Journal of Chromatography A, 1218 (2011) 3994–4001

Contents lists available at ScienceDirect

Journal of Chromatography A

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

Study of retention behaviour and mass spectrometry compatibility in zwitterionic hydrophilic interaction chromatography for the separation of modified nucleosides and nucleobases

Encarnación Rodríguez-Gonzalo*, Diego García-Gómez, Rita Carabias-Martínez

Departamento de Química Analítica, Nutrición y Bromatología, Facultad de Química, Universidad de Salamanca, 37008 Salamanca, Spain

ARTICLE INFO

Article history:
Received 22 March 2011
Received in revised form 14 April 2011
Accepted 3 May 2011
Available online 11 May 2011

Keywords:
Zwitterionic hydrophilic interaction chromatography (ZIC-HILIC)
Sulfobetaine stationary phase
Modified nucleosides and nucleobases
Methylguanines
Hydroxyguanosines
Oxidative stress

ABSTRACT

A study has been made of the chromatographic behaviour of modified nucleosides and nucleobases using different stationary phases with functional groups of polar nature, all of them compatible with aquoorganic mobile phases. The stationary phases assayed were a pentafluorophenylpropyl (PFP) column for reverse phase separation, and another two for hydrophilic interaction chromatography (HILIC) separation. Six modified nucleosides and nucleobases (hydroxylated and methylated derivatives) were chosen as the target analytes. In the study, chromatographic resolution as well as the sensitivity in detection by mass spectrometry were taken into account. The results obtained showed that the zwitterionic (ZIC-HILIC) column was the most suitable one for the separation of these analytes. From the study of the different parameters affecting separation it may be concluded that in the ZIC-HILIC column separation is based on a mechanism of partition and interaction through weak electrostatic forces.

1. Introduction

Oxidative stress is defined as cellular damage caused by reactive oxygen species (ROS). These species are continually being produced as a result of the cell's metabolism and their production may be increased as a result of exposure to xenobiotics or to radiation [1]. ROS interact with and modify several biomolecules, especially DNA, lipids and proteins [1]. DNA lesions are of special importance because they can alter the sequence of nucleotides. The natural mechanisms for DNA repair involve the excision of the damaged bases or nucleosides [2]. The products thus released cannot be reused and circulate freely through the blood until they are eliminated in the urine [3]. Additionally, the action of hydrolytic enzymes such as ribonucleases and phosphatases releases normal and modified nucleosides during RNA turnover [4], a process that is enhanced by the presence of different types of tumours [5]. Thus, modified nucleosides and nucleobases (MNN) arise mainly from the post-transcriptional modification of the nucleosides forming transfer RNA (tRNA) [6]. Around 100 MNN related to these metabolic processes have been detected to date [7].

Studies have addressed the potential of MNN as markers of several diseases [8,9], and, mainly, as early markers of different types of cancer [10–14]. Other studies have related the urinary concentration of MNN to different life styles [15].

Among the most widely studied MNN are the methylation or hydroxylation products of guanosine; this is because they are the most abundant [16–18]. Simultaneous determination of both groups of MNN (hydroxylated and methylated) is of great interest since the mechanisms of mutagenesis and carcinogenesis brought about by the oxidation and methylation processes are different [19].

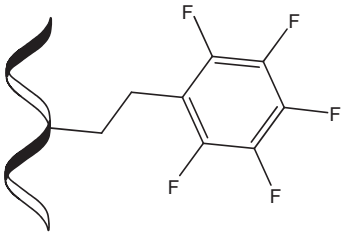
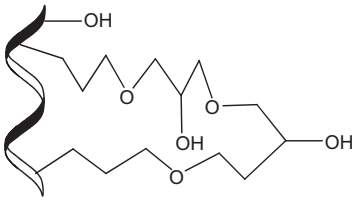
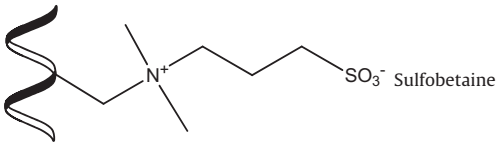
The analytical techniques most widely employed for the simultaneous determination include capillary electrophoresis [20], immunoassay [21] and, mainly, liquid chromatography (HPLC) [22]. UV detection [23] has been replaced by mass spectrometry (MS) [13,24], which allows a surer identification and confirmation as well as affording structural information about the compounds being assayed.

The analysis of highly polar compounds, such as MNN, involves important difficulties in reverse phase liquid chromatography (RPLC) owing to the poor retention of these analytes in the stationary phase. Normal phase liquid chromatography (NPLC) would afford a better retention but is not desirable owing to the difficulty involved in coupling this chromatographic technique with mass spectrometry. Hydrophilic interaction chromatography (HILIC) is a viable alternative to RPLC for the separation of polar compounds. HILIC, a term coined by Alpert [25], is based on the use of polar stationary phases combined with mobile phases with a high organic content and a small amount of water. In addition, HILIC has the

* Corresponding author. Fax: +34 923 294 574.
E-mail address: erg@usal.es (E. Rodríguez-Gonzalo).

0021-9673/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2011.05.003

Table 1
Characteristics of the chromatographic columns used for the analysis of the modified nucleosides and nucleobases.

Chromatographic column	Dimensions (mm)	Pore size (Å)	Particle size (μm)	Stationary phase
Luna PFP(2)	150 × 4.6	100	3	 Pentafluorophenylpropyl
Luna HILIC	150 × 4.6	200	3	 Cross-linked diol
ZIC-HILIC	150 × 4.6	100	3.5	 Sulfobetaine

advantage of enhanced detection sensitivity when used in conjunction with MS due to the high organic content of the mobile phase, which allows efficient spraying and desolvation in electrospray ionization (ESI). The applications of HILIC include the analysis of different polar molecules, especially those of biological nature [26]. In recent years, new possibilities have emerged with the development of stationary phases specific to HILIC, such as zwitterionic hydrophilic interaction chromatography (ZIC-HILIC) columns [27].

The aim of the present work was to study the chromatographic behaviour shown by different MNN, compounds of similar and very high polarity, using a pentafluorophenylpropyl (PFP) reverse phase column and two polar columns based on cross-linked diol (Luna-HILIC) and sulfobetaine (ZIC-HILIC). The MNN selected were 8-hydroxy-guanine, 8-hydroxy-guanosine, 8-hydroxy-2′-deoxyguanosine, 1-methyl-guanine, 7-methyl-guanine and 9-methyl-guanine. The ZIC-HILIC column showed the highest selectivity. The optimum conditions were chosen bearing in mind resolution and sensitivity in later detection by means of mass spectrometry. Using this column, a detailed study was performed to elucidate the mechanisms involved in the retention of the analytes.

In sum, this study explores the benefits and limitations of using HILIC for the analysis of MNN. To our knowledge, no other work has attempted to study the mechanisms of retention, for the separation of MNN using ZIC-HILIC.

2. Experimental

2.1. Chemicals

Analytical standards of *8-hydroxy-guanine* (8OHGua), CAS RN [5614-64-2] and *8-hydroxy-guanosine* (8OHG), CAS RN [3868-31-3] were purchased from Cayman Chemical (Michigan, USA). *8-hydroxy-2′-deoxyguanosine* (8OH2dG), CAS RN [88847-89-6]; *9-methyl-guanine* (9mGua), CAS RN [5502-78-3]; *1-methyl-guanine* (1mGua), CAS RN [938-85-2] and *7-methyl-guanine* (7mGua), CAS RN [578-76-7] were obtained from Sigma–Aldrich (Steinheim, Germany).

The organic solvents – acetonitrile (ACN) and methanol (MeOH) – were of HPLC grade (Merck, Darmstadt, Germany) and were used as received. Ultra-high quality (UHQ) water was obtained with a Wasserlab (Noain, Spain) Ultramatic water purification system. All other chemicals were of analytical reagent grade.

2.2. Instrumentation

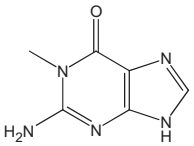
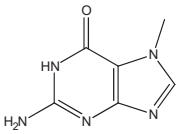
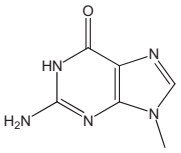
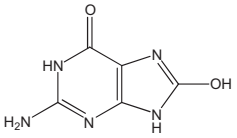
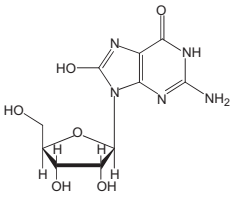
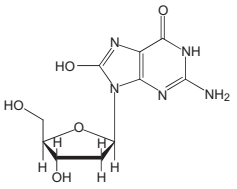
HPLC analyses were performed on a HP 1100 Series chromatograph from Agilent (Waldbronn, Germany). The Diode Array Detector (DAD) recorded the spectra in the 190–400 nm range. The Agilent single quadrupole LC/MSD SL mass spectrometer was equipped with an electrospray (ESI) source. The analytical columns were a reverse phase Luna PFP(2) packed with 3 μm particles and a Luna HILIC packed with 3 μm particles from Phenomenex (Torrance, CA, USA) and a ZIC-HILIC packed with 3.5 μm particles from Merck (Darmstadt, Germany). The characteristics of the chromatographic columns used are shown in Table 1. ${}_{w}{}^{w}$pH values were determined with a 691 pH Meter from Metrohm (Herisau, Switzerland).

2.3. Preparation of standards

The initial stock solutions for the 1mGua, 7mGua, 9mGua (500 μg mL^{-1}) and for the 8OH2dG and 8OHG standards (150 μg mL^{-1}) were prepared in acidified UHQ water. The stock solution for 8OHGua (150 μg mL^{-1}) was prepared in 0.1 M sodium hydroxide. These stock solutions were stored at 4 °C in brown glass bottles. Working solutions were prepared daily at 5 μg mL^{-1} for 1mGua, 7mGua and 9mGua, and 3 μg mL^{-1} for 8OH2dG, 8OHG and 8OHGua.

The mobile phase was a mixture of organic solvent and an aqueous medium at different pH values and with different concentrations of salts. To measure ${}_{w}{}^{w}$pH values, the pH meter was calibrated using aqueous buffers and pH measurements were performed before the addition of organic solvent. The apparent ${}_{w}{}^{s}$pH, after adding the organic solvent, was not measured.

Table 2
Physical properties of the modified nucleosides and nucleobases analyzed.

Modified nucleosides and nucleobases	Mw	log $D^{a}_{pH\,3}$	Structure
1-Methyl-guanine (1mGua)	165.07	−1.4	
7-Methyl-guanine (7mGua)	165.07	−1.8	
9-Methyl-guanine (9mGua)	165.07	−1.4	
8-Hydroxy-guanine (8OHGua)	167.04	−1.5	
8-Hydroxy-guanosine (8OHG)	299.09	−1.9	
8-Hydroxy-2′-deoxy-guanosine (8OH2dG)	283.09	−1.3	

[a] Calculated using ChemBioDraw Ultra 12.0 from CambridgeSoft by Crippen's and Viswanadhan's fragmentations and Advanced Chemistry Development (ACD/Labs) software.

3. Results and discussion

The high number of known MNN (approx. 100) means that some of them must be selected as a model to study their behaviour in HILIC. In the present case, we chose hydroxylated and methylated forms because these are the commonest modifications. As the base structure, we chose guanine, the most studied and abundant base as regard a possible relationship between the modified nucleosides and different diseases. Some of the physical and chemical properties of the six MNN studied are shown in Table 2. The pK_a values reported in the literature for these compounds lie within a broad interval. This is because the nucleobases and their derivatives present multiple tautomers with different site-specific microscopic pK_a values. Thus, values of 3.1–3.3 for guanine and values of −0.1 to 0.2 for hydroxylated-MNN were found [28,29]. In addition, pK_a values were experimentally calculated by UV spectrophotometry [30], measuring absorbance at different $_w{}^w$pH values in a 80% ACN: 20% H_2O medium; values of 3.2, 3.3 and 3.9 for 7mGua, 9mGua and 1mGua, respectively, were found. However, it was not possible to determine the pK_a values for the hydroxylated-MNN.

3.1. Reversed phase chromatography and HILIC for the separation of modified nucleosides and nucleobases

Using the three chromatographic columns indicated in Section 2, a study was made of the chromatographic behaviour of the six analytes selected (Fig. 1).

A mobile phase with an aqueous phase of 90% in RPLC (Fig. 1a$_1$) did not afford a satisfactory separation of the analytes, retention being almost completely absent ($k < 0.8$) for all the MNN.

It was necessary to increase the aqueous phase up to 95% (Fig. 1a$_2$) to obtain significant retentions, and even under these conditions the coelution of two sparingly retained analytes occurred ($0.2 < k < 2.2$).

When a Luna HILIC column was used employing 20% formic acid 2.6 mM ($_w{}^w$pH = 3.1): 80% ACN, the analytes exhibited greater retention than in RPLC ($k < 1.2$), but the resolution was not satisfactory (Fig. 1b$_1$). A decrease in the proportion of aqueous solvent to 10% (Fig. 1b$_2$) allowed appropriate retentions to be obtained ($1.6 < k < 3.0$), although two pairs of MNN coeluted.

The ZIC-HILIC column with a mobile phase containing 20% of aqueous phase (Fig. 1c) afforded suitable retentions ($1.6 < k < 3.2$).

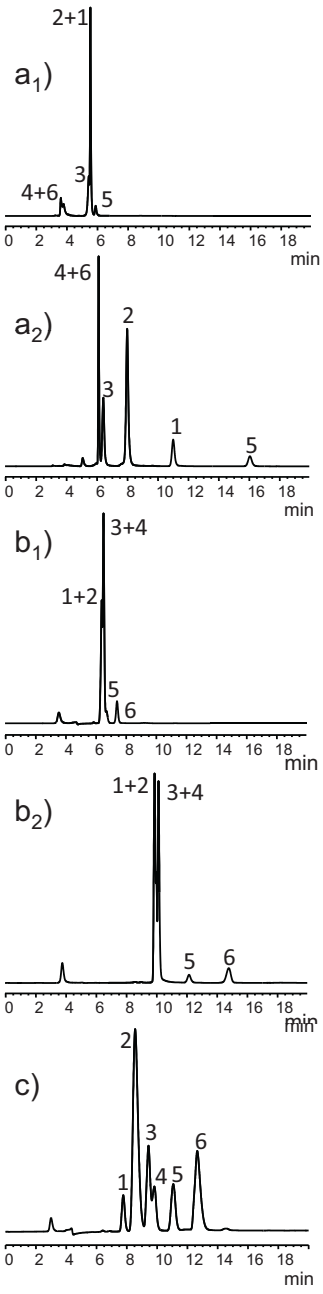


Fig. 1. UV chromatograms obtained by the analysis of the modified nucleosides and nucleobases using isocratic elution. By RPLC using 90% formic acid 2.6 mM $_{w}^{w}$pH = 3.1: 10% ACN (a_1) and 95% formic acid 2.6 mM $_{w}^{w}$pH = 3.1: 5% ACN (a_2). By Luna HILIC using 20% formic acid 2.6 mM $_{w}^{w}$pH = 3.1: 80% ACN (b_1) and 10% formic acid 2.6 mM $_{w}^{w}$pH = 3.1: 90% ACN (b_2). By ZIC-HILIC using 20% formic acid 2.6 mM $_{w}^{w}$pH = 3.1: 80% ACN (c). Analyte identification: (1) 8OH2dG, (2) 9mGua, (3) 1mGua, (4) 8OHGua, (5) 8OHG and (6) 7mGua. Column temperature: 20 °C. Chromatographic flow rate: 0.5 mL min^{-1}. Detection: DAD 254 nm.

The high selectivity of the ZIC-HILIC column should be noted, since it resolved the six MNN in a total analysis time of less than 14 min.

For all six compounds studied, the order of elution in RPLC was different from that observed in Luna HILIC and ZIC-HILIC columns. In the latter two, hydrophilic interactions play an important role in the separation. No correlation was observed between the number of hydroxyl groups of the molecule and the retention observed, a relationship, however, that has been found for other molecules [31].

For the analytes studied, the best conditions as regard separation were obtained with RPLC (separation of five MNN) and with ZIC-HILIC (separation of all six MNN). However, with RPLC it was necessary to use a high water content in the mobile phase, usually above 90% (Fig. 1a_2). This has a negative effect in mass spectrometry when it is used as the method of detection because solvents with a high water content produce a less efficient ionization in the electrospray ionization (ESI). Table 3 shows the signal ratio obtained, using mass spectrometry with a single quadrupole, when the chromatographic eluent has a water content of 20% (ZIC-HILIC separation) or of 95% (RPLC separation). The relationship between both signals varied by one order of magnitude, between 10- and 23-fold (ratio I/II). Likewise, the signal/noise ratios observed (data not shown) were between 8- and 26-fold higher with ZIC-HILIC than with RPLC.

3.2. Separation of modified nucleosides and nucleobases in ZIC-HILIC

In light of the results obtained, the ZIC-HILIC column was selected for all the later studies since it seems to offer the optimum possibilities for analysis of the MNN. Alpert proposed a partition mechanism between aqueous layer associated with the stationary phase, and the organic phase to explain separation in HILIC [25]. However, later studies have shown that the mechanism of retention involves more complex equilibria [32]. Moreover, the presence of charged sites in the stationary phase in ZIC-HILIC would propitiate the appearance of other retention mechanisms. All these factors prompted us to study the different factors that could affect the separation of the MNN.

3.2.1. Effect of the nature and content of the organic phase

A study was made of analyte separation in a typical protic polar solvent, in this case MeOH, and an aprotic polar solvent, ACN. MeOH can act as a donor and an acceptor of hydrogen bonds, whereas ACN can act as an acceptor of hydrogen bonds and furthermore provides stronger dipole–dipole interactions. It has been suggested that protic polar solvents are able to compete for the polar sites of the stationary phase disturbing the formation of the aqueous layer required for the partition mechanism [33]. This leads to a more hydrophobic aqueous stationary phase, whose consequence is a poor retention of analytes with a high capacity to form hydrogen bonds. The results obtained, upon varying composition of the mobile phase for ACN or MeOH/2.6 mM formic acid (pH: 3.1) mixtures, show that for all the analytes MeOH acted as a stronger solvent than ACN. Thus the retention factors were always lower with MeOH, although the order of elution persisted in both cases. Optimum separation was achieved with a mobile phase made up of 80% ACN: 20% formic acid 2.6 mM ($_{w}^{w}$pH = 3.1). For higher percentages of ACN (>80%), very strong retentions occurred and the analysis time being more than 40 min for a percentage of 85% of ACN and 75 min for a percentage of 90%.

3.2.2. Effect of buffer pH

The nature of the stationary phase in ZIC-HILIC restricts the range that can be used to pH 3–8. We assessed its performance from $_{w}^{w}$pH = 3.1 (2.6 mM formic acid) to 6.7 (2.5 mM ammonium formate) with intermediate points at 3.8, 4.7 and 5.7 (2.5 mM formic acid/ammonium formate buffer, setting ammonium concentration to 2.5 mM with ammonium perchlorate). Fig. 2 shows the chromatograms obtained at $_{w}^{w}$pH: 3.1, 4.8 and 6.7. In these experiments, confirmation of the identity of the analytes was achieved with ESI-MS. Comparison of the chromatograms at $_{w}^{w}$pH = 3.1 (Fig. 2a) and 4.8–6.7 (Fig. 2b and c) allowed us to conclude that hydroxylated-MNN were not charged in the range of pH from 3.1 to 6.7. However, at $_{w}^{w}$pH = 3.1, methylated-MNN were positively charged as can be deduced from the variation in their retention times. This theory is supported by the pK_a values found in the literature [28,29], and by those pK_a values obtained experimentally by us. The retention of these MNN is greater in the positively

Table 3
Comparison between mass spectrometric signals of the modified nucleosides and nucleobases studied using different elution solvents in ZIC-HILIC and RPLC separation.

Modified nucleosides and nucleobases	MS signal[a]			Ratio I/II	Ratio I/III
	ZIC-HILIC acid[b] (I)	RPLC acid[c] (II)	ZIC-HILIC neutral[d] (III)		
8OH2dG	4.6×10^7	2.0×10^6	4.6×10^6	23	10
9mGua	3.0×10^9	3.0×10^8	4.1×10^8	10	7.3
1mGua	7.2×10^8	3.2×10^7	5.1×10^7	22	14
8OHGua	1.2×10^8	8.5×10^6	1.3×10^7	14	9.4
8OHG	2.5×10^8	1.1×10^7	3.0×10^7	23	8.3
7mGua	4.0×10^8	2.6×10^7	3.3×10^7	15	12

[a] ESI settings: capillary voltage: +3500 V; drying gas flow: 10 L min^{-1}; temperature: 350 °C; nebulizer pressure: 50 psi. Signals as chromatographic areas in Selected Ion Monitoring (SIM) mode for: 284, 166, 168 and 300 *m/z*.
[b] Mobile phase: 20% 2.6 mM formic acid ($_w{}^w$pH = 3.1): 80% ACN.
[c] Mobile phase: 95% 2.6 mM formic acid ($_w{}^w$pH = 3.1): 5% ACN.
[d] Mobile phase: 20% 2.6 mM ammonium formate ($_w{}^w$pH = 6.7): 80% ACN.

charged form (Fig. 2a) than in their neutral form (Fig. 2b and c). This behaviour is consistent with a mechanism based on partition, since the protonated forms would be more soluble in the aqueous phase retained on the stationary phase. Likewise, the electrostatic interactions with the residue of sulfobetaine of the stationary phase would be greater in the case of positively charged molecules. The electrostatic interactions would thus occur mainly with the terminal sulfonic group and not with the interior ammonium group, which is clearly sterically hindered.

Regarding the separation of the MNN, it can be accomplished at any $_w{}^w$pH values from 3.1 to 6.7, but better resolution was obtained at more acidic pHs. Moreover, the electrospray ionization is much more favourable if the analytes arrive at the ionization system at a $_w{}^w$pH where they are easily charged. Thus, it is seen that a change in $_w{}^w$pHs from 6.7 to 3.1, maintaining the same concentration of salts, elicited an increase in the signal varying between 7.3- and 14-fold (Table 3, ratio I/III).

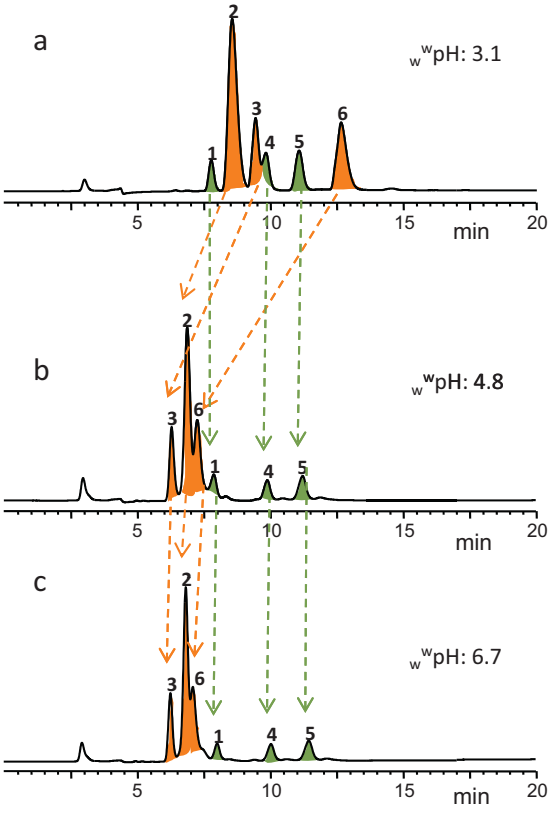


Fig. 2. UV chromatograms, normalized to the highest peak, obtained using isocratic elution 20% formic acid: 80% ACN at $_w{}^w$pH = 3.1 (a); 20% formic acid/ammonium formate buffer 2.5 mM: 80% ACN at $_w{}^w$pH = 4.8 (b); and 20% ammonium formate 2.5 mM: 80% ACN at $_w{}^w$pH = 6.7 (c). Analyte identification as in Fig. 1. Column temperature: 20 °C. Chromatographic flow rate: 0.5 mL min^{-1}. Detection: DAD 254 nm. Peak identification confirmed by ESI-MS.

3.2.3. *Effect of the salt concentration*

Owing to the high organic content of the mobile phase in ZIC-HILIC, the number of salts available is limited to those showing an acceptable solubility in organic medium, like perchlorate or organic salts.

In HILIC columns, the increase in salt concentration usually leads to an increase in retention [34]. Additionally, other effects may appear in ZIC-HILIC [35,36], since when working at low salt concentrations a displacement equilibrium is established between charged analytes and the cations of the mobile phase in the stationary phase (ion exchange).

These effects were assessed varying the salt concentration from 0.25 to 200 mM for ammonium perchlorate and from 0.25 to 5.0 mM for potassium perchlorate (because of its poor solubility) at $_w{}^w$pHs of 3.1 and 6.7.

At high salt concentrations, above 50 mM (Fig. 3a), an increase in retention occurs for all MNN at any $_w{}^w$pHs, in agreement with the characteristics of a partition mechanism. The high organic content of the mobile phase led the salts to be located mainly in the aqueous phase bound to the stationary phase of the column. Thus, high concentrations could have produced an increase in the volume or hydrophilicity of the aqueous layer, attracting the solvated MNN and causing a greater retention of the analytes.

At low salt concentrations, methylated-MNN at a $_w{}^w$pH where they are not charged (Fig. 3a: 7mGua, $_w{}^w$pH = 6.7) and hydroxylated-MNN at any $_w{}^w$pH (Fig. 3a and b: 8OH2dG) keep relatively constant retention values. However, for positively charged methylated-MNN (Fig. 3a and b: 7mGua, $_w{}^w$pH = 3.1), an increase in retention occurs, indicating that if analytes are in its protonated form, a electrostatic interaction occurs. Starting out from equilibrium (1), whose equilibrium constant is K, where BH^+ are the charged analytes, whose acid–base ionization constant is K_a, and M^+ represents the cations of the salts present:

$$BH^+ + RSO_3{}^-M^+ \leftrightarrow RSO_3{}^-BH^+ + M^+ \quad (1)$$

It has been reported [32] that electrostatic interactions fit Eq. (2):

$$k_{el} = A\frac{1}{[M^+]} \quad (2)$$

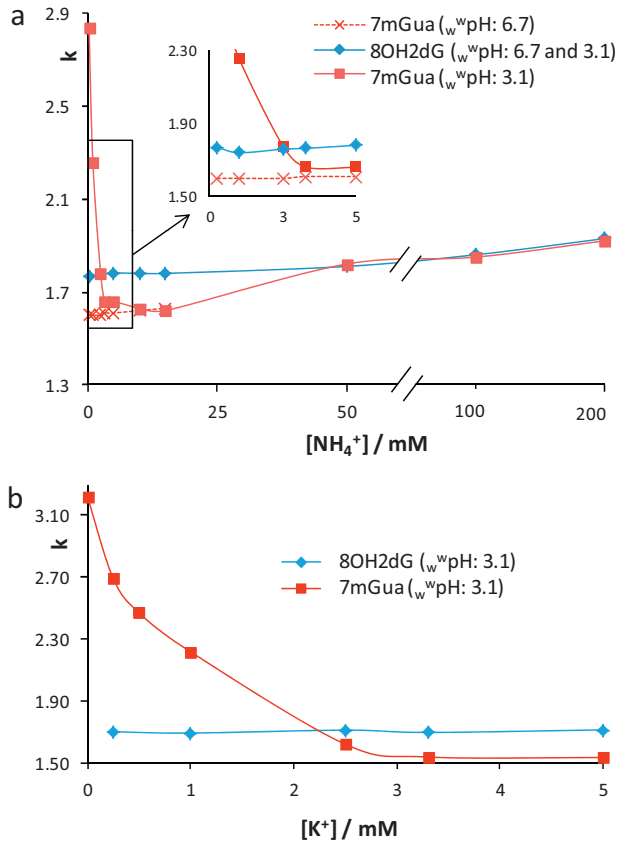


Fig. 3. Retention factors for 7mGua and 8OH2dG, plotted versus salt concentration, obtained using 20% ammonium perchlorate: 80% ACN (a) and 20% potassium perchlorate: 80% ACN (b). Column temperature: 20 °C. Chromatographic flow rate: 0.5 mL min^{-1}.

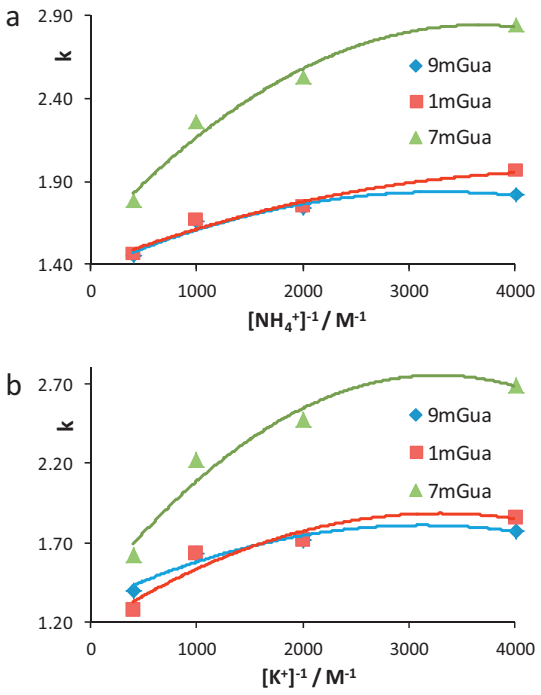


Fig. 4. Retention factors for methylated-MNN, plotted versus the inverse of salt concentration, obtained using 20% ammonium perchlorate (with formic acid, 2.6 mM at $_w$^{w}pH = 3.1): 80% ACN (a), and 20% potassium perchlorate (with formic acid 2.6 mM at $_w$^{w}pH = 3.1): 80% ACN (b). Column temperature: 20 °C. Chromatographic flow rate: 0.5 mL min^{-1}.

where A is a constant similar to that described by McCalley [32]:

$$A \propto \frac{K[\mathrm{RSO_3^-M^+}]}{1 + Ka/[\mathrm{H^+}]} \quad (3)$$

Accordingly, a plot of the experimental retention factors (k_{exp}) against the inverse of the salt concentration ($[M^+]^{-1}$) should be a straight line, proportional to A, with the ordinate at the origin being zero if electrostatic interactions are the only retention mechanism (Eq. (2)). Experimentally, the values found for ammonium and potassium salt concentrations of 0.25–2.5 mM (corresponding to $[M^+]^{-1}$ values ranging from 4000 to 400 M^{-1}) fitted a second-order polynomial equation (Fig. 4). The presence of other non-electrostatic retention mechanisms (e.g. partition mechanism) was indicated by the fact that values for the ordinate at the origin are different from zero and a straight line was not obtained.

The results obtained show that the presence of salt and the increase in its concentration do not elicit any significant improvement in the separation of the MNN. If it is also considered that the presence of salts can increase ion suppressions in the possible detection by mass spectrometry, sensitivity thus decreasing, it is possible to set 2.6 mM formic acid in the absence of salts as the most appropriate medium for these analytes.

3.2.4. Effect of temperature

The Van't Hoff equation has been proposed as a way to relate the retention factor and temperature. Likewise, it has been suggested that if the retention mechanism in HILIC is a mechanism of partition, then the Van't Hoff equation must be fulfilled [37]. Table 4 shows the results obtained upon modifying the column temperature between 20 °C and 60 °C (5 calibration points). A good correlation was obtained for the nucleobases modified by methylation, negative enthalpy values being observed for the three analytes. Accordingly, the retention process is exothermic, and its values seem to be compatible with a partition mechanism. However, the nucleosides and the nucleobases modified by hydroxylation do not fit the Van't Hoff equation. This can be related to the presence of hydroxyl groups and their ability to form hydrogen bonds. Thus, the presence of a greater number of hydroxyl groups (one in 8OHGua, three in 8OH2dG and four in 8OHG) produces a larger deviation from the ideal behaviour of a partition mechanism that could be described by the Van't Hoff equation.

Regarding separation, variations in temperature did not seem to be critical. Neither the efficiency nor the selectivity of the separation was significantly affected. Thus, a temperature of 20 °C can be considered optimum for performing the separation of the MNN studied.

Table 4
Effect of temperature on ZIC-HILIC separation of the modified nucleosides and nucleobases analyzed. Thermodynamic parameters calculated from the Van't Hoff equation.[a]

Modified nucleosides and nucleobases	r^2	Slope (K)	ΔH° (kJ mol^{-1})
8OH2dG	0.430	–	–
9mGua	0.987	480	−4.0
1mGua	0.900	325	−2.7
8OHGua	0.739	–	–
8OHG	0.288	–	–
7mGua	0.970	440	−3.7

[a] Isocratic elution: 80% ACN: 20% formic acid 2.6 mM ($_w$^{w}pH = 3.1). Temperature: variable from 20 to 60 °C. Flow rate: 0.5 mL min^{-1}. Injection: 50 μL in ACN.

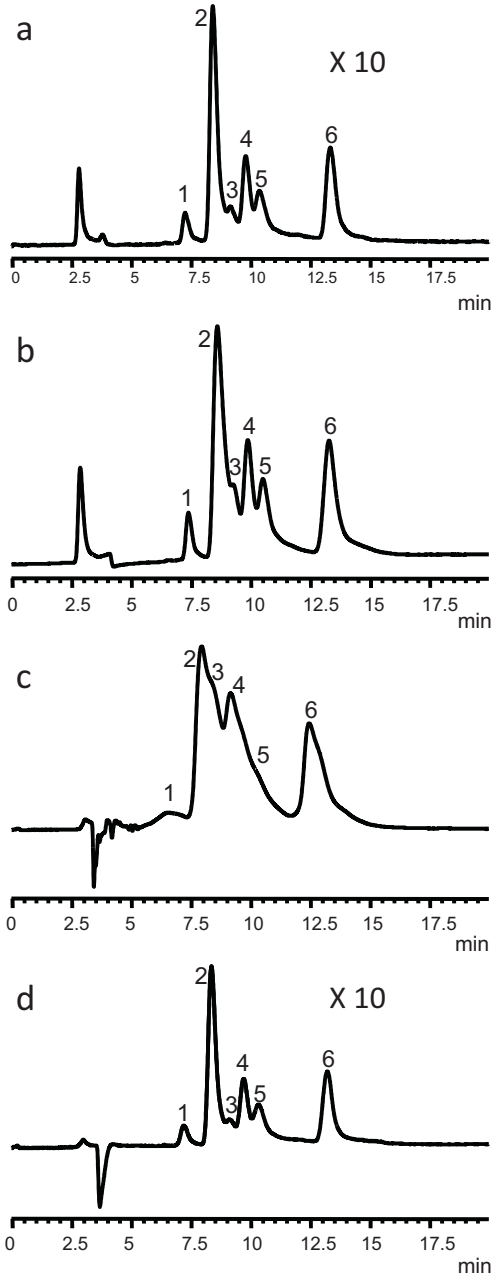


Fig. 5. UV chromatograms obtained using isocratic elution 20% formic acid 2.6 mM $_{w}^{w}$pH = 3.1: 80% ACN for a working standard solution at 5 μg mL^{-1} for methylated-MNN and 3 μg mL^{-1} for hydroxylated-MNN. Column temperature: 20 °C. Chromatographic flow rate: 0.5 mL min^{-1}. Detection: DAD 254 nm. Injection of 5 μL in ACN (a); 50 μL in ACN (b); 50 μL in 50% ACN: 50% water (c); and 5 μL in water (d). Analyte identification as in Fig. 1.

3.2.5. Effect of chromatographic flow rate

The flow rate directly affects the efficiency of the column, as may be deduced from the equation of Van Deemter. For two of the analytes (9mGua and 8OHG), a study was made of the effect of the chromatographic flow rate, analyzing 22 flow rates ranging from 0.1 mL min^{-1} to 2 mL min^{-1}. The correlation coefficients (r^2) for the theoretical curve (HETP = A + B/u + C·u) were 0.947 for 9mGua and 0.955 for 8OHG. For both analytes, the minimum experimental value for the HETP was seen at 0.5 mL min^{-1}. As a side effect, the increase in flow produced a corresponding increase in column pressure. Thus, for a flow rate of 0.5 mL min^{-1} the pressure was 55 bar; for 1.0 mL min^{-1} it was 121 bar, and for 2.0 mL min^{-1} it was 204 bar. These observations allowed a flow rate of 0.5 mL min^{-1} to be selected as optimum for separation. With this flow rate value, HETP of around 20 μm were obtained (equivalent to 50,000 N/m) with a total analysis time of 15 min.

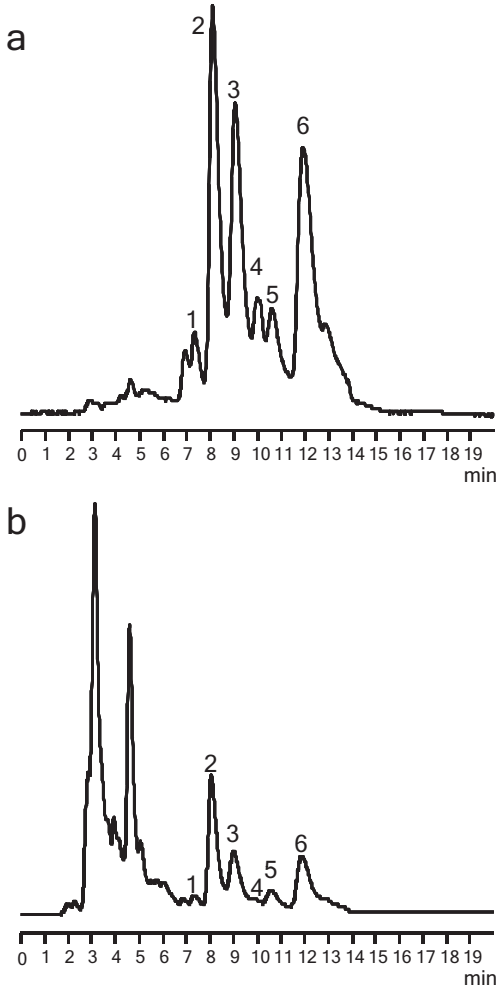


Fig. 6. (a) Total Ion Chromatogram (TIC) and (b) UV chromatogram obtained from a urine sample fortified at 5 μg mL^{-1} for methylated-MNN and 3 μg mL^{-1} for hydroxylated-MNN using isocratic elution 20% formic acid 2.6 mM $_{w}^{w}$pH = 3.1: 80% ACN. Column temperature: 20 °C. Chromatographic flow rate: 0.5 mL min^{-1}. Analyte identification as in Fig. 1.

3.3. Injection in ZIC-HILIC

Another important advantage of HILIC as compared with RPLC is the possibility of injecting samples with a high content of organic solvent, without this affecting the separation and the symmetry of the chromatographic peaks. The usual procedures of extraction, preconcentration and sample cleaning, which are essential for the analysis of trace amounts of compounds in complex samples, as is the case of the analysis of MNN [38], generate a solution that contains the analytes dissolved in predominantly organic media, which means that it is necessary to incorporate an evaporation/redissolution step. This step can be sidestepped by injecting the organic extract directly in HILIC. As organic solvent, ACN has been suggested as the best choice although ACN/isopropanol mixtures could be used in case of solubility issues. In addition, proportion of water greater than 10% in the sample diluent should be avoided to maintain sharp peaks [39].

The separation and symmetry of peaks obtained were satisfactory when injections of 5 μL and 50 μL were performed in a 100% organic medium (Fig. 5a and b) thus showing the injection

volume capacity of the column. The correlation coefficients (r^2) between the volume injected and the peak area obtained (6 calibration points) for the analytes studied varied between 0.986 and 0.997.

Injections of 50 μL with different water contents showed a deterioration in the separation and symmetry of the peaks as expected (Fig. 5c). However, when 5 μL were injected in a 100% aqueous medium (Fig. 5d), the separation and symmetry of the peaks were satisfactory and very similar to those obtained in totally organic medium. Nevertheless, injection of 5 μL in ACN afforded a better separation and peak shape.

Fig. 6 shows the injection of 5 μL of a real urine sample purified following the procedure proposed by Li et al. [40], eluting the SPE cartridge with mobile phase (80% ACN: 20% formic acid 2.6 mM) and sidestepping the evaporation/redissolution step.

3.4. Reproducibility in ZIC-HILIC

One of the best-known disadvantages of NPLC is its lack of reproducibility [34]. To determine the precision of the separation achieved with the ZIC-HILIC column, three samples (prepared daily) were analyzed along three consecutive days, performing 7 injections per day (21 injection in all). The relative standard deviations (RSD) were lower than 2.5% for the retention times and lower than 3.5% for the adjusted retention times. Regarding the analytical signal obtained, measured as chromatographic peak areas, the corresponding RSD remained below 4%.

4. Conclusions

It has been showed that ZIC-HILIC is a suitable alternative for the chromatographic analysis of MNN. A mobile phase containing 80% ACN and 20% of formic acid (2.6 mM) allowed appropriate resolution to be achieved. Additionally, the characteristics of ZIC-HILIC mean that the compatibility between chromatographic separation and detection by mass spectrometry is very high, allowing the injection of volumes up to 50 μL in a completely organic medium, leading to high sensitivity and compatibility with the usual methods of extraction/preconcentration used for the analysis of MNN. The retention of these compounds in ZIC-HILIC occurs through a mechanism of partition between the aqueous phase bound to stationary phase and the organic component of the mobile phase; however, the mechanism is complex, and it presumably involves other processes such as retention of the MNN with hydroxyl groups by hydrogen bonding or electrostatic interactions, for positively charged analytes.

Acknowledgements

The Ministerio de Ciencia e Innovación, Spain, (Project CTQ 2008-02200/BQU) and Junta de Castilla y León (Grupo de Excelencia GR-65) are gratefully acknowledged for financial support of this work. D.G.G. gratefully acknowledges financial assistance from Ministerio de Educación, Spain (FPU grant).

References

[1] S.M. Cooke, R. Olinski, S. Loft, Cancer Epidemiol. Biomarkers Prev. 17 (2008) 3.
[2] M. Harri, H. Kasai, T. Mori, J. Tornaeus, K. Savela, K. Peltonen, J. Chromatogr. B 853 (2007) 242.
[3] H.M. Liebich, S. Müller-Hagedorn, F. Klaus, K. Meziane, K.R. Kim, A. Frickenschmidt, B. Kammerer, J. Chromatogr. A 1071 (2005) 271.
[4] H. Topp, R. Duden, G. Schöch, Clin. Chim. Acta 218 (1993) 73.
[5] E. Borek, B.S. Baliga, C.W. Gehrke, C.W. Kuo, S. Belman, W. Troll, T.P. Waalkes, Cancer Res. 37 (1977) 3362.
[6] D. Jégourel, R. Delépée, F. Breton, A. Rolland, R. Vidal, L.A. Agrofoglio, Bioorg. Med. Chem. 16 (2008) 8932.
[7] B. Kammerer, A. Frickenschmidt, C.E. Müller, S. Laufer, C.H. Gleiter, H. Liebich, Anal. Bioanal. Chem. 382 (2005) 1017.
[8] S.H. Lee, I. Kim, B.C. Chung, Clin. Biochem. 40 (2007) 936.
[9] G. la Marca, S. Malvagia, B. Casetta, E. Pasquini, I. Pela, M. Hirano, M.A. Donati, E. Zammarchi, J. Mass Spectrom. 41 (2006) 586.
[10] D. Bullinger, H. Fröhlich, F. Klaus, H. Neubauer, A. Frickenschmidt, C. Henneges, A. Zell, S. Laufer, C.H. Gleiter, H. Liebich, B. Kammerer, Anal. Chim. Acta 618 (2008) 29.
[11] W.Y. Hsu, W.T. Chen, W.D. Lin, F.J. Tsai, C.T. Lin, W.Y. Lo, L.B. Jeng, C.C. Lai, Clin. Chim. Acta 402 (2009) 31.
[12] K. Roszkowski, D. Gackowski, R. Rozalski, T. Dziaman, A. Siomek, J. Guz, A. Szpila, M. Foksinski, R. Olinski, Int. J. Cancer 123 (2008) 1964.
[13] H.Y. Li, S.M. Wang, H.M. Liu, S.S. Bu, J. Li, D. Han, M.Z. Zhang, G.Y. Wu, J. Mass Spectrom. 44 (2009) 641.
[14] L.B. Jeng, W.Y. Lo, W.Y. Hsu, W.D. Lin, C.T. Lin, C.C. Lai, F.J. Tasi, Rapid Commun. Mass Spectrom. 23 (2009) 1543.
[15] K. Tamae, K. Kawai, S. Yamasaki, K. Kawanami, M. Ikeda, K. Takahashi, T. Miyamoto, N. Kato, H. Kasai, Cancer Sci. 100 (2009) 715.
[16] R. Mateos, L. Bravo, J. Sep. Sci. 30 (2007) 175.
[17] I.H. Lin, M.R. Chao, C.W. Hu, M.L. Ho, J.Y. Huang, H.S. Lee, C.C. Chen, R.H. Wong, Toxicology 260 (2009) 1.
[18] M.R. Chao, C.J. Wang, H.H. Yang, L.W. Chang, C.W. Hu, Rapid Commun. Mass Spectrom. 19 (2005) 2427.
[19] A. Seidel, S. Brunner, P. Seidel, G.I. Fritz, O. Herbarth, Br. J. Cancer 94 (2006) 1726.
[20] Y. Jiang, Y. Ma, Anal. Chem. 81 (2009) 6474.
[21] A.J. Sasco, F. Rey, C. Reynaud, J.Y. Bobin, M. Clavel, A. Niveleau, Cancer Lett. 108 (1996) 157.
[22] R. Tuytten, F. Lemière, E.L. Esmans, W.A. Herrebout, B.J. van der Veken, B.U.W. Maes, E. Witters, R.P. Newton, E. Dudley, Anal. Chem. 79 (2007) 6662.
[23] D.L. Walters, D.L. Jacobs, J.E. Tomaszewski, S.J. Graves, J. Pharm. Biomed. Anal. 19 (1999) 955.
[24] R. Tuytten, F. Lemière, W. Van Dongen, E. Witters, E.L. Esmans, R.P. Newton, E. Dudley, Anal. Chem. 80 (2008) 1263.
[25] A.J. Alpert, J. Chromatogr. 499 (1990) 177.
[26] F. Qin, Y.Y. Zhao, M.B. Sawyer, X.F. Li, Anal. Chem. 80 (2008) 3404.
[27] S. Mysling, G. Palmisando, P. Højrup, M. Thaysen-Andersen, Anal. Chem. 82 (2010) 5598.
[28] Y.H. Jang, W.A. Goddard III, K.T. Noyes, L.C. Sowers, S. Hwang, D.S. Chung, Chem. Res. Toxicol. 15 (2002) 1023.
[29] V. Verdolino, R. Cammi, B.H. Munk, H.B. Schlegel, J. Phys. Chem. B 112 (2008) 16860.
[30] A. Albert, E.P. Serjeant, The Determination of Ionization Constants: A Laboratory Manual, 3rd edition, Chapman & Hall, London, 1984.
[31] A. Yanagida, H. Murao, M. Ohnishi-Kameyama, Y. Yamakawa, A. Shoji, M. Tagashira, T. Kanda, H. Shindo, Y. Shibusawa, J. Chromatogr. A 1143 (2007) 153.
[32] D.V. McCalley, J. Chromatogr. A 1217 (2010) 3408.
[33] M. Liu, J. Ostovic, E.X. Chen, N. Cauchon, J. Chromatogr. A 1216 (2009) 2362.
[34] Y. Guo, S. Gaiki, J. Chromatogr. A 1074 (2005) 71.
[35] J.A. Alpert, Anal. Chem. 80 (2008) 62.
[36] G. Marrubini, B.E.C. Mendoza, G. Massolini, J. Sep. Sci. 33 (2010) 803.
[37] Z. Hao, B. Xiao, N. Weng, J. Sep. Sci. 31 (2008) 1449.
[38] E. Dudley, S. El-Shakawi, D.E. Games, R.P. Newton, Nucleosides Nucleotides Nucleic Acids 19 (2000) 545.
[39] J. Ruta, S. Rudaz, D.V. McCalley, J.L. Veuthey, D. Guillarme, J. Chromatogr. A 1217 (2010) 8230.
[40] H.Y. Li, S.M. Wang, H.M. Liu, J. Li, D. Han, S.S. Bu., M.Z. Zhang, Rapid Commun. Mass Spectrom. 22 (2008) 3161.