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LIQUID CHROMATOGRAPHY AND ATMOSPHERIC PRESSURE
IONISATION MASS SPECTROMETRY IN THE ANALYSIS OF DRUG
SEIZURES

by

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Academic dissertation

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CONTENTS

LIST OF ORIGINAL PUBLICATIONS	4
ABBREVIATIONS AND SYMBOLS	5
ABSTRACT	6
1. INTRODUCTION	8
2. REVIEW OF THE LITERATURE	10
2.1. Drugs and poisons	10
2.2. Forensic analyses of seized drugs	10
2.2.1. Screening and identification	10
2.2.2. Quantitation	13
2.2.3. Chiral analyses.....	14
2.3. Atmospheric pressure ionisation/mass spectrometry in forensic drug analyses	22
2.3.1. Ionisation techniques used in the study	22
2.3.2. Applications.....	23
3. AIMS OF THE STUDY	31
4. MATERIALS AND METHODS	32
4.1. Chemicals	32
4.2. Instrumentation	32
4.3. LC–ESI/MS/MS (I, II, IV)	36
4.4. AP-MALDI/MS and AP-DIOS/MS (III)	36
4.5. Chiral LC–UV–ESI/MS (IV)	37
5. RESULTS AND DISCUSSION	38
5.1. LC–ESI/MS/MS in fast analysis of a complex mixture (I)	38
5.2. LC–ESI/MS/MS in fast analysis of mass equivalent substances (II)	40
5.3. High-throughput screening by AP-MALDI/MS/MS and AP-DIOS/MS/MS (III)	41
5.4. Effect of eluent in direct chiral LC separation of amphetamine derivatives (IV)	46
5.4.1. Reversed-phase and polar ionic modes with vancomycin column	46
5.4.2. Reversed-phase and polar ionic modes with β -cyclodextrin column	50
5.4.3. Effect of molecular structure on resolution and comparison of methods	50
5.4.4. Chiral LC–ESI/MS	51
6. CONCLUSIONS	53
ACKNOWLEDGEMENTS	55
REFERENCES	56

LIST OF ORIGINAL PUBLICATIONS

This doctoral thesis is based on the following four articles, hereafter referred to by their Roman numerals (**I–IV**):

- I** K. Pihlainen, E. Sippola, R. Kostiainen, Rapid identification and quantitation of compounds with forensic interest using fast liquid chromatography–ion trap mass spectrometry and library searching. *J. Chromatogr. A*, 994 (2003) 93–108.

- II** K. Pihlainen, L. Aalberg, M. Tepponen, R.C. Clark, R. Kostiainen, The identification of 3,4-MDMA from its mass equivalent isomers and isobaric substances by using fast LC–ESI/MS/MS. *J. Chromatogr. Sci.*, 43 (2005) 92–97.

- III** K. Pihlainen, K. Grigoras, S. Franssila, R. Ketola, T. Kotiaho, R. Kostiainen, Analysis of amphetamines and fentanyls by atmospheric pressure desorption ionisation on silicon/MS and atmospheric pressure matrix assisted laser desorption ionisation/MS and its application to forensic analysis of drug seizures. *J. Mass Spectrom.*, 40 (2005) 539–545.

- IV** K. Pihlainen, R. Kostiainen, Effect of the eluent on enantiomer separation of controlled drugs by liquid chromatography–ultraviolet absorbance detection–electrospray ionisation tandem mass spectrometry using vancomycin and native β -cyclodextrin chiral stationary phases. *J. Chromatogr. A*, 1033 (2004) 91–99.

ABBREVIATIONS AND SYMBOLS

[M+H] ⁺	Protonated molecule
α-CHCA	α-Cyano-4-hydroxycinnamic acid
ACN	Acetonitrile
AP	Atmospheric pressure
APCI	Atmospheric pressure chemical ionisation
CD	Cyclodextrin or circular dichroism
CSP	Chiral stationary phase
DIOS	Desorption ionisation on silicon
ESI	Electrospray ionisation
FA	Formic acid
GC	Gas chromatography
HOAc	Acetic acid
LC	High-performance liquid chromatography
ISTD	Internal standard
k	Capacity factor $((t_R - t_0)/t_0)$
LOD	Limit of detection
m/z	Mass-to-charge ratio
MALDI	Matrix assisted laser desorption ionisation
MeOH	Methanol
MRM	Multiple reaction monitoring
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
NH ₄ OAc	Ammonium acetate
PIM	Polar ionic mode
POP	Polar organic phase
PrOH	Propanol
QIT	Quadrupole ion trap
QTOF	Quadrupole time-of-flight mass spectrometer
RP	Reversed-phase
R _s	Resolution (chromatography $R_s = 1.176(t_{r,2} - t_{r,1})/(w_{1/2,1} + w_{1/2,2})$ if the peak widths (w) are determined at half height, or MS $R_s = m/\Delta m$)
t ₀	Column dead time
TEA	Triethylamine
TLC	Thin-layer chromatography
TOF	Time-of-flight mass spectrometry
t _R	Absolute retention time
TSP	Thermospray ionisation
UV	Ultraviolet

Common abbreviations of the analytes studied:

AM	Amphetamine
LSD	Lysergic acid diethylamide
MA	Methamphetamine
MDA	3,4-Methylenedioxyamphetamine
MDEA	3,4-Methylenedioxy-N-ethylamphetamine
MDMA	3,4-Methylenedioxymethamphetamine
NE	Norephedrine
PCA	<i>para</i> -Chloroamphetamine
PMA	<i>para</i> -Methoxyamphetamine
PMMA	<i>para</i> -Methoxymethamphetamine
3-MF	3-Methylfentanyl

ABSTRACT

Liquid chromatography–mass spectrometry (LC–MS) with electrospray ionisation (ESI), atmospheric pressure matrix assisted laser desorption ionisation/mass spectrometry (AP-MALDI/MS) and atmospheric pressure desorption ionisation on silicon/mass spectrometry (AP-DIOS/MS) in positive ion mode were applied for analysis of seized drug units. Use was made of fast chromatographic separations on monolithic column, chiral chromatographic separations with two different chiral stationary phases (CSP), tandem mass spectrometry (MS/MS) and spectral comparison with in-house libraries.

A fast LC–MS/MS method relying on monolithic column, gradient elution and an ion trap mass spectrometer was developed for quick identification and quantitation of 14 forensically interesting but chemically different compounds. Not only basic but also neutral and acidic compounds were efficiently ionised with ESI in the positive ion mode. All mass spectra showed an abundant protonated molecule, which was chosen as the precursor ion. The combination of retention time and MS/MS spectral information with automated library search enabled highly reliable identification of compounds. Results for 476 standard samples and 50 authentic samples showed that the compounds of interest could be unambiguously identified with the library searching. One characteristic and abundant product ion was chosen for quantitative analysis. All compounds were eluted within 2.5 min and the total analysis time was 5 min including the stabilisation time required for the next injection. The relative standard deviations (RSD) were typically below $\pm 20\%$, which can be considered acceptable for forensic semiquantitative analysis of drug seizures. The evaluation process showed good linearity ($r > 0.993$) of the method. Limits of detection (LODs) ranged from 10.0 ng/ml to 50.0 ng/ml.

In another approach, a fast LC–MS/MS method with automated library search was developed and validated for distinguishing the controlled drug compound 3,4-methylenedioxymethamphetamine (3,4-MDMA, “ecstasy”) from 17 of its isomeric and closely related isobaric substances. 3,4-MDMA is a controlled substance whereas in many countries the other mass equivalent compounds are not. With single MS the compounds produced an intense protonated molecule, $m/z = 194$, and some characteristic fragments, but tandem mass spectrometry (MS/MS) was applied to enhance specificity. The MS/MS fragmentation was studied in order to distinguish 3,4-MDMA from the other 17 compounds. However, the MS/MS spectra of 3,4-MDMA and six other compounds were closely similar and the six compounds interfered in the identification of 3,4-MDMA. A fast LC–MS/MS method was accordingly developed for the unambiguous identification of 3,4-MDMA. The use of monolithic column and an MS/MS spectral library with retention times allowed automated confirmation of the presence of the controlled compound within five minutes. This qualitative method was tested with 49 ecstasy samples seized by the police. All results were congruent with results obtained by other methods.

A preliminary study was conducted on the feasibility of AP-MALDI/MS and AP-DIOS/MS techniques in the identification of amphetamines and fentanyls in forensic samples. With both ionisation techniques, the MS spectra showed abundant protonated molecules, and the background did not disturb the analysis. The use of tandem mass spectrometry (MS/MS) allowed the identification of the amphetamines and fentanyls. AP-MALDI/MS/MS and AP-DIOS/MS/MS were also successfully applied to identification of the compounds of interest in drug seizures. Common diluents and tablet material did not disturb the analysis and

compounds were unequivocally identified. The LOD for amphetamines and fentanyl with AP-DIOS/MS/MS were 100–1000 ng/ml indicating excellent sensitivity of the method. The LODs with AP-MALDI/MS/MS were about 5 to 10 times higher.

Enantiomer separation of nine amphetamine derivatives, methorphan and propoxyphene was studied with use of macrocyclic antibiotic vancomycin and native β -cyclodextrin (β -CD) as chiral stationary phases. Effects of 46 eluent compositions on enantiomer separation in reversed-phase (RP) and polar ionic modes (PIM) were investigated. β -CD was found to be more suitable for amphetamine derivatives in general and vancomycin for methorphan and propoxyphene. An eluent system capable of separating the enantiomers of all amphetamines in one run was developed. In addition methods are reported for separation of enantiomers of methorphan and propoxyphene. Suitability of the eluent systems for electrospray ionisation is discussed, and methods employing tandem mass spectrometric (MS/MS) detection were developed. Suitability of chiral LC-ESI/MS/MS was tested with seized drug samples. Repeatability of the methods was good and LODs with mass spectrometric detection were 25–100 ng/ml for most compounds.

The methods developed and tested with seized sample material confirm that fast LC-ESI/MS/MS, AP-MALDI/MS/MS and AP-DIOS/MS/MS and direct chiral LC-ESI/MS/MS can provide valuable and novel tools for analysing drug seizures.

1. INTRODUCTION

Forensic science produces scientific and technical evidence for purposes of justice. The tasks of the forensic drug laboratory can be briefly summarised as detection and identification, interpretation, and reporting of the results. Samples investigated in a forensic drug laboratory commonly contain illicit drugs, prescription drugs of licit origin, and illegal cocktails of licit and/or illicit drugs seized by local authorities. Matrices of the samples are various: tablets, capsules and powders, herbal or oily material and blotter paper into which the drug is impregnated. Concentrations of the drugs range from relatively low to nearly one hundred per cent. From the analytical point of view, however, concentrations of the drugs in seized samples are usually high and sensitivity of the method is seldom an issue. Occasionally trace analyses are needed for example of the various materials used for smoking, inhaling, injecting, storing or wrapping the drugs.

The market for street drugs, that is drugs and poisons of abuse, is continually expanding and a wide variety of illicit drugs have established themselves in the past 40 years. New drugs not controlled under legal statutes continue to appear. Explanations offered for this are increased availability of the scientific literature (through the internet), the availability of precursors, and the growing expertise of clandestine chemists. The range of relevant substances to be analysed expands in pace, and the physical and chemical properties of the drugs, such as volatility, polarity and stability, vary widely. Side by side with this, analytical methods for detecting and identifying known compounds and for characterising the structures of new molecules are in rapid development both for law enforcement purposes and for protection of public health (toxicology).

The main aim in forensic drug analysis is the detection and unequivocal identification of possible illicit components in a seized drug unit. Analytical techniques that are of value in forensics must offer high selectivity, sensitivity, and tolerance of contaminants, and they should be suitable for their purpose. The routine analytical separation techniques in use today are gas chromatography (GC), thin-layer chromatography (TLC), liquid chromatography (LC) and capillary electrophoresis (CE). The introduction of mass spectrometric techniques has revolutionised the work of forensic laboratories, allowing them to quickly identify unknowns in a variety of sample materials. The mass spectrometer combines good sensitivity with high selectivity. The single most important technique for the identification of active substances in forensic drug analyses is gas chromatography–electron ionisation mass spectrometry (GC–EI/MS). However, GC–MS only allows detection, as such, of drugs that are thermally stable, volatile, non-ionic and within the reach of the mass range of the instrument. LC separation, in contrast, can be considered more or less universal in applicability and particularly when LC is used in combination with electrospray ionisation (ESI) interface, even thermolabile, non-volatile and ionic molecules become available to mass spectrometric analysis. LC–MS is simple, rapid, highly specific and sensitive, and in scan mode it produces unequivocal MS data. Furthermore, since methods offering improved sensitivity, selectivity, speed and cost are required for all drugs of abuse, and since MS can supply all these needs, new MS techniques are of great interest for forensic drug analysis.

This thesis addresses the potential of atmospheric pressure ionisation mass spectrometry to solve the analytical problems associated with seized drugs. Three atmospheric pressure ionisation techniques were studied: electrospray ionisation (ESI), matrix assisted laser desorption ionisation (MALDI) and desorption ionisation on silicon (DIOS). In all these

techniques, the analytes are ionised at normal pressure outside the mass analyser. The advantages of this are simple introduction of the sample and direct on-line coupling of the sample or the analytical separation technique to the MS. The soft nature of some atmospheric pressure ionisation techniques and particularly ESI often results in only a protonated or deprotonated molecule; moreover the selectivity of single MS is not very high. Tandem mass spectrometry (MS/MS) with an ion trap mass spectrometer was therefore applied, and the fragmentation of the molecules under investigation under these conditions is briefly discussed. With all methods in-house mass spectral libraries were exploited for identification.

The study comprised several parts. **(I)** Since a seized drug unit may contain numerous components – the principal drug of action as well as various adulterants and diluents of differing chemical character – a fast separation, detection and identification method was developed for compounds with different physical and chemical properties. As well the suitability of the method for quantitation of the compounds was investigated. **(II)** The growing expertise among clandestine chemists is resulting in the appearance of new molecules in street markets, molecules with structures only slightly modified from those of known illicit drugs. Standard analytical data are required for these previously unknown drugs of abuse and new homologue or analogue drugs. As a contribution to this direction, an LC–ESI/MS/MS study was carried out, aimed at identification of the controlled substance 3,4-methylenedioxymethamphetamine (3,4-MDMA, “ecstasy”) and its differentiation from a group of its mass equivalent and structurally similar substances. **(III)** As new, fast and cost effective techniques are continually in demand in analytical laboratories, a preliminary study was carried out on the potential of atmospheric pressure desorption ionisation techniques for the identification of illicit drugs. The new atmospheric pressure techniques: MALDI and DIOS with MS/MS were investigated, with amphetamine derivatives and fentanyls used as model compounds. **(IV)** The determination of chirality is sometimes important in determining the licit or illicit nature of a sample and useful in profiling drug batches. This is especially true for amphetamine-related compounds. A study was made of the effect of eluent on the chiral separation of different amphetamines, methorphan and propoxyphene by direct chiral LC with two different chiral stationary phases (CSP), and chiral LC–ESI/MS/MS methods were developed. The results of the four studies confirm the potential of LC–ESI/MS/MS, AP-MALDI/MS/MS and AP-DIOS/MS/MS techniques for identification as well as quantitation of illicit substances in drug seizures.

2. REVIEW OF THE LITERATURE

In the following review of the literature the compounds of interest to a forensic drug laboratory and the analytical techniques employed are briefly discussed. In all cases, analyses of drug seizure samples include screening and identification of the possible drug compound(s). Quantitation and chiral analyses are sometimes of interest. The chiral analyses of amphetamine derivatives are discussed in some detail, since their chiral separation was studied in this research. Development of LC-ESI/MS/MS, AP-MALDI/MS/MS and AP-DIOS/MS/MS methods for forensic analysis was the main part of the work. The ionisation techniques are introduced and the previous applications of them to forensic and related analyses are discussed.

2.1. Drugs and poisons

The samples arriving in forensic laboratories contain illicit drugs, or pharmaceuticals or industrial chemicals that are subject to abuse or encountered in illicit markets. Mostly, the compounds of interest are controlled substances listed in the United Nations (UN) three Conventions of 1961, 1971 and 1988 [1]. The EU classifies illicit drugs according to these three UN Conventions. Other compounds of interest are chemical analogues of the controlled substances and compounds used for doping purposes. Commonly, the substances are classified as morphine and related narcotics, cannabinoids, cocaine, amphetamines, benzodiazepines, barbiturates, lysergic acid diethylamide (LSD), γ -hydroxybutyrate (GHB) and miscellaneous substances [2,3]. The miscellaneous substances include hallucinogens and anabolic steroids, growth hormone and other substances used for doping purposes. Each group contains frequently encountered compounds of similar chemical character or compounds commonly found in the same sample (e.g. cannabinoids). Classifications differ, of course, and are updated from time to time as the prevalence of substances changes and new substances appear.

The compounds studied in this work included from among the controlled substances morphine and related narcotics, amphetamines, benzodiazepines, barbiturates and LSD and various anabolic steroids, psilocybin, and several other misused drugs from the miscellaneous group. In addition, since drug analogues are of great importance, a series of these molecules were included. Although most drugs of abuse are basic, there are also a few acidic and some neutral compounds. All these types were represented in this study. Larger molecules (MW > 600) such as used for doping purposes were excluded. The compounds were chosen to broadly represent the analytical challenges in forensic drug analysis today and so represent stable and labile compounds, polar and nonpolar compounds, compounds found in only very low concentrations in seized samples, and isomeric and isobaric compounds.

2.2. Forensic analyses of seized drugs

2.2.1. Screening and identification

The primary aim in forensic drug analyses is the unequivocal identification of illicit components in a seized drug unit. Typically various screening analyses or presumptive tests such as colour tests are carried out first. After the preliminary screening come the

confirmatory analyses, which are preferably based on different physico-chemical principles, are at least as sensitive as the screening analysis and provide the highest level of confidence in the result [4]. This two-step procedure is also employed in toxicology [5] and by international organisations like the World Anti-Doping Agency (WADA) [6].

For laboratories involved in the forensic analysis of seized drugs there are several international organisations and committees charged with encouraging national laboratories to comply with best practice and international standards for quality and competence assurance. In Europe the pertinent body is the Drug Working Group (DWG) of the European Nation Forensic Science Institute (ENFSI) [7]. Internationally, the Scientific Working Group for the Analysis of Seized Drugs (SWGDRUG) Core Committee has made various recommendations for forensic laboratories analysing seized drugs [8]. Consisting of more than 20 forensic scientists from around the world, the Core Committee recommends minimum standards for the forensic examination of seized drugs. The recommendations encompass three main areas: education and training, quality assurance and methods of analysis. *Minimum standards* for the forensic examination of commonly seized drugs are presented, and analytical techniques for the identification of drugs are categorised. The categorisation of the techniques and the recommendations for their use are presented in **Table 1**. While this cannot be considered the only procedure for accomplishing reliable analyses, agreed methodologies and common criteria for identification of the compounds of interest, as well as inter-laboratory comparisons and proficiency tests, certainly improve the credibility of laboratories performing analyses.

The spectroscopic techniques in group A (**Table 1**) provide the best resolving power for unequivocal identification of molecular structure. As analytical techniques they can be used alone or, more commonly, in combination with a separation technique from group B. The combination of LC with nuclear magnetic resonance spectroscopy (NMR) perhaps provides most information on the molecular structure [9,10], but the technique is not particularly sensitive. NMR is also expensive and beyond the reach of most analytical laboratories.

Category B includes analytical separation techniques (electrophoretic and chromatographic techniques). LC and thin-layer chromatography (TLC) can be considered suitable for most analytes of forensic interest. TLC is often very simple and cost effective and can also be automated, but it suffers from poor separation efficiency and is of limited use in quantitation. Capillary electrophoretic (CE) techniques have high separation efficiency and selectivity and are particularly suitable for analysing ionic and inorganic species [11]. CE techniques suffer, however, from low sensitivity and poor reproducibility of the migration times. GC is often the most sensitive technique, but it is not suitable for polar, non-volatile, thermally unstable and ionic compounds, or at least not without time-consuming sample preparation. In some cases, the chromatographic and electrophoretic techniques lack the specificity required for characterising isomeric compounds or identifying new molecules and they then need to be used in combination with spectroscopic or spectrometric techniques.

The techniques in category C are mainly used in screening and for determining whether a compound belongs to some specific group of drugs. Ultraviolet (UV) and fluorescence (FL) spectroscopy are also commonly used as means of detection in combination with LC or other separation technique.

Table 1. Categories and examples of analytical techniques according to SWGDRUG recommendations for the analysis of seized drugs, and the guidelines for their use. The selectivity of the technique diminishes from A to C. (Reproduced from the SWGDRUG report [8] with minor modifications.)

Category A	Category B	Category C
Infrared spectrophotometry	Capillary electrophoresis	Colour tests
Mass spectrometry	Gas chromatography	Fluorescence Spectroscopy
Nuclear magnetic resonance spectroscopy	Ion mobility spectrometry	Immunoassay
Raman spectroscopy	Liquid chromatography	Melting point
	Microcrystalline tests	Ultraviolet spectroscopy
	Pharmaceutical identifiers	
	Thin layer chromatography	
	Cannabis only:	
	Macroscopic examination	
	Microscopic examination	

1. When a validated category A technique is incorporated into an analytical scheme, then at least one other technique (from category A, B or C) must be used.
 - 1.1. This combination must identify the specific drug present and must preclude a false positive identification.
 - 1.2. When sampling size allows, the second technique should be applied on a separate sampling, for quality assurance reasons. When sample size is limited, additional measures should be taken to assure that the results correspond to the correct sample.
 - 1.3. All category A techniques must have data that are reviewable.
2. When a category A technique is not used, then at least three different validated methods must be employed.
 - 2.1. These in combination must demonstrate the identity of the specific drug present and must preclude a false positive identification.
 - 2.2. Two of the three methods must be based on uncorrelated techniques from category B.
 - 2.3. A minimum of two separate samplings should be used in these three tests. When sample size is limited, additional measures should be taken to assure that the results correspond to the correct sample.
 - 2.4. All category B techniques must have data that are reviewable.
3. For the use of any method to be considered of value, the test must be considered “positive”. While “negative” tests provide useful information for ruling out the presence of a particular drug or drug class, these results have no value toward establishing the forensic identification of a drug.
4. In cases where hyphenated techniques are used (e.g. gas chromatography–mass spectrometry, liquid chromatography–diode array ultraviolet spectrophotometry), they will be considered as separate techniques provided that the results from each are used.
5. Cannabis exhibits tend to have characteristics that are visually recognisable. Thus, macroscopic and microscopic examinations of cannabis will each be considered as Category B techniques when observations include documented details of botanical features. Additional testing must follow the scheme outlined in sections 1 and 2.
 - 5.1. For exhibits of cannabis that lack sufficient observable macroscopic and microscopic botanical detail (e.g. extracts or residues), Δ^9 -tetrahydrocannabinol (THC) or the other cannabinoids must be identified utilising the principles set forth in sections 1 and 2.
6. Examples of reviewable data are:
 - 6.1. printed spectra, chromatograms and photographs or photocopies of TLC plates
 - 6.2. contemporaneous documented peer review of the tests for microcrystalline tests
 - 6.3. recording of detailed descriptions of morphological characteristics for cannabis
 - 6.4. reference to published data for pharmaceutical identifiers

Gas chromatography combined with electron ionisation/mass spectrometry (GC–EI/MS) is considered the “golden standard”, or the reference method for confirmation of positive screening tests [12,13]. GC–MS is recommended as *the* confirmatory technique in the Forensic Toxicology Laboratory Guidelines [5]. In both systematic toxicological analyses and forensic analyses of drug seizures, GC–MS in scan mode is also commonly used in screening. Scan mode allows the detection and identification of many drugs or potential drugs at the same time [4,14].

The advantage of mass spectrometry over other analytical techniques common in drug analysis (**Table 1**) is that MS as such can be considered a universal detector [15] and the amount of information obtained is very large. Often a single spectrum offers the means for identification, and even quantitation of the components can be accomplished with a minute amount of sample, owing to the high sensitivity and specificity of the technique. The identification of a compound on the basis of its mass spectrum is accomplished by comparing the recorded spectrum against the mass spectrum of a reference compound or spectra in a spectral library. An identification in confirmation analyses can also be based on just a few selected structure-specific ions. Suggestions have been made, and also official guidelines drawn up, for the criteria for the identification of compounds with use of different MS techniques [5,6,16,17].

As a means to widening the application range of mass spectrometry, and since no single chromatographic technique is capable of separating all possible components LC–MS has found an increased number of forensic applications during the past few years. Interest in LC–MS increased markedly after the introduction of commercial ESI and atmospheric pressure chemical ionisation (APCI) interfaces. Already several broad-scale drug screening applications have been developed [18–29]. In these atmospheric pressure ionisation (API) interfaces the ionisation of the analytes takes place at normal pressure outside the mass analyser. The ionisation at normal pressure makes the introduction of a sample and direct on-line coupling of the analytical separation techniques to the MS simple, and the analyses are more stable. LC separation is considered an almost universal technique, and LC–MS allows the analysis of thermally labile, non-volatile, polar and ionic compounds which cannot be directly analysed by GC–MS.

2.2.2. Quantitation

Because a fast qualitative response is often all that is needed for the immediate decision-making of the police or court, the main emphasis in the analysis of seized drugs is on qualitative analysis. In some instances, however, determination of the purity of the drug seizure is of importance. The court’s primary interest then is to make the legal distinction between possession and trafficking of substances, or to grade offences of possession as minor or serious. Sometimes mere weighing of the sample can be considered a quantitative analysis, but no country within the EU definitively uses quantity to decide who is a user and who a trafficker [30].

The methods used in chemical quantitation of the active substance of a sample are mostly the same as used in qualitative confirmation methods after the primary screening: that is, LC, GC and nowadays also capillary electrophoretic techniques (CE) [11,31]. Quantitation is commonly performed after the identification and the methods are targeted to and optimised for specific analyte(s) and matrices.

2.2.3. Chiral analyses

Chiral analyses can be important for compounds appearing in different enantiomeric forms. Amphetamine derivatives, especially 3,4-methylenedioxymethamphetamine (3,4-MDMA) and methamphetamine (MA), are chiral compounds and among the most important drugs produced in clandestine laboratories [32]. The methods of synthesis may be achiral or chiral, which means that chiral analysis of a seizure can provide valuable information for investigative purposes [33]. This “profiling”, as it is called, is one of the most intriguing areas in forensic analysis today, because of the possibility to establish links between clandestine laboratories and drug distributors. At present the most common method for profiling does not, however, involve chirality determinations but rather determinations of chemical impurities and profiling of by-products in a specific drug batch [34].

In the case of some drugs, the optical isomers possess different pharmacological and pharmacokinetic properties and therefore different toxicological properties [35], which makes the chiral information important as a national health issue. For example, the S-(+)-enantiomers of amphetamine (AM) and MA possess higher psychostimulant activity than do the R-(-)-enantiomers. For law enforcement purposes the most critical reason for doing chiral analyses in a forensic laboratory is where only one optical isomer is controlled under legal statutes. EU legislation, for example, criminalises only one isomer of propoxyphene and methorphan, while in the United States the possession of crystal S-(+)-MA (known as “ice”) will usually lead to more severe penalties [36].

A collection of methods that have proved useful in analysing the enantiomers of amphetamine derivatives in drug seizures, over-the-counter or other medicaments, herbal products and biological material are listed in **Table 2**. Emphasis is on the chiral analysis of drug seizures and on chiral screening analysis of several amphetamines. Chiral separations can be performed with the use of chiral stationary phases (CSPs) or with achiral columns and diastereomer formation through derivatisation with a chiral reagent. Also, chiroptical detectors can be used. Today, amphetamine derivatives are mostly enantiomer separated by GC with derivatisation, by CE with a chiral selector in the running buffer, or by direct LC.

GC technique is mainly targeted to the analysis of biological samples because of its sensitivity (see **Table 2**). The disadvantage is the time-consuming derivatisation with a possible source of error. The derivatising reagents can lack stereochemical purity and stability and may undergo unpredictable racemisation [37,38]. In addition, the evaporation steps may lead to loss of the volatile amphetamine derivatives.

CE has become a popular technique for separation of the enantiomers of amphetamine derivatives, performed either indirectly by diastereomer formation or, more commonly, directly by addition of a chiral selector to the background electrolyte. Native or modified β -cyclodextrin (β -CD) has proven to be the most successful chiral selector to date (**Table 2**). α - and γ -CDs have been applied without much success, though γ -CD is an efficient selector in its more water soluble, highly sulphated form (**Table 2**). The poor reproducibility of the CE migration times can be overcome with the use of internal standards or MS detection. While, the sensitivity continues to be fairly low, it can be improved through the use of derivatising reagents that introduce a fluorescing group to the analyte and the use of a sensitive fluorescence (FL) detector. Derivatisation has also been attempted with LC [39–42]. The main advantage of CE is its high efficiency, which means that the actual analytical runs can

be accomplished quickly. A good example of this is the method of Wallenborg et al.[43] in which on-chip CE is used with highly sulphated γ -CD (HS- γ -CD) and laser induced fluorescence (LIF) detection. Enantiomer separation was achieved for five amphetamine-related compounds in four minutes and for six in seven minutes. A fluorescent tagging reaction with 4-fluoro-7-nitrobenzofurazane (NBD-F) was also accomplished in a few minutes. Belder et al.[44] were able to baseline separate the enantiomers of norephedrine (NE) in just 2.5 seconds by microchip CE with HS- γ -CDs and linear imaging UV detection. This, they believed, was record time for a chiral separation.

Chiral LC has been used for amphetamine derivatives in both indirect and direct mode. Achiral LC with on-line circular dichroism (CD) detection is a technique that does not require the separation of enantiomers in order to identify them. It is less sensitive than UV, however, it requires a chromophore and, with complex mixtures, it frequently requires some separation of compounds [45]. In direct chiral LC analyses, the column is coated with a chiral stationary phase (CSP) and the analytes are separated without prior derivatisation. Time is saved and errors related to derivatisation are avoided. Direct LC separations have become more popular with the availability of new, more selective and more rugged CSPs, as can be seen in **Table 2**.

In direct chiral LC analyses the most widely used CSP for separating the amphetamine derivatives has been native or modified β -CD (**Table 2**). Crown ether has been used as well, but it does not form a complex with a secondary amine and thus MA was not separated with it [46]. Protein [47–49] and specifically prepared immunoaffinity [51] columns have also been successfully applied. Vancomycin chiral selector had not been applied to amphetamine derivatives before the present work, probably because it had been found more suitable for the separation of acidic compounds [51]. Vancomycin has many of the separation characteristics of protein based stationary phases, including exceptional stability and higher sample capacity [52]. Vancomycin is a glycopeptide that belongs to macrocyclic antibiotic phases. Structures of the macrocyclic antibiotics are highly complex; for example the vancomycin molecule produced by *Streptomyces orientalis* contains 18 chiral centres with various functional groups surrounding its three pockets or cavities [53]. Possible interactions with the analyte include π - π complexation, hydrogen-bonding, inclusion complexation, dipole interactions, steric interactions, and anionic and cationic binding. The strengths of these interactions depend on the type of mobile phase employed [53]. Vancomycin has been used with different techniques and for many different analytes since its introduction [54]. It has been used not only in LC, but in CE, in thin-layer chromatography (TLC)[55], enhanced fluidity liquid chromatography (EFLC)[56], supercritical fluid chromatography (SFC)[57] and capillary electrochromatography (CEC)[58]. With mass spectrometric detection, it has been used at least with CE-ESI/MS [59] and with LC-APCI/MS [60,61]. Earlier studies have demonstrated the utility of the vancomycin column in LC analyses of also basic compounds (e.g. ergot alkaloids [62]).

The chiral separation methods for enantiomers of AM and MA developed by Katagi et al. in 1994 with thermospray ionisation (TSP) [63] and later with ESI [64] interfaces were among the very first LC-MS applications for these analytes. Still, chiral LC in combination with mass spectrometry has rarely been used, even though it can provide the sensitivity and specificity often needed and MS offers a more universal detector than FL. In the case of amphetamine derivatives, for example, FL detection is not always effective for those that are non-fluorescing. As is presented in **Table 2** the LC methods with FL detection provide similar limits of detection (LODs) to LC-MS methods, but the more common UV detection is clearly

less sensitive. It should be noticed that most of the LC methods listed in **Table 2** are not directly suitable for ESI and APCI mass spectrometry because of the use of nonvolatile phosphate buffer. Some modifications are necessary.

Table 2. Selection of GC, CE and LC methods for the chiral separation of amphetamine derivatives and related compounds.

Compounds ^a	Matrix	Derivatisation ^b	Separation, comments ^c	Detection and Details	LOD (ng/ml)	Linearity (ng/ml)	Ref.	
EP, PS, MA, MC	Stds	MTPA	GC: DB-5 (15 m x 0.25 mm x 0.25 μm), 190°C (1 min)–2°C/min–220°C (1 min)–20°C/min–275°C, inj. V 1 μl, split 1/25, runtime 14 min	FID, MS (¹ H-NMR; offline)	n.d.	n.d.	[65]	
MDMA, MDA, metabolites	Stds, synthesis products, urine	(S)-HFBPCI+ MSTFA+MBTF A -> N-(S)-HFBP--O-TMS	GC: DB-5MS (15 m x 0.25 mm x 0.25 μm), 105°C(7.5min)–10°C/min–200°C (10min)–20°C/min–280°C	MS (SIM) QIT	EI, PCI (i-Bu)	n.d.	n.d.	[38]
MDMA, MDA, AM, metabolites	Urine plasma	(R)-MTPCI and (S)-MTPCI	GC: DB17 (30 m x 0.25 mm x 0.25 μm) (urine) HP Ultra 1 (25 m x 0.2 mm x 0.11 μm) (plasma), 100 °C (3 min)–15 °C/min–285 °C(5 min)	NPD (U), MS (PI)	SIM, MRM	LOQ 0.1 (U) and 0.05 (PI) for MDMA (MS)	0.1–80 (pl) 0.025–8 (u) for MDMA	[37]
AM, MA, MDA, MDMA, MDEA	Urine	<i>l</i> -TPC	GC: DB-17 or ZB-50 (15 m x 0.25 mm x 0.25 μm), 120°C-30°C/min-210°C-6°C/min-260°C (1 min), splitless, and HP-1 (12 m x 0.2 mm x 0.33 μm), 130°C-4°C/min-190°C-25°C/min-250°C (2 min), splitless. Runtimes 15 and 20 min, respectively	MS	SIM	LOQ 10 (AM, MA), 25 (MDA, MDMA, MDEA)	5–10000	[66]
MDA, MDMA, HMA, HMMA	Stds, urine	1) (R)-MTPCI +NH ₄ OH 2) HMDS	GC: Ultra 2 AT (12 m x 0.22 mm x 0.33 μm), 150°C(1 min)-20°C/min-290°C (7 min), split 1/10, inj. V 2 μl	MS	SIM (3 ions, 1 quant.)	2.4/3.1 (R/S-MDMA), 0.2/0.3 (R/S-MDA)	12.5–200 (MDMA) 1.25–20 (MDA)	[67]
AM, MA, NE, NP, EP, PS	Stds, forensic samples	GITC	MECC: DM-β-CD + 200 mM SDS, 20% MeOH, T=30°C, 15 kV, runtime 25 min	UV	210 nm	n.d.	n.d.	[68]
DM-4-MA, EP, PS, NE, AM, MA, BDMA, MDA, MDMA, MDEA	Stds	No	CE: 120 mM HP-β-CD, 100 mM citric acid-19.27 mM Na ₂ HPO ₄ , pH 2.5, NE, DM-4-MA, BDMA no chiral R _s , runtime for all 50 min	UV	200 nm	n.d.	n.d.	[69]
AM, NE, NP, CA, MrCA, cocaine, MA, EP, MC, MrCAT, Propoxyphene	Stds, forensic samples	No	CE: Mixture of anionic and neutral CDs <i>e.g.</i> DM-β-CD and β-CD-SBE(IV), MeOH/25 mM Tris-H ₃ PO ₄ buffer, pH 2.45 <i>e.g.</i> 1.2:98.8, 30 kV, T=30°C runtimes 14 to 28 min	UV	210 nm	n.d.	n.d.	[36]

Table 2. (Continued)

Compounds ^a	Matrix	Derivatisation ^b	Separation, comments ^c	Detection and Details	LOD (ng/ml)	Linearity (ng/ml)	Ref.
EP, PS, N-MEP, N-MPS, NE, NP, AM, MA	Stds, herbal prep.	No	CE: 70 mM HP- β -CD – 30 mM TMAC –10 mM SDS, pH 2.0, 28 kV, T=31°C, NE no chiral R _s , runtime 41 min	UV	210 nm	4000	4000–100000 [70]
MA, AM, 4-HA, 4-MA, MDMA, MDEA, DMA, DMMA, BDMA	Stds	No (yes; Marfey's reagent tested but it brought no extra advantage)	CE: 10 mM native β -CD, DM- β -CD, TM- β -CD, HP- β -CD and/or CM- β -CD, 50 mM NaH ₂ PO ₄ , 20 kV, 20°C, at least partial separation for all with native β -CD, analysis times different, all < 32 min, most < 12 min	UV	214 nm	n.d.	n.d. [71]
AM, MA, MDA, MDMA, MDEA	Stds, urine	No	CE: 20 mM HP- β -CD (α - and other β -CDs also tested), 200 mM NaH ₂ PO ₄ 25 kV, 15°C runtime for mixture 30 min	UV (DAD)	200 nm	n.d.	500–10000 [72]
AM, MA, selegiline	Stds	Yes and no	GC: DM- β -CD (Chiraldex) (with and without derivatisation) LC: HP- β -CD, S-NEC- β -CD, DMP- β -CD with AQC deriv. CE: HP- β -CD	FID, UV	254 nm 214 nm (MA)	n.d.	n.d. [42]
MDA, MDMA, MDEA	Stds, synthesis products	No	CE: α -, β -, γ -CD tested; β -CD the only that provided separation in 3 diff. buffer systems, runtime 20 min	ESI+,MS (SIM)	[M+H] ⁺ s	3000	n.d. [73]
AM, MA, PE	Stds	No	CE: α -, β -, γ -CD and DM- β -CD tested → 12 mM DM- β -CD, 50 mM Tris-H ₃ PO ₄ , pH 2.3, 22 kV, ambient T, runtime 10 min	UV	210 nm	n.d.	n.d. [74]
AM, MA, EP	Stds, urine, hair	No	CE: α -, β -, γ -CD tested → 15 mM β -CD, 150 mM phosphate buffer, pH 2.5, 10 kV, T=17.5°C, runtime 30 min	UV	200 nm	300	625–20000 [75]
MA, AM, EP, NE, DMa, N-MEP, MDMA, MDA, MDEA	Stds, urine	No	CE: β -CD, DM- β -CD, TM- β -CD, HP- β -CD examined → mixture only choice: 3 mM β -CD+10 mM DM- β -CD, pH 2.5, +30 kV, 75mM Tris, T=25° C, 3 s injection, runtime 33 min	UV	195 nm	100	200–500000 [76]
NE,EP,CA,PS, MC,AM,MA	Stds	NBD-F	Microchip CE (MCE): Best: highly sulphated γ -CD (10mM)+1.4 mM SDS buffer phosphate 50 mM, pH 7.35, 8 kV, runtime 7 min	LIF	488nm	n.d.	n.d. [43]
EP, NE, NP, PS	Stds, herbal material	No	CE: 10 mM l-leucine as chiral selector, 20 mM phosphate buffer, pH 9.0, 20% <i>i</i> -PrOH, 15 kV, T=25°C, compounds separated from one another in 7 min	UV	190 nm	n.d.	3330–20000 [77]
NE, NP, EP, PS, AM, MA, MDA, MDMA,MDEA	Stds	No	CE: HS- γ -CD, (SU(XIII)- γ -CD). Optimised to high CD conc. (10mM), 50 mM phosphate background electrolyte at pH 2.6, -12 kV, cap. T=15°C.	UV	195 nm	0.012 (NE)	n.d. [78]
EP, PS, MA, phentermine (ISTD)	Stds, forensic samples	No	CE: 15 mM β -CD, 300 mM NaH ₂ PO ₄ , pH 2.5, 20 kV, 30°C runtime 30 min	UV	200 nm	3000	5000–25000 [79]

Table 2. (Continued)

Compounds ^a	Matrix	Derivatisation ^b	Separation, comments ^c	Detection and Details	LOD (ng/ml)	Linearity (ng/ml)	Ref.	
MA, MC, PS, EP (ketamine)	Stds, forensic samples, urine	No	CE: 17.5 mM native β -CD, H ₂ O/ACN 95:5+150 mM phosphate, pH 2.5, 20 kV, T=15 °C, runtime <20 min GC: HP-5 MS (30 m x 0.32 μ m) 70°C (1min)–15°C/min–200°C (2 min)–20°C/min–260°C (12.3 min), splitless, runtime 27 min CE: 2.5 mM HS- γ -CD, (SU(XIII)- γ -CD), RP mode, cap. T=15°C.	UV MS (GC)	210 nm full scan 40–450 m/z	1.0 (GC)	n.d.	[80]
NE, NP, EP, PS, AM, MA, MDA, MDMA, MDEA MA, PS, EP	Stds, synthesis products Stds, forensic samples	No, d-isoproterenol (ISTD) GITC	LC: Bondapak C ₁₈ (30 x 3.9 mm), 1) THF/H ₂ O/HOAc 35:70:1; 2) MeOH/H ₂ O/HOAc 50/49/1; 3) phosphate buffer, pH 3.0/MeOH 5/1, 1.5 ml/min, ambient T, runtime 30 min LC: Pirkle covalent phenylglycine or Pirkle ionic phenylglycine (25 x 4.5 cm), various amounts of <i>i</i> -PrOH in hexane, inj. V 20 μ l	(+)ESI/MS/MS UV	[M+H] ⁺ _s (QTOF) 254 nm 280 nm	10 000 n.d. ^d	n.d.	[81] [82]
MA, AM	Stds	<i>l</i> -TPC	LC: Pirkle covalent phenylglycine or Pirkle ionic phenylglycine (25 x 4.5 cm), various amounts of <i>i</i> -PrOH in hexane, inj. V 20 μ l	moving belt-EI/MS UV	full scan m/z 71–450 254 nm	n.d.	n.d.	[83]
MA, AM	Urine	No	LC: Phenylcarbamate- β -CD (Ultron ES-PhCD), ACN/MeOH/50 mM KPO ₄ 10/30/60, pH 6.0 (UV), and ACN/MeOH/100 mM NH ₄ OAc 10/30/60, pH 6.0 (TS-MS), 1.0 ml/min, T=25°C LC: Native β -CD (250 x 4.0 mm x 5 μ m) + achiral for derivatised compounds, 0.1 M NH ₄ OAc, pH 7 and 5 to 15% MeOH, 0.4/0.5 ml/min; only partial separation for DE-4-EA, runtime 39 min	TS–MS UV	SIM 220 nm	0.2–0.5 (SIM) 3–10 (scan) 50–100 (UV)	n.d.	[63]
AM, MA, 4-HA, 4-MA, MDMA, MDEA, DMA, DMMA, DM-4-EA, BDMA EP, MA, selegiline AM, MA, NE	Stds, forensic samples Stds	No or PITC, NITC, Marfey's, AQC	LC: Native β -CD (250 x 4.0 mm x 5 μ m) + achiral for derivatised compounds, 0.1 M NH ₄ OAc, pH 7 and 5 to 15% MeOH, 0.4/0.5 ml/min; only partial separation for DE-4-EA, runtime 39 min LC: Native β -CD, Chiradex (250 x 4.0 mm x 5 μ m), 500 M TEA with H ₂ SO ₄ , pH 3.5, no organic, 0.8 ml/min, runtime 39 min LC: Chiral crown ether CROWNPAK CR(+) (150 x 4.0 mm), 1.0 ml/min, aq. HClO ₄ , pH 1.8	UV (DAD)	non-deriv. 254 nm	n.d.	n.d.	[84] [85]
MA, AM, metab.	Stds, urine	No	LC: Phenylcarbamate- β -CD column (Ultron ES-PhCD) (150 x 6.0 mm x 5.0 μ m) ACN/MeOH-50 mM KPO ₄ (UV), 100 mM NH ₄ OAc/MeOH/ACN (60/30/10), pH 6.0 (TS), 1 ml/min, native β -CD as reference column, runtime 20 min	UV TS-MS	220 nm SIM, [M+H] ⁺ _s	50 (UV), 0.5 (MS) (AM), 100 (UV) (MA), 1.0 (MS) (MS)	200–20000 (UV)	[86]
MDA, MDMA, MDEA, MBDB	Stds, forensic samples, blood	No	LC: Native- β -CD, Astec Cyclobond I 2000 (250 x 4.6 mm x 5 μ m) or HPE- β -CD, Astec Cyclobond 2000-RSP (same dim.), KH ₂ PO ₄ /K ₂ HPO ₄ 0.1 M, pH 6.5, 1.0% TEA/ACN 95/5 v/v T=30°C, 1.0 ml/min runtime 36 min	FL	ex 285 nm em 320 nm	HPE- β -CD 11–24 LOQ 37–80	HPE- β -CD 50–3000	[87]

Table 2. (Continued)

Compounds ^a	Matrix	Derivatisation ^b	Separation, comments ^c	Detection and Details		LOD (ng/ml)	Linearity (ng/ml)	Ref.
AM, EP, PS	Stds, tablets	FMOC or FMOC-L-Pro	LC: FMOC derivatives: native β -CD ChiraDex (250 x 4 mm x 5 μ m) Achiral for FMOC-L-Pro derivatives, RP (ACN/MeOH, phosphate or NH ₄ OAc) and NP (hexane/PrOH, ACN/EtOAc) and PIM (ACN/HOAc/TEA)	FL UV	ex 264 nm em 313 nm UV 254 nm	FMOC-L-Pro: 10 (AM), 25-50 (EP, PS) (FL) 100 (AM, EP), 250-500 (PS) (UV)	500–20000	[40]
MA, AM	Urine	No	LC: Phenylcarbamate- β -CD column (150 x 2.0 mm), 10 mM NH ₄ OAc/MeOH/ACN (60/30/10), pH 5.0, 150 μ l/min, 25°C, total runtime 30 min	(+)ESI-MS	scan and SIM: [M+H] ⁺ s	20 and 50 (scan), 0.5 and 1.0 (SIM) (MA, AM)	1.0–5000	[64]
MDEA, MDA, metab., MDMA (ISTD)	Plasma	No	LC: 1) ChiraDex (250 x 4 mm) runtime 25 min, 0.8 ml/min, 40 mmol KH ₂ PO ₄ , pH 5.5/ACN 91/9, inj. V 20 μ l 2) LiChroCart Superspher 60 RP-select B (250 mm x 4 mm x 5 μ m) and 0.01 M β -CD in 40 mmol KH ₂ PO ₄ , pH 3.0/ACN 98/2, 0.5 ml/min, inj. V 20 μ l, runtime 33 min 3) Chiral CBH 150 mm x 4 mm (metabolites)	FL (MDA, MDEA) ECD (HME)	ex 286 nm em 322 nm nm (MDEA, MDA)	LOQ 5.0 (FL)	1.2–20.3 (MDA). 10.1–175.9 (MDEA)	[47]
AM, NE, NOE, MDA	Stds, urine, plasma	OPA+NAC	LC: Lichrospher 100 RP ₁₈ (125 x 4 mm, 5 μ m), MeOH/OAc buffer, pH 4.5, 50/50 (60/40 for NOE), 0.75 ml/min, runtime 45 min, 60/40 MeOH/OAc buffer, pH 4.5, 1.0 ml/min, runtime 25 min	FL	em 231 nm ex 425 nm	25 (NE, NOE) 50 (AM, MDA)	LOQ 100 and 250–750(?)	[41]
MA, AM	Stds, urine	No and yes with GC-MS	LC: Anti-D-MA monoclonal Ab column, fraction collection (<i>i.e.</i> complicated) \rightarrow Syva Emit d.a.u. or derivatisation and GC-MS	EMIT, GC-MS	SIM	1100 (LC- (offline)-GC-MS)	n.d.	[50]
MA	Stds, forensic samples	No	LC: Chiralcel OB-H, <i>n</i> -hexane/ <i>i</i> -PrOH 9/1 v/v, 1.0 ml/min, runtime < 15 min	UV	220 nm	n.d.	n.d.	[48]
MDEA, MDA, metab.	Plasma, urine	No	LC: Chiral CBH column (150 x 4 mm x 5 μ m) with CBH guard column (10 x 3 mm, 5 μ m), 20 mM NaH ₂ PO ₄ , 50 mM Na ₂ EDTA+ 7% <i>i</i> -PrOH, pH 6.44, 0.7 ml/min, 17.5°C, runtime 22 min	FL	em 286 nm ex 322 nm	LOQ 5	5–100–800 (MDEA), 5–200 (MDA); U	[49]
AM, yohimbine	Stds, seized drugs	No	LC: C ₈ (250 x 4.6 mm, 3 μ m), A: phosphate buffer pH 3.7, B: ACN, 0-10 min 10–30% B, 10- 20 min 30% B, 1.0 ml/min, T=25°C, inj. V 10 μ l, runtime 20 min. CD less sensitive than UV, but chiral separation not required.	UV/Vis CD ^e	258 nm	n.d.	n.d.	[45]

Table 2. (Continued)

Compounds ^a	Matrix	Derivatisation ^b	Separation, comments ^c	Detection and Details	LOD (ng/ml)	Linearity (ng/ml)	Ref.
AM, MA, NE, PMA, PCA, PMMA, MDA, MDMA, MDEA	Stds, seized drugs	No	LC: Native β -CD (150 x 4.6 mm x 5 μ m), 95/5 100 mM NH ₄ OAc/MeOH, 0.8 ml/min, 15°C, inj.V 20 μ l, runtime 55 min, NE no chiral R _s . Vancomycin (150 x 4.6 mm x 5 μ m), MeOH/HA/TEA 100/0.03/0.02, 0.8 ml/min, 15°C, inj.V 20 μ l, runtime 20 min: MDEA, NE no chiral R _s , other 0.5 < R _s < 1.5, peaks overlapping → MS required	(+)ESI/MS/MS MRM [M+H] ⁺	100, 1000 (AM) (β -CD)	n.d.	IV

^aAbbreviations for compounds not already mentioned: 4-HA: 4-Hydroxyamphetamine; 4-MA: 4-Methoxyamphetamine; BDMA: 4-Bromo-2,5-dimethoxyamphetamine; CA: Cathinone; DM-4-EA: 2,5-Dimethoxy-4-ethyl amphetamine; DM-4-MA: 2,5-Dimethoxy-4-methyl amphetamine; DMA: 2,5-Dimethoxyamphetamine; DMeA: Dimethylamphetamine; DMMA: Dimethoxymethamphetamine; EP: Ephedrine; MBDB: *N*-Methyl-1-(1,3-benzodioxol-5-yl)-2-butanamine; MC: Methcathinone; MrCA: Merucathinone; MrCAT: Merucathine; N-MEP: *N*-Methylephedrine; N-MPS: *N*-Methylpseudoephedrine; NOE: Norepinephrine; NP: Norpseudoephedrine; PE: Phenylephrine; PS: Pseudoephedrine.

^bThe abbreviations used for the derivatisation reagents: AQC: *6-Aminoquinolyl-N-hydroxysuccinimidyl carbamate*; DPIBCl: *4-(4,5-Diphenyl-1H-imidazol-2-yl)benzoyl chloride*; Fmoc-L-Pro: *9-Fluorenylmethyl chloroformate-L-proline*; (R)-MTPCl: *(R)-(-)- α -Methoxy- α -trifluoromethylphenylacetyl chloride*; Mosher's reagent, (S)-MTPCl: *(S)-(+)- α -Methoxy- α -trifluoromethylphenylacetyl chloride*; GITC: *2,3,4,6-Tetra-O-acetyl- β -D-glucofuranosyl isothiocyanate*; HMDS: *1,1,1,3,3,3-Hexamethyldisilazane*; Marfey's reagent: *N- α -(2,4-Dinitro-5-fluorophenyl)-L-alaninamide*; *l*-TPC: trifluoroacetyl-*l*-prolyl chloride; MBTFA: *N-Methylbis(trifluoroacetamide)*; MSTFA: *N-Methyl-N-trimethylsilyltrifluoroacetamide*; MTPA: *(R)-(+)- α -Methoxy- α -(trifluoromethyl)phenylacetic acid*; N-(S)-HFBCl: *N-Heptafluorobutyryl-(S)-prolyl chloride*; NAC: *N-Acetyl-L-cysteine*; NBD-F: *4-Fluoro-7-nitro-2,1,3-benzoxadiazole, 4-fluoro-7-nitrobenzofurazane*; NITC: *Naphthyl isothiocyanate*; OPA: *o*-Phthaldialdehyde; PFPP: *Pentafluorophenylpropyl*; PITC: *Phenyl isothiocyanate*; S-TFPC: *(S)-(-)-N-(Trifluoroacetyl)-prolyl chloride*; TMAC: *Tetramethylammoniumchloride*.

^cOther abbreviations in the table, if not already mentioned: U: Urine; Pl: Plasma; β -CD-SBE(IV): Anionic, randomly substituted sulfobutyl ether β -CD; DM- β -CD: Heptakis(2,6-di-O-methyl)- β -cyclodextrin; DMP- β -CD: Dimethylphenylcarbamate- β -cyclodextrin; EMIT: Enzyme multiplied immunoassay; HP- β -CD: Hydroxypropyl- β -cyclodextrin; HS- γ -CD: Highly sulphated- γ -cyclodextrin; S-NEC- β -CD: S-Naphthylethylcarbamate- β -cyclodextrin; SDS: Sodium dodecyl sulphate; PCI: Positive chemical ionisation; SIM: Selected ion monitoring.

^dn.d. = not determined/reported,

^eCD = circular dichroism.

2.3. Atmospheric pressure ionisation/mass spectrometry in forensic drug analyses

Many of the compounds that are of interest for forensic analysis cannot be directly analysed by GC–MS, the “golden standard” technique for confirmation of compound identity. Some compounds break into smaller compounds in the heat of the GC injector, an obvious problem for unequivocal identification. For this, and other reasons already discussed, the combination of LC and MS and especially the introduction of the API interfaces have been important for forensic drug analyses. The following sections briefly describe the three API techniques that were used in this study and describe some existing applications for analysing the analytes of interest.

2.3.1. Ionisation techniques used in the study

The atmospheric pressure ionisation techniques used in this study were electrospray ionisation (ESI), matrix assisted laser desorption ionisation (MALDI) and laser desorption ionisation on silicon (DIOS). In MALDI, α -cyano-4-hydroxy cinnamic acid (α -CHCA) was used as matrix, while DIOS was performed using a porous silicon platform without any matrix. The desorption ionisation techniques are not integrated with LC in a dynamic way. Some attempts to accomplish automated coupling have been reported, however [88,89]. Other atmospheric pressure ionisation techniques available and used in analysing for illicit drug compounds include atmospheric pressure chemical ionisation (APCI) [90] and markedly less frequently sonic spray ionisation (SSI) [91] and atmospheric pressure photoionisation (APPI)[92].

Electrospray ionisation was chosen for the method development owing to its capability to efficiently produce ions from a variety of compounds and for its soft nature; it mostly produces a protonated molecule without fragment ions. ESI [93–95] involves the nebulisation of a liquid stream into charged droplets through the action of the high electric field applied to the (metal) capillary. A pneumatic nebulisation gas (nitrogen) is commonly added to assist the droplet formation. The liquid subsequently breaks into smaller charged droplets through solvent evaporation with the help of heat or drying gas or both. At the Rayleigh limit, the small, densely charged droplets undergo Coulombic explosion. After several successive fissions the radii of the highly charged droplets are very small (<10–20nm) and the droplets are finally transformed into gas-phase ions. The generation of gas-phase ions from the microdroplets is a result of either desorption of the ions from the droplet surface or the desolvation of preformed ions, or both. These two models are called the ion-evaporation model (IEM) and the charged-residue model (CRM), respectively. The formed gas-phase ions are then directed into an orifice leading to the vacuum of the mass analyser.

As a soft ionisation technique, ESI is suitable for the analysis of thermally labile, otherwise fragile and non-volatile polar or moderately nonpolar organic molecules [93]. Because ESI can produce multiply charged ions from large biomolecules, the analysis even of whole proteins is possible. ESI is compatible with LC separations and is especially suitable for compounds that can be ionised in solution. The solution chemistry is sometimes critical as surface tension, conductivity and volatility of the solvent, among others, play an important role in the ionisation process.

MALDI was initially introduced for the analysis of high molecular mass compounds [96,97]. As a soft ionisation technique, its utility lies in an ability to provide molecular weight

information on intact molecules, good sensitivity and suitability to analysis of complex mixtures. In MALDI the analyte is mixed with a matrix and the mixture then co-crystallises on the target plate. A pulsed laser beam irradiates the co-crystal causing the accumulation of high-density energy within the crystal, which causes both the analyte and the matrix to vaporise. The matrix helps transfer the required energy of the pulsed laser beam to the analyte in order to vaporise and ionise it [96,98]. It also reduces the required desorption energy. The exact desorption ionisation mechanism is not known.

MALDI is commonly performed in vacuum, but recently an atmospheric pressure (AP)-MALDI ion source was developed [99]. Ionisation at atmospheric pressure makes the analyses easier to perform and the ionisation is even softer than in vacuum [100]. More importantly, the AP-MALDI source is compatible with all types of mass analysers, whereas vacuum-MALDI is typically used with the time-of-flight (TOF) analyser. Commercial AP-MALDI sources are easily interchangeable with ESI sources.

Even more recent than AP-MALDI is the AP-DIOS technique [101]. In this MALDI-related but matrix-free, soft laser desorption ionisation technique, analyte and solvent molecules are trapped on the high surface area of porous silicon [102–104]. From the silicon surface the analytes are directly desorbed and ionised through irradiation with a laser. Porous silicon has proven to be an effective medium for desorbing compounds and generating intact ions in the gas phase [102]. The lack of matrix allows the analysis of small molecules ($MW < 500$) with low background because there are no ions from the matrix to interfere the analysis. The lack of matrix also allows more uniform deposition of aqueous samples [98]. Sensitivity at the femtomole level with little or no fragmentation has been achieved [98], and protonation and deprotonation have been reported to be the favoured ionisation process [104,105,106]. AP-DIOS uses the AP-MALDI source design. AP-MALDI/MS and AP-DIOS/MS have high throughput and automation capability for the rapid analysis of samples. The rate of analysis is currently limited by the data acquisition systems.

2.3.2. Applications

Table 3 presents selected LC–MS applications, and their operational parameters, involving the analysis of the substances of interest of this study. Except for the information-dependant acquisition (IDA) method of Decaestecker et al.[107], the methods are targeted at identifying and/or quantifying predefined compounds. LC, or some separation is commonly used together with ESI although, e.g. Weinmann and Svoboda [108] successfully quantitated illicit drugs in serum and urine using only solid-phase extraction (SPE) and flow-injection analysis (FIA)/ESI/MS/MS. And Selby et al. [109] successfully quantitated the alkaloids present in illicit heroin samples by FIA/ESI/MS/MS. Nowadays, some separation in combination with ESI is nevertheless considered essential owing to the problems of direct sample introduction. The problems arise from the matrix, which can suppress the ionisation efficiency. The suppression can be unpredictable and even originate from compounds lacking electrospray response [110]. In addition, compounds that have the same mass equivalent precursor ion can be problematic if they are not separated before the mass analysis. The CE methods are not included in **Table 3**, although a number of applications of CE/MS in forensic analysis have been published [111–115].

Vacuum-MALDI has been used in analysing benzodiazepines quantitatively from tablets [116], vacuum-DIOS has been applied to the analysis of small molecules, such as catecholamines [117]. Vacuum-MALDI [118] and vacuum-DIOS [119,120] have been applied in forensics only in the analysis of synthetic polymers. AP-MALDI [121] and AP-DIOS [122,123] have been used for both qualitative and quantitative analysis of small drug and biogenic molecules, but so far they have not been used in practical casework in forensic drug analyses.

Many recent reviews [15,124–130] have described the operational parameters and applications of LC–MS in analysing drugs of abuse and other toxic compounds in forensic toxicology and related areas. Only methods reported in the last four years are included in **Table 3**. Unfortunately LC–MS methods continue to be underrepresented in the analysis of drug seizure samples [2,3,131,132]. In the three latest reviews of this area [3,131,132], less than 3% of the references cover atmospheric pressure ionisation–, TS–, particle beam– or fast atom bombardment ionisation–MS techniques. Applications of LC–MS in forensic toxicology can be considered comparable in many respects. The different matrix, the higher concentration of the analytes and the absence of metabolites are the main differences between analyses in forensic toxicology and analyses of drug seizures. The legal demands are the same.

Table 3 shows that the reversed-phase (RP) octadecylsilyl (C_{18}) column is still the most widely used analytical column with MS detection. LC methods are continuing to evolve, however, because the high selectivity of tandem mass spectrometry, accurate mass measurement and developments in acquisition rates are making the complete chromatographic separation of different compounds unnecessary. The development of pumps suitable for fast gradient chromatography, high-throughput columns like short and monolithic columns and the fast ultra-high pressure separations allow shorter LC run times. For example, Jeanville et al. [133] quantitated cocaine and its primary metabolites in 3.2 minutes using a 3 cm column, and Wu et al. [134] separated six benzodiazepines within 90 s using ultrahigh pressure LC–TOF. Such short analysis times mean that the sample preparation step can become the rate-limiting step, particularly in the analysis of biological samples.

The fast development of LC–MS instrumentation is clearly evident in the recent reviews, as in **Table 3**. By 1997 the atmospheric pressure ionisation interfaces ESI and APCI were beginning to find wider use, and by 2000 they were virtually the only ionisation techniques in use. Although the mass analysers used in routine applications are still mostly single quadrupoles (Q), triple quadrupoles (QQQ), and quadrupole ion traps (QIT), now the use of TOF and quadrupole time-of-flight (QTOF) analysers is increasing. Recently, for example, TOF was used in an identification of street drugs based on accurate mass and a target library of exact monoisotopic masses [135]. The quantitation was performed with chemiluminescence nitrogen detection (CLND) and the use of primary reference standards was, therefore, avoided with both detectors. While the widely used single quadrupole technique has limitations, it has proven to be useful in certain applications. The triple quadrupole instrument operated in MRM mode is a more sensitive technique than QIT or TOF, as is also seen in **Table 3**. In QIT and TOF, however, a full spectrum is recorded with better sensitivity in comparison to triple quadrupoles. Owing to its very high acquisition rate, QTOF can be the most sensitive technique for the acquisition of product ion spectra [136].

Table 3. Operational parameters of selected LC–MS methods for analysing illicit drugs published in 2000–2004. The compounds of interest were given special emphasis in the selection of methods.

Compounds	Matrix	Sample preparation	Separation	Detection and Details		LOD (ng/ml)	Linearity (ng/ml)	Ref.	Year
Psilocin, psilocybin	Honey	MeOH extraction	LC: C ₁₈ : (125 x 3 mm), ACN/50 mM NH ₄ COOH pH 3.0 5/95, 0.6 ml/min	APCI+, Q	MRM, [M+H] ⁺ s	n.d.	n.d.	[137]	1997
Psilocin	Mushrooms	Ethyl acetate extraction, Derivatisation DBD-Pro-COCl, Bufotenin ISTD	LC: RP-18 (100 x 2 mm, 3 μm) 50 mM NH ₄ OAc/ACN (73/27), 0.15 ml/min, inj. V 5 μl, runtime 35 min	ESI+, QIT	SIM [M+H] ⁺ and MRM	LOQ: 6.3 ng/mg mushroom	1.6–4.08	[138]	2004
15 1,4-benzodiazepines, +metabolites	Stds, hair	Soxhlet (hair), ESTD	LC: C ₁₈ (150 x 4.6 mm)+similar guard (30 mm), H ₂ O/ACN/MeOH 30/5/65 (15 benzos) ACN/H ₂ O 65/35 (for indiv. cmpds+metab.), 0.25 ml/min, inj. V 20 μl, runtime 18 min. Also CE methods developed.	ESI+, QIT	MRM, [M+H] ⁺	16.7 (temazepam)	22.5–150	[139]	1999
Temazepam, + 6 other benzodiazepines	Buffer solution, urine, serum	In-tube SPME, ESTD	LC: C ₁₈ (50 x 2.1 mm, 3 μm), isocr. 50 mM NH ₄ OAc/MeOH 40/60, 0.3 ml/min, T = 25°C, run time 12 min	ESI+, Q	SIM, [M+H] ⁺	1.0 (temazepam)	2.0–500 (temazepam)	[140]	2000
Temazepam, + 3 other	Dog plasma	Automated SPE, d ₅ -diazepam ISTD	LC: C ₁₈ (50 x 2.1 mm, 5 μm), A: 0.01M NH ₄ OAc, B: ACN, 0-3 min 30% B, 3-5 min 30-100% B, 0.25 ml/min, ambient T, run time 5 min	ESI+, QQQ	MRM, [M+H] ⁺	0.3 (LOQ)	0.3–150	[141]	2001
11 benzodiazepines+metab.	Urine	Hydrolysis+filtration+on-line SPE	LC: C ₁₈ (150 x 1.5 mm, 5 μm), 10 mM NH ₄ OAc/ACN, non-linear gradient with elution of trapped analytes and separation, 0.15 ml/min, T = 30°C, run time 38 min	APCI+, Q	SIM, [M+H] ⁺	2–10 (SIM), 20–50 (scan)	2–10–500	[142]	2002
Temazepam, + 9 other benzodiazepines	Plasma	LLE, d-analogues ISTD	LC: RP phenyl column (150 x 2.1 mm, 5 μm), A: ACN/MeOH/20 mM NH ₄ OAc 10/10/80, B: ACN/20 mM NH ₄ OAc 95/5, nonlinear, stepped gradient, 0.25 ml/min, inj. V 10 μl, run time 15 min	ESI+, QQQ	MRM, [M+H] ⁺ s	0.4	1–800	[143]	2002
Benzodiazepines, buprenorphine, LSD, + metabolites	Blood, urine	LLE or SPE, d-analogues ISTD	LC: n.d.	n.d.	scan, MRM	10, heroin metabolites, blood, <0.1, buprenorphine, urine	n.d.	[144]	2002
Temazepam, 3 other compounds	Plasma	High-flow on-line extr., alprazolam ISTD	LC: monolithic C ₁₈ (100 x 4.6 mm), A: 0.1% FA, B: ACN+0.1% FA, 0-1.2 min 10-90% B, 4 ml/min, split 0.4ml/min to MS	ESI+, QQQ	MRM, [M+H] ⁺	n.d.	2.5–5000	[145]	2003

Table 3. (Continued)

Compounds	Matrix	Sample preparation	Separation	Detection and Details		LOD (ng/ml)	Linearity (ng/ml)	Ref.	Year
Temazepam + 32 benzodiazepines/ metabolites	Blood	LLE Methyl- bromazepam ISTD	LC: C ₁₈ (150 x 2.1 mm, 3.5 μm) A: 0.006 FA, pH 3, B: MeOH, 0–5 min 30-40% B, 5–25 min 40-50% B, 25–30 min 50-60% B, 30–35 min 60% B, 36–45 min 30% B, 0.2 ml/min. Inj. V 50 μl, T=30°C, cycle time 45 min	APCI+, QIT	SIM [M+H] ⁺ or MRM	0.1–12.6	5–2000 (temazepam)	[146]	2004
Temazepam + 22 benzodiazepines + 4 other	Plasma	LLE 4 d-analogue ISTDs	LC: Spherical RP (125 x 2.0 mm, 6 nm pore size) + similar guard column, A:5 mM NH ₄ formate, pH 3, B: ACN, gradient and flow-rate programming, inj. V. 5 μl, runtime 10 min.	APCI+, Q	Scan (screening) SIM (quantit.)	5.0 (scan), 10 (SIM;LOQ) (temazepam)	10–1250 (temazepam)	[147]	2004
Temazepam + 3 other benzodiazepines	Blood	Restricted- access SPME	LC: C ₁₈ (50 x 4.6 mm, 5 μm)+ C ₁₈ (10 x 4.6 mm) guard, H ₂ O/MeOH, 0.6 ml/min, runtime 15 min	ESI+, Q	SIM [M+H] ⁺	30 (temazepam)	50–1000	[148]	2004
DMeA, + metabolites: MA, AM	Urine	SPE, ESTD	LC: SCX col. (150 x 2.0 mm) 5 mM NH ₄ OAc, pH 4.0/ACN 35/65, 0.2 ml/min, runtime 25 min	ESI+, Q	SIM, [M+H] ⁺	50 (AM), 10 (MA)	100–5000 (AM), 50–5000 (MA)	[149]	2000
AM, MA, MDA, MDMA, MDEA +11 other	Serum	SPE, d- analogue ISTDs	LC: C ₁₈ (125 x 3 mm, 4 μm), ACN/50 mM NH ₄ OCOH, pH 3.0 25/75, 0.3/0.4/0.8 ml/min depending on compound	APCI+, Q	SIM, [M+H] ⁺ s + 1 or 2 fragments	1.0–2.0	5–500	[150]	2000
AM, + 1 other	Hair	Digestion + LLE, d-analogue ISTDs	LC: C ₁₈ (300 x 2.0 mm, 5 μm), isocr. 0.1% FA/MeOH 80/20 (AM), runtime 3.0 min	ESI+, QQQ	MRM, [M+H] ⁺ s	n.d.	16–8000	[151]	2001
AM, MA, MDA, MDMA, MDEA	Plasma	LLE, d-analogue ISTDs	LC: C ₁₈ (100 x 2.1 mm, 3.5 μm), 10 mM NH ₄ OAc/ACN 75/25, isocratic, 0.3 ml/min, inj. V 10 μl, runtime 4.5 min	ESI+, QQQ	MRM, [M+H] ⁺ s	n.d.	0.1–500	[152]	2001
AM, MA, MDA, MDMA, MDEA	Saliva	LLE, d-analogue ISTDs	LC: C ₁₈ (100 x 2.1 mm, 3.5 μm), 10 mM NH ₄ OAc/ACN 90/10, isocratic, 0.3 ml/min, inj. V 10 μl, runtime 3.5 min	ESI+, QQQ	MRM, [M+H] ⁺ s	1.0 (in saliva)	0.1–500	[153]	2001
AM, MA, MDA, MDMA, MDEA, morphine, cocaine, + 2 other	Oral fluid	SPE, MDMPA, 2'- methylcocaine ISTD (3 ISTDs)	LC: RP phenyl column (100 x 2.1 mm, 3 μm) and phenyl guard (7.5 x 2.1 mm), MeOH+10 mM NH ₄ OCOH/10 mM NH ₄ OCOH, pH 5.6 –41.2% MeOH in 20 min, 0.2 ml/min, total runtime 28 min	ESI+, QTOF	MRM, [M+H] ⁺ s, morphine not fragmented	0.22–1.07	2.0–100	[154]	2002

Table 3. (Continued)

Compounds	Matrix	Sample preparation	Separation	Detection and Details	LOD (ng/ml)	Linearity (ng/ml)	Ref.	Year	
DMeA, + metabolites: MA, AM	Urine	Direct injection, ESTD	LC: Highly cross-linked polyvinyl alcohol gel (Asahipak GS-320HQ), (300 x 7.6 mm, 1 µm prefilter), ACN/20 mM NH ₄ OAc, pH 8.5 30/70, 0.6 ml/min, split 0.07 ml/min to MS, runtime 60 minutes Chiral LC for DMeA: Chiral DRUG (150 x 2 mm, 1 µm prefilter), ACN/20 mM NH ₄ OAc, pH 5, 0.1 ml/min, runtime 30 min	ESI+, Q Scan (qual), SIM, (quant), [M+H] ⁺ s	20 (MA), 60 (AM) (SIM)	60–3000 (MA), 180–9000 (AM)	[155]	2002	
AM, MA, EP, MDMA, MDA, MDEA	Plasma, oral fluid	LLE, d-analogue ISTDs	LC: C ₁₈ (100 x 2.1 mm, 3.5 µm), 10 mM NH ₄ OAc/ACN 75/25, 0.3 ml/min, inj. V 10 µl, runtime 4 min, total analysis time 20 min (including sample preparation)	ESI+, QQQ	MRM, [M+H] ⁺	0.2–2.0	0.5–500	[156]	2003
AM, MDMA, cocaine, 9 other drugs	Seized drugs	Dissolution to MeOH, dil. with MeOH/ 0.1% FA, no standard	LC: C ₁₈ (100 x 20 mm, 3 µm) + 4 x 20 mm precolumn, A: 5 mM NH ₄ OAc in 0.1% FA, B: 5 mM NH ₄ OAc in ACN, 0–10 min 10–40% B, 10–13.5 min 40–75% B, 13.5–16 min 75–80% B, 16–19 min 80% B, 0.3 ml/min, T=40°C, inj. V 10 µl, total cycle time 24 min (TOF); A: 0.1% FA, B: MeOH, 0–7min 10–40% B, 7–12 min 40–75% B, 12–15.5 75–90%B, 15.5–19 90% B, 0.25 ml/min, T=40°C, inj. V 10 µl, total cycle time 26 min	ESI+, TOF Quant. CLND	Exact monoisot. mass (TOF) equimolar N response (CLND)	n.d.	n.d.	[135]	2004
Pentobarbital, (Phenobarbital as ISTD)	Dog food	SPE, phenobarbital ISTD	LC: Polystyrene-divinylbenzene (150 x 2.1 mm, 5 µm) A: water, B: ACN 0-4 min 20% B – 50% B, 4-7 min 50% B, step to 90% B, 7-9 min 90% B, 0.3 ml/min, total cycle time 25 min	APCI-, QIT (no signal in APCI+/ ESI+, ESI- yes)	MS/MS [M-H] ⁻ s scan+SIM	40 (pentobarb.)	10–400	[157]	2000
Cocaine, + 1 metab.	Urine	1/10 dilution, d ₃ -cocaine ISTD	LC: PFPP bonded silica column (30 x 2.1 mm, 5 µm), ACN/5 mM NH ₄ OCOH, pH 3.0, 90/10, 0.6 ml/min, inj. V 10 µl, runtime 4.5 min	ESI+, QQQ	MRM, [M+H] ⁺ s	0.16	1.0–1000 LOQ 5.3	[158]	2000
Cocaine, + 15 metab.	Meconium	LLE + SPE, d ₃ -cocaine ISTD	LC: C ₈ (150 x 2.1 mm, 5 µm), A:20 mM NH ₄ OAc, pH 2.7, B:MeOH/ACN 50/50, stepped gradient, 0.27 ml/min, T=38°C, run time 20 min, total cycle time 25 min	ESI+, QQQ	MRM, [M+H] ⁺ s	1.0	1.0–1000	[159]	2000
Cocaine, + 12 metab.	Blood, amniotic fluid, placental & fetal tissue	SPE, d ₃ -cocaine ISTD	LC: C ₈ (150 x 2.1 mm 5 µm), A: 20 mM NH ₄ OAc, B: MeOH/ACN 50/50, 0- 2 min 0% B, 2-7 min 0-15% B, 7-15 15% B, 15-23 min 15-100% B, 0.27 ml/min, T=37 °C, runtime 23 min	ESI+, QQQ	MRM, [M+H] ⁺ s	0.5 (cocaine)	10–250	[160]	2001

Table 3. (Continued)

Compounds	Matrix	Sample preparation	Separation	Detection and Details		LOD (ng/ml)	Linearity (ng/ml)	Ref.	Year
Cocaine, + 1 metab.	aCSF	In vivo microdialysis	LC: C ₁₈ (125 x 3 mm, 5 μm), + guard col., A: 0.05% HOAc, pH 3.55, B: ACN+0.05% HOAc, 0-4 min 15%B, 4-10 min 15-65% B, 0.5 ml/min, inj. V 10 μl, total cycle time 20 min	ESI+, Q	SIM, [M+H] ⁺ s	1.5 (cocaine)	3–3000	[161]	2001
Cocaine, + 2 metab.	Urine	SPE on-line, d-analogue ISTDs	LC: Allure basix® (30 x 2.1 mm, 5 μm) + guard col., A: 50 mM FA+100mM NH ₄ OCOH in water, B: ACN/acetone 60/40, total runtime 3.2 min	ESI+, QQQ	MRM, [M+H] ⁺ s	0.25 (cocaine)	7.5–1000	[133]	2001
Cocaine, + 4 metab.	Hair	Digestion + SPE, d-analogue ISTDs	LC: Metasil basic (100 x ? mm, 3 μm), isocr. 0.1% FA/MeOH 75/25, 0.25 ml/min, T=30°C, runtime 15 min	ESI+, Q	SIM, [M+H] ⁺ s	n.d.	7.2–18000	[162]	2001
Cocaine, + 1 other	Plasma	SPE, d-analogue ISTDs	LC: C ₁₈ (100 x 2.0 mm, 5 μm), isocr. MeOH/0.1% FA 50/50, 0.15 ml/min, inj V. 20 μl, runtime 5 min	APCI+, QQQ	MRM, [M+H] ⁺ s	n.d.	2.5–750	[163]	2001
Cocaine, + metab.	Plasma	SPE, d-analogues ISTD	LC: C ₈ (150 x 2.1 mm, 5 μm), A: 20 mM NH ₄ OAc, B: MeOH/ACN 1/1, 0-1 min 1% B, 1-3 min 1-80% B, 3-15 min 80% B, 0.3 ml/min, ambient T, 1/7 split, runtime 15 min	ESI+, QQQ	MRM, [M+H] ⁺ s	4.1 (cocaine)	5–1000 LOQ 14.4	[164]	2001
Cocaine, + 3 metabolites	Plasma	SPE, d-analogue ISTDs	LC: C ₁₈ (100 x 2 mm, 5 μm), 0.1% FA/MeOH 55/45, 0.15 ml/min, runtime 5 min	ESI+ or APCI+, QQQ	MRM, [M+H] ⁺	2.5 (LOQ)	2.5–750 (n.d.)	[165]	2003
Cocaine, + 3 biogenic amines	aCSF	Microdialysis	LC: C ₁₈ (150 x 2.1 mm, 3 μm or 150 x 2.0 mm, 3 μm), A: 0.1% FA, B: ACN+0.1% FA 0–0.5 min 0% B, 0.5–4.0 min 0–100% B, 0.3 ml/min, T=40°C, inj. V 10 μl	ESI+, QQQ	MRM [M+H] ⁺	0.001 or 0.0001 (dep. on mass analyser)	5 decades	[166]	2004
Morphine, MDMA, cocaine, 14 other (GUS)	Urine	LLE, Butorphanol ISTD	LC: C ₁₈ (100 x 2.1 mm, 3.5 μm), from 90% H ₂ O/MeOH/ACN (82/9/9) + 0.045 M NH ₄ OAc to 90% MeOH/ACN (18/41/41) + 0.045 M NH ₄ OAc, 0.2 ml/min, inj. V 50 μl, total runtime 23 min	ESI+, QTOF	MRM, [M+H] ⁺ s (mostly) (IDA)	n.d.	200–2/4000 200–2000 (cocaine)	[107]	2000
Morphine, + 6 other opiates	Blood	SPE, nalorphine ISTD	LC: C ₁₈ (150 x 4.6 mm, 3 μm), A: 0.1% FA, B: MeOH, 0–2 min 5% B, 2–10 min to 90% B, until 20 min in 90% B, 0.5 ml/min, T=50°C, inj. V 10 μl, runtime 20 min	ESI+, QIT	MRM, [M+H] ⁺ s	LOQ <10	50–750	[167]	2001
Morphine, + 2 metab.	Plasma	Automated SPE, d-analogue ISTDs	LC: Silica (50 x 3.0, 5 μm), H ₂ O/ACN/trifluoroacetic acid 9/91/0.01 Inj. V 5 μl, 0.7 ml/min, runtime 3.5 min	ESI+, QQQ	MRM, [M+H] ⁺ s 286→152	0.5 (LOQ)	0.5–50	[168]	2002

Table 3. (Continued)

Compounds	Matrix	Sample preparation	Separation	Detection and Details		LOD (ng/ml)	Linearity (ng/ml)	Ref.	Year
Morphine, cocaine, propoxyphene, + 27 other drugs/metabolites	Oral fluid	ACN protein precipitation, 14 d-analogue ISTDs	LC: Ether-linked phenyl with polar end-capping, (150 x 2.0 mm, 4 µm)+ similar guard (4.0 x 2.0 mm), A:10 mM NH ₄ formate+0.001% FA, pH 4.5, B: ACN, 0-13 min 5-26% B, 13-22 min 26-90% B, 22-24 min 90% B, 24-27 min 90-5% B, 0.3 ml/min, inj V. 50 µl, T=25°C, total analysis time 35 min	APCI+, QQQ	MRM, [M+H] ⁺	0.5, 5.0 (propoxyphene)	1–500 or 10–500 (propoxyphene)	[169]	2003
Morphine, + gluc.	Plasma	SPE d ₃ -ISTD	LC: C ₁₈ (150 x 2.1 mm, 5 µm) and C ₈ guard (4 mm x 2 mm), isocratic ACN/MeOH/10 mM OCOH, pH 3, 2.2/2.5/95%, flow rate 0.2 ml/min, runtime 10 min	ESI+, Q	SIM [M+H] ⁺	0.25 (signal/noise 2/1)	0.5–200	[170]	2003
Morphine, cocaine + gluc., + 4 other	Meconium	SPE Nalorphine ISTD	LC: C ₈ (150 x 4.6 mm), 1% HOAc/ACN 97/3 to 73/27 in 11 min, 1 ml/min, inj. V 20 µl, T=30°C, runtime 15 min, cycle time 25 min	ESI+, Q	SIM m/z 286, 227, 209 m/z 304, 212, 182	1.2 (morphine), 0.9 (cocaine)	5.0–1000	[171]	2003
Morphine, MDMA, cocaine, 14 other (GUS)	Blood	LLE, SPE, Butorphanol ISTD	LC: C ₁₈ (100 x 2.1 mm, 3.5 µm), from 100% H ₂ O/MeOH/ACN (80/10/10) + 5 mM NH ₄ OAc (=A) to 50% H ₂ O/MeOH/ACN (20/40/40) + 5 mM NH ₄ OAc (=B), in 7 min and 7 min hold + 1.5 min 100% B. 0.3 ml/min, inj. V 25 µl, total run time 24 min	ESI+, QTOF	MRM, [M+H] ⁺ s (mostly) (IDA)	60–160 e.g. for morphine, 8–16 for cocaine	modifications of ref. [107]	[172, 173]	2004
Morphine + 2 glucuronides	Dog and monkey plasma	SPE, nalorphine ISTD	LC: C ₁₈ (150 x 2.1 mm, 5 µm), ACN/0.05% HOAc (1/24) or MeOH/0.01% FA (1/19), T=40°C, 0.1 ml/min, inj. V 10 µl, runtime 9 min	ESI+, QQQ	MRM [M+H] ⁺	0.5 (LOQ)	0.5–50 or 100	[174]	2004
Morphine, buprenorphine, temazepam, phenobarbital, AM, MDMA, 64 other stimulants	Plasma	Protein precipitation	LC: C ₁₈ (50 x 2.0 mm, 3µm), C ₈ (10 x 2.0 mm) guard column, A: 10 mM NH ₄ OAc+5% ACN, pH 5.0, B: 10 mM NH ₄ OAc+90% ACN, pH 5.0, 0–2 min 0–20%B, 2–12 min 20–60%B, 12–14 min 60–100%B, 14–16 min 100%B, , 0.2 ml/min, inj. V 20 µl; two injections: screening and confirmation, total cycle time 21 min	ESI+, QIT, DAD	1) MS: [M+H] ⁺ 2) in-source CID 230–300 nm	5 (AM, morphine, temazepam), 1.0 (buprenorphine), 0.5 (MDMA), phenobarbital only DAD	n.d.	[175]	2004
Morphine, cocaine, LSD, 9 other drugs	Plasma, urine	SPE, d-analogue ISTDs	LC: C ₁₈ (150 x 2 mm, 3 µm)+ polar-RP guard, A: 4 mM NH ₄ OAc, pH 4.6, B: ACN, 0–1 min 0%B, 1–13 min 0–60%B, 13–15 min 60%B, 15–17 min 60-0%B, 17–23 min 0%B, post column: ACN 0.05 ml/min (basic drugs), phenylhexyl column (50 x 2.0 mm, 3 µm) + Polar-RP guard (4 x 2.0 mm), A: 5 mM NH ₄ OAc, pH 6.5, B: ACN, 0–0.4 min 30% B, 0.4–7.8 min 30–90%B, 7.8–8.2 min 90%B, 8.2–8.5 90–30%B, 8.5–11 min 30%B, (Cannabinoids), 0.25 ml/min, T=40°C, inj. V 20 µl	ESI+, QQQ	MRM [M+H] ⁺ + 2 qualif. ions	4.0 (morphine), 1.5 (cocaine)	6.3–250 (morphine) 4.7–500 (cocaine)	[176]	2004

Table 3. (Continued)

Compounds	Matrix	Sample preparation	Separation	Detection and Details	LOD (ng/ml)	Linearity (ng/ml)	Ref.	Year
Morphine, cocaine, 7 other	Hair	LLE, SPE, d-analogue ISTDs	LC: C ₁₈ 150 x 2.0 mm, 4 μm) c ₁₈ (4 x 2.0 mm) guard, A: 10 mM NH ₄ OAc+0.001% FA, pH 4.5, B: ACN, 0–20 min 10–90%B, 20–22 min 90%B, 0.2 ml/min, T=30°C, Inj. V 20 μl, cycle time 30 min	APCI+, MRM QQQ [M+H] ⁺	16.6 (morphine), 3.4 (cocaine)	33.2–10000 (morphine) 6.8–200 (cocaine)	[177]	2004
LSD, iso-LSD, + 7 metab.	Blood, plasma, urine	LLE, d ₃ -LSD ISTD	LC: Spherisorb 5 RP 8S C ₈ (100 x 2.1 mm, 5 μm), 40% H ₂ O+60% ACN + 0.1% FA+ 2 mM NH ₄ OCOH, 0.4 ml/min, runtime 8 min	ESI+, MRM, QQQ [M+H] ⁺ ; neutral loss scan for metab. ID	0.2 (LOQ)	0–4.0	[178]	2001
LSD	Urine	LLE, d ₃ -LSD ISTD	LC: C ₁₈ , other conditions not reported.	ESI+, Q	n.d.	0.05–10	[179]	2001
Buprenorphine, + 2 metab.	Plasma	SPE, d-analogue ISTDs	LC: Inertsil ODS-3 (100 x 3 mm, 3 μm), A: 2 mM NH ₄ OCOH + 0.1% FA, B: ACN/H ₂ O 99.5/0.5 +2 mM NH ₄ OCOH + 0.1% FA, 0-0.1 min 16% B, 0.1-5.1 min 30% B, 5.1-7.6 min 30% B, total runtime 12.7 min	ESI+, MRM QQQ Surviving [M+H] ⁺	0.1 (LOQ)	0.1–50	[180]	2001
Buprenorphine, cocaine, dextro-propoxyphene, DXM, LSD, MDMA, MA, morphine, temazepam + 229 other	Blood	LLE Dibenzepine and enalapril ISTDs	LC: C ₁₈ ACN/10 mM NH ₄ OAc, pH 3.2 with FA 20/80 to 100/0 in 10 min, 3 min isocratic, 0.2 ml/min, inj. V 30 μl, T=35°C, run time 13 min, cycle time 18 min	ESI+, MRM, QQQ [M+H] ⁺ s	10, < 20, <20, <20, <20, 20, 50, 100, <20	n.d.	[28]	2003
Buprenorphine, 1 metabolite	Plasma	SPE clonazepam ISTD	LC: C ₁₈ (55 x 4 mm, 3 μm), MeOH/50 mM NH ₄ OAc, pH 4.5 50/50, 0.8 ml/min, T=30°C	APCI+, MRM QQQ [M+H] ⁺ (surviving parent ion)	0.002, 0.01 (LOQ)	0.01-5.0	[181]	2003
AM, MDMA, buprenorphine, LSD, morphine, phenobarbital, psilocybin, temazepam, 6 other	Seized drugs	LLE ESTD	LC: C ₁₈ (50 x 4.6 mm, monolith), A: 0.1% FA/MeOH+0.1% FA 95/5, B: MeOH+0.1% FA/0.1% FA 95/5, 0-0.5 min 5–50% B, 0.5-2.5 min 50–100%B, 2.5 ml/min, inj V. 50 μl, T=30 °C, runtime 2.5 min, cycle time 5 min	ESI+, MRM, QIT [M+H] ⁺ s	10–50	100–30000	I	2003

Special abbreviations used in the table: DMmA: Dimethylamphetamine; EP: Ephedrine; MDMPA: 3,4-Methylenedioxypropylamphetamine; aCSF: Artificial cerebrospinal fluid; Gluc: Glucuronide/-s; GUS: General unknown screening; LLE: Liquid-liquid extraction; SPE: Solid phase extraction; DBD-Pro-COCl: 4-(2-Chloroformylpyrrolidin-1-yl)-7-(N,N-dimethylaminosulfonyl)-2,1,3-benzoxadiazole; ESTD: External standard; Q: Single quadrupole; QQQ: Triple quadrupole; CLND: Chemiluminescence nitrogen detection; LOQ: Limit of quantitation; n.d.: Not determined/reported

3. AIMS OF THE STUDY

The aims of this study were to evaluate the applicability of liquid chromatographic, liquid chromatography–electrospray ionisation/mass spectrometric, and atmospheric pressure laser desorption ionisation/mass spectrometric techniques in qualitative and quantitative analyses of drugs of abuse in drug seizures.

The more specific aims of the research were

- to study the applicability of fast liquid chromatography–electrospray ionisation/tandem mass spectrometry (LC–ESI/MS/MS) and library searching to fast detection, identification and quantitation of forensically relevant compounds with different physical and chemical properties **(I)**,
- to study the mass spectrometric behaviour of mass equivalent isomers and isobaric substances of 3,4-MDMA and to develop a fast confirmation method for the presence of 3,4-MDMA in order to quickly distinguish it from the non-controlled substances by fast LC–ESI/MS/MS and library searching **(II)**,
- to evaluate in a preliminary way the feasibility of atmospheric pressure matrix assisted laser desorption ionisation (AP-MALDI) and atmospheric pressure desorption ionisation on silicon (AP-DIOS) mass spectrometry for fast detection and identification of small drug molecules encountered in forensic drug seizures **(III)**,
- to study the effect of eluent composition on enantiomer separation of selected basic drugs using vancomycin and native β -cyclodextrin chiral stationary phases and to develop chiral liquid chromatographic separation methods for forensic purposes using ESI/MS detection **(IV)**.

4. MATERIALS AND METHODS

The major experimental features are described in this section. For more detailed descriptions the reader is referred to the original publications **I–IV**.

4.1. Chemicals

The standards and chemicals that were used are listed in **Table 4**. The structures of the studied compounds are presented in **Figure 1**.

4.2. Instrumentation

All instrumentation is presented in **Table 5**. Helium (4.6, 99.996%) was used in the ion traps as damping and collision gas.

The LC separations in studies **I** and **II** were carried out with a Chromolith Speedrod monolith column (50 x 4.6 mm, C₁₈ RP_e Merck, Germany). The chiral LC separations in study **IV** were carried out with Chirobiotic V vancomycin chiral column and Cyclobond I 2000 native β -cyclodextrin chiral column (both 150 x 4.6 mm, 5 μ m particle size, Advanced Separation Technologies Inc., USA). The column eluent was split and one tenth was directed to MS. In study **IV**, one tenth of the eluent was directed to MS and nine tenths to a diode array detector (DAD).

The control software versions were Chemstations 08.03 (**I**) and 09.04 (**II, IV**) for the LC and LC/MSD Trap software versions 4.1 (**I**) and 4.2 (**I, II, IV**) for the MS. The control software versions used in **III** were EsquireControl version 5.0 for the MS and Target software version 3.4 for the AP-MALDI source.

Table 4. Standard compounds and chemicals used in the study.

Standard/chemical	Purity and comments	Supplier/Manufacturer	Paper
α -Cyano-4-hydroxycinnamic acid (α -CHCA)	Purum grade, $\geq 99\%$	Sigma-Aldrich (USA)	III
β -Hydroxy-3-methylfentanyl	Standard	United Nations (Austria)	III
β -Hydroxyfentanyl	Standard	United Nations (Austria)	III
α -Methylfentanyl	Standard	United Nations (Austria)	III
17 isomeric and isobaric substances to 3,4-MDMA	Synthesised reference material, purified by recrystallisation [182]	Dr. L.A. Aalberg, Auburn University (USA)	II
3-Methylfentanyl (3-MF)	Standard	United Nations (Austria)	III
Acetic acid	Analytical grade	Riedel-de Haën (Germany)	IV
Acetonitrile	LC grade	Rathburn (Scotland)	II, IV
Acetylfentanyl	Standard	United Nations (Austria)	III
Alfentanil	Standard	United Nations (Austria)	III
Ammonium acetate (NH ₄ OAc)	Reagent plus grade	Sigma-Aldrich (USA)	IV
Amphetamine	Standard	Sigma (USA) or Lipomed AG (Switzerland)	I, III, IV
Buprenorphine	Reference material	USP Inc. (USA)	I
Clenbuterol	Standard	Sigma (USA)	I
Dextropropoxyphene	Standard	Sigma (USA)	IV
Ethanol	AA	Primalco Ltd (Finland)	III
Fentanyl	Standard	Sigma (USA)	III
Formic acid	Analytical grade	Merck (Germany)	I
Hydrofluoric acid	48 % (w/v) in water, 99.99+%	Sigma-Aldrich (Germany)	III
Isopropyl alcohol	LC grade	Rathburn (Scotland)	IV
Levomethorphan	Standard	University Pharmacy (Finland)	IV
Levopropoxyphene	Standard	USP Inc. (USA)	II
LSD	Standard	USP Inc. (USA)	I
MDA	Standard	United Nations (Austria)	III, IV
MDEA	Standard	United Nations (Austria)	III, IV
MDMA	Standard	RBI (USA) or United Nations (Austria)	I – IV
Metandienone	Standard	Steraloids Inc. (USA)	I
Methamphetamine	Standard	Lipomed AG (Switzerland)	III, IV
Methanol	LC grade	Rathburn (Scotland)	I, IV
Morphine	Standard	University Pharmacy (Finland)	I
Nandrolone	Standard	Steraloids Inc. (USA)	I
Norephedrine	Standard	Knoll AG (Germany)	III, IV
PCA	Standard	Sigma (USA)	III, IV
Phenobarbital	Standard	Radian International (USA)	I
PMA	Standard	Lipomed AG (Switzerland)	III, IV
PMMA	Standard	Lipomed AG (Switzerland)	III, IV
Psilocybin	Standard	Lipomed AG (Switzerland)	I
Rasemethorphan	Reference material	University Pharmacy (Finland)	IV
Salbutamol	Standard	Sigma (USA)	I
Stanozolol	Standard	Sigma (USA)	I
Sufentanil	Standard	United Nations (Austria)	III
Temazepam	Standard	Radian International (USA)	I
Testosterone	Standard	Fluka (Switzerland)	I
Triethylamine	Analytical grade	Fluka (Switzerland)	IV
Trifluoroacetic acid	Analytical grade	Sigma-Aldrich (USA)	III
Water	Deionised, Milli Q 18 Ω	Milli-Q, Millipore (USA)	I – IV

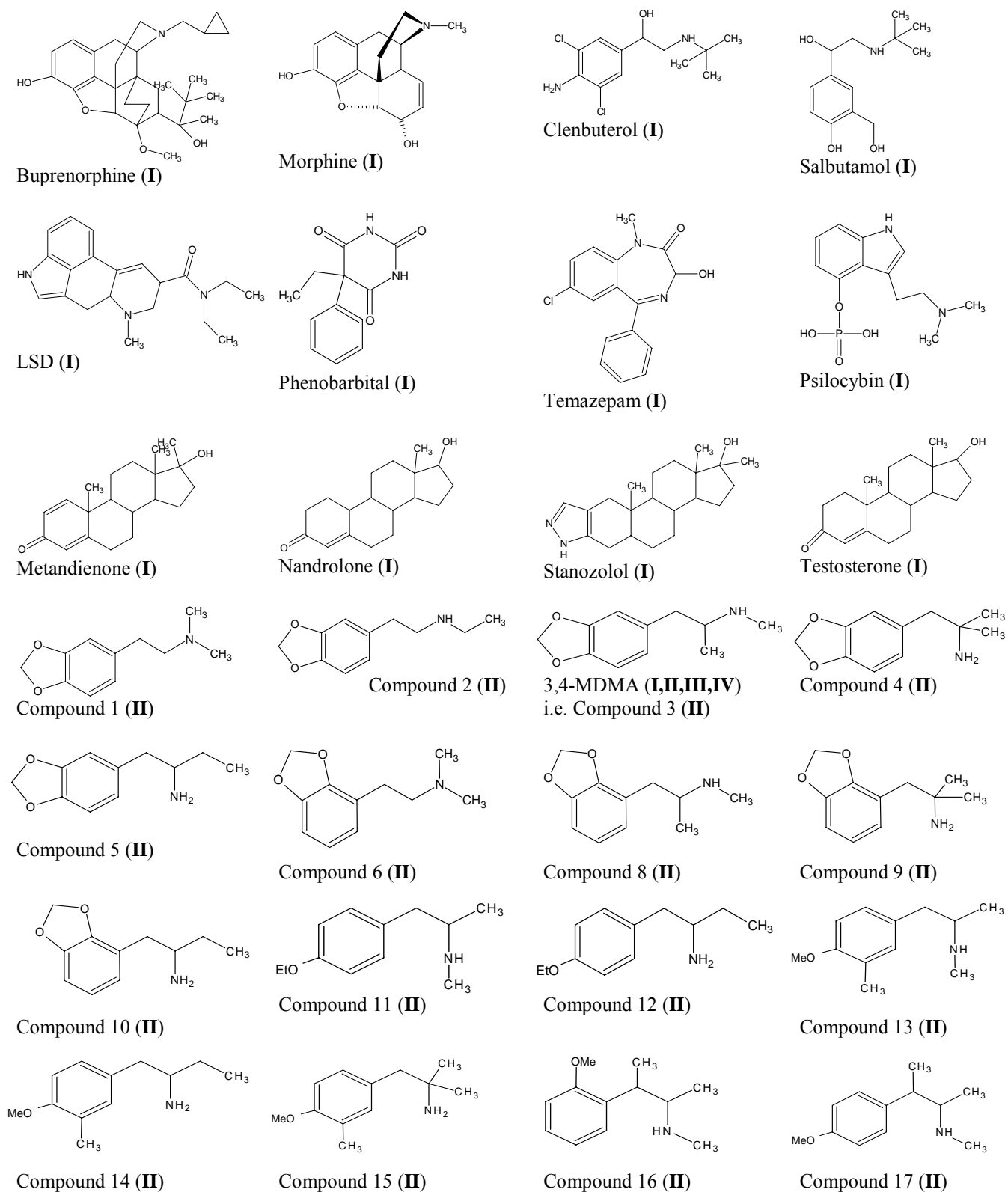


Figure 1. Chemical structures of the studied compounds (continues).

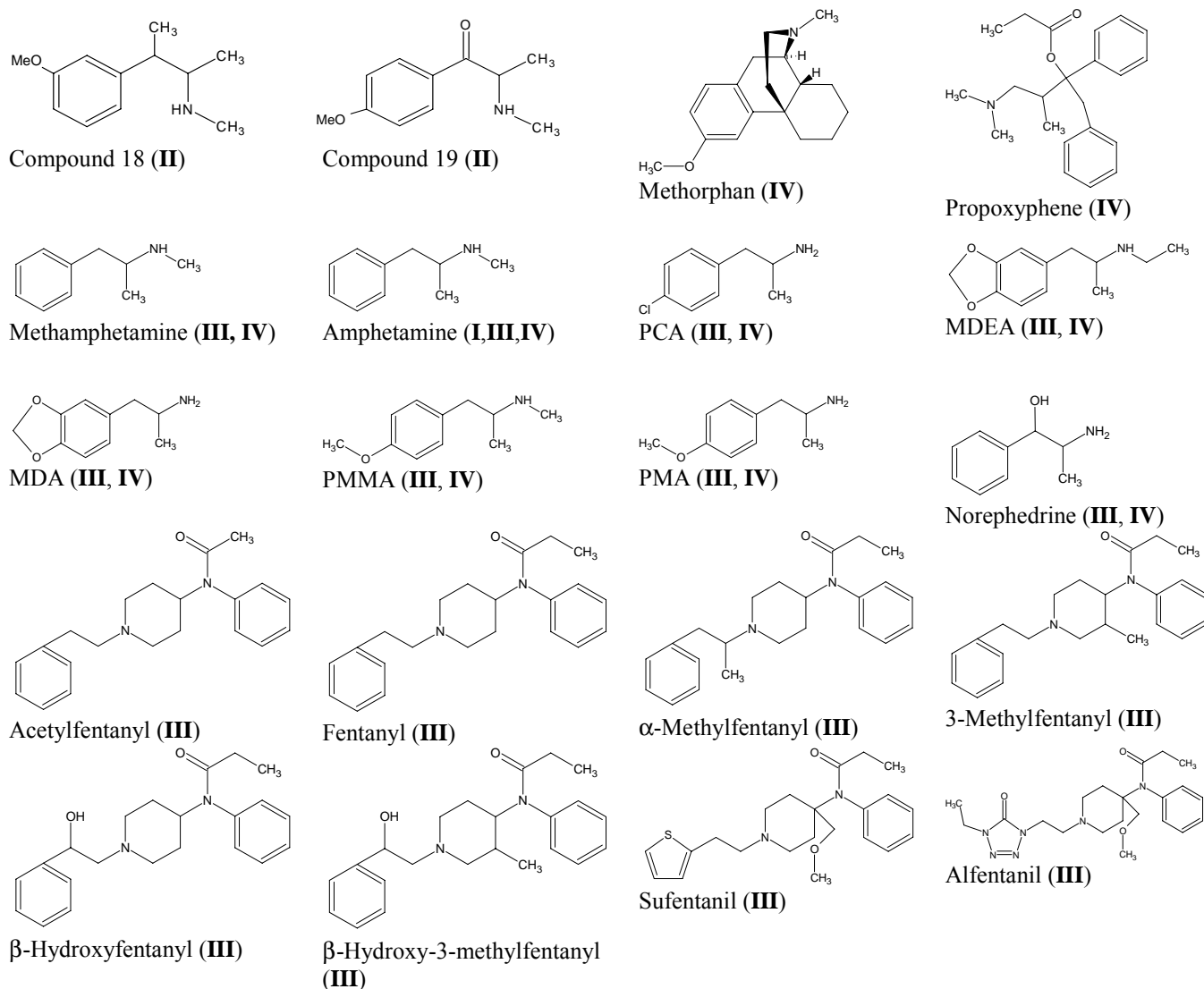


Figure 1. Chemical structures of the studied compounds (continued).

Table 5. Instrumentation used in the experimental work.

Instrument	Model/type	Manufacturer	Paper
Liquid chromatograph	LC 1100 with vacuum degasser, autosampler and DAD	Agilent Technologies (USA)	I, II, IV
ESI/ion trap mass spectrometer	Agilent 1100 Series LC/MSD Trap, SL	Agilent Technologies (Germany)	I, II, IV
AP-MALDI/ion trap mass spectrometer	Esquire 3000+ with AP-MALDI ion source	Bruker Daltonics (Germany) and Agilent Technologies (USA)	III
Eluent flow splitter	Accurate, with 1/10 and 1/100 split ratio	LC Packings (USA)	I, II, IV
Nitrogen generator	Model 75–72, for drying and/or nebulising gas	Whatman (USA)	I, II, III, IV
pH meter	MP 220, Inlab 422 electrode	Mettler Toledo GmbH (Switzerland)	IV

4.3. LC-ESI/MS/MS (I, II, IV)

For LC-ESI/MS/MS, the stock solutions of drugs and synthesis products (1.0 to 7.0 mg/ml) were prepared in either methanol (MeOH) (**I, IV**) or in MeOH/H₂O (1/1, v/v) (**II**). The dilutions for LC analyses were made with 0.1% formic acid (0.1% FA) (**I**) or deionised water (**II, IV**). Dilutions of 10–50 µg/ml were used in optimising the separations (**I, II, IV**) and in fragmentation studies of the compounds (**I, II**), and further dilutions were used in quantitative method development (**I**), testing of methods and determining the LODs (**I, II, IV**).

The actual sample material (**I, II, IV**) was dissolved in MeOH, sonicated for 5–10 minutes, diluted with deionised water, and filtered (GHP Acrodisc, Pall Gelman Laboratory, Ann Arbor, USA) into autosampler vials. All solutions were stored at -20°C. In quantitative analyses the samples were weighed; in qualitative analyses an appropriate amount, e.g. 15 mg, was taken for the analysis.

The achiral gradient LC separations (**I, II**) were initially modelled with chromatography simulation software (DryLab 2000 chromatography optimisation software v3.00.06, LC Resources Inc., Walnut Creek, CA, USA). The data obtained from two model runs with different linear gradients with all compounds to be separated was used as input data for the software. The final gradient conditions were developed with the help of the modelling.

The MS/MS mode in which the fragmentation energy is increased during the MS/MS spectral acquisition, the so-called "SmartFrag" option, was adopted as the technique for acquiring MS/MS spectra. In this mode the fragmentation energy is increased in the selected range, either to the set upper limit or until there is no more precursor ion left in the ion trap. In this work the selected voltage was 1.0 V and the range was set to 30%–200% of this voltage. This technique was used to collect MS/MS library spectra and was used in all MS/MS methods in this work (**I–IV**) with the above-mentioned settings.

4.4. AP-MALDI/MS and AP-DIOS/MS (III)

A gold-coated 96-target plate was used in the MALDI experiments. For DIOS the unmodified porous silicon (pSi) spots were prepared by electrochemical etching as described in detail by Tuomikoski et al.[106] The DIOS chips were rinsed with hydrofluoric acid, stored in glass containers under ethanol and were allowed to dry in air before sample application.

The stock solutions of amphetamines and fentanyls were prepared in MeOH (1 mg/ml) and the dilutions were made with deionised water and MeOH to obtain MeOH/water 1/1 (v/v) solutions. The MALDI matrix, α -cyano-4-hydroxycinnamic acid (α -CHCA), was used as a saturated solution dissolved in ACN/MeOH/0.1% trifluoroacetic acid (TFA) in water (2/1/0.1 v/v/v). Samples of 0.4 µl were applied to the spots on the DIOS plate and samples of 0.6 µl (including the matrix) to the MALDI plate. The spots were allowed to dry in ambient air before analysis.

4.5. Chiral LC–UV–ESI/MS (IV)

Reversed-phase (RP) and polar ionic mode (PIM) eluents were prepared daily. The pH of the RP eluents was adjusted with acetic acid. Whenever a new eluent system was introduced the columns were stabilised for 30 to 90 minutes.

The chromatographic behaviour of the compounds was initially studied using stock solutions and UV detection ($\lambda = 233, 258$ and 285 nm). The best chromatographic separations for methorphan, propoxyphene and amphetamines were optimised for higher injection volume ($20 \mu\text{l}$) and the buffer systems were optimised for ESI/MS detection.

5. RESULTS AND DISCUSSION

The main results of the studies are shortly described in this chapter. LC–ESI/MS/MS methods were developed for quick analysis of a complex mixture of analytes of differing chemical character and for discrimination of mass equivalent substances. High throughput tandem mass spectrometric screening methods relying on AP-MALDI and AP-DIOS interfaces were successfully applied to amphetamines and fentanyl and a direct chiral LC–MS system based on β -CD was found for the chiral separation of amphetamines. For more details the reader is kindly asked to refer to the original publications **I–IV**.

5.1. LC–ESI/MS/MS in fast analysis of a complex mixture (I)

A seized drug unit is likely to contain numerous components: the principal drug of action along with various adulterants and diluents of different chemical character. To test the suitability of LC–ESI/MS in forensic analysis, a representative heterogeneous group of 14 compounds was selected: acidic, basic and neutral compounds, stable and labile compounds, and compounds with the same nominal masses (**Figure 1**). The drugs included thermolabile compounds such as LSD, temazepam, psilocybin and buprenorphine, which, without derivatisation, tend to be difficult or impossible to analyse by conventional GC–MS.

Positive ion mode was chosen, since with suitable mobile phase composition, it is then possible to ionise not only basic compounds but also neutral and acidic compounds. All the compounds of interest were efficiently ionised with the chosen eluent system of MeOH, water and 0.1% FA. All mass spectra showed an abundant $[M+H]^+$ ion, which was selected as the precursor ion for MS/MS analysis. In addition, the mass spectra of the neutral compounds, *i.e.* testosterone, nandrolone and metandienone, showed relatively intense sodium adduct ion, $[M+Na]^+$. A weak $[M+Na]^+$ ion was recorded for temazepam. The mass spectra of salbutamol, phenobarbital, AM and 3,4-MDMA showed some fragment ions. Tandem mass spectrometry was chosen because the chromatography was designed to be fast, without complete separation of the compounds.

The MS/MS spectra of all compounds showed characteristic behaviour allowing identification of the compounds. One characteristic and abundant product ion was chosen for quantitative analysis. The use of monolithic column, non-linear gradient elution and MS/MS allowed the analysis of all 14 compounds within five minutes, including stabilisation time before the next injection. Although many of the compounds were not fully separated in the LC step, as can be seen in **Figure 2**, all compounds were unambiguously identified with MS/MS. Gradient elution was necessary because of wide variation in the polarity of the compounds. The capacity factors, k , with the LC method employed were acceptable, ranging from 2.1 to 9.7 ($t_0 = 0.212$ min). This meant that salts and any other compounds eluting without retention were separated from the compounds of interest and the suppression caused by these other compounds in ESI was minimised. The relative standard deviation (RSD) of the retention times was 0.45–4.8%, indicating good reproducibility of the LC system.

Suitability of the MS/MS spectra and retention times in the library for the identification of the compounds was tested with 476 injections of standard samples with concentrations varying between 0.1 and 40 $\mu\text{g/ml}$. The search algorithm compares the unknown spectrum with the spectra in the library (Fit) and the spectra in the library with the unknown (Rfit), and from this

it generates a purity value. The maximum purity value is 1000, indicating perfect correlation between measured and library spectra. The purity values were clearly better for concentrations above than below 0.3 $\mu\text{g/ml}$ (640–900 above and 354–868 below). This was mainly due to higher background interference and lower repeatability of the relative abundances of the product ions at concentrations below 0.3 $\mu\text{g/ml}$. The results showed the necessity of including retention times to the library search, though they were less critical for the compounds producing several abundant product ions (morphine, buprenorphine, testosterone, stanozolol) than for compounds producing one very intense product ion and only a few other weak ions (amphetamine, 3,4-MDMA). Combining retention time and MS/MS spectral information in the library search improved the reliability of the identification significantly. Owing to good reproducibility of the LC separation, all the compounds were found within a very narrow retention time window (± 2.5 seconds). The library matches were also evaluated with 50 seized samples and pharmaceutical preparations with concentrations varying from 0.2 $\mu\text{g/ml}$ to a very high 3.75 mg/ml. The compounds, which had first been identified by conventional methods were all identified unambiguously by the new LC–MS/MS method with library search. For all samples, the correct compound was the first one in the MS/MS library hit list and was found within $\pm 3\%$ retention time window. The retention time window was larger for authentic sample material because of the wider concentration range.

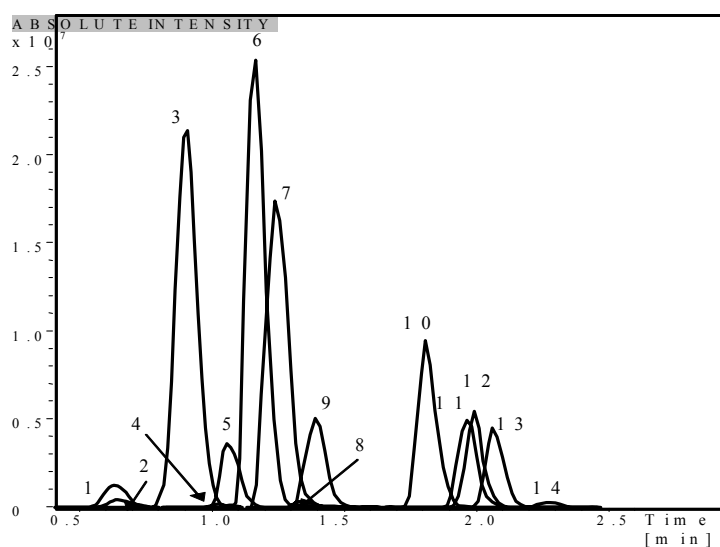


Figure 2. One point smoothed extracted ion chromatograms of the target (MRM) ions. Compounds are 1 Psilocybine, 2 Morphine, 3 Salbutamol, 4 Amphetamine, 5 3,4-MDMA, 6 Clenbuterol, 7 LSD, 8 Phenobarbital, 9 Buprenorphine, 10 Temazepam, 11 Nandrolone, 12 Metandienone 13 Testosterone, 14 Stanozolol. The concentration of each substance is 12 – 15 $\mu\text{g/ml}$.

Additionally, the suitability of the method was investigated for quantitation purposes. An external standard method was chosen in view of the widely varying chemical and physical properties of the compounds. The method was evaluated by determining linearity, repeatability and limits of detection (signal-to-noise ratio ≥ 3). The correlation coefficients (r) were between 0.993 and 0.999 within the concentration range of 0.1–30 $\mu\text{g/ml}$ ($n=6$) indicating good linearity of the method. The linearity of LSD, nandrolone and buprenorphine began to deteriorate at concentrations above 40 $\mu\text{g/ml}$ owing to saturation of the surface of the electrically charged droplets in ESI [183]. LODs for all compounds ranged between 10 and 50 ng/ml, which was considered a sufficient level for this type of forensic analysis. Within-day reproducibility of the method was evaluated at three concentration levels (3.0, 10 and 30

µg/ml; five injections each). The RSDs were typically below $\pm 20\%$, but clearly higher for phenobarbital ($\pm 28.8\%$) and AM ($\pm 25.9\%$). The repeatability of the method was considered acceptable for purposes of forensic analysis, although it suffered from a limited number of data points due to co-eluting compounds and peaks that were only two to seven seconds wide. With the settings employed, one data point was collected in about 400 ms for one parent ion [i.e. accumulation time (up to 10 ms) + the scan time for m/z 50–500 (30.4 ms) + isolation time (290 ms) + fragmentation time (40 ms) + scan time (30.4 ms)]. If more than one compound is analysed within same cycle, the number of data points is decreased and may not be sufficient for reproducible integration of the peaks. With the ion trap employed, the time needed for one data point cannot be decreased from the values mentioned.

5.2. LC-ESI/MS/MS in fast analysis of mass equivalent substances (II)

A method relying on fast liquid chromatographic separation and tandem mass spectrometry was developed to distinguish the main ecstasy component 3,4-MDMA from a set of 17 mass equivalent isomers and isobaric substances including side chain regioisomers and different ring substitution patterns (**Figure 1**). Isobaric substances are defined as compounds of the same nominal mass but of different elemental composition [184]. The original hope was to develop a method not requiring prior LC separation, but this did not prove to be possible.

The mass spectra of the 18 compounds (numbered 1–6 and 8–19, **Figure 1**) showed a strong protonated molecule at m/z 194 and one or two fragment ions. No adduct ions were seen. The main fragment ion depended on the length of the alkyl chain at nitrogen and was at m/z 177, 163 or 149. As an exception, compound 19 produced a main fragment at m/z 176. The controlled drug 3,4-MDMA (numbered compound 3) produced m/z 194 and a fragment ion at m/z 163, as did compounds 8, 11, 13, 16, 17 and 18. Thus, compounds 8, 11, 13, 16, 17 and 18 had the same main fragment ion as 3,4-MDMA.

Tandem mass spectrometry (MS/MS) was applied to enhance specificity, with the strong protonated molecule, $[M+H]^+$, at m/z 194 chosen as the precursor ion. The MS/MS library spectra were produced and the experiments carried out by increasing the fragmentation amplitude during the spectral acquisition. Increasing the fragmentation energy made comparison of the stabilities of the molecules impossible and 3,4-MDMA could only be distinguished from the other compounds on the basis of unique product ions or distinct intensity patterns.

Similarly to the mass spectra the MS/MS experiments allowed a division of the compounds into four groups according to the main product ion appearing in the MS/MS spectra: 1) compounds that produced an ion at m/z 177 by the loss of ammonia, 2) compounds 3 (*i.e.* 3,4-MDMA), 8, 11, 13, 16, 17 and 18 which produced an ion at m/z 163 through the loss of methylamine, 3) compounds that produced an ion at m/z 149 through the loss of primary ethylamine or secondary dimethyl amine, and 4) the keto-compound 19 that produced an ion at m/z 176 through the loss of water. Thus, 3,4-MDMA was distinguished from the compounds in groups 1, 3 and 4 according to the MS/MS spectra. Within the group 2, 3,4-MDMA could be distinguished from compounds 16 and 17, which showed an additional product ion at m/z 121, most likely due to migration of the methyl group in the benzylic species to form the methoxybenzyl or methoxytropylium ion. 2,3-MDMA (compound 8), which was structurally the most similar to 3,4-MDMA, produced a product ion at m/z 135 which was not seen for 3,4-MDMA, and thus these compounds were distinguished. When

3,4-MDMA was analysed with the settings used in the library search (Sect. 5.1.) but with direct introduction of the sample the library gave seven different hits, including all the compounds of group 2, which produced the same intense product ion from the same precursor (m/z 194 \rightarrow m/z 163).

Misidentifications occurred because the main product ion was the only abundant ion in the MS/MS spectra of the compounds in group 2 and it affected the search algorithm. Hence, with the library settings employed, unambiguous identification of 3,4-MDMA requires chromatographic separation before MS/MS analysis. A fast LC separation was accordingly optimised to separate 3,4-MDMA from the six other compounds of group 2. The separation was designed so as to give the greatest possible resolution (R_S) for 3,4-MDMA within a few minutes runtime. **Figure 2** shows the LC separation of the seven compounds of group 2 in black. The other 11 compounds studied are shown in grey.

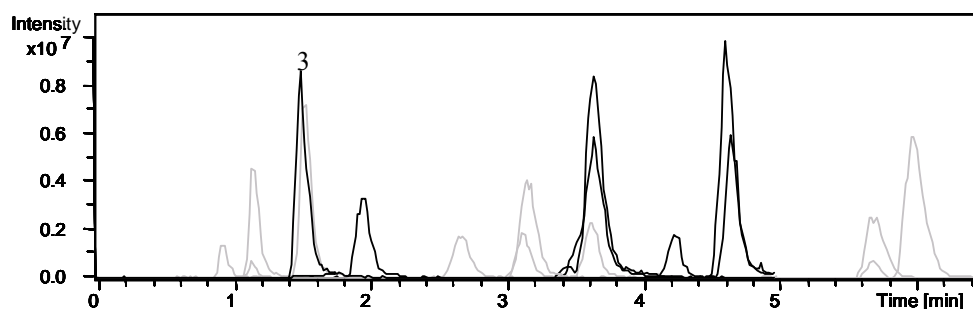


Figure 3. LC separation of the 18 compounds with equivalent mass, including 3,4-MDMA. The chromatogram for transition m/z 194 \rightarrow m/z 163 for the seven compounds in group 2 is shown in black and the other transitions in grey. The peak of 3,4-MDMA is marked with number 3. The concentrations of the compounds were 10 or 20 μ g/ml.

Although not all of the substances could be identified, application of the developed LC–ESI/MS/MS method and automated library search allowed confirmation of the presence of 3,4-MDMA in the presence of 17 of its mass equivalent substances. From 11 of the compounds it was quickly distinguished by MS/MS with library search. From the six interfering compounds with similar fragmentation pattern, 3,4-MDMA ($t_R = 1.51$ min) was separated within a five minute LC run. Compound 19 co-eluted with 3,4-MDMA, and co-elution can cause a mixture spectrum. However, compound 19 was easily recognised as the only compound that produced unique product ions with intensive fragment ion at m/z 176.

5.3. High-throughput screening by AP-MALDI/MS/MS and AP-DIOS/MS/MS (III)

With the continuing demand of analytical laboratories for new, fast and cost effective techniques, a preliminary study was carried out on the applicability of new atmospheric pressure desorption ionisation techniques in detection and identification of illicit drugs. The new techniques matrix assisted laser desorption ionisation (MALDI) and desorption ionisation on silicon (DIOS) at atmospheric pressure were investigated with MS/MS. MALDI and DIOS techniques are attractive to analytical laboratories requiring high sample throughput, because analyses are extremely fast and are easily automated. In view of their current importance, the investigation was carried out with amphetamine derivatives and fentanyl as model compounds.

The AP-MALDI/MS and AP-DIOS/MS parameters were optimised to maximise the absolute abundance of the protonated molecule. The effect of temperature of the drying gas in the ion source was tested between 45 and 150°C, and the maximal absolute abundance of the protonated molecule was found at 45°C. Although MDA, PMA and NE fragmented strongly even at this low temperature, the protonated molecule was still abundant. Increase of the temperature to 100°C increased fragmentation but still had no significant effect on the absolute abundances of the protonated molecules. The fragmentation increased further when the temperature was raised to 150 °C. High temperatures in the AP-MALDI ion source or ion optics have been applied in peptide analyses to prevent analyte–matrix cluster ion formation [27,185]. AP-MALDI spectra of the test compounds measured at 45 °C did not show any cluster ions, however, and the same conditions were used for both MALDI and DIOS techniques. Under these optimised conditions and with the plate operated in fixed position, the signal of the protonated molecule of 3-MF (18 pmol) lasted for about 10 minutes in DIOS experiments and about 5 minutes in MALDI experiments. The plate can also be moved spirally during the laser pulses. In spiral mode the signal lasted over 30 minutes in DIOS experiments and over 20 minutes in MALDI experiments. Such a long-lasting signal allows sequential analysis, such as is needed in optimisation of laser pulse energy and in MS and MSⁿ analysis from a single spot.

All mass spectra of the compounds exhibited abundant protonated molecules. With AP-DIOS the background disturbances were minimal. Although the relative abundances of the matrix background ions (e.g. m/z 379, 172, 144) were clearly higher with AP-MALDI, they still did not interfere with the analyses. The α -CHCA matrix is known to interfere with analyses of some low molecular mass compounds [186]. All the compounds studied have high proton affinity and their ionisation efficiency in DIOS and MALDI was very good, therefore. The high ionisation efficiency of such compounds was in good agreement with earlier studies [122]. The fragmentation was minimal for all amphetamine derivatives except MDA, PMA and NE, which showed a few intense fragment ions. Likewise, among the fentanyls, 3-MF, α -methylfentanyl, sufentanil, alfentanil, and β -hydroxyfentanyl showed little dissociation with DIOS/MS.

Mixtures of all nine amphetamine derivatives were analysed by AP-MALDI/MS/MS and AP-DIOS/MS/MS to determine the applicability of the techniques in detection and identification of a single analyte in a sample containing several compounds of the same type. The protonated molecules of the compounds (**Figure 4**) were isolated automatically in sequence and fragmented by MS/MS during one run from one spot. The MS/MS spectra obtained with AP-MALDI and AP-DIOS (**Figure 5**) were compared against an in-house reference ESI–MS/MS spectral library created earlier with use of the same type of mass spectrometer and similar fragmentation conditions.

The AP-MALDI/MS/MS and AP-DIOS/MS/MS spectra of the amphetamine derivatives are presented in **Figure 5**. The MS/MS fragmentation of the protonated molecule was similar to that produced by ESI (**IV**). The AP-MALDI/MS/MS and AP-DIOS/MS/MS spectra showed the same intense product ion due to the alkylamine loss as was described for 3,4-MDMA isomers in Sect. 5.2. Fentanyls fragmented in MS/MS experiments mainly through neutral loss of the amide moiety (route A) and neutral loss of the amine moiety (route B). The third important fragmentation route was the cleavage of the piperidine ring and loss of the propionyl group (route C) (**Figure 3** in **III**). The fentanyls including a hydroxy group, i.e. β -hydroxyfentanyl and β -hydroxy-3-methylfentanyl, produced abundant product ions upon loss

of water, m/z 335 and m/z 349, respectively. Sufentanil produced abundant product ion with m/z 355 upon loss of sulphur.

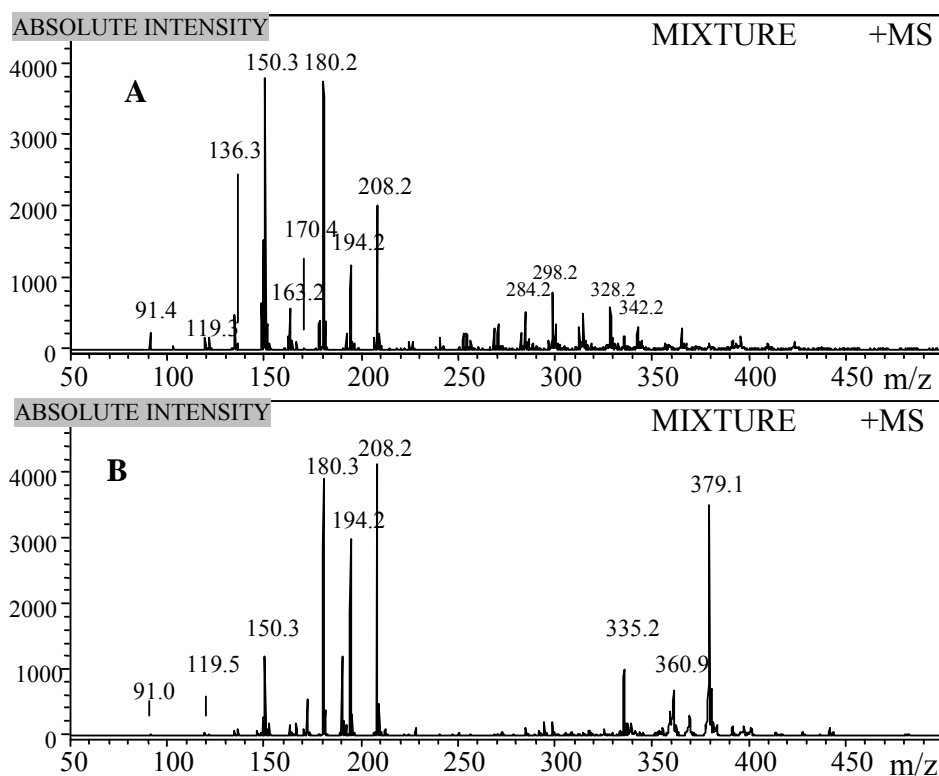


Figure 4. (A) AP-DIOS and (B) AP-MALDI mass spectra for a mixture of amphetamine derivatives: AM (m/z 136), MA (m/z 150), PMA (m/z 166), PCA (m/z 170), PMMA (m/z 180), MDA (m/z 180), MDMA (m/z 194), MDEA (m/z 208)(120 pmol) and NE (m/z 152)(290 pmol)

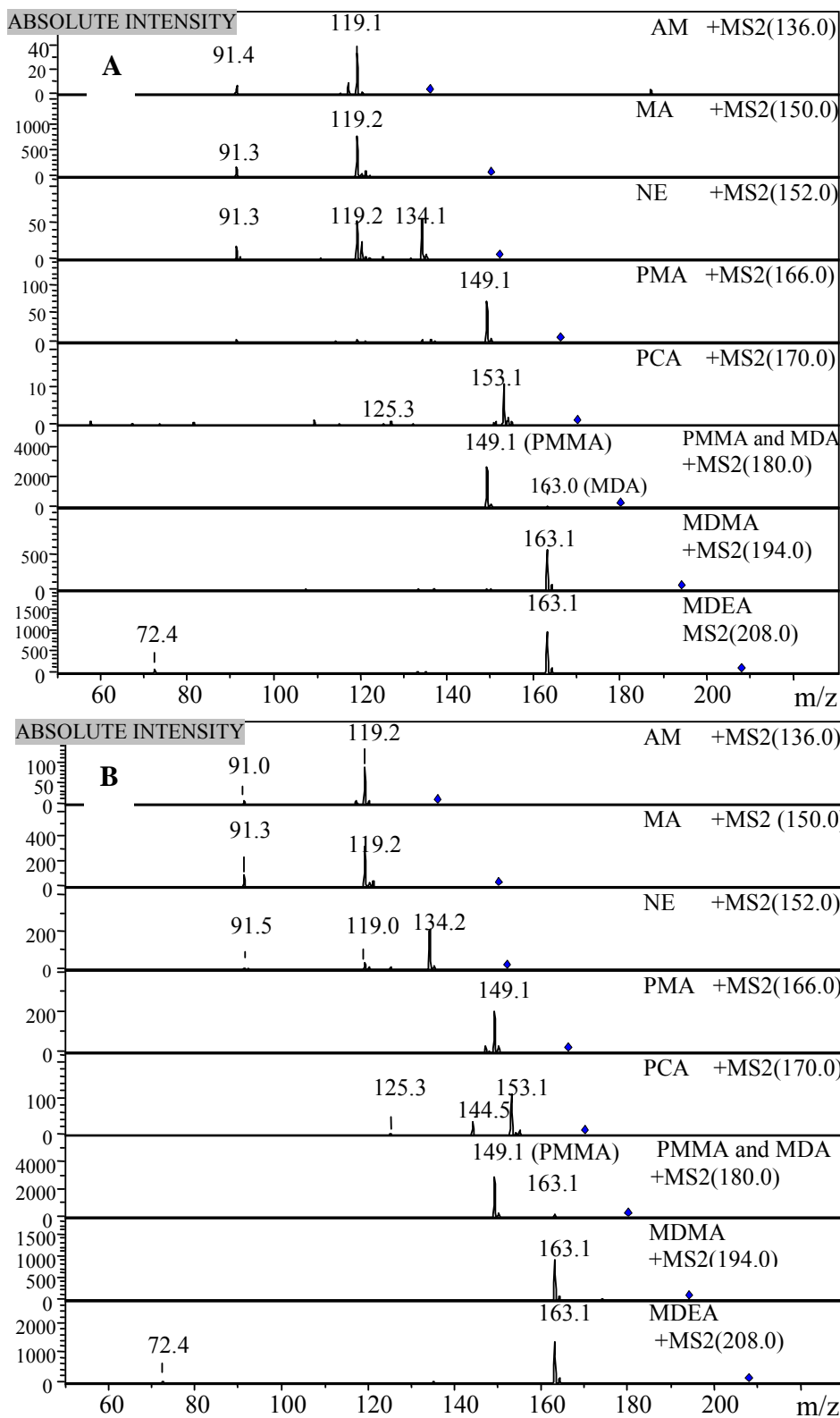


Figure 5. MS/MS spectra measured by AP-DIOS (**A**) and AP-MALDI (**B**), for a mixture of amphetamine derivatives: AM, MA, PMA, PCA, PMMA, MDA, MDMA, MDEA concentrations of 120 pmol and NE concentration of 290 pmol. The diamonds indicate the precursor ion.

All MS/MS spectra measured in this work corresponded well with the library spectra obtained by ESI under similar conditions. Although PMMA and MDA have a common precursor ion (m/z 180), they could be identified unambiguously by means of their specific product ions, m/z 163 for MDA and m/z 149 for PMMA. The difference in the intensities of the product ions (**Figure 5**) is evidently due to the dissociation of MDA in the ion source, which also contributed to its higher LOD. The MS/MS spectra of fentanyls showed more diagnostic and specific product ions than those of amphetamine derivatives, and also all fentanyls studied were identified unambiguously. Even 3-MF and α -methylfentanyl, which have a common precursor ion (m/z 351), were distinguished by MS/MS, as they produced different and specific product ions: 3-MF produced m/z 230 and α -methylfentanyl m/z 216, both by route B. Where compounds have the same precursor and the same product ions, as some of the compounds in study **II**, these techniques cannot provide sufficient selectivity.

The LODs (signal-to-noise ratio 10) for amphetamine derivatives and fentanyls measured by AP-DIOS/MS/MS were between 100 and 1000 ng/ml (1 and 3 pmol) indicating excellent sensitivity of the technique. The good ionisation efficiency is due to high proton affinity of the amino groups of the compounds. The same was recognised in earlier work where propranolol was investigated by AP-DIOS [122]. For MA, 3,4-MDMA and 3-MF, the LODs were also measured by AP-MALDI, and they were five to ten times higher than with DIOS indicating better sensitivity of AP-DIOS. The quantitative repeatability of AP-DIOS/MS/MS was only tentatively investigated by measuring the absolute abundance of the main product ion of 3-MF (m/z 351 \rightarrow m/z 202). The relative standard deviation (RSD) from five (3+2) sample spots (110 pmol) on two DIOS chips was relatively good at 16%. This level of repeatability is quite sufficient for qualitative forensic target analyte screening.

Also seized samples of fentanyls and amphetamines were analysed to assess the suitability of the techniques for the analysis of real forensic samples. An example of an analysis of an ecstasy tablet is shown in **Figure 6**. The AP-MALDI/MS and AP-DIOS/MS spectra of the tablet were clean and showed a signal at m/z 194 and no signals from other active substances. The matrix constituents in the tablet did not disturb the analyses, nor did the α -CHCA matrix in AP-MALDI. The MS/MS spectrum showed an abundant ion at m/z 163 and a minor ion at m/z 58, and the compound was identified by library search as 3,4-MDMA.

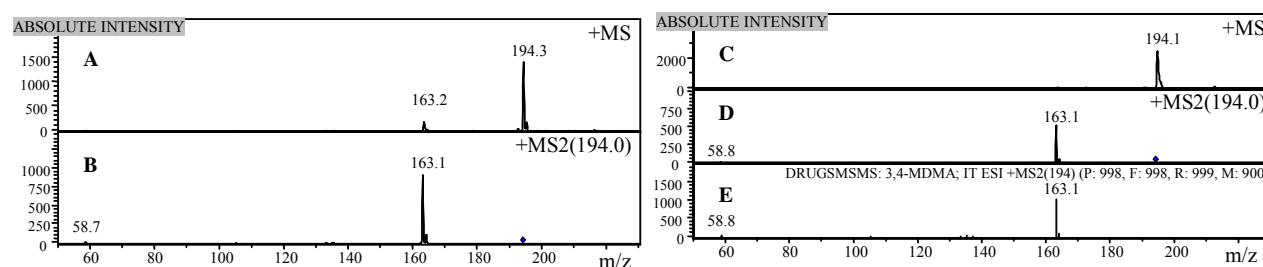


Figure 6. AP-DIOS/MS (**A**), AP-DIOS/MS/MS (**B**), AP-MALDI/MS (**C**) and AP-MALDI/MS/MS (**D**) spectra of a powder sample containing 3,4-MDMA, and MS/MS library spectrum of 3,4-MDMA (**E**).

The limits of detection obtained with AP-DIOS/MS/MS varied between 0.1 and 1 μ g/ml, which means that with the sample preparation technique employed (only 1 mg was weighed) the lowest detectable amount of the analyte in drug seizure samples is between 0.5 and 5 μ g/mg. The concentration of the active substance in ecstasy tablets is commonly between 200

to 600 µg/mg, that is more than 80 times higher than the limit of detection. This and the analysis of the seized samples clearly show that AP-DIOS/MS/MS is suitable for the analysis of amphetamines and fentanyl present in seized samples at low concentrations.

5.4. Effect of eluent in direct chiral LC separation of amphetamine derivatives (IV)

Because some compounds are controlled only as one optical isomer, determining the licit or illicit nature of a sample sometimes depends on chiral separation. Within the EU, however, all of the studied amphetamine derivatives and their enantiomers are controlled drugs, except NE which is controlled only as a precursor for preparing illicit drugs [187].

Methorphan, propoxyphene and MA were used as model compounds in a comparison of 46 eluent compositions tested in the development of chiral LC–MS methods. Vancomycin, a novel chiral stationary phase for these compounds and the more commonly used β-CD were evaluated as chiral stationary phases (CSP). Both can be used in normal-phase, reversed-phase or polar organic phase modes. Because only polar eluent systems with high enough conductivity are suitable for ESI, nonpolar eluent systems were not tested.

Only the enantiomer separation of amphetamine derivatives is discussed here; the results for methorphan and propoxyphene can be found in the original manuscript (IV). Development of a method for chiral separation is a time-consuming and highly empirical process because it is difficult to predict the selectivity of analytes on commercial CSPs. The results for MA were used in choosing suitable conditions for the eight other amines, but it was kept in mind that closely related molecules do not necessarily behave similarly in chiral separation systems. The eluent systems investigated are presented in **Table 6**.

5.4.1. Reversed-phase and polar ionic modes with vancomycin column

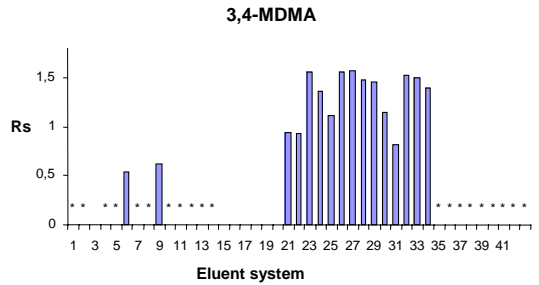
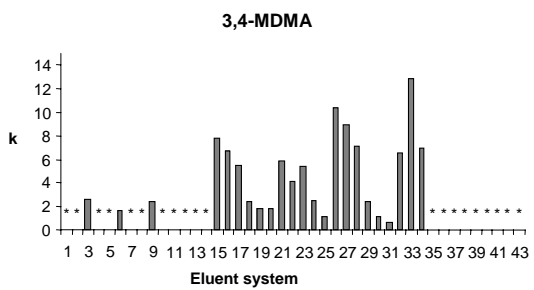
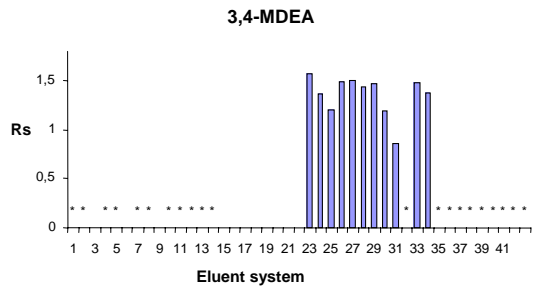
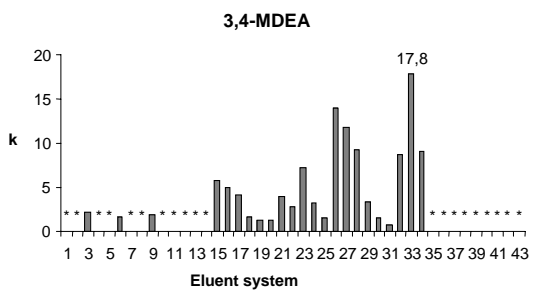
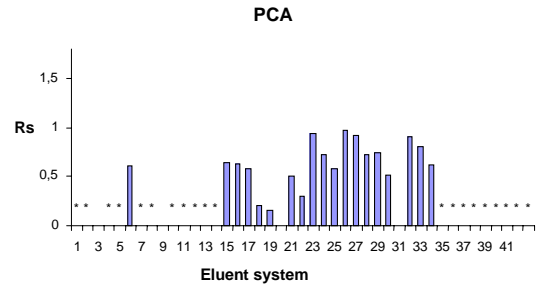
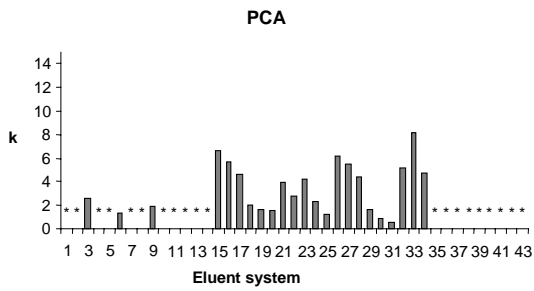
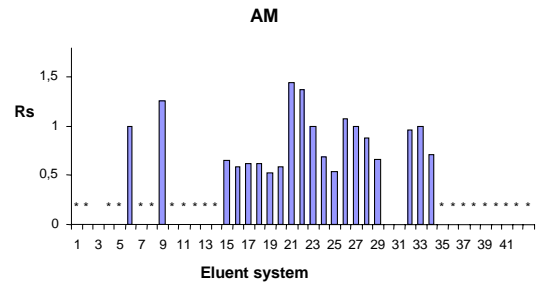
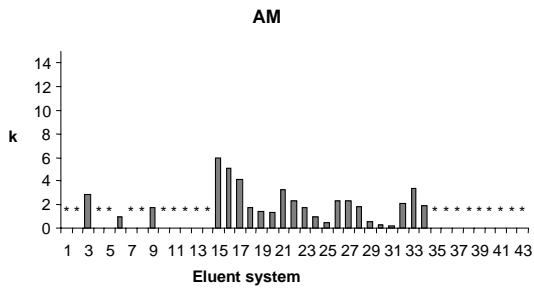
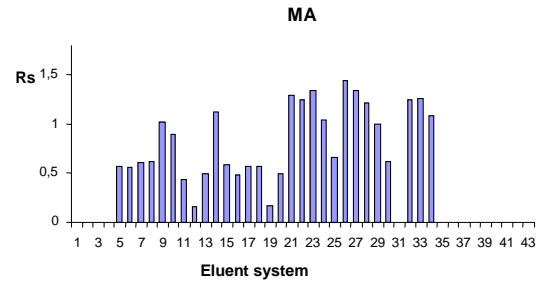
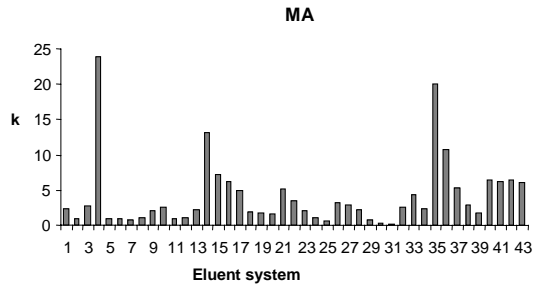
The effect of the eluents 1–14 (**Table 6**) on the resolution and retention of enantiomers of MA was studied in the reversed phase (RP) mode with the vancomycin column. The results indicated that MeOH and *i*-PrOH provide enantiomer separation, while ACN does not. The result for ACN was confirmed by running all the amphetamine derivatives with 70/30 ACN/0.1% TEAA. As with MA, none of the enantiomers of the amphetamine derivatives were separated. Plots showing the effect of the amount of organic modifier on retention were U-shaped as is typical for glycopeptide columns (**Figure 7**)[54].

The best enantiomer separation for MA was obtained with the eluents 9, 10 and 14. However, owing to the more nonpolar character of *i*-PrOH (14) than of MeOH, the retention times were significantly longer than those with the eluents 9 and 10, and also the backpressure was higher. The best enantiomer separation for MA with a reasonable retention factor ($k = 2.1$) was achieved with eluent 9 (80% MeOH), and this was chosen for further studies. Eluent 9 also showed acceptable resolution for all the other amphetamines, except for the enantiomers of PCA, MDEA and NE. The latter two compounds were not enantiomer separated on the vancomycin column with any solvent composition. The best separation for PCA was obtained with eluent 6 ($R_s = 0.6$). The resolution and retention factors for the amphetamines with all eluent systems are presented in **Figure 7**.

Table 6 The eluent systems investigated in reversed-phase (RP) and polar ionic modes (PIM) with vancomycin and β -CD columns.

No.	Vancomycin (RP)	No.	β -CD (RP)
1	ACN/0.1% TEAA 30/70, pH 4	23	ACN/1.0% TEAA 5/95, pH 4
2	ACN/0.1% TEAA 50/50, pH 4	24	ACN/1.0% TEAA 10/90, pH 4
3	ACN/0.1% TEAA 70/30, pH 4	25	ACN/1.0% TEAA 15/85, pH 4
4	ACN/0.1% TEAA 90/10, pH 4	26	MeOH/1.0% TEAA 5/95, pH 4
5	MeOH/0.1% TEAA 30/70, pH 4	27	MeOH/1.0% TEAA 10/90, pH 4
6	MeOH/0.1% TEAA 40/60, pH 4	28	MeOH/1.0% TEAA 15/85, pH 4
7	MeOH/0.1% TEAA 50/50, pH 4	29	i-PrOH/1.0% TEAA 5/95, pH 4
8	MeOH/0.1% TEAA 70/30, pH 4	30	i-PrOH/1.0% TEAA 10/90, pH 4
9	MeOH/0.1% TEAA 80/20, pH 4 (5, 6, 7)	31	i-PrOH/1.0% TEAA 15/85, pH 4
10	MeOH/0.1% TEAA 90/10, pH 4	32	ACN/100 mM NH ₄ OAc 5/95, pH 4
11	i-PrOH/0.1% TEAA 30/70, pH 4	33	MeOH/100 mM NH ₄ OAc 5/95, pH 4
12	i-PrOH/0.1% TEAA 50/50, pH 4	34	MeOH/100 mM NH ₄ OAc 15/85, pH 4
13	i-PrOH/0.1% TEAA 70/30, pH 4		
14	i-PrOH/0.1% TEAA 90/10, pH 4		
No.	Vancomycin (PIM)	No.	β -CD (PIM)
15	MeOH/ACN/HOAc/TEA 70/30/0.03/0.02	35	MeOH/ACN/HOAc/TEA 10/90/0.03/0.02
16	MeOH/ACN/HOAc/TEA 70/30/0.03/0.04	36	MeOH/ACN/HOAc/TEA 15/85/0.03/0.02
17	MeOH/ACN/HOAc/TEA 70/30/0.06/0.02	37	MeOH/ACN/HOAc/TEA 20/80/0.03/0.02
18	MeOH/ACN/HOAc/TEA 70/30/0.5/0.05	38	MeOH/ACN/HOAc/TEA 25/75/0.03/0.02
19	MeOH/ACN/HOAc/TEA 70/30/0.6/0.2	39	MeOH/ACN/HOAc/TEA 30/70/0.03/0.02
20	MeOH/ACN/HOAc/TEA 70/30/0.9/0.1	40	MeOH/ACN/HOAc/TEA 15/85/0.3/0.2
21	MeOH/HOAc/TEA 100/0.03/0.02	41	MeOH/ACN/HOAc/TEA 15/85/0.3/0.4
22	MeOH/HOAc/TEA 100/0.06/0.02	42	MeOH/ACN/HOAc/TEA 15/85/0.6/0.2
		43	MeOH/ACN/HOAc/TEA 15/85/0.9/0.1

The mode in which acid and base are used to adjust the ionisation state of the chiral selector has recently been designated the polar ionic mode (PIM) to distinguish it from polar organic phase (POP) mode where no acid or base is added to the organic eluent [188]. We use the new term here, though the old term was used in the original manuscript (**IV**). The polar ionic mode (PIM) was tested using the vancomycin column and eight different mobile phase compositions (eluent 15–22, **Table 6**). The initial mobile phase (eluent 15) was selected on the basis of an earlier study of clenbuterol [189] and this was modified by varying the acid/base ratio, and finally the MeOH/ACN ratio. In PIM, the acid/base ratio and the concentrations of acid and base are the most important parameters in controlling resolution and retention, respectively.



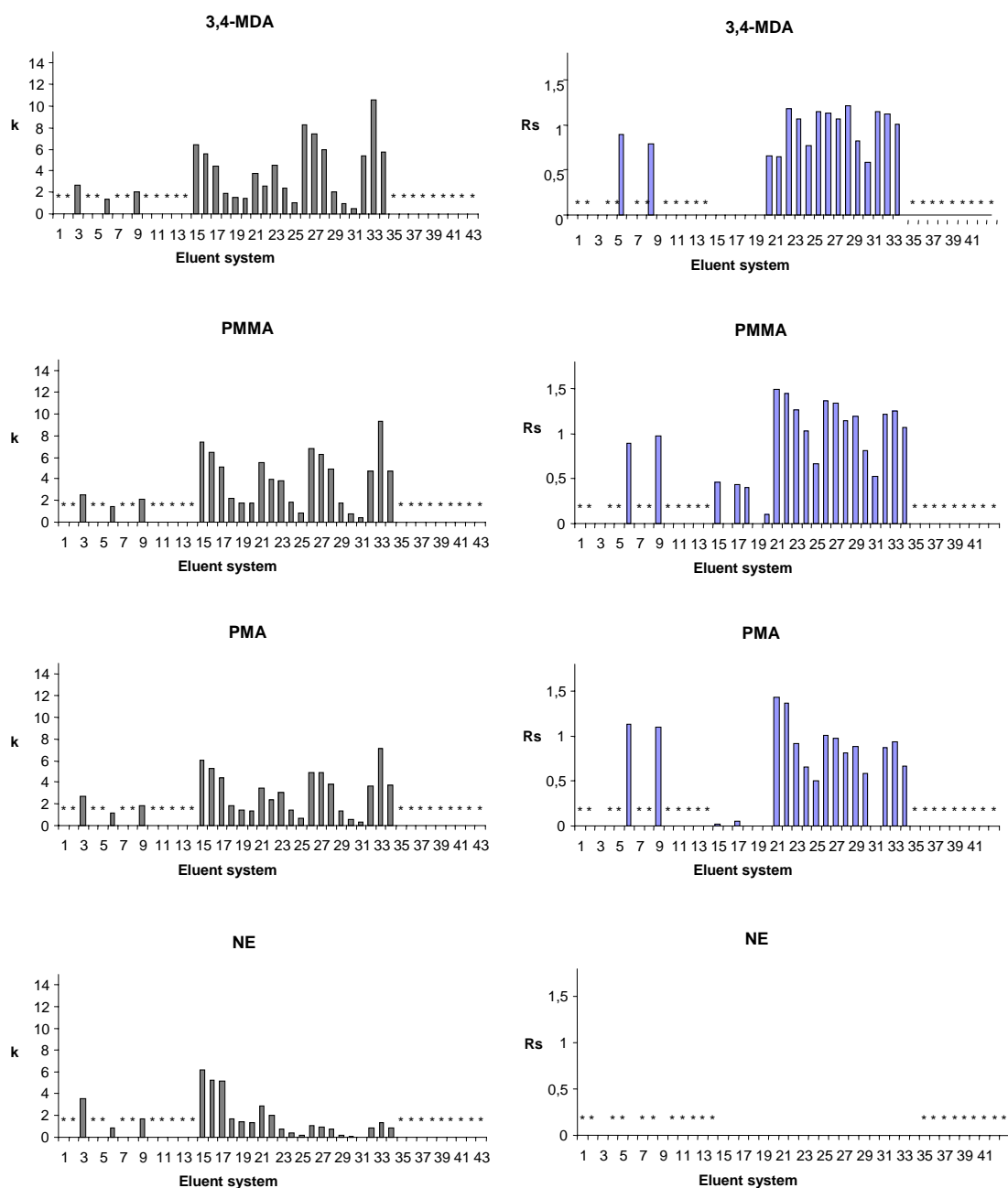


Figure 7. Retention factors and resolution of the amphetamine derivatives with the tested eluent systems. For explanation of the eluent systems, see **Table 6**. Systems 1–22 were run with vancomycin CSP and systems 23–43 with β -CD CSP. Eluent systems marked with an asterisk were not run.

The best resolution for MA ($R_s = 1.3$) with reasonable retention ($k=5.1$) was obtained with eluent 21. As can be seen, the resolution of the enantiomers of MA was decreased with an addition of acetonitrile (eluent 15–20) indicating the importance of hydrogen bonding capability of the hydroxyl group of MeOH.

The results obtained with MA were confirmed by running all the amphetamine derivatives with the eluents 15–22. Eluent 21 provided the best resolution, although MDEA and NE were not separated with any of these eluents (**Figure 7**). The importance of using 100% MeOH

instead of MeOH/ACN mixture was evident particularly for MDMA, MDA and PMA, which were not enantiomer separated with any of the eluents containing ACN but were resolved with 100% MeOH (eluents 21 and 22). An exception to other amphetamines was PCA which showed better separation with an increased amount of ACN (**Figure 7**).

5.4.2. Reversed-phase and polar ionic modes with β -cyclodextrin column

Use of the RP eluents 23–34 with the β -CD column provided an acceptable separation for MA. As in the earlier studies [84], it was clear that the amount of the organic modifier must be low (<15%) and the buffer concentration relatively high (1% TEAA) in order to achieve an acceptable resolution for amphetamine derivatives. The retention increased together with the resolution when the polarity of the solvent system was increased. The order of the elution strength of the organic modifier was *i*-PrOH > ACN > MeOH (**Figure 7**) indicating that the β -CD column, unlike vancomycin, behaves as an RP column.

The best results for MA were obtained with MeOH as organic modifier (eluents 26, 27 and 28). The best resolution with reasonable retention was achieved with eluent 26, which also provided acceptable resolution for all the other amphetamine derivatives except NE (**Figure 7**). Furthermore, this eluent enabled a chromatographic separation of the amphetamines and their enantiomers from each other allowing their analysis as a mixture by UV detection. The amphetamine derivatives eluted from the β -CD column in increasing order of size. Likewise, in an earlier study, with β -CD as a background electrolyte in CE, the migration order corresponded with increasing molecular mass (MDA < MDMA < MDEA) [73]. Sadeghipour et al. [87] and Brunnenberg and Kovar [47] reported that with RP mode and native β -CD, the R(–)-enantiomer always eluted before S(+). This was evidently also true in our experiments with β -CD.

Since MA showed no enantiomer separation on the β -CD column in PIM (**Figure 7**), the other amphetamine derivatives were not run in this mode.

5.4.3. Effect of molecular structure on resolution and comparison of methods

The effect of length of an alkyl chain near the chiral centre of the amphetamine derivatives on the enantiomer separation was studied by comparing MDA, MDMA and MDEA with each other, PMA and PMMA with each other and AM and MA with each other (**Figure 7**). The comparison with the vancomycin column in RP and PIM showed that, with two exceptions the enantiomer resolution decreases as the length of the alkyl chain increases and the ethyl substituent (MDEA) prevents the chiral separation totally. The reason may be that a longer alkyl chain causes increased steric hindrance for interaction between the amino groups of amphetamines and the acidic groups of vancomycin. The exceptions were MDMA and MDA in PIM. The effect of the alkyl chain length was not significant in enantiomer separation with β -CD. However, the size of the molecule had a significant effect on the retention, as the amphetamines eluted from the β -CD column in increasing order of size.

The effect of the electrophilic substituents in the aromatic ring of the amphetamine derivatives was studied by comparing AM, PMA, MDA and PCA with each other and MA, PMMA and MDMA with each other. With the vancomycin column in RP and PIM, the enantiomer

separation of the amphetamine derivatives was clearly influenced by the electrophilic substituents at the aromatic ring. The compounds without substituents (AM, MA) showed best resolution, but the methoxy compounds (PMA, PMMA) showed almost as good resolution. The dimethoxy substitution in MDA and MDMA decreased the resolution significantly, and chlorine totally prevented the separation of enantiomers of PCA with eluent 9 in the vancomycin column. These results suggest that π - π interactions between the aromatic ring of the amphetamines and vancomycin have an effect on the resolution. With β -CD column, the electrophilicity of the substituents in the phenyl ring affected the resolution only a little. Interestingly, NE, which has a hydroxyl group at the carbon adjacent to the chiral centre, did not show enantiomer separation with any of the methods tested.

The best single method for at least partial separation of most of the amphetamine derivatives was based on the β -CD column with RP eluent 26 (MeOH/1.0% TEAA 5/95). All amphetamine derivatives were separated except NE. The β -CD column with RP eluent 26 was also able to separate the amphetamines from each other far better than the vancomycin column with any eluent. However, the vancomycin column provided better enantiomer separation for AM, PMMA, and PMA as individual compounds (**Figure 7**).

5.4.4. Chiral LC-ESI/MS

After determination of the β -CD column with eluent system 26 as the best combination for the separation of enantiomers of amphetamine derivatives in a mixture, the suitability of this system was tested in LC-ESI/MS/MS detection. The method was not directly suitable since the high TEAA concentration (1%) caused severe suppression of ionisation and led to lower sensitivity and rapid contamination of the ion source. Therefore, 1% TEAA (72 mM) was replaced by 100 mM ammonium acetate (eluent 33), which showed comparable resolution with somewhat increased retention (**Figure 7**). Sensitivity was better with ammonium acetate than with TEAA due to decreased suppression. **Figure 8** shows extracted product ion chromatograms for enantiomers of amphetamine derivative.

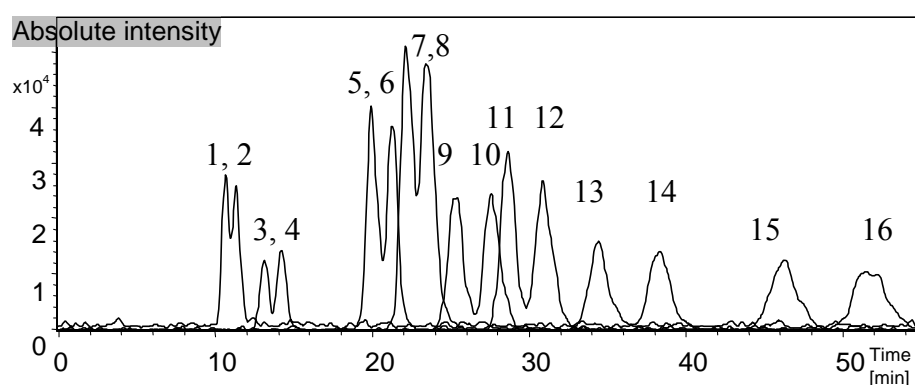


Figure 8. Extracted product ion chromatograms of eight amphetamine derivatives. Method: MeOH/100 mM NH₄OAc 5/95 (eluent 33) and β -CD column. Compounds: **1** R-AM, **2** S-AM, **3** R-MA, **4** S-MA, **5** R-PMA, **6** S-PMA, **7** R-PCA, **8** S-PCA, **9** R-PMMA, **10** S-PMMA, **11** R-MDA, **12** S-MDA, **13** R-MDMA, **14** S-MDMA, **15** R-MDEA, **16** S-MDEA. Sample concentrations 35 – 130 μ g/ml.

The LC–ESI/MS/MS method was evaluated by determining the LODs, repeatability of the peak areas of the product ion chromatograms and retention times. Since identification of the compounds was all that was intended a complete quantitative evaluation was not performed. The relative standard deviations of the peak areas were between 2.4 and 11.4% and those of the retention times between 0.2 and 1.3%. The repeatability of the method was thus acceptable. The LODs were between 25 and 1000 ng/ml, which can be considered acceptable for forensic analysis.

Figure 9 shows an analysis of amphetamine derivatives from an ecstasy tablet by LC–UV–ESI/MS/MS using the β -CD column and the RP eluent 33. The extracted product ion chromatograms show that the sample included a high concentration of MDMA, with MDA and amphetamine as impurities. The high concentration of MDMA overloaded the column causing peak tailing and a shift in the retention times. A more dilute sample had to be injected separately to get reasonable peak shapes for AM and MDMA and a more concentrated sample to detect MDA.

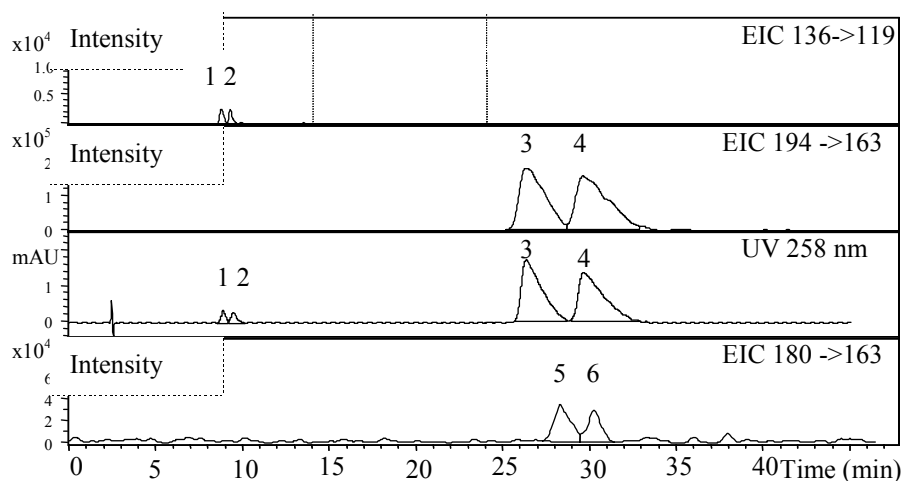


Figure 9. An ecstasy tablet analysed on β -CD column with eluent 33 (see **Table 6**). Extracted product ion chromatograms of 1 and 2 AM, 3 and 4 MDMA and their corresponding UV trace. The lowest trace is from a second injection of a more concentrated sample, 5 and 6 MDA.

6. CONCLUSIONS

The fast LC–ESI/MS/MS methods developed in this work were shown to be applicable for detection, confirmation and quantitation of a variety of forensically interesting compounds. The results showed that chemically very different compounds – basic, acidic and neutral – can be ionised by positive ion ESI and quantitatively analysed in one run. All 14 compounds were quantitatively analysed on monolithic analytical column within 2.5 minutes. Moreover, the technique proved suitable for analysing compounds that are not easily analysed by conventional GC–MS. It was also shown that the method can be used in distinguishing a controlled drug from chemically closely similar compounds, *i.e.* from mass equivalent regioisomers and isobaric substances. A controlled drug was distinguished within five minutes from 17 similar compounds generating the same precursor ion. It was clear, however, that selective and fast LC of any mixture is appropriate only with tandem mass spectrometers. The quadrupole ion trap (QIT) mass spectrometer was used throughout the study.

In-house libraries including MS and MS/MS spectra and also the retention times of the compounds of interest provided fast identification. Comprising LC separation, tandem mass spectrometry and spectral comparison against in-house libraries, the methods developed were highly specific and reliable. Automated library search was considered essential because of the vast amounts of data generated in faster analyses with a specific detector with fast acquisition rate. With automated library search, the data handling process is kept simple even for the less experienced user. The speed, specificity, ruggedness and possibility to automate the data analysis make the new methods highly attractive for forensic routines.

Preliminary studies on the use of AP-MALDI/MS/MS and AP-DIOS/MS/MS for detection and identification of drugs of abuse confirmed that both techniques are suitable for very fast screening of selected amphetamine derivatives and fentanyls in samples obtained in drug seizures. In neither technique did the background ions disturb the analysis, since in both cases these appeared at higher or different mass numbers than the analytes. The LODs demonstrated high sensitivity for both techniques, suggesting their potential in trace analysis as well. In future, the work could be automated to allow the analysis of up to 100 samples on a single MALDI or DIOS plate. After initial investment, both techniques should also be highly cost effective. For all these reasons, AP-MALDI/MS/MS and AP-DIOS/MS/MS are of great interest for forensic purposes. An important finding was that the MS/MS spectra of the compounds studied were similar regardless of the ionisation technique: AP-MALDI, AP-DIOS or ESI. Thus, library spectra to be used in identifications could be generated with any of these techniques. The spectra must be generated under the same conditions, however, and with the same type of MS instrument.

The study on eluent composition in chiral separations provided information on the chiral separation mechanisms. Evaluation of a selection of reversed-phase and polar ionic mode eluent systems compatible with ESI/MS showed that even small changes in eluent composition may affect the resolution significantly. Although very similar molecules can act very differently in chiral systems some predictions of the chiral separation of amphetamine derivatives could be made on the basis of analyses conducted with methamphetamine. Several amphetamine derivatives was separated within a single run, a feature that would be beneficial when determining the chiral composition of drug seizures containing several amphetamine derivatives or in successive analyses of different amphetamine samples.

The β -CD column with reversed-phase eluent system was more suitable for chiral analysis of amphetamine derivatives in general than the vancomycin column. The β -CD column not only allowed the separation of enantiomers of the amphetamine derivatives, but also nearly baseline separation of the derivatives from each other. Non-volatile TEAA, which caused severe signal suppression in ESI/MS, could be replaced by ammonium acetate without reduction of the resolution. Evidently vancomycin has not previously been used as a chiral selector for amphetamine derivatives, methorphan or propoxyphene. The performance of vancomycin as a chiral selector was only reasonable. However, when comparison was made of the β -CD column and vancomycin with the several eluent systems tested the vancomycin selector was more enantiomer selective for amphetamine, *p*-methoxyamphetamine, *p*-methoxymethamphetamine, methorphan and propoxyphene. In future, this selector could provide valuable applications for these analytes. Neither of the chiral selectors could separate the enantiomers of norephedrine. Furthermore, since eluent splitting was used in this work, and the LODs were sufficient for forensic contexts, it is clear that chiral LC-MS will benefit in future when smaller diameter chiral columns become widely available.

Even though the suitability of the new methods was also tested with sample material seized by local authorities, more work is needed before AP-MALDI/MS/MS and AP-DIOS/MS/MS methods can be considered reliable tools for routine forensic casework. Given the importance of reliability in forensic casework, expert knowledge of the operational parameters affecting the performance of sophisticated mass spectrometric techniques will be critical both in the introduction of new techniques and in developing and maintaining new methods for forensic purposes.

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