Talanta 130 (2014) 475–494

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Review

Recent developments in DNA adduct analysis by mass spectrometry: A tool for exposure biomonitoring and identification of hazard for environmental pollutants

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ARTICLE INFO

Article history:
Received 11 April 2014
Received in revised form
19 June 2014
Accepted 22 June 2014
Available online 30 June 2014

Keywords:
DNA adduct analysis
Mass spectrometry
Exposure biomarkers
Environmental pollutants
Hazard identification

ABSTRACT

DNA adducts represent an important category of biomarkers for detection and exposure surveillance of potential carcinogenic and genotoxic chemicals in the environment. Sensitive and specific analytical methods are required to detect and differentiate low levels of adducts from native DNA from *in vivo* exposure. In addition to biomonitoring of environmental pollutants, analytical methods have been developed for structural identification of adducts which provides fundamental information for determining the toxic pathway of hazardous chemicals. In order to achieve the required sensitivity, mass spectrometry has been increasingly utilized to quantify adducts at low levels as well as to obtain structural information. Furthermore, separation techniques such as chromatography and capillary electrophoresis can be coupled to mass spectrometry to increase the selectivity. This review will provide an overview of advances in detection of adducted and modified DNA by mass spectrometry with a focus on the analysis of nucleosides since 2007. Instrument advances, sample and instrument considerations, and recent applications will be summarized in the context of hazard assessment. Finally, advances in biomonitoring applying mass spectrometry will be highlighted. Most importantly, the usefulness of DNA adducts measurement and detection will be comprehensively discussed as a tool for assessment of *in vitro* and *in vivo* exposure to environmental pollutants.

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Abbreviations: 1,2-DHN, 1,2-dihydroxynaphthalene; 1,2-NQ, 1,2-naphthoquinone; DEB, 1,2,3,4-diepoxybutane; N1HX-N7G-DB, 1-(hypoxanth-1-yl)-4-(guan-7-yl)-2,3-butanediol; εAde, 1,N^6-ethenoadenine; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; 2,7-DiNF, 2,7-dinitrofluorene; PhIP, 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine; IP-1,2-O, 2-amino-3-methylimidazo[4,5-f]quinoline (IQ), 2-amino-3,8-dimethylmidazo[4,5-f]quinoxaline (MeIQx), 2-amino-9H-pyrido[2,3-b]indole (AαC), 2-ethenyl-2-methyloxirane; M1dG, 3-(2-deoxy-β-D-erythro-pentofuranosyl)pyrimido[1,2-α]purin-10(3H)-one; NMOR, 3-hydroxy-N-nitrosomorpholine; εCyt, 3,N4-ethenocytosine; 3-NBA, 3-nitrobenzanthrone; ABP, 4-aminobiphenyl; HNE, 4-hydroxynonenal; PHB, 4-(3-pyridyl)-4-hydroxybut-1-yl; fapy-adenine, 4,6-diamino-5-formamidopyrimidine; NNAL, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; 4-OHE, 4-oxo-2(E)-hexenal; N^7-EtG, 7-ethylguanine; BPDE, 7,8-dihydroxy-9,10-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene; 8-oxo-dG, 8-hydroxy-7,8-dihydro-2'-deoxyguanosine; E2, 17β-estradiol; AMS, accelerator mass spectrometry; A, acrolein (AC), adenine; Ado, adenosine; AA, aristolochic acid; S_NAr, aromatic nucleophilic substitution; B[b]F, benzo[a]pyrene (B[a]P), benzo[b]fluoranthene; B[k]F, benzo[k]fluoranthrene; S_N2, bimolecular nucleophilic substitution; BD, butadiene; ct-DNA, calf thymus DNA; CE, capillary electrophoresis; CNL, constant neutral loss; C, cytidine; Cyt, cytosine; dA, deoxyadenosine; dC, deoxycytosine; dG, deoxyguanosine; dI, deoxyinosine; dT, deoxythymidine; DES, diethylstilbestrol; ESI, electrospray ionization; ε, etheno-; EA, ethoxyacetaldehyde; EO, ethylene oxide; Rad, free radical; FT-ICR, Fourier transform ion cyclotron resonance; GC, gas chromatography; GSH, glutathione; GA, glyceraldehyde; G, guanine; Gua, guanosine; HSDB, Hazardous Substances Data Bank; Hε, heptone-etheno-; HAA, heterocyclic aromatic amine; HES, hexestrol; HE, hydroxyethyl; ICP, inductively coupled plasma; IARC, International Agency for Research on Cancer; IT, ion trap; LIF, laser-induced fluorescence; LIT, linear ion trap; LPO, lipid peroxidation products; Luc, lucidin; LuP, lucidin-3-O-primeveroside; MDA, malondialdehyde; MS, mass spectrometry; MALDI, matrix-assisted laser desorption/ionization; MA, Michael addition; MN, micronucleus test; MTBE, methyl *tert*-butyl ether; MeCyt, methylcytosine; MMS, methylmethane sulfonate; MOA, mode of action; N^2-ethylidene-dG, N^2-ethylidene-2′-deoxyguanosine; N^7-HEG, N^7-(2′-hydroxyethyl)guanine; MNU, N-methyl-N-nitrosourea; NDMA, N-nitrosodimethylamine; NNN, N′-nitrosonornicotine; NPYR, N-nitrosopyrrolidine; NICI, negative ion chemical ionization; NOEL, no observable effect limit; ODN, oligodeoxynucleotide; PAH, polyaromatic hydrocarbon; PUFA, poly unsaturated fatty acids; PGC, porous graphitized carbon; PAEKI, pressure-assisted electrokinetic injection; IP-3,4-O, propen-2-yloxirane; POB, pyridyloxobutyl; QTOF, quadrupole-time-of-flight; Rub, rubicin; SB, Schiff base; SPE, solid phase extraction; N^7-MeG, N^7-methylguanine; N3-MeA, N3-methyladenine; PDE-dA, (±)-*anti*-DB[a,l]; G, O6-Me-; TBA, *tert*-butyl alcohol; dTp(Me)dT, thymidyl(3′,5′)thymidine; TOF, time-of-flight; UHPLC, ultra-performance liquid chromatography; S_N1, unimolecular aliphatic nucleophilic substitution; VC, vinyl chloride; (AA), HNE, and AC, Insert abbreviations

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http://dx.doi.org/10.1016/j.talanta.2014.06.050
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Contents

1. Introduction

The interactions of toxicants with DNA, including non-covalent binding, covalent adduct formation, oxidative damage, or cross-linking may lead to the formation of apurinic site and exocyclic adducts resulting in an alteration of the DNA structure, and consequently mutation, carcinogenicity or cell death when repair is ineffective [1–3]. Study of DNA adducts can contribute to understanding the mechanisms by which environmental pollutants exhibit genotoxic properties [4]. Some chemicals are able to directly interact with DNA to form adducts while others require metabolic activation to a reactive form prior to DNA adduct formation. In the environment, genotoxins come from a wide variety of exogenous sources such as pollutants in the environment, occupational exposure and life-style choices, such as smoking. Some genotoxins have endogenous sources produced as by-products of metabolism [5]. General classes of chemicals capable of forming adducts include polycyclic aromatic hydrocarbons (PAHs) [6], heterocyclic aromatic amines (HAAs) [7], estrogens [8], nitrosamides [9], and epoxides [10]. Alternatively, adduct-forming chemicals can also be categorized based on their sources such as cooked-meat carcinogens [11]; tobacco-specific carcinogens [12]; diesel exhaust [13]; plant toxins such as aristolochic acid [14]; and lipid peroxidation (LPO) products, such as 4-hydroxynonenal and acrolein [15].

Modifications of DNA by chemicals can be at one or more DNA bases or sites on DNA bases as shown in Fig. 1. DNA adducts can also be formed by chemicals with the DNA phosphate backbone as well as the DNA bases. In this review, only DNA adducts modified on DNA bases will be reviewed. The adduct formation mechanism and chemical structures usually determine the modification sites. For example, the electrophilic reaction chemistry involved in the formation of covalent DNA adducts has been recently reviewed by Enoch et al. [16] to identify structural alerts in computational modeling. The authors described seven mechanistic domains: acylation, Michael addition (MA), Schiff base formation (SB), aromatic nucleophilic substitution (S_NAr), unimolecular aliphatic nucleophilic substitution (S_N1), bimolecular aliphatic nucleophilic substitution (S_N2) and reactions involving free radicals (Rad). In each domain, chemical classes are assigned and the associated chemical mechanisms are discussed. The authors also detailed currently available mechanistic knowledge and identified 57 structural alerts that can be applied to models for mutagenicity and genotoxicity. In another recent review, Boysen et al. [17] described the formation of N^7-guanine adducts and their biological significance due to higher abundance compared to other adducts. The formation of adducts in animal models from nitrosourea compounds, nitrosamines, hydrazines and olefins was discussed while some experiments were conducted to demonstrate the mutagenic properties of N^7-guanine adducts. Other reviews have discussed reaction mechanisms and adduct structures for well-studied chemicals, such as benzo[*a*]pyrene (B[a]P) [18,19] and acetaldehyde [20], and chemical classes, such as LPO

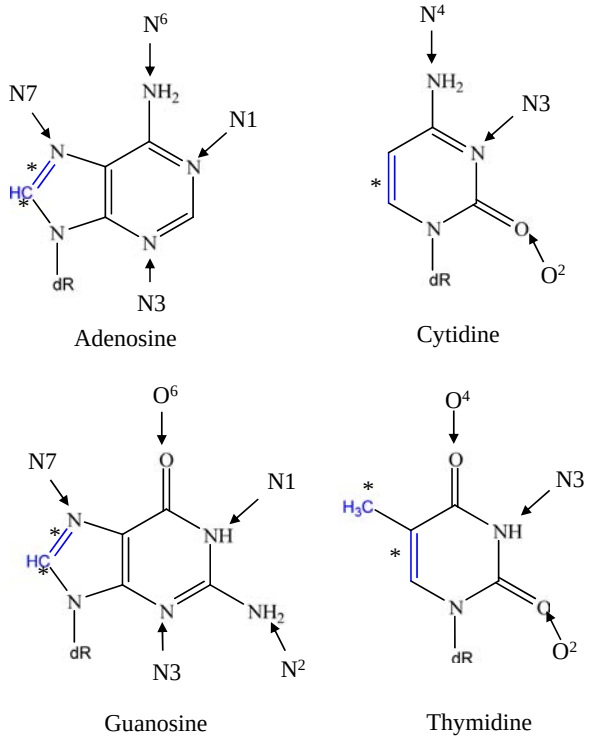


Fig. 1. Potential sites of damage on DNA bases including adduction (arrow) and oxidation (blue and asterisk). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

products [15,21–23], HAAs [7,11], tobacco-specific carcinogens [24] and methylating agents [25].

DNA adducts are of great interest since they can serve as biomarkers for the detection and surveillance of potential mutagenic and genotoxic chemical exposure. Swenberg et al. [26] have reviewed such biomarkers in toxicology and risk assessment context, where DNA adducts served as biomarkers of exposure and mutations. In this review, the authors emphasized that there was a significant difference between biomarkers of the effect and mutation. For biomarkers of exposure, such as DNA adducts, dose-response curves were typically linear throughout, even at low doses, whereas dose-response curves for biomarkers of mutation were linear only to the background number of mutations. Thus, they concluded that the relationship between exposure and mutation response should be further investigated to enable better application of biomarkers in risk assessment. Similar opinions were also expressed in another review regarding the use of DNA adducts in cancer risk assessment by Jarabek et al. [27]. The authors emphasized the importance of integrating information of DNA adducts with other information. Indeed, to validate a DNA adduct as a suitable biomarker, data on DNA adducts must include stability, repair, reproducibility, repeatability, dose-response, and intra-individual variability. A selection of validated biomarkers for environmental carcinogens has been reviewed by Gallo et al. [28].

Generally speaking, the level of DNA adducts found in human tissues following administration of single doses of genotoxic compounds is in a range of $1/10^{11}$ nucleotides (1 modification per 10^{11} nutcleotides). The level of adducts that may be formed in experimental systems after chronic treatment with a carcinogenic dose of a compound is approximately $1/10^4$ nucleotides. The background level of alkylated adducts in human DNA is usually more than 1 modification per 10^7 nucleotides [29–31]. Therefore, analytical methods should be sensitive enough to detect DNA adducts at such a low level in humans. Common methods for detecting DNA adducts include ^{32}P-postlabelling, immunochemical techniques, laser-induced fluorescence (LIF), and mass spectrometry. The ^{32}P-postlabelling assay has been widely applied for determination of DNA adducts with a detection limit of as low as 1 in 10^{10} unmodified bases [32]. However, ^{32}P-postlabelling methods are labor intensive and require radioactive materials. Consequently, these methods suffer from non-specific labeling and lack of structure information. Immunological assays may be time consuming in development but could be easier and quicker by offering high sample throughput than other techniques in practice. In addition, method sensitivity is limited to the specificity of the antibodies. Similarly, although LIF can potentially offer yoctomolar detection limits [33], sensitivity is limited by the specificity and efficiency of the derivatization reaction between target adducts and the fluorophore. These methods have been reviewed [1,34–37]. Compared to the aforementioned methods, MS can offer not only high sensitivity, for example, 1 in 10^8 unmodified bases with conventional mass spectrometry-based methods and to one adduct per 10^{12} unmodified bases with accelerator mass spectrometry [32,38], but also structural information for DNA adducts. Furthermore, mass spectrometry is still improving its sensitivity as advances in new technologies for ionisation, transmission and detection of ions are continuing. The fundamental principles and recent advances in MS have been extensively reviewed [39–44]. Combination of MS and separation techniques in DNA adduct analysis have also been previously reviewed [29,45–48]. This review will describe the main advances in MS for DNA adduct analysis during the past six years (2007–2013). Considering that ICP-MS is usually classified as an inorganic mass spectrometry, this review will not introduce the advances and applications of ICP-MS on DNA adduct analysis although the technique has been extensively used to detect cisplatin DNA adducts. Furthermore, the potential use of DNA adduct analysis as a tool for hazard assessment and identification of environmental contaminants will be discussed.

2. Advances in mass spectrometry

2.1. Accelerator mass spectrometry (AMS)

Accelerator MS (AMS) is currently considered the most sensitive MS analyzer that can accurately measure target compounds at atto-to-zeptoomole (10^{-18}–10^{-21}) levels and analyze hundreds of samples per day [42]. As a mass spectrometer designed only for quantitation of radioactive isotopes, AMS is similar to ^{32}P-postlabelling techniques and cannot provide structural information for DNA adducts. The ^{14}C- or ^{3}H-labelled compounds are commonly reported in the use of radioactive isotope labeled targets. Other isotopes that are used in biomedical applications are ^{26}Al, ^{41}Ca, ^{32}Si, and ^{10}Be [49]. Due to the ultralow detection limit, AMS is gaining popularity in biomedical applications. It was reported that AMS can detect DNA adducts as low as a level of 1 adduct in 10^{12} unmodified bases [38]. Therefore, it has been employed to perform metabolomic, kinetic, and/or dosimetry studies for environmental contaminants at real world concentrations. AMS has proven to be very useful in investigations of DNA adducts with chemicals of identified source. Some representative examples and their exposure scenario (*in vitro* or *in vivo* exposure) are summarized in Table 1. For example, Marsden et al. [50] treated rats with ^{14}C-ethylene oxide (^{14}C-EO) to determine whether DNA damage by ethylene oxide was from endogenous or exogenous sources. It was found that doses of ^{14}C-EO were correlated to non-labeled N^7-(2′-hydroxyethyl) guanine (N^7-HEG), which therefore suggested exogenous EO was producing endogenous N^7-HEG. With this technique, 1-aminocyclopropane-1-carboxylic acid was further identified as the precursor to EO *in vivo*. AMS is also helpful in elucidating the binding mechanism of chemicals with DNA and monitoring *in vivo* metabolism. For example, Yuan et al. [51] compared adduct formation in mice exposed to ^{14}C-labeled methyl tert-butyl ether (MTBE), a gasoline additive, and its metabolite ^{14}C tert-butyl alcohol (TBA). With the application of this technique in *in vivo* study, the authors found that the methyl group of MTBE and tert-butyl alcohol formed adducts with DNA in mouse liver, lung, and kidney. The methyl group of MTBE was the predominant binding group in liver, while the methyl group and the tert-butyl group gave comparable contributions to the adduct formation in lung and kidney. The results also confirmed that TBA in *in vivo* system was able to form DNA adducts via its metabolite MTBE. This study demonstrated the advantages of AMS on revealing of the formation mechanism of MTBE–DNA adducts by using doubly ^{14}C-labeled MTBE. This was also the first report of TBA adducts. Another example for AMS analysis of DNA adducts is a recent study on monitoring *in vivo* metabolism and elimination of the endogenous DNA adduct, M_1dG (3-(2-deoxy-β-D-erythropentofuranosyl) pyrimido[1,2-α]purin-10 (3H)-one) by Knutson et al. [52] in Tannenbaum's lab at MIT. The authors reported the metabolic processing of M_1dG at concentrations 4–8 orders of magnitude lower in concentration than previously analyzed, by the use of AMS analysis with isotope labeling on the principle metabolite 6-oxo-[^{14}C]M_1dG. They also revealed that 6-oxo-M_1dG excreted in urine could be a useful biomarker for endogenous oxidative damage through the investigation on the recovery of the ^{14}C in urine and feces corresponding to the metabolite 6-oxo-M_1dG [52]. Recent advancements in AMS are rapidly increasing the number of applications on AMS instrumentation. Advancements include coupling HPLC to AMS and the reduction in size of the instrumentation allowing the instrument to be placed in a conventional laboratory rather than requiring a

Table 1
Examples of mass spectrometry based detection methods for DNA adducts

Adduct	Mass spectrometry	Species	Source	Detail	Ref
N^7-HEG	AMS	Rat	Tissue	*in vivo*	[50]
MTBE-DNA	AMS	Mouse	Liver, kidney, and lung	*in vivo*	[51]
M1dG	AMS	Rat	Urine	*in vivo*	[52]
5′-CHO-dA, 5′-CHO-dG, 5′-CHO-dC, 5′-CHO-dT	AMS	Mouse	Liver	*in vivo*	[53]
N^2-ethyl-dG, CPr-dG	TOF	–	Calf Thymus DNA	ESI-LC–MS/MS	[54]
DNEHTHP-N^2-dG,NPDE-N^2-dG, DNN-acetylamino PDE-N^7-dG,TNPDE-N^6-dA	TOF	Fish	Bile	ESI	[55]
N^2-methylguanine,N^2-ethylguanine, N^2-furan-2-yl-methylguanine, N^2-tetrahydrofuran-2-yl-methylguanine, N^4-methylcytosine	TOF	Mouse	–	ESI, *in vitro* ESI-LC–MS/MS	[56]
dA-AL-I	TOF	Mouse, human	Liver and kidney kidney	ESI-UHPLC	[61]
N^1-acetylspermidine	TOF	mouse	Urine	ESI-UHPLC	[62]
dG-C8-PhIP, dG-C8-ABP	TOF		Carcinogenic DNA	MALDI	[67]
B[a]PDE-2'-Deoxynucleosides	TOF	–	–	MALDI	[68]
dC-mech-dC	TOF	Mouse	DNA duplex	ESI-MS/MS	[69]
M1-dG	TOF	Human	blood	MALDI	[70]
isomeric C-8 deoxyguanosine adducts	MS^n	–	–	–	[80]
4-ABP, MeIQx, AαC	LIT/MS^3	–	calf Thymus DNA	–	[81]
dG-C8-PhIP, dGC8-AαC, dG-C8-MeIQx,dG-C8-4-ABP	LIT/MS^n	Human	saliva	LC-ESI	[82]
dG-C8-HAAs, dG-C8-4-ABP	MS/MS	Human	hepatocyte	ESI-UHPLC	[83]
B[a]P-7,8-transdihydrodiol	MS/MS	Human	Lung	LC	[84]
N^2-propano-Dg, N^2-ethyl-dG	MS/MS	Human	Lung	LC-ESI	[85]
N^2-ethyl-dG, εdA, 1,N^2-PdG1, 1,N^2-PdG2, 1, 8-OH-PdG, 6-OH-PdG	MS/MS	Human	Lung and esophagus	LC-ESI	[86]
8-oxodG, Gh, Ox, NitroIm, Sp, M1dG; dO, 1,N^6-etheno-deoxyadenosine, 1,N^2-ethenodeoxyguanosine	MS/MS	Mouse	Spleen, liver and Kidney	LC	[87]
2′-deoxyuridine, 2′-deoxyxanthosine, 2′-deoxyinosine, 8-oxo-2′-deoxyguanosine, 1, N^2-etheno-2′-deoxyguanosine, 1,N^6-etheno-2′-deoxyadenosine, 3,N^4-etheno-2′-deoxycytidine	MS/MS	Mouse	Spleen, liver and kidney	LC	[88]
B[a]PDE-N^2-dG,B[b]FDE-N^2-Dg, B[b]FDE-N^6-dA,B[b]FDE-N^4-dC, DB[a,l]PDE-N^2-dG, DB[a,l]PDE-N^6-dA, DB[a,l]PDE-N^4-dC,	MS/MS	–	Calf thymus DNA	Column-switching LC-ESI	[89]
HεdC, HεdA, BεdC, HεdG	MS/MS	Human	Colon, heart, kidney, liver, lung, pancreas, small intestine, and spleen	LC	[90]
8-MOP-induced ICL	MS/MS	Human	HEK293T	LC	[91]
2-Amino-9H-pyrido[2,3–b]indole, 4-aminobiphenyl	MS/MS^3	Human	Hepatocyte	ESI-UHPLC	[92]
1,2-GpG, 1,2-ApG, and 1,3-GpXpG cisplatin intrastrand cross-links	MS/MS	-	Oligodeoxyribonucleotide	LC	[93]
dG-N^7-IQ, dA-N^6-IQ, dG-C8-PhIP,	MS^n	–	–	–	[75]
7-Ethyl-Gua	MS/MS	Human	Leukocyte	Nanospray	[76]
HPB-releasing DNA adducts	Orbitrap MS	Human	Exfoliated oral mucosa cell	–	[77]
N7-(2,3,4-trihydroxybut-1-yl)-guanine	Orbitrap MS	Human	Leukocyte	–	[78]
Octenal-related dA and dC adducts	Orbitrap MS	–	–	–	[79]

Abbreviations: accelerator mass spectrometry (AMS); N^7-(2′-hydroxyethyl)guanine (N^7-HEG);, Methyl tert-butyl ether (MTBE); tert-butyl alcohol (TBA); 3-(2-deoxy-β-d-erythropentofuranosyl) pyrimido[1,2-α]purin-10(3*H*)-one (M_1dG); 5-formylated 2′-deoxyadenosine (5′-CHO-dA); 5-formylated 2′-deoxyguanosine (5′-CHO-dG); 5-formylated 2′-deoxycytidine (5′-CHO-dC); 5-formylated thymidine (5′-CHO-dT); N^2-Ethyl-2′-deoxyguanosine (N^2-ethyl-dG); N^2-propano-2′-deoxyguanosine (CPr-dG); quadrupole time-of-flight (QTOF); dinitro-7,8-epoxy-9-hydroxytetrahydro phenanthrene-N^2-deoxyguanosine (DNEHTHP-N^2-dG); nitrophenanthrenediolepoxide-N^2-deoxyguanosine (NPDE-N^2-dG); dinitro N-acetylaminophenanthrene diol epoxide-N^7-deoxyguanosine (DNN-acetylamino PDE-N^7-dG); trinitrophenanthrene diol epoxide-N6-deoxyadenosine (TNPDE-N6-dA); 7-(deoxyadenosin-N6-yl) aristolactam I (dA-AL-I); 2-amino-1-methyl-6-phenylimidazo[4,5–b]pyridine (dG-C8-PhIP); dG-C8-4-aminobiphenyl (dG-C8-ABP); $CH_2CH_2N(CH_3)CH_2CH_2$ (mech); malondialdehyde-deoxyguanosine adducts (M1-dG); 4-aminobiphenyl (4-ABP); 2-amino-3,8-dimethylimidazo[4,5–f]quinoxaline (MeIQx); 2-amino-9H-pyrido[2,3–b]indole (AαC); N-(deoxyguanosin-8-yl) (dG-C8); 2-amino-1-methyl-6-phenylimidazo[4,5–b]pyridine (PhIP); 2-amino-3,8-dimethylimidazo[4,5–f]quinoxaline (MeIQx); heterocyclic aromatic amines (HAAs); benzo[a]pyrene-7,8-*trans*-dihydrodiol (B[a]P-7,8-transdihydrodiol); 1, N^6-etheno-2′-deoxyadenosine (εdA); N^2-propano-2′-deoxyguanosine (1,N^2-PdG1, 1,N^2-PdG2); 3-(2′-deoxyribosyl)-5,6,7,8-tetrahydro- 8-hydroxy-pyrimido[1,2–a]purine-(3H)-one (8-OH-PdG); 3-(2'-deoxyribosyl)-5,6,7,8-tetrahydro-6- hydroxypyrimido[1,2–a]purine-(3*H*)-one, (6-OH-PdG); 8-oxo-7,8-dihydro-2′deoxyguanosine (8-oxodG); guanidinohydantoin (Gh);oxazolone (Ox);guanidino-4-nitroimidazole (NitroIm); spiroiminodihydantoin (Sp);2'deoxyxanthosine (dO), heptanone-etheno-2′-deoxycytidine (HεdC); heptanone-etheno-2′-deoxyadenosine (HεdA); butanone-etheno-2′-deoxycytidine (BεdC);heptanone-etheno-2′-deoxyguanosine (HεdG); 8-methoxypsoralen-induced DNA interstrand cross-links (8-MOP-induced ICL); Human embryonic kidney 293T cells (HEK293T); 7-ethylguanine (7-EtG); hydroxy-1-(3-pyridyl)-1-butanone (HPB).

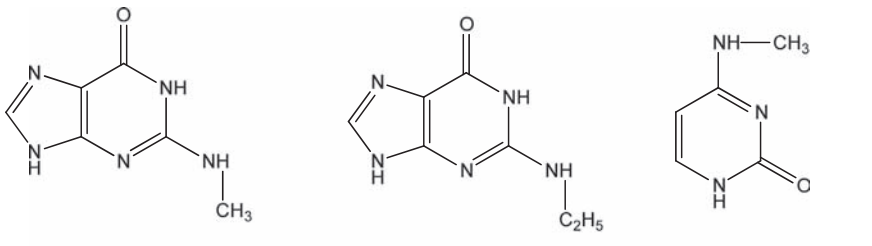
dedicated building. For example, a method using AMS associated with high performance liquid chromatography (HPLC) was reported to investigate the contribution of incorporation and/or adduction of formic acid with liver DNA in mouse. The authors demonstrated that a combination of HPLC with AMS was an essential means for the evaluation of DNA adduction [53]. In this study, four kinds of 5′-formylated adducts prepared by the reaction of formic acid and deoxyribonucleosides *in vitro* were used as references for the HPLC–AMS analysis of *in vivo* adduction. A precise analysis of the hydrolysate by the HPLC–AMS method indicated that a majority of formic acid incorporated directly into DNA, whereas less than 1.5% might form instable formylated DNA adducts *in vivo*. This evidence greatly supported the important perspective that formic acid is not carcinogenic. However, due to the fact that radioactive isotope labeled targets may not be available for all desired applications, AMS is limited to investigations of known DNA adducts with labelled chemicals that can be easily synthesized, which is a disadvantage, compared to other types of mass spectrometer.

2.2. Time-of-flight mass spectrometry (TOFMS)

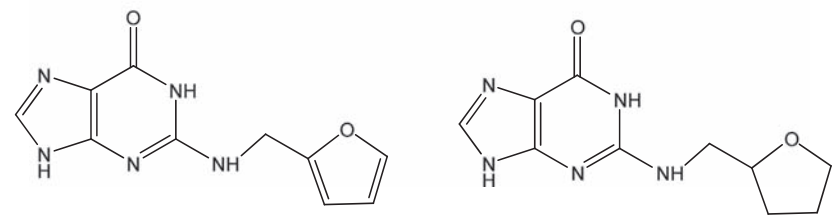
Time-of-flight mass spectrometry (TOFMS) is a method of mass spectrometry in which an ion's mass-to-charge ratio is determined

via a time measurement. It is the most commonly used high resolution mass analyzer with high mass accuracy and a large mass range. Both electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI) can be used as the ion source for TOF. TOF instrumentation has previously been used mainly for confirming the structural identity of DNA adducts of previously uncharacterised adducts. Some representative examples using TOF mass spectrometry for DNA adduct analysis and their exposure scenarios (*in vitro* or *in vivo* exposure) are listed in Table 1. For example, Inagaki et al. [54] used ESI-TOF as a complementary technique to confirm masses of adducts that were observed with LC–MS/MS (triple quadrupole) method. Wahidulla and Rajamanickam [55] used quadrupole time-of-flight (QTOF) to detect DNA damage in fish that were co-exposed to phenanthrene and nitrite. Using high resolution ESI-TOF mass spectrometry, Li et al. [56] demonstrated that AlkB had the biochemical capability to repair *in vitro* all simple N-alkyl adducts occurring at the Watson – Crick base pairing interface of the four DNA bases, including N^2-methylguanine, N^2-ethylguanine, N^2-furan-2-yl-methylguanine, N^2-tetrahydrofuran-2-yl-methylguanine, and N^4-methylcytosine in ss-DNA but not in ds-DNA (Fig. 2). This was important in confirming that AlkB was a versatile gatekeeper of genomic integrity under alkylation stress. HPLC is being increasingly applied for separation prior to the ESI-TOF methods for DNA adduct analysis. One example is the study of aristolochic acids (AAs) which are the plant derived compounds once used as an herbal remedy [57–60]. Study by LC-ESI-TOF has provided insight into the carcinogenic mechanism of AAs by identifying the structures of new DNA–AA adducts. Another study also found that DNA adducts of AAs were measured in formalin-fixed paraffin embedded (FFPE) tissues [61]. More recently, Manna et al. [62] has developed a method for rapid screening and stratification of subjects after exposure using a UHPLC–ESI-QTOFMS which is an integral part of determining countermeasures against radiation. In this study, the effect of aging and repeated exposure was examined on the metabolic response to sublethal irradiation in mice.

Matrix-assisted laser desorption/ionization (MALDI) is a soft ionization technique used in mass spectrometry which allows the analysis of biomolecules and biopolymers such as DNA, proteins, peptides and sugars, and large organic molecules such as polymers, dendrimers and other macromolecules that tend to be fragile and fragment when ionized by more conventional ionization methods. Compared to electrospray ionization (ESI), MALDI produces far fewer multiply charged ions. Benefiting from this merit, it is commonly and widely applied in TOF-MS for accurate mass measurement, allowing the optimization of structural information for analysis of large biomolecules such as proteins [63,64], DNA oligonucleotides [44], DNA sequencing [65], and determining DNA polymorphisms [66]. Recently, it has been extended to the analysis of DNA adducts. Barnes and Chiu [67] did an exploratory study to characterize the fragmentation pathways of carcinogenic DNA adducts dG-C8-PhIP (2-amino-1-methyl-6-phenylimidazo[4,5–b]pyridine) and dG-C8-ABP (dG-C8-4-aminobiphenyl) using MALDI-TOF/TOF. By using a higher collision energy (1 keV), they achieved higher accuracy for the characterization of selected dG adducts and observed more extensive precursor ion dissociation and fragmentation of adducts compared to other MS/MS techniques with lower collision energies. This was due to the fact that the small matrix ions and its cluster ions did not interfere with the measurements of both selected dG adducts. Fragmentation allowed the authors to identify DNA adducts as well as the position of adduction. This method also revealed previously unreported fragment ions of dG-C8-PhIP, demonstrating that MALDI-TOF/TOF was a good tool for analysis of DNA adducts. Garaguso et al. [68] used MALDI-TOF with 2,5-dihydroxybenzoic acid (DHB) matrix layer (ML) sample preparation to identify PAH–DNA adducts. This method was robust and simple with a sensitivity of <100 fmol and a mass accuracy of <10 ppm that was enough to enable molecular specification and characterization of adducted nucleotides and of the alkylating agent. Compared to the ^{32}P-postlabeling assay, a distinct advantage of the method was the capability for unambiguous identification of DNA adducts and the identification of the chemical nature of the alkylating agent. Rojsitthisak et al. [69] used MALDI-TOF and ESI-MS to analyze the mechlorethamine DNA crosslink at C–C using double-stranded DNA oligonucleotides as the probe. With this technique, the authors were able to determine the atomic connectivity of the C–C crosslink and identified a DNA adduct at the N^3 position of cytosine for the first time. Further applications of TOF have made it possible to determine qualitative information regarding previously unknown adducts at low levels. For instance, Wang et al. [63] reported a new, advantageous method for mass spectrometry which allowed for nontargeted analysis of modified nucleotides in DNA (and RNA) through labeling with benzoylhistamine (BH) by MALDI-TOF/TOFMS. The method provided deoxynucleotide-specific detection, accurate measurement of molecular ions, high sensitivity, semiquantitation, and, to the extent studied to date, normalization of response within a factor of <3. For analysis of potentially labile



N^2-methylguanine N^2-ethylguanine N^4-methylcytosine



N^2-furan-2-yl-methylguanine N^2-tetrahydrofuran-2-yl-methylguanine

Fig. 2. The structures of N-alkyl–four DNA base-adducts

adducts with MALDI-TOF, Bono et al. [70] utilized reduction of adducts with $NaBH_4$ to increase the stability of the adduct precursor prior to MALDI plating. In this work, MALDI-TOF was used to analyze reference standards from the reaction of malondialdehyde (MDA) with ct-DNA for formaldehyde exposure studies. The authors were able to identify 6 M_1dG adducts, including M_3mdC which had not been previously reported.

Compared to TOF and MS/MS technique, the Orbitrap MS is a new generation of high resolution mass spectrometer first described in 2000. It consists of an outer barrel-like electrode and a coaxial inner spindle-like electrode that forms an electrostatic field with quadro-logarithmic potential distribution [71–73]. Ions become trapped because of electrostatic attraction to the inner electrode which is balanced by centrifugal forces. Image current from dynamically trapped ions is detected, digitized and converted using Fourier transform into frequency and then mass spectra. It can be operated in full scan mode, and therefore does not require MS/MS optimization for each DNA adduct which makes the Orbitrap highly amenable for DNA adduct screening. The Orbitrap instrument also provides high mass accuracy (1–2 ppm) coupled with very rapid scan times, high resolving power (up to 200,000 amu), large dynamic range (around 5000 amu) and is comparable in sensitivity to triple quadrupole instruments. These characteristics make it a powerful addition to the arsenal of mass spectrometric techniques available for probing biological systems and increases selectivity and confidence of routine analyses. Front-end separation techniques such as reversed-phase HPLC and UHPLC can also be coupled to the Orbitrap MS to further increase the selectivity and have been recently reviewed by Makarov and Scigelova [74]. In the application of LC techniques hyphenated to Qrbitrap MS, multi-dimensional LC separations have been applied, usually in proteomics applications, while an UHPLC front-end is more frequently encountered in the area of metabolomics and metabolite analysis. Recently, special chromatographic techniques such as hydrophilic interaction chromatography and its variations have also been cited with the Orbitrap detection. Despite its capacity to obtain high quality information, the studies using Orbitrap MS for DNA adducts analysis have been reportedin only the past two years, which is likely due to high purchase and maintenance costs for the instrumentation. In one report by Jamin et al. [75], the heterocyclic aromatic amine (HAA) metabolites NHOH-PhIP and NHOH-IQ were reacted with deoxynucleotides (dNTs) to form adducts. Attribution of ions was supported by accurate mass measurements performed on an Orbitrap MS. The structural characterization of five adducts, including two new compounds dG-N^7-IQ and dA-N^6-IQ, was improved by applying high resolution MS^n methods. In particular, accurate mass measurements of fragment ions observed on MS^3 spectra allowed determination of elemental composition. This work clearly showed the usefulness of exact mass data and extensive high resolution data for supporting the overall fragmentation patterns. This study has demonstrated that combining Orbitrap instrumentation with MS^n experiments can generate new information for both known adducts and newly discovered adducts. Furthermore, using diagnostic fragment ions to detect adducts will be useful in future studies pertaining to the formation of HAA-DNA adducts *in vitro* or *in vivo*. In another report, Orbitrap was coupled to a LC–nanospray system by Balbo et al. [76] to develop a liquid chromatography-nanoelectrospray-high resolution tandem mass spectrometry-selected reaction monitoring (LC–NSI-HR-MS/MS-SRM) method for quantitatively monitoring 7-ethylguanine (7-EtG) in leukocyte DNA from smokers and nonsmokers against a [$^{15}N_5$]7-EtG standard. The detection of adducts was accomplished by selected reaction monitoring (SRM) as well as accurate mass determination. Other applications with Orbitrap MS include confirmation of 4-hydroxy-1-(3-pyridyl)-1-butanone (HPB)-releasing DNA adducts such as 7-pyridyloxobutyl-deoxyguanosine (7-POB-dGuo) in human exfoliated oral mucosa cells [77] (Fig. 3), quantitation of N^7-(2,3,4-trihydroxybut-1-yl)-guanine adducts of 1,3-butadiene in human leukocyte DNA [78] (Fig. 3), and identification of octenal-related dA and dC adducts including 8-(2-oxoheptyl)-3,N^4-etheno-dC and 11-(2-oxoheptyl)-1,N^6-etheno-dA [79] (Fig. 3). Continued development in this area will undoubtedly give rise to exciting new applications as Orbitrap instruments are becoming more widespread. Some representative applications of Qrbitrap mass spectrometry and their exposure scenario (*in vitro* or *in vivo* exposure) are also outlined in Table 1.

2.3. *Tandem mass spectrometry (MS/MS) and MS^n*

Tandem mass spectrometry, also known as MS/MS or MS^2, involves multiple steps of mass spectrometry selection with some form of fragmentation occurring in between the stages. It can be regarded as another important tool for DNA adducts analysis since the increased selectivity allows identification of specific adducts within complex mixtures. The use of MS/MS such as triple

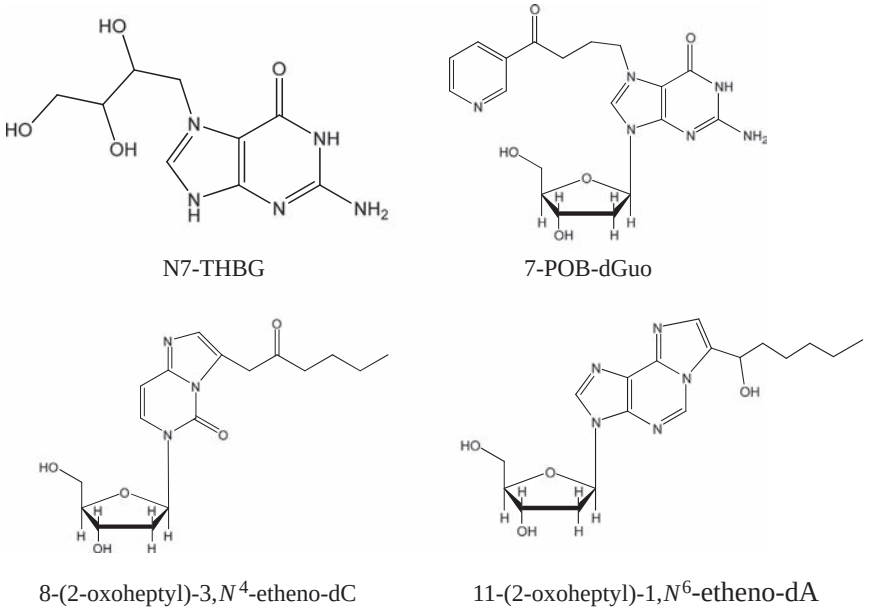


Fig. 3. The structures of dG, dA and dC adducts.

quadrupole, QTRAP, and QTOF, is very common in LC-based MS methods. Multi-stage tandem MS (MS^n) is a technique that allows the further fragmentation of product ions and can only be performed using ion-trap (IT). Recently, Sagoo et al. [80] employed IT-MS to study the tautomerization in gas-phase ion chemistry and fragmentation pathways of C8-dG adducts from phenol-induced DNA damage. With MS^n, the authors demonstrated that charge distribution through the N-7 site is critical in guanosine adduct fragmentation. The modification of the C-8 site of dG alters the reactivity of adducts for fragmentation by charge redistribution which allows for the formation of a ket-tautomer. Different reaction pathways were therefore observed for 8-(4″-hydroxyphenyl)-2′-deoxyguanosine and 8-(2″-hydroxyphenyl)-2′-deoxyguanosine. This method showed the ability of tandem mass spectrometry to completely differentiate between the isomeric dG adducts. Linear ion-trap (LIT) is a type of two-dimensional (2D) ion-trap that has higher injection efficiencies and higher ion storage capacities. LIT is also widely employed in DNA adducts analysis. Bessette et al. [81] developed a LIT/MS method for DNA adducts analysis using constant neutral loss (CNL) followed by triple-stage MS (CNL-MS^3). CNL of the deoxyribose from DNA adducts was selected in the first dimension and the second dimension triggered MS^3. The method was able to screen for adducts of 4-aminobiphenyl (4-ABP), 2-amino-3,8-dimethylimidazo[4,5–f]quinoxaline (MeIQx), 2-amino-9H-pyrido[2,3–b]indole (AαC), B[a]P, PhIP, AC, HNE in human and rat hepatocytes. The method detection limit approached 1 adduct in 10^8 unmodified DNA bases. Subsequently, the same group developed an LC-ESI-LIT-MS/MS^n method for detecting tobacco and cooked-meat adducts in human saliva [82] and formation of DNA adducts with 4-ABP and HAAs in human hepatocytes [83]. The authors demonstrated that PhIP was a damaging agent. Adduct levels were found from 3.4 to 140 adducts per 10^7 DNA bases with a rank of AαC > 4-ABP > PhIP > MeIQx > IQ in relative amounts. It was also found that human hepatocytes could form some dG-C8-adducts at a level of 100-fold greater than those of rats. This result indicated that use of rats as a study model may not be suitable for human risk assessment. Huang et al. [84] used a LC–MS/MS method to investigate the formation of B[a]P-7,8-dione-DNA adducts in human lung adenocarcinoma A549 cells, human bronchoalveolar H358 cells, and immortalized human bronchial epithelial HBEC-KT cells. With aid of MS^n spectra, authors found that in A549 cells the B[a]P-7,8-dione-DNA adducts were identified as hydrated-B[a]P-7,8-dione-N^2-2′-deoxyguanosine and hydrated-B[a]P-7,8-dione-N^1-2′-deoxyguanosine (Fig. 4) while in HBEC-KT cells, they were hydrated-B[a]P-7,8-dione-2′-deoxyadenosine, hydrated-B[a]P-7,8-dione-N^1- or N^3-2′-deoxyadenosine, and B[a]P-7,8-dione-N^1- or N^3-2′-deoxyadenosine. The major recent advancements in the DNA adducts field is development of screening or DNA adductomics methods which allow the detection of multiple adducts rather than a single adduct from the analysis of a single sample. Kanaly et al. [85,86] developed a DNA adduct detection method using LC/ESI/MS/MS to detect multiple DNA adducts in human lung tissue. The authors designed an adductome analysis strategy to detect the neutral loss of 2′-deoxyribose from positively ionized 2′-deoxynucleoside adducts in multiple reaction ion monitoring mode (MRM) transmitting the $[M+H]^+ > [M+H-116]^+$ transition over a total of 374 transitions in the mass range from m/z 228.8 to m/z 602.8. With this quick screening method, seven DNA adducts, N^2-ethyl-2′-deoxyguanosine (N^2-ethyl-dG), 1,N^6-etheno-2′-deoxyadenosine (εdA), α-*S*- and α-*R*-methyl-γ-hydroxy-1,N^2-propano-2′-deoxyguanosine (1,N^2-PdG1, 1,N^2-PdG2), 3-(2′-deoxyribosyl)-5,6,7,8-tetrahydro- 8-hydroxy-pyrimido[1,2–a]purine-(3H)-one (8-OH-PdG) and the two stereoisomers of 3-(2′-deoxyribosyl)-5,6,7,8-tetrahydro-6-hydroxypyrimido[1,2–a]purine-(3*H*)-one (6-OH-PdG) were unambiguously detected in all tissue DNA samples. Using HPLC–MS/MS and immunoblot techniques, Pang et al. [87] contributed to defining the prevalent DNA damage chemistry associated chronic inflammation and quantified 12 DNA damage products in tissues from the SJL mouse model of nitric oxide (NO) overproduction. The authors found that oxidative and nitrosative stresses associated with inflammation affected tissues at a distance from the activated macrophages responsible for NO overproduction during chronic inflammation and revealed the complexity of NO chemistry *in vivo*. In analysis of damage products as biomarkers of inflammation, it is therefore necessary to have a good understanding of the chemical biology of inflammation, sensitive analytical methods and ability to focus on multiple chemicals instead of on single chemical as surrogates for inflammation. To achieve these goals, Taghizadeh et al. [88] developed a general and sensitive LC–MS/MS method to quantify, in a single DNA sample, the nucleoside forms of seven DNA lesions reflecting the range of chemistries associated with inflammation: 2′-deoxyuridine, 2′-deoxyxanthosine and 2′-deoxyinosine from nitrosative deamination; 8-oxo-2′-deoxyguanosine from oxidation; and 1,N^2-etheno-2′-deoxyguanosine, 1,N^6-etheno-2′-deoxyadenosine and 3,N^4-etheno-2′-deoxycytidine arising from reaction of DNA with lipid peroxidation products. In another study, a two-dimensional linear quadrupole ion trap mass spectrometer (LIT-MS) was employed by Bessette *et al.* [81] to simultaneously screen for DNA adducts of environmental, dietary, and endogenous genotoxicants at levels of adduct modification approaching 1 adduct per 10^8 unmodified DNA bases, when 10 μg of DNA was employed for the assay, by data-dependent constant neutral loss scanning followed by triple-stage mass spectrometry (CNL-MS^3). In this method, the acquisition of MS^3 product ion spectra of the aglycone adducts $[BH_2]^+$ was triggered by the loss of the deoxyribose (dR) from the protonated DNA adducts ($[M+H-116]^+$) in the MS/MS scan mode and five DNA adducts of the tobacco

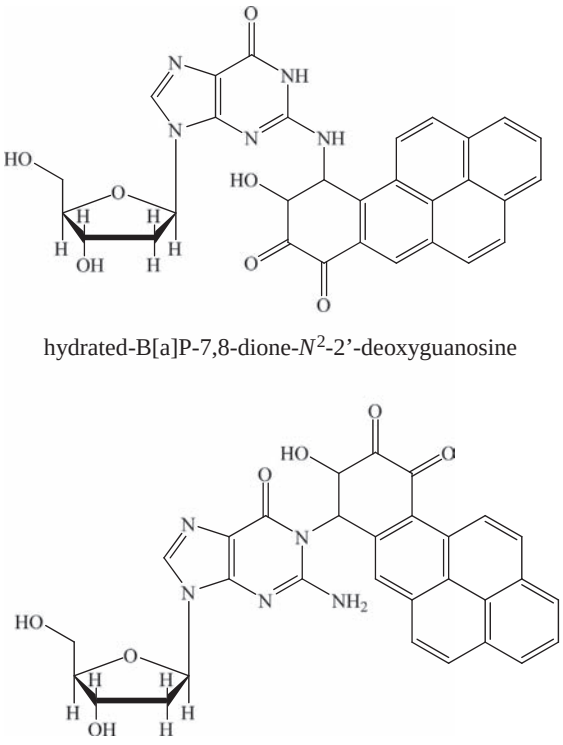


Fig. 4. The structures of hydrated-B[a]P-7,8-dione-N^2-2′-deoxyguanosine and hydrated-B[a]P-7,8-dione-N^1-2′-deoxyguanosine

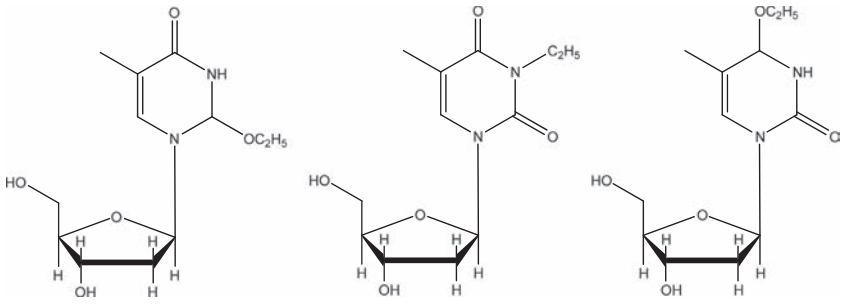
carcinogen 4-aminobiphenyl (4-ABP) were detected in human hepatocytes treated with 4-ABP, and three DNA adducts of the cooked-meat carcinogen 2-amino-3,8-dimethylimidazo[4,5–f]quinoxaline (MeIQx) were identified in the livers of rats exposed to MeIQx. Subsequently, a targeted DNA adductomic approach using LC–MS/MS incorporating software-based peak picking and integration for the assessment of human exposure to mixtures of PAHs from sources such as industrial or urban air pollution, tobacco smoke and cooked food was developed by Singh et al. [89]. With constant neutral loss scanning or SRM $[M+H-116]^+$ transitions plus product ions derived from the PAH moiety for improving detection sensitivity, different PAH DNA adducts were identified. More recently, Chou et al. [90] reported a LC–MS/MS method for DNA adductome analysis of several human specimens of pulmonary DNA as well as various LPO-induced DNA adducts in 68 human autopsy tissues, including colon, heart, kidney, liver, lung, pancreas, small intestine, and spleen. With this method, DNA adducts derived from 4-ONE and 4-OHE, namely, heptanone-etheno-2′-deoxycytidine (HεdC), heptanone-etheno-2′-deoxyadenosine (HεdA), and butanone-etheno-2′-deoxycytidine (BεdC), were identified as major adducts in one human pulmonary DNA. The quantitative analysis in this study further revealed that 4-ONE-derived HεdC, HεdA, and heptanone-etheno-2′-deoxyguanosine (HεdG) were ubiquitous in various human tissues with median values of 10, 15, and 8.6 adducts per 10^8 bases. Another recent screening method for DNA adducts with unknown chemical structures was developed by Inagaki et al. [54] using LC–ESI-MS/MS with precursor ion scan analysis of these fragment ions. The authors successfully discovered new guanine adducts with the screening method. Other recent applications using ion-trap MS includes quantitation of 8-methoxypsoralen-induced DNA interstrand cross-links and monoadducts [91], DNA adducts of the tobacco carcinogens 2-amino-9H-pyrido[2,3–b]indole and 4-aminobiphenyl [92] and characterization of cisplatin adducts [93]. Some representative examples and their exposure scenario (*in vitro* or *in vivo* exposure) are listed in Table 1.

3. Separation techniques

3.1. HPLC and UHPLC

HPLC is the most widely used separation technique coupled to MS for DNA adduct analysis and has been comprehensively reviewed [29,45,46,48]. In order to avoid redundant description for conventional HPLC, capillary and nanoLC, we will mainly describe recent developments, including new applications of ultra-high performance LC (UHPLC) and new online pre-concentration approaches. The nanoelectrospray source developed as the interface of MS is now commonly used to couple capillary HPLC or nano-UHPLC to further provide the best sensitivity for detection of DNA adducts. Recently, Chen and Liu [94] used the nanospary source and have developed a stable isotope dilution capillary LC–nanospray ionization-MS/MS method to sensitively measure N^3-ethyladenine and N^7-ethylguanine in human leukocyte deoxyribonucleic acid as potential biomarkers for smoking-related cancers. The authors achieved the quantification limit of 50 and 100 fg of 3-EtAde and 7-EtGua, corresponding to 4.7 and 8.6 adducts in 10^9 normal nucleotides for the entire assay. Subsequently, Chen and Lee [95] used the same method to quantitate three smoking-related ethylthymidine adducts in human salivary DNA, O^2-ethylthymidine (O^2-edT), N^3-ethylthymidine (N^3-edT) and the promutagenic O^4-ethylthymidine (O^4-edT) (structures are shown in Fig. 5) as potential biomarkers for exposure to ethylating agents and possibly for cancer risk assessment. The results indicated that these three adducts were non-detectable in nonsmokers but they were clearly observed in smokers' salivary DNA samples.

UHPLC has become commercially available by the Waters Corporation in early 2004 and offers advantages of shorter separation times, higher flow rates, increased resolution and sensitivity over conventional HPLC [96]. UHPLC was first coupled to QTOF for analysis of drug metabolites in bile by Plumb et al. [97]. Since then, more UHPLC devices have been commercially developed by other manufacturers and consequently, numerous UHPLC–MS-based methods have been developed. However, it has only been in the past five years that UHPLC–MS has been applied to DNA adduct analysis. In 2009, Feng et al. [98] employed UHPLC–MS/MS to separate 4-stereoisomers of 7,8-dihydroxy-9,10-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene (BPDE)-dG, in their *trans*-(+), *trans*-(−), *cis*-(+) and *cis*-(−) forms in lung cells within 2–4 min. The method achieved a detection limit less than 0.7 fmol ($S/N=3$) for the four stereoisomers of anti-BPDE-N^2dG and a dynamic range of 2 orders of magnitude (2.3–630 fmol, $R^2 >$ or $=0.997$). Other than the DNA adducts, the authors also found that a number of key optical intermediates were formed during activation of B[a]P in A549 cells, including *trans*-(+)-B[a]P-7,8-dihydrodiol and *trans*-(−)-B[a]P-7,8-dihydrodiol and their corresponding downstream metabolites (+)-anti-BPDE and (+)-syn-BPDE. In 2011, Lu et al. [99] developed a highly sensitive nano-UHPLC–MS method for determining the dosimetry of N^2-hydroxymethyl-dG adducts in rats exposed to $[^{13}CD_2]$-formaldehyde. The authors found that exogenous DNA adducts formed in a non-linear fashion and endogenous adducts were dominant at low exposures (> 99%). However, exogenous adducts were reported non-detectable in bone marrow of rats even at high exposure doses. The same nano-UPLC method was employed by Moeller et al. [100] to determine N^2-OHMe-dG adducts in the nasal epithelium and bone marrow of nonhuman primates exposed to $[^{13}CD_2]$-formaldehyde. Successively, the method was improved using heat-assisted ESI (HESI) in the system to eliminate interferences and improve assay performance and sensitivity for analysis of 8-oxo-7,8-dihydro-2′-deoxyguanosine



O^2-edT N^3-edT O^4-edT

Fig. 5. Structures of thymidine adducts.

(8-oxo-dG) by Boysen et al. [101]. A detection limit of 0.2 fmol/μg DNA, or ~0.4 8-oxo-dG adducts per 10^6 dG, was achieved. More recently, Yin et al. [102] developed ammonium bicarbonate-enhanced stable isotope dilution UHPLC–MS/MS method Acr-DNA adducts of three nucleotides (dG, dA, and dC) in human leukocytes from acrolein (Acr), a ubiquitous environmental pollutant. The authors found that ammonium bicarbonate as an additive to the mobile phase not only improved the protonation of AcrdG adducts but also suppressed the formation of MS signal-deteriorating metal-AcrdG complexes during electrospray ionization. This leads to the enhancement of MS detection enabling them to achieve a detection limit ($S/N=3$) of about 40–80 amol. Other applications with UHPLC include the determination of aristolochic acid (AA) derived DNA adducts [103], depurinating estrogen adducts [104], methylated DNA [105] and recently the cytosine derived DNA modifications [106].

Online pre-concentration prior to DNA adduct analysis, usually by solid phase extraction (SPE), can increase the method sensitivity of LC–MS in comparison with the ^{32}P-postlabelling assay. SPE can also separate unmodified bases and unnecessary salts from adducted-nucleosides to reduce the matrix interference and increase ionization efficiency of MS [103]. In addition to SPE, ultra-centrifugation, spin filters, packed-tips and preparative LC have been employed as the online pre-concentration method to isolate adducts from digested DNA [107–109]. Online pre-concentration, however, is always preferred for ease of automation. Doergeet al. [110] pioneered the use of online desalting in quantitative analysis of 4-aminobiphenyl-C8-deoxyguanosyl DNA adducts produced *in vitro* and in *vivo* using HPLC–ES-MS. Commercially available micro-LC chips can be used for online sample enrichment and have been applied to determine 4-ABP-DNA adducts with a LOD of 5 adducts in 10^9 normal bases [111]. Another recent strategy has been the development of a column switching system to perform online SPE [112]. In this approach, the sample was first loaded onto a trap column where it was retained and washed with wash buffer similar to HPLC elution conditions. The column was then flushed with loading buffer and then loaded onto the separation column by valve-switching (elution position). Finally, after injection, valves were switched again and the trap column was washed and conditioned. This approach has been applied to measurement of DNA adducts of 3-nitrobenzanthrone (3-NBA) [113] and PhIP [112] with a detection limit of 2.0 fmol and 2.5 fmol (1.5 adducts per 10^8 bases), respectively. Other applications of this approach include determination of 8-oxo-dG and 8-oxo-dA [114], N^2-ethylidene-2′-deoxyguanosine (N^2-ethylidene-dG) adducts [115], N^7-methylguanine (N^7-MeG) and N^7-EtG in mosquito fish [116], N^3-MeA [117] and 8-oxo-dG in mice [118], and urinary 8-oxo-G [119–121]. The advantages for methods utilizing online pre-concentration are increased sensitivity and decreased matrix effect and ion suppression due to using nano-LC with nanoelectrospray ionisation. In addition to single adsorbent, the use of several adsorbents in the trap column, including silica, ZIC® –HILIC, polystyrene–divinylbenzene, diol and PGC, were also employed for online enrichment of glyoxal-dG to focus the adduct without carrying-over [122]. With this procedure, the authors were able to obtain an absolute detection limit of 15 fmol for the glyoxal-dG standard, with the mass limit of detection was 15 pg, corresponding to a concentration limit of detection of 75 fg μl^{-1} DNA hydrolysate solution. This further corresponded to 48 adducts per 10^6 normal nucleosides by means of capillary LC sample enrichment column switching coupled to ESI-MS detection. This detection limit was higher when compared with the column switching methods previously reported due to the poor electrospray efficiency of glyoxal-dG, but the sensitivity was still sufficient to detect biologically relevant concentrations of glyoxal-dG (1 adduct in 10^6 normal nucleosides). Some other column-switching methods have also been developed for analysing exocylic dA adducts [123,124], and urinary N^7-HEG [125]. It is necessary to note that the trap column does not always necessarily have to be a SPE column and can also be HPLC column containing for example a C18 stationary phase. Also, centrifugation and spin filters are normally used for the purification of thermally labile depurinating DNA adducts.

3.2. Capillary electrophoresis (CE)

Capillary electrophoresis (CE) is another separation technique with the advantages of speed, low-cost, and high resolution for both charged and neutral analytes. There are many reports of CE–MS for DNA adduct analysis that have been reviewed by Bakry et al. [126]. Compared to HPLC, however, the use of CE–MS for DNA adduct analysis still lags in widespread uptake due in part to lower sensitivity associated with its small injection volume, typically in the nanoliter range which is 3 orders of magnitude less than the usual injection amount (μL) of high performance liquid chromatography (HPLC). In order to solve this problem, Feng et al. [127] have developed an online sample pre-concentration technique in CE, the pressure-assisted electrokinetic injection (PAEKI), which dramatically increased the sample loading amount. PAEKI was easily coupled to a CE–MS system for sensitive online PAEKI-CE–MS determination of both single stranded and duplex oligonucleotide-BPDE adducts and adducts of oligonucleotide with styrene-7,8-oxide (SO) and phenyl glycidyl ether (PGE) [127,128] (Fig. 6). This study demonstrated that CE–MS could still contribute to the study of genotoxic compounds by detecting interactions [129]. A CE–ICP-MS method was developed to probe the interactions of two inorganic Sb species (Sb^{III} and Sb^{V}) with herring fish DNA [130]. The CE–ICP-MS method allowed the authors to determine the reaction stoichiometry, the thermodynamics of the reaction as well as the rate constant for the reaction. It is expected that CE–MS may play greater roles in future studies focused on determining hazards that may result in covalently bound adducts.

3.3. Gas chromatography (GC)

The gas chromatography–mass spectrometry (GC–MS) has also been used for the detection of DNA adducts and the use of this technique on analysis of DNA adducts has been recently reviewed [131]. DNA adducts levels at ~1 adduct per 10^9 nucleotides typically can be detected by GC-MS due to the advantages of high resolution and high sensitivity. The major disadvantages of GC–MS for DNA adducts analysis include the requirement of derivatization to convert non-volatile DNA adducts to volatile material. Further, GC–MS is unsuitable for thermally labile adducts which are better suitable to LC methods. Although in recent years there have been fewer GC–MS reports for DNA adduct analysis due to the increasingly popularity of LC-based methods, GC–MS has proved most useful in analysis of volatile, thermally stable, depurinating DNA adducts (*e.g.* guanine, adenine adducts). Recent applications of GC–MS have employed negative chemical ionization (NCI), the most sensitive mode for DNA-adduct analysis, and have focused on quantitation of depurinating adducts that can be readily analyzed from bodily fluids or tissue. In recent work, DNA adducts of *o*-toluidine and 4-ABP in human bladder were measured by gas chromatography/mass spectrometry [132]. The result from this study found that 4 and 11 of 12 tumour samples contained adducts of 4-ABP and o-toluidine, respectively, in epithelial and submucosal bladder tissues of sudden death victims were above background and lower adduct levels were present in both epithelial and submucosal bladder tissues of SDV (4-ABP and o-toluidine-releasing DNA adducts), supporting a carcinogenic role for *o*-toluidine. In other recent applications, Yong *et al.* [133] detected N^7-HEG adducts in the granulocytes of hospital workers who were exposed to EO. In this study, adducts were detected even in

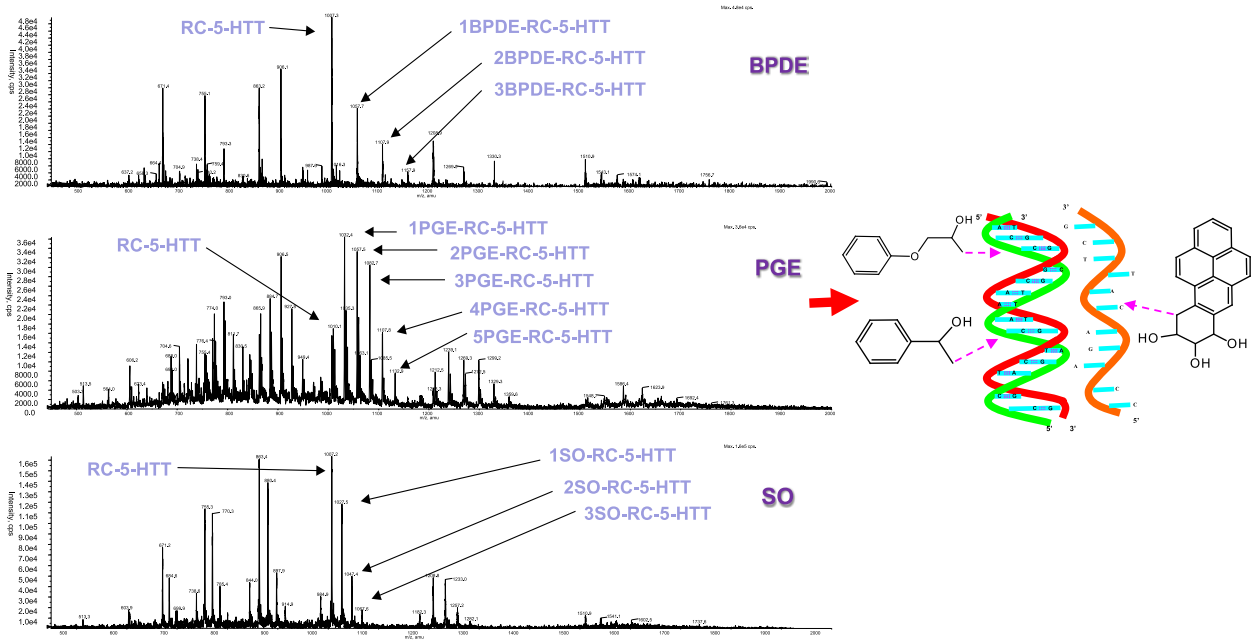


Fig. 6. The mass spectra of adducts of DNA oligonucleotides with three chemicals and the structural image of adducts.

workers who were exposed at levels below the regulated limit. Using isotope dilution GC/MS and negative ion chemical ionization (NICI) source, Chen et al. [134] found that the urinary etheno(ε)-adducts of adenine and cytosine [1,N^6-ethenoadenine (εAde) and 3, N^4-ethenocytosine (εCyt)] were associated with gender. Min and Ebeler [135] studied the effect of exposure to flavonoids on DNA oxidation at concentrations relevant to physiological levels. In this proof-of-principle study, ct-DNA was exposed to flavanoids under oxidizing conditions ($Fe^{2+/3+}$). The adducts 8-OH-dG and 4,6-diamino-5-formamidopyrimidine (fapy-adenine) were measured with GC–EI-MS. Another study with gas chromatography–mass spectrometry-selective ion monitoring (GC–MS-SIM) was performed by Chan et al. [136] to detect several oxidative adducts including 8-hydroxy guanine (7,8-dihydro-8-oxo-2′-deoxyguanosine; 8-OH guanine) from calf thymus DNA treated with different flavonoids (*e.g.*, catechin, quercetin, myricetin, luteolin, morin and cyanidin). The results showed that flavonoids acted as antioxidants at low concentrations relevant to physiological levels. However measuring only one oxidative DNA adduct as a biomarker may result in misleading conclusions regarding antioxidant activities of natural products and it should be noted that the European Standards Committee on oxidative DNA Damage (ESCODD) advise against using GC–MS for the detection of 8-oxodG in DNA samples. This is due to the high temperatures employed in the derivatisation step in which there is potential for the artifactual formation of 8-oxodG.

4. Advances in off-line sample preparation

Modified nucleosides are the most common target for analysis of DNA adducts by MS. The sample preparation prior to the measurement with MS is quite various depending on the sample matrix. Adducts in urine can be analyzed without any pre-treatment, or after being released by either thermal or enzymatic hydrolysis [137,138]. For all other types of human or animal samples, a digestion process is usually needed to hydrolyze the DNA adducts to their single nucleosides. In the hydrolysis of DNA or DNA adducts, a single enzyme usually does not lead to a complete hydrolysis and therefore multiple enzymes are often needed. The most common protocol used was first published by Crain in 1990 [138] and used a combination of 3 enzymes: nuclease P_1, phosphodiesterase I and alkaline phosphatase. Most MS approaches apply this protocol with minor variations, such as the addition of an ultrafiltration step to remove enzymes prior to analysis [101,105]. However, in order to better hydrolyze DNA containing bulky adducts, mixtures of different enzymes have been proposed. In 2008, Neale et al. [139] compared the effectiveness of micrococcal nuclease, nuclease P_1, DNase I, snake venom phosphodiesterase, spleen phospodiesterase, and alkaline phosphatase in different combinations. The purpose of the assay was to optimize digestion parameters to maximize adduct detection in capillary LC–nanospray-MS/MS. In this assay the authors found that a mixture of four enzymes (nuclease P1, DNase I, phosphodiesterase, and alkaline phosphatase) provided the best hydrolysis results for the test compounds studies. This study demonstrated the possibility of optimizing hydrolysis schemes which could potentially allow lower levels of bulky adducts to be detected by MS. Also, the different sources of tissue available for measuring DNA adducts in humans may need different enzyme hydrolysis process. For example, Bessette et al. [82] employed a hydrolysis process with DNase I for 1.5 h and with nuclease P1 for another 3 h followed by treatment with alkaline phosphatase and phosphodiesterase for 18 h to hydrolyze the DNA adducts in human saliva prior to the detection with linear quadrupole ion trap/multistage tandem mass spectrometry. Balbo et al. [140] recently investigated levels of N^2-ethylidene-dG, the major DNA adduct of acetaldehyde, by LC–ESI-MS/MS in DNA from human oral cells extracted after drinking alcohol. The authors used DNase I (type II, from bovine pancreas), phosphodiesterase I (type II, from Crotalus adamanteus venom), and alkaline phosphatase to digest the DNA adducts. The digested sample was then desalted and purified using a solid-phase extraction cartridge [Strata-X 33 mm, 30 mg/1 mL (Phenomenex)] and then purified using a mixed mode, anion exchange reversed phase extraction cartridge (Oasis MAX, 30 mg/cartridge, Waters). The results provided the conclusive evidence linking alcohol drinking and the kinetics of acetaldehyde–DNA adduct formation in the human oral cavity. In another study, Chen and Lin [141] used a stable isotope dilution nanoflow LC–nanospray-MS/MS method to measure the multiple exocyclic DNA adducts in human saliva. The authors compared the enzyme hydrolysis methods

on the releasing efficiency of DNA adducts and found that the hydrolysis method with the enzymes (A) [micrococcal nuclease (from Staphylococcus aureus), phosphodiesterase II (from bovine spleen), adenosine deaminase (from bovine spleen) and alkaline phosphatase nuclease P1] were much more effective than enzymes (B) [phosphodiesterase I (from Crotalus adamanteus venom), adenosine deaminase (from bovine spleen) and alkaline phosphatase]. Enzyme mixture (A) released 5 times more AdG and 2 times more 1,N^2-εdGuo than the hydrolysis method (B). On the other hand, the levels of CdG and εdCyd were comparable using both methods and the level of εdAdo was 18% higher using method (B) than using method (A). The results clearly demonstrated that hydrolysis of the adducted nucleosides from DNA greatly depended on the types and amounts of the hydrolytic enzymes used as well as the pH and incubation time of the hydrolysis. The digested samples were cleaned up with solid extraction (SPE) column with Bond Elut C18 cartridge prior to the analysis with the nanoLC–NSI-MS/MS analysis. Successively, the method was applied to quantification of ethylpurine adducts in human urine [142].

Another advancement in off-line sample preparation is the application of a chemical reduction with $NaBH_3CN$ for Schiff base type adducts to reduce labile adducts to more stable forms and improve detection of DNA adducts in MS analysis. This has been applied to the study of acetaldehyde [115,143], ethanol [144] and nitrosamine adducts [145–148], for example, by LC–ESI-MS/MS which allowed for previously undetected adducts to be identified and quantitated by MS methods. However, this strategy is not amenable to all labile adducts.

5. Application of DNA adduct analysis by MS to exposure biomonitoring, identification of genotoxic chemicals and hazard assessment

As discussed above, MS provides sensitive and highly specific detection capabilities as well as structural characterization. It is therefore becoming more popular in adductomic approaches that have been applied to identification and assessment of chemical hazards in the environment. From 2007 to 2011, over 150 studies have been published which focused on analysis of DNA nucleoside adducts using mass spectrometry. A wide range of chemical classes were covered with the majority of the studies focusing on determining mechanisms of toxicity and metabolic activation, carcinogenesis and interference with DNA repair. These studies have helped identify genotoxic chemicals and their mechanism of toxicity. Study of DNA adducts can provide chemical support for determining carcinogenic mechanism and includes determining DNA sequence preference for adduction [143], the effect of gene heterogeneity on adduct formation [149], or mechanisms of prevention such as the role of GSH for DNA adduction against electrophilic species including 4-hydroxynonenal (HNE) as well as other chemicals [150]. A large number of studies have also focused on method development in order to lower limits of detection and increase selectivity for biomonitoring applications.

In order to assess the level of hazard a chemical may pose, measurement of DNA adduct levels may be useful for indicating genotoxicity. Currently, risk assessment guidelines define chemical hazards in more general terms of estimated daily intakes and tolerable daily intakes. The formation of DNA adducts from reaction with a chemical pollutant indicates that the contaminant may pose a chemical hazard. It stands to reason that the formation of more DNA adducts from a single contaminant would indicate greater potential toxicity due to the greater difficulty for repair of larger, structurally diverse quantities of damaged DNA. Furthermore, the number and type of body compartments (*e.g.* organs, fluids) containing adducts from a single contaminant may provide useful information for hazard assessment, such as potential for elimination (*e.g.* in urine), route of exposure (*e.g.* high adduct levels in lung) or metabolic activation (*e.g.* high adduct levels in liver). For example, B[a]P, a well-studied PAH, is a known carcinogenic environmental contaminant. At least 9 different DNA adducts have been characterized and isolated from body fluids (saliva, urine, blood), lung, and liver cells in humans and animals. A cursory survey of this data would indicate the B[a]P is metabolically activated (adducts found in liver), and can therefore be eliminated (found in urine). However, the observation of adducts in lung could also indicate a significant route of exposure to B[a]P likely through inhalation or from systemic uptake of B[a]P or its metabolites as well. The different adduct types identified by mass spectrometry indicate multiple target sites on DNA and indicate that there are likely multiple metabolically active forms. Indeed, fundamental studies have shown that DNA adducts from B[a]P exposure can result from metabolites such as BPDE, benzo[a]pyrene-7,8-dione (BPQ) [151], B[a]P-7,8-dihydro-7,8-diol [152], as well as from radical pathways [153]. Biomonitoring studies have further shown a correlation between individuals exposed to urban air pollution by benzene and polycyclic aromatic hydrocarbons (PAHs) and the general DNA adducts levels using the ATP-γ ^{32}P postlabeling method [154]. In order to properly assess a genotoxic chemical hazard, both fundamental studies, to identify genotoxins and understand mechanisms of toxicity, and biomonitoring studies, to determine the relationship between adduct levels and exposure levels, are necessary.

There are many challenges, however, associated with the use of DNA adducts in exposure biomonitoring. One aspect is identifying the exposure source through linking the DNA adducts to the contaminants in a variety of environments (*e.g.* occupational, air, water) and lifestyle choices (food, medicine, supplements/herbal remedies) that exist for human activities. Since chemical exposure can occur from more than one type of environment it can therefore be difficult to accurately assess exposure levels. It is also a challenge to have commercially available standards for quantitation of biomarkers. For some lesions, such as the oxidative lesion 8-oxo-dG, both standards and isotopic standards are commercially available. However, for many new environmental pollutants and their metabolites, especially for the corresponding DNA adducts, identification and the synthesis of standards for quantitation are needed.

In addition, another challenge hindering the use of DNA adducts as biomarkers for hazard assessment is the lack of reference or background values. Quantitation of background or reference adduct levels in humans is necessary for biomonitoring since some adducts can be formed from endogenous processes as well as exogenous environmental exposures. Background DNA damage in healthy individuals, stemming from endogenous processes, can be as high as 1 lesion per 10^5 bases [155]. Furthermore, a specific DNA-adduct may also arise from exposure to more than one chemical source, *i.e.* it may be formed by more than one reaction pathway. For example, 8-oxo-dG is an oxidative lesion endogenously from LPO, but it can also arise from the exposure to B[a]P [156,157], carbon tetrachloride [114] or can be produced by superoxide anion and other ROS. In order to determine the effect of exogenous exposure and establish appropriate threshold values for hazard assessment, a baseline DNA adduct level must be accurately measured in human or animal models.

Finally, we may conclude that measurement of DNA adducts may not only provide an additional endpoint for traditional exposure biomonitoring, but with the use of MS-based methods, may provide a useful tool for surveillance as DNA adducts may provide unique chemical information about unknown parent compounds which individuals may be exposed to. However, determination of exposure levels (*e.g.* environmental concentrations) for chemicals which form DNA adducts is outside the scope of this review. A series of excellent reviews related to MS of environmental contaminants have been previously published [158–161].

Table 2
Results with MS technique from *in vitro* and *in vivo* exposoure situation

Exposure	Adducts	Details	Results	Ref.
in vivo	Formaldehyde adducts, crosslinks, NNN-dA and NNN-dT	NNK, NNAL, NDMA, NNN as stable precursors	Implying the potential for these chemicals to act as bidentate carcinogens; pathway of NNN hydroxylation.	[164–166]
in vitro	O^2-POB-dT	In CHO cell lines	Repair enzymes were slower to repair these adducts which could be responsible for associated higher AT to TA transverse mutations	[167]
in vitro	O^6-POB-dG	–	Repair kinetics were 1.5-fold faster for the O^6-POB-dG:T pair than O^6-POB-dG:C pair	[168]
in vivo	7,8-Butanoguanine adducts, mitochondrial adducts	Treated with carcinogens NNK and NDMA	Provided a potential link between nitrosamines and cancer development through identifying preferential adducts distribution as potential new biomarkers	[147,145,169–171]
in vitro	7-EtG, acrolein, 4-ABP, formaldehyde, PhIP	Compare DNA adduct levels between smokers and non-smokers	No significant differences for 7-EtG, acrolein and 4-ABP adducts; No correlation between adduct levels of 4-ABP and smoking habits; formaldehyde adduct levels higher in smokers than non-smokers	[82,76,109,111,172–175]
in vivo	HE DNA adducts, Alkyl-DNA adduct	Alkylation, oxidative damage	Alkyl-DNA adduct levels were observed remaining elevated for 1 day after exposure then gradually decrease, and returned to background levels within 4-days post-exposure	[176,176]
in vivo	N^2-BPDE-dG	Capillary LC-ESI-MS/MS	C-5 methylation on cytosine increases the yields of N^2-BPDE-dG lesions at the base paired guanine mostly by facilitating the formation of pre-covalent intercalative complexes with BPDE	[188]
in vitro	MPdG, MPdA	in liver, lung, and kidney	Probenecid-altered DNA adducts distribution in rat tissues direct inhibition of hepatic anion transport proteins	[189]
in vivo	BPDE-N^2-dGuo	–	The genotoxicity of B[a]P in binary mixtures could be modulated by other PAHs	[190,191]
in vivo	B[*a*]PDE-dGuo, B[*a*]PDE-GSH-adducts	In H358 lung and HepG2 liver cells	A detoxification pathway had been up-regulated rather than an activation pathway	[152]
in vivo	BPDE-N^2-dGuo	Environmental and industrial air samples	Industrial extracts produced more BPDE-N2-dG adducts than strand breaks, whereas the opposite was observed with environmental extracts	[192]
in vitro	HεdC, HεdA, and HεdG	In human autopsy tissues	It provide a prime example of how endogenous adducts can contribute to total adduct levels	[195]
in vivo	N^3-Me-A, N^7-Me-G, O^6-Me-G, dTp(Me)dT, N^7-MeG, O^6-MeG	–	Methylation that can alter DNA which is important in carcinogenesis and mutagenesis, but also a native cellular process for controlling gene expression	[25,207–210,188]
in vitro	DNA–E_2 adducts	–	E_2 undergoes oxidation by P450 1B1 and under aerobic conditions to form quinones that can form DNA adducts *in vitro*	[212]
in vivo	DNA–E_2 adducts	In urine	It proved the formation pathway of DNA adducts from E_2	[213]
in vivo	4-OHE1(E_2)-1-N^3-A, 4-OHE-(E_2)-1-N^7-G, N^3-A, N^7-G	–	It identified the link between synthetic and natural estrogens as quininone metabolites which react with DNA	[214]
in vivo	Estrogen–DNA markers	–	Estrogens undergo the same mechanism of metabolic activation as other weak carcinogens and adduct formation could be modulated	[215–217]
in vitro	AL-dA, AA-dC, Luc-N^2-dG, Luc-N^6-dA	For biomonitoring purposes	It identified the new adducts to understand the chemical and biological mechanisms	[57,58,220,226]
in vivo	Acrylamide-G, A adducts	CHO cells	A correlation was found between these adducts and sister chromatid exchange, suggesting that formation of DNA adducts and the toxicity of acrylamide are related to the processes	[54,221]
in vivo	HAA-DNA adducts	HAA exposure in humans and animals from cooked meat	PhIP in human hair showed an accumulation over time with a correlation with DNA adducts	[230]
in vitro	8-Oxo-dG	In kidney and LC-PUFA, in spleen tissue and 18:2n-6 and n-6 PUFA intake	It is to assess oxidatively-generated DNA damage as a function of diet	[231]

Abbreviations: α-acetates of 4-(methylnitrosamino)-1-(3- pyridyl)-1-butanone (NNK), 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL), N-nitrosodimethylamine (NDMA), N′-nitrosonornicotine (NNN), O^2- pyridyloxobutyl-dT (O^2-POB-dT), Chinese hamster ovary (CHO), 1-sulfooxymethylpyrene (1-SMP), 1-hydroxymethylpyrene (1-HMP), 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), heptone-etheno-dC (HεdC), thymidyl(3′,5′)thymidine (dTp(Me)dT), 17β-estradiol (E2), long chain poly unsaturated fatty acids (LC-PUFA), 2-(1-methylpyrenyl)-2-deoxyguanosine (MPdG), N6-(1-methylpyrenyl)-2-deoxyadenosine (MPdA).

The remainder of this section will discuss recent studies which apply MS for DNA adduct analysis. Due to the need for volatile analytes in GC and the lower sensitivity of CE, the majority of recent studies have been based on LC/MS instrumentation. We will therefore highlight LC/MS applications that have been published from 2007 to 2013. New developments in two of the major classes of adduct forming chemicals are discussed, followed by a summary of two major applications of DNA adduct analysis. Some representative results with MS techniques have been outlined in Table 2.

5.1. DNA adducts of nitrosamines and tobacco genotoxins

Much of the work in this area has been done by Hecht et al. who have promoted understanding of metabolism and reactivity of tobacco specific nitrosamines to elucidate the mechanism of genotoxic chemicals found in tobacco [24,162,163]. Their tool of choice in these studies has been HPLC or capillary LC with ESI-MS/MS to detect and characterize DNA adducts from both *in vitro* and *in vivo* exposures. In these studies, *ct*-DNA and deoxynucleosides have been reacted with carcinogenic nitrosamine metabolites in order to gain insight into the formation of formaldehyde adducts [164]. α-Acetates of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL), and N-nitrosodimethylamine (NDMA), and N′-nitrosonornicotine (NNN) [164–166] were used as stable precursors for their metabolites formed *in vivo*. Significantly, in addition to previously identified formaldehyde adducts, crosslinks were also identified, implying the potential for these chemicals to act as bidentate

carcinogens. dA and dT adducts resulting from interaction with NNN were also characterized for the first time, providing insight into the pathway of NNN hydroxylation and further evidence of its genotoxic potential.

Further genotoxic evidence for NNK was reported in an *in vitro* study conducted by Li et al. [167] in Chinese hamster ovary (CHO) cell lines. The authors measured O^2-pyridyloxobutyl-dT (O^2-POB-dT) adducts by LC–MS/MS and found that the repair enzymes were slower to repair these adducts which could be responsible for associated higher AT to TA transverse mutations. Kotandeniya et al. [168] further evaluated the influence of adduct positioning within the DNA duplex on the ability of enzymes to repair O^6-POB-dG lesions and found the repair kinetics were 1.5-fold faster for the O^6-POB-dG:T pair than O^6-POB-dG:C pair. These fundamental studies aided in understanding the susceptibility of DNA to sustained damage by formation of DNA adducts.

Following their *in vitro* studies, Wang et al. [147] was the first to report evidence of formaldehyde adducts in rats treated with carcinogens NNK and NDMA formed by intermediates such as cyclic oxoniums in the NNK→NNAL pathway. These *in vivo* studies have led to the report of new adducts such as 7,8-butanoguanine adduct 2-amino-6,7,8,9-tetrahydro-9-hydroxypyrido[2,1–f]purine-4(3H)-one from NPYR exposure [145]; the finding that mitochondrial adducts steadily accumulate and are not well repaired in rats treated with NNK and NNAL [169]; and adduct concentration peak times after exposure [170,171]. Collectively, these studies provided a potential link between nitrosamines and cancer development through identifying preferential adduct distribution as potential new biomarkers for biomonitoring studies.

Other recent studies utilizing MS detection have been conducted to compare DNA adduct levels between smokers and non-smokers in order to evaluate the dose-response relationship of tobacco carcinogens. Potential biomarkers included 7-EtG (by a potential ethylating agent in cigarette smoke) [76,172], acrolein [173,174], 4-ABP [109,111], formaldehyde [175], and PhIP [82]. No significant differences were observed between adduct levels from smokers and non-smokers for 7-EtG, acrolein and 4-ABP adducts. Consequently it was assumed that defense mechanisms such as glutathione conjugation protected leukocytes from DNA damage by acrolein and cigarette smoke. No correlation was found between adduct levels of 4-ABP and smoking habits [109,111], however, formaldehyde adduct levels were found to be higher in smokers than non-smokers, 179 ± 205 fmol/μmol dA in smokers compared to 15.5 ± 33.8 fmol per μmol dA in non-smokers, indicating a role of formaldehyde in smoking induced cancer [175].

LIT-multistage-MS was previously described to be a useful tool in identifying adducts of tobacco smoke [82]. With this tool, tobacco-specific adducts and cooked meat carcinogens were measured in human saliva of smokers and non-smokers. PhIP was found to be the most significant adduct forming chemical and therefore a good potential biomarker (Fig. 7).

It was reported that HE lesions and HE adducts were formed from ethoxyacetaldehyde (EA) [176]. Significantly, the adduct profile for HE lesions derived from α-hydroxynitrosomorpholine was found to be different from damage profiles previously reported for α-substituted cyclic nitrosamines. In addition to alkylation, N-nitrosoalkylamines were found to cause oxidative damage to DNA *in vivo* [177]. Alkyl-DNA adduct levels were observed remaining elevated for 1 day after exposure then gradually decrease, while 8-oxo-dG was rapidly excised from DNA. The levels of alkyl-DNA eventually returned to background levels within 4-days post-exposure which provided information potentially useful for estimating initial exposure levels. The results on DNA adducts of nitrosamines and tobacco genotoxins achieved with MS techniques have been summarized in Table 2.

5.2. Advances in PAH–DNA adduct biomarkers

PAHs are well-known environmental pollutants and many studies have focused on analyzing DNA adducts derived from PAH exposure. Recent reports range in directions from the investigation of mechanisms of adducts formation and discovery of new biomarkers to development of sensitive quantitation methods. Some recent examples of MS applications summarized in Table 2 are as follows: the mechanism of adduct formation in naphthalene carcinogenesis, investigated by Cavalieri et al. who used UHPLC–tandem-MS to determine a 1,4-Michael addition reaction mechanism for N^7-G and N^3-A adduct formation from 1,2-naphthoquinone (1,2-NQ) or 1,2-dihydroxynaphthalene (1,2-DHN) exposure [178]; decomposition kinetics, where Chiron *et al.* measured dA and dG etheno-adducts of 1,3-dinitropyrene and 1,4-dinitrophenol [179]; repair of 1-sulfooxymethylpyrene adducts [180]; and internal isotope standardization for quantitation of B[a]P-G adducts (LOD of 239 fmol for B[a]P-N^7-G) [181].

MS has also been used in the determination of PAH–DNA adducts for dose-response studies in animal models including: 2-acetylaminofluorene adducts and gender specific epigenetic changes [182]; 2,7-dinitrofluorene (2,7-DiNF) and 9-oxo-2,7-DiNF adducts in mammary glands as potentially-causative agents of breast cancer [183]; exposure monitoring using 1-hydroxypyrene and 8-oxo-dG biomarkers in the urine [153]; (±)-*anti*-DB[a,l]PDE-dA adducts in oral tissues of mice treated with dibenzo[a,l]pyrene [184]; and B[a]P adducts in mouse sperm for monitoring occupational exposure to PAHs [185].

5-methylcytosine (MeCyt) is an important endogenous DNA modification that plays a central role in epigenetic regulation, chromatin structure and DNA repair [186] and the metabolites of PAHs such as B[a]P, BPDE and other diol epoxides. These are produced upon bioactivation of PAHs present in tobacco smoke that exhibit an enhanced reactivity towards the N^2-position of guanine in MeCyt:G base pairs [187]. Therefore, an important application of PAH–DNA adducts was on the investigation of the effect of DNA regulation on DNA adduct formation. Guza et al. analyzed oligonucleotides containing C-5 alkylcytosines by capillary LC–ESI-MS/MS to determine the effect of cytosine methylation (MeCyt) on adduct formation by BPDE [188]. The authors found that C-5 alkylcytosines and related structural analogs specifically enhanced the reactivity of the base-paired guanine towards BPDE and modified the diastereomeric composition of N^2-BPDE-dG adducts. Significantly, it was found that endogenous systems using regulatory cytosine methylation also produced an effect on susceptibility of the regulated DNA sequence to BPDE adduct formation. The data from this study aided in elucidating how BPDE adduct interactions were modified by MeCyt because the relative reactivity of guanine towards BPDE increases as the size of the C-5 alkyl group at the base paired cytosine is increased in the alkyl series. As a result, C-5 methylation on cytosine increased the yields of N^2-BPDE-dG lesions at the base paired guanine mostly by facilitating the formation of pre-covalent intercalative complexes with BPDE.

Another important application of MS for DNA adduct analysis was determining the influence of co-exposures and mixtures on DNA adduct formation. This is especially relevant for proper risk assessment of chemical hazards as environmental pollutants such as PAHs are usually present in mixtures. A fundamental study was conducted to measure DNA adducts in animals co-treated with 1-sulfooxymethylpyrene (1-SMP) or 1-hydroxymethylpyrene (1-HMP) and probenecid with the ^{32}P-postlabeling and LC–MS/MS MRM methods [189]. The results suggested that probenecid altered DNA adduct distribution in rat tissues including kidney, liver, plasma and hepatic tissue through direct inhibition of hepatic anion transport proteins, which mediated biliary excretion of 1-SMP in renal tubules and from the liver into the bile. Tarantini

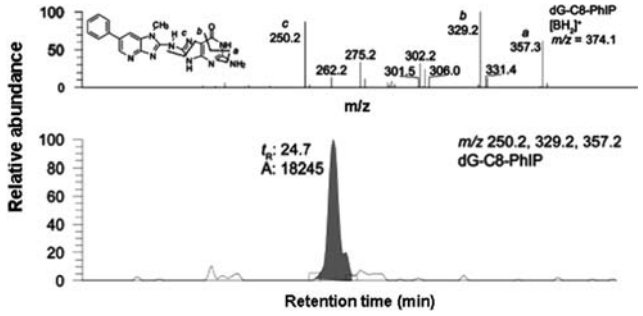


Fig. 7. Representative MS^3 product ion spectra (top) and reconstructed LC-ESI/MS/MS3 ion chromatogram (bottom) of dG-C8-PhIP modified from Bessette et al. [82]. Reprinted with permission from the American Chemical Society.

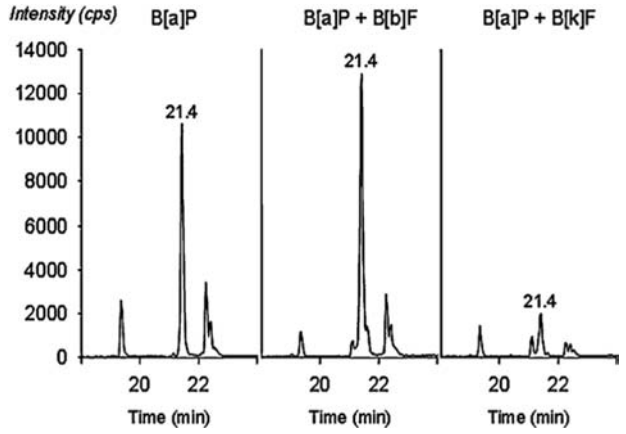


Fig. 8. HPLC-tandem-MS detection of BPDE-N^2-dGuo DNA adducts in HepG2 hepatocytes exposed to B[a]P alone or in binary mixtures with B[b]F or B[k]F at equimolar where the left panel represents the single exposure control. The chromatograms represent the total ion current of the three monitored fragmentations. The retention time was 21.4 min. Reprinted from Tarantini et al. [191] with permission from Elsevier.

et al. used LC–MS to measure B[a]P adducts formed from exposure to pure B[a]P and B[a]P in an environmental mixture and found a 6-fold enhancement in the number of adducts when exposed to the environmental mixture [157]. On the contrary, chrysoeriol was found to have a chemopreventive effect when co-administered as a binary mixture with B[a]P [190]. Subsequently, binary mixtures with B[a]P were also studied by the same group [191]. Direct competition or differences in the formation kinetics of BPDE-N^2-dGuo DNA adducts were not observed between B[a]P and the studied PAHs, but it was found that the genotoxicity of B[a]P in binary mixtures was modulated by other PAHs, resulting in the most often observation of a potentiation of BPDE-N^2-dGuo adduct formation with exception of B[k]F. As can be seen in Fig. 8, binary mixtures with B[k]F resulted in a concentration-dependent inhibition of adduct formation whereas co-treatment with B[b]F lead to an increase in adduct formation. Similarly, Gelhaus et al. [152] found that when the cells were co-administered with B[a]P and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), B[a]P adducts were increased in human liver cells and decreased in H358 lung cells, suggesting that a detoxification pathway had been up-regulated rather than an activation pathway that had been down-regulated in the cells [152].

Successively, real mixtures, extracted from environmental and industrial air samples, were investigated in a similar manner by the same group to examine the effect of environmental chemical composition on the genotoxicity of PAH mixtures to human hepatocytes [192]. It was found that industrial extracts produced more BPDE-N^2-dG adducts than strand breaks, whereas the opposite was observed with environmental extracts. These studies highlight the importance of evaluating chemical hazard in relation to other possible chemical hazards.

5.3. Discerning external from endogenous adducts

Quantification of endogenous DNA adducts represents an important analytical challenge since endogenous adducts (i) contribute to background DNA adduct levels and (ii) may have the same structure or pathway of DNA adducts resulting from external exposure. Swenberg et al. [5] recently summarized the relationship between DNA adducts from endogenous and exogenous sources and mutagenicity for formaldehyde, vinyl chloride and ethylene oxide. Broadly, the challenge with endogenous DNA adducts can be divided into external chemicals that share an endogenous mechanism, such as oxidation or methylation, or external chemicals that are also produced endogenously, such as estrogen. Endogenous adducts therefore also include chemicals which are products or by-products of metabolism.

DNA adducts resulting from oxidation can be difficult to monitor due to endogenous sources, particularly from LPO which leads to ε-adducts. Efforts have been made to monitor DNA adducts to understand the chemistry of chronic inflammation and LPO [87], detect lesions reflective of inflammation [88] as well as determine background levels of LPO derived adducts [193]. Background levels for ε-adducts were found in humans to be 28.2, 44.1, 8.5 adducts per 10^8 normal bases in placenta and 16.2, 11.1, 8.6 per 10^8 normal bases in blood for adducts 1,N^6-etheno-2'-deoxyadenosine (εdA), 3,N^4-etheno-2'-deoxycytidine (εdC), and 1,N^2-etheno-2′-deoxyguanosine (1,N^2-εdG) respectively [194]. LC–MS has been used to perform adductome analysis to measure adduct levels of LPO-induced DNA adducts in human autopsy tissues [195]. Adducts heptone-etheno-dC (HεdC), HεdA, and HεdG, known to form from LPO products 4-OHE and 4-ONE *in vitro*, were found to be ubiquitous in all tissues studied (8.6–15 adducts per 10^8 normal bases). The high levels of these adducts signify that they were products of normal human metabolic processes and provide a prime example of how endogenous adducts can contribute to total adduct levels. Establishment of such baseline levels is therefore important in planning exposure studies in order to determine the threshold for a positive response.

Pentachlorophenol (PCP) is a ubiquitous environmental pollutant with broad applications, including as a biocide, wood preservative, insecticide, and disinfectant. A final quinone metabolite of pentachlorophenol is tetrachlorobenzoquinone (Cl_4BQ). Cl4BQ may undergo oxidation to quinone which can react with DNA to form adducts. Recent applications exploring new oxidation adducts as potential biomarkers and endpoints include dG and dC adducts formed from tetrachlorobenzoquinone (Cl_4BQ) (Fig. 9) [196,128].

Other studies have also been conducted to explore the carcinogenicity of vinyl chloride by identifying the 7-(2-oxoethyl)-2′-dG formed from 3,4,7,8-tetrahydro-7-hydroxy-4-oxopteridine-5(6H)-carbaldehyde, an epoxide from N-[2-amino-6-[(2-deoxy-β-D-erythro-pentofuranosyl)amino]-3,4-dihydro-4-oxo-5-pyrimidinyl]-N-(2-oxoethyl)-formamide, an intermediate of vinyl chloride (VC) [197]; dG and GSH adducts of 4HNE [150,198,199]; and S-[1-(N^2-deoxyguanosinyl)methyl]glutathione induced by formaldehyde [200].

Mass spectrometry using labeled compounds for exposure has been applied to assess the contribution of endogenous oxidation processes to formed DNA adducts. This has been discussed in the context of AMS, but can also be performed with other mass analyzers using more common labels. Studies with $^{13}CD_2$-formaldehyde have revealed that formaldehyde can induce N^2-hydroxymethyl-dG mono-adducts and dG-dG cross-links in DNA near

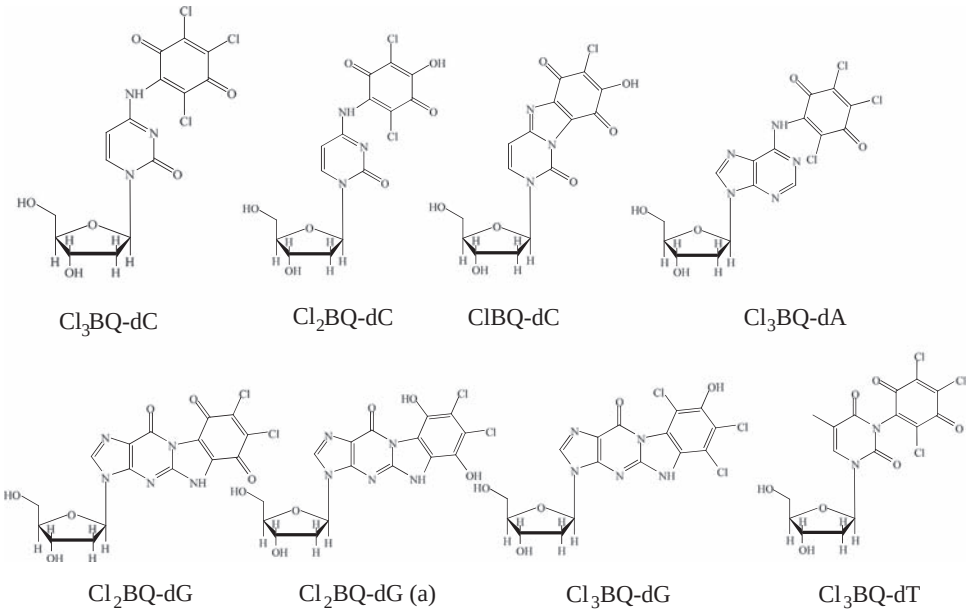


Fig. 9. The structures of DNA adducts formed from tetrachlorobenzoquinone (Cl_4BQ).

the portal of entry and that endogenous formaldehyde-dG and -dA mono-adducts were present in all tissues [201]. The study with $^{13}C_2$-VC revealed that genotoxicity of VC in tissues was from the process of metabolism as opposed to a circulating metabolite [202], and the studies with ^{14}C-labelled EO confirmed previous studies that N^7-HEG [203,204] was not a suitable marker for EO exposure since levels in exposed individuals were consistent with endogenous background levels [50,205,206].

Another major process that can alter DNA is methylation which is important in carcinogenesis and mutagenesis [25], but also a native cellular process for controlling gene expression [207]. Most of the recent applications in this area have been improvement of methodology for lowered detection limits so that low levels of exposure could be evaluated. Chadt et al. developed a LC–MS method for monitoring and screening of N^3-Me-A, N^7-Me-G and O^6-Me-G and achieved an absolute detection limit of 0.1–0.2 ng/ml for adducts [208]. Zhang et al. determined thymidyl(3′,5′)thymidine (dTp(Me)dT) in cultured cells exposed to low levels of N-methyl-N-nitrosourea (MNU) and MMS by a sensitive method with a detection limit of 0.1 ng/mL (6.4 adducts per 10^8 bases) [209]. Pottenger et al. reported a LC–MS method to explore dose-response relationships in mouse lymphoma cells exposed to MMS and MNU and operational thresholds (NOELs – no observed effect levels,), 10 kM and 0.69 kM respectively, by detecting N^7-MeG and O^6-MeG adducts [210]. In addition to adduct levels, it would be of value to be able to measure endogenous methylation levels, since methylation may modulate the extent to which adducts form [188].

Estrogen–DNA adducts can also be endogenously formed due to the rise in exogenous sources of estrogen and have been monitored in breast cancer tissues [211]. An *in vitro* study was conducted to establish a direct connection between the parent hormone (17β)-estra-1,3,5(10)-triene-3,17-diol (E_2) and DNA adduct formation. The results indicated that E_2 underwent oxidation by P450 1B1 (CYP1B1) and under aerobic conditions to form quinones that were able to form DNA adducts *in vitro* [212]. This formation pathway of DNA adducts from E_2 has been proved by the indemnification of DNA–E_2 adducts in urine in an *in vivo* study [213]. In another study, Saeedet al. [214] showed that exogenous synthetic estrogens diethylstilbestrol (DES) and hexestrol (HES) formed depurinating DNA adducts, 4-OHE1(E_2)-1-N^3-A and 4-OHE-(E_2)-1-N^7-G, as well as N^3-A and N^7-G adducts. Importantly, this study identified the link between synthetic and natural estrogens as quinone metabolites which reacted with DNA. Zahid et al. further showed that adduct formation could be modulated since co-exposure with resveratrol was able to protect MCF-10 breast cells from carcinogenic estrogen metabolites [215]. Estrogens undergo the same mechanism of metabolic activation as other weak carcinogens, such as naphthalene in PAHs to their oxidized quinones [216], which may explain why the same types of adducts formed by the quinones of both chemicals were often observed. Estrogen–DNA markers are further available in urine as well as blood and tissue for exposure and biomonitoring purposes. 4-OHE_2-7-G, 8-oxo-dG and a formamidopyrimidine analogue have been monitored in human urine [217]. Depurinating estrogen–DNA adducts in human urine in the context of breast and other human cancers have been recently reviewed [213]. Some results on endogenous adducts determined with MS techniques have been listed in Table 2.

5.4. Quantitation of DNA adducts for exposure monitoring and surveillance

Another important area of application of the measurement of DNA adducts is for monitoring exposure in individuals potentially at risk through *e.g.* occupational exposure, as well as for routine surveillance such as contaminants in food. Dietary sources of genotoxicants may come from the accidental ingestion of harmful toxicants, residues from drugs or food packaging, or result from food preparation, such as those formed during the cooking of meat [11,218]. Although many studies have been done *in vivo* for biomonitoring purposes, there have been some studies *in vitro* in order to discover or establish appropriate biomarkers and to understand the chemical and biological mechanisms. Some representative examples on quantitation of DNA adducts for exposure monitoring and surveillance with MS techniques have been outlined in Table 2. Two recent examples are the application of synthesized oligonucleotides. A QTOF-MS system was used to determine the adducting site of aristolochic acid (AA) and sequence specificity of the adduction and identity of a new dC adduct [57,58]. The same system was also used to determine AL-dA adducts in mammalian cells and the adenine was found the

principal DNA base subjected to electrophilic attack by metabolically activated aristolochic acid [219]. Another example is to use the MS to identify two adducts, Luc-N^2-dG and Luc-N^6-dA, which could serve as markers for lucidin-3-O-primeveroside (LuP) commonly used in dyes and food, and therefore to elucidate the biological mechanisms underlying the carcinogenicity of the components of madder root (MR) [220]. The metabolism of LuP was found to generate genotoxic compounds such as lucidin (Luc) and rubiadin (Rub) [220].

Acrylamide, a process-induced chemical in food, has been recently studied due to concerns about its health effects. Inagakiet al. employed a LC-ESI-TOF-MS system to screen for new adducts of acrylamide [54]. In this study, several new dG adducts, 7GA-Gua and "product A", were identified. With the help of a LC–ESI-MS/MS system, Gasper et al. observed depurinating adducts of G and A adducts when CHO cells were exposed to acrylamide and glyceraldehyde (GA) [221]. A correlation was found between these adducts and sister chromatid exchange, suggesting that formation of DNA adducts and the toxicity of acrylamide were related to the processes.

Food chemicals that have been under recent study for their potential impact on human health include ethanol and HAAs from cooked meats [7,222]. Noteworthy, in the context of DNA adducts from food exposures, it was found that food itself could be a potential source of damaged DNA nucleosides [223]. This finding emphasizes the importance of measuring the background and the exposure levels. In a recent study, MS was used to identify DNA adducts from AA, a carcinogen found in some herbs [59]. Other DNA adducts were reported from food sources, including pyrrolizidine [224,225], estragole [226] *trans*-2-hexenal [227], and alcohol [228,229]. As for HAA exposure in humans and animals from cooked meat, Bessette et al. [230] found that among those HAA-DNA adducts identified, only PhIP in human hair showed an accumulation over time with a correlation with DNA adducts and therefore could be used as a non-invasive biomarker for human exposure.

In addition, apart from DNA adducts formed by reaction with ingested chemicals, it is worth noting that LC–MS can also be used to assess oxidatively-generated DNA damage as a function of diet. For rainbow trout fed different lipid sources, a correlation was found between 8-oxo-dG in kidney and long chain poly unsaturated fatty acids (LC-PUFA) as well as between εdA levels in spleen tissue and 18:2n-6 and n-6 PUFA intake [231]. These adducts are also formed endogenously by LPO in normal metabolic processes. This example further emphasizes the challenges in identification and quantitation of exogenous DNA adducts from endogenous DNA adducts.

For assessing exposure, MS has predominantly been applied to identify potential biomarkers and to measure levels of adducts in exposed cells, animals or individuals. Recent exposure markers that have been identified by MS include gluteraldehyde adducts [232]; N^7-[2-[(2-hydroxyethyl)thio]-ethyl]guanine (HETEG) as a biomarker for exposure to sulfur mustard (mustard gas) [233]; N^7-hydroxypropylguanine (N^7-HPGua) from inhalation of polypropylene [234]; ochratoxin A-dG adducts [235]; identification of major dG adducts of brevetoxin in rats [236]; *S*-[2-(N^7-guanyl)-ethyl]glutathione–DNA adducts channel catfish (*Ictalurus punctatus*) exposed to ethylene dichloride [237]; and adducts of isoprene [2-ethenyl-2-methyloxirane (IP-1,2-O) and propen-2-yloxirane (IP-3,4-O)], *R,S*-C1-N^6-dA, *R*-C2-N^6-dA,and *S*-C2-N^6-dA; adducts of IP-3,4-O are *S*-C3-N^6-dA, *R*-C3-N^6-dA, *R,S*-C4-N^6-dA, *S*-C4-N^1-dI, *R*-C4-N^1-dI, *R*-C3-N^1-dI, *S*-C3-N^1-dI, and C3-N^7-A [10].

One occupational hazard that has been studied in great detail is butadiene (BD), an air pollutant with industrial and domestic sources that poses specific hazard to factory workers [123]. BD can form a number of metabolites and at least five BD related DNA adducts have been identified including 1,4-bis-(guan-7-yl)-2,3,-butanediol (bis-N7G-BD), 1-(guan-7-yl)-4-(aden-1-yl)-2,3-butanediol (N7G-N1A-BD), 1,N^6-(2-hydroxy-3-hydroxymethylpropan-1,3-diyl)-2′-deoxyadenosine (1,N^6-γ-HMHP-dA), 1-(hypoxanth-1-yl)-4-(guan-7-yl)-2,3-butanediol (N1HX-N7G-DB) and 1,N^6-(1-hydroxymethyl-2-hydroxypropan-1,3-diyl)-2′-deoxyadenosine (1,N^6-R-HMHP-dA) [123,238–243]. These studies have provided mechanistic information for elucidating the formation of the identified adducts, uncovered unique markers for quantitatively monitoring BD exposure [124,241], and confirmed a correlation between BD exposure and BD-DNA adducts levels in test animals [239].

5.5. Identification of biological consequences of DNA adducts

In addition to identifying DNA adduct structures and quantifying their levels in biological samples for assessing human exposure to toxic contaminants in the environment, another important application of mass spectrometry is to determine the biological consequences of DNA damage. For example, mass spectrometry has been recently used to assess how DNA lesions compromise replication and transcription *in vitro* and *in vivo* and to investigate DNA repair mechanisms and adduct persistence in tissues. Recently, Tretyakovaet al. has comprehensively reviewed major applications on accurate sequencing primer extension products, the determination of the DNA site-specific mutagenesis and DNA adduct repair *in vitro* and *in vivo* with mass spectrometry-based methods [244].

Since the focus of this review is on advances in detection of adducted and modified DNA bases by mass spectrometry, this section will, therefore only briefly review the applications of mass spectrometry on the biological consequences of DNA adducts with representative examples. N-nitroso compounds (NOCs) are usually present in many sources such as air, food, and tobacco smoke at a low level. Many of these NOCs are known carcinogens and can result in DNA alkylation after metabolic activation. Alkylation at nitrogen atoms and at oxygen atoms on nucleobases has been shown to induce difference biological consequences. It was reported that the former alkylation can induce transversions, frameshift mutations, and small deletions, while the later alkylation primarily produces point mutations by Andersen *et al.* [245]. Therefore, in order to understand how DNA lesions compromise DNA replication *in vitro*, the authors developed a liquid chromatography-tandem mass spectrometry method to analyze primer extension products, 7-mer unextended primer d(AATTCTC), the +1 products, the 10-mer deletion product, and the full-length extension products. O^2- and O^4-methylthymidine (O^2-MdT and O^4-MdT) induced in tissues of laboratory animals exposed with N-methyl-N-nitrosourea were found to be poorly repaired and likely contributed to the mutations arising from exposure to DNA methylating agents. The study results revealed that the exonuclease-free Klenow fragment of *Escherichia coli* DNA polymerase I (Kf^-) and *Saccharomyces cerevisiae* DNA polymerase η (pol η) preferentially incorporated dAMP opposite O^2-MdT, while O^4-MdT primarily directed dGMP misincorporation. As a result, human DNA polymerase κ (pol κ) was demonstrated to favorably incorporate the incorrect dGMP opposite both lesions. In another study, You et al. used a LC–MS/MS method to identify the transcription products of S-cdA and S-cdG to investigate how endogenous and exogenous DNA damage compromised transcription in cells [246]. Mass spectrometry provided an accurate assessment of transcriptional mutagenesis occurring at or near the lesion site through identification of mutant transcripts *in vitro* and in mammalian cells. The authors demonstrated that lesion 8,5′-cyclo-2′-deoxyadenosine (cdA) and lesion 8,5′-cyclo-2′-deoxyguanosine (cdG), but not N^2-(1-carboxyethyl)-2′-deoxyguanosine (N^2-CEdG), induced transcriptional mutagenesis *in vitro* and *in vivo* and all

examined lesions were primarily repaired by transcription-coupled nucleotide excision repair in mammalian cells when located on the template DNA strand. Subsequently, the same group used the same mass spectrometry technique to identify the transcription products of 6-thioguanine (^{S}G) and S^{6}-methylthioguanine (S^{6}mG) *in vitro* and in human cells [247]. Through monitoring the fragmentation of the $[M–3H]^{3-}$ ions of the complementary 14-mer fragments (d(GCAAAMCTAGAGCT) (M=A, T, C, or G), the authors confirmed the identity of mutant transcript and also found that only the wild-type sequence (d(GCAAAGCTAGAGCT)) could be detected in the restriction mixtures arising from the *in vitro* transcription of ^{S}G-containing substrates, suggesting that S^{6}mG was a possible contributor of thiopurine-mediated cytotoxicity. Fig. 10 is an example diagram of MS/MS fragment ions for identification of DNA extension products.

LC–MS/MS can also be used to sequence the *in vitro* replication bypass and extension products [248,249]. For example, Maddukuri et al. analyzed hPol κ-tatalyzed *in vitro* replication products on unmodified DNA and the bypass of M$_1$dG-modified DNA using a LC–MS/MS sequencing method [248]. It was verified that hPol κ extended template-primers in the order M$_1$dG:dC > M$_1$dG:dG > M$_1$dG:dT ~ M$_1$dG:dA but neither hPol ι nor Rev1 extended M$_1$dG-containing template-primers in the 3′-GXC-5′ template sequence. Christovet al. [249] used a LC–ESI-MS/MS sequencing method to analyze the bypass and extension of 7-(2-oxoheptyl)-ε-dGuo lesion in the 5′TXG-3′ and 5′CXG-3′ templates. With the aid of this LC–MS/MS method, two major extension products from translesions synthesis of the 1,N^{2}-ε-dGuo lesion derived from the misinsertion of Ade and a one-base deletion were identified and sequenced. The results of this study suggested that the products from replication of the etheno lesions in a 5-CXG-3′ local sequence context were the result of misinsertion of Ade, a one-base deletion, and error-free bypass.

Interstrand cross-links (ICLs) are highly toxic DNA lesions that block transcription and replication by preventing strand separation, which is very important to understand the biological consequences of environmental carcinogens on DNA damages and DAN mutations. More recently, Liu and Wang [91] utilized a LC–MS/MS method together with isotope dilution technique to assess the repair of 8-methoxypsoralen (8-MOP)-induced DNA ICLs, as well as monoadducts (MAs), in cultured mammalian cells. DNA was isolated from the cell lysate and then digested with nuclease P1 for LC–MS/MS analysis. After 8-MOP/UVA treatment, the levels of ICL and MAs in repair-competent cells were found substantially decreased, but little repair of 8-MOP-ICLs and -MAs in xeroderma pigmentosum was observed. The 8-MOP photoadducts were proved by the substrates for nucleotide excision repair in mammalian cells. In another study, Kirkali et al. wanted to find the evidence for upregulated repair of oxidatively induced DNA damage in human colorectal cancer [250]. They applied a gas chromatography/isotope-dilution mass spectrometry method to measure the levels of oxidatively induced DNA lesions including 4,6-diamino-5-formamidopyrimidine (FapyAde), 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyGua), and 8-hydroxyguanine (8-OH-Gua) and a liquid chromatography/isotope-dilution tandem mass spectrometry method to measure other oxidatively induced DNA lesions including 4,6-diamino-5-formamidopyrimidine, 2,6-diamino-4-hydroxy-5-formamidopyrimidine, 8-hydroxyguanine (5′S)-8,5′-cyclo-2′-deoxyadenosine (S-cdA) and (5′R)-8,5′cyclo-2′-deoxyadenosine (R-cdA). The results strongly suggested that upregulation of DNA repair in malignant colorectal tumors likely contributed to the resistance to therapeutic agents affecting the disease outcome and patient survival.

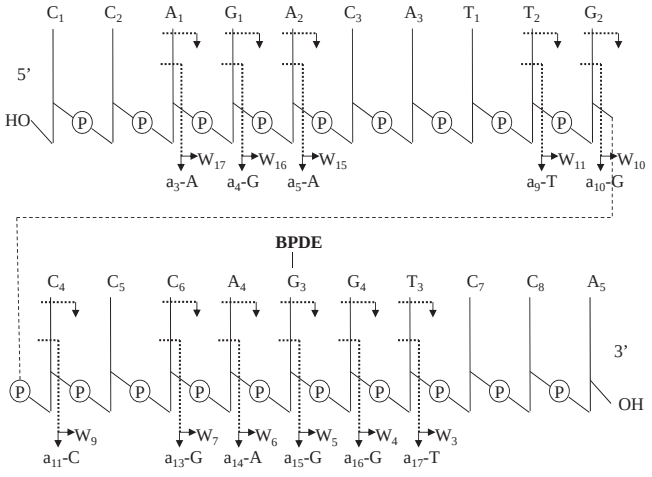


Fig. 10. The typical diagram of MS/MS fragment ions for identification of DNA extension products. W$_n$ and a$_n$-base indicate different fragment ions from the DNA extension products.

6. Conclusions

The increase in use of new MS methods for sensitive and selective DNA-adduct analysis has been made possible by advancements in MS instrumentation and advances in separation techniques and sample preparation. Using MS, one can perform biomonitoring or exposure surveillance for known genotoxins, discover new biomarkers for use in future biomonitoring studies, study the mechanisms behind carcinogenesis, evaluate new chemicals to determine their relative genotoxic hazard and identify biological consequences of DNA adducts. The ability to perform these types of studies has been highlighted in this review. A major roadblock to using MS analysis of DNA adducts in hazard assessment is the lack of a universal regulatory protocol for risk assessment that incorporates DNA adduct data. The absence of such protocol is due, in part, to the need to validate adducts as biomarkers and to link detected DNA adducts to biological outcomes, such as onset of disease. Furthermore, there is a need to establish background values and define detrimental adduct thresholds since low levels of adducts are typically found endogenously. Currently, MS may find its greatest application supporting risk assessment activities by providing rapid access to dose-response data, internal doses of chemicals, biologically effective doses as well as indicators for adverse biological events, such as pro-mutagenic lesions.

Acknowledgement

Dr. Jennilee M. A. Gavina and Dr. Chunhe Yao would like to thank NSERC Canada and Health Canada for financial support in the form of post-doctoral fellowship. The authors acknowledge financial support from the Canadian Government under the Chemicals Management Plan (CMP).

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