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REVIEW ARTICLE

Urinary biomarkers of smokers' exposure to tobacco smoke constituents in tobacco products assessment: a fit for purpose approach

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Abstract

There are established guidelines for bioanalytical assay validation and qualification of biomarkers. In this review, they were applied to a panel of urinary biomarkers of tobacco smoke exposure as part of a ''fit for purpose'' approach to the assessment of smoke constituents exposure in groups of tobacco product smokers. Clinical studies have allowed the identification of a group of tobacco exposure biomarkers demonstrating a good doseresponse relationship whilst others such as dihydroxybutyl mercapturic acid and 2-carboxy-1-methylethylmercapturic acid – did not reproducibly discriminate smokers and non-smokers. Furthermore, there are currently no agreed common reference standards to measure absolute concentrations and few inter-laboratory trials have been performed to establish consensus values for interim standards. Thus, we also discuss in this review additional requirements for the generation of robust data on urinary biomarkers, including toxicant metabolism and disposition, method validation and qualification for use in tobacco products comparison studies.

Keywords

Biomarker qualification, biomarker validation, comparative studies, tobacco products assessment

History

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Introduction

One of the most effective ways to estimate smokers' exposure to tobacco smoke constituents is the measurement of biomarkers, which has the advantage of estimating integrated exposure over a period of time, without having to take into account smoking behaviour or counting the number of tobacco articles smoked (Gregg et al., 2006; Hatsukami et al., 2003; Scherer, 1999). Body fluids such as blood (Foulds et al., 1992), saliva (Jarvis et al., 1992) and excreted products such as exhaled breath (Wald et al., 1981) and urine (Carmella et al., 1997) have been used to measure biomarkers of smoke exposure but the collection of urine is potentially the least invasive of these approaches. Further, sufficient urine sample may be collected on a frequent basis, allowing typical analytical assay validation, including linearity, accuracy, precision, repeatability and reproducibility of measurements, to be conducted.

After the publication of the report from the Institute of Medicine (IOM) on tobacco harm reduction (Institute of Medicine, 2001), there has been resurgence in interest in the measurement of biomarkers obtained from smokers. Additionally, the World Health Organization (WHO) Study Group on Tobacco and Product Regulation (TobReg) suggested that the yields of some smoke toxicants should be regulated and lowered (Burns et al., 2008). More recently, Hecht and colleagues discussed the use of tobacco toxicant biomarkers for potential product regulation and cancer prevention and they concluded that ''the methods are now sufficiently routine that their application in large studies is feasible'' (Hecht et al., 2010). This conclusion has received some endorsement for a more recent IOM report concerning scientific standards for evaluating modified risk tobacco products (Institute of Medicine, 2012).

Along with chemical characterization of the product and estimation of the yield of toxicants from the product in use, measurement of biomarkers of exposure to tobacco toxicants will play an important role in the evaluation of any new types of tobacco product aimed at reducing smokers' exposure to toxicants (Ashley et al., 2007; Hatsukami et al., 2006; Institute of Medicine, 2001). The IOM originally introduced the term ''potential reduced-exposure product'' (PREP) for such products (Institute of Medicine, 2001) and, more recently, the US Food and Drug Administration (FDA) and also the IOM have used ''modified risk tobacco product'' (MRTP) to describe them (Institute of Medicine, 2012; O'Connor, 2012). Throughout this article, the term ''reduced toxicant prototype'' (RTP) is used to designate novel products that are being evaluated because it is recognized that claims about the potential for risk modification cannot be made until the actual human exposure to toxicants from these products and long-term data from their use in populations becomes available. Partial data informing such an assessment may be obtained by using suitable biomarkers

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of exposure. Biomarkers other than exposure (effect, potential harm, risk, susceptibility, etc.) are not considered in this review.

In other non-tobacco use situations, guidelines for the evaluation of biological measurements, e.g. those of the FDA (Food and Drug Administration, 2001) or the International Organization for Standardization (ISO) (International Organization for Standardization, 2005, 2007), are applied before their widespread use in diagnostic or clinical settings, and so it is of interest to apply existing guidelines to tobacco exposure biomarkers. This article reviews the application of urinary biomarkers in comparisons between groups of smokers of different products and groups of non-smokers. Monitoring environmental exposure to tobacco smoke is not considered. To make these comparisons, currently available data on urinary biomarkers of exposure to tobacco smoke toxicants, taken from the lists published by WHO TobReg (Burns et al., 2008) and Hecht and colleagues (Hecht et al., 2010), is reviewed. The alignment of these data with existing guidelines is summarized and, when available, the key data are presented to demonstrate how well each criterion is met and to show where expected data are not available. Assuming the purpose of measuring biomarkers is to evaluate groups of smokers of RTPs in comparison with conventional cigarette smokers and with non-smokers, this comparative approach allows biomarker validation, qualification and ''fitness for purpose'' to be assessed. Any gaps in the data are highlighted as high-priority activities for tobacco biomarkers research, and for RTP assessment. Completion of these activities would enhance science-based manufacturing stewardship and regulatory scrutiny of RTPs.

Methods

A list of urinary biomarkers for smoke constituents taken from those provided by TobReg (Burns et al., 2008) and by Hecht and colleagues (2010) was compiled and assessed against general guidelines on biomarker and bioanalysis: those outlined by the FDA (Food and Drug Administration, 2001), ISO (International Organization for Standardization, 2005, 2007), the IOM (Institute of Medicine, 2010), Scherer (2005) and Chau & colleagues (2008). The approach taken was to gather information on the analytical techniques from recently published studies and to cross-check these data against the guidelines. For most biomarkers examined the recently applied analytical techniques typically use gas chromatography (GC) or liquid chromatography (LC) followed by mass spectrometry (MS) or tandem mass spectrometry (MS/MS). Thus, the literature reviewed was not exhaustive and was based on these recent publications and those cited within the biomarker list publications that used the same techniques for biomarker analysis. For each potential biomarker, the method of analysis, limit of detection (LOD), lower limit of quantification (LLOQ), precision, accuracy, recovery and sample stability under assay and storage conditions were recorded (Food and Drug Administration, 2001). This subset of the published guidelines is highlighted because, if this information is not available, it is unlikely that other data such as upper limit of quantification and assay linearity could be determined from the published literature. Thus, any laboratory wishing to use the biomarker might expect more difficulty in validating the assay before performing a study. Furthermore, in the absence of a standard reference material, inter-laboratory comparisons are required to assign absolute values to specific analyte measurements (International Organization for Standardization, 2005). Unless reference standards or a consensus value for a standard has been assigned by appropriate inter-laboratory comparisons, then biomarker values should only be used with caution; for example, in within laboratory relative comparisons rather than absolute value assignment or after taking into consideration the methods, size, design and overall quality of the studies being compared.

Assay validation is a necessary but not sufficient step to determine whether a biomarker is qualified for use in particular circumstances. For biomarker qualification, an approach has been outlined by others to assess overall ''fitness for purpose'' in a pharmaceutical environment (Chau et al., 2008; Lee et al., 2006). In a tobacco context, several distinct uses of biomarkers for RTP evaluation can be envisaged: (1) a small study of short duration (up to a few days) in which RTP users are directly compared with conventional cigarette smokers under conditions of clinical confinement and all other variables (diet, etc.) are controlled; (2) short-term evaluation (1–12 weeks) of RTP users compared to conventional cigarette smokers and to non-smokers, with periodic episodes of clinical confinement; (3) long-term assessment (>12 weeks) of RTP users compared to conventional cigarette smokers and to non-smokers, with periodic episodes of clinical confinement; (4) cross-sectional and population studies of several groups including RTP users. The specific requirement to qualify a biomarker as fit for purpose would be different in each set of circumstances and depends on the objectives of the study. For example, biomarkers of exposure to smoke constituents with other known dietary or environmental sources could be suitable for use under controlled conditions, like those in clinical confinement, but may be unsuitable in study designs where such variables are not controlled, such as cross-sectional studies.

A comparison of specific biomarker concentrations in smokers and non-smokers should give the greatest magnitude of change that might be found in an RTP study, and could be used for power calculations. However, if a smoking cessation study has been conducted and the biomarker evaluated, then both a practical degree of change and the kinetics of that change can be used to inform study design. Further information about a biomarker's performance can be obtained from product switching studies in which individuals or groups have experimentally changed their tobacco product use for defined periods of time. All such data were sought for the biomarkers included in this review.

Other biological considerations may affect biomarker performance and influence the practical aspects of a study design; e.g. diurnal variation in enzyme activity may affect smoke constituent metabolism and the biomarker elimination half-life. This could be of importance in studies where an early morning spot urine sample, but not the first void, is collected for a biomarker with a short elimination half-life. Thus, a combination of all of these properties, and the

objectives of a study, should be considered to ascribe overall fitness for intended purpose.

Results

The data gathered on a series of urinary biomarkers of exposure to tobacco smoke constituents are summarized in Tables 1–3. In Table 1, features of the analytical techniques (limits of detection and quantification, accuracy and precision) and sample handling, namely stability on storage and freezing, are presented. In Table 2 the reported ranges in groups of smokers and non-smokers and other characteristics that address expected uncertainty in these measurements are shown. Typically, at least a 2-fold change in biomarker concentrations between groups of smokers and non-smokers would be expected for practical application. However, this level of difference can be affected by the design of the study (controlled versus non-controlled), the study setting (clinical confinement versus unrestricted subject movement) and the reproducibility characteristics of the assay over the short and long term. In some circumstances, a difference of less than 2-fold might be suitable; whereas, for uncontrolled, cross-sectional studies a difference of 5- or even 10-fold might be required. Other parameters that can affect the interpretation of biomarker data are captured in Table 3 and, together, these data are all used in determining fitness for purpose. This approach was not intended to capture every urinary biomarker assay that has been performed for smoke constituent exposure but to focus on the types of assays typically being used by current bioanalytical laboratories, often using chromatography followed by MS or MS/MS. For ease of reading, the tables are presented in the same general format and order, with the first column listing the smoke constituent and the second column the relevant biomarkers. When data are available, a summary value from the published study is given along with a reference to that publication. Blank cells indicate that relevant data were not found in the published literature. There is no definitive manner in which to split these data and an overall judgment about a specific biomarker requires data from all tables, as well as a consideration of the intended study application.

Tobacco-specific nitrosamines (TSNAs) are a group of N′-nitrosamines derived from tobacco alkaloids such as nicotine, nornicotine, anabasine and anatabine during the curing and processing of tobacco as well as during the pyrrolysis process (Scherer & Richter, 1997; Stepanov & Hecht, 2005). There are few or no other known sources. There are four main TSNAs, 4-(methylnitrosoamino)-1-(3-pyridyl)-1-butanone (NNK), N-nitrosonornicotine (NNN), N-nitrosoanabasine (NAB) and N-nitrosoanatabine (NAT). From the literature the most widely studied of these is 4-(methylnitrosoamino)-1-(3-pyridyl)-1-butanone (NNK). The biomarker of choice to measure NNK exposure is total urinary 4-(methylnitrosoamino)-1-(3-pyridyl)-1-butanal (NNAL), for which assay validation data (Bhat et al., 2011; Church et al., 2010a; Kavvadias et al., 2009b; Shah et al., 2009; Xia et al., 2005; Yuan et al., 2009), ranges in smokers and non-smokers (Anderson et al., 2003; Carmella et al., 1997; Hecht et al., 1999, 2010; Meger et al., 2000), use in short- and long-term studies (Sarkar et al., 2008), and details of mass balance, half-life and use in product comparison and smoking cessation studies (Carmella et al., 2009; Goniewicz et al., 2009; Kavvadias et al., 2009a; Sarkar et al., 2008), are available. The only criterion that does not appear to have been met for NNAL is that of a formal, published, inter-laboratory comparison, which would be required to set a consensus value on a suitable reference material, in the absence of a reference standard.

The biomarkers for exposure to other TSNAs, N′-nitrosonornicotine (NNN), N′-nitrosoanabasine (NAB) and N′-nitrosoanatabine (NAT) are not as well-characterized as NNAL but assay validation data (Kavvadias et al., 2009b; Stepanov & Hecht, 2005) and ranges in smokers and non-smokers are available (Kavvadias et al., 2009b; Sarkar et al., 2008; Stepanov & Hecht, 2005) as well as some smoking product switching studies, which give comparative data using the same laboratory for analysis (Sarkar et al., 2008). However, data from long-term studies with product switching or smoking cessation, the elimination half-life in humans and inter-laboratory comparisons were not found.

Polycyclic aromatic hydrocarbon (PAHs) are chemically diverse and they are formed during the incomplete combustion of organic materials, such as tobacco. They are found in ambient air, cooked foods, and in numerous occupational settings. For PAH exposure, two biomarkers are widely used in smoking studies: 1-hydroxypyrene (1-OHP) and 3-hydroxybenzo[a]pyrene (3-OHBaP). Assay validation data are available (Carmella et al., 2004; Feng et al., 2006; Jongeneelen et al., 1986; Lafontaine et al., 2006; Scherer et al., 2007a; Suwan-ampai et al., 2009), but the qualification data for these biomarkers is equivocal. Both 1-OHP and 3-OHBaP give approximately a twofold difference between smokers and non-smokers in some studies (Lindner et al., 2011; Sarkar et al., 2010), but the ranges of values for smokers and non-smokers overlapped in other studies (Lafontaine et al., 2006; Scherer et al., 2007a; Suwan-ampai et al., 2009). For 1-OHP there was variability in the longer term data (Carmella et al., 2004), but this was not found in a more recent study of smokers over a 6-month follow up (Sarkar et al., 2008). Also for 1-OHP, a reduction in mean values in groups using an electrically heated cigarette compared to a group continuing to smoke conventional cigarettes was reported (Feng et al., 2006; Frost-Pineda et al., 2008a, b; Roethig et al., 2007), but another study did not report an alteration in mean values on smoking cessation (Carmella et al., 2009). For 3-OHBaP a reduction on switching from conventional cigarettes to snus oral tobacco consumption and on smoking cessation was observed (Sarkar et al., 2010), but long-term data comparisons in tobacco product switching or smoking cessation studies and other properties like urinary accumulation kinetics were not found.

Aromatic amines, also known as arylamines, are usually encountered industrially in the manufacture of dyes but also in rubber processing and pesticide production and biomarkers of exposure to aromatic amines have been widely studied in occupational settings, but less so in tobacco exposure studies. Biomarker assays based on their measurement in urine samples are available, along with precision, accuracy, recovery and stability in sample matrix (Grimmer et al., 2000; Riedel et al., 2006; Weiss & Angerer, 2002). Differences in

Table 1. Smoke constituents and their biomarkers in urine: assay validation parameters summary.

Smoke constituent	Biomarker	Analytical technique*	Limit of detection [in matrix] (Ref)	Lower limit of quantification (Ref)	Storage studies in matrix temp. duration (Ref)	Precision‡ (Ref)	Accuracy (Ref)	Recovery (Ref)
NNK†	Total NNAL†	GC-TEA	0.04 pmol/ml [8.4 pg/ml] (Yuan et al., 2009) 0.1 pmol/ml [20.9 pg/ml] (Church et al., 2010a)		−20 °C 4 years (Yuan et al., 2009)	10.9% intra-day RSD (Yuan et al., 2009) 6.4% (Church et al., 2010a)	>95% (Church et al., 2010a)	
		LC-MS/MS	2.0 pg/ml (Xia et al., 2005), (Kavvadias et al., 2009b)	5.0 pg/ml (Kavvadias et al., 2009b) 20.0 pg/ml (Bhat et al., 2011)	21 °C 24 h, 10 °C 6 days, −20 °C 8 months (Kavvadias et al., 2009b) Freeze–thaw 3 cycles (Kavvadias et al., 2009b)	6.0–11.9% (Kavvadias et al., 2009b)	94.1–103.0% (Bhat et al., 2011) 100.4–110.7% (Kavvadias et al., 2009b)	30.3–31.7% (Shah et al., 2009) 32.1–45.5% (Kavvadias et al., 2009b)
NNN†	NNN	GC-TEA	5.7 pg/ml (Stepanov & Hecht, 2005)					
		LC-MS/MS	0.8 pg/ml (Kavvadias et al., 2009b)	2.0 pg/ml (Kavvadias et al., 2009b)	21 °C 24 h, 10 °C 6 days, −20 °C 8 months (Kavvadias et al., 2009b) Freeze–thaw 3 cycles (Kavvadias et al., 2009b)	1.9–4.4% (Kavvadias et al., 2009b)	90.3–91.1% (Kavvadias et al., 2009b)	37.9–56.8% (Kavvadias et al., 2009b)
NAT†	Total NAT	GC-TEA	2.65 pg/ml (Stepanov & Hecht, 2005)					
		LC-MS/MS	0.7 pg/ml (Kavvadias et al., 2009b)	2.0 pg/ml (Kavvadias et al., 2009b)	21 °C 24 h, 10 °C 6 days, −20 °C 8 months (Kavvadias et al., 2009b) Freeze–thaw 3 cycles (Kavvadias et al., 2009b)	1.4–13.2% (Kavvadias et al., 2009b)	93.5–103.9% (Kavvadias et al., 2009b)	46.3–77.4% (Kavvadias et al., 2009b)
NAB†	Total NAB	GC-TEA	3.44 pg/ml (Stepanov & Hecht, 2005)					
		LC-MS/MS	1.1 pg/ml (Kavvadias et al., 2009b)	5.0 pg/ml (Kavvadias et al., 2009b)	21 °C 24 h, 10 °C 6 days, − 20 °C 8 months (Kavvadias et al., 2009b) Freeze–thaw 3 cycles (Kavvadias et al., 2009b)	1.1–11.7% (Kavvadias et al., 2009b)	88.2–97.2% (Kavvadias et al., 2009b)	42.0–76.3% (Kavvadias et al., 2009b)
Pyrene	1-OHP†	HPLC-FD	5 pg/ml (Lafontaine et al., 2006) 0.05 pmol/ml (Carmella et al., 2004)		−20 °C >12 month (Feng et al., 2006)	4.6% (Feng et al., 2006) 4.2% 30 replicates RSD (Carmella et al., 2004)	88.6–91.7% (Feng et al., 2006) 90% (Scherer et al., 2007a)	50% (Carmella et al., 2004)
		GC-MS	2.0–3.3 pg/ml (Suwan-ampai et al., 2009)			1. 5–5.7% (n > 2500) (Suwan-ampai et al., 2009)		
Benzo[a]pyrene	3-OHBaP†	HPLC	134 pg/ml (Jongeneelen et al., 1986)					
		HPLC-FD	0.05 pg/ml (Lafontaine et al., 2006)					
		GC-MS	10.5 pg/ml (Suwan-ampai et al., 2009)			2.4–7.9% Inter-assay CoV (n > 2500) (Suwan-ampai et al., 2009)		
		LC-MS/MS		50 fg/ml (Sarkar et al., 2010)		3.6–10.9% Inter-day CoV (Sarkar et al., 2010)	96.4–100.2% (Sarkar et al., 2010)	
Aromatic amines	1-AN	GC-MS	0.05 pg/ml (Grimmer et al., 2000) 150 pg/ml (Weiss & Angerer, 2002)		stable on >1 freeze-thaw cycles (Weiss & Angerer, 2002)	7.9% (Grimmer et al., 2000) 2.8–8.2% Within series RSD (Weiss & Angerer, 2002)	70–90% (Weiss & Angerer, 2002)	93.9% (Grimmer et al., 2000)
	2-AN	GC-MS	0.05 pg/ml (Grimmer et al., 2000) 75 pg/ml (Weiss & Angerer, 2002) 4 pg/ml (Riedel et al., 2006)		stable on >1 freeze-thaw cycles (Weiss & Angerer, 2002)	6.8% (Grimmer et al., 2000) 2.6–6.3% Within series RSD (Weiss & Angerer, 2002)	90% (Weiss & Angerer, 2002)	92.5% (Grimmer et al., 2000)
	4-ABP	GC-MS	0.05 pg/ml (Grimmer et al., 2000) 50 pg/ml (Weiss & Angerer, 2002) 4 pg/ml (Riedel et al., 2006)		stable on >1 freeze–thaw cycles (Weiss & Angerer, 2002)	3.3% (Grimmer et al., 2000) 2.7–5.9% Within series RSD (Weiss & Angerer, 2002)	100–108% (Weiss & Angerer, 2002)	96.8% (Grimmer et al., 2000)
		GC-MS/MS		0.9 pg/ml (Seyler & Bernert, 2011)				
	o-tol	GC-MS	50 pg/ml (Weiss & Angerer, 2002) 1 pg/ml (Riedel et al., 2006)		stable on >1 freeze-thaw cycles (Weiss & Angerer, 2002)	7.0–7.3% Within series RSD (Weiss & Angerer, 2002)	95–106% (Weiss & Angerer, 2002)	90% (Weiss & Angerer, 2002)
1,3-Butadiene	DHBMA†	GC-MS/MS	5 ng/ml (van Sittert et al., 2000)					

		LC-MS/MS	76 ng/ml (Urban et al., 2003)	10 ng/ml (Schettgen et al., 2009)	−24 °C 9 months (Urban et al., 2003)			
		HPLC-MS/MS	12 pmol/ml (Carmella et al., 2009) 0.14 ng/ml (Ding et al., 2009)		10 Freeze–thaw cycles (Ding et al., 2009)	8.7% (Carmella et al., 2009) 3.5–5.3% (Ding et al., 2009)	109% (Carmella et al., 2009) 103% (Ding et al., 2009)	
	MHBMA†	GC-MS/MS	0.1 ng/ml (van Sittert et al., 2000)					
		LC-MS/MS	2.7 ng/ml (Urban et al., 2003)	2.0 ng/ml (Schettgen et al., 2009)	−24 °C 9 months (Urban et al., 2003)			
		HPLC-MS/MS	3.2 pmol/ml (Carmella et al., 2009) 0.05 ng/ml (Ding et al., 2009)		10 Freeze–thaw cycles (Ding et al., 2009)	16.0% (Carmella et al., 2009) 1.7–2.5% (Ding et al., 2009)	58.9% (Carmella et al., 2009) 100–106% (Ding et al., 2009)	
	1-MHBMA	UPLC-HILIC-MS/MS	0.05 ng/ml (Sterz et al., 2012)	0.15 ng/ml (Sterz et al., 2012)		2.7-6–1% (Sterz et al., 2012)	98–103% (Sterz et al., 2012)	
	2-MHBMA	UPLC-HILIC-MS/MS	0.24 ng/ml (Sterz et al., 2012)	0.72 ng/ml (Sterz et al., 2012)		3.9–7.4%(Sterz et al., 2012)	85.3–107% (Sterz et al., 2012)	
	THBMA†	HPLC-MS/MS	<0.1 ng/ml (Kotapati et al., 2011)	1.0 ng/ml (Kotapati et al., 2011)	3 Freeze–thaw cycles (Kotapati et al., 2011)	0.9–6.5% Intraday RSD (Kotapati et al., 2011)	96.9–113% (Kotapati et al., 2011)	54.5% (Kotapati et al., 2011)
Benzene	tt-MA†	GC-MS	3 ng/ml (Scherer et al., 2007a), (Ruppert et al., 1995)		−20 °C >12 month (Feng et al., 2006)	3% (Feng et al., 2006)	92% (Scherer et al., 2007a)	83–92% (Feng et al., 2006)
	SPMA†	GC-MS	0.03 pg/ml (Kim et al., 2006)					
		HPLC-MS/MS	0.03 ng/ml (Scherer et al., 2007a) 0.013 pmol/ml (Carmella et al., 2009) 0.01 ng/ml (Feng et al., 2006) (Ding et al., 2009)		−20 °C >12 month (Feng et al., 2006) 10 Freeze–thaw cycles (Ding et al., 2009)	<5% (Scherer et al., 2007a) 7.6% (Carmella et al., 2009) 2.8–7.5% (Ding et al., 2009)	96.2% (Carmella et al., 2009) 101–103% (Ding et al., 2009)	97.6–100.4% (Feng et al., 2006)
Catechol	catechol	GC-MS	< matrix background (Kerzic et al., 2010), (Waidyanatha et al., 2004)	< matrix background (Kerzic et al., 2010), (Waidyanatha et al., 2004)		10.5% (urine) (Waidyanatha et al., 2004) 4.6 % (serum) (Kerzic et al., 2010)		
Acrolein	3-HPMA†	LC-MS/MS	50 ng/ml (Mascher et al., 2001)	0.9 ng/ml (Carmella et al., 2007)		1.6% Six measurements RSD (Carmella et al., 2007)	125% (Carmella et al., 2007)	Circa 40% (Carmella et al., 2007)
		LC-MS/MS	6.0 ng/ml (Scherer et al., 2007a)			<2.5% (Scherer et al., 2007a)		
		HPLC-MS/MS	2.3 pmol/ml (Carmella et al., 2009) 0.2 ng/ml (Ding et al., 2009)		10 Freeze–thaw cycles (Ding et al., 2009)	9.4% (Carmella et al., 2009) 2.6–3.0% (Ding et al., 2009)	100% (Carmella et al., 2009) 95–109% (Ding et al., 2009)	
Crotonaldehyde	HMPMA†	LC-MS/MS	28 ng/ml (Scherer et al., 2007b)	92 ng/ml (Scherer et al., 2007b)		1.8–3.5% (Scherer et al., 2007b)	105–8% (Scherer et al., 2007b)	
	CMEMA†	LC-MS/MS	32 ng/ml (Scherer et al., 2007b)	104 ng/ml (Scherer et al., 2007b)		4.6–7.3% (Scherer et al., 2007b)	101–2% (Scherer et al., 2007b)	
Acetaldehyde	Mainly DNA adducts							
Formaldehyde	Mainly DNA adducts							
Ethylene Oxide	HEMA†	HPLC-MS/MS	0.024 pmol/ml (Carmella et al., 2009) 0.03 ng/ml (Ding et al., 2009)		10 Freeze–thaw cycles (Ding et al., 2009)	12.1% (Carmella et al., 2009) 3.1–3.6% (Ding et al., 2009)	93.4% (Carmella et al., 2009) 103% (Ding et al., 2009)	
Acrylamide	Acrylamide	HPLC-MS		0.5 ng/ml (Fuhr et al., 2006)				
	Glycidamide	HPLC-MS		2.5 ng/ml (Fuhr et al., 2006)				
	AAMA†	HPLC-MS/MS	0.8 ng/ml (Urban et al., 2006)	5 ng/ml (Fuhr et al., 2006)		1.3–1.6% (Urban et al., 2006)	98.2–99.5% (Urban et al., 2006)	83.5% (Urban et al., 2006)
	GAMA†	HPLC-MS/MS	0.5 ng/ml (Urban et al., 2006)	5 ng/ml (Fuhr et al., 2006)		0.8–1.3% (Urban et al., 2006)	80.0–96.3% (Urban et al., 2006)	87.2% (Urban et al., 2006)
Acrylonitrile	CEMA†	LC-MS/MS	0.5 ng/ml (Schettgen et al., 2009) 0.06 ng/ml (Minet et al., 2011a)	1.0 ng/ml (Schettgen et al., 2009) 0.17 ng/ml (Minet et al., 2011a)		4.9% Intraday RSD (Schettgen et al., 2009) 2.0–2.6% (Minet et al., 2011a)	94–109% (Schettgen et al., 2009) 92–105% (Minet et al., 2011a)	110% (Schettgen et al., 2009)
Cadmium	Cadmium	ICP-MS	0.01 ng/ml (Huang & Yang, 1997)					

(*continued*)

Table 1. Continued

Smoke constituent	Biomarker	Analytical technique*	Limit of detection [in matrix] (Ref)	Lower limit of quantification (Ref)	Storage studies in matrix temp. duration (Ref)	Precision‡ (Ref)	Accuracy (Ref)	Recovery (Ref)
		AAS	0.1 ng/ml (Paschal et al., 2000) 0.05 ng/ml (Hoffmann et al., 2000)			1–18% relative measurement error (Hoffmann et al., 2000)	100 ± 5% (Paschal et al., 2000) Between labs <10% difference (Paschal et al., 2000)	

†Abbreviations: 1-AN, 1-aminonaphthalene; 1-OHP, 1-hydroxypyrene; 2-AN, 2-aminonaphthalene; 3-OHBaP, 3-hydroxy-benzo[a]pyrene; 3-HPMA, 3-hydroxypropylmercapturic acid; 4-ABP, 4-aminobiphenyl; AAMA, N-acetyl-S-(2-carbamoylethyl)-L-cysteine; CEMA, N-acetyl-S-(2-carboxyethyl)-L-cysteine; CMEMA, 2-carboxy-1-methylethylmercapturic acid; DHBMA, dihydroxybutyl mercapturic acid; GAMA, N-(R,S)-acetyl-S-(2-carbamoyl-2-hydroxyethyl)-L-cysteine; HEMA, N-acetyl-S-(2-hydroxyethyl)-L-cysteine; HMPMA, 3-hydroxyl-methylpropylmercapturic acid; MHBMA, monohydroxybutenyl mercapturic acid; NAB, nitrosoanabasine; NAT, nitrosoanatabine; NNAL, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol; NNK, 4-(methylnitrosoamino)-1-(3-pyridyl)-1-butanone; SPMA, S-phenylmercapturic acid; THBMA, trihydroxybutyl mercapturic acid; tt-MA, *trans,trans*-muconic acid; Total NAB, NAB + NAB-N-glucuronide measured after sample glucuronidase treatment; Total NAT, NAT + NAT-N-glucuronide, measured after sample glucuronidase treatment; Total NNAL, NNAL + NNAL-glucuronide measured after sample glucuronidase treatment.

*Techniques: AAS, atomic absorption spectroscopy; FD, fluorescence detection; GC, gas chromatography; HILIC, hydrophilic interaction chromatography; HPLC, high-performance liquid chromatography; ICP, inductively coupled plasma; LC, liquid chromatography; MS, mass spectrometry; MS/MS, tandem mass spectrometry; TEA, thermal energy analysis; UPLC, ultra-performance liquid chromatography.

‡Precision (intra-day coefficient of variation, unless stated) and Accuracy are as defined by the FDA (Food and Drug Administration, 2001) as the closeness of individual measures of an analyte when the procedure is applied repeatedly to multiple aliquots of a single homogeneous volume of biological matrix. Generally, precision was measured using pooled urine samples sometimes spiked and several replicates were measured to achieve this. Whereas accuracy was generally was measured in most papers using spiked samples at several levels. However, in some paper it is merely stated that these guidelines were followed and no information on how samples were prepared for accuracy and precision measures were described.

urinary concentrations between smokers and non-smokers were not always found (Grimmer et al., 2000), but at least three studies did report differences for 2-aminonaphthalene (2-AN), 4-aminobiphenyl (4-ABP) and *ortho*-toluidine (o-Tol) (Lindner et al., 2011; Riedel et al., 2006; Seyler & Bernert, 2011). Data from product switching studies are becoming available (Frost-Pineda et al., 2008a; Sarkar et al., 2010). However, additional work on stability under assay conditions and further characterization of half-life of elimination for these chemicals is required. For 1-aminonaphthalene (1-AN), in addition to these criteria, use in products switching or smoking cessation studies were not found. Further, for all these aromatic amine biomarkers, no reports in long-term smoking studies and no inter-laboratory comparisons were found. Thus, additional data on urinary aromatic amine measurements, especially from long-term studies, are required to establish their utility as biomarker of exposure in smoker studies. It has to be noted that DNA and haemoglobin adducts of aromatic amines have been used in a number of studies prior to the development of urinary methods. The adducts were sensitive enough to distinguish between smokers and non-smokers but not non-smokers environmentally exposed to tobacco smoke and not between low 'tar' and higher 'tar' cigarette smokers (Bartsch et al., 1990; Bernert et al., 2005; Bryant et al., 1988; Vineis et al., 1996). Confounding factors from food and chemicals such as hair dyes are limiting factors for the detection of discrete differences between non-smokers with and without environmental exposure to tobacco smoke (Ambrosone et al., 2007).

1,3-Butadiene is an industrial petrochemical used in the production of polymers, polybutadiene, styrene-butadiene rubbers and nitrile-butadiene rubbers (International Agency for Research on Cancer, 1992). It is also a product of incomplete combustion of wood and vegetable matter and is a component of vehicle exhaust fumes. 1,3-butadiene is in IARC Group 1 (''carcinogenic to humans'') (International Agency for Research on Cancer, 2012). For exposure to 1,3-butadiene exposure, the assay for the biomarker monohydroxybutenyl mercapturic acid (MHBMA) has been validated (Carmella et al., 2009; Ding et al., 2009; Schettgen et al., 2009; Sterz et al., 2012; Urban et al., 2003; van Sittert et al., 2000) and used in smoker product switching and cessation studies (Carmella et al., 2009; Sarkar et al., 2008). With the exception of inter-laboratory comparison studies, the data support MHBMA as qualified for use in tobacco studies. Furthermore, a recent study has described a method to quantify two isomers of MHBMA (Sterz et al., 2012). In that study, 2-MHBMA was the more abundant isomers but the 1-MHBMA isomer could be detected with higher sensitivity, specificity and accuracy. The biomarker trihydroxybutyl mercapturic acid (THBMA) has been used in one study and the assay characteristics showed that it would be suitable for use as a biomarker of tobacco smoke exposure (Kotapati et al., 2011). An assay for the biomarker dihydroxybutyl mercapturic acid (DHBMA) is available and has characteristics that validate its use (Carmella et al., 2009; Ding et al., 2009; Schettgen et al., 2009; Urban et al., 2003; van Sittert et al., 2000). However, DHBMA did not differentiate between smokers and non-smokers (Ding et al., 2009; Urban et al., 2003) and did not change on smoking cessation

Table 2. Biomarker qualification summary.

Smoke constituent†	Biomarker†	Analytical technique†	Ranges in smokers (Ref)	Ranges in non-smokers (Ref)	Short-term repeatability‡ (Ref)	Long-term study (Ref)	Inter-laboratory proficiency test (Ref)
NNK	Total NNAL	GC-TEA	99.8–1420.8 ng/24 h urine (Hecht et al., 1999) 0.6–1.8 pmol NNAL/mg creatinine 0.5–6.7 pmol NNAL glucuronide/mg creatinine (Carmella et al., 1997)	0.0016–0.0094 ng/mg creatinine (Meger et al., 2000), (Anderson et al., 2003)	13.7% inter-day RSD (Yuan et al., 2009) 7.8% (Church et al., 2010a)		
		LC-MS/MS	153 ± 132 pg/ml mean ± SD (Kavvadias et al., 2009b) 189.5 ± 161.1 ng/24 h mean ± SD (Lindner et al., 2011)	2.7 ± 3.1 pg/ml mean ± SD (Kavvadias et al., 2009b) 0–24.4 pg/ml (Bernert et al., 2010) 6.06 ng/24 h mean (Sarkar et al., 2008)	3.9–11.5% (Kavvadias et al., 2009b)	Statistically non-significant changes over 6 months in control group smokers (Sarkar et al., 2008)	
NNN	Total NNN	GC-TEA	0.182 ± 0.12 pmol/mg creatinine mean ± SD (Stepanov & Hecht, 2005) 8.51 pg NNN-glucuronide/mg creatinine mean (Stepanov & Hecht, 2005)				
		LC-MS/MS	7.20 ± 6.2 pg/ml mean ± SD (Kavvadias et al., 2009b)	<LOD (Kavvadias et al., 2009b) 0.49 ng/24 h mean (Sarkar et al., 2008)	5.1–11.4% (Kavvadias et al., 2009b)		
NAT	Total NAT	GC-TEA	0.187 ± 0.11 pmol/mg creatinine mean ± SD (Stepanov & Hecht, 2005)				
		LC-MS/MS	161 ± 150.9 pg/ml mean ± SD (Kavvadias et al., 2009b)	< LOD (Kavvadias et al., 2009b) 0.04 ng/24 h mean (Sarkar et al., 2008)	2.5–8.8% (Kavvadias et al., 2009b)		
NAB	Total NAB	GC-TEA	0.041 ± 0.04 pmol/mg creatinine Mean ± SD (5)				
		LC-MS/MS	47.0 ± 47.5 pg/ml mean ± SD (Kavvadias et al., 2009b)	< LOD (4) 1.21 ng/24 h mean ± SD (Sarkar et al., 2008)	3.5–6.5% (Kavvadias et al., 2009b)		
Pyrene	1-OHP	HPLC-FD	0.346 μg/24 h mean (Scherer et al., 2000) 0.196 ± 145 μg/24 h mean ± SD (Scherer et al., 2007a) 0.129–1.157 μg/24 h (Lafontaine et al., 2006) 1.36 ± 0.78 nmol/24 h mean ± SD (Carmella et al., 2009)	0.157 μg/24 h mean (Scherer et al., 2000) 0.101 ± 0.070 μg/24 h mean ± SD (Scherer et al., 2007a) 0.024–0.523 μg/24 h (Lafontaine et al., 2006) 1.09 ± 1.97 nmol/24 h[§] mean ± SD (Carmella et al., 2009)	4.6% (Scherer et al., 2007a) 23–130% 7-day variability RSDs ($n = 13$) (Carmella et al., 2004)	No seasonal effect detected (Carmella et al., 1997) 6-weeks individual RSDs 8.6–71% ($n = 13$) (Carmella et al., 2004)	G-EQUAS^{++}
		HPLC-MS/MS	291 ± 123 ng/24 h mean ± SD (Lindner et al., 2011) [LC-MS/MS]	123 ± 101 ng/24 h mean ± SD (Lindner et al., 2011) [LC-MS/MS]		Non-significant changes over 6 months in control group smokers (Sarkar et al., 2008) [LC-MS/MS]	
		GC-MS	circa 150 ± 80 pg/ml mean ± SD (Suwan-ampai et al., 2009)	circa 50 ± 30 pg/ml mean ± SD (Suwan-ampai et al., 2009)	1.5–5.7% Inter-assay CoV ($n > 2500$) (Suwan-ampai et al., 2009)		
Benzo[a] pyrene	3-OHBaP	HPLC-FD	< 40–367 pg/24 h urine (Lafontaine et al., 2006)	< 44–224 pg/24 h urine (Lafontaine et al., 2006)			
		GC-MS	>50% sample <LOD (smokers & non-smokers) (Suwan-ampai et al., 2009)	>50% sample <LOD (smokers & non-smokers) (Suwan-ampai et al., 2009)	2.4–7.9% inter-assay CoV ($n > 2500$) (Suwan-ampai et al., 2009)		
		LC-MS	193.1 ± 100.6 pg/24 h mean ± SD (Riedel et al., 2006)		3.6–10.9% (Scherer et al., 2010)		
Aromatic amines	1-AN	GC-MS	506.7 ± 670.0 ng/24 h (Grimmer et al., 2000) mean ± SD	68.9 ± 105.4 ng/24 h (Grimmer et al., 2000) mean ± SD	12.6–18.6% Inter-day RSD (Weiss & Angerer, 2002)		
	2-AN	GC-MS	84.5 ± 102.7 ng/24 h mean ± SD (Grimmer et al., 2000) 17.5 ± 30.9 ng/24h mean ± SD (Lindner et al., 2011) [LC-MS/MS]	120.8 ± 272.2 ng/24 h mean ± SD (Grimmer et al., 2000) 1.79 ± 3.6 ng/24 h mean ± SD (Lindner et al., 2011) [LC-MS/MS]	12.9–13.0% Inter-day RSD (Weiss & Angerer, 2002)		
	4-ABP	GC-MS	78.6 ± 85.2 ng/24 h mean ± SD (Grimmer et al., 2000) 24.7 ± 75.4 ng/24 h mean ± SD (Lindner et al., 2011) [LC-MS/MS]	68.1 ± 91.5 ng/24 h mean ± SD (Grimmer et al., 2000) 2.77 ± 11.8 ng/24 h mean ± SD (Lindner et al., 2011) [LC-MS/MS]	9.8–15.1% Inter-day RSD (Weiss & Angerer, 2002)		

(*continued*)

Table 2. Continued

Smoke constituent†	Biomarker†	Analytical technique†	Ranges in smokers (Ref)	Ranges in non-smokers (Ref)	Short-term repeatability‡ (Ref)	Long-term study (Ref)	Inter-laboratory proficiency test (Ref)
		GC-MS/MS	8.69 (7.43–10.16) pg/mg creatinine geo mean (95% CI) (Seyler & Bernert, 2011)	1.64 (1.30–2.07) pg/mg creatinine geo mean (95% CI) (Seyler & Bernert, 2011)			
	o-tol	GC-MS			10.6–14.1% Inter-day RSD (Weiss & Angerer, 2002)		
		LC-MS/MS	179 ± 491 ng/24 h mean ± SD (Lindner et al., 2011)	63.5 ± 128 ng/24 h mean ± SD (Lindner et al., 2011)			
1,3-Butadiene	DHBMA	LC-MS/MS	''not diff from non-smokers'' (Urban et al., 2003)	''not different from smokers'' (Urban et al., 2003)			G-EQUAS
		HPLC-MS/MS	1038 ± 514 nmol/24 h mean ± SD (Carmella et al., 2009)	662 ± 248 nmol/24 h§ mean ± SD (Carmella et al., 2009)	8.7% (Carmella et al., 2009)		
		LC-MS/MS	113–1830 ng/ml (Ding et al., 2009) 166–1092 μg/g creatinine (Ding et al., 2009)	ND-329 ng/ml (Ding et al., 2009) ND-582 μg/g creatinine (Ding et al., 2009)		7 month QC inter-day 12–13% (Ding et al., 2009)	
	MHBMA	LC-MS/MS	ND-132 ng/ml (Ding et al., 2009) ND-59.7 μg/g creatinine (Ding et al., 2009) 3.27 ± 3.02 μg/24 h mean ± SD (Lindner et al., 2011)	ND-73.4 ng/ml (Ding et al., 2009) ND-122 μg/g creatinine (Ding et al., 2009) 0.30 ± 0.40 μg/24 h mean ± SD (Lindner et al., 2011)		7 month QC Inter-day 10–12% (Ding et al., 2009) NS changes over 6 months in control group smokers (Sarkar et al., 2008)	G-EQUAS
		HPLC-MS/MS	66.1 ± 69.4 nmol/24h mean ± SD (Carmella et al., 2009)	3.66 ± 2.41 nmol/24 h§ mean ± SD (Carmella et al., 2009)	16.0% (Carmella et al., 2009)		
	THBMA	HPLC-MS/MS	21.6 ± 10.2 ng/mg creatinine mean ± SD (Kotapati et al., 2011)	13.7 ± 7.9 ng/mg creatinine mean ± SD (Kotapati et al., 2011)	1.6–7.9% Inter-day RSD (Kotapati et al., 2011)		
Benzene	tt-MA	GC-MS	165 ± 126 μg/24 h mean ± SD (Scherer et al., 2007a)	113 ± 139 μg/24 h mean ± SD (Scherer et al., 2007a)	3% (Scherer et al., 2007a)		G-EQUAS
	SPMA	HPLC-MS/MS	3.20 ± 3.80 nmol/24h mean ± SD (Carmella et al., 2009) ND-37.7 ng/ml ND-18.4 μg/g creatinine (Ding et al., 2009) 4.13 ± 3.36 μg/24h mean ± SD (Lindner et al., 2011)	0.21 ± 0.21 nmol/24h§ mean ± SD (Carmella et al., 2009) ND-0.26 ng/ml ND-0.45 μg/g creatinine (Ding et al., 2009) 0.39 ± 0.27 μg/24h mean ± SD (Lindner et al., 2011)	<5% (Scherer et al., 2007a) 7.6% (Carmella et al., 2009)	7 month QC Inter-day 8% (Ding et al., 2009) Non-significant changes over 6 months in control group smokers (Sarkar et al., 2008) [LC-MS/MS]	G-EQUAS
Catechol	catechol	GC-MS	2.07 μg/ml mean (Waidyanatha et al., 2004)	1.69 μg/ml mean (Waidyanatha et al., 2004) 1.27 (0.3–11.9) μg/ml median (range) (59) [All subjects inc smokers]			
Acrolein	3-HPMA	LC-MS/MS	2.8 mg/24 h urine mean (Mascher et al., 2001) 1297 ± 1164 μg/24 h mean ±SD	0.8 mg/24 h urine mean (Mascher et al., 2001) 337 ± 383 μg/24 h mean ± SD	<2.5% (Scherer et al., 2007a)	Non-significant changes over 6 months in	4 labs (Minet et al., 2011b)

			(Scherer et al., 2007a) 1.81 ± 1.26 mg/24 h mean ± SD (Lindner et al., 2011) 0.68, 1.13, 1.81 mg/24 h medians (1 mg, 4 mg, 10 mg products) (Minet et al., 2011a)	(Scherer et al., 2007a) 0.63 ± 0.80 mg/24 h mean ± SD (Lindner et al., 2011) 0.23 mg/24 h median (Minet et al., 2011a)		control group smokers (Sarkar et al., 2008)	
			1.3 mg/24 h mean (Scherer et al., 2006)	0.3 mg/24 h urine mean (Scherer et al., 2006)			
			80.9–4030 ng/ml (Ding et al., 2009) 75-3678 µg/g creatinine (Ding et al., 2009)	ND-128 ng/ml (Ding et al., 2009) ND-245 µg/g creatinine (Ding et al., 2009)		7-month QC Inter-day 8–12% (Ding et al., 2009)	
		HPLC-MS/MS	10020 nmol/24 h (Carmella et al., 2009) 53.5–3795 ng/ml 104–4423 µg/g creatinine (Eckert et al., 2011)	1500 nmol/24 h[§] (Carmella et al., 2009) 32.6–2325 ng/ml 39.3–1562 µg/g creatinine (Eckert et al., 2011)	9.4% (Carmella et al., 2009) 6.7–8.5% (Eckert et al., 2011)		
Croton-aldehyde	HMPMA	LC-MS/MS	2122 ± 1286 µg/24h urine mean ±SD (Scherer et al., 2007b)	752 ± 457 µg/24 h mean ± SD (Scherer et al., 2007b)	1.1–2.0% (Scherer et al., 2007b)		
	CMEMA	LC-MS/MS	1970 ± 2500 µg/24h urine mean ± SD (Scherer et al., 2007b)	1071 ± 513 µg/24 h urine mean ± SD (Scherer et al., 2007b)	1.7–4.1% (Scherer et al., 2007b)		
Ethylene Oxide	HEMA	HPLC-MS/MS	ND-20.8 ng/ml ND-16.0 µg/g creatinine (Ding et al., 2009) <LOD–49.4 ng/ml 1.1–67.7 µg/g creatinine (Eckert et al., 2011)	ND-1.4 ng/ml ND-1.1 µg/g creatinine (Ding et al., 2009) <LOD–5.0 ng/ml 0.6–8.1 µg/g creatinine (Eckert et al., 2011)	4.2–5.6% (Eckert et al., 2011)	7 month QC Inter-day 11–15% (Ding et al., 2009)	G-EQUAS
Acrylamide	Acrylamide	HPLC-MS					
	Glycidamide,	HPLC-MS					
	AAMA	HPLC-MS/MS	185.7 ± 107.8 µg/24 h mean ± SD (Urban et al., 2006)	73.1 ± 46.6 µg/24 h mean ± SD (Urban et al., 2006)	6.3–6.6% (Urban et al., 2006)		G-EQUAS
	GAMA	HPLC-MS/MS	27.6 ± 20.1 µg/24 h mean ± SD (Urban et al., 2006)	15.9 ± 13.0 µg/24 h mean ± SD (Urban et al., 2006)	2.7–5.9% (Urban et al., 2006)		G-EQUAS
Acrylonitrile	CEMA	LC-MS/MS	240 ng/ml median, 2.0–1382 ng/ml range (Schettgen et al., 2009) 75.4, 140, 187 µg/24 h medians (1 mg, 4 mg, 10 mg product smokers) (Minet et al., 2011a)	2.0 ng/ml median, <LOQ–21.3 ng/ml range (Schettgen et al., 2009) 1.1 µg/24 h median (Minet et al., 2011a)	6.6% Inter-day RSD (Schettgen et al., 2009) 2.9–5.6% (Minet et al., 2011a)		G-EQUAS
Metals	Cadmium	ICP-MS	0.59 µg/g creatinine mean (McElroy et al., 2007b)	0.30 µg/g creatinine mean (McElroy et al., 2007b)			G-EQUAS
		AAS	0.412 (0.392-0.434) ng/ml geometric mean (95% CI) (Hoffmann et al., 2000) Smokers > non-smokers at every age group >20 years (Paschal et al., 2000)	0.226 (0.216–0.236) ng/ml geometric mean (95% CI) (Hoffmann et al., 2000)	1–18% relative measurement error (Hoffmann et al., 2000)		

†Abbreviations: as for Table 1.
‡This parameter is sometimes described as ''inter-day precision'' and we have captured many variations in its presentation. The inter-day coefficient of variation, unless stated.
§Value from Smokers 56 days after cessation (>100 half-lives).
++G-EQUAS, German External Quality Assessment Scheme (see www.g-equas.de).

Table 3. Other biomarker parameters.

Smoke constituent †	Biomarker†	Direct comparison of tobacco products or user groups? (Ref)	Half-life‡ (Ref)	Metabolic enzymes (Ref)
NNK	total NNAL	Switching study (Sarkar et al., 2008) Product yield study (Kavvadias et al., 2009a) Reduction on smoking cessation (Carmella et al., 2009)	40–45 d (Hecht et al., 1999, Hecht et al., 2004) 10–18 d (Goniewicz et al., 2009)	CYP1B1, CYP2A13, CYP1A2, HSD11D1, AKR1B10, AKR1C1, AKR1C2, AKR1C4, CBR1, DCXR, UGT1A9, UGT1A4, UGT2B7, UGT2B10, UGT2B17, MRP1, MRP2 (Leslie et al., 2001, Carmella et al., 2002, Breyer-Pfaff et al., 2004, Wiener et al., 2004, Bao et al., 2005, Lazarus et al., 2005, Martin et al., 2006, Church et al., 2010b, Chiang et al., 2011, Ter-Minassian et al., 2012)
NNN	NNN	Switching study (Sarkar et al., 2008) Product yield study (Kavvadias et al., 2009a)	45 min (Upadhyaya et al., 2002) patas monkey	CYP2A6, CYP2A13, CYP2E1, CYP3A4, UGT2B10 (Chen et al., 1980, Patten et al., 1997, Wong et al., 2005)
NAT	total NAT	Switching study (Sarkar et al., 2008) Product yield study (Kavvadias et al., 2009a)	90 min (Li et al., 2006) rabbit	
NAB	total NAB	Switching study (Sarkar et al., 2008) Product yield study (Kavvadias et al., 2009a)	30 min (Li et al., 2006) rabbit	
Pyrene	1-OHP	Reduction in EHC vs CC (Feng et al., 2006, Roethig et al., 2007, Frost-Pineda et al., 2008a) No statistically significant reduction on smoking cessation (Carmella et al., 2009)	6 h in 8 US smokers (St Helen et al., 2012)	CYP1A1, CYP1B1, GSTM1, GSTP1, UGT1A6, UGT1A7, UGT1A9 (Nerurkar et al., 2000, Pal et al., 2000, Luukkanen et al., 2001, Chuang & Chang, 2007)
Benzo[a]pyrene	3-OHBaP	Reduction on switching to snus versus CC and on smoking cessation (Sarkar et al., 2010)		CYP1A1, CYP1B1, CYP1A2, CYP3A4, UGT1A1, UGT1A7, UGT1A10, UGT1A8, UGT2A1, SULT1B1, EPHX1 (Kim et al., 1998, Shimada et al., 1999, Zheng et al., 2002, Lodovici et al., 2004, Shimada & Fujii-Kuriyama, 2004, Bushey et al., 2011)
Aromatic amines	1-AN			NAT1, NAT2, CYP1A2, SULTs, UGTs (Frederickson et al., 1992, Badawi et al., 1995, Kimura et al., 1999, Finel et al., 2005, Al-Zoughool & Talaska, 2006, Sarkar et al., 2006, Butler et al., 2011)
	2-AN	Reduction in EHC versus CC (Frost-Pineda et al., 2008a)		
	4-ABP	Reduction on smoking restriction and on switching to smokeless product (Sarkar et al., 2010) and EHC (Frost-Pineda et al., 2008a)	15 h (Frederickson et al., 1992)	
	o-tol	Reduction in EHC versus CC (Frost-Pineda et al., 2008a)		
1,3-Butadiene	DHBMA	No statistically significant reduction on smoking cessation (Carmella et al., 2009)	5–9 h (van Welie et al., 1992)	CYP2E1, CYP3A4, CYP2A6, EPHX1, GSTT1, GSTM1, MPO (Seaton et al., 1995, Himmelstein et al., 1996, Nieusma et al., 1998, Fustinoni et al., 2002, Abdel-Rahman et al., 2005, Tan et al., 2010)
	MHBMA	Reduction in Test versus CC (Sarkar et al., 2008) Reduction on smoking cessation (Carmella et al., 2009)	5–9 h (van Welie et al., 1992)	
	THBMA	25–50% reduction at 56 days following smoking cessation (Kotapati et al., 2011)		

Benzene	tt-MA	Reduction in EHC versus CC and non-smoker versus smoker (Kim et al., 2006)	6–12 h (Ruppert et al., 1995, Kim et al., 2006)	CYP2E1, CYP2B1, CYP2F1, GSTT1, GSTM1, COMT, NQO1, EPHX1, AKR1C1, SULT1A1, UGT1A6, MPO (Rossi et al., 1999, Dougherty et al., 2008, Manini et al., 2010, Angelini et al., 2011, Mansi et al., 2012)
	SPMA	Reduction in EHC versus CC and non-smoker versus smoker (Feng et al., 2006, Roethig et al., 2007) Reduction on smoking cessation (Carmella et al., 2009)	6–12 h (Kim et al., 2006)	
Catechol	Catechol		3–7 h Metabolite of benzene	[See benzene]
Acrolein	3-HPMA	Reduction in EHC versus CC and nonsmoker versus smoker (Roethig et al., 2007) Reduction on smoking cessation (Carmella et al., 2009)	5–9 h (Scherer et al., 2006)	ALDH, ALR, GSTM1, GSTP1, GSTA1, GGT1, GGT2, GGT3, NAT1, NAT2 (Berhane et al., 1994, Pal et al., 2000, Mascher et al., 2001)
Crotonaldehyde	HMPMA	Significantly reduced on cessation or switch to lower toxicant cigarette (Scherer, 2005, Scherer et al., 2007b) Reduction on smoking cessation (Carmella et al., 2009)	5–9 h (van Welie et al., 1992, Scherer et al., 2006)	GSTP1 (Pal et al., 2000)
	CMEMA	No significant change on cessation or lower toxicant cigarette (Scherer, 2005, Scherer et al., 2007b)	5–9 h (van Welie et al., 1992, Scherer et al., 2006)	
Acetaldehyde				ALDH2, ALDH1B1 (Weiner & Wang, 1994, Stagos et al., 2010)
Formaldehyde				ALDH2, ADH5 (Hedberg et al., 2001, Wang et al., 2002b, Thompson et al., 2010)
Ethylene oxide	HEMA	Reduction on smoking cessation (Carmella et al., 2009) Significantly reduced on cessation or switch to lower toxicant cigarette (Scherer, 2005, Scherer et al., 2010)	<5 h (Haufroid et al., 2007)	GSTT1 (Muller et al., 1998, Fennell et al., 2000)
Acrylamide	Acrylamide		2.4 h (Fuhr et al., 2006)	CYP2E1, EPHX1, CYPs (Doroshyenko et al., 2009, Huang et al., 2011a, Huang et al., 2011b)
	Glycidamide, AAMA		17.4 h (Fuhr et al., 2006)	
	GAMA		25.4 h (Fuhr et al., 2006)	
Acrylonitrile	CEMA	Significantly reduced on cessation or switch to lower toxicant cigarette (Scherer, 2005, Scherer et al., 2010)		CYP2E1, GSTP1 (Kedderis et al., 1993, Thier et al., 2001, Thier et al., 2002, Wang et al., 2002a, Suhua et al., 2010)
Metals	Cadmium		Months (Huang & Yang, 1997, Hoffmann et al., 2000, Paschal et al., 2000, McElroy et al., 2007a, McElroy et al., 2007b)	

†Abbreviations as in Table 1. CC, conventional cigarette; EHC, electrically heated cigarette.
‡Approximate half-life based on elimination from body fluid or measurement in urine.

(Carmella et al., 2009); therefore, it is not qualified for use as a biomarker in tobacco smoke exposure or products assessment studies.

Benzene is used as a reagent for the polymer industry. It also occurs ubiquitously in the environment with petrochemical, vehicle and combustion processes being important sources. Benzene is in IARC Group 1 (''carcinogenic to humans'') (International Agency for Research on Cancer, 2012). For exposure to benzene, two biomarkers, *trans, trans*-muconic acid (tt-MA) and S-phenyl mercapturic acid (SPMA) have been widely used. Both assays have been validated (Carmella et al., 2009; Ding et al., 2009; Feng et al., 2006; Kim et al., 2006; Ruppert et al., 1995; Scherer et al., 2007a) and used in a variety of tobacco product studies (Feng et al., 2006; Kim et al., 2006; Roethig et al., 2007) but only SPMA differentiated between groups of smokers and non-smokers with a consistently greater than 2-fold difference between group means across studies (Scherer et al., 2007a) and showed a difference between groups on smoking cessation (Carmella et al., 2009). However, a review on tt-MA by Scherer et al. (1998) reported significant differences between smokers and non-smokers with seven of the 14 studies reviewed showing a greater than 2-fold difference. This article also indicated that it is known that tt-MA is also formed from the metabolism of sorbic acid, which is widely used in foods, possibly interfering with studies in smokers and that if this urinary biomarker is used it is recommended that ingestion of sorbic acid should be taken into account (Scherer et al., 1998). Additionally, SPMA assay precision has been characterized in long-term studies (Ding et al., 2009). Catechol is also a metabolite of benzene; however, while it has been used as a biomarker of benzene exposure (Kerzic et al., 2010; Waidyanatha et al., 2004), a 2-fold separation between smokers and non-smokers was not observed (Waidyanatha et al., 2004). Additional data would be required to qualify this biomarker for use in tobacco smoke exposure studies.

Acrolein, also known as propenal, is the simplest unsaturated aldehyde. It is a chemically reactive compound found in the environment as a by-product of overheated organic matter (oils), plastics, and fossil fuel combustion and can also be formed by lipid peroxidation and oxidative stress in normal mammalian tissues (Chung et al., 1996; Esterbauer et al., 1991). For biomarker assays of exposure to acrolein, 3-hydroxypropylmercapturic acid (3-HPMA) has been validated (Carmella et al., 2007, 2009; Ding et al., 2009; Mascher et al., 2001; Scherer et al., 2007a) and showed a difference in group mean concentration between smokers and non-smokers with a ratio of greater than 2 (Lindner et al., 2011; Mascher et al., 2001; Minet et al., 2011a). 3-HPMA has been used in product switching studies and smoking cessation studies (Carmella et al., 2009; Roethig et al., 2007). Furthermore, it has been used in short-term and long-term studies in groups of smokers (Lindner et al., 2011; Sarkar et al., 2008) and an inter-laboratory comparison study has been published (Minet et al., 2011b; Shepperd et al., 2009).

Crotonaldehyde, like acrolein, is an unsaturated aldehyde which is produced through the combustion of carbon-containing fuels and is therefore an important environmental pollutant (Budiawan, 2001). In addition, it is commonly found in foodstuffs such as fish, meat, fruit and vegetables and alcoholic beverages including wine and whisky (Budiawan, 2001). It is also reported to be produced endogenously through lipid peroxidation (Chung et al., 1996; Hecht, 2001). The assays for the biomarkers of crotonaldehyde exposure, 3-hydroxyl-methylpropylmercapturic acid (HMPMA) and 2-carboxy-1-methylethylmercapturic acid (CMEMA), have been characterized to a similar extent (Scherer et al., 2007b), but data on sample storage stability and long-term evaluation in studies of smokers were not found. However, while HMPMA could differentiate groups of smokers from non-smokers CMEMA could not (Scherer et al., 2007b). These data show that HMPMA would be suitable for exposure assessment in smoker studies but that CMEMA would not; although further work on HMPMA is still required.

Ethylene oxide is used as an intermediate in the production of several industrial chemicals and is used as a fumigant or sterilizing agent. Exposure to ethylene oxide in the general population is through medical, food, clothing and cosmetics that have been sterilized with the compound and it has also been detected in tobacco smoke and automotive exhaust fumes (U.S. Department of Health and Human Services Public Health Service National Toxicology Program, 2011). The compiled data on N-acetyl-S-(2-hydroxyethyl)-L-cysteine (HEMA) as a biomarker for ethylene oxide exposure show a validated assay (Carmella et al., 2009; Ding et al., 2009), differences between groups of smokers and non-smokers (Ding et al., 2009; Eckert et al., 2011) and reductions in group mean values in tobacco products switching studies (Scherer, 2005; Scherer et al., 2010). An elimination half-life of <5 h (Haufroid et al., 2007) could be problematic in study design (e.g. time since last product use would be a critical datum) and, although the sample is stable to repeated freeze–thaw cycles (Ding et al., 2009), extended storage at assay temperature in the urine matrix was not reported.

Acrylamide is an industrial chemical used in a wide range of applications including water treatment, oil extraction, biotechnology and paper manufacturing. It is also formed in the heating process of starch-containing/carbohydrate-rich food (Tareke et al., 2002). For acylamide exposure, more data are available on the mercapturic acid metabolites, N-acetyl-S-(2-carbamoylethyl)-L-cysteine (AAMA) and N-(R,S)-acetyl-S-(2-carbamoyl-2-hydroxyethyl)-L-cysteine (GAMA) than the parent molecule or its metabolite glycidamide (Fuhr et al., 2006; Urban et al., 2006). Glycidamide was included in one study because the investigators considered it to be a marker of the toxicity pathway based on rodent studies (Fuhr et al., 2006). An approximate 2-fold difference between group means in smokers and non-smokers for AAMA and GAMA was reported (Urban et al., 2006). Nonetheless, sample stability data and long-term studies were not found and further work would be required to qualify AAMA and GAMA for use as tobacco smoke exposure biomarkers.

The main source of exposure to acrylonitrile is occupational, since it is primarily used in industry, where it is used to make other chemicals such as plastics, synthetic rubber and acrylic fibers. It has also been detected in food which has been stored in containers manufactured from plastics constructed with acrylonitrile, such as acrylonitrilebutadiene-styrene (ABS). For acrylonitrile, data on the biomarker N-acetyl-S-(2-carboxyethyl)-L-cysteine (CEMA)

assay validation and some comparisons of smokers and non-smokers are available (Minet et al., 2011a; Schettgen et al., 2009), but sample storage stability, use in long-term studies of smokers and inter-laboratory comparisons were not found.

The main exposure to cadmium, in people, occurs through the consumption of foods and drinking water, the inhalation of cadmium particles from ambient air or cigarette smoke, and the incidental ingestion of contaminated dust or soil. Urinary cadmium has been measured in several large studies conducted over different time periods and much of the assay validation and biomarker qualification data are available (Hoffmann et al., 2000; McElroy et al., 2007a, b; Paschal et al., 2000). However, the cross-sectional studies reported that urinary cadmium concentration increased as the subjects' age increased in both smokers and non-smokers (McElroy et al., 2007a) and this observation suggests that longitudinal studies of tobacco products use would require data to be presented as individual changes over time rather than group mean changes. Of course, statistical techniques such as age-adjustment may also be applied to cross-sectional data, to facilitate inter-group comparisons.

Discussion

The intended study application is fundamental when considering whether a biomarker is fit for purpose. Aspects of the assay validation, such as linearity, accuracy, precision, repeatability and reproducibility of measurements (Food and Drug Administration, 2001), may be considered as basic required information, but such data alone do not qualify a biomarker for use in a particular application. Lee et al. (2006) described fitness for purpose as ''[the] notion that assay validation should be tailored to meet the intended purpose of the biomarker study, with a level of rigor commensurate with the intended use of the data'' and, although their description was for a pharmaceutical application, we contend that this description applies to considerations of different biomarker measurement study designs that might be applied to tobacco products. For example, if the purpose of a study is to compare the absolute amount of a biomarker in a sample across several different testing laboratories, then a reference standard should be available or, at the least, a consensus value for a standard material based on an inter-laboratory trial (Food and Drug Administration, 2001, International Organization for Standardization, 2005, 2007). Further, if a single spot urine sample is to be collected for biomarker measurement in a cross-sectional study, then the metabolic pathway leading to the biomarker formation or destruction and the kinetics of its appearance in urine should be known, along with a measure of time since subjects' last exposure. Clearly, a situation with rapid elimination of a biomarker into urine, a short urinary half-life and a long time, or even a variable time, between exposure and urine collection would compromise any data collected.

The approach taken to collect urine samples is also an important consideration for study design. While many studies have used a 24 h collection period, this is difficult to achieve in studies that are conducted without subject confinement. If a spot sample is taken, then the time of sample collection and the approach taken to correct biomarker concentration for subjects' hydration and urine volume output over any defined period will also affect the biomarker measurement variability. The time of collection of spot urine samples was found to affect the variability in studies of sex hormones, with a morning spot sample being less variable than an overnight collection in a group of normotensive women (Muti et al., 2000). However, in studies of urinary electrolyte concentrations, overnight samples were better predictors of 24-h calcium excretion than were daytime collections (Cirillo et al., 1993), and afternoon spot urine samples, adjusted for creatinine concentration, correlated better with 24-h sodium excretion than did morning spot samples (Mann & Gerber, 2010). Correction of spot urine samples for creatinine concentration is an adjustment that is widely used throughout the biomedical scientific literature (Arndt, 2009; Cote et al., 2008). Previous studies of smokers reported that adjustment of urinary biomarkers for creatinine concentration was itself highly variable and could be improved further by correction for urinary specific gravity (Heavner et al., 2006). From this, it appears that the use of any spot urine sample for biomarker measurement in tobacco product comparison studies would require a separate investigation to qualify the biomarker for use.

In many tobacco studies, nicotine exposure biomarkers are considered to give the best objective measure of tobacco exposure but they were not included in this review because another recent summary is available (Tricker, 2006). However, it is appropriate to summarize the characteristics of nicotine as a biomarker here, to allow comparison with other putative tobacco smoke exposure biomarkers. Nicotine is present in milligram per gram quantities in tobacco and approximately 10% transfers to mainstream smoke. Upon inhalation of mainstream smoke, nicotine is rapidly absorbed into the bloodstream and rapidly metabolized by several enzyme systems. Nicotine elimination from the plasma has a half-life of approximately 2 h in man and little unchanged nicotine is recovered from the urine of smokers. The major metabolites, cotinine and *trans*-3′-hydroxycotinine are eliminated into urine more slowly and, together with nicotine and all their glucuronide conjugates (nicotine + 5), urinary measurements account for approximately 80% of the initial mass of nicotine absorbed into the body. When another four metabolites (nornicotine, norcotinine, nicotine N-oxide and cotinine N-oxide) are considered (nicotine + 9), urinary measurements account for approximately 90–95% of the initial mass of nicotine absorbed. Collectively, these metabolites have elimination half-lives of <24 h and so their measurement mainly reflects very recent and the previous 2–3 days smoking activity. Nicotine metabolites are often expressed as ''total nicotine equivalents'' based on calculations allowing for the molecular mass of each metabolite converted back to nicotine and usually expressed in milligrams. Numerous studies have used nicotine metabolites as biomarkers of smoke exposure for both products switching and smoking cessation. Despite this extensive characterization, nicotine would not be fit for purpose as a biomarker of toxicant exposure, in studies comparing RTPs in which nicotine levels were maintained, while other toxicants were reduced, an approach suggested more than 30 years ago (Russell, 1976). More recently, it has been proposed that the

toxicant to nicotine ratio could be used as one measure for the potential harm reduction of RTPs use (Burns et al., 2008). However, the use of this ratio would require an understanding of the other toxicant biomarker elimination kinetics, in relation to nicotine, if urinary biomarkers were the means of assessment.

In considering other putative biomarkers of smoke constituent exposure, few are as well characterized and as widely used as nicotine metabolites. From this review, the biomarkers DHBMA (Urban et al., 2003), catechol (Waidyanatha et al., 2004) and CMEMA (Scherer et al., 2007b) were not able to distinguish between groups of smokers and non-smokers and thus are not fit for purpose in studies of smoking cessation or tobacco products switching, such as in RTP assessment. Further, while urinary cadmium concentration was elevated in cross-sectional and long-term studies of smokers compared to non-smokers (Hoffmann et al., 2000; McElroy et al., 2007a, b; Paschal et al., 2000), all subjects showed an increased urinary cadmium concentration with age (McElroy et al., 2007a). Therefore, careful study design considerations would be needed to allow this putative biomarker to be used for RTP assessment, as it would be important to differentiate between product use and bioaccumulation with age.

For the great majority of biomarkers considered, it is clear that assay validation and biomarker qualification has been performed for only short-term studies, typically of less than one-week duration. Thus, the TSNAs other than NNK (NNN, NAB and NAT), 3-OHBaP, 2-AN, 4-ABP, o-tol, THBMA, HMPMA, AAMA, GAMA and CEMA may only be described as fit for purpose, as urinary biomarkers of tobacco smoke exposure, if the study duration does not exceed 1 week. With this group of putative tobacco smoke exposure biomarkers, several details required for assay validation and biomarker qualification were not found in the literature. It is likely that some of these data, such as assay linearity and recovery after extraction from the biological matrix, may exist within the originating laboratory but were not reported.

Long-term studies (typically of 6–7 months duration) were only found in the literature for NNAL (Sarkar et al., 2008), 1-OHP (Carmella et al., 1997, 2004; Sarkar et al., 2008), MHBMA (Ding et al., 2009; Sarkar et al., 2008), SPMA (Ding et al., 2009; Sarkar et al., 2008), 3-HPMA (Ding et al., 2009; Sarkar et al., 2008) and HEMA (Ding et al., 2009) but, even then, sample storage data and other basic assay characteristics were often not reported. This would be a concern if, for example, the analysis plan called for storage of all samples so that they could be analysed as a single batch within the laboratory. The FDA guidance on full validation of bioanalytical method recommends that freeze and thaw stability, short-term stability and long-term stability should be established for the analytes (Food and Drug Administration, 2001). In particular, the short-term temperature stability test should be conducted for a time period reflecting the expected duration at which a sample will be kept at room temperature and at 4 °C, for instance holding time in the autosampler. In the panel of studies we have reviewed, some laboratories have conducted a stability assessment at least at room temperature and for frozen samples, some have only conducted the stability assessment for frozen samples, and others did not report stability testing (Table 1).

As a PAH biomarker, 1-OHP has been widely studied and it has been used in several smoking studies, with mixed results. While it can be used to show differences between the type of smoking product used in a controlled study (Feng et al., 2006; Roethig et al., 2007), the magnitude of change and potential confounding by external influences such as diet and vehicle exhaust exposure (Chuang & Chang, 2007; Menzie et al., 1992) limit its use. A greater concern is that pyrene is not a carcinogen and it is more hydrophilic than most other PAH. Therefore, 1-OHP is unlikely to be a good surrogate biomarker of other, carcinogenic, PAH exposure. Some investigators have used 3-OHBaP as a biomarker of benzo[a]pyrene (Lafontaine et al., 2006; Sarkar et al., 2010) although, as noted above, some of the assay validation and biomarker qualification data for the long-term use of 3-OHBaP were not found. Recent data have suggested that urinary concentrations of other PAH may also give clear and statistically significant differences between smokers and non-smoker groups. For example, in one study of 622 spot urine samples taken in the USA and Poland, 1-hydroxyfluorene and 2-naphthol were reported to be more selective of tobacco smoke exposure than 1-hydroxypyrene and hydroxyphenanthrenes (St Helen et al., 2012). In addition, a further study by St Helen et al. (2013) showed that racial differences in urinary 2-napthol and total PAH levels indicating regional, international and racial variations are also significant considerations for these and other biomarkers proposed for possible RTP assessment. Further studies may qualify these PAH metabolites as useful biomarkers for use in tobacco products switching and in smoking cessation studies.

For several of the biomarkers included in this review, other methods of analysis such as DNA and haemoglobin adducts are available. 4-ABP is typically measured as haemoglobin adducts (Bartsch et al., 1990; Bernert et al., 2005; Bryant et al., 1988) and formaldehyde and acetaldehyde are typically measured as DNA adducts in leukocytes (Chen et al., 2007; Lu et al., 2009; Wang et al., 2000, 2009). A recent study quantified NNK-derived DNA adducts in the oral mucosa of smokers and non-smokers (Stepanov et al., 2013). DNA-adduct from tissue biopsies, white blood cells, and haemoglobin adducts biomarkers, accumulate over a prolonged period of time, depending on the specific matrix used. For instance T-lymphocytes have a half-life of a few months to a few years (Vrisekoop et al., 2008) whilst red blood cells have a half-life of 60 days (Berlin et al., 1959). Hair and nails have also been proposed as matrices to measure the cumulative exposure to smoke toxicants over months (Avila-Tang et al., 2013). Those matrices also have their specific limitations, for instance the NNK-DNA adducts are well correlated with smoking in mouth epithelial cells (Stepanov et al., 2013), however in other studies conducted in tissue biopsies, myosmine, which is found in tobacco and food was suspected to be a significant confounding factor (Schlöbe et al., 2008). Acrolein DNA adducts were also well correlated with smoking status when DNA was extracted from mouth epithelial cells (Nath et al., 1998) but not from white blood cells (Zhang et al., 2011). One explanation is that the formation of mercapturic acid from the reaction of acrolein

with blood glutathione subsequently excreted in urine is sufficiently efficient to protect leukocytes from DNA damage (Zhang et al., 2011). Hair contamination by sweat and environmental toxicants and hair pigmentation also have an impact on the reliability of the data collected from this matrix (Avila-Tang et al., 2013). These examples illustrate that biomarkers should be carefully selected according to the purpose of the study to take into account half-life and matrix.

The difficulties in validating, qualifying and establishing adduct assays as fit for purpose should not be overlooked. Indeed, it could be expected that such assays would be inherently less reproducible and give greater intra- and inter-assay imprecision and greater variability in long-term sample storage stability than the urinary assays described here (Angerer et al., 2007; Chen et al., 2007; Lu et al., 2009; Wang et al., 2000, 2009).

Some investigators have used urinary mutagenicity as a biomarker of exposure to genotoxic chemicals. Studies have confirmed that cigarette smokers have higher levels of urinary mutagenicity than non-smokers, even when both groups were maintained on a low-mutagenic, boiled food diet (Doolittle et al., 1990). Since then, several studies have confirmed that urinary mutagenicity was decreased in cigarette smokers who switched to using an electrically heated cigarette (Frost-Pineda et al., 2008a; Rahn et al., 1991; Roethig et al., 2005, 2007, 2008; Smith et al., 1996) or the use of oral snus (Sarkar et al., 2010). However, the urinary mutagenicity assay is not based on chemical standards and its characteristics, such as linearity, precision, accuracy, limits of detection, etc., cannot be compared with the other assays discussed in this review. It gives a relative assessment of the mutagenicity of samples made in a direct comparison test and its use as a regulatory action standard would be problematic, requiring reference cultures, standards and frequent inter-laboratory comparisons to ensure robust data. Further, the output of urinary mutagenicity testing is a combination between the exposure dose to the mutagenic agent and the metabolism of those toxicants within the exposed subject. Metabolic interindividual differences are a source of assay variation and specific genotypes have been associated with an increase risk of tobacco related diseases (Daly et al., 1994). However our current understanding of the metabolic pathway variations limits our ability to factor genotype and phenotype in the interpretation of urine mutagenicity studies.

Nonetheless, well-controlled data generated with such assays could help to inform the process of RTP assessment.

In compiling these data on putative tobacco smoke exposure biomarkers, we considered that the metabolic pathways and kinetic of the pathways leading to the biomarker appearance in the urine were also of importance. Although much information on the metabolic pathways was available, fewer formal calculations of the mass balance for specific metabolites were found. Also, little information on diurnal variation in metabolic enzymes was retrieved; although this is reported not to affect the mercapturic acids (van Welie et al., 1992), which are the biomarkers for several of the smoke constituents considered in this review. Also, from the elimination half-lives of the biomarkers examined, most exposures, with the exception of NNAL and cadmium, would be expected to change in relatively short-term studies. While this is helpful to confirm a toxicant reduction in a controlled RTP assessment study, a biomarker with a short elimination half-life would be of less use in cross-sectional or population studies, where an occasional cigarette smoker could give high readings based on smoking one or two cigarettes shortly before urine sample collection. There remains a need for the availability of biomarkers other than NNAL that have half-lives in the days to weeks range, to give better estimates of long-term exposure to smoke toxicants.

Overall, if biomarkers measurements are intended for the long-term assessment of RTPs or for the setting of regulatory action levels, as proposed by some groups (Ashley et al., 2007; Burns et al., 2008; Hecht et al., 2010), then this would require measurements to be made across bio-analytical laboratories. It is not clear that sufficient information is available for any biomarkers on the proposed lists, for these purposes. Indeed the only published data on an inter-laboratory comparison that were found was for 3-HPMA across four testing laboratories (Minet et al., 2011b) and a study by Biber et al. (1987) on nicotine and cotinine in serum and urine conducted in 11 laboratories. A larger inter-laboratory study for this biomarker has been conducted recently through CORESTA but this was a laboratory proficiency trial and was not intended to set a consensus value for a standard material, and the data have not been submitted for publication yet. Some proficiency testing schemes, such as the German External Quality Assessment Scheme (G-EQUAS: see, http://www.g-equas.de), are available. This scheme includes occupation and environmental levels of many biomarkers discussed in this review but it does not include several, such as total nicotine equivalents and TSNAs. Thus, by registering with such a scheme, testing laboratories should be able to determine whether their measurements for specific biomarkers fall within a group consensus, which would facilitate obtaining reproducible results across laboratories. Ideally, any schemes would include the ranges of concentrations of biomarkers found in smokers, to establish assay validity as one of the criteria of fitness for purpose.

A key characteristic for any candidate biomarker is it's specificity for tobacco-related exposures, and the investigator's ability to discriminate between the contributions of tobacco smoke exposure from those of non-tobacco exposures. Common dietary and environmental exposures to precursor compounds, such as PAH (Chuang & Chang, 2007; Scherer et al., 2000), benzene (Scherer et al., 1998) or acrylamide (Fuhr et al., 2006) pose a continuing challenge for otherwise promising urinary biomarkers with regard to the design and interpretation of investigations intended to address the exposures that result from the smoking of tobacco in all of its diverse forms.

As a final remark, we need to consider that one of the key purpose of a biomarker in the context of tobacco smoke exposure and product risk assessment is the predictive nature of such biomarkers for the tobacco-related diseases. Unfortunately very little data are currently available regarding the disease predictivity of biomarkers of exposure, but some interesting results are starting to emerge from prospective clinical studies. Dose-dependent association between urinary biomarkers of cotinine, total NNAL, and

tetrahydrophenanthrene were associated with risk of lung cancer in a Chinese cohort (Yuan et al., 2011). A similar association was found for NNAL in the serum of smokers (Church et al., 2009). In contrast, urinary metabolites of 1,3-butadiene, ethylene oxide, benzene, and acrolein, were not independent risk predictors for lung cancer (Yuan et al., 2012). Finally the haemoglobin adduct of 4-aminobiphenyl has also been identified as a good risk predictor for bladder cancer but this has only been shown in non-smokers (Tao et al., 2013). Since prospective clinical studies are conducted over many years, it is likely that the ultimate goal of a comprehensive understanding of the predictivity of urinary biomarkers for the tobacco-related disease will require a significant effort over a prolonged period of time.

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