

**HIGH RESOLUTION NMR INVESTIGATIONS OF THE OXIDATIVE
CONSUMPTION OF SALIVARY BIOMOLECULES BY A TOOTH-
WHITENING MOUTHRINSE**

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ABSTRACT

A multicomponent evaluation of the oxidative consumption of salivary biomolecules by a tooth-whitening mouthrinse preparation has been performed using high resolution proton nuclear magnetic resonance spectroscopy. Unstimulated human saliva samples (n = 10) were treated with aliquots of a solution derived from the above product and 600 MHz ^1H NMR spectra acquired on these samples demonstrated that hydrogen peroxide (H_2O_2) and/or peroxodisulphate ($\text{S}_2\text{O}_8^{2-}$) effected the oxidative decarboxylation of the salivary electron-donor pyruvate (to acetate and CO_2). Experiments conducted on chemical model systems confirmed the consumption of pyruvate by this product, and also revealed that the amino acids cysteine and methionine (precursors to volatile sulphur compounds responsible for oral malodour) were oxidatively transformed to cystine and methionine sulphoxide respectively. In conclusion, high field proton 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 health care products containing these agents. The biochemical, periodontal and potential therapeutic significance of the results obtained are discussed.

1 INTRODUCTION

The incorporation of hydrogen peroxide (H_2O_2), carbamide peroxide and peroxy-adducts of inorganic anions such as peroxoborate, peroxodisulphate and peroxocarbonate as agents for the bleaching of discoloured teeth in commercially-available gels, toothpastes and oral rinses has evoked much interest concerning their modes of action and redox activity in oral environments^{1,2}. However, the analysis of salivary biomolecules with the capacity 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 generally offer only a limited characterisation of the redox reactivity of H_2O_2 and/or related peroxy- adducts.

The multicomponent analytical ability of high resolution nuclear magnetic resonance (NMR) spectroscopy, however, offers major advantages over the above conventional techniques (reviewed in ^{3,4}). The development of high field NMR spectrometers with increased resolution, dynamic range and sensitivity has given rise to rapid advances in the analysis of 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 (^1H)] present in the ^1H 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 ^1H nuclei in a well characterised way. 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 components present in biofluids (e.g., human plasma^{5,6}, urine^{7,8}, synovial fluid⁹ and salivary supernatants¹⁰) 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 spin-echo pulse sequences, a procedure generating spectra containing well-resolved, sharp signals ascribable to a multitude of low-molecular mass components (endogenous or exogenous), 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 has little or no requirement for the pre-treatment of samples. Moreover, it does not necessarily require knowledge of sample composition prior to analysis.

In this investigation we describe the application of high resolution ¹H NMR spectroscopy to the determination of the oxidising actions of a commercially-available carbamide peroxide- and peroxodisulphate-containing tooth-whitening formulation towards biomolecules present in human saliva. The reactions of oxidising 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, periodontal, aesthetic and biochemical significance of the results obtained are discussed in detail.

2 *MATERIALS AND METHODS*

2.1 Sample Collection and Preparation

Unstimulated human saliva samples were obtained from a total of 10 healthy volunteers (6 male, 4 female). Subjects were seated comfortably and then asked to collect all saliva into a cup for a period of 10 min. Immediately after collection, all samples were centrifuged at 16,000 x g for 30 min. (4°C) to remove debris.

The tooth-whitening oral rinse product investigated here (Janina mouthrinse, Wisdom Dental Corporation.) contained glycerol, sorbitol, trisodium citrate, sodium chloride, magnesium sulphate, peroxodisulphate, carbamide peroxide and sodium fluoride in order of decreasing percentage content (unspecified).

Aliquots (0.60 ml) of each salivary supernatant were removed and 0.10 ml aliquots of the above tooth-whitening formulation were then added. The mixtures were equilibrated at a temperature of 37°C for a 30 min. period and then stored at -20°C for a duration of 18 hr. prior to ¹H NMR analysis. Further 0.60 ml aliquots of each salivary supernatant sample treated with 0.01-0.03 ml volumes of HPLC-grade H₂O (previously sparged with helium gas for a 30 min. period) and then incubated and stored in the same manner served as controls.

Aqueous solutions containing sodium pyruvate (5.00×10^{-3} mol. dm⁻³), L-cysteine (5.00×10^{-3} mol. dm⁻³) or L-methionine (5.00×10^{-3} mol. dm⁻³) (Sigma Chemical Co. Ltd., Poole, Dorset, U.K.) were prepared in 4.00×10^{-2} mol. dm⁻³ phosphate buffer (pH 7.00) which was rigorously deoxygenated by purging with helium gas prior to use (30 min. at ambient temperature). 1.00 ml aliquots of these solutions were treated with 0.010 ml of dentifrice formulation as described above, the samples equilibrated at a temperature of 37°C for 30 min. and stored at -20°C for a period of 18 hr. prior to ¹H NMR analysis. Matching reductant-containing solutions treated with equivalent volumes of HPLC-grade H₂O in place of the mouthrinse, equilibrated at 37°C (30 min.) and stored in the same manner, served as controls.

2.2 Proton NMR Measurements

Proton (¹H) NMR measurements on the above samples were conducted in Bruker AMX-400 [University of London Intercollegiate Research Services (ULIRS), Kings College Facility, University of London, U.K.] or Bruker AMX-600 (ULIRS, Queen Mary and Westfield College Facility, University of London, U.K.) spectrometers operating at frequencies of 400.13 and 600.13 MHz respectively 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 $^2\text{H}_2\text{O}$ was added to provide a field frequency lock.

The intense water signal ($\delta = 4.8$ ppm) was suppressed by presaturation via gated decoupling during the delay between pulses. Where appropriate, the broad protein resonances present in control and dentifrice supernatant-treated salivary supernatant samples were suppressed by the Hahn spin-echo sequence (D[$90^\circ\text{x-t-}180^\circ\text{y-t-collect}$])¹¹ which was repeated 128 times ($t = 68$ ms). Chemical shifts were referenced to external sodium 3-trimethylsilyl [2,2,3,3- $^2\text{H}_4$] propionate (TSP ; $\delta = 0.00$ ppm). Where present, the methyl group resonances of alanine ($\delta = 1.487$ ppm) and lactate ($\delta = 1.330$ ppm) served as secondary internal references for the saliva samples examined.

For single-pulse (1D) ^1H NMR spectra acquired on control and dentifrice supernatant-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°C 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 ; 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 ^1H 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, 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 broadening of the TSP resonance which arises from its binding to salivary proteins or alternative macromolecules.

Two-dimensional shift-correlated ^1H - ^1H NMR (COSY) spectra of human salivary supernatants were acquired on the Bruker AMX-400 facility using the standard sequence of Aue *et. al.* with 2.048 data points in the t_2 dimension, 256 increments of t_1 , a 3.00 s relaxation delay, and 64 transients.

3 RESULTS

3.1 High Resolution ^1H NMR Analysis of Human Salivary Supernatants

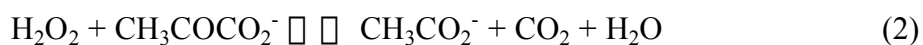
As previously documented¹⁰, 600 MHz single-pulse ^1H NMR spectra of control (untreated) human salivary supernatant samples, contained many prominent, sharp resonances ascribable to wide range of low-molecular-mass components. Indeed, signals assignable to short-chain organic acid anions (e.g., acetate, *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 1(a) and (c) respectively]. The organic acid anions detectable are, of course, products derived from microbial metabolic pathways and hence these agents (either individually or two or more in concert), and/or their salivary concentrations, may serve as chemotaxonomic indicators of microbial activity in the oral environment. For example, the pathogenic micro-organism *P. gingivalis* generates high levels of *n*-butyrate¹².

Also notable is a broad resonance located at 2.04 ppm which is assignable to the acetamido methyl group protons ($-\text{NHCOCH}_3$) of N-acetylsugars located in the molecularly-mobile branching carbohydrate side-chains of “acute phase” glycoproteins (i.e., salivary mucins)¹³. This broad resonance overlaps several sharper acetamido- CH_3 group ^1H resonances attributable to low-molecular-mass N-acetylsugars such as N-acetylneuraminate and N-acetylglucosamine saccharide fragments which conceivably arise from the actions of bacterial-derived neuraminidase and hyaluronidase respectively¹⁴. In addition, both ethanol and methanol were detected in a large proportion of the human saliva samples subjected to ^1H NMR analysis. Although ethanol is a bacterial-derived catabolite (for example, arising from carbohydrate metabolism by *Streptococcus mutans*)¹⁵, the methanol present is derived from the passive or direct inhalation of cigarette smoke in which this alcohol is present, a consequence of the combustion of tobacco lignin which contains many methoxy aromatic substituents in its complex, macromolecular structure.

Two-dimensional ^1H - ^1H 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 $-\text{CH}_2$ group signals (triplet, $\delta = 1.04$ ppm and quartet, $\delta = 2.17$ ppm respectively), the *n*-butyrate- CH_3 , α - CH_2 and β - CH_2 group resonances (triplet, $\delta = 0.91$ ppm, multiplet, $\delta = 1.56$ ppm and triplet, $\delta = 2.15$ ppm respectively), the ethanol- CH_3 and $-\text{CH}_2$ groups (triplet, $\delta = 1.21$ ppm and quartet, $\delta = 3.68$ ppm) and the tyrosine aromatic ring protons (doublets located at 6.88 and 7.17 ppm) (data not shown).

3.2 Multicomponent ^1H NMR Evaluations of the Oxidative Consumption of Salivary Components by Peroxoborate Present in the Dentifrice Formulation

Typical 600 MHz single-pulse ^1H NMR spectra of a human saliva sample acquired prior and subsequent to *in vitro* treatment with an aliquot of the oral rinse preparation are shown in Figure 2. Clearly, addition of the carbamide peroxide and peroxodisulphate-containing product gives rise to the complete disappearance of the pyruvate- CH_3 group signal (singlet, $\delta = 2.388$ ppm), an observation reproducible in all saliva samples tested in this manner ($n = 10$). These data are fully consistent with the oxidative consumption of salivary pyruvate by peroxodisulphate and/or hydrogen peroxide (H_2O_2) present in the formulation (equations 1 and 2 respectively). Indeed, 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 depicted in equation 2.



Peroxodisulphate is an extremely powerful oxidising agent [i.e., the redox potential (E^0) for the system depicted in equation (3) is +2.01 V]



But its reactions are mechanistically very complex. For example, hydrolysis of peroxodisulphate generates peroxomonosulphate (equation 4), also a powerful oxidant which can oxidatively decarboxylate pyruvate to acetate and CO_2 .



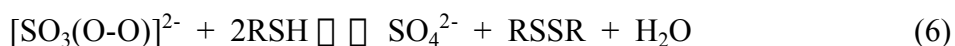
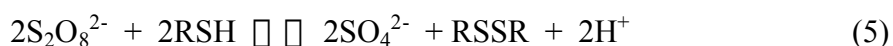
As expected, spectra of the product-treated salivary supernatant contained many intense resonances from components present in this product (e.g., glycerol, citrate, methyl paraben, saccharin) together with marked elevations in the intensities of those present in both matrices (specifically formate and citrate). Of course, the small rise in the acetate- CH_3 group signal intensity observed following treatment is attributable to the oxidant-mediated oxidative decarboxylation of pyruvate described above.

3.3 Chemical Model Studies of the Reactions of Salivary Electron Donors with Dentifrice-containing Peroxoborate

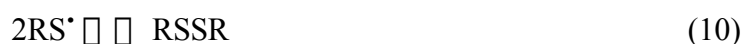
Aqueous standard solutions of pyruvate were treated with aliquots of solutions derived from the tooth-whitening formulation (section 2) in order to provide further information concerning the nature and mechanism of the peroxodisulphate- and H_2O_2 -mediated oxidation of this α -keto acid anion in human saliva. ^1H NMR analysis revealed that reaction of pyruvate (5.00×10^{-3} mol. dm^{-3}) with 0.050 ml of the above solution gave rise to an almost complete conversion of the α -keto acid anion substrate to acetate and CO_2 (Figure 3). Indeed, > 95% of the pyruvate was oxidatively decarboxylated to acetate and CO_2 under the experimental conditions utilised. Moreover, a singlet resonance ascribable to an unidentified impurity present in the commercially-available pyruvate sample employed for these investigations ($\delta = 1.50$

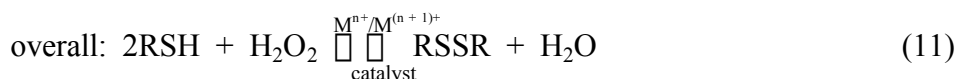
ppm) was also eliminated from the spectrum subsequent to treatment with the product, indicating that this species supplies electron donating equivalents towards peroxodisulphate and/or H₂O₂.

L-cysteine and L-methionine were employed as model thiol and thioether compounds, respectively, for these investigations since they are present as amino acid residues in many salivary proteins and, following the bacterially-mediated proteolysis of such macromolecules, serve as precursors to volatile sulphur compounds (VSCs) which are predominantly responsible for oral malodour [e.g. methyl mercaptain (CH₃SH) which accounts for approximately 60% of the VSCs detectable]. Reaction of L-cysteine with peroxodisulphate or peroxomonosulphate present in the dentifrice formulation tested yielded its corresponding disulphide cystine as a major oxidation product, consistent with equations 5 and 6 (data not shown).

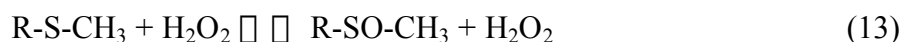


Of course, H₂O₂ can also effect the oxidation of thiols, a complex reaction system catalysed by the presence of trace levels of redox-active transition metal ions, notably those of iron and copper (equations 7 - 11).





¹H NMR analysis also demonstrated that the thiomethyl group (-S-CH₃)-containing amino acid L-methionine was oxidatively consumed by oxidants present in the dentifrice preparation examined, yielding methionine sulphoxide (-SO-CH₃ group singlet, $\delta = 2.725$ ppm) as the predominant product (Figure 5). These processes are consistent with the reactions depicted in equations 12 and 13.



DISCUSSION

Multicomponent ¹H NMR investigations of the oxidising (“therapeutic”) ability of the tooth-whitening formulation investigated here demonstrated that critical salivary electron donors are readily consumed by peroxodisulphate and H₂O₂ therein, together with any peroxomonosulphate derived from hydrolysis of the former. Indeed, on consideration of the total oxidant concentration of the product examined here, it is clear that in concentration terms the oxidants available will effectively “swamp” all salivary electron donor activity available. Indeed, the mean salivary concentrations of pyruvate (a two electron donator) and thiols (a single electron supplier) are 9.0×10^{-5} ¹⁷ and 3.6×10^{-5} mol.dm⁻³ ¹⁸ respectively. 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 also be fully, or at least partially consumed during oral health care programmes involving this tooth-whitening preparation.

Consumption of cysteine and methionine by peroxoborate species is of much importance to oral hygiene and clinical periodontology since both CH_3SH and H_2S are generated from these amino acids via metabolic pathways operational in gram-negative micro-organisms, one involving the enzymes cystine reductase (reduction of cystine to cysteine) and serine sulphhydrase (desulphuration of cysteine yielding H_2S and serine).

In addition to the major aesthetic problems caused by oral malodour (halitosis), there is currently much evidence available suggesting that VSCs adversely contribute towards the periodontal disease process. Indeed, as might be expected from its ability to participate in thiol-disulphide interchange reactions, CH_3SH influences enzymic and immunologic activities in manners that give rise to periodontal tissue destruction¹⁹ and also enhances the permeability of oral mucosa, the latter representing a process which facilitates the penetration of hazardous components such as endotoxins into tissues²⁰. 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, alteration of collagen metabolism and interference with protein synthesis¹⁹. Furthermore, VSCs have been found to retard wound healing processes²¹.

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 multicomponent 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 utility concerning multicomponent assessments of the interactions of therapeutically-active agents present in commercially-available oral health care products with human saliva biomolecules, and the oxidative decarboxylation of salivary pyruvate by peroxoborate adducts in the tooth-whitening mouthrinse evaluated in this study 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 ($K_a = 3.20 \times 10^{-3} \text{ mol. dm}^{-3}$)²², it may play a role in facilitating tooth demineralisation processes. In view of the ready diffusion of such organic acids into enamel²³, the consumption of salivary pyruvate by peroxoborate oxidants present in the tooth-whitening product examined here may suppress the development and progression of primary root caries lesions. Hence, peroxodisulphate and H_2O_2 oxidants 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 health care products reflects the oxidising capacity of such materials, a phenomenon of much significance with regard to their therapeutic and aesthetic roles. Therefore, high resolution ^1H NMR analysis of isolated human saliva or appropriate chemical model systems serves as a very useful method for the *in vitro* testing of oral health care products.

^1H NMR analysis of human saliva samples obtained prior and subsequent to the administration of dentifrices or oral rinses to patients with periodontal diseases (i.e., *in vivo* investigations) may demonstrate an oral health care product-mediated alleviation in levels of bacterial-derived salivary components (e.g., short-chain non-volatile carboxylic acid anions such as acetate, *n*-butyrate, formate, fumarate, lactate, propionate, pyruvate etc) reflecting the removal of potentially pathogenic micro-organisms following treatment regimens, and recent pilot studies conducted by the

authors have shown that the technique is readily applicable to assessments of this nature.