The Influence of Oxidative Stress, Carcinogens and Cloning on DNA methylation determined by Capillary Electrophoresis

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I certify that I was a representative in this PhD research for the Beni-Sweif University in Egypt and I have carried out the work at the Bergische Universität Wuppertal in Germany.

I certify that I have carried out this work independently, have used no sources or tools other than those specified and have marked all citations.

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To my parents

List of abbreviation

The following acronyms are used in the thesis:

°C	Degree celsius
λ_{ex}	Excitation wavelength
λ_{em}	Emission wavelength
µg	Microgram
µL	Microliter
µm	Micrometer
3-NBA	3-Nitrobenzanthrone
4-ABP	4-Aminobiphenyl
5-meC	5'-methylcytosine
5-me-dCMP	5'-methyl-2'-deoxycytosine-3'-monophosphate
8-oxo-dGMP	8-oxo-2'-deoxyguanosine-3'-monophosphate
8-OH-dG	8-hydroxy-2'-deoxyguanosine
8-oxo-dG	8-oxo-2'-deoxyguanosine
A	Adenine
AA(s)	Aristolochic acid (s)
AAN	Aristolochic acid nephropathy
AP	Apurinic site, 2'-deoxyribose-3'-monophosphate
ART	Assisted reproduction technologies
B[*a*]P	Benzo[*a*]pyrene
Bar	Pressure unit
BEN	Balkan endemic nephropathy
BER	Base excision repair
BODIPY	4,4-Difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-*s*-indacen-3-propionylethylendiamine hydrochloride
Bp	Base pair
BW	Body weight
C	Cytosine
CE	Capillary electrophoresis

CE-LIF	Capillary electrophoresis with laser induced fluorescence detector
CE-UV	Capillary electrophoresis with ultraviolet detection
CGE	Capillary gel electrophoresis
CEC	Capillary electrochromatography
CH_2CL_2	methylene chloride
CHN	Chinese Herbs nephropathy
CIEF	Capillary isoelectric focusing
CITP	Capillary isotachophoresis
cm	Centimeters
COBRA	Combined bisulphite restriction analysis
CpG-Island	5'-CG-3'-dinucleotides
CT-DNA	Calf thymus-DNA
CZE	Capillary zone electrophoresis
dAMP (or 3'-dAMP)	2'-deoxyadenosine-3'-monophosphate
dA-AA I,II	7-(2'-deoxyadenosine-N6-yl)-aristolactam I,II
dC-AAII	7-(2'-deoxycytosine-N6-yl)-aristolactam II
dG-AA	7-(2'-deoxyguanosine-N2-yl)-aristolactam
dCMP (or 3'-dCMP)	2'-deoxycytidine-3'-monophosphate
dG	2'-deoxyguanosine
dGMP (or 3'-dGMP)	2'-deoxyguanosine-3'-monophosphate
dTMP (or 3'-dTMP)	Thymidine-3'-monophosphate
Da	Daltons
DNA	Deoxyribonucleic acid
DNase I	Deoxyribonuclease I
Dnmt	DNA-methyl transferase
DZ	Dizygotic twins
e.g.	Example
EDC	1-ethyl-3-(3'-*N,N'*-dimethylaminopropyl)-carbodiimide hydrochloride
EDTA	Ethylene diamine tetraacetic acid
ESI-MS	Electrospray ionisation mass spectrometry
EtOH	Ethanol
f	Corrrection factor

FDA	Food and drug administration
Fe^{2+}	Ferrous ion
Fig	Figure
G	Guanine
h	Hours
H_2O	Water
H_2O_2	Hydrogen peroxide
HAAs	Heterocyclic aromatic amines
HBO	Hyperbaric oxygen
HC	Holstein clones
HCL	Hydrochloric acid
HEPES	N-(2-hydroxyethyl)-piperazine-N'-2-ethanesulphonic acid
Hpa II	*Hpa* II Methyl transferase
HPLC	High performance liquid chromatography
HPLC-ESI-MS	High performance liquid chromatography with electrospray ionisation mass spectrometry
HPLC-MS	High performance liquid chromatography with mass spectrometry
HPLC -UV	High performance liquid chromatography with ultraviolet detection
Hupki	Human p53 knock in
ICSI	Intracytoplasmic sperm injection
ID	Inner diameter
ITP	Isotachophoresis
IVF	*In vitro*-Fertilisation
kV	Kilovolts
L	Liter (s)
L_D	Length of capillary to the detector
LIF	Laser induced fluorescence
LOS	Large Offspring Syndrome
L_T	Total capillary length
mbar	Millibar
MEKC	Micellar electrokinetic chromatography

MEKC-LIF	Micellar electrokinetic chromatography with laser induced fluorescence detector
MeOH	Methanol
mL	Milliliter(s)
mm	Millimeter
MN	Micrococcal nuclease
MS	Mass spectrometry
MSREs	Methylation sensitive restriction endonuclease
mW	Milliwatt
MZ	Monozygotic twins
m/z	Mass to charge ratio
N.B.	Nota bene
Na_2HPO_4	Disodium hydrogen phosphate
NaH_2PO_4	Sodium dihydrogen phosphate
$NaHCO_3$	Sodium bicarbonate
NaOH	Sodium hydroxide
Na_2SO_3	Sodium sulphite
ng	Nanogram
nL	Nanoliter(s)
nm	nanometer
NADPH-oxidase	Nicotinamide adenine dinucleotide phosphate oxidase
NER	Nucleotide excision repair
$OH^{\cdot}$	Hydroxyl free radical
O_2	Oxygen
$O_2^{\cdot-}$	Superoxide ion
O_3	Ozone
OPSL	Optically pumped semiconductor laser
P	Pressure
PA	Peak area
PAEKI	Pressure assisted electrokinetic injection
PAHs	Polycyclic aromatic hydrocarbons
PCR	Polymerase chain reaction
psi	Pound per square inch
RFU	Relative fluorescence unit

RIA	Radioimmunoassay
ROS	Reactive oxygen species
RP	Reversed phase
Rpm	Revolutions per minute
RT	Room temperature
R.t.	Retention time
SAM	S-adenosyl methionine
SC	Simmental clones
SCNT	Somatic cell nuclear transfer
S.D.	Standard deviation
SDS	Sodium dodecyl sulphate
s	Seconds
s.e.m.	Standard error of the mean
S.I.	Small intestine
S/N	Signal to noise ratio
SPD	Spleen phosphodiesterase
SPE	Solid phase extraction
ST	Simmental twins
T	Thymine
Tab.	Table
TCM	Traditional Chinese medicine
TLC	Thin layer chromatography
t_m	Migration time
U	Uracil in case of DNA
	Unit of enzyme activity in case of description MN and SPD enzymes
UV	Ultraviolet
UV-VIS	Ultraviolet-visible
V	Volt
V	Voltage
VECSEL	Vertically external cavity surface emitting of laser

Index

1 Introduction

Be less curious about people
and more curious about ideas

Marie Curie

1.1 Epigenetics

The term *epigenetics*[*] defines all mitotically and meiotically heritable changes in gene expression that are not coded by the DNA sequence [1, 2]. Epigenetics literally means 'on top of genetics'. In addition to the DNA sequencing[*], epigenetics explains the contribution of another system, one that is responsible for the direct translation of the genetic information to control the genes influencing our growth and development [2]. Additionally, it explains why different cell types of an organism share identical DNA sequences but show amazingly diverse functions and phenotypes [2-4]. Epigenetics is important for development and cell differentiation but could contribute to congenital disorders and health problems [3]. Several epigenetic processes have been described in humans. DNA methylation and histone modifications, main components of epigenetic code, are the mechanisms involved side by side for the regulation of epigenetic processes [5].

1.1.1 Histone modification

DNA[*] is coiled around histones (the main protein components), which means that both together compose the chromatin [6]. DNA is wrapped around nucleosomes (core histones) [7] and fixed in place with small proteins called linker histones (**Fig. 1**). The supercoiling of this necklace helps the fitting of DNA into the tiny nucleus [6]. Additionally, chromatin controls the gene expression, DNA replication and cell functions. Histone modifications including methylation, acetylation, phosphorylation and others affect gene expression, DNA replication, mitosis and DNA repair mechanism [8-10].

[*]: See Appendix

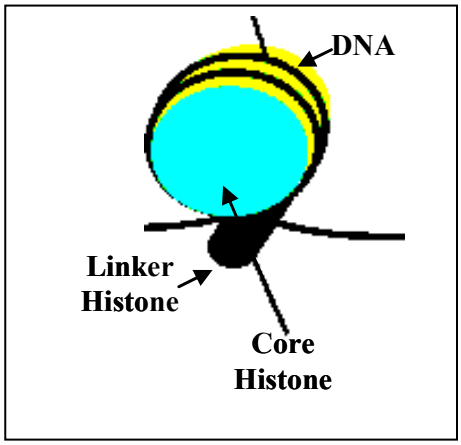


Figure 1: Nucleosome unit of chromatin structure [6]

1.1.2 DNA methylation

DNA methylation is an enzyme-mediated chemical modification involving the covalent addition of a methyl group to the cytosine nucleotide at the carbon 5' position of the cytosine ring within CpG dinucleotides [11-13] (**Fig. 2**). 5-methylcytosine is sometimes considered as the fifth base of DNA structure. DNA methylation is the unique natural modification of DNA revealing that about 3-4% of all cytosines in mammalian DNA are methylated [14].

Three methyltransferase enzymes are involved in this process: Dnmt3a and Dnmt3b function as *de novo* methyltransferases to introduce methyl group to cytosines as well as Dnmt1 responsible for maintenance of methylation patterns by acting at the replication forks to introduce CH_3 groups on the newly synthesized DNA.

CpG sites are regions of DNA where a cytosine nucleotide is located next to a guanine nucleotide with the nucleosides linked together by a phosphodiester bond (p). The "CpG islands" are largely localized in the gene promoter* regions of housekeeping genes (which are essential for general cell functions) or other genes* frequently expressed in a cell noting their evidence in small areas of 300-3000 Bp [12]. Almost 50% of the human genes contain CpG islands in their promoter regions [15]. CpG sites in human DNA are methylated (70-80 %) but only in areas where CpG density is low [13].

For the majority of genes, especially housekeeping genes, the CpG islands in their 5' regions are protected from methylation as CpG density is high, and their expressions are switched-on [16, 17]. That means the methylation-free pattern is important for the transcription of associated genes except in case of X-chromosome inactivation and gene imprinting.

Most CpG islands are reported to be unmethylated in healthy tissue [18, 19]. In contrast, the CpG sequences in inactive genes as tumour-suppressor genes are usually methylated to suppress their expression [20, 21]. In consequence, methylation of CpGs in the DNA of humans and other vertebrates distinguishes tissues and is involved in the regulation of gene expression and cell differentiation [22]. The mechanism of switch off of inactive genes leads to permanent silencing of associated genes and then this silencing state is transmitted through mitosis*. From this point, DNA methylation represents a basic process of epigenetics by inheriting these changes of the gene expression without any alteration of DNA.

On the other side, DNA methylation plays a fundamental role in carcinogenesis by contributing to the hypermutability of CpG sequences as there is high probability of the conversion of cytosine (C) in the methylated CpGs to thymine (T) by deamination [23]. Unlike the cytosine to uracil (U) mutation, which is efficiently repaired, the cytosine to thymine mutation can be corrected only by the mismatch repair [24]. But this repair is inefficient and the conversion of the methylated CG sequence to the TG sequence is of high frequency (**Fig. 2**).

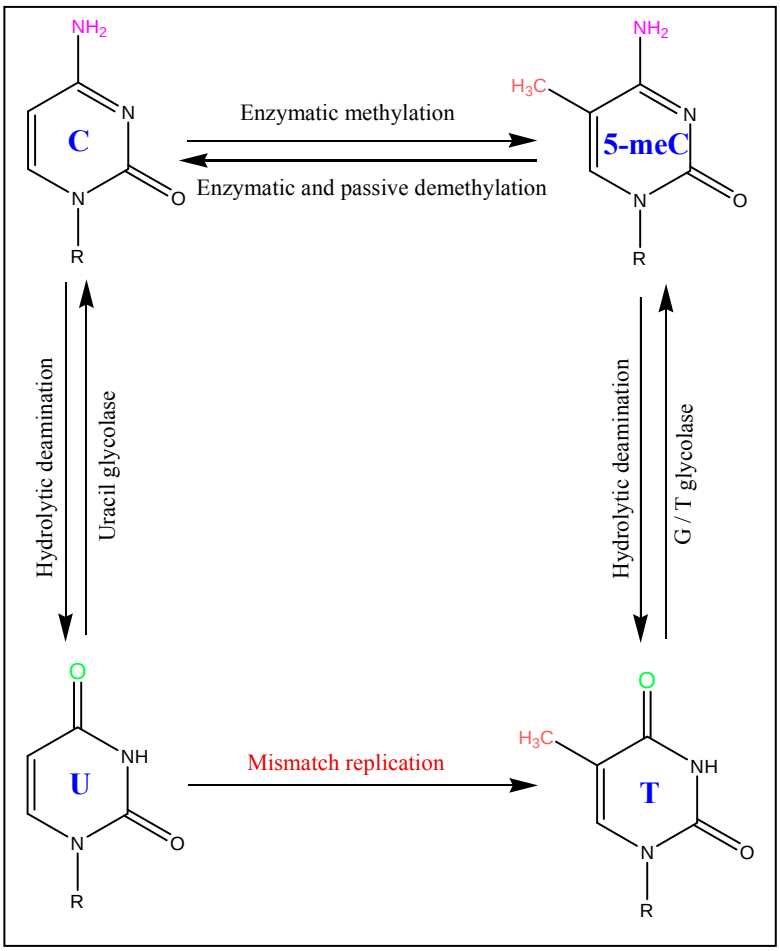


Figure 2: Genetic mutation caused by DNA-methylation. C: Cytosine; 5-meC: 5-methylcytosine; U: Uracil; T: Thymine [24]

1.2 The role of DNA methylation

Methylation of DNA is the most extensively studied epigenetic modification in mammals [25]. It is an essential mechanism for regulating embryonic development [26] and influences the chromatin structure [27], X-chromosome inactivation [26, 28], imprinting parental genes and protection of the host organism against expression of undesired sequences, like non-coding or repetitive ones [29]. It is also essential for epigenetic control of gene expression and the maintenance of genomic integrity [30-32]. **Figure 3** summarises its functions in normal cells.

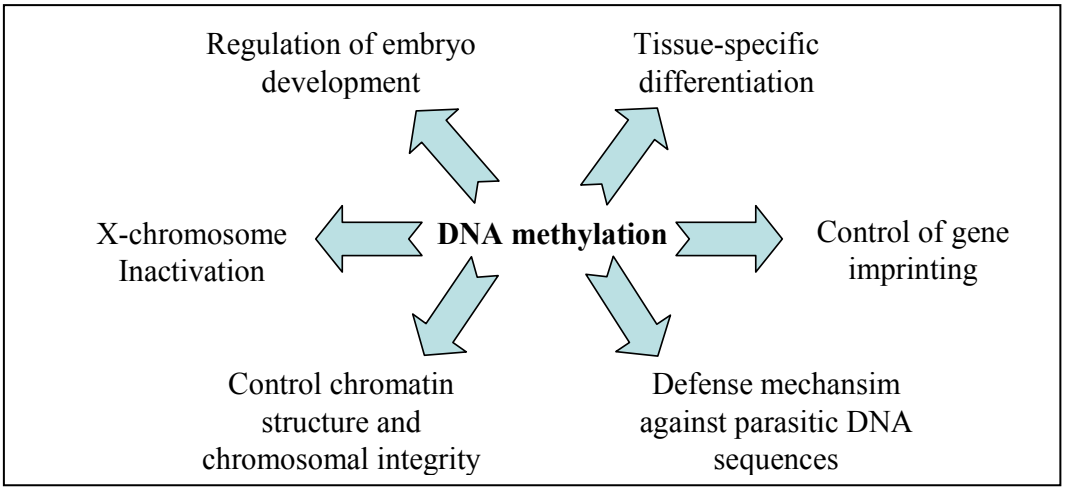


Figure 3: The multiple roles of DNA methylation in normal cells

1.2.1 Gene expression

Gene expression is a continuous mechanism throughout the life stages of an organism, which makes the cell to gain the control over its morphological and functional characteristics [33]. It illustrates how our genes work and how they are regulated. Gene expression plays an important role in cell differentiation noting some genes are turned on (active) in certain tissues or at certain developmental stages and others are turned off (inactive). The alteration of any step of the genetic expression process (transcription, translation, posttranslational modification of proteins) will lead to change of the function of the gene [33, 34]. Different studies like restriction enzyme analysis have established an inverse relationship between gene methylation and gene expression [35, 36]. In other words, DNA methylation and gene silencing are two faces of one coin [37] affecting in consequence the other epigenetic processes [25].

Between 70-80 % of the CpG sites in the human genome are methylated [38] and the unmethylated CpG regions are found in somatic cells [16]. The methylation of CpG islands in promoter

regions can suppress the gene expression of the associated gene [34, 39]. This gene silencing takes place as the methylation within promoter regions is associated with transcriptional repression of the gene [40] and behaves as a switch off for the affected gene.

1.2.2 Chromatin structure and transcription repression

Both DNA methylation and histone modification are the two major mechanisms that regulate gene expression, thus it is very important to understand how the histone modification affects the chromatin structure that dictates the gene expression pattern emerging from the human genome.

The unmethylated CG dinucleotides in gene promoter regions are free to act as receptors for the transcription factors and enable the initiation of the transcription process. However, methylation of CpGs is catalyzed by several DNA methyltransferases as DNMT1 (DNA (cytosine-5-)-methyltransferase-1), DNMT3a and DNMT3b [41] and interferes with the binding of many transcription factors [21, 42]. But, methylated CpGs facilitate the assembly of transcription repressor complexes that include histone deacetylases (HDAC), histone methyl transferases and chromatin remodeling ATPase [43]. Finally, this represents a connection between three mechanisms CpG methylation, chromatin structure modification and gene silencing. Clearly, DNA methylation is involved with silencing of the gene, which is suspected to induce a higher density of the chromatin structure. This modification of chromatin limits the accessibility to the promoter sites leading to transcription repression [21, 27, 44].

This complicated mechanism can be clarified in **figure 4**. The methylated CpG sites offer binding points for m-CpG binding proteins involved in gene repression such as MBD1, MBD2 (methyl-CpG binding domain-1 and 2) and MeCP2 (methyl CpG binding protein 2) [45]. These proteins in turn offer binding sites to transcriptional co-repressors, which finally associate with transcription repression mediated factors (histone deacetylase, histone methylases and chromatin ATPases) [46]. The deacetylation of histone [47-49] restricts the access to promoter regions due to more densely packed nucleosomes and more condensed chromatin structure, leading to repression of gene activity and stop of the transcription [50-52].

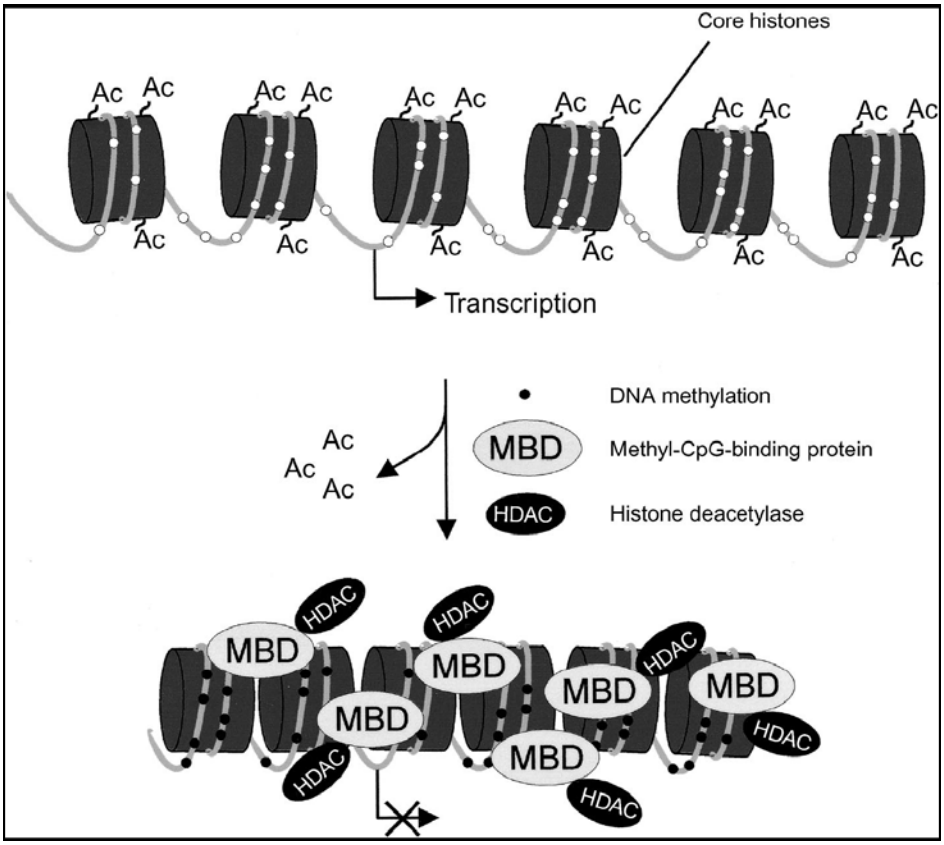


Figure 4: Model for methylation-dependent transcription repression [43]

1.2.3 Genomic imprinting

Genomic imprinting is one of the epigenetic phenomena which occur when both maternal and paternal alleles are present, but one allele* will be expressed while the other remains inactive. The gender of the parent controls the gene expression according to the associated allele [53]. The mechanism of imprinting of parental genes could be due to the gene silencing by methylation [54]. In case of genomic imprinting, either the maternal or paternal homologue of approximately 0.1 to 1 % of mammal genes is repressed via methylation while the other is expressed [55]. Genomic imprinting process plays a critical role in the regulation of growth in the embryo and neonate and is necessary for the development [56]. In some cases, aberrant methylation of certain gene results in the expression of the other allele with the susceptibility of cancer. This epigenetic phenomenon could be explained by the embryos produced by nuclear transplantation, either with two paternal genomes (androgenotes) or with two maternal genomes (gynogenotes). Although the zygotes could be formed, neither type was able to undergo further development [57, 58].

This is due to the natural state which implies a complementary relation between the maternal and paternal genomes for the mammalian development and reproduction of species [59]. Each genome contains different vital characters and necessary properties [60]. Large offspring syndrome (LOS) is a major problem concerning cloning as well as *in vitro* fertilization. The oversize phenomenon of produced animals or humans respectively can be explained by the genomic imprinting alteration. Some imprinted genes were reported to be connected to human diseases [61] when their inactive alleles were expressed.

1.2.4 X-chromosome inactivation

A related mechanism to imprinting is the X chromosome inactivation via gene silencing and methylation. The X-chromosome inactivation occurs when one of the two X chromosomes (either the maternally or paternally derived X) in every cell in a female is randomly inactivated shortly after fertilization in embryogenesis of females [62]. This change is inherited and all descendent cells will have the same X-chromosome inactivated as the cell from which they arose. Consequently, the male and the female have equal gene expression carried on the X-chromosome. Thus, the new female population is a mosaic type, composed of two genetically different types of cells, one which expresses only the paternal X-chromosome, and another which expresses only the maternal X-chromosome. The inactive X chromosome does not express the majority of its genes [63] due to its silencing by repressive heterochromatin* [64]. It was found that the inactive X-chromosome is associated with hyper-methylation and histone hypoacetylation [65]. X-linked genetic diseases are representative to this epigenetic phenomenon [66, 67].

1.2.5 Cancer

Cancer is one of the diseases that had attracted the attention of the people as well as the scientists in different fields. Excessive researches in oncology had been done in the last decades to understand the nature of this disease, recognise its types and to control its invasion.
Cancer is categorised as a genetic disease [42, 68] caused by somatic mutations* [69], contrary to the other genetic diseases caused mainly by germ line mutations* [70]. Moreover, the accumulation of mutation* plays a definite role in the progression of the disease. Cancer is mainly characterized by multiplication of the cells, cell proliferation and migration from the original site to distant sites in case of malignant forms [71].

Cancer is classified into benign and malignant* tumours. Metastasis differentiates between malignant and benign* tumours [70], noting that in the malignant tumours, the cancer cells escape through newly formed specific blood vessels and the lymphatic system to spread throughout the body and locate in other organs forming metastases. There are different types of malignant tumours named according to the affected site: carcinoma*; adenocarcinoma*; melanoma*; leukemia*; lymphoma*; sarcoma* and giloma*.

Although several studies tried to anticipate the mechanism of carcinogenesis, there is no definite mechanism agreed by all. However, a common argument revealed that cancer is a multistep process [72, 73] starting with 'Initiation Phase' to 'Promotion Phase' and end with 'Progression Phase' [74]. These stages are characterised by dynamic changes in the genome as mutation, chromosome translocation* and gene amplification [75].

Tumour initiation is the primary phase characterised by the exposure of normal cells to different types of carcinogens* that lead to genetic and epigenetic damage in these affected cells. If DNA damage is not repaired, an irreversible mutation stage will be developed and in parallel, the excessive proliferation of the initiated cells will induce the tumour promotion phase [76] provoking changes in the expression of genome [77]. The repetitive exposure to carcinogens gives raise to the second stage of carcinogenesis to be developed and the accumulation of the mutations leads to more expansion of the tumour cells with uncontrollable cell growth. The final stage of progression is characterised by malignant growth, invasion of tumour to the surrounding tissues and the development of metastasis.

Two target genes, oncogenes and tumour suppressor genes, have been clearly identified through their involvement in the stages of tumour formation [78]. The genetic alterations affect the proto-oncogenes (normal cellular genes) or oncogenes (activated counterparts) by excessive gain of their function and the tumour suppressor genes by loss of their function.

Oncogenes are the activated counterparts of normal cellular genes (proto-oncogenes) formed by genomic alterations affecting proto-oncogenes such as point mutation* [79], gene amplification or gene fusion. Proto-oncogenes are responsible for the regulation of cell growth, cell division and replication. Cell growth is initiated normally by growth factors that are encoded by genes and bind to receptor, the trans-membrane protein, giving rise to different signals and transcription factors that activate transcription and gene expression. Any mutation of the genes involved in this cycle turns them into oncogenes modifying their expressions and functions. Consequently, the cell division could get out of control leading to the transformation of a normal cell to a tumour cell [77]. Over 80 oncogenes have been defined, among which ras-oncogene is the most famous one, being identified in third of all human cancers [80].

Tumour suppressor genes code for anti-proliferation proteins that suppress mitosis and cell growth play a fundamental role in the repairing of DNA mistakes and in apoptosis*. The name of these genes refers to their role as brake to prevent cellular proliferation and stop cell division through growth inhibitory signals sent by the surrounding cells. Various mechanisms are involved in the inactivation of tumour suppressor genes as point mutation, chromosomal deletion or gene conversion. In consequence, they do not work properly and cells can grow out of control provoking cancer. Among the tumour suppressor genes, p53 protein is one of the excessively studied genes, encoded as *P53* gene detected in more than 50 % of human cancer, e.g. associated with colon, lung, skin, brain tumours and others.

1.2.5.1 DNA methylation: Effective biomarker for tumourigenesis

DNA methylation [81] has a specific meaning and echo in the medical field referring to its fingerprint in several cancer diagnosis researches that conclude DNA methylation patterns in tumour cells are altered relative to those of normal cell [82-84]. It was interesting to report in pioneer works from two decades ago that 5-methylcytosine is identified as a mutational hotspot in *Escherichia coli* [14]. Subsequently, the studies had illustrated that abnormal methylation is closely related to carcinogenesis [85, 86] noting that tumour cells exhibit global hypomethylation of the genome accompanied by region-specific hypermethylation of tumour suppressor genes [68, 87]. Both have an active effect for the onset of cancer [88, 89] and have been reported in a variety of neoplasm, namely, of the colon and rectum [82, 90-93], lung [94], uterine [95], cervix [96] and gastric carcinoma [97]. The effect of DNA methylation in cancer cell is illustrated in **figure 5**.

In case of cancer, hypermethylation occurs to CpG islands in the promoter regions of tumour-suppressor genes which are then transcriptionally silenced [21]. The 'two-hit hypothesis' implies both alleles of tumour suppressor gene should be mutated to manifest the tumourogenesis [98]. Gene hypermethylation could be one of these hits contributing to malignancy development due to the inactivity of tumour suppressor genes [99]. In particular, different studies have found relation between the hypermethylation of specific genes involved in DNA repair, cell cycle regulation and apoptosis, and certain human tumours [81]. In other words, gene hypermethylation can result in inactivation of DNA repair, defect of signal transmission between cells and loss of apoptosis.

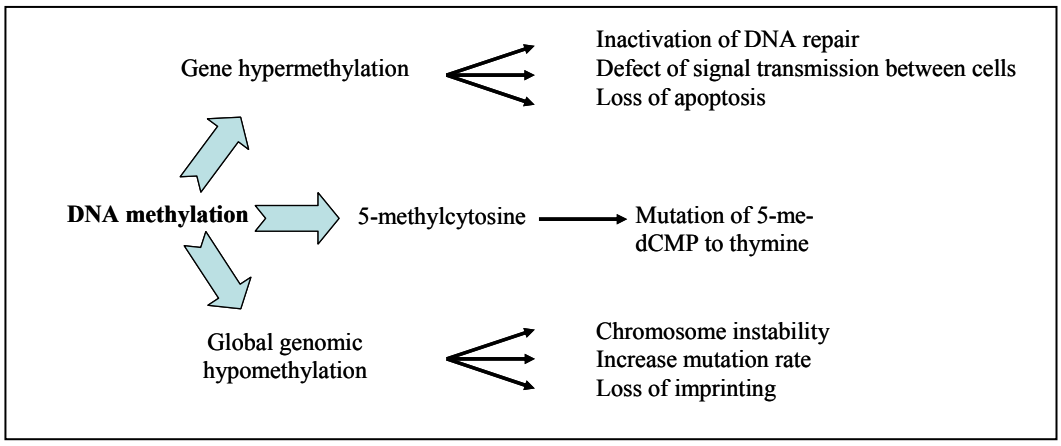


Figure 5: The effect of methylation level in tumour cells

On the other hand, the overall genomic hypomethylation was associated with few human malignancies such as colonic neoplasias [100, 101], lung carcinoma [102] and metastatic prostate carcinoma [103]. DNA hypomethylation, however, was detected as an important spot in breast carcinoma [88]. Global hypomethylation affects DNA in repetitive sequences as well as centromeres* resulting in chromosome instability, loss of imprinting and increased mutation rate. A reduction in the genomic content of 5-meC varied from 8% to 31% according to carcinogen type was determined in other specific studies concerning bronchial epithelial cells treated with a broad range of chemical carcinogens [104].

Medical researches illustrated that hypomethylation is present at early stages of chronic lymphocytic leukaemia (B-CLL) [105].

In conclusion, DNA methylation can be used as biomarker for diagnosis of cancer because the profiles of DNA alterations characterizing a certain cancer type were helpful as cancer diagnostics, moreover the inactivated genes by promoter region hypermethylation provide a fundamental link of these inactivation patterns corresponding to specific tumours [106]. Importantly, the altered DNA methylation-caused tumour is a reversible epigenetic mutation offering targets for therapeutic drugs. DNA-methyltransferase inhibitor drugs as 5'-azacytidine, decitabine and histone deacetylase inhibitors are being used as chemotherapeutic agents to treat some kinds of tumours [1, 107].

1.2.6 In-vitro fertilization (IVF)

Several assisted reproduction technologies (ART) were established to solve the infertility problems [108]. *In vitro*-fertilisation (IVF), one of the most significant ART had shown success to conceive baby in 1976 then the development of its expanded techniques, most importantly to be cited the intracytoplasmic sperm injection (ICSI) [109], had really helped the infertile couples to conceive infants. However, different birth defects [110, 111] had been remarked in some cases like chromosomal abnormalities, cardiovascular defects, growth abnormalities [112, 113] as well as high risk of low birth weight. Hansen *et al* [114] had declared that the babies concieved by IVF have twice the rate to develop major birth defects as compared to naturally conceived children. Additionally, IVF was reported to be associated with diseases like Angelman syndrome (AS) [115] and Beckwith-Wiedemann syndrome (BWS) [116, 117] that etiologically related to aberrant genomic imprinting [118, 119]. The epigenetic modifications of the genome including DNA methylation and histone modification are linked through their functions and synchronously affecting each other. DNA methylation as an important factor in modeling the structural patterns of genome was investigated in IVF in different studies. Different methylation status was reported [120, 121] depending on the protocol of *in vitro* embryo formation as well as tissue specificity [120]. IVF could affect the DNA methylation via imprinting errors that happened during this *in vitro* reproduction technique [122, 123].

1.2.7 Cloning

Somatic cell nuclear transfer (SCNT), or cloning, is a technique used to create genetically identical animals by somatic cell nuclear transfer into unfertilized eggs. In this technique, the nuclear genetic material is transferred from the animal that should be cloned (known as genetic donor) into a recipient cell matured oocyte or unfertilised egg (cytoplast) from which the genetic material has been removed. Then, the somatic cell taken from genetic donor is inserted into the enucleated egg and the fusion of two cells is accomplished by electricity. The fused egg is activated for the reproduction. Then the activated egg is placed in a culture medium. The cellular division is watched till an early-stage embryo (blastocyst) is formed and then is transferred to a recipient female (sometimes referred to as 'surrogate mother') for the subsequent development [124].

1.2.7.1 Cloning efficiency

The birth of the first mammal cloned from an adult donor cell, 'Dolly', proved the success of cloning [125] and has initiated a stream of researches to improve cloning technology. However the overall efficiency of this technology is low [126-128] noting a success rate ranging from 0.1 % to 3 % with an exception of 15-20 % success recorded by cattle as a model extensively used for mammalian cloning [129]. It was estimated that the clones show frequently high mortality rates *in utero* due to the incomplete reprogramming of nuclear activities [127].

Normally, somatic nuclei acquire highly specialized DNA and chromatin modifications during the differentiation stage in order to support early development [130]. Upon somatic nuclear transfer into oocytes, the donor nucleus should be remodelled from highly differentiated somatic patterns to a totipotent embryonic pattern (zygote that can develop into any sort of cell) then to pluripotent pattern (embryo state that gains the capacity to develop to many cell types). The end of these development stages is referred to 'terminal differentiation' when cells gain specific characteristics that allow them to do a specific function. This process is called 'epigenetic reprogramming' occurring in mammalian embryos at two times during their development, once soon after fertilization known as preimplantation reprogramming and the other takes place during gametogenesis and known as gametogenic reprogramming (**Fig. 6**). It is usually associated with some changes in epigenetic features similar to those that occur

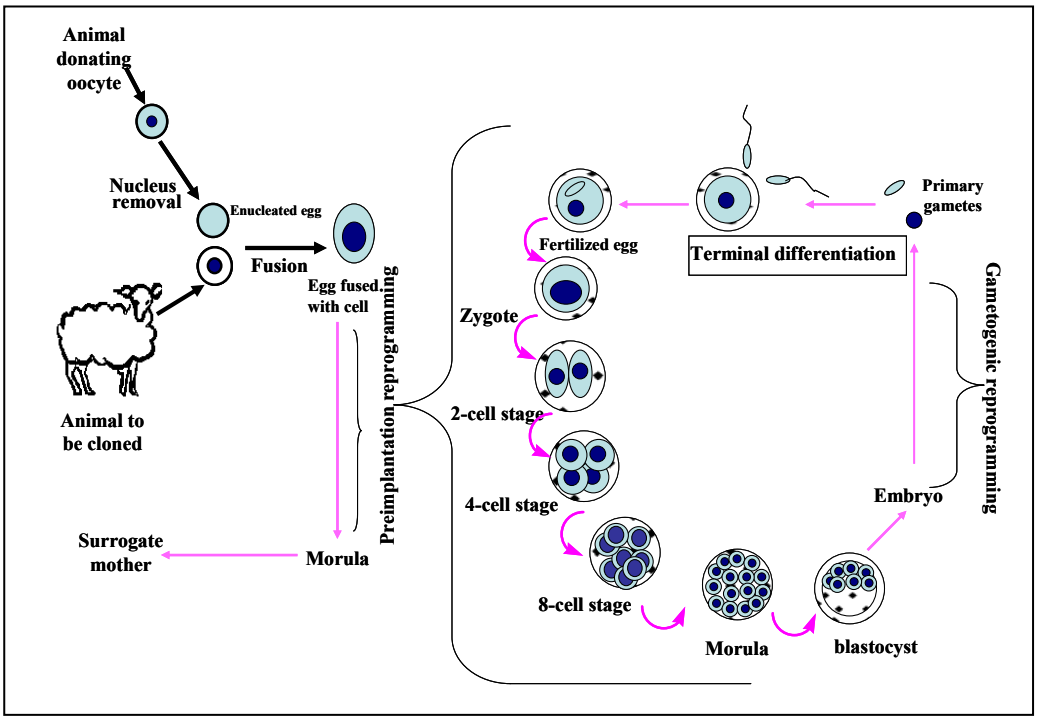


Figure 6: Overview of epigenetic reprogramming in cloning process and embryo development (Figure modified from www.fda.gov/ucm/groups)

in normal embryos during early development [131, 132]. However, different studies showed an evidence that incomplete or inappropriate epigenetic reprogramming of donor cell nuclei is the main cause behind the low cloning efficiency, high embryonic and foetal losses after SCNT and developmental abnormalities [133, 134]. At different stages, inappropriate reprogramming occurs leading to failure of cloning.

Simply talking, other logical reasons rather than the failure of epigenetic reprogramming can explain the unsuccessful cloning procedure in the embryonic stage. These reasons could be the incompatibility between the enucleated egg and the transferred nucleus, or an egg with a newly transferred nucleus may fail to divide or develop properly, or simply the implantation of the embryo into the surrogate mother might be impossible. On the other side, the surviving clones can exhibit congenital defects [135] such as LOS [126], kidney or brain malformations and impaired immune systems.

1.2.7.2 Effect of cloning on epigenetics

High rates of embryonic and foetal mortality [127, 136], and an increased incidence of congenital defects [135, 136] have been associated with perturbations in developmentally important epigenetic marks [127] such as DNA methylation and histone modifications [137-139].

The methylation of DNA is involved in long-term epigenetic silencing of specific sequences [43], including transposons*, imprinted genes and pluripotency-associated genes in the cloning process [140].

Various studies had demonstrated that SCNT procedure can impact on the global DNA methylation status of cloned bovine embryos by insufficient active de-methylation of the nuclear donor DNA after transplantation, by delayed or attenuated further passive de-methylation, and by precocious *de novo* methylation before the 16-cell stage [134, 137, 141, 142].

Other studies had revealed aberrations in global DNA methylation status of cloned embryos and fetuses in different species [137, 143-145] . In general, an increase in DNA methylation level in cattle clones has been correlated with poor developmental potential of cloned preimplantation embryos [137] and with compromised foetal overgrowth [144]. The global changes in methylation states have also been described for specific genes and non-coding DNA sequences under epigenetic control [127, 133, 138, 139, 142, 146]. Although the overview of these reports shows a relation between the aberrant methylation level and the poor success for the cloning process, the genomic methylation status of live offspring was not studied so far.

1.2.8 Twins and epigenetics overview

Monozygotic (MZ) twins originate from the same zygote and split into two eggs in the gestational period and are supposed to share identical epigenomes when the blastocyst splits. Studies had concluded that the time frame of splitting plays a role whether identical twins or conjoined twins (splitting after 12 days) are conceived. Surely, dizygotic (DZ) twins developed from different zygotes carrying different epigenetic profiles. It was observed that MZ twins could show varying behaviour in the incidence of psychiatric diseases such as schizophrenia and bipolar disorders as well as other diseases like Parkinson's and cancer [147-150]. This differential susceptibility to the diseases was returned to the role of the environmental agents in affecting the epigenome [151] and then the twins manifest a difference in phenotype. However, the accumulating evidence [152, 153] had recently highlighted that the change in DNA methylation and chromatin structure of mammalian genome induced due to exposure to the environment significantly alters the gene function associated with different phenotypic outcomes [149, 154]. Additionally, Kaminsky *et al.* [155] had proved that the epigenetic profiles are not fully determined by DNA sequence, otherwise MZ twins should not show epigenetic differences. Most MZ twins showed the same methylation status, whereas few others showed aberrant methylation profiles. This epigenetic variation between twins explain the etiology of the complicated diseases developed by one of the traits [149]. The correlation between the diversion of the epigenetic profile in the late adolescent and the development of the disease despite the same epigenetic status after twin separation is cleared by epigenetic model of twin discordance for psychosis represented by Petronis *et al.* [147]. In conclusion, the DNA methylation, this dynamic process throughout the life time, affects the gene expression by shutting some genes off or increasing the role of others. The accumulation of these variations of gene expression in MZ twins contributes to their discordance and their response to the diseases. Additionally, in the recent years there is doubt concerning the assumption that MZ twins are genetically identical organisms due to findings of the researchers about the change on the genetic level associated with the gene sequence. This issue is still under investigation.

1.3 DNA adducts

1.3.1 DNA adducts and cancer

The formation of DNA adducts is resulted from the covalent interaction of genotoxic carcinogens with DNA, derived from exogenous and endogenous sources, either directly or following metabolic activation [156, 157]. If this DNA modification is not repaired, it will lead to mutations in critical genes such as those involved in the regulation of cellular growth and subsequently to cancer development [158] (**Fig. 7**).

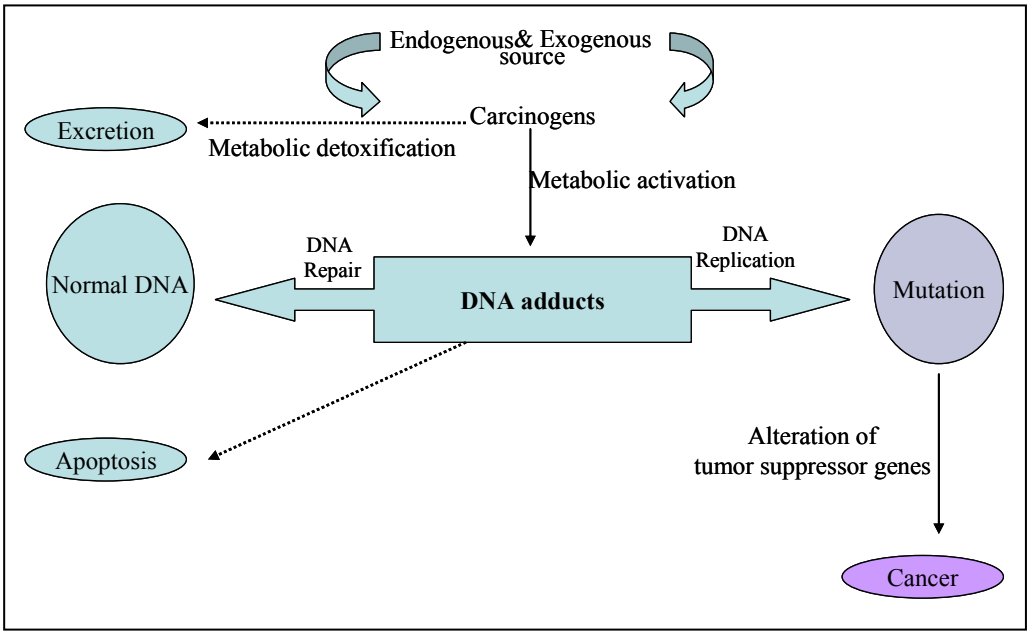


Figure 7: The pathway of DNA adducts formation

As shown by the Salmonella test established by Ames [159] and by other tests [160-162], most carcinogens are either mutagens (electrophilic reactants that covalently bind to DNA bases) or premutagens (chemicals that undergo metabolic conversion to such electrophilic compounds). However, the majority of the genotoxic carcinogens require metabolic activation in order to form electrophilic reactive species that bind covalently to nucleophilic sites in DNA [161]. Generally speaking, nitrogen and oxygen centres are the prefered nucleophilic sites on DNA to be attacked by alkylating and arylating agents [163].

DNA damage is induced either by normal cellular processes or by some environmental agents. Either endogenous or exogenous mediated-DNA damage share similarities in occurrence as well as in cell response. The damage is occurred initially either by involvement of free radicals (ionizing radiation, UV light, oxidative stress) or adduct formation (mutagenic

chemicals, alkylating agents, arylating agents). Unrepaired DNA damage will accumulate and contribute to alterations in the DNA sequence leading to mutation upon DNA replication. In addition, alterations in the DNA sequence may occur when adducts are subjected to erroneous repair. All DNA damage types, especially chemicals-induced DNA damage, are likely being the major cause of cancer, aging or other diseases [159, 164]. A variety of DNA repair systems are involved to keep the integrity of the genetic material. Persistent DNA damage can result in induction of both transcription and signal transduction pathways, replication errors and genomic instability. All these processes are equally associated with cancer. In other words, the cellular response to DNA damage could be either positively by repairing approaches or negatively by replication of errors with the induction of diseases.

Both DNA adducts and DNA methylation that are considered as DNA modifications play an important role in monitoring cancer formation. Most studies that consider the association between cancer at different sites and adduct levels at these sites have shown that cancer cases have higher levels of adducts than non-cancer controls [165]. These carcinogen-DNA adducts in humans are considered as biomarkers of individual exposure and of potential risk for cancer development [162]. The conclusions illustrated by Buss *et al.* [166] and Poirier and Beland [167] confirm that the adduct formation is directly proportional to the dose, and subsequently any traces of adduct exposure present definitely a carcinogenic risk. However, a universal value could not be given to such an acceptable level of adducts because their mutational effectiveness varies according to the nature of the carcinogen as well as the chemical structure of the DNA adduct. This was proven by Saffhill *et al.* [168] as *N*-7 alkylguanine adducts have considerably less mutagenic potential than O^6 alkylguanine adducts. Lastly, different studies had argued that genotoxic carcinogens are site selective, for example hepatocellular tumour is associated with the exposure to aflatoxin B_1 which leads to a specific mutation at codon 249 of the *p53* tumour suppressor gene [169, 170]. Furthermore, the spectrum of mutations induced by polycyclic aromatic hydrocarbons (PAHs) in the *p53* tumour suppressor gene in bronchial epithelial cells has been reported to be similar to the major mutational hotspots in human lung cancers, indicating that PAHs may be involved in lung carcinogenesis [171-173].

1.3.2 Endogenous adducts

Endogenous mutagens that cause DNA damage are major contributing factors to the onset of aging and other degenerative diseases associated with aging, such as cancer [157, 174].

Oxidation, *methylation, depurination* and *deamination* are the endogenously causative processes to significant DNA damage. Jackson and Loeb [175] had shown that the endogenous DNA damage occurs at a high frequency compared with exogenous damage and the majority of damage types induced by normal cellular processes are identical to those caused by some environmental agents. The major endogenous sources of DNA damage are those produced by ROS and aldehydes derived from lipid peroxidation [176, 177]. The former will be discussed later in details (Section oxidative stress). The major aldehyde products of lipid peroxidation are crotonaldehyde, acrolein and malondialdehyde (MDA) [176, 178]. Equally, etheno- [179-182], propano- [178, 183], and malondialdehyde- [175, 184] derived DNA adducts are produced by the reaction of DNA with epoxyaldehydes, aldehydes and MDA, respectively. These later exocyclic adducts are categorized also as examples of adducts formed by exogenous mutagens referring to acrolein and crotonaldehyde as exogenous environmental contaminants [175, 185]. In addition to oxygen and lipid peroxidation products, DNA damage could occur in living cells through other small reactive molecules like *alkylating agents* or through *spontaneous reactions*.

Methylation of DNA bases can occur through the action of environmental chemicals (exogenous) or intracellular (endogenous) agents. For example, *N*-methyl-*N*-nitrosoamine which is a component of tobacco smoke with powerful alkylating property elevates the mutation rates in cultured cells [186]. S-adenosylmethionine (SAM) is a reactive methyl group donor found inside every cell for normal cellular metabolism [187]. It could contribute to methylate inappropriate targets, such as adenine [188], guanine [187] and to less extent thymine leading also to mutation [175, 187]. The nitrogen atoms of the purine bases (N_3 of adenine and N_7 of guanine) and the oxygen atom of guanine (O^6) are particularly susceptible sites to methylation. So the main adducts formed are illustrated in **figure 8** referring to O^6-methylguanine as the most mutagenic adduct [187]. A minority of endogenous methylation agents such as betaine, choline and others, could be generated from endogenous cellular precursors or from exogenous sources such as diet, tobacco smoke or environmental pollution [170] and lead to DNA damage [189].

Spontaneous damage contributes to the hydrolysis of DNA with the loss of a base, or deamination, or sugar ring deterioration, or tautomeric shift* [189-191].

Abasic sites* are among the most frequent endogenous lesions found in DNA, with an estimated 10000 lesions/human cell/day [192]. These apurinic / apyrimidinic sites are produced from the cleavage of the glycosidic bond present between bases and deoxyribose in DNA due to heating, alkylation of bases or the action of N-glycosylases [190].

AP sites are not only produced by spontaneous depurination / depyrimidation but also to a large extent by ROS (reactive oxygen species) [193]. AP sites can be rapidly and efficiently [192] repaired, but mutations and chromosome aberrations occurred in the case when there is no damage repair [194].

Finally, cytosine and its homologue 5-methylcytosine are the main base targets for the hydrolytic deamination [192]. The fifth distinct base in DNA, 5-methylcytosine, is prone to spontaneous deamination more rapidly than cytosine [185] provoking a high degree of mutagenesis which results in CG → TA transitions [195].

It should be mentioned that X-rays could be not only exogenously but also endogenously involved in DNA damage by free radical-mediated reactions and lead to single strand break.

Of course, endogenous DNA damage can be repaired by 'Base Excision Repair' system (BER) by removing the damaged base via DNA glycolase then re-synthesising and sealing the base by DNA polymerase and DNA ligase, respectively [196].

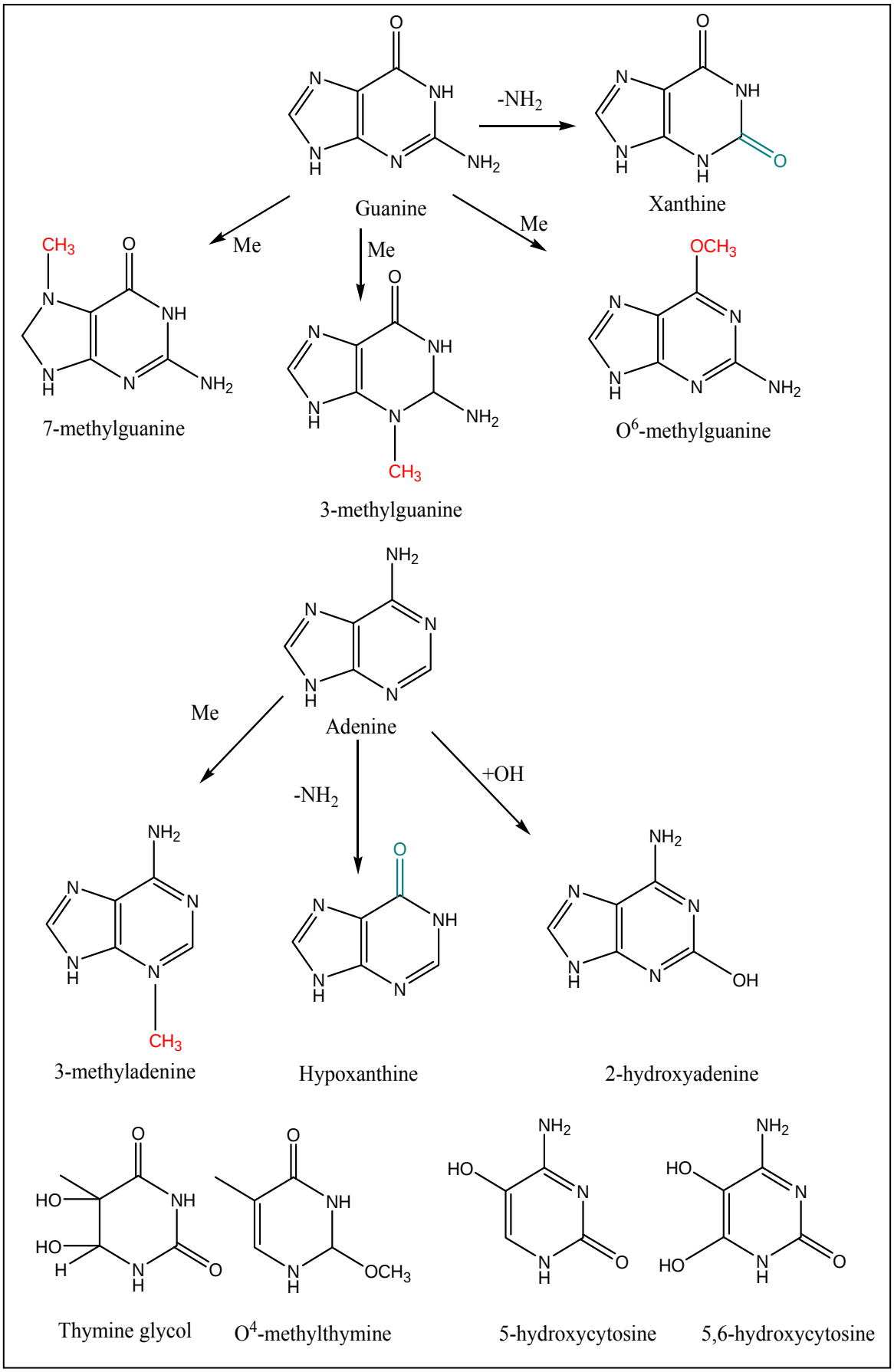


Figure 8: Examples of endogenous DNA adducts produced by methylation, deamination and oxidation of corresponding bases (N.B.: They can be also produced by exogenous agents).

1.3.2.1 Oxidative stress

Oxidative stress occurs due to an imbalance between the generated and exogenously produced oxidants, and the body's natural antioxidant defense mechanisms and different DNA repair systems [197]. Recently, oxidative stress is defined as altered homeostatic balance resulting from oxidant insult [198].

The reactive oxygen substances (ROS) are the well known drivers of the oxidative stress as well as the reactive nitrogen species [199]. ROS are continuously formed in living cells as a consequence of metabolic and other biochemical reactions [200] as well as by influence of external factors. Most of the ROS species are free radicals (e.g.: $O_2^{\cdot-}$, $OH^{\cdot}$) characterized by possessing unpaired free electrons defining their high reactivity. The non free radicals (e.g.: H_2O_2) are able to produce easily free radicals under certain circumstances [201]. Among all ROS, hydroxyl free radical ($OH^{\cdot}$) is the most reactive species which diffuses easily towards potential targets.

The free radicals are produced through reactions involving molecular oxygen catalysed either by oxidative enzymes or metals. ROS are a double-edged sword as they play an important role in physiological processes as in mediating inter- and intra-cellular signaling to highlight the pathways regulating proliferation, cell growth and apoptosis [202] as well as in defense mechanism against pathogens. When produced in excess, they modify cellular macromolecules by oxidizing DNA, proteins and lipids [199, 203-206], evoking vital damage to these biomolecules [207]. ROS are implicated in a plenty of pathological processes [208] such as chronic fatigue, skin disorders, hormonal imbalance, ageing [209], Alzheimer [210], cancer [211], atherosclerosis [212], heart diseases and autoimmune disorders [213].

The ROS-mediated damage gives rise to different mutation mechanisms which take place according to the reactive species involved in the damage [214]. Gamma-radiation, superoxide and hydrogen peroxide lead to the most mutagenic lesion G:C → A:T transitions. However G:C → C:G transversions are arising from transition metals and peroxyl radicals [215]. Aging [216], as well as the associated degenerative diseases, is attributed to the attack of free radicals on different macromolecules and cell constituents [217] and the accumulation of mutations at mitochondrial DNA, which can be determined in tissues characterised with many post-mitotic cells, like the brain [218]. The “Free radical theory” of ageing established by Harman [219] had assumed that the generation of intracellular oxygen species is the major limiting factor for the life expectancy [220]. In agreement with this theory, several studies had confirmed diet in-

take [221, 222] and the supplementation of antioxidants [223, 224] reduce the aging process by affecting the rate of production of mitochondrial ROS.

So far even under normal aerobic metabolism, active and potentially dangerous oxidants are produced leading to vital cellular damage, represented by the production of hundreds of hits per cell per day [225, 226]. This high endogenous level of oxidative adducts confirms that both deficiency of antioxidants and mitogenesis [227] are important risk factors for cancer [199, 228]. Two sources are responsible for producing ROS.

- Endogenous ROS

Endogenous ROS are produced in the human body by normal physiological processes as by-products of several metabolic and enzymatic reactions [217]. O_2 is of fundamental necessity for the life, essential for the respiration in all aerobic organisms as well as for the photosynthesis in the plants but it contributes in the production of active and potentially dangerous oxidants during normal aerobic metabolism [220]. All the biological functions in our body need energy which is produced via the electron transport chain in the mitochondria [229]. However, dangerous metabolic intermediates or by-products are obtained in the electron transfer reactions, such as superoxide ion ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2). **Figure 9** illustrates the main endogenous source of reactive oxygen species (ROS) [230]. Moreover, superoxide radicals and H_2O_2 are particularly produced in peroxisomes during the ROS decomposition, β-oxidation of fatty acids and the decontamination of xenobiotics [200].

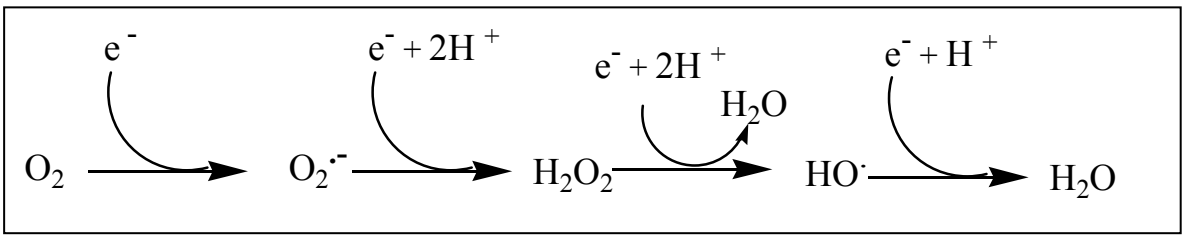


Figure 9: Electron transport chain as source of ROS [230]

Some pathologic systems contribute to oxidative stress by the excessive generation of free radicals as result of virus- or bacteria-infected cell phagocytosis process [231]. A respiratory burst of phagocytes is introduced by NADPH-oxidase generating superoxide ions [213, 231, 232]. The immune system generates peroxide, superoxide, and singlet oxygen to attack different microbes and destroy attacking pathogens. Additionally, the biotransformation of some cytotoxic drugs [233] could be accompanied by the generation of toxic intermediates and ROS.

Finally, flavo- and heme proteins, involved in the cellular energy processes, give rise to ROS derived from molecular oxygen after leak of electrons [197].

- Exogenous ROS

ROS are exogenously introduced by exposure to environmental contaminants like transition metals, oxidants and free radicals or exposure to other forms of pollutants. Lifestyle factors [234], ultraviolet light (UV) [235], ionising radiations [236], chemotherapeutic agents [237], psychological stress [238] are the major source to produce oxidants.

Not only a negative lifestyle, like smoking is as a promoter for oxidative stress but also a positive habit can be causative as well. During muscular exercise, the requirement of the body for O_2 is increased to fulfil the energy demands of this physical activity. Oxygen undergoes a four-electron transfer reaction producing ROS. Although it is highly recommended to perform muscular exercises to improve a healthy life because the produced ROS are beneficially used by the immune system and enhance its activity, the excess of exercises reveal serious adverse effects [239]. Surprisingly, it was concluded that intense long duration exercises reduce the immune function [240] and are associated with high frequency of other diseases as arteriosclerosis, cancer and premature ageing [241-243]. In contrast, both acute exhaustive and prolonged moderate exercises are usually related to the low risk of development of oxidative DNA damage signs and related diseases [244]. These conflicts had attracted Poulsen *et al.* [245] to frame the relation between exercise and oxidative DNA modifications.

The exposure to ionizing radiations and UV light increases the frequency of oxidative stress. The tropospheric ozone, found close to the ground, is created through the interactions of volatile organic compounds (VOCs) and nitrogen oxides in the presence of heat and sunlight producing harmful health effects. An increased level of oxygen free radical (specially 8-hydroxy-2'-deoxyguanosine: 8-OH-dG) was recorded in ozone-exposed plants [246, 247], explaining the mechanism of ozone-mediated plant injury. Although chemotherapy (anthracyclines, cyclophosphamide, cisplatin, cytarabine, mitomycin, fluorouracil and bleomycin) help in the improvement of the health of cancer patients, oxidative stress–mediated injury of normal tissues is a significant side effect. Some chemotherapeutic agents, such as bleomycin [248], introduce oxidative stress as a mechanism to kill cancer cells. However, other chemotherapeutic agents, such as the anthracyclines [249, 250], are more harmful by inducing oxidative stress in non-targeted tissues.

1.3.2.2 8-oxoguanosine

Among DNA oxidation, one of the most commonly occurred is the hydroxylation of guanine in the 8-position and formation of 7,8-dihydro-8-oxoguanosine [225]. This oxo-adduct gives rise to the most frequent somatic mutation found in human cancers [251] and described by lacking of base pairing and misreading of the modified base, whereas 8-OH-dG can pair with C or A. G:C → T:A is a transversion pathway (**Fig. 10**) which occurs often in association with the existence of 8-OH-dG adduct *in vivo* and *in vitro* and is confirming the diversity of the mutation forms detected with oxidative stress [252]. In most cases, oxidative stress is detected in parallel with aging, cancer and other related neurodegenerative diseases [251, 253-255] with relevant level of this adduct. Additionally, increased level of 8-OH-dG had been found in different pathological conditions as hepatitis, cardiovascular diseases, skin disorders, breast, lung and colon cancers [208]. From another side, the detection of 8-OH-dG in urine reflects the oxidative stress case [256] as well as the possible repair by a cellular N-glycolase [257]. Therefore, it is the most often studied oxidative species as a marker for human biomonitoring of carcinogenesis [163, 234, 253, 258].

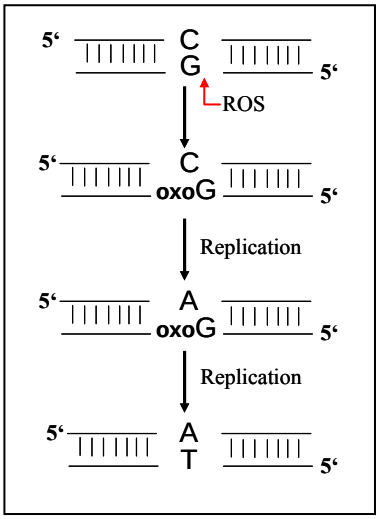


Figure 10: Mutation by 8-oxoguanosine

1.3.2.3 Methods of analysis of 8-oxoguanosine

Determination of 8-OH-dG levels in DNA is of vital importance and requires a sensitive detection system due to its presence in nanomolar concentration range. Various methods are described for the detection and quantification of 8-OH-dG, such as high performance liquid

chromatography with electrochemical detection (HPLC-ECD) [234, 238, 245, 259, 260], ^{32}P post-labeled HPLC [261], ^{32}P post-labeled-TLC [262], gas chromatography-mass spectrometry (GC-MS) [263, 264] and LC-MS methods [265, 266]. HPLC-ECD and GC-MS had high reputation for the quantification of oxo-adducts. However, the levels of 8-OH-dG estimated by the latter method were found to be 10-50 folds higher than the levels estimated by the former method. The sensitivity recorded by GC-MS is about 1 molecule 8-OH-dG per 10^6 molecules deoxyguanosine [245]. However, this technique requires specialized personal and expensive instrumentation in addition to the need of derivitization step prior to the analyses which can induce an insult of artificially oxidative products explaining the high level of adducts recorded with GC-MS. The silylation step and the non-availability of isotopically labeled internal standards are the major disadvantages limiting the application of GC-MS technique for the determination of DNA base modifications. The requirement of specific antibody induces a certain difficulty to apply immunologically based methods. Consequently, HPLC-ECD, the pioneer method in this field, is considered so far the only method employed for analysis of 8-OH-dG due to its simplicity, sensitivity and availability of its equipments. However, the only drawback is the difficulty to estimate exact concentration of 8-OH-dG.

1.3.3 Exogenous DNA adducts

They are formed due to the exposure to genotoxic carcinogens such as polycyclic aromatic hydrocarbons (PAHs), UV photoproducts and aflatoxins [267-271] that react with one or more DNA bases producing gene mutation [160].

1.3.3.1 Environmental chemicals

Polycyclic aromatic hydrocarbons are the most extensively studied species as they are widely found, for example in cigarette smoke [272]. They are strongly implicated as causative agents in the development of lung cancer [171].

Among the PAHs, benzo[*a*]pyrene (B[*a*]P), which occurs in amounts of 20 to 40 ng per cigarette, is one of the most potent carcinogens. As previously mentioned, the chemicals are not the main causative agents for cancers, but they are metabolically activated by cytochrome P450 enzyme leading to electrophilic species that can bind to DNA [175]. Following this mechanism, B[*a*]P, a bulky adduct, proceeds in a certain metabolic pathway to produce the ultimate carcinogenic metabolite of the isomers of 7,8-Diol-9,10-epoxide (BPDE) [273], which

is an electrophilic metabolite that attacks preferentially the nitrogen sites of nucleobase resulting in the formation of N^2-guanine adducts [171, 226] (**Fig. 11**). Thus, B[*a*]P leads to the mutations in growth regulatory proto-oncogenes and tumour-suppressor genes and results in cancer formation [171, 274, 275]. B[*a*]P was detected along *P53* tumour suppressor genes which represents the major mutational hotspots in two third of detected lung cancers in humans [171].

Importantly in the pharmaceutical field, B[*a*]P is present in a high content in coal tars, which are the major incorporated ingredients in the preparation of therapeutic ointments used in severe cases of psoriasis [276, 277]. Recently, coal tars were estimated as being highly involved in the carcinogenicity in humans [278]. Different studies performed *in vitro* as *in vivo* to provide an evident conclusion for the contribution of PAHs in carcinogenesis [171, 274, 277].

Recently, nitro-PAHs were classified as new mutagens found in some environmental components such as diesel exhaust and airborne particles [279].

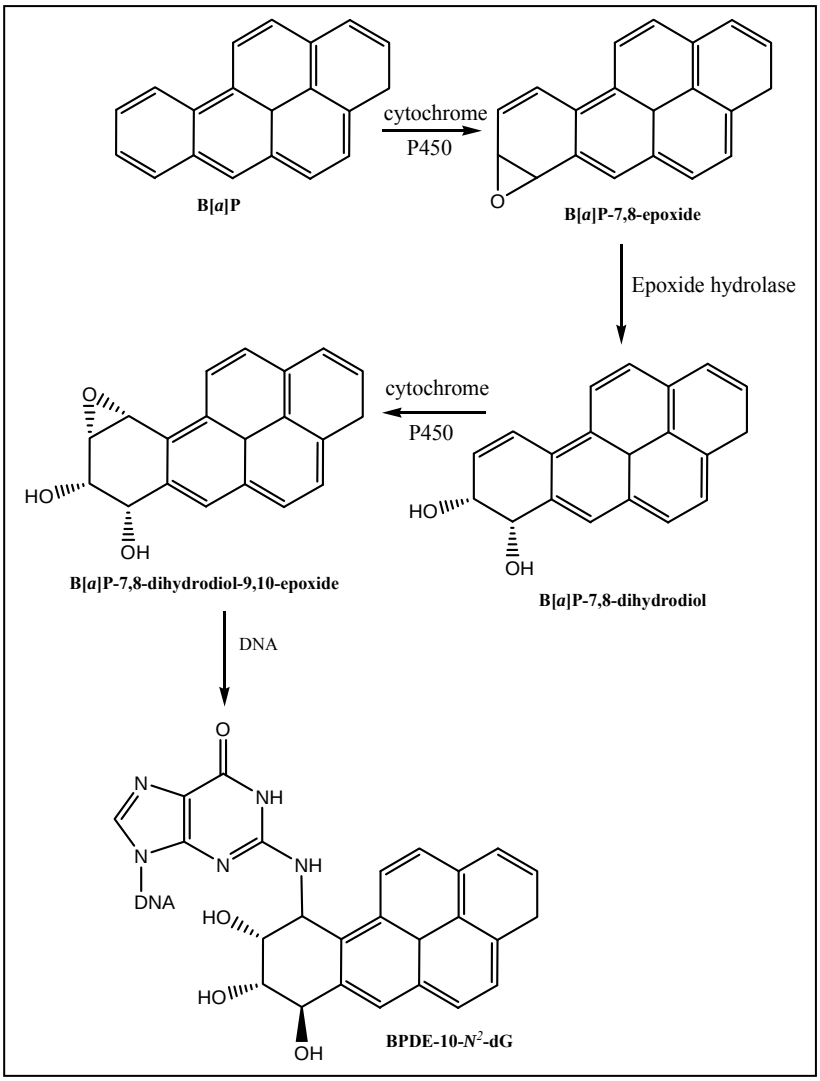


Figure 11: Proposed metabolic activation pathway of B[*a*]P [273]

One of the most strong and dangerous mutagens reported by Ames test and belonging to nitro-PAHs class is 3-nitrobenzanthrone (3-NBA) noting its mutagenic strength 10000 times more than B[*a*]P. This polycyclic aromatic nitro-ketone is mainly identified in air pollution, fuel combustion and soil particles [280, 281]. It is metabolically activated by different enzymes either *in vivo* or *in vitro* [282, 283] producing covalent DNA adducts [279] with subsequent mutations [284] mainly at purine sites [282] at the C8 and N^2 position of guanine and at the C8 and N^6 position of adenine [285]. Nitroreduction is the initial step of activation of 3-NBA to produce N-hydroxy-3-aminobenzanthrone (N-OH-3-ABA) then different pathways can be followed. N-OH-3-ABA is metabolised by O- acetyltransferase [286] or sulfotransferase [287] to produce N- nitrinium ion yielding non-acetylated DNA adducts [281] or it exerts N-acetylation [286] followed by O-esterification to produce N-acetyl-nitrinium ion yielding acetylated DNA adducts [288]. Or simply, it is reduced to 3-aminobenzanthrone that involved indirectly in the formation of both adduct types (**Fig. 12**). 3-NBA adducts were detected along *p53* mutations in Hupki mice as well as in lung tumours in mice [289] and rats [171].

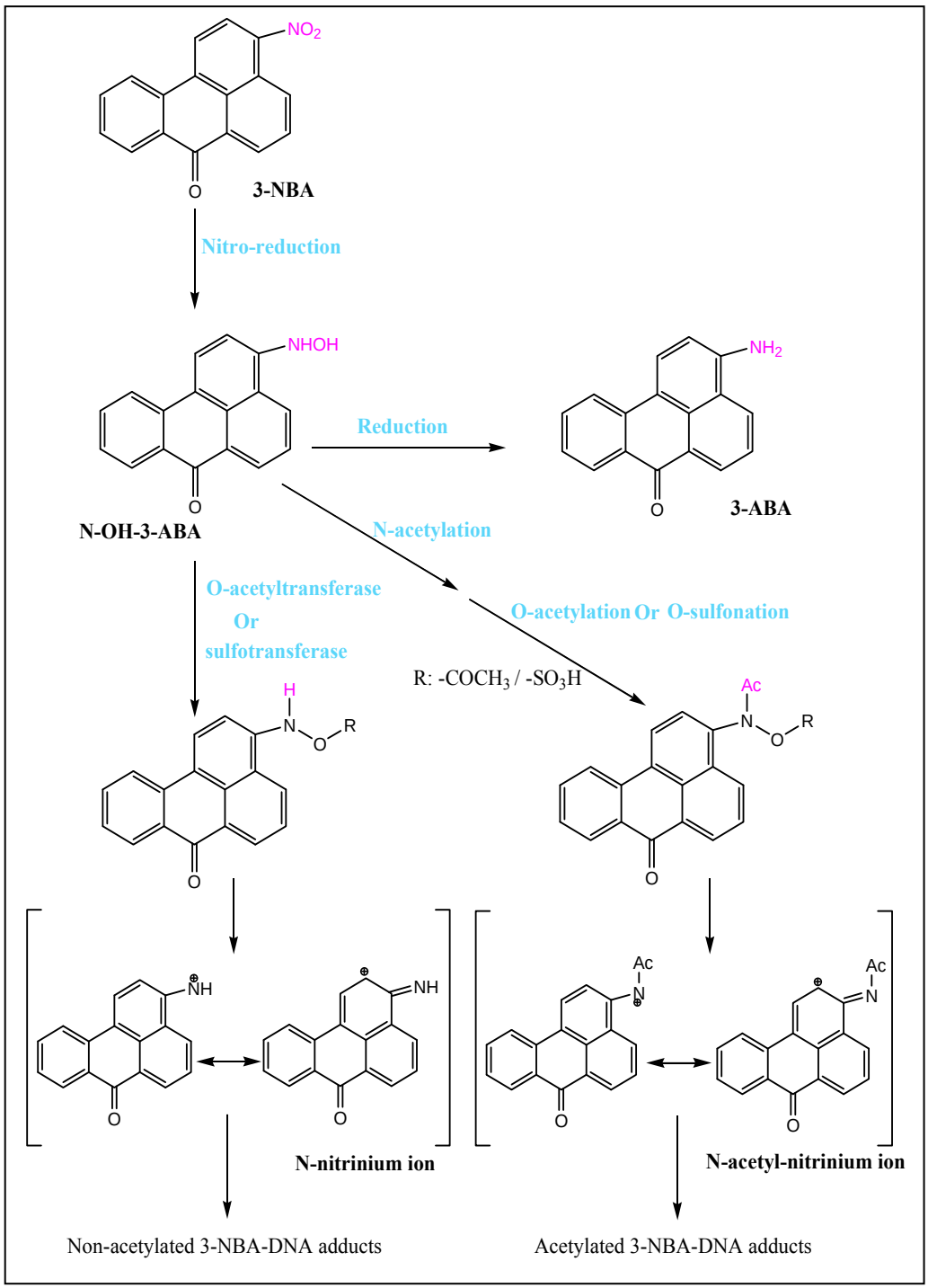


Figure 12: Proposed metabolic activation pathway of 3-NBA [281]

Heterocyclic aromatic amines (HAAs) represent the second equally important class of exogenous DNA adducts that contribute to human cancer risk [290]. They are formed in consequence of cooking foods at high temperatures and/or for long period of time [291], especially frying or grilling proteinaceous foods such as meat [291, 292], fish and poultry, as products of protein pyrolysis [293, 294]. They are also generated from occupational source [295], environmental source [296] and tobacco smoking [290, 297]. Many of these HAAs were used in laboratory experiments as well as color additives in pharmaceutical preparations and cosmetics, antioxidant in manu-

facturing of rubbers and plastics and finally in dyes and paints industry. Among 19 structurally determined HAAs [290], 4-Aminobiphenyl (4-ABP) is a major agent investigated for its role in the etiology of mainly bladder cancer [298, 299] and breast cancer [297, 300]. 4-ABP occurs in amounts of 0.2 and 140 ng per cigarette noting the formation of 4-ABP-hemoglobin adducts in smokers [301].

It is metabolically activated in a manner like 3-NBA by the cytochrome P450 [294] in liver to N-hydroxy-4 ABP which forms covalent DNA adduct and induce mutations at multiples sites [302] e.g. at the C8 position of guanine by their exocyclic amino group. The latter activated form enters the circulation and produce haemoglobin adducts or is further transported to urinary bladder to react either directly with DNA or after N-acetylation (**Fig. 13**). N-sulphation and N-glucorination are other reported metabolic activation pathways [303].

4-ABP is also associated with cancer in the fatty sites in the human body as adipose tissue of breast due to its lipophilic nature via its activation by peroxidises enzymes in the mammary gland following O-esterification pathway to produce adducts with DNA [304] (**Fig. 13**).

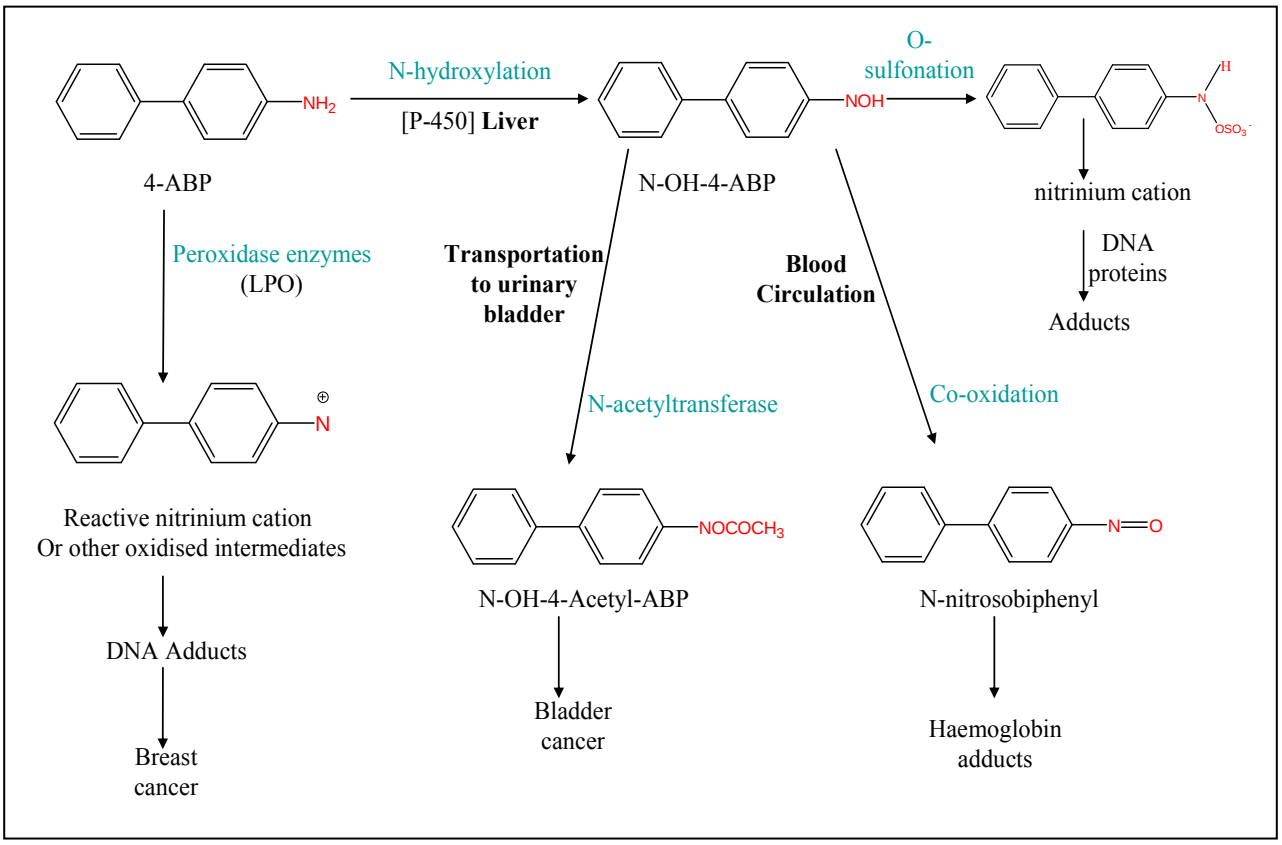


Figure 13: Proposed metabolic activation pathway of 4-ABP

1.3.3.2 Aristolochic acid (AA)

Not only the environmental polluants or food derivative heterocyclic amines form DNA adducts, but also some fungal products contribute to carcinogenesis [305] such as aflatoxin B1 which is a fungal metabolite produced by *Aspergillus flavus* and related fungi that grow on improperly stored foods [306-309]. In addition**,** aristolochic acid (AA) is a natural product recorded for its carcinogenicity.

Chinese herbs nephropathy (CHN) [310, 311] is a recently described subacute interstitial fibrosis which progress rapidly causing permanent kidney damage, including end-stage of kidney failure and upper urothelial cancer. It is named also AA nephropathy [312, 313] characterising nephrotoxicity disease which is associated with the prolonged use of chinese herbs in the form of slimming pills [312, 314-316]. This syndrome has been traced to aristolochic acid (AA) which is a major alkaloid of isochinoline group extracted from Aristolochica species [316] and other plant species. It is consisting of a mixture of structurally related nitrophenanthrene derivatives, AAI and AAII (**Fig. 14**). They form DNA adducts after metabolic activation by simple reduction of the nitro group [315, 316].

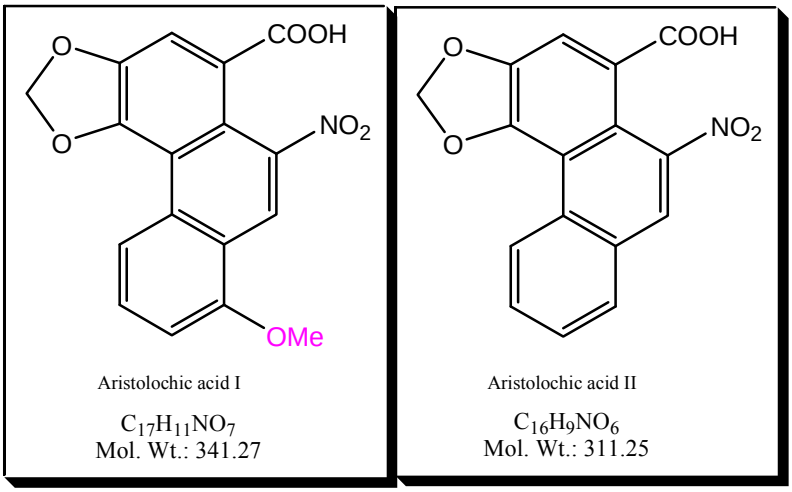


Figure 14: Chemical structures of aristolochic acid I (8-methoxy-6-nitro-phenanthro[3,4 - d][1,3]dioxole-5-carboxylic acid) and aristolochic acid II (6-nitro-phenanthro[3,4-d][1,3]dioxole-5-carboxylic acid)

Aristolochia is a large genus of plants with over 500 species belonging to the Birthwort family. At least 180 kinds of the plant family *Aristolochiaceae* are well-known over the world. It was of great interest by the ancient Egyptians, Greeks and Romans [317, 318]. Its nomenclature was developed from Greek words (Aristos: best) and (locheia: birth) meaning 'correct delivery'. In the Indian folk medicine, it was given to women in labour to expel the placenta [319,

[320]; but due to its poisonous effect, it can lead to the death of the mother. Also, it is highly used in traditional Chinese medicine (TCM) [321]. AA containing herbs were used for healing wounds due to the disinfectant effect of AA which drains off the fluid from the wound [316]. Consequently, the herbal drugs containing AA were used in the prophylaxis and treatment of snake bites [322]. It has anti-inflammatory effect [323] and was used for the therapy of chronic inflammations such as arthritis, gout and rheumatism [316, 324]. In 2001, FDA had released a warning to the consumers to stop using herbal medicines containing AA due to the history of diseases associated with the use of AA.

During the 1990s in Belgium, a nephrotoxicity syndrome was initially recorded in group of women after ingesting slimming pills. Then importantly, several cases of irreversible renal failure and malignant tumours of the urinary tract were reported. This was triggered by chinese herbal drug containing AA used during a slimming regimen [312] with different severity of reported cases depending on the taken dosage. This renal disorder was initially called Chinese Herbs Nephropathy (CHN), referring to the origin of herbs used in these remedies imported to Belgium [325, 326]. Then this syndrome was also named Aristolochic acid nephropaty (AAN) referring to its etiology.

Identical cases of AAN have been reported in other places out Belgium, especially in Asian countries, where traditional medicines are very popular [316, 326]. Later, more cases were reported in other European countries [311, 327] as well as Taiwan, China, Japan [311, 328] and in the USA.

The spread of the disease had initiated a stream of researches that had later supported these observations as identical renal interstitial fibrosis and urothelial malignancy were reported in rabbits and rats treated with AA [325, 329]. Finally, the similarity of etiological conditions between CHN and the case due to AA exposure such as tubular proteinuria, renal glucosuria, a progressive rise in serum creatinine [330, 331] and severe anemia at early stage with a great possibility of development of urothelial tumours [314] had lead to rename the CHN disease as Aristolochic acid nephropathy (AAN).

Based on clinical characteristics and morphological findings, AAN [321] has major similarities with another renal fibrosis disease called Balkan endemic nephropathy (BEN) [332, 333] noting the same causative factor later discovered for both diseases [321, 334]. BEN was recorded in the 1920s as the agriculture of *Aristolochia clematitis* was common in the region of Balkan Mountains along the Danube River basin. The oral intake of flour obtained from the growing wheat beside which *Aristolochia* species had grown could explain the exposure of the humans in the Balkan regions to AA due to contamination of bread with *Aristolochia* seeds [335]. Mor-

phologically, both diseases showed extensive hypocellular interstitial sclerosis, tubular atrophy, glomerulosclerosis and urothelial atypia associated with the possibility of malignant transformation of the urothelium [314, 336, 337]. Both are linked to the exposure to AA either through the intake of herbal remedies prepared from *Aristolochia* plants or the ingestion of contaminated wheat-made products.

Aristolochic acids are nephrotoxic and carcinogenic nitroaromatic compounds via the production of AA- DNA adducts which were detected in the kidney and ureter of AAN patients [338]. Different studies had reported AA as nephrotoxic, potent carcinogen in laboratory animals [339-342] and man [343] as well as genotoxic mutagen [341, 344, 345]. After enzymatic activation (cytochrome P450 and peroxidase) [346] aristolactams [347] are the activated metabolites formed by AA nitroreduction [348] and form major DNA adducts [349] through the attack of aristolactam-nitrinium ion to the exocyclic amino group of adenosine and guanosine [315, 349] (**Fig. 15**). The AA–DNA adducts can be used as potential biomarkers of AA toxicity [167].

Certain genes are associated with different mutations in response to AA exposure, such as TP53 [350]. This gene was found to be mutated in 50% of the recorded human cancers [351] and was recently identified in some AA-induced tumours [343, 351, 352]. The p53 mutational spectra of these cancers are dominated by the type of AT-TA transversions [284], resembling the mutational behaviour observed in urothelial cancer patient [353] as well as in H-ras oncogene in rodents exposed to AA dosage [354, 355].

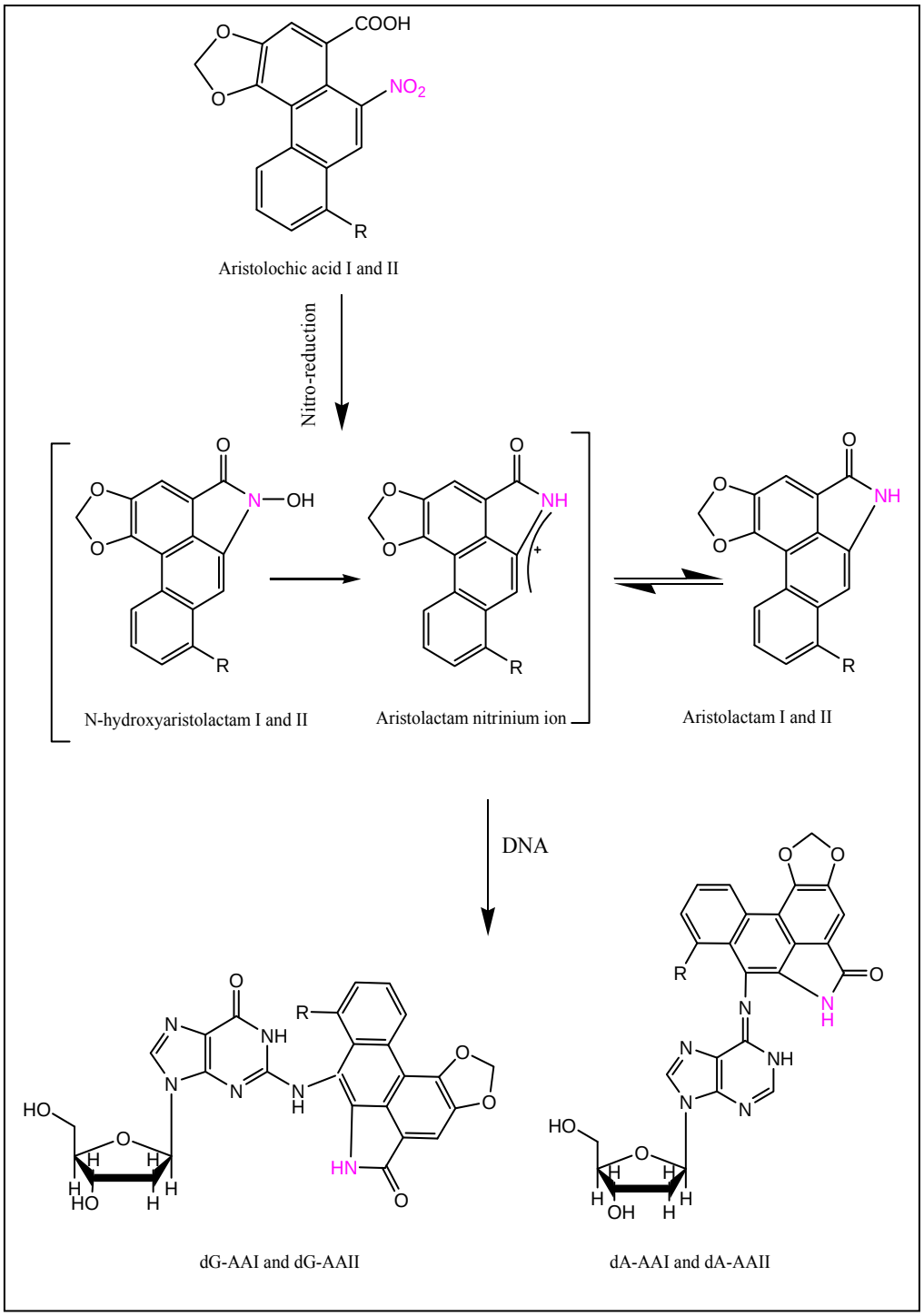


Figure 15: Mechanism of AA-DNA adducts formation (AAI, R : OCH3) and (AAII, R : H) [333]

1.3.3.3 Methods for the determination of AA

Various chromatographic methods were applied for the determination of aristolochic acid in various selected herbs and dietary slimming products such as HPLC [356-359] and TLC [360, 361]. CZE was successfully applied to determine AA in real samples of herbs and dietary natural products as an alternative method to HPLC due to its simplicity. CZE-cyclic voltametry

method had been used for the determination of AA in medicinal plants using phosphate and / or borate as separation buffers [332, 362]. Another CE method had been recently published to perform the analysis of AA within minutes without any sample pre-treatment [363]. The advantage of MEKC principles was also achieved by proposing an on-line concentration method using field-enhanced sample injection (FESI) mode for the detection of AAs in chinese medicine preparations [364]. Capillary electrophoresis with laser-induced fluorescence detection (CE-LIF) were also used for the analysis of the reduced AAs that fluoresce at 477 nm when excited at 405 nm using a solid-state blue laser [365]. Different detection principles, such as UV, MS [366] and NMR [367] were recorded for the analysis of AA in dietary slimming supplements too. DNA adducts formed by AAI and AA II were analysed *in vitro* and *in vivo* to study their contribution in cancer formation using ^{32}P- postlabelling method [348, 368-370].

1.4 Methods for determination of the DNA-methylation level

Some researchers were interested to set different techniques to study the occurrence of methyl cytosine and its localization in the genome. Others were interested to measure the level of methylcytosine in the genomic DNA either by high-performance separation techniques or by enzymatic or chemical means. Although the formers are more sensitive than the latter, enzymatic and chemical techniques are favoured due to their simplicity and cheapness.

1.4.1 Gene-specific methylation analysis methods

The analysis of gene specific methylation content implies initially a basic amplification of the target sequence. This is achieved by so-called polymerase chain reaction (PCR). Primers which are short DNA fragments possessing a sequence complementary to the target sequence and DNA polymerase enzymes which are the elemental components of this well-known technique. By applying PCR specifically to amplify sequence within CpGs, the information on 5-methylcytosine will be erased as DNA polymerases could not discriminate between cytosine and 5-methylcytosine. From here, all the following described methods for the analysis of gene specific methylation are focusing on modifying one of these bases to allow differentiation between them [371].

- Methylation Sensitive Restriction Endonucleases (MSREs)

The principle of this technique was obtained from the role of 5-meC in prokaryotes as defence mechanism against foreign DNA. Simply explained, the 'bacterial Restriction/modification' system is involving the methylation of the host DNA by DNA methyltransferase enzymes to protect it from restriction enzymes which check any DNA in the cell for this 'methylation signature' and cut the sequence that lacks the correct methylation pattern. In this way, most foreign DNA are identified and cleaved before its expression [372]. Isoschizomers restriction endonucleases have been used to study methylation changes within their specific recognition sites noting their different sensitivities to 5-meC. Essentially, one enzyme is insensitive to 5-meC (e.g.*Msp*I) but the other (e.g.*Hpa*II) does not cut DNA if its cleavage site is occupied by 5-meC (**Fig. 16**).

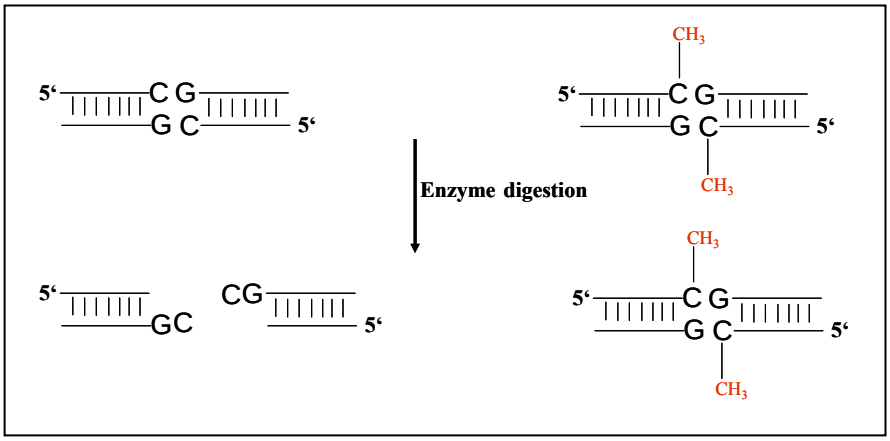


Figure 16: Gene-specific methylation analysis using MSREs [371]

The output of this simple technique studying the methylation status can be further identified by a southern hybridization gel-electrophoresis technique (MSRE-Southern analysis) involving the digestion of genomic DNA with the restriction enzyme *Hpa*II and in parallel, another part of genomic DNA is digested by *Msp*I enzyme [373]. The conclusion is identified from the size of the hybridising bands related to the digestion of both enzymes. If the bands are of the same size, then this sequence was not methylated. However, larger band detected with *Hpa*II means the sequence was methylated (**Fig. 17**). The drawback of the method is obvious in case of incomplete digestion leading to false positive results of restriction-enzyme based methods. Moreover, the technique is limited to specific restriction sites and it requires suitable amount of pure DNA. This technique can be coupled with PCR (MSRE-PCR) [373] to increase the sensitivity by 1000-fold revealing the amplification of the sequence site if it is not cleaved. The inability of restriction enzyme to digest methylated sequences yields longer fragments, indi-

cating a methylated CpG dinucleotide. The choice of the identification tools either southern blot techniques or PCR is dependent on the length of the digested DNA fragment.

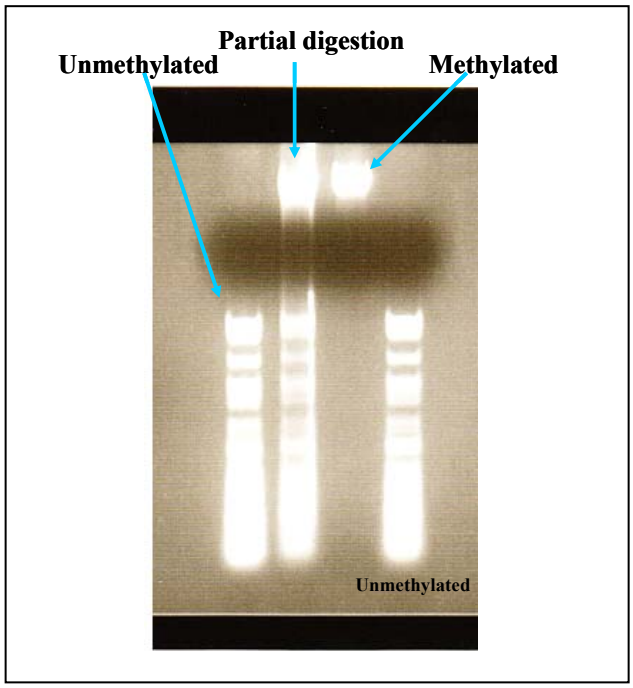


Figure 17: Representative model of combined MSRE-Southern Blot gel electrophoresis analysis

The electrophoresis was carried out in 0.8 % (w/v) Agarose in 1 X TAE buffer (triacetic acid + EDTA) + Ethidium bromide gel for one hour at 120 V using BioRad Mini –Sub Cell GT Gel electrophoresis apparatus. The resulting gel was dried and exposed to x-ray film (Polaroid MP-4 Land Camera; UV transluminator VVP San Gabriel USA) for 5 min.

- Sodium bisulphite reaction

Treatment of DNA with sodium bisulphite (Na_2SO_3) will result in a reaction between Na_2SO_3 and pyrimidine base [374-376]. The sulphonation occurs at position 6 of the pyrimidine ring [376, 377] and the reaction is reversible (**Fig. 18**). The amino group at position 4 of C and 5-meC becomes non stable by the attack of bisulphite group. Thus, the deamination of these bases occurs to either U or T, respectively [374, 376]. However, the kinetic rate of deamination reaction of both bases distinguishes between C and 5-meC in DNA as the former reaction is faster than the latter [378]. The reaction is principally dependent on the sequence differences due to deamination of unmethylated cytosines to uracil under conditions that methylated cytosines escape the reaction [374] (**Fig. 19**).

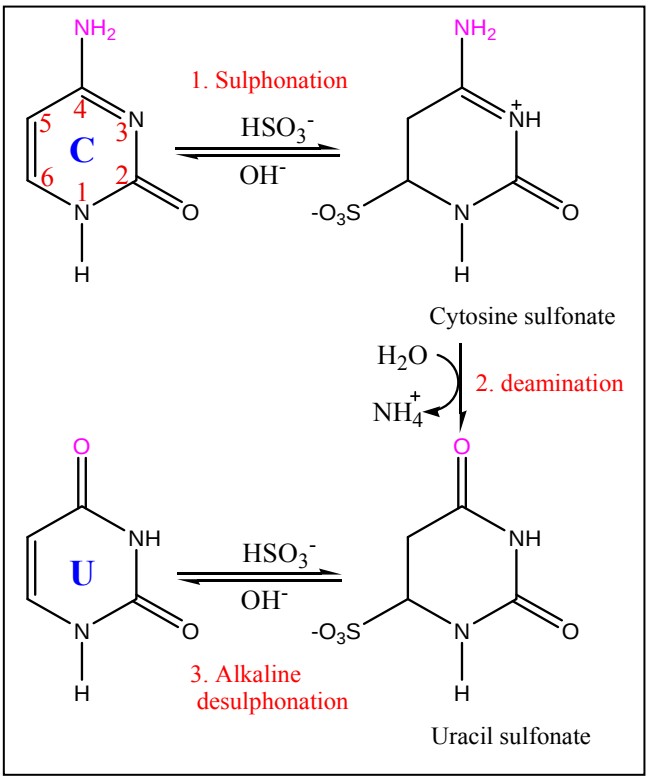


Figure 18: Bisulphite conversion reaction [374]

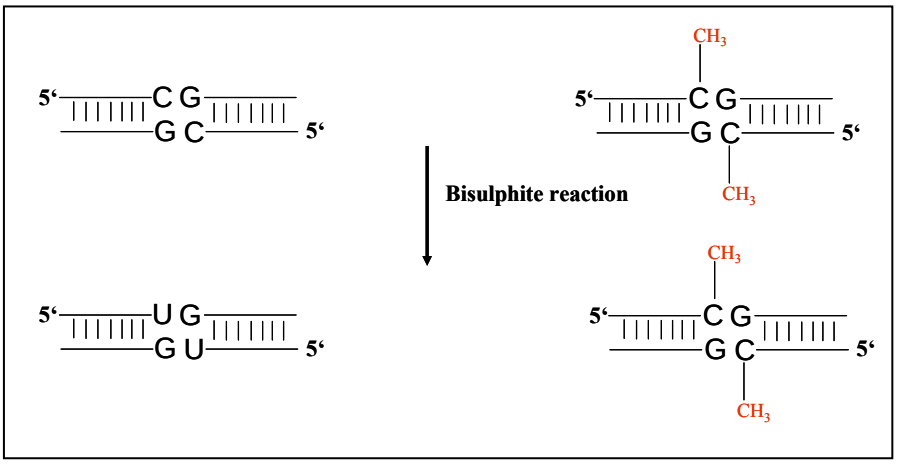


Figure 19: Gene specific methylation analysis using sodium bisulphite reaction [371]

The difference in methylation status revealed by bisulphite reactivity can be determined and quantified by PCR-based technique. Bisulfite sequencing was the universal methodology used in the last years providing accurate information about the methylation status. Its limitation is dependent on the incomplete conversion of C to U as SO_3^{2-} reacts only with cytosine bases not involved in base pairing [371].

Finally, other methods were literally cited, such as combined bisulphite restriction analysis (COBRA) [371, 379, 380], methylation specific PCR (MSP) [381], methylation sensitive single nu-

cleotide primer extension (Ms-SNuPE) [382, 383] whereas the analysis could be done by means of ion pair RP HPLC [383], capillary electrophoresis [384] or MALDI TOF [385], methyl light [386] and RNase-T1-MALDI-TOF [387] methods.

1.4.2 Genomic-wide methylation analysis methods

The analysis of genome wide methylation pattern can be performed directly on raw DNA samples after its isolation. Its principle is based on the determination of ratio between 5-methyl cytosine and cytosine.

- Instrumental analysis

Before the determination of DNA methylation by the proposed instrumental technique, it is highly required to hydrolyse the DNA molecule to its mononucleotides. This process is successfully done by the enzymatic hydrolysis of the genomic DNA by using firstly either Deoxyribonuclease I (DNase I), nuclease P1 [388] or snake venom phosphodiesterase [389] to produce deoxyribonucleotides, followed by alkaline phosphatase treatment. The finally produced deoxyribonucleosides are subsequently separated by standard reverse-phase HPLC [388, 390-394], TLC or CE [395, 396]. The separated deoxyribonucleosides can be identified by combining HPLC with UV [388, 389, 391, 394] as well as CE with UV [397] or by hyphenating HPLC-MS to increase the sensitivity [393].

HPLC is the oldest and preferred technique for the determination of the genome methylation levels of DNA [388]. But, HPLC method suffers from the known problems related to the elution buffer as well as the low amount available of DNA samples. HPLC-UV determination method requires at least 10 µg DNA sample, however 1 µg is required for CE-UV [397] and HPLC-ESI-MS [393] determination. Alternative method as TLC was prone to the lack of specificity.

In the last 10 years, a reproducible electrophoretic method was established and modified [398] for accurate determination of the methylation level [394] as well as for detection of DNA adducts [399]. CE-LIF technique had offered an opportunity to analyse 100 ng DNA sample [400] after its hydrolysis with micrococcal nuclease and phosphodiesterase II.

- Radioactive-mediated SssI methyltransferase assay

This assay is a simple modification of de novo methylation process [376] catalysed by the enzyme *Sss*I DNA methyltransferase using S-adenosyl methionine (SAM) as methyl donor. Its principle is depending on using the *Sss*I enzyme to transfer a tritium-labelled methyl group from SAM to unmethylated cytosines in CpG sites of genomic DNA [401, 402] (**Fig. 20**). The amount of radioactive incorporated labels can be quantified by a scintillation counter. The radioactivity is inversely proportional to the CpG methylation and can reverse the extent of global changes in methylation pattern. However, the variability of counts recorded from experiment to experiment and from one day analysis to another had influenced the use of this assay due to the instability of both SAM and the methyltransferase enzyme.

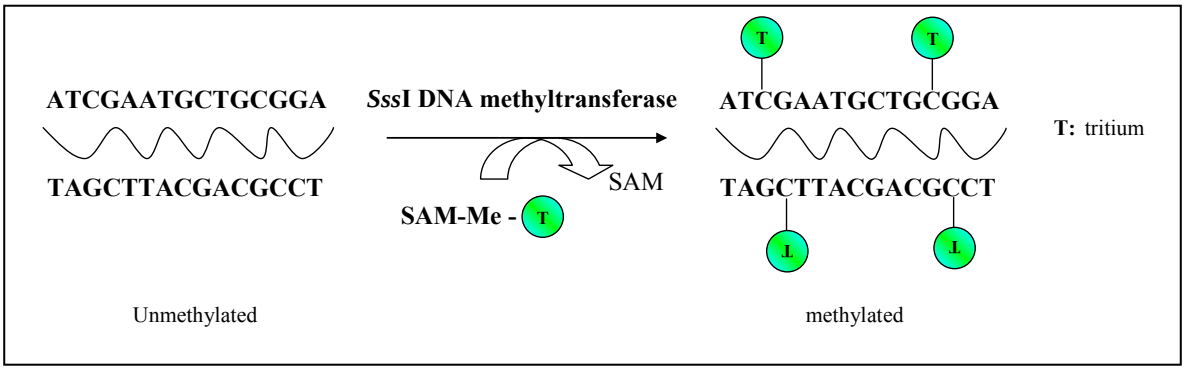


Figure 20: Pathway of radioactive-mediated methyltransferase assay.

- Chloracetaldehyde fluorescent assay

Oakeley *et al.* [403] had developed a method to monitor the changes in genome-wide methylation levels by labelling the modified DNA by fluorescent tags using chloracetaldehyde. The DNA was first depurinated by treatment with sulphuric acid, then the purine bases were removed by precipitation with silver or by column chromatography [404]. Then, the depurinated DNA was treated with sodium bisulphite that converts C into U leaving the 5-methylcytosine unreacted [378]. At the end, the sample was incubated with chloroacetaldehyde to produce the intensely fluorescent etheno-cytosine derivative of 5-meC, which was quantified by a fluorimeter. Thus, this method is providing a tool for the measurement of the level of 5-meC in the genome [403]. The chloracetaldehyde reaction–dependent assay is a good alternative to other methods used for determination of genome-wide methylation because of the non-fluorescence property of the chloracetaldehyde as well as the DNA molecule. However, the toxicity of chloracetaldehyde and the time consumed in this assay could limit its use. The

depurination is an important step to prevent the interference from the adenosine purine base by forming a fluorescent adduct after reaction with chloracetaldehyde.

1.4.3 Capillary Electrophoresis (CE)

Capillary electrophoresis is one of the most important instrumental techniques applied for the determination of genomic methylation level and specifically used in the research work performed in this thesis. It is a powerful separation technique performing the analysis dependently on a unique physicochemical characteristic 'electrophoretic mobility'. The term electrophoresis, of Greek origin, means electron (electron) and phoresis (carrying) and reflects the definition of the process as: the movement of ions under the influence of an electric field across buffer-filled capillaries. This phenomenon of electrophoretic transport in an electric field has been known by the pioneering work of Kohlrausch [405] in 1897. The history of the development of CE is briefly described in the appendix [405-410]. CE was popularised after introduction of narrow glass capillaries by Jorgenson and Lukacs enabling the use of high voltage with good detection of separated peaks [409-411]. Depending on the types of capillary and electrolyte used for the analysis, the CE technology can be categorised into several separation modes [Capillary Zone Electrophoresis (CZE) [412], Micellar Electrokinetic Chromatography (MEKC) [412, 413], Capillary Gel Electrophoresis (CGE), Capillary Isotachophoresis (CITP) [414-421], Capillary Isoelectric Focusing (CIEF) and Capillary Electrochromatography (CEC)] [422]. CE is a flexible technique compatible with several detection modes such as: UV, LIF, MS, amperometric, voltammetric and other detectors. CE promises to become an important analytical technique, complementary to HPLC. Its applicability covers a broad range of entities, from simple ions to high molecular weight species such as DNA and even intact living cells. The analysis of ionic species and separation of compounds such as chiral drugs, pesticides, amino acids, peptides, proteins, oligonucleotides and DNA restriction fragments [411, 423-428] could be performed with CE.

.

2 Aim and objectives of the study

The main objective of this work is the determination of genomic methylation level by capillary electrophoresis so as to better define the molecular-biological relationships associated with cancer and other cell abnormalities.

The first aim of this study is concerned with the assessment of the influence of oxidative damage on the methylation level. The CE-LIF method used in the whole work for the analysis of DNA modifications requires the presence of the phosphate group in the 3' position of 2'-deoxyribose of the nucleotide to be analysed. A synthesis of the modified 8-oxo-2'-deoxyguanosine-3'-monophosphate (8-oxo-dGMP) nucleotide, its characterisation with ESI-MS and its detection with CE-LIF should be realised.

The second aim is the determination of the change in DNA methylation level by CE-LIF in several DNA samples treated with carcinogens - aristolochic acid (AA), 3-nitro-benzanthrone (3-NBA), benzo[*a*]pyrene B[*a*]P and 4-aminobiphenyl (4-ABP) - in either *in-vivo* or *in-vitro* studies in order to find whether there is a correlation between the methylation level and the carcinogen-adduct formation.

Finally, a great part of this work focuses on the determination of the DNA methylation level in clones and twins to clarify some epigenetic approaches toward cloning. Successful somatic cell nuclear transfer (SCNT) cloning is compatible with the birth of live offspring in a wide range of mammalian species; however the low overall efficiency of the technology could be related to the failure of epigenetic reprogramming. Clones that survive into adulthood, in contrast, are judged to be normal, and the epigenetic marks required for their normal development are assumed to have been retained; however, the epigenetic status of such healthy adult clones has never been investigated. The aim of this study is to evaluate the genome-wide 5-meC levels in healthy adult female SCNT clones generated from different genotypes of the Holstein and Simmental cattle breeds. In parallel, the genome wide 5-meC levels of experimentally generated monozygotic (MZ) twins will be investigated. MZ twins are chosen as comparative models in the study because their methylation status is highly similar in adult humans across populations.

3 Results and Discussion

3.1 Spectrophotometeric determination of DNA concentration

The concentration of all DNA samples which were provided and analysed in the course of this work was adjusted to give the appropriate quantity for each study and was either 1 or 10 µg. This adjustment was accomplished spectrophotometrically. Each dried DNA sample was dissolved in 500 µL distilled water. A transmission spectrum of the solution was recorded against water in the wavelength region from 200 to 800 nm (See **Appendix. Fig. 58**). DNA shows a relevant absorbance in the UV range. The computation of the DNA concentration [µg/mL] was calculated by the Lambert Beer law with the extinction value at 260 nm. The purity of the DNA was calculated from the relationship of the difference of the extinction values at 260 nm and 320 nm to the extinction value at 280 nm.

3.2 Determination of genome wide methylation level with CE-LIF

Schmitz *et al*. [395, 400, 429-432] had developed a sensitive method for the determination of the genome-wide methylation level in real samples by CE-LIF. This method was used in all the analyses done in this work. The method involved the hydrolysis of the DNA extracted from different tissues to modified or unmodified nucleotides (2'-deoxynucleoside-3'-monophosphate), the derivatisation of the product with BODIPY® FL EDA as fluorescence marker, and then the analysis by micellar electrokinetic chromatography using laser induced fluorescence detector (**Fig. 21**).

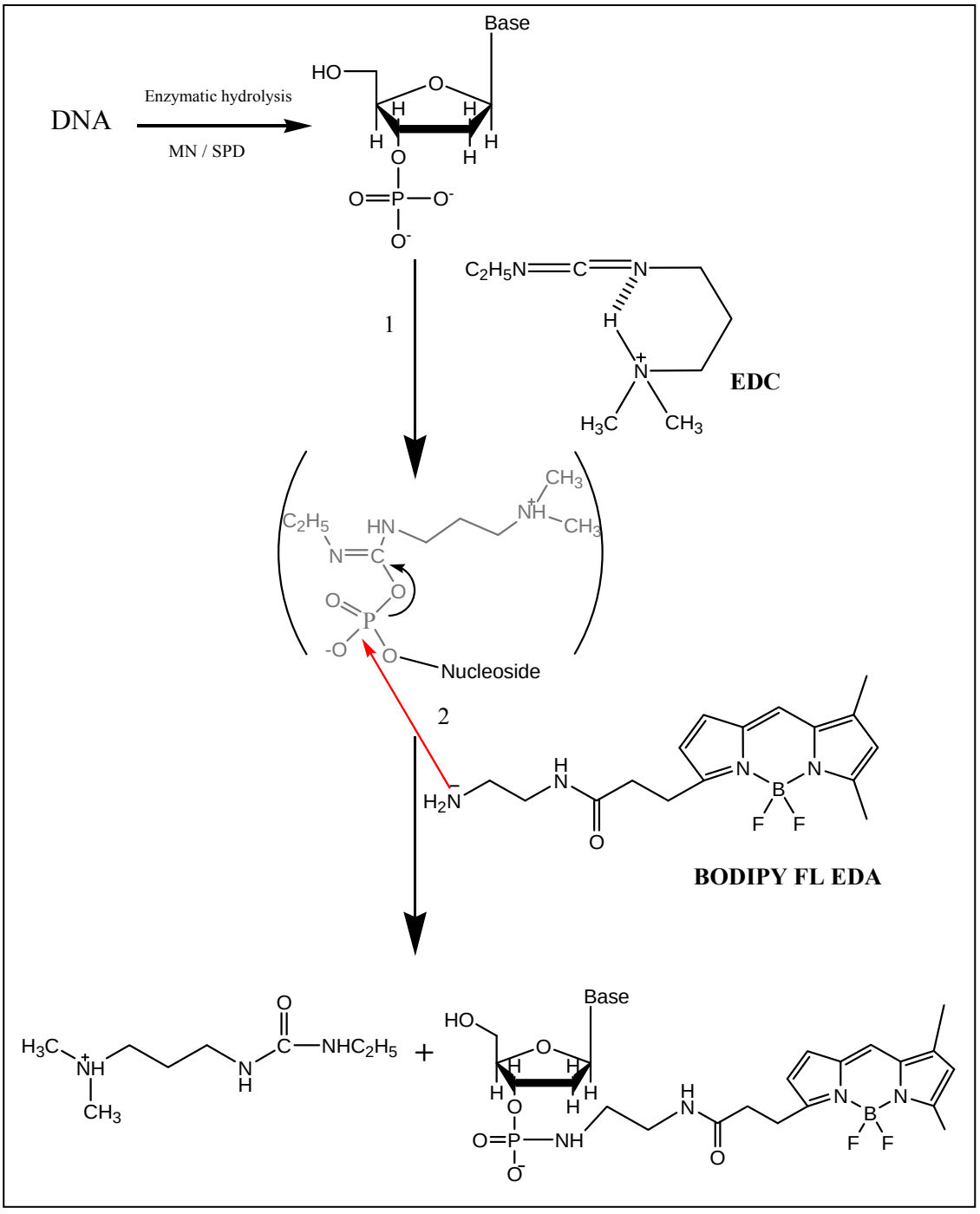


Figure 21: **Outline of the procedure used for the analysis of DNA modifications by means of CE-LIF**

1: Formation of the intermediate product by activation of phosphate moiety of 2'-deoxynucleotide with EDC.

2: Nucleophilic attack by the primary amine of BODIPY on the activated phosphorus moiety.

A mixture of the enzymes micrococcal nuclease (MN: endo- exonuclease enzyme) and spleen phosphodiesterase (SPD: exonuclease enzyme) was used to digest DNA to yield mononucleotides with terminal 3'-phosphates [433, 434]. Calcium ions were included in the hydrolysis buffer

in order to offer a suitable medium for the activity of MN then the hydrolysis took place for 3 h at 37°C.

The fluorescence derivatisation of mononucleotides was done with BODIPY FL EDA hydrochloride to allow detection of non-fluorescing analytes. The spectroscopic characteristics of BODIPY (maximum absorption of 503 nm and maximum emission of 510 nm) make this marker suitable for use with the two types of lasers utilised in this study (wave laser excitation at 488 nm) [435] and also overcome any interference by native fluorescent matrix compounds [432].

The covalent coupling of the fluorescence marker to mononucleotides took place only in presence of water-soluble carbodiimide, as this is essential for the activation of phosphate moiety of mononucleotide toward nucleophilic attack by the amino group of the fluorescence marker [436]. At neutral pH, EDC (1-ethyl-3-(3'-*N,N'*-dimethylaminopropyl)-carbodiimide hydrochloride) possesses a strongly basic tertiary amino group which can be activated through intramolecular proton transfer from the tertiary nitrogen atom to the imide nitrogen [436]. The phosphoamidate is then formed by the nucleophilic attack of the amino linker of BODIPY on the phosphorus of the activated phosphate group (**Fig. 21**). However, this derivatisation requirement excludes the use of fluorescence markers or buffer systems with carboxylic acid, primary amino, or phosphate groups [437]. Therefore, N-(2-hydroxyethyl)-piperazine-N-2-ethane sulfonic acid (HEPES) was the only suitable buffer system used for the derivatisation [399].

At the end, a precipitation reaction was mandatory for the stabilisation of the samples to get rid of the excess of added BODIPY and EDC. It must be mentioned that the procedure for the derivatisation reaction had called for the use of EDC in excess because some of it is lost by reaction with water to a urea derivative, and BODIPY must also be used in excess to ensure high reaction yield [398]. However, this excess of reagents led to great interference from fluorescent signals of BODIPY decomposition products [432].

Sodium phosphate was chosen as a separation buffer because its high buffer capacity at pH 9.0 is suitable for a high EOF. The data are displayed as an electropherogram that reports detector response as a function of time. A representative electropherogram of the analysed CT-DNA sample (10 μg) is illustrated in **figure 22**. The separated chemical species appeared as peaks with different migration times in the electropherogram, i.e., five major signals of the derivatised nucleotides in the following order: 2'-deoxyadenosine-3'-monophosphate (dAMP), 2'-deoxyguanosine-3'-monophosphate (dGMP), thymidine-3'-monophosphate (dTMP), 2'-deoxycytidine-3'-monophosphate (dCMP) and 5'-methyl-2'-deoxycytidine-3'-

monophosphate (5-me-dCMP). In the early part of the electropherogram, an oligo-zone appears, representing DNA which was not completely digested by the enzyme mixture (MN/SPD). The oligo-zone is dependent on the quantity of SPD used in the enzyme mixture as well as the purity of each particular enzyme charge [398].

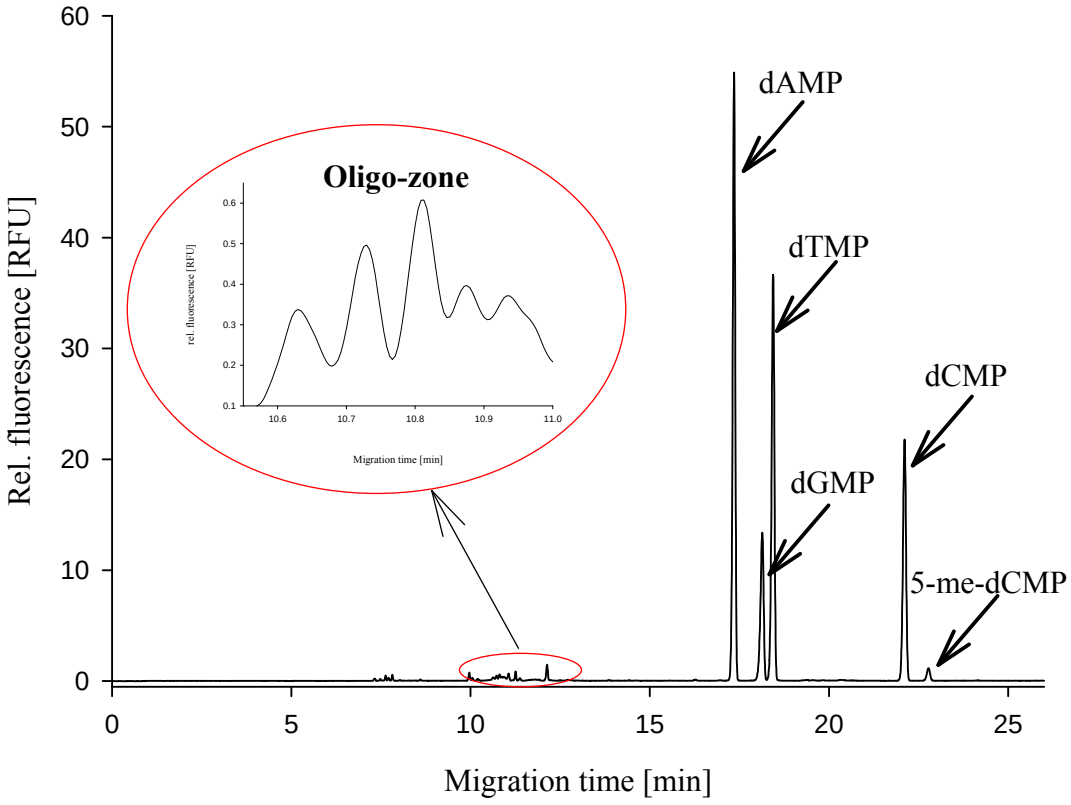


Figure 22: Representative electropherogram for the determination of 5-methylcytosine in a 10 µg DNA sample by CE-LIF. MEKC operating conditions: separation voltage: 20 kV; T: 20°C; running buffer: 90 mM SDS in 18 mM sodium phosphate buffer (pH 9.0) containing 10 % v/v methanol; fused-silica capillary (L_T =50 cm; L_D =40 cm; ID =50 mm).

The methylation level was determined from the following equation:

$$\%5-\mathrm{meC} = \frac{\mathrm{meC} \bullet f_1}{\left(\mathrm{meC} \bullet f_1 + \mathrm{C} \bullet f_2\right)} \times 100 \qquad (1)$$

Where % 5-meC: methylation level
meC: corrected peak area of 5-me-dCMP
C : corrected peak area of dCMP
f_1 : correction factor of 5-me-dCMP
f_2 : correction factor of dCMP

Integrated peak areas were used for the routine determination of methylation level [438, 439]. The correction factor was determined in our group [398] and previously by Stach *et al.* [395] by the analysis of Lambda DNA (unmethylated and enzymatically methylated). This correction factor was calculated to be 0.678 ± 0.001 S.D. for dAMP, 1.761 ± 0.001 S.D. for dGMP, 0.980 ± 0.001 S.D. for TMP, 1.075± 0.006 S.D.for dCMP (f_2) and 0.910 ± 0.04 S.D. for 5-me-dCMP (f_1) [398].

3.3 Enhancement of the sensitivity of the CE-LIF method

3.3.1 Comparison of two different LIF-detectors

The sensitive laser-induced-fluorescence detector is the main detection system on which our laboratory is dependent for all the CE analyses. However, two detectors are used the argon-ion laser (λ_{em} = 488 nm) and the Sapphire ™ solid laser.
First of all, a comparison study was done to choose the more sensitive detector.

- Argon-Ion laser

In different parts of the whole work, the argon-ion laser with power output of 10 mW was used for determination of the methylation level (**Fig. 23**).
The laser consists of a tube filled with the noble gas argon at a pressure of approximately 1 mbar as the laser gain medium. In order to generate the blue laser light, the gas is exposed to an electrical discharge. The Ar^+ and Ar^{2+} ions are produced by this electrical load and pass through a laser transition to a lower steady state [440, 441].

Figure 23: Picture of Beckman Coulter™ Argon-Ion Laser

- Sapphire ™ solid laser

The Sapphire ™ solid laser possesses a maximum power output of 20 mW. **Figure 24** shows the solid laser with its control unit. The laser is placed on a thermoelectric cooler, which ensures an effective removal of the excess heat developed and also allows a stabilizing state for the diode laser and the resonator (442).

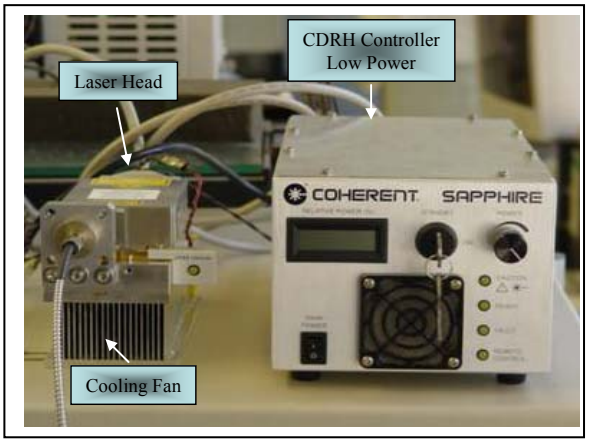


Figure 24: Picture of Sapphire solid laser (left) and the control unit (right)

The Sapphire ™ solid laser belongs to the class of 'Optically Pumped Semiconductor Lasers' (OPSL) class. This is similar to the group 'Vertical-external-cavity surface-emitting of Laser' (VECSEL). The difference between the two groups is that VECSELs are electrically pumped while the Sapphire ™ solid laser works with optical energy. The optical pumping has the advantage of producing higher power outputs than the power produced by an injection current-dependent laser (442).

A laser is constructed of three components. The energy source, referred to as the pump source or diode, provides energy to the laser system. A gain medium, the major determining factor of the wavelength of operation, is excited by the pump source to produce a spontaneous and stimulated emission of photons leading to the amplification or optical gain. The optical resonator has two parallel mirrors (focusing optics in OPS); one functions as a high reflector and the other as a partial reflector for the fundamental wavelength of the OPS.

The heart of this laser is an OPS chip (Optically Pumped Semiconductor) that functions not only as laser medium but also as one of the final mirrors of the linear resonator. The output beam of the pump diode is focused onto the OPS chip by a lens. The light from the medium, produced by spontaneous emission, is reflected by the mirrors back into the medium, where it may be amplified by stimulated emission. The light may reflect from the mirrors and thus pass through the gain medium many hundreds of times before exiting the cavity. A curved

mirror that is externally arranged completes the resonator for the IR radiation [443]. **Figure 25** shows schematically the structure of the resonator containing the gain material (OPS-chip), a frequency-doubling crystal and the output coupler.

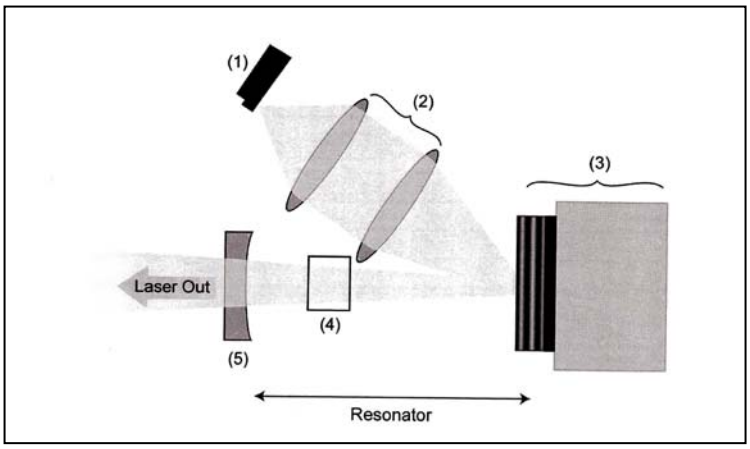


Figure 25: Schematic diagram of optically pumped semiconductor laser (OPSL) [443]. It is composed of: 1. Pump diode; 2. Focusing optics; 3. OPS chip**;** 4. Doubling crystal; 5. Output coupler

The pumping radiation of the 808 nm diode laser focuses on the OPS chip. The radiation is absorbed by the designed structure of the chip (**Fig. 25**). Thus the OPS chip, as laser medium, strengthens the IR wavelength of 976 nm. A non-linear crystal doubles the frequency of the IR light to a wavelength of 488 nm [442]. The blue beam passes through lenses and exits through the window of the output coupler.

In order to achieve the required detection limit of DNA adducts, a sensitive detection system and/or an off-line or on-line enrichment concentration technique is required. Therefore, it was necessary to study the characteristics of the Sapphire ™ solid laser and to compare its sensitivity with the Beckman laser (Argon ion laser) which was normally used.

Basically, the Sapphire laser already has advantages over the argon laser. The Sapphire ™ solid laser has a life time eight times that of the argon laser [444]. In addition to this economic advantage of the former laser, the Sapphire ™ solid laser is relatively small in size which is considered an important advantage in the practical work because it is easy either to transfer of the laser (if it should be necessary for the work to couple it with other CE equipment in the laboratory) or to re-build its units. In order to reach a good decision for the choice of lasers, the two lasers: Sapphire laser and Beckman Laser were tested for their sensitivity. Therefore, the signal to noise ratios of the lasers were first determined then compared, and comparative measurements of standard samples were also performed.

3.3.1.1 Determination of signal to noise ratio

Before the two lasers could be compared, the intensity of the Sapphire ™ solid laser had to be adjusted to be the same as that of the Beckman Coulter ™ argon ion laser. This was done by analysing the Beckman Coulter test solution (fluorescein solution) as a test solution with the argon-ion laser. Then, the same test solution was measured with the Sapphire ™ solid laser.
The power output of the control unit of the solid state laser was adjusted to deliver the same signal intensity as obtained from the Beckman laser.
An equivalency of the two signals was reached with a power output of 16 mW. Both lasers experience a power loss within the light conductor as well as within the inter-connections. For example, the power loss of the Beckman laser amounted to 6.6 mW (of 10 mW to 3.4 mW) within the light conductor and 2 mW (of 3.4 mW to 1.4 mW) in the inter-connections [444].
After the adjustment of the lasers, the analysis of hydrolysed and derivatised standard CT-DNA was performed in order to compare accurately the two lasers and the application for the analysis of real samples. The results showed good agreement between the two lasers. The methylation level of the standard samples deviated less than 1 % between the two lasers [Beckman Coulter ™ laser 6.07 % ± 0.08 S.D. (n=10) and Sapphire ™ laser 6.06 % ± 0.15 S.D. (n=12)].
The signal to noise ratio (S/N) is an important measurement that should be defined for the detector. For the determination of S/N ratio, the fluorescein solution was analysed by both lasers with sodium tetraborate buffer (20 mM, pH 7.0) for separation. A signal was obtained after approximately 4-6 min, and the S/N ratio was computed according to the following formula **2** [445]:

$$\frac{S}{N} = \frac{2H}{h_n} \qquad (2)$$

Where S/N: signal to noise ratio
H : peak height of the signal (equivalent to RFU)
h_n : height of the noise

In order to identify the noise level, the width of the fluorescein signal at its half length was recorded ($b_{1/2}$). The multiplication of $b_{1/2}$ by 20 resulted in the noise range (t_n) (**Fig. 26A**).

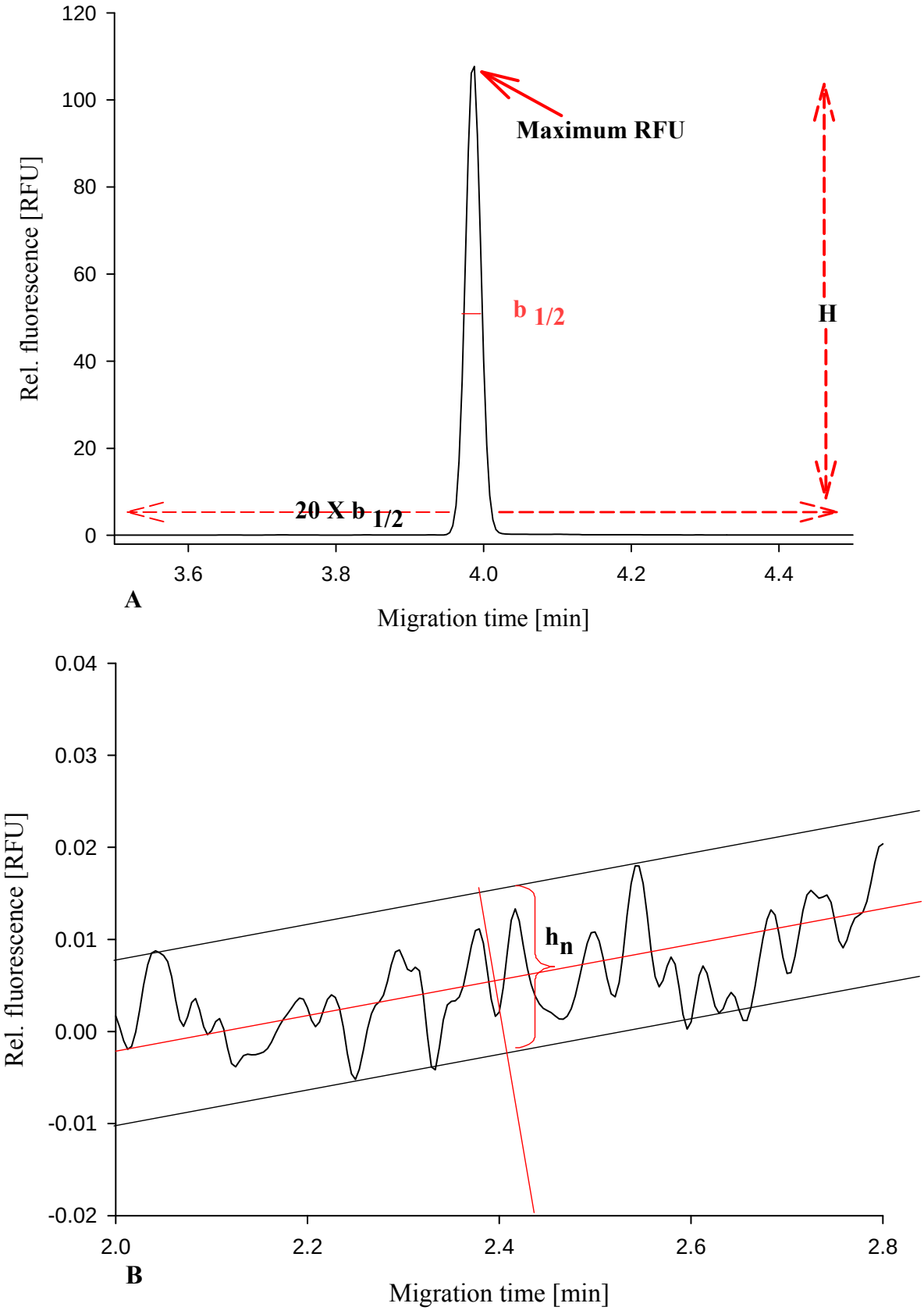


Figure 26 (A and B): Electropherogram of fluorescein solution to test the sensitivity of laser induced fluorescence detector. It shows the calculation of signal (A) to noise (B) ratio (S/N)

In this range, two lines are drawn through the highest peaks points and lowest through points. Then at half distance of t_n, a perpendicular line is drawn two peak-through lines, and then the point in the middle distance at y axis is taken to calculate accurately h_n (**Fig. 26B**).
The signal height and the noise strength were measured and the lasers could thus be compared objectively with one another. **Table 1** shows the calculated S/N ratios of the two lasers.
The results revealed that the two lasers deviate slightly from each other in the S/N relationship. The Sapphire ™ solid laser has a better S/N relationship and therefore gives higher sensitivity than the Beckman ™ argon ion laser.

Laser Type	Signal to noise ratio (S/N) ± S.D.	n
Sapphire ™ solid laser	35903 ± 2682	3
Beckman Coulter ™ Argon-Ion laser	31945 ± 835	3

Table 1: Signal to noise ratios of Sapphire ™ solid laser and Beckman ™ Coulter Argon-Ion laser

3.3.1.2 Reproducibility of the measurements

With the exchange of a fundamental component of the established MEKC-LIF system, the reproducibility of the methodology had to be tested. Two aliquots of the standard CT-DNA were hydrolysed and derivatised and each was analysed six to eight times with the Sapphire solid laser. The methylation levels determined are listed in **table 2**. The results of the analyses showed a very small deviation from each other (S.D. < 1.41 %). This confirms the reproducibility of the method after exchanging the laser detectors.

Sample	Methylation level (%) ± S.D.	n
CT (1)	6.20 ± 0.07	8
CT (2)	6.22 ± 0.07	6

Table 2: Determination of the methylation level of CT-DNA samples (10 µg) using Sapphire ™ solid laser

In summary, both laser systems, the solid laser and the argon ion laser, were coupled with CE equipment and tested for S/N ratios. The results confirmed the benefit of replacing of argon-ion laser by the Sapphire solid laser to be used with CE for its higher sensitivity. Also, the reproducibility of the measurements recommended the use of the Sapphire laser. In addition, the longer life time of the solid laser is definitely a profit, and the analyses costs can be drastically lowered.

3.4 Synthesis of 8-oxo-2'-deoxyguanosine-3'-monophosphate (8-oxo-dGMP)

The imbalance occurring in the triangle of oxidants, antioxidant systems and DNA repair mechanisms favours the status of oxidative stress. Among 20 major defined oxidative DNA adducts [260, 446], the 8-oxo-2'-deoxyguanosine adduct is the most investigated oxidative modification in DNA. It is associated with specific cellular repair mechanism (DNA glycolase enzyme: OGG1) [255]. Therefore, its existence in urine as a repair product could be used as a biomarker for some inflammatory diseases and to lesser extent for carcinogenesis. Its presence in diverse cancer sites, however, had suggested the role of oxidative stress in the initiation of carcinogenesis. Oxidative stress and aberrant CpG methylation are each associated with carcinogenesis. This makes it of interest to find whether there is a correlation between oxidative stress and DNA methylation. Firstly, it was important to synthesise 8-oxo-2'-deoxyguanosine-3'-monophosphate (8-oxo-dGMP) because the analysis of DNA adducts by the proposed CE-LIF method needs the availability of well-characterised standards of these adducts in the form of 2'-deoxynucleoside-3'-monophosphates. Unfortunately, very few DNA adducts are commercially provided in this form. Therefore, different synthesis routes to 8-oxo-dGMP were tried before its detection and the determination of methylation level in real cancer-mediated samples were undertaken.

3.4.1 Optimisation of the synthesis

Because guanine is the nucleobase most prone to oxidation [447], various synthesis pathways were tried. First, 25 or 100 µg DNA was dissolved in 330 µL of the freshly prepared ferrous sulphate reagent, 1 mM $FeSO_4 \cdot 7\ H_2O$ in 10 mM ammonium acetate that was adjusted to pH 4 with diluted acetic acid buffer, then treated with 13.8 µL of 35 % H_2O_2. A purification step was done in accordance with the Qiagen genomic DNA purification protocols appropriate to

the amount of DNA. Unfortunately, the purification was not successful. Therefore, a second synthesis pathway was tried using 1 mM hydrogen peroxide with different amounts of 2'- deoxyguanosine-3'-monophosphate (10, 20 and 100 µg) as starting material. The reaction mixture was shaken for 6 hours at 25°C in a covered thermomixer, and kept overnight in the dark at the same temperature without shaking. The solutions were then dried using speed vacuum, derivatised with BODIPY and precipitated for CE-LIF analysis. Since the analysis of 8-oxo-dGMP seemed to be very difficult with the CE-LIF due to the unavailability of an internal standard, the reaction was compared with blank sample using water instead of hydrogen peroxide. No difference was observed revealing the detection of a single peak related to the starting material. Moreover, the relative fluorescence units (RFU) were found to be (25, 50 and 153 units) for 10, 20 and 100 µg of 3'-dGMP, respectively (**Fig. 27**).

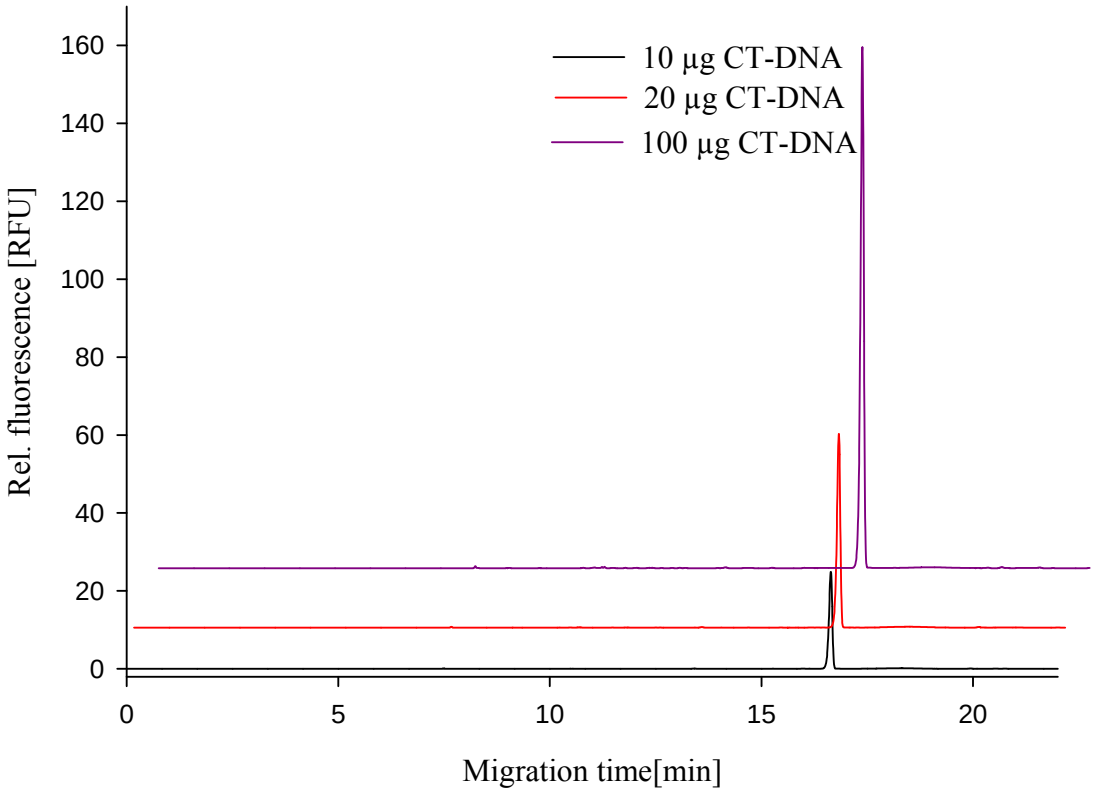


Figure 27: CE-LIF analyses of 10 µg, 20 µg and 100 µg of dGMP treated with 1 mM hydrogen peroxide. Analysis was done after hydrolysis, derivatisation, precipitation and dilution by a factor 1:100. MEKC operating conditions: separation voltage: 20 kV; T: 20°C; running buffer: 90 mM SDS in 18 mM sodium phosphate buffer (pH 9.0) containing 10 % v/v methanol; fused-silica capillary (L_T =50 cm; L_D =40 cm; ID =50 mm).

Different portions of the base line of electropherograms of 10 µg dGMP in presence of either peroxide or water were expanded and showed no significant difference in the base line (**Fig. 28**). The CE measurements were performed either directly after sample preparation or after 24 h and no extra-peak could be detected to prove the success of the oxidation reaction. The electropherograms in **figures 27** and **28** are representative of immediate measurements.

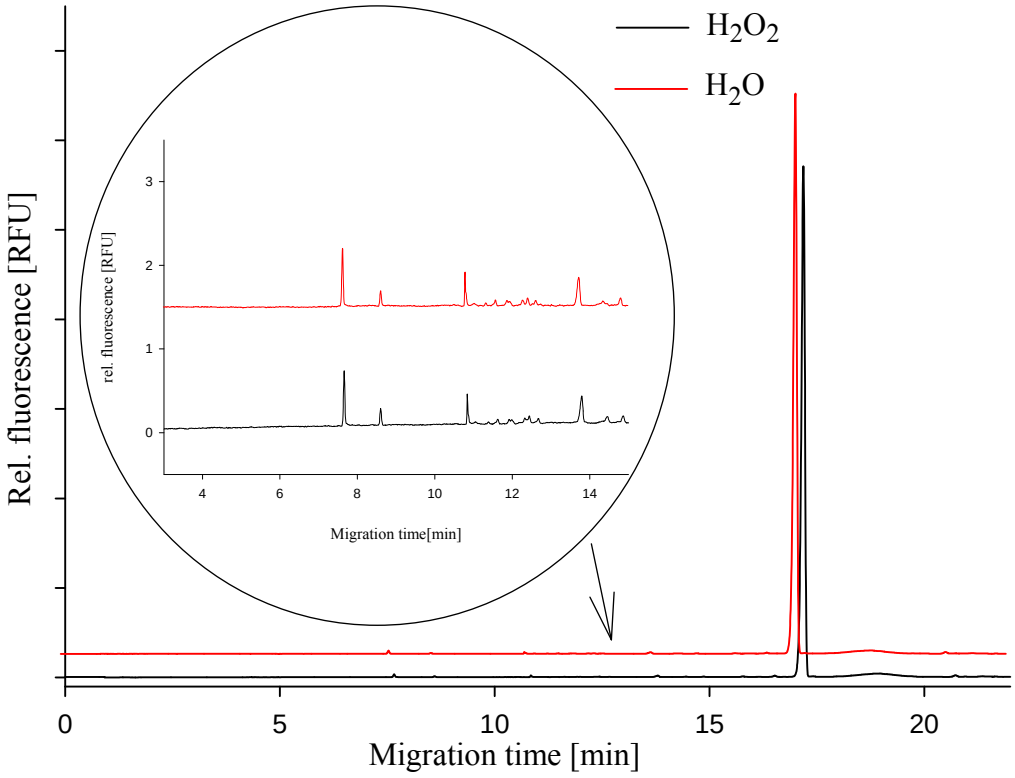


Figure 28: CE-LIF analyses of 10 µg dGMP treated with hydrogen peroxide or water as control sample. Analysis was done after hydrolysis, derivatisation, precipitation, and without dilution. MEKC operating conditions: the same as previously mentioned.

The simple correlation in **figure 27** and the comparison in **figure 28** had revealed that the starting material 2'-deoxyguanosine-3'-monophosphate (3'-dGMP) was not oxidised by H_2O_2. This suggested pathway was not successful until a catalyst was used because H_2O_2, despite its strong oxidative property, has no hydroxylation activity [448].

The method of Schuler *et al.* [258] is another synthesis scheme which we applied to the synthesis of 8-oxo-dGMP with the substitution of $CuSO_4$ for $FeSO_4$. 2'-Deoxyguanosine-3'-monophosphate was used as the starting material in presence of hydrogen peroxide, copper sulphate and sodium ascorbate to synthesise 8-oxo-dGMP (**Fig. 29**).

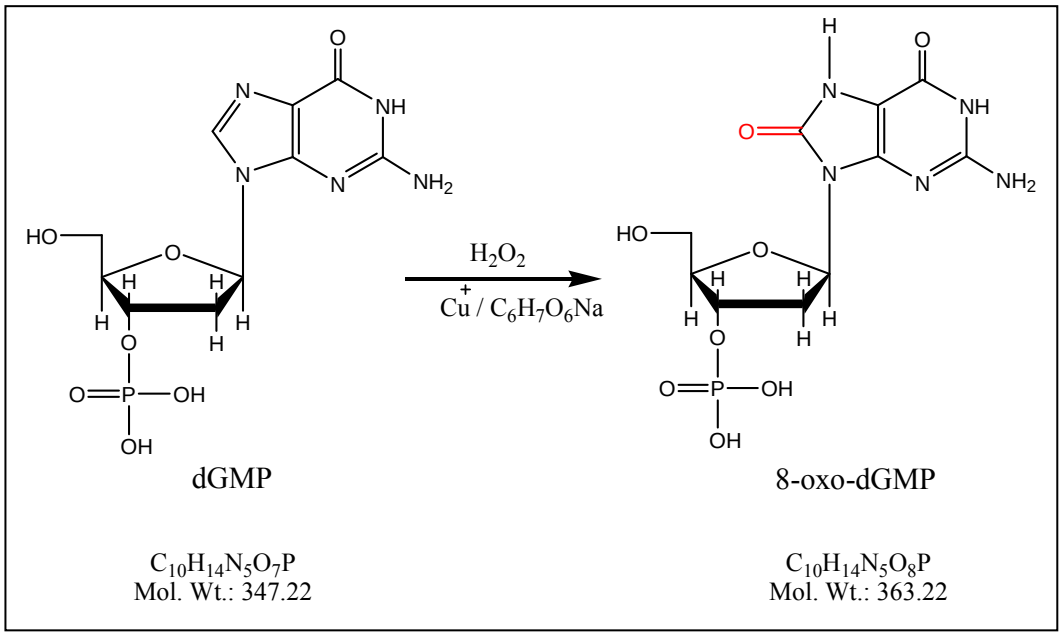


Figure 29: Proposed scheme of 8-oxo-2'-deoxyguanosine-3'-monophosphate synthesis

Different reaction mechanisms were followed to synthesise the 8-hydroxy-2'-deoxyguanosine (8-OH-dG). The Udenfriend system [449] (ascorbic acid, Fe^{2+}- EDTA and O_2) has been used effectively for hydroxylation of dG, and Fenton's reaction has also been recorded. Hydrogen peroxide can react with transition metal (Fe^{2+} or Cu^{+}) in the Fenton's reaction to produce reactive hydroxyl radical [450, 451].

$$M^{n+} + H_2O_2 \rightarrow M^{(n+1)+} + OH^- + OH\bullet$$

Hydrogen peroxide can interact with the resulting OH• to form superoxide anion radical, which in turn generates the hydroxyl radical from H_2O_2 via Haber-Weiss reaction [452]. Other schemes have been suggested to provide an explanation model for the mechanism of this reaction [453].

$$H_2O_2 + OH\bullet \rightarrow H_2O + O_2^{-\cdot} + H^+$$
$$H_2O_2 + O_2^{-\cdot} \rightarrow O_2 + OH^- + OH\bullet$$

Then the guanine nucleobase can be attacked at its different sites C (4), C (5) or C (8) by hydroxyl radical generated from the incubation of Cu^{+} / Na ascorbate to produce 4-OH-, 5-OH- and 8-OH-dGMP, respectively [454]. The isormers 4-OH and 5-OH-dGMP are transformed back to guanine by loss of a molecule of water [455]. However, 8-OH–dGMP can be either reduced with further opening of the imidazole ring to yield 2,6–diamino-5-formamidopyrimidine (FAPy-G) or oxidised with further loss of a proton forming 8-OH-dG [456] or its tautomer 7,8–dihydro-8-oxo-2'-deoxyguanosine (8-oxo-dG) [455] (**Fig. 30**).

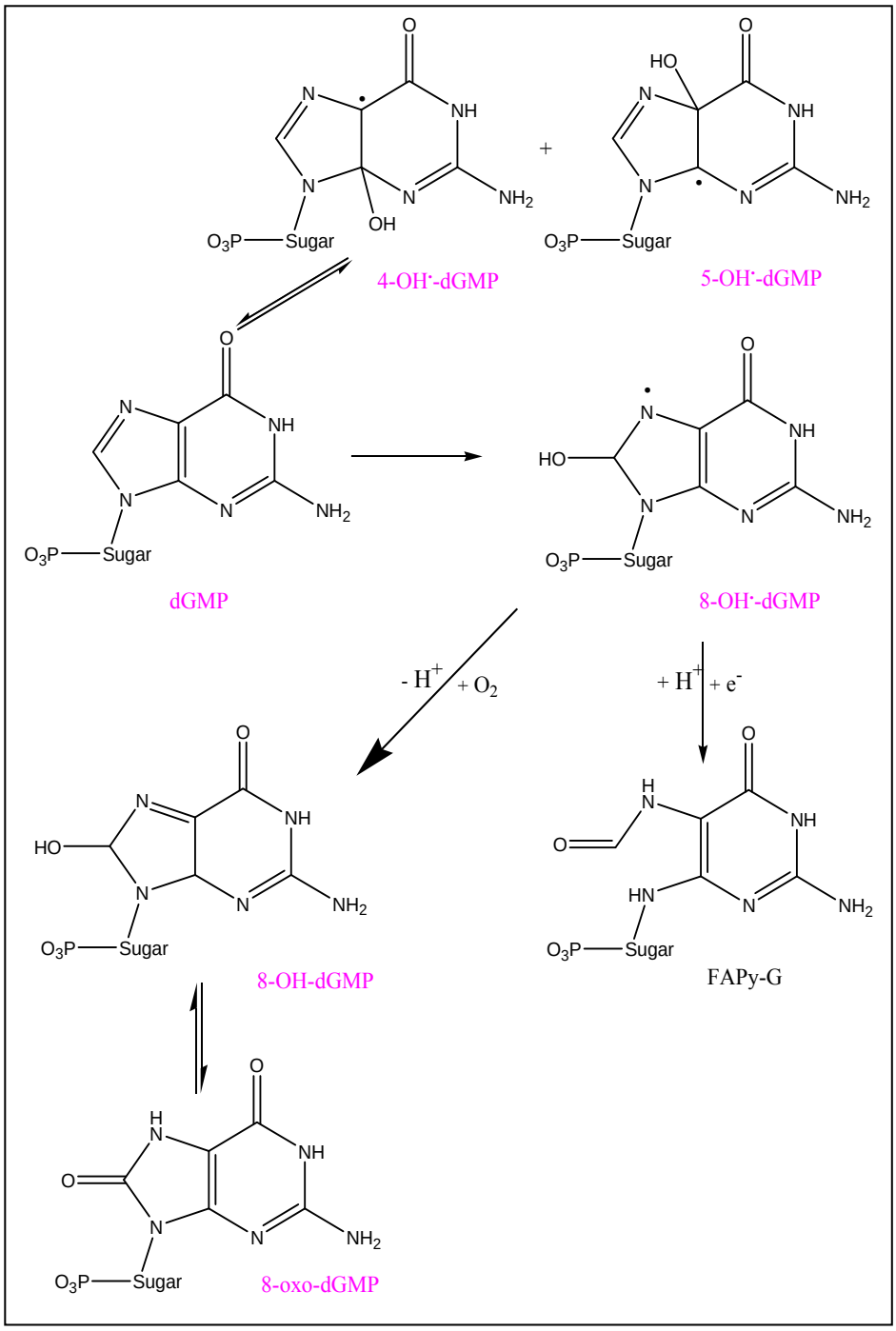


Figure 30: Scheme of oxo-adduct formation after attack of hydroxyl radical on 2'-deoxyguanosine-3'-monophosphate [455]

Iron [457], copper [458] and chromium [459] are the most common transition metals that participate in Fenton-like reactions to catalyse DNA oxidation. The oxygen can be obtained by bubbling oxygen through the reaction mixture or by adding hydrogen peroxide (H_2O_2) [460]. H_2O_2 was chosen in this study to stimulate the production of hydroxyl radical in presence of metal because it is an easier procedure with minimum precautions necessary.

Various reducing agents such as hydroxylamine, hydrazine, dihydroxymaleic acid, sodium bisulphite, sodium ascorbate and acetol were investigated for the hydroxylation reaction [461]. Sodium ascorbate was chosen for the reaction because the mixture of H_2O_2 gives the highest recorded yield of the oxidised product 8-OH-dG when ascorbic acid is used [460]. Sodium ascorbate is used in its acid form or its sodium salt to accelerate the production of 8-oxo-dGMP. However, it should be used in low concentration for the reaction because it possesses an antioxidant character [461] which has an inhibiting activity on UV-induced photochemical oxidation.

The reaction mixture was protected from light at low temperature to limit further oxidation [462] and the first portion of the solution was injected into the HPLC for the separation after 15 min reaction. Each injection introduced 100 µL onto the Lichrospher RP-18 HPLC column, and the peaks corresponding to dGMP and 8-oxo-dGMP were detected by UV system and then collected separately. All portions were collected over two and half hours calculated from zero reaction time. This time interval was suitable with the yield of 8-oxo-dGMP, which is favourably formed in the equilibrium reaction with 3'-dGMP [462].

The complete separation of 8-oxo-dGMP from 3'-dGMP and its purification were of tremendous importance not only for CE analysis but also for hindrance of the spontaneous oxidation of dGMP [463]. Equally, the same precautions (low temperature, no light) were observed in the storage of collected fractions of 8-oxo-dGMP after its synthesis and separation from the reactants. The preparation of the sample to be analysed by CE-LIF was performed as soon as possible after collection and purification.

3.4.2 Purification of 8-oxo-2'-deoxyguanosine-3'-monophosphate

Several experiments were performed for the separation of 8-oxo-dGMP from 3'-dGMP and the elution was performed at flow rate 0.8 mL/min.

The time of sample collection was calculated according to the following equation:

$$V = \Pi\, r^2 l \qquad (3)$$

$$V = 3.14.\ (0.025)^2.\ 58\ cm = 0.11\ cm^3$$

Where V: volume of capillary from detector to collected fraction

r: radius of capillary

l: length of capillary

As the flow rate is 0.8 mL/min, the eluted fraction is collected 8 sec after the peak appearance. Both fractions were identified by their distinctive spectrum (λ_{max} 247 nm).

Eluent system l consisted of 25 mM ammonium formate buffer that was adjusted to pH 4.7 with diluted formic acid. This buffer has proved its ability to separate 8-oxo-dGMP from 3'-dGMP with both TLC [464] and HPLC [258, 465]. The 2'-deoxyguanosine-3'-monophosphate (dGMP) fraction was collected between 12 and 14 min and the 8-oxo-2'-deoxyguanosine-3'-monophosphate (8-oxo-dGMP) fraction between 16 and 18 min with this buffer system. Fractions of approximately 2 mL dGMP and 1.5 mL 8-oxo-dGMP were collected from each run (**Fig. 31**).

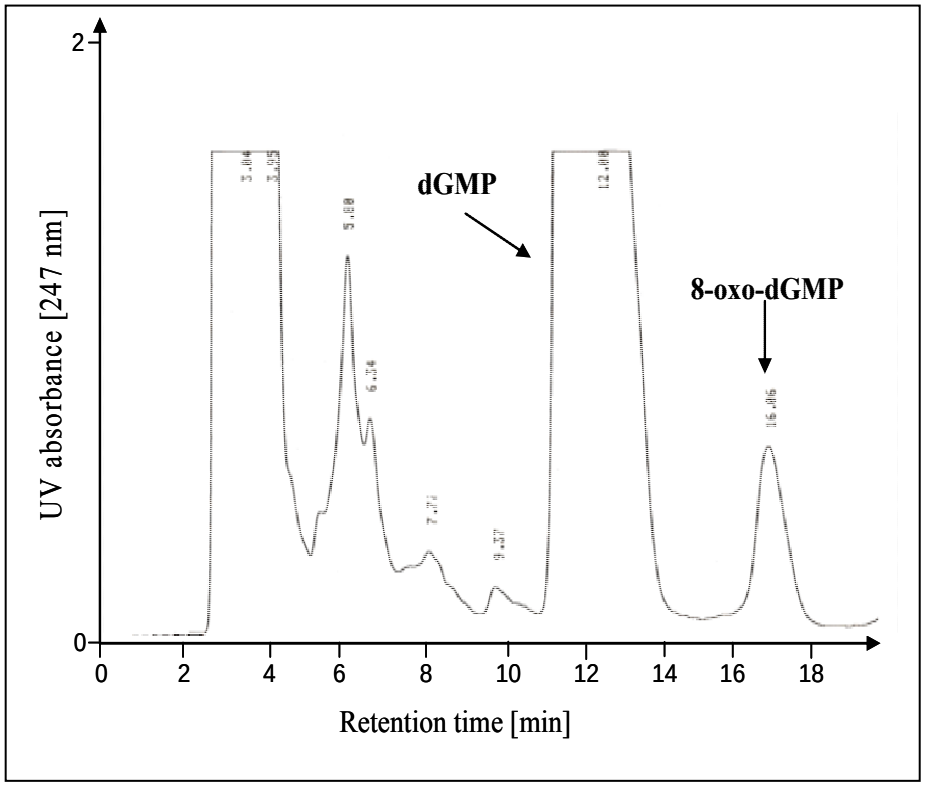


Figure 31: Representative HPLC chromatogram for the separation of 8-oxo-dGMP from its starting material 3'-dGMP (Eluent: 25 mM ammonium formate buffer pH 4.7). Chromatographic conditions: HPLC analysis was carried out on a reversed phase C 18 column with UV detection at 247 nm, flow rate (0.8 mL/min).

The collected fractions from several runs were pooled, evaporated by speed vacuum to 2 mL, then applied to RP-18 solid-phase extraction columns (SPE) which had previously been conditioned with 5 mL methanol (MeOH) followed by 5 mL water (H_2O) and finally eluted with 1 mL H_2O and 1 mL MeOH. The SPE step is important for cleaning up and removal of the remaining reactants or side products. First, the collected waste and water fractions (SPE) of the portion suspected to contain 3'-dGMP were injected for mass spectrometry (MS) detec-

tion in the ESI positive mode. Identical mass spectra were observed for the two fractions. The ESI-MS has molecular ion peaks at *m/z* 348.2 $[M+H]^+$ and at *m/z* 370.2 $[M+Na]^+$, indicating the molecular formula to be $C_{10}\,H_{14}\,N_5\,O_7\,P$. This confirms that the peak detected in **figure 31** (retention time 12-14 min) corresponds to dGMP, which can be collected and lyophilised for further utilisation. Also, fragmentation is observed. The N-glycosidic bond is cleaved, so that daughter peaks result. These intense peaks were observed at *m/z* 152.1 and 174.1 corresponding to the protonated guanine nucleobase and the sodium adduct of the guanine nucleobase, respectively (**Fig. 32**), and representing the loss of the neutral 2'-deoxyribose moiety (116 Da) and the phosphate group (80 Da) [465, 466].

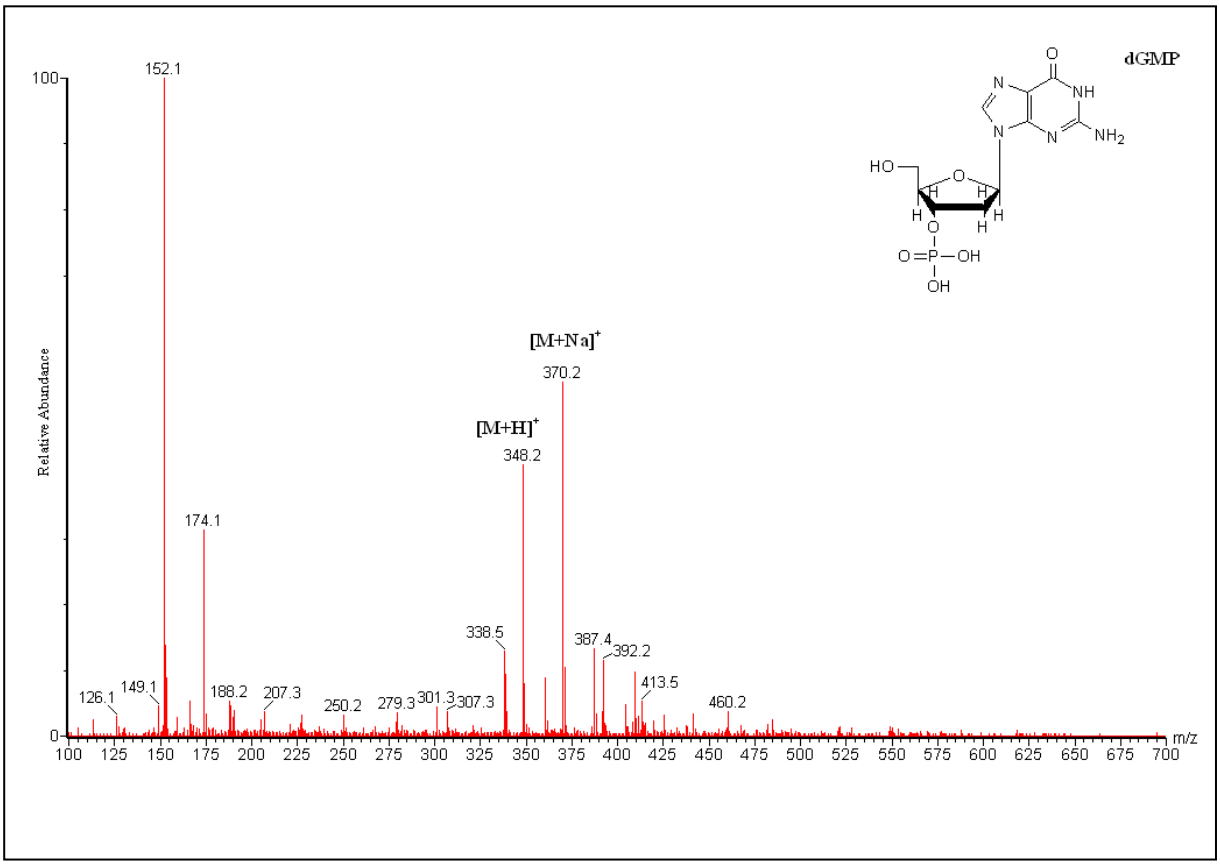


Figure 32: Mass spectrum ESI-MS positive injection mode of 2'-deoxyguanosine-3'-monophosphate (dGMP) in the collected fraction from HPLC (Mobile Phase: 25 mM ammonium formate) at *m/z* 348

Then, the collected waste, water and methanol fractions (SPE) of the portion suspected to contain 8-oxo-dGMP were injected for MS detection in the ESI positive mode (**Fig. 33**). The MS analysis had shown that the synthesised 8-oxo-dGMP can be detected in waste and water fractions, and its concentration in the former fraction was greater than in the latter one. Methanol fractions contain other degraded products which interfere with the determination of

8-oxo-dGMP. Therefore, waste and water fractions were further analysed by MEKC to investigate the electropherograms obtained for the presence of a peak corresponding to 8-oxo-dGMP.

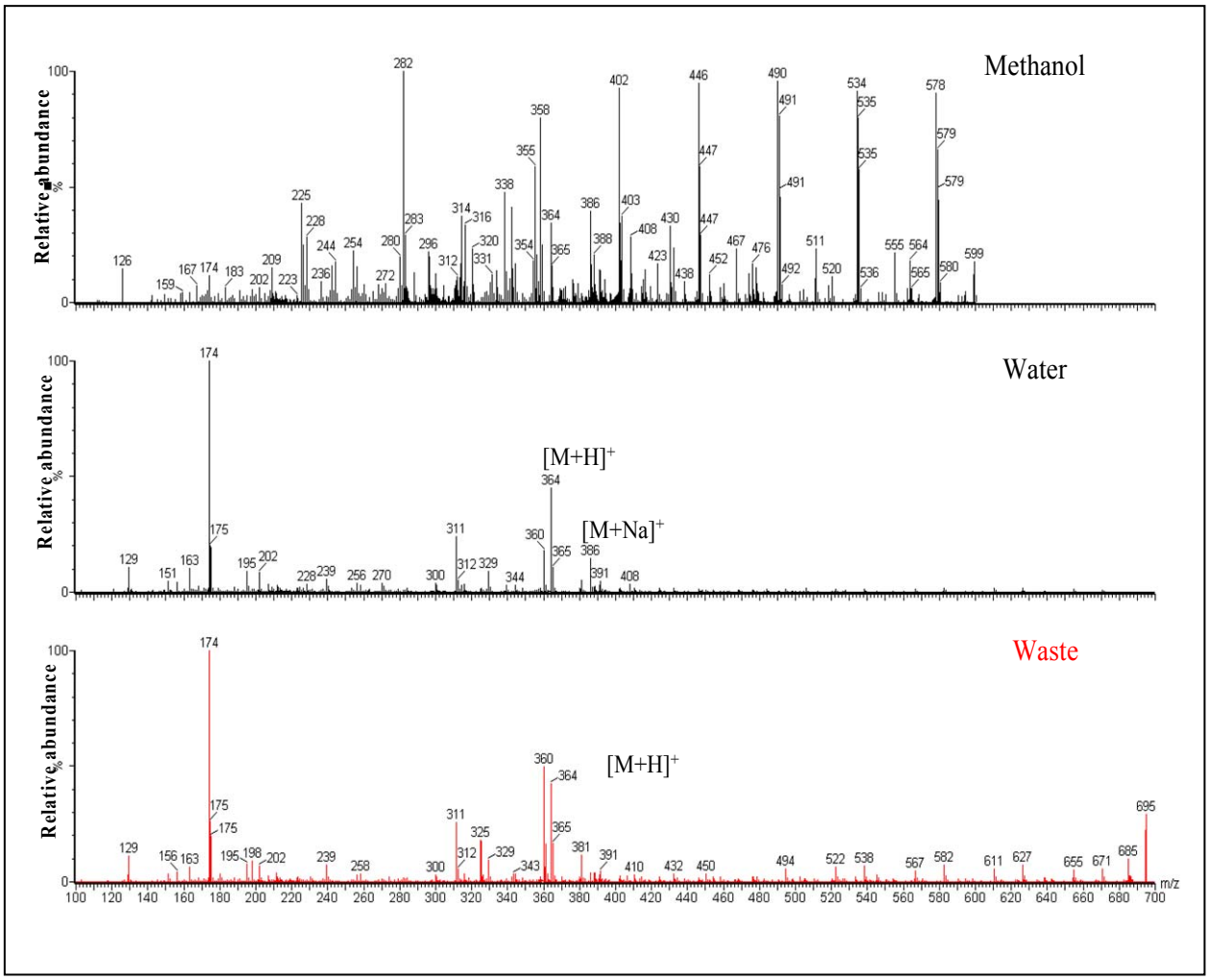


Figure 33: Mass spectra. ESI-MS positive ionization mode of 8-oxo-2'-deoxyguanosine-3'-monophosphate in several collected fractions from SPE (waste, water and methanol fractions)

50 µL of each of the collected fractions containing 8-oxo-dGMP from waste and water after SPE-mediated purification was added in separate experiments to 10 µg CT-DNA, then hydrolysed, derivatised, precipitated and finally measured with CE-LIF. The electropherograms of 10 µg CT-DNA (CT10) spiked with 8-oxo-dGMP aliquoted from either the waste or water solutions after SPE were similar and are illustrated in **figure 34**. A similar result was observed for the electropherograms of CT10 spiked with dGMP fractions from either waste or water collections (**Fig. 35**). Surprisingly, both CT-DNA samples spiked with the two fractions containing 8-oxo-dGMP had revealed two peaks at migration times 12.5 and 16.5 min in addition

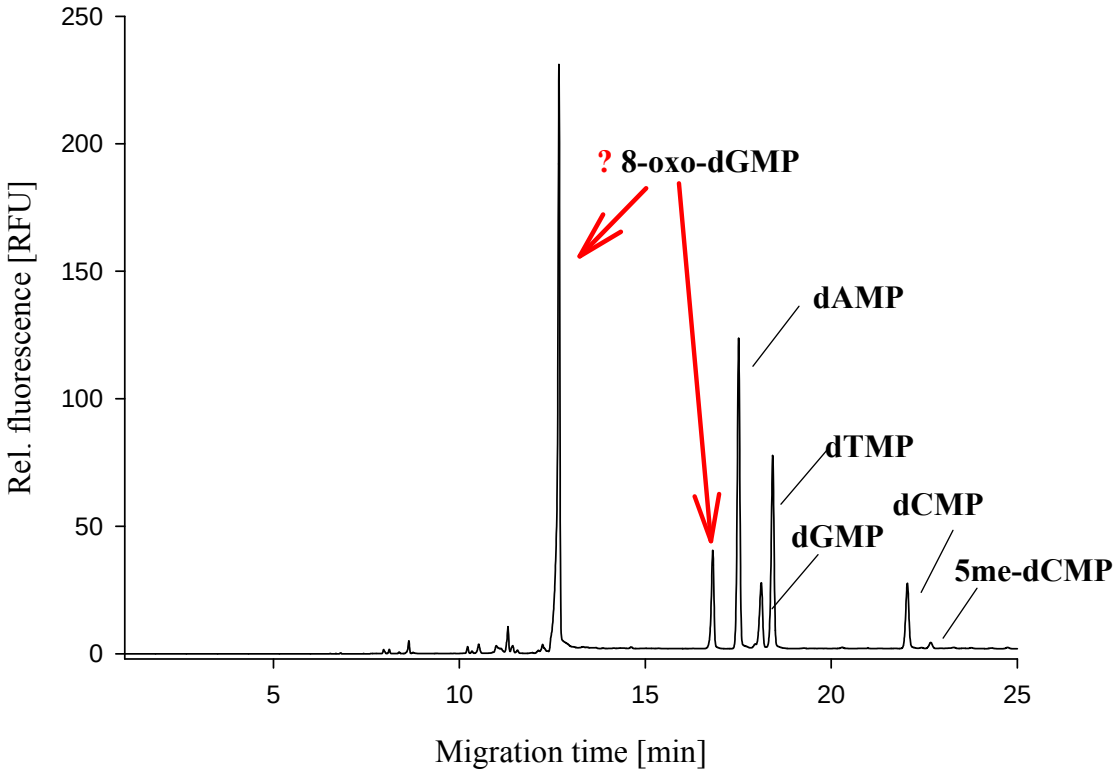


Figure 34: CE-LIF analysis of 10 µg CT-DNA spiked with 8-oxo-dGMP (water fraction) after hydrolysis, derivatisation and precipitation. Dilution (1:100). Experimental conditions: the same as previously mentioned**.**

to the peaks of nucleotides. The appearance of the common peak at 12.5 min either in the treated 8-oxo-dGMP fraction or the treated dGMP fraction (**Figures 34** and **35**) hinders the detection of synthesised 8-oxoguanosine. This peak could be related to the ammonium formate buffer, which leads to great interference, since carboxylic acid groups can be also labelled by the fluorophore molecule [467]. Spiking CT10 sample with dGMP leads to great difference in the electropherograms.

Although the synthesis of 8-oxo-dGMP and its complete separation from the starting material by HPLC was successful with ammonium formate as eluent, the collected 8-oxo-dGMP was not pure enough to be detected as a single peak with the sensitive LIF detector. Also, further purification with various SPE columns was not successful (SPE set is illustrated in **Appendix. Fig. 59**).

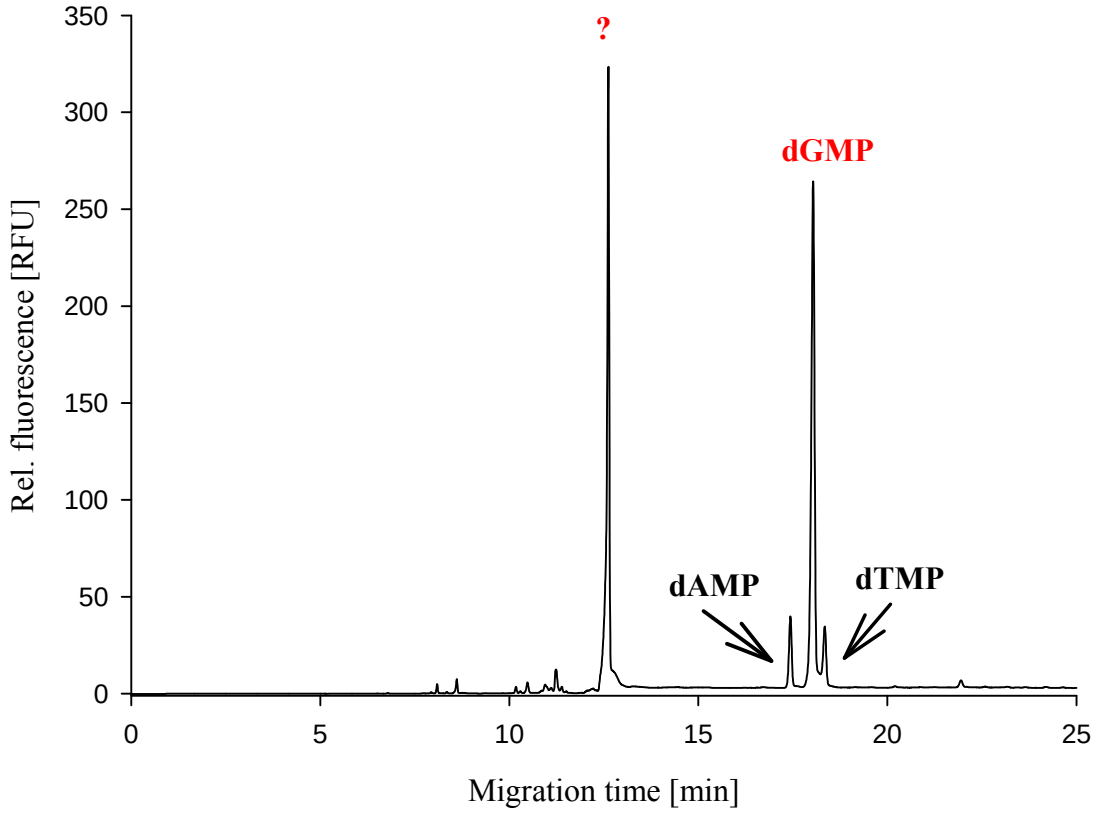


Figure 35: CE-LIF analysis of 10 µg CT-DNA spiked with dGMP after hydrolysis, derivatisation and precipitation. Dilution (1:100). MEKC operating conditions: the same as previously mentioned.

Therefore, other eluents were tried to achieve better separation as well as to obtain the 8-oxo-dGMP in pure form. Among the tried mobile phases, eluent system 2 consisting of 15% methanol:85 % water, pre-degassed with helium was found to give optimum separation in HPLC. Three peaks were detected in the HPLC chromatogram (**Fig. 36**). Previous studies [258, 448] had shown that the 8-oxo-dGMP peak is eluted in most cases after the dGMP peak. The first broad peak was taken to be the starting material (dGMP) because of its surface area. The two successive peaks at migration times 4.53 and 4.90 min were separately collected, evaporated to dryness with the speed vacuum rotator, then detected by MS. 8-Oxo-dGMP was detected at *m/z* 364 in the MS spectrum of one of these two peaks, confirming that the peak collected at R.t. 4.90 min corresponds to 8-oxo-dGMP. However, the electropherogram obtained from the analysis of 10 µg CT-DNA spiked with 8-oxo-dGMP still reveals two peaks that were confusing and are still unidentified as previously illustrated in **figure 34**.

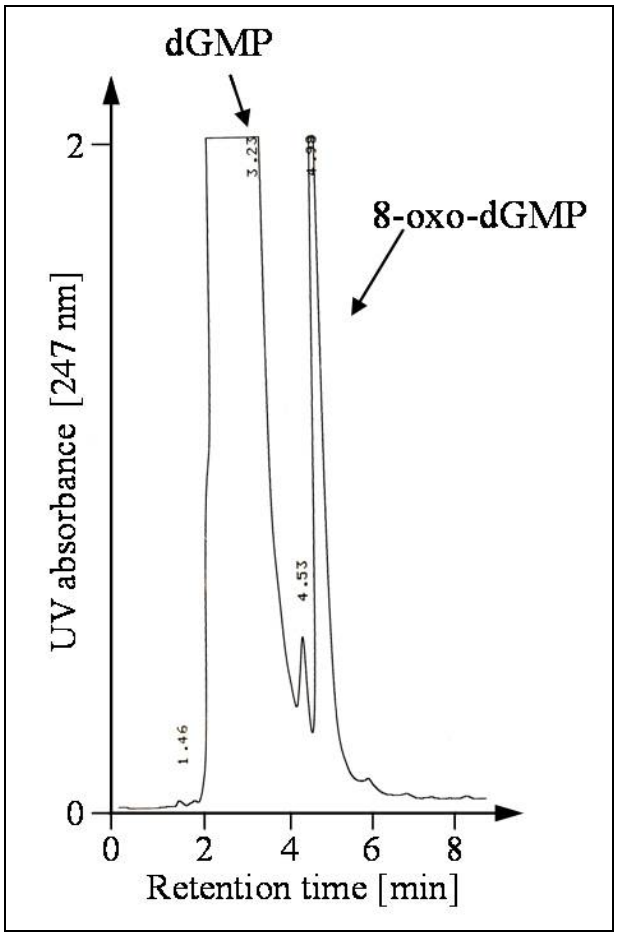


Figure 36: HPLC chromatogram for the detection of 8-oxo-dGMP in the reaction mixture with its starting material dGMP (Eluent: 15 % methanol in 85 % water). Chromatographic conditions: the same as previously described.

That means the fraction of 8-oxoguanosine (8-oxo-dGMP) is still incompletely pure in that more than one peak is detected in MEKC analysis.

Finally, the best separation was obtained with pure 8-oxoguanosine fraction by using dual elution system. It started with 25 mM ammonium formate buffer adjusted to pH 4.7 with formic acid (eluent system 1). 100 µL of reaction sample was injected onto HPLC column. All peaks were detected by UV-VIS photodiode array detector. Guanosine (dGMP) had UV absorbance at λ_{max} 247 nm and its peak was eluted at time interval 12-14 min and 8-oxo-dGMP peak was eluted at time interval 16-18 min (λ_{max} 247 nm).

Then the fractions of 8-oxo-2'-deoxyguanosine-3'-monophosphate pooled from several runs were gathered together then evaporated by vacuum speed to 2 mL (8 times concentration).

After concentration, this fraction was re-injected on the RP-18 column and re-eluted with 15 % methanol: 85 % water (eluent system 2). 8-Oxo-dGMP peak had UV absorbance at λ_{max} (MeOH) 278 nm and two peaks were detected at retention times 2.87 and 3.25 (**Fig. 37**). The two obtained peaks were separately collected, evaporated till dryness, reconstituted in water (500 µL), dried again and stored for further MS detection and CE measurement.

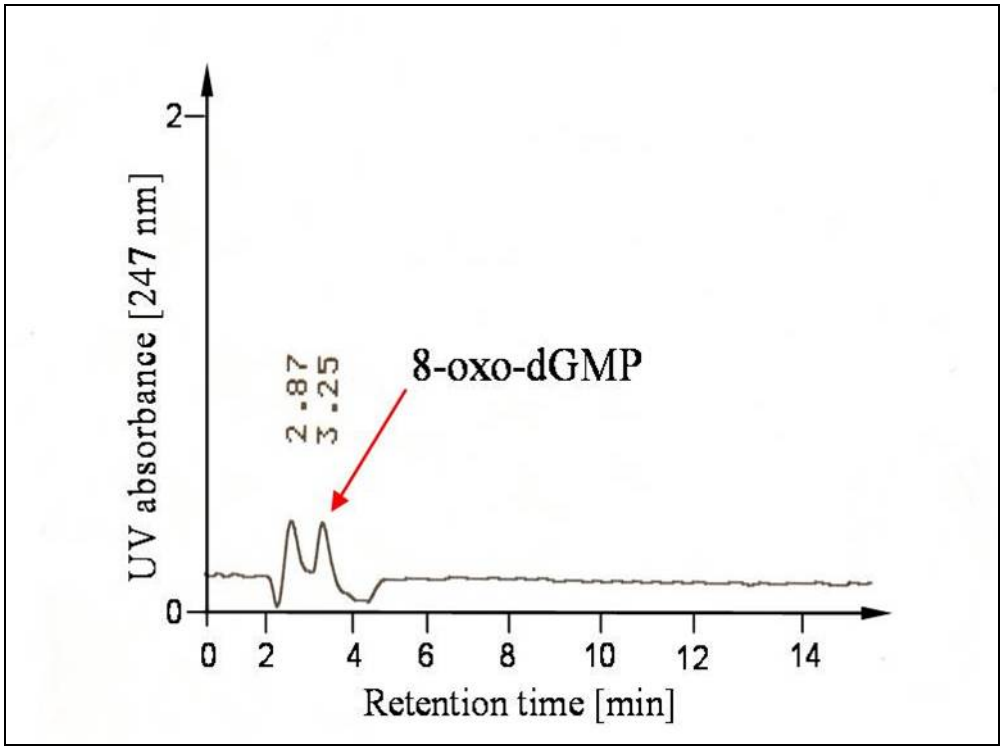


Figure 37: HPLC chromatogram of 8-oxo-dGMP in its previously collected HPLC fraction with 25 mM ammonium formate buffer (R.t. 16 min) and re-eluted with 15 % methanol:85 % water. Chromatographic conditions: the same as previously described.

3.4.3 Characterisation of 8-oxo-2'-deoxyguanosine-3'-monophosphate

Dried aliquots of the two collected peaks re-eluted with methanol:water (15:85) were dissolved (water:acetonitrile (50:50) + 0.1 % formic acid), then analysed by ESI-MS. The MS spectrum of the first collected peak at 2.87 min does not reveal any base peak assigned to 8-oxo-dGMP (not shown). On the other hand, the ESI-MS of the second collected peak at 3.25 min shows molecular ion peaks at *m/z* 364.3 $[M+H]^+$ (base peak) and at *m/z* 386.2 $[M+Na]^+$, indicating the molecular formula $C_{10}\,H_{14}\,N_5\,O_8\,P$, which can be assigned to 8-oxo-dGMP (**Fig. 38**). The comparison of the mass spectrum of 8-oxo-dGMP eluted only with elu-

ent system 1 (See appendix. **Fig. 62**) with that of 8-oxo-dGMP eluted twice first with ammonium formate buffer then with 15 % methanol:85 % water reveals a smoother trace with higher signals for the latter spectrum (**Fig. 38**). This finding easily confirms the success of this dual HPLC elution, first with ammonium formate buffer then with 15 % methanol:85% water to obtain pure synthestic 8-oxo-2'-deoxyguanosine-3'-monophosphate. This step can be considered a more successful clean-up step than the experiments with various types of SPE.

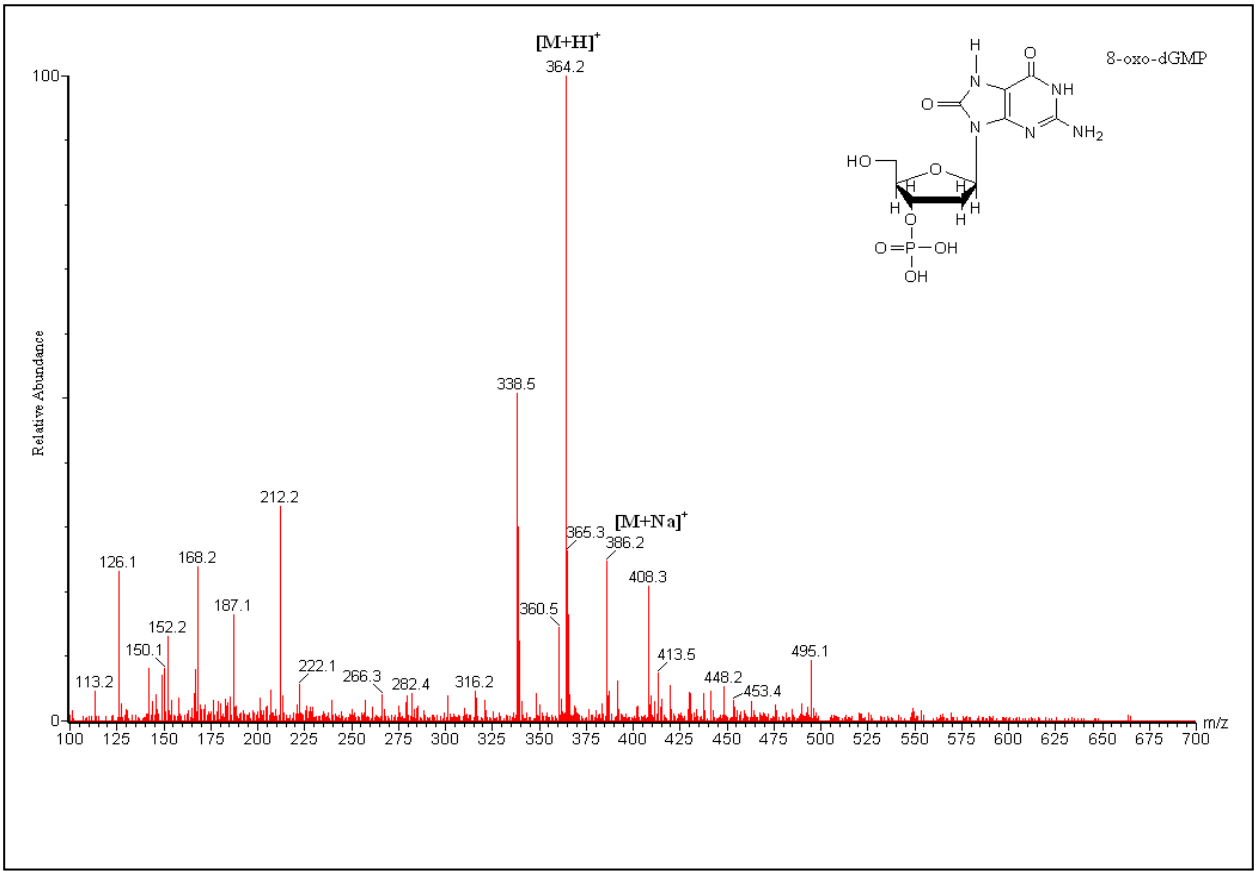


Figure 38: Mass spectrum ESI-MS positive injection mode of 8-oxo-2'-deoxyguanosine-3'-monophosphate (8-oxo-dGMP) in the collected fraction from HPLC (Mobile Phase: 25 mM ammonium formate then 15 % MeOH:85 % water) at *m/z* 364

3.4.4 Analysis of 8-oxo-2'-deoxyguanosine-3'-monophosphate by CE-LIF

A dried aliquot of the collected peak containing 8-oxo-dGMP was derivatised with BODIPY, then precipitated and analysed by CE-LIF.

Finally, the peak corresponding to 8-oxo-dGMP was successfully detected without any interference from other degraded compounds or impurities (**Fig. 39, pink electropherogram**: t_m 21.92 min). To test the accuracy of the determination of 8-oxo-dGMP, the signal of

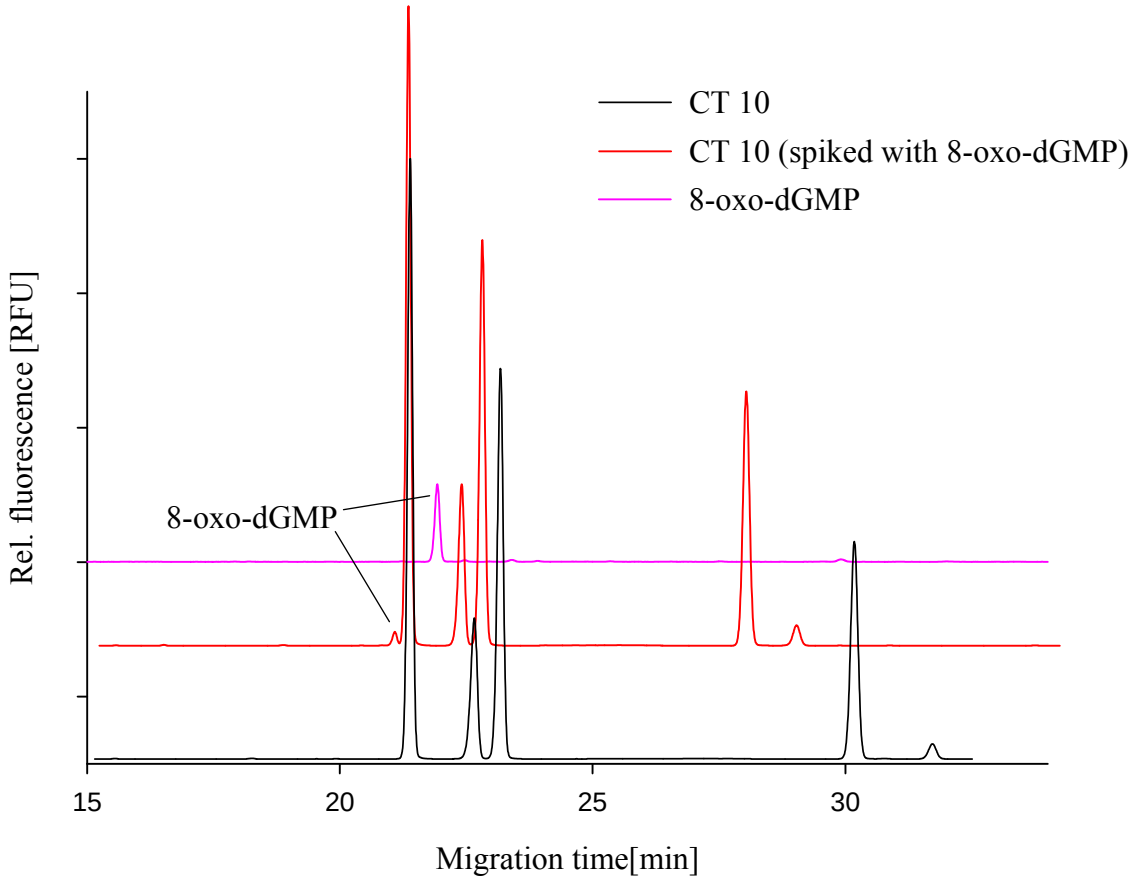


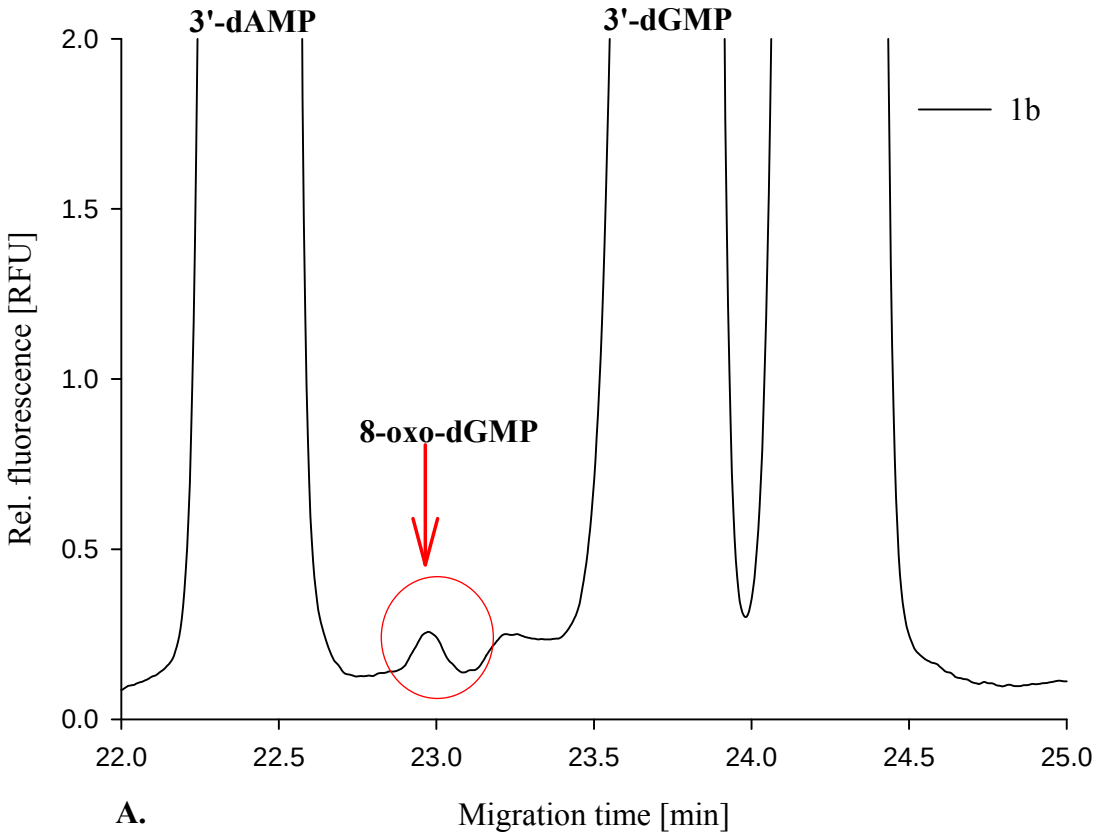
Figure 39: MEKC-LIF detection of 8-oxo-dGMP analysed in pure form (pink electropherogram) or spiked with 10 µg CT-DNA (red electropherogram) in comparison to analysis of 10 µg CT-DNA (black electropherogram). MEKC operating conditions: the same as previously mentioned.

synthesised and purified 8-oxo-dGMP was defined by spiking the 8-oxo-dGMP with standard CT-DNA. Then, the sample was analysed with the routine CE-LIF after hydrolysis, derivatisation and precipitation. The peak corresponding to 8-oxo-dGMP can easily be detected at t_m: 20.84 min (**Fig. 39, red electropherogram**). The analysis of the authentic CT-DNA was also carried out under the same experimental conditions acting as reference for both previous analyses (**Fig. 39, black electropherogram**). The comparison showed that pure CT-DNA is free of the peak detected in the analysis of CT-DNA spiked with the new synthesised 8-oxo-dGMP confirming the success of synthesis of 8-oxo-dGMP.

3.4.5 Detection of 8-oxo-dGMP and determination of methylation level in real samples with CE-LIF

It was not possible to use the synthesised 8-oxo-dGMP in standard addition procedure for the precise determination of the oxo-adduct in real samples, because the modified nucleotide could not be quantified due to different response factors as a result of both the derivatisation and the quenching yield [395, 399, 400, 430, 431]. But for qualitative analysis, the standard could be used. As a practical proof, it was proposed in a double blind study to analyse 8-oxo-dGMP in real DNA samples provided from army combat divers. It was found that there is a high risk to develop cancer due to oxidative stress during their diving exercises, in which closed-circuit re-breathing devices are used. Several NAVY forces over the world use "Closed-Circuit Oxygen Rebreathing Diving Apparatus" (CCORDA). This oxygen re-breather system enables the divers to inhale oxygen under high pressure that is suitable for long and deep diving and has the additional advantage of low noise with minimal gas escape in comparison with open-circuit SCUBA. There had been a study involving the navy soldiers who took part in the underwater exercises with the closed-circuit oxygen re-breather equipment for 25 h/week during the first year, then 15 h/week during the following three years. Blood samples were taken from the soldiers before and after five months training. In this study, samples were taken from two soldiers: control samples taken before training and two samples taken five months after training. All samples were analysed eight to ten times for accurate results. The 8-oxo-dGMP peak was expected to be easily detected, since the previous observations had revealed that it appeared earlier than the 3'-dAMP peak. The migration time of 8-oxo-dGMP detection ranges from 20-22 min because of changes in the capillary or buffer charge from one analysis to another and/or whether the 8-oxo-dGMP is analysed alone or spiked with CT10. Therefore, the location of the peak was referred to 3'-dAMP peak for the easier detection. The electropherograms of samples *1a* and *2a* are free of the modified nucleotide peak and concluded to be control samples. The previously described observation that the 8-oxo-dGMP peak appeared before 3'-dAMP peak made it easy to detect 8-oxo-dGMP peak in the electropherograms of samples from soldiers after five months training (*1b* and *2b*). Surprisingly, the peak could not be detected in the sample *1b* at the matched migration time observed in the analysis of sample *2b*. It seems that the peak is shifted to higher migration time between 3'-dAMP and 3'-dGMP peaks (**Fig. 40A**).

The most important observation is concerning the sample *2b* because of the appearance of the 8-oxo-dGMP peak before 3'-dAMP peak (**Fig. 40B**) as well as the probability of peak detection in the position between 3'-dAMP and 3'-dGMP peaks (**Fig. 40C**). The shift of 8-oxo-dGMP peak by time in the electropherograms of the analysed sample *D24* may be due to the formation of two chemical forms, present mainly in 8-oxo-deoxyguanosine and also in its minor tautomer 8-hydroxy-deoxyguanosine form [456, 468]. Another idea is that 8-oxo-dGMP is further oxidised and this product migrates between 3'-dAMP and 3'-dGMP.



A.

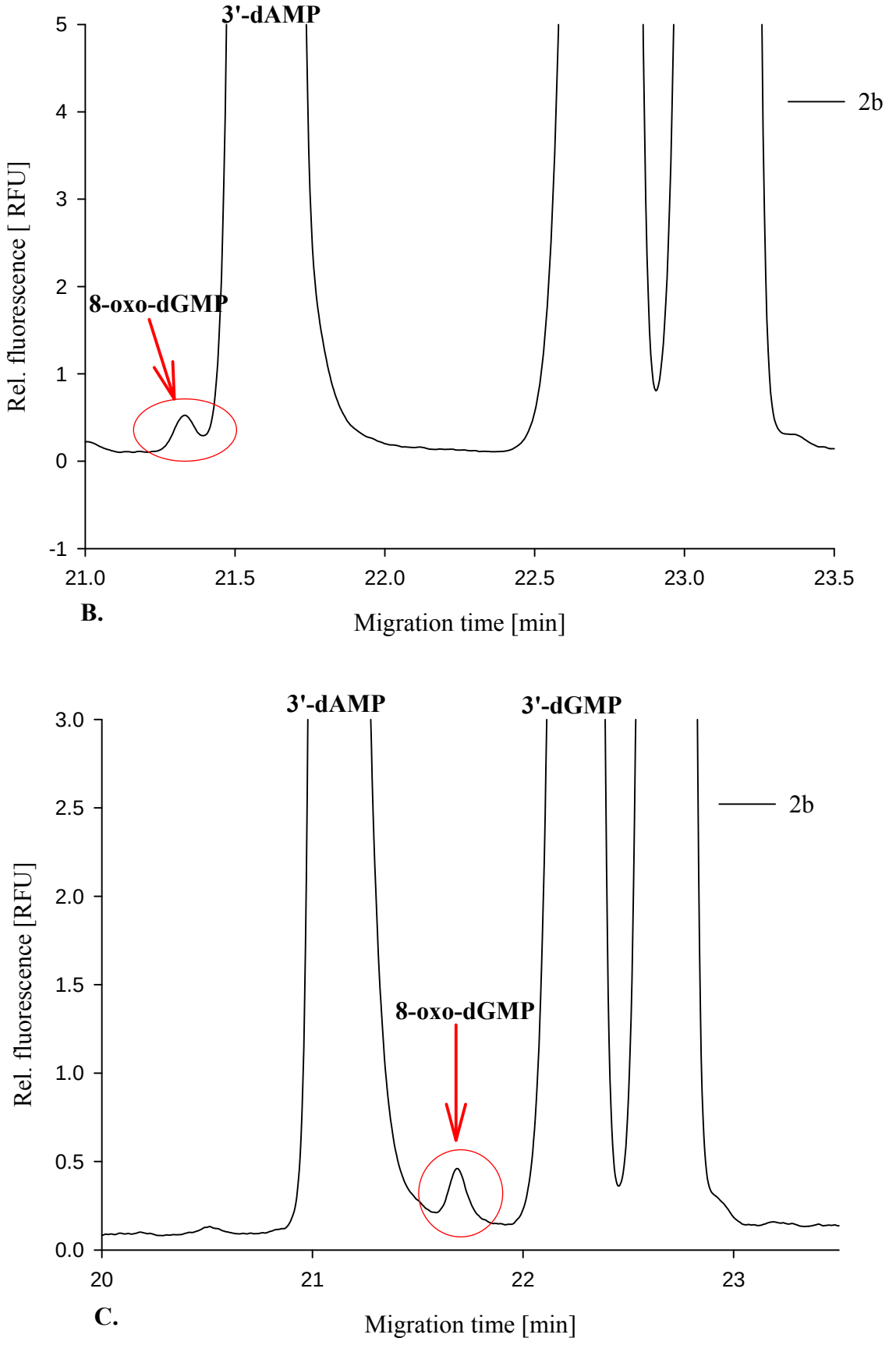


Figure 40 (A-C): MEKC-LIF electropherograms for the detection of 8-oxo-dGMP in real samples [1b (A); 2b (B and C)]. MEKC operating conditions: the same as previously mentioned.

3.4.6 Study of the electrophoretic mobility of 8-oxo-dGMP

In consequence of the observation of two peaks for the oxidated dGMP at different migration times, it was obviously important to monitor the change in the migration time of the peak in subsequent analyses of the same sample. The separation buffer is composed of 20 mM sodium phosphate buffer, 95 mM SDS buffer with 10 % MeOH as organic modifier and adjusted at pH 9.0. At this basic pH, the keto form of 8-oxo-3'dGMP can be easily converted to the enol form through an intermediate step by loss of a proton from N^7 of imidazole ring [469]. The intermediate enolate anion is therefore stabilised by resonance. Therefore the appearance of two peaks with two different migration times can be explained by keto-enol tautomerism phenomena [456, 468] (**Fig. 41**). The fluorescence responses of the two tautomers [470] were

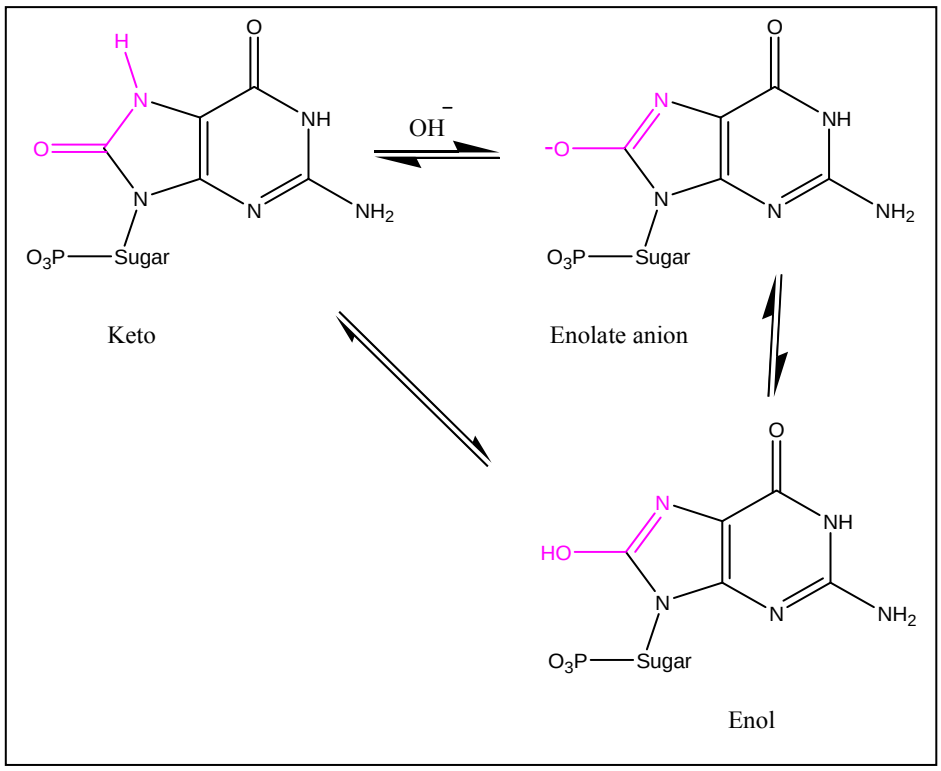


Figure 41: Scheme of keto-enol tautomers of 8-oxoguanosine-3'-monophosphate

found to be different; this may be due to effect of the surfactant (SDS) on keto and enol forms. But normally, a signal with two peaks is expected for keto and enol forms of one compound. Therefore, a further oxidation process should be a possible explanation.

For further investigation, a standard addition of definite volume of synthesised and derivatised 8-oxo-dGMP to a derivatised 10 µg CT-DNA sample was performed. The sample was analysed in subsequent individual runs. Each run was carefully examined for the existence of a small peak corresponding to the oxo-adduct. The 3'-dAMP peak was used as reference for

the location of both peaks. The peak defined as 8-oxoguanosine-3'-monophosphate was numbered (1) and the peak related to 3'-dAMP was numbered (2) for easily monitoring of the migration profile of 8-oxo-dGMP and linking its position to 3'-dAMP peak (**Fig. 42**). The migration times of both peaks are presented in **table 3**.

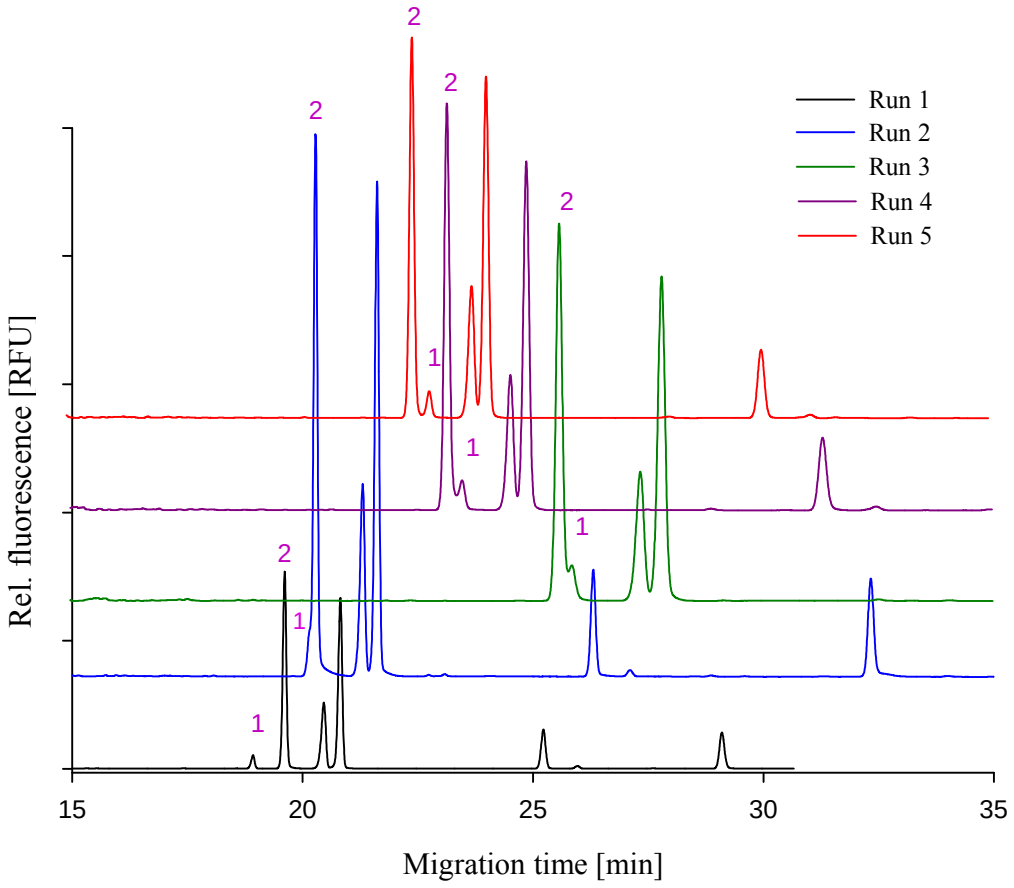


Figure 42: Analysis of 10 µg CT-DNA spiked with 8-oxo-dGMP in successive runs to monitor migration times. MEKC operating conditions: the same as previously mentioned.

On analysis of the first run, peak 1 is detected prior to the 3'-dAMP peak in the order of the electropherogram as expected from the previous comparative analysis of CT10 spiked with 8-oxoguanosine to CT10 sample (**Fig. 39, red electropherogram**).

The disappearance of peak 1 in the second run (or perhaps a shoulder in front of peak 2) and its re-appearance as a shoulder in the third run perhaps can confirm the presence of 8-oxoguanosine in its tautomeric forms. A number of tautomers can be drawn for 8-oxoguanine because of the diversity of sites on the molecule that can be either protonated or ionised. Thus, 128 potential tautomeric and ionised forms can be drawn for this oxidised base [468].

In the third run and successive runs (4 and 5), the re-appearance of peak 1 is observed in another position following the 3'-dAMP peak in the electropherogram. The repeatability of this

peak in this position in the electropherogram had suggested that this form may represent the major tautomer [468] that corresponds to the detected peak of the pure synthetised 8-oxo-3'dGMP represented in **figure 39** (**pink electropherogram**).

The peak areas of peaks 1 and 2 calculated with adjustment for their migration times were integrated to correlate the detected 8-oxo-dGMP peak with the 3'-dAMP peak. Accordingly, the time-corrected peak area of 8-oxo-dGMP is divided by the time-corrected peak area of 3'-dAMP and the area/area ratios were calculated as percents as in the determination of methylation level (**Table 3**). It is observed that the area percentage of the peak detected prior to 3'-dAMP is lower than that of the peak detected between 3'-dAMP and 3'-dGMP. This can be explained by a difference in quenching yields between the two oxidised forms.

Run number	Migration time (min)	PA_1 / PA_2 %
	t_m Peak 1 / t_m Peak 2	n=3
Run 1	**0.962 ± 0.02**	**0.11 ± 0.07**
Run 2	**-**	**-**
Run 3	**1.011 ± 0.06**	**-**
Run 4	**1.015 ± 0.03**	**0.14 ± 0.05**
Run 5	**1.016 ± 0.03**	**0.14 ± 0.06**

Table 3: **Corrected migration times of 8-oxo-dGMP and corrected peak areas percentage in subsequent individual runs. (PA: Peak area. 1: 8-oxo-dGMP. 2: 3'-dAMP)**

3.4.7 Correlation between the methylation level, oxidative stress and carcinogenesis

Several *in-vivo* studies had detected the production of high levels of ROS in divers or patients treated with hyperbaric oxygen (HBO) upon their exposure to HBO [471, 472]. Oxidative stress mediated by ROS has been attributed to the increased risk of cancer development and diseases related to neurological impairment [473]. Accordingly, it was hypothesised in this study that cancer development and changes in the genome-wide methylation level could be related to the oxidative stress generated by hypoxia-reoxygenation during the severe diving training [474-476]. For this study, four samples were chosen from a set of samples provided to our laboratory; these were investigated for the oxo-adduct and the methylation level. As previously demon-

strated, the 8-oxo-dGMP was found in the samples taken after training (*1b* and *2b*). These samples and the samples before training (*1a* and *2a*) were derivatised and analysed eight times by CE-LIF. The methylation level percentages of the combat divers are calculated and presented in **figure 43**. A hypomethylation is obvious for the divers after training, since diver (1) has higher methylation status in both situations: before and after training than diver (2). These analyses highlight an inverse relation between the presence of 8-oxo-dGMP and the 5-meC level. This finding correlates with the results of the study by Guz *et al.* [477] study. They had suggested that the high level of 8-oxo-dG may induce cancer via the mutagenic potential arising through the mispairing of the modified base as well as via its influence on gene expression by effecting DNA methylation. The explanation of this observation was well illustrated by Weizman *et al.* [478] who concluded that the oxygen radical injury dramatically altered the methylation level. The replacement of guanosine in DNA with 8-oxo-dG had an impact on the activity of the Dnmt1 enzyme responsible for hemimethylation of DNA and subsequently of the Dnmt3a and Dnmt3b enzymes responsible for maintenance of DNA methylation during replication [479] by methylating cytosine targets. This effect on enzyme activity leads to an aberrant methylation level (hypomethylation) which is frequently associated with various diseases including ageing, neuro-degenerative diseases and cancer [254, 255].

In conclusion, our study supports a model linking oxidative stress to the change in methylation level and cancer development. To verify this conclusion, further analysis of more samples is necessary.

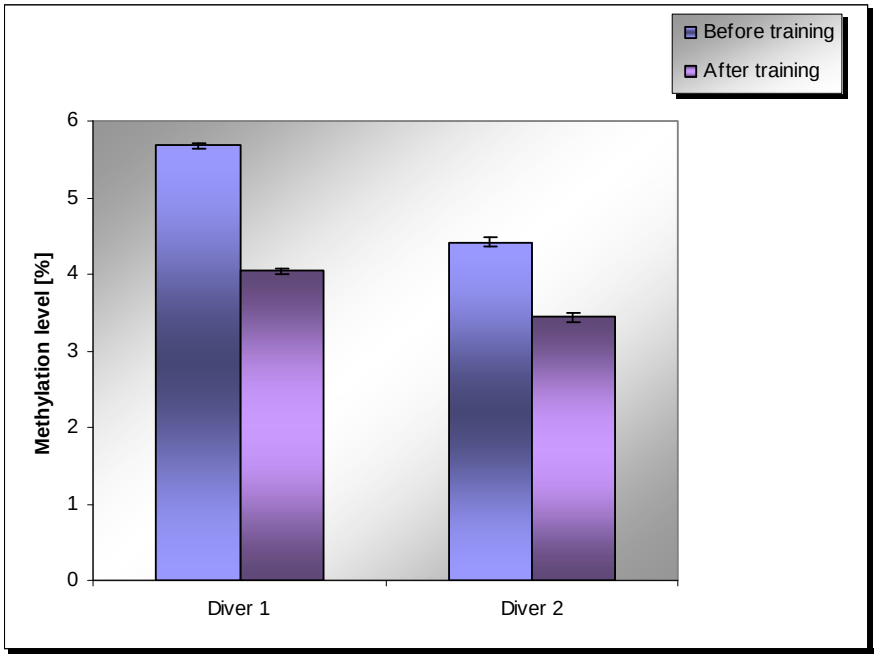


Figure 43: Methylation level of two divers before and five months after training

3.5 Influence of carcinogens on the genomic methylation level

Several carcinogen-DNA adducts have been used as reflectors of their mutagenic and carcinogenic effects. DNA modifications have been generally used as biomarkers of exposure to these carcinogens and risk of cancer development. In this work, a number of carcinogens such as AA, 3-NBA, B[*a*]P and 4-ABP were investigated for their effect on the level of 5-meC in DNA samples. Although these genotoxic carcinogens belong to different classes of exogenous pollutants, they share a common pathway to induce cancer by performing first a metabolic activation that results in DNA-bound chemicals and then affecting the functions of the cell and possibly leading to cancer. The correlation between the exposure to these different carcinogens and the methylation level will contribute to understanding the involvement of the environmental pollutants in cancer development via epigenetic modification.

3.5.1 Influence of aristolochic acid treatment on Sprague-Dawley rats

The old herbal drug aristolochic acid (AA), derived from the Aristolochiaceae family [480], has recently been associated with the development of nephropathy, designated aristolochic acid nephropathy (AAN), as well as urothelial cancer in AAN patients [337, 343, 481]. AAN syndrome was first reported in a hospital in Brussels, where two young Belgian women were undertreatment for renal failure. Two years before, both had followed a slimming regimen and had taken slimming herbal pills containing AA. The source of the AA in the herbal pills could have been the accidental substitution of *Stephania tetrandra* (Hanfangji or Fenfangji) by *Aristolochia fangchi* (Guangfangji) during the packaging of the herbal preparations or during the marketing of the drugs. The phonetic similarity of fangji and fangchi with the Chinese accent was probably the cause of the improper substitution of these Chinese herbs [482]. Additionally, the roots belonging to the same family (aristolochia) appear very similar, and that could also induce confusion (**Fig. 44**).

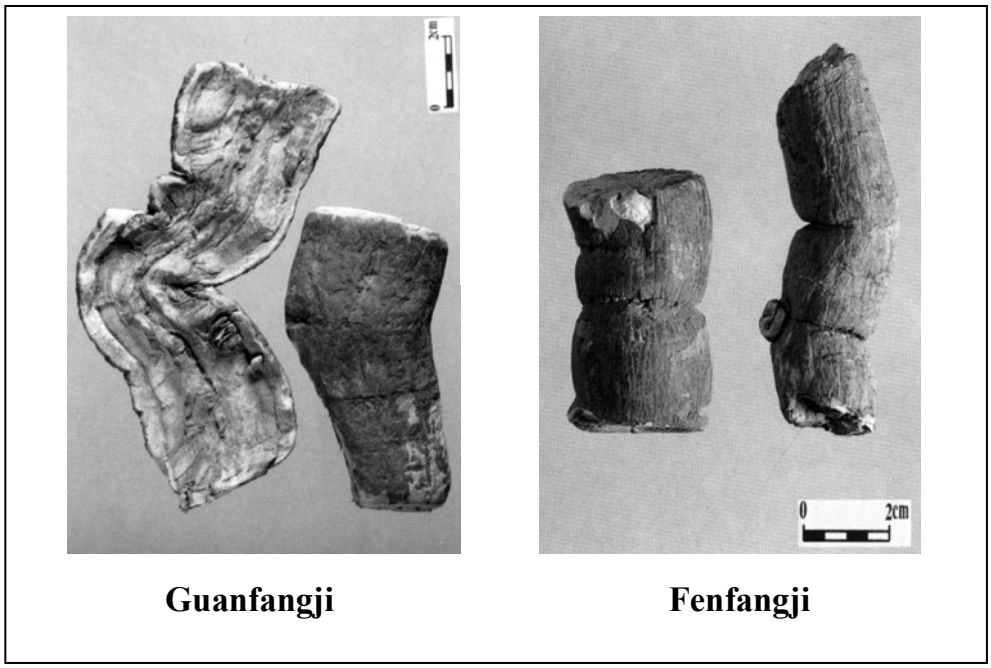


Figure 44: The roots of two herbs used in the traditional Chinese medicine [483]

It was clearly confirmed in the course of research into the AA toxicity that ingestion of these phytotoxins results in nephrotoxicity at mg/kg doses as well as cancers in different tissues. Moreover, Grollman *et al.* [484] have reported the detection of dA and dG aristolactam adducts in the renal cortex of the endemic nephropathy patients [484-486]. The adducts formed following AA exposure were associated with cell cancers. Therefore, it is interesting to test the effect of AA on the methylation level by our sensitive method. In this study, DNA methylation was determined in several organs taken from rats dosed with aristolochic acid at a daily dose of 30 mg/kg Body weight (BW, discussed in this section) or from mice dosed with 5 mg AA/kg BW (discussed in section 3.5.2).

3.5.1.1 Quantification of DNA in AA-dosed rat samples

The male Sprague-Dawley rats were treated with a single oral dose of 30 mg AAI/kg BW. At 24 h after the dosage of AA, the rats were sacrificed. Various tissue samples were collected and stored at -80°C. DNA samples were extracted from these tissues, purified and delivered from Hong Kong to our laboratory in Wuppertal. 24 DNA samples were quantified spectrophotometrically as previously described under section (3.1), and the calculated DNA quantities together with the corresponding purity values of the AA-treated samples and control samples are presented in **table 4**. A glance reveals that the colon samples from treated animals are the least pure of all the samples because of the impurities probably caused by pro-

teins. The dissolved samples were aliquoted in 10 µg, lyophilised and kept frozen for further CE-LIF analysis.

Tissue	Control			Treatment with 30 mg AA/kg BW		
	Sample Code	DNA-amount [µg]	Purity	Sample Code	DNA-amount [µg]	Purity
Fore-stomach	S 43	41.13	1.42	S 38	24.84	1.43
	S 45	44.84	1.46	S 39	52.67	1.40
Colon	Co 11	36.16	1.49	Co 15	35.34	1.33
	Co 12	41.68	1.47	Co 17	23.97	1.37
Kidney	K 34	53.75	1.56	K 29	52.18	1.50
	K 35	50.04	1.48	K 32	43.81	1.57
Small intestine	S.I. 2	37.23	1.52	S.I. 7	25.52	1.48
	S.I. 5	53.27	1.43	S.I. 8	66.83	1.48
Liver	L 21	22.26	1.49	L 19	57.79	1.51
	L 26	40.65	1.59	L 27	45.68	1.53
Bladder	B 51	20.92	1.52	B 46	18.00	1.41
	B 52	22.31	1.52	B 54	9.70	1.51

Table 4: DNA quantification in AA-dosed rats and control samples and their purities

3.5.1.2 CE-LIF analysis of methylation level in rats dosed with AA

In order to analyse the samples with CE-LIF, DNA samples were hydrolysed and derivatised by the procedure described in the experimental section (section 5.3.1). At that time of the study, a special new charge of enzyme mixture was prepared in the laboratory for the analysis of these samples. In addition, a mixture of 15 fold enzyme concentration (composition is described in the experimental section) was chosen after testing several prepared concentrations (5, 10 and 15 fold) that had shown the activity of this chosen mixture was suitable for the hydrolysis of AA-DNA-adduct samples. These fundamental changes in the procedure of the experiment meant that a correction factor should be reset to overcome the fluctuations that could arise from hydrolysis and derivatisation steps. Unfortunately, for reasons of cost, a new correction factor with Lambda DNA (unmethylated and enzymatically methylated) could not be established during this work. We therefore used the correction factors of dCMP and 5-me-dCMP previously established in our group for the calculation of the methylation level. With

these, a deviation of 8.41 ± 0.05 % (n=8) was found for the methylation level of CT-DNA analysed with the new enzyme charge. Therefore, an experiment was performed to estimate the methylation level values of all analysed control and AA-treated samples, as well as CT-DNA samples. The values were calculated using the previous correction factor illustrated in equation **1** (above in section (3.2)). Then, a further correction factor was determined by dividing the average value of methylation level of the CT-DNA previously calculated with the established correction factor (equivalent to 6.50) by the arithmetic mean value of the methylation levels of CT-DNA which were analysed in the subsequent hydrolysis sequences. This second correction factor was then applied for the calculation of the methylation level values of all the analysed (control and AA-dosed) samples in this study. The calculated correction factors for each hydrolysis sequence are listed in **table 5** for the accurate determination of the methylation levels of the samples.

Hydrolysis sequence	Correction factor
1	0.772
2	0.781
3	0.774
4	0.767
5	0.768
6	0.778
7	0.775
8	0.771

Table 5: **Calculated correction factors of CT-DNA analysed through MEKC sequences in AA-dosed rat study** (hydrolysis was done with 15- fold enzyme mixture)

In each sequence, CT-DNA samples as well as three dosed-rats and/or control-rats samples were hydrolysed, derivatised, analysed with CE-LIF to achieve an accurate analysis. Moreover for accuracy, each sample was analysed six to eight times via a sequence carried out over about 24 h (difficulties arising influenced the time needed). From each organ, four samples were analysed: two AA-treated rat samples with 30 mg AA/kg BW and two control samples. The results of the determination of the methylation level are illustrated in **table 6**, whereby the values represent the arithmetic means of the methylation levels of the individual measurements for each sample and the standard deviations of the individual measurements. The

equations used for the calculation of the arithmetic mean (Equation **4**) and standard deviation (Equation **5**) can be summarised as follow [487] :

$$\bar{x} = \frac{\sum_{i=1}^{n} x_i}{n} \qquad (4)$$

$$S = \sqrt{\frac{\sum_{i=1}^{n} \left(x_i - \bar{x}\right)^2}{n}} \qquad (5)$$

Where $\bar{x}$: arithmetic mean of methylation level
S : standard deviation
x_i : methylation level of single measurement
n: number of measurements

Organ	AA Treatment	Methylation level [%] ± S.D.	n
Forestomach	*Control*	**2.95 ± 0.05**	**5**
		2.96 ± 0.08	**4**
	30 mg/kg AA	3.91 ± 0.02	6
		3.91 ± 0.06	4
Colon	*Control*	**4.07 ± 0.02**	**5**
		3.95 ± 0.02	**6**
	30 mg/kg AA	4.39 ± 0.05	8
		4.52 ± 0.04	8
Kidney	*Control*	**3.40 ± 0.07**	**5**
		3.44 ± 0.03	**7**
	30 mg/kg AA	4.25 ± 0.05	6
		3.74 ± 0.06	7

Bladder	*Control*	**3.48 ± 0.03**	**6**
		3.49 ± 0.04	**8**
	30 mg/kg AA	3.69 ± 0.02	8
		3.60 ± 0.05	5
Small intes-tine	*Control*	**3.57 ± 0.03**	**5**
		3.93 ± 0.04	**6**
	30 mg/kg AA	3.73 ± 0.06	6
		3.89 ± 0.03	6
Liver	*Control*	**3.62 ± 0.03**	**5**
		3.73 ± 0.03	**6**
	30 mg/kg AA	3.84 ± 0.04	8
		3.73 ± 0.03	6

Table 6: **Methylation levels of DNA isolated from different organs of AA-dosed rats (n:** Number of measurements**)**

3.5.1.3 Computation and evaluation of data

In the majority of the measurements, the data points do not contribute equally to the final average. In addition, the illustrated genomic methylation levels in **table 7** involve the arithmetic means of series of each sample under different sample sizes (number of measurements). The overall situation had imposed the necessity of calculating the weighted mean to compare accurately the methylation level data of treated samples with that of control samples. Thus, both the weighted mean of the average values of two treated samples and the weighted mean of the average values of two control samples for each organ were calculated according to equation **6**. And, the weighted standard deviation, the square root of the variance, was calculated within the series of measurements according to equation **7**. The average weighted methylation levels of the different organs are listed in **table 7**.

$$\overline{\overline{X}} = \frac{\sum_{i=1}^{k} n_i \, \overline{x_i}}{\sum_{i=1}^{k} n_i} \qquad (6)$$

$$S_{within} = \sqrt{\frac{\sum_{i=1}^{k} S_i^2 (n_i - 1)}{\left[\sum_{i=1}^{k} n_i\right] - k}} \qquad (7)$$

Where
$\overline{\overline{X}}$: weighted mean of methylation level
$\overline{x}$: arithmetic mean of methylation level
S_{within}: standard deviation of weighted mean within the series
S: standard deviation of single measurements
k: number of series (animals)
n: number of measurements

Organ	Methylation level [%] ± S.D.	
	Control	Treated
Forestomach	2.95 ± 0.06	3.90 ± 0.04
Colon	4.00 ± 0.01	4.46 ± 0.04
Kidney	3.42 ± 0.05	3.97 ± 0.05
Bladder	3.49 ± 0.03	3.65 ± 0.03
Small intestine	3.77 ± 0.03	3.80 ± 0.04
Liver	3.68 ± 0.03	3.80 ± 0.03

Table 7: Weighted means of methylation levels in different organs from AA-dosed rats

A different methylation level behaviour can be observed in the results from the tested organs. The methylation values had clearly shown that forestomach, colon and kidney of AA-dosed rats are hypermethylated (**Fig. 45**). In a cooperation with the research group of Prof. Cai in the Department of Chemistry at the Hong Kong Baptist University, a parallel study was performed with the same set of samples and tissues with the LC-ESI-MS technique [488] to detect DNA adducts. A variety of AA-DNA adducts were quantified, characterised and detected by LC-MS, namely dA-AAI, dA-AAII in kidney and liver, dC-AAII in kidney of rats dosed with AA at high levels and to a lesser extent dG-AA adduct (only in kidney). Chan *et al.* [488] provided the explanation that the exo-cyclic amino group of the aristolactam moiety of the guanosine adduct was situated in the narrow minor groove of the DNA strand, in contrast to that of adenosine adduct, which was located in the major groove and so easily accessible, so that the yield of dA-AA adducts was higher. This is a similar explanation for the A:T to T:A mutation scheme associated with the study of mutagenicity of AA in Muta ™ Mouse [489].
Additionally, a previous study performed on rats treated with 0.1, 1.0 or 10 mg AA/kg BW over a period of three to six months, had revealed squamous cell carcinoma of the forestomach as well as benign and malignant tumours of kidneys and urinary tract [339, 340]. It seems that AA stimulate cell proliferation in the forestomach and leads to cancer promotion [490]. This finding confirms our results that the hypermethylation is a valuable biomarker for the possible finding of tumours in both stomach and kidney organs after exposure to AAs. A similar approach with the study of Mengs [318] on the effect of AA on the forestomach had shown squamous cell carcinoma in the forestomach of 39 female mice treated with 5 mg/kg of AA and only one recorded case [318] of adenocarcinoma in the glandular stomach as well. This statement affirms our results revealing the risk of tumour development in the stomach after AA exposure, whether the experiments were performed on rats or mice.
Surprisingly, the colon had presented an obvious hypermethylation in the AA-dosed rats, although this is not target tissue for AA, but is a representative organ recommended in the comparative studies of normal and tumour cells. Since the colon-cell kinetics in these two cases are similar, this represents an advantage in such studies specifically in the determination of DNA methylation [491]. A similar hypermethylation pattern had been detected in mapping of the methylation pattern of specific promoter gene to be associated with the incidence of colon cancer [492]. This comparison could help to establish a correlation of the probability of the development of cancer of the colon with its exposure to AA, in dependence on the dose and the time of exposure.

On the other hand, neither bladder, nor small intestine nor liver exhibited a tendency toward more or less methylation (**Fig. 46**).

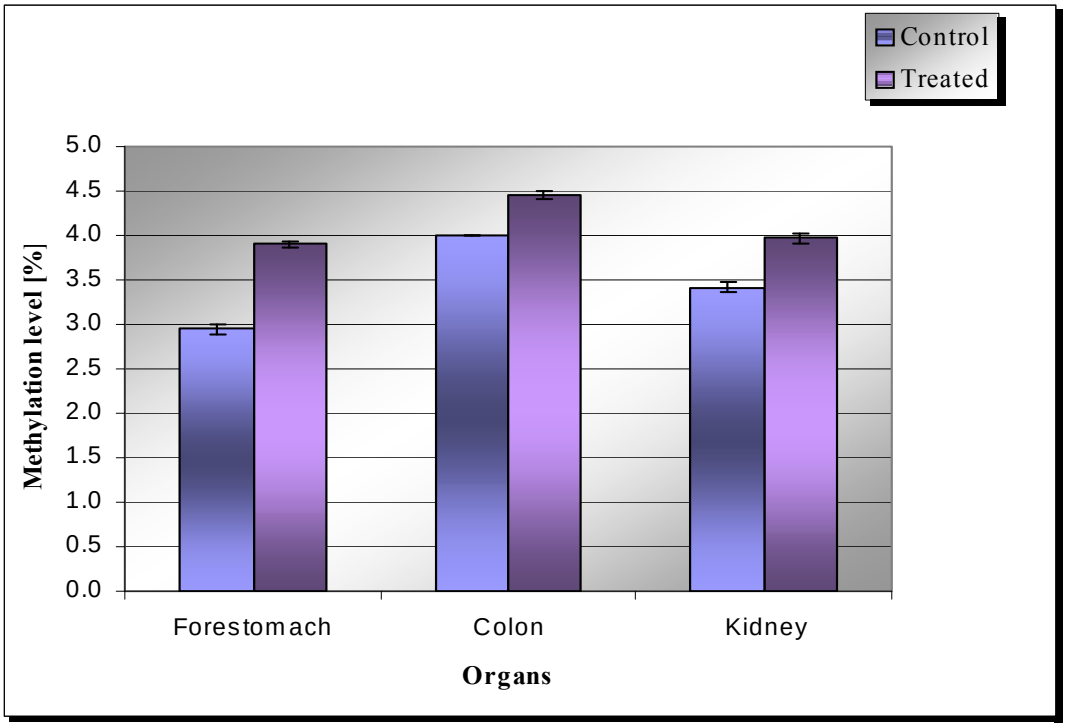


Figure 45: Aberrant methylation level of certain organs taken from rats after AA exposure. The values are the weighted mean of the average values ± S.D.

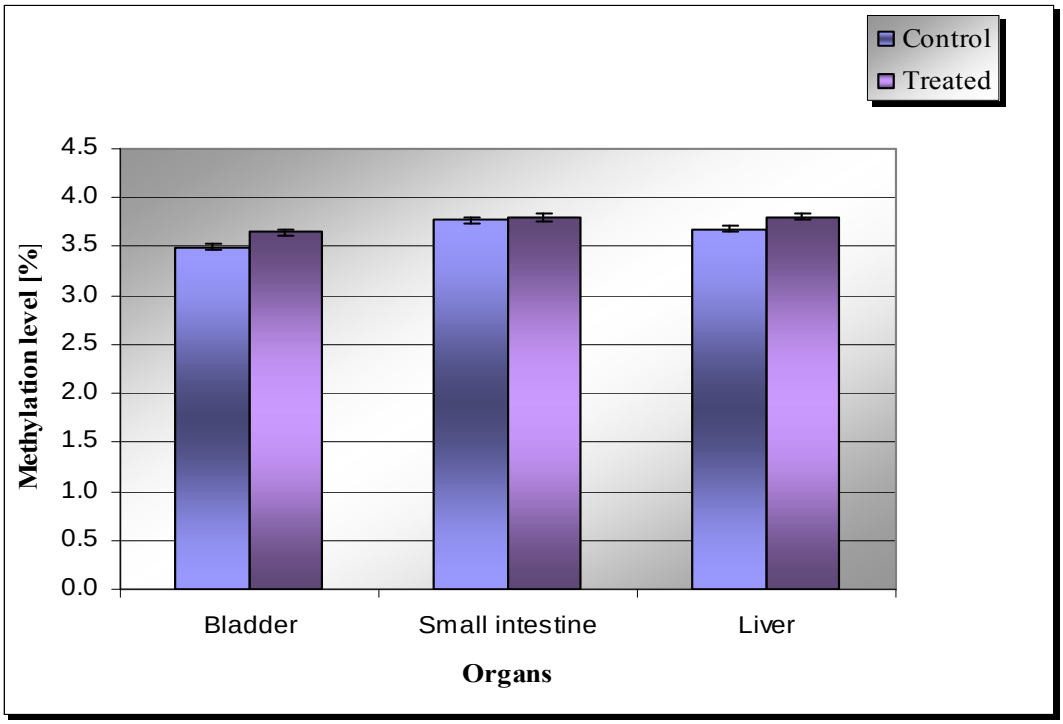


Figure 46: Stable methylation level of some organs taken from rats after AA exposure. The values are the weighted mean of the average values ± S.D.

There have been few reports of liver carcinoma cases due to AA exposure, although hepatic foci and nodules formed in consequence to the exposure of rats to 10 mg/kg AA under specific conditions have been recorded [493]. This is consistent with our data received from CE-LIF analysis, which manifests no change of the methylation status in the rat liver tissue although, the AA-DNA adducts, mainly dA-AAI, were detected in liver by the parallel assay performed for these samples with LC-ESI-MS [488]. The findings of the hypermethylation level in kidney in comparison with control and that there was no change in the methylation degree in liver are identical to the gene expression patterns reported in the study of Arlt *et al.* [333], confirming the alteration of the expression levels of more genes in kidney than in the liver following 10 mg AA/kg BW treatment of Big Blue rats. In addition, Arlt *et al.* [333] declared that a variety of biological processes related to defence response and immune response were changed in kidney, but not in liver.

Urothelial cell tumours can be induced by a number of chemical carcinogens through environmental and/or occupational exposure [295, 494]. Recently, AA was also recorded as a causative factor in the development of urothelial cancer [336, 484, 485]. However, the A:T mutations caused by specific AA-adenine base adduct were not reported in urothelial cancer. Because of this, it was necessary in our study to test the pattern of methylation level in the bladder due to its exposure to the carcinogen AA to see whether another part of the urinary tract also undergoes a change in methylation, as previously mentioned in case of kidney. The analysis of the samples taken from the bladder shows no significant difference in the methylation level between the animals treated with AA and the control animals (**Fig. 55**). This data is found to be consistent with the previous clinical finding that bladder cancer is an invasive form of urothelial cancer [495], and, among urothelial tumours, cancers caused by AA had shown a tendency to develop in the upper urinary tract [496]. Perhaps the exposure period of the rats used in the experiment to AA was not long enough to affect the genomic methylation level. It is highly recommended that further study be carried out to monitor the effect of AA in bladder and related tissues for longer exposure time. In conclusion, these differences clearly suggest that a correlation can be established between AA exposure and aberrant methylation level in different tissues, which is reported to be associated with gene expression leading to cancer formation.

3.5.2 Influence of aristolochic acid treatment on Hupki mice

3.5.2.1 CE-LIF analysis of methylation level in mice dosed with AA

P53 is one of the most important genes in carcinogenesis and for this study, Hupki mice were used, which are produced by insertion of human p53 gene in place of some parts of murine *P53* gene in mice [497]. The DNA methylation levels in 48 DNA samples from different organs (pancreas, stomach, spleen, colon, liver, lung and kidney) of Hupki mice were determined by CE-LIF. The Hupki mice were treated daily with an oral dose of 5 mg AA/kg BW. One group of mice was sacrificed three weeks after the AA dosage i.e., 24 h after the last treatment with AA, and another group was sacrificed 52 weeks after the first treatment. Several DNA samples were extracted from the collected organs, purified and delivered in aliquots of 10 µg from the group of Dr. Arlt at the Institute of Cancer Research in England. The analysis of the samples was done by Schelle [498] in our Analytical Chemistry department in Wuppertal

In that study, the hydrolysis of DNA samples involved with AA-adduct formation was obviously done with a high concentration of enzyme mixture, as previously performed in the hydrolysis of AA-dosed rat samples. The factor was calculated for these analysed samples, following the same protocols as were clarified in section 3.5.1.2. The accuracy of measurements was controlled by analysing 16 CT-DNA samples in parallel with the 48 Hupki DNA samples provided. Each sequence, consisting of a CT-DNA sample and three real samples, was carried over a period of 36-45 h, and each sample was analysed twelve to fifteen times. So, the accuracy of measurements was definitely assured by this analysis procedure.

From each organ, the analysis was done for three AA-treated mice samples with 5 mg AA/kg BW and three control samples. The methylation levels determined by CE-LIF are illustrated in **table 8**, whereby the values are the arithmetic means of the methylation level of the individual measurements for each sample according to equation **4**. The errors in the methylation level given by the standard deviations of all individual measurements of AA-treated or control sampls were calculated with equation **5**.

Organ	**Treatment with AAI**	**Methylation level [%] ± S.D.**	**n**
Pancreas	*Control*	**4.01 ± 0.10**	**10**
		3.44 ± 0.11	***11***
		4.00 ± 0.06	**12**

	3 Weeks	*3.64 ± 0.08*	*12*
		4.26 ± 0.05	*12*
		*	*
Glandular Stomach	*Control*	***4.30 ± 0.08***	***11***
		4.02 ± 0.09	***10***
		*	*
	3 Weeks	*4.02 ± 0.05*	*12*
		3.95 ± 0.07	*12*
		4.06 ± 0.06	12
Spleen	*Control*	**4.31 ± 0.09**	**10**
		4.23 ± 0.05	**10**
		4.51 ± 0.04	**9**
	3 Weeks	*3.86 ± 0.06*	*10*
		4.24 ± 0.08	11
		4.26 ± 0.06	13
Kidney	*Control*	**4.13 ± 0.05**	**12**
		4.25 ± 0.09	**12**
		4.33 ± 0.08	**9**
	3 Weeks	4.24 ± 0.06	12
		4.23 ± 0.05	12
		4.28 ± 0.06	10
Colon	*Control*	**4.31 ± 0.06**	**12**
		4.21 ± 0.13	**12**
		4.49 ± 0.08	**11**

	3 Weeks	3.87 ± 0.04	11
		3.92 ± 0.05	10
		4.72 ± 0.09	*15*
Liver	*Control*	**4.08 ± 0.10**	**9**
		4.09 ± 0.09	**12**
		4.46 ± 0.06	***7***
	3 Weeks	*	*
		4.29 ± 0.12	11
		4.35 ± 0.07	10
Lung	*Control*	***4.37 ± 0.07***	***8***
		*	*
		3.96 ± 0.06	***10***
	3 Weeks	3.68 ± 0.09	12
		4.15 ± 0.07	*11*
		3.74 ± 0.10	9
Kidney	*Control*	**3.76 ± 0.10**	**12**
		3.93 ± 0.05	**9**
		3.98 ± 0.04	**10**
	52 Weeks	4.25 ± 0.07	9
		4.36 ± 0.10	12
		4.30 ± 0.11	11

Table 8: **Methylation level values of DNA isolated from different organs of AA-dosed Hupki mice (n:** Number of measurements**, *** Rejected data**)**

Some values had shown a large deviation and thus were not taken into consideration in the further statistical evaluation of the data. (These values are marked with different colour and in italic writing style in **table 8**).

3.5.2.2 Computation and evaluation of data

The mean values of measurements made for each organ were handled for better evaluation of data by calculating the weighted mean values from equation **6**. In addition, the standard deviation within the series of measurements was calculated from equation **7**, taking in consideration the different number of random tests as well as the number of animals. The data presented in **table 9** are obtained on the basis of the formulae used for the weighted methylation levels without the red marked values and for the standard deviations.

Organ	**Methylation level [%] ± S.D.**	
	Control (3 weeks)	**Treated (3 weeks)**
Pancreas	*	*
Glandular Stomach	*	*
Spleen	4.34 ± 0.06	4.25 ± 0.06
Kidney	4.23 ± 0.07	4.25 ± 0.05
Colon	4.33 ± 0.09	3.89 ± 0.04
Liver	4.09 ± 0.09	4.32 ± 0.09
Lungs	*	*
	Control (52 Weeks)	**Treated (52 Weeks)**
Kidney	3.88 ± 0.07	4.31 ± 0.09

Table 9: **Weighted means of methylation level average values of different organs of AA-dosed mice (*** Rejected data due to extreme deviation from the mean**)**

For a better overview, the methylation levels determined as weighted means of the individual values of various organs are presented also in **figure 47**. This shows clearly that the response of several organs in their methylation levels is different upon AA exposure. This difference

declares that tissue specificity of the organs plays a fundamental role in the aberrant behaviour of the methylation level of the studied organs that distinguish the normal tissues from abnormal [499, 500]. The colon taken from mice dosed with AAI had shown a hypomethylation, while the liver had shown a hypermethylation. Interestingly, both colon and liver had shown a great difference between AA-treated samples and controls but in opposite directions. Unfortunately, there are too few measured values for the pancreas, glandular stomach and lungs to allow a reasonable conclusion.

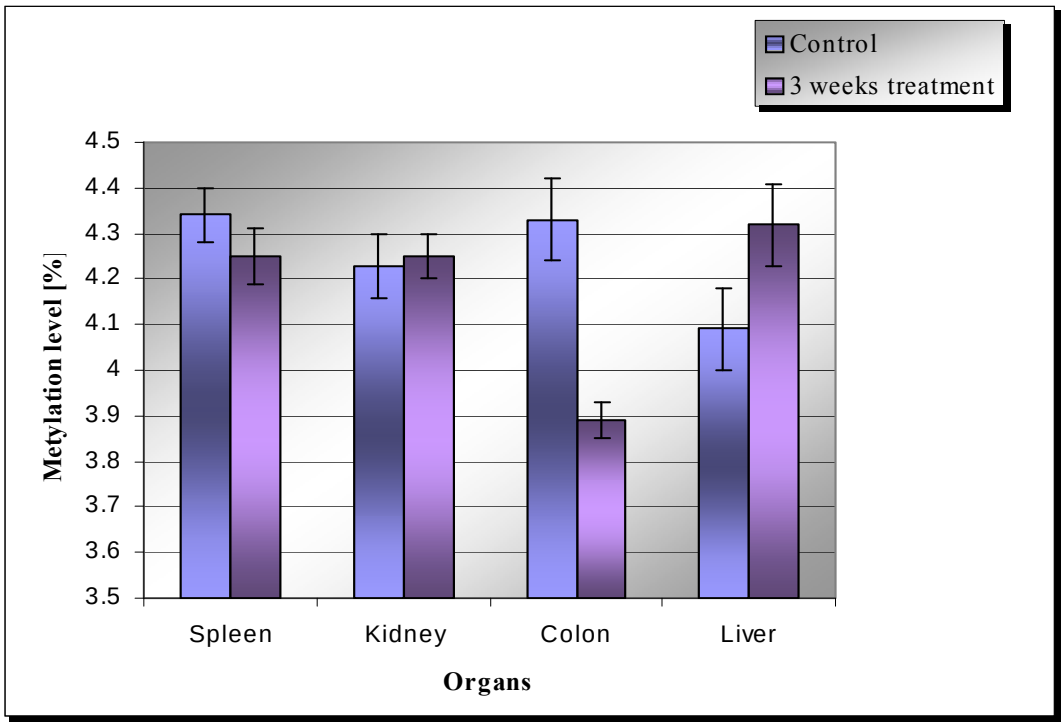


Figure 47: Methylation level of the individual studied organs taken from AA-dosed mice. The values are the weighted mean of the average values ± S.D.

3.5.2.3 Effect of the length of treatment

The change of the average methylation level in the kidney as a function of length of exposure of the mice to AA is illustrated in **figure 48**. No clear deviation in the methylation level can be observed between mice treated for three weeks and the untreated mice; while an extreme deviation could be determined in case of 52 weeks of mice treatment with AAI. Strong similarity is found to the reported histopathological observations of Mengs [318], that no neoplastic signs had been detected in AA-treated mice for weeks, till the manifestation of cystic papillary adenomas in the renal cortex beginning in the 26th week. Equally, this reported observation supports the dose dependence of the AA effect in the DNA of kidney [488] in terms of length of exposure to AA. In addition, it is probable that the clearance of AA-adduct from the

body of the mice was variable, since the group treated for three weeks was sacrificed 24 h after stoppage of treatment, while the group treated for 52 weeks was sacrificed immediately after last AA dosage.

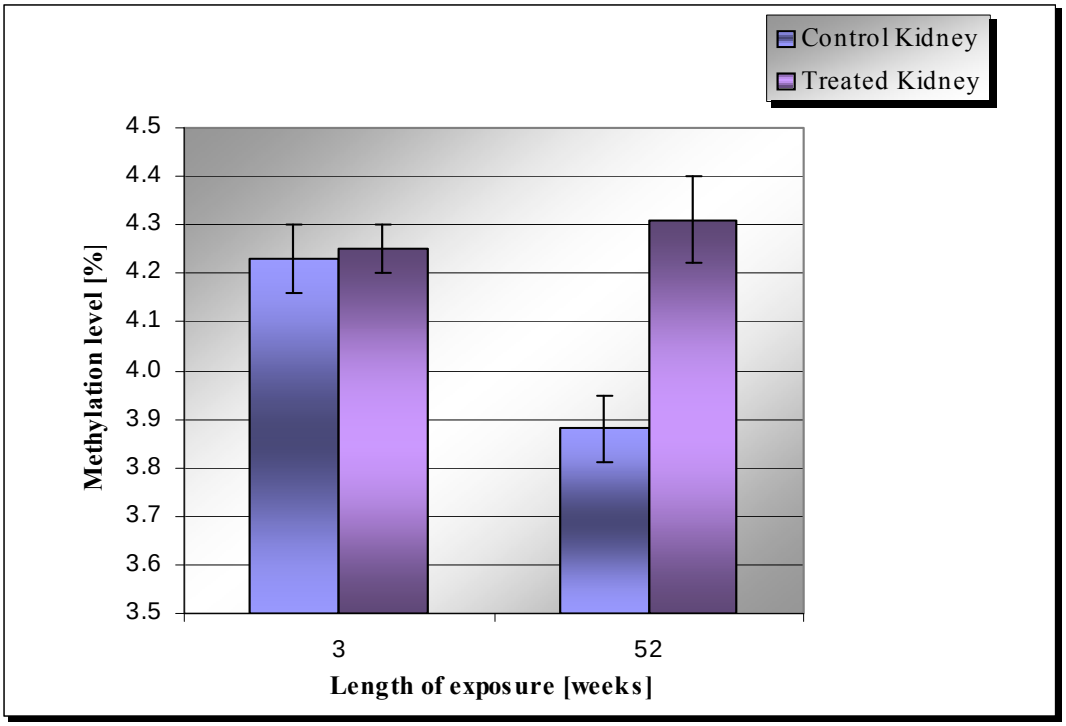


Figure 48: The effect of length of AA-exposure on methylation level of dosed mice. The values are the weighted mean of the average values ± S.D.

Additionally, it is remarkable that the genomic methylation level is inversely related to the age of control animals. This comparison of data between both controls is congruent with the conclusion of research that had found a decline of global DNA methylation with ageing [501], while a hypermethylation was determined in gene-specific methylation patterns [502]. In my opinion, this finding is reinforced by the aberrant methylation level of mice under the influence of AA. Because the samples taken from treated animals do not agree with this conclusion [501], but exhibit a slight hypermethylation of 52-weeks samples in comparison with 3-weeks studied samples. The alteration of methylation pattern by the effect of AA had compensated the decrease of methylation level that should be caused by ageing. Additionally, this difference might be related to the nutrition regime [503] followed for a longer time for the group treated for 52 weeks. It is concluded that AA is a highly dangerous mutagen that vigorously damages DNA and leads to higher methylation levels.

3.5.2.4 Correlation between the adduct level and the methylation level

A parallel study was performed by the institute of cancer research in London for the determination of AA-DNA adducts in the various organs. It is remarkable how different the adduct levels, determined per 10^8 nucleotides, are in the individual organs (**Fig. 49**). Among adduct level patterns of the different organs, the kidney is the outlier, with the highest adduct level after three weeks treatment of the mice with aristolochic acid. We found that the kidney samples are exceedingly highly methylated after 52 weeks treatment of mice with aristolochic acid. Although the duration of AA exposure is different in the two findings, both high levels of adducts and methylation level are related specifically to kidney.

It is found that the hypomethylation level detected in case of colon correlates with the low adduct levels identified by the ^{32}P-postlabeling assay. On the other hand, the liver also shows a low adduct level, but a hypermethylation level is obvious. Thus, a correlation between adduct levels and the genomic methylation level is unlikely and was indeed not found. In conclusion, it should be useful to examine the methylation level of the other organs after 52 weeks AA treatment in future studies.

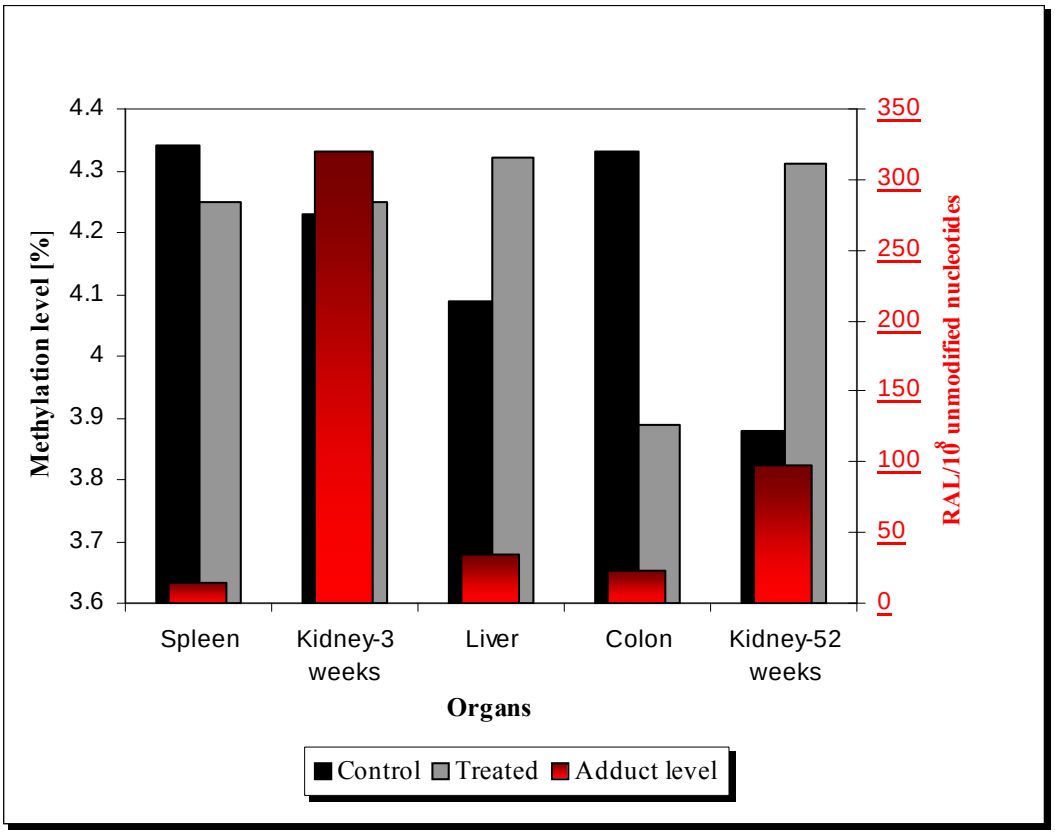


Figure 49: Methylation levels (CE-LIF method) and adducts levels (^{32}P-postlabeling assay) of various organs of Hupki mice dosed with AA

3.5.3 *In-vivo* study of the influence of 3-nitrobenzanthrone treatment (3-NBA) on Sprague-Dawley rats

3.5.3.1 Quantification of DNA in 3-NBA-dosed rat samples

In this study, the female Sprague-Dawley rats were treated with a single dose of 0.2 or 2.0 mg 3-NBA/kg BW by intratracheal instillation under ether anaesthesia. The rats were sacrificed 48 h after 3-NBA administration [504]. Seven organs were removed (pancreas, heart, kidney, urinary bladder, small intestine, liver and lungs) and immediately frozen in liquid nitrogen and stored at -80°C; then DNA samples were isolated from these organs.
For the present work, 36 DNA samples were provided by the German Cancer Research Centre in Heidelberg (Deutschen Krebsforschungszentrum Heidelberg, DKFZ) and analysed by Schiewek [505]. The samples were first analysed by UV-VIS spectrophotometry to quantify the samples as previously described (section 3.2.). The calculated DNA quantities and the extent of purities of the 3-NBA-treated and control samples are summarised in **table 10**.

Organ	Control			Treatment with 3-NBA		
	Sample	DNA amount [µg]	Purity	Sample from animal	DNA amount [µg]	Purity
Left Lung (0.2 mg/kg BW)	**Control**	10.4	1.64	**4**	20.4	1.68
				5	27.6	1.74
Right Lung (0.2 mg/kg BW)				**4**	32.5	1.76
				5	32.2	1.76
Small Intestine (0.2 mg/kg BW)	**Control**	29.2	1.83	**4**	34.9	1.76
				5	37.8	1.77
Pancreas (2 mg/kg BW)	**Control**	28.6	1.79	**1**	20.0	1.75
				2	23.0	1.76
				3	35.1	1.83
Heart (2 mg/kg BW)	**Control**	33.10	1.82	**1**	32.5	1.76
				2	25.3	1.76
				3	-	-
				1	27.5	1.84

Kidney (2 mg/kg BW)	**Control**	31.00	1.84	**2**	25.5	1.83
				3	9.3	1.79
Bladder (2 mg/kg BW)	**Control**	15.30	1.84	**1**	28.50	1.86
				2	16.70	1.79
				3	14.00	1.78
Small intestine (2 mg/kg BW)	**Control**	29.2	1.83	**1**	25.1	1.82
				2	28.7	1.81
				3	23.9	1.82
Liver (2 mg/kg BW)	**Control**	30.0	1.80	**1**	19.2	1.69
				2	22.0	1.67
				3	11.0	1.84
Lungs (2 mg/kg BW)	**Control**	10.4	1.64	**1**	28.6	1.83
				2	30.1	1.79
				3	39.2	1.84

Table 10: DNA quantification in 3-NBA-dosed rats and control samples and their purities [505]

The dissolved samples were aliquoted in 10 µg then lyophilised under speed vacuum and stored at -25°C. 28 samples were taken from three experimental animals (numbered 1, 2 and 3) treated with 2.0 mg 3-NBA/kg BW and a control animal. In addition, eight samples: six were taken from left lung, right lung and small intestine of two experimental animals (numbered 4 and 5) treated with the lower dose of 0.2 mg 3-NBA/kg BW and two control samples from lung and small intestine were analysed.

3.5.3.2 CE-LIF analysis of methylation level in rats dosed with 3-NBA dosed rats

For the accurate evaluation of measurements, CT-DNA was used as external standard to be analysed in parallel with the samples in the same sequence. The accuracy of measurements was attained by nine analyses of each sample. The values of methylation level and the standard deviation of the mean values of individual measurements of each organ for each animal were calculated, and the statistic analysis of the methylation levels of the samples was done by PD. Dr. Scherer in the Analytical-Biological Research Laboratory in Munich (Analytisch-Biologisches Forschungslabor München). The mean methylation levels from the experimental study for the low and high dose in different tissues are illustrated in **tables 11** and **12**, respectively.

Organ	Animal	Methylation level [%] ± S.D.
Left lung	*4*	3.97 ± 0.15
	5	4.19 ± 0.24
Right lung	*4*	4.06 ± 0.20
	5	4.06 ± 0.20
Lungs	***(control)***	4.01 ± 0.22
Small intestine	*4*	*
	5	4.03 ± 0.32
	(control)	4.08 ± 0.30

Table 11: **Methylation levels in two organs of rats dosed with 0.2 mg/kg BW 3-nitrobenzanthrone (* No sample was available)**

Organ	Animal	Methylation level [%] ± S.D.
Pancreas	*1*	3.67 ± 0.04
	2	3.65 ± 0.15
	3	3.75 ± 0.12
	(control)	3.62 ± 0.09
Heart	*1*	3.56 ± 0.04
	2	3.53 ± 0.09
	3	*
	(control)	3.53 ± 0.17
Kidney	*1*	3.71 ± 0.05
	2	3.72 ± 0.04
	3	*
	(control)	3.71 ± 0.09
Bladder	*1*	3.38 ± 0.13
	2	3.36 ± 0.08
	3	3.38 ± 0.08
	(control)	3.42 ± 0.18
	1	3.68 ± 0.14

Small intestine	*2*	3.65 ± 0.14
	3	3.72 ± 0.08
	(control)	3.85 ± 0.09
Liver	*1*	4.29 ± 0.09
	2	4.32 ± 0.05
	3	4.30 ± 0.10
	(control)	4.29 ± 0.05
Lungs	*1*	3.59 ± 0.10
	2	3.72 ± 0.06
	3	3.71 ± 0.06
	(control)	3.83 ± 0.05

Table 12: **Methylation levels in various organs of rats dosed with 2 mg/kg BW 3-nitrobenzanthrone (* No sample was available)**

3.5.3.3 Computation and statistical analysis of data

Statistical analysis of the results obtained by the CE-LIF analysis has been carried out. No significant difference was found between controls and the samples of the different tissues examined, including the samples of lung and small intestine treated with 0.2 mg/kg BW of 3-NBA.

In the high-dose experiment, a significant difference was interestingly observed between the samples and controls in case of small intestine and lung; a hypomethylation was revealed with a confidence limit of 95 % (**Table 12**). The appearance of hypomethylation in both S.I. and lungs may be explained by the 3-NBA-adduct formation. The presence of such a bulky carcinogen (3-NBA) could result in a local change in the structure of the DNA strand similar to that caused by BPDE-dG adduct formed in CpG dinucleotide and lead to steric hindrance [506, 507]. Because of the reciprocal hydrophobic effect that arises from the aromatic moiety of 3-NBA adduct and the methyl groups of the neighbouring 5-methylcytosines, the 5-meC could stand out from the DNA strand and thus serves as the substrate of isoforms of the DNA methyltransferases in the rats, whereby an aberration could consequently occur to the methylation level.

In conclusion, the lungs and small intestine in the high-dose study differ most in their methylation level from all other tissues after intratracheal exposure to 3-NBA. Accordingly, a com-

parative overlook between the mean of the methylation levels [%] of the controls in case of low-dose analysed samples in small intestine and lungs (4.08 ± 0.30 S.D. and 4.01 ± 0.22 S.D., respectively) and that of high-dose analysed samples (3.85 ± 0.09 S.D. and 3.83 ± 0.05 S.D., respectively) had surprisingly revealed a great difference, about 5 %. Although the control animals were identical in the two cases, this significant difference originates from an external factor, i.e. the two different charges of enzyme mixture used for DNA digestion in the two cases due to the differing times of analyses of the two sample sets. The low-dosage sample set was hydrolysed much later with a fresh charge of the enzyme mixture charge, since the charge used for the high-dosage sample set had unfortunately run out. The use of another enzyme charge changed the characteristics of the sample matrix and led to larger fluctuations in the methylation levels determined. It is highly probable that the diverse enzyme sources gave rise to a different distribution in the yield of the individual nucleotides and different data for the same controls.

3.5.3.4 Correlation between the adduct level and methylation level

The DNA samples examined in this section were ^{32}P-postlabeled by Bieler *et al.* [504] for the determination of 3-NBA-adduct in the studied organs. All the data obtained from the two studies are listed in **table 13** in order to establish a meaningful statistical comparison between treated samples, control samples and the adduct level. The weighted methylation levels shown in the table were computed with equation **6**, and the corresponding standard deviation values with equation **7,** taking in consideration the number of rats used in this experiment. The adduct pattern was identical in all the different tissues described according to the identified adducts, however different adduct levels were found in all organs.

It is interesting to observe that the hypomethylation degree detected in small intestine and lung does not correlate with the adduct level in these organs (**Fig. 50**). Although the small intestine shows the second lowest adduct level (98.1 ± 5.7 S.D. adducts per 10^8 unmodified nucleotides) determined among the organs examined, the lung tends to show the second highest concentration at DNA adduct level [504]. Moreover, the highest level of 3-NBA–DNA adducts was detected in pancreas (619.9 ± 372.2 S.D. adducts per 10^8 nucleotides), in contrast to the lowest level of 3-NBA-DNA adducts, which was detected in liver (59.1 ± 31.1 S.D. adducts per 10^8 nucleotides). However, there is no difference in methylation levels between

control and treated samples in the two tissues. Hence, it is concluded that no definite correlation could be established between the adduct concentration in different tissues and the determined genomic methylation level.

Organ	Adducts / 10^8 unmodified nucleotide	5-meC [%] of control ± S.D.	Weighted mean of 5-meC [%]of samples ± S.D.
Small intestine	98.1 ± 5.7	3.85 ± 0.09	3.68 ± 0.01
Pancreas	619.9 ± 372.2	3.62 ± 0.09	3.69 ± 0.02
Heart	220.0 ± 101.1	3.53 ± 0.17	3.55 ± 0.01
Kidney	334.8 ± 50.6	3.71 ± 0.09	3.72 ± 0.002
Bladder	215.2 ± 56.4	3.42 ± 0.18	3.37 ± 0.004
Lungs	349.6 ± 138.5	3.83 ± 0.05	3.67 ± 0.02
Liver	59.1 ± 31.1	4.29 ± 0.05	4.30 ± 0.01

Table 13: **DNA-adduct concentration [504] and methylation levels in different organs in the 3-NBA-dosed rats study [505]**

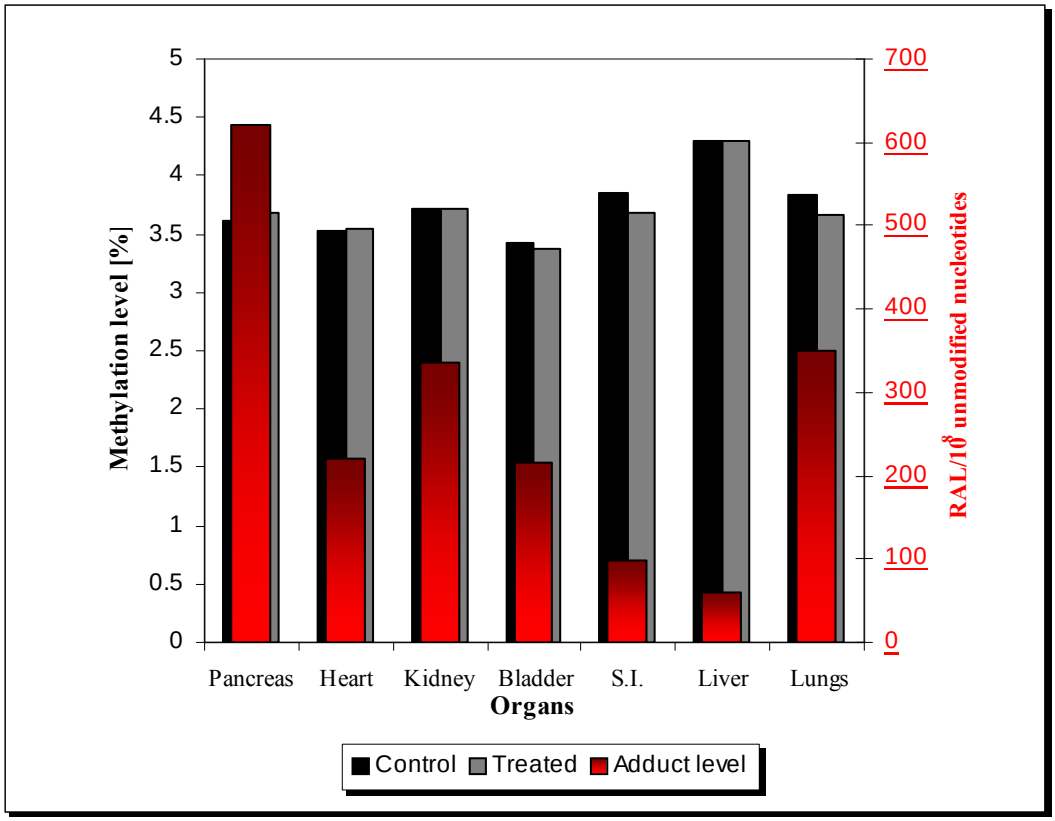


Figure 50: Methylation levels (CE-LIF method) and adducts levels (^{32}P-postlabeling assay) of various organs of Sprague-Dawley rats dosed with AA [398]

3.5.3.5 Comparison of the effect of various exogenous carcinogens on the genomic methylation level of Sprague-Dawley rats

Two studies were performed independently on Sprague-Dawley rats to investigate the effect of carcinogens belonging either to natural product group (AA) or chemical group (3-NBA) on the methylation level of the DNA samples isolated from different organs from the animals. The studies were different in some parameters such as the dose and route of administration of carcinogens and other factors related to the rats (sex, weight, and age). However, the most striking conclusion that imposes itself from this comparison is the tissue specificity of the DNA adducts formed, in meaning, first that 3-NBA-DNA adducts are preferentially formed in the lung after intratracheal exposure and second that the kidney is the susceptible target tissue of AA-DNA adduct formation. Moreover, a hypomethylation level was determined in the case of 3-NBA-DNA adducts in lung, in contrast to the hypermethylation level determined in the case of AA-DNA adducts in kidney. This observation helps to highlight how useful the aberrant genomic methylation level, either hypo- or hyper-status, is as a biomarker for carcinogenesis and other related dangerous diseases that are due to exogenous chemicals, so that it can be a valuable tool to prevent cancer risk. Finally, this comparison reinforces the conclusion that the extent of methylation of cytosine residues varies strongly between tissues and can be used as marker to distinguish healthy from unhealthy ones [(499)].

3.5.4 *In-vitro* study of various carcinogens on the genomic methylation level

3.5.4.1 Quantification of DNA in Caco-2 cell samples

In previous parts of this work, the study of the effect of exogenous carcinogens on the methylation level was performed with samples treated *in-vivo*. Equally important, the study in this part was focused on the determination of methylation level in *in vitro* samples that were under the influence of carcinogens for different lengths of time. DNA samples were isolated from Caco-2 cells incubated with different carcinogens (B[*a*]P, 4-ABP or 3-NBA) in the two concentrations of 2.5 and 5 µg/kg for 24, 48 and 72 h. Caco-2 cells are derived from human adenocarcinoma of the colon and they differentiate much as epithelial cells of the small intestine do [(508-510)]. Therefore, Caco-2 cells are used for *in-vitro* studies as models of the role of the small intestine i.e., drug absorption, transportation [(511)] and metabolism as well as models to investigate the cytotoxic functions of carcinogens [(512)].

The 24 DNA samples were quantified by UV-VIS spectrophotometery as described before (section 3.2). The calculated DNA quantities and the extent of purities of the carcinogen-treated samples and control samples are shown in **table 14**. The dissolved samples were aliquoted in 1 µg, lyophilised under speed vacuum and stored at -25°C.

Sample treatment	48 hours treatment			72 hours treatment		
	Sample Code	**DNA-amount [µg]**	**Purity**	**Sample Code**	**DNA-amount [µg]**	**Purity**
Control A	**7A**	9.1	1.76	**13A**	4.2	1.66
Control B	**7B**	10.4	1.75	**13B**	7.3	1.72
Control C	**7C**	3.4	1.67	**13C**	6.6	1.58
B[*a*]P 2.5 µg/kg A	**8A**	7.8	1.75	**14A**	7.7	1.58
B[*a*]P 2.5 µg/kg B	**8B**	8.0	1.79	**14B**	8.9	1.66
B[*a*]P 2.5 µg/kg C	**8C**	7.4	1.76	**14C**	5.5	1.58
4-ABP 5 µg/kg A	**9A**	7.9	1.74	**15A**	6.0	1.60
4-ABP 5 µg/kg B	**9B**	8.3	1.74	**15B**	5.6	1.61
4-ABP 5 µg/kg C	**9C**	7.8	1.79	**15C**	6.1	1.60
3-NBA 5 µg/kg A	**11A**	9.1	1.74	**17A**	7.1	1.67
3-NBA 5 µg/kg B	**11B**	6.5	1.77	**17B**	7.3	1.62
3-NBA 5 µg/kg C	**11C**	6.1	1.77	**17C**	7.5	1.61

Table 14: **DNA quantification in Caco-2 cells samples and their purities (**A,B,C: three different Caco-2 cells**)**

3.5.4.2 CE-LIF analysis of methylation level in Caco-2 cells incubated with various carcinogens

All the samples were subjected to CE–LIF analysis after hydrolysis, derivatisation and precipitation of the excess of the fluorescent marker as in the routine method described in the experimental section. The accuracy of measurements was guaranteed by carrying the analysis procedure so that eight CT-DNA samples were analysed in parallel with the 24 Caco-2 cells samples. All the samples were analysed by Klink [513] through successive sequences over 36-45 hours each. The methylation levels determined are plotted in **table 15** as arithmetic

means of individual measurements. The correction factor used for the calculation of methylation level was estimated as previously mentioned (3.5.1.2). The methylation levels and the standard deviation of the individual measurements were calculated with equations **4** and **5**, respectively.

Sample Code	Carcinogen treatment	Dose of treatment	Duration of treatment	Methylation level [%] ± S.D.	n
7A	Control A	0	48 h	3.68 ± 0.06	14
7B	Control B			3.64 ± 0.09	13
7C	Control C			3.55 ± 0.04	11
8A	B[*a*]P	2.5 µg/kg A		3.57 ± 0.09	15
8B		2.5 µg/kg B		3.62 ± 0.05	14
8C		2.5 µg/kg C		3.48 ± 0.09	10
9A	4-ABP	5 µg/kg A		3.63 ± 0.05	15
9B		5 µg/kg B		3.59 ± 0.08	14
9C		5 µg/kg C		3.72 ± 0.06	14
11A	3-NBA	5 µg/kg A		3.63 ± 0.07	15
11B		5 µg/kg B		3.78 ± 0.11	15
11C		5 µg/kg C		3.72 ± 0.07	12
13A	Control A	0	72 h	3.54 ± 0.11	12
13B	Control B			3.76 ± 0.08	14
13C	Control C			3.48 ± 0.08	15
14A	B[*a*]P	2.5 µg/kg A		3.54 ± 0.07	15
14B		2.5 µg/kg B		3.66 ± 0.07	15
14C		2.5 µg/kg C		3.49 ± 0.08	15
15A	4-ABP	5 µg/kg A		3.50 ± 0.14	15
15B*		5 µg/kg B		-	-
15C		5 µg/kg C		3.62 ± 0.06	15
17A	3-NBA	5 µg/kg A		3.60 ± 0.09	15
17B		5 µg/kg B		3.72 ± 0.06	15
17C		5 µg/kg C		3.49 ± 0.09	14

Table 15: **Methylation level values of DNA isolated from carcinogens-treated Caco-2 cells (n:** Number of measurements, * Rejected data. A, B and C: Three different Caco-2 cells) [513]

3.5.4.3 Computation and statistical analysis of data

No accurate data analysis could be carried out depending on the comparison of arithmetic means of samples with those of control due to different sample size (number of measurements). Therefore, the calculation of the weighted mean is helpful to better estimate the probability of the distribution of data. The weighted means of the methylation level values of control and carcinogen-incubated Caco-2 cells were calculated with equation **6** as described before (3.5.1.3). The standard deviation values within the series of measurements were corrected by equation **7** under the assumption that the variance of the measurements can be regarded as constant because the samples A, B and C are from the same origin. All weighted means of methylation levels are plotted in **table 16**.

Sample description	Treatment duration	Methylation level [%] ± S.D
Control	48 h	3.63 ± 0.06
B[*a*]P (2.5 µg/kg)		3.57 ± 0.08
4-ABP (5 µg/kg)		3.65 ± 0.07
3-NBA (5 µg/kg)		3.71 ± 0.09
Control	72 h	3.59 ± 0.09
B[*a*]P (2.5 µg/kg)		3.56 ± 0.08
4-ABP (5 µg/kg)		3.56 ± 0.10
3-NBA (5 µg/kg)		3.61 ± 0.08

Table 16: Weighted means of methylation level average values of Caco-2 cells incubated with different carcinogens

The weighted average values of the methylation level determined in DNA of control and Caco-2 cell samples incubated with carcinogens (B[*a*]P, 4-ABP and 3-NBA) for 48 and 72 h are graphically represented in **figure 51**. Surprisingly, a higher methylation level could be observed after 48 h incubation with 4-ABP and 3-NBA than that after 72 h incubation with the same carcinogens. The explanation may be that DNA repair system plays a role after short-term damage by such persistent DNA adducts. Therefore, it is worthwhile in future studies to monitor the effect of 3-NBA after incubation times longer than 72 h for the methylation status of Caco-2 cells.

Among all comparisons between the measurements, the 3-NBA-incubated samples had shown the highest deviation of methylation level from that of the corresponding control. Therefore, a

further statistical evaluation of control and 3-NBA treated samples after 48 h was performed to investigate whether a significant difference exists or not.

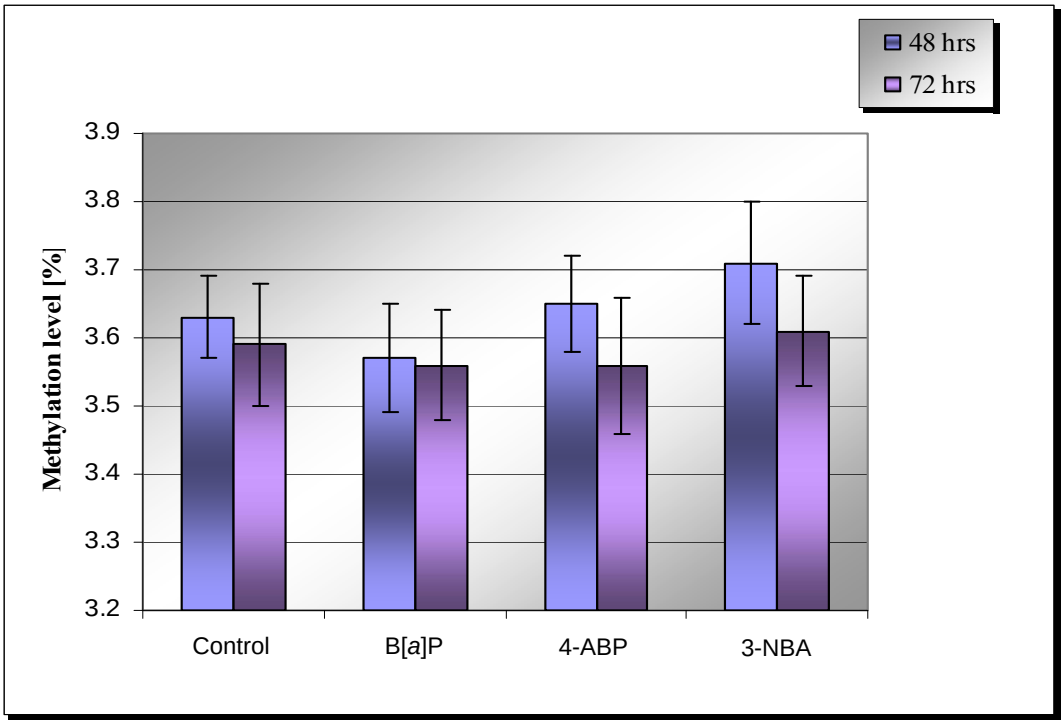


Figure 51: Methylation level of the studied DNA from Caco-2 cells incubated with different carcinogens. The values are the weighted mean of the average values ± S.D.

With the software graph PAD Prism® for comparison of the mean values for the control and 3-NBA samples, no significant difference between the control and 3-NBA treated Caco-2 cells (unpaired t test, $P < 0.05$) was obtained. However on the level of comparing the individual measurements of the two sample sets and not the means (three samples of each control and treated cells, the test does reveal a significant difference between the control and 3-NBA treated Caco-2 cells. This confusion raises the important question whether the difference between the two mean values is due to an insufficient number of studied cells or due to an insufficient number of measurements, especially since the F test which compares variances had indicated that there was no significance difference between the control and the carcinogenic-mediated Caco-2 cells (**Tab. 17**).

Description	Mean values comparison	Individual measurements comparison
Mean ± s.e.m. of control	3.623 ± 0.03762 (n= 3)	3.629 ± 0.01352 (n= 38)
Mean ± s.e.m. of 3-NBA-treated Caco-2 cells	3.711 ± 0.04307 (n= 3)	3.710± 0.0169 (n= 42)
Unpaired t test ($P < 0.05$)	ns difference	**
P value	0.1998 (two tailed)	0.1998 (two tailed)
t, df	t = 1.534 , df = 4	t = 3.713 , df = 78
F test	ns	ns
P value	0.8655	0.111

Table 17: **Statistical analysis of 48 hours 3-Nitrobenzanthrone study**
(ns: non significant. ** significant difference**)**

3.6 Influence of biological reproduction on DNA methylation

Nuclear transplantation or cloning is a new technology that attracted great interest not only from the researchers but also from people in general because of its potential applications in nutrition [514], biomedicine and husbandry [125, 514]. Inappropriate or incomplete epigenetic re-programming has been considered as the major cause for the low success of cloning [127, 135, 515] as well as for the abnormalities seen in developed clones. Simply explaining, during the resetting of the epigenetic marks of terminally differentiated somatic cell, aberrant epigeno-types of embryos and foetuses could be formed leading to abnormalities such as placental dysfunctions or foetal overgrowth phenotypes. Reprogramming of global DNA methylation during preimplantation development presents a window for epigenetic perturbation that may affect gene expression and phenotype throughout pre-embryo and foetus stages and postnatal life [142] from neonatal to adult. DNA methylation is the epigenetic mark of interest in the cloning process, and, accordingly, abnormal DNA methylation patterns have been examined

in the majority of the studies about epigenetic changes in animal clones. Despite the finding of epigenetic perturbations in these studies, the majority of cloned cattle beyond a critical period of about six months after birth, if they do not exhibit any health issues of concern, are assumed to be normal [515-517]. However, the DNA methylation status of the apparently healthy adult clones derived from the same nuclear donor has not been yet investigated. Therefore, the quantification of global 5-methylcytosine level of healthy adult cloned cattle was performed in this double-blinded study by using the former discussed CE technique [395, 399] to measure the 5-meC content in DNA extracted from different tissues.

In cooperation with the Institute of Molecular Animal Breeding and Biotechnology Gene Centre at the University of Munich, 84 DNA samples of bovine twins and clones (Simmental breed, 1µg) were provided. Also, in cooperation with the Laboratory of Development Biology and Reproduction (INRA) in France, 45 DNA samples (1 µg) of bovine clones (Holstein breed, 1 µg) were provided.

3.6.1 Accuracy of the measurements

The accuracy of the determination of the methylation level in the provided clones and twins samples can be proved by analysing each sample several times. In order to define the minimum number of single measurements that should be applied for all the analyses in this study, several random samples were chosen in advance to be hydrolysed, derivatised and analysed several times for the determination of the methylation level. The methylation level values of the individual measurements of a representative sample from the chosen set are shown in **figure. 52**.

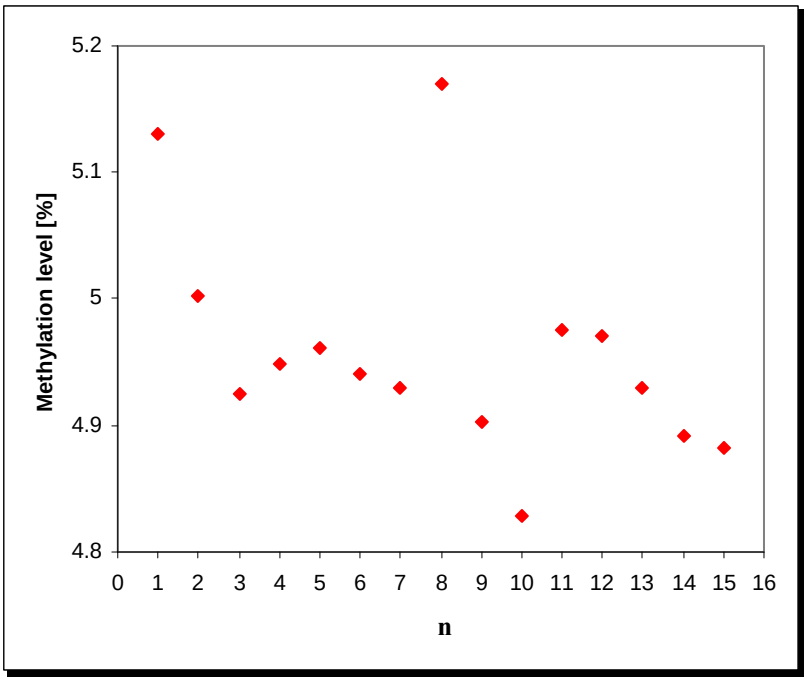


Figure 52: **Multiple measurements of a chosen hydrolysed and derivatised real sample**

The mean value of the methylation level of this series of measurements (4.96 ± 0.09 %, n=15) was calculated. It was concluded that eight or more individual measurements at least should be accomplished in order to obtain a reliable value for the methylation level.

3.6.2 Determination of 5-methyl cytosine level in blood cells of healthy Simmental and Holstein clones

The determination of the methylation level of 51 leukocytes samples isolated either from bovine twins or clones of Simmental breed and of 22 leukocytes samples isolated from bovine clones of Holstein breed was done by the established CE-LIF method. Each sample was hydrolysed, derivatised and precipitated according to the preparation of 1 µg DNA samples and finally analysed 8-15 times. The analysed methylation levels in DNA of leukocytes (white blood cells) from 42 healthy female clones at 1- 8 years of age of two different breeds and 13 pairs of Simmental twins are recorded in **tables 18**, **19** and **20**, noting that the indicated values represent the arithmetic means of the methylation level of the individual measurements for each sample. The errors of the methylation level are corrected by the factor established by Thiemann [398]. Statistical analysis was performed to evaluate the efficacy of the method.

Sample Code	Methylation level [%] ± S.D.	n
T1	5.35 ± 0.08	10
T2	5.26 ± 0.10	14
T3	5.07 ± 0.15	20
T4	5.10 ± 0.09	14
T6	5.60 ± 0.12	11
T9	5.43 ± 0.08	15
T10	5.29 ± 0.07	13
T11	5.51 ± 0.11	18
T12	5.47 ± 0.08	14
T13	5.00 ± 0.11	16
T14	5.53 ± 0.08	8
T15	5.32 ± 0.05	11
T16	5.20 ± 0.07	12
T17	5.38 ± 0.08	11
T18	5.54 ± 0.08	14
T19	5.43 ± 0.07	13
T20	5.52 ± 0.08	14
T21	5.36 ± 0.10	12
T22	5.35 ± 0.12	12
T23	5.61 ± 0.12	16
T24	5.46 ± 0.12	16
T25	5.35 ± 0.06	15
T26	5.64 ± 0.08	13
T27	5.46 ± 0.13	14
T28	5.57 ± 0.12	12
T29	5.36 ± 0.08	13
T30	5.21 ± 0.21	18

Table 18: Determination of methylation level in DNA of leukocytes of Simmental twins

Sample Code	Methylation level [%] ± S.D.	n
K1	6.83 ± 0.09	12
K2	6.38 ± 0.14	6
K3	6.48 ± 0.13	12
K4	6.48 ± 0.12	12
K5	6.47 ± 0.15	10
K6	6.93 ± 0.13	13
K7	6.79 ± 0.04	11
K8	6.36 ± 0.08	9
K10	6.54 ± 0.12	9
K11	6.42 ± 0.08	9
K12	5.94 ± 0.11	10
K13	6.84 ± 0.11	11
K14	6.18 ± 0.16	12
K15	6.69 ± 0.13	13
K16	6.75 ± 0.15	8
K18	6.54 ± 0.13	9
K19	6.26 ± 0.13	13
K20	6.35 ± 0.07	11
K21	6.35 ± 0.17	11

Table 19: Determination of methylation level in DNA of leukocytes of Simmental clones

Sample Code	Type	Methylation level± S.D.	n
S2251	Donor cell mother	5.09 ± 0.15	7
S402	Clones genotype 2251	4.76 ± 0.09	9
S357		4.80 ± 0.08	8
S438		5.61 ± 0.13	8
S448		5.03 ± 0.06	10
S401		4.43 ± 0.04	7
S477		5.09 ± 0.06	10

S439		4.99 ± 0.05	10
S474		5.25 ± 0.05	10
S0029	Donor cell mother	4.80 ± 0.08	11
S309	Clones genotype 0029	4.81 ± 0.05	12
S437		5.31 ± 0.06	13
S447		5.13 ± 0.03	15
S0333	Donor cell mother	4.56 ± 0.04	12
S0531	Clones genotype 0333	4.78 ± 0.06	10
S0541		5.29 ± 0.06	10
S0542		5.09 ± 0.07	13
S0543		5.66 ± 0.07	13
S0547		5.61 ± 0.07	10
S0007	Clones genotype 7711	5.69 ± 0.06	13
S0034		4.43 ± 0.07	12
S139		5.01 ± 0.07	13

Table 20: Determination of methylation level in DNA of leukocytes of Holstein clones

3.6.2.1 Comparative study of DNA methylation status between clones of the Simmental breed and the Holstein breed

The cloned animals were generated by SCNT [518, 519] using fibroblasts from nine adult donors that yielded two to nine clones per donor. Of the nine clone genotypes studied, five were from the Simmental breed (SC) and four from the Holstein breed (HC) (**Fig. 53A-C**).

It was observed that individual 5-meC levels in clones were ranged from 4.4 % to 6.9 %. The 5-meC content in DNA was ranged from 4.43 % - 5.09 % in clones with Holstein genetics (n=19) to 5.94 % - 6.93 % in clones with Simmental genetics (n=19). ANOVA showed unexpected significant differences in the mean 5-meC levels of Simmental and Holstein clones (mean ± s.e.m. 6.50 % ± 0.01 % and 5.09 % ± 0.02 %, two tailed *t*-test $P < 0.001$) (**Fig. 53A, orange and red crosses**). Although the different means of methylation levels were observed, the two clones' breeds exhibit similar mode of variability from their means.

The variance is the best studied factor to estimate variability, therefore exact restricted likelihood ratio tests were computed on the basis of linear mixed effects models [520] to test for the

presence of variability between genotypes. Firstly, the observed variances between clone genotypes of both breeds were similar (0.0204 and 0.0164). The distribution of the values around the mean is same for both clone breeds (**Fig. 53A**). Strikingly, the estimated variance in 5-meC level *within* clone genotypes from both breeds (0.104) was markedly higher than the estimated variance *between* clone genotypes ($< 10^{-10}$), which was not significantly different from 0 (exact restricted likelihood ratio test $P > 0.99$). This estimated variance opposes the assumption that clones of the same genotype will show less difference in methylation level. The existence of high variability of DNA metylation status in cloned cattle was obvious.

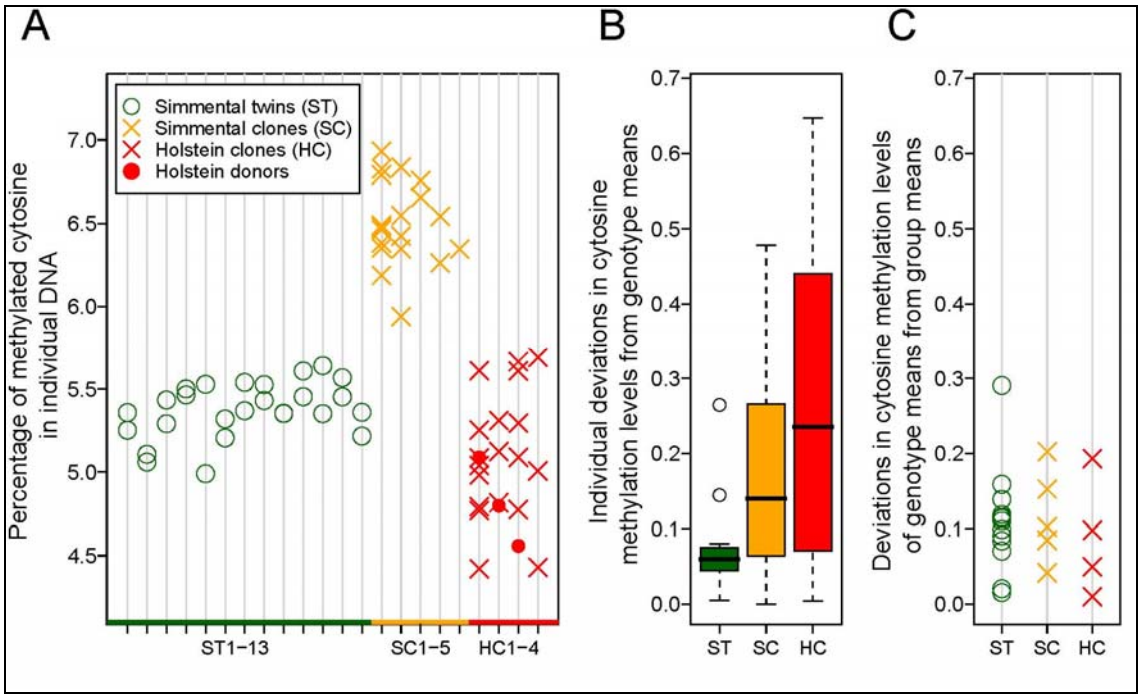


Figure 53: **Variability in cytosine methylation (5-meC%) of DNA from adult monozygotic twins and adult healthy somatic cell nuclear transfer clones**

(**A**) 5-meC levels as per cent of total cytosine in the DNA of white blood cells from individual Simmental breed twins (genotypes ST1-13, n=26), Simmental clones (genotypes SC1-5, n=19), and Holstein breed clones (genotypes HC1-4, n=19). Nuclear donors for Holstein clone genotypes HC1-3 are indicated as non-cloned references in the respective genotypes.

(**B**) Box plots of the absolute deviations of individual monozygotic Simmental twins (ST, n=26), Simmental clones (SC, n=19), and Holstein clones (HC, n=19), from their respective genotype means. The two ST outliers represent a twin pair.

(**C**) Dot plots of the absolute deviations of genotype means for Simmental twin pairs ST1-13, Simmental clone genotypes SC1-5, and Holstein clone genotypes HC1-4, from their respective group mean.

3.6.3 Individual and comparative study of DNA methylation status between the nuclear mother donor and its clones in Holstein breed.

Clone is assumed to be identical twin of the genetic donor. Consequently, the clones of Holstein (HS) breed were compared in this study to nuclear donor animals. Three Holstein individuals that served as nuclear donors were analysed for the 5-meC level of their genome together with their clones. The 5-meC levels of first control group used in this study ranged from 4.56 % - 5.09 % in Holstein nuclear donor animals (n=3). Two nuclear donors (*Clones genotype 029 and 0333*) showed the lowest 5-meC levels of the respective genotypes (**Fig. 53A**). This provides further evidence that reprogramming of nuclear function by SCNT is frequently associated with global DNA hypermethylation that extends from the embryonic [137, 143] and foetal stages [144] into adulthood. The 5-meC level of the third nuclear donor (*Clones genotype 2251*) was identical to only one of its eight healthy clones but higher than five of them and lower than the two remaining ones. This clearly demonstrates that a fully functional reprogramming of a given donor genotype is compatible with a highly flexible methylation status of its DNA and that genomic copies [521] of adult animals have to be considered as epigenome variants. There is an important question which proposed itself after this comparison of adult cloned animals with their adult nuclear donors: Is the observation of aberrant methylation status in clones resulting from incomplete epigenetic reprogramming of the early SCNT embryo or from other factors that affected maintenance methylation during later developmental stages [139, 146]. In this study, our data offer new insights to this question by showing that variability in DNA methylation levels in live clones is not correlated with the potential to provide consistently full term reprogramming into live calves as illustrated by the comparison between HC1 and HC2 genotypes (5-meC individual levels from 4.43 ± 0.04 S.D. to 5.61 ± 0.13 S.D. with 38.77 % blastocysts (per fused embryos) and 8.22 % born calves (per transferred embryos), and from 4.81 ± 0.05 S.D. to 5.31 ± 0.06 S.D. with 35.6 % blastocysts and 2.4 % born calves, respectively) (**Fig. 53A**).

Most importantly, the only true indication of 'reprogramming' is the birth of normal offspring, as discussed by Campbell [521]. Many changes are involved in the mechanisms responsible for the control of gene expression which makes the reprogramming process unclear.

Moreover, there was no correlation observed between the proportions of leukocyte subfractions and the global methylation level, excluding the possibility that the observed variability in DNA methylation levels are caused by variation of leukocyte subfractions.

3.6.4 Comparative study of DNA methylation status between clones and monozygotic twins of the Simmental breed

Individual genome-wide 5-meC levels were recently shown to be similar in adult human across populations [522]. Accordingly, monozygotic (MZ) twins are chosen as standard models of genetically identical traits as clones concerning the investigation of the methylation status. The analyses were performed on several sets of monozygotic twins of HS breed to create a reference on the extent of mutations and epigenetic changes occurring during embryonic and foetal development of a given genotype produced by sexual production.

The monozygotic twins used in this study were generated by microsurgical splitting of fertilised embryos [523]. Simmental clones were compared with female Simmental monozygotic twins to estimate the contribution of SCNT to the observed variability between clones (**Fig. 62A-C**). The 5-meC levels of this second chosen control group used in this study ranged from 5.07% - 5.64% in Simmental MZ twins (n=24) and as previously mentioned the 5-meC levels in Simmental clones (n=19) are 5.94 % - 6.93 %.

The estimated variability of 5-meC levels within 5 genotypes of Simmental clones (0.0636, n = 19) was obviously higher than in 13 Simmental twin pairs (0.0193, n=26). When an outlier twin pair was excluded, the variability in twins was even lower (0.0091, n=24) (**Fig. 53B**). The variability of 5-meC levels in twins especially detected with the outlier pair could be due to either external or internal inherited factors.

In Simmental clones, the estimated variability *within* genotypes (0.0636) was thus higher than *between* ($< 10^{-10}$) clone genotypes. The same finding was previously observed for both breeds. In contrast, the variability *within* twin genotypes (0.0091) was lower than *between* twin genotypes (0.0136), as expected.

More importantly, the absolute deviations of 5-meC values of individual SCNT clones from their respective genotype means were five-fold increased as compared with twins. (**Figure 53B, illustrated as the fractions under the black line in green and light orange boxes**). While the deviations of genotype means from the respective group means were similar for clones and twins (**Fig. 53C**).

On the other hand, when comparison was performed between Holstein clones and twins, the difference in absolute deviations of individual 5-meC values from genotype means was even more pronounced than the difference between Simmental clones and twins (**Fig. 53B, illustrated as the fractions under the black line in green and red boxes**). The comparison with twins had additionally confirms a SCNT cloning effect illustrated by a clone-specific variability of DNA methylation. In other words, these epigenome variants highlight that clones are only genomic copies of adult animals and not epigenomic ones [521].

In conclusion, significant genomic DNA hypermethylation in clones was detected in Simmental clones as compared with Simmental twins (mean ± s.e.m. 6.50 % ± 0.01 % and 5.38 % ± 0.01 %, two-tailed *t*-test $P < 0.001$). Thus, a conclusion can be illustrated in terms that SCNT procedure has been shown to affect the DNA methylation status of cloned embryos [137, 145], and the effect is remained throughout development till adulthood (**Fig. 53A**).

3.6.5 Effect of the age, nutrition and cloning process on the epigenetic variability of clones

Our study observed epigenetic differences in monozygotic twins that can be due to external and internal factors. The former factors can take place through exposure to different environmental conditions that lead to different physiological activities. The internal factors can be due to the epigenetic transmission defects through cell divisions that produce an accumulation of epigenetic differences by "epigenetic drift" associated with ageing [524], as observed in MZ twins in humans. Interestingly, the analysis in this study reveals that the ageing process and associated epigenetic drift could act differently in clones.

The data demonstrate significant differences in means of 5-meC levels between Simmental and Holstein clones that arise from breed-specific differences in poorly understood reprogramming efficiency and/or differences in donor cell preparation and SCNT procedure. In particular, the possibility that these differences were age-related [524] is excluded noting the overlapping of the age of Simmental and Holstein clones (unlike DNA methylation levels). Furthermore, the mean age of the Simmental clone genotype SC4 (80.5 months ± 1.5 months s.e.m.) and the mean age of Holstein clone genotype HC4 (72.0 months ± 5.8 months s.e.m.) were very similar (**Fig. 54**).

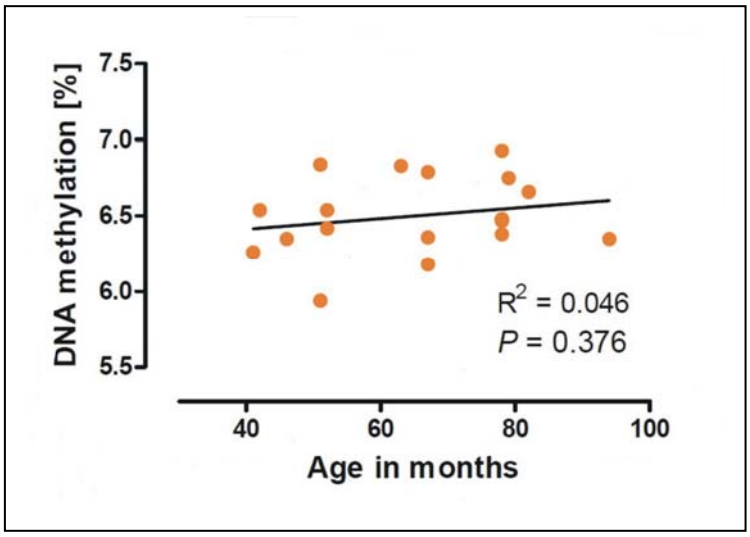


Figure 54: Correlation between the methylation level and the age of Simmental and Holstein clones

The comparison between LMU and INRA cloning procedures [133, 144] shows several differences. In the LMU cloning procedure, donor cells are subconfluent when used for nuclear transfer whereas they are quiescent in the INRA protocol. After fusion between the donor cell and the recipient oocyte, the activation process is different and delayed for 1 hour and a half in the former protocol compared to the latter. Finally, embryo culture conditions in the LMU and INRA protocols were different in terms SOF medium + 10 % serum and O_2 control was used for the LMU clones and B2 medium + 2.5 % serum and no O_2 control for INRA clones. This could impact on sequence-specific methylation status and developmental gene expression in mammals [525, 526]. Representative photographs for both breeds are illustrated in **figures 55** and **56**.

Figure 55: Holstein clones from INRA

Figure 56 : Simmental clones from LMU

Based on the current data, it is hypothesised that the SCNT cloning procedure induces a "hypermethylation drift" protocol in global DNA methylation levels that is within surviving and health compatible limits and this deviation is potentially related to breed-specific effects. The obtained data do not confirm a normalisation of DNA methylation differences between clones and non-clones with advancement of age, as suggested by a study with a limited number of SCNT cloned mice [527]. Instead, these data support the alternative hypothesis proposed by Senda *et al.* [527] that only cloned animals with a more appropriate methylation status can survive to adulthood. This assumption is supported by the data from gene-specific analyses that suggested more severely altered epigenetic marks in clones that died soon after birth [528] as compared with animals that died as juveniles [138].

3.6.6 Determination of 5-methyl cytosine level in liver tissue of healthy and abnormal Holstein clones

Researches involving the biochemical properties of food products from cloned and non cloned animals had not detected any significant differences [517]. Cloned animals and their products have recently been declared safe for human consumption by United States FDA [514]. It is obvious that the determination of methylation level of critical tissues involved in economically important traits (milk and meat) should be included in our study.

Therefore, the analysis of methylation level in DNA of the liver and muscle samples from HC was performed by CE-LIF. The determination of methylation level was done for 10 DNA samples extracted from liver cells of female Holstein clones. The evaluated data are recorded in **table 21** noting that the values are calculated as previously mentioned.

Sample Code	Type	State	Methylation level [%] ± S.D.	n
F6648	Genetic mother	Adult mother	4.88 ± 0.12	7
F2251	Donor cell mother	Control	4.04 ± 0.08	10
F402	Clones genotype 2251(M/C)	YHC	3.98 ± 0.08	8
F357		YHC	4.33 ± 0.06	8
F401		YHC	4.83 ± 0.11	7
F406		PaC	4.12 ± 0.08	7
F739		PaC	4.13 ± 0.08	13
F853		PaC	4.64 ± 0.13	9
F411		PaC	4.82 ± 0.07	8
F828		PaC	4.93 ± 0.05	13

Table 21: **Determination of methylation level in DNA of liver of healthy and abnomal Holstein clones (**YHC: Young healthy clones. PaC: Pathological clones**)**

The determination of methylation level in the liver cells confirms the epigenetic differences in clones. Variable and altered DNA methylation status was detected in young healthy adult clones (3 samples) as well as in unhealthy clones (5 samples).

As in case of leukocytes study, a variability in liver cells of 5-meC between clones of the same genotype was also observed. In contrast, the variability is greater with a hypermethylation in abnormal clones. This finding reflects that living clones without any health problems should not be considered normal [515-517]. Our data revealed that both normal and abnormal clones exhibit an epigenetic variation.

3.6.7 Determination of 5-methyl cytosine level in muscles of healthy and abnormal Holstein clones

Muscles are the second important tissue for the economic profit of cattle clones in milk production. Therefore, the analysis of methylation level of 13 DNA samples extracted from voluntary muscles of healthy clones as well as involuntary muscles (heart) of abnormal clones of Holstein breed was performed (later clones were died). The data are evaluated and recorded in **table 22**.

Sample Code	Type	State	Methylation level [%] ± S.D.	n
M6648	Genetic mother	Adult mother	4.66 ± 0.12	9
M2251	Donor cell mother	Control	4.74 ± 0.04	10
M477	Clones genotype 2251(M/C)	YHC	4.65 ± 0.09	8
M438		YHC	4.74 ± 0.04	5
M474		YHC	4.77 ± 0.15	8
M448		YHC	4.77 ± 0.11	10
M401		YHC	4.84 ± 0.06	8
M439		YHC	4.85 ± 0.08	9
C739		HPC	4.25 ± 0.02	12
C853		HPC	4.67 ± 0.04	11
C406		HPC	4.74 ± 0.04	13
C411		HPC	4.76 ± 0.04	14
C828		HPC	5.09 ± 0.11	7

Table 22: **Determination of methylation level in DNA of muscles of healthy and abnormal Holstein clones (**YHC: Young healthy clones. PHC: Heart muscles of pathological clones**)**

The quantification of 5-meC level in muscle samples taken from healthy clones had revealed a different observation than the previous studied methylation status in blood samples. No significant difference could be detected in healthy adult clones.

In case of heart muscles, it was observed a significant difference of methylation level between the phenotypes (C739 and C828) and the other abnormal clones (C406, C411 and C853). However, there is no detected variability between clones when outlier of these 2 phenotypes was excluded. In general, the observed variability in muscles is relatively small compared to the aberrant methylation level in case of blood and liver samples. The comparison of data obtained from the study of methylation status in muscles is not beneficial noting the difference in muscle types between normal clones and abnormal clones as the analysed DNA samples are extracted from legs muscles from the former and heart muscle from the latter. The heart samples were insufficient to obtain valuable data due to death of clones.

In conclusion, the reprogramming process establishes first the fully totipotent state and then the pluripotent state in the embryo; changes in gene expression during development are associated with changes in epigenetic modifications. The reprogramming process could be altered

through nuclear transfer procedures, since perturbed DNA methylation patterns could thus be responsible for the observed changes in gene expression and may set the embryo or foetus on a developmental path that is not compatible with a live and healthy offspring [127, 138, 143]. However, due to the dynamic nature of DNA methylation that plays a fundamental role in the regulation of cellular processes and transgenerational effects, it was not clear how and when the methylation pattern is affected in compromised clones. Therefore, long term effects of SCNT reprogramming were evaluated by quantifying global epigenetic plasticity in adult healthy cloned cattle.

In fact, clones surviving into adulthood are judged to be normal [515, 529] and their epigenetic marks required for normal development are assumed to be maintained. On the other side, the clones dying around birth showed a sequence specific variations in DNA methylation [525, 526, 528]. In contrast to this hypothesis, our data highlight SCNT effects on DNA methylation of healthy adult clones that are resilient to epigenetic reprogramming during development and postnatal exposure to environmental effects. It was concluded that through SCNT cloning procedures, functional reprogramming of a donor genome into healthy adults is compatible with a highly flexible methylation status of its DNA. The DNA methylation levels in the great majority of clones were higher than in the nuclear donors or monozygotic twins (study of blood samples). In the Simmental breed, it was detected a 25 % increase in DNA methylation levels of clones in comparison with twins. The Clones (mother-genotypes) as well as the clone-twin comparisons had provided further confirmation of the previously observed global DNA hypermethylation in embryonic [143] and fetal SCNT stages [144] to healthy adults.

The global 5-meC variations of such percentage could not be logically originated only from the limited number of differentially methylated imprinted genes [530, 531] but other sequence elements such as transposons or satellite DNA should be involved in these alteration of DNA methylation levels.

Moreover, the recent data obtained from monozygotic twin studies have highlighted the important role of epigenome variations as the basis for differences in heritable complex traits [155] and new epigenome perspectives show the dynamic interplay of chromatin and DNA sequence in heritable traits [532].

4 Summary

DNA methylation is an epigenetic modification established during embryogenesis and reset during development. It is an essential process for cell differentiation, gene expression control, regulation of vital cell functions and successful mammalian development. Aberrant DNA methylation patterns have frequently been associated with carcinogenesis, with a general loss of genomic 5-methylcytosine and a hypermethylation of particular genes. Therefore, the analysis of the genomic methylation level would provide a comprehensive tool for the correlation between 5-meC-mediated biological variations and cancer development as well as other cell abnormalities. DNA adducts (another chemical modification of DNA formed via attack of exogenous or endogenous chemical reactive agents) have frequently been found to be the first step in the development of a cancerous cell and to contribute to tumour aetiology. Thus the analysis of DNA modifications is of great importance, as these represent valuable biomarkers for exposure to pollutants and carcinogens as well as reflectors of the risk of cancer formation or other aging diseases.

The first section in this study focused on the synthesis of 8-oxo-2'-deoxyguanosine-3'-monophosphate (8-oxo-dGMP), its detection by the sensitive method CE-LIF and the determination of the effect of this oxo-compound on the methylation level. The CE-LIF method developed by Schmitz *et al* [395, 399] involves the determination of modified or unmodified nucleotides in form of 2'-deoxyribose-3'-phosphate. Therefore, the hydroxy-nucleotide form of the guanine nucleobase was synthesised to enable its detection with CE-LIF via its derivatisation with the fluorescent marker BODIPY. 8-Oxo-dGMP was successfully synthesised from the authentic 3'-dGMP compound by the method of Schuler *et al.* [258], then was separated from the starting material on a C18 column (250 x 4.6 mm, 5 µM) with an ammonium formate buffer (25 mM , pH 4.7). After isolation of the desired fraction, a further purification step with 15 % methanol:85 %water was carried out. The pure synthesised 8-oxo-dGMP product was characterised with ESI-MS ($[M+H]^+$ at *m/z* 364.3). A sequence CE-LIF analysis of 8-oxo-dGMP had revealed that it exists either in tautomeric forms or a further oxidation product of the synthesised 8-oxo-dGMP is formed by time. A double-blind study was carried out on leukocytes taken from combat divers who had been subjected to oxidative stress during their five months diving training by their exposure to hyperbaric oxygen (HBO). The 8-oxo-dGMP was detected in the samples from divers after training showing an obvious hypomethylation in comparison to control samples taken from same divers before training. The formation of the oxoguanosine adduct possibly affects the role of DNA methyltransferase enzymes

and leads to a decrease in the genomic methylation level. This study was successful in establishing a link between the aberrant methylation level, oxidative stress (8-oxo-dGMP) and promotion of carcinogenesis, but further studies are necessary to verify these results.

The second aim of this work was the study of the change of methylation level in response to several carcinogens either natural products or environmental pollutants. The effect of aristolochic acid (AA) on the genomic methylation level of treated rats and mice was first examined. A single oral dose of 30 mg AA/kg BW (body weight) was applied to male Sprague-Dawley rats, which were sacrificed by decapitation at 24 h after the AA treatment. In addition, an oral dose of 5 mg AA/kg BW was given daily to female Hupki mice (knock-in mice with human p*53* gene). One group of mice was sacrificed three weeks after the AA dosage i.e., 24 h after the last treatment with AA, and another group was sacrificed 52 weeks after the first treatment. DNA samples were extracted from several collected tissues from both studies and analysed by CE-LIF. The results showed different patterns in the methylation level according to the tested organ, confirming a specificity of the methylation level that can distinguish different tissues. In cooperation with a research group in Hong Kong, the highest AA-DNA adduct concentration was detected in kidneys of the AA-dosed rats (HPLC-ESI-MS method). Similarly, a research group in England had detected the highest AA-DNA adduct concentration in kidneys of AA-dosed hupki mice among several tissues analysed by the ^{32}P-postlabeling method. An obvious hypermethylation state was observed in kidney in both treated rats (24 h treatment) and treated mice (52 weeks treatment). A hypermethylation was also detected with the CE-LIF method in stomach and colon in the former study as well as in liver in the latter study (in comparison with their respective controls). Moreover, the comparison of data between both controls of mice killed after 3 and 52 weeks had revealed an expected inverse relation between the animal age and methylation level. In contrast, AA-dosed mice did not reveal any hypomethylation related to the exposure length, thus confirming that the effect of AA on methylation level had overcome the age effect. Our study had highlighted the beneficial use of the aberrant methylation level as helpful biomarker for the promotion of carcinogenesis due to AA exposure and had additionally confirmed that kidney is the most targeted tissue of AA toxicity.

Other exogenous chemicals were studied for their carcinogenic effect on the genomic methylation level in both *in-vivo* and *in-vitro* studies. In the former study, DNA samples were extracted from various organs of Sprague-Dawley rats dosed intra-tracheally with 0.2 and 2.0 mg/kg BW of 3-nitrobenzanthrone [3-NBA] and scarificed 48 h after the treatment. The ^{32}P-postlabeling method had revealed various DNA-adduct concentrations in specific tissues.

The same samples were analysed by CE-LIF and revealed a hypomethylation in small intestine and lungs in the high-dosage study. In the *in-vitro* study, DNA samples extracted from Caco-2 cells incubated with several carcinogens [3-NBA, benzo[*a*]pyrene (B[*a*]P) and 4-amino-biphenyl (4-ABP)] in two concentrations of 2.5 and 5 µg/kg for 24, 48 and 72 h were analysed for their methylation level. No significant difference in the methylation level could be detected in case of treated Caco-2 cells. This comparison suggests the importance of *in-vivo* study of the effect of carcinogens on the methylation level for a reliable conclusion. Moreover, the variability of the methylation level among different analysed tissues provides insight into the role of related factors such as the cell proliferation rate or the efficiency of DNA-repair system in cancer formation via epigenetic modification.

Finally, DNA methylation as an epigenetic mark was studied in this work for its effect in the process of cloning by somatic cell nuclear transfer (SCNT). Epigenetic perturbations are responsible for foetal and postnatal abnormalities that are frequently observed after SCNT. Conversely, the epigenetic status of healthy adult clones is generally considered to be normal, although it has never been investigated. The analysis was performed by CE-LIF to determine the methylation level from the whole genome of individual animals of each genotype. In order to evaluate long-term effects of SCNT reprogramming, the genome-wide methylation level was determined in blood samples of 38 healthy adult female SCNT clones generated from nine genotypes of the Holstein and Simmental breeds of cattle. Individual methylation levels ranged from 4.4 % to 6.9 % with significant differences between the mean 5-meC levels of Holstein and Simmental clones (6.50 % ± 0.01 % and 5.09 % ± 0.02 %, $P < 0.001$). The variability of methylation level was also found in the case of liver samples in a parallel study performed in ten adult Holstein clones. In addition, in order to judge the contribution of SCNT to the variability of individual 5-meC levels, further analysis was carried out to compare Simmental clones with 12 sets of similarly aged female monozygotic Simmental twins experimentally generated from bisected fertilised embryos. In this study, it is concluded that global DNA methylation levels of circulating white blood cells of healthy adult bovine clones are in fact highly variable between individuals of the same genotype. Also, the methylation level of clones is higher than the nuclear donor animals or in contemporary monozygotic twins. The absolute deviations of 5-meC values of individual SCNT clones from their genotype means were five times as high as in twins. Finally, this study revealed unexpected DNA methylation differences between healthy adult clones of the same genotype and between genotypes of different breeds that call for an in-depth analysis of genetic and epigenetic risks associated with SCNT cloning and clear guidelines for the use of cloned epigenetics [514].

5 Materials and Methods

5.1 Chemicals

5.1.1 General laboratory chemicals

1-ethyl-3-(3´-*N, N´*-dimethylamino-propyl) -carbodiimide hydrochloride (EDC)	Fluka, Steinheim, Germany
2'-thymidine-3'- monophosphate (TMP; Na salt)	Sigma, Steinheim, Germany
4,4-difluoro-5, 7-dimethyl-4-bora-3a, 4a-diaza -s-indacene-3-propionyl ethylenediamine hydrochloride (Bodipy-FL-EDA)	Invitrogen, Molecular Probes, Eugene, USA
Acetone	Merck, Darmstadt, Germany
Calcium chloride dihydrate	Fluka, Steinheim, Germany
Calf-thymus DNA (CT-DNA) (deoxyribonucleic acid Na salt)	Sigma-Aldrich, Steinheim, Germany
Dichloromethane HPLC grade	Fischer Scientific, Loughborogh, United Kingdom
Disodium hydrogen phosphate	Merck, Darmstadt, Germany
Fluorescein, Test-mixture, Na salt (water)	Beckman Coulter, Krefeld, Germany
HEPES: N-(2-Hydroxyethyl)-piperazine -N´-2-ethane sulphonic acid.	Merck, Darmstadt, Germany
Hydrochloric acid	Merck, Darmstadt, Germany
Isopropanol	Chemical unit in Bergische Universität Wuppertal
Methanol	Merck, Darmstadt, Germany
Micrococcal nuclease (MN) (Staphylocous aureus)	Sigma-Aldrich, Steinheim, Germany
Paraffin oil	Fluka, Steinheim, Germany
Sodium borate	Fluka, Steinheim, Germany
Sodium dihydrogen phosphate-dihydrate	Merck, Darmstadt, Germany
Sodium dodecyl sulphate, 99 %	Merck, Darmstadt, Germany
Sodium hydroxide	Carl Roth GmbH Co., Karlsruhe, Germany

Sodium tetraphenylborate	Fluka, Steinheim, Germany
Spleen phosphodiesterase (SPD)	Calbiochem, Darmstadt, Germany
Water HPLC analysed for preparation of derivitisation buffer	JTBaker, Deventer, Holland

Distilled water was purified through TKA- GenPure water distillation apparatus.

5.1.2 Chemicals for the synthesis of 8-oxo-2'-deoxyguanosine-3'-mono-phosphate

2'-deoxyguanosine-3'- monophosphate (dGMP; Na salt)	Sigma, Steinheim, Germany
Ammonium acetate	Fluka, Steinheim, Germany
Ammonium formate	Acrös organics; Geel, Belgium
Copper sulphate pentahydrate ($CuSO_4 \cdot 5\ H_2O$)	Merck, Darmstadt, Germany
Ferrous Sulphate heptahydrate ($FeSO_4 \cdot 7\ H_2O$)	Merck, Darmstadt, Germany
Hydrogen peroxide solution (35 % H_2O_2)	Merck; Darmstadt, Germany
L-Ascorbic acid Na salt ($C_6H_{17}O_6Na$)	Acrös organics; Geel, Belgium

5.1.3 Solvents for HPLC separation of 8-oxo-2'-deoxyguanosine-3'-mono-phosphate from its starting material

Acetonitrile LC-MS grade	Fisher Scientific, Loughborough, UK
Acetic acid	JTBaker, Deventer, Holland
Formic acid	Fluka, Steinheim, Germany
Methanol HPLC grade	Acrös Organics, New Jersey, USA

5.2 Experimental preparations

5.2.1 Standard Preparations

- **Hydrolysis buffer (250 mM HEPES in 100 mM $CaCl_2$, pH 6.0)**

3.0 g HEPES and 0.7 g $CaCl_2 \cdot 2H_2O$ were weighed then transferred in a 100-mL screw-cap bottle and dissolved in 50 mL water. The solution was then titrated with 1 M NaOH to pH 6.0.

- **Derivatisation buffer (50 mM HEPES, pH 6.4)**

Firstly, a stock solution of 800 mM HEPES (pH 6.5) was prepared as the following:
9.5 g HEPES were weighed in a 100-mL beaker and dissolved in 50 mL water. The solution was then titrated with 1 M NaOH to pH 6.5.
For the preparation of 50 mM HEPES derivatization buffer, 1 mL of the stock solution (800 mM) was transferred in a 50-mL centrifuge tube then 15 mL water were added.

- **Separation buffer for MEKC-LIF**

90 mM SDS was dissolved in a solution of 90 % v/v sodium phosphate buffer (20 mM, pH 9.0) and then 10 % v/v MeOH was added as organic modifier.
The sodium phosphate buffer was prepared as follow:
A solution of 20 mM di-sodium hydrogen phosphate (Na_2HPO_4) and a solution of 20 mM sodium dihydrogen phosphate (NaH_2PO_4) were prepared. The pH of Na_2HPO_4 solution was approximately 10.9 and that of NaH_2PO_4 solution was approximately 5.6. The desired pH 9.0 was obtained by pouring NaH_2PO_4 solution to Na_2HPO_4 while the solution was continuously stirred and monitored with a pH meter at room temperature. The buffer was degassed by sonication before use.

- **Separation buffer for CE-LIF (20 mM sodium phosphate buffer, pH 9.0)**

A solution of 20 mM di-sodium hydrogen phosphate (Na_2HPO_4) and a solution of 20 mM sodium dihydrogen phosphate (NaH_2P_4) were prepared. Then, the latter was added portion-wise on to Na_2HPO_4 till pH 9.0 was reached for the solution and finally 10 % v/v MeOH was added.

- **Sodium tetraphenyl borate**

1.8 g $C_{24}H_{20}B$ Na were weighed and dissolved in 100 mL disodium hydrogen phosphate buffer (1 mM, pH 6.0). The solution was stored in a 250-mL screw-cap bottle.

- **Sodium tetraborate buffer (20mM)**

1.8 g sodium tetraborate were weighed and dissolved in 100 mL disodium hydrogen phosphate buffer (1 mM, pH 7.0). The solution was stored in a 250-mL screw-cap bottle.

5.2.2 Preparation of washing solutions for CE

- **Preparation of 1M NaOH**

8 g NaOH pellets were weighed and dissolved in 100 mL water. The solution was stored in a 250-mL screw-cap bottle.

- **Preparation of 1M HCL**

210 mL water (measured in a 250-mL graduated cylinder) was poured in a 250-mL screw-cap bottle, and then 20 mL of 36 % HCL were added. The solution was shaken after the bottle had been sealed.

- **Preparation of 200 mM SDS solution**

11.5 g SDS were weighed, transferred to a 250-mL screw-cap bottle, and then 200 mL water was added. The solution was stirred slowly after the bottle had been sealed, then allowed to stand in order to be used when no foam is seen.

5.2.3 Specific preparation for the synthesis and characterisation of 8-oxo-2'-deoxyguanosine-3'-monophosphate

- **Preparation of 10 mM ammonium acetate buffer:**

0.771 g of ammonium acetate were weighed then transferred to a 1-L volumetric flask and dissolved in 1 L distilled water, and then the pH was adjusted to 4 with diluted acetic acid. The buffer was degassed before its use.

- **Preparation of 25 mM ammonium formate buffer:**

1.576 g of ammonium formate were weighed then transferred to a 1-L volumetric flask and dissolved in 1 L distilled water, and then the pH was adjusted to 4.7 with diluted formic acid. The buffer was degassed prior to its use.

- **Preparation of 1 mM ferrous sulfate:**

0.0278 g $FeSO_4 \cdot 7H_2O$ were weighed then transferred to a 150-mL beaker and dissolved in 100 mL 10 mM ammonium acetate buffer (pH 4.0).

- **Preparation of 20 mM copper sulfate:**

0.5 mg $CuSO_4 \cdot 5\ H_2O$ were weighed on micro-plates of aluminium foil using sensitive balance then transferred to an eppendorf capsule and dissolved in 1 mL distilled water. The solution was freshly prepared before the reaction.

- **Preparation of 170 mM sodium ascorbate:**

0.034 mg $C_6H_{17}O_6Na$ were weighed on micro-plates of aluminium foil using sensitive balance then transferred to an eppendorf capsule and dissolved in 1 mL distilled water. The solution was freshly prepared before the reaction.

- **Preparation of 1 mM hydrogen peroxide (H_2O_2):**

800 µL of H_2O_2 (35 %) were transferred from bottle to an eppendorf capsule and diluted with 200 µL water. The solution should be freshly prepared before the reaction.

- **Preparation of 2 % ammonia:**

8 mL concentrated ammonia (25 %) were diluted with water and the volume was then completed till 100 mL with water.

5.2.4 Enzyme preparations

The enzyme mixture, consisting of spleen phosphodiesterase (SPD) and micrococcal nuclease (MN) enzymes for the hydrolysis, was prepared according to the following steps:

5.2.4.1 Dialysis of the spleen phosphodiesterase enzyme (SPD)

The enzyme solution (approx. 1 mL), containing a concentration of 10 U SPD enzyme, was drawn up with a 2-mL syringe with 0.4 x 20 mm needle (For single use only). Then, the solution was carefully injected between the diaphragms of the dialysis framework (Slide A Lyzers, 0.5-3 mL volume). The vial of enzyme was washed with water. Then the washing solution was transferred to the dialysis membrane by means of a needle. Air must be withdrawn carefully from the dialysis chamber. After the removal of the syringe, the injection point was marked afterwards on the dialysis framework with a water resistant marker. The dialysis

framework was fastened to a spongy floater with its marked corner in order to avoid the danger of the enzyme loss during dialysis. This assembly was transferred to 10-L glass beaker filled with millipore cold water. Dialysis was accomplished for 24 h with 4°C in the refrigerating chamber. The water was changed after approximately 6 h. One day (24 h) is a sufficient time for dialysis to remove low molecular weight compounds. After dialysis, the solution was removed with a 5-mL syringe with 0.4 x 20 mm needle from the dialysis membrane and then the chamber was rinsed with 500 µL millipore water. The whole collected protein enzyme solution was divided on two 1.5-mL eppendorf caps and immediately frozen in liquid nitrogen and finally lyophilised in the speed vacuum.

5.2.4.2 Dialysis of the Micrococcal nuclease enzyme (MN)

The dialysis of the Micrococcal nuclease was prepared similar to the dialysis of the SPD enzyme, with exception that 500 U MN were dissolved in 500 µL water and were taken with a 1-mL syringe to be transferred to the dialysis membrane.

5.2.4.3 Production of the MN/SPD enzyme mixture

- **Production of the MN-solution:**

The lyophilised protein (500 U) was dissolved in 1667 µL redistilled water; that results in a concentration 300 mU/µL.

- **Production of the SPD-solution:**

According to the required final concentration of SPD enzyme, the volume of water was calculated to loosen the lyophilised enzyme described as follow:

- **Production of the enzyme mixture**

a) MN/SPD mixture with 5 times concentrated SPD:

A final concentration (12.5 mU/µL SPD and of 150 mU/µL MN) was calculated to prepare 5 times concentrated enzyme-mixture. The lyophilised SPD was first dissolved in 400 µL water to produce SPD solution of concentration 25 mU/µL. Then, equal volumes of SPD (25 µL) and MN (25 µL) were mixed. In such a way, the prepared 50 µL aliquot of the enzyme mixture was stored at -20°C.

b) MN/SPD mixture with 15 times concentrated SPD:

In order to prepare 15 times concentrated enzyme-mixture, a final concentration of 37.5 mU/µL SPD was calculated. Then, the lyophilised SPD was dissolved in 133 µL water (SPD concentration: 75 mU/µL). Finally, equal volumes of SPD (25 µL) and MN (25 µL) were mixed. Then, the prepared 50 µL aliquot of the enzyme mixture was stored at -20°C.

5.2.5 Other preparations

Aristolochic acid study

A. Male Sprague-Dawley rats (n=3, BW 180-200 g) were kept in a temperature and humidity controlled room with dark-light cycles. A single oral dose of 30 mg/kg BW of AA in 1 % $NaHCO_3$ was given to rats for 24 h. Then, the rats were sacrificed by decapitation. Different organs were removed and stored at -80°C then DNA extraction was done using Trizol reagent according to the instructions prescribed by the manufacturer (Invitrogen, CA, USA). The samples were handled in the department of Chemistry of Prof. Zongwei Cai at Hong Kong Baptist University in Hong Kong, China.

B. Female Hupki (human *p53* knock-in) mice were treated orally with a daily dose of 5 mg/kg BW of AA according to protocols described to induce tumors in various mouse organs. Group of mice was sacrificed three weeks after the AA dosage (24 h after the last treatment) and another group was sacrificed 52 weeks after the first treatment. Different DNA samples were extracted from the collected organs. The samples were handled in institute of Cancer Research by Dr. Volker Arlt, Section of Molecular Carcinogenesis in London, United Kingdom.

3-Nitrobenzanthrone study

Female Sprague-Dawley rats (BW 300-380 g) were treated with dose of 0.2 or 2.0 mg/kg BW of 3-NBA by intra-tracheal instillation under ether anaesthesia (3 rats/dose). The dose was prepared by procedure of Bieler *et al.*[504]. The rats were sacrificed 48 h after 3-NBA administration. Different organs were collected, frozen immediately in liquid nitrogen and stored at -80°C then DNA isolation was done following Qiagen genomic DNA Purification procedure (Blood & Cell Culture DNA kit, Qiagen, Germany). DNA samples were precipitated by iso-

propanol and dissolved in distilled water. The samples were handled in the German Cancer Research Center, Section of Molecular Toxicology in Heidelberg, Germany

SCNT and twin study

Animal experiments

Somatic cell nuclear transfer (SCNT) cloned animals were generated by two standard SCNT procedures [133, 144, 518, 519] with fibroblasts from nine donors that yielded two to nine clones per donor. Monozygotic twins were generated by microsurgical bisection of the fertilised embryos [523]. All animal experiments were carried out in accordance with German or French legislation on animal ethics and welfare.

DNA samples

DNA samples of Holstein Clones were prepared in INRA (Biologie du dévelopement et Reproduction, Jouy en Josas) in France. DNA samples of Simmental Clones and Twins were prepared in Chair for Molecular Animal Breeding and Biotechnology, and Laboratory for Functional Genome Analysis (*LAFUGA*), Gene Center, LMU Munich, Munich) in Germany.

DNA extraction from blood cells

EDTA blood samples were obtained by jugular vein puncture and then leukocytes separation was performed by centrifugation (25 min, 1200 x g). The cell fraction was diluted in 10 mL PBS and further centrifuged (10 min, 530 x g).
Cell subfraction counts were performed using the Malassey cell counting method before DNA extraction (Blood & Tissue DNA Kit, Qiagen, Germany). DNA samples were dissolved in distilled water and stored at -20°C.

DNA extraction from Liver of Holstein Clones

Liver biopsies were taken by surgical needles, cut into very small pieces with a scalpel and then crushed using special adapted mixer to enable more efficient lyses. Finally, further cell lyses was carried out by addition of Buffer ATL and proteinase Kinase solution followed by incubation of the mixture at 56°C. During the incubation time, vortex was occasionally done to disperse the sample. The lysate was then treated with 4 µL of RNase (A) to get rid of the RNA and incubated for 2 min at RT. Then, Buffer AL and ethanol were added to the sample and the mixture was immediately mixed by vortex and applied to DNA easy Mini spin col-

umn. After centrifugation, the filtrate was discarded. Then, the column was washed first with AW-1 buffer followed by centrifugation. The second wash was done with AW-2 buffer followed by centrifugation at maximum speed to dry the DNeasy membrane from residual ethanol. DNA was finally eluted from the column membrane by addition of small volume of AE buffer to increase the final DNA concentration. The column was incubated for 1 min at RT and then centrifuged to collect the filtrate containing the required DNA.

DNA extraction from muscles of Holstein Clones

The specimens from muscle tissues were crushed, immediately frozen in liquid nitrogen and stored in aluminium foil at -80°C. In order to extract the DNA, the tissues were crushed in liquid nitrogen. Then, the powder was incubated overnight in SLB buffer and Proteinase K at 42°C. The sample was continuously agitated during the incubation period. Then the sample was treated with RNase (A) solution (10 mg/mL) and incubated for 1 h at 37°C. The sample is treated again with PK in SLB buffer for 1 h and half at 42°C. After incubation, the sample was extracted with a mixture of phenol, chloroform and iso-amelic acid, then agitated carefully and centrifuged for 6 min at 5000 rpm. After extraction, the DNA was carefully precipitated with NaOH and ethanol. The precipitate was washed 3 times with 70 % EtOH then dried (UV lamp, 10 min). Then, the solution was re-suspended in TE (10 mM Tris, 1mM EDTA, pH 8.0) and left for agitation at RT over night.

N.B: All the samples analysed in the whole research work were quantified by UV-VIS spectrophotometery and subsequently aliquoted in either 1 or 10 µg.

5.3 Methods

5.3.1 Common methodology for the preparation of DNA samples (1 µg or 10 µg)

5.3.1.1 Hydrolysis of genomic DNA samples (1 µg or 10 µg)

1 µg or 10 µg of dried DNA was dissolved in 5 µL water and then hydrolysed to 2'-deoxynucleoside-3'-monophosphate by incubation for 3 h at 37°C with 5 µL of the enzyme solution. This enzyme solution consists of 5 µL of the enzyme mixture (150 mU/µL MN and

12.5 mU/µL SPD) and 1 µL of the hydrolysis buffer (250 mM HEPES in 100 mM $CaCl_2$, pH 6.0). The incubation was done while shaking (800 rpm).

5.3.1.2 Derivatisation of genomic DNA samples (1 µg or 10 µg)

DNA hydrolysate (1 µg) was derivatised with 15 µL of 1.8 M EDC (dissolved in 50 mM HEPES, pH 6.4), 15 µL of 27 mM Bodipy FLEDA dissolved in the same buffer and 15 µL HEPES buffer (50 mM, pH 6.4). Then the sample was incubated for 21 h in the dark at 25°C. The incubation was done while shaking (800 rpm). In the case of 10 µg DNA, the hydrolysed solution was derivatised with 1.8 M EDC, 27 mM Bodipy FLEDA (20 µL each) and 20 µL HEPES buffer. The sample was incubated under the same conditions described for 1 µg DNA sample.

5.3.1.3 Precipitation of the excess of Bodipy and EDC (1 µg or 10 µg)

Precipitation of 1 µg DNA sample

55 µL of the derivatised sample were transferred into a 15-mL cap and then diluted with 425 µL water. To the solution, 550 µL of 52.5 mM sodium tetraphenylborate (dissolved in 1 mM sodium phosphate buffer, pH 6.0) were added portion-wise while shaking for the precipitation of nucleotide. After mixing, 11 mL methylene chloride (CH_2Cl_2) were added to the solution, well mixed and finally centrifuged for 4 min at 3000 rpm and 20°C. The aqueous phase was isolated and then centrifuged again for 10 min at 13000 rpm. The supernatant was transferred to 1.5-mL cap and stored at -25°C to be analysed by CE.

Precipitation of 10 µg DNA sample

70 µL of the derivatised sample were transferred into a 50-mL cap and diluted with 630 µL water. To the solution, 770 µL sodium tetraphenylborate (52.5 mM solution in 1 mM sodium phosphate buffer, pH 6.0) were added portion-wise while shaking for the precipitation of nucleotide. After mixing, 15 mL CH_2Cl_2 were added to the solution, well mixed and finally centrifuged for 4 min (3000 rpm, 20°C). The aqueous phase was isolated and then centrifuged again for 10 min (13000 rpm). The supernatant was transferred to 1.5-mL cap and stored at -25°C to be analysed by CE.

5.3.1.4 CE-LIF measurement

A definite volume (37.5 or 42 µL) of DNA sample (1 or 10 µg) was transferred to 200 µL PCR vial respectively then diluted by a factor 1:100 with distilled water (162.5 or 158 µL) which was pre-degassed in case of long sequence-measurements. Finally, two drops of paraffin oil were poured on the surface of the sample to prevent its evaporation during long time analysis by CE-LIF. The PCR vial was placed in specific CE vial then closed with rubber cap and finally placed in the sample rack of CE (25°C). The sample is hydrodynamically injected into the capillary and an electric field is supplied to the electrodes by a high-voltage power supply. Then, the ions move through the capillary in the direction of electroosmotic flow to the detector placed at the outlet end of the capillary. The ions are separated according to their size (r) to charge (q) ratio in terms of their migration due to the electrophoretic velocity. Finally, the separated ions flow into the laser induced fluorescence detector and their signals are transferred to the data system which results in a plot of relative fluorescence unit (RFU) versus migration time (t_m) and the electropherogram is recorded.

According to the CE mode chosen for the analysis, CZE or MEKC technique, the working conditions of CE were adjusted. **Tables 23** and **24** summarise the conditions of the used CE or MEKC separation techniques respectively.

Condition	Value	Duration (min)	Solution
Rinse (Pressure)	20 psi	1.00	SDS
Rinse (Pressure)	20 psi	1.50	NaOH
Rinse (Pressure)	20 psi	1.00	H_2O
Rinse (Pressure)	20 psi	2.00	Separation buffer for MEKC
Injection (Pressure)	0.5 psi (forward)	5.00 (sec)	Sample
Separation (Voltage)	20 kV	45	Separation buffer for MEKC

Table 23: Method of separation of derivatised nucleotide with MEKC-LIF

Condition	Value	Duration (min)	Solution
Rinse (Pressure)	20 psi	1.50	NaOH
Rinse (Pressure)	20 psi	1.00	H_2O
Rinse (Pressure)	20 psi	2.00	Separation buffer for CZE
Injection (Pressure)	0.5 psi (forward)	5.00 (sec)	Sample
Separation (Voltage)	28 kV	20	Separation buffer for CZE

Table 24: Method of separation of derivatised nucleotide with CZE-LIF

5.3.2 Synthesis of 8-oxo-2'-deoxyguanosine-3'- monophosphate

4 mg of 2'-deoxyguanosine- 3'-monophosphate (dGMP) were dissolved in 400 µL distilled water. Then, 160 µL sodium ascorbate freshly prepared solution (170 mM), 160 µL copper sulphate pentahydrate solution (20 mM) and 100 µL hydrogen peroxide (28 % H_2O_2) were added. The reaction was left for 15 min at room temperature. Then, the mixture was separated on RP-18 column (Lichrospher^R 100, 250 x 4.6 mm ID, 5 µm). Different eluents were tried: 25 mM ammonium formate (pH 4.7), acetonitrile:water (different proportions), ammonium formate:methanol mixture (1:1) and methanol:water (different percentages). The retention times of guanosine and oxoguanosine were identified according to the eluent system (flow rate 0.8 mL/min). The HPLC column was prepared at the beginning of every working day; the buffer was firstly purged for 2 min (valve is opened) to get rid of air bubbles in the injection part then flowed for 20 min to clean the column. The wavelength of UV detector was adjusted at 247 nm. Once a stable state of pressure was attained (116 bar), 100 µL of the sample was injected onto the column (4 times).

5.3.3 Electrospray ionisation mass spectrometry (ESI-MS) of 8-oxo-2'-deoxy-guanosine-3'- monophosphate

The purified and dried 8-oxo-dGMP fraction was dissolved in water:acetonitrile (50:50, 0.1 % formic acid) then injected into the ion source where the molecules are ionized.

5.3.4 Preparation of the capillary for CE measurement

The fused-silica capillary for the capillary electrophoresis system was prepared from polyimide coated capillaries (type TSP Tubing Fused Silica) with an inner diameter of 50 µm and an outside diameter of 375 µm. The steps of the preparation of the capillary were described as follows: (See appendix **Fig. 57**)

- **Preparation of the detector window**

The capillary of length more than 50 cm was cut from the stainless steel wire provided in meters using a ceramic capillary cutter. Then, the effective length was measured and the detector window was marked within this range. The polyimide sheath covering the capillary should be burned in the location of the window which estimated to be 3 mm. To achieve this burning, the capillary was inserted into a device designed for the capillary preparation (See Appendix). Finally the marked detector window was burned with the gas burner for few seconds to not break the fragile glass; afterwards the window was properly cleaned with methanol. The capillary was inserted into the cartouche, and then the inlet and the outlet were cut to the same length. In order to avoid high injection fluctuations, about 2 mm of the polyimide coating was burned away at the capillary inlet. This heat curing technique improves the long-term stability of the capillary by preventing the gradual build-up of welled and frayed polyimide coating rests in front of the capillary inlet or outlet after longer contact with CE buffer [533].

- **Conditioning of the capillary**

The capillary should be conditioned before its first use. It was rinsed with 1 M NaOH (15 min, 20 psi), 1 M HCL (15 min, 20 psi), 1 M NaOH (15 min, 20 psi), water (5 min, 20 psi) and electrolyte (10 min, 20 psi). Then finally, the electrolyte buffer was injected (5 sec, 0.5 psi). The run was performed for 50 min and good base line was obtained by applying voltage of 20 kV at 20°C.

- **Washing of the capillary**

At the beginning of every working day and between each run, the capillary was rinsed with 200 mM sodium dodecyl sulphate (1 min, 20 psi), 1 M NaOH (1.5 min, 20 psi), water (1 min, 20 psi) and finally with electrolyte (2 min, 20 psi). Then the electrolyte buffer was injected (5 sec, 0.5 psi).

At the end of the working day, capillary was washed with water (2 min, 100 psi), then both inlet and outlet of the capillary is kept overnight in water vials.

5.4 Instrumentation

5.4.1 CE-LIF system specifications are described as follows:

Capillary electrophoresis: P/ACE^{TM} MDQ system with a laser-induced-fluorescence (LIF) detector (Beckman Coulter, Krefeld, Germany).
32Karat^{TM} Software, Version 7 (Beckman Coulter, Krefeld, Germany)

LIF detection system: includes LIF detector module (dual wavelength), power supply, LIF cartridge plug and probe stabilizer, fibre optic cable, 520 nm emission filter and 488 nm notch filter (Beckman Coulter, Krefeld, Germany)

Laser 1: Argon-ion laser with λ_{ex} =488 nm (Power: 10 mW output) Beckman Coulter (Krefeld, Germany).

Laser 2: Sapphire solid-Laser with λ_{ex} =488nm (Power: 20 mW output) Coherent (Dieburg, Germany).

Capillary: Fused silica capillary (50 µm ID, 375 µm OD, L_T =50 cm, with the detection window at L_D =40 cm) (BGB-Analytik, Schloßböckelheim, Germany).

5.4.2 UV-VIS spectrophotometer specifications for DNA quantification are described as follows:

Specord^{R} 205 UV/VIS spectrophotometer (Analytik Jena, Jena, Deutschland)
Software WinAspect (Analytik Jena, Jena, Deutschland)
0.5 mm Suprasil 115-OS Quartz glass cuvettes (Hellma, Mühlheim, Deutschland), measurement at 254 nm

5.4.3 General laboratory instruments

Centrifugation:

Mini spin-Table centrifuge: Max. 13400 U/min (Eppendorf, Hamburg, Germany)

Allegra^{TM} 25 R centrifuge: Beckman Coulter^{TM}, Krefeld, Germany

Hydrolysis and derivitisation:

Thermomixer: Thermomixer comfort inclusive 0.5 mL- and 2 mL-table with aluminium foil cover (Eppendorf, Hamburg, Deutschland)

DNA dryness:

Speed vacuum: Jouan SA RC 1022 (Jouan, Saint Herblain, France)

DNA purification: Qiagen Genomic-Tip 100 / G for mini-preparation volumes
Qiagen Genomic-Tip 20 / G for midi-preparation volumes

Enzyme preparation:

Dialysis membrane: Slide-A-Lyzer dalysis cassette (10.000 Da), 0.5-3 mL capacity (Perbio Science, Germany)
Syringes: 1 mL-, 2 mL-, 5 mL- syringe (Braun, Melsungen, Germany)
Needles: 0.4 x 20 mm needle (Braun, Melsungen, Germany)
Water resistant marker, spongy floater, glass beaker (10 L)

pH adjustment of buffers:

pH-meter: Beckman 350, (Beckman Coulter, Krefeld, Germany)
pH-standard electrode: 511275-AB (Beckman Coulter, Krefeld, Germany)

Water preparation:

1. Milli-Q synthesis A10 water ultrapure laboratory water system (Millipore, Schwalbach or Eschborn, Germany), and the water quality was 18.2 MOhms × cm with TOC 5-10 ppb.
2. TKA-GenPure 08.2207 UV-TOC/UF water distillation apparatus (TKA, Niederelbert, Germany).

Others equipments:

Balances: Sensitive balance BP 221S [Max 220 g, d = 0.1 mg] (Sartorius, Göttingen, Germany)
Analytical microbalance M2P [Max 2 g, d = 1µg] (Sartorius, Göttingen, Germany)
Beakers: 10 mL, 50 mL, 100 mL, 200 mL, 500 mL, 800 mL beakers.
Centrifuge tubes: 15 and 50 mL Centrifuge tubes (Sarstedt, Nümbrecht, Germany)
Gas burner: MICROFLAM-burner MFB/E (Proxxon, Niersbach, Germany)

Graduated cylinders:	25 mL, 50 mL, 100 mL, 250 mL graduated cylinders
Magnetic stirrer:	IKAMAG RCT (IKA)
Pipettes:	Transfer pipettes in different sizes [0.5 – 10 µL, 10 – 100 µL, 100 – 1000 µL, 1-5 mL] (Brand, Wertheim, Germany) Pasteur pipettes [0.5 and 3 mL] (Sarstedt, Nümbrecht, Germany) Pipette micro-tips 0.1-10 µL (Roth, Karlsruhe, Germany) Pipette yellow- tips, 200 µL (Sarstedt, Nümbrecht, Germany) Pipette blue- tips, 1000 µL (Sarstedt, Nümbrecht, Germany) Pipette white- tips, 1250 µL long (Sarstedt, Nümbrecht, Germany) Plastibrand 5-mL tips (Roth, Karlsruhe, Germany)
Reaction capsules:	Eppendorf capsules (Caps 0.5, 1.5 mL), Hamburg, Germany
Sample vials:	2 mL CE glass vials (Beckmann Coulter, Krefeld, Germany) 200 µL PCR vials (Neolab, Heidelberg, Germany) Rubber caps REV AA and REV AB for glass and PCR vials (Beckman Coulter, Krefeld, Germany)
Sonicator:	Qualilab USR 54H (Merck Eurolab, Bruchsal, Germany)
Storage containers:	250 mL screw-cap vials of Duran (C, No: A 359.1)
Syringe:	Glass syringe Luer Lock [20 mL], Fortuna®, (Poulten & Graf, Wertheim, Germany)
Volumetric flasks:	50 mL, 100 mL, 200 mL, 500 mL volumetric flasks.
Vortexer:	REAX 2000 (Heidolph, Schwabach, Germany)

5.4.4 LC-UV system specifications for HPLC separation of 8-oxo-2'-deoxyguanosine-3'-monophosphate from its starting material are described as follows:

Pump:	Merck Hitachi L-6000 A (Tokyo, Japan)
Column:	Lichrospher^{R} 100 column (250 x 4 mm, ID), RP- 18 E (5 µm) (Darmstadt, Germany)
Detector:	Merck Hitachi L-4250 UV- visible detector: (absorbing band λ max 247) (Tokyo, Japan)

5.4.5 SPE system specifications are described as follows:

Solid phase extraction (SPE) vacuum manifold (Merck, Lichrolut) consists of:

1. Glass chamber to allow easy visual monitoring.
2. Adjustable racks to accommodate a variety of vials and small-capacity beakers.
3. Flow control valves to allow easy control of flow through SPE columns.
4. Vacuum gauge and valve to monitor and release the vacuum during processing.
5. Different Stationary phase types for SPE columns were used:

   1) Lichrolut^{R} RP-18 E (40-63 μm) in plastic column, 3 mL capacity (Merck, Darmstadt, Germany).

   2) Oasis^{R} HLB (Waters Corporation) solid phase extraction column, a copolymer consisting of lipophilic divinylbenzene and hydrophilic N-vinylpyrrolidone, capacity 60 mg/3 mL (Waters, Eschborn, Germany)

   3) SPE: Strata^{TM}-X-AW, a Polymeric mixed mode weak anion exchange sorbent (modified surface of styrene divinylbenzene sorbent), capacity 200 mg/3 mL (phenomenex, USA).

5.4.6 Mass spectrophotometer:

Micromass Q-Tof ultima^{TM} API (Waters corporation, Milford massachussets, USA) Capillary voltage: 3 kV, source T: 95°C, desolvation T: 180°C, collision Energy: 10 eV.

5.5 Statistical methods

SCNT and twin study

Separate models for the 3 experimental groups were run to estimate inter-individual and inter-genotype variances and another linear mixed effects model for the combined measurements of all Simmental animals to estimate the difference in average methylation levels between clones and twins. Data analysis was carried out with the statistical software environment R 2.6.2 [(534)]. Specifically, the lme4 package [(535)] was used for the estimation of the linear mixed effects models [(536)] and the RLRsim package [(537)] to compute exact restricted likelihood ratio tests [(538)] for the presence of between-genotype variation. Reported *P*-values for the latter are based on Monte Carlo samples of 10000 values from the exact sample distribution of the likelihood ratio.

3-Nitrobenzanthrone-In-vitro study

Data analysis was carried out with the statistical software graph PAD Prism® version 5.02 for comparison of the mean values for the control samples and that of 3-NBA–incubated Caco-2 cells.

5.6 Software

- CE measurements: 32 Karat™ version 7.0 (2003), Beckman Coulter, Krefeld, Germany
- UV measurement: WinAspect version 2.2.1.0 (2006), Analytik Jena, Jena
- Mass spectrum: MassLynx version 4.0
- Chemical structures : ChemDraw Ultra version 7.0.1
- Calculations: Excel-Microsoft Office 2003
- Text: Word-Microsoft Office 2003
- Electropherograms: Sigma plot 2004 version 9.0
- HPLC chromatograms: Paint.NET, Version 3.36

6 Appendix

Glossary

Abasic sites: Sites in DNA from which purine or pyrimidine bases have been lost by cleavage of the deoxy ribose N-glycosidic linkage.

Adenocarcinoma: A tumour developed in an organ or gland.

Allele: Alternative form of a gene.

Apoptosis: Programmed cell death.

Benign tumour: referring to tumour possessing cells that closely resemble normal cells and is localised in the tissue where it originates. See also malignant tumour.

Carcinogen: Any chemical or physical agent that can cause cancer when cells or organisms are exposed to it.

Carcinoma: A malignant tumour derived from epithelial cells.

Centromere: Constricted portion of a mitotic chromosome where sister chromatides are attached, it is required for proper chromosome segregation during mitosis and meiosis.

Chromosome translocation: Abnormality caused to the chromosome by rearrangement of parts between non homologous chromosomes.

DNA: Deoxyribonucleic acid; the molecule that carries genetic information.

DNA adducts: Active metabolites bound covalently to DNA

DNA sequencing: Methods to determine the order of nucleotide bases.

Epigenetics: On top of genetics.

Gene: Fundamental unit of heredity (specific section of DNA within a chromosome).

Gene conversion: one allele of gene is converted to another during meiotic recombination

Genome: A full set of chromosomes carried by a particular organism.

Genotype: The genetic makeup of an individual, also the alleles at one or more specific Loci. See also allele.

Germ line mutation: mutation concerning lineage of germ cells, which give rise to gametes and thus participate in formation of the next generation of organisms, and this mutation will be transmitted from one generation to the next through gametes. See also mutation.

Giloma: A malignat tumour derived from nerve tissues.

Heterochromatin: Regions of chromatin those remain highly condensed and transcriptionally inactive during interphase.

***In-vitro*:** Denoting a reaction or process taking place in an isolated cell-free extract; sometimes used to distinguish cells growing in culture from those in an organism.

***In-vivo*:** In an intact cell or organism.

Leukemia: Cancer of white blood cells and its precursors.

Lymphoma: A malignant tumour developed in lymph glands.

Malignant tumour: referring to tumour that can invade surrounding normal tissue. See also benign tumour.

Meiosis: Cell division in sexually reproducing organisms that reduces amount of genetic information by half.

Melanoma: Cancer involving pigment cells of the skin.

Mitosis: Simple cell division without a reduction in chromosome number.

Mutation: A permenant and heritable change in the nucleotide sequence of a chromosome, usually in a single gene; commonly leads to change in or loss of the normal function of the gene product.

Phenotype: Physical characteristics of an individual.

Point mutation: Change of a single nucleotide in DNA, especially in a region coding for protein, can result in formation of a codon specifying a different amino acid or a stop codon.

Promoter: Regulatory regions of DNA located towards 5' region of gene and provide a control point for regulated gene transcription.

Sarcoma: A malignant tumour of connective and supporting tissues (blood vessels, bones and muscles)

Somatic mutation: mutation concerning any plant or animal cell other than a germ cell. See also mutation.

Tautomeric shift: spontaneous rearrangement to transient alternative form

keto (standard) ↔ ***enol*** forms (**G** &**T amino** (standard) ↔ ***imino*** forms of **A** & **C**)

Transposon: a relatively long mobile DNA element, in prokaryotes and eukaryotes, that moves in the genome by a mechanism involving DNA synthesis and transposition.

N.B: Most of the definitions were structurally modified from the book 'Molecular Cell Biology', 4th Edition.

A brief history of Capillary electrophoresis

1897: **Kohlrausch** introduced of the basis of the essential characters of the electrophoretic transport processes by its law ''independence of ion migration'' in and proofed mathematically that the passage of an electric current does not change the concentration in a system with uniform electrolyte distribution [405].

1930s: **Arne Tiselius** introduced the free solution-moving boundary electrophoresis in its classical forms [406] for the analysis of complex protein mixtures.

1948: **Tiselius** was awarded Nobel Price in Chemistry for his work concluding that the direction and the rate of migration of the components were determined by their charge and their mobility.

1960s: Introduction of classical techniques employing stabilizing media of paper and gels of polyacrylamide and agarose. Gel electrophoresis was designed as a molecular sieve for the size-dependent separation of nucleic acid and proteins.

1967: **Hjertén** performs electrophoresis in free solution without any supporting media to overcome the convection problem and named after him 'free-zone-electrophoresis'[407].

1974: **Virtane** applied electrophoresis employing open glass tubes of 200-500 µm ID capillaries.

End of the70's: First steps for the actual development of capillary electrophoresis by **Mikkers**, **Everearts** and **Verheggen** [408] using thin capillaries (200 µm ID) made from Teflon for zone electrophoresis.

1981: Rapid advance in CE was achieved by **Jorgenson** and **Lukacs** [409-411] and described electrophoretic separations of peptides using free zone electrophoresis (FZE) in glass capillaries of 75 mm ID employing high voltage of separation (30 kV) .

1984: **Terabe** [539] introduces micellar electrokinetic chromatography (MEKC)

1985: Introduction of capillary isoelectric focusing (CIEF) by **Hjerten.**

From begining of 1950: Researches about capillary electrochromatography (CEC)

Laboratory Document

Hydrolysis and Derivatisation of DNA Samples

Date: 20.10.08 Operator(s) (Name/Abbreviation): DZ

Sample(s)-Abbreviation: S.I. 2, S.I. 7, S.I. 8 and CT-10

Description:

Samples:
1. Hong Kong samples: S.I. 2,7,8 (10µg)
2. CT-DNA: 10 µg

1. Hydrolysis

Hydrolysis method: I
Preparation of DNA at: 01.09.08 Amount: 10 µg

Remarks:

Hydrolysis with 15 folds enzyme mixture

Preparation of DNA by (Operator): Hong Kong and DZ

Enzyme mixture MN/SPD (150mU/ml MN, 2.5U/ml SPD):

Date of preparation: 29.05.08 / 12.08.08 Operator: DZ
Date of first use of respective aliquot: 20.10.08

Hydrolysis buffer (250 mM HEPES, 100 mM $CaCl_2$, pH 6.0):

Description: HB250 DZ (3) Preparation Date: 16.07.08

Operator: DZ

Production of used water: 16.07.08 Charge: G-08-01

Was the hydrolysis buffer warmed up to R.T. before hydrolysis? Yes

Water for DNA-dilution (Tridest.):

Manufactured by processing of demineralized water by means of a water treatment device of the company Millipore

Equipment designation and technical Data:

Milli-Q SYNTHESIS A-10 ( )	TKA Genpure (x)
Charge: H-08-02	

Information of water quality (See Water-Document**)**

Hydrolysis conditions:

Temperature: 37°C

Exact Hydrolysis duration: 3 h

h x ν: Yes ( ) No (x)

2.Derivatisation:

Derivatisation method: DVI MT

Derivatisation buffer:

Description: DDZ (3) Composition: 50 mM HEPES, pH 6.36

Preparation Date: 28.04.08 Operator: DZ

Production of used water: 21.02.08 Charge: Water for HPLC

Was the derivatisation buffer warmed up to R.T. before derivatisation? Yes

BODIPY and EDC:

BODIPY = 1.25 mg + 125 µL buffer DDZ (3)

Lot & Filling code (Manufacturer): 479175

1. Date of opening of used aliquot: 131008

Remark:

EDC = 42.6 mg + 121.5 µL buffer DDZ (3)

Lot & Filling code (Manufacturer): 1251845 / 30307089

Internal charge / Aliquot description: 02.10.07 / 010 DZ

1. Date of opening of used aliquot: 10.10.08

Subsequent aliquoting: Yes (x) No ( )

Derivatisation conditions:

Temperature: 25°C

Derivatisation duration: 21 h Reaction volume [µL]: 70 µL

h x ν: yes ( ) No (x)

Pipettes (Transfer pipette ®, Fa. Brand):

Which Pipettes for hydrolysis were used??

- Production of enzyme solution: 10-100µL (yellow) (x) 0.5-10µL (grey) ( )

-DNA-transfer and enzyme addition: 10-100µL (yellow) ( ) 0.5-10µL (grey) (x)

Which Pipettes for derivatisation were used?

- HEPES addition to hydrolysate:	10-100µL (yellow)	(x)	0.5-10µL (grey)	( )
-Production BODIPY-solution:	10-100µL (yellow)	(x)	0.5-10µL (grey)	( )
- Production EDC-solution:	10-100µL (yellow)	(x)	0.5-10µL (grey)	( )

Last calibration of pipettes is done at: March 2007

Subsequent treatment of derivatised sample(s):

1. Aliquot:

---

2. Storage (Temp. / Location): - 20°C / Laboratory H11.16

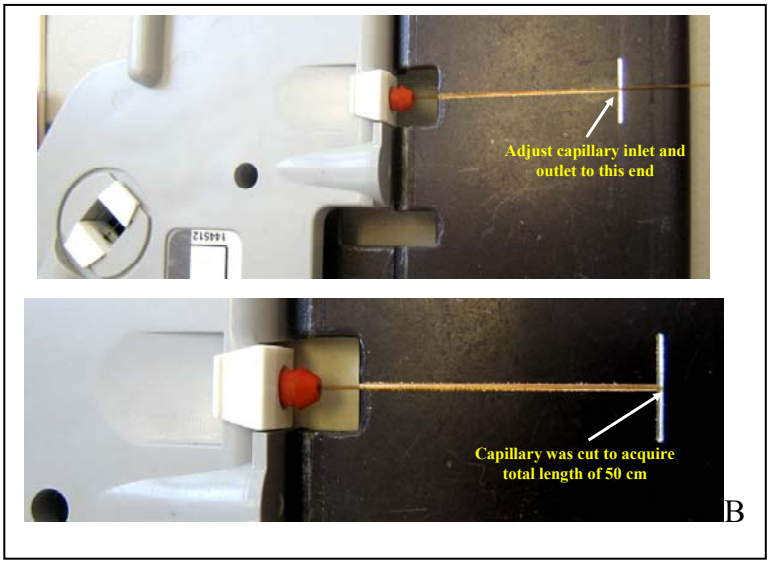
Conclusion:

After derivatisation, precipitation of sample was done by tetraphenylborate. Then centrifugation was done for 10 min.

Date: 21.10.08 Signature of Operators: …….

Description of sequence

Run number	Sample	Capillary	Laser	Dil.	Buffer	Data file
001-003	T1	WNI 05A DZ 140309	Sapphire solid laser	1:100	20 mM sodium phosphate buffer (pH 9.0) in 10 % MeOH	140309DZ001-003
004-006	T2	same	same	same	same	140309DZ004-006
007-009	T3	…	…	…	…	140309DZ007-009
010-012	CT 1	…	…	…	…	140309DZ010-012



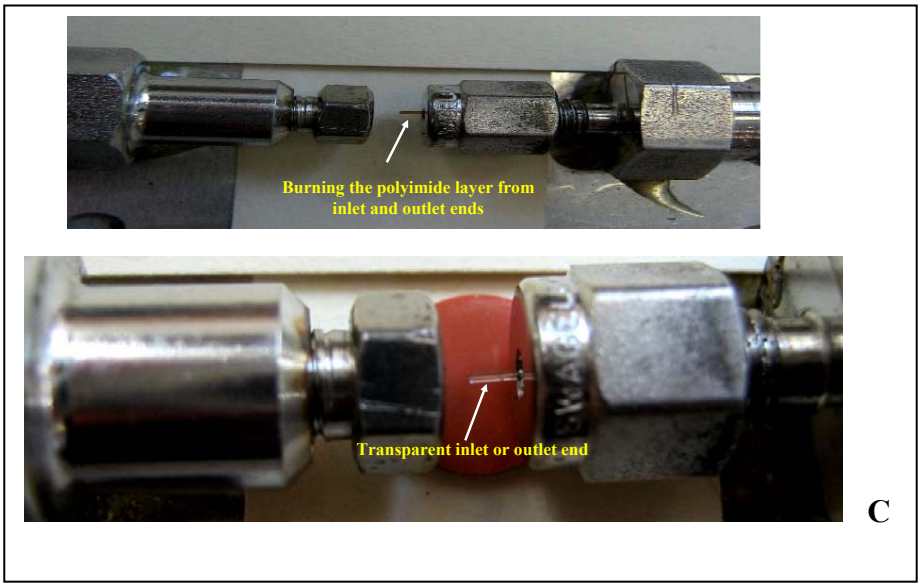


Figure 57: Steps of capillary preparation

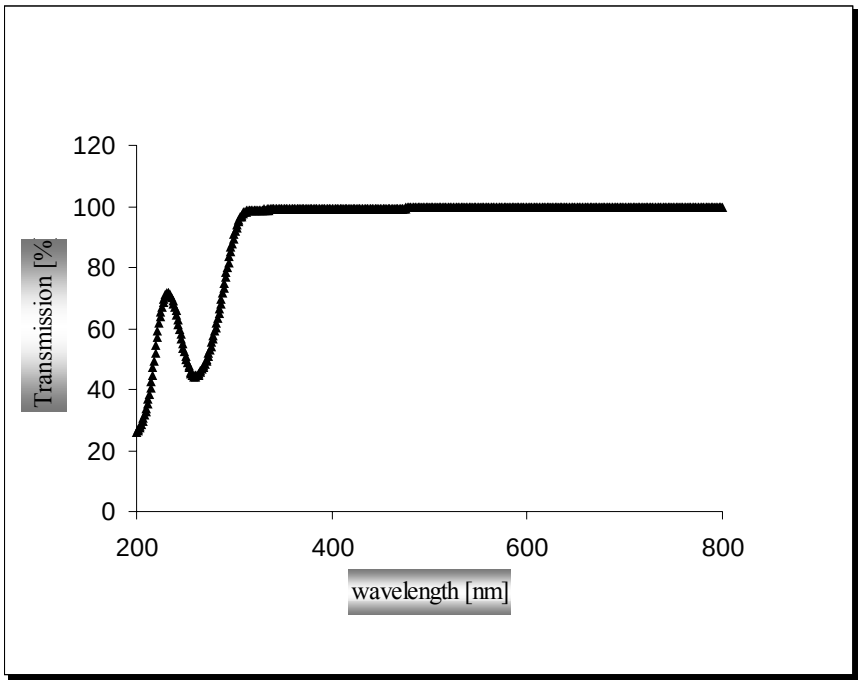


Figure 58: Transmission spectrum of CT-DNA

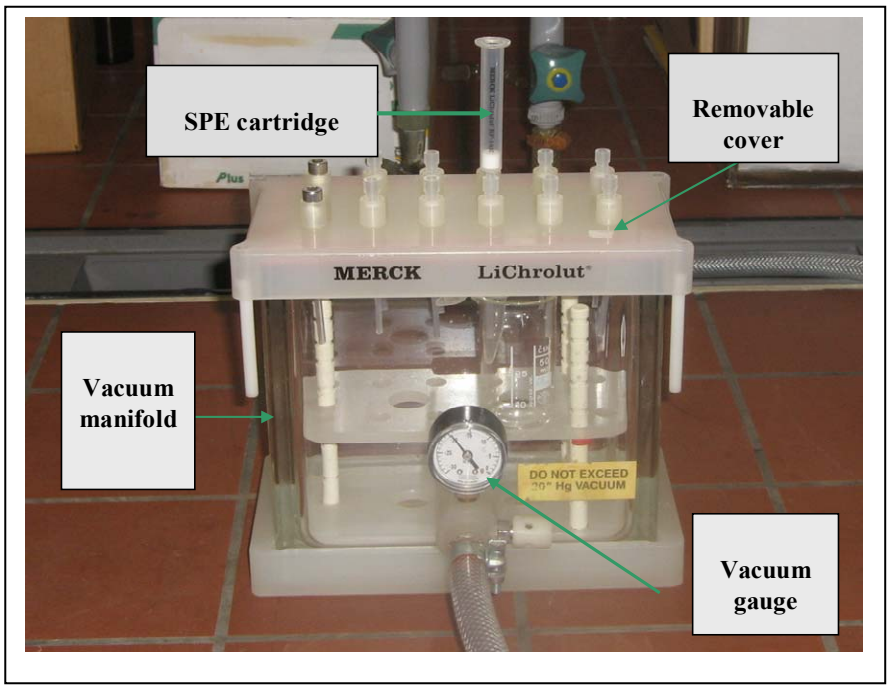


Figure 59: Solid phase extraction manifold

The illustrated solid phase extraction (SPE) set was used for cleaning purposes of the synthesised 8-oxo-dGMP.

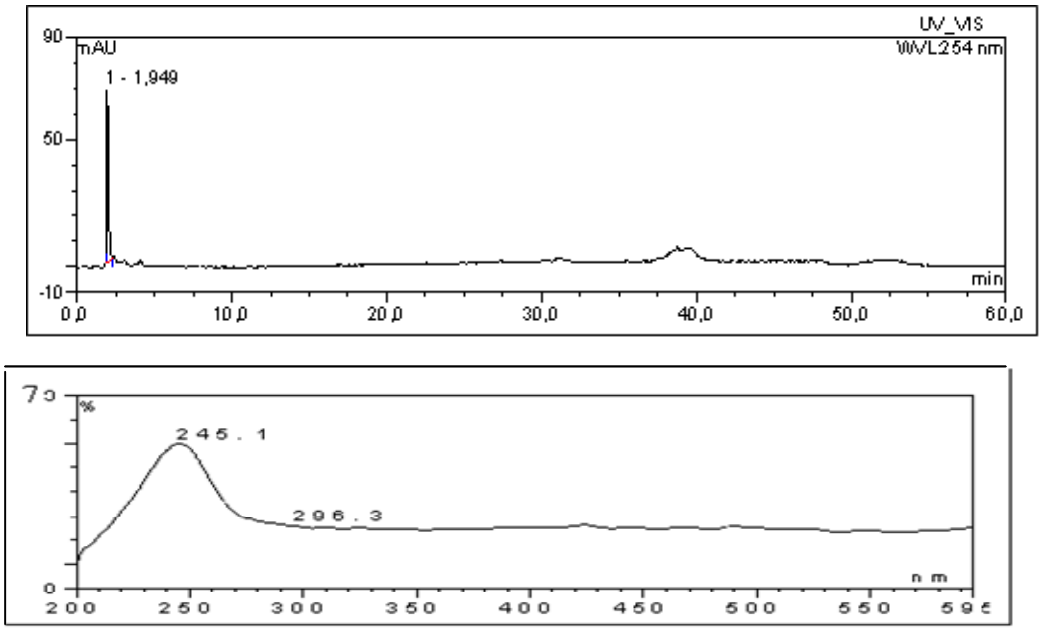


Figure 60: LC chromatogram-UV–VIS spectrum of 2'-deoxyguanosine-3'-monophosphate (3'-dGMP). Chromatographic conditions: The analysis was performed on Knauer column (125 X 4 mm, ID), prepacked with Eurosphere 100 C18 (5 µm), with integrated Knauer pre-column (Berlin, Germany)**,** Dionex pump P580A LPG , Dionex Photodiode Array Detector.

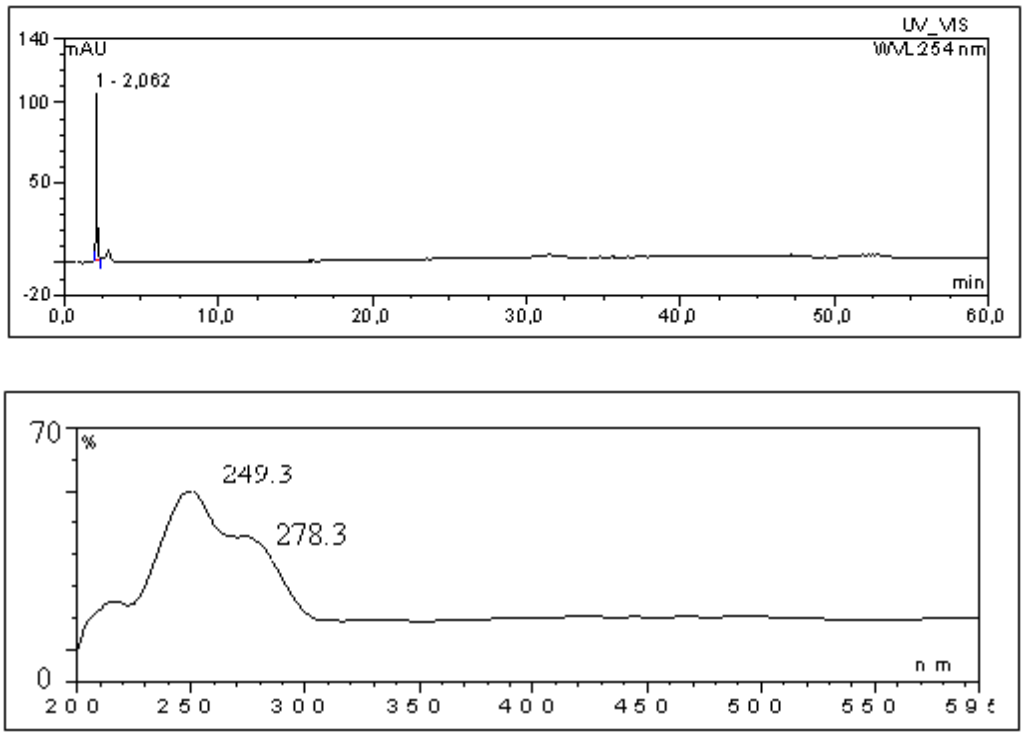


Figure 61: LC chromatogram-UV–VIS spectrum of 8-oxo-2'-deoxyguanosine-3'-monophosphate (8-oxo-dGMP). Chromatographic conditions: same as in previous figure

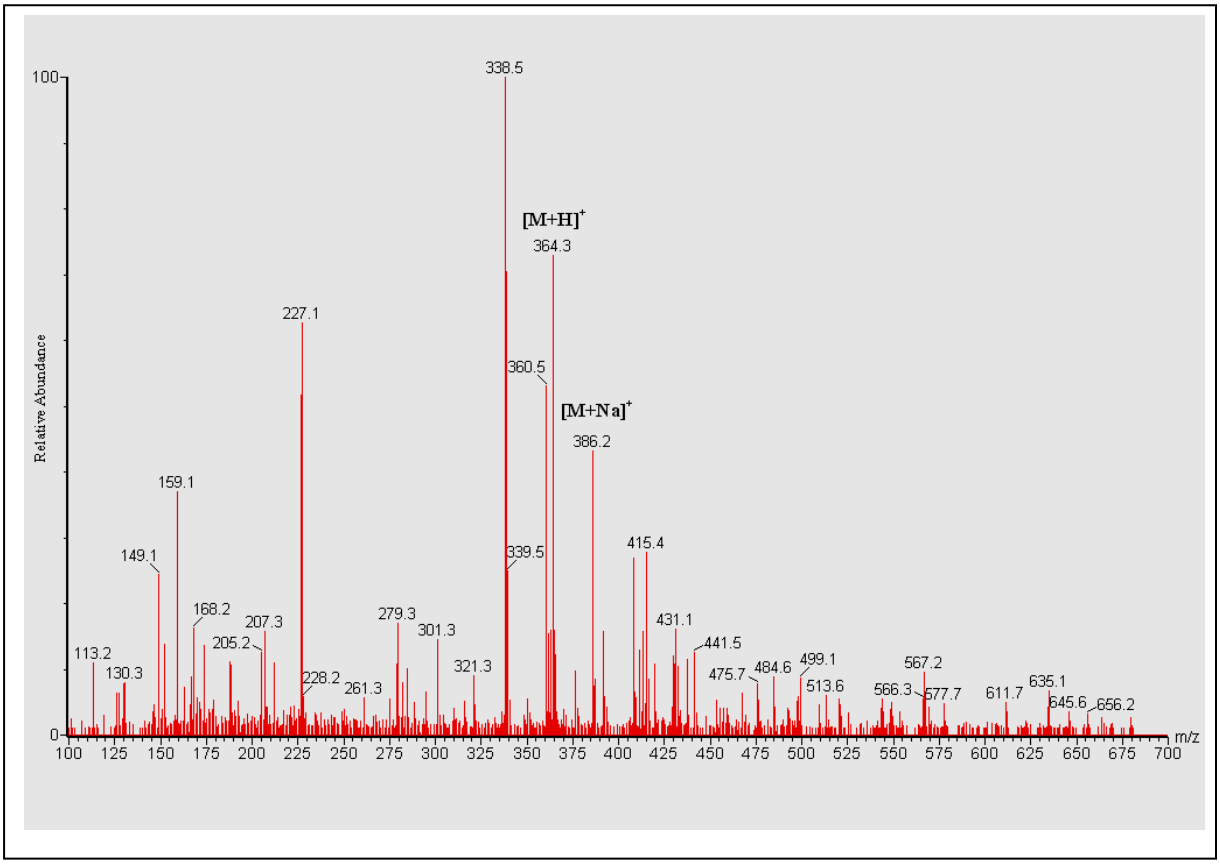


Figure 62: Mass spectrum ESI-MS positive injection mode of non purified 8-oxo-dGMP in the collected fraction from HPLC after its separation from the starting material (Mobile Phase: 25 mM ammonium formate)

7 List of figures

8 List of tables

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Curriculum Vitae

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Education

- Courses in Instrumental analysis at Institut für Naturwissenschaft, Fachbereich C - Analytische Chemie, Bergische Universität Wuppertal, Germany.
- Chemometry Seminar at Bergische Universität Wuppertal, Germany.
- Master degree of Pharmaceutical Sciences in Analytical Chemistry, Faculty of Pharmacy, Cairo University; with general grade very good, in 2004.
- Postgraduate studies in the department of Analytical Chemistry at the Faculty of Pharmacy, Cairo University, Egypt, with general grade very good, in 1999.
- Bachelor of Pharmaceutical sciences, Faculty of Pharmacy, Cairo University, Egypt, with general grade Excellent with honors, in 1998.
- Graduation with secondary school certificate 'Thanaweya Amma', Frensh School ''Notre Dame Des Apôtres", Cairo, Egypt, in1993.
- Certificate de l'Alliance française, 1991-1992.
- Diplôme de Tourisme de la Chambre du Tourisme Française, Paris, in 1991-1992.
- Preparatory School, "Notre Dame Des Apôtres", Cairo, Egypt, from 1986-1989.

Professional and work experience

- **Februar 2007 – now:** PhD student at the ''Institut für Naturwissenschaft'', Fachbereich C -Analytische Chemie, Bergische Universität Wuppertal, Germany.
- **August 2004–Mai 2006:** Assistant lecturer at the department of Analytical Chemistry, Faculty of Pharmacy, Cairo University-Beni Sweif Branch, Egypt
- **June 1999–July 2004:** Demonstrator at the department of Analytical Chemistry, Faculty of Pharmacy, Cairo University-Beni Sweif Branch, Egypt
- **February 2003–December 2005:** Assistant lecturer at the department of Pharmacognosy and Phytochemistry, Faculty of Pharmacy, 6 October University, Egypt.
- **August 1998- May 2005:** Pharmacist, part-time in In- and Out- patient Pharmacies.

List of courses taught:

- Teaching practical courses in Analytical Chemistry (Water Analysis) for fifth semester students at the"Institut für Naturwissenschaft", Fachbereich C -Analytische Chemie, Bergische Universität Wuppertal, Germany.
- Teaching practical courses in analytical chemistry for first year and second year pharmacy students in the department of Analytical Chemistry at Cairo University, Egypt. Courses taught are: Qualitative analysis (Cations and Anions). Quantitative analysis: Titrimetry, Spectrophotomety, Electrochemistry, Quality control analysis of oils and fats.Water analysis
- Teaching practical courses in Phytochemistry for third year and forth year pharmacy students in the department of Pharmacognosy and Phytochemistry, 6 October University. Courses taught are: Chromatographic separations of HPLC and TLC. Qualitative determination and quantitative analysis of volatile oils. Quantitative analysis of carbohydrates. Qualitative identification and determination of alkaloids.

List of publications:

1. **Stability-Indicating Methods for the Determination of Indapamide in the Presence of its Degradation Product**, *Bull.Fac.Pharm*. Cairo Univ., 2003, 41, 269-283
2. **Quantification Of Leukocyte Genomic 5-Methylcytosine Levels Reveals Epigenrtic Plasticity in Healthy Adult Cloned Cattle**. Accepted article in *Cloning and stem cells* 2009

Conferences

- **Highly variable epigenomes in healthy adult clones**. Poster presented in 34th International symposium on High-Performance Liquid Separations and Related Techniques, June 28- July 2nd, 2009 in Dresden, Germany. Among 628 Posters, the poster awarded the **third price** given in pharmaceutical and bioanalysis that sponsored by Pfizer.

- **A strategy to determine the contribution of epigenetics to phenotypical variations using adult cows with the same genotype**. Poster presented in International Embryogenomics meeting, October 17-20th, 2007 in Paris, France.

- Lifecom symposium at March 12th-14th 2007 (Heinrich Heine Universität, Düsseldorf)