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Review

Liquid chromatography–mass spectrometry in nucleoside, nucleotide and modified nucleotide characterization

E.L. Esmans*, D. Broes, I. Hoes, F. Lemière, K. Vanhoutte

University of Antwerp (RUCA-UIA) Department of Chemistry, Nucleoside Research and Mass Spectrometry Unit, Groenenborgerlaan 171, B-2020 Antwerp, Belgium

Abstract

Macromolecules such as deoxyribonucleic acid (DNA) and ribonucleic acid as well as their constituents play an important role in all kinds of biochemical reactions in nature. Hence their isolation and identification plays a major role in biochemical analysis. Mass spectrometry (MS) has gained an important position in this field because of the development of soft ionization techniques such as fast atom bombardment (FAB), liquid secondary mass spectrometry, thermospray ionization (TSP), the atmospheric pressure ionization techniques, electrospray ionization and atmospheric pressure chemical ionization and matrix assisted laser desorption. Because of their polar nature, mixtures of nucleosides, nucleotides and oligonucleotides are wel separated by liquid chromatography (LC) and electrophoretic techniques. Therefore it is not surprising to note that a lot of effort has been put into the development of LC–MS methods for the analysis of these compounds. In this review, covering the period 1990–1996, the LC–MS analysis of nucleobases, nucleosides, nucleotides, oligonucleotides and DNA adducts by TSP, continuous flow FAB and electrospray MS is discussed. © 1998 Elsevier Science B.V.

Keywords: Reviews; Liquid chromatography–mass spectrometry; Interfaces, LC–MS; Nucleosides; Nucleotides

Contents

*Corresponding author.

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1. Introduction

It is common knowledge that macromolecules such as deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) as well as their constituents play an important role in a variety of biochemical processes. Fundamental investigations on their structure, the search for modifications, their occurrence e.g., are therefore needed because the results will lead to a better understanding of these processes. Analysis of compounds isolated from complex biological matrices implies the analysis of compounds in complex mixtures usually at low concentrations. As such a lot of effort has been spent in developing methodologies capable of analyzing nucleobases, nucleosides and nucleotides using mass spectrometry (MS) because the technique can provide structural information of minute amounts of compound and can be hyphenated to a variety of separation techniques such as gas chromatography (GC), high-performance liquid chromatography (HPLC), capillary electrophoresis (CE) e.g., allowing an efficient on-line analysis. More recently, especially since the introduction of electrospray ionization (ESI) and matrix assisted laser desorption (MALDI), MS really became popular in these fields where in the past mass spectral analysis of both low- and high-molecular-mass compounds was hampered by their polarity. More specifically in the area of nucleic acid research this offered new alternatives, for example, in the field of oligonucleotide analysis.

Although mixtures of a variety of nucleobases and nucleosides can be analyzed by combined gas chromatography–mass spectrometry (GC–MS), derivatization to more volatile derivatives is a prerequisite. Most of the time trimethylsilylation is used because TMSi-derivatives provide a lot of structural information under electron impact (EI) conditions. However, the GC–MS analysis of more complex nucleosides i.e., nucleosides containing more polar modifications in the carbohydrate or aglycon, nucleotides and oligonucleotides fails [1].

Fortunately, due to their chemical nature, underivatized nucleosides, nucleotides and related compounds are perfect molecules for analysis by HPLC and/or CE coupled to mass spectrometry (LC–MS) techniques.

In this review the period 1990–1996 is covered focusing on the three major LC–MS interfaces used in this field of research i.e., the continuous flow or dynamic fast atom bombardment (CF-FAB), the thermospray (TSP) and the electrospray (ES) interfaces [2]. We want to emphasize that in the case of FAB-MS all articles dealing with static FAB have been discarded and that in the case of LC–ES-MS the articles dealing with the measurement of oligonucleotides introducing them by flow injection analyses (FIA) have been included.

2. Nucleobases, nucleosides and nucleotides

2.1. Continuous flow fast atom bombardment mass spectrometry

The feasibility of LC–CF-frit-FAB-MS for the structural characterization of modified nucleosides in tRNA at low microgram levels was investigated [3]. The resulting nucleoside mixture was separated by using a microbore reversed-phase LC column (Develosil ODS-5 500 μm I.D.) with an aqueous ammonium acetate–methanol gradient at a flow–rate of approximately 6 μl/min. Mass spectral data were obtained from 30–100 ng of modified nucleoside material. Approximately 3–10 μg of tRNA was sufficient for the acquisition of good mass spectral data.

In 1993 Takeda et al. [4] described a successful attempt for differentiation of isomeric dinucleotides using the same interface. Samples were separated by gradient elution on a 500 μm I.D. capillary column filled with ODS-5 using ammonium acetate–methanol. Both solvents were spiked with 0.8% glycerol. An *A S. cerevisiae* $tRNA^{Phe}$ hydrolysate was analyzed and the hypermodified nucleoside wybutosine (yW) was identified together with a dinucleotide (estimated less than 320 p*M*) composed of adenosine (A) and yW ($[M-H]^-$ at m/z 836). Its sequence was determined as yWpA.

Takeda and his group also investigated D-ribose-methylated dinucleotides of the type NmpN′ [5] by an analogous method. Methylation of the 2′-OH of the D-ribofuranosyl moiety is one of the types of structural modifications found in tRNA. It occurs at the polynucleotide level after the transcription of the tRNA genes, as in the case of base modifications.

NmpN′ dinucleotides (and NmpN′mpN′′) in tRNA were screened in tRNA hydrolysates from the extremely thermophilic archaeon *Pyrodictium occultum*. Structural information on the NmpN′ compounds was obtained from the mass spectra without the need to compare the data with those of authentic standards because the mass spectra contained molecular mass information in addition to sequence-specific fragment ions.

Langridge et al. [6] applied CF-FAB to cyclic nucleotide biochemistry. The modification and optimization of a CF-FAB system for the mass spectrometric analysis of cyclic nucleotides was described. Cyclic nucleotides were introduced by means of a 75 μm I.D. fused-silica capillary in water–methanol–glycerol (70:20:10) at 6.7 μl/min. Modification of the probe tip was necessary to facilitate solvent evaporation and solvent degassing and the introduction of filter system were found to be essential. In comparison with static FAB a 50 to 80 fold increase in sensitivity for cCMP was observed. CID/MIKE (mass-analyzed ion kinetic energy) spectra of a 3.2 μg/μl solution of cCMP was obtained and allowed differentiation between isomeric 3′,5′- and 2′,3′-cyclic nucleotides. This approach was also used to identify endogenous cyclic nucleotides in tissue extracts and to elucidate the structure of both naturally occurring and synthetic cyclic nucleotide derivatives. A quantitative application of the CF-FAB/MIKES technique in cyclic nucleotide analysis described a kinetic study of cyclic nucleotide phosphodiesterases and cyclic nucleotide-responsive protein kinases. Cyclic nucleotide-related enzymes were investigated [7]. The implementation of a modified probe tip and the realization of a optimum flow-rate of 2–3 μl/min by introducing a longer, narrower capillary were necessary to obtain good results. Subsequent studies showed excellent reproducibility and sensitivity for injections of standard cyclic nucleotides and confirmed that there is a sensitivity gain for CF-FAB analysis of cyclic nucleotides as has been reported for other compounds. FIA of 3′,5′-cyclic adenosine monophosphate (cAMP) and 3′,5′-cyclic cytidine monophosphate (cCMP) phosphodiesterase incubates, monitored both the time course of the enzymatic reaction and the effect of enzyme concentration upon rate and also provided quantitative data, which in turn produced enzyme kinetics data that correlate well with radiometric studies. Initial attempts to utilize CF-frit-FAB in the ''on-line'' mode of operation, allowed direct monitoring of substrate and product levels from an ongoing phosphodiesterase reaction, and the increased selectivity provided by MS allowed unambiguous identification of substrate, product and potential intermediates, thus providing new capabilities with which to study these enzyme reactions. Although stability appears to be a problem for any quantitative ''on-line'' work, the possibility of utilizing a ''coaxial'' interface, where the separate capillaries are used to carry the analyte and the matrix solutions, may provide better stability and the potential to analyze more complex enzyme systems such as adenylyl cyclase.

2.2. Thermospray liquid chromatography–mass spectrometry

Nucleosides and nucleobases are often used as test compounds for evaluating the performance of different LC–MS systems as shown by Voyksner et al. [8]. Chace and Gallery [9] used 2′,3′-dideoxycytidine (ddC) as an example in a study towards the use of diamine reagents in TSP-MS as an alternative to the more commonly used ammonium salts. Due to its higher proton affinity diaminomethane has a stronger tendency to form adducts with most analytes than ammonium. The (+) TSP mass spectrum ddC, in the presence of ammonium acetate (0.1 *M*) was characterized by a protonated molecule $[M+H]^+$ at *m/z* 212 and the base peak at *m/z* 112 ($[BH+H]^+$). Using diaminomethane the same ions were observed but accompanied by major ions at *m/z* 272 and 172, the corresponding diaminomethane adducts.

The influence of the eluent pH on the creation of molecular ion species of nucleosides was described by Ashton et al. [10]. The $[M+H]^+$ ions of fifteen nucleosides and analogues were studied under varying eluent composition (pH acidic–neutral, organic modifier) correlating the pK_a values, log *k* values (hydrophobicity) with the ion abundance under different conditions. The hydrophobic and acidic properties of compounds had an important impact on the $[M+H]^+$ formation in (+)TSP in the presence of ammonium acetate and less than 5% organic modifier. Under these conditions, the abundance of the

protonated molecule of hydrophobic nucleosides with a low pK_a could be enhanced by lowering the pH. The presence of organic modifier significantly influenced the ionization process.

LC–MS becomes of increasing importance in drug metabolism studies and quality control. Patanella et al. [11,12] studied the metabolism of the guanine analogue carbovir. The metabolite, isolated from the urine of two female marmosets (*Callithrix jaccus*), was analyzed by injecting 20 μl onto a C_{18} column (4.6 mm I.D.) using acetonitrile–0.01 *M* ammonium acetate (pH 5.5) (10:90) at a flow-rate of 1 ml/min. The compound was identified as the corresponding 5′-glucuronide. A major route of metabolism of carbovir is the oxidation of the methylene hydroxyl on the cyclopentene ring to a carboxylic acid function. The intermediate aldehyde was never detected before. By trapping this metabolite with methoxylamine and subsequent LC–MS analysis its occurrence was proven.

The quantification of ddC, a nucleoside analogue evaluated for the treatment of AIDS was investigated by Jajoo et al. [13] in plasma samples. The analysis was done by reversed-phase LC on a ODS column (4.6 mm I.D) again using methanol–0.05 *M* ammonium acetate at 1 ml/min. A multiple ion monitoring procedure, using an internal standard with four labeled atoms (two ^2H and two ^{15}N) yielded a linear calibration curve from 0.25 to 20 mg/ml (25 μl injection) with a detection limit of 50 pg. This procedure was used to monitor plasma levels after oral administration of the drug. A maximum level was reached after 2 h. No derivatization, high sensitivity, high specificity and a short analysis time were reported as major advantages of this method.

The nucleoside-drugs gemcitabine and cladribine (Fig. 1) were studied by Shipley et al. [14] and Weber et al. [15], respectively. The pharmacokinetics, metabolism and disposition of gemcitabine was examined in mice, rats and dogs. The compound and its uracil metabolite were identified by e.g., comparison of retention times and the observation of the correct protonated molecule. In the case of cladribine TSP was used to identify synthesis related by-products in the bulk drug. 50 to 100 μl cladribine samples (4 μg/μl) were separated on a C_{18} column using 0.1 *M* ammonium acetate–acetonitrile at 1.2 ml/min.

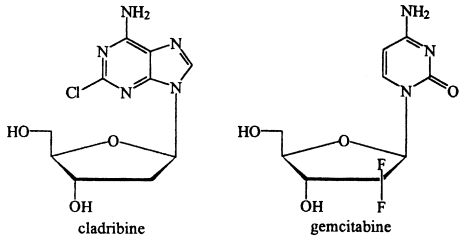

Fig. 1. Structures of gemcitabine and cladribine (adapted from Refs. [14,15]).

As nucleic acids, nucleotide nucleosides and nucleobases intervene in all kind of natural processes it is of no surprise that they are analyzed in a variety of biological matrices. For the unambiguous detection of nucleotides (mono-, di- and triphosphates) in retina homogenates from *Octopus vulgaris* LC–MS was used to identify their structure in addition to LC–UV techniques [16]. Samples were analyzed on a C_{18} column (4.6 mm I.D.) using 0.1 *M* ammonium acetate (pH 3)–acetonitrile–methanol (0.05 *M* ammonium acetate) at 1 ml/min. The TSP mass spectra contained, in varying abundance, a small protonated molecule, the nucleoside fragment and the base moiety. In total thirteen nucleotides (guanosine monophospate: GMP, guanosine diphosphate: GDP, adenosine monophospate: AMP etc.) were detected and identified.

TSP and ES methods were developed for the analysis of the three coenzymes riboflavine, riboflavin 5′-monophosphate and flavin adenine dinucleotide (FAD) in sugar beet root extracts [17]. This revealed two novel riboflavin compounds: riboflavin 3′-monosulfate and riboflavin 5′-monosulfate. These appeared to be the major flavins in roots. Either positive and negative ion mode were used in this study and molecular mass information ($[M-H]^-$; $[M+H]^+$; $[M+Na]^+$) could be obtained for all compounds (except for FAD) under (+)TSP conditions when eluted from a Spherisorb ODS-2 column (4.6 mm I.D.) using a gradient elution [(0.05 *M* ammonium acetate (1% formic acid) (A) and 0.05 *M* ammonium acetate–methanol (B)] at 1 ml/min. The fragmentation in (+)TSP proved to be useful for the characterization of the isoalloxazine moiety in flavine and its derivatives. The detection of the riboflavin sulfates is easily done using LC–TSP-MS. Complete characterization however required also

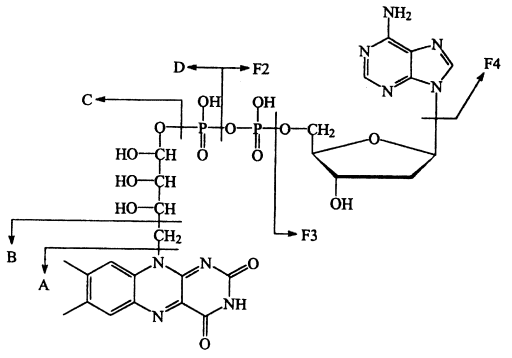

Fig. 2. Major fragment ions in the TSP mass spectra of flavins (adapted from Ref. [17]).

ES-, nuclear magnetic resonance (NMR)- and inductively coupled plasma (ICP)-MS (Fig. 2).

Serrano et al. [18] published an analytical procedure and quality assurance criteria for the determination of major and minor deoxynucleosides in fish tissue. The composition of DNA from liver of three species of fish i.e., rainbow trout (*Onchorynchus mykis*), medaka (*Oryzias latipes*) and brown bullhead (*Ictalurus nebulosus*) was investigated. DNA was isolated from fish livers and enzymatically hydrolyzed before (−)/(+) LC–TSP-MS analysis on a C_{18} column (4.6 mm I.D.) using acetonitrile–ammonium acetate (pH 6) at 1.2 ml/min.

As already mentioned earlier, an excellent review on the LC–MS analysis of RNA hydrolysates was written by Pomerantz and McCloskey [19]. The principal fragmentation of nucleosides under TSP conditions as published by these authors is given in Fig. 3.

The reaction catalyzed by an activity, originally discovered in the species *Xenopus Leavis* and which modifies adenosine to inosine within double-stranded RNA (dsRNA) was studied [20] under (+)TSP conditions. The analysis was performed on a C_{18} column (4.6 mm I.D.) using 250 m*M* ammonium acetate–acetonitrile at 2 ml/min. In biological systems two possible pathways are known by which Ade is converted to Ino: hydrolytic deamination and hypoxanthine insertion. By using dsRNA with uniformly ^{13}C labeled adenosine it was possible to confirm that the carbons of the original base were retained during the Ade→Ino conversion. The spectral data of the modification reaction using $H_2^{18}O$ demonstrated that water is the oxygen donor. These data excluded a base replacement mechanism and suggest a hydrolytic deamination. Mass spectra were characterized by the presence of $[M+H]^+$; $[S-H]\cdot H_2O\cdot NH_4^+$; $[BH+H]^+$ and $[BH+NH_4]^+$ ions.

Three studies were published on the methylation of RNA: the work of Cong et al. [21], the study of Diamond et al. [22] and finally the publication of Takeda et al. [23]. Whether analyzing nucleobases, nucleoside or nucleotides, all used comparable experimental parameters i.e., reversed-phase chromatography using methanol–ammonium acetate as mobile phase at 1 ml/min.

Cong et al. [21] were able to detect nine methylated nucleobases (3-methyluracil, 1-, 2-, 3- and 7-methylguanine, 1-, 2-, 3- and 6-methyladenine) in the RNA from calf and rat liver, baker's yeast and *Torula* and *Euglena* cells. Diamond et al. [22] studied the influence of selenium on the methylation of selenocysteine tRNA. Two isoacceptors for selenocysteine (tRNA) from rats fed with a normal and a selenium deficient diet were isolated and analyzed by LC–TSP-MS. The tRNAs differ only by 2′-O-methylation of the 5-methylcarboxymethyl uridine (mcm^5U) which occurred in the wobble position of the anticodon. Both modified nucleosides

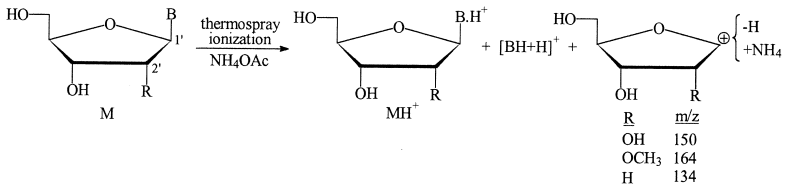

Fig. 3. Principle fragment ions found in the TSP mass spectra of nucleosides (adapted from Ref. [19]).

were detected in the hydrolysates and their identity was confirmed by comparison with the known mcm^5U or the synthesized mcm^5Um. The retention times matched and the TSP spectra were identical. Major ions were observed at *m/z* 317 and 185 (mcm^5U: $[M+H]^+$ and $[BH+H]^+$) and *m/z* 331 and 185 (mcm^5Um: $[M+H]^+$ and $[BH+H]^+$).

Methylation of the 2′-OH of D-ribose in RNA is a well known and documented modification of RNA which plays a role in a variety of functional roles such as the enhancement of structural stability e.g. Since the LC–UV procedures may lead to an incorrect assignment of the structure, Takeda et al. [23] implemented LC–TSP-MS for the analysis of ribose methylated dinucleotides of the type NmpN′ in the enzymatic digests of tRNA. TSP is complementary to conventional HPLC and provided nanogram sensitivity and is applicable to complex mixtures. As a model, the results of a small $tRNA^{Phe}$ (76 nucleotides) containing eleven different modified residues were discussed. In some of the hydrolysates several dinucleotides were found. The TSP mass spectra gave the $[M+H]^+$ and $[BH+H]^+$ ions of the respective nucleosides linked to each other by the 3′–5′-phosphodiester bond.

2.3. Electrospray liquid chromatography–mass spectrometry

Witters et al. [24] used microbore LC coupled to ESI-MS–MS to quantitate cyclic nucleotides in chloroplasts of the leaves of *Nicotiana tabacum*, urine and blood samples. Microbore ion suppression HPLC was carried out on a 2.1 mm I.D. column filled with Adsorbosphere C_{18} material using methanol–10 m*M* ammonium acetate–0.25% acetic acid (pH 3.5) at 200 μl/min. A higher sensitivity was obtained in (−)ESI mode. The cyclic nucleotides cAMP, 3′,5′-cyclic inosine monophosphate (cIMP), 3′,5′-cyclic guanosine monophosphate (cGMP) and cCMP were quantified using external standards with a detection limit of 100 f*M* for cAMP under multiple reaction monitoring (MRM) using the $[M-H]^- \rightarrow B^-$ transition. On-line monitoring of enzymatic peroxidation of NAD^+ was carried out by Zhao et al. [25]. In this study both molecular mass and structural information was obtained for the unstable intermediates formed by the degradation of NAD^+ by using capillary isotachophoresis coupled to ESI-MS and ESI-MS–MS. Measurements were carried out in (+)ESI mode with a sheath flow of 1 μl/min [water (0.1% HCOOH)–MeOH (75:25)]. Another type of CE, i.e., capillary zone electrophoresis (CZE), was used by Wang et al. [26] to analyze the reaction products of N-(O,O-diisopropyl) phosphoryl-threonine and uridine. The hypothesis was made that N-(O,O-dialkyl)phosphorylated amino acids (DAP-aa) could be the basis of prebiotic synthesis of nucleic acids and proteins. CZE was carried out using a 100 m*M* Tris buffer adjusted to pH 7.5 with glacial acetic acid. A sheath flow of isopropanol–water–acetic acid (60:40:1) at 3 μl/min was delivered at the probe tip. Nucleotides were detected as products of the reaction with uridine giving experimental evidence for this hypothesis. Franco et al. [27] identified the metabolite resulting from the incubation of ADP ribose with yeast phospho-riboisomerase, as ADP ribulose by using micro LC–(−)ESI MS. Compounds were separated on a reverse phase Hypersil BDS column (1 mm I.D.) by gradient elution using 3 m*M* tetrapropylammonium hydroxide (TPA)–formic acid pH 4 (A) and acetonitrile–water–formic acid pH 4 (B) at 50 μl/min. Ions were observed at $[M-H]^-$ and $[M+TPA-2H]^-$. Nine modified nucleosides and nucleoside monophosphates were analyzed by Reddy and Iden [28] under (+) and (−)ESI (Fig. 4). 2′-Deoxynucleosides were dissolved in acetonitrile–water (50:50) containing 0.5% formic acid at a concentration of 15 p*M*/μl for (+)ESI. 2′-Deoxynucleosides and their monophosphates were dissolved in acetonitrile–water (50:50) or methanol–water

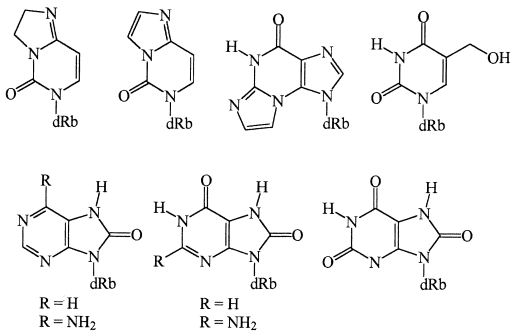

Fig. 4. Structures of some modified nucleosides analyzed under (+) and (−) ESI conditions (adapted from Ref. [28]).

(90:10) containing 1% ammonia at a concentration of 150 p*M*/μl. All compounds were introduced by FIA. Under high cone voltage conditions collision induced dissociation (CID) spectra were obtained characterized by the presence of $[BH+H]^+$; $[S]^+$, $[M-BH]^-$ and $[B]^-$ ions.

The correlation between peak intensity of the $[M+H]^+$ ion and the analyte concentration was investigated in concentrations ranging from 0.5 up to 50 p*M*/μl, giving a linear curve over the full concentration range with a correlation coefficient of 0.998. 2′-Deoxyadenosine was used as internal standard. The detection limit was said to be <3 p*M*. Various nucleoside-like molecules were analysed by ESI-MS as well. The oxidation products of paralytic shellfish poisoning toxins were analyzed by microbore HPLC–(+)ESI-MS and MS–MS (Quilliam and Janacek [29]) on a 2.1 mm I.D. filled with LiChrospher RP18 using acetonitrile–10 m*M* heptafluorobutyric acid in a gradient elution at 200 μl/min. The anti-influenza agent 1,3,4-thiadiazol-2-ylcyanamide and its D-ribose metabolite were identified by ESI-MS and MS–MS by Ehlhardt et al. [30]. Compounds were isolated by off-line chromatography, dissolved in water acidified with glacial acetic acid and introduced by FIA. Banoub et al. [31] used low energy collision activated dissociation (CAD) ESI-MS–MS on the $[M+H]^+$ ions for the structural characterisation of azidothymidine (AZT), 3′-azido-2′,3′,4′-trideoxy-4′-thio-5-halogenouridines and their α-anomers. This MS–MS study provided characteristic fingerprint patterns permitting a differentiation between the α-en β-anomers in the 3′-azido-2′,3′,4′- trideoxy-4′-thio-5-halogenouridine series. Compounds (1 μg/μl) were dissolved in methanol–water (1:1) containing 1% formic acid and introduced by FIA at 5 μl/min.

Nucleosides have also been analyzed by ultrasonically assisted ESI [32]. This ionization method was evaluated and its applicability was illustrated by the identification of nucleosides from as little as 150 f*M* of RNA (prior to digestion). Analysis was done on a capillary HPLC column (250 μm I.D.) filled with C_{18} reversed-phase material using a gradient elution (methanol–water) at 1.8 μl/min. Several methylated nucleosides were identified.

In the area of medicinal chemistry atmospheric pressure chemical ionization (APCI) has been used to identify reduction compounds of AZT with dithiothreitol (DTT) [33]. LC–MS analysis was performed on a classical 4.6 mm I.D. column filled with Partisil SAX-10 using ammoniumphosphate–methanol in a gradient elution.

3. Oligonucleotides

In the domain of molecular biology, biochemistry and medicinal chemistry the analysis of oligonucleotides is a topic of increasing importance. Establishing the size, purity and sequence of nucleic acids is crucial in these fields and the analysis of natural or synthetic oligonucleotides and derivatives such as phosphorothioate analogues (used in the field of anti-sense oligonucleotide studies) are a only few of the numerous applications found in the literature. Significant progress in the area of accurate mass determination, the sequencing and the study of non-covalent interactions was made possible by introducing mass spectrometric techniques such as MALDI-time of flight (TOF) and ESI coupled to quadrupole, TOF and Fourier transform MS (FT-MS). In this review we will only focus on the ES data obtained by both flow injection analysis and on-line LC–MS.

3.1. Electrospray liquid chromatography–mass spectrometry

Due to the production of multiply charged ions under ESI conditions the analysis of higher molecular mass compounds becomes possible since the *m*/*z* values of these ions fall within the mass range of e.g., commercial quadrupole systems. ESI mass spectra of oligonucleotides (ONs) and related compounds are usually recorded under (−)ESI conditions and often suffer from the occurrence of both low-intense multiply charged ions and Na^+ and K^+ adduct signals with a Gaussian like distribution. Since the quasi-molecular ion can have a poor signal intensity accurate mass determination can be difficult [34]. As we mentioned earlier [35] Pommerantz and McCloskey [19] acknowledged this problem and discussed the importance of determining the mass of such ONs within ±0.01% to infer the composition of oligonucleotides from RNA and DNA.

As it often is the case, sample preparation is of utmost importance to realize good data. The main object in sample preparation is the removal of Na^+ and K^+ ions which strongly interact with the polyionic backbone of the oligonucleotides [36,37]. General methods used are based on precipitation of ONs from ammonium acetate solutions: it is a simple and efficient method but loss of some of the oligonucleotide during precipitation remains a matter of concern. Reverse phase HPLC removes bound cations from standard DNA and RNA but seems less effective in the case for modified oligomers such as phosphorothioates [38]. Another strategy consisting of the removal of bound cations is the addition of chelators such as cyclohexyl diaminetetraacetic acid (CDTA) (3,4,5). Bleicher and Bayer [39] recorded a 10-mer (M_r~3000). Compounds were introduced by FIA at 5 μl/min at a concentration of 10^{-4} *M*. In order to show the effect of K^+ adduction on the spectrum quality the compound was dissolved in triethylamine (TEA) and stored for 6 h. Additional peaks at $[M-(n+m)H+mK]^{n-}$ were found (n= number of protons; m=number of additional K^+ ions). Also the influence of the organic solvent on the signal intensity was discussed. Compared to methanol, ethanol and acetone, acetonitrile gave superior results. pH effects on the quality of the ESI mass spectra were found to be compound dependent. It was shown that a dT10-mer was more prone to pH changes than a dC10-mer. Cheng et al. [40] studied the charge state reduction of oligonucleotides by the addition of acids and diamines. The latter approach has the advantage that the stability of the oligonucleotides was not affected. Prior to measurements oligonucleotides were desalted by exchange with NH_4^+ ions by repetitive precipitation from a 1 *M* $NH_4$0Ac solution. About 12 μ*M* of oligonucleotide solutions were infused at 0.2 μl/min. In the case of the addition of acids (acetic acid, formic acid, HCl etc.) HCOOH and CH_3COOH were suitable for reducing the charge state, however their addition to the aqueous solution of the oligonucleotide also reduced ion intensity substantially. For a d(pT)12-oligomer the charge state was reduced from 7- to 4- and 3- (Fig. 5).

Formation of oligonucleotide–diamine complexes by addition of 1,4-butanediammonium diacetate showed charge shifting towards higher m/z values.

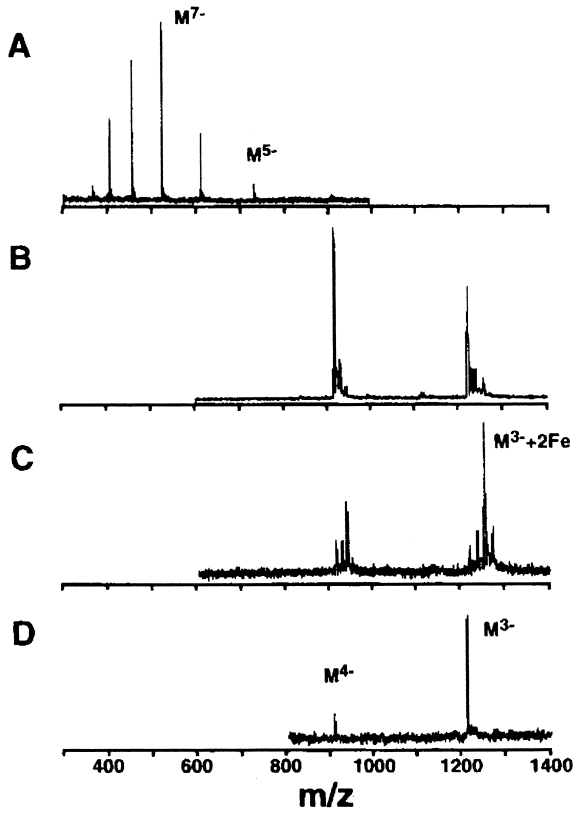

Fig. 5. ESI mass spectra of a $d(pT)_{12}$–NH_4^+ salt in (A) deionized water (pH 7); (B) 0.5 *M* formic acid (pH 1.9); (C) 2.5 *M* formic acid (pH 1.6); (D) 2.5 *M* formic acid–50 m*M* 1,10-phenantroline (pH 1.7) (adapted from Ref. [40]).

In 1996 the same group [41] published the influence of the addition of acids and bases such as imidazole and piperidine on the shift of charge states of oligonucleotides. For example for a d(pT)18-mer cocktails of imidazole–piperidine–acetic acid in 80% acetonitrile not only reduced the charge state from 6-; 7- and 4- to 3- but also substantially suppressed the Na-adduction. The authors also underlined the utility of sample preparation after which the oligonucleotides were dissolved in the appropriate solvent (concentrations from 10 to 90 p*M*/μl) and introduced by FIA at a flow-rate as described above.

Greig and Griffey [38] already showed in 1995 that the addition of strong bases to the oligonucleotide solution such as TEA, piperidine reduced the Na^+/K^+ adduct signals most effectively but decreased the total ion current as well. They claimed

that addition of imidazole not only resulted in a modest suppression of the Na^+/K^+ adduction but also gave up to a 4-fold improvement in sensitivity. In the case of phosphorothioate or phosphodiester oligomers co-addition of imidazole and triethylamine or piperidine produced high ion abundances and a good suppression of cation adducted species. In most cases samples were dissolved in 100 μl of a 1:1 mixture of aqueous buffer and isopropanol and injected by FIA at a flow-rate of 6 μl/min.

In the field of polymerase chain reaction (PCR) amplified DNA products the amplified fragments are typically analyzed by electrophoretic techniques (slab gels or gel-filled capillaries). In this approach mixtures of nucleic acids are separated by molecular mass and are usually detected by fluorescence or radioisotopic labeling. Mass spectrometric analysis of these oligomers is complicated by the presence of various salts, nucleoside triphosphates, primers and other materials used in the PCR process. Therefore efficient sample preparation is a prerequisite. Synthetic oligo's were prepared on a 0.2– 1 μ*M* scale and separated by preparative gel electrophoresis and precipitated from NH_4OAc. A 75-mer was analyzed by ESI-ion trap MS [42] by FIA in methanol at a flow-rate of 3–5 μl/min. Although the measured mass was greater than the calculated the differentiation could be made between two PCR products a 75- and a 72-mer. ESI-MS has also been used for the analysis of post-synthetically modified ODNs. A modified 12-mer (4-thio-2′-deoxyuridine) was analyzed with a mass accuracy of 1 u on a quadrupole instrument. 10 μl samples were introduced by FIA in isopropanol–water pH 9.5 (20:80) at 7 μl/min [43].

The interest in anti-sense oligodeoxynucleotides (ODNs) as potential therapeutic drugs in the field of anti-viral or anti-cancer research has increased. Phosphodiester and phosphorothioate (SODNs) were analyzed by (−)ESI by FIA in acetonitrile–0.5% NH_3 (50:50). The molecular mass of a 25-mer phosphorothioate was measured with an accuracy of 0.001% (standard error 0.05 u) [44] at a concentration of 12 p*M*/μl. In order to obtain these results 1 m*M* ODN -solutions were desalted by RP-HPLC on a C_{18} column prior to mass measurements. (−)ESI-MS was also used for the investigation of the so-called ''$n-1$'' impurity present in synthetic SODNs obtained by the solid-phase β-cyanoethyl phosphoramidate method [45]. These SODNs were isolated by preparative polyacrylamide gel electrophoresis (PAGE) and afterwards desalted on ODN purification columns. SODNs in the $NH4^+$ form (20–50 μ*M* solutions) were mixed with acetonitrile and infused at 3–4 μl/min.

In 1995 Valaskovic et al. [46] showed that full mass spectra could be obtained at high resolving power by electrospraying 0.2 nl samples of large (M_r >10 000) nucleotides in combination with FT-MS. In order to achieve extreme high sensitivity a special laboratory-made ''nano-electrospray '' tip was made: the total amount of analyte loaded was 8.6 f*M* and 216 a*M*. A 50-mer DNA was desalted by HPLC and a 20 μ*M* solution in acetonitrile–0.015% TEA (70:30) was introduced at a flow-rate of only 3 nl/min.

Not too many data were available showing the power of combined LC–MS techniques in this area. In 1994 Bleicher et al. [47] showed the analysis of synthetic ODNs (5 to 24-mers) on a 2 mm I.D. HPLC column directly coupled to ESI-MS triple quadrupole system. The HPLC column was filled with Nucleosil C_{18} and operated at a flow-rate of 200 μl/min of which 70 μl/min was introduced into the ESI-source. Gradient elution was used using mixtures of ammonium acetate and acetonitrile. The success of the HPLC analysis was related to the presence of a 5′-dimethoxytrityl protecting group which makes the ODNs more hydrophobic. Spectra were run under (−)ESI: for the protected 24-mer a mass accuracy of 0.001% was found.

Iden et al. [48] determined the molecular mass of a 17-mer and a 22-mer under (−)ESI conditions with a sensitivity of 20 to 100 p*M*/μl. The mobile phase consisted of isopropanol–water (1% TEA). The measurement of the molecular mass allowed detection of modifications on the bases. Reddy et al. [49] presented (−)ESI-MS spectra of ONs (10 to 24-mers) containing modifications with a sensitivity of 50 p*M*/μl. They used a mobile phase of MeOH–water. Although most papers report FIA of ONs, Bothner et al. [50] used micro LC–ESI-MS to determine the molecular mass of antisense ONs. Separations of the antisense ONs (17 to 20-mers) were performed on a PLRP-S 5 μm 100 Å column (500 μm I.D.) with a mobile phase of acetonitrile–200 m*M* diisopropylamine–water (pH=7.2, adjusted

with acetic acid) at 50 μl/min. 7 n*M* of each antisense ON was injected. Barry et al. [51] coupled CE to ESI-MS to separate and detect modified ONs (4 to 6-mers, 100 f*M*/μl). CE was coupled to ESI-MS using a sheath flow of 20 m*M* NH_4OAc (pH= 9.8)–2-propanol (75:25) at 3 μl/min. CE was performed in a polyvinylpyrrolidone matrix (PVP) which behaves as a pseudo-phase in which the ONs with hydrophobic modifications are retained longer than their normal unmodified ONs. The charge state of the multiple charged negative ions was determined using the m/z difference (Δm) between the deprotonated molecule ion and the first sodiated ion. Herron et al. [52] determined the charge state of product anions with a proton transfer from protonated pyridine to the product anions on an ion trap mass spectrometer. This is an alternative to measuring the spacing in mass-to-charge between adjacent peaks in the isotopic distribution (high resolution) or measuring Δm between the deprotonated molecule and the first sodiated ion.

Another field in the analysis of ONs with ESI-MS is nucleotide sequencing. The basis was led in the early 1990s by McLuckey and co-workers [53,54]) who presented a generally accepted nomenclature. This is shown in Fig. 6. taken into account the variation adopted by Barry et al. [55].

Ni et al. [56] developed a computer algorithm based on the fragmentation scheme proposed by McLuckey to determine the sequence of ONs using a triple quadrupole mass spectrometer. This method was demonstrated for ONs up to 15-mers and was primarily based on the recognition of the w- and a-series. Iden et al. [48] applied the same strategy to a synthetic modified hexamer. The use of an ion trap mass spectrometer was preferred to a triple quadrupole, since in this case w_n and a_n were predominant [53,55]. In 1994 Little and coworkers [36,57] investigated ESI coupled to FT-MS, which is a technique which routinely achieves sufficient high mass accuracy (≤20 ppm) and a resolving power of 10^5. Base composition and sequence information from ODNs as large as 25 bases was obtained. Full sequence information of 8–14 oligomers and extensive sequence information of 21–25 mers were obtained by nozzle-skimmer dissociation. Samples were desalted by HPLC on a C_{18} column using triethylammonium acetate–acetonitrile. ODNs were dissolved in methanol–water (10:1) and 14–40 μ*M* solutions were electrosprayed at a flow-rate of 1.5–2.0 μl/min. A year later the same group published the sequencing of a 50-mer ON. Nozzle-skimmer fragmentation and infrared multiphoton dissociation produced complementary fragment ions. Boshenok and Sheil [58] examined a series of nucleotide oligomers (2–6 mers) under a different range of collision and ionization conditions. In general abundant peaks due to the free bases and phosphate groups were observed along with some sequence ions. Samples were introduced at 5–10 μl/min in 50% aqueous isopropanol [(−)ES] or water [(+)ES] at a concentration of 10–200 p*M*/μl.

An alternative approach in the sequence determination of ONs was demonstrated by Kowalak et al. [59] and Limbach et al. [60]. Here a combination of enzymatic digestion and ESI-MS was used to elucidate the position and nature of post-transcriptional modifications in 16S rRNA as depicted in Fig. 7.

An analogous approach was used by Glover et al. [61]. A 10-mer ODN was cleaved by both endo-en exonucleoases and aliquots were taken at different time intervals. Samples were purified and separated off-line by HPLC on a C_{18} column and introduced by FIA in a mobile phase consisting of acetonitrile–water (1:1) at a flow-rate of 50 μl/min. The sample itself was dissolved in acetonitrile–50 m*M* imidazole (7:3) in order to decrease sodium adductation. Mass spectra were run under (−)ESI.

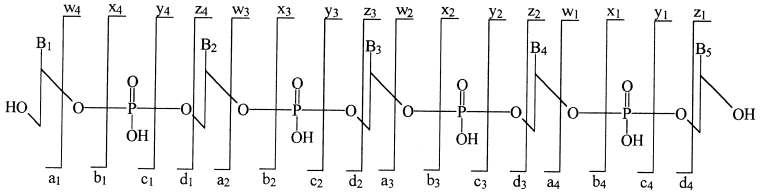

Fig. 6. Oligonucleotide fragmentation nomenclature (adapted from Refs. [55,58]).

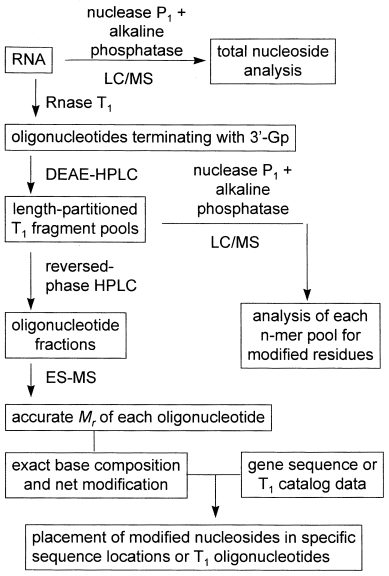

Fig. 7. Sequencing of polynucleotides by a combining enzymatic and MS procedures to elucidate the position and nature of post-transcriptional modifications in 16S-rRNA (adapted from Ref. [59]).

Baker et al. [62] determined the mass and sequence of a methylphosphonate antisense ON (18-mer). Noteworthy is the fact that this occurred in the (+)ESI mode. Therefore formic acid (0.1%) was added to the mobile phase which consisted of methanol (or acetonitrile)–water (50:50). The sequence determination was based on the y series ions resulting from heterolytic cleavages at the sugar–phosphonate bond.

A final topic in the field of ONs is the study of non-covalent complexes with ESI-MS. Experiments on dilute samples are conducted under gentle interface conditions to avoid non-specific associations and dissociation of non-covalent complexes. In addition to these requirements ESI compatible solutions must be used. For detailed information on experimental conditions we refer to Smith et al. [63]. Gale and Smith [64] studied the non-covalent interaction of minor groove binding molecules and duplex ONs such as distamycin A, pentamidine and Hoechst 33258 under (−)ESI conditions. Cheng et al. [65] determined the binding stoichiometry of gene V protein to several ONs. Both groups introduced the samples, dissolved in 10 m*M* ammonium acetate (pH 7.0) by flow injection at a flow-rate of 200 nl/min. The non-covalent interaction of cytidylic acids with ribonuclease A was investigated by Camilleri and Haskins [66] under (+)ESI conditions in aqueous methanol in order to probe the binding to the enzyme while Ganguly and co-workers [67,68] studied the non-covalent GDP/GTP human H-ras complexes. The presence of non-covalent complexes of ras-GDP and ras-GTP were confirmed. Finally, duplex and even quadruplex formation of ONs were studied by Light-Wahl et al. [69] and Goodlett et al. [70], respectively. Specific and non-specific non-covalent dimers of ODNs were observed by Ding and Anderegg [71] when mixtures of complementary and noncomplementary strands were analyzed by (−)ES. The appearance of the dimers was concentration dependent.

4. DNA adducts

Exposure to carcinogens may occur from the environment, dietary exposure, physiological processes and also endogenous sources.

Chemical carcinogens can be divided into two groups: genotoxic compounds which cause damage to DNA and the non-genotoxic which cause no direct damage but indirectly initiate DNA damage because of their metabolic pathway. Most of the these compounds can react with this macromolecule with the formation of a new covalent bond giving so-called DNA adducts. This process is believed to be the event necessary for chemical carcinogenesis provided the adducts are not removed by DNA-repair mechanisms.

Major problems encountered in this area are related to the detection, structural identification and quantitation of such adducts especially in in vivo samples where they are present in only minute amounts (1 base modified out of 10^6–10^{11} bases). This means that one may expect ca. 10 pg of adduct in 1 mg of DNA, in other words, one has to be able to detect a needle in a haystack. A solution to this problem is the development of suitable sample clean-up systems which allow the separation of modified from unmodified material prior to analysis.

Since the introduction of MS in this area an excellent review covering the period from 1980 to

1991 has been written by Chiarelli and Lay [72]. Selected examples have been reviewed by Farmer and Sweetman [73].

4.1. Continuous flow-fast atom bombardment mass spectrometry

While structural studies of DNA adducts formed by in vitro reactions are invaluable, particularly those employing MS–MS, the sensitivity of FAB-MS was rather disappointing especially with regard to sensitivity required for in vivo studies. Wellemans et al. [74] showed that the sensitivity of FAB for the analysis of arylamine–deoxynucleoside adducts was greatly enhanced by trimethylsilylation. To overcome the limitation of background ions these authors investigated the analysis of polyaromatic hydrocarbon (PAH)–2′-deoxynucleoside adducts under CF-FAB conditions. Samples in DMSO were introduced in glycerol–heptafluorobutyric acid by FIA through a 10 μm capillary. Coaxially, a matrix of water–glycerol (75:25) was introduced. Four derivatization schemes (acetylation, methylation, trimethylsilylation and pentafluorobenzylation) were evaluated and the improvements in FAB sensitivity for guanine were compared. It was observed that the pentafluorobenzylated derivative could be measured at the lowest detection limit achieved in the static FAB mode i.e., 2.5 ng. The lowest amounts that could be successfully pentafluorobenzylated were 50 ng and 10 ng, for guanine and adenine, respectively. A significant improvement sensitivity (full scan) was observed in the coaxial CF-FAB approach: 50 pg pentafluorobenzylated adenine compared to 500 pg in static FAB under full scan conditions.

1,3-Butadiene is one of the most commonly produced petrochemicals in the world and has recently been identified as a strong rodent carcinogen. Butadiene is metabolized to reactive 3,4-epoxy-1-butene which may bind to DNA. The reaction between 3,4-epoxy-1-butene and 2′-deoxyadenosine was studied by Kostiainen et al. [75] in trifluoroethanol–triethylamine and the reaction mixture was analyzed by LC–CF-FAB-MS on a NovaPak C_{18} column (4.6 mm I.D.) using water–glycerol (90:10) as the mobile phase. About 6 μl/min was introduced into the ion source. The recorded total ion chromatogram showed four peaks. The spectra of the peaks exhibited a $[M+H]^+$ of 3,4-epoxy-1-butene adducts of 2′-deoxyadenosine, indicating formation of different isomers of the adducts. One alkylation site was identified as the exocyclic amino group of 2′-deoxyadenosine (dAdo), another site was suggested to be N-3.

Wolf et al. [76] exploited the increased sensitivity of CF-FAB for the analysis of PAH–2′-deoxynucleoside adducts. Full scan CF-FAB spectra were recorded using less than 50 ng of sample. Tandem mass spectral data were obtained from only 20–40 ng of adduct. This represents a 20–30 fold increase in sensitivity over single-scan spectra recorded under static FAB conditions. The same group also investigated LC–MS to analyze the reaction products of N-acetoxy-N-acetyl-2-aminofluorene (AAF) with calf thymus DNA [77]. After enzymatic digestion adducts were isolated by solid-phase extraction (SPE) and analyzed on a 320 μm I.D. capillary column filled with C_{18} material using methanol–water spiked with 1% glycerol as mobile phase. Full-scan and MS–MS data [constant neutral loss (CNL), MRM] provided useful structural information on the adducts for low-nanogram (low-p*M*) quantities. The detection limit under LC–MS operation and using MRM was below 25 pg (50 f*M*). This illustrated the potential of CF-FAB to detect one 2′-deoxynucleoside–AFF adduct in about 10^{-6} unmodified deoxynucleosides in approximately 1 mg of DNA (Fig. 8).

Recently studies involving the coupling of CE to CF-FAB for nucleotide adducts analysis from the in vitro reaction between N-acetoxy-N-acetyl-2-aminofluorene (AAF) and DNA [78] have been published. Good overall performance for mixture analysis and low-level adduct detection has been demonstrated.

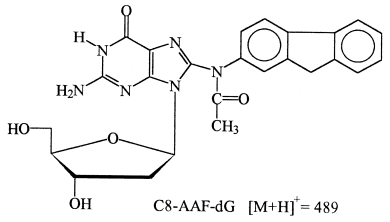

Fig. 8. Structure of the adduct formed by the interaction of N-acetoxy-N-acetyl-2-aminofluorene (AAF) with calf thymus DNA as identified by CF-FAB-MS and CF-FAB-MS–MS. (adapted from Ref. [77]).

Sample stacking was used to improve the detection limits as much as by 3 orders of a magnitude (10^{-8} *M* range). A 40 cm×50 μm I.D. capillary was used to transfer materials eluting from the CZE capillary along with a make- up flow of CF-FAB matrix solution into the CF-FAB probe. Stacking techniques are expected to work only with negatively charged species under typical CZE conditions. For DNA adducts, this means the analysis of the corresponding nucleotides. An AAF–dGMP standard was used to examine the stacking-CZE–CF-FAB-MS system. It was determined that a 3.5 nl injection through the CZE–CF-FAB-MS system of a $5 \cdot 10^{-4}$ *M* C_8–AAF–dGMP solution (or 1.0 ng) provided a good quality normal full scan signal under the conditions employed. Using the stacking technique, it was found that a 35 nl injection of a $5 \cdot 10^{-5}$ *M* solution and a 350 nl injection of a $5 \cdot 10^{-6}$ *M* solution, both providing 1.0 ng of C_8–AAF–dGMP, resulted in spectra of equivalent quality. Hence, the stacking method lowered the concentration detection limit by two-orders of a magnitude for this analyte. C_8–AAF–dGMP and to a lesser extent the isomeric adduct, N^2–AAF–dGMP were identified. Before analysis adducts were separated on a polystyrene–divinylbenzene (PS–DVB) SPE column from the bulk unreacted 2′-deoxynucleotides.

4.2. Thermospray liquid chromatography–mass spectrometry

Meersman et al. [79] studied the adduct formation of 2-bromoacrolein (2BA) with calf thymus DNA using HPLC, NMR and LC–TSP-MS. After enzymatic hydrolysis, the nucleoside mixture was analyzed by reversed-phase chromatography. The 2BA–thymidine adduct was isolated by semi-preparative HPLC and injected on a Microsorb C_{18} column (4.6 mm I.D.) coupled to the TSP interface. The mobile phase consisted of methanol–100 m*M* ammonium formate (20:40, pH 4) at a flow-rate of 1 ml/min. The spectrum obtained revealed $[M+H]^+$, $[M+Na]^+$, $[M+K]^+$ adducts (*m*/*z* respectively, 315, 337, 353). The $[BH+H]^+$ fragment was detected as *m*/*z* 199. These ions together with the absence of the bromine isotope pattern indicated that the 2BA–thymidine adduct was 3-(2′′-bromo-3′′-oxopropyl)-thymidine.

Most studies on DNA adducts dealt with carcinogens from exogenous sources (environment, diet). Malondialdehyde (MDA) is a product of the oxidative lipid metabolism, a by-product of prostaglandin biosynthesis and an end-product of lipid peroxidation. Although endogenous it is a mutagen in bacterial and mammalian test systems and carcinogenic in rodents. The adduct found upon treatment of DNA with MDA was identified by TSP-MS and TSP-MS–MS as 1-methylguanosine (M_1G) [80] (Fig. 9).

M_1G can be quantified in urine samples. Preliminary HPLC analyses with electrochemical detection indicated the presence of M_1G in urine in a concentration as high as 5 p*M*/ml. A LC–TSP-MS method was developed to determine whether HPLC–electron-capture detection (ECD) results could be confirmed hence providing extra structural information on the adduct. M_1G and $[^2H_2]M_1G$ were synthesized by reaction of MDA with guanine and purified by HPLC. M_1G was reduced with $NaBH_4$ to dihydro-M_1G (DHM_1G) which has a higher retention time. This proved useful for the separation of M_1G from interfering substances in the biological matrix. The TSP spectra showed protonated molecules and simple fragmentation under CAD MS–MS. M_1G was isolated from urine samples in a procedure including internal standard addition, C_{18} Sep-Pak extraction, HPLC separation and $NaBH_4$ reduction followed by a second HPLC purification. These samples, with and without internal standard addition, were analyzed by LC–TSP-MS single ion recording (SIR) on a C_{18} column (4.6 mm I.D.) using methanol–ammonium acetate (20:80) at a flow-rate of 1.5 ml/min. The results indicated that the levels of M_1G in urine of healthy volunteers were below the limit of detection (500 f*M*/ml). The original HPLC results using electrochemical detection were reinvestigated and, as expected, an electrochemical active substance

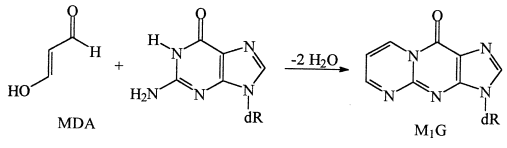

Fig. 9. Structure of the M_1G-adduct formed by the interaction of malondialdehyde and DNA (adapted from Ref. [80]).

co-eluting with DHM_1G was present in the urine samples. The concentrations of DHM_1G were also found to be under the limit of detection of the HPLC–electron capture detection (ECD) method.

Guanine adducts of two polyaromatic compounds were characterized by Herrenzo-Saenz and coworkers [81,82]. Calf thymus DNA was reacted with 3-nitrobenz[*a*]pyrene and 3-aminochrysene. The resulting adducts were isolated and purified from a hydrolysate. LC–TSP-MS spectra confirmed the molecular mass of the 6-(deoxyguanosine-N^2-yl)-3-aminobenzo[*a*]pyrene and N- (deoxyguanosyin-8-yl)-3-aminochrysene. Further characterization was done by NMR.

Kuehl et al. [83] investigated the in vitro reaction of calf thymus DNA with allyl halogenides which are potential mutagenic contaminants in the aquatic environment. DNA-hydrolysates were analyzed by reversed-phase chromatography on a C_{18} column using acetonitrile–0.05 *M* ammonium acetate using gradient elution at a flow-rate of 1.4 ml/min. The in vitro dose/adduct formation relation was studied using the ratio of the peak areas $[M+H]^+_{adduct}/[BH+H]^+_{guanosine}$. The formation of N7 and O^6 guanosine adducts (localization of alkyl group was based on HPLC behavior) increased with the amount of allylbromide added. Since the ratio N-7/O^6 alkylation is an estimate for the propensity to be carcinogenic, these data were also studied. However, for the determination of an accurate ratio internal standards are required which were not used in the present investigation. This study indicates the potential of LC–MS techniques for the chemospecific detection of mutagenic environmental contaminants. The sensitivity of the technique as described allows the detection of approx. 1 modified guanine per 10000 guanine residues.

Esmans and coworkers [84,85] discussed the nucleoside adducts formed with two industrial epoxides: phenylglycidyl ether (PGE) and bis-phenol(A) diglycidyl ether (BPADGE). Adducts were analyzed on a Select B column (4 mm I.D.) using methanol–0.1 *M* ammonium acetate at a flow-rate of 0.8 ml/min. The influence of the source parameters on the mass spectra is discussed and 500 pg of PGE/dUrd could be detected by multiple ion monitoring of *m/z* 263 and 379. For the Urd and dCyd adducts information on the alkylation site can be found in the spectra. Attention is drawn to the hydrolytic deamination of the N-3 Cyd adduct resulting in the formation of the corresponding dUrd adduct in both the TSP source and solution prior to analysis. The dGuo adduct also showed depurination and imidazole ring opening reactions which was illustrated by the data found for the BPADGE adduct of 2′-deoxyguanosine (dGuo).

Another way DNA adduct formation takes place under the influence of UV irradiation and was investigated with (+)/(−)TSP by Bérubé et al. [86]. Photoproducts of the dinucleoside monophosphates dTpdU and dTpdT (cyclobutane dimers, photohydrates, 6–4 photoadducts and their Dewar equivalents). Compounds were separated on a C_{18} column (C_{18}) using acetonitrile–0.1 *M* ammonium acetate, in a gradient elution at 1.0 ml/min. Molecular ion species and fragment ions were observed in all cases. Photoproducts could be detected at the low p*M* level using SIR and both (+) and (−)TSP recordings were comparable in terms of sensitivity. Ion abundance is enhanced using the ''filament-on'' mode, and was more pronounced in the negative ion mode.

4.3. Electrospray mass spectrometry

In our research group [87] LC–ESI-MS and LC–ESI-MS–MS was investigated for the structural identification of 2′-deoxynucleotide/bis phenol A diglycidyl ether adducts. The adducts were separated using conventional HPLC on a 5RP8 select B (4.6 mm I.D.) column using NH_4OAc (0.01 *M*)–MeOH, at 0.8 ml/min. By installing a post-column splitter 40 μl/min of the total eluent was led to the source of the mass spectrometer. (−)ESI low-energy collision activated decomposition (CAD) spectra allowed the differentiation between phosphate- alkylation and base-alkylation. Further information on base-alkylation was obtained when (+)ESI low-energy CAD spectra were investigated and compared with the spectra of the 2′- deoxynucleoside/bis phenol A diglycidyl ether adducts. PGE adducts of dC and dA were investigated by Lemière et al. [88] and their stability in solution was studied. ES-MS and MS–MS data were presented showing the ability of low-energy CAD product ion scans to determine the exact alkylation site of the heterocyclic moiety under either (+)ESI or (−)ESI conditions (Fig. 10). Liu et

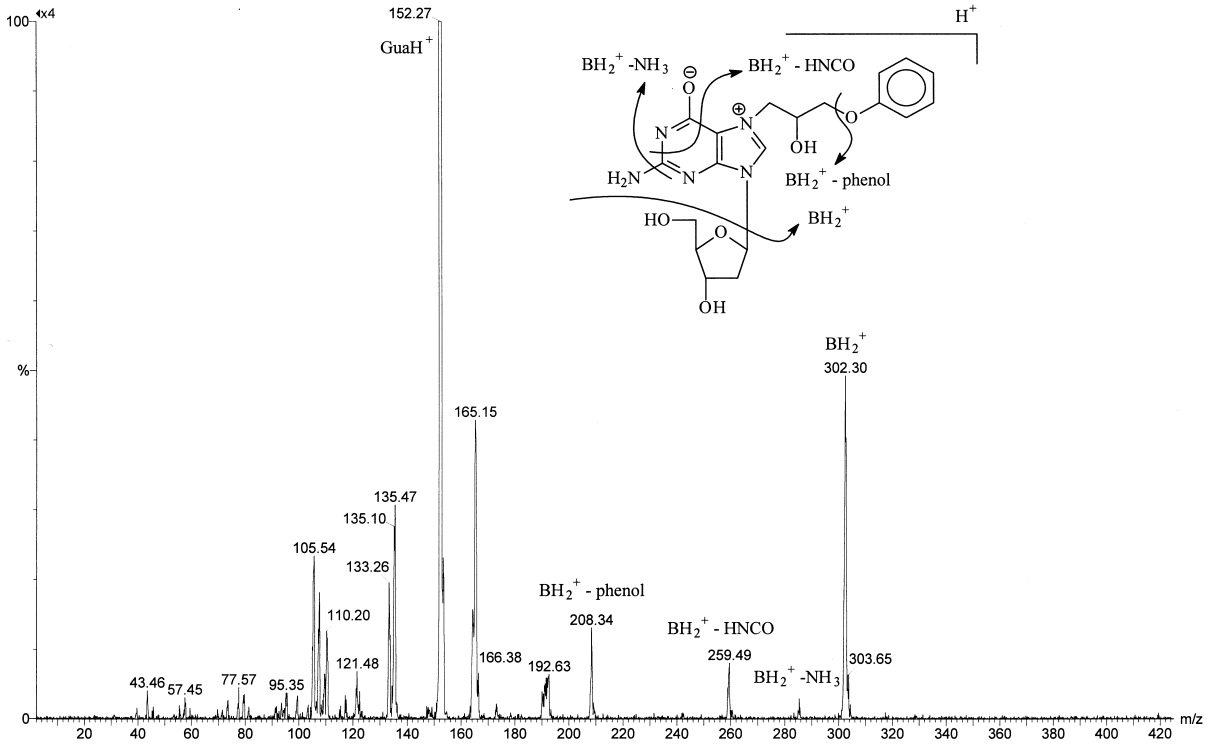

Fig. 10. Low energy CAD product ion spectrum of the $[M+H]^+$ of the N-7 adduct of PGE/dGuo (E.L. Esmans, unpublished results).

al. [89] coupled conventional HPLC (C_{18}, CH_3CN–water, 0.6 or 1 ml/min, 150 μl/min was led to the source of the mass spectrometer) to ESI-MS for the detection of dGuo adducts formed in the reaction of dGuo and DNA with *cis*-4-oxo-pentenal and α-acetoxy-N-nitrosopiperidine. Adducts were enriched using off-line HPLC prior to on-line LC–ESI-MS and were monitored under SIR ($[M+H]^+$ and $[M+Na]^+$) and MRM ($[M+H]^+ \Rightarrow [BH+H]^+$). A detection limit under MRM of 0.3 p*M* was obtained.

Miniaturised HPLC techniques were coupled to ESI-MS in order to improve the mass sensitivity (amount injected on column). Chaudhary and coworkers [90] coupled microbore HPLC (C_8, NH_4OAc–MeOH, 200 μl/min) to ESI-MS for the characterisation of an N^6-oxopropenyl–2′-deoxyadenosine adduct. The adducts were localised in the reaction mixture by monitoring the loss of a 2′-deoxyribose unit (dRib: 116 u) using a constant neutral loss (CNL) scan. In order to get more structural information of the formed adducts, source induced dissociation was effected to produce $[BH+H]^+$ type ions, which were then further fragmented under low-energy CAD conditions. Several research groups coupled capillary LC to ESI-MS. Yen et al. [91] hyphenated micro LC and capillary LC with ESI-MS to quantify N^2,3-ethenoguanine (εGua) isolated from in vitro (calf thymus DNA) and in vivo (human and rat liver) sources. Micro LC (C_{18}, MeOH–water, 25 μl/min) gave the best results, i.e., less carry-over and better sensitivity when large volume injections (4 μl or greater) were performed. The internal standard method ($[^{13}C_4]$εGua) was used to quantify the adducts under SIM conditions. The adducts were enriched using SPE prior to LC–ESI-MS analysis. An absolute detection limit of 50 f*M* adduct isolated out the biological samples was obtained. Our research group [92] compared capillary LC to conventional HPLC for the detection of 2′-deoxynucleoside–bis phenol A diglycidyl ether adducts. Introduction of capillary LC, improved the absolute detection limits by a factor 167. Detection limits of 1 pg of a 2′-deoxyadenosine–bis phenol A diglycidyl ether adduct injected onto the capillary

LC–ESI-MS system (C_8, NH_4OAc (0.01 *M*)–MeOH, 5 μl/min) under SIM ($[M+H]^+$) conditions were presented. Full scan (+)ESI-MS spectra of 110 pg adduct and full scan (+)ESI-MS–MS (low-energy CAD) spectra of 550 pg adduct were recorded. Rindgen et al. [93] coupled capillary LC [C_8, water (0.05% TFA)–MeOH (0.05% TFA), 7 μl/min] to ESI–MS for the determination of in vitro DNA adducts of 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine. Several scan modes were evaluated in order to detect the 2′-deoxynucleoside adducts in the (+)ESI mode. In full scan ESI-MS, 1.1 ng adduct could be detected. The MRM scan mode ($[M+H]^+ \Rightarrow [BH_2]^+$) further enhanced the sensitivity to 78 f*M* (38 pg). The CNL scan mode (loss of dRib) was used to localise several known and unknown adducts in the reaction mixtures.

Wickham et al. [94] studied a covalent hedamycin-d$(CACGTG)_2$ adduct by flow injection in the (−)ESI mode. Barry et al. [95] coupled CZE to (−)ESI-MS (3 μl/min sheath flow consisting of rinsing buffer–2-propanol) for the detection and identification of benzo[*a*]pyrene diol epoxide (BP) DNA adducts. The adducts were formed in the in vitro reaction of calf thymus DNA with BP and were enriched prior to CZE–ESI-MS analysis using SPE. Full scan ESI-MS spectra of 3.4 ng of a BP-dGMP standard could be obtained. Low- energy CAD data of 8.5 ng of the same standard were presented. In order to enhance sensitivity, MRM functions were defined ($-H_3PO_4$ and $-H_2O$). In this scan mode, 130 f*M* (85 pg) adduct could be measured. Sample stacking improved the concentration sensitivity so that four adducts in 10^7 unmodified bases could be detected (10^{-8} *M*).

The endogenous DNA adduct formed by the reaction between malondialdehyde (MDA) and DNA has been identified by Chaudhary et al. [96] and established that the adduct formed with 2′-deoxyguanosine (dG) was a constituent of human liver. Analysis was carried out by direct coupling of a 2.1 mm I.D. microbore C_8 column to the ESI interface. The mobile phase composition was methanolic ammonium acetate–acetic acid at a flow-rate of 200 μl/min. The adduct was isolated from 9.6 mg DNA from human liver and structurally identified as dG modified at the purine base. CNL and low energy CAD data were presented. The former compound was also identified as the major constituent in MDA-treated calf thymus DNA. By monitoring the $[M+H]^+ \rightarrow [BH+H]^+$ transition it was possible to detect 10 pg of the adduct with a S/N ratio >15.

Schrader and Linscheid [97] studied the interaction of styrene oxide with DNA constituents by coupling of CZE to (−)ESI-MS. In this case sample stacking was used as sample loading technique. CZE capillaries of 75 μm I.D. were coupled ammoniumcarbonate as the eluent. A liquid sheath of isopropanol or methanol was used in order to get a stable flow of approximately 5 μl/min into the ESI-source. Exocyclic (N2 or 06) adducts of dG and N-7 adducts were identified. An analogous approach was followed by Deforce et al. [98] studying the interaction of epoxides such as phenylglycidyl ether with calf thymus DNA.

Adducts formed by the interaction of 1,2,3-trichloropropane (TCP), an environmental genotoxic compound, have been analyzed [99]. Adducts were induced in vivo by intra peritoneal administration of TCP to rats at 300 mg/kg. The adduct was identified as S-[1- hydroxymethyl]-2-(N7-guanyl)-ethyl] gluthathione by (+)ESI-MS, ESI-MS–MS and by comparison of these results with an authentic sample. Samples were separated on a capillary HPLC column (320 μm I.D.) filled with a C_{18} stationary phase using trifluoroacetic acid and acetonitrile in a gradient elution at 5 μl/min. Quantitative data were presented.

5. Conclusions

One cannot deny that the structural analysis of both naturally occurring and synthetic nucleic acid constituents in complex mixtures has made a significant progress by the introduction of LC–MS methods. This progress is due to innovating developments in both areas of LC and MS which have been elegantly hyphenated. To date LC–TSP-MS and CF-FAB have largely been replaced by ESI and APCI. From a LC–MS point of view we feel that ESI-LC–MS is the method of choice because of its compatibility with different chromatographic techniques and the way this ionization technique copes with a large variety of nucleic acid compounds of both high- and low-molecular-mass and this with a sen-

sitivity which allows the analysis of nucleic acid material in biological samples. However if structural identification of unknowns is needed or quantitative measurements are planned tandem mass spectrometry is a requirement because protonated or deprotonated molecules are the main species produced under ESI conditions.

In case were small amounts of sample have to be analyzed or where the nucleic acid compounds are present in a complex matrix we are convinced that the coupling of capillary LC (column 300 μm I.D.) and nano-HPLC (column 75 μm I.D.) to ESI or nano-ESI will become the method of choice [100]. These separation techniques can be combined with column switching techniques in order to remove unwanted material (e.g., salts) and to enhance the concentration sensitivity prior to LC–MS analysis. Furthermore when these nano- technologies are coupled to FT-MS or Q-TOF instruments an even more sensitive detection of polar high-molecular-mass compounds is achieved.

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