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Volatile sulphur compounds in morning breath of human volunteers

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ABSTRACT

Objective: Morning breath contains elevated concentrations of volatile sulphur components (VSCs). Therefore, morning breath is recognised as a surrogate target for interventions on breath quality. Nevertheless, factors influencing morning breath are poorly understood. Our aim was to evaluate concentrations of VSC at the time of awakening.

Methods: A procedure was developed to collect breath samples at home. Intra- and inter-person variations were determined in two small studies based on measurements of hydrogen sulphide, methyl mercaptan and dimethyl sulphide in healthy volunteers.

Results: Highest levels of VSC were found directly after waking up, followed by a significant decline afterward. Considerable day-to-day variation was found, but could not be linked to dietary intake. A significantly higher concentration of H₂S and CH₃SH was observed in the group of female subjects compared to males.

Conclusions: When morning breath is used as a target for interventions, breath collected at the time of or shortly after waking up is preferred over breath collected later during the morning. Gender plays an important role in VSC levels, and should be taken into account.

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Breath malodor or halitosis is an important negative factor in social communication and therefore a problem to many individuals. Although most adults suffer from breath malodor only occasionally, an estimated 10–30% has problems more regularly.^{1–3} A good correlation between organoleptic measurements and monitoring of volatile sulphur compounds (VSCs) was found in studies where breath malodor was evaluated by both techniques (reviewed by [1]). Therefore, VSCs are considered to be the most important contributors to breath malodor, and thereby provide a suitable biomarker for breath quality evaluation.⁴

The tongue is the major site for production of VSCs.^{3,5,6} VSC production on the tongue is dependent on microbial

activity by mostly Gram-negative proteolytic bacteria. This activity results in degradation of organic substrates from either endogenous or dietary origin. Degradation of the sulphur-containing amino acids cysteine and methionine greatly contributes to the production of VSCs, generating the end-products hydrogen sulphide (H₂S), methyl mercaptan (CH₃SH) and dimethyl sulphide ((CH₃)₂S), which are renowned contributors to the malodor perceived.^{2,4,7,8} Most breath clinics make use of the Halimeter to measure the VSCs and to detect oral malodor. However, the Halimeter cannot differentiate between the three VSCs. Moreover, it is more sensitive to H₂S than to CH₃SH and it is almost insensitive to (CH₃)₂S.⁹ The OralChroma™ forms a good

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alternative that enables differentiation between the three major VSCs.¹⁰

Determination of VSCs in morning breath is generally recognised as a surrogate measure to evaluate the efficacy of therapeutic interventions on breath malodor. Several studies have examined the impact of various treatments on the quality of morning breath.^{11–13} Nevertheless, to the best of our knowledge, systematic studies describing the VSC levels in morning breath in comparison to other times of day are limited to one study with 8 healthy adults.¹⁴ Except for this study, morning breath has been determined not directly after awakening but by inviting subjects at a fixed time point in the morning to the laboratory.

We hypothesised that VSCs were the highest directly after awakening. To test this hypothesis, a method was applied to collect breath samples immediately after awakening for later analysis in the laboratory. This method was used in a small study to evaluate morning breath composition at different key moments during the morning. In addition, we were interested to determine whether variation in morning breath after awakening could be correlated with factors such as age, gender, or diet. Therefore, this newly developed method was applied in a study in which morning breath was determined in 50 volunteers on three subsequent days.

1. Materials and methods

1.1. Breath composition during the morning (Study I)

The study was approved by the Medical Ethical Committee of Wageningen University. Twelve subjects of mixed gender were recruited through the staff of NIZO Food Research on the basis of their willingness to participate only. They were instructed to collect three morning breath samples: (1) directly after waking up while still in bed, (2) shortly before breakfast, and (3) directly after breakfast. This gives insight in the effect of light physical activity and food consumption. Morning breath samples were collected according to a standard procedure, and all brought to the laboratory before 9 a.m. In short, all volunteers were provided with three 10 ml type Injekt syringes (B. Braun Medical BV, Oss, The Netherlands) since these syringes gave a good recovery of VSCs.¹⁵ Subjects were carefully instructed to place the syringe between their closed lips and maintain the syringe in position for 1 min. During that time, they could breathe normally through their nose. After exactly 1 min, using the syringe-plunger, they collected 10 ml air from their oral cavity and locked the syringe as fast as possible with a 3-way combi-lock (Codan BV, Deventer, The Netherlands) to allow analysis of the sample without directly opening the syringe. They were further instructed to restrain from oral hygiene procedures like tooth-brushing until they had collected all three samples. There were no restrictions with respect to diet, neither on the days before sampling or during breakfast. Samples were brought to the research facility and analysed within 3 h.

1.2. Breath composition at awakening (Study II)

The study protocol was approved by the Medical Ethical Committee of Wageningen University. Twenty-five male

and twenty-five female volunteers were recruited from the staff of NIZO Food Research, and their families, friends and neighbours. Inclusion was based on a short questionnaire in which they indicated to be without dentures, braces, piercings in the oral cavity. In addition, they stated that they were not under treatment of oral diseases or disorders such as periodontitis, and had no history of major surgery in the oral cavity. Volunteers were not using mouth-rinse solutions, antibiotics, probiotic products, or medication which may interfere with breath quality. All gave their informed consent.

The volunteers were instructed not to use tongue scrapers, and restrain from yoghurt products or garlic-containing foods during the entire study period. For a period of the three consecutive days of the study, they were asked to register their consumed food products in a diary, followed by collection of a morning breath sample the next morning. Directly after waking up in the morning, while still in bed, they were asked to collect morning breath according to a standard procedure as described in Study I.

1.3. Sample analysis

All breath samples from Study I and Study II were analysed for three volatile sulphur compounds, hydrogen sulphide, methyl mercaptan and dimethyl sulphide, using a portable gas chromatography device (OralChroma™, Abilit Corporation, Japan) as indicated by the manufacturer. A 1 ml syringe was connected to the 3-way combi-lock associated with the syringe containing the breath sample. One ml of the breath sample was transferred to the syringe and thereafter directly injected into the gas chromatograph. One ml was used instead of a halve ml as recommended by the manufacturer, because of a higher sensitivity.¹⁰ After 8 min, the chromatogram was checked. Data were calculated from the display values since a double amount of gas was used. These display values were subsequently corrected by comparison with values obtained by injected standards, since the OralChroma™ cannot be calibrated manually.¹⁰ Detection limits were 4 ppm for each of the three VSCs.¹⁰

1.4. Data analysis

Data from samples of Study I were averaged, and time points were compared. For Study II, the average VSC concentration of each subject was taken for further analysis. The parameters gender and age were analysed for their influence on breath composition at awakening. For many other parameters (e.g. smoking or intake of specific dietary components) the number of subjects within the study population was not sufficient to draw solid conclusions. Data were analysed using either the Student's t-test, or the Mann–Whitney *U* test if parameters were not normally distributed, using the Statistica™ software package. *P*-values < 0.05 were considered as statistically significant. Pearson product moment correlation coefficients were determined using the statistical software package Statistica™.

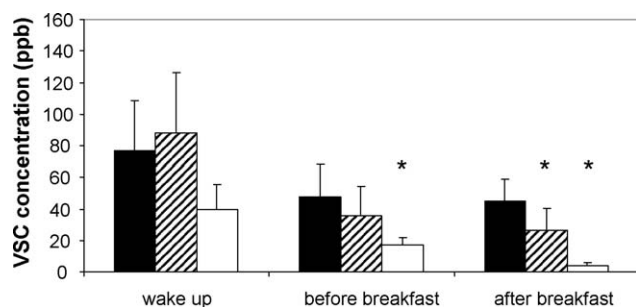


Fig. 1 – VSCs in breath samples of volunteers ($n = 12$) collected at three key moments during the morning (Study I). H₂S (black bars), CH₃SH (dashed bars) and (CH₃)₂S (white bars) were analysed. Average values \pm standard error of the mean are presented. * Value is statistically significant from value at time of waking up (Student's t-test, two-sided, $p < 0.01$).

2. Results

2.1. Volatiles in breath samples during the morning (Study I)

Samples from twelve volunteers in a pilot study at 3 different times during the morning were collected. Although a time schedule for sample collection was not provided, volunteers were instructed to hand in samples before 9 a.m. Therefore, we estimate that samples were collected in a time span shorter than 1 h. Analysis of these 36 samples revealed detectable levels of at least one VSC in all but one sample. The highest concentrations of VSCs were found directly after awakening (Fig. 1). A subsequent decline in VSC concentrations over time was found for all VSC levels evaluated, with the most drastic reduction observed for (CH₃)₂S followed by CH₃SH. The differences between samples that were collected before and after breakfast were not statistically significant.

2.2. Baseline characteristics for morning breath at awakening (Study II)

The twenty-five male and twenty-five female subjects of the study of morning breath at awakening were 40 ± 7 and 40 ± 9 years (average \pm standard deviation) for female and male subjects, respectively. Females ranged from 28 to 55 years of

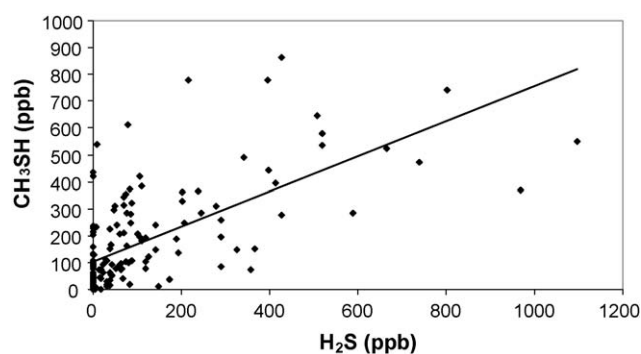


Fig. 2 – Correlation between H₂S and CH₃SH in samples from breath at awakening (Study II; $n = 150$). Pearson correlation coefficient = 0.65 ($p < 0.001$).

age (median 41 years), and men ranged from 27 to 52 years of age (median 41 years). All volunteers collected morning breath samples on three consecutive days and reported the food products they consumed during the day. Smoking was reported by 1 male and 2 female participants, use of coffee during the study by 18 male and 19 female participants, and use of alcohol during the study by 12 male and 7 female participants. A total of 150 samples were analysed for H₂S, CH₃SH and (CH₃)₂S. Methyl mercaptan was found in almost 90% of the samples whereas hydrogen sulphide and dimethyl sulphide were only present in two-thirds of all samples (Table 1). Nine samples (6%) were free of detectable levels of all three VSCs. Positive correlations were found between the sampling days, especially when comparing sampling days 2 and 3. Significant Pearson correlation coefficients for H₂S were 0.33 (day 1 versus day 3; $p < 0.05$) and 0.56 (day 2 versus day 3; $p < 0.001$). For CH₃SH, correlation coefficient was 0.67 (day 2 versus day 3; $p < 0.001$).

A positive correlation (correlation coefficient = 0.65) was also found between H₂S and CH₃SH concentrations (Fig. 2). No such correlation was found between (CH₃)₂S and either H₂S or CH₃SH.

2.3. Correlations with gender and age

The concentrations of VSCs in morning breath were compared for male and female subjects. A significantly higher concentration of H₂S and CH₃SH was observed in the group of female subjects (Fig. 3). No correlation with age was found within the

Table 1 – Basic parameters in the baseline study.

	H ₂ S	CH ₃ SH	(CH ₃) ₂ S
Total number of analyses	150	150	150
Number of positive samples (%)	97 (65%)	133 (89%)	95 (63%)
Concentration mean \pm SD (ppb)	115 \pm 192	178 \pm 193	35 \pm 45
Concentration median (ppb)	39	102	20
Low-high range (ppb)	0–1097	0–863	0–217
Subjects positive at 0 occasions	7/50	1/50	4/50
Subjects positive at 1 occasions	11/50	2/50	11/50
Subjects positive at 2 occasions	10/50	10/50	21/50
Subjects positive at 3 occasions	22/50	37/50	14/50

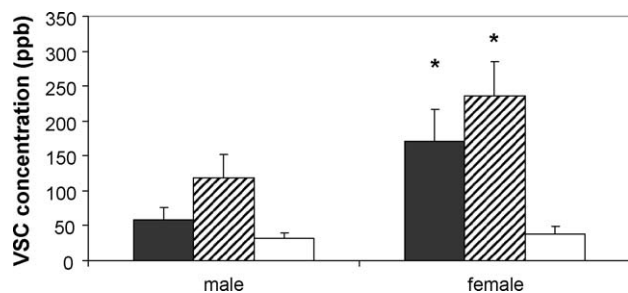


Fig. 3 – Volatile sulphur compounds in breath samples of male ($n = 25$) and female volunteers ($n = 25$) collected directly after wake up. H_2S (black bars), CH_3SH (dashed bars) and $(CH_3)_2S$ (white bars) were analysed. Average values \pm standard error of the mean are presented. * Values in females that are statistically different from values in men (Mann–Whitney U test, one-sided, $p < 0.05$).

age range of this study, even when gender was taken into account (Fig. 4). For many other parameters (e.g. intake of specific dietary components) the size of the study population was too limited to draw proper conclusions.

3. Discussion

The results of this study demonstrated the normal values for three of the most important VSCs in bad breath at the time of waking up. This was possible by using a method that allowed volunteers to collect breath samples at home. In addition, we used a fast analysis method that has demonstrated its reliability in breath analysis of both patients and healthy subjects.^{10,16,17} Several other studies have determined morning breath in samples collected in the laboratory,^{11–13} which makes it likely that subjects were active for a substantial period of the morning. By using our sampling technique, we have demonstrated that VSC concentrations rapidly decline following waking up and initiation of daily life routines. This is in line with the only other study where samples were analysed directly upon awakening.¹⁴ These authors concluded that VSC concentrations tended to decline appreciably in the first hour after subjects awoke.

Reported threshold values of objectionability of VSCs are 95, 12 and 24 ppb for H_2S , CH_3SH and $(CH_3)_2S$, respectively.⁴ The majority of the samples in our study were above these threshold levels, especially for CH_3SH , as can be deduced from the median values in Table 1. A similar observation was made for the odor recognition threshold. The median value for CH_3SH in the present study was almost 3 times higher compared to the 100% odor recognition threshold (35 ppb).⁴ It is therefore concluded that a majority of the samples that were collected directly after awakening contained noticeable odors. As inclusion of participants was based on a questionnaire but not on breath measurements during the day, the inclusion of subjects with halitosis cannot be fully excluded.

As earlier described, H_2S and CH_3SH are not present in nose breath.⁴ The positive correlation found between H_2S and CH_3SH means that both VSCs are often simultaneously elevated, e.g. as a result of bacterial degradation of both

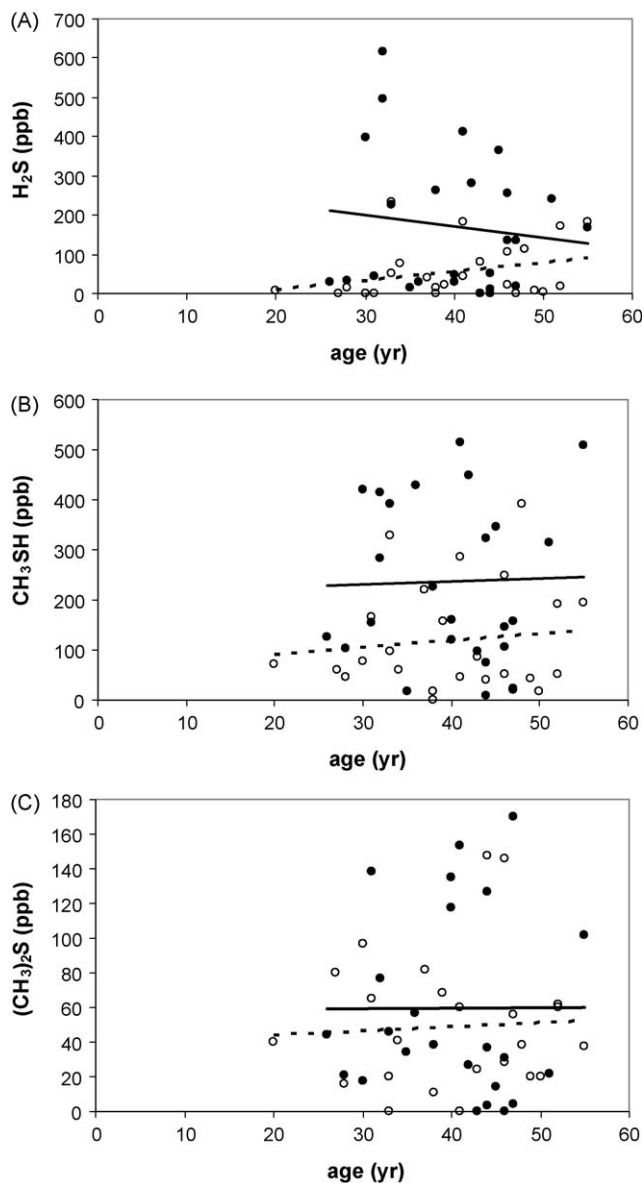


Fig. 4 – Correlations between age and VSC concentrations for H_2S (A), CH_3SH (B) and for $(CH_3)_2S$ (C) in female (●) and male (○). Trend lines are presented for males (dashed line) and females (solid line). Within the age group recruited, no correlation was found between age and VSC concentration.

cysteine and methionine within the oral cavity. No such correlation was found between these ‘oral’ VSCs and $(CH_3)_2S$. In patients with halitosis, the origin of $(CH_3)_2S$ lies outside the mouth, the majority coming from the blood.⁴ However, the much higher concentration of $(CH_3)_2S$ directly after waking up, compared to that after breakfast, implies that the condition on waking up differs from that found in halitosis patients during their visit to the breath clinic. Most of the $(CH_3)_2S$ on waking up probably also has its origin within the oral cavity (built up during sleep). This is in contrast to the situation after breakfast, when the origin of most of the remaining $(CH_3)_2S$ lies outside the mouth, in particular the blood.⁴

According to the manufacturer of the OralChroma™, one can speak of oral malodour when levels of H₂S or CH₃SH pass 112 and 26 ppb, respectively. For most volunteers, the levels for H₂S were lower than this threshold value whereas values for CH₃SH were in many cases substantially higher. As we have instructed volunteers to collect a sample after 1 min of closing their mouth instead of 30 s, to increase sensitivity,¹⁰ our values are not fully comparable to these thresholds. Nevertheless, we can conclude that a large part of the volunteers suffer from morning halitosis. Our first study demonstrates that this is only a transient phenomenon that in most volunteers disappears after breakfast, even without direct dental hygiene.

In the 3-day study presented here, the data for VSC collected at day 1 correlated significantly to days 2 and 3, but we observed the best correlation between days 2 and 3. Although chance finding could not be excluded, one explanation would be that learning is involved in the process of breath sample collection since this is not a habitual procedure, especially not as the first thing of the day. If this is indeed the case, a potential solution to reduce non-biological variation in future studies would be to include repeated sampling and ignore the first sampling point.

One of the surprising outcomes of the present study is the significantly higher VSC concentrations in the female population. Gender effects on VSCs are controversial, and higher concentrations in women as we describe may be limited to morning breath only. In a large group of 2672 Japanese adults sampled during the day, no significant differences were observed in the VSC between males and females in any age group,¹⁸ and were even better in female periodontal patients when analysed during the day.¹⁹ Nevertheless, a recent study described higher VSC concentrations in morning breath of women compared to men in line with our findings, and could link this to phases in the menstrual cycle.²⁰ We have no reasons to believe that differences in compliance are the cause of gender differences in VSC concentrations in morning breath. Moreover, if compliance were to play a role, it is expected that women, as being more conscious about their breath,²¹ would intentionally or unintentionally provide samples with lower, rather than higher VSC levels. Therefore, we consider the outcome as a true biological finding that is likely limited to morning breath.

The exact reason for gender differences in morning breath is not clear. Exogenous factors such as dietary intake are not likely to differ between the sexes, although the diaries for the group studied here were not conclusive. It is more plausible that endogenous factors play a role, e.g. salivary flow rate differences. Salivary flow rates are reduced during the night²² and may be the reason for higher VSC concentrations in morning breath, together with the numbers and metabolic activity of the oral microbiota. Besides, several studies show that salivary flow rates are higher in males.^{20,23–25} A recent study has focused on gender-related gene expression levels in the salivary glands²⁶ that potentially explain how gender modulates saliva production.

An additional aim of this study was to identify potential dietary components that may influence morning breath quality, by using food questionnaires. We did not find exogenous factors of importance, although food is known to

influence breath quality. Garlic preparations are the best known examples for their almost immediate effect on breath, although the volatiles mainly responsible for this are allyl mercaptan and allyl methyl sulphide.²⁷ These compounds were not determined in our assay since volunteers were asked to restrain from garlic. Reasons why we did not observe effects of diet are likely related to the power of the study and the exploratory approach: only 50 volunteers were included that reported a large variety of different food products they had consumed. The level of detail in these diaries is not sufficient to calculate nutrient intake, and food intake surveys are known for underreporting.²⁸ In addition, we could only focus on direct effects of food intake, whereas it cannot be excluded that VSC are influenced by nutrient intake on the long term.

In conclusion, we present a practical method for collecting morning breath samples and have demonstrated that VSC levels were elevated in such samples compared to samples taken at later time points. This method is promising for use in clinical intervention studies aiming to modulate the oral microbiota by mouth-rinses, tongue cleaning, tooth-brushing or other antimicrobial strategies.

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Competing interests: None declared.

Ethical approval: Ethical approval for this study was given by the local ethics committee (Commissie Mensgebonden Onderzoek regio Wageningen).

REFERENCES

- van den Broek AM, Feenstra L, de Baat C. A review of the current literature on aetiology and measurement methods of halitosis. *J Dent* 2007;35(8):627–35.
- Tangerman A. Halitosis in medicine: a review. *Int Dent J* 2002;52(Suppl 3):201–6.
- Winkel EG. Halitosis control. In: Lindhe J, Karring T, Lang NP, editors. *Clinical periodontology and implant dentistry*. Blackwell Munksgaard; 2008. p. 1325–40.
- Tangerman A, Winkel EG. Intra- and extra-oral halitosis: finding of a new form of extra-oral blood-borne halitosis caused by dimethyl sulphide. *J Clin Periodontol* 2007;34(9):748–55.
- Waler SM. On the transformation of sulfur-containing amino acids and peptides to volatile sulfur compounds (VSC) in the human mouth. *Eur J Oral Sci* 1997;105(5 Pt 2):534–7.
- Allaker RP, Waite RD, Hickling J, North M, McNab R, Bosma MP, et al. Topographic distribution of bacteria associated with oral malodour on the tongue. *Arch Oral Biol* 2008;53(Suppl 1):S8–12.
- Schmidt NF, Missan SR, Tarbet WJ. The correlation between organoleptic mouth-odor ratings and levels of volatile sulfur compounds. *Oral Surg Oral Med Oral Pathol* 1978;45(4):560–7.

8. Tonzetich J. Production and origin of oral malodor: a review of mechanisms and methods of analysis. *J Periodontol* 1977;**48**(1):13–20.
9. Rosenberg M, Kulkarni GV, Bosy A, McCulloch CA. Reproducibility and sensitivity of oral malodor measurements with a portable sulphide monitor. *J Dent Res* 1991;**70**(11):1436–40.
10. Tangerman A, Winkel EG. The portable gas chromatograph OralChroma: a method of choice to detect oral and extra-oral halitosis. *J Breath Res* 2008;**2**:017010.
11. Carvalho MD, Tabchoury CM, Cury JA, Toledo S, Nogueira-Filho GR. Impact of mouthrinses on morning bad breath in healthy subjects. *J Clin Periodontol* 2004;**31**(2):85–90.
12. Peruzzo DC, Jandiroba PF, Nogueira Filho Gda R. Use of 0.1% chlorine dioxide to inhibit the formation of morning volatile sulphur compounds (VSC). *Braz Oral Res* 2007;**21**(1):70–4.
13. Roldan S, Herrera D, Santa-Cruz I, O'Connor A, Gonzalez I, Sanz M. Comparative effects of different chlorhexidine mouth-rinse formulations on volatile sulphur compounds and salivary bacterial counts. *J Clin Periodontol* 2004;**31**(12):1128–34.
14. Suarez FL, Furne JK, Springfield J, Levitt MD. Morning breath odor: influence of treatments on sulfur gases. *J Dent Res* 2000;**79**(10):1773–7.
15. Winkel EG, Tangerman A. Appropriate sample bags and syringes for preserving breath samples in breath odour research: a technical note. *J Breath Res* 2008;**2**:017011.
16. Tsai CC, Chou HH, Wu TL, Yang YH, Ho KY, Wu YM, et al. The levels of volatile sulfur compounds in mouth air from patients with chronic periodontitis. *J Periodontol Res* 2008;**43**(2):186–93.
17. van den Velde S, Quirynen M, van Hee P, van Steenberghe D. Halitosis associated volatiles in breath of healthy subjects. *J Chromatogr B Analyt Technol Biomed Life Sci* 2007;**853**(1–2):54–61.
18. Miyazaki H, Sakao S, Katoh Y, Takehara T. Correlation between volatile sulphur compounds and certain oral health measurements in the general population. *J Periodontol* 1995;**66**(8):679–84.
19. Morita M, Wang HL. Relationship between sulcular sulfide level and oral malodor in subjects with periodontal disease. *J Periodontol* 2001;**72**(1):79–84.
20. Calil CM, Lima PO, Bernardes CF, Groppo FC, Bado F, Marcondes FK. Influence of gender and menstrual cycle on volatile sulphur compounds production. *Arch Oral Biol* 2008;**53**(12):1107–12.
21. Almas K, Al-Hawish A, Al-Khamis W. Oral hygiene practices, smoking habit, and self-perceived oral malodor among dental students. *J Contemp Dent Pract* 2003;**4**(4):77–90.
22. Dawes C. Circadian rhythms in human salivary flow rate and composition. *J Physiol* 1972;**220**(3):529–45.
23. Tulunoglu O, Demirtas S, Tulunoglu I. Total antioxidant levels of saliva in children related to caries, age, and gender. *Int J Paediatr Dent* 2006;**16**(3):186–91.
24. Percival RS, Challacombe SJ, Marsh PD. Flow rates of resting whole and stimulated parotid saliva in relation to age and gender. *J Dent Res* 1994;**73**(8):1416–20.
25. Inoue H, Ono K, Masuda W, Morimoto Y, Tanaka T, Yokota M, et al. Gender difference in unstimulated whole saliva flow rate and salivary gland sizes. *Arch Oral Biol* 2006;**51**(12):1055–60.
26. Srivastava A, Wang J, Zhou H, Melvin JE, Wong DT. Age and gender related differences in human parotid gland gene expression. *Arch Oral Biol* 2008.
27. Rosen RT, Hiserodt RD, Fukuda EK, Ruiz RJ, Zhou Z, Lech J, et al. Determination of allicin, S-allylcysteine and volatile metabolites of garlic in breath, plasma or simulated gastric fluids. *J Nutr* 2001;**131**(3s):968S–71S.
28. Braam LA, Ocke MC, Bueno-de-Mesquita HB, Seidell JC. Determinants of obesity-related underreporting of energy intake. *Am J Epidemiol* 1998;**147**(11):1081–6.