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Quantification of modified nucleotides and nucleosides by isotope dilution mass spectrometry

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Abstract

Epigenetic modifications are closely related to certain disorders of the organism, including the development of tumors. One of the main epigenetic modifications is the methylation of DNA cytosines, 5-methyl-2′-deoxycycytidine. Furthermore, 5-mdC can be oxidized to form three new modifications, 5-(hydroxymethyl)-2′-deoxycytidine, 5-formyl-2′-deoxycytidine, and 5-carboxy-2′-deoxycytidine. The coupling of liquid chromatography with tandem mass spectrometry has been widely used for the total determination of methylated DNA cytosines in samples of biological and clinical interest. These methods are based on the measurement of the free compounds (e.g., urine) or after complete hydrolysis of the DNA (e.g., tissues) followed by a preconcentration, derivatization, and/or clean-up step. This review highlights the main advances in the quantification of modified nucleotides

Abbreviations: 1D, one-dimensional; 2D, two-dimensional; 4-APC, 4-(2-(trimethylammonio) ethoxy)benzenaminium halide; 5mdCMP, 5-methyldeoxycytidine monophosphate; 5-aza, 5-aza-2′-deoxycytidine; 5-caC, 5-carboxylcytosine; 5-cadC, 5-carboxyl-2′-deoxycytidine; 5-fC, 5-formylcytosine; 5-fCm, 2′-O-methyl-5-formylcytidine; 5-fdC, 5-formyl-2′-deoxycytidine; 5-fdU, 5-formyl-2′-deoxyuridine; 5-foC, 5-formylcytidine; 5-fU, 5-formyluridine; 5-fUm, 2′-*O*-methyl-5-formyluridine; 5-gmdC, β-glucosyl-5-hydroxymethyl-2′-deoxycytidine; 5-gmdC, β-glucosyl-5-hydroxymethyl-2′-deoxycytidine; 5-hdmU, 5-hydroxymethyl-2′-deoxycytidine; 5-hmC, 5-hydroxymethylcytosine; 5-hmdC, 5-hydroxymethyl-2′-deoxycytidine; 5-hmrC, 5-hydroxymethylcytidine; 5-hmU, 5-hydroxymethyluracil; 5-mC, 5-methylcytosine; 5-mdC, 5-methyl-2′-deoxycytidine; 5-mrC, 5-methylcytidine; 6-mA, N^6-methyl-2′-adenosine; 6-mdA, 6-methyldeoxyadenosine; 8-OHdG, 8-hydroxy-2-deoxyguanosine; 8-OHG, 8-hydroxyguanosine; BDAPE, 2 bromo-1-(4-dimethylamino-phenyl)-ethanone; BEH, bridged ethyl hybrid column; BER, base excision repair; BPAP, 2-bromo-4′-phenylacetophenone; CE, capillary electrophoresis; cfDNA, circulating cell-free DNA; CGIs, cytosine guanine dinucleotide islands; CpG, cytosine guanine dinucleotide; CRC, colorectal cancer; dAMP, 2′-deoxyadenosine monophosphate; dC, 2′-deoxycytidine; dCMP, 2′-deoxycytidine monophosphate; dG, 2′-deoxyguanosine; dGMP, 2′-deoxyguanosine monophosphate; DIPEA, diisopropyl ethylenediamine; DMAP, 4-dimethylaminopyridine; DMPA, *N*,*N*-dimethyl-*p*-phenylenediamine; DNA, deoxyribonucleic acid; DNMT, DNA methyltransferase; DNSH, dansylhydrazine; dU, deoxyuridine; EDC, *N*-(3-(dimethylamino)propyl)-*N*′-ethylcarbodiimide hydrochloride; ESI, electrospray ionization; GC, gas chromatography; gDNA, genomic DNA; HBOT, 1-hydroxybenzotriazole hydrate; HCC, hepatocellular carcinoma; HILIC, hydrophilic-interaction liquid chromatography; HPLC, high-performance liquid chromatography; IBD, inflammatory bowel disease; IDA, isotope dilution analysis; IDMS, isotope dilution mass spectrometry; LC, liquid chromatography; LOD, limit of detection; MARs, matrix attachment regions; MRM, multiple reaction monitoring; MS, mass spectrometry; MS/MS, tandem mass spectrometry; MTBSTFA, N-tert-Butyldimethylsilyl-*N*-methyltrifluoroacetamide; N3-mA, N3-methyladenine; N7-mG, N7-methylguanine; NER, nucleotide excision repair; QqQ, triple quadrupole mass spectrometer; q-TOF, quadrupole-time of flight mass spectrometer; RNA, ribonucleic acid; RP, reverse phase; RSD, relative standard deviation; SPE, solid-phase extraction; SPME, solid-phase microextraction; SRM, selected reaction monitoring; SSc, systemic Sclerosis; TBDMS, tert-butyldimethylsilyl derivative; TBDMSCl, tert-butyldimethylchlorosilane; TET, ten-eleven translocation methylcytosine dioxygenase; TMCS, chlorotrimethylsilane; TMP, thymidine monophosphate; UPLC, ultrahigh-performance liquid chromatography.

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and nucleosides by isotope dilution using isotopically labeled analogs combined with liquid or gas chromatography coupled to mass spectrometry reported in the last 20 years. The different possible sources of labeled compounds are indicated. Special emphasis has been placed on the different types of chromatography commonly used (reverse phase and hydrophilic interaction liquid chromatography) and the derivatization methods developed to enhance chromatographic resolution and ionization efficiency. We have also revised the application of bidimensional chromatography and indicated significant biological and clinical applications of these determinations.

KEYWORDS

gas and liquid chromatography, isotope dilution, mass spectrometry, methylated and modified nucleotides and nucleosides

1 | INTRODUCTION

Epigenetics is the branch of biology that studies how the variations in a chromosome, that do not entail modifications in the underlying DNA sequence, affect the regulatory mechanisms of gene expression. Different epigenetic variations have been described, such as covalent modifications of nucleic acids, posttranslational modification of histone proteins, structural and functional variants of histones, remodeling of chromatin, and RNA-associated changes (Gibney & Nolan, 2010). The methylation of DNA bases is one of the most studied epigenetic mechanisms for regulating gene expression (Heikkinen et al., 2022). It occurs naturally according to given patterns and it is essential for the correct development of the organisms. However, it can be easily dysregulated by the effect of some external factors such as the diet, lifestyle, or the environment (Jaenisch & Bird, 2003). The aberrant variations in the DNA methylation patterns are known to play an important role in aging (Pal & Tyler, 2016) as well as in the onset and progression of several age-related diseases such as type 2 diabetes (Raciti et al., 2021), cancer (Nebbioso et al., 2018; Wilson et al., 2007), multiple sclerosis (Webb & Guerau-de-Arellano, 2017), autoimmune (Li et al., 2021), and neurodegenerative disorders (Martínez-Iglesias et al., 2020).

Cytosine methylation is the best-known and predominant epigenetic DNA modification in mammals (Dupont et al., 2009), and the most studied, due to its close relation with cancer. It is a reversible covalent chemical modification, which involves the addition of a methyl group to the carbon-5 of a cytosine residue in a cytosine-guanine dinucleotide (CpG) site (de novo methylation) and its maintenance during DNA replication (maintenance methylation) by the action of different DNA methyltransferase (DNMT) enzymes (Liu et al., 2007).

5mC demethylation may occur following an active or passive pathway. Passive demethylation takes place due to the inactivity of the DNMT enzyme involved in the maintenance of the methylated cytosines during the replication, whereas active demethylation is an enzymatic process. Cytosine enzymatic demethylation in mammals is mainly mediated by a ten-eleven translocation methylcytosine dioxygenase (TET) protein that stepwise oxidizes cytosine to 5-hydroxymethylcytosine (5-hmC), 5-formylcytosine (5-fC), and 5-carboxylcytosine (5-caC), with 5-hmC being the most prevalent modification (Fu & He, 2012; Kohli & Zhang, 2013).

In mammals, CpG sites are typically highly methylated (70%–80%) throughout the genome, but the methylation rate significantly decreases when the CpG dinucleotides are in the so-called CpG islands (CGIs) (Li & Tollefsbol, 2021). The CGIs are regions with a high level of CpG dinucleotides located within and close to gene promoter sequences. The hypermethylation of cytosines in the CGIs is typically linked to repression in the transcription and gene silencing, whereas the hypomethylation can potentially lead to the overexpression of genes (Campuzano et al., 2019; Das & Singal, 2004). Both, hypermethylation and hypomethylation of cytosines, may affect the onset and progression of certain diseases. For example, hypermethylation of tumor suppressor genes and hypomethylation of oncogenes are common events in carcinogenesis. However, the methylation levels may vary both, between different types of cancer and for different stages of the disease (Wilson et al., 2007). Consequently, such variations are under continuous investigation in the search of disease biomarkers (Celarain & Tomas-Roig, 2020; Li et al., 2021; Martínez-Iglesias et al., 2020).

Besides cytosine methylation, other DNA modifications have been recently discovered in mammals, but their function is still under study, such as N^6-methyl-2′-

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adenosine (6-mA) or 5-hydroxymethyluracil (5-hmU) (Dai et al., 2021; Fu et al., 2015; Greer et al., 2015; Lyu et al., 2023; Zhang et al., 2015). These new modifications could be also potential biomarkers but first their variations need to be properly assessed.

2 | MASS SPECTROMETRY FOR THE DETERMINATION OF NUCLEIC ACID MODIFICATIONS

Considering the relevance of nucleic acid modifications in DNA and RNA, an increasing interest in the development of reliable analytical methods for their precise and accurate determination has arisen (Chen et al., 2017). The availability of analytical methods for the reliable determination of DNA and RNA modifications has become essential to reveal or better understand their function, being also a very valuable tool for the early detection, prognosis, prevention, and therapy monitoring of cancer as well as other prevalent diseases.

Several methods are reported in the literature to perform global DNA methylation analysis and they have been reviewed from different perspectives (Chen et al., 2019; Dai et al., 2021; Kurdyukov & Bullock, 2016; Lai et al., 2019; Li & Tollefsbol, 2021; Li et al., 2018; Yuan, 2020). In the beginning, this methylation was assessed using high-performance separation technologies (Fisher & Giese, 1988; Havlis et al., 2001; Ramsahoye, 2002) and UV detectors (Berdasco et al., 2009; Guz et al., 2008; Toraño et al., 2012), but it has been replaced by protocols that use mass spectrometry due to the increased selectivity and sensibility that leads to more accurate and reliable identifications and quantifications (Chowdhury et al., 2017).

Mass spectrometric detection is usually performed under positive electrospray ionization (ESI) mode and the analytes can be monitored by multiple reaction monitoring (MRM) or by high-resolution MS using linear ion traps or orbitraps (Cao et al., 2021; Chilakala et al., 2017; Dudley & Bond, 2014; Fu et al., 2015; Guo et al., 2018; Jiang et al., 2017; Quinlivan & Gregory, 2008; Schmid et al., 2015; Tang et al., 2013, 2015; Xie et al., 2022; Ye et al., 2017; Zhang et al., 2012).

Currently, the determination DNA and its modifications is enabled using MS/MS. 5-mC and 5-hmC quantification has been reported using methodological calibrations constructed with standards of increasing amounts of each analyte and the same amount of hydrolyzed cytosine. Then methylation percentages for mC and hmC are obtained by interpolation from the respective linear calibration sources (Fernandez et al., 2018; Le et al., 2011).

High-resolution mass spectrometry (HRMS) can be used to obtain structural information and it has been applied in previous works for different purposes within this field such as the determination of DNA modifications (5-fC and 5-caC) in the DNA of mice (Ito et al., 2011; Pfaffeneder et al., 2011), the simultaneous detection of 5-mC, 5-hmC, 5-fC, and 5-caC by quadrupole TOF mass spectrometer to search for potential biomarkers for determining the stage of breast cancer (Guo et al., 2017), the 5-hmC distribution in human liver tumor (Chen et al., 2013), the dysregulation of 5-hmC in kidney cancer tissues using a QTRAP (Chen et al., 2016) and the study of the dysregulation of cytidine modification and its relation to a variety of human diseases by orbitrap (Zhang et al., 2019).

3 | LIQUID CHROMATOGRAPHY (LC) AND ISOTOPE DILUTION MASS SPECTROMETRY (IDMS) FOR THE DETERMINATION OF MODIFIED NUCLEIC ACIDS

3.1 | IDMS

Mass spectrometry for the determination of nucleic acids modifications allow the use of isotope-labeled analogs as internal standards, enabling the development of isotope dilution-based quantification approaches. IDMS is considered as a primary measurement method that provide highly accurate and precise quantifications directly traceable to the International System of Units. Fundamentals and general applications of IDMS for elemental and molecular analysis have been published elsewhere (Garcia Alonso & Rodriguez-Gonzalez, 2013). Such kind of methodologies are highly desirable for an accurate assessment of the function of the different DNA modifications and their use as disease biomarkers.

IDMS is one of the most advanced techniques for the quantitative analysis of DNA modifications and it gives information about the mechanisms and/or the biological importance of these modifications (Olinski et al., 2021). The stable isotopically labeled analogs, commonly ^{2}H, ^{15}N ^{13}C, of each nucleoside could be used as an internal standard for accurate quantifications in relatively short run times providing high sensitivity and selectivity without compromising quality and validation criteria (Foksinski et al., 2017). Some of these isotopically enriched compounds are commercially available whereas most of them must be chemically or biologically synthesized (Burdzy et al., 2002). Table 1 summarizes selected applications of isotopically labeled compounds that have been employed for the determination of native

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TABLE 1 Isotopically labeled compounds employed for the IDMS determination of native and modified nucleotides and/or nucleosides.

Compounds	Labeling isotopes	Source	References
2′-Deoxycytidine	$^{15}N_3$	Commercial (Synthèse AptoChem)	Chilakala et al. (2017)
Adenosine Uracil	$^{15}N_5$ $^{15}N^{13}C_2$	Commercial (Cambridge Isotope Laboratories)	Neubauer et al. (2012)
5-Methyl-2′-deoxycytidine O^6-Methylguanine	$^2H_3$	Commercial (Toronto Research Chemicals)	Hu et al. (2012) Hu et al. (2013)
2′-Deoxycytidine	$^{15}N_3$	Commercial (Cambridge Isotope Laboratories)	
N3-Methyladenine	$^2H_3$		
8-Oxo-7,8-dihydro-2′-deoxyguanosine 2′-deoxyguanosine	$^{15}N_5$		
N7-Methylguanine 8-Oxo-7,8-dihydroguanine	$^{15}N_5$	Synthesized	
5-Methylcytosine	$^2H_3$		
5-Methyl-2-deoxycytidine 5-Hydroxymethylcytosine-—2-deoxycytidine	$^2H_3$	Commercial (Toronto Research Chemicals)	Yin et al. (2015)
Cytidine5′-triphosphate	$^{15}N_3$	Commercial (Sigma-Aldrich)	Fu et al. (2015)
Cytidine	$^{15}N_3$	Synthesized	
Ribose-cytidine	$^{13}C_5$	Commercial (Cambridge Isotope Laboratories)	
5-Methyl-cytidine adenosine 2′-O-methyl-cytidine 2′-O-methyl-adenosine	$^{13}C_5$	Synthesized	
N^6-methyl-2′-adenosine	$^2H_3$	Synthesized	
8-Oxo-2′-deoxyguanosine	$^{15}N_{51}$	Commercial (Cambridge Isotope Laboratories)	Gackowski et al. (2016)
2′-Deoxythymidine	$^{15}N^{13}C$		
2′-Deoxyuridine	$^{15}N^{13}C_2$	Commercial (Medical Isotopes)	
5-(Hydroxymethyl)—2′-deoxycytidine	$^2H_3$	Commercial (Toronto Research Chemicals)	
5-Methyl-2′-deoxycytidine 5-Formyl-2′-deoxycytidine 5-Carboxy-2′-deoxycytidine 5-(Hydroxymethyl)—2′-deoxyuridine	$^{15}N_2{}^{13}C_{10}$	Synthesized	
2′-Deoxyadenosine	$^{15}N_5$	Commercial (Cambridge Isotope Laboratories)	Liu et al. (2017)
5-Methyl-2′-deoxycytidine 5-Hydroxymethyl-2′-deoxycytidine	$^2H_3$	Commercial (Toronto Research Chemicals)	Guo et al. (2018)
5 -Hydroxymethyl-cytidine	$^{13}C^2H_2$		
5-Methyl-cytidine	$^{13}C_5$	Synthesized	
Cytosine	$^{13}C^{15}N_2$	Commercial (Toronto Research Chemicals)	Ye et al. (2017)
Adenine	^{13}C	Commercial (CDN Isotopes)	
Cytosine	$^{13}C_2{}^{15}N_3$	Commercial (Sigma Aldrich)	Rossella et al. (2009)
5-methyl-2′-deoxycytidine	$^2H_3$	Commercial (CDN Isotopes)	
Thymidine 5-methyl-2′-deoxycytidine	^{13}C or $^2H_4$ or $^{15}N_2$	Synthesized	Burdzy et al. (2002)

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TABLE 1 (Continued)

Compounds	Labeling isotopes	Source	References
Deoxyguanosine	$^{15}N_5$	Commercial (Cambridge Isotope Laboratories)	Tsuji et al. (2014)
5-Methyldeoxycytidine 5-hydroxymethyldeoxycytidine	$^{2}H_3$	Commercial (Toronto Research Chemicals)	
8-Hydroxy2′-deoxyguanosine	$^{15}N_5$	Commercial (Cambridge Isotope Laboratories)	Chen et al. (2020)
8-Hydroxyguanosine 8-hydroxy-2-deoxyguanosine	$^{13}C^{15}N_2$	Commercial (Toronto Research Chemicals)	

and modified nucleotides and/or nucleosides by IDMS. As it can be observed, there are several companies that market these compounds isotopically labeled with ^{2}H, ^{13}C, and/or ^{15}N. Compounds doubly labeled on more than one type of atom are also available. Unfortunately, isotopically labeled compounds are often expensive, not commercially available, or very difficult to synthesize, hindering the widespread use of IDMS. Nevertheless, in the case of modified nucleosides, some research has been done to synthetize them. For instance, Gackowski et al. have synthesized 5-formyl-2′-deoxycytidine, 5-carboxy-2′-deoxycytidine and 5-(hydroxymethyl)-2′-deoxyuridine labeled with $^{15}N_2{}^{13}C_{10}$ oxidizing $^{15}N^{13}C$ labeled 5-methyl-2′-deoxycytidine and 2′-deoxythymidine with $Na_2S_2O_8$ (Gackowski et al., 2016). To do that [$^{15}N_2$,$^{13}C_{10}$]-5-mdC was synthesized as described by Divakar and Reese (1982) using [^{15}N–uridine,^{13}C–uridine]-2′-deoxythymidine as a substrate. Chromatographically purified [$^{15}N_2$,$^{13}C_{10}$]–5-mdC and [^{15}N–uridine,^{13}C–uridine]-2′-deoxythymidine (5 mg) were further oxidized with $Na_2S_2O_8$ (25 mg/mL in 0.1 M phosphate buffer pH 7.0) to obtain [$^{15}N_2$,$^{13}C_{10}$]-5-fdC and [$^{15}N_2$,$^{13}C_{10}$]-5-cadC (12 min at 60°C) and [$^{15}N_2$,$^{13}C_{10}$]-5-hmdU (20 min at 60°C), respectively, using the optimized method of Abdel Rahman et al. (2001).

The preparation of isotopically labeled native and modified nucleotides and/or nucleosides can be performed by biosynthesis in an isotopically labeled medium using model organisms in combination with preparative LC. Thus, deoxycytidine and 5-methyl-2′-deoxycitidine labeled with ^{15}N have been obtained from *Escherichia coli* grown in a medium labeled with $^{15}NH_4^+$ (Quinlivan & Gregory, 2008). In our laboratory, we have employed *Chlamydomonas reinhardtii* grown with the same labeling reagent (Carcelén et al., 2017) to prepare a suite of ^{15}N-labeled native and modified nucleosides. After nucleic acid extraction and hydrolysis, preparative LC was employed for the purification of the different nucleosides and, finally, the purified compounds characterized by LC-MS/MS. Table 2 shows the compounds obtained and its characteristics. As it can be observed, highly enriched compounds could be obtained by this procedure (Carcelén, 2023).

TABLE 2 ^{15}N labeled compounds, ion formula, and isotopic enrichments determined by HPLC-MS for the nucleosides synthesized in our laboratory (Carcelén, 2023).

Compound	Molecular formula	Isotopic enrichment (atom%)
Cytidine	$C_9H_{13}{}^{15}N_3O_5$	99.35 ± 0.25
Desoxycytidine	$C_9H_{13}{}^{15}N_3O_4$	99.42 ± 0.35
Uridine	$C_9H_{12}{}^{15}N_2O_6$	99.21 ± 0.10
5-Methyldeoxycytidine	$C_{10}H_{15}{}^{15}N_3O_4$	99.17 ± 0.08
Adenosine	$C_{10}H_{13}{}^{15}N_5O_4$	99.31 ± 0.02
Desoxyadenosine	$C_{10}H_{13}{}^{15}N_5O_3$	99.35 ± 0.08
Guanosine	$C_{10}H_{13}{}^{15}N_5O_5$	99.42 ± 0.02
Desoxyguanosine	$C_{10}H_{13}{}^{15}N_5O_4$	99.18 ± 0.05
Thymidine	$C_{10}H_{14}{}^{15}N_2O_5$	99.50 ± 0.09
N^6-Methyl-2′-adenosina	$C_{11}H_{15}{}^{15}N_5O_4$	99.30 ± 0.02

3.2 | Reversed-phase (RP) LC

Many methods have been developed for the accurate measurement of 5-mC, 5-hmC, 5-fC, and 5-caC (Kellner et al., 2017; Liu, Dunwell, et al., 2013; Liu, Wang, et al., 2013; Romerio et al., 2005; Rossella et al., 2009; Wang et al., 2011; Xie et al., 2018; Yin et al., 2015). Also, metabolic pathways could be studied in vivo using metabolic isotope tracing with LC-MS/MS (Dai et al., 2021; Kellner et al., 2014; Liu et al., 2017).

LC-MS/MS is heavily dependent on the ionization (mostly protonation) of the analytes in solution. It has been observed that DNA hydrolysate buffer and residual coeluted nucleosides might greatly suppress the protonation of 5-hmdC but ammonium bicarbonate can eliminate suppression caused by both factors (Mo et al., 2020).

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Ammonium bicarbonate increases the protonation capacity in the gas phase and facilitates proton transfer to the nucleosides. However, in real complex matrices (e.g., biological or clinical samples), accurate quantitation of modified bases at low concentrations relies on the use of isotopically-labeled internal standards to correct for analyte loses during sample preparation or matrix effects in the analysis of modified bases by mass spectrometry (Hu et al., 2012).

During the past decade, chromatography coupled with MS/MS has played an important role in the identification of the TET-induced oxidation products of cytosine. The use of stable isotope-labeled internal standards will offer unambiguous identification and more accurate measurements of levels of intermediates that are proposed to be involved in the cytosine modifications (Liu et al., 2013).

High-performance LC (HPLC)-based methods have been frequently used as they permit the separation of the different nucleobases, nucleosides, and nucleotides after the DNA hydrolysis (Hu et al., 2013, Rossella et al., 2009, Yin et al., 2016, 2018), being the only technique able to provide true measures of global methylation as originally defined (Vryer & Saffery, 2017). As was mentioned above, HPLC has been used for the quantification of DNA methylation in combination with UV or fluorescence detection (Li & Franke, 2011). However, in the last years, HPLC coupled to MS is the preferred detection technique, as it offers an efficient and reliable quantification of the different DNA base modifications due to its increased sensitivity and selectivity. In addition, when using tandem mass spectrometry (MS/MS), structural information can be also obtained (Dai et al., 2021). HPLC-MS/MS in the selected reaction monitoring mode (SRM) also shows the advantage of providing unequivocal detection and so an accurate quantification of the already known and the emerging DNA modifications. LC−tandem mass spectrometry (LC-MS/MS) has also become a powerful technology that can overcome typical sensitivity and selectivity issues associated to the determination of modified DNA bases (Hu et al., 2012; Yin et al., 2015).

The ultrahigh-performance LC-tandem mass spectrometry (UPLC-MS/MS) technique can be also applied for the accurate determination of the abundance and biological functions of epigenetic DNA modifications. For example, Le et al. (2011) have reported a fast, reliable, and robust method for the simultaneous quantification of 5-hmC and 5-mC in small samples of digested DNA. In this study, only 5 µg of genomic DNA are used. Using reverse phase (RP)-UPLC with MS/MS the analysis time was reduced to 6 min per sample. Liu et al. (2017) reported the development and application of stable isotope-labeled deoxynucleoside $[^{15}N_5]$-2′-deoxyadenosine ($[^{15}N_5]$-dA) as an initiation tracer, combined with RP-UPLC-MS/MS analysis, for accurate and rapid identification and detection of target 6-mdA. Lai et al. (2018) developed a method in which RP-UPLC-MS/MS detection was improved due to the implementation of a vertical-ultracentrifugation that inhibits inorganic salts widely used in DNA digestion. These innovative approaches enable the rapid, sensitive, and robust UPLC-MS/MS determination of methylated DNA, demethylation intermediates, and other DNA modifications. Zhang et al. (2021) developed an IDMS method combined with RP-UPLC-MS/MS for the measurement of epigenetic DNA modifications in the subchromatin structures. In this study, a simultaneous quantification of the 3 analytes of interest is not carried out. On the one hand, they quantify dC and 5-mdC, and on the other hand 5-hmdC. The isotopically labeled analogs used were $^{15}N_3$-dC, $[D_3]$−5-mdC and $[D_3]$−5-hmdC. Hu et al. (2012) reported a sensitive and reliable LC-MS/MS method for the direct and simultaneous determination of 5-mC and 5-mdC with the use of isotopically labeled internal standards (Figure 1).

Tsuji et al. (2014) developed a sensitive RP-UPLC-QqQ method for quantifying 5-hmdC, 5-mdC, and dG levels using isoptopically labeled internal standards and applied the method to estimate the global level of 2 modified cytosines in genomic DNA. In this work, the isotopically labeled compounds chosen were $^{15}N_5$-dG, $[D_3]$-5-mdC, and $[D_3]$-5-hmdC. Chen et al. (2020) developed and validated an accurate and robust solid-phase extraction (SPE) method coupled with RP-UPLC-

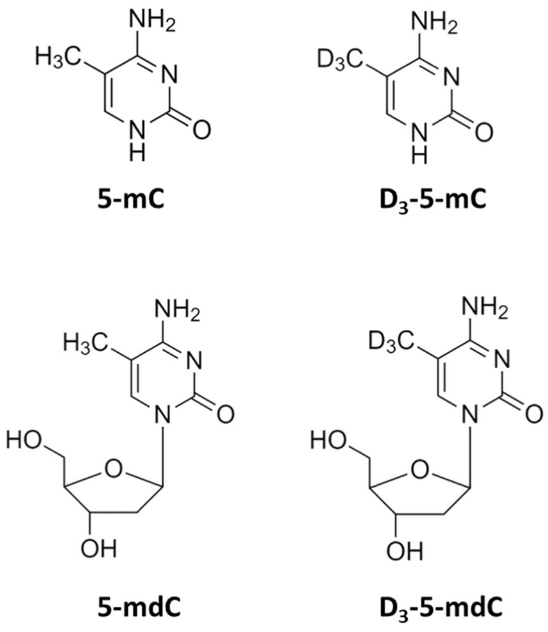

FIGURE 1 Chemical structure of 5-mC and 5-mdC and their corresponding isotopically labeled analogs employed by Hu et al. (2012).

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FIGURE 2 Chemical structures of 8-hydroxy-2′-deoxyguanosine (8-OHdG), 8-hydroxyguanosine (8-OHG), and the corresponding isotope-labeled internal standards employed by Chen et al. (2020).

MS/MS for the simultaneous quantification of 8-hydroxy-2-deoxyguanosine (8-OHdG) and 8-hydroxyguanosine (8-OHG). Quantification was carried out using $[^{15}N_5]$-8-OHdG and $[^{13}C^{15}N_2]$-8-OHG as internal standards. Figure 2 shows the natural and isotopically labeled structures used in this work.

Other studies of interest using RP-HPLC-MS/MS are the work proposed by Liang et al. (2016) to study the regulation of 6-mA in mammalian cells and tissues and the work presented by Wang et al. (2017) where they report a method to evaluate 5-hmdC rapidly, sensitively and specifically in rice with IDMS using $[D_3]$-5-hmdC as labeled analog. Schmid et al. (2015) also developed a method with limits of detection (LOD) in the range of hundreds of attomol for the detection of 5-mdC in commercial synthetic DNA, using RP-HPLC-MS/MS and $[D_3]$-5-mdC as internal standard.

As mentioned above, most of the methods apply a RP-LC separation employing a C18 stationary phase with column lengths from 50 to 250 mm, internal diameters between 2.0 and 4.6 mm, and particle sizes between 1.6 and 5.0 µm.

3.3 | Hydrophilic-interaction LC (HILIC)

However, HILIC has emerged as an alternative to RP owing to its good resolution for polar compounds and the higher compatibility of the mobile phases with the electrospray source. Zhang et al. (2012) developed and validated a HILIC-MS/MS method for the simultaneous determination of cytosine, 5-mC, and 5-hmC levels in biological samples. The total assay time, including hydrolysis and LC-MS/MS analysis, was relatively short, requiring less than 2 h. HILIC was proven to be a suitable option for the determination of cytosine, 5-mC, and 5-hmC. The method was applied to the measurement of these compounds at a low amount (picograms per microgram) of DNA in cerebrum, cerebellum, testis, and liver. Another study using HILIC-MS/MS was reported by Guo, Xie, et al. (2018) who developed a novel malic acid-enhanced HILIC-MS/MS method for sensitive and simultaneous measurement of the modified cytosine nucleosides in human urine. The use of malic acid increased the sensitivity of the method for 5-mdC y 5-hmdC, resulting in LODs of 0.025 fmol for 5-mdC and 5-hmdC (20–40 times lower than those obtained without using malic acid). They successfully quantified 5-mdC y 5-hmdC in urine samples from 90 colorectal cancer (CRC) patients and 90 healthy subjects. Quantitative determination was achieved with a separation time of less than 5 min and isotopically labeled analogs $[D_3]$-5-mdC and $[D_3]$-5-hmdC for IDMS quantifications. Chen et al. (2013) reported the simultaneous detection of 5-mdC and 5-hmdC in genomic DNA by using hydrophilic poly(NAHAM-co-PETA) monolith coupled with high-resolution q-TOF mass spectrometry and an online trapping system to improve sensitivity. They assessed 5-mC and 5-hmC contents in 143 hepatocellular carcinoma (HCC) tissues, which include 75 tumor tissues and 34 matched pairs of tumor and adjacent tissues. With this methodology, they obtained LODs of 0.06 and 0.19 fmol and LOQs of 0.20 and 0.64 fmol for 5-mdC and 5-hmdC, respectively. In addition, Ye et al. (2017) developed a robust method for detecting DNA methylation level over targeted genomic regions using nucleobases quantification in bisulfite amplicons by isotope dilution HILIC-MS/MS. For all HILIC separations, the most common column used was a Bridged Ethyl Hybrid (BEH) with dimensions of (100 mm × 2.1 mm i.d. × 1.7 µm).

4 | CHEMICAL DERIVATIZATION FOR THE DETERMINATION OF MODIFIED NUCLEIC ACIDS BY MASS SPECTROMETRY

4.1 | Derivatization reactions for gas chromatography

Nucleotides, nucleosides, and nucleobases are efficiently and specifically detectable using HPLC-MS. This technique is highly sensitive but, in some cases, the

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chromatographic resolution obtained is not enough to avoid spectral interferences. As an alternative, gas chromatography coupled to mass spectrometry (GC-MS) provides higher chromatographic resolution and, in some cases, better sensitivity due to the lower matrix effect in the electron ionization source while maintaining the information obtained by MS (Romerio et al., 2005). The analytes of interest in this review, nucleotides, nucleosides, and nitrogenous bases are low-volatile compounds. Therefore, their determination by GC requires a previous derivatization step to increase their volatility and thermal stability. Silylating agents are particularly suitable for GC analysis due to their compatibility with the stationary phase of most common capillary columns. MTBSTFA (*N*-tert-butyldimethylsilyl-*N*-methyltrifluoroacetamide) is the preferred derivatization reagent for silylation (Schummer et al., 2009). Rossella et al. (2009) developed a GC/MS procedure for the detection and quantification of total DNA methylation as a ratio of cytosine to 5-mC by derivatizing with MTBSTFA + 1% TMCS. The derivatization process was optimized by adding acetonitrile and pyridine to the derivatizing reagent, increasing the signal by 9–10 times compared to the derivatizing reagent alone. The highest sensitivity was obtained with a mixture of MTBSTFA + 1%TMCS with pyridine (1:1), without acetonitrile. However, in this case, a nonhomogeneous increase was observed for 5-mC and its internal standard [D_3]-5-mC. Accuracy and precision of this method was between 96.7% and 101.2% and the intra- and inter-day RSD were both less than 4%. Romerio et al. (2005) developed a simple, highly selective and sensitive method to quantify DNA methylation extracted from human peripheral blood mononuclear cells with GC/MS. $^{13}C_2$-cytosine and $^{13}C_2$-5-mC were used as internal standards since the quantification of methylation was calculated as a 5-methylcytosine/total cytosine ratio. Each sample, calibration standard, and matrix-added standard were dried under N_2 and derivatized by adding 50 μL of a solution of MTBSTFA + 1% TBDMSCl and acetonitrile (1:1) to form the *tert*-butyldimethylsilyl (TBDMS) derivative. The separation process was achieved in less than 6 min and the calibration curve was linear in the range 1–10 μg/mL for cytosine and in the range 0.05–0.4 μg/mL for 5-mC.

4.2 | Derivatization reactions for LC

Derivatization reactions can be also applied in LC-MS approaches to improve analyte ionization and/or chromatographic retention in RP separations to avoid coelution between target compounds. For example, Tang et al. (2015) developed a selective derivatization of cytosine moieties with 2-bromo-1-(4-dimethylamino-phenyl)-ethanone (BDAPE) coupled with RP-HPLC-ESI-MS/MS for the simultaneous determination of cytosine modifications in genomic DNA. The chemical derivatization notably improved the LC separation due to the hydrophobic phenyl group and increased ionization of 5-mdC, 5-hmdC, 5-fdC, and 5-cadC in the ESI source as the derivatized compound contains an easily chargeable tertiary amine group (Figure 3). The bromoacetonyl group of BDAPE can readily react with the 3-N and 4-N positions of cytosine to form a stable pentacyclic structure.

The derivatization with BDAPE was carried out at 60°C for 6 h with 4 mM of BDAPE using 4 mM of triethylamine as the catalyst, obtaining a yield higher than 99%. The limits of detection (LOD) of 5-mdC, 5-hmdC, 5-fdC, and 5-cadC derivatives were 0.10, 0.06, 0.11, and 0.23 fmol, respectively. Girard's reagents GirD, GirT, and GirP were used to derivatize 5-fdC and 5-cadC to enhance their ionization in the ESI source (Hong & Wang, 2007; Tang et al., 2014). As shown in Figure 4, a hydrazide moiety reacts with aldehydes to give hydrazone derivatives with easily chargeable moieties (quaternary ammonium/pyridinium/tertiary ammonium).

The derivatization must be performed separately for 5-fdC and 5-cadC because the 5-fdC does not react in the presence of 1-chloro-4-methylpyridinium iodide which is an activator of the derivatization of 5-cadC. The derivatization temperature for both compounds was 40°C, derivatization time was 5 min for 5-fdC and 40 min for 5-cadC. The molar ratio of Gird/Analyte was 50/1 for 5-fdC and 150/1 for 5-cadC obtaining derivatization efficiencies of 99% and 95%, respectively.

Table 3 shows the improvement of the detection limits when using Girard's reagents for 5-fdC and 5-cadC.

A similar chemical derivatization process for 5-fdC was used by Jiang et al. (2017) who developed a strategy combining chemical labeling and in-tube SPME-UPLC-ESI-MS/MS analysis for the measurement of DNA and

FIGURE 3 Derivatization with BDAPE (Tang et al., 2015). BDAPE, 2-bromo-1-(4-dimethylamino-phenyl)-ethanone.

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FIGURE 4 Chemical derivatization using Girard's reagents for the determination of 5-fdC and 5-cadC by LC/ESI-MS/MS analysis (Hong & Wang, 2007; Tang et al., 2014). [Color figure can be viewed at wileyonlinelibrary.com]

TABLE 3 Limits of detection (LOD) of 5-fdC and 5-cadC with and without derivatization by Girard's reagents (Tang et al., 2014).

	5-fdC		5-cadC	
Girard's reagent	LOD (fmo)	Sensitivity increase (*x*-fold)	LOD (fmol)	Sensitivity increase (*x*-fold)
None	7.8		103.9	
GirD	0.15	52	0.42	247
GirT	0.09	87	0.77	135
GirP	0.03	260	0.75	139

RNA formylation. Using this method, they simultaneously measured six formylated nucleosides, 5-formyl-2′-deoxycytidine (5-fdC), 5-formylcytidine (5-foC), 5-formyl-2'-deoxyuridine (5-fdU), 5-formyluridine (5-fU), 2′-*O*-methyl-5-formylcytidine (5-fCm), and 2′-*O*-methyl-5-formyluridine (5-fUm), in DNA and RNA of cultured human cells and several mammalian tissues. They used three labeling reagents including GirP, GirT, and 4-(2-(trimethylammonium) ethoxy)benzenaminium halide (4-APC) that carry hydrazide or amine group to label 5fdC and 5foC under mild conditions. The schematic illustration of the analytical procedure is shown in Figure 5.

For the analyte 5fdC, after derivatization the signal increased between 120 and 310-fold, and LODs decreased from 9.2 fmol without derivatization to 0.03 fmol by derivatization with GirP. As in the previous study, the best reagent for 5fdC is GirP, obtaining the same LOD. This result was achieved with the same optimal conditions except for the temperature, which in this case was reduced to 30°C. A strategy has been developed to selectively transfer a glucosyl group to the hydroxymethyl moiety of 5-hmdC to form a more hydrophilic residue (β-glucosyl-5-hydroxymethyl-2′-deoxycytidine, 5-gmdC) by using T4 β-glucosyltransferase (Figure 6A) (Liu et al., 2014, Tang et al., 2013). The more hydrophilic 5-gmdC was enriched by using NH_2-silica via hydrophilic interaction before LC–MS/MS analysis, to avoid matrix effects and improve LOD (Figure 6B).

Using this method, the 5-hmdC content in genomic DNA of three human cell lines and seven yeast strains was quantified. They also evaluated the conversion rate of 5-hmdC to 5-gmdC and the results showed that β-GT can convert 5-hmdC residues to 5-gmdC with almost 100% efficiency. They further assessed the extraction selectivity and efficiency of NH_2-silica toward 5-gmdC by hydrophilic interaction. The recoveries of 5-gmdC were higher than 80% in different amounts of nucleoside mixtures. Good linearities within the range of 5–2000 fmol of 5-gmdC were observed with coefficient values (R) being greater than 0.99. The LOD and LOQ were 1.5 and 5.0 fmol for 5-gmdC and 6.7 and 20.0 fmol for 5-hmdC. In 2016, Zhang et al. (2016) developed a novel strategy by oxidation–derivatization combined with MS analysis for the determination of 5-hmdC and 5-fdC in both DNA and RNA. The strategy employed in this work was the oxidation of the hydroxy methylated group of 5-hmdC to form 5-fdC and subsequent derivatization of both formylates with dansylhydrazine (DNSH) (Figure 7).

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FIGURE 5 The schematic illustration of an analytical procedure based on the combination of chemical labeling and in-tube-SPME-UPLC-ESI-MS/MS analysis for the measurement of DNA and RNA formylation (Jiang et al., 2017). [Color figure can be viewed at wileyonlinelibrary.com]

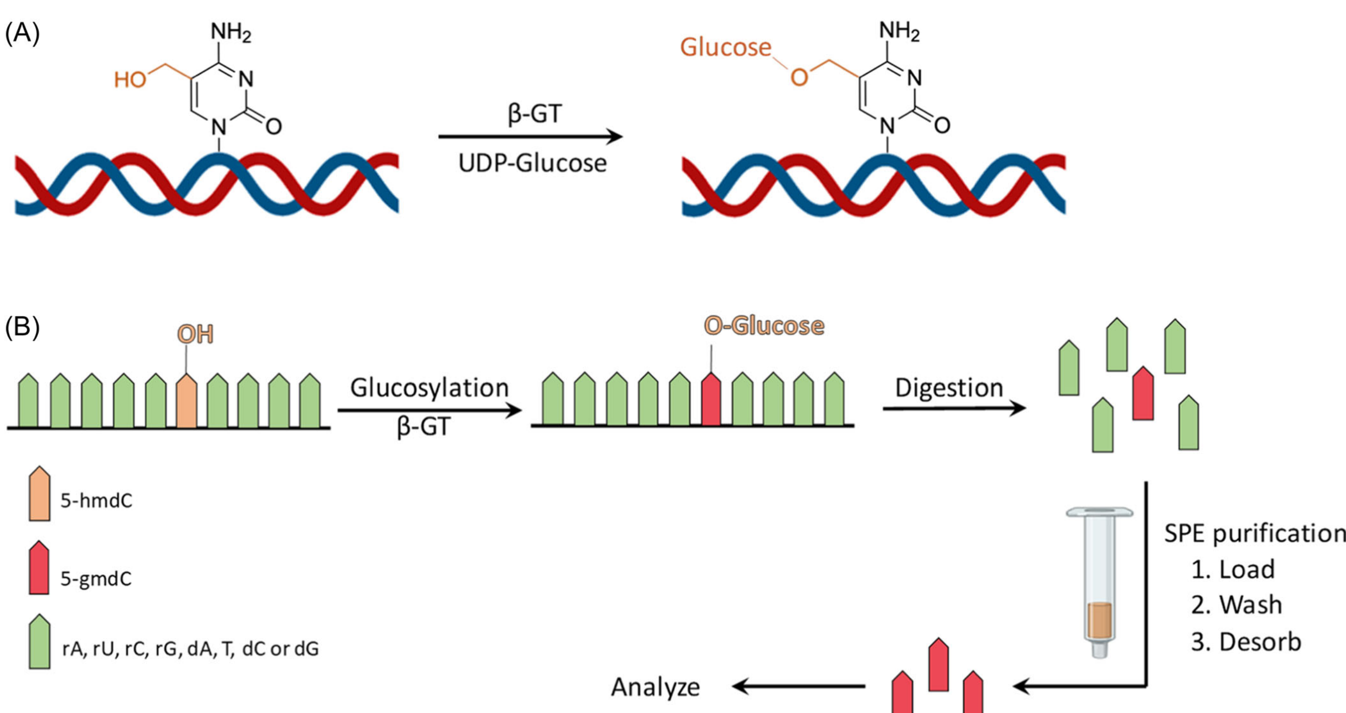

FIGURE 6 (A) Glucosylation of the hydroxyl group of 5-hmdC by β-GT in double-stranded DNA to form 5-gmdC using UDP-glucose as a cofactor. (B) Quantification of 5hmdC content in genomic DNA by SPE-LC-MS/MS (Liu et al., 2014, Tang et al., 2013). [Color figure can be viewed at wileyonlinelibrary.com]

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FIGURE 7 Oxidation step with MnO_2 followed by chemical derivatization with DNSH (Zhang et al., 2016). DNSH, dansylhydrazine.

DNSH contains a hydrazide moiety that can react with aldehyde to give hydrazone derivatives with an easily chargeable moiety (tertiary ammonium). Thus, the ionization efficiencies of target analytes analyzed by LC-ESI-MS/MS were increased. An oxidation efficiency of 99% was obtained at 40°C for 1 h using 40 mg of MnO_2 for 200 pmol of 5-hmdC dissolved in 360 µL of acetonitrile. On the other hand, the optimal conditions for derivatization with DNSH were 40°C during 1 h with a derivatization concentration of 0.4 mM resulting in an efficiency of more than 95%. Finally, it was shown that the LOD for 5-hmdC was reduced by this strategy 363-fold from 14.5 to 0.04 fmol. Guo et al. (2017) developed a RP-HPLC-ESI-TOF methodology with derivatization to quantify 5-mC and its oxidation products in genomic DNA. 4-dimethylamino benzoic anhydride was use as derivatization agent improving the separation and ionization in the ESI source. The 4-(dimethylamino) benzoic anhydride was used to react with the amino group of the cytosine to form an amido bond in the fourth position to add the 4-(dimethylamino) benzoic group to the cytosine. The entire reaction was carried out under the catalysis of DIPEA (diisopropyl ethylenediamine). The introduced 4-(dimethylamino) benzoic group provided a higher retention time of the four cytosine oxidation products on the RP column and hence a higher chromatographic resolution. Moreover, the addition of a dimethylamino group enhances the protonation in mass spectrometry, resulting in increased detection sensitivity. The optimum simultaneous derivatization conditions for 5-mC, 5-hmC, 5-fC, and 5-caC by 4-(dimethylamino) benzoic anhydride were 90°C for 3 h with 20 folds of 4-(dimethylamino) benzoic anhydride using 2.5 µL DIPEA and 6.5 µL of DMAP (4-dimethylaminopyridine) at 1 mg/mL as the catalyst. Using these derivatization conditions, four oxidation products of cytosine were efficiently derivatized (>95%). Their results demonstrated that the derivatization efficiencies did not change despite the content of the four cytosine modifications. The LOD and LOQ of 5-mC, 5-hmC, 5-fC, and 5-cadC were in the range 1.2–2.5 and 3.7–7.6 fmol, respectively. Another study using chemical derivatization to improve ionization and sensitivity in ESI was reported by Yu et al. (2019) who developed an analytical method for the sensitive and accurate quantification of modified cytosines by LC-MS/MS. In this research, several novel hydrazine-based labeling reagents were first evaluated for the chemical derivatization of modified cytosines. Each of the labeling reagents included a hydrazine group, a hydrophobic triazine group, and two easily ionizable tertiary amine groups. The different derivatization reagents tested were Me_2N, Et_2N, and i-Pr_2N. The analyte 5-fC was measured with all three reagents and the best results were obtained with i-Pr_2N, so 5-caC was only measured with this reagent. Optimum conditions for 5-fC are 5 mM of i-Pr_2N for 50 nM of 5-fC, vortexing for 10 s at room temperature achieving a yield of 99%. In the case of 5-caC, it took 30 min at 37°C to react 5 mM i-Pr_2N with 50 nM of 5-caC with the addition of 4 mg/mL HBOT (1-hydroxybenzotriazole hydrate) dissolved in acetonitrile and 50 mg/mL EDC (*N*-(3-(dimethylamino) propyl)-N′-ethylcarbodiimide hydrochloride) dissolved in water. By derivatizing with i-Pr_2N, the LODs decreased from 1.25 to 2.5 fmol for 5-fC and 5-caC to 10 and 25 amol, decreasing 125 and 100 times, respectively. The most recent work published so far using LC-MS/MS combined with chemical derivatization was published by Cao et al. (2021). They used 2-bromo-4′-phenylacetophenone (BPAP) as reagent for the derivatization of dC, 5-mdC, and 5-hmdC. BPAP also reacts with adenine and 6-mA. The optimal reaction conditions were different for cytosine and adenine derivatives. In the case of cytosine, the derivatization was carried out for 4 h at 80°C with 15 mg/mL BPAP and 0.02 µL acetic acid. For adenine derivatives the derivatization was carried out for 11 h at 80°C with 4 mg/mL BPAP and 2 mg/mL triethylamine. The LODs of the dC, 5-mdC, and 5-hmdC were reduced by 6, 2.5, and 5 times, respectively. If nucleotides are to be measured, Zeng et al. (2017) established a method by chemical labeling coupled with

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TABLE 4 Comparison of the limits of detection (LODs) of the BDAPE chemical derivatization method for nucleotides with a previous method.

	LODs (fmol)	
Nucleotide	**Study (Zeng et al. (2017)**	**Previously (Zhang et al. (2011)**
dCMP	0.42	30
5-mdCMP	0.30	-
dAMP	0.13	3
dGMP	0.38	30
TMP	0.13	6

LC−ESI-MS/MS for sensitive and simultaneous determination of 10 nucleotides, including dCMP, 5-mdCMP, dGMP, TMP, dAMP and their RNA analogs. The authors used DMPA (*N*,*N*-dimethyl-*p*-phenylenediamine) that contains a hydrophobic phenyl group and an easily chargeable tertiary amine group to simultaneously label the phosphate group in 10 nucleotides. Consequently, the detection sensitivities of these DMPA-labeled products increased when they are measured by LC−ESI-MS/MS. Moreover, the introduced hydrophobic phenyl group in DMPA increased the retention of these nucleotides on reversed-phase LC and boost the detection sensitivities. DMPA labeling was applied at 50°C for 1.5 h in 1 mM imidazole buffer (pH 6.0), and the molar ratios of DMPA and EDC over nucleotides were set as 40,000 and 5000, respectively. The EDC acted as an activator. Under these optimized reaction conditions, the detection sensitivities of nucleotides increased by 88–372-fold. The LODs obtained for the nucleotides from DNA are shown in Table 4, where they compare with the LODs obtained in a previous publication.

Finally, to summarize, Table 5 shows all the derivatization methods presented in this review together with the target analytes and their limits of detection.

5 | BIDIMENSIONAL LC FOR THE DETERMINATION OF MODIFIED NUCLEIC ACIDS BY IDMS

Advances in chromatographic separation technology have enabled the development of methodologies using two-dimensional (2D) LC for this type of studies. Gackowski et al. (2015) published one of the first methods using a rapid, specific, and sensitive isotope dilution automated online 2D-UPLC-MS/MS to measure 5-mdC, 5-hmdC, 5-fdC and 5-cadC in DNA isolated from various rat and porcine tissues. The chromatographic columns used in both dimensions were C18. At-column dilution technique was employed between the first and second dimension-LC to improve the retention at a trap/transfer column. This system operated in a heart-cutting mode, so selected aliquots of effluent from the first dimension were led to the trap/transfer column using a 6-port valve switch, which served as an “injector” for the second-dimension. With this methodology they were able to conclude that the 5-hmdC was the only modification and depended mostly on the cell proliferation status, irrespective of the species origin. In 2017, using this methodology Foksinski et al. (2017) showed that different cell lines had different epigenetic profiles. Additionally, different types of malignant cells show characteristic profiles of DNA epigenetic marks which differ significantly. Gackowski et al. (2016) applied isotope-dilution automated online 2D-UPLC-MS/MS for direct determination of the 5-mdC, 5-hmdC, 5-fdC, 5-cadC, 5-hmdU, dU, and 8-OHdG in human samples of colorectal carcinoma (CRC) tissue. Although both columns were C18, the number of unmodified nucleosides reaching the MS decreased (lower noise), and narrower peaks were obtained compared to 1D approaches. The sensitivity obtained by 1D- and 2D-UPLC-MS/MS were compared, and, in all cases, 2D chromatography provided higher signal-to-noise ratios for unlabeled and labeled compounds. In the calibration curves, R^2 values higher than 0.99 were obtained for all analytes in the expected concentration range for each analyte. In a different study, Rozalski et al. (2016) developed a specific and sensitive, isotope-dilution, automated, online, 2D-UPLC–MS/MS to measure 5-mdC, 5-hmdC, 5-fdC, 5-cadC, 8-OHdG and 5-hdmU in the same urine sample from healthy subjects and CRC patients. Both columns were C18 and the chromatographic system operated in a heart-cutting mode with trap/transfer column. As usual in 2D-LC, the second column was significantly shorter than the first. With this method, they obtained LODs and LOQs between 0.05 and 3 fmol and 0.13–9 fmol, respectively. A highly significant difference in the urinary excretion of 5-hmdC in healthy subjects and CRC patients was observed in this work.

Dziaman et al. (2018) determined, using isotope-dilution automated online 2D-UPLC-MS/MS, the levels of epigenetic DNA modifications and 8-OHdG in tissues from patients with inflammatory bowel disease (IBD), benign polyps, and CRC. Like previous studies, they reported that 5-hmdC levels in CRC were several times lower than in normal colonic tissues, with the level of this compound in benign polyps and CRC being essentially the same, that is, approximately four times lower than in normal colonic tissues. However, this study shows for the first time that the levels of 5-hmdC in IBD

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TABLE 5 Summary of the derivatizing reagents used in this review for cytosine-related modifications.

Reference	Target	Derivatizing reagent	LOD (fmol)	LOD without derivatization (fmol)
Tang et al. (2015)	5-mdC 5-hmdC 5-fdC 5-cadC	BDAPE	0.10 (5-mdC) 0.06 (5-hmdC) 0.11 (5-fdC) 0.23 (5-cadC)	3.5 (5-mdC) 5.6 (5-hmdC) 9.8 (5-fdC) 28.5 (5-cadC)
Tang et al. (2014)	5-fdC 5-cadC	Girard D Girard T Girard P	0.15, 0.09, 0.03 (5-fdC) 0.42, 0.77, 0.75 (5-cadC)	7.8 (5-fdC) 103.9 (5-cadC)
Jiang et al. (2017)	5-fdC	4-APC Girard T Girard P	0.03 (GirP) 0.08 (GirT) 0.05 (4-APC)	9.2
Tang et al. (2013)	5-hmdC 5-gmdC	T4 β-glucosyltransferase	6.7 (5-hmdC) 1.5 (5-gmdC)	Not given
Zhang et al. (2016)	5-hmdC 5-fdC	DNSH + MnO2	0.04 (5-hmdC) 0.04 (5-fdC)	14.5 (5-hmdC) Not given
Guo et al. (2017)	5-mC 5-hmC 5-fC 5-caC	4-dimethylamino benzoic anhydride	1.2-2.5 for all	Not given
Yu et al. (2019)	5-fC 5-caC	Me2N Et2N i-Pr2N	Me_2N: 0.050 (5-fodC) Et_2N: 0.0125 (5-fodC) i-Pr_2N: 0.010 (5-fodC), 0.025 (5-cadC)	1.25 (5-fC) 2.5 (5-caC)
Cao et al. (2021)	dC 5-mdC 5-hmdC adenine 6-mA	BPAP	0.22 (dC) 0.33 (5-mdC) 0.23 (5-hmdC) 1.48 (adenine) 0.67 (6-mA)	1.32 (dC) 0.83 (5-mdC) 1.15 (5-hmdC) Not given Not given
Zeng et al. (2017)	dCMP 5-mdCMP dAMP dGMP TMP	DMPA	0.42 (dCMP) 0.30 (5-mdCMP) 0.13 (dAMP) 0.38 (dGMP) 0.13 (TMP)	44.2 (dCMP) 32.3 (5-mdCMP) 25.7 (dAMP) 33.6 (dGMP) 48.4 (TMP)

were lower than in normal colonic tissues. Yakovlev et al. (2019) employed a sensitive 2D-UPLC–MS/MS method to examine the levels of noncanonical DNA bases in the buds of Norway spruce (*Picea abies*). As in previous studies, 2D-LC was carried out with two C18 columns in the heart-cutting mode with a trap/transfer column. They measured 5-mdC, 5-hmdC, 5-fdC, 5-cadC, 8-OHdG, dU, and 5-hmdU, obtaining limits of detection between 0.05 and 3 fmol and limits of quantification between 0.13 and 10 fmol. Finally, Dal-Beckar et al. (2022) carried out the first quantification of epigenetic DNA modification products in systemic sclerosis. In this study, 5-mdC, 5-hmdC, 5-fdC, 5-cadC, and 5-hmdU were quantified by 2D-UPLC-MS/MS system in patients with systemic sclerosis (SSc). They showed significantly increased 5-hmdU while lower 5-hmdC in SSc compared to the healthy individuals. The evaluation of epigenetic alterations at the systems level has the potential to elucidate the underlying mechanisms of SSc as well as developing new therapeutic strategies.

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6 | BIOLOGICAL AND CLINICAL APPLICATIONS: SEEKING NEW BIOMARKERS

Due to the loss of DNA methylation enzymes from its genome, the research model organism *Caenorhabditis elegans* was thought to be completely devoid of DNA methylation. However, Hu et al. (2015) first demonstrated that 5-mdC is present in *C. elegans* genomic DNA using LC-MS/MS but 5-hmdC was not detectable. Moreover, it was observed that the DNA of this microorganism was hypo- or hyper-methylated in a dose-dependent manner by the DNA methyltransferase (DNMT)-inhibiting drug decitabine (5-aza-2′-deoxycytidine) or cadmium, respectively.

A relevant discovery was reported by Ito et al. (2011) who demonstrated that Tet proteins can generate 5-fC and 5-caC from 5-mC enzymatically. Also, they revealed the presence of 5-fC and 5-caC in genomic DNA of mouse embryonic stem cells and mouse organs. Furthermore, it was demonstrated that the genomic content of 5-hmC, 5-fC, and 5-caC could be increased or reduced through overexpression or depletion of Tet proteins. This study concluded that DNA demethylation occur through Tet-catalyzed oxidation followed by deglycosylation. Hu et al. (2012) studied the relationship of the active DNA demethylation and base excision repair (BER) and nucleotide excision repair (NER) pathways. The determination of 5-methylcytosine and 5-methyl-2′-deoxycytidine in human urine by isotope dilution LC-MS/MS showed that mean urinary 5-mC and 5-mdC concentrations were 28.4 ± 14.3 and 7.04 ± 7.2 ng/mg creatinine, respectively, supporting the possibility of DNA demethylation through BER and NER pathways. The levels of 5-mC were significantly positively correlated with N7-mG, N3-mA, and 8-OHdG. Therefore, the results indicate a good correlation between 5-mC and oxidized and methylated DNA lesions, which may implicate the underlying link between genetic (DNA lesions) and epigenetic (DNA methylation) alterations arising from exogenous exposure and/or endogenous cellular processes in humans. 5-hmC is an emerging biomarker for disease diagnosis, treatment, and prognosis. By the analysis of 13 volunteers, the presence of 5-hmC in human urine was first demonstrated by Yin et al. (2015). Unexpectedly, it was observed that the level of 5-hmC was comparable to that of its precursor 5-mC in human urine. Since the abundance of 5-hmC (as a rare DNA base) is 1 or 2 orders of magnitude lower than 5-mC in genomic DNA, this finding probably involves a much greater turnover of 5-hmC than 5-mC in mammalian genomic DNA and underscores the significance of DNA demethylation in daily life. Huang et al. (2016) developed a method using chemical labeling and LC-MS for the sensitive and simultaneous determination of 5-mC oxidative products, discovering the presence of 5-caC in the RNA of mammals. Tang et al. (2012, 2013), reported for the first time the presence of 5-hmC in the yeast model organism. Furthermore, the 5-hmC contents in two *Schizosaccharomyces pombe* yeast strains were even higher than those of 5-mC, showing that 5-hmC may play an important role in the physiological functions of yeast.

5-hydroxymethylcytidine was first discovered in human urine employing a new malic acid-enhanced HILIC-MS/MS method (Guo et al., 2018). Monomethylations of cytidine and adenosine are common post-transcriptional modifications in RNA. Results showed that the distributions of 5-methylcytidine, 2′-*O*-methylcytidine, N^6-methyl-2′-adenosine and 2′-*O*-methyladenosine are tissue-specific. Additionally, the concentrations of 2′-*O*-methylated ribonucleosides are higher than the corresponding methylated nucleobase products (5-methylcytidine and N^6-methyladenosine) in total RNA isolated from mouse brain, pancreas, and spleen but not in mouse heart. Also, they found that the levels of 5-methylcytidine, 2′-*O*-methylcytidine, and 2′-*O*-methyladenosine were significantly lower (by 6.5–43-fold) in mRNA than in total RNA isolated from HEK293T cells, whereas the level of N^6-methyl-2′-adenosine was slightly higher (Fu et al., 2015). Liu et al. (2017) used stable isotope-labeled deoxynucleoside [$^{15}N_5$]–2′-deoxyadenosine as an initiation tracer and first developed a metabolically differential tracing code to monitor DNA 6-mdA in human cells. This study concludes that mammalian DNA 6-mdA may be potentially differentiated from that produced by infecting mycoplasmas.

Also, a specific measurement of epigenetic DNA modifications in matrix attachment regions (MARs) has been carried out. By this approach, it was first shown that the 5-hmdC concentration in MARs decreased significantly (41.09%) in four tested cell lines in comparison with the concentration in genomic DNA. This means that MAR DNA is less sensitive than genomic DNA to DNA demethylation (Zhang et al., 2021). Jiang et al. (2017) discovered in cultured human cells and tissues the existence of 5-fU, 5-fCm, and 5-fUm which had not been reported before.

LC-MS methods have been extensively used to quantify the different methylation products in clinical implication studies. Epigenetic status can be assessed noninvasively based on the urinary excretion of a broad spectrum of epigenetic modifications. 2D ultrahigh performance LC with tandem mass spectrometry was carried out to measure epigenetic DNA modifications in urine samples and evaluate the whole-body epigenetic status in healthy subjects and CRC patients. From

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measurements of 5-hmC, 5-fC, 5-caC, and their corresponding 2′-deoxynucleosides, it was concluded that human urine contains all the modifications except from 5-fdC and 5-cadC. Moreover, a highly significant difference in the urinary excretion of 5-hmdC was found between healthy subjects and CRC patients (Rozalski et al., 2016). Furthermore, another study showed that the concentrations of 5-mdC, 5-hmdC, 5-mrC, and 5-hmrC in urine were significantly lower in CRC (Guo et al., 2018). Guo et al. employed human urine of breast cancer patients for the quantification of 5-hmdC, 5-fdC, and 5-cadC. Results demonstrated that the level of 5-hmdC decreased in breast cancer patients, while the levels of 5-fdC and 5-cadC increased, which contribute to the clinical diagnosis. Urinary excretion of 8-oxo-7,8-dihydro-2′-deoxyguanosine and 8-oxo-7,8-dihydroguanine have been proposed as molecular markers of cancer (Roszkowski et al., 2011). More than 200 cancer patients and healthy volunteers were included in the analysis using methodologies which involve HPLC pre-purification followed by GC with isotope dilution mass spectrometry detection. Significantly elevated levels of these compounds excreted in daily urine were observed in cancer patients as compared with healthy subjects, with considerable statistical significance. Lyu et al. (2022) have showed that the generation of misincorporated DNA 6-mA in associated with the cellular stresses-caused release of RNA N^6-methyl-2′-adenine nucleoside. Furthermore, their data support that DNA 6-mA is a hallmark of poor prognosis of isocitrate dehydrogenase mutation-absent glioblastoma patients.

On the other hand, tissues are widely used as samples in disease research studies. In 2005, a method for quantification of 5-mC was described using LC-ESI-MS/MS applying it in the detection of archived tumors due to its sensitive limit of detection (Song et al., 2005). A LC-UPLC-ESI-QTOF method was reported for the accurate quantification of 5-mC levels, along with its oxidation products in tumor tissue and tumor-adjacent normal tissue. In this study, it was concluded that 5-fC and 5-caC are increased in tumor tissue. Furthermore, the levels of 5-mC, 5-hmC, 5-fC, and 5-caC measured in tumor tissue and tumor-adjacent tissue were observed to be different using different classifications, suggesting that cytosine modifiers may be used as potential biomarkers for determining the stage of breast cancer development, as well as prognosis (Guo et al., 2017). Tang et al. (2015) employed human CRC tissues and tumor-adjacent normal tissues for the determination of 5-methylcytosine and its oxidation products. Analyses of DNA extracted showed that the oxidation products were lower in colorectal carcinoma tumor compared to controls. Gackowski et al. (2016) concluded the same analysis using 2D chromatography for the determination of the analytes. The complete automated 2D separation is extremely useful for analysis of samples containing high amounts of coeluting interferents by MS. Jiang et al. (2017) were able to simultaneously measure six formylated nucleosides, including 5-fdC, 5-foC, 5-fdU, 5-fU, 5-fCm, and 5-fUm, from DNA and RNA of cultured human cells and different mammalian tissues. A significant increase of 5-foC and 5-fU in RNA and 5-fdU in DNA in human thyroid carcinoma tissues compared to normal tissues adjacent to the tumor were observed. These results showed that abnormal DNA and RNA formylation can contribute to the generation and development of the tumor. Additionally, the measurement of DNA and RNA formylation can also be used as an indicator for the cancer diagnosis. A nanoflow LC-nanoelectrospray ionization-tandem mass spectrometry and isotope-dilution method were used for the simultaneous measurement of oxidatively induced DNA modifications in a rat model of human Wilson's disease (Yu et al., 2016). The level of 5-hmdC was significantly lower in the liver tissues of the rats used in this model, although no differences were found for the concentrations of 5-mdC. These results indicate that abnormal accumulation of copper can disturb genomic stability by elevating oxidatively induced DNA lesions and by modifying epigenetic pathways of gene regulation. Liu et al. (2019) have demonstrated that global 5-hmC and 5-fC contents were decreased significantly in the very early stage of hepatocellular carcinoma. The decrease of 5-hmC and 5-fC was mainly due to the decrease of 5-mC and associated with hepatitis B virus infection, decreased TET enzyme activity, and uncoordinated expression of DNA methylation-related enzymes.

Also, it is of paramount importance to develop effective and minimal invasive methods for diagnosis and prognosis of cancer using blood samples. CE-ESI-MS was employed to study the genome-wide profiles of 5-hmC in circulating cell-free DNA (cfDNA) and in genomic DNA (gDNA) of matched tumors and adjacent tissues collected from 260 patients diagnosed with colorectal, gastric, pancreatic, liver or thyroid cancer and normal tissues from 90 healthy subjects. The use of 5-hmC as biomarker of circulating cfDNA were much higher predictive of colorectal and gastric cancers than conventional biomarkers from tissue biopsies (Li et al., 2017). The quantitative determination of blood-modified nucleosides in the development of diabetic nephropathy was studied by Guo et al. (2019). Serum samples were collected from 43 healthy volunteers and 156 patients. It was concluded that the 5-mdC/cytosine and 5-mC/cytosine ratios in the diabetic nephropathy group were not statistically different from the healthy volunteers and early renal injury diabetes groups. A GC/MS method for the quantification of total DNA methylation, as cytosine/5-mC ratio, was reported (Rossella

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et al., 2009). The DNA methylation level from peripheral blood leukocytes of healthy subjects, the bone marrow of leukemia patients and from myeloma cell lines was carried out. Median methylations of 5.45, 3.58, and 2.74 were found in DNA from healthy individuals, bone marrow of leukemia patients, and myeloma cell lines, respectively. Therefore, these last two groups were statistically different, according to the analysis of variance test ($p < 0.001$). In a recent article, a systemic investigation of the effects of adolescent alcohol exposure on two DNA and 12 RNA modifications in peripheral blood of rats by LC-ESI-MS/MS have been demonstrated for the first time (Chen et al., 2022).

7 | CONCLUSIONS

The understanding of modified nucleic acids' role in living organisms is one of the cutting-edges of the current science. Tandem mass spectrometry can be considered as the gold standard technique for the determination of modified nucleic acids in biological and clinical samples. However, the complexity of the samples and the variety of the target compounds require efficient separations (mainly reverse phase or HILIC) and adequate internal standardizations for accurate quantifications. The use of isotopically labeled analogs is the best strategy to correct for matrix effects that lead to suppression and/or enhancement of the ESI ionization during the chromatographic runs. Soon, the number of methods based on 2D chromatography are very likely to increase considering the differences in the range of concentrations found among the modified and unmodified nucleosides. In any case, the use of isotopically labeled analogs as internal standards is mandatory to obtain reliable results. Unfortunately, most of the labeled analogs of the target analytes are still not commercially available so much more effort must be spent on the development of (bio) synthesis procedures that ultimately will boost the improvement of the accuracy and precision of modified nucleic acids determinations by mass spectrometry.

REFERENCES

Abdel Rahman AAH, Wada T, Saigo K. Facile methods for the synthesis of 5-formylcytidine. *Tetrahedron Letters*. 2001;*42*:1061–1063.

Berdasco M, Fraga MF, Esteller M. Quantification of global DNA methylation by capillary electrophoresis and mass spectrometry. *Methods in Molecular Biology*. 2009;*507*:23–34.

Burdzy A, Noyes KT, Valinluck V, Sowers LC. Synthesis of stable-isotope enriched 5-methylpyrimidines and their use as probes of base reactivity in DNA. *Nucleic Acids Research*. 2002;*30*:4068–4074.

Campuzano S, Pedrero M, Yánez-Sedeño P, Pingarrón, JM. Advances in electrochemical (bio)sensing targeting epigenetic modifications of nucleic acids. *Electroanalysis*. 2019;*31*:1409–1425.

Cao X, Lintelmann J, Padoan S, Bauer S, Huber A, Mudan A, Oeder S, Adam T, Bucchianico SD, Zimmermann R. Adenine derivatization for LC-MS/MS epigenetic DNA modifications studies on monocytic THP-1 cells exposed to reference particulate matter. *Analytical Biochemistry*. 2021;*618*:114127.

Carcelen JN. Doctoral Thesis. University of Oviedo, Spain. March 2023. https://digibuo.uniovi.es/dspace/handle/10651/5573/discover

Carcelén JN, Marchante-Gayón JM, Rodriguez-González P, Valledor L, Cañal MJ, Garcia Alonso JI. A cost-effective approach to produce ^{15}N-labelled amino acids employing Chlamydomonas reinhardtii CC503. *Microbial Cell Factories*. 2017;*16*:146.

Celarain N, Tomas-Roig J. Aberrant DNA methylation profile exacerbates inflammation and neurodegeneration in multiple sclerosis patients. *Journal of Neuroinflammation*. 2020;*17*:21.

Chen B, Yuan BF, Feng YQ. Analytical methods for deciphering RNA modifications. *Analytical Chemistry*. 2019;*91*:743–756.

Chen K, Zhang J, Guo Z, Ma Q, Xu Z, Zhou Y, Xu Z, Li Z, Liu Y, Ye X, Li X, Yuan B, Ke Y, He C, Zhou L, Liu J, Ci W. Loss of 5-hydroxymethylcytosine is linked to gene body hypermethylation in kidney cancer. *Cell Research* 2016;*26*:103–118.

Chen ML, Shen F, Huang W, Qi JH, Wang Y, Feng YQ, Liu SM, Yuan BF. Quantification of 5-methylcytosine and 5-hydroxymethylcytosine in genomic DNA from hepatocellular carcinoma tissues by capillary hydrophilic-interaction liquid chromatography/quadrupole TOF mass spectrometry. *Clinical Chemistry*. 2013;*59*:824–832.

Chen MY, Gui Z, Chena KK, Dinga JH, Heb JG, Xionga J, Li JL, Wang J, Yuan BF, Feng YQ. Adolescent alcohol exposure alters DNA and RNA modifications in peripheral blood by liquid chromatography-tandem mass spectrometry analysis. *Chinese Chemical Letters*. 2022:*33*:2086–2090.

Chen Q, Hu Y, Fang, Z, Ye M, Li J, Zhang S, Yuan Y, Guo C. Elevated levels of oxidative nucleic acid modification markers in urine from gastric cancer patients: quantitative analysis by ultra performance liquid chromatography-tandem mass spectrometry. *Frontiers in Chemistry*. 2020;*8*:1–7.

Chen Y, Hong T, Wang S, Mo J, Tian T, Zhou X. Epigenetic modification of nucleic acids: from basic studies to medical applications. *Chemical Society Reviews*. 2017;*46*:2844–2872.

Chilakala S, Li L, Feng Y, Xu Y. Microwave-assisted enzymatic hydrolysis of DNA for mass spectrometric analysis: a new strategy for accelerated hydrolysis. *Analytical Biochemistry*. 2017;*546*:28–34.

Chowdhury B, Cho IH, Irudayaraj J. Technical advances in global DNA methylation analysis in human cancers. *Journal of Biological Engineering*. 2017;*11*:1–12.

Dai Y, Yuan BF, Feng YQ. Quantification and mapping of DNA modifications. *RSC Chemical Biology*. 2021;*2*:1096–1114.

Dal-Bekar NE, Siomek-Gorecka A, Gackowski D, Köken-Avşar A, Yarkan-Tuğsal H, Birlik M, İşlekel H. Global hypomethylation pattern in systemic sclerosis: an application for absolute quantification of epigenetic DNA modification products by 2D-UPLC-MS/MS. *Clinical Immunology*. 2022;*239*:108997.

10982787, 0, Downloaded from https://analyticalsciencejournals.onlinelibrary.wiley.com/doi/10.1002/mas.21865 by Readcube (Labtiva Inc.), Wiley Online Library on [05/03/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

Das PM, Singal R. DNA methylation and cancer. *Journal of Clinical Oncology*. 2004;*22*:4632–4642.

Divakar KJ, Reese CB. 4-(1,2,4-Triazol-1-yl)- and 4-(3-nitro-1,2,4-triazol-1-yl)-1-(β-D-2,3,5-tri-O-acetylarabinofuranosyl)pyrimidin-2(1H)-ones. Valuable intermediates in the synthesis of derivatives of 1-(β-D-arabinofuranosyl)cytosine (ara-C). *Journal of Chemical Society Perkin Transactions 1*. 1982:1171–1176.

Dudley E, Bond L. Mass spectrometry analysis of nucleosides and nucleotides. *Mass Spectrometry Reviews*. 2014;*33*:302–331.

Dupont C, Armant DR, Brenner CA. Epigenetics: definition, mechanisms and clinical perspective. *Seminars in Reproductive Medicine*. 2009;*27*:351–357.

Dziaman T, Gackowski D, Guz J, Linowiecka D, Bodnar M, Starczak M, Zarakowska E, Modrzejewska M, Szpila A, Szpotan J, Gawronski M, Labejszo A, Liebert A, Banaszkiewicz Z, Klopocka M, Foksinski M, Marszalek A, Olinski R. Characteristic profiles of DNA epigenetic modifications in colon cancer and its predisposing conditions-benign adenomas and inflammatory bowel disease. *Clinical Epigenetics*. 2018;*10*:1–11.

Fernandez AF, Valledor L, Vallejo F, Cañal MJ, Fraga MF. Quantification of global DNA methylation levels by mass spectrometry. *Methods in Molecular Biology*. 2018;*1708*:49–58.

Fisher, DH, Giese RW. Determination of 5-methylcytosine in DNA by gas chromatography-electron-capture detection. *Journal of Chromatography*. 1988;*452*:51–60.

Foksinski M, Zarakowska E, Gackowski D, Skonieczna M, Gajda K, Hudy D, Szpila A, Bialkowski K, Starczak M, Labejszo A, Czyz J, Rzeszowska-Wolny J, Olinski R. Profiles of a broad spectrum of epigenetic DNA modifications in normal and malignant human cell lines: proliferation rate is not the major factor responsible for the 5-hydroxymethyl-2′-deoxycytidine level in cultured cancerous cell lines. *PLoS One*. 2017;*12*:1–13.

Fu L, Amato NJ, Wang P, McGowan SJ, Niedernhofer LJ, Wang Y. Simultaneous quantification of methylated cytidine and adenosine in cellular and tissue RNA by nano-flow liquid chromatography-tandem mass spectrometry coupled with the stable isotope-dilution method. *Analytical Chemistry*. 2015;*87*: 7653–7659.

Fu Y, He C. Nucleic acid modifications with epigenetic significance. *Current Opinion in Chemical Biology*. 2012;*16*:516–524.

Fu Y, Luo GZ, Chen K, Deng X, Yu M, Han D, Hao Z, Liu J, Lu X, Dore LC, Weng X, Ji Q, Mets L, He C. N6-methyldeoxyadenosine marks active transcription start sites in Chlamydomonas. *Cell*. 2015;*161*:879–892.

Gackowski D, Starczak M, Zarakowska E, Modrzejewska M, Szpila A, Banaszkiewicz Z, Olinski R. Accurate, direct, and high-throughput analyses of a broad spectrum of endogenously generated DNA base modifications with isotope-dilution two-dimensional ultraperformance liquid chromatography with tandem mass spectrometry: possible clinical implication. *Analytical Chemistry*. 2016;*88*:12128–12136.

Gackowski D, Zarakowska E, Starczak M, Modrzejewska M, Olinski R. Tissue-specific differences in DNA modifications (5-hydroxymethylcytosine, 5-formylcytosine, 5-carboxylcytosine and 5-hydroxymethyluracil) and their interrelationships. *PLoS One*. 2015;*10*:1–12.

Garcia-Alonso JI, Rodriguez-Gonzalez P. 2013. *Isotope Dilution Mass Spectrometry*. Royal Society of Chemistry.

Gibney ER, Nolan CM. Epigenetics and gene expression. *Heredity*. 2010;*105*: 4–13.

Greer EL, Blanco MA, Gu L, Sendinc E, Liu J, Aristizábal-Corrales D, Hsu CH, Aravind L, He C, Shi Y. DNA methylation on N6-adenine in C. elegans. *Cell*. 2015;*161*:868–878.

Guo C, Xie C, Chen Q, Cao X, Guo M, Zheng S, Wang Y. A novel malic acid-enhanced method for the analysis of 5-methyl-2′-deoxycytidine, 5-hydroxymethyl-2′-deoxycytidine, 5-methylcytidine and 5-hydroxymethylcytidine in human urine using hydrophilic interaction liquid chromatography-tandem mass spectrometry. *Analytica Chimica Acta*. 2018;*1034*;110–118.

Guo M, Li X, Zhang L, Liu D, Du W, Yin D, Lyu N, Zhao G, Guo C, Tang D. Accurate quantification of 5-methylcytosine, 5-hydroxymethylcytosine, 5-formylcytosine, and 5-carboxylcytosine in genomic DNA from breast cancer by chemical derivatization coupled with ultra-performance liquid chromatography-electrospray quadrupole time. *Oncotarget*. 2017;*8*:91248–91257.

Guo M, Liu D, Sha Q, Geng H, Liang J, Tang D. Succinic acid enhanced quantitative determination of blood modified nucleosides in the development of diabetic nephropathy based on hydrophilic interaction liquid chromatography mass spectrometry. *Journal of Pharmaceutical and Biomedical Analysis*. 2019;*164*:309–316.

Guo M, Zhang L, Du Y, Du W, Liu D, Guo C, Pan Y, Tang D. Enrichment and quantitative determination of 5-(hydroxymethyl)-2′-deoxycytidine, 5-(formyl)-2′-deoxycytidine, and 5-(carboxyl)-2′-deoxycytidine in human urine of breast cancer patients by magnetic hyper-cross-linked microporous polymers based on polyionic liquid. *Analytical Chemistry*. 2018;*90*:3906–3913.

Guz J, Foksinski M, Siomek A, Gackowski D, Rozalski R, Dziaman T, Szpila A, Olinski R. The relationship between 8-oxo-7,8-dihydro-2'-deoxyguanosine level and extent of cytosine methylation in leukocytes DNA of healthy subjects and in patients with colon adenomas and carcinomas. *Mutation Research*. 2008:*640*:170–173.

Havlis J, Madden JE, Revilla AL, Havel J. High-performance liquid chromatographic determination of deoxycytidine monophosphate and methyldeoxycytidine monophosphate for DNA demethylation monitoring: experimental design and artificial neural networks optimisation. *Journal of Chromatography B*. 2001;*755*:185–194.

Heikkinen A, Bollepalli S, Ollikainen M. The potential of DNA methylation as a biomarker for obesity and smoking. *Journal of Internal Medicine*. 2022;*292*:390–408.

Hong H, Wang Y. Derivatization with Girard reagent T combined with LC-MS/MS for the sensitive detection of 5-formyl-2′-deoxyuridine in cellular DNA. *Analytical Chemistry*. 2007;*79*: 322–326.

Hu CW, Chen JL, Hsu YW, Yen CC, Chao MR. Trace analysis of methylated and hydroxymethylated cytosines in DNA by isotope-dilution LC–MS/MS: first evidence of DNA methylation in Caenorhabditis elegans. *Biochemical Journal*. 2015;*465*:39–47.

Hu CW, Lee H, Chen JL, Li YJ, Chao MR. Optimization of global DNA methylation measurement by LC-MS/MS and its application in lung cancer patients. *Analytical and Bioanalytical Chemistry*. 2013;*405*:8859–8869.

Hu CW, Liu HH, Li YJ, Chao MR. Direct analysis of 5-methylcytosine and 5-methyl-2′-deoxycytidine in human

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urine by isotope dilution LC-MS/MS: correlations with N-methylated purines and oxidized DNA lesions. *Chemical Research in Toxicology*. 2012;*25*:462–470.

Huang W, Lan MD, Qi CB, Zheng SJ, Wei SZ, Yuan BF, Fenga YQ. Formation and determination of the oxidation products of 5-methylcytosine in RNA. *Chemical Science*. 2016;*7*:5495–5502.

Ito S, Shen L, Dai Q, Wu SC, Collins LB, Swenberg JA, He C, Zhang Y. Tet proteins can convert 5-methylcytosine to 5-formylcytosine and 5-carboxylcytosine. *Science*. 2011;*333*: 1300–1303.

Jaenisch R, Bird A. Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. *Nature Genetics*. 2003;*33*:245–254.

Jiang HP, Liu T, Guo N, Yu L, Yuan BF, Feng YQ. Determination of formylated DNA and RNA by chemical labeling combined with mass spectrometry analysis. *Analytical Chimica Acta*. 2017;*981*:1–10.

Kellner S, DeMott MS, Cheng CP, Russell B, Cao B, You D, Dedon PC. Oxidation of phosphorothioate DNA modifications leads to lethal genomic instability. *Nature Chemical Biology*. 2017;*13*:888–894.

Kellner S, Ochel A, Thüring K, Spenkuch F, Neumann J, Sharma S, Entian KD, Schneider D, Helm M. Absolute and relative quantification of RNA modifications via biosynthetic isotopomers. *Nucleic Acids Research*. 2014;*42*:1–10.

Kohli RM, Zhang Y. TET enzymes, TDG and the dynamics of DNA demethylation. *Nature*. 2013;*502*:472–479.

Kurdyukov S, Bullock M. DNA methylation analysis: choosing the right method. *Biology*. 2016;*5*:3.

Lai W, Lyu C, Wang H. Vertical ultrafiltration-facilitated DNA digestion for rapid and sensitive UHPLC-MS/MS detection of DNA modifications. *Analytical Chemistry*. 2018;*90*:6859–6866.

Lai W, Mo J, Yin J, Lyu C, Wang H. Profiling of epigenetic DNA modifications by advanced liquid chromatography-mass spectrometry technologies. *Trends in Analytical Chemistry*. 2019;*110*:172–182.

Le T, Kim KP, Fan G, Faull KF. A sensitive mass spectrometry method for simultaneous quantification of DNA methylation and hydroxymethylation levels in biological samples. *Analytical Biochemistry*. 2011;*412*:203–209.

Li J, Li L, Wang Y, Huang G, Li X, Xie Z, Zhou Z. Insights into the role of DNA methylation in immune cell development and autoimmune disease. *Frontiers in Cell and Developmental Biology*. 2021;*9*:757318.

Li QY, Yuan BF, Feng YQ. Mass spectrometry-based nucleic acid modification analysis. *Chemical Letters*. 2018;*47*:14531459.

Li S, Tollefsbol TO. DNA methylation methods: global DNA methylation and methylomic analyses. *Methods*. 2021;*187*: 28–43.

Li X, Franke AA. High-throughput and cost-effective global DNA methylation assay by liquid chromatography-mass spectrometry. *Analytica Chimica Acta*. 2011;*703*:58–63.

Li W, Zhang X, Lu X, You L, Song Y, Luo Z, Zhang J, Nie J, Zheng W, Xu D, Wang Y, Dong Y, Yu S, Hong J, Shi J, Hao H, Luo F, Hua L, Wang P, Qian X, Yuan F, Wei L, Cui M, Zhang T, Liao Q, Dai M, Liu Z, Chen G, Meckel K, Adhikari S, Jia G, Bissonnette MB, Zhang X, Zhao Y, Zhang W, He C, Liu J. 5-Hydroxymethylcytosine signatures in circulating cell-free DNA as diagnostic biomarkers for human cancers. *Cell Research*. 2017;*27*:1243–1257.

Liang D, Wang H, Song W, Xiong X, Zhang X, Hu Z, Guo H, Yang Z, Zhai S, Zhang LH, Ye M, Du Q. The decreased N6-methyladenine DNA modification in cancer cells. *Biochemichal and Biophysical Research Communications*. 2016;*480*:120–125.

Liu B, Liu X, Lai W, Wang H. Metabolically generated stable isotope-labeled deoxynucleoside code for tracing DNA N6-methyladenine in human cells. *Analytical Chemistry*. 2017;*89*: 6202–6209.

Liu J, Jiang J, Mo J, Liu D, Cao D, Wang H, He Y, Wang H. Global DNA 5-hydroxymethylcytosine and 5-formylcytosine contents are decreased in the early stage of hepatocellular carcinoma. *Hepatology*. 2019;*69*:196–208.

Liu S, Jin W, Yijing S, Candace G, Yaxue Z, Devarati M, Philip JB, David EF, Hongjun S, Yinsheng W. Quantitative assessment of Tet-induced oxidation products of 5-methylcytosine in cellular and tissue DNA. *Nucleic Acids Research*. 2013;*41*: 6421–6429.

Liu S, Ji D, Cliffe L, Sabatini R, Wang Y. Quantitative mass spectrometry-based analysis of β-D-glucosyl-5-hydroxymethyluracil in genomic DNA of Trypanosoma brucei. *Journal of American Society of Mass Spectrometry*. 2014;*25*:1763–1770.

Liu S, Dunwell TL, Pfeifer GP, Dunwell JM, Ullah I, Wang Y. Detection of oxidation products of 5-methyl-2′-deoxycytidine in Arabidopsis DNA. *PLoS ONE*. 2013;*8*:8–13.

Liu S, Wang J, Su Y, Guerrero C, Zeng Y, Mitra D, Brooks PJ, Fisher DE, Song H, Wang Y. Quantitative assessment of Tet-induced oxidation products of 5-methylcytosine in cellular and tissue DNA. *Nucleic Acids Research*. 2013;*41*: 6421–6429.

Liu Z, Liu S, Xie Z, Blum W, Perrotti D, Paschka P, Klisovic R, Byrd J, Chan KK, Marcucci G. Characterization of in vitro and in vivo hypomethylating effects of decitabine in acute myeloid leukemia by a rapid, specific and sensitive LC-MS/MS method. *Nucleic Acids Research*. 2007;*35*:e31.

Lyu C, Niu Y, Lai W, Wang Y, Wang Y, Dai P, Ma C, Chen S, Li Y, Jiang G, Liang Z, Ma W, Gao Z, Tong WM, Wang H. Rare and misincorporated DNA N(6)-methyladenine is a hallmark of cytotoxic stresses for selectively stimulating the stemness and proliferation of glioblastoma cells. *Cell Discovery*. 2022;*8*:39.

Lyu C, Wang HD, Lai W, Wang H. Identification and quantification of DNA N6-methyladenine modification in mammals: a challenge to modern analytical technologies. *Current Opinion in Chemical Biology*. 2023;*73*:102259.

Martínez-Iglesias O, Carrera I, Carril JC, Fernández-Novoa L, Cacabelos N, Cacabelos R. DNA methylation in neurodegenerative and cerebrovascular diseases disorders. *International Journal of Molecular Sciences*. 2020;*21*:2220.

Mo J, Liang Z, Lu M, Wang H. Protonation−suppression-free LC-MS/MS analysis for profiling of DNA cytosine modifications in adult mice. *Analytical Chemistry*. 2020;*92*:7430–7436.

Neubauer S, Rugova A, Chu DB, Drexler H, Ganner A, Sauer M, Mattanovich D, Hann S, Koellensperger G. Mass spectrometry-based analysis of nucleotides, nucleosides, and nucleobases—application to feed supplements*Analytical and Bioanalytical Chemistry*. 2012;*404*:799–808.

10982787, 0, Downloaded from https://analyticalsciencejournals.onlinelibrary.wiley.com/doi/10.1002/mas.21865 by Readcube (Labtiva Inc.), Wiley Online Library on [05/03/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

Nebbioso A, Tambaro FP, Dell'Aversana C, Altucci L. Cancer epigenetics: moving forward. *PLoS Genetics*. 2018;*14*:e1007362.

Olinski R, Rozalski R, Gackowski D. Mass spectrometry-based analysis of DNA modifications: potential applications in basic research and clinic. *Methods in Molecular Biology*. 2021;*2198*: 27–35.

Pal S, Tyler JK. Epigenetics and aging. *Science Advances*. 2016;*2*:e1600584.

Pfaffeneder T, Hackner B, Truß M, Münzel M, Müller M, Deiml CA, Hagemeier C, Carell T. The discovery of 5-formylcytosine in embryonic stem cell DNA. *Angewandte Chemie International Edition*. 2011;*50*:7008–7012.

Quinlivan EP, Gregory JF. DNA methylation determination by liquid chromatography-tandem mass spectrometry using novel biosynthetic [U-15N]deoxycytidine and [U-15N] methyldeoxycytidine internal standards. *Nucleic Acids Research*. 2008;361–367.

Raciti GA, Desiderio A, Longo M, Leone A, Zatterale F, Prevenzano I, Miele C, Napoli R, Beguinot F. Dna methylation and type 2 diabetes: novel biomarkers for risk assessment? *International Journal of Molecular Sciences*. 2021;*22*:11652.

Ramsahoye BH. Measurement of genome-wide DNA methylation by reversed-phase high-performance liquid chromatography. *Methods*. 2002;*27*:156–161.

Romerio AS, Fiorillo G, Terruzzi I, Senesi P, Testolin G, Battezzati A. Measurement of DNA methylation using stable isotope dilution and gas chromatography-mass spectrometry. *Analytical Biochemistry*. 2005;*336*:158–163.

Rossella F, Polledri E, Bollati V, Baccarelli A, Fustinoni S. Development and validation of a gas chromatography/mass spectrometry method for the assessment of genomic DNA methylation. *Rapid Communications in Mass Spectrometry*. 2009;*23*:2637–2646.

Roszkowski K, Jozwicki W, Blaszczyk P, Mucha-Malecka A, Siomek A. Oxidative damage DNA: 8-oxoGua and 8-oxodG as molecular markers of cancer. *Medical Science Monitor*. 2011;*17*:329–333.

Rozalski R, Gackowski D, Siomek-Gorecka A, Banaszkiewicz Z, Olinski R. Urinary measurement of epigenetic DNA modifications: a non-invasive assessment of the whole-body epigenetic status in healthy subjects and colorectal cancer patients. *ChemistryOpen*. 2016;*5*:550–553.

Schmid K, Thüring K, Keller P, Ochel A, Kellner S, Helm M. Variable presence of 5-methylcytosine in commercial RNA and DNA. *RNA Biology*. 2015;*12*:1152–1158.

Schummer C, Delhomme O, Appenzeller BMR, Wennig R, Millet M. Comparison of MTBSTFA and BSTFA in derivatization reactions of polar compounds prior to GC/MS analysis. *Talanta*. 2009;*77*:1473–1482.

Song L, James SR, Kazim L, Karpf AR. Specific method for the determination of genomic DNA methylation by liquid chromatography-electrospray ionization tandem mass spectrometry. *Analytical Chemistry*. 2005;*77*:504–510.

Tang Y, Chu JM, Huang W, Xiong J, Xing XW, Zhou X, Feng YQ, Yuan BF. Hydrophilic material for the selective enrichment of 5- hydroxymethylcytosine and its liquid chromatography-tandem mass spectrometry detection. *Analytical Chemistry*. 2013;*85*:6129–6135.

Tang Y, Gao XD, Wang Y, Yuan BF, Feng YQ. Widespread existence of cytosine methylation in yeast DNA measured by gas chromatography/mass spectrometry. *Analytical Chemistry*. 2012;*84*,7249–7255.

Tang Y, Xiong J, Jiang HP, Zheng SJ, Feng YQ, Yuan BF. Determination of oxidation products of 5-methylcytosine in plants by chemical derivatization coupled with liquid chromatography/tandem mass spectrometry analysis. *Analytical Chemistry*. 2014;*86*:7764–7772.

Tang Y, Zheng SJ, Qi CB, Feng YQ, Yuan BF. Sensitive and simultaneous determination of 5-methylcytosine and its oxidation products in genomic DNA by chemical derivatization coupled with liquid chromatography-tandem mass spectrometry analysis. *Analytical Chemistry*. 2015;*87*: 3445–3452.

Toraño EG, Petrus S, Fernandez AF, Fraga MF. Global DNA hypomethylation in cancer: review of validated methods and clinical significance. *Clinical Chemistry and Laboratory Medicine*. 2012;*50*:1733–1742.

Tsuji M, Matsunaga H, Jinno D, Tsukamoto H, Suzuki N, Tomioka Y. A validated quantitative liquid chromatography-tandem quadrupole mass spectrometry method for monitoring isotopologues to evaluate global modified cytosine ratios in genomic DNA. *Journal of Chromatography B*. 2014;*953–954*: 38–47.

Vryer R, Saffery R. What's in a name? Context-dependent significance of 'global' methylation measures in human health and disease. Clinical *Epigenetics*. 2017;*9*:2.

Wang J, Yuan B, Guerrero C, Bahde R, Gupta S, Wang Y. Quantification of oxidative DNA lesions in tissues of long-evans cinnamon rats by capillary high-performance liquid chromatography-tandem mass spectrometry coupled with stable isotope-dilution method. *Analytical Chemistry*. 2011;*83*: 2201–2209.

Wang X, Guo T, Wang S, Chen X, Chen Y, Yuan J, Zhao R. Determination of 5-hydroxymethyl-2′-deoxycytidine in rice by high-performance liquid chromatography–tandem mass spectrometry with isotope dilution. *Analytical Letters*. 2017;*50*: 2351–2358.

Webb LM, Guerau-de-Arellano M. Emerging role for methylation in multiple sclerosis: beyond DNA. *Trends in Molecular Medicine*. 2017;*23*:546–562.

Wilson AS, Power BE, Molloy PL. DNA hypomethylation and human diseases. *Biochimica et Biophysica Acta - Reviews on Cancer*. 2007;*1775*:138–162.

Xie NB, Wang M, Ji TT, Guo X, Ding JH, Yuan BF, Feng YQ. Bisulfite-free and single-nucleotide resolution sequencing of DNA epigenetic modification of 5-hydroxymethylcytosine using engineered deaminase. *Chemical Science*. 2022:*13*: 7046–7056.

Xie Q, Wu TP, Gimple RC, Li Z, Prager BC, Wu Q, Yu Y, Wang P, Wang Y, Gorkin DU, Zhang C, Dowiak AV, Lin K, Zeng C, Sui Y, Kim LJY, Miller TE, Jiang L, Lee CH, Huang Z, Fang X, Zhai K, Mack SC, Sander M, Bao S, Kerstetter-Fogle AE, Sloan AE, Xiao AZ, Rich JN. N6-Methyladenine DNA Modification in glioblastoma. *Cell*. 2018;*175*:1228–1243.

Yakovlev IA, Gackowski D, Abakir A, Viejo M, Ruzov A, Olinski R, Starczak M, Gunnar CG Fossdal, Krutovsky KV. Mass

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spectrometry reveals the presence of specific set of epigenetic DNA modifications in the Norway spruce genome. *Science Reports*. 2019;*9*:1–7.

Ye X, Zhang L, Chen B, Li J. Yang Q, Huang Q, Zhang J, Gao Y, Li Z, Cai C. A quantitative method for detecting DNA methylation over targeted genomic regions using isotope dilution liquid chromatography-tandem mass spectrometry. *Talanta*. 2017;*169*:136–140.

Yin J, Chen S, Zhang N, Wang H. Multienzyme cascade bioreactor for a 10 min digestion of genomic DNA into single nucleosides and quantitative detection of structural DNA modifications in cellular genomic DNA. *ACS Applied Materials & Interfaces*. 2018;*10*:21883–21890.

Yin J, Xu T, Zhang N, Wang H. Three-enzyme cascade bioreactor for rapid digestion of genomic DNA into single nucleosides. *Analytical Chemistry*. 2016;*88*:7730–7737.

Yin R, Mo J, Lu M, Wang H. Detection of human urinary 5-hydroxymethylcytosine by stable isotope dilution HPLC-MS/MS analysis. *Analytical Chemistry*. 2015;*87*:1846–1852.

Yu Y, Guerrero CR, Liu S, Amato NJ, Sharma Y, Gupta S, Wang Y. Comprehensive assessment of oxidatively induced modifications of DNA in a rat model of human Wilson's disease. *Molecular & Cellular Proteomics*. 2016; *15*:810–817.

Yu Y, Yuan F, Zhang XH, Zhao MZ, Zhou YL, Zhang XX. Ultrasensitive determination of rare modified cytosines based on novel hydrazine labeling reagents. *Analytical Chemistry*. 2019;*91*:13047–13053.

Yuan BF. Assessment of DNA epigenetic modifications. *Chemical Research in Toxicology*. 2020;*33*:695–708.

Zeng H, Qi CB, Liu T, Xiao HM, Cheng QY, Jiang HP, Yuan BF, Feng YQ. Formation and determination of endogenous methylated nucleotides in mammals by chemical labeling coupled with mass spectrometry analysis. *Analytical Chemistry*. 2017;*89*:4153–4160.

Zhang HY, Xiong J, Qi BL, Feng YQ, Yuan BF. The existence of 5-hydroxymethylcytosine and 5-formylcytosine in both DNA and RNA in mammals. *Chemical Communications*. 2016;*52*:737–740.

Zhang G, Huang H, Liu D, Cheng Y, Liu X, Zhang W, Yin R, Zhang D, Zhang P, Liu J, Li C, Liu B, Luo Y, Zhu Y, Zhang N, He S, He C, Wang H, Chen D. N6-methyladenine DNA modification in Drosophila. *Cell*. 2015;*161*:893–906.

Zhang L, Zhang L, Zhou K, Ye X, Zhang J, Xie A, Chen L, Kang JX, Cai C. Simultaneous determination of global DNA methylation and hydroxymethylation levels by hydrophilic interaction liquid chromatography-tandem mass spectrometry. *Journal of Biomolecular Screening*. 2012;*17*:877–884.

Zhang R, Lai W, Wang H. Quantification of epigenetic DNA modifications in the subchromatin structure matrix attachment regions by stable isotope dilution UHPLC-MS/MS analysis. *Analytical Chemistry*. 2021;*93*:15567–15572.

Zhang W, Tan S, Paintsil E, Dutschman GE, Gullen EA, Chu E, Cheng YC. Analysis of deoxyribonucleotide pools in human cancer cell lines using a liquid chromatography coupled with tandem mass spectrometry technique. *Biochemical Pharmacology*. 2011;*82*:411–417.

Zhang YF, Qi CB, Yuan BF, Feng YQ. Determination of cytidine modifications in human urine by liquid chromatography–mass spectrometry analysis. *Analytica Chimica Acta*. 2019;*1081*:103–111.

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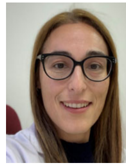

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