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ORIGINAL ARTICLE

High-resolution ^{1}H NMR investigations of the oxidative consumption of salivary biomolecules by oral rinse peroxides

HUBERT CHANG[1], CHRISTOPHER J. L. SILWOOD[2], EDWARD LYNCH[3] & MARTIN GROOTVELD[2,3]

[1]*Willesden Green, London, UK,* [2]*Institute for Materials Research and Innovation (IMRI), University of Bolton, Bolton, UK, and* [3]*Warwick Dentistry, Warwick Medical School, University of Warwick, Warwick, UK*

Abstract

Background. A multicomponent evaluation of the oxidative consumption of salivary biomolecules by a tooth-whitening oral rinse preparation has been performed using high-resolution proton (^{1}H) nuclear magnetic resonance spectroscopy (NMR). ***Methods.*** Unstimulated human saliva samples ($n = 12$) were treated with aliquots of the oral rinse tested and 600 MHz ^{1}H NMR spectra acquired on these samples demonstrated that hydrogen peroxide (H_2O_2) and/or peroxodisulphate ($S_2O_8^{2-}$) present in this product gave rise to the oxidative decarboxylation of the salivary electron-donor pyruvate (to acetate and CO_2), and also oxidized methionine (a precursor to volatile sulphur compounds responsible for oral malodour), and malodourous trimethylamine to methionine sulphoxide and trimethylamine-N-oxide, respectively (reductions observed in the salivary concentrations of each biomolecular peroxide-scavenging agent were all extremely statistically significant, $p < 0.005$). ***Results.*** Experiments conducted on chemical model systems confirmed the consumption of pyruvate by this product, and also revealed that the amino acids cysteine and methionine were oxidatively transformed to cystine and methionine sulphoxide, respectively. ***Conclusions.*** High-field ^{1}H NMR analysis provides much valuable molecular information regarding the fate of tooth-whitening oxidants in human saliva and permits an assessment of the mechanisms of action of oral healthcare products containing these agents. The biochemical and potential therapeutic significance of the results obtained are discussed.

Key Words: *^{1}H NMR analysis, human saliva, oral rinse, oral healthcare product, hydrogen peroxide, peroxodisulphate, salivary electron-donors*

Introduction

The incorporation of hydrogen peroxide (H_2O_2), carbamide peroxide and further peroxo-adducts of inorganic anions such as peroxoborate, peroxodisulphate and peroxocarbonate as agents in commercially-available gels, toothpastes and oral rinses, as microbicidal and/or teeth-whitening agents, has stimulated much interest regarding their modes of action and redox activity in the oral environment [1–4]. Human saliva contains a wide range of biomolecular species, and many of those available with electron-donating properties have the capacity to directly or indirectly react with such peroxides and hence play a role in neutralizing their therapeutic or cosmetic actions *in vivo*. However, the analysis of salivary biomolecules with the ability to react with such peroxide-based oxidants by conventional methods (for example, those involving gas- or high-performance liquid-chromatographic techniques) generally requires the time-consuming, labour-intensive determination of pre-selected components. Indeed, these methods also require much information regarding the identity of particular salivary biomolecules prior to analysis and hence they generally offer only a limited characterization of the redox reactivity of H_2O_2 and/or related peroxo-adducts.

The multicomponent analytical ability of high-resolution nuclear magnetic resonance (NMR) spectroscopy, however, offers major advantages over the above conventional techniques [5,6]. The development of high-field NMR spectrometers with increased resolution, dynamic range and sensitivity has given rise to rapid advances in the analysis of

Correspondence: Martin Grootveld, Institute for Materials Research and Innovation (IMRI), University of Bolton, Deane Road, Bolton BL3 5AB, UK. E-mail: mg6@bolton.ac.uk

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complex, multicomponent samples such as human biofluids, pharmaceutical formulations, dentifrices and foodstuffs.

NMR spectroscopy is a technique which involves the absorption of energy from the radiofrequency region of the electromagnetic spectrum to detect changes in the alignment of nuclear magnets during exposure to a powerful external magnetic field. The absorption frequencies of such nuclei (e.g. those of biologically-ubiquitous hydrogen nuclei (^{1}H)) present in the ^{1}H NMR spectrum of a particular chemical species is critically dependent on their magnetic (and, therefore, chemical) environment and the appearance (multiplicity) of a resonance (signal) is influenced by neighbouring ^{1}H nuclei in a well-characterized manner. Furthermore, the intensity of each signal is directly proportional to the product of the number of magnetically-equivalent protons in the structural/functional group giving rise to it and the concentration of the molecule containing that group. Hence, much valuable molecular information regarding the nature and concentrations of a wide range of endogenous (and exogenous) components present in biofluids (e.g. human plasma [7,8], urine (some notable recent applications are shown in [9,10], (synovial fluid [11] and salivary supernatants [12]) can be simultaneously obtained from high-field, high-resolution NMR investigations. The broad overlapping resonances arising from any macromolecules (e.g. proteins) present in biofluid samples are routinely suppressed by the application of selected spin-echo pulse sequences, a process generating spectra containing well-resolved, sharp signals ascribable to many low-molecular-mass components (both endogenous and xenobiotic in origin) and the molecularly-mobile portions of macromolecules such as 'acute-phase' glycoprotein carbohydrate side-chains and lipoprotein-associated triacylglycerols. Biomedical NMR spectroscopy is a virtually non-invasive technique since it generally has little or no requirement for the pre-treatment of biofluid samples. Furthermore, it does not necessarily require knowledge of sample composition prior to analysis.

In this investigation we describe the application of high-resolution ^{1}H NMR analysis for the purpose of monitoring the oxidizing actions of an experimental carbamide peroxide (CP)- and peroxodisulphate ($S_2O_8^{2-}$)-containing oral rinse formulation towards biomolecules present in human saliva. The reactions of oxidizing components in this dentifrice preparation with appropriate, single-component chemical model systems (buffered aqueous solutions containing the salivary electron-donors pyruvate, L-methionine and L-cysteine) were also examined. The therapeutic, aesthetic and biochemical significance of the results obtained are discussed in detail.

Materials and methods

Sample collection and preparation

Unstimulated human saliva samples were obtained from a total of 12 healthy volunteers (seven male, five female). In order to avoid interferences arising from the introduction of exogenous agents into the oral environment, participants were requested to collect all saliva available, i.e. 'whole' saliva expectorated from the mouth, into a plastic universal collection tube immediately after waking in the morning on a pre-selected day. Each participant was also requested to refrain completely from oral activities (i.e. eating, drinking, tooth-brushing, oral rinsing, smoking, etc.) during the short period between awakening and sample provision (ca. 5 min). Full ethics approval for this investigation was obtained prior to its commencement, and informed consent was obtained for the participation of each volunteer involved.

Each collection tube contained sufficient sodium fluoride (15 μmol) in order to ensure that metabolites were not produced or consumed via the actions of micro-organisms or their enzymes present in whole saliva during periods of sample preparation and/or storage. Saliva specimens were transported to the laboratory on ice and then centrifuged immediately (16,000 g for 30 min) on their arrival to remove cells and debris, and the resulting supernatants were stored at –70°C for a maximum duration of 18 h prior to analysis. The pH values of each salivary supernatant were determined prior to ^{1}H NMR analysis (these ranged from 6.79–7.32).

The experimental oral rinse product investigated here (Janina Mouthrinse, Wisdom Dental Corporation) contained glycerol, sorbitol, trisodium citrate, sodium chloride, magnesium sulphate, methyl paraben, sodium saccharin, sodium peroxodisulphate, carbamide peroxide and sodium fluoride in order of decreasing percentage content (unspecified). However, the tooth-whitening agents disodium peroxodisulphate and carbamide peroxide (CP) were present at added levels of 0.20 and 0.19% (w/v), respectively.

Aliquots (0.60 ml) of each salivary supernatant were removed and 0.10 ml volumes of the above oral rinse formulation were then added *in vitro*. The mixtures were equilibrated at a temperature of 37°C for a 30 min period and then stored at –20°C for a maximum duration of 18 h prior to ^{1}H NMR analysis. Further 0.60 ml aliquots of each salivary supernatant sample treated with 0.10 ml volumes of HPLC-grade H_2O (previously 'sparged' with helium (He) gas for a 30 min period) and then equilibrated and stored in the same manner served as controls.

Aqueous solutions containing sodium pyruvate, L-cysteine or L-methionine (all 5.00×10^{-3} mol/dm^3) (Sigma Chemical Co. Ltd., Poole, Dorset, UK) were prepared in 4.00×10^{-2} mol/dm^3 phosphate buffer (pH 7.00), which was rigorously deoxygenated

by purging with He gas prior to use (30 min at ambient temperature); 1.00 ml aliquots of these solutions were treated with 0.10 ml volumes of the above oral rinse formulation as described above, the samples equilibrated at a temperature of 37°C for 30 min and then stored at a temperature of –20°C for a period of 18 h prior to ^{1}H NMR analysis. Matching salivary biomolecule-containing solutions treated with equivalent volumes of HPLC-grade H_2O in place of the oral rinse, equilibrated at 37°C (30 min) and stored in the same manner served as controls.

Proton (^{1}H) NMR measurements

Proton (^{1}H) NMR measurements on the above samples were conducted on a Bruker Avance AX-600 spectrometer (Queen Mary University of London facility, London, UK) operating at a frequency of 600.13 MHz and a probe temperature of 298 K. Typically, 0.60 ml of sample was placed in a 5-mm diameter NMR tube and 0.07 ml of ^2H_2O was added to provide a field frequency lock. The intense water signal (δ = 4.80 ppm) was suppressed by presaturation via gated decoupling during the delay between pulses. Where appropriate, the broad protein resonances present in control and oral rinse-treated salivary supernatant samples were suppressed by the Hahn spin-echo sequence (*D*[90°*x*-*t*-180°*y*-*t*-collect])[11], which was repeated 128 times (t = 68 ms). Chemical shifts were referenced to external sodium 3-trimethylsilyl [2,2,3,3–$^2H_4$] propionate (TSP; δ = 0.00 ppm). Where present, the methyl group resonances of acetate (singlet, δ = 1.920 ppm), alanine (doublet, δ = 1.487 ppm) and lactate (doublet, δ = 1.330 ppm) served as secondary internal references for the saliva samples investigated.

For single-pulse, one-dimensional (1D) ^{1}H NMR spectra acquired on control and oral rinse-treated solutions of pyruvate and the amino acids L-cysteine and L-methionine, typical pulsing conditions were 64 free induction decays (FIDs) using 32,768 data points, 72° pulses and a 3 s pulse repetition rate to allow full spin-lattice (T_1) relaxation of the protons in the samples investigated. Chemical shifts were referenced to TSP (internal; final concentration 2.50×10^{-4} mol/dm^3) and exponential line-broadening functions of 0.30 Hz were employed for purposes of processing.

The identities of biomolecule resonances present in the salivary ^{1}H NMR spectra acquired were routinely assigned by a consideration of chemical shift values, coupling patterns and coupling constants. The relative intensities of selected signals therein were determined by electronic integration via the spectrometer's proprietary software (XWIN-NMR), and the concentrations of components detectable were determined by comparisons of their resonance areas with that of a 4.20×10^{-2} mol/dm^3 standard solution of TSP located within a coaxial NMR tube insert. This procedure was employed to avoid any broadening of the TSP resonance which may arise from its binding to salivary proteins or alternative macromolecules. Maintenance of the exact integral regions for each spectrum acquired was ensured.

Two-dimensional (2D) shift-correlated ^{1}H–^{1}H NMR (COSY) spectra of human salivary supernatants were acquired using the standard sequence of Aue et al. [13] with 2048 data points in the t_2 dimension, 256 increments of t_1, a 3.00 s relaxation delay and 64 transients.

Statistical analysis of salivary biomolecule concentration data

The statistical significance of differences observed between salivary pyruvate, methionine and trimethylamine concentrations was determined by a paired sample t-test performed on untransformed data (based on 11 degrees-of-freedom for n = 12 participants).

Results

High-resolution ^{1}H NMR analysis of human salivary supernatants

As previously observed [12], 600 MHz single-pulse ^{1}H NMR spectra of control (untreated) human salivary supernatant samples contained many prominent, sharp resonances ascribable to a wide range of low-molecular-mass biomolecules. Indeed, signals assignable to short-chain organic acid anions (e.g. acetate, *iso*- and *n*-butyrates, formate, fumarate, lactate, propionate, pyruvate, succinate and 3-D-hydroxybutrate), carbohydrates such as glucose and galactose (both α- and β-anomers) are observable (the high- and low-field regions of a typical spectrum acquired in this investigation are shown in Figures 1A and B, respectively). The organic acid anions detectable are, of course, microbial catabolic products [14], and hence the salivary concentrations of these agents (either individually, two or more in concert or many in a multivariate context), are likely to serve as chemotaxonomic indicators of microbial activity in the oral environment. For example, the pathogenic micro-organism *P. gingivalis* generates high concentrations of *n*-butyrate, propionate and acetate, and smaller levels of *iso*-butyrate, *iso*-valerate, phenylacetate and succinate [15]. Also of notable interest is a broad resonance located at 2.04 ppm which arises from the acetamido methyl group protons (-NHCOCH_3) of N-acetyl sugars present in the molecularly-mobile branching carbohydrate side-chains of 'acute phase' glycoproteins [16]. This broad resonance overlaps several sharper acetamido-CH_3 group ^{1}H signals attributable to low-molecular-mass

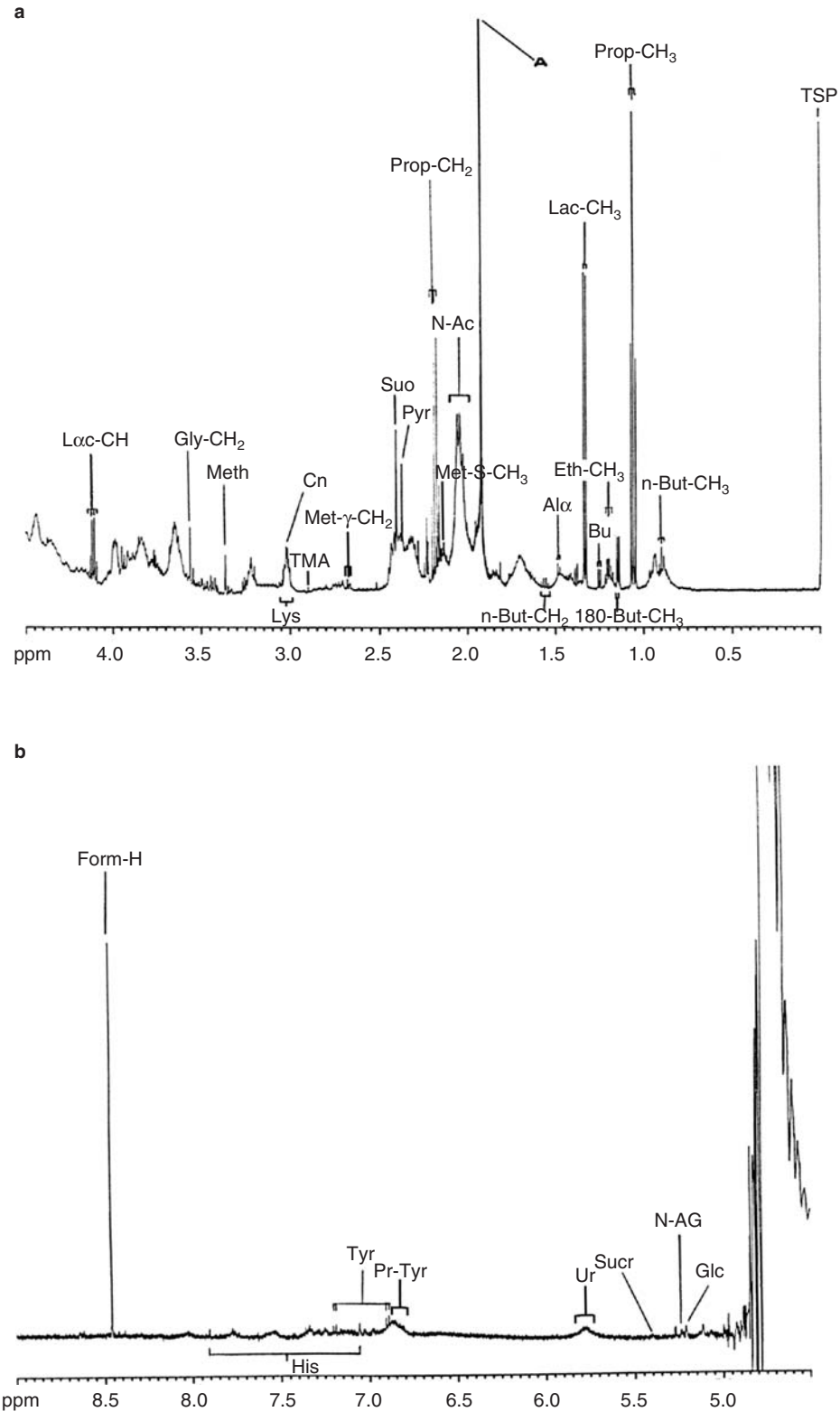

Figure 1. (A) High- and (B) low-field regions of a 600 MHz ^{1}H NMR spectrum of a human salivary supernatant sample (a typical spectrum is shown). Abbreviations: A, acetate-CH_3; Ala, alanine-CH_3; Bu, 3-D-hydroxybutyrate-CH_3; *iso*-But, *iso*-butyrate-CH_3; *n*-But-CH_3, *n*-butyrate-CH_3; *n*-But-CH_2, *n*-butyrate-CH_2; Cn, creatinine-N-CH_3; Eth, ethanol-CH_3; Form, formate-H; Gluc, α-glucose anomeric proton; Gly, glycine-CH_2; His, histidine imidazole ring protons; Lac-CH_3 and ϵ-CH, lactate-CH_3 and -CH protons, respectively; Lys, lysine -CH_2 group; Met-S-CH_3 and γ-CH_2, methionine-S-CH_3 and γ-CH_2 protons, respectively; Meth, methanol-CH_3; N-Ac, spectral region for acetamido methyl groups (i.e. –$NHCOCH_3$) of N-acetylsugars (the sharper signals are conceivably ascribable to free N-acetylglucosamine and/or N-acetylneuraminate or to low-molecular-mass oligosaccharide fragments containing these sugars, the broader one(s) to those present in the molecularly-mobile portions of N-acetylated glycoproteins); N-AG, N-acetylglucosamine anomeric ring proton; Phe, phenylalanine aromatic ring protons; Prop-CH_3 and -CH_2, propionate-CH_3 and -CH_2, respectively; Pyr, pyruvate-CH_3; Suc, succinate-CH_2; Sucr, sucrose-H1 glucose residue proton; Tyr, 'free' tyrosine aromatic ring protons; Ur, urea H_2N-CO-NH_2; Pr-Tyr, aromatic ring protons of protein tyrosine residue(s); TSP, sodium 3-trimethylsilyl [2,2,3,3-$^2H_4$] propionate-Si$(CH_3)_3$.

N-acetylsugars such as N-acetylneuraminate and N-acetylglucosamine saccharide fragments which may arise from the actions of bacterial-derived neuraminidase and hyaluronidase, respectively [17].

Also noteworthy is the ability of this technique to detect a range of amino acids (including alanine, glycine, histidine, lysine, methionine, phenylalanine and tyrosine), some of which, in view of their relatively low concentrations, were only clearly visible in expanded partial spectra of the human salivary supernatant specimens. Methionine, detectable by its characteristic side-chain -SCH_3 group signal (singlet, δ = 2.13 ppm, Figure 1A) is of particular interest to this investigation since it is one of the ^{1}H NMR-detectable biomolecules which was found to be affected by addition of the oral rinse formulation.

Furthermore, micromolar (μM) concentrations of a series of malodourous amines (specifically methylamine, dimethylamine and trimethylamine, singlet signals located at 2.59, 2.75 and 2.92 ppm, respectively) were also monitorable in the spectra acquired, and the trimethylamine resonance is also of particular relevance to this study since it is oxidizable by peroxides and/or peroxo-adducts present in the oral rinse formulation tested.

Both ethanol and methanol were detectable in a large proportion of the human saliva samples subjected to ^{1}H NMR analysis. Although ethanol is a bacterial-derived catabolite (for example, arising from carbohydrate metabolism by *Streptococcus mutans*) [18], the methanol present arises from the passive or direct inhalation of cigarette smoke in which this exogenous alcohol is present, a consequence of the combustion of tobacco lignin which contains many methoxy aromatic substituents in its macromolecular structure. Urea was also detectable in all salivary supernatant spectra acquired (prominent broad resonance located at δ = 5.80 ppm).

The aromatic (δ = 6.50–8.00 ppm) regions of these spectra contained clearly visible resonances ascribable to the amino acids phenylalanine and tyrosine and, for some of the samples, histidine (Figure 1B). Also visible were two broad signals centred at ca. 6.90 and 7.55 ppm, the former of which is assignable to the aromatic ring protons of one or more protein-incorporated tyrosine residues (M Grootveld, unpublished data).

The 2D ^{1}H–^{1}H COSY NMR technique served to facilitate the identification of molecular 'backbones'. Indeed, the COSY spectroscopic technique permits the transfer of magnetization from an ^{1}H nucleus (or magnetically-equivalent group (two or three) of such nuclei) bonded to one specific carbon atom (C2 position), to one or two classes of magnetically-distinct nuclei located at one carbon position further along a molecular chain (i.e. C1 and C3 position, etc.). Specifically, the latter nucleus/nuclei is/are directly coupled to the C2-bearing ^{1}H nucleus.

2D ^{1}H–^{1}H COSY NMR spectra of typical human salivary supernatant samples showed clear connectivities between the multiplet resonances present, for example, linkages between the propionate-CH_3 and -CH_2 group signals (triplet, δ = 1.04 ppm and quartet, δ = 2.17 ppm, respectively), the *n*-butyrate-CH_3, β-CH_2 and γ-CH_2 group resonances (triplet, δ = 0.91 ppm, multiplet, δ = 1.56 ppm and triplet, δ = 2.15 ppm, respectively), the ethanol-CH_3 and -CH_2 groups (triplet, δ = 1.21 ppm and quartet, δ = 3.68 ppm) and the 'free' tyrosine aromatic ring protons (doublets located at δ = 6.88 and 7.17 ppm) (data not shown).

Multicomponent ^{1}H NMR evaluations of the oxidative consumption of salivary components by H_2O_2 and $S_2O_8^{2-}$ present in the oral rinse formulation

Typical 600 MHz single-pulse ^{1}H NMR spectra of a human saliva sample acquired prior and subsequent to its *in vitro* treatment with an aliquot of the oral rinse preparation are shown in Figure 2. Clearly, addition of the CP- and $S_2O_8^{2-}$-containing product gives rise to a marked reduction in the intensity of the pyruvate-CH_3 group signal (singlet, δ = 2.388 ppm), an observation reproducible in all saliva samples tested in this manner (n = 12). These data are fully consistent with the oxidative consumption of salivary pyruvate by $S_2O_8^{2-}$ and/or hydrogen peroxide (H_2O_2) present in the formulation (equations 1 and 2, respectively) [19]. Consistent with these observations, a resonance assigned to the -CH_3 group protons of pyruvate hydrate (singlet, δ = 1.50 ppm) was also substantially diminished in intensity subsequent to treatment of the salivary supernatants with the oral rinse product.

$$S_2O_8^{2-} + CH_3COCO_2^- + H_2O \rightarrow 2SO_4^{2-} + CH_3CO_2^- + CO_2 + 2H^+ \quad (1)$$

$$H_2O_2 + CH_3COCO_2^- \rightarrow CH_3CO_2^- + CO_2 + H_2O \quad (2)$$

A paired sample *t*-test revealed an extremely highly significant difference between the mean salivary pyruvate concentrations determined before and after treatment with the oral rinse product ($p < 10^{-12}$, Table I).

Although not observed in all ^{1}H NMR spectra acquired on salivary supernatant samples before treatment with the oral rinse formulation (7 out of the 12 examined), also particularly notable was the oxidative transformation of the amino acid methionine to its corresponding sulphoxide species by oxidants present in the oral rinse formulation examined here. Indeed, L-methionine's characteristic -S-CH_3 group ^{1}H resonance (singlet, δ = 2.13 ppm) was substantially diminished in intensity subsequent to treatment with this

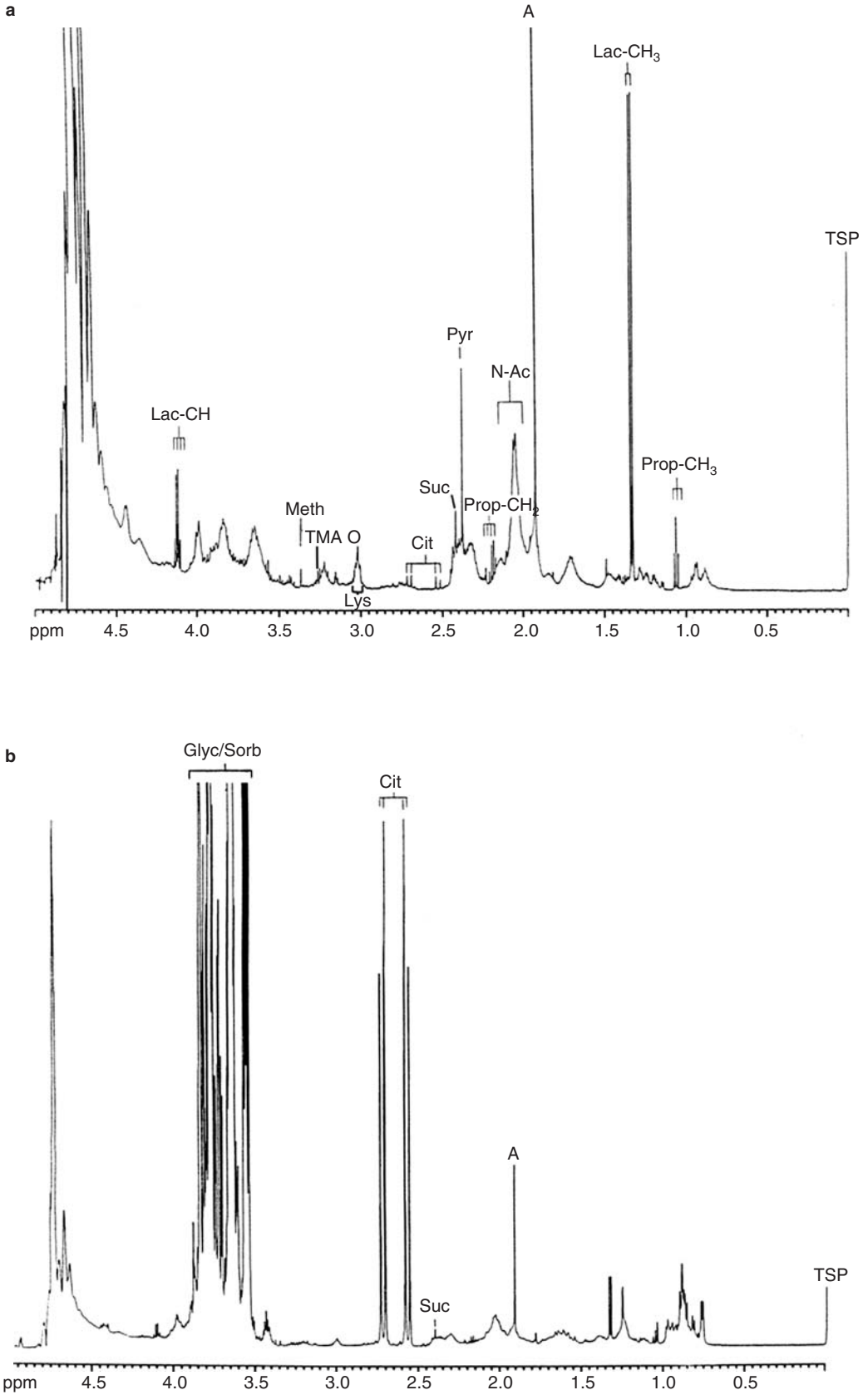

Figure 2. 0.00–5.00 ppm regions of 600.13 MHz single-pulse ^{1}H NMR spectra of an unstimulated human salivary supernatant sample (pH 6.91) acquired (A) before and (B) after treatment with the H_2O_2- and $S_2O_4^{2-}$-containing oral rinse formulation investigated. The control spectrum shown in (A) was treated with an equivalent volume of de-aerated doubly-distilled H_2O prior to analysis. Corresponding 5.00–10.00 ppm regions of the spectra exhibited in (A) and (B) are shown in (C) and (D), respectively. Typical spectra are shown. Abbreviations: as Figure 1, with Cit, citrate-CH_2CO_2 group; Glyc, glycerol-CH_2OH and -CHOH group protons; MP, methylparaben (4-hydroxybenzoic acid methyl ester) aromatic ring protons; Sac, saccharin aromatic ring protons; Sorb, sorbitol-CH_2OH and -CHOH protons.

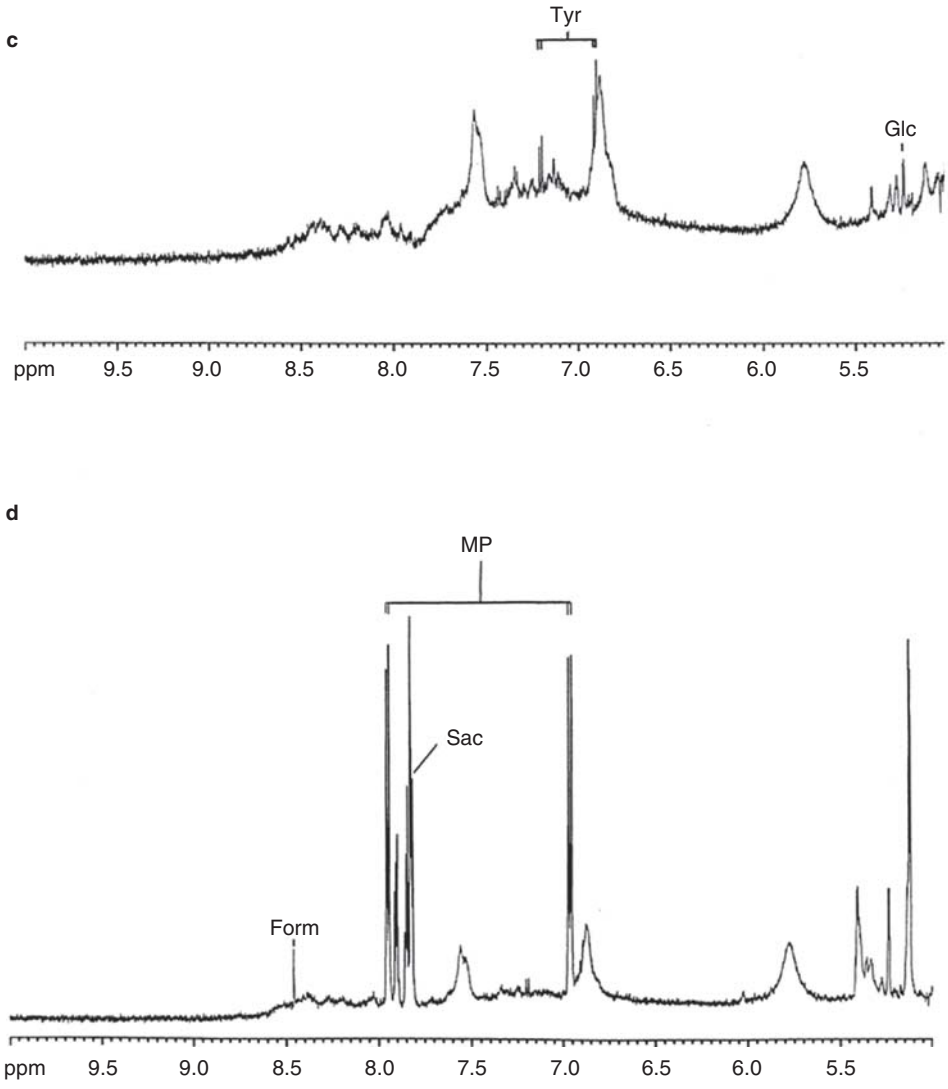

Figure 2. (*Continued*).

product, as outlined above. These decreases in its salivary concentration were matched by corresponding elevations in that of its sulphoxide (monitored by the intensity of its -SO-CH_3 group signal located at δ = 2.725 ppm (equations 3 and 4)), as shown in Figure 3. The mean (pre- minus post-treatment) difference between these salivary methionine concentrations was very highly significant ($p < 10^{-8}$, Table I).

$$S_2O_8^{2-} + {}^{+}H_3NCH(CH_2CH_2SCH_3)CO_2^{-} + H_2O \rightarrow 2SO_4^{2-} + {}^{+}H_3NCH(CH_2CH_2SOCH_3)CO_2^{-} + 2H^{+} \quad (3)$$

$$H_2O_2 + {}^{+}H_3NCH(CH_2CH_2SCH_3)CO_2^{-} \rightarrow {}^{+}H_3NCH(CH_2CH_2SOCH_3)CO_2^{-} + H_2O \quad (4)$$

Additionally, a highly-significant decrease in the intensity of the salivary trimethylamine (TMA) resonance (singlet, δ = 2.92 ppm) was observed subsequent to treatment with the oral rinse product tested ($p < 0.005$, Table I). This observation is explicable by the oxidation of this malodourous biomolecule by oxidants present in the oral rinse tested to its non-malodourous oxidation product trimethylamine-N-oxide (clearly identifiable by increases in its ON-$(CH_3)_3$ group ^{1}H NMR signal located at 3.24 ppm, equations 5 and 6)). Typical ^{1}H NMR results which demonstrate this are also exhibited in Figure 3.

$$S_2O_8^{2-} + N(CH_3)_3 + H_2O \rightarrow 2SO_4^{2-} + ON(CH_3)_3 + 2H^{+} \quad (5)$$

$$H_2O_2 + N(CH_3)_3 \rightarrow ON(CH_3)_3 + H_2O \quad (6)$$

Moreover, the salivary lactate resonances (doublet, δ = 1.33 and quartet, δ = 4.13 ppm, -CH_3 and -CH protons respectively) were also found to be diminished in intensity following the treatment of salivary supernatants with the oral rinse formulation in the manner outlined above; the mean percentage

Table I. Mean (± SEM) salivary supernatant concentrations of pyruvate, methionine and trimethylamine prior to and subsequent to treatment of this biofluid with the oral rinse product investigated. The *p*-value represents the significance of the differences in these biomolecule concentrations (paired sample *t*-test performed on untransformed data).

	Pyruvate	Methionine	Trimethylamine
Sample size, *n*	12	7	11
Mean pre-treatment concentration, mol/dm^3	$4.33 \pm 0.68 \times 10^{-3}$	$1.49 \pm 0.26 \times 10^{-4}$	$9.60 \pm 1.70 \times 10^{-5}$
Mean post-treatment concentration, mol/dm^3	$1.10 \pm 0.37 \times 10^{-4}$	$3.40 \pm 0.78 \times 10^{-5}$	$6.70 \pm 1.39 \times 10^{-5}$
p-value (paired sample *t*-test)	$< 10^{-12}$	$< 10^{-8}$	< 0.005

decrease in the normalized intensity of these signals was ± 21 ± 6% (mean ± SEM).

Further oral rinse-mediated modifications to the salivary ^{1}H NMR profiles included the oxidation of both free tyrosine and also its salivary protein residues, which were monitorable by reductions in the intensities of their resonances (6.88 and 7.17 ppm (free tyrosine) and a broad resonance located at 6.90 ppm (protein-incorporated tyrosine)).

As expected, spectra of product-treated salivary supernatants contained many intense resonances assignable to components present in this product (e.g. glycerol, citrate, methyl paraben, saccharin, etc.), together with marked elevations in the intensities of those present in both matrices (specifically formate and acetate). Of course, the small rise in the acetate-CH_3 group signal intensity observed following oral rinse treatment is ascribable to the $S_2O_8^{2-}$ and/or H_2O_2–mediated oxidative decarboxylation of pyruvate as described above.

Of further interest to this investigation was the observation that the urea signal at $\delta = 5.80$ ppm increased in intensity following treatment with the oral rinse product in all salivary supernatant specimens examined (Figure 2). This observation was, of course, expected since the H_2O_2 in this formulation is added as CP ($H_2NCONH_2.H_2O_2$), a crystallisable 1:1 addition product of this oxidant with urea.

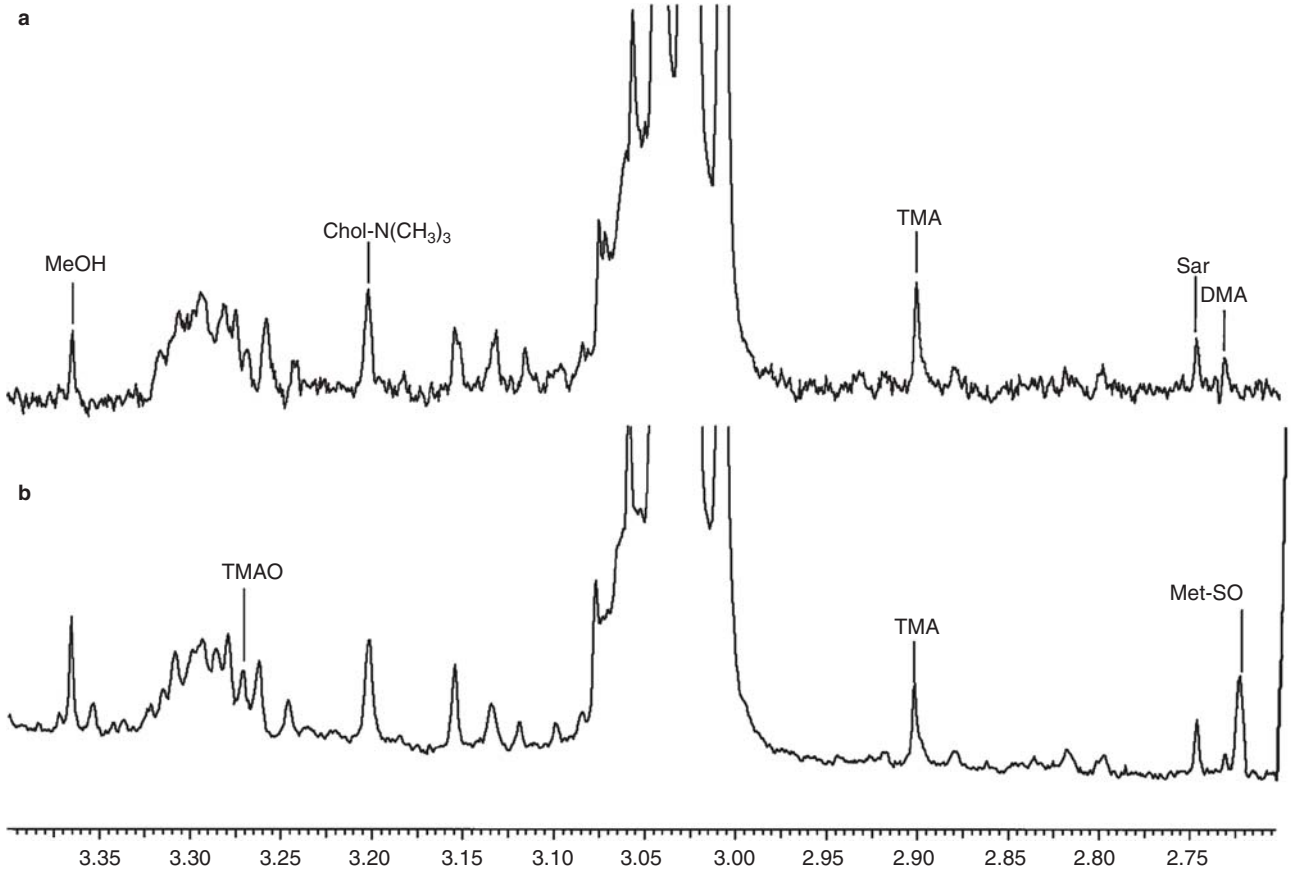

Figure 3. Expanded 2.70–3.40 ppm regions of an unstimulated human salivary supernatant sample (pH 7.06) acquired (A) prior to and (B) subsequent to treatment with the H_2O_2- and $S_2O_4^{2-}$-containing oral rinse formulation investigated. Typical spectra are shown. Abbreviations: as Figure 1, with Chol-N$(CH_3)_3$, choline-N$^+(CH_3)_3$; DMA, dimethylamine-N$(CH_3)_3$; Met-SO, methionine sulphoxide-CH_3; Sar, sarcosine-NH-CH_3; TMA, trimethylamine-N$(CH_3)_3$; TMAO, trimethylamine-N-oxide-ON$(CH_3)_3$.

Chemical model studies of the reactions of salivary electron donors with oral rinse-containing oxidants

Aqueous standard solutions of pyruvate were treated with aliquots of the oral rinse product in order to provide further information concerning the nature and mechanism of the $S_2O_8^{2-}$- and H_2O_2-mediated oxidation of this α-keto acid anion in human saliva. ^{1}H NMR analysis revealed that reaction of a 1.00 ml volume of pyruvate solution (5.00×10^{-3} mol/dm^3) with 0.10 ml of the above oral rinse formulation gave rise to an almost complete conversion of the α-keto acid anion scavenger to acetate and CO_2 (Figure 4). Indeed, >95% of the pryuvate was oxidatively decarboxylated to acetate and CO_2 under the experimental conditions utilized. Moreover, as expected, a singlet resonance ascribable to pyruvate hydrate ($CH_3C(OH)_2CO_2^-$, methyl group singlet at δ = 1.50 ppm) was also eliminated from the spectrum subsequent to treatment with the product tested here.

Reaction of L-cysteine with peroxodisulphate (or peroxomonosulphate arising from its hydrolysis) and/or H_2O_2 present in the oral rinse tested yielded its corresponding disulphide cystine as a major oxidation product (data not shown), an observation consistent with equations (7) and (8), where RSH represents $^+H_3NCH(CH_2SH)CO_2^-$.

$$2S_2O_8^{2-} + 2RSH \rightarrow 2SO_4^{2-} + RSSR + 2H^+ \quad (7)$$

$$\left[SO_3(O-O)\right]^{2-} + 2RSH \rightarrow SO_4^{2-} + RSSR + H_2O \quad (8)$$

^{1}H NMR analysis also demonstrated that the thiomethyl group (-S-CH_3)-containing amino acid L-methionine was oxidatively consumed by oxidants present in the oral rinse product examined, yielding methionine sulphoxide (-SO-CH_3 group singlet, δ = 2.725 ppm) as the predominant product (Figure 5). Indeed, under our experimental conditions, treatment of this solution with the added oral rinse gave rise to an ~ 40% conversion of methionine to its sulphoxide, the latter of which was completely undetectable in ^{1}H NMR spectra of the untreated amino acid. These processes are fully consistent with the reactions depicted in equations (3) and (4) above.

Discussion

Multicomponent ^{1}H NMR investigations of the oxidizing ('therapeutic') ability of the oral rinse formulation investigated here demonstrated that critical salivary electron-donors are readily consumed by $S_2O_8^{2-}$ and H_2O_2 therein, together with any further oxidants derived from hydrolysis of the former. Indeed, on consideration of the total oxidant concentration of the product examined here, it might be expected that, in concentration terms, the oxidants available will effectively 'swamp' most of the salivary electron donor activity available. Indeed, the mean salivary concentrations of pyruvate (a two-electron donor) and thiols (a single electron provider) are 4.33×10^{-3} (this work) and 3.6×10^{-5} mol/dm^3 [20], respectively.

Previous investigations have demonstrated that biofluid pyruvate acts as a powerful endogenous electron-donor (i.e. a water-soluble antioxidant) and is oxidatively decarboxylated to acetate and CO_2 on reaction with hydrogen peroxide (H_2O_2), as represented in equation (2) [19].

$S_2O_8^{2-}$ is an extremely powerful oxidizing agent (i.e. the redox potential (E_0) value for the system depicted in equation (9) is +2.01 V). However, its reactions are mechanistically very complex. For example, hydrolysis of $S_2O_8^{2-}$ generates peroxomonosulphate ($[SO_3(O\text{-}O)]^{2-}$) (equation 10), a further powerful oxidant which can also oxidatively decarboxylate pyruvate to acetate and CO_2.

$$S_2O_8^{2-} + 2e^- \rightarrow 2SO_4^{2-} \quad (9)$$

$$S_2O_8^{2-} + H_2O \rightarrow SO_4^{2-} + \left[SO_3(O-O)\right]^{2-} + 2H^+ \quad (10)$$

The extent of oxidative consumption of each of the three major electron-donating biomolecules found to be involved in the scavenging of the two available oral rinse oxidants was pyruvate (97.5%) > methionine (77.2%) > TMA (30.5%). Therefore, in view of the much greater salivary concentration of pyruvate over that of both methionine and TMA, it is clear that the major H_2O_2- and $S_2O_8^{2-}$-scavenger present in human saliva is this α-keto acid anion.

The consumption of salivary lactate by oral rinse oxidants observed here can be rationalized by consideration of the availability of trace levels of low-molecular-mass, catalytic iron(II), copper(I) or cobalt (II) complexes in these specimens, which react with oral rinse H_2O_2 to generate a hydroxyl radical ($^\bullet$OH) (equation 11) which can directly oxidize this microbial catabolite to pyruvate, which, in turn, can then directly be further oxidized (by $S_2O_8^{2-}$, H_2O_2 or further $^\bullet$OH radical, predominantly $S_2O_8^{2-}$ and H_2O_2) to acetate and CO_2 (equations 1, 2 and 12); these particular redox-active metal ions may be retained in their lower oxidation states (reduced forms) by the availability of a range of electron-donors in human saliva (notably ascorbate, urate, thiocyanate, thiols, etc.). Similarly, electron donation to $S_2O_8^{2-}$ and/or its peroxomonosulphate hydrolysis product by these 'catalytic' metal ions can also generate reactive radical species with the ability to oxidize lactate to pyruvate and, subsequently, the latter to acetate and CO_2.

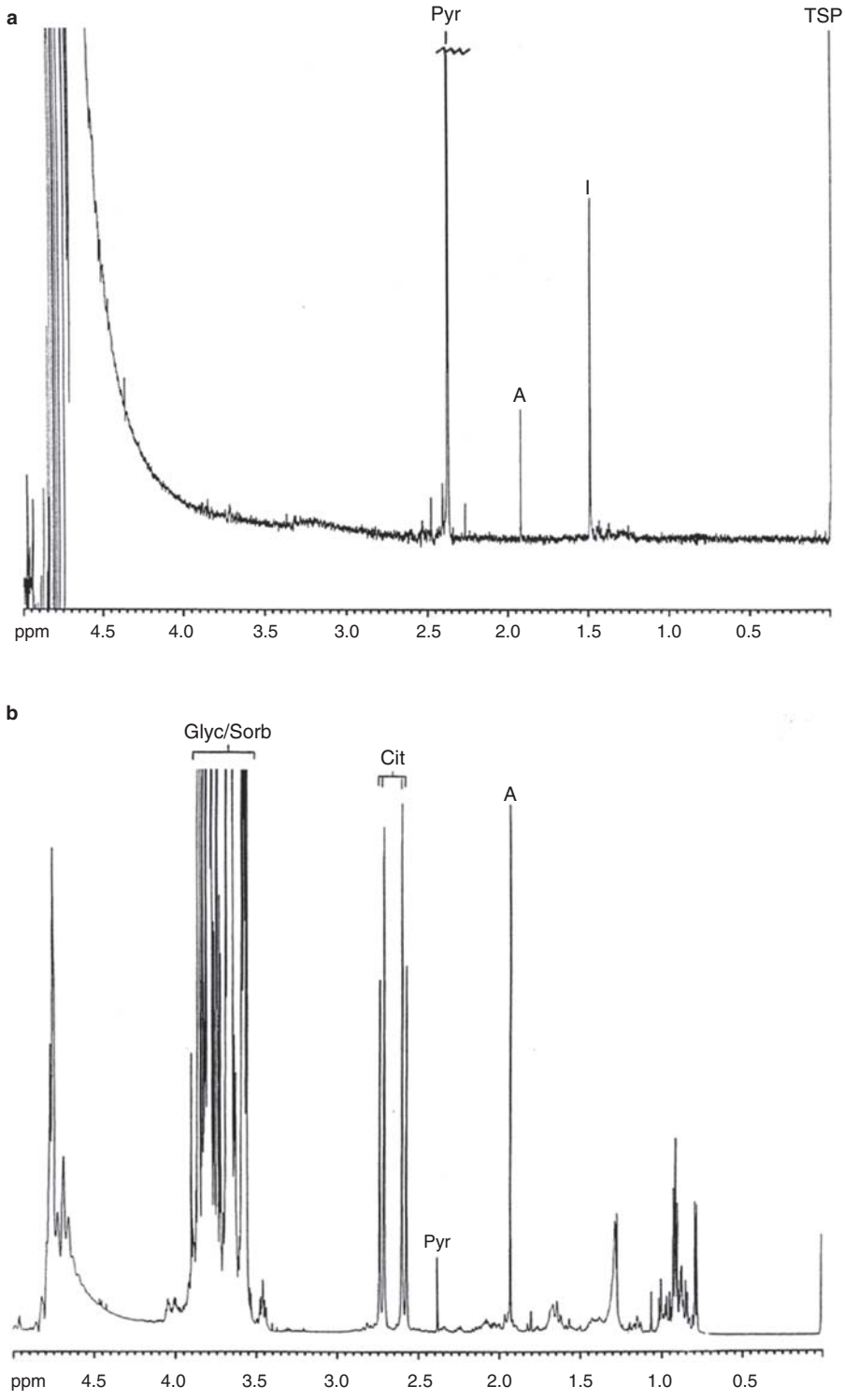

Figure 4. 0.00–5.00 ppm regions of 600.13 MHz ^{1}H NMR spectra of a 5.00×10^{-3} mol/dm^3 aqueous solution of sodium pyruvate (in 4.00×10^{-2} mol/dm^3 phosphate buffer, pH 7.00) acquired subsequent to treatment with 0.10 ml volumes of (A) de-aerated HPLC-grade H_2O (control sample) and (B) the oral rinse formulation investigated here. Typical spectra are shown. Abbreviations: as Figures 1 and 2, with I representing pyruvate hydrate-CH_3.

$$M^{n+} + H_2O_2 \rightarrow M^{(n+1)+} + {}^{\bullet}OH + OH^- \quad (11)$$

$$CH_3CHOHCO_2^- + 2{}^{\bullet}OH \rightarrow CH_3COCO_2^- + 2H_2O \quad (12)$$

After making appropriate allowances for thermodynamic equilibria and the rate of each reaction involved under physiological conditions, this indicates that reductants present at relatively low salivary levels (e.g. the amino acids cysteine and methionine) will

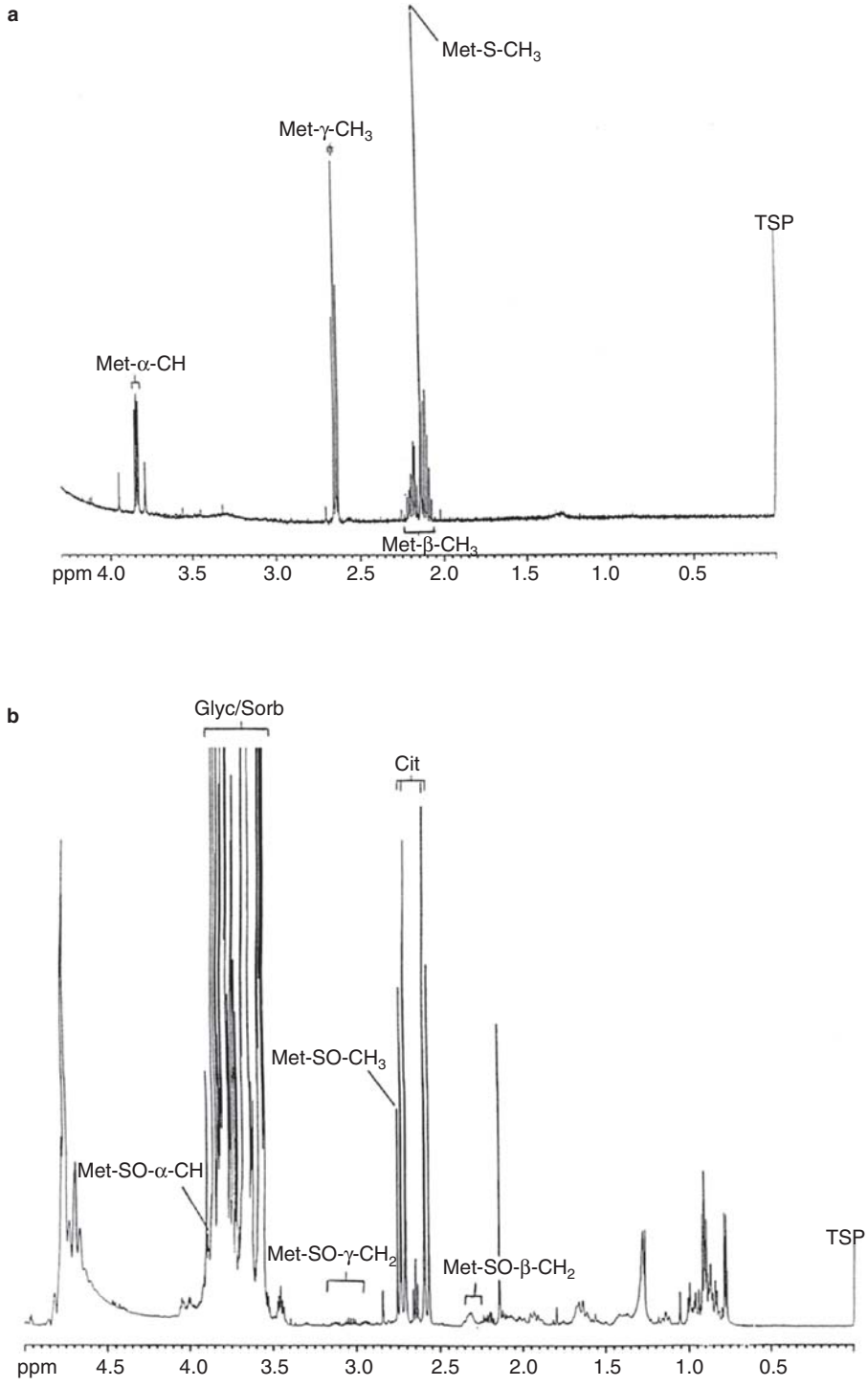

Figure 5. Partial (0.00–4.30 ppm regions of) 600.13 MHz ^{1}H NMR spectra of a 5.00×10^{-3} mol/dm^3 aqueous solution of L-methionine (in 4.00×10^{-2} mol/dm^3 phosphate buffer, pH 7.00) obtained following treatment with 0.10 ml volumes of (A) de-aerated HPLC-grade H_2O (control sample) and the oral rinse product investigated here. Typical spectra are shown. Abbreviations: as Figure 2, with Met-S-CH_3, -α-CH, β-CH_2 and γ-CH_2, methionine-S-CH_3, -α-CH, β-CH_2, and γ-CH_2 group proton resonances, respectively; Met-SO-CH_3, -α-CH, β-CH_2 and γ-CH_2, methionine sulphoxide-SO-CH_3, -α-CH, β-CH_2 and γ-CH_2 group proton resonances, respectively.

also be oxidatively consumed during oral health-care programmes with oral rinse products which contain H_2O_2 or alternative peroxo-adducts such as $S_2O_8^{2-}$ present the oral rinse preparation investigated here.

Of course, H_2O_2 can also give rise to the oxidation of available salivary thiols, a relatively complex reaction system which can be catalysed by the presence of trace levels of redox-active transition metal ions, notably those of iron and copper (equations 13–17).

$$M^{(n+1)+} + e^- \rightarrow M^{n+} \qquad (13)$$

$$M^{n+} + H_2O_2 \rightarrow M^{(n+1)+} + {}^{\bullet}OH + OH^- \quad (14)$$

$${}^{\bullet}OH + RSH \rightarrow RS^{\bullet} + H_2O \quad (15)$$

$$2RS^{\bullet} \rightarrow RSSR \quad (16)$$

$$M^{(n-1)+} \leftrightarrow M^{n+} \text{ catalyst overall}: 2RSH + H_2O_2 \rightarrow RSSR + H_2O \quad (17)$$

Consumption of cysteine and methionine by H_2O_2 and $S_2O_8^{2-}$ species is of much importance to oral hygiene and clinical periodontology since, following the bacterially-mediated proteolysis of proteins containing these amino acids, both the malodourous VSCs methyl mercaptan (CH_3SH) and hydrogen sulphide (H_2S) are generated from them via metabolic pathways operational in gram-negative micro-organisms, one involving the enzymes cystine reductase (reduction of cystine to cysteine) and serine sulphydrase (desulphydration of cysteine yielding H_2S and serine) [21]. CH_3SH accounts for ~ 60% of the VSCs detectable.

In addition to the major aesthetic problems caused by oral malodour (halitosis), there is currently much evidence available to suggest that VSCs adversely contribute towards the periodontal disease process. Indeed, since it has the ability to take part in thiol-disulphide interchange reactions (equation 18), CH_3SH influences enzymic and immunological activities in manners that give rise to periodontal tissue destruction [22] and also enhances the permeability of oral mucosa, the latter representing a process which promotes the penetration of hazardous components such as endotoxins into tissues [23]. At concentrations similar to those generated in periodontal pockets, CH_3SH has the ability to exert effects regarding the activity and integrity of cells, e.g. the modification of cell shape and cytoskeletal patterns, diminishment of cell proliferation and migration, the alteration of collagen metabolism and interference with protein synthesis [22]. Additionally, VSCs have been found to inhibit wound healing processes [24].

$$R_1SH + R_2SSR_2 \leftrightarrow R_2SH + R_1SSR_2 \quad (18)$$

In addition to serving as an essential precursor to VSCs, it is also of much importance to note that, since methionine is one of only two amino acids encoded by a single codon (AUG) in the standard genetic code (tryptophan being the other), its consumption by oxidants present in the oral rinse formulation evaluated here is of much bactericidal significance. Indeed, an N-formylmethionine derivative serves as a primary amino acid adduct for this purpose in bacteria [25].

Trimethylamine (TMA) is an extremely malodourous agent and its excessive excretion into human breath is responsible for the odour of rotting marine fish characteristic of subjects with 'fish-breath disorder' (trimethylaminuria) [26]. We have previously reported that TMA is readily detectable in saliva acquired from subjects without this condition [17] and, in view of the known ability of H_2O_2 to oxidize this agent to its corresponding non-malodourous N-oxide, it was not unexpected that CP and $S_2O_8^{2-}$ present in the oral healthcare product tested here gave rise to its oxidation in human saliva.

These results demonstrate that peroxo-adducts present in the oral rinse tested oxidized salivary TMA to its corresponding N-oxide, data providing evidence for the ability of this product to combat recalcitrant oral malodour arising from this agent.

However, the limited (30%) decrease in its salivary concentration observed presumably reflects the more powerful capacity of further salivary electron-donors (predominantly pyruvate, methionine and perhaps cysteine) to scavenge H_2O_2 and $S_2O_8^{2-}$.

High-resolution, high-field ^1H NMR spectroscopy is a technique which offers many advantages over alternative time-consuming, labour-intensive analytical methods since (1) it permits the rapid, non-invasive and simultaneous examination of a very wide range of components present in biofluids (e.g. human saliva as outlined in this study) and (2) generally requires little or no knowledge of sample composition prior to analysis. Furthermore, chemical shift values, coupling patterns and coupling constants of resonances present in ^1H NMR spectra of such multi-component systems provides much valuable information regarding the molecular nature of both endogenous and exogenous chemical species therein.

As demonstrated here, the technique is of much value and utility concerning multicomponent assessments of the interactions of therapeutically-active agents present in commercially-available oral healthcare products with human salivary biomolecules, and the oxidative decarboxylation of salivary pyruvate by H_2O_2 and peroxosulphate adducts in the oral rinse evaluated serves as an important fundamental example of this, which may be of some relevance to its mechanisms of action. Indeed, since pyruvic acid is a very powerful proton donor ($K_a = 3.20 \times 10^{-3}$ mol/dm^3) [27], it may play a role in facilitating tooth demineralization processes. In view of the ready diffusion of such organic acids into enamel [28], the consumption of salivary pyruvate by peroxo-oxidants present in the product examined here may suppress the development and progression of primary root caries lesions. Hence, $S_2O_8^{2-}$ and H_2O_2 may exert caries-preventative actions, and further experiments to explore this are currently underway.

The nature, rate and extent of salivary electron donor consumption (e.g. that of VSCs, their amino acid precursors and pyruvate) by oxidants present in oral healthcare products reflects the oxidizing capacity of such materials, a phenomenon of much significance

with regard to their therapeutic, aesthetic and cosmetic roles. Therefore, high-resolution ^{1}H NMR analysis of isolated human saliva or appropriate chemical model systems serves as a very useful method for the *in vitro* testing of oral healthcare products.

Indeed, ^{1}H NMR analysis of human saliva samples obtained prior to and subsequent to the administration of oral rinses or toothpastes to patients with periodontal diseases (i.e. in *in vivo* investigations) may demonstrate oral healthcare product-mediated modifications in the levels of microbial-derived salivary catabolites (e.g. short-chain non-volatile carboxylic acid anions such as acetate, *n*-butyrate, formate, fumarate, lactate, propionate, pyruvate, etc.), which, in turn, may reflect the removal of potentially pathogenic micro-organisms following treatment regimens and recent pilot studies conducted by the authors have revealed that, when linked with multivariate chemometric methods, this technique is readily applicable to this class of investigation.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

References

[1] Thomas EL, Milligan TW, Joyner RE, Jefferson MM. Antibacterial activity of hydrogen peroxide and the lactoperoxidase-hydrogen peroxide-thiocyanate system against oral streptococci. Infect Immun 1994;62:529–35.

[2] Miyasaki KT, Genco RJ, Wilson ME. Antimicrobial properties of hydrogen peroxide and sodium bicarbonate individually and in combination against selected oral, gram-negative, facultative bacteria. J Dent Res 1986;65:1142–8.

[3] Lynch E, Samarawickrama DYD, Claxson AWD, Hawkes J, Atherton M, Naughton DP, et al. Safety aspects concerning the therapeutic and cosmetic applications of hydrogen peroxide (H_2O_2)-containing gels, whiteners, oral rinses and dentifrices. J Ir Dent Assoc 1994;40:78–82.

[4] Lynch E, Sheerin A, Atherton M, Hawkes J, Haycock P, Grootveld MC. Molecular mechanisms associated with the bleaching actions of commercially-available whitening oral health care products. J Ir Dent Assoc 1996;41: 94–102.

[5] Grootveld M, Sheerin A, Atherton M, Millar AD, Lynch EJ, Blake DR, et al. Applications of high resolution NMR analysis to the study of inflammatory diseases at the molecular level. In Clark RJH, Hester RE, editors. Biomedical applications of NMR spectroscopy, advances in spectroscopy volume 25. Chicester, UK: John Wiley and Sons; 1996. p 295–327.

[6] Evans JNS. Biomolecular NMR spectroscopy. Biophys J 1996;70:2996–7.

[7] Nicholson JK, Foxall PJ, Spraul M, Farrant RD, Lindon JC. 750 MHz ^{1}H and ^{1}H-^{13}C NMR spectroscopy of human blood plasma. Anal Chem 1995;67:793–811.

[8] Savorani F, Kristensen M, Larsen FH, Astrup A, Englesen SB. High throughput prediction of chylomicron triglycerides in human plasma by nuclear magnetic resonance and chemometrics. Nutr Metab 2010;7:43–9.

[9] Zhang S, Zheng C, Lanza IR, Sreekumaran Nair K, Raftery D, Vitek O. Interdependence of signal processing and analysis of urine. ^{1}H NMR Spectra for metabolic profiling. Anal Chem 2009;81:6080–8.

[10] Willem GEJ, Schoonen C, Kloks PAM, Ploemen J-P HTM, Smit MJ, Zandberg P, et al. Uniform procedure of ^{1}H NMR analysis of rat urine and toxicometabonomics part II: comparison of NMR profiles for classification of hepatotoxicity. Toxicol Sci 2007;98:286–97.

[11] Naughton D, Whelan M, Smith EC, Williams RB, Blake DR, Grootveld M. An investigation of the abnormal metabolic status of synovial fluid from patients with rheumatoid arthritis by high field proton nuclear magnetic resonance spectroscopy. FEBS Lett 1993;317:135–8.

[12] Grootveld M, Silwood CJ. ^{1}H NMR analysis as a diagnostic probe for human saliva. Biochem Biophys Res Commun 2005;329:1–5.

[13] Aue WP, Bartholdi E, Ernst RR. Two-dimensional spectroscopy. Application to nuclear magnetic resonance. J Chem Phys 1976;64:2229–47.

[14] Guerrant GO, Lambert MA, Moss CW. Analysis of short-chain acids from anaerobic bacteria by high-performance liquid chromatography. J Clin Microbiol 1982;16:355–60.

[15] Gibson FC, Genco A. The genus porphyromonas. In Dworkin M, Falkow S, Rosenberg E, Schleifer K-H, Stackebrandt E, editors. The Prokaryotes volume 7: proteobacteria: delta, epsilon subclass (part 6). Singapore: Springer; 2006. p 428–54.

[16] Bell JD, Brown JCC, Nicholson JK, Sadler PJ. Assignment of resonances for 'acute-phase' glycoproteins in high resolution proton NMR spectra of human blood plasma. FEBS Lett 1987;215:311–15.

[17] Silwood CJL, Lynch E, Claxson AWD, Grootveld MC. ^{1}H and ^{13}C NMR spectroscopic analysis of human saliva. J Dent Res 2002;81:422–7.

[18] Williams RAD, Elliot JC. Basic and applied dental biochemistry. Edinburgh: Churchill Livingstone; 1979.

[19] Herz H, Blake DR, Grootveld M. Multicomponent investigations of the hydrogen peroxide- and hydroxyl-radical scavenging antioxidant capacities of biofluids: the roles of endogenous pyruvate and lactate. Free Radic Res 1997;26: 19–35.

[20] Grigor J, Roberts AJ. Reduction in the levels of oral malodor precursors by hydrogen peroxide: *in vitro* and in vivo assessments. J Clin Dent 1992;III:111–15.

[21] Tonzetich G, Carpenter PAW. Production of volatile sulphur compounds from cysteine, cysteine and methionine by human dental plaque. Arch Oral Biol 1971;16:599–607.

[22] Brunette DM. Effects of baking-soda-containing dentifrices on oral malodour. Compend Contin Educ Dent 1996;17 (Suppl 19):225–35.

[23] Ng W, Tonzetich J. Effect of hydrogen sulphide and methyl mercaptan on the permeability of oral mucosa. J Dent Res 1984;63:994–7.

[24] Yaegaki K. Oral malodour and periodontal disease. In Rosenberg M, editor. Bad breath: research and perspectives. Tel Aviv: Ramot; 1995. p 88–108.

[25] Thach RE, Dewey KF, Brown JC, Doty P. Formylmethionine codon AUG as an initiator of polypeptide synthesis. Science 1966;153:416–18.

[26] Mitchell S. Trimethylaminuria (fish-odour syndrome) and oral malodour. Oral Dis 2005;11(Suppl 1):10–13.

[27] Martell AE, Motekaitis RJ. The determination and use of stability constants. Weinheim: VCH; 1988.

[28] Featherstone JDB, Rodgers BE. Effect of acetic, lactic and other organic acids on the formation of artificial caries lesions. Caries Res 1981;15:377–85.