ASSESSMENT OF THE GENOTOXICITY OF DIETARY ACRYLAMIDE

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Abstract

Assessment of the genotoxicity of dietary acrylamide

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The identification of the chemical acrylamide (AA) in food was followed by intense research which led to the discovery that AA forms in starch-rich foodstuff when roasted, fried and baked, due to the reaction of the amino acid asparagine and reducing sugars. Animal studies have shown the carcinogenic effect of AA leading to tumours in multiple sites and in 1994 the IARC classified AA as a probable human carcinogen.

After ingestion AA is metabolised to the reactive epoxide glycidamide (GA) that is able to react with bio-macromolecules such as DNA and haemoglobin (Hb). Three major DNA adducts of GA have been reported of which the *N*7-(2-carbamoyl-2-hydroxyethyl)guanine (*N*7-GA-Gua) is the most abundant.

The aim of this project is to develop a sensitive method for the detection of the *N*7-GA-Gua adduct in human leukocyte DNA and urine with two different analytical techniques, namely mass spectrometry, applying LC-MS/MS, and the immunoassay ELISA.

A mass spectrometric method was developed utilising online column-switching achieving a LOD of 7 adducts/10^8 nucleotides and a LOQ of 9 adducts/10^8 nucleotides for the detection of *N*7-GA-Gua in human leukocyte DNA. The developed method was applied to analyse the leukocyte DNA of 32 healthy volunteers. In a few samples peaks were detectable, indicating the presence of the *N*7-GA-Gua adduct but below the LOD and with a high variability. For 10 samples the AA- and GA-Hb adducts were analysed; AA-Hb adducts correlated with AA intake 24 hours prior to donation but there was a non-significant correlation between Hb adducts and *N*7-GA-Gua adduct levels. It was not feasible to develop a method for the detection of *N*7-GA-Gua in urine due to the high salt concentration of this matrix and difficult clean-up procedures prior to LC-MS/MS.

Two polyclonal antibodies were raised against *N*7-GA-Gua to develop a competitive ELISA but due to very strong binding with low sensitivity towards the *N*7-GA-Gua adduct the antibodies were not suitable for use in an ELISA assay.

Aiming for a better sensitivity might allow detecting the *N*7-GA-Gua in human samples.

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Abbreviations

A	Absorbance
AA	Acrylamide
AA-Val-FTH	AA-fluorescein-5-[4-isopropyl-3-(2-carbamoylethyl)-2-thioxo-imidazolidin-5-one]
AAMA	*N*-acetyl-S-(2-carbamoyl-2-hydroxyethyl)-L-cysteine
ACN	Acetonitrile
AFB-*N*7-Gua	Aflatoxin B1-*N*7-guanine
ALARA	As low as reasonably achievable
AP	Apurinic or apyrimidinic site
Aq.	Aqueous
ATP	Adenosine triphosphate
BAM	Biotin-anti-mouse
BAR	Biotin-anti-rabbit
BCA	Bicinchoninic acid
BER	Base excision repair
Biotin-PEG-NHS	Biotin-polyethylene glycol-*N*-hydroxysuccinimide ester
BSA	Bovine serum albumin
Bw	Body weight
C	Carbon
CO_2	Carbon dioxide
CT	Calf thymus
CYP2E1	Cytochrome P450 2E1
dc	Direct current
(d)dH_20	(Double-)distilled water
CID	Collision induced dissociation
CTAB	Cetyl trimethylammonium bromide
DCM	Dichloromethane

DMF	N,N-dimethylformamide
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EH	Epoxide hydrolase
ELISA	Enzyme-linked immunosorbent assay
EMCS	6-Maleimidohexanoic acid *N*-hydroxysuccinimide ester
EPIC	European Prospective Investigation into Cancer and Nutrition
ESI	Electrospray ionisation
Et_3SiH	Triethylsilane
FA	Formic acid
FFQ	Food frequency questionnaire
FIRE	FITC reagent used for measurement of adducts (R) formed from electrophilic compounds with a modified Edman
FITC	Fluorescein isothiocyanate
fmol	Femtomole
g	*g* force
GA	Glycidamide
GA-Val-FTH	GA-fluorescein-5-[4-isopropyl-3-(2-carbamoylethyl)-2-thioxo-imidazolidin-5-one]
GAMA	*N*-(R/S)-acetyl-S-(2-carbamoyl-2-hydroxyethyl)-L-cysteine
GC-MS	Gas-chromatography mass spectrometry
GMP	Guanosine monophosphate
GSH	Glutathione
H	Hydrogen
H_2O_2	Hydrogen peroxide
Hb	Haemoglobin
HCEC	Human colonic epithelial cells
HCHO	Formaldehyde

HEPES	4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC	High-performance liquid chromatography
Hprt	Hypoxanthine-phospho-ribosyl-transferase
hr(s)	Hour(s)
IARC	International Agency for Research on Cancer
ILMA	Immunoluminometric assay
IQR	Interquartile range
IS	Internal standard
Iso-GAMA	*N*-acetyl-S-(1-carbamoyl-2-hydroxyethyl)-cysteine
I.U.	International units
k	Thousand
K_2HPO_4	Potassium phosphate dibasic
KLH	Keyhole limpet hemocyanin
L	Litre
(L)LOD	(Lower) Limit of detection
(L)LOQ	(Lower) Limit of quantification
M	Molar
µ	Micro
MA	Mercapturic acid
MALDI	Matrix-assisted laser desorption ionisation
MeOH	Methanol
$MgCl_2$	Magnesium chloride
min	Minute(s)
mL	Millilitre
MLC	Mouse lymphoma cells
MS	Mass spectrometry
m/z	Mass-to-charge
*N*1-GA-dA	*N*1-(2-carboxy-2-hydroxyethyl)-2'-deoxyadenosine

*N*3-GA-Ade	*N*3-(2-carbamoyl-2-hydroxyethyl)-adenine
*N*7-GA-Gua	*N*7-(2-carbamoyl-2-hydroxyethyl)-guanine
NaCl	Sodium chloride
Na_2CO_3	Sodium carbonate
$NaH_2PO_4*2H_2O$	Sodium dihydrogen orthophosphate
NaOH	Sodium hydroxide
NEM	*N*-ethylmaleimide
ng	Nanogram
NH_4HCO_2	Ammonium formate
NMR	Nuclear magnetic resonance
OD	Optical density
Ov	Ovalbumin
PAH	polycyclic aromatic hydrocarbons
PBS	Phosphate buffered saline
Pd	Palladium
PhiP	2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine
pmol	Picomole
polyAA	Polyacrylamide
PS	Protamine sulphate
rf	Radiofrequency
RSD	Relative standard deviation
RT	Room temperature
s	Seconds
S/N	Signal-to-noise
SOP	Standard operating procedure
SRM	Selected reaction monitoring
TBAF	Tetra-n-butylammonium fluoride
TBDMSCl	Tert-Butyl(chloro)dimethylsilane

TFA	Trifluoroacetic acid
TGB	Thyroglobulin
THF	Tetrahydrofuran
Tk	Thymidine kinase (gene)
TLC	Thin layer chromatography
TOF	Time of flight
U	Units
UoL	University of Leicester
US	United States

Chapter 1

1 Introduction

There was an estimated 12.7 million cases of cancer in the world in 2008 with the most common cancers across the world being lung, breast, colorectal, stomach and prostate [1,2]. Approximately 30 - 40% of all cancers are preventable and the World Cancer Research Fund (WCRF) published a list of recommendations for the prevention of cancer including [3],

- Eating a greater variety of vegetables, fruits, wholegrains and pulses
- Limiting the consumption of red and processed meats
- Limiting the consumption of salty foods and foods which were processed with salt
- Limiting the consumption of energy-dense foods and avoiding sugary drinks
- Limiting intake of alcoholic drinks and avoid the use of tobacco products
- Being as lean as possible but not underweight

The WCRF based these recommendations on epidemiological studies such as the European Prospective Investigation into Cancer and Nutrition (EPIC) [3].

Additionally, environmental factors are of importance to the development of cancer illustrated by migration studies, where people's risk to develop certain types of cancer adapts to the prevalence in the region where they immigrated to, rather than developing the cancers they are predisposed to when living in the country where they emigrated from, showing that genetic disposition can be "overruled" by external factors. A study observed the cancer mortality of Polish Americans in comparison to the mortality rates in Poland and the United States (US). Mortality of stomach cancer was low and similar to Poland whereas the low risk of breast, colon and rectum cancer in Poland was not observed for Polish immigrated to the US. Male Polish immigrants had increased cancer of oesophagus and larynx, higher even than for both countries, Poland and the US [4]. In 1973 Buell reported about breast cancer incidence of Japanese American women compared to Japanese and white Americans. Between 1950 and 1960 the breast cancer incidence was slightly increased but still similar to the Japanese numbers whereas by the time 1969 to 1973 the incidence seemed to adapt

to numbers of the US. Even though Polish and Japanese immigrants arrived in the US at similar times, Polish women had already an increase in breast cancer incidence similar to white Americans at the first analysis compared to Japanese women suggesting that Japanese held on longer to their traditional way of life [5]. A more recent study examined incidence rates of colorectal cancer for Japanese, Chinese and Filipinos immigrants in comparison to US white born. Japanese men born in the US had incident rates two fold higher compared to those born in Japan but about 60% higher rates compared to US born white men. Japanese women born in the US had a 40% higher incidence than both women born in Japan or US born white. US born white men had a similar colorectal cancer incidence to men born in China, whereas Chinese men born in the US had a lower incidence. Both Chinese born in China and Chinese born in the US had a reduction between 30 and 40% of colorectal cancer incidence compared to US white women. Filipinos, no matter if born in the Philippines or in the US had 20 to 50% reduced rates in comparison to US white born [6].

1.1 Cancer

Cancer is a multi-stage process [7,8] and the development can take many years, involving the steps of initiation, promotion and progression [8,9]. Vogelstein and Kinzler reviewed in 1993 the multi-step nature of cancer illustrating that cancer evolves from the accumulation of multiple mutations in a cell. The long process and the multiple numbers of steps involved can be pointed out by the fact that cancer incidence increases significantly with increasing age. The multi-stage theory is further proven by looking at results of transformations and then tumours in both animals and humans, and observing the involvement of oncogenes and suppressor genes as a function of mutations [10]. Hanahan and Weinberg reported hallmarks which are distinct for the formation of cancer, i.e. “sustaining proliferative signalling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis” [11]. The essential step of the chemical induced genotoxic carcinogenesis is the reaction of a reactive compound with DNA, leading to DNA adducts that can cause mutations. Once these mutations are

established in the cell after replication, these cells are referred to as initiated cells. This mutation is not reversible and only apoptosis of the cell can remove the damage. In promotion, the initiated cell is proliferated by a chemical agent or directly by gene expression. Promotion is also threshold dependent as well as dose dependent but compared to initiation is a reversible step. Progression involves genotoxic and clastogenic events which then transform the pre-neoplastic form into neoplastic stage [8].

1.1.1 Biomarkers

The International Agency for Research on Cancer (IARC) defines a biomarker as ‘‘Any substance, structure or process that can be measured in the body or its products and may influence or predict the incidence or outcome of disease’’ [12]. In molecular epidemiology, biomarkers can be divided into three different groups, i.e. biomarker of exposure, effect and susceptibility. Figure 1.1 shows the different types of biomarkers and the stages of genotoxic carcinogenesis, where they can be used to provide more detailed information.

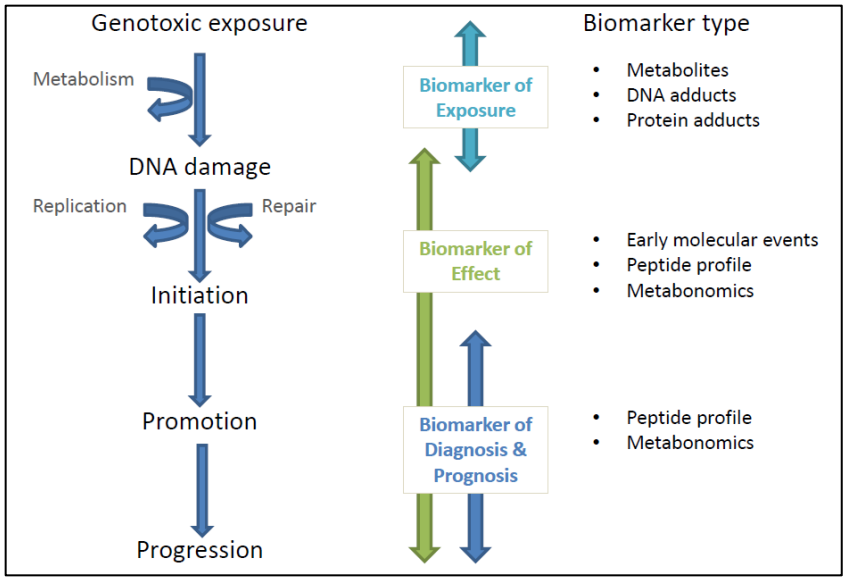


Figure 1.1: Schematic diagram showing the different types of biomarkers on the genotoxic carcinogenesis.

DNA adducts and haemoglobin (Hb) adducts amongst others belong to the group of biomarkers of exposure. Micronuclei, chromosomal aberrations, mutations and aneuploidy are considered as biomarkers of early effect [7,12].

1.1.1.1 DNA adducts

1.1.1.1.1 General information

A DNA adduct, formed at nucleophilic sites in DNA, is defined as a compound formed by the covalent reaction of a parent chemical or its reactive metabolites with DNA [7,13,14]. DNA adducts can form on different atoms within the four bases of which the ring nitrogens and the exocyclic oxygens are preferred positions for alkylation. *N*7-guanine adducts, often the most abundant form, are less mutagenic than for example adducts at O^6-guanine and O^4-thymine due to the mispairing caused by the latter two adducts. There are chemicals that can directly react with DNA and others that need to be metabolically activated, i.e. the activation of AA to the epoxide GA and hence the division into direct-acting and metabolically activated carcinogens [13,15,16]. DNA adducts are an essential step in the carcinogenicity process but the formation of adducts is not sufficient for tumourigenesis [14,17]. While DNA adducts form both in target tissue and in non-target tissue, organ specificity cannot be proved alone by its detection [16]. In double stranded DNA, *N*7-guanine adducts have half-lives between 2 - 150 hrs due to their chemical instability. An enzymatic removal of *N*7-guanine adducts rather than spontaneous depurination can decrease the half-life to minutes (min) [14]. After enzymatic repair adducts get rapidly eliminated and can be detected in urine, especially for *N*7-guanine and *N*3-adenine adducts [18]. Adducts can also be formed with phosphate groups in DNA due to the reaction with alkylating agents. There are no known repair mechanisms for the formed DNA-phosphate adducts, leading to accumulation. Alkylation causes the esterification of the phosphate oxygen and the formation of the phosphotriester configuration, which are chemically stable under physiological conditions [19].

1.1.1.1.2 DNA adducts utilised as biomarkers

One of the best known and first example of a DNA adduct used as a biomarker linking DNA adducts to a specific type of cancer is the aflatoxin B1-*N*7-guanine (AFB-*N*7-Gua) adduct. Aflatoxin is a mycotoxin, mostly occurring on staple foods like maize and peanuts due to storage in warm and humid climates [20,21]. The AFB-*N*7-Gua adducts formed in tissue depurinate from DNA and are excreted in urine [22]; it has been shown in all tested animals that aflatoxin is a liver carcinogen [23] and AFB-*N*7-Gua causes G:C → A:T transitions and G:C → T:A transversions [24]. In 1992, a prospective study with men in Shanghai showed that participants who had detectable levels of AFB-*N*7-Gua adducts in urine had a 7.6 fold higher risk for developing liver cancer and after adjusting for hepatitis B antibodies and smoking the risk increased to 9.1 [25-27]. Today AFB-*N*7-Gua adducts are considered as biomarkers of exposure to aflatoxins and over the years this exposure established it to be a risk factor for liver cancer development [21]. Additionally to the AFB-*N*7-Gua adducts, DNA adducts with, for example, tamoxifen, ethylene oxide and benzidine have been detected in humans [16]. Philips reviewed amongst other DNA adduct concerns, the influence of diet on DNA adducts levels. In a group of fire-fighters polycyclic aromatic hydrocarbons (PAH) DNA adduct levels corresponded to their intake of barbecued burger rather than their fire-fighting activity and exposure to fire [22]. A recent link between DNA adducts and disease, the Balkan Endemic Nephropathy, which is caused by exposure to aristolochic acid, a constituent in plants of the Aristolochiaceae family was described by Jelakovic *et al*. [28]. After metabolism, aristolochic acid forms aristolactam DNA adducts which are concentrated in the renal cortex. These DNA adducts serve as biomarker of exposure and were linked to urothelial cancer. Tumour tissue from 67 patients, living in an endemic nephropathy area was examined and compared to 10 control cancer patients living in a non-endemic region. Adduct levels were detectable in 70% of patients from endemic areas but not in any of the control patients living in non-endemic regions [28].

1.1.1.1.3 Analytical techniques for DNA adduct detection

DNA adducts can be analysed with a huge array of analytical techniques which require a high sensitivity, good specificity and the ability to quantify identified adducts. There are immunoassays and immunohistochemistry, mass spectrometry coupled with gas-chromatography (GC) or high performance liquid chromatography (HPLC), ^{32}P-postlabelling, fluorescence spectroscopy, electrochemical conductance and accelerator mass spectrometry [26,29]. Sensitivity and specificity for these different analytical techniques vary, but most techniques can detect between 0.1 – 1 adduct/10^8 nucleotides. Poirier reported in 1997 that immunoassay and ^{32}P-postlabelling were the most frequently used methods. The sensitivity of radiolabelled immunoassays and enzyme linked immunoassay (ELISA) was 1 adduct/10^8 nucleotides. In comparison to immunoassays, ^{32}P-postlabelling has a higher sensitivity by detecting up to 1 adduct/10^9-10^{10} nucleotides and requires less DNA (2 – 10 µg instead of 200 µg). A disadvantage of the ^{32}P-postlabelling technique is the underestimation of DNA adducts levels due to incomplete radiolabelling of some adducts. The same can happen with immunoassays where an overestimation can occur due to cross-reactivity of antibodies. The specificity of ELISA methods can be improved by *a priori* HPLC steps [22,26,29]. Fluorescence techniques can only be applied to compounds that have fluorescent properties. Coupled with HPLC it can achieve sensitivity of 1 adduct/10^8 nucleotides. Electrochemical conductance assays are also commonly coupled to HPLC but as with the former example can only be performed when the analyte of interest shows electrochemical properties. The most sensitive method is the accelerator mass spectrometry analysis which can detect adducts in the range of 1 - 10 adduct/10^{12} nucleotides, although this has the disadvantage that one has to work with compounds labelled with certain isotopes, such as ^{14}C [13,29].

1.1.1.2 Protein adducts

Proteins contain various nucleophilic sites, like amine and thiol groups within amino acids, that are able to react with reactive electrophiles to form adducts such as those found in either serum albumin or Hb. There are no repair processes to these formed

adducts. One mL of blood contains about 150 mg Hb, 30 mg serum albumin and 0.003 – 0.008 mg DNA, indicating that proteins are a much more available matrix than DNA for analysis of adducts. Serum albumin has a half-life of 20 days whereas that of Hb adducts is 63 days. The adduct formation leads to a steady state level and decreases with the degradation of Hb [15,18,30]. Electrophiles preferably react with Hb and serum albumin on thiol groups in cysteine and nitrogens in histidine, tryptophan, the amino group in lysine and N-terminal amino groups [15]. Therefore Hb adducts are a measure for an internal dose of exposure to a reactive chemical and can serve as long term biomarkers of exposure due to the long life time of erythrocytes (approximately 120 days) [31,32]. Examples of adduct level investigations in humans are aflatoxin B1, PAH, benzene and ethylene oxide [15].

1.1.2 DNA repair

DNA is exposed to a variety of harmful conditions, like chemicals, environmental substances, radiation and also endogenous metabolites which can cause damage to the DNA double helix structure. There are different positions in the DNA bases that are prone to reaction with electrophiles like alkylating agents, as shown in Figure 1.2.

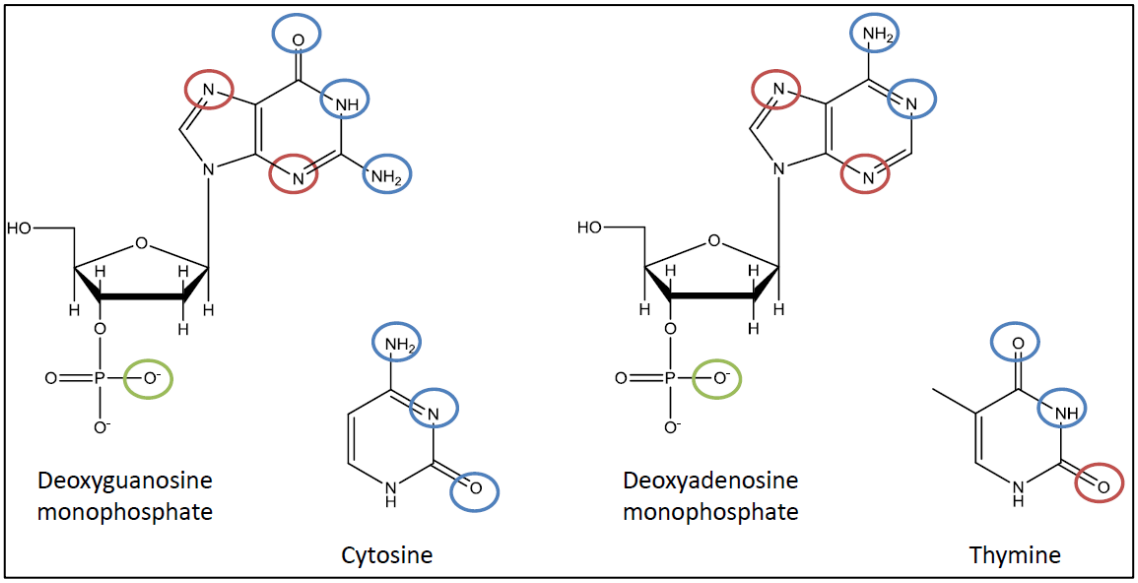


Figure 1.2: Preferred sites in DNA for alkylating agents. The nitrogen and oxygen atoms circled in blue are involved in base pairing. The nitrogen and oxygen atoms circled in red are positions within the base that are not involved in base pairing. Oxygen marked in green are positions for the formation of phosphotriesters (not shown for cytosine and thymine) (modified from [33]).

Generally, nitrogen atoms within the bases are more nucleophilic than the oxygen atoms. The *N*7-guanine and *N*3-adenine are the most reactive positions [33]. There are different DNA repair mechanisms to ensure the integrity and stability of the genome is safeguarded. The main aim of DNA repair is to identify and remove DNA damage and to verify replication of error free DNA. There are approximately 150 genes known that are involved in DNA repair [34] and there are five DNA repair mechanisms, depending on the nature of the DNA damage:

- BASE EXCISION REPAIR (BER) will take place when DNA bases are alkylated, or when oxidative damage, apurinic or apyrimidic (AP) sites are present.
- NUCLEOTIDE EXCISION REPAIR occurs when the DNA has bulky adducts incorporated or when intra-strand linking occurs.
- MISMATCH REPAIR fixes single bases that were mispaired and looped intermediates during replication.
- DIRECT REVERSAL is undertaken by a single enzyme which removes groups from modified DNA bases and incorporates them in their own structure.
- DOUBLE STRAND BREAK REPAIR is of high importance due to the cytotoxic nature of double strand breaks that leads to mutation and cancer.

BER is the main mechanism for damage caused by metabolites within the cell and it can be performed as short-patch BER (single modified base gets removed) or long-patch BER (removal of more than one nucleotide) [35,36]. Different enzymes are involved in this multi-step process of BER. In the process of short-patch BER repair, a DNA glycosylase hydrolyses the N-glycosidic bond to cleave the modified base leading to an AP site. An AP-endonuclease cleaves the phosphodiester backbone which causes a single strand break. Following this, the AP site is removed, the correct single nucleotide inserted and replaced by a DNA polymerase. The DNA ligase reseals the lesion [36-39]. The basic principle for the long-patch process is the same but it involves different enzymes. It appears that the adenosine triphosphate (ATP) concentration has an influence on which patch the repair mechanism follows. If the ATP level is low, it is more likely that the long-patch repair mechanism is followed whereas the short-patch is preferred when ATP levels are higher. Additionally, it depends on the removal of the phosphodiester, if it cannot effectively be removed, the long-patch BER will take place [36].

1.2 Acrylamide

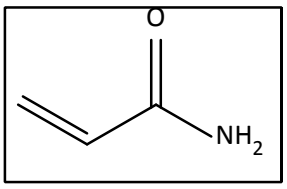


Figure 1.3: AA structure

Acrylamide (AA) (Figure 1.3) is an α,β-unsaturated compound of high reactivity due to two reactive sites in the molecule; firstly the conjugated double bond and secondly the amide group. AA is an odourless and colourless [8,40] vinyl chemical with a wide range of applications mainly in industrial processes like wastewater treatment, as a grouting agent and soil conditioner or in processes in the paper, textile and cosmetic industry. It is also used as a monomer for the polymerisation to polyAA which is commonly used in laboratories, i.e. for gel electrophoresis [40].

1.2.1 Carcinogenicity and genotoxicity of acrylamide

Many *in vitro* and *in vivo* studies have shown evidence of the carcinogenic and genotoxic effect both AA and GA exhibit. The AA metabolite GA (Figure 1.4) is believed to be the main initiator of the genotoxic and carcinogenic effect caused by AA.

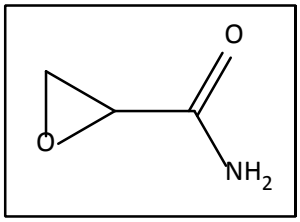


Figure 1.4: GA structure

Carcinogenicity was evaluated in two lifetime studies on rats [41,42] in 1995 and 1986, respectively. Friedman *et al*. observed an increase in tumours of the thyroid, mammary glands and mesotheliomas of testis. Johnson *et al*. reported tumour increases of mammary gland, central nervous system, thyroid gland, oral tissue, clitoral gland and uterus in female and thyroid and scrotal mesothelial tumours in male rats [41]. A more recent two year life time study was undertaken by Beland *et al*. on B6C3F1 mice and F344/N rats dosing the animals with AA in drinking water. After termination of the study there was an increase in harderian gland tumours and lung cancer for both sexes in mice. Male mice had an increase in forestomach cancer and female mice showed an increase in mammary gland, ovary and skin cancer. Rats of both sexes had an increase in thyroid neoplasms. Male rats showed increases in testis, heart and pancreas tumours whereas female rats had higher incidences in clitoral gland, mammary gland, oral cavity and skin tumours [43].

AA is not mutagenic in the Ames test whereas GA shows mutagenic effects. Observed genotoxic effects for AA detected *in vitro* are sister chromatid exchanges, chromosomal aberrations, micronuclei, polyploidy and aneuploidy without application of a metabolic system [44,45].

GA treatment of V79 cells caused DNA damage, detectable after 1 hr in the comet assay. A 4 hr treatment with 300 µM led to DNA damage whereas 100 µM was sufficient to damage DNA following 24 hrs treatment. When applying the DNA repair enzyme formamidopyrimidine-DNA-glycosylase (as a means to detect DNA damage) the DNA damage could be measured at a concentration of 10 µM and GA induced strand breaks decreased about 80% within 8 hrs which might be due to BER

mechanisms. In the Hprt assay 800 µM GA induced mutations, which was a rather high concentration used. The DNA damage caused by GA appeared to be repaired fast when treated in sub-mutagenic concentrations whereas at mutagenic concentrations DNA strand breaks seemed to persist longer suggesting that the AA derived genotoxicity at high concentrations leads to the formation of *N*7-GA DNA adducts overriding the DNA repair process [46].

Treating mouse lymphoma cells (MLC) with AA and GA showed both compounds to be cytotoxic and mutagenic. However, GA already showed mutagenicity at lower concentrations than AA. Testing for loss of heterozygosity revealed that the mutations are caused by chromosome damage, suggesting a clastogenic mode of action. A difference in mutation spectra between AA and GA might give evidence that chromosomal mutations are caused by different mechanisms. The mutagenic effect of AA might be caused by induction of reactive oxygen species that cause a depletion of glutathione (GSH) in the cell whereas GA is a direct mutagen by the formation of DNA adducts [45].

The genotoxicity of AA *in vitro* was tested by taking CYP2E1 expressing cell lines with addition of S9 mix. The metabolic transformation of AA to GA could not be detected with or without S9. In the GA treated bacterial strain TA1535/pSK1002 with and without S9 an increase of DNA damage was noticeable in the *umu* assay showing that the enzymes contained in the S9 did not inhibit this effect. In human lymphoblastoid cells AA treatment only led to traces of *N*7-GA-Gua adduct whereas the GA treatment led to high levels of the *N*7-GA-Gua adduct [47].

GA caused micronuclei by chromosome breakage *in vivo* in mice and rats. The mutagenic and clastogenic properties of AA could be shown in MLC [45]. For all doses given, GA was more mutagenic than AA [48].

Mutagenicity was shown in the liver *cII* gene of both AA and GA treated Big Blue mice, both leading to G:C → T:A transversions and frameshift mutations. Further, both AA and GA led to an increase of mutant frequencies in the lymphocyte Hprt test [49]. The findings of Besaratinia and Pfeifer were that an AA or GA treatment of cells led to

more A → G transitions and G → C transversions whereas GA treatment caused mainly G → T transversions suggesting different formation pathways of AA and GA. AA can react directly with DNA following a Michael reaction, the reaction between a nucleophile and an α,β-unsaturated carbonyl group, whereas GA follows the oxidative pathway [48].

Another study also found increased lymphocyte Hprt mutation frequencies after AA and GA treatment of rats but neither AA nor GA treatment significantly increased liver, testes or mammary cell *cII* mutation frequency. The high dose however, increased the mutation frequency in bone marrow and thyroid, but not significantly in male rats. In peripheral blood no micronuclei increase was detected for either compound. Mei *et al*. suggested their results showed that AA and GA are more likely gene mutants than clastogens or aneugens [50].

Genotoxicity of AA was further shown by Koyama *et al*. [51] in a study where a group of young and adult rats was treated with AA in drinking water. The group investigated DNA damage with the comet assay in liver, micronucleus assay in bone marrow, *N*7-GA-Gua adduct levels were measured in liver, mammary and thyroid glands and testis as well as the gpt mutation assay in liver and testis. In this study the AA intake of the young animals was about 1.5 times higher than that of adults due to a higher water intake. There was a dose response in the comet assay for young and adult animals. The two highest treatment group adult animals showed significantly induced DNA damage but in young rats, only for the highest dose was an increased damage noticeable. For the highest dose there was an increase in micronuclei frequency for the young rats but none for all doses in adult animals. The *N*7-GA-Gua adduct showed a dose dependent increase for all tissues, without difference in mammary or thyroid tissue between young and adult groups. However, the adduct levels in liver and testes varied between groups with young rats having higher levels, especially in testes, where levels were 6 times more than that of adult animals. The gpt-mutation frequency was insignificantly lower in young control animals compared to adult control animals. With the exception of the highest dose for both groups, which doubled the increase of the gpt-mutation

frequency in testis, no increase was observed. This study showed that young rats were especially at risk in terms of genotoxicity of the testis.

As can be seen from lifetime carcinogenicity bioassays, AA treatment of animals did not lead to tumours of a specific target organ but to many different tumours, including tumours of the hormonal system. These findings in addition to results of epidemiologic studies, where it appears that there is an increased risk of endometrial and ovarian cancer associated with dietary AA intake suggest that there are also non-genotoxic mechanisms involving a hormonal route for AA carcinogenicity. Studies showed that AA interfered with prolactin, progesterone, testosterone and estrogen. In female rats, interference with thyroid hormones was observed but not in male rats. This hormonal mechanism has yet to be investigated [30]. A study undertaken by Von Tungeln *et al*. looked at the genotoxic effects of neonatally treated mice. Mice were either treated on postnatal days 1, 8 and 15 or starting on day 1 up to day 8. The latter treatment group showed 2 – 3 fold higher levels of DNA damage with the low GA dose (0.14 mmol) than the treatment group with 3 dosing days. Von Tungeln *et al*. suggest that DNA adduct formation other than GA derived could be causing the Tk and Hprt mutant induction possibly by the formation of reactive oxygen species leading to oxidative damage [52].

The above mentioned alternative mechanisms were observed at high dosage treatment and should not be of high importance when studying the influence of dietary AA intake.

1.2.2 Acrylamide in foodstuff – history, background and formation

While building a tunnel in Hallandsås, Sweden in 1997, polyAA was part of the sealing agent to prevent leakage of water but the polymerisation process was not completed entirely and hence, AA leaked into the water which then entered a creek. Shortly after, cows showed neurotoxicological symptoms and fish in a pond died, both being exposed to the contaminated water. Blood samples of tunnel workers were examined and they revealed high Hb adduct levels of AA [53]. Surprisingly the control group,

without known exposure to AA, also showed levels of the AA-Hb adduct. In subsequent investigations, Swedish researchers at the National Food Agency in Sweden discovered the existence of AA in foodstuff.

In 1997, Bergmark analysed Hb adducts from a non-smoking population, smokers (as cigarettes contain AA) and workers exposed to AA. The mean AA-Hb level for non-smokers was 31 pmol/g globin, for AA exposed laboratory workers 54 pmol/g globin and 116 pmol/g globin for smokers. The adduct levels of smokers correlated with numbers of cigarettes smoked but no explanation could be given to why non-exposed people showed such a high background level [54].

After detecting the AA-Hb adduct in occupational studies, with background levels of approximately 40 pmol/g globin in a group thought to be unexposed to AA, in 2000, Tareke *et al*. analysed the AA-Hb adduct in rats being fed with either a fried or non-fried (control) diet to test the hypothesis that AA exposure occurs through food. In the study the adduct levels of rats fed "fried" food were highly increased compared to the control group and the adduct levels were similar to AA-Hb adduct level found in a non-smoking population [55].

It was in 2002 that Mottram *et al*. and Stadler *et al*. discovered that AA is formed from a Maillard reaction between amino acids and reducing sugars. Stadler *et al*. showed, using asparagine and [$^{13}C_6$]-labelled glucose that asparagine was the carbon donor for the AA formation. A further experiment, where [^{15}N]-amide labelled asparagine was reacted with glucose showed that the amino acid was the nitrogen source for AA [56,57]. Figure 1.5 shows the schematic formation of AA following the Maillard reaction.

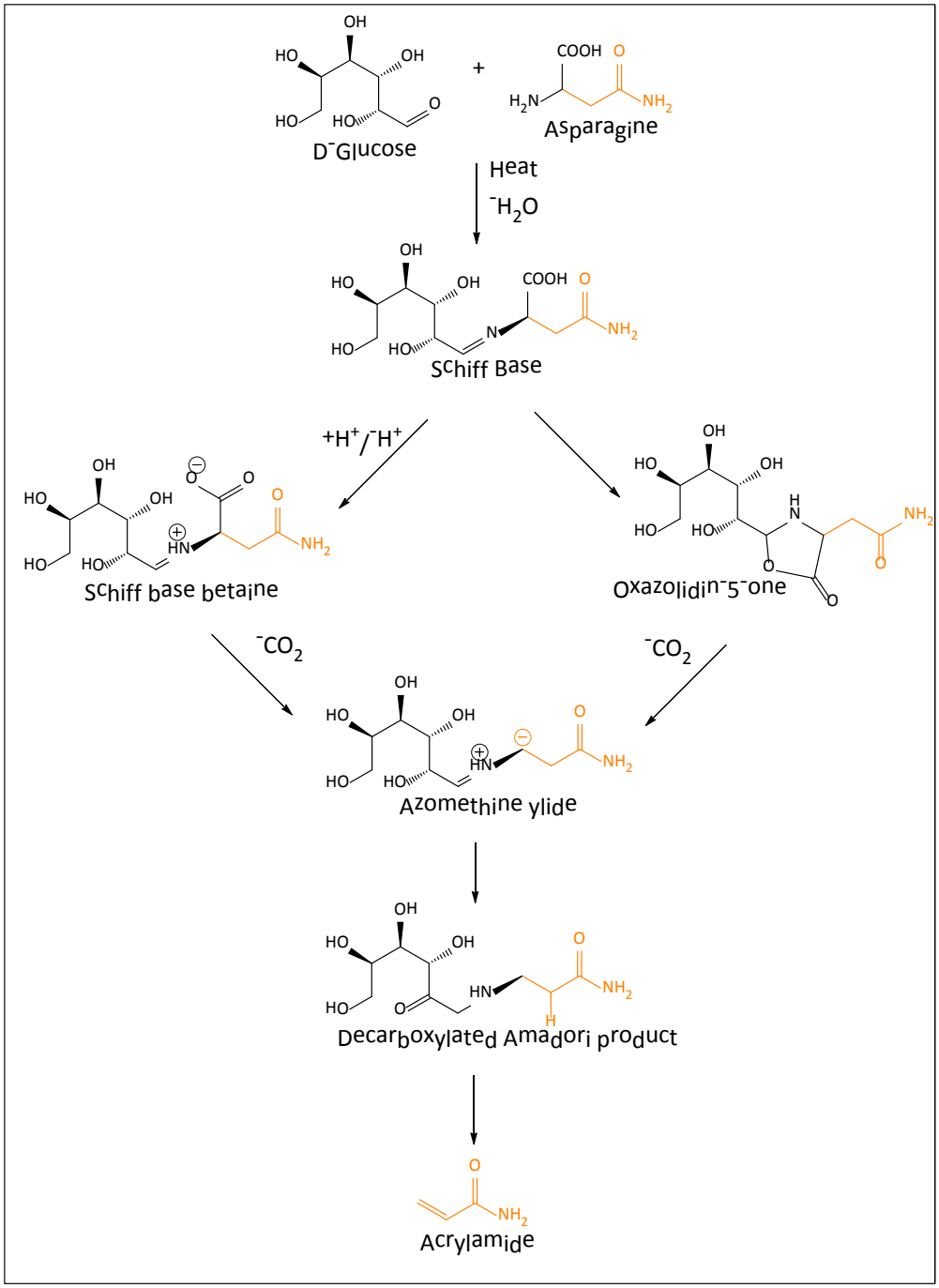


Figure 1.5: Formation of AA in the Maillard reaction. Modified from Blank [58].

The Maillard reaction, a non-enzymatic browning reaction is responsible for the formation of colour, odour and flavour in a variety of foods, like bread crust. The reaction starts with temperatures above 120°C and AA formation is dependent on duration and temperature [59,60]. Hence, temperatures should be reduced and

heating times shortened in order to reduce AA levels in food [61]. Boiled and microwaved food do not contain AA [62].

Therefore, the most AA rich food are potato based foodstuff like crisps and chips, but also biscuits, cereals, coffee, bread and crisp bread (see Table 1.1). With such a high prevalence in a wide range of food it is believed that about 30% of the calorie intake is from food that contains AA [48,62,63].

Table 1.1: AA content of commonly consumed foodstuff based on values from the Food Standards Agency, Food Survey Information sheet Number 01/13 April 2013 on acrylamide and furan [64]

Product	Number of samples	Mean AA amount (µg/kg)	Min AA amount (µg/kg)	Max AA amount (µg/kg)
French fries	20	193	55	775
Potato crisps	10	852	172	2231
Soft white bread	12	12	6	42
Cereals	2	98	57	139
Coffee (instant)	6	771	656	878
Biscuits	10	316	30	1044

Although the formation of AA occurs while cooking, it cannot be considered a food toxicant but is rather classified as a food borne toxicant [46]. After the findings that AA is formed in starch containing food through processing by the reaction of the amino acid asparagine with glucose, food producers have tried to reduce AA levels following the principle of ALARA (as low as reasonably achievable) which is based on a voluntary level [46,60].

1.2.3 Human exposure to AA

Based on results of animal studies the IARC classified AA as a category 2a carcinogen, probably carcinogenic in humans [65]. The Food and Agriculture Organisation of the United Nations and the World Health Organisation estimated in 2002 an AA intake between 0.3 - 0.8 µg/kg body weight (Bw)/day. Mills *et al*. reported a daily AA intake in the UK and Ireland of 0.61 µg/kg Bw/day and for a high intake 1.29 µg/kg Bw/day [66].

There are a variety of estimates for the US and Europe of which all are within the same range. It is believed that the AA intake for children is about 1.5 fold higher due to the higher AA intake in relation to Bw and body surface [62,67,68]. Pingarilho *et al*. report of an average daily intake of AA between 0.5 – 1.0 µg/kg Bw for adults with twice as much for 13 year olds having a western diet [69]. Heudorf *et al*. calculated a median AA intake of 0.54 µg/kg Bw/day for 110 children taking part in their study [67].

A result of the NewGeneris Study, a European prospective mother-child cohort, discovered that AA and GA intake was associated with a reduced birth weight of babies as well as a decrease in head circumference illustrating an *in utero* exposure due to consumption of AA containing food during pregnancy. The AA-Hb and GA-Hb adducts were analysed and associated with the AA intake estimated by food frequency questionnaire (FFQ) and there was a statistically significant reduction in head circumference and birth weight after adjusting for gestational age and country [70]. The Norwegian Mother and Child Cohort Study investigated the relationship of dietary AA intake and foetal growth and found a negative association, concluding that reducing AA containing food during pregnancy could improve foetal growth [71].

The EPIC study enrolled almost half a million people in 27 centres across 10 different European countries. One of their findings was that AA intake were about 2 – 3 fold higher in northern European countries compared to countries in southern Europe. The food groups that contributed most to the AA intake were “bread, crisp bread, rusk” and “coffee” followed by “potatoes”. The first two groups accounted for at least 50% of AA intake, and in some countries even more. The “potato” food contribution for the UK centres was 15% [72]. Mestdagh *et al*. investigated the eating habits of staff and students in a university canteen in Belgium. The intervention group was offered a free portion of fruit and vegetable and these volunteers had approximately 10% (not significant) less AA intake than the study group. Their finding was that snack food, which was consumed in between main meals, contributed about 40% towards the AA intake. For lunch, the consumption of chips was the main AA source and for breakfast and dinner, bread contributed mostly towards the AA intake [61]. Beside dietary exposure smokers are additionally exposed to AA due to its presence in cigarette

smoke with an approximate 1 – 2 µg AA/cigarette [73,74] which can lead to an additional approximate 3.1 µg/kg Bw/day intake, caused by smoking [52]. Additionally, occupational exposure, like factory workers in polyAA production (about 1.4 µg/kg Bw/day) and people working in laboratories using AA for gels, increase their AA intake due to this additional exposure [52].

1.2.4 Metabolism of acrylamide

AA can be absorbed through ingestion, inhalation and also by skin contact [40]. AA is a small molecule, which is rapidly absorbed by the gastrointestinal tract and due to its hydrophilicity it can passively diffuse through cell membranes within the body [75,76]. In pregnant women, AA passes the placenta exposing the foetus, additionally it can also be found in breast milk [77].

There are two pathways within the AA metabolism. Firstly, the activating reaction by Phase I enzymes and secondly the detoxifying reaction catalysed by Phase II enzymes. Figure 1.6 gives an overview of possible pathways of the AA metabolism.

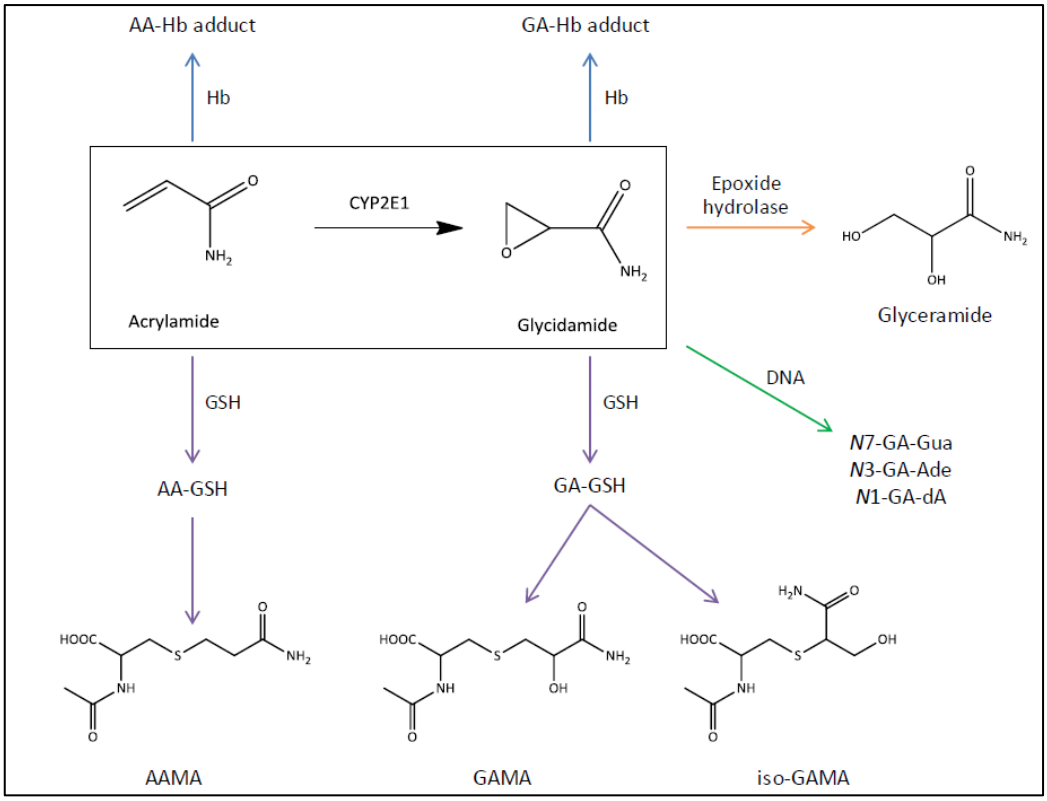


Figure 1.6: AA metabolism. GSH: glutathione, Hb: haemoglobin, (modified from [78]).

1.2.4.1 Phase I reactions - Activating metabolism – CYP2E1

Once AA is ingested, inhaled or absorbed by dermal contamination it gets metabolised to the reactive epoxide GA *via* an enzymatic reaction by cytochrome P450 2E1 (CYP2E1). The enzyme CYP2E1 belongs to the activating Phase I enzymes cytochrome P450 family, capable of oxidising substrates. The liver, with about 7% of all CYP isoforms, has the highest CYP2E1 enzyme activity but it is expressed in other tissues as well. There are no activity and distribution differences in genders for CYP2E1 and it is polymorphic in humans. In the foetus CYP2E1 is not expressed but becomes activated just after birth [79].

The involvement of CYP2E1 *in vivo* was firstly discovered when Sumner *et al*. dosed AA and acrylonitrile to wild-type, CYP2E1-null and wild-type mice treated with a total CYP 450 blocking agent. Only the wild-type mice showed urinary metabolites of direct GSH conjugation and metabolites of GSH conjugation to GA whereas CYP2E1-null and treated wild-type mice only excreted the direct GSH conjugate metabolites [80].

Rather than looking at the urinary metabolites to investigate the involvement of CYP2E1 in the metabolism of AA to GA, Ghanayem *et al*. treated wild-type and CYP2E1-null mice with AA and analysed the GA-DNA and Hb adduct levels. The plasma AA levels in AA treated CYP2E1-null mice were 137 fold higher than in wild-type mice, whereas the latter had a 19 fold higher GA plasma level compared to the CYP2E1-null mice. The control mice showed background levels of *N*7-GA-Gua in liver and testes. However, the control CYP2E1-null mice had no detectable *N*7-GA-Gua levels, whereas the wild-type mice had significantly higher *N*7-GA-Gua and *N*3-(2-carbamoyl-2-hydroxyethyl)-adenine (*N*3-GA-Ade) adduct levels of similar amounts in all organs. The treated CYP2E1-null mice had a 52 – 66 fold lower *N*7-GA-Gua adduct level detectable compared to the wild-type. Hb adducts could be detected for AA and GA for the treated and control wild-type animals and the GA-Hb adduct was about 33 fold higher than for CYP2E1-null mice treated with AA, whereas they had twice as high AA-Hb adduct levels than wild-type mice [32]. Following this Settels *et al*. and Kraus *et al*. verified that CYP2E1 is also the major enzyme in humans to metabolise AA to GA [76,77]. It is GA that is considered to be responsible for the observed carcinogenicity of AA [76]. The finding of traces of the *N*7-GA-Gua adduct in CYP2E1-null mice suggests that there are other pathways involved in the metabolism of AA [32], confirmed by the finding of Kraus *et al*. who suggested that CYP1A1, CYP1A2, CYP2C19 and CYP2D6 might play a negligible role in AA metabolism [77]. Studies were undertaken to investigate the influence of polymorphism in metabolising genes on the amounts of AA- and GA-Hb adducts detected. The polymorphisms in CYP2E1, GST and microsomal epoxide hydrolase (mEH) were analysed and compared with Hb adduct levels of workers with AA exposure. When adjusting for covariates, significant associations were found for CYP2E1, GSTM1 and mEH4 genotypes and the AA- and GA-Hb levels [81]. Duale *et al*. also found a significant association between GSTM1 and GSTT1 and the ratio of GA-Hb/AA-Hb adduct levels. Also for other combinations of CYP2E1, GSTM1, GSTP1, GSTT1 and mEH genotypes associations could be found [82].

1.2.4.2 Phase II reactions - Detoxification metabolism – Mercapturic acids

The mercapturic acid (MA) products of AA and GA can be considered biomarkers of short-term exposure, based on their half-lives that exist within hrs up to a few days, and the fact that they are excreted within urine [31]. AA and GA both react with GSH to form the MAs

- *N*-acetyl-S-(2-carbamoyl-2-hydroxyethyl)-L-cysteine (AAMA),
- *N*-(R/S)-acetyl-S-(2-carbamoyl-2-hydroxyethyl)-L-cysteine (GAMA),
- *N*-acetyl-S-(1-carbamoyl-2-hydroxyethyl)-cysteine (iso-GAMA) and
- glyceramide (2,3-dihydroxy-propionamide)

[78,83]. GA is hydrolysed by the EH to form glyceramide which is then excreted in urine [69]. Smokers show higher levels for AAMA and GAMA [67]. Boettcher *et al*. gave a single dose of d_3-AA (0.99 mg) to a healthy volunteer. In urine samples prior to dosage neither d_3-AAMA nor d_3-GAMA was detectable. In the following 46 hrs, 51.7% of the given dose was excreted as AAMA and only 4.6% as GAMA, showing clearly that AAMA was the main metabolite. Within this study it appeared that AA gets metabolised in two stages, the first one was fast, having a half-life of 3.5 hrs and the second one was slower with a half-life of 10 hrs. The GAMA/AAMA ratio was 0.1 and is similar to the ratio found in rats [83]. A study by Hartmann *et al*., also dosed 0.99 mg d_3-AA to a single volunteer, detected the MAs of the oxidative metabolism, e.g. the GAMA, iso-GAMA and glyceramide. After 1.5 hrs the adduct was already detectable in urine with the majority being excreted between 8 – 22 hrs. After 46 hrs, 5.4% of the given dose was excreted as d_3-glyceramide, and the total of urinary metabolites of the oxidative pathway accounted for 11% of the given dose [78]. In both studies [78,83] there was an instant increase of the AAMAs in urine whereas the GAMA levels were delayed and started to increase about 4 hrs after dosing. This result fits the model of AA being metabolised to GA first. A different study undertaken by Boettcher *et al*. [31] investigated the influence of 48 hrs fasting on the level of MAs. After the fasting period the MA levels were reduced to a level even below a mean for non-smokers concluding that diet is the main source of AA intake besides smoking.

One study assessed the impact of diet on the urinary metabolites of AA, AAMA and GAMA in children. A significant association could be seen between the metabolites and the intake of French fries, where children with a higher intake showed higher levels [67].

Watzek *et al*. reported a half-life for AAMA of 14 hrs and 22 hrs for GAMA in their study, where volunteers consumed self-made potato crisps, and 12 hrs for AAMA, where volunteers consumed shop bought potato crisps instead [84]. In comparison to an aqueous AA solution a bread crust based consumption of AA in rats led to a minimally reduced bioavailability as shown as AA-Hb adducts and a 20% reduction for AAMA. The ratio for the GAMA/AAMA for rats in the AA in drinking water group was about 30% lower than ratios for the rats being fed with matrix based food [85].

1.2.4.3 Interspecies differences

The metabolism of AA in rats, mice and humans differs between species.

Mice show higher ratios of GA-Hb adducts to AA-Hb adducts compared to rats suggesting that mice metabolise AA faster. It seems that the GA epoxide group is more reactive towards Hb than the double bond of the AA molecule [40].

Sumner *et al*. dosed mice and rats with ^{13}C-labelled AA and analysed the urinary metabolites by ^{13}C-NMR. The study revealed that rats excreted 67% of total urinary metabolites as AA-GSH conjugate whereas for mice it was only 41%. However, rats excreted 31% as GA-GSH conjugates and mice 50% GA-GSH conjugates showing that mice metabolised AA more effectively to GA than rats. The metabolite glyceramide was detected in rats (2%) and mice (5%) [86]. However, the AA metabolism between rats and humans differs. For the oxidation from AA to GA, rats, given a dose of 3 mg/kg excreted 41% as MA of GA whereas this was only 14% in humans given the same dose. Increasing the AA dose for rats to 50 mg/kg (no human data available for this dose) showed that only 28% of the urinary metabolites were GAMA, suggesting a dose-dependent oxidation decreased with increasing AA dose. After these findings in rats, it was suggested that humans show similar effects. Summarising the difference in the

oxidation pathway rats oxidised about three fold more AA to GA than humans. Given a dose of 3 mg/kg AA 59% was excreted as AA-GSH conjugate in rats whereas it was about 30% more in humans, and 86% of the total urinary metabolites were excreted as AA-GSH. Hence, humans produce less GA. With the same dose, rats excreted about 20% as GA-GSH conjugates but for humans the values were below the limit of quantification (LOQ). The differences in metabolism were quite severe for the EH. Whereas for rats only 1.2% was excreted as glyceramide, humans seemed to hydrolyse GA more effectively and excreted approximately 11% in the urinary metabolites. Summarising the findings of the EH results, rats oxidised more AA to GA and had a higher GA-GSH level than humans. Humans hydrolysed GA more effectively to glyceramide and hence might be less exposed to the carcinogen GA due to lower concentrations [9].

1.2.4.4 AA related DNA adducts

AA reacts with DNA very slowly whereas GA has a high affinity towards DNA and reacts with DNA to mainly form 3 different DNA adducts. *N*7-GA-Gua was the first DNA adduct, discovered by Segerbäck *et al*. [69,87]. After this initial discovery two adducts to adenine, i.e. *N*3-GA-Ade and *N*1-(2-carboxy-2-hydroxyethyl)-2'-deoxyadenosine (*N*1-GA-dA) were reported [73] (Figure 1.7). *N*6-(2-carboxy-2-hydroxyethyl)-deoxyadenosine (*N*6-GA-dA) is a product derived from the *N*1-GA-dA after re-arrangement [88].

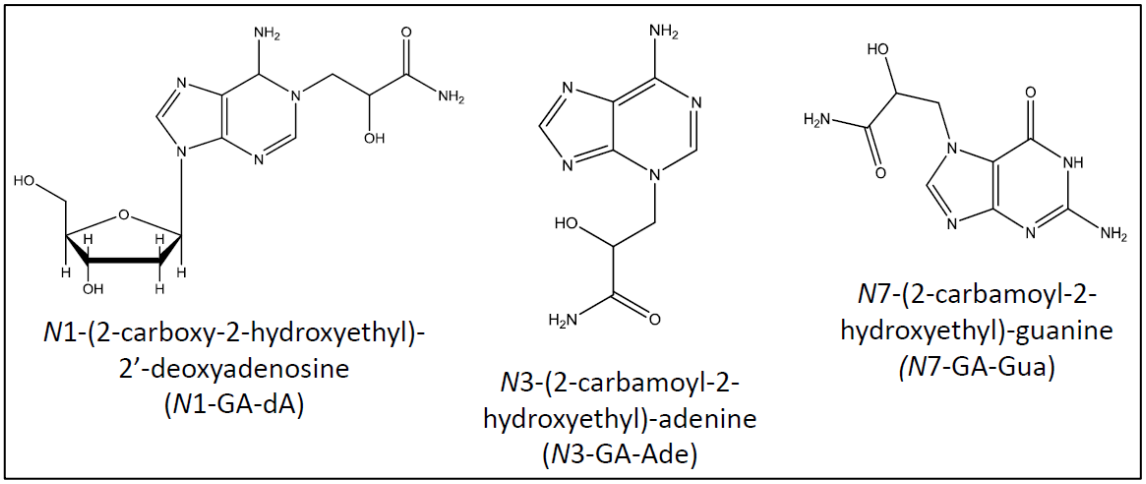


Figure 1.7: DNA adducts formed by GA.

The *N*7-GA-Gua adduct is the most abundant one and was detected *in vivo* in mice and rats alongside the *N*3-GA-Ade adduct [73,87,88]. The *N*6-GA-dA adduct was detected in GA modified DNA and GA treated cells but not in mice treated with AA. *In vitro*, the ratio of *N*7-GA-Gua:*N*3-GA-Ade:*N*1-GA-dA was 100:22:1.4 [32,52,88]. Mei *et al*. found a 60 fold excess of *N*7-GA-Gua adduct compared to the *N*3-GA-Ade adduct when treating MLC with GA. Following AA treatment no GA-DNA adducts were detectable, corroborating the suggestion that AA needs metabolic activation to form DNA adducts; and MLC do not express CYP2E1 [45]. Doerge *et al*. found the DNA adduct formation in mice reached a steady state approximately 14 days after continuous AA dosage in water, with a formation half-life of about 4 days. There was a notable difference in the formation half-life in female and male rats, with 4 days for female and 3 days for male rats but both reached a steady state level of adduct approximately after 14 days continuous dosing [89]. Maniere *et al*. on the other hand reported a maximum *N*7-GA-Gua value between 5 and 24 hrs with a decrease after 72 hrs. The elimination rate for the *N*3-GA-Ade adduct was faster and it had a half-life of 20 hrs for the high dose (54 mg/kg Bw) and the half-life increased to 20 - 30 hrs for the low dose (18 mg/kg Bw) [90].

The *N*7-GA-Gua and *N*3-GA-Ade are promutagenic adducts and can depurinate spontaneously leading to abasic sites which are mutagenic [17,48,73]. When DNA gets replicated AP sites can lead to incorporation of 2'-deoxyadenosine, that in turn can

lead to G → T transversions [17]. The spontaneous depurination of *N*3-GA-Ade was quicker than that of *N*7-GA-Gua. Additionally, *N*3-GA-Gua led to sister chromatid exchange, S-phase arrest, chromosomal aberrations and cytotoxicity. With the alkylation on the *N*1 atom, the *N*1-GA-dA adduct is highly promutagenic due to its involvement of the base pairing atoms which can cause miscoding [17,48,73].

1.2.4.5 AA related Hb adducts

Both AA and GA can react with the N-terminal valine in Hb via a Michael addition to form Hb adducts [55]. The first mentioning of AA-Hb adducts analysis was in 1993 by Bergmark *et al*. when they determined Hb adducts of AA and acrylonitrile of factory workers in polyAA production by a modified Edman degradation using GC-MS [91]. Increasingly, LC-MS/MS, due to its higher throughput, is being employed for analysis of protein adducts of AA [32,81,92-94]. A further improvement to the Hb adduct analysis was developed by von Stedingk *et al*. [95]. This "adduct FIRE procedure" allows the detection of AA- and GA-Hb adducts amongst others in small volumes of whole blood (250 µL per analysis) without prior extraction of red blood cells. FIRE stands for "FITC reagent used for measurement of adducts (R) formed from electrophilic compounds with a modified Edman procedure". The advantages are that it is fast and does not require large volumes of blood [95] (for further details see 5.3.2).

The analysis of Hb adducts of AA and GA can firstly be used as a measure of bioavailability due to continuous exposure and secondly for a measure of bioactivation of AA to its genotoxic metabolite GA [85]. The ratio of GA-Hb adducts to AA-Hb adducts can be used to get an impression of the metabolism of AA in different species but even within species there are measurable differences that suggest that different people might have different ways of metabolising AA [32]. Within the EPIC study AA-Hb and GA-Hb adducts were analysed and compared to FFQ data. The study found that alcohol intake decreased the GA- to AA-Hb adduct ratio probably due to a competitive inhibition of the CYP2E1 enzyme caused by alcohol [96]. Both AA and GA are more reactive towards thiol groups in Hb than the amino groups and additionally GA is more reactive towards the N-terminal valine than AA [40,85].

Smokers have higher Hb adduct levels than non-smokers, where the mean AA biomarker concentration can be 4 – 5 times higher for smokers [74]. Kütting *et al*. found a high association between reported number of smoked cigarettes and the analysed Hb adduct levels with self-reported questionnaires [97].

1.2.5 Epidemiology – AA intake and the risk of cancer

Long since the discovery of the occurrence of AA in commonly consumed food, epidemiological studies focused on analysing the link between the dietary AA intake and the risk of various types of cancer. Table 1.2 provides an overview of epidemiological studies that have assessed dietary AA intake and the risk of cancer.

Table 1.2: Epidemiological studies undertaken to investigate the risk between specific types of cancer and the dietary AA intake. Most studies did not find an association between the dietary AA intake and the risk of cancer. Increased risk or a positive association are highlighted in red writing

Study	Cancer	Results	Assessment
Mucci *et al*. (2003) [98]	large bowel cancer kidney cancer bladder cancer	no association - reanalysed no association	FFQ
Pelucchi *et al*. (2006) [99]	oral cavity and pharynx cancer oesophagus cancer large bowel cancer larynx cancer breast cancer ovarian cancer prostate cancer	no association	FFQ
Hogervorst *et al*. (2007) [100]	endometrial cancer ovarian cancer breast cancer	increased risk for postmenopausal endometrial cancer increased risk for ovarian cancer no association	FFQ
Olesen *et al*. (2008) [101]	breast cancer ER+	no association adjustment for smoking: positive association	AA-Hb adducts
Hogervorst *et al*. (2008) [102]	colorectal cancer gastric cancer pancreatic cancer oesophagus cancer	no association	FFQ
Wilson *et al*. (2009) [63]	prostate cancer	no association	FFQ, AA-Hb adducts
Wilson *et al*. (2009) [103]	premenopausal breast caner	no association	FFQ
Larsson *et al*. (2009) [104]	prostate cancer	no association	FFQ
Larsson *et al*. (2009) [105]	colorectal cancer	no association	FFQ

Larsson *et al.* (2009) [106]	epithelial ovarian cancer	no association	FFQ
Larsson *et al.* (2009) [107]	breast cancer	no association	FFQ
Hogervorst *et al.* (2009) [75]	brain cancer	no association	FFQ
Hogervorst *et al.* (2009) [108]	lung cancer	no association in men inverse association in women	FFQ
Wilson *et al.* (2010) [109]	endometrial cancer ovarian cancer	increased risk overall non-significant increase	FFQ
Pedersen *et al.* (2010) [110]	overall breast cancer ER- ER+, PR-, joint receptor +	no association no association non-significant increase for never-smoking women	FFQ
Hirvonen *et al.* (2010) [111]	cancer among Finnish male smokers	association found for lung cancer	FFQ
Burley *et al.* (2010) [112]	breast cancer	no overall association possible weak association for premenopausal breast cancer	FFQ
Pelucchi *et al.* (2011) [113]	pancreatic cancer	no association	FFQ
Bongers *et al.* (2012) [59]	lymphatic malignancies	may increase risk of multiple myeloma and follicular lymphoma in men no association for women	FFQ
Wilson *et al.* (2012) [114]	prostate cancer	no association	FFQ
Xie *et al.* (2013) [115]	ovarian cancer	no association	FFQ AA- and GA-Hb adducts
Obon-Santacana *et al.* (2013) [116]	pancreatic cancer	no association	FFQ

Most studies evaluated the AA intake with FFQ whereas some of the more recent studies included the analysis of Hb adducts of AA and GA as a representative biomarker for the AA exposure. Wirfält *et al*. were the first group to combine a diet history questionnaire and the measurement of Hb adducts to investigate the association between the dietary AA intake and AA-Hb adduct levels of the Malmö Diet and Cancer cohort study concluding that both AA from food and tobacco are sources of exposure to AA [117].

There are perceived limitations to the use of FFQs due to various factors. Firstly, there is a high variability of the AA content in foodstuff between different brands and secondly, variability within the same batch of one company's food product can also differ greatly [59,63]. Another aspect when using FFQ, is that they cover long periods of time where participants need to remember what they ate which might lead to a less

accurate provision of information [30,96]. Home cooking is an important aspect to consider because it is less controlled than industrial processes and people have different preparation techniques, which might lead to highly varied AA levels in food [96]. Another aspect to consider when working with FFQ is that people tend to underestimate foods that are considered unhealthy whereas they overestimate foods considered to be healthy [97]. A review of the epidemiologic studies pointed out that earlier studies were mostly case-control studies, whereas later studies tended to be more often prospective cohort studies. The prospective cohort studies have more data on AA in food and provide a better assessment of the AA exposure of the population studied.

Smoking can be a confounder, and hence it is important to control for smoking when assessing the dietary AA intake and risk of cancer. Ideally it would be better to have smoker-free participants' [30].

1.3 Mass spectrometry

Mass spectrometry is becoming the preferred analytical technique used for the analysis of DNA adducts with increasing numbers of applications and continuous development of more sensitive spectrometers.

A mass spectrometer consists of an inlet for the sample, an ion source, a mass analyser, a vacuum and a detecting system (Figure 1.8) [118,119].

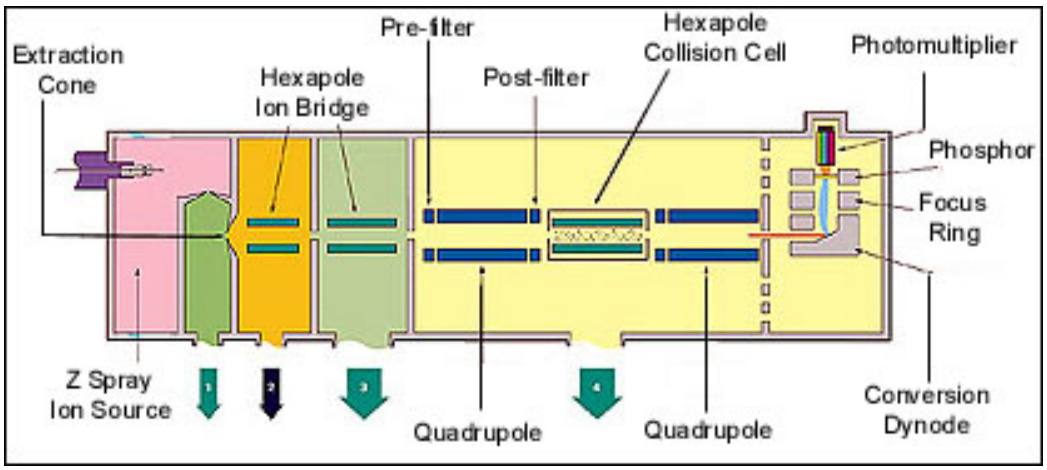


Figure 1.8: Schematic overview of a mass spectrometer consisting of the Z spray ion source, extraction cone, hexapoles, quadrupoles and the detector.

- The INLET SYSTEM prepares the sample to be introduced to the source for ionisation.
- The ION SOURCE ionises the compound of interest before it enters the mass spectrometer. Thus molecules previously found in solution are transferred to gas phase and are ionised. Once ions are produced, molecules can be transmitted using a combination of electromagnetic fields into the analyser under a strong vacuum. For the analysis of adducts electrospray ionisation (ESI) is the predominant ionisation modality but electron and chemical ionisation are also used.
- The MASS ANALYSER separates ions by mass-to-charge (m/z) and focuses them before detection. There are different mass analysers available, e.g. quadrupole, ion trap or TOF (Time of flight).
- The VACUUM ensures that ions fly without eliminating each other and hence, it prevents collisions and discharges.
- The DETECTION SYSTEM measures the abundance of ions which is then transformed into a signal by the computer system shown as spectra.

With the focus of this project to detect the DNA adduct by LC-MS/MS, ESI, quadrupole and tandem mass spectrometry will be explained further.

ESI is a soft ionisation method, where the dissolved analyte, injected from a liquid chromatography system, gets transferred into the gas phase by spraying through a

needle which is held under a voltage. The occurring droplets move towards the source and vacuum where the solvent is further eliminated until the charge on the droplets gets too dense and ions are released, also called Coulombic fission. Characteristic for ESI is the proton that is attached to generate ions. The Z-spray configuration helps to remove ions from neutral molecules as by applying an electrical field so the ions get deflected and follow a Z-formed route whereas neutral molecules follow their initial straight line and are separated without entering the mass spectrometer.

A QUADRUPOLE consists of 4 rods which are arranged in an accurate square. At any one time two diagonally opposite rods have the same positive direct current (dc) voltage whereas the other remaining rods run in the same but negative dc voltage. Additionally, for each pair of rods a radiofrequency (rf) voltage is imposed, which between rod pairs is 180° out of phase. For a given dc and rf voltage only given m/z ions are able to exit the quadrupole on a stable trajectory without being discharged when hitting the rods. Changing either the rf or dc voltage, when rf frequency is constant allows to scan a broad *m/z* range.

TANDEM MASS SPECTROMETRY (MS/MS) involves the use of multiple mass analysers. Commonly used are triple quadrupole mass spectrometers, applied for the LC-MS/MS analysis, consisting of Q1qQ3. Quadrupole Q1 generates precursor ions which get fragmented into product ions and neutral fragments in quadrupole q, the collision cell under a stream of inert gas before subsequent separation in the quadrupole Q3, the second analyser.

COLLISION INDUCED DISSOCIATION (CID) of an ionised molecule leads to reproducible fragmentation. The fragmentation products of the molecule can be used to increase the specificity of the assay. Monitoring of specific fragmentation transitions (Selected reaction monitoring – SRM) can enable improved levels of sensitivity and much greater specificity.

Incorporating ONLINE COLUMN-SWITCHING (Figure 1.9) into the method helps to improve sensitivity by retaining the analyte of interest on a trap column while impurities are

washed to waste. When the switching valve changes positions, the analyte gets back-flushed and transferred onto the analytical column for separation.

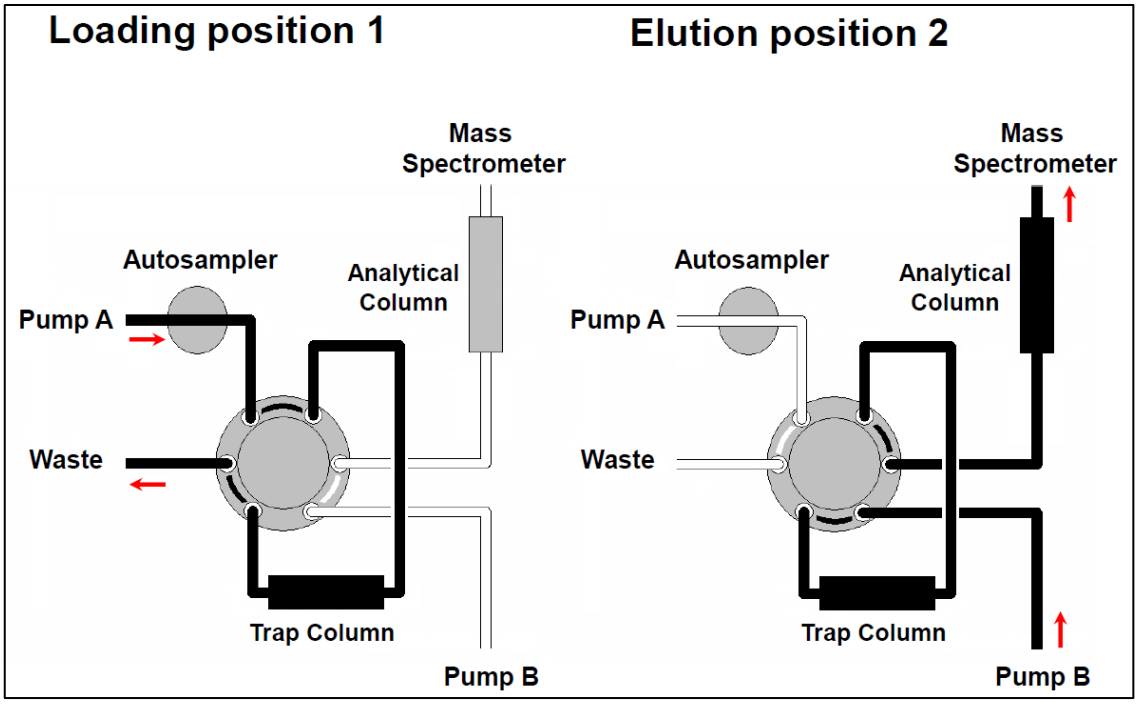


Figure 1.9: Scheme for the online column-switching.

1.4 Enzyme-linked immunosorbent assay

ELISA is an analytical technique, incorporating the use of antibodies, for the detection of analytes in a variety of samples with high specificity and high sample throughput. This type of immunoassay can be performed in different variations.

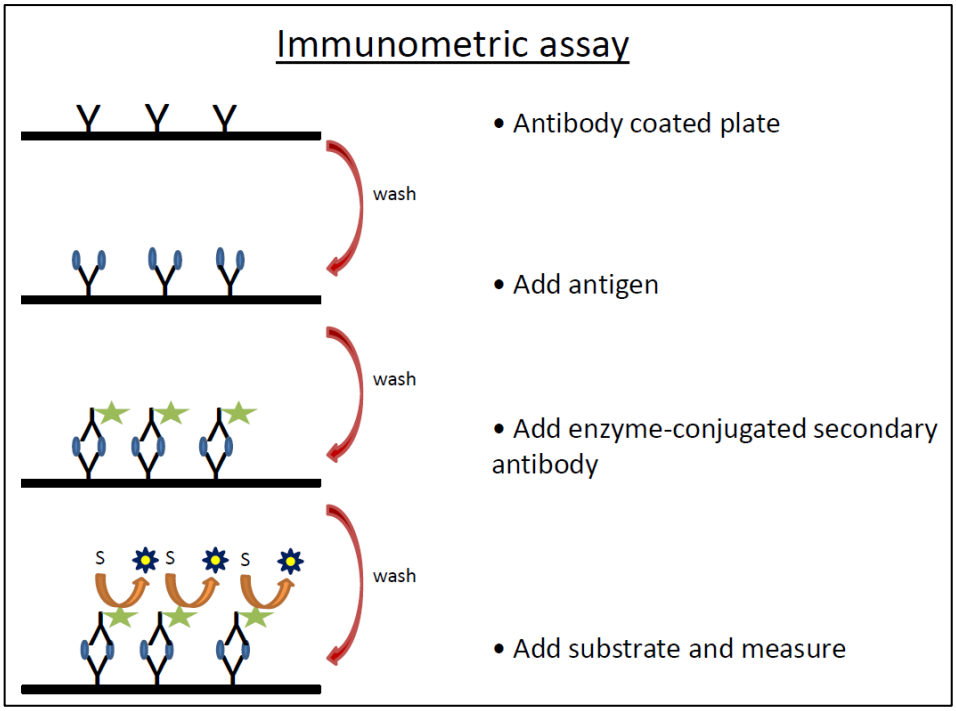


Figure 1.10: Schematic overview of the steps involved in an immunometric ELISA design.

In the classic immunometric assay, also called sandwich ELISA, an antibody is coated to a surface, mostly in form of a 96 well plate. The antigen is added followed by an enzyme-linked secondary antibody that generates a signal, e.g. change of colour or light emission, upon addition of a substrate allowing quantification of the analyte within the sample (Figure 1.10). When performing an ELISA experiment the plates are washed after each step to avoid false signals by unspecific binding [120].

Depending on the properties of the analyte different ELISA designs can be applied. Small molecules are usually detected by the variation called competitive ELISA (Figure 1.11).

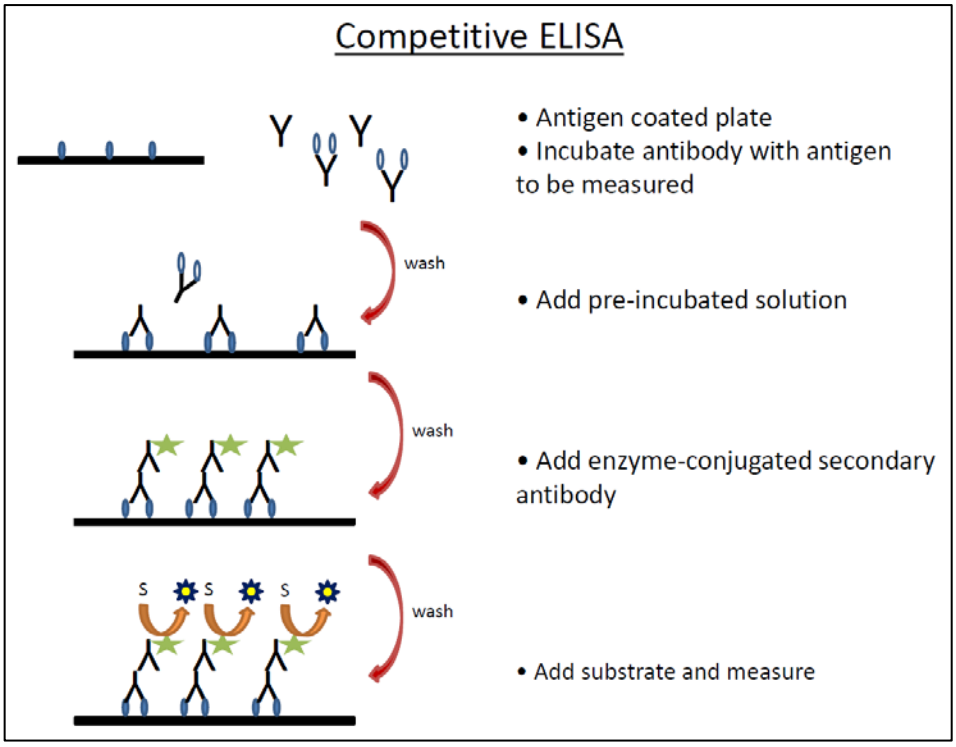


Figure 1.11: Schematic overview of a competitive ELISA.

The antigen is coated to the surface of the plate while the antibody and an antigen containing sample are pre-incubated at the same time. This pre-incubated solution is then applied to the plate where the free antibodies bind to the pre-coated antigen. An enzyme-linked secondary antibody is added that reacts with the substrate upon addition to generate a signal used for quantification of the sample. If the sample contains high amounts of analyte less free antibody binds to the plate and hence the signal response is going to be small. If there is hardly any analyte in the sample the antibodies bind to the coated antigen to generate a high signal in the competitive ELISA system [120].

1.5 Human volunteer study

In a human volunteer study levels of *N*7-GA-Gua adduct were analysed in leukocyte DNA and urine samples and then correlated to estimated dietary AA intake determined by FFQ.

Healthy human volunteers, from within our and related departments, were recruited via e-mail distribution. After consenting to participate, volunteers donated a sample of whole blood and urine. After the extraction of leukocyte DNA from whole blood, samples were analysed to detect the *N*7-GA-Gua adduct with the developed and validated methods, namely LC-MS/MS and ELISA.

A subset of samples was analysed for AA- and GA-Hb adducts by Henrik Carlsson in Stockholm University, Sweden using the adduct FIRE procedure (see section 5.3.2).

1.6 Hypothesis

This project will test the hypothesis that the *N*7-GA-Gua adduct is detectable in human samples. If the adduct is present in human samples it will be further tested if there is a correlation between the dietary AA intake, evaluated through FFQ and the obtained adduct levels.

1.7 Aims and objectives

AIMS:

- To verify if the *N*7-GA-Gua DNA adduct is formed in humans and if it is detectable by analysing leukocyte DNA and urine.
- To investigate whether the *N*7-GA-Gua adduct can be used as a biomarker of exposure to AA contained within food.

OBJECTIVES:

- To develop and validate methods for the quantification of *N*7-GA-Gua by LC-MS/MS and ELISA
- The project will examine blood and urine from human volunteers who will be asked to complete a FFQ. The samples will be assessed for *N*7-GA-Gua levels and compared to estimated AA intake from FFQs.

Chapter 2

2 Method Development – Mass spectrometry

2.1 Introduction

Method development and validation is requisite for the reliable and confident measurement of analytes. Previous publications have reported the measurement of the *N*7-GA-Gua adduct. Segerbäck *et al*. detected the formation of *N*7-GA-Gua in both mouse and rat DNA after a single treatment with ^{14}C-labelled AA [87].

The majority of research focussed on the detection of *N*7-GA-Gua using animal models. Additionally, most studies used relatively high doses of AA or GA for treatment of rats or mice compared to the estimated dietary intake of humans, but recently Watzek *et al*. dosed Sprague-Dawley rats orally with a single dose between 0.1 – 10000 µg/kg Bw. For the dose of 0.1 µg/kg Bw AA they did not detect the *N*7-GA-Gua adduct in liver, kidney and lung. Following an increase to 1 µg AA/kg Bw they could detect the adduct in kidney (1.1 ± 0.4 adducts/10^8 nucleotides) and lung (0.6 ± 0.1 adducts/10^8 nucleotides) tissue but not in liver. 1 – 2 adducts/10^8 nucleotides could be detected in all three tested organs by increasing the dosage to 10 and 100 µg/kg Bw with a dose dependent increase in adduct levels [121].

The main aim of the work described in this chapter is the development of an improved method by applying column-switching LC-MS/MS to detect the most abundant DNA adduct of GA with guanine, *N*7-GA-Gua, in human leukocyte DNA and urine samples.

2.2 Materials and methods

2.2.1 Materials

2.2.1.1 General chemicals

All chemicals were purchased from Sigma Aldrich, Dorset, UK unless otherwise stated. Acetic acid, HPLC fluorescence grade methanol, ammonium formate, methanol, LC-MS Optima water and LC-MS Optima methanol were obtained from Fisher Scientific, Loughborough, UK. 2'-deoxyguanosine monohydrate and formic acid were obtained

from Fluka, UK. 30% (w/w) hydrogen peroxide was obtained from VWR Prolabo, UK. [$^{15}N_5$]-2'-deoxyguanosine monohydrate was obtained from Cambridge Isotope Laboratories, Andover, USA.

2.2.1.2 Equipment

HPLC columns, Synergi 4 µm Fusion-RP 80Å 30 x 2.0 mm, Synergi 4 µm Fusion-RP 80Å 50 x 3.0 mm, Synergi 4 µm Fusion-RP 80Å 50 x 2.0 mm, Synergi 4 µm Fusion-RP 80Å 50 x 2.0 mm and Synergi 4 µm Fusion-RP 80Å 250 x 2.0 mm, Synergi 4 µm Fusion-RP 80Å 250 x 4.6 mm column were all purchased from Phenomenex, Macclesfield, UK, as well as the KrudKatcher Classic HPLC In-Line Filters (0.5 µm) and Security Guard Cartridge Kit. Thermo Hypersil 250 x 21.2 mm 5 µm Hypersil® BDS C18 column was purchased from ThermoScientific, UK. Amicon® Ultra 0.5 mL 3 k and Amicon® Ultra 0.5 mL 10 k were purchased from Millipore, Cork, Ireland. Strata X-C, 33 µm cation mixed-mode polymer 100 mg/6 mL, Strata X-A, 33 µm Polymeric Strong Anion 30 mg/1 mL and Strata X-C, 33 µm Poymeric Strong Cation 30 mg/1 mL sample were purchased from Phenomenex, Macclesfield, UK. Oasis HLB 6 cc and 1cc columns were purchased from Waters Ltd., Manchester, UK. Acrodisc Syringe Filters 0.2 µm supor Membrane Low Protein Binding were purchased from Pall Corporation, MI, USA. Eppendorf Centrifuge 5415R was from Eppendorf, UK. Allegra® X-12R Centrifuge was from Beckman Coulter, UK. inoLab, pH meter, UK. Speed vac® Plus SC210A were from Thermo Electron Savant, UK. Block heater SBH130D, stuart®, UK. WhirliMixer were from Fisherbrand, Fisher Scientific, Loughborough, UK. Blood & cell culture DNA midi and maxi kits were purchased from Qiagen, Manchester, UK. BD Vacutainer LH 102 I.U. tubes were purchased from Bunzl, UK. Spectrophotometer U-0310 was purchased from Hitachi, Japan. GeneQuant RNA/DNA Calculator was purchased from Pharmacia Biotech. Waters HPLC vials and insert 150 µl with plastic spring were purchased from Waters Ltd., Manchester, UK.

2.2.2 Methods

A number of steps in the method development procedure, described in the following section, were evaluated and optimised (Figure 2.1).

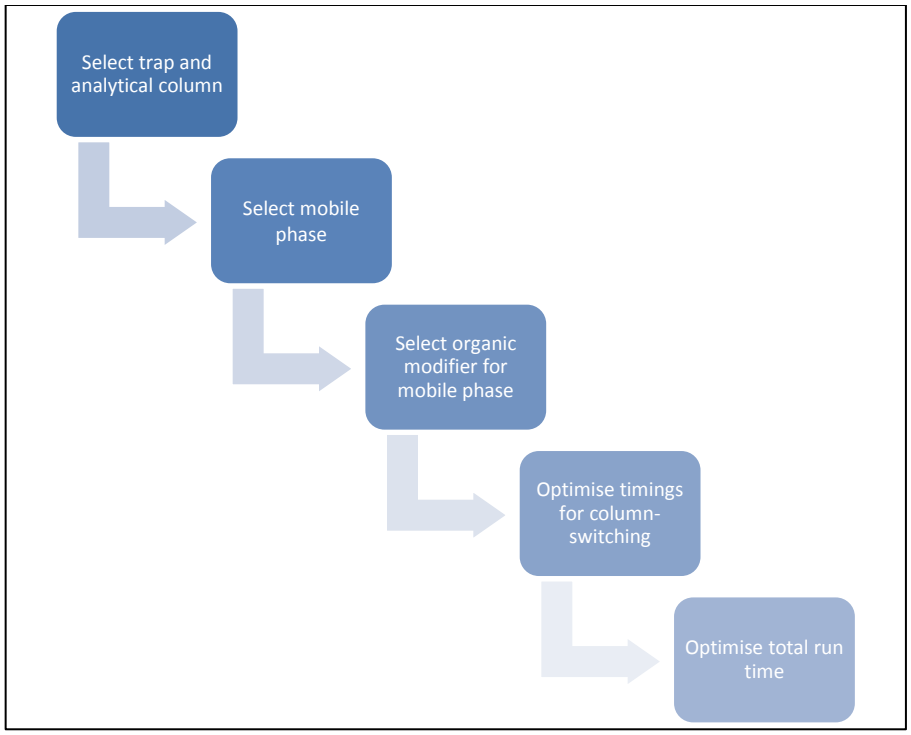


Figure 2.1: Flow chart of the steps involved in the process of method development.

2.2.2.1 Standards

2.2.2.1.1 Synthesis of the standards *N*7-GA-Gua and [$^{15}N_5$]-*N*7-GA-Gua

*N*7-GA-Gua and the internal standard (IS) [$^{15}N_5$]-*N*7-GA-Gua were synthesised using a two-step chemical synthesis as described in detail in section 4.2.2.2.

2.2.2.1.2 Purification of the standards *N*7-GA-Gua and [$^{15}N_5$]-*N*7-GA-Gua

The synthesised *N*7-GA-Gua adduct was purified using a semi-prep HPLC (Varian, UK) with a Thermo Hypersil 250 x 21.2 mm 5 µm Hypersil® BDS C18 column. The mobile phase consisted of 90:10 ddH_2O/methanol (MeOH) (*v/v*) and the flow rate was 10 mL/min. The isocratic run finished after 20 min. Injection volume was 200 µL. The

chromatogram showed three peaks and each one of the peaks was collected into a separate falcon tube. All three collected peaks were analysed in the Chemistry Department, University of Leicester (UoL) on a Micromass Quattro (Waters Ltd., Manchester, UK) by mass spectrometry to check which fraction contained the *N*7-GA-Gua adduct.

Using identical chromatographic conditions to the ones described above the labelled [$^{15}N_5$]-*N*7-GA-Gua adduct showed the same chromatogram.

2.2.2.1.3 Quantification of *N*7-GA-Gua and [$^{15}N_5$]-*N*7-GA-Gua

The purity of the *N*7-GA-Gua adduct was determined by UV absorbance from 240 nm to 320 nm to obtain an UV spectrum. The *N*7-GA-Gua adduct standard was diluted 1:10 in 10 mM ammonium formate pH 7.0 and the absorbance values for 285 nm were used for the calculation of its concentration, following the equation for the Beer-Lambert law:

$$A = \varepsilon * c * L$$

where A is the absorbance, ε the extinction coefficient, c the concentration of the solution and L the path length (1 cm). For the determination of the concentration of the *N*7-GA-Gua adduct the extinction coefficient 7.1 $mM^{-1}cm^{-1}$ was used [73].

Two IS [$^{15}N_5$]-*N*7-GA-Gua standards were prepared in solvent. The second was a 1:1 dilution of the first. These were both measured to determine their final concentration by plotting them against a calibration line of purified *N*7-GA-Gua adduct. By applying the equation gained from the *N*7-GA-Gua calibration line analysed by LC-MS the concentration of [$^{15}N_5$]-*N*7-GA-Gua was determined.

2.2.2.1.4 Calculation of *N*7-GA-Gua adduct levels in biological samples

For determining the levels of the *N*7-GA-Gua adduct in biological samples the adduct can be calculated and expressed as numbers of adducts/10^8 nucleotides. The calculation was done as follows using the following three equations:

EQUATION 1: Determination of the moles of adduct detected

$$Adduct\ moles = \frac{\text{peak area analyte}}{\text{peak area IS}} \times IS\ amount\ \ in\ fmol \times 1 \times 10^{-15}$$

EQUATION 2: Moles of DNA analysed (assuming that 1 µg of DNA equals 3240 pmol)

$$DNA\ moles = 100\ \mu g \times 3240 \times 1 \times 10^{-12}$$

EQUATION 3: Level of adducts per 10^8 nucleotides

$$\frac{\frac{Adduct\ moles}{DNA\ moles}}{1 \times 10^{-8}} = adducts\ per\ 10^8\ nucleotides$$

2.2.2.2 Biological samples – Whole blood – HCEC cells – CT and ST DNA

The aim for this project was to analyse human leukocyte DNA and urine samples. For method development both of these matrices were used. As later described in the validation chapter (Chapter 3), due to the high volume of human blood needed for the validation process, experiments were performed with calf thymus (CT) DNA or salmon testis (ST) DNA as matrix. In the beginning of the project Human colonic epithelial (HCEC) cell DNA was also evaluated as a matrix. Urine was obtained from healthy volunteers.

Figure 2.1 gives a brief overview of the steps involved from whole blood to analysis by LC-MS.

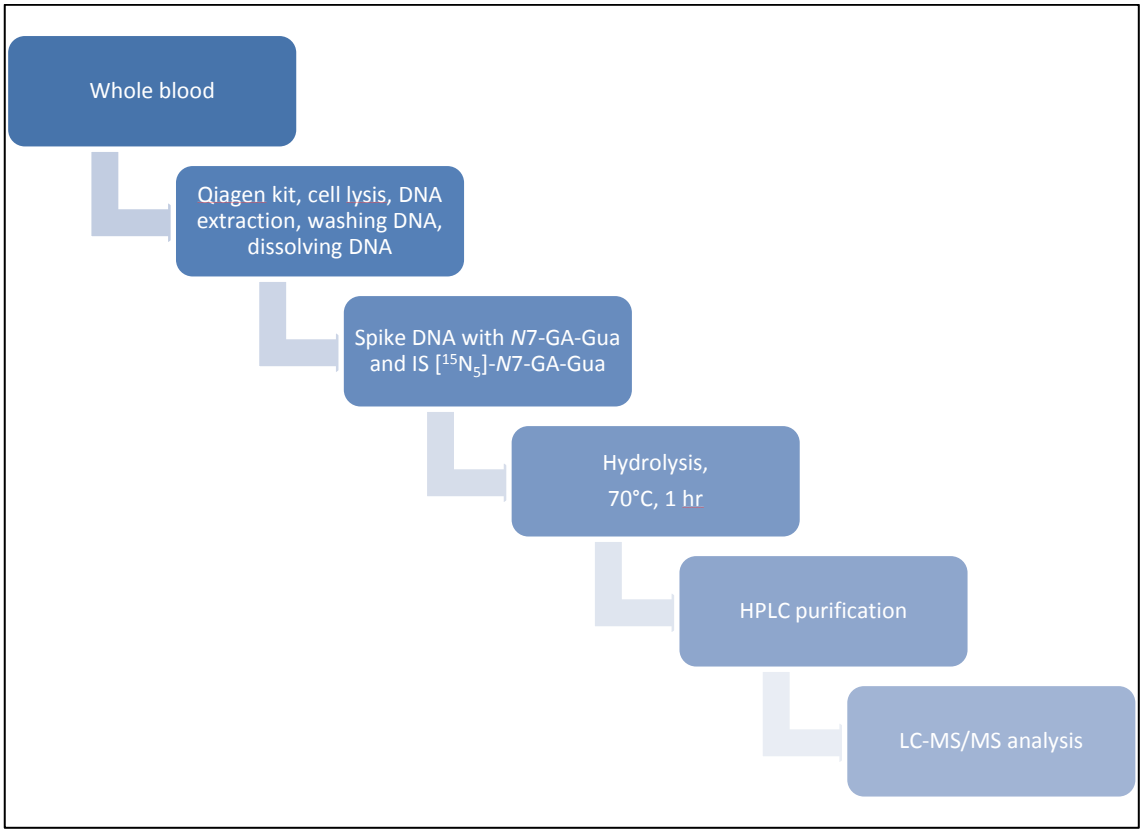


Figure 2.2: The flow chart gives a schematic overview of the work-up procedure from the step of taking whole blood until its final analysis by LC-MS.

2.2.2.2.1 DNA extraction from whole blood

The leukocyte DNA from whole blood was extracted by using the Qiagen kit.

Lysis protocol

Blood was collected in BD Vacutainer LH 102 I.U. tubes (6 mL). The blood was transferred to a 50 mL falcon tube and mixed with one volume of ice-cold buffer C1 (6 mL) and 3 volumes of ice-cold ddH_2O (18 mL). The falcon tubes were mixed by inverting several times until the suspension became translucent. The mixed solution was then kept on ice for 10 min before the lysed blood was centrifuged at 4°C for 15 min at 1300*g*. The supernatant was discarded. To the tubes was added ice-cold buffer C1 (1 mL) and ice-cold ddH_2O (3 mL) and the pelleted nuclei re-suspended by vortexing. The tubes were again centrifuged at 4°C for 15 min at 1300*g*. After centrifugation the supernatant was discarded. The pellet was then stored at -20°C.

Qiagen isolation procedure

The pellet was re-suspended in buffer G2 (5 mL) by vortexing at maximum speed for 1 min before 20 U of RNase A (Sigma) and 2 U of Ribonuclease T_1 were added. The tubes were mixed by inversion and incubated in the water bath for 30 min at 37°C before Proteinase K (10 µL/mL H_2O) (10 µL) was added. The tubes were mixed by inversion before being placed in the water bath and incubated for another 2.5 hrs at 37°C.

The Qiagen genomic tips (midi) were equilibrated with buffer QBT (4 mL) and allowed to empty by gravity flow.

The tubes containing the DNA were vortexed at maximum speed for 1 min (or longer if the pellet was not re-suspended) and applied to the equilibrated Qiagen genomic tip. The solution was allowed to enter the column by gravity flow. The Qiagen tips were then washed with buffer QC (2 x 7.5 mL) and the buffer was eluted by gravity flow. The DNA was eluted with buffer QF (pre-warmed to 37°C) (5 mL). The columns were emptied by gravity flow. The eluate was mixed with 0.7 volumes of ice-cold isopropanol (3.5 mL) and the DNA precipitated by inverting the tubes gently. The tubes were centrifuged at 3500*g* at 4°C for 25 min and then stored at -20°C.

Washing DNA / UV spec DNA

Following storage at -20°C the tubes were centrifuged at 4°C for 25 min at 3500*g* and the supernatant was carefully removed. Into each tube, absolute ethanol (EtOH) (500 µL) was added and then transferred into a smaller eppendorf tube. The eppendorf tubes were centrifuged at 4°C at 15700*g* for 5 min before the EtOH was discarded and 70:30 EtOH/H_2O (*v/v*) (500 µL) was added. The eppendorf tubes were centrifuged at 4°C at 15700*g* for 5 min before the aqueous EtOH was discarded. The remaining EtOH in the tubes was allowed to evaporate into the air before ddH_2O (500 µL) was added. The pellet was re-dissolved by vortexing and kept on the rotating mixer for 4 – 5 hrs to re-dissolve.

For determination of the DNA content a 1:10 dilution (10 µL DNA solution plus 90 µL Tris base pH 7.4) was prepared. The UV absorbance was determined at 260 and 280

nm. The concentration was calculated by assuming that A_{260nm} = 1 is equivalent to 50 µg/mL for double stranded DNA.

Following the DNA concentration determination, hydrolysis was carried out (see section 2.2.2.2.2).

2.2.2.2.2 Thermal hydrolysis of DNA

Thermal hydrolysis was used to depurinate the *N*7-GA-Gua adduct.

DNA (152.38 µg) (equals 100 µg DNA on column) was pipetted into a 1.5 mL eppendorf tube and the labelled [$^{15}N_5$]-*N*7-GA-Gua adduct was added. The tubes were vortexed, centrifuged for 30 s at 16100*g* and evaporated to dryness in a large centrifugal evaporator. After drying, ddH_2O (100 µL) was added, vortexed and centrifuged for 30 s at 16100*g* before the tubes were placed in the heating block at 70°C for 1 hr. Amicon molecular weight cut-off filters were conditioned by applying ddH_2O (200 µL) and centrifuging for 20 min at 16100*g*. Following incubation at 70°C the tubes were centrifuged for 5 min at 16100*g* before ice-cold absolute EtOH (80 µL) was added to each tube. The tubes were vortexed and centrifuged for 30 s at 16100*g*. The solution was transferred to the pre-conditioned filters and centrifuged for 1 hr at 16100*g*. The filters were discarded and the tubes evaporated to dryness in a large centrifugal evaporator.

For mass spectrometric analysis 20 µL 0.1% FA was added and the sample re-dissolved by vigorous vortexing. The tubes were centrifuged for 1 min at 16100*g* and the solution was transferred to a HPLC vial, containing a low volume insert, ready for LC-MS/MS analysis (injection volume 15 µL).

2.2.2.2.3 HPLC purification of DNA samples following hydrolysis

Adenine and guanine are released by thermal hydrolysis. Due to the expected low amounts of adducted bases the unmodified bases are in vast excess which may have a detrimental influence on the chromatographic separation of the adduct base. In

addition, interferences, mainly salts that might still be carried over from the Qiagen process, can have a negative influence on the mass spectrometric results.

In samples measured after thermal hydrolysis some interfering peaks could be seen in the LC-MS/MS chromatogram. In some traces it was hard to determine the peak corresponding to the adducted base (as seen in Figure 2.5); therefore an UV-HPLC purification step was included.

The Waters Alliance HPLC system was used for purification of the DNA adducts derived from blood DNA and CT DNA matrix based samples. A Phenomenex Fusion RP column 250 x 4.6 mm connected to a guard column was used with an isocratic flow using 90:10 ddH_2O/MeOH (*v/v*). The flow rate was set to 1 mL/min and the total run time was 20 min.

Firstly, a guanine standard solution and then a [$^{15}N_5$]-*N*7-GA-Gua standard solution were injected onto the HPLC purification system to verify their retention times. The low concentration of *N*7-GA-Gua in samples falls below the limit of detection (LOD) for the HPLC-UV thus the chromatographic window (approximately 1.5 min either side of the retention time) for the adduct and IS was collected in the absence of any tangible chromatographic peak. The *N*7-GA-Gua adduclimit of t had a chromatographic window of about 3 – 4 min. Following collection, the *N*7-GA-Gua adduct was concentrated in a centrifugal evaporator and dried down completely following transfer into a small 2 mL eppendorf tube for mass spectrometric analysis.

For this HPLC purification step, LC-MS Optima water (40 µL) was added to the evaporated filtrate of the final hydrolysis step and the sample re-dissolved by vigorous vortexing. The tubes were centrifuged for 1 min at 16100*g* and the solution was transferred to a HPLC vial, containing a low volume insert, ready for HPLC purification (injection volume 35 µL).

2.2.2.3 Mass spectrometry

2.2.2.3.1 Mass spectrometer

All mass spectrometry was carried out on a Micromass Quattro Ultima tandem quadrupole mass spectrometer, Waters Ltd., Manchester, UK. Raw data was processed using MassLynx software purchased from Waters Ltd., Manchester, UK.

The consistent parameters used for the Quattro Ultima mass spectrometer are shown in Table 2.1.

Table 2.1: Parameters of the mass spectrometer that were kept on these consistent settings

Parameter	Setting
Collision energy	19 eV
Source temperature	110°C
Desolvation temperature	350°C
Cone gas flow	25 L/hr
Desolvation gas flow	650 L/hr

2.2.2.3.2 General information about the chromatograms

Figure 2.3 shows typical chromatograms for analysis of the *N*7-GA-Gua adduct using LC-MS/MS SRM. The information that can be taken from a chromatogram is shown and aids the understanding of chromatograms presented in this and following chapters.

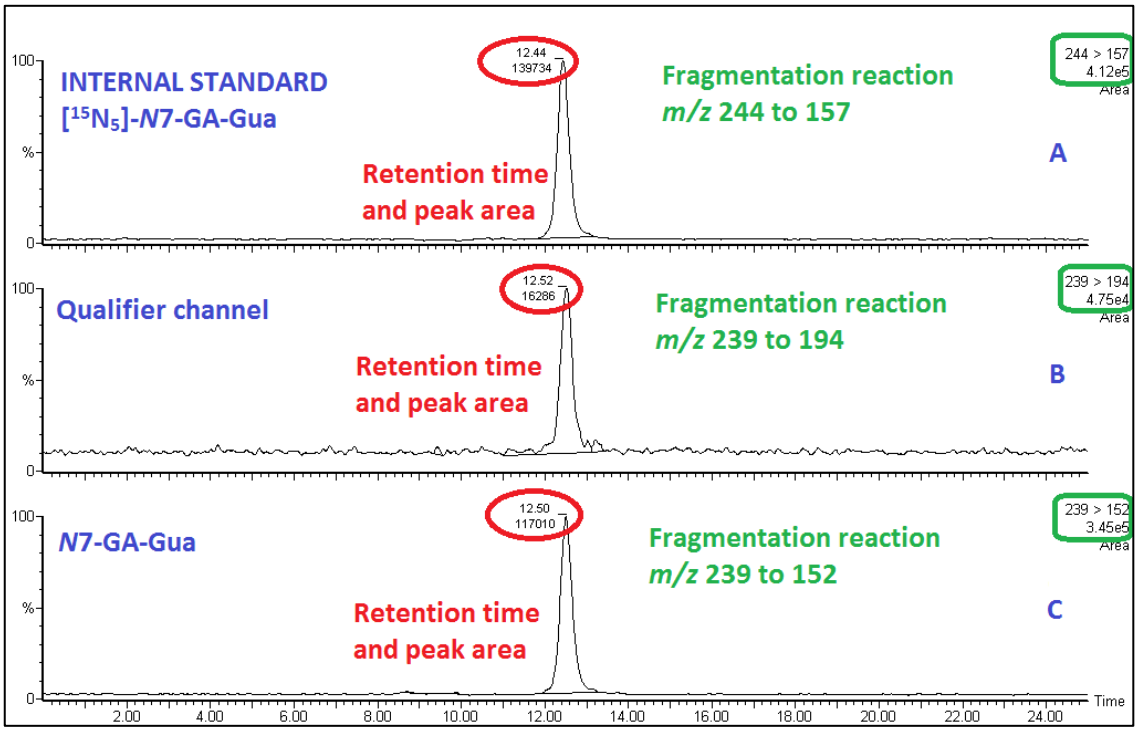


Figure 2.3: Typical chromatogram obtained for the analysis of the *N*7-GA-Gua adduct and its labelled standard using LC-MS/MS SRM. Details that can be taken from LC-MS/MS SRM chromatograms presented in this chapter. The presented LC-MS/MS SRM chromatograms reflects one sample injection by LC-MS to give the 3 monitored channels where the blue labelling explains what compound is detected in this channel, red states the peaks retention time (top value in circle) and area (bottom value in circle) and the green label points out which fragmentation is being monitored. LC-MS/MS SRM chromatogram A represents the channel monitoring the IS [$^{15}N_5$]-*N*7-GA-Gua fragmentation reaction *m/z* 244 to 157. LC-MS/MS SRM chromatogram B shows the qualifier channel with monitored fragmentation of the *N*7-GA-Gua adduct *m/z* 239 to 194 which is not used for quantification. LC-MS/MS SRM chromatogram C monitors the *N*7-GA-Gua fragmentation reaction *m/z* 239 to 152 which is used to quantify the adduct. The IS has a shorter retention time than the *N*7-GA-Gua and this alignment assists in assigning adduct peaks.

2.2.2.3.3 Development of LC-MS

Optimisation of the mass spectrometer tuning parameters

The signal for the *N*7-GA-Gua adduct was optimised by infusing 10 µL/min of a *N*7-GA-Gua adduct standard (typically 1 pmol/µL solution 5:95 MeOH/0.1% formic acid (FA) (*v/v*)). Several parameters were altered to optimise the signal including capillary and cone voltage, hexapole 1 and 2 plus aperture (all in V), ion energy 1 and 2, entrance and exit as well as the collision energy.

Selection of trap and analytical column

For the development of the column-switching, a trap column, which only retained the analyte and not the impurities, and an analytical column, which separated the analyte after back-flushing it from the trap column onto the analytical column, were chosen. Different trap columns varying in length and diameter (30 x 2.0 mm, 50 x 2.0 mm and 50 x 3.0 mm) were tested. The main aim was to maximise the purification of the analyte and reduce the sample derived interferences.

Selection of mobile phase

A LC-MS/MS method requires a mobile phase that is compatible with the mass spectrometer and also provides good chromatographic separation. Therefore two different aqueous phases (10 mM ammonium formate and 0.1% FA) and organic modifiers (MeOH and acetonitrile (ACN)) were tested. Also the proportions of organic modifier within the aqueous phase had to be tested to assure optimum chromatography. For pump A (serving the trap column) 5%, 3% and 1% organic content were tested and the auxiliary pump (serving the analytical column) was set up with 5%, 7.5%, 10%, 12.5% and 15% organic content.

Switching valve timing

The timings for the switching valve setting for the loading to the elution positions (Figure 1.9) to optimise purification were also assessed.

Run time optimisation

The run time was optimised to efficiently separate the analyte, as well as enabling washing and equilibration of the column for the next injection. In the process of developing the LC-MS/MS method for the detection of the *N*7-GA-Gua adduct different total run times were evaluated.

SRM selection

Previous work [73,87] has established the major fragmentation transition for the *N*7-GA-Gua adduct of *m/z* 239 to 152. To increase the specificity of detection, further multiple transitions were also monitored, i.e. *m/z* 239 to 88 and *m/z* 239 to 194.

The ion at *m/z* 88 represents the GA fragment after the loss of guanine. The fragmentation *m/z* 239 to 194 represents the breaking of the bond between the amide group and the *N*7-GA-Gua molecule (see Figure 2.13).

2.2.2.4 Biological sample analysis

2.2.2.4.1 Calibration line in pooled leukocyte DNA

In the first batch of analysed human leukocyte DNA samples the *N*7-GA-Gua adduct could not be detected. This experiment was set up to test if different concentrations of spiked *N*7-GA-Gua could be observed when spiked into extracted and pooled leukocyte DNA. The extracted leukocyte DNA from the different volunteers was pooled and a calibration line was determined. The *N*7-GA-Gua adduct was spiked into the DNA to give amounts of 100, 250, 500, 750, 1000 and 1250 fmol on column and analysed in duplicate. Each sample was spiked with [$^{15}N_5$]-*N*7-GA-Gua to give 750 fmol on column in a 15 µL injection.

2.2.2.4.2 Glycidamide treatment of HCEC cells and whole blood

This experiment was set up to test if the *N*7-GA-Gua adduct is detectable when whole blood and HCEC cells are treated with various amounts of GA and to verify if the GA treatment of whole blood and HCEC cells leads to a linear dose response of *N*7-GA-Gua adduct levels.

HCEC cells and cell culture

HCEC cells were treated with GA as described by Martins *et al*. [122]. Approximately 7 – 8 million cells provided one injection on the LC-MS (100 µg DNA on column).

CELL CULTURE – GA TREATMENT

GA was dissolved in PBS to give a 500 µM solution and diluted 1:1 in PBS to give 250 µM and further diluted 1:5 in PBS to give 100 µM solutions. The GA solutions were sterilised by passage through Acrodisc® filters. The media of the cells was removed.

New cell culture media was mixed with foetal calf serum and 23 mL added to the flasks. Each of the GA solutions (1 mL) were added into the appropriate flasks and the cells were incubated at 37°C and 5% CO_2. For the control samples, 1 mL PBS was added to the cells.

CELL CULTURE – HARVESTING CELLS FOR DNA

After 24 hrs the cells were removed from the incubator, the media removed and cells rinsed with PBS (2 x 10 mL). A 1:10 dilution of trypsin in PBS (4 mL) was added to each flask and incubated for 4 – 5 min at 37°C until the cells detached from the flask. Media (4 mL) mixed with foetal calf serum was added to the cells to neutralise the trypsin. The cells were pipetted into a centrifuge tube and suspended. The tubes were kept on ice until all flasks were processed and then they were centrifuged at 500*g* for 5 min at 4°C. The supernatant was discarded and the pellet re-suspended in PBS (10 mL). This procedure was repeated two more times, the supernatant discarded and the cells re-suspended in PBS (2 mL). DNA was then extracted following the Qiagen procedure as described in section 2.2.2.2.1.

Whole blood

Whole blood was treated with different amounts of GA (duplicate with 100, 250 and 500 µM and duplicates with 100, 500 and 1000 µM) for 24 hrs at 4°C. The 24 hr treatment of whole blood seemed to have an influence on the DNA content of blood that appeared to degrade within this time. Thus another experiment was set up, where the whole blood was treated with GA for 6 hrs.

2.2.2.4.3 Recovery of *N*7-GA-Gua

The *N*7-GA-Gua adduct recovery of the non-optimised method was evaluated using CT DNA, HCEC cell DNA and human leukocyte DNA spiked with the *N*7-GA-Gua adduct before hydrolysis and adding the IS [$^{15}N_5$]-*N*7-GA-Gua after hydrolysis and prior to analysis by LC-MS. For the calculation of the recovery as a percentage, the same amounts of unlabelled and labelled adduct in standard solutions are measured without

going through the hydrolysis procedure and compared to the set that went through hydrolysis. For this experiment the switching valve was set from 2 – 8 min, with a total run time of 25 min. The recovery of the most recent method, after including the gradient on the analytical column was covered within the process of validation and is described in Chapter 3.

2.2.2.4.4 Different amounts of leukocyte DNA and the "Concentrating effect"

After the DNA extraction using Qiagen kit the leukocyte DNA was washed, dissolved in 500 µL ddH_2O and stored at -20°C in an eppendorf tube. DNA from one volunteer (10 Lithium heparin tubes each containing 6 mL of whole blood DNA extracted to give 10 tubes each containing 500 µL washed and dissolved DNA) was combined and stored in tubes containing 5 mL DNA solution. After processing the data for the GA treated whole blood, where reaction tubes were kept at 4°C for 24 hrs as well as for 6 hrs it appeared that the DNA degraded within this treatment period. Hence most analyses were not performed with 100 µg DNA on column but instead with the amount of remaining DNA (ranged between 56 µg to 100 µg) but still a dose response was noticeable.

Therefore an experiment was set up, using different DNA amounts on column to see whether having less matrix might be advantageous for the detection of *N*7-GA-Gua in human samples. Hence, different amounts of DNA were pipetted, spiked with IS and hydrolysed. After all aliquots were pipetted (the "remaining solution" did not give a full aliquot anymore), the tube was centrifuged briefly and the remaining content pipetted into an eppendorf tube, also spiked with IS, and hydrolysed (illustrated in Figure 2.4).

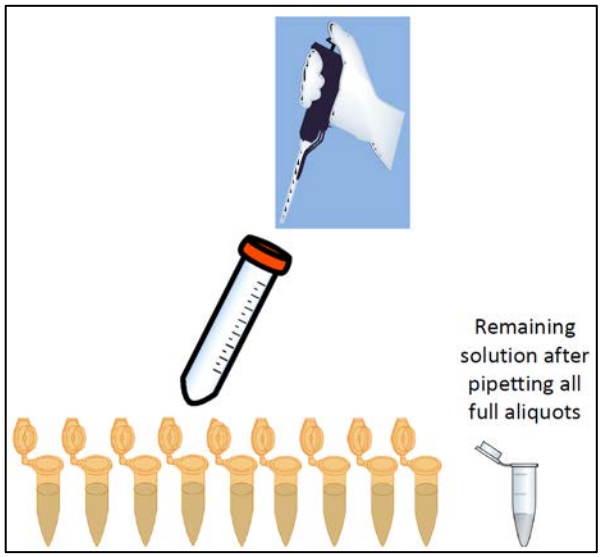


Figure 2.4: The 5 mL leukocyte DNA solution in the falcon tube was pipetted into aliquots with fixed amounts of DNA on column (shown in the Figure by orange eppendorf tubes). At the end, when no full aliquot could be pipetted the tube was centrifuged and the "remaining solution" also placed in an eppendorf tube (displayed as a grey eppendorf). Every tube was spiked with a fixed amount of IS [$^{15}N_5$]-*N*7-GA-Gua, hydrolysed and analysed by LC-MS.

Smoker's leukocyte DNA

Cigarette smoke contains 1 – 2 µg AA per cigarette [73] and it is shown that the AA-Hb adduct levels for smokers are approximately 4 times higher than for non-smokers [123]. Thus it was investigated whether the *N*7-GA-Gua adduct could be detected in smoker's blood DNA. The DNA from whole blood collected from a smoker for method development was extracted and dissolved before they were combined into a 15 mL falcon tube to give a total volume of 5 mL DNA solution. Then different amounts of the leukocyte DNA were pipetted (duplicates for 25, 37.5, 50, and 75 µg DNA on column; single injection for 100 µg on column and the "remaining solution" in the tube added up to 20.5 µg DNA on column all in a 15 µL injection), spiked with [$^{15}N_5$]-*N*7-GA-Gua to give 794 fmol on column, hydrolysed and analysed by LC-MS.

Non-smoker's leukocyte DNA

Non-smokers leukocyte DNA (5 mL) was defrosted and aliquoted (470 µL aliquots equals 133.33 µg DNA to give 100 µg DNA on column). The remaining 215 µL solution gave an injection with 45.7 µg DNA on column. Every tube was spiked with [$^{15}N_5$]-*N*7-GA-Gua to give 794 fmol on column in a 15 µL injection, hydrolysed and tested for the "concentrating effect" by LC-MS.

Assessment of storage volume

In a different experiment it was tested whether the storage volume of leukocyte DNA influenced the "concentrating effect". Eppendorf tubes containing leukocyte DNA solution (1 mL, 1.5 mL and 2 mL) were stored and 100 µg DNA on column aliquots were pipetted. The 1 mL tube gave one 100 µg DNA on column aliquot and the "remaining solution" was 78 µg DNA on column. The 1.5 mL tube resulted in two 100 µg DNA on column aliquots and one with 75 µg DNA on column whereas the 2 mL tube resulted in four 100 µg DNA on column aliquots and one with 101 µg DNA on column. Each tube was spiked with [$^{15}N_5$]-*N*7-GA-Gua to give 794 fmol on column in a 15 µL injection, hydrolysed and analysed by LC-MS.

2.2.2.4.5 Matrix effect on different DNA quantities

In order to decide how much DNA on column per injection to use, different CT DNA amounts were pipetted and spiked with labelled and unlabelled adduct to see what influence the matrix has on the signal response of both standards.

Different amounts of CT DNA (triplicate) were spiked with *N*7-GA-Gua to give 500 fmol on column and [$^{15}N_5$]-*N*7-GA-Gua to give 315 fmol on column, hydrolysed and analysed by LC-MS.

2.2.2.4.6 Interfering peaks in the chromatogram

The transition *m/z* 239 to 152 showed a lot of interfering peaks around the retention time of the *N*7-GA-Gua adduct when analysing matrix containing samples which made it difficult to detect the adduct peak at low concentrations. Therefore, the source of these impurity peaks was investigated.

Switching valve

The switching valve was set to switch to the elution position after 2 min and back to the loading position after 4.5 min instead of the previous setting of 8 min.

Qiagen procedure for CT DNA and ST DNA

The Qiagen kit procedure for the extraction of the leukocyte DNA from whole blood was assessed to see if this was contributing to the interferences associated with the separation of the *N*7-GA-Gua adduct. The kit uses buffers with a high concentration of salts which might cause signal suppression once the samples are subjected to LC-MS. During the first drying step of hydrolysis, a white precipitate was observed, whereas dried down CT DNA mostly appears as a clear film in the tubes. The CT DNA and ST DNA were purchased from Sigma who use a membrane procedure for its purification and hence leads to less salt contaminated DNA.

The extent of contamination provided by the Qiagen kit was evaluated by subjecting CT DNA and ST DNA to the Qiagen procedure. For each DNA sample a low dose (CT DNA 0.43 mg, ST DNA 0.54 mg) and a high dose (CT DNA 0.89 mg, ST DNA 0.98 mg) weighted DNA sample was applied to the Qiagen columns (maxi) to see if the amount of DNA passed through the columns had an influence on the extraction purity. The Qiagen processed samples were compared with the same batch of CT DNA and ST DNA without purification, respectively analysed after being hydrolysed.

Amicon filters

Hydrolysis of the DNA involved the use of Amicon molecular weight cut-off filters. These filters were also assessed to see if they were the source of interfering substances.

Firstly, ddH_2O, 0.1% FA and a standard solution, containing both *N*7-GA-Gua and [$^{15}N_5$]-*N*7-GA-Gua (each to give 630 fmol on column in a 15 µL injection) were subjected to the hydrolysis process, without matrix. The chromatograms were then checked for any impurities being washed off from the filters that eluted in a 1 min window around the expected retention time of the *N*7-GA-Gua adduct.

Secondly, the size of the molecular weight cut-off filter was assessed. Two different types were tested, the 3 k and 10 k Amicon molecular weight cut-off filters. CT DNA (triplicate) was hydrolysed and spiked with [$^{15}N_5$]-*N*7-GA-Gua to give 794 fmol on column in a 15 µL injection.

Thirdly, the SOP for the hydrolysis procedure instructed to equilibrate the Amicon filters for 20 min with 200 µL ddH_2O at 15700*g*. Da Costa *et al*. equilibrated the Amicon filters for 45 min at 21°C at 13400*g* [73]. CT DNA was hydrolysed and spiked with [$^{15}N_5$]-*N*7-GA-Gua to give 794 fmol on column in a 15 µL injection. Also 200 µL ddH_2O was applied to the Amicon filter for testing using the procedure described by Da Costa *et al*. [73].

Solid phase extraction (SPE) for DNA samples

CT DNA and ST DNA were subjected to the SPE process in a similar manner to Chao *et al*. [124]. Each sample was spiked with [$^{15}N_5$]-*N*7-GA-Gua to give 1135 fmol on column. HLB 1cc columns were used for the SPE procedure described in Table 2.2. The loading solution was the evaporated hydrolysis filtrate re-dissolved in ddH_20 (1 mL). The tubes were spiked with the [$^{15}N_5$]-*N*7-GA-Gua standard at different points in the protocol. One set was spiked with [$^{15}N_5$]-*N*7-GA-Gua before hydrolysis, the second tube before the SPE process. From the loading step onwards all fractions were collected and dried down before analysing them by LC-MS.

Table 2.2: Solvents used for the process of HLB 1cc columns and Strata X columns for the purification of hydrolysed ST DNA and CT DNA samples, * modified the method described by Chow *et al.* [124]

	HLB 1cc column	**Strata X - optimised**
Condition	1 mL MeOH	1 mL MeOH
Equilibration	1 mL ddH_2O	1 mL ddH_2O
Loading	hydrolysis filtrate	hydrolysis filtrate
Wash 1	1 mL 5:95 MeOH/ddH_2O (*v/v*)	1 mL ddH_2O
Wash 2		1 mL 5:95 MeOH/ddH_2O (*v/v*)
Elution	1 mL 40:60 MeOH/ddH_2O (*v/v*)	1 mL 70:30 MeOH/ddH_2O (*v/v*) *

Method optimisation was attempted by purifying hydrolysed CT DNA on Strata X columns. Samples were spiked with *N*7-GA-Gua and [$^{15}N_5$]-*N*7-GA-Gua (each to give 630 fmol on column in a 15 µL injection) and hydrolysed before processing them on SPE Strata X cartridges. For one set the hydrolysed filtrate was applied to the column

and for the other set the filtrate was placed in a centrifugal evaporator for 30 min to evaporate the EtOH. After evaporating the EtOH 100 µL ddH_2O was added and this was applied on the cartridges. Both sets underwent SPE with conditions described in Table 2.2 for Strata X columns. All fractions were dried down in a centrifugal evaporator.

Leukocyte DNA and CT DNA – Hydrolysis - SPE

Leukocyte DNA (duplicate) was taken through Strata X columns and compared to a duplicate set just hydrolysed. All tubes were spiked with [$^{15}N_5$]-*N*7-GA-Gua to give 794 fmol on column. The SPE cartridges were conditioned (1 mL MeOH), equilibrated (1 mL ddH_2O) and the sample loaded (180 µL hydrolysis filtrate diluted with 320 µL ddH_2O). The columns were washed (1 mL ddH_2O) and the adduct eluted (1 mL 40:60 MeOH/ ddH_2O (*v/v*)).

DNA containing low amounts of N7-GA-Gua – Hydrolysis vs. SPE

Low amounts of the *N*7-GA-Gua adduct were spiked into CT DNA to establish how much is detectable to aid the validation afterwards. Therefore CT DNA was spiked with *N*7-GA-Gua (to give 19, 45, 94 and 188 fmol on column) and [$^{15}N_5$]-*N*7-GA-Gua to give 794 fmol on column. A duplicate of each concentration was obtained. One set was analysed by LC-MS after hydrolysis and the other set was hydrolysed and processed with SPE (Strata X). The SPE cartridges were conditioned (1 mL MeOH), equilibrated (1 mL ddH_2O) and the sample loaded (180 µL hydrolysis filtrate diluted with 320 µL ddH_2O). The columns were washed (1 mL ddH_2O) and the adduct eluted (1 mL 40:60 MeOH/ddH_2O (*v/v*)).

Reversing the role of IS and unlabelled standard

CT DNA was used to set up a calibration line by spiking the labelled standard in varying amounts (to give 5, 10, 15, 20, 25, and 30 fmol on column in a 15 µL injection) and using the *N*7-GA-Gua adduct as IS (to give 563 fmol on column in a 15 µL injection). Each chromatogram showed a huge artefact peak in the channel *m/z* 239 to 152 which made it hard to observe even the spiked unlabelled adduct. In most of the chromatograms (Figure 2.5) it is just one poorly resolved peak which makes discriminating the *N*7-GA-Gua peak challenging.

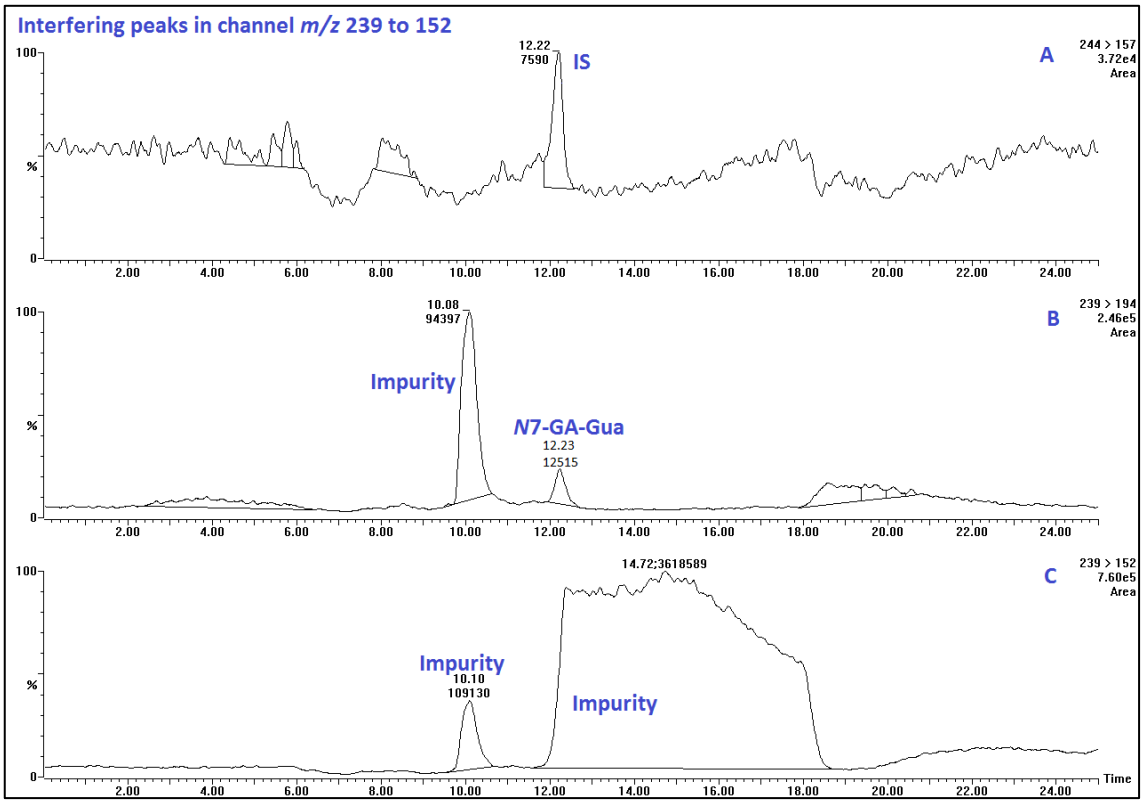


Figure 2.5: LC-MS/MS SRM of *N*7-GA-Gua and IS spiked in CT DNA with the adduct as a surrogate IS. LC-MS/MS SRM chromatogram A shows the transition of the labelled standard with a retention time of 12.22 min. In LC-MS/MS SRM chromatogram B the transition *m/z* 239 to 194 gave a resolved peak whereas the impurity peak in the *m/z* channel 239 to 152 (LC-MS/MS SRM chromatogram C) meant the detection of the *N*7-GA-Gua adduct was infeasible.

Following these observations it was decided to purify the DNA by using HPLC.

2.2.2.5 Optimised method for the detection of the *N*7-GA-Gua adduct

The parameters for the optimised final method are presented in Table 2.3.

Table 2.3: The parameter for the optimised method developed for the detection of the *N*7-GA-Gua adduct

Sample preparation		**Sample analysis (mass spectrometer)**	
Qiagen kit for DNA extraction from whole blood	100 µg DNA on column	**Trap column:**	Synergi 4 µm Fusion-RP 80Å 50 x 2.0 mm
Hydrolysis:	70°C, 1 hr	**Analytical column:**	Synergi 4 µm Fusion-RP 80Å 250 x 2.0 mm
	10 k Amicon filter	**Timings for column-switching:**	2 - 4.5 min
HPLC purification	**Column**: Synergi 4 µm Fusion-RP 80Å 250 x 4.6 mm	**Mobile phase:**	**trap column**: 99:1 0.1% FA/MeOH (*v/v*)
	Mobile Phase: 90:10 LC-Optima water/ MeOH (*v/v*)		**analytical column**: 95:5 0.1% FA/MeOH (*v/v*)
	Run time: 20 min	**Flow rate:**	0.12 mL/min
	Flow rate: 1 mL/min	**Run time:**	40 min
		Gradient for trap and analytical column:	See Table 2.11 and Table 2.12

2.2.2.6 Development of a method to detect *N*7-GA-Gua in urine

An attempt was made to develop a method for the measurement of the *N*7-GA-Gua adduct in urine. A SPE purification step had to be applied due to the high salt concentration in urine prior to mass spectrometric analysis. Different SPE columns were tested to try and find an optimal procedure for the *N*7-GA-Gua detection in this matrix.

2.2.2.6.1 SPE – HLB columns

Standards through HLB 6cc columns – generic method

*N*7-GA-Gua standards were used to test the Waters Oasis HLB 6cc columns before analysing urine samples. The generic method provided by the manufacturer (Table 2.4) was tested with a set of acidic standard solutions and basic standard solutions. The acidic solution was prepared by mixing ddH_2O (1 mL) with 15 M H_3PO_4 (40 µL) whereas for the basic solution ddH_2O (1 mL) was mixed with 14.5 M NH_4OH (40 µL). The loading solution (5 mL: 2.5 mL sample + 2.5 mL acidic/basic solution) was spiked with 10 pmol *N*7-GA-Gua. The SPE procedure was followed as described in Table 2.4. The eluates were evaporated to dryness and re-dissolved in 200 µL 0.1% FA and spiked with the IS [$^{15}N_5$]-*N*7-GA-Gua and of this solution 20 µL was injected onto LC-MS, containing theoretically 750 fmol on column of *N*7-GA-Gua and 800 fmol on column [$^{15}N_5$]-*N*7-GA-Gua per 15 µL injection.

Table 2.4: The different conditions used for the Waters Oasis HLB 6cc columns. The generic, optimised acidic and optimised basic variations were provided by the manufacturer

	Generic	Optimised acidic	Optimised basic
Condition	3 mL MeOH	3 mL MeOH	3 mL MeOH
Equilibration	3 mL ddH_2O	3 mL ddH_2O	3 mL ddH_2O
Load	5 mL sample	5 mL sample	5 mL sample
Wash 1	4 mL 5:95 MeOH/ddH_2O (*v/v*)	4 mL 5:95 MeOH/ddH_2O (*v/v*)	4 mL 5:95 MeOH/ddH_2O (*v/v*)
Wash 2		4 mL 2% FA in 1:1 MeOH/ddH_2O (*v/v*)	4 mL 5% NH_4OH in 1:1 MeOH/ddH_2O (*v/v*)
Elution	4 mL MeOH	4 mL 5% NH_4OH in 1:1 MeOH/ddH_2O (*v/v*)	4 mL 2% FA in 1:1 MeOH/ddH_2O (*v/v*)

Standards through HLB 6cc columns – optimised method

The next step was to follow the optimised method which contained an additional washing step and used a different eluting solvent (see Table 2.4). The loading solution (5 mL: 2.5 mL sample + 2.5 mL acidic/basic water) was spiked with 10 pmol *N*7-GA-Gua. SPE procedure was followed as described in Table 2.4. The evaporated eluates were re-dissolved in 200 µL 0.1% FA and spiked with the IS [$^{15}N_5$]-*N*7-GA-Gua. 20 µL of this solution was injected onto LC-MS, containing theoretically 750 fmol on column of *N*7-GA-Gua and 800 fmol on column [$^{15}N_5$]-*N*7-GA-Gua per 15 µL injection.

2.2.2.6.2 SPE – Strata X-C columns

Strata X-C purification of N7-GA-Gua standards

*N*7-GA-Gua standards were applied to ion exchange columns (Strata X-C 100 mg/6 mL) without matrix. Two sets of columns were used where for one set 2 mL each for washing, loading and eluting were applied and for the other set 4 mL for all steps was used. The standard solutions (6 mL) were acidified with H_3PO_4. The SPE procedure was followed as described in Table 2.5 using 2 mL and 4 mL for each step, respectively. All fractions were collected and evaporated to dryness in a centrifugal evaporator. The eluate was spiked with the IS [$^{15}N_5$]-*N*7-GA-Gua and re-dissolved in 205 µL 0.1% FA. Per

15 µL injection onto the LC-MS there was 730 fmol on column *N*7-GA-Gua and 886 fmol on column [$^{15}N_5$]-*N*7-GA-Gua present.

Table 2.5: Solvents used for the SPE process with Strata X-C (100 mg/6 mL) and a new method to work with Strata X-C (30 mg/1 mL)

	Strata X-C (100 mg/6 mL)	**Strata X-C (30 mg/1 mL)**
Condition	MeOH	1 mL MeOH
Equilibration	ddH_20	1 mL ddH_2O
Load	Sample acidified with 20 µL 15 M H_3PO_4	1 mL Sample (pH 6-7)
Wash 1	0.1% H_3PO_4	1 mL 0.1 M HCl in ddH_2O
Wash 2	MeOH	1 mL 0.1 M HCl in MeOH
Elution	5:95 NH_4OH/MeOH (*v/v*)	2 x 500 µL 5:95 NH_4OH/MeOH (*v/v*)

Analysis of urine by Strata X-C SPE

The urine samples were passed through Acrodisc syringe filters, diluted 1:1 with ddH_2O and acidified with 20 µL FA/mL before loading onto the columns. A set of urine samples went through the SPE process non-spiked and another set was spiked with *N*7-GA-Gua and [$^{15}N_5$]-*N*7-GA-Gua (to give 630 fmol on column each in 15 µL injection). Each set of non-spiked and spiked urine was processed with 4 mL and 6 mL for the conditioning (MeOH), equilibrating (ddH_2O), loading and washing (first [0.1% FA] and second wash [MeOH]) step before eluting (5:95 NH_4OH/MeOH (*v/v*)) the analyte.

Phenomenex SPE method

A SPE method was suggested by Phenomenex Ltd. using Strata X-C (30 mg/1 mL). The SPE process was followed as shown in (Table 2.5). At first 1 pmol *N*7-GA-Gua (1 mL) was passed through the SPE column. All fractions were collected and evaporated to dryness in a centrifugal evaporator. 794 fmol on column [$^{15}N_5$]-*N*7-GA-Gua in 15 µL injection was added when re-dissolving the eluates in 20 µL 0.1% FA before analysis by LC-MS.

In a second experiment only 500 fmol *N*7-GA-Gua adduct underwent the SPE process. The same columns and method was used as described in Table 2.5 for Strata X-C (30

mg/1 mL). Before analysis by LC-MS [$^{15}N_5$]-*N*7-GA-Gua to give 794 fmol on column in a 15 µL injection was added.

2.2.2.6.3 SPE – Strata X columns

Different volumes of urine (100, 200, 300, 400 and 500 µL) were diluted 1:1 with ddH_2O and spiked with standards both *N*7-GA-Gua and [$^{15}N_5$]-*N*7-GA-Gua (each to give 630 fmol on column in a 15 µL injection). The SPE procedure shown in Table 2.6 was followed. The eluates were evaporated to dryness using a centrifugal evaporator.

Table 2.6: Solvents used for processing the samples going through the work-up procedure of SPE Strata X columns

	Strata X
Condition	1 mL MeOH
Equilibration	1 mL ddH_2O
Load	1:1 diluted and spiked urine
Wash	1 mL ddH_2O
Elution	1 mL 40:60 MeOH/ddH_2O (*v/v*)

The evaporated eluates were re-dissolved in 20 µL 0.1% FA and a 15 µL injection analysed by LC-MS.

2.2.2.6.4 SPE – Strata X-A columns

For the method suggested by Phenomenex, 1 pmol *N*7-GA-Gua standard (1 mL) was taken through the SPE process (anion exchange columns Strata X-A). The SPE procedure was followed as described in Table 2.7.

Table 2.7: Solvents for the SPE procedure of Strata X-A columns

	Strata X-A (30 mg/1 mL)
Condition	1 mL MeOH
Equilibration	1 mL ddH_2O
Load	1 mL Sample (pH 6 -7)
Wash 1	1 mL 25 mM ammonium acetate (pH 6 -7)
Wash 2	1 mL MeOH
Elution	2 x 500 µL 5:95 FA/MeOH (*v/v*)

The eluate was evaporated to dryness in a centrifugal evaporator and re-dissolved in 20 µL 0.1% FA containing 794 fmol on column [$^{15}N_5$]-*N*7-GA-Gua in a 15 µL injection.

The experiment was repeated where only 500 fmol *N*7-GA-Gua adduct underwent the SPE procedure (Table 2.7). Before analysis by LC-MS 794 fmol on column IS [$^{15}N_5$]-*N*7-GA-Gua was added.

2.2.2.6.5 Alternative SPE protocols

Different methods for ion exchange SPE namely, the Strata X-C and Strata X-A were investigated. Throughout this section all experiments were done by applying the method described in Table 2.8 however, for the small cartridges (30 mg/1 mL) only 1 mL for each step was used.

Standards only

Firstly only standard solutions were applied to both Strata X-C and Strata X-A columns.

Table 2.8: Recommended protocols for Strata X-A and Strata X-C SPE columns

	Strata X-A (200 mg/3 mL)	Strata X-C (200 mg/3 mL)
Condition	3 mL MeOH	3 mL MeOH
Equilibrate	3 mL of 100 mM K_2HPO_4	3 mL 0.1 M HCl
Load	diluted sample	diluted sample
Wash 1	3 mL of 100 mM K_2HPO_4	3 mL 0.1 M HCl
Wash 2	3 mL MeOH	3 mL MeOH
Dry	5 min under full vacuum	5 min under full vacuum
Elute	3 mL of (5:95) FA:MeOH (*v/v*)	2 x 1.5 mL (5:95) FA:MeOH (*v/v*)

500 fmol of *N*7-GA-Gua standard was mixed with 100 mM K_2HPO_4 pH 12.7 (for Strata X-A, 1 mL) or 0.1 M HCl pH 1 – 2 (for Strata X-C, 1 mL and 3 mL), respectively. The pH of the solutions was checked with litmus paper. The sample for Strata X-A was centrifuged for 10 min at 2000*g* at 15°C. Every fraction of the SPE process was collected and evaporated to dryness.

Urine

The 1 mL and 3 mL Strata X-C and Strata X-A cartridges were investigated. For the 1 mL cartridges 0.5 mL urine was applied and diluted 1:1 with 0.5 mL 100 mM K_2HPO_4 (pH 12.7) or 0.1 M HCl (pH 1 – 2), respectively. The 3 mL columns were used by applying 2.5 mL urine and diluting it 1:1 with 100 mM K_2HPO_4 or 0.1 M HCl, respectively. The pH of the solutions was tested with litmus paper. The samples were processed as described in Table 2.8 with exception of the 1 mL cartridges where only 1 mL solution was used for every step. After evaporating the eluate to dryness and re-dissolving in 20 µL 0.1% FA, a non-dissolvable precipitate prevented their analysis by LC-MS.

Analysis of different urine volumes

To investigate whether the precipitation could be avoided, an experiment was performed using different urine volumes, ranging between 100 µL and 2500 µL (100, 250, 500, 1000, 1500, 2000 and 2500 µL), each spiked to give 500 fmol on column *N*7-GA-Gua in a 15 µL injection. All urine samples were diluted 1:1 with either 100 mM K_2HPO_4 (pH 12.7) or 0.1 M HCl (pH 1 - 2). The samples for the Strata X-A columns were centrifuged for 10 min at 2000*g* and the urine applied to Strata X-C columns was

filtered with Acrodisc filters. The samples were processed as described in Table 2.8 using 1 mL cartridges. All fractions were collected and dried down in a centrifugal evaporator. Samples were spiked to give 750 fmol on column [$^{15}N_5$]-*N*7-GA-Gua and re-dissolved in 20 µL 0.1% FA before analysis by LC-MS.

Analysis using smaller urine volumes – different sample dilution

An experiment was set up with a smaller range of urine volumes between 100 µL and 1000 µL (100, 250, 500, 750 and 1000 µL) and a 1:2 dilution of urine and 100 mM K_2HPO_4 (pH 12.7) or 0.1 M HCl, respectively. Each sample was spiked with 5 pmol *N*7-GA-Gua and followed the SPE procedure shown in Table 2.8 for 1 mL cartridges. All urine samples were centrifuged for 10 min at 2000*g*. Before re-dissolving the fractions in 20 µL 0.1% FA and injecting 15 µL by LC-MS they were spiked with [$^{15}N_5$]-*N*7-GA-Gua to give 750 fmol on column. All eluates from the Strata X-C columns were analysed but the Strata X-A eluates from the 750 µL and 1000 µL urine had a non-dissolvable precipitate.

Calibration line with urine matrix

Different amounts of *N*7-GA-Gua were spiked into urine to see if a linear dose response could be established. The same amounts of *N*7-GA-Gua were spiked as was used for the validation of the adduct in CT DNA ranging from 25 fmol to 150 fmol on column (to give 25, 50, 75, 100, 125 and 150 fmol on column). The *N*7-GA-Gua solution were spiked into 250 µL urine. Samples were diluted 1:2 (urine and K_2HPO_4 pH 12.7 or 0.1 M HCl pH 1 - 2, respectively) following the SPE procedure described in Table 2.8. Each eluate was spiked with 750 fmol on column [$^{15}N_5$]-*N*7-GA-Gua, re-dissolved in 20 µL 0.1% FA and a 15 µL injection analysis by LC-MS.

pH changes

To improve the *N*7-GA-Gua recovery for the adduct in SPE columns the pH for 100 mM K_2HPO_4 was changed to 13.2 and a 1 M HCl solution was used instead of the previously used 0.1 M HCl. Different urine volumes were diluted with different volumes of 100 mM K_2HPO_4 or 1 M HCl, respectively as shown in Table 2.9. *N*7-GA-Gua was spiked to give 500 fmol on column in a 15 µL injection.

Table 2.9: The table shows how much urine was diluted with either 1 M HCl for the Strata X-C columns or 100 mM K_2HPO_4 pH 13.2 for the Stata X-A columns

Strata X-C		Strata X-A	
Urine	**1 M HCL**	**Urine**	**100 mM K_2HPO_4**
100 µL	300 µL	250 µL	750 µL
100 µL	4900 µL	250 µL	4750 µL
250 µL	750 µL	2000 µL	3000 µL
250 µL	4750 µL	1000 µL	4000 µL
2000 µL	3000 µL		
1000 µL	4000 µL		

All fractions were collected and evaporated to dryness in a centrifugal evaporator and spiked with [$^{15}N_5$]-*N*7-GA-Gua to give 500 fmol on column before re-dissolving in 20 µL 0.1% FA and analysing a 15 µL injection by LC-MS.

HPLC purification of small urine volumes

The raw recovery, just looking at the *N*7-GA-Gua adduct recovery was very low for Strata X-A columns in the previous experiment and henceforth only Strata X-C columns were used. Urine (100 µL) per cartridge was diluted with 300 µL 1 M HCl and spiked with *N*7-GA-Gua to give 437.5 fmol on column (triplicate) before processed by SPE (Table 2.8 using 1 M HCl). The eluates were evaporated to dryness in a centrifugal evaporator before they were re-dissolved in 40 µL LC-MS Optima water and purified on the HPLC (Waters HPLC system) following HPLC purification as described in section 2.2.2.2.3 for DNA samples. The collected fraction from the HPLC run was dried down in a centrifugal evaporator, spiked with [$^{15}N_5$]-*N*7-GA-Gua to give 500 fmol on column and re-dissolved in 0.1% FA before analysis of a 15 µL injection by LC-MS.

HPLC purification of N7-GA-Gua from increased urine volumes

This experiment was set up to see whether it is possible to analyse increased urine volumes when the HPLC purification step is included. Only Strata X-C columns were used for this experiment. Urine (100 µL) was mixed with 300 µL 1M HCl and urine (2500 µL) was mixed with 2500 µL 1M HCl. One set was spiked with only [$^{15}N_5$]-*N*7-GA-Gua to give 500 fmol on column and the other set with both labelled and unlabelled

standard to give 500 fmol on column. The SPE process was undertaken for Strata X-C columns as mentioned in Table 2.8 (using 1 M HCl). Eluates were evaporated to dryness and re-dissolved in 40 µL LC-MS Optima water before purification on the HPLC. The HPLC purification fraction was evaporated to dryness on a centrifugal evaporator, spiked with [$^{15}N_5$]-*N*7-GA-Gua and re-dissolved in 20 µL 0.1% FA before analysis by LC-MS.

HPLC purification of different urine volumes

Applying 2.5 mL of urine influenced the response of the *N*7-GA-Gua signal compared to 100 µL of urine. Hence different urine volumes (500, 1000, 1500 and 2000 µL) were taken through SPE and in addition HPLC purified. Only Strata X-C columns were used and the SPE protocol mentioned in Table 2.8 was followed using 1 M HCl. *N*7-GA-Gua adduct to give 500 fmol on column was spiked. After SPE and HPLC the eluate fraction was evaporated to dryness in a centrifugal evaporator, spiked with [$^{15}N_5$]-*N*7-GA-Gua to give 750 fmol on column and re-dissolved in 20 µL 0.1% FA before analysis of a 15 µL injection by LC-MS.

2.2.2.6.6 Urine: Dilute and inject

Urine was diluted 1:1000, 1:100 and 1:10 with 0.1% FA. One set of urine for each dilution was spiked with [$^{15}N_5$]-*N*7-GA-Gua to give 500 fmol on column and the other set was spiked with both *N*7-GA-Gua and [$^{15}N_5$]-*N*7-GA-Gua to give 500 fmol on column in a 15 µL injection and analysed by LC-MS.

2.2.2.7 Heating block experiment

The heating step in the hydrolysis protocol was considered to be a potential source of variation in the analysis of the adduct. Thus this step was systematically assessed. The DNA is heated at 70°C for one hr in this step. The laboratory equipment provided a heating block with three different metallic inserts in which tubes can be placed. This experiment served the purpose of investigating if there is a difference between these various inserts and the results obtained in the validation (Chapter 3). In Block 1 the 1.5

mL eppendorf tubes were entirely submerged. Block 2 held the 1.5 mL eppendorf tubes, but still protruded. Block 3 had cone shaped bottoms in the notches where the 1.5 mL tubes fit in but protruded slightly less than block 2. CT DNA was spiked with 20 µL of standard solution *N*7-GA-Gua and [$^{15}N_5$]-*N*7-GA-Gua (to give 600 fmol on column each in a 15 µL injection). Triplicate per heating block were hydrolysed, then purified by HPLC and analysed by LC-MS.

2.3 Results

2.3.1 Standards

2.3.1.1 Synthesis of *N*7-GA-Gua and [$^{15}N_5$]-*N*7-GA-Gua

The syntheses of both labelled and unlabelled adduct resulted in products that were analysed in positive and negative ESI on an open access instrument (Chemistry Department, UoL) and preliminary evidence of the adduct was attained. Further structural confirmation was obtained using ^{1}H-NMR (appendix section 8.1).

2.3.1.2 Purification and concentration determination of the *N*7-GA-Gua and [$^{15}N_5$]-*N*7-GA-Gua

The UV absorption spectrum was obtained for the unlabelled adduct to determine its final concentration and also to check the purity of the standard (Figure 2.6). Using the Beer-Lambert law, the purified *N*7-GA-Gua standard solution has a concentration of 1142 pmol/µL.

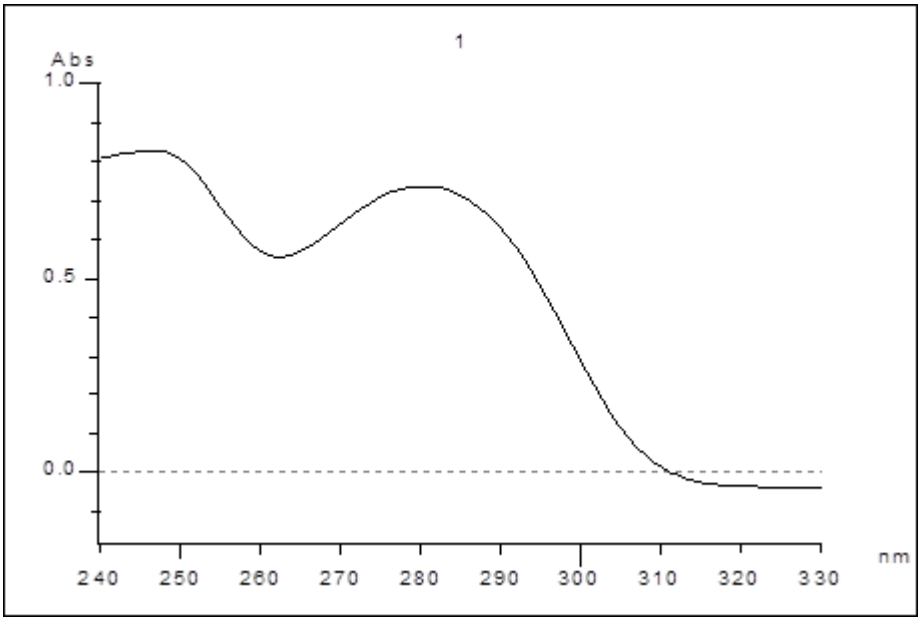


Figure 2.6: UV spectrum for a 1:10 dilution of *N*7-GA-Gua standard in 10 mM ammonium formate pH 7.0. Abs = absorbance, nm = wavelength.

A calibration line of different *N*7-GA-Gua concentrations was used to determine the concentration of the [$^{15}N_5$]-*N*7-GA-Gua adduct by LC-MS analysis of a high and low concentration solution. The concentration of [$^{15}N_5$]-*N*7-GA-Gua was determined to be 151.3 pmol/µL (Figure 2.7).

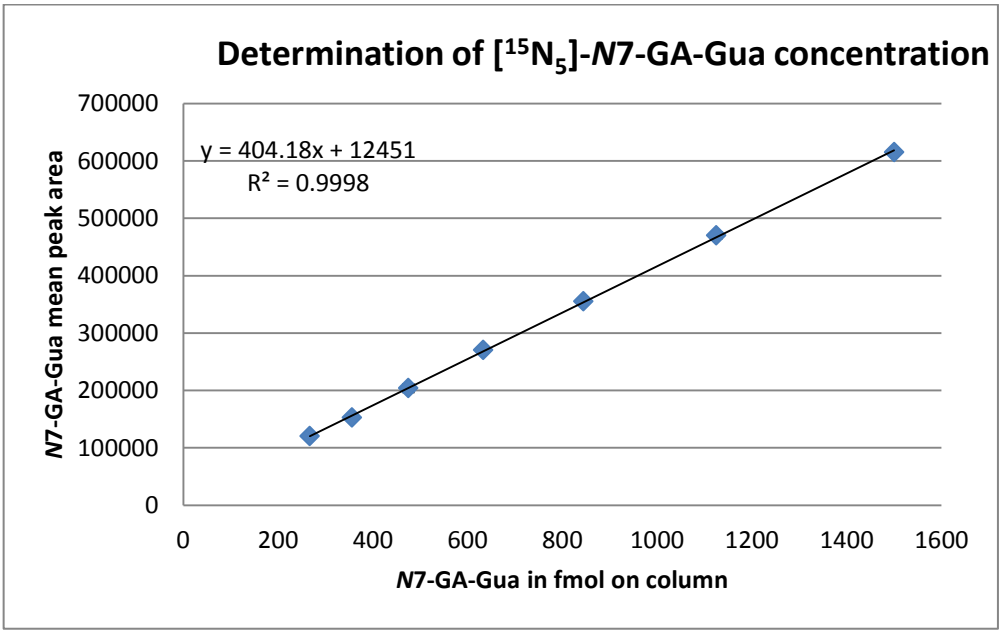


Figure 2.7: Calibration line with purified *N*7-GA-Gua solution to determine the concentration of the IS [$^{15}N_5$]-*N*7-GA-Gua solution obtained by LC-MS.

2.3.2 Biological samples - HPLC purification of DNA samples after hydrolysis

Due to the very small amounts expected for the *N*7-GA-Gua adduct in human samples, and the low sensitivity limit of the UV detection system, the fraction which contained the adduct had to be collected blind (without a visible peak). Once the retention times of a guanine standard and pure [$^{15}N_5$]-*N*7-GA-Gua standard (Figure 2.8) were verified the fraction containing the adduct could be collected purified from any guanine contamination.

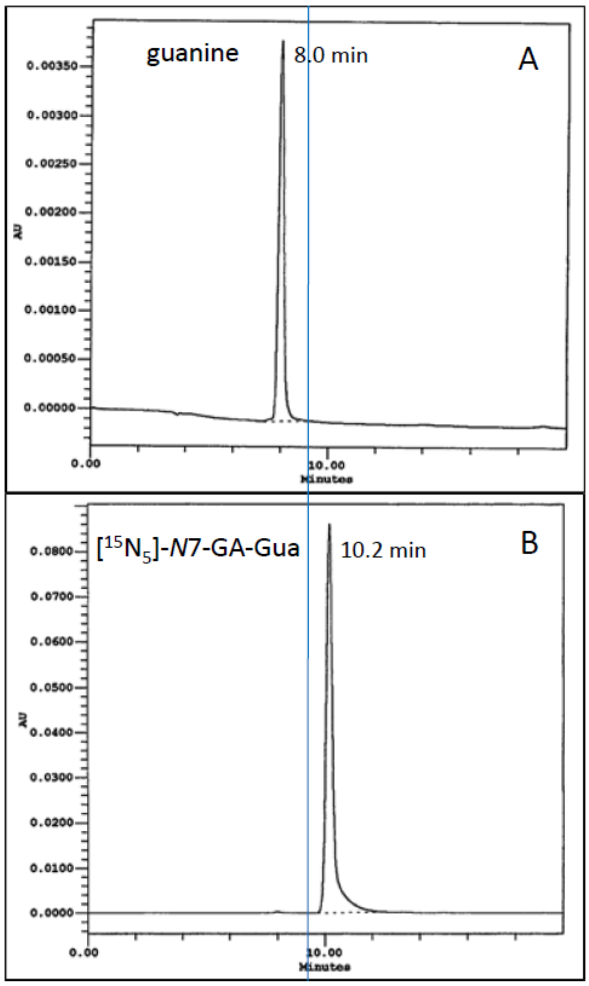


Figure 2.8: Analysis of a guanine standard solution (A) onto the HPLC UV purification system with a retention time of 8.0 min. B shows an injection of the IS [$^{15}N_5$]-*N*7-GA-Gua solution onto the HPLC system. The retention time for the IS peak was 10.2 min. The blue line shows sufficient separation time between the *N*7-GA-Gua adduct and the guanine peak.

For each purified sample run, a chromatographic window of 3 – 4 min was collected for the fraction that contained the adduct. Analysis by LC-MS confirmed if the adduct was present.

The inclusion of the HPLC purification step reduced the effect of any artefactual responses in the *m/z* 239 to 152 channel. There were still impurities detectable in this channel but were resolved from the *N*7-GA-Gua adduct. The area of the IS [$^{15}N_5$]-*N*7-GA-Gua increased by this HPLC purification step due to less signal suppression as shown in a typical chromatogram in Figure 2.9. The chromatograms shown in Figure 2.9 also show that the *N*7-GA-Gua background level of CT DNA is not easily discernible in the sample that was only hydrolysed whereas it is resolved in the HPLC purified sample set.

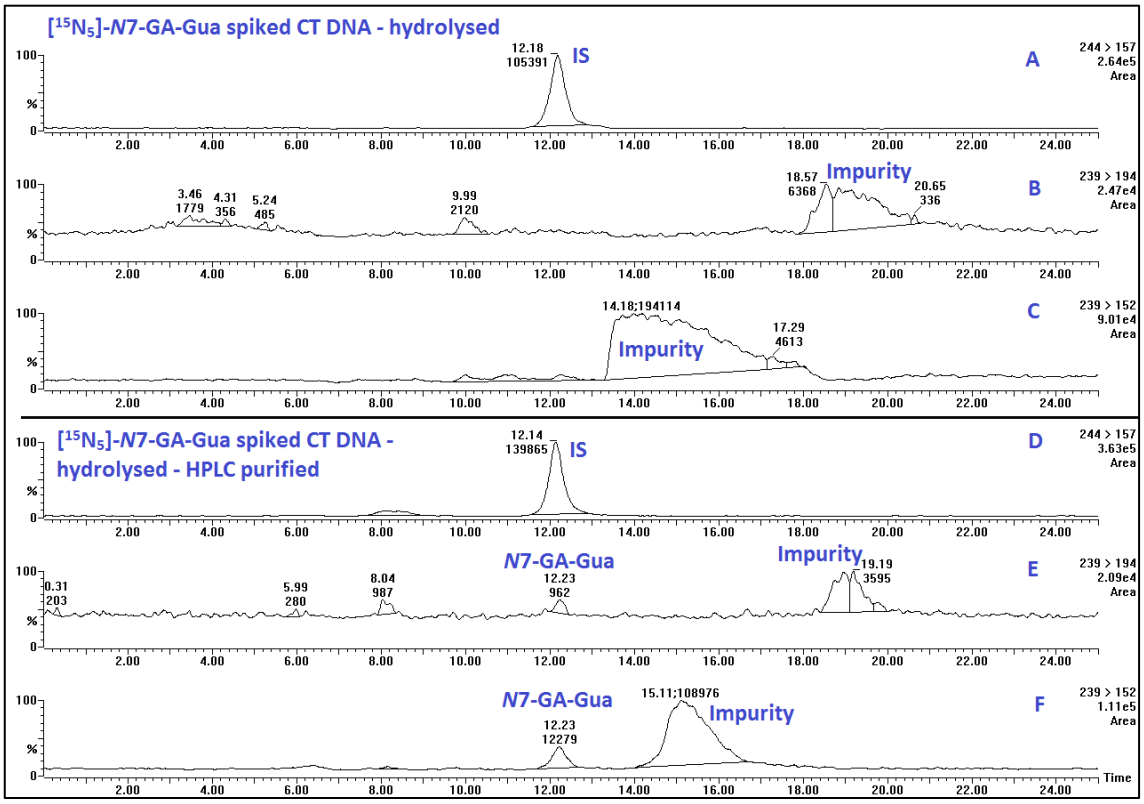


Figure 2.9: LC-MS/MS SRM analysis of the effect of HPLC purification after the hydrolysis procedure. Comparison between typical LC-MS/MS SRM chromatograms of [$^{15}N_5$]-*N*7-GA-Gua spiked CT DNA that was subjected to the hydrolysis procedure (A - C) and [$^{15}N_5$]-*N*7-GA-Gua spiked CT DNA that was hydrolysed and HPLC purified before analysed by LC-MS (D – F). LC-MS/MS SRM chromatograms A and D show the IS monitoring the fragmentation of *m/z* 244 to 157, LC-MS/MS SRM chromatograms B and E show the transition for the *N*7-GA-Gua adduct fragmentation *m/z* 239 to 194 and LC-MS/MS SRM chromatograms C and F showing the fragmentation of *m/z* 239 to 152 for the *N*7-GA-Gua adduct.

2.3.3 Development of LC-MS

Trap and analytical column

Table 2.10 presents the effects of different amounts of organics (5%, 2%, 1% MeOH and ACN) in the mobile phase (0.1% FA) on the retention time of the *N*7-GA-Gua adduct on the 50 x 3.0 mm and the 30 x 2.0 mm trap column.

Table 2.10: Effects of different organic modifier proportions on two different trap columns. The table shows the different retention times for the *N*7-GA-Gua adduct on the 50 x 3.0 mm and 30 x 2.0 mm trap columns with 0.1% FA and varying amounts of MeOH and ACN, respectively. Using the 50 x 3 mm column with 5% MeOH led to a retention time for *N*7-GA-Gua of 3.94 min, 2% MeOH led to 5.11 min and 1% MeOH led to 5.49 min). Using the 30 x 2.0 mm column with 5% ACN led to a retention time for the *N*7-GA-Gua adduct of 1.64 min, 2% ACN led to 1.90 min and 1% ACN led to 2.15 min)

50 x 3.0 mm trap column, 0.1% FA		**30 x 2.0 mm trap column, 0.1% FA**	
MeOH	**Retention time in min**	**ACN**	**Retention time in min**
5%	3.94	**5%**	1.64
2%	5.11	**2%**	1.90
1%	5.49	**1%**	2.15

Applying *N*7-GA-Gua on the 50 x 2.0 mm trap column and changing the organic modifier ACN from 1% to 2% and then 5% led to different retention times for the *N*7-GA-Gua adduct as shown in Figure 2.10.

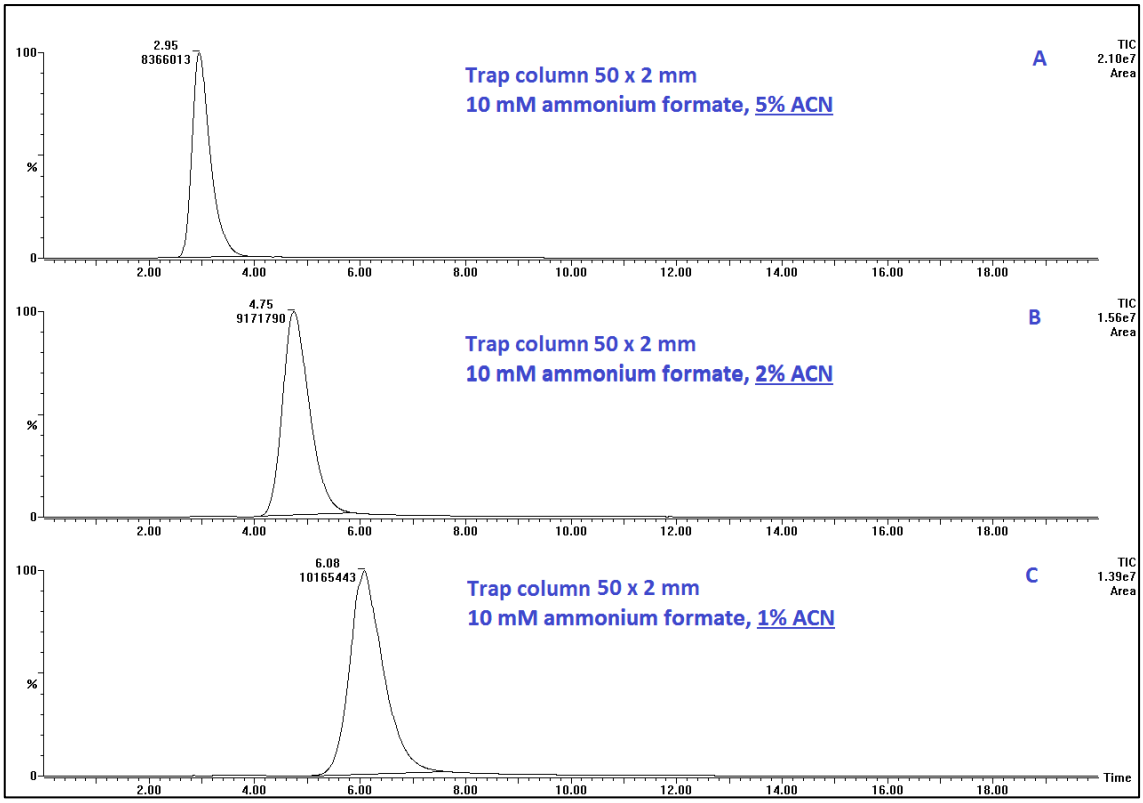


Figure 2.10: Shows the different retention times for the *N*7-GA-Gua adduct on the 50 x 2.0 mm trap column with 10 mM ammonium formate pH 5.4 and varying amounts of ACN. In LC-MS/MS chromatogram A 5% ACN was used (retention time of *N*7-GA-Gua was 2.95 min), 2% ACN in LC-MS/MS chromatogram B (retention time of *N*7-GA-Gua was 4.75) was used and 1% ACN in the run presented in LC-MS/MS chromatogram C (retention time of *N*7-GA-Gua was 6.08 min).

Figure 2.11 shows the chromatogram for applying both *N*7-GA-Gua and [$^{15}N_5$]-*N*7-GA-Gua on a 50 x 2.0 mm trap column using 1:99 MeOH/0.1% FA (*v/v*).

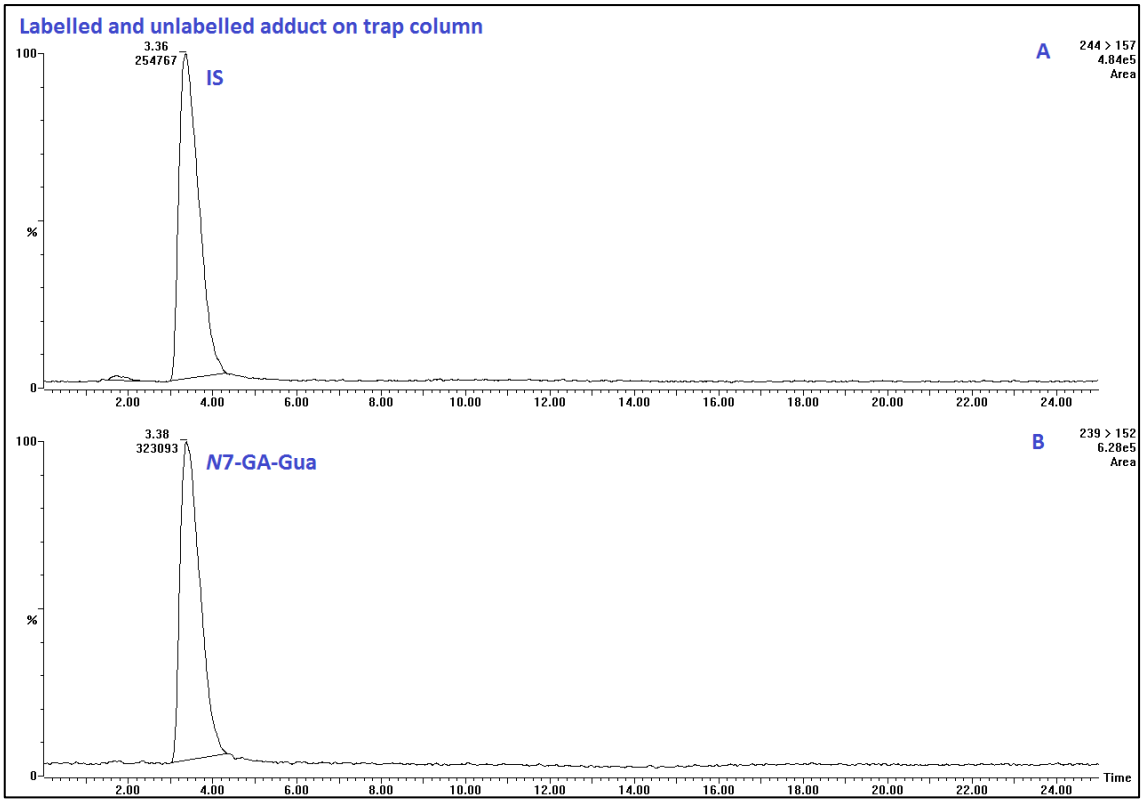


Figure 2.11: The LC-MS/MS SRM chromatogram for labelled and unlabelled *N*7-GA-Gua standard separated on the 50 x 2.0 mm trap column only. The retention time for the labelled [$^{15}N_5$]-*N*7-GA-Gua adduct is 3.36 min (LC-MS/MS SRM chromatogram A) and for the *N*7-GA-Gua adduct (LC-MS/MS SRM chromatogram B) 3.38 min. The mobile phase consisted of 99:1 0.1% FA/MeOH (*v/v*).

The switching system was advantageous because following elution from the trap to the analytical column the trap column was washed and then re-equilibrated off-line during the remainder of the run. The gradient for washing and re-equilibrating the trap column is shown in Table 2.11.

Table 2.11: Gradient serving the trap column to wash off any remaining impurities before injection of the next sample

Min	A (%)	B (%)
0.0	99	1
4.5	99	1
4.6	0	100
10.0	0	100
10.1	99	1
25.0	99	1

The gradient washing and equilibrating the analytical column is described in Table 2.12.

Table 2.12: Gradient for the analytical column to wash off any remaining impurities before injection of the next sample

Min	A (%)	B (%)
0.0	95	5
15.0	95	5
15.1	25	75
20.0	25	75
20.1	95	5
40.0	95	5

For the final method a 50 x 2.0 mm trap column and a 250 x 2.0 mm analytical column was chosen.

Mobile phase

Using 10 mM ammonium formate led to a decrease of signal over time possibly caused by ions coating the source and ion tunnel and leading to a drop in sensitivity. This effect did not occur when applying 0.1% FA which therefore was chosen for the method.

Table 2.13 shows the retention times of the *N*7-GA-Gua adduct with varying amounts of organic modifier in the mobile phase serving the auxiliary pump and the analytical column.

Table 2.13: Impact of different percentages of organic modifier (MeOH) in the mobile phase on the retention time of the *N*7-GA-Gua adduct

% Organics	Retention times in min
5	12.37
7.5	11.09
10	10.37
12.5	9.8
15	9.5

The chosen mobile phase consisted of 99:1 0.1% FA/MeOH (*v/v*) for pump A (trap column) and 95:5 0.1% FA/MeOH (*v/v*) for the auxiliary pump (analytical column).

Switching valve timings

The LC-MS/MS total ion count chromatograms shown in Figure 2.12 show the results for determining the optimum time settings to change the switching valve positions. The retention time of the *N*7-GA-Gua adduct decreased from 21.62 min to 12.48 min. An increase in the peak area for the adduct was observed by adjusting the switching valve time settings.

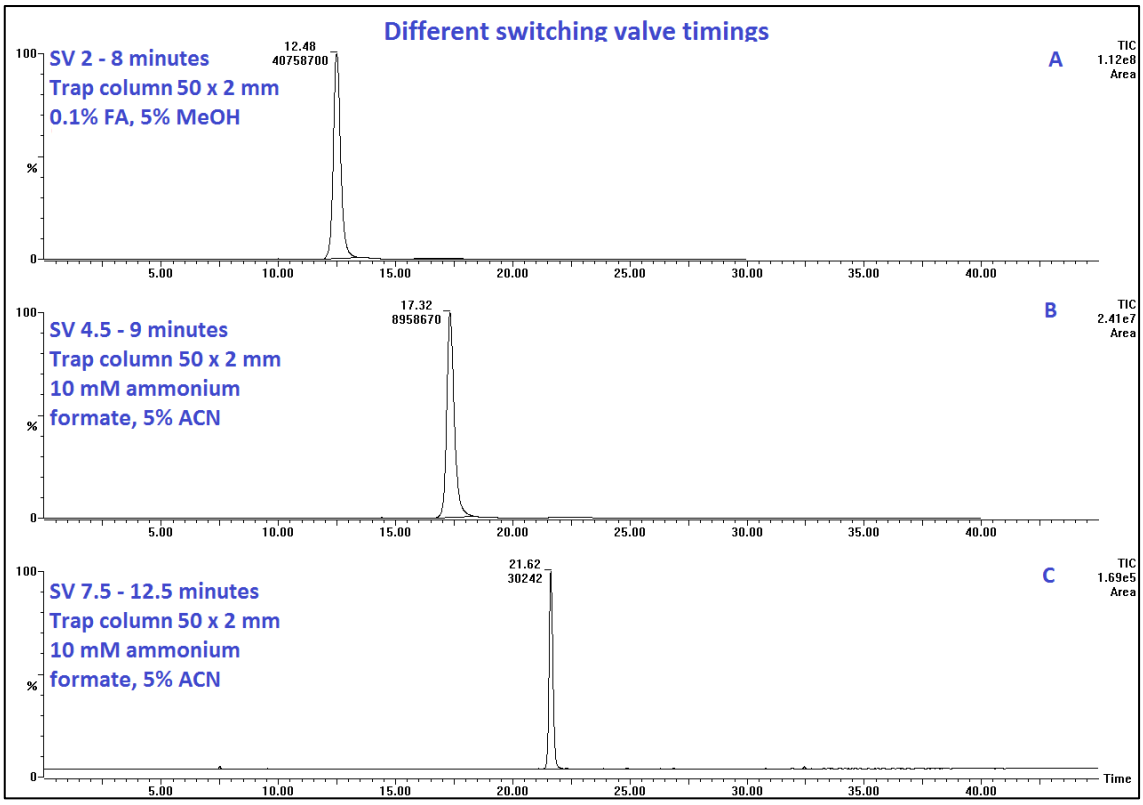


Figure 2.12: The effect of different timings for the switching valve positions. In chromatogram A the switching valve was set to 2 and 8 min, the retention time of the *N*7-GA-Gua adduct was 12.48 min and the total run time was 30 min. In chromatogram B the switching valve changed positions on 4.5 and 9 min, the *N*7-GA-Gua adduct had a retention time of 17.32 min in a total run time of 40 min. In chromatogram C the first column-switching was set up with 7.5 – 12.5 min, the retention time of the *N*7-GA-Gua adduct was 21.62 min in a total run time of 50 min. For the runs presented in chromatograms B and C the mobile phase consisted of 10 mM ammonium formate pH 5.4 and 5% ACN for the auxiliary pump. The run presented in chromatogram A was analysed using 0.1% FA and 5% MeOH. For all runs the *N*7-GA-Gua adduct to give 100 pmol on column was applied.

The valve switched from position 1 (analyte gets loaded onto the trap column while the impurities go to waste) to position 2 (analyte gets back flushed off the trap column

onto the analytical column) after 2 min (Figure 1.9). After 8 min the switching valve turned back to position 1. The total run time was 25 min. For the majority of experiments in the method development chapter these timings were used but altered when impurities made it unclear if the adduct was present or absent in an analysis. These alterations are mentioned if used. The switching valve was switched back to position 2 at 4.5 min instead of 8 and the total run time was 25 min. An adaption of the method meant that a gradient on the auxiliary pump was applied and the switching valve timings from 2 and 4.5 min were kept but the total run time increased to 40 min.

Run time

Within the process of the method development the total run time was changed a few times to assure the best chromatographic separation possible whilst balancing the requirements of throughput. The total run time was optimised at 40 min.

SRM selections

Figure 2.13 shows possible positions where positive ionisation ESI MS/MS CID fragmentation of the *N*7-GA-Gua adduct could occur overlaid with a LC-MS/MS spectrum of a *N*7-GA-Gua tuning standard injection. The major fragmentation transition is *m/z* 239 to 152.

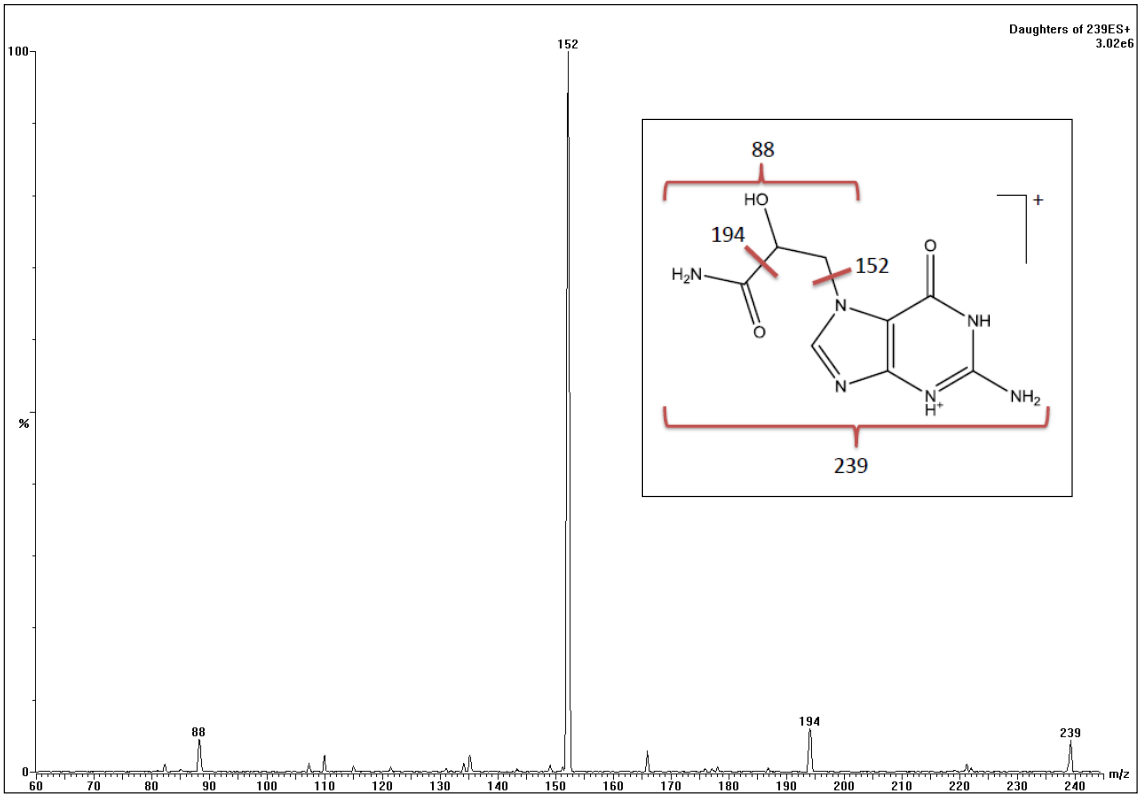


Figure 2.13: Possible sites in the molecule for fragmentation of the *N*7-GA-Gua adduct on the mass spectrometer. The LC-MS/MS spectrum shows peaks for the fragmentation products with *m/z* 88, *m/z* 152 and *m/z* 194 and the un-fragmentised adduct ion with *m/z* 239 can also be observed.

The fragmentation transition *m/z* 239 to 88 was monitored but dismissed due to background interferences when biological matrix samples were analysed (shown in Figure 2.14).

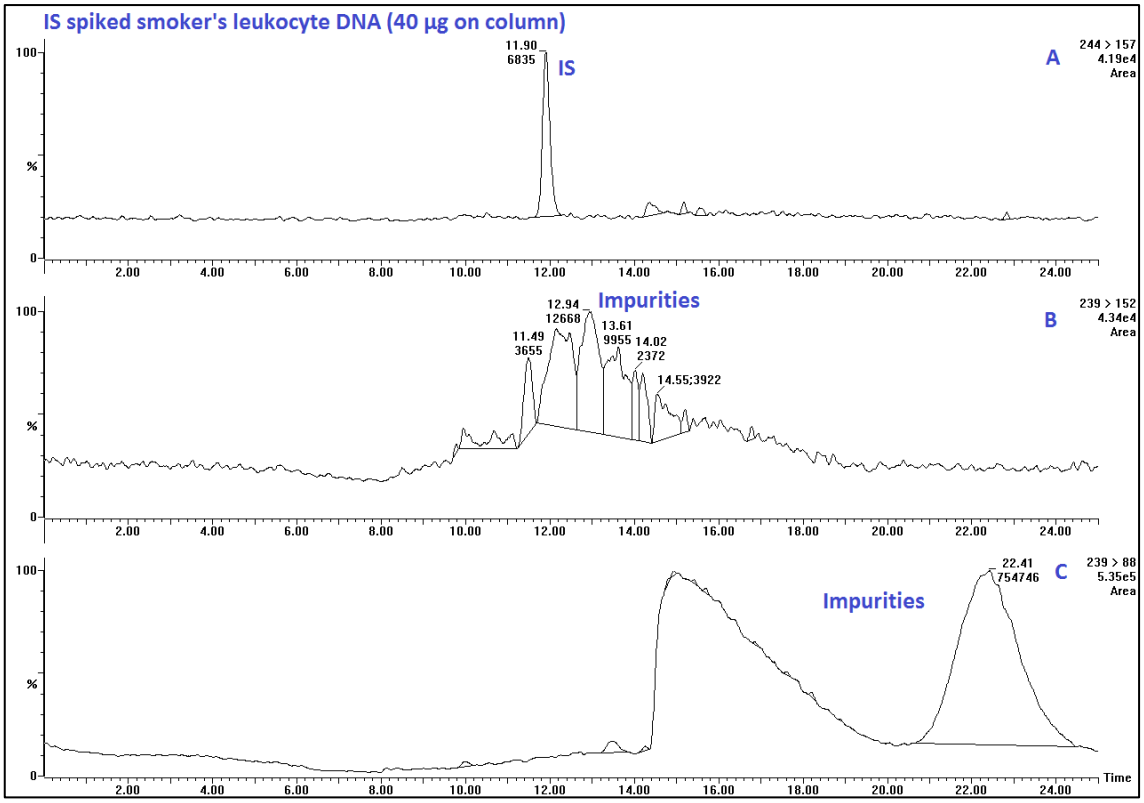


Figure 2.14: LC-MS/MS SRM chromatograms for IS spiked smoker's leukocyte DNA sample, 40 µg DNA on column with SRM transitions monitoring the fragmentation of *m/z* 244 to 157 for the [$^{15}N_5$]-*N*7-GA-Gua adduct (A), *m/z* 239 to 152 for the *N*7-GA-Gua adduct (B) and *m/z* 239 to 88 also for the *N*7-GA-Gua adduct (C).

The small mass of the GA fragment led to difficulties for its detection which was most probably due to solvent and salt ions being eluted within the same mass range and masked the GA detection. The fragmentation of the transition *m/z* 239 to 194 was determined in a separate experiment and showed less impurities in the chromatogram and therefore that channel was included in the analysis to increase the specificity of the detection of the *N*7-GA-Gua adduct (Figure 2.15).

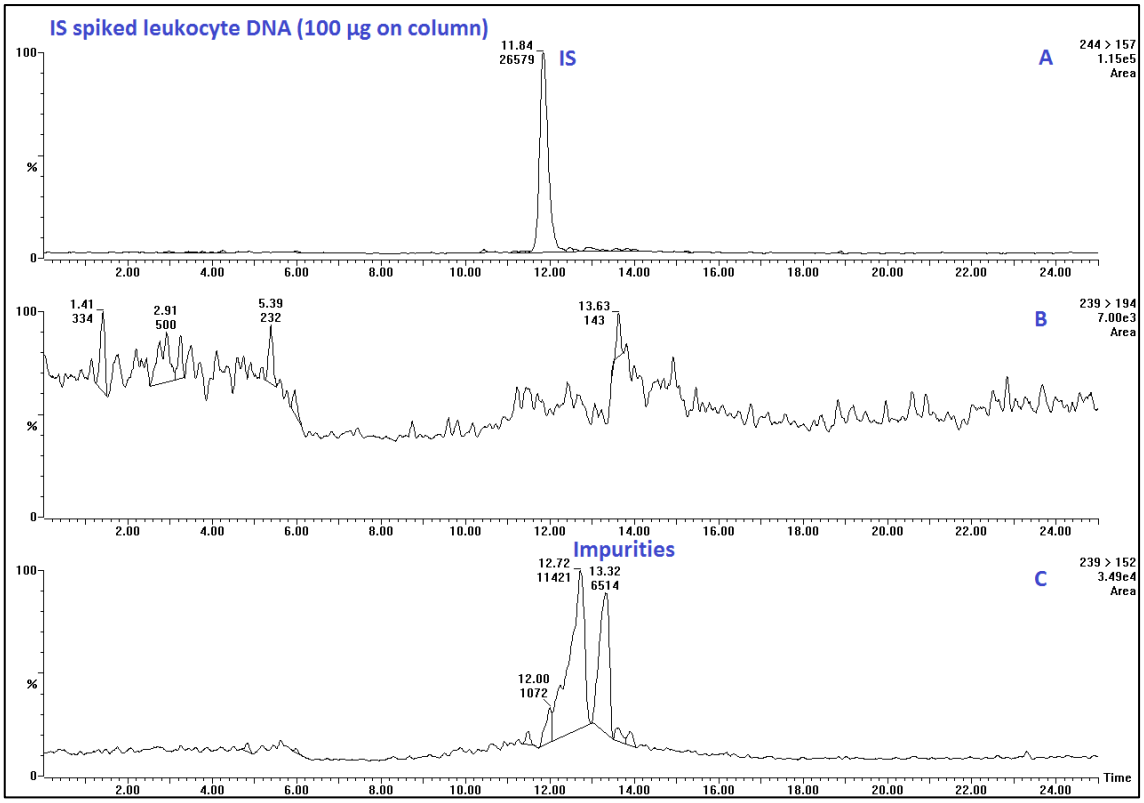


Figure 2.15: LC-MS/MS SRM chromatograms for IS spiked leukocyte DNA (100 µg on column) with SRM transitions monitoring the fragmentation of *m/z* 244 to 157 for the [$^{15}N_5$]-*N*7-GA-Gua adduct (A), *m/z* 239 to 194 for the *N*7-GA-Gua adduct (B) and *m/z* 239 to 152 for the *N*7-GA-Gua adduct (C).

When analysing very low levels of *N*7-GA-Gua the transition *m/z* 239 to 194 may not be detected as the peak is approximately 10% of the area detected for the transition *m/z* 239 to 152.

2.3.4 Biological sample analysis

2.3.4.1 Calibration line in pooled leukocyte DNA

Figure 2.16 shows a linear response for spiking varying amounts of the *N*7-GA-Gua adduct to extracted and pooled leukocyte DNA (duplicate), leading to a R^2 value of 0.9728.

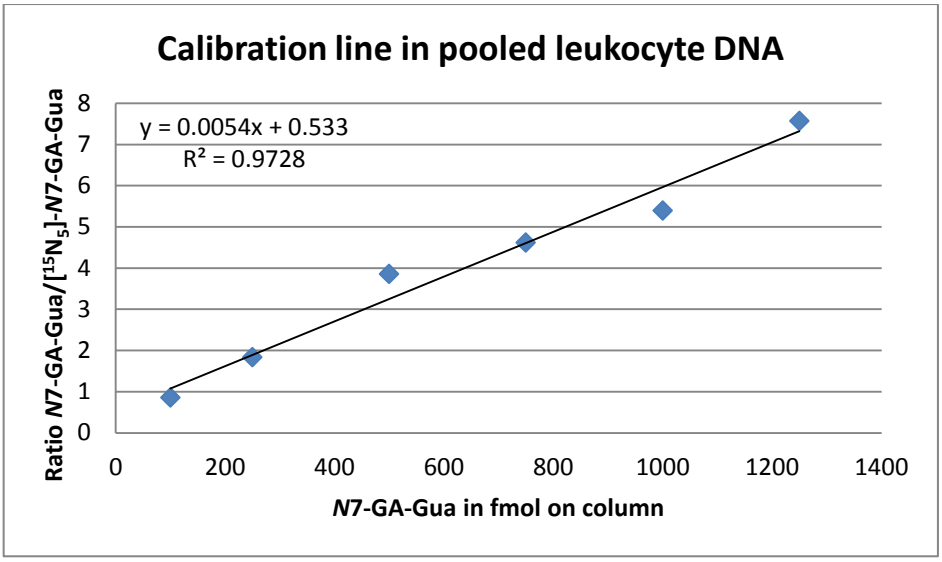


Figure 2.16: Calibration line obtained by spiking different amounts of *N*7-GA-Gua (from 100 to 1250 fmol on column) into pooled human leukocyte DNA (133.33 µg giving 100 µg on column). Each sample was spiked with 4.2 µL [$^{15}N_5$]-*N*7-GA-Gua solution (0.15 pmol/µL) to give 477 fmol on column in a 15 µL injection.

2.3.4.2 Glycidamide treatment of HCEC cells and whole blood

After 24 hrs there was a dose-response in the *N*7-GA-Gua adduct levels to be seen both in the cell experiment (Figure 2.17) and in the GA treated whole blood (6 hrs) (Figure 2.18).

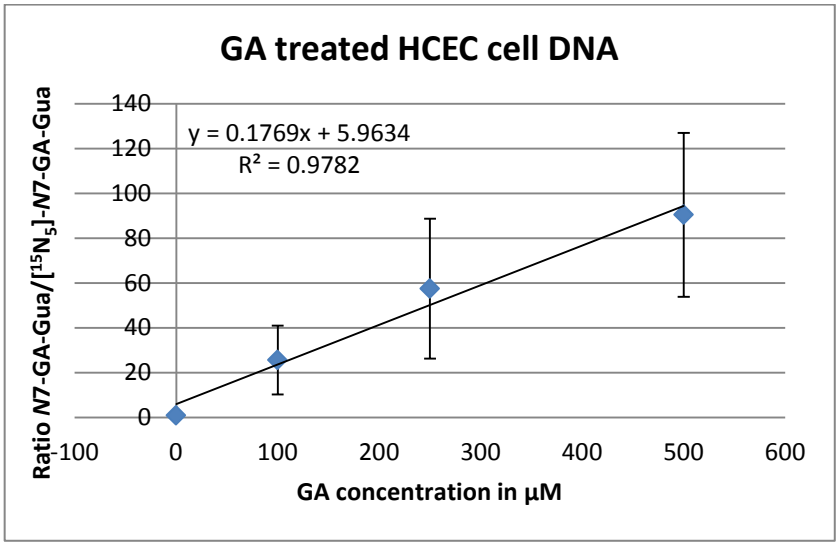


Figure 2.17: Dose response following the treatment of HCEC cell DNA with 100, 250 and 500 µM of GA for 24 hrs at 37°C.

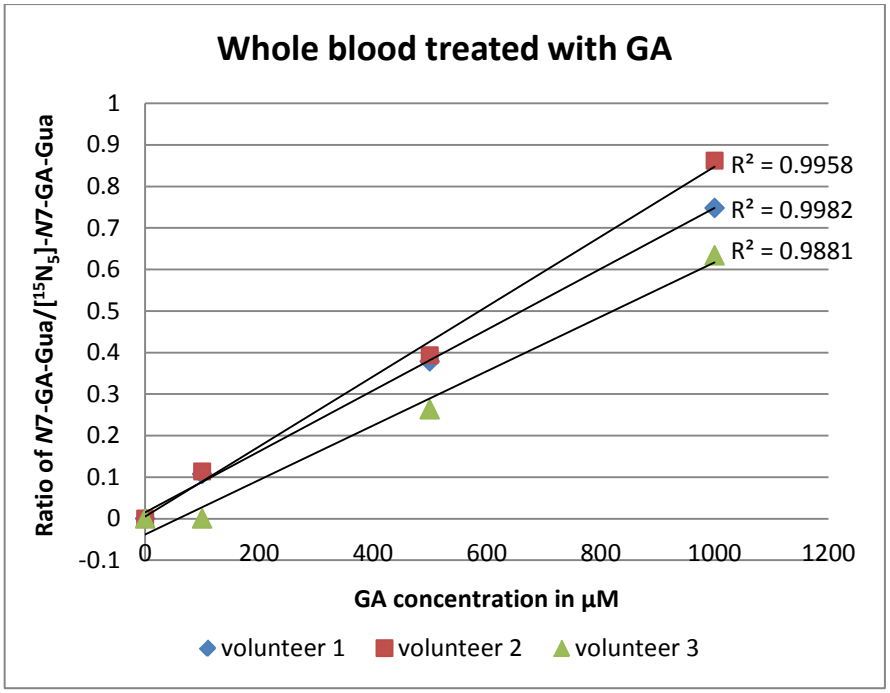


Figure 2.18: Dose response following the treatment of whole blood with 100, 500 and 1000 µM of GA for 6 hrs at 4°C.

The GA treatment for 6 hrs showed a linear dose-response for two samples tested. In one sample only the 500 and 1000 µM led to a *N*7-GA-Gua adduct peak but none for the 100 µM treatment. The amount of DNA from whole blood seemed to decrease during treatment and the remaining DNA after extraction (unlike the usual 100 µg on column) was used completely for the analysis.

2.3.4.3 Recovery of *N*7-GA-Gua

In the early stages of method development the *N*7-GA-Gua adduct recovery was analysed in three different matrices, i.e. CT DNA, human leukocyte DNA and HCEC cells DNA. The recoveries for the ratio (*N*7-GA-Gua peak area/[$^{15}N_5$]-*N*7-GA-Gua peak area) were 86% in CT DNA, 89% in HCEC cells DNA and 49% in leukocyte DNA. The raw recoveries for just the *N*7-GA-Gua and the [$^{15}N_5$]-*N*7-GA-Gua peak areas for all three matrices were lower and ranged between 26% - 46% and 29% - 66%, respectively (Figure 2.19).

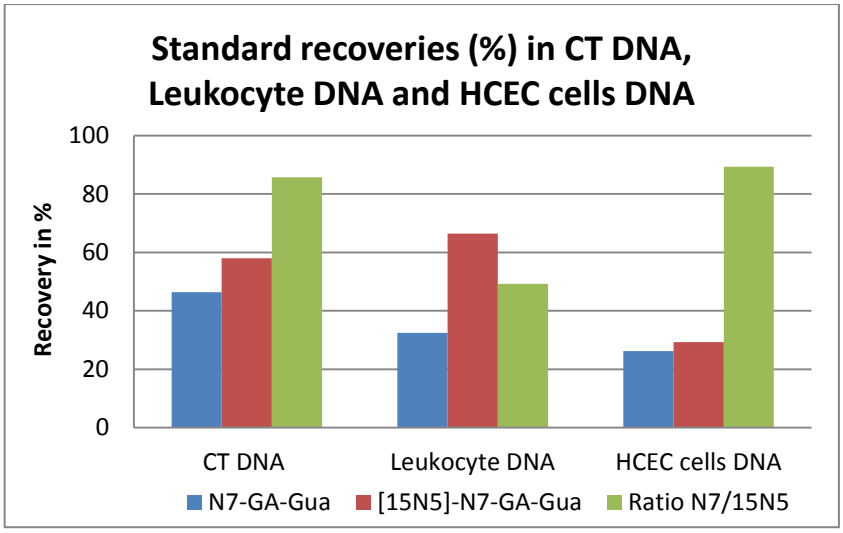


Figure 2.19: Recoveries for the *N*7-GA-Gua adduct in CT DNA, leukocyte DNA and HCEC cells DNA.

2.3.4.4 Different amounts of leukocyte DNA and the "concentrating effect"

Smoker's leukocyte DNA

A *N*7-GA-Gua peak could be detected in the "remaining solution" that was left in the tube after aliquoting all samples from a smoker's leukocyte DNA. There were indications that the adduct was present in the other runs but interfering peaks in the channel *m/z* 239 to 152 (Figure 2.20) made a conclusion difficult. It appeared that with increasing amounts of DNA the peak area for the [$^{15}N_5$]-*N*7-GA-Gua standard decreased maybe caused by the presence of biological matrix.

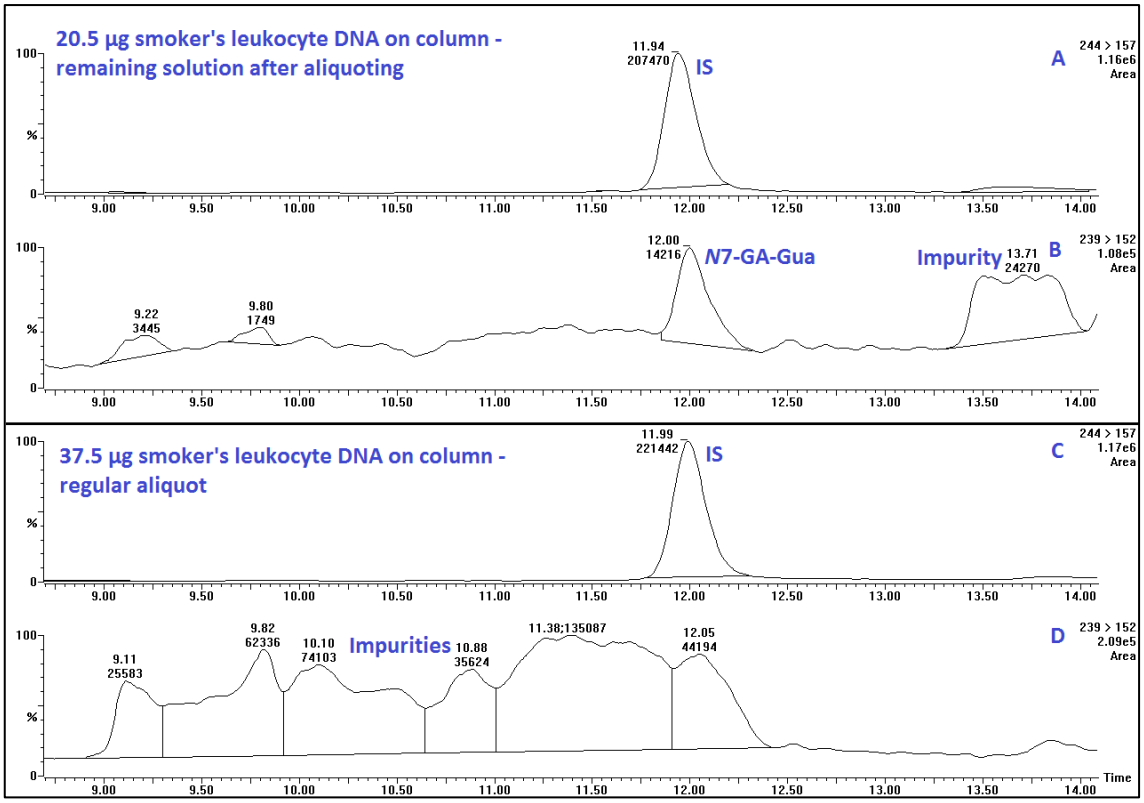


Figure 2.20: The LC-MS/MS SRM chromatograms show the IS [$^{15}N_5$]-*N*7-GA-Gua peak (A) as well as a peak for the *N*7-GA-Gua adduct (B) in the smoker's leukocyte DNA injection that was made up from the "remaining solution" (equals 20.5 µg DNA on column which equals 82 adducts/10^8 nucleotides) after aliquoting DNA solution to inject different DNA amounts on column. The LC-MS/MS SRM chromatograms C and D show the same smoker's leukocyte DNA for a 37.5 µg DNA on column injection. The LC-MS/MS SRM chromatogram C shows the IS [$^{15}N_5$]-*N*7-GA-Gua peak. The impurity peaks in LC-MS/MS chromatogram D monitoring the transition *m/z* 239 to 152 for the *N*7-GA-Gua adduct made the detection of the adduct infeasible.

Non-smoker's leukocyte DNA

Pipetting aliquots giving 100 µg non-smoker's DNA on column and analysing the "remaining solution" of 45.7 µg DNA on column injection showed a small peak at the retention time where *N*7-GA-Gua eluted (Figure 2.21) which equals 52 adducts/10^8 nucleotides.

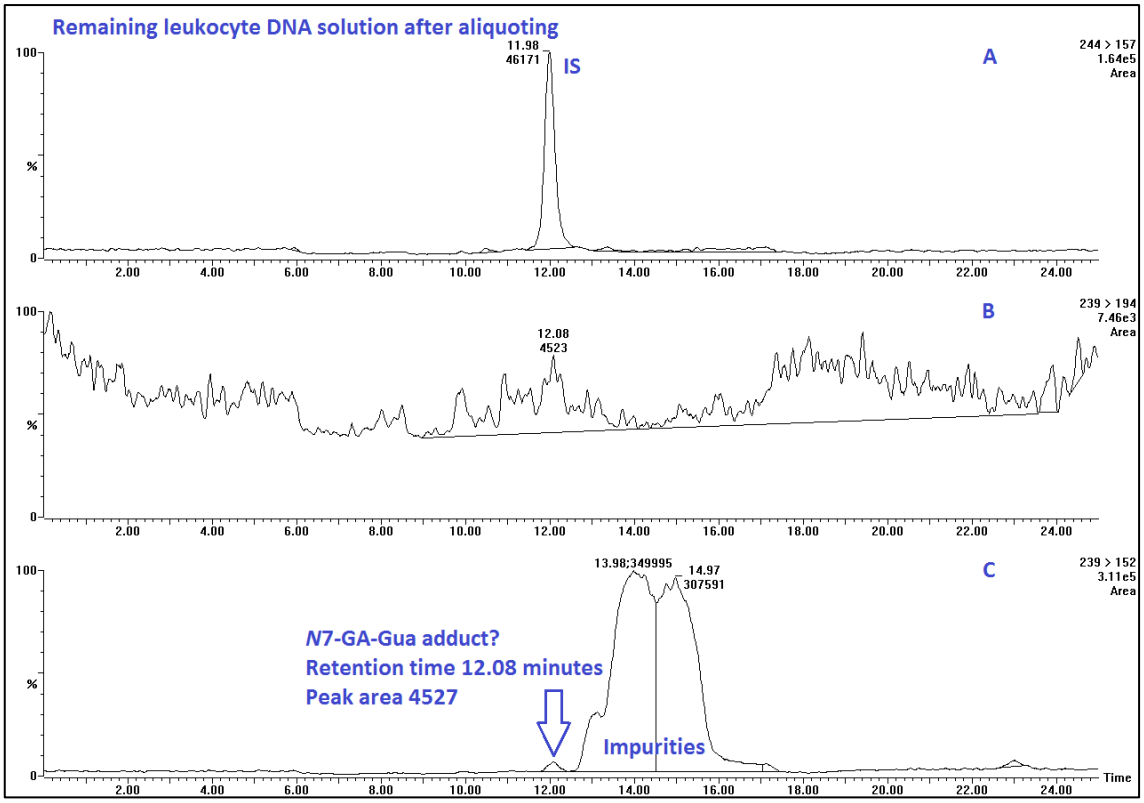


Figure 2.21: LC-MS/MS SRM chromatogram of the "remaining solution" of non-smoker's leukocyte DNA (equals 45.7 µg DNA on column) after aliquoting 100 µg on column. LC-MS/MS SRM chromatogram A monitored the fragmentation of *m/z* 244 to 157 for the [$^{15}N_5$]-*N*7-GA-Gua adduct, *m/z* 239 to 194 for the *N*7-GA-Gua adduct was monitored in LC-MS/MS SRM chromatogram B and *m/z* 239 to 152 for the *N*7-GA-Gua adduct is shown in LC-MS/MS SRM chromatogram C. A peak (equals 52 adducts/10^8 nucleotides) was observed at the retention time consistent for the *N*7-GA-Gua adduct (chromatogram C).

The 100 µg on column DNA samples showed evidence that the *N*7-GA-Gua adduct was present in this person's blood sample. With the exception of one sample (out of 7) where no peak could be detected all the remaining six showed *N*7-GA-Gua ranging from peak area 377 up to 9706 (shown in Figure 2.22) which for the first equals 3 adducts/10^8 nucleotides and for the latter equals 72 adducts/10^8 nucleotides.

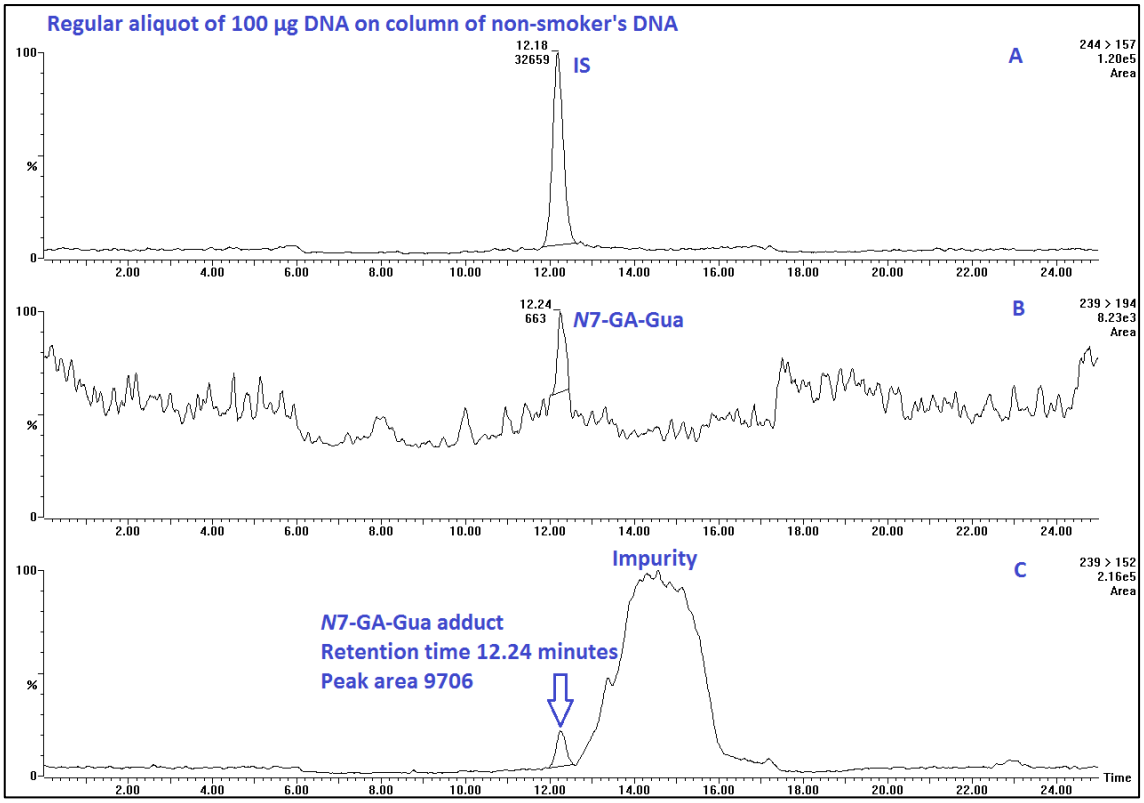


Figure 2.22: LC-MS/MS SRM chromatograms of an aliquot (133.33 µg DNA giving 100 µg DNA on column) of non-smoker's DNA for testing the "concentrating effect". LC-MS/MS SRM chromatogram A shows the peak for the IS. LC-MS/MS SRM chromatogram B shows the fragmentation reaction *m/z* 239 to 194 for the *N*7-GA-Gua adduct. There was a peak in the transition *m/z* 239 to 152 (LC-MS/MS SRM chromatogram C) with a retention time that was consistent with the *N*7-GA-Gua adduct.

Assessment of storage volume

This experiment aimed to test whether this "concentrating effect" can be seen when using leukocyte DNA aliquots stored in smaller volumes than 5 mL by taking a 1 mL, 1.5 mL and 2 mL leukocyte DNA solution. The "concentrating effect" could not be detected on these samples (Figure 2.23).

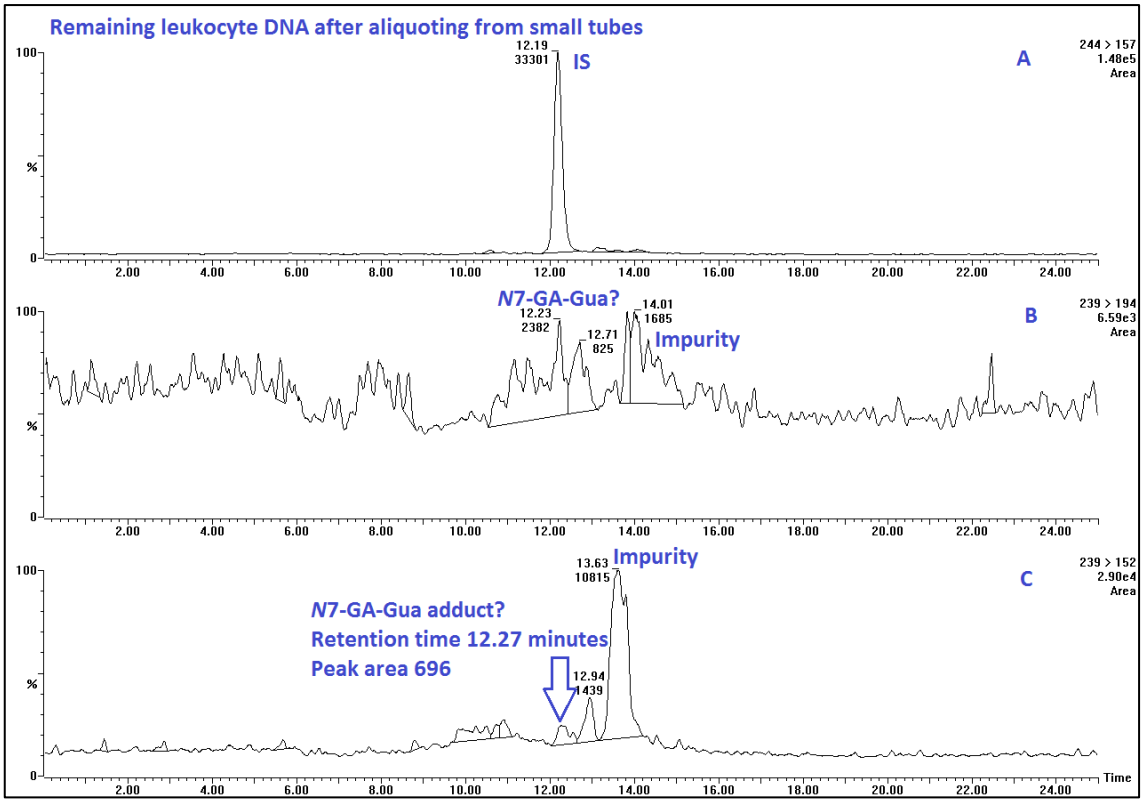


Figure 2.23: LC-MS/MS SRM chromatogram of the "remaining solution" after aliquoting from small volumes. LC-MS/MS SRM chromatogram A shows the transition monitoring the fragmentation of *m/z* 244 to 157 for the IS [$^{15}N_5$]-*N*7-GA-Gua, *m/z* 239 to 194 for the *N*7-GA-Gua adduct (LC-MS/MS chromatogram B) and *m/z* 239 to 152 for the *N*7-GA-Gua adduct (LC-MS/MS chromatogram C). There was a peak at the retention time of the *N*7-GA-Gua adduct (LC-MS/MS SRM chromatogram C) but due to impurities in the same transition it was difficult to confidently determine its presence.

The channel *m/z* 239 to 152 showed interfering peaks that make it difficult to confirm the presence of the *N*7-GA-Gua adduct.

2.3.5 Matrix effect on different DNA quantities

Figure 2.24 shows that taking different CT DNA amounts on column spiked with labelled and unlabelled adduct did not seem to have a significant influence on the signal response of both standards.

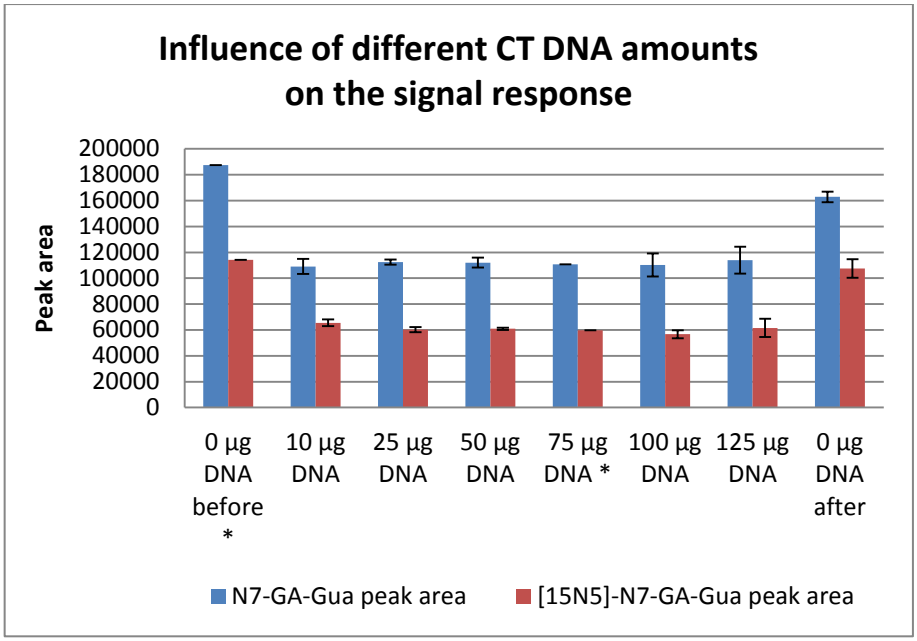


Figure 2.24: Influence of different CT DNA amounts on column on the signal response of the *N*7-GA-Gua and [$^{15}N_5$]-*N*7-GA-Gua adduct standard. * marked values are based on duplicate injections, all other samples were analysed in triplicate.

Peak areas of standard solutions analysed following the analysis of biological samples decreased compared to the standard solutions analysed before DNA samples were measured. This effect was seen on all mass spectrometric analysis days when biological samples were analysed. The signal response was smaller for the DNA samples compared to the standards (0 µg DNA) but different DNA amounts did not influence the response. In previous runs analysing leukocyte DNA no *N*7-GA-Gua adduct was detectable. Therefore, 100 µg of DNA on column was chosen as the optimum amount for biological samples to increase the chance of detecting the adduct.

2.3.6 Interfering peaks in the chromatogram

In order to improve separation of the *N*7-GA-Gua adducts from any interfering substances various aspects of the method were modified and assessed.

Switching valve

Adjusting to an earlier switching time to position 1 helped in getting less impurities washed from the trap column onto the analytical column. The final method used the switching valve times of 2 min and 4.5 min.

Qiagen procedure for CT DNA and ST DNA

Two different amounts of unspiked CT DNA and ST DNA were weighed and dissolved in buffer G2 and then processed using the Qiagen procedure as described in section 2.2.2.2.1. Applying two different amounts was used to test if the Qiagen columns gave different results when low and high DNA amounts were processed.

In both hydrolysed ST DNA and CT DNA samples a *N*7-GA-Gua adduct peak was detectable. The low amount ST DNA sample (Qiagen processed) showed an adduct peak in one run but not in the second run. In all three samples of the high amount ST DNA a *N*7-GA-Gua peak was detectable. All Qiagen processed CT DNA samples show peaks for the *N*7-GA-Gua adduct. The adduct peak appeared to be bigger in the Qiagen processed samples than in the hydrolysed samples.

The comparison between the chromatograms of the hydrolysed CT DNA and ST DNA and the Qiagen processed CT DNA and ST DNA does not favour one specific method over the other (typical chromatograms shown in Figure 2.25).

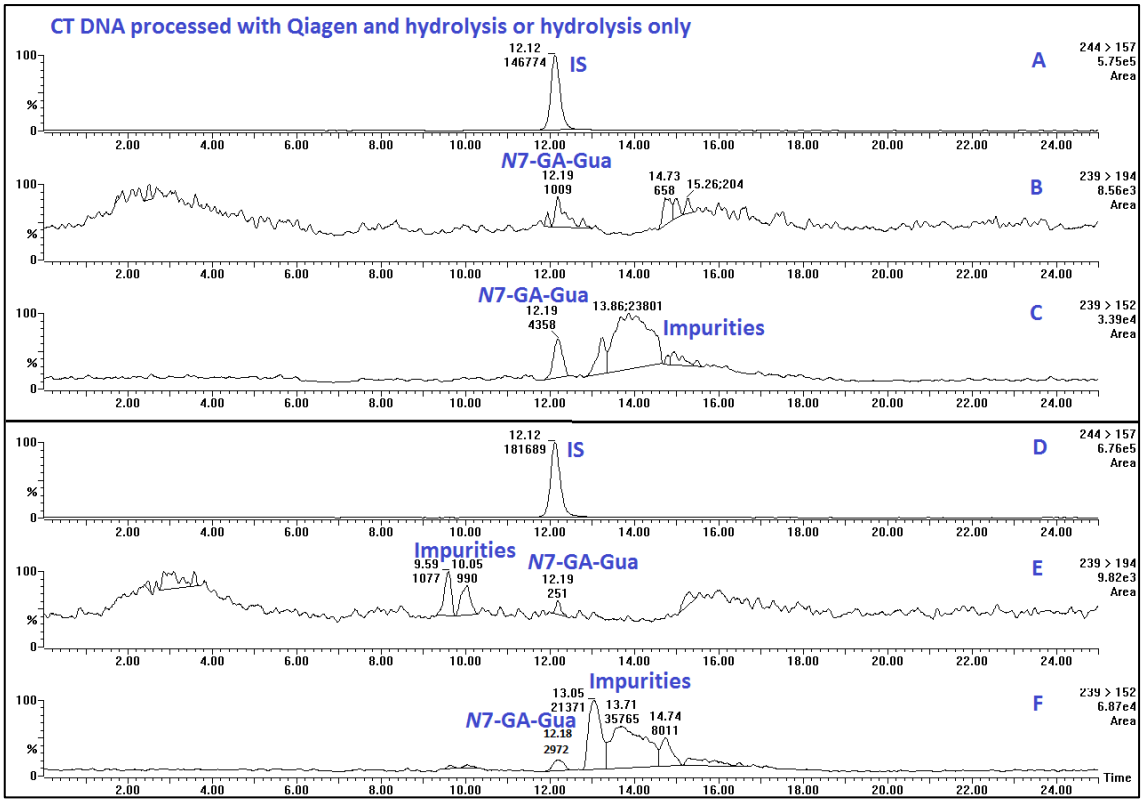


Figure 2.25: Comparison of typical LC-MS/MS SRM chromatograms for CT DNA subjected to the Qiagen process before hydrolysis (A – C) and CT DNA that was hydrolysed without being Qiagen processed (D – F). LC-MS/MS SRM chromatograms A and D monitored the fragmentation of *m/z* 244 to 157 for the [$^{15}N_5$]-*N*7-GA-Gua adduct, *m/z* 239 to 194 for the *N*7-GA-Gua adduct (B and E) and *m/z* 239 to 152 for the *N*7-GA-Gua adduct (C and F).

Amicon filters

Using ddH_2O, 0.1% FA and both labelled and unlabelled standards through the hydrolysis procedure without any added matrix the influence of the Amicon filters was tested. The impurity peaks seemed to be less in the sample where 0.1% FA (Figure 2.26) was hydrolysed compared to hydrolysed ddH_2O (Figure 2.27) and the unlabelled and labelled standards (Figure 2.28).

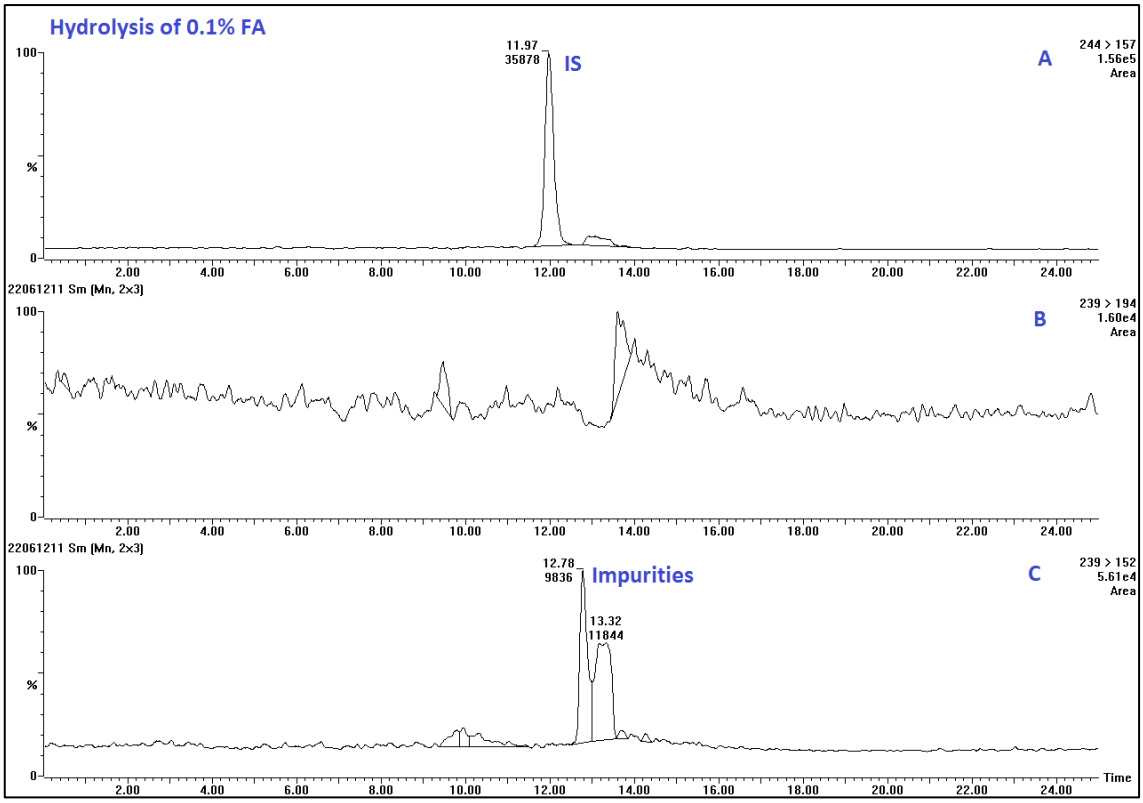


Figure 2.26: The LC-MS/MS SRM chromatograms show the result for the injection of 0.1% FA that underwent the hydrolysis process. LC-MS/MS SRM chromatogram A shows the IS peak. LC-MS/MS SRM chromatograms B monitored the fragmentation of *m/z* 239 to 194 for the *N*7-GA-Gua adduct and LC-MS/MS SRM chromatogram C shows the transition for *m/z* 239 to 152 for the *N*7-GA-Gua adduct.

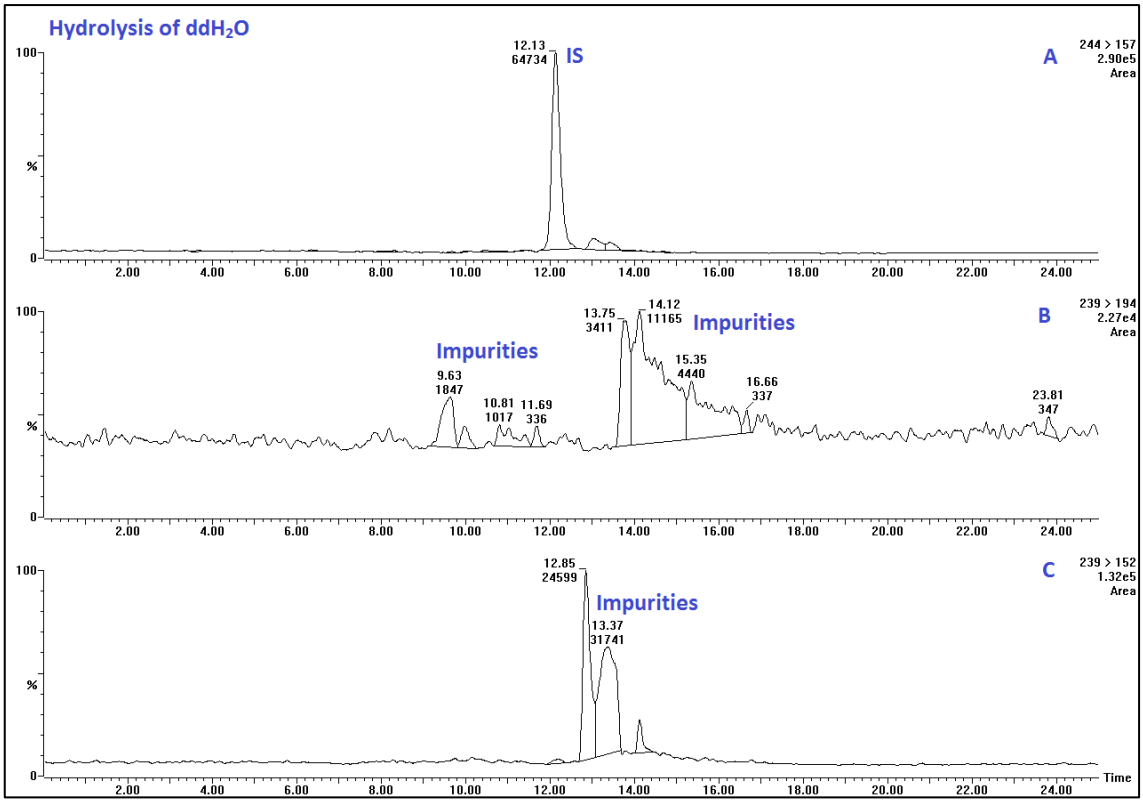


Figure 2.27: The LC-MS/MS SRM chromatograms show the result for the injection of ddH_2O that underwent the hydrolysis process. The LC-MS/MS SRM chromatogram A shows the IS peak. LC-MS/MS SRM chromatogram B monitored the fragmentation of *m/z* 239 to 194 for the *N*7-GA-Gua adduct and LC-MS/MS SRM chromatogram C shows the transition for *m/z* 239 to 152 for the detection of the *N*7-GA-Gua adduct.

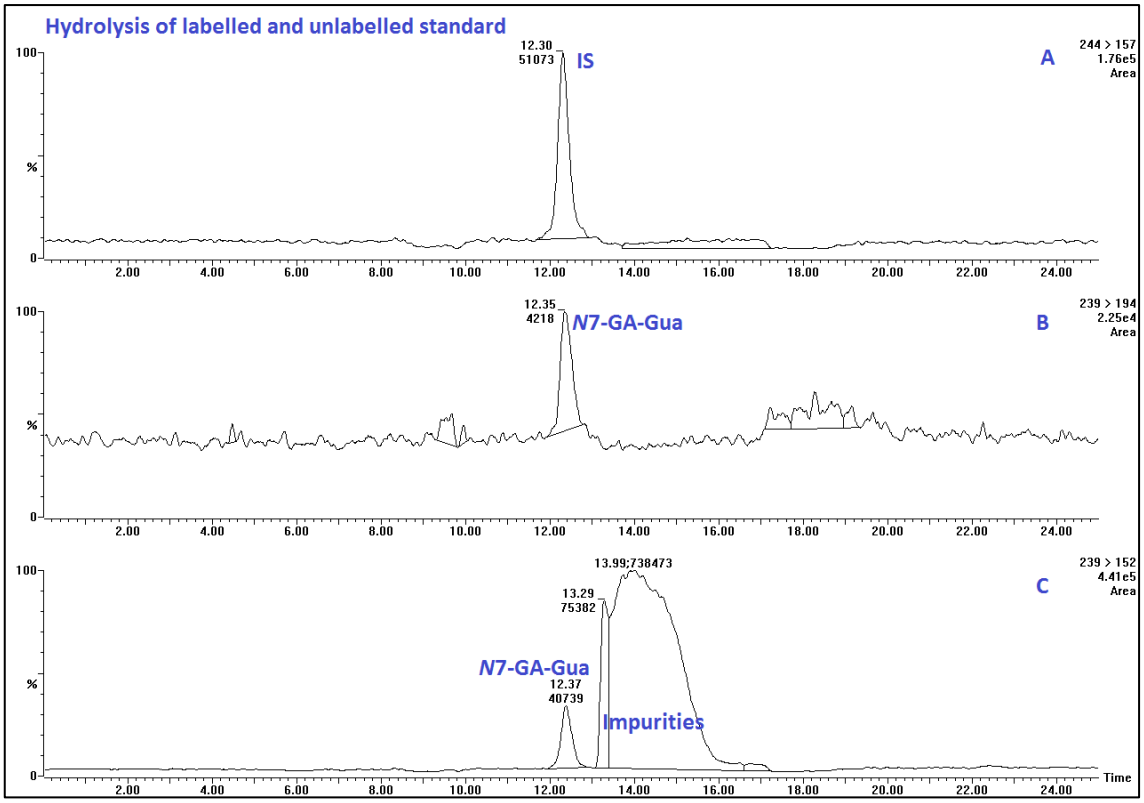


Figure 2.28: The LC-MS/MS SRM chromatograms show the result for the injection of labelled and unlabelled standards that underwent the hydrolysis process. The LC-MS/MS SRM chromatogram A shows the IS peak. LC-MS/MS SRM chromatogram B monitored the fragmentation of *m/z* 239 to 194 for the *N*7-GA-Gua adduct and LC-MS/MS SRM chromatogram C shows the transition for *m/z* 239 to 152 for the *N*7-GA-Gua adduct.

The difference in retention time allowed the resolution of the adduct peak from the impurity shown in Figure 2.28 for the transition *m/z* 239 to 152 but the artefact peak was considerable and therefore a reduction would be desirable.

The LC-MS/MS SRM chromatograms for Amicon 3 k vs. 10 k molecular weight cut-off filters (Figure 2.29) displayed a high degree of similarity to each other. The calculated Relative standard deviation (RSD) for the [$^{15}N_5$]-*N*7-GA-Gua adduct was 5.5% for the 3 k filters and 4.2% for the 10 k filters. The mean [$^{15}N_5$]-*N*7-GA-Gua peak area for the triplicate analysis done with the 3 k filters was lower than for the mean peak areas of the IS that underwent the processing by the 10 k filters. The impurity peaks in the *m/z* 239 to 152 channel might be sufficiently large to interfere with the *N*7-GA-Gua adduct (Figure 2.29) and thus may hinder the assessment of the LOD.

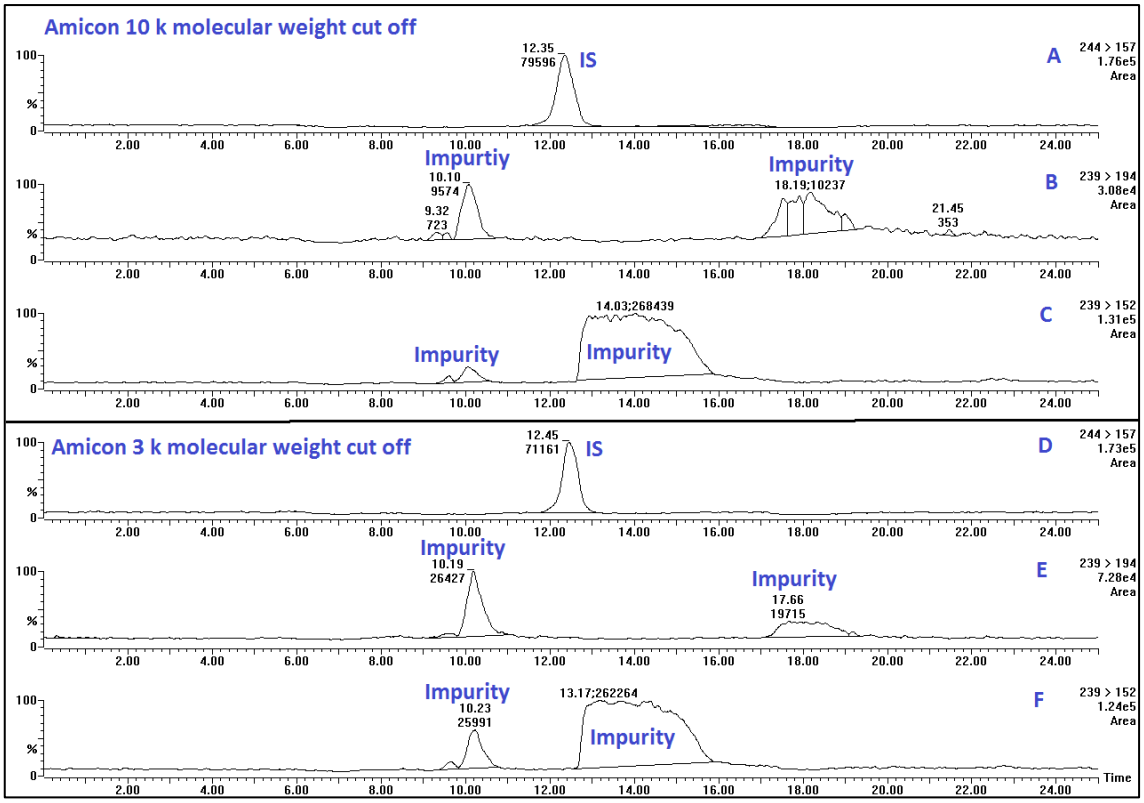


Figure 2.29: Comparison of using 10 k and 3 k Amicon molecular weight cut-off filters. The LC-MS/MS SRM chromatograms A – C were obtained by applying 10 k filters and the LC-MS/MS SRM chromatograms D – E were obtained by using 3 k filters. LC-MS/MS SRM chromatograms A and D show the IS peak. LC-MS/MS SRM chromatograms B and E monitored the fragmentation of *m/z* 239 to 194 for the *N*7-GA-Gua adduct and LC-MS/MS SRM chromatograms C and F show the transition for *m/z* 239 to 152 for the detection of the *N*7-GA-Gua adduct.

Equilibrating the Amicon filters for 20 min or 45 min did not interfere with the peak area of the spiked IS [$^{15}N_5$]-*N*7-GA-Gua or change the impurity peak detected in the channel monitoring *m/z* 239 to 152 (shown in Figure 2.30).

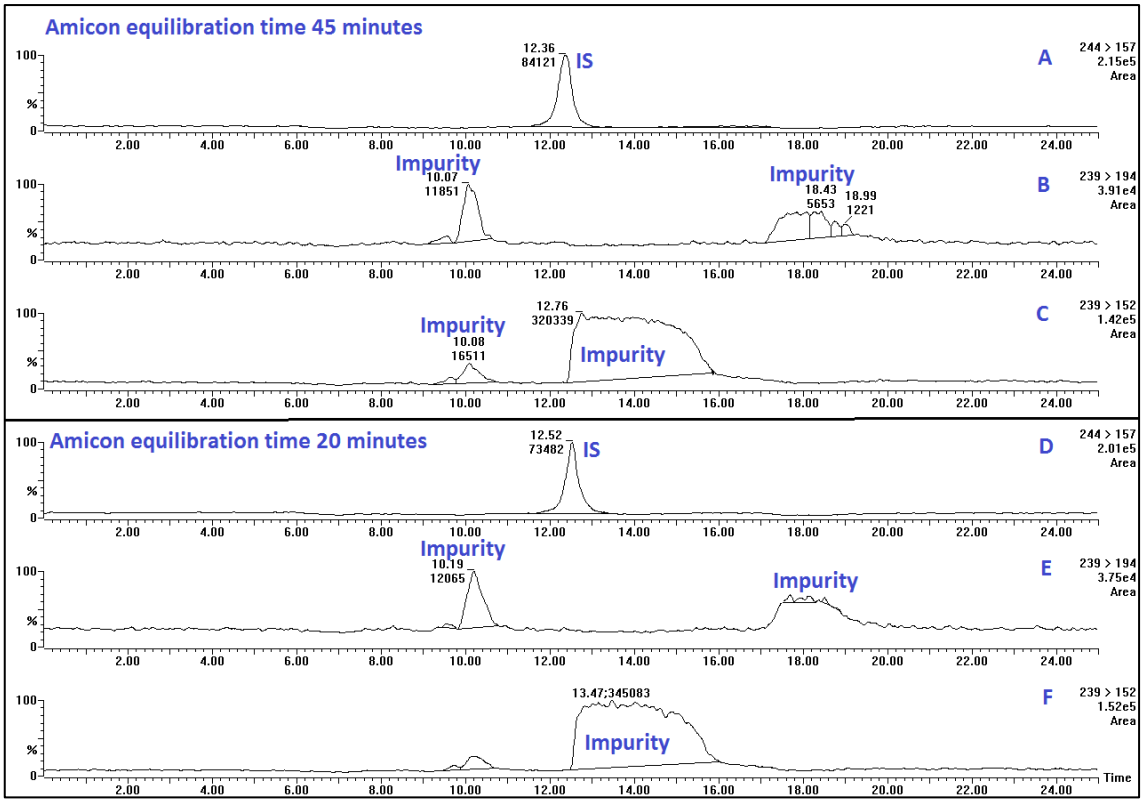


Figure 2.30: Different equilibration times for Amicon molecular weight cut-off filters. The LC-MS/MS SRM chromatograms A – C show the equilibration of the filters with 200 µL ddH_2O at 21°C at 13400*g* for 45 min and the LC-MS/MS SRM chromatograms D - F show the equilibration of the filters with 200 µL at 18°C at 16100*g* for 20 min. LC-MS/MS SRM chromatograms A and D show the IS peak. LC-MS/MS SRM chromatograms B and E monitored the fragmentation of *m/z* 239 to 194 for the *N*7-GA-Gua adduct and LC-MS/MS SRM chromatograms C and F show the transition for *m/z* 239 to 152 for the detection of the *N*7-GA-Gua adduct.

SPE for DNA samples

There was no discernible difference between the eluates for CT DNA and ST DNA that underwent SPE, and both failed to reveal a *N*7-GA-Gua peak. Table 2.14 informs about the losses of the IS within the SPE process. The biggest portion of the spiked [$^{15}N_5$]-*N*7-GA-Gua adduct was found in the washing fraction. In the loading solution, [$^{15}N_5$]-*N*7-GA-Gua adduct was also detectable which means that during loading some of the adduct was already lost. In the transition *m/z* 239 to 152 was an impurity peak at around the retention time 14 min but it should not interfere with the *N*7-GA-Gua adduct detection.

Table 2.14: Table shows the proportion of spiked IS [$^{15}N_5$]-*N*7-GA-Gua that remained after each step when the hydrolysed CT DNA and ST DNA samples were subject to the SPE process

	CT DNA - spiked before hydrolysis (%)	CT DNA - spiked before SPE (%)	ST DNA – spiked before hydrolysis (%)	ST DNA – spiked before SPE (%)
Eluate	11	15	15	16
Wash	80	72	76	78
Load	9	13	9	6

The spiked CT DNA samples were processed with the optimised method for Strata X columns for purification (Table 2.2). The eluate where the EtOH was not evaporated showed peaks below the LOD for *N*7-GA-Gua (peak area 1759) and [$^{15}N_5$]-*N*7-GA-Gua adduct (peak area 3818) detectable, also below LOD. No adduct was detected for the other eluate whereas the highest amounts of spiked adducts were lost in the first washing step and about half as much again within the second washing step.

Leukocyte DNA – Hydrolysis - SPE

Leukocyte DNA (duplicate) samples where a set underwent SPE (Strata X) was compared to a set of leukocyte DNA (duplicate) that was hydrolysed (no SPE). One sample of each set showed a peak for the *N*7-GA-Gua adduct. Both samples going through the SPE cartridges had smaller [$^{15}N_5$]-*N*7-GA-Gua peak areas compared to the duplicates that were hydrolysed but did not undergo SPE.

Low dose spiking of N7-GA-Gua – Hydrolysis vs. SPE

In the experiment where low doses of the *N*7-GA-Gua adduct were spiked into CT DNA and hydrolysed were compared to the same concentrations in CT DNA that went through the SPE procedure following hydrolysis.

The IS was observed at all concentrations analysed, but the analyte was absent in all analyses for the Strata X samples. The CT DNA that was hydrolysed showed both the *N*7-GA-Gua and [$^{15}N_5$]-*N*7-GA-Gua peaks. The *N*7-GA-Gua gave a linear dose response with a R^2 = 0.998. The signal-to-noise (S/N) ratio for the spiked amount of 94 fmol on

column was 5.6. The typical chromatograms for the amount of 94 fmol are shown in Figure 2.31.

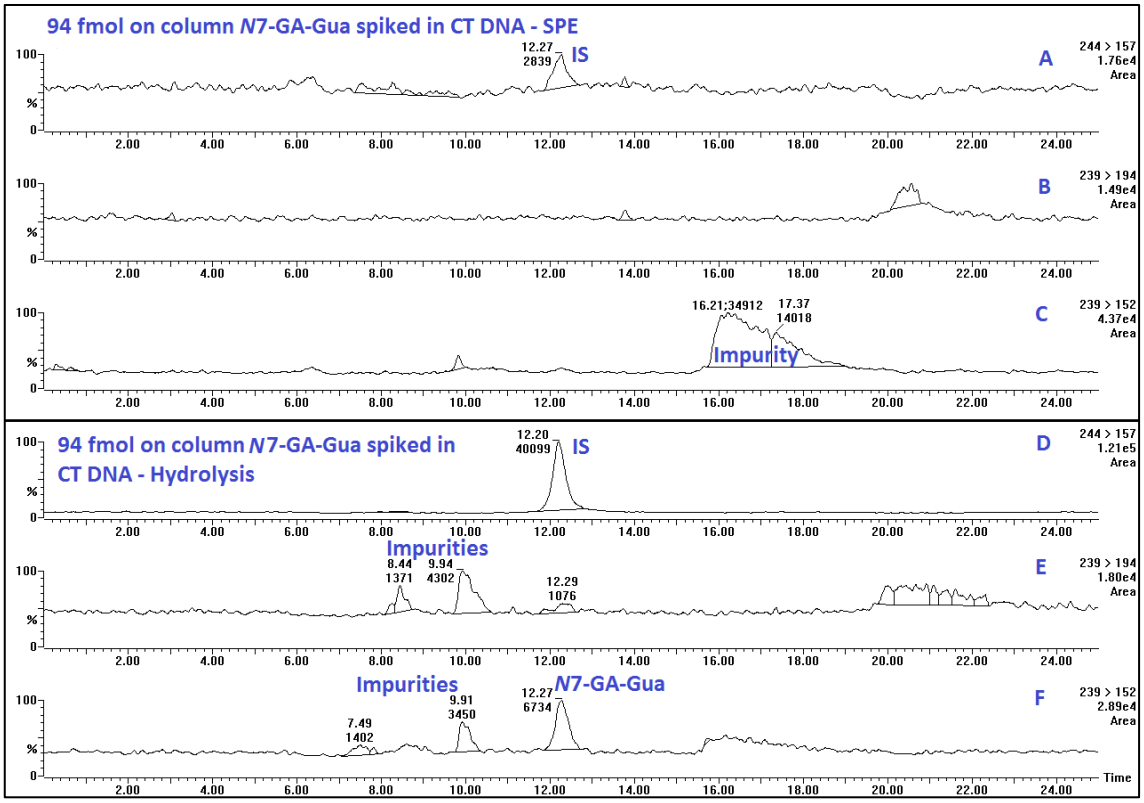


Figure 2.31: The typical LC-MS/MS SRM chromatogram for a spiked CT DNA sample (94 fmol on column *N*7-GA-Gua and 794 fmol on column [$^{15}N_5$]-*N*7-GA-Gua) that was hydrolysed and afterwards underwent SPE (Strata X) are shown in the LC-MS/MS SRM chromatograms (A - C) whereas the LC-MS/MS SRM chromatograms (D – F) show a spiked CT DNA sample (94 fmol on column *N*7-GA-Gua and 794 fmol on column [$^{15}N_5$]-*N*7-GA-Gua) which was analysed after hydrolysis. LC-MS/MS SRM chromatograms A and D show the IS peak. LC-MS/MS SRM chromatograms B and E monitored the fragmentation of *m/z* 239 to 194 for the *N*7-GA-Gua adduct and LC-MS/MS SRM chromatograms C and F show the transition for *m/z* 239 to 152 for the detection of the *N*7-GA-Gua adduct.

2.3.7 Development of a method to detect *N*7-GA-Gua in urine

2.3.7.1 SPE – HLB columns

Standards through HLB 6cc columns – generic and optimised method

After analysing the HLB column eluates of acidified and basified *N*7-GA-Gua standards of the generic as well as the optimised method, the *N*7-GA-Gua adduct was not detected in either sample. The [$^{15}N_5$]-*N*7-GA-Gua (794 fmol on column) spiked after the SPE process was detectable. The acidic fractions could not be evaporated to dryness;

loading and washing solutions for the basic sample were analysed and showed that the *N*7-GA-Gua was already lost in these steps. For the generic method the majority of the *N*7-GA-Gua adduct was lost in the loading step and a smaller fraction was detectable in the solution of the washing step. Also for the optimised basified sample the majority of the *N*7-GA-Gua adduct was lost in the loading step and less so in the first washing step. In both cases the *N*7-GA-Gua adduct lost in the washing step was about 3.6 fold less compared to the loss in the loading step.

2.3.7.2 SPE – Strata X-C columns

Standards through Strata X-C

Applying standards to the Strata X-C columns and using 2 mL and 4 mL, respectively for the conditioning, equilibrating, washing and eluting step there were *N*7-GA-Gua adduct and [$^{15}N_5$]-*N*7-GA-Gua adduct peaks detectable (Figure 2.32).

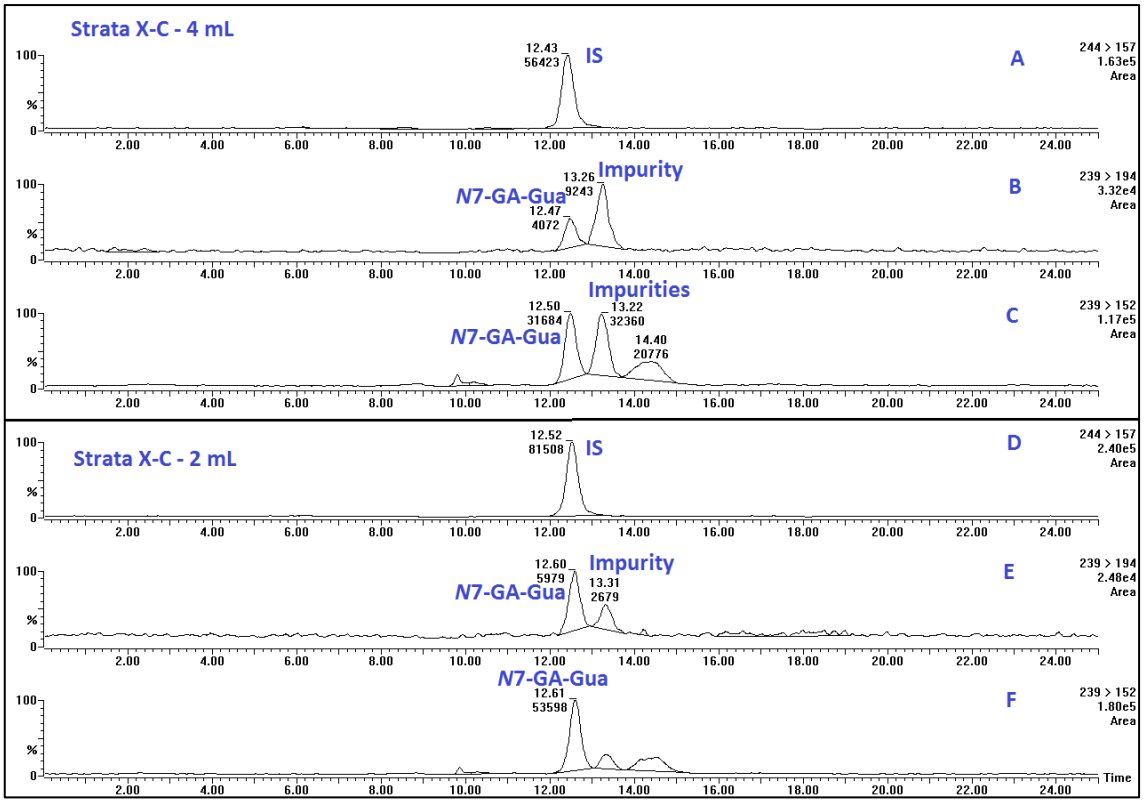


Figure 2.32: Applying standards through Strata X-C columns and using either 2 mL or 4 mL for every step of conditioning, equilibration, loading, washing and eluting. LC-MS/MS SRM chromatograms A – C show the results for the eluate applying 4 mL solutions and LC-MS/MS SRM chromatograms D – F show the results for the eluate taking 2 mL for every step of the SPE process. LC-MS/MS SRM chromatograms A and D show the transition *m/z* 244 to 157 for the IS. LC-MS/MS SRM chromatograms B and E monitored the fragmentation of *m/z* 239 to 194 for the *N*7-GA-Gua adduct and LC-MS/MS SRM chromatograms C and F show the transition for *m/z* 239 to 152 for the detection of the *N*7-GA-Gua adduct.

The loading and wash 1 fraction could not be evaporated to dryness so they were not analysed by LC-MS. The unlabelled *N*7-GA-Gua adduct was not detectable in the wash 2 fraction. The *N*7-GA-Gua peak areas were higher for the 2 mL method compared to the 4 mL method and the ratio of *N*7-GA-Gua peak area/[$^{15}N_5$]-*N*7-GA-Gua peak area for the 2 mL was 0.63 compared to the ratio of 0.57 for the 4 mL method.

Urine through Strata X-C

For the spiked and non-spiked urine samples no *N*7-GA-Gua adducts could be detected for either the use of 4 mL or 6 mL loading solution. The [$^{15}N_5$]-*N*7-GA-Gua peak area for the first run was high then dropped quite rapidly but increased again with the last run and can be detected in all runs. Due to an impurity peak in the transition *m/z* 244 to 157 it was difficult to identify the IS peak but the retention time of the smaller peak was close to the [$^{15}N_5$]-*N*7-GA-Gua retention time of the standard solution that was

analysed before the biological samples (Figure 2.33). The areas of the interfering peaks are higher for the sample where 6 mL solution was applied compared to the 4 mL sample.

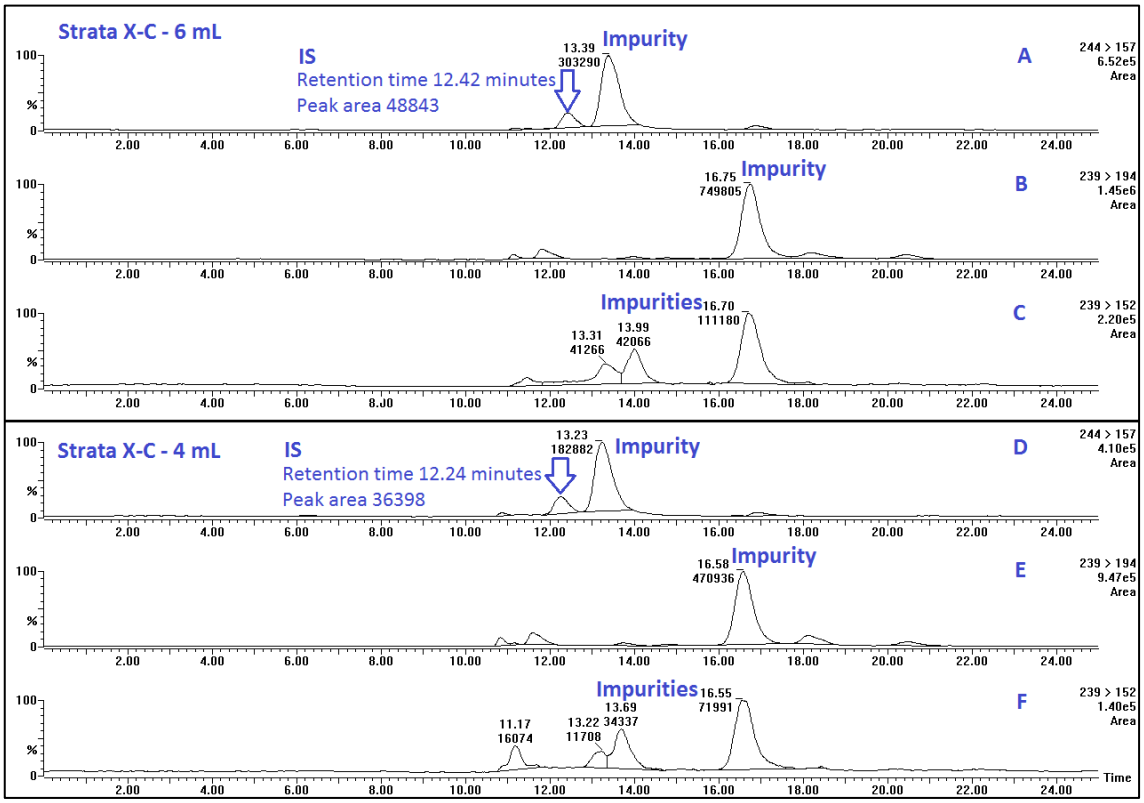


Figure 2.33: Applying urine spiked with labelled and unlabelled adduct through Stata X-C columns with either 6 or 4 mL for every step of conditioning, equilibration, loading, washing and eluting. LC-MS/MS SRM chromatograms A – C show the results for the eluate applying 6 mL solutions and LC-MS/MS SRM chromatograms D – F show the results for the eluate taking 4 mL for every step of the SPE process. LC-MS/MS SRM chromatograms A and D show the transition *m/z* 244 to 157 for the IS. LC-MS/MS SRM chromatograms B and E monitored the fragmentation of *m/z* 239 to 194 for the *N*7-GA-Gua adduct and LC-MS/MS SRM chromatograms C and F show the transition for *m/z* 239 to 152 for the detection of the *N*7-GA-Gua adduct.

Phenomenex consultant method

Following the new protocol for the Strata X-C columns there was a *N*7-GA-Gua peak (1 pmol spiked) in the eluate and a small adduct peak detected in the wash 2 fraction. No *N*7-GA-Gua peak was detected in the loading and the first washing fraction. The *N*7-GA-Gua adduct peak detectable in the eluate was about 10% of the area compared to the control solutions (no matrix and no SPE procedure) measured within the same run.

The adduct was also detectable in the eluate after processing 500 fmol *N*7-GA-Gua with Strata X-C. Approximately a third of the *N*7-GA-Gua adduct was washed off in the

second washing step. The loading and the first washing solution were free of *N*7-GA-Gua adduct.

2.3.7.3 SPE – Strata X columns

The analysed samples, spiked with the *N*7-GA-Gua and [$^{15}N_5$]-*N*7-GA-Gua adduct where different amounts of urine underwent SPE showed that an increasing amount of urine led to a decreased signal for the labelled and unlabelled adduct. In the 100 µL spiked urine sample, peaks for *N*7-GA-Gua and [$^{15}N_5$]-*N*7-GA-Gua were detectable (Figure 2.34). In the 200 µL spiked urine sample there were very small peaks detectable below LOD for both labelled and unlabelled standards. The *N*7-GA-Gua peak area was about 10 fold smaller for the 100 µL injection whereas the IS [$^{15}N_5$]-*N*7-GA-Gua peak area only decreased about half. For 300, 400 and 500 µL spiked urine samples neither of the spiked adducts were detected.

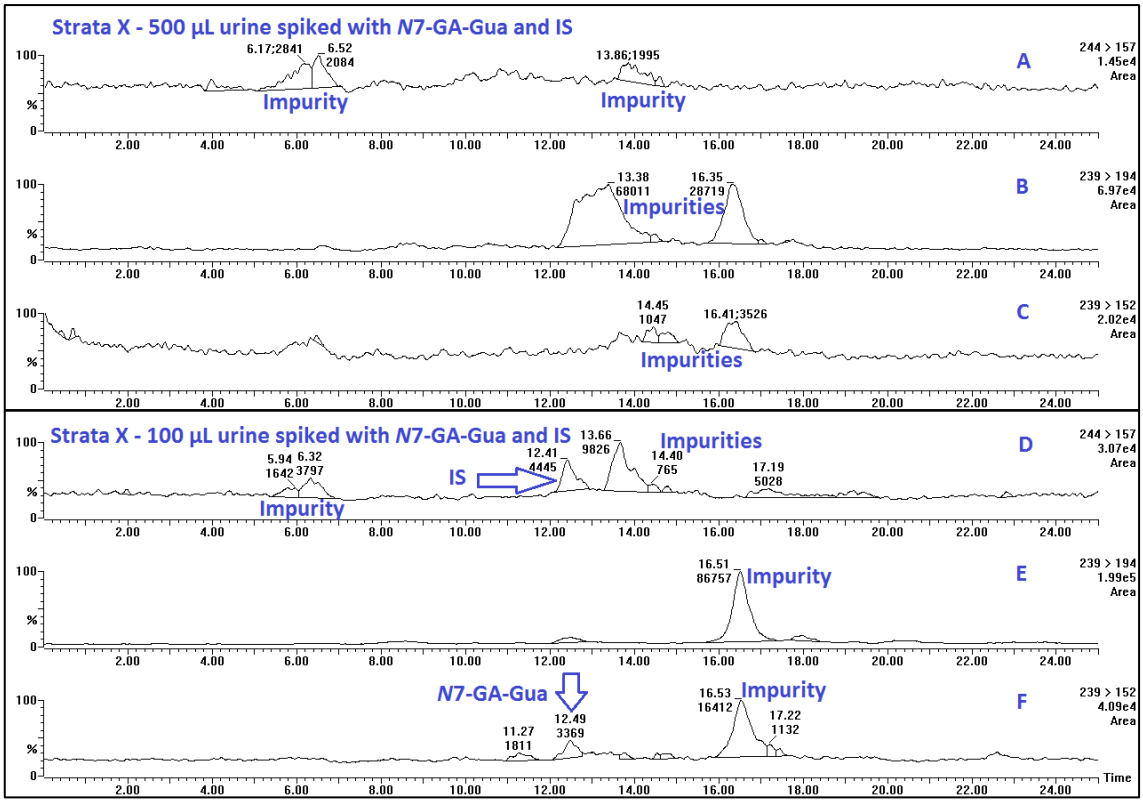


Figure 2.34: LC-MS/MS SRM chromatograms following injections of different volumes of spiked urine onto the Strata X columns. 500 µL urine (LC-MS/MS SRM chromatograms A – C) and 100 µL urine (LC-MS/MS SRM chromatograms D – F) were spiked with *N*7-GA-Gua and [$^{15}N_5$]-*N*7-GA-Gua to give 630 fmol on column each. LC-MS/MS SRM chromatograms A and D show the transition *m/z* 244 to 157 for the IS. LC-MS/MS SRM chromatograms B and E monitored the fragmentation of *m/z* 239 to 194 for the *N*7-GA-Gua adduct and LC-MS/MS SRM chromatograms C and F show the transition for *m/z* 239 to 152 for the detection of the *N*7-GA-Gua adduct. There is only a small fraction of the spiked adduct detectable for the 100 µL spiked urine sample as shown in LC-MS/MS SRM chromatograms D and F.

2.3.7.4 SPE – Strata X-A columns

Following the protocol for the new SPE method for Strata X-A columns there was no *N*7-GA-Gua peak detectable in the eluate but it was present in the loading fraction and both wash fractions. The highest peak area was found in the first washing step. After repeating the experiment a small *N*7-GA-Gua signal was detectable in the eluate (below LOD) and also in the loading and both washing fractions. Adding all *N*7-GA-Gua adduct peak areas from each step of a single SPE procedure and calculating the proportions of *N*7-GA-Gua in each fraction showed percentages of 1.4% for the eluate, 7.3% for loading, 19.5% washing 1 and 71.6% for the second washing step.

No *N*7-GA-Gua adduct was detectable in the eluate when 500 fmol *N*7-GA-Gua adduct underwent the Strata X-A process but it was detectable in the loading and both washing fractions. The loading fraction contained 17.4%, the first washing fraction contained 68.8% and the second washing fraction contained 13.7% of the detectable *N*7-GA-Gua adduct.

2.3.7.5 Alternative SPE protocols

Standards only

After the standard solution was processed using the Strata X-C and Strata X-A the evaporated fractions were spiked with [$^{15}N_5$]-*N*7-GA-Gua to give 567 fmol on column and analysed by LC-MS. It was not possible to analyse all the collected fractions from the Strata X-A purification due to the presence of an insoluble precipitate. *N*7-GA-Gua was detectable in the eluates of both columns. In the first wash step for the Strata X-C columns there was a small peak for *N*7-GA-Gua detectable which was about 20 fold smaller than the peak in the eluate. The other fractions were free of *N*7-GA-Gua adduct. The recoveries for the ratio of *N*7-GA-Gua peak area/[$^{15}N_5$]-*N*7-GA-Gua peak area for Strata X-C was 115% (1 mL), Strata X-C (3 mL) 88% and for Strata X-A 70% (1 mL). The *N*7-GA-Gua recovery for Strata X-C was 85% (1 mL) and 52% (3 mL) and for Strata X-A 51%.

Urine

The evaporated eluates could not be analysed due to the presence of an insoluble precipitate.

Analysis of different urine volumes

In the experiment with varying amounts of urine there was almost none of the *N*7-GA-Gua recovered after the SPE process for Strata X-C columns. *N*7-GA-Gua was only detected in the 250 µL and 1000 µL fractions. The Strata X-A eluates from 1000 µL onwards were not analysed due to an insoluble precipitate being present. The *N*7-GA-Gua recovery for the Strata X-A columns using 250 µL urine was the highest with 57% compared to 17% and 20% for the 100 µL and 500 µL urine, respectively but the ratio

recovery of *N*7-GA-Gua peak area/[$^{15}N_5$]-*N*7-GA-Gua peak area was greater than 300%. The Strata X-C column only provided a recovery of only 1% for both the ratio recovery as well as the raw *N*7-GA-Gua recovery.

Analysis using smaller volumes – different sample dilution

A decrease in *N*7-GA-Gua peak area with increasing volumes of urine was observed when applied to Strata X-C columns with a smaller range for different urine volumes (100 µL to 1000 µL) (Figure 2.35). Ratio recovery dropped from 88% (100 µL) to 60% (1000 µL). The 250 µL urine sample had the highest *N*7-GA-Gua peak area of the Strata X-A samples.

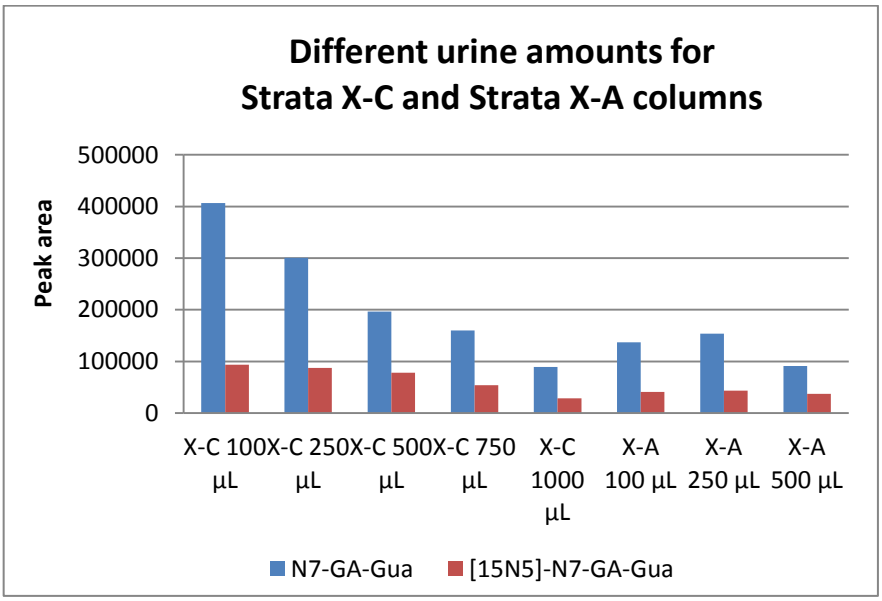


Figure 2.35: Peak areas of *N*7-GA-Gua going through the SPE procedure with Strata X-C and Strata X-A columns using different amounts of urine as shown in µL indication on the x-axis. The IS [$^{15}N_5$]-*N*7-GA-Gua was spiked before the analysis by LC-MS and was not taken through the procedure of SPE.

Calibration line with urine matrix

Spiking different amounts of the *N*7-GA-Gua adduct into urine gave a poor linear response. The R^2 for Strata X-C was 0.4211 and R^2 for Strata X-A was 0.7777.

pH changes

The pH of the K_2HPO_4 and HCl solutions were changed and different urine volumes were spiked with *N*7-GA-Gua to give 500 fmol on column prior to the SPE procedure and with [$^{15}N_5$]-*N*7-GA-Gua to give 500 fmol on column before LC-MS analysis. The Strata X-A eluates 250/4750, 2000/3000 and 1000/4000 (referring to urine in

µL/K_2HPO_4 in µL; Table 2.9) could not be analysed due to an insoluble precipitate. In the 2000/3000 and 1000/4000 samples for Strata X-C *N*7-GA-Gua was not detectable. *N*7-GA-Gua was detectable in the remaining eluates and recoveries for the ratio of *N*7-GA-Gua peak area/[$^{15}N_5$]-*N*7-GA-Gua peak area varied between 28 to 88% with raw recoveries for the *N*7-GA-Gua adduct ranging between 2 and 29% (Figure 2.36).

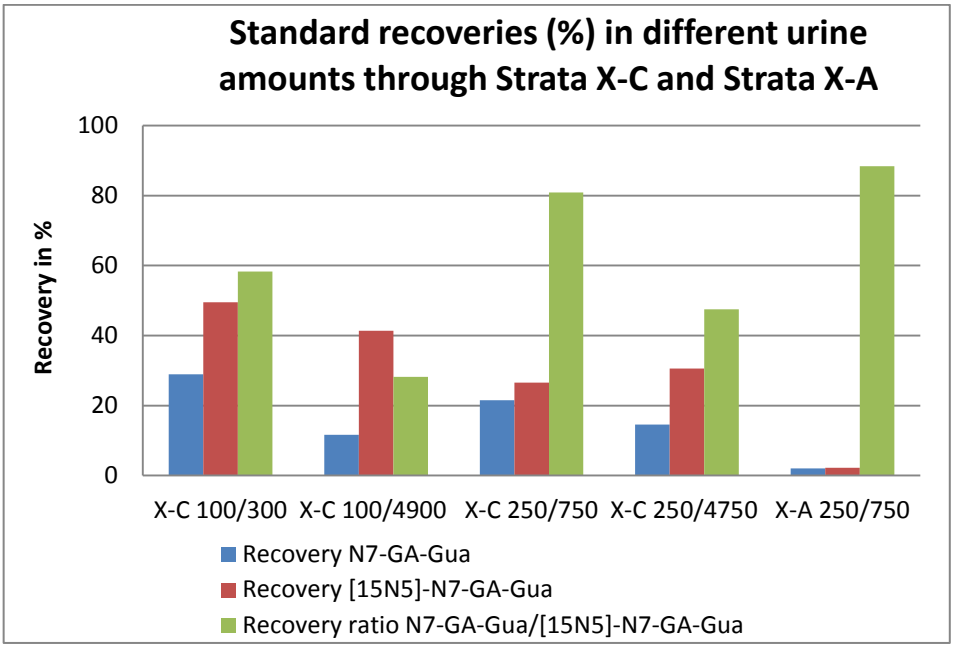


Figure 2.36: Recoveries for the *N*7-GA-Gua adduct spiked into different amounts of urine, the IS [$^{15}N_5$]-*N*7-GA-Gua and the ratio of *N*7-GA-Gua peak area/[$^{15}N_5$]-*N*7-GA-Gua peak area.

HPLC purification of small urine volumes

Including the HPLC purification step to the analysis of urine samples (triplicate) after SPE (Strata X-C) the recovery for the ratio of *N*7-GA-Gua peak area/[$^{15}N_5$]-*N*7-GA-Gua peak area increased to 77.6%, 78% and one sample showed a recovery of 109%. The raw recovery for the *N*7-GA-Gua adduct was 72.5%, 72.7% and 102%.

HPLC purification of N7-GA-Gua from increased urine volumes

A *N*7-GA-Gua peak was detectable in two of the three 100 µL non-spiked urine. For the 2.5 mL non-spiked urine samples no *N*7-GA-Gua could be detected and only one sample showed a peak for the IS [$^{15}N_5$]-*N*7-GA-Gua due to contaminating peaks in the chromatogram. The samples analysed with both spiked standards showed that for the high urine volume the peak areas for both standards were substantially lower compared to the low urine volume samples (Table 2.15).

Table 2.15: Peak areas ± standard deviation of the spiked *N*7-GA-Gua and the IS [$^{15}N_5$]-*N*7-GA-Gua in urine samples processed in a set of 100 µL urine diluted with 300 µL 1M HCl and 2.5 mL urine diluted with 2.5 mL 1M HCl applied to Strata X-C columns

	***N*7-GA-Gua**	**[$^{15}N_5$]-*N*7-GA-Gua**
X-C 100 µL/300 µL	73595 ± 19160	93800 ± 4711
X-C 2.5 mL/2.5 mL	5088 ± 718	53735 ± 3016

HPLC purification of different urine volumes

Purifying different amounts of spiked urine with the Strata X-C columns and purifying the samples by HPLC did result in the detection of *N*7-GA-Gua. The control standard solutions gave a mean peak area and standard deviation for *N*7-GA-Gua of 253058 ± 8851 and for the IS [$^{15}N_5$]-*N*7-GA-Gua 356657 ± 7201. In the subsequently analysed urine samples it was difficult to confirm if both the IS and unlabelled adduct were present. In the blank urine sample spiked only with IS [$^{15}N_5$]-*N*7-GA-Gua the peak with retention time 11.17 min for the IS was 6 fold higher than the [$^{15}N_5$]-*N*7-GA-Gua peak area for the standard solutions analysed beforehand so the peak with retention time 10.66 min was predicted to be the IS peak (Figure 2.37).

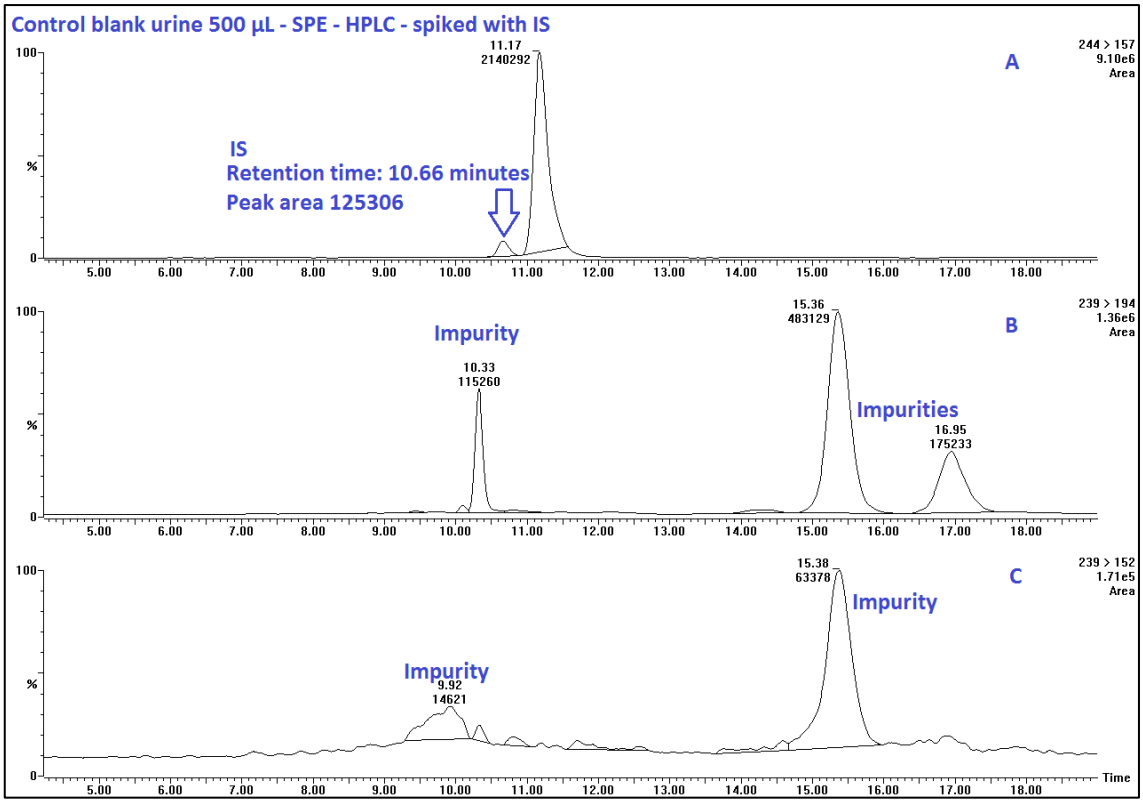


Figure 2.37: The LC-MS/MS SRM chromatograms for 500 µL unspiked urine. The sample was spiked with IS prior to injection and underwent SPE and HPLC purification before analysis by LC-MS. LC-MS/MS SRM chromatogram A shows the transition *m/z* 244 to 157 for the IS. LC-MS/MS SRM chromatogram B monitored the fragmentation of *m/z* 239 to 194 for the *N*7-GA-Gua adduct and LC-MS/MS SRM chromatogram C shows the transition for *m/z* 239 to 152 for the detection of the *N*7-GA-Gua adduct.

The chromatograms showed two peaks of which the one with longer retention time had an increased peak area compared to the peak with the earlier retention time (Figure 2.37). The same was observed when analysing higher urine amounts (Figure 2.38, Figure 2.39). The LC-MS/MS SRM chromatograms in Figure 2.38 show the results of the analysis of a 500 µL spiked urine taken through the SPE procedure and HPLC purification.

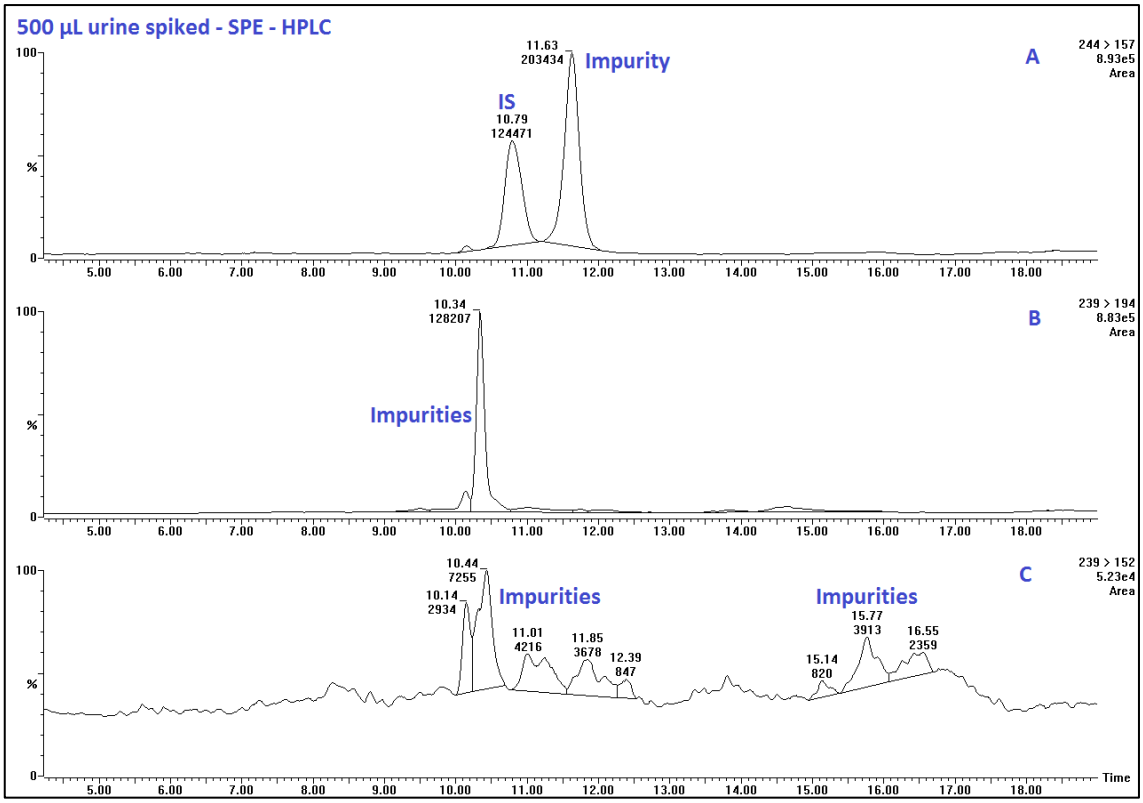


Figure 2.38: The LC-MS/MS SRM chromatograms for 500 µL spiked urine. The sample was spiked with IS prior to injection and underwent SPE and HPLC purification before analysis by LC-MS. LC-MS/MS SRM chromatogram A shows the transition *m/z* 244 to 157 for the IS. LC-MS/MS SRM chromatogram B monitored the fragmentation of *m/z* 239 to 194 for the *N*7-GA-Gua adduct and LC-MS/MS SRM chromatogram C shows the transition for *m/z* 239 to 152 for the detection of the *N*7-GA-Gua adduct.

The LC-MS/MS SRM chromatograms for the 1500 µL (Figure 2.39) and 2000 µL spiked urine samples showed the presence of more peaks with similar areas to the *N*7-GA-Gua standards (not subjected to SPE or HPLC, no matrix). This made the assignment of the IS challenging and consequently the assignment of the adduct peak impossible.

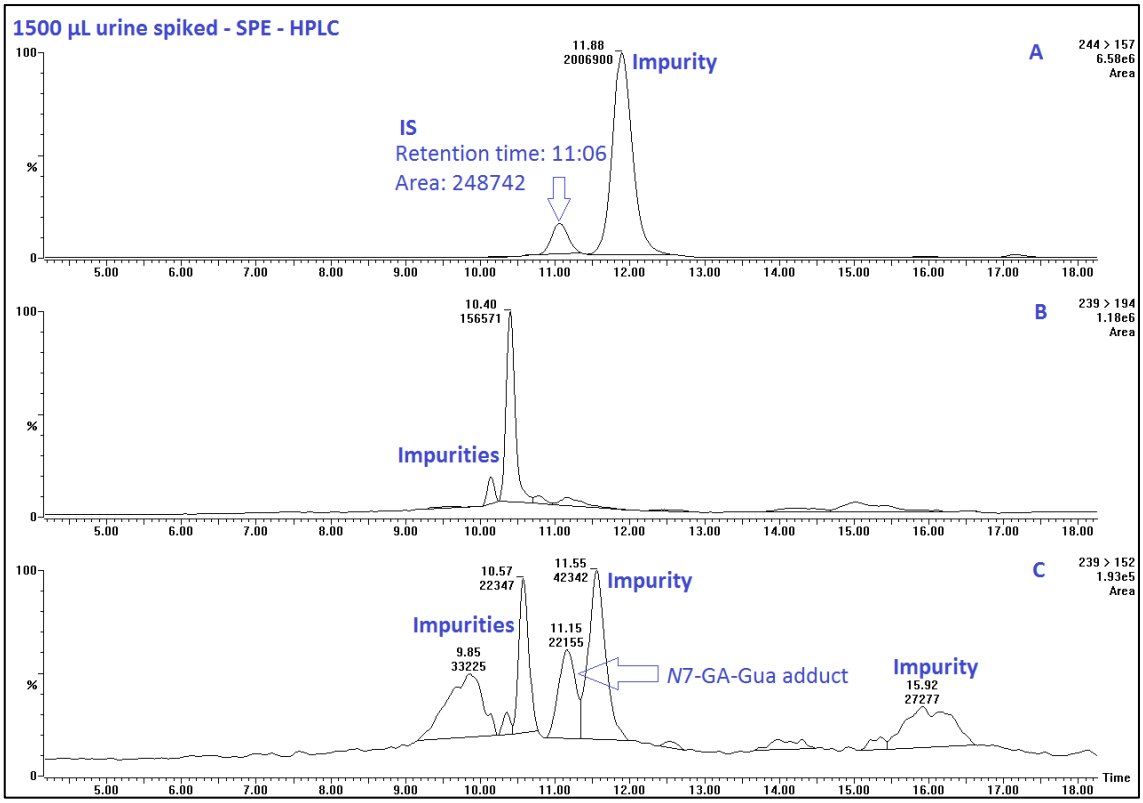


Figure 2.39: The LC-MS/MS SRM chromatograms for 1500 µL spiked urine. The sample was spiked with IS prior to injection and underwent SPE and HPLC purification before analysis by LC-MS. LC-MS/MS SRM chromatogram A shows the transition *m/z* 244 to 157 for the IS. LC-MS/MS SRM chromatogram B monitored the fragmentation of *m/z* 239 to 194 for the *N*7-GA-Gua adduct and LC-MS/MS SRM chromatogram C shows the transition for *m/z* 239 to 152 for the detection of the *N*7-GA-Gua adduct.

2.3.7.6 Urine: Dilute and inject

Urine was diluted 1:1000, 1:100 and 1:10 with 0.1% FA and analysed by LC-MS without undergoing SPE purification. The urine samples spiked with only the IS [$^{15}N_5$]-*N*7-GA-Gua did not show a peak for the *N*7-GA-Gua adduct. In the samples that were spiked with both standards a decrease in average peak area for the *N*7-GA-Gua adduct was noticeable for a more concentrated solution. The mean *N*7-GA-Gua peak area values and standard deviation were 116785 ± 4297 (1:1000), 103437 ± 678 (1:100) and 74108 ± 1205 (1:10). The chromatogram for the 1:1000 dilution appeared very similar to the standard control injection without matrix. Interfering peaks in the chromatogram increased with increasing amounts of the urine matrix. The peak area of the IS [$^{15}N_5$]-*N*7-GA-Gua decreased from highest to lowest dilution with mean peak area and

standard deviation of 125522 ± 6560 (1:1000), 112850 ± 3707 (1:100) and 87149 ± 886 (1:10).

2.3.8 Heating block

Testing the influence of which type of heating block was used to hydrolyse the samples revealed that there were differences between various insert blocks. Samples in Block 1 had a RSD value of 1.2%, in Block 2 21.9% and in Block 3 the RSD was 8.6%.

2.4 Discussion

A method for the detection of the *N*7-GA-Gua adduct by LC-MS/MS SRM applying online column-switching was developed. After validation (Chapter 3) the method was applied to the analysis of human volunteers samples (Chapter 5).

To summarise the optimised mass spectral analysis: the analyte was loaded on a trap column (50 x 2.0 mm) with 99:1 0.1% FA/MeOH (*v/v*) as mobile phase before being washed onto the analytical column (250 x 2.0 mm) with a mobile phase consisting of 95:5 0.1% FA/MeOH (*v/v*) and final detection on the mass spectrometer with a total run time of 40 min. After separation of the analyte both columns were washed with a high percentage of organic and equilibrated (amounts and times in Table 2.11 and Table 2.12) for the next analysis.

The use of column-switching is novel for the detection of the *N*7-GA-Gua adduct and has not been described previously. Da Costa *et al*. and Watzek *et al*. analysed *N*7-GA-Gua with a LC-MS/MS method but the online column-switching method was not applied [73,121]. Hence both methods vary in different aspects, either having another protocol for hydrolysis, using different equipment and changes in mobile phase and total run time.

The method described in this chapter is complex and time-consuming with the inclusion of HPLC purification but allows detection of small amounts of *N*7-GA-Gua in a biological matrix with the ultimate aim to analyse human samples. Since the discovery

that AA occurs in food, all published analyses regarding the *N*7-GA-Gua adduct were undertaken using animal studies. These *in vivo* studies use higher doses than humans typically consume within their diet so it is possible that the adduct levels in humans are low (if detectable at all) which necessitates a high sensitivity methodology which can exhibit a low LOD. In order to achieve this sensitivity the method involves analytical steps that prolong the work flow, i.e. HPLC purification. The methodology is time consuming and thus has poor throughput. It takes 6 days from the time of taking whole blood from volunteers to the final analysis by LC-MS and allows the measurement of about 8 volunteers' samples in triplicate (24 single injections). The HPLC step limits the sample number but the samples can be stored at -20°C after purification and hence more samples can be purified to increase the sample throughput.

A further limitation was the challenging analysis of the *N*7-GA-Gua adduct in urine and more particularly the difficulties in its retention on SPE cartridges. No results were obtained when following the methods described by Balbo *et al*. and Chao *et al*. which might be explained by the fact that their methods aimed to detect 7-ethylguanine which is less polar than the *N*7-GA-Gua adduct and easier to retain on SPE columns [124,125].

This work can be seen as a good foundation and a basis to further improve and work on the method for the detection of the *N*7-GA-Gua adduct towards a high sample throughput.

2.5 Summary

This chapter described the work for the development of a sensitive method for the detection of the *N*7-GA-Gua adduct incorporating a novel online column-switching method.

After choosing the trap and the analytical column, the mobile phase and organic modifier were selected. With the settings for both the timings of the switching valve and the total run time optimised for the best chromatographic separation of the *N*7-

GA-Gua adduct standard. Detection and quantification became challenging when biological matrixes were introduced to the method process. The sensitivity of detection was decreased and the separation of the *N*7-GA-Gua peak was problematic due to interfering co-eluting matrix derived peaks. Urine was especially problematic with a complete lack of detection with or without SPE purification. Hence for urine, no satisfactory method for the detection of the *N*7-GA-Gua adduct was developed. However, in DNA (leukocyte DNA as well as CT DNA that was used as an interchangeable matrix) several analytical steps, i.e. hydrolysis and HPLC purification improved the detection of the *N*7-GA-Gua adduct. After testing numerous parameters a final method was chosen and validation experiments were conducted.

Chapter 3

3 Validation of the Mass Spectrometric Method for *N*7-GA-Gua Detection in DNA

3.1 Introduction

This chapter describes the work that was undertaken for the validation of the method which was developed and described previously (Chapter 2). In order to apply a developed analytical method for the analysis of samples it needs to be verified that the method can reproducibly analyse the compound of interest. Thus, the validation of an analytical method provides confirmation that the analyte of interest can be detected within the given setting with a certainty determined by the parameters of the validation process.

This project aimed at developing a method for the detection of the *N*7-GA-Gua adduct in human leukocyte DNA and urine. Since the method for the detection of the *N*7-GA-Gua adduct in urine was not established (Chapter 2) this chapter describes the validation of the detection of *N*7-GA-Gua in human leukocyte DNA.

Thus far there have been no publications on the detection of the *N*7-GA-Gua adduct in human tissue DNA. Therefore the existing work will be discussed mainly in respect to results achieved by animal studies and the comparison of validation methods undertaken for different DNA adducts. Several methods for the detection of *N*7-GA-Gua have been partially validated as described in da Costa *et al*., Doerge *et al*. and Watzek *et al*. [73,89,121]. Balbo *et al*. analysed a different DNA adduct, *N*7-ethylguanine and described this method and its validation [125].

The aim of the experiments reported in this chapter was to validate the newly developed method for the detection of the *N*7-GA-Gua adduct in leukocyte DNA with the objective to then apply the validated method to analyse human volunteer DNA samples for the determination of *N*7-GA-Gua levels.

3.2 Materials and methods

3.2.1 Materials

General chemicals, equipment and mass spectrometer used for the validation procedure were the same as mentioned in Chapter 2, see sections 2.2.1.1, 2.2.1.2 and 2.2.2.3.1.

3.2.2 Methods

The validation process followed the "Guidance for Industry, Bioanalytical Method Validation of the US Department of Health and Human Services, Food and Drug Administration from May 2001" and the publication "Key Elements of Bioanalytical Method Validation for Small Molecules" by S. Bansal *et al.*, published in 2007 [126,127]. Figure 3.1 gives a brief overview of the steps involved for the procedure in processing all samples needed to validate the method.

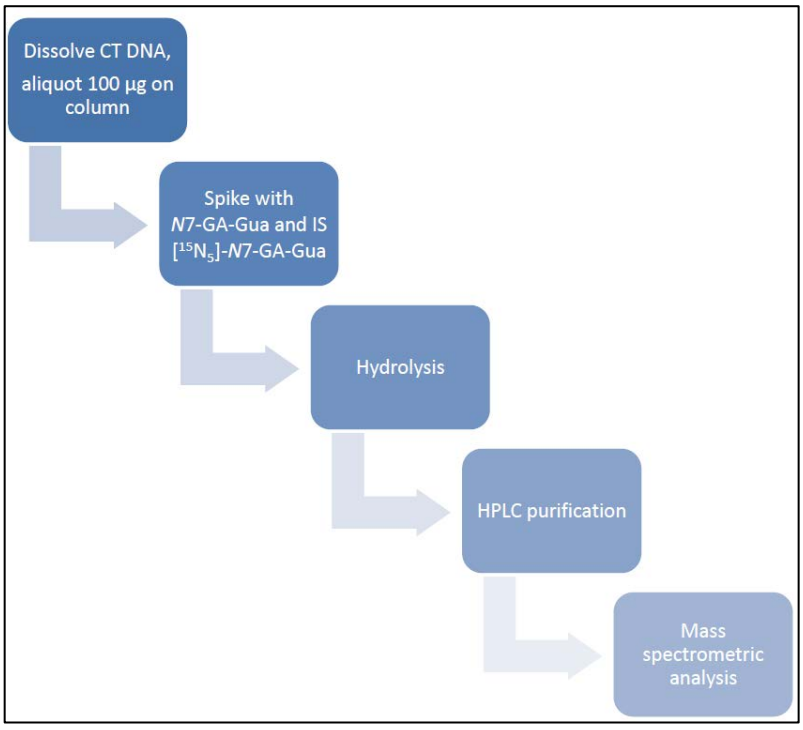


Figure 3.1: Flow chart illustrating the steps involved in the validation process from dissolving CT DNA to the final analysis by LC-MS.

CT DNA served as the matrix for the validation and was dissolved in ddH_2O. After determination of the DNA concentration by UV spectrophotometry, 152.38 µg DNA aliquots were pipetted that equalled 100 µg DNA on column for each sample injection by LC-MS. The CT DNA aliquots were spiked with labelled and unlabelled adduct standards before going through the hydrolysis procedure, with the exception of the samples to determine the recovery of the *N*7-GA-Gua adduct. The recovery samples were spiked with the *N*7-GA-Gua adduct and the labelled IS [$^{15}N_5$]-*N*7-GA-Gua was spiked at the last step, just prior to measurement by LC-MS. Following hydrolysis the samples were HPLC purified and analysed by LC-MS.

3.2.2.1 The Validation process

Within the process of validation for the *N*7-GA-Gua adduct the calibration line, accuracy, precision, LOD and the recovery was determined on 6 different days. The standard injections (standard solution in 0.1% FA) and the CT DNA based samples were pipetted on the same day. The CT DNA samples were spiked with the appropriate amounts of labelled and unlabelled adduct, dried down in a centrifugal evaporator and hydrolysed (see section 2.2.2.2.2). The filtrate from the hydrolysis step was dried down in a centrifugal evaporator and re-dissolved in 40 µL of LC-MS Optima water for HPLC purification. Following HPLC the collected fraction was dried down in a centrifugal evaporator and then re-dissolved in 20 µL 0.1% FA (the recovery samples were spiked with [$^{15}N_5$]-*N*7-GA-Gua at this stage and re-dissolved in 0.1% FA to give 20 µL).

Each day of validation involved analysis of a total of 81 samples, of which 27 were standards tubes and 54 were CT DNA. Due to standard samples not going through the process of hydrolysis and HPLC purification they were kept at -20°C for four days and analysed with the processed CT DNA based samples. Figure 3.2 gives a schematic overview on how many standard samples were pipetted for each concentration for one day of validation. Each cross equals one eppendorf tube that gives one injection by LC-MS.

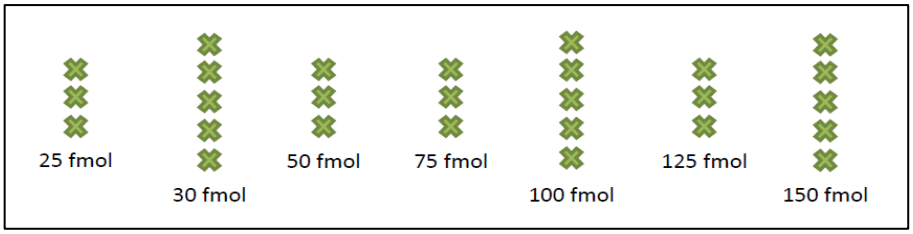


Figure 3.2: Schematic overview of number of standard sample tubes within the validation process for one day. Each cross represents one injection by LC-MS.

Figure 3.3 shows how many CT DNA sample tubes were used for the validation of this method. One cross resembles one CT DNA aliquot (100 µg DNA on column) in one eppendorf tube that was firstly spiked, secondly hydrolysed before HPLC purification and final analysis by LC-MS. The amounts shown under the set of crosses in Figure 3.2 and in Figure 3.3 reflect the spiked amount of *N*7-GA-Gua adduct on column. Into each eppendorf tube (with exception of the recovery samples) was spiked IS [$^{15}N_5$]-*N*7-GA-Gua (500 fmol on column).

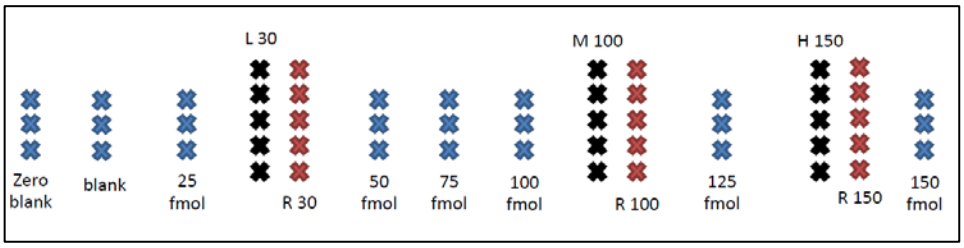


Figure 3.3: Schematic overview of all CT DNA samples included in the validation process for the determination of one validation day. Each cross represents one injection by LC-MS after hydrolysis and HPLC purification. Blue crosses (triplicate) represent the samples for determination of the calibration line, black crosses (5 replicates) reflect the samples for the determination of the accuracy and precision (L = low, M = medium and H = high amount) of the method and the red crosses (5 replicates) resemble the recovery (denoted as R) of the method. All numbers refer to the spiked amounts of the *N*7-GA-Gua adduct on column in fmol. With the exception of the recovery samples all tubes were spiked with 500 fmol on column IS [$^{15}N_5$]-*N*7-GA-Gua. Recovery samples were spiked with IS before analysis by LC-MS.

The calibration line was determined by triplicate injections of spiked CT DNA represented by the blue crosses in Figure 3.3. Zero blank denotes that the CT DNA samples were neither spiked with the *N*7-GA-Gua adduct nor the IS [$^{15}N_5$]-*N*7-GA-Gua whereas the blank CT DNA samples were spiked only with the IS [$^{15}N_5$]-*N*7-GA-Gua. The amounts mentioned in Figure 3.2 and Figure 3.3 refers to the spiked amount of *N*7-GA-Gua on column. All black crosses represent the amounts low (30 fmol on column = L 30), medium (100 fmol on column = M 100) and high (150 fmol on column = H 150) in

5 replicates for the determination of the accuracy and precision of the method. The red crosses are the samples for the determination of the recovery, also 5 replicates with the same low, medium and high amounts as for accuracy and precision. With the exception of the recovery samples (referred to as R 30, R 100 and R 150) all tubes were spiked with both the *N*7-GA-Gua adduct and the IS [$^{15}N_5$]-*N*7-GA-Gua prior to hydrolysis. All sample tubes for the determination of the recovery were spiked with the *N*7-GA-Gua adduct before hydrolysis and the IS [$^{15}N_5$]-*N*7-GA-Gua was added just before measurement by LC-MS, thus added after hydrolysis and HPLC purification.

3.2.2.2 Calculations of DNA and standard adduct amounts on column

Within this chapter the standard adduct amounts are given as amount in fmol on column. The DNA amount used for each injection was 100 µg on column. This section illustrates the calculations for DNA and standards amounts applied in the validation procedure.

3.2.2.2.1 DNA amounts on column

For every injection 100 µg DNA on column was used in 15 µL injection volume for the final mass spectrometric analysis:

100.00 µg in 15 µL

→ 133.33 µG in 20 µL (volume to re-dissolve dried down HPLC purified fraction)

For the HPLC purification, which preceded mass spectrometric analysis, the hydrolysis filtrate was re-dissolved in 40 µL of which 35 µL were injected:

133.33 µg in 35 µL

→ 152.38 µG in 40 µL

Hence, to reach a final amount of 100 µg DNA on column 152.38 µg DNA was needed to be aliquoted at the beginning of the analysis.

3.2.2.2.2 [$^{15}N_5$]-*N*7-GA-Gua

The spiking amounts for the IS varied between the recovery and remaining samples. The stock solution had a concentration of 151.3 pmol/µL of which a 1:1000 dilution prepared in 0.1% FA was used for spiking.

SAMPLES:

Each sample was spiked with 500 fmol IS on column, in a 15 µL injection by LC-MS:

500.00 fmol in 15 µL

→ 666.67 fmol in 20 µL (volume to re-dissolve dried down HPLC purified fraction)

For the HPLC purification, which preceded mass spectrometric analysis, the hydrolysis filtrate was re-dissolved in 40 µL of which 35 µL were injected:

666.67 fmol in 35 µL

→ 761.91 FMOL in 40 µL

For the spiking volume of the IS the required fmol was divided by the concentration of the standard solution:

761.91 fmol/151.3 fmol/µL = 5.0 µL

Each sample was spiked with 5.0 µL 1:1000 dilution of IS.

RECOVERY:

The spiking volume for the recovery samples was different because the IS was spiked at the very last step of the analysis without going through the work-up procedure.

Each sample was spiked with 500 fmol on column IS, in a 15 µL injection by LC-MS:

500.00 fmol in 15 µL

→ 666.67 fmol in 20 µL (volume to re-dissolve before analysis by LC-MS)

For the spiking volume of the IS the required fmol was divided by the concentration of the standard solution:

666.67 fmol/151.3 fmol/µL = 4.4 µL

Each sample was spiked with 4.4 µL 1:1000 dilution of IS.

3.2.2.2.3 *N*7-GA-Gua

Different amounts of the *N*7-GA-Gua adduct were spiked into CT DNA for the calibration line and the low (30 fmol on column), medium (100 fmol on column) and high (150 fmol on column) amount samples to determine accuracy and precision.

The stock solution of *N*7-GA-Gua was 1142 pmol/µL. A 1:1000 dilution in 0.1% FA was used to prepare the stock solution (11.43 fmol/µL) which gave 150 fmol on column when 20 µL were spiked and 15 µL injected onto LC-MS.

11.43 fmol/µL * 20 = 228.6 fmol/20 µL

	228.6 fmol	in	40 µL (re-dissolving for HPLC purification)
→	200.03 FMOL	in	35 µL (injection volume on HPLC for purification)
	200.03 fmol	in	20 µL (re-dissolving for LC-MS analysis)
→	150.0 FMOL	in	15 µL (injection by LC-MS)

The stock solutions for the other concentrations were prepared by diluting the 150 fmol on column solution as shown in Table 3.1.

Table 3.1: Dilutions for the *N*7-GA-Gua spiking solutions for the calibration line. For each concentration the 150 fmol on column solution (11.43 fmol *N*7-GA-Gua/µL) was diluted with 0.1% FA. 20 µL of the final dilution was then spiked to the corresponding CT DNA tube

fmol on column	150 fmol stock in µL (11.43 fmol *N*7-GA-Gua/µL)	0.1% FA in µL
150	20 µL per sample for spiking	-
125	200	40
100	300	150
75	100	100
50	150	300
30	50	200
25	40	200

3.2.2.3 Calibration line

CT DNA (100 µg on column per injection) was spiked with varying amounts of *N*7-GA-Gua in the range of 25 fmol on column to 150 fmol on column (25, 50, 75, 100, 125, 150 fmol on column) as shown in Table 3.1.

Every calibration point was analysed in triplicate. Each tube was spiked with [$^{15}N_5$]-*N*7-GA-Gua (to give 500 fmol on column). The calibration line was established by taking the ratio of the *N*7-GA-Gua peak area to [$^{15}N_5$]-*N*7-GA-Gua peak area plotted against the amount of *N*7-GA-Gua in fmol on column and checked for linearity. Furthermore, on every validation day, a standard calibration line without matrix was included. The *N*7-GA-Gua adduct was pipetted in various amounts (to give 25, 30, 50, 75, 100, 125, 150 fmol on column) and mixed with 500 fmol on column of the labelled adduct [$^{15}N_5$]-*N*7-GA-Gua prior to injection. All measurements were done in triplicate with the exception of 30, 100 and 150 fmol that were done in 5 replicates. These low, medium and high injections of standard solutions were needed to gain the results for the recovery of the *N*7-GA-Gua adduct.

3.2.2.4 Accuracy

The accuracy of a method describes how close the measured mean value lies to the one that was theoretically calculated. To calculate this, the equation established when plotting the calibration line was used.

The accuracy was determined by 5 injections of low (30 fmol *N*7-GA-Gua on column), medium (100 fmol *N*7-GA-Gua on column) and high (150 fmol *N*7-GA-Gua on column) amounts of *N*7-GA-Gua, each spiked with [$^{15}N_5$]-*N*7-GA-Gua (to give 500 fmol on column). The accuracy of the method was determined in regards to the achieved calibration line. The value for the theoretical accuracy was calculated with the linear equation of the calibration line for the three concentrations low, medium and high. Taking the measured value for the three concentrations in relation to the calculated ones gives the value for the accuracy of the method.

3.2.2.5 Precision and inter-day precision

The precision of a method describes how close one value lies within the determination of multiple aliquots of the same concentration and is expressed as RSD in %.

The precision was determined by 5 injections of low (30 fmol *N*7-GA-Gua on column), medium (100 fmol *N*7-GA-Gua on column) and high (150 fmol *N*7-GA-Gua on column) amounts of *N*7-GA-Gua spiked with [$^{15}N_5$]-*N*7-GA-Gua (to give 500 fmol on column). The RSD for the ratio of *N*7-GA-Gua peak area to the [$^{15}N_5$]-*N*7-GA-Gua peak area determined the value for the precision for each concentration.

3.2.2.6 Recovery

The recovery reflects the value of how much analyte is recovered after processing through the method procedure.

The recovery was determined by 5 injections of low (30 fmol *N*7-GA-Gua on column), medium (100 fmol *N*7-GA-Gua on column) and high (150 fmol *N*7-GA-Gua on column) amounts of *N*7-GA-Gua spiked into CT DNA. The *N*7-GA-Gua adduct was spiked into

the samples and underwent the process of hydrolysis and HPLC purification. The IS was not added until the samples were analysed by LC-MS ([$^{15}N_5$]-*N*7-GA-Gua to give 500 fmol on column). The recovery was determined by comparing the matrix based samples to standard injections of equal amounts of *N*7-GA-Gua and [$^{15}N_5$]-*N*7-GA-Gua but without going through the process of sample work-up.

3.2.2.7 Lower limit of detection (LLOD) and lower limit of quantification (LLOQ)

For this method the LLOD was defined as a S/N ratio = 3 and a S/N ratio = 7 was used for the LLOQ. The quantification expressed as adducts/10^8 nucleotides can be found in section 2.2.2.1.4.

3.2.2.8 Stability

It is essential that data is collected on the effect of storage on the measured molecule. Thus both the analyte and IS were assessed for stability over 10, 20 and 27 weeks at -20°C and room temperature (RT). The effects of freeze-thaw and short term temperature stability were also assessed.

3.2.2.8.1 Internal standard stability

The stability of the IS was tested by preparing 5 samples of 500 fmol [$^{15}N_5$]-*N*7-GA-Gua on column in 0.1% FA ready for measurement by LC-MS and storing them for 6 hrs at RT prior to analysis. The IS stability was tested against 5 freshly pipetted IS samples with equal amount of [$^{15}N_5$]-*N*7-GA-Gua.

3.2.2.8.2 Freeze-thaw stability

Triplicate of low (30 fmol *N*7-GA-Gua on column) and high (150 fmol *N*7-GA-Gua on column) amounts spiked into CT DNA were thawed for 30 min at RT and refrozen at –

20°C for 24 hrs. This was repeated two more times and the samples were analysed on the third cycle against freshly prepared samples in CT DNA.

3.2.2.8.3 Short term temperature stability

Triplicate of low (30 fmol *N*7-GA-Gua on column) and high (150 fmol *N*7-GA-Gua on column) amounts spiked into CT DNA were kept at RT for 24 hrs prior to processing them and short term temperature stability was compared against freshly pipetted samples in CT DNA.

3.2.2.8.4 Long term stability

Triplicate of low (30 fmol *N*7-GA-Gua on column) and high (150 fmol *N*7-GA-Gua on column) amounts spiked into CT DNA were kept at -20°C. After 10, 20 and 27 weeks the stored samples were processed and the influence of long term storage tested by comparison to freshly pipetted samples spiked into CT DNA. The last batch of long term stability samples were analysed after 27 weeks instead of the scheduled 30 weeks due to relocation of the laboratory.

3.2.2.8.5 Limitations of the validation study

The method was developed to detect the *N*7-GA-Gua adduct in human leukocyte DNA. However, the validation was done in CT DNA. Using the same matrix for validation would have required large and impracticable amounts of whole blood for a single day of validation. One 6 mL Lithium-Heparin blood collection tube gives approximately one analysis by LC-MS. Each day of validation would have needed 56 blood sampling tubes, adding up to 324 mL whole blood per day leading to almost 2 litres of blood for the whole validation. Due to this constraint the use of CT DNA as an alternative matrix for method validation is considered a well-established tool in DNA adduct analysis. Additionally, using human whole blood would be costly and take considerable time to carry out the DNA extraction. However, CT DNA does have a background level of *N*7-

GA-Gua which was detected in each validation day with varying amounts. As a consequence the background level masked the lowest amounts making assessment within this range challenging.

3.3 Results

The data that forms the basis of this results section was analysed in three different ways.

- The first analysis was done by using the raw values given for each integrated peak area for every run measured by LC-MS. This is referred to as "RAW".
- The second set was analysed by adjusting for the background level of *N*7-GA-Gua on all CT DNA based samples. The average *N*7-GA-Gua adduct area for the blank CT DNA sample injection was subtracted from all CT DNA based samples. In the following tables presenting the results this data is referred to as "ADJUSTED".
- On the third set of analysis, the data was adjusted further by removing outliers, mainly negative values that occurred as a consequence of subtraction of the background level of *N*7-GA-Gua. In the following tables, presentation of these results is referred to as "ADJUSTED, NO NEGATIVES".
- The excluded values for the data "adjusted, no negatives" were tested for outliers as described in section 3.3.1.

3.3.1 Outliers

A value within a data set is classified as an outlier if this value is either very low or very high compared to the remaining values within the same data set. The FDA guidelines allow excluding outliers from the validation data set following statistical confirmation [127].

After evaluating the data for the validation in the three different ways as explained in section 3.3 the excluded values were then statistically tested to verify if these values were a matter of outliers.

The IQR rule for outliers was performed with Windows Excel®. The minimum (Quartile 0 = Q_0) and maximum (Quartile 4 = Q_4) value as well as Quartile 1 (Q_1), Quartile 2 (Q_2) and Quartile 3 (Q_3) were calculated for each tested data set. Q_1 indicates that 25% of the values are below this value and 75% are above this given value. Q_2 equals the mean value of a data set. Q_3 indicates that 75% of the values are below this value and 25% are above this given value. The IQR (Inter Quartile Range) is the difference between Q_3 and Q_1. A value is classified as an outlier when its value is higher than

$$Q_3 + 1.5(IQR)$$

or if the value is lower than

$$Q_1 - 1.5(IQR)$$

An example for the calculation of outliers is presented in Table 3.2 for the low amount (30 fmol on column) of Validation day 1.

Table 3.2: Result for the statistical test for outliers in the data set for the low amount (30 fmol on column) for the recovery analysis on Validation day 1. Q_0 denotes the minimum value, Q_1 the first quartile, Q_2 is the mean, Q_3 is the third quartile and Q_4 equals the maximum of the data set. Q_1 marks the level where 25% of the values are below this level and 75% are above this value. Q_3 denotes that 75% of all values are below this limit and 25% of the values are above this level. IQR stands for Inter Quartile Range and is the difference between Q_3 and Q_1. Every value being higher than the High level counts as an outlier and every value that is smaller than the Low level is also considered an outlier. Calculations for High level and Low level are as follows: High level = Q_3 +1.5(IQR) and Low level = Q_1 – 1.5(IQR). The value for the last replicate, R30-5, labelled in red is determined as an outlier

Sample	*N*7-GA-Gua area adjusted	Outlier calculation			
R 30-1	-1591.33	min = Q_0	-3876.33		
R 30-2	-3876.33	Q_1	-1993.33	IQR	4654.00
R 30-3	2660.67	Q_2	-1591.33	High level	9641.67
R 30-4	-1993.33	Q_3	2660.67	Low level	-8974.33
R 30-5	11952.67	max = Q_4	11952.67		

3.3.2 Calibration line

All calibration lines for the injections of the standard solutions gave a linear dose response for the range of 25 fmol up to 150 fmol on column with a R^2 value of at least 0.99. All calibration lines established with CT DNA samples were linear within the range of 25 fmol to 150 fmol on column with a R^2 value of at least 0.84.

A typical calibration line in CT DNA with the *N*7-GA-Gua background levels subtracted can be seen in Figure 3.4.

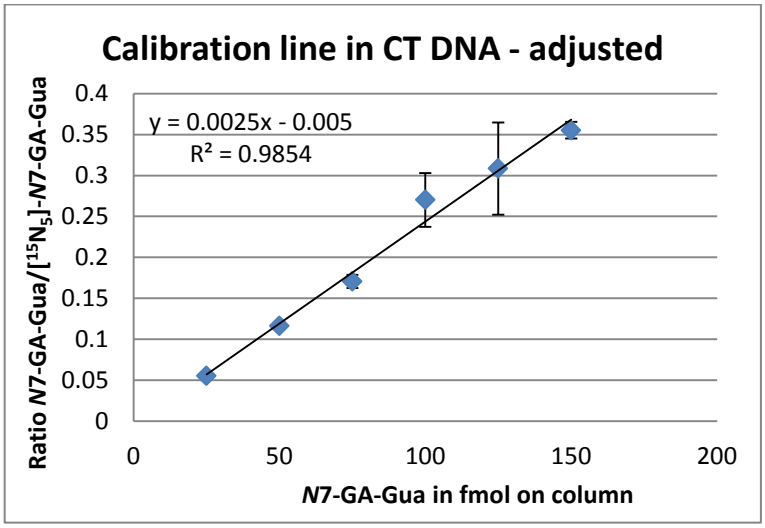


Figure 3.4: A typical calibration line for *N*7-GA-Gua spiked into CT DNA, hydrolysed and HPLC purified before analysis by LC-MS. Each sample was spiked with 500 fmol on column IS [$^{15}N_5$]-*N*7-GA-Gua. The *N*7-GA-Gua background levels were subtracted before plotting the ratio of *N*7-GA-Gua peak area/[$^{15}N_5$]-*N*7-GA-Gua peak area (data from Validation day 4).

Once the values were adjusted for the background levels R^2 values were at least 0.80 with one exception where 0.37 was achieved. **Table 3.3** shows the R^2 values for each day of validation in detail, listing both standard and CT DNA values.

Table 3.3: All R^2 values for the *N*7-GA-Gua standard calibration lines and the CT DNA based calibration lines measured for all 6 validation days. The table shows the R^2 – raw (non-adjusted), R^2 adjusted (*N*7-GA-Gua background subtracted) and the R^2 adjusted, no negative values, where the *N*7-GA-Gua background was subtracted and the negative values were not considered. The coloured values have values that were excluded for evaluation of the R^2, and reasons are explained at the bottom of the table

	R^2 standards	R^2 CT DNA, raw	R^2 CT DNA adjusted	R^2 CT DNA adjusted, no negatives
Validation day 1	0.997	0.976	0.9224	
Validation day 2	0.9982	0.8481	0.8009[a]	
Validation day 3	0.9995	0.9663	0.3759	0.8608[b]
Validation day 4	0.9994	0.9861	0.9854	
Validation day 5	0.9985	0.9208	0.8733	
Validation day 6	0.9977	0.9969	0.9973	

[a]**Calibration point 75 fmol on column (triplicate) has large variation (RSD = 79%)**
[b]**Calibration point 75 fmol on column was deleted due to two negative values**

Analysis of the standard calibration lines achieved excellent linearity and good R^2 values. As expected, the biological matrix influenced the results and produced R^2 values that were slightly less ideal than achieved with standards. The low R^2 value for the calibration line of the second day of validation was a result of one inaccurate concentration within the calibration. The variation within the triplicate analysis of the amount of 75 fmol on column was large (RSD of ratio *N*7-GA-Gua peak area/[$^{15}N_5$]-*N*7-GA-Gua peak area = 78.7%). Without considering the triplicate analysis of the 75 fmol on column amount the R^2 value would improve to a value of 0.9815. On the third day of validation the adjusted R^2 value was 0.3759. This value was not acceptable but can be explained by two values of the triplicate analysis for the amount of 75 fmol on column. These negative values were due to subtracting the *N*7-GA-Gua background level. If the entire calibration point of 75 fmol on column was not considered when plotting the calibration line the R^2 value improved to 0.8608.

3.3.3 Accuracy

For an acceptable level of accuracy of the method the mean value should lie within the range of 85 - 115% of the theoretical value with it being raised to a range from 80 to 120% for the LLOQ.

Table 3.4 lists all values for the accuracy for the low, medium and high amounts of the validated method. The results are split into raw, adjusted and adjusted, no negatives.

Table 3.4: Accuracy of the method for all 6 days of validation listed for the amounts low (30 fmol on column), medium (100 fmol on column) and high (150 fmol on column) as Accuracy - raw (non – adjusted), Accuracy - adjusted (after *N*7-GA-Gua background level subtraction) and Accuracy – adjusted, no negatives (*N*7-GA-Gua background subtraction and removal of negatives). The coloured values have values that were excluded in the determination of the accuracy, and reasons explained at the bottom of the table

	Accuracy – raw (%)			Accuracy - adjusted (%)			Accuracy - adjusted no negatives (%)		
	30 fmol	**100 fmol**	**150 fmol**	**30 fmol**	**100 fmol**	**150 fmol**	**30 fmol**	**100 fmol**	**150 fmol**
Validation day 1	101.6	99.9	104.1	53.4	98.8	103.2	113.3[a]	98.8	103.2
Validation day 2	99.9	79.0	77.2	-14.6	83.8	88.5	91.0[b]	83.8	88.5
Validation day 3	86.7	95.0	104.1	-475.6	140.3	127.6	89.5[c]	110.0[c]	119.4[c]
Validation day 4	102.8	96.8	97.2	102.9	96.5	96.2			
Validation day 5	102.8	95.1	98.1	47.0	95.8	94.4	96.9[d]	95.8	94.4
Validation day 6	94.4	104.2	113.4	89.6	103.2	112.0			

[a]4 replicates, one negative value deleted → statistically proven outlier

[b]4 replicates, one negative value deleted → statistically proven outlier

[c]All values are based on an adjusted calibration line where the 75 fmol on column amount was deleted due to two negative values → no statistically proven outliers within the 75 fmol on column amount

[d]4 replicates, one negative value deleted → no statistically proven outlier

With the exception of Validation day 2 all values for the measured Accuracy (non-adjusted) for the amounts low, medium and high were within the requirements given by the FDA guidelines. Adjusting for the *N*7-GA-Gua background levels left only Validation day 4 and Validation day 6 within the acceptable range. The biggest deviation of the allowed range was seen for the lowest amount of 30 fmol (on column).

If on Validation day 1 one negative value was not considered within the 30 fmol on column amount the accuracy changed from 53.4% to 113.3%. On the second day of validation one negative value within the 30 fmol on column amount was not considered. This changed the Accuracy - adjusted from -14.6% to 91.0% for Accuracy – adjusted, no negatives. For both the first and second day of validation the negative value was an outlier as was statistically proven and was therefore excluded. Validation day 3 showed a huge variation in the 75 fmol on column amount (RSD for the ratio of *N*7-GA-Gua peak area/[$^{15}N_5$]-*N*7-GA-Gua peak area = -171%) and for the calibration line that amount was deleted due to two negative values. With taking this adjusted calibration line to calculate the accuracy the values were within the allowed range for the low and medium amounts (89.5% for low and 110.0% for medium amount) but not for the high amount (119.4%). The 150 fmol on column amount should not exceed the value of 115%. Testing the triplicate values for the amount of 75 fmol on column with the huge variation and two negative values for outliers revealed that the data set did not contain any outliers. In the low amount of Validation day 5 one negative value was not considered which changed the Accuracy – adjusted from 47.0% to 96.9% for Accuracy – adjusted, no negatives. When the low amount – adjusted data set was tested for outliers it revealed that the negative value was not considered to be an outlier.

3.3.4 Precision and inter-day precision

For the values to be eligible to pass the validation regulations the precision of the mean value must be within the range of variation of ±15% of the RSD or ±20% of the RSD for the LLOQ. Intraday precision is the precision calculated for every day.

Combining all the values for the intraday precision gives the data for the inter-day precision.

The precision for the runs of the standard solutions were calculated and are shown in Table 3.5.

Table 3.5: Precision of the method for the analysed standard solutions for the low (30 fmol on column), medium (100 fmol on column) and high (150 fmol on column) amounts

	Precision – raw (RSD%)			Precision - adjusted (RSD%)		
	30 fmol	**100 fmol**	**150 fmol**	**30 fmol**	**100 fmol**	**150 fmol**
Validation day 1	12.5	2.7	1.3			
Validation day 2	25.8	2.2	1.7	3.3[a]	2.2	1.7
Validation day 3	3.8	2.2	0.9			
Validation day 4	2.2	1.8	3.3			
Validation day 5	2.1	2.6	2.3			
Validation day 6	6.0	6.3	1.6			

[a]4 replicates, one value deleted due to almost double the *N*7-GA-Gua peak area compared to the other injections → statistically proven outlier

All measured standard precisions were within the allowed range with the exception of one value for the Validation day 2 for the low amount. One value of the 5 replicates had almost twice the *N*7-GA-Gua peak area compared to the other 4 samples. If this value was excluded the precision for this concentration decreased from 25.8% to 3.3%. The statistical test revealed it to be an outlier.

Table 3.6 lists the precision for each day of validation for each amount of CT DNA based samples non-adjusted and after adjustment for *N*7-GA-Gua background levels and negatives.

Table 3.6: Precision of the method for the analysed CT DNA based samples for the low (30 fmol on column), medium (100 fmol on column) and high (150 fmol on column) amounts. Values are listed for Precision - raw (non-adjusted), Precision – adjusted (*N*7-GA-Gua background levels subtracted) and Precision – adjusted, no negatives (*N*7-GA-Gua background levels subtracted and negatives removed). The coloured values have values that were excluded in the determination of the precision, as explained at the bottom of the table

	Precision – raw (RSD%)			Precision - adjusted (RSD%)			Precision – adjusted, no negatives (RSD%)		
	30 fmol	100 fmol	150 fmol	30 fmol	100 fmol	150 fmol	30 fmol	100 fmol	150 fmol
Validation day 1	21.4	3.1	6.9	275.8	11.1	8.1	62.4[a]	11.1	8.1
Validation day 2	24.0	2.9	1.8	-1616.8	3.7	2.2	5.2[b]	3.7	2.2
Validation day 3	13.7	9.4	7.8	29.8	9.5	5.5	6.4[c]	9.5	6.1
Validation day 4	8.3	8.0	4.1	12.6	8.8	4.4			
Validation day 5	16.9[d]	4.5	3.6	246.2	5.2	3.7	34.7[e]	5.2	3.7
Validation day 6	9.3	9.8	19.9	12.9	11.2	20.7	12.9	11.2	5.2[f]

[a] **4 replicates, one negative value deleted → statistically proven outlier**

[b] **Triplicate, one negative value and highest *N*7-GA-Gua area value deleted → statistically proven outliers**

[c] **Triplicate, two values almost half as much as other samples → no statistically proven outliers**

[d] **This amount had a big variation (RSD mean *N*7-GA-Gua peak area = 62%)**

[e] **4 replicates, one negative value deleted; the low amount replicates had a big variation (Ratio RSD = 246%) → no statistically proven outlier**

[f] **4 replicates, deleted one value where the IS area was less than half of the other 4 injections areas → statistically proven outlier**

The values for the non-adjusted precision of the developed method were within the allowed range except for two 30 fmol on column amounts on Validation day 1 and Validation day 2 and for the 150 fmol on column amount on Validation day 6. The RSD for mean *N*7-GA-Gua peak area for those three days showed a big variation with it being 25.9% for Day 1, 41.0% for Day 2 and 39.4% for Day 6. The 16.9% for the low amount of Day 5 is still within the allowed range but the variation within the *N*7-GA-Gua peak areas was high as shown in the RSD for the mean *N*7-GA-Gua peak area = 61.9%.

When adjusting for the *N*7-GA-Gua background levels the values for the low amounts increased above the allowed 20% for Validation day 1, 2, 3 and 5. The medium 100 fmol on column amount on all 6 validation days was within the allowed range. The background level adjustment did not influence the results of the high amount (150 fmol on column) of Validation day 6.

Excluding one negative value for the low amount of Validation day 1 and 2 improved the precision on the second day of validation but not on the first, where precision of 62.4% exceeded the allowed range of 20%. The excluded values for the low amounts of Day 1 and Day 2 were statistically proven outliers. The variation for the mean area of the *N*7-GA-Gua adduct peaks (30 fmol on column) of the 5 replicates of Validation day 3 is big (RSD for mean *N*7-GA-Gua area = 30.3%). If this amount was adjusted by deleting two values where the *N*7-GA-Gua peak areas were almost half as big as the remaining three the precision changed to 6.4%. Removing one negative value for the 30 fmol on column amount of Validation day 5 decreased the precision from 246.2% to 34.7% but was still not within the allowed range. Running the statistical tests on the low amounts for Validation day 3 and Validation day 5 revealed that these values were not considered as outliers. The 5 replicates of the high amount of Validation day 6 had a big variation (RSD for mean peak area of *N*7-GA-Gua =42.3%, RSD for the mean peak area of [$^{15}N_5$]-*N*7-GA-Gua = 32.9% and RSD for ratio of *N*7-GA-Gua peak area/[$^{15}N_5$]-*N*7-GA-Gua peak area = 20.7%). One value for the IS [$^{15}N_5$]-*N*7-GA-Gua peak area was less than half as big as the ones of the remaining 4 replicates and excluding that value changed the precision – adjusted, no negatives to 5.2%. This run also showed the

lowest *N*7-GA-Gua area peak within the 5 replicates. Statistical tests confirmed this value as an outlier.

Table 3.7 shows the data for the inter-day precision of all 6 separate days of validation combined, listed as non-adjusted, adjusted for background and adjusted, no negatives.

Table 3.7: Inter-day precision calculated for the method and presented for the low (30 fmol on column), medium (100 fmol on column) and high (150 fmol on column) amounts. Values are listed for Inter-day precision –raw (non-adjusted), Inter-day precision – adjusted (*N*7-GA-Gua background levels subtracted) and Inter-day precision – adjusted, no negatives (*N*7-GA-Gua background levels subtracted and negative values removed)

Inter-day precision - raw (RSD%)			**Inter-day precision - adjusted (RSD%)**			**Inter-day precision Adjusted, no negatives (RSD%)**		
30 fmol	**100 fmol**	**150 fmol**	**30 fmol**	**100 fmol**	**150 fmol**	**30 fmol**	**100 fmol**	**150 fmol**
47.8	16.5	12.5	227.5	13.6	13.9	31.0	13.6	13.9

The combined non-adjusted data for the precision used to establish the inter-day precision of the method led to higher values than the guidelines allow in respect to the low and medium amounts. After adjusting for the *N*7-GA-Gua background levels the inter-day precision deteriorated for the low but improved on the medium amount, whereas the high amount only showed a minor change. Taking away the negative values within the low adjusted amount (one replicate each for Validation day 1, 2 and 5) improved the RSD for the inter-day precision to 31.0%. Considering that the negative value of Validation day 5 was not considered as an outlier would increase the inter-day precision of 30 fmol on column to 58.1%. Removing the outlier of the data set for the high amount (Validation day 6) only increased the inter-day precision for the 150 fmol on column amount from 13.86% to 13.76%.

3.3.5 Recovery

Table 3.8 presents the recoveries of the *N*7-GA-Gua adduct in CT DNA determined in the validation process.

Table 3.8: Recovery for the *N*7-GA-Gua adduct for the method, shown as low (30 fmol on column), medium (100 fmol on column) and high (150 fmol on column) amounts. Values are listed for Recovery - raw (non-adjusted), Recovery – adjusted (*N*7-GA-Gua background levels subtracted) and Recovery – adjusted, no negatives (*N*7-GA-Gua background levels subtracted and negative values removed). The coloured values have values removed for the analysis of the recovery for that given amount and reasons are explained at the bottom of the table

	Recovery – raw (%)			Recovery - adjusted (%)			Recovery Adjusted, no negatives (%)		
	30 fmol	**100 fmol**	**150 fmol**	**30 fmol**	**100 fmol**	**150 fmol**	**30 fmol**	**100 fmol**	**150 fmol**
Validation day 1	234.6	140.5	103.7	11.8	79.3	59.4	65.9[a]	79.3	59.4
Validation day 2	275.3	90.8	81.0	194.9	62.3	61.4	53.4[b]	62.3	61.4
Validation day 3	109.7	45.3	74.0	56.5	26.0	60.1	56.5	48.0[c]	60.1
Validation day 4	109.6	83.2	82.5	57.6	69.4	71.1	57.6	69.4	71.1
Validation day 5	94.1	59.2	77.3	67.9	51.2	71.8	67.9	54.4[d]	71.8
Validation day 6	85.7	78.0	65.0	60.2	69.7	59.5	60.2	69.7	59.5

[a]**Duplicate, three negative values deleted → no statistically proven outliers, instead the highest *N*7-GA-Gua peak area was confirmed to be a statistically outlier**

[b]**Triplicate, values for highest and lowest areas deleted → statistically proven outliers**

[c]**Triplicate, two negative values deleted → no statistically proven outliers**

[d]**Triplicate, highest and lowest values deleted → statistically proven outliers**

The calculated *N*7-GA-Gua recovery in CT DNA showed big variation for both the data sets of Recovery (non-adjusted) and the Recovery - adjusted. The data set Recovery, adjusted – no negatives showed recovery values between 48.0% and 79.3%.

Removing three negative values for the low amount for Validation day 1 changed the recovery from 11.8% to 65.9%. However, these values were statistically not outliers. The excluded values for the highest and lowest peak areas for *N*7-GA-Gua on Validation day 2 for the lowest amount changed the recovery from 194.9% to 53.4% and after testing were considered as outliers. The Validation day 3 had two negative values within the analysis of the replicates for the 100 fmol on column amount. Removing these values increased recovery from 26.0% to 48.0% but after statistically testing they were considered not to be outliers. Within the 100 fmol on column amount for Validation day 5 the highest and the lowest *N*7-GA-Gua peak area values were excluded and the values were confirmed to be outliers.

Averaging the data of the 6 validation days gave for each amount (low, medium, high) the mean recovery ± standard deviation of 60.3% ± 5.6 (for 30 fmol on column), 63.9% ± 11.4 (100 fmol on column) and 63.9% ± 5.9 (150 fmol on column). This shows that the values for the recovery of the *N*7-GA-Gua adduct between different amount on column are comparable.

3.3.6 Limit of detection and limit of quantification

The LOD for this validated method is 25 fmol (7 adducts/10^8 nucleotides) and the LOQ 30 fmol (9 adducts/10^8 nucleotides). The adduct/10^8 nucleotides values were obtained by using the data that was adjusted for background.

3.3.7 Stability

3.3.7.1 Internal standard stability

Leaving the IS [$^{15}N_5$]-*N*7-GA-Gua at RT for 6 hrs did not have any impact on its stability. The recovery of the IS after staying at RT for 6 hrs prior to analysis was 100.0%.

3.3.7.2 Freeze-thaw stability

The recoveries for the freeze-thaw experiment for the low (30 fmol on column) and high (150 fmol on column) amounts are summarised in Table 3.9.

Table 3.9: Freeze thaw stability for the low (30 fmol on column) and high (150 fmol on column) amount reported for as freeze thaw stability - raw (non-adjusted), freeze thaw stability – adjusted (*N*7-GA-Gua background levels subtracted) and freeze thaw stability – adjusted, no negatives (*N*7-GA-Gua background levels subtracted and negative values removed). The coloured value had one value that was excluded in the calculation for the freeze thaw stability as explained at the bottom of the table

Freeze thaw stability – raw (%)		Freeze thaw stability – adjusted (%)		Freeze thaw stability - adjusted, no negatives (%)	
30 fmol	**150 fmol**	**30 fmol**	**150 fmol**	**30 fmol**	**150 fmol**
275.7	99.5	465.4	99.8	140.1[a]	99.8
[a]**Duplicate, lowest value deleted → no statistically proven outlier**					

All values, from “non-adjusted” to “adjusted, no negatives” gave similar values for the high amount (150 fmol on column). The triplicate analysis for the low amount (30 fmol on column) had a variation of RSD = 97% for the ratio of *N*7-GA-Gua peak area/[$^{15}N_5$]-*N*7-GA-Gua peak area for the non-adjusted data set. RSD deteriorated with subtraction of the background levels to 121%. The adjusted, no negatives stability value of 140.1% was achieved by excluding the lowest *N*7-GA-Gua peak area within the triplicate. However, statistically this value was not an outlier. It seems that the recovery of the *N*7-GA-Gua adduct in the setting of the freeze thaw stability achieved a higher value compared to the recovery determined within each validation day.

3.3.7.3 Short term temperature stability

The result for the short term temperature stability for the low (30 fmol on column) and high (150 fmol on column) amount is shown in Table 3.10.

Table 3.10: Short term temperature stability for the low (30 fmol on column) and high (150 fmol on column) amount reported as short term temperature stability – raw (non-adjusted), short term temperature stability – adjusted (*N*7-GA-Gua background levels subtracted) and short term temperature stability – adjusted, no negatives (*N*7-GA-Gua background levels subtracted and negative values removed). The coloured value had one value that was excluded in the calculation for the freeze thaw stability as explained at the bottom of the table

Short term temperature stability – raw (%)		Short term temperature stability – Adjusted (%)		Short term temperature - adjusted, no negatives (%)	
30 fmol	**150 fmol**	**30 fmol**	**150 fmol**	**30 fmol**	**150 fmol**
114.0	101.3	126.7	103.0	94.8[a]	103.0
[a]Duplicate, highest value deleted → no statistically proven outlier					

All values, from "non-adjusted" to "adjusted, no negatives" gave similar values for the high amount (150 fmol on column). The variation within the triplicate injection for the low amount (30 fmol on column) was slightly high, with a RSD = 23.1%. After adjusting for the *N*7-GA-Gua background levels the recovery changed to 126.7% for the *N*7-GA-Gua adduct standard. After excluding the highest *N*7-GA-Gua peak area value in the triplicate the recovery decreased to 94.8%. This highest value was not statistically confirmed as outlier. As seen for the freeze thaw experiment it appeared that recovery was increased compared to the recovery experiments determined on every day of validation.

3.3.7.4 Long term stability

The CT DNA samples (triplicate) for the low and high amount were kept at -20°C for 10, 20 and 27 weeks prior to analysis and compared to freshly pipetted samples. After analysis the ratio recovery for 10 weeks non-adjusted was 114.5% whereas the high dose had a non-adjusted recovery of 117.3%. The recovery for the 30 fmol on column amount after storing the samples for 20 weeks was 121.1% and 120.5% for the 150 fmol on column amount. The raw low dose recovery of the samples stored for 27 weeks was 46.7% and 100.7% for the high amount (see Table 3.11).

Table 3.11: Long term stability for the low (30 fmol on column) and high (150 fmol on column) amount reported for as long term stability - raw (non-adjusted), long term stability – adjusted (*N*7-GA-Gua background levels subtracted) and for the 27 weeks stability the long term stability – adjusted, no negatives (*N*7-GA-Gua background levels subtracted and the one high value removed)

	Long term stability - raw		Long term stability – adjusted		Long term stability - adjusted, no negatives
	30 fmol	**150 fmol**	**30 fmol**	**150 fmol**	**30 fmol**
10 Weeks	114.5	117.3	113.1	117.2	
20 Weeks	121.1	120.5	121.2	120.4	
27 Weeks	46.7[a]	100.7	24.8[a]	103.6	82.7[b]
[a]**Mean ratio RSD values (*N*7-GA-Gua/[$^{15}N_5$]-*N*7-GA-Gua) of the control 30 fmol on column amount samples were very high: 87.2%**					
[b]**Deleted one value, highest *N*7-GA-Gua area**					

Adjusting the data by subtracting the *N*7-GA-Gua adduct background level of the blank CT DNA injections spiked with only [$^{15}N_5$]-*N*7-GA-Gua resulted in a recovery of 113.1% and 117.2% for low and high amount after storage for 10 weeks respectively. The 20 weeks storage led to an adjusted recovery of 121.1% and 120.4% for low and high amount respectively. The adjusted data for week 27 gave a recovery of 24.8% and 103.6% for the low and high amount respectively. The low values of the 30 fmol on column amount for both raw and adjusted data are due to a high variability in the *N*7-GA-Gua peak areas (RSD of ratio 88.0%) of the control 30 fmol on column samples. One peak area had almost a 4 fold higher area than the remaining two samples. Deleting this value increased the recovery for the 30 fmol on column amount to 82.7%.

3.4 Discussion

Following method development for the determination of the *N*7-GA-Gua adduct in leukocyte DNA, the validation of the method was undertaken on 6 different days. On each of the days the linearity, accuracy, precision, and recovery was analysed. LOD and LOQ were determined for every day of validation and additionally the stability of the standard in the matrix as well as the stability of the IS was tested.

Comparing this validation to previous publications in the literature, working either with the same analyte or on different DNA adducts, variations within the validation process are notable and often it is not stated which guidelines were followed. The validation process described by da Costa *et al*. was shorter than the one described in this chapter [73]. They treated ST DNA with two different concentrations of GA and used the resulting modified DNA to validate the accuracy and precision. They also validated a second time but spiked both labelled and unlabelled adduct to untreated ST DNA and again reported values for accuracy and precision. Other validation schemes will be discussed within the following sections.

3.4.1 Limitations of the validation study

The reason for using CT DNA instead of human leukocyte DNA as matrix was done due to insufficient availability of large amounts of whole blood. As mentioned before the process for this validation would have required a large volume of whole blood. The most problematic part of the method validation that occurred by using CT DNA as a matrix was the presence of a detectable background level of the *N*7-GA-Gua adduct. Adjusting for this background by subtracting its average *N*7-GA-Gua peak area (determined within the triplicate blank injections, see Figure 3.3) from all CT DNA based samples improved the values towards the expected ranges for the majority of the data sets. However, in some cases subtraction resulted in negative values. In addition some values appeared to be outliers, the reason for this being unknown. If the data were adjusted for these outliers nearly all values lay within the range of the given values of the FDA guideline. The possibility of using ST DNA instead of CT DNA, as done by da Costa *et al*. was also assessed in preliminary work (see section 2.2.2.4.6), but ST DNA also gave a background reading for the *N*7-GA-Gua adduct [73]. Another option for an analyte free matrix would have been the use of oligonucleotides. This way it would definitely have been an analyte free matrix without background but due to costs of these oligonucleotides it was not feasible for such a big validation assessment.

For the detection of the *N*7-ethylguanine adduct in leukocyte DNA described by Balbo *et al*. background levels of this adduct in both CT DNA and human buffy coat DNA was detectable [125]. As described for the validation of the method for the detection of *N*7-GA-Gua in leukocyte DNA they also subtracted their background levels from all samples in the validation process. In a study where the DNA adduct of 1,3-butadiene was analysed detectable endogenous levels were also subtracted [128].

Within the triplicate analysis of the background levels in the blank CT DNA injections for every day of validation there was already a variation in the *N*7-GA-Gua peak areas detectable. Subtracting these background levels from each CT DNA based samples an additional variation was added to the non-adjusted result. This had a bigger impact on the low amounts compared to the high ones, which can be seen especially at the low amount (30 fmol on column) for the results of accuracy and precision.

Before undertaking the validation, a trial validation day was processed to familiarise with the analysis and to check on the concentration range to use. No background *N*7-GA-Gua adduct was observed in the trial validation day. For the validation the same batch of CT DNA was used as for the trial validation day. One possibility of having varying background levels of *N*7-GA-Gua might be down to the sensitivity of the mass spectrometer on the day of analysis.

3.4.2 Outliers

For data analysis of each validation day, negative values resulted from subtracting the *N*7-GA-Gua background levels, and the sporadic higher or lower peak area in a data set were excluded. Afterwards those removed values were statistically tested to see if they were outliers. For most of the excluded values the statistical test verified that they are outliers but in some cases the negative values were not classified as outliers.

An example for the outlier calculation is presented in Table 3.2 for the low amount (30 fmol on column) of Validation day 1 where three out of 5 values are negative. In none of the analytical runs of the validation process are negative peak area values expected and would not occur in an ideal case (no background levels). Nevertheless testing for

outliers on the low amount recovery experiment of Validation day 1 did not recognise these negative values as outliers but the run that achieved the highest *N*7-GA-Gua peak area.

Also the data set for the determination of accuracy and precision of Validation day 5 for the low amount did not label the negative value as an outlier. Testing triplicate data sets did not lead to a value marked as an outlier as seen for the determination of the 75 fmol on column amount for Validation day 1, and the results for freeze thaw and short term temperature stability test. Maybe more values are needed for a more reliable result as increasing sample numbers decrease variability. The example of the inter-day precision for the high amount of Validation day 6 shows that outliers have less influence on the variability of higher amounts compared to low amounts and thus are more robust. Due to a small [$^{15}N_5$]-*N*7-GA-Gua area peak the value was an outlier. However, removing this value from the data set only increased the inter-day precision by 0.1%.

3.4.3 Calibration lines

All standard calibration lines were linear and had a R^2 value of almost 1 whereas the variation from 1 was greater for the calibration lines gained with the CT DNA samples. This is attributable to the presence of biological matrix. Matrix impurities can co-elute and cause ionisation suppression during LC-MS analysis. Most papers publish R^2 matrix based calibration lines closer to 1, for example Watzek *et al*. giving a R^2 value of at least 0.999 for both the high and low dose experiment [121]. For the validation of the detection of *N*7-ethylguanine by Balbo *et al*. R^2 of at least 0.99 were stated [125].

3.4.4 LOD and LOQ

The determined LOD and LOQ for this validated method are 25 fmol and 30 fmol, being 7 adducts/10^8 nucleotides and 9 adducts/10^8 nucleotides respectively after *N*7-GA-Gua background level subtraction. Retrospectively, a different concentration range for the calibration line that determined the LOQ should have been chosen. The average S/N

for all 25 fmol on column injections was 9.0 whereas the average S/N for the 30 fmol on column injections was 9.3. Both determinations have a high RSD value of 42% and 45%, respectively and both values are close to each other and exceed the S/N = 3 that was aimed for by far. Considering this aspect of actually having LOD and LOQ within the same concentration, with the exception of Validation day 5, where the 50 fmol on column amount only gave a S/N = 6.6, a 10 – 15 fmol on column injection would have most likely been still detectable, leading to a S/N = 3. To then minimise the impact of having a background level on such a low spiked amount it would be advisable to use the IS to spike varying amounts for the calibration line and to use the unlabelled adduct as an IS.

Assessing the achieved LOD and LOQ with values reported previously does not result in a more sensitive method than reported in previous studies for the detection of the *N*7-GA-Gua adduct. The LOD for the method described by da Costa *et al*. for the *N*7-GA-Gua adduct was 0.5 adducts/10^8 nucleotides and the LOQ for the *N*7-GA-Gua adduct was 2 adducts/10^8 nucleotides whereas the group of Doerge *et al*. report a LOD for the *N*7-GA-Gua adduct of even 0.5 adducts/10^8 nucleotides and a LOQ for the *N*7-GA-Gua adduct of 1 adduct/10^8 nucleotides [73,89]. Watzek *et al*. reported two different LOD and LOQ values for a high and low dose experiment [121]. The experiment where they fed rats high doses of AA had a LOD of 8 pmol and a LOQ of 17 pmol that equals 1 adduct/10^8 nucleotides and 3 adducts/10^8 nucleotides, respectively. The LOD and LOQ for the low dose experiment was 1 and 2 fmol, being 0.15 adducts/10^8 nucleotides and 0.25 adducts/10^8 nucleotides, respectively.

3.4.5 Accuracy and precision

Two values within this validation process did not fit the range given by the FDA guidelines that was followed. The high amount (150 fmol on column on Validation day 3) had an accuracy of almost 120% (value for Accuracy - adjusted, no negatives) which should not exceed 115%. The precision for the low amount (30 fmol on column on Validation days 1 and 5) was 64% and 35% (both values within Accuracy - adjusted, no

negatives group) and should not exceed 20%. No obvious explanation for why they deviate can be given.

3.4.6 Recovery

Due to the detectable *N*7-GA-Gua background levels some values for recovery were exceptionally high and hence they do not reflect the true value for the recovery of the spiked amounts of the *N*7-GA-Gua adduct. The adjustment of subtracting the background levels improved these values. However, only the additional adjustment by excluding the negative values and outliers achieved a less varied result for the recoveries of all validation days. Averaging all concentrations from all validation days gives a mean plus/minus standard deviation *N*7-GA-Gua recovery of 62.7% ± 7.8%. Neither Watzek *et al*., Doerge *et al*. nor da Costa *et al*. reported on their recovery for the *N*7-GA-Gua adduct of their methods [73,89,121].

3.4.7 Stability tests

The results for the freeze thaw, short term temperature and long term stability tests showed recoveries of more than 100%. This might be down to an error that was made while setting these experiments up. Rather than just spiking the CT DNA samples with the unlabelled *N*7-GA-Gua adduct and adding the IS [$^{15}N_5$]-*N*7-GA-Gua at the very end before LC-MS analysis both the labelled and unlabelled adduct were added and sample tubes stored at -20°C (samples for long term stability), kept at RT (samples for short term temperature stability) or frozen and thawed (samples for freeze thaw stability). This mistake was bypassed by spiking an additional 500 fmol on column [$^{15}N_5$]-*N*7-GA-Gua prior to analysis by LC-MS so that the samples were spiked in total with 1000 fmol on column [$^{15}N_5$]-*N*7-GA-Gua.

The non-adjusted RSD values for the ratio of *N*7-GA-Gua peak area/[$^{15}N_5$]-*N*7-GA-Gua peak area for the low amount (30 fmol on column) of both the freeze thaw stability and the short term temperature stability were high, with 97.2% and 23.1% respectively leading to extremely high values for recovery. In comparison the RSD values for the

high amounts (150 fmol on column) were 2.2% (freeze thaw stability) and 2.9% (short term temperature stability). The adjusted, no outliers RSD values for the low amount were 19% (both freeze thaw and short term temperature stability), 5% (freeze thaw) and 4% (short term temperature) for the high amount. Instead of doing triplicate analysis it would have been better to analyse the stability tests by taking 5 replicates and thus try to minimise the variation by increasing the number of samples. However, although the variation was high within the samples of the low amount compared to the samples of the high amount they led to similar values for the recovery, which suggests that there is a different reason for the fact that the stability test recoveries of the *N*7-GA-Gua adduct led to higher values than the 63% achieved (averaged all recoveries for the adjusted, no outliers values of the 6 validation days) within the validation process. A likely explanation is that the recoveries within the validation were assessed by comparing the values achieved in CT DNA based samples to pure standard solutions in 0.1% FA in the absence of matrix whereas the recoveries of the stability tests were achieved by comparing against freshly pipetted samples in CT DNA. This way the matrix factor is eliminated and does not impact the result. However, this makes comparing the recoveries and stability test recoveries problematic.

An explanation for the high recovery of the short term temperature stability could be due to the samples being at RT for 24 hrs so that the solubility product of the *N*7-GA-Gua adduct had more time to equilibrate and dissolve more and hence led to a higher recovery compared to the samples being processed straight away where solubility of the *N*7-GA-Gua adduct might be a concern.

Looking at the results of the long term stability it is surprising that the *N*7-GA-Gua adduct level seems to increase with storage. The general assumption would be that the *N*7-GA-Gua adduct levels decrease with increasing storage time. But adverse effects can be seen when studying the results of the 10, 20 and 27 weeks results long term stability experiments. One possibility might be that within the process of the whole work-up procedure possible co-eluted interferences suppress the *N*7-GA-Gua adduct signal on the mass spectrometer. Due to storage these might be eliminated/degraded so that the *N*7-GA-Gua adduct can be measured without those

interfering compounds. The CT DNA used for the long term stability experiment, the spiked, stored samples as well as the freshly pipetted samples was dissolved and aliquoted on the same day. This should eliminate matrix factors because the blank as well as the freshly pipetted samples used for comparison is the same CT DNA. Another explanation could be that the mistake of pipetting the IS at the beginning and storing it cannot be bypassed as easily. With the matrix being the same for all samples another conclusion suggests itself, that it is down to the standard but in case the *N*7-GA-Gua adduct degrades during storage the recovery should be less.

3.4.8 Outliers within validation process

For clarification of why single values within the analyses of triplicate or 5 replicates vary strongly without an obvious mistake occurring during process and analysis, all involved steps in the protocol were assessed. The equipment involved in the heating step within the hydrolysis process (samples for 1 hr at 70°C) was considered to have an influence on the result and hence the heating block inserts were tested (see section 2.2.2.7). For the different heating blocks 1, 2 and 3 the RSD of the ratio of *N*7-GA-Gua peak area/[$^{15}N_5$]-*N*7-GA-Gua peak area were 1.2%, 21.9% and 8.6% respectively showing that the samples undergo a different heat distribution depending on heating block which can impact the final results by adding an additional variation. Block 1 was filled up first and only if there were more samples Block 2 or 3 were topped up but it was not marked which samples were placed in Block 2 or 3.

3.4.9 Pipetting and mass spectrometer variation

Pipetting was considered to be one of the causes for the huge variation on the low amount points in this validation. Low doses are affected more by additional variation than higher doses. The mass spectrometer variation is also an aspect to consider. Although having an IS to adjust for changes in its sensitivity as well as work-up procedure, when analysing samples close to the LOD additional influences want to be

eliminated. The RSD of the ratio of *N*7-GA-Gua peak area/[$^{15}N_5$]-*N*7-GA-Gua peak area for a standard solution injected 5 times on to LC-MS from the same vial was 12.5%.

3.5 Summary

The validation described in this chapter was not a straightforward process mainly due to the existing *N*7-GA-Gua background levels in the CT DNA that made the process more challenging. Exchanging the matrix from human leukocyte DNA to CT DNA is a valid step when the method matrix is of limited availability [126]. Adjusting the data by not including values, negative values or values that appear to be outliers in the analysis leads to a statistical problem. In some sets the RSD calculation was based only on two values but it improved the value for the experiment.

One possibility to change the outcome of this validation would have been to switch the roles of labelled and unlabelled adduct but it is not yet a well-established method. With pipetting the [$^{15}N_5$]-*N*7-GA-Gua adduct in varying amounts (knowing there will be no background levels) and using the unlabelled *N*7-GA-Gua adduct as an IS the *N*7-GA-Gua background levels would not have had such a huge influence on the variability of the analysis for the lower amounts (25 and 30 fmol on column) applied. A final change would be to adjust the concentration range of the calibration line to lower levels to improve the LOD.

Chapter 4

4 Method Development - ELISA

4.1 Introduction

ELISAs are a very useful group of immunoassays for the specific detection of low levels of analytes. There are several ways in applying antibodies to analytical methods for the detection of analytes, and for small molecules the competitive ELISA is the method of choice to assure the antibodies are not sterically hindered and are able to attach to the analyte.

ELISA is a well-established analytical tool in clinical diagnostics, for example in HIV diagnostics [129], and due to the high selectivity of antibodies ELISAs can be very sensitive detection and quantification methods. It has a lot of advantages, as it is a cheap method and allows high sample throughput. ELISAs are also used for the detection of food contaminants like AA, DNA adducts of estrogen, aflatoxin B1 and benzo[a]pyrene and even insect pests for detection in grain samples [130-133].

ELISA is a versatile method where numerous alterations can be applied into the experimental design to ensure sensitivity, reliability, quantification and high throughput where required. Zhou *et al.* produced a hapten for the detection of AA in foods without using the actual analyte, AA, but a bigger molecule, *N*-acryl oxysuccinimide where parts cleave off during the antigen synthesis to leave the molecule of interest, AA connected to the protein [134].

The main aim of this project was to develop a competitive ELISA for the detection of the *N*7-GA-Gua adduct. This chapter describes the tests and experiments undertaken for its development.

4.2 Materials and methods

4.2.1 Materials

4.2.1.1 General chemicals

All chemicals were purchased from Sigma Aldrich, Poole, UK unless otherwise stated. Biotin-PEO-NHS, Water-Soluble and Pierce BCA Protein Assay Kit were purchased from ThermoScientific, UK.

4.2.1.2 Antibodies

The production of polyclonal antibodies raised in rabbits against the hapten derived from the *N*7-GA-Gua adduct was carried out by Davids Biotechnologie GmbH, Regensburg, Germany. The serum of two immunised rabbits was provided.

4.2.1.3 Equipment

Microlite ™ 2+ plates (96 well plate) were purchased from Dynex Technologies, West Sussex, UK. MLX (Microtiter Plate Luminometer) and MRW (multi-reagent microplate strip washer) were purchased from Dynatech, UK. HPLC was purchased from Varian, UK. Eppendorf Centrifuge 5415R was purchased from Eppendorf, UK. Sonic bath FS100b was purchased from Decon, UK. Titramax 100 (shaker) was purchased from Heidolph Instruments, UK. Auto lumat, Multi-Tube Luminometer was purchased from Berthold Technologies, Harpenden, UK. The HPLC column Aqua 5 µm C18 200 Å 250 x 4.6 mm 5 µm micron column was purchased from Phenomenex, Macclesfield, UK. Shimadzu spectrophotometer was purchased from Shimadzu, UK.

4.2.2 Methods

4.2.2.1 Antibodies

Prior to the start of this project antibodies were synthesised using a similar method. These were two monoclonal antibodies from hybridoma mouse cells and two

polyclonal antibodies, produced from rabbit sera, referred to throughout this chapter as M1, M2 and R1, R2 respectively. For this project two new polyclonal antibodies were produced, referred to as Ab1 and Ab2.

4.2.2.2 Chemistry – Hapten synthesis

*N*7-GA-Gua is a small molecule and consequently would not give an immune response in a host animal. Therefore it needs to be conjugated to a carrier protein before immunisation to elicit an immunogenic response. This is achieved by chemically modifying the *N*7-GA-Gua adduct to incorporate a linker which can be used to conjugate to a carrier protein.

The underlying synthesis shown in Figure 4.1 was developed by Dr R. Britton (Chemistry Department, UoL).

Characterisation of the products produced at each step was done by NMR and mass spectrometry. For NMR data see appendix section 8.1.

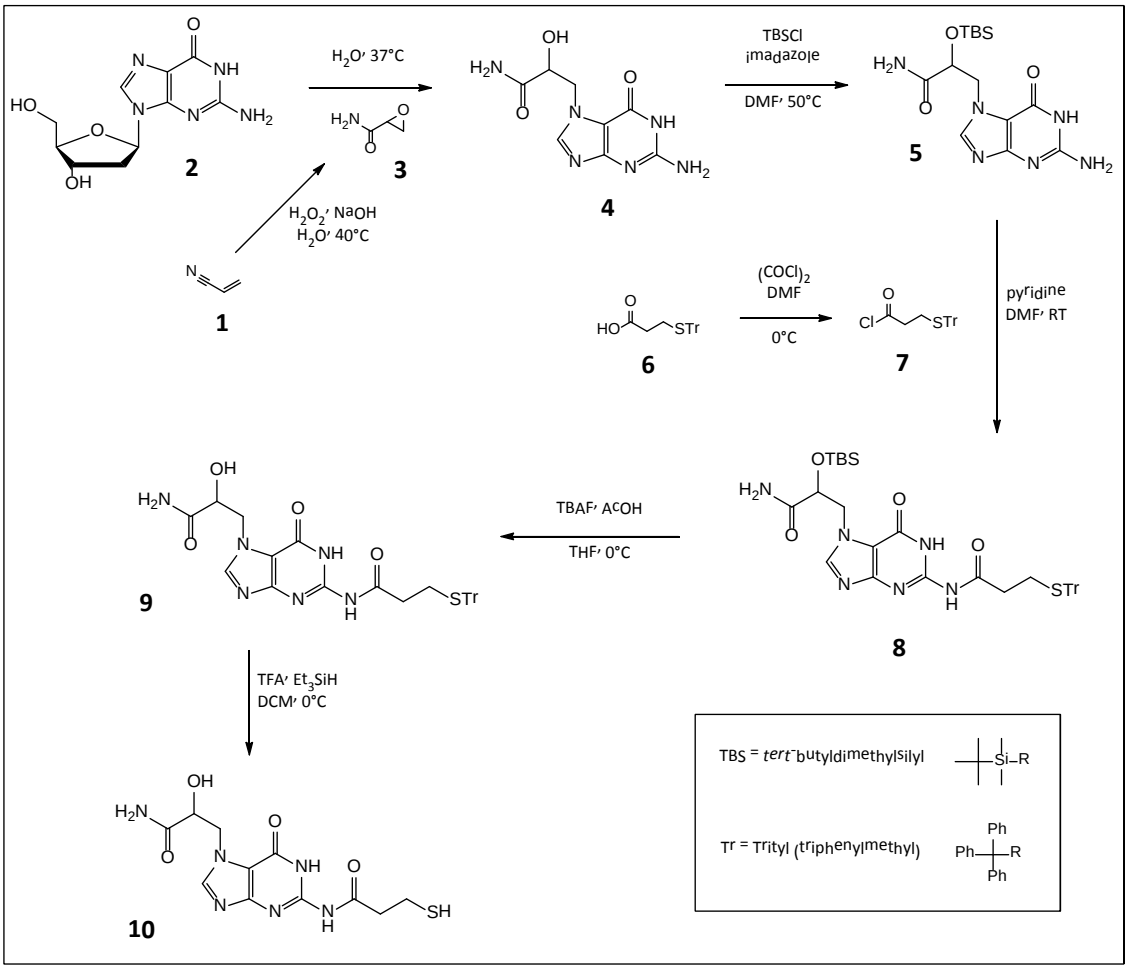


Figure 4.1: Hapten synthesis. **1** acrylonitrile, **2** deoxyguanosine monohydrate, **3** GA, **4** *N*7-GA-Gua, **5** *O*TBS-*N*7-GA-Gua, **6** *S*-Trityl-Thiopropionic acid, **7** *S*-Trityl-Thiopropanoylchloride, **8** *S*Tr-*O*TBS-Hap, **9** STr Hap, **10** Hapten

Glycidamide (3)

NOTE: GA (**3**) is toxic and may cause cancer and heritable genetic damage so exercise with caution when handling!

Acrylonitrile (**1**) (13.2 mL, 200 mmol) was mixed with ddH_2O (100 mL) and to this was added H_2O_2 (30%) (22.3 mL). This solution was stirred for 5 min. NaOH solution (1 M) was added drop wise, and the pH was monitored with a pH probe and kept between 7.3 and 7.8. When the pH reached 7.8 no more NaOH solution was added. Palladium (Pd) (0.2 g) on activated charcoal (10%) was added to the solution which was then kept stirring at RT for approximately 24 hrs. The Pd/C was removed by filtration through Celite filter aid. Acetone (100 mL) was cooled down on ice (0°C) and added to the filtrate which was then filtered again. The solvents were removed *in vacuo* using a

rotary evaporator. GA (**3**) remained as a yellow viscous liquid. The flask was left in the fridge to crystallise GA (**3**).

*N7-GA-Gua (**4**)*

Deoxyguanosine monohydrate (**2**) (1.0 g, 3.51 mmol) was dissolved in pre-warmed (37°C) ddH_2O (180 mL). GA (**3**) (3.1 g, 35.59 mmol) was dissolved in ddH_2O (20 mL) and added to the stirred solution. The solution was kept stirring for 7 days at 37°C. During this time a white precipitate was formed. The precipitate was filtered and washed with ddH_2O (3 x 10 mL). The *N*7-GA-Gua (**4**) (380 mg) was air-dried before drying in a desiccator.

*N*7-GA-Gua (**4**) yield: 45%

*OTBS-N7-GA-Gua (**5**)*

*N*7-GA-Gua (**4**) (1.9 g, 7.98 mmol) was transferred into a 250 mL round bottom flask. Anhydrous pyridine (25 mL) was added via a syringe. The suspension was then sonicated in a sonic bath to produce a more fine suspension before the pyridine was evaporated. The remaining *N*7-GA-Gua was suspended in pyridine (25 mL) and co-evaporated twice more. Anhydrous DMF (150 mL) was added followed by imidazole (2.7 g, 39.9 mmol) and TBDMSCl (6.02 g, 39.9 mmol). Then the flask was placed in an oil bath at 50°C and stirred for 18 hrs. After this time the completion of the reaction was checked by TLC. DMF was removed *in vacuo* and the brown solid remaining in the flask was dissolved in acetone (20 mL), stirred and heated. The precipitate was washed three times with hot acetone (20 mL) and twice with a mixture of 1:1 acetone/MeOH (*v/v*) (10 mL) then air dried. The dry *O*TBS-*N*7-GA-Gua (**5**) (1.33 g) was kept in the fridge and NMR performed to check the structure and purity.

*O*TBS-*N*7-GA-Gua (**5**) yield: 47%

*3-(Tritylthio)propanoyl chloride (**7**)*

S-Trityl-thiopropionic acid (**6**) (3.97 g, 11.3 mmol) was suspended in DCM (100 mL). DMF (0.2 mL) was added and the solution kept stirring at 0°C. Once the solution had been cooled down, oxalyl chloride (4.8 mL, 15.1 mmol) was slowly added to the

reaction. The suspension cleared and after one hr the reaction was finished. The volatiles were removed *in vacuo* to yield the acid chloride which was used without further purification.

*STr-OTBS-Hap (**8**)*

*O*TBS-*N*7-GA-Gua (**5**) (1.33 g, 3.78 mmol) was dried by co-evaporating with anhydrous pyridine (3 x 50 mL). The flask was flushed with nitrogen before pyridine (190 mL) was added to suspend the solid. The cloudy solution was stirred on ice at 0°C. 3-(Tritylthio) propanoyl chloride (**7**) was dissolved in dry DMF (40 mL) to produce a clear yellow solution. This solution of 3-(tritylthio)propanoyl chloride (**7**) was added to the stirred suspension of *O*TBS-*N*7-GA-Gua (**5**). After the addition was complete, the solution was allowed to warm to RT. The reaction mixture was kept stirring for 18 hrs. MeOH (100 mL) was added to the reaction and kept stirring for 30 min at RT. The solvents were removed using a rotary evaporator and a brown viscous liquid was obtained. TLC comparison verified reaction completion. The product was then purified by column chromatography. Silica 60 was used as a stationary phase and the mobile phase consisted of 93:7 DCM/EtOH (*v/v*). After collection of all fractions containing the *S*Tr-*O*TBS-HAP (**8**) (329 mg) the solvents were removed and yielded a yellowish coloured solid in the flask.

*S*Tr-*O*TBS-HAP (**8**) yield: 13%

*STr HAP (**9**)*

*S*Tr-OTBS-HAP (**8**) (0.32 g, 0.47 mmol) was dissolved in THF (15 mL) and acetic acid (81 µL) was added to this stirred solution. The flask was placed on ice and cooled down to 0°C before tetra-n-butylammonium fluoride (TBAF) in THF (1 M) (1.42 mL, 1.42 mmol) was added. The solution cleared to produce a yellow-orange coloured solution. This solution was stirred for 30 min at 0°C. Solvents were removed *in vacuo* and TLC verified that the product was formed in this reaction. *S*Tr HAP was purified by column chromatography. Silica 60 was used as a stationary phase. The mobile phase consisted of 85:15 DCM/EtOH (*v/v*) and was changed to 80:20 DCM/EtOH (*v/v*) to elute the product (137.8 mg).

*S*Tr HAP (**9**) yield: 52%

*Hapten (**10**)*

*S*Tr HAP (**9**) (137.8 mg, 0.242 mmol) was suspended in anhydrous DCM (10 mL) and cooled down to 0°C on ice. To this solution TFA (2 mL) was slowly added. The solution turned clear and yellow. Et_3SiH (85 µL) was added drop wise. The solution was stirred at 0°C for 30 min. The completion of the reaction was checked by TLC before the volatiles were evaporated. The residue was stirred with 0.5% TFA aq. (10 mL) and the volatiles were evaporated. This was repeated one more time. The compound (75mg) was triturated with n-hexane (10 x 1 mL) and then dried *in vacuo*. The purity was checked by NMR.

Hapten (10) yield: 95%

4.2.2.3 General information for the ELISA procedure

The following experimental parameters were constant throughout this chapter.

- PLATE: All described experiments were performed on a 96 well plate, Microlite ™ 2+ (96 well plate) by Dynex Technologies
- WASHING SOLUTION C: PBS pH 7.2
- WASHING SOLUTION B: PBS including 0.05% Tween
- WASHING STEP: Every plate wash was performed three times with 300 µL
- BSA: 0.5% BSA in PBS; for blocking the non-specific binding sites of the wells
- BIOTIN-ANTI-RABBIT (BAR)/BIOTIN-ANTI-MOUSE (BAM) ANTIBODIES: BAR was used in a 1:100000 and BAM in a 1:20000 dilution

4.2.2.4 Protein conjugation to hapten

Performance of experiment

For the antibody production the synthesised hapten was conjugated to a carrier protein for the animal to elicit an immunogenic response after injection of this antigen.

Three different proteins were used, keyhole limpet hemocyanin (KLH), ovalbumin and thyroglobulin (TGB).

TGB: TGB (10.8 mg) was dissolved in 0.1 M sodium dihydrogen orthophosphate (NaH_2PO_4*$2H_2O$) pH 8 (1080 µL). The thiol groups were blocked with an *N*-ethylmaleimide (NEM) solution (4.66 mg dissolved in 665.7 µL ddH_2O). The NEM solution (16.2 µL) was added to the TGB solution and incubated on the shaker for 10 min at RT. 6-Maleimidohexanoic acid *N*-hydroxysuccinimide ester (EMCS) (6 µmol) was dissolved in DMF. The TGB solution (1 mL) was mixed with the dissolved EMCS solution and kept on the shaker for 30 min at RT. A Sephadex G25 column was prepared and equilibrated with 2 column volumes of PBS before it was conditioned with 0.1 M NaH_2PO_4*$2H_2O$ pH 6.5. Then 1 mL of the TGB solution was put on the column and run through by gravity flow. The column was washed using 0.1 M NaH_2PO_4*$2H_2O$ pH 6.5 (2.4 mL). The protein fraction was eluted using 0.1 M NaH_2PO_4*$2H_2O$ pH 6.5 (3 mL). The hapten (3.77 mg) was dissolved in 10 µL DMSO and added to the TGB eluate and mixed quickly to prevent precipitation. This final solution was kept on the shaker for 5 hrs at RT before it was transferred into a dialysis membrane and stirred for 4 days at 4°C in 1 L PBS, with one change of PBS daily.

The dialysed TGB solution was then aliquoted and centrifuged for 10 min at 16100*g*, the protein concentration was determined before the conjugate was used in an experiment.

KLH AND OVALBUMIN: The conjugation of KLH and ovalbumin onto the hapten was performed the same way as described for the TGB conjugate.

Testing the conjugates

ON DAY 1 the plate was coated with 100 µL protein per well (100 ng protein/well) and kept on the shaker at RT overnight.

ON DAY 2 the plate was washed with solution C and blocked with 150 µL BSA solution/well. After incubation on the shaker for 1 hr the plate was washed with solution B before 100 µL antibody solution (100 ng/well) was added to each well. After

incubation on the shaker at RT for 2 hrs the plate was washed with solution B and 100 µL BAM/BAR per well was added and kept on the shaker for 1 hr at RT. The plate was washed with solution B and 100 µL streptavidin solution was pipetted into each well. After incubation for 1.5 hrs the plate was washed with solution B and analysed on the plate reader.

4.2.2.5 Antigen-Antibody-Titration

This experiment was set up in order to check which concentration of antigen and antibody gives the best competition to then apply in further experiments. By monitoring the changes in competition it should be possible to obtain the best sensitivity. This experiment was set up with the previously produced antibody M1.

ON DAY 1 the plate was coated with different amounts of ovalbumin-hapten conjugate per well. Concentrations of ovalbumin-hapten conjugate decreased in double dilutions from the top to the bottom of the plate (100, 50, 25 and 12.5 ng ovalbumin-hapten conjugate; green values in Table 4.1). Per well 100 µL per concentration was pipetted and incubated on the shaker at RT overnight.

Table 4.1: Plate design for the antibody-antigen-titration with decreasing concentration of ovalbumin-hapten conjugate (as shown in green) from top to bottom and decreasing antibody concentrations from the left to the right hand side of the plate. Wells in row A, C, E and G are blank measurements where only antibody was added, whereas rows B, D, F and H contained the antibody-*N*7-GA-Gua pre-incubated solution. Ov = ovalbumin, ab = antibody

	1	2	3	4	5	6	7	8	9	10	11	12
A	100 ng ov/ 100 ng ab		100 ng ov/ 80 ng ab		100 ng ov/ 60 ng ab		100 ng ov/ 40 ng ab		100 ng ov/ 20 ng ab		100 ng ov/ 10 ng ab	
B												
C	50 ng ov/ 100 ng ab		50 ng ov/ 80 ng ab		50 ng ov/ 60 ng ab		50 ng ov/ 40 ng ab		50 ng ov/ 20 ng ab		50 ng ov/ 10 ng ab	
D												
E	25 ng ov/ 100 ng ab		25 ng ov/ 80 ng ab		25 ng ov/ 60 ng ab		25 ng ov/ 40 ng ab		25 ng ov/ 20 ng ab		25 ng ov/ 10 ng ab	
F												
G	12.5 ng ov/ 100 ng ab		12.5 ov/ 80 ng ab		12.5 ng ov/ 60 ng ab		12.5 ng ov/ 40 ng ab		12.5 ng ov/ 20 ng ab		12.5 ng ov/ 10 ng ab	
H												

Controls were prepared with M1 concentrations and for samples the antibody solutions were mixed with 1 pmol *N*7-GA-Gua. Antibody concentrations decreased from the left plate side to the right hand side (100, 80, 60, 40, 20 and 10 ng antibody per well) (Table 4.1). The antibody-antigen solutions were incubated at RT overnight.

ON DAY 2 the plate was washed with solution C before 180 µL BSA solution/well was added and left on the shaker for 1 hr before the plate was washed with solution B. 100 µL of the pre-incubated antibody-antigen solution was pipetted into each designated well. The plate was kept on the shaker for incubation at RT overnight.

ON DAY 3 the plate was washed with solution B and 100 µL BAM was added into each well. After incubation on the shaker at RT for 1 hr the plate was washed with solution B. 100 µL streptavidin solution was pipetted per well and kept in the dark for incubation at RT for 1.5 hrs. The plate was washed with solution B and analysed on the plate reader.

4.2.2.6 Ovalbumin conjugation to *N*7-GA-Gua (linker glutaraldehyde)

Antibodies were raised against a hapten-TGB conjugate using EMCS as a linker. In order to avoid false positive binding of the antibodies to TGB a new conjugate was prepared using the protein ovalbumin. Ovalbumin was conjugated onto *N*7-GA-Gua using glutaraldehyde as a linker. The purpose of this experiment was to have a shorter linker between molecule and protein in an attempt to create a conjugate from which the antibodies could be displaced more easily without binding too strongly. The conjugate was then used to coat the plate when working with the competitive ELISA design. Using a different linker and protein, hopefully increases the specificity of the antibodies towards the antigen *N*7-GA-Gua.

Ovalbumin-N7-GA-Gua conjugation

Ovalbumin (49.98 mg) was dissolved in PBS (1 mL), glutaraldehyde solution (25%) (10 µL) was added and the solution left on a rotating table for 1 hr at RT. *N*7-GA-Gua (11.4 mg) was dissolved in 1:1 TFA/trifluorethanol (TFE) (*v/v*) (300 µL). The Sephadex G25

column was prepared by washing with three column volumes of PBS. The ovalbumin solution was applied to the column and washed with PBS (2.4 mL) before the ovalbumin-glutaraldehyde conjugate was eluted with PBS (3 mL). The eluate was combined with the *N*7-GA-Gua solution but formed a precipitate. TFE (2 mL) was added and the solution placed briefly in a sonic bath. The solution was kept on a rotating table for 3 hrs before lysine (10 mg) was added and the solution placed in a dialysis membrane in 1 L PBS at 4°C. The PBS solution was changed twice a day for 2 days.

The conjugate solution was dialysed and the protein content determined by BCA assay (see section 4.2.2.8).

Second Ovalbumin-N7-GA-Gua conjugation

Ovalbumin (49.5 mg) was dissolved in PBS (1 mL), glutaraldehyde solution (25%) (10 µL) was added and the solution left on the rotating table for 1 hr at RT. *N*7-GA-Gua (10.8 mg) was dissolved in 1:1 TFA/TFE (*v/v*) (200 µL). The Sephadex G25 column was prepared by washing with three column volumes of PBS. The ovalbumin solution was applied to the column and washed with PBS (2.4 mL) before the ovalbumin-glutaraldehyde conjugate was eluted with PBS (3 mL). The eluate was mixed with 2 M sodium carbonate (Na_2CO_3) solution (500 µL) before it was combined with the *N*7-GA-Gua solution. Some precipitate formed, TFE (2 mL) was added and the solution placed briefly in a sonic bath. The solution was kept on the rotating table for 3 hrs before lysine (10 mg) was added and the solution placed in a dialysis membrane in 1 L PBS at 4°C. The PBS solution was changed twice a day, for 2 days.

The conjugate solution was dialysed and the protein content determined by BCA assay (see section 4.2.2.8).

Testing of the conjugates

ON DAY 1 the plate was coated with 100 µL ovalbumin conjugate (500 ng protein/well) and incubated overnight at RT.

ON DAY 2 the plate was washed with solution C and 180 µL BSA solution pipetted into each well. After incubation for 1 hr the plate was washed with solution B and 100 µL antibody solution (M1, M2, R1, R2) was added (100 ng ab/well). After incubation for 2 hrs the plate was washed with solution B and 100 µL BAR/BAM per well was pipetted. The plate was incubated for 1 hr and washed with solution B before 100 µL streptavidin solution was added and the plate was incubated for 2 hrs before washing with solution B and reading on a luminometer.

4.2.2.7 Protein A column - Purification of antibodies

The IgG fraction of the polyclonal antibodies was purified from the rabbit serum using a Protein A column.

Antibody purification

The Protein A solution was diluted with 100 mM Tris-HCl buffer and divided evenly on two columns. The columns were then equilibrated with six times 100 mM Tris-HCl pH 8 (2 mL). 1 mL of each rabbit serum was mixed with 200 mM Tris-HCl pH 8 (1 mL) and applied to the columns. To improve the binding the solution was re-circulated through the column twice. The column was washed ten times with 100 mM Tris-HCl pH 8 (2 mL). To reduce the buffering capacity, the column was washed with 10 mM Tris-HCl pH 8 (2 mL). Before elution of the IgG fraction the OD 280 nm was measured on a spectrophotometer to assure it was zero. Then 2 M Tris-HCl (400 µL) was placed in a bijoux tube and the antibody eluted into Tris-HCL with two times 100 mM glycine-HCl pH 2.5 (1 mL). The columns were washed with 100 mM Tris-HCl pH 8 and azide was added for storage in the fridge.

Testing the antibodies

ON DAY 1 the plate was coated with 100 µl ovalbumin-hapten conjugate (100 ng/well) and kept on the shaker at RT overnight.

ON DAY 2 the plate was washed with solution C before 180 µL BSA/well was pipetted and incubated for 1 hr. The plate was washed with solution B and 100 µL/well different

antibody concentrations were added (1000, 500, 250, 125, 62.5, 31.25, 15.6 and 7.8 ng/well) and incubated on the shaker for 2 hrs. The plate was washed with solution B before 100 µL BAR was added into each well. After 1 hr on the shaker at RT the plate was washed with solution B and 100 µL streptavidin solution was added. The plate was incubated for 1.5 hrs, washed with solution B and read on a luminometer.

4.2.2.8 Protein quantification – BCA assay

The BCA assay was used to measure the protein content of the protein conjugates.

The Pierce BCA Protein Assay Kit was used for the protein quantification. 100 µL BSA stock solution (2 mg/mL) was used to prepare the calibration line. Table 4.2 shows the pipetting scheme for the BSA solution for the standard calibration line.

Table 4.2: Pipetting scheme for the preparation of the standard calibration line made with a BSA stock solution with a concentration of 2 mg/mL

Vial	ddH_2O in µL	BSA stock in µL	Final concentration in µg/µL
A	0	30	2
B	12.5	37.5	1.5
C	32.5	32.5	1
D	17.5	17.5 of B	0.75
E	32.5	32.5 of C	0.5
F	32.5	32.5 of E	0.25
G	32.5	32.5 of F	0.125
H	40	0	Blank

The volume of the required working reagent was prepared based on the following equation:

$$Volume\ of\ working\ reagent\ (mL) = (8 + number\ of\ samples) \times 3 \times 0.2$$

8 is the number of standards, 3 the number of replicates and 0.2 the volume of working reagent per sample in mL. After pipetting the working reagent it was mixed

with 50 parts of reagent A into 1 part of reagent B and vortexed to give a light green colour.

10 µL of each standard and blank concentration was pipetted into a 96 well plate in triplicate before adding 200 µL working reagent into each well. The plate was mixed for 30 s, covered and incubated at 37°C for 30 min. After the plate cooled down to RT the absorbance at 595 nm was measured on a plate reader. The mean value of the blank was subtracted from all standards and sample measurements and a standard curve was prepared by plotting the mean value for each standard against its concentration in µg/µL. The equation obtained from the standard calibration curve was used to calculate the protein content of the unknown samples.

4.2.2.9 Competitive ELISA experiments

Throughout this method development work for accomplishing a competitive ELISA the following two competitive ELISA designs were applied.

Competitive ELISA design 1 (working with tracer)

- ON DAY 1 the plate was coated with 100 µL antibody (100 ng antibody/well) and kept on the shaker for incubation overnight.
- ON DAY 2 the plate was washed with solution C before 180 µL BSA solution per well was added. After 2 hrs incubation at 4°C the plate was washed with solution B before 100 µL antigen solution in different concentrations were pipetted into the wells. The plate was kept at 4°C overnight.
- ON DAY 3 the plate was washed with solution B before 50 µL tracer solution (1:1000; biotinylated hapten or biotinylated *N*7-GA-Gua) was pipetted and incubated at 4°C overnight.
- ON DAY 4 the plate was washed with solution B before 100 µL streptavidin solution was pipetted into each well. After 2 hrs incubation the plate was washed with solution B and read on a luminometer.

Competitive ELISA design 2

- ON DAY 1 the plate was coated with 100 µL ovalbumin-hapten conjugate (100 ng protein/well). An antigen and antibody containing solution was pre-incubated overnight. The plate and the antigen-Ab-solutions were kept on the shaker at RT for incubation.
- ON DAY 2 the plate was washed with solution C. Each well was blocked by pipetting 180 µL BSA. The plate was incubated for 1 hr and washed with solution B before 100 µL pre-incubated solutions were pipetted into the wells and left for incubation overnight at RT.
- ON DAY 3 the plate was washed with solution B and 100 µL BAR solution was pipetted into each well, incubated for 1 hr, and washed with solution B before 100 µL streptavidin solution was added per well. After incubation for 1.5 hrs the plate was washed with solution B and read on a luminometer.

4.2.2.10 Biotinylation of hapten and *N*7-GA-Gua (Tracer production)

Biotin was conjugated to the hapten and the *N*7-GA-Gua adduct in order to produce a tracer (biotinylated antigen) for ELISA experiments.

Hapten biotinylation

ON DAY 1 Hapten (2.18 mg) was dissolved in DMSO (70.9 µL) (to give 100 nmol/µL). PBS (100 µL) was placed in an eppendorf tube and hapten solution (2.5 µL) (equals 250 nmol) added. In a different eppendorf tube, PBS (40 µL) was pipetted and tributylphosphine (1 µL) added. This solution (2.5 µL) was added to the hapten-PBS solution and left for 30 min. 200 nmol Biotin-PEO-NHS (biotin-polyethylen glycol-*N*-hydroxysuccinimide ester) was dissolved in PBS (50 µL) and added to the hapten solution. The tube was kept on the shaker at RT overnight.

ON DAY 2 Cysteine (0.63 mg) dissolved in ddH_2O (20 µL) was added to the biotinylation solution. The HPLC was set up and the acidified biotinylation solution was injected and fractions collected. The HPLC column used was a Phenomenex Aqua 5 µm C18 200Å 250 x 4.6 mm 5µm micron. The gradient (0 to 80% ACN in 40 min) had a total run time

of 45 min and a flow rate of 1 mL/min. The aqueous phase consisted of water with 0.1% TFA and the organic phase was ACN with 0.1% TFA added. A standard solution of both the hapten and the *N*7-GA-Gua solutions were prepared to have them as a comparison for the injection of the biotinylated solution. After injection of the biotinylated solution 16 different peak fractions were collected. In a second injection of the same solution again 16 fractions with similar retention times were collected.

N7-GA-Gua biotinylation

ON DAY 1 TFE (50 µL) was mixed with 400 nmol *N*7-GA-Gua (2 µL). Two aliquots, each 400 nmol Biotin-PEO-NHS were dissolved in DMF (20 µL). This was then added to the *N*7-GA-Gua solution and kept on the shaker overnight at RT.

ON DAY 2 Lysine (10 µL of 10 mg/mL) was added to bind the non-reacted biotin and the solution purified on the HPLC. Ten fractions were collected.

Testing the biotinylated fractions – plate design

Fractions collected by HPLC were tested in a plate design.

ON DAY 1 the plate was coated with 100 µL antibody solution/well (100 ng/well) and incubated on the shaker overnight at RT.

ON DAY 2 the plate was washed with solution C and each well was blocked with 180 µL BSA solution and kept on the shaker for 1 hr. After washing the plate with solution B, 100 µL of diluted fraction solution (0.5 µL fraction solution in 300 µL ILMA) was added to the wells. The plate was left on the shaker overnight at RT.

ON DAY 3 the plate was washed with solution B and 100 µL streptavidin solution was added per well. After 2 hrs incubation time the plate was washed with solution B and read on a luminometer.

Testing the biotinylated fractions – tube design

Fractions collected by HPLC were tested in a tube design.

ON DAY 1 100 µL antibody solution (100 ng/tube) was added to the tube and 100 µL of collected fractions (1:1000 in ILMA) were added. They were then incubated overnight at 4°C.

ON DAY 2 100 µL Bio Mag Goat Anti-Rabbit IgG suspension was added and kept on the shaker to incubate for 2 hrs. The tubes were transferred into a magnetic rack. After 5 min when the magnetic beads settled the liquid was discarded and the tubes were washed with solution B. 100 µL Streptavidin solution was added per tube. After incubation for 2 hrs the tubes were placed in the magnetic rack, washed with solution B and measured on Auto Lumat®.

Competitive ELISA

ON DAY 1 the plate was coated with 100 µL Ab1 and Ab2 solution (100 ng/well) and kept on the shaker for incubation at 4°C overnight.

ON DAY 2 the plate was washed with solution C and blocked with 180 µL BSA/well. After incubation for 2 hrs at 4°C the plate was washed with solution B and 100 µL antigen solution (hapten, non-acetylated and acetylated *N*7-GA-Gua in concentrations of 100, 25, 6.25, 1.56, 0.39, 0.098, 0.0244 and 0 pmol/well) was added. The plate was kept on the shaker at 4°C overnight.

ON DAY 3 the plate was washed with solution B, 50 µL tracer (1:1000) was added and the plate incubated at 4°C overnight.

ON DAY 4 the plate was washed with solution B before 100 µL streptavidin solution was pipetted per well. The plate was incubated for 2 hrs at RT, washed with solution B and read on a luminometer.

4.2.2.11 Acetylation and succinylation of *N*7-GA-Gua

Antibodies (M1, M2, R1, R2) which were produced prior to the start of this project were tested in experiments before the new polyclonal antibodies arrived. These old antibodies did not bind very well to the tracer or the adduct. Therefore, the *N*7-GA-Gua adduct was acetylated and succinylated. Connecting this "bridge" onto the

molecule makes it appear more similar to the hapten with the aim to have the antibodies recognise the "bridge" on the molecule to bind onto it. The "Product information, cGMP Enzyme Immunoassay Kit; Technical bulletin" by Sigma Aldrich was used as a reference for the acetylation.

Acetylation of N7-GA-Gua

Acetic anhydride and triethylamine (1+4) was used as the acetylating reagent. 100 mM sodium phosphate buffer (20 µL) and *N*7-GA-Gua (1 µL of 1032 pmol/µL) was pipetted into an eppendorf tube. Acetylating reagent (0.5 µL) was added to the tube and this solution was kept on the shaker for 3 hrs at RT before it was stored in the fridge overnight and used in the experiment to test the acetylated *N*7-GA-Gua.

Succinylation of N7-GA-Gua

Succinic anhydride (1000 nmol) was dissolved in DMF (140 µL) before the addition of 1 nmol *N*7-GA-Gua (105 µg/mL) and a mixture of 100 mM $NaHCO_3$/100 mM Na_2CO_3 pH 9 (20 µL). The final solution consisted of 100 mM $NaHCO_3$/100 mM Na_2CO_3 pH 9 (41 µL), *N*7-GA-Gua (1 µL) and succinic anhydride solution (2 µL). The mixture was rotated on the shaker for 5 hrs and stored in the fridge overnight for use in the experiment the following day.

Experiment with acetylated and succinylated antigen – Competitive ELISA design 1

ON DAY 1 the plate was coated with 100 µL Ab1 and Ab2 solutions (100 ng/well) and kept on the shaker at 4°C overnight.

ON DAY 2 the plate was washed with solution C, blocked with 180 µL BSA/well and left at 4°C for 2 hrs. After washing with solution B, 100 µL antigen solutions (acetylated and succinylated *N*7-GA-Gua adduct and hapten in 666, 166.5, 41.63, 10.41, 2.6, 0.65, 0.163 and 0 pmol/well) were added. The plate was kept at 4°C to incubate overnight.

ON DAY 3 the plate was washed with solution B, 50 µL tracer (1:1000) solution was added per well before the plate was incubated at 4°C overnight.

ON DAY 4 the plate was washed with solution B, 100 µL streptavidin solution per well was added and left on the shaker for 2 hrs at RT. The plate was washed with solution B and read on a luminometer.

4.2.2.12 *N*7-GA-Gua for coating the plate

The *N*7-GA-Gua adduct was used to coat the plate to assess whether the antibodies recognise it as antigen. *N*7-GA-Gua was diluted in PBS and 0.1% TFA to check which one led to better results.

ON DAY 1 100 µL *N*7-GA-Gua (0.1 pmol/µL) were pipetted per well and the plate was incubated on the shaker overnight at RT.

ON DAY 2 the plate was washed with solution C before 180 µL BSA/well was added and left for 1 hr at RT. After washing the plate with solution B, 100 µL (100 ng/well) prepared antibody solutions (M1, M2, R1, R2) were pipetted into the designated wells. The plate was incubated overnight on the shaker at RT.

ON DAY 3 the plate was washed with solution B and 100 µL BAR/BAM were pipetted into the designated wells. After 1 hr incubation the plate was washed with solution B and 100 µL streptavidin solution was added into each well. The plate was washed with solution B before the plate was read on a luminometer.

4.2.2.13 DNA for coating in competitive ELISA models

The project aimed to analyse *N*7-GA-Gua in human leukocyte DNA and urine. Therefore it was tested whether the antibodies recognise the *N*7-GA-Gua adduct in a model biological matrix DNA. CT DNA was treated with GA (900 µg CT DNA in 1 mL ddH_2O treated with 1 mg GA).

4.2.2.13.1 NaCl and CTAB

Two procedures were used to attach the DNA to the plate, one using sodium chloride (NaCl) and the other using cetyl trimethylammonium bromide (CTAB).

NaCl

The loading solution consisted of DNA solution (195 µL), 3 M NaCl (700 µL), PBS (505 µL). As control the NaCl solution was pipetted. The final NaCl concentration was 1.5 M.

CTAB

The loading solution consisted of DNA solution (195 µL), 1 mM CTAB (70 µL), PBS (500 µL) and ddH_2O (635 µL). As control the CTAB solution was used. The final CTAB concentration was 0.05 mM.

ON DAY 1 100 µL of each loading solution (12.5 µg DNA/well) was pipetted into the designated wells and left on the shaker overnight at RT.

ON DAY 2 the plate was washed with solution C, each well was blocked with 180 µL BSA solution and incubated for 1 hr. The plate was then washed with solution B before 100 µL antibody (100 ng/well) was added to the wells. After incubation for 3 hrs the plate was washed with solution B and 100 µL BAM/BAR, respectively were pipetted in its designated wells. After 1 hr the plate was washed with solution B and 100 µL streptavidin solution was added per well. After incubation for 1 hr the plate was washed with solution B and read on a luminometer.

Non-treated DNA as control

NACL: The loading solution consisted of GA treated CT DNA solution (222.4 µL), 3 M NaCl (500 µL), PBS (277.6 µL) and the final mix for control DNA consisted of CT DNA solution (194.4 µL), 3 M NaCl (500 µL), PBS (305.6 µL). The final NaCl concentration was 1.5 M.

CTAB: The loading solution consisted of GA treated CT DNA solution (222.4 µL), 1 mM CTAB (50 µL), PBS (500 µL) and ddH_2O (227.6 µL) and the final mix for control DNA consisted of CT DNA solution (194.4 µL), 1 mM CTAB (50 µL), PBS (500 µL) and ddH_2O (255.6 µL). The final CTAB concentration was 0.05 M.

ON DAY 1 100 µL of each loading solution (20 µg DNA/well) was then pipetted into the designated wells and left on the shaker overnight at RT.

ON DAY 2 the plate was washed with solution C, each well was blocked with 180 µL BSA solution and incubated for 1 hr. The plate was then washed with solution B before 100 µL antibody (100 ng/well) were added to the wells. After incubation for 3 hrs the plate was washed with solution B and 100 µL BAR were pipetted in its designated wells. After 1 hr the plate was washed with solution B and 100 µL streptavidin solution was added per well. After incubation for 1 hr the plate was washed with solution B and read on a luminometer.

4.2.2.13.2 Protamine sulphate

Protamine sulphate (PS) 1

This experiment was conducted following instructions from the paper by Brinkman *et al*. [135].

ON DAY 1 PS (4.9 mg) was dissolved in ddH_2O (9.8 mL) on the rotating table (to give 0.5 mg/mL). 150 µL/well were pipetted and left on the shaker for 2 hrs at RT. The plate was washed with solution C before 150 µL GA treated CT DNA (900 µg/mL) and CT DNA were pipetted into designated wells. The plate was kept on the shaker at RT overnight.

ON DAY 2 the plate was washed with solution B and blocked with 250 µL BSA/well. After 1 hr incubation time the plate was washed with solution B before 150 µL antibody solution (100 ng/well) was pipetted per well. The plate was kept on the shaker for 3.25 hrs and washed with solution B. 150 µL/well BAR was added and kept on the shaker for 1 hr and washed with solution B before 150 µL streptavidin solution was added into each well. The plate was kept for reaction for 1.25 hrs before the plate was washed with solution B and read on a luminometer.

PS 2

This experiment followed the method published by Stokes *et al*. [136].

PS (40.88 mg) was dissolved in ddH_2O (1% aq. solution; 4.1 mL) on the rotating table. PS (200 µL) was pipetted per well and left for 30 min at RT on the shaker. The plate was washed with solution C before GA treated (200 µL) and non-treated control CT DNA (200 µL) were pipetted per well and kept on the shaker for 30 min at RT. The plate was then washed with solution B and blocked with 250 µL BSA/well. After 1 hr incubation time the plate was washed with solution B before 200 µL antibody solution (100 ng/well) was pipetted. The plate was kept on the shaker for 3.25 hrs and washed with solution B. 200 µL BAR was added per well and kept on the shaker for 1 hr. The plate was washed with solution B before 200 µL streptavidin solution was added into each well and the plate was kept incubating for 1.25 hrs. Before the plate was read on a luminometer it was washed with solution B.

4.2.2.13.3 BSA

ON DAY 1 BSA (12.93 mg) was dissolved in PBS (1 mL). GA treated CT DNA (200.17 µL of 900 µg/mL) was mixed with formaldehyde (37%) (HCHO) solution (24.3 µL), BSA solution (139.21 µL) and PBS (536.32 µL). The control solution consisted of CT DNA (174.96 µL of 1028.83 µg/mL), HCHO solution (24.3 µL) (37%), BSA (139.21 µL) and PBS (561.53 µL). The prepared solutions were mixed and allowed to react for 1 hr on the rotating table at RT. 100 µL of each DNA-BSA solution was pipetted in its designated wells and the plate was kept on the shaker overnight at RT.

ON DAY 2 the plate was washed with solution C and blocked with 180 µL BSA per well. After 1 hr incubation time the plate was washed with solution B before 100 µL antibody solution (100 ng/well) was pipetted per well. The plate was kept on the shaker for 3.25 hrs and washed with solution B. 100 µL BAR/well was added and incubated on the shaker for 1 hr. The plate was washed with solution B before 100 µL streptavidin solution was added per well. The plate was left to react for 1.25 hrs and washed with solution B before it was measured on a luminometer.

4.2.2.13.4 Chitosan

Chitosan

Chitosan (37.76 mg) was dissolved in 0.5 M acetic acid (1.5%) (2.52 mL) on the shaker. 100 µL of this solution was pipetted per well and evaporated in an oven at 60°C. 0.3 M NaOH (100 µL/well) was pipetted to neutralise the acid and kept on the shaker for 45 min. The plate was then washed with solution C before the GA treated and non-treated CT DNA (20 µg DNA/well) was pipetted in the designated wells and left for incubation on the shaker overnight at RT.

ON DAY 2 the plate was washed with solution B and blocked with 250 µL BSA/well. After 1 hr incubation time the plate was washed with solution B before 100 µL antibody solution (100 ng/well) was pipetted into each well. The plate was kept on the shaker for 3.25 hrs and washed with solution B. 100 µL BAR/well was added and kept on the shaker for 1 hr. The plate was washed with solution B before 100 µL streptavidin solution was added into each well and the plate was incubated for 1.25 hrs before washed with solution B and measured on a luminometer.

Chitosan and sonicated DNA

Before applying the CT DNA in this experiment it was sonicated to break into smaller fragments so that the antibodies can access the adduct easier.

ON DAY 1 chitosan (39.05 mg) was dissolved in 0.5 M acetic acid (2.6 ml) on the rotating table and then 100 µL was pipetted per well. The acetic acid was then evaporated in an oven at 62.5°C. The wells were neutralised by adding 0.3 M NaOH (100 µL) and shaking it for 45 min. The plate was washed with solution C before the DNA was pipetted. The GA treated and non-treated CT DNA solutions were sonicated for 25 min before applying 100 µL/well. The plate was left on the shaker at RT overnight.

ON DAY 2 the plate was washed with solution C and blocked for 1 hr with 180 µL BSA/well. The plate was washed with solution B before 100 µL antibody solutions (100 ng/well) were applied. After 3.5 hrs incubation, the plate was washed with solution B and 100 µL BAR added per well. The plate was washed after 1 hr with solution B and

100 µL streptavidin solution was pipetted and kept on the shaker for incubation for 1.5 hrs. Before reading the plate it was washed with solution B.

4.2.2.14 Conjugation of BSA with guanosine and GMP (linker formaldehyde)

Guanosine

ON DAY 1 the linker HCHO was synthesised from paraformaldehyde. Paraformaldehyde (201.55 mg), NaH_2PO_4 (2.26%) (415 µL) and NaOH (2.52%) (85 µL) was mixed and placed in the heating block at 70°C to dissolve and turn clear. After 2 hrs NaH_2PO_4 (2.26%) (415 µL) and NaOH (2.52%) (85 µL) was added.

BSA (10 mg) was dissolved in PBS and added to guanosine (49.79 mg) dissolved in PBS (approximately 19 mL). The activated HCHO solution was added plus additional HCHO (37%) (1.89 mL). Due to the big volume the tube was kept on the rotating table overnight.

ON DAY 2 the BSA-formaldehyde-guanosine solution was transferred into a dialysis bag and placed into 1 L of PBS at 4°C. PBS was changed twice a day for two days before the content of the dialysis bag was transferred into a falcon tube and centrifuged for 5 min. The supernatant was transferred into a new falcon tube and the precipitate discarded. GA (150 mg) was dissolved in ddH_2O (500 µL) and added to the BSA-HCHO-guanosine solution. The solution was mixed before the tube was placed in the water bath at 37°C for 1 week. The solution was transferred into a dialysis membrane and dialysed in 1 L PBS at 4°C. The PBS was changed twice a day for two days. The solution was aliquoted into smaller tubes and centrifuged at 15700*g* for 5 min. In case the tubes contained a precipitate, the supernatant was transferred into a new tube. Protein content was determined with the BCA assay (see section 4.2.2.8).

Guanosine monophosphate (GMP)

BSA (20.83 mg) was dissolved in PBS (5 mL) and GMP (45.83 mg) was added and dissolved. For the activated HCHO solution paraformaldehyde (2.5 g) was mixed with

ddH_2O (7.5 mL), 1 M NaOH (150 µL) added and the mixed tube were warmed at 60°C for 30 min. The tube was shaken every 5 min. The solution was filtered and 1 M HCl (150 µL) was added. The pH was adjusted to pH 7.0 before an equal volume 40 mM HEPES/20 mM $MgCl_2$ was added. Activated HCHO solution (5 mL) was mixed with the BSA-GMP solution and kept on the rotating table at RT for 2 hrs. The solution was transferred into a membrane and dialysed in 1 L PBS at 4°C. The PBS was changed twice a day over 2 days. The content of the membrane was transferred into a tube and GA (100 mg) dissolved in ddH_2O (200 µL) was added for the GA treatment. The tube was mixed and placed in a water bath at 37°C for 1 week before the solution was dialysed in 1 L PBS at 4°C. The PBS was changed twice a day for two days before the content of the membrane was aliquoted and centrifuged briefly. The solution was transferred to a new tube if there was a precipitate. The aliquoted solutions were stored at -20°C. The protein content of the solution was analysed with the BCA assay (see section 4.2.2.8).

4.2.2.15 Conjugation of BSA with guanosine and GMP (linker glutaraldehyde)

Guanosine

ON DAY 1 BSA (31.05 mg) was dissolved in PBS (1 mL), glutaraldehyde solution (25%) (10 µL) was added and kept on the rotating table for 1 hr at RT. A Sephadex G25 column was washed with 3 column volumes of PBS before the BSA-glutaraldehyde solution was applied and ran through by gravity flow. The column was washed with PBS (2 mL) and eluted with PBS (3 mL). Guanosine (49.65 mg) was dissolved in PBS (approximately 18.5 mL). The eluate (1 mL) was mixed with the guanosine solution and kept on the rotating table overnight at RT.

ON DAY 2 the BSA-glutaraldehyde-guanosine solution was transferred into a dialysis bag and placed into 1 L of PBS at 4°C. PBS was changed twice a day for two days before the content of the dialysis bag was transferred into a falcon tube and centrifuged for 5 min. The supernatant was transferred into a new falcon tube and the precipitate

discarded. GA (150 mg) was dissolved in ddH_2O (500 µL) and added to the BSA-glutaraldehyde-guanosine solution. The solution was mixed before the tube was placed in the water bath at 37°C for 1 week and transferred into a dialysis membrane and dialysed in 1 L PBS at 4°C. The PBS was changed twice a day for two days. The solution was aliquoted into smaller tubes and centrifuged at 15700*g* for 5 min. If the tubes contained a precipitate the supernatant was transferred into a new tube. Protein content was then determined with the BCA assay (see section 4.2.2.8).

GMP

BSA (41.83 mg) was dissolved in PBS (1 mL), glutaraldehyde solution (25%) (10 µL) was added and kept on the rotating table for 1.5 hrs at RT. The Sephadex G25 column was prepared and washed with 3 column volumes of PBS before the BSA-glutaraldehyde solution was applied and ran through by gravity flow. The column was washed with PBS (2.6 mL). The conjugate was then eluted with PBS (2.2 mL) and the eluate collected into the tube containing GMP (61.8 mg). The GMP was dissolved by vortexing and left on the rotating table for 2 hrs at RT. Then, the solution was gel filtrated on the Sephadex G25 column again. Once the protein-GMP solution was applied to the column it was washed with PBS (2.6 mL) before the conjugate was eluted with PBS (2.2 mL). This was done twice, every time 1.1 mL BSA-GMP solution was loaded on the column. GA (170 mg) was dissolved in ddH_2O (300 µL). The total volume of eluate and GA solution (4.7 mL) was aliquoted into 5 eppendorf tubes and kept in the heating block at 70°C for 2 days. Then the tubes were centrifuged before the content was combined into a membrane and placed in 1 L PBS for dialysis at 4°C. The PBS was changed twice a day for two days before the content of the membrane was aliquoted and centrifuged briefly. The solution was transferred to a new tube if there was a precipitate. The protein content of the solution was analysed with the BCA assay and the solutions were stored at -20°C.

4.2.2.15.1 Testing the guanosine and GMP conjugates

ON DAY 1 the plate was coated with 100 µL/well BSA-HCHO-guanosine-GA, BSA-glutaraldehyde-guanosine-GA conjugates, BSA-HCHO-GMP-GA and BSA-glutaraldehyde-GMP-GA (500 ng/well) conjugates respectively and incubated overnight at RT.

ON DAY 2 the plate was washed with solution C and 180 µL/well BSA added to block the plate for 1 hr. The plate was washed with solution B and 100 µL antibody solution (100 ng/well) was pipetted into the wells and incubated for 3.5 hrs. After washing with solution B, 100 µL BAM/BAR solution was pipetted into the designated wells and the plate incubated for 1 hr. The plate was washed with solution B before 100 µL streptavidin solution was added and kept for incubation for 1.5 hrs. The plate was washed with solution B and measured on a luminometer.

4.2.2.15.2 Testing for displacement of guanosine and GMP conjugates

The displacement of the BSA-glutaraldehyde-guanosine conjugate was tested with the M1 antibody. The displacement of the antibodies against the BSA-glutaraldehyde-GMP conjugate was tested using the antibodies Ab2 and M1.

ON DAY 1 the plate was coated with 100 µL glutaraldehyde conjugate (500 ng/well) and kept on the shaker overnight at RT. A *N*7-GA-Gua and antibody solution was prepared for pre-incubation. The *N*7-GA-Gua amounts ranged from 10 pmol to 2.4 fmol/well, diluted 1:4 (10 pmol, 2.5 pmol, 625 fmol, 156 fmol, 39 fmol, 9.8 fmol, 2.4 fmol per well). 50 µL antibody solution (100 ng/well) was mixed with 50 µL *N*7-GA-Gua concentration per well. The antibody-antigen solutions were incubated overnight on the shaker at RT.

ON DAY 2 the plate was washed with solution C and blocked with 180 µL BSA. After 1 hr the plate was washed with solution B and 100 µL pre-incubated solutions were pipetted into their designated wells. Incubation took place overnight at RT.

ON DAY 3 the plate was washed with solution B before 100 µL BAM/BAR was pipetted into the wells. After 1 hr incubation, the plate was washed with solution B and 100 µL streptavidin solution was added. The plate was incubated for 1.5 hrs before it was washed with solution B and read on a luminometer.

4.2.2.16 Working with antibodies in a standard/blood experiment (replacing Qiagen procedure)

This experiment was set up to test whether the antibodies can be used to concentrate the adduct from a blood sample leading to the possibility that no Qiagen kit procedure (see section 2.2.2.2.1) would be required.

Antibodies-standard-experiment

ON DAY 1 four tubes were prepared each containing 50 pmol *N*7-GA-Gua/500 µL ddH_2O. The tubes were mixed and 100 µg antibody solution R1 (526.32 µL), R2 (36.5 µL), Ab1 (56.85 µL) and Ab2 (60.94 µL) was added, mixed and kept on the rotating table at 4°C overnight.

ON DAY 2 Bio Mag Goat Anti Rabbit magnetic beads (500 µL) were added into each tube. The tubes were kept on the shaker for 1 hr before being placed in the magnetic rack and settled for 5 min. The supernatant was discarded and the pellet washed with 2 mL 50 mM ammonium bicarbonate. The tubes were placed in the magnetic rack again, settled and the supernatant discarded. Two more washing steps using 1 mL and one using 500 µL 50 mM ammonium bicarbonate were done before the beads were dried briefly by inverting the magnetic rack. The antibodies were denatured by re-suspending in TFA (1%) (350 µL) for 5 min. The solution was transferred in pre-conditioned Amicon filters (400 µL ddH_2O centrifuged for 20 min at 16100*g*) and centrifuged for 1 hr at 16100*g*. The filtrate was dried down in a centrifugal evaporator overnight.

The samples were spiked with 7 µL (0.15 pmol/µL) [$^{15}N_5$]-*N*7-GA-Gua and re-dissolved in 13 µL 0.1% FA prior to analysis by LC-MS (injection volume 15 µL).

Antibodies-standard-experiment – low dose

ON DAY 1 1 pmol *N*7-GA-Gua/100 µL ddH_2O was mixed with 100 ng antibody solution R1 (5.263 µL), Ab1 (0.569 µL) and Ab2 (0.609 µL) and 1 pmol *N*7-GA-Gua/100 µL ddH_2O was mixed with 100 µg antibody solution Ab1 (56.85 µL) and Ab2 (60.94 µL) and all solutions were kept on the rotating table at 4°C overnight.

ON DAY 2 Bio Mag Goat Anti Rabbit magnetic beads (500 µL) were added into each tube containing 100 µg antibody solution and Bio Mag Goat Anti Rabbit magnetic beads (50 µL) were added to the 100 ng antibody solution containing tubes. The tubes were kept on the shaker for 1 hr before placed in the magnetic rack and settled for 5 min. The supernatant was discarded and the pellet of the 100 µg antibody solution washed with 50 mM ammonium bicarbonate (600 µL) four times and the tubes containing 100 ng antibody solution were washed with 50 mM ammonium bicarbonate (200 µL) four times. The tubes were placed in the magnetic rack again, settled and the supernatant discarded. The antibodies were denatured by re-suspending in TFA (1%) (350 µL) for 5 min. The solution was transferred into pre-conditioned Amicon filters (400 µL ddH_2O centrifuged for 20 min at 16100*g*) and centrifuged for 1 hr at 16100*g*. The filtrate was dried down in a centrifugal evaporator overnight.

The samples were spiked with 7 µL (0.1513 pmol/µL) [$^{15}N_5$]-*N*7-GA-Gua and re-dissolved in 20 µL 0.1% FA prior to analysis by LC-MS (injection volume 15 µL).

Antibodies-blood-experiment

ON DAY 1 whole blood (20 mL) was taken (mixed with 100 µL 1 M EDTA) from a volunteer and divided into two 50 mL falcon tubes, each containing 10 mL blood. Ice-cold buffer C1 (10 mL) and ice-cold ddH_2O (30 mL) were added to the blood and gently mixed until it became translucent. The tubes were kept on ice for 10 min before they were centrifuged for 15 min at 4°C at 2435*g*. The supernatant was disposed and ice-cold buffer C1 (2 mL) and ice-cold ddH_2O (6 mL) was added. The tubes were centrifuged again for 15 min at 4°C at 2435*g*. They were vortexed briefly and the contents were combined into one tube and centrifuged for 15 min at 4°C at 2435*g*. The supernatant was disposed and ice-cold (2 mL) buffer C1 and ice-cold ddH_2O (6 mL) was

added. The pellet was re-dissolved by vortexing and then centrifuged for 15 min at 4°C at 2435*g*. The supernatant was discarded and the pellet stored at -20°C overnight.

ON DAY 2 G2 buffer (200 µL) was added and the pellet re-suspended by vortexing. 20 U RNase A (32.92 µL) and 2 U Ribonuclease T1 (37.65 µL of 1:1000 dilution) were added and the tube mixed before it was incubated in the water bath at 37°C for 30 min. Then Proteinase K (40 µL of 10 mg/mL) was added, the tube mixed and incubated in the water bath at 37°C for 2.5 hrs. Due to the salts and detergents contained in the G2 buffer used for lysing the cells in blood, the solution was diluted 10 fold with ddH_2O and split evenly between two eppendorf tubes. After vortexing, the tubes were placed in the heating block at 70°C for 1 hr. At this stage a control DNA extracted from human blood was also placed in the heating block for 1 hr to verify that the G2 buffer had no negative effect on the result. The tubes were cooled down before 100 µg Ab2 (60.94 µL) was added, and the tubes kept on the rotating table at 4°C overnight.

ON DAY 3 Bio Mag Goat Anti Rabbit IgG beads (500 µL) was added and kept on the shaker for 1 hr at RT. Amicon molecular weight cut-off filters 10 k were conditioned with ddH_2O (400 µL) and centrifuged for 20 min at 16100*g*. After incubation the tubes were placed into a magnetic rack and the content settled for 15 min. The supernatant was discarded and the pellet washed with 50 mM ammonium bicarbonate (1 mL). The tubes were placed in the magnetic rack and after settling down the supernatant discarded. The pellet was washed with 50 mM ammonium bicarbonate (500 µL). This was repeated two more times. The antibodies were denatured by adding TFA (1%) (350 µL), placed briefly in a sonic bath to re-suspend properly and left for 5 min to react. The solution was then transferred to the filters and centrifuged for 1 hr at 16100*g*. The filtrate was dried down in a centrifugal evaporator overnight.

ON DAY 4 the samples were re-dissolved in 0.1% FA (11 µL) and [$^{15}N_5$]-*N*7-GA-Gua (10 µL 1:1000). Due to the G2 buffer that was used, 1 µL of this solution was analysed by a matrix-assisted laser desorption ionisation (MALDI) mass spectrometer to verify that there was no residual detergent left. After confirmation by MALDI that no interfering detergents were present, the sample was analysed using LC-MS.

4.3 Results

4.3.1 Chemistry – hapten synthesis

The successful synthesis of the hapten was achieved and the characterisation and purity of all intermediates during the hapten synthesis was checked by NMR and mass spectrometry and compared to previously synthesised compounds (in house).

4.3.2 Protein conjugation to hapten

The hapten was connected to different carrier proteins of which the TGB conjugate gave the highest response (Figure 4.2) and was used as antigen for the production of the two polyclonal antibodies.

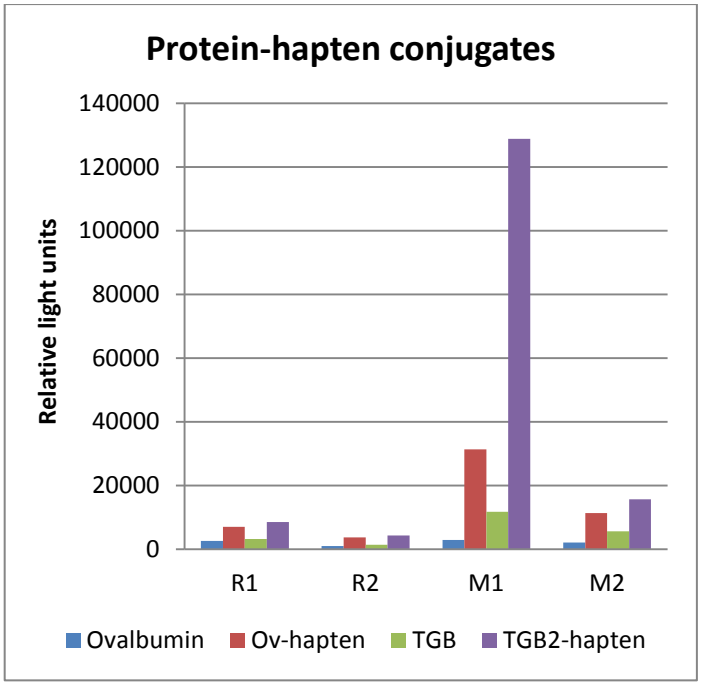


Figure 4.2: Signal response of ovalbumin and TGB as well as the ovalbumin-hapten and TGB-hapten conjugate 2 for the different antibodies R1, R2, M1 and M2 to find the best conjugate for eliciting the immune response in the rabbits and production of the polyclonal antibodies.

4.3.3 Antigen-Antibody-Titration

The results for the antigen-antibody-titration are shown in Figure 4.3 but there was no competition between the antigen *N*7-GA-Gua and the antibody M1.

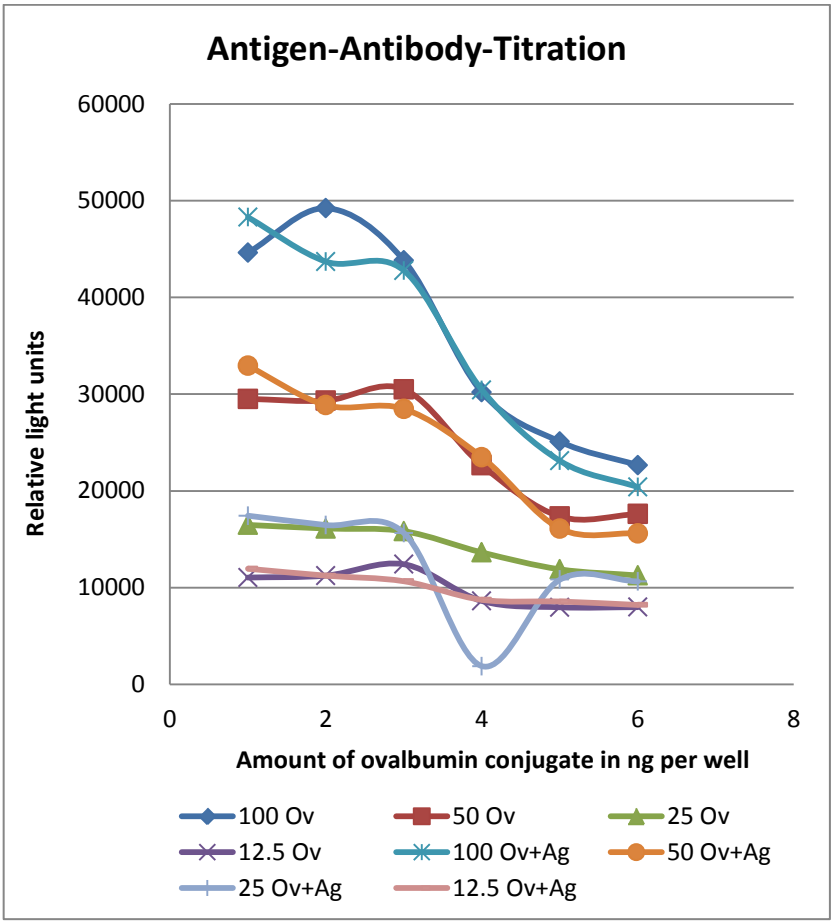


Figure 4.3: Response of the antigen-antibody-titration. The antigen-antibody-titration showed no competition between the antibody M1 and the antigen *N*7-GA-Gua. Each curve represents the response for a given amount of ovalbumin conjugate in ng/well (denoted as number and Ov, whereas number and Ov+Ag refer to the ovalbumin conjugate and the antigen).

4.3.4 Ovalbumin conjugation to *N*7-GA-Gua

Connecting the *N*7-GA-Gua adduct to the protein ovalbumin and testing it against the antibodies M1, M2, R1 and R2 yielded a signal for the second ovalbumin conjugation but with a value of just below 7000 Relative light units where ideally the value should lie above 100000 Relative light units (Figure 4.4).

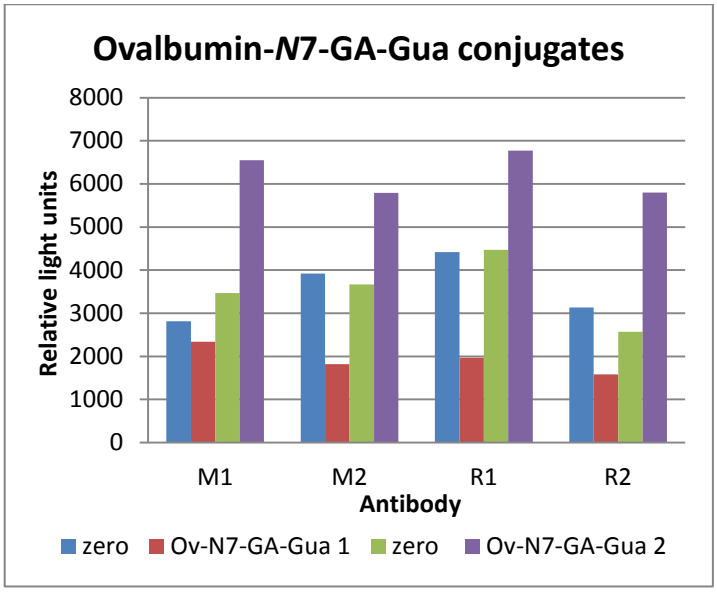


Figure 4.4: Response of the antibodies against the two ovalbumin-*N*7-GA-Gua conjugates. Zero was the ovalbumin control. Ov = ovalbumin.

4.3.5 Protein A purification – Testing the purified antibodies

Testing the Protein A purified polyclonal antibodies showed that they are binding at all concentrations tested (1000, 500, 250, 125, 62.5, 31.25, 15.6 and 7.8 ng) (Figure 4.5).

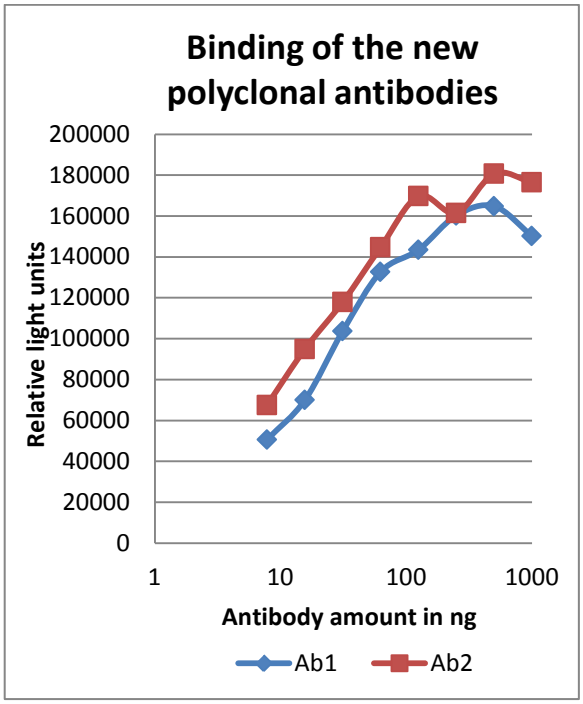


Figure 4.5: Response of the new polyclonal antibodies purified from rabbit serum and tested against the ovalbumin-hapten conjugate. Both Ab1 and Ab2 bound at even lower amounts.

4.3.6 BCA assay

The protein content of every protein-conjugation onto the hapten and the *N*7-GA-Gua adduct as well as the BSA conjugation onto GMP was determined by BCA assay. The protein content of the synthesised conjugates is shown in Table 4.3.

Table 4.3: Protein content of all protein conjugations onto the hapten and the *N*7-GA-Gua as well as the BSA conjugate onto GMP

Conjugate	Protein content in µg/µL
TGB-hapten	1.0515
TGB2-hapten	0.992
KLH-hapten	0.131
Ovalbumin1-*N*7-GA-Gua	0.5883
Ovalbumin2-*N*7-GA-Gua	3.495
BSA-glutaraldehyde-GMP-GA	5.034
BSA- HCHO-GMP-GA	1.4395

A typical calibration line achieved by BCA assay is shown in Figure 4.6 for the determination of the protein content of the BSA-guanosine-HCHO-GA and BSA-guanosine-glutaraldehyde-GA conjugates.

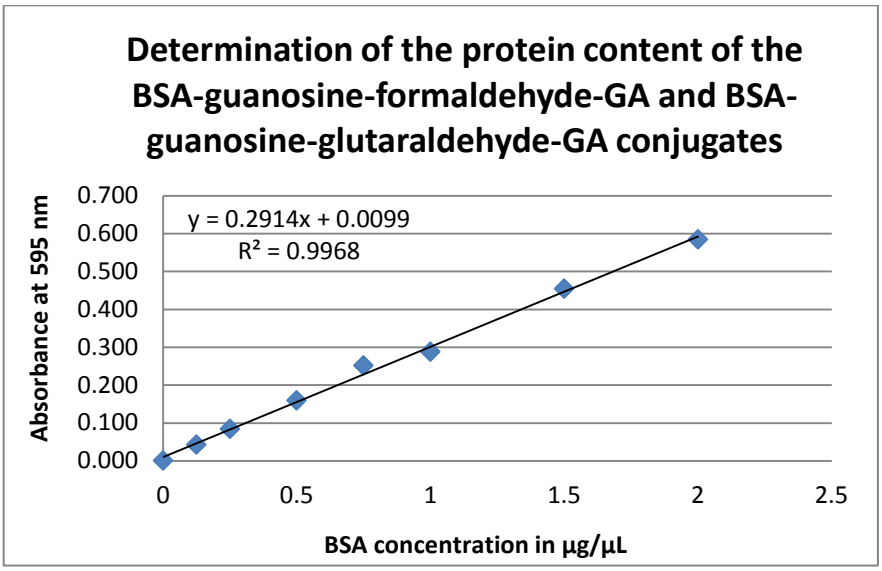


Figure 4.6: Typical calibration line for the determination of the protein content of protein conjugates by applying the BCA assay by taking different concentrations of a BSA standard solution. The presented calibration line was obtained for the quantitation of the BSA-guanosine-formaldehyde-GA and BSA-guanosine-glutaraldehyde-GA conjugates.

4.3.7 Acetylation and succinylation of *N*7-GA-Gua

The *N*7-GA-Gua structure was modified by acetylation and succinylation with the aim of producing a more similar structure to the hapten. This was then used to try and displace the antibody in the competition reaction of the experiment but both polyclonal antibodies bound strongly to the hapten without competition. No competition between antigen and antibody was seen when applying these modified antigens in a competitive ELISA assay (Figure 4.7).

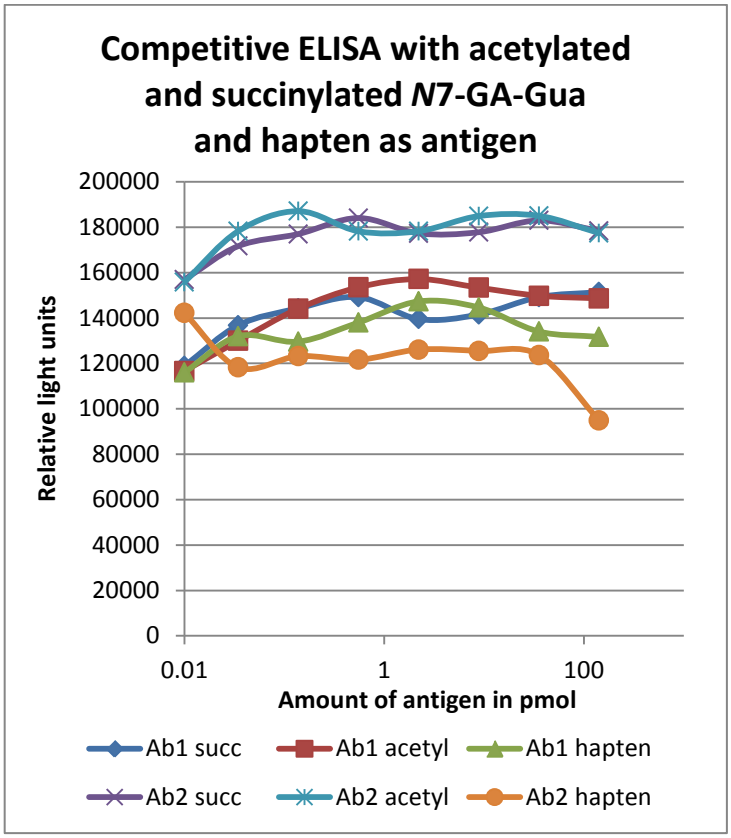


Figure 4.7: Results for the competitive ELISA with acetylated and succinylated *N*7-GA-Gua and hapten used as antigen. Antibodies were binding but there was no competition between the antigen and antibody.

4.3.8 Biotinylation of hapten and *N*7-GA-Gua

After biotinylation of the hapten and the adduct the solutions were purified by HPLC and the collected fractions tested. Figure 4.8 shows the response of the collected fractions.

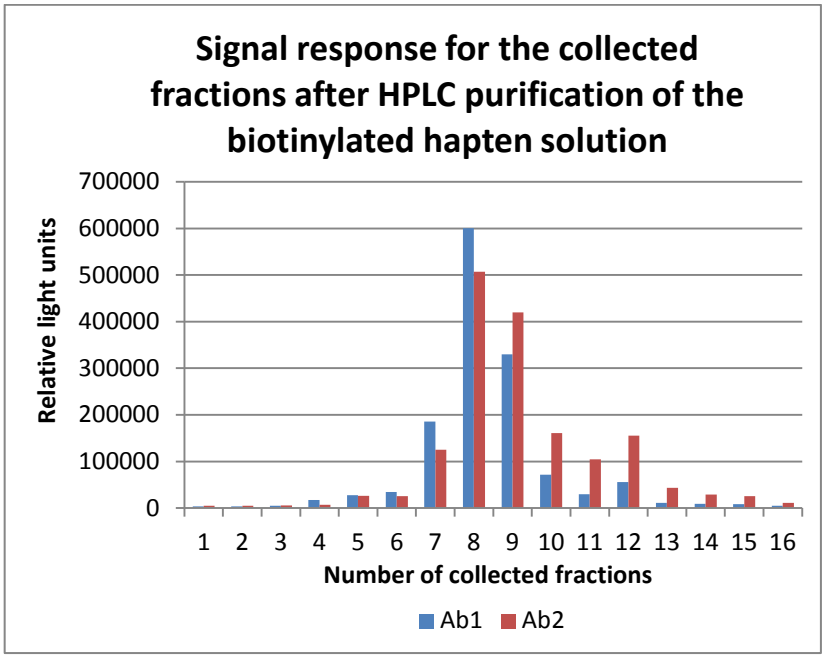


Figure 4.8: The signal response for the collected fractions after HPLC purification of the biotinylated hapten solution. The fractions 8, 9 and 12 looked promising and were tested further.

The fractions giving the highest response in the testing experiment were then used in a competitive ELISA experiment.

Competitive ELISA

Applying the hapten-biotin-tracer in the competitive ELISA design 1 did not lead to a competition between the antibody and the antigen *N*7-GA-Gua. Ab2 against the hapten showed some competition as shown by the orange line in Figure 4.9 but there was not sufficient competition to construct a competitive ELISA, as there was still substantial binding even with a high concentration of unlabelled analyte.

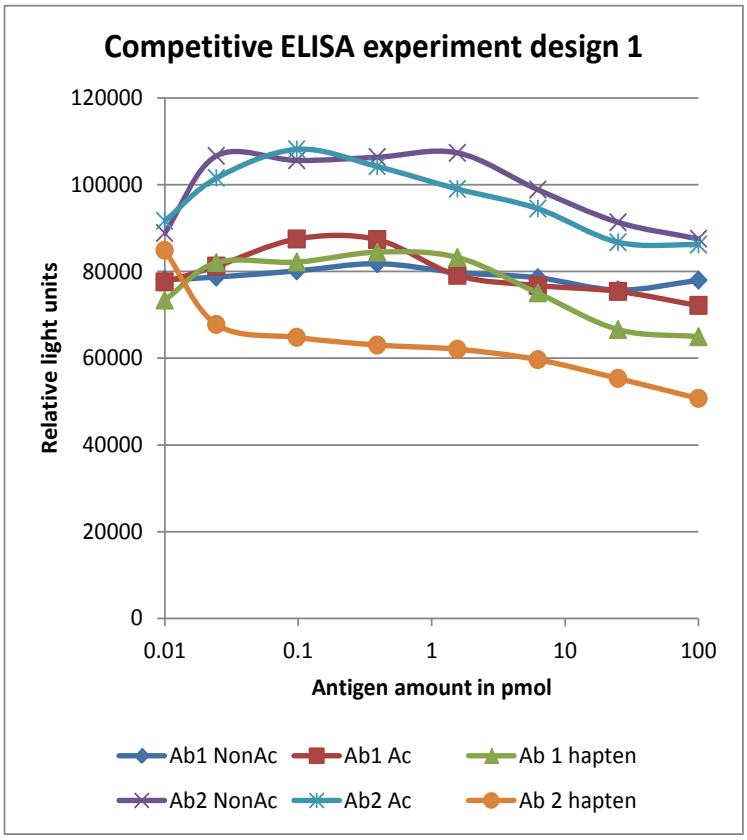


Figure 4.9: Competitive ELISA results for varying amounts of antigen. Result for applying the produced hapten-biotin-tracer in a competitive ELISA experiment design 1. The antibodies did not recognise the non-acetylated nor the acetylated *N*7-GA-Gua adduct. The response to the hapten for Ab2 is the best (orange coloured curve).

4.3.9 *N*7-GA-Gua for coating the plate

Coating the plate with *N*7-GA-Gua adduct gave very low readings which demonstrates that there was no competition between antibodies and antigen (Figure 4.10).

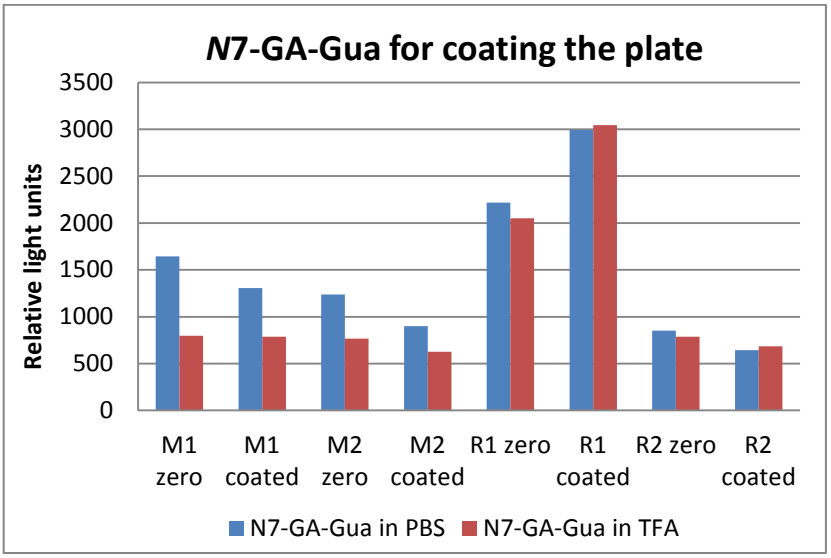


Figure 4.10: Results for the experiment where the antigen *N*7-GA-Gua was used for coating the plate. There was no competition between the antigen and the antibodies. The *N*7-GA-Gua was diluted in PBS (blue bars) and in TFA (red bars). All Relative light unit signals are low and there was no real difference between the control (no DNA) and DNA coated wells.

4.3.10 DNA for coating in competitive ELISA models

Mass spectrometric analysis confirmed the presence of the *N*7-GA-Gua adduct in GA treated CT DNA and a smaller peak in non-treated CT DNA. When GA treated CT DNA was coated on the plate with NaCl and CTAB the signal response was good for Ab1 and Ab2 but not sufficient to construct a competitive ELISA (Figure 4.11).

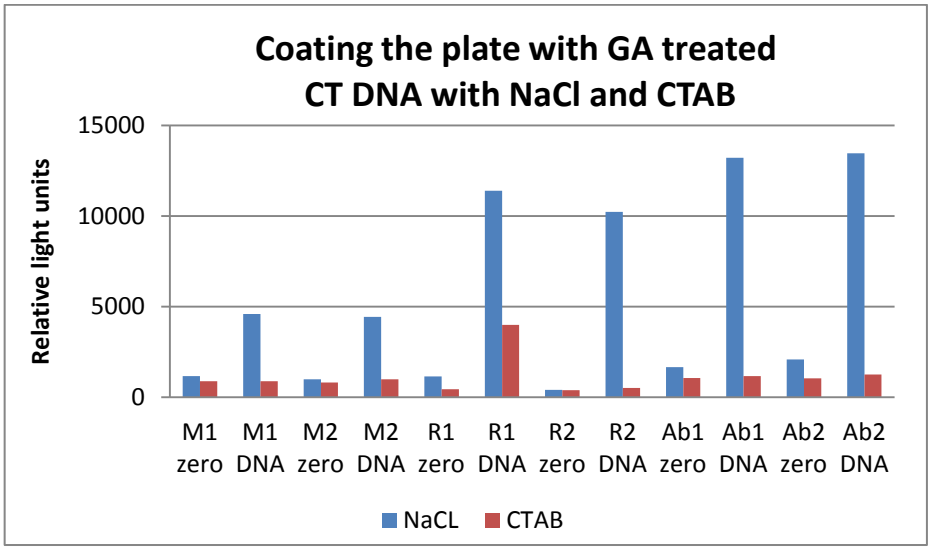


Figure 4.11: GA treated CT DNA was coated on the plate by means of NaCl and CTAB. NaCl and CTAB solution was applied to the control wells (labelled as zero). The response was higher for the DNA samples coated onto the plate using NaCl and yielded a signal significantly higher than background, suggesting antibody binding to the hapten or antigen for antibodies R1, R2, Ab1 and Ab2.

The signal response changed when the experiment was repeated and non-treated DNA as control was applied (Figure 4.12). It appears that the DNA was not binding to the plate appropriately. There was no incremental signal of the treated DNA compared to untreated DNA which suggests that antibodies were just binding non-specifically.

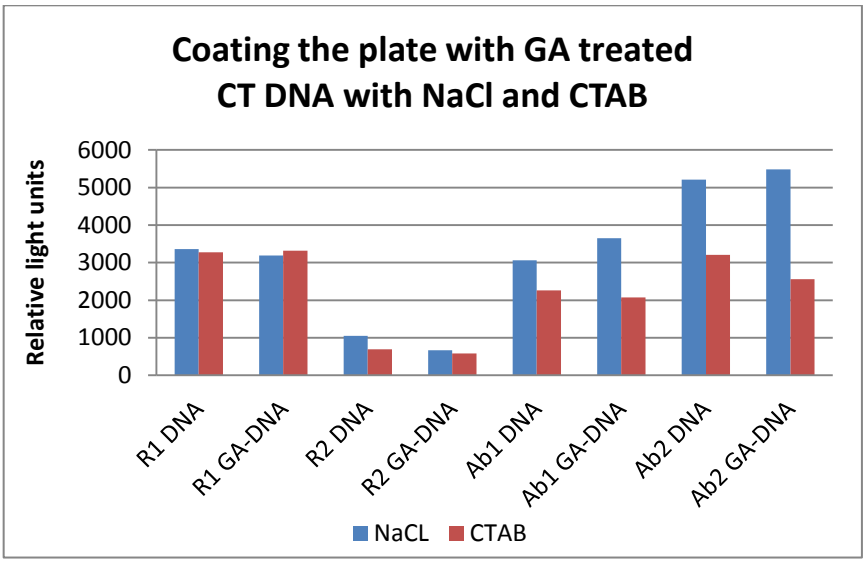


Figure 4.12: GA treated CT DNA was coated onto the plate with NaCl and CTAB (labelled as GA-DNA) and non-treated CT DNA was applied as control (labelled as DNA). There was no difference between NaCl and CTAB for the antibodies R1 and R2 and the Relative light unit readings were very low. Ab1 and Ab2 seemed to coat better to the plate when NaCl was used. The Relative light unit readings increased from Ab1 to Ab2 but were still low.

Protamine sulphate

Using the different methods for the PS to coat DNA onto a plate did not lead to a high signal reading. There was no incremental difference between treated and untreated DNA which suggests non-specific binding.

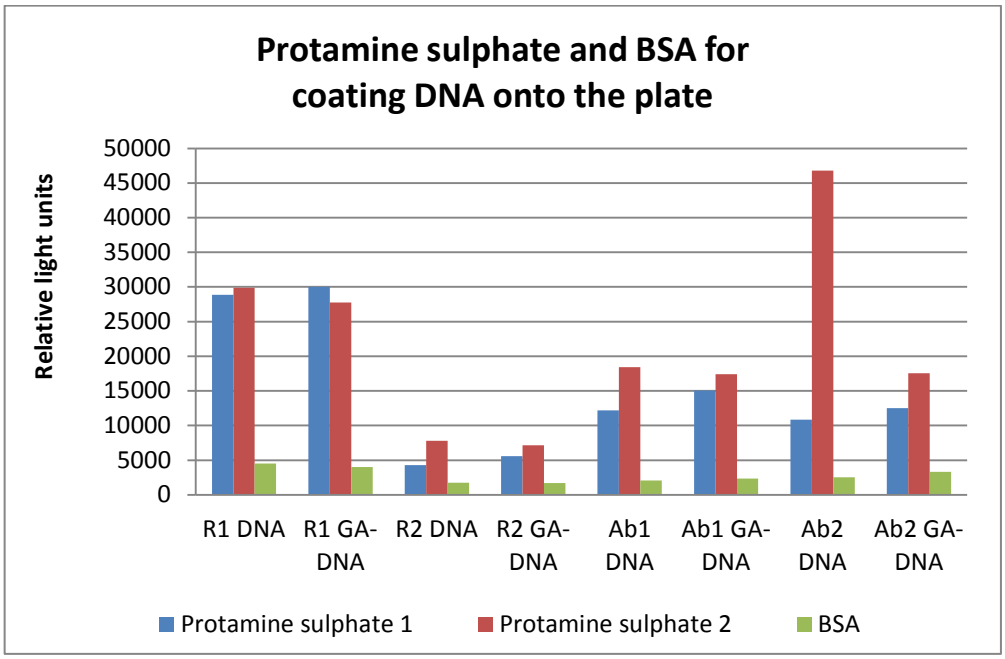


Figure 4.13: Results for the two experiments with protamine sulphate and BSA to coat DNA onto the plate. With the exception of antibody R1 GA-DNA the Relative light units readings were slightly higher for the second protamine sulphate experiment. For Ab2 there was a high difference between the first protamine sulphate experiment and the second. The Relative light units readings for the BSA experiment were all consistently very low whereas the readings for both protamine sulphate experiments with antibody R1 were the highest within this experiment only outreached by the protamine sulphate experiment 2 with Ab2 that had a reading of just over 45000 Relative light units.

BSA

Using BSA for coating the DNA onto the plate did not improve binding of DNA to the wells (Figure 4.13).

Chitosan

In the chitosan experiments there was very little difference between the non-treated and treated DNA and the signal readings were very low and ideally should have exceeded a value of 100000 Relative light units (Figure 4.14).

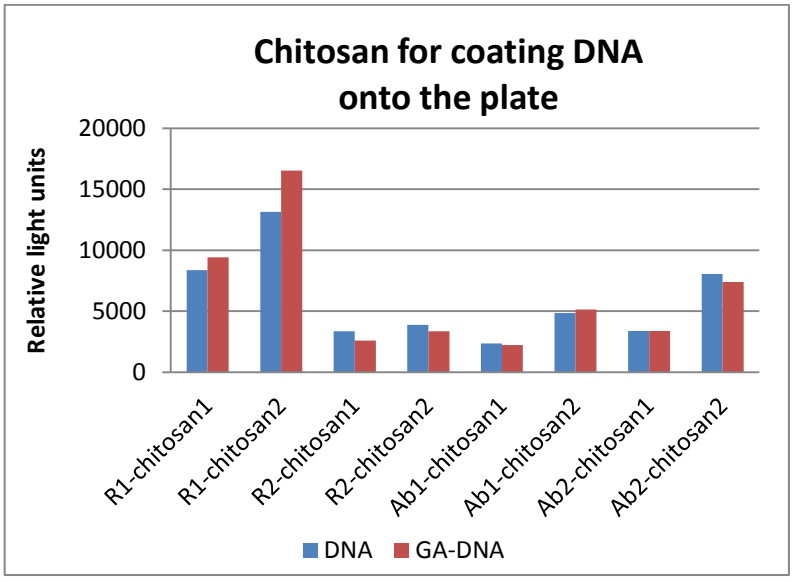


Figure 4.14: Results for the two experiments where chitosan was used to coat DNA onto the plate. The blue bars represent the non-treated CT DNA and the red bars stand for the GA treated DNA. For the experiment antibodies R1, R2, Ab1 and Ab2 were used. There was no difference in signal readings for control DNA and GA treated DNA.

Chitosan and sonicated DNA

Placing DNA in a sonic bath prior to application to the chitosan coated plate only resulted in non-specific binding (Figure 4.15). DNA was stuck to the plate.

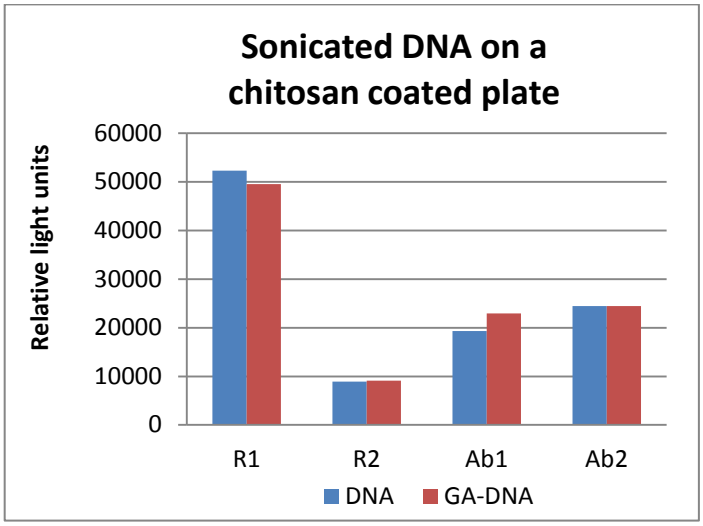


Figure 4.15: Results for applying sonicated DNA on a chitosan coated plate only gave non-specific binding. The blue bars reflect non-treated DNA and the red bars the treated DNA.

4.3.11 Conjugation of BSA onto guanosine and GMP

Testing the conjugates

The signal values for the guanosine conjugates were very low (Figure 4.16) but the signal for the M1 antibody against the glutaraldehyde conjugate might have been a real signal which had to be verified in a following experiment. Figure 4.16 B shows the response against the BSA-HCHO-guanosine-GA conjugate but the signal response was very low and there was no difference in signal from control BSA to BSA conjugate with the exception of antibody M1. However, the Relative light units were too low to generate a competitive ELISA.

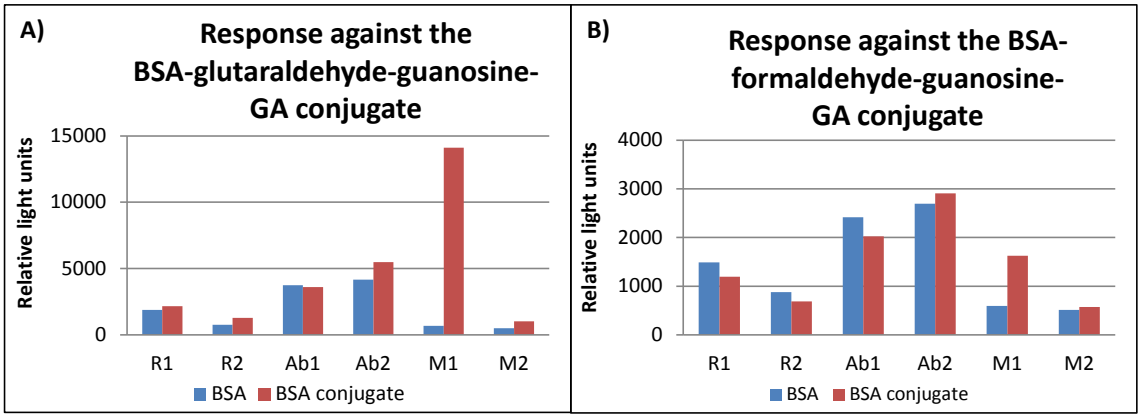


Figure 4.16: A) Response against the BSA-glutaraldehyde-guanosine-GA conjugate of antibodies R1, R2, Ab1, Ab2, M1 and M2. The blue bars represent the control BSA signal and the red bars reflect the response against the conjugate. The signal of antibody M1 yielded a signal significantly higher than background, suggesting antibody binding to the conjugate. B) Response against the BSA-formaldehyde-guanosine-GA conjugate of antibodies R1, R2, Ab1, Ab2, M1 and M2. The blue bars represent the control BSA signal and the red bars reflect the response against the conjugate. All Relative light units readings were very low and with the exception of the BSA conjugate there seemed to be no difference between the conjugate and the BSA control.

The response of the antibodies against the BSA-glutaraldehyde-GMP-GA conjugate is shown in Figure 4.17 A. The signals had very low values, suggesting that there was insufficient binding to construct a useful ELISA. The response against the BSA-HCHO-GMP-GA conjugate is shown in Figure 4.17 B but there was no difference in signal between the control BSA and the BSA conjugates for all antibodies.

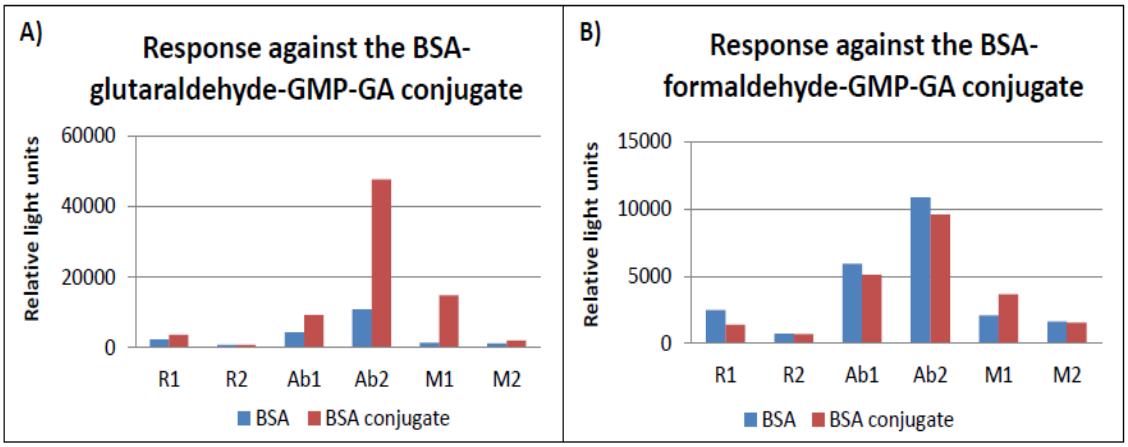


Figure 4.17: A) Response against the BSA-glutaraldehyde-GMP-GA conjugate of antibodies R1, R2, Ab1, Ab2, M1 and M2. The blue bars represent the control BSA signal and the red bars reflect the response against the conjugate. The response of Ab2 and M1 might have been a real signal whereas the other antibodies showed very low signal readings. B) Response against the BSA-formaldehyde-GMP-GA conjugate of antibodies R1, R2, Ab1, Ab2, M1 and M2. The blue bars represent the control BSA signal and the red bars show the response against the conjugate. There was no difference in signal response for the conjugates compared to the controls for all antibodies.

TESTING FOR DISPLACEMENT OF GUANOSINE AND GMP CONJUGATES

A noticeable displacement was not observed for either the BSA-glutaraldehyde-guanosine-GA or the BSA-glutaraldehyde-GMP-GA conjugate. There was no competition between antigen and antibody.

4.3.12 Working with antibodies in a standard/blood experiment (replacing Qiagen procedure)

Antibodies-standard-experiment

For the antibodies Ab1 and Ab2 a *N*7-GA-Gua peak was detectable as shown in Figure 4.18 highlighted by the arrow. No *N*7-GA-Gua peak was detected for the antibodies R1 and R2.

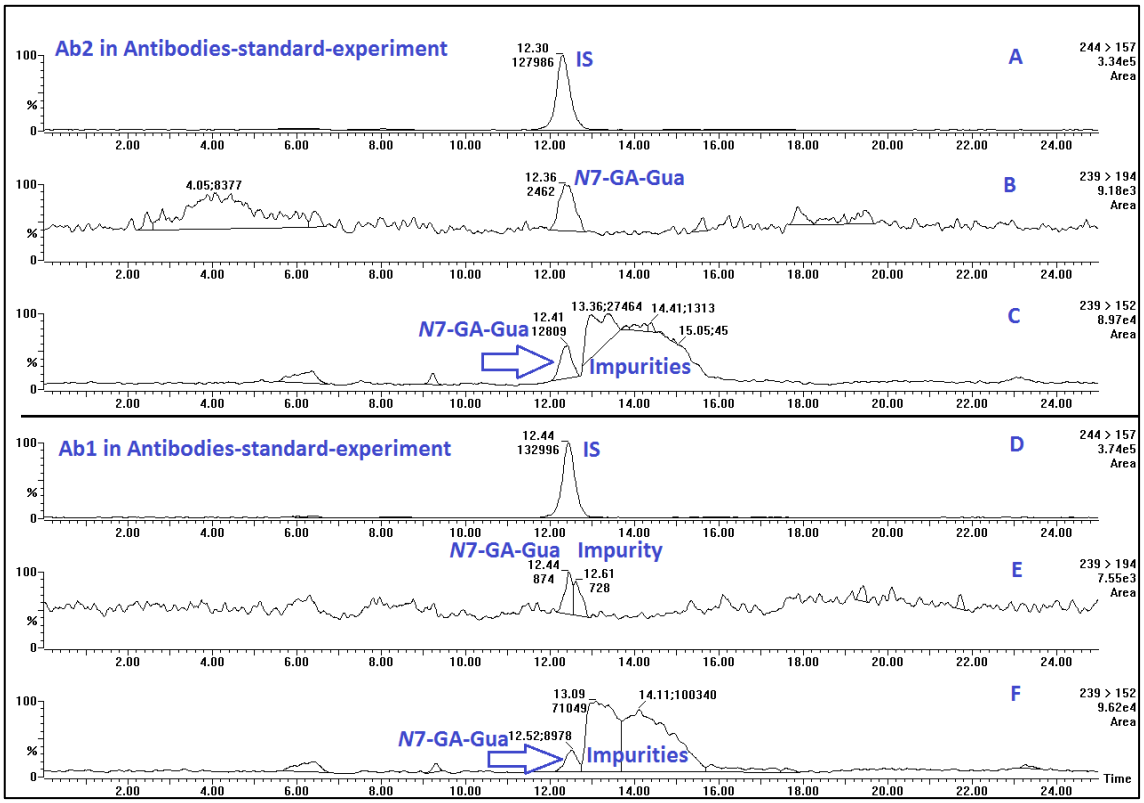


Figure 4.18: LC-MS/MS SRM chromatograms A – C show the results for the use of Ab2 and LC-MS/MS SRM chromatograms D – F show the results for the use of Ab1. LC-MS/MS SRM chromatograms A and D show the peak for the IS [$^{15}N_5$]-*N*7-GA-Gua, the transition *m/z* 244 to 157. LC-MS/MS SRM chromatograms B and E monitored the fragmentation of *m/z* 239 to 194 for the *N*7-GA-Gua adduct and LC-MS/MS SRM chromatograms C and F show the transition for *m/z* 239 to 152 for the detection of the *N*7-GA-Gua adduct.

Antibodies-standard-experiment – low dose

After analysing the samples by LC-MS, no *N*7-GA-Gua peak was detectable for all antibodies used.

Antibodies-blood-experiment

No *N*7-GA-Gua peaks were detectable.

4.4 Discussion

The main aim was to develop a successful ELISA method for the detection of the *N*7-GA-Gua DNA adduct in human volunteers samples. Unfortunately, this aim was not realised due to failure in getting a displacement of antibodies and hence no competition between the antibodies and the antigen, the *N*7-GA-Gua adduct.

4.4.1 Protein conjugation to the hapten and *N*7-GA-Gua

The hapten synthesis was successful as could be seen by comparison of the final product against previously generated mass spectra (in house). Rather than doing the hapten synthesis and using the hapten-protein conjugate for the antibody production it might have been possible to treat DNA with GA and use the reacted *N*7-GA-Gua containing DNA to elicit an immunogenic response in animals as described in Okahashi *et al*. where they immunised animals with estrogen-DNA adduct modified single stranded DNA conjugated to BSA [131].

Three different proteins were conjugated onto the hapten of which the one with the highest response (TGB-hapten conjugate) was used as an immunogen and the ovalbumin-hapten conjugate was applied in experiments to coat the plate. The protein conjugation to the *N*7-GA-Gua with glutaraldehyde aimed to use the antigen rather than the hapten (which was used to immunise the animals) for coating the plates. This way the binding of the antibodies to the adduct was supposed to be enhanced rather than binding to the bridge and the connected protein. The glutaraldehyde molecule has a shorter C-chain compared to the EMCS which should have helped to increase binding of antibodies onto the antigen. The antibodies are binding but they cannot be displaced in a competitive ELISA experiment.

In the work described by Nayak *et al*. the plate was coated with an ovalbumin conjugate and urine was applied to detect the AFB1-*N*7-guanine adduct [132]. The urine was purified with immunoaffinity columns. Since it was not possible to develop a mass spectrometric method for the detection of *N*7-GA-Gua in urine (see section 2.2.2.6), had time and progress permitted, an investigation could be carried out into the application of these antibodies to capture the adduct in urine prior to LC-MS analysis.

4.4.2 Polyclonal antibodies

The newly produced antibodies Ab1 and Ab2 are binding but they could not be displaced within the ELISA experiments. Perhaps the *N*7-GA-Gua adduct does not have sufficient epitope groups, which may lead to antibodies with little affinity towards the molecule or the affinity is towards the conjugated protein and the linker used to attach the protein. It is possible that removing the anti-TGB antibodies from the IgG fraction may enhance the affinity of the remaining IgG towards the adduct as described by Zhou *et al*. where they applied the IgG fraction onto a BSA-immobilised immunoaffinity column in order to gain a more pure antibody fraction. Zhou *et al*. also showed that increasing the ratio of hapten to protein and giving the animal additional booster injections can increase the affinity of antibodies against a small molecule antigen [134].

4.4.3 Biotinylation of hapten and *N*7-GA-Gua (tracer production)

When the different fractions of the biotinylation experiments were tested the conjugates with the highest response were applied to the final ELISA experiment but they did not lead to successful detection of the *N*7-GA-Gua adduct due to a lack of competition. Another explanation might be that the tracer was not stable in the fridge from the time of collection to the time of application in the final plate experiment.

4.4.4 Acetylation and succinylation of *N*7-GA-Gua

Modifying the antigen structure by acetylation and succinylation of *N*7-GA-Gua also did not increase the competition between antigen and antibody. The antibodies were binding but could not be displaced. This might imply that the antibodies have no affinity towards the bridge (ECMS) that was used to conjugate the hapten with the protein, or that the structure is too different to be recognised as such. The degree of acetylation and succinylation of the *N*7-GA-Gua was not assessed.

4.4.5 Coating DNA onto the plate

Using the DNA to coat the plate to then detect the *N*7-GA-Gua might have avoided the necessity of having to hydrolyse the DNA samples prior to mass spectrometric analysis. An example is described by Okahashi *et al*. when detecting the estrogen-DNA adduct, 4-Hydroxyequilenin with immunoassay [131]. However, they used a monoclonal antibody for their ELISA method and a concentration of 0.003% PS to pre-coat the DNA onto the plate whereas in the experiments described in section 4.2.2.13.2 the PS concentration was higher.

Georgiadis *et al*. developed an immunochemical assay for the detection of the O^6-methylguanine adduct where they used restriction enzymes to get shorter DNA fragments of defined length and adduct level [130]. Placing the DNA in a sonic bath did not improve the results. The results for DNA and GA treated DNA gave very similar results and this indicates that the IgG fraction has an affinity towards the guanine group rather than the desired GA moiety.

4.4.6 Conjugation of BSA on guanosine and GMP

Originally, guanine should have been applied in this experiment but due to its poor solubility which would have led to a large volume, guanosine and GMP were conjugated with BSA and reacted with GA. Two different linkers were used, glutaraldehyde and HCHO. For the GA reaction the protocol of the hapten synthesis was followed and the reaction tubes were kept in the water bath at 37°C for 1 week. This reaction might not have been successful due to GA reacting with amino acids of the protein and hence not leading to the anticipated *N*7-GA-Gua fragment within the molecule.

4.4.7 Summary

This chapter described a variety of experiments that were undertaken in order to successfully develop a method for the detection of the *N*7-GA-Gua adduct.

Besides testing,

- different concentrations of antibodies and antigen
- applying different dilutions of tracer
- changing incubation times and temperatures
- trying to modify the antigen
- coating plates with DNA and antigen-protein conjugate
- using the antibodies to detect the adduct in blood DNA

unfortunately, no positive results were achieved. The diversity of tested experiments shows that a successful ELISA is most of all dependent on the antibodies. The use of the antibodies in a competitive ELISA was discontinued because of the lack of competition on addition of the antigen even in the presence of strong binding of hapten by the antibodies. The antibodies could possibly be used as an immunocapture reagent, but time did not permit the testing of this.

Chapter 5

5 Human Volunteer Study

5.1 Introduction

In this chapter, the hypothesis that the *N*7-GA-Gua adduct is found in humans following consumption of dietary AA will be tested. After method development (Chapter 2) and method validation (Chapter 3) an important part of this project was the completion of the human volunteer study to verify firstly if the *N*7-GA-Gua adduct can be detected in human samples and secondly, if so, at what levels. Moreover can a link be established between AA levels in diet and DNA adducts? This chapter explains the process of ethics and recruitment, before human data are discussed with the results obtained by the DNA adduct analysis of volunteer human leukocyte DNA samples and the Hb adducts of AA and GA for a subset of samples.

5.2 The human volunteer study

5.2.1 Ethics application process

Every research study that involves participation of human volunteers needs ethical permission by the UoL. The ethics application form was submitted to the research ethics review site for human subjects. Included in this application were the questionnaire that volunteers were asked to fill in, the participant information sheet and the consent form (see appendix section 8.2). A standard assessment process was used to consider the application. All data was subject to the provisions of the Data Protection and Freedom of Information Acts. The project references for the successful ethics application for Study 1 and Study 2 are ss610-0058b and ss610-9d750, respectively. They were completed on 22/03/13 and 20/05/2013.

5.2.2 Recruitment of volunteers

After the ethical application was approved healthy volunteers were recruited by distributing an email around the department of Cancer Studies and Molecular Medicine, UoL and associated scientists also from the UoL. The volunteers were asked

to donate blood and urine and fill in a short questionnaire about their dietary intake of common foodstuff that contains AA. The collected samples were anonymised prior to analyses.

5.2.3 Study design

To participate, subjects needed to be over 18 years and healthy. Volunteers were required to give their consent following reading the participant information sheet. A blood and a urine donation were collected from all participants. All volunteers filled in the FFQ about their average weekly intake of AA containing food as well as the intake 24 hrs prior to donation.

The changes in ethics between Study 1 and Study 2 are important in regards to the additional permission to proceed with supplemental analytical methods, i.e. the analysis of Hb adducts.

5.2.3.1 Study 1

For Study 1, 22 volunteers were recruited, 9 male and 13 female. One volunteer was an occasional smoker (1 cigarette every three days). Each participating volunteer donated 30 mL of whole blood and 50 mL of urine. From all volunteers there were 5 people where specimen collection of whole blood was inadequate to provide sufficient DNA to enable triplicate injection by LC-MS. One volunteer's sample only gave a single injection.

5.2.3.2 Study 2

11 people volunteered for Study 2, 7 male and 4 female including one smoker (15 cigarettes per day). Ethical permission for Study 2 allowed taking 35 mL blood and collecting 50 mL urine from each participant. Furthermore, the changes in ethics for Study 2 gave permission for the analysis of Hb adducts in whole blood as well as MA metabolites of AA and GA in urine. The Hb adduct analyses were done by collaborators

in Sweden (Margareta Törnqvist, Henrik Carlsson; University of Stockholm, Sweden) but the MA metabolites have not yet been analysed. One volunteer's blood donation did not lead to enough material for a single injection and therefore this volunteer was excluded from the study.

On the day of specimen collection the whole blood was processed, the nuclei pellet frozen and the leukocyte DNA extracted at a later stage. Urine samples were aliquoted and stored at -20°C.

5.2.4 Anonymisation of participants' samples

A senior biomedical scientist within the department kept a databank on the volunteers who donated blood and urine and anonymised their participation. Each volunteer was assigned a number by a random number generator in Excel that was placed on the blood collection tubes, urine collection cup as well as on the FFQ. Processing the collected specimens commenced after collection and anonymisation within 2 hrs.

5.2.5 Specimen collection

Blood was collected in BD Vacutainer®, LH 102 I.U. (6 mL) purchased from Bunzl, London, UK. Sterile polypropylene urine collection cups (120 mL) were purchased from Beckton, Dickinson and Company, Oxford, UK.

5.2.6 Questionnaire data for dietary AA intake

The volunteers' average weekly AA intake in µg and the AA intake 24 hrs prior to donation in µg were calculated based on the FFQ every volunteer filled in. The weekly AA intake was used to provide the daily AA intake in µg for each person.

The "Food Survey Information Sheet Number 01/13 April 2013 from the Food Standards Agency (FSA), A Rolling Programme of Surveys on Process Contaminants in UK Retail Foods, Interim Report Covering Sampling During 2011 - 2012, Acrylamide & Furan: Survey 5" was used to estimate the AA levels of various foods asked for in the

FFQ to ensure AA levels were based on UK data rather than European or US data [64]. The report stated the AA concentrations in µg/kg of various food categories for three different deliveries received between November 2011 and December 2012. These values were averaged to calculate the AA content of the food stated in the FFQ. The FFQ did not specify bread consumption of white and brown bread so these values were averaged. Some values are based on two or even one delivery value only. All averaged values were then used for the calculation of the AA intake of the participating volunteers.

The FFQ is based on serving sizes, slices or mugs. In order to calculate the AA value per portion, piece or slice the weight of biscuits, crisps, chips and bread slices was estimated.

Biscuits from different brands and type vary in weight and taking this into account an average of 18 g per biscuit was used. The mass per serving of crisps also varied between brands and 30 g was defined as weight per portion of crisps unless volunteers stated their commonly consumed brand, in which case the serving size for the mentioned brand was used if applicable and listed in the FSA report. There is also variation in the weight for a slice of bread, e.g. thin or thick cut and variation by brand and to calculate the AA content a slice of bread was defined as 47 g. An averaged value was used for cereals unless volunteers stated a certain brand. If the brand was listed in the FSA report then the AA content calculation was based on that value. If volunteers stated a brand that was not mentioned in the FSA report the averaged value was taken for the calculation.

Of all 32 volunteers two people stated they were smokers. The literature states AA levels between 1 – 2 µg of AA/cigarette [73]. Therefore the average of 1.5 µg AA/cigarette was used to approximate the AA intake through smoking.

Coffee samples (fresh brew and instant granulates) were weighed and the values used to calculate the AA content per mug. If volunteers did not indicate if they consumed fresh brew or instant coffee an average of both values was used.

Table 5.1: The table lists the AA in µg/kg levels for the food categories specified in the FSA report. The second column shows the weight in g per portion, slice or mug and the AA value in µg per portion/slice/mug is listed in the last column for all food categories within the FFQ [64]

	AA in µg/kg	Weight in g per portion/slice/mug	AA in µg per portion/slice/mug
Crisps - general average	756.0	30.0	22.7
Crisps (Kettle)	280.0	40.0	11.2
Crisps (Walkers)	432.0	30.0	13.0
Chips	201.0	150.0	30.2
Biscuits	316.0	18.0	5.7
Coffee-fresh brew	239.0	10.0	2.4
Coffee-instant	771.0	3.5	2.7
Chocolate	161.0	100.0	16.1
Bread (other)	22.5	47.0	1.1
Crisp bread	190.0	10.0	1.9
Dark rye crisp bread (Ryvita)	350.0	10.0	3.5
Cereals	168.5	45.0	7.6
Weetabix organic	121.0	37.5	4.5
Bran flakes	415.0	30.0	12.5
Cigarettes	1.0 – 2.0 µg/cig	1.5	1.5

Once the weight in g per portion/slice/mug was determined the AA content in µg per portion/slice/mug was calculated (Table 5.1). These values were then used to calculate the weekly AA intake in µg per volunteer, from which an average daily intake was derived. The same calculation process was followed for the FFQ information 24 hrs prior to donation.

5.3 Analysis of human volunteers samples

5.3.1 DNA adducts

The developed (Chapter 2) and validated (Chapter 3) method was used to analyse the *N*7-GA-Gua adduct in human leukocyte DNA.

5.3.2 Hb adducts

The AA- and GA-Hb adducts were analysed at Stockholm University, Sweden by Henrik Carlsson.

METHOD

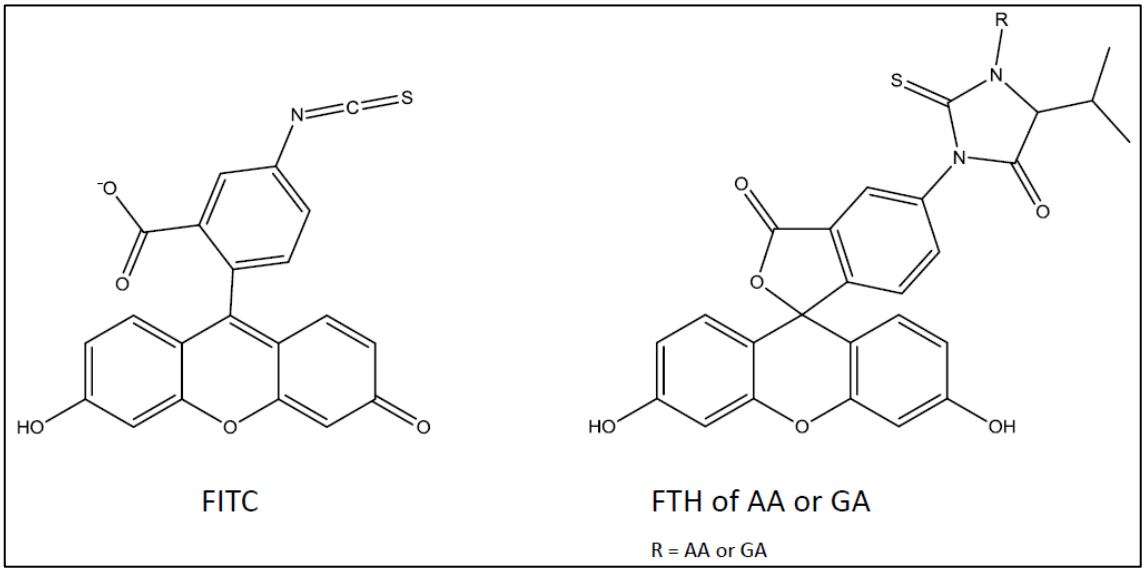


Figure 5.1: The reagent FITC was used for derivatising the AA- and GA-Hb adducts for the detection as AA-Val-FTH (AA-fluorescein-5-[4-isopropyl-3-(2-carbamoylethyl)-2-thioxo-imidazolidin-5-one]) or GA-Val-FTH (GA-fluorescein-5-[4-isopropyl-3-(2-carbamoylethyl)-2-thioxo-imidazolidin-5-one]) by LC-MS/MS. Modified from von Stedingk *et al*. [95].

Each volunteer's Hb level was determined with HemoCue before 250 µL of whole blood was transferred into a 2 mL eppendorf tube. 1 M $KHCO_3$ (15 µL) and fluorescein isothiocyanate (30 µL) (FITC; 5 mg dissolved in 30 µL DMF per sample) (Figure 5.1) were added into each tube and mixed. The samples for the calibration line were prepared by pipetting 250 µL cows blood and mixing it with 1 M $KHCO_3$ (15 µL) and FITC solution (30 µL). The samples were placed in an Eppendorf Thermomixer Comfort,

mixed and incubated at 37°C overnight. The next day IS (50 µL, d_7-AA-Val-FTH and d_7-GA-Val-FTH, 0.1 pmol/µL) was added. Additionally, the calibration line samples were spiked with unlabelled adduct. To each tube ACN (1.5 mL) was added, the tubes mixed and centrifuged for 10 min at 3000*g*. Oasis Max columns (60 mg, Waters) were conditioned with ACN (2 mL) and equilibrated with 0.01 M ammonium hydroxide (1 mL). Into each sample 1 M ammonium hydroxide solution (50 µL) was added before applying onto the SPE cartridges. The columns were washed with ACN (2 mL), dH_2O (2 mL) and 0.5% cyanoacetic acid in dH_2O (2 mL). The samples were eluted into HPLC vials with 0.25% cyanoacetic acid in 6:4 ACN:dH_2O (*v/v*) (1.4 mL). The vials were placed in a rack and the eluate evaporated to dryness under a gentle stream of air. The samples were re-dissolved in 7:3 dH_2O:ACN (*v/v*) (100 µL) and analysed by LC-MS (HPLC system: Shimadzu; mass spectrometer: ABSciex 3200 QTrap). The mobile phase consisted of 95:5 0.1% FA/ACN (*v/v*) (A) and 5:95 0.1% FA/ACN (*v/v*) (B) with a flow rate of 0.12 mL/min. The column used was a 150 x 2.1, 3 µm Supelco Discovery HS, C18 from Sigma. The run finished after 25 min of which the first 5 min were directed to waste. The gradient was linear, starting at 0.5 min to 100% B, holding B at 100% for 5 min before the column was equilibrated to starting conditions for 5 min.

5.4 Volunteers data – Results

5.4.1 Volunteers AA intake

The data for each volunteer's AA intake can be found in the appendix (section 8.3).

Table 5.2 informs about minimum, maximum, average, median and standard deviation of AA intake in µg per week, per day and for the period 24 hrs prior to donation. The data includes all volunteers and the same analysis was done excluding the data of the smoking volunteers.

Table 5.2: Volunteers AA intake listed as minimum, maximum, average, standard deviation and median AA intake in µg per week, daily AA intake in µg and for the 24 hrs period prior to donation

	AA intake in µg (all volunteers)			AA intake in µg (smokers excluded)		
	weekly	**daily**	**24 hrs prior to donation**	**weekly**	**daily**	**24 hrs prior to donation**
Minimum	0.00	0.00	5.60	0.00	0.00	5.60
Median	172.35	24.62	24.75	172.35	24.62	23.73
Maximum	462.90	66.13	88.29	324.80	46.40	88.29
Average	177.36	25.34	33.19	172.52	24.64	32.45
Standard deviation	92.62	13.23	19.72	75.20	10.74	19.98

The weekly AA intake for all volunteers ranged from 0.00 to 462.90 ± 91.70 µg AA/week. The average daily AA intake was between 0.00 to 66.13 ± 13.10 µg AA/day. 24 hrs prior to donation AA intake values ranged from 5.60 to 88.29 ± 25.50 µg AA/24 hrs.

Table 5.3 informs about minimum, maximum, average and median AA intake in µg per week, per day and for the period 24 hrs prior to donation divided into male and female volunteers.

Table 5.3: Volunteers AA intake, listed for male and female volunteers, reported as minimum, maximum, average, standard deviation and median AA intake in µg per week, daily AA intake in µg and for the 24 hrs period prior to donation separately listed for all male and female volunteers

	AA intake in µg (female volunteers)			AA intake in µg (male volunteers)		
	average week	**average day**	**24 hrs prior to donation**	**average week**	**average day**	**24 hrs prior to donation**
Minimum	0.00	0.00	5.60	73.80	10.54	13.23
Median	139.15	19.88	22.18	234.30	33.47	32.20
Maximum	214.50	30.64	75.10	462.90	66.13	88.29
Average	127.91	18.27	28.99	226.80	32.40	37.38
Standard deviation	63.37	9.05	18.22	92.19	13.17	20.83

The weekly, daily and 24 hrs prior to donation AA intake in µg was smaller for female volunteers compared to male volunteers.

The daily AA intake was derived from the weekly data and was used throughout this chapter. Figure 5.2 shows the distribution of the average daily AA intake in µg (in blue) and for the period 24 hrs prior to donation of blood and urine sample for each volunteer (shown in red).

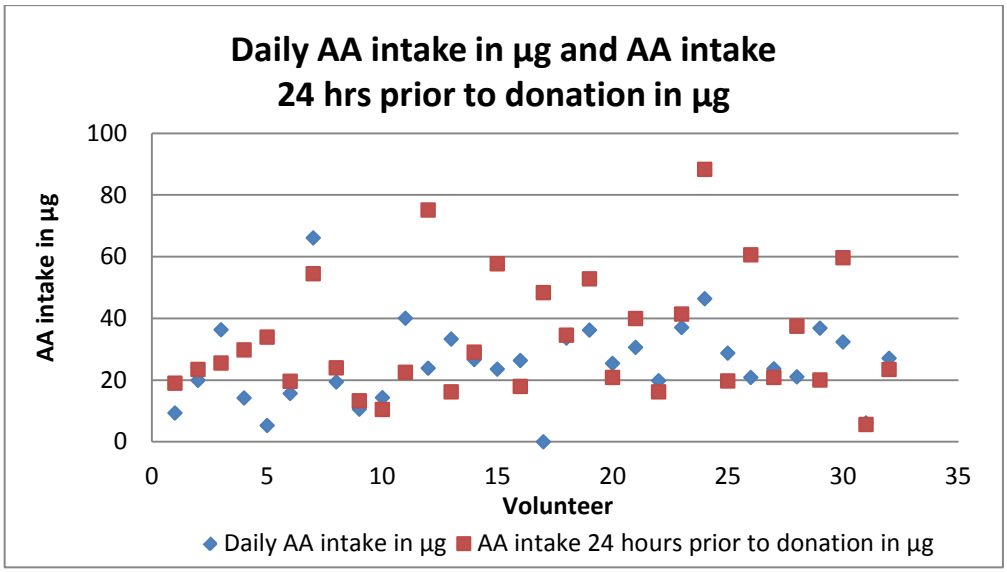


Figure 5.2: Volunteers daily AA intake in µg (shown in blue) and AA intake 24 hrs prior to sample donation (shown in red).

The daily AA intake for all volunteers was widely distributed ranging from 0.00 to 66.13 ± 13.10 µg with the highest value obtained from the 15 cigarettes/day smoking volunteer (Volunteer 7). The difference in estimated AA intake between this smoker to the next highest volunteer's estimated AA intake value was 19.7 µg AA.

The distribution changed when looking at the food consumption 24 hrs prior to donation. The AA intake ranged from 5.60 to 88.29 ± 25.50 µg. The volunteer with a typical AA free week consumed chocolate within the 24 hrs period prior to donation of blood and urine leading to an AA intake of 48.30 µg AA. The highest AA intake was 88.29 µg (Volunteer 24) due to consumption of crisps, chips, biscuits and coffee.

5.4.2 Influence of individual foodstuff on total AA intake

Figure 5.3 shows the proportion for each food item in relation to the total AA intake of all volunteers.

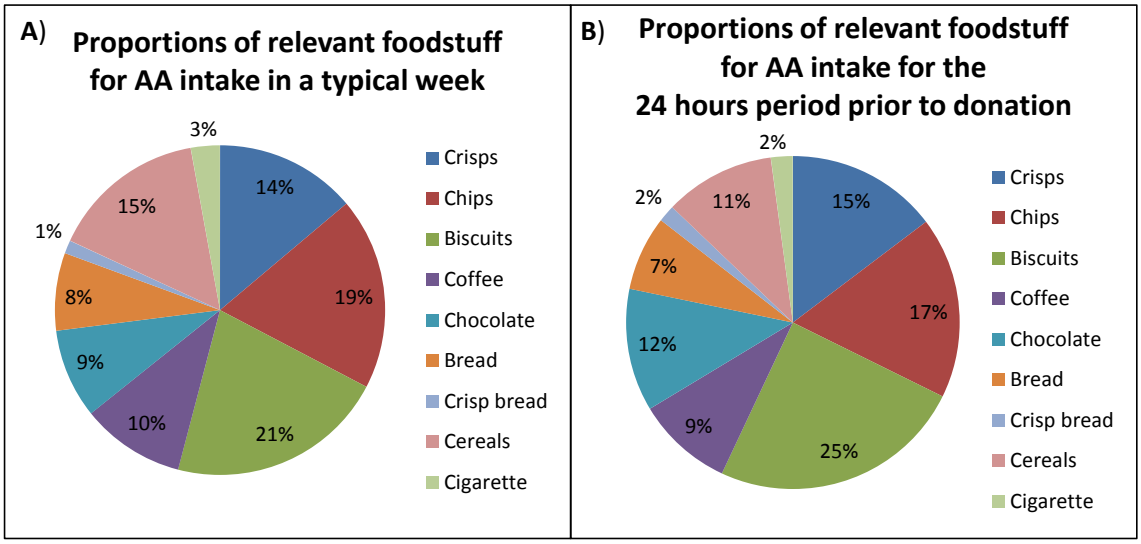


Figure 5.3: The proportion of each foodstuff listed in the FFQ in proportion to the total AA intake of all volunteers. Figure A) shows the proportion in regard to the weekly AA consumption and Figure B) reflects the data for the period 24 hrs prior to sample donation.

Difference between daily AA intake and 24 hrs prior to donation AA intake

For some volunteers the daily AA intake was in agreement to the AA intake 24 hrs prior to donation whereas for others there was a big difference between the two values (Figure 5.2).

Percentage of all food categories on an individual's level based on the AA intake data 24 hrs prior to donation

The percentage of the AA intake on an individual's level caused by the food types listed in the FFQ for the 24 hr period are shown in Table 5.4 as proportions.

Table 5.4: The range in percentages of all listed foodstuff in the FFQ on an individual's level to the total AA intake 24 hrs prior to donation

	Percentage in %
Crisps	14.7 - 66.9
Chips	20.3 - 87.3
Biscuits	19.8 - 70.4
Coffee	5.4 - 32.6
Chocolate	1.5 - 100
Bread	6.3 - 100
Crisp bread	43.3 - 100
Cereals	10.1 - 80.4

Most volunteers who ate chips 24 hrs prior to donation obtained a higher estimated level of AA intake than volunteers that did not consume chips. For this study population the biscuits consumption had a high contribution towards the AA intake of volunteers. Bread contains 1.1 µg AA/slice and hence was not of high impact to total AA intake when consuming 1 or 2 slices/day. Although the original value per slice appears small it contributed greatly towards the AA intake if bread was consumed as a staple food on a regular basis.

5.4.3 *N*7-GA-Gua adduct detection in leukocyte DNA

The collected volunteers whole blood was processed as described in section 2.2.2.2.1.

The samples of 32 volunteers were analysed of which 26 volunteers were performed in triplicate, 5 volunteers in duplicate and one volunteer's sample was a single injection, giving a total of 89 injections by LC-MS. The table listing the mean *N*7-GA-Gua peak areas, mean [$^{15}N_5$]-*N*7-GA-Gua peak areas and mean ratio (*N*7-GA-Gua peak area/[$^{15}N_5$]-*N*7-GA-Gua peak area) peak areas is shown in the appendix, section 8.4.

In three quarters of all samples there was apparently a peak detectable that elutes within a few seconds to the IS (Figure 5.4).

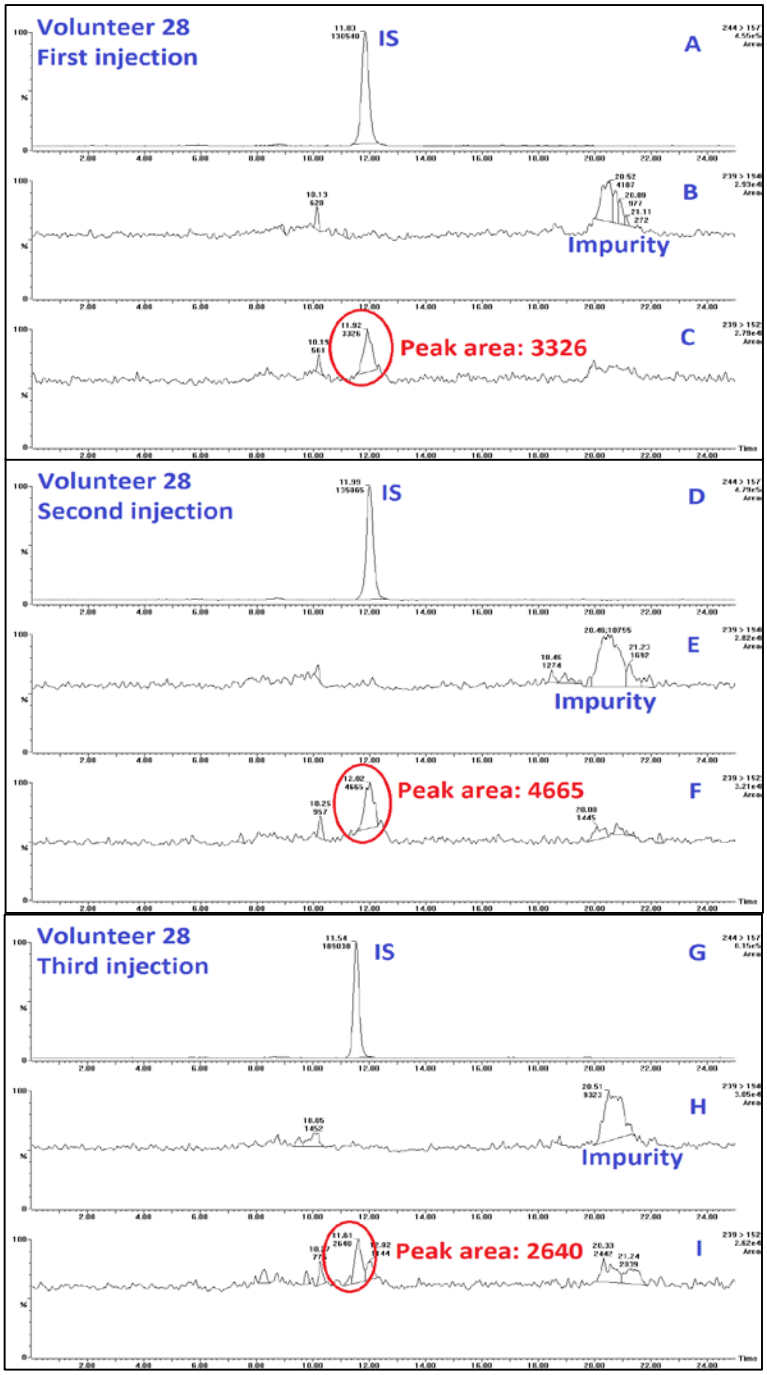


Figure 5.4: LC-MS/MS SRM chromatograms for the triplicate analysis of one volunteer's samples where the *N*7-GA-Gua peak areas were quite close to each other. LC-MS/MS SRM chromatograms A – C represent the first injection, LC-MS/MS SRM chromatograms D – F represent the second injection and LC-MS/MS SRM chromatograms G – I represent the third injection. LC-MS/MS SRM chromatograms A, D and G show the transition *m/z* 244 to 157 for the IS. LC-MS/MS SRM chromatograms B, E and H monitored the fragmentation of *m/z* 239 to 194 for the *N*7-GA-Gua adduct. LC-MS/MS SRM chromatograms C, F and I show the transition for *m/z* 239 to 152 for the detection of the *N*7-GA-Gua adduct.

Hence, there is evidence to suggest that the *N*7-GA-Gua adduct was present. Figure 5.5 shows the mean peak areas for the *N*7-GA-Gua adduct peaks for all volunteers samples and the mean peak areas for the IS [$^{15}N_5$]-*N*7-GA-Gua of all volunteers.

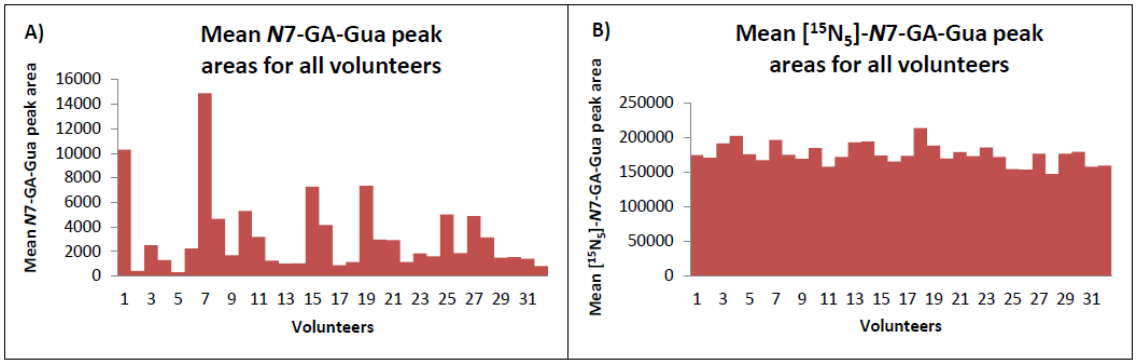


Figure 5.5: Mean *N*7-GA-Gua peak areas and mean [$^{15}N_5$]-*N*7-GA-Gua peak areas. Figure A) presents the mean *N*7-GA-Gua peak areas for the analysed leukocyte DNA samples for all volunteers and Figure B) shows the mean [$^{15}N_5$]-*N*7-GA-Gua peak areas for the leukocyte DNA samples analysed for all volunteers.

The high variability within analysed duplicate/triplicate samples can be seen in Figure 5.6 where *N*7-GA-Gua peak areas of one volunteer's triplicate analysis ranged from 3987 to 12057.

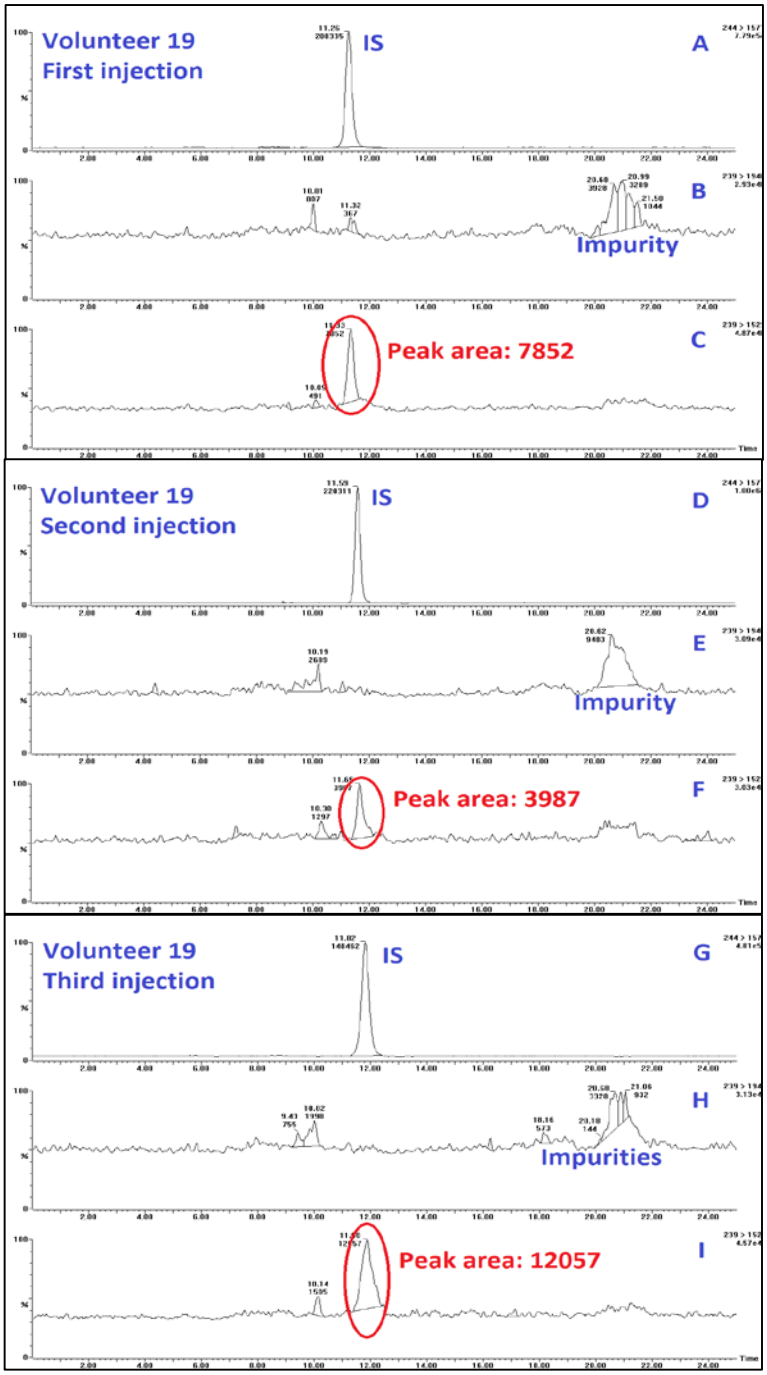


Figure 5.6: LC-MS/MS SRM chromatograms for the triplicate analysis of one volunteer's samples where the *N*7-GA-Gua peaks had widespread areas. LC-MS/MS SRM chromatograms A – C represent the first injection, LC-MS/MS SRM chromatograms D – F represent the second injection and LC-MS/MS SRM chromatograms G – I represent the third injection. LC-MS/MS SRM chromatograms A, D and G show the transition *m/z* 244 to 157 for the IS. LC-MS/MS SRM chromatograms B, E and H monitored the fragmentation of *m/z* 239 to 194 for the *N*7-GA-Gua adduct. LC-MS/MS SRM chromatograms C, F and I show the transition for *m/z* 239 to 152 for the detection of the *N*7-GA-Gua adduct.

The high variability can further be seen in Table 5.5 which lists all minimum, maximum, average, median and standard deviation values for the RSD of the mean IS [$^{15}N_5$]-*N*7-

GA-Gua peak, RSD of the mean *N*7-GA-Gua peak for all runs and the RSD for the mean ratio of the *N*7-GA-Gua peak area/[$^{15}N_5$]-*N*7-GA-Gua peak area.

Table 5.5: Values for minimum, maximum, average, median and standard deviation for the RSD in % of the IS [$^{15}N_5$]-*N*7-GA-Gua, the *N*7-GA-Gua adduct peak and the ratio of the *N*7-GA-Gua peak area/[$^{15}N_5$]-*N*7-GA-Gua peak area

	RSD [$^{15}N_5$]-*N*7-GA-Gua (%)	RSD *N*7-GA-Gua (%)	RSD Ratio *N*7-GA-Gua/[$^{15}N_5$]-*N*7-GA-Gua (%)
Minimum	0.66	5.95	1.38
Maximum	23.20	173.21	173.21
Average	11.74	77.59	80.16
Median	12.59	69.27	77.25
Standard deviation	6.17	45.10	43.65

5.4.4 Relationship between *N*7-GA-Gua adduct levels and the AA intake of volunteers

All volunteers sample analysis

The relationship between the *N*7-GA-Gua adduct levels and the estimated daily AA intake for all volunteers is shown in Figure 5.7. The relationship between the *N*7-GA-Gua adduct levels and the AA intake 24 hrs prior to donation is presented in Figure 5.8. In both, Figure 5.7 and Figure 5.8 all analytical runs were included in the bar graph for the *N*7-GA-Gua adduct levels.

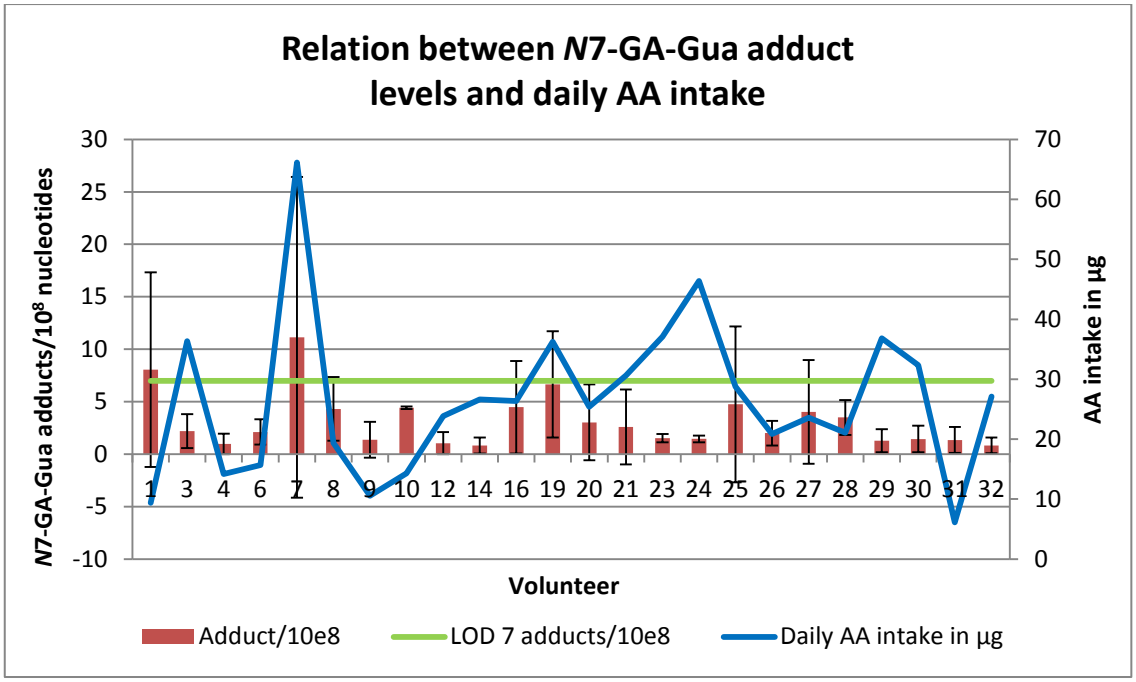


Figure 5.7: Relationship between the mean *N*7-GA-Gua adduct levels (in adducts/10^8 nucleotides) and the daily AA intake in µg for all volunteers. The red coloured bars represent the *N*7-GA-Gua adduct levels on the left hand y-axis and the blue coloured line represents the daily AA intake in µg on the right hand y-axis. The green line represents the LOD for the detection of *N*7-GA-Gua with the developed method of 7 adducts/10^8 nucleotides.

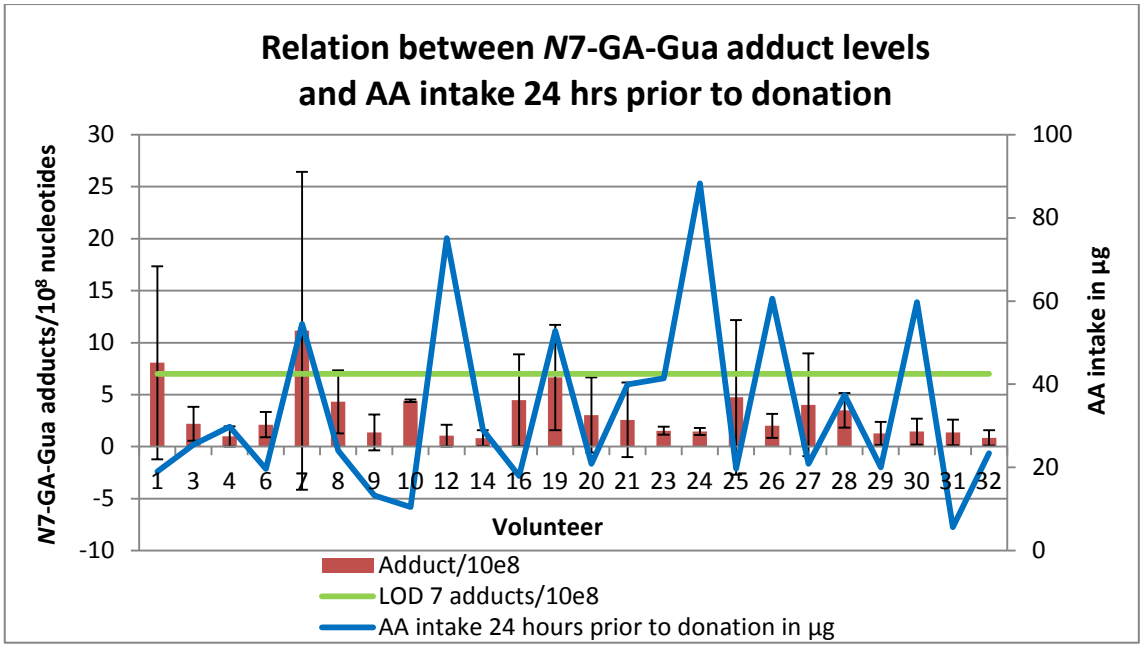


Figure 5.8: Relationship between mean *N*7-GA-Gua adduct levels (in adducts/10^8 nucleotides) and the AA intake in µg 24 hrs prior to donation for all volunteers. The red coloured bars represent the mean *N*7-GA-Gua adduct levels on the left hand y-axis and the blue coloured line represents the 24 hrs prior to donation AA intake in µg on the right hand y-axis. The green line represents the LOD of 7 adducts/10^8 nucleotides of the method.

Both graphs showed a huge standard deviation for the *N*7-GA-Gua adduct levels and only for some volunteers the estimated AA intake agreed with the level of *N*7-GA-Gua adduct.

Only triplicate analysis of volunteers

When only volunteers were considered with *N*7-GA-Gua adduct levels detectable in all three runs (triplicate analysis) there was a better relationship to be seen between AA intake and *N*7-GA-Gua adduct levels as shown in Figure 5.9 for daily AA intake and in Figure 5.10 for the AA intake 24 hrs prior to donation.

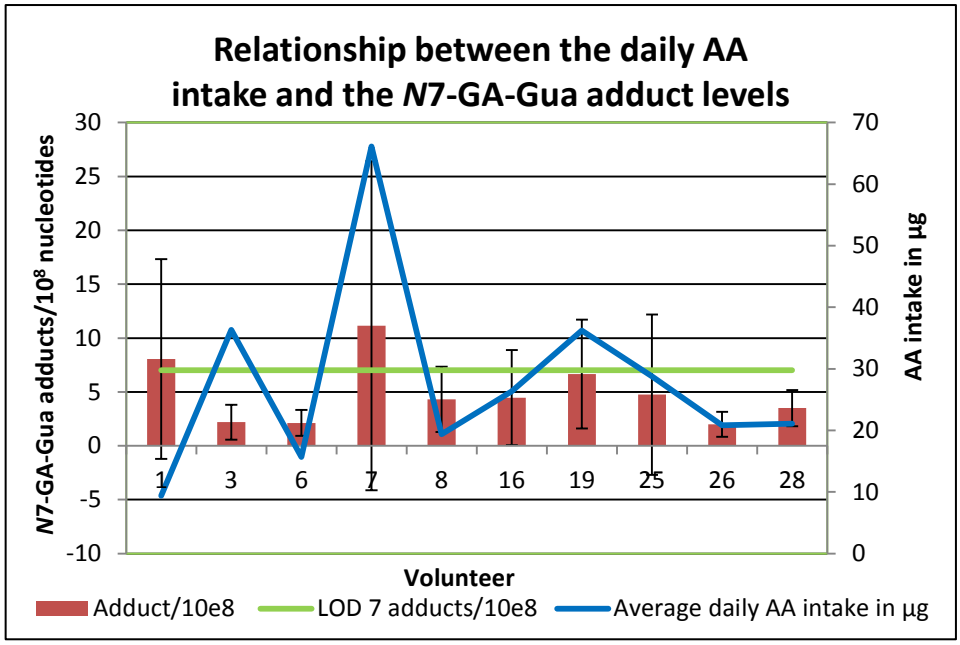


Figure 5.9: Relationship between mean *N*7-GA-Gua adduct levels (in adducts/10^8 nucleotides) and the daily AA intake in µg for 10 volunteers with measurable adduct levels in all three analytical runs. The red coloured bars represent the *N*7-GA-Gua adduct levels on the left hand y-axis and the blue coloured line represents the daily AA intake in µg on the right hand y-axis. The green line represents the LOD of 7 adducts/10^8 nucleotides for the method of *N*7-GA-Gua adduct detection.

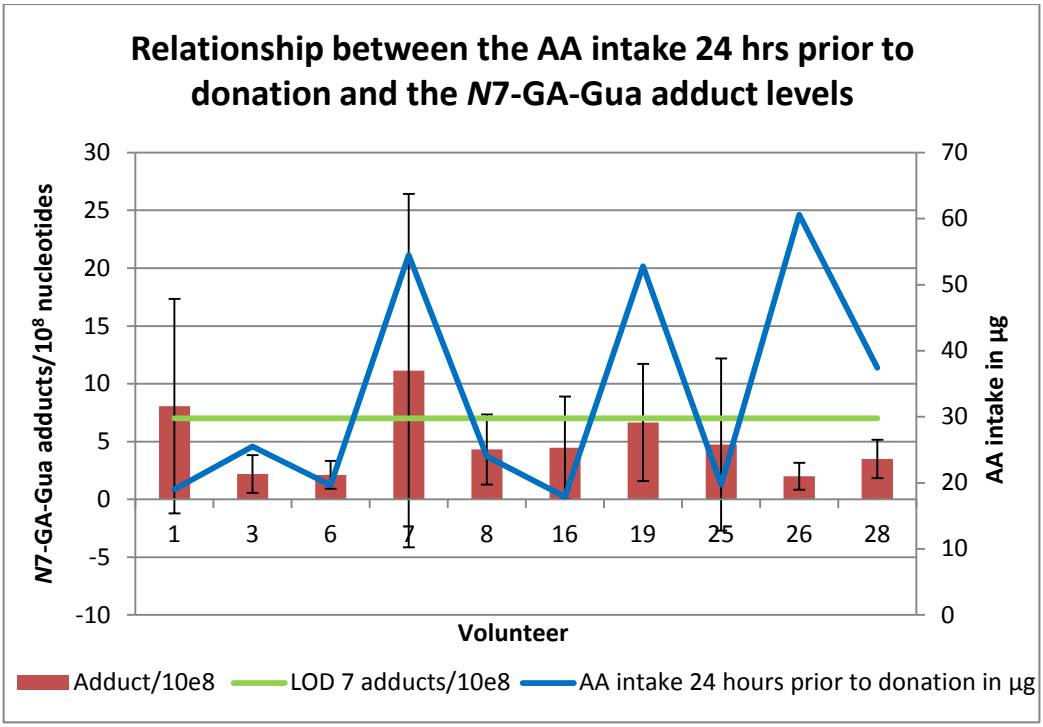


Figure 5.10: Relationship between mean *N*7-GA-Gua adduct levels (in adducts/10^8 nucleotides) and the AA intake in µg 24 hrs prior to donation for 10 volunteers with detectable adduct levels in all three analyses. The red coloured bars represent the mean *N*7-GA-Gua adduct levels on the left hand y-axis and the blue coloured line represents the 24 hrs prior to donation AA intake in µg on the right hand y-axis. The green line represents the LOD of 7 adducts/10^8 nucleotides for the method of *N*7-GA-Gua adduct detection.

The relationship between AA intake and *N*7-GA-Gua adduct levels appeared to be stronger when only volunteers with triplicate analysis were presented.

5.4.5 Hb adduct detection in whole blood – Study 2

AA- and GA-Hb adducts were detectable in all volunteers samples and Figure 5.11 shows a typical chromatogram for the LC-MS/MS analysis of a smoker's blood sample.

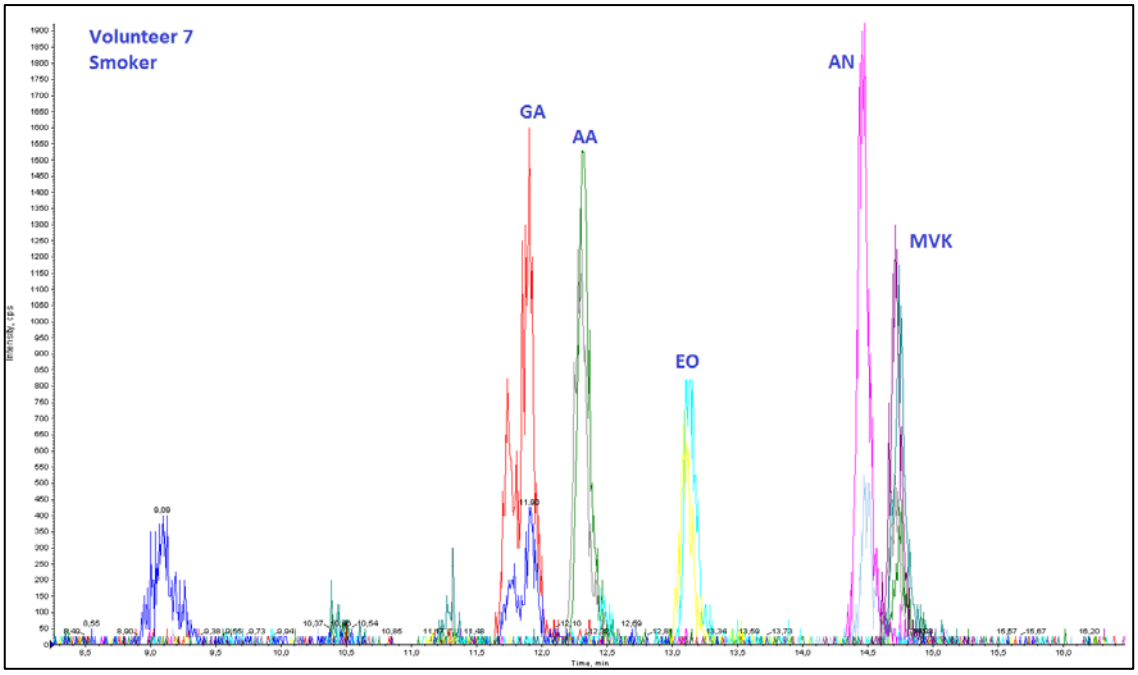


Figure 5.11: A typical chromatogram for the LC-MS/MS analysis of AA- and GA-Hb adduct levels obtained for the smoking volunteer. EO = ethylene oxide, AN = acetonitrile, MVK = methyl vinyl ketone were included in the Hb analysis method but are not of relevance to this project.

Table 5.6 lists the results for the AA- and GA-Hb adduct analysis. Every volunteer's analysis was done in triplicate.

Table 5.6: Minimum, maximum, average, median and standard deviation for the AA- and GA-Hb adduct analysis in pmol/g Hb. The values are presented for all volunteers and additionally for non-smokers only

	Including Smoker		Excluding Smoker	
	AA-Hb (pmol/g Hb)	**GA-Hb (pmol/g Hb)**	**AA-Hb (pmol/g Hb)**	**GA-Hb (pmol/g Hb)**
Minimum	19	11	19	11
Maximum	264	51	97	49
Average	70	28	56	26
Median	58	26	54	25
Standard deviation	50	11	21	9

The values for the AA-Hb adducts ranged between 19 and 264 pmol/g Hb and the GA-Hb adducts varied between 11 and 51 pmol/g Hb. Excluding the values of the smoker lowered the range of AA-Hb adducts to 19 to 97 pmol/g Hb but the difference for GA-Hb adducts was still very similar with 11 to 49 pmol/g Hb.

Relationship between AA- and GA-Hb adducts and estimated AA intake

Figure 5.12 shows the relationship between the AA- and GA-Hb adducts and the daily AA intake in µg as well as the AA intake for the 24 hrs prior to donation in µg.

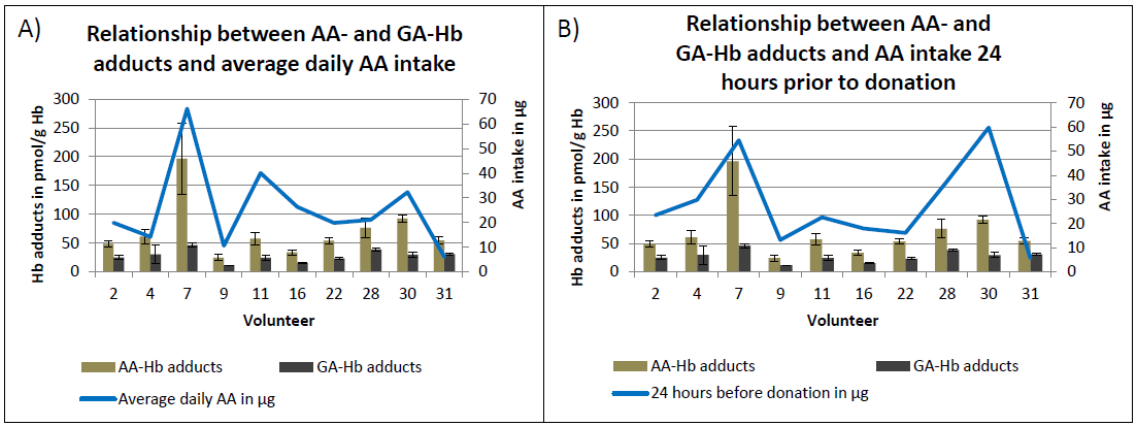


Figure 5.12: Relationship between Hb adducts and AA intake. Figure A) shows the relationship between the AA- and GA-Hb adduct levels and the daily AA intake in µg and Figure B) shows the relationship between the AA- and GA-Hb adduct levels and the AA intake in µg for the period 24 hrs prior to donation for the 10 volunteers donating blood in Study 2.

The AA intake for both daily and 24 hrs prior to donation and the Hb adducts showed a good relationship with the exception of two volunteers for daily AA intake and four volunteers for the 24 hrs prior to donation period.

Relationship between AA- and GA-Hb adducts and N7-GA-Gua adduct levels

The relationship between the AA- and GA-Hb adducts and the *N*7-GA-Gua adduct levels is shown in Figure 5.13.

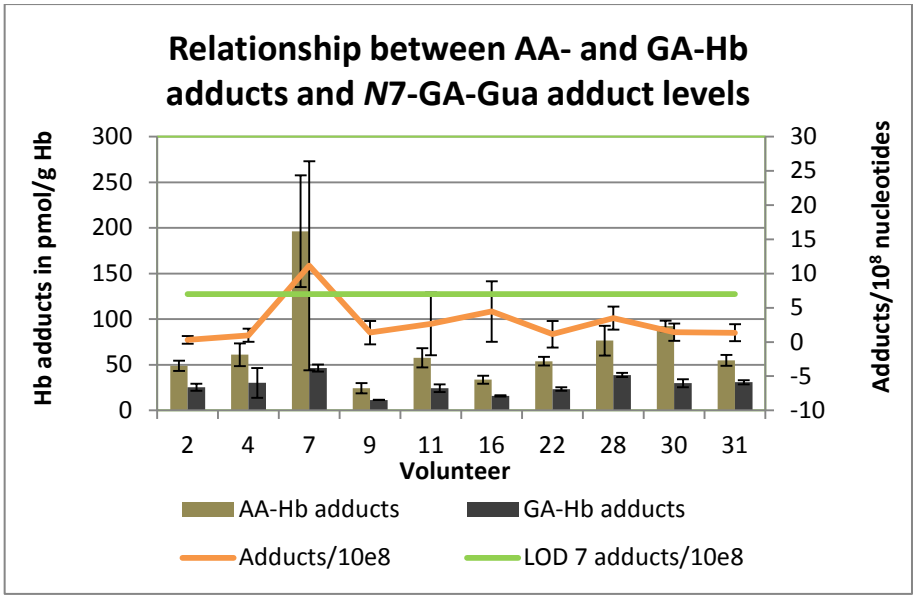


Figure 5.13: Relationship between the AA- and GA-Hb adduct levels and the *N*7-GA-Gua adduct levels. The light coloured bars show AA-Hb adducts, the dark coloured bars the GA-Hb adducts and the orange line represents the *N*7-GA-Gua adduct/10^8 nucleotides value. The green line represents the LOD of the method for the DNA adduct analysis with 7 adducts/10^8 nucleotides. Volunteer 7 is a smoker.

There was a relationship between the DNA and Hb adducts which was supported by the high correlation coefficients obtained in the Spearman correlation (see Table 5.7) but did not reach significance.

5.4.6 Statistical analysis

Histograms

For the statistical analysis Stata version 13 was used.

The distribution of the median *N*7-GA-Gua adduct/10^8 nucleotides and the AA intake obtained from the FFQ is shown in Figure 5.14.

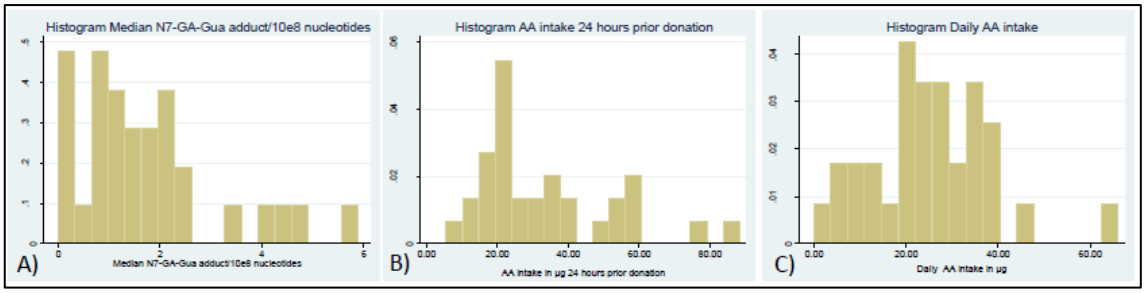


Figure 5.14: Histograms for A) Median *N*7-GA-Gua adduct/10^8 nucleotides B) Daily AA intake in µg and C) AA intake 24 hrs prior to donation in µg.

Neither the *N*7-GA-Gua nor either of the AA intakes was normally distributed.

Spearman correlation

The results for the Spearman's rank correlation coefficient test are presented in Table 5.7. Statistically significant results are marked with an * and p-values printed in red.

Table 5.7: Spearman's rank correlation coefficients. The first row presents rho values, the second row presents numbers of observations and the third row presents the p values. Results are statistically significant if $p < 0.05$ and are labelled with *

	Median *N*7-GA-Gua adduct	Daily AA intake	AA intake 24 hrs prior to donation	Median AA-Hb adducts	Median GA-Hb adducts	Sex	Smoker	Age
Median *N*7-GA-Gua adduct	1.0000							
	32							
Daily AA intake	0.0646	1.0000						
	32	96						
	0.7253							
AA intake 24 hrs prior to donation	-0.0191	0.3448*	1.0000					
	32	96	96					
	0.9174	0.0006						
Median AA-Hb adducts	0.5092	0.5394	0.7939*	1.0000				
	10	10	10	10				
	0.1327	0.1076	0.0061					
Median GA-Hb adducts	0.4970	0.4667	0.6121	0.8061*	1.0000			
	10	10	10	10	10			
	0.1439	0.1739	0.0600	0.0049				
Sex	0.1288	0.5754*	0.2336*	0.3419	-0.0380	1.0000		
	32	96	96	10	10	96		
	0.4822	0.0000	0.0220	0.3336	0.9170			
Smoker	-0.0420	0.0140	0.1958	0.5222	0.5222	0.0000	1.0000	
	32	96	96	10	10	96	96	
	0.8194	0.8924	0.0559	0.1215	0.1215	1.0000		
Age	0.2287	0.1487	-0.1768	-0.2031	-0.2893	0.0409	-0.1056	1.0000
	32	96	96	10	10	96	96	96
	0.2081	0.1481	0.0848	0.5736	0.4176	0.6924	0.3060	

There were no significant correlations regarding the *N*7-GA-Gua adduct. However, the AA intake 24 hrs prior to donation correlated with the daily AA intake. Further correlations existed between the median AA-Hb adduct levels and the AA intake 24 hrs

prior to donation, the median GA-Hb adduct levels with the median AA-Hb adduct levels and sex (male) with daily AA intake as well as AA intake 24 hrs prior to donation.

The correlation coefficient for the median *N*7-GA-Gua adduct and the Hb adducts were quite high (AA-Hb: 0.5092 and GA-Hb: 0.4970) but statistically non-significant. The daily AA intake showed high correlation coefficients for the AA-Hb adducts (0.5394) and GA-Hb adducts (0.4667) but was non-significant. The Hb adducts had a high correlation coefficient (0.5222) with smoker but were not statistically significant.

Since there was no statistically significant result in the Spearman correlation test for the *N*7-GA-Gua adduct no further statistical tests were performed.

5.5 Discussion

The human volunteers' leukocyte DNA samples were analysed and the results will be discussed in respect to analysis, AA intake, FFQ and Hb adducts.

5.5.1 Recruitment of volunteers

The main aim of this study was to perform a preliminary measurement of the *N*7-GA-Gua adduct present in human leukocyte DNA following dietary exposure to AA. Therefore the recruiting process was straightforward and volunteers could participate if they were healthy and aged 18 or over. Using a different study design could make the recruiting process more difficult.

5.5.2 Questionnaire and AA intake

The FFQ asked for volunteers' gender, age, if they smoke and consumed quantities of different AA containing foodstuff for a typical week's consumption and the 24 hrs prior to donation of blood and urine samples. It would have been advantageous to include weight so that the AA intake could have been calculated as µg/kg Bw as commonly stated in literature. Recruiting volunteers from within the department where

laboratory work partly involves the use of AA would have made it useful asking volunteers if they are exposed to this additional AA exposure. Missing this information could lead to an underestimation of the AA intake. Ethanol induces the human CYP2E1 enzyme and hence alcohol consumption might have been of interest. Vikström *et al*. found a negative correlation between GA-Hb/AA-Hb adduct ratios and alcohol intake [137].

Regarding the volunteer who only smokes one cigarette every 3 days the questionnaire should have had a section included within the table for foodstuff to see if the person smoked and/or how many cigarettes within the period 24 hrs prior to donation. This does not have a big impact on the person smoking 15 cigarettes a day. Von Tungeln *et al.* stated the exposure to AA from cigarette smoke to about 3.1 µg/kg Bw/day [52]. Using this value to do the calculation and assuming an average of 70 kg per person the AA intake through smoking would lead to 217 µg/day. The smoker in this study has an AA intake through smoking of 22.5 µg/day by calculating 1.5 µg AA/cigarette which is marginally less and might underestimate the AA intake through smoking. On the other hand, looking at the highest daily AA intake which was accomplished by this smoker and seeing that there is a difference of almost 20 µg AA to the next highest daily AA intake shows that the way the AA intake was calculated matches up with the data. The FFQ only asked for AA containing food. Since there is no information about the remaining food intake no calculation based on the energy intake can be performed like Duarte-Salles *et al*. reported. In a second analysis they did the calculation based on the AA exposure relative to Bw [71]. Wirfält *et al*. reported an AA intake between 3.5 – 189 µg/day with a median of 45 µg/day for all subgroup of samples from the Malmö Diet and Cancer cohort. The median AA intake for the low intake group was 9.2 µg/day (n = 20), the high intake group 69 µg/day (n = 82) and for randomly picked subjects 25 µg/day (n = 40), which is close to the reported median of 24.62 µg/day in this study [117]. Bjellaas *et al*. on the other hand reported median AA intake levels of 13.5 µg/kg in non-smokers, 18.3 µg/kg for smokers and also noticed higher AA intake values in males than females [94].

5.5.3 AA in foodstuff

The evaluation of the AA intake of volunteers is based on approximation and averages. It is difficult to determine an AA value for a certain type of food because AA levels of the same batch vary and additionally there are differences in AA levels between brands [60,63]. This is illustrated in the FSA report, where AA content for three different deliveries of foodstuff from the same brand bought in different towns at three different time points showed huge variation, e.g. Co-operative own brand crisps had an AA level of 150 µg/kg in November 2011, 1244 µg/kg for the batch analysed in March 2012 and 222 µg/kg in November 2012 [64]. Temperature has a big influence on the AA content of food as shown by Sörgel *et al*. Preparing crisps at 140°C for 2 min led to an AA level of 36.3 µg/kg whereas an increase in temperature to 180°C increased the AA level to 2557.9 µg/kg. Increasing the cooking time to 4 min led to an AA level of 53.3 µg/kg at 140°C and 7678.3 µg/kg at 180°C. Similar results were obtained for French fries, showing increased AA levels with increasing temperature from 140°C to 160°C and finally 180°C with a further increase in AA levels when cooking time was doubled [138]. Additionally, the FSA report stated AA values for French fries as ready sold between 41 – 888 µg AA/kg. French fries pre-cooked for home cooking have a bigger variation with a range between 17 – 2908 µg AA/kg [64]. Home cooked food varies in AA levels because the cooking temperature, preferences and taste differs between people. Additionally, AA levels in food prepared at home are not controlled. Svensson *et al*. listed AA values in commonly consumed food in Sweden which are higher than FSA values used for this FFQ for crisps, bread, cereals and French fries. Similar values were obtained for biscuits and crisp bread. The AA content of coffee was only 25 µg/kg whereas this study estimated 771 µg/kg for instant coffee and 239 µg/kg for fresh brew coffee [139]. A further aspect where no data is yet published is the bioavailability of AA from different food items which would be interesting to include in AA containing food databanks.

5.5.4 Statistical analysis

The statistical correlation between the daily AA intake and the 24 hrs prior to donation AA intake shows that there was not a big difference in people's AA intake for a week and the AA intake 24 hrs prior to donation.

Hb adducts are a marker for long term exposure of the last 4 months and the AA intake 24 hrs prior to donation should not be of high relevance to the levels but yet the Spearman correlation showed a significant result for the median AA-Hb adducts and the AA intake 24 hrs prior to donation. The significant correlation between median AA-Hb and median GA-Hb adduct was expected since AA is metabolised to GA. The correlation between the Hb adducts and the AA intake indicates that the evaluation of volunteers' AA intake based on the FSA data of AA containing foodstuff is valid.

The significant correlation for male volunteers to daily and 24 hrs prior to donation AA intake can be explained by higher AA intake compared to females. The higher AA intake again can be due to a higher food intake.

The correlation between the median *N*7-GA-Gua adduct and the median AA- and GA-Hb adducts are high but not statistically significant. The same for smoker and median AA- and GA-Hb adducts, where high coefficient numbers are obtained but without showing a statistically significant result which is most likely caused by not having had enough volunteers. Doubling the numbers could have shifted the p values into significance.

5.5.5 *N*7-GA-Gua detection in human leukocyte DNA

The analysis of the volunteers' leukocyte DNA samples had a high variation which can be seen in the RSD values ranging between 0.66% and 23.20% for the IS (Table 5.5). The same amount of IS [$^{15}N_5$]-*N*7-GA-Gua was spiked into each sample and considering the specifications of the validation process published in the FDA guidelines the variation of the RSD values should not exceed 15% [127]. The detection of the *N*7-GA-Gua adduct proved to be a challenge and was marked by a great inconsistency throughout the entire analysis. The high RSD values for the *N*7-GA-Gua adduct peak

illustrate the huge variation in the leukocyte DNA samples data (RSD values range between 5.95% and 173.21%). Looking at triplicate or duplicate data, respectively, there is a consistent variation for each sample. Only in six samples there is a larger than LOD sized *N*7-GA-Gua peak detectable but only for one out of triplicate injections. The *N*7-GA-Gua peak in the remaining two samples is small and below LOD for all six samples. Another aspect that might cause the high variation in people's samples can be due to the fact that with the exception of some runs all samples are below the LOD. Analytes below the LOD are seen in the chromatograms. These are very small peaks which are absent in blanks but are just visible and share the transition characteristics of the analyte as well as the retention time of the IS. They allow the assumption that the *N*7-GA-Gua adduct is present but not reproducibly detectable.

5.5.6 Relationship between *N*7-GA-Gua adduct levels and the AA intake and Hb adducts

Overlaying the *N*7-GA-Gua adduct/10^8 nucleotides for all volunteers with the daily AA intake and the AA intake 24 hrs prior to donation did not show a matching relationship. When only volunteers were plotted where the *N*7-GA-Gua adduct was detected in each of the triplicate samples there was a good relationship between adduct levels and AA intake with one exception for the daily AA intake and three exceptions for the 24 hrs prior to donation AA intake. The AA- and GA-Hb adducts on the other hand showed a better relationship with AA intake both daily and 24 hrs prior to donation. The relationship between DNA adduct and Hb adducts was good. A possible future direction is to use the Hb adducts as a way to normalise the DNA adduct levels.

5.5.7 Hb adduct analysis

Looking at the results of the Hb adduct analysis clearly shows which volunteer smokes with it being the highest values for both the AA- and GA-Hb adducts levels. There was no trend to be seen when comparing the daily AA intake with the values of the AA- and

GA-Hb adducts. The lower AA intakes do not correlate to the lower AA- and Hb-adduct levels. A deviation of the expected values can be caused due to a higher or lower AA intake. An underestimation can be caused by an unexpected, additional exposure like laboratory work, second hand smoke or wrong estimations in the FFQ. An overestimation can occur when food categories are averaged and the consumed food would contain less AA than estimated. Additionally, the AA metabolism from volunteers could have an influence on adduct levels. Hagmar *et al*. state that smoking 10 cigarettes/day leads to an additional 0.06 nmol adduct/g globin [53]. In the initial FIRE procedure publication by von Stedingk *et al*. the AA-Hb value was 47.7 and 34.5 pmol/g Hb for two volunteers and the reported GA-Hb value was 18.4 and 19.9 pmol/g [95]. These values are lower than the ones reported here and might be explained by firstly only triplicate analysis for two volunteers having been carried out and secondly an improving of the method since the first publication. The detected AA- and GA-Hb adduct values in maternal blood using the same procedure by von Stedingk *et al*. range from 12 – 160 pmol/g Hb, with a mean of 33 pmol/g Hb and 7.6 – 160 pmol/g Hb with a mean of 28 pmol/g Hb for AA-Hb and GA-Hb adducts, respectively. The mean AA-Hb value for smoker is 110 pmol/g Hb and for GA-Hb adducts 102 pmol/g Hb [140]. In the Norwegian Mother and Child Cohort Study mean maternal AA-Hb adduct levels of 31 pmol/g Hb and 23 pmol/g Hb for GA-Hb adducts were found [71]. The values obtained in this study are higher for AA-Hb adduct levels but GA-Hb adduct levels are very similar to the results obtained by the studies of von Stedingk *et al*. and Duarte-Salles *et al* [71,140]. Variation in results can be explained by different factors as described by Rappaport *et al*. who report a 5 – 10 fold difference in Hb adduct levels after a given benzene exposure which is partly caused by methodological variation and also due to genetic differences between individuals and lifestyles [15]. This also shows that the influence of diet is significant. In a study comparing a vegetarian diet to a non-vegetarian diet Hb adducts of AA and GA and the frequency of micronuclei were analysed. There was a significant difference between groups for the GA- to AA-Hb ratio, probably caused by differences in metabolism and micronuclei frequency was significantly lower in vegetarians than non-vegetarians showing, that a vegetarian diet

could be beneficial to reduce the genotoxic risk. Between the two groups there was no big difference in AA intake established by FFQ (Frostne *et al*., Talk at ICEM 2013).

5.6 Summary

After recruitment of volunteers and collection of FFQ data, volunteers provided blood and urine samples. The blood was analysed for the detection of the *N*7-GA-Gua adduct and a subset of samples (10 of 32 volunteers) where the AA- and GA-Hb adducts were also analysed. The AA intake for each volunteer was established by using AA levels in relevant foodstuff published by the FSA for a typical weekly consumption and the 24 hr period prior to donation of blood and urine. The relationship between AA intake and the DNA adduct levels determined by mass spectrometric analysis was obtained. This study demonstrated the tantalising possibility that the *N*7-GA-Gua adduct was found in humans following exposure to AA. Improvements to analytical workflows could help prove this finding and a more accurate FFQ would help to improve the estimation of the AA exposure. The analysis of the AA-Hb adducts from 10 volunteers led to a statistically significant correlation in respect to the AA intake 24 hrs prior to donation whereas the correlation between the AA- and GA-Hb adducts and the DNA adducts had a high correlation coefficient but was non-significant.

Chapter 6

6 Final Discussion

6.1 Final discussion

After the discovery of AA in food stuff a decade ago research focused on the impact of AA on the carcinogenesis process. There is clear evidence from animal studies that high levels of AA lead to cancer but the mechanism is still not entirely verified [41-43]. The formation of the metabolite GA leads to DNA adducts and hence is a strong indicator that there is a genotoxic pathway for the carcinogenicity of AA. The analytical methods, i.e. mass spectrometry and ELISA applied in this project in combination with the analyte *N*7-GA-Gua adduct has not previously been used successfully for the detection in human samples. Thus far the *N*7-GA-Gua adduct was detected in tissues of treated animals. For the detection of the *N*7-GA-Gua adduct within animal studies most commonly LC-MS/MS was used, as described by Da Costa *et al*. and Watzek *et al*. [73,121]. No publication was found, where ELISA was applied for *N*7-GA-Gua detection. Hence, the object of this project was to establish a method for the detection of the *N*7-GA-Gua adduct applying LC-MS/MS and ELISA in order to identify and measure the adduct in humans.

The developed and applied method is linear in the range between 25 and 150 fmol on column (25 fmol equals 7 adducts/10^8 nucleotides). The analysis of human samples demonstrated high variation for the peaks eluting at the retention time where IS [$^{15}N_5$]-*N*7-GA-Gua elutes. Despite these co-eluting species, the first observation of the *N*7-GA-Gua adduct in humans is present.

The major disadvantages of the method are a long work-up process from whole blood to final analysis by LC-MS and the use of relative high amounts of DNA. The inclusion of the HPLC purification step increased the final DNA amount required to 152 µg/sample injection. In comparison to other publications, the DNA amount is within a similar range, with Balbo *et al*. applying 180 µg DNA in their method whereas for the detection of heterocyclic aromatic amines in hair different DNA amounts were used, ranging between 25 and 100 µg [125,141]. In another study, undertaken by von Tungeln *et al*. 10 – 250 µg for the detection of *N*7-GA-Gua and *N*3-GA-Ade were used

[52]. Using the correct amount of DNA is a balancing act between increasing sensitivity for the analyte and increasing potentially interfering signal suppressants. Therefore, it would be advantageous to increase the sensitivity of the method to then decrease the DNA amount on column.

There are little differences between published methods and procedures for the detection of the *N*7-GA-Gua adduct where details vary in respect mainly on manufacturer and type of mass spectrometers, chromatographic equipment and the hydrolysis protocol [73,121]. When comparing the sensitivities and LOD with methods in the literature the detection of *N*7-GA-Gua adduct shows a higher LOD than previously published by other groups as already discussed in Chapter 3. One explanation for this might be the validation process where this project followed the full FDA guidelines which are strict and work intense. As was seen and discussed in Chapter 3, the LOD could have been set at a lower level and having a background free matrix would have simplified the validation process and subsequent data analysis. A possible solution to this would have been to switch roles between IS and analyte, where the IS would be used in varying amounts and the analyte taken as IS with a fixed amount (also discussed in Chapter 3). Even in animal studies, background levels are reported as in a study undertaken by Ghanayem *et al*. where mice were treated with a single dose of AA, and both GA-DNA adducts and Hb adducts were measured to investigate the influence of the enzyme CYP2E1. They detected low levels of *N*7-GA-Gua in the control wild-type mice. These background levels were caused by diet and reached approximately 9 adducts/10^8 nucleotides in liver with a LOQ of 0.2 adducts/10^8 nucleotides. Unless AA levels in rodent feed are very high this suggests that background levels in humans due to diet could be detectable in a similar range without considering metabolic differences [32]. Young *et al.* on the other hand predicted a liver *N*7-GA-Gua background level caused by dietary AA intake of only 0.06 – 0.26 adducts/10^8 nucleotides which is significantly lower and would make a detection of the adduct with this method unlikely [142].

In order to verify where the variation in the analysed human samples came from it would have been advantageous to analyse positive controls, i.e. tissues obtained from

animal studies. AA treated animal organs would expectedly have high *N*7-GA-Gua adduct levels which should exceed the LOD of this method and hence show less variation. High levels of variation in this instance would indicate an unreliable method.

One aspect that was not tested when developing the method was an alternative to the Qiagen procedure for the extraction of DNA where phenol chloroform extraction as described by Kotova *et al*. and Watzek *et al*. was performed [88,121]. However, Da Costa *et al*. found that DNA isolation using either the phenol chloroform or Qiagen kit extraction led to comparable results [73]. Further, the toxicity of the reagents used for the phenol chloroform extraction means that the Qiagen kit offers a good alternative.

For quantification of the DNA adduct levels in samples it might have been worth considering the inclusion of a calibration line on each day of analysis, rather than taking the adducts/10^8 nucleotides calculation. This way any problems associated with the chemical stability of the IS would be accounted for by determining the analytes concentration by calibration line and analysis within the same run. A calibration line appears to be more accurate than using just the IS peak area and the analyte peak area which is similar to a one-point calibration. Taking the adduct/10^8 nucleotide calculation is often chosen when working with labelled standards because of their cost and limited availability. When using the IS to do the quantification as shown in Chapter 3 it would be beneficial to establish that the concentration of the standard has not changed. Additionally, including long term stability test for the IS would be advisable.

DNA adducts of different compounds are also analysed using LC-MS for detection with varying sensitivity, e.g. the DNA adduct of ethylene oxide is well reported. In a described analytical method detecting 5 hydroxyethylated DNA adducts simultaneously, the ethylene oxide DNA adduct, *N*7-hydroxyethyl-guanine, was detected with a LOD of 0.5 fmol and *N*3-hydroxyethyl-2-deoxyuridine adduct had a LOD of 25 fmol [143]. Another food borne toxicant, the heterocyclic aromatic amine, 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhiP) occurs when protein rich food is grilled, so its levels can be detected in grilled meat. PhiP, amongst other heterocyclic aromatic amines forms guanine DNA adducts on the C8-position upon metabolism. Animal treatment with PhiP led to tumours of the prostate, mammary gland and colon.

Applying a column-switching method for the detection of the PhiP-C8-Gua adduct in colon samples of treated mice (50 mg PhiP/kg Bw) levels of about 15 adducts/10^6 2'-deoxynucleosides were detected. The method was 10 fold more sensitive than the developed method for *N*7-GA-Gua (Chapter 2), with a LOD of 2.5 fmol, using only 50 µg DNA on column [144].

The development of a successful ELISA method essentially depends on the affinity of the antibodies against the antigen. Changing different parameters will not improve the assay if the antibodies are lacking specificity. Monoclonal antibodies seem to have a better affinity towards their antigen due to the specific epitope they are binding to. A further advantage of monoclonal antibodies is that the clones can be grown and hence they are not limited as the polyclonal antibodies are. However, the production of monoclonal antibodies is more time and cost consuming compared to polyclonal ones. Having good antibodies, ELISA is a method that can compete with mass spectrometric analysis in terms of sensitivity and certainly amounts of DNA required. It is also a method that allows a high throughput of samples. The method described by Okahashi *et al*. has a LOD of 5 adducts/10^8 nucleotides with applying 1 µg DNA per sample having a LOD that is comparable to methods using a Quattro Ultima mass spectrometer [131]. Georgiadis *et al*. describes an even more sensitive method applying ELISA and gaining a LOD of 1.5 adducts/10^9 nucleotides only using 10 µg of DNA per well [130].

This projects' human volunteer study recruited healthy volunteers aged 18 and over who provided dietary information in respect to AA containing foodstuff. A link between AA intake and DNA adduct levels was then investigated. No statistically significant correlation was found for the level of *N*7-GA-Gua adduct and AA intake. Since the correlation coefficient is quite high, a larger population may increase the chance of reaching significance. Thus, the study may have been underpowered to deliver the required statistical vigour. However, an alternative approach, taking a cross study design where volunteers have a washout period followed by a diet with a high portion of AA containing food would have been of advantage to see if there are any differences in adduct levels. After another washout period volunteers consume a diet

with food that is low in AA again followed by a washout period. Blood donation would follow after every washout step in which AA containing food is to be avoided. The cross study design is a good way to investigate what influence diet has because every participant acts as their own control. A disadvantage of this method is that it is time consuming and it involves more specimen collections which makes blood collection for volunteers more logistically difficult and involved. To increase the compliance of participating volunteers' and to monitor their exact AA intake it might be useful to provide the foodstuff for every meal of which the AA content can be analysed prior to the study.

6.2 Summary

Analysing human volunteer samples indicates the presence of the *N*7-GA-Gua adduct in leukocyte DNA. To date no publication has reported the detection of the *N*7-GA-Gua adduct in human samples.

A sensitive LC-MS/MS method incorporating online column-switching for the detection of the AA derived *N*7-guanine adduct in human leukocyte DNA was developed and validated. The DNA extraction process and the hydrolysis step take four days followed by a HPLC purification step which allows handling approximately 25 samples per day. The analytical method consists of a total LC-MS/MS run time of 40 min per sample. The LOD for this method is 7 adducts/10^8 nucleotides and the LOQ is 9 adducts/10^8 nucleotides.

After analysis of the human volunteer samples there is an indication for the existence of the *N*7-GA-Gua adduct. For the majority of samples where the *N*7-GA-Gua adduct level from the triplicate analysis was below the LOD, the variability is high. For 10 volunteers the AA-Hb and GA-Hb adduct levels were analysed and were detectable in every volunteer's sample. The AA- and GA-Hb adducts showed high correlation coefficients with both AA intakes, daily and 24 hrs prior to donation but only AA-Hb adducts were statistically significant in relation to the AA intake 24 hrs prior donation.

Furthermore, the correlation coefficients were strong for the AA- and GA-Hb adducts and the *N*7-GA-Gua adducts but not statistically significant.

For the detection of the *N*7-GA-Gua adduct in urine no method could be developed. This might be due to very small amounts of adduct present in respect to the total volume as well as the high salt concentration of the urine matrix.

The development of a competitive ELISA using newly prepared antibodies for the detection of the *N*7-GA-Gua adduct was challenging and was dismissed due to very strong binding of the antibodies without competing with the *N*7-GA-Gua adduct.

Generally, more steps in a work-up procedure are accompanied by an increase in variability to the final result of an analysis. In order for this project to be successful, ideally the sensitivity should be increased. With having a purification step included already, there are not any more options for increasing sensitivity on a work-up level. Recent developments in mass spectrometry for example high resolution SRM and a new generation of triple quadrupole mass spectrometer instruments may have benefitted overall sensitivity. However, different mass spectrometers would also involve the whole procedure of method development since methods cannot easily be transferred between machines.

The most crucial step in the sample work-up is the washing step of the extracted DNA followed by hydrolysis. In order to achieve the most sensitive result with the described method, these are the steps that need careful handling because the solubility of DNA can be difficult if in the washing step the organic was not dried off or if the DNA was dried too much. In addition for quantification of the analysed sample the exact amount of dissolved and hydrolysed DNA is required.

For a high sample throughput it will be advantageous to reduce the analysis time for one sample.

6.3 Further work

MASS SPECTROMETRY

- If access allows, it will be of advantage to try and develop a method for a high definition accurate-mass mass spectrometer which combines the advantages of a quadrupole with an ion trap or TOF analyser. The sensitivity should be similar or better even though the column-switching advice would not be applied.
- Analysis of positive control samples (provided, access to AA treated animal organs is available) from animal studies carried out by collaborators.

ELISA

- The polyclonal antibodies produced with the aim of developing an ELISA could be used to prepare immunoaffinity columns to purify the *N*7-GA-Gua adduct from blood or urine prior to analysis by LC-MS.

HUMAN VOLUNTEER STUDY

- Smoking is a known source of AA and considering that a smoker in this study had higher Hb adduct levels than non-smokers it can be assumed that their DNA adduct levels should be higher. It was noted that the smoker had the highest levels of the *N*7-GA-Gua adduct. Therefore it would be of interest to undertake a study where a few smokers are recruited, to verify if the adduct is detectable with less variation. Additionally, if these smoking volunteers would be willing to cease smoking for a certain period of time and then start smoking again the influence of smoking on the *N*7-GA-Gua adduct level should be visible.

References

7 References

1. WCRF. http://www.wcrf.org/cancer_statistics/world_cancer_statistics.php. In; 2013
2. CRUK. http://www.cancerresearchuk.org/cancer-info/cancerstats/keyfacts/worldwide/. In; 2013
3. WCRF. http://www.wcrf-uk.org/cancer_prevention/recommendations.php. In; 2013
4. Staszewski J, Haenszel W. Cancer mortality among the Polish-born in the United States. J Natl Cancer Inst 1965; 35: 291-297
5. Buell P. Changing incidence of breast cancer in Japanese-American women. J Natl Cancer Inst 1973; 51: 1479-1483
6. Flood DM, Weiss NS, Cook LS et al. Colorectal cancer incidence in Asian migrants to the United States and their descendants. Cancer Causes Control 2000; 11: 403-411
7. Jarabek AM, Pottenger LH, Andrews LS et al. Creating context for the use of DNA adduct data in cancer risk assessment: I. Data organization. Crit Rev Toxicol 2009; 39: 659-678
8. Klaunig JE. Acrylamide carcinogenicity. Journal of Agricultural & Food Chemistry 2008; 56: 5984-5988
9. Gargas ML, Kirman CR, Sweeney LM et al. Acrylamide: Consideration of species differences and nonlinear processes in estimating risk and safety for human ingestion. Food & Chemical Toxicology 2009; 47: 760-768
10. Vogelstein B, Kinzler KW. The multistep nature of cancer. Trends in genetics : TIG 1993; 9: 138-141
11. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell 2011; 144: 646-674
12. Knudsen LE, Hansen AM. Biomarkers of intermediate endpoints in environmental and occupational health. International Journal of Hygiene & Environmental Health 2007; 210: 461-470
13. Himmelstein MW, Boogaard PJ, Cadet J et al. Creating context for the use of DNA adduct data in cancer risk assessment: II. Overview of methods of identification and quantitation of DNA damage. Crit Rev Toxicol 2009; 39: 679-694
14. Boysen G, Pachkowski BF, Nakamura J et al. The formation and biological significance of N7-guanine adducts. Mutation Research-Genetic Toxicology and Environmental Mutagenesis 2009; 678: 76-94
15. Rappaport SM, Li H, Grigoryan H et al. Adductomics: characterizing exposures to reactive electrophiles. Toxicol Lett 2012; 213: 83-90
16. Hemminki K, Koskinen M, Rajaniemi H et al. Dna adducts, mutations, and cancer 2000. Regul Toxicol Pharmacol 2000; 32: 264-275
17. Besaratinia A, Pfeifer GP. DNA adduction and mutagenic properties of acrylamide. Mutat Res 2005; 580: 31-40
18. Scheepers PT. The use of biomarkers for improved retrospective exposure assessment in epidemiological studies: summary of an ECETOC workshop. Biomarkers 2008; 13: 734-748

19. Haglund J, Van Dongen W, Lemiere F et al. Analysis of DNA-phosphate adducts in vitro using miniaturized LC-ESI-MS/MS and column switching: phosphotriesters and alkyl cobalamins. Journal of the American Society for Mass Spectrometry 2004; 15: 593-606
20. Shuker DE. The enemy at the gates? DNA adducts as biomarkers of exposure to exogenous and endogenous genotoxic agents. Toxicol Lett 2002; 134: 51-56
21. Egner PA, Groopman JD, Wang JS et al. Quantification of aflatoxin-B1-N7-Guanine in human urine by high-performance liquid chromatography and isotope dilution tandem mass spectrometry. Chem Res Toxicol 2006; 19: 1191-1195
22. Phillips DH. DNA adducts as markers of exposure and risk. Mutat Res 2005; 577: 284-292
23. Wogan GN, Hecht SS, Felton JS et al. Environmental and chemical carcinogenesis. Seminars in cancer biology 2004; 14: 473-486
24. Woo LL, Egner PA, Belanger CL et al. Aflatoxin B1-DNA adduct formation and mutagenicity in livers of neonatal male and female B6C3F1 mice. Toxicol Sci 2011; 122: 38-44
25. Ross RK, Yuan JM, Yu MC et al. Urinary aflatoxin biomarkers and risk of hepatocellular carcinoma. Lancet 1992; 339: 943-946
26. Poirier MC. DNA adducts as exposure biomarkers and indicators of cancer risk. Environ Health Perspect 1997; 105 Suppl 4: 907-912
27. Qian GS, Ross RK, Yu MC et al. A follow-up study of urinary markers of aflatoxin exposure and liver cancer risk in Shanghai, People's Republic of China. Cancer Epidemiol Biomarkers Prev 1994; 3: 3-10
28. Jelakovic B, Karanovic S, Vukovic-Lela I et al. Aristolactam-DNA adducts are a biomarker of environmental exposure to aristolochic acid. Kidney international 2012; 81: 559-567
29. Farmer PB, Singh R. Use of DNA adducts to identify human health risk from exposure to hazardous environmental pollutants: The increasing role of mass spectrometry in assessing biologically effective doses of genotoxic carcinogens. Mutation Research - Reviews in Mutation Research 2008; 659: 68-76
30. Hogervorst JG, Baars BJ, Schouten LJ et al. The carcinogenicity of dietary acrylamide intake: a comparative discussion of epidemiological and experimental animal research. Crit Rev Toxicol 2010; 40: 485-512
31. Boettcher MI, Bolt HM, Angerer J. Acrylamide exposure via the diet: influence of fasting on urinary mercapturic acid metabolite excretion in humans. Arch Toxicol 2006; 80: 817-819
32. Ghanayem BI, McDaniel LP, Churchwell MI et al. Role of CYP2E1 in the epoxidation of acrylamide to glycidamide and formation of DNA and hemoglobin adducts. Toxicol Sci 2005; 88: 311-318
33. Errol C. Friedberg GCW, Wolfram Siede, Richard D. Wood, Roger A. Schultz, Tom Ellenberger. DNA Repair and Mutagenesis. Second Edition. Aufl; 2006
34. Wood RD, Mitchell M, Lindahl T. Human DNA repair genes, 2005. Mutat Res 2005; 577: 275-283
35. P. KMS. Introduction to the Cellular and Molecular Biology of Cancer: Oxford University Press; 2005

36. Robertson AB, Klungland A, Rognes T et al. DNA repair in mammalian cells: Base excision repair: the long and short of it. Cellular and molecular life sciences : CMLS 2009; 66: 981-993
37. Wilson DM, 3rd, Thompson LH. Life without DNA repair. Proceedings of the National Academy of Sciences of the United States of America 1997; 94: 12754-12757
38. Wood RD, Mitchell M, Sgouros J et al. Human DNA repair genes. Science (New York, NY) 2001; 291: 1284-1289
39. Fortini P, Pascucci B, Parlanti E et al. The base excision repair: mechanisms and its relevance for cancer susceptibility. Biochimie 2003; 85: 1053-1071
40. Friedman M. Chemistry, biochemistry, and safety of acrylamide. A review. J Agric Food Chem 2003; 51: 4504-4526
41. Friedman MA, Dulak LH, Stedham MA. A lifetime oncogenicity study in rats with acrylamide. Fundamental and applied toxicology : official journal of the Society of Toxicology 1995; 27: 95-105
42. Johnson KA, Gorzinski SJ, Bodner KM et al. Chronic toxicity and oncogenicity study on acrylamide incorporated in the drinking water of Fischer 344 rats. Toxicol Appl Pharmacol 1986; 85: 154-168
43. Beland FA, Mellick PW, Olson GR et al. Carcinogenicity of acrylamide in B6C3F(1) mice and F344/N rats from a 2-year drinking water exposure. Food and Chemical Toxicology 2013; 51: 149-159
44. Dearfield KL, Douglas GR, Ehling UH et al. Acrylamide: a review of its genotoxicity and an assessment of heritable genetic risk. Mutat Res 1995; 330: 71-99
45. Mei N, Hu J, Churchwell MI et al. Genotoxic effects of acrylamide and glycidamide in mouse lymphoma cells. Food and Chemical Toxicology 2008; 46: 628-636
46. Baum M, Loeppky RN, Thielen S et al. Genotoxicity of glycidamide in comparison to 3-N-nitroso-oxazolidin-2-one. J Agric Food Chem 2008; 56: 5989-5993
47. Koyama N, Yasui M, Oda Y et al. Genotoxicity of acrylamide in vitro: Acrylamide is not metabolically activated in standard in vitro systems. Environ Mol Mutagen 2011; 52: 11-19
48. Besaratinia A, Pfeifer GP. Genotoxicity of acrylamide and glycidamide. J Natl Cancer Inst 2004; 96: 1023-1029
49. Manjanatha MG, Aidoo A, Shelton SD et al. Genotoxicity of acrylamide and its metabolite glycidamide administered in drinking water to male and female Big Blue mice. Environmental & Molecular Mutagenesis 2006; 47: 6-17
50. Mei N, McDaniel LP, Dobrovolsky VN et al. The Genotoxicity of Acrylamide and Glycidamide in Big Blue Rats. Toxicol Sci 2010; 115: 412-421
51. Koyama N, Yasui M, Kimura A et al. Acrylamide genotoxicity in young versus adult gpt delta male rats. Mutagenesis 2011; 26: 545-549
52. Von Tungeln LS, Churchwell MI, Doerge DR et al. DNA adduct formation and induction of micronuclei and mutations in B6C3F1/Tk mice treated neonatally with acrylamide or glycidamide. Int J Cancer 2009; 124: 2006-2015

53. Hagmar L, Tornqvist M, Nordander C et al. Health effects of occupational exposure to acrylamide using hemoglobin adducts as biomarkers of internal dose. Scand J Work Environ Health 2001; 27: 219-226
54. Bergmark E. Hemoglobin adducts of acrylamide and acrylonitrile in laboratory workers, smokers and nonsmokers. Chem Res Toxicol 1997; 10: 78-84
55. Tareke E, Rydberg P, Karlsson P et al. Acrylamide: a cooking carcinogen? Chem Res Toxicol 2000; 13: 517-522
56. Stadler RH, Blank I, Varga N et al. Acrylamide from Maillard reaction products. Nature 2002; 419: 449-450
57. Mottram DS, Wedzicha BL, Dodson AT. Acrylamide is formed in the Maillard reaction. Nature 2002; 419: 448-449
58. Blank I. Current status of acrylamide research in food: measurement, safety assessment, and formation. Ann N Y Acad Sci 2005; 1043: 30-40
59. Bongers ML, Hogervorst JG, Schouten LJ et al. Dietary acrylamide intake and the risk of lymphatic malignancies: the Netherlands Cohort Study on diet and cancer. PLoS One 2012; 7: e38016
60. Pedreschi F, Mariotti MS, Granby K. Current issues in dietary acrylamide: formation, mitigation and risk assessment. J Sci Food Agric 2014; 94: 9-20
61. Mestdagh F, Lachat C, Baert K et al. Importance of a canteen lunch on the dietary intake of acrylamide. Mol Nutr Food Res 2007; 51: 509-516
62. Mucci LA, Wilson KM. Acrylamide intake through diet and human cancer risk. Journal of Agricultural & Food Chemistry 2008; 56: 6013-6019
63. Wilson KM, Balter K, Adami H-O et al. Acrylamide exposure measured by food frequency questionnaire and hemoglobin adduct levels and prostate cancer risk in the Cancer of the Prostate in Sweden Study. International Journal of Cancer 2009; 124: 2384-2390
64. FSA. A Rolling Programme of Surveys on Process Contaminants in UK Retail Foods, Interim Report Covering Sampling During 2011-2012, Acrylamide & Furan: Survey 5. In; April 2013
65. [Anonym]. Acrylamide. IARC monographs on the evaluation of carcinogenic risks to humans / World Health Organization, International Agency for Research on Cancer 1994; 60: 389-433
66. Mills C, Tlustos C, Evans R et al. Dietary acrylamide exposure estimates for the United Kingdom and Ireland: comparison between semiprobabilistic and probabilistic exposure models. Journal of Agricultural & Food Chemistry 2008; 56: 6039-6045
67. Heudorf U, Hartmann E, Angerer J. Acrylamide in children -exposure assessment via urinary acrylamide metabolites as biomarkers. International Journal of Hygiene & Environmental Health 2009; 212: 135-141
68. Bjellaas T, Stolen LH, Haugen M et al. Urinary acrylamide metabolites as biomarkers for short-term dietary exposure to acrylamide. Food & Chemical Toxicology 2007; 45: 1020-1026
69. Pingarilho M, Oliveira NG, Martins C et al. Induction of sister chromatid exchange by acrylamide and glycidamide in human lymphocytes: Role of polymorphisms in detoxification and DNA-repair genes in the genotoxicity of

glycidamide. Mutation Research-Genetic Toxicology and Environmental Mutagenesis 2013; 752: 1-7
70. Pedersen M, von Stedingk H, Botsivali M et al. Birth Weight, Head Circumference, and Prenatal Exposure to Acrylamide from Maternal Diet: The European Prospective Mother-Child Study (NewGeneris). Environmental Health Perspectives 2012; 120: 1739-1745
71. Duarte-Salles T, von Stedingk H, Granum B et al. Dietary Acrylamide Intake during Pregnancy and Fetal Growth-Results from the Norwegian Mother and Child Cohort Study (MoBa). Environmental Health Perspectives 2013; 121: 374-379
72. Freisling H, Moskal A, Ferrari P et al. Dietary acrylamide intake of adults in the European Prospective Investigation into Cancer and Nutrition differs greatly according to geographical region. Eur J Nutr 2013; 52: 1369-1380
73. Gamboa da Costa G, Churchwell MI, Hamilton LP et al. DNA adduct formation from acrylamide via conversion to glycidamide in adult and neonatal mice. Chem Res Toxicol 2003; 16: 1328-1337
74. Vesper HW, Bernert JT, Ospina M et al. Assessment of the relation between biomarkers for smoking and biomarkers for acrylamide exposure in humans. Cancer Epidemiology, Biomarkers & Prevention 2007; 16: 2471-2478
75. Hogervorst JGF, Schouten LJ, Konings EJM et al. Dietary acrylamide intake and brain cancer risk. Cancer Epidemiology, Biomarkers & Prevention 2009; 18: 1663-1666
76. Settels E, Bernauer U, Palavinskas R et al. Human CYP2E1 mediates the formation of glycidamide from acrylamide. Arch Toxicol 2008; 82: 717-727
77. Kraus D, Rokitta D, Fuhr U et al. The role of human cytochrome P450 enzymes in metabolism of acrylamide in vitro. Toxicol Mech Methods 2013; 23: 346-351
78. Hartmann EC, Latzin JM, Schindler BK et al. Excretion of 2,3-dihydroxy-propionamide (OH-PA), the hydrolysis product of glycidamide, in human urine after single oral dose of deuterium-labeled acrylamide. Arch Toxicol 2011; 85: 601-606
79. Bolt HM, Roos PH, Thier R. The cytochrome P-450 isoenzyme CYP2E1 in the biological processing of industrial chemicals: consequences for occupational and environmental medicine. Int Arch Occup Environ Health 2003; 76: 174-185
80. Sumner SC, Fennell TR, Moore TA et al. Role of cytochrome P450 2E1 in the metabolism of acrylamide and acrylonitrile in mice. Chem Res Toxicol 1999; 12: 1110-1116
81. Huang YF, Chiang SY, Liou SH et al. The modifying effect of CYP2E1, GST, and mEH genotypes on the formation of hemoglobin adducts of acrylamide and glycidamide in workers exposed to acrylamide. Toxicology Letters 2012; 215: 92-99
82. Duale N, Bjellaas T, Alexander J et al. Biomarkers of human exposure to acrylamide and relation to polymorphisms in metabolizing genes. Toxicol Sci 2009; 108: 90-99
83. Boettcher MI, Bolt HM, Drexler H et al. Excretion of mercapturic acids of acrylamide and glycidamide in human urine after single oral administration of deuterium-labelled acrylamide. Arch Toxicol 2006; 80: 55-61

84. Watzek N, Scherbl D, Feld J et al. Profiling of mercapturic acids of acrolein and acrylamide in human urine after consumption of potato crisps. Mol Nutr Food Res 2012; 56: 1825-1837
85. Berger FI, Feld J, Bertow D et al. Biological effects of acrylamide after daily ingestion of various foods in comparison to water: a study in rats. Mol Nutr Food Res 2011; 55: 387-399
86. Sumner SC, MacNeela JP, Fennell TR. Characterization and quantitation of urinary metabolites of [1,2,3-13C]acrylamide in rats and mice using 13C nuclear magnetic resonance spectroscopy. Chem Res Toxicol 1992; 5: 81-89
87. Segerback D, Calleman CJ, Schroeder JL et al. Formation of N-7-(2-carbamoyl-2-hydroxyethyl)guanine in DNA of the mouse and the rat following intraperitoneal administration of [14C]acrylamide. Carcinogenesis 1995; 16: 1161-1165
88. Kotova N, Juren T, Myohanen K et al. (32)P-HPLC analysis of N1-(2-carboxy-2-hydroxyethyl)deoxyadenosine: A DNA adduct of the acrylamide-derived epoxide glycidamide. Toxicology Letters 2011; 207: 18-24
89. Doerge DR, da Costa GG, McDaniel LP et al. DNA adducts derived from administration of acrylamide and glycidamide to mice and rats. Mutation Research-Genetic Toxicology and Environmental Mutagenesis 2005; 580: 131-141
90. Maniere I, Godard T, Doerge DR et al. DNA damage and DNA adduct formation in rat tissues following oral administration of acrylamide. Mutat Res 2005; 580: 119-129
91. Bergmark E, Calleman CJ, He F et al. Determination of hemoglobin adducts in humans occupationally exposed to acrylamide. Toxicol Appl Pharmacol 1993; 120: 45-54
92. Chevolleau S, Jacques C, Canlet C et al. Analysis of hemoglobin adducts of acrylamide and glycidamide by liquid chromatography-electrospray ionization tandem mass spectrometry, as exposure biomarkers in French population. Journal of Chromatography 2007; A. 1167: 125-134
93. Brisson B, Ayotte P, Normandin L et al. Relation between dietary acrylamide exposure and biomarkers of internal dose in Canadian teenagers. Journal of exposure science & environmental epidemiology 2013, DOI: 10.1038/jes.2013.34
94. Bjellaas T, Olesen PT, Frandsen H et al. Comparison of estimated dietary intake of acrylamide with hemoglobin adducts of acrylamide and glycidamide. Toxicol Sci 2007; 98: 110-117
95. von Stedingk H, Rydberg P, Tornqvist M. A new modified Edman procedure for analysis of N-terminal valine adducts in hemoglobin by LC-MS/MS. J Chromatogr B Analyt Technol Biomed Life Sci 2010; 878: 2483-2490
96. Ferrari P, Freisling H, Duell EJ et al. Challenges in estimating the validity of dietary acrylamide measurements. Eur J Nutr 2013; 52: 1503-1512
97. Kutting B, Uter W, Drexler H. The association between self-reported acrylamide intake and hemoglobin adducts as biomarkers of exposure. Cancer Causes & Control 2008; 19: 273-281

98. Mucci LA, Dickman PW, Steineck G et al. Dietary acrylamide and cancer of the large bowel, kidney, and bladder: absence of an association in a population-based study in Sweden. Br J Cancer 2003; 88: 84-89
99. Pelucchi C, Galeone C, Levi F et al. Dietary acrylamide and human cancer. International Journal of Cancer 2006; 118: 467-471
100. Hogervorst JG, Schouten LJ, Konings EJ et al. A prospective study of dietary acrylamide intake and the risk of endometrial, ovarian, and breast cancer. Cancer Epidemiology, Biomarkers & Prevention 2007; 16: 2304-2313
101. Olesen PT, Olsen A, Frandsen H et al. Acrylamide exposure and incidence of breast cancer among postmenopausal women in the Danish Diet, Cancer and Health Study. International Journal of Cancer 2008; 122: 2094-2100
102. Hogervorst JGF, Schouten LJ, Konings EJM et al. Dietary acrylamide intake is not associated with gastrointestinal cancer risk. Journal of Nutrition 2008; 138: 2229-2236
103. Wilson KM, Mucci LA, Cho E et al. Dietary acrylamide intake and risk of premenopausal breast cancer. Am J Epidemiol 2009; 169: 954-961
104. Larsson SC, Akesson A, Wolk A. Dietary acrylamide intake and prostate cancer risk in a prospective cohort of Swedish men. Cancer Epidemiology, Biomarkers & Prevention 2009; 18: 1939-1941
105. Larsson SC, Akesson A, Bergkvist L et al. Dietary acrylamide intake and risk of colorectal cancer in a prospective cohort of men. European Journal of Cancer 2009; 45: 513-516
106. Larsson SC, Akesson A, Wolk A. Long-term dietary acrylamide intake and risk of epithelial ovarian cancer in a prospective cohort of Swedish women. Cancer Epidemiology, Biomarkers & Prevention 2009; 18: 994-997
107. Larsson SC, Akesson A, Wolk A. Long-term dietary acrylamide intake and breast cancer risk in a prospective cohort of Swedish women. Am J Epidemiol 2009; 169: 376-381
108. Hogervorst JGF, Schouten LJ, Konings EJM et al. Lung cancer risk in relation to dietary acrylamide intake. Journal of the National Cancer Institute 2009; 101: 651-662
109. Wilson KM, Mucci LA, Rosner BA et al. A Prospective Study on Dietary Acrylamide Intake and the Risk for Breast, Endometrial, and Ovarian Cancers. Cancer Epidemiology Biomarkers & Prevention 2010; 19: 2503-2515
110. Pedersen GS, Hogervorst JG, Schouten LJ et al. Dietary acrylamide intake and estrogen and progesterone receptor-defined postmenopausal breast cancer risk. Breast Cancer Res Treat 2010; 122: 199-210
111. Hirvonen T, Kontto J, Jestoi M et al. Dietary acrylamide intake and the risk of cancer among Finnish male smokers. Cancer Causes Control 2010; 21: 2223-2229
112. Burley VJ, Greenwood DC, Hepworth SJ et al. Dietary acrylamide intake and risk of breast cancer in the UK women's cohort. Br J Cancer 2010; 103: 1749-1754
113. Pelucchi C, Galeone C, Talamini R et al. Dietary acrylamide and pancreatic cancer risk in an Italian case-control study. Ann Oncol; 22: 1910-1915
114. Wilson KM, Giovannucci E, Stampfer MJ et al. Dietary acrylamide and risk of prostate cancer. International Journal of Cancer 2012; 131: 479-487

115. Xie J, Terry K, Poole E et al. Acrylamide hemoglobin adducts levels and ovarian cancer risk in the Nurses' Health Study. Am J Epidemiol 2013; 177: S5-S5
116. Obon-Santacana M, Lujan-Barroso L, Slimani N et al. Dietary intake of acrylamide and pancreatic cancer risk in the European Prospective Investigation Into Cancer and Nutrition (EPIC) cohort. Ann Nutr Metab 2013; 63: 1204-1204
117. Wirfalt E, Paulsson B, Tornqvist M et al. Associations between estimated acrylamide intakes, and hemoglobin AA adducts in a sample from the Malmo Diet and Cancer cohort. Eur J Clin Nutr 2008; 62: 314-323
118. Roboz J. Mass spectrometry in cancer research. CRC Press LLC; 2002
119. de Hoffmann E, Stroobant V. Mass Spectrometry Principles and Applications. Third Edition. Aufl. Wiley; 2007
120. Wild D. The Immunoassay Handbook; 2001
121. Watzek N, Bohm N, Feld J et al. N7-Glycidamide-Guanine DNA Adduct Formation by Orally Ingested Acrylamide in Rats: A Dose-Response Study Encompassing Human Diet-Related Exposure Levels. Chemical Research in Toxicology 2012; 25: 381-390
122. Martins C, Oliveira NG, Pingarilho M et al. Cytogenetic damage induced by acrylamide and glycidamide in mammalian cells: correlation with specific glycidamide-DNA adducts. Toxicol Sci 2007; 95: 383-390
123. Boettcher MI, Schettgen T, Kutting B et al. Mercapturic acids of acrylamide and glycidamide as biomarkers of the internal exposure to acrylamide in the general population. Mutat Res 2005; 580: 167-176
124. Chao MR, Wang CJ, Chang LW et al. Quantitative determination of urinary N7-ethylguanine in smokers and non-smokers using an isotope dilution liquid chromatography/tandem mass spectrometry with on-line analyte enrichment. Carcinogenesis 2006; 27: 146-151
125. Balbo S, Villalta PW, Hecht SS. Quantitation of 7-ethylguanine in leukocyte DNA from smokers and nonsmokers by liquid chromatography-nanoelectrospray-high resolution tandem mass spectrometry. Chem Res Toxicol 2011; 24: 1729-1734
126. Bansal S, DeStefano A. Key elements of bioanalytical method validation for small molecules. The AAPS journal 2007; 9: E109-114
127. U.S. Department of Health and Human Services FaDA, CDER, CVM. Guidance for Industry Bioanalytical Method Validation. In; May 2001
128. Sangaraju D, Villalta P, Goggin M et al. Capillary HPLC-accurate mass MS/MS quantitation of N7-(2,3,4-trihydroxybut-1-yl)-guanine adducts of 1,3-butadiene in human leukocyte DNA. Chem Res Toxicol 2013; 26: 1486-1497
129. Iweala OI. HIV diagnostic tests: an overview. Contraception 2004; 70: 141-147
130. Georgiadis P, Kovacs K, Kaila S et al. Development and validation of a direct sandwich chemiluminescence immunoassay for measuring DNA adducts of benzo[a]pyrene and other polycyclic aromatic hydrocarbons. Mutagenesis 2012; 27: 589-597
131. Okahashi Y, Iwamoto T, Suzuki N et al. Quantitative detection of 4-hydroxyequilenin-DNA adducts in mammalian cells using an immunoassay with a novel monoclonal antibody. Nucleic acids research 2010; 38: e133

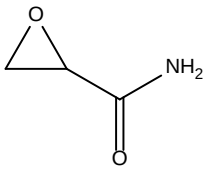
132. Nayak S, Sashidhar RB, Bhat RV. Quantification and validation of enzyme immunoassay for urinary aflatoxin B1-N7-guanine adduct for biological monitoring of aflatoxins. Analyst 2001; 126: 179-183
133. Krizkova-Kudlikova I, Hubert J. Development of polyclonal antibodies for the detection of Tribolium castaneum contamination in wheat grain. J Agric Food Chem 2008; 56: 8035-8040
134. Zhou S, Zhang C, Wang D et al. Antigen synthetic strategy and immunoassay development for detection of acrylamide in foods. Analyst 2008; 133: 903-909
135. Brinkman K, Termaat R, Van den Brink H et al. The specificity of the anti-dsDNA ELISA. A closer look. J Immunol Methods 1991; 139: 91-100
136. Stokes RP, Cordwell A, Thompson RA. A simple, rapid ELISA method for the detection of DNA antibodies. Journal of clinical pathology 1982; 35: 566-573
137. Vikstrom AC, Wilson KM, Paulsson B et al. Alcohol influence on acrylamide to glycidamide metabolism assessed with hemoglobin-adducts and questionnaire data. Food Chem Toxicol 2010; 48: 820-824
138. Sorgel F, Weissenbacher R, Kinzig-Schippers M et al. Acrylamide: increased concentrations in homemade food and first evidence of its variable absorption from food, variable metabolism and placental and breast milk transfer in humans. Chemotherapy 2002; 48: 267-274
139. Svensson K, Abramsson L, Becker W et al. Dietary intake of acrylamide in Sweden. Food Chem Toxicol 2003; 41: 1581-1586
140. von Stedingk H, Vikstrom AC, Rydberg P et al. Analysis of Hemoglobin Adducts from Acrylamide, Glycidamide, and Ethylene Oxide in Paired Mother/Cord Blood Samples from Denmark. Chemical Research in Toxicology 2011; 24: 1957-1965
141. Bessette EE, Yasa I, Dunbar D et al. Biomonitoring of carcinogenic heterocyclic aromatic amines in hair: a validation study. Chem Res Toxicol 2009; 22: 1454-1463
142. Young JF, Luecke RH, Doerge DR. Physiologically based pharmacokinetic/pharmacodynamic model for acrylamide and its metabolites in mice, rats, and humans. Chemical Research in Toxicology 2007; 20: 388-399
143. Tompkins EM, Jones DJ, Lamb JH et al. Simultaneous detection of five different 2-hydroxyethyl-DNA adducts formed by ethylene oxide exposure, using a high-performance liquid chromatography/electrospray ionisation tandem mass spectrometry assay. Rapid Commun Mass Spectrom 2008; 22: 19-28
144. Singh R, Arlt VM, Henderson CJ et al. Detection and quantitation of N-(deoxyguanosin-8-yl)-2-amino-1-methyl-6-Phenylimidazo 4,5-b pyridine adducts in DNA using online column-switching liquid chromatography tandem mass spectrometry. Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences 2010; 878: 2155-2162

Appendix

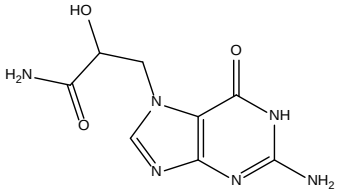
8 Appendix

8.1 NMR data

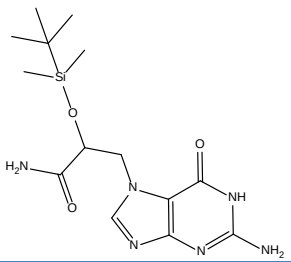
- GA → Solvent: D_2O

δ_H [400 MHz]	Proton	
		
2.86	C3-H_a**H_b**	1 H, dd
3.01	C3-**H_a**H_b	1 H, t
3.48	C3-**H**	1 H, dd

- N7-GA-Gua → Solvent: D_6-DMSO

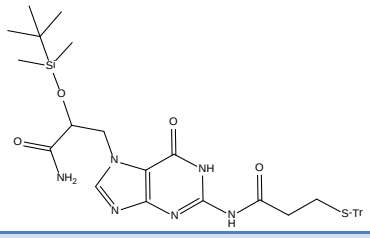
δ_H [400 MHz]	Proton	
		
4.12	N7-C**H_a**H_b	1 H, dd, J = 8.9, 13.4
4.23	N7-CH_2**C**H-OH	1 H, ddd, J = 3.3, 6.0, 8.9
4.56	N7-CH_a**H_b**	1 H, dd, J = 3.3, 13.4
5.91	N7-CH_2CH-O**H**	1 H, d, J = 6.0
6.08	N2**H_2**	2 H, s
7.28	CO-N**H_a**H_b	1 H, s
7.30	CO-NH_a**H_b**	1 H
7.78	C8**H**	1 H, s
10.72	N1**H**	1 H, br s

- OTBS- N7-GA-Gua → Solvent: D_6-DMSO

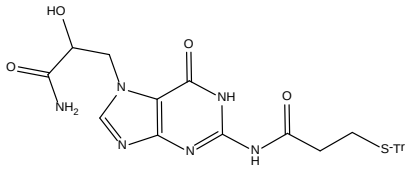
δ_H [400 MHz]	Proton
	

0.88	$SiC(CH_3)_3$	9 H, s
4.33	N7-C**H**$_a$H$_b$	1 H, dd, J = 8.4, 13.1
4.49	N7-CH$_a$**H**$_b$ N7-CH$_2$C**H**-OTBS	2 H, m
6.27	N2-**H**$_2$	2 H, s
7.11	CO-N**H**$_a$H$_b$	1 H, s
7.53	CO-NH$_a$**H**$_b$	1 H, s
7.67	C8-**H**	1 H, s
10.82	N1-**H**	1 H, s

- STr-OTBS-Hap → Solvent: D_6-DMSO

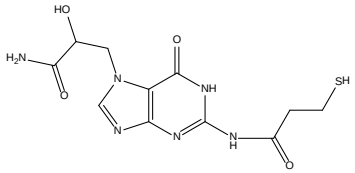
δ_H [400 MHz]	**Proton**	
		
-0.21	Si**CH**$_3$	3 H, s
0.85	$SiC(C\mathbf{H}_3)_3$	9 H, s
2.45	-**CH**$_2$-CH$_2$-S-	2 H, t, J = 6.8
2.61	-CH$_2$-**CH**$_2$-S-	2 H, t, J = 6.8
4.47	N7-C**H**$_a$H$_b$ N7-CH$_2$-C**H**-OTBS	2 H, m
4.62	N7-CH$_a$**H**$_b$	1 H, dd, J = 1.7, 11.5
7.13	CO-N**H**$_a$H$_b$	1 H, s
7.43 – 7.48	S-$C(Ph)_3$	15 H, m
7.55	CO-NH$_a$**H**$_b$	1 H, s
8.13	C8-**H**	1 H, s
11.65	N2-**H**	1 H, s
12.06	N1-**H**	1 H, s

- STr-Hap → Solvent: D_6-DMSO

δ_H [400 MHz]	**Proton**	
		
2.37	-**CH**$_2$-CH$_2$-S-	2 H, t, J = 7.2
2.52	-CH$_2$-**CH**$_2$-S-	2 H, t, J = 7.2
4.27	N7-CH$_2$-C**H**-OH N7-C**H**$_a$H$_b$	2 H, m
4.65	N7-CH$_a$**H**$_b$	1 H, m
5.91	CO-N**H**$_a$H$_b$	1 H, s
5.95	CO-NH$_a$**H**$_b$	1 H, s

7.20 - 7.34	S-C(Ph)$_3$	15 H, m
8.04	C8-**H**	1 H, s
11.54	N2-**H**	1 H, br s
11.93	N1-**H**	1 H, br s

- Hapten → Solvent: D_6-DMSO

δ_H [400 MHz]	Proton	
		
2.09	-CH_2-CH_2-S**H**	1 H, s
2.78	-C**H**$_2$-C**H**$_2$-SH	4 H, m
4.29	N7-C**H**$_a$H$_b$ N7-CH_2-C**H**-OH	2 H, m
4.66	N7-CH_a**H**$_b$	1 H, m
7.32	CO-N**H**$_2$	2 H, 2 x s
7.96	C8-**H**	1 H
11.65	N2-**H**	1 H, br s
12.08	N1-**H**	1 H, s

8.2 Participant information sheet, consent form and questionnaire

8.2.1 Participant information sheet – Study 1

PARTICIPANT INFORMATION SHEET **(**Version 01 22/03/2013)

Title of Research:
Assessment of exposure of DNA to dietary acrylamide.
You are being asked to provide samples to aid future research studies. Before you decide, it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information and discuss it with others if you wish. Ask if there is anything that is unclear or if you would like more information. Take time to decide whether or not you wish to take part.

Purpose of the research:
To determine if a constituent of diet, acrylamide, which is known to be produced by high temperature cooking of some foods, causes any DNA damage in humans.

Why have I been chosen?
We require samples from volunteers of differing dietary habits to assess if there is a relationship between our DNA assay results and consumption of particular foods. Volunteers will have no relevant medical conditions and will not be on any medication.

Do I have to take part?
It is up to you whether or not you decide to take part. If you do decide to take part you will be given this information sheet to keep, and be asked to sign a consent form. If you decide to take part, you are still free to withdraw at any time and without giving a reason. If you wish to withdraw, you can do so by contacting Peter Farmer (pbf1@le.ac.uk), Don Jones (djlj1@le.ac.uk, 01162523169), Rob Britton (rgb6@le.ac.uk), Leong Ng (lln1@le.ac.uk), or Sandra Schumacher (ss610@le.ac.uk, 01162231835).

What will happen to me if I take part?
If you decide to volunteer, you will be required to give a blood (30 mL) and urine (50 mL) sample, and complete a questionnaire to summarise your diet. The blood and urine will be analysed to see if there is any evidence of an interaction between acrylamide and DNA. This will be done by analysis of the adduct of the active metabolite of acrylamide, glycidamide, with the DNA base guanine.

Will any further tests be carried out on my samples?
No.

What are the risks in taking part?

The collection of blood will be carried out by personnel trained for doing so.

What are the benefits of taking part?
There is no financial benefit of taking part. The research however will be of great value as it should allow us to give dietary advice to control human exposure to the chemical acrylamide.

What if there is a problem?
If any problems arise during your participation in this study, please contact Peter Farmer, Don Jones, Rob Britton, Leong Ng or Sandra Schumacher using the contact details above.

What if something goes wrong?
It is very unlikely for you to be harmed during this study, however if you are harmed by taking part in this research project, there are no special compensation arrangements. If you are harmed due to someone's negligence, then you may have grounds for a legal action but you may have to pay for it. Regardless of this, if you wish to complain, or have any concerns about any aspect of the way you have been approached or treated during the course of this study, the normal University complaints mechanisms should be available to you.

Will my taking part in this study be kept confidential?
Yes. If you consent to take part in this research project we will retain your consent form, but we will not collect, or keep any additional details about you. Any remaining material from your sample will be destroyed appropriately, when the study ends.

What will happen to the results of the research study?
The results of the study will be published in scientific journals and also presented at local and international meetings.

Who has reviewed the study?
The study has been reviewed by an ethics officer at the University of Leicester.

THANK YOU FOR READING THIS VOLUNTEER INFORMATION SHEET

If you need to ask any questions please contact: Peter Farmer, Don Jones, Rob Britton, Leong Ng or Sandra Schumacher using the contact details above.

8.2.2 Participant information sheet – Study 2

PARTICIPANT INFORMATION SHEET **(**Version 02 13/05/2013**)**

Title of Research:
Assessment of biomarkers to dietary acrylamide.
You are being asked to provide samples to aid future research studies. Before you decide, it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information and discuss it with others if you wish. Ask if there is anything that is unclear or if you would like more information. Take time to decide whether or not you wish to take part.

Purpose of the research:
To determine if a constituent of diet, acrylamide, which is known to be produced by high temperature cooking of some foods, causes any damage to macromolecules such as DNA and haemoglobin in humans.

Why have I been chosen?
We require samples from volunteers of differing dietary habits to assess if there is a relationship between our acrylamide biomarker results and consumption of particular foods.
Volunteers will have no relevant medical conditions and will not be on any medication.

Do I have to take part?
It is up to you whether or not you decide to take part. If you do decide to take part you will be given this information sheet to keep, and be asked to sign a consent form. If you decide to take part, you are still free to withdraw at any time and without giving a reason. If you wish to withdraw, you can do so by contacting Peter Farmer (pbf1@le.ac.uk), Don Jones (djlj1@le.ac.uk, 01162523169), Rob Britton (rgb6@le.ac.uk), Leong Ng (lln1@le.ac.uk), or Sandra Schumacher (ss610@le.ac.uk, 01162231835).

What will happen to me if I take part?
If you decide to volunteer, you will be required to give a blood (35 mL) and urine (50 mL) sample, and complete a questionnaire to summarise your diet. The blood will be analysed to see if there is any evidence of an interaction between acrylamide with DNA and haemoglobin. This will be done by analysis of the adducts of the active metabolite of acrylamide, glycidamide, with the DNA base guanine or of acrylamide and/or glycidamide with the amino acid valine in hemoglobin. The urine will be analysed to see if there is any evidence of DNA repair which would reflect by the presence of the DNA adduct that can be measured. Also detectable in urine are mercapturic acids that show that both acrylamide and its metabolite glycidamide are being excreted and therefore detoxified.

Will any further tests be carried out on my samples?
No.

What are the risks in taking part?
The collection of blood will be carried out by personnel trained for doing so.

What are the benefits of taking part?
There is no financial benefit of taking part. The research however will be of great value as it should allow us to give dietary advice to control human exposure to the chemical acrylamide.

What if there is a problem?
If any problems arise during your participation in this study, please contact Peter Farmer, Don Jones, Rob Britton, Leong Ng or Sandra Schumacher using the contact details above.

What if something goes wrong?
It is very unlikely for you to be harmed during this study, however if you are harmed by taking part in this research project, there are no special compensation arrangements. If you are harmed due to someone's negligence, then you may have grounds for a legal action but you may have to pay for it. Regardless of this, if you wish to complain, or have any concerns about any aspect of the way you have been approached or treated during the course of this study, the normal University complaints mechanisms should be available to you.

Will my taking part in this study be kept confidential?
Yes. If you consent to take part in this research project we will retain your consent form, but we will not collect, or keep any additional details about you. Any remaining material from your sample will be destroyed appropriately, when the study ends.

What will happen to the results of the research study?
The results of the study will be published in scientific journals and also presented at local and international meetings.

Who has reviewed the study?
The study has been reviewed by an ethics officer at the University of Leicester.

THANK YOU FOR READING THIS VOLUNTEER INFORMATION SHEET

If you need to ask any questions please contact: Peter Farmer, Don Jones, Rob Britton, Leong Ng or Sandra Schumacher using the contact details above.

8.2.3 Consent form – Study 1

CONSENT FORM

Title of Research:
Assessment of exposure of DNA to dietary acrylamide

Principle Investigator: Don Jones

	Please initial box
1) I confirm that I have read and understood the information sheet Version 01 22/03/2013 and have had the opportunity to ask questions.	
2) I understand that my participation is entirely voluntary and that I am free to withdraw at any time.	
3) I agree to take part.	

Name of volunteer	Date	Signature

Name of researcher	Date	Signature

8.2.4 Consent form – Study 2

CONSENT FORM

Title of Research:
Assessment of biomarkers to dietary acrylamide

Principle Investigator: Don Jones

	Please initial box
1) I confirm that I have read and understood the information sheet Version 02 13/05/2013 and have had the opportunity to ask questions.	
2) I understand that my participation is entirely voluntary and that I am free to withdraw at any time.	
3) I agree to take part.	

Name of volunteer	Date	Signature
Name of researcher	Date	Signature

8.2.5 Questionnaire

QUESTIONNAIRE Volunteer number:

1. Please state your gender
   - o Male
   - o Female
2. Do you smoke?
   - o Yes → how many cigarettes/day? .....
   - o No

3. Acrylamide is a compound that is found in a variety of foodstuffs. The most commonly consumed are crisps, chips, biscuits, coffee, chocolate, bread and cereals.
   Please state how often you consume food of these categories on regular/general basis and within the last 24 hours before donating blood/urine samples for this study.
   Please indicate number of packs, pieces, mugs, slices or portions.
   Please write down if predominantly a specific brand is consumed.

	Based on a typical weeks consumption			**24-hours before donation**
Crisps	.....	bags/week	.....brand	.....bags
Chips	.....	portions		.....portions
Biscuits	.....	pieces		.....pieces
Coffee	.....	mugs	fresh brew/instant	.....mugs
Chocolate	.....	grams		.....grams
Bread	.....	slices		.....slices
Crisp bread	.....	slices	.....brand	.....slices
Cereals	.....	portions	.....brand	.....portion

8.3 Volunteers data

Table 8.1: Volunteers daily AA intake in µg, AA intake 24 hours prior to donation in µg, gender and smoking status

AA intake in µg					
Volunteer	**Daily AA intake**	**AA intake 24 hours prior to donation**	**Gender**	**Smoker**	**Age in years**
1	9.38	19.00	female	0	32
2	19.91	23.45	female	0	31
3	36.35	25.50	male	0	21
4	14.18	29.79	male	0	37
5	5.29	33.95	female	Smoker	25
6	15.67	19.65	female	0	66
7	66.13	54.50	male	Smoker	30
8	19.36	24.00	female	0	25
9	10.54	13.23	male	0	25
10	14.31	10.50	female	0	35
11	40.03	22.55	male	0	24
12	23.83	75.10	female	0	25
13	33.29	16.20	male	0	32
14	26.62	29.01	female	0	34
15	23.56	57.71	female	0	30
16	26.34	17.92	male	0	35
17	0.00	48.30	female	0	23
18	33.65	34.60	male	0	35
19	36.22	52.80	male	0	34
20	25.41	20.90	female	0	23
21	30.64	39.95	female	0	26
22	19.85	16.15	female	0	25
23	37.09	41.45	male	0	37
24	46.40	88.29	male	0	57
25	28.77	19.74	female	0	31
26	20.84	60.60	male	0	24
27	23.61	20.90	female	0	43
28	21.09	37.43	male	0	24
29	36.82	20.06	male	0	52
30	32.32	59.71	male	0	25
31	6.12	5.60	female	0	32
32	27.11	23.45	male	0	25

8.4 Analytical results from volunteers DNA samples

Table 8.2: Results for the analysed volunteers samples shown as mean *N*7-GA-Gua peak areas, mean [$^{15}N_5$]-*N*7-GA-Gua peak areas and mean ratios of *N*7-GA-Gua peak areas/ [$^{15}N_5$]-*N*7-GA-Gua peak areas

Volunteer	Mean *N*7-GA-Gua	Mean [$^{15}N_5$]-*N*7-GA-Gua	Mean ratio *N*7-GA-Gua/[$^{15}N_5$]-*N*7-GA-Gua
1	10290.0000	174763.3333	0.0523
2	398.0000	170973.0000	0.0021
3	2507.0000	191662.6667	0.0142
4	1290.0000	202595.0000	0.0064
5	304.6667	175922.6667	0.0015
6	2245.0000	167386.0000	0.0137
7	14878.3333	196733.0000	0.0722
8	4658.3333	175049.3333	0.0280
9	1686.0000	169298.0000	0.0089
10	5288.5000	184758.5000	0.0286
11	3182.3333	157826.3333	0.0172
12	1255.0000	172179.0000	0.0068
13	1006.5000	193027.0000	0.0059
14	1022.3333	194173.0000	0.0053
15	7273.3333	174080.6667	0.0377
16	4156.3333	165217.0000	0.0290
17	876.5000	173580.5000	0.0050
18	1137.0000	214202.0000	0.0053
19	7353.6667	188384.0000	0.0431
20	2975.6667	169715.6667	0.0196
21	2923.0000	179107.0000	0.0167
22	1146.0000	173034.3333	0.0073
23	1843.0000	185593.0000	0.0099
24	1607.0000	171868.5000	0.0094
25	5006.0000	154377.3333	0.0307
26	1880.0000	153347.6667	0.0130
27	4884.0000	176642.0000	0.0261
28	3150.0000	147320.3333	0.0226
29	1477.6667	176660.6667	0.0083
30	1545.3333	179404.6667	0.0094
31	1388.3333	157769.6667	0.0088
32	818.6667	159581.6667	0.0054