

MEDETOX

Innovative Methods of Monitoring of Diesel Engine Exhaust Toxicity in Real Urban Traffic
(LIFE10 ENV/CZ/651)

ANALYSIS OF DNA ADDUCTS OF POLYCYCLIC AROMATIC HYDROCARBONS BY ³²P-POSTLABELLING

SUMMARY

1. ³²P-postlabelling method
2. B(a)P-DNA standards
3. Cell culture A549 and CT DNA incubation
4. DNA isolation
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8. DNA adducts separation by thin layer chromatography (TLC)
9. Visualisation and quantification of DNA adduct maps
10. HPLC nucleotides concentration analysis
11. DNA adduct quantification

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1. ^{32}P -postlabelling method

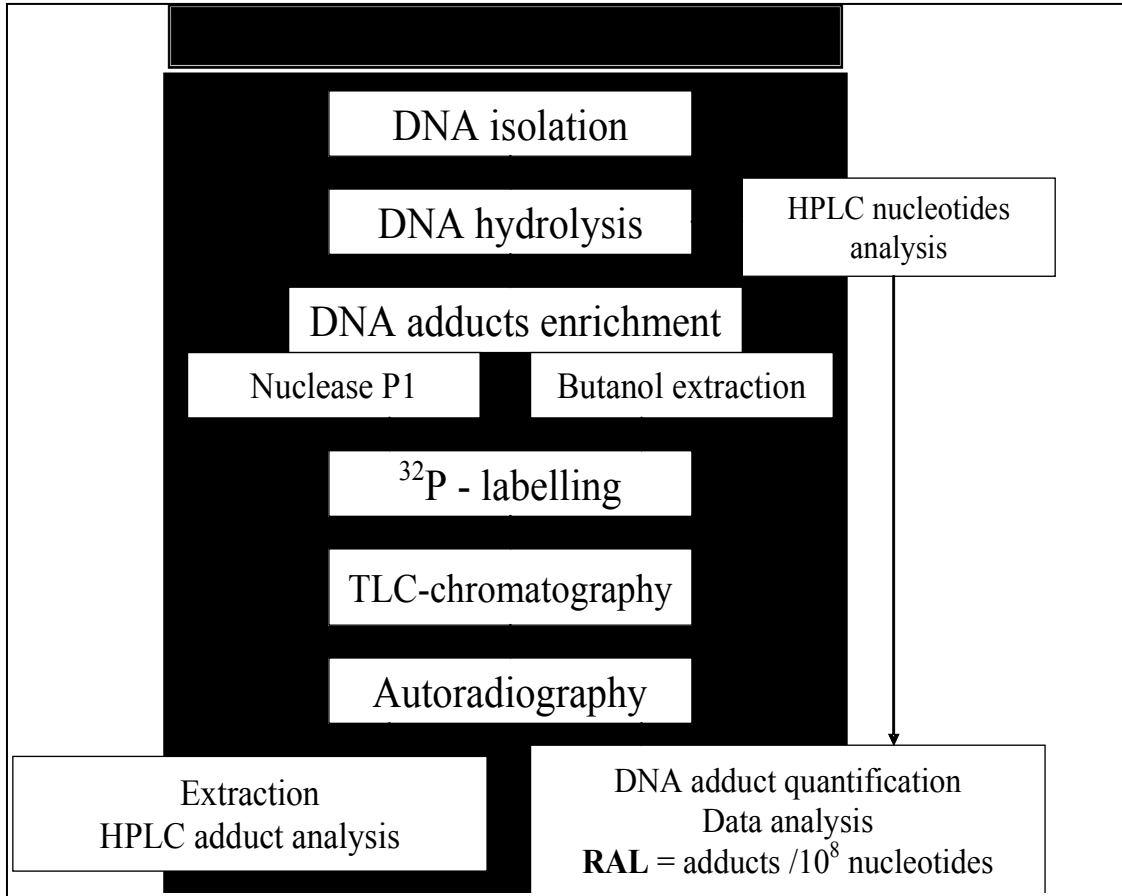
The ^{32}P -postlabelling protocol is shown in **Fig.1**. Carcinogen-modified DNA is digested enzymatically to deoxyribonucleoside 3'-monophosphates with endonuclease (micrococcal nuclease) and exonuclease (spleen phosphodiesterase). In order to increase the sensitivity of the method the enhancement procedure is used to enrich adducts. This procedure uses an enzymatic postincubation of DNA digests with nuclease P1 (from *Penicillium citrinum*). Nuclease P1 dephosphorylates deoxyribonucleoside 3'-monophosphates of normal nucleotides only to deoxyribonucleosides but not of adducted nucleotides. Deoxyribonucleosides do not serve as substrates of T4-polynucleotide kinase for the transfer of [^{32}P]phosphate from [α - ^{32}P]ATP.

Then DNA hydrolysates are converted to 5'- ^{32}P -labelled 3',5'-bisphosphates by incubation with [α - ^{32}P]ATP in the presence of carrier ATP and T4-polynucleotide kinase at pH 9.5. This alkaline pH is used in order to minimize the 3'-phosphatase activity of the polynucleotide kinase. ^{32}P -labelled adducts are separated and resolved from the excess of labelled non-modified nucleotides in two dimensions by multidirectional anion-exchange thin layer chromatography (TLC) on polyethyleneimine (PEI) cellulose plates (**Fig. 2**). During the first elutions (D1 and D2 directions) with aqueous electrolyte labelled unmodified nucleotides and [^{32}P]phosphate are removed from the origin for subsequent resolution using different solvent systems (D3, D4 directions). Location of the adducts is carried out by screen enhanced autoradiography and visualized as dark distinct spots on X-ray films. These areas are then excised for quantitation by liquid scintillation. Adduct levels are calculated as relative adduct labelling (RAL) values, which represent the ratio of count rates of adducted nucleotides over count rates of total (adducted and normal) nucleotides. Utilizing the standard protocol, DNA adducts present at levels of 1 adduct in 10^7 normal nucleotides (0.3 fmol adduct/ μg DNA) can be detected.

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Fig. 1



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2. B(a)P DNA standards

B(a)P-DNA standard - 4 adducts/10⁸ nucleotides- was prepared by DNA isolation from rat liver after oral administration of B[a]P 100 mg/kg body weight of rats. Aliquots (20 µl) of the standard are stored at -80°C. Standard as a positive DNA control is analyzed in triplicate in each postlabelling experiment to check variability between experiments.

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3. Cell culture A549 and CT DNA incubation

Calf thymus DNA (CT DNA 1mg/ml) is incubated with various EOM samples (EOM = extractable organic mass) for 24 h at 37 °C with and without metabolic activation using the S9 fraction (1 mg protein/ml). B[a]P and DMSO treated calf thymus DNA samples are used as positive and negative controls, respectively.

A549 cells are grown at 37 °C in 72 cm² flasks with 15-30 ml Dulbecco's modified Eagle's medium (with 10% FBS) to 70-80% confluency. Then we treat cells in application medium (with 1% FBS) with EOM samples for 24h at 37 °C. After that we harvest cells.

Chemicals and solutions

calf thymus DNA incubation

Microsomal S9 fraction from rat liver homogenate – (Institute organických syntéz a.s., Rybitví)

Calf thymus DNA (Sigma D 1501)

Glukoso-6-phosphate (Sigma G 7879)

NADP (Sigma N 0505)

DMSO - Dimethyl sulfoxide (Sigma D2650)

Pufr A: 1.65 M KCl + 0.4 M MgCl₂

Pufr B: Phosphate buffer 0.2 M, pH = 7.4: 60 ml 0.2 M NaH₂PO₄ + 440 ml 0.2M Na₂HPO₄

A549 cells incubation

Dulbecco's modified Eagle's medium (DMEM) with 1.0 g/L glucose, with pyruvate, without Lglutamine (LONZA BE12-707F/12)

Fetal bovine serum (FBS), EU standard (LONZA DE14-801F/12)

L-glutamine (200 mM) (LONZA BE17-605E)

Gentamicin sulfate 10 mg/ml (LONZA 17-519L)

Trypsin/EDTA (1x) contains 0.5 g/L trypsin 1:250 and 0.2 g/L Versene® (EDTA) (LONZA BE17-161E)

DMSO (Sigma D2650)

Cell harvesting

1. After exposure wash cells with cold PBS and scrape into the 15 ml labeled with the name of the project Medetox and sample number.
2. Wash the cell culture flasks with 4 ml PBS and add into the 15 ml tubes. Centrifuge at 3000 rpm for 5 min.
3. Discard the supernatant, homogenize the cell pellet, add 13 ml of PBS and centrifuge at 3000 rpm for 5 min. Repeat this step twice.

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4. Finally centrifuge at 3000 rpm for 5 minutes, discard the supernatant, homogenize the cell pellet and continue with DNA isolation or store the samples in a freezer at – 80 °C.

4. DNA isolation

The cell pellets are homogenized in a solution of 10 mM Tris-HCl, 100 mM EDTA and 0.5% SDS, pH 8.0. DNA is isolated using RNAses A and T1 and proteinase K treatment followed by phenol/chloroform/isoamylalcohol. DNA concentration is estimated spectrophotometrically by measuring the UV absorbance at 260 nm. DNA samples are stored at – 80 °C until analysis.

Chemicals and solutions

Extraction buffer: 1 % SDS, 10 mM EDTA, 20mM Tris

Solution – 1 litre (store at room temperature):

2,42 g Tris (M.W 121.1)

3,723 g EDTA (M.W.372.3)

10 g Sodiumdodecylsulphate-SDS (M.W.268.4)

1 M Tris, pH=8 (store at room temperature)

12,1 g/100 ml H₂O, pH adjust with HCl

5M NaCl (store at room temperature)

29,23 g/ 100 ml H₂O

50 mM Tris, pH 7.4, for CIP a CI solutions

1.21 g / 200 ml, pH adjust with HCl

CIP - chloroform : isoamylalkohol : phenol (24 : 1 : 25)

250 g phenol, redistilled (Sigma, 328111)

240 ml chloroform (Merck, 1.02445)

10 ml isoamylalcohol (Sigma I 0640)

Melt phenol in a water bath at 50°C. Then mix all chemicals and shake thoroughly with 80 ml 50 mM Tris pH=7.4. Store the solution in the fridge overnight – Tris will make the layer on the CIP solution.

CI - chloroform : isoamylalkohol (24 : 1)

240 ml chloroform (Merck 1.02445)

10 ml isoamylalcohol

Mix and shake thoroughly with 80 ml 50 mM Tris pH=7.4

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RNA mix = Ribonuclease A 10mg/ml (Sigma, R-5125), **Ribonuclease T1 5000 U/ml** (Sigma R-1003)

Prepare a solution of Ribonuclease A 10mg/ml in 50 mM Tris pH=7.4. Heat this solution at 80 °C for 10 minut and then let it cool down. Gently mix 1 ml of this solution with Ribonuclease T1 (5000 U/ampulla). Make aliquots (200 µl) keep in a freezer at -20 °C.

Proteinase K (20 mg/ml H₂O) (Sigma, P-6556)

Prepare fresh before use.

Material

15 ml tubes with a gel (PLG Light, Eppendorf 2302840)

Equipment

pH-metr, Beckman

Centrifuge Savant Speed fuge HSC 10K, centrifuge Eppendorf 5810 R

Vortex MS2 Minishaker IKA, vortex Bio vortex V1 BIOSAN

Thermoblock Thermodual Liebisch, thermoblock DRI-BLOCK DB.3D

Water bath GFL 1086

Spectrophotometer Nanodrop

Evaporater Jouan RC10.22., RCT 90

DNA isolation

1. Homogenize the cell pellets in 1,5 ml of an extraction buffer (1% SDS,10 mM EDTA, 20 mM Tris; pH = 8.0) in 15 ml plastic tubes with covers.
2. Add **30 µl** of **RNA-mix** (10 mg/ml RNAza A, 5000 U/ml RNAza T), mix gently and incubate in water bath 1.5 hours at 37 °C.
3. Add **30 µl** of **proteinase K** (20 mg/ml), mix and incubate in water bath 2 hours at 37 °C.
4. Use a pipette to transfer the mixture to a 15 ml tubes with a gel and add **75 µl** of **1 M Tris, pH=8, 150 µl** of **5M NaCl** and **1.5 ml** of **CIP**. Put the cap on the tube and manually shake – at least one hundred times. Centrifuge at 3000 rpm for 5 minutes.
5. Recover the upper aqueous phase into new 15 ml tubes with a gel, add **1.5 ml** of **CI**. Put the cap on the tube and manually shake – at least one hundred times. Centrifuge at 3000 rpm. for 5 minutes.
6. Recover the upper aqueous phase into 5 ml tubes and add **150 µl** of **5M NaCl**. Precipitate the nucleic acid by **1.5 ml** of **ice-cold absolute ethanol** (Merck, 1.11727). Centrifuge at 3000 rpm for 5 minutes. Discard the ethanol and dry the DNA in the evaporater.
7. Resuspend the DNA pellet in proper dH₂O (optimal is the final concentration 0.8-1.5 mg/ml). Mix thoroughly and keep dissolving at room temperature overnight.

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8. Measure the DNA concentration on the spectrophotometer (Nanodrop). Optimal concentration is 1 – 2ug/ul. Absorbance A260/A280 ratio is used for controlling the purity of the DNA - the number higher than 1.8 means the RNA contamination, lower the protein contamination.
9. Pipette the samples to 1.5 ml tubes and sign them with the name of the study MEDETOX, sample number, DNA concentration and the date of the DNA isolation. Keep in the freezer at - 80 °C.

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5. DNA hydrolysis

Carcinogen-modified DNA is digested enzymatically to deoxyribonucleoside 3'-monophosphates with endonuclease (micrococcal nuclease) and exonuclease (spleen phosphodiesterase).

Chemicals

Micrococcal nuclease (MN, Sigma N 3755; 1 bal MN = 200 U)
Spleen phosphodiesterase (SPD, MP Biomedicals 0210097701)
Other chemicals Sigma (analytical grade)
Slide –A-Lyzer 10K Dialysis Cassettes 0.5 - 3ml sample volume (Pierce Biotechnology, Thermo scientific No. 66380)
1,5 ml tubes (Eppendorf, Axygen)

Solutions

MN/SPD mix: 50 U MN/ml a 1mg SPD/ml (2 U/ml)

Use a Slide-A-Lyzer 10K Dialysis Cassettes to remove low molecular weight contaminant from SPD. For more details see the Detailed Procedure Instruction List for Adding and Removing Samples. Dialyse SPD in dH₂O 2 hours in a fridge at 4°C, then in the fresh dH₂O another 2 hours . Adjust the volume to 4 ml with dH₂O, pipette to the vial with MN (1 vial MN=200U) and mix gently. Keep aliquots in a freezer at – 20 °C (max. 4 months).

Hydrolysis buffer

100 mM Na Succinate pH = 6.0
50 mM CaCl₂
Solution - 100 ml: 2.7 g Na Succinate (M.W.= 270.1), 0.368 g CaCl₂ (M.W.=147), pH adjust with HCl.
100 µl aliquots store at – 20 °C.

Hydrolysis mix

MN/SPD mix : Hydrolysis buffer = 4 : 1
Make always fresh!

Equipment

Centrifuge Savant Speed fuge HSC 10K, centrifuge Spectrafuge 24D Labnet
Vortex MS2 Minishaker IKA, vortex Bio vortex V1 BIOSAN
Thermoblocks Thermoblock Liebis, thermoblock DRI-BLOCK DB.3D

Hydrolysis conditions

DNA amount / sample: 6 µg

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Final DNA concentration in the mixture	0.4 µg/µl
MN / sample:	0.3 U
SPD / sample:	12 mU
pH	6.0
Total volume:	15 µl
Incubation time:	4 hours
Temperature:	37 °C

DNA hydrolysis

1. Pipette the volume containing 6 µg DNA to 1.5 ml microtubes, complete to 7.5 µl with dH₂O.
2. Add 7.5 µl of MN/SPD mix to each sample, mix and briefly centrifuge (5000 rpm, 20 sec).
3. Incubate the samples in the thermoblock at 37°C for 4 hours.
4. Centrifuge briefly (5000 rpm, 20 sec). Make aliquots of each sample – pipette 2.5 µl of each sample to new microtubes and store in a freezer at – 20 °C until the HPLC analysis (analysis of individual nucleotides - dC, dG, dT, dA).
5. An enzymatic postincubation of “DNA digest“ (12.5 µl contain approximately 5 µg DNA) with nuclease P1 is following – immediately or the next day (keep the aliquots (12.5 µl) in a freezer at – 20 °C).

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6. Nuclease P1 adduct enrichment

This procedure uses an enzymatic postincubation of DNA digests with nuclease P1 (from *Penicillium citrinum*). Nuclease P1 dephosphorylates deoxyribonucleoside 3'-monophosphates of normal nucleotides only to deoxyribonucleosides but not of adducted nucleotides. Deoxyribonucleosides do not serve as substrates of T4-polynucleotide kinase for the transfer of [³²P]phosphate from [γ -³²P]ATP. After this step proceed with ³²P-ATP labelling, never freeze the samples after the incubation with nuclease P1.

Chemicals

Nuclease P 1 from *Penicillium cintrinum* 500 U (Yamasa corporation 8801)
Other chemicals Sigma (analytical grade)

Solutions

0.64 M Na-acetate pH = 5.0

2.178 g Na-acetate (M.W.=136.1) dissolve in 20 ml of HPLC H₂O, pH adjust with dilute acetic acid. Complete to volume 25 ml. 150 μ l aliquots store at - 20 °C.

3.2 mM ZnCl₂

10.9 mg ZnCl₂ (M.W.=136.3) dissolve in 25 ml HPLC H₂O.
150 μ l aliquots store at - 20 °C.

1.36 M Tris pH = 9.5

4.12 g Tris (M.W.=121.1) dissolve in 25 ml HPLC H₂O.
150 μ l aliquots store at - 20 °C.

10 mM Na acetate pH = 5.0

Dilute 0.64 M Na-acetate pH = 5.0
(100 μ l of solution + 6.3 ml HPLC H₂O)
200 μ l aliquots store at - 20 °C.

Nuclease P1

1 mg dissolve in the original ampulla in 150 μ l HPLC H₂O and immediately make P1-mix. Residue store at - 20 °C, max. for 4 weeks.

Equipment

Centrifuge Savant Speed fuge HSC 10K, centrifuge Spectrafuge 24D Labnet
Vortex MS2 Minishaker IKA, vortex Bio vortex V1 BIOSAN
Thermoblocks Thermoblock Liebsch, thermoblock DRI-BLOCK DB.3D

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NUCLEASE P1 conditions

DNA hydrolysate amount:	12.5 µl "DNA digest"
Nuclease P1 / sample :	2.4 U
pH:	5.0
Total volume of sample (nuclease P1 enrichment):	18.5 µl
Incubation time:	30 min
Incubation temperature:	37 °C
Reaction stop:	1 µl 1.36 M Tris
Total volume at the end of the procedure:	16.5 µl

Nuclease P1 enrichment

1. Prepare P1-mix for all samples:

For 1 sample: 1 µl nuclease P1
 1 µl 0.64 M Na acetate, pH=5.0
 1 µl 3.2 mM ZnCl₂

2. Add 3 µl of P1-mix to each sample (12.5 µl DNA digest), vortex gently and centrifuge briefly (5000 rpm, 30 sec).
2. Incubate samples 30 minutes at 37 °C in the thermoblock.
3. Centrifuge briefly (5000 rpm, 30 sec) and add 1 µl of 1.36 M Tris to each sample.
4. Store the samples at 4 °C until the ³²P-labelling.

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7. ³²P-ATP labelling

In this step DNA hydrolysates (normal and modified deoxyribonucleoside 3'-monophosphates) are converted to 5'-³²P-labelled 3',5'-bisphosphates by incubation with [γ -³²P]ATP in the presence of carrier ATP and T4-polynucleotide kinase at pH 9.5. This alkaline pH is used in order to minimize the 3'-phosphatase activity of the polynucleotide kinase.

Chemicals

³²P-ATP 3000 Ci/mmol; 1 mCi/100 μ l (Perkin Elmer BLU0024)
T4 Polynucleotide kinase (PNK) 30 U/ μ l (USB, #70031)
Other chemicals Sigma (analytical grade)

Solutions

Labelling buffer - pH=9.0

230 mM Bicine
115 mM Magnesium Chloride
115 mM Dithiothreitol (DTT; D-0632)
5.8 M Spermidine (S-2626)
For 25 ml we weight: 0.9385 g Bicine (M.W.=163.2);
0.274 g Mg Cl₂; 0.443 g DTT (M.W.=154.2); 0.2105 g Spermidine
(M.W.=145.2). Adjust pH with 1 N NaOH.
150 μ l aliquots at - 80 °C

Equipment

Thermoblocks Thermoblock Liebsch, thermoblock DRI-BLOCK DB.3D
Protective equipment for radiation

Labelling conditions

DNA amount:	5 μ g
T4 PNK / sample:	3.6 U
γ - ³² P-ATP / sample:	24.2 μ Ci
pH:	9.5
Total volume during the labelling:	24 μ l (P1)
Incubation time:	30 min
Incubation temperature:	37 °C

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³²P-ATP labelling protocol

1. Prepare ATP-mix : For one package of ATP (activity 1 mCi = 37 MBq) and volume 100 µl:

Labelling buffer	60 µl
T4 P N K	8 µl
HPLC H ₂ O	142 µl
³² P-ATP	100 µl
Total volume	310 µl (for 40 samples)

Do not vortex, mix only with a tip (approx. 10 x).

2. Add 7.5 µl of ATP-mix to each sample and mix gently with a tip. Centrifuge briefly and incubate 30 minutes at 37 °C in thermoblock.
3. Pipette the samples on the origin of the chromatographic plates (**Fig. 2**) and put them into the chromatographic tanks with the D1 solution.

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8. DNA adduct separation by thin layer chromatography (TLC)

³²P-labelled adducts are separated and resolved from the excess of labelled non-modified nucleotides in two dimensions by multidirectional thin layer chromatography (TLC) on 10x10 cm polyethyleneimine (PEI) cellulose plates. During the first elutions (D1 and D2 directions) with aqueous electrolyte labelled unmodified nucleotides and ³²P-phosphate are removed from the origin onto a paper wick while aromatic hydrophobic adducts are retained at the origin for subsequent resolution using different solvent systems - D3 (pH=3.5) and D4 (pH=8.0) directions (Fig. 2).

Chemicals and materials

Chemicals from Sigma (analytical grade)
POLYGRAM CEL 300 PEI – polyethyleneimine cellulose plates, 20x20 cm, 0.1 mm (Macherey-Nagel, Germany)
Filter paper Whatman č. 3
Fluorescence pen (Autoradiography pen Sigma Z36,351-0)
Roentgen films 35x43 cm a 20.3x25.4 cm (Kodak BioMax MRfilm)
Developer G153, Fixer G354 (Agfa)
Autoradiographic cassettes (Hypercassette™ 35x 43 cm a 20.3x25.4 cm, Amersham)

Solutions

D1: 1 M Sodium phosphate, pH = 6.8

For 2 litres:

276 g NaH₂PO₄·H₂O (M.W.=138) or

240 g NaH₂PO₄ (M.W.=119.98) solute in 1.5 l HPLC H₂O

Adjust pH with conc. NaOH to 6.8 and complete the volume to 2 l.

D2: 2.5 M Ammonium formate, pH = 3.5

For 500 ml:

50 ml formic acid (99%) mix with 300 ml HPLC H₂O and

approx. 20 - 30 ml 25% NH₄OH (27%) to pH = 3.5

Complete the volume to 500 ml.

D3: 3.5 M LiF, 8.5 M Urea, pH = 3.5

For 2 l:

1020 g of Urea (M.W.=60.16) dissolve in 1.5 l HPLC H₂O and add 277.4 ml of formic acid (99%)

Adjust pH with LiOH to pH=3.5

Complete the volume to 2 l.

D4-A: 0.5 M Tris, pH = 8.0

For 1 l:

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60.55 g of Tris (M.W.=121.1) dissolve in 900 ml HPLC water
Adjust pH with 1 N HCl a complete the volume to 1 l.

D4: 0.8 M LiCl, 0.5 M Tris, 8.5 M Urea, pH = 8.0

For 2 l:
1020 g urea
121.1 g Tris (M.W.=121.1)
67.84 g LiCl (m.W.=42.4)
Dissolve in 1,5 l HPLC H₂O
Adjust pH pwith conc. HCl to pH=8.0
Complete the volume to 2 l.

D5 = D1: 1 M Sodium phosphate, pH = 6.8

Equipment

Chromatographic tanks
Hairdryers
Daylight light box Kaiser prolite basic (KFB 2176)
Hypercassette™ 35x 43 cm a 20.3x25.4 cm , Amersham, USA
CURIX 60 machine, Agfa – for developing the films
Protective equipment for radiation

Chromatography conditions

Chromatography D1 - D5 (scheme in the **figure 2**).

- D1 overnight, to the end of the filter paper (10x20 cm, Whatman č.3) stapled to the plate, min. 15 hours
- D2 developing to origin (OR) (approximately 1 minute)
- D3 3.5 hours
- D4 3 hours
- D5 overnight, to the end of the filter paper (10x7 cm, Whatman no.3) stapled to the plate, min. 15 hours

Chromatography protocol

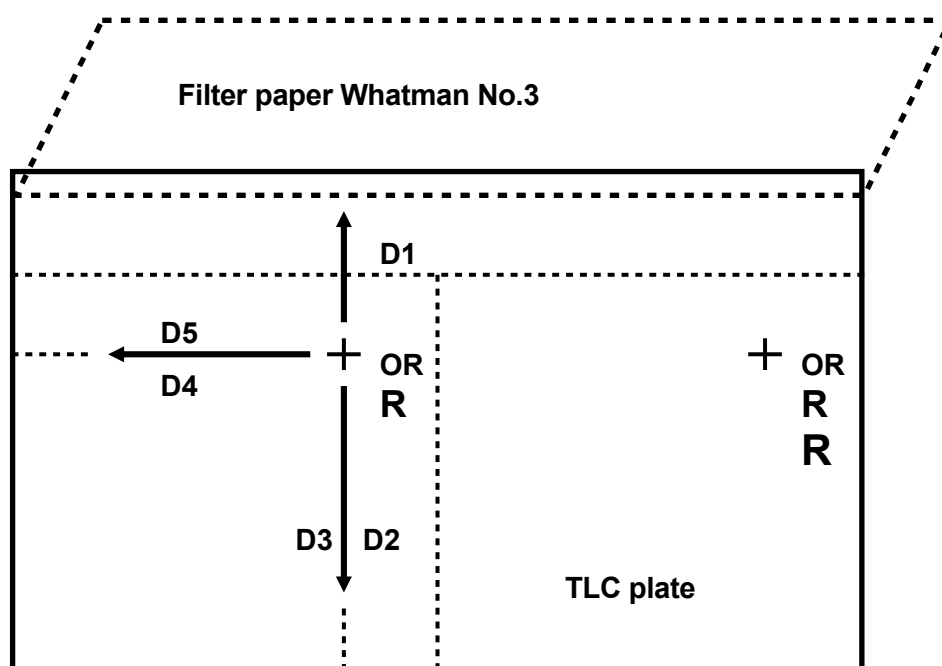
1. Draw the borders with a pencil according the schema (**Fig.2**). Cut away the margins and staple the filter papers (10x20 cm).
2. Pipette the samples on the origin of the chromatographic plates (**Fig. 2**) and put them into the chromatographic tanks with the D1 solution (tank for 16 samples – 180 ml of D1) – avoid splashing. Seal the tank with its lid. Let develop overnight (min. 15 hours).

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3. In the morning – cut away the filter papers and wash the plates in the tanks with dH₂O. Let the water slowly run through the tanks for 20 minutes. Drain the plates under the hairdryers for 15 – 20 minutes.
4. Dried plates dip to D2 solution and let develop to OR (approx. 1 minute). Then put the plates into the chromatographic tanks with D3 solution (tank for 16 sample – 180 ml of D3) and let develop 3,5 hours.
5. Cut away the top 2 cm part of the plate and cut the plate in the middle. Wash and drain the plates as described in step 3.
6. Dried plates briefly dip to D4-A solution and immediately put into the tanks with D4 solution (tank for 16 samples - 180 ml of D4) and let develop 3 hours.
7. Wash and drain the plates as described before (20 min). Staple the filter paper (10x7 cm) to the end part of D4 chromatography, put the plates into the chromatographic tanks with D5 and let develop overnight.
8. In the morning – cut away the filter papers, wash the plates in the tanks with dH₂O and drain them. Mark each plate in the corners with the fluorescent pen. Place the plates into clean autoradiography cassettes, then put the X-ray KODAK films on it in the dark room and let expose in a freezer at – 80 °C for 24 – 72 hours.
9. Develop the films in the CURIX 60 machine, Agfa.

Fig. 2



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9. Visualisation and quantification of DNA adduct maps

The DNA adduct levels are evaluated from the distinct adduct spots and from the diagonal radioactives zones (DRZ) on thin layer chromatograms. The radioactivity is measured by liquid scintillation counting.

Chemicals and material

Scintillation cocktail LSC SigmaFluo™ (Sigma L8286)
Scintillation vials (Greiner)

Equipment

LS counter, Wallac (Pharmacia)
Protective equipment for radiation
Daylight light box Kaiser prolite basic (KFB 2176)
Dispenser of the scintillation cocktail

Chromatography evaluation

1. Choose the proper template of map of spots and diagonal radioactive zones (DRZ) for the cutting. Draw the template on the films.
2. Draw the template on the plates using light box and with the aid of fluorescent marks. Cut with the scissors all spots and DRZ from each plate (blanks included) and put into marked scintillation counter vials. Add scintillation cocktail to vials (5ml per vial). Also prepare a vial with only scintillation cocktail as a control. Measure on LS counter – always edit the reference date for γ -³²P-ATP. Dispose counted vials and scint. cocktail as hazardous waste.

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10. HPLC nucleotides concentration analysis

To determine the exact amount of DNA in each sample, aliquots of the DNA enzymatic digest (1 µg of DNA hydrolysate) are analyzed by HPLC for nucleotide content, which simultaneously allow for controlling the purity of the DNA.

Chemicals

Nucleotides standards:

dG- 2'-deoxyguanosine 3'-monophosphate, sodium salt, Sigma D 4147 (2 mg)

dA- 2'-deoxyadenosine 3'-monophosphate, sodium salt, Sigma D 3139 (1 mg)

dC- 2'-deoxycytidine 3'-monophosphate, sodium salt, Sigma D 3389 (1 mg)

dT- thymidine 3'-monophosphate, sodium salt, Sigma T 3512 (5 mg)

Acetonitrile, HPLC grade

Membrane filters for mobile phase, Waters

Chromatographic column SGX C18 5 µm, 4 x 250 mm, TESSEK, č.141 300013 001

Pre-column SGX C18 5 µm, 4 x 40 mm, TESSEK, ČR

Solutions

25 mM NaH₂PO₄, pH=4.0 with 2% acetonitrile- mobile phase

For 2 l - solute in 1.5 l HPLC water:

6.0 g anhydric NaH₂PO₄ (M.W.= 120) or

6.9 g NaH₂PO₄.H₂O (M.W.= 138)

Adjust pH with dilute H₃PO₄. Add 40 ml of acetonitrile and complement to 2 l.

Before use filter solution on the filtration equipment Waters.

Store at 4 °C up to 1 month.

Standard solutions for the calibration (concentration 100 ng dC, dG, dT a dA/µ)

Dissolve standards in proper volume of HPLC water (final concentration 1mg/µl). Dilute 10x to concentration 100 ng/µl. Store aliquots (100 µl) in a freezer at -80°C.

Equipment

Centrifuge Savant Speed fuge HSC 10K, centrifuge Spectrafuge 24D Labnet

Vortex MS2 Minishaker IKA, vortex Bio vortex V1 BIOSAN

Filtration equipment for mobile phase, Waters

HPLC system Waters:

HPLC pumpa typ 600

Diode-array detektor typ 966

Autosampler typ 717

Personal computer Digital

Chromatography software Millenium

Laser printer HP 5P

HPLC analysis conditions

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Innovative Methods of Monitoring of Diesel Engine Exhaust Toxicity in Real Urban Traffic (LIFE10 ENV/CZ/651)

Isocratic mode

Mobile phase: 25 mM NaH₂PO₄ with 2% acetonitrile, pH=4.0

Elution flow rate: 1 ml/min

Analysis time: 14 min

Injection volume of a sample: 5 µl

Column: Analytic, 4 x 250 mm, 5 µm, ODS 18

Detection: Detection in range 230 -320 nm; processing at 260 nm

Calibration curves: Standards dC, dG, dT a dA-5'-monophosphates. Calibration for each nucleotide in range 10-30 ng.

Retention time: dC - 3.7 min; dG - 5.5 min; dT - 6.3 min; dA - 10.9 min

HPLC analysis protocol

1. Mix the aliquots of the nucleotides in the ratio 1:1:1:1, the final concentration of each nucleotide will be 25 ng/µl. Dilute 10x. Injection volume is 4 µl = 10 ng of each nucleotide.
2. Thaw the aliquots of DNA of the samples stored after hydrolysis (2.5 µl) and add 37.5µl of HPLC water. Vortex and briefly centrifuge. Pipette the samples to HPLC vials and analyze for nucleotide content by reverse-phase HPLC with UV detection, which simultaneously allow for controlling the purity of the DNA as well.

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11. DNA adducts quantification

Adduct levels are calculated as relative adduct labelling (RAL) values, which represent the ratio of count rates of adducted nucleotides over count rates of total (adducted and normal) nucleotides.

The final number of DNA adducts **Z** is:

$$Z [\text{adduct} / 10^8 \text{ nucleotid}] = 0.00501 \cdot \frac{A_{CPM}}{X_{\mu\text{gDNA}}}$$

To determine the exact amount of DNA in each sample, aliquots of the DNA enzymatic digest (1 μg of DNA hydrolysate) is analyzed for nucleotide content (**X**) by reverse-phase HPLC with UV detection, which simultaneously allow for controlling the purity of the DNA as well.

The correction of CPM of analyzed samples is performed according to CPM of blank samples.

A B(a)P-DNA adduct standard is run in triplicate in each postlabeling experiment to control for interassay variability and to normalize the calculated DNA adduct levels.