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Review

Applications of mass spectrometry for quantitation of DNA adducts

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Abstract

DNA adducts are formed when electrophilic molecules or free radicals attack DNA. ^{32}P-postlabeling has been the most commonly used assay for quantitation of DNA adducts due mainly to its excellent sensitivity that allows quantitation at concentrations as low as ~1 adduct per 10^9 normal bases. Such methods, however, do not have the specificity desired for accurate and reliable quantitation, and are prone to produce false positives and artifacts. In the last decade, mass spectrometry in combination with liquid and gas chromatography has presented itself as a good alternative to these techniques since it can satisfy the need for specificity and reliability through the use of stable isotope-labeled internal standards and highly specific detection modes such as selected reaction monitoring and high-resolution mass spectrometry. In this article, the contribution of mass spectrometry to the quantitation of DNA adducts is reviewed with special emphasis on unique applications of mass spectrometry in the area of DNA adduct quantitation and recent applications with improvements in sensitivity.

Keywords: DNA adducts

Contents

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1. Introduction

DNA is prone to alteration by electrophilic reagents due to the presence of nucleophilic sites on its purine and pyrimidine bases, guanine, adenine, thymine, and cytosine. The most active of these nucleophilic sites are nitrogen atoms at position 7 of guanine and position 3 of adenine [1,2]. Electrophilic compounds can react with one of the DNA bases, acting as a nucleophile, causing a covalent modification of the DNA base. This covalently modified DNA base is referred to as a DNA adduct. The electrophilic reagents that produce these DNA adducts may be introduced from either exogenous sources or produced in vivo endogenously through the processes such as lipid peroxidation and oxidative stress [3]. Many of these carcinogenic chemicals are not electrophilic initially, i.e. are not directly reactive with DNA, but become so upon metabolic activation. A good example of such a metabolic activation reaction is conversion of alkenes to electrophilic epoxides by cytochrome P450. Most of the DNA adducts formed do not persist in DNA. There are repair mechanisms such as base and nucleotide excision repair and dealkylation that can remove many of these modifications from DNA. However, if not repaired efficiently, the DNA adducts can cause miscoding during transcription and lead to mutations and to cancer eventually. Therefore, DNA adduct formation is widely believed to be a critical step in carcinogenesis.

DNA adducts represent excellent biomarkers for determining the extent of damage to the genetic material, which has long been of interest in understanding the mechanism of carcinogenesis and in the assessment of cancer risk posed by various chemicals or processes. In earlier studies, this has mostly been done indirectly by measurement of the exposure at high levels and by use of theoretical models. In doing so, one has to consider factors such as absorption, metabolism, detoxification and DNA repair in order to accurately estimate the damage that a particular chemical may cause in DNA. On the other hand, measurement of the concentration of DNA adducts provides one with the dose in the tissue, thus integrating all these factors that can vary significantly from one individual to another. For this reason, DNA adducts are indispensable tools in the development of biomarkers for specific substances or processes, and can be used for improving our understanding of the mechanism of carcinogenesis and accuracy of the process of risk assessment. Therefore, accurate quantitation of DNA adducts is essential and constitutes an important area of research.

Various methods have been developed for quantitation of DNA adducts. Of these methods, the most commonly used ones are ^{32}P-postlabeling (PPL), high-performance liquid chromatography (HPLC) with electrochemical detection, and various chromatographic techniques utilizing mass spectrometric detection. HPLC with fluorescence detection, immunoassays, and accelerator mass spectrometry (AMS) are also used for quantitation of DNA adducts. The methods used for quantitation of DNA adducts can be grouped into two major categories in terms of sensitivity and specificity. In one category, methods such as PPL and AMS have tremendously high sensitivity, but lack specificity. The other category includes those that are highly specific, but not as sensitive as the methods in the first category. The mass spectrometric techniques, which fall into

the latter, are highly specific owing to the capability of mass spectrometry to provide structural information and unambiguous quantitation through the use of stable isotope labeled internal standards. The sensitivity of the mass spectrometric methods, despite being lower than those of the techniques in the first group, is improving constantly through new developments in the design of instrumentation and in the methods of sample introduction. In light of these continuing developments, mass spectrometric techniques appear to have the potential to become the most complete method for the analysis of DNA adducts by providing both sensitivity and specificity. Furthermore, mass spectrometers are being utilized more widely than ever as a result of the decrease in the cost of the instrumentation that is much more compact and can easily outperform the earlier models.

The purpose of this article is to present a review of currently available mass spectrometric methods used for quantitation of DNA adducts. Studies employing mass spectrometry as a qualitative tool, mainly in characterization of novel DNA adducts, will be left out of the scope of this review. The methods employing LC–MS techniques will be given more attention than the other mass spectrometric methods due mainly to the continual improvements being made in this area.

2. Methods for analysis of DNA adducts

The methods for analysis of DNA adducts include, as briefly discussed above, the methods using radioactivity and scintillation counting (following administration of radiolabeled compounds), ^{32}P-post-labeling, accelerator mass spectrometry, immunoassays, liquid chromatography coupled with electrochemical detection, and mass spectrometric methods. Several reviews covering these methods have been published. A comprehensive review of the role of mass spectrometry in the analysis of DNA adducts has been done by Chiarelli et al. [4]. Application of electron capture mass spectrometry (EC-MS) to the analysis of DNA adducts, with a special emphasis on the sample preparation, was covered in great detail by Giese [5]. The latest developments in the area of EC-MS were presented by the same author with extensive experimental details and model applications [6]. Applications of capillary liquid chromatography and capillary electrophoresis were recently reviewed by Apruzzese et al. [7]. Liquid chromatography and electrophoresis techniques using electrospray ionization mass spectrometry (ESI-MS) as the method of detection have been discussed briefly in relation to the analysis of DNA adducts [8]. Application of high-performance liquid chromatography with electrochemical detection for the determination of the biomarkers of oxidative stress has been covered by Hensley et al. [9]. Recently, Phillips et al. [10] discussed the strengths and the weaknesses of the methods employed in the assessment of genotoxicity of a compound.

3. Considerations for sample preparation and handling

Sample preparation is considered by many scientists to be the most critical step in the quantitation of DNA adducts by mass spectrometry. DNA adducts are present in a complex matrix in which there could be up to 10^8-fold excess of unmodified bases. In addition, there often are other contaminants such as proteins, RNA and glycogen present in significant amounts depending on the purity of DNA. It is, therefore, important to employ a good sample preparation strategy to successfully extract and enrich adducts of interest from this complex DNA matrix. This is critical to reduce the potential negative effects of interfering compounds on the sensitivity of the method, and also to improve the reliability of data generated. For example, inorganic salts and other highly polar compounds are common contaminants in the DNA matrix, and can adversely affect the sensitivity of electrospray ionization (ESI) by suppressing the ionization process itself even when they are present in low amounts. Other contaminants may simply increase the baseline and result in loss of sensitivity. Therefore, effort should be spent to remove materials other than the analyte(s) of interest from the matrix. This is the ultimate goal of the whole sample preparation process employed prior to the mass spectrometric analysis and it should be taken into consideration when the procedures for sample preparation are designed. Ideally, the pro-

cedures employed should be as specific to the analyte as possible.

The type of mass spectrometric technique employed for detection of the DNA adduct (e.g. LC–MS, GC–MS, etc.) determines the nature and amount of sample preparation needed. It is of course most desirable to select a method with minimal sample preparation requirements for the reasons that will be discussed below. Time and labor can be a factor as well, especially when the number of samples that need to be analyzed, is significant. From the sample preparation point of view, the LC–MS techniques would be preferred, as they offer the most efficient and simple methodology as long as they provide the satisfactory sensitivity for the application at hand.

Quantitation of DNA adducts by mass spectrometry consists of three major steps; DNA isolation, hydrolysis of the DNA and enrichment of the adduct of interest in the hydrolysate, and finally analysis of the DNA hydrolysate by LC–MS or GC–MS. A generalized scheme for the analysis of DNA adducts by mass spectrometry is given in Fig. 1. The quality of the DNA obtained from DNA isolation is of great importance, since contaminants present in the DNA can cause interference or signal suppression during the mass spectrometric analysis as discussed above. DNA isolation is also a step where some modifications could be introduced artifactually into the DNA if precautions to avoid such modifications are not taken. These modifications may not be of concern if they do not interfere with the analysis of the DNA adduct being studied. It does, however, present a problem for some particular adducts as will be discussed in detail below. Another concern besides the purity of DNA is the stability of the DNA adduct under investigation. The DNA isolation procedure should be designed so that the DNA adduct is preserved in DNA throughout the whole isolation procedure.

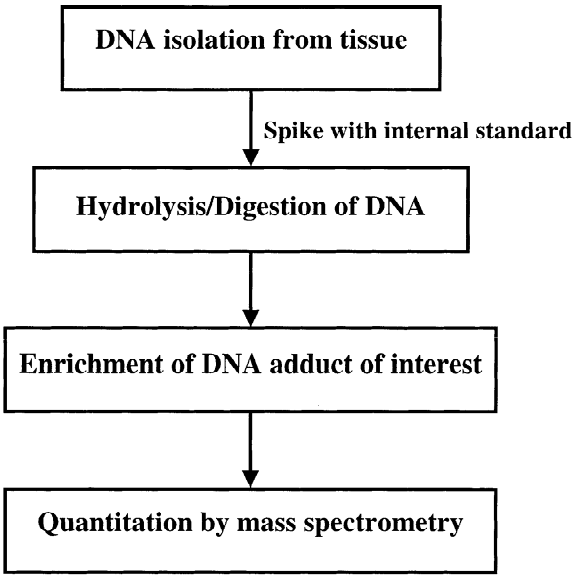

Fig. 1. Scheme for quantitation of DNA adducts by mass spectrometry.

Following DNA isolation is the hydrolysis or digestion of the DNA to release the DNA adduct of interest. The method of choice in this step is determined by the desired form of the DNA adduct for mass spectrometric analysis, i.e. nucleobase, 2-deoxyribonucleoside, or 2-deoxyribonucleotide. Enzymatic digestion could typically be used to obtain the DNA adducts in the form of nucleotides and nucleosides. However, there is not much specificity in this type of hydrolysis since all the unmodified 2=-deoxyribonucleotides/2=-deoxyribonucleosides are cleaved from DNA along with the targeted adducts. Therefore, the resulting hydrolysate contains the normal and adducted nucleosides or nucleotides. Mild acid hydrolysis results in a somewhat cleaner matrix because it cleaves only the purine bases, leaving the pyrimidine bases intact on the DNA backbone. The DNA backbone and associated pyrimidine bases still attached can easily be separated from the purines by filtration or precipitation. It is, of course, most desirable to remove only the DNA adduct of interest from DNA and obtain a perfectly clean sample. There are applications for certain types of adducts that approach this ideal situation. *N*7 and *N*3 purine adducts for example can easily be removed from DNA by neutral thermal hydrolysis (NTH), leaving the backbone of the DNA, other unmodified bases, and bases modified at locations other then *N*7 and *N*3. There are reports of utilization of repair enzymes to selectively remove modified bases. One such study was done by Dizdaroglu et al. [11] to cleave 8-OH-Gua selectively prior to LC–MS analysis using *E. coli* Fpg protein. In the analysis of DNA adducts by PPL, ^{32}P-labeled bases can also be cleaved from DNA very selectively.

After hydrolysis there may be a need for further clean-up depending on the level of adducts in the sample and type of analysis used for quantitation. In the case of mass spectrometric analysis, typically some sort of enrichment of the adducts of interest is needed, since the concentration of the modified bases in real matrices is usually very low relative to the unmodified bases (on average 1 adducted base/10^6–10^8 unmodified bases). The choice of enrichment technique is determined by the characteristics of the DNA adduct and resources available. It could be as simple as solid-phase extraction (SPE) or as sophisticated as immunoaffinity chromatography, or most desirably, the isolation of the adduct from the DNA could itself be highly selective as in the case of hydrolysis by *E. coli* Fpg protein to isolate 8-OH-Gua.

It should be kept in mind that as more and more steps are included in the sample preparation procedure, there will be an increase in the loss of sample during the processes employed in these additional steps such as sample transfer and solvent exchange, and increase in the losses due to adsorption of the analyte to surfaces. Furthermore, the possibility for introduction of artifacts increases as the sample processing becomes more complex. Therefore, one has to decide whether the step being added results in a net gain in terms of sensitivity and/or specificity. On-line sample clean-up techniques, that have recently started to be used in DNA adduct analysis [12], have great promise for future applications as they cause minimal sample loss due to elimination of sample transfer.

The creation of artifacts during sample processing and storage is also of great concern. One should always exercise precaution to avoid artifactual formation of the DNA adduct of interest during the sample processing and handling. However, some adducts are more likely to form an artifact than others due to their relative ease of formation under the conditions used in sample preparation and handling. A good example of such a DNA adduct is 8-hydroxyguanine (8-OH-Gua), a well-studied biomarker of oxidative DNA damage. There have been reports suggesting the artifactual formation of this adduct in almost every step of the analysis including isolation and hydrolysis of DNA, repetitive thawing of DNA, ^{32}P-postlabeling, and even during electrospray ionization [13]. Hydroxy radicals arising from Fenton chemistry are believed to be responsible for the artifactual formation of 8-OH-Gua from unmodified dGuo present in large amounts in the sample. The addition of radical scavengers, such as BHT (2,4-Di-*tert.*-butyl-4-methylphenol) and TEMPO (2,2,6,6-Tetramethyl-1-piperidinyloxy, free radical), appears to minimize artifactual formation if present in the solution throughout the whole sample processing procedure. Recognizing that some adducts could form as an artifact during sample preparation, this possibility should be investigated for each adduct of interest as part of the method development. In situations where there is artifactual formation, the contribution from the artifacts should be reduced to levels below the detection limit of the method for a successful analysis.

The presence of large amounts of matrix components due to insufficient enrichment of the analyte can result in significant increase in the detection limit of the method. This could be in the form of either an increase in the baseline, making measurements below this elevated baseline impossible, or an overall loss in the sensitivity due to the suppression of ionization as in the case of electrospray ionization. It is therefore very critical to ensure efficient removal of these interfering compounds prior to detection. Since the compounds that create these problems are usually very similar in chemical behavior to the DNA adduct under investigation, their removal from the sample using separation techniques such as reverse phase chromatography can present a great challenge. However, techniques like immunoaffinity chromatography that are much more specific to the DNA adduct of interest could be used to help eliminate the interfering compounds from the sample. One can also use automated switching valves to divert these interfering compounds to the waste instead of ionization source to reduce the suppression effect. Although one could incorporate such a procedure in GC–MS methods as well, the LC–MS methods are better suited for these kinds of applications.

4. On-line sample clean-up

Multidimensional chromatography is useful in the separation of complex mixtures of biomolecules where a single chromatographic separation is not

sufficient. It could be performed as different combinations of chromatographic techniques such as ion-exchange, reverse-phase, normal-phase, size exclusion, affinity, immunoaffinity, etc. Various forms of multidimensional chromatography are also commonly utilized for the purpose of removing undesired compounds from the sample prior to the final analysis. In LC–MS experiments, this is typically done by performing an extra chromatographic separation of the same or different chemistry before the existing separation in order to isolate a desired portion of the sample. These types of two-dimensional chromatographic on-line separations are made possible through the use of automated switching valves. For this reason, they are commonly referred to as column-switching techniques. The most commonly employed forms of two dimensional chromatography as applied to the on-line sample clean-up are on-line salt removal (desalting), sample preconcentration or stacking, and ''heart-cut'' injection of desired compounds, where only the compounds in a selected window of retention from the first chromatographic separation (first dimension) is allowed on to the second chromatographic separation (second dimension), while the compounds out of the selected window are diverted to the waste. A generalized scheme for a column-switching setup that could be used for these purposes is depicted in Fig. 2. In these column-switching applications, typically a smaller column is used as a trap column prior to separation on an analytical column. The sample is loaded onto the trap column while the analytical column is being conditioned with the starting mobile phase. The effluent from the trap column is directed to the waste until shortly before the analyte of interest elutes. The retained analytes in the trap column are then back flushed onto the analytical column until all of the desired compounds are carried onto the analytical column. Flow of the trap column is again diverted to the waste until the next run. After the analyte is transferred from the trap column onto the analytical column, it is eluted with the choice of mobile phase, and is detected.

On-line desalting techniques using column-switching have proven extremely useful, especially for LC–ESI-MS methods. Most biological samples contain some salt, which can suppress electrospray ionization. Salts and any other poorly retained compounds can be easily removed by on-line desalting. This process typically adds 1–2 min to the run time, which is very minimal compared to hours that could easily be spent when this is done off-line, for example, using solid-phase extraction.

Trap columns can also be packed with a media highly specific to the DNA adduct studied. The best example of this is on-line immunoaffinity clean-up using columns packed with resins containing antibodies raised against the analyte. There have been numerous examples of on-line immunoaffinity chromatography in the area of bioanalysis. Recently, it

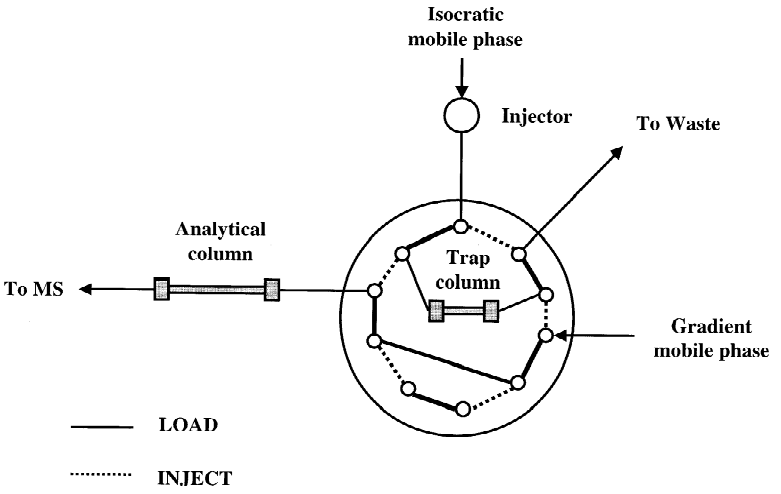

Fig. 2. A general scheme of a column-switching setup that can be used for on-line sample preparation and clean-up.

has also been employed in quantitation of DNA adducts [12,14].

5. Unique features of mass spectrometry in quantitative analysis of DNA adducts

The main reason for a scientist to use mass spectrometry in a quantitative method is the high level of specificity it provides in the detection process. In a typical mass spectrometric detection, ions at a mass/charge (m/z) within a fixed window of atomic mass unit (amu) (typically 0.5–1 amu) are registered. This mode is referred to as selected ion monitoring (SIM). SIM enhances the specificity of a method significantly, especially, when it is combined with a chromatographic separation prior to the mass spectrometric detection. The degree of specificity for the mass spectrometric detection can be further increased by performing even more specific modes of mass spectrometric detection. Two important examples of these highly specific modes are high-resolution mass spectrometry (HRMS) and tandem mass spectrometry in selected reaction monitoring (SRM) mode. In HRMS, the window of detection is even narrower than that in SIM, which results in significant reduction of the contribution from possible interfering ions at the m/z's close to that of the analyte. SRM, typically performed using an instrument capable of tandem mass spectrometry such as triple quadrupole and quadrupole–time of flight mass spectrometers, is the most specific mode of mass spectrometric detection. In order to register a response in an SRM experiment, the analyte has to have the right mass and also produce a predetermined fragment ion upon fragmentation by collision-induced dissociation (CID). The increased specificity that SRM and HRMS provide is highly valuable in reducing or eliminating interferences from complex biological matrices. Our laboratory [15] has demonstrated the importance of increased specificity provided by HRMS in trace level quantitation in a study that investigated the effectiveness of increasing the resolving power of the mass spectrometer in reduction of the contribution from an interfering compound during quantitation of *N*7-(2-hydroxyethyl)guanine present endogenously in rat spleen DNA. It was concluded in this study that at resolving powers of 10 000 and above the contribution from the interfering compound present in the biological matrix was negligible.

Mass spectrometric detection also permits one to conduct isotope dilution mass spectrometry (IDMS) experiments through its unique capability to differentiate compounds based on the difference in m/z. This capability could also be exploited to characterize compounds that co-elute or are not completely resolved in a chromatographic separation. In a typical IDMS experiment, standard compounds that are prepared by replacing several atoms in that compound with their stable isotopes such as ^{13}C or ^{15}N are used as internal standards. The internal standard prepared this way is almost identical chemically to the analyte. When added to the sample before the sample preparation procedure, these stable isotope internal standards can be used to correct for losses during the sample preparation and variations in the mass spectrometric response, since the analyte and its corresponding stable isotope internal standard behave extremely similar in the processes to which the sample is subjected. It is now almost a standard practice among scientists in this area to use these kinds of internal standards in quantitation when mass spectrometry is used as the method of detection. In our opinion based on personal experience, a quantitative mass spectrometric method becomes much less reliable as a quantitative tool without the stable isotope internal standards due to significant fluctuations observed in the sensitivity of the mass spectrometric response. This is true especially in LC–MS techniques using atmospheric pressure ionization techniques such as electrospray and atmospheric pressure chemical ionization. Ionization efficiency in these atmospheric pressure ionization processes are widely recognized to be highly sensitive to even slight changes in experimental conditions such as solvent composition, condition of the source (e.g. contamination of the source from unknown chemicals present in samples), and presence of salts in the solution or in the ionization source.

Another unique application of mass spectrometry in the area of DNA adduct quantitation is found in studies where the origin of the DNA adduct is under investigation. As we know, some DNA adducts can be induced by both endogenous and exogenous chemicals [3]. Through the use of stable isotope

analogs, mass spectrometry can allow one to quantitate the DNA adducts originating from different sources by taking advantage of the mass difference between the chemicals that induce the DNA adduct and its stable isotope analog. In one such study by Morinello et al. [16], male Sprague–Dawley rats were exposed to $^{13}C_2$-labeled vinyl chloride (VC) by inhalation in order to determine the source of N^2,3-ethenoguanine (N^2,3-εGua) in the brain and hepatocyte DNA. Using a highly specific and sensitive immunoaffinity-gas chromatography-isotope dilution high resolution mass spectrometry (IA-GC–IDHRMS) method, they have analyzed the DNA samples from hepatocytes and brain for both N^2,3-εGua and [$^{13}C_2$]-N^2,3-εGua. By administering [$^{13}C_2$]-VC, they could distinguish the endogenously formed N^2,3-εGua from the VC-derived N^2,3-εGua since the VC-derived N^2,3-εGua would be 2 mass units greater. They showed that the amount of [$^{13}C_2$]-N^2,3-εGua in hepatocyte DNA increased from background levels to 60 ± 10 N^2,3-εGua adducts/10^8 normal guanine bases, while there was no change in endogenous levels in liver or brain DNA. In contrast, [$^{13}C_2$]-VC did not cause an increase in the concentration of [$^{13}C_2$]-N^2,3-εGua in the brain. The fact that [$^{13}C_2$]-N^2,3-εGua was not detected in the brain DNA suggested that the VC-specific adduct did not form in the brain. In a similar recent study, a stable isotope-labeled ethyl linoleate ([$^{13}C_{18}$]-EtLA) was used to investigate whether or not N^2,3-εGua was formed by products of lipid peroxidation [17]. Reactions of 2′-deoxyguanosine with [$^{13}C_{18}$]-EtLA and 4-hydroxy-2-nonenal (HNE) were conducted and the resulting reaction products were analyzed by both IA-GC–EC-ID-HRMS and IA-LC–ESI-IDMS–MS for ^{13}C-labeled and unlabeled N^2,3-εGua. It was demonstrated that there was ~90% incorporation of ^{13}C in N^2,3-εGua formed while the unlabeled N^2,3-εGua increased to a lesser extent. These results suggested that two separate mechanisms existed for the formation of N^2,3-εGua by oxidative processes involving both HNE and EtLA. Studies such as those summarized above would not have been possible if it was not for the unique capability of mass spectrometry to discriminate chemically similar compounds based on a slight difference in mass.

6. Application of mass spectrometric methods for quantitation of specific DNA adducts

The last decade witnessed a tremendous increase in utilization of mass spectrometry in the area of DNA adduct analysis. Most of these applications have been in a qualitative manner, i.e. identification and characterization of novel DNA adducts or confirmation of the identity of known adducts. Recently, more and more laboratories have also begun studies to develop mass spectrometric assays for quantitation. This is largely due to decreasing cost of mass spectrometers, improved sensitivity of the new generation instrumentation, the use of stable isotope internal standards, the new approaches in clean-up procedures, and the development of more user-friendly data stations for mass spectrometers that once required a dedicated mass spectrometrist to operate. Development of new interfaces such as ESI, matrix assisted laser desorption ionization, and thermospray ionization for coupling liquid chromatography to mass spectrometry has also started a new era in analysis of biomolecules. Through these interfaces, polar biomolecules that could not be introduced into a mass spectrometer without some kind of derivatization can now be analyzed directly. In addition, electrospray ionization has eliminated the need for mass analyzers with a large range of mass because of the phenomenon that it generates multiply charged ions from most high molecular mass biomolecules such as proteins and oligonucleotides. Since mass spectrometers measure m/z, a 20 000 Da polypeptide that carries 20 charges upon ionization in an ESI source would have an m/z of 1000 and is therefore easily detected by affordable mass analyzers like quadrupole mass analyzers.

Mass spectrometric methods such as GC–MS, LC–MS and capillary zone electrophoresis–mass spectrometry (CE–MS) are developing rapidly to become the methods of choice in quantitation of DNA adducts. The most commonly used mass spectrometric techniques are however gas chromatography with negative ion chemical ionization mass spectrometry (GC–EC-MS) and liquid chromatography with electrospray mass spectrometry (LC–ESI-MS).

In the following sections, we will review quantita-

tive methods utilizing mass spectrometry as the method of detection. The methods will be grouped according to the origin of the DNA adduct or adducts under consideration. This review of quantitative mass spectrometric methods for DNA adducts is also presented in a tabular format for quick reference (Table 1). Accelerator mass spectrometry, even though it is referred as a mass spectrometric technique, is different from the rest of the mass spectrometric techniques in that it provides no information on chemical structures of the isotopic compounds it measures. Therefore, the methods using this mass spectrometric technique are not covered in this review. Interested readers are referred to the excellent work that has been published on applications of accelerator mass spectrometry for quantitation of DNA adducts [10,18–20].

6.1. Reactive oxygen species

Reactive oxygen species (ROS) are reactive free radicals formed from molecular oxygen during metabolic pathways. ROS and other free radicals cause a number of lesions in DNA, the best known and well studied of them being 8-OH-Gua. This adduct has been quantitated mainly by HPLC with electrochemical detection (See Ref. [9] for a review). GC–MS methods were also developed and broadly used for quantitation of 8-OH-Gua as well as other ROS-induced lesions [21–25]. Anson et al. [21] developed such an assay for simultaneous quantitation of lesions resulting from damage by radical oxygen species using the GC–MS method developed earlier by the same laboratory [22]. The lesions studied were 5-hydroxyuracil (5-OH-Ura), 5-hydroxyhydantoin (5-OH-Hyd), 8-OH-Gua, 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyGua), 4,6-diamino-5-formamidopyrimidine (FapyAde), 8-hydroxyadenine (8-OH-Ade), 5,6-dihydroxyuracil (5,6-diOH-Ura), 5-hydroxy-5-methylhydantoin (5-OH-5-Me-Hyd), 5-(hydroxymethyl)uracil (5-OH-Me-Ura), and 5-hydroxycytosine (5-OH-Cyt). Authors used this method to study differences in the amount of these adducts in mitochondrial and nuclear DNA from livers of Wistar rats. Significant differences were detected only for 5-OH-Ura, 8-OH-Ade, and 5-OH-Cyt. For the rest of the DNA adducts, the concentrations in both types of DNA were similar.

Recently, quantitation by LC–MS of 8-OH-Gua, both as base and nucleoside, has been shown to be an attractive alternative to the earlier GC–MS methods. Serrano et al. [26] developed the first LC–ESI-MS–MS method for quantitation of 8-OH-dGuo and was able to quantitate it in adriamycin-exposed Sprague–Dawley rats at 0.2 adducts/10^6 normal dGuo. However, no stable isotope internal standard was used in the method. The LC–MS methods appear to be well suited, particularly for 8-OH-Gua, because they minimize the artifactual formation due to simplicity of the required sample preparation. An interesting phenomenon has been observed during detection of 8-OH-dGuo by LC–ESI-MS. Ravanat et al. [13] have first suggested a partial conversion of normal dGuo to 8-OH-dGuo during electrospray ionization, as evidenced by the existence of a peak in the 8-OH-dGuo channel at the same retention time as that of dGuo. We have also observed this partial conversion of dGuo to 8-OH-dGuo when we analyzed rat liver for 8-OH-dGuo. Fig. 3 shows a SRM trace for a rat liver DNA. The first peak in this chromatogram corresponds to the 8-OH-dGuo that is believed to originate from conversion of dGuo to 8-OH-dGuo in the ESI source after it eluted from the HPLC column. This conversion however does not interfere with the quantitation as the two compounds are well separated on the HPLC column prior to ionization.

Ravanat et al. [13], in the study just mentioned, have evaluated the sensitivity of the detection modes SIM and SRM during the development of an LC-IDMS method for quantitation of 8-OH-dGuo. A stable isotope-labeled internal analog, [$^{15}N_3$, $^{13}C_1$]-8-OH-dGuo, was synthesized and used as internal standard. The sensitivity in the SIM mode was extremely poor with a detection limit of 5 pmol while the detection limit was lowered to ~20 fmol in the SRM mode. They applied the newly developed LC–ESI-IDMS–MS method to the analysis of DNA from control pig liver and calf thymus, and urine samples.

Renner et al. [27] developed an LC–MS method for the quantitation of urinary 8-OH-dGuo. Off-line SPE was used for initial removal of some urinary

Table 1
Summary of applications of mass spectrometry to quantitation of DNA adducts

Compound	Adduct	Method	Refs.
Reactive oxygen species	8-OH-Gua, 8-OH-Ade, 5-OH-Ura, 5-OH-Hyd, FapyGua, FapyAde, 5,6-diOH-Ura, 5-OH-5-Me-Hyd, 5-OH-MeUra, 5-OH-Cyt	GC–IDMS	[21–25]
	8-OH-Gua	LC–ESI-IDMS	[11,13]
	8-OH-dGuo	LC–ESI-IDMS–MS	[13,27]
	8-OH-dGuo, 8-OH-dAdo	LC–ESI-MS–MS	[28]
		LC–ESI-IDMS–MS	[29]
Lipid peroxidation products	N^2,3-ϵGua	GC–EC-IDMS	[33,34]
		GC–EC-IDHRMS	[16,17,36–38]
	N^2,3-ϵGua, 1,N^2-åGua	GC–EC-IDHRMS	[38]
	1,N^2-ϵGua, OH-Ethano-Gua	LC–ESI-IDMS–MS	[41,42]
	1,N^6-ϵAde	LC–ESI-IDMS	[80]
		LC–ESI-IDMS–MS	[43]
	N^2,3-ϵGua	LC–ESI-IDMS	[39]
	1,N^6-ϵdAdo, 3,N^4-ϵdCyt	LC–ESI-IDMS–MS	[12]
	3,N^4-ϵdCyt	LC–ESI-IDMS–MS	[14]
	M_1G	GC–EC-IDMS	[44–47]
		LC–ESI-IDMS–MS	[49]
	Cr-dGuo,HX-dGuo,HNE-dGuo	LC–ESI-MS–MS	[48]
Polycyclic aromatic hydrocarbons	BP-6-*N*7-Gua, BP-6-*N*7-Ade	LC–ESI-MS	[51]
	BPDE-dGMP adduct	CE–ESI-MS–MS	[53]
	BPDE-tetraols	CE–EC-IDMS	[52]
Olefins	N7-Gua adducts of butadiene	LC–ESI-IDMS–MS	[54–56]
	*N*7-Gua adducts of ethylene	GC–EC-IDMS–MS	[15,57,59,60,81]
		LC–ESI-MS	[61]
		LC–ESI-IDMS–MS	[62]
	*N*7-Gua adducts of propylene	GC–EC-IDMS–MS	[64,65]
	*N*7-Gua adducts of styrene	ICP-MS	[66]
		LC–ESI-MS	
		LC–ESI-IDMS–MS	[67]
Alkylating agents	*N*3-alkyladenines	GC–IDMS	[69,70]
Radiation	ThdGly, 5-OH-dUrd, 5-HMdUrd, 5-For-dUrd, 8-OH-dGuo, and 8-OH-dAdo.	LC–ESI-IDMS–MS	[77]
	dβF-8-OH-dGuo, 8-OH-dGuo-dβF	LC–ESI-MS–MS	[78]
Bisphenol A diglycidyl ether	Bisphenol A diglycidyl ether-dGMP adducts	LC–ESI-MS	[79]
Heterocyclic aromatic amines	dGuo-*C*8-PhIP	LC–ESI-MS–MS	[75]
	dGuo-*C*8-IQ, dGuo-*N*2-IQ	LC–ESI-MS–MS	[76]
4-aminobiphenyl	dGua-C8-4-ABP adducts	GC–EC-IDMS	[71,72]
	dGuo-*C*8-4-ABP adducts	LC–ESI-IDMS	[73]
Mephalan	dAMP adducts of melphalan	LC–ESI-MS–MS	[82]

Abbreviations used in the methods column are given at the end of the article. Abbreviations for adducts: 5-hydroxyuracil (5-OH-Ura), 5-hydroxyhydantoin (5-OH-Hyd), 8-hydroxyguanine (8-OH-Gua), 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyGua), 4,6-diamino-5-formamidopyrimidine (FapyAde), 8-hydroxyadenine (8-OH-Ade), 5,6-dihydroxyuracil (5,6-diOH-Ura), 5-hydroxy-5-methylhydantoin (5-OH-5-Me-Hyd), 5-(hydroxymethyl)uracil (5-OHMeUra), and 5-hydroxycytosine (5-OHCyt) 1,N^6-ethenoadenine (1,N^6-ϵAde), 3,N^4-ethenocytidine (3,N^4-ϵCyt), 1,N^2-ethenoguanine (1,N^2-ϵGua), and N^2,3-ethenoguanine (N^2,3-ϵGua), Pyrimido[1,2-alpha]purin-10(3H)-one (M_1G), Crotonaldehyde-modified dGuo (Cr-dGuo), 2-hexenal-modified dGuo (HX-dGuo), and 4-hydroxy-2-nonenal -modified dGuo (HNE-dGuo), 7-(benzo[a]pyren-6-yl)guanine (BP-6-*N*7-Gua), 7-(benzo[a]pyren-6-yl)adenine (BP-6-*N*7-Ade),*Anti*-7,8,9,10-tetrahydrobenzo-[a]pyrene-7,8-diol 9,10-epoxide (BPDE), 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP), *N*-(deoxyguanosin-8-yl)-2-amino-3-methylimidazo[4, 5-f]quinoline (dGuo-*C*8-IQ), 5-(deoxyguanosin-N(2)-yl)-2-amino-3-methylimidazo[4,5-f]quinoline (dGuo-*N*2-IQ), *N*-(2-deoxy-β-D-*erythro*-pentofuranosyl)formylamine-8-oxo-7,8-di-hydro-dβ-deoxyguanosine (dβF-8-oxodGuo) and its isomer (8-oxodGuo-dβF), 5-hydroxy-2′-deoxyuridine (5-OH-dUrd), 5,6-dihydroxy-5,6-dihydrothy-midine (ThdGly), 5-formyl-2′-deoxyuridine (5-For-dUrd), and 5-(hydroxymethyl)-2′-deoxyuridine (5-HM-dUrd).

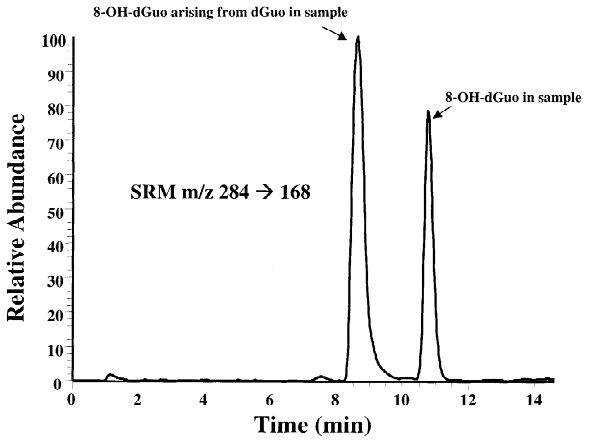

Fig. 3. Product ion chromatogram of a rat liver hydrolysate analyzed for 8-OH-dGuo using LC–ESI-MS–MS in SRM (m/z 284–168). The LC–MS system consisted of Magic 2002 HPLC unit coupled to a Finnigan TSQ 7000 triple quadrupole mass spectrometer with API2 ESI source. The LC separation was done on an Aquasil C_{18} column (2×150 mm, 3 micron). The mobile phase was 5% acetonitrile isocratically supplied at 200 μl/min.

components. An interesting finding they reported was that acetonitrile did not elute 8-OH-dGuo from SPE cartridges packed with LiChrolute EN. Therefore, they were able to use acetonitrile conveniently as a washing solvent for selective removal of the other urinary components. The limit of detection was 0.2 ng/ml or 7 fmol of standard ($S/N=3$). Mass spectrometric detection was done in the SRM mode using the transition m/z 284.1–167.8. The method however lacked an internal standard. Simultaneous quantitation of 8-OH-dAdo and 8-OH-dGuo by LC–ESI-MS–MS in commercially obtained calf thymus DNA was recently reported [28], producing 8-OH-dGuo data comparable to those obtained by the LC-ECD in the same study. Weimann et al. [29] have also developed a similar method capable of quantitation of both adducts, with the addition of ^{15}N-labeled standards as internal standards.

Recently an LC–ESI-IDMS method using only a single stage mass spectrometer that can detect 8-OH-Gua at ~$1/10^6$ normal Gua bases using ~2 μg DNA was developed by Dizdaroglu et al. [11]. They achieved a sensitivity of ~50 fmol with standard using the SIM mode, a significant improvement when compared to the sensitivity reported earlier by others in the same detection mode [13].

6.2. Lipid peroxidation products

6.2.1. Ethenobases

Ethenobases are exocyclic adducts that are formed through reaction of DNA bases with metabolites of compounds such as vinyl chloride or urethane, or with lipid peroxidation products endogenously. The commonly known ethenobases are 1,N^6-ethenoadenine (1,N^6-ϵAde), 3,N^4-ethenocytidine (3,N^4-ϵCyt), 1,N^2-ethenoguanine (1,N^2-ϵGua), and N^2,3-ethenoguanine (N^2,3-ϵGua). Their structures are shown in Fig. 4. The ethenobases have been shown to be promutagenic, causing both transversion and transition mutations. Human and animal tumors associated with vinyl chloride exposure contain mutations consistent with these miscoding properties [30–32].

Fedtke et al. [33,34] developed a gas chromatography negative ion chemical ionization isotope dilution mass spectrometry (GC–EC-IDMS) method for quantitation of N^2,3-ϵGua in preweanling and adult Sprague–Dawley rats exposed by inhalation to vinyl chloride. An electrophore labeling technique [35] based on converting the analyte into its dipentafluorobenzyl derivative was used for highly sensitive detection by electron capture mass spectrometry of negatively charged ions produced in chemical ionization of the pentafluorobenzyl derivative. Sensitivities

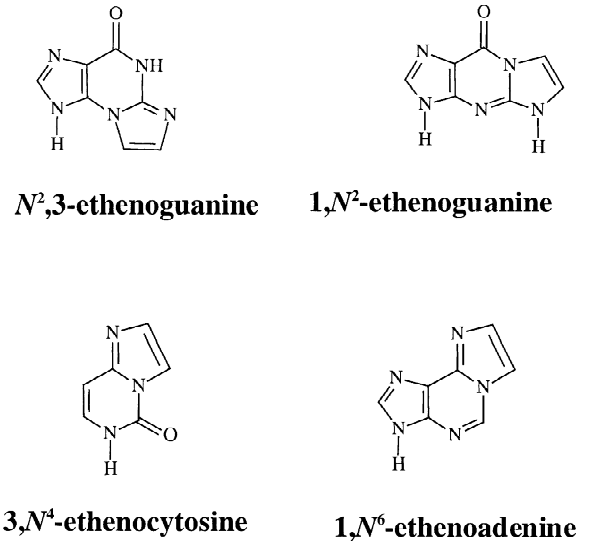

Fig. 4. Structures of etheno adducts.

of ~300 amol were achieved with pure standards using electrophore labeling. A ^{13}C-labeled analog of N^2,3-ϵGua, [$^{13}C_4$]-N^2,3-ϵGua, was used as internal standard to assure accurate quantitation. N^2,3-ϵGua released from DNA by mild acid hydrolysis was further enriched using low-pressure strong cation-exchange chromatography. The method detection limit, however, was much higher (60 fmol N^2,3-ϵGua/μmol Gua) for real samples apparently due to the suppression caused by impurities in the DNA hydrolysate.

Immunoaffinity chromatography has recently been utilized by us and others as a highly specific method of enrichment of low level adducts, including endogenous etheno adducts, therefore making detection limits as low as 1 adduct per 10^8 nucleotides possible. Ham et al. [36] have applied the immunoaffinity clean-up off-line prior to quantitation of N^2,3-ϵGua by GC–HRMS. Immunoaffinity columns were built using polyclonal antibodies raised against N^2,3-ϵGua. The columns were highly stable when stored at 4 °C and used for multiple clean-ups. There were no unmodified bases detectable in the sample after the immunoaffinity chromatography clean-up. By combining this highly specific enrichment technique with sensitive detection by electron capture mass spectrometry, they have been able quantitate N^2,3-ϵGua endogenously present in 100 μg rat liver DNA at 8.9 N^2,3-ϵGua adducts/10^8 normal guanine bases. Fig. 5 shows representative GC–HRMS chromatograms from endogenous rat liver DNA samples.

The method of Ham et al. [37] was later modified to allow simultaneous quantitation of both N^2,3-ϵGua and 1,N^2-ϵGua [38]. The immunoaffinity columns contained polyclonal antibodies raised against N^2,3-ϵGua and 1,N^2-ϵGua, and therefore retained both etheno adducts from the DNA hydrolysate. Using ~250 μg DNA, detection limits for N^2,3-ϵGua and 1,N^2-ϵGua were estimated to be 5.0 and 8.7 adducts/10^8 normal guanine bases, respectively. This study clearly demonstrated that the immunoaffinity columns specific for multiple DNA adducts could be prepared for their simultaneous analysis. Besides saving time and labor, simultaneous analysis of several adducts allows one to make more accurate comparisons of adducts under investigation, since conditions for analysis of these adducts are nearly identical.

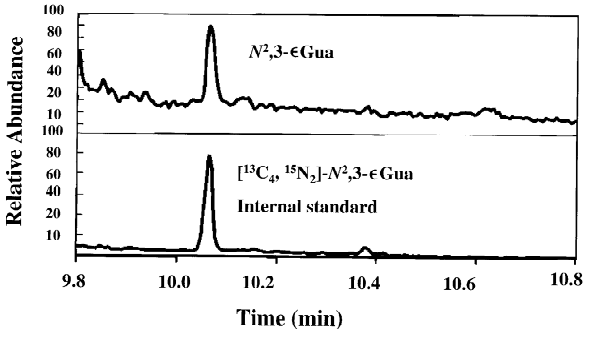

Fig. 5. SIM traces for N^2,3-ϵGua and its internal standard ([$^{13}C_4$]-N^2,3-ϵGua) in a control rat liver DNA. Reprinted with permission from [36].

Around the mid 1990s, several researchers started to make the transition to LC–MS techniques for quantitation of ethenobases for several reasons, the most apparent being the elimination of tedious and sometimes artifact-prone derivatization procedures that the GC–MS methods with electrophore labeling require. Our laboratory has reported the development of such a method for N^2,3-ϵGua using strong cation-exchange chromatography for removal of unmodified bases followed by analysis by LC-IDMS in the SIM mode [39]. The detection limits achieved by this method were 5 and 50 fmol for pure standard and biological sample, respectively. The method was applied to analysis of chloroethylene oxide-treated calf thymus DNA, liver DNA from rats exposed to chloroethylene oxide by portal vein injection, and unexposed human liver DNA. Due to the low levels of N^2,3-ϵGua in unexposed human liver DNA, high amounts of DNA needed to be processed. This was clearly the method of choice for analysis of DNA with relatively high levels of N^2,3-ϵGua due to its simplicity. Yen et al. have used a similar method for quantitation of 1,N^6-ϵAde in urine of rats exposed to chloroethylene oxide [39,40]. Immunoaffinity chromatography was again used for highly efficient and specific extraction of urinary 1,N^6-ϵAde prior to LC–MS analysis.

Muller et al. have employed LC–ESI-IDMS–MS for analysis of chloroethylene oxide-treated DNA for 1,N^2-ϵGua and 5,6,7,9-N^2-(2-oxoethyl)guanine (OH-Ethano-Gua) [41]. Limits of quantitation for 1,N^2-ϵGua and OH-Ethano-Gua were approximately 3 pmol because of the suppression due to the presence

of excessive amounts of normal guanine and adenine bases even after an HPLC clean-up. They were able to improve the sensitivity of the method when they used the 2′-deoxyribonucleosides instead of bases. The method using the nucleosides had detection limits as low as 10 pmol OH-Ethano-dGuo/μmol dGuo in chloroethylene oxide-treated DNA samples. Attempts to measure 1,N^2-ϵGua in the same samples however were not successful due to an interfering compound that co-eluted with the analyte. An LC–ESI-IDMS–MS method was developed for quantitation of 1,N^2-ϵdGuo, and has been applied to analysis of calf thymus DNA treated with *trans,trans*-2,4-decadienal [42]. The method utilized ^{15}N-labeled internal standard and demonstrated a reasonable sensitivity for detection of 1,N^2-ϵdGuo, as low as 20 fmol. The baseline level of this adduct was, however, relatively high compared to other etheno adducts (~3/10^6 dGuo). A recent study in our laboratory has revealed that 1,N^2-ϵGua could easily be formed during sample processing at alkaline pH [38]. Both studies have shown the formation of this adduct in vitro, however the in vivo formation of this adduct has not been reported. The latter study also suggested that the previously detected 1,N^2-ϵGua was probably an artifact due to its ease of formation under basic conditions.

Chen et al. have developed both GC–EC-IDMS and LC–ESI-IDMS–MS methods for quantitation of 1,N^6-ϵAde in human placental DNA [43]. Data from both methods were very similar, ~2.3 1,N^6-ϵAde/10^6 normal adenine bases in human placenta. Even though the sensitivity of their GC–MS method was comparable to those developed earlier, the LC–MS method did not have the satisfactory sensitivity for routine analysis. The authors have stated that poor peak shape in HPLC separation was responsible for the low sensitivity.

Recently, extremely sensitive LC–MS methods using on-line sample clean-up techniques have been developed. Doerge et al. [12] incorporated on-line SPE extraction into the LC–ESI-IDMS–MS and achieved detection limits as low as 1 adduct/10^8 normal nucleotides for 1,N^6-ϵdAdo using ~100 μg DNA (~ 4 fmol of the adduct in 100 μg DNA) from control and urethane-exposed mouse livers. Due to the interference from 2′-deoxyadenosine, which has the same SRM transition as 3,N^4-ϵdCyt, endogenous 3,N^4-ϵdCyt could not be detected. This problem was later overcome in a follow-up study from the same laboratory [14]. After usual enzymatic hydrolysis to obtain 2′-deoxynucleosides, the hydrolysate was treated with adenosine deaminase to convert dAdo to deoxyinosine in order to minimize the interference from dAdo as reported earlier. Immunoaffinity chromatography and solid-phase extraction were performed on-line prior to LC–MS analysis. An HPLC column with graphitized carbon media as packing, which has a unique selectivity for the separation of adducts, was utilized. This material was also very stable at elevated temperatures. Complete baseline separation of 3,N^4-ϵdCyt from the dAdo remaining after the conversion was possible at 85 °C. This allowed the detection of N^4-ϵdCyt at 5 adducts/10^8 normal nucleotides. Furthermore, at elevated temperatures, backpressure that HPLC pumps are subjected to becomes much less due to decreased viscosity of the heated solvent. The decreased load on the pumps make multidimensional LC separations more feasible.

6.2.2. Malondialdehyde

Malondialdehyde (MDA) is one of the major aldehydic compounds endogenously formed upon peroxidation of membrane lipids. MDA has been of interest in the area of carcinogenesis since it reacts with DNA and therefore could play a role in formation of cancer from natural causes. Chaudhary et al. were first to develop a quantitative assay for analysis of a cyclic adduct formed by reaction of MDA with *N*1 and *N*2 of guanine, pyrimido[1,2-α]purin-10(3H)-one (M_1G), by taking advantage of electron capture mass spectrometry [44]. Prior to the GC–EC-IDMS analysis, the aldehydic adduct was reduced with sodium borohydride and isolated from the DNA matrix. The reduced adduct was derivatized to its pentafluorobenzyl derivative and then to the corresponding *tert*-butyldimethylsilyl derivative. The internal standard used for IDMS was [$^2H_2$]-M_1G. The detection limit was ~30 fmol ($S/N>5$) injected on column. The method was used for quantitative measurements of M_1G in control rat livers. Similar methodology was used for analysis of DNA from human liver and pancreas for M_1G [45,46]. An average amount of 9 M_1G/10^7 was determined in the human livers analyzed.

An improved version of this method was later developed by incorporating an immunoaffinity clean-up procedure [47]. Recovery of the whole sample preparation process prior to the GC–EC-IDMS analysis was ~40%. The detection limit of the improved assay was 100 fmol/sample or 3 adducts/10^8 normal bases. This improved method was applied to analyses of DNA from human blood. The concentration of M_1G in these blood samples was 6.2 ± 1.2 adducts/10^8 normal bases.

In addition to the GC–EC-MS methods, an LC–ESI-MS method was developed for dGuo adducts of the lipid peroxidation products malondialdehyde, crotonaldehyde, 2-hexenal, and 4-hydroxy-2-nonenal in order to validate the results obtained by PPL [48]. They have reported a detection limit of ~30 fmol for pure standards of each adduct injected on column, which would theoretically allow 1 adduct/10^7 normal nucleotides using ~100 μg DNA. More recently, Hakala et al. have also developed an assay using LC–ESI-IDMS–MS [49]. This method was tested by analyzing CT-DNA treated in vitro with MDA. Based on the measurements in these samples and the sensitivity obtained with standards (50 fmol), it was concluded that this method promised to be useful for quantitation in biological samples that have ~1 adduct/10^6 guanine bases using samples of ~100 μg of DNA.

6.3. Polycyclic aromatic hydrocarbons

Polycyclic aromatic hydrocarbons (PAH) are a major class of compounds that pose a threat to human health due to their presence in polluted air and cigarette smoke. Methods using chromatographic techniques such as gas chromatography, liquid chromatography, capillary electrophoresis (CE), and capillary electrochromatography coupled to mass spectrometry have been utilized for measurement of DNA adducts related to the PAH's. Giese et al. have applied electrophore labeling to quantitation by GC–MS of DNA adducts of PAH (See [50] for a review).

Casale et al. developed an LC–ESI-MS method for the quantitation of 7-(benzo[a]pyren-6-yl)guanine (BP-6-*N*7-Gua) and 7-(benzo[a]pyren-6-yl)adenine (BP-6-*N*7-Ade) adducts originating from the reaction of the benzo[a]pyrene (BP) radical cation with DNA. These adducts were measured in urine of smokers and coal-smoke exposed women, as well as controls [51]. Their method allowed quantitation of 1 pmol of BP-6-*N*7-Ade that was added in 400 mg of creatinine equivalents (ca 400 ml) of urine with recovery ranging between 70 and 75%. The detection limit was around 0.1 pmol.

A methodology for quantitation of BP-tetraols in DNA that were introduced by the reaction with benzo[a]pyrene diolepoxides (BPDE) has been developed [52]. The method is based on measurement of the BP-tetraols by electron capture mass spectrometry as their tetramethyl ethers. The BP-tetraols bound to DNA by the reaction with benzo[a]pyrene diolepoxides were released by mild acid hydrolysis, converted to methyl ethers, and analyzed by GC–EC-MS. This method was validated by ^{3}H-postlabeling method that was developed previously by the same laboratory. Results from both methods were comparable to those from conventional radiochemical methods. Despite the fact that structural information related to site of binding by BPDE is lost after hydrolysis, this technique provides a universal tool to determine the extent of adduct formation by BP and diolepoxy metabolites of other PAH's. This assay is not limited to measurement of the tetraols in DNA. The authors used the same methodology to quantitate the tetraols in globin as well.

Barry et al. have used CE–ESI-MS–MS for quantitation of BPDE adducts with deoxyguanosine monophosphate [53]. Combined with solid-phase extraction clean-up, they were able to achieve detection limits of 130 fmol of the standard or 4 adducts/10^7 normal bases. Since the nucleotides were negatively charged under the conditions used in the method, sample stacking (accumulation of the sample at the inlet of the column using low elution strength) was successfully applied to improve sensitivity.

6.4. Olefins

6.4.1. Butadiene

Butadiene is used in large quantities in the production of rubber and plastic and is present in air mainly from automobile exhaust and cigarette smoke. It is carcinogenic in both mouse and rat and

workers in the styrene–butadiene rubber industry have an increased incidence of leukemia.

Our laboratory [54] and others [55,56] have developed LC–MS methods for quantitation of *N*7-guanine adducts induced by DNA-active metabolites of BD, epoxybutane, diepoxybutane, and epoxybutanediol. Our method allowed simultaneous quantitation of all four adducts, racemic and meso forms of *N*7-(2,3,4-trihydroxybut-1-yl)-guanine (THB-Gua), *N*7-(2-hydroxy-3-buten-1-yl)guanine (EB-Gua I), and *N*7-(1-hydroxy-3-buten-2-yl)guanine (EB-Gua II). The LC–MS method developed by our research group has also been applied to molecular dosimetry studies in rodents. Fig. 6 shows a typical product ion chromatogram obtained from analysis of lung DNA from a rat exposed to BD by inhalation for 4 weeks at 625 ppm. Neutral thermal hydrolysis (NTH) was used to cleave the *N*7-Gua adducts. A DNA backbone was separated from the hydrolysate by filtration through Centricon-10 filters. Salts were removed from the sample by off-line SPE prior to LC–MS analysis. Method detection limits for THB-Gua and EB-Gua adduct were 1 and 0.1 adducts/10^6 normal Gua bases, respectively. A similar method was developed by Oe et al. for racemic and meso THB-Gua and was applied to study its formation and persistence in rats with similar capabilities [56]. The method of Oe, however, used acid hydrolysis for isolation of the DNA adducts from DNA instead of NTH and no desalting was performed. The method allowed measurement of THB-Gua at ~0.1 adducts/10^6 normal bases (~0.4 THB-Gua adducts/10^6 normal guanine bases).

6.4.1.1. Ethylene. Ethylene, a petrochemical, is metabolized to ethylene oxide which can bind to DNA forming *N*7-(2-hydroxyethyl)guanine (7HEG). A GC–EC-IDHRMS method has been developed in our laboratory for highly sensitive and specific quantitation in rodents and humans [15,57]. 7HEG isolated from DNA by NTH was converted to its pentafluorobenzyl derivative for sensitive detection by EC-HRMS as originally developed by Saha et al. [58]. The method was applied to quantitative measurement of endogenous as well as ethylene-induced 7HEG in various types of tissues (liver, lung, spleen, and brain) from rodents [59]. For endogenous 7HEG in human lymphocytes and control animals, relatively high amounts of DNA (300 μg) were needed. Eide et al. have used both the method of Wu and their ^{32}P-postlabeling method to analyze tissues from control and ethylene exposed rats [60]. Results from

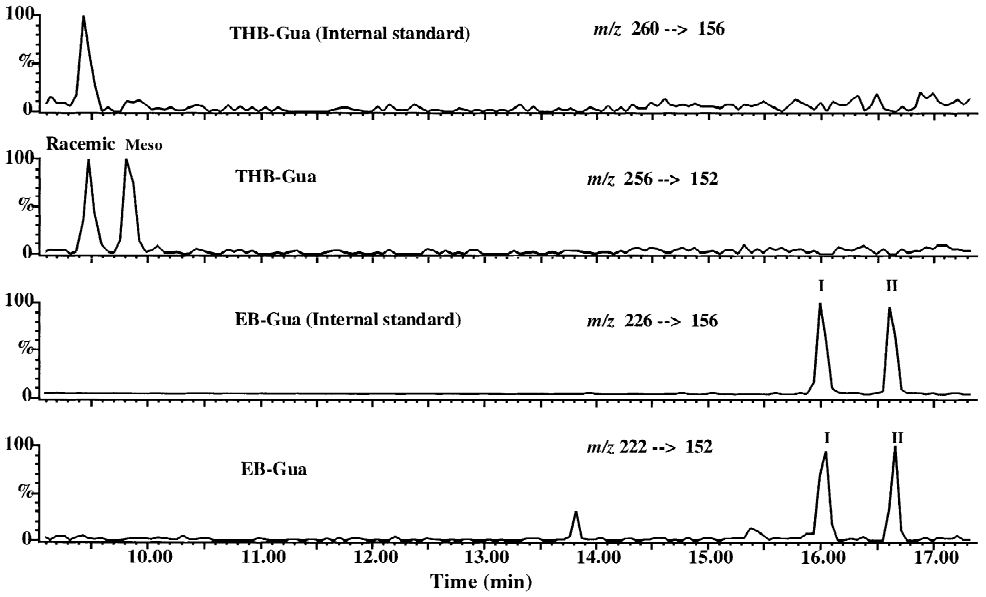

Fig. 6. SRM traces for N7 guanine adducts of 1,3-butadiene. Reprinted with permission from [54].

both methods were in good agreement as determined by regression analysis.

An LC–MS method using SIM detection of $[M+H]^+$ ion of 7HEG (m/z 196) has been reported by Leclercq et al. [61]. The method was applied to quantitation of 7HEG in calf thymus DNA and lymphocyte DNA from blood treated with ethylene oxide. They were able to detect 4 7HEG/10^7 normal nucleotides. The method was very sensitive with pure standard (~1 fmol on column). Authors however reported problems with reproducibility at even low pmol levels, which required generation of the calibration curve at the beginning of each session.

Our laboratory has recently developed an LC–ESI-IDMS–MS method for quantitation of 7HEG in human and rodent DNA [62]. The detection was done with a highly sensitive and specific mode of SRM using the transition m/z 196–152, which corresponded to the loss of the 2-hydroxyethyl moiety at the *N*7 position. A ^{13}C-labeled stable isotope of 7HEG, [$^{13}C_4$]-7HEG, was used as internal standard, which corrected for sample losses and variations in sensitivity. With this method, we were able to quantitate endogenous 7HEG in liver DNA from both rats and mice. The detection limit was ~5 adducts/10^8 normal guanine bases in ~150 μg of rodent liver DNA injected. Fig. 7 shows SRM traces for 7HEG and [$^{13}C_4$]-7HEG for a control rat liver hydrolysate. The amount of 7HEG in this particular sample was determined to be 8.3 ± 0.2 ($n=3$) adducts/10^8 normal guanine bases. The excellent sensitivity of this technique could be attributed mainly to efficient separation of the analyte from relatively large amounts of guanine that remained even after neutral thermal hydrolysis. Separation was done on a polar end-capped C_{18} reverse phase column that allowed the use of highly polar mobile phases, which was needed for a good separation of 7HEG from guanine. Slight heating of the column proved to be helpful in improving the sensitivity by producing a narrower and in turn a more concentrated peak. In addition, in order to reduce suppression of ESI, the LC effluent was diverted to the waste rather than to the mass spectrometer during elution of unwanted peaks.

6.4.1.2. Propylene. Propylene, which exists either naturally in the environment or is introduced from

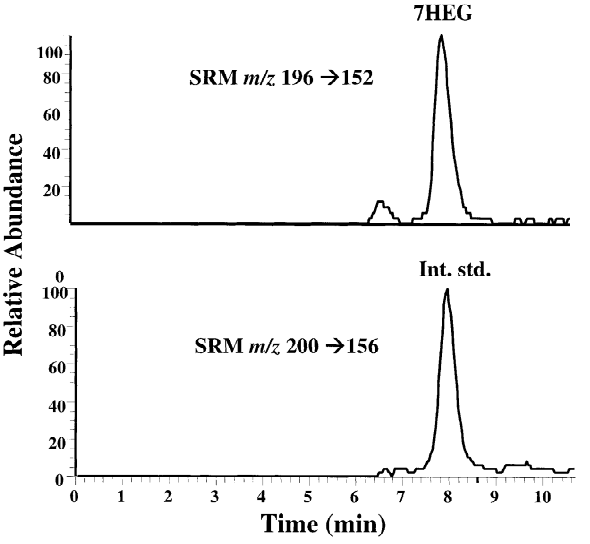

Fig. 7. Product ion chromatograms of 7HEG (SRM m/z 196–152) and [$^{13}C_4$]-7HEG (SRM m/z 200–196) from a control liver DNA. The LC–MS system consisted of a Magic 2002 HPLC unit coupled to a Finnigan TSQ 7000 triple quadrupole mass spectrometer with API2 ESI source. The LC column, Aquasil C_{18} column (2×150 mm, 5 micron), was eluted with a water (A)–acetonitrile (B) gradient (0% B for 5 min, to 10% B in 5 min, to 80% B in 5 min, to 0% B in 3 min, 0% B for 12 min). The flow rate of LC mobile phase was 300 μl/min. The column was kept at 35 °C.

industrial emissions, can be metabolized to propylene oxide, a DNA-reactive compound [63]. Propylene oxide is also an industrial chemical used in production of plastic and synthetic materials. Humans are exposed to propylene oxide mostly in the workplace.

A GC–EC-IDHRMS assay similar to the one used for 7HEG has been developed in our laboratory for quantitation of the *N*7-guanine adduct of propylene oxide, *N*7-(2-hydroxypropyl)guanine (7HPG), in various tissues (nasal respiratory, nasal olfactory, lung, spleen, lymphocytes, liver, testis) of male F344 rats exposed to propylene oxide [64,65]. The nasal tissues, the target tissue for propyleneoxide related carcinogenesis, obtained 7 h after the cessation of exposure, had the highest level of 7HPG (~600 adducts/10^6 Gua bases). The concentration of 7HPG in the nasal tissues obtained 3 days after cessation of exposure was, on the other hand, only half as much. The concentration in the other tissues varied between

10 and 70 adducts/10^6 Gua bases. The amount of 7HPG was also measured in lymphocytes obtained by combining blood from four rats killed immediately after cessation of the exposure. The lymphocytes had 39.6 adducts/10^6 Gua bases. The same tissues were also analyzed by PPL for validation by Segerback et al. [63]. The agreement between the two methods was excellent, with values from both methods being within 20% of each other. The amount of 7HPG in control rats was below the detection limit, 50 fmol in the whole sample.

6.4.1.3. Styrene. As in the case of propylene, styrene upon metabolic activation by enzymes such as cytochrome P450 forms styrene oxide, that can react with DNA. The major source of styrene oxide however is industrial emission during production of reinforced plastic.

In an interesting study, a combination of UV, inductively coupled plasma-mass spectrometry (ICP-MS), and ESI-MS detection of LC effluent was used to achieve both qualitative and quantitative information in analysis of adducts formed from reaction of styrene oxide DNA bases [66]. DNA that was treated in vitro with styrene oxide was digested enzymatically to the nucleotides for analysis. ICP-MS detection of phosphorus (m/z 31) was less sensitive than ESI-MS in SIM mode.

A GC–EC-IDHRMS method was developed in our laboratory for quantitation of styrene oxide-induced *N*7-guanine adducts, *N*7-(2-hydroxy-1-phenylethyl)guanine (HPEG-I) and *N*7-(2-hydroxy-2-phenylethyl)guanine (HPEG-II) [67]. The DNA adducts were converted to their pentafluorobenzyl derivatives in order to make them amenable to GC and to allow sensitive detection by EC-MS in SIM mode. This conversion was, however, inefficient with an overall yield of ~10%. The method had a detection limit of approximately 50 fmol (1 fmol injected). Selected reaction monitoring was also evaluated as the detection method and found to be ~3-fold less sensitive. Internal standards were ^{13}C-labeled stable isotopes. As an application of the method, B-lymphoblastoid human cell lines were exposed to varying concentrations (0.05–1 m*M*) of styrene oxide in vitro and analyzed for HPEG-I and HPEG-II. A linear dose–response relationship was obtained for both adducts.

6.4.1.4. Alkylating agents. *N*-nitroso alkylating agents form a variety of DNA adducts, most of them being at *N*7 guanine and *N*3 of adenine [68]. Interestingly, there has not been much interest toward the development of quantitative assays using mass spectrometry for this important class of compounds. A GC–IDMS method has been developed for quantitation of *N*3-alkyladenines [69,70]. Urinary *N*3-alkyladenines, *N*3-methyladenine, *N*3-ethyladenine, *N*3-(2-hydroxyethyl)adenine, and *N*3-benzyladenine, were isolated by immunoaffinity chromatography using monoclonal antibodies immobilized onto Protein A-Sepharose media and quantitated by GC–IDMS as their *tert*-butyl-dimethyl derivatives. Deuterated standards served as internal standards for the alkyladenines studied. Analysis of urine samples for *N*3 alkyladenines showed that both *N*3-methyladenine and *N*3-(2-hydroxyethyl)adenine originated from diet. The concentration of *N*3-ethyladenine was very low and did not change with diet, and *N*3-benzyladenine was not detected at all (below the detection limit of 1 pmol/ml).

6.4.1.5. 4-Aminobiphenyl. 4-Aminobiphenyl (4-ABP) is a urinary bladder carcinogen found mostly in cigarette smoke. DNA adducts are formed mainly by its electrophilic metabolite formed via *N*-hydroxylation. The DNA adduct formed between this electrophilic metabolite and guanine, Gua-C8–4-ABP, was measured in urinary bladder and lung by a GC–EC-IDMS assay developed by Lin et al. [71]. The assay was highly sensitive, with the detection limits for the whole method being as low as 0.32 adducts/10^8 normal nucleotides. DNA was hydrolyzed in 0.05 N NaOH and electrophore labeled with pentafluoropropionic anhydride for sensitive detection by EC-MS. This GC–MS method was later used in an interlaboratory study to validate data on levels of DNA adducts of 4-ABP measured by immunochemical assay and PPL [72]. Data obtained by the GC–MS method were in good agreement with those by immunochemical method for two selected samples.

An LC–ESI-IDMS method with on-line sample concentration and clean-up has been recently made available for quantitation of the nucleoside form of the adduct, dGuo-C8-4-ABP, in hepatic DNA from

mice treated in vivo with 4-ABP [73]. Methods such as this one demonstrate the potential that LC–MS techniques have for the development of simple, sensitive, and specific assays. The technique of column-switching has been successfully applied for on-line sample preparation. Isotope dilution mass spectrometry was also utilized through use of a deuterated analog of dGuo-C8-4-ABP, dGuo-C8-4-ABP-d_9, as the internal standard. Combination of effective sample clean-up and sensitive SRM detection has resulted in a method detection limit of 23 fmol injected amount on column or 7 dGuo-C8-4-ABP adducts/10^8 normal nucleotides in 100 μg DNA. The simplicity of the method is obviously the major improvement over the GC–MS methods developed earlier. In addition to the automation of sample clean-up, the derivatization that was required in the GC–MS method was eliminated.

6.4.1.6. Melphalan. Nano LC coupled to nano ESI –MS detection was evaluated for sensitivity and compared to a micro LC–ESI-MS method for analysis of dAMP adducts of melphalan, L-phenylalanine mustard [74]. Approximately a 10-fold increase in sensitivity was gained with the nano version due to its higher mass sensitivity. Both SRM and SIM modes were used for mass spectrometric detection, with the latter being surprisingly more sensitive. In the SRM mode, 50 fmol was the detection limit using the capillary setup as opposed to 7 fmol with the nano setup. A detection limit of slightly lower than 1 fmol ($S/N=14$) was achieved for the melphalan-dAMP adduct. This research has demonstrated that smaller scale chromatographic separations with nano ESI-MS detection could provide better sensitivity.

6.4.1.7. Heterocyclic aromatic amines. Heterocyclic aromatic amines are carcinogenic chemicals found in cooked meat. Several MS methods have been developed for the analysis of adducts that are formed from the reactions of the heterocyclic aromatic amines with DNA. A capillary LC–ESI-MS–MS method was used for quantitative analysis of the adduct of 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) with 2′-dGuo, *N*-(deoxyguanosyl-8-yl)-PhIP (dGuo-C8-PhIP). The method was used for measurement of PhIP adducts in in vitro reaction mixtures. The detection limit was 80 fmol of the dGuo-C8-PhIP using SRM [75]. Recently, the same laboratory has developed a similar methodology for 2′-dGuo adducts of 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ), *N*-(deoxyguanosin-8-yl)-2-amino-3-methylimidazo[4,5-*f*]quinoline (dGuo-C8-IQ) and 5-(deoxyguanosin-*N*2-yl)-2-amino-3-methylimidazo-[4,5-*f*]quinoline (dGuo-*N*2 -IQ) [76]. The DNA adducts were detected in kidney tissues of chronically treated cynomolgus monkeys using SRM. The detection limit in tissue samples was ~1 adduct/10^7 normal bases when 300 μg DNA was used.

6.4.1.8. Radiation. Frelon et al. have developed an assay using LC–ESI-IDMS–MS for simultaneous measurement of radiation-induced adducts 5,6-dihydroxy-5,6-dihydrothymidine, 5-hydroxy-2′-deoxyuridine, 5-(hydroxymethyl)-2′-deoxy-uridine, 5-formyl-2′-deoxyuridine, 8-oxo-7,8-dihydro-2′-deoxyadenosine, 8-oxo-7,8-dihydro-2′-deoxyguanosine, 4,6-diamino-5-formamidopyrimidine, and 2,6-diamino-4-hydroxy-5-formamidopyrimidine [77]. They were quantified in isolated and cellular DNA exposed to gamma-radiation. Detection limits varied between 10 and 200 fmol depending on the DNA adduct.

Tandem base lesions *N*-(2-deoxy-β-D-*erythro*-pentofuranosyl)formylamine-8-oxo-7,8-di-hydro-dβ-deoxyguanosine (dβF-8-oxodGuo) and its isomer 8-oxodGuo-dβF produced by ·OH radicals were measured by an LC–ESI-MS–MS in aerated solutions that were subjected to γ-radiation [78]. SIM detection of $[M-1]^-$ in negative ion ESI produced sensitive measurement with detection limit at ~10 fmol.

6.4.1.9. Bisphenol A diglycidyl ether. The potential of nano LC–ESI-MS to improve detection limits in analysis of DNA adducts has been shown by Vanhoutte et al. in a study in which in vitro reaction mixtures from treatment of 2′-dGMP with Bisphenol A diglycidyl ether, a bifunctional epoxide used in epoxy resin industry [79]. They have compared sensitivities of conventional, micro LC coupled to

conventional ESI-MS and nano LC with nano flow ESI-MS. The improvement using the nano system was ~3000 compared to conventional methods.

7. Concluding remarks

Mass spectrometry adds a high degree of specificity to a quantitative method. This contribution is even more dramatic when it is used in combination with isotope dilution mass spectrometry and selected reaction monitoring. The only disadvantage of mass spectrometry when used in a quantitative method is that it still is not as sensitive as some of the most sensitive techniques for quantitation of DNA adducts such as PPL. The gap in sensitivity, however, is getting smaller as MS technology is continually improving. GC–EC-HRMS and LC–ESI-MS–MS are the most promising of all mass spectrometric methods for DNA adduct quantitation. Low amol detection limits are already a routine practice with GC–ESI-MS. The LC–ESI-MS–MS methods developed recently are reporting detection limits of low fmol and even a fraction of a fmol. Especially in LC–MS techniques, there are areas in which improvements or modifications can be made in order to lower detection limits. One such area that was discussed above is automation of the sample clean-up process. As the number of off-line sample preparation procedures, which usually require transfer of the sample from one tube to another or solvent exchange by completely drying and redissolving, is reduced, sample losses will also be reduced. This will translate into increased sensitivity.

Apart from developments in the processes of ionization and detection, there are continuing efforts to discover new types of HPLC columns or column materials as well. Any improvement in the chromatographic behavior of the analyte should also improve sensitivity. For instance, we find that LC separations at elevated temperatures improves the peak shape and consequently the sensitivity. It is our belief that assays using techniques such as nano LC–MS, on-line sample preparation/clean-up become more feasible for routine analysis, the LC–MS techniques could be the ideal method for DNA adduct quantitation.

8. Nomenclature

PPL	^{32}P-postlabeling
GC	Gas chromatography
LC	High-performance liquid chromatography
CE	Capillary zone electrophoresis
ESI	Electrospray ionization
EC	Electron capture
MS	Mass spectrometry
MS–MS	Tandem mass spectrometry
SRM	Selected reaction monitoring
SIM	Selected ion monitoring
IDMS	Isotope dilution mass spectrometry
IDMS–MS	Isotope dilution tandem mass Spectrometry
CID	Collision induced dissociation

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