The 32P-post-labelling assay has emerged as a major tool for detecting bulky DNA adducts in subjects exposed to carcinogens, especially aromatic compounds. However, the 32P-post-labelling protocol still requires the use of high amounts of radioactivity, i.e. 25–50 μCi per sample, an obstacle that limits its use in large studies. The characterization of the DNA adducts measured is also limited. Methodological improvements and increased DNA adduct characterization are necessary to make this assay capable of achieving higher throughput. A new protocol was tested to ensure efficient hydrolysis to reduce the use of radioactive material and to obtain higher chromatography resolution. Different chromatography systems based on high-urea or ammonium hydroxide systems were also employed to characterize the adducts being measured. Improvements were tested by re-analysing DNA adducts in a group of police officers and urban residents in Genoa, Italy. The analysis of carcinogen-modified DNA standards was also included in the study for qualitative and quantitative comparison. An efficient DNA digestion was obtained using a method involving hydrolysis by micrococcal nuclease and a mixture of two spleen phosphodiesterases at fixed concentrations. A 72% reduction of the amount of radioactivity used for labelling was achieved in respect to the non-modified protocol without loss of DNA adduct sensitivity. An improved chromatography resolution was obtained by reducing the volume of sample to be spotted on the chromatogram. Lower volume of spotting sample can decrease sample diffusion and the formation of unresolved spots on the thin-layer chromatography plate. The amount of output produced using a single batch of carrier-free [γ-32P]ATP was increased by about 3.5-fold. A complex pattern of DNA adducts was observed in leukocytes using both high-urea or isopropanol–ammonium hydroxide systems, two techniques effective in the detection of aromatic DNA adducts. The above observations indicate that DNA adducts being measured are likely to have been induced by aromatic compounds.

Introduction

Over the last two decades, the 32P-post-labelling DNA assay has emerged as a major tool for detecting bulky DNA adducts in subjects exposed to complex mixtures of environmental carcinogens (1–8), especially polycyclic aromatic hydrocarbons (PAHs), a well-known class of carcinogens (9). Higher levels of PAH-related DNA adducts have been reported in tobacco smokers and among subjects heavily exposed to air pollution (2–4). A meta-analysis of 13 DNA adduct-based studies on occupational cohorts exposed to PAH has shown that the association between DNA adducts and air pollution is significant in heavily exposed industrial workers and in less severely exposed urban workers (2).

A meta-analysis of cancer and DNA adducts has found that DNA adducts are predictive of lung cancer, particularly in tobacco smokers (5). In this study, current smokers presented a significant difference between cases and controls, with cases having higher levels of DNA adducts than controls. There are also three studies in which prospective measurements of DNA adducts have been found to be predictive of cancer risk (6–8). In each study, 32P-post-labelling assay was employed to analyse the levels of bulky DNA adducts in leukocyte DNA and associated to the subsequent risk to develop lung cancer.

The 32P-post-labelling technique has many advantages for monitoring humans exposed to environmental carcinogens, it requires few micrograms of DNA per analysis, is ultra sensitive, e.g. one adduct per 107–1010 normal nucleotides and applicable to a wide variety of DNA adduct structures, including PAH and/or aromatic amine DNA adducts (3,10). Despite its sensitivity, the characterization of the bulky DNA adducts being measured is still limited (11).

Interlaboratory trials have been also performed to develop a recommended 32P-post-labelling protocol (10). However, this protocol still requires the use of significant quantities of radioactive material, i.e. 25–50 μCi per sample (10), an obstacle that limits its use in large molecular epidemiology studies. Methodological improvements and increased DNA adduct characterization are necessary to make the 32P post-labelling capable of achieving higher throughput.

The present study focuses on different 32P-post-labelling improvements with the primary aim to increase the applicability of this assay to larger molecular epidemiological studies. A new protocol was tested to ensure efficient DNA hydrolysis, obtain higher chromatography resolution and reduce the use of radioactive material necessary for adduct labelling. The development of novel parameters was performed following the recommendations of previous 32P-post-labelling optimization trials (10,12).

Different chromatography systems, including high-urea or ammonium hydroxide systems, effective in the detection of aromatic DNA adducts (13) were also employed to acquire more insights on the nature of the bulky DNA adducts being measured by the 32P-post-labelling technique.

Improvements were tested by re-analysing the presence of DNA adducts in a group of police officers and urban residents in Genoa, Italy, in part obtained from a previous study (14).
The analysis of carcinogen-modified DNA standards was also included in the study for qualitative and quantitative comparison. The standards were added DNA from Epstein Barr virus (EBV)-immortalized cells treated with anti-benzo(a)pyrene-7,8-dihydrodiol-9,10-epoxide (BPDE), from BPDE-treated calf thymus DNA and from livers of mice treated intraperitoneally (i.p.) with benzo(a)pyrene [B(a)P].

Materials and methods

Chemicals

Ribonuclease A, ribonuclease T1, proteinase K, micrococcal nuclease (MN), spleen phosphodiesterase (SPD), nuclease P1 (Nu.P1), B(a)P and cyclosporin A were purchased from Sigma (St Louis, MO, USA). SPD was also purchased from Worthington (Lakewood, NJ, USA). Carrier-free [γ-32P]ATP (3000 Ci/ mmol) and ficoll-hypaque were from Amersham (Buckinghamshire, UK). T4-poly nucleotide kinase (PNK) was from Epicentre Technologies (Madison, WI, USA). Swiss CD1 mice, 8–10 weeks old and weighing 30–40 g were obtained from Charles River (Como, Italy). Polyethyleneimine (PEI) cellulose thin-layer chromatography (TLC) plates were from Macherey-Nagel (Postfach, Germany) and Merek (Darmstadt, Germany). BPDE was purchased from NCI Chemical Carcinogen Reference Standard Repository, Midwest Research Institute (Kansas City, MO, USA).

Preparation of carcinogen-modified DNA

Samples of DNA containing bulky DNA adducts were prepared as follows:

BPDE-modified EBV cell DNA. Peripheral blood mononuclear cells (PBMCs) from an healthy donor were purified by ficoll–hypaque gradient centrifugation and cultured overnight in the presence of EBV-transformed B95-8 marmoset cell line supernatant and 0.5 μg/ml cyclosporin A. The cells were washed twice by repeated centrifugation in RPMI 1640 and re-suspended in RPMI 1640 supplemented by 10% fetal bovine serum. The cells were cultured for about 3 weeks adding fresh medium every 3 days and the immortalized lymphoblastoid cell line was obtained when the signs of transformation, including cell aggregates, increased size and rate of growth, were observed. EBV-immortalized cells were supplemented by 10% fetal bovine serum. The cells were cultured for about 3 weeks adding fresh medium every 3 days and the immortalized lymphoblastoid cell line was obtained when the signs of transformation, including cell aggregates, increased size and rate of growth, were observed. EBV-immortalized cells were cultured at a density of 0.5 × 10^6 cells per ml in complete medium in the presence of 1.0 μM BPDE for 30 min. Untreated cells were used as controls. After washing the treated cells twice with phosphate-buffered saline by centrifugation, the pellet was stored at −80°C until DNA isolation and purification.

BPDE-modified calf thymus DNA. BPDE-modified DNA was prepared as standard material for a previous interlaboratory trial (10).

B(a)P-modified mouse liver DNA. Three mice were injected i.p. with 0.06 mg/kg B(a)P in 1:3 dimethyl sulfoxide/olive oil. Vehicle and untreated animals were used as controls (15). After 24 h, the mice were killed, their tissues immediately frozen in liquid nitrogen and stored at −80°C until DNA isolation and purification.

Study subjects

Leukocytes were obtained from a group of 23 caucasian smoker and non-smoker police officers and urban residents in Genoa, Italy. A detailed description of the study and part of the 32P-post-labelling data from the analysis of DNA adducts using a non-modified method is reported elsewhere (14).

DNA isolation and purification

DNA was isolated and purified using a method that requires digestion with ribonuclease A, ribonuclease T1 and proteinase K and extraction with saturated phenol, phenol/chloroform/soyam oil alcohol (25:24:1), chloroform/soyam oil alcohol (24:1) and ethanol precipitation (7). DNA was gently dissolved in sterile distilled water and its concentration was determined using a Beckman DU 800 spectrophotometer considering 1 A260 = 50 μg. Measurements of A260/A280 and A230/A260 ratios were employed to check DNA purity. DNA was stored at −80°C until laboratory analysis.

32P-post-labelling DNA adduct assay

DNA hydrolysis. A new protocol was developed to improve DNA hydrolysis and to avoid incomplete hydrolysis due to batch differences in phosphodiesterase efficiencies (Table I). A digestion method based on the DNA hydrolysis by MN and a mixture of two SPDs at fixed concentrations was tested. In these experiments, MN and SPD concentrations and incubation time were varied to determine the optimal digestion conditions at 37°C.

<table>
<thead>
<tr>
<th>Table I. Modified protocol of the 32P-DNA-post-labelling technique</th>
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<tbody>
<tr>
<td>Post-labelling steps</td>
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<tr>
<td>DNA hydrolysis</td>
</tr>
<tr>
<td>DNA (evaporated to dryness)</td>
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<tr>
<td>MN [143 mU/μl]</td>
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<tr>
<td>SPD mixture [6 mU/μl]</td>
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<tr>
<td>Na succinate [20 mM], CaCl2 [10 mM], pH 6.0</td>
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<tr>
<td>Final volume</td>
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<tr>
<td>Nu.P1 treatment</td>
</tr>
<tr>
<td>Samples (evaporated to dryness)</td>
</tr>
<tr>
<td>H2O</td>
</tr>
<tr>
<td>ZnCl2 [2.2 mM]</td>
</tr>
<tr>
<td>Na acetate [0.42 M], pH 5.0</td>
</tr>
<tr>
<td>Nu.P1 [1U/μl]</td>
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<tr>
<td>Final volume</td>
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<tr>
<td>Tris base solution</td>
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<tr>
<td>[0.16 mM] (unbuffered)</td>
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<tr>
<td>32P post-labelling</td>
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<tr>
<td>H2O</td>
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<tr>
<td>T4-PNK [10 μl/μl]</td>
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<tr>
<td>[γ-32P]ATP (10 μCi/μl)</td>
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<tr>
<td>Bicine buffer, pH 9.0</td>
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<tr>
<td>Final volume</td>
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<tr>
<td>Nu.P1 treatment</td>
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<tr>
<td>Hydrolyzed samples, evaporated to dryness, were treated with Nu.P1 (0.1 μl/μl, final concentration) in 46.6 mM sodium acetate, pH 5.0, and 0.24 mM ZnCl2 at 37°C for 30 min (final volume 9.0 μl)</td>
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<tr>
<td>Modified 32P-post-labelling reaction. The activity of radioactivity necessary for adduct labelling and the total volume of post-labelling reaction was reduced (Table I), in keeping with that reported from Szyfter et al. (12). In detail, the modified Nu.P1-resistant nucleotides, evaporated to dryness, were incubated with 7 μCi of carrier-free [γ-32P]ATP (3000 Ci/mM) and T4-PNK (0.75 U/μl) to generate 32P-labelled adducts in bicine buffer (final concentrations), 20 μM MgCl2, 10 μM dithiothreitol and 0.5 mM spermidine.</td>
</tr>
<tr>
<td>Non-modified 32P-post-labelling reaction. The modified Nu.P1-resistant nucleotides, evaporated to dryness, were incubated with 25 μCi of carrier-free [γ-32P]ATP (3000 Ci/mM) and T4-PNK (2.0 U/μl) to generate 32P-labelled adducts in bicine buffer, pH 9.0, at 37°C for 30 min, final volume 5.0 μl (7).</td>
</tr>
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</table>

In detail, DNA (1.0 or 5.0 μg, evaporated to dryness) was hydrolyzed by incubation with MN [21.45 mU/μl] and a mixture of two SPDs (5.1, Sigma/ Worthington) [6.0 mU/μl] in 5.0 mM Na succinate, 2.5 mM calcium chloride, pH 6.0 at 37°C for 4.5 h (final volume 10.0 μl). Appropriately diluted digests were subjected to 32P labelling and unidimensional chromatographic analyses using 280 mM (NH4)2SO4 and 50 mM NaH2PO4.

DNA hydrolysis

Samples of DNA (evaporated to dryness) were added to 2.5 mM MgCl2, 10 mM dithiothreitol and 0.5 mM spermidine.

bicine buffer (final concentrations): 20 mM bicine, 10 mM MgCl2, 10 mM dithiothreitol and 0.5 mM spermidine.

Nu.P1 treatment. Hydrolyzed samples, evaporated to dryness, were treated with Nu.P1 (0.1 U/μl, final concentration) in 46.6 mM sodium acetate, pH 5.0, and 0.24 mM ZnCl2 at 37°C for 30 min (final volume 9.0 μl). After Nu.P1 treatment, 1.8 μl of 0.16 mM Tris base was added to the sample (Table I).

Modified 32P-post-labelling reaction. The activity of radioactivity necessary for adduct labelling and the total volume of post-labelling reaction was reduced (Table I), in keeping with that reported from Szyfter et al. (12). In detail, the modified Nu.P1-resistant nucleotides, evaporated to dryness, were incubated with 7 μCi of carrier-free [γ-32P]ATP (3000 Ci/mM) and T4-PNK (0.75 U/μl) to generate 32P-labelled adducts in bicine buffer (final concentrations), 20 mM MgCl2, 10 mM dithiothreitol, 0.5 mM spermidine, pH 9.0, at 37°C for 30 min (final volume 2 μl).

Spotting sample. After labelling reaction, the 2 μl of solution was applied on a PEI cellulose TLC plate (Table I). At the end of labelling, a subset of 32P-labelled samples were analysed on PEI cellulose sheet in 280 mM (NH4)2SO4 and 50 mM NaH2PO4 to ensure the presence of an excess of carrier-free [γ-32P]ATP over substrates during the labelling reaction.

Non-modified 32P-post-labelling reaction. The modified Nu.P1-resistant nucleotides, evaporated to dryness, were incubated with 25 μCi of carrier-free [γ-32P]ATP (3000 Ci/mM) and T4-PNK (2.0 U/μl) to generate 32P-labelled adducts in bicine buffer, pH 9.0, at 37°C for 30 min, final volume 5.0 μl (7).

Spotting sample. After labelling reaction, the 5.0 μl of solution was deposited on the chromatogram.

High-urea system. DNA adduct analysis was carried out using high-urea system according to published conditions (7,10). Plates were developed
overnight using 1.0 M sodium phosphate, pH 6.8, direction (D1). There was no development in D2 after D1 (10). DNA adduct resolution was achieved using 4.0 M lithium formate, 7.5 M urea, pH 3.5 (D3), 0.65 M lithium chloride, 0.45 M Tris base and 7.7 M urea, pH 8.0 (D4). TLC plates were finally developed using 1.7 M sodium phosphate, pH 5.0 (D5).

Isopropanol–ammonium hydroxide system. Adduct analysis was also carried out using the isopropanol–ammonium hydroxide system. After an overnight development using 1.0 M sodium phosphate, pH 6.8 (D1), DNA adducts were separated using 4.0 M lithium formate, 7.5 M urea, pH 3.5 (D3), 2-propanol and 10 M ammonium hydroxide (8:12; vol/vol) (D4). Plates were then developed using 1.7 M sodium phosphate, pH 5.0 (D5).

A solvent at alkaline pH was employed for D3 direction, e.g. 0.65 M lithium chloride, 0.45 M Tris base and 7.7 M urea, pH 8.0. A solvent at higher molarity was also tested for D4 direction, e.g. 12 M ammonium hydroxide (8:12; vol/vol).

Much caution has to be used when handling isopropanol–ammonium hydroxide solvents since they are toxic and highly volatile. The use of non-urea solvents in sealed tanks in a fume cupboard was found to be more labour intensive than the use of urea solvents.

DNA adduct quantification. Detection and quantification of DNA adducts and total nucleotides was obtained by storage phosphor imaging techniques employing intensifying screens from Molecular Dynamics (Sunnyvale, CA, USA) at room temperature for 0.15–48 h. The screens were scanned using a Typhoon 9210 (Amersham). Software used to process the data was ImageQuant (version 5.0) from Molecular Dynamics. After background subtraction, the levels of DNA adducts were expressed as relative adduct labelling (RAL) = screen response (screen pixel) in adducted nucleotides/screen response (screen pixel) in total nucleotides. To calculate the levels of screen response (screen pixel) in total nucleotides, samples of DNA hydrolysates were appropriately diluted and reacted in mixtures as described above. The obtained 32P-labelled total nucleotides were separated on PEI cellulose using 280 mM (NH4)2SO4 and 50 mM NaH2PO4.

Results

DNA hydrolysis

Digestion of DNA was studied by varying enzyme concentrations and incubation time at 37°C. The enzymatic range tested was between 10 and 80 mU/μl for MN and between 2 and 15 mU/μl for SPD mixture. The completeness of DNA hydrolysis was verified by the base composition analysis of DNA according to Reddy et al. (16). The use of a combination of MN (21.45 mU/μl) and a mixture of two SPDs (3.6 mU/μl) was required to obtain about the same amounts of A and T as those of G and C, as well as the expected values for the four major bases. Incubation time was at 37°C for 4.5 h. DNA hydrolysis efficiency was not influenced by longer incubation time. Conversely, at lower enzyme concentrations, digestion was not complete in 4.5 h.

32P-post-labelling reaction

A 72% reduction of the amount of carrier-free [γ-32P]ATP necessary for labelling reaction was achieved in respect to our previous protocol (7), according to Szyfter et al. (12), except for the pH of bicine buffer, e.g. pH 9.0 instead of pH 9.6. The optimum pH for post-labelling reaction involving T4-PNK has been reported to be between 8.5 and 9.0 to avoid phosphatase activity of this enzyme (10). The presence of an excess of carrier-free [γ-32P]ATP over substrates was chromatographically verified at the end of labelling reaction in a set of samples by unidimensional chromatography.

The reduction of the amount of radioactivity material necessary for adduct labelling increased the throughput of the assay. The amount of DNA adduct analyses performed using, for example, a single batch of carrier-free [γ-32P]ATP, 500 μCi,
Discussion

Future molecular epidemiology studies will require the analysis of thousands of subjects. To achieve this realistically, significant improvements in the throughput capability of the \( ^{32}P \)-post-labelling assay are necessary.

An efficient and reliable DNA hydrolysis was obtained by incubating DNA with MN and a mixture of two SPDs at fixed concentrations. Conditions for the digestion were evaluated by analysing the base composition of DNA (16). Defined enzyme concentrations were necessary to obtain a regular relationship among the concentration of the four major bases in respect to the Chargaff’s rules. The accuracy of these determinations is dependent on obtaining quantitative hydrolysis of DNA and quantitative labelling of the digestion products (10,16). Our improvement can be useful to avoid an incomplete DNA hydrolysis due to the presence of batch variations in phosphodiesterase efficiencies. An incomplete hydrolysis of DNA prior to labelling has been also associated with adduct lost and higher chromatographic background (10).

The next finding shows that a significant reduction of the amount of radioactive material necessary for DNA adduct labelling was achieved using the modified labelling protocol without loss of adduct sensitivity, as confirmed by the results obtained using carcinogen-modified DNA and human samples. This was performed in agreement with a previous study (12), but keeping the bicine buffer at pH 9.0 to avoid potential phosphatase activity of T4-PNK (10).

The decreased use of radioactive material allows the achievement of an higher throughput. Indeed, the amount of output, in terms of DNA adduct measurements, produced using a single batch of carrier-free \( [\gamma-^{32}P]ATP \) was increased by about 3.5-fold in respect to the previous labelling protocol. The use of lower amounts of carrier-free \( [\gamma-^{32}P]ATP \) also reduces the level of radioactivity exposure of laboratory researchers and technicians.

Our results indicate that an improved resolution of adduct spots was obtained by reducing the volume of sample to be deposited on the chromatogram after labelling reaction (Figure 2). The choice of the appropriate volume of sample to be spotted on the chromatogram is a critical step in the \( ^{32}P \)-post-labelling technique and, generally, in chromatography. The application of a large volume of sample on the chromatogram induces high sample diffusion and the formation of unresolved adduct spots. Conversely, the use of a low volume of spotting sample increases the chromatographic resolution of adduct spots by reducing the value of height equivalent to a theoretical plate (HETP). A HETP reduction induces an increment of the number of theoretical plates allowing an increased adduct spot separation. The application of low volume of sample on the chromatogram is equivalent to performing a more rapid sample injection in an high-performance liquid chromatography system.

A comparison of the present pattern of bulky DNA adducts with that obtained using the non-modified \( ^{32}P \)-labelling procedure confirms that an improved resolution of DNA adducts was achieved using the modified \( ^{32}P \)-labelling protocol in terms of spot resolution (Figure 2 and ref. 14). The previous pattern of DNA adducts was generally constituted by a DRZ, with few or no spots, while a complex pattern of well-resolved adduct spots was now detected. The levels of DNA adducts using the modified \( ^{32}P \)-labelling procedure were comparable to those previously detected in the same set of samples, e.g. \( 2.1 \pm 0.3 \) (SE) DNA adducts per \( 10^8 \) normal nucleotides (14). A significant correlation between the two measurements was found \( (r = 0.7, P < 0.05) \).

Our attempts to acquire more insights on the nature of the adduct spots being detected by the \( ^{32}P \)-post-labelling assay show that a complex pattern of bulky DNA adducts can be measured in leukocyte DNA using both high-urea or isopropanol–ammonium hydroxide solvents (Figure 2), two systems effective in the detection of aromatic DNA adducts (13). A similar average recovery of DNA adducts was also obtained under both the chromatography systems. The above observations indicate that leukocyte DNA adducts are likely to be induced by aromatic compounds, such as PAH and/or aromatic amines.

Comparative considerations of remarkable characteristics of the pattern of DNA adducts with those of leukocyte DNA adducts detected using the two chromatography systems would be speculative since the migration rate of carcinogen-adducted DNA standard was different in isopropanol–ammonium hydroxide in respect to high-urea system (Figure 1).

Knowledge of the nature of human DNA adducts gives valuable information regarding the mutational effects that results from human exposure to environmental carcinogens. When unrepaired, aromatic DNA adducts can cause mutations,
including mutational hot spots in the p53 tumour suppressor gene (17). Three studies have attempted to address the characterization of 32P-post-labelling adducts by analysing adducts both by 32P-DNA post-labelling and fluorescence methods (18). The level of B(a)P DNA adducts has been found to represent the 10–118% of adducts detected using the 32P-DNA post-labelling technique (18).

An analysis of DNA adduct studies on humans performed using isopropanol–ammonium hydroxide systems shows that a number of aromatic DNA adducts, including 4-ABP-DNA adducts, have been generally detected in oral and bladder mucosa (19, 20). Some conflicting results over the presence of aromatic DNA adducts in lung tumor tissues have been also reported (11). This discrepancy can be due to the differences in the chromatography systems and/or to variations in 32P-post-labelling protocols. The presence of isopropanol–ammonium hydroxide, by neutralizing the anion-exchange properties of PEI-TLC plates, can increase adduct mobility and induce adduct losses (13). Reduced recovery of some PAH-related DNA adducts, including benzo(k)fluoranthene-, 7,12-dimethylbenz(a)anthracene- and cyclopenta(cd)pyrene-DNA adducts, have been reported using non-urea systems (13).

In summary, our study shows the development of a new 32P-post-labelling protocol capable of ensuring efficient DNA hydrolysis, reducing the radioactivity necessary for adduct labelling and increasing DNA adduct resolution. Our improvements support an easier use of the 32P-post-labelling assay in large molecular epidemiology studies, where a biomarker of internal dose is needed.

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References