

Multicomponent Spectroscopic Investigations of Salivary Antioxidant Consumption by an Oral Rinse Preparation Containing the Stable Free Radical Species Chlorine Dioxide (ClO_2^\cdot)

EDWARD LYNCH^a, ANGELA SHEERIN^b, ANDREW W. D. CLAXSON^b, MARTIN D. ATHERTON^b, CHRISTOPHER J. RHODES^c, CHRISTOPHER J. L. SILWOOD^b, DECLAN P. NAUGHTON^b, and MARTIN GROOTVELD^{b,*}

^aDepartment of Conservative Dentistry and ^bInflammation Research Group, St. Bartholomew's and the Royal London Hospitals School of Medicine and Dentistry, Turner Street, London E1 2AD, U.K.; ^cDepartment of Chemistry, Queen Mary and Westfield College, University of London, Mile End Road, London E1 4NS, U.K.

Accepted by Professor C. Rice-Evans

(Received 24 November 1995; Revised 30 August 1996)

A multicomponent evaluation of the oxidative consumption of salivary biomolecules by a commercially-available oral rinse preparation containing an admixture of the stable free radical species chlorine dioxide (ClO_2^\cdot) with chlorite anion (ClO_2^-) has been investigated using high resolution ^1H NMR spectroscopy. The results obtained demonstrated that ClO_2^\cdot and/or ClO_2^- present in this preparation effected the oxidative decarboxylation of salivary pyruvate (to acetate and CO_2). Experiments conducted on chemical model systems confirmed the oxidative decarboxylation of pyruvate by this oral rinse, and also demonstrated that urate, thiocyanate anion, and the amino acids cysteine and methionine (precursors to volatile sulphur compounds responsible for oral malodour), were oxidatively consumed. The biochemical, periodontal and therapeutic significance of the results are discussed.

Keywords: Chlorine dioxide, chlorite anion, human saliva, ^1H NMR, periodontology, halitosis

INTRODUCTION

Chlorine dioxide (ClO_2^\cdot) is a stable free radical species which, unlike NO_2^\cdot , exhibits little or no tendency to dimerise in view of an effective delocalisation of its unpaired electron. It is readily soluble in water forming a yellow-coloured solution (λ_{max} 360 nm, $\epsilon = 1,150 \text{ M}^{-1} \text{ cm}^{-1}$ [1,2]) in which it can remain intact for considerable periods of time, a phenomenon critically dependent upon pH and the availability of appropriate "stabilisers".[3]

ClO_2^\cdot is a powerful oxidant (redox potential + 0.96V at pH 4–7[3]) with potent bactericidal,[4] viricidal,[5,6] sporocidal,[7] cysticidal,[8] algicidal,[9] fungicidal[9] and organoleptic[10] properties, and has found much use as an effective deodorising

*Corresponding author.

and bleaching agent.^[11] Its excellent biocidal actions are more effective than those of chlorine (Cl_2) in aqueous solution [i.e., hypochlorous acid (HOCl)/hypochlorite anion (OCl^-)] since it is a stronger oxidant when present at an equivalent concentration.^[12] Indeed, it has also frequently been employed for purposes of water disinfection and, unlike the applications of aqueous Cl_2 in this context, does not appear to give rise to a range of chlorinated organic products (e.g., chlorophenol and chloroform) on reaction with trace levels of phenolic adducts and alternative organic precursors present (chloroform is generated via haloform reactions involving the attack of OCl^- on humic and fulvic acid derivatives^[13]); such chlorinated organic components pose a major hazard to human health.

In view of its powerful oxidising properties, ClO_2^- has a rich chemistry. In aqueous solution, it readily accepts an electron from single electron-transfer reductants to form chlorite anion (ClO_2^-) which also has the ability to act as a reactive oxidant.^[14] Previous investigations have established that ClO_2^- readily oxidises a variety of biomolecules, including products derived from bacterial metabolism. Notable examples are the oxidations of (1) the carbon-carbon double bonds of unsaturated aliphatic organic compounds, including unsaturated fatty acids;^[15] (2) aldehydes to their corresponding carboxylic acids;^[3] (3) thiols such as the amino acid cysteine together with inorganic and hydrogen sulphides;^[3,17] (4) phenols including the amino acid tyrosine, and thiophenols (producing the corresponding disulphide, disulphoxide and sulphonic acid derivatives—the disulphides also react with ClO_2^- to generate further disulphoxide and sulphonic acid);^[3,18] (5) secondary and tertiary amines (both aliphatic and aromatic);^[3,19] (6) alcohols and carbohydrates (relatively slow reactions which can yield carboxylic acid functional groups);^[3,20] (7) 1,4-dihydroxybenzenes (hydroquinones) which form corresponding quinones without any detectable aromatic ring chlorination;^[3,21] (8) Further amino acids such as methionine, proline, hydroxypro-

line, histidine and tryptophan.^[25] Clearly, the oxidative consumption of critical biomolecules by ClO_2^- accounts for its broad-spectrum biological activity, and specific permutations of the above processes (with each reaction system involved operating either individually or in concert) serve as potential mechanisms for one or more of its many biocidal properties.

ClO_2^- exerts a powerful pathogenic action towards micro-organisms which contaminate fish and plant-derived foodstuffs,^[3] and, of special reference to this investigation, is frequently utilised in toothpastes and oral rinses, together with deodorant/detergent and antiseptic preparations. Oral rinses containing ClO_2^- have been utilised in dental practices for more than 30 years and recently, a number of patients have used twice daily a 0.10% (w/v) ($1.48 \times 10^{-2} \text{ mol.dm}^{-3}$) ClO_2^- oral rinse solution (RetarDEX®) in combination with a toothpaste (RetarDENT®) containing the same ClO_2^- content. In a clinical report on pocket reduction, 67% of 2,085 pockets were reduced from $\geq 4\text{mm}$ to $\leq 3\text{mm}$ in a mean period of 3.4 months.^[23] Moreover, 71.85% of bleeding observed at probing sites was curtailed between two dental hygiene visits with a mean value of 6.9 months ($p < 0.01$). In addition to exerting a powerful antimicrobial activity, the above oral rinse formulation has also been shown to (1) oxidatively consume volatile sulphur compounds (VSCs) responsible for halitosis, (2) elevate the O_2 tension in both saliva and plaque, (3) remove residual organic solutes and (4) suppress the activity of bacterial proteolytic enzymes.^[23] Therefore, the therapeutic application of ClO_2^- —containing oral health care products appear to serve as an effective means of preventing or combating periodontitis and maintaining a high level of oral hygiene.

To date, no adverse effects associated with the use of ClO_2^- have been reported. Indeed, Lubbers *et. al.*^[24] conducted an increasing dose level tolerance investigation in which 50 human volunteers drank an aqueous solution containing $7.41 \times 10^{-5} \text{ mol.dm}^{-3}$ ClO_2^- for a period of 12 consecutive

weeks, and a range of appropriate clinical and laboratory indices were found to be unaffected by its ingestion.

In this study we have (1) employed a variety of analytical techniques [electronic absorption, electron spin resonance (ESR) and nuclear magnetic resonance (NMR) spectroscopies] to ascertain the chemical composition of a commercially-available ClO_2^- -containing oral rinse preparation (RetarDEX®) and (2) conducted a multicomponent evaluation of the oxidising actions of this therapeutic agent towards biomolecules present in human saliva using high resolution proton (^1H) NMR spectroscopy. Where appropriate, the reactions of oxidising components present in this oral rinse preparation with salivary electron donors (the α -keto acid anion pyruvate and the amino acids cysteine and methionine, thiocyanate anion and urate) were also investigated in chemical model systems. To the best of our knowledge, this is the first scientific report detailing a multicomponent biomolecular evaluation of the therapeutic actions of a stable free radical species in the treatment of periodontal diseases and dental caries.

2. MATERIALS AND METHODS

2.1 Sample Collection and Preparation

Unstimulated human saliva samples were obtained from a total of 10 healthy volunteers (7 male, 3 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 g for a 30 min. period to remove debris.

1.00 ml aliquots of each salivary supernatant were removed, and an equivalent volume of RetarDEX® oral rinse (D.D.S. Ltd, Scottsdale, Arizona, U.S.A.) was added. The mixture was incubated 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 1.00 ml aliquots of each salivary supernatant sample

treated with an equivalent volume of doubly-distilled H_2O (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 1.00×10^{-2} mol.dm $^{-3}$ sodium pyruvate, L-cysteine or L-methionine (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 then treated with an equivalent volume of RetarDEX® oral rinse, 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 as described above for control and oral rinse-treated salivary supernatants. Additional 1.00 ml aliquots of each solution treated with an equivalent volume of doubly-distilled H_2O and then incubated and stored in the same manner served as controls.

Similarly, aqueous solutions containing individual or mixtures of electron donors (antioxidants) known to be present in human saliva (pyruvate, cysteine, thiocyanate anion and urate) were prepared in de-oxygenated phosphate buffer solution (pH 7.10) and aliquots of each sample were treated with appropriate volumes of RetarDEX® oral rinse to yield a range of initial reactant concentrations (details given in Table II), equilibrated at 37°C for a period of 30 min. and then stored at -20°C for a duration of 18 hr. prior to ^1H NMR analysis. Matching antioxidant solutions treated with equivalent volumes of doubly-distilled water in place of the oral rinse formulation, equilibrated at 37°C and stored in the same manner, served as controls.

Potassium thiocyanate and uric acid were also obtained from Sigma, and sodium chlorite [80% (w/w)] and sodium chlorate were purchased from Aldrich Chemical Co Ltd (Gillingham, Dorset, UK). All other chemicals were of the highest possible grade and obtained from commercially-available sources.

2.2 Proton NMR Measurements

Proton (^1H) NMR measurements on the above samples were conducted on Bruker AMX-600 [University of London Intercolleigate Research Services (ULIRS), Queen Mary and Westfield College Facility, University of London, UK] or Bruker AMX-400 (ULIRS, King's College Facility, University of London, UK) spectrometers operating at frequencies of 600.13 and 400.13 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 was suppressed by presaturation via gated decoupling during the delay between pulses. For control and oral rinse-treated salivary supernatants, the broad protein resonances were suppressed by the Hahn spin-echo sequence ($\text{D}[90^\circ\text{x-t-}180^\circ\text{y-t-collect}]$)^[27] 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) spectra acquired on control and oral rinse-treated solutions of pyruvate, L-cysteine and L-methionine, typical pulsing conditions were 32 free induction decays (FIDs) using 32,768 data points, 72° pulses, and a 3 s pulse repetition rate, the latter to allow full spin-lattice (T_1) relaxation of the protons in the samples investigated. Exponential line-broadening functions of 0.20 Hz were employed for purposes of processing.

The relative intensities of selected signals were determined by electronic integration, and the concentrations of components detectable in a typical sample of the oral rinse preparation were determined by comparing their resonance areas with that of an added TSP internal standard (final concentration 3.03×10^{-5} mol.dm $^{-3}$).

Two dimensional shift-correlated NMR (COSY) spectra of human saliva were recorded on the Bruker AMX-400 facility using the standard sequence of Aue *et al.*,^[28] with 2,048 data points in the t_2 dimension, 256 increments of t_1 , a 3.00 s relaxation delay, and 64 transients.

150 MHz ^{13}C NMR spectra of control (untreated) and RetarDEX[®] oral rinse-treated aqueous thiocyanate solutions were acquired on the Bruker AMX-600 spectrometer. Pulsing conditions were 6,144 FIDs using 65,536 data points, 67.5° pulses, a 0.655 s acquisition time and a 1.00 s pulse delay.

2.3 Spectrophotometric and ESR Analysis of Oral Rinse Preparations

Zero-order and second-derivative electronic absorption spectra of RetarDEX[®] oral rinse, together with aqueous solutions of sodium chlorite and chlorate were recorded on a PC-Controlled Unicam UV-2 spectrophotometer (scan rates 120 and 30 nm/min. for absorption and second-derivative spectra respectively). The pH of these samples was adjusted to the desired value prior to analysis with aqueous HCl or NaOH.

Electron spin resonance spectra of oral rinse samples were recorded using a Varian E-9 spectrometer at a temperature of 77 K in a liquid N_2 -filled finger Dewar. Where appropriate, the pH of the sample was adjusted to values of 2.90 or 1.20 with aqueous HCl prior to ESR analysis.

2.4 HPLC Determination of Salivary Urate

Control and RetarDEX[®] oral rinse-treated salivary supernatant samples were ultrafiltered at $5,000 \times g$ for 30 min. at 5°C using Millipore ultrafiltration devices with a molecular mass cut-off of 5 kDa, and the ultrafiltrate obtained subjected to urate analysis by HPLC according to a modification of the method of Grootveld and Halliwell.^[36] HPLC was conducted using an Applied Chromatography Systems (ACS) 351 isocratic pump cou-

pled to a Rheodyne injector fitted with a 20 μ l loop. A Spherisorb S5-ODS 1 guard column was used to separate urate from other detectable salivary supernatant components. The mobile phase was 88% (v/v) 3.00×10^{-2} mol.dm⁻³ sodium citrate/ 2.77×10^{-2} mol.dm⁻³ sodium acetate buffer (pH 4.75) and 12% (v/v) methanol at a flow rate of 1.00 ml/min. continuously sparged with He gas during elution. Urate was detected with a variable wavelength electronic absorption detector (ACS 750/16), at 292 nm. Identification of the urate peak in chromatograms of human salivary supernatant ultrafiltrates was based on its wavelength-dependence (plots of peak height versus wavelength for salivary supernatant ultrafiltrates closely matched those of authentic urate calibration standards in the 220–320 nm wavelength range). Calibration standards of urate were injected into the sample loop by microsyringe, and plots of peak height versus calibration standard concentration were linear with zero intercept ($r = 0.9998$).

2.5 Time-Dependent Spectrophotometric Monitoring of the Reaction of Salivary Urate with RetarDEX® Oral Rinse Oxohalogen Oxidants

The rate of the reaction between salivary urate and oxohalogen oxidants present in the oral rinse preparation investigated was monitored by difference spectrophotometry at a temperature of 22°C and pH 5.96 (a value very close to the mean pH of unstimulated human saliva³¹). For this purpose, a twin reference quartz cell was employed. To the first split reference cell compartment, a mixture of 0.40 ml of salivary supernatant and 0.60 ml of 4.00×10^{-2} mol.dm⁻³ phosphate buffer (pH 5.96) was added; to the second cell compartment, a mixture of 0.40 ml of RetarDEX® oral rinse and 0.60 ml of 4.00×10^{-2} mol.dm⁻³ phosphate buffer (pH 5.96) was added. Subsequently, 0.40 ml of the same salivary supernatant sample and 1.20 ml of the above phosphate buffer solution were added to the sample (reaction) cell and the reaction initiated by the

addition of 0.40 ml of RetarDEX® oral rinse [this reaction mixture was thoroughly mixed prior to acquisition of the first difference spectrum (ca. 10 s after mixing)]. Difference spectra (230–330 nm wavelength range) were recorded on a Unicam UV-2 PC-controlled spectrophotometer at 30 min. intervals thereafter for a total period of 9.5 hr. The rate of the ClO₂⁻/ClO₂[•]-mediated oxidative consumption of salivary urate was spectrophotometrically monitored in a total of 5 separate salivary supernatant samples.

3 RESULTS

3.1 Spectrophotometric Analysis of Oral Rinse Preparations

Figure 1a shows the electronic absorption spectrum of a typical intact sample of the oral rinse preparation investigated here (pH 6.5), together with those of 1.00×10^{-2} mol.dm⁻³ aqueous solutions of sodium chlorite and sodium chlorate in 4.00×10^{-2} mol.dm⁻³ phosphate buffer (pH 7.00). Clearly, little or no ClO₂[•] (λ_{max} . 360 nm) is detectable in the spectrum of the oral rinse preparation which has broad absorption maxima centered at ca. 260 and 290 nm. This spectrum is very similar to that of aqueous chlorite solution (λ_{max} 262 nm, $\epsilon = 84 \text{ M}^{-1} \text{ cm}^{-1}$, with a broad overlapping side-band at ca. 285 nm) and, without allowing for absorbance contributions arising from any alternative components present [e.g., hypochlorite anion (OCl⁻)], the absorbance of the oral rinse preparation at 262 nm corresponds to a ClO₂⁻ concentration of 1.34×10^{-2} mol.dm⁻³, a value close to that of its specified ClO₂⁻ level of 0.10% (w/v) (1.48×10^{-2} mol.dm⁻³). These data indicate that ClO₂⁻ is the predominant oxidant present in this commercially-available oral rinse. However, the electronic absorption spectrum of this material deviates from aqueous solutions of chlorite in that it (1) has significant absorption in the 320–380 nm wavelength range (presumably ascribable to very low levels of ClO₂[•] present, Section 3.2) and (2) appears to contain additional absorption bands located at

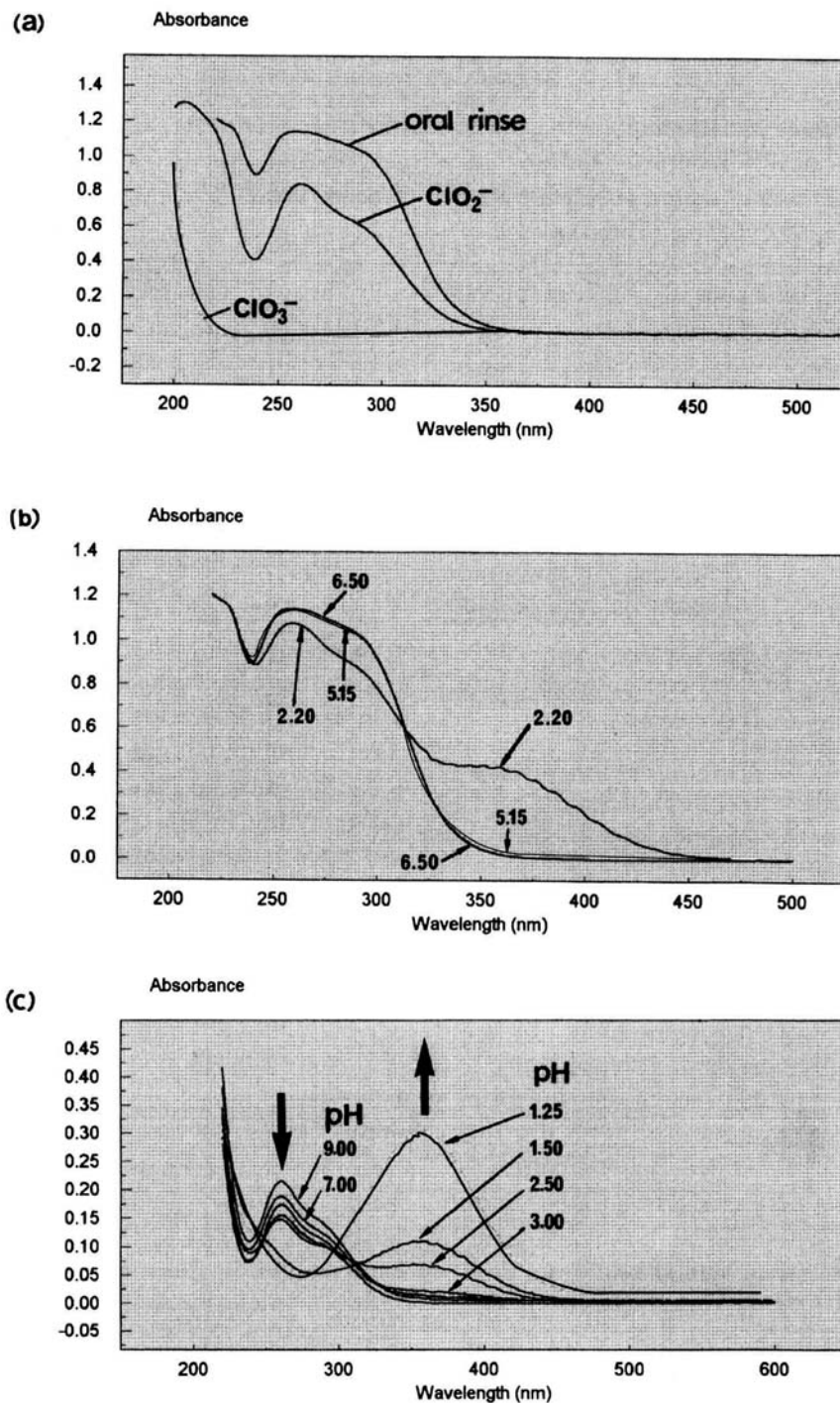
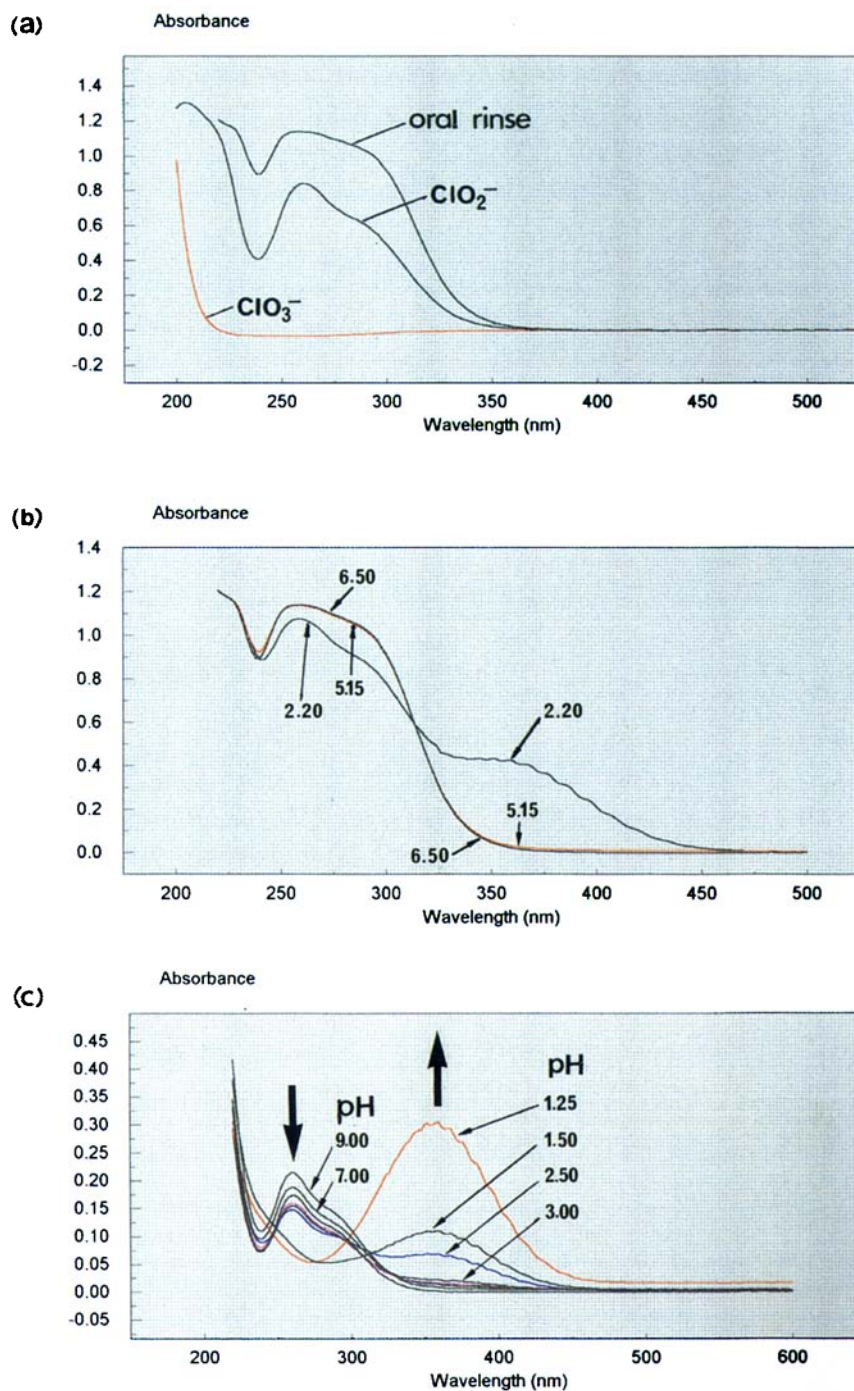


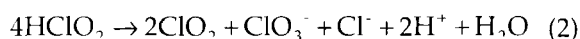
FIGURE 1 Electronic absorption spectra of (a) intact RetarDEX® oral rinse (pH 6.50), and $1.00 \times 10^{-2} \text{ mol.dm}^{-3}$ aqueous solutions of sodium chlorite and sodium chlorate in $4.00 \times 10^{-2} \text{ mol.dm}^{-3}$ phosphate buffer (pH 7.00); (b) RetarDEX® oral rinse obtained at pH values of 6.50 (untreated), 5.15 and 2.20; (c) $1.00 \times 10^{-2} \text{ mol.dm}^{-3}$ aqueous solutions of sodium chlorite adjusted to pH values of 9.00, 7.00, 5.50, 4.00, 3.00, 2.50, 1.50 and 1.25. The pH of these samples was modified with aqueous HCl or NaOH and, for the results shown in (b) and (c), samples were equilibrated at ambient temperature for a period of 60 min. prior to recording spectra. Abbreviations: ClO_2^- , chlorine dioxide (λ_{max} 360 nm); ClO_2^- , chlorite anion; ClO_3^- , chlorate anion. The arrows in (c) denote decreases or increases in absorbance observed with decreasing pH value. (See Color Plate I at the back of this issue.)



Color Plate I (See page 214, Figure 1) Electronic absorption spectra of (a) intact RetarDEX® oral rinse (pH 6.50), and $1.00 \times 10^{-2} \text{ mol.dm}^{-3}$ aqueous solutions of sodium chlorite and sodium chlorate in $4.00 \times 10^{-2} \text{ mol.dm}^{-3}$ phosphate buffer (pH 7.00); (b) RetarDEX® oral rinse obtained at pH values of 6.50 (untreated), 5.15 and 2.20; (c) $1.00 \times 10^{-2} \text{ mol.dm}^{-3}$ aqueous solutions of sodium chlorite adjusted to pH values of 9.00, 7.00, 5.50, 4.00, 3.00, 2.50, 1.50 and 1.25. The pH of these samples was modified with aqueous HCl or NaOH and, for the results shown in (b) and (c), samples were equilibrated at ambient temperature for a period of 60 min. prior to recording spectra. Abbreviations: ClO_2^- , chlorine dioxide (λ_{max} 360 nm); ClO_2^- , chlorite anion; ClO_3^- , chlorate anion. The arrows in (c) denote decreases or increases in absorbance observed with decreasing pH value.

ca. 235 and 292 nm which are conceivably ascribable to detectable levels of HOCl (λ_{max} 235 nm, $\epsilon = 100 \text{ M}^{-1} \text{ cm}^{-1}$) and OCl^- (λ_{max} 292 nm, $\epsilon = 350 \text{ M}^{-1} \text{ cm}^{-1}$ at pH 9.5) present, predominantly the former in view of the pK_a value of HOCl (7.40). Indeed, a corresponding second-derivative spectrum of the oral rinse preparation contained a small minimum located at 292 nm, a result further suggesting that this material contains trace levels of aqueous Cl_2 . Consistent with these observations, Fukayama *et al.*^[26] have reported that commercially-available preparations of stabilised ClO_2^- also contain HOCl, OCl^- and small amounts of molecular Cl_2 , together with ClO_2^- , chlorate (ClO_3^-) and chloride.

As expected, treatment of the oral rinse preparation with HCl (pH 2.20) gave rise to an absorption spectrum with an intense 360 nm band attributable to ClO_2^\cdot liberated from chlorite present via the disproportionation of chlorous acid (equations 1 and 2).



The stoichiometry, kinetics and mechanism of this process are very complex^[2,29] and the reaction depicted in equation 2 is catalysed by chloride ion, a reaction product (equation 2). The pK_a value of chlorous acid is 2.31,^[30] and hence at pH 2.20 there are only relatively small concentrations of unprotonated chlorite present. However, small but significant levels of unstable HClO_2 are also expected at pH values of ca. 5 and we were able to detect low levels of ClO_2^\cdot generation (i.e., small increases in absorbance at 360 nm) when the pH of the oral rinse preparation was adjusted to 5.15. The 240–315 nm region of the spectrum of this material obtained at pH 2.20 is clearly more similar to that of chlorite anion, indicating the consumption of one or more interfering components present, presumably the protonation of OCl^- present (λ_{max} 292 nm) to form HOCl.

Figure 1c shows a spectrophotometric titration of an aqueous solution containing 1.67×10^{-3}

mol.dm^{-3} chlorite with hydrochloric acid (HCl). Clearly, as the pH of the sample is lowered from 9.00 to 1.25, high levels of ClO_2^\cdot (λ_{max} 360 nm) are generated with a corresponding reduction in the level of ClO_2^- (the latter represented by a decrease in absorbance in the 250–300 nm wavelength range). In the spectrum acquired at a pH value of 1.25, the absorbance at 360 nm corresponds to a ClO_2^\cdot concentration of $2.61 \times 10^{-4} \text{ mol.dm}^{-3}$ (i.e., 31% of the total level expected from equation 2). Interestingly, the spectra shown in this figure demonstrate that small but significant levels of ClO_2^\cdot are generated from aqueous chlorite at pH values of 5.5 or more, data indicating that this free radical species is likely to be liberated from the oral rinse preparation in the acidotic oral environment (the mean pH value of unstimulated human saliva is 5.98³¹).

3.2 ESR Analysis of Oral Rinse Preparations

The results outlined in Section 3.1 were confirmed by electron spin resonance (ESR) spectroscopic analysis of the oral rinse preparation. The ESR spectrum of the untreated (pH 6.50) material [Fig. 2a] contained a weak ClO_2^\cdot signal, confirming its presence at a very low concentration. As expected, acidification of this sample gave rise to a prominent ClO_2^\cdot signal which was markedly intensified on further decreasing the pH from 2.90 to 1.20 [Fig. 2b and c]. These ESR data further demonstrate the liberation of this stable free radical species from the disproportionation of unstable chlorous acid (HClO_2). The ClO_2^\cdot signal present in the spectra exhibited in Figs. 2b and c shows clear hyperfine couplings to both ^{35}Cl and ^{37}Cl nuclei, the intensities of which reflect their isotopic ratio of approximately 3:1 ($^{35}\text{Cl}:^{37}\text{Cl}$).

3.3 ^1H NMR Analysis of RetarDEX® Oral Rinse

The 600 MHz ^1H NMR spectrum of a typical sample of the $\text{ClO}_2^-/\text{ClO}_2^\cdot$ -containing oral rinse is shown in Figure 3. As expected, this spec-

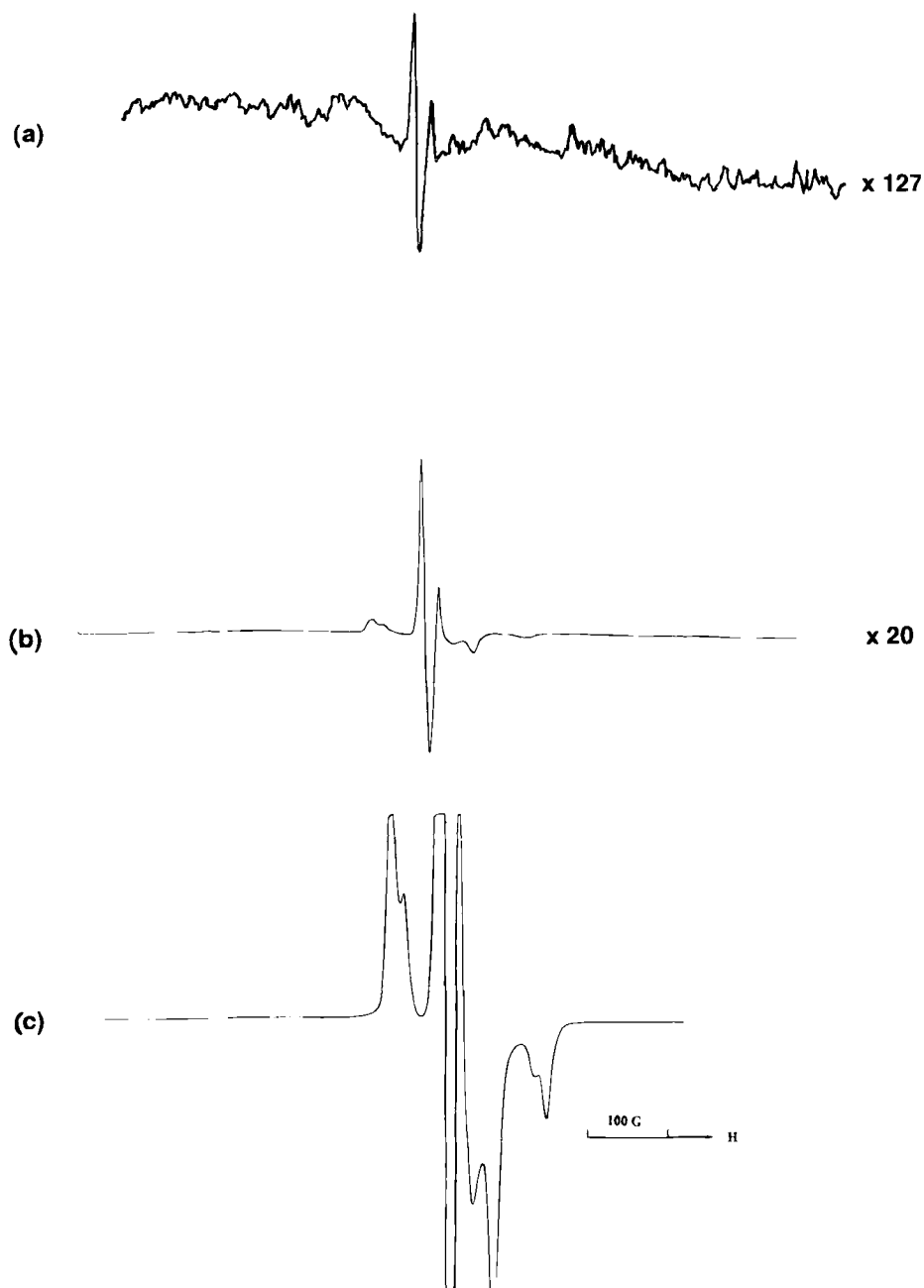


FIGURE 2 ESR spectra of RetarDEX® oral rinse acquired at pH values of 6.50 (untreated), 2.90 and 1.20. The pH of these samples was adjusted as described in Figure 1. Clear hyperfine couplings to ^{35}Cl and ^{37}Cl nuclei are indicated in spectra (b) and (c).

trum contains intense resonances arising from citrate [characteristic AB coupling system (doublet of doublets, $\delta = 2.63$ ppm)]. However, further ^1H resonances are also notable, i.e. ethanol- CH_3 and $-\text{CH}_2$ group signals (triplet and quartet located at $\delta = 1.20$ and 3.66 ppm respectively) together with acetate- CH_3 and formate-H group singlets ($\delta = 1.92$ and 8.45 ppm respectively). Moreover, a prominent singlet at 3.37 ppm assignable to the $-\text{CH}_3$ group protons of methanol is also present in the spectrum. The acetate, formate, ethanol and

methanol detectable are presumably impurities present in the commercial samples of $\text{ClO}_2^-/\text{ClO}_2^{\cdot}$, citric acid, trisodium orthophosphate ($\text{Na}_3\text{PO}_4 \cdot 12\text{H}_2\text{O}$) and/or flavour and colour added to the oral rinse by the manufacturer. The concentrations of the above components in a typical RetarDEX® oral rinse preparation are given in Table I. The presence of low levels of ESR-detectable ClO_2^{\cdot} radical in this oral rinse was not found to significantly increase the line-widths of any of the resonances present in its ^1H NMR spectrum.

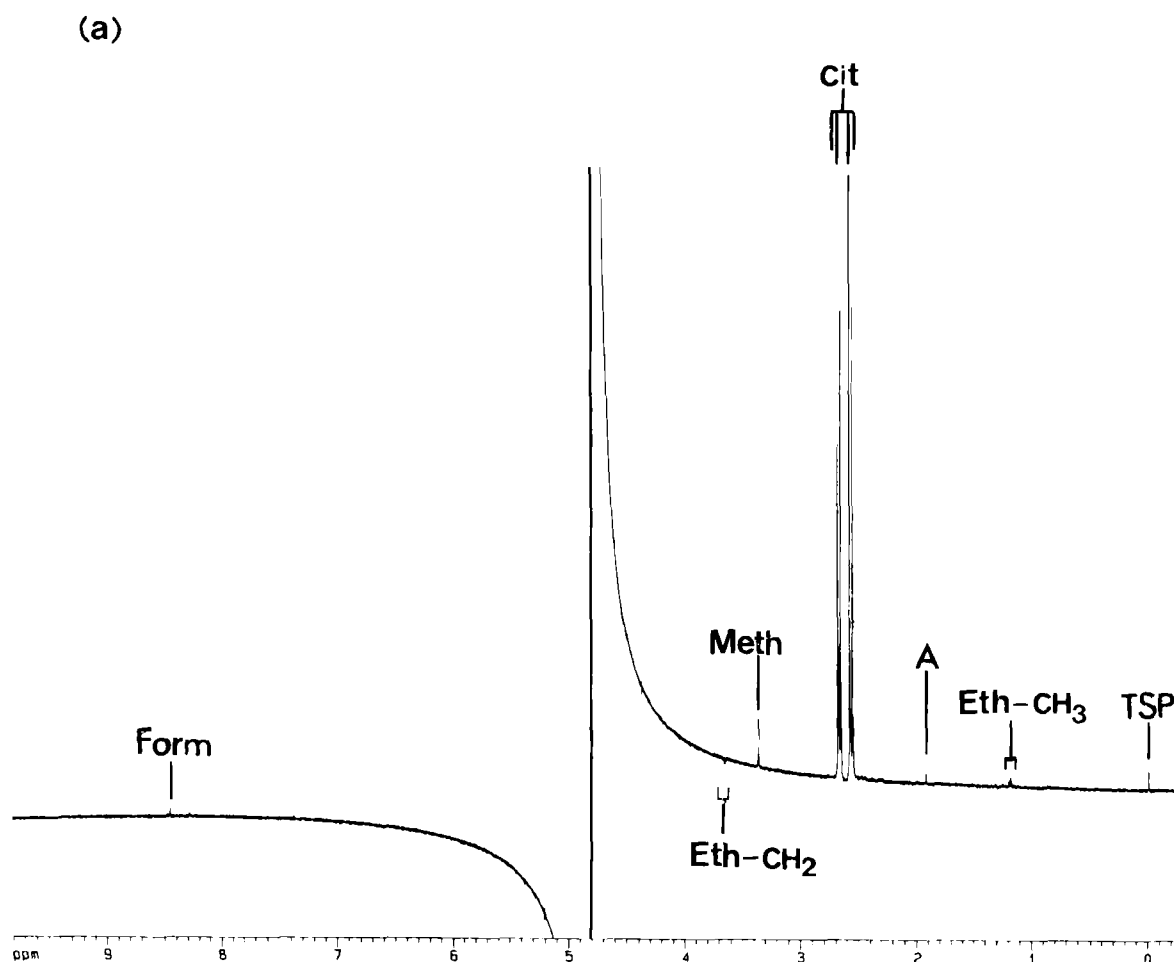


FIGURE 3 (a) Complete, and (b) expanded 0.50–4.00 ppm regions of a 600 MHz ^1H NMR spectrum of a sample of RetarDEX® oral rinse. A typical spectrum is shown. Abbreviations: A, acetate- CH_3 ; Cit, Citrate- CH_2 ; Eth, ethanol- CH_3 ; Meth, methanol- CH_3 ; Form, Formate-H; TSP, Sodium-3-trimethylsilyl-[2,2,3,3- $^2\text{H}_4$]-propionate (added as an internal standard).

(b)

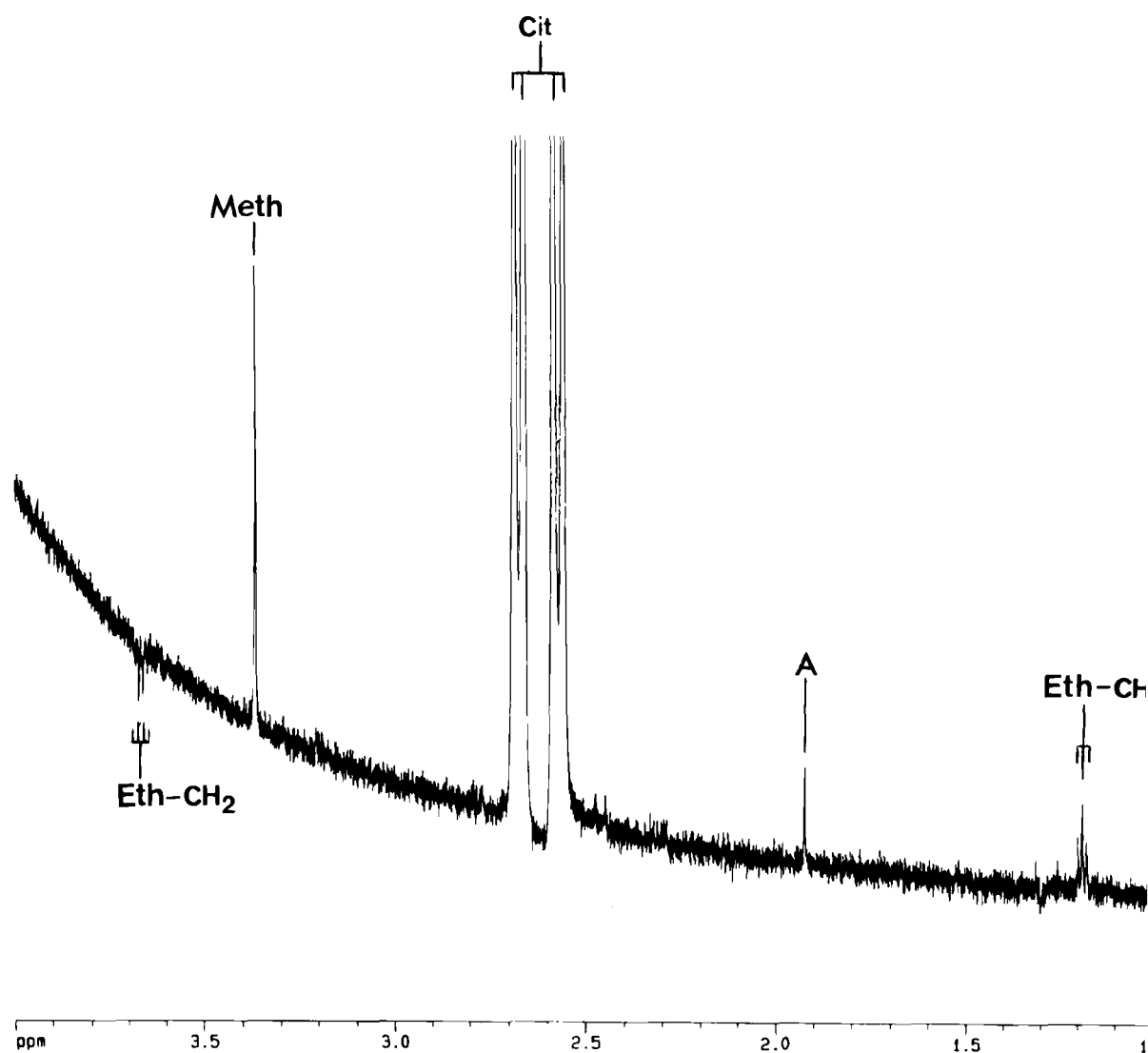


FIGURE 3 Continued

3.4 Multicomponent Evaluations of the Oxidative Consumption of Salivary Components by ClO_2^- and/or ClO_2^- Present in the Oral Rinse Preparation

Figure 4a shows the ¹H Hahn spin-echo NMR spectrum of a typical control (untreated) human saliva sample. This spectrum contains

many sharp, prominent resonances which arise from a wide variety of low-molecular-mass components. Indeed, signals assignable to *n*-butyrate, propionate, *iso*-butyrate, 3-D-hydroxybutyrate, lactate, acetate, pyruvate, succinate, creatinine and glycine are present in the high field (0.00–4.50 ppm), aliphatic regions of spectra acquired, and those of fumarate, formate,

TABLE I Concentrations of citrate, acetate, formate, methanol and ethanol present in a typical sample of RetarDEX® oral rinse, determined by ^1H NMR spectroscopy.

Component	Concentration (mol.dm^{-3})
Citrate	6.134×10^{-3}
Acetate	4.31×10^{-5}
Formate	$\text{ca. } 6 \times 10^{-5}$
Methanol	1.71×10^{-4}
Ethanol	7.64×10^{-5}

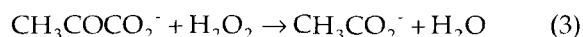
histidine and tyrosine are detectable in the low field (6.00–9.00 ppm), aromatic regions. The organic acid anions detectable (e.g. *n*-butyrate, propionate etc.) reflect, in whole or in part, the growth of potentially pathogenic micro-organisms such as *P. gingivalis*^[16] and hence serve as chemotaxonomic markers of disease activity.

A further notable feature of these spectra is a broad resonance centred at 2.04 ppm which is attributable to the acetamido methyl group protons ($-\text{NHCOCH}_3$) of N-acetylsugars present in the molecularly-mobile, branching carbohydrate side-chains of 'acute-phase' glycoproteins (i.e. salivary mucins). This resonance underlies a number of sharper acetamido- CH_3 group signals assignable to low-molecular-mass N-acetylsugars such as sialates and N-acetylglucosamine-containing saccharide fragments which conceivably arise from the actions of bacterial-derived neuraminidase and hyaluronidase respectively. Moreover, both methanol and ethanol were detectable in the great majority of human saliva samples examined by ^1H NMR spectroscopy. Although ethanol is a bacterial-derived catabolite, we have recently demonstrated that the methanol present is derived from the inhalation of cigarette smoke (i.e. direct or passive) via the combustion of tobacco lignin which contains many methoxy groups in its complex, macromolecular structure.

Two-dimensional COSY ^1H NMR spectra of typical human saliva samples [Fig. 4b] showed clear connectivities between the multiplet resonances present, e.g. 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 ethanol- CH_3 and $-\text{CH}_2$ groups and the tyrosine aromatic ring protons (doublets located at 6.88 and 7.17 ppm).

600 MHz ^1H Hahn spin-echo NMR spectra of a human saliva sample obtained prior and subsequent to treatment with RetarDEX® oral rinse *in vitro* (Section 2) are shown in Figure 5. Clearly, addition of the $\text{ClO}_2^-/\text{ClO}_2^{\cdot-}$ -containing oral rinse gives rise to the complete disappearance of the pyruvate- CH_3 group signal, an observation reproducible in all samples examined ($n = 10$). These data are consistent with the oxidative consumption of salivary pyruvate by ClO_2^- and/or $\text{ClO}_2^{\cdot-}$ radical. Indeed, pyruvate acts as a powerful endogenous reductant (i.e., a water-soluble antioxidant) and is oxidatively decarboxylated to acetate and CO_2 on reaction with hydrogen peroxide^[22] (equation 3), a reactive oxygen species present in certain alternative commercially-available oral rinses.



Further modifications to the salivary ^1H NMR profiles observed on addition of the $\text{ClO}_2^-/\text{ClO}_2^{\cdot-}$ -containing oral rinse included the oxidative consumption of 3-D-hydroxybutyrate in samples in which it was NMR-detectable prior to treatment ($n = 3$). Moreover, in two of the saliva samples investigated, a singlet resonance of unknown identity ($\delta = 1.72$ ppm) was generated in spectra following *in vitro* treatment with the oral rinse.

As expected, elevations in the intensities of resonances arising from components also present in the oral rinse preparation (acetate, citrate, formate, methanol and ethanol) were also observed, those of the citrate and methanol signals being substantial. However, the increase in the acetate- CH_3 group signal intensity is also partially ascribable to the $\text{ClO}_2^-/\text{ClO}_2^{\cdot-}$ -mediated oxidative decarboxylation of pyruvate described above.

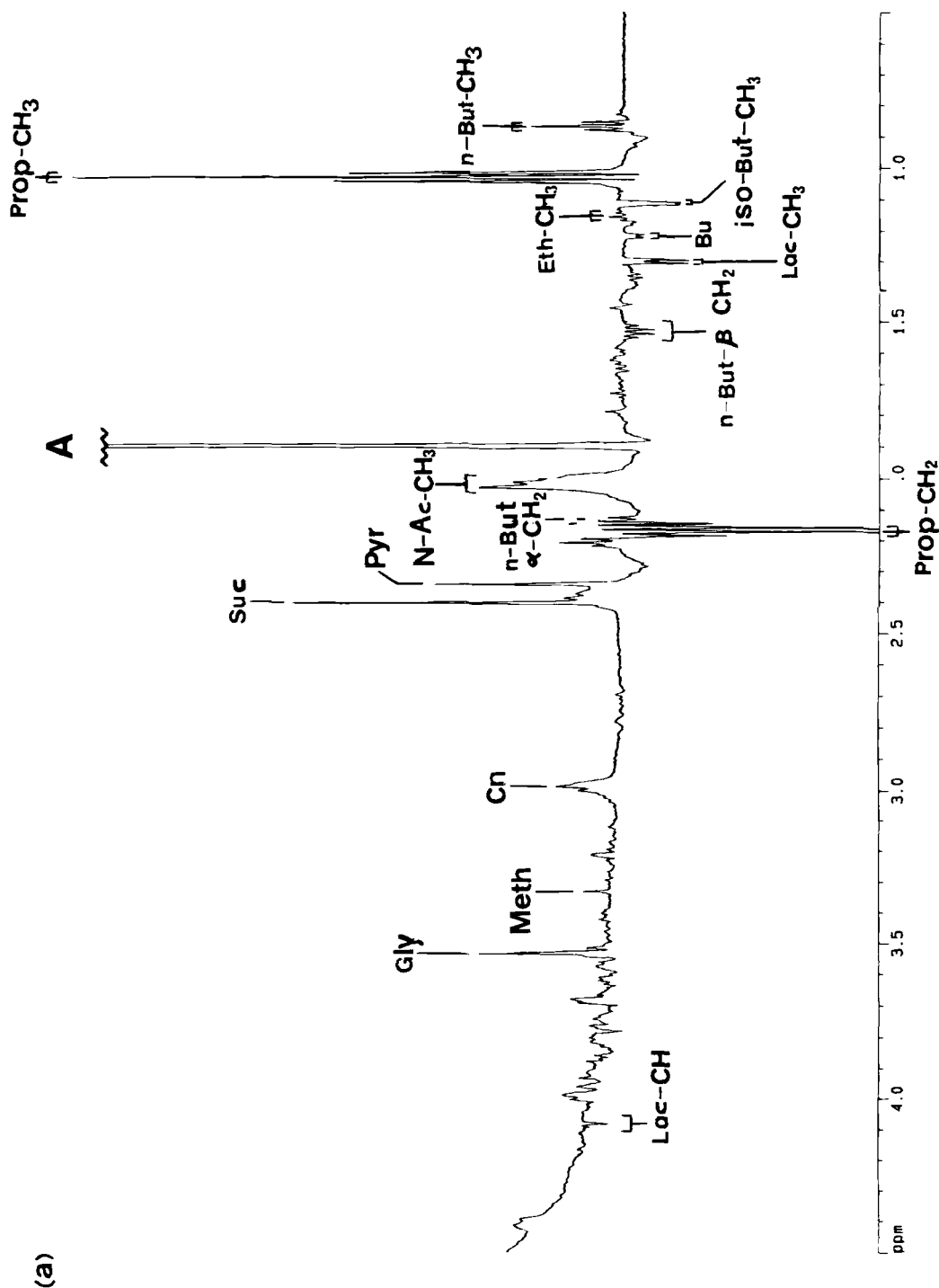


FIGURE 4 (a) High Field (aliphatic) region of the 600 MHz ^1H Hahn spin-echo NMR spectrum of a human salivary supernatant, and (b) 400 MHz two-dimensional shift-correlated ^1H NMR (COSY) spectrum of the supernatant obtained from a further human saliva sample. Typical spectra are shown. Abbreviations, as Figure 3, with Bu, 3-D-hydroxybutyrate-CH₃; *n*-But-CH₃, β - and α -CH₂, *n*-butyrate-CH₃, β - and α -CH₂ groups respectively; Gly, glycine- α -CH₂; *iso*-But, *iso*-butyrate-CH₃; Lac-CH₃ and -CH, lactate-CH₃ and -CH; N-Ac-CH₃, acetamido methyl groups of both high- and low-molecular-mass components (e.g., *N*-acetylsugars presents in the molecularly-mobile, branching carbohydrate side-chains of *N*-acetylated glycoproteins, and oligosaccharides derived from the depolymerisation of hyaluronate, respectively); Prop-CH₃ and -CH₂, propionate-CH₃ and -CH₂; Pyr, pyruvate-CH₃; Suc, succinate-CH₂; Cn, creatinine N-CH₃; Tyr, tyrosine aromatic ring protons; His, histidine imidazole ring protons; Phen, phenylalanine aromatic ring protons; Form, formate-H; Ala, alanine-CH₃.

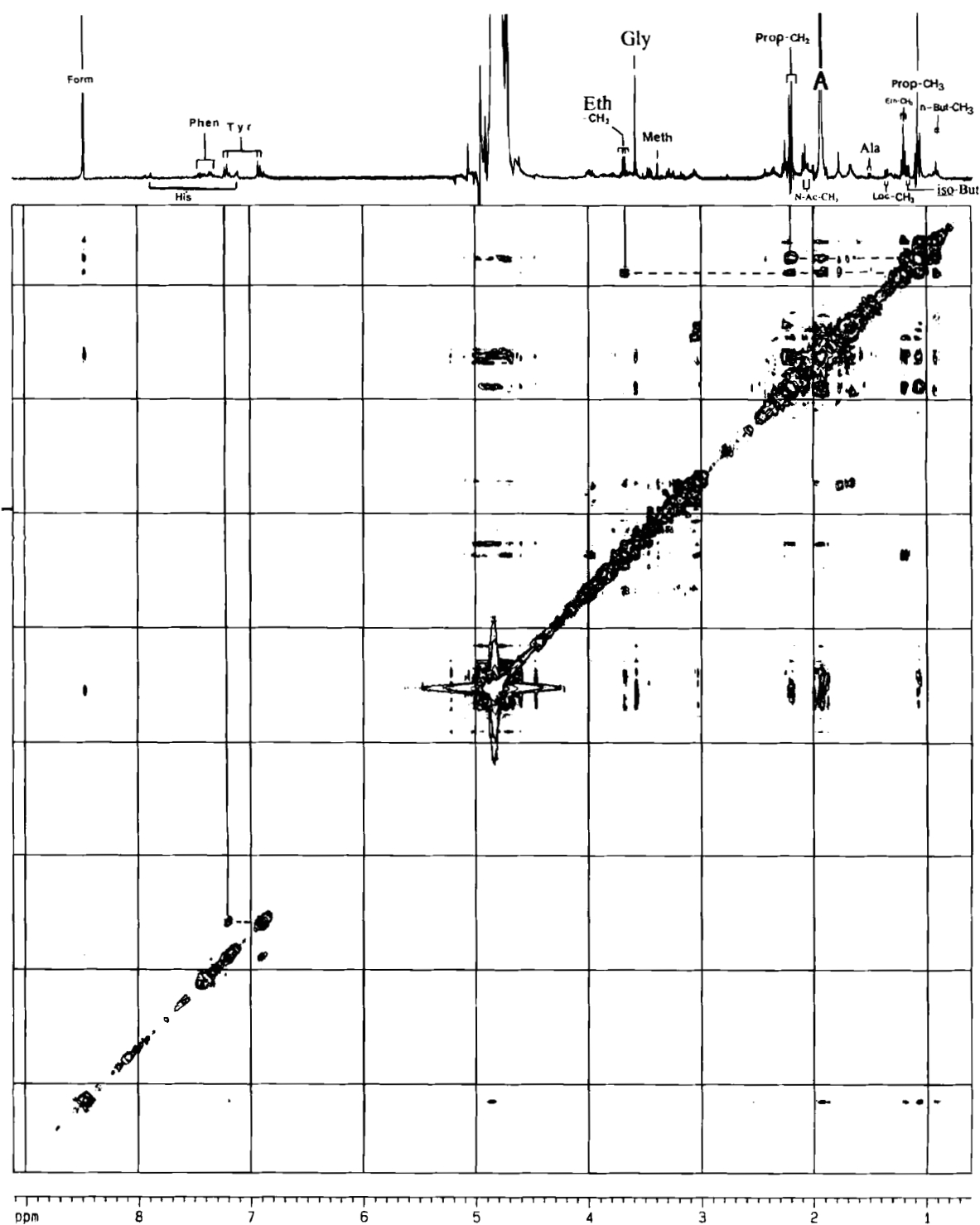
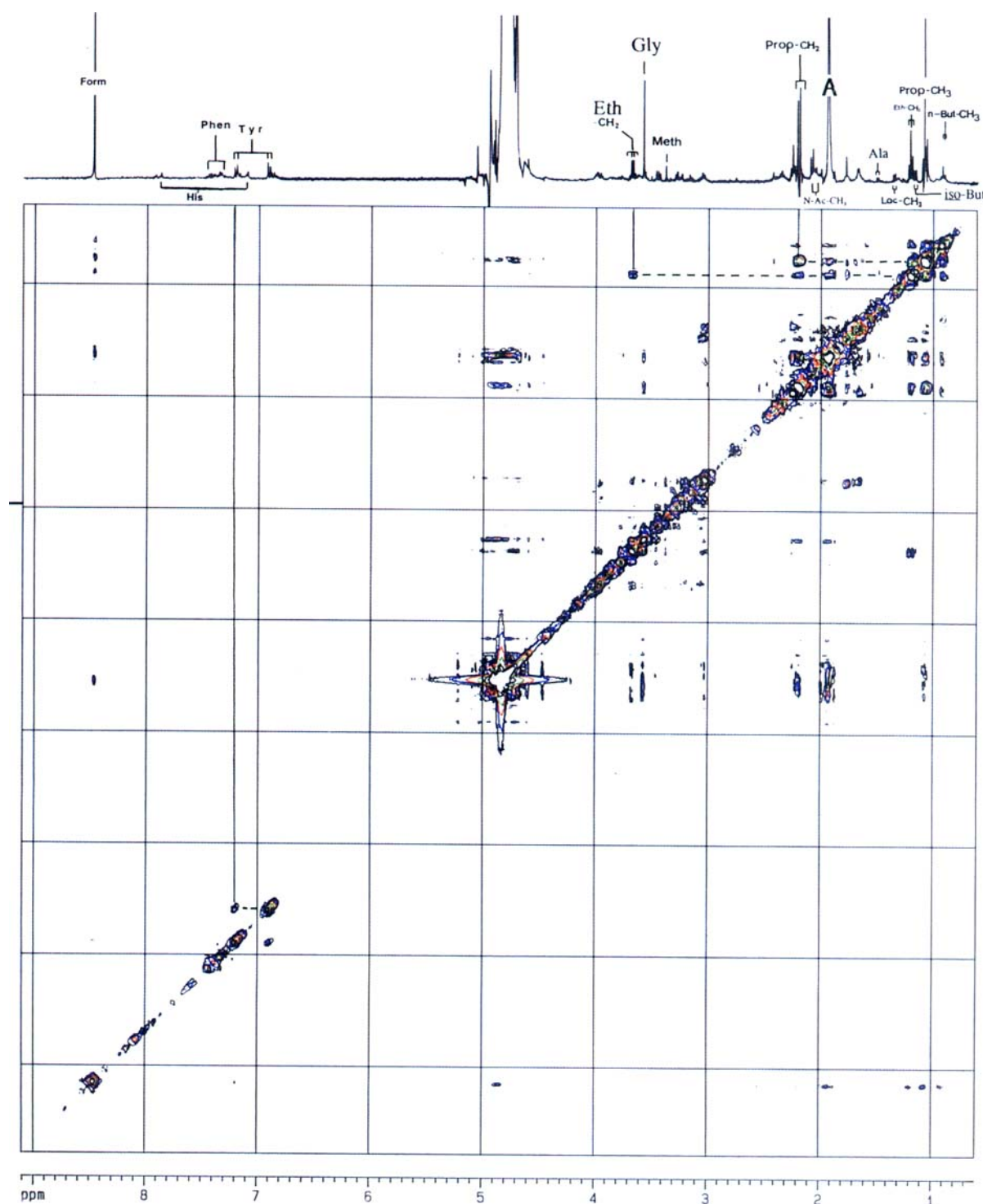


FIGURE 4 Continued (See Color Plate II at the back of this issue.)



Color Plate II (See page 221, Figure 4) (a) High Field (aliphatic) region of the 600 MHz ^1H Hahn spin-echo NMR spectrum of a human salivary supernatant, and (b) 400 MHz two-dimensional shift-correlated ^1H NMR (COSY) spectrum of the supernatant obtained from a further human saliva sample. Typical spectra are shown. Abbreviations, as Figure 3, with Bu, 3-D-hydroxybutyrate- CH_3 ; *n*-But- CH_3 , β - CH_2 and α - CH_2 , *n*-butyrate- CH_3 , β - and α - CH_2 groups respectively; Gly, glycine- α - CH_2 ; *iso*-But, *iso*-Butyrate- CH_3 ; Lac- CH_3 and -CH, lactate- CH_3 and -CH; N-Ac- CH_3 , acetamido methyl groups of both high- and low-molecular-mass components (e.g., N-acetylsugars presents in the molecularly-mobile, branching carbohydrate side-chains of N-acetylated glycoproteins, and oligosaccharides derived from the depolymerisation of hyaluronate, respectively); Prop- CH_3 and - CH_2 , propionate- CH_3 and - CH_2 ; Pyr, pyruvate- CH_3 ; Suc, succinate- CH_2 ; Cn, creatinine N- CH_3 ; Tyr, tyrosine aromatic ring protons; His, histidine imidazole ring protons; Phen, phenylalanine aromatic ring protons; Form, formate-H; Ala, alanine- CH_3 .

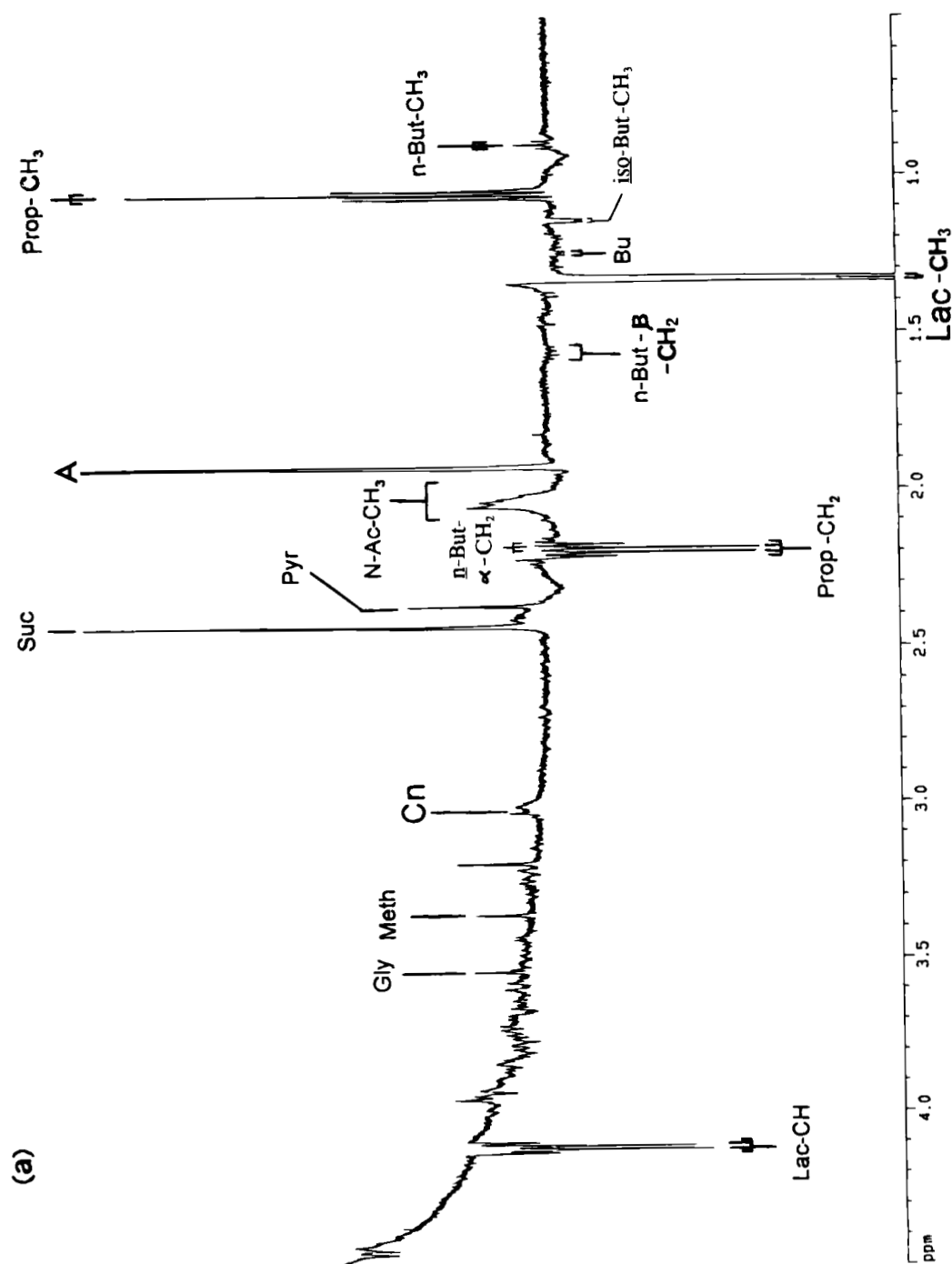


FIGURE 5 Partial (0.50–4.50 ppm regions of) 600 MHz ^1H Hahn spin-echo NMR spectra of a salivary supernatant sample obtained after treatment with equivalent volumes of (a) de-gassed doubly-distilled H_2O (control) and (b) RetarDEX® oral rinse. Typical spectra are shown. Abbreviations: as Figures 3 and 4.

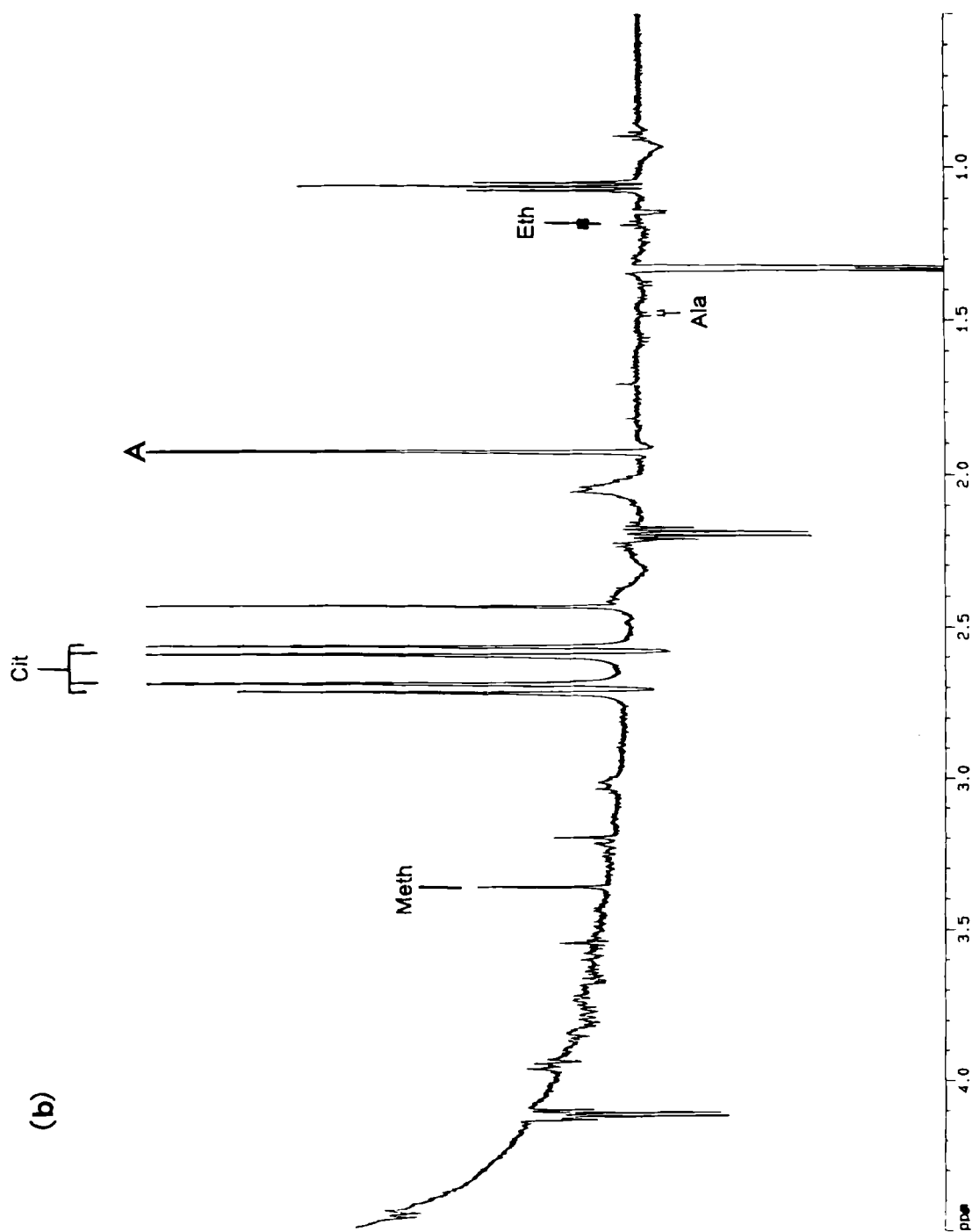
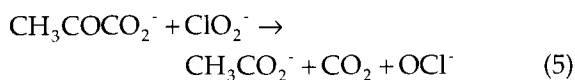
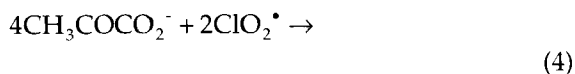


FIGURE 5 Continued

3.5 Chemical Model Studies of the Reactions of Salivary Electron Donors with ClO_2^- and ClO_2^\bullet Present in the Oral Rinse Preparation

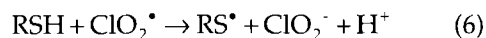
Aqueous solutions of pyruvate were treated with the RetarDEX® oral rinse to provide further information regarding the nature and mechanism of the ClO_2^- - and ClO_2^\bullet -mediated oxidation of this α -keto acid anion in human saliva. ^1H NMR analysis demonstrated that reaction of pyruvate in an aqueous phosphate-buffered solution with $\text{ClO}_2^-/\text{ClO}_2^\bullet$ present in the above oral rinse at pH 7.20 gave rise to an almost complete transformation of the α -keto acid anion substrate to acetate and CO_2 (Fig. 6). Indeed, >99% of the pyruvate was oxidatively decarboxylated to acetate and CO_2 under the experimental conditions employed, and a singlet resonance attributable to an impurity present in the commercially-available pyruvate sample utilised ($\delta = 1.50$ ppm) was eliminated from the spectrum following treatment with the oral rinse. Intriguingly, this reaction was accompanied by the liberation of copious amounts of green-coloured chlorine gas (Cl_2) and samples containing mixtures of pyruvate and the oral rinse preparation had the characteristic malodour of this halogen. Hence, the above data appear to be consistent with the reactions depicted in Equations 4 and 5.



Of course, dissolution of Cl_2 in aqueous systems yields hypochlorous acid/hypochlorite anion (HOCl/OCl^-) together with chloride ion, and the HOCl/OCl^- generated can also effect the oxidation of many endogenous or bacterial-derived salivary biomolecules. For example, chlorination of the β -amino acid taurine (detectable in the ^1H NMR spectra of many of the human salivary supernatant samples examined

in this investigation) yields N-*mono*- and N-*dichloro*amines.

L-cysteine was chosen as a model thiol compound for these investigations since it is present as an amino acid residue in many salivary proteins and hence results obtained from such experiments serve to provide further useful information regarding the reaction mechanisms of, and the molecular nature of products arising from the interactions of ClO_2^- and ClO_2^\bullet with thiol compounds which contribute substantially towards oral malodour^[32] [e.g. methylmercaptan (CH_3SH) which accounts for ca. 60% of the volatile sulphur compounds (VSC) detectable]. Reaction of L-cysteine with ClO_2^- and/or ClO_2^\bullet present in the commercially-available oral rinse preparation (Section 2) yielded the disulphide cystine as a major product (Fig. 7), providing evidence for the oxidation of thiols by the consecutive, two-step reaction sequence involving ClO_2^\bullet (Equations 6 and 7), and/or a reaction involving ClO_2^- (Equation 8), the latter presumably representing the predominant process. However, further resonances present in the 3.25–3.35 and 3.45–3.80 ppm regions of 600 MHz ^1H NMR spectra of oral rinse-treated L-cysteine solutions (Fig. 7c) demonstrated that alternative, minor oxidation products are also generated. Such products may include cysteine sulphinic acid, cystine disulphoxide and cysteate, and further experiments to determine their precise chemical nature are currently in progress.



^1H NMR analysis also revealed that the thiomethyl group ($-\text{S}-\text{CH}_3$)-containing amino acid L-methionine was also oxidatively consumed by ClO_2^- and/or ClO_2^\bullet present in the oral rinse preparation, yielding a wide range of products, predominantly methionine sulfoxide ($-\text{SO}-\text{CH}_3$ group singlet, $\delta = 2.725$ ppm) with lower levels of methionine sulphone (data not shown).

TABLE II ^1H NMR-determined $[\text{acetate}] \cdot ([\text{acetate}] + [\text{pyruvate}])^{-1}$ concentration ratios of reaction mixtures containing specified levels of pyruvate, cysteine, urate, thiocyanate anion and $\text{ClO}_2^-/\text{ClO}_2^*$, the latter oxohalogen oxidants added as RetarDEX® oral rinse formulation. The above reaction mixtures also contained $7.60 \times 10^{-3} \text{ mol.dm}^{-3}$ phosphate buffer (pH 7.10), and samples were equilibrated at 37°C for a period of 30 min. Acetate concentrations were corrected for trace levels of this component present in the added oral rinse preparation (Table I). Control samples for reaction mixtures (5)–(8) (i.e., those with an equivalent volume of doubly-distilled H_2O added in place of RetarDEX® oral rinse) gave $[\text{Acetate}] \cdot ([\text{Acetate}] + [\text{Pyruvate}])^{-1}$ values of 0.00–0.03.

Sample Code	Composition of Reaction Mixture	[Acetate]
		[Acetate] + [Pyruvate]
(1)	$4.00 \times 10^{-4} \text{ mol.dm}^{-3}$ pyruvate	0.00, 0.01
(2)	$1.60 \times 10^{-3} \text{ mol.dm}^{-3}$ pyruvate	0.01, 0.03
(3)	$4.00 \times 10^{-4} \text{ mol.dm}^{-3}$ pyruvate; $2.96 \times 10^{-3} \text{ ClO}_2^-/\text{ClO}_2^*$	1.00, 1.00
(4)	$1.60 \times 10^{-3} \text{ mol.dm}^{-3}$ pyruvate; $2.96 \times 10^{-3} \text{ mol.dm}^{-3} \text{ ClO}_2^-/\text{ClO}_2^*$	0.98, 0.99
(5)	$1.60 \times 10^{-3} \text{ mol.dm}^{-3}$ pyruvate; $8.00 \times 10^{-4} \text{ mol.dm}^{-3}$ cysteine; $4.00 \times 10^{-4} \text{ mol.dm}^{-3}$ urate; $2.96 \times 10^{-3} \text{ mol.dm}^{-3} \text{ ClO}_2^-/\text{ClO}_2^*$	0.65
(6)	$1.60 \times 10^{-3} \text{ mol.dm}^{-3}$ pyruvate; $8.00 \times 10^{-4} \text{ mol.dm}^{-3}$ cysteine; $4.00 \times 10^{-4} \text{ mol.dm}^{-3}$ urate; $8.00 \times 10^{-3} \text{ mol.dm}^{-3} \text{ SCN}^-$; $2.96 \times 10^{-3} \text{ mol.dm}^{-3} \text{ ClO}_2^-/\text{ClO}_2^*$	0.32
(7)	$1.43 \times 10^{-3} \text{ mol.dm}^{-3}$ pyruvate; $1.43 \times 10^{-3} \text{ mol.dm}^{-3}$ cysteine; $3.57 \times 10^{-4} \text{ mol.dm}^{-3}$ urate; $4.23 \times 10^{-3} \text{ mol.dm}^{-3} \text{ ClO}_2^-/\text{ClO}_2^*$	0.66, 0.58
(8)	$1.43 \times 10^{-3} \text{ mol.dm}^{-3}$ pyruvate; $1.43 \times 10^{-3} \text{ mol.dm}^{-3}$ cysteine; $3.57 \times 10^{-4} \text{ mol.dm}^{-3}$ urate; $1.43 \times 10^{-3} \text{ mol.dm}^{-3} \text{ SCN}^-$; $4.23 \times 10^{-3} \text{ mol.dm}^{-3} \text{ ClO}_2^-/\text{ClO}_2^*$	0.08, 0.08

3.6 Multicomponent *In Vitro* Evaluations of the Reactions of Salivary Electron Donors with Oral-Rinse-Containing $\text{ClO}_2^-/\text{ClO}_2^*$

High resolution NMR spectroscopy was also employed to determine the $\text{ClO}_2^-/\text{ClO}_2^*$ scavenging capacity of competing antioxidants/electron donors present in human saliva. For this purpose, selected reaction mixtures containing pyruvate (0.40 , 1.43 or $1.60 \times 10^{-3} \text{ mol.dm}^{-3}$), urate (3.57 or $4.00 \times 10^{-4} \text{ mol.dm}^{-3}$), cysteine (0.80 or $1.43 \times 10^{-3} \text{ mol.dm}^{-3}$), thiocyanate anion (0.00 , 1.43 or $8.00 \times 10^{-3} \text{ mol.dm}^{-3}$), and RetarDEX oral rinse preparation [initial $\text{ClO}_2^-/\text{ClO}_2^*$ concentration 2.96 or 4.23

$\times 10^{-3} \text{ mol.dm}^{-3}$ (predominantly ClO_2^-)] in $7.60 \times 10^{-3} \text{ mol.dm}^{-3}$ phosphate buffer (pH 7.10) were prepared and equilibrated at 37°C for a 30 min. period. Results obtained from these experiments are documented in Table II. These data clearly indicate that the oxidative consumption of pyruvate by $\text{ClO}_2^-/\text{ClO}_2^*$ oxidants present in the oral rinse formulation is inhibited by cysteine and thiocyanate anion (SCN^-). Indeed, the ^1H NMR spectra acquired on these reaction mixtures confirmed the complete oxidation of cysteine to its corresponding disulphide (cystine) by the above added oxohalogen compounds, in accordance with Figure 7. When expressed relative to the

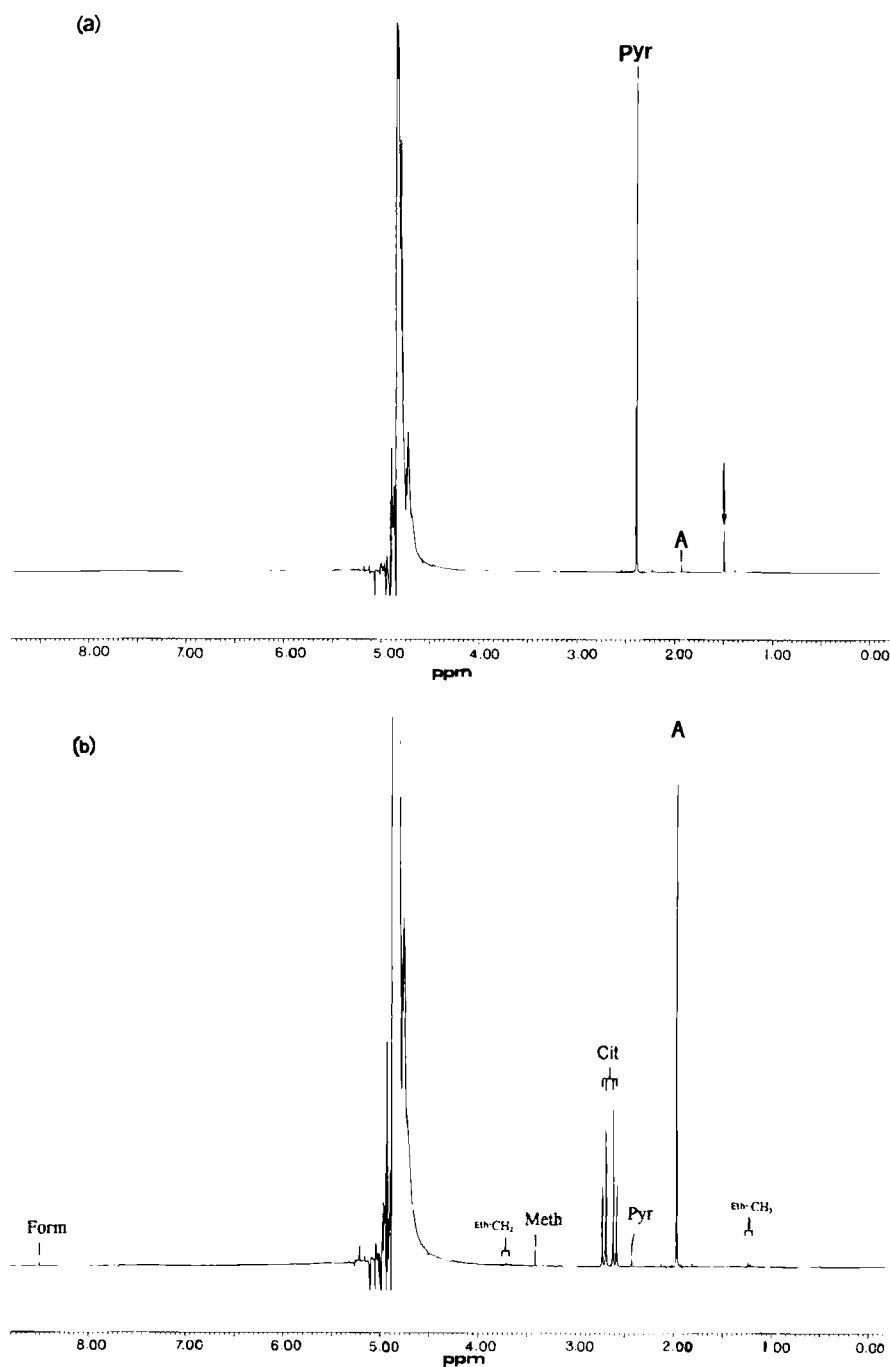


FIGURE 6 0.00–4.00 ppm regions of 400 MHz ^1H NMR spectra of a $1.00 \times 10^{-2} \text{ mol.dm}^{-3}$ aqueous solution of sodium pyruvate in $4.00 \times 10^{-2} \text{ mol.dm}^{-3}$ phosphate buffer (pH 7.10) obtained subsequent to treatment with equivalent volumes of (a) de-gassed doubly-distilled H_2O (control) and (b) RetarDEX® oral rinse. Typical spectra are shown. Abbreviations: as Figures 3 and 4. The arrow in spectrum (a) denotes a singlet resonance ($\delta = 1.50 \text{ ppm}$) ascribable to an impurity present in the commercially-available sample of pyruvate utilised.

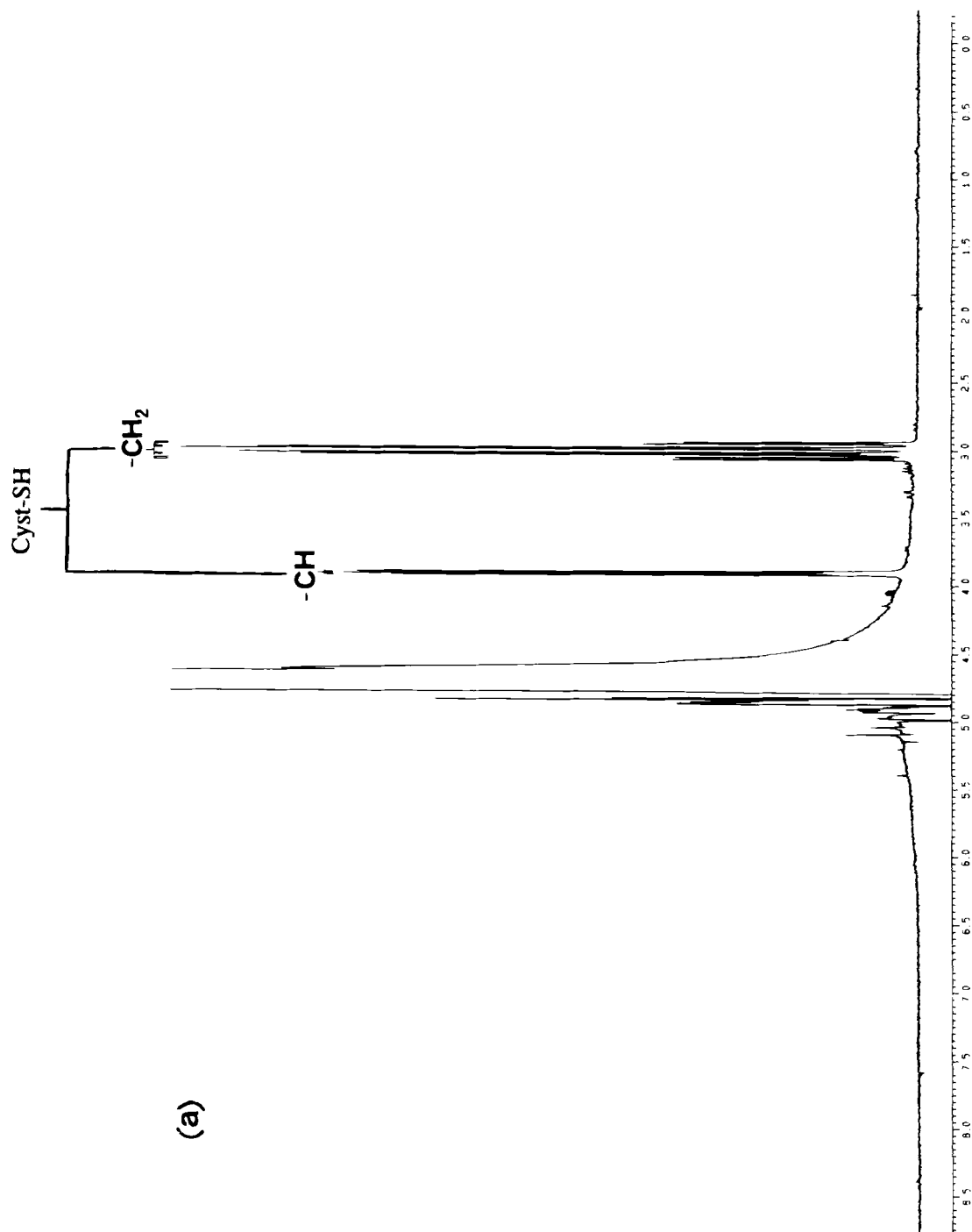


FIGURE 7 High resolution ^1H NMR spectra of a $1.00 \times 10^{-2} \text{ mol.dm}^{-3}$ aqueous solution of L-cysteine in $4.00 \times 10^{-2} \text{ mol.dm}^{-3}$ phosphate buffer (pH 7.10) obtained following treatment with equivalent volumes of (a) de-gassed doubly-distilled H_2O (control; spectrometer operating frequency 400.13 MHz) and (b) RetarDEX® oral rinse (spectrometer operating frequency 600.13 MHz). (c). Expanded 0.50–4.50 ppm region of the spectrum shown in (b). Typical spectra are shown. Abbreviations: as Figure 3, with Cyst-SH and Cyst-S-S-Cyst representing L-cysteine and cystine respectively.

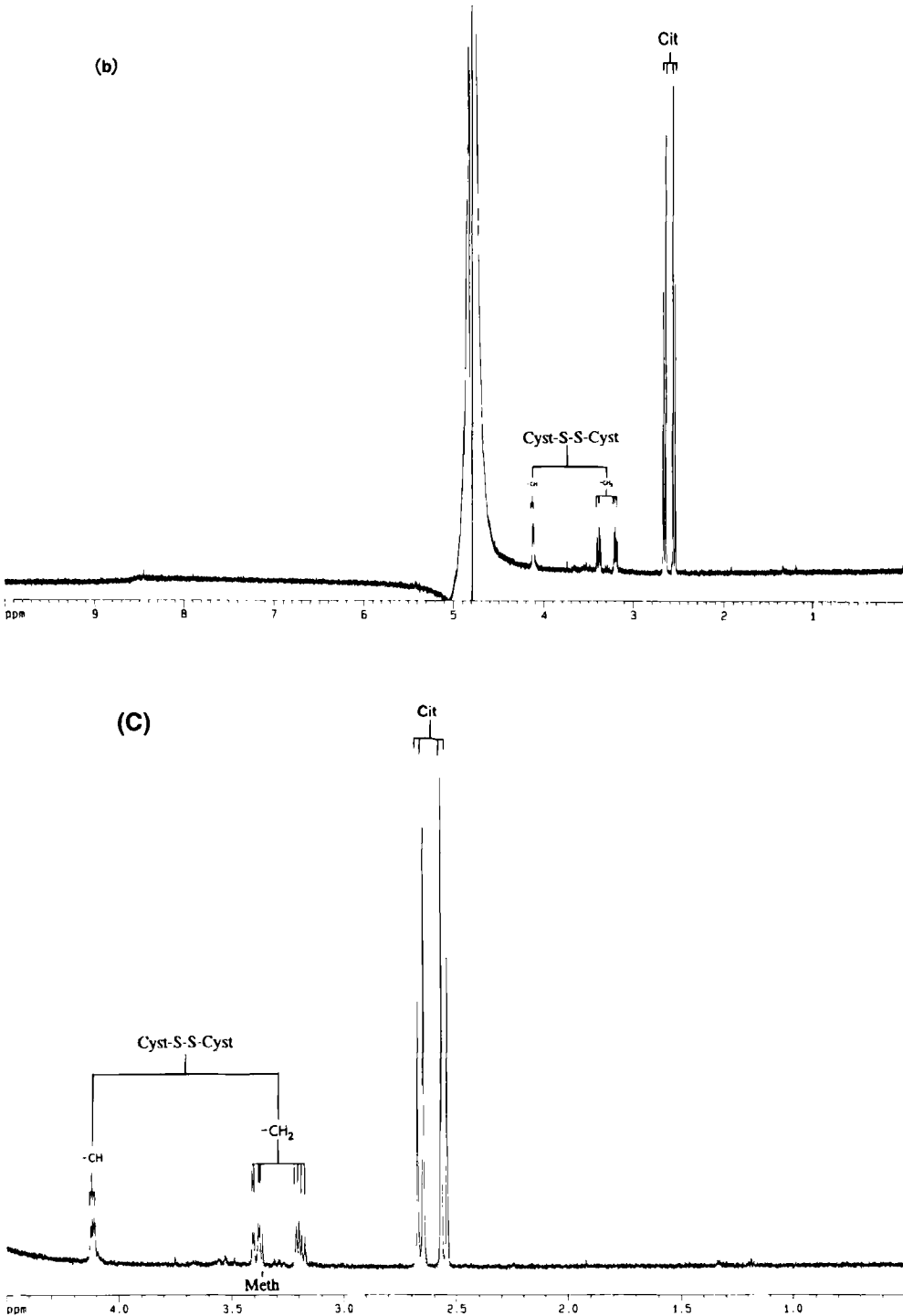


FIGURE 7 Continued

$\text{ClO}_2^-/\text{ClO}_2^{\cdot}$ scavenging capacity of pyruvate, cysteine is preferentially consumed in redox reactions involving these oral rinse oxidants. However it should be noted that in view of the relatively low salivary levels of thiols (cf. oral malodour precursors, mean value apparently $3.6 \times 10^{-5} \text{ mol.dm}^{-3}$),^[34] this class of antioxidant is unlikely to offer pyruvate [mean \pm standard error (s.e.) salivary level $9.0 \pm 0.7 \times 10^{-5} \text{ mol.dm}^{-3}$ ($n = 12$)] a significant level of protection against $\text{ClO}_2^-/\text{ClO}_2^{\cdot}$ -mediated oxidative damage *in vivo*.

Further experiments involving ^{13}C NMR analysis of aqueous reaction mixtures containing the salivary electron donor thiocyanate anion (present in human saliva at concentrations of up to 1.24 and $2.74 \times 10^{-3} \text{ mol.dm}^{-3}$ in non-smokers and smokers, respectively^[35]) and RetarDEX® oral rinse (initial SCN^- and $\text{ClO}_2^-/\text{ClO}_2^{\cdot}$ concentrations 5.00×10^{-2} and $7.40 \times 10^{-3} \text{ mol.dm}^{-3}$, respectively) demonstrated the oxidative consumption of SCN^- after a 30 min. equilibration period at 37°C . Indeed, reproducible reductions in the intensity of the characteristic SCN^- ^{13}C resonance at 220.5 ppm were accompanied by the generation of new signals located at 112.7 and 120.8 ppm (Fig. 8). These new signals do not arise from citrate present in the oral rinse preparation [aqueous solutions of trisodium citrate in $^2\text{H}_2\text{O}$ have ^{13}C NMR signals located at 48.5 ($-\text{CH}_2\text{CO}_2^-$), 78.0 ($\text{HO}-\text{C}-\text{CO}_2^-$), 182.2 ($-\text{CH}_2\text{CO}_2^-$) and 184.8 ppm ($\text{HO}-\text{C}-\text{CO}_2^-$) when expressed relative to TSP ($\delta = 0.00 \text{ ppm}$)].

No ^1H NMR-detectable products arising from the $\text{ClO}_2^-/\text{ClO}_2^{\cdot}$ -mediated oxidative damage to urate were observed in the above samples, nor simple reaction mixtures containing urate and RetarDEX® oral rinse (initial urate and $\text{ClO}_2^-/\text{ClO}_2^{\cdot}$ concentrations 4.00×10^{-4} and $2.96 \times 10^{-3} \text{ mol.dm}^{-3}$, respectively) in $7.60 \times 10^{-3} \text{ mol.dm}^{-3}$ phosphate buffer (pH 7.10). Therefore, HPLC utilising a reversed-phase column was employed to monitor the consumption of this salivary antioxidant by oxohalogen oxidants present in Retardex® oral rinse. For this purpose, aliquots (0.50 ml) of human salivary super-

natants ($n = 5$) were treated with an equivalent volume of the oral rinse, equilibrated at a temperature of 37°C for a 24-hr. period, ultrafiltered, and the ultrafiltrates obtained subjected to HPLC analysis (corresponding 0.50 ml aliquots of salivary supernatants treated with an equivalent volume of doubly-distilled water and then equilibrated and ultrafiltered in the same manner served as matched controls for each sample investigated). Table III displays salivary urate levels prior and subsequent to treatment with RetarDEX® oral rinse. Clearly, the endogenous antioxidant urate is oxidatively consumed by $\text{ClO}_2^-/\text{ClO}_2^{\cdot}$, the mean (\pm standard error) percentage decrease in its salivary concentration being $74 \pm 14\%$ (range 32–100%). The wide range of these percentage values is ascribable to “between sample” variance (“scatter”) of (1) the initial urate concentration, and (2) those of alternative, competing salivary antioxidants (e.g., thiols, SCN^- , pyruvate etc). The time-dependence of the $\text{ClO}_2^-/\text{ClO}_2^{\cdot}$ -mediated oxidative consumption of salivary urate at ambient temperature (22°C) and pH 5.96 was monitored by difference spectrophotometry, and clear decreases in absorbance at 292 and 239 nm (λ_{max} values for an authentic standard sample of urate^[37]) with increasing time were notable (Fig. 9). Minimum wavelength values (λ_{min}) for the decrease in urate absorbance observed were confirmed by first- and second-derivative spectrophotometry [the complexity of the decreases in absorbance in the 230–320 nm wavelength range is explicable by synchronous spectral modifications arising from the salivary electron donor-mediated reduction of oxohalogen compounds present in the oral rinse preparation (predominantly ClO_2^-)]. Under our experimental conditions, half-life ($t_{1/2}$) values for the $\text{ClO}_2^-/\text{ClO}_2^{\cdot}$ -mediated oxidative consumption of salivary urate ranged from 5.1–6.9 hr ($n = 5$). These results indicate that endogenous urate has the ability to offer salivary pyruvate only a low level of protection against attack by $\text{ClO}_2^-/\text{ClO}_2^{\cdot}$ oxidants present in the oral rinse preparation investigated.

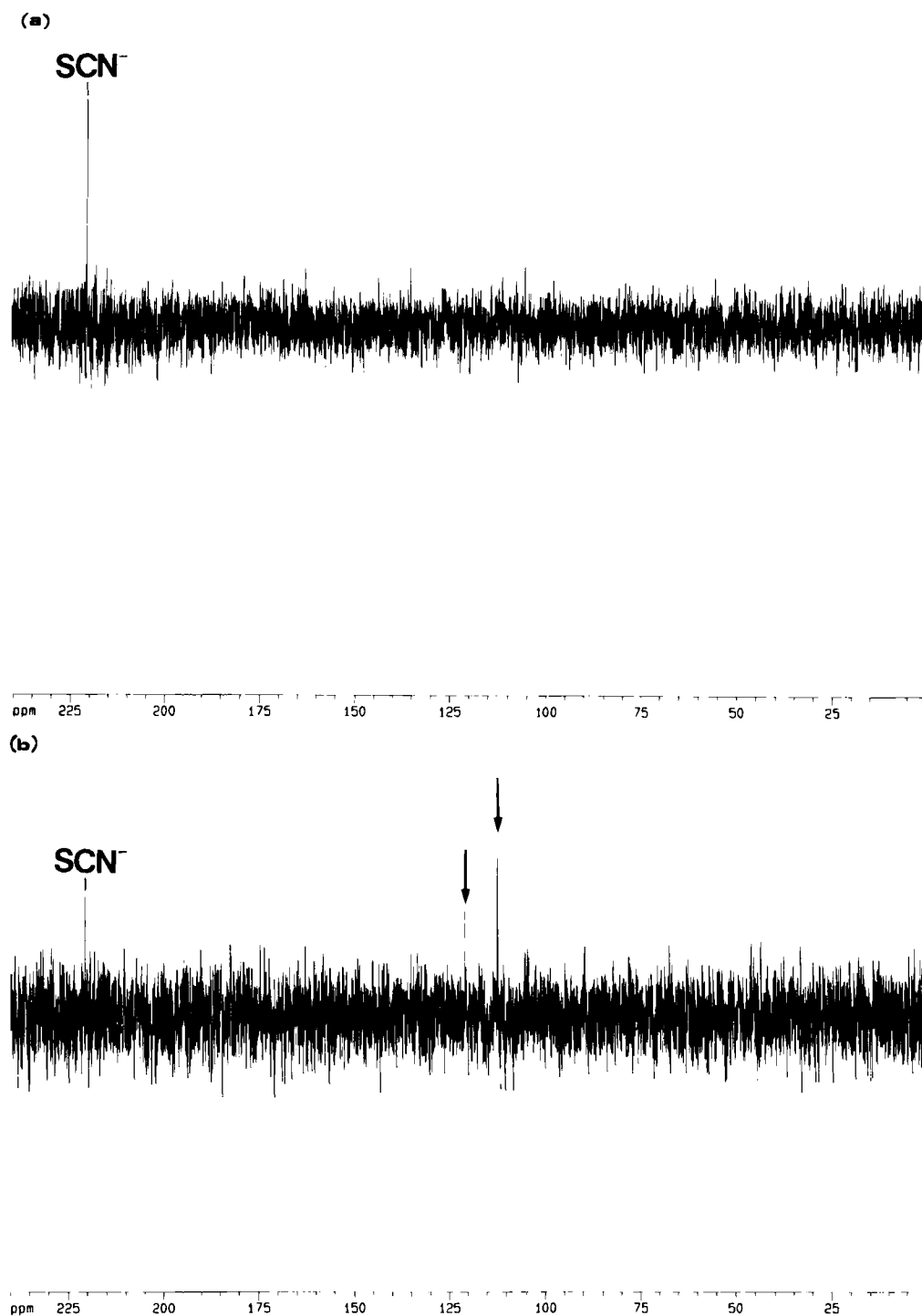


FIGURE 8 150 MHz ^{13}C NMR spectra of 0.10 mol.dm^{-3} aqueous solutions of potassium thiocyanate in $1.52 \times 10^{-2} \text{ mol.dm}^{-3}$ phosphate buffer (pH 7.10) obtained after treatment with equivalent volumes of (a) de-gassed doubly-distilled H_2O (control sample) and (b) RetarDEX® oral rinse. Typical spectra are shown. The arrows in spectrum (b) indicate resonances of products arising from the reaction of $\text{ClO}_2^-/\text{ClO}_2^\bullet$ with thiocyanate anion (SCN^-).

TABLE III Salivary urate concentrations prior and subsequent to treatment with RetarDEX® oral rinse as described in Section 2.4 (initial $\text{ClO}_2^-/\text{ClO}_2^{\cdot-}$ levels for the oral rinse-treated samples were $7.40 \times 10^{-3} \text{ mol.dm}^{-3}$). Urate concentrations were determined by HPLC (Section 2.4) and each value documented represents the mean of two separate determinations. The "between determination" coefficient of variation for this assay system was 1.4%.

Sample code	Salivary urate concentration prior to treatment (baseline control) ($10^{-6} \text{ mol.dm}^{-3}$)	Salivary urate concentration subsequent to treatment with RetarDEX® oral rinse ($10^{-6} \text{ mol.dm}^{-3}$)
1	41	21
2	47	3
3	165	112
4	2	0
5	75	2

4 DISCUSSION

Multicomponent ^1H NMR investigations of the oxidising ('therapeutic') capacity of the $\text{ClO}_2^-/\text{ClO}_2^{\cdot-}$ -containing oral rinse preparation examined here demonstrated that salivary electron donors are readily consumed by the above oxohalogen oxidants. Indeed, with regard to data presented, all further endogenous salivary reductants tested offer pyruvate selective levels of protection against $\text{ClO}_2^-/\text{ClO}_2^{\cdot-}$ -mediated oxidative damage, the amino acid cysteine being more effective than SCN^- and urate in this context. However, in view of the mean concentrations of these electron donors in human saliva ($\text{SCN}^- \gg \text{urate} > \text{cysteine}$), SCN^- is expected to play a major role in neutralising the oxidising actions of oral rinse-derived ClO_2^- and $\text{ClO}_2^{\cdot-}$. Hence, in facile thermodynamic terms, human salivary SCN^- (although less reactive towards ClO_2^- and $\text{ClO}_2^{\cdot-}$ than cysteine) is present at concentrations sufficient to effectively compete with alternative biological reductants therein such as pyruvate, offering them a significant level of protection against oxidative consumption by oral rinse oxohalogen oxidants. Nevertheless, a 1:1 (v/v) mixture of oral rinse to human saliva yields a total $\text{ClO}_2^-/\text{ClO}_2^{\cdot-}$ concentration of 7.40×10^{-3} (predominantly ClO_2^-), and, assuming, a similar dilution of this mouthwash preparation immedi-

ately subsequent to oral application, it is clear that in concentration terms, the oxohalogen oxidants present will effectively "swamp" all salivary electron donor activity available. Indeed, after making allowances for thermodynamic equilibria and the rate of each reaction involved under the appropriate physiological conditions, this indicates that reductants present at relatively low salivary levels (e.g., cysteine, urate) will also be consumed (either fully or partially) during oral rinsing programmes involving the RetarDEX® oral hygiene product examined in this study.

Consumption of cysteine and methionine by ClO_2^- and/or $\text{ClO}_2^{\cdot-}$ is of further importance to clinical periodontology since CH_3SH and H_2S are generated from these amino acids via metabolic pathways operational in gram-negative microorganisms³² (Schemes I and II), Scheme I being a process which involves the enzymes cystine reductase (reduction of cystine to cysteine) and serine sulphydrase (desulphydration of cysteine yielding H_2S and serine).

Interestingly, a preliminary investigation conducted by Tozentich³³ showed that therapeutic administration of an oral rinse preparation containing 0.01% (w/v) ($1.48 \times 10^{-3} \text{ mol.dm}^{-3}$) $\text{ClO}_2^{\cdot-}$ significantly diminished the concentrations of VSCs detectable in early morning mouth air samples obtained from human subjects with

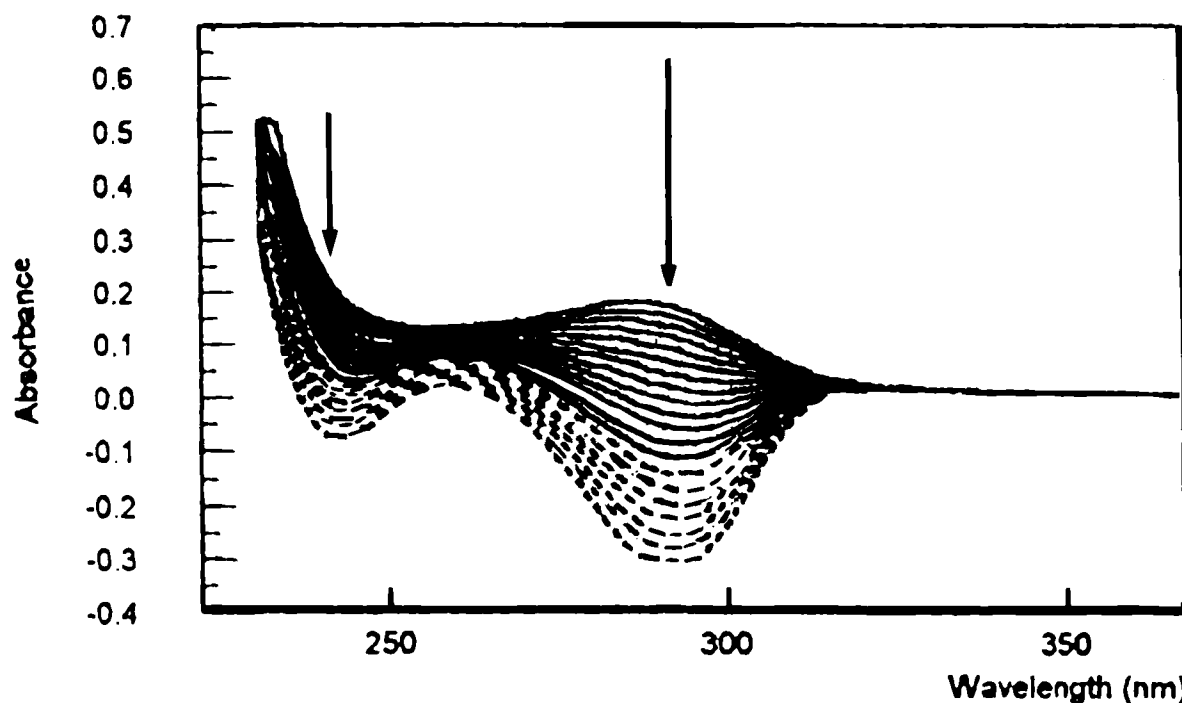
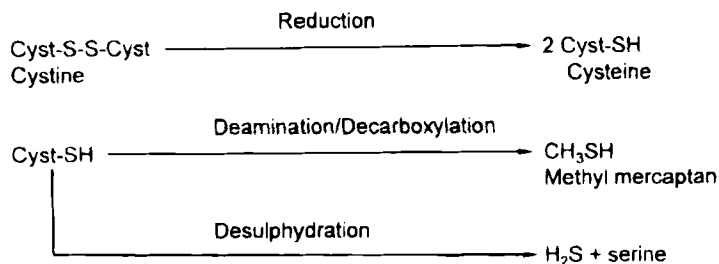


FIGURE 9 Time-dependent modifications in the difference electronic absorption spectrum of a reaction mixture containing a human saliva supernatant (0.40 ml), RetarDEX® oral rinse (0.40 ml) and $4.00 \times 10^{-2} \text{ mol.dm}^{-3}$ phosphate buffer, pH 5.96 (1.20 ml) at a temperature of 22°C. The initial concentration of $\text{ClO}_2^-/\text{ClO}_2^\cdot$ in the reaction mixture was $2.96 \times 10^{-3} \text{ mol.dm}^{-3}$ and the composition of solutions present in the two compartments of the twin reference cell are given in Section 2.5. The first spectrum was recorded immediately after mixing the added solutions (i.e., within 10 s), and subsequent spectra were recorded at 30 min. intervals. Typical data are shown.

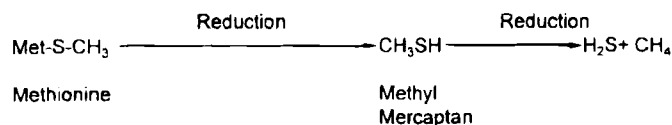
objectionable levels of these malodorous agents (i.e., >5 mg CH_3SH per 100 ml mouth air), an observation of much pertinence to the results obtained here.

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 allows the rapid, non-invasive and simultaneous study of a multitude of components present in biological samples (eg, biofluids such as human saliva as outlined in this study) and (2) generally requires little or no knowledge of sample composition prior to analysis. Moreover, chemical shift values, coupling patterns and coupling constants of resonances present in ^1H NMR spectra of such multicomponent systems provide much valuable molecular information regarding both endogenous and exogenous chemical species detectable.

As demonstrated in this investigation, the technique is of much utility concerning multicomponent evaluations of the interactions of therapeutically-active agents present in commercially-available mouthwash preparations with human saliva [or gingival crevicular fluid (GCF)] biomolecules, and the oxidative decarboxylation of salivary pyruvate by an admixture of ClO_2^- and ClO_2^\cdot radical in the oral rinse preparation investigated here serves as a fundamental, *prima facie* example of this which may be of some relevance to one or more of its mechanisms of action. The rate and extent of salivary electron donor consumption (e.g., that of VSCs, their amino acid precursors, and pyruvate) by oxidants present in oral rinse preparations reflects the oxidising capacity of such commercially-available materials, a phenomenon of much significance with regard to their therapeutic and aesthetic roles. Hence, high resolution



SCHEME I Generation of CH_3SH and H_2S via metabolism of the amino acids cysteine and cystine by enzymes present in gram-negative micro-organisms.



SCHEME II Generation of CH_3SH and H_2S via metabolism of the amino acid methionine by enzymes present in gram-negative micro-organisms.

^1H NMR analysis of human saliva or appropriate chemical model systems serves as a very useful method for the *in vitro* testing of oral rinses, and we have recently found that it also provides much useful information concerning the molecular mechanisms underlying the therapeutic actions of active agents present in commercially-available dentifrices. Indeed, the multicomponent analytical ability of high field ^1H NMR spectroscopy permits evaluations of the nature, rate and extent of chemical modifications arising on equilibration of human saliva or GCF samples with intact dentifrice preparations, data which will be presented in detail elsewhere.

^1H NMR analysis of human saliva samples collected prior and subsequent to the administration of oral rinses or dentifrices to patients with periodontal diseases (i.e., *in vivo* assessment) may demonstrate a reduction in the levels of bacterial-derived salivary components (eg, short-chain non-volatile carboxylic acid anions such as *n*-butyrate, propionate, fumarate, formate, etc), reflecting the removal of potentially pathogenic micro-organisms following treatment, and recent pilot studies conducted by the authors have shown that the technique is readily applicable to investigations of this nature.

Acknowledgements

We are very grateful to the University of London Intercollegiate Research Services for the provision of NMR facilities, and to Janet Shaw and Jean Gatehouse for typing the manuscript.

References

- [1] T. Chen (1967). Spectrophotometric determination of microquantities of chlorate, chlorite, hypochlorite and chloride in perchlorate. *Analytical Chemistry*, **39**, 804–813.
- [2] R. G. Kieffer and C. Gordon. (1968). Disproportionation of chlorous acid. I. Stoichiometry. *Inorganic Chemistry*, **7**, 235–239.
- [3] W. J. Massenchelein (1979). *Chlorine Dioxide*, Ann Arbor Science, Ann Arbor, M.I.
- [4] S. Harakeh, A. Illescas and A. Matin (1988). Inactivation of bacteria by Purogene. *Journal of Applied Bacteriology*, **64**, 459–463.
- [5] M. Alvariz (1982). Mechanisms of inactivation of poliovirus by chlorine dioxide and iodine. *Applied Environmental Microbiology*, **44**, 1064–1071.
- [6] Y. S. Chen and J. M. Vaughn (1990). Inactivation of human and simian rotaviruses by chlorine dioxide. *Applied Environmental Microbiology*, **56**, 1363–1366.
- [7] P. M. Foegeding, V. Hemstapat and F. G. Giesbrecht (1986). Chlorine dioxide inactivation of *Bacillus* and *Clostridium* spores. *Journal of Food Science*, **51**(1), 197–201.
- [8] D. G. Korich, J. R. Mead, M. S. Madore, N. A. Sinclair and C. R. Sterling (1990). Effects of ozone, chlorine dioxide, chlorine and monochloroamine on *cryptosporidium parvum* oocyst viability. *Applied Environmental Microbiology*, **56**, 1423–1428.
- [9] N. Takahashi (1985). Chlorine dioxide as an algicide and fungicide for cultured lava. *Japan Kokar Tokyo Koho J.P.*

- 60, 184,002 (85,184,002 CCI.AOIN59100), Appl. 84/37, 327, pp. 1–3.
- [10] Y. Sano (1985). Deodorizing activity and effect of a stable chlorine oxide (ClO_2) *Fragrance Journal*, **13**(3), 51–53.
- [11] G. C. White (1972). Chlorine Dioxide. In *Handbook of Chlorination*, (ed. G. C. White), Van Nostrand Reinhold Co., New York, pp. 596–627.
- [12] J. E. Smith and J. L. McVey (1973). Virus inactivation by chlorine dioxide and its application to storm water overflow. *Preparatory Paper, National Meeting of the Division of Environmental Chemistry*, **13**, 177.
- [13] J. J. Rook (1974). Formation of haloforms during chlorination of natural waters. *Water Treatment and Examination*, **23**, 234–243.
- [14] R. Thompson and G. Gordon (1966). Stoichiometry of the reaction between chromium(II) and the chlorine oxidants in aqueous perchloric acid. *Inorganic Chemistry*, **5**, 557–562.
- [15] B. O. Lindgren and C. M. Svahn (1966). Reactions of chlorine dioxide with unsaturated compounds. *Acta Chemica Scandinavica*, **20**, 211–218.
- [16] G. O. Guarrant, M. A. Lambert and C. W. Moss (1982). Analysis of short-chain acids from anaerobic bacteria by high performance liquid chromatography. *Journal of Clinical Microbiology*, **16**, 355–360.
- [17] E. Schmidt and K. Brunsdorf (1922). Zur kenntnis der natürlichen Eiweißstoffer, I Mitteilung: Verhalten von chlordinoxyd gegenüber organischen verbindungen. *Chemische Berichte*, **55A**, 1529–1534.
- [18] K. Paluch (1965). Reaction of chlorine dioxide with phenols. III. Influence of temperature on the reaction of ClO_2^* with phenol. *Roczniki Chemistry*, **39**, 1539–1545.
- [19] G. Gordon, R. G. Kieffer and D. H. Rosenblatt (1972). The chemistry of chlorine dioxide. *Progress in Inorganic Chemistry*, **15**, 201–276.
- [20] F. S. Becker, J. K. Hamilton and W. E. Lucke (1965). Cellulose oligosaccharides as model compounds in chlorine dioxide bleaching. *Tappi*, **48**, 60–64.
- [21] K. Paluch (1965). The reaction of chlorine dioxide with phenols. II. Hydroquinone, chloro-derivatives of hydroquinone, and nitrophenols. *Roczniki Chemistry*, **38**, 43–46.
- [22] H. Herz, D. R. Blake and M. Grootveld (1994). Multicomponent investigations of the hydrogen peroxide- and hydroxyl radical-scavenging antioxidant capacities of biofluids: the roles of endogenous pyruvate and lactate. *Free Radical Research* (in press).
- [23] C. W. Chapek, O. K. Reed and P. A. Ratcliff (1994). Management of periodontitis with oral-care products. *Compendium of Continuing Dental Education*, **15**(6), 740–746.
- [24] J. R. Lubbers, S. Chauhan, J. K. Miller and J. R. Blanchine (1984). The effects of chronic administration of chlorine dioxide, chlorite and chlorate to normal healthy male volunteers. *Journal of Environmental Pathology, Toxicology and Oncology*, **4**/5, 229–238.
- [25] H. K. Tan (1987). Reaction of chlorine dioxide with amino acids and peptides: kinetics and mutagenicity studies. *Mutation Research*, **188**, 259–266.
- [26] M. Y. Fukayama, H. Tan, W. B. Wheeler and C.-I. Wei. (1986). Reactions of aqueous chlorine and chlorine dioxide with model food compounds. *Environmental Health Perspectives*, **69**, 267–274.
- [27] E. Hahn (1950). Spin echoes. *Physics Review*, **80**, 580.
- [28] W. P. Aue, E. Bartholdi and R. R. Ernst (1976). Two-dimensional spectroscopy: application to nuclear magnetic resonance. *Journal of Chemical Physics*, **64**, 2229–2246.
- [29] R. G. Kieffer and G. Gordon (1967). Disproportionation of chlorous acid II. Kinetics. *Inorganic Chemistry*, **7**(2), 239–244.
- [30] F. Tachiki (1944). *Nippon Kagaku Zasshi*, **65**, 346.
- [31] R. H. Oster, *et al.* (1953). Human salivary buffering rate measured *in situ* in response to an acid stimulus found in some common beverages. *Journal of Applied Physiology*, **6**, 348–354.
- [32] S. M. Waler (1992). Halitosis. In: *Clinical and Biological Aspects of Dentifrices*, (Eds G. Embery and G. Rolla). Oxford University Press, Oxford/New York/Tokyo, PP. 21–28.
- [33] J. Tozentich (1977). Production and origin of oral malodor: A review of mechanisms and methods of analysis. *Journal of Periodontology*, **48**, 13–20.
- [34] J. Grigor and A. J. Roberts (1992). Reduction in the levels of oral malodor precursors by hydrogen peroxide: *in-vitro* and *in-vivo* assessments. *The Journal of Clinical Dentistry*, **III**(4), 111–115.
- [35] J. Tenovuo and K. K. Mäkinen (1976). Concentration of thiocyanate and ionizable iodine in saliva of smokers and non-smokers. *Journal of Dental Research*, **55**, 661–663.
- [36] M. Grootveld and B. Halliwell (1987). Measurement of allantoin and uric acid in human body fluids. A potential index of free radical reactions *in vivo*. *Biochemical Journal*, **29**, 289–299.