Short Communication

Detection and Determination of Methanol and Further Potential Toxins in Human Saliva Collected from Cigarette Smokers: A ^{1}H NMR Investigation

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Abstract

Introduction/Objectives: The deleterious health effects of tobacco smoking are now widely recognized and documented. High-resolution ^{1}H NMR analysis of human saliva provides a high level of valuable molecular information regarding the nature and levels of a wide range of both endogenous and exogenous agents therein. This investigation focused on the detection of molecular modifications to the salivary ^{1}H NMR profiles of cigarette smokers following the smoking of a single cigarette product.

Methods: Cigarette-smoking human participants (6 female, 7 male) provided saliva samples both prior and subsequent to smoking a single cigarette (the former following a 12 hr. overnight fasting/smoking-abstention period). A group of n = 7 non-smoking controls also provided saliva samples before and after a 4.0 min. 'smoking mimic' time period. ^{1}H NMR analysis of supernatants derived therefrom was conducted at an operating frequency of 400 MHz.

Results: ^{1}H NMR analysis revealed that single cigarette smoking episodes gave rise to substantial increases in the salivary concentrations of methanol ($p<10^{-6}$) and propane-1,2-diol ($p = 2.0 \times 10^{-4}$), i.e. ca. 40- and 3.2-fold escalations in their mean levels respectively; the identity of methanol was confirmed by GC-MS analysis. As expected, there were no modifications to these tobacco smoking marker levels in control group participants following a corresponding 4.0 min. non-smoking period.

Conclusions: ^{1}H NMR analysis of human saliva provided much valuable information on the infiltration of toxins and further agents from cigarette smoke into this biofluid. The marked elevations in salivary methanol levels observed are of much concern in view of its documented toxicological properties and adverse health effects.

ABBREVIATIONS

NMR: Nuclear Magnetic Resonance; TSP: Sodium 3-(trimethylsilyl)propionate-2,2,3,3-d$_4$; WHO: World Health Organisation; CRUK: Cancer Research UK; IARC: International Agency for Research on Cancer; AD: Alcohol Dehydrogenase; FD: Formaldehyde Dehydrogenase; ETS: Environmental Tobacco Smoke

INTRODUCTION

The hazardous influence of tobacco smoking on global healthcare, economics and the environment are now extensively documented, and in 2012 the WHO reported that 12% of all deaths globally (in those aged >30 years) were attributable to tobacco smoking [1]. In 2012, this gave rise to a total of 5.7% of global healthcare spending being attributed to smoking-related diseases, and this equated to $467 million in total purchasing power parity [2]. There is an estimated 8 million smokers in the UK (reflecting 16% of the UK population) [3], and CRUK has a major goal to decrease the prevalence of smoking to 5% of the population or below by 2035 [2]. In order to enhance this process, further educational outputs, together with the study of toxins present in cigarette smoke, may serve to deter smoking, or indeed encourage smokers to terminate this highly addictive habit.

There are *ca.* 4,000 toxic chemical agents in tobacco smoke, and more than 50 carcinogens [4]. Indeed, gas phase constituents include carbon monoxide (CO), methanol, aldehydes such as acetaldehyde, acrolein and crotonaldehyde, further carbonyl compounds, hydrogen cyanide (HCN), acrolein, ammonia, acetone, hydrogen sulphide (H_2S), hydrocarbons and nitrosamines [5].

Methanol intoxication can give rise to a broad spectrum

of deleterious health effects, which include gastrointestinal distress and ocular disturbances, in addition to neurological complications [6]. Although common causes of methanol toxicity involve the ingestion of tainted alcohols such as 'moonshine' or commercial products containing this agent, i.e. anti-freeze [7], its inhalation and dermal absorption ascribable to occupational exposure or abuse are less common causes [7]. Safe workplace exposure limits for methanol in the atmosphere are 200 and 250 ppm for long and short-term exposures respectively [8].

Methanol is also a common product of the combustion of tobacco lignin. *In vivo*, it is oxidised by hepatic alcohol dehydrogenase (AD) to generate formaldehyde [6], which exerts severe toxicological and carcinogenic actions in humans in view of its aggressive chemical reactivity [9-11]. Moreover, formaldehyde is then sequentially metabolised to formate via the actions of formaldehyde dehydrogenase (FD); excessive levels of this secondary metabolite *in vivo* give rise to metabolic acidosis and blindness [12,13]. Formaldehyde is classified by the IARC as a group 1 carcinogen [14]. Moreover, there is evidence available that this toxin can target the brain via its ability to cross the blood-brain barrier, and it has been reported that exposure to it gives rise to dose-dependent neurotoxic effects in both humans and animals [15-18].

Interestingly, the same class 1 AD oxidises both methanol and ethanol, and this enzyme exhibits a 10-fold increase in its affinity for the latter [19]. Therefore, metabolism of methanol is much prolonged when expressed relative to that of ethanol. Moreover, if ethanol concentrations remain > 0.2g/l, methanol is not metabolised [19,20].

Our research group has previously documented the applications of high-resolution proton (^{1}H) NMR spectroscopy to the detection and quantification of biomolecules present in human saliva, e.g. [21,22]. This technique offers many advantages over alternative time-consuming and labour-intensive analytical methods since (1) it permits the rapid, non-invasive and simultaneous study of a very wide range of endogenous biomolecules and exogenous agents present in biofluids, and (2) generally requires little or no knowledge of sample composition prior to analysis. Moreover, chemical shift values, coupling patterns and coupling constants of resonances present in ^{1}H NMR spectra of such complex, multicomponent systems provide much valuable supporting molecular information regarding all chemical species detectable. At operating frequencies of 400-600 MHz, this technique has a sensitivity of $\leq 1 \times 10^{-5}$ mol.dm^{-3}, and where required, broad overlapping resonances arising from macromolecules such as proteins present may be routinely suppressed by the application of pre-selected spin-echo pulse sequences.

The above investigations have also previously reported the detection of methanol in human salivary supernatant samples. Since one source of methanol is the combustion of tobacco lignin species during cigarette smoking episodes, in this study we have employed high-resolution ^{1}H NMR analysis to detect and determine this toxin in intact human saliva arising from the smoking of a single tobacco cigarette. This study was performed in order to provide reliable estimates of smoking-induced increases the salivary methanol levels. Further cigarette smoking-derived

agents were simultaneously identified and, where appropriate, quantified. The bioanalytical advantages of the technique employed, and the toxicological ramifications of results acquired, are discussed with special reference to smoking-driven and passive exposures of humans to cigarette smoke.

MATERIALS AND METHODS

Fasted, unstimulated saliva specimens were collected from n = 13 healthy human participants (6 female, 7 male) with a mean ± SEM age of 27.2 ± 1.3 years (range 19-34), and who smoked a range of <1 to 40 cigarettes daily (although 40 was an unusual extreme), i.e. 12.3 ± 3.2 per day (mean ± SEM); a 95% confidence interval for this mean estimate was 5.2-19.3 cigarettes per day. The non-smoking control group (n = 7) had a mean age of 28.7 ± 4.1 years (range 20-54). Written informed consent was acquired from all participants, and this investigation was performed in accordance with the Declaration of Helsinki of 1975 (revised in 1983). It was approved by the Faculty of Health and Life Sciences Research Ethics Committee, De Montfort University, Leicester UK. Smoking participants (n=13) primarily underwent a 12.0 hr. overnight fasting/smoking abstention period (including a 7-8 hr. sleeping pattern), and provided two saliva samples, the first immediately after awakening in the morning and prior to the specified tobacco smoking episode, the second (matched) one immediately following the smoking of a single cigarette product (specifically, within 20 s of smoking termination). Estimates from a small sample of smoking participants revealed that the single cigarette smoking exercise took 3.8 ± 0.14 min. (mean ± SD).

Non-smoking (control) participants also underwent the above 12.0 hr. fasting period, and also provided two corresponding saliva samples, the first again immediately after awakening in the morning, the second following a 4.0 min. non-smoking period.

Saliva samples were immediately transported to the laboratory and prepared for ^{1}H NMR analysis by centrifuging at 10,000 rpm for a period of 10 min. at 4°C, and removal of the clear supernatant. Aliquots (0.50 ml) of these supernatants were then treated with 0.06 ml of pH 7.00 phosphate buffer (Acros Organics) containing 0.04% (w/v) sodium azide (Sigma-Aldrich) as a microbicide, and also 0.050 ml of ^{2}H$_2$O (Sigma-Aldrich) containing 0.050% (w/v) sodium 3-trimethylsilyl-(2,2,3,3-^{2}H$_4$)-1-propionate (TSP, Sigma-Aldrich), δ = 0.00 ppm. These mixtures were then thoroughly rotamixed and transferred to newly-purchased NMR tubes (Norell) ready for analysis. TSP served as an internal chemical shift reference and quantitative calibration standard (final concentration 2.38 x 10^{-4} mol.dm^{-3}), and ^{2}H$_2$O served as a field frequency lock. Where required, samples were stored at a temperature of -80°C for a maximum duration of 18 hr. prior to analysis.

^{1}H NMR spectra were acquired on a 400 MHz Bruker AV NMR spectrometer (Leicester School of Pharmacy, De Montfort University, Leicester, UK) operating at a frequency of 399.93 MHz. Each human salivary supernatant sample was analysed using water suppression (noesygppr1d) with irradiation at the water frequency (δ = 4.80 ppm) during the recycle and mixing time delays. 32K data points were acquired using 128 scans and 2 dummy scans, 3 μs pulses over a sweep width of 4,844 Hz, and a receiver gain of 128. An NMR autosampler device ensured that

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samples were continuously delivered throughout the analysis period.

Two-dimensional (2D) shift-correlated ^{1}H-^{1}H COSY spectra of human salivary supernatants were also acquired on this NMR facility using a modification of the standard sequence of Aue et al. [23].

Spectra were routinely assigned via considerations of chemical shift values, coupling patterns and coupling constants, together with comparisons with established literature values and reference spectra available on the the Human Metabolome Database [24].

Salivary methanol and propane-1,2-diol concentrations were determined by comparisons of their resonance areas with that of added TSP using ACD software. Since the protein concentration of human saliva (1.40-6.40 g.dm^{-3} [25]) is much lower than that of human blood plasma (65-83 g.dm^{-3} [26]), the very minimal broad macromolecule resonance envelope was found not to interfere with the observation and integration of the great majority of sharp low-molecular-mass resonances, and hence all salivary methanol and propane-1,2-diol concentrations documented represent those arising from such spectra.

Gas chromatographic-mass spectrometric (GC-MS) analysis of methanol in human salivary supernatants was performed on a Bruker 450 GC and 300 quadrupole MS facility. Methanol standards (Optima™ LC/MS Grade, Fisher Chemical) and the supernatant samples (1.00 μl) were injected using a Bruker CP-8400 autosampler. The GC Oven temperature was set at 40°C for a 5.0 min. period, was ramped up to 300°C at a rate of 10°C/min., and then held for a period of 20 min. at this temperature with a capillary flow rate of 1.50 ml/min. Separation was performed on a Zebron ZB-5MSI (30 m x 0.25 mm x 0.25 μm) column. The ion source temperature and the transfer line between the GC and MS facilities was set at 200°C, and the MS scan range at 20-200 m/z values. All data were analysed using a Bruker MS workstation version 7.0 software.

Paired Student's t-tests, and both Pearson and partial linear correlation analyses, were performed on untransformed ^{1}H NMR resonance bucket intensity data using XLSTAT2016 or 2017 software.

Additionally, a more detailed statistical analysis of the experimental ^{1}H NMR data acquired was performed using a multifactorial analysis-of-variance (ANOVA) experimental design (the mathematical model for this is provided in equation 1). In this model, T_i, S_j, $P_{(j)k}$ and G_l represent the between-sampling timepoint, between-smoking group (i.e. smokers *vs.* non-smokers), between-participant and between-gender factors, TS_{ij} the first-order T_i x S_j interaction effect, and e_{ijklm} the residual (unexplained error) variance component. All sources of variation in this model were fixed effects, with the exception of the between-participants one ($P_{(j)k}$), which was random and 'nested' within each smoking group (S_j).

$$y_{ijklm} = \mu + T_i + S_j + P_{(j)k} + G_l + TS_{ij} + e_{ijklm} \quad (1)$$

Since it was considered the most important, the sampling timepoint x smoking group first-order interaction effect (TS_{ij}) was the only one incorporated into this statistical analysis

model. Such an interaction effect was expected to be highly significant, since mean salivary methanol and propane-1,2-diol concentrations were expected to markedly increase following the smoking of a single cigarette product in the smoker participant group, but not so in the non-smoking control (smoking mimic) one.

A further experimental design was utilized in order to explore possible relationships beween both background and post-cigarette smoking salivary levels of methanol and propane-1,2-diol, and the estimated number of cigarettes smoked daily by participants within the smoking group. This form of analysis-of-covariance (ANCOVA) was performed for both the pre- and post-smoking sets of samples individually, and the mathematical model for this design is provided in equation 2, where C_i, G_j and CG_{ij} represent the estimated mean daily smoking frequency (quantitative variable), the between-gender (qualitative variable), and smoking frequency x gender interaction sources of variation respectively, and e_{ijk} the unexplained experimental error variance.

$$y_{ijk} = \mu + C_i + G_j + CG_{ij} + e_{ijk} \quad (2)$$

RESULTS AND DISCUSSION

Expanded 0.70-4.50 ppm regions of the 400 MHz single-pulse ^{1}H NMR spectra of typical pre-smoking (control) human salivary supernatant samples (Figure 1) contained many prominent, sharp resonances ascribable to a wide range of low-molecular-mass components. Indeed, signals assignable to short-chain organic acid anions, amino acids, amines and carbohydrates, etc. were readily observable. All resonances present in this higher field spectral region, together with their coupling patterns and assignments, are presented in Table 1.

^{1}H NMR analysis of salivary supernatants clearly demonstrated substantial increases in the concentrations of both methanol (s, δ = 3.38 ppm) and propane-1,2-diol (d, δ = 1.13 ppm; dd, δ = 3.45 and 3.54 ppm; and m, δ = 3.87 ppm) within 20 s of completing the smoking a single tobacco cigarette by study participants. Figure 1 shows the expanded 0.70-4.50 ppm regions of typical ^{1}H NMR spectra of these samples collected from 2 typical participants both before and after smoking a single cigarette product. Methanol is derived from the combustion of tobacco lignin, whereas propane-1,2-diol is commonly added as a humectant to cigarettes. Two-dimensional ^{1}H-^{1}H COSY spectra acquired on selected samples confirmed the identity of propane-1,2-diol as a salivary marker of tobacco smoking (i.e. clearly-linked δ = 1.13 (d), 3.45 (dd), 3.54 (dd) and 3.87 ppm (m) resonances were observed (data not shown). Also detectable in a small number of the post-smoking salivary supernatant samples (n = 2) was dihydroxyacetone (s, δ = 4.40 ppm), which is known to be a flavouring agent added to cigarette products.

GC-MS analysis confirmed the presence of methanol in saliva samples collected from participants following their single cigarette smoking exercise (Figure 2). Indeed, this analyte had the same retention time (1.31 min.) and m/z (32.1 for $CH_3OH^{•+}$) values as those observed for an authentic methanol calibration standard sample.

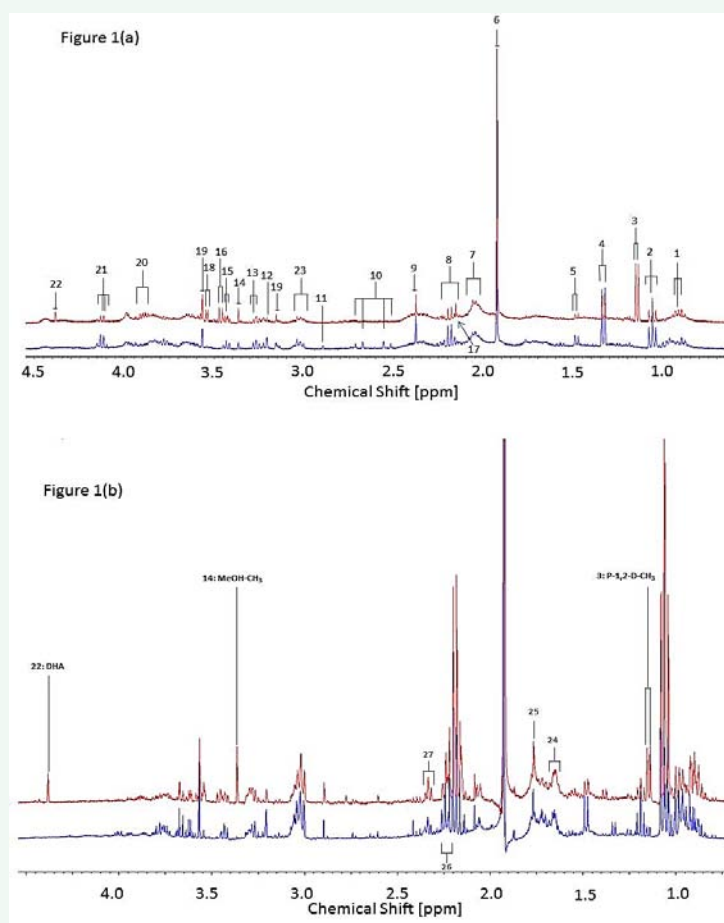Mean ± SEM salivary methanol concentrations determined prior and subsequent to performance of the single cigarette

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Figure 1 (a) Partial (0.70-4.50 ppm regions of) 400 MHz ^{1}H NMR spectra of human salivary supernatant samples collected from a human participant smoker prior (blue) and subsequent (red) to the smoking of a single cigarette product. Corresponding prior (blue) and subsequent (red) spectra from a second participant are shown in **(b).** Typical spectra are shown.

Abbreviations: Numerical abbreviation codes correspond to those provided in Table 1. The triplet and quartet resonances visible at 1.19 and 3.64 ppm respectively arise from the -C$\underline{\text{H}}_3$ and -C$\underline{\text{H}}_2$OH function protons of ethanol respectively.

Table 1: Chemical shift values, coupling patterns and assignments of resonances present in the 0.70-4.50 ppm regions of 400 MHz^{1}H NMR spectra acquired on the human salivary supernatant samples investigated. Spectral assignment labels correspond to those visible in Figure 1.

Spectral assignment label (Figure 1)	^{1}H NMR resonance chemical shift value (δ)/ppm	Coupling pattern	Assignment
1	0.92	*t*	*n*-Butyrate-CH$_3$
2	1.06	*t*	Propionate-CH$_3$
3	1.13	*d*	Propane-1,2-diol-CH$_3$
4	1.33	*d*	Lactate-CH$_3$
5	1.48	*d*	Alanine-CH$_3$
6	1.92	*s*	Acetate-CH$_3$
7	2.04	*s*	Glycoprotein-, Hyaluronate-, Oligosaccharide- and Free Aminosugar-NHCOCH$_3$ functions
8	2.18	*q*	Propionate-CH$_2$
9	2.38	*s*	Pyruvate-CH$_3$
10	2.62	AB coupling pattern	Citrate -CH$_2$CO$_2^-$
11	2.90	*s*	Trimethylamine-N(CH$_3$)$_3$
12	3.21	*s*	Choline-N(CH$_3$)$_3$
13	3.26	*t*	Taurine-CH$_2$NH$_3^+$
14	3.37	*s*	Methanol-CH$_3$
15	3.43	*t*	Taurine-CH$_2$SO$_3^-$

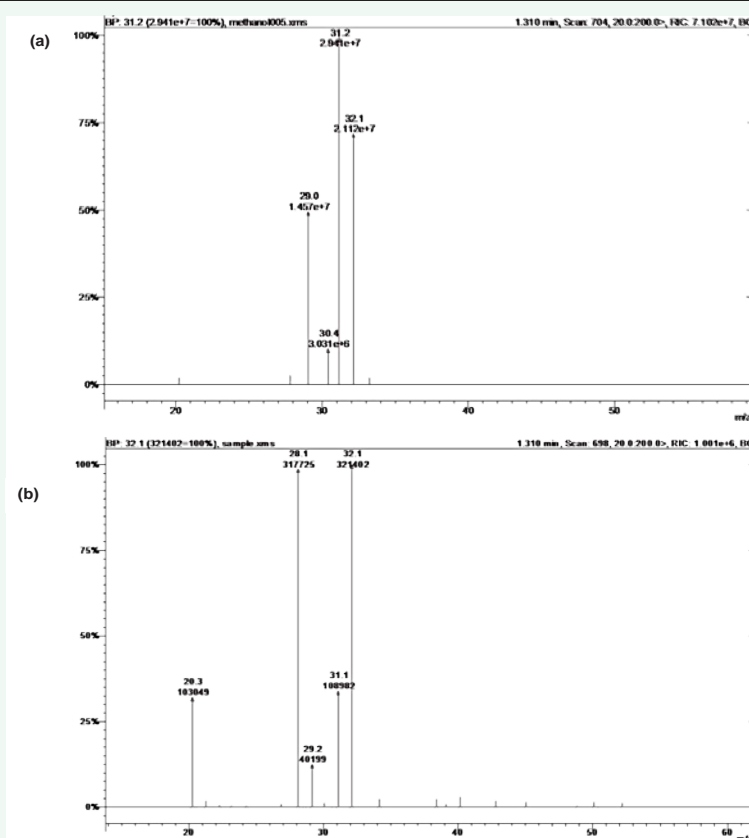| 16	3.45	*dd*	Propane-1,2-diol-CH_2OH
17	2.13	*s*	Methionine-SCH_3
18	3.54	*dd*	Propane-1,2-diol-CH_2OH
19	3.57	*s*	Glycine-CH_2
20	3.87	*m*	Propane-1,2-diol-CHOH
21	4.13	*q*	Lactate-CH
22	4.40	*s*	Dihydroxyacetone-CH_2OH
23	3.00-3.06	*t/s*	5-Aminopentanoate-5-CH_2/Creatinine-$N(CH_3)$
24	1.64	*m*	5-Aminopentanoate-3,4-CH_2
25	1.77	*s*	Senecioate-CH_3
26	2.23	*t*	5-Aminopentanoate-2-CH_2
27	2.36	*m*	Glutamate-γ-CH_2

Figure 2 GC-MS analysis and detection of methanol in human saliva (all experimental conditions are provided in the Materials and Methods section). (a) Mass spectrum of an authentic methanol standard solution (retention time 1.31 min.); the molecular ion of methanol ($CH_3OH^{\bullet+}$) has an m/z value of 32.1. (b) Corresponding mass spectrum of peak eluting at a retention time 1.31 min. in a corresponding GC-MS analysis of a typical human salivary supernatant specimen, confirming the presence of methanol with a molecular ion m/z value of 32.1.

smoking episode were 11 ± 3 (range 0-31: 6/13 samples had values below the limit of quantification, LOQ) and 410 ± 134 (range 33-1,800) $\mu mol.dm^{-3}$ respectively, and those for propane-1,2-diol were 134 ± 34 (range 12-506) and 427 ± 62 (range 116-854) $\mu mol.dm^{-3}$ respectively. These increases observed were both highly statistically significant (p = $<10^{-6}$ and 2.0×10^{-4} for methanol and propane-1,2-diol respectively: paired sample t-tests). 95% confidence intervals for corresponding matched mean increases were 140-658 and 147-437 $\mu mol.dm^{-3}$ for methanol and propane-1,2-diol respectively.

However, for the non-smoking control group, in which participants (n = 7) provided saliva samples both before and after a 4.0 min.'smoking mimic' period, mean ± SEM salivary methanol concentrations were only 4 ± 2.4 (range 0-14: 5/7 samples had values below the LOQ value) and 5 ± 2.6 (range 0-18) $\mu mol.dm^{-3}$ respectively. Corresponding non-smoking participant mean ± SEM salivary concentration values for propane-1,2-diol were 46 ± 28 (range 0-195: 4/7 samples had values below the LOQ value) and 23 ± 11 (range 0-78: 4/7 samples had values below the LOQ value) $\mu mol.dm^{-3}$ for the before and after smoking mimic delay timepoints, respectively. 95% confidence intervals for corresponding paired mean modifications in salivary methanol
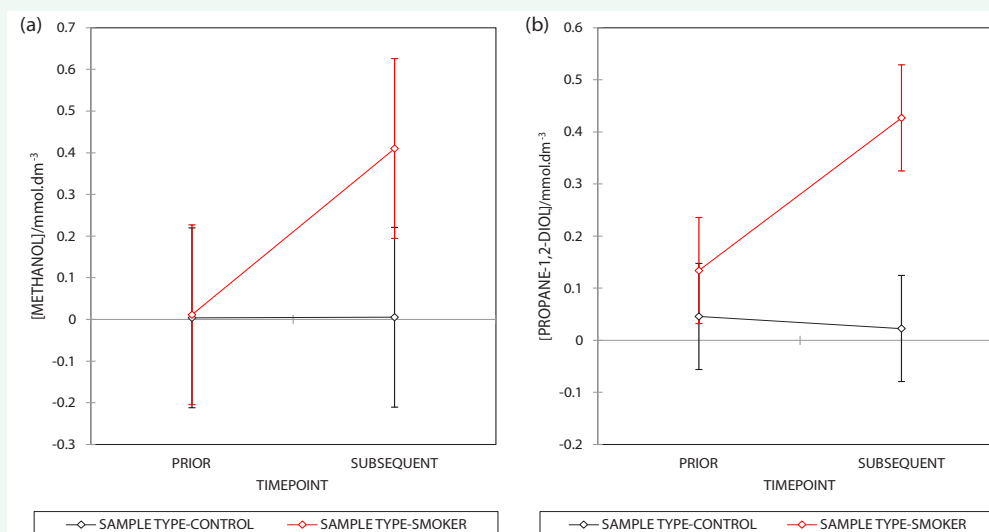
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Figure 3 Plots of means± 95% confidence intervals for salivary methanol and propane-1,2-diol concentrations [(a) and (b) respectively, mmol.dm^{-3}] prior and subsequent to perfoming a single cigarette smoking exercise by smokers (red), or to completing a 4.0 min. smoking mimic delay period by non-smokers (black).

and propane-1,2-diol levels were negligible (0 to 3 and -61 to 15 µmol.dm^{-3} respectively). Plots of mean ± 95% confidence intervals for salivary methanol and propane-1,2-diol levels for both the smoking and non-smoking control participants (i.e. before and after completing the single cigarette smoking regimen for the former group, and before and after the 4.0 min. smoking-mimic period for the latter one) are shown in Figure 3.

As expected, for the n = 13 smoking participants, there were no significant linear Pearson or partial correlations between the pre- and post-smoking salivary concentrations of these two alcohols (i.e. $p>0.05$), and nor were there significant positive ones between the post-smoking levels of methanol and propane-1,2-diol. These observations presumably reflect the variable [methanol]:[propane-1,2-diol] concentration ratios of inhalable cigarette smoke, which are expected to differ between differing cigarette tobacco products used by the smoking participants.

However, for the non-smoking control group, there was a very good agreement and strong correlations between between the pre- and post-smoking mimic exercise salivary supernatant levels of propane-1,2-diol (r = 0.9915, $p = 1.27 \times 10^{-5}$), as indeed we might expect from the short time period (*ca.* 4 min.) between these participants' saliva sample donations. It was not possible to explore such correlations for salivary methanol levels since most of these were below the LOQ value of this analyte with our ^{1}H NMR technique.

Statistical analysis of only the smoking participant group (n = 13) according to the model given in equation 2 demonstrated that there was no significant influence exerted by the estimated daily number of cigarettes smoked by participants on salivary methanol, nor propane-1,2-diol concentrations, both for the pre-and post-cigarette smoking specimens collected. These observations are not unexpected in view of the lengthy fasting/smoking cessation period of 12.0 hr. instigated for the purpose of this study. However, further investigations may be required to in

order to explore associations between salivary marker levels and cigarette smoking frequencies, especially in view of the limited number of smoking participants recruited for the purpose of this pilot study.

Our results demonstrate the value of high-resolution ^{1}H NMR analysis for the multicomponent investigations of human saliva, particularly for evaluating the effects of cigarette smoking on its molecular profile. Indeed, these results have revealed that this bioanalytical strategy is valuable for the detection and determination of agents derived from the inhalation of cigarette smoke, particularly toxic methanol, the mean salivary levels of which are elevated *ca.* 40-fold after participants completed the single cigarette smoking exercise.

Considerations of the numbers of cigarettes consumed on a daily basis and associated diurnal variations, together with further factors such as the volume of human saliva generated *in vivo* (estimates range from 0.75-1.50 litres daily) and its diurnal dependence, intra-oral access of this biofluid to cells and tissues, and salivary flow rates, etc. will serve to provide more detailed information on the quantities of cigarette smoking-derived methanol available to exert toxic actions in humans.

A single tobacco cigarette smoking exercise was selected for this study in order to facilitate estimations of the mean daily intakes of methanol and propane-1,2-diol toxins for light, medium, heavy and/or very heavy smokers (i.e. ≤ 3, 5-9, 10-19 and ≥ 20 cigarettes per day, respectively) in a sampled UK population. Typically, for an individual smoking a total of 10 cigarettes daily, the total estimated daily smoking-mediated salivary loads of methanol and propane-1,2-diol would be [(410-11) x 10 x (10/1000)] = 39.9 µmoles (equivalent to 1.3 mg) and [(427-134) x 10 x (10/1000)] = 29.3 µmoles (equivalent to 2.2 mg) respectively, assuming a total available salivary volume of 10.0 ml for each smoking episode.

Methanol levels in saliva have been previously found to be

useful for the indirect detection of alcohol intoxication [27]. In principle, determination of trace levels of this toxin in human saliva collected from verified non-smokers may conceivably be at least partially ascribable to the passive inhalation of cigarette smoke (i.e. ETS exposure), and experiments to explore this are currently in progress in our laboratory.

The determination of reliable biofluid markers remains one of the most objective and frequently employed strategies for determining smoking behavior and exposure to cigarette smoke, especially nicotine. Although cotinine determinations in saliva, blood and urine have been traditionally employed for this purpose, this approach has been hindered by selected complications. For example, only marginal correlations were found between salivary cotinine concentrations and questionnaire estimates of ETS exposure [28]. Therefore, the simultaneous analysis of a range of cigarette smoke-derived constituents in human saliva (particularly toxins such as methanol) in a metabolomics context may offer major opportunities and advantages for such explorations. Such metabolomics investigations should also be fully supported by the application of analytical techniques of a higher sensitivity than ^{1}H NMR analysis, e.g. GC-MS as utilized here for the confirmation of methanol's identity, and liquid chromatographic-mass spectrometric (LC-MS) approaches.

Recently, da Fonsecu et al. [29]. developed a method for the simultaneous determination of nicotine, cotinine and trans-3'-hydroxycotinine in oral fluid specimens using a GC-MS/MS strategy. This approach only required a 0.20 ml sample volume, and employed deuterated analogues as internal standards; standard calibrations were linear within the 0.50-1000 μg.dm^{-3} range.

However, with the exception of the upper extremes of these ranges, such concentrations are unfortunately beyond the sensitivity of high-resolution 'state-of-the-art' ^{1}H NMR analysis, and the higher levels readily quantifiable with the above LC-MS/MS technique may only be detectable using very high operating frequency NMR facilities (e.g., ≥ 700 MHz) coupled with cryogen-enhanced probe facilities. However, the application of techniques such a solid-phase extraction (SPE) strategies in order to pre-concentrate cigarette smoking-derived markers from larger salivary sample volumes than those employed here will undoubtedly enhance the detectability and monitoring of such analytes, and hence facilitate such investigations. Further experiments to explore this are currently in progress.

Furthermore, simpler and more cost-effective analytical approaches available employing colourimetric methods for the determination of salivary thiocyanate anion (SCN$^-$) are also available, although this marker analyte is putatively less specific in view of its availability in many foods, together with further cyanogens [30]. However, Pulishery et al. [31] found that the mean salivary levels of this marker were 175 ± 55 and 204 ± 46 mg.dm^{-3} (mean ± SD) in tobacco smokers and tobacco chewers respectively, values far exceeding those of non-tobacco users (98 ± 18 mg.dm^{-3}).

Moreover, the establishment of potentially valuable multicomponent bioanalytical strategies in order to establish multivariate correlations between salivary levels of tobacco smoke-derived agents via multidimensional statistical analysis techniques, such as principal component, correlated component regression or orthogonal partial least squares regression analyses, or alternatively via the application of suitable computational intelligence techniques (CITs), may indeed serve to distinguish between smokers and non-smokers, and potentially also be used to predict frequency of tobacco cigarette use of cigarettes by the former. Moreover, such metabolomics and associated pathway analyses of human saliva or oral biopsy specimens may also serve to provide valuable information regarding the nature and extent of any oral tissue damage induced by human smoking habits.

CONCLUSIONS

In conclusion, ^{1}H NMR analysis of human saliva provided valuable molecular information regarding the nature and concentrations of agents arising from the smoking of a single cigarette by human smoker participants, particularly methanol, propane-1,2-diol and, where present, dihydroxyacetone. Of critical importance is the observation of substantial increases in salivary methanol levels, which is a major concern in view of its high level of toxicity. These studies will pave the way for future studies focused on distinguishing between smokers and non-smokers, monitoring of the smoking frequencies of individuals, and also perhaps the exposure of humans to ETS. Further experiments are required to explore these enthralling research avenues.

ACKNOWLEDGEMENTS

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