DNA-Adduct Analysis by NanoFlow™ Electrospray.

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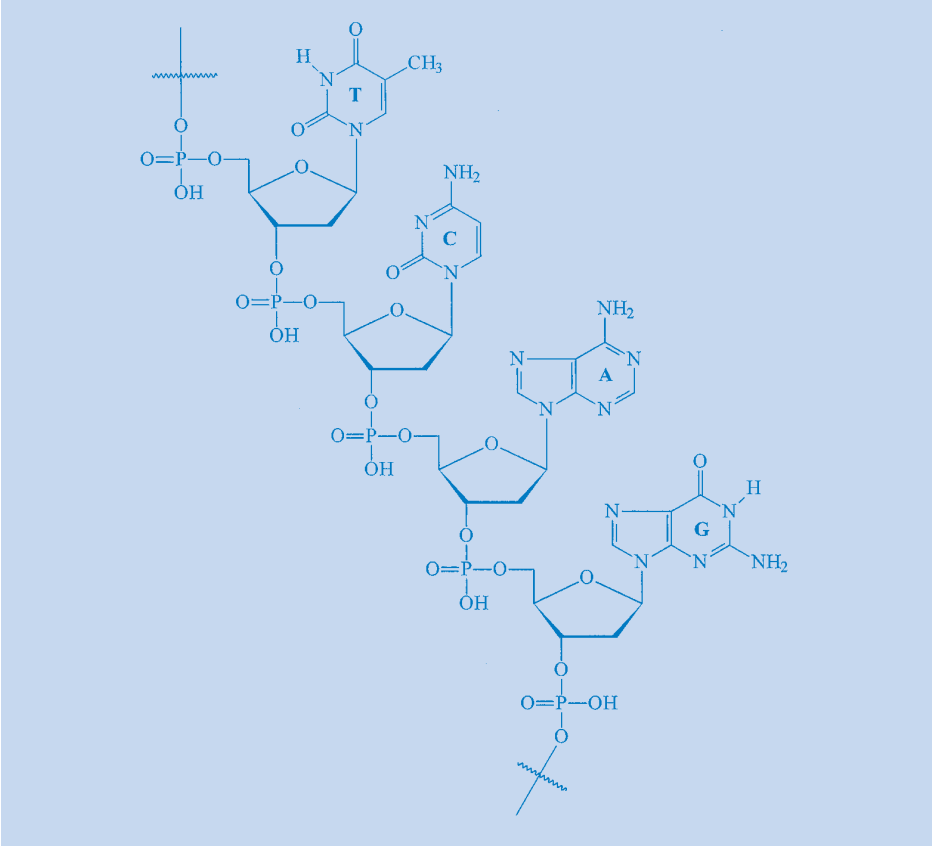
Introduction

Deoxyribonucleic acid (DNA) is the genetic blueprint for all known life and serves as the template for the production of structural and regulatory proteins. DNA has a double helix structure, with each helix consisting of a linear polymeric chain which incorporates alternating phosphate groups and deoxyribose sugar units. In addition each sugar is attached to one of four possible organic nitrogen bases: guanine, cytosine, adenine, and thymine. These bases hydrogen-bond to a partner base (cytosine to guanine, and adenine to thymine) at the centre of the double helix and thus maintain the structural integrity of the molecule.



Chemical carcinogens or their metabolites can interact with DNA resulting in the formation of covalently bonded DNA-adducts. If these adducts are not enzymatically repaired, they may cause mutations and in the extreme case carcinogenesis[1]. After exposure to a chemical carcinogen approximately one in 10^{10} nucleotides is modified and the resulting DNA-adducts can be isolated in very small quantities (pg)[2,3]. It is therefore essential that the mechanism of DNA binding is fully researched and understood.

Several analytical techniques, including ^{32}P post-labelling, immunoassay, and fluorescence spectroscopy, have been developed to detect DNA-adducts at these low levels, but such techniques do not give any structural information. The relatively recent advent of electrospray (ES) mass spectrometry (MS), with its high sensitivity, amenability to polar organic molecules and compatibility with Liquid Chromatography (LC), provides the potential for a rapid, sensitive analysis of this type of sample while simultaneously generating molecular weight and structural information[4,5,6].

This Application Note describes the successful analysis of a standard *in vitro* reaction mixture of 2'-deoxyguanosine-5'-monophosphate (dGMP) with bis-phenol A diglycidyl ether (BPADGE, see Box 1) using NanoFlow™ ES-MS. BPADGE[7] is a bifunctional epoxide which is used in the epoxy resin industry, and may be a potent mutagen and carcinogen. The aim of the project was to establish methodology that is sufficiently sensitive for future studies which would involve the detection of DNA-adducts from *in vivo* sources using Nano-LC-MS. The benefits of Nano-LC[8], and a comparison of injection methods are discussed.

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Experimental

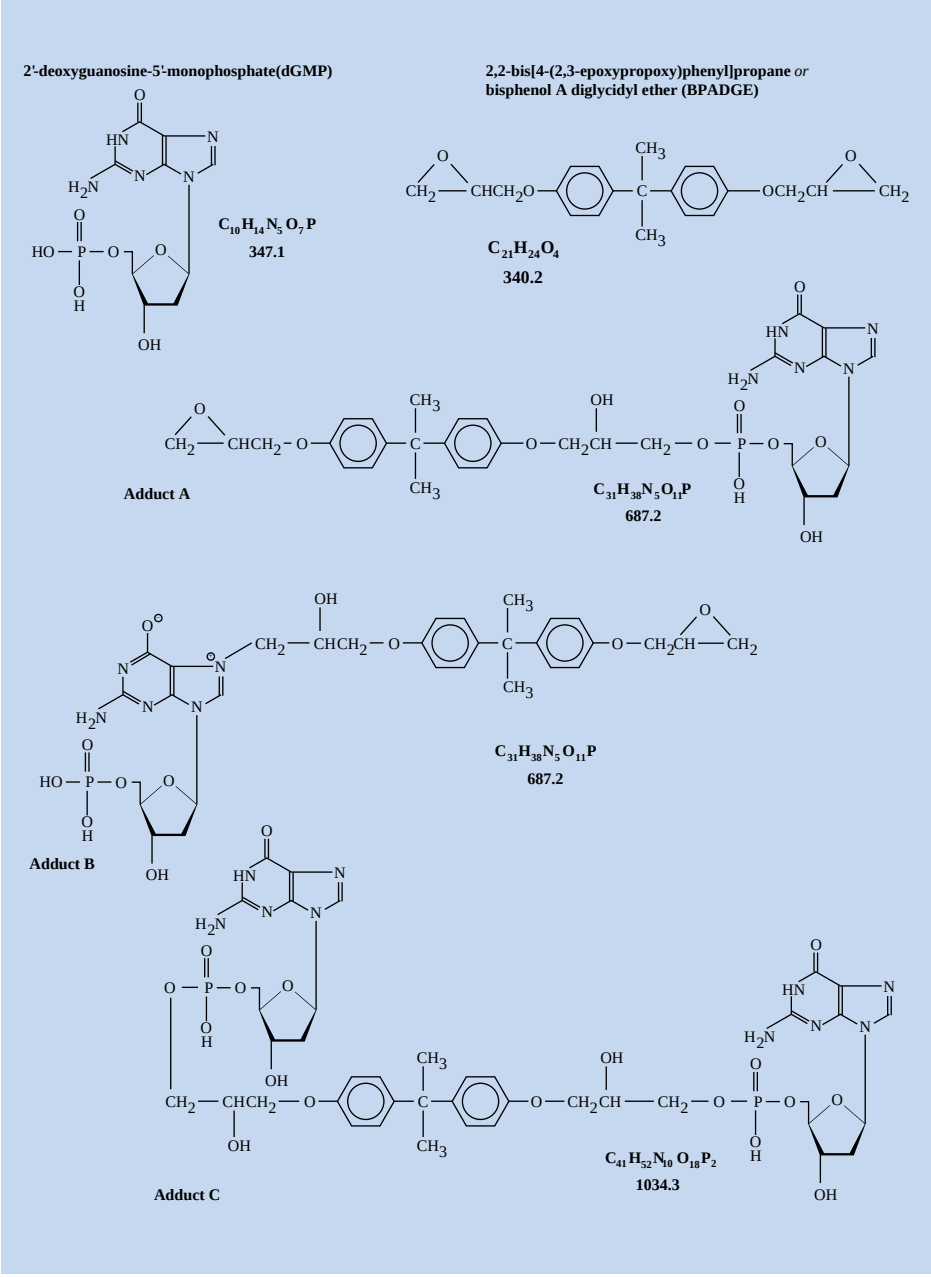
Synthesis of dGMP/BPADGE adducts[9]

To a solution of dGMP in water (2 mg/mL), a solution of BPADGE (1 mL; 0.5 M in tetrahydrofuran) and tetrahydrofuran (1.5 mL) were added. The resulting mixture was stirred at 37°C for 48 h in a Pierce Reacti-vial™. The crude reaction mixture was then evaporated to dryness and redissolved in water (1 mL). This aqueous solution was extracted with diethyl ether (2 x 4 mL) to remove the excess BPADGE, and the remaining aqueous layer evaporated to dryness, redissolved in water (2 mL) and used for analysis without any further purification.

Instrumentation

ES-MS was performed on a Quattro II tandem quadrupole mass spectrometer (Micromass UK Ltd., Altrincham, Cheshire, UK) equipped with an ES source operating in negative ionisation mode. A NanoFlow™ ES probe was used exclusively in the continuous flow mode spraying with a fused silica tip (20 µm i.d.). The probe tip was held at 3.0 kV, and a cone voltage of 40 V was found to be optimal for these samples. The counter electrode was removed from the source, and the source temperature held at 45°C. The drying gas flow was set to 40 L/h. The operating conditions of the mass spectrometer were optimised using a solution of dGMP (10^{-4} M in the mobile phase).

Tandem mass spectrometry (MS-MS) was achieved using argon as collision gas at a collision cell pressure of 3 x 10^{-3} mbar. For the product ion spectra, the precursor ions (either the $(M-H)^-$ or the $(M-2H)^{2-}$ ions, depending on which were the more intense) were passed through the first quadrupole analyser and into the collision cell. Fragmentation was optimised with a collision energy of 36 eV, and the fragment, or product, ions were analysed by scanning the second quadrupole analyser.



Box 1. The chemical structure of BPADGE and the DNA-adducts

The MassLynx (Micromass UK Ltd., Altrincham, Cheshire, UK) suite of software programs was used to control the mass spectrometer, data acquisitions and data processing.

Chromatography

On-line LC-MS was performed using a Kontron 325 HPLC solvent delivery pump and a Kontron 332 UV detector (Kontron Instruments, Milan, Italy) which had been retrofitted with a U-Z View™ 3 nL internal volume flow cell (LC Packings, Amsterdam, The Netherlands). The UV wavelength was set at λ = 260 nm.

Automatic injections and automatic column switching experiments were performed using a Fully Automated Micro Autosampler (FAMOS™, LC Packings, Amsterdam, The Netherlands). The set-up for the different experiments is shown in Figure 1. Splitting of the mobile phase flow was achieved prior to sample injection by using an Acurate™ microflow processor (LC Packings, Amsterdam, The Netherlands).

For the split injection experiments (3 nL injections) an additional split was installed between the injector and the analytical column (NAN75-15-05-C8, 75 µm i.d., 15 cm length, 5 µm particle size, Hypersil C8 BDS, LC Packings, Amsterdam, The Netherlands).

A micro pre-column (300 µm i.d., 5 mm length, C8 packing material, LC Packings, Amsterdam, The Netherlands) was used for the column switching experiments. A second HPLC solvent delivery pump (Model 422, Kontron, Milan, Italy) was used to load the sample on the pre-column, and a second UV detector (Model 332, Kontron, Milan, Italy) equipped with a capillary U-shaped flow cell (35 nL, LC Packings, Amsterdam, The Netherlands) was used to monitor the waste eluent from the outlet of the pre-column.

Samples were eluted from the analytical column using an isocratic mobile phase of 1:1 (v/v) methanol : 10 mM aqueous ammonium acetate at pH 7, flowing at 200 nL/minute.

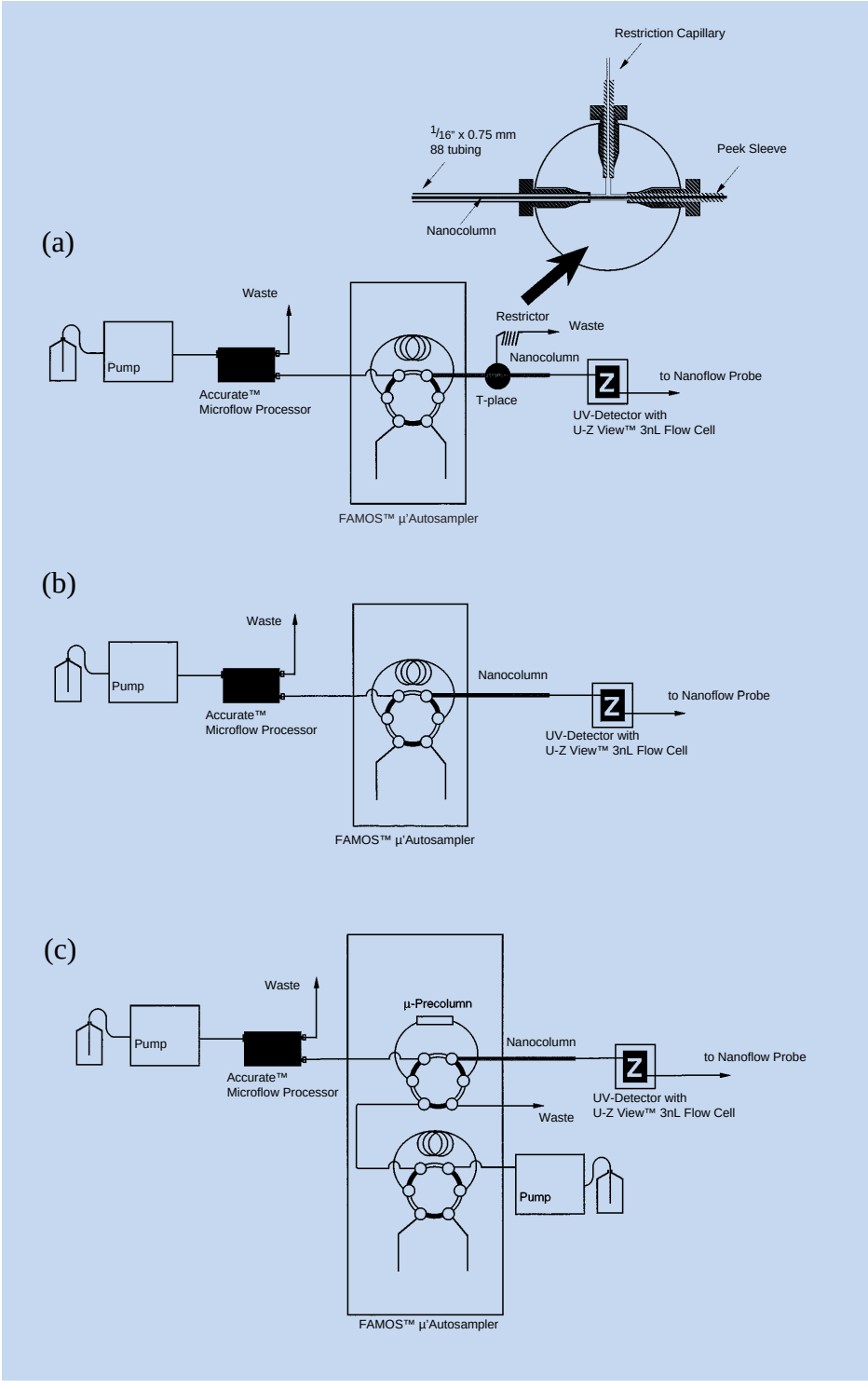


Figure 1. Experimental set-up of the different injection methods: (a). split injections (b). large volume injections, (c). column switching.

Results and Discussion

NanoFlow™ Electrospray

Conventional electrospray is optimised with liquid flow rates ranging from 5 µL/minute to 1 mL/minute, and is thus compatible with a wide range of microbore and standard bore HPLC columns with internal diameters ranging from 0.3 to 4.6 mm. Electrospray has emerged to be the technique of choice for on-line HPLC-MS analyses of mixtures.

As the amount of material under investigation is usually very limited, more and more demand is put on the limits of detection and so packed capillary columns (25 to 100 µm i.d.) have been designed[8] to cope with smaller (nL) sample injection volumes. These columns also demand lower mobile phase flow rates (200 nL/minute or less).

As a result, microelectrospray ionisation sources[10,11] have been developed to cater for these much reduced solvent flow rates, and have been found to be particularly beneficial for separations using mobile phase flow rates up to 1000 nL/minute.

The NanoFlow™ electrospray probe described in this Application Note is designed to yield high quality data at reduced flow rates of between 5 and 1000 nL/minute, and can be configured either as a continuous flow interface[12] for on-line HPLC analyses by spraying from a fused silica capillary, or for "single shot" analyses by spraying from gold-coated borosilicate capillary tips at the very low flow rates. For the DNA-adduct experiments described here, the probe was used in continuous mode for high sensitivity on-line HPLC-MS analyses.

Split Injections

From the LC-ES-MS analysis of the reaction mixture from 2'-deoxyguanosine-5'-monophosphate (dGMP) with bis-phenol A diglycidyl ether (BPADGE), both UV and mass spectrometric data were obtained from one 3 nL sample injection (Figure 2).

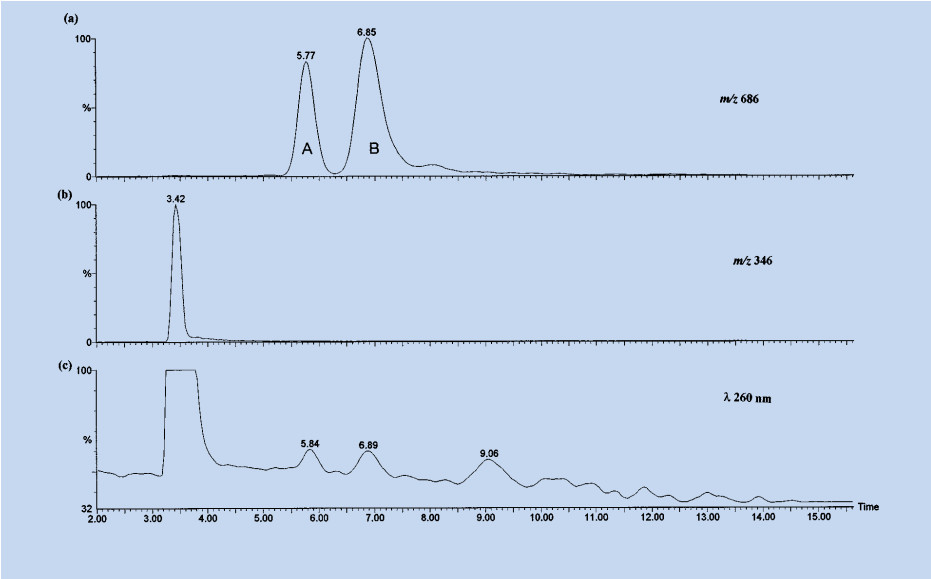


Figure 2. NanoFlow™ LC-ES-MS analysis of a dGMP/BPADGE reaction mixture obtained by injecting 3 nL of the mixture on-column using the split injection method. (a). Mass chromatogram of m/z 686, with the two adducts A and B labelled; (b). Mass chromatogram of m/z 346; (c). In-line UV detection at λ 260 nm.

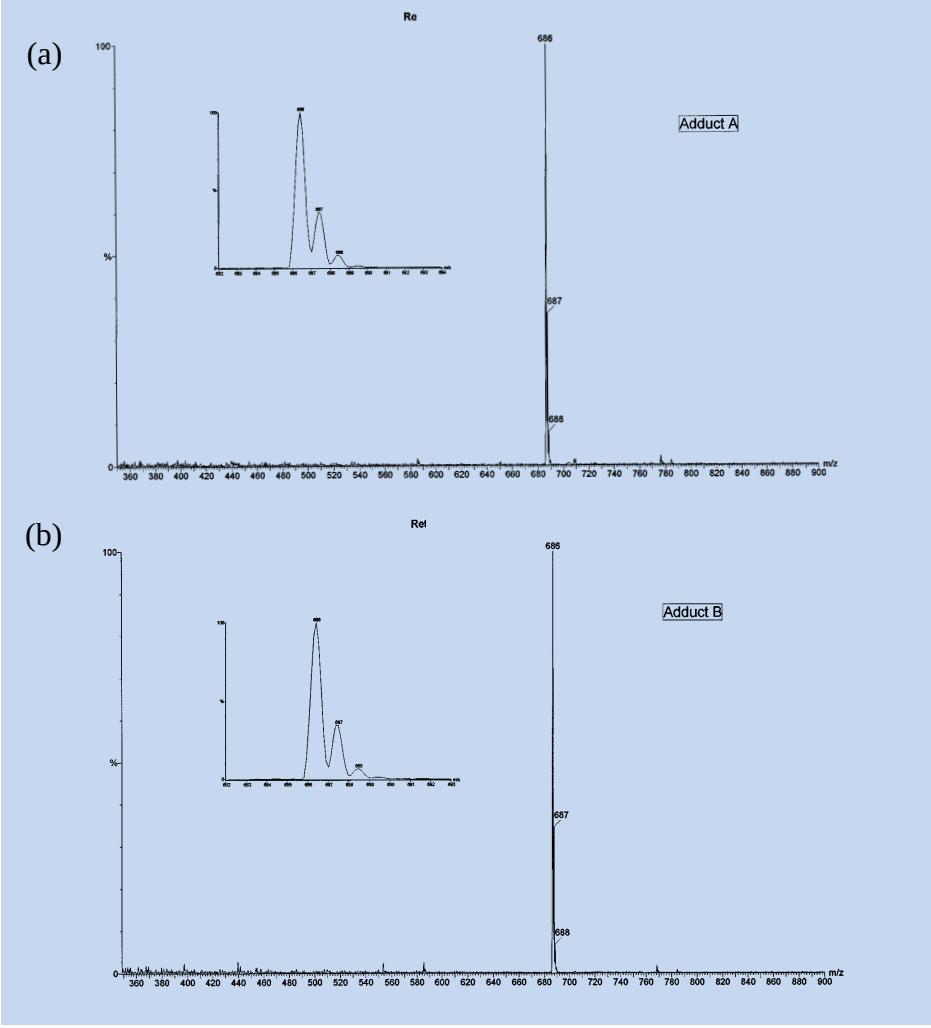


Figure 3. NanoFlow™ LC-ES-MS full scan spectra of : (a). adduct A; (b). adduct B.

The UV chromatogram shows several components which can be identified on inspection of their mass spectra. The mass chromatograms of *m/z* 346 and 686 highlight the presence of unreacted dGMP (eluting at 3.42 minutes) and two isomeric dGMP-BPADGE adducts (eluting at 5.77 and 6.85 minutes). The adducts arise directly from the addition of one molecule of dGMP across one of the epoxide rings of a molecule of BPADGE (Box 1).

ES-MS spectra from the two adducts are shown in Figure 3. The spectra are dominated by $(M-H)^-$ ions resulting from deprotonation of the intact molecules under the negative ionisation electrospray conditions, and so are strikingly similar.

In order to establish structural information regarding these two isomers which could lead to their differentiation, MS-MS product ion spectra were acquired by allowing the $(M-H)^-$ ions to undergo fragmentation in the collision cell, and then detecting the resulting fragment ions formed.

Figure 4 shows the two ES-MS-MS spectra obtained under identical conditions, and it can be seen that adduct A has fragmented to produce significant ions at *m/z* 535, 437, 283, 195, 153, 150, and 79, while adduct B produced a spectrum dominated by ions at *m/z* 195, accompanied by less intense ions at *m/z* 490, 177 and 79. From this information adduct A has been identified as the phosphate-alkylated adduct (Box 2), and adduct B as the guanosine base-alkylated adduct (Box 3)[9].

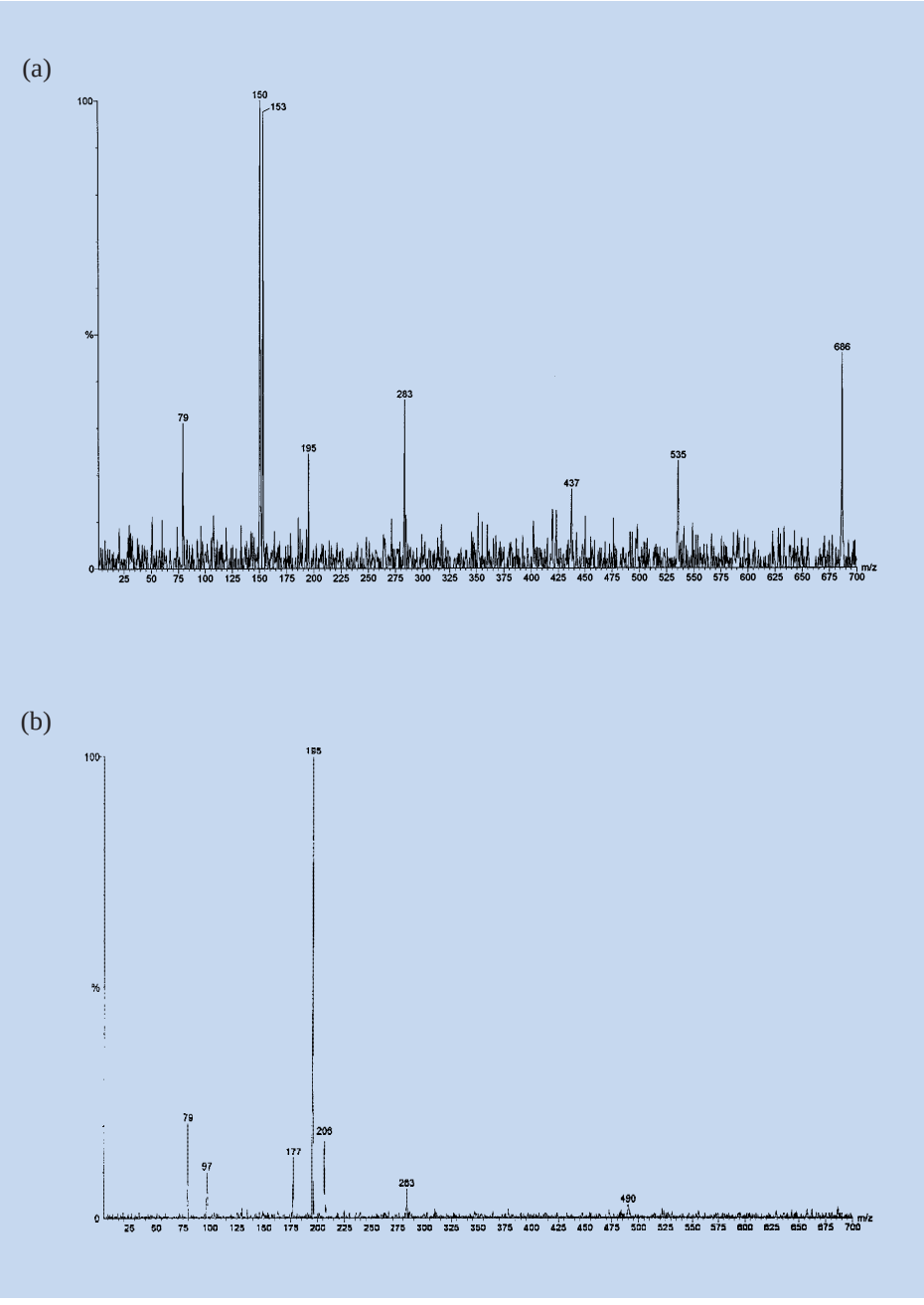
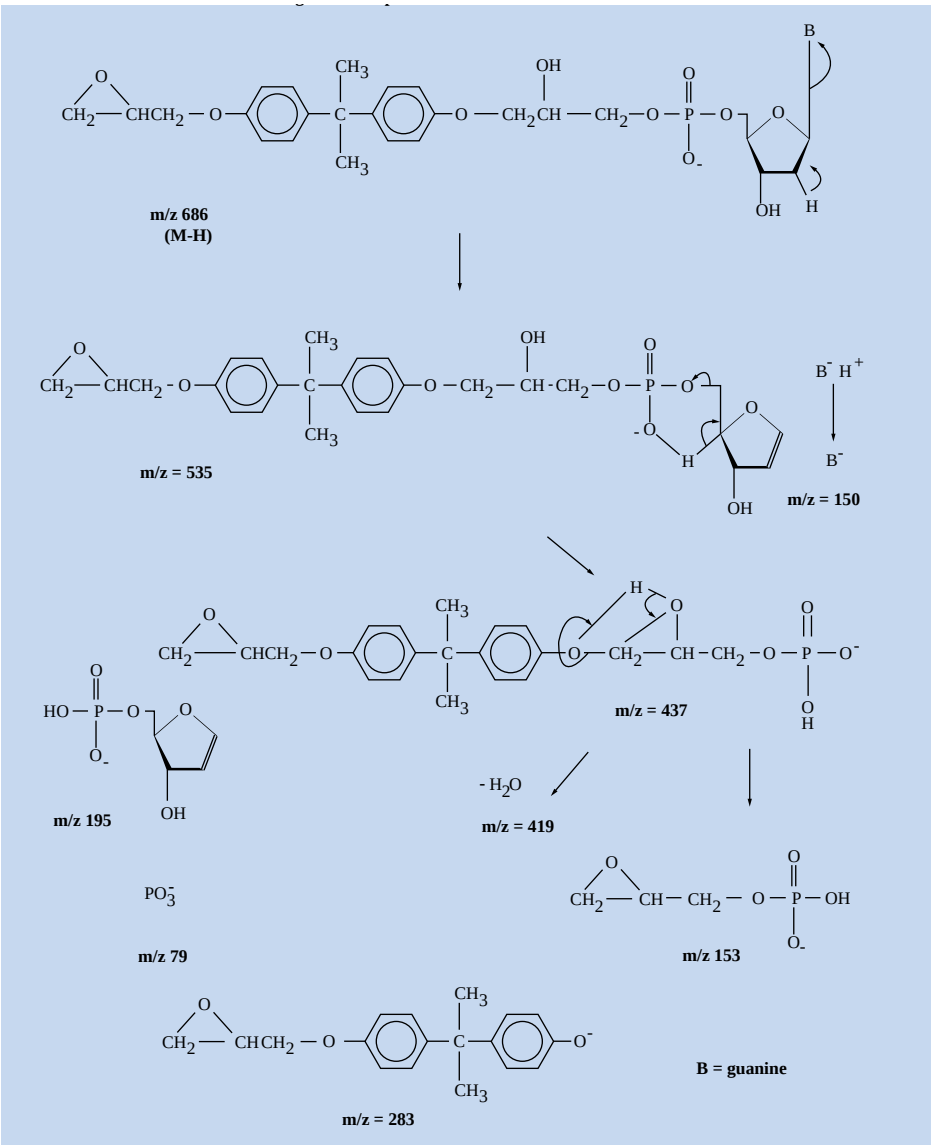


Figure 4. NanoFlow™ LC-ES-MS-MS product ion spectra of : (a). adduct A; (b). adduct B.

Large volume injections

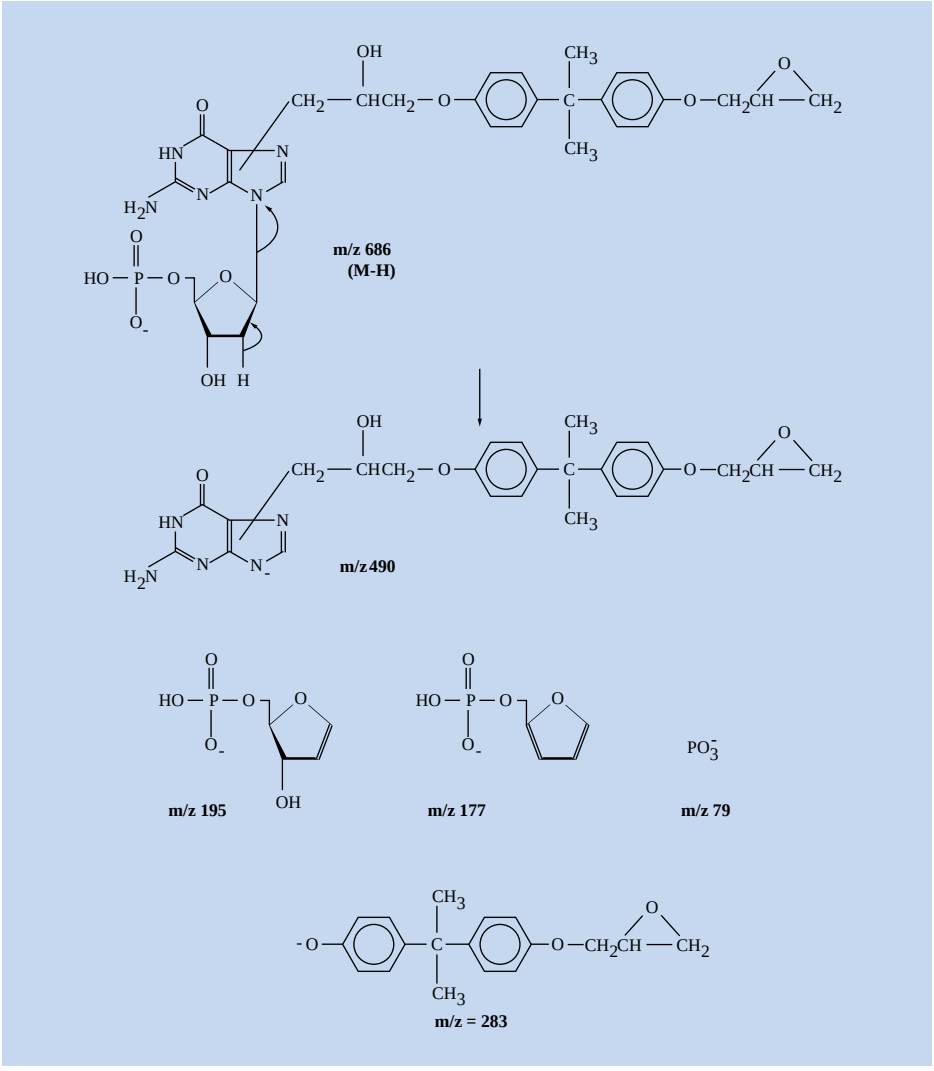
For enhanced sensitivity, large injection volumes are preferred. In these cases the additional split between the injection valve and the analytical column was removed and in this configuration volumes between 20 and 100 nL were injected directly onto the analytical column (Figure 1).

The sample should be loaded in aqueous solution, and there should be a difference in solvent strength between the mobile phase and the sample solution. This results in an increase in sensitivity compared with the split injections, and Table 1 documents the sensitivity achieved when using the different types of injection methods for adduct A.



Box 2. Fragmentation pattern of adduct A

From Table 1 it can be seen that an 8-fold increase in MS sensitivity is obtained when a 20 nL injection is made compared with a 3 nL injection, while an injection of 100 nL produces a 64-fold increase in MS sensitivity. From this it is apparent that the response seen is directly related to the amount of sample injected, and it is therefore beneficial to make a large injection, if sufficient sample is available and if the chromatographic resolution can be maintained.



Box 3. Fragmentation of adduct B.

Injection type	Injection volume (relative increase)		MS (full scan) (relative response)	UV (relative response)
split	3 nL	(1)	1	1
large volume	20 nL	(7)	8	3
large volume	100 nL	(33)	64	32
column switch	1 µL	(333)	211	278

Table 1. A Comparison of Sensitivity between the Different Injection Methods

Column switching

In the column switching experiment, see Figure 1, 1 µL of sample was concentrated on the pre-column, which was a significantly larger volume than in the earlier analyses. Additionally the pre-column can be used to "cut" early eluting components which are not of interest, or endogenous material which may contaminate the source; in this experiment it was used to send the unmodified nucleotide dGMP to waste. Thus the adducts alone were passed onto the analytical column, and Figure 5 compares the chromatograms (λ = 260 nm in both cases) from the two UV detectors; the upper trace shows the output from the analytical column and the lower trace the output from the pre-column *i.e.* the waste.

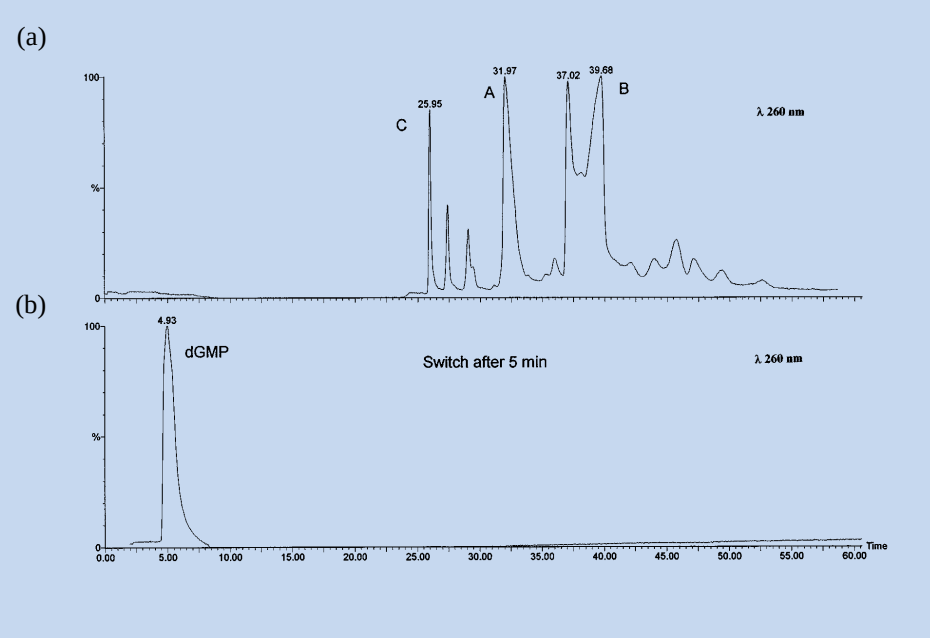


Figure 5. Nano-LC column switch experiments showing: (a). UV detection at λ 260 nm monitoring the eluent from the analytical column with enrichment of adducts A and B, and the appearance of a new compound C; (b). UV detection at λ 260 nm monitoring the eluent from the pre-column which was switched to waste after 5 minutes in order to prevent unreacted dGMP being applied to the analytical column.

The preconcentration and column switching experiment resulted in a large increase in sensitivity (Table 1), which was reflected in the enhanced quality of the spectra generated. Figure 6 shows the MS-MS product ion spectrum monitoring fragment ions from the $(M-H)^-$ ions of adduct A, and can be compared directly with the spectrum shown in Figure 4a. The increase in sensitivity is apparent.

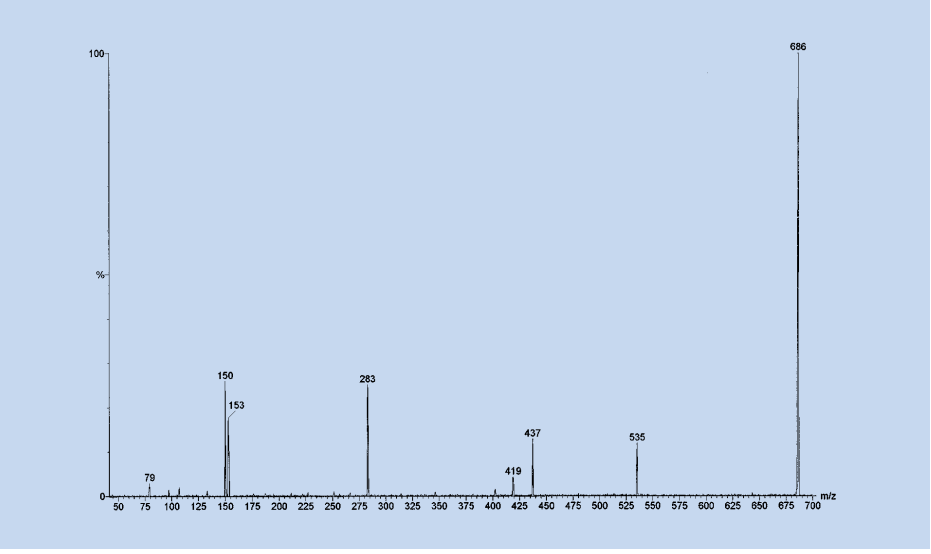


Figure 6. NanoFlow™ LC-ES-MS-MS product ion spectrum of adduct A, resulting from the column switching experiment.

Using this methodology minor components that were undetected by the other injection techniques can be localised and identified in the LC-ES-MS run. One such component, labelled C in Figure 5, exhibits a full scan spectrum dominated by doubly charged ions appearing at *m/z* 516 (Figure 7).

The MS-MS product ion spectrum from the doubly charged quasi-molecular ions $(M-2H)^{2-}$ of adduct C displayed a fragmentation pattern which showed some similarities to that of adduct A (Figure 8). In particular the ions at *m/z* 153 and 150 confirmed that adduct C had been formed by addition of BPADGE through the phosphate group of the nucleotide, and the compound was identified as the cross-linked adduct C resulting from the addition of a molecule of dGMP across each of the two epoxide rings on one molecule of BPADGE (Box 1).

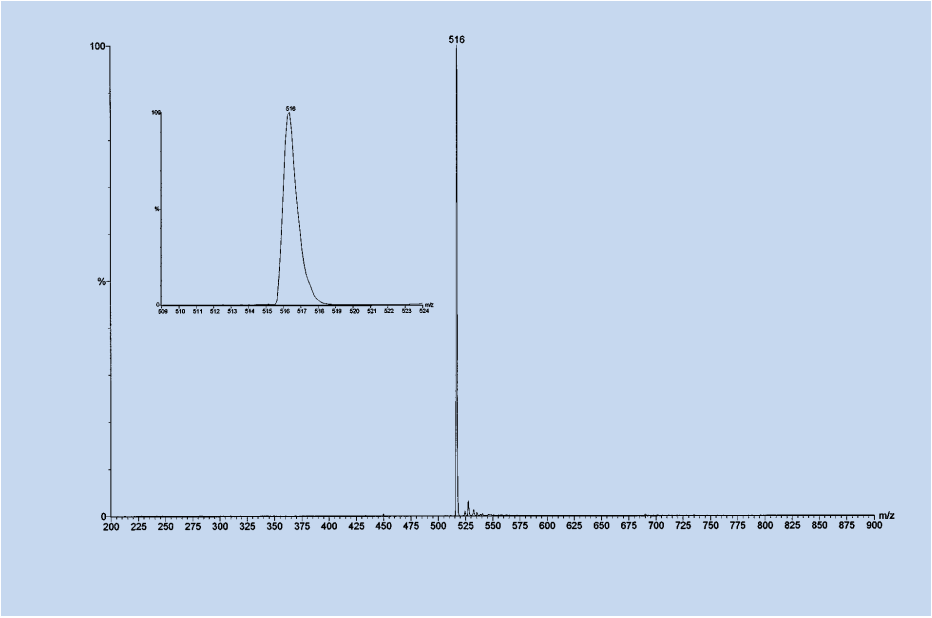


Figure 7. NanoFlow™ LC-ES-MS full scan spectrum of the cross-linked adduct C.

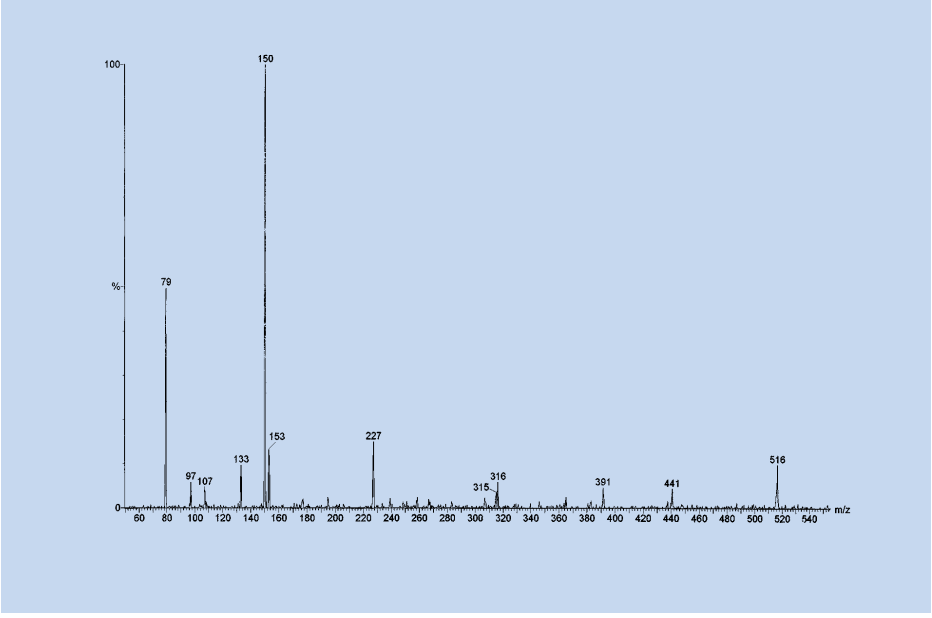


Figure 8. NanoFlow™ LC-ES-MS-MS product ion spectrum of adduct C, resulting from the column switching experiment.

Summary

Nano-LC has been coupled successfully to electrospray mass spectrometry using the NanoFlow™ interface probe, and different Nano-LC injection methods have been demonstrated.

The column switching method produced the highest sensitivity and hence most information concerning the samples. In this configuration the unmodified nucleotide was sent to waste, and the reaction products were enriched on the pre-column before entering the analytical column. The technique is sufficiently sensitive for it to be extended to the examination of DNA adducts from *in vivo* reactions.

DNA adducts have been identified and characterised by the molecular mass information obtained from their MS spectra and from the structural information displayed in their MS-MS product ion spectra. The DNA adducts resulting from phosphate alkylation can be distinguished from those resulting from base alkylation by examination of their fragmentation patterns.

Acknowledgements

The University of Antwerp would like to acknowledge the National Fund for Scientific Research (NFWO/LOTTO), the Flemish Institute for Promotion of Scientific and Technological Research in Industry (IWT), and the Flemish government (GoA action) for their support of this work. K.V. thanks the IWT for a research grant.

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Keywords:
NanoFlow™ Electrospray, Nano-LC, LC column switching, tandem mass spectrometry, DNA adducting, dGMP, BPADGE.

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