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ORIGINAL ARTICLE

Chemometric analysis of the consumption of oral rinse chlorite (ClO_2^-) by human salivary biomolecules

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Abstract
Objectives Oral rinse formulations containing chlorite anion (ClO_2^-) as an active agent exert a range of valuable oral healthcare activities. However, salivary biomolecules which chemically react with this oxidant can, at least in principle, serve as potentially significant barriers to these therapeutic properties in the oral environment. Therefore, in this investigation, we have explored the extent of ClO_2^- consumption by biomolecules which scavenge this agent in human salivary supernatants (HSSs) in vitro.
Materials and methods HSS samples were equilibrated with oral rinse formulations containing this active agent (30 s at 35 °C in order to mimic oral rinsing episodes). Differential spectrophotometric and ion-pair reversed-phase high-performance liquid chromatographic analyses were employed to determine residual ClO_2^- in these admixtures.
Results Bioanalytical data acquired revealed the rapid consumption of ClO_2^- by biomolecular electron donors and/or antioxidants present in HSS samples. Mean±95 % confidence interval (CI) consumption levels of 7.14±0.69 and 5.34±0.69 % of the total ClO_2^- available were found for oral rinse products containing 0.10 and 0.40 % (*w*/*v*) ClO_2^-, respectively. A mixed model analysis-of-variance performed on experimental data acquired demonstrated highly-significant differences between oral rinse ClO_2^- contents ($p<0.0001$), trial participants ($p<0.001$) and sampling days–within-participants ($p<0.001$), and also revealed non-additive ClO_2^--scavenging responses of participants' HSSs to increases in the oral rinse content of this oxidant ($p<0.0001$). A slower, second phase of the reaction process ($t_{1/2}$=1.7–2.8 h) involved the oxidative consumption of salivary urate.
Conclusions These data clearly demonstrate that for recommended 30 s oral rinsing episodes performed at physiological temperature, <10 % of the total oral rinse ClO_2^- available is chemically and/or reductively consumed by HSS biomolecules for both oral rinse formulations investigated.
Clinical relevance These observations are of much clinical significance in view of the retention of these products' active agent, i.e. <10 % of ClO_2^- is consumed by HSS biomolecules within recommended 30 s oral rinsing episodes, and hence, the bulk of this oxyhalogen oxidant (>90 %) may effectively exert its essential microbicidal, anti-periodontal and oral malodour-neutralising actions.

Keywords Oral rinse · Human saliva · Chlorite · Chlorine dioxide · Salivary biomolecules · Antioxidants

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Introduction

A range of commercially available oral rinse formulations contain the active agent sodium chlorite (Na^+/ClO_2^-), which is commonly referred to as 'stabilised' chlorine dioxide ($ClO_2^{\bullet}$). ClO_2^- can be converted to active $ClO_2^{\bullet}$ by lowering the pH value of aqueous solutions containing the former agent, a process which involves its prior protonation to form unstable chlorous acid (the pK_a value for this transformation is 2.31), which then decomposes to form $ClO_2^{\bullet}$ (stoichiometrically, 0.50 of a molar equivalent of this free radical

species is liberated per mole of ClO_2^- consumed), together with chloride and chlorate anions (Eqs. 1 and 2).

Notwithstanding, selected reactions of ClO_2^- with salivary biomolecules may also serve to generate $ClO_2^\bullet$ from ClO_2^-. However, although the beneficial therapeutic actions of oral healthcare products containing 'stabilised' $ClO_2^\bullet$ (specifically ClO_2^-) have been previously and predominantly ascribable to much lower levels of this free radical species putatively generated from ClO_2^- in the oral environment, ClO_2^- itself can exert a high level of therapeutic actions, which are of both a microbicidal and (favourable) biochemical nature [2].

Both $ClO_2^\bullet$ and ClO_2^- oxidatively consume volatile sulphur compounds (VSCs), together with their amino acid precursors in the oral cavity [2]. Indeed, human saliva contains a wide range of biomolecules [1], many of these being $ClO_2^\bullet$-reactive, with at least some of them also being ClO_2^--reactive. Such reactive biomolecules therefore act as a 'load' on ClO_2^--containing oral rinse preparations, which may prevent such oral healthcare products from being effective in critical areas such as the teeth and gingival tissues.

$$ClO_2^- + H^+ \rightarrow HClO_2 (pK_a = 2.31) \quad (1)$$

$$4HClO_2 \rightarrow 2ClO_2^\bullet + ClO_3^- + Cl^- + H_2O \quad (2)$$

The abilities of $ClO_2^\bullet$ and its ClO_2^- precursor to act as powerful microbicidal agents have been attributed to both chemical and physiological modes of action [6]. However, the precise mechanisms of action(s) for these agents have been debated for many years.

$ClO_2^\bullet$ is highly soluble in water, and its redox chemistry involves its ability to readily accept electrons (i.e. it is an extremely powerful oxidant), a process giving rise to the generation of ClO_2^-, chlorate (ClO_3^-) and chloride anions. Indeed, it readily oxidises a wide variety of biomolecules, including alkenes, amines, phenols, carbonyl compounds, alcohols, carbohydrates and amino acids, the latter including cysteine and methionine which serve as VSC precursors [2, 7–19].

It has been suggested that $ClO_2^\bullet$'s bactericidal actions arise from a disruption of protein synthesis (these experiments involved the exposure of a faecal strain of *Escherichia coli* to three different concentrations of $ClO_2^\bullet$ at four temperatures) [20]. However, another study indicated that this may not be the case [21], and further investigations found that $ClO_2^\bullet$ effectively disrupted the permeability of the outer bacterial membrane (a process involving its ability to oxidatively damage cell membrane proteins and lipids) [22]. Furthermore, it has been found that $ClO_2^\bullet$ reacted with poliovirus RNA and also impaired RNA synthesis [23], and that it inactivates influenza virus A/H1N1 hemagglutinin and neuraminidase [24], and hence it is likely that protein denaturation also serves as a mechanism for its virus-neutralising activities.

Two clinical studies performed in order to assess the effect of a 0.10 % (*w*/*v*) 'stabilised' $ClO_2^\bullet$ (predominantly chlorite, ClO_2^-) oral rinse formulation on both gingivitis and oral soft tissues have revealed that this product was effective at improving periodontal health [25, 26]. Further, more recent investigations of ClO_2^--containing oral healthcare products have involved assessments of their abilities to oxidatively consume and/or suppress the adverse generation of VSCs responsible for oral malodour, and all of these studies have demonstrated a high level of effectiveness for these formulations in this context [27–29]. Moreover, further studies have focused on the microbicidal activities of such products [30], and an additional recent investigation evaluated the microbicidal activity of such a product with regard to the clinical management of chronic atrophic candidiasis [31].

However, much of the therapeutic activity of oral rinse ClO_2^- (and also $ClO_2^\bullet$ derivable therefrom) is likely to be determined by the availability of a minimum effective concentration of this agent in the oral environment in vivo, a parameter which, in turn, is expected to be attenuated by the salivary concentrations of biomolecules which can serve to scavenge this biocidal agent. Indeed, this biofluid contains significant levels of electron-donating metabolites [2].

Notwithstanding, there also remains the possibility that ClO_2^- can be transformed to the more bioactive free radical species $ClO_2^\bullet$ by virtue of its reaction with selected amino acids present in this biofluid [such as the β-amino acid taurine in neutral to acidic aqueous media (Eq. 3) [32]].

$$H_3{}^+NCH_2CH_2SO_3{}^- + 3ClO_2{}^- \rightarrow Cl(H)NCH_2CH_2SO_3{}^- + 2ClO_2{}^\bullet + 2OH^- \quad (3)$$

Therefore, this investigation was focused on the in vitro equilibration of oral rinse formulations containing 0.10 and 0.40 % (*w*/*v*) sodium chlorite (Na^+/ClO_2^-) with HSS samples at a therapeutically relevant volume ratio [specifically, a 5:1 oral rinse/HSS (*v*/*v*) ratio], temperature (35 °C) and time period (30 s), and these reaction mixtures were then subjected to the analysis of residual ClO_2^- with a newly developed differential spectrophotometric technique. The validity and reliability of this technique was established via direct comparisons of residual ClO_2^- concentration data derived therefrom with those arising from corresponding ClO_2^- determinations made on a series of these equilibrated admixtures using an ion-pair reversed-phase high-performance liquid chromatographic (IP-RP-HPLC) technique involving the diode-array spectrophotometric identification of this 'active' oxyhalogen oxidant.

The null hypothesis of these investigations was that none of the ClO_2^- present in the oral rinse products tested was significantly consumed by HSS biomolecules following equilibration in the above manner.

Materials and methods

Oral rinse formulations and reagents

ClOSY II oral rinse formulations containing 5.26×10^{-3} mol dm^{-3} trisodium phosphate [0.20 % (*w*/*v*), as $Na_3PO_4{\cdot}12H_2O$], 4.11×10^{-3} mol dm^{-3} citric acid [0.079 % (*w*/*v*)] and sodium chlorite (Na^+/ ClO_2^-) at added levels of either 0.10 or 0.40 % (*w*/*v*), i.e. 1.106×10^{-2} or 4.42×10^{-2} mol dm^{-3}, respectively, were supplied (Rowpar Pharmaceuticals, Scottsdale, AZ, USA). The final pH value of these oral rinse products was 6.50–6.65. Anhydrous sodium chlorite [$NaClO_2$] at a purity level of 80 % (*w*/*w*) was purchased (Sigma-Aldrich Chemical, Gillingham, Dorset, UK); 2.00×10^{-2} mol dm^{-3} solutions of phosphate buffer (pH 6.50) were prepared in HPLC grade water.

Recruitment of participants, participant population and saliva sample collection

A series of non-medically compromised participants (n=20) aged 21–35 years (11 male, 9 female) without any form of active periodontal disease or active dental caries were recruited to the study. 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 tube immediately after waking in the morning on three separate, pre-selected days. 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 collection (<5 min). Each collection tube contained sufficient sodium fluoride (1.5×10^{-5} mol) in order to ensure that metabolites were not generated 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 on their arrival to remove cells and debris (3,500 rpm at 4 °C for a period of 20 min), and the resulting supernatants (i.e. HSS samples) then stored at −70 °C for a maximum duration of 18 h prior to the performance of differential spectrophotometric and/or RP-IP-HPLC experiments. The pH values of selected HSS samples were determined both prior and subsequent to their in vitro treatment with each oral rinse formulation with a pH metre (HI 1270 combination pH electrode and meter, Hanna, Leighton Buzzard, UK).

As noted above, for each participant selected for the study, the above saliva collection process was repeated over a period of 3 days in order to determine the reproducibility of data acquired and also to enhance the experiment's precision. Ethical approval for this investigation was obtained from the London South Bank University Research Ethics Committee.

Differential spectrophotometric determination of the extent of ClO_2^- consumption by human salivary biomolecules

Three equivalent (0.10 ml) aliquots of each HSS sample were added to separate sample tubes. To the first and second of these was added 0.50 ml volumes of oral rinses I and II, respectively, whereas the third was treated with an equivalent volume of 2.00×10^{-2} mol dm^{-3} phosphate buffer (pH 6.50) and hence served as an untreated control. Each reaction mixture was then individually equilibrated at 35 °C for a 30-s period immediately prior to differential spectrophotometric and/or HPLC analysis.

A newly developed spectrophotometric method was employed for the determination of residual ClO_2^- in each of the HSS specimens collected and subsequently equilibrated with each oral rinse products as outlined below (and hence its level of consumption by biomolecules therein during this process). Indeed, the above 0.10-ml aliquots of each HSS specimen were treated with the above fixed volumes (0.50 ml) of each oral rinse formulation, and following appropriate further dilution with 2.00×10^{-2} mol dm^{-3} phosphate buffer (pH 6.50), difference electronic absorption spectra were acquired after each addition, i.e. differential spectrophotometric experiments involving a split reference cell with oral rinse/-human salivary supernatant mixtures in the sample (reaction) cell, and (separated) overall equivalent contents and concentrations of these samples in the split reference cell compartments (oral rinse in the first compartment, and salivary supernatant in the second) so that the total reference cell absorbance value at pre-selected wavelengths represented those of the unreacted oral rinse/salivary supernatant mixture (i.e. that at a time-point of 0.0 s), whilst that of the sample cell represents that observed subsequent to any chemical reactions involved at the 30.0-s time-point. Specifically, the first split reference cell compartments (of path length 0.50 cm) contained exactly twice the concentrations of ClO_2^- added to the reaction (sample cell) mixture in the 1.0-cm path length cell, whilst the second 0.50-cm path length reference cell compartment contained exactly twice the HSS sample volume content of that present in the corresponding reaction mixture sample cell, i.e. for equilibration mixtures containing a 5:1 (*v*/*v*) ratio of oral rinse to HSS, the first reference cell compartment contained a 0.50-ml volume of oral rinse but made up to a final volume of 0.60 ml with 2.00×10^{-2} mol dm^{-3} phosphate buffer solution (pH 6.50), whilst the second contained a 0.10-ml volume of the corresponding HSS sample, but also diluted to a final 0.60 ml volume with this phosphate buffer solution. ClO_2^- has a wavelength of maximum absorbance (λ_{max}) at a wavelength of 262 nm (ε=163 $M^{-1}cm^{-1}$, this work) and also absorbs significantly at 305, 310 and 320 nm (ε=69, 54 and 29 $M^{-1}cm^{-1}$ respectively, also this work).

A spectrophotometric facility (ATI Unicam UV2, Cambridge, UK) was employed for the determination of residual

ClO_2^- in each of the salivary supernatants collected in order to determine its level of consumption by biomolecules therein on equilibration. Aliquots (0.10 ml) of each HSS specimen pre-treated with a 0.50-ml volume of the oral rinse formulation containing 0.10 % (*w*/*v*) ClO_2^- (oral rinse I) were thoroughly rotamixed, equilibrated at a temperature of 35 °C for a 30-s period and subsequently diluted to a final volume of 1.20 ml with 2.00×10^{-2} mol dm^{-3} phosphate buffer solution (pH 6.50) (which yields an absorbance value for ClO_2^- of approximately 1.0 at a wavelength of 262 nm). Spectrophotometric scans were made against the split reference cuvette (containing 0.60 ml volumes of the solutions prepared as described above) throughout a wavelength range of 190–400 nm, and residual ClO_2^- concentrations in these mixtures were monitored by the differential decrease in absorbance at wavelengths of 305, 310 and 320 nm (Δ_{305}, ΔA_{310} and ΔA_{320}, respectively). All 5:1 (*v*/*v*) oral rinse/HSS reaction mixtures were analysed in triplicate in order to evaluate the 'between-determination' reproducibility of these ClO_2^- measurements. Each reaction mixture was equilibrated separately and then analysed immediately following the 30-s equilibration period.

For reaction mixtures containing 0.10 ml aliquots of each HSS specimen treated with a 0.50-ml aliquot of the oral rinse product containing a ClO_2^- concentration of 0.40 % (*w*/*v*) (oral rinse II), once thoroughly rotamixed and equilibrated at 35 °C for 30 s as described above, a 0.135-ml aliquot of this reaction admixture was diluted to a final volume of 1.20 ml with 2.00×10^{-2} mol dm^{-3} phosphate buffer solution (pH 6.50) since this oral rinse contained exactly four times the ClO_2^- concentration of oral rinse I. The reference cell oral rinse compartment contained a 0.135-ml volume of oral rinse II diluted to a final volume of 0.60 ml with the above pH 6.50 phosphate buffer solution (so that its final concentration was exactly equivalent to twice that of the reaction sample cell at a time-point of 0.00 s), whereas the HSS compartment contained an exactly corresponding 22.5-μl volume of salivary supernatant diluted to 0.60 ml with this phosphate buffer solution so that the percent HSS volume in this cell compartment (of 0.50 cm path length) was exactly twice that of the HSS–oral rinse reaction mixture in the 1.0-cm length sample cell. Again, residual ClO_2^- (and correspondingly, that consumed by HSS sample biomolecules) was determined via measurement of ΔA_{305}, ΔA_{310} and ΔA_{320} values, and each sample was analysed in triplicate in order to ensure reproducibility of the analytical ClO_2^- concentration data acquired.

For corresponding control experiments, the sample cell contained the 5:1 (*v*/*v*) mixture of 2.00×10^{-2} mol dm^{-3} phosphate buffer solution (pH 6.50)/HSS sample which was again diluted to a final volume of 1.20 ml with further phosphate buffer; the first reference cell compartment contained a 0.60-ml aliquot of only the above phosphate buffer solution, whereas the second contained a 0.10-ml volume of the corresponding HSS sample diluted to a final volume of 0.60 ml with this phosphate buffer solution.

A small number of HSS specimens which had a final absorbance value >0.03 at wavelengths ≥310 nm [when acquired with only 2.00×10^{-3} mol dm^{-3} phosphate buffer (pH 6.50) as the diluent at a 5:1 (*v*/*v*) ratio, this solution also serving as the spectrophotometric reference blank] were discarded, and the participants from which they were collected were requested to provide one or more further (replacement) specimens.

HPLC monitoring of the interaction of the oral rinse ClO_2^- with HSS samples

In order to monitor the accuracy and validity of the above differential spectrophotometric technique for the determination of residual ClO_2^- concentration in the pre-equilibrated 5:1 (*v*/*v*) oral rinse/HSS mixtures, its level remaining in each HSS sample was also determined using a novel HPLC technique employing a reversed-phase C18 column with the ion-pair reagent hexadecyl-trimethylammonium bromide present in the mobile phase. A Millenium HPLC system (Waters, Milford, MA, USA), consisting of a model 626 Pump, a model 096 Photodiode Array Detector and an in-line degasser (Waters) remotely operated using unique Millennium software (Waters), was employed.

Samples were prepared via the treatment of 0.10 ml volumes of HSSs with 0.50 ml aliquots of either oral rinses I or II, as outlined above. Once thoroughly rotamixed and then equilibrated at a temperature of 35 °C for 30 s as described above in 'Differential spectrophotometric determination of the extent of ClO_2^- consumption by human salivary biomolecules', reaction admixtures were immediately diluted 1/40 with 2.00×10^{-2} mol dm^{-3} phosphate buffer (pH 6.50) and then 10.0 μl aliquots of these diluted solutions were injected onto a reversed-phase 4.6×75 mm Spherisorb C18 ODS Column (Waters). A Spherisorb S5-ODS 1 guard column (Waters) was employed to remove any potential analytical column contaminants. The reaction mixtures were again equilibrated separately prior to their immediate direct injection onto the HPLC column system.

The mobile phase was de-gassed using the above in-line degasser and consisted of 2 % (*w*/*v*) borate/gluconate buffer with 2 % (*v*/*v*) butan-1-ol and 12 % (*v*/*v*) acetonitrile (final pH 7.20), which operated at a flow rate of 1.10 ml/min. The ion-pair reagent hexadecyl-trimethylammonium bromide (Sigma-Aldrich Chemical) was added at a final concentration of 5.00×10^{-2} mol dm^{-3} in order to ensure that ClO_2^- is readily separable from interfering salivary components. This analyte was identified via the examination of its electronic absorption spectrum generated by the photo-diode array detector and comparisons with that of an authentic reference standard (i.e. λ_{max} 262 nm, Fig. 1).

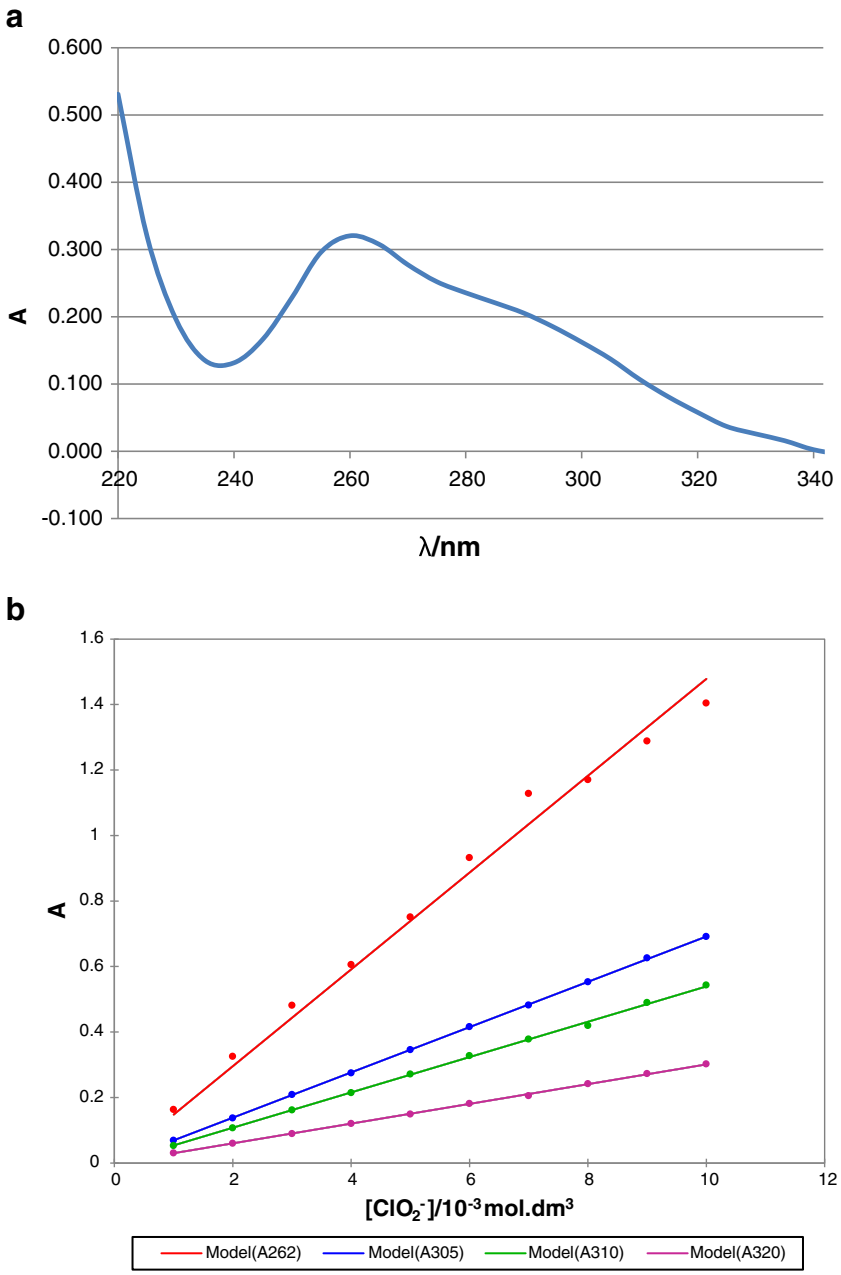

Fig. 1 **a** Electronic absorption spectrum of a 2.00×10^{-3} mol dm^{-3} aqueous solution of sodium chlorite (Na^{+}/OCl_2^{-}) in 2.00×10^{-2} mol dm^{-3} phosphate buffer solution (pH 6.50); **b** plots of absorbance at 262, 305, 310 and 320 nm versus ClO_2^{-} concentration for the spectrophotometric analysis of a series of calibration standards in the $1.00–10.00\times10^{-3}$ mol dm^{-3} concentration range ($r\geq0.9960$)

Time-dependent differential spectrophotometric monitoring of the reaction of salivary urate with oral rinse ClO_2^{-}

The rate of the reaction of salivary urate with added oral rinse ClO_2^{-} was investigated by differential spectrophotometry at a temperature of 35 °C and a pH value of 6.50. In order to achieve this, the twin silica reference cuvette was also utilised. Indeed, to the first split reference cell compartment, thoroughly rotamixed mixtures of 0.10 ml of the HSS sample and 0.50 ml of 1.00×10^{-2} mol dm^{-3} phosphate buffer solution (pH 6.50) was added, whilst to the second compartment, a mixture containing a 0.50 ml volume of oral rinse I and 0.10 ml of the above phosphate buffer solution was added. Following this procedure, a 0.10 ml volume of the same HSS specimen and 0.60 ml of the above phosphate buffer solution were added to the sample (reaction) cuvette, and the reaction was then triggered by the addition of a 0.50 ml aliquot of oral rinse I; these reaction mixtures were, of course, thoroughly rotamixed immediately prior to acquisition of the first difference spectrum approximately 1 min later (spectra were recorded at a scan rate of 120 nm min^{-1}).

Difference spectra in the 220–380 nm range were recorded on the above PC-controlled spectrophotometer equipped with a thermostatted cell holder at a temperature of 35.0 °C at 30 min intervals for a total period of 10.00 h (scan rate 120 nm min^{-1}). The rates of salivary urate consumption (i.e. $t_{1/2}$ values) were determined by monitoring decreases in the λ_{max} values of urate (specifically 292 and 236 nm) in a total of 15 HSS samples.

Absorbance values at 292 nm (A_{292}) for corresponding control HSS samples diluted in a 1:11 (*v*/*v*) ratio with the above phosphate buffer solution (i.e. those corresponding to values at the 0.00 min time-point in the reaction mixtures) ranged from 0.11 to 0.32 for the 15 samples investigated in this manner. First- and second-derivative spectra of the completed reaction cell solutions were also recorded on the above spectrophotometer, but at a flow-rate of 30 nm min^{-1}.

Experimental design

This investigation had an analysis-of-variance (ANOVA)-based experimental design, with each of the HSS samples being treated with both oral rinse formulations (I and II). ANOVA was employed to determine the statistical significance of each component of variance for the ClO_2^- consumption dataset. Indeed, the aim of this procedure was to determine the significance of the 'between-treatments (oral rinses)', 'between-participants' and 'between sampling days–within-participants' effects, and also further components-of-variances involved, specifically that arising from the treatment × participant interaction effect.

This experimental design is classified as a mixed model, three-factor system with treatments (two oral rinses) being a fixed effect at two levels, and participants (n=20 in total) and 'sampling days–within-participants' (n=3) being random effects. Mixed model component analysis for each ClO_2^- consumption level determined comprised the three main effect factors, the treatment×participant interaction effect, and both fundamental and 'between-replicates' errors. The 'between sampling days–within-participants' component of variance was 'nested' within each participant (Table 1).

The treatments × participants interaction effect was included in order to evaluate whether or not the HSS samples of selected participants responded differently to the two treatment regimens [0.10 versus 0.40 % (*w*/*v*) ClO_2^-] than the remainder, i.e. the significance of this variance component represents non-additivity in the mathematical model for this experimental design.

This experimental design further reduced the error mean square value and hence improved the precision of the experiment. Data were $\log_e$-transformed prior to the performance of statistical analysis in order to satisfy assumptions of normality and homogeneity of sample variances; indeed, this transformation is particularly appropriate for concentration data in view of the knowledge that the variability of such measurements increases proportionately with their magnitude.

Results

Differential spectrophotometric determination of ClO_2^- consumption by salivary biomolecules

Primarily, a series of ClO_2^--containing calibration standards (prepared in 2.00×10^{-2} mol dm^{-3} phosphate buffer, pH6.50) were subjected to spectrophotometric analysis, and the electronic absorption spectrum of an authentic sample of this oxyhalogen anion in phosphate buffer solution (pH6.50) is shown in Fig. 1a. Plots of its absorbance at 262, 305, 310 and 320 nm (A_{262}, A_{305}, A_{310} and A_{320}, respectively) versus its concentration were linear throughout the $1.00–10.00\times10^{-3}$ mol dm^{-3} concentration range [$r\geq0.9960$, Fig. 1b]. After making allowances for its commercial purity from the supplier [80.0 % (*w*/*w*), Sigma-Aldrich Chemical], its estimated molar extinction coefficients (ε values) at wavelengths of 262, 305, 310 and 320 nm were 163±7.0, 69±2.9, 54±2.3 and 29±1.3 $M^{-1}cm^{-1}$ [mean±95 % confidence intervals (CIs)] respectively, the first being very similar to a previously reported value of 155 $M^{-1}cm^{-1}$ at a wavelength of 260 nm [33].

Following establishment of the above ClO_2^- extinction coefficient values at the above four wavelengths, differential spectrophotometric analysis of this oxyhalogen oxidant in each of the 5:1 (*v*/*v*) oral rinse/HSS reaction admixtures was performed in order to determine its level of consumption by

Table 1 Experimental design for the analysis of the differential spectrophotometric ClO_2^- consumption dataset acquired, representing a mixed model three-factor ANOVA system with sampling days (n=3 per participant) 'nested' within each participant

Source of variation	Levels	Degrees of freedom (*df*)	Nature	Parameters estimated for the mixed model
Between-treatments	2	1	Fixed	$\sigma^2+9\sigma_{TP}^2+180_{KT}^2$
Between-participants	20	19	Random	$\sigma^2+6\sigma_{D(P)}^2+18\sigma_P^2$
Treatment–participant interaction	40	19	Fixed	$\sigma^2+9\sigma_{TP}^2$
Between sampling days–within-participants	3 per volunteer	40	Random	$\sigma^2+6\sigma_{D(P)}^2$
Error (residual)	n/a	295	n/a	σ^2
Between-replicates	3 per treatment	4	Random	σ_R^2

Abbreviations: σ^2, error (residual) mean square (variance); ${}_{KT}^2$, 'between-treatments' factor component; σ_P^2, 'between-participants' component of variance; $\sigma_{D(P)}^2$, 'between-sampling days–within-participants' component of variance; σ_{TP}^2, treatment × participant interaction component; σ_R^2, between-replicates (n=3) mean square

salivary biomolecules therein on equilibration. Since selected salivary amino acids (tryptophan, phenylalanine, tyrosine and, to a much lesser extent, cystine) [29, 30] and also urate [31, 32] absorb at the λ_{max} value of ClO_2^- (262 nm), we elected to monitor the differential decrease in the absorbance of this oxyhalogen agent at 305, 310 and 320 nm since it still absorbs significantly at all of these lower energy wavelengths, and the great majority of electronic absorption spectra acquired on untreated HSS samples in this investigation revealed that this biofluid exhibits negligible absorbance at wavelengths ≥310 nm. Indeed, salivary tryptophan, phenylalanine, tyrosine and urate have λ_{max} values of 279 ($\varepsilon = 5.60\times10^3\,M^{-1}cm^{-1}$ [34, 35]), 257 ($\varepsilon = 2.00\times10^2\,M^{-1}cm^{-1}$ [29, 30]), 275 ($\varepsilon = 1.40\times10^3\,M^{-1}cm^{-1}$ [34, 35]), and 236 and 292 nm ($\varepsilon = 8.59\times10^3$ and $1.14\times10^4\,M^{-1}cm^{-1}$ [36, 37]), respectively, at pH values at or close to neutrality, i.e. at values very similar to 6.50 as selected for our investigations.

Subsequently, each Δ_{305}, ΔA_{310} and ΔA_{320} value determined from the differential spectral profiles were then converted to the parameter micromole ClO_2^- per millilitre of HSS via employment of the corresponding ε values determined by the method outlined above [the 'overall' biofluid hypothetically representing a mean ('pooled') HSS specimen]. There were no statistically significant differences between the wavelengths employed (i.e. 305 versus 310 versus 320 nm) for the determination of residual ClO_2^- concentrations in the 5:1 (*v*/*v*) oral rinse/HSS admixtures [randomised blocks (two-way) ANOVA performed on $\log_e$-transformed data ($n=60$), $p=0.762$]. Indeed, there was an excellent agreement and very strong correlation between these three sets of values ($r\geq0.9876$), an observation which provides much evidence for the equality of these wavelengths when employed as means to determine residual ClO_2^- in these equilibration admixtures.

As expected, all control samples in which 0.10 ml volumes of HSS samples were diluted with 0.50 ml of $2.00\times10^{-2}\,mol\,dm^{-3}$ phosphate buffer solution and then equilibrated at 35 °C for a period of 30 s in the same manner as the test admixtures showed no changes in differential absorbance at both wavelengths (mean±95 % CIs of ΔA_{305}, ΔA_{310} and ΔA_{320} values for this set of control sample mixtures were −0.002±0.003, 0.001±0.002 and −0.001±0.002, respectively).

The pH values of the HSS samples collected ranged from 6.87 to 7.28, and the addition of these samples to oral rinse formulations I and II in a 1:5 (*v*/*v*) ratio, followed by equilibration of these reaction admixtures at 35 °C for a period of 30 s, did not significantly influence the pH value of the oral healthcare products employed (6.50–6.65).

Ion-paired reversed-phase HPLC analysis of ClO_2^- in 5:1 (v/v) oral rinse/HSS equilibration mixtures

Experiments involving alteration of the ion-pair reagent concentration from 0.50 to $5.00\times10^{-3}\,mol\,dm^{-3}$ revealed that a concentration of $5.00\times10^{-2}\,mol\,dm^{-3}$ gave rise to an effective resolution of ClO_2^- from salivary components (e.g. urate) in all samples investigated. Measurement of the retention time of the ClO_2^- peak was established via the injection of a series of authentic Na^+/ClO_2^- calibration standards ($1.00–10.00\times10^{-3}\,mol\,dm^{-3}$), as shown in Fig. 2. As noted in 'Materials and methods', identification of the ClO_2^- peak was based on its retention time (6.90 min) and also the diode-array spectrum of this HPLC peak (λ_{max} 262 nm). IP-RP-HPLC analysis of the above Na^+/ClO_2^- calibration standards (in triplicate) demonstrated a clear linear relationship between peak intensity and concentration. Indeed, plots of mean peak area versus ClO_2^- concentration were linear [correlation coefficient, $r=0.9965$, Fig. 2d], and repeated chromatograms had reproducible retention times.

There was a high level of agreement between these HPLC determinations of ClO_2^- and those determined by the differential spectrophotometric technique described in 'Differential spectrophotometric determination of ClO_2^- consumption by salivary biomolecules'. Indeed, for samples in which both techniques were employed for the analysis of the 5:1 oral rinse/HSS mixtures subsequent to their equilibration ($n=37$), there was a very strong correlation between ClO_2^- concentrations determined by the former technique and those measured by the latter ($r=0.9890$, data not shown), and no significant differences were found between these two sets of values (paired sample *t* test performed on untransformed data).

Statistical analysis of ClO_2^- consumption dataset

Statistical analysis of data acquired from the differential spectrophotometric determination of ClO_2^- consumption by salivary biomolecules [i.e. multifactorial ANOVA] revealed highly significant differences between (1) the ClO_2^- content of each oral rinse investigated ($p<0.0001$), (2) participants ($p<0.01$) and (3) 'sampling days-nested–within-participants' ($p<0.001$). Moreover, the treatment (oral rinse composition)–participant interaction mean square value was also found to be very highly significant ($p<0.0001$). Table 2 lists *p* values determined for the significance of each factor/component of variance tested in this ANOVA-based model.

In order to further investigate the highly significant treatment×participant interaction mean square value (Table 2), plots of the mean ClO_2^- consumption level (micromole per millilitre of HSS sample) versus its oral rinse product concentration were made for each of the participants recruited to the investigation (Fig. 3); the participant mean values determined were those of $n=3$ separate saliva samples collected from each donor and $n=3$ replicate ClO_2^- determinations made for each one. Three of the participants' HSS samples demonstrated a much greater response to the higher ClO_2^- content oral rinse product than that anticipated from the non-

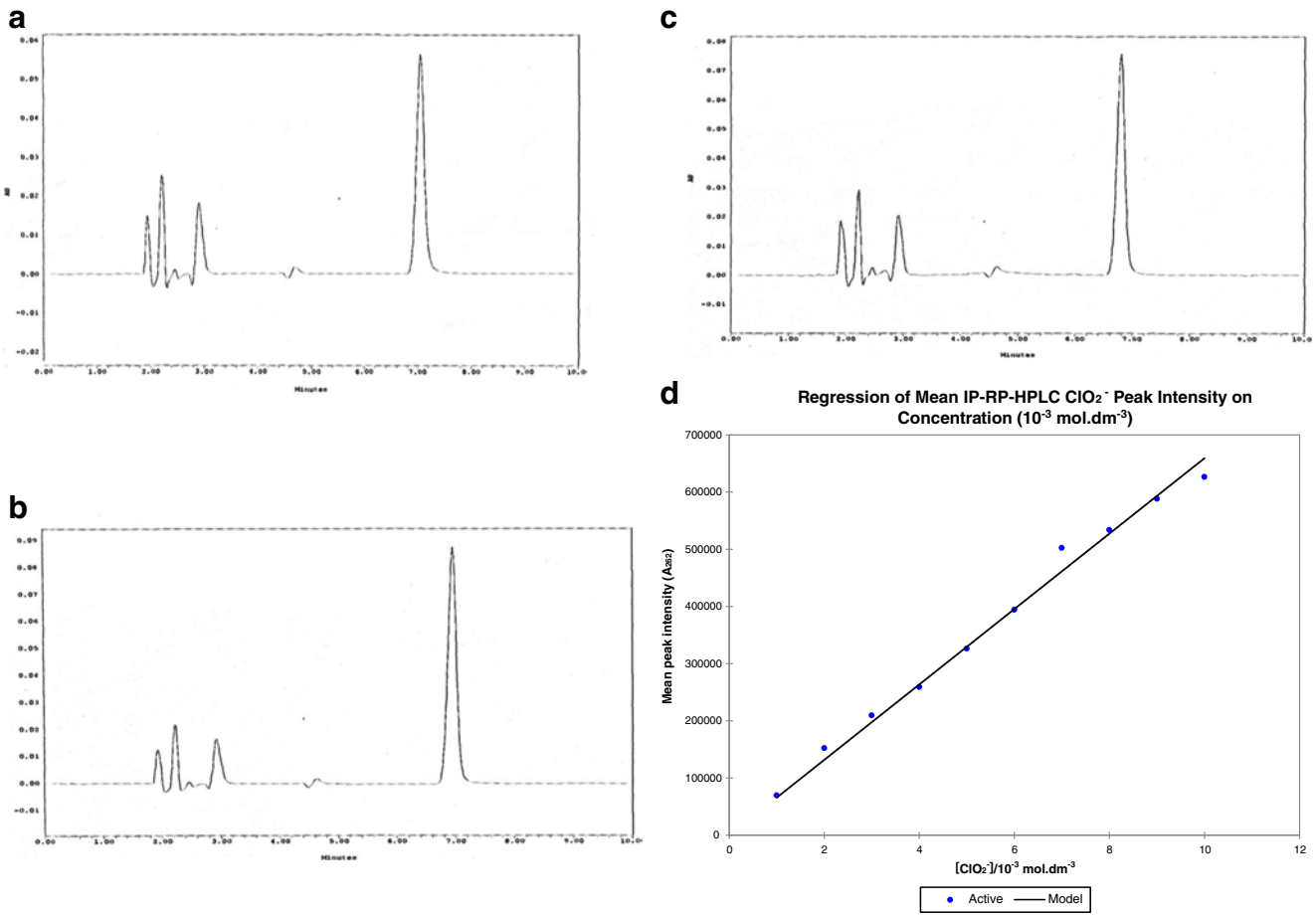

Fig. 2 Ion-pair reversed-phase HPLC (IP-RP-HPLC) chromatograms of **a** 1.00×10^{-3} mol dm^{-3} Na^+/ClO_2^- standard solution, **b** oral rinse formulation II containing 0.40 % (*w*/*v*) ClO_2^- [diluted 1/40 with 2.00×10^{-2} mol dm^{-3} phosphate buffer (pH 6.50) prior to injection] and **c** a typical salivary supernatant sample (0.10 ml) pre-treated with a 0.50-ml volume of the above oral rinse product [this reaction admixture was also diluted 1/40 with 2.00×10^{-2} mol dm^{-3} phosphate buffer (pH 6.50) prior to analysis]. The retention time of ClO_2^- was 6.90 min. **d** Plot of ClO_2^- peak area (microvolt per second) versus its concentration obtained from the IP-RP-HPLC analysis of a series of ClO_2^- calibration standards according to the technique described in section 2.4 'HPLC monitoring of the interaction of the oral rinse ClO_2^- with HSS samples' (r=0.9965)

interactive (additive) model for the dataset, and two further ones surprisingly gave rise to a lower level of consumption with this higher ClO_2^- concentration formulation (albeit only a slightly lower ClO_2^- consumption level). Hence, the highly significant treatment × participant interaction effect is ascribable to differences in both (1) the magnitude of the response to the treatments applied (visible as non-parallel lines in Fig. 3) and (2) the direction of this response for some participants, on increasing the ClO_2^- content of the oral rinse from a level of 0.10 to 0.40 % (*w*/*v*). Indeed, Fig. 3 reveals clear participant-dependent differences in the magnitude of the increase in ClO_2^- consumption level on elevating the oral rinse content of this agent.

Table 2 Significance of factors/components of variance for analysis of variance (ANOVA) performed for the experimental design model applied to the salivary ClO_2^- consumption dataset

Source of variation	p values
Between treatments (oral rinses)	<0.0001
Between participants	<0.001
Treatment–participant interaction	<0.0001
Between sampling days–within-participants	<0.001

Estimates of the overall mean consumption of ClO_2^- determined for reaction mixtures containing a 5:1 (*v*/*v*) ratio of oral rinse/HSS were 3.94±0.38 and 11.80±1.52 (mean±95 % CI) μmol ml^{-1} of HSS for the 0.10 % (*w*/*v*) and 0.40 % (*w*/*v*) ClO_2^--containing oral rinses, respectively, as determined by the differential decreases in absorbance observed at wavelengths of either 305 (Δ_{305}), 310 (ΔA_{310}) or 320 nm (ΔA_{320}). Hence, for the oral rinse product containing four times the available ClO_2^-, [the 0.40 % (*w*/*v*) one], approximately three times the amount of this oxyhalogen species is scavenged by a hypothetical mean 'pooled' HSS sample (i.e. one that is hypothetically 'pooled' both 'between-participants'

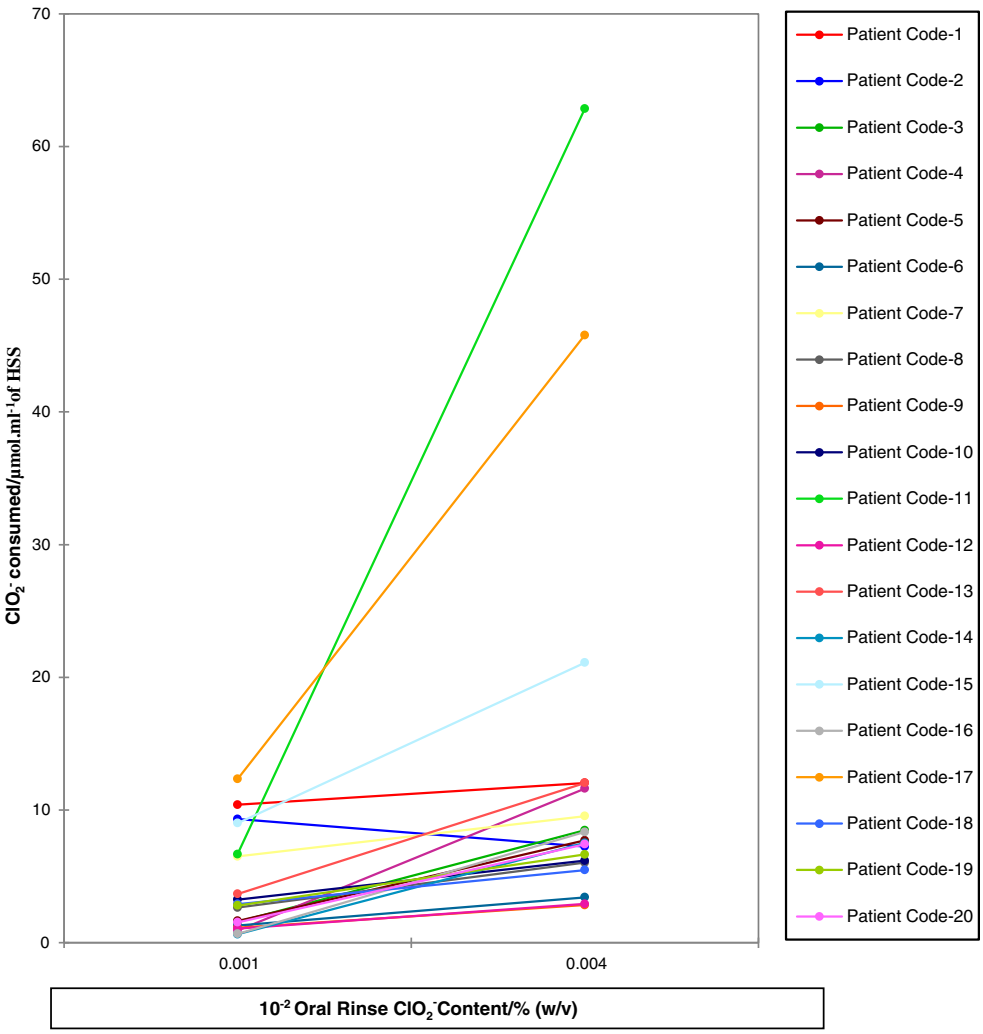

Fig. 3 Plots of the mean level of ClO_2^- consumed (micromole per millilitre) by HSS specimens versus its concentration [percent (*w*/*v*)] in the oral rinse product tested for each of the participants recruited to the investigation (each data point represents the mean value of $n=3$ separate saliva samples and $n=3$ replicate ClO_2^- determinations made for each one)

and 'between sampling days–within-participants') than that consumed from the formulation with a lower ClO_2^- content [0.10 % (*w*/*v*)].

The above mean consumption estimates correspond to 7.14±0.69 % (mean±95 % CI, range 0.00–37.74 %) of the total ClO_2^- available in the oral rinse product containing 0.10 % (*w*/*v*) of this oxyhalogen oxidant and 5.34±0.69 % (mean±95 % CI, range 0.00–48.73 %) of that for the formulation containing 0.40 % (*w*/*v*) of this agent.

The 'between-replicates' mean square value was only 1.266×10^{-4}, indicating a high level of reproducibility for repeated (triplicate) determinations conducted on each sample tested. Indeed, 'between-replicates' coefficient of variation (CV) values ranged from 2 to 8 %, with an overall mean CV value of 5.3 %.

A plot of the observed extent of ClO_2^- consumption (micromole per millilitre of HSS) from oral rinse I versus that derived from oral rinse II (Fig. 4) revealed a significant correlation between these two series of levels [$r=0.5376$ ($p<0.0001$)]. However, as expected in view of the non-additive antioxidant response of individual participants' HSS samples to increases in the oral rinse ClO_2^- content, this correlation was not strong. Polynomial regression analysis confirmed, however, that there was no evidence for a quadratic regression of the HSS ClO_2^- consumption level for the 0.40 % (*w*/*v*) oral rinse on that for the product containing 0.10 % (*w*/*v*) of this agent, i.e. that with an x^2 ([0.10 % (*w*/*v*) content HSS consumption level]^2) component; the intercept for this polynomial regression analysis was not significantly different from zero.

Time-dependent spectrophotometric monitoring of the interaction of oral rinse ClO_2^- with HSS urate

The time dependence of the oxidative attack of oral rinse ClO_2^- (i.e. that in oral rinse I) on the UV-absorbing and -detectable salivary biomolecule urate (a known antioxidant) was also investigated in intact HSS samples ($n=15$) using differential spectrophotometry. For these investigations, the oral rinse formulation containing 0.10 % (*w*/*v*) ClO_2^- was added to HSS samples in the manner described in 'Materials and methods' [i.e. at an added 5:1 (*v*/*v*) oral rinse/HSS ratio], and subsequent to the primary ClO_2^- concentration-dependent rapid decreases

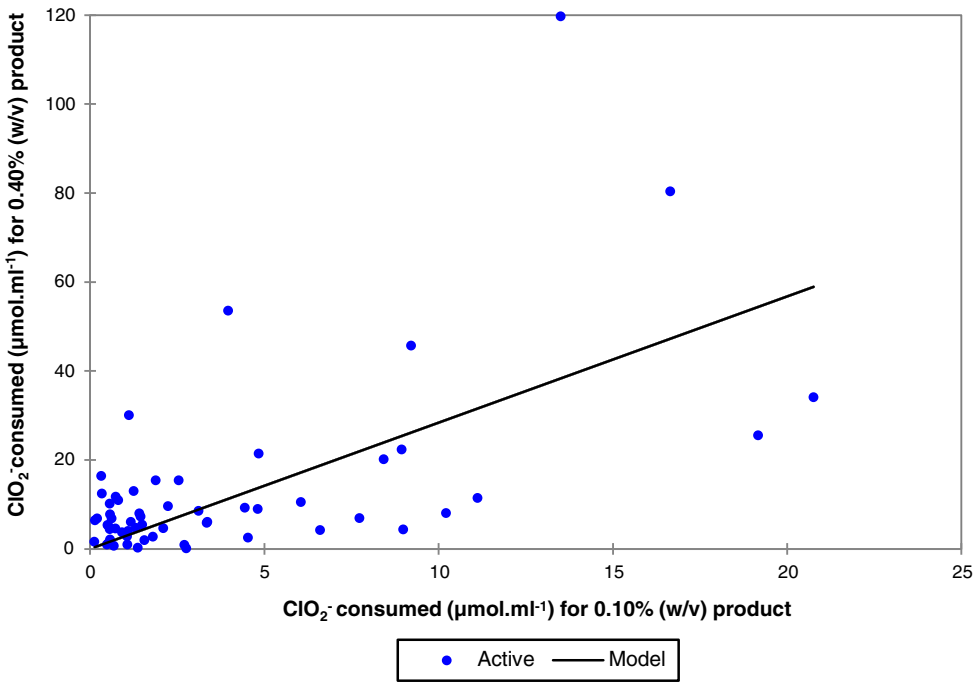

Fig. 4 Plot of the concentration of ClO_2^- consumed by HSS samples for the oral rinse formulation containing 0.40 % (*w*/*v*) ClO_2^- versus that measured for the product containing 0.10 % (*w*/*v*) of this active agent [r=0.5376 (p<0.0001)]

in A_{305}, A_{310} and A_{320} observed during the 30-s equilibration period (as noted above), a series of differential spectral scans (which monitored modifications in absorbance in the 220–350 nm wavelength range) were acquired for n=15 of these reaction mixtures at a spectrophotometer reaction cell temperature of 35 °C. These experiments revealed a slower, second phase of time-dependent decreases in UV absorbance, notably at 236 and 292 nm (which represent the λ_{max} values for commercially available, authentic samples of urate [36, 37]). Of course, the differential spectrophotometric technique permitted the synchronous monitoring of spectral modifications in the sample (reaction) cell relative to those of the (0.00 min time-point) absorbance values of salivary biomolecule/ClO_2^- admixtures.

The final absorption spectrum recorded [following a 10.0-h equilibration period at physiological temperature (35 °C)] represents a composite of residual ClO_2^- (i.e. λ_{max} 262 nm), and HSS urate, tyrosine, tryptophan and, to a lesser extent, phenylalanine remaining in the equilibration mixtures, together with that of any products arising from the reactions of these biomolecules with the added oxyhalogen oxidant which may also absorb in the spectral region monitored (data not shown). Hence, employment of this differential spectrophotometric technique yielded valuable kinetic data regarding the relatively slow rate of urate consumption in this medium (at the above temperature). Under our experimental conditions, estimated half-life ($t_{½}$) values for the attack of ClO_2^- on salivary urate varied from 1.7–2.8 h for the 15 HSS samples investigated in this manner. The above final (differential) minimum wavelength values (λ_{min}) of urate (236 and 292 nm) were confirmed via the acquisition of both first- and second-derivative spectra on completion of this second equilibration period (10.0 h).

As expected, for each of these differential spectrophotometric kinetic profiles acquired, the decrease in absorbance observed at a wavelength of 262 nm demonstrated both extremely rapid (i.e. that complete within a 30-s equilibration period at 35 °C) and much slower phases, the latter having $t_{1/2}$ values similar to those observed at 236 and 292 nm. The primary, rapid phase of this decrease is attributable to ClO_2^- loss within 30 s in accordance with the Δ_{305}, ΔA_{310} and ΔA_{320} datasets acquired as described above, i.e. that occurring within a recommended 30-s oral rinsing episode (or, in this case, a corresponding 30-s in vitro biomimetic process), whereas the second (much slower) reaction stage corresponds to the reaction of this oxyhalogen oxidant with salivary urate (and perhaps also further slowly reacting salivary biomolecules which also absorb significantly at 262 nm). As expected, the decrease in A_{262} values observed for the second, slower phase of the reaction process was much less that those observed at wavelengths of 236 and 292 nm, a reflection of the much higher ε values for urate at these wavelengths than that of ClO_2^- at 262 nm, even on consideration of the higher levels of the latter species present in the equilibrium admixtures (although urate itself also absorbs at 262 nm, its ε value at this wavelength is much lower than those of this biomolecule at wavelengths of 236 and 292 nm [36, 37]).

Discussion

The major objective of this investigation was to monitor the level of consumption of ClO_2^- via chemical reactions with salivary biomolecules (oxidative or otherwise) for both oral

rinses examined in order to determine whether or not their therapeutic activities will be limited by such reactions and, in this manner, provide valuable information regarding the concentration of this active agent remaining in this biofluid during recommended 30 s oral rinsing episodes with these products. Clearly, this information is of much importance with regard to the microbicidal activities of these oral rinse products, together with their capacities to combat oral malodour conditions [38, 39] and periodontal diseases [25, 26], for example.

For this purpose, both differential spectrophotometric and IP-RP-HPLC techniques were employed. For the former (which was based on determinations of the absorbance of ClO_2^- at the near-UV wavelengths of 305, 310 and 320 nm), concentrations of the potentially interfering UV-absorbing biomolecules tryptophan, phenylalanine, tyrosine and urate in human saliva have been previously reported by Hawkins et al. [40] (mean levels of tyrosine of 3.97 and 2.87×10^{-4} mol dm^{-3}, tryptophan 1.52 and 1.13×10^{-4} mol dm^{-3} and urate 1.43 and 1.49×10^{-4} mol dm^{-3} for two separate groups), Alagendran et al. [41] (mean levels of phenylalanine of $2.3–6.5\times10^{-5}$ mol dm^{-3}, tyrosine $1.2–5.1\times10^{-5}$ mol dm^{-3} and tryptophan $1.3–6.8\times10^{-5}$ mol dm^{-3} for women during different stages of their menstrual cycles) and Makinen et al. [42] (mean ± SEM levels of phenylalanine of $5.3\pm3.0\times10^{-5}$ mol dm^{-3} and tyrosine $6.2\pm4.8\times10^{-5}$ mol dm^{-3} in human palatine gland secretions).

Although urate exhibits a very low level of absorbance at wavelengths of 305 and 310 nm (and is zero at 320 nm), the rate of its oxidative consumption by ClO_2^- was found to be extremely slow under the experimental conditions employed here ('Time-dependent spectrophotometric monitoring of the interaction of oral rinse ClO_2^- with HSS urate'), and hence, its very minor contribution to the absorbance of ClO_2^- monitored at these particular wavelengths, was found not to interfere with determinations of the residual level of ClO_2^- in the 5:1 (*v*/*v*) oral rinse/HSS reaction admixtures following their equilibration at 35 °C for only a 30-s period. Moreover, although the amino acid tryptophan also displays a very low absorbance at 305 nm (at neutral or near-neutral pH values), in view of its low salivary concentration [40, 41]), and also the consideration that its HSS level (prior to treatment) is effectively six times lower than that of the intact biofluid supernatant in view of the 5:1 (*v*/*v*) oral rinse/HSS reaction admixtures subjected to analysis in this manner, its contribution to the ΔA_{305} values monitored here for ClO_2^- consumption level determinations is also clearly negligible. Both tyrosine and phenylalanine do not absorb at wavelengths ≥305 nm.

Computations on the dataset acquired revealed that for oral rinse I [containing 0.10 % (*w*/*v*) ClO_2^-], there is a mean percentage consumption of 7.14±0.69 % (mean±95 % CI) of the total ClO_2^- available following reaction with salivary biomolecules, whereas for oral rinse II [containing 0.40 % (*w*/*v*) ClO_2^-], this value is 5.34±0.69 %. These percentage values are equivalent to 3.94±0.38 and 11.80±1.52 (mean± 95 % CI) μmol ClO_2^- ml^{-1} of HSS for oral rinses I and II, respectively. Therefore, it is clear that a recommended 30-s oral rinsing episode with either of the two products tested gives rise to a >90 % retention of the active ClO_2^- component, i.e. <10 % of it is consumed by chemical reactions with HSS biomolecules within this pre-selected equilibration period.

Interestingly, pyruvate is readily consumed by oral rinse ClO_2^- (Eq. 4) [2], and this α-keto acid anion represents the major source of salivary ClO_2^--consuming capacity, especially since it is present in this biofluid at up to millimolar concentrations [43].

$$2CH_3COCO_2^- + ClO_2^- \rightarrow 2CH_3CO_2^- + 2CO_2 + Cl^- \quad (4)$$

Also of particular relevance to the therapeutic activities of these ClO_2^--containing oral rinse formulations are the possible reactions of this active agent with a range of salivary amino acids, which serve as processes for generating the more potent microbicidal agent $ClO_2^{\bullet}$ from its ClO_2^- precursor, reaction systems which are, of course, expected to be more favourable for the oral rinse product with the higher ClO_2^- content. Indeed, the documented reaction of ClO_2^- with taurine [27] (Eq. 3), and plausibly also a range of further amino acids (including glycine and alanine), has a stoichiometry which involves the consumption of 3 molar equivalents of ClO_2^- per mole of taurine to generate 1 of taurine's *N*-monochloroamine [$Cl(H)NCH_2CH_2SO_3^-$] and 2 of $ClO_2^{\bullet}$ (the production of *N*-monochlorotaurine is rapid when expressed relative to that of $ClO_2^{\bullet}$ accumulation); at the lower pH values investigated by these researchers, *N*-monochlorotaurine disassociates to taurine and *N*-dichlorotaurine. Moreover, production of the reactive $HOCl/OCl^-$ species during the induction period observed for the reaction of ClO_2^- with this β-amino acid (and also presumably salivary α-amino acids) will also serve to oxidatively consume available salivary biomolecules. Even if this mechanistic process only proceeds for selected 'free' amino acids (or those located at the N-termini of salivary proteins), the $HOCl/OCl^-$ generated will, of course, be available to react with a much wider range of 'scavenger' species in a (relatively) unselective manner to form *N*-α-monochloroamines and *N*-dichloroamines.

However, since many N-α-monochloroamines generated in this manner are unstable at physiological temperature [44] and decompose to corresponding aldehydes (Eq. 5), further investigations focused on the detection and quantification of such products which correspond to the side chains of α-amino acids (e.g. formaldehyde from salivary glycine, acetaldehyde from alanine, etc.) are required in order to

demonstrate this. Hence, specific alsehydes may serve as valuable biomarkers of $HOCl/OCl^-$ activity in this context.

$$Cl(H)N - CHR - CO_2^- + H_2O \rightarrow RCHO + NH_3 + CO_2 + Cl^- \quad (5)$$

However, formaldehyde (HCHO), and presumably further aldehydes, are oxidisable by the ClO_2^- precursor in both mildly acidic and alkaline media [45], a reaction that also gives rise to $ClO_2^\bullet$ as a product.

Hence, generation of the aggresively reactive oxidants $ClO_2^\bullet$ and $HOCl/OCl^-$ from their ClO_2^- precursor in this manner will render a much wider range of salivary biomolecules vulnerable to attack and hence oxidative damage, a postulate that may account for a higher level of salivary antioxidant consumption than that which would be attributable to the actions of ClO_2^- alone.

The sulphur-containing amino acids cysteine and methionine, and the phenolic amino acid tyrosine, each with redox-active side chains can, of course, also be oxidatively modified by ClO_2^- (and also $ClO_2^\bullet$ and $HOCl/OCl^-$ produced via its reaction with these and/or further amino acids) to cysteine sulphonate (and cystine), methionine sulphoxide (Eqs. 6 and 7) [2] and a tyrosine-derived quinone species, respectively.

$$H_3N^+CH(CH_2CH_2SCH_3)CO_2^- + ClO_2^- \rightarrow H_3N^+CH(CH_2CH_2SOCH_3)CO_2^- + OCl^- \quad (6)$$

$$2H_3N^+CH(CH_2CH_2SCH_3)CO_2^- + ClO_2^- \rightarrow 2H_3N^+CH(CH_2CH_2SOCH_3)CO_2^- + Cl^- \quad (7)$$

The total salivary levels of free thiols (e.g. cysteine itself, peptides containing this amino acid such as glutathione, protein cysteinyl residues, etc.) are 3.6×10^{-5} mol dm^{-3} [46], a value which is very low when expressed relative to that of added ClO_2^- for the above specified equilibration processes with both oral rinse formulations tested here. An investigation of the oxidative attack of ClO_2^- on *N*-acetylcysteine [47] that revealed that the final product generated from this reaction was *N*-acetylsulphonate, and the mechanism of this process involved the rapid formation of $ClO_2^\bullet$ without a monitorable induction period.

Moreover, in view of their extremely low concentrations (<100 ppb in exhaled breath for human subjects without halitosis, and >150 ppb for those with this condition) [3–5] and boiling points (e.g. 5.95 °C for methyl mercaptan), VSCs will, of course, exert a negligible contribution to the reductive consumption of ClO_2^- (and $ClO_2^\bullet$) via electron-transfer reactions in human saliva, unlike their amino acid precursors (L-cysteine and L-methionine).

In view of the restrictively slow rate of reaction between ClO_2^- and urate (mean salivary concentrations ca. 7×10^{-5} mol dm^{-3} [2] and 1.5×10^{-4} mol dm^{-3} [40]), it appears that this salivary antioxidant will offer little or no contribution to the total oxyhalogen oxidant scavenging capacity of human saliva, especially on consideration of the short (30 s), recommended length of oral rinsing episodes with these products. Indeed, for this system, the differential spectrophotometric kinetic data acquired were similar to those acquired in a previous investigation [2], although, as expected, the rate of this process was more rapid in the investigations described here since the experiments were performed at a temperature of 35 °C rather than at 22 °C as documented in [2]. Moreover, the reaction mixtures investigated here contained 2.5 times the added oral rinse ClO_2^- concentration than that employed in [2], and their HSS content in the final reaction mixture was only 8.33 % (*v*/*v*) rather than 20 % (*v*/*v*) as in the experiments performed in [2].

Further salivary biomolecules which potentially play a role in the scavenging of oral rinse ClO_2^- (together with lower levels of $ClO_2^\bullet$ and/or $HOCl/OCl^-$ derivable as by-products from its reaction with amino acids) are trimethylamine and also perhaps SCN^-, the latter present at millimolar levels in this biofluid, and which is significantly elevated in tobacco smokers [2].

Information arising from this investigation regarding the ability of salivary biomolecules to scavenge significant levels of oral rinse ClO_2^- have provided valuable insights for the design of further studies, which have recently been performed in our laboratory (in these experimental systems, we employed high-resolution ^{1}H NMR analysis in order to determine the ability of a range of salivary biomolecules to consume added oral rinse ClO_2^-). Such investigations have confirmed the scavenging of ClO_2^- by a range of amino acids (including those without electron-donating side chains, as well as methionine and tyrosine), pyruvate and additional α-keto acid anions, trimethylamine, and further metabolites; these studies will be reported in detail elsewhere.

As expected, there was a significantly elevated level of ClO_2^- consumption by HSS samples when the 0.40 % (*w*/*v*) oral rinse formulation was employed in place of the 0.10 % (*w*/*v*) product, the former being approximately three times greater than the latter. Notwithstanding, it should be noted that the treatment × participant interaction effect was highly significant ($p<0.0001$). This interaction is explicable by the consideration that selected HSS specimens have only a limited ability to scavenge (and hence offer 'protection' against) added ClO_2^-, i.e. if all the ClO_2^--scavenging antioxidants available are consumed at its concentration present in equilibration admixtures involving the addition of the 0.10 % (*w*/*v*) oral rinse product, then no further consumption will be expected on treatment and equilibration of these particular HSS samples in the same manner with the product containing the higher ClO_2^- content. Indeed, reference to Fig. 3 shows that for at least several participants, there was

little or no increase in the mean level of ClO_2^- consumed on increasing the added oral rinse ClO_2^- content four-fold.

These observations also partially explain the significant but nevertheless poor correlation observed between the levels of ClO_2^- consumption by HSS samples (i.e. scavenging biomolecules therein) and the content of this active agent in the added oral rinse formulations [i.e. 0.10 versus 0.40 % (*w*/*v*)] (Fig. 4), and this is ascribable to the differential response of individual study participants' samples to increases in added oral rinse ClO_2^- concentration.

It is also highly likely that if intact human saliva (i.e. that directly collectable from human participants) was employed as a test medium for these investigations rather than HSSs, then the 'debris' which is removed by the preliminary centrifugation step during the prior experimental sample preparation stage of the experiments (performed here in order to generate clear supernatants) will also offer ClO_2^--scavenging activity. Indeed, since this debris includes a range of both human and microbial cells (which potentially also contain a wealth of ClO_2^--scavenging biomolecules such as α-keto acid anions, thiols such as glutathione and a wide range of amino acids, proteins and glycosaminoglycans), then this matrix may also serve as a source of ClO_2^--neutralising capacity, and further experiments to investigate this influence are currently in progress in our laboratory.

Conclusions

Our results clearly demonstrate the consumption of oral rinse ClO_2^- by salivary biomolecules (electron donors, antioxidants or otherwise) when added to HSSs and then equilibrated at 35 °C for 30 s, a period recommended for oral rinsing episodes with these products. Data acquired revealed that only 7.14 and 5.34 % of the total ClO_2^- available was consumed for oral rinse formulations containing 0.10 and 0.40 % (*w*/*v*), respectively, of this active agent and therefore confirm that >90 % of it remains so that it may exert its therapeutic (e.g. microbicidal and anti-periodontal) actions. Our data also demonstrated that the magnitude of the ClO_2^--scavenging 'antioxidant' response of HSS samples differed substantially 'between-participants' on increasing the oral rinse content of this oxyhalogen oxidant from 0.10 to 0.40 % (*w*/*v*); for example, HSS samples collected from some of the participants recruited to the investigation were much more responsive to the oral rinse with the higher [0.40 % (*w*/*v*)] ClO_2^- content (i.e. they scavenged much more ClO_2^-) than those of others. Salivary biomolecules which are vulnerable to attack by (and putatively serve as scavengers of) ClO_2^- include α-keto acid anions such as pyruvate (present in this biofluid at up to millimolar levels), SCN^-, methylamines, and amino acids with electron-donating side chains (L-cysteine, L-methionine and L-tyrosine, including those present in salivary proteins), together with amino acids/peptides in general (including the β-amino acid taurine). The latter reaction systems involve the generation of N-chlorinated amino acid adducts and $ClO_2^{\bullet}$, both of which have more potent microbicidal actions than ClO_2^- itself.

Conflict of interest The authors declare that they have no conflicts of interest.

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