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Abstract

Objectives: To compare a new mouthwash (SB12®) containing 0.025% chlorhexidine and 0.3% zinc for oral malodour reduction against four commercially available mouthwashes and negative control. **A secondary aim was to compare the two methods for measuring volatile sulphur compounds (VSC) by halimetry and OralChroma.**

Methods: Organoleptic scale, halimeter and the OralChroma were used to assess oral malodour and VSC. The effects of five test formulations and water (negative control) were assessed after 30-, 60-, 90- and 180-minutes, with one week between the treatments to avoid any cross-over effect.

Results: Reduction in H_2S by halimetry and malodour levels by organoleptic assessment ranged from, **slight** (LacerFresh®) ($P > 0.05$), moderate (BreathRx®, SmartMouth®) ($P < 0.01$) to **marked** effect (SB12 ®, Listerine®) ($P < 0.001$) at all time points compared to water. The largest differences were observed at 30-minutes and decreased with time. SB12® showed separation from Listerine® at 180-minutes, using ANOVA plus Bonferroni's Multiple Comparison post-test ($P < 0.05$). Relationships between organoleptic, halimeter and OralChroma were between $R^2 = 0.795$ to 0.926.

Conclusion: SB12 shows a consistent and reproducible inhibitory effect on oral malodour parameters which in turn **correlate well with each other**.

Introduction

A significant source of oral malodour is from organisms on the surface of the tongue with microbes inhabiting the tongue biofilm being responsible for approximately 80% of cases of oral malodour (Yeagaki and Sanada, 1992; Rosenberg and Leib, 1995; Van den Broek ***et al.,*** 2008). The particular papillary surface of the tongue with its large number of crypts and fissures allows it to harbour a high number of bacteria in a relatively anaerobic environment (Tonzetich, 1977). The extremely diverse microflora particularly Gram-negative anaerobes possess enzymes that allow biotransformations of sulphur-substrates (cysteine, methionine and glutathione) into volatile sulphur compounds (VSC) (Kleinberg and Westbay, 1990: Scully *et al*., 1997). The main VSC in oral malodour is believed to be hydrogen sulphide (H_2S), although methyl mercaptan (CH_3SH) (Tonzetich, 1971; Yaegaki and Sanada, 1992) and dimethyl sulphide $(CH_3)_2S$ may also play a role (Quirynen, 2003; Suarez *et al*., 2000). In addition to producing bad breath, VSC produced by periodontopathogens in the gingival crevice have been implicated in the aetiology of periodontal disease resulting in tooth loss if left untreated (Shapiro *et al*., 1977; Radcliff and Johnson, 1999). Other volatile organic compounds (VOC) contribute to an unknown extent to oral malodour and they are thought to include indole, amines and acids (Kostlec *et al*., 1980; Goldberg *et al*., 1994; Radcliff and Johnson, 1999).

Numerous mouthwashes are available for use as part of a daily oral hygiene routine. The formulations contain actives that may inhibit microbial growth, enzymatic reactions or may react directly with VSC to reduce their levels in the breath. In addition, these formulations may include flavour-compounds, which can mask the effects of odiferous compounds.

Certain metal ions, in particular zinc (Zn), are well known to reduce or inhibit the formation of VSC (Tonzetich, 1971; Yaegaki and Suetaka, 1989; Young *et al*., 2002) as do certain antibacterial agents such as chlorhexidine (CHX) and cetylpyridinium chloride (CPC) with a subsequent reduction in oral malodour (Denton, 1991; Loe and Schiott, 1970; Lang *et al*., 1973; Grossman *et al*., 1996; Young *et al*., 2002; Winkel *et al*., 2003; **Roldan *et al*., 2003**). The combination of low concentrations of Zn and CHX seems to be particularly effective (Young *et al*., 2002; Winkel *et al*., 2003). More evidence is emerging for the efficacy of this combination including double-blind comparisons with several widely used formulations against halitosis. The studies have in the main used gas chromatography (GC) to measure VSC (Tonzetich *et al*., 1991; Yaegaki and Sanada, 1992; Rosenberg, 1996).

Two common approaches to assessing oral malodour include halimetry and organoleptic measurements by a trained odour judge (Rosenberg *et al*., 1991; De Boever *et al*., 1994). More recently another instrument has been commonly employed; a portable GC system (OralChroma®). Organoleptic assessments by a trained judge have been shown to correlate with halimetry (Rosenberg *et al*., 1991; De Boever *et al*., 1994) but the relationship between these measurements and Oral Chroma have not been widely studied.

The aim of this study was to compare a combination of low concentrations of Zn and CHX (SB12®) with several commercially available mouthwash

preparations and a negative control using both organoleptic measures and a halimeter. A secondary aim was to compare these results with those obtained using an OralChroma®.

The hypothesis to be tested in this study was that the combination of Zn and CHX in low concentrations is at least as effective as a selection of other currently used antibacterial agents/mouth rinses against malodour and VSC concentrations. By testing the active formulations against a negative control (water), information could be gained as to the efficacy of test compounds in terms of their immediate (within 30 minutes) and intermediate (3 hour) effects.

Materials and methods

Human subjects

Fourteen volunteers from the University of the West of England were selected from a database of volunteers previously screened for inclusion in malodour trials. The panel included 8 female and 6 male with a mean age at onset of 39 years (range 23 – 64). They were all healthy adults with no sign of oral disease.

Study design

The study was double-blind and neither judge, technician or panellists knew which product was administered for all test days. Test days were one week apart. Each subject was randomly assigned a label 1 to 14. The mouthwashes were assigned letters A to F. All products were dispensed into **15 ml** volumes by an independent technical member of staff. The volunteers rinsed for 2 minutes for each mouthwash. Each subject received all test products in random order thereby acting as their own control.

Eligibility criteria included informed consent and availability at the specified study intervals and sampling times plus a baseline organoleptic malodour score of >2 on each study morning. Exclusion criteria included; medical history of infectious diseases (e.g. hepatitis, HIV, tuberculosis); **obvious gingival inflammation, active or severe caries, gingivitis or advanced periodontitis and oral thrush**; antibiotic medication within 1 month prior to the start of the trial or during the trial period; consumption of medicated sweets containing antimicrobial agents; changes in oral hygiene practices during the trial; consumption of foods associated with oral malodour (such as garlic, spices or alcohol) on the day prior to, and on the sampling day; wearing of strongly perfumed cosmetics on the sampling day and substantial false dentition. On the evening prior to the test day volunteers were instructed to continue their normal oral hygiene habit but on the morning of their assessments they were asked to avoid oral hygiene (brushing their teeth) and food intake.

All participants were provided with their individual protocol, a diary and appointment dates/times for attending the laboratory. An adverse reaction form was available on request from the principal investigator. With the exception of the treatment mornings, subjects were not asked to alter their normal oral hygiene regime throughout the 6-week study.

Oral test rinses
Five oral rinses, all of which are commercially available, were compared along with water as the negative control; SB12®, Listerine®, BreathRX®, Smarth Mouth® and Lacer Fresh®. Table 1 lists the mouthrinses, the manufacturers and a summary of their ingredients (and amounts) as far as this information is available.

Ethics and study conduct
The protocol and informed consent form were approved by the local Ethics Committee. The study was conducted in a manner consistent with the ethics encompassed within the 'Declaration of Helsinki'.

Organoleptic assessment
One trained odour judge scored breath odour levels using the 0 to 5 organoleptic scale as outlined by Rosenberg *et al*., (1991) and modified in term of odour descriptives by Greenman *et al*. (2004), 0 = no odour, 1 = barely noticeable, 2 = slight odour, 3 = moderate odour, 4 = strong odour, 5 = very strong odour (saturation).

Instrumental analysis:
Measurements with Halimeter and OralChroma were taken according to the manufacturer's instructions. Two halimeter readings were taken and the calculated average was recorded as ppb. OralChroma readings were taken using a 1ml gas sample from a "2-minute" closed mouth via plastic syringe. **H_2S was obtained by measurements of area-under-curve (AUC) of the separated chromatographic peaks from the output trace. However, due to the 10 minute time period required for running samples, only one sample per person per time point was taken.**

Trial procedures
On the test day, volunteers reported to the breath odour judge who carried out a baseline breath assessment. Two assessments were taken within 5 minutes and an average value calculated for each time point. Following organoleptic assessment, the laboratory technician undertook baseline halimeter and OralChroma readings. The volunteers where then given **15 ml** of one of six test mouthrinses, in a randomised and double-blind manner and instructed to rinse the mouth for two-minutes. The breath assessments and instrumental readings were repeated at 30, 60, 90 and 180 minutes following test or control "treatment". The volunteers were not allowed to eat or drink between sampling.

Statistical analysis
***O*rganoleptic scores, VSC concentrations (by halimetry) and H_2S (by OralChroma) were taken at baseline, 30, 60, 90 and 3 hours per person, per treatment. GraphPad Prism (GraphPad Software, San Diego, Ca, USA) was used to log transform, plot (as change in readings from time zero) and statistically analyse the data using ANOVA plus Bonferroni's multiple comparison post test. Correlation tests were performed using**

Excel Microsoft and goodness of fit expressed as the coefficient of determination (r^2).

Results

Table 2 shows the range and overall average readings for the pre-treatment (baseline) conditions for the three measured parameters. Since this involved readings from 14 individual trialists on 6 different occasions (5 treatments and control) the total data points are n = 84. The mean and range are suitable for a designed study to show reductions in malodour parameters. Efficacy in terms of reduction in VSC compared to control substance F (water) as measured by the Halimeter (figure 1 and table 3) varied amongst the mouthrinse formulations, ranging from **no significant** effect with product D (LacerFresh®), moderate effect for products B (BreathRX®) and C (SmartMouth®) p≤0.01, to good **marked** with products E (SB12 ®) and A (Listerine®) p≤0.001.

Comparing the Halimeter™ readings between products, SB12 ® (p≤0.01) and Listerine® (p≤0.05) both showed statistical separation from LacerFresh at all time points **using ANOVA plus Bonferroni's Multiple Comparison post test**. The separation for SB12 was larger and was maintained throughout the 3 hour observation period.

Efficacy as measured by reduction in breath odour using the organoleptic scale showed water to have a very slight breath reduction at 30 minutes, but then odour levels increase to above the initial, time zero, pre-treatment level. All products separated statistically from water at all time points (range p ≤ 0.05 – 0.001). As can be seen in figure 2, product D (LacerFresh®) had the least benefit, products A (Listerine®), B (BreathRX®) and C (SmartMouth®) show more marked reductions whilst product E (SB12®) reduced breath odour levels to a measurably greater extent than all other products, and maintained the reduction up to 180 minutes.

SB12®, (figure 2, table 3), showed statistical separation from LacerFresh® at all time points (p<0.001), from SmartMouth® at 90 minutes (p≤0.05) and at 180 minutes (p<0.001), and from Listerine® and BreathRX® by 180 minutes (p<0.01). Listerine® showed statistical separation from LacerFresh® at all time points (p≤0.05). Smart Mouth® and BreathRX® separated from LacerFresh® from 60 minutes (p<0.001).

The organoleptic data support the Halimeter™ results with all products maintaining their positions of efficacy F<D<C<B<A<E. Figure 3 shows the results obtained for H_2S using the OralChroma™. **These data followed a similar profile of reduction and recovery over time as halimetry or organoleptic scores.** Relationships between organoleptic scores, Halimeter™, and OralChroma were between R^2 = 0.795 to 0.926 as seen in figures 4-6.

Discussion

Five oral rinses, SB12® (containing a low concentration of Zn and CHX), Listerine®, BreathRX®, SmartMouth® and Lacer Fresh®, all of which are commercially available, were compared along with water as the negative control. The odour-inhibiting capacity of the mouthwash formulations was determined using the organoleptic scale, the Halimeter and the OralChroma. Malodour levels of 14 orally healthy volunteers were assessed at baseline and at the same time periods during the day. The organoleptic assessment of individuals prior to and after treatment was performed by one trained odour judge in a completely double-blind manner. It is well accepted that humans have the capacity to determine the strength (i.e. concentration) of odour molecules. Models relating the organoleptic score to the occupancy of odour binding sites (degree of receptor saturation) have been proposed (Greenman *et al*., 2004; 2005). Judges can be trained to score the strength of odour (0 to 5) and it is clear that to have a useful meaning, a zero score must relate to no detectable odour and a 5 must be as strong as it gets. When subjected to pure odour compounds of known concentrations, judges are able to discriminate and respond in a dose dependant manner even when the order of concentrations is randomised. Moreover, the judges can repeat their measurements at a later date and be shown to give similar (reproducible) responses. Another useful method to validate the organoleptic judge is to see how their scores compare with other objective measurements of the same or similar samples, using an instrumental gas sensor (e.g. Halimeter) or GC.

In this study, it was important to see whether any correlations between sensory and instrumental measurements existed so that one type of measure could be used to validate the other. Although some reports have shown a relationship between organoleptic score and either halimeter or GC (Rosenberg *et al*., 1991; Doran *et al*., 2007; Winkel and Tangerman 2005; Van del Velde *et al*., 2009), no relationships between all three methods have been reported. In the current work, it was noticed that whether an inhibitory effect from an active mouthwash was calculated as a change in malodour value from time zero or as an absolute measurement at each time point, the correlations between the three methods of breath measurement were high. This finding implies that all methods are equally capable of assessing oral malodour and that any method on its own might also be sufficient.

The inhibitory effects on H_2S and oral malodour can be descrbied as follows: slight effect (Lacer Fresh®), a moderate effect (BreathRX®, SmartMouth®) and a marked effect (Listerine®, SB12®). However, in comparison with a clinically proven mouthwash such as Listerine (Pitts *et al*., 1983), SB12 was shown to be numerically and at some time points, statistically, superior.

Chlorhexidine, a cationic bis-biguanide with low mammalian toxicity and broad spectrum activity against Gram-negative and Gram-positive bacteria (Denton, 1991) has been used for *in vitro* and *in vivo* studies (Kinniment *et al*., 1996: Jones, 1997). The cationic properties of CHX explain how its electrostatic attraction by the anionic bacterial surfaces may lead to membrane disruption, increased permeability and cell death and as a result, to a reduction in bacterial load (Jones, 1997; Kuyyakanond, 1992, Quirynen and Zhao, 2002)

and malodour. CHX is also known for its high substantivity to buccal surfaces and has been shown to reduce gingival inflammation and dental plaque (Cummins and Creeth, 1992; Addy and Moran, 1997; Bollen and Quirynen, 1996). The strong antimicrobial action and increased substantivity in the mouth of CHX justify its use for malodour reduction (Bosy *et al*, 1994; De Bover *et al*., 1996). More recently, CHX has been used in association with other anti-malodour agents such as CPC and Zn and the efficacy of this combination was shown to be more effective than CHX alone (Quirynen *et al*., 2002; Roldan *et al*., 2000) suggesting a more synergistic effect by CHX when present with other active compounds.

The efficacy of CHX against microbes has been shown to be both dose and time-dependant (Quirynen **and Zhao**, 2002) and different product formulations may use CHX at different concentrations which might explain the variability of side effects such as discolouration of the oral mucosa and teeth as well as an alteration of taste (Bossy *et al*., 1994; Flötra *et al*., 1971; Quirynen **and Zhao**, 2002).

From the 1970's onwards, zinc has been extensively studied either on its own or in association with other compounds used to control oral malodour, (Tonzetich, 1977; Schmidt and Tarbet, 1978; Wåler, **1978**; Young *et al*., 2001). In addition to its antimicrobial properties, zinc is relatively non-toxic, non-cumulative and gives no visible coloration (Quirynen *et al*., 2002). It is believed that zinc binds to the membrane of microorganisms, interfering with, and reducing cell growth rate (Sugman, 1983; Radke *et al*., 1994). It has also been suggested that zinc reacts with VSC by forming an insoluble complex (ZnS) which is non-volatile and thus non-odiferous (Boulware *et al*., 1985).

In previous clinical trials using mouthwashes containing zinc, volunteers have reported an unpleasant metallic taste **(Young *et al*., 2003).** It has also been shown that low concentrations of zinc alone do not produce an unpleasant taste but are not very effective against oral malodour. Likewise, CHX at high concentrations produces taste effects as well as staining. Young *et al* showed that low concentrations of CHX still maintained an effect over time. It could be that a low concentration of CHX may reduce the staining of the teeth without loosing all of its anti malodour properties. **A synergistic effect between low zinc and low CHX, previously observed by others (Thrane *et al*., 2007; Young *et al*., 2003) may reduce oral malodour and decrease the above mentioned side-effects. It is likely that zinc and CHX have different high-affinity binding sites within the cell, and that occupation of one type of site makes the cell more sensitive to the inhibitory or cidal effects of the other type of ligand.**

In conclusion, a combination of CHX and Zn in low concentration, such as SB12, was shown to reduce significantly (for up to 3 hours) H_2S **in the oral cavity** which is considered to be the main contributor to oral malodour.

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Table 1: Mouthrinse, ingredients and code

Code	Mouthrinse	Ingredients
A	Listerine antibacterial Mouthwash-Total Care® **Pfizer Consumer Healthcare**	Aqua, Alcohol, Sorbitol, Aroma, Poloxamer 407, Benzoic Acid, Eucalyptol, Methyl salicylate, Thymol, Menthol, Sodium Fluoride, Zinc Chloride, Sucralose, Sodium saccharin, Sodium benzoate, Benzyl alcohol, Cl 16035, Cl 42090. Contains Sodium fluoride (0.022% w/v 100ppm F).
B	BreathRX® **Discus Dent Inc USA**	Aqua, Sorbitol, Propylene glycol, PEG-40, hydrogenated Castor oil, Polaxamer 407, Xylitol, Aroma (Mint, thymol and Eucalyptus oil), Zinc gluconate, Cocamidopropyl Betaine, Cetylpyridinium Chloride, Sodium Saccharin, Citric Acid, Cl 42090.
C	SmartMouth Wash® **Triumph Pharmaceutical Inc US**	Solution 1: Purified water, Sodium Benzoate, Benzoic Acid, and Sodium Chlorite. Solution 2: Purified water, glycerine, Polaxamer 407, Propylene Glycol, Benzoic acid, Flavour, Polaxamer 124, Sodium Benzoate, Sodium Chloride, Sodium Saccharin, Zinc Chloride, D&C Yellow No 10, and FD&C Blue No 1.
D	LacerFresh Mouthwash® **Lacer, S.A Sardenya Barcelona**	Triclosan 0.15%, Zinc Chloride 0.05%, Sodium Fluoride 0.05% (225ppm), Xylitol 1%
E	SB12® **Antula Healthcare Stockholm**	Zinc acetate (0.3%), chlorhexidine diacetate (0.025%), sodium fluoride (0.05%), mint/menthol flavour (in alcohol)
F	Control	Water

Table 2: Average, minimum and maximum values of baseline readings (pre-treatment measurements) for organoleptic scores, VSC and H_2S (from OralChroma) recorded for 14 trialists

Measurements	Average (± SD; n=84)	Minimum (± SD)	Maximum (± SD)
Organoleptic score	3.55 (0.23)	2.50 (0.00)	4.33 (0.25)
Halimeter readings	156 (49.21)	36.16 (3.65)	379.50 (86.00)
H_2S OralChroma	416.40 (213)	50.75 (27.80)	544 (61.09)

Table 3: Summary of ANOVA plus Bonferroni statistical data

Products	Time	P values Halimeter	P values Organoleptic
A and B	All Time Points	-	-
A and C	All Time Points	-	-
A and D	30 60 90 180	**P < 0.05** **P < 0.01** **P < 0.01** **P < 0.05**	**P < 0.05** **P < 0.001** **P < 0.001** **P < 0.001**
A and E	30 60 90 180	- - - -	**-** **-** **-** **P < 0.01**
A and F	30 60 90 180	**P < 0.001** **P < 0.001** **P < 0.001** **P < 0.001**	**P < 0.001** **P < 0.001** **P < 0.001** **P < 0.001**
B and C	All Time Points	-	-
B and D	30 60 90 180	- - - -	**-** **P < 0.001** **P < 0.001** **P < 0.001**
B and E	30 60 90 180	- - - -	**-** **-** **-** **P < 0.01**
B and F	30 60 90 180	**P < 0.01** **P < 0.01** **P < 0.01** **P < 0.01**	**P < 0.001** **P < 0.001** **P < 0.001** **P < 0.001**
C and D	30 60 90 180	**-** **-** **-** **-**	**-** **P < 0.001** **P < 0.001** **P < 0.001**
C and E	30 60 90 180	- - - -	**-** **-** **P < 0.05** **P < 0.01**
C and F	30 60 90 180	**P < 0.01** **P < 0.01** **P < 0.01** **P < 0.01**	**P < 0.001** **P < 0.001** **P < 0.001** **P < 0.001**
D and E	30 60 90 180	**P < 0.01** **P < 0.001** **P < 0.001** **P < 0.001**	**P < 0.001** **P < 0.001** **P < 0.001** **P < 0.001**
D and F	30 60 90 180	**-** **-** **-** **-**	**P < 0.001** **P < 0.001** **P < 0.05** **-**
E and F	30 60 90 180	**P < 0.001** **P < 0.001** **P < 0.001** **P < 0.001**	**P < 0.001** **P < 0.001** **P < 0.001** **P < 0.001**

(-) Not Significant

A=Listerine; B=BreathRX; C=SmartMouth; D=Lacerfresh; E=SB12; F=Water

List of figures

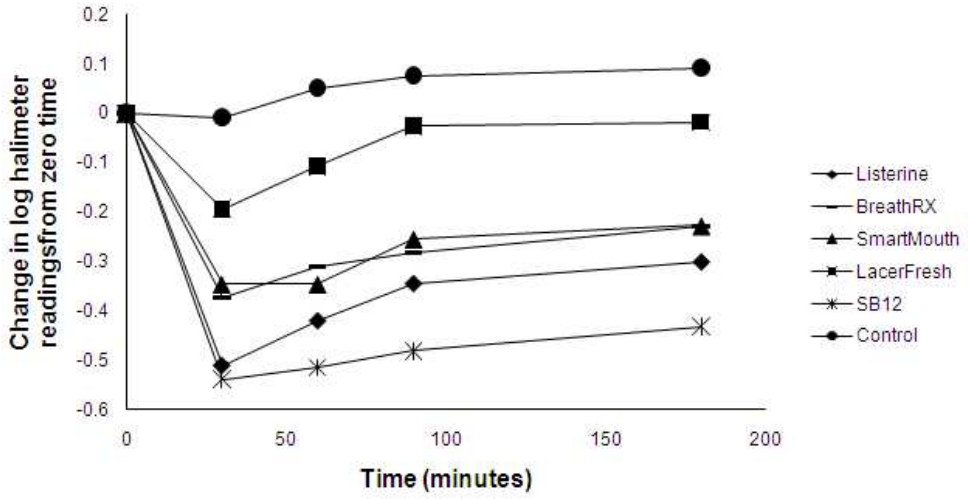

Figure 1: Log10 halimeter changes for 5 products plus control
170x101mm (96 x 96 DPI)

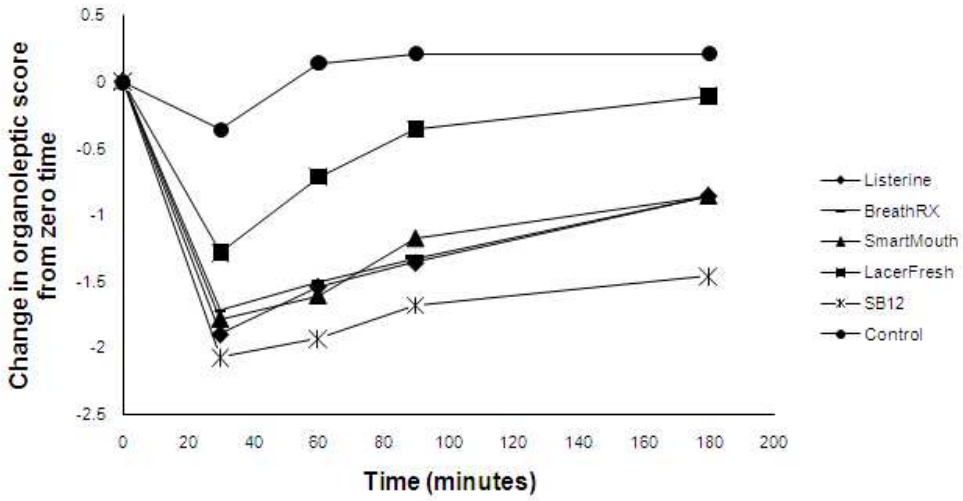

Figure 2: Organoleptic changes for 5 products plus control
169x101mm (96 x 96 DPI)

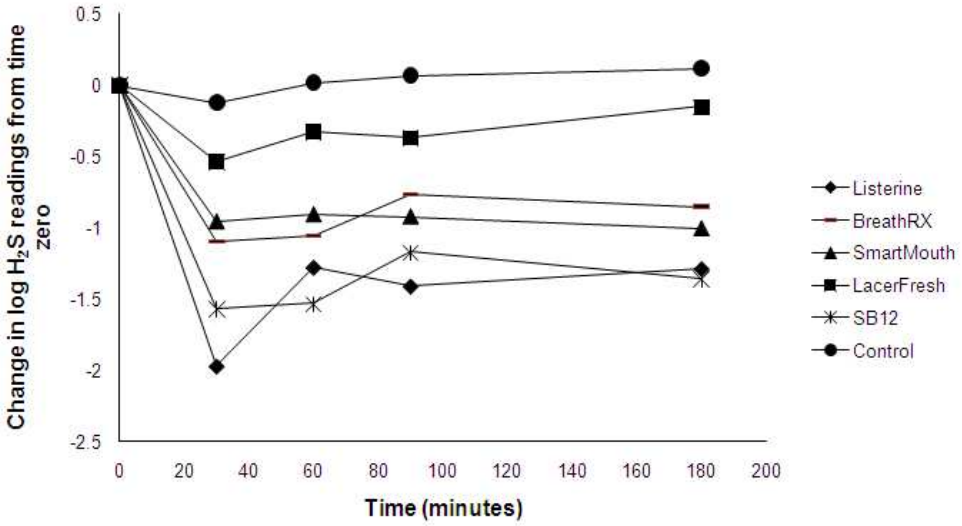

Figure 3: Log10 Hydrogen sulphide changes for 5 products plus control
185x102mm (96 x 96 DPI)

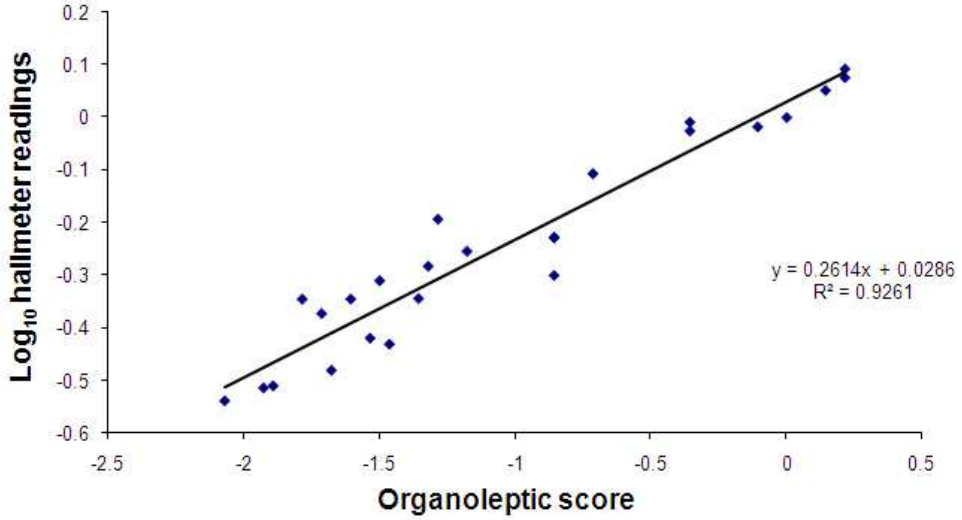

Figure 4: Correlation between organoleptic score and Log10 halimeter readings
177x103mm (96 x 96 DPI)

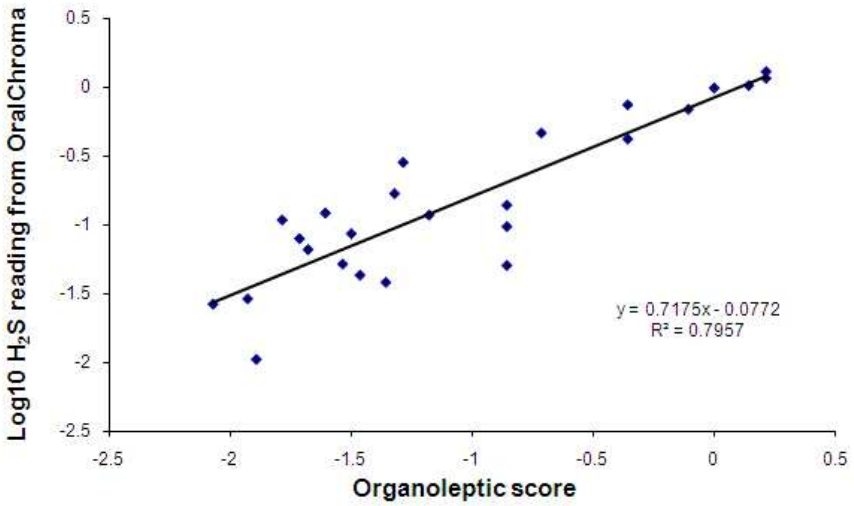

Figure 5: Correlation between organoleptic score and Log10 H2S readings from Oral ChromaTM
177x102mm (96 x 96 DPI)

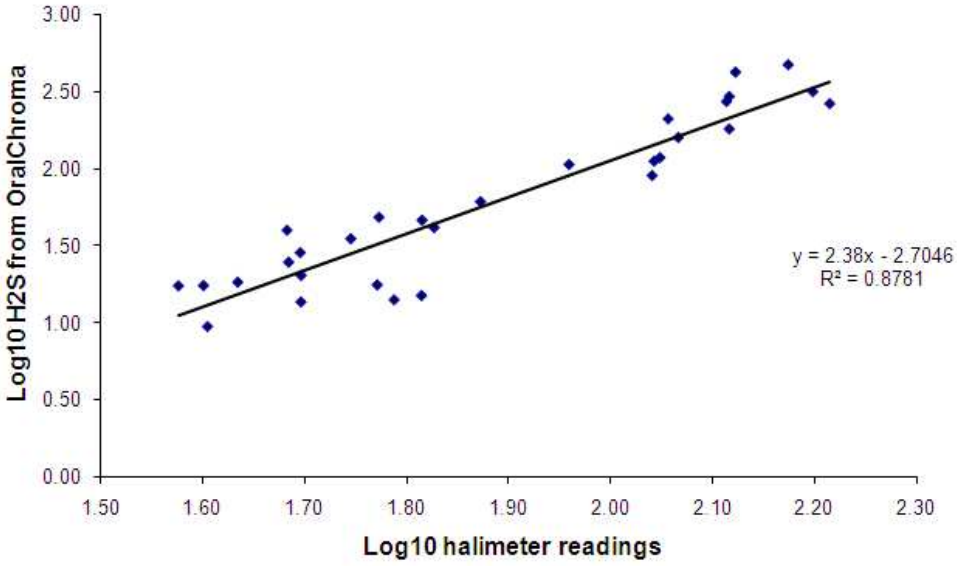

Figure 6: Correlation between Log10 Halimeter readings and Log10 H2S from Oral ChromaTM

170x111mm (96 x 96 DPI)