J. Am. Soc. Mass Spectrom. (2011) 22:1242–1251
DOI: 10.1007/s13361-011-0126-8

RESEARCH ARTICLE

A Nano-Chip-LC/MS^n Based Strategy for Characterization of Modified Nucleosides Using Reduced Porous Graphitic Carbon as a Stationary Phase

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Abstract

LC/MS analysis of ribonucleosides is traditionally performed by reverse phase chromatography on silica based C18 type stationary phases using MS compatible buffers and methanol or acetonitrile gradients. Due to the hydrophilic and polar nature of nucleosides, down-scaling C18 analytical methods to a two-column nano-flow setup is inherently difficult. We present a nano-chip LC/MS ion-trap strategy for routine characterization of RNA nucleosides in the fmol range. Nucleosides were analyzed in positive ion mode by reverse phase chromatography using a 75 µ×150 mm, 5 µ particle porous graphitic carbon (PGC) chip with an integrated 9 mm, 160 nL trapping column. Nucleosides were separated using a formic acid/acetonitrile gradient. The method was able to separate isobaric nucleosides as well as nucleosides with isotopic overlap to allow unambiguous MS^n identification on a low resolution ion-trap. Synthesis of 5-hydroxycytidine (oh^{5}C) was achieved from 5-hydroxyuracil in a novel three-step enzymatic process. When operated in its native state using formic acid/acetonitrile, PGC oxidized oh^{5}C to its corresponding glycols and formic acid conjugates. Reduction of the PGC stationary phase was achieved by flushing the chip with 2.5 mM oxalic acid and adding 1 mM oxalic acid to the online solvents. Analyzed under reduced chromatographic conditions oh^{5}C was readily identified by its MH$^+$ *m/z* 260 and MS^n fragmentation pattern. This investigation is, to our knowledge, the first instance where oxalic acid has been used as an online reducing agent for LC/MS. The method was subsequently used for complete characterization of nucleosides found in tRNAs using both PGC and C18 chips.

Key words: Nucleosides, Nano-Chip Ion-Trap LC/MS^n, Porous Graphitic Carbon, tRNA

Introduction

Ribonucleic acids and their modified derivatives play an important role in a variety of fundamental biological processes. At present more than 100 modified RNA nucleosides are known, where each type of cellular RNA is modified to a different extent [1]. tRNA is the most heavily modified among the different types of RNA with up to 25% of nucleosides showing modifications in higher eukaryotes. In RNA, modifications are formed post-transcriptionally at the standard nucleosides adenosine, guanosine, uridine, and cytidine by enzymes such as specific methyl transferases and pseudouridine synthetases [2, 3]. Nucleosides are subjected to simple modifications like base or ribose methylation, base

Electronic supplementary material The online version of this article (doi:10.1007/s13361-011-0126-8) contains supplementary material, which is available to authorized users.

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Received: 16 December 2010
Revised: 7 March 2011
Accepted: 10 March 2011
Published online: 15 April 2011

isomerization, reduction, and thiolation, but also hypermodifications such as wybutosine, a hypermodified guanosine found in phenylalanine tRNA from *Saccharomyces cerevisiae* [4].

Reverse phase high performance liquid chromatography (RP-HPLC), in combination with UV detection or mass spectrometry or both, is commonly used to investigate nucleic acids and their modifications [5–7]. The majority of LC methods typically employ silica-based C18 reverse phase columns, low ionic strength acidic buffers, and acetonitrile or methanol gradients. Gehrke and Kou [8] discussed RP HPLC/UV methods for analysis of ribonucleic acids, and were able to resolve 65 nucleosides in a single analysis. In a classic LC/MS paper, Pommerantz and McCloskey [9] presented a reverse phase method for identification of 64 RNA derived nucleosides. Identification of individual nucleosides was based on relative chromatographic retention using a C18 column and thermospray MS. Spectra primarily contained the *m/z* of the nucleoside pseudomolecular ion and of the protonated base moiety following neutral loss of the ribose due to in-source fragmentation. In a series of papers, R. P. Newton and coworkers presented LC/MS methods for detection of purine and pyrimidine nucleosides in urine based on reverse phase separation [10–14]. Kammerer et al. [15] presented an LC-Ion Trap MS^n method for identification of modified nucleosides in urine. Thirty-six nucleosides were separated by RP/C18 using an ammonium formate/methanol gradient and identified by their characteristic MS^4 fragmentation.

Due to their polar and hydrophilic nature, nucleosides generally elute with poor retention and resolution under aqueous or low organic conditions on RP C18 stationary phases. As an alternative to silica based C18 type stationary phases, porous graphitic carbon (PGC) stationary phase provide markedly greater retention and selectivity for polar compounds and, due to its physical and chemical nature, tolerates extreme chromatographic conditions compared with silica based sorbents (reviewed in [16] and [17]). Several studies report the use of PGC for analysis of nucleosides and nucleotides and their modifications. Xing et al. [18] presented a LC/MS method for analysis of 16 nucleosides and their mono, di-, and triphosphates using a PGC stationary phase, and Monser [19] used PGC to separate and quantify four closely related purine bases in gonads of sea urchins.

However, irrespective of the chromatographic mode applied, papers describing LC/MS of ribonucleosides under nano-flow conditions appear sparse. Nano-LC/MS offers significant improvements in sensitivity and thus requires lesser amount of sample, but at the same time also necessitate careful sample preparation and more attention to the analytical instrumentation. Nano-LC/MS applications generally use a two-column setup with a short enrichment column placed in front of the analytical column to reduce sample-loading time and to desalt and focus analytes at the top of the analytical column prior to gradient separation and MS analysis. The use of C18 type stationary phases for enrichment of nucleosides is not likely to succeed due to their hydrophilic nature. Nucleosides such as dihydrouridine, pseudouridine, and cytidine that elute isocratically under aqueous conditions on C18 stationary phases are not retained on the enrichment column and will thus be lost during sample loading.

We have recently presented a chip-based nano-LC/MS method specifically developed to unambiguously identify 8-methyladenosine (m^{8}A) as a new modification in ribosomal RNA [20]. Mass analysis was performed using an Agilent Chip Cube nano-LC/ESI ion-trap system. The automated chip handler, the 'chip-cube', is mounted directly in front of an ion trap and contains a micro valve for flow-path switching, providing ultra low dead volume and minimal peak dispersion. The chip consists of an integrated enrichment column and a 75 μ (i.d.) analytical column, a nanospray emitter, as well as frits and electrical contacts imbedded in a polyimide film [21]. Using a PGC chip, we were able to successfully resolve m^{8}A from 2-methyladenosine (m^{2}A), thereby enabling structural confirmation based on their MS^5 fragmentation characteristics [20]. Similar baseline separation of m^{2}A and m^{8}A could not be achieved using C18 chromatography.

Here we present a general chip based nano-LC/MS^n strategy for characterization of RNA nucleosides in the fmol range. As mentioned earlier, PGC provides markedly greater retention and selectivity for polar and hydrophobic compounds compared with C18. Furthermore, PGC is known to have a redox catalytic capacity [17]. Thus, due to the unique retention characteristics of PGC, special attention was given to the redox state of the stationary phase as well as analysis of large hydrophobic hyper-modified nucleosides such as wybutosine (yW) found in tRNA^{Phe} from *Saccharomyces cerevisiae* and queuosine (Q) and 2-methylthio-*N*^{6}-isopenthyladenosine (ms^{2}i^{6}A) found in tRNA^{TyrII} form *Escherichia coli*.

Experimental

A method development nucleoside test-mixture was prepared by mixing of a commercially available nucleoside standard (Sigma, Denmark) and a synthetic 5-hydroxycytidine (oh^{5}C, vide infra). The test-mixture contained the following 13 nucleosides in varying amounts (Table 1, Figure 1): cytidine (C), 3-methylcytidine (m^{3}C), 5-methylcytidine (m^{5}C), 2′-*O*-methylcytidine (Cm), 2-thiocytidine (s^{2}C), 5-hydroxycytidine (oh^{5}C), uridine (U), pseudouridine (Ψ), 5-methyluridine (m^{5}U), 1-methyladenosine (m^{1}A), N^6-methyladenosine (m^{6}A), inosine (I), guanosine (G). The test mixture was diluted 20 times in 0.1% formic acid (FA) prior to analysis and injected as is. All other chemicals, enzymes, and solvents were purchased from Sigma, Denmark or Merck, Germany, unless otherwise stated, and used as received. Water was taken from an in-house Elga Purelab Ultra Water System.

Table 1. Nucleosides Test Mixture Analyzed on PGC

Nucleoside	Retention time (min) pmol Injected	Oxidized[a]	Reduced	MH^+ *m/z*	Fragment ions MS^n [b]
3-Methylcytidine (m^3C)	1	9.1	10.7	258	**126**, 109, 95, 83, 69
Cytidine (C)	0.5	9.5	10.6	244	**112**, 95, 69
1-Methyladenosine (m^1A)	0.2	11.7	13.0	282	**150**, 133, 109, 94
2′-*O*-methylcytidine (Cm)	0.2	11.8	12.9	258	**112**, 95, 83, 69
5-Hydroxycytidine (ho^5C)	0.6	12.0[c]	12.9	260	**128**, 111, 101, 100, 85, 72, 57
5-Methylcytidine (m^5C)	1	12.3	13.3	258	**126**, 109, 108, 83, 81
Pseudouridine (Ψ)	0.3	12.4	12.0	245	209, 191, 163,
Uridine (U)	0.3	13.8	13.5	245	**113**, 96, 70
2-Thiocytidine (s^2C)	0.1	17.2	17.5	260	**128**, 111, 101, 94, 69
5-Methyluridine (m^5U)	0.5	17.0	16.5	259	**127**, 110, 82, 54
3-Methyluridine[d] (m^3U)	NA	17.0	16.9	259	**127**, 109, 96, 68
Guanosine (G)	0.2	20.5	19.7	284	**152**, 135, 128, 110
N^6-methyladenosine[d] (m^6A)	NA	23.3	22.2	282	**150**, 133, 123, 94

[a] Oxidized refers to PGC in its native oxidative state.
[b] MS^2 BH_2^+ ion is marked in bold.
[c] Observed as *m/z* 294 and 322.
[d] Non-certified component.

Synthesis of 5-Hydroxycytidine

Enzymes used in the synthesis of oh^5C: ribokinase (*rbs*K, EC 2.7.1.15) [22], 5-phospho-D-ribosyl-α-1-pyrophosphate synthetase (*prs*A, EC 2.7.6.1) [23], uracil phosphoribosyltransferase (*ura*P, EC 2.4.2.9) [24], uridylate kinase (*pyr*H, EC 2.7.4.22) [25], inorganic pyrophosphatase (*hpp*A, EC 3.6.1.1) [26], and CTP synthetase (*pyr*G, EC 6.3.4.2) [27], were prepared from overexpression strains described. All standard analytical HPLC methods and stepwise purification methods are described in detail in Scott et al. [28].

Synthesis of oh^5C was achieved through a three-step enzymatic process. Step 1: to a 500 mL round bottom flask, D-ribose (300 mg, 2.0 mmol) and 5-hydroxyuracil (256 mg, 2.0 mmol) were dissolved in 200 mL of pH 7.5 water containing: 0.008 mM dATP, 0.05 mM kanamycin, 0.15 mM ampicillin, 0.4 mM dAMP, 20 mM $MgCl_2$, 20 mM dithiothreitol, 50 mM potassium phosphate, 100 mM creatine phosphate. The synthesis was started with the addition of 10 units of ribokinase (*rbs*K, EC 2.7.1.15), 15 units of 5-phospho-D-ribosyl-α-1-pyrophosphate synthetase (*prs*A, EC 2.7.6.1), 20 units of uracil phosphoribosyltransferase (*ura*P, EC 2.4.2.9), 20 units of adenylate kinase (*pls*A, EC 2.7.4.3), 25 units of uridylate kinase (*pyr*H, EC 2.7.4.22), 65 units of inorganic pyrophosphatase (*hpp*A, EC 3.6.1.1), and 125 units of creatine phosphokinase (*ckm*T, EC 2.7.3.2). After 48 h, the synthesis of 5-hydroxyuridine-5′-triphosphate (oh^5UTP) appeared complete by analytical HPLC. The reaction was brought to 0.5 M ammonium bicarbonate and pH 9.5 with addition of ammonium hydroxide and purified by boronate-affinity chromatography. Step 2: the oh^5UTP (1.6 mmol) was then dissolved in 200 mL of pH 8.5 water containing: 0.008 mM dATP, 0.05 mM kanamycin, 0.15 mM ampicillin, 1.0 mM dAMP, 20 mM $MgCl_2$, 20 mM dithiothreitol, 50 mM creatine phosphate, and 100 mM NH_4Cl. The second step of the synthesis was started with the addition of 10 units of adenylate kinase (*pls*A, EC 2.7.4.3), 250 units of CTP synthetase (*pyr*G, EC 6.3.4.2), and 500 units of creatine phosphokinase (*ckm*T, EC 2.7.3.2). The reaction was again monitored by HPLC, whereby after 24 h the synthesis of 5-hydroxycytidine-5′-triphosphate (oh^5CTP) appeared complete. The reaction was again brought to 0.5 M ammonium bicarbonate and pH 9.5 with addition of ammonium hydroxide, and purified by boronate-affinity chromatography. The oh^5CTP (1.0 mmol) was then dissolved in 100 mL of pH 9.5 water containing: 0.1 mM zinc acetate, 10 mM $MgCl_2$. The third and final step of the synthesis was started with the addition of 200 units of calf intestinal alkaline phosphatase (Promega Corp.). After 24 h the synthesis of 5-hydroxycytidine (oh^5C) appeared complete. The oh^5C was purified by reverse phase C18 chromatography (0.6 mmol, 30% overall isolated yield) [29] and verified by ^1H NMR and ESI. Data: ^1H NMR (600 MHz, D_2O) δ (mult.) 6.62 (s, H-6), 5.81 (d, H-1′), 4.16 (dd, H-2′), 4.06 (dd, H-3′), 3.92 (ddd, H-4′), 3.72 (dd, H-5′), 3.64 (dd, H-5″). A sub ppm accurate mass of *m/z* 260.0877 (MH^+, resolution 158,000) was determined by direct infusion of a 100 pmol/μL standard in 50% acetonitrile, 0.1% FA on an LTQ Orbitrap (Thermo, Germany).

Hydrolysis of tRNAs

Thirty pmol $tRNA^{phe}$ from *Saccharomyces cerevisiae* (Sigma, Denmark) and 50 pmol $tRNA^{TyrII}$ from *Escherichia coli* (Sigma) were hydrolyzed to nucleosides using nuclease P1, phosphodiesterase I, and alkaline phosphatase according to Crain [30]. Enzymes were precipitated with eight times (by vol.) methanol and centrifuged at 14,000*g* for 15 min. The supernatant was collected, evaporated to dryness, and resuspended in 20 μL 0.1% FA and subjected to LC/MS^n analysis.

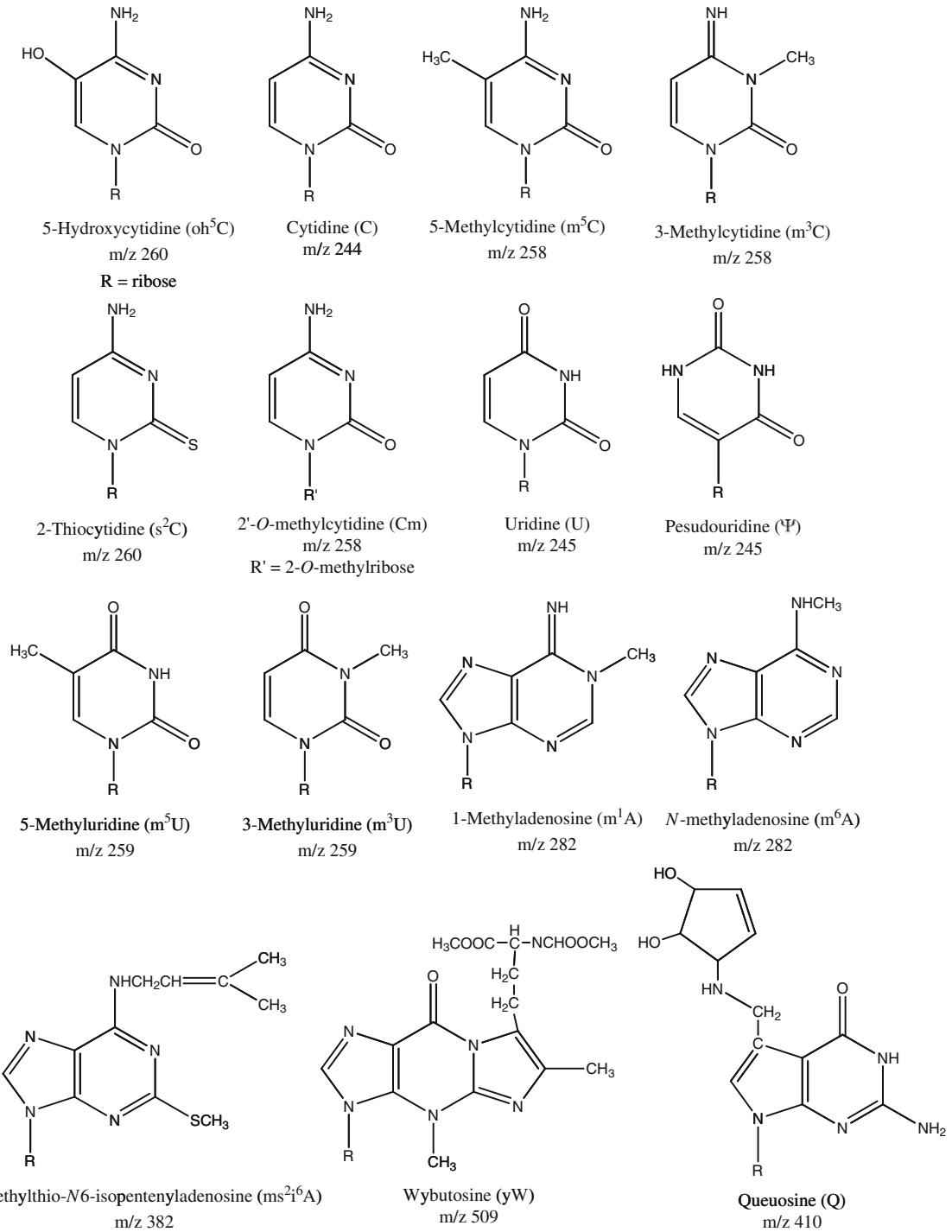

Figure 1. Chemical structures of the discussed nucleosides drawn in their uncharged form. Masses are given as their expected *m/z* in positive ion-mode

Nano-Chip ESI-ITMS^n

Mass analysis was performed using an Agilent Chip Cube nano-LC/ESI ion-trap system consisting of a loading pump operating at 4 µL/min and an analytical pump operating at 500 nL/min. The automated chip handler, the 'chip-cube', is mounted directly in front of an Agilent XCT Ultra 6340 ion trap with a micro valve for flow-path switching. The chip consists of an integrated 9 mm, 160 nL PGC, 5 µm particles enrichment column, a 150 mm×75 µm PGC, 5 µm particles analytical column and a nano-spray emitter. Samples were loaded onto the enrichment column in 0.1% formic acid. Gradient separation using 0%–90% acetonitrile with 0.1% FA and tandem MS detection were achieved using the method described in Giessing et al. [20]. For analysis of nucleosides under reduced conditions, the two columns of

the chip were initially flushed for 30 min with 2.5 mM oxalic acid; 1 mM oxalic acid was subsequently added to both of the aqueous eluents to maintain the PGC in a reduced state during sequence analysis. To obtain CID fragment spectra in the low *m/z* range, the fragmentation amplitude was manually adjusted to 0.5 V for MS and 0.6 V for MS^n to secure a potential well deep enough to avoid ejection of fragment ions from the ion trap.

For identification of large hypermodified nucleosides in tRNA, the PGC Chip was exchanged with a 75 µm, 43 mm Zorbax C18 chip, and gradient time was adjusted to the shorter chip configuration (see Figure 4 legend for details). No changes were made to the solvent composition.

Results and Discussion

The preparation of 5-hydroxycytidine (oh^{5}C) was carried out using an adapted *in vitro* enzymatic synthesis method for 5-fluorouridine-5′-triphosphate [31]. The synthesis of oh^{5}C began with the C-5-phosphorylation of ribose by ribokinase (*rbs*K), to give ribose-5-phosphate (R5P) that is further phosphorylated at the C-1 position by 5-phospho-D-ribosyl-α-1-pyrophosphate synthase (*prs*A) forming 5-phospho-D-ribosyl-α-1-pyrophosphate (PRPP). Uracil phosphoribosyltransferase (*upp*A) efficiently couples 5-hydroxyuracil to PRPP, forming 5-hydroxyuridine-5′-monophosphate (oh^{5}UMP). The monophosphate oh^{5}UMP was subsequently converted to oh^{5}UTP by sequential phosphorylation of uridylate kinase (*pyr*H) and creatine phosphokinase (*cpk*). The second step of the synthesis involved the specific intraconversion of oh^{5}UTP to oh^{5}CTP catalyzed by CTP synthetase (*pyr*G). To our knowledge, this is the first example of a C-5 substituted uridine analog being an active substrate for *pyr*G. The final step of the synthesis involves the complete dephosphorylation of oh^{5}CTP by calf intestine alkaline phosphatase to produce oh^{5}C in a 30% overall yield. Key features of this synthesis include the use of dATP as the phosphate donor, and dATP being easily separated from the ribonucleotide analog by boronate affinity chromatography.

The nucleosides in the test mix were successfully separated on the nano-flow PGC chip and identified using tandem mass spectrometry, Table 1. Chemical structures of the discussed nucleosides are presented in Figure 1. The majority of nucleosides were readily identified by extracted ion chromatograms (XIC) of their pseudo-molecular ion mass (MH^+) and neutral loss of either *m/z* 132 or 146 of ribose or methyl-ribose respectively, Table 1. For XICs see Figure S-1 in supplemental material, which can be found in the on-line version of this article. Pseudouridine (Ψ) was identified by its MH^+ of *m/z* 245 and distinctive fragmentation pattern with subsequent losses of water [15], and only oh^{5}C was not observed by its MH^+ at the expected *m/z* 260.

Contrary to LC with UV detection, LC/MS does not require complete resolution of all compounds for unique identification of each nucleoside. The selectivity of the mass detector adds a valuable second dimension besides retention time for identification purposes at a speed by which up to five tandem MS spectra can be acquired in the ion trap. This allows ample time in a chromatographic peak for full characterization of several co-eluding precursor ions. In the nucleoside test mix, Cm and m^{1}A co-elute in time at roughly 11.7 min (Table 1), but are easily separated based on the difference in precursor ion mass $[M+H]^+$ of *m/z* 258 and 282, respectively. Furthermore, the nucleoside test mix contains both isobaric compounds (e.g., m^{3}C, Cm and m^{5}C as well as s^{2}C and oh^{5}C) in addition to compounds with isotopic overlap (e.g., C, U and Ψ). A prerequisite for MS^n identification of isobaric compounds is sufficient chromatographic resolution, especially when working on a low resolution MS instrument such as a 3D ion trap. Separating nucleosides using a PGC stationary phase, we were able to resolve and identify the three methylated cytidines at *m/z* 258 from m^{5}U at *m/z* 259 as well as C at *m/z* 244 from U and Ψ at *m/z* 245, by their characteristic MS^n fragmentation patterns, Table 1. As a curiosity, we also identified two nucleosides, m^{6}A and m^{3}U, which are not part of the certified nucleoside test mix obtained from Sigma. m^{6}A was observed under both native and reduced conditions and identified based on its neutral loss of ribose (*m/z* 282→*m/z* 150) and its MS^n fragmentation pattern [15], Table 1. m^{3}U was observed as a split peak of m^{5}U at 16.9 min when analyzed under reduced conditions, Figure 2. Both methyluridines were identified based on their neutral loss of ribose (*m/z* 259→*m/z* 127) and their subsequent MS^n fragmentation pattern [15], Table 1.

In order to achieve sufficient intensity to obtain MS^n identification of each nucleoside in the commercial test mixture, the injected amount was kept between 0.1–1 pmol on-column, Table 1. Nucleosides such as m^{5}U and s^{2}C that ionize poorly in positive ESI also have low concentrations in commercial standard mixture, thus requiring larger amounts injected for complete characterization. Furthermore, injection of larger amounts also provides broader chromatographic peaks, thereby providing ample time to record up to MS^5 of several co-eluting precursor ions. If only concerned with detection of nucleosides based on the initial characteristic MS/MS neutral loss of ribose, 40 fmol or less was sufficient to routinely identify most of the nucleosides in the test mixture (data not shown).

As mentioned previously, 5-hydroxycytidine was not observed at *m/z* 260 in the test mixture when operating the PGC chip in native state using 0.1% FA and acetonitrile as eluents (see Figure S-1). The nucleobase of oh^{5}C has a relative low oxidation potential compared with the natural base cytosine (70 mV and >700 mV, respectively [32]) and is here used to test the effect of the redox state of the PGC stationary phase. Single injection of the oh^{5}C standard using the same chromatographic conditions yielded a single peak eluding at 12.0 min containing two dominant masses of *m/z* 294 and 322 and not the expected *m/z* of 260 (see Figure S-2). Tandem ms of *m/z* 294 and 322 revealed that both ions in MS/MS produce a dominating ion of *m/z* 276 indicating loss

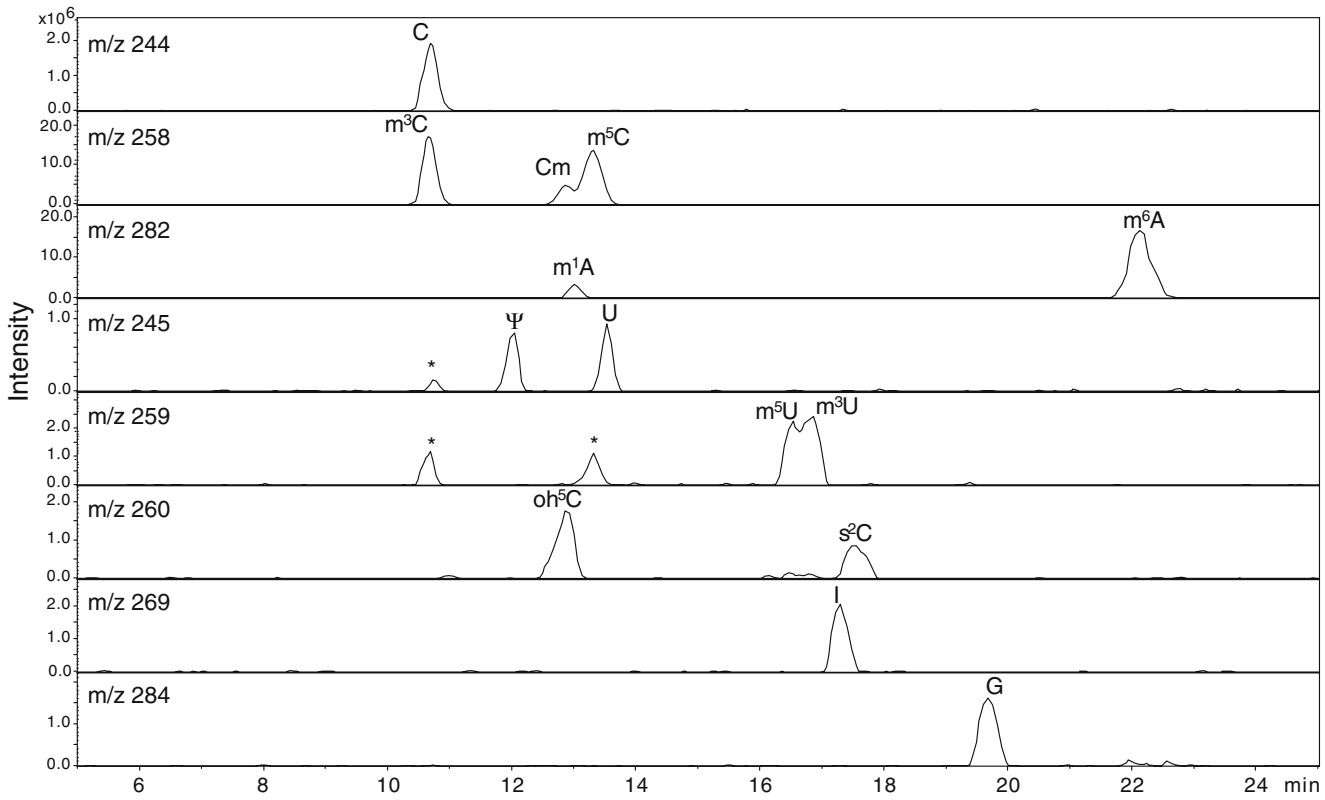

Figure 2. Extracted ion chromatogram (XIC) of nucleoside test mixture analyzed on reduced PGC using oxalic acid as an online reducing agent. oh^{5}C is observed as at 12.9 min in XIC of *m/z* 260. Asterisks indicate peaks of isotopic overlap. See Table 1 for MS^n details

of H_2O and CHOOH, respectively. Subsequent loss of H_2O was observed in MS^3 of *m/z* 276 from both precursor ions (data not shown). Loss of water is a characteristic feature observed in MS spectra of cytidine and uridine glycols [33, 34]. Rivière et al. [35] proposed that oxidation of the DNA analogue 5-hydroxy-2′-deoxycytidine (oh^{5}dC) initially gives isodialuric or dialuric acid derivatives of oh^{5}dC and that these intermediate products subsequently undergo deamination to produce isodialuric or dialuric acid derivatives of 5-hydroxy-2′-deoxyuridine (oh^{5}dU). In the case of oxidation of oh^{5}C by PGC, adhering to the nitrogen rule, the even masses of the observed oxidation products and fragment ions in this experiment indicate intact cytidine glycols and not deamination. Hiraoka et al. [36] observed lack of deamination when the cytosine base was hydroxylated at C-5 or C-6, further corroborating the intact cytidine glycols observed here. Substituting formic acid with acetic acid in the eluents gave a single chromatographic peak with dominating ions at *m/z* 294 and 336 corresponding to oxidation to a glycol and an acetic acid conjugate (data not shown). Direct infusion of the oh^{5}C standard gave a single peak at *m/z* 260 with the expected neutral loss of 132 in MS/MS and no evidence of either *m/z* 294 or 322, indicating that oxidation does not occur during the electrospray process.

Based on the analytical evidence presented above, we propose that oh^{5}C is oxidized to its corresponding glycols and acid conjugates when analyzed on PGC. Oxidation reactions of nucleobases in DNA is hypothesized to play a major role in mutagenesis and cancer, and as many as 30 modifications of cytosine have been characterized from studies of the nucleobase, the nucleoside, and DNA [32].

PGC has known redox characteristics and has been shown to be oxidizing in its native state [17, 37]. Reduction of PGC is usually achieved by adding sodium sulfite to the eluents, and PGC has been used for on-column electrochemical redox derivatization to enhance separation selectivity [38, 39]. The use of a sodium salt in eluents for LC/MS is not desirable and the oxidizing activity of PGC is recovered once the reducing agent is removed, eliminating the option of off-line treatment of the column with sodium sulfite prior to analysis of several samples in sequence. We found that flushing of the PCG chip continuously for 30 min with a 2.5 mM oxalic acid and subsequently adding 1 mM oxalic acid to the two aqueous online buffers can keep PGC in a reduced state, thus preventing oxidation of oh^{5}C. When reanalyzing the nucleoside test mix under reduced chromatographic conditions, oh^{5}C is readily identified in the XIC of *m/z* 260 with a retention time of 12.9 min, Figure 2 and Table 1. Initial fragmentation of *m/z* 260 gave neutral loss of ribose to the single ion of the nucleobase with *m/z* 128, Figure 3. In MS^3, three dominating ions of *m/z* 111, 101, and 100 corresponding to loss of NH_3, HCN, and CO,

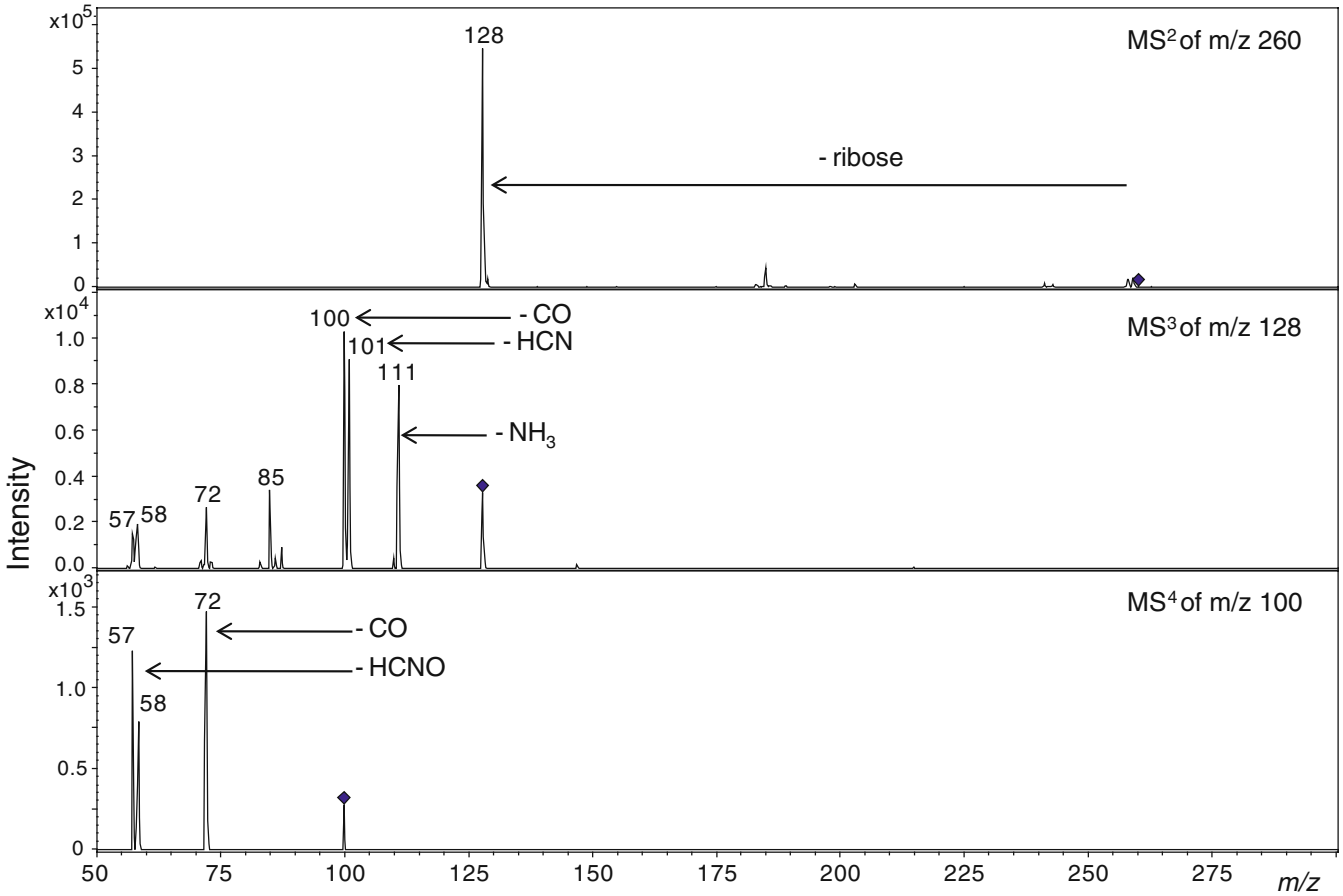

Figure 3. Tandem mass spectra of oh^{5}C analyzed on reduced PGC using oxalic acid as an online reducing agent

respectively, were observed along with minor ions of *m/z* 85, 72, and 57/58. The ion-trap only allows selection of one precursor ion for MS stages greater than two. MS^4 of *m/z* 100, the most intense ion from MS^3, gave product ions of *m/z* 72 and 57 corresponding to loss of CO and HNCO, respectively. This online MS^4 fragmentation pattern is identical to the one observed for *m/z* 100 of the nucleobase of oh^{5}dC in a direct infusion experiment reported by Cao and Wang [40].

To our knowledge, this is the first time oxalic acid has been used as an online reducing agent for LC/MS. As evident from Table 1, reducing conditions influenced chromatographic retention characteristics of all components in the test mixture. The overall retention mechanism on PGC is determined by the balance of two factors: a hydrophobic or dispersive interaction as for reverse phases sorbents and charged-induced interactions of a polar analyte with the polarizable surface of the graphite [16, 41]. The second effect is often referred to as the "polar retention effect by graphite (PREG)" [42]. The explanation for PREG is still quite vague, but it probably depends on the orientation of polar functional groups at the surface of the graphite and, therefore, on the three-dimensional structure of the analyte [17]. Adding 1 mM oxalic acid to the eluents did not seem to cause ion suppression as commonly observed when using acetic acid or TFA. On the contrary, an overall increase in intensities of XIC was observed, possibly due to improvements in peak shape brought on by reduction of secondary chromatographic effects by reduction of PGC with oxalic acid.

To explore the general applicability of the method, two commercially available tRNAs, tRNA^{Phe} and tRNA^{TyrII}, were hydrolyzed and submitted to LC/MS, Table 2. Differences in observed retention times between nucleosides common to both tRNA^{Phe} and tRNA^{TyrII} reflect day-to-day analytical variability. Fourteen of the 15 known tRNA^{Phe} nucleosides were readily identified by their distinct retention time on PGC and MS^n fragmentation characteristics, Table 2; only wybutosine (yW) was not observed. yW is a member of the tricyclic 'wye' family of ribonucleosides originating from tRNA [4, 43]. yW is one of the most complexly modified guanosine residues. It contains a three-ringed aromatic 'wye' base substituted at C7 with a methionine related side chain. We hypothesize that the polar and aromatic structure of yW makes it strongly retained on PGC so that it does not elute during a single gradient run. After prolonged flushing of the PGC chip with 90% acetonitrile yW was observed as a continuously eluting low intensity peak at *m/z* 509 (data not shown) further supporting the strong retention hypothesis. Increasing the

Table 2. Nucleosides in tRNA^{Phe} (*S. cerevisiae*) and tRNA^{TyrII} (*E. coli*)

Nucleoside	Retention time min tRNA^{Phe}	Retention time min tRNA^{TyrII}	MH$^+$ *m/z*	Fragment ions MS^n [a]
5,6-Dihydrouridine (D)	8.0		247	**115**, 98, 97, 73, 69
Cytidine (C)	10.6	10.5	244	**112**, 95, 69
Pseudouridine (Ψ)	12.0	11.6	245	209, 191, 163
4-Thiouridine (s^{4}U)		12.3	261	**129**[b]
2′-*O*-methylcytidine (Cm)	12.9		258	**112**, 95, 83, 69
1-Methyladenosine (m^{1}A)	13.0		282	**150**, 133, 109, 94
5-Methylcytidine (m^{5}C)	13.3		258	**126**, 109, 108, 83, 81
Uridine (U)	13.5	13.1	245	**113**, 96, 70
2-Methylguanosine (m^{2}G)	15.5		298	**166**, 149, 124
5-Methyluridine (m^{5}U)	16.5	15.9	259	**127**, 110, 82
Queuosine (Q)		17.3	410	295, 163, 121, 93,67
Adenosine (A)	18.1	18.0	268	**136**, 119, 109, 94
Guanosine (G)	19.7	20.4	284	**152**, 135, 110, 107, 80
2′-*O*-methylguanosine (Gm)	21.0	20.9	298	**152**, 135, 128, 110
7-Methylguanosine (m^{7}G)	23.7		298	**166**, 149, 121, 94
N^2,N^2-Dimethylguanosine (m$_2^2$G)	25.5		312	**180**, 162, 153, 137, 122, 110
Wybutosine (yW)	6.2[c]		509	**377**, 345, 216, 201, 173, 120
2-Methylthio-N^6-isopentenyladenosine (ms^{2}i^{6}A)		8.9[c]	382	**250**, 194, 182, 165

[a] MS^2 BH$_2$+ ion is marked in bold.
[b] low intensity peak.
[c] Analyzed on C18 chip.

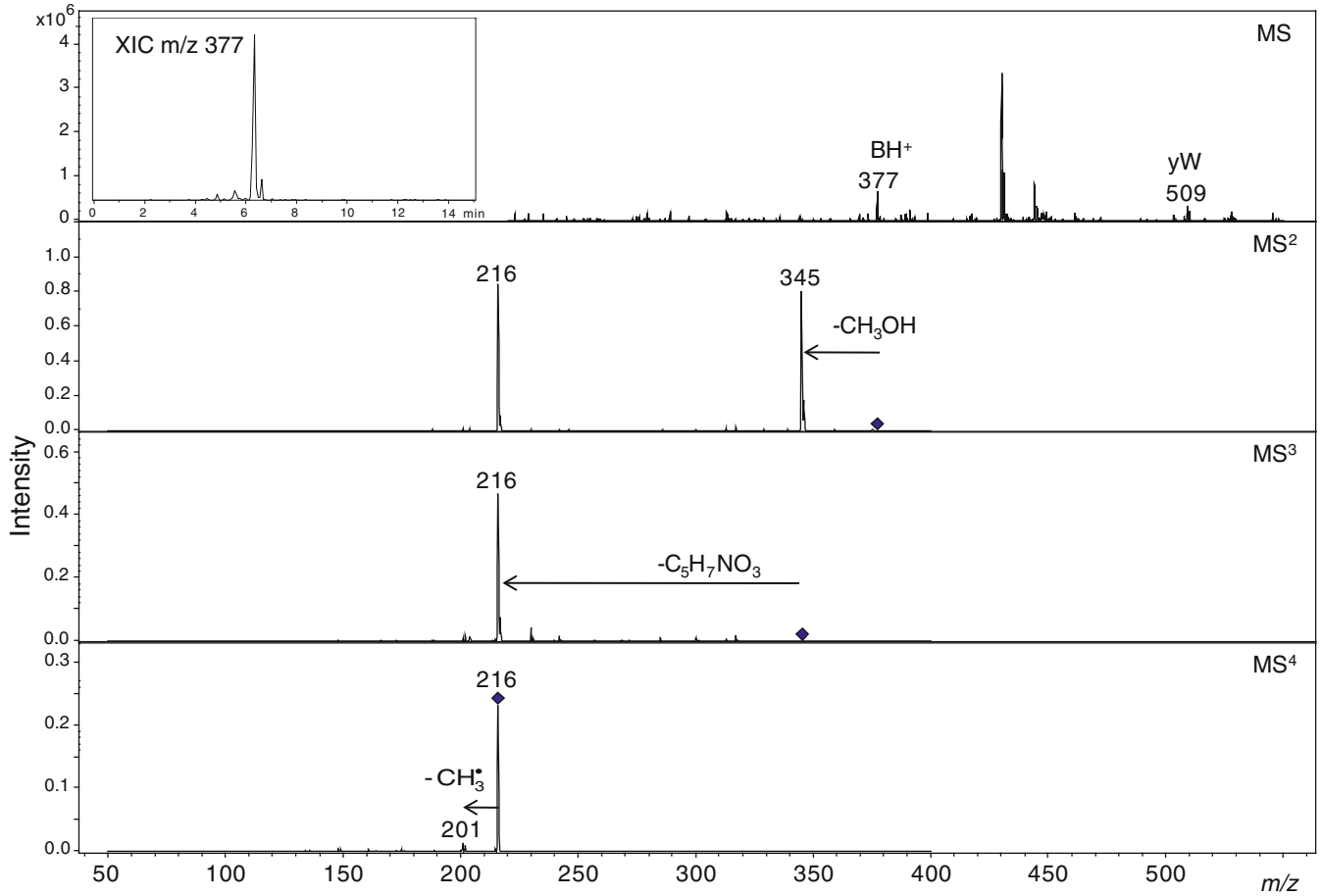

Figure 4. Hydrolyzed tRNA^{phe} analyzed on a 43 mm Zorbax C18 chip. Flow rate 300 nL/min, gradient 2%–90% acetonitrile, 0.1% FA over 15 min. All other settings as for PGC, see text for details

elutropic strength of the organic solvent by substituting acetonitrile with acetone did not solve the problem (data not shown). Acetone is a three times stronger solvent than acetonitrile and can, if UV detection is not required, successfully replace acetonitrile and methanol as organic modifier in LC/MS [44]. Neither adding 5% tetrahydrofuran (THF) nor operating the PGC chip under oxidized conditions nor at pH 6 could elute yW as a distinct chromatographic peak from the PGC chip.

Identification of yW was achieved by re-injecting the hydrolyzed tRNA on a Zorbax SB C18 small-molecule chip, Figure 4. For ease of use, the acidic solvent system was maintained to avoid flushing and re-equilibrating the LC system and chip with a buffer system at neutral pH. Since yW is acid labile [4], it is predominantly observed as the nucleobase at *m/z* 377 eluting at 6.2 min with a low intensity peak of the intact nucleoside at *m/z* 509. Sequential ion-trap fragmentation of the nucleobase *m/z* 377 gave peaks at *m/z* 345 in MS^2 and *m/z* 216 in MS^3 corresponding to loss of CH_3OH and $C_5H_7NO_3$ respectively, Figure 4. In MS^4, homolytic cleavage with loss of CH_3-radical is observed from *m/z* 216. Zhou el al. [45] observed *m/z* 345 and 216 in fragment ion spectra of wybutosine from tRNA^{Phe} from yeast using a single stage MS/MS triple quadrupole instrument. The authors also observed facile loss of a CH_3-radical from other protonated wye bases and explained this uncommon loss by charge stabilization and radical delocalization through the nitrogen-rich tricyclic ring [45].

Nine of the 10 known tRNA^{TyrII} nucleosides were identified by their distinct retention time on PGC and MS^n fragmentation characteristics, Table 2. As observed for yW, 2-methylthio-*N*^{6}-isopenthyladenosine (ms^{2}i^{6}A) in tRNA^{TyrII} was not observed when analyzed using reduced PGC but required reanalysis on C18 for complete MS^4 identification (see Figure S-3). Queuosine a hypermodified guanosine nucleoside present in tRNA^{TyrII} with *m/z* 410 was identified by MS^4 (see Figure S-4) using PGC, indicating that the bimodal retention mechanism of graphite is not entirely controlled by molecular size and aromatic carbon content of the analyte.

Conclusion

We have shown that the different retention and separation properties provided by PCG makes it a suitable stationary phase for characterization of modified nucleosides by ion-trap LC-MS^n under nano-flow conditions. Resolution and sensitivity of the nano-chip system provided complete characterization of isobaric nucleosides in the low fmol range. Furthermore, we have shown that online reduction of PGC with oxalic acid has significant impact on chromatography as well as analysis of easily oxidized nucleosides without compromising resolution or sensitivity. A combination of PGC and C18 reverse phase chromatography is required for complete characterization of both hydrophilic and hydrophobic nucleosides derived from a sample such as a tRNA. However, the ease of use of the automated chip system and the identical solvent configuration used, combined with high sensitivity, makes it an ideal system for routine analysis of nucleosides. Furthermore, adapting a neutral loss data analysis strategy makes it an ideal system for mapping of unknown modifications from biological matrices were limited sample is obtainable.

Acknowledgments

The authors thank Jesper Havelund and Anette Rasmussen for invaluable laboratory assistance, and Frank Kjeldsen for assisting with the Orbitrap analysis. The authors also thank Dayin Lin and Rudolf Grimm at Agilent for providing chips and support.

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