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Short communication

Time profile of putrescine, cadaverine, indole and skatole in human saliva

M. Cooke[a,*], N. Leeves[b], C. White[c]

[a]*Centre for Chemical Sciences, Royal Holloway, University of London, Egham, Surrey TW20 OEX, UK*
[b]*26 Norfolk Chase, Warfield, Bracknell, Berkshire RG42 3XN, UK*
[c]*Procter & Gamble Technical Centres Limited, Rusham Park, Whitehall Lane, Egham, Surrey TW20 9NW, UK*

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Saliva; Putrescine; Cadaverine; Indole; Skatole; Time profile

Summary The concentrations of putrescine, cadaverine, indole and skatole were determined in the saliva of healthy human volunteers upon waking and at time points during the day. Putrescine was found to be the most abundant of the amines studied, followed by cadaverine then indole. Skatole could not be detected in the saliva samples at any time point. The amines were found in the highest concentrations immediately upon waking (mean concentrations (μg/ml): putrescine 33.0 ± 19.0, cadaverine 17.6 ± 16.7 and indole 0.4 ± 0.4) with a rapid decrease following consumption of breakfast and brushing their teeth (mean concentrations (μg/ml): putrescine 7.0 ± 6.4, cadaverine 3.1 ± 4.7 and indole 0.04 ± 0.09). Putrescine and cadaverine then increased in concentration during the day apart from a decrease post-lunch caused by increased salivary flow and mechanical cleaning due to mastication. An analytical method based on high performance liquid chromatography and fluorescence detection has been developed to quantify amines in human saliva. Sodium fluoride has been shown to be an effective inhibitor of amine formation in saliva at room temperature allowing samples to be collected and kept without requiring cold storage.

Bacterial putrefaction of saliva in the oral cavity consists of essentially two processes. The first is the breakdown of proteins and peptides, the second is the degradation of the resulting amino acids (e.g. L-arginine, L-ornithine, L-lysine and cysteine) by enzymes, into volatile compounds such as hydrogen sulphide, methyl mercaptan, indole, skatole, putrescine and cadaverine.

The majority of research work focuses on the formation of sulphur compounds, as these are associated with halitosis. The in vitro and in vivo work on these sulphur compounds is well summarised by Greenman,[1] with most research focusing on identifying and quantifying the compounds in the gaseous phase by headspace analysis (in vitro) or captured exhaled breath (in vivo).

The formation of amine compounds by putrefaction in the oral cavity is less well reported, presumably as Tonzetich et al.[2] reported their inability to increase the odour of saliva due to their lack of volatility, thus reducing their importance in malodour. However, Goldberg et al.[3] report a strong association between the amount of cadaverine in saliva and oral malodour. Putrescine has it's name derived from putrefying flesh and indole analogues are associated with bitterness by Goodman and Temussi.[4] Therefore the formation of amine compounds in saliva may also provide a change in taste and influence the perception of oral cleanliness.

*Corresponding author. Tel.: +44-1398-332103; fax: +44-1398-332103.
E-mail address: mcooke@moorymeadow.freeserve.co.uk (M. Cooke).

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The majority of the reported quantitative work on amines is based on in vitro studies using saliva that has been incubated for periods up to 48 h. In vivo quantitative work is limited to the studies by Goldberg et al.,[3] Gopalakrishna and Nagarajan[5] and Venza et al.[6] They report single time point values for putrescine and cadaverine. For compounds such as indole and skatole no in vivo quantification in saliva could be found. This study reports, we believe for the first time, the variation in putrescine, cadaverine, and indole concentrations in saliva from healthy subjects over a 10 h period.

Twelve subjects (nine males aged 18–45 years and three females aged 27–38 years) in good general and oral health (albeit that no plaque or gingivitis scores were recorded), provided saliva samples. They brushed with a commercial sodium fluoride based toothpaste twice a day for 7 days prior to and on the day of saliva sample collection. Saliva was provided by the subjects on 1 day only. The subjects also had a controlled diet the evening before sample collection and during the saliva collection day. This was an attempt to reduce the potential influence of dietary proteins and amino acids on the results.

Saliva samples (1 ml) were collected immediately on the subjects waking, after eating breakfast and brushing, 9.00 a.m., 11.00 a.m., 20 min post-lunch, 3.00 p.m. and 5.00 p.m. The unstimulated saliva was expectorated into a glass vial coated with 5 mg of NaF to inhibit further amine formation. The concentration of NaF in the saliva sample is 5 mg/ml.

Cadaverine and putrescine were analysed by high performance liquid chromatography following derivatisation. Saliva samples (100 μl) were mixed with di-sodium tetraborate buffer (200 μl, 0.025 M, pH = 9.5) and 100 μl of derivatising reagent (made from 50 mg *o*-phthaldialdehyde dissolved in 4.5 ml of ethanol, 0.5 ml of 0.1 M di-sodium tetraborate buffer (pH = 9.9) followed by 50 μl of ethanethiol and stored at −20 °C prior to use). The mixture was shaken for 90 s, 200 μl of ethyl acetate added, shaken for a further 60 s, and centrifuged (1000 g, 60 s). The upper ethyl acetate layer was injected (20 μl) onto the high performance liquid chromatography column. Separations were performed on a Waters high performance liquid chromatography system and a Model 470 fluorescence detector.

The column (15 cm × 4.5 mm i.d., Capital Analytical, Leeds, UK) contained a low activity octadecylsilane (5 μm), was eluted with 91% methanol/9% pH 7.0 Tris buffer (0.02 M Tris base, 0.02 M HCl) at a flow rate of 1.2 ml/min. Detection was done by fluorescence at $\lambda_{Ex} = 340$ nm, $\lambda_{Em} = 450$ nm. Typical retention times for putrescine were 8.00 min and 9.5 min for cadaverine. The retention times of standard putrescine and cadaverine *o*-phthaldialdehyde derivatives and spiked saliva samples were used to identify the amines.

Indole and skatole were analysed by gas chromatography–mass spectrometry following ethyl acetate extraction of the same saliva sample as that used for cadaverine and putrescine. The pH of 600 μl of saliva was raised to about 10 using KOH (2 M) and then mixed with 300 μl of ethyl acetate, shaken for 60 s and then centrifuged (1000 g, 60 s). The upper ethyl acetate layer was injected (10 μl) onto the gas chromatography column, separations were performed on a Hewlett-Packard gas chromatography–mass spectrometry system. The column (15 m × 0.25 mm i.d. × 0.25 μm d.f.) containing Carbowax 20 M was eluted with the following temperature programme: injector and detector temperatures: 200 °C, initial column temperature: 100 °C (5 min) then warmed up to 200 °C at 10 °C/min. Typical retention time for indole was 8.6 min and for skatole 9 min. Detection was performed by selective ion monitoring (117, 90 and 89 *m/z* for indole and 131, 132 and 77 *m/z* for skatole).

The use of NaF coated vials was shown to inhibit any further cadaverine and putrescine formation when stored at 25 °C for 48 h when compared to a non-NaF containing control as shown in Fig. 1. Suzuki[7] also reported an inhibitory effect by sodium fluoride for phenol and indole in saliva. The mechanism of inhibition is not clear from our study or from that of Suzuki.[7] Ekstrand et al.[8] suggest that fluoride can effect bacteria in several ways, for example a change in normal membrane function or effecting positively charged enzyme pathways.

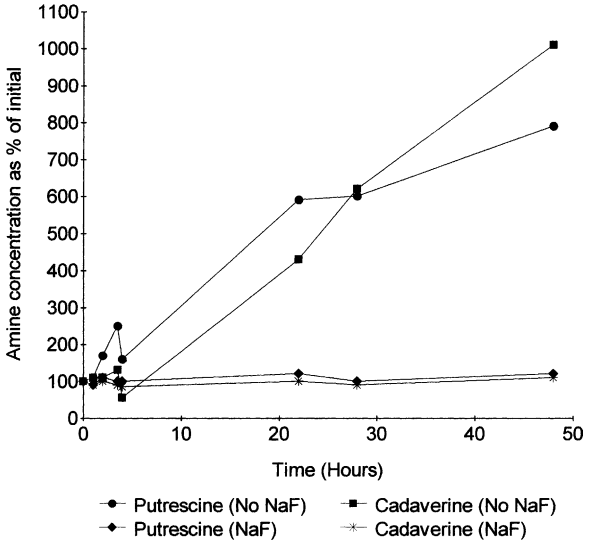

Figure 1 Comparison of putrescine and cadaverine levels in saliva, incubated in vitro, with and without NaF added, expressed as percentage of the initial concentration of amine in saliva.

This method provides a simple way to collect and ''freeze'' saliva samples, reducing potential analytical interference from the use of organic preservatives such as chlorhexidine gluconate or a time consuming cryogenic method. The presence of free amino acids in saliva samples also makes the use of cryogenics a potential source of error as they may degrade to amines during the sample warming up phase.

Although the analytical method used was essentially that used in many previous reports (separation by isocratic high performance liquid chromatography, detection by fluorescence of the derivatised amines), we have made several modifications to improve both analytical performance and method reliability. The optimum pH for separation is 7. Three buffers can supply this, they are phosphate, acetate and Tris. Previously, it has been suggested that phosphate is the best choice because it gives a greater fluorescence yield than acetate for a given amine derivative. We have measured the fluorescence yield for equimolar amounts of putrescine and cadaverine in phosphate and acetate buffers and find no significant difference in fluorescence yield. In Tris buffer the fluorescence yield is reduced by around 10% with consequent minor loss of sensitivity and limit of detection of the method.

However the concentrations in saliva are sufficient to permit the use of Tris. We therefore rejected the use of phosphate buffer because it presents no analytical advantage and is aggressive to the high performance liquid chromatography equipment. Initially we used acetate buffer but it tends to grow biofilms readily at ambient laboratory temperature ($20 \pm 2\ ^{\circ}C$) which block the solvent filters, hence Tris was selected. We have also measured the fluorescence maxima of the diamines of ethylenediamine, diaminopropane, putrescine, cadaverine and diaminohexane. All maximise at 450 ± 10 nm confirming that this is a suitable choice for multiderivative detection. Previously this method has used ethanethiol in the derivatisation process. This produces a derivative with an exposed hydroxyl group, which confers hydrophilicity and reduces retention, which in turn, limits the separations which may be developed, especially if multicomponent detection is desired. By using ethanethiol a hydrophobic derivative results giving increased retention for a given solvent ratio. Being a less polar derivative improved peak shape also results. Should additional retention be required then a longer alkyl side chain should be used. For example, use of butanethiol more than doubles the retention for a given solvent ratio. Use of gradient elution then reveals the presence of other minor components which produce a fluorescence response and are thus likely to be amines. Finally column selection is important for method performance. A low activity silica column (low residual metal content) combined with a relatively high carbon loading (>10%) and base deactivation chemistry produces the best results both in terms of chromatographic performance and column durability. We used methanol and not acetonitrile as eluant solvent because it made no difference to chromatographic performance and has lower mammalian toxicity.

The profile of the mean concentrations of the amines in saliva over the 10 h period is shown in Fig. 2. The standard deviations at all time points are high for all the amines. The profile for each

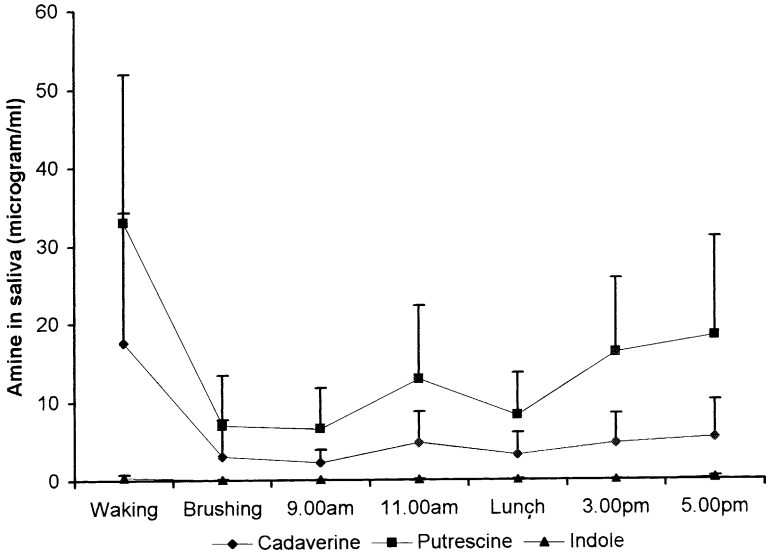

Figure 2 Mean concentration of cadaverine, putrescine and indole in saliva throughout the day.

Table 1 Comparison of mean concentration of putrescine and cadaverine in human saliva.

	Sample time	Putrescine (μg/ml)	Cadaverine (μg/ml)
This study	On waking	33.0 ± 19.0	17.6 ± 16.7
	Post-brushing	7.0 ± 6.4	3.1 ± 4.7
Goldberg et al.[3]	Not defined	9.6 ± 8.2	37.9 ± 31.4
Golpalakishna and Nagerajan[5]	Not defined	5.8 ± 3.1	n.d.
Venza et al.[6]	Not defined	0.026 ± 0.002	n.d.

n.d.: not determined.

individual subject is however consistent with the mean profiles. The amines are most abundant when the subjects first awake and then decrease rapidly after breakfast and brushing. There is then an increase in putrescine concentration during the day along with a slower increase in cadaverine, with both these amines there is a reduction post eating lunch. Indole levels are again highest on waking and then essentially absent for the rest of the time period. No skatole was detected in any saliva samples during this study. At all time points the mean concentrations of each amine follows the order putrescine > cadaverine > indole, although one subject did have a higher cadaverine concentration than putrescine on waking but then reverted to putrescine > cadaverine for the rest of the sampling period.

Our results are compared to those reported by other workers in Table 1. Our results are similar to those found by Goldberg et al.[3] and by Gopalakrishna and Nagarajan.[5] Both these studies, as in our study, show large variance in amine concentrations between subjects. The results presented by Venza et al.[6] for putrescine are several orders of magnitude lower in concentration than those reported in this paper. In his study, proteins and cellular elements are removed by centrifugation prior to the organic solvent extraction step, but in our study we extract with ethyl acetate and then centrifuge. It is likely that their method measures the free amines in saliva whereas our method determines both the free amines and the amines loosely bound with proteins and the cellular elements. Our results in combination with Venza et al.[6] indicate strongly that the vast majority of the diamines are associated in some way with components in saliva. It is not clear at this stage of the research which amine measurement, total or soluble, is the most biologically relevant. The low levels of free amines means it's likely that they play little part in any changes in taste perception during the day. The wide variation seen in the amine concentrations between subjects in our study is not unexpected for a complex biological system such as that in the oral cavity and is also in line with the wide variations seen by Suarez et al.[9] in their study of sulphur gases from the oral cavity. It is also noted that our time profile of amines in saliva are also consistent with the concentration of sulphur gases in breath over time. Tonzetich[10] demonstrated that oral parameters vary across the menstural cycle, this may contribute to variations in the amine levels between female subjects. In contrast Venza et al.[6] concluded that putrescine saliva concentrations are independent of sex or age. The post-lunch reduction in amine levels can also be explained by the cleaning action of ingesting food. The dominance of putrescine over cadaverine could result from many factors but is most likely to be explained by amino acid substrate availability. Putrescine can be formed by the decarboxylation of L-ornithine which is an intermediate in the degradation of L-argine, compared to cadaverine being formed by the decarboxylation of L-lysine. The literature values for the concentration of these amino acids found in saliva supports the predominance of putrescine with L-argine and L-ornithine reported at 15.8 and 37.4 μmol/l, respectively, by Gopalakrishna and Nagarajan,[5] compared to only 3.2 μmol/l reported for L-lysine by Dreyfus et al.[11] Interestingly, Goldberg et al.[3] reported, in contrast to our findings, that cadaverine is more abundant than putrescine in saliva. We can offer no explanation at this stage for the difference in the results other than it may be related to a different dietary intake during the sampling period.

The presence of indole in saliva and the absence of skatole is consistent with the work of Claus et al.[12] who found indole to be present in both saliva and tongue scrapping samples but only found skatole in tongue scrapings. He did not quantify any of the amines so we cannot make a comparison with our results.

The apparent high diamine concentrations in saliva may provide a source of amines to covalently bind to proteins, Yao et al.[13] employed dansyl cadaverine in place of a peptide lysyl donor and shown that it can be enzymatically coupled to protein glutamine to give a fluorescent dansylated protein derivative. The reaction of amines would change the 3D structure of the salivary proteins resulting in a change their rheology and lubrication

properties, with the amine concentrations being highest in the morning it is hypothesised that ''morning mouth'', often associated with high volatile sulphur levels, is also likely to be a tactile phenomenon.

The results of our study show that the amines putrescine, cadaverine and indole are found in their highest concentration in saliva upon waking, they are rapidly reduced by the combined action of eating breakfast and oral cleaning. The amines slowly increase in concentration during the day but can again be reduced by the mechanical action of chewing involved in the ingestion of lunch. No skatole was found in any of the saliva samples.

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