



Oral fluid as an alternative matrix to determine ethanol for forensic purposes



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ABSTRACT

The present work aimed to evaluate whether it is possible to use oral fluid to monitor alcohol in drivers.

In a control experiment the subjects ingested beer with an alcoholic percentage of 4.7%, in an amount that furnished 0.5 g ethanol per kg of body weight. Volunteer's urine, oral fluid, and breath were collected at 10, 30, 60, and 90 min after alcohol intake. Urine and oral fluid were analyzed by gas chromatography with FID (Flame Ionization Detector); breath was analyzed by Alcotest 7410 (Dräger).

The absorption profiles correlated well. The Pearson correlation value between samples of oral fluid and urine, and oral fluid and exhaled air, was close to 1, showing that oral fluid is a promising matrix to monitor drivers in traffic or involved in accidents.

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1. Introduction

Ethanol is widely consumed in most societies. Despite being a psychotropic drug, its use is not proscribed, so it is available for any individual of legal age to consume [1].

Alcohol acts on the central nervous system and elicits side effects that are particularly harmful to drivers: it impairs motor coordination, vision and reaction time, negatively affecting the driver's ability to conduct vehicles [1,2]. Therefore, ethanol is often the underlying cause of traffic accidents [3–7]. This causal relationship has led many countries to adopt laws that discourage intake before driving [8].

In Brazil, the Brazilian Traffic Code establishes the limit of ethanol allowed in drivers blood and exhaled air, a measure known as Dry Law. This code makes driving under the influence of any blood ethanol concentration and ethanol concentration in breath equal to or higher than 0.05 mg of ethanol per liter of exhaled air a criminal offense. In addition to ethanol, the use of psychoactive substances prohibited by law, such as cocaine, marijuana and

amphetamines, is not allowed, either. In all cases, the driver receives punishment [9].

To monitor drivers and collect evidence, police officers use portable alcometers and blood alcohol tests. If none of these procedures are feasible the police officer must rely on other ways to prove that drivers are intoxicated with alcohol, i.e., clinical examinations, videos, testimonial evidence, or other strategies allowed by law [9].

Regarding tests in biological samples, bodily fluids such as breath, oral fluid, and urine offers advantages over blood: collection is not invasive and does not require specialized personnel either. Ethanol analysis in breath and urine are well known and often applied to screen ethanol in drivers [10–14], however, oral fluid is not commonly used, despite the fact that literature studies have evaluated the validity of its use to investigate ethanol [14–18]. Oral fluid is a good choice once it has a non-invasive way of collection and besides does not entail privacy invasion if an assisted collection is required to avoid sample adulteration.

Devices that can analyze ethanol in breath are very useful to estimate blood alcohol concentration in drivers when it is needed to monitor them in traffic, due to the fact that it is a portable measure instrument. The disadvantage of such dispositive is that the analysis can't be repeated, but when driver's oral fluid is collected, different laboratories can confirm the driver's abuse of

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alcohol by repeating the determination of ethanol in oral fluid if the sample chain of custody is preserved.

Oral fluid, or oral fluid secretion, involves three major oral fluid glands: parotid, submaxillary and sublingual. The degree to which each gland contributes to oral fluid composition depends on several factors such as the type, duration and intensity of stimulation; time of day; diet; age; gender; and physical status. Furthermore, the pH of oral fluid varies among individuals and within the subject. Variation of pH does not constitute a problem for ethanol analysis, unlike other drugs, alcohol distribution depends on the amount of water in the fluid, which is not a function of pH [19].

It is possible to obtain oral fluid samples in different ways: allowing oral fluid to flow from the oral cavity to a recipient, aspirating the sample through a vacuum tube, or placing a small cotton roll in the mouth to stimulate the formation of oral fluid and retain the liquid [20]. In addition to cotton, other stimulants can be used, such as paraffin and citric acid [21,22]. Generally, about 1–5 mL of oral fluid is available for collection, which is a small volume compared with the amounts of urine that can be collected (greater than 50 mL) [23].

It is possible to conduct analysis in oral fluid, urine, or blood by gas chromatography using the headspace technique which consists of heating the sample inside a glass vial equipped with a sealed cap and a septum. The volatile analyte evaporates inside the vial, to form a vapor phase called headspace. With a syringe, one pierces the septum with a needle and collects the analyte free from sample interferences and injects it into the gas chromatography port. Hence, preparing oral fluid and urine samples by headspace methodology to determine ethanol does not demand complicated procedures, the alcohol vaporizes easily under slight heating, and the resulting vapor phase without interferences can be injected into the equipment [14,24].

When oral fluid or exhaled air samples are collected near the time of drug or alcohol consumption, it is probable that the sample will contain ethanol residues that remained in contact with the oral cavity [21,25]. This could increase the concentration of the analyte in the sample and generate erroneous results.

To assess whether it is feasible to use oral fluid samples to determine the presence of ethanol in the body for forensic purposes, we performed a study in which, healthy volunteers consumed alcohol in a controlled manner. Then, oral fluid, urine, and exhaled air samples were collected at different times; the ethanol concentrations in these samples were determined and compared.

2. Materials and methods

2.1. Materials

Ethanol (J.T. Baker, Mexico), HPLC grade, was used for calibration; isobutanol (Merk, Germany), analytical grade, was the internal standard; NaCl (Synth, São Paulo), analytical grade, was used to increase ethanol and isobutanol concentration in the vapor phase by salting-out effect. The 20 mL headspace vials were purchased from Sun Sri, Spain and the Salivette[®] devices from Sarstedt AG & Co, Germany.

Working standard solutions (10 and 100 g L⁻¹) were prepared by appropriate dilution of ethanol in deionized water. Internal standards were prepared in 500 mL of deionized water with 150 µL of isobutanol and 1 g of NaCl.

2.2. Methods

2.2.1. Validation procedures

To validate the method, parameters such as linearity, precision (intra-day and inter-day) and accuracy, carryover, selectivity,

sensitivity, and analyte stability were evaluated. The method was validated using oral fluid and urine.

2.2.2. Linearity

Seven calibration points were used to perform the linearity study. Ethanol-free oral fluid and urine were spiked with ethanol (HPLC grade) in order to obtain concentrations of 0.1, 0.2, 0.5, 1.0, 2.0, 3.0 and 4.0 g of ethanol per liter of bodily fluid. These solutions were added in a headspace vial with internal standard solution in amounts of 100 µL and 500 µL respectively. After heating the vial at 80 °C for 10 min, 400 µL of the headspace were collected for analysis.

The linearity was expressed as the coefficient of determination (R^2), and it was evaluated from a least square regression line calculated from all the standard concentrations. The calibrator concentrations were required to be within $\pm 15\%$ of the target when calculated against the full calibration curve.

2.2.3. Precision and accuracy

It was used, to determine the precision and accuracy, three Quality Controls (QC) samples, QC1 (low), QC2 (medium) and QC3 (high). The QC ethanol concentrations were, respectively, 0.5, 2.0 and 3.0 g L⁻¹.

The precision and accuracy assay were performed by analyzing five replicates of each QC samples. Inter-assay precision was determined on three consecutive days ($n = 15$), and intra-assay precision was calculated from 5 replicate determinations per concentration in one assay batch. Data were evaluated by examining the variance in each group (intra- and inter-day), which were established by the RSD%.

Precision was measured as the Relative Standard Deviation (RSD%) which values should be better than 15%. Accuracy was determined by comparing measured concentrations with target values and expressed as the percentage of the target concentration. Accuracy values should fall within $\pm 15\%$.

2.2.4. Carryover

Carryover was determined by analysis of a blank oral fluid and urine specimen immediately after the analysis of the highest point of the calibration curve (4.0 g L⁻¹).

2.2.5. Selectivity

The method conditions were tested to see if it allows good separation of ethanol from other volatiles that may be present in oral fluid and urine, such as methanol and ketone. Oral fluid and urine blank samples were fortified with ethanol, methanol and ketone at concentration of 0.2 g L⁻¹. Their retention times had to be within ± 0.2 min of the mean calibrator retention time.

2.2.6. Sensitivity

It was defined by lower limits of detection (LLOD) and lower limits of quantification (LLOQ); it was determined empirically through oral fluid and urine specimens fortified with different decreasing concentrations of ethanol. LLOD and LLOQ were established at a signal-to-noise ratio of at least 3:1 and 10:1 respectively.

Precision and accuracy at LLOQ concentrations were according to acceptable criteria (RSD $\pm 20\%$).

2.2.7. Analyte stability

The stability assay was assessed using ethanol-free urine and oral fluid samples in triplicate fortified with ethanol at 0.5 g L⁻¹ (QC low) and 3.0 g L⁻¹ (QC high) both were storage in the respective sample collection devices, Salivette[®] for oral fluid and 80 mL plastic container for urine. The storage conditions studied were: room temperature for 24 h; three freeze–thaw cycles (12, 24 and 36 h),

Table 1

Description of the volunteers, by gender, body mass, beer volume, and amount of ingested ethanol.

Volunteers	Gender	Body weight (kg)	Volume of beer ingested (mL)	Amount of ethanol (g)
1	Female	64	838	32.0
2	Female	54	707	27.0
3	Female	67	877	33.5
4	Female	66	864	33.0
5	Female	71	930	35.5
6	Male	90	1178	45.0
7	Male	95	1244	47.5
8	Male	74.5	975	37.2
9	Male	89	1165	44.5
10	Male	53	694	26.5

each cycle 100 μL of QC was taken for analysis, and storage at $-5\text{ }^{\circ}\text{C}$ for 7 days. The concentrations obtained after each assayed condition were compared with those from freshly prepared samples.

2.2.8. Volunteers

The individuals who participated in the study were in good health and had exhaled air alcohol concentrations equal to zero, as revealed by a Breathalyzer test before alcohol intake. All the participants signed an informed consent form. For the volunteer's safety, their release from the experimental site was allowed only after the Breathalyzer test confirmed zero ethanol concentration. Ten volunteers, five men and five women, aged 20–31 years and weighing 53–95 kg weight were included in the study.

2.2.9. Ethanol administration

The amount of ethanol that each individual ingested was restricted to 0.5 g ethanol per kg of body weight. The alcoholic beverage was beer with 4.7% alcohol. The volunteers consumed beer with an empty stomach after fasting for at least 2 h. They were allowed 20 min to ingest the beverage but the average duration of alcohol intake was 10 min. Table 1 provides information such as gender, weight, volume, and quantity of beer and ethanol consumed by the volunteers.

2.2.10. Sample collection

Oral fluid samples were collected using a commercial collection device Salivette[®] (Fig. 1). The device consists of two tubes – one contained an orifice in the bottom as well as a piece of cotton that is placed in the mouth to stimulate the formation of oral fluid and absorb the secreted oral fluid. After sample collection, the cotton soaked with oral fluid was centrifuged at 2500 rpm for 5 min; the oral fluid leaked to the second tube through the orifice.

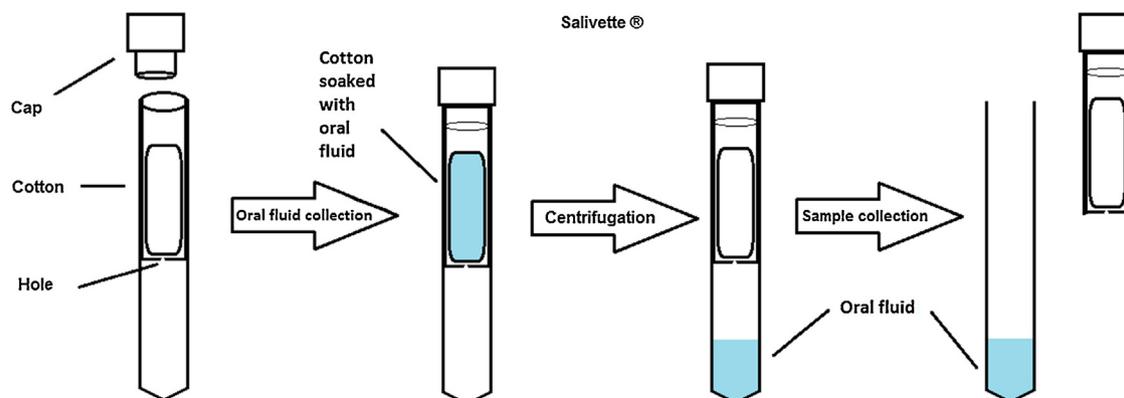


Fig. 1. Representation of the device used to collect oral fluid (Salivette[®]).

Urine samples were collected in a 80 mL plastic collector; exhaled air was collected and analyzed directly on the breathalyzer. Sampling occurred at 30, 60, and 90 min after ingestion of the beer. The oral fluid and urine samples were stored at $-20\text{ }^{\circ}\text{C}$ until analysis.

2.2.11. Sample preparation

Ethanol was extracted from oral fluid and urine by headspace technique. The bodily fluids were prepared for analysis adding 100 μL of the sample and 500 μL of the internal standard solution in 20 mL vial sealed with silicon/PTFE septa and steel caps. The vial was heated at $80\text{ }^{\circ}\text{C}$ for 10 min and a volume of 400 μL of the vapor phase was collected for injection.

2.2.12. Equipment and analytical conditions

Ethanol analysis in oral fluid and urine was accomplished using the gas chromatograph model 7890 A (Agilent Technologies) with Flame Ionization Detector (FID).

A fused silica capillary column from Ohio Valley, 30 m \times 0.25 mm \times 0.25 μm in polyethylene glycol was used. Nitrogen was the carrier gas at a flow rate of 1 mL min^{-1} . The detector and injector were maintained at $300\text{ }^{\circ}\text{C}$ and $250\text{ }^{\circ}\text{C}$, respectively. The sample was introduced in the split injection mode. The following column temperature program was employed: $50\text{ }^{\circ}\text{C}$ for 2 min; temperature rise to $200\text{ }^{\circ}\text{C}$ at a heating rate of $20\text{ }^{\circ}\text{C min}^{-1}$.

The ethanol concentration in breath was measured with an Alcotest 7410 (Dräger, Germany) breathalyzer. The volunteers provided a sample by blowing up directly at the instrument using a disposable blow device, the concentration of ethanol in breath was available immediately but the instrument need an interval between to measurements to get ready for another sample. It was observed that the instrument fails to measure ethanol concentration in breath when the test is performed right after drink ingestion, due the residual alcohol in mouth as demonstrated by Barquin et al. [26]. To avoid wrong interpretation results, the measurements are presented at 30 min after beer ingestion.

3. Results and discussion

Calibration curves were obtained from 0.1 to 4.0 g L^{-1} of ethanol, linearity was observed under concentration 10 g L^{-1} . The linear regression equations and coefficients of correlation were: oral fluid: $y = 0.217x - 0.0153$, $r^2 = 0.999$; urine: $y = 0.1885x - 0.0014$, $r^2 = 0.999$.

Lower limits of detection and quantification, intra-assay and inter-assay precision and accuracy were adequate for the purposes of the present study, Table 2 presents the corresponding data. Intra-assay precision ranged from 1.0 to 2.2% (RSD) for oral fluid

Table 2

Lower limits of detection (LLOD), lower limits of quantification (LLOQ), precision (RSD%) and accuracy of intra ($n=5$) and inter-assays ($n=15$).

	Oral fluid		Urine	
	LLOD	LLOQ	LLOD	LLOQ
	0.005 g L ⁻¹		0.005 g L ⁻¹	
	0.1 g L ⁻¹		0.1 g L ⁻¹	
	Oral fluid		Urine	
	RSD%	Accuracy%	RSD%	Accuracy%
Intra-assay				
CQ1	1	7.8	1.3	2.1
CQ2	2.2	0.1	1.1	-0.4
CQ3	1.7	0.4	1.1	0.9
Inter-assay				
CQ1	3.1	11.6	5.3	6.2
CQ2	1.7	-0.5	7.3	-0.6
CQ3	0.2	0.6	4.1	2.9

and from 1.1 to 1.3% for urine. Inter-assay precision ranged from 0.2 to 3.1% for oral fluid and from 4.1 to 7.3 for urine. Intra-assay accuracy ranged from -0.4 to 2.1% and inter-assay from -0.6 to 6.2.

There was no interference at the same retention time of ethanol when methanol or ketone was present and no carry over was observed. The samples demonstrated to be stable at all conditions studied.

Fig. 2 shows a representative chromatogram of the urine analysis for volunteer 7 after 30 min of beer ingestion. Using a method based at the one described in De Martinis et al. [24], the peaks of the target analyte (ethanol) and internal standard (isobutanol) are well resolved. Table 3 lists ethanol concentrations in exhaled breath, oral fluid, and urine vs. time after ingestion of alcoholic drinks.

To illustrate the ethanol absorption and elimination profile in the body, we associated the average concentrations with the time elapsed since ingestion. For comparison purposes, Figs. 3 and 4 present the ethanol concentrations in oral fluid and breath, and Figs. 5 and 6 depict the ethanol concentrations in oral fluid and urine for men and women volunteers.

Gubala and Zuba [18] analyzed ethanol concentrations in oral fluid and breath after controlled intake of beverages. The ethanol concentration curves vs. time for the oral fluid and exhaled air samples were similar. In the present study, we obtained the same profile for oral fluid and breath, and for oral fluid and urine, with differences between genders.

Table 3 reveals that the maximum mean ethanol concentration in urine occurs at 30 min, reaching values of 0.39 g L⁻¹ and

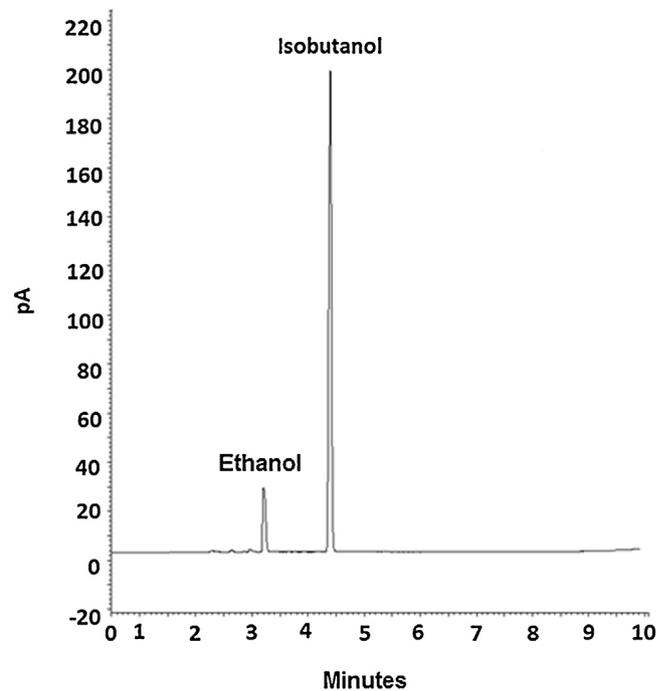


Fig. 2. Chromatographic profile for ethanol analysis a urine sample obtained from volunteer 7 after 30 min of beer ingestion.

0.44 g L⁻¹ for women and men respectively. In a study by De Martinis et al. [24] in which volunteers ingested the same amount of ethanol (0.5 g ethanol/kg body weight), the maximum ethanol concentrations in the urine sample were lower, 0.35 g L⁻¹ and 0.28 g L⁻¹ for women and men respectively and besides it was achieved 30 min later: between 90 and 120 min of beer ingestion. De Martinis et al. [24] conducted the experiment after the volunteers had ingested food, which may have delayed ethanol absorption by the small intestine.

According to Baselt [2], the presence of food in the stomach decreases the ethanol concentration and it takes longer to reach the ethanol concentration peak in the blood because ethanol absorption is slower. Thus, the presence of food can also influence the ethanol concentration in other bodily fluids, since the occurrence of ethanol in other parts of the body depends on the presence of ethanol in the blood.

Maximum ethanol concentrations in oral fluid occur at different times for men and women: 0.14 g L⁻¹ at 30 min and 0.21 g L⁻¹ at 60 min, respectively (Table 3).

Table 3

Concentration of ethanol in breath, urine, and oral fluid at 30, 60, and 90 min after beer intake and before the beginning of the experiment (time 0), for female (1–5) and male volunteers (6–10).

	Ethanol concentration according to the time of sample collection (min)											
	Breath (mg L ⁻¹)				Urine (g L ⁻¹)				Oral fluid (g L ⁻¹)			
	0	30	60	90	0	30	60	90	0	30	60	90
1	0	0.27	0.19	0.10	0	0.25	0.51	0.19	0	0.01	0.03	0.04
2	0	0.25	0.23	0.20	0	0.42	0.32	0.18	0	0.20	0.39	0.06
3	0	0.25	0.23	0.20	0	0.37	0.23	0.14	0	0.15	0.08	0.02
4	0	0.26	0.19	0.20	0	0.38	0.41	0.27	0	0.02	0.16	0.09
5	0	0.20	0.21	0.20	0	0.52	0.18	0.26	0	0.40	0.40	0.29
Average	0	0.26	0.21	0.18	0	0.39	0.33	0.21	0	0.16	0.21	0.10
6	0	0.27	0.15	0.10	0	0.12	0.11	0.17	0	0.39	0.26	0.05
7	0	0.27	0.26	0.20	0	0.57	0.61	0.49	0	0.21	0.12	0.15
8	0	0.27	0.23	0.20	0	0.54	0.26	0.32	0	0.05	0.15	0.02
9	0	0.25	0.22	0.20	0	0.41	0.46	0.29	0	0.01	0.07	0.06
10	0	0.25	0.16	0.10	0	0.56	0.26	0.34	0	0.04	0.07	0.01
Average	0	0.25	0.20	0.16	0	0.44	0.34	0.32	0	0.14	0.13	0.05

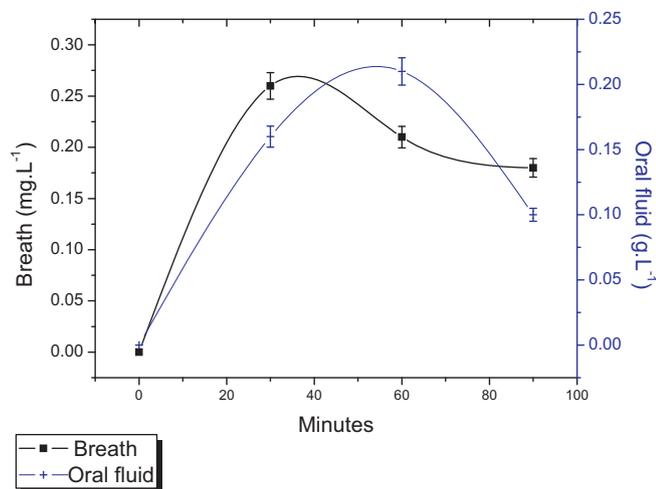


Fig. 3. Ethanol concentration in breath and oral fluid at 30, 60, and 90 min, after beer ingestion for women (volunteers 1–5).

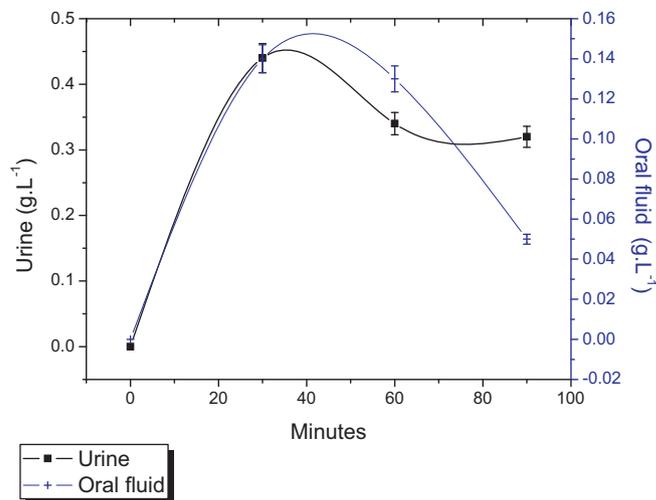


Fig. 6. Ethanol concentration in urine and oral fluid at 30, 60, and 90 min after beer ingestion for men (volunteers 6–10).

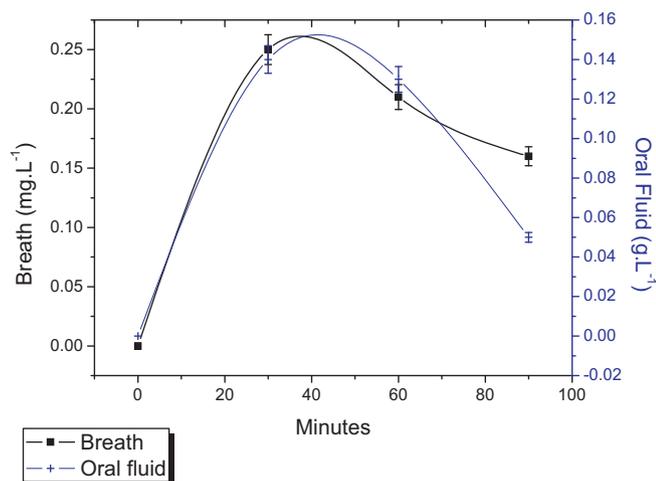


Fig. 4. Ethanol concentration in breath and oral fluid at 30, 60, and 90 min, after beer ingestion for men (volunteers 6–10).

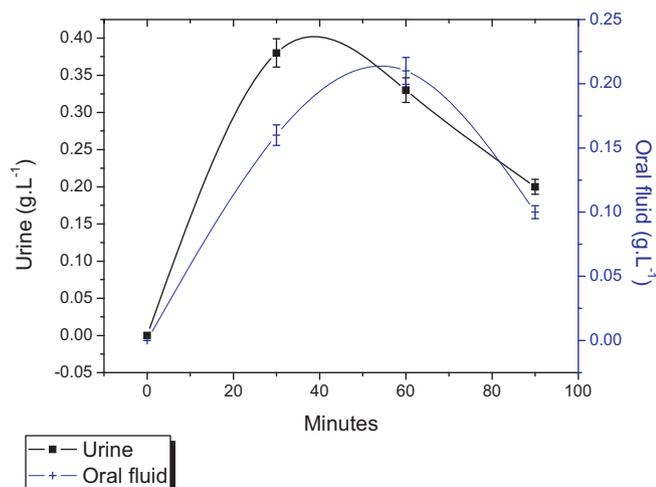


Fig. 5. Ethanol concentration in urine and oral fluid at 30, 60, and 90 min after beer ingestion for women (volunteers 1–5).

Jones [25] detected maximum ethanol concentration in oral fluid at 40 min after ethanol intake of 0.68 g/kg in men who had been fed prior to the experiment. Giang [14] achieved maximum ethanol concentration after 60 min for both genders, when volunteers also ingested food together with a larger ethanol concentration (0.8 g/kg for women and 1.00 g/kg for men).

For men, on average, the maximum ethanol concentration was reached in a shorter time as compared with data reported by Jones [25] and Giang [14], whose studies involved the presence of food. However, the time that was necessary to achieve maximum alcohol concentration for women took place at 60 min as in the study by Giang [14]. The composition of oral fluid depends on several parameters such as gender, individual's conditions and type, and duration of oral fluid secretion as cited previously [19].

To verify how the ethanol concentration in oral fluid and breath, and oral fluid and urine correlated, we investigate whether a linear association existed between the concentrations using the Pearson correlation. Oral fluid significantly correlated with urine and breath. The closer to 1 unit (in module) the value of the correlation, the greater the degree of linear statistical dependence between the variables [27]. Hence, the ethanol concentrations in oral fluid strongly correlate with the ethanol concentrations in breath and urine – the Pearson correlation values between oral fluid and breath were 0.88 and 0.96 and for oral fluid and urine were 0.93 and 0.91 for women and men respectively.

4. Conclusion

The ethanol absorption profile of oral fluid resembles the profile of exhaled air and urine in both genders as demonstrated by Pearson's correlation.

The ethanol concentration in oral fluid depends on the ethanol concentration in the blood. However, the legislation permits ethanol level equal to zero in driver's blood: Therefore, the oral fluid sample is applicable to monitor drivers, since it may indicate alcohol intake and, thus, blood alcohol levels above the zero limit.

Compared with blood, oral fluid provides a noninvasive way of collecting sample for alcohol analysis and even for analysis of other drugs prohibited by law, when employing the proper technique. Furthermore, compared with breath, saliva allows repeating the analysis if storage properly to preserve the ethanol concentration level at the time of collection, which indicates that oral fluid is a promising matrix to monitor traffic and drivers involved in accidents. The use of oral fluid to determine ethanol intake should

be interpreted as an independent analysis to evaluate a driver's sobriety and it cannot exclude the alcohol dosage in blood.

To better assess the validity of using oral fluid as a biological sample to investigate ethanol in the forensic field, it is necessary to evaluate other products such as food (chocolate candies containing liquor), medicines containing alcohol in the formulation, and oral care products such as mouthwashes, because they could affect ethanol levels in breath and oral fluid.

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