Short communication

Separation of oxidatively damaged DNA nucleobases and nucleosides on packed and monolith C18 columns by HPLC-UV-EC

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Abstract

This study involves the incorporation of a commercially available Phenomenex Onyx C18 monolith column into the separation and detection of oxidative DNA damage. It includes thorough investigation of monolith performance and a comparison of the performance of monolith columns with a commercially available packed Restek reverse phase Ultra C18 column for the separation of DNA bases and nucleosides. The performance of the monolith was examined using efficiency, resolution, plate height, asymmetry and retention times, and each case showed improved or at least comparable results in the separation of a mix of DNA bases and nucleosides. A 90% reduction, from just under 40 min to just under 4 min, was obtained in the elution time of this separation. To the best of our knowledge, this is the first report of a fast monolith column separation successfully coupled to both a UV–vis and EC detector, which is especially useful for the analysis of oxidative DNA damage. The determination of 8-oxoG and 8-OH-dG, oxidation products of guanine and 2′-deoxyguanosine, respectively, may be compromised by their ease of oxidation and therefore the fast separation, selective and sensitive detection, with no artifactual oxidation, detailed in this report, is ideal.
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Keywords: HPLC-UV-EC; Oxidative DNA damage; Guanine; 8-Oxo-7,8-dihydroguanine; 2′-Deoxyguanosine; 8-Oxo-7,8-dihydro-2′-deoxyguanosine; Monolith

1. Introduction

Sensitive and selective detection and quantification of oxidative DNA damage is an important topic in modern science. There is much research into methods of detection and elucidation of the mechanisms by which our DNA is attacked by various oxidants, including endogenous reactive oxygen species (ROS) [1–5]. It is the understanding of these mechanisms of oxidative stress that will lead to the elucidation of the mechanisms of disease initiation and propagation. Oxidative stress has been linked with numerous important diseases, such as cancer, neurodegeneration and heart disease [6].

Artifactual oxidation, both in sample preparation and analysis is a major obstacle when trying to accurately measure oxidative stress products such as 8-oxo-7,8-dihydroguanine (8-oxoG) and 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-OH-dG) [7,8]. It is fast becoming evident, however, that these are not the final products of oxidative stress, but rather intermediates in a complicated scheme involving many possible reactions and resulting in numerous potential final products [9–11]. The determination of these highly oxidisable intermediate species is, however, still as important as ever in order to fully comprehend mechanisms of oxidative DNA damage.

One area that has not been researched in enough depth is thorough minute-by-minute analysis of *in vitro* and *in vivo* oxidative DNA damage by ROS in order to conduct comprehensive analysis of all oxidative lesions involved. This type of analysis is necessary but can be time consuming with long separation times, especially in the analysis of nucleosides, which can be up to 45 min long. Thorough analysis, therefore, can be a laborious task [8,12]. In addition, the ease at which products 8-oxoG and 8-OH-dG can be further oxidised [13], means that analysing samples in duplicate and triplicate may be compromised due to degradation over the long intervals between sample analyses. This degradation can potentially create large error ranges between injections as well as inaccurate readings.

In the interest of fully and accurately elucidating and comparing the mechanisms of oxidative DNA damage to both DNA bases and nucleosides, it is essential to have a method that is thorough, accurate and fast, with minimal artifactual oxidation [14].

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One recognised method of determination of products of oxidative stress is HPLC coupled to both ultraviolet and electrochemical detection (HPLC-UV-EC) [15–17]. EC detection allows for a specific determination of oxidation products 8-oxoG and 8-OH-dG, that is not possible with simple UV detection of oxidation products [18]. UV detection allows for simultaneous detection of the unmodified products. The separation of DNA bases and nucleosides using the same isocratic method is uncommon, due to the long elution times. Nucleoside separations are usually carried out using gradient elution; the use of gradient elution is not necessary for the separation of DNA bases [7].

This study incorporates the use of a commercially available endcapped silica C18 reverse phase Phenomenex Onyx monolith for the separation of both DNA bases and nucleosides on the same fast, simple and isocratic HPLC method, coupled to EC detection for the determination of oxidative DNA damage. Monolith columns, since their discovery have been at the height of recent discussion in separation science, as they exhibit superior or at least comparable separation ability over regular particle packed columns [19,20]. There are various types of monolith columns, silica, organic polymer columns [21] and a number of methods of monolith preparation, including the sol–gel process for silica columns [22]. In HPLC, they show low back pressure with high-flow rates not previously viable for use in HPLC with no compromise in separation performance, allowing for excellent fast separations, even with complex large biomolecules [23,24]. Applications of monolith columns are not limited to just HPLC. Silica monoliths have been applied to capillary-HPLC-MS [24]. Organic monoliths have been applied to solid-phase extraction, preconcentration, and on a large plant scale for purification [25,26] and both silica and organic monoliths have been applied to capillary electrochromatography [27]. Monoliths do, however, suffer from some drawbacks including poor tolerance to alkaline mobile phases, high solvent consumption and in some cases need heating or cooling [28].

This study compares the performance of the monolith against the performance of a regular particle packed column by examining efficiency, resolution, peak symmetry and retention time. The high speed, isocratic monolith separation, which allows for the simultaneous determination of DNA bases and nucleosides by UV, was then coupled with EC detector for the specific and sensitive detection of oxidation products. The method was modified to reduce the flow rate by splitting the flow to the EC detection cell, in order to reduce noise and pressure in the EC detection. This separation results in a significant decrease in temporal resolution and therefore has the potential to facilitate elucidation of DNA damage mechanisms with fast analysis and reduced artifactual oxidation and degradation of products.

2. Experimental

2.1. Reagents

Deionised water was purified using a MilliQ system to a specific resistance of greater than $18.2\,\text{M}\Omega\,\text{cm}$. All chemicals including the DNA bases and nucleosides guanine (G0381, $\geq$99%), adenine (A8626, $\geq$99%), thymine (T0376,

$\geq$99%), cytosine (C3506, $\geq$99%), and uracil (U0750, $\geq$99%), 7,8-dihydro-8-oxoguanine (R288608), 8-oxo-7,8-dihydro-2′-deoxyguanosine (H5653) 2′-deoxycytidine, 2′-deoxyguanosine, 2′-deoxyadenosine and 2′-deoxyuridine, ammonium acetate, and glacial acetic acid were purchased from Sigma–Aldrich (Tallaght, Dublin, Ireland). Ethanol and methanol were obtained from Labscan Ltd. (Dublin, Ireland).

2.2. Chromatographic conditions

All HPLC buffers and mobile phases were filtered through a 47 mm, 0.45 μm polyvinylidene fluoride (PVDF) micropore filter (Sigma–Aldrich) prior to use. Fresh solutions of all standards were prepared weekly, with the exception of 8-oxoG, which was prepared on the day of use. HPLC analysis was performed using a Varian ProStar HPLC system and an injection volume of 20 μl, with Varian ProStar 230 Solvent Delivery Module and Varian ProStar 310 UV-VIS Detector with data acquisition rate of 10 Hz and detector time constant of 1 s. The column temperature was ambient, and the detector wavelength was set at 254 nm. For packed column analysis, this system was coupled with a Restek reverse phase Ultra C18 5 μm 4.6 mm × 250 mm column (Restek, Belfast, U.K.), equipped with Ultra C18 4 mm × 10 mm guard column with a 3% acetonitrile (ACN), 50 mM ammonium acetate, pH 4.6 mobile phase (pH was adjusted with glacial acetic acid).

For monolith separations, the HPLC system was coupled to a Phenomenex Onyx RP-18 monolith column (Phenomenex, Cheshire, U.K.) of dimensions 4.6 mm × 100 mm coupled to an Onyx monolith C18 guard column (5 mm × 4.6 mm). Plate height, H was calculated using the following equation:

$$H = \frac{L}{N}$$

where L is column length and N is the number of theoretical plates as calculated by

$$N = \frac{5.55t_r^2}{w_{1/2}^2}$$

The adjusted plate height, generally used for the Knox plot, takes into account the size of particles in the column [30]. Plate height, without any adjustment, was used for the monolith column.

The resolution was calculated as

$$R_s = \frac{\Delta t_r}{w_{av}}.$$

2.3. Electrochemical detection

Electrochemical detection was performed using a CC-4 electrochemical cell (BAS) comprising of glassy carbon working electrode, stainless steel auxiliary electrode and Ag/AgCl reference electrode. Amperometric current–time plots were generated using a CHI800B potentiostat with accompanying software. EC chromatograms were recorded at a detection potential of 600 mV vs. Ag/AgCl. UV and EC chromatograms were

exported and analysed using Microsoft Excel or Sigma Plot Version 8.0.

2.4. Control experiments

Controlled incubations were performed, with both G and 8-oxoG to ensure that no artifactual oxidation was caused by the reaction conditions themselves, as reported previously [29].

3. Results

3.1. HPLC-UV

This study builds significantly on a Restek C18 packed column method that has been previously used for HPLC-UV-EC analysis of DNA bases and their oxidative DNA damage products. The method was advanced to separate both DNA bases and nucleosides and their oxidative DNA damage products simultaneously [29]. The techniques and parameters were modified for use on a Phenomenex Onyx monolith RP-18 column, and the flow rate adjusted to allow for higher sample throughput, and hence a more comprehensive study, while still being able to perform sensitive electrochemical detection. The chromatography of the packed column and the monolith column were compared for their performance.

Using a packed Restek Ultra C18 reverse phase column, a 3% ACN, 85 mM ammonium acetate, 50 mM acetic acid was determined to be optimum for the separation of G, C, A, T, dG, dC and dA. Separation using 5, 10 or 15% ACN or 5% methanol caused co-elution of the earlier eluting compounds, and therefore each of these mobile phase compositions was deemed inadequate. The optimised separation resulted in baseline separation for all peaks, with the exception of 2′-deoxyuridine and thymine. This co-elution was also observed on the monolith column. 2′-Deoxyuridine is, however, only present in RNA, and not in DNA. Therefore, its co-elution with thymine did not present a problem in this study of oxidative DNA damage, and it was not used for the remaining analyses. It should be noted that the separation time using flow rate $1.0\,\mathrm{ml\,min^{-1}}$ was of approximately 40 min duration, as shown in Fig. 1.

The separation of DNA bases and nucleosides was then optimised using a Phenomenex Onyx monolith RP-18 endcapped column. Uridine was not used in this separation, due to the previous co-elution issues that were faced. Using the same conditions as those used with the packed column, an injection of a 1 mM G, C, A, T, dG, dC and dA mixed standard into a $1.0\,\mathrm{ml\,min^{-1}}$ eluent stream of 3% ACN, 85 mM acetic acid and 50 mM ammonium acetate resulted in G and dC co-eluting. The organic content of the mobile phase was adjusted step-wise to a lower ACN content, in order to improve the separation. 1.2% ACN showed optimal resolution between the G and dC peaks. This separation is shown in Fig. 2.

The flow rate was then increased in $0.5\,\mathrm{ml\,min^{-1}}$ increments with 1.0 and $4.0\,\mathrm{ml\,min^{-1}}$ shown in the inset in Fig. 2. The quality of the separation was analysed at each of these flow rates. It was evident that the performance of a monolith column was at its best at the higher flow rates, with no significant loss in
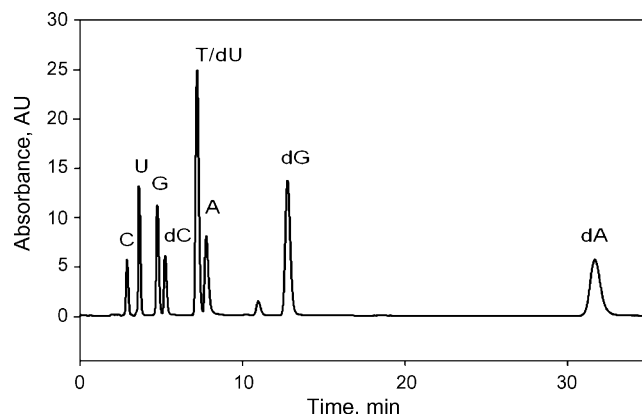

Fig. 1. HPLC separation of DNA nucleosides and bases with UV detection at 254 nm using a Restek C18 5 μm packed column with mobile phase of 3% acetonitrile, 85 mM ammonium acetate, 50 mM acetic acid at a flow rate of $1\,\mathrm{ml\,min^{-1}}$, detection at 254 nm. Elution order: cytosine, uracil, guanine, 2′-deoxycytidine, thymine and 2′-deoxyuridine, adenine, 2′-deoxyguanosine, 2′-deoxyadenosine.

efficiency and comparable or better asymmetry, as illustrated in Figs. 3–5. The benefit of increasing the flow rate was especially noticeable for adenine. Fig. 3 showed a significant decrease in asymmetry, and Figs. 4 and 5, the Van Deemter and Knox plots, illustrated the decrease in plate height with increasing linear velocity. Fig. 4 also illustrates a comparison between the Restek packed column at $1.0\,\mathrm{ml\,min^{-1}}$ and the Phenomenex monolith at both 1.0 and $4.0\,\mathrm{ml\,min^{-1}}$. There was a higher tailing factor in the monolith at $1.0\,\mathrm{ml\,min^{-1}}$ in comparison to the packed column, though in most cases, at $4.0\,\mathrm{ml\,min^{-1}}$ this tailing was reduced to a level comparable to that of the packed column, indicating the improvement of the separation with increased flow. There was no significant increase in tailing or asymmetry for any of the other separation components. The separation time, even at
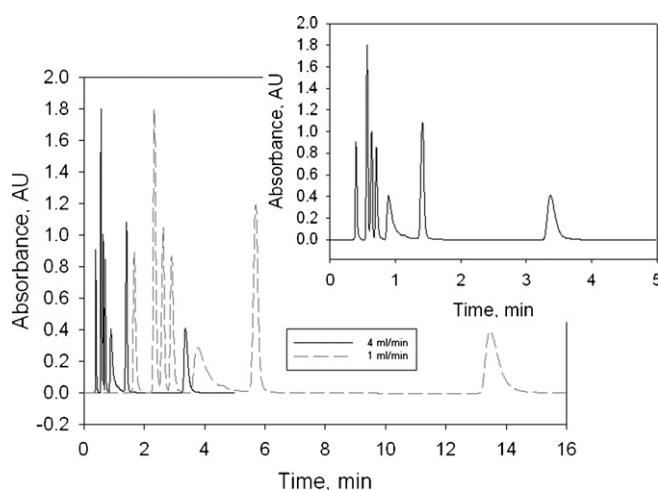

Fig. 2. HPLC separation of DNA nucleosides and bases with UV detection at 254 nm using a Phenomenex Onyx monolith RP-18 column with mobile phase of 1.2% acetonitrile, 85 mM ammonium acetate, 50 mM acetic acid at a flow rate of $1\,\mathrm{ml\,min^{-1}}$ (dashed line) and at a flow rate of $4\,\mathrm{ml\,min^{-1}}$ (solid line), detection at 254 nm. Elution order: cytosine, guanine, 2′-deoxycytidine, thymine, adenine, 2′-deoxyguanosine, 2′-deoxyadenosine. *Inset*: Separation obtained at flow rate of $4\,\mathrm{ml\,min^{-1}}$.
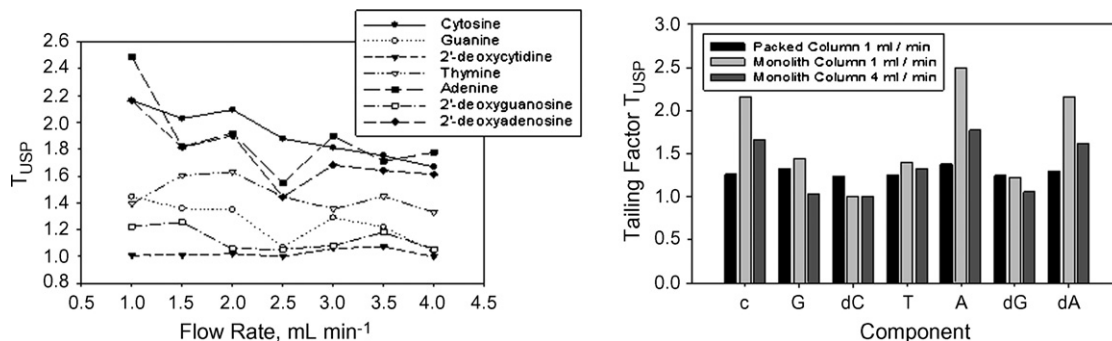
Fig. 3. *Left*: Effect of increasing flow rate on peak asymmetry (USP Tailing, T_{USP}) for each of the separation components, cytosine, guanine, 2′-deoxycytidine, thymine, adenine, 2′-deoxyguanosine, 2′-deoxyadenosine on a Phenomenex Onyx RP-18 monolith 4.6 mm × 100 mm. *Right*: Comparison between packed (Restek reverse phase Ultra C18 5 μm 4.6 mm × 250 mm) and monolith (Phenomenex Onyx RP-18 monolith 4.6 mm × 100 mm) columns for each separation component.

1.0 ml min^{-1} using the monolith with 1.2% ACN mobile phase was just 14 min, and this was reduced to under 4 min. Therefore, overall, there was a 90% decrease in runtime from 40 min on a packed column to 4 min on a monolithic column with no significant loss in resolution.

3.2. HPLC-UV-EC

A major issue in applying a high-speed monolith separation to the analysis of oxidative DNA damage is the effect of the high flow on the electrochemical detection. Use of inline flow cell electrochemical (EC) detection is ideal for low flow rate separations, but at 4.0 ml min^{-1}, baseline noise as well as high pressure in the lines and leaks may become a problem. In order to use such high-flow streams, a splitting of the eluent stream was necessary. Flow-splitting apparatus can be expensive; however, in this study the flow splitting was accomplished using a simple t-piece coupled with PEEK tubing, the inlet was 0.254 mm I.D., as was the waste outlet, and the outlet to the EC detector was 0.178 mm I.D. The high pressure in the lines caused by the high-flow rate meant that there was a constant flow through the smaller diameter tubing and the stream did not just go to the larger diameter waste line.
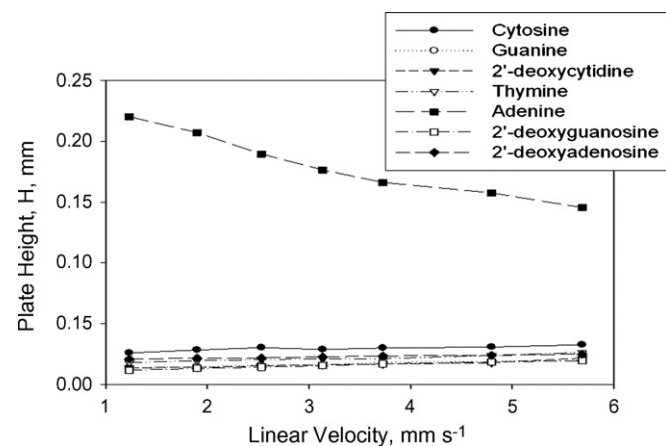
The high pressure in the line created by the fast flowing eluent caused enough pressure to drive the split in the flow so that 3.3 ml min^{-1} was sent to waste and 0.7 ml min^{-1} flowed through the EC detector cell. The noise level on the EC detector was in the range of 10^{-10} A while the guanine damage product 8-oxoG was still easily quantified. The selective detection of 8-oxoG and 8-OH-dG was carried out at 600 and 700 mV, respectively, and was linear with good correlation coefficients of 0.99 or greater recorded for concentrations in both the micromolar and millimolar ranges. The simultaneous separation of 8-oxoG and 8-OH dG was carried out at 650 mV and is shown in Fig. 6. The limit of detection was in the nanomolar range, at approximately 50 nM. This was comparable to the LOD obtained with the traditional HPLC-UV-EC which utilised the Restek C18 column.

4. Discussion

The separation ability of a monolith column was evident at a higher flow rate in this study. The reduction of separation time from over 40 min to under 4 min is a dramatic 10-fold reduction in separation time for the simultaneous analysis of DNA bases, nucleosides and oxidative lesions such as 8-oxoG.

It should be noted that due to co-elution, uridine could not be used as an internal standard for any future studies, nonetheless
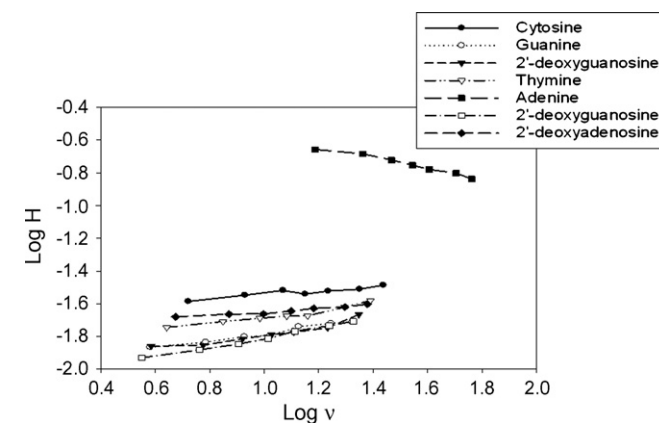

Fig. 4. Van Deemter Plot of Plate Height, H for each of the separation components, cytosine, guanine, 2′-deoxycytidine, thymine, adenine, 2′-deoxyguanosine, 2′-deoxyadenosine on the monolith column (Phenomenex Onyx RP-18 monolith 4.6 mm × 100 mm) against eluent linear velocity.

Fig. 5. Knox plot of log of plate height, *H* for each of the separation components, cytosine, guanine, 2′-deoxycytidine, thymine, adenine, 2′-deoxyguanosine, 2′-deoxyadenosine on the monolith column (Phenomenex Onyx RP-18 monolith 4.6 mm × 250 mm) against the log of the reduced linear velocity.
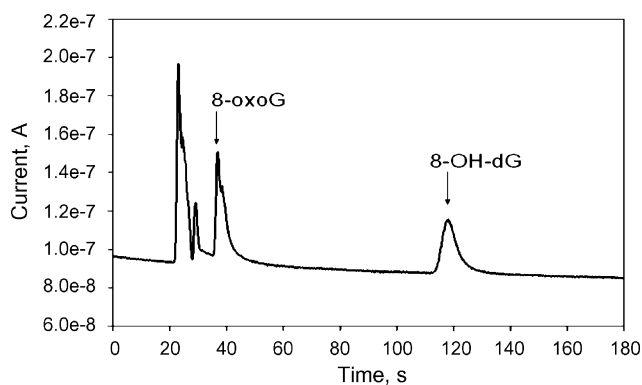
Fig. 6. Electrochemical chromatogram illustrating the separation of 8-oxoG and 8-OH-dG, separation carried out using a Phenomenex Onyx monolith RP-18 column with mobile phase of 1.2% acetonitrile, 85 mM ammonium acetate, 50 mM acetic acid at a flow rate of 4 ml min^{-1}, detection at 650 mV.

the separation is suitable for the separation of the DNA bases and nucleosides, as the nucleoside uridine is only present in RNA. For internal standard purposes, however, uracil, the DNA base equivalent was completely baseline resolved, eluting between cytosine and guanine on both the packed column and the monolith column (data not shown) and therefore could be used if an internal DNA standard is needed.

At a flow rate of 1.0 ml min^{-1}, the monolith showed reduced retention times, while still retaining good peak shape and baseline resolution between components. A 1.0 ml min^{-1} separation was compared for the packed and monolith columns and the result is illustrated in Fig. 2. There was a general increase in peak symmetry, with increasing flow rate, evident for all peaks, but most especially for adenine and 2′-deoxyadenosine. The peak widths for the monolithic column were greatly reduced by increasing the flow rate through the column. At the higher flow rate, the level of asymmetry was reduced for most peaks until they were comparable with those of the packed column. This change in asymmetry, presented in Fig. 3, was most noticeable for cytosine, adenine and 2′-deoxyadenosine, which showed the most problematic tailing on the monolith separation. The separation efficiencies were reduced slightly for the early elution compounds with increasing flow using the monolithic column. However, this reduction was primarily due to the decreased elution time for these components. The separation efficiency for adenine was reduced on transfer of the separation to the monolithic column. This may be due to increased silanol activity often observed with monoliths; to try to improve the efficiency of adenine, in future analysis silanol masking agents such as triethylamine will be added to the mobile phase.

The pressure in the packed column, at 1.0 ml min^{-1} was approximately 2.03×10^7 Pa (approx 3000 psi), whereas in the monolith column was just 2.53×10^6 Pa (367 psi). As the flow rate, and hence back pressure increased, peak shapes were improved or not changed significantly, as was evident from the improvement in symmetry with flow rate in Fig. 3. The symmetry, measured as tailing, was reduced slightly or comparable for each of the components with increasing flow velocity. There were no significant changes in asymmetry that would indicate a compromise in separation quality. There was a reduction in

tailing for adenine which would be the most problematic peak, where tailing is concerned.

The efficiencies, of each of the DNA and nucleoside mixture were comparable over the entire range of flow rates. There was no dramatic change, as the retention times were reduced along with the peak width at half height. Some components, especially the early eluting compounds did show a decrease in efficiency, most likely due to extra column effects, though this change was not significant enough to alter the integrity of the separation. For adenine there was, however, a very significant decrease in peak height, shown in the Van Deemter plot in Fig. 4, illustrating that with increasing flow rate the chromatography was improving for this peak. Each of the other peaks showed an increase in plate height, though this was not significant, suggesting that there was a comparable separation for these across the range of flow rates. The resolution remained comparable for each of the components as the flow rate increased. Baseline resolution (>1.7 for all peaks) was maintained between all adjacent components of the DNA nucleoside and bases indicative, therefore, that even with the dramatic runtime reduction and resulting closely eluting peaks, the separation was not compromised. Comparing the packed Restek column at 1.0 ml min^{-1} to the optimum monolithic flow rate of 4.0 ml min^{-1}, the resolution values were significantly higher for the Restek column. However, this was due to a dramatic increase in the elution time when using the Restek column. With the monolithic column, all components were still baseline resolved, and there was no decrease in the overall separation quality.

The simultaneous separation of 8-oxoG and 8-OH dG resulted in a limit of detection which was in the nanomolar range, at approximately 50 nM. This was comparable to the LOD obtained with the traditional HPLC-UV-EC which utilised the Restek C18 column. However, the faster runtime reduced the length of time between when a sample was reconstituted and when it was analysed in triplicate. This is significant as samples may degrade or be further oxidised by trace oxidants present between repeat analysis, thus increasing sample deviation. One of the recommendations of ESCODD was that further work was needed to develop procedures that prevent oxidation from occurring during sample preparation for chromatographic analysis [15]. Previous analysis has concentrated on simplifying sample preparation and clean up techniques to reduce this artifactual oxidation [31]. The decreased analysis time of this protocol may help to reduce artifactual oxidation by optimising the chromatographic parameters themselves.

The afore-mentioned disadvantages of monolith columns, sensitivity to high pH, high solvent consumption and need for heating or cooling [28], did not really apply to this study. The eluent used in the separation was at a low pH (4.6), the organic concentration was reduced dramatically to just 1.2% ACN. There was also no need for heating or cooling for this particular separation.

5. Conclusion

This paper describes the separation of both nucleosides and DNA bases. This separation is important for the analysis of

DNA damage by a range of oxidants, chemical reactions and other stresses. The major problems associated with determination of oxidative damage are the intermediate nature of oxidation products, especially those of G and dG, 8-oxoG and 8-OH-dG, respectively. The reactive and unstable nature of these compounds means that their detection should be carried out in a fast manner, with minimal stresses that could result in the artifactual oxidation of these intermediate species.

The use of monolith reverse phase separations minimises back pressure, while allowing for fast and efficient separations of DNA components. The subsequent analysis of their oxidation products by electrochemical detection was achieved with splitting the flow to minimise high-flow strain on the electrochemical detector, while still obtaining a fast separation. With the analysis optimised in this study, samples may be analysed in just 10% of the time previously required, with no compromise in separation performance, and in some cases improved peak shape (for example for adenine). This fast analysis, just 4 min, ensures minimal degradation of damaged DNA samples between injections, and hence allows for a more accurate as well as a more in-depth, comprehensive study of oxidative DNA damage, with the potential for assisting in elucidation of the important mechanisms of oxidative stress.

Acknowledgement

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