2-Nonenal-Ovulatory Specific Volatiles in Human Saliva throughout Menstrual Cycle by Gas Chromatography and Mass Spectrometry Analysis

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Abstract: Problem statement: The present investigation carry out a pilot study of a novel method to identify the salivary volatiles in different phases of menstrual cycle for the assessment of ovulation detection using gas chromatography and mass spectrophotometer. **Approach:** The profiles from follicular phase (6-12 days); ovulatory phase (13-14 days) and luteal phase (15-26 days) of menstrual cycle samples were compared to establish any qualitative and quantitative differences that might have potential value in human olfactory communication. Dichloromethane was used as the solvent for extraction of the compounds. **Results:** Fifteen compounds were identified. They include organic compounds like, acid, aldehyde, amine and alcohol. The most important constituent was 2-nonenal, which usually comprised 75% or more of the total volatiles observed in ovulatory phase. The concentration of many constituents varied widely. This appeared to be periodically in three cycles for five of the constituents, with a period of a few weeks and with pronounced maxima at the peak of ovulatory period of which only two were common to all the chromatograms. The chemical profile of ovulatory phase saliva was distinguished by the presence of two specific compounds, viz. 2-Nonenal, Acetic acid and Acetaldehyde that were not found in the other reproductive phases of saliva sample in women. Apparently these compounds are 2-nonenal, dodecanol, acetic acid and acetaldehyde. One or more of these compounds may have pheromonal activity in human body odor. **Conclusion:** Differentiation of the volatile patterns among reproductive phases in women may help to find the diagnostic marker for ovulation detection.

Key words: volatiles, ovulation, menstrual cycle, GC-MS

INTRODUCTION

Ovulation detection of the reproductive phase of a woman is an important decisive factor for applying fertility revealing in human, since the occurrence and duration of the fertile period in reproductive age women are strictly related to the time of ovulation. Based on the previous reports and importance of biochemical constituents in the saliva, the present study is aimed to detect a biomarker in the human saliva for predicting ovulation. Recent year's attention has been paid on the biochemical importance of saliva. Hormones used to measure in blood have now been estimated in saliva, though the quantities are comparatively less

(Braat *et al*., 1998). Hence, saliva is considered as the best non-invasive source for chemical and biochemical study (Freundl *et al*., 1996).

Saliva is a complex body fluid containing several different electrolytes including salts of sodium, potassium chloride and non-electrolyte components including several proteins, enzymes and immunoglobulins (Alagendran *et al*., 2007). There has for many years been a need to detect and diagnose the precise time of ovulation in a given female mammal. It can be of great importance, for example, to pinpoint the time of ovulation to insure that fertilization occurs. Alternatively, it may be important for other medical reasons to diagnose ovulation (Huggins and Preti,

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1976). There is a limited understanding of how the body produces the volatile organic compounds present in human odor. Most of the relevant scientific research pertaining to human scent has evaluated the contents of axillary (armpit) and plantar (foot) sweat. Compounds present in both male (Zeng *et al*., 1991) and female (Zeng *et al*., 1996) pH-adjusted axillary secretion extracts have been isolated and identified through preparative gas chromatography, analysis by gas chromatography/mass spectrometry (Preti and Leyden, 2010).

Human odor components have been studied through headspace GC-MS for compounds specific to age (Haze *et al*., 2001). Compound classes such as hydrocarbons, alcohols, acids, ketones and aldehydes were present in human odor. This study also presented 2-nonenal as a compound that is only present in the odor of individuals over 40 year of age. 2-Nonenal, as well as other aldehydes, was produced through oxidative degradation of monosaturated fatty acids, such as palmitoleic acid and vaccenic acid. Human body odor consists of various odor components. Studies examining the volatile components by various methods have detected the presence of alcohols, ketones, aldehydes, esters, ethers, hydrocarbons and other substances in body odor (Bernier *et al*., 2000). The key components of body odor present in the sweat, the axillary region, or foot has been determined and the mechanisms by which such odors are formed have been examined (Kanda *et al*., 1990; Zeng *et al*, 1991). It is well known that the lipids and their derivatives are involved in the olfactory communication. Here we investigated the changes in human saliva during the period of menstrual cycle found a specific component that is characteristic of the salivary volatiles.

Furthermore, the present study revealed that the change of the monosaturated fatty acid composition of skin surface lipids and the increase in the formation of this characteristic odor component.

MATERIALS AND METHODS

Collection of whole Saliva: Whole and stimulated parotid saliva samples were collected using sweet free chewing gum for saliva secretion and spitting through the use of a custom-made Lashley Cup. As a part of the collection procedure, subjects were required to undergo a full plaque indexing and oral examination (excluding periodontal probing). Only subjects with good oral hygiene were selected for the study. The salivary samples were collected from 50 different female volunteers having normal menstrual cycle during the age of 20-40 years. The samples were collected during

(follicular phase (6-12 day); Ovulatory phase (13-14 day); luteal phase (15-26 day) with help of the day of menses. The samples were screened through nylon mesh (16-120 µm) at the time of collection and were stored at-70°C for further use (Navazesh and Christensen, 1982).

Out of the twenty subjects, volunteered to contribute their stimulated parotid saliva samples. A drop of lemon juice was applied to the tongue of the subjects at 5-10 min intervals to stimulate parotid saliva secretion. Samples collected were stored at-70°C until further analysis.

Separations of samples: About 1 mL of saliva was collected from each individual and was isolated in the ratio chloroform and methanol (2:1) and centrifuged at 10,000 rpm for 10 min. The separated sample was used for extraction of salivary volatiles using Dichloromethane (1:1 ratio). The tubes were tightly closed and kept for 30 min at 100°C in a water bath. About 2 mL of methylation reagent was added to each tube and kept again in a water bath at 80°C for 20 min. The tubes were closed down to the ambient temperature. Extracting solvent (1.25 mL) was added to each tube, closed tightly and shaken thoroughly for 10 min. About 2/3 of the organic phase (upper layer) containing the fatty acid methyl esters were transferred into screw cap glass vials.

Gas chromatography: The supernatant was filtered through a silica-gel column (60-120 mesh) and concentrated under vacuum (at 30°C) for fractionation and chemical identification by (a) gas chromatography-mass spectrometry (GC-MS; QP-5000, Schimadzu, Japan). Two microlitres of extract was injected into the GC-MS system on a 30 m glass capillary column with a ¢lm thickness of 0.25 mm (30 m 60.2 mm i.d. coated with UCON HB 2000) using the following temperature programme: initial oven temperature of 408°C for 4 min, increasing to 250°C at 158°C/min and then held at 250°C for 10 min. GC-MS was run under computer control at 70 eV. Chemical ionization was performed using ammonia as the reagent gas at 95 eV. The solvent (dichloromethane) peak was seen at 2.4 min during the GC-MS analysis. (b) From each vial 1 µl of the Fatty Acid Methyl Ester (FAME) was injected into the Gas Chromatography (GC) Column (DEGS) using column temperature of 180°C and injection port temperature was 200°C. Flame Ionization Detector (FID) was used as a detector with 230°C. Nitrogen was used as reagent gas and then used as a sample for the GC-MS analysis (Kumar *et al*., 2000).

RESULTS

The analysis of human saliva using GC shows 15 different volatiles. Some of the volatile compounds such as carbonic acid, 1-methylcyclopentene and diphenylamine were present in follicular phase. Among these 2-Nonenal were found to be in higher concentration in ovulatory phase when compared to other phases. The two volatiles, carbonic acid and 1-methyl-cyclo pentane were elevated in preovulatory phase and these volatiles were comparatively less in phase. And also butanoic acid, 2-propenyl ester and n-tetradecane were found in both preovulatory and postovulatory phase while exclusively absent in the ovulatory phase (Table 1).
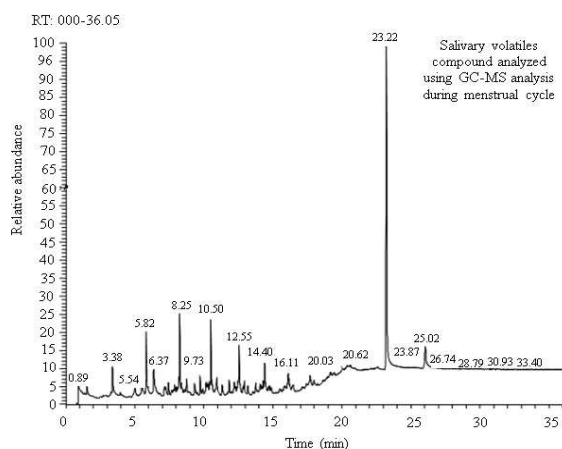
The reproductive phase of menstrual cycle was consistently cyclical changes in volatile components like diphenyl amine (RT value = 6.37) and phenol (RT value = 12.55) are set forth in Fig. 1c and 1g are remarkably high during follicular phase. Indeed, the relatively high concentrations of diphenylamine have been found at follicular and ovulatory phase, that sharp drops in that concentration have been found at the day prior to ovulation and that concentrations returned to higher levels at the time of ovulation. Concentration variations for over the period of one menstrual cycle to other showing steroids hormone dependent. Such compounds may be used in combinations with other indicators, to confirm the particular menstrual phase of a given subject. It is currently believed that the metabolic pathways which lead to the detection of a large dodecanol spike at the time of ovulation are also likely to cause the spiking of other relatively long-chain alcohols having in the range of 10-16 carbon atoms, particularly 11-14 carbon atoms. The concentration change for dodecanol, as indicated in Fig. 1 was remarkable in light of the fact that a sharp 4-fold change in concentration of this compound occurred precisely at the time of ovulation was indicated.
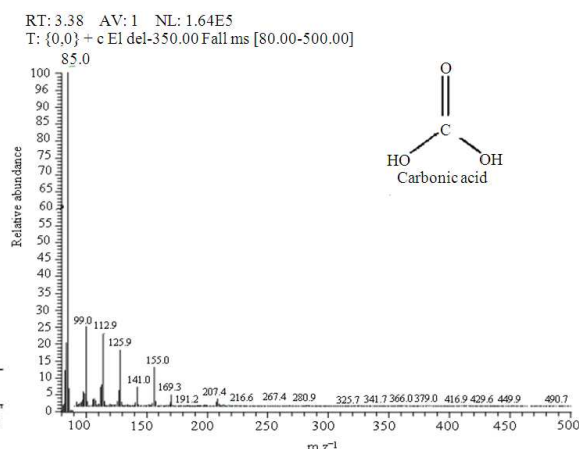
Table 1: Salivary volatiles examined for changes across a menstrual cycle and their nature of compounds, molecular formula/weight

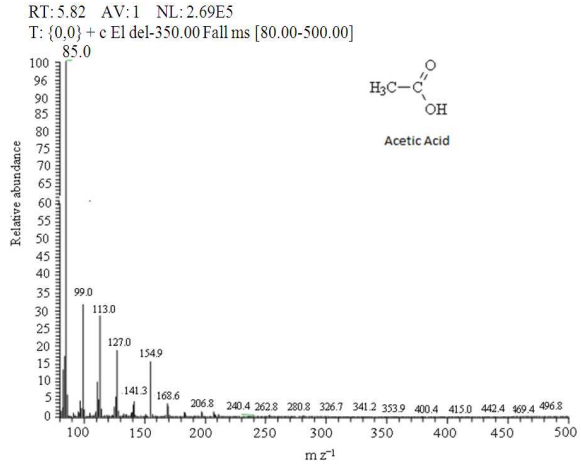| Compound name	Retention tine	Follicular phase (15-26 days)	Ovulatory phase (13-14 days)	Luteal phase (6-12 days)	Nature of compound	Molecular formula	Molecular weight
Carbonic acid	3.38	++	-	-	Carboxylic acid	H_2CO_3	62.02
Acetic acid	5.82	-	++	-	Carboxylic acid	$C_2H_4O_2$	60.00
Diphenylamine	6.37	+++	+	-	Amine	$C_{12}H_{11}N$	169.24
2-Nonenal	8.25	-	+++	-	Aldehyde	$C_9H_{16}O$	140.12
butanoic acid, 2-propenyl ester	9.73	+	-	+	Ester	$C7 H1_2O_2$	164.00
Dodecanol	10.50	+	++	-	Alcohol	$CH_3 (CH_2)_{11}OH$	186.34
Phenol	12.55	+	++	-	Alcohol	C_6H_5OH	94.11
Acetaldehyde	14.40	-	++	-	Aldehyde	CH_3CHO	44.06
4-Methyl phenol	16.11	-	-	++	Alcohol	$C9 H_{12}O$	136.00
1-methyl-cyclopentane	20.03	++	-	-	Alkane	C_6H_{12}	84.16
Skatole	20.87	+	-	+	Alcohol	C_9H_9N	131.17
pentanoic acid	23.22	-	+	++	Carboxylic acid	$C5 H_{10}O_2$	102.00
n-tetradecane	23.87	+	-	+	Alkane	$C_{14}H_{30}$	198.39
3-hexanol	26.02	+	-	+	Alcohol	$C6 H_{14}O$	102.00
propionic acid	28.79	-	-	++	Carboxylic acid	$C3 H_6O_2$	130.00

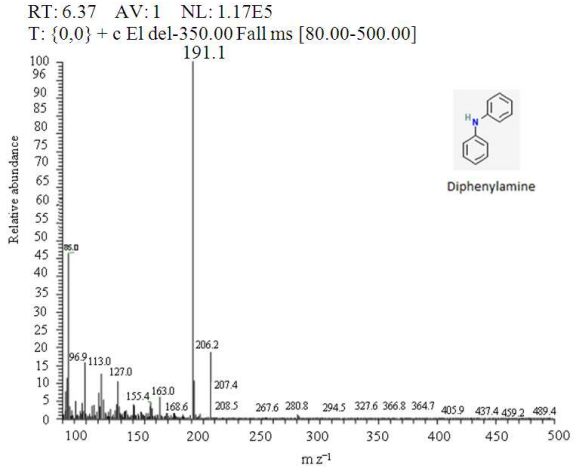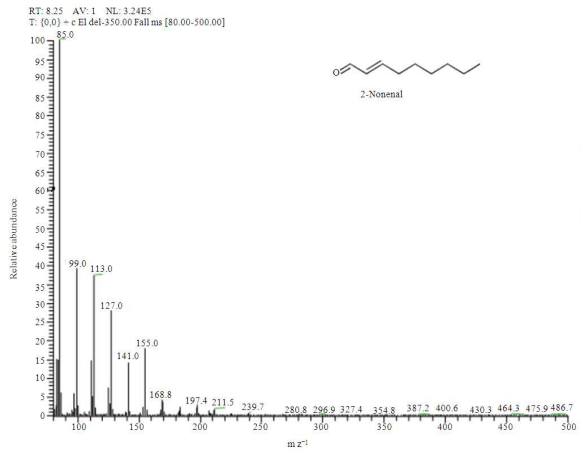+++: Presently high in triplicate analysis; ++: Raise moderately; +: Present; -: Absent

(a)

(b)

RT: 5.82   AV: 1   NL: 2.69E5
T: {0,0} + c El del-350.00 Fall ms [80.00-500.00]

(c)

RT: 6.37   AV: 1   NL: 1.17E5
T: {0,0} + c El del-350.00 Fall ms [80.00-500.00]

(d)

RT: 8.25   AV: 1   NL: 3.24E5
T: {0,0} + c El del-350.00 Fall ms [80.00-500.00]

(e)

RT: 9.73   AV: 1   NL: 7.85E4
T: {0,0} + c El del-350.00 Fall ms [80.00-500.00]

(f)

RT: 10.50   AV: 1   NL: 2.64E5
T: {0,0} + c El del-350.00 Fall ms [80.00-500.00]

(f)
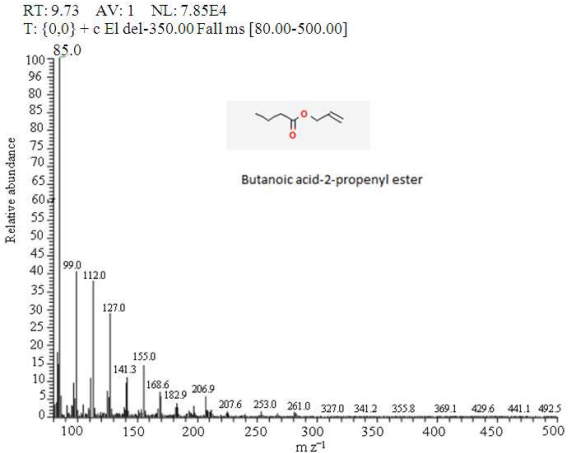
RT: 12.55   AV: 1   NL: 1.61E5
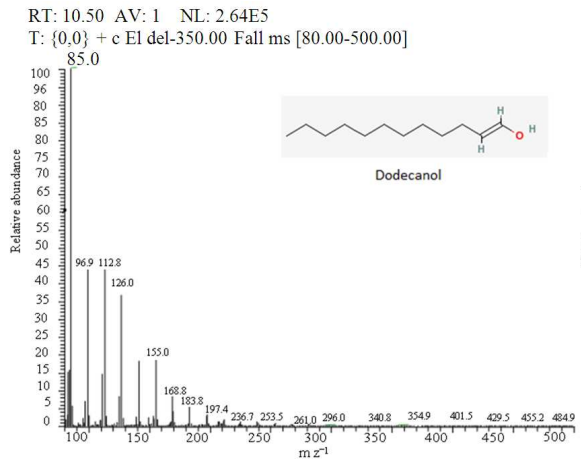T: {0,0} + c El del-350.00 Fall ms [80.00-500.00]
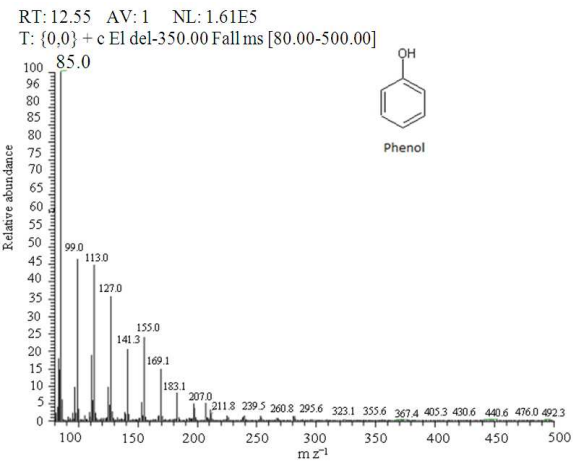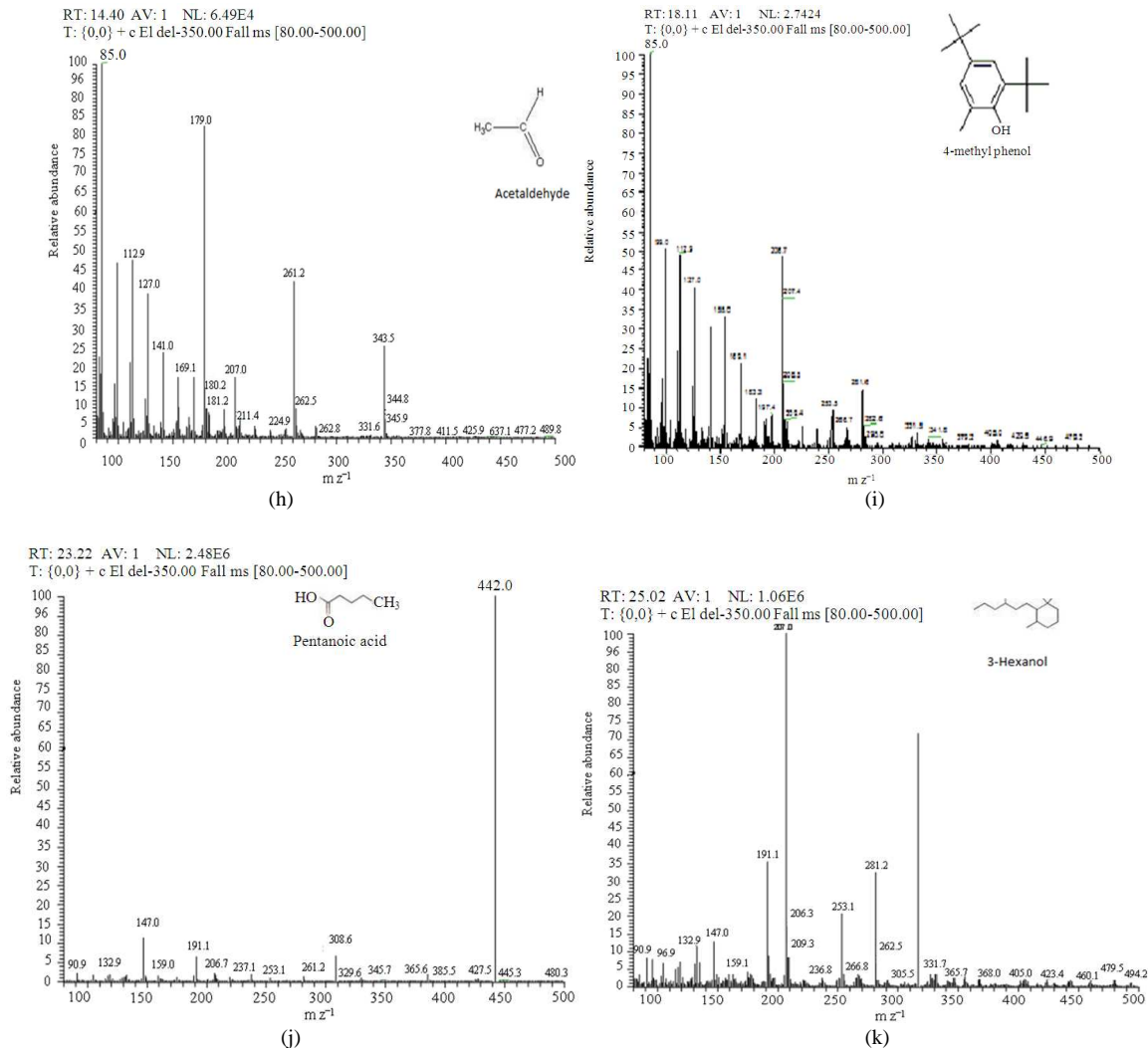

(g)

(h)

(i)

(j)

(k)

Fig. 1: The salivary volatiles compound during menstrual cycle

Unlike the other compounds examined in a quantitative manner, this sharp increase at mid-cycle is the only elevation in concentration which was observed for 2-nonenal (RT value: 8.25), acetic acid (RT value: 5.82), acetaldehyde (RT value: 14.40) and dodecanol (RT value: 10.50) are ovulatory specific volatiles in women reproductive periods. Previous report determines that acetaldehyde was the estrus specific volatiles in cow milk (Denhard *et al.*, 1991), urine (Denhard and Claus, 1996; Kumar *et al.*, 2000) and now in saliva was reported as the odor specific volatiles during menstrual cycle.

The data presented for dodecanol is, however, unique and may prove to be superior even to the detection of other compounds which are known to cycle, such as various volatile aldehyde, acidic and alcohol compounds, form a sharp spike in the concentration of such compounds was detected for every subject at the time of ovulation and is believed to occur only once during the entire menstrual cycle (Doty *et al.*, 1975). The reliable method of diagnosing ovulation in a cycling female mammal is disclosed wherein the concentration of 2-nonenal specifically present in human body as odorous substances provide a olfactory signals in human.

DISCUSSION

The present study was predicated upon the finding that certain long chain alcohols, such as dodecanol, exhibit a unique cyclical behavior during the course of a female's menstrual cycle and accordingly, may be utilized to provide a novel method of diagnosing

ovulation. By monitoring the concentration of such alcohols, particularly dodecanol, during the menstrual cycle beginning after menses and by providing an indicator means for qualitatively and quantitatively responding to the concentration of such alcohol(s), a method is provided wherein significant increases in the concentration of such aldehydes and alcohol(s) is the biomarker of ovulation (Preti *et al*., 2009). Generally, the method of the present invention comprises the monitoring of saliva for the concentration of certain long-chain alcohols, particularly those having 10-14 carbon atoms and more particularly dodecanol, by providing an indicator means for qualitatively and quantitatively responding to concentration of said alcohol compounds in saliva, whereby the means for indicating that concentration diagnosis the precise time of ovulation.

Further, the difference in salivary volatiles content across various reproductive phases clearly indicates that these volatiles have got specific role in human olfactory communication depending upon the physiological change of the women reproductive status. The mechanism of fixing volatile molecules by "lipids" has been observed in *Tupaia belangeri* (Shrew), one of the most primitive mammals (Miyazawa *et al*., 2008). In certain cases lipids itself act as pheromones. For instance, our previous study also indicated that (Kannan *et al*., 1998) lipids play a major role in the sexual attraction in *Rattus norvegicus*. This study focused on the analysis of the GC-MS profiles of the volatile compounds of women saliva in three different phases of follciular, ovulatory and luteal phases has provided evidence that different salivary compounds are present in the different stages. In particular, two peaks present during the ovulatory phase, relating to acetaldehyde and acetic acid, were not found in the other two phases. The appearance of specific peaks in the ovulatory saliva may represent behaviorally important chemical signals. The discovery of substances that appear to be related to ovulation offers a promising approach to this problem. Such substances have been identified in equine urine and reported to act as markers for detection of estrus (Ma and Klemm, 1997). There are also reports that indicate that the oestrus-related odors present in cows' urine can be identified by rats (Denhard and Claus, 1988) and that trained dogs are capable of recognizing characteristic odors in milk during the estrus stage in the cow (Hawk *et al*., 1984). Furthermore, Weidong *et al*. (1996) have reported that unique markers for oestrus can be detected in bovine milk. The present findings provide additional support to the possibility of identifying the estrus phase of the cow by detecting urinary pheromonal compounds. Ovulation specific chemical cues might also be useful as sexual stimulants

(Kumar *et al*., 2000). Both qualitative and quantitative differences were observed among the males and females studied. Various types of compounds were extracted from males and females for odor detection, sinus, axillary, sweat fluids, including a variety of aldehydes, organic fatty acids, ketones and alkanes, which agrees with aging-Haze *et al*. (2001); sweat- (Ostrovskaya *et al*. 2002), human odor (Martin *et al*. 2005), human axilla- Wysocki *et al*. (2009) and nasal fluid Preti and Leyden (2010) analyzed volatile components using GC-MS.

The present study revealed that the salivary volatile compounds such as butanoic acid, skatole and 3-hexanol were present in follicular and luteal phases of women reproductive cycle. Among these, 2-nonenal, dodecanol, acetic acid and acetaldehyde are predominantly present in higher concentration in ovulatory phase. Diphenylamine and phenol also present in follciular and ovulatory phase as a chemical stimulants during the period of LH surge. Indeed, it also indicates that the appearances of these volatiles like alcohol, amine and acids in ovulatory and luteal phases are due to the high circulation of steroidal hormones. The difference in the fatty acid composition may be due to the alterations of lipid metabolism especially of metabolic events affecting ketogenesis and would have reflected in the chemical alteration of saliva. The presence of salivary volatiles like 2-nonenal, acetic acid, acetaldehyde and dodecanol with uniform concentration throughout the menstrual cycle showed that these volatiles molecules are influenced by the hormonal changes during menstrual cycle.

Huggins and Preti (1976) support the present investigation which provides a simple, reliable method of detecting ovulation by detecting a single "peak" or "spike" of such alcohol which occurs at the time of ovulation. The aldehyde and alcohol, particularly 2-Nonenal and dodecanol, content of saliva, was detected in the gas chromatogram headspace of an incubated sample thereof, is believed to be a secondary characteristic which is responsive to elevated levels of female sex hormones. Unlike other such salivary compounds, dodecanol remains at a relatively constant base level throughout the menstrual cycle, exhibiting a single peak or spike precisely corresponding to ovulation. Unfortunately, the biochemical mechanisms and pathways leading to the production of elevated amounts of dodecanol precisely at the time of ovulation are not currently understood.

Curran *et al*. (2005) carry out the study as overall, the GC/MS headspace method developed for the analysis of human odor profiles allows for identification of the volatile organic compounds present in human

scent samples. Through this method, a combination of the relative ratios of common compounds and the presence of differing compounds allows for the chromatographic distinction among individuals. The authors have defined human scent to be the most abundant VOCs present in the headspace above scent samples; however, other substances that have a relatively low volatility or are present in low concentrations may also make contributions to human odor.

CONCLUSION

The notable amount of 2-nonenal and dodecanol during ovulatory phase in comparison to that of other stages suggests that it may be involved in chemical communication during menstrual periods. Since the ovulatory saliva has the capacity to attract the opposite partner through chemical communication, this volatile present in ovulatory phase appears to play a role in sex conspecifies. Further, the unique correspondence of such dodecanol spikes to the time of ovulation and only to the time of ovulation. The primary object of the present study reveals that the provision of a simple, safe, reliable method of diagnosing ovulation by monitoring the concentration of aldehydes and certain long-chain alcohols in saliva over the course of a given menstrual cycle, a marked increase in the concentration of such aldehydes and particularly 2-nonenal, being diagnostic of ovulation.

ACKNOWLEDGEMENT

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