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Review

Review on the analysis of 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine and its phase I and phase II metabolites in biological matrices, foodstuff and beverages

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ABSTRACT

The heterocyclic aromatic amine, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP), has been shown to be carcinogenic in rodents, mice and rats. Following phase I N-hydroxylation and phase II esterification PhIP exerts its carcinogenic effect by binding to DNA purines. Quantitative and qualitative analysis of its bioactivated metabolites as well as it detoxification products is important in studying its biological effects and inter- and intra-individual exposures. A review is presented with an extensive coverage of publications specifically reporting on the analysis of PhIP and its phase I and II metabolites in biological matrices, foodstuff and beverages. Analytical techniques such as liquid and gas chromatography coupled with various detection techniques (mass spectrometry, ultraviolet or fluorescence detection) were mostly applied. We conclude that since the initial identification of PhIP in 1986 a large set of assays has been developed for the analysis of PhIP and its phase I and phase II metabolites in a wide range of matrices, these included food products and biological samples such as plasma, urine and faeces. In addition, it was shown that numerous metabolites were recovered and identified. Thus, we conclude that liquid chromatography coupled to mass spectrometry is clearly the method of choice for sensitive qualitative as well as quantitative analysis with high selectivity and reaching lower quantification levels in the sub pg/mL range. The main aim of this review is that it can be used by other researchers as a resource for method development and optimization of analytical methods of PhIP and its carcinogenic or detoxification products.

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1. Introduction

Heterocyclic aromatic amines (HAAs) are a class of carcinogenic compounds found in proteinaceous foods such as cooked meats and fish [1]. 2-Amino-1-methyl-6-phenylimidazo[4-5-*b*]pyridine (PhIP) is one of the most abundant HAAs and has been identified and isolated for the first time by Felton et al. [2]. PhIP is carcinogenic in rodents and induces lymphomas in mice, mammary carcinomas in female rats and colon and prostate carcinomas in male rats [3–7]. PhIP is formed from phenylalanine, creatinine and glucose as a by-product of the Maillard reaction during cooking or frying of protein-rich foods at high temperatures [8,9].

In order to exert its carcinogenic effect, it is generally assumed that PhIP requires bioactivation, mediated by N-hydroxylation at the amine group by CYP1A1 and CYP1A2 [10–15]. This results in the formation of 2-hydroxyamino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (N^2-OH-PhIP). The phase II metabolites, N^2-acetoxy-PhIP and N^2-sulfonyloxy-PhIP are subsequently formed from N^2-OH-PhIP by acetyltransferases [16–18] and sulfotransferases [16,18–21], respectively. Heterolytic cleavage of the sulfate or acetate group results in nitrenes and/or nitrenium ions which can form adducts with DNA purines depending on the delocalization of the positive charge of the nitrenium ion or of the electron deficiency of the nitrene [22–24]. Glucuronidation, being a major metabolic pathway in the biotransformation of xenobiotics also plays an important role in the detoxification of PhIP [25]. PhIP and its phase I metabolites: N^2-OH-PhIP, 4′-OH-PhIP and 5-OH-PhIP are substrates for various UDP-glucuronosyltransferases (UGTs) and sulfotransferases (SULTs) [25–28]. Predominant positions for glucuronidation and sulfatation are the N^2, *N*3 and 4′-position (Table 1). Whereas 4′-OH-PhIP and its sulfated conjugate are considered as readily excreted detoxification metabolites [29–33], 5-OH-PhIP is thought to be only formed via activation of PhIP to N^2-OH-PhIP and subsequent esterification. It is therefore considered a biomarker for activation of PhIP [11,34–36].

Quantitative and qualitative analysis of PhIP's bioactivated metabolites as well as its detoxification products is important in studying inter- and intra-individual exposures. A review is now presented consisting of a comprehensive overview of analytical publications describing the analysis of PhIP and its phase I and II metabolites in biological matrices, foodstuff and beverages. PhIP and its metabolites were recovered from food products (e.g. meat, fish, beer and wine) and in urine, blood, faeces and hair. Analyses were performed after intravenous or regular (i.e. food intake) administration of PhIP to organisms such as mice, rats and humans.

To facilitate the analysis of PhIP and its metabolites, various analytical assays were developed of which some also include other HAAs. In these publications techniques were used such as liquid and gas chromatography or capillary electrophoresis, coupled with predominantly mass spectrometry (MS), or ultraviolet (UV) and fluorescence (Flu) detection. A discussion is presented on the performance of various sample preparation procedures and separation and detection techniques including their pros and cons.

A relatively large part of the described assays focus on the analysis of PhIP and its metabolites in plasma and urine after food intake. Although PhIP including its hydroxylated, glucuronidated and sulfated forms are thoroughly investigated, the activation of PhIP is generally agreed to be preceded by the formation of acetyl and sulfonyl esters, but these two PhIP esters have not been recovered from any matrix hitherto. This might very well be due to their instability. DNA-PhIP adduct formation, however, has been studied extensively and correlations were seen between decreased levels of DNA adducts and increased levels of N^2-OH-PhIP-N^2-glucuronide [26]. Irrefutably, analytical assays for the analysis of PhIP and its metabolites are crucial to gain profound insight into the disposition of PhIP and its carcinogenic or detoxification products.

This review includes a summary of the analytical techniques used for the analysis of PhIP and its phase I and II metabolites (with their chemical structures).

2. Sample pretreatment

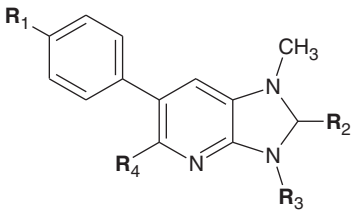
The analyses of PhIP and its metabolites have been performed in a wide range of matrices, including: urine, faeces, whole blood, plasma, hair, milk, tissue, microsomal incubate, meat and fish. Each of these matrices is complex and requires pretreatment before injection into, e.g. a liquid chromatograph. Thus, PhIP and its polar metabolites need to be extracted, while simultaneously endogenous compounds need to be restrained from extraction. This prevents them from interfering with chromatographic separation and detection and negatively affecting the sensitivity, accuracy and precision of the analysis. The removal of endogenous compounds such as proteins, lipids and salts results in an overall increased performance of an analytical assay.

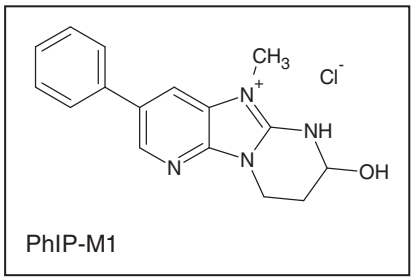
Standard sample pretreatment techniques such as protein precipitation (PP), solid phase extraction (SPE) and liquid–liquid extraction (LLE) have been applied in most publications. Exceptions were the use of Blue Cotton/Chitin, hollow-fibre supported liquid membrane (HF-SLM) extraction [37–39], SPE by use of a molecular imprinted polymer (MIP) [11,34] and conversion of samples to graphite for high resolution mass spectrometric analysis [40,41].

PP is a simple and straightforward technique in which organic modifier, acids or highly concentrated salt solutions are used to precipitate proteins in, e.g. plasma, tissue or food product homogenate. This was typically followed by centrifugation of the precipitated proteins. Subsequent dilution of the supernatant into a weak organic modifier results in a chromatography compatible matrix. When samples are prepared by use of PP, only the proteins are removed and endogenous compounds other than proteins largely remain. Especially in complex samples such as tissue or meat homogenate this can, however, cause interferences in the form of matrix effects (e.g. ion suppression in mass spectrometry) or contamination of analytical columns decreasing their overall performance and lifetime. For higher extraction selectivity and cleaner extracts, SPE and LLE are the methods of choice for PhIP analysis. These sample pretreatment techniques were preferred over PP with a relatively long total analysis time as a major disadvantage, as most protocols consisted of multiple clean-up steps. A wide range of extraction solvents was used for LLE. Extraction was often preceded with either an alkalizing step to increase the extraction recovery and selectivity, or hydrolysis to convert glucuronides and sulfates into their respective aglycones. To improve recovery, LLE often consists of multiple steps (Table 2

) or is combined with PP or SPE. LLE was typically followed by evaporation of the organic modifier and reconstitution in a weak solvent, e.g., the mobile phase eluent. SPE was frequently used as sample preparation for the analysis of PhIP and its metabolites with a wide variety of solid phases, such as: Bond Elut [42–44], Amberlite [45], Blue Chitin/Cotton [46–48], Isolute [11,34] and Extrelut [42,44,49,50]. Extrelut is used in sample preparation methods as originally described by Gross and Grüter [44]. This method is often applied for the analysis of HAAs, including PhIP, in cooked foods and consists of LLE on a solid support, followed by SPE with cation exchange and Extrelut columns. Extrelut columns are predominantly used for the analysis of multiple HAAs in meat and food products [42,44,50] or urine and faeces [49]. They contain a robust stationary phase of wide-pore diatomaceous earth which can be used within a pH range of 1–13. An alternative SPE technique for PhIP and metabolite analysis uses a blue pigment: copper phtalocyanine trisulfonate, a common blue pigment widely used as a dye which appeared to have a high affinity for aromatic compounds

Table 1
Trivial names, chemical structures and molecular masses of PhIP and its metabolites.





Trivial name	R_1	R_2	R_3	R_4	Formula	Mol. mass
PhIP	H	NH_2	–	H	$C_{13}H_{12}N_4$	224.1
2-OH-PhIP	H	OH	–	H	$C_{13}H_{11}N_3O$	225.1
N^2-methyl-PhIP	H	NH-CH_3	–	H	$C_{14}H_{14}N_4$	238.1
4′-OH-PhIP	OH	NH_2	–	H	$C_{13}H_{12}N_4O$	240.1
N^2-OH-PhIP	H	NH-OH	–	H	$C_{13}H_{12}N_4O$	240.1
5-OH-PhIP	H	NH_2	–	OH	$C_{13}H_{12}N_4O$	240.1
2-nitro-PhIP	H	NO_2	–	H	$C_{13}H_{10}N_4O_2$	254.1
N^2-methyl-4′-OH-PhIP	OH	NH-CH_3	–	H	$C_{14}H_{14}N_4O$	254.1
PhIP-M1	See inlay	–	–	–	$C_{16}H_{17}N_4O$	281.1
N^2-acetoxy-PhIP	H	NH-acetate	–	H	$C_{15}H_{14}N_4O_2$	282.1
N^2-sulfonyloxy-PhIP	H	NH-sulfate	–	H	$C_{13}H_{12}N_4O_4S$	320.1
4′-OH-PhIP-sulfate	Sulfate	NH_2	–	H	$C_{13}H_{12}N_4O_4S$	320.1
5-OH-PhIP-sulfate	H	NH_2	–	Sulfate	$C_{13}H_{12}N_4O_4S$	320.1
N^2,4′-diOH-PhIP-sulfate	Sulfate	NH-OH	–	H	$C_{13}H_{12}N_4O_5S$	336.1
5,4′-diOH-PhIP-sulfate	Sulfate	NH_2	–	OH	$C_{13}H_{12}N_4O_5S$	336.1
PhIP-*N*3-glucuronide	H	=NH	Gluc	H	$C_{19}H_{20}N_4O_6$	400.1
PhIP-N^2-glucuronide	H	NH-gluc	–	H	$C_{19}H_{20}N_4O_6$	400.1
4′-OH-PhIP-glucuronide	O-Gluc	NH_2	–	H	$C_{19}H_{20}N_4O_7$	416.1
5-OH-PhIP-glucuronide	H	NH_2	–	O-Gluc	$C_{19}H_{20}N_4O_7$	416.1
4′-OH-PhIP-N^2-glucuronide	OH	NH-gluc	–	H	$C_{19}H_{20}N_4O_7$	416.1
N^2-OH-PhIP-N^2-glucuronide	H	N(OH)-gluc	–	H	$C_{19}H_{20}N_4O_7$	416.1
N^2-OH-PhIP-*N*3-glucuronide	H	=N-OH	Gluc	H	$C_{19}H_{20}N_4O_7$	416.1
N^2,4′-diOH-PhIP-glucuronide	O-Gluc	NH-OH	–	H	$C_{19}H_{20}N_4O_8$	432.1

The molecular mass is based on the monoisotopic mass. '=' is used to indicate the presence of a double bond between the amine and the imidazole moiety. If R_3 is: '–', a C=N double bond is present between the 2 and 3 position of the imidazole moiety. Gluc: glucuronide; PhIP: 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine. R_1: 4′-position; R_2: N^2-position; R_3: *N*3-position.

with three or more fused rings in their structure. The planar structure can form a 1:1 hydrophobic complex with the blue pigment that has a large planar structure in the molecule [51]. This pigment can be covalently linked to a support matrix of Cotton (Blue Cotton) [46] or chitin (Blue Chitin) [47,48]. Extractions appear to be simple with high recoveries ranging from 60 to 100%. PhIP and 4′-OH-PhIP were recovered from meat and acid-hydrolyzed urine using Blue Cotton and subsequent derivatization for gas chromatography–mass spectrometry (GC–MS) analysis reaching a limit of detection (LOD) of 2.5 pg/mL [46]. Hashimoto et al. were the first to report the use of Blue-Chitin to extract PhIP from dried hair using column-switching liquid chromatography–mass spectrometry (LC–MS) [47]. An LOD as low as 47 pg/mL was reached by miniaturization of an analytical set-up for the determination of PhIP in human urine [48]. Miniaturization was accomplished by micro-SPE in capillaries filled with Blue Chitin.

MIPs are synthetic polymers having a predetermined selectivity for a given analyte, or group of structurally related compounds, that make them ideal materials to be used in separation processes [52]. They are formed in the presence of a molecule that is extracted afterwards, thus leaving complementary cavities behind. A MIP-SPE set-up was successfully used for the clean-up of urine after consumption of cooked chicken, extracting PhIP and 6 of its phase I and II metabolites [34].

One commonly known membrane extraction technique consists of the use of a supported liquid membrane (SLM), where a flat-sheet or a hollow-fibre (HF) membrane is utilized in a three-phase system. The three-phase (aqueous–organic–aqueous) HF-SLM procedure consists of an organic solvent-impregnated membrane forming one phase, another acceptor aqueous phase placed inside the HF lumen, and a third phase; the aqueous sample itself. If a weakly basic compound, for example PhIP ($pK_a = 5.6$, $\log P = 1.2$), is to be extracted, the sample (donor phase) pH is made alkaline and the acceptor pH is adjusted to an acidic value. A review on membrane-based techniques for sample enrichment was published by Jönsson et al. [53]. HF-SLM was successfully used for the extraction and quantification of heterocyclic amines, including PhIP, from urine and plasma [37–39]. HF-SLM extraction is usually applied to liquid and semi-solid samples reaching limits of detection as low as 25 pg/mL in urine and 11 pg/mL in plasma [39].

To study the bioavailability and fate of PhIP in, e.g. mice, radioactive PhIP (e.g. [^{14}C]PhIP) can be administered after which the radiocarbon concentration is followed. Measurement of ^{14}C levels, originating from [^{14}C]PhIP was successfully performed by use of accelerator mass spectrometry (AMS) (Section 4.1). For this technique it is required that samples should be in a form compatible with the AMS ion source. It was shown that graphite was ideal for this purpose, as it gives high ion outputs. Therefore, the samples were converted into filamentous graphite before analysis by AMS. Mauthe et al. performed a protein precipitation on milk samples before analyses by HPLC after which the fractions were collected [40]. HPLC fractions were converted to graphite and subsequent analysis by AMS allowed for sensitive determination of the radiocarbon concentration in each fraction. By defining which metabolite elutes in each fraction, a sensitive quantification was accomplished of PhIP metabolites [40,41]. Although the range in polarity between PhIP and its metabolites is relatively wide, the above described sample preparation techniques have proven to

Table 2
Bioanalytical assays for PhIP and its metabolites.

Matrix (volume)	Species	Analyte	LLQ	Sample prep.	Further sample pretreatment (v/v)	Column (dimensions)	Mobile phase (v/v)	Detection (ionization/wavelength)	Val.	Remarks	Ref.
Plasma (100 μL)	Mouse	PhIP	0.978 ng/mL	PP	Homogenization of intestinal content, faeces and tissues in 4% (m/v) BSA. Dilution of bile in plasma. Dilution of urine in ACN/3.5 mM NH_4Ac (3:7). Plasma, homogenate and diluted bile: PP with ACN. Centrif. Dilution in ACN/3.5 mM NH_4Ac (3:7).	Synergi Hydro C18 (150 mm × 2.0 mm)	A: 3.5 mM NH_4Ac buffer pH 3.5	MS (ESI-QqQ)	Yes	Tissues: brain, colon tissue, cecum tissue, kidney, liver, small intestine tissue, spleen and testis	[61]
Urine (20 μL) Bile (10 μL) Intestinal content, faeces and tissue (100 μL homogenate)	Human	N^2-OH-PhIP	1.11 ng/mL				B: ACN				
Urine (1.0 mL)	Human	PhIP	5 pg/mL	PP	Ur: acidified with FA (20 μL, 88%)	Zorbax-SB-C18 (250 mm × 0.3 mm)	A: 0.01% FA in H_2O	MS	No	Biosynthesis of metabolites	[54]
		4′-OH-PhIP	5 pg/mL		Centrifugation. SPE: HyperSep Retain CX			(ESI-QqQ)			
M. inc. (1 mg/mL)	Rabit liver	PhIP-N^2-gluc	50 pg/mL	SPE	SPE: washed: 2% FA in H_2O, 2% FA in MeOH,		B: 0.01% FA + 5% H_2O in ACN			Profile urine metabolites in omnivores after meat consumption	
		PhIP-$N3$-gluc	50 pg/mL		H_2O, 5% NH_4OH. Dried. eluted with 1% NH_4OH in MeOH. evap. conc.: vacuum			^1H NMR			
		N^2-OH-PhIP-N^2-gluc	50 pg/mL								
M. inc (2 mg/mL)	Human liver	N^2-OH-PhIP-$N3$-gluc	50 pg/mL		β-Glucuronidase + sulfatase + acid (HCl)						
Urine (3 mL)	Human	PhIP	2 pg/g	HF-SLM	Add: propyl gallate in EtOH + EDTA in H_2O	Symmetry C8 (150 mm × 2.1 mm)	A: 30 mM NH_4Ac	MS	No	Application of: [39]	[37]
		4′-OH-PhIP	5 pg/g		up to 2 mM. Centrif. Multiple step		buffer pH 4.5	(ESI-IT)			
		5-OH-PhIP	9 pg/g		hollow-fibre liquid membrane extraction. Three different extraction conditions studied.		B: ACN			Also included: MeIQx and Norharman	
Urine (0.5 mL)	Human	PhIP	5 pg/mL	SPE	Ur: chilled MeOH/acetone (1:1).	Zorbax-XDB-C18 (250 mm × 0.3 mm)	A: 0.01% FA +	MS	Part.	(Bio)synthesis of metabolites using	[32]

		4′-OH-PhIP	50 pg/mL		Centrif. evap. acidified		5% ACN in H_2O	(ESI-QqQ)		microsomes or the nitro derivative of PhIP. Glucuronide concentrations determined from their UV absorption spectra. Quantification of metabolites using calibration curves of purified PhIP metabolites from incubates
		5-OH-PhIP	50 pg/mL		with FA. SPE: HyperSep Retain CX.					
M. inc (2 mg/mL)	Human liver and rat	N^2-OH-PhIP	50 pg/mL		Washed: 2% FA in H_2O, 2% FA in MeOH,		B: 0.01% FA +			
		PhIP-4′-O-gluc	50 pg/mL		H_2O, 5% NH_4OH. Eluted with		5% H_2O in ACN			
		PhIP-N^2-gluc	50 pg/mL		1% NH_4OH in MeOH.					
		PhIP-N3-gluc	50 pg/mL		Evap. Recon: H_2O:MeOH (1:1)					
		N^2-OH-PhIP-N^2-gluc	50 pg/mL							
		N^2-OH-PhIP-N3-gluc	50 pg/mL							
Hair (50 mg)	Human	PhIP	65 pg/g	LLE	Wash (3×) with 1 mL 0.1 M HCl. Centrif.	Aquasil C18 (250 mm × 0.5 mm)	A: H_2O + 0.01%	MS	Part.	Also includes: analysis of AαC and MeIQx and formation of DNA adducts. [62]
Fur (10 mg)	Rodent			SPE	Wash (3×) with MeOH. Dried. Add: 1 mL 1 M NaOH. Heating. LLE: 2× 5 mL Et. ac. SPE: Oasis MCX. Recon: 0.1% FA:MeOH (1:1)		+ 10% ACN B: ACN + 0.01% FA + 5% H_2O	(ESI-QqQ)		
Urine (3 mL)	Human	PhIP	1 ng/mL	HF-SLM	Dilution with 0.5 M NaOH. hollow-fibre immersion in urine. Strirring. Acceptor phase transferred for analysis.	Rp-Ace C18 (250 mm × 4.6 mm)	A: 30 mM NH_4Ac buffer pH 4.5 B: ACN	UV (315 nm)	Part.	Also includes: analysis of 10 other HAAs Includes an extraction time profile. [38]
Urine (1.4 mL) Plasma (0.3 mL)	Human	PhIP	Ur: 25 pg/mL Pl: 11 pg/mL	HF-SLM	Dilution with 0.5 M NaOH. Hollow-fibre immersion in urine. Strirring. Acceptor phase transferred for analysis.	Rp-Ace C18 (250 mm × 4.6 mm)	A: 30 mM NH_4Ac buffer pH 4.5 B: ACN	Flu (Ex: 315 nm) (Em: 390 nm)	Part.	Introduction of the LPME method. Optimization of extraction parameters [39]
Faeces (5 mL slurry)	Human	PhIP	Not. sp	SPE	Add: 0.5 mL 6 M NaOH + 5 g diatom. earth.	Symmetry C18 (150 mm × 2.1 mm)	A: 0.01% FA in H_2O	MS	No	Includes synthesis of PhIP-M1 and its trideuterated derivate [49]
		PhIP-M1			Placed into Extrelut-20 cartridge: elute: 30 (ur)		B: ACN	(ESI-IT)		

Table 2 (*Continued*)

Matrix (volume)	Species	Analyte	LLQ	Sample prep.	Further sample pretreatment (v/v)	Column (dimensions)	Mobile phase (v/v)	Detection (ionization/wavelength)	Val.	Remarks	Ref.
					and 60 (fa) mL DCM. Oasis MCX. Elution: 10%					Acid hydrolysis to release	
Urine (5 mL)					NH_4OH in ACN. Evap. Recon: ACN: 5 mM FA (3:1). Hydrolysis: HCl + heat. Add: NaOH.					Phase II conjugates.	
Urine (0.05% of 16 h urine)	Human	PhIP	Not sp.	SPE (-MIP)	Isolute 101 column. Elution with MeOH. Evap.	Zorbax SB-C3 (150 mm × 3 mm)	A: 0.01% FA in H_2O	MS	No	Biosynthesis of 4′-OH-PhIP	[34]
		4′-OH-PhIP			Recon. in 50 mM HCl. Add: hydrazine hydrate.		B: ACN	(ESI-IT)		and 5-OH-PhIP	
	Chicken	5-OH-PhIP			Incubate: 4 h 60 °C. Purify: Isolute 101.					Synthesis of molecular imprinted polymer columns.	
Meat (not sp.)					Hydrolysis: β-glucuronidase + sulfatase. Purified: Isolute 101 and MIP.						
Meat (2 g)	Beaf	PhIP	0.03 μg/kg	SPE	Hom. 1 M NaOH (4 mL/g). Mixed: Extrelut-20	Aquasil C18 (200 mm × 1 mm)	A: 1 mM NH_4Ac	MS	No	Includes the analysis of 12	[50]
	Chicken				resin. Elution with 5% toluene in DCM to		/ACN/FA (90:10:0.1)	(ESI-QqQ)		other HAAs.	
	Pork				Oasis MCX LP. Elution: 5% NH_4OH in MeOH. Evap. Recon. DMSO: 0.1% FA (1:1)		B: ACN/H_2O/FA (95:4.9:0.1)	^1H NMR			
Urine (50 μL)	Mouse	PhIP	Not sp.	PP	Ur: acetonitrile followed by centrifugation.	Acquity BEH C18 (Not sp.)	A:	MS	No	Principal component analysis of results. Includes: human recombinant P450 reactions, antibody inhibition of PhIP metabolism and PhIP-DNA adduct analysis.	[13]
		N^2-methyl-PhIP		SPE	M. inc: loaded onto Oasis column.		0.01% FA in H_2O	(ESI-QTOF)			
M. inc	Mouse liver	4′-OH-PhIP			Elution with 1 mL MeOH. Evap.						
		5-OH-PhIP			Recon. ACN/H_2O (1:1).		B:	LSC			
		N^2-OH-PhIP					0.01% FA in ACN				
		N^2-methyl-4′-OH-PhIP									
		4′-OH-PhIP-sulfate								LSC of ^{14}C-PhIP in urine	
		5-OH-PhIP-sulfate									
		N_2,4′-diOH-PhIP-sulfate									

		5,4′-diOH-PhIP-sulfate PhIP-N^2-gluc PhIP-$N3$-gluc 4′-OH-PhIP-gluc 5-OH-PhIP-gluc 4′-OH-PhIP-N_2-gluc N^2-OH-PhIP-N^2-gluc N^2-OH-PhIP-$N3$-gluc N^2,4′-diOH-PhIP-gluc								
Tissue (100 mg)	Mouse	PhIP 4′-OH-PhIP N^2-OH-PhIP	Not sp.	LLE	Homogenized in PBS. Extraction with Et. ac./MTBE (1:1). Evap. Recon. in MeOH/0.1% FA in H_2O (7:3)	Luna C18 (50 mm × 4.6 mm)	0.1% FA in MeOH/H_2O (70:30)	MS (ESI-QqQ)	No	Includes PhIP-DNA adduct analysis and a study on PhIP metabolism in extrahepatic organs. Analyzed tissues: lung, stomach, colon, small intestine and mammary gland.	[63]
M. inc	Rabbit liver	PhIP	Not sp.	SPE	(1) urine applied to Isolute 101 column. Elution: MeOH. Evap. (2) Hydrolysis: β-glucuronidase + sulfatase. (3) Hydrazine hydrate incubation. Purified: Isolute 101 + MIP.	Zorbax SB-C3 (150 mm × 3 mm)	A: 0.01% FA in H_2O B: ACN	MS (ESI-IT)	No	Deconjugation of phase II metabolites.	[11]
Urine (1 mL)	Human	4′-OH-PhIP 5-OH-PhIP N^2-OH-PhIP PhIP-gluc N^2-OH-PhIP-N^2-gluc 4′-OH-PhIP-gluc									
Meat based infant food (1 g)	Beef Chicken Horse Lamb Rabbit liver	PhIP	26 ng/g	LLE SPE	LLE: 2× with acetone. Mixing. Centrif. PP during storage in freezer. Centrif. SPE: Benzenesulfonic-SCX bonded silica. LLE extract loaded + 2 M NH_4Ac/Acetone. (1:1). Concentrated by evap. Filtered.	TSK-Gel ODS-80TS (250 mm × 2.0 mm)	A: NH_4Ac buffer pH 2.8 B: ACN	MS (ESI-QqQ)	Yes	Includes 6 other HAAs.	[64]
Milk (5 mL)	Human	PhIP	0.68 pg/mL	LLE	Add: 10% TCA/ACN (1:1). Mixed. Add: 0.1 M	Electron	A: 0.1% FA in H_2O	MS	No	Meat extraction adjusted from: [42]	[65]

Table 2 (*Continued*)

Matrix (volume)	Species	Analyte	LLQ	Sample prep.	Further sample pretreatment (v/v)	Column (dimensions)	Mobile phase (v/v)	Detection (ionization/wavelength)	Val.	Remarks	Ref.
Meat (4 g)	Chicken				HCl. Heated. Centrif. LLE: hexane. Aq. layer: 1 M NaOH: pH 10. Extr: Et.ac. Add: anhydr. Na_2SO_4. Add: 0.1 M HCl. Recon: 0.1% FA/ACN (9:1)	Acquasil C18 (250 mm × 1 mm)	B: ACN	(ESI-QqQ)			
Meat (2 g crust)		PhIP	0.02 ng/g	SPE	Crust: freeze-dried, grounded.	Symmetry C8 (150 mm × 2.1 mm)	A: 30 mM NH_4Ac	MS	No	Includes: effect of red wine marinades	[71]
		4′-OH-PhIP	0.08 ng/g		Mixed with 1 M NaOH. Hom. SPE: [76]. Evap. Recon: MeOH.		buffer pH 4.5 B: ACN	(ESI-IT)		on the formation of 4′-OH-PhIP and its MS^n fragmentation.	
Urine (1.4 mL)	Human	PhIP	47 pg/mL	PP	Urine mixed with $ZnSO_4$. Vortexed. Cenrtif.	Blue Chitin filled capillary (50 μm I.D.)	Not sp.	MS (nESI-IT)	No		[48]
Faeces	Human	PhIP	Not sp.	SPE	Hom. faeces with PBS (0.1 M, pH 7) + 1 g/L	Genesis C18 (150 mm × 4.6 mm)	A: 0.01% FA	MS	No	Identification of a new PhIP	[72]
(1 mL digested fecal slurry)		PhIP-M1			Na-triglycolate. Centrif. 1 mL applied to Strata C18 SPE. Centrif. Loaded: 200-mg C18-U. Elution: 0.1 mM. pH 3.5 NH_4Ac:ACN (1:4). Dried. Recon: 0.1 mM pH 3.5 NH_4Ac:ACN (1:4)	Zorbax SB-C3 (150 mm × 3 mm) Omnisphere 250 (250 mm × 21.4 mm)	B: ACN A: 0.01% FA B: ACN 0.05% FA in H_2O/ ACN (85:15)	(ESI-IT/HRMS) UV (315 nm) Flu (Ex: 316 nm) (Em: 370 nm) NMR		metabolite: PhIP-M1 formed by intestinal microbiota Various types of 1D and 2D NMR are used.	
Meat (4 g)	Beef	PhIP	<1 pg on column	SPE	Hom. 16 mL NaOH. mixed with Extrelut-20 resin. Elution with DCM. Load: Bond-Elut PRS. Eluted: MCX LP by DCM/toluene	Aquasil C18 (150 mm × 1 mm)	A: 0.1% FA in H_2O B: ACN	MS (ESI-QqQ)	Part.	Also includes the analysis of 9 other HAAs.	[42]
Beef extract paste + meat scrapings (1 g)	Chicken (grilled)										

					(95:5). Elute: 5% NH_4OH in MeOH. Evap. Recon. Mob. phase. buffer.					
Urine (5 mL)	Human	PhIP	Not sp.	SPE	Strata X SPE. Elution with MeOH. Evap	YMC ODS-A (250 mm × 3 mm)	A:	MS	No	[66]
		PhIP-N^2-gluc			Recon: 0.01 M HCl. YM-3 centrifugal filter.		H_2O/MeOH/HAc	(ESI-QqQ)		
		N^2-OH-PhIP-N^2-gluc			Apply: Benzenesulfonic acid SPE. Eluted to		(97:2:1)			
		N^2-OH-PhIP-N3-gluc			C18 SPE: 0.05 M NH_4Ac pH 8. Eluted by		B:MeOH/H_2O/HAc			
		4′-OH-PhIP-sulfate			5 mL MeOH/H_2O (6:4). Evap to 20 μL.		(95:4:1)			
Urine (1 mL)	Human	PhIP	3.7 pg/mL	LLE	Alkalinize by 0.1 mL 10 M NaOH. LLE: Et. ac.	Symmetry C18 (100 mm × 1 mm)	A: NH_4Ac buffer	MS	Part.	TLC combined with UV, DEP-EI-MS	[67]
				SPE	Centrif. Acidify by HAc. Apply to MCX SPE. Elution by 5% NH_4OH in MeOH. Evap. Recon.: 5 mM NH_4Ac/ACN (9:1) pH 7.85		B: ACN	(ESI-QqQ)		and NMR used for reaction product identification. Also includes: the analysis of 11 other HAAs.
Hair (3 g)	Human	PhIP	50 pg/g	SPE	Washed: 0.1% SDS. 4× H_2O + EtOH. Dried.	Mercury MS	A: 40 μM NH_4Ac	MS	Part.	Spectrophotometric characterization of melanin. Correlation observed between PhIP levels and melanin content in hair.	[47]
					Alkalanize: 1 M NaOH. Centrif. Filtrated. Add: 6 M HCl. SPE: Blue Chitin. Elute: MeOH–NH_4OH (50:1) Conc. Dissolved: MeOH. Centrif. Conc. Dissolved: 0.1 M HCl. LLE: n-hexane pH (>10 by 28% NH_4OH). LLE: 2× DCM. Conc. Recon: 40 μM NH_4Ac/MeOH (1:1). Filtered.	Luna C18 (20 mm × 2 mm)	buffer pH 4	(ESI-Q)		
							B: MeOH			
M. inc	Human liver	PhIP	Not sp.	PP	Seeded cells are scraped and combined with the	Supelco C18 DB (250 mm × 4.6 mm)	A: 20 mM DEA-Ac	MS		Investigation of the differential	[33]
	Rat liver	4′-OH-PhIP			culture medium. Add: acetonitrile. Centrif. Evap.		buffer pH 5	(ESI-QqQ)		metabolism of PhIP in rat and human hepatocytes. N-OH-PhIP and 5-OH-PhIP are synthesized.	
		5-OH-PhIP									
		4′-OH-PhIP-sulfate					B: MeOH	UV			
		PhIP-N^2-gluc						(spectra)			

Table 2 (*Continued*)

Matrix (volume)	Species	Analyte	LLQ	Sample prep.	Further sample pretreatment (v/v)	Column (dimensions)	Mobile phase (v/v)	Detection (ionization/wavelength)	Val.	Remarks	Ref.
		PhIP-*N*3-gluc								Contains online UV absorbance spectra and product ion mass spectra of PhIP metabolites.	
		4′-OH-PhIP-gluc						NMR			
		N^2-OH-PhIP-N^2-gluc						LSC			
		N^2-OH-PhIP-*N*3-gluc						([2-^{14}C]PhIP)			
Milk (5 mL)	Human	PhIP	3 pg/mL	SPE	Sonication (5 min). Add: 5 mL 0.1 M HCl.	IB-SIL C18 BDS (250 mm × 4.6 mm)	A: 10 mM NH_4Ac	MS	No		[68]
					Heated: 5 min 50 °C. Apply to MCX cartridge.		pH 4/ACN (95:5)	(ESI-QqQ)			
					Elution by ACN/NH_4OH (95:5).		B: ACN/	UV (263 nm)			
					Evap. Recon: 10 mM NH_4Ac pH 4 or MeOH.	Symmetry Shield C18 (100 mm × 2.1 mm)	10 mM NH_4AC	Flu (ex: 315 nm)			
							pH 4 (95:5)	(em: 390 nm)			
Urine (1 mL)	Human	PhIP	1 pg/mL	LLE	Alkalinize: 2 mL 0.2 M sodium phosphate buffer	HP1 fused silica (25 m × 0.2 mm)	Carrier gas: helium	MS	No	Application of this method in [57]	[55]
				SPE	pH 8. Mixing. LLE: 2× 3 mL Et. ac. Evap. Recon.			(CI-Q)			
					MeOH + 0.01 M Na-PO_4 buf. pH 7. Mixing. Apply:	film: 0.33 μm				where phase II metabolites	
					C18 SPE. Elution: MeOH/H_2O (6:4)					are included.	
					Evap. Hydrolysis: 1 M NaOH 12 h at 100 °C.					Derivatization using BPFB.	
Urine (5 mL)	Human	PhIP	Not sp.	SPE	Apply to macroporous polymeric SPE column.	YMC ODS-A (250 mm × 3 mm)	A: H_2O/MeOH/HAc	MS	No	Synthesis of deuterated	[73]
		4′-OH-PhIP-sulfate			Elution: 5 mL MeOH. Evap. Recon. 0.01 M HCl.		(97:2:1)	(ESI-IT)		N^2-OH-PhIP-N^2-glucuronide.	
		PhIP-N^2-gluc			Filter (YM-3). Benzenesulfonic acid column.		B:MeOH/H_2O/HAc			Determination of human variation in carcinogen metabolism. Appl. of [29]	
		N^2-OH-PhIP-N^2-gluc			Elute onto C18 SPE with 0.05 M NH_4Ac pH 8.		(95:4:1)				
		N^2-OH-PhIP-*N*3-gluc			Eluted by MeOH/H_2O (1:1). Evap.						
M. inc	Human liver	PhIP	1 ng/mL	PP	Ice-cold MeOH. Mixing. Centrif. Loaded:	Supelcosil C18 (750 mm × 2.1 mm)	A: 0.5 mM NH_4Ac/	MS	Yes	Mobile phase optimization	[74]
		N^2-OH-PhIP	10 ng/mL	SPE	CPE-C18. Elution: 0.1 mM NH_4AC pH 3.5/		MeOH/THF	(ESI-IT)			

					MeOH (1:4). Evap. Recon. MeOH:H_2O (9:1). Mixing. Centrif.		(80.5:19:0.5) B: MeOH/H_2O (65:35)	UV (200–400 nm)			
Urine (5 mL)	Human	PhIP 4′-OH-PhIP-sulfate PhIP-N^2-gluc N^2-OH-PhIP-N^2-gluc N^2-OH-PhIP-N3-gluc	Not sp.	SPE	Apply to macroporous polymeric SPE column. Elution: 5 mL MeOH. Evap. Recon. 0.01 M HCl. Filter (YM-3). Benzenesulfonic acid column. Elute onto C18 SPE with 0.5 M NH_4Ac pH 8. Eluted by MeOH/H_2O (1:1). Evap.	YMC basic (250 mm × 3 mm)	A: H_2O/MeOH/HAc (97:2:1) B:MeOH/H_2O/HAc (95:4:1)	MS (ESI-IT)	No	Synthesis of deuterated N^2-OH-PhIP-N^2-glucuronide Method applied in [73]	[29]
Meat (whole sample)	Beef	PhIP 4′-OH-PhIP	Not sp.	SPE	Different SPE procedures were compared: to establish the best conditions for the determination of HAAs in beef extracts.	TSK-Gel ODS-80T (25 mm × 4.6 mm)	A: 0.01 M TEA pH 3.3 by H_3PO_4 B: ACN	UV (200-300 nm spectra)	Part.	Analysis of PhIP, 4′-OH-PhIP and 12 other HAAs.	[76]
Urine (10 mL)	Human	PhIP	4 pg/mL	LLE	Acidify with 6 M HCl, incubate 4 h, 70 °C. Neutralize: 6 M NaOH. Alkalanize: Na_2CO_3. LLE: 2× Et. ac. Freeze out residual H_2O. LLE: 2× 0.1 M HCl. Evap.	Micro-bore C18-RP (150 mm × 1 mm) Narrow bore (150 mm × 2.1 mm)	A: 40 μM NH_4Ac pH 4/MeOH (90:10) B: MeOH/40 μM NH_4Ac pH 4 (98:2) MeOH/H_2O (50:50) + 0.1% FA	MS (ESI-Q) MS (ESI-QqQ)	No	Determination of variation in excretion of PhIP in urine from White, African-American and Asian-American men. Also includes PhIP: creatinine ratios. Two different LC–MS set-ups were used.	[56]
Plasma (2 mL)	Human	PhIP 4′-OH-PhIP-sulfate PhIP-N^2-gluc	Not sp.	PP	Ur: ^{14}C content determination by LSC. 6000–8000 dpm concentrated. Centrifugal filtered and injected.	TSK-Gel ODS-80TM (220 mm × 4.6 mm)	A: 0.1% TEA B: MeOH	MS (ESI-QqQ)		Application of this method in [70] Four step purification and concentration of metabolites.	[69]
Urine (1 mL)	Human	N^2-OH-PhIP-N^2-gluc			Plasma: PP with ice-cold MeOH.	Amberlite XAD-2	MeOH and MeOH/	UV (315 nm)			

Table 2 (*Continued*)

Matrix (volume)	Species	Analyte	LLQ	Sample prep.	Further sample pretreatment (v/v)	Column (dimensions)	Mobile phase (v/v)	Detection (ionization/wavelength)	Val.	Remarks	Ref.
		N^2-OH-PhIP-*N*3-gluc 5 unidentified metab			Centrif. Concentrated.		NH_4OH (90:10)			Individual metab. peaks treated: β-glucuronidase, sulfatase, HCl	
M. inc	Human	PhIP	0.04	PP	M. inc terminated by addition of	μBondapack C18 (300 mm × 3.9 mm)	A: 0.1% DEA	UV (313 nm)		2-OH-PhIP prepared from hydrolysis of	[14,77]
		2-OH-PhIP	0.5		ice-cold MeOH. Centrif.		pH 4 by HAc			2-nitro-PhIP. Absorbance and flu detectors used in tandem	
		4′-OH-PhIP	0.05					Flu			
		N^2-OH-PhIP	1.5				B: MeOH	(Ex: 316 nm)		Contains excitation spectra of PhIP and its metabolites. Metabolism study	
		2-nitro-PhIP	Not sp.					(Em: 370 nm)			
		Unknown metabolite	Not sp.							of PhIP by CYP 1A1, 1A2 and 1B1	
Tissue (5–10 mg)	Rat	PhIP	Not sp.	PP	Milk: mixed with MeOH, mixed, centrif. Evap.	Econoshere C18 (100 mm × 4.6 mm)	A: 0.1% TFA	MS	No	$[^{14}C]/[^{13}C]$ ratio measured and	[40]
		4′-OH-PhIP		Converted to graphite	Recon in 0.1% TFA. LC fraction collection.		B: ACN	(ESI-QqQ)		normalized to $[^{14}C]/[^{12}C]$ ratio	
Blood (20 μL)		4′-OH-PhIP-sulfate			AMS measurements: tissue, blood, milk or HPLC fractions dried. Add: tributyrin.					of a carbon standard.	
Milk		N^2-OH-PhIP-*N*3-gluc				Zorbax C18 SB (150 mm × 1 mm)	A: 0.1% HAc	UV		Tissues: Liver and mammary gland	
(100–500 μL)					Converted to graphite.		B: ACN	(variable λ)		and stomach contents	
Urine (10 mL)	Human	PhIP	2.5 pg/mL	LLE	Ur: Hydrolysis: 1 M HCl, heating 2 h 100 °C.	Nova-Pak C18 (100 mm × 8 mm)	A: Not sp.	MS		Derivatization using HFBAA.	[46]
Meat (2 g)	Beef	4′-OH-PhIP		SPE	Neutralize (pH 6–7): 10 M NaOH. LLE: [79,80]			(EI-Q)			
					SPE: Blue Cotton. Elution: $MeOH/NH_4OH$. Evap.	Ultra-2 (25 m × 0.2 mm)	B: MeOH			LC combined with UV	
					Meat: Hom in 0.25 N HCl. LLE: DCM.			UV (320 nm)		GC combined with low and high resolution MS. Also includes:	
					Centrif. Alkalize. LLE: Et. ac.	CP-Sil 5 CB (25 m × 0.25 mm)					

					Evap. Recon: H_2O.					analysis of MeIQx and DiMeIQx.	
Bile (not sp.)	Dog	PhIP	Not sp.	LLE	M. inc: EtOH:phenol (99:1). Centrif. Recon in H_2O.	Supelco C18 (250 mm × 4.6 mm)	A: 20 mM DEA-Ac	MS		DNA conc. determined in DNA after	[45]
M. inc (not sp.)	Rat	N^2-OH-PhIP		PP	LLE: *n*-butanol and phenol respect. PP: EtOH.		buffer pH 5.0	(FAB-QqQ)		M. inc. by UV abs. DNA-associated	
Urine (not sp.)	Human	N^2-OH-PhIP-N^2-gluc		SPE	Bile and ur: Amberlite XAD-2. Elute: MeOH			UV (260 nm)		radioactivity determined by LSC.	
		N^2-OH-PhIP-$N3$-gluc			Concentrated. Eluting peaks collected and evap.		B: MeOH	LSC		Use of: [ring-^{3}H]PhIP and [2-^{14}C]PhIP	
Urine	Rat	PhIP	1 ng/g	LLE	Ur: dissolved in H_2O. Fa: lyophilized. Grounded.	HP1 fused silica (12 m × 0.2 mm)	Carrier gas: Not sp.	MS	No	Ur: aliquots representing 0.5% (24-, 48-	[58]
Faeces (see remarks)			5 ng/g		Hom. Dissolved: H_2O pH: 9–10 (Na_2CO_3)			(CI-Q)		and 72-h samples) or 2% (96-h samples). Faeces: 0.2% (24-h and 48-h 48-h samples). 0.8% (72-h and 96-h samples). BPFB derivatives.	
					(ur and fa). LLE (2×): Et. ac. Centrif.						
					LLE (2×): 0.1 M HCl. Add: 1 M Na_2CO_3.	(25 m × 0.2 mm)					
					LLE (2×): Et. ac. Centrif. Evap.						
Meat (2 g)	(See remarks)	PhIP	0.2 ng/g	LLE	Hom. in 0.25 M (meat) or	DB5 fused silica (15 m × 0.25 mm)	Carrier gas: helium	MS	No	Food: fried beef patties,	[59]
					0.5 M (foodstuff) HCl.			(CI-Q)		steal fatty bacon.	
Foodstuff (0.5 g)					Centrif. LLE (2×): DCM. Centrif. Alkalize					lean bacon. BBQd pork and chicken.	
					(Na_2CO_3). LLE (2×): Et. ac. Evap.					Beef stock-cube. Food grade beef	
					Recon in MeOH. Derivatization:					extract and condensed consommé.	
					3,5-Bis(trifluoromethyl)benzyl bromide					Includes: MeIQx/DiMeIQx	
Beer (30 mL)	Ten brands	PhIP	Not sp.	LLE	Condensed to 25 mL. Add: 1 M HCl. LLE (2×):	Asahipack ES-502C (100 mm × 7.6 mm)	20 mM $NH_4H_2PO_4$/	MS		MS: Direct insertion probe	[43]

Table 2 (*Continued*)

Matrix (volume)	Species	Analyte	LLQ	Sample prep.	Further sample pretreatment (v/v)	Column (dimensions)	Mobile phase (v/v)	Detection (ionization/wavelength)	Val.	Remarks	Ref.
Wine (30 mL)				SPE	DCM at pH ~ 10 by NH_4OH. Condensed partly. SPE: Bond Elut SI. Elute: 0.1 M HCl in MeOH. Evap. Recon: 20 mM $NH_4H_2PO_4$/20 mM H_3PO_4/ ACN (45:45:10)	LC ODS-300 (250 mm × 7.5 mm)	20 mM H_3PO_4/ACN (45:45:10) A: 10 mM H_3PO_4 B: ACN	(EI-double focusing) Flu (ex: 336 nm) (em: 388 nm)		Contains excitation and emission spectra of PhIP.	
Faeces (1 mg paste)	Mouse	[2-^{14}C]PhIP	Not sp.	Converted to graphite	Tissues: cut while frozen. Fecal pellets mixed with: H_2O/MeOH (1:1). Hom. urine, fecal paste and tissue slices: dried under vacuum. Converted to filamentous graphite.	–	–	AMS	No	First study of the bioavailability and fate of PhIP at a human equivalent dose. Tissue: fat, lung, liver, intestine, stomach, kidney, thymus, spleen, heart, muscle and pancreas. $[^{14}C]/[^{13}C]$ ratio measured + normalized to $[^{14}C]/[^{12}C]$ ratio of a carbon standard.	[41]
Tissue (5–20 mg)											
Urine (50–500 μL)											
Whole blood (50 μL)											
Meat (3 g)	Not sp.	PhIP	1 ng/g	SPE	Alkalize: 1 M NaOH. Extrelut column. Elute: DCM to Bond-elut PRS. Dried. Elution to C18 column with 0.5 M NH_4Ac. pH 8. Dried. Eluate containing apolar amines: add: NH_4OH and H_2O. Apply: Bond-Elut C18. Dried. Apolar amines eluted by MeOH:NH_4OH (9:1). Polar and apolar extracts: evap. Recon.	TSK-Gel ODS-80 (250 mm × 4.6 mm)	A: 0.01 M TEA pH 3.2 (H_3PO_4) B: 0.01 M TEA pH 3.6 (H_3PO_4) C: ACN	UV (spectra) Flu (ex: 315 nm) (em: 390 nm)		Extraction effiency determination of amines. LLQ reverts to fluorescence detection.	[44]
Fish (10 g)	Salmon										

Fried ground meat (116.5 kg)	Beef	PhIP	Not sp.	LLE	Patties formed. Fried. Outer part removed + chopped	PRP-1 S-DVB (350 mm × 20 mm)	A: 0.1% DEA in H_2O	MS	Isolation and identification of the new	[2]
				SPE	Divided. Hom. Add: 0.01 M HCl pH 2.0. Centrif. Supernatant saved. Pellet: washed in acid + centrif.		B: 0.1% DEA in ACN	(DIP-Q) (double focusing)	mutagen: PhIP	
					Combined supernatant: pH 7.0 by NaOH. SPE: XAD-2 Amberlite. Elute: acetone and MeOH. Evap. Dilute: H_2O. adjust pH: 2.0. LLE (3×) DCM.	PRP-1 (350 mm × 9.4 mm)	A: 0.1% DEA in H_2O B: 0.1% DEA in MeOH	^{1}H-NMR	Multiple purification steps on preperative LC with fraction collections.	
					Aq. phase: adjust pH to 7.0. SPE: XAD-2 column. Elution with acetone. Concentrated to small volume.	Nucleosil C18 (300 mm × 7.8 mm)	A: 0.1% DEA in H_2O B: 0.1% DEA in MeOH pH 6.0 by HAc	Salmonella assay		
						Lichrosorb C18 (250 mm × 4.6 mm)	A: 0.1% DEA in H_2O B: 0.1% DEA in MeOH pH 6.0 by HAc			
						Econsphere CN (250 mm × 4.6 mm)	25% MeOH in H_2O + 0.1% DEA pH 6.0			

^{1}H-NMR: proton-nuclear magnetic resonance; AαC: 2-amino-9*H*-pyrido[2,3-*b*]-indole; ACN: acetonitrile; AMS: accelerator mass spectrometry; BBQd: barbecued; BPFB: *bis*-(pentafluorobenzyl); BSA: bovine serum albumine; Centrif.: centrifugation; $CHCl_3$: chloroform; CI: chemical ionization; DCM: dichloromethane; DEA: diethylamine; DEP: direct exposure probe; DiMeIQx.: 2-amino-3,4,8-trimethylimidazo[4,5-*f*]quinoxaline; DIP: direct inlet probe; DMSO: dimethyl sulfoxide; EI: electron impact; Em: emission; ESI: electrospray ionization; Et.ac.: ethyl acetate; EtOH: ethanol; Evap.: evaporation; Ex: excitation; fa: faeces; FA: formic acid; FAB: fast atom bombardment; Flu: fluorescence; For: formiate; Gluc: glucuronide; H_2O: water; H_3PO_4: phosphoric acid; HAA: heterocyclic Aromatic Amine; HAc: acetic acid; HFBAA: heptafluorobutyric anhydride; Hom.: homogenization; I.D.: internal diameter; IT: ion trap; LLE: liquid–liquid extraction; LLQ: lower limit of quantification; LPME: liquid-phase microextraction; LSC: liquid scintillation counting; MeIQx: 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline; MeOH: methanol; Met.: metabolite; M. inc: microsomal incubate; Mob. Phase: mobile phase; MS: mass spectrometry; MTBE: methyl *tert*-butyl ether; NH_4Ac: ammonium acetate; NH_4OH: ammonium hydroxide; Not sp.: not specified; Part: partial; PhIP: 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine; PhIP-M1: 7-hydroxy-5-methyl-3-phenyl-6,7,8,9-tetrahydropyrido[3′,2′:4,5]imidazo[1,2-*α*]pyrimidin-5-ium chloride; pl: plasma; PP: protein precipitation; Q: single quadrupole; QqQ: triple quadrupole; Recon.: reconstitution; S-DVB: styrene-divinylbenzene; se: serum; SPE: solid phase extraction; TCA: trichloroacetic acid; TEA: triethylamine; TFA: trifluoroacetic acid; THF: tetrahydrofuran; ti: tissue; TLC: thin layer chromatography; TOF: time of flight; ur: urine; UV: ultraviolet.

be successful for the simultaneous extraction of both PhIP and its metabolites from complex matrices like biomatrices.

During sample pretreatment, incubation of biological matrices was effected with any of the following reagents: acid, β-glucuronidase, sulfatase or hydrazine hydrate to hydrolyse, e.g. glucuronides, sulfates and alcohols to their respective parent compound [11,34,35,46,49,54–57]. By analyzing the parental PhIP, N^2-OH-PhIP, 4′-OH-PhIP and 5-OH-PhIP concentration before and after hydrolysis, an estimation can be made at the concentration of the corresponding phase II metabolites. However, this provides no information on the position of the hydroxyl, sulfate or glucuronide group. Styczynski et al. demonstrated the use of β-glucuronidase to discriminate between the N^2- and *N*3-glucuronide of PhIP [27]. The former is a substrate for β-glucuronidase (from *Escherichia coli*) whereas the latter is not. H. Frandsen investigated the use of hydrazine hydrate for the hydrolysis of PhIP-glucuronides [11]. Incubation of PhIP-glucuronide with hydrazine hydrate resulted in the complete cleavage of the glucuronide moiety from the PhIP aglycone.

3. Chromatography

3.1. Liquid chromatography

Reversed phase chromatography was successfully applied in most reports on the analysis of PhIP and its metabolites. Sufficient selectivity for the baseline separation of PhIP metabolites is often required in both mass spectrometry and UV/Flu spectroscopy detection as some metabolites have the same precursor and product ions having the same *m*/*z* values and have overlapping absorption and emission regions. Analytical columns consisting of alkyl chain silica-bonded reversed phases were typically combined with eluent of an organic modifier (e.g. methanol or acetonitrile) and a formic or acetic acid buffer. Before the end of the 20th century ion pairing reagents like diethylamine (DEA), triethylamine (TEA) and trifluoroacetic acid (TFA) were often added to the mobile phase to enhance selectivity and resolution of the separation. One of the many contaminants present in the silica of older generation columns are metal ions. These ionic groups provide ion-exchange sides for ionized acids, resulting in tailing peaks for acidic solutes. Secondly, these metals enhance ionization of the silanol groups at high pH, thereby activating them after which they interact strongly as cation-exchange sites for ionized bases. Strong retention and tailing peaks are subsequently seen for basic solutes. The addition of ion-pairing reagents like DEA, TEA and TFA largely suppresses these problems. Analytical silica based reversed phase columns from recent dates consist of highly pure, metal-free silica. Using these columns, the addition of ion-pairing agents is needless and therefore MS compatible eluents can be used. This transition in eluent composition was clearly seen in reported analytical assays for the analyses of PhIP and its metabolites (Table 2). In the contrast of standard high-performance liquid chromatography, Chen et al. developed a rapid, high resolution ultra-performance liquid chromatography (UPLC) assay [13]. A 4.5 min run-time was used for the analysis of 17 PhIP metabolites using an Acquity BEH C18 column coupled to a quadrupole-time-of-flight mass spectrometer. Although UPLC offers significant theoretical advantages in resolution, speed, and sensitivity for analytical methods, particularly when coupled with mass spectrometers capable of high-speed acquisitions, so far only one article reported on the use of UPLC for the analysis of PhIP and its metabolites [13].

3.2. Gas chromatography and capillary electrophoresis

Neither PhIP nor its metabolites are volatile. Application of gas chromatography (GC) is therefore not obvious as it requires a labour intensive sample pretreatment consisting of derivatization to volatile derivatives. GC was used for the analysis of PhIP and its metabolites in urine, faeces and food products after conversion to their *bis*(pentafluorobenzyl) (BPFB) [55,57,58], heptafluorobutyric anhydride (HFBAA) [46] or di-bistrifluoromethylbenzyl (TFMB) [59] derivatives. Detection was performed using chemical ionization or electron impact ionization coupled to a single quadrupole mass spectrometer. Viberg et al. developed an on-line capillary-based quantitative assay for the analysis of PhIP in urine using an in-line extraction-based on Blue Chitin coupled to nano-electrospray ionization mass spectrometry [48].

4. Detection

4.1. Mass spectrometry

At the interface of the liquid chromatograph and the mass spectrometer liquid is converted to the gas phase. The preferred method of choice for the liquid–gas conversion in LC–MS analysis is electrospray ionization (ESI); a form of ionization where liquid containing the analytes is dispersed at atmospheric pressure by an electrospray into a fine aerosol, facilitating the liquid–gas conversion. Exceptions to the use of an ESI source are: fast atom bombardment (FAB) [45], chemical ionization (CI) [55,57–59] and electron impact (EI) ionization [43,46]. CI and EI are complementary ionization techniques used in gas chromatography coupled to mass spectrometry for the ionization of gases and volatile organic molecules [60]. EI leads to the (full) fragmentation of the molecular ion in contrast to CI which produces ions with less energy yielding spectra with less fragmentation in which the molecular ion can be identified. FAB is an ionization technique which is performed using a non-volatile liquid matrix. The matrix is bombarded under vacuum with a high energy beam of neutral atoms/molecules or ions. Like ESI, it is a relatively soft ionization technique and produces primarily intact protonated molecules [60]. FAB was used for the identification of N^2-OH-PhIP glucuronides formed by hepatic microsomes from human, dog and rat [45].

After ions are formed, they are subsequently transferred into the mass spectrometer and separated based on their mass to charge ratio (*m*/*z*). For the analyses of PhIP and its metabolites various techniques were used for the separation of ions, formed upon ionization: a single quadrupole mass spectrometer [47,56], a quadrupole-time-of-flight [13], a triple quadrupole [32,33,40,42,50,54,56,61–70], an ion trap [11,29,34,37,48,49,71–74], a double focussing instrument [43,72] and an accelerator mass spectrometer [41]. Low quantification limits (pg/g or pg/mL) were reached using mass spectrometry as a detection technique. The choice for a mass spectrometer depends strongly on the application of the analysis. Single or triple quadrupoles with or without a time-of-flight tube are typically used for quantitative analysis of PhIP metabolites. Ion trap mass spectrometry additionally allows acquisition of qualitative data by extensive fragmentation (MS^n) of ions. Fragmentation spectra can be used to elucidate the molecular structure of unknown metabolites. It was successfully used for the identification of urinary PhIP metabolites after meat consumption [29,34,48] or formed by intestinal microbiota in faeces [49,72]. Accelerator mass spectrometry (AMS) is a low energy nuclear physics technique that separates and directly counts the nuclei of long-lived isotopes. AMS measures the concentration of radio-isotopes, originating from, e.g. [^{14}C]PhIP relative to a stable isotope of the same element (e.g. [^{14}C]/[^{13}C] ratio measured and normalized to [^{14}C]/[^{12}C] ratio of a carbon standard). AMS is often used for sensitive, high resolution analysis of long-lived radio-isotopes.

A double focussing mass spectrometer is used for very high resolution analysis. It is a sector instrument in which ion beams are focused in both direction and velocity. The high resolution spec-

tra and accurate mass allow for the identification of unknown compounds, such as PhIP in 1986 [2] or a newly identified PhIP metabolite formed by intestinal microbiota [72].

To compensate for matrix effects during sample pretreatment and mass spectrometry detection a stable isotope labelled internal standard can be added. To the best of our knowledge, thus far, the only commercially available stable isotope labelled internal standard is PhIP deuterated at the methyl moiety (i.e. PhIP-d3). Deuterated forms of PhIP metabolites can be obtained by converting PhIP-d3 into N^2-OH-[$^2H_5$-phenyl]PhIP and subsequent incubation with, e.g. liver microsomes to obtain deuterated phase II metabolites of PhIP. Kulp et al. and Walters et al. generated the stable isotope labelled internal standards N^2-OH-[$^2H_5$-phenyl]PhIP-N^2-glucuronide and N^2-OH-[$^2H_5$-phenyl]PhIP-*N*3-glucuronide using this approach [29,75].

4.2. Ultraviolet and fluorescence

Ultraviolet and fluorescence detection require baseline separation for quantitation purposes. Total analysis time of an LC-UV/Flu assay is often long. In terms of reproducibility and robustness, ultraviolet and fluorescence detection have the advantage over mass spectrometry, however, methods are less sensitive and specific compared to MS methods. UV and Flu were used for quantification of PhIP and its metabolites in various matrices [14,38,39,44,76,77] or for identification purposes additionally to mass spectrometry analyses [33,40,43,45,46,68–70,72,74]. The minor influence of the addition of a hydroxyl group to the imidazole moiety, terminal amine or phenyl group on the UV absorbance is demonstrated in [77].

5. Conclusion

Since PhIP was first identified and isolated, numerous publications have reported on its presence in food, in biological matrices after food consumption, its activation and detoxification pathways and the formation of phase I and phase II metabolites. Analytical assays for the identification and quantification of these metabolites play a crucial role in understanding the bioavailability, distribution, excretion and toxicology of PhIP metabolites.

This review presents an overview of the analytical publications reporting on the analysis of PhIP and its phase I and phase II metabolites. Liquid chromatography coupled to mass spectrometry has been by far the method of choice for sensitive and selective identification and quantification of these metabolites. (Triple) quadrupoles have been used for quantitative analysis of known metabolites or double focussing and ion trap mass spectrometry for the identification of metabolites formed by, e.g. microsomal incubate. Surprisingly, only a limited number of the described assays have been fully validated according to FDA-guidelines [78]. This requires further attention in studies to come in the field of PhIP bioanalysis.

References

[1] A.M. Sanz, J.H. Ayala, V. Gonzalez, A.M. Afonso, J. Chromatogr. B: Analyt. Technol. Biomed. Life Sci. 862 (2008) 15.
[2] J.S. Felton, M.G. Knize, N.H. Shen, P.R. Lewis, B.D. Andresen, J. Happe, F.T. Hatch, Carcinogenesis 7 (1986) 1081.
[3] N.J. Gooderham, S. Creton, S.N. Lauber, H. Zhu, Toxicol. Lett. 168 (2007) 269.
[4] H. Esumi, H. Ohgaki, E. Kohzen, S. Takayama, T. Sugimura, Jpn. J. Cancer Res. 80 (1989) 1176.
[5] N. Ito, R. Hasegawa, K. Imaida, S. Tamano, A. Hagiwara, M. Hirose, T. Shirai, Mutat. Res. 376 (1997) 107.
[6] T. Shirai, M. Sano, S. Tamano, S. Takahashi, M. Hirose, M. Futakuchi, R. Hasegawa, K. Imaida, K. Matsumoto, K. Wakabayashi, T. Sugimura, N. Ito, Cancer Res. 57 (1997) 195.
[7] W. Zheng, D.R. Gustafson, R. Sinha, J.R. Cerhan, D. Moore, C.P. Hong, K.E. Anderson, L.H. Kushi, T.A. Sellers, A.R. Folsom, J. Natl. Cancer Inst. 90 (1998) 1724.
[8] M. Shioya, K. Wakabayashi, S. Sato, M. Nagao, T. Sugimura, Mutat. Res. 191 (1987) 133.
[9] M. Murkovic, J. Chromatogr. B: Analyt. Technol. Biomed. Life Sci. 802 (2004) 3.
[10] R. Reistad, H. Frandsen, S. Grivas, J. Alexander, Carcinogenesis 15 (1994) 2547.
[11] H. Frandsen, Food Chem. Toxicol. 45 (2007) 863.
[12] N.J. Gooderham, S. Murray, A.M. Lynch, M. Yadollahi-Farsani, K. Zhao, A.R. Boobis, D.S. Davies, Drug Metab. Dispos. 29 (2001) 529.
[13] C. Chen, X. Ma, M.A. Malfatti, K.W. Krausz, S. Kimura, J.S. Felton, J.R. Idle, F.J. Gonzalez, Chem. Res. Toxicol. 20 (2007) 531.
[14] F.G. Crofts, T.R. Sutter, P.T. Strickland, Carcinogenesis 19 (1998) 1969.
[15] C. Cheung, X. Ma, K.W. Krausz, S. Kimura, L. Feigenbaum, T.P. Dalton, D.W. Nebert, J.R. Idle, F.J. Gonzalez, Chem. Res. Toxicol. 18 (2005) 1471.
[16] H. Suzuki, J.S. Morris, Y. Li, M.A. Doll, D.W. Hein, J. Liu, L. Jiao, M.M. Hassan, R.S. Day, M.L. Bondy, J.L. Abbruzzese, D. Li, Carcinogenesis 29 (2008) 1184.
[17] R.F. Minchin, P.T. Reeves, C.H. Teitel, M.E. McManus, B. Mojarrabi, K.F. Ilett, F.F. Kadlubar, Biochem. Biophys. Res. Commun. 185 (1992) 839.
[18] M.A. Malfatti, M.H. Buonarati, K.W. Turteltaub, N.H. Shen, J.S. Felton, Chem. Res. Toxicol. 7 (1994) 139.
[19] H.C. Chou, N.P. Lang, F.F. Kadlubar, Cancer Res. 55 (1995) 525.
[20] H. Glatt, FASEB J. 11 (1997) 314.
[21] H. Glatt, Chem. Biol. Interact. 129 (2000) 141.
[22] E.L. Jamin, D. Arquier, C. Canlet, E. Rathahao, J. Tulliez, L. Debrauwer, J. Am. Soc. Mass Spectrom. 18 (2007) 2107.
[23] D. Wild, A. Dirr, Mutagenesis 4 (1989) 446.
[24] D. Lin, K.R. Kaderlik, R.J. Turesky, D.W. Miller, J.O. Lay Jr., F.F. Kadlubar, Chem. Res. Toxicol. 5 (1992) 691.
[25] M.A. Malfatti, J.S. Felton, Carcinogenesis 22 (2001) 1087.
[26] R.W. Dellinger, G. Chen, A.S. Blevins-Primeau, J. Krzeminski, S. Amin, P. Lazarus, Carcinogenesis 28 (2007) 2412.
[27] P.B. Styczynski, R.C. Blackmon, J.D. Groopman, T.W. Kensler, Chem. Res. Toxicol. 6 (1993) 846.
[28] M.A. Malfatti, E.A. Ubick, J.S. Felton, Carcinogenesis 26 (2005) 2019.
[29] K.S. Kulp, M.G. Knize, M.A. Malfatti, C.P. Salmon, J.S. Felton, Carcinogenesis 21 (2000) 2065.
[30] K.R. Kaderlik, R.F. Minchin, G.J. Mulder, K.F. Ilett, M. Daugaard-Jenson, C.H. Teitel, F.F. Kadlubar, Carcinogenesis 15 (1994) 1703.
[31] M.H. Buonarati, K.W. Turteltaub, N.H. Shen, J.S. Felton, Mutat. Res. 245 (1990) 185.
[32] J.M. Fede, A.P. Thakur, N.J. Gooderham, R.J. Turesky, Chem. Res. Toxicol. 22 (2009) 1096.
[33] S. Langouet, A. Paehler, D.H. Welti, N. Kerriguy, A. Guillouzo, R.J. Turesky, Carcinogenesis 23 (2002) 115.
[34] H. Frandsen, Food Chem. Toxicol. 46 (2008) 3200.
[35] J. Alexander, R. Reistad, H. Frandsen, S. Grivas, Mutat. Res. 376 (1997) 7.
[36] H. Frandsen, E.S. Rasmussen, P.A. Nielsen, P. Farmer, L. Dragsted, J.C. Larsen, Mutagenesis 6 (1991) 93.
[37] R. Busquets, J.A. Jonsson, H. Frandsen, L. Puignou, M.T. Galceran, K. Skog, Mol. Nutr. Food Res. 53 (2009) 1496.
[38] F.U. Shah, T. Barri, J.A. Jonsson, K. Skog, J. Chromatogr. B: Analyt. Technol. Biomed. Life Sci. 870 (2008) 203.
[39] J. Lezamiz, T. Barri, J.A. Jonsson, K. Skog, Anal. Bioanal. Chem. 390 (2008) 689.
[40] R.J. Mauthe, E.G. Snyderwine, A. Ghoshal, S.P. Freeman, K.W. Turteltaub, Carcinogenesis 19 (1998) 919.
[41] K.W. Turteltaub, J.S. Vogel, C.E. Frantz, N. Shen, Cancer Res. 52 (1992) 4682.
[42] R.J. Turesky, J. Taylor, L. Schnackenberg, J.P. Freeman, R.D. Holland, J. Agric. Food Chem. 20 (53) (2005) 3248.
[43] S. Manabe, H. Suzuki, O. Wada, A. Ueki, Carcinogenesis 14 (1993) 899.
[44] G.A. Gross, A. Gruter, J. Chromatogr. 592 (1992) 271.
[45] K.R. Kaderlik, G.J. Mulder, R.J. Turesky, N.P. Lang, C.H. Teitel, M.P. Chiarelli, F.F. Kadlubar, Carcinogenesis 15 (1994) 1695.
[46] R. Reistad, O.J. Rossland, K.J. Latva-Kala, T. Rasmussen, R. Vikse, G. Becher, J. Alexander, Food Chem. Toxicol. 35 (1997) 945.
[47] H. Hashimoto, T. Hanaoka, M. Kobayashi, S. Tsugane, J. Chromatogr. B: Analyt. Technol. Biomed. Life Sci. 803 (2004) 209.
[48] P. Viberg, K.G. Wahlund, K. Skog, J. Chromatogr. A 1133 (2006) 347.
[49] L. Vanhaecke, M.G. Knize, H. Noppe, H. De Brabander, W. Verstraete, W.T. Van de, Food Chem. Toxicol. 46 (2008) 140.
[50] W. Ni, L. McNaughton, D.M. LeMaster, R. Sinha, R.J. Turesky, J. Agric. Food Chem. 56 (2008) 68.
[51] H. Hayatsu, T. Oka, A. Wakata, Y. Ohara, T. Hayatsu, H. Kobayashi, S. Arimoto, Mutat. Res. 119 (1983) 233.
[52] F.G. Tamayo, E. Turiel, A. Martin-Esteban, J. Chromatogr. A 1152 (2007) 32.
[53] J.A. Jonsson, L. Mathiasson, J. Chromatogr. A 902 (2000) 205.
[54] D. Gu, L. McNaughton, D. Lemaster, B.G. Lake, N.J. Gooderham, F.F. Kadlubar, R.J. Turesky, Chem. Res. Toxicol. 19 (23) (2010) 788.
[55] M.D. Friesen, N. Rothman, P.T. Strickland, Cancer Lett. 173 (2001) 43.
[56] L.C. Kidd, W.G. Stillwell, M.C. Yu, J.S. Wishnok, P.L. Skipper, R.K. Ross, B.E. Henderson, S.R. Tannenbaum, Cancer Epidemiol. Biomarkers Prev. 8 (1999) 439.
[57] P.T. Strickland, Z. Qian, M.D. Friesen, N. Rothman, R. Sinha, Mutat. Res. 506–507 (163–73) (2002) 163.
[58] M.D. Friesen, L. Garren, J.C. Bereziat, F. Kadlubar, D. Lin, Environ. Health Perspect. 99 (179–81) (1993) 179.
[59] S. Murray, A.M. Lynch, M.G. Knize, M.J. Gooderham, J. Chromatogr. 616 (1993) 211.
[60] E. de Hoffmann, V. Stroobant, Mass Spectrometry—Principles and Applications, Wiley, 2007.

[61] S.F. Teunissen, M.L. Vlaming, H. Rosing, J.H. Schellens, A.H. Schinkel, J.H. Beijnen, J. Chromatogr. B: Analyt. Technol. Biomed. Life Sci. 878 (2010) 2353.
[62] E.E. Bessette, I. Yasa, D. Dunbar, L.R. Wilkens, L. Le Marchand, R.J. Turesky, Chem. Res. Toxicol. 22 (2009) 1454.
[63] X. Ma, J.R. Idle, M.A. Malfatti, K.W. Krausz, D.W. Nebert, C.S. Chen, J.S. Felton, D.J. Waxman, F.J. Gonzalez, Carcinogenesis 28 (2007) 732.
[64] F. Calbiani, M. Careri, L. Elviri, A. Mangia, I. Zagnoni, Food Addit. Contam. 24 (2007) 833.
[65] K.A. Scott, R.J. Turesky, B.C. Wainman, P.D. Josephy, Chem. Res. Toxicol. 20 (2007) 88.
[66] K.S. Kulp, M.G. Knize, N.D. Fowler, C.P. Salmon, J.S. Felton, J. Chromatogr. B: Analyt. Technol. Biomed. Life Sci. 802 (2004) 143.
[67] R.D. Holland, J. Taylor, L. Schoenbachler, R.C. Jones, J.P. Freeman, D.W. Miller, B.G. Lake, N.J. Gooderham, R.J. Turesky, Chem. Res. Toxicol. 17 (2004) 1121.
[68] L.S. DeBruin, P.A. Martos, P.D. Josephy, Chem. Res. Toxicol. 14 (2001) 1523.
[69] M.A. Malfatti, K.S. Kulp, M.G. Knize, C. Davis, J.P. Massengill, S. Williams, S. Nowell, S. MacLeod, K.H. Dingley, K.W. Turteltaub, N.P. Lang, J.S. Felton, Carcinogenesis 20 (1999) 705.
[70] N.P. Lang, S. Nowell, M.A. Malfatti, K.S. Kulp, M.G. Knize, C. Davis, J. Massengill, S. Williams, S. MacLeod, K.H. Dingley, J.S. Felton, K.W. Turteltaub, Cancer Lett. 143 (1999) 135.
[71] R. Busquets, L. Puignou, M.T. Galceran, K. Wakabayashi, K. Skog, J. Agric. Food Chem. 55 (2007) 9318.
[72] L. Vanhaecke, N. Van Hoof, W. Van Brabandt, B. Soenen, A. Heyerick, N. De Kimpe, D. De Keukeleire, W. Verstraete, W.T. Van de, J. Agric. Food Chem. 54 (2006) 3454.
[73] M.G. Knize, K.S. Kulp, M.A. Malfatti, C.P. Salmon, J.S. Felton, J. Chromatogr. A 914 (2001) 95.
[74] S. Prabhu, M.J. Lee, W.Y. Hu, B. Winnik, I. Yang, B. Buckley, J.Y. Hong, Anal. Biochem. 298 (2001) 306.
[75] D.G. Walters, P.J. Young, C. Agus, M.G. Knize, A.R. Boobis, N.J. Gooderham, B.G. Lake, Carcinogenesis 25 (2004) 1659.
[76] F. Toribio, L. Puignou, M.T. Galceran, J. Chromatogr. A 836 (1999) 223.
[77] F.G. Crofts, P.T. Strickland, C.L. Hayes, T.R. Sutter, Carcinogenesis 18 (1997) 1793.
[78] U.S. Food and Drug Administration - Center for Drug Evaluation and Research - Guidance for Industry Bioanalytical Method Validation, http://www.fda.gov/CDER/GUIDANCE/4252fnl.pdf, 2008.
[79] S. Murray, N.J. Gooderham, A.R. Boobis, D.S. Davies, Carcinogenesis 10 (1989) 763.
[80] S. Murray, N.J. Gooderham, A.R. Boobis, D.S. Davies, Carcinogenesis 9 (1988) 321.