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Use of Monoclonal Δ-9-Tetrahydrocannabinol Antibodies Chemically Bound to a Polystyrene Surface Using Glutaraldehyde for the Purpose of Extracting Δ-9-Tetrahydrocannabinol and Δ-9-Tetrahydrocannabinol Carboxylic Acid from Postmortem Whole Blood Samples for Analysis by Gas Chromatography Mass Spectrometry

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USE OF MONOCLONAL Δ-9-TETRAHYDROCANNABINOL ANTIBODIES CHEMICALLY BOUND TO A POLYSTYRENE SURFACE USING GLUTARALDEHYDE FOR THE PURPOSE OF EXTRACTING Δ-9-TETRAHYDROCANNABINOL AND Δ-9-TETRAHYDROCANNABINOL CARBOXYLIC ACID FROM POSTMORTEM WHOLE BLOOD SAMPLES FOR ANALYSIS BY GAS CHROMATOGRAPHY MASS SPECTROMETRY

by

Thomas Sidney Pittman

A Dissertation
Submitted to the Graduate Studies Office
of The University of Southern Mississippi
in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy

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ABSTRACT

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Quantitations of drugs and their respective metabolites in postmortem blood samples using gas chromatographic instrumentation is a primary analytical practice used to determine if drugs played a role in or were the cause of a victim's death. Postmortem blood samples often prove difficult to work with due to interfering substances formed during the putrefaction process. Attempts to eliminate interfering substances with present day extraction methods can be time consuming, costly and often ineffective when dealing with drugs that exhibit toxicity or impairment at very low concentrations. This study was conducted using monoclonal antibodies chemically bound to a polystyrene surface to extract Δ-9-Tetrahydrocannabinol and its major carboxylic acid metabolite from postmortem blood samples. The device was a Falcon® cell culture flask with 12.5 cm^2 of surface area. To each flask was added a 5% solution of glutaraldehyde followed by 5 μg/ml of antibody. Binding studies for THC and THCA using ELISA reagents resulted in an average binding capacity of ≥ 200 ng/ml for individual analytes. When both analytes were added at equal concentrations, binding capacity for THCA fell as the concentration

for THC was increased. Percent yield studies demonstrated an average 54% yield for THC and an average of 49% yield for THCA. Paired t test for THC demonstrated a significant difference in two runs where $t_{0.1(8)} = 3.355$ and paired t values were 4.384 and 6.034. Two runs for THCA were $t_{.01(27)} = 2.771$ had paired t values of 9.596 and 8.827 which also demonstrate a significant difference. All samples reported as "None Detected" for THC or THCA by MCL were found to contain no THC or THCA by this extraction method. Ten samples reported by MCL as "unable to report due to interfering substances" for THC were run twice with two samples showing no THC detected and eight of the samples showed no interferences present when using this extraction device.

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CHAPTER I

INTRODUCTION

Chromatographic analysis for and quantitations of drugs along with their respective metabolites in postmortem blood samples is a primary analytical practice used in forensic toxicology to determine whether drugs played a role in or were the cause of a victim's death because drug testing is the only objective means to determine use (Niedbala et al., 2001). Postmortem blood samples often prove a difficult matrix to analyze due to interfering substances produced during the natural putrefaction process that begins shortly after death. Other natural constituents of whole blood or other drugs can also cause problems when working with postmortem blood samples.

Liquid/liquid extraction schemes of drugs from biological matrices where first devised in the 1850s (Levine, 2003) and the process and chemistry of such extractions of drugs from postmortem biological samples remain basically the same today. Procedures with added steps can be used in efforts to remove interfering substances such as back extractions of samples into acids or alkaline buffers and then a second extraction at an acid or alkaline pH, but these steps add time and cost to the process. The following is an example of how a back extraction would proceed. To a 16x125 screw cap glass tube, 1-2 ml of sample would be buffered to pH 4.5 with 1-2 ml of an acetate buffer, vortexed and then 8-10 ml of an appropriate organic solvent added. The sample would then be shaken or rotated from 15 to 30 min followed by centrifugation for 20 min. The organic phase would be transferred to a clean tube and 1-2 ml of an alkaline buffer added to the organic solvent. The solvent would again be shaken or rotated and centrifuged. The organic phase this time would be discarded and an acidic buffer added to the aqueous phase. An

appropriate organic solvent would be added, 8-10 ml, and the sample shaken or rotated for a third time followed by centrifugation. The organic layer would be transferred to a new tube and evaporated to dryness. The extract would be dissolved in a desired injection solvent for analysis. There have now been three extraction steps, several milliliters of buffer and up to 20 ml of an organic solvent used in this procedure. Depending on the number of calibrators, controls and samples, a back extraction procedure may take 4-6 hours to complete.

Percent recovery of drugs becomes an issue with the use of multiple extraction steps. Many drugs encountered today result in impairment or toxicity with concentrations in the ng/ml range and few extraction procedures achieve 100% recovery. With each extraction step, a portion of the drug is lost which may result in not being able to detect, confirm or quantitate a drug during analysis.

Solid phase extraction techniques (SPE) provide an alternative to liquid/liquid extractions for drugs in biological matrices. Forensic SPE procedures were initially designed for extraction of illicit drugs from urine samples for confirmation testing required by employee drug testing programs. The use of SPE columns is also used to extract drugs from serum or plasma, but these two matrices do not present the same type problems found with postmortem whole blood samples.

SPE is done by either negative or positive pressure devices. Negative pressure systems have a vacuum chamber that pulls the sample through the columns. A positive pressure system simply pushes the sample through the columns. Both type SPE systems possess inherent problems of clogging when postmortem whole blood is the sample being extracted. Degradation products in whole blood often cannot be centrifuged into a pellet or filtered out of the sample and it is these components that clog SPE columns.

Once a column is clogged, it can no longer be used and the sample is lost.

There are critical steps in SPE that makes the procedure work most efficiently. One is the wetting of the columns with water and an alcohol prior to adding the sample. Next is the controlling of how fast the sample flows through the column to ensure the analyte of interest is retained. The third step is the drying of the column prior to elution. If any of the steps is not strictly controlled, the extraction procedure will fail.

Time consuming steps such as the need to make fresh reagents for daily use and maintenance of equipment add cost to SPE methods, and elution of interfering substances remain problematic as in liquid/liquid extractions.

Another issue that arises from present extraction procedures is maintenance of chromatographic instrumentation. The principle behind gas chromatography is to volatilize an injected sample and use a gas to move the constituents through the column achieving separation. Heated injection liners of gas chromatographs and chromatographic columns are subject to contamination from various breakdown products of blood and other type biological samples that do not volatilize and adhere to the injection liner and the front several inches of the column. Analytes of interest that adhere to the these contaminants often fail to elute, show a reduction in concentration or poor peak shape making quantitations difficult at best. The glass liners often have to be discarded and the at least 18 inches from the front of the column cut to restore quality chromatography. Once a column has been cut, retention times of all analytes will change requiring altering SIM windows or gas chromatographic settings changed to maintain established retention times.

Degradation products that do flow through the column will eventually result in a loss of mass spectral sensitivity. Compounds are deposited on the metal lenses of the

mass spectral source causing a film to develop that interferes with the electronic tuning of the mass spectrometer. Once the internal stainless steel lenses have become sufficiently dirty and the instrument cannot be Autotuned to specifications, a cleaning of the lenses is necessary. Cleaning requires shutting down the instrument for at least one day to accomplish. After cleaning, the instrument must be recalibrated to ensure the cleaning was successful. If not, the cleaning must be repeated. When many whole blood samples are routinely analyzed, cleaning becomes a common routine that requires an inventory of expensive replacement parts that are of one time use or are not amenable to cleaning. Loss of sensitivity due to dirty extractions is a major concern when analyzing drugs such as marijuana that play a role in accidental deaths due to impairment of an individual's central nervous system.

Therefore, there is a need for a simple, inexpensive and robust device for extraction of drugs from postmortem blood samples that does not suffer from or that will significantly reduce problems with interfering substances. The chemical binding of antibodies to a polystyrene surface for the purpose of extracting a drug from postmortem blood samples is a feasible theory to the approach, possibly reducing many if not all the problems discussed above.

CHAPTER II

REVIEW OF LITERATURE

Marijuana

Marijuana is the common name given to the plant *Cannabis sativa.* There are reportedly some 400 chemicals in marijuana with approximately 61 being classified as cannabinoids (Marnell, 2006). Historically marijuana has been used by man for thousands of years. The plant has provided fibers from the branches and stems for ropes, clothing, and sails for ships as well as food from seeds and oils found in the flowering parts of the plant. Marijuana was used as medicine in China and India as early as 3000 B.C.

Marijuana is not a native plant to North America (Marnell, 2006). The plant was introduced by Europeans colonizing North America and grown as a cash crop. The plant grew well in most all areas of North America and thus successfully spread across what is today the United States. Marijuana continued to be grown as a cash crop in the United States until the around 1937 when the Federal government became concerned about its abuse as a drug and passed the Marijuana Tax Act in an attempt to stop the growing and The psychoactive constituent in marijuana is Δ-9-tetrahydrocannabinol (Δ-9-THC) and is classified mainly as a central nervous system hallucinogenic although the psychological effect of the drug varies from person to person and by dosage (Huestis, 2003). The concentration of Δ-9-THC considered to cause central nervous system impairment resulting in an individual's inability to safely operate a motor vehicle is considered 2 ng/ml and above in the United States and other countries (Garriott et al., 1986; McBay, 1988; Samyn et al., 2002; Lin & Lin, 2005). This means on any given analysis of marijuana for purposes of determination of impairment of an individual a GC/MS with

ectron impact (EI) mass spectral instrument the limit of detection (LOD) and limit of quantitation (LOQ) must be less than 2 ng/ml to ensure that a quantitative result of 2 ng/ml is accurate since marijuana is the most abused illegal drug in the majority of countries including the United States (Mason & McBay, 1984; Crouch et al., 1993; Niedbala et al., 2001).

The pharmacodynamics of marijuana show several modes of action (Hollister, 1992). The human brain demonstrates high stereospecific receptors for the levo (-) isomer of Δ-9-THC with some five times the potential effect on the user when compared to the dextro (+) isomer. Five distinct areas of the brain have been identified with the levo specific receptors.

Δ-9-THC is highly lipophilic and produces effects on cell membranes similar to several other drugs of different classifications such as ethanol, LSD, amphetamines and morphine. These differing actions make it difficult to classify marijuana within a drug group.

Smoking is the most common means of introducing Δ-9-THC into the central nervous system. Peak concentrations of 19-26 ng/ml were reached within 10 min of smoking a cigarette containing 10 mg of Δ-9-THC (Agurell et al., 1973). These levels decreased to less than 5 ng/ml within 2 hours. Other studies have shown peak concentrations of Δ-9-THC ranging from 46-188 ng/ml following smoking of a single marijuana cigarette (Rosenfeld, Bowins, Roberts, Perkins & MacPherson, 1974; Mason, A.P., Perez-Reyes, M., McBay, A.J. & Foltz, R.L., 1983 & Kelly et al., 1993). In these studies, the concentration levels of Δ-9-THC fell to or below 5 ng/ml within a two hour period.

Oral ingestion of marijuana after being cooked shows much slower absorption

rates via the gastrointestinal tract with peak blood concentrations being reached in 1-3 hours. The peak concentrations are lower than levels seen in smoking only averaging 6 ng/ml when 20 mg of Δ-9-THC was ingested (Baselt, 1982).

A major problem that exists in the extraction of marijuana for analysis at these levels is the inability to eliminate interfering substances during the extraction process in many samples. Δ-9-THC is extracted most efficiently in a liquid/liquid extraction at an acid pH of 4.5 (Chu & Drummer, 2002). Acidic extracts tend to produce "dirty" extracts due to interaction with and denaturing of proteins. Neutral compounds and lipids are also extracted more efficiently into organic solvents at an acid pH adding to the possible range of interfering substances in the final extract. This is the rational behind using a back extraction to reduce unwanted compounds when performing an extraction for acidic drugs. Back extractions will improve the chromatography by eliminating some contaminants, but the problem arises whenever extraction efficiency of a drug is poor and the expected concentration is in the ng/ml range.

SPE extractions where both Δ-9-THC and Δ-9-THCA are extracted using the same column also use an acidic elution solvent for the Δ-9-THCA resulting in the same problems. Some SPE procedures have proven successful for extracting both analytes from the same column (Moeller, M.R., Doerr, G. & Warth, S., 1992; D'Asaro, 2000; Gustafson, R.A., Moolchan, E.T., Barnes, A., Levine B., & Heutis, M.A., 2003) but with plasma samples and not whole blood samples. Compounds that remain on the column following initial washing steps at a neutral pH for Δ-9-THC are eluted by the acidic solvent into the final extract of Δ-9-THCA. These interfering substances are often responsible for failed analyses in samples containing low concentrations of Δ-9-THC.

A key requirement of confirmations is ion ratios that must fall within a specified

range to be considered acceptable and the drug confirmed present. Interfering substances are the reason most analyses fail in not meeting the ion ratio criteria. Methods for whole blood extractions that have proven reliant for confirmation and meeting ion ratio criteria often combine extraction protocols of liquid/liquid and SPE with GC/MS analysis (Thompson & Cone, 1987; Felgate & Dinan, 2000). The need to use to separate methods is time consuming and expensive.

Antibodies

Antibodies are "Y" shaped glycoproteins made up of two heavy chains and two light chains bound together by disulfide bonds (Kindt, Goldsby & Osborne, 2007). The light chains and heavy chains form two binding regions located at their two amino terminal ends that are highly variable in their amino acid sequence. These highly variable regions provide specificity for binding to most any molecule, including metals (Wylie et al., 1992; Love et al., 1993).

Antibodies treated with either the enzymes pepsin or papain result in particular fragments. Antibody treated with pepsin result in fragments called "Fab" and "Fc". The Fab fragments consist of the light and heavy chains that form the upper area containing the two binding sites as individual pieces. The Fc fragment is the lower portion or tail of the antibody consisting of two heavy chains. When treated with papain, the only fragment recovered is the "$F(ab')_2$" which is the two amino terminal ends still attached by disulfide bonds. The biological role served by antibodies is to bind a specific area or epitope located on an antigen and aid in its' destruction or removal from the body.

B cells are formed within and released from bone marrow with each B cell carrying antibodies bound to their cell membranes. The B cells are referred to naïve since they have yet to encounter antigen that has an epitope that will bind their specific

antibody. When these B cells come into contact with an antigen possessing an epitope that matches the binding area of the antibody, the B cells are up regulated and begin to divide. The new cells produced become either memory B cells or plasma cells.

Plasma cells secrete antibodies identical to the ones found on the original B cell. These cells can be isolated and used for the purpose of producing antibodies against specific analytes of interest such as drugs or classes of drugs.

For a substance to be immunogenic it must have a molecular weight around 2000 daltons or larger. Smaller molecules will not evoke an immune response that will result in the production of antibodies. Most drugs do not have molecular weights in that range and must be bound to a larger protein structure (Smith, 1999). Bovine serum albumin (BSA) and bovine thyroid globulin (BTG) are two proteins used for attachment of drugs through linking molecules used to produce an immune response adequate for the production of antibodies.

To facilitate the drug to act as the epitope, spacer molecules are placed between the drug and protein so the drug is not located directly on the protein thereby increasing the chances of the drug itself binding antibodies on B cells. The protein-drug complex is injected into a suitable host such as a goat or rabbit and the immune response of the host animal is allowed to proceed. Some portion of the drug or the entire drug molecule may act as an epitope that binds an antibody with sufficient affinity resulting in cellular division of plasma cells that will produce antibodies specific for that drug structure.

Plasma cells that produce the most specific antibodies are isolated from the host animal and mixed with mouse tumor cells to form hybridomas. These hybridoma cells are cultivated and continue the production of antibodies. Next the hybridoma cells are separated and screened to determine which cell line is producing the most specific

antibody. This specific cell line is isolated for culturing and further purification of its antibodies. Antibodies that come from a single cell line are called monoclonal antibodies and demonstrate the best specificity for drug assays.

Immunoassay Methods

Catt and Niall (1967) were one of the first to report on using a solid support for immobilization of antibodies for use in radio-immunoassays (RIA). The technique of binding antibodies to a solid surface for the isolation or testing of presence of specific analytes of interest has since found widespread use in various scientific disciplines. Most of these immunoassay or affinity techniques were initially used for the purification of proteins, hormones and other ligands of interest in biochemistry and diagnostic and clinical chemistry testing (Hage, 1998). An example of a clinical test is the analysis of fibrinogen in plasma. Here a specific anti-fibrinogen antibody is used to capture fibrinogen dissolved in an aqueous solution similar to plasma as it flows through a column. Other constituents that are not bound by the ligand are washed from the column prior to switching solvents to elute the fibrinogen. The fibrinogen can now be quantitated by a UV/VIS detector.

RIA methods led to the development of several other immunoassay techniques. All these methods take advantage of specific antibodies to achieve the desired result of screening for the presence and/or quantitation of specific analytes. Enzyme multiplied immunoassay technique (EMIT), fluorescent polarization immunoassay (FPIA), cloned enzyme drug immunoassay (CEDIA) and enzyme linked Immunosorbent assay (ELISA) are all techniques used in the forensic field as screening assays for illicit and therapeutic drugs. The forensic field uses such techniques for the expressed purpose of screening for the presence of illicit drugs and not for quantitation or confirmation as seen in the clinical

area.

Affinity Chromatography

Affinity chromatography has been an analytical method since around 1910 (Axen, Porath & Ernback, 1967). The purpose of the technique is to separate specific molecules from all others within a complex matrix. A key to using antibodies bound to a solid surface is that the system can be used over and over (Kim, H.O., Durance, T.D., & Li-Chan, E.C., 1999). Such devices used in a forensic setting could only be used once which is a reason for development of a simplified device at low cost.

Initially, the method was used to isolate and purify large biomolecules such as proteins, carbohydrates and nucleic acids. The first solid supports for affinity ligands were polysaccharides activated using cyanogen bromide (Axen et al., 1967). More recently affinity chromatography has been applied to the isolation of drugs from either humans or in food products from animals. Haagsma and van de Water (1992) used affinity chromatography for isolating antibiotic residues in food products generated from animals. Identification of drugs in human urine using an online immunoaffinity method coupled with high performance liquid chromatography mass spectrometry was performed by Rule and Henion (1992).

Webb et al. (1996) developed an immunoaffinity extraction technique for the analysis of LSD which was also coupled with a high performance liquid chromatographic system, but the experiments demonstrated poor consistency between analyses. Their study used an affinity gel made from Protein A Sepharose CL-4B containing antiserum against LSD. Each cartridge contained 0.36 g of the gel and 0.2 ml urine was added for analysis. The column was rinsed prior to elution of LSD with phosphate buffer, water and 0.5 ml absolute ethanol. LSD was eluted with 1.5 ml ethanol, dried and then

reconstituted with 100 uL of mobile phase. The internal standard, methysergide, was added following extraction which may account for the inconsistent results. The only publication using gas chromatography with an EI mass-spectrometer found to date is Bagnati et al. (1991) who identified zeronal and the metabolite β-zeronal in calf urine using a gel based extraction method.

There are several methods that have been developed for the binding of molecules to a solid matrix such as a polystyrene surface for the subsequent binding of specific types of ligands (Hermanson et al., 1992). The manner and orientation of ligand attachment to the matrix is one of the most important questions to consider prior to developing an affinity based extraction device. The best type of attachment is covalent bonding between the surface and the ligand (Nisnevitch & Firer, 2001). This ensures the ligand remains in place during wash steps that remove unwanted substances. Secondly the ligand must also remain attached during the elution of the analyte of interest so that the ligand itself does not become a contaminant. Hale (1995) used an iminodiacetate resin in conjunction with $CoCl_2$ and H_2O_2 for attachment of antibody specifically using the Fc region to ensure proper orientation of binding sites.

Many of the methods developed for use by affinity chromatography for subsequent purification and identification use some form of a high performance liquid chromatography system (HPLC) coupled with various types of detectors such UV/VIS, diode array or multiple mass spectrometer techniques (Johns et al., 1996; Hage, 1998; Thompson & Cone, 1987).

Polystyrene is a matrix that possesses a hydrophobic surface that contains no functional groups for direct attachment of either spacer molecules or ligands (Hermanson et al., 1992). The surface may be treated with alkaline buffers in the pH range of 9.0 to

9.5 making the surface amenable to passive, noncovalent absorption of affinity ligands such as antibodies. The problem that arises with this type preparation is there is no way to ensure the ligand will not be removed from the surface during experiments.

Glutaraldehyde has been used for many years as a linking molecule for attaching proteins to polystyrene surfaces (Klasen et al., 1982). Polystyrene plates can be modified with glutaraldehyde to provide a covalent linking molecule for attachment of proteins. The chemical reaction of binding one end of glutaraldehyde to the polystyrene is accomplished at an acidic pH by producing radical vinyl bonds between the polystyrene and one end of the glutaraldehyde molecule (Hermanson et al., 1992). Antibodies are then attached to this layer of glutaraldehyde molecules at an alkaline pH (8-9.5) with chemical bonds formed between the free aldehyde groups with amino groups located on the antibody. This is a simple procedure that does not require any specialized instrumentation or chemicals and can be carried out in most all laboratories with ease. The one drawback to the procedure is the orientation of antibodies cannot be controlled. Thus, a sufficient concentration of antibodies and surface area are required to ensure sufficient numbers of binding sites will be available for drug capture.

Another consideration of orientation is positioning of antibody in a device. The issue becomes how far an antigen may have to diffuse when dealing with a flat surface (Stenberg & Nygren, 1988). Once antibodies in a given area are bound with antigen, then unbound antigens must be able to diffuse to non-bound antibodies. If this distance is too far, then a reduction in percent yield can be expected.

Enzyme Linked Immunosorbent Assay

Enzyme linked immunosorbent assays were initially developed for special clinical applications followed in time for specific drug quantitations in serum or plasma and for

drug screening in urine and whole blood for drugs of abuse. ELISA assays work based on competitive binding between analyte and enzyme tagged analyte for antibody binding sites in reaction wells. In general, the more analyte present the more antibody sites will be bound by analyte. An enzyme tagged with the same analyte of interest will bind antibody sites that are free. A color reagent is added as a substrate for the enzyme to create a color change. The reaction is stopped using a solution designed to inhibit the enzyme and an absorbance reading taken of each well at a specified wave length of light. The lower the absorbance value the higher the concentration of analyte in the sample. Conversely, the higher the absorbance value the lower the concentration of the analyte. Each assay kit has a cutoff threshold standard at a given concentration or set of calibrators for determination of a positive sample and concentration in clinical testing.

A clinical ELISA test developed to quantitate mannose-6-phospahte receptors in cells is an example of using immobilized antibodies for the capture and purification of proteins of interest (Suresh et. al, 2002). In this study, goat antibodies specific for protein receptors MPR 300 and MPR 46 were bound to each well of 96 well microtiter plates. Purified membrane extracts were added to designated wells and then washed prior to adding anti-goat IgG conjugated to alkaline phosphatase. Methods such as this use an enzyme and substrate to generate a color which is measured at specific wave lengths of light for quantitative purposes. Clinically it is acceptable to rely on such an assay without further testing that the assay is actually measuring the analyte of interest based solely on the specificity of the antibodies produced. Such is not the case in forensic science.

ELISA testing in forensic science is used for the purpose of screening biological samples for the presence of specific drugs or drug classes (Levine, 2003). The assays use

monoclonal or polyclonal antibodies developed against a specific drug or specific drug class that demonstrate sufficient cross reactivity to other drugs within the same class. This type testing is used strictly for drug screening due to the phenomenon of cross reactivity. As an example immunoassay kits designed for amphetamines will cross react with several over-the-counter medications that contain drugs very similar in molecular structure to the amphetamines resulting in presumptive positive results when present in biological samples being tested (Kelly et al., 1993).

When testing is performed for a class of drugs such as opiates, barbiturates or benzodiazepines a positive result only indicates the possible presence of one or more of the drugs within the class, but does not indicate which drug or drugs is/are actually present. This is the reason behind confirmatory testing so that the drug(s) may be positively identified by a more specific methodology such as gas chromatography-mass spectroscopy.

Quantitations of drugs by ELISA as well as other immunoassay techniques is forensically unacceptable due to cross reactivity when it is not known which specific drug(s) of a given class of drugs is present. Each drug within a class cross reacts at a different binding percentage based on its concentration as compared to the drug for which the antibody was specifically designed against. This makes quantitations of drugs basically impossible when using ELISA test kits as a testing method in forensic settings.

CHAPTER III

MATERIALS AND METHODS

Phase I

Experimental Protocol

This project was designed to develop an extraction device that can be easily adopted for use with a gas-chromatograph coupled to an EI+ mass-spectrometer along with other typical chromatographic instrumentation used in forensic laboratories.

An experimental protocol was developed to test the hypothesis that Δ-9-THC within a complex biological matrix could be bound by antibodies specific for Δ-9-THC, remain bound during several washing steps and subsequently recovered from a 96 well ELISA microtiter plate for qualitative analysis by GC/MS.

The second step in the research protocol was immobilization of sufficient Δ-9-THC monoclonal antibodies onto a solid surface of polystyrene using glutaraldehyde as the linking molecule. One goal of this project was to develop a device of sufficient surface area to which antibody specific for Δ-9-THC could be chemically bound without any sophisticated equipment. The device had to be capable of holding 1 to 2 ml of a biological matrix in a manner to ensure exposure of analyte to antibody.

Third a rinsing protocol designed to eliminate interfering substances was established without releasing the bound drug from antibodies. Once interfering substances were removed, the final step in preparation for analysis was the release of all analyte from antibodies using a minimum volume of an organic solvent. At this point, a minimum volume of solution containing primarily the drug and/or metabolites of interest was evaporated and prepared for analysis by GC/MS. The purified extraction was accomplished using a short incubation period, limited number of rinse steps, and a

minimum volume of organic solvent.

Drug Standards

Standard solutions of Δ-9-THC at a concentration of 1.0 mg/ml and Δ-9-THCA at a concentration of 1 mg/ml in methanol were purchased from Cerilliant (Round Rock, TX). These standard solutions were used to make stock and working standard solutions for use in all experiments. Deuterated standards of Δ-9-THC-D3 and Δ-9-THCA-D3 were also purchased from Cerilliant containing 100 μg/ml of analyte for use in quantitation Δ-9THC and Δ-9-THCA. Stock solutions for all analytes were made from the purchased standards. Stock drug solutions where used to prepare working solutions of individual analytes for spiking all calibrators and controls. Postmortem blood samples from the Mississippi Crime Laboratory were spiked with internal standard solutions.

ELISA Kit Testing

An ELISA Cannabinoid kit was purchased from Immunalysis (Pomona, CA) for the initial phase of the project. The ELISA kit contained five 96 well plates with 12 removable rows of cannabinoid antibody coated wells with eight wells per row. Each coated well had the capacity to hold approximately 300 μL of total fluid. Individual plates were designated based on concentration of analyte added and incubation time during initial experiments. The kit also came with THC derivative solution conjugated to horseradish peroxidase, synthetic negative blood, 3, 3', 5, 5' tetramethylbenzidine (TBM chromogenic substrate) and 1 N hydrochloric acid as a stopping agent.

The first experiment was run to establish that the kit would perform as described by the manufacturer. Synthetic blood was spiked with Δ-9-THC at a concentration of 25 ng/ml and added to seven rows with the first row representing a negative sample.

The second experimental protocol followed was based on the manufacture's

instructions with the exception of sample dilution prior to analysis. Volumes of sample (125 µL) and THC-conjugate added to each well were also altered to achieve approximately 1 ml of sample per eight wells for binding studies. The decision not to dilute the samples was necessary for later determination of analyte percent yield by GC/MS analysis. The protocol used for the first two experiments were as follows:

1. 125 µL of sample was added to a designated well.
2. Plates were incubated for 1 hr at room temperature.
3. Plates were washed five times with 250 µL DI water using an automated plate washer.
4. Following initial wash steps, 125 µL of THC-conjugate derivative was added to each well.
5. Plates were incubated for 1 hr at room temperature and then washed five times with DI water using an automated plate washer.
6. Plates were inverted and slapped on dry paper towels to ensure no THC-conjugate remained and wells were dry of fluid.
7. 125 µL of TMB chromogenic substrate was added to each well and incubated in the dark at room temperature for 30 min.
8. 125 µL of 1N HCl (stop reagent) was added to each well and the plates tapped several times to insure complete mixing.
9. Developed color of all wells was measured using a Victor 3 (Perkin Elmer, Shelton, CT) automated plate reader at 450 nm and absorbances recorded.

The protocol for the analyte recovery experiments was as follows:

1. Perform steps 1-6 as described above.

2. 250 uL of acidic methanol, hexane or N-butyl chloride was added to each well of their respective plates and incubated for 1 hr.
3. Recovery of solvent from a single row of eight wells was collected and placed in a single labeled 16x125 mm screw cap tube.
4. Tubes were refrigerated until ready for analysis.

The third experiment performed was addition of Δ-9-THC, Δ-9-THCA and their respective deuterated internal standards at 25 ng/ml each to duplicate rows in order to determine the antibodies would bind and be retained through the method protocol.

Incubation Period Determination for Percent Yield Study

An optimum incubation period for analyte recovery was performed by incubating spiked samples for 15, 30 and 60 min in the microtiter plates. All analytes were added to specified rows of wells at a concentration of 25 ng/ml and run through the method.

Once the optimum time frame was established, this time was used for the remainder of the ELISA experiments. Four drug free synthetic blood samples of 1 ml each were spiked individually with Δ-9THC, Δ-9-THCA and their respective deuterated internal standards at concentrations of 25 ng. Plates were then run through the recovery protocol as described above.

A 0.1% methanolic HCL solution was prepared by adding 0.1 ml of concentrated HCL to 90 ml methanol, mixing and then brought to a final volume of 100 ml. Hexane and N-butyl chloride solvents were used directly from stock bottles. These three solutions were used to release the analytes from the antibody following the washing procedure. Into each well was pipetted 250 μL of a releasing solution and allowed to stand for 30 min. Solvent from each well was collected and the contents of each

designated row were placed in a labeled 16x125 mm screw cap tube for storage at 4° C until ready for analysis by GC/MS.

Tubes containing the different releasing solutions were evaporated to dryness. To each tube, 40 uL of O-Bis (trimethylsilyl) trifluroacetamide (BSTFA) was added for derivatization of analytes. Each tube was then capped and incubated for 60 minutes at 70° C. Derivatized samples were analyzed by GC/MS operating in the Selected Ion Mode (SIM). Ions chosen for Δ-9-THC were 386, 343 and 303 amu. The ions chosen for the internal standard of Δ-9-THC were 389 and 306. The ions chosen for Δ-9-THCA were 371, 473 and 488 amu with 374 and 476 amu as the internal standard ions.

Phase II

Experimental Protocol

Phase 2 of the research began with developing a protocol to immobilize drug specific Δ-9-THC monoclonal antibodies within a polystyrene cell culture flask. Two methods were chosen for binding of antibodies to the polystyrene surface; 1) passive absorption and 2) chemical linkage with glutaraldehyde. Two methods were chosen to first establish if one was more efficient to prepare and secondly which one would be more robust in performing the experiments.

Passive Immobilization of Antibody

Antibodies were mixed in an alkaline buffer and allowed to incubate in the extraction flask for absorption to take place prior to a blocking agent being added to the device: Devices were made fresh for each batch run. The protocol for the absorption experiment is given below:

1. A 0.1 M buffer of sodium carbonate (Na_2CO_3-H_2O), pH 11.3, was prepared by accurately weighting out 12.4 g and dissolving in 950 ml

Type III water. The pH was checked with an Accumet XL 25 (Fisher Scientific, Houston, TX) and the solution was brought to a final volume of 1000 ml.

2. A 0.1 M buffer of sodium bicarbonate ($NaCHO_3$), pH 8.3, was prepared by accurately weighting out 8.4 g and dissolving in 950 ml Type III water. The pH was checked as above and the solution brought to a final volume of 1000 ml.
3. 400 ml of the 0.1 M carbonate buffer was measured out and the pH was brought to 9.25 by addition of the sodium bicarbonate buffer up to a volume of 500 ml.
4. 0.5 mg of Δ-9-THC antibody solution was added to 100 ml of the carbonate buffer for a concentration of 5 μg antibody per ml of buffer, pH 9.25.
5. 1 ml of the buffered antibody solution was placed in each extraction device and allowed to incubate overnight at room temperature.
6. The following day each device was rinsed five times with phosphate buffered saline (PBS), pH 7.2.
7. A 0.1M solution of l-lysine was added to each device and allowed to incubate for 3 hrs at room temperature: L-lysine served as a blocking agent for any remaining active sites.
8. The devices were then washed 5 times with PBS and allowed to air dry.
9. 1 ml synthetic blood samples were spiked with 25 ng Δ-9-THC and added to 10 devices and incubated for 1 hr on a platform rotator

moving at 25 revolutions per min.

Following the respective incubation periods, the devices were removed from the platform rotator and the contents poured into a biohazard waste container. Each device was then washed 5 times with sodium phosphate buffer (pH 7.2) to remove any remaining contaminants. The devices were allowed to drain for 30 min followed by addition of 2 ml of the releasing solvent. The devices were incubated in the releasing solvent for a period of 30 min. The 2 ml of releasing agent from each device was decanted into a clean 16 x 125 mm screw cap tube and labeled. Great care was taken not to collect any remaining traces of water or buffer from any devices during this step.

Collected aliquots of solvent were dried at 60° C under vacuum. Forty (40) µL of BSTFA with 1% TMS was added to each tube. The tubes were then capped, vortexed and incubated at 70° C for 1 hr for derivatization prior to analysis by GC/MS.

Glutaraldehyde Immobilization of Antibody

The second method used glutaraldehyde as the linking molecule to chemically bind the antibody to the polystyrene surface. The protocol for gluteraldehyde bonding to a polystyrene surface is given below:

1. A 0.1 M solution of sodium phosphate dibasic (Na_2HPO_4), pH 9.5, was made by accurately weighting out 14.2 g and dissolving in 950 ml Type III water and brought to a final volume of 1000 ml.
2. A 0.1 M solution of sodium phosphate monobasic (NaH_2PO_4), pH 4.5, was made by accurately weighting out 12.0 g dissolving in 950 ml Type III water and brought to a final volume of 1000 ml.
3. 450 ml of the monobasic buffer was measured and the pH adjusted to 5.0 using the 0.1 M solution of sodium phosphate dibasic solution and

brought to a final volume of 500 ml. Final pH of 5.0 was checked using an Accumet 25 XL pH meter.

4. 10 ml of a 25% glutaraldehyde solution was added to 40 ml of sodium phosphate buffer (pH 5.0) for 50 ml of a 5% glutaraldehyde solution.
5. A sodium phosphate buffer at pH 8.0 was made by using the two buffer solutions above. 400 ml of the sodium dibasic buffer was measured out and brought to the proper pH as necessary using 0.1 M of sodium phosphate monobasic solution and brought to a final volume of 500 ml.
6. 1 ml of the glutaraldehyde solution was pipetted into 10 devices and allowed to incubate at room temperature for a minimum of 4 hrs.
7. Following incubation, each device was thoroughly washed with sodium phosphate buffer, pH 5.0.
8. 0.5 mg of Δ-9-THC monoclonal antibody was added to 100 ml of a sodium phosphate buffer, pH 8.0, for a concentration of 5 μg antibody per ml of buffer.
9. 1 ml of antibody solution was added to an extraction device and incubated at 37° C for a minimum of 3 hrs.
10. Devices were allowed to cool to room temperature and washed with pH 8.0 sodium phosphate buffer 3 times.
11. Each device was drained and washed twice with phosphate buffer, pH 7.2, made from 0.1M solution of sodium phosphate monobasic and 0.1M sodium phosphate dibasic followed by 2 washes with Type III water.

12. 1 ml synthetic blood samples were spiked with 25 ng Δ-9-THC.
13. While vortexing, 1 ml of acetonitrile was added drop-wise using a variable pipettor.
14. The samples were then centrifuged for 25 min at 3000 rpm and then the upper layer was transferred to a clean 15x125 mm screw cap tube.
15. The acetonitrile layer was evaporated to approximately 250 mcl and then reconstituted with 1 ml of a 0.5% saline solution, vortexed and centrifuged at 3000 rpm for 25 min.
16. The saline solution was transferred to prepared device and capped.
17. The devices were incubated for 120 min on a platform rotator moving at 25 revolutions per min.

The devices were treated as described under the "Passive Immobilization" section above.

Monoclonal Δ-9-THC Antibodies

Monoclonal drug antibodies produced against Δ-9- THC were purchased in 1 mg lots from BioDesign Co (Saco, ME). The purchase of a 1 mg lot of monoclonal antibodies was sufficient to make 200 extraction devices at a concentration of 5 μg of antibody per device. Description of the antibody purchased is as follows:

1. Catalog # G82922M
2. Description – monoclonal antibody to Δ-9-THC
3. Clone D81 from Mouse ascites
4. Immunogen – 8-THC-BSA
5. Isotype IgG_1
6. Specificity to Δ-9-THC and metabolites

7. Buffer – PBS pH 7.2
8. Lot number 8D09407

Extraction Device

A Falcon® tissue flask (Thermo-Fisher Scientific, Houston, TX) with 12.5 cm^2 bottom surface area along with a canted neck and screw cap was chosen as the prototype container. The flask could be sealed with a non-vented screw cap and had a large enough orifice to easily add 1 to 2 ml of buffered sample and to pour off 2 to 3 ml of rinse solution and drug releasing solvent. The flasks have a flat bottom and were easy to stack for incubation on a platform rotator.

Analyte Releasing Solvents

The releasing solvents tested were an organic solvents and an acidic alcohol normally used in forensic laboratories in general extraction procedure for Δ-9-THC and Δ-9-THCA extraction purposes. The solvents chosen were hexane and 0.1% methanolic HCl. Each solvent was used individually to determine which was the most efficient. The releasing solution found optimal in Phase 1 of the study was tested first. Releasing solutions were collected in 16X125 mm borosilicate glass screw cap tubes and placed in a RapidVap® evaporator for drying under vacuum at 50°C. The dried extracts were reconstituted with 40 uL BSTFA plus 1% TMS, capped, vortexed and incubated for 60 min at 70°C for derivatization.

Gas Chromatograph and Mass Spectrometer Parameters

The gas chromatograph injection port was set up in the splitless mode for injection of 2 uL of extracted samples using an autoinjector. The mass spectrometer was set up in the SIM mode to monitor 3 characteristic ions of each analyte and two ions for each internal standard. Secondary ion ratios were monitored to assure compliance within

the ± 20% criteria for confirmation. Gas chromatograph parameters were set as follows:

1. GC was a Clarus 600 manufactured by Perkin Elmer, (Shelton, CT)
2. Injection port temperature 250° C
3. Initial oven temperature 120° C with an initial hold of 1.00 min
4. Following 1 min hold, temperature was ramped 15° C/min to 320° C for a total run time of 14.33 min
5. Column was a MS 5, 30 m in length with a 250 um diameter purchased from Perkin Elmer, (Shelton, CT) containing 5% phenyl and 95% polysiloxane

Mass spectrometer parameters were set as follows:

1. Mass Spectrometer was a Clarus 600 electron impact (EI+) quadrapole manufactured by Perkin Elmer, (Shelton, CT)
2. Solvent delay from 0 to 10.62 min
3. THC ions monitored were 303, 386 and 387 (BSTFA+1% TMS derivative)
4. THCA ions monitored were 371, 473 and 488 (BSTFA+1% TMS derivative)
5. Δ-9-THC-D3 and Δ-9-THCA-D3 ions monitored were 306, 389 and 374, 476 respectfully.
6. Window for THC was 10.63 to 11.63 min
7. Window for THCA was 12.57 to 14.33 min
8. Dwell times were 0.050 s for each ion
9. Ion mode was EI+
10. Inter channel delay was 0.01 s for each ion monitored

ELISA Testing of Glutaraldehyde Prepared Devices

Extraction devices were prepared as described under the glutaraldehyde antibody immobilization section. One of the devices was treated as a negative with no analyte added. Δ-9-THC at concentrations of 10, 50, 100, 200, 500 and 1000 ng/ml Δ-9-THC were added to the remaining six devices. A total of five batch runs were performed.

Following initial incubation and wash steps, 1 ml of conjugated THC-derivative was added to each device and incubated for 1 hr. The devices were washed six times with DI water following this second incubation to remove all traces of conjugated THC-derivative. Next 1 ml of TMB color reagent was added to each device. Each device was mixed well by gentle swirling and allowed to incubate in the dark for 30 min. Stop reagent (1 N HCl) was added to each device following the 30 min incubation and the devices were thoroughly mixed again by gentle swirling to ensure reaction between TMB and conjugate was completely stopped.

The contents from each device were transferred to labeled, 2 ml polystyrene cuvettes for reading absorbance values of each solution using a Lambda 35 UV/VIS spectrophotometer (Perkin Elmer, Shelton, CT) at 450 nm.

Percent Yield Studies

Percent yield of Δ-9-THC was determined by spiking ten, 1 ml aliquots of synthetic whole blood with 25 ng of the analyte and extracting these samples using devices prepared the day of analysis. A 25 ng/ml direct standard was used to determine the results of the extracted samples. A total of 5 experiments using 10 devices for each analyte were conducted on different days for a total of 50 experiments Δ-9-THC and Δ-9-THCA. Percent yield data from experiments were compared using a Paired t test to determine if data demonstrated any significant statistical differences among the different

daily experiments.

A second round of experiments to determine binding specificity was conducted by adding both Δ-9-THC and Δ-9-THCA to the same extraction device in order to determine if there was an appreciable effect on the recovery of THCA or both of the analytes. Both analytes were added to prepared extraction devices at equal concentrations of 10, 50, 100, and 150 ng/ml. Effect on binding specificity for THCA was determined by direct comparison of results from the respective analytes. The experiment was conducted five times.

Linearity of Single Extracted Analytes

One ml whole blood aliquots ranging from 3 to 50 ng/ml of each analyte were analyzed by the glutaraldehyde procedure and judged for acceptability. Criteria designated as acceptable linearity was a line having a correlation coefficient of $r^2 > 0.985$. This procedure was conducted daily using five devices per run for a minimum of 50 samples run over a 5 day period to test within run and day-to-day consistency of device preparation.

Linearity of Extracted Analytes with Deuterated Internal Standards

Deuterated internal standards (IS) were added to the next phase of the study. Whole blood samples were spiked at 25 ng/ml Δ-9-THC-D3 and 50 ng/ml Δ-9-THCA-D3 with appropriate concentrations of Δ-9- THC and Δ-9-THCA to prepare a set of five calibrators ranging from 2.0 to 50 ng/ml for Δ-9-THC and 2.0 to 50 ng/ml for Δ-9-THCA. These standards were prepared for the purpose of demonstrating linearity ($r^2 > 0.985$).

Establishing limits of quantitation (LOQ) and limits of detection (LOD) for each analyte was accomplished by extracting 10 samples at designated concentrations. Ten

samples were spiked at a concentration of 0.5 ng/ml Δ-9-THC for LOD determination. Ten samples at a concentration of 1.0 ng/mlΔ-9-THC were extracted to determine LOQ. The same protocol was followed for Δ-9-THCA with concentrations of 2 and 5 ng/ml for LOD and LOQ, respectfully.

Once linearity was established, quality control samples were prepared using standards from a separate lot of drug standards from the same vendor with concentrations that tested the low and high portions of the calibration curve; 5 and 30 ng/ml for Δ-9-THC and 10 and 40 ng/ml for Δ-9-THCA. Acceptable criteria of controls were set at ± 20% of expected concentration.

Analysis of Postmortem Samples

The final step in Phase 2 of the study was the analysis of postmortem blood samples secured from the Mississippi Crime Laboratory that had been previously analyzed by a reputable laboratory and extracting these samples using the new device. All experiments were conducted twice on different days to ensure the devices were consistent between runs. This experiment step consisted of two parts: 1) extraction of samples with known reported concentrations of one or both analytes, and 2) extraction of samples where concentrations of Δ-9-THC could not be reported due to interfering substances. Appropriate statistical tests were performed to evaluate the validity of the new extraction procedure where reported sample concentrations were known. Samples where values were not reported due to interfering substances were compared to the new extraction procedure to evaluate if interfering substances had been removed, thus allowing reporting of quantitative results. Δ-9-THCA was analyzed on all 30 samples following the same protocol.

CHAPTER IV

EXPERIMENTAL RESULTS

Preparation of Drug and Internal Standards

Drug standards of Δ-9-THC, Δ-9-THCA, Δ-9-THC-D3 and Δ-9-THCA-D3 were purchased from Cerilliant Corp. (Round Rock, TX). Δ-9-THC and Δ-9-THCA were purchased in concentrations of 1 mg/ml while the two deuterated internal standards were purchased in concentrations of 100 μg/ml. Stock standards of Δ-9-THC and Δ-9-THCA were made by diluting 1 ml of the respective analyte with 9 ml methanol for final concentrations of 100 μg/ml each. Stock standards for internal standards were 0.5 and 1.0 mcg/ml for Δ-9-THC-D3 and Δ-9-THCA-D3 respectfully (Liu, et al., 1995). See Tables 1 and 2 for complete dilution protocols for making working solutions.

Results of Phase 1 ELISA Experiments

The first experiment was conducted by spiking separate 1.0 ml aliquots of synthetic blood in 16x125 mm screw cap tubes with 25 ng/ml of Δ-9-THC. As stated in the method protocol, 125 μL of spiked solution was placed in 56 designated wells and 125 μL of negative blood was placed in first eight wells (see Figure 1) to establish the method worked in my laboratory as described by the manufacturer. Absorbance readings were measured for all wells using a Victor 3, 96 well plate-reader operating at 450 nm.

Figure 1 demonstrates the cannabinoid ELISA kit worked as described by the manufacturer. The synthetic negative sample gave negative results as expected and the spiked samples at 25 ng/ml Δ-9-THC gave positive results. Analysis of the absorbance

Table 1

Dilution protocol for Making Stock and Working Standards

Stock Standards

Δ-9-THC – 1 ml of a 1 mg/ml Standard + 9.0 ml MEOH = 100 mcg/ml: Stock Std 1
1 ml of Stock Std 1 + 9.0 ml MEOH = 10 mcg/ml: Stock Std 2

Δ-9-THC Working Standards

1 ml of Stock Std 2 + 9.0 ml MEOH = 1 mcg/ml: Working Std 1
0.4 ml Stock Std 2 + 9.6 ml MEOH = 0.4 mcg/ml: Working Std 2
0.2 ml Stock Std 2 + 9.8 ml MEOH = 0.2 mcg/ml: Working Std 3
0.1 ml Stock Std 2 + 9.9 ml MEOH = 0.1 mcg/ml: Working Std 4

Calibrators

50 µL Working Std 1 in 1 ml blood = 50 ng/ml Δ-9-THC
50 µL Working Std 2 in 1 ml blood = 20 ng/ml Δ-9-THC
50 µL Working Std 3 in 1 ml blood = 10 ng/ml Δ-9-THC
30 µL Working Std 4 in 1 ml blood = 3 ng/ml Δ-9-THC

Stock Standards

Δ-9-THCA – 1 ml of a 1 mg/ml Standard + 9.0 ml MEOH = 100 mcg/ml: Stock Std 3
1 ml of Stock Std 3 + 9.0 ml MEOH = 10 mcg/ml: Stock Std 4

Δ-9-THCA Working Standards

1 ml Stock Std 2 + 9.0 ml MEOH = 1 µg/ml: Working Std 5
0.4 ml Stock Std 2 + 9.6 ml MEOH = 0.4 µg/ml: Working Std 6
0.2 ml Stock Std 2 + 9.8 ml MEOH = 0.2 µg/ml: Working Std 7
0.1 ml Stock Std 2 + 9.9 ml MEOH = 0.1 µg/ml: Working Std 8

Calibrators

50 µL Working Std 5 in 1 ml blood = 50 ng/ml Δ-9-THCA
50 µL Working Std 6 in 1 ml blood = 20 ng/ml Δ-9-THCA
50 µL Working Std 7 in 1 ml blood = 10 ng/ml Δ-9-THCA
50 µL Working Std 8 in 1 ml blood = 5 ng/ml Δ-9-THCA

Table 1: Calibrators of Δ-9-THC and Δ-9-THCA were made by adding amounts shown in the table to 1 ml aliquots of postmortem blood. Calibrators were made new for each run of 10 samples from the Mississippi Crime Laboratory.

Table 2

Dilution Protocol for Making Internal Standard Spiking Solutions

Stock Standard Δ-9-THC-D3

1 ml of 100 μg Δ-9-THC-D3 + 9 ml MEOH = 10 μg/ml: Stock Solution
1 ml of 10 μg/ml Δ-9-THC-D3 + 9 ml MEOH = 1 μg/ml: Working Solution

25 μL of Working Solution to 1 ml sample = 25 ng/ml Δ-9-THC-D3

Stock Standard Δ-9-THCA-D3

1 ml of 100 Δ-9-THCA-D3 + 9 ml MEOH = 10 μg/ml: Stock Solution
1 ml of 10 mcg/ml Δ-9-THCA-D3 + 4 ml MEOH = 2 mcg/ml: Working Solution

25 μL of Working Solution to 1 ml sample = 50 ng/ml Δ-9-THCA-D3

Table 2: 25 μL of each internal standard is added to all calibrators, controls and samples to be used for quantitation curves and determination of control and sample values.

Figure 1

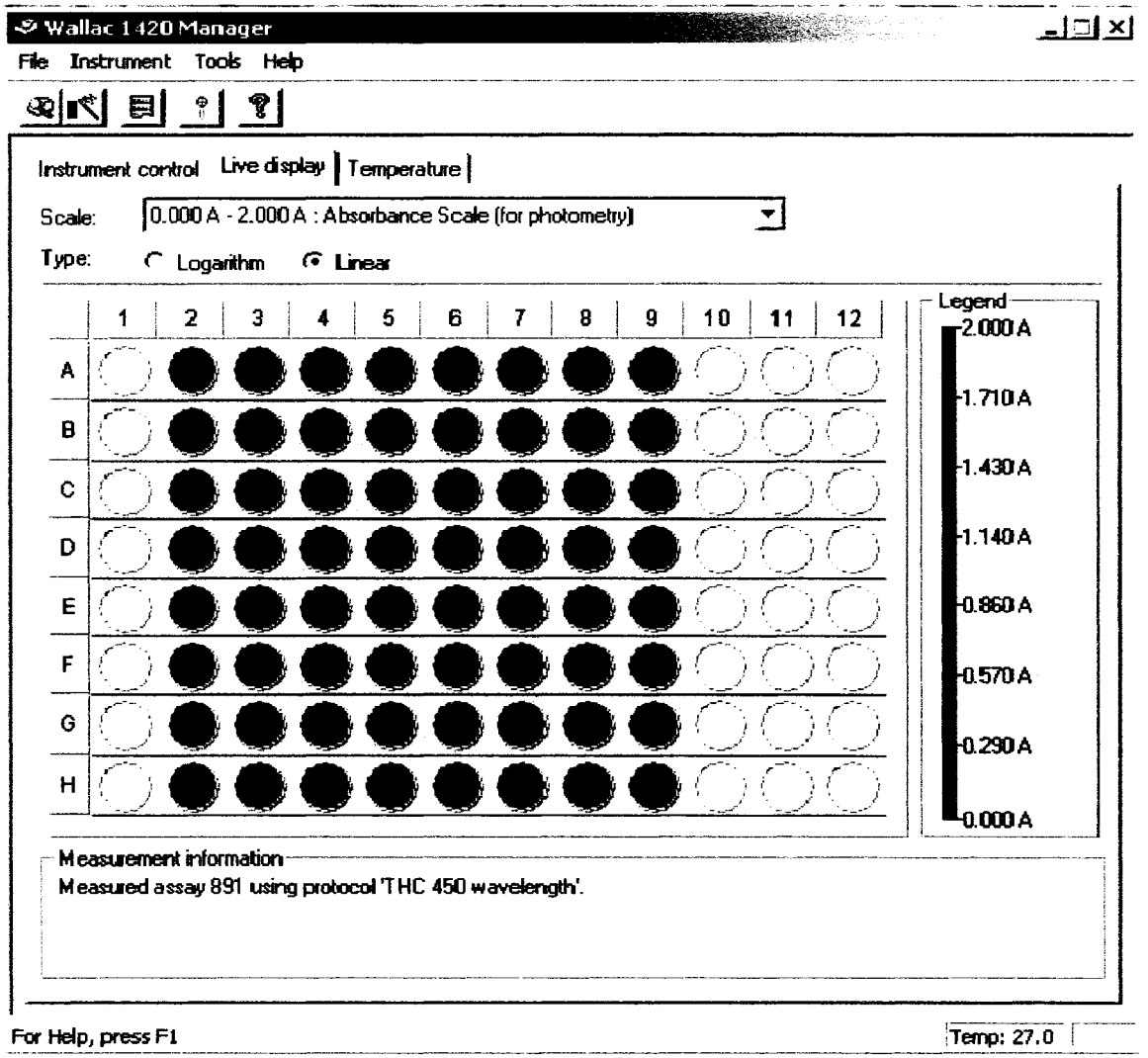

Figure 1: Display from Victor 3 showing the results from an ELISA run. The first row in red is a negative whole blood sample. The blue samples are positive results from whole blood samples spiked with 25 ng/ml Δ-9-THC. The figure demonstrates the ELISA kit worked as described by the manufacturer in my laboratory.

Figure 2

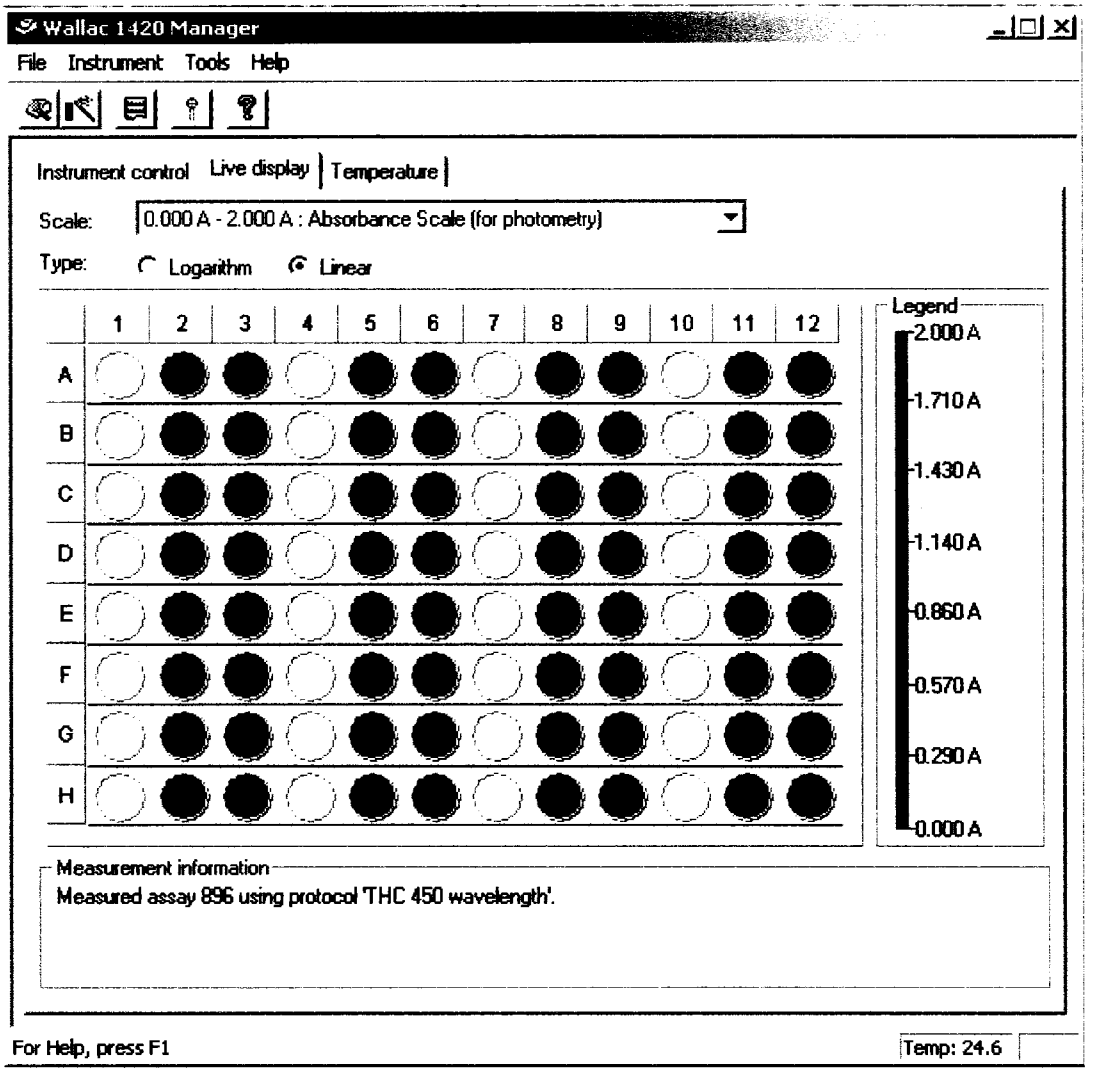

Figure2: Display shows all four analytes added to separate 1 ml whole blood samples at a concentration of 25 ng/ml and run in duplicate. The figure demonstrates that all analytes are bound by the antibodies and retained including the deuterated internal standards Δ-9-THC-D3 and Δ-9-THCA-D3.

values for the eight negative readings gave a mean of 1.824 with a standards deviation of 0.231. The positive samples resulted in a mean of 0.224 and a standard deviation of 0.023. The values achieved indicate that the pipetting was precise and the automated washer worked as expected in removing all unbound traces of the conjugated THC-derivative. The results also demonstrated that Δ-9-THC remained attached to the antibody during the washing procedure and was not released.

Results of Second ELISA Experiment

Synthetic blood aliquots spiked individually with 25 ng of all four analytes were pipetted into 16 different wells and results shown in Table 3 demonstrated that the antibody cross-reacted with all four analytes tested with basically equal affinity given the closely related absorbance values. The results also indicate that the three other analytes of interest were held by the antibody during the washing steps and not released from the antibody.

Results of Releasing Agent Experiment

Synthetic blood aliquots of 1 ml were spiked with 25 ng Δ-9-THC and carried through the ELISA protocol as described for the recovery experiments. The appropriate volume (125 μL) of a selected solution was added to designated wells and allowed to incubate for the times stated above. The 0.1% acidic methanol resulted in the best recovery of Δ-9-THC averaging 22%. Hexane was the second best with an average recovery of 15%. N-butyl chloride reacted with the polystyrene resulting in no recovery of analytes. The 25 ng/ml spiked solutions did have analyte present from both releasing agents as shown in Figure 3. Two major ions were present with no interfering peaks present.

Table 3

ELISA ASSAY on Δ-9-THC; Δ-9-THCA; Δ-THC-D3; Δ-9-THCA-D3

Plate	Repeat	End time	Start temp.	End temp.	BarCode
1	1	1:07:14 PM	24.5	24.5	N/A

Absorbance @ 450 (1.0s) (A)

0.000

	0.200	0.224		0.203	0.200		0.422	0.419		0.249	0.254
	0.200	0.209		0.220	0.197		0.427	0.444		0.255	0.254
	0.211	0.209		0.199	0.234		0.451	0.522		0.261	0.260
	0.208	0.204		0.197	0.233		0.514	0.436		0.259	0.261
	0.235	0.226		0.227	0.207		0.498	0.498		0.277	0.263
	0.206	0.207		0.212	0.205		0.598	0.409		0.257	0.262
	0.204	0.205		0.204	0.200		0.584	0.420		0.266	0.258
	0.207	0.237		0.203	0.204		0.482	0.414		0.267	0.267
Avg. Abs	0.209	0.215		0.208	0.210		0.497	0.445		0.261	0.260

	0.198	0.220		0.200	0.196		0.412	0.409		0.249	0.254
	0.197	0.208		0.213	0.193		0.416	0.434		0.256	0.254
	0.207	0.204		0.206	0.229		0.441	0.513		0.262	0.259
	0.204	0.199		0.193	0.227		0.503	0.426		0.259	0.260
	0.230	0.222		0.223	0.212		0.487	0.482		0.279	0.263
	0.202	0.203		0.209	0.201		0.587	0.499		0.257	0.261
	0.201	0.201		0.200	0.197		0.574	0.418		0.267	0.258
	0.203	0.231		0.200	0.202		0.472	0.403		0.266	0.268
Avg. Abs	0.205	0.211		0.205	0.207		0.487	0.448		0.262	0.260

Table 3: Absorbance values from ELISA analysis of all four analytes of interest at 25 ng/ml. From left to right the analytes are Δ-9-THC, Δ-9-THCA, Δ-9-THCA-D3 and Δ-9-THC-D3. All have consistent readings with the exception of Δ-9-THCA-D3 which shows absorbance values approximately two times the other three analytes.

The samples collected of each solution from the designated row of wells representing a negative sample, were evaporated to dryness with air in a 60^0 C water bath. To each tube 40 μL of O-Bis (trimethylsilyl) trifluroacetamide (BSTFA) was added for derivatization. Each negative sample tube was analyzed by GC/MS in the same manner as those containing analyte. Figure 3 shows that no Δ-9-THC was found in the tubes representing the negative sample. This finding indicates that the pipettor, saline solution, derivatization solvent and ELISA plate and reagents were not contaminated with Δ-9-THC.

Figure 4 shows an example of Δ-9-THC that was extracted using the ELISA plates. The three ions were all present and met ratio criteria for confirmation. The recovery of Δ-9-THC using these plates is poor as noted by the high baseline. Δ-9-THCA recovery was also poor using the ELISA plates. The three ions of interest did meet the confirmation criteria.

Acceptable calibration curves could not be demonstrated using the ELISA plates for either Δ-9-THC or Δ-9-THCA. Poor recovery of analytes using the plates would explain the inability to recover analytes with a degree of precision. Internal standards of Δ-9-THC-D3 or Δ-9-THCA-D3 were not used in any of the extractions procedures from the ELISA plates or to see if an acceptable calibration curve could be obtained given the ELISA plates were not the device of choice for final experiments.

Figure 3

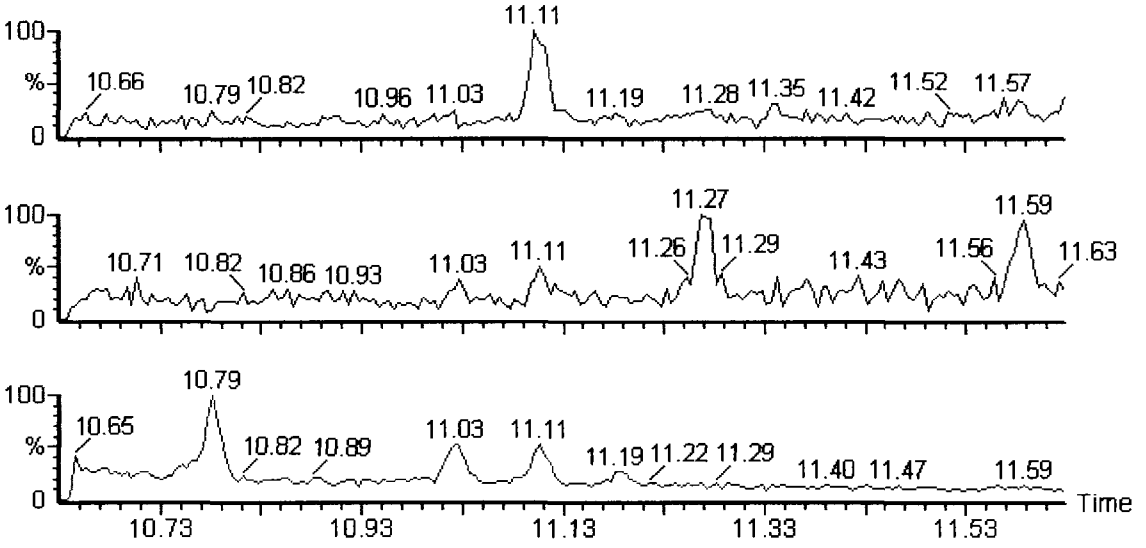

Figure 3: Negative sample of Δ-9-THC showing that the three ion of interest are not present and would not meet ratio criteria for being considered acceptable. The figure also shows none of the items used to prepare the sample were contaminated with Δ-9-THC.

Figure 4

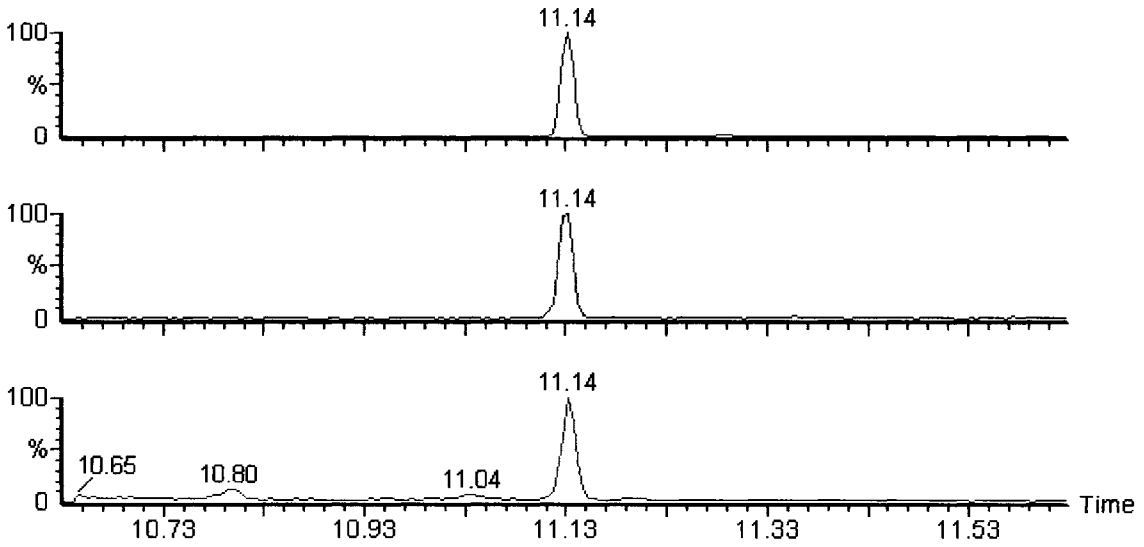

Figure 4: Shows the three ions monitored for Δ-9-THC by GC/MS analysis. The figure represents a 25 ng/ml sample that was extracted from an ELISA plate using 0.1% acidic methanol. Recovery is poor as demonstrated by the low baseline levels. Ions peaks pictured for Δ-9-THC are 386, 303 and 387 from top to bottom.

Falcon® Cell Culture Flask Chosen As Extraction Device

A 25 ml Falcon® cell culture flask was chosen as the device for binding of antibodies and extraction of samples (see Figure 5). The device was easy to handle and had several qualities that may it a good choice for initial experiments.

Sample Treatment with Acetonitrile

As described under the Methods and Material chapter, postmortem samples had to be treated with acetonitrile prior to use with the extraction devices. Figure 6 shows the difference in sample appearance following the acetonitrile treatment. Δ-9-THC or Δ-9-THCA could not be extracted from postmortem samples using untreated blood samples.

Results of Passive Immobilization of Antibodies

The passive method for immobilizing the antibodies was not deemed successful to the point of continuing experiments using this method. Recovery of Δ-9-THC was poor only averaging 20% with a range of 0 to 32%. There was no recovery of analyte in some 40% of the experiments conducted.

One of the problems was the l-lysine used as a blocking agent. The acidic methanol releasing solution would extract this compound leaving a white film of l-lysine in the tubes following evaporation. The l-lysine would dissolve in the BSTFA leaving the BSTFA solution white and opaque. This created chromatographic problems requiring cleaning or changing of the glass injection inserts following approximately every 15 injections. Removing the step of adding the lysine reduced the chromatographic problems but did not improve recovery of analyte using the acidic methanol or hexane.

Use of hexane as the releasing solution did not appear to remove the lysine from the extraction devices given there was no white film visible in evaporated tubes and did not result in any chromatographic problems or required cleaning of injection inserts.

Figure 5

Figure 5: Photographs of 25 ml Falcon® cell culture flasks used as extraction devices. The flasks have 12.5 cm^2 surface area on the bottom surface which was adequate for chemically binding 5 mcg of antibody.

Figure 6

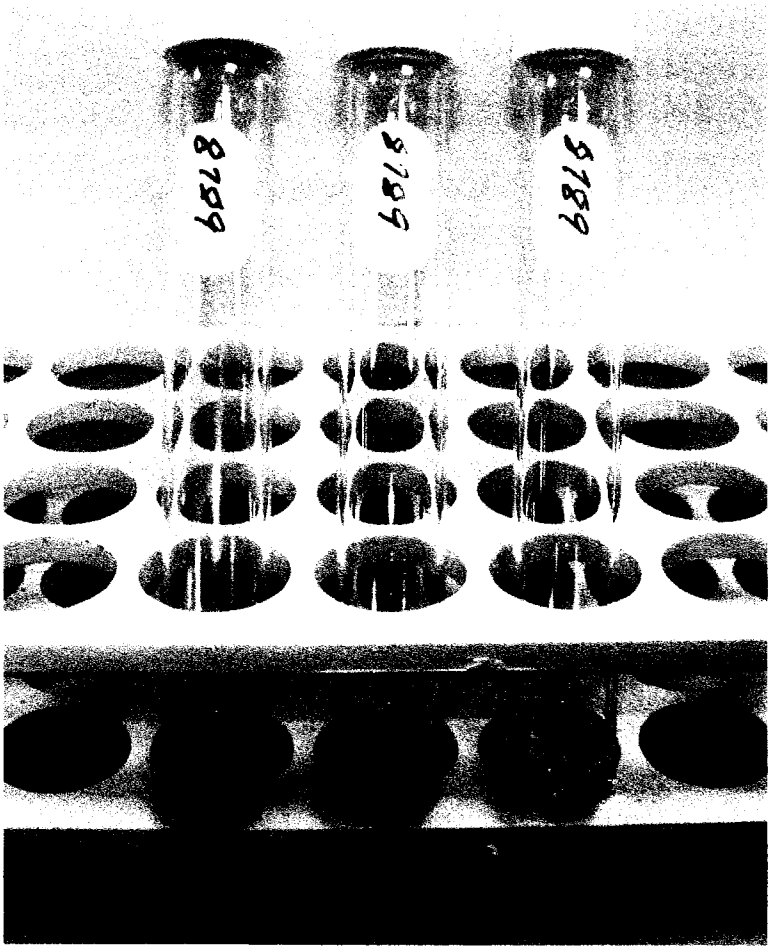

Figure 6: Tube on the left is a postmortem sample prior to any treatment with acetonitrile. The middle tube represents the sample after treatment and centrifugation and decanting into a clean tube. The tube on the right is the final sample following evaporation of the acetonitrile and addition of 1 ml buffered saline.

However, the recovery of analyte did not improve as compared to the acidic methanol and many devices showed no recovery of analytes.

Given that these two releasing solutions both demonstrated poor if any recovery of analyte using this method of immobilization of antibody, lead to two conclusions as to why this method did not work. One was that there was insufficient antibody bound to the extraction devices and/or that the antibody was removed during the washing steps. This experimental protocol was abandoned with no further attempts to improve its performance.

Results of ELISA Testing of Glutaraldehyde Bound Antibody

Five experiments were conducted where flasks treated with glutaraldehyde for antibody immobilization had 1 ml samples at concentrations of Δ-9-THC of 0, 10, 50, 100, 200, 500 and 1000 ng added and allowed to incubate for 1 hour. The flasks were washed as described in the method protocol and dried. ELISA reagents were then used to estimate binding capacity of the flasks for Δ-9-THC as described in the ELISA method (see Figure 7). All experiments showed an inverse relationship of absorbance to concentration as expected. A concentration ≥ 200 ng Δ-9-THC approached the limit of the flask to show any further binding capacity (see Figures 8, 9, 10, 11 and 12). Average absorbance values from the five experiments were 3.1323 for 0 ng, 2.5765 for 10 ng, 1.8673 for 50 ng, 0.6063 for 100 ng, 0.2172 for 200 ng, 0.0229 for 500 ng and 0.0015 for 1000 ng (see Table 4 and Figure 13).

Percent Yield Results with Glutaraldehyde Immobilized Antibodies

Ten flasks were then carried through the antibody attachment protocol on five different days for % yield studies. Each sample was spiked with 25 ng Δ-9-THC and carried through the extraction process. The average yield for Δ-9-THC was 54% with a

range of 50 to 61%. The same study was performed using Δ-9-THCA resulting in an average % yield of 47% with a range of 37 to 52% (see Table 5 and Tables 6-15).

Paired *t* test statistical analyses were run on the percent yield results for Δ-9-THC to see if there was a significant difference between the groups. Each group was compared statistically to all other groups during these analyses. There was a statistically significant difference found between all the groups demonstrating the difference did not occur by chance.

The same statistical analyses were conducted on the Δ-9-THCA results for the five experiments. The statistical analyses showed a significant difference between each group. Again these analyses show that the device is working as designed for both Δ-9-THC and Δ-9-THCA and the production from day-to-day is consistent.

Experiments were conducted as above when adding both Δ-9-THCand Δ-9-THCA at 25 ng each to determine ant difference in recovery when both analytes were present. There was no significant difference in recovery at this concentration of analytes. Next devices were made according to the glutaraldehyde protocol and both analytes were added to a single device at increasing concentrations. The concentrations were 10, 20, 40, 60, 80, 100 and 150 ng/ml. Recovery of both analytes was consistent from 10 to 60 ng/ml. The recovery of Δ-9-THCA began to fall when the concentration of Δ-9-THC began to approach 80 ng (see Figure 14). Concentrations of Δ-9-THC above 80 ng/ml showed continued recovery while the Δ-9-THCA recovery fell at each rise in concentration Δ-9-THC.

Figure 7

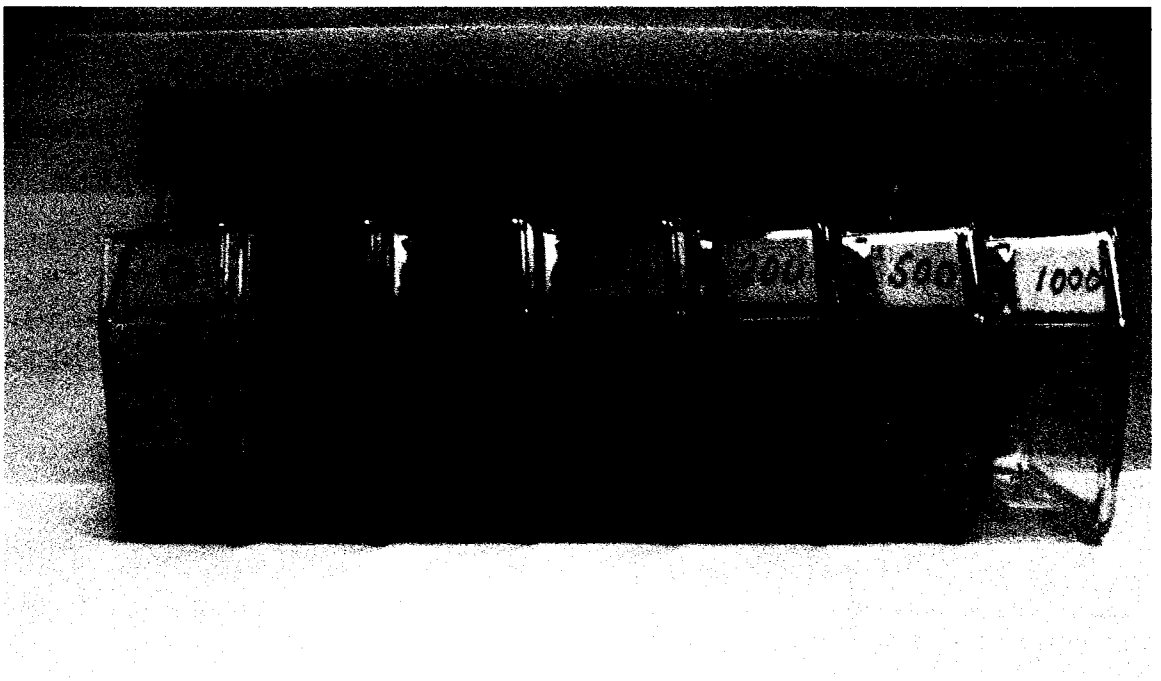

Figure 7: Flasks treated with glutaraldehyde to immobilize monoclonal THC antibodies subjected to ELISA reagents to determine binding capacity of Δ-9-THC. Concentrations range from 0 to 1000 ng/ml. Note that the color diminishes as the Δ-9-THC concentration rises. The 1000 ng/ml flask shows no color indicating no antibody binding sites were available for THC-derivative to bind.

Figure 8

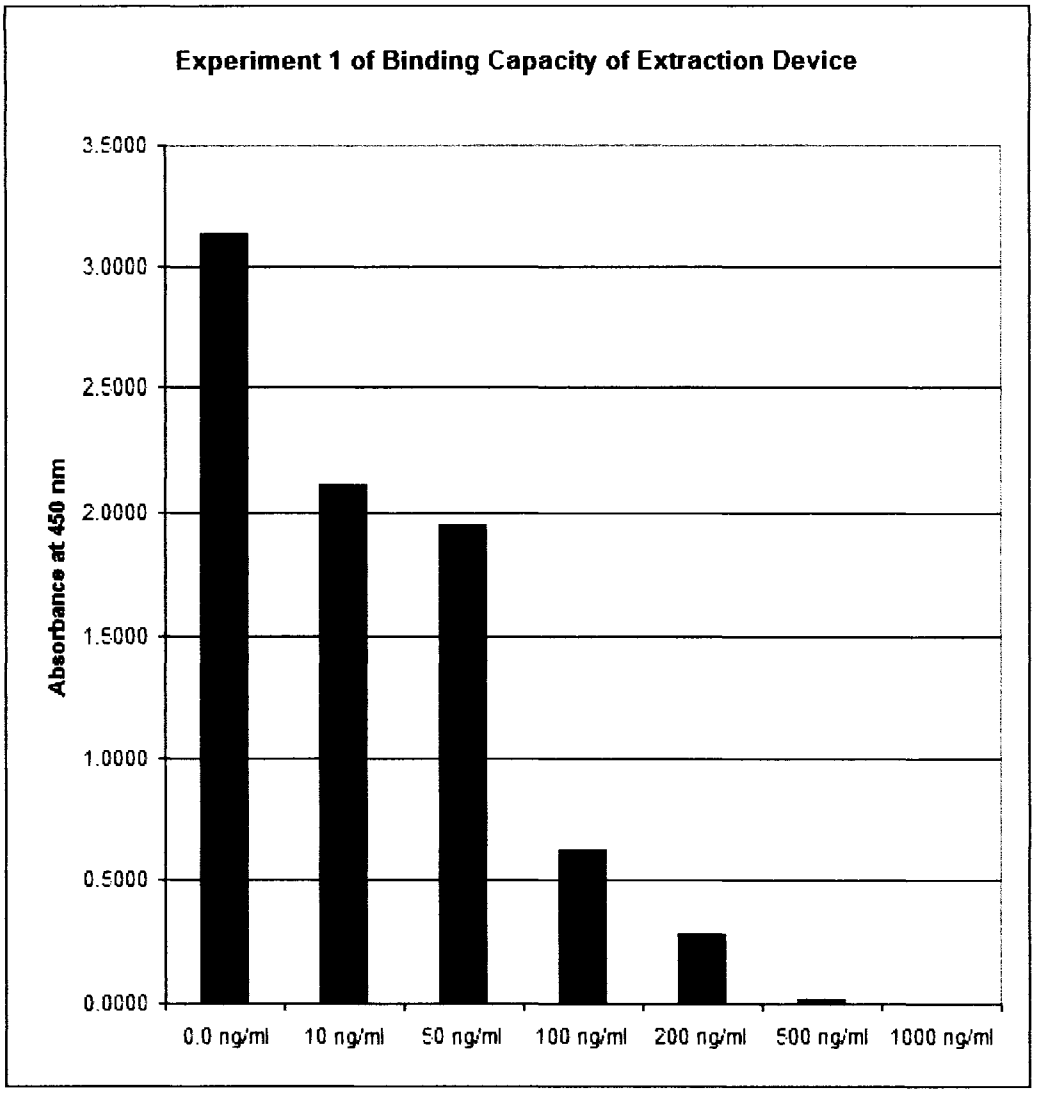

Figure 8: Bar graph shows the absorbance values from flasks treated with glutaraldehyde for immobilization of antibodies and subjected to ELISA reagents to determine binding capacity of Δ-9-THC.

Figure 9

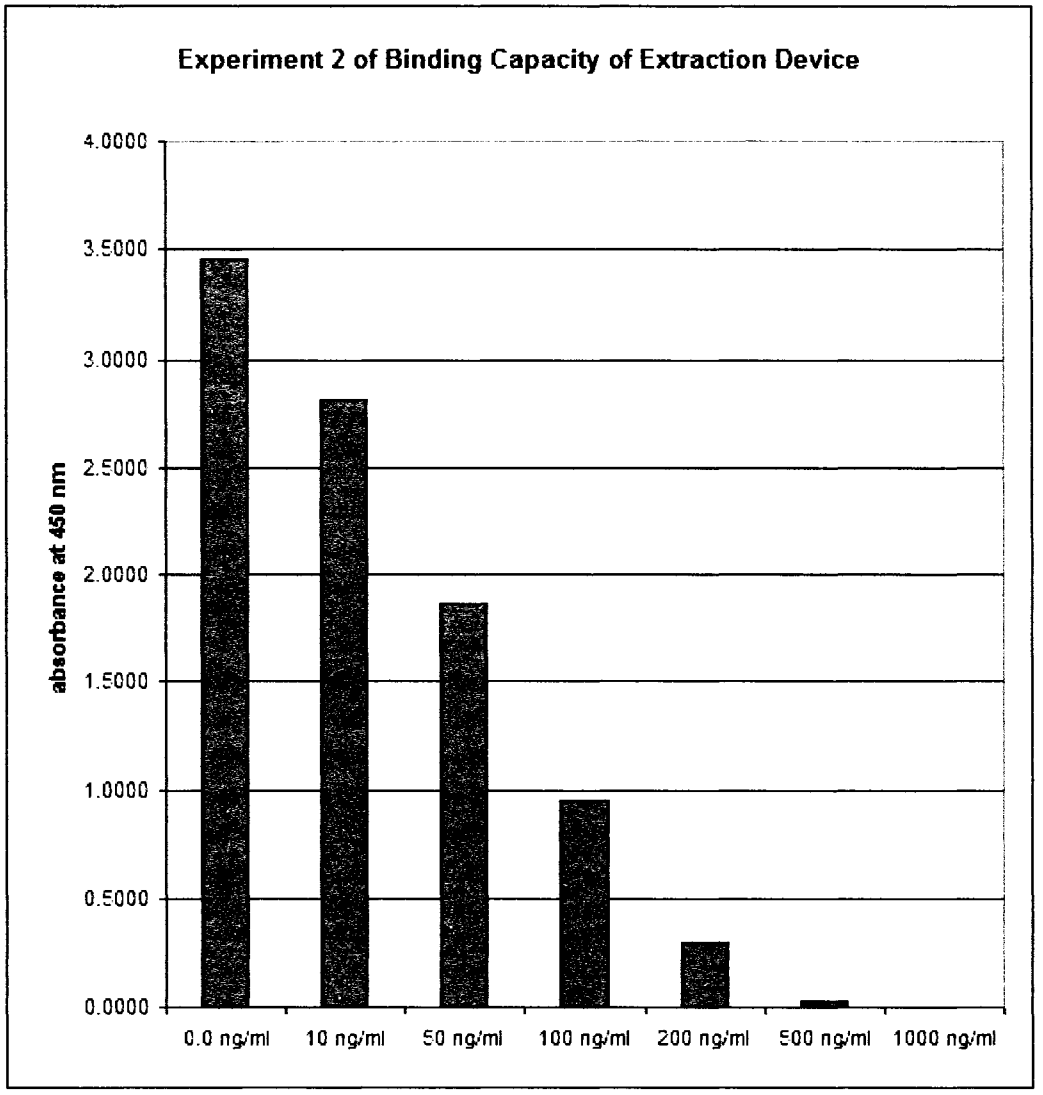

Figure 9: Bar graph shows the absorbance values from second experiment of flasks treated with glutaraldehyde for immobilization of antibodies and subjected to ELISA reagents to determine binding capacity of Δ-9-THC.

Figure 10

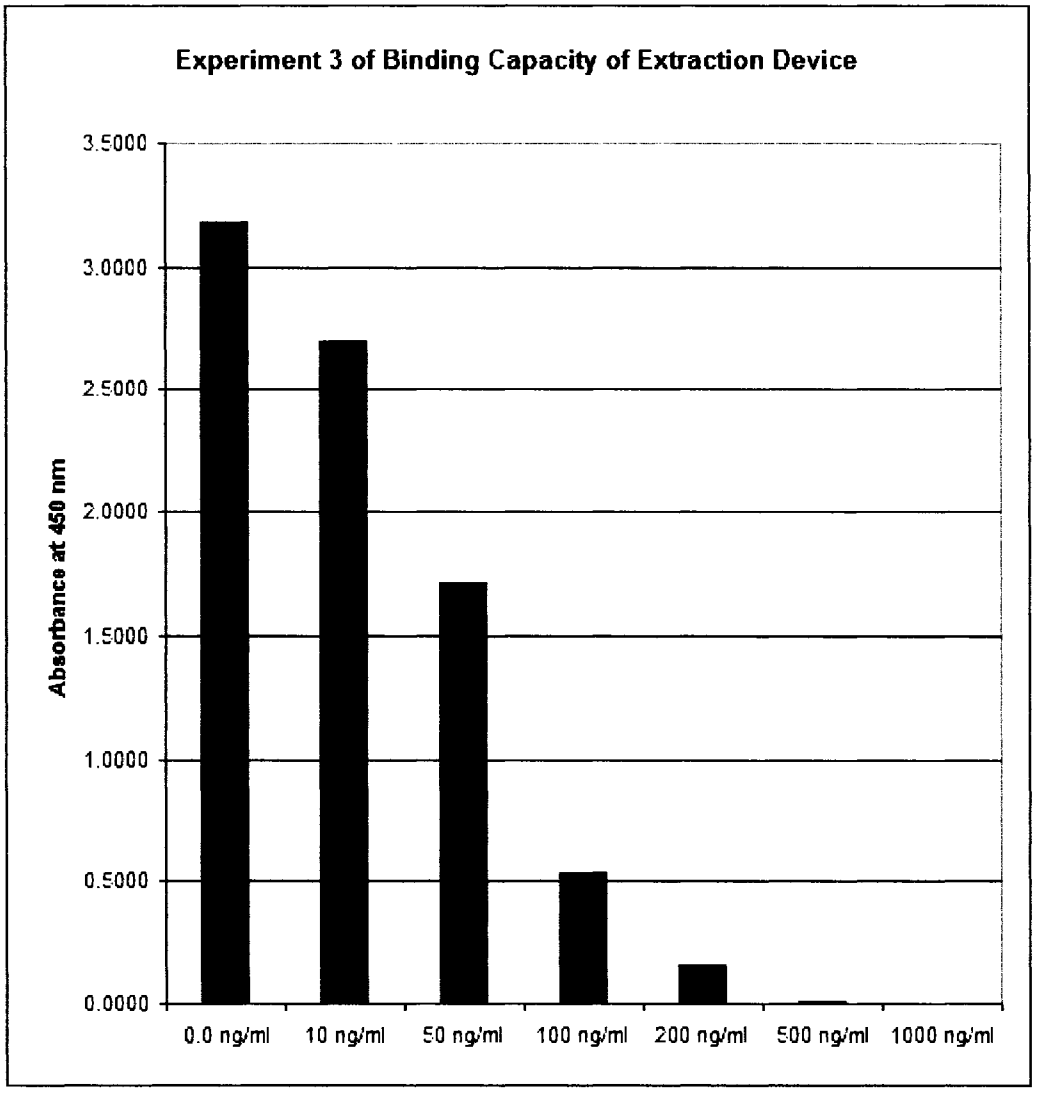

Figure 10: Bar graph shows the absorbance values from third experiment of flasks treated with glutaraldehyde for immobilization of antibodies and subjected to ELISA reagents to determine binding capacity of Δ-9-THC.

Figure 11

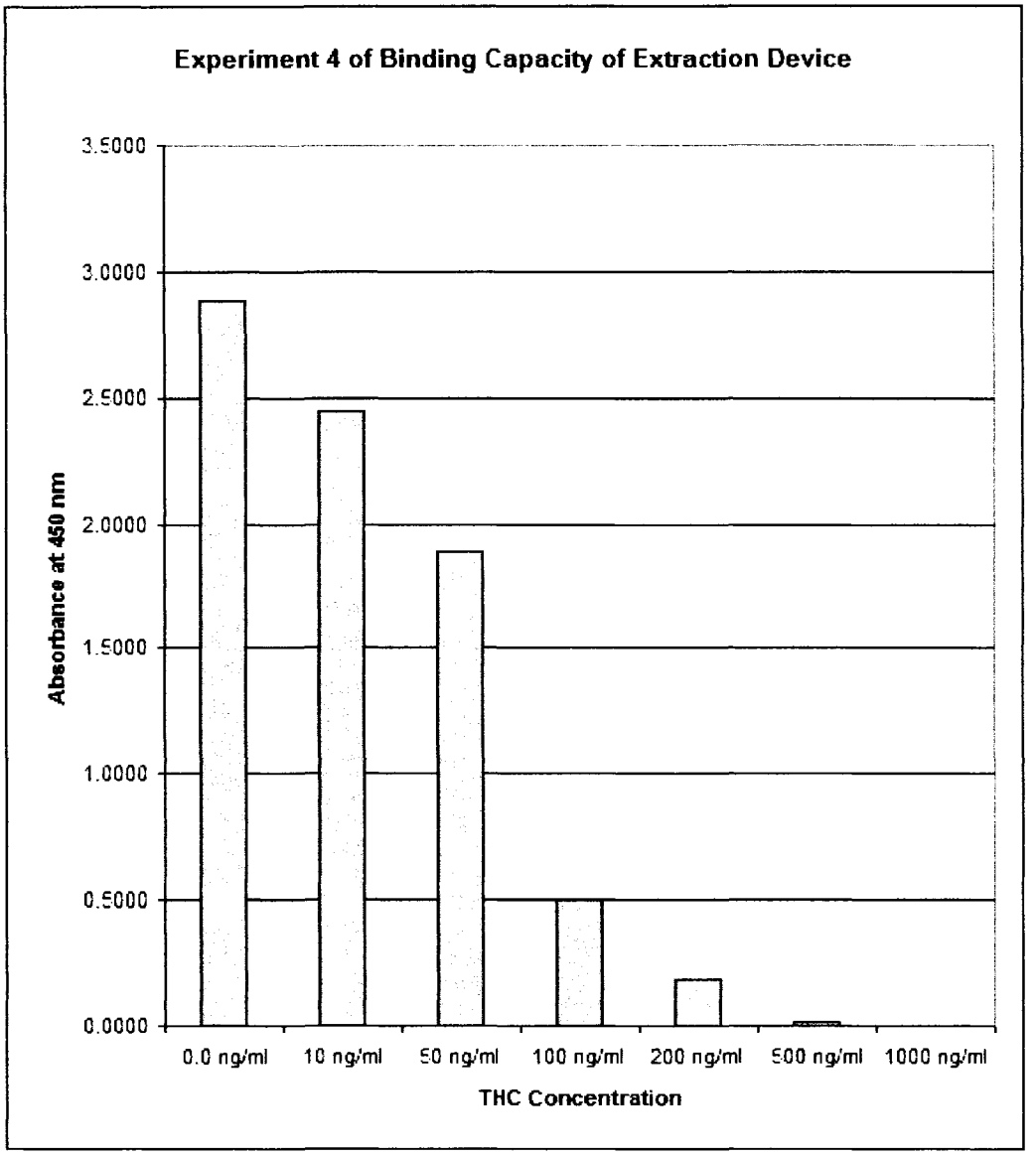

Figure 11: Bar graph shows the absorbance values from fourth experiment of flasks treated with glutaraldehyde for immobilization of antibodies and subjected to ELISA reagents to determine binding capacity of Δ-9-THC.

Figure 12

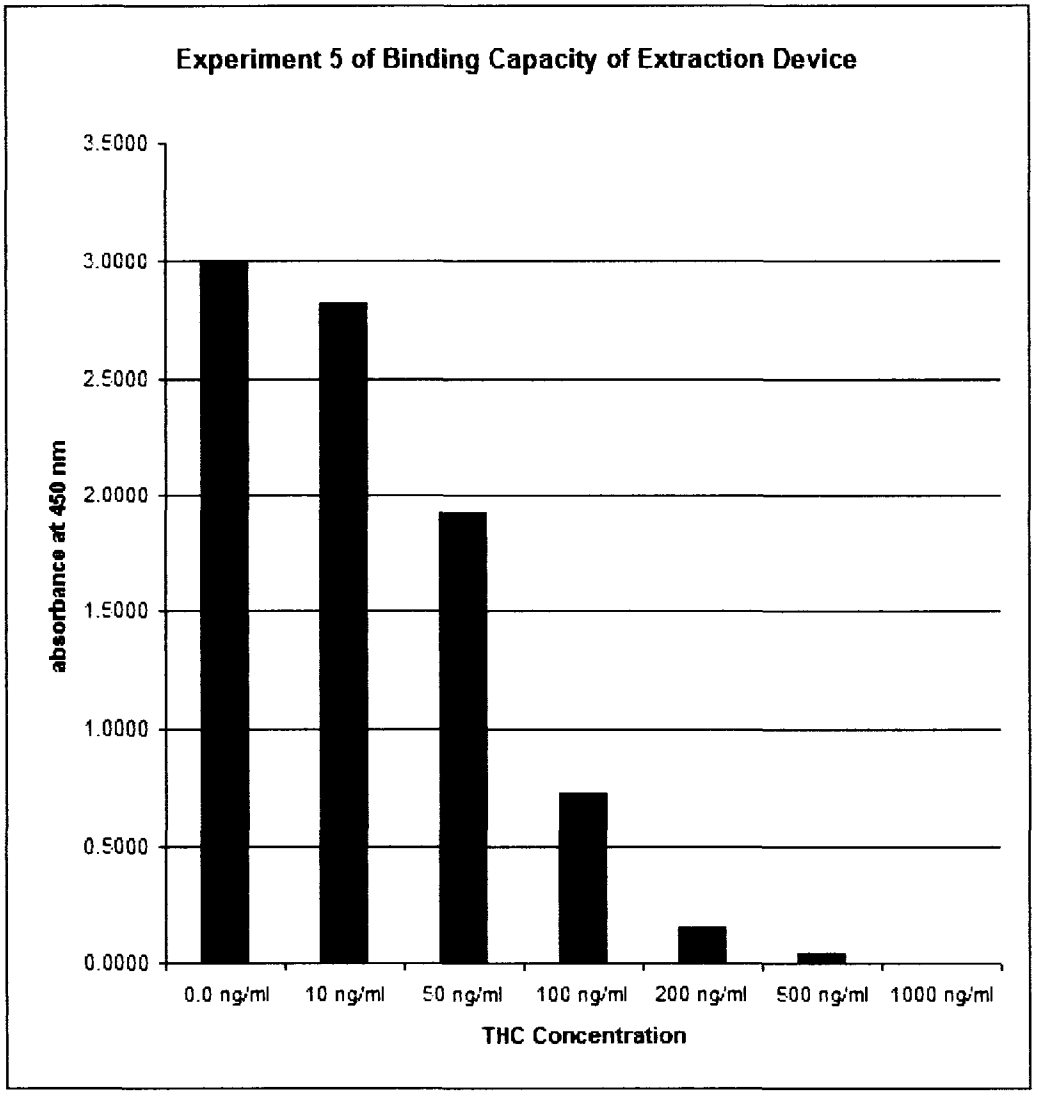

Figure 12: Bar graph shows the absorbance values from fifth experiment of flasks treated with glutaraldehyde for immobilization of antibodies and subjected to ELISA reagents to determine binding capacity of Δ-9-THC.

Table 4

	0.0 ng/ml	10 ng/ml	50 ng/ml	100 ng/ml	200 ng/ml	500 ng/ml	1000 ng/ml
	3.1321	2.1056	1.9555	0.6201	0.2860	0.0145	0.0023
	3.4588	2.8121	1.8663	0.7452	0.2956	0.0289	0.0010
	3.1855	2.6996	1.7063	0.5332	0.1569	0.0097	0.0005
	2.8851	2.4439	1.8850	0.5002	0.1877	0.0180	0.0013
	3.0001	2.8211	1.9232	0.5330	0.1596	0.0433	0.0023
Mean Abs. Values	3.1323	2.5765	1.8673	0.6063	0.2172	0.0229	0.0015

Table 4: Original absorbance values from five experiments of flasks treated with ELISA reagents in determining capacity of Δ-9-THC to be bound by extraction devices.

Figure 13

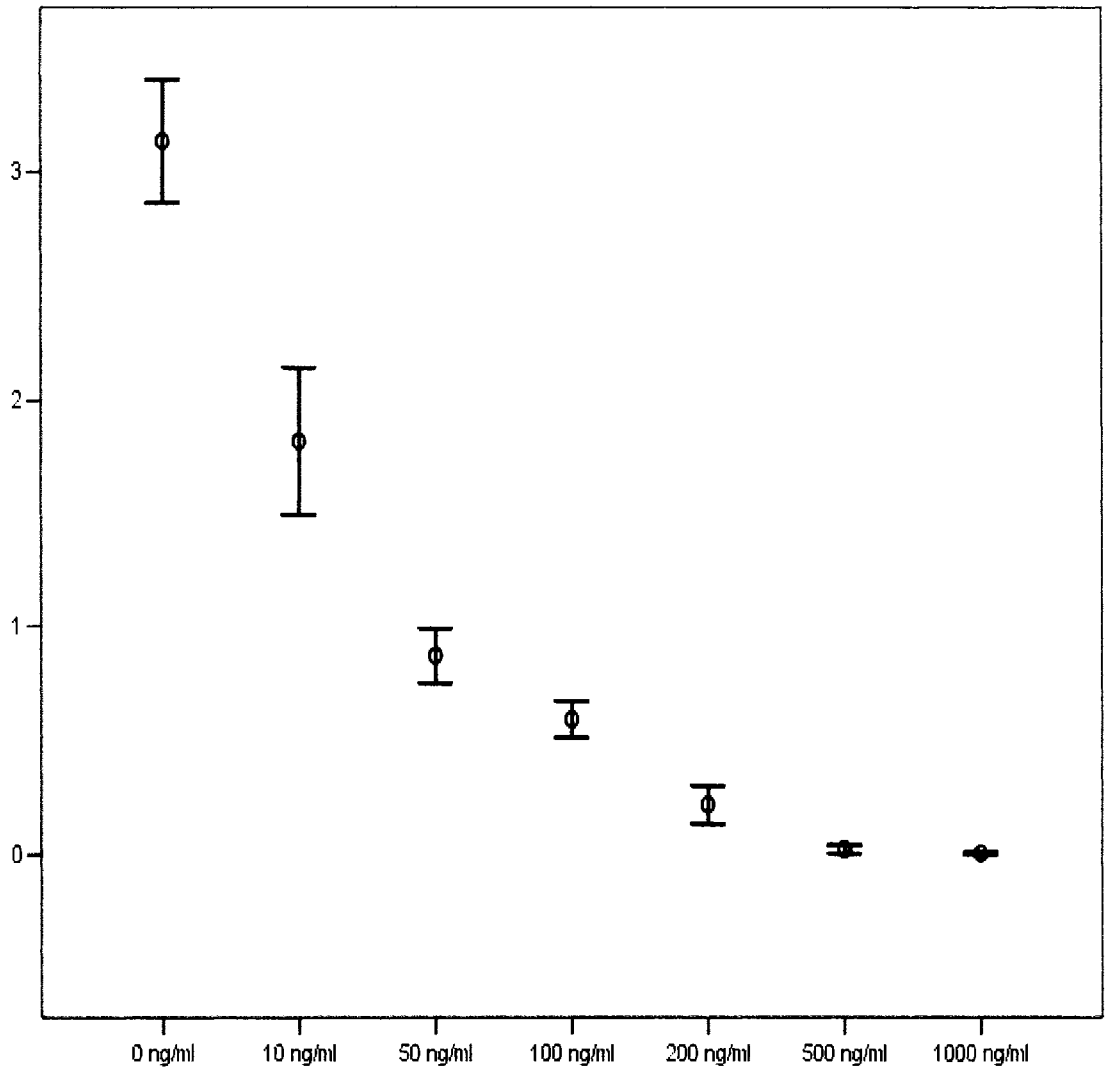

Figure 13: Error bars show variations of absorbance readings from binding capacity experiments using the extraction flasks. As the concentration of Δ-9-THC increases, the variation becomes smaller as more binding sites are unavailable for binding the THC derivative that generates the color change.

Table 5

PERCENT YIELD DATA for THC EXPERIMENTS

	Mean ng/ml	% Yield
EXP 1	15.3	0.61
EXP 2	13.1	0.52
EXP 3	13.0	0.52
EXP 4	12.6	0.50
EXP 5	13.8	0.55
	Avg. % Yield	0.54

PERCENT YIELD DATA for THCA EXPERIMENTS

	Mean ng/ml	% Yield
EXP 1	12.9	0.52
EXP 2	9.3	0.37
EXP 3	12.6	0.50
EXP 4	11.5	0.46
EXP 5	12.8	0.51
	Avg. % Yield	0.47

Table 5: Shows the average percent recovery in ng/ml of Δ-9-THC and Δ-9-THCA when compared to a direct standard at a concentration of 25 ng/ml. Data is from five experiments of 10 samples individually spiked and carried through the glutaraldehyde antibody bound procedure.

Table 6

Quantify Sample Summary Report

Sample List: **C:\TurboMass\USMFSC.PRO\SampleDB\072707A**
Last Modified: **Monday Jul 27 14:48:35 2007**
Method: **C:\TurboMass\USMFSC\MethDB\THC**
Printed: **Fri Jul 27 10:48:51 2007**

	Sample RT	Qualifiers P/F	Conc. ng/ml
Sample Name:072707A1	Sample ID: 25 ng/ml CAL1		
THC	11.13	Pass	25
Sample Name: 072707A2	Sample ID: Spike 1		
THC	11.14	Pass	14.5
Sample Name: 072707A3	Sample ID: Spike 2		
THC	11.13	Pass	17.3
Sample Name: 072707A4	Sample ID: Spike 3		
THC	11.13	Pass	13.4
Sample Name: 072707A5	Sample ID: Spike 4		
THC	11.14	Pass	15.9
Sample Name: 072707A6	Sample ID: Spike 5		
THC	11.13	Pass	18.2
Sample Name: 072707A7	Sample ID: Spike 6		
THC	11.13	Pass	14.9
Sample Name: 072707A8	Sample ID: Spike 7		
THC	11.13	Pass	13.7
Sample Name: 072707A9	Sample ID: Spike 8		
THC	11.13	Pass	18.8
Sample Name: 072707A10	Sample ID: Spike 9		
THC	11.13	Pass	11.6
Sample Name: 072707A11	Sample ID: Spike 10		
THC	11.13	Pass	15.1

Table 6: Experiment 1 of percent yield studies for Δ-9-THC. The first injection was a direct 25 ng/ml standard. The 10 subsequent injections were compared to the direct standard for determination of Δ-9-THC yield from extraction devices.

Table 7

Quantify Sample Summary Report

Sample List:	**C:\TurboMass\USMFSC.PRO\SampleDB\072807A**
Last Modified:	**Monday Jul 27 14:48:35 2007**
Method:	**C:\TurboMass\USMFSC\MethDB\THC**
Printed:	**Sat Jul 28 13:18:50 2007**

	Sample RT	**Qualifiers P/F**	**Conc. ng/ml**
Sample Name: 072807A1	Sample ID: 25 ng/ml CAL2		
THC	11.13	Pass	25
Sample Name: 072807A2	Sample ID: Spike 11		
THC	11.14	Pass	15.4
Sample Name: 072807A3	Sample ID: Spike 12		
THC	11.13	Pass	12.9
Sample Name: 072807A4	Sample ID: Spike 13		
THC	11.13	Pass	15.0
Sample Name: 072807A5	Sample ID: Spike 14		
THC	11.14	Pass	14.7
Sample Name: 072807A6	Sample ID: Spike 15		
THC	11.13	Pass	16.1
Sample Name: 072807A7	Sample ID: Spike 16		
THC	11.13	Pass	15.6
Sample Name: 072807A8	Sample ID: Spike 17		
THC	11.13	Pass	12.4
Sample Name: 072807A9	Sample ID: Spike 18		
THC	11.13	Pass	12.7
Sample Name: 072807A10	Sample ID: Spike 19		
THC	11.13	Pass	15.2
Sample Name: 072807A11	Sample ID: Spike 20		
THC	11.13	Pass	17.1

Table 7: Experiment 2 of percent yield studies for Δ-9-THC. The first injection was a direct 25 ng/ml standard. The 10 subsequent injections were compared to the direct standard for determination of Δ-9-THC yield from extraction devices.

Table 8

Quantify Sample Summary Report
Sample List: **C:\TurboMass\USMFSC.PRO\SampleDB\073007A**
Last Modified: **Monday Jul 27 14:48:35 2007**
Method: **C:\TurboMass\USMFSC\MethDB\THC**
Printed: **Tue Jul 30 19:32:30 2007**

	Sample RT	Qualifiers P/F	Conc. ng/ml
Sample Name: 073007A1	Sample ID: 25 ng/ml CAL3		
THC	11.13	Pass	25
Sample Name: 073007A2	Sample ID: Spike 21		
THC	11.14	Pass	15.0
Sample Name: 073007A3	Sample ID: Spike 22		
THC	11.13	Pass	15.3
Sample Name: 073007A4	Sample ID: Spike 23		
THC	11.13	Pass	12.1
Sample Name: 073007A5	Sample ID: Spike 24		
THC	11.14	Pass	12.9
Sample Name: 073007A6	Sample ID: Spike 25		
THC	11.13	Pass	17.2
Sample Name: 073007A7	Sample ID: Spike 26		
THC	11.13	Pass	11.0
Sample Name: 073007A8	Sample ID: Spike 27		
THC	11.13	Pass	15.8
Sample Name: 073007A9	Sample ID: Spike 28		
THC	11.13	Pass	13.0
Sample Name: 073007A10	Sample ID: Spike 29		
THC	11.13	Pass	14.3
Sample Name: 073007A11	Sample ID: Spike 30		
THC	11.13	Pass	17.5

Table 8: Experiment 3 of percent yield studies for Δ-9-THC. The first injection was a direct 25 ng/ml standard. The 10 subsequent injections were compared to the direct standard for determination of Δ-9-THC yield from extraction devices.

Table 9

Quantify Sample Summary Report
Sample List: **C:\TurboMass\USMFSC.PRO\SampleDB\073107A**
Last Modified: **Monday Jul 27 14:48:35 2007**
Method: **C:\TurboMass\USMFSC\MethDB\THC**
Printed: **Tue Jul 31 16:12:33 2007**

	Sample RT	Qualifiers P/F	Conc. ng/ml
Sample Name:073107A1	Sample ID: 25 ng/ml CAL 4		
THC	11.13	Pass	25
Sample Name: 073107A2	Sample ID: Spike 31		
THC	11.14	Pass	12.6
Sample Name: 073107A3	Sample ID: Spike 32		
THC	11.13	Pass	11.8
Sample Name: 073107A4	Sample ID: Spike 33		
THC	11.13	Pass	13.3
Sample Name: 073107A5	Sample ID: Spike 34		
THC	11.14	Pass	12.0
Sample Name: 073107A6	Sample ID: Spike 35		
THC	11.13	Pass	15.9
Sample Name: 073107A7	Sample ID: Spike 36		
THC	11.13	Pass	14.1
Sample Name: 073107A8	Sample ID: Spike 37		
THC	11.13	Pass	12.6
Sample Name: 073107A9	Sample ID: Spike 38		
THC	11.13	Pass	13.4
Sample Name: 073107A10	Sample ID: Spike 39		
THC	11.13	Pass	11.6
Sample Name: 073107A11	Sample ID: Spike 40		
THC	11.13	Pass	14.2

Table 9: Experiment 4 of percent yield studies for Δ-9-THC. The first injection was a direct 25 ng/ml standard. The 10 subsequent injections were compared to the direct standard for determination of Δ-9-THC yield from extraction devices.

Table 10

Quantify Sample Summary Report
Sample List: **C:\TurboMass\USMFSC.PRO\SampleDB\080107A**
Last Modified: **Monday Jul 27 14:48:35 2007**
Method: **C:\TurboMass\USMFSC\MethDB\THC**
Printed: **Wed Aug 01 17:12:49 2007**

	Sample RT	Qualifiers P/F	Conc. ng/ml
Sample Name:080107A1	Sample ID: 25 ng/ml CAL 5		
THC	11.13	Pass	25
Sample Name: 080107A2	Sample ID: Spike 41		
THC	11.14	Pass	16.1
Sample Name: 080107A3	Sample ID: Spike 42		
THC	11.13	Pass	12.0
Sample Name: 080107A4	Sample ID: Spike 43		
THC	11.13	Pass	13.6
Sample Name: 080107A5	Sample ID: Spike 44		
THC	11.14	Pass	13.6
Sample Name: 080107A6	Sample ID: Spike 45		
THC	11.13	Pass	13.2
Sample Name: 080107A7	Sample ID: Spike 46		
THC	11.13	Pass	14.1
Sample Name: 080107A8	Sample ID: Spike 47		
THC	11.13	Pass	16.2
Sample Name: 080107A9	Sample ID: Spike 48		
THC	11.13	Pass	13.7
Sample Name: 080107A10	Sample ID: Spike 49		
THC	11.13	Pass	14.2
Sample Name: 080107A11	Sample ID: Spike 50		
THC	11.13	Pass	16.3

Table 10: Experiment 5 of percent yield studies for Δ-9-THC. The first injection was a direct 25 ng/ml standard. The 10 subsequent injections were compared to the direct standard for determination of Δ-9-THC yield from extraction devices.

Table 11

Quantify Sample Summary Report
Sample List: **C:\TurboMass\USMFSC.PRO\SampleDB\072707A**
Last Modified: **Monday Jul 27 14:48:35 2007**
Method: **C:\TurboMass\USMFSC\MethDB\THCA**
Printed: **Fri Jul 27 10:48:51 2007**

	Sample RT	**Qualifiers P/F**	**Conc. ng/ml**
Sample Name: 072707A12	Sample ID: 25 ng/ml CAL 1		
THCA	13.07	Pass	25
Sample Name: 072707A13	Sample ID: Spike 1		
THCA	13.08	Pass	12.2
Sample Name: 072707A14	Sample ID: Spike 2		
THCA	13.07	Pass	10.5
Sample Name: 072707A15	Sample ID: Spike 3		
THCA	13.07	Pass	11.7
Sample Name: 072707A16	Sample ID: Spike 4		
THCA	13.07	Pass	12.0
Sample Name: 072707A17	Sample ID: Spike 5		
THCA	13.07	Pass	10.3
Sample Name: 072707A18	Sample ID: Spike 6		
THCA	13.07	Pass	11.3
Sample Name: 072707A19	Sample ID: Spike 7		
THCA	13.07	Pass	14.0
Sample Name: 072707A20	Sample ID: Spike 8		
THCA	13.07	Pass	14.6
Sample Name: 072707A21	Sample ID: Spike 9		
THCA	13.07	Pass	10.2
Sample Name: 072707A22	Sample ID: Spike 10		
THCA	13.07	Pass	12.9

Table 11: Experiment 1 of percent yield studies for Δ-9-THCA. The first injection was a direct 25 ng/ml standard. The 10 subsequent injections were compared to the direct standard for determination of Δ-9-THCA yield from extraction devices.

Table 12

Quantify Sample Summary Report
Sample List: **C:\TurboMass\USMFSC.PRO\SampleDB\072807A**
Last Modified: **Monday Jul 27 14:48:35 2007**
Method: **C:\TurboMass\USMFSC\MethDB\THCA**
Printed: **Sat Jul 28 13:18:50 2007**

	Sample RT	Qualifiers P/F	Conc. ng/ml
Sample Name:072807A12	Sample ID: 25 ng/ml CAL 2		
THCA	13.07	Pass	25
Sample Name: 072807A13	Sample ID: Spike 11		
THCA	13.07	Pass	15.4
Sample Name: 072807A14	Sample ID: Spike 12		
THCA	13.08	Pass	12.9
Sample Name: 072807A15	Sample ID: Spike 13		
THCA	13.07	Pass	15.0
Sample Name: 072807A16	Sample ID: Spike 14		
THCA	13.07	Pass	14.7
Sample Name: 072807A17	Sample ID: Spike 15		
THCA	13.07	Pass	16.1
Sample Name: 072807A18	Sample ID: Spike 16		
THCA	13.07	Pass	15.6
Sample Name: 072807A19	Sample ID: Spike 17		
THCA	13.08	Pass	12.4
Sample Name: 072807A20	Sample ID: Spike 18		
THCA	13.07	Pass	12.7
Sample Name: 072807A21	Sample ID: Spike 19		
THCA	13.07	Pass	15.2
Sample Name: 072807A22	Sample ID: Spike 20		
THCA	13.07	Pass	17.1

Table 12: Experiment 2 of percent yield studies for Δ-9-THCA. The first injection was a direct 25 ng/ml standard. The 10 subsequent injections were compared to the direct standard for determination of Δ-9-THCA yield from extraction devices.

Table 13

Quantify Sample Summary Report

Sample List: **C:\TurboMass\USMFSC.PRO\SampleDB\073007A**
Last Modified: **Monday Jul 27 14:48:35 2007**
Method: **C:\TurboMass\USMFSC\MethDB\THCA**
Printed: **Mon Jul 30 16:12:33 2007**

	Sample RT	Qualifiers P/F	Conc. ng/ml
Sample Name: 073007A12	Sample ID: 25 ng/ml CAL 3		
THCA	13.07	Pass	25
Sample Name: 073007A13	Sample ID: Spike 21		
THCA	13.07	Pass	13.6
Sample Name: 073007A14	Sample ID: Spike 22		
THCA	13.08	Pass	14.0
Sample Name: 073007A15	Sample ID: Spike 23		
THCA	13.07	Pass	13.7
Sample Name: 073007A16	Sample ID: Spike 24		
THCA	13.07	Pass	12.6
Sample Name: 073007A17	Sample ID: Spike 25		
THCA	13.08	Pass	13.1
Sample Name: 073007A18	Sample ID: Spike 26		
THCA	13.08	Pass	14.9
Sample Name: 073007A19	Sample ID: Spike 27		
THCA	13.08	Pass	15.2
Sample Name: 073007A20	Sample ID: Spike 28		
THCA	13.07	Pass	13.4
Sample Name: 073007A21	Sample ID: Spike 29		
THCA	13.07	Pass	10.3
Sample Name: 073007A22	Sample ID: Spike 30		
THCA	13.07	Pass	11.6

Table 13: Experiment 3 of percent yield studies for Δ-9-THCA. The first injection was a direct 25 ng/ml standard. The 10 subsequent injections were compared to the direct standard for determination of Δ-9-THCA yield from extraction devices.

Table 14

Quantify Sample Summary Report

Sample List:	**C:\TurboMass\USMFSC.PRO\SampleDB\073107A**
Last Modified:	**Monday Jul 27 14:48:35 2007**
Method:	**C:\TurboMass\USMFSC\MethDB\THCA**
Printed:	**Tue Jul 31 19:32:303 2007**

	Sample RT	Qualifiers P/F	Conc. ng/ml
Sample Name: 073107A12	Sample ID: 25 ng/ml CAL 4		
THCA	13.07	Pass	25
Sample Name: 073107A13	Sample ID: Spike 31		
THCA	13.08	Pass	10.6
Sample Name: 073107A14	Sample ID: Spike 32		
THCA	13.07	Pass	11.4
Sample Name: 073107A15	Sample ID: Spike 33		
THCA	13.07	Pass	11.5
Sample Name: 073107A16	Sample ID: Spike 34		
THCA	13.07	Pass	12.0
Sample Name: 073107A17	Sample ID: Spike 35		
THCA	13.07	Pass	13.2
Sample Name: 073107A18	Sample ID: Spike 36		
THCA	13.08	Pass	11.9
Sample Name: 073107A19	Sample ID: Spike 37		
THCA	13.07	Pass	10.6
Sample Name: 073107A20	Sample ID: Spike 38		
THCA	13.07	Pass	13.0
Sample Name: 073107A21	Sample ID: Spike 39		
THCA	13.07	Pass	11.2
Sample Name: 073107A22	Sample ID: Spike 40		
THCA	13.08	Pass	11.5

Table 14: Experiment 4 of percent yield studies for Δ-9-THCA. The first injection was a direct 25 ng/ml standard. The 10 subsequent injections were compared to the direct standard for determination of Δ-9-THCA yield from extraction devices.

Table 15

Quantify Sample Summary Report

Sample List: C:\TurboMass\USMFSC.PRO\SampleDB\080107A
Last Modified: Monday Jul 27 14:48:35 2007
Method: C:\TurboMass\USMFSC\MethDB\THCA
Printed: Wed Aug 01 17:12:49 2007

	Sample RT	Qualifiers P/F	Conc. ng/ml
Sample Name: 080107A1	Sample ID: 25 ng/ml CAL 5		
THCA	13.07	Pass	25
Sample Name: 080107A2	Sample ID: Spike 41		
THCA	13.07	Pass	12.4
Sample Name: 080107A3	Sample ID: Spike 42		
THCA	13.08	Pass	11.7
Sample Name: 080107A4	Sample ID: Spike 43		
THCA	13.11	Pass	11.9
Sample Name: 080107A5	Sample ID: Spike 44		
THCA	13.07	Pass	13.3
Sample Name: 080107A6	Sample ID: Spike 45		
THCA	13.08	Pass	13.2
Sample Name: 080107A7	Sample ID: Spike 46		
THCA	13.07	Pass	12.1
Sample Name: 080107A8	Sample ID: Spike 47		
THCA	13.07	Pass	13.1
Sample Name: 080107A9	Sample ID: Spike 48		
THCA	13.08	Pass	11.0
Sample Name: 080107A10	Sample ID: Spike 49		
THCA	13.07	Pass	13.2
Sample Name: 080107A11	Sample ID: Spike 50		
THCA	13.08	Pass	13.8

Table 15: Experiment 5 of percent yield studies for Δ-9-THCA. The first injection was a direct 25 ng/ml standard. The 10 subsequent injections were compared to the direct standard for determination of Δ-9-THCA yield from extraction devices.

Figure 14

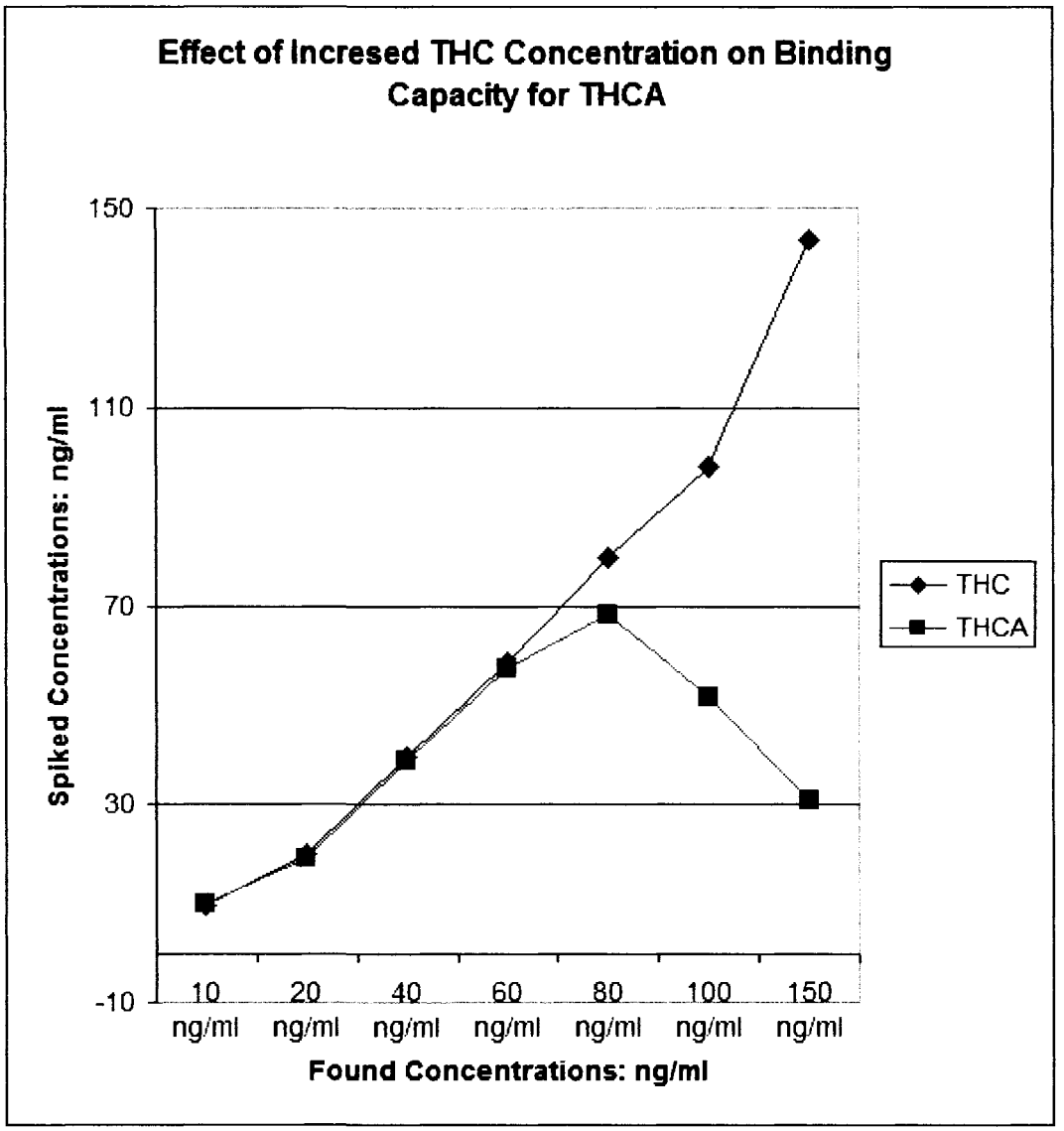

Figure 14: Demonstrates the effect of increasing the concentration of Δ-9-THC on the binding capacity of Δ-9-THCA by the glutaraldehyde bound antibody in an extraction device. The antibody is monoclonal for Δ-9-THC and has a greater affinity for that analyte over Δ-9-THCA.

Linearity of Individual Extracted Analytes

One ml of negative synthetic blood was added to one treated flask as a negative sample and 1 ml aliquots of spiked synthetic blood with concentrations of 5, 10, 30, and 50 ng of Δ-9-THC were added to glutaraldehyde treated flasks. The extraction of these spiked samples produced a linear curve with an r^2 value of 0.998 (see Figure 15). The same experiment was run with Δ-9-THCA at 2, 5, 20, 50 ng/ml which also produced a linear curve with an r^2 value of 0.997 (see Figure 16).

Linearity Using Deuterated Internal Standard

Seven 1 ml synthetic blood aliquots were spiked with 25 ng of Δ-9-THC-D3 as an internal standard and then with Δ-9-THC at concentrations of 0, 2, 5, 10, 25 and 50 ng/ml to test extraction of both analytes and determine linearity. The extraction produced a linear curve with a r^2 value of 0.995 (see Figure 17).

The same experiment was run with Δ-9-THCA-D3 and Δ-9-THCA, with the exception of a 1 ng sample. Results were also linear to 50 ng/ml.

Analyses of Postmortem Samples

The first group of postmortem samples analyzed was those secured from the Mississippi Crime Laboratory were those samples reported as "None Detected". These 10 samples were analyzed on two different days using the extraction. All samples were found to be negative for Δ-9-THC. These findings are in agreement with the results reported by the Mississippi Crime Laboratory (see Tables 16 and 17). The negative findings also demonstrate that none of the solutions or hardware used during the experiments were or became contaminated with analytes of interest.

Figure 15

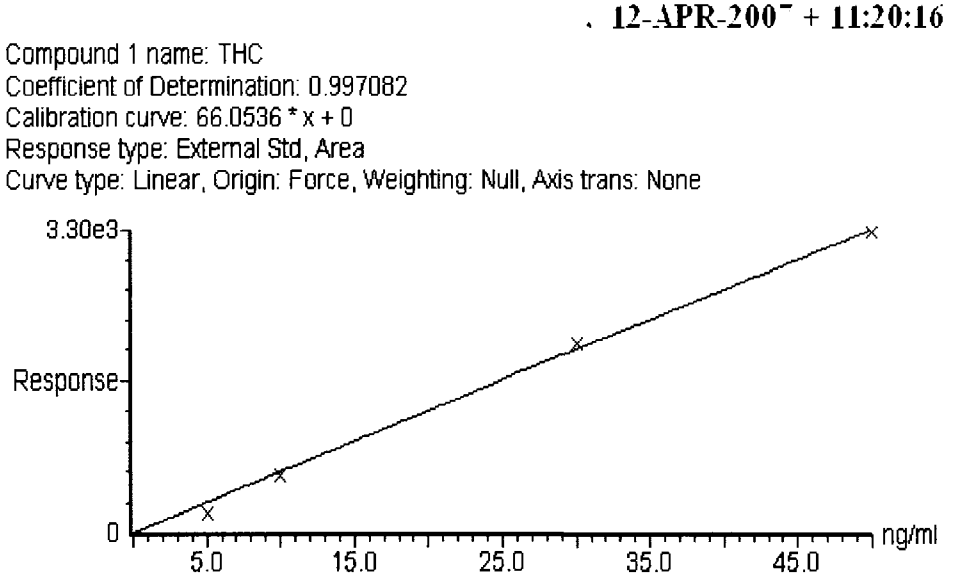

Figure 15: Calibration curve of Δ-9-THC extracted as a single analyte. The r^2 value is 0.997082 which exceeds the criteria of ≥ 0.985 to be considered linear. The range is linear to 50 ng/ml.

Figure 16

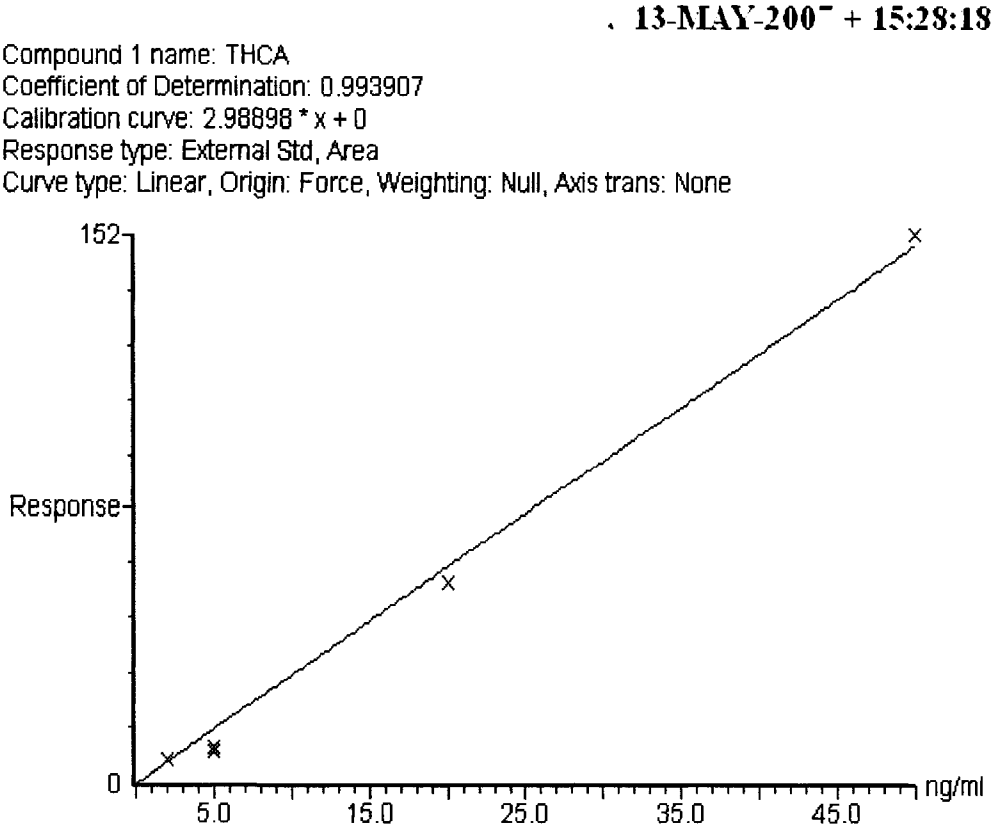

Figure 16: Calibration curve of Δ-9-THCA extracted as a single analyte. The r^2 value is 0.993907 which exceeds the criteria of ≥ 0.985 to be considered linear. The range is linear to 50 ng/ml.

Figure 17

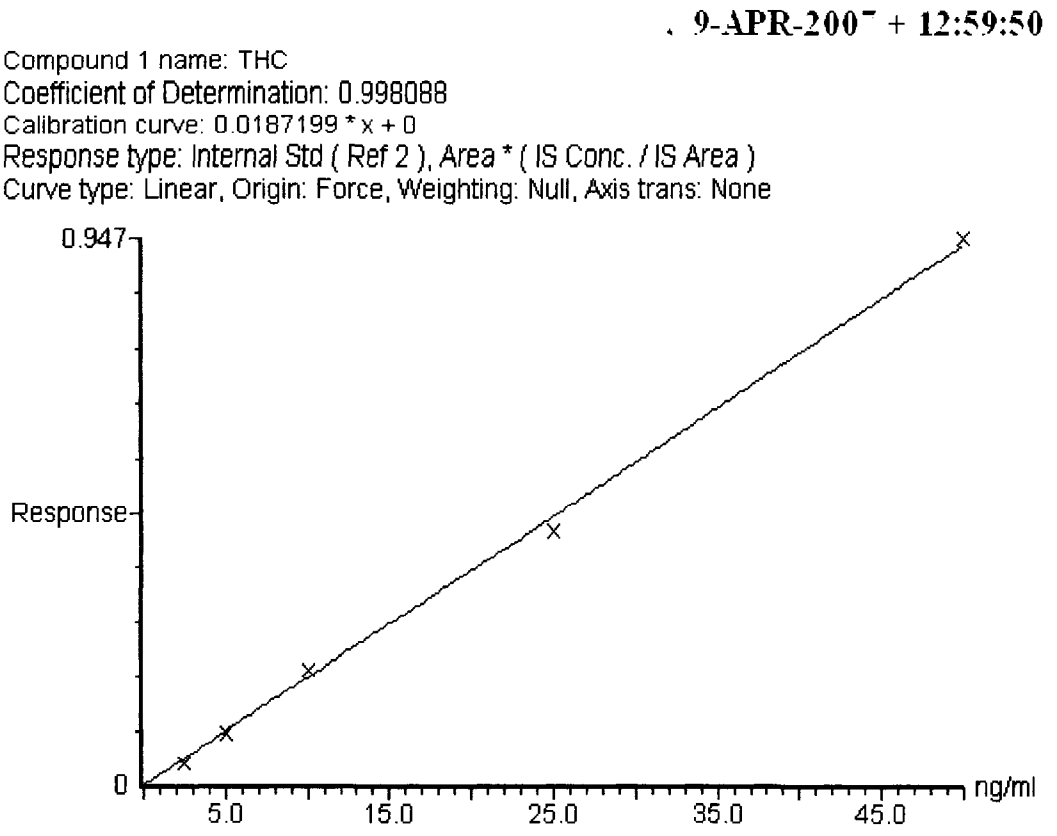

Figure 17: Shows a linear curve of Δ-9-THC using the internal standard method. Extracted calibrators ranged from 2 – 50 ng/ml.

Table 16

Quantify Sample Summary Report
Sample List: **C:\TurboMass\USMFSC.PRO\SampleDB\083007A**
Last Modified: **Monday Bug 27 14:48:35 2007**
Method: **C:\TurboMass\USMFSC\MethDB\THC**
Printed: **Fri Bug 31 16:40:21 2007**

	Sample RT	Qualifiers P/F	Conc. ng/ml
Sample Name: 083007A1	Sample ID: 0 ng/ml BLK		
THC	NF	Fail	0.0
THC-D3	11.12	Pass	1.0
Sample Name: 083007A2	Sample ID: 3 ng/ml CAL		
THC	11.11	Pass	2.8
THC-D3	11.12	Pass	1.0
Sample Name: 083007A3	Sample ID: 10 ng/ml CAL		
THC	11.11	Pass	10.8
THC-D3	11.09	Pass	1.0
Sample Name: 083007A4	Sample ID: 20 ng/ml CAL		
THC	11.11	Pass	19.6
THC-D3	11.08	Pass	1.0
Sample Name: 083007A5	Sample ID: 50 ng/ml CAL		
THC	11.11	Pass	49.6
THC-D3	11.08	Pass	1.0
Sample Name: 083007A6	Sample ID: 5 ng/ml CTRL		
THC	11.11	Pass	4.9
THC-D3	11.08	Pass	1.0
Sample Name: 083007A7	Sample ID: FSC 1A (13995)		
THC	11.11	Pass	5.2
THC-D3	11.07	Pass	1.0
Sample Name: 083007A8	Sample ID: FSC 2A (8516)		
THC	NF	Fail	0
THC-D3	11.08	Pass	1.0
Sample Name: 083007A9	Sample ID: FSC 3A (13298)		
THC	NF	Fail	0
THC-D3	11.08	Pass	1.0
Sample Name: 083007A10	Sample ID: FSC 4A (14876)		
THC	NF	Fail	0
THC-D3	11.08	Pass	1.0
Sample Name: 083007A11	Sample ID: FSC 5A (14886)		
THC	NF	Fail	0
THC-D3	11.08	Pass	1.0
Sample Name: 083007A12	Sample ID: FSC 6A (6342)		
THC	NF	Fail	0
THC-D3	11.09	Pass	1.0

Table 16, (continued)

Sample Name: 083007A13	Sample ID: FSC 7A (8011)		
THC	NF	Fail	0
THC-D3	11.08	Pass	1.0
Sample Name: 083007A14	Sample ID: FSC 8A (8106)		
THC	NF	Fail	0
THC-D3	11.09	Pass	1.0
Sample Name: 083007A15	Sample ID: FSC 9A (8245)		
THC	NF	Fail	0
THC-D3	11.10	Pass	1.0
Sample Name: 083007A16	Sample ID: FSC 10 (8464)		
THC	NF	Fail	0
THC-D3	11.09	Pass	1.0
Sample Name: 083007A17	Sample ID: 30 ng/ml CTRL		
THC	NF	Pass	28.6
THC-D3	11.08	Pass	1.0

Table 16: Represents first run of samples from the Mississippi Crime Laboratory reported as "None Detected" for Δ-9-THC. Page 1 shows the calibrators, low control and 5 samples. Page 2 shows 5 samples and the high control. Results match results reported from the Mississippi Crime Laboratory.

Table 17

Quantify Sample Summary Report
Sample List: **C:\TurboMass\USMFSC.PRO\SampleDB\083007B**
Last Modified: **Monday Bug 27 14:48:35 2007**
Method: **C:\TurboMass\USMFSC\MethDB\THC**
Printed: **Fri Bug 31 16:52:33 2007**

	Sample RT	Qualifiers P/F	Conc. ng/ml
Sample Name: 083007B1	Sample ID: 0 ng/ml BLK		
THC	NF	Fail	0.0
THC-D3	11.12	Pass	1.0
Sample Name: 083007B2	Sample ID: 3 ng/ml CAL		
THC	11.11	Pass	3.0
THC-D3	11.12	Pass	1.0
Sample Name: 083007B3	Sample ID: 10 ng/ml CAL		
THC	11.11	Pass	10.1
THC-D3	11.09	Pass	1.0
Sample Name: 083007B4	Sample ID: 20 ng/ml CAL		
THC	11.11	Pass	19.8
THC-D3	11.08	Pass	1.0
Sample Name: 083007B5	Sample ID: 50 ng/ml CAL		
THC	11.11	Pass	50.3
THC-D3	11.08	Pass	1.0
Sample Name: 083007B6	Sample ID: 5 ng/ml CTRL		
THC	11.11	Pass	5.1
THC-D3	11.08	Pass	1.0
Sample Name: 083007B7	Sample ID: FSC 1B (13995)		
THC	11.11	Fail	0
THC-D3	11.07	Pass	1.0
Sample Name: 083007B8	Sample ID: FSC 2B (8516)		
THC	NF	Fail	0
THC-D3	11.08	Pass	1.0
Sample Name: 083007B9	Sample ID: FSC 3B (13298)		
THC	NF	Fail	0
THC-D3	11.08	Pass	1.0
Sample Name: 083007B10	Sample ID: FSC 4B (14876)		
THC	NF	Fail	0
THC-D3	11.08	Pass	1.0
Sample Name: 083007B11	Sample ID: FSC 5B (14886)		
THC	NF	Fail	0
THC-D3	11.08	Pass	1.0
Sample Name: 083007B12	Sample ID: FSC 6B (6342)		
THC	NF	Fail	0
THC-D3	11.09	Pass	1.0

Table 17, (continued)

Sample Name: 083007B13	Sample ID: FSC 7B (8011)		
THC	NF	Fail	0
THC-D3	11.08	Pass	1.0
Sample Name: 083007B14	Sample ID: FSC 8B (8106)		
THC	NF	Fail	0
THC-D3	11.09	Pass	1.0
Sample Name: 083007B15	Sample ID: FSC 9B (8245)		
THC	NF	Fail	0
THC-D3	11.10	Pass	1.0
Sample Name: 083007B16	Sample ID: FSC 10B (8464)		
THC	NF	Fail	0
THC-D3	11.09	Pass	1.0
Sample Name: 083007B17	Sample ID: 30 ng/ml CTRL		
THC	NF	Pass	32
THC-D3	11.08	Pass	1.0

Table 17: Represents second run of samples from the Mississippi Crime Laboratory reported as "None Detected" for Δ-9-THC. Page 1 shows the calibrators, low control and 5 samples. Page 2 shows 5 samples and the high control. Results match results reported from the Mississippi Crime Laboratory.

The next group of samples from the Mississippi Crime Laboratory run was those reported with quantitative values for Δ-9-THC. These ten samples were run on two separate occasions along with calibrators and controls (see Table 18). All samples were found to contain Δ-9-THC using the extraction device and all met the criteria for considered reportable (see Figure 18).

Statistical analysis was performed using the Paired *t* test. Group A was compared to the values reported by the Mississippi Crime Laboratory and a significant difference was found. The critical value for $t_{.01\,(9)}$ is 3.250. The statistical analysis found the Paired *t* value to be 4.384 (see Table 20). The average difference between the groups was 0.32 ng/ml. The largest difference was 0.7 ng/ml while the smallest difference was 0.2 ng/ml.

A second run of the same samples using a different set of devices was performed and analyzed statistically as stated above (see Table 19). Again a significant difference was found were the Paired *t* value was calculated to be 6.034 (see Table 21). The average difference in concentration was 0.35 ng/ml with a range of 0.1 to 0.6 ng/ml.

All samples received from the Mississippi Crime Laboratory were run for Δ-9-THCA (see Figure 19). No Δ-9-THCA was found in two of the samples which agreed with values reported by the crime laboratory (see Tables 22-24). A second run was performed with similar results (see Tables 25-27).

A Paired *t* test was performed for the Δ-9-THCA results in the same manner as for Δ-9-THC. The first group showed a significant with a Paired *t* value of 9.596 where $t_{.01(27)}$ is 2.771 (see Table 28). The second run was subjected to the same statistical analysis also demonstrating a significant difference with the critical *t* value being 8.827 (see Table 29).

Table 18

Quantify Sample Summary Report
Sample List: **C:\TurboMass\USMFSC.PRO\SampleDB\082707A**
Last Modified: **Monday Jul 27 14:48:35 2007**
Method: **C:\TurboMass\USMFSC\MethDB\THC**
Printed: **Fri Aug 31 14:48:51 2007**

	Sample RT	Qualifiers P/F	Conc. ng/ml
Sample Name: 082707A1	Sample ID: 0 ng/ml CAL 1		
THC	11.13	Fail	0
THC-D3	11.12	Pass	1.0
Sample Name: 082707A2	Sample ID: 3 ng/ml CAL		
THC	11.14	Pass	3.11
THC-D3	11.12	Pass	1.0
Sample Name: 082707A3	Sample ID: 10 ng/ml CAL		
THC	11.13	Pass	8.71
THC-D3	11.12	Pass	1.0
Sample Name: 082707A4	Sample ID: 20 ng/ml CAL		
THC	11.13	Pass	19.93
THC-D3	11.12	Pass	1.0
Sample Name: 082707A5	Sample ID: 50 ng/ml CAL		
THC	11.14	Pass	49.88
THC-D3	11.12	Pass	1.0
Sample Name: 082707A6	Sample ID: 5 ng/ml CTRL		
THC	111.14	Pass	5.3
THC-D3	11.12	Pass	1.0
Sample Name: 082707A7	Sample ID: FSC 11A (3330)		
THC	11.14	Pass	1.8
THC-D3	11.12	Pass	1.0
Sample Name: 082707A8	Sample ID: FSC 12 A (4086)		
THC	11.13	Pass	10.3
THC-D3	11.12	Pass	1.0
Sample Name: 082707A9	Sample ID: FSC 13 A (6279)		
THC	11.13	Pass	1.4
THC-D3	11.12	Pass	1.0
Sample Name: 082707A10	Sample ID: FSC 14 A (6349)		
THC	11.13	Pass	2.6
THC-D3	11.12	Pass	1.0
Sample Name: 082707A11	Sample ID: FSC 15 A (6352)		
THC	11.13	Pass	3.3
THC-D3	11.12	Pass	1.0

Table 18, (continued)

Sample Name: 082707A12	Sample ID: FSC 16 A (8573)		
THC	11.13	Pass	5.0
THC-D3	11.12	Pass	1.0
Sample Name: 082707A13	Sample ID: FSC 17 A (1778)		
THC	11.13	Pass	2.8
THC-D3	11.12	Pass	1.0
Sample Name: 082707A14	Sample ID: FSC 18 A (14275)		
THC	11.13	Pass	3.1
THC-D3	11.12	Pass	1.0
Sample Name: 082707A15	Sample ID: FSC 19 A (2946)		
THC	11.13	Pass	1.8
THC-D3	11.12	Pass	1.0
Sample Name: 082707A16	Sample ID: FSC 20 A (15667)		
THC	11.13	Pass	8.1
THC-D3	11.12	Pass	1.0
Sample Name: 082707A17	Sample ID: 30 ng/ml CTRL		
THC	11.13	Pass	27.0
THC-D3	11.12	Pass	1.0

Table 18: Run B of Δ-9-THC samples from Mississippi Crime Laboratory reported with quantitated results. Table shows calibrators, controls and individual sample results. All results are from analyses conducted using the extraction device bound with monoclonal Δ-9-THC antibodies. All calibrators, controls and samples met criteria for confirmation and statistical analysis.

Table 19

Quantify Sample Summary Report
Sample List: **C:\TurboMass\USMFSC.PRO\SampleDB\082707A**
Last Modified: **Monday Jul 27 14:48:35 2007**
Method: **C:\TurboMass\USMFSC\MethDB\THC**
Printed: **Fri Aug 31 14:48:51 2007**

	Sample RT	Qualifiers P/F	Conc. ng/ml
Sample Name:082707B1	Sample ID: 0 ng/ml CAL 1		
THC	11.13	Fail	0
THC-D3	11.12	Pass	1.0
Sample Name: 082707B2	Sample ID: 3 ng/ml CAL		
THC	11.14	Pass	2.96
THC-D3	11.12	Pass	1.0
Sample Name: 082707B3	Sample ID: 10 ng/ml CAL		
THC	11.13	Pass	8.95
THC-D3	11.12	Pass	1.0
Sample Name: 082707B4	Sample ID: 20 ng/ml CAL		
THC	11.13	Pass	20.23
THC-D3	11.12	Pass	1.0
Sample Name: 082707B5	Sample ID: 50 ng/ml CAL		
THC	11.14	Pass	49.91
THC-D3	11.12	Pass	1.0
Sample Name: 082707B6	Sample ID: 5 ng/ml CTRL		
THC	111.14	Pass	4.6
THC-D3	11.12	Pass	1.0
Sample Name: 082707B7	Sample ID: FSC 11B (3330)		
THC	11.14	Pass	1.9
THC-D3	11.12	Pass	1.0
Sample Name: 082707B8	Sample ID: FSC 12 B (4086)		
THC	11.13	Pass	10.6
THC-D3	11.12	Pass	1.0
Sample Name: 082707B9	Sample ID: FSC 13 B (6279)		
THC	11.13	Pass	1.4
THC-D3	11.12	Pass	1.0
Sample Name: 082707B10	Sample ID: FSC 14 B (6349)		
THC	11.13	Pass	2.5
THC-D3	11.12	Pass	1.0
Sample Name: 082707B11	Sample ID: FSC 15 B (6352)		
THC	11.13	Pass	3.1
THC-D3	11.12	Pass	1.0

Table 19, (continued)

Sample Name: 082707B12	Sample ID: FSC 16 B (8573)		
THC	11.13	Pass	4.8
THC-D3	11.12	Pass	1.0
Sample Name: 082707B13	Sample ID: FSC 17 B (1778)		
THC	11.13	Pass	2.6
THC-D3	11.12	Pass	1.0
Sample Name: 082707B14	Sample ID: FSC 18 B (14275)		
THC	11.13	Pass	3.1
THC-D3	11.12	Pass	1.0
Sample Name: 082707B15	Sample ID: FSC 19 B (2946)		
THC	11.13	Pass	2.0
THC-D3	11.12	Pass	1.0
Sample Name: 082707B16	Sample ID: FSC 20 B (15667)		
THC	11.13	Pass	7.9
THC-D3	11.12	Pass	1.0
Sample Name: 082707B17	Sample ID: 30 ng/ml CTRL		
THC	11.13	Pass	31.0
THC-D3	11.12	Pass	1.0

Table 19: Run B of Δ-9-THC samples from Mississippi Crime Laboratory reported with quantitated results. Table shows calibrators, controls and individual sample results. All results are from analyses conducted using the extraction device bound with monoclonal Δ-9-THC antibodies. All calibrators, controls and samples met criteria for confirmation and statistical analysis.

Table 20

Direct-Difference Statistical Evaluation of THC Values: Paired t Ratio Testing Group A				
Sample Identification	MCL Results X1	EXP- A Results X2	Difference D X1-X2	Difference Squared D2
3330	2	1.8	0.2	0.04
4086	11	10.3	0.7	0.49
6279	1.6	1.4	0.2	0.04
6349	2.9	2.6	0.3	0.09
6352	3.6	3.3	0.3	0.09
8573	5.4	5	0.4	0.16
1778	3	2.8	0.2	0.04
14275	3.5	3.1	0.4	0.16
2946	2.1	1.8	0.3	0.09
15667	8.3	8.1	0.2	0.04
	$\sum X1 = 43.4$	$\sum X2 = 40.2$		$\sum D2 = 1.5$
Group Means	MCL 4.340	EXP- A 4.020		
Mean Difference Between Groups (MD)	0.320			
Standard Deviation of Difference (SDD)		$SD^2 =$	$\sqrt{(\sum D2/N)-MD^2}$	
	$\sum D2 / N = .15$	$MD2 = 0.102$	$\sqrt{.05} = 0.22$	
Standard Error of Differences (SED)		SED =	SDD / ($\sqrt{}$ N-1)	0.073
Paired *t* Ratio =	MD/SED = 4.384			
$t_{.01(9)}$ 3.250	(Significant Difference)			

Table 20: Direct Difference Test (Paired t Test) of first run of THC samples. The test identifies a significant difference between the groups indicating differences in results are not attributable to chance. The average difference between the groups is 0.32 ng/ml.

Table 21

Direct-Difference Statistical Evaluation of THC Values: Paired t Ratio Testing Group B				
Sample Identification	MCL Results X1	EXP- B Results X2	Difference D X1-X2	Difference Squared D2
3330	2	1.9	0.1	0.01
4086	11	10.6	0.4	0.16
6279	1.6	1.4	0.2	0.04
6349	2.9	2.5	0.4	0.16
6352	3.6	3.1	0.5	0.25
8537	5.4	4.8	0.6	0.36
1778	3	2.6	0.1	0.01
14275	3.5	3.1	0.4	0.16
2946	2.1	2	0.1	0.01
15667	8.3	7.9	0.4	0.16
	$\sum X1 = 43.4$	$\sum X2 = 39.9$		$\sum D2 = 1.5$
Group Means	MCL 4.340	EXP- B 3.990		
Mean Difference Between Groups (MD)	0.350			
Standard Deviation of Difference (SDD)		$SD^2 =$	$\sqrt{(\sum D2/N)-MD^2}$	
	$\sum D2 / N = .15$	$MD2 = 0.123$	$\sqrt{0.03} = 0.173$	
Standard Error of Differences (SED)		SED =	$SDD / (\sqrt{N-1})$	0.058
Paired t Ratio =	MD/SED = 6.034			
$t_{.01(9)}$ 3.250	(Significant Difference)			

Table 21: Direct Difference Test (Paired t Test) of second run of THC samples. The test identifies a significant difference between the groups indicating the differences in results are not attributable to chance. The average difference between the groups is 0.35 ng/ml.

Figure 18

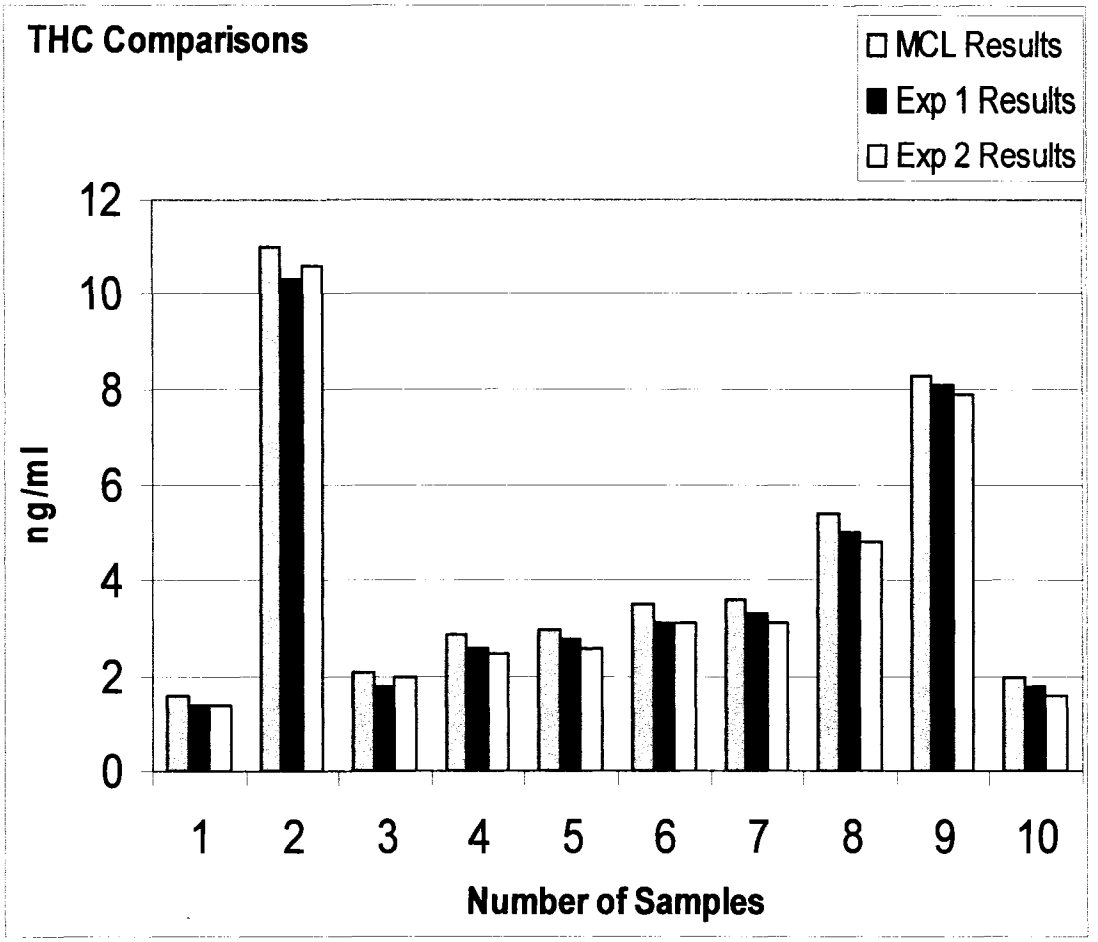

Figure 18: Comparison of Δ-9-THC results reported by the Mississippi Crime Laboratory versus separate extractions using the glutaraldehyde treated flasks with Δ-9-THC monoclonal antibodies. The light blue bar represents results from the Mississippi Crime Laboratory while the purple and white bars represent results using the extraction device. Note there is a slight negative bias using the extraction device.

Table 22

Quantify Sample Summary Report
Sample List: **C:\TurboMass\USMFSC.PRO\SampleDB\082707A**
Last Modified: **Monday Jul 27 14:48:35 2007**
Method: **C:\TurboMass\USMFSC\MethDB\THCA**
Printed: **Fri Aug 31 15:03:44 2007**

	Sample RT	Qualifiers P/F	Conc. ng/ml
Sample Name: 082707A1	Sample ID: 0 ng/ml CAL 1		
THCA	NF	Fail	0
THCA-D3	13.05	Pass	1.0
Sample Name: 082707A2	Sample ID: 5 ng/ml CAL		
THCA	13.07	Pass	4.80
THCA-D3	13.05	Pass	1.0
Sample Name: 082707A3	Sample ID: 10 ng/ml CAL		
THCA	13.07	Pass	10.21
THCA-D3	13.05	Pass	1.0
Sample Name: 082707A4	Sample ID: 20 ng/ml CAL		
THCA	13.07	Pass	18.98
THCA-D3	13.05	Pass	1.0
Sample Name: 082707A5	Sample ID: 50 ng/ml CAL		
THCA	13.07	Pass	49.91
THCA-D3	13.05	Pass	1.0
Sample Name: 082707A6	Sample ID: 10 ng/ml CTRL		
THCA	13.07	Pass	9.0
THCA-D3	13.05	Pass	1.0
Sample Name: 082707A7	Sample ID: FSC 1 A (3330)		
THCA	NF	Pass	13
THCA-D3	13.05	Pass	1.0
Sample Name: 082707A8	Sample ID: FSC 2 A (4086)		
THCA	13.07	Pass	49
THCA-D3	13.05	Pass	1.0
Sample Name: 082707A9	Sample ID: FSC 3 A (6279)		
THCA	13.07	Pass	37
THCA-D3	13.05	Pass	1.0
Sample Name: 082707A10	Sample ID: FSC 4 A (6349)		
THCA	13.07	Pass	24
THCA-D3	13.05	Pass	1.0
Sample Name: 082707A11	Sample ID: FSC 5 A (6352)		
THCA	13.07	Pass	60
THCA-D3	13.05	Pass	1.0

Table 22, (continued)

Sample Name: 082707A12	Sample ID: FSC 6 A (8573)		
THCA	13.07	Pass	49
THCA-D3	13.05	Pass	1.0
Sample Name: 082707A13	Sample ID: FSC 7 A (1778)		
THCA	13.07	Pass	58
THCA-D3	13.05	Pass	1.0
Sample Name: 082707A14	Sample ID: FSC 8 A (14275)		
THCA	13.07	Pass	9
THCA-D3	13.05	Pass	1.0
Sample Name: 082707A15	Sample ID: FSC 9 A (2946)		
THCA	13.07	Pass	22
THCA-D3	13.05	Pass	1.0
Sample Name: 082707A16	Sample ID: FSC 10 A (15667)		
THCA	13.07	Pass	34
THCA-D3	13.05	Pass	1.0
Sample Name: 082707A17	Sample ID: 30 ng/ml CTRL		
THCA	13.07	Pass	42.0
THCA-D3	13.05	Pass	1.0

Table 22: Run A of ten Δ-9-THCA samples from Mississippi Crime Laboratory reported with quantitated results. Table shows calibrators, controls and individual sample results. All results are from analyses conducted using the extraction device bound with monoclonal Δ-9-THCA antibodies. All calibrators, controls and samples met criteria for confirmation and statistical analysis.

Table 23

Quantify Sample Summary Report
Sample List: **C:\TurboMass\USMFSC.PRO\SampleDB\083007A**
Last Modified: **Monday Jul 27 14:48:35 2007**
Method: **C:\TurboMass\USMFSC\MethDB\THCA**
Printed: **Fri Aug 31 16:40:21 2007**

	Sample RT	Qualifiers P/F	Conc. ng/ml
Sample Name:083007A1	Sample ID: 0 ng/ml CAL 1		
THCA	NF	Fail	0
THCA-D3	13.05	Pass	1.0
Sample Name: 083007A2	Sample ID: 5 ng/ml CAL		
THCA	13.07	Pass	4.86
THCA-D3	13.05	Pass	1.0
Sample Name: 083007A3	Sample ID: 10 ng/ml CAL		
THCA	13.07	Pass	10.10
THCA-D3	13.05	Pass	1.0
Sample Name: 083007A4	Sample ID: 20 ng/ml CAL		
THCA	13.07	Pass	19.41
THCA-D3	13.05	Pass	1.0
Sample Name: 083007A5	Sample ID: 50 ng/ml CAL		
THCA	13.07	Pass	50.17
THCA-D3	13.05	Pass	1.0
Sample Name: 083007A6	Sample ID: 10 ng/ml CTRL		
THCA	13.07	Pass	10.5
THCA-D3	13.05	Pass	1.0
Sample Name: 083007A7	Sample ID: FSC 11 A (13995)		
THCA	NF	Fail	0
THCA-D3	13.05	Pass	1.0
Sample Name: 083007A8	Sample ID: FSC 12 A (8516)		
THCA	13.07	Pass	21
THCA-D3	13.05	Pass	1.0
Sample Name: 083007A9	Sample ID: FSC 13 A (13298)		
THCA	13.07	Pass	16
THCA-D3	13.05	Pass	1.0
Sample Name: 083007A10	Sample ID: FSC 14 A (14876)		
THCA	13.07	Pass	13
THCA-D3	13.05	Pass	1.0
Sample Name: 083007A11	Sample ID: FSC 15 A (11457)		
THCA	13.07	Pass	20
THCA-D3	13.05	Pass	1.0

Table 23, (continued)

Sample Name: 083007A12	Sample ID: FSC 16 A (6342)		
THCA	13.07	Pass	7
THCA-D3	13.05	Pass	1.0
Sample Name: 083007A13	Sample ID: FSC 17 A (8011)		
THCA	13.07	Pass	8
THCA-D3	13.05	Pass	1.0
Sample Name: 083007A14	Sample ID: FSC 18 A (8106)		
THCA	13.07	Pass	10
THCA-D3	13.05	Pass	1.0
Sample Name: 083007A15	Sample ID: FSC 19 A (8245)		
THCA	13.07	Pass	5
THCA-D3	13.05	Pass	1.0
Sample Name: 083007A16	Sample ID: FSC 20 A (8464)		
THCA	13.07	Pass	7
THCA-D3	13.05	Pass	1.0
Sample Name: 083007A17	Sample ID: 30 ng/ml CTRL		
THCA	13.07	Pass	41.0
THCA-D3	13.05	Pass	1.0

Table 23: Run A of ten Δ-9-THCA samples from Mississippi Crime Laboratory reported with quantitated results. Table shows calibrators, controls and individual sample results. All results are from analyses conducted using the extraction device bound with monoclonal Δ-9-THCA antibodies. All calibrators, controls and samples met criteria for confirmation and statistical analysis.

Table 24

Quantify Sample Summary Report
Sample List: **C:\TurboMass\USMFSC.PRO\SampleDB\090407A**
Last Modified: **Monday Jul 27 14:48:35 2007**
Method: **C:\TurboMass\USMFSC\MethDB\THCA**
Printed: **Fri Sep 7 17:22:11 2007**

	Sample RT	Qualifiers P/F	Conc. ng/ml
Sample Name: 090407A1	Sample ID: 0 ng/ml CAL 1		
THCA	NF	Fail	0
THCA-D3	13.05	Pass	1.0
Sample Name: 090407A2	Sample ID: 5 ng/ml CAL		
THCA	13.07	Pass	5.06
THCA-D3	13.05	Pass	1.0
Sample Name: 090407A3	Sample ID: 10 ng/ml CAL		
THCA	13.07	Pass	9.70
THCA-D3	13.05	Pass	1.0
Sample Name: 090407A4	Sample ID: 20 ng/ml CAL		
THCA	13.07	Pass	21.19
THCA-D3	13.05	Pass	1.0
Sample Name: 090407A5	Sample ID: 50 ng/ml CAL		
THCA	13.07	Pass	49.99
THCA-D3	13.05	Pass	1.0
Sample Name: 090407A6	Sample ID: 10 ng/ml CTRL		
THCA	13.07	Pass	10.3
THCA-D3	13.05	Pass	1.0
Sample Name: 090407A7	Sample ID: FSC 21 A (14621)		
THCA	13.07	Pass	51
THCA-D3	13.05	Pass	1.0
Sample Name: 090407A8	Sample ID: FSC 22 A (16804)		
THCA	13.07	Pass	6
THCA-D3	13.05	Pass	1.0
Sample Name: 090407A9	Sample ID: FSC 23 A (1662)		
THCA	13.07	Pass	20
THCA-D3	13.05	Pass	1.0
Sample Name: 090407A10	Sample ID: FSC 24 A (6482)		
THCA	13.07	Pass	5
THCA-D3	13.05	Pass	1.0
Sample Name: 090407A11	Sample ID: FSC 25 A (6701)		
THCA	13.07	Pass	11
THCA-D3	13.05	Pass	1.0

Table 24, (continued)

Sample Name: 090407A12	Sample ID: FSC 26 A (8789)		
THCA	NF	Fail	0
THCA-D3	13.05	Pass	1.0
Sample Name: 090407A13	Sample ID: FSC 27 A (9500)		
THCA	13.07	Pass	5
THCA-D3	13.05	Pass	1.0
Sample Name: 090407A14	Sample ID: FSC 28 A (9861)		
THCA	13.07	Pass	16
THCA-D3	13.05	Pass	1.0
Sample Name: 090407A15	Sample ID: FSC 29 A (9873)		
THCA	13.07	Pass	23
THCA-D3	13.05	Pass	1.0
Sample Name: 090407A16	Sample ID: FSC 30 A (11461)		
THCA	13.07	Pass	5
THCA-D3	13.05	Pass	1.0
Sample Name: 090407A17	Sample ID: 30 ng/ml CTRL		
THCA	13.07	Pass	41.0
THCA-D3	13.05	Pass	1.0

Table 24: Run A of ten Δ-9-THCA samples from Mississippi Crime Laboratory reported with quantitated results. Table shows calibrators, controls and individual sample results. All results are from analyses conducted using the extraction device bound with monoclonal Δ-9-THCA antibodies. All calibrators, controls and samples met criteria for confirmation and statistical analysis.

Table 25

Quantify Sample Summary Report
Sample List: **C:\TurboMass\USMFSC.PRO\SampleDB\082807B**
Last Modified: **Monday Jul 27 14:48:35 2007**
Method: **C:\TurboMass\USMFSC\MethDB\THCA**
Printed: **Fri Aug 31 15:03:33 2007**

	Sample RT	Qualifiers P/F	Conc. ng/ml
Sample Name: 082807B1	Sample ID: 0 ng/ml CAL 1		
THCA	NF	Fail	0
THCA-D3	13.05	Pass	1.0
Sample Name: 082807B2	Sample ID: 5 ng/ml CAL		
THCA	13.07	Pass	5.91
THCA-D3	13.05	Pass	1.0
Sample Name: 082807B3	Sample ID: 10 ng/ml CAL		
THCA	13.07	Pass	9.93
THCA-D3	13.05	Pass	1.0
Sample Name: 082807B4	Sample ID: 20 ng/ml CAL		
THCA	13.07	Pass	20.49
THCA-D3	13.05	Pass	1.0
Sample Name: 082807B5	Sample ID: 50 ng/ml CAL		
THCA	13.07	Pass	50.62
THCA-D3	13.05	Pass	1.0
Sample Name: 082807B6	Sample ID: 10 ng/ml CTRL		
THCA	13.07	Pass	9.0
THCA-D3	13.05	Pass	1.0
Sample Name: 082807B7	Sample ID: FSC 1 B (3330)		
THCA	NF	Pass	11
THCA-D3	13.05	Pass	1.0
Sample Name: 082807B8	Sample ID: FSC 2 B (4086)		
THCA	13.07	Pass	51
THCA-D3	13.05	Pass	1.0
Sample Name: 082807B9	Sample ID: FSC 3 B (6279)		
THCA	13.07	Pass	34
THCA-D3	13.05	Pass	1.0
Sample Name: 082807B10	Sample ID: FSC 4 B (6349)		
THCA	13.07	Pass	22
THCA-D3	13.05	Pass	1.0
Sample Name: 082807B11	Sample ID: FSC 5 B (6352)		
THCA	13.07	Pass	55
THCA-D3	13.05	Pass	1.0

Table 25, (continued)

Sample Name: 082807B12	Sample ID: FSC 6 B (8573)		
THCA	13.07	Pass	52
THCA-D3	13.05	Pass	1.0
Sample Name: 082807B13	Sample ID: FSC 7 B (1778)		
THCA	13.07	Pass	59
THCA-D3	13.05	Pass	1.0
Sample Name: 082807B14	Sample ID: FSC 8 B (14275)		
THCA	13.07	Pass	9
THCA-D3	13.05	Pass	1.0
Sample Name: 082807B15	Sample ID: FSC 9 B (2946)		
THCA	13.07	Pass	24
THCA-D3	13.05	Pass	1.0
Sample Name: 082807B16	Sample ID: FSC 10 B (15667)		
THCA	13.07	Pass	32
THCA-D3	13.05	Pass	1.0
Sample Name: 082807B17	Sample ID: 30 ng/ml CTRL		
THCA	13.07	Pass	38.0
THCA-D3	13.05	Pass	1.0

Table 25: Run B of ten Δ-9-THCA samples from Mississippi Crime Laboratory reported with quantitated results. Table shows calibrators, controls and individual sample results. All results are from analyses conducted using the extraction device bound with monoclonal Δ-9-THCA antibodies. All calibrators, controls and samples met criteria for confirmation and statistical analysis.

Table 26

Quantify Sample Summary Report

Sample List: C:\TurboMass\USMFSC.PRO\SampleDB\083007B
Last Modified: Monday Jul 27 14:48:35 2007
Method: C:\TurboMass\USMFSC\MethDB\THCA
Printed: Fri Aug 31 16:52:33 2007

	Sample RT	Qualifiers P/F	Conc. ng/ml
Sample Name: 083007B1	Sample ID: 0 ng/ml CAL 1		
THCA	NF	Fail	0
THCA-D3	13.05	Pass	1.0
Sample Name: 083007B2	Sample ID: 5 ng/ml CAL		
THCA	13.07	Pass	4.02
THCA-D3	13.05	Pass	1.0
Sample Name: 083007B3	Sample ID: 10 ng/ml CAL		
THCA	13.07	Pass	11.10
THCA-D3	13.05	Pass	1.0
Sample Name: 083007B4	Sample ID: 20 ng/ml CAL		
THCA	13.07	Pass	18.85
THCA-D3	13.05	Pass	1.0
Sample Name: 083007B5	Sample ID: 50 ng/ml CAL		
THCA	13.07	Pass	49.81
THCA-D3	13.05	Pass	1.0
Sample Name: 083007B6	Sample ID: 10 ng/ml CTRL		
THCA	13.07	Pass	8.8
THCA-D3	13.05	Pass	1.0
Sample Name: 083007B7	Sample ID: FSC 11 B (13995)		
THCA	NF	Fail	0
THCA-D3	13.05	Pass	1.0
Sample Name: 083007B8	Sample ID: FSC 12 B (8516)		
THCA	13.07	Pass	19
THCA-D3	13.05	Pass	1.0
Sample Name: 083007B9	Sample ID: FSC 13 B (13298)		
THCA	13.07	Pass	17
THCA-D3	13.05	Pass	1.0
Sample Name: 083007B10	Sample ID: FSC 14 B (14876)		
THCA	13.07	Pass	12
THCA-D3	13.05	Pass	1.0
Sample Name: 083007B11	Sample ID: FSC 15 B (11457)		
THCA	13.07	Pass	21
THCA-D3	13.05	Pass	1.0

Table 26, (continued)

Sample Name: 083007B12	Sample ID: FSC 16 B (6342)		
THCA	13.07	Pass	6
THCA-D3	13.05	Pass	1.0
Sample Name: 083007B13	Sample ID: FSC 17 B (8011)		
THCA	13.07	Pass	6
THCA-D3	13.05	Pass	1.0
Sample Name: 083007B14	Sample ID: FSC 18 B (8106)		
THCA	13.07	Pass	8
THCA-D3	13.05	Pass	1.0
Sample Name: 083007B15	Sample ID: FSC 19 B (8245)		
THCA	13.07	Pass	5
THCA-D3	13.05	Pass	1.0
Sample Name: 083007B16	Sample ID: FSC 20 B (8464)		
THCA	13.07	Pass	6
THCA-D3	13.05	Pass	1.0
Sample Name: 083007B17	Sample ID: 30 ng/ml CTRL		
THCA	13.07	Pass	37.0
THCA-D3	13.05	Pass	1.0

Table 26: Run B of ten Δ-9-THCA samples from Mississippi Crime Laboratory reported with quantitated results. Table shows calibrators, controls and individual sample results. All results are from analyses conducted using the extraction device bound with monoclonal Δ-9-THCA antibodies. All calibrators, controls and samples met criteria for confirmation and statistical analysis.

Table 27

Quantify Sample Summary Report
Sample List: **C:\TurboMass\USMFSC.PRO\SampleDB\090407B**
Last Modified: **Monday Jul 27 14:48:35 2007**
Method: **C:\TurboMass\USMFSC\MethDB\THCA**
Printed: **Fri Sep 7 17:22:11 2007**

	Sample RT	Qualifiers P/F	Conc. ng/ml
Sample Name: 090407B1	Sample ID: 0 ng/ml CAL 1		
THCA	NF	Fail	0
THCA-D3	13.05	Pass	1.0
Sample Name: 090407B2	Sample ID: 5 ng/ml CAL		
THCA	13.07	Pass	5.38
THCA-D3	13.05	Pass	1.0
Sample Name: 090407B3	Sample ID: 10 ng/ml CAL		
THCA	13.07	Pass	11.33
THCA-D3	13.05	Pass	1.0
Sample Name: 090407B4	Sample ID: 20 ng/ml CAL		
THCA	13.07	Pass	19.92
THCA-D3	13.05	Pass	1.0
Sample Name: 090407B5	Sample ID: 50 ng/ml CAL		
THCA	13.07	Pass	50.79
THCA-D3	13.05	Pass	1.0
Sample Name: 090407B6	Sample ID: 10 ng/ml CTRL		
THCA	13.07	Pass	10.4
THCA-D3	13.05	Pass	1.0
Sample Name: 090407B7	Sample ID: FSC 21 B (14621)		
THCA	13.07	Pass	49
THCA-D3	13.05	Pass	1.0
Sample Name: 090407B8	Sample ID: FSC 22 B (16804)		
THCA	13.07	Pass	7
THCA-D3	13.05	Pass	1.0
Sample Name: 090407B9	Sample ID: FSC 23 B (1662)		
THCA	13.07	Pass	22
THCA-D3	13.05	Pass	1.0
Sample Name: 090407B10	Sample ID: FSC 24 B (6482)		
THCA	13.07	Pass	5
THCA-D3	13.05	Pass	1.0
Sample Name: 090407B11	Sample ID: FSC 25 B (6701)		
THCA	13.07	Pass	8
THCA-D3	13.05	Pass	1.0

Table 27, (continued)

Sample Name: 090407B12	Sample ID: FSC 26 B (8789)		
THCA	NF	Fail	0
THCA-D3	13.05	Pass	1.0
Sample Name: 090407B13	Sample ID: FSC 27 B (9500)		
THCA	13.07	Pass	5
THCA-D3	13.05	Pass	1.0
Sample Name: 090407B14	Sample ID: FSC 28 B (9861)		
THCA	13.07	Pass	14
THCA-D3	13.05	Pass	1.0
Sample Name: 090407B15	Sample ID: FSC 29 B (9873)		
THCA	13.07	Pass	25
THCA-D3	13.05	Pass	1.0
Sample Name: 090407B16	Sample ID: FSC 30 B (11461)		
THCA	13.07	Pass	5
THCA-D3	13.05	Pass	1.0
Sample Name: 090407B17	Sample ID: 30 ng/ml CTRL		
THCA	13.07	Pass	37.0
THCA-D3	13.05	Pass	1.0

Table 27: Run B of ten Δ-9-THCA samples from Mississippi Crime Laboratory reported with quantitated results. Table shows calibrators, controls and individual sample results. All results are from analyses conducted using the extraction device bound with monoclonal Δ-9-THCA antibodies. All calibrators, controls and samples met criteria for confirmation and statistical analysis.

Table 28

Direct Difference Statistical Evaluation of THCA Values: Paired *t* test
Testing Group A

Sample Identification	MCL Results	EXP-A Results	Difference D = X1-X2	Difference Squared (D^2)
8516	26	21	5	25
13298	21	16	5	25
14876	17	13	4	16
11457	28	20	8	64
6342	10	7	3	9
8011	10	8	2	4
8106	14	10	4	16
8245	6	5	1	1
8464	11	7	4	16
3330	18	13	5	25
4086	60	49	11	121
6279	43	37	6	36
6349	30	24	6	36
6352	69	60	9	81
8573	58	49	9	81
1778	68	58	10	100
14275	13	9	4	16
2946	27	22	5	25
15667	40	34	6	36
14621	63	51	12	144
16804	11	6	5	25
1662	28	20	8	64
6482	7	5	2	4
7601	13	11	2	4
9500	7	5	2	4
9861	21	16	5	25
9873	30	23	7	49
11461	6	5	1	1
	$\sum X1 = 755$	$\sum X2 = 604$		$\sum D^2 = 1053$

Group Means	MCL = 26.964	EXP-A = 21.571
Mean Difference of Groups - MCL – EXP-A		26.964-21.571= 5.393
Standard Deviation of Difference (SDD)		SDD = $\sqrt{(\sum D^2/N) - MD^2}$
		$\sqrt{37.607-29.083} = 2.920$
Standard Error of Difference (SED)		SED = SDD/($\sqrt{N-1}$) = 0.562

Paired *t* Ratio = MD/SED = 9.596

$t_{.01(27)} = 2.771$ Significant Difference Found

Table 28: Paired *t* test on the first run of Δ-9-THCA results reported from the Mississippi Crime Laboratory. The test shows a significant difference indicating the differences were not by chance.

Table 29

Direct Difference Statistical Evaluation of THCA Values: Paired *t* test
Testing Group B

Sample Identification	MCL Results	EXP-B Results	Difference D = X1-X2	Difference Squared (D^2)
8516	26	19	7	49
13298	21	17	4	16
14786	17	12	5	25
11457	25	21	7	49
6342	10	6	4	16
8011	10	6	4	16
8106	14	8	6	36
8245	6	5	1	1
8464	11	6	5	25
3330	18	11	7	49
4086	60	51	9	81
6279	43	34	9	81
6349	30	22	8	64
6352	69	55	14	196
8573	58	52	6	36
1778	68	59	9	81
14275	13	9	4	16
2946	27	24	3	9
15667	40	32	8	64
14621	63	49	14	196
16804	11	7	4	16
1662	28	22	6	36
6482	7	5	2	4
7601	13	8	5	25
9500	7	5	2	4
9861	21	14	7	49
9873	30	25	5	25
11461	6	5	1	1
	$\sum X1 = 755$	$\sum X2 = 589$		$\sum D^2 = 1266$

Group Means	MCL = 26.964	EXP-A = 21.036
Mean Difference of Groups - MCL – EXP-A		26.964 – 21.036 = 5.929
Standard Deviation of Difference (SDD)		$SDD = \sqrt{(\sum D^2/N) - MD^2}$
		$\sqrt{45.214 - 35.148} = 3.173$
Standard Error of Difference (SED)		$SED = SDD/(\sqrt{N-1}) = 0.611$

Paired *t* Ratio = MD/SED = 8.827

$t_{.01(27)} = 2.771$ Significant Difference Found

Table 29: Paired *t* test on the second run of Δ-9-THCA results reported from the Mississippi Crime Laboratory. The test shows a significant difference indicating the differences were not by chance.

Figure 19

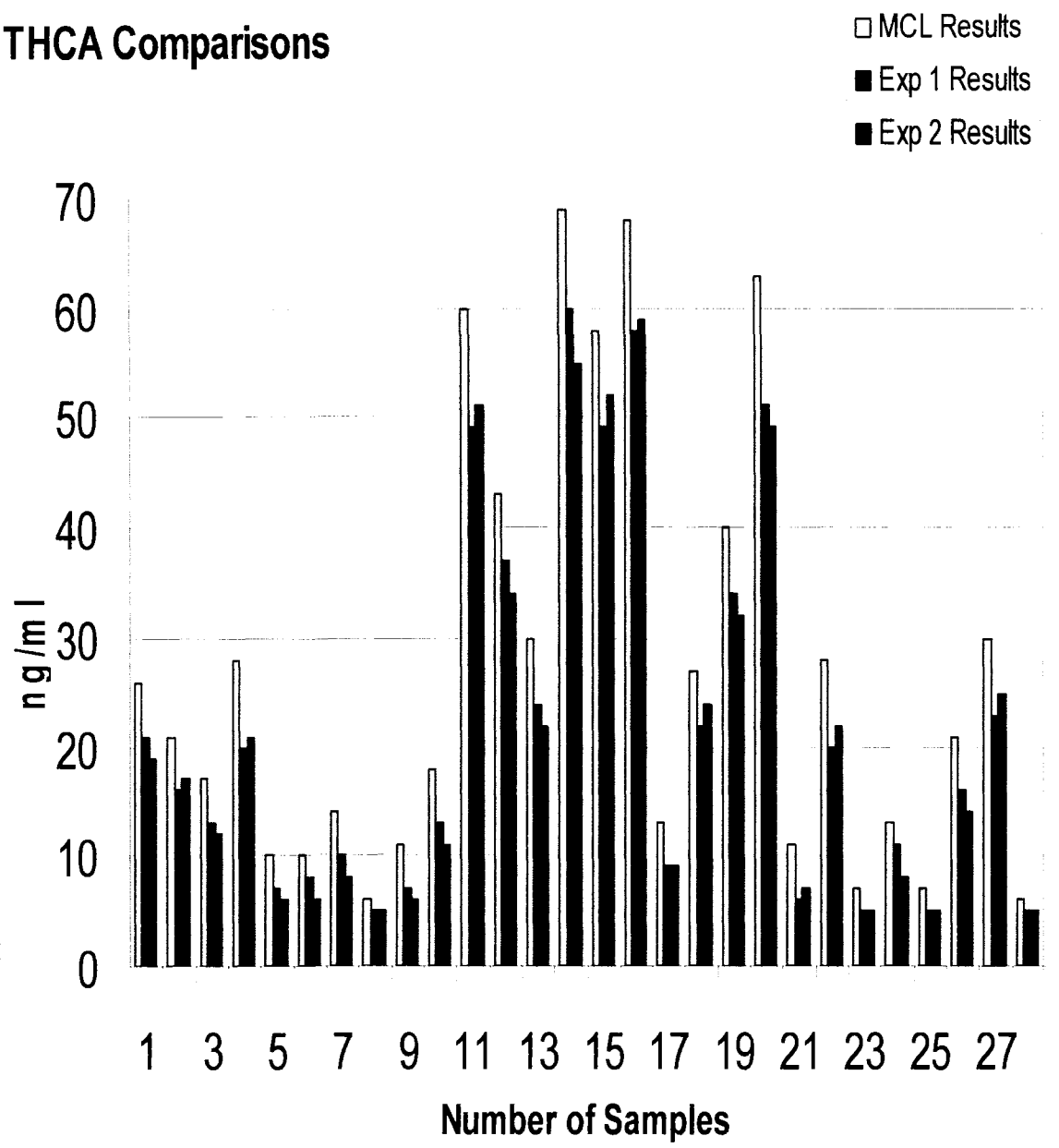

Figure 19: Comparison of Δ-9-THCA Mississippi Crime Laboratory results versus results from two separate extractions using the glutaraldehyde treated flasks with Δ-9-THC monoclonal antibodies. The white bars represent the results reported by the Mississippi Crime Laboratory while the blue and red bars represent results from the extraction device. Note there is a negative bias using the extraction devices.

The last set of samples analyzed from the Mississippi Crime Laboratory was those Δ-9-THC samples reported as "None Detected Due to Interfering Substance". These ten samples were treated the same as all other samples when analyzed using the extraction device. The ten samples were all run twice on separate days.

Two of the samples were found to be "none detected" at 1 ng/ml. Eight of the samples were found to contain Δ-9-THC at various concentrations. The remaining eight samples met the requirements for confirmation and quantitation (see Tables 30 and 31).

A Paired *t* test was not run for statistical comparison since there were no results from the Mississippi Crime Laboratory for comparison. A statistical evaluation of the groups themselves did not reveal a significant difference between the groups using the Paired *t* test. Mean values were 10.13 ng/ml for group A and 8.75 ng/ml for group B.

These results are significant in that the extraction method using bound antibodies appears to have removed any interfering substances and allow quantitative results to be obtained (see Figure 20). Any interfering substances would have been removed during the washing steps while the analytes remained bound prior to release for analysis.

A liquid/liquid extraction was performed in my laboratory to see if any interfering peaks were found (see Figure 21). There are two unidentified peaks located to the right of the Δ-9-THC peak in this extraction. These two peaks may account for the interference noted in the original report. The two peaks were clearly separated in this analysis which may be due to the fact a new column was used for the analysis and a different oven temperature program. A column used on a daily basis for routine analyses over a few months time would probably not separate these peaks at some point.

Table 30

Quantify Sample Summary Report

Sample List: C:\TurboMass\USMFSC.PRO\SampleDB\090407B
Last Modified: Monday Jul 27 14:48:35 2007
Method: C:\TurboMass\USMFSC\MethDB\THC
Printed: Fri Sep 7 17:22:11 2007

	Sample RT	Qualifiers P/F	Conc. ng/ml
Sample Name: 090407A1	Sample ID: 0 ng/ml CAL 1		
THC	NF	Fail	0
THC-D3	11.12	Pass	1.0
Sample Name: 090407A2	Sample ID: 3 ng/ml CAL		
THC	11.13	Pass	2.87
THC-D3	11.12	Pass	1.0
Sample Name: 090407A3	Sample ID: 10 ng/ml CAL		
THC	11.14	Pass	9.46
THC-D3	11.12	Pass	1.0
Sample Name: 090407A4	Sample ID: 20 ng/ml CAL		
THC	11.13	Pass	20.09
THC-D3	11.12	Pass	1.0
Sample Name: 090407A5	Sample ID: 50 ng/ml CAL		
THC	11.14	Pass	49.79
THC-D3	11.12	Pass	1.0
Sample Name: 090407A6	Sample ID: 10 ng/ml CTRL		
THC	11.13	Pass	8.8
THC-D3	11.12	Pass	1.0
Sample Name: 090407A7	Sample ID: FSC 11 B (14621)		
THC	11.13	Pass	7.3
THC-D3	11.12	Pass	1.0
Sample Name: 090407A8	Sample ID: FSC 12 B (16804)		
THC	11.14	Pass	1.6
THC-D3	11.12	Pass	1.0
Sample Name: 090407A9	Sample ID: FSC 13 B (1662)		
THC	11.13	Pass	4.1
THC-D3	11.12	Pass	1.0
Sample Name: 090407A10	Sample ID: FSC 14 B (6482)		
THC	NF	Fail	0
THC-D3	11.12	Pass	1.0
Sample Name: 090407A11	Sample ID: FSC 15 B (6701)		
THC	11.13	Pass	3.1
THC-D3	11.12	Pass	1.0

Table 30, (continued)

Sample Name: 090407A12	Sample ID: FSC 16 B (8789)		
THC	11.13	Pass	13.0
THC-D3	11.12	Pass	1.0
Sample Name: 090407A13	Sample ID: FSC 17 B (9500)		
THC	11.13	Pass	2.3
THC-D3	11.12	Pass	1.0
Sample Name: 090407A14	Sample ID: FSC 18 B (9861)		
THC	11.14	Pass	2.4
THC-D3	11.12	Pass	1.0
Sample Name: 090407A15	Sample ID: FSC 19 B (9873)		
THC	11.13	Pass	2.6
THC-D3	11.12	Pass	1.0
Sample Name: 090407A16	Sample ID: FSC 20 B (11461)		
THC	NF	Fail	0
THC-D3	11.12	Pass	1.0
Sample Name: 090407A17	Sample ID: 30 ng/ml CTRL		
THC	11.14	Pass	37.0
THC-D3	11.12	Pass	1.0

Table 30: Run A of ten Δ-9-THC samples from Mississippi Crime Laboratory reported as "Unable to Report Due to Interfering Substances". Table shows calibrators, controls and individual sample results. All results are from analyses conducted using the extraction device bound with monoclonal Δ-9-THC antibodies. All calibrators, controls and samples met criteria for confirmation and statistical analysis. All samples were reportable using the extraction device.

Table 31

Quantify Sample Summary Report

Sample List: C:\TurboMass\USMFSC.PRO\SampleDB\090507B
Last Modified: Monday Jul 27 14:48:35 2007
Method: C:\TurboMass\USMFSC\MethDB\THC
Printed: Fri Sep 7 17:42:46 2007

	Sample RT	Qualifiers P/F	Conc. ng/ml
Sample Name: 090507B1	Sample ID: 0 ng/ml CAL 1		
THC	NF	Fail	0
THC-D3	11.12	Pass	1.0
Sample Name: 090507B2	Sample ID: 3 ng/ml CAL		
THC	11.13	Pass	2.87
THC-D3	11.12	Pass	1.0
Sample Name: 090507B3	Sample ID: 10 ng/ml CAL		
THC	11.14	Pass	9.46
THC-D3	11.12	Pass	1.0
Sample Name: 090507B4	Sample ID: 20 ng/ml CAL		
THC	11.13	Pass	20.09
THC-D3	11.12	Pass	1.0
Sample Name: 090507B5	Sample ID: 50 ng/ml CAL		
THC	11.14	Pass	49.79
THC-D3	11.12	Pass	1.0
Sample Name: 090507B6	Sample ID: 10 ng/ml CTRL		
THC	11.13	Pass	8.8
THC-D3	11.12	Pass	1.0
Sample Name: 090507B7	Sample ID: FSC 11 B (14621)		
THC	11.13	Pass	7.3
THC-D3	11.12	Pass	1.0
Sample Name: 090507B8	Sample ID: FSC 12 B (16804)		
THC	11.14	Pass	1.6
THC-D3	11.12	Pass	1.0
Sample Name: 090507B9	Sample ID: FSC 13 B (1662)		
THC	11.13	Pass	4.1
THC-D3	11.12	Pass	1.0
Sample Name: 090507B10	Sample ID: FSC 14 B (6482)		
THC	NF	Fail	0
THC-D3	11.12	Pass	1.0
Sample Name: 090507B11	Sample ID: FSC 15 B (6701)		
THC	11.13	Pass	3.1
THC-D3	11.12	Pass	1.0

Table 31, (continued)

Sample Name: 090507B12	Sample ID: FSC 16 B (8789)		
THC	11.13	Pass	13.0
THC-D3	11.12	Pass	1.0
Sample Name: 090507B13	Sample ID: FSC 17 B (9500)		
THC	11.13	Pass	2.3
THC-D3	11.12	Pass	1.0
Sample Name: 090507B14	Sample ID: FSC 18 B (9861)		
THC	11.14	Pass	2.4
THC-D3	11.12	Pass	1.0
Sample Name: 090507B15	Sample ID: FSC 19 B (9873)		
THC	11.13	Pass	2.6
THC-D3	11.12	Pass	1.0
Sample Name: 090507B16	Sample ID: FSC 20 B (11461)		
THC	NF	Fail	0
THC-D3	11.12	Pass	1.0
Sample Name: 090507B17	Sample ID: 30 ng/ml CTRL		
THC	11.14	Pass	37.0
THC-D3	11.12	Pass	1.0

Table 31: Run B of ten Δ-9-THC samples from Mississippi Crime Laboratory reported as "Unable to Report Due to Interfering Substances". Table shows calibrators, controls and individual sample results. All results are from analyses conducted using the extraction device bound with monoclonal Δ-9-THC antibodies. All calibrators, controls and samples met criteria for confirmation and statistical analysis. All samples were reportable using the extraction device.

Figure 20

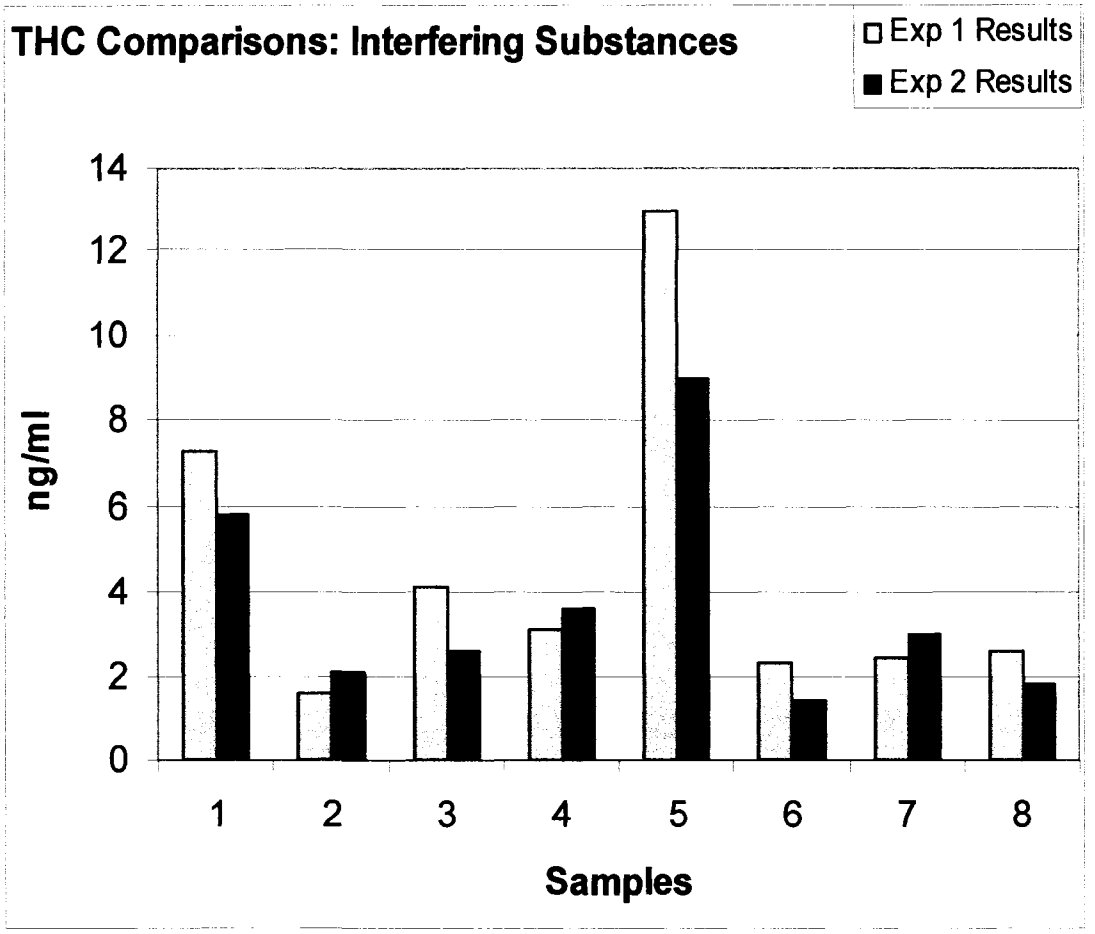

Figure 20: Graph represents 8 of the 10 samples reported from the Mississippi Crime Laboratory as "Unable to Report Due to Interfering substances". Two of the samples were found to be negative for Δ-9-THC. The other 8 samples extracted using the device could have been quantitated and reported as shown above.

Figure 21

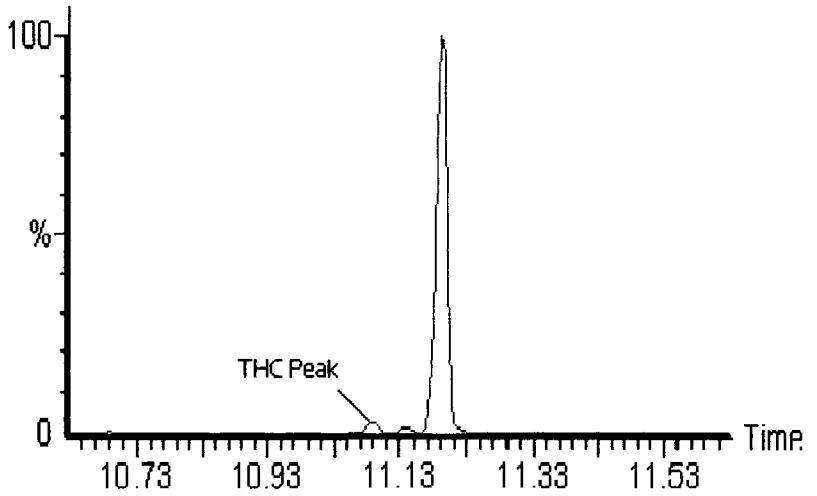

Figure 21: Chromatogram of sample reported with an interfering substance following a liquid/liquid extraction in my laboratory. Note the two peaks to the right of the THC peak which may have caused the interference in the original report.

CHAPTER V

DISCUSSION

This project was designed to prove the concept of using monoclonal antibodies chemically bound to a solid polystyrene surface to extract Δ-9-THC, Δ-9-THCA and their respective internal standards from postmortem blood samples for analysis by EI+ GC/MS. A self-contained extraction device capable of extracting these analytes and removing interfering substances from postmortem blood samples would improve the reporting percentage of Δ-9-THC. Fewer reports stating "None Detected Due to Interfering Substance" for the presence of Δ-9-THC would be advantageous for medical examiners and toxicologists in determining if the cause of a victim's death was related in some manner to the use marijuana.

Antibodies have been used for many years in various methods for both purification and detection of analytes of interest. Both monoclonal and polyclonal antibodies play a vital role in both clinical and forensic testing. A major difference between the disciplines is that the clinical field can use data generated directly from immunoassay testing. Forensic analyses require a second more specific test to confirm any positive immunoassay result. The most common instrument used in forensic laboratories to accomplish confirmations is an EI+ GC/MS.

Monoclonal antibodies are considered more specific for the analyte of interest with fewer problems of cross reactivity with compounds similar in structure even those that belong to the same drug class. Polyclonal antibodies demonstrate the most cross reactivity and are used more in drug screening assays which must be followed by a confirmation analysis. Monoclonal antibodies were chosen for this project because of the interest was mainly in the extraction of Δ-9-THC after removal of interfering substances.

Polyclonal antibodies were considered for use but no vendor was found that could supply the polyclonal antibodies at a known concentration.

Gas Chromatography Mass Spectrometry

Gas chromatography mass spectrometry is a combination of two methods each providing unique information about an analyte. A gas chromatograph (GC) provides information in the form of retention times due to its ability to separate a complex mixture into constituent components within samples. The ability to separate such mixtures is accomplished by use of a mobile phase in the form of a gas and a stationary phase within a chromatographic column. As the gas moves the mixture through the column, the interaction of constituents between both phases brings about the separation. Some constituents are retained in the system longer than others resulting in differences in elution times.

An electron impact (EI+) mass spectrometer is an instrument that provides information about the structure of molecules such as drugs through a fragmentation process. As molecules elute from the GC into the mass spectrometer, they collide with electrons and fragment in a specific pattern. This fragmentation pattern is consistent from instrument to instrument as long as the EI parameters are held the same. Such consistency is why this type of mass spectrometer has been chosen for performing drug confirmations in general and has been accepted by the legal community as the gold standard method.

Two key pieces of information are given by the fragmentation pattern. First is the pattern itself which can be placed in mass spectral libraries for searching and identification purposes. Second specific ion fragments can be measured against one another to provide a ratio for further confidence in the results. It is these three pieces of

information, retention time, fragmentation pattern and ion ratios, that give the GC/MS the ability to positively identify a compound.

One of the major problems with a GC/MS is the need to maintain the system so that the chromatography and the mass spectral patterns are free of unwanted contaminants. Such contaminants are compounds from extractions that tend to stay in the chromatographic column or co-elute with compounds of interest making fragmentation patterns difficult to discern. The development of an extraction device that eliminates most or all such interfering substances is one of the key reasons for pursuing this project.

Initial ELISA Experiments

Initial experiments in this project using ELISA 96 well plates demonstrated that antibody bound Δ-9-THC could be subsequently recovered for analysis by GC/MS following several wash steps to remove possible interfering substances. The ELISA experiments also showed that antibody bound Δ-9-THCA and both deuterated internal standards for GC/MS analysis could be recovered in a single extraction process. These experimental results gave credence to the concept of producing a device using Δ-9-THC antibodies that could be used to extract these analytes from postmortem whole blood while removing interfering substances. The extraction results from the ELISA plates were both positive but did point out some issues that had to be over come.

One positive point as stated above was mainly that the analytes of interest remained bound during the protocol and could be recovered for analysis by GC/MS. This meant that a larger device built on the same principle should perform in the same manner. Analytes could be introduced, bound by antibody, washed to remove interfering substances and then the analyte of interest recovered for analysis.

One of the negative issues noted was that recovery of analytes was poor and

inconsistent at best. Since the samples were not diluted, as would be the case normally, poor recovery may have stemmed from an analytes inability to diffuse within the blood sample to the sides or bottom of the wells where the antibodies were located: ELISA plates are held static during the incubation period. Another reason may be that whole blood contains many large and complex substances that may have played a role in steric hindrance preventing analyte from binding the antibody. Dilution of the sample in ELISA testing would reduce to some degree the inability for an analyte to diffuse within the sample by removing many of the large molecules and at the same time increase the ratio of antibody to analyte enhancing chances for binding.

Two steps were put in the extraction protocol to overcome these issues. First an excess of antibody was placed in each device, five mcg of antibody per device. Secondly, the device was rotated to facilitate the movement of analyte within the device to increase the likelihood of contacting antibody for binding. These steps did improve the percent yield, 54% for Δ-9-THC when using the device as compared to the 28% from ELISA plates. The percent yield was much more consistent with the extraction device.

Extraction Device Chosen for Experiments

The device chosen for main portion of this project was a 25 ml Falcon® cell culture flask. The flask's bottom measured 12.5 cm^2 which 1 ml of fluid would just cover. The shallow depth of a solution ensured the glutaraldehyde would contact the surface for binding and that the antibody solution would come into contact with the glutaraldehyde for chemical binding. The flask had a screw cap for closure and was large enough to easily decant sample into and out of the flask as well as wash and releasing solutions. The flat bottom was ideal for stacking the flasks on the platform

rotator during experiments without tilting or falling. There were some problems observed with this flask during the project.

First the 90^{o} angle formed on three sides served as a collection area for all solutions during rotation. A solution would naturally go to the sides as the flask rotated no matter how slow the rotation rate. Flasks that contain an internal angle along the sides may prevent fluid build up of solutions and samples around the edges and provide consistent coverage of the bottom area during rotation.

A second solution would be to use a rocker to ensure a solution covered the entire bottom area during an experiment as it moved back and fourth. However, a means of securing the flasks to the rocker would have to be devised in order to place several flasks on at one time.

Another problem seen with the flasks was cracking that occurred during the experiments. Hairline cracks would begin to appear when a releasing solvent was added to the flasks. The cracks were seen in the top of the flask, along the sides, but not in the bottom area of the flasks. At no time were the cracks of sufficient width to leak any of the releasing solution during incubation.

The worst area for cracking noted was around the neck area under the cap. After removing the cap, often small pieces of polystyrene came off while pouring the releasing solvent into the glass tubes. These small pieces of polystyrene would then interfere with the analysis by GC/MS by clogging the Autosampler needle and/or dissolving in the derivatization solvent coating the injection liner and column which on occasion would alter the retention times of the analytes. The hairline cracks were believed due to pressure changes within the flasks from the heating step and the various types of aqueous and organic solutions used during an extraction.

The cracks under the cap were found to come more from over tightening the cap. When the cap was allowed to remain lose, the cracking was much less severe which in turn eliminated the chromatographic problems encountered from this problem. This same device is also sold with a vented cap which should be tried in future experiments.

The device overall proved to be a good choice for initial experiments. There was sufficient surface area for antibody binding and pipetting reagents into or decanting reagents out of the opening was adequate. Once the problems with cracking under the caps were resolved, no other issues arouse from using this device.

Antibody Binding Methods

Two methods for attaching antibody to the device were chosen; 1) Passive attachment and 2) chemical attachment with glutaraldehyde. Two methods were chosen to test which one would be most efficient for preparing the devices, which method would provide the best percent yield of analyte and the one demonstrating the best robustness during the extraction protocol.

First the passive adhesion was attempted by simply allowing 1 ml of the antibody solution containing 5 mcg of antibody at a pH of 9.2 to incubate overnight at ambient temperature. The premise for adherence to the polystyrene surface passively is the alkaline pH would cause the antibody to slightly unfold exposing some hydrophobic regions. These regions would tend to adhere to the hydrophobic polystyrene. The devices were then washed three times with pH 7.2 sodium phosphate buffer followed by two washing of Type III water. Synthetic blood solutions containing 25 ng/ml of Δ-9-THC were added to individual extraction flasks and allowed to incubate on a platform rotator for 1 hr. The flasks contents were poured into a biohazard container and the flasks rinsed with PBS. Two ml of Hexane and 0.1% acidic methanol were added to

separate flasks as releasing solvents to test which releasing solvent worked best with the devices. The releasing solvents were collected, prepared and analyzed by GC/MS.

Results of these experiments were considered unsuccessful given recovery of any analyte occurred only 60 percent of the time. Even when the experiments did result in analyte recovery, percent yield of analyte was inconsistent to the point linearity, LOQ or LOD could not be determined. The main problem with this type of immobilization is leaching of the antibody during removal of the analyte of interest (Nisnevitch and Frier, 2001). After several attempts to improve passive antibody binding by longer incubation times, use of a detergent and altering pH values, this procedure was abandoned.

The second method chosen was to bind gluteraldehyde to the flask bottom surface using the method of Hermanson, et al. (1992). The initial protocol called for using 2% glutaraldehyde in a pH 5.0 sodium phosphate buffer. One ml of this solution was added to four extraction devices and allowed to incubate for 4 hours at room temperature. The devices were then washed 5 times with the pH 5.0 phosphate buffer. Each device was tapped over paper towels to remove excess liquid and then allowed to drain for 10 minutes.

The second step was adding one ml of the antibody solution containing 5 mcg of antibody per ml of a sodium phosphate buffer, pH 8.0, was added to each device and incubated at 37° C for a minimum of 3 hr. Each device was then washed 3 times with a sodium phosphate buffer, pH 8.0, tapped to remove excess buffer and allowed to drain for 10 min.

One ml samples of synthetic blood containing no drug were added to two devices and 1 ml samples containing 25 ng/ml Δ-9-THC were added to two other devices. All four devices were carried through the extraction protocol. The four devices were washed

5 times with a sodium phosphate buffer, pH 7.2 and allowed to drain for 30 min. Two ml of hexane was added to one of the negative and one of the spiked devices and 0.1% acidic methanol was added to the other two devices in order to determine which solvent produced the best recovery of analyte using the flasks as well as which resulted in the least interference with the GC/MS assay.

Hexane proved to be the best releasing reagent with an analyte yield of 28% as compared to a 17% yield with acidic methanol. The two negative samples did not show any recovery of Δ-9-THC. This experiment was repeated three times with similar results.

In order to raise the percent yield, the glutaraldehyde solution was increased to a 5% solution for binding the antibodies within the device. This change did increase the percent yield from 28% to an average of 38% using hexane as the releasing solvent. Again hexane was a better releasing solvent than 0.1% acidic methanol. Hexane also provided a cleaner extract when compared to the 0.1% acidic methanol when inspecting glass injection liners following a series of injections. The difference may be in the manner by which the antibodies are bound to the polystyrene. The binding of antibodies in ELISA plates from Immunalysis is proprietary and probably does not release any antibody during the releasing step whether using hexane or acidic methanol. The same may not be true for antibodies bound to polystyrene using glutaraldehyde.

A concern using this type of attachment is the orientation and spread of the antibodies over the bottom surface of the flasks. The antibodies can orient in any direction given the attachment between the glutaraldehyde and the antibody uses amine groups. Amine groups are available for bonding in many areas of the antibody including the terminal ends where the binding sites are located. There is also no way to control exactly where the antibodies bind the glutaraldehyde as to position in the 12.5 cm^2

surface area of the flask bottom. This is the reason for having to add what is considered an excess of antibody, 5 mcg, to each device to extract approximately 100 – 200 ng of analytes.

Experiments Using Synthetic Whole Blood

Initial experiments used synthetic whole blood purchased from Immunalysis with the ELISA kits. The lypholized whole blood was prepared according to instructions received in the ELISA kits and used for initial experiments of percent yield and linearity studies with the new devices. The synthetic blood had a homogeneous consistency with no clots and showed no precipitation when analytes in methanol solutions were added.

Linearity was established from 0 to 50 ng/ml using internal standards for both analytes. Limit of Detection (LOD) for Δ-9-THC was calculated to be 1.0 ng/ml. The Limit of Quantitation (LOQ) was found to be 1.0 ng/ml which was higher than desired but at an acceptable level for reporting of Δ-9-THC. LOD for Δ-9-THCA was found to be 2.0 ng/ml with a LOQ of 3.0 ng/ml. The values for Δ-9-THCA are excellent for this analyte for most laboratories do not report Δ-9-THCA present or give a quantitative value below 5 ng/ml.

Use of the synthetic blood bolstered the concept that the device was working as designed and that future research would only make the device better and more efficient. This was until testing of actual samples began.

Experiments Using Actual Postmortem Samples

Thirty postmortem samples were secured from the Mississippi Crime Laboratory that had been previously analyzed for Δ-9-THC and Δ-9-THCA. Ten of the samples had been found to contain no Δ-9-THC at 0.5 ng/ml, ten of the samples had been reported with Δ-9-THC present plus quantitated and ten samples had been reported as "None

Detected Due to Interfering Substance". Twenty eight of the samples reported Δ-9-THCA as present and quantitated with two samples being reported as "None Detected at 5 ng/ml". This group of samples provided an excellent challenge to using the new device for they covered all aspects of the original experimental design.

Initially five devices were made and 1 ml of postmortem samples added to each and run through the protocol simply to establish both analytes would be recovered. Surprisingly, only two of the five had any recovery of analytes and the percent yield was approximately half that seen with the synthetic blood samples. Five more devices were prepared and the experiment repeated after each individual postmortem sample was mixed over night. The results of the second experiment were basically the same as the first with the exception of Δ-9-THCA being recovered from a third sample. No internal standards were added for quantitations during these first two experiments so no information was gained concerning those two analytes during the initial experiments with postmortem samples.

New batches of all buffers and the 5% glutaraldehyde were made for a third experiment on the postmortem samples. Two devices were prepared and 1 ml of a postmortem sample reported as "None Detected" was spiked with 25 ng/ml Δ-9-THC and placed in one device and 1 ml of a postmortem sample reported as "None Detected" was spiked with 25 ng/ml Δ-9-THCA and placed in the second device. The two devices were carried through the protocol with fresh reagents. Recovery of the Δ-9-THC was still poor and the sample spiked with Δ-9-THCA demonstrated no recovery of analyte.

At this point, experiments were conducted again using the synthetic blood to reaffirm original results and test the antibody solution for deterioration of the antibody. Two devices were used spiked as described in the previous paragraph. Analytes from

both devices were recovered as expected using the synthetic blood. This meant that the problem was with the postmortem samples themselves and not any reagents, antibody or prepared devices.

Wong et al. (1982) did a study on the effect of long term storage of Δ-9-THC in blood and serum. Their study looked at the storage of spiked samples of Δ-9-THC in whole blood and serum in several storage environments and at several temperatures ranging from -20^0 to 60° C over a 25 week period. No appreciable loss of Δ-9-THC was found at any storage temperature or environment until around week 17. After this point, recovery of Δ-9-THC from blood or serum became inconsistent and was not achievable after week 25. The article's authors concluded that the inability to recover the analyte was not due to degradation of the molecule or surface absorption onto the container, but rather a binding of the molecule to degrading proteins in the sample.

A method to separate the drug from any protein had to be developed to release any bound Δ-9-THC. Two solvents were chosen to attempt to denature proteins and allow recovery of both Δ-9-THC and Δ-9-THCA; 1) methanol and 2) acetonitrile.

A 1 ml sample of the synthetic blood spiked with 25 ng/ml Δ-9-THC was placed in a 16X125 mm screw cap borosilicate glass tube. While vortexing the sample, 1 ml methanol was slowly added one drop at a time. Vortexing continued for 30 seconds after the last drop of methanol was added. The sample was then centrifuged at 3000 rpm for 25 minutes. The sample was removed and checked to see if a pellet was present and the supernatant was fairly transparent. The supernatant was then transferred to a clean 16X125 mm tube and stored at 4° C for later analysis. The same procedure was conducted using acetonitrile in place of methanol. The acetonitrile supernatant was more transparent than the methanol sample. The acetonitrile sample was handled in the same

manner and stored for later analysis.

Two devices were prepared to use with the samples prepared above and taken through the protocol. Recovery of analyte was achieved using both solvents with more analyte being recovered with acetonitrile, however recovery of analytes were less than with previous runs. This raised the question of were the organic solvents were reacting with the antibodies resulting in the poor recovery of analytes.

The procedure was performed two more times using acetonitrile only with spiked synthetic blood samples. A new step was added following centrifugation and transfer of the supernatant to a clean tube. The acetonitrile was evaporated almost to dryness under a vacuum at 50° C. To each of the two tubes was added 1 ml of a 0.5% saline solution and the tubes vortexed a second time. The tubes were then centrifuged at 3000 rpm for 25 minutes. The supernatant was transferred into a device which was carried through the protocol. Recovery of the analyte from both tubes improved and was found to be in the 50% range as compared to 38% in the initial experiments using the synthetic blood.

Two postmortem negative samples were taken and spiked with 25 ng/ml Δ-9-THC and treated with the acetonitrile procedure. The samples were then carried through the extraction protocol. Results matched percent yield studies conducted with the synthetic blood samples using this same procedure. The percent yield increase held true for Δ-9-THCA experiments using the acetonitrile procedure prior to extraction. From this point forward all experiments were conducted using the acetonitrile procedure to prepare the samples.

Antibody Binding Studies Using Extraction Device

Seven extraction devices were prepared using the glutaraldehyde protocol and then tested using ELISA reagents to determine the approximate binding capacity for Δ-9-

THC and Δ-9-THCA of a device. This experiment was conducted five different times. Samples spiked from 10 to 1000 ng/ml were used plus a negative sample. Absorbance values from the experiments followed an inverse curve as expected. Those devices containing the lowest concentration of analyte showed the largest range of absorbance readings while increasing the concentration, narrowed the absorbance values seen.

As the antibody sites became saturated with analyte, fewer of the enzyme-tagged THC derivatives were able to be bound and subsequently washed out of the devices prior to adding the color reagent. The fewer the THC derivatives bound within a device the expectation would be decreased to no color change. This is exactly what is shown by the experiments. One would also expect the absorbance value ranges to become narrower as the concentration of analyte increased for the same reasons. This is also shown in the experimental results.

The devices showed the ability to bind a concentration of Δ-9-THC of between 200 and 500 ng/ml. At 1000 ng/ml, the device was found to be totally saturated with no color change noted. The absorbance range at 500 ng/ml was 0.0097 to 0.0433 which indicates that this concentration probably saturates most all antibody sites since the absorbance values differ by only 0.0336. At the 200 ng/ml concentration, absorbance demonstrated a range difference of 0.1360 which indicates that binding sites were still available.

For each of the 35 devices to show a binding capacity of 200 ng/ml or more is adequate for performing extractions on postmortem blood samples. The concentration of Δ-9-THC usually seen in postmortem blood samples is less than 20 ng/ml: The highest value that this researcher has seen in a postmortem blood sample was 53 ng/ml in an individual who was smoking marijuana at the time of death. Taking into account the

addition of 25 ng/ml of Δ-9-THC-D3 and 50 ng/ml of Δ-9-THCA-D3, this totals less than 100 ng/ml which leaves sufficient binding capacity for extracting Δ-9-THCA. The 35 devices made for this experiment demonstrates that this protocol is consistent from batch to batch for extractions of both analytes.

Percent Yield Studies

Percent yield studies were conducted to determine the average concentration recovered from a spiked 1 ml sample as compared to a direct standard. These experiments were conducted for Δ-9-THC and Δ-9-THCA at 25 ng/ml separately using 100 prepared devices (50 for each analyte) over a ten day timeframe. The average percent yield for Δ-9-THC was 54% with a range of 50 – 61%. Δ-9-THCA had a 47% yield with a range of 37 – 52%. These experiments were conducted using the acetonitrile clean up protocol which had previously been shown to provide the best percent yield from extraction devices. These results are fairly consistent in regards again to the making of extraction devices but need to be improved with future research. A standard statistical test of reliability was performed for both analytes to ensure the values obtained met Cronbach's alpha for reliability within each experiment. Both Δ-9-THC and Δ-9-THCA met the criteria having alpha scores of 0.954 and 0.803 respectfully.

Research using different chemical linking molecules or choosing a different device that would allow for consistent orientation of antibodies, possess fewer variables for spread of bound antibodies and/or are more resistant to organic solvents and heat are all areas for future research in drug extractions from difficult matrices. Improvement of extraction efficiency would also allow lower detection and quantitation levels to be achieved.

Effect of Δ-9-THC Concentration on Binding of Δ-9-THCA

A study was conducted where both Δ-9-THC and Δ-9-THCA were added together in the same device at equal concentrations to establish if the there was an effect on the binding capacity of Δ-9-THCA. Concentrations ranged from 10 – 150 ng for each analyte. Percent yield of both analytes were practically the same until the concentration of Δ-9-THC was greater than 60 ng. The percent yield of Δ-9-THCA began to fall above this level and continued to fall as the concentration of Δ-9-THC was further increased. When the concentration of Δ-9-THC was 150 ng approximately 30 ng of Δ-9-THCA was recovered even though 150 ng of Δ-9-THCA had been added to the device.

These results speak to the specificity of the antibody for Δ-9-THC as compared to Δ-9-THCA. However, this finding would have limited impact on using the device as an extraction method for both analytes. It must be pointed out again that these levels of both analytes would not be found in a real sample.

Linearity Studies

Two types of linearity studies were performed during this project. The first was extracting each analyte singly and the second was an extraction of both analytes along with their respective internal standards.

Negative blood samples were spiked at concentrations of 5, 10, 25 and 50 ng/ml of Δ-9-THC. The samples were extracted and then analyzed by GC/MS. Extraction of Δ-9-THC gave a linear curve with an r^2 of 0.998. Extraction of Δ-9-THCA gave a linear curve with a r^2 of 0.997. No attempt was made to extend the curve using the single analytes since no real extraction would be done without using deuterated internal standards.

The linear curves for both analytes with their internal standards was performed by

spiking negative blood samples at 2, 5, 10, 25 and 50 ng/ml of Δ-9-THC and Δ-9-THCA along with 25 ng/ml Δ-9-THC-D3 and 50 ng/ml Δ-9-THCA-D3. Limiting the curve to 50 ng/ml was due to the loss of Δ-9-THCA when Δ-9-THC concentration went above 60 ng/ml. Both analytes were extracted simultaneously in runs using the extraction device. Results of the curves were r^2 of 0.995 for Δ-9-THC and 0.997 for Δ-9-THCA.

These experiments demonstrate the ability to extract both analytes of interest from a single device which means that real samples can be handled in the same manner with both analytes being extracted in one run. This is the only extraction method this researcher is aware of where both analytes are extracted in a single step.

First Analysis of True Postmortem Samples

Analyses of postmortem samples received from the Mississippi were divided into four groups. The first group analyzed for Δ-9-THC was those samples reported as "None Detected". The ten samples were prepared using the acetonitrile protocol and then extracted using prepared devices the same day. A set of calibrators ranging from 3 – 50 ng/ml and two controls at 5 and 30 ng/ml were also run with the samples. The calibration curve met the criteria for being linear and the controls were within ± 20% of expected values. The samples were run twice on separate days under the same conditions.

Both groups agreed with the findings reported by the Mississippi Crime Laboratory. There was no Δ-9-THC detected in any of the 10 samples during either run (see Figures 22 and 23). Internal standards added were recovered and met the criteria for being acceptable (see Figures 24 and 25).

Figure 22

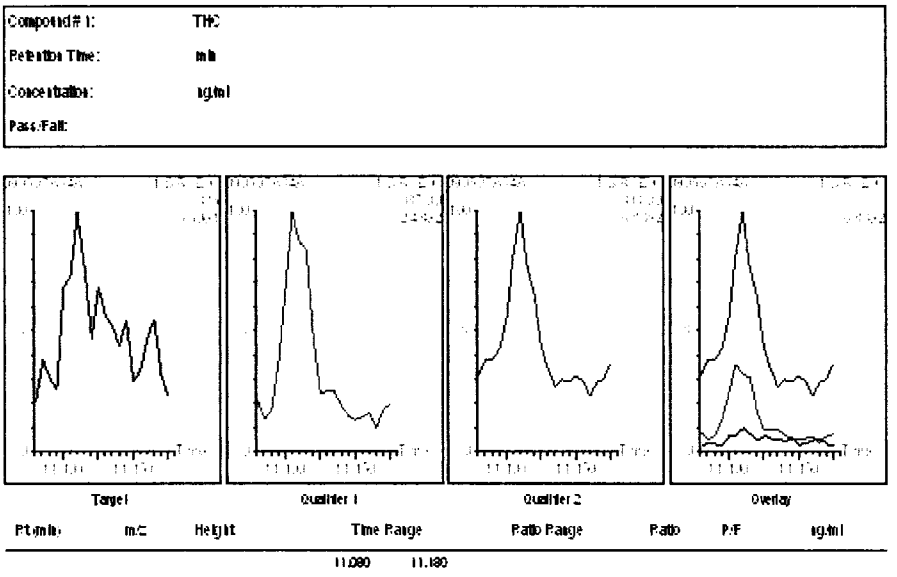

Figure 22: Represents a negative sample of Δ-9-THC as reported by the Mississippi Crime Laboratory. The three required ions are not present as indicated by failure of the computer to integrate and would not fit any confirmation criteria.

Figure 23

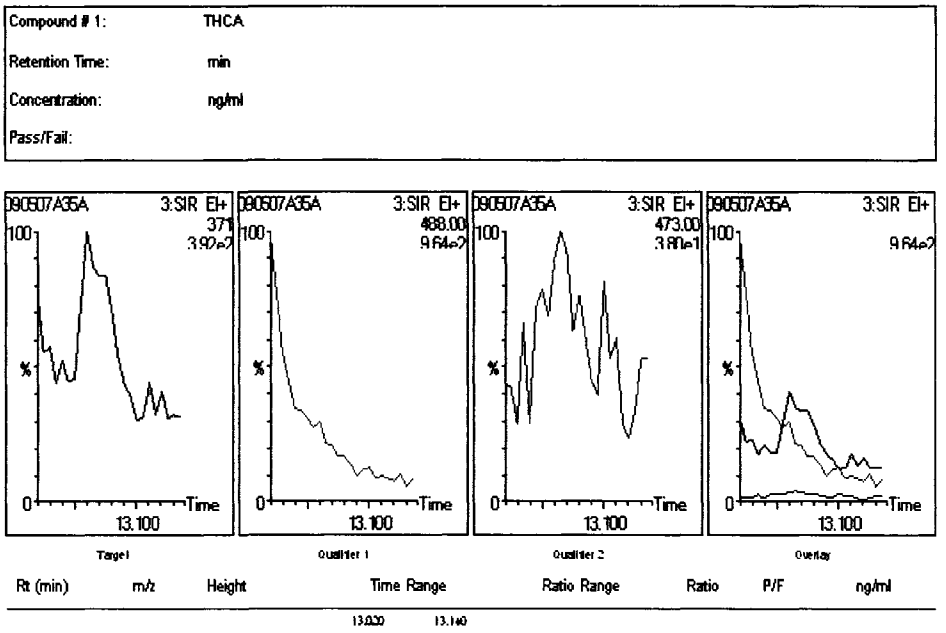

Figure 23: Represent a negative sample from the Mississippi Crime Laboratory for Δ-9-THCA. The three ions are obviously not present and would not meet criteria for confirmation.

Figure 24

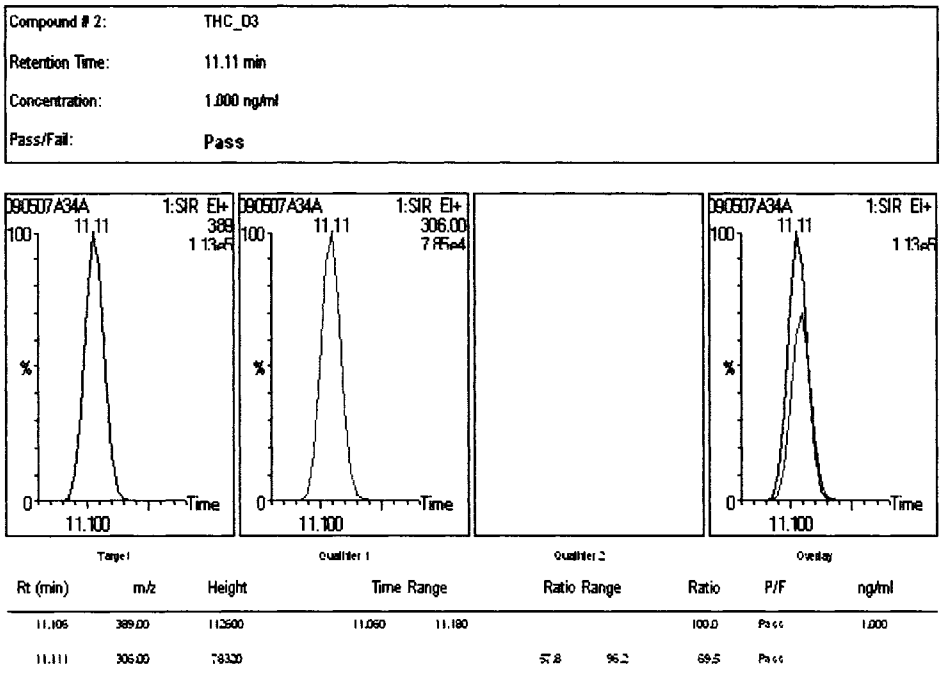

Figure 24: Internal standard ions recovered from extraction of negative Δ-9-THC sample seen in figure 22. Note the ions passed criteria for being acceptable.

Figure 25

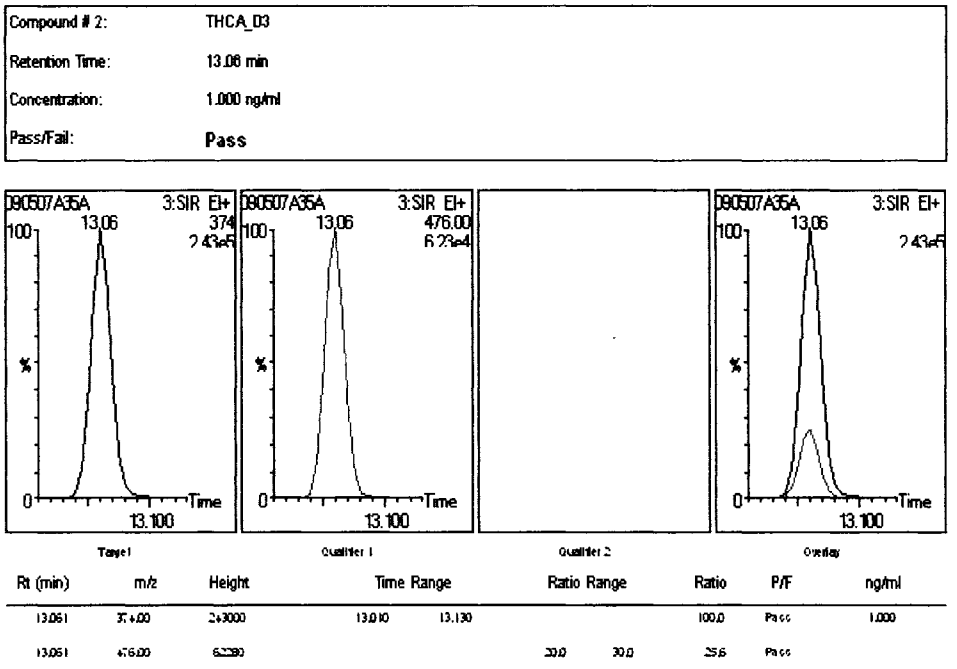

Figure 25: Internal standard ions recovered from negative Δ-9-THCA sample seen in figure 23. Note the ions met criteria for being considered acceptable.

The second group of samples analyzed was those reported with Δ-9-THC present and quantitated (see Figures 26 and 27). Paired *t* results from the first run were compared to a critical value for $t_{.01(9)}$ was 3.250. The Paired *t* value found was 4.384 indicating a significant difference between the results. The samples extracted with the device showed a negative bias averaging 0.32 ng/ml. The second group analyzed also showed a significant difference between the groups as well with a Paired *t* value of 6.034. A negative bias was noted again with a slight average difference of 0.35 ng/ml.

These results indicate the device is capable of extracting Δ-9-THC and the results obtained are comparable to results from another laboratory using a different extraction technique. This is a critical element of the project for it clearly demonstrates the ability to accurately quantitate Δ-9-THC that is important in postmortem blood samples because this is the compound that is responsible for impairment of the central nervous system.

The third group of samples to discuss is the finding for Δ-9-THCA which was analyzed twice for all 30 samples. Two of the 30 samples were found to be negative which agrees with the reports from the Mississippi Crime Laboratory. The other 28 samples were reported to contain Δ-9-THCA (see Figures 28 and 29). Those samples that had a reported value above 50 ng/ml were diluted in order to bring them in the linear range of the assay and then multiplied by the dilution factor for their final result.

The Δ-9-THCA samples were analyzed 10 at a time along with their respective Δ-9-THC analyses. Statistical analysis of the first group of 28 showed a significant difference between the results obtained with the extraction device and the results reported by the Mississippi Crime Laboratory as was described in the results chapter.

Figure 26

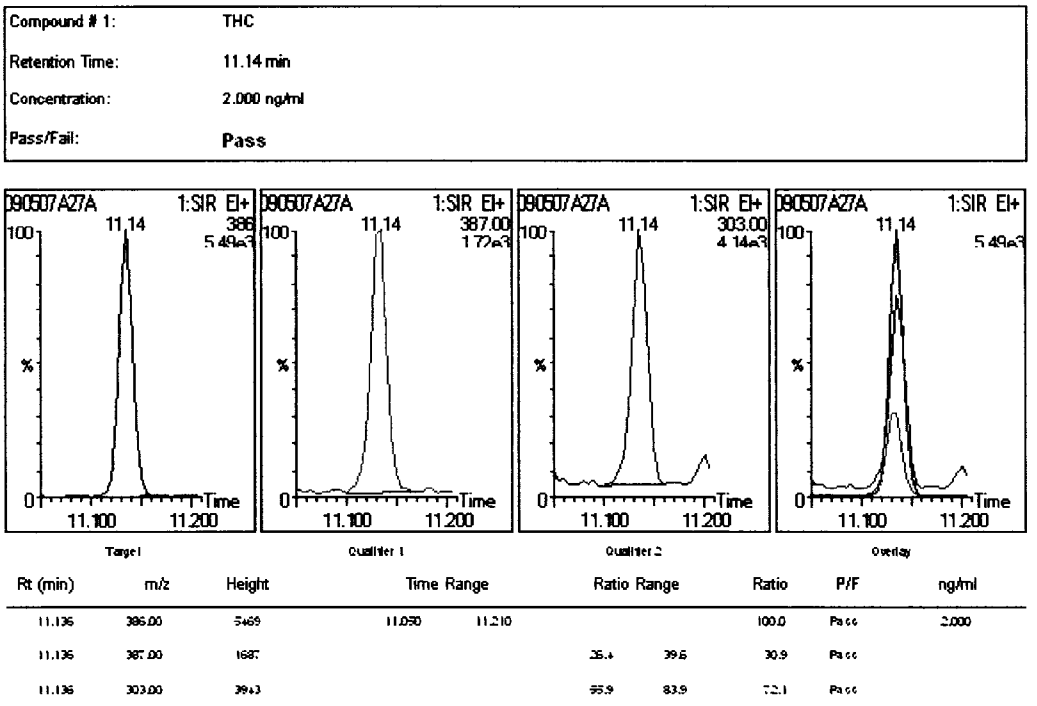

Figure 26: Shows the three ions present from a sample whose concentration was calculated to be 2.0 ng/ml of Δ-9-THC. All three ions meet ration criteria for being considered confirmed and quantitation acceptable. This is sample 2946 from Mississippi Crime Laboratory.

Figure 27

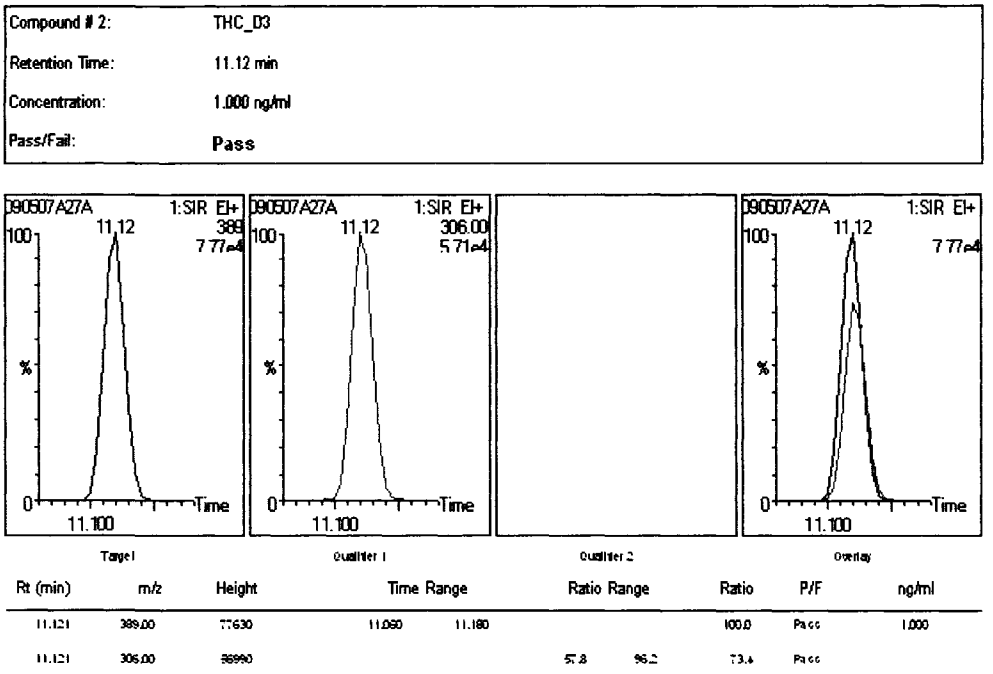

Figure 27: Internal standards ions recovered with sample seen in figure 26.

Figure 28

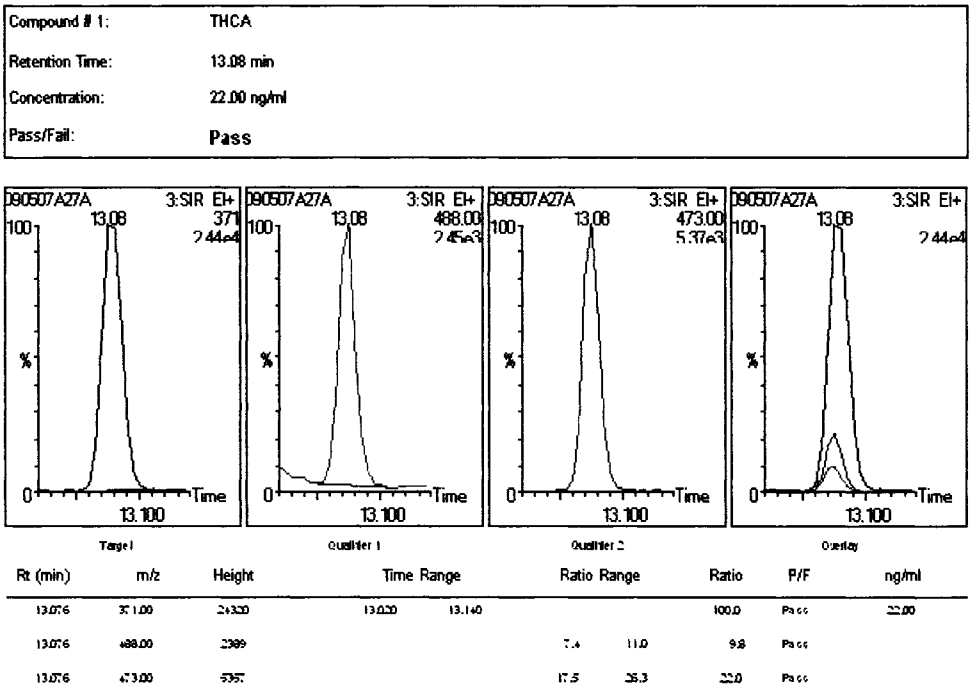

Figure 28: Δ-9-THCA extracted from Mississippi Crime Laboratory sample 2946. Note all three ions are present and the result is 22 ng/ml. This analyte was extracted at the same time as the Δ-9-THC extracted from the same device shown in Figure 26.

Figure 29

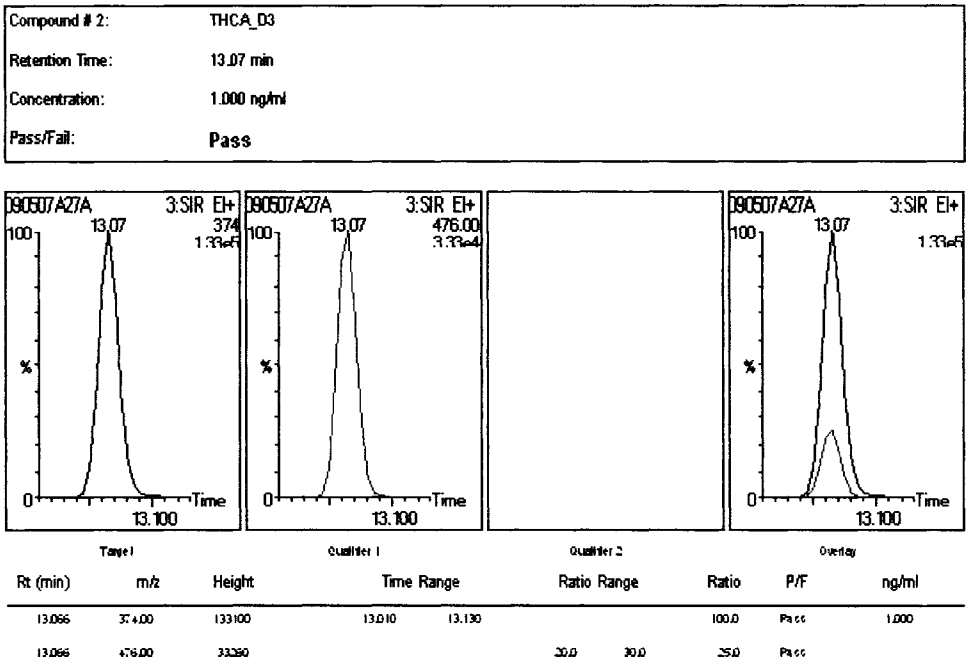

Figure 29: Δ-9-THCA internal standard ions from sample 2946 shown in figure 28.

There was a negative bias noted averaging 5.4 ng/ml less than the reported values. The calculated mean for the first group was 21.6 with a range of 5 – 60 ng/ml. The second analytical run also showed a significant difference between the results with a mean of 21 ng/ml again with a negative bias of 5.9 ng/ml. The mean value for the reported values was 27.0 with a range of 6 – 69 ng/ml.

There were four samples that had to be diluted X2 because of values above the linear range of 50 ng/ml. All eight results came back well within 20% of the reported value. Given Δ-9-THCA only indicates previous use and does not apply to impairment, these values would be acceptable for reporting.

The last Δ-9-THC group to be discussed was the samples reported as "None Detected Due to Interfering Substance". This group is considered the most critical of all the analyses run for it tests whether the extraction process actually removed the interfering substances.

Two of the ten samples were found to contain no Δ-9-THC at a concentration of 1 ng/ml. The other eight samples did confirm the presence of and give quantitative results for Δ-9-THC. These findings indicate that any substance that interfered with the analysis of these samples when performed by another laboratory using a different extraction technique had been removed.

The extraction device performed as anticipated proving an extraction approach using such device with bound antibody for a specific drug is a viable option. The device proved to be simple to use, of low cost and not require the multiple steps encountered with today's common extraction procedures.

One of the main obstacles encountered during the project was the time required to bind the antibodies to the polystyrene flask in preparation for extractions. The initial step

was a four hour incubation to bind the glutaraldehyde to the flask followed by a three hour incubation period of binding the antibodies to the glutaraldehyde. Take into account the time for washing steps in between the two incubation periods along with the minimum of one hour for incubation of the sample, 20 min wash in hexane to release the analytes, 20 min evaporation of the hexane and one hour incubation for derivatization, the total time from start to instrument was approximately 10 hours. Research should be performed to establish if any of these times could be shortened by using different binding chemistries or different type devices.

Future projects for other drugs using this approach could be LSD, 6-monoacetylmorphine, morphine, oxycodone, alprazolam and others that demonstrate impairment or toxicity at concentrations in the ng/ml range. Extraction devices that would hold specific antibodies to different drugs within the same device need to be investigated.

Such projects are anticipated thesis projects and research for students pursuing graduate degrees in the field of forensic science with an emphasis in the areas of Toxicology or Implied Consent.

The new device and process could bring to the field of toxicology the advances enjoyed in biochemistry and molecular biology in separation and purification of proteins and DNA from complex matrices using the technique of affinity chromatography. With no or reduced interfering substances to deal with, instrumentation maintenance and supply cost would be reduced by as much as 50% and analytical extraction procedures could be shortened by several hours, thus, improving turn around time for data reporting.

Training of laboratory personnel on this new device would be extremely simple with basically no learning curve involved. The only steps are adding the buffered sample,

incubation, rinsing the device and then releasing the drug for analysis. From that point forward, the steps are the same as any other extraction protocol for analyzing drugs on chromatographic instruments.

There is an excellent opportunity for commercial production of the device and making it available for purchase and use by all laboratories dealing with postmortem drug testing. The device as envisioned is not costly to produce. The device also eliminates the use and disposal of high volumes of organic solvents, buffers and glassware presently used for extraction procedures. Savings on instrument maintenance and supplies would offset costs even further.

The device also lends itself to extraction of substances other than drugs from complex matrices provided a specific antibody can be produced against the antigen of interest. Furthermore, the device is small and portable which makes it practical for use in the field for collection of suspected toxins or chemicals for later analysis.

APPENDIX

Figure 30

Compound 1 name: THC
Coefficient of Determination: 0.994990
Calibration curve: 0.0377391 * x + 0
Response type: Internal Std (Ref 2), Area * (IS Conc. / IS Area)

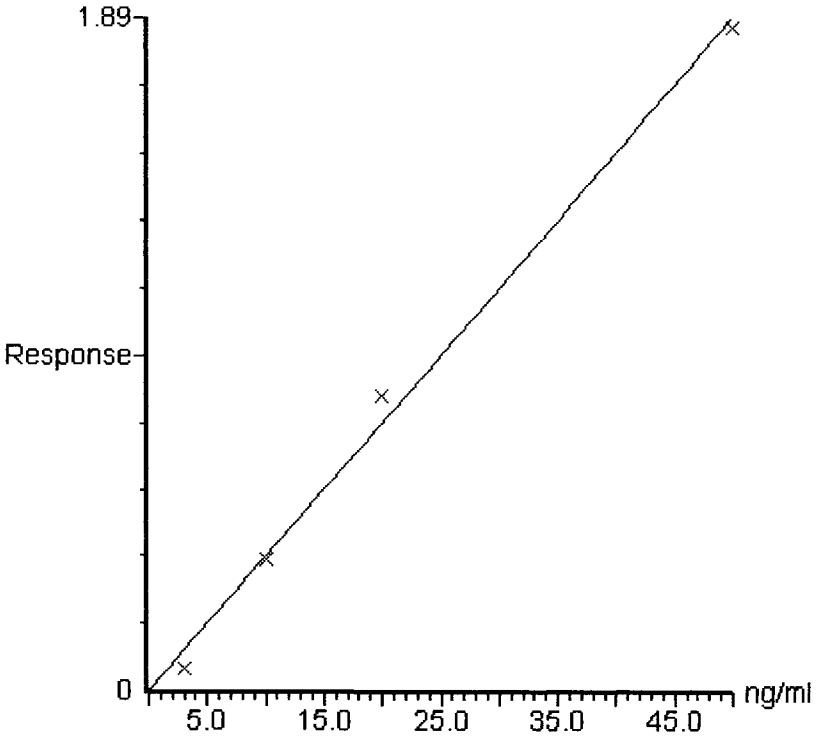

Figure 30: A calibration curve for Δ-9-THC from run 083007A.

Figure 31

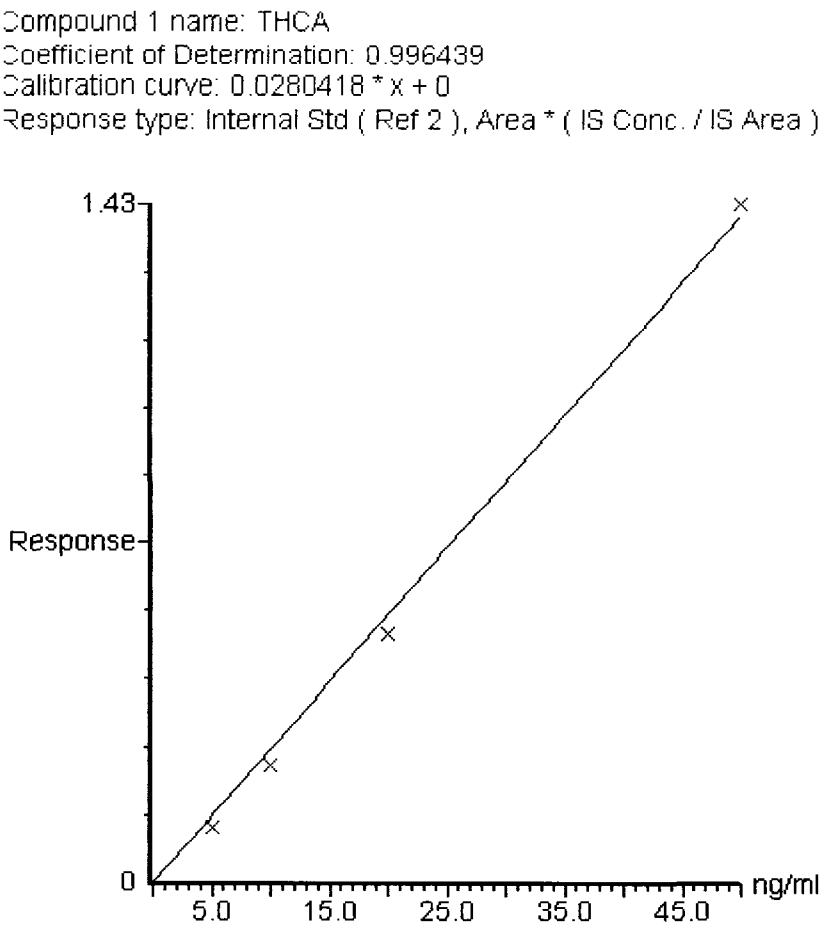

Figure 31: A calibration curve for Δ-9-THCA from run 082707A.

Table 32

Quantify Sample Summary Report
Sample List: **C:\TurboMass\USMFSC.PRO\SampleDB\061207**
Last Modified: **Monday May 27 08:18:39 2007**
Method: **C:\TurboMass\USMFSC\MethDB\THCA**
Printed: **Wed Jun 12 09:10:30 2007**

	Sample RT	Qualifiers P/F	Conc. ng/ml
Sample Name:0612071	Sample ID: 2 ng/ml CAL		
THC	11.11	Pass	2.0
THC-D3	11.12	Pass	1.0
Sample Name: 0612072	Sample ID: Spike 1		
THC	11.10	Pass	0.6
THC-D3	11.12	Pass	1.0
Sample Name: 0612073	Sample ID: Spike 2		
THC	NF	Fail	0
THC-D3	11.12	Pass	1.0
Sample Name: 0612074	Sample ID: Spike 3		
THC	11.09	Pass	0.2
THC-D3	11.12	Pass	1.0
Sample Name: 0612075	Sample ID: Spike 4		
THC	NF	Fail	0
THC-D3	11.12	Pass	1.0
Sample Name: 0612076	Sample ID: Spike 5		
THC	NF	Fail	0
THC-D3	11.12	Pass	1.0
Sample Name: 0612077	Sample ID: Spike 6		
THC	11.11	Pass	0.4
THC-D3	11.12	Pass	1.0
Sample Name: 0612078	Sample ID: Spike 7		
THC	NF	Fail	0
THC-D3	11.12	Pass	1.0
Sample Name: 0612079	Sample ID: Spike 8		
THC	NF	Fail	0
THC-D3	11.12	Pass	1.0
ample Name: 06120710	Sample ID: Spike 9		
THC	NF	Fail	0
THC-D3	11.12	Pass	1.0
Sample Name: 06120711	Sample ID: Spike 10		
THC	NF	Fail	0
THC-D3	11.12	Pass	1.0

Table 32: Experiment for LOD at 0.5 ng/ml. The first injection was a 2.0 ng/ml extracted standard. The 10 subsequent injections were to determine the Limit of Detection for Δ-9-THC. Samples failed 7 out of 10 injections.

Table 33

Quantify Sample Summary Report
Sample List: **C:\TurboMass\USMFSC.PRO\SampleDB\061207**
Last Modified: **Monday May 27 08:18:39 2007**
Method: **C:\TurboMass\USMFSC\MethDB\THCA**
Printed: **Wed Jun 12 19:21:00 2007**

	Sample RT	Qualifiers P/F	Conc. ng/ml
Sample Name:06120712	Sample ID: 2 ng/ml CAL		
THC	11.11	Pass	2.0
THC-D3	11.12	Pass	1.0
Sample Name: 06120713	Sample ID: Spike 1		
THC	11.10	Pass	0.8
THC-D3	11.12	Pass	1.0
Sample Name: 06120714	Sample ID: Spike 2		
THC	11.11	Pass	1.1
THC-D3	11.12	Pass	1.0
Sample Name: 06120715	Sample ID: Spike 3		
THC	11.09	Pass	0.9
THC-D3	11.12	Pass	1.0
Sample Name: 06120716	Sample ID: Spike 4		
THC	11.10	Pass	1.2
THC-D3	11.12	Pass	1.0
Sample Name: 06120717	Sample ID: Spike 5		
THC	11.10	Pass	1.2
THC-D3	11.12	Pass	1.0
Sample Name: 06120718	Sample ID: Spike 6		
THC	11.11	Pass	1.1
THC-D3	11.12	Pass	1.0
Sample Name: 06120719	Sample ID: Spike 7		
THC	11.10	Pass	0.9
THC-D3	11.12	Pass	1.0
Sample Name: 06120720	Sample ID: Spike 8		
THC	11.10	Pass	0.8
THC-D3	11.12	Pass	1.0
Sample Name: 06120721	Sample ID: Spike 9		
THC	11.11	Pass	1.1
THC-D3	11.12	Pass	1.0
Sample Name: 06120722	Sample ID: Spike 10		
THC	11.10	Pass	1.1
THC-D3	11.12	Pass	1.0

Table 33: Experiment for LOQ at 1.0 ng/ml. The first injection was a 2.0 ng/ml extracted standard. The 10 subsequent injections were to determine the Limit of Quantitation for Δ-9-THC. Analysis gave a mean of 1.02 ng/ml. All samples met criteria 10 out of 10 injections.

Table 34

Quantify Sample Summary Report

Sample List: C:\TurboMass\USMFSC.PRO\SampleDB\061207
Last Modified: Monday May 27 08:18:39 2007
Method: C:\TurboMass\USMFSC\MethDB\THCA
Printed: Wed Jun 12 09:41:07 2007

	Sample RT	Qualifiers P/F	Conc. ng/ml
Sample Name: 0612071	Sample ID: 5 ng/ml CAL		
THCA	13.09	Pass	5.0
THCA-D3	13.07	Pass	1.0
Sample Name: 0612072	Sample ID: Spike 1		
THCA	13.09	Pass	0.8
THCA-D3	13.07	Pass	1.0
Sample Name: 0612073	Sample ID: Spike 2		
THCA	13.08	Pass	1.5
THCA-D3	13.07	Pass	1.0
Sample Name: 0612074	Sample ID: Spike 3		
THCA	13.09	Pass	0.9
THCA-D3	13.06	Pass	1.0
Sample Name: 0612075	Sample ID: Spike 4		
THCA	13.09	Pass	1.7
THCA-D3	13.07	Pass	1.0
Sample Name: 0612076	Sample ID: Spike 5		
THCA	13.09	Pass	1.1
THCA-D3	13.07	Pass	1.0
Sample Name: 0612077	Sample ID: Spike 6		
THCA	13.08	Pass	2.1
THCA-D3	13.06	Pass	1.0
Sample Name: 0612078	Sample ID: Spike 7		
THCA	13.10	Pass	1.9
THCA-D3	13.08	Pass	1.0
Sample Name: 0612079	Sample ID: Spike 8		
THCA	13.09	Pass	0.8
THCA-D3	13.07	Pass	1.0
Sample Name: 06120710	Sample ID: Spike 9		
THCA	13.09	Pass	1.1
THCA-D3	13.07	Pass	1.0
Sample Name: 06120711	Sample ID: Spike 0		
THCA	13.09	Pass	1.5
THCA-D3	13.06	Pass	1.0

Table 34: Experiment for LOD at 2.0 ng/ml. The first injection was a 5.0 ng/ml extracted standard. The 10 subsequent injections were to determine the Limit of Detection for Δ-9-THCA. All samples met criteria 10 out of 10 injections.

Table 35

Quantify Sample Summary Report
Sample List: **C:\TurboMass\USMFSC.PRO\SampleDB\061207**
Last Modified: **Monday May 27 08:18:39 2007**
Method: **C:\TurboMass\USMFSC\MethDB\THCA**
Printed: **Wed Jun 12 09:41:07 2007**

	Sample RT	Qualifiers P/F	Conc. ng/ml
Sample Name:06120712	Sample ID: 5 ng/ml CAL		
THCA	13.09	Pass	5.0
THCA-D3	13.07	Pass	1.0
Sample Name: 06120713	Sample ID: Spike 1		
THCA	13.09	Pass	2.9
THCA-D3	13.07	Pass	1.0
Sample Name: 06120714	Sample ID: Spike 2		
THCA	13.08	Pass	3.2
THCA-D3	13.07	Pass	1.0
Sample Name: 06120715	Sample ID: Spike 3		
THCA	13.09	Pass	2.8
THCA-D3	13.06	Pass	1.0
Sample Name: 06120716	Sample ID: Spike 4		
THCA	13.09	Pass	3.1
THCA-D3	13.07	Pass	1.0
Sample Name: 06120717	Sample ID: Spike 5		
THCA	13.09	Pass	3.1
THCA-D3	13.07	Pass	1.0
Sample Name: 06120718	Sample ID: Spike 6		
THCA	13.08	Pass	3.0
THCA-D3	13.06	Pass	1.0
Sample Name: 06120719	Sample ID: Spike 7		
THCA	13.10	Pass	2.8
THCA-D3	13.08	Pass	1.0
Sample Name: 06120720	Sample ID: Spike 8		
THCA	13.09	Pass	2.9
THCA-D3	13.07	Pass	1.0
Sample Name: 06120721	Sample ID: Spike 9		
THCA	13.09	Pass	3.1
THCA-D3	13.07	Pass	1.0
Sample Name: 06120722	Sample ID: Spike 10		
THCA	13.09	Pass	3.2
THCA-D3	13.06	Pass	1.0

Table 35: Experiment for LOQ at 3.0 ng/ml. The first injection was a 5.0 ng/ml extracted standard. The 10 subsequent injections were to determine the Limit of Quantitation for Δ-9-THCA. All samples met criteria 10 out of 10 injections.

Figure 32

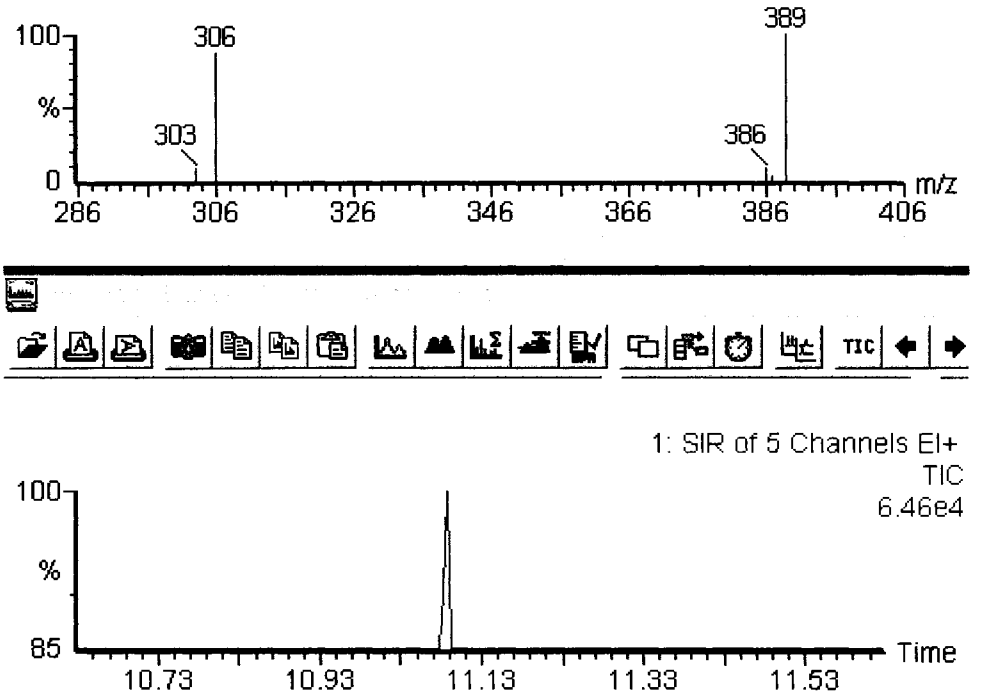

Figure 32: Chromatographic peak and selected ions for Δ-9-THC from sample 1662. This sample was reported with interfering substances and unable to quantitate. Using the device, there were no interfering substances noted and 4.1 ng/ml was found present.

Figure 33

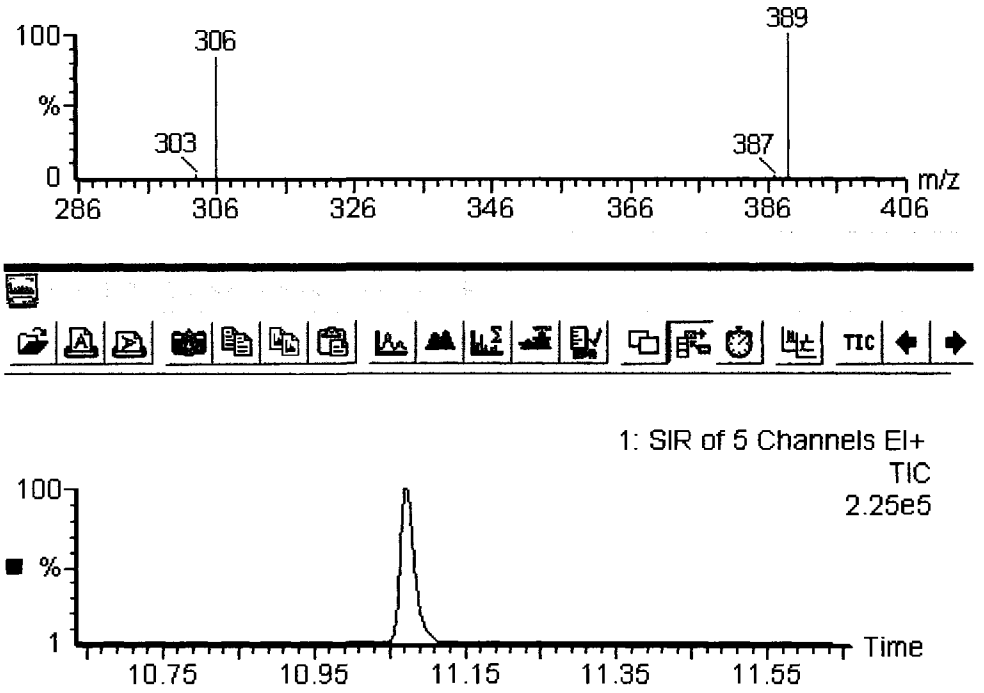

Figure 33: A sample reported with interfering substances. This is sample 6482 from the Mississippi crime Laboratory. There are no interfering substances noted and the sample was found to be negative for Δ-9-THC.

Figure 34

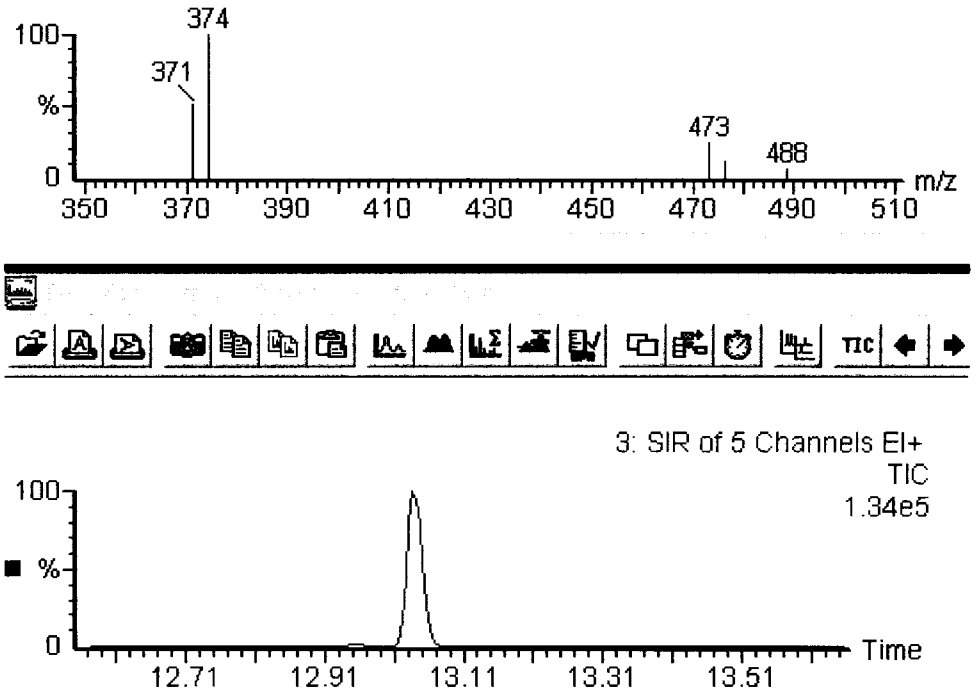

Figure 35: Sample 2946 for Δ-9-THCA. 24 ng/ml were found present in this analysis as compared to 27 ng/ml reported by the Mississippi Crime Laboratory.

BIBLIOGRAPHY

Argurell, S., Hallidin, J., Lindgren, E., Ohlson, A., Widman, M., Gillespie, H., & Hollister, L. (1973). Pharmocokinetics and Metabolism of Δ-9-Tetrahydrocannabinol and other Cannabinoids with emphasis on man. *Pharmacol. Rev.* 38: 21 – 43.

Axen, R., Porath, J. & Ernback, S. (1967). Nature. 214: 1302 – 1312.

Bagnati, R., Oriundi, M.P., Russo, V., Danese, M., Berti, F. & Fanelli, R. (1991). Determination of Zeronal and β-zeronal in Calf Urine by Immunoaffinity Extraction and Gas Chromatography-Mass Spectrometry After Repeated Administration of Zeronal. *J Chromatgr.* 564: 493 – 502.

Bandet, F., Theze, J. & Zovali, M. (1991). UV-treated Polystyrene Microtiter Plates for use in an ELISA to Measure antibodies against Synthetic Peptides. *J. Immunol. Methods.* 142, 73-82.

Baselt, R.C. (1982). Disposition of Toxic Drugs and Chemical in Man. 2nd Ed. (pp 715 - 719). Biomedical Publications, Davis, CA.

Brennand, D.M., Danson, M.J. & Hough, D.W. (1986). A Comparison of ELISA Screening Methods for Production of Monoclonal Antibodies against Soluble Protein Antibodies. *J. Immunol. Methods.* 93, 9-16.

Burns, M. (2003). Medical-Legal Aspects of Drugs. (pp 235 – 251). Lawyers &Judges Publishing Company, Inc., Tucson, AZ.

Catt, K. & Niall, H.D., (1967). Nature. 213: 825 – 827.

ChiChu, M.H. & Drummer, O.H. (2002). Determination of Δ-9-Tetrahydrocannabinol in Whole Blood using Gas Chromatography Mass Spectrometry. *J. Anal. Toxicol.* 26, 575-581.

Cook, C.E., Seltzman, H.H., Schindler, Tallent, V.H., Chin, K.M. & Pitt, C.G. (1992). Radioimmunoassays for Cannabinoids. (NIDA Research Monograph No. 42, 19-32): Washington D.C.: U.S. Government Printing Office.

Crouch, D.J., Birky, M.M., Gust, S.W., Rollins, D.E., Walsh, J.M., Moulden, J.V., Quilan, K.E. & Beckel, R.W. (1993). The prevalence of drugs and alcohol in fatally injured truck drivers. *J. Forensic Sci.* 38: 1342-1353.

D'Asaro, J.A. (2000). An automated and simultaneous solid-phase extraction of Δ-9-tetrahydrocannabinol and 11-nor-9-carboxy-Δ-9-tetrahydrocannabinol in whole blood using the Zymark Rapid Trace™ with confirmation and quantitation by GC-EI-MS. *J. Anal. Toxicol.* 24: 289-295.

Drummer, O.H. & Gerostamoulos, J,. (2002). Postmortem Drug Analysis: Analytical and Toxicological Aspects. *Therap. Drug Monit.* 24(2): 199-209.

Dufresne, C., Favette, P., Paradis, C. & Bonlieu, R. (2001). Comparative Study of Liquid-Liquid Extraction and Solid-phase Extraction Methods for the Separation of Sufentanil from Plasma Befoe Gas Chromatography Mass Spectrometric Analysis. *Clin. Chem.* 47, 600-602.

Felgate, P.D. & Dinan, A.D. (2000). The determination of Δ-9-tetrahydrocannabinol and 11-nor-9-carboxy-Δ-9-tetrahydrocannabinol in whole blood using solvent extraction combined with polar solid-phase extraction. *J. Anal. Toxicol.* 24: 127-132.

Gardas, A. & Lewartowskey, A. (1988). Coating of Proteins to Polystyrene ELISA Plates in the Presence of Detergents. *J. Immunol. Methods.* 106, 251-255.

Garriott, J.C, Di Maio, V.J. & Rodriquez, R.G. (1986). Detection of cannabinoids in homicide and motor vehicle fatalities. *J. Forensic Sci.* 37(4): 969-983.

Gerber, C.E., Bruchelt, G., Gotze-Speer, B. & Speer, C.P. (2000). Detection By ELISA of Low Transferrin Levels in Bronchioalveolar Secretions of Preterm Infants. *J. Immunol. Methods.* 233, 42-45.

Graves, H.C.B. (1988). The Effect of Surface Charge on Non-specific Binding of Rabbit Immunoglobulin G in Solid-Phase Immunoassay. *J. Immunol. Methods.* 111, 157-166.

Gude, T., Preiss, A., & Rubach, K. (1995). Determination of chloramphenicol in muscle, liver, kidney and urine of pigs by means of immunoaffinity chromatography and gas chromatography with electron capture detection. *J. Chromatogr. B* 673(2): 197-204.

Gustafson, R.A, Moolchan, E.T., Barnes, A., Levine,B. & Huestis, M.A. (2003). Validated method for the simultaneous determination of Δ-9-tetrahydrocannabinol (THC), 11-hydroxy-THC and 11-nor-9-carboxy-THC in human plasma using solid phase extraction and gas chromatography-mass spectrometry with positive chemical ionization. *J Chromtogr. B* 798: 145-154.

Haagsma, N. & van de Water, C. (1992). Monoclonal Antibody Mediated clean-up Procedure for the High Performance Liquid Chromatographic Analysis of Chloramphenicol in Milk and Eggs. *J. Chromatogr.* 478: 205 - 216

Hage, D.S., (1998). Periodate Oxidation of Antibodies for Site-selective Immobilization in Immunoaffinity Chromatography. *J. Biochem. Biophys. Methods.* 147: 69 – 82.

Hale, J. (1995). Irreversible, oriented immobilization of antibodies to cobalt-iminodiacetate resin for use as immunoaffinity media. *Anal. Biochem.* 231: 46-49.

Hawks, R.L. (1992). Analysis of Cannabinoids: Introduction and Review. (NIDA Research Monograph No. 42, 1-6) Washington D.C.: U.S. Government Printing Office.

Hollister, L.E. (1992). Drugs of Abuse: In B.G Katzung (Ed.). Basic and Clinical Pharmacology (pp 445-448). Appleton and Lange, East Norwalk, CT.

Huang, W., Moody, D.E., Andrenyak, D.M., Smith, E.K., Foltz, R.L., Huestis, M.A. & Newton, J.F. (2001). Determination of Δ-9-Tetrahydrocannabinol and 11-Nor-9-Carboxy-Δ9-Tetrahydrocannabinol in Human Plasma by Solid-Phase Extraction and Gas Chromatography-Negative Ion Chemical Ionization Mass Spectrometry. *J. Anal. Toxicol.* 25, (531-537).

Heustis, M.A. (2003). Principles of Forensic Toxicology. 2nd Ed. B. Levine, Ed. (pp 246 – 264). AACC, Washington, D.C.

Huestis, M.A., Mithcell, J.M., & Cone, E.J. (1995). Detection times of marijuana metabolites in urine by immunoassay and GC-MS. *J. Anal. Toxicol.* 19: 443-449.

Johns, M.A., Rosengarten, M.J. & Regnier, F.E. (1996). Enzyme-linked immunosorbent assays in a chromatographic format. *J. Chromatogr. A* 73(1): 195-206.

Josten, A., Haalck, L. Spener, F. & Meusel, M. (2000). Use of Microbial Transglutmase for Biotinylation of Antibodies. *J. Immunol. Methods,* 240, 47-54.

Kelly, P., & Jones, R.T., (1993). Metabolism of Tetrahydrocannabinol in Frequent and Infrequent Marijuana Users. *J. Anal. Toxicol.* 85: 51 – 56.

Kelly, T.H., Foltin, R.W., Emurian, C.S. & Fischman, M.W. (1993). Performance based testing for drugs of abuse: dose and time profiles of marijuana, amphetamine, alcohol and diazepam. *J. Anal. Toxicol.* 17: 264-272.

Kindt, T.J., Goldsby, R.A. & Osborne, B.A. (2007). Immunology 6th Ed. (pp 12 – 20). W.H. Freeman & Co., New York, NY.

Klasen, E.A., Rigutti, A., Bos, A. & Bernini, L.F. (1982). Development of a Screening System for Detection of Somatic Mutations. I. Enzyme Immunoassay for Detection of Antibodies against Specific Hemoglobin Determinants. *J. Immunol. Methods*, 54, 241-250).

Klasen, E.A., Rigutti, A., Bos, A. & Bernini, L.F. (1983). Development of a Screening System for Detection of Somatic Mutations. I. The use of Peptides and Insoluble Protein Fragments in a Non-competitive Solid-Phase Enzyme Immunoassay. *J. Immunol. Methods*, 59, 281-289.

Kim, H.O., Durance, T.D. & Li-Chan, E.C. (1999). Reusability of avidin-biotinylated immunoglobulin Y columns in immunoaffinity chromatography. *Anal. Biochem.* 164: 383-397.

Levine, B.L., 2003. Principles of Forensic Toxicology. 2nd Ed. (pp 185-201). AACC Press, Washington D.C.

Lin, D-L. & Lin, R-L. (2005). Distribution of 11-Nor-9-Carboxy-Δ-9-Tetrahydrocannabinol in Traffic Fatality Cases. *J. Anal. Toxicol.* 29: 58 – 61.

Love, R.A., Villafranca, J.E., Aust, R.M., Nakamura, K.K., Jue, R.A., Major, J.G., et al. (1993). How the anti-(metal chelate) antibody CHA255 is specific for the metal ion of its antigen: X-ray structures for two Fab'/Hapten complexes with different metals in the chelate. *Biochemistry* 32: 10950-10959.

Manno, J.E., Manno, B.R., Kemp, P.M., Alford, D.D., Abukhalef, I. K., McWilliams, M.E., Hagaman, F.N. & Fitzgerald, M.J. (2001). Temporal Indication of Marijuana Use Can be Estimated from Plasma and Urine Concentrations of Δ-9-Tetrahydrocannabinol 11-Nor-Δ-9-Tetrahydrocannabinol Carboxylic Acid. *J. Anal. Toxicol.*, 25, 538-549.

Marnell, T., 2006. Drug Identification Bible. 5th Ed. (pp 289 – 304). Amera-Chem, Inc., Grand Junction, CO.

Mason, A.P., Perez-Reyes, M., McBay, A.J. & Foltz, R.L. (1983). Cannabinoid concentrations in plasma after inhalation of marijuana smoke. *J. Anal. Toxicol.* 7: 172-174.

Mason, A.P. & McBay, A.J. (1984). Ethanol, marijuana and other drug use in 600 drivers killed in single-vehicle crashes in North Carolina, 1978-1981. *J. Forensic Sci.* 29: 987-1026.

McBay, A.J. (1988). Interpretation of blood and urine cannabinoid concentrations. *J. Forensic Sci.* 33(4): 875-883.

Moeller, M.R., Doerr, G. & Warth, S. (1992). Simultaneous quantitation of delta-9-tetrahydrocannabinol (THC) and 11-nor-carboxy-delta-9-tetrahydrocannabinol (THC-COOH) in serum by GC/MS using deuterated internal standards and its application to a smoking study and forensic cases. *J. Forensic Sci.* 37: 969-983.

Moody, D.E., Crouch, D.J., Smith, R.P., Cresalia, C.W., Francom, P., Wilkins, D.G. & Rollins, D.E. (1991). Drug and alcohol involvement in railroad accidents. *J. Forensic Sci.* 36:1474-1484.

Moody, D.E., Monti, K.M., & Crouch, D.J. (1992). Analysis of forensic specimens for cannabinoids. II. Relationship between blood Δ-9-tetrahydrocannabinol and blood and urine 11-nor-Δ-9-tetrahydrocannabinol-9-carboxylix acid concentrations. *J. Anal. Toxicol.* 16: 302-306.

Neidbala, R.S., Kardas, K.W., Fritch, D.F., Kardo,S., Fries, T., Waga, J., Robb, J., & Cone, E.J. (2001). Detection of Marijuana Use by Oral Fluid and Urine Analytes Following Single Dose Administration of Smoked and Oral Marijuana. *J. Anal. Toxicol.* 25, (289-301).

Ordronneau, P., Abdullah, L.H. & Petrisz, P. (1991). An Efficient Enzyme Immunoassay for Glutamate using Glutaraldehyde Coupling of the Hapten to Microtiter Plates. *J. Immunol. Methods.* 93, 169-176.

Peat, M.A., Finkle, B.S. & Deyman, M.F. (1982). Laboratory Evaluation of Immunoassay Kits for the Detection of Cannabinoids in Biological Fluids. (NIDA Research Monograph No. 42, 85-92) Washington, D.C.: U. S. Government Printing Office.

Rosenfeld, J., Bowins, B., Roberts, J., Perkins, J. & MacPherson, A.S. (1974). Loss of Cannabinoids by Adhesion to Glass and Plastic Containers. *Anal. Chem.* 46: 2232 – 2238.

Rosenthal, D. & Brine, D. (1979). Quantitative Determination of Δ-9-Tetra-Hydrocannabinol in Cadaver blood. *J. Forensic Sci,* 24, 282-290.

Rotmans, J.P. & Delwel, H.R. (1983). Cross-linking of *Schistisoma mansoni* Antigens and their Covalent Bindng on the Surface of Polysyrene Microtitration Trays for use in ELISA. *J. Immunol. Methods*, 59, 281-287).

Rule, G. & Henion, J. (1992). Determination of Drugs from Urine On-Line immunoaffinity chromatography-high-performance Liquid Chromatography-Mass Spectrometry 582: 103 – 112.

Samyn, N., De Boeck, G., & Verstraete, A.G. (2002). The use of Oral Fluid and Sweat Wipes for the Detection of Drugs of Abuse in Drivers. *J. Forensic Sci.* 47(6): 1380 – 1387.

Smith, M.L. (1999). Immunoassay: In B. Levin (Ed.), Principles of Forensic Toxicology (pp 117-137). AACC Press, Washington D.C.

Sprinthall, R.C. (1994). Basic Statistical Analysis, 4th Ed. (pp372 – 389). Allyn and Bacon, Needham Heights, MA.

Stenberg, M. & Nygren, H. (1981). Kinetic of Antigen-Antibody Reactions at Solid-Liquid Interfaces. *J. Immunol. Methods.* 113, 3-15.

Suresh, K., Ramanadham, M. & Nadimpalli, S.K. (2002). An ELISA Method to Quantify the Mannose-6-Phophate receptors. *J. Biochem, Biophys.* 52, (111-119).

Suter, M. (1982). A Modified ELISA Technique for Anti-hapten antibodies. *J. Immunol. Methods.* 53, 103-110.

Thompson, L.K. & Cone, E.J. (1987). Determination of delta-9-tetrahydrocannabinol in human blood and saliva by high-performance liquid chromatography with amperometric detection. *J. Chromatogr.* 421: 91-97.

Suter, M. (1982). A Modified ELISA Technique for Anti-hapten antibodies. *J. Immunol. Methods.* 53, 103-110.

Wassell, J., Reed, P., Kane, J. & Weinkove, C. (1999). Freedom from Drug Interferences in New Immunoassay for Urinary Catecholamines and Metanephrines. *Clin. Chem.* 45, 2216-2233.

Webb, K.S., Baker, P.B., Cassells, N.P., Francis, J.M., Johnston, D.E., Lancaster, S.L., Reed, G.D. & White, S.A. (1996). The Analysis of Lysergide (LSD): The Development of a Novel Enzyme Immunoassay and Immunoaffinity Affinity Extraction Procedures Together with a HPLC-MS Confirmation Procedure. *J. Forensic Sci* 41: 938 – 946.

Wong, A.S., Orbanosky, M.W., Reeve, V.C. & Beede, J.D. (1992). Stability of Delta-9-Tetrahydrocannabiol in Stored Blood and Serum. (NIDA Research Monograph No. 42, 119 – 124): Washington D. C.: U.S. Government Printing Office.

Wylie, D.A., Lu, D., Carlson, R., Babacan, K.F., Schuster, S.M. & Wagher, F.W. (1992). Monoclonal Antibodies Specific for Mercuric Ions. *Proc Natl Acad Sci* 89: 4101 – 4108.

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EDUCATIONAL BACKGROUND

2008 University of Southern Mississippi
Hattiesburg, MS
Major: Biology
Degree: PhD
Dissertation Topic: Use of Δ-9-Tetrahydrocannabinol Monoclonal Antibodies Chemically Bound to Polystyrene Using Glutaraldehyde for the Purpose of Extracting Δ-9-Tetrahydrocannabinol and Δ-9-Tetrahydrocannabinol Carboxylic Acid from Postmortem Whole Blood Samples for Analysis by Gas Chromatography Mass Spectrometry

1989 University of Southern Mississippi
Hattiesburg, MS
Major: Biology
Degree: MS

1978 University of Southern Mississippi
Hattiesburg, MS
Drgree: BS
Major: Biology
Minor: Chemistry

1976 Jones County Junior College
Ellisville, MS
Degree: AA
Major: Chemistry

EMPLOYMENT HSTORY

August 2005 to present
University of Southern Mississippi
Instructor for the Department of Administration of Justice Forensic Science Division. Responsibilities include class lectures and laboratory instruction to undergraduate students seeking degrees in Administration of Justice or Forensic Science. Establish class curriculum criteria, graduation criteria and advise students pursuing a Forensic Science degree. Pursuit of research in the field of Forensic Science especially relating to the specialty of Forensic Toxicology.

August 2000 - present
Self- Employed Consultant: ToxConsulting, LLC
Consultant for law firms dealing with interpretation of drug and alcohol impairment as they relate to probable cause of accidents and/or injury(s). Serve as consultant for major instrumentation manufacturer. Duties are instructor for operation and maintenance of gas chromatographic and mass spectroscopy instrumentation and Beta site tester of new products and software.

August 2000 - August 2005
Mississippi Crime Laboratory Section Chief-Toxicology Responsibilities include supervision and training of forensic scientists as well as day-to-day operations of the Toxicology Section. Method development, verification and equipment purchases and performance of analytical testing are part of my duties.

August 1998 -2000
Self-employed Consultant and President of Diversified Laboratory Services, LLC. Responsibilities included set up of all aspects of a Toxicology screening laboratory. Establish and validate esoteric testing methods and training of employees in client laboratories.

April 1984 – July 1998
Puckett Laboratory Assistant Laboratory Manager
Duties included oversight of the day-to-day operations of the main clinical laboratory and three regional laboratories. Method development, procedure manuals, supervision of employees, accreditation and budgeting comprised the specific responsibilities.

Puckett Laboratory Director of Clinical Toxicology Responsible for supervision and training of toxicology employees, method development, CAP-FUDT accreditation and client resource for issues dealing with all aspects of drug and alcohol testing in the workplace.

Puckett Laboratory Positive Certifying Scientist
Duties included training of employees in NIDA laboratory and review of analytical data plus chain-of-custody for drug and alcohol results.

Puckett Laboratory Supervisor of Special Chemistry Duties included direct supervision of employees; maintain accreditation, procedure manuals and equipment maintenance.

Puckett Laboratory Research and Development Assistant Responsible for updating drug testing procedures and equipment to secure accreditation from CAP-FUDT and NIDA.

TEACHING EXPERIENCE

William Carey College

Pharmacology for Nurses
Chromatography
Medical Genetics
Comparative Anatomy
Clinical Chemistry

University of Southern Mississippi

Physical Science
Biology
Medical Genetics
Cellular Physiology
Forensic Toxicology
Arson and Explosives
Drug Identification
Introduction to Forensic Science
Forensic Analysis

GRANTS

Concept Proposal Submitted to National Institute of Justice, November, 2005 Accepted February, 2006; Full Proposal submitted March, 2006. Not funded.

Forensic Science Initiative Department of Justice, September 2006. $2 M fully funded.

PRESENTATIONS

Coroner's 2001 Fall Meeting Topic: Proper Collection and Storage of Postmortem Samples

Coroner's 2002 Fall Meeting Topic: Instrumental Analyses of Drugs in Postmortem Samples

Coroner's 2003 Spring Meeting Topic: How the MCL Toxicology Section helps you.

Grand Rounds UMC Pathology Department: Forensic Toxicology (Sept. 2003)

Municipal Prosecutors Conference: June 2004
Topic: Basic Toxicology for Prosecutors

Prosecutor's 2004 Spring Meeting
Topic: An Overview of Forensic Toxicology

Grand Rounds UMC Pathology Department: Postmortem Drug Testing (October 2004)

Prosecutor's Fall Meeting: October 2004
Topic: Forensic Toxicology - What it is and What it is not.

Perkin Elmer International Sales Meeting July 2005
Topic: Use of GC/MS SIFI Mode in Forensic Toxicology

2006 ASCLS-MS/LSCLS Annual Meeting April 2006
Topic: Forensic toxicology; "What it is and What it is not."

COURT/EXPERT WITNESS TESTIMONY

Gave expert testimony on cocaine metabolite result in vitreous fluid by Immunoassay. Testimony given via video tape at offices of Baker Donelson in Jackson, MS August 7, 2007. (Wanda Collins vs Ford Motor Co.)

Gave expert testimony by deposition concerning blood testing for Cannabinoids by immunoassay. Jerry Cooper Payne, Administrator vs Stephen R. Thomas, Does 1-10 and Doe 11. March 15, 2007. (Case pending Panola County Circuit Court).

Testified as expert witness concerning a person's ability to drive a motor vehicle with a blood alcohol concentration of 0.22% and the approximate number of drinks required to obtain said blood alcohol concentration. (Adams County Circuit Court, February, 2007, Yancey vs Ford Motor Co.)

Testified as expert witness on interpretation of ethyl alcohol in combination with SOMA in motion to suppress drug evidence. (Circuit Court, Pearl River County, State of MS vs D. Bordelon, March, 2006)

Testified as expert witness on amphetamine drug toxicology. (Circuit Court, Warren County: State of MS vs R. Vaughn, December, 2005)

Testified as expert witness on the effects of ethyl alcohol on a person's ability to operate a motor vehicle. (Circuit Court, Harrison County, January, 2005)

Gave testimony in deposition concerning blood/serum alcohol assay using a VITROS 950 dry slide instrument.. (November, 2004 - Deposition taken at Baker Donelson, Jackson, MS - Case of Sandra Coleman vs. Ford Motor Co., et. al. # 2002-0209-CV-1).

Testified as expert witness on effects of cocaine on victim in homicide case. (Circuit Court, George County, December, 2004)

Testified as expert witness on negative alcohol and drug test results of homicide victim. (Circuit Court, October, 2003)

Testified as expert witness on Etomidate and Laudanosine. (Circuit Court, Forrest County, State of MS vs Stephanie Stevens, September, 2003)

Testified as expert witness on urine marihuana confirmation. (Justice Court, June 2003)

Testified as expert witness on urine marihuana confirmation. (Justice Court, May, 2003)

Testified as expert witness on methods for analysis of "crack" cocaine. (Municipal Court, May 1996)

Testified as expert witness in arbitration case concerning methods used in screening and confirmation of marihuana in urine. (May, 1992)

Testified as expert witness on the validity of methods used for analysis of blood alcohol. (Circuit Court, January 1992)

Testified as expert witness in case concerning passive inhalation of "crack" cocaine as well as methods used for the detection of cocaine and cocaine metabolites in urine. (Federal Court, April1991)

Testified as expert witness in DUI case concerning ingestion of ethyl alcohol over a 12 hour period and the effects on subsequent blood alcohol levels. (Circuit Court, April 1991)

Testified as expert witness on chain-of-custody procedures for receiving, testing, storage and return of marihuana exhibits for Metro Narcotics Force. (Youth Court, April 1991)

Testified as expert witness on methods used for the detection and quantification of ethyl alcohol, marihuana and cocaine metabolite in blood. (Circuit Court, November 1989)

PUBLICATIONS: Peer-reviewed journals, abstracts and presentations/posters

Broussard, L., Broussard, A., Pittman, T., & Lirette, D. (2000). Case Report: Death Due to inhalation of Ethyl Chloride. Journal Forensic Sciences, 45(1), 223-225.

Broussard, L., Broussard, A., Lirette, D., & Pittman, T. (1999, February). Death due to inhalation of ethyl chloride. Paper presentation at the American Academy of Forensic Sciences Annual Meeting, Orlando, FL.

Broussard, L., Broussard, A., Pittman, T., & Lierette, D. (1999). Death due to inhalation of ethyl chloride. Proceedings of the American Academy of Forensic Sciences, V, 269.

Broussard, L., Lafferty, D., Pittman, T., & Presley, L. (1998, February). Headspace Gas Chromatographic Method for the Measurement of Difluoroethane in Blood. Paper presented at the American Academy of Forensic Sciences Annual Meeting, San Francisco, CA.

Atkins, K. D., Broussard, L., Broussard, A., Brustowicz, T., Lafferty, D., Lierette, D., Pittman, T., & Presley, L. (1998, May) Inhalant Abuse in the New Orleans Area: 2 Case Studies. Paper presentation at the Southern Association of Forensic Scientists (SAPS) Spring Meeting; New Orleans, LA.

Broussard, L., Pittman, T., Lafferty, D. & Presley, L. (1998). Headspace Gas Chromatographic Method for Measurement of Difluoroethane in Blood. Proceedings of the American Academy of Forensic Sciences. IV, 248

Broussard, LA, Presley L, Pittman T, Clouette R, Wimbish GH. The Simultaneous Identification and Quantification of Codeine, Morphine, Hydrocodone and Hydromorphone in Urine as Trimethylsilyl and Oxime Derivatives. Clin Chem 1997:43 (6) 1029-32

Broussard LA, Brustowicz T, Pittman T, Atkins K, Presley, L. (1997). Case Report: Two Traffic Fatalities Related to the Use of Difluoroethane. Journal of Forensic Sciences. vol. 42(6); 1184-86

Broussard LA, Blakeney J. Pittman T, Presley L. (1997). False Negative Methadone Results in Diabetic Patient. AACC Southeast section and Clinical Chemists of Georgia Spring Meeting Program, Atlanta, GA March (abstract)

Broussard LA, Brustowicz T, Pittman T, Atkins K, Presley L. Measurement of Difluroethane in Blood by Gas Chromatography. (1997) AACC Southeast section and Clinical Chemists of Georgia Spring Meeting, Atlanta, GA (abstract)

Broussard LA, Blakeney J, Pittman T, Presley L. False Negative Results for Drugs of Abuse in Diabetic Ketoacidosis. Clinical Chemistry, 43(6) S207 (abstract)

Blakeney, J., Broussard, L., Pittman, T., & Presley, L. (1997, March). False Negative Methadone Results in a Diabetic Patient. Paper Presentation at the Annual Meeting of the AACC. Atlanta, GA.

Pittman, Thomas S. Characterization of dictytine granules and associated matrices in species of Cribrariacea: Myxomycetes. University of Southern Mississippi, Hattiesburg, MS Thesis, May 1989