Recovery of spiked Δ^9-tetrahydrocannabinol in oral fluid from polypropylene containers

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Abstract

Oral fluid is currently used by Australian and international law enforcement agencies and employers to detect recent use of cannabis and other drugs of abuse. The main psychoactive constituent of cannabis, Δ^9-tetrahydrocannabinol (THC), is highly lipophilic and losses occur when in contact with plastic, possibly due to its adsorption onto the plastic surface. This study aims to investigate factors governing the interaction of THC with plastic and search for ways of overcoming such interaction so to improve THC recovery. As polypropylene is one of the most common types of plastic used in collection devices, it was the focus of this study. All experiments were done by preparing neat oral fluid samples spiked with THC in 2-mL polypropylene centrifuge tubes. Samples were transferred with or without prior addition of Triton® X-100 (0.25%) to glass tubes containing d_3-THC as internal standard and 0.1 M phosphate buffer was then added. Samples were extracted by liquid-liquid extraction using hexane/ethyl acetate (9:1 v/v), dried and analysed by gas chromatography-mass spectrometry (GC–MS) after derivatisation. No significant difference was found in terms of THC loss to plastic when the concentration ranged from 25–1000 ng/mL in the same volume of oral fluid. Varying the oral fluid volume (0.5–1.5 mL) while keeping THC at a constant concentration showed an upward trend with more loss associated with lower volumes. The use of Triton® X-100 significantly decreased the adherence of THC to the plastic tubes and increased the THC transfer (>96%) at all volumes tested. Degradation of THC during storage was also studied over a 4-week period and it was found that azide did not seem to play a significant role in preserving THC in oral fluid.

1. Introduction

THC is one of the major drugs of concern in police roadside testing programs as well as in workplace drug testing due to its high prevalence around the world. Oral fluid is an increasingly popular matrix to use in drug testing for a number of reasons, including its non-invasive collection, reduced risk of adulteration, a shorter window of detection and thus a more useful indicator of very recent ingestion than urine [1-5].

Many commercial oral fluid collection devices are available, several of them containing some form of stabilising buffer which dilutes the oral fluid collected. Previous studies have found that these devices often have difficulty collecting consistent volumes of oral fluid and accurate quantification of THC can be challenging [2, 3]. Expectoration is also problematic due to issues such as 'dry mouth' and foaming but it is still a viable collection technique, especially since it is the only way to analyse an accurate volume of oral fluid. Hence, it is important to know what interactions THC may have with the containers in which the samples are stored.

Sample containers are commonly made from polypropylene and such containers have been used in recent studies involving oral fluid [6-9]. Polypropylene was chosen for this study to investigate the adsorptive properties of THC to plastic surfaces when in the oral fluid matrix and also to observe any losses that occur during storage for up to 4 weeks.

2. Materials and methods

2.1. Materials

Capped polypropylene centrifuge tubes of 2-mL volume were obtained from Scientific Specialties Inc. (Lodi, CA, USA). THC-free oral fluid was provided from volunteers and used for the study on the day of collection. The absence of THC in collected oral fluid was

confirmed by following the sample preparation and analysis procedures described in the following paragraphs.

All solvents and chemicals used were analytical grade or higher. Methanol (MeOH) and ethyl acetate was obtained from RCI Labscan Limited (Bangkok, Thailand). N-Hexane was obtained from Scharlab (Barcelona, Spain). All water used was purified using an Arium Milli-Q system from Sartorius AG (Göttingen, Germany). THC (1 mg/mL in MeOH) and d_3-THC (0.1 mg/mL in MeOH) were purchased from Cerilliant (Austin, TX, USA). These reference standards were diluted with methanol to obtain working stock solutions for THC at 50 µg/mL and d_3-THC at 25 µg/mL. Di-sodium hydrogen orthophosphate anhydrous (Na_2HPO_4) and sodium dihydrogen orthophosphate monohydrate (NaH_2PO_4), used to make 0.1 M Sørensen's phosphate buffer were sourced from Ajax Chemicals (Auburn, Australia) and mixed to achieve pH of approximately 6. *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) with 1% trimethylchlorosilane (TMCS) was sourced from United Chemical Technologies (Bristol, PA, USA). Triton® X-100 was obtained from Sigma–Aldrich (Louis, MO, USA), sodium azide was obtained from BDH Laboratory Supplies (Poole, England), and Cozart® DDS buffer was obtained from Alere (Brisbane, Australia).

2.2. Sample preparation and liquid-liquid extraction (LLE)

Aliquots of freshly collected drug-free oral fluid were transferred into 2-mL polypropylene centrifuge tubes and spiked with THC standards in methanol at various specified concentrations. The absolute concentration of methanol in these spiked oral fluid samples was less than 4%. After capping, the sample tubes were vortex-mixed to ensure complete contact between sample and plastic surface. Unless otherwise specified, samples thus prepared were decanted into 10-mL screw-cap glass test tubes to avoid further contact with additional plastic surfaces. The centrifuge tubes were weighed before and after sampling to account for liquid loss from the decanting process. Deuterated internal standard solution (5 µg/mL) was added to the glass test tubes followed by addition of Sørensen's phosphate buffer

(1 mL). Hexane/ethyl acetate (2.5 mL; 9:1 v/v) was then added and the tubes placed on a roller mixer on moderate speed for 60 min. The organic upper layers (2 mL) were transferred into high recovery GC–MS vials (1.5 mL; PM Separations, Brisbane, Australia) and evaporated under a gentle stream of N_2 gas at 40 °C. Residues were reconstituted in 75 µL ethyl acetate and 50 µL BSTFA with 1% TMCS and heated at 75 °C for 20 min before analysis by GC–MS.

2.3. GC–MS

Chromatographic analyses were carried out using an Agilent 7890A/5975C GC–MS system with an Agilent 19091S capillary column (30 m × 250 µm × 0.25 µm). Splitless mode was used for injection and 2 µL were injected. The injector temperature was 280 °C. The oven was operated at an initial temperature of 140 °C for one minute, then increased at 40 °C/min to 300 °C and held for 2 min. MS was operated in SIM mode monitoring the TMS derivatives of THC and d_3-THC at *m/z* 386, <u>371</u> and 303 for THC and *m/z* 389, <u>374</u> and 306 for d_3-THC. The underlined ions were used for quantification. All calibration standards and samples were run in triplicate.

Data acquisition and analysis were performed using the included Agilent MSD ChemStation software package.

2.4. Validation studies

Serial dilution of the working stock solution of THC gave eight concentrations of THC in MeOH: 50, 25, 10, 5, 2.5, 1, 0.5, 0.25 µg/mL. 10 µL of each of these eight were spiked into a separate tube containing 500 µL drug-free oral fluid resulting in concentrations of 1000, 500, 250, 100, 50, 25, 10, and 5 ng/mL. Internal standard was then added to each tube (10 µL) giving a concentration of 500 ng/mL of d_3-THC. After addition of 1 mL of 0.1 M Sørensen's phosphate buffer (pH ~5.7), the LLE procedure was followed as previously described. Linearity of the calibration curve was calculated using a line of best fit with the acceptable

correlation factor set at >0.99. Inter- and intra-day precision and accuracy studies were carried out using quality control (QC) samples spiked at 15, and 75 ng/mL (n = 5). Accuracy was determined to be acceptable if the calculated concentrations fell within 15% of the concentration spiked (expressed as a percentage mean relative error or MRE). Precision was deemed acceptable if the percentage relative standard deviation (RSD) was <15%. The limit of detection (LOD) was the lowest concentration of analyte that was observed as a peak in the chromatograms at all monitored ion fragments. The limit of quantification (LOQ) was the lowest concentration of analyte that could be quantified with a %RSD of <20% and a MRE of <20%.

3. Results

3.1. Method validation

Linearity of the GC–MS method was achieved over the range of 5–1000 ng/mL with a correlation coefficient of 0.9990. The intra- and inter-day precision and accuracy of the method were satisfactory and are summarised in Table 1. The RSD values were 2.52–8.57% and the MRE ranged from 1.38% to 6.48%.

The LOD was determined to be 1 ng/mL. The LOQ was found to be 5 ng/mL. Precision and accuracy at this concentration level was determined to be 3.14% and 9.73% respectively.

3.2. Plastic surface and THC recovery

When THC was spiked into 1.5 mL neat oral fluid at six different concentrations (25, 50, 100, 250, 500 and 1000 ng/mL), all samples experienced a similar degree of THC loss to the polypropylene tubes, ranging from 22.8% to 29.3% (Figure 1). The concentration of THC in oral fluid samples of equal volume did not appear to affect the degree of adsorption to the plastic surface.

In contrast, when different volumes (0.5, 1.0 and 1.5 mL) of oral fluid containing the same concentration of THC (100 ng/mL) were tested, there was an apparent trend of increasing loss in lower oral fluid volumes (Figure 2).

3.3. Surfactant and THC recovery

THC loss to the plastic was found to be significantly minimised during repeated experiments in which Triton® X-100 (0.25%, approximately 10 times the critical micelle concentration or CMC) was mixed with the THC-spiked oral fluid samples before transfer to the glass test tubes for liquid-liquid extraction. As shown in Figure 2, the use of Triton® X-100 resulted in >96% recovery of THC from the polypropylene containers at all oral fluid volumes tested.

It was also noted that the absolute signal intensities of d_3-THC following extraction from the glass test tubes increased significantly when Triton® X-100 was present compared to the control experiments in which Triton® X-100 was not utilised (Figure 3).

3.4. Oral fluid proteins and THC recovery

In order to investigate the role of oral fluid proteins in THC recovery from the plastic surface, THC-fortified oral fluid samples were centrifuged at 7000 rpm for 10 min and the resulting protein pellets were collected, reconstituted in phosphate buffer and assayed for THC content. It was found that 51.7% of THC was recovered from the protein pellet fraction, while 28.8% of THC was recovered from the supernatant, giving a total THC recovery of 80.5%. In a parallel experiment in which Triton® X-100 was added into the polypropylene tube that contained the protein pellet, an additional 14.7% of THC was recovered, raising the total THC recovery to 95.2% (Figure 4).

To further investigate the role of proteins in THC recovery from plastic surfaces, THC was spiked into Sørensen's phosphate buffer and its recovery was compared with that fortified in oral fluid. When THC (100 ng/mL) was spiked in phosphate buffer (0.5 and 1.5 mL), a very

low recovery was observed: 24.5% in 0.5 mL buffer and 48.1% in 1.5 mL buffer (Figure 5). The extraction efficiency of the liquid-liquid extraction method was also determined for THC spiked into phosphate buffer with and without the addition of Triton® X-100. These extractions were carried out directly in the glass test tubes and did not involve any transfer from plastic tubes. The results showed a high recovery of THC even without the additive. From the plain phosphate buffer extractions approximately 90% of THC was recovered; when Triton® X-100 was present, the recovery increased to 106% when compared to control samples which had not undergone extraction.

3.5. Storage conditions and THC recovery

The effect of sodium azide on THC recovery following various storage protocols was investigated. Oral fluid (250 µL) fortified with THC (50 ng/mL) in polypropylene centrifuge tubes was mixed with 750 µL of either Sørensen's phosphate buffer alone, Sørensen's phosphate buffer with azide (1%), or the Cozart® DDS buffer containing 0.1% azide. These samples were stored either at 4 °C in a refrigerator or at 20 °C in a cabinet for up to 4 weeks. After addition of Triton® X-100, aliquots (500 µL) of these samples were analysed for THC content.

As shown in Figure 6, samples treated with the Cozart® buffer suffered only a minimal loss of THC over the 4-week period while refrigerated, but even at room temperature the losses were small. More loss was observed in samples treated with phosphate buffer and azide, however these losses were still minimal up to 3 weeks into storage in a refrigerator. The samples kept at room temperature however, showed a larger loss and both treatments resulted in 40–50% loss of THC by the end of the 4 weeks. Whilst the phosphate buffer treated samples lost more THC by the fourth week, up until then there was minimal difference between the concentrations of THC found in these samples and the samples treated with azide.

4. Discussion

THC is known to be highly lipophilic and poorly water soluble, having a high octanol/water partition coefficient (log P = 6.97) [10]. It is therefore generally accepted that THC can interact with the non-polar plastic material via non-covalent interactions and adsorb to plastic container surfaces. It is also possible that THC degrades readily in oral fluid by way of metabolic action of microorganisms present in the matrix. Both these forces can be overcome by use of buffers containing surfactants and preservatives, a practice followed by some commercial manufacturers supplying oral fluid collecting devices. However for the purpose of drug testing, these buffers significantly dilute the oral fluid and no collection device can accurately measure the absolute volume of oral fluid collected, making concentrations detected difficult to interpret.

In our study we have shown that storage of THC-containing oral fluid in plastic containers led to a poor recovery of THC during quantitative analysis, supporting the view that THC binds with plastic surfaces. We have demonstrated that the loss of THC to plastic surfaces when in the neat oral fluid matrix is relative to the surface area to volume ratio with larger ratios resulting in a greater loss. Choi et al. [7] made a similar observation when they found that a higher THC loss over 6 days occurred in polypropylene containers of a larger internal diameter. This can be explained by the larger surface area to volume ratio of the containers with larger internal diameter. We anticipated that lower concentrations of THC would lead to a higher loss as there might be less competition for adsorbing space on the plastic surface; however this was not observed under our experimental conditions. This may be due to the presence of proteins or other materials in oral fluid which binds with THC and help keep it from adsorbing to the container surface. This was shown to be likely since the supernatants of centrifuged samples had such a low recovery of THC compared to the protein-rich reconstitute (Figure 4). Additionally, a very low recovery was found from plain phosphate buffer when compared to oral fluid spiked at the same THC concentration in the same

volumes, which further supports the possible role of proteins in binding THC in oral fluid. Protein binding of THC also explains the low absolute recovery of both THC and d_3-THC observed from oral fluid during the liquid–liquid extraction process conducted in glass test tubes compared to the much higher recovery when extracting from plain phosphate buffer. It is noteworthy that at this stage we do not know what specific component or components in the protein-rich fraction of oral fluid are responsible for binding THC. During the course of our investigation, it was demonstrated that the THC had not degraded in the short term since the addition of non-ionic surfactant Triton® X-100 increased recovery of the spiked THC to almost 100%. Other researchers have previously published papers describing loss of THC over time [8, 11], but none have given data on immediate losses.

We have previously reported that THC has excellent stability in the Cozart® buffer with an average recovery of 89% when stored in a refrigerator (4 °C) for 13–18 months [12]. The current study found that even at room temperature (20 °C, in the dark), there is minimal loss over a 4-week period. Ventura et al. [13] reported that adding sodium azide (0.1%) to oral fluid helped to prevent degradation of several illicit drugs during up to 7 days of storage at 25 °C and 37 °C and up to 2 months at 4 °C and –20 °C. Sodium azide is also utilised in many commercial oral fluid collecting buffers including the Cozart® DDS buffer which contains 0.1% sodium azide. In our study, sodium azide did not seem to give any benefit over phosphate buffer as an additive to reduce loss as both sets of samples showed a loss of almost 25% from refrigerated samples and over 50% from the room temperature samples after a 4-week period of time. Our results show that azide has no protective effect on THC in oral fluid, at least not over the 4-week period studied, although its long term (>4 weeks) effect remains to be further studied. Conversely, the samples stored in Cozart® DDS buffer were only observed to lose 10% of added THC when refrigerated and 15% at room temperature after four weeks of storage. This study avoided the adsorption issue by adding Triton® X-100 during sample analysis since we have shown this significantly increases recovery of THC

from plastic surfaces. Therefore we can assume that the losses seen in our study are due to the degradation of THC and not adsorption. We can also assume that since the addition of sodium azide in large excess would have prevented microbial growth, the degradation losses of THC observed under our experimental conditions are not due to microbial action but to other factors such as oxidative degradation. Moore et al. [14] previously reported that THC loss during storage may be exacerbated by chemical reactions induced by oxygen or other environmental factors such as fluorescent lighting. Exposure to light and oxygen was minimised during the current study by storage in the dark and the relatively small space available in the storage tubes used. This may explain the difference between these results and our previous finding that more than 50% of the spiked THC was lost in just 1 day after storage at room temperature when larger tubes were used and left on the bench [12]. These results suggest that addition of anti-oxidants rather than azide into oral fluid may be more beneficial in THC preservation. Given the importance of detecting THC in oral fluid to society, further research into understanding other factors that govern the behaviour of THC in the biological matrix is warranted.

5. Conclusions

It was demonstrated that THC had the tendency to bind to polypropylene surfaces, leading to poor extraction recovery in neat oral fluid. The recovery of THC is dependent on the oral fluid volume to inner surface area ratio; smaller oral fluid volume in larger containers suffered from a higher loss of THC. Use of Triton® X-100 can significantly increase the THC recovery from polypropylene containers.

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Tables and Figures

Table 1. Intra- and inter-day precision and accuracy results for the LOQ and QC samples (n = 5).

Concentration level (ng/mL)	Intra-day precision (RSD%)	Intra-day accuracy (MRE%)	Inter-day precision (RSD%)	Inter-day accuracy (MRE%)
5	3.14	9.73	1.81	8.32
15	8.57	6.48	8.18	1.99
75	2.52	1.38	6.37	5.37

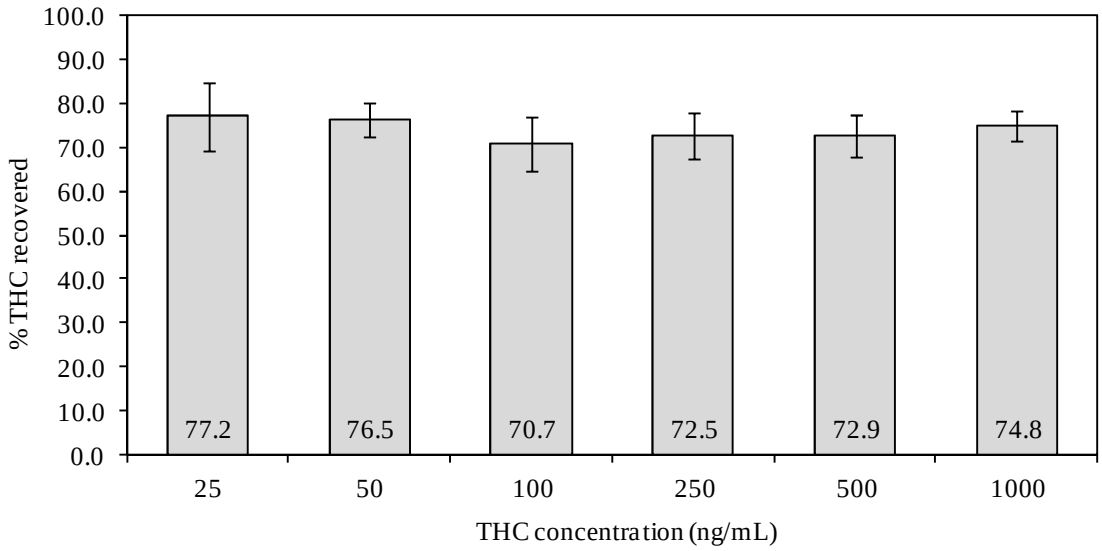

Figure 1. Recovery of THC from polypropylene tubes at various concentrations in 1.5 mL oral fluid. Data values represent the mean; error bars represent the standard deviation (n = 3).

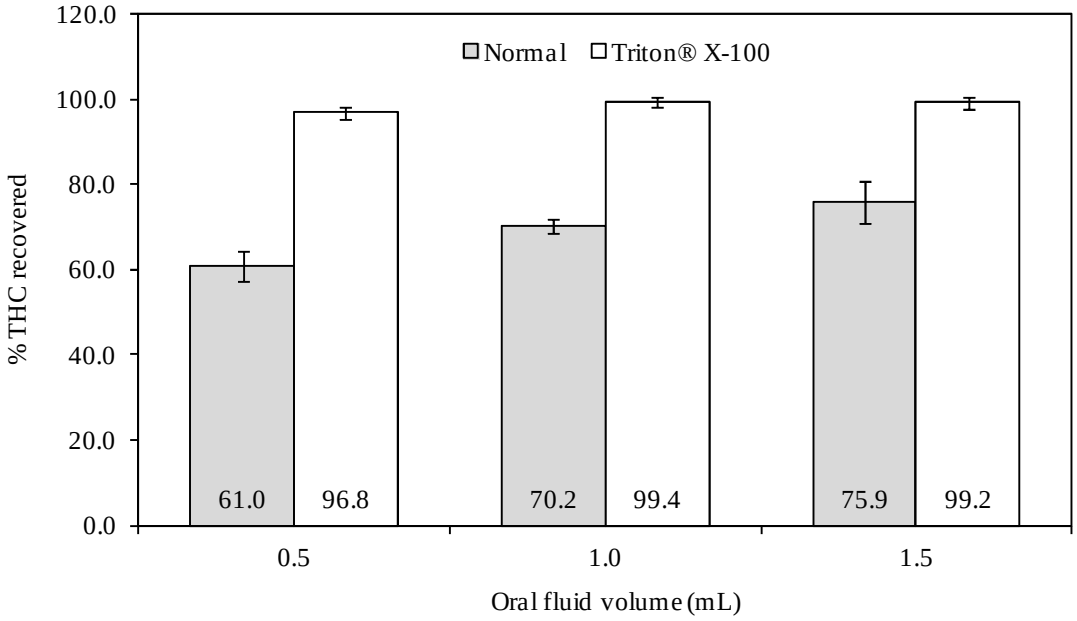

Figure 2. Recovery of THC from oral fluid volumes of 0.5, 1.0 and 1.5 mL not treated (normal) and treated with 0.25% Triton® X-100, all spiked at 100 ng/mL THC in polypropylene tubes. Data values represent the mean; error bars represent the standard deviation (n = 5).

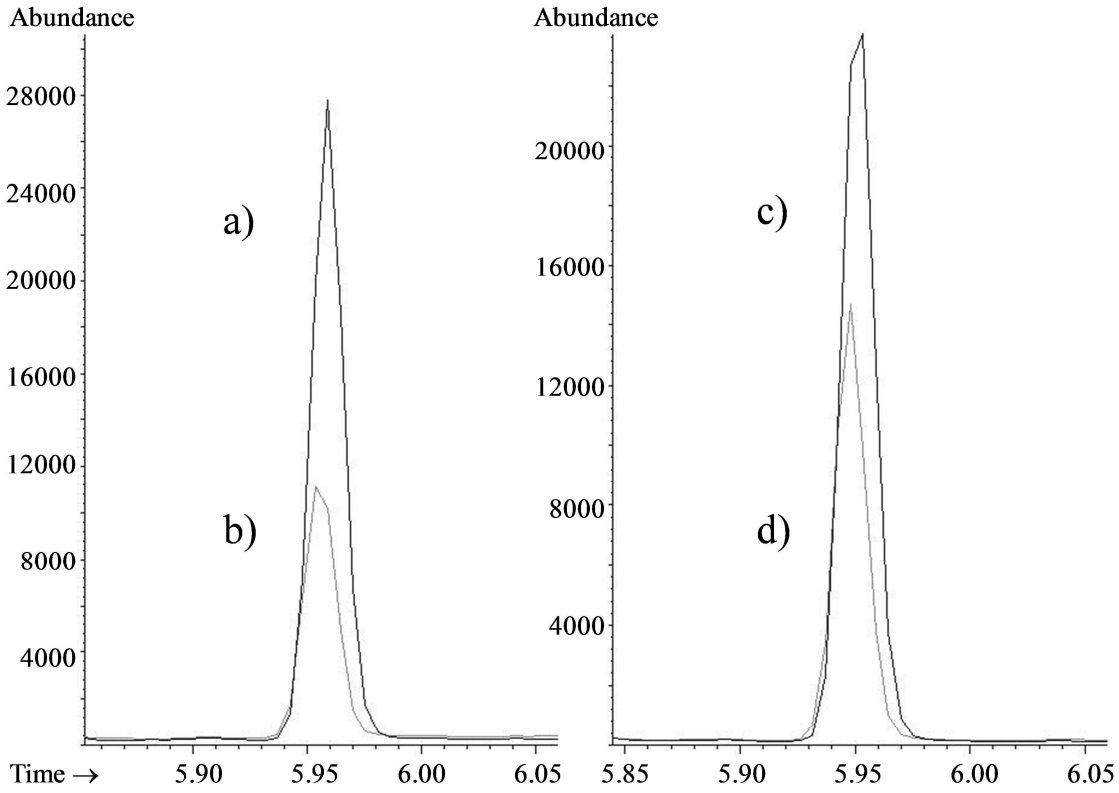

Figure 3. GC–MS chromatograms showing increased signal intensity from 1 mL oral fluid samples spiked with 100 ng/mL THC and internal standard after transfer and extraction: (a) *m/z* 371 (THC) from a Triton® X-100-treated sample; (b) *m/z* 371 from an untreated sample; (c) *m/z* 374 (d_3-THC) from a Triton® X-100-treated sample; (d) *m/z* 374 from an untreated sample.

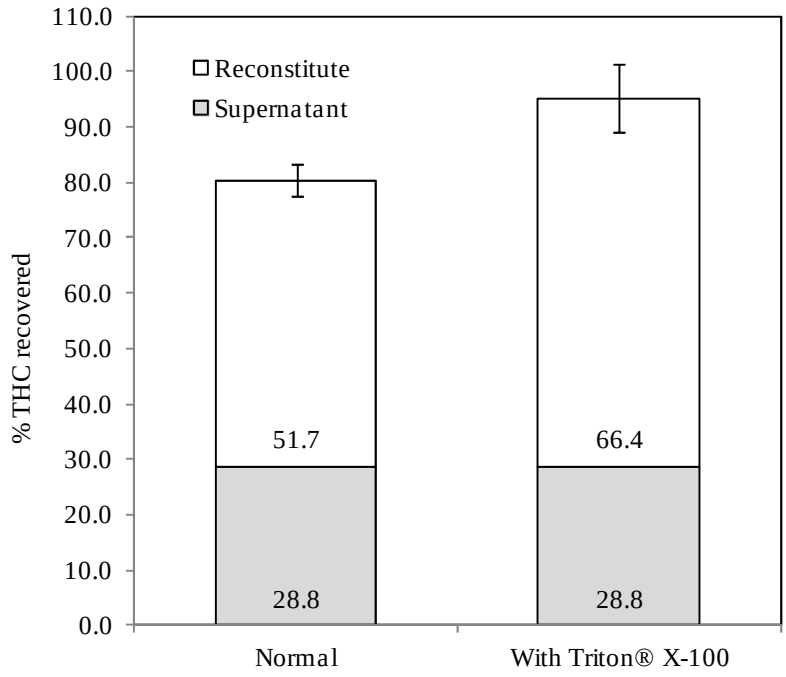

Figure 4. Recovery of THC from the supernatant and reconstitute of protein pellets after centrifugation of oral fluid samples. THC was spiked at 100 ng/mL in polypropylene tubes. Samples without treatment of Triton® X-100 (normal) are represented on the left, on the right are the samples treated with Triton® X-100 after supernatant was removed. Data values represent the mean; error bars represent the standard deviation (n = 5).

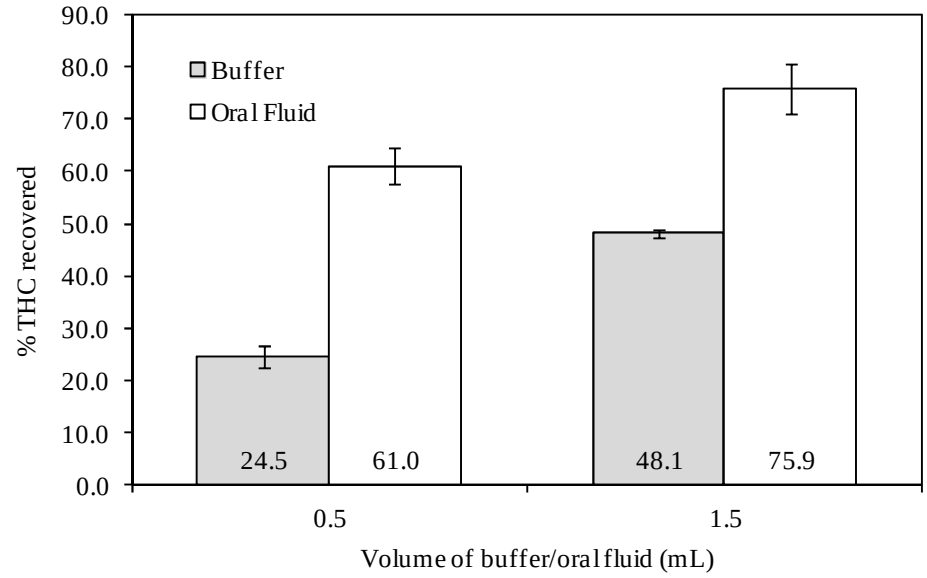

Figure 5. Comparison of THC recovered from phosphate buffer and oral fluid spiked into 0.5 and 1.5 mL at 100 ng/mL concentration in polypropylene tubes. Data values represent the mean; error bars represent the standard deviation (n = 3).

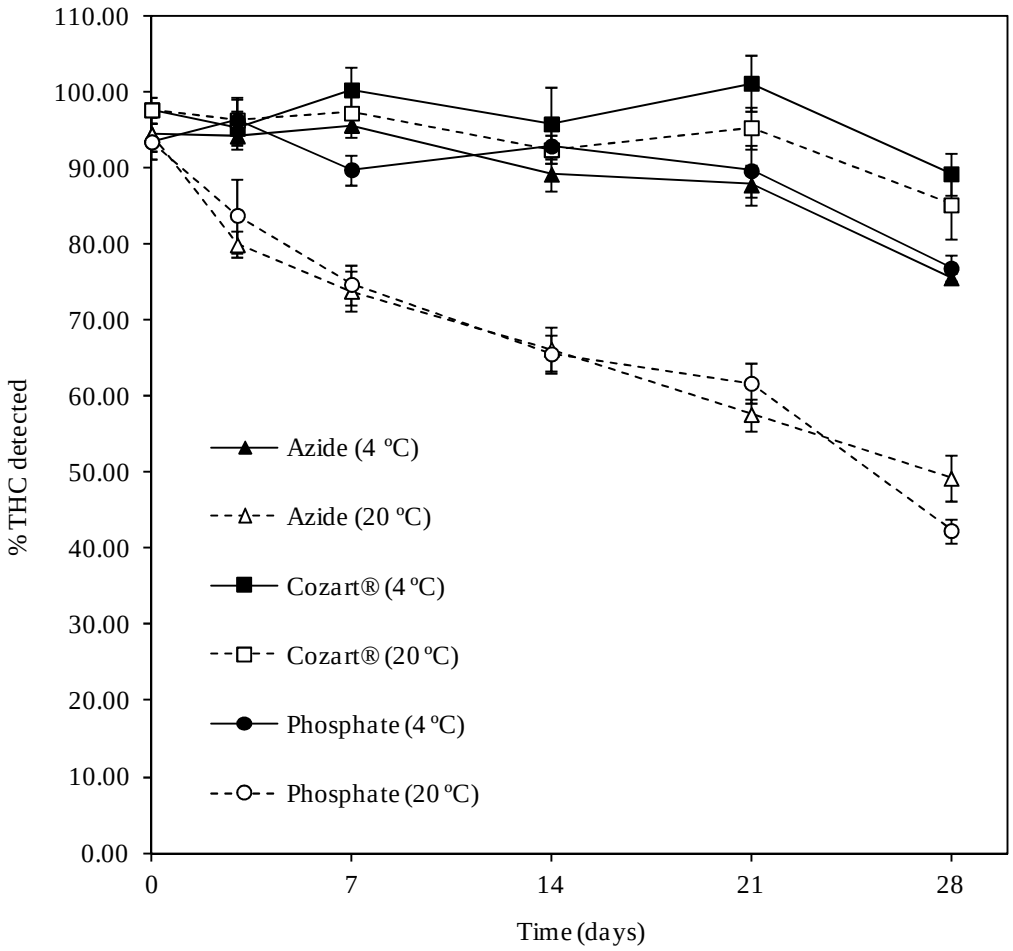

Figure 6. Percentage of THC remaining in oral fluid during storage from 3 to 28 days in the presence of 0.1 M phosphate buffer, 0.75% sodium azide solution and Cozart® DDS buffer solution. Results were means from 4 repeated experiments (n = 4) with standard deviations represented by the error bars.