantitative analyses of GSR patterns by LC–MS.

Future Aspects

The most important question at present is: to what extent can LC–API-MS be applied to systematic toxicological analysis; that is, in the search for unknown substances? The role of GC–EI-MS as the *gold standard* for toxicological screening is unquestionable, because of the availability of large databases of mass spectra containing usually more than 8 peaks per spectrum. Could the further development of LC–API-MS change this situation? In the last years some studies have been performed which demonstrated the very high potential of LC–ESI-MS for systematic toxicological screening.

The inter-laboratory comparison of ESI- and APCI-generated mass spectra shows a large variability in fragmentation intensities and sometimes in fragmentation patterns. Therefore, the standardization of MS procedures is of critical importance to further broaden the application of an LC–API-MS-based screening system. A novel approach to enhance the identification potential of LC–API-MS is the introduction of orthogonal-acceleration TOF instruments, which may enable the mass measurement of ions with an accuracy better than 5 ppm. When the problem of inter-laboratory reproducibility of mass spectra is solved, LC–API-MS may become a *gold standard*, both for identification and quantification.[1]

In addition to the impressive mass precision achieved by the "all-ion-detection" capability of orthogonal-acceleration reflectron TOF (oa-TOF) mass spectrometers, these instruments can provide in the region of a 20- to 100-fold improvements in sensitivity compared with triple-quadrupole systems when operated in product ion scan mode. In contrast, triple quadrupole instruments provide better sensitivity in MRM mode, at the expense of information content. The combination of a quadrupole, a collision cell and an oa-TOF (Q-TOF) leads to unsurpassed sensitivity and specificity that greatly facilitates the identification of unknown compounds.

However, a limitation of oa-TOF is its limited dynamic range, which could be compensated for in the future by hardware and software developments. In the meantime, triple quadrupole instruments and Q-TOF are bound to co-exist.[19]

Another important part of the future is the miniaturization of LC–MS techniques to develop "LC–MS on a Chip" analytical capabilities.

Chip-based quantitative capillary electrophoresis (CE)–MS determination of drugs in human plasma was described for the on-chip separation and electrospray detection of selected small drug molecule compounds. The chip-based CE system was microfabricated from glass and coupled to a micro ion spray device constructed in-house. The results from this work demonstrate the feasibility of performing rapid (i.e., 30 s) chip-based quantitative CE–MS determinations of samples containing small molecule compounds.[21]

Conclusions

The combination of HPLC and mass spectrometry has been used for many years. However, since the introduction of robust API interfaces there has been a tremendous increase in the popularity of the technique amongst scientists from a wide variety of disciplines. LC–MS–MS has evolved into a robust and reliable tool that also offers versatility, specificity and sensitivity.

In the field of forensic sciences in particular, the use of LC–MS–MS has changed considerably. Where it was once a technique used only infrequently; that is, as an alternative to GC–MS for more 'troublesome' analyses (e.g. highly polar or thermolabile compounds), LC–MS has now emerged as a technique that is a worthy contender for the status of *gold standard*. The high sensitivity in combination with the ease at which structural analysis can be performed makes LC–MS–MS a practical problem-solving tool.

Already popular amongst forensic toxicologists, it seems likely that the next few years will continue to see an increase in the use of LC–MS–MS within the other branches of forensic analysis especially sports doping and the analysis of chemical and biological agents.

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