

High-Resolution ^1H NMR Investigations of the Oxidative Consumption of Salivary Biomolecules by a Tooth-Whitening Formulation: Relevance to Safety Issues

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ABSTRACT

A multicomponent evaluation of the oxidative consumption of salivary biomolecules by a hydrogen peroxide (H_2O_2)-containing tooth-whitening formulation has been performed using high-resolution proton (^1H) nuclear magnetic resonance (NMR) spectroscopy. Unstimulated human saliva samples ($n = 10$) were treated with aliquots of supernatants derived from 1) the H_2O_2 -containing whitening gel, 2) the corresponding tooth-whitening accelerant solution containing an amino-alcohol activator, and 3) a combination of these product agents pre-mixed in the recommended manner. 600 MHz ^1H NMR spectra acquired on these samples demonstrated that H_2O_2 present in the whitening gel gave rise to the oxidative decarboxylation of salivary pyruvate (to acetate and CO_2), the direct oxidation of trimethylamine and methionine (to trimethylamine-N-oxide and methionine sulphoxide respectively), and the indirect oxidative consumption of lactate and carbohydrates in general. Experiments conducted on a chemical model system confirmed the consumption of pyruvate by added H_2O_2 , and also revealed that this method could be employed for determinations of the H_2O_2 content of tooth-whitening products. In conclusion, high-resolution ^1H NMR analysis provides much valuable molecular information regarding the fate of tooth-whitening oxidants in human saliva, and also permits an assessment of the ability of endogenous antioxidants therein to protect against any soft tissue damage arising from the possible leakage of H_2O_2 from tooth-whitening application trays.

Keywords: Biomedical ^1H NMR Analysis; Human Saliva; Tooth-Whitening Product; Hydrogen Peroxide; Salivary Antioxidants

1. Introduction

The incorporation of hydrogen peroxide (H_2O_2), carbamide peroxide (CP) and peroxo-adducts of inorganic anions such as peroxoborate, peroxodisulphate and peroxocarbonate as agents for the bleaching of discoloured teeth in commercially-available tooth-whitening products such as gels, toothpastes and oral rinses has evoked much interest regarding their modes of action, redox activity in oral environments, and adverse effects and safety considerations [1,2]. However, our understanding of the capacities of salivary biomolecules to chemically react with (and hence neutralise) such peroxide-based tooth-whitening oxidants remains limited. In principal, salivary biomolecules which can act as scavengers of H_2O_2 (or other peroxo-adducts) serve to protect oral soft tissues against any deleterious effects exertable by these oxidants which are sometimes prone to escape from customised application trays or alternative application devices and hence make con-

tact with these and further sensitive oral environments.

Moreover, the analysis of salivary biomolecules 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 identities 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 peroxo-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]). Indeed, 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 (intact or otherwise), pharmaceutical formulations, dentifrices and

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foodstuffs, etc. 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 are critically dependent on their magnetic (and therefore, chemical) environment. Moreover, the appearance (multiplicity) of a resonance (signal) is influenced by neighbouring ^1H nuclei in a well characterised 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 components present in biofluids (e.g., human plasma [5,6], urine [7,8], knee-joint synovial fluid [9] and salivary supernatants [10]) can be simultaneously obtained from high-field, high-resolution NMR investigations. The broad overlapping resonances arising from any macromolecules (e.g. proteins, polysaccharides, etc.) present in biofluid samples are routinely suppressed by the application of spin-echo pulse sequences, a procedure which generates 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 a detailed knowledge of sample composition prior to analysis.

Since 1) small levels of H_2O_2 are released into saliva during tooth-whitening treatments [the quantity being dependent on its content in the product employed, the product class, the product’s application method (such as customised trays), and also partially on the amount of saliva generated], and 2) this oxidant has the ability to chemically react with chemical species present in this and/or other oral fluids present on tooth surfaces, in this manuscript we describe the application of high-resolution ^1H NMR analysis to simultaneously evaluate the oxidising actions of a commercially-available H_2O_2 -containing tooth-whitening formulation¹ towards a multitude of biomolecules present in (intact) human salivary supernatants. The reactions of H_2O_2 with a single-component chemical model system containing a buffered aqueous solution of the H_2O_2 -scavenger pyruvate (which is readily ^1H NMR-detectable in human saliva) was also e-

xamined. The significance of the results acquired is discussed with special reference to the capacity of endogenous antioxidants present in human saliva to offer protection against sensitivity or further adverse effects induced by H_2O_2 which inadvertently escapes from bleaching application trays and comes into contact with soft tissues. The mechanisms involved in the scavenging of H_2O_2 and/or more reactive oxygen radical species arising therefrom by salivary antioxidants/electron-donors are also 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 participants (6 male, 4 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). Approval for the collection of saliva specimens from human participants in this study was obtained from the University of Bolton Research Ethics Committee.

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 (4000 r.p.m. for 30 min.) on their arrival to remove cells and debris, and the resulting supernatants were stored at -20°C for a maximum duration of 18 hr prior to treatment with tooth-whitening product supernatants and/or solutions, further storage at -20°C as detailed below, and ^1H NMR analysis. The pH values of each salivary supernatant were determined prior to ^1H NMR analysis (these ranged from 6.45 - 7.44).

The tooth-whitening product investigated here consisted of a dual-syringe system, the first of which contained 12.5% (w/w) H_2O_2 (the active bleaching agent) and 2.50% (w/w) carbopol, and the second 95.0% (v/v) of an amino-alcohol bleaching accelerant, the remaining 5.0% (v/v) being water (**Figure 1(a)**). For the purpose of clinical application, the components in the first syringe are pre-mixed with those of the second in a 4:1 volume ratio so that the final applied H_2O_2 content is 10.0% (w/v). The pH value of the undiluted accelerant solution was 12.56.

¹Get2smile Home-Whitening System (Wyten Technology Ltd., UK).



(a)



(b)

Figure 1. (a) Double-barrelled opaque syringe with mixing tip, Dappen's dish for mixing and brush for application [the larger syringe chamber contains the 12.50% (w/w) H_2O_2 -containing tooth-whitening gel, and the smaller one contains the 95.0% (v/v) amino-alcohol tooth-whitening activator]; (b) A thin coat of the resulting 4:1 (v/v) tooth-whitening gel: amino-alcohol tooth-whitening activator solution admixture (similar to varnish) is painted onto the tooth enamel surface.

For the clinical application of this dual-syringe tooth-whitening product, a specially-designed mouth retractor is employed to retract the lips away from the teeth, and then the wy10 bleaching gel/activator mixture is painted onto tooth surface enamel as a thin coat of varnish [Figure 1(b)] in order to ensure that the bleaching agent does not gain access to areas outside the tooth surface (adequate further steps are taken to ensure that the bleaching agent components are isolated from all exposed dentine and soft tissues). This tooth-whitening product is employed with a thermal diffusion system² which is mounted over the retractor and serves to elevate the temperature of the bleaching gel on the enamel surfaces up to 38°C. The application is applied once-daily (of total length 40 min, and involving a further bleaching episode

²S2Power Thermal Diffuser (Wyten Technology Ltd., UK).

after 20 min with a repeated application (replenished volume of the 4:1 tooth-whitening gel:activator solution mixture). The final pH value of the resulting 4:1 (v/v) tooth-whitening gel:amino-alcohol activator product admixture is 10.0.

2.2. Preparation of Aqueous Supernatants/Solutions Derived from the H_2O_2 Tooth-Whitening Gel and Tooth-Whitening Accelerant Formulations

Approximately 100 mg pre-recorded masses of the 12.5% (w/w) H_2O_2 bleaching gel formulation present in syringe 1 were added to a 10.00 ml volume of HPLC-grade water, the mixture rotamixed and then centrifuged (3500 r.p.m. at ambient temperature) for a 30 min. period. The clear supernatant (of pH value 5.53) was removed and then employed to treat salivary supernatant samples and aqueous solutions containing sodium pyruvate in the manners outlined below.

The 95.0% (v/v) bleaching activator (accelerant) material present in syringe 2 was diluted 1:10 with HPLC-grade water so that its final concentration was 9.50% (v/v), and this solution was then directly utilised for additions to human salivary supernatant specimens. This 9.50% (v/v) activator solution had a pH value of 11.24, and the addition of only 38 μl of this to 0.18 ml volumes of human salivary supernatants (the mixture made up to a final volume of 0.594 ml with $^2\text{H}_2\text{O}$ and H_2O as detailed below) did not elevate the final pH value of the latter and the final reaction mixture above 7.70, *i.e.* the buffering capacity of the much larger volume salivary supernatant effectively countered the non-neutral (alkaline) pH value of the activator.

Three separate aliquots (0.18 ml) of each salivary supernatant sample were removed and 76 μl volumes of the supernatant arising from the above 12.5% (w/v) H_2O_2 (syringe 1) formulation was then added to the first, 38 μl of the above 9.50% (v/v) amino-alcohol accelerant formulation solution was added to the second, and a combination of 76 μl of the above H_2O_2 -containing supernatant and 38 μl of the accelerant solution were added to the third; to each of these mixtures, a 0.30 ml aliquot of $^2\text{H}_2\text{O}$ (field frequency lock) was then added and their final volume was then adjusted to 0.594 ml with HPLC-grade H_2O . The mixtures were thoroughly rotamixed and then equilibrated at a temperature of 37°C for a 30 min. period and then stored at -20°C for a maximum duration of 72 hr prior to ^1H NMR analysis. Further 0.18 ml aliquots of each salivary supernatant sample treated with a 0.30 ml volume of $^2\text{H}_2\text{O}$ and 0.114 ml of HPLC-grade H_2O [both 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 ($5.00 \times 10^{-3} \text{ mol}\cdot\text{dm}^{-3}$) (Sigma Chemical Co. Ltd., Poole, Dorset, UK) were prepared in $5.00 \times 10^{-2} \text{ mol}\cdot\text{dm}^{-3}$ phosphate buffer (pH 7.00) which was rigorously deoxygenated by purging with helium (He) gas prior to use (30 min at ambient temperature). 1.00 ml aliquots of these solutions were treated with 0, 19, 38, 57, 76 and 95 μl volumes of the above H_2O_2 solution, the samples thoroughly rotamixed and equilibrated at a temperature of 37°C for 30 min, and then stored at a temperature of -20°C for a period of 72 hr prior to ^1H NMR analysis. Matching pyruvate-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. This technique served as an effective means to estimate the content of this active tooth-whitening agent present in the tooth-whitening gel (syringe 1) formulation.

2.3. Proton (^1H) NMR Measurements

Typically, the total 0.594 ml volume of each sample was placed in a 5-mm diameter NMR tube, and then 6.0 μl of a $1.00 \times 10^{-2} \text{ mol}\cdot\text{dm}^{-3}$ solution of trimethylsilyl-[2,2,3,3- $^2\text{H}_4$] propionate (TSP) in $^2\text{H}_2\text{O}$ [serving as an internal chemical shift reference ($\delta = 0.00$ ppm) and quantitative ^1H NMR standard] was added.

Proton (^1H) NMR measurements on the above pre-prepared human salivary supernatant 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 in quadrature detection mode and a probe temperature of 300 K. Each spectrum corresponded to 32 free induction decays (FIDs), 5.6 μs pulses, a 1 s pulse repetition rate, 32,768 (subsequently zero-filled to 65,536) data points and spectral width 10,500 Hz. The intense residual $\text{H}_2\text{O}/\text{HOD}$ signal ($\delta = 4.80$ ppm) was suppressed via gated decoupling during the delay between pulses. Exponential linebroadening functions of 0.30 Hz were routinely applied to the FIDs prior to Fourier transformation, and all spectra were manually-phased and baseline-corrected. Spectra were acquired in an automated manner using a sample changer for continuous sample delivery.

Where appropriate, the broad protein resonances present in control and tooth-whitening product-treated salivary supernatant samples were suppressed by the Hahn spin-echo sequence (D[$90^\circ\text{x-t-180}^\circ\text{y-t-collect}$]) which was repeated 128 times ($t = 68$ ms). Chemical shifts were referenced to internal TSP. Where present, the methyl group resonances of acetate (singlet, $\delta = 1.920$ ppm), alanine (doublet, $\delta = 1.487$ ppm) and lactate (doublet, $\delta = 1.330$ ppm) served as secondary internal chemical shift references for the saliva samples investigated.

For single-pulse, one-dimensional (1D) ^1H NMR spectra acquired on control and H_2O_2 -containing tooth whitening gel supernatant-treated solutions of pyruvate, samples were prepared by the addition of a fixed volume (0.10 ml) of a solution of TSP in $^2\text{H}_2\text{O}$ ($1.00 \times 10^{-2} \text{ mol}\cdot\text{dm}^{-3}$) to a 0.50 ml volume of the reaction mixture. 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; final concentration $1.67 \times 10^{-3} \text{ mol}\cdot\text{dm}^{-3}$), and exponential line-broadening functions of 0.30 Hz were again 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, and those of the phosphate-buffered aqueous pyruvate solutions treated with increasing volumes (0, 19, 38, 57, 76 and 95 μl) of the above solution prepared from the H_2O_2 -containing tooth-whitening gel, 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 TSP (final concentration $1.00 \times 10^{-4} \text{ mol}\cdot\text{dm}^{-3}$ in the salivary supernatants). Maintenance of the exact integral regions for each spectrum acquired was ensured.

Since the protein concentration of human saliva ($1.40 \text{ g}\cdot\text{dm}^{-3}$ - $6.40 \text{ g}\cdot\text{dm}^{-3}$ [11]) is much lower than that of human blood plasma ($65 \text{ g}\cdot\text{dm}^{-3}$ - $83 \text{ g}\cdot\text{dm}^{-3}$ [12]), the minimal macromolecular broad resonance envelop was not found to interfere with the observation and integration of each of the sharp low-molecular-mass resonances, and hence all of the salivary metabolite concentrations documented here represent those derived from such spectra. Furthermore, it should also be noted that the biomolecule concentrations determined in this investigation reflect only the non-macromolecular-bound portion of these components and hence are expected to be somewhat lower than their total salivary levels.

Two-dimensional shift-correlated ^1H - ^1H 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.

2.4. Statistical Analysis of Salivary Biomolecule Concentration Data

The statistical significance of differences observed between salivary pyruvate, methionine, lactate and trimethylamine concentrations was determined by a paired sam-

ple t-test performed on untransformed data (based on 9 degrees-of-freedom for $n = 10$ participants).

3. Results

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

Figure 2(a) shows a typical high-field (0.72 ppm - 2.46 ppm) region of the 600 MHz single-pulse ^1H NMR spectrum of an untreated human salivary supernatant sample, and corresponding partial spectra acquired subsequent to the *in vitro* treatment of this sample with specified aliquots of the tooth-whitening amino-alcohol activator so-

lution, bleaching gel formulation supernatant, and a combination of equivalent added volumes of the activator and bleaching gel supernatant solutions, are shown in Figures 2(b)-(d) respectively (the volumes of each solution or supernatant added are specified above in Section 2).

As previously documented [10], 600 MHz single-pulse ^1H NMR spectra of control (untreated) human salivary supernatant samples contained many prominent, sharp resonances ascribable to a 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, *n*-valerate and 3-D-hydroxybutrate), carbohydrates

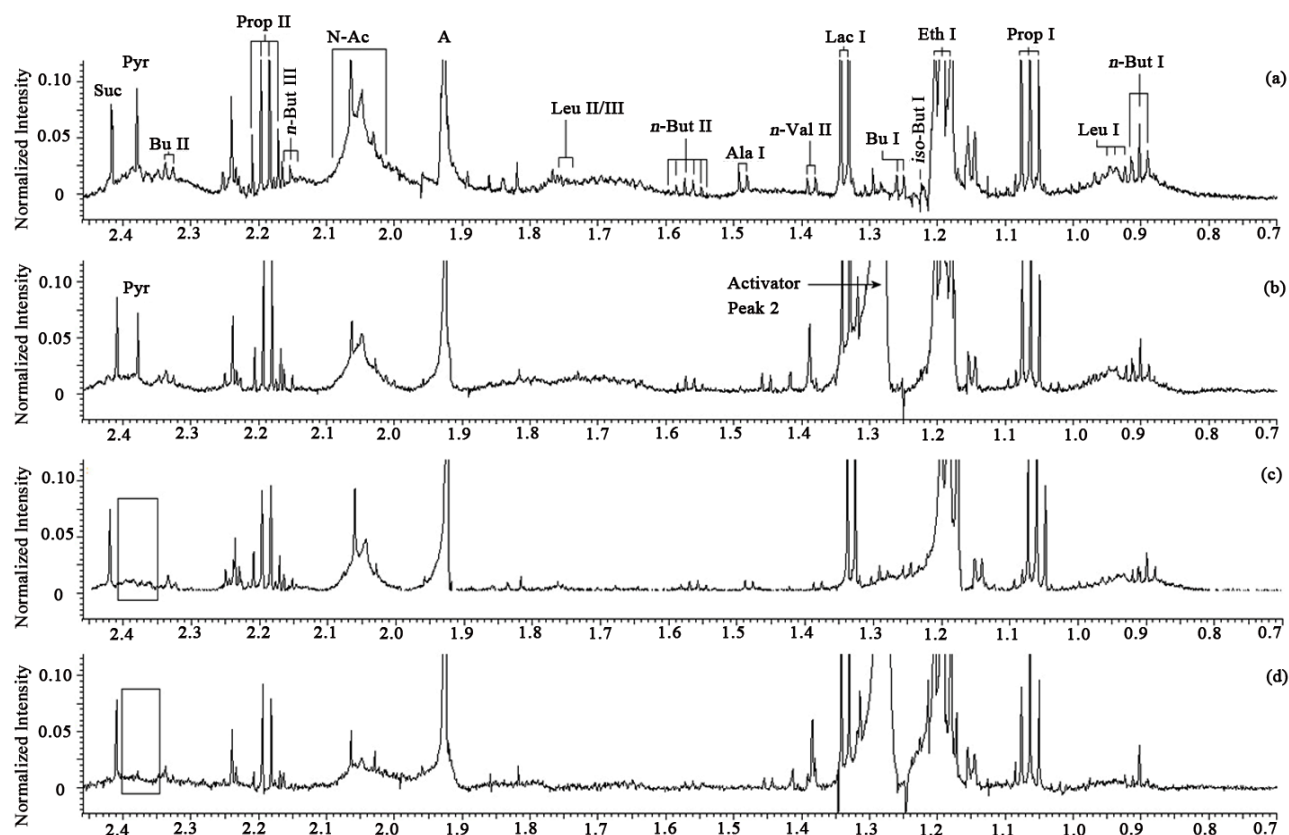


Figure 2. Expanded 0.72 ppm - 2.46 ppm regions of the 600.13 MHz single-pulse ^1H NMR spectra of a human salivary supernatant specimen (pH value 6.84). (a) Untreated; (b) Treated with the 9.50% (v/v) amino-alcohol accelerant solution; (c) Treated with the supernatant derived from the H_2O_2 -containing tooth-whitening gel, and (d) treated with a combination of the 9.50% (v/v) amino-alcohol accelerant solution and the supernatant derived from the H_2O_2 -containing tooth-whitening gel (as described in Section 2). For these samples, 38 μl and/or 76 μl aliquots of the amino-alcohol accelerant solution and the supernatant obtained from the tooth-whitening gel, respectively, were added to a 0.18 ml volume of human saliva, and subsequently 0.30 ml of $^2\text{H}_2\text{O}$ was added and the reaction mixture was made up to a final volume of 0.594 ml with HPLC-grade water (samples were then equilibrated at a temperature of 37°C for a 30 min period). The disappearance of the pyruvate- CH_3 resonance in the spectra shown in (c) and (d) is highlighted by rectangles. Typical spectra are shown. Abbreviations: A, Acetate- CH_3 ; Ala I, alanine- CH_3 , group protons; Bu I and Bu II 3-D-hydroxybutyrate γ - CH_3 and α/α' - CH_2 group protons respectively; *iso*-But I and II, *iso*-butyrate- CH_3 and - CH group protons respectively; *n*-But I, II and III, *n*-butyrate γ - CH_3 , β - and α - CH_2 protons respectively; Eth I, ethanol- CH_3 ; Lac I, lactate- CH_3 group protons; Leu I and II/III, leucine δ - CH_3 's and β - CH_2/γ - CH respectively; N-Ac, spectral region for acetamido methyl groups of N-acetyl sugars; Prop I and II, propionate- CH_3 and - CH_2 group protons respectively; Pyr, pyruvate- CH_3 ; Suc, succinate- CH_2 ; *n*-Val II, *n*-valerate γ - CH_2 protons. Activator Peak 2 represents a major high-field region resonance arising from the added amino-alcohol tooth-whitening activator/accelerant.

such as glucose and galactose (both α - and β -anomers) are observable. The organic acid anions detectable are products arising from microbial metabolic pathways, and hence these agents (either individually or several or more in concert), particularly their salivary concentrations, may serve as chemotaxonomic indicators of microbial activity in the oral environment. Indeed, the pathogenic micro-organism *P. gingivalis* generates high levels of acetate, *n*-butyrate and propionate, together with smaller amounts of *iso*-butyrate, *iso*-valerate, phenylacetate and succinate [14,15].

Also present are one or more broad resonances located at 2.04 ppm which are assignable to the acetamido methyl group protons (-NHCOCH₃) of N-acetylsugars located in the molecularly-mobile branching carbohydrate side-chains of salivary glycoproteins. This assignment is consistent with those previously made for the ¹H NMR spectra of other biofluids [16]. This broad resonance overlaps several sharper acetamido-CH₃ 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 respectively, the latter towards ground substance hyaluronate [17]. These resonances may also be assignable to N-acetyl amino acids such as N-acetyl glycine or N-acetylglutamate.

In addition, both ethanol and methanol were detectable 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*) [18], the methanol present is putatively 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 substituents in its complex, macromolecular aromatic structure.

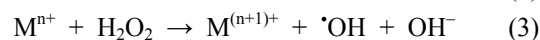
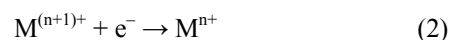
Two-dimensional ¹H-¹H COSY NMR spectra of typical human salivary supernatant samples showed clear connectivities between the multiplet resonances present. For example, linkages between the alanine-CH₃ and α -CH signals (doublet, δ = 1.487 ppm and quartet, δ = 3.765 ppm respectively), propionate-CH₃ and -CH₂ group signals (triplet, δ = 1.04 ppm and quartet, δ = 2.17 ppm respectively), the *n*-butyrate-CH₃, β -CH₂ and α -CH₂ group resonances (triplet, δ = 0.91 ppm, multiplet, δ = 1.56 ppm and triplet, δ = 2.15 ppm respectively), the ethanol-CH₃ and -CH₂ groups (triplet, δ = 1.21 ppm and quartet, δ = 3.68 ppm), the lactate-CH₃ and -CHOH group resonances (doublet, δ = 1.330 ppm and quartet, δ = 4.130 ppm respectively) 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 H₂O₂ Present in the Tooth-Whitening Formulation Investigated

Clearly, addition of the H₂O₂-containing product gives rise to the complete disappearance of the pyruvate-CH₃ group signal (singlet, δ = 2.388 ppm), an observation reproducible in all saliva samples tested in this manner (*n* = 10) [Figures 2(a) and (c)]. Further expanded 1.88 ppm - 2.46 ppm regions of these spectra are exhibited in Figures 3(a) and (c) respectively. These data are fully consistent with the oxidative consumption of salivary pyruvate by H₂O₂ present in the formulation (Equation (1)). Indeed, previous investigations have demonstrated that pyruvate acts as a powerful biofluid electron-donor (*i.e.*, a water-soluble antioxidant) and is oxidatively decarboxylated to acetate and CO₂ on reaction with H₂O₂. The mean (\pm SEM) pre-treatment salivary pyruvate concentration was $2.19 (\pm 1.07) \times 10^{-3} \text{ mol} \cdot \text{dm}^{-3}$ for the *n* = 10 samples collected in this study.



An alternative mechanism for the generation of acetate and CO₂ from pyruvate involves 1) the prior generation of [•]OH radical from the interaction of "catalytic" redox-active low-molecular-mass metal ions such as Fe(II), Cu(I) and Co(II) present in human saliva with the H₂O₂ tooth-whitening agent, and 2) direct reaction of this aggressively-reactive oxygen radical species with this α -keto acid anion "scavenger" (Equations (2) and (3)), although with much larger concentrations of available H₂O₂, this oxidation process is, of course, much more likely to proceed in accordance with Equation (1). In addition to both endogenous and exogenous (dietary) sources of these metal ions, the mean concentrations of copper and cobalt ions in human saliva are $1.25 (\pm 0.20) \times 10^{-6}$ (mean \pm SEM) [19] and $3.7 \times 10^{-7} \text{ mol} \cdot \text{dm}^{-3}$ [20] respectively, the latter representing the mean of 10 out of 37 salivary determinations in which it was detectable, and presumably predominant as Co(II) in view of thermodynamic considerations and the availability of Co(III)-reducing electron donors in this biofluid.



Since ¹H NMR-detectable concentrations of acetate were detectable as a minor contaminant present in the tooth-whitening gel (syringe 1) supernatant, spectra of the H₂O₂-containing bleaching product-treated salivary supernatants showed elevations in the intensities of resonances ascribable to this agent. Of course, smaller increases in the acetate-CH₃ group signal intensity observed

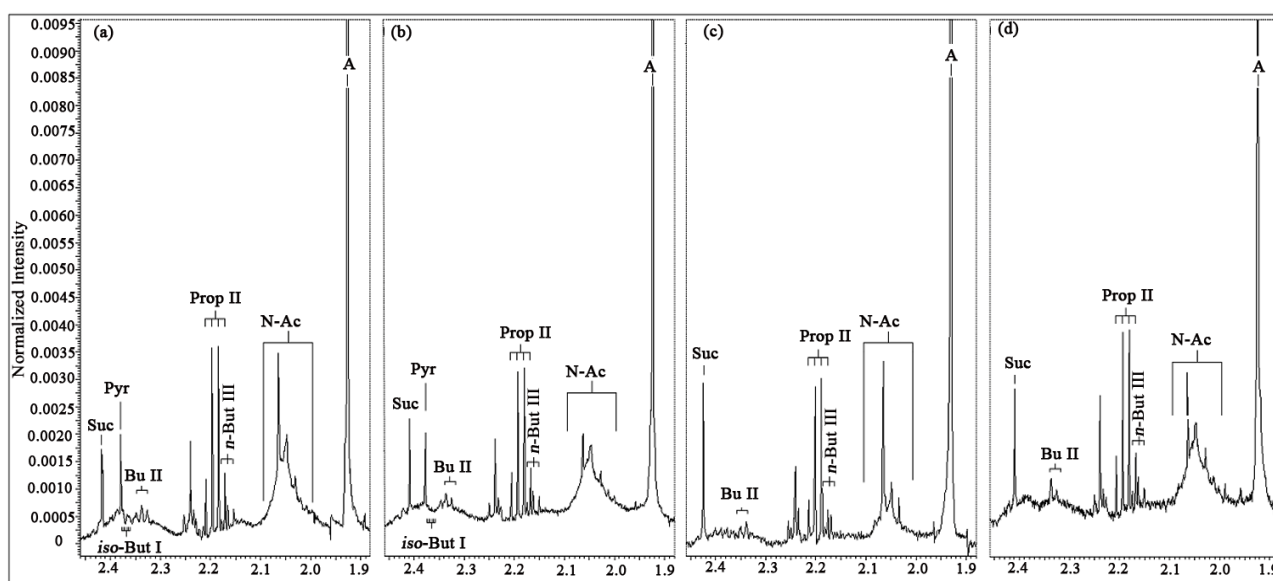
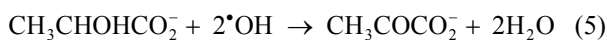


Figure 3. Corresponding expanded 1.88 ppm - 2.46 ppm regions of the spectra shown in Figure 2. Abbreviations: as Figure 2.

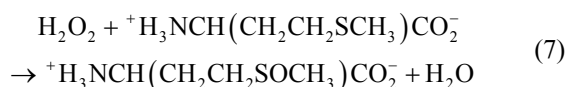
following treatment are attributable to the H_2O_2 -mediated oxidative decarboxylation of pyruvate described above.

Further H_2O_2 -induced modifications to the ^1H NMR profiles of human saliva included 1) the oxidative consumption of lactate ($-\text{CH}_3$ group doublet, $\delta = 1.33$ ppm, $-\text{CHOH}$ proton quartet, $\delta = 4.13$ ppm), which is explicable by the attack of $\cdot\text{OH}$ radical on this metabolite to primarily generate pyruvate (Equation (5)) which is then consumed by excess H_2O_2 (Equation (1)), or further $\cdot\text{OH}$ radical (Equation (4)) [as noted above, $\cdot\text{OH}$ radical can be generated from Fenton or pseudo-Fenton reaction processes involving trace levels of “catalytic” metal ions (Equations (2) and (3)); 2) significant elevations in the salivary concentrations of formate, an organic acid anion which is known to arise from oxidative damage to carbohydrate species such as glucose, and/or hyaluronate or N-acetyl glycoprotein N-acetylsugar species by $\cdot\text{OH}$ radical produced in the same manner as that described in 1) above; 3) the oxidation of trimethylamine (TMA) (singlet, $\delta = 2.91$ ppm) to its corresponding N-oxide, a process represented by equation 6. Mean \pm SEM percentage decreases observed in the TSP-normalised intensities of the lactate- CH_3 and trimethylamine- $\text{N}(\text{CH}_3)_3$ resonances were $21\% \pm 5\%$ ($p < 0.05$) and $45\% \pm 9\%$ ($p < 0.01$) respectively, whereas that for the increase in the formate signal was $42\% \pm 11\%$ ($p < 0.02$). The mean \pm SEM before-treatment concentrations of lactate, trimethylamine and formate were $(4.43 \pm 2.17) \times 10^{-3}$, $(9.17 \pm 3.04) \times 10^{-5}$ and $(5.72 \pm 2.51) \times 10^{-3} \text{ mol}\cdot\text{dm}^{-3}$ respectively.



Moreover, for a limited number of the salivary super-

natant samples examined ($n = 3$), we also found that the amino acid methionine ($-\text{S}-\text{CH}_3$ group singlet resonance, $\delta = 2.13$ ppm) was also consumed, a process which generated its primary oxidation product methionine sulphoxide (with a characteristic $-\text{SO}-\text{CH}_3$ group singlet signal located at $\delta = 2.725$ ppm) as previously described [21] and fully consistent with Equation (7). For these 3 samples, the mean \pm SEM methionine concentration was $1.60 \pm 0.44 \times 10^{-4} \text{ mol}\cdot\text{dm}^{-3}$, and the mean percentage decrease in its 2.13 ppm $-\text{S}(\text{CH}_3)$ group signal was $93\% \pm 4\%$ ($p < 0.005$ for $n = 3$ specimens).



Treatment of human salivary supernatant specimens with a combination of the H_2O_2 -containing bleaching gel supernatant (derived from syringe 1) with the diluted bleaching accelerant solution (from syringe 2) gave rise to similar modifications to the ^1H NMR profile of this biofluid [Figures 2(d) and 3(d)], specifically the complete oxidative consumption of pyruvate, accompanied by smaller reductions in the intensities of the lactate signals and increases in that of formate, together with even smaller decreases in that of the methionine- $\text{S}-\text{CH}_3$ resonance, although the latter observation was again limited to the small number of samples in which it was ^1H NMR-observable ($n = 3$).

As expected, addition of the tooth bleaching amino-alcohol accelerant alone to each salivary supernatant sample did not give rise to the ^1H NMR spectral modifications which were observed in those treated with either the H_2O_2 -containing bleaching gel supernatant or a combination of an equivalent volume of this solution (76 μl) with

an additional 38 μl of the 9.50% (v/v) solution of the tooth-whitening accelerant (apart, of course, from the appearance of signals ascribable to the added accelerant and an acetate contaminant also present in its solution). A Typical example of the spectra acquired from these samples is shown in **Figures 2(b)** and **3(b)**.

However, a sharp singlet resonance located at *ca.* 2.06 ppm was markedly and reproducibly diminished in intensity on treatment of each saliva sample with the bleaching activator solution [**Figures 2(b)** and **3(b)**], and this observation was also notable in samples that were treated with a combination of this activator solution and the H_2O_2 gel-derived supernatant [**Figure 1(d)**], but not in those to which the H_2O_2 -containing supernatant was added alone [**Figures 2(c)** and **3(c)**]. As noted above, this particular signal is ascribable to the acetamido group ($-\text{NHCOCH}_3$) protons of low-molecular-mass N-acetyl-sugars or N-acetyl amino acids. This observation is not simply explicable, but if this resonance is assignable to an N-acetyl amino acid, the decrease in its intensity may arise from a condensation reaction between the carboxylate group (s) of these biomolecules (including the side-chain carboxylate group of N-acetylglutamate if this species is responsible for the signal) and the free amino group of the bleaching accelerant added, although this reaction is unlikely to proceed at the 37°C equilibration temperature; however, it may be assisted by one or more salivary enzymes.

Moreover, also noteworthy were elevations in the electronically-integrated intensities of a resonance assignable to the malodorous biomolecule dimethylamine (singlet, $\delta = 2.78$ ppm), an observation which is presumably attributable to the displacement and hence mobilisation of this species (which is protonated and hence positively-charged at salivary pH values which are close to neutrality) from negatively-charged binding sites present in salivary macromolecules by a relatively large excess of this added positively-charged amino-alcohol accelerating agent. Such binding-sites could include the carboxylate groups of aspartate or glutamate residues in salivary proteins, and/or that of glucuronyl residues in salivary polysaccharides or glysoaminoglycans such as soft tissue-derived hyaluronate.

3.3. Chemical Model Studies of the Reaction of Pyruvate with H_2O_2 Present in the Tooth-Whitening Gel

Aqueous 5.00×10^{-3} mol·dm $^{-3}$ standard solutions of pyruvate ($n = 3$) were treated with a 76 μl volume of the supernatant solution derived from the H_2O_2 -containing tooth-whitening gel (Section 2) in order to provide further information regarding the nature and mechanism of this agent's ability to oxidise this α -keto acid anion in human saliva. ^1H NMR analysis revealed that reaction of pyruvate (initially 5.00×10^{-3} mol·dm $^{-3}$) with this blea-

ching agent gave rise to an essentially stoichiometric transformation of the α -keto acid anion substrate to acetate and CO_2 . Indeed, >54% of the pyruvate was oxidatively decarboxylated to acetate and CO_2 under the experimental conditions employed, and after making allowances for the presence of a small amount of pyruvate as pyruvate hydrate ($\text{CH}_3\text{C}(\text{OH})_2\text{CO}_2^-$) in aqueous solution, and assuming that the reaction goes to completion, the expected level of acetate production from Equation (1) was 55.9%.

Pyruvate hydrate has a singlet resonance ($\delta = 1.50$ ppm) of relatively low intensity which was also present in all ^1H NMR spectra acquired on aqueous pyruvate solutions. This resonance was also completely removed from the spectrum subsequent to treatment with the H_2O_2 -containing product evaluated here.

Plots of the ratio of the concentration of acetate generated to that of the initial (pre- H_2O_2 -treated) pyruvate plus pyruvate hydrate concentrations versus the added tooth-whitening product supernatant volume or corresponding (predicted) added H_2O_2 concentration ($[\text{H}_2\text{O}_2]$) were linear with a zero intercept (**Figure 4**), data demonstrating that this technique serves as an excellent method for the determination of this agent in such commercially-available tooth-whitening products. Indeed, the correlation coefficient (*r* value) obtained was 0.9988. The relationship between this ratio and added H_2O_2 concentration is described by equation 8, where $[\text{acetate}]_0$, $[\text{pyruvate}]_0$ and $[\text{pyruvate hydrate}]_0$ represent the initial concentrations of acetate, pyruvate and pyruvate hydrate respectively [the initial concentration of pyruvate hydrate was also determined by the electronic integration of its singlet resonance ($\delta = 1.50$ ppm) and normalisation of its intensity to that of the TSP internal standard of known concentration]. The $[\text{acetate}]_0$ value (the initial, untreated pyruvate solution acetate concentration) was corrected for a contribution from small amounts of an acetate contaminant present in the H_2O_2 bleaching gel supernatant solution employed for these studies (*i.e.* this contribution was dependent on the volume of supernatant added).

$$[\text{H}_2\text{O}_2] = \frac{([\text{acetate}]_{\text{final}} - [\text{acetate}]_0)}{([\text{pyruvate}]_0 + [\text{pyruvate hydrate}]_0)} \quad (8)$$

The estimated percentage H_2O_2 content of the gel present in syringe 1 of the tooth-whitening formulation investigated was found to be 12.37% \pm 0.35% (w/w) (mean \pm SEM) for $n = 5$ repeat determinations, a value in close agreement with that of its specified value [12.50% (w/w)].

4. Discussion

4.1. H_2O_2 -Scavenging Capacity of Salivary Biomolecules

Multicomponent ^1H NMR investigations of the oxidising

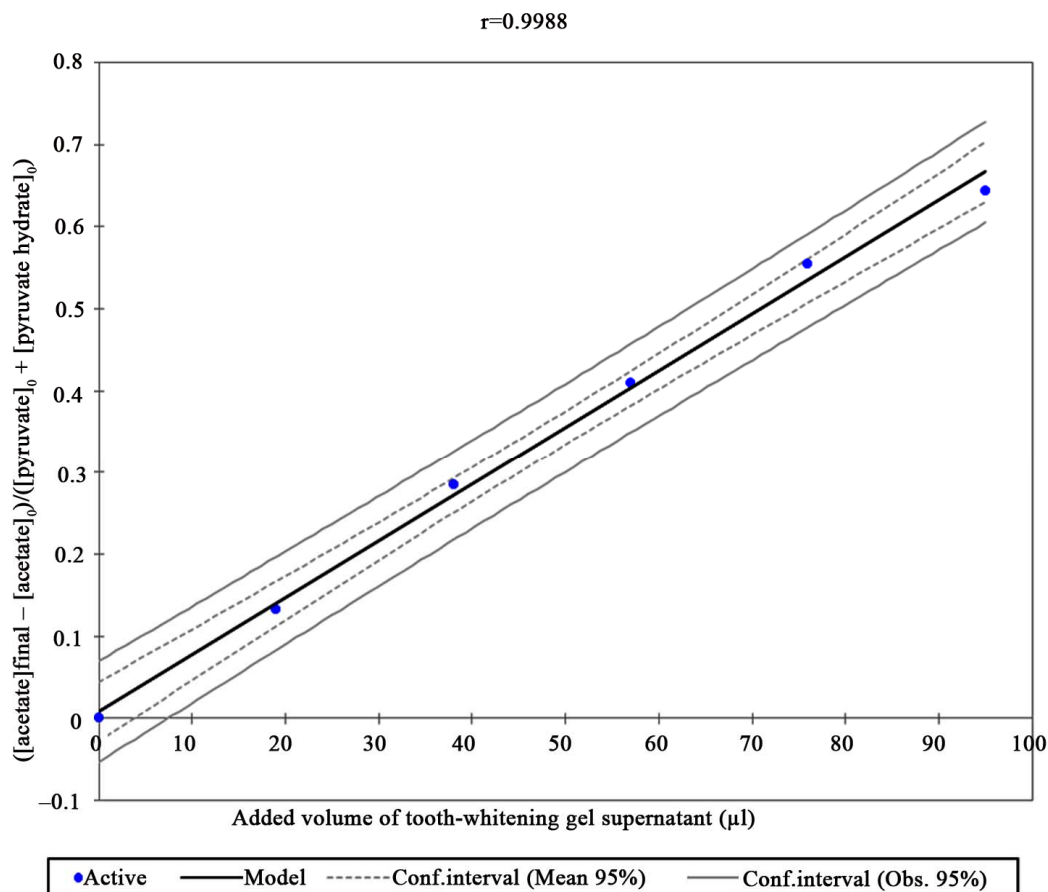


Figure 4. Plot of the ^1H NMR-determined ratio of acetate concentration generated to that of initial pyruvate. Specifically, $[\text{Acetate}]/[\text{Pyruvate}]_0$, as represented by the right-hand side of equation 8 (*i.e.* $([\text{acetate}]_{\text{final}} - [\text{acetate}]_0)/([\text{pyruvate}]_0 + [\text{pyruvate hydrate}]_0)$) was plotted against microliter (μl) volumes of added supernatant derived from the H_2O_2 -containing tooth-whitening gel formulation. The added supernatant volumes were 0, 19, 38, 57, 76 and 95 μl , and the resonance intensities and corresponding estimated H_2O_2 concentrations were adjusted to allow for the small level of dilution arising from the added H_2O_2 solution. The 95% confidence intervals shown represent those for the mean values and for the regression estimates, the latter the estimate of $([\text{acetate}]_{\text{final}} - [\text{acetate}]_0)/([\text{pyruvate}]_0 + [\text{pyruvate hydrate}]_0)$, *i.e.* H_2O_2 concentration, for each added volume of tooth-whitening gel supernatant.

ability of the tooth-whitening formulation investigated here towards salivary biomolecules demonstrated that critical salivary electron-donors are readily consumed by H_2O_2 therein. Indeed, it is clear that any H_2O_2 which “escapes” from the tooth-whitening site to saliva (or alternative oral fluids), or that which inadvertently comes into contact with this oral fluid, will be effectively consumed by salivary electron-donors which therefore serve to protect soft tissues against any sensitivity or damage inducible by this tooth-whitening agent or any reactive oxygen radical species derivable therefrom. These further oxidants include hydroxyl radical ($\cdot\text{OH}$) arising from Fenton-type reactions [22] involving any low-molecular-mass redox-active transition metal ions available in human saliva and serving as catalytic sources, mono-deprotonated H_2O_2 (HO_2^-), which is more bleaching-active than its protonated precursor [23], (although is only generated in significant amounts at alkaline pH values sin-

ce H_2O_2 's first pKa value is 11.65), and perhaps also superoxide anion (O_2^-) generated from the single-electron oxidation of H_2O_2 and/or HO_2^- .

Indeed, the salivary concentrations of pyruvate (a two electron donor) range from $(0.10 - 10.60) \times 10^{-3} \text{ mol}\cdot\text{dm}^{-3}$ [17], and the mean level of thiols (a single electron supplier) therein is $3.6 \times 10^{-5} \text{ mol}\cdot\text{dm}^{-3}$ [24]. After making appropriate allowances for thermodynamic equilibria, and the rate of each reaction involved under physiological conditions, data acquired here indicates that further reductants present in human saliva (*e.g.*, urate, ascorbate, thiocyanate anion and the amino acids cysteine and methionine), those at relatively low concentrations, will also be at least partially effective with regard to the neutralisation of any adverse effects exertable by H_2O_2 during tooth-whitening episodes conducted with this particular product.

However, it should be noted that selected single ele-

electron-donors present in human saliva such as thiols and ascorbate also have the capacity to reduce higher oxidation state metal ions to their lower ones [for example, reduction of Fe(III) to Fe(II)] in accordance with Equation (2), so that the latter can then take part in Fenton or pseudo-Fenton reaction systems which generate the aggressively-reactive $\cdot\text{OH}$ radical (Equation (3)).

All of the above salivary metabolites serve to act as biomolecular protectants against any adverse sensitivity reactions and/or toxic effects exertable by the leakage of H_2O_2 to soft tissue areas which, without an adequate form of control, are accessible by this particular biofluid.

Interestingly, methionine is known to be an essential precursor required for the production of volatile sulphur compounds (VSCs) by Gram-negative anaerobic bacteria in the oral environment; *i.e.*, it plays a major role in the development of halitosis. Moreover, malodorous TMA has been implicated as a major factor involved in recalcitrant oral malodour. However, their involvement in these conditions will not be discussed further here.

4.2. Administration and Effectiveness of Tooth-Whitening Products

Currently, there are four methods for the administration of such tooth-whitening agents, specifically 1) that administered by a dental clinician, a process which can involve the employment of high H_2O_2 concentrations, which commonly range from 35% - 40% (w/w)—this treatment is frequently supplemented with the use of a light and/or heat source, 2) that supervised by a dental clinician in which a customised bleaching tray containing high levels of CP, often ranging from 35% - 45% (w/w), is placed into the mouth of the patient for periods of up to 1.00 hr in the clinician's surgery, 3) a home-use tooth-whitening kit provided by a dentist (otherwise known as "Home" or "Nightguard" bleaching) and applied by the patient [usually a 10% - 22% (w/w) CP formulation] present in a customised application tray, and 4) products which are purchasable by consumers in pharmacies and other retail outlets (known as "over-the-counter" products); these often contain H_2O_2 or CP [up to an EU legal limit of 0.10% (w/w or w/v)], or peroxoborates or peroxosulphates, but more recently this range of products has expanded to include those which contain chlorine dioxide (ClO_2)-generating systems, especially those which involve the acidulation of aqueous chlorite solutions. The product investigated here can be directly applied in the dental surgery [under 1) and 2) above], but is predominantly for "at-home" use as in 3) above.

Both a series of small clinical investigations and case studies have provided evidence that a 10% (w/w) CP-containing gel formulation applied in a bleaching tray overnight, (*i.e.* the "Nightguard" vital bleaching technique), gave rise to a predictable level of tooth whitening

[25-32], as did H_2O_2 -containing strips [33] and a "Power-bleaching" technique employing 35% (w/v) H_2O_2 coupled with or without activation by *UV* light and/or heating stages [34,35].

4.3. Deleterious Tooth-Sensitivity Effects of H_2O_2 -Containing Tooth-Whitening Products

Tooth sensitivity is a common deleterious effect of external tooth-whitening processes [36], and particularly noteworthy in this context is the observation that with the use of products containing 10% (w/v) CP, 15% - 65% of patients reported an enhanced level of tooth sensitivity [37,38]. Moreover, increased incidences of tooth sensitivity (from 67% - 78%) have been noted following tooth-whitening episodes with H_2O_2 coupled with a thermal enhancement process [39].

It is well known that tooth sensitivity usually lasts for up to four days post-bleaching [39,40]. However, this adverse effect can be prolonged for up to 39 days [37, 38]. Furthermore, a clinical study that involved comparisons of two differing 10% (w/w) CP brands revealed that 55% of a large number of patients investigated found tooth sensitivity and/or gingival irritation, and 20% of those who had experienced such deleterious effects discontinued the treatment in view of their discomfort [37].

Although mechanisms that could account for such tooth sensitivities during or following such external tooth-whitening episodes remain speculative, one *in vitro* study revealed that H_2O_2 can penetrate enamel and dentine, and also penetrate the pulp chamber [41].

4.4. Advantages of ^1H NMR Analysis for Evaluations of the Fate of H_2O_2 in the Oral Environment

High-resolution, high-field ^1H NMR spectroscopy is a technique which offers many advantages over alternative time-consuming, labour-intensive analytical methods for evaluating the fate of H_2O_2 (or alternative peroxo-adduct tooth-whitening agents) in oral fluids since 1) it permits the rapid, non-invasive and simultaneous examination of a very wide range of components present in biofluids, and 2) it 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 the ^1H NMR spectra of such multicomponent systems provide much valuable information regarding the molecular nature of both endogenous and exogenous agents therein.

5. Conclusions

In conclusion, high-resolution ^1H NMR analysis is a technique of much utility regarding multicomponent as-

assessments of the interactions of active agents present in commercially-available tooth-whitening products with human salivary biomolecules, and the oxidative decarboxylation of salivary pyruvate, together with the oxidation of lactate, trimethylamine, methionine and carbohydrates in general, by H₂O₂ present in a tooth-whitening formulation as demonstrated here, serves as an important fundamental example of this which is clearly of some relevance to the ability of human saliva to offer protection against any adverse effects arising from the leakage of this oxidant onto sensitive oral environments such as gums and further soft tissue environments.

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