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An approach based on ultra-high pressure liquid chromatography–tandem mass spectrometry to quantify O^6-methyl and O^6-carboxymethylguanine DNA adducts in intestinal cell lines

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ABSTRACT

O^6-methylguanine (O^6-MeG) and O^6-carboxymethylguanine (O^6-CMG) are characteristic promutagenic and toxic DNA adducts formed by nitrosated glycine derivates and *N*-nitrosopeptides. Since endogenous nitrosation has been hypothesised as a plausible origin for the association between red and processed meat intake and colorectal cancer, a highly sensitive, fast and specific quantitative assay is needed to correlate the dose of individual DNA adducts with the effects of food consumption and individual digestive and metabolic processes. An ultra-high pressure liquid chromatography–tandem mass spectrometry (UHPLC–MS/MS) assay for quantitation of O^6-MeG and O^6-CMG, using the deuterated analogues as internal standards (ISTD), was developed. Samples of calf thymus DNA containing O^6-MeG and O^6-CMG were purified by acid hydrolysis and solid phase extraction prior to quantification by UHPLC–MS/MS in the selected reaction monitoring mode. The method was successfully validated in terms of repeatability (RSD < 10%), reproducibility (RSD < 15%) and linearity (99.9%) by incubating 0.1 mg calf thymus DNA with the known *N*-nitroso compound potassium diazoacetate (KDA). The limit of quantitation was 30 fmol mg^{-1} DNA for O^6-MeG or 1 adduct per 10^8 nucleotides and 50 fmol mg^{-1} DNA for O^6-CMG or 1.7 adducts per 10^8 nucleotides. Subsequently, the method was applied to human colon carcinoma cell lines, Caco-2 and HT-29, treated with KDA to illustrate its capability to quantify O^6-MeG and O^6-CMG DNA adducts using biological relevant models *in vitro*. This method will support further research to unravel the mechanistic basis of endogenous nitrosation processes upon consumption of red and processed meat products.

1. Introduction

Detection of DNA adducts is widely used for monitoring the exposure of cellular DNA to genotoxic agents. Knowledge of the nature and amounts of DNA adducts formed *in vivo* or *in vitro* provides valuable information regarding the mutational effects that may result from particular exposures. Epidemiological and clinical studies have consistently demonstrated that the consumption of meat and in particular red and processed meat is associated with an increased risk of colorectal cancer [1]. The formation of the DNA adducts O^6-carboxymethyl-2′-deoxyguanosine (O^6-CMdG) and O^6-methyl-2′-deoxyguanosine (O^6-MedG) has been associated with increased colorectal cancer risk upon intake of red and processed meat.

These particular DNA adducts (O^6-CMdG and O^6-MedG) are the result of endogenously formed alkylating *N*-nitroso compounds (NOCs), which are metabolised to highly reactive methylating agents that interact with the nucleophilic centres of DNA bases [2,3]. In turn, the formation of these endogenous NOCs has been hypothesised as a plausible origin for increased colon cancer risk, since a dose–response relation with the faecal excretion of NOCs for red and processed meat but not for white meat intake has been established [4–6]. Evidence of human exposure to NOCs,

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reinforcing the presence of alkylating agents in the human gastrointestinal (GI) tract [7,8] is based on the fact that O^6-CMdG and O^6-MedG are indeed detectable in colonic biopsies and human blood DNA samples [9–11].

The identification and quantification of very low DNA adduct concentrations, *in vivo* or *in vitro* requires ultrasensitive methodologies. This is particularly true for the analysis of human samples or *in vitro* applications on cell lines, where only small amounts of sample, and therefore DNA, are available. The methods currently used for DNA adduct determination include immunoassays [12,13] and capillary electrophoresis-laser induced fluorescence immunoassays [14], ^{32}P-postlabeling [15], GC/ECD [16] and HPLC with fluorescence detection [17,18]. In recent years there has been a continuous improvement in the analytical approaches used to study these adducts, both qualitatively and quantitatively, with mass spectrometric detection playing an increasing role in these developments [19–22]. HPLC coupled to mass spectrometric detection achieves a perfect balance between a high specificity and sensitivity. Specificity is further improved when utilising the selected reaction monitoring (SRM) mode of tandem triple quadrupole mass spectrometers. LC–MS has already been applied to both O^6-MeG and O^6-MedG adducts [21,23], however to the best of our knowledge no studies so far have reported MS-based methods with combined detection of O^6-methyl adducts and O^6-carboxymethyl adducts either as the bases or the nucleosides.

For this reason, the aim of our present study was to investigate the potential of triple quadrupole mass spectrometry (QqQ-MS) coupled to ultra-high pressure liquid chromatography (UHPLC) to quantify in a highly sensitive and specific manner the DNA adducts of interest, O^6-MeG and O^6-CMG, *in vitro* in colonic cell lines treated with the known NOC potassium diazoacetate (KDA), and for future experiments *in vivo* in human exfoliated colonocytes. To this purpose, a sample pre-treatment and UHPLC–MS/MS detection method were developed followed by an extensive validation study performed with a standard reference DNA; calf thymus (CT) DNA. This newly developed UHPLC–MS/MS method was then applied *in vitro* to measure the generated adduct levels in NOC-treated human colon carcinoma cell lines (Caco-2 and HT-29).

2. Materials and methods

2.1. Reagents and chemicals

Caution: KDA is carcinogenic. It should be handled in a well ventilated fume hood with extreme care and with personal protective equipment.

The chemical standard O^6-MeG was purchased from Sigma–Aldrich (St. Louis, MO, USA) and the internal standard O^6-methyl-d3-guanine (O^6-Me-d3-G) was obtained from Toronto Research Chemicals Inc. (Toronto, Canada). O^6-CMG was derived *via* 0.1 M formic acid hydrolysis at 70 °C for 1 h, from O^6-CMdG with subsequent purification [12]. The stock solutions of the chemical standards O^6-MeG and O^6-CMG were prepared in ethanol at a concentration of 6.06 μmol mL^{-1} and 4.8 μmol mL^{-1}, respectively, and diluted with deionised water to give working solutions of 818/81.8/8.18 fmol μL^{-1} and 646/64.6/6.46 fmol μL^{-1}, respectively. A work solution of O^6-Me-d3-G (59.4 fmol μL^{-1}) in deionised water was prepared, as internal standard. All solutions were stored in dark glass bottles at −20 °C.

Phosphate-buffered saline buffer (PBS) was prepared with chemicals purchased from Sigma–Aldrich (St. Louis, MO, USA) and consisted of 137 mM of NaCl, 2.5 mM KH_2PO_4 and 7 mM K_2HPO_4 in deionised water and adjusted to a pH of 7.3. TE buffer was made up with Tris (10 mM) and EDTA (1 mM, ethylenediaminetetraacetic acid) in deionised water and adjusted to a pH of 8.

Reagents were of analytical grade (VWR International, Merck, Darmstadt, Germany) when used for extraction and purification steps, and of Optima® MS grade for MS application (Fisher Scientific UK, Loughborough, UK), respectively.

Validation experiments and initial DNA adduct discovery was carried out with CT-DNA (Rockland Immunochemicals, Gilbertsville, PA, USA).

KDA was synthesised *via* alkaline hydrolysis of ethyl diazoacetate (EtDA, Sigma–Aldrich, St. Louis, MO, USA) [18]. The stock solution of 800 mM KDA was made up of 1.14 g EtDA and 11.4 mL 1.8 M KOH, and mixed for 4 h at room temperature in the dark, working solutions were obtained through dilution with PBS.

2.2. Optimisation of adduct-DNA acid hydrolysis

CT-DNA (1 mg mL^{-1}) was treated with 5 mM KDA overnight at 37 °C and 150 rpm in an Innova® 42 Incubator Shaker (New Brunswick Scientific, Edison USA) to generate DNA adducts. To quantify the adduct levels an acid hydrolysis of the KDA-treated CT-DNA is required to free purines, deoxynucleotides and deoxynucleosides. The hydrolytic potencies of 0.1 M hydrochloric acid [12,24] and formic acid [12] for 30 min at 80 °C or for 60 min at 70 °C were compared. Reactions were carried out in triplicate for each of the compound and treatment combinations.

2.3. Sample extraction and purification

In brief, 100 μg DNA supplemented with O^6-Me-d3-G (50 μL, 2.97 pmol) was dissolved in 2 mL of 0.1 M formic acid and hydrolysed by heating (80 °C for 30 min). The hydrolysate was cooled on ice and then applied to an Oasis HLB cartridge (SPE) (30 mg, 1 mL), which was conditioned with 2 mL of 100% MeOH and equilibrated with 2 mL deionised water. After loading the hydrolysate, a vacuum suction was applied on the SPE cartridge, followed by the elution step with 2 mL of 100% MeOH. The collected fraction was evaporated to dryness (90 min, 20 °C) using a SpeedVac® Plus (Savant, Holbrook, NY, USA). Finally, the dried residue was redissolved in a total volume of 100 μL of mobile phase consisting of 95:5 0.05% aqueous acetic acid:MeOH.

2.4. UHPLC–MS/MS analysis

The LC system consisted of a Thermo Electron (San Jose, USA) Accela UHPLC pumping system, coupled with an Accela Autosampler and Degasser. Chromatographic separation of the DNA-adducts was achieved by reversed phase chromatography and gradient elution. Separation of the DNA-adducts was carried out on an Acquity UPLC BEH C18 column (Ethylene Bridged Hybrid) (1.7 μm, 100 mm × 2.1 mm, Waters, Milford, MA, USA), kept at 30 °C. The mobile phase was pumped at a flow rate of 0.3 mL min^{-1}. Optimised separation of the analytes was obtained using a linear gradient starting with a mobile phase of 0.05% aqueous acetic acid:MeOH (95:5). After 0.85 min, the amount of methanol was increased to 50% in 3.15 min, immediately followed by a 100% MeOH and kept there for 1 min. The column was allowed to re-equilibrate for 2 min at initial conditions before the next run, giving a total run time of 7.2 min. Analysis was performed on a triple quadrupole mass analyser (TSQ Vantage, Thermo Electron, San Jose, USA), fitted with a heated electrospray ionisation (HESI II) source operating in the positive ion mode with the following working conditions: spray voltage of 4.5 kV; vaporiser and capillary temperature of 180 °C and 250 °C, respectively; sheath and auxiliary gas of 25 and 5 arbitrary units (a.u.), respectively; cycle time of 3.0 s. Argon pressure in the collision cell (Q2) was set at 1.5 mTorr and the mass resolution at the first (Q1) and third (Q3) quadrupole was set at 0.7 Da at full width at half maximum (FWHM). Precursor ion,

Table 1
Collected SRM transitions and compound specific mass spectrometric parameters.

Analyte	tR	Precursor ion (*m/z*)	Product ions (*m/z*)	S-lens (RF amplitude) (V)	Collision energy (eV)
O^6-carboxymethylguanine	1.72	210.0	107.0	83	37
			110.0		33
			135.0		30
			152.0		18
O^6-methylguanine	2.79	166.1	67.1	91	33
			121.1		25
			134.1		23
			149.1		19
O^6-methyl-d3-guanine	2.80	169.2	70.2	90	31
			107.1		29
			134.1		24
			152.1		19

S-lens RF amplitude, and collision energy (CE) in Q2 were optimised individually per compound (Table 1). Quantification and confirmation data were acquired in the selected reaction monitoring (SRM) mode, the transitions followed are displayed in Table 1. Instrument control and data processing were carried out with Xcalibur Software (version 2.1, Thermo Fisher Scientific, San Jose, USA).

2.5. Validation

Initial DNA adduct characterisation was carried out by incubating the reference matrix, *i.e.* CT-DNA, *in vitro* directly with 5 mM KDA to produce the promutagenic DNA adducts O^6-MeG and O^6-CMG [18]. The analytical procedure was validated by evaluating specificity, selectivity, linearity, repeatability, intra-laboratory reproducibility, recovery and limit of quantification (LOQ) with CT-DNA as the reference matrix.

2.5.1. Specificity and selectivity

The specificity of the method was demonstrated by analysis of untreated CT-DNA samples and samples fortified with each analyte separately or with a mixture of the two DNA adducts at different concentration levels, *i.e.* calibration curves.

Selectivity was demonstrated by identifying the analytes based on their relative retention time, *i.e.* the ratio of the chromatographic retention time of the analyte to that of the internal standard, which for liquid chromatography was set at a tolerance level of 2.5% (according to 2002/657/EC) [25].

2.5.2. Linearity

The linearity of response for the developed method was tested by assaying blank matrix samples, *i.e.* untreated CT-DNA, fortified at ten different concentration levels of both NOC-specific DNA adducts, O^6-MeG and O^6-CMG, ranging from 0 to 242 and 0 to 191 pmol mg^{-1} DNA, respectively. Linear regression analysis was carried out by plotting the peak area ratios of the analyte against the internal standard *vs.* the analyte concentrations, in order to determine the slope, intercept and correlation coefficient (R^2).

2.5.3. Mean recovery

To investigate the influence of sample clean-up and the prevalence of matrix elements, recoveries of O^6-MeG and O^6-CMG were assessed. The recoveries of the quantitative method were individually determined at nine different fortification levels (cfr. Section 2.5.2) and compared to the corresponding concentration of the selected calibration curve in order to calculate the recovery values ((measured value/fortification level) × 100).

2.5.4. Precision

To evaluate the precision of the method, repeatability and within-laboratory reproducibility were determined. Repeatability was evaluated by calculating the coefficients of variance (CV). To this purpose, eight calibration curves comprising of nine fortification levels in blank matrix were analysed on 2 consecutive days, carried out by the same analyst under repeatable conditions. For the within-laboratory reproducibility four additional calibration curves (nine fortification levels) were produced, by another analyst under varying environmental conditions.

2.5.5. Limit of quantification

LOQs were determined using spiked matrix samples and were defined as the concentration below which the method could not operate with acceptable precision and accuracy and at which the signal-to-noise ratio was greater than ten.

2.6. Cell culture

The human colon carcinoma cell lines Caco-2 and HT-29 (American Type Culture Collection, Manassas, VA, USA) were cultured as monolayers in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% foetal calf serum, 1% non-essential amino acids, penicillin (100 U mL^{-1}) and streptomycin (0.1 mg mL^{-1}) (all from Gibco Invitrogen cooperation, Merelbeke, Belgium), at 37 °C in a humidified 5% CO_2 incubator. Cells were passaged weekly by trypsinisation. For the experiments the colonic cells were harvested at 80% confluency.

2.7. DNA extraction

Before harvesting DNA, the colonic cell lines were grown in a monolayer culture (80% confluency), and collected after a 5 min incubation at 37 °C by trypsinisation. Prior to cell lysis, a cell viability rate of 99% was confirmed by trypan blue exclusion. For the DNA extraction, cells were centrifuged for 5 min at 10,000 × *g* at 4 °C to obtain a pellet. Subsequently 1 mL of DNAzol® reagent (Molecular Research Centre Inc., Cincinnati, OH, USA) was added per 1×10^7 of cells. This ready to use reagent is based on the use of a novel guanidine-detergent lysing solution that hydrolyses RNA and at the same time allows selective DNA precipitation. Lysis of the cells was obtained by gently pipetting the mixture or inverting the assay tube. The DNA was precipitated from the lysate by adding 0.5 ml of 100% ethanol per ml of DNAzol® reagent used for isolation. Next, the sample was mixed by inversion and kept at room temperature for 1–3 min. DNA, visible as a cloudy precipitate was collected by spooling with a pipette tip while carefully decanting the supernatant. Where the resulting sheared DNA would not spool, 5 min centrifugation at 5000 × *g* was applied to obtain a DNA pellet. Finally the pellet was resuspended in 250–500 μL TE buffer. The concentration of the collected DNA was determined by a Nanodrop ND-1000 Spectrophotometer (Isogen Lifescience, IJsselstein, The Netherlands).

2.8. In vitro and in vivo application

The validated method was applied to simultaneously quantify the levels of O^6-MeG and O^6-CMG in a series of *in vitro* KDA-treated DNA samples of different origin (CT-DNA, Caco-2 and HT-29). Different concentrations of KDA (0, 1, 2.5 and 5 mM) were supplemented to the monolayer cultured cell lines of Caco-2 and HT-29 during a 24 h incubation step, and for the HT-29 cells an additional experiment was performed with a shorter incubation time of two h. In a parallel experiment, DNA was extracted from the cultured cell lines (referred to as pre-extracted (p.e.) DNA), prior to KDA incubation. Calibration curves were in the range of 0–121 pmol mg^{-1} DNA (O^6-MeG) and 0–96 pmol mg^{-1} DNA (O^6-CMG) using untreated CT-DNA to produce the standards.

In vivo application of our method was performed after collection of fresh stool samples by means of a Fecotainer® (AT Medical, The Netherlands) from four healthy volunteers (three males and one female), aged between 28 and 38 years old. The volunteers did not take any antibiotics in the past six months and followed their normal Western diet pattern, with no restrictions applied. After collection, the stool samples were processed immediately as described by Lewin et al. to remove the exfoliated colonocytes from the stool surface [10]. For each volunteer, the colonocytes were collected and lysed (DNAzol®) in order to collect the DNA, which was subsequently analysed for the targeted DNA adducts O^6-MeG and O^6-CMG by UHPLC–MS/MS.

2.9. Quality assurance and data analysis

Prior to sample analysis, a standard mixture of the target compounds was injected to check the operational conditions of the chromatographic device. To every sample, an internal standard (ISTD) was added at a concentration of 59.4 fmol μL^{-1} (O^6-Me-d3-G), prior to the extraction. Identification of the DNA adducts was based on the retention time relative to the ISTD and on the ion ratios of the product ions (four per compound). Upon identification, the analyte concentration was calculated by fitting its area ratio in a nine-point calibration curve, established in CT-DNA fortified with O^6-MeG and O^6-CMG in the range of 0–242 and 0–191 pmol mg^{-1} DNA, respectively with the ISTD at 59.4 fmol μL^{-1}. Area ratios were determined by integration of the area of an analyte under the specific SRM chromatograms in reference to the integrated area of the internal standard. Positive and negative QC samples were prepared by dissolving 100 μg CT-DNA in TE buffer, with and without the addition of 5 mM KDA, respectively.

3. Results

3.1. Development of sample clean-up

To evaluate DNA adduct recovery, a proper execution of the DNA hydrolysis step is required. Mild acid hydrolysis of DNA samples with 0.1 M formic acid (F.A.) and hydrochloric acid (HCl) was compared for its purine base-releasing capacity [12]. Besides the type of acid used, a different hydrolysis time and temperature were investigated, respectively, 30 min at 80 °C and 60 min at 70 °C. The hydrolysed CT-DNA samples were analysed by UHPLC–MS/MS to investigate the O^6-MeG and O^6-CMG content. The rate of O^6-MeG formation upon KDA treatment was not as high as for O^6-CMG, in line with previous literature findings [3,10]. For this reason, the hydrolysis method with the highest ratio of O^6-MeG towards O^6-CMG was chosen, *i.e.* F.A. for 30 min at 80 °C with a ratio of 0.070. HCl hydrolysis at identical conditions obtained a slightly lower ratio of 0.062, which was better than F.A. (ratio of 0.059) and HCl for 1 h at 70 °C (ratio of 0.045) (Fig. 1).

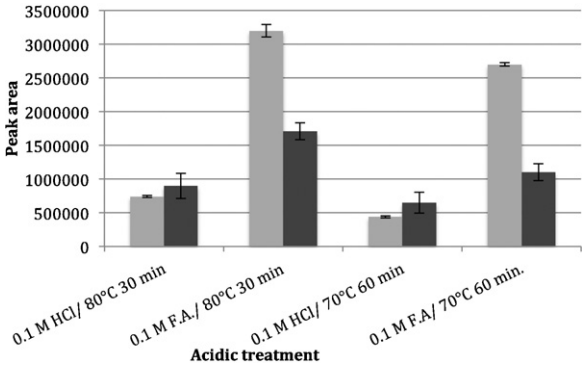

Fig. 1. Absolute comparison (area) of the purine base-releasing capacity of different acidic treatments with respect to O^6-methyl guanine (□) and O^6-carboxymethylguanine (■).

For the sample clean-up, we started from the protocol of Upadhyaya et al. [24], who used Strata X SPE cartridges (Phenomex, Torrance, USA). Because this protocol only focussed on O^6-MeG, we also evaluated different types of SPE cartridges, of which the Oasis HLB SPE cartridge (Waters Corporation, Milford, MA, USA) provided a higher extraction yield and better signal-to-noise ratios for O^6-CMG. In addition, the washing step was omitted and replaced by a vacuum suction, since rinsing with 10% MeOH significantly decreased the recovery of O^6-CMG with about 30% (data not shown).

3.2. U-HPLC–MS/MS parameters

To determine the optimal MS conditions, the default parameters for the HESI probe, capillary temperature, vaporising temperature, sheath gas pressure, and auxiliary gas pressure were optimised by individually infusing the analytical standards. During this tuning step acetic acid, formic acid and ammonium acetate were evaluated as candidate mobile phase additives to enhance ionisation. Addition of formic acid at 0.1% in the mobile phase provided the most optimal compromise between ionisation and peak geometry.

Besides the MS conditions, chromatographic separation and resolution of the target compounds had to be achieved. To this extent a whole range of UHPLC columns (nine in total) were tested, *i.e.* Acquity BEH C18 (1.7 μm, 50–100 mm × 2.1 mm, Waters), HSS C18 (1.8 μm, 50 mm × 2.1 mm, Waters), Hypersil Gold (1.9 μm, 50–100 mm × 2.1 mm, Thermo Scientific), Nucleodur C18 Pyramid (1.8 μm, 100 mm × 2.1 mm, Machery-Nagel), Nucleodur C18 gravity (1.8 μm, 50 mm × 2.1 mm, Machery-Nagel) and Nucleodur Isis (1.8 μm, 50–100 mm × 2.0 mm, Machery-Nagel). Finally, based on the retention time, base peak separation, and peak efficiency measured as peak width at baseline, the Acquity BEH C18 column was selected. Different mobile phase compositions (ACN, MeOH, H_2O), ionisation enhancers (acetic acid, formic acid and NH_4OAc), flow rates (300–500 μL min^{-1}) and column temperatures (30–45 °C) were tested leading to the selection of a final mobile phase consisting of 0.05% aqueous acetic acid and methanol at 30 °C and a flow rate of 0.3 mL min^{-1}.

3.3. Validation

3.3.1. Specificity and selectivity

The targeted DNA adducts, O^6-MeG and O^6-CMG were not detected in the blank matrix, untreated CT-DNA. For each analyte spiked however, chromatograms showed a significant increase in peak area and intensity at the analyte's retention time compared to the blanks, taking a S/N of at least 3 into account (Fig. 2). No other matrix substances interfered at these retention times. Therefore,

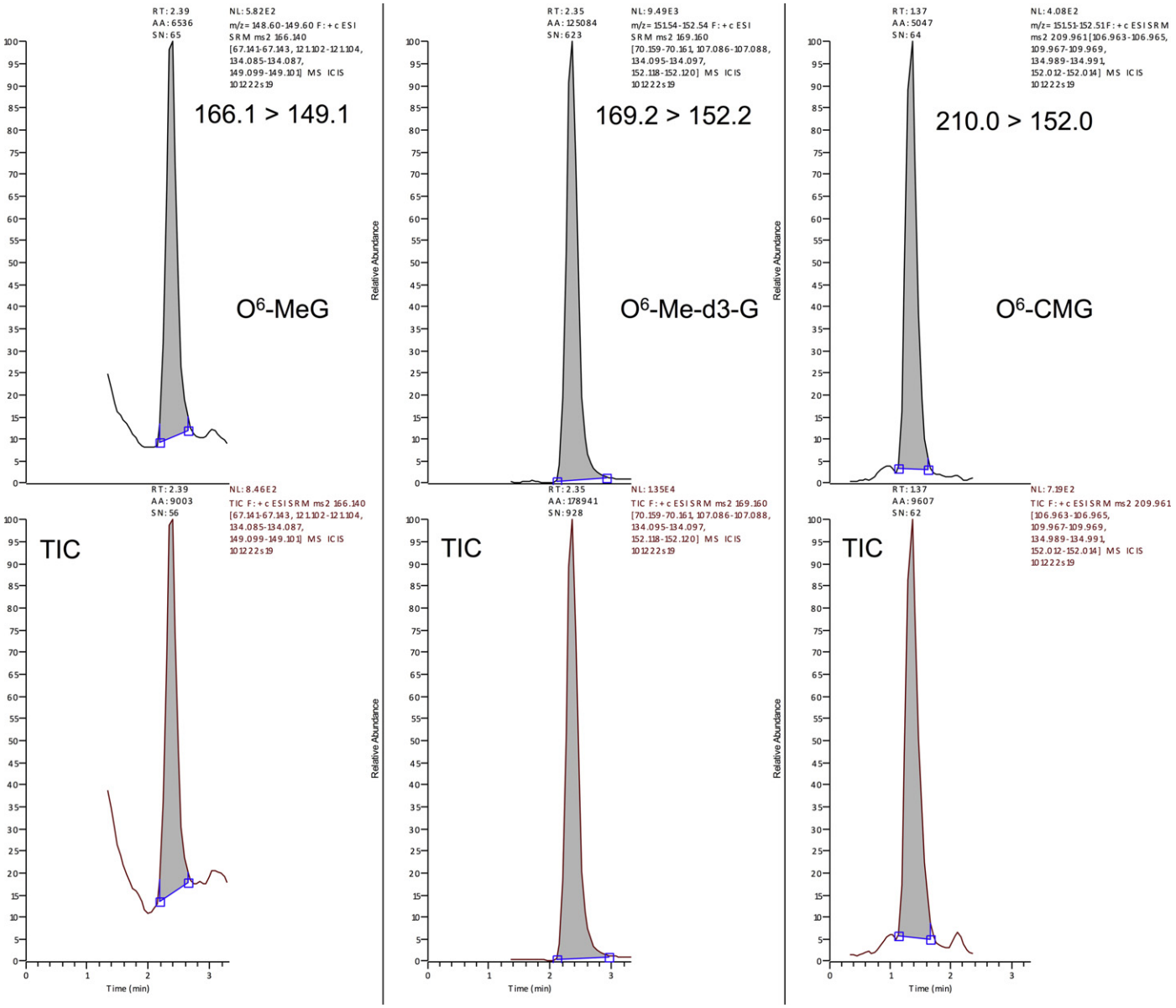

Fig. 2. SRM chromatogram of CT-DNA fortified with O^6-methyl- and O^6-carboxymethylguanine at 0.95 pmol mg^{-1} DNA and the internal standard O^6-methyl-d3-guanine at 29.7 pmol mg^{-1} DNA, with the main transition and sum of all transitions (TIC).

the newly developed method was found to be specific for O^6-MeG and O^6-CMG in the presence of matrix components.

The individual relative retention times ($n = 10$) of O^6-MeG and O^6-CMG extracted from blank matrix, 0.987 min ± 0.021 and 0.616 min ± 0.013 respectively, both displayed a coefficient of variation of 2.1%. This falls well within the stated tolerance level of 2.5% for liquid chromatography (2002/657/EC) [25]. As a result, the identification of the two DNA adducts from CT-DNA samples proved to be unambiguous.

3.3.2. Linearity, recovery, precision and limit of quantification

The UHPLC–MS/MS nine-point calibration curves for O^6-MeG and O^6-CMG in matrix samples (CT-DNA) showed excellent linearity. Over the concentration range of 0–242 pmol mg^{-1} DNA for O^6-MeG and 0–191 pmol mg^{-1} DNA for O^6-CMG, the correlation coefficients (R^2) were 0.998 ± 0.002 ($n = 12$).

Recovery values, representing the average of twelve determinations ($n = 12$) per fortification level and expressed as a percentage of the measured concentration at a certain fortification level divided by the fortification level were calculated for each fortification level (O^6-MeG: 0.9–242 pmol mg^{-1} DNA; O^6-CMG: 0.8–191 pmol mg^{-1} DNA) (data not shown). The overall recovery (Table 2) for O^6-MG and O^6-CMG, 105.5% ± 9.2 and 100.7% ± 10.0, respectively, indicate that the sample clean-up procedure and the prevalence of matrix interferences did not interfere with the analytical detection.

To evaluate the precision of the method, repeatability and within-laboratory reproducibility were determined. For both DNA adducts good repeatability was obtained, since the individual overall CVs for each analyte were below 10% (Table 2). The within-laboratory reproducibility, calculated over 108 measurements also displayed a more than acceptable intra-laboratory coefficient of variation well below 15% (Table 2) (2002/657/EC) [25].

LOQs (Table 2) were determined using spiked matrix samples and were set on the lowest quantifiable concentrations of the calibration curve below which the method could not operate with acceptable precision and accuracy and at which the signal-to-noise ratio was greater or equal to 10.

Table 2
The limit of quantification (LOQ), recovery and precision of the developed method for O^6-carboxymethylguanine and O^6-methylguanine measured in calf thymus DNA.

Analyte	LOQ (pmol mg^{-1} DNA)	Recovery[a]		Repeatability[a]	Within-laboratory reproducibility[b]
		Mean ± SD (%)	RSD (%)	RSD (%)	RSD (%)
O^6-carboxymethylguanine	0.05	100.7 ± 10.0	9.9	8.2	8.3
O^6-methylguanine	0.03	105.5 ± 9.2	10.1	9.5	10.7

[a] 72 aliquots of CT-DNA and 9 fortification levels, resulting in 8 measurements per fortification level.
[b] 108 aliquots of CT-DNA and 9 fortification levels, resulting in 12 measurements per fortification level.

3.3.3. Application in vitro and in vivo

Two types of human colon carcinoma cell lines (Caco-2 and HT-29) were treated *in vitro* with different concentrations of KDA. Additionally, different KDA incubation times (2 and 24 h) were tested on the HT-29 cell line to compare the formation yield. As expected, the highest DNA adduct formation was formed upon 24 h of incubation (Fig. 3). Only O^6-CMG and no O^6-MeG DNA adducts were formed during the KDA incubation of the colonic cell lines. The detected O^6-CMG concentrations showed a high repeatability and a linear ($R^2 > 0.9$) dose–response curve for the different KDA concentrations, however a significant interbatch variation between different passages of cells was observed, especially in case of the HT-29 cells (Fig. 3).

In an attempt to simplify the procedure, DNA was extracted from the cultured cells prior to KDA incubation. From these results it could be concluded that a similar concentration of O^6-CMG adducts was formed, as compared to the previous experiments in which the cell lines were directly exposed to KDA (Fig. 3). As a result of the simplification, the unacceptable interbatch variation seen between the different passages of the cell cultures was eliminated, which was particularly high for the HT-29 cell lines. Therefore it was decided to perform future incubation experiments with pre-extracted DNA instead of whole cells. Table 3 summarises the obtained DNA adduct concentrations when treating pre-extracted DNA of different origin (CT, Caco-2 and HT-29) with KDA.

In vivo application of our method was performed on exfoliated colonocytes, collected from the outer layer of each individual stool sample of four healthy volunteers. From each stool sample, 4–13 μg of DNA was collected and subsequently screened for the presence of the targeted DNA adducts. The UHPLC–MS/MS analysis detected traces of O^6-CMG below the LOQ level (0.05 pmol mg^{-1} DNA) in only two of the four samples (Fig. 4).

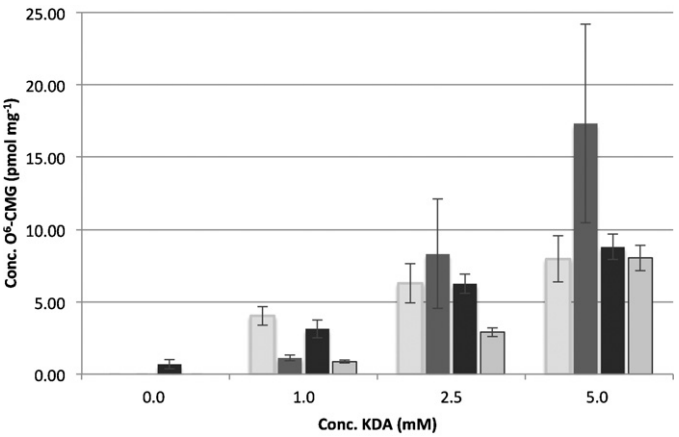

Fig. 3. The dose response plot (±standard error) for the cell lines HT-29 after 2 h (□) and 24 h (■) of incubation and Caco-2 after 24 h (■) of incubation with various concentrations of KDA (mM), *vs.* pre-extracted (p.e.) Caco-2 DNA (□) after 24 h incubation with KDA.

4. Discussion

The present study describes the optimisation and validation of a high-throughput, accurate and robust analysis method for the combined detection of O^6-carboxy and O^6-methylguanine DNA adducts. This quantitative analytical method has been subsequently applied to measure the DNA adduct formation *in vitro* on the human colonic carcinoma cell lines HT-29 and Caco-2 (and their pre-extracted DNA) after incubation with the known gastro-intestinal *N*-nitroso compound KDA and *in vivo* on human colonocytes in healthy individuals.

A proper validation procedure requires a reference matrix to assure a scientifically correct outcome. To this end, CT-DNA was chosen because no background lesions for O^6-methylguanine and O^6-carboxymethylguanine were detected in a preliminary screening. A number of sample pre-treatment steps including acid hydrolysis and solid phase extraction, needed to be optimised to ensure maximal adduct recovery. Formic acid obtained better hydrolysis results, as confirmed by Moore et al. [12], whereas a hydrolysis time of 30 min at 80 °C was preferred in line with Upadhyaya et al. [24].

The detection of DNA adducts, more specifically of O^6-MeG and O^6-CMG may be realised using a whole range of analytical techniques, *i.e.* immunoassays [12,13], capillary electrophoresis-laser induced fluorescence immunoassays [14], ^{32}P-postlabeling [15], GC/ECD [16] and HPLC/fluorescence [17,18]. Throughout recent years, the use of mass spectrometric detection has become more popular, despite the amount of DNA required 10–100 μg *vs.* 1 μg to several milligrams for the other detection techniques [22], more popular. The great advantage of MS compared to other analytical methods is that it provides information about the structural identity of the compounds under investigation, thus confirming that the correct DNA adduct is targeted. This technique also allows accurate and reproducible data when labelled internal standards are used. Furthermore working in the selected reaction monitoring mode of a triple quadrupole MS provides additional specificity to the assay. Instead of coupling our MS detection to HPLC [21,23], UHPLC was selected, which significantly increased sample throughput by using sub-2 micron particle sized columns with low dead volume that are able to withstand high-pressure LC equipment, thus drastically shortening analysis time without loss of separation efficiency while increasing sensitivity [26]. Accurate quantitation was achieved by the use of calibration curves and deuterated internal standards, identical in extraction and chromatographic behaviour to the DNA adduct of interest but slightly differing in mass, resulting in mass-based separation. For both DNA adducts a deuterated version was available, namely O^6-Me-d3-G and O^6-CM-d2-G [27]. Preliminary tests ($n = 12$) indicated a 11.8% ± 0.02 decomposition of O^6-CM-d2-G to O^6-CMG, whereas for O^6-Me-d3-G to O^6-MeG only 0.7% ± 0.02. This is thought to be due to the position of the deuterium atoms on the α-C of the carboxymethyl moiety making them more readily exchangeable in O^6-CM-d2-G than the deuterium atoms of O^6-Me-d3-G. For this reason O^6-Me-d3-G was retained as internal standard for both DNA adducts. As for the calibration

Table 3
Levels of O^6-methylguanine and O^6-carboxymethylguanine detected with the U-HPLC–MS/MS analysis method upon 24 h of KDA treated DNA, derived from different origin (CT, Caco-2 and HT-29 DNA); ND: not detected.

Conc. KDA (mM)	CT-DNA (pmol mg^{-1} DNA)		Caco-2 DNA (pmol mg^{-1} DNA)		HT-29 DNA (pmol mg^{-1} DNA)	
	O^6-MeG	O^6-CMG	O^6-MeG	O^6-CMG	O^6-MeG	O^6-CMG
0.5	0.5 ± 0.0	9.6 ± 1.1	0.1 ± 0.0	0.6 ± 0.0	ND	0.4 ± 0.1
1.0	0.6 ± 0.0	11.7 ± 0.9	0.1 ± 0.0	0.9 ± 0.1	ND	1.0 ± 0.1
2.5	1.0 ± 0.0	22.4 ± 1.5	0.3 ± 0.0	2.9 ± 0.3	ND	1.7 ± 0.3
5.0	1.4 ± 0.0	52.4 ± 1.5	0.7 ± 0.1	8.1 ± 0.9	ND	4.3 ± 0.3

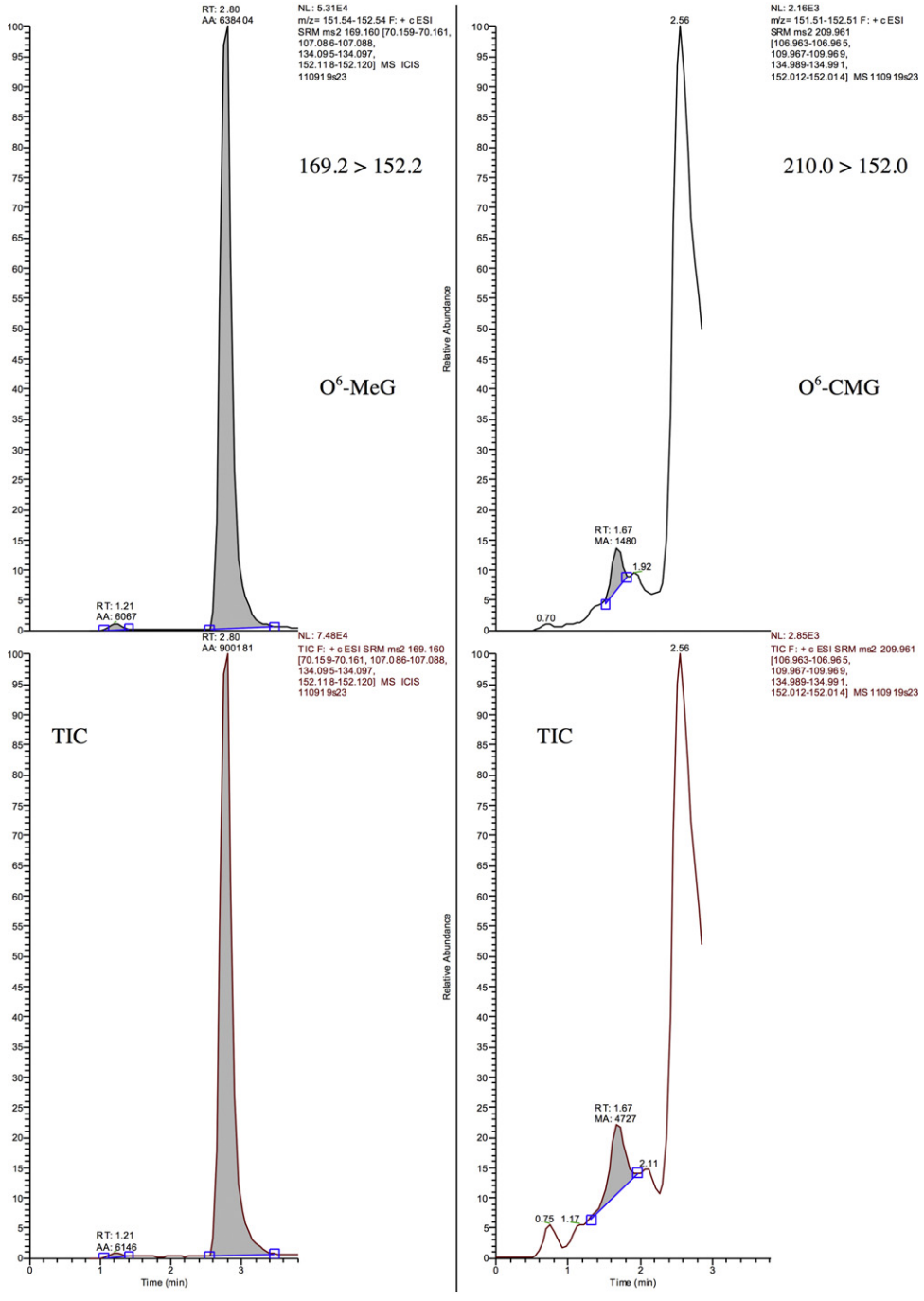

Fig. 4. The detection of O^6-carboxymethylguanine at LOD concentration level (<0.05 pmol mg^{-1} DNA) in exfoliated colonocytes of the outer layer of one stool sample.

curves, a wide concentration range of 0–242 pmol mg^{-1} for O^6-MeG and 0–191 pmol mg^{-1} DNA for O^6-CMG was selected during the validation study, which was adjusted when analysing the KDA-treated cell lines or their pre-extracted DNA to a more appropriate range of 0–121 pmol mg^{-1} DNA for O^6-MeG and of 0–91 pmol mg^{-1} DNA for O^6-CMG.

To the best of our knowledge this is the only mass spectrometry based method combining the detection of O^6-MeG and O^6-CMG reported so far. For this reason comparison of our newly developed analytical procedure with previously published methods is not straightforward. When comparing the LOQs of previously reported LC–MS methods for O^6-MeG, LOQs of 0.36 pmol mL^{-1} and 3.1 pmol mL^{-1} were reported [21,23], both higher than our LOQ of 0.061 pmol mL^{-1}. Upadhyaya et al. [24], reached the lowest LOQ of 3 fmol mg^{-1} DNA compared to our 30 fmol mg^{-1} DNA (Table 2). However if the detection of O^6-CMG had not been prioritised during our UHPLC–MS/MS optimisation, a lower LOQ could have been obtained for O^6-MeG. Nevertheless, compared to other reports the LOQ of our newly developed UHPLC–MS/MS method was found highly satisfactory and gave comparable, albeit slightly higher, value for O^6-CMG (50 fmol mg^{-1} DNA).

Applying our newly developed method to KDA-treated CT-DNA resulted in the detection of O^6-CMG and to a lesser extent O^6-MeG (Table 3). These findings correspond with previous observations indicating that carboxymethylation with concomitant methylation at the O^6 atom of 2′-deoxyguanosine appears to be a common feature when nitrosated glycine derivatives, including KDA, react with DNA [3,18,28]. The DNA adduct O^6-MedG has been previously reported to be found in colonic biopsies [9,10]. Additionally, O^6-CMG appears to be a potential biomarker for DNA carboxymethylation, since it cannot be repaired by O^6-alkylguanine-DNA alkyltransferase [3]. For these reasons, we opted to incorporate both NOC-specific DNA adducts, O^6-CMG and O^6-MeG in our quantitative UHPLC–MS/MS method, however the emphasis was put on the detection of O^6-CMG.

Analyses of KDA incubated CT-DNA indeed resulted in the detection of the DNA adducts O^6-CMG and to a lesser extent O^6-MeG, as described by Shuker et al. [3]. However when incubating pre-extracted Caco-2 DNA with KDA, only a limited amount of O^6-MeG was identified, whereas upon incubation of pre-extracted HT-29 DNA no O^6-MeG was found, coherent with literature [10]. This might suggest that carboxylation is a more common feature within Caco-2 cell lines compared to HT-29, or that the repair mechanism of O^6-MeG, the O^6-alkylguanine-DNA alkyltransferase, is more present or active in the HT-29 cell line. The number of DNA adducts formed upon KDA incubation in CT-DNA was significantly higher than that in pre-extracted Caco-2 and HT-29 DNA. This may be explained by the fact that pre-extracted Caco-2 and HT-29 DNA did not undergo any kind of clean-up or wash step, resulting in unpurified DNA contaminated with a large amount of unidentified peptides, able to interfere with the KDA incubation as opposed to the purified commercially purchased CT-DNA. Working with pre-extracted DNA significantly simplified the elaborate procedure to obtain DNA adduct results, and reduced the large interbatch variation for the HT-29 cell line. Both cell lines underwent an identical number of passages (25–29) and period of growth (7 days), nevertheless the colonic carcinoma cells might not have been at the same stage of the cell cycle and thus might react differently to the KDA treatment, which could explain the observed differences in interbatch variation.

The *in vitro* experiment indicated a good correlation between the KDA treated colonic cell lines and the pre-extracted DNA, since in both cases similar amounts of the DNA adducts O^6-MeG and O^6-CMG were formed. For this reason, a small *in vivo* experiment was conducted to measure the targeted DNA adducts in exfoliated colonocytes, to acknowledge the implementation potential of this newly developed method for future studies. The analysis of the DNA collected from the exfoliated colonocytes showed trace levels of O^6-CMG. The obtained signal was relatively low, which is explained by the fact that the volunteers were on a normal diet and the amount of faeces was limited (<100 g). Nevertheless, this experiment indicates a good applicability of the UHPLC–MS/MS method for future *in vitro* and *in vivo* studies.

5. Conclusion

The results of the present study acknowledge the excellent applicability of triple quadrupole mass spectrometry coupled to ultra-high pressure liquid chromatography for the simultaneous detection of DNA adducts, more specifically O^6-methylguanine and O^6-carboxymethylguanine, in a accurate, sensitive, robust and high throughput manner. The performance characteristics (*i.e.* specificity, selectivity, repeatability, linearity, precision, recovery and limit of quantification) of this validated UHPLC–MS/MS method are highly satisfactory when applied to blank CT-DNA matrix, but also when applied to KDA-treated pre-extracted DNA samples of different origin (CT-DNA, Caco-2 and HT-29 DNA). The latter demonstrates the potential for applying this detection method in future mechanistic studies evaluating the *in vitro* or *in vivo* (geno)toxic activity of NOCs in the human gastrointestinal tract.

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