



## CHEMICAL CHARACTERIZATION OF VOLATILES CUE IN WOMEN SALIVA

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### ABSTRACT

The present study was designed to identify the volatile compounds across different phases of menstrual cycle in order to detect ovulation. The human saliva was extracted with dichloromethane (1:1 ratio, v/v) and analyzed by gas chromatography linked mass-spectrometry (GC-MS). Alkanes, aliphatic acids, esters and ketones were identified in the secretions. Numerous salivary volatile compounds were identified during the phases of menstrual cycle are constituted as preovulatory (6-12 days), ovulatory (13-14 days) and postovulatory phases (15-26 days) in the age group of 20-30 years. Among these, the compounds, namely, Trimethylamine, Aristolochic acid, acetic acid, phenol, dodecanol, limonene and propionic acid were more specific to ovulatory phases rather than other phases. Moderately increased levels at midcycle were probably related to interovulatory follicular growth. A salivary volatile compound seems to possess an olfactory changes and endocrine regulation might be implausible due to signaling pheromones. The result conclude that saliva contain more than fifteen volatiles fraction with olfactory changes in maintaining the reproductive status. Out of these volatile compounds Trimethylamine and aristolochic acid seems to be present maximum time in the secretions near the time of ovulatory phase which is considered as a marker for ovulation prophecy. None of these compounds to our knowledge has been reported on ovulatory saliva and one or more may possibly be considered as putative sex pheromones.

**KEYWORDS:** Volatile organic compounds, menstrual cycle, saliva, women.

### INTRODUCTION

To assess the time of conception in vivo and carry out simultaneous biomonitoring, ovulation must be identified prospectively. We tale on the development and validation of a simple, prospective method, the Peak day procedure, to determine apt day of ovulation based upon daily observations of body fluids.<sup>[31]</sup> Based on the available facts, it has been anticipated that using symptom biomarkers for ovulation may bear out as the accurate and effective as using hormonal biomarkers, at a much more cost effective ratio for large population-based studies.<sup>[32]</sup> The exact tme for prediction and detection of the fertile phase of the menstrual cycle is of immense value for those engaged in either promoting or controlling fertility. This period is defined as the time of the menstrual cycle when conception is possible. The duration of the fertile phase will depend on the lifespan of the gametes in the female genital tract and this may vary from couple to couple and even from cycle to cycle. The fertile phase of the menstrual cycle is strictly related to the time of ovulation.<sup>[1]</sup> In the majority of animal

species the fertile phase is limited to a relatively short period and becomes evident by the phenomenon of estrus. Changes in sexual behavior and attractiveness are synchronized with ovulation to maximize chances for fertilization.<sup>[2]</sup> The human female, however, does not show these cyclical changes externally. Therefore it has been necessary to develop alternative methods to determine the time of fertility during the menstrual cycle.<sup>[3]</sup> These methods rely on the identification of substances or biological phenomena which in a direct or indirect way reflect the physiological changes that take place during the process of follicular maturation and ovulation.

Most up-to-date information shows that saliva was a staggeringly good quality in cooperation with change of hormones and biochemical's also their intensity changes in accordance with the menstrual cycle. In fact, saliva is a remarkably complex biological fluid with an extraordinary natural history and it addresses a laundry list of obvious. Many volatiles are produced in the oral

cavity through putrefactive action of microorganisms on proteinaceous substrates in whole saliva.<sup>[4,5,6]</sup> which consists primarily of secretions from the parotid, submaxillary, and sublingual glands, but also contains gingival exudate, leukocytes, exfoliated epithelial cells, microorganisms, food debris, and skin lipids.<sup>[7,8,9]</sup> The organization of termite societies depends predominantly on intra-specific chemical signals (pheromones) produced by saliva which induce and modulate individual behavioral responses.<sup>[10]</sup> For instance, sexually experienced males preferred the saliva of estrus females to that of non-estrus females in Mongolian gerbils.<sup>[11]</sup> In boars, pheromones are secreted in saliva to cause estrous sows to take up the mating stance.<sup>[12]</sup> The integration of various compounds in specific ratios may contribute to the formation of a specific odour.<sup>[13]</sup> Therefore, a successful artificial insemination program must incorporate efficient and accurate detection of oestrus and timely insemination relative to ovulation.<sup>[14]</sup>

Chemical investigations on mammalian pheromones have been relatively less. However, in the last two decades, there has been a considerable study on the chemistry of mammalian pheromone identification in mouse,<sup>[15,16]</sup> rat,<sup>[17]</sup> bobcat,<sup>[18]</sup> tiger,<sup>[19]</sup> white-tailed deer,<sup>[20]</sup> horse,<sup>[21]</sup> bovine,<sup>[22]</sup> elephant,<sup>[23]</sup> and human.<sup>[24]</sup> These findings indicate that mammalian pheromones may be a single compound or a mixture of compounds and that each of the major fractions are authentically involved in assigning specific cue related to reproductive and communal behaviors.

Unpredictable odor (volatiles) from the opposite sexual characteristics conspecifics contribute mate recognition in several mammalian species, by following earlier reports.<sup>[25,26]</sup> To date, no chemical investigations have been performed on saliva from different phases of menstrual cycle and its biological functions. Detection of ovulation in human is one of the major problems in artificial insemination. The reason behind this study was to identify the ovulatory specific volatile compounds in women and make it possible to develop a biochemical marker to detect ovulation accurately. Therefore, the present study was to examine the salivary volatile profiles across the menstrual cycle in possess an olfactory changes and endocrine regulation might be due to pheromonal regulation which compared the reproductive phases in order to elucidate the ovulatory specific volatiles in women.

#### MATERIAL AND METHODS

Twenty normally menstruating women, around 20-30 years of age, participated as subjects in this serial study. The subjects were selected on the basis of normal menstrual cycle ( $28 \pm 2$  days cycle). The saliva sample was taken from each subject during preovulatory (6-12 days), ovulatory (13-14 days) and postovulatory phases (15-26 days). The volunteers were instructed to abstain from eating, smoking and drinking 10hrs prior to testing.

And also the volunteers were asked for tooth brushing to prevent minimal gingival bleeding.

The determination of different phases and the procedures of collection of saliva during these phases have been explained earlier.<sup>[27,28,29]</sup> In brief 5 ml of saliva was collected during 8.00-9.00 A.M in each phase. Period of ovulation was judged by daily oral body temperature recordings, ferning pattern and determination of salivary hormones was carried out on the same day collection by standard methods. The saliva sample were centrifuged was 5000 rpm at 4°C for 10 min. The clear fluid was concentrated and used for the salivary volatiles testing in triplicate manner. Directly after screening, the samples were stored at frozen at -20°C and analyzed by gas chromatography mass spectrometry.<sup>[30]</sup>

#### Sample analysis

The samples collected from the particular stage as per the experimental protocol were pooled to minimise the effect of individual variation. Dichloromethane was used to extract the compounds from the saliva samples. Triplicate 5 ml of samples were taken from the pooled samples and separately mixed with 5 ml of dichloromethane. The supernatant was filtered through a silica-gel column (60-120 mesh) for 30 min at room temperature. The filtered extract was reduced to 1/5 of its original volume by cooling with liquid nitrogen to condense.

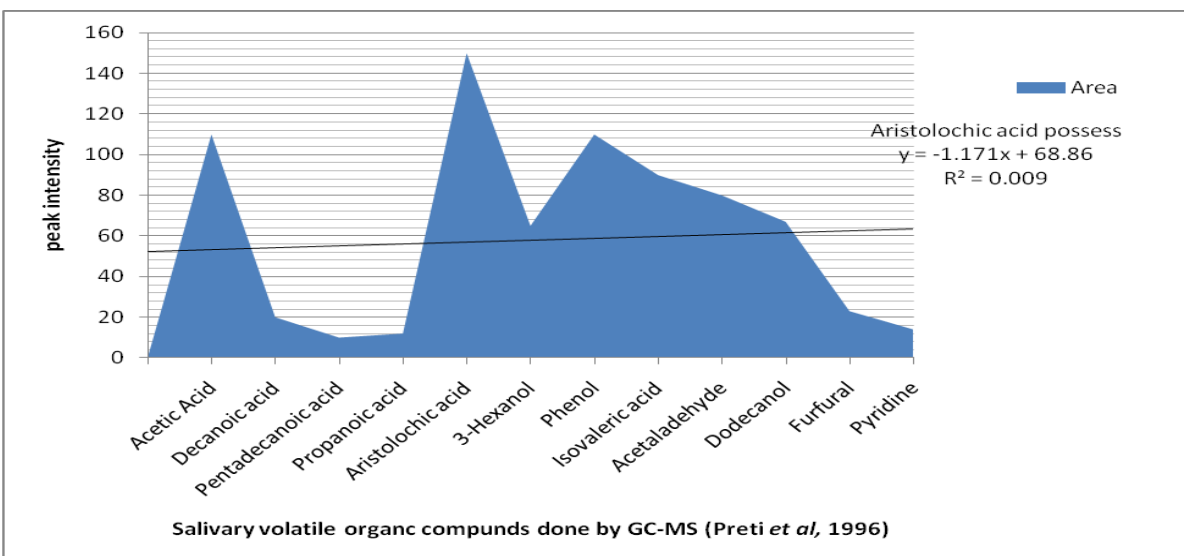
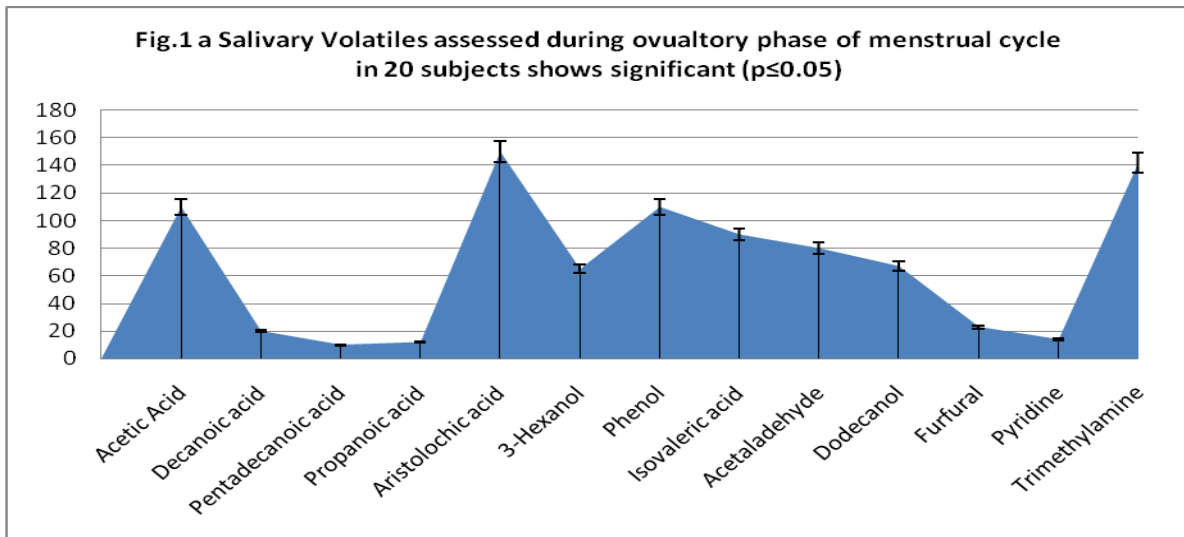
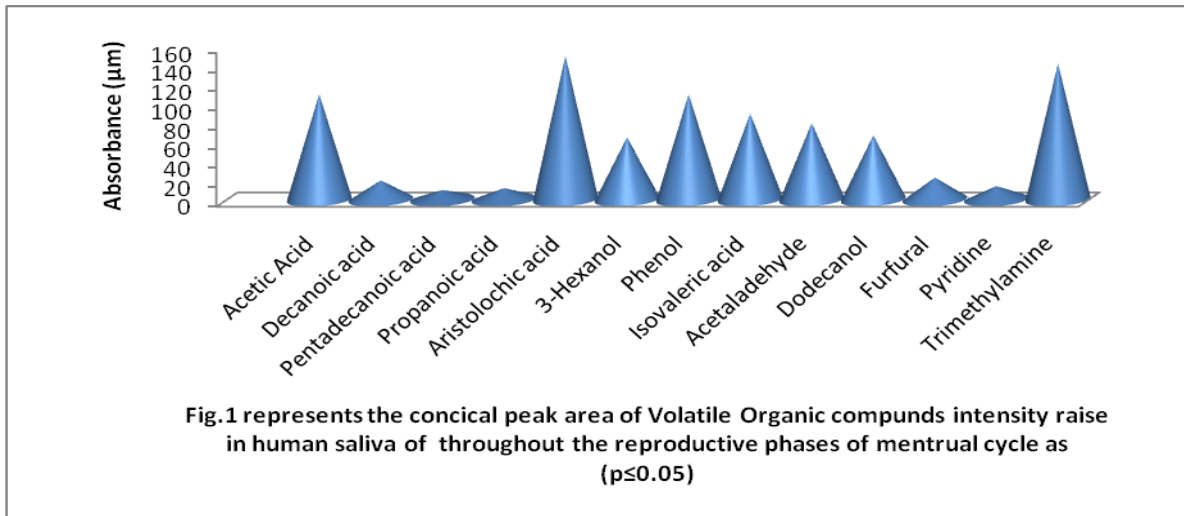
The sample was fractionated and chemical compounds were identified by GC-MS (QP-5050, Shimadzu). Two microlitres of extract were injected into the GC-MS system on a 30m glass capillary column with a film thickness of 0.25  $\mu\text{m}$  (30 x 0.2 mm i.d. coated with UCON HB 2000) using the following temperature program me: Initial oven temperature of 40°C for 4 min, increasing to 250°C at 15°C/min, and then held at 250°C for 10 min. The GC-MS was run under computer control at 70-eV. The solvent (dichloromethane) peak was seen at 4.0 min. The saliva was analyzed repeatedly six times and subjected to cross checking and confirmation. The identified compounds were then compared with the standard run under the same conditions<sup>[30]</sup>. These data were already stored in a compact library of chemical substance (NIST 6221B).

#### RESULTS

The GC-MS profiles shown in Figs. 1 and 1a, b are the representative of human saliva obtained in the preovulatory (6-12 days), ovulatory (13-14 days) and postovulatory phases (15-26 days). The human saliva of ovulatory phase showed six peaks and of preovulatory exhibited four peaks of postovulatory phases exhibited four peaks. Twelve different peaks were noted in the human saliva of three different phases (Fig. 1). Of these, Aristolochic acid, acetic acid, propionic acid, limonene, dodecanol and phenol were unique in the ovulatory phase but were absent in the other phases (Fig.1). However, the compounds, Decanoic acid,  $\alpha$ -

Anderstenol, 3-methyl-3-hexanol and furfural were found only in preovulatory and pyridine, Isovaleric acid, pentanoic acid and octadecan-1-ol are present in post

ovulatory phases (Table 1). Acetaldehyde present in ovulatory and also in postovulatory phases.



**Fig.1b represents the salivary volatile compounds aristolochic acid followed by trimethylamine during Ovulatory phase of menstrual cycle shows 5% levels significant ( $p \leq 0.05$ )**

## DISCUSSION

In the present study, numerous volatile compounds were identified in the human saliva at different reproductive phases, which qualitatively differed from one phase to the other. The present results revealed that the aristolochic acid, acetic acid, propionic acid and phenol appeared during ovulatory phase but were not found in the other reproductive phases. Among the compounds identified in ovulatory phase saliva, the acetic and propionic acids belong to fatty acids, the aristolochic acid is in acid group and phenol is in phenol group. Among this Aristolochic acid shows moderately high in ovulatory phase compare to other phases due to olfactory changes due to their pheromonal activity

Estrus saliva is known to contain chemo-signals, the compound identified specifically in this period may be considered as behaviourally important chemical signals that might attract males (Archunan, 2003). Smith and Block (1991) reported that adult female Mongolian gerbils were preferentially attracted to saliva from adult non-sibling males when paired with saliva from their male siblings. This study confirms that saliva is an important oral cue used by females in the assortment of socio-sexual buddies in human. Hence, the present study indicates that the compound, trimethylamine and aristolochic acid, may be the reputed compound peaks become visible subsequent to ovulatory phase (Table-1).

Further, the identified compounds in estrus and to activate of the sex that can beget offspring by fertilization or insemination appear to be sex attractants. It is to be noted that during the mating behavior, certain

compounds cause a neurological response that relaxes the penis muscles and releases blood flow into corpus cavernosum penis in preparation for erection (Walker, 1984). The present findings further confirm that the identified compounds in ovulatory phase are particularly involved to activate in the food debris of the male, it may be skin complaint as sex attractants. Once such substances have been identified, they could be used as biomarkers for estrus. It might then be possible to develop simple non-invasive methods for prediction of ovulation.

## CONCLUSION

An innovative method for the improvement of saliva profiles which provides in sequence harmonizing to on hand analyses has been developed. Physical investigations illustrate the aim of basal body temperature, vaginal and salivary ferning test are the detection of cyclic periods to perception of LH surge. Application of the developed protocol toward the investigation of saliva as a standard for the non-invasive detection of ovulation/estrus specific volatiles for the evasion of assisted reproductive technology or artificial insemination. The results of the salivary volatiles estimate during the period of menstrual cycling are presumably LH dependent which elicits the method provides a reliable, reproducible method for metabolic profiling. Gas chromatographic-mass spectrometric analysis of the volatile constituents endow with positive detection of 15 compounds. Out of these compounds trimethylamine, aristolochic acid, acetaldehyde and phenol are detected as volatile signal throughout the cyclic phase of human estrus/ovulation.

**Table. 1: represents the salivary volatile compounds during menstrual cycle.**

Serial No	Nature of compounds	Name of the compounds	Mass	Reproductive phases			Biological significance
				Preovp	Ovp	Povp	
1	Acid	Acetic Acid	60.0		++		Olfactory stimulator in human
2	Acid	Decanoic acid	172.3	++	+	+	Sex attractants in mammals
3	Acid	Pentadecanoic acid	420.8	++		++	Sex attractants in mammals
4	Acid	Propanoic acid	130.5	+++	+	++	Sex attractants in mammals specially in rats and cows
5	Acid	Aristolochic acid	341.27		+++		Olfactory stimulator in human
6	Alcohol	3-Hexanol	102.5				Sex attractants
7	Alcohol	Phenol	94.11	+++	++		Sex attractants
8	Fatty acids	Isovaleric acid	102.1			++	Behavior activity
9	Aldehyde	Acetaldehyde	44.05		++		Sex attractants in cows
10	Alcohol	Dodecanol	186.34		++	+	Sex attractants in human vaginal mucus
11	Aldehyde	Furfural	96.09	++		+	Food inducers
12	Amine	Pyridine	79.09	+++		+	Behavior activity
13	Amine	Trimethylamine	75.1		+++	+	Mouth Odor

+ Less ++ High +++ Moderately high.

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