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Research and Development in Forensic Toxicology

Development and Production of Reference Materials for Control and Calibration of Hair Drug Testing

Final Report

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ABSTRACT

Hair testing for drugs of abuse needs matrix-matched control materials to help ensure the laboratory's process and authenticity of its results.

This project developed and produced four reference materials of hair fortified with controlled substances for use as controls and/or calibrators. These reference materials can be used to identify and measure drugs of abuse in hair for forensic purposes.

RTI International's[1] (RTI's) Center for Forensic Sciences surveyed laboratories that perform hair analyses to determine which controlled substances would have the best utility, and these analytes were included in the reference materials. Head hair strands (14–20 g) were washed with deionized water to remove potential surface contaminants. Fortification solutions were prepared with appropriate analytes, and the intact, whole head hair strands were completely submerged in the solution at room temperature for a period of time that was dependent on the analyte and the concentration of that analyte in the fortifying solution. Following successive isopropanol-phosphate buffer decontamination washes, the hair was homogenized and divided into 100-110 mg aliquots. RTI validated these materials through random sampling testing by multiple forensic laboratories using their validated testing procedures to obtain realistic reference ranges. Both gas and liquid chromatographies, coupled with mass spectral technologies, were used. At least two analytical techniques and two reference laboratories analyzed each reference material to provide representative reference values using numerous techniques. Uncertainty measurements [2*SQRT (Variance)] were determined for all reference materials to estimate the intra- and inter-laboratory variability for the analytical testing. The results are shown in the following table:

Reference Material	Analyte	Target Concentration (pg/mg)	Reference Range (pg/mg)
RM-RTI-CFS-2407-THCA-1	11-Nor-delta-9-THC-9-carboxylic acid (THCA)	0.30	0.25 ± 0.17
RM-RTI-CFS-2407-MOR-2	Morphine	500	627 ± 320
RM-RTI-CFS-2407-COC-3	Cocaine	1500	2212 ± 672
RM-RTI-CFS-2407-AMPS-4	Amphetamine Methamphetamine Methylenedioxymethamphetamine (MDMA, Ecstasy)	750 (each analyte)	1352 ± 600 1507 ± 473 1294 ± 294

These reference materials will directly impact policy implementation for death investigation, workplace drug testing, crime scene analysis, and other uses for hair testing by providing validated external control materials at relevant concentrations. The reference materials will assist forensic laboratories to improve the defensibility of their analytical results by documenting their performance to accurately measure drugs of abuse in hair.

[1] RTI International is a trade name of Research Triangle Institute.

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EXECUTIVE SUMMARY

Statement of the Problem

Although hair testing has many forensic applications, including death investigations, workplace drug testing, drug-facilitated crimes, and violation of probation or parole, many unresolved issues have historically limited its widespread use. Among these issues is the lack of consistent matrix-matched control materials (Ropero-Miller, 2007a). As with all analytical testing, the reliability and quality of the test results largely depend upon the quality control (QC) and calibration of the analysis. Control materials can be used to validate a method, calibrate an analytical procedure, and continuously verify a laboratory's performance for a given protocol. Ideally, these reference materials should be produced and validated outside of the laboratory's control to remove bias and improve authenticity of results. Moreover, using a matrix-matched control, which is sufficiently similar to the matrix of the samples being tested, helps to detect the presence of matrix effects. Laboratory quality assurance (QA)/QC programs often incorporate matrix-matched controls to help monitor inter-laboratory variability and intra-laboratory precision.

There are two types of hair control materials available to forensic laboratories to assist them with their QA/QC programs: proficiency hair samples and hair reference materials (HRMs). Proficiency hair samples are provided to a laboratory for testing at a scheduled time as part of a program that evaluates laboratory performance among the system of laboratories. Three hair proficiency programs for drugs of abuse testing include the National Laboratory Certification Program (NLCP), the international Society of Hair Testing (SOHT), and the German Society of Toxicological and Forensic Chemistry (GFTCh). The NLCP uses drug-free hair soaked in drug analyte solutions and decontaminated to simulate drug concentrations within a drug-user's head hair, whereas SOHT use a large homogenized pool of drug-users' hair. The GFTCh uses both drug-user pooled hair samples and drug-fortified hair. Each type of proficiency sample has its strengths and weaknesses. Hair proficiency testing (PT) samples are provided in limited quantity and can only be used briefly by that laboratory to evaluate its sample preparation and analytical procedures. Although it is important for a laboratory to participate in a PT program for the matrices it routinely tests, it is not required, and these samples are generally not available to laboratories for troubleshooting, method validation, or other times that require a testing procedure evaluation. However, external HRMs can be purchased by a forensic laboratory for "at will" use for its QA/QC program.

Presently, reference materials for drugs of abuse testing are mostly available for blood and urine. The College of American Pathologists (CAP) provides urine drug screening and confirmation proficiency samples, serum drug confirmation, serum volatiles or alcohols, and whole-blood forensic toxicology confirmatory samples. CAP is not currently providing hair reference materials, but many commercial companies provide blood- and urine-based reference materials. The National Institute of Standards and Technology (NIST) is the only organization that provides Standard Reference Materials (SRMs) with certified concentrations of drugs of abuse in hair. NIST hair SRMs contain drug concentrations that are substantially higher than concentrations of interest for most forensic applications, thereby limiting the use of these HRMs. For example, the 0.99 ng/mg of delta-9-tetrahydrocannabinol (THC) in SRM 2380 is 4,000 times higher than established confirmatory cut-off concentrations currently used by forensic hair

testing laboratories for this drug analyte in hair. SRM 2380 contains THC, whereas its metabolite, 11-Nor-delta-9-THC-9-carboxylic acid (THCA), is the analyte of interest in most forensic applications in the United States. These samples are prepared using drug-free hair that is fortified (multiple days in a spiking solution) with the drug analytes. Gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-mass spectrometry (LC-MS) were used for analysis of the reference materials (Welch et al., 2003). Measurements for the reference material concentrations with both analytical techniques ranged from 4% to 16%.

Laboratory-certifying organizations, such as the American Board of Forensic Toxicology (ABFT) and the American Society of Crime Laboratory Directors Laboratory Accreditation Board (see ASCLD LAB's Web site at www.ascld-lab.org/legacy/indexlegacy.html), support QA measures, such as matrix-matched calibrators and controls, to help laboratories provide better overall analytical services (see ABFT's Web site at www.abft.org/Documents.asp; ASCLD LAB's Web site at www.ascld-lab.org/legacy/indexlegacy.html). The American Academy of Forensic Sciences (AAFS)/Society of Forensic Toxicology (SOFT) Forensic Toxicology Joint Guidelines state that reference materials should be certified by methods that have been approved by the scientific community for the analysis of the analyte of interest (AAFS/SOFT, 2006). Forensic laboratories cannot currently follow these guidelines because relevant reference materials do not exist for testing for controlled substances in hair. At present, hair testing laboratories are obliged to produce their own calibrators and controls for which they have limited or no external validation. Thus, the results of the most sensitive and advanced analytical techniques are only produce results that are as defensible and appropriate as the control and calibration materials that were used in their determination. Laboratories may put exceptional efforts into their analytical methods and procedures and still produce results that could potentially be challenged and defeated in court based on the testing controls and calibrators.

Hence, forensic laboratories have a current need for matrix-matched HRMs at concentrations relevant to the concentrations found in hair. Commercial availability of such reference materials will provide an external source of QC material to forensic laboratories. Use of these HRMs will improve the quality of laboratory results, producing results that are more applicable for policy decisions and more defensible in judicial proceedings.

Project Purpose and Goals

The primary purpose of this project was to develop and produce a reference material reflective of the current state of hair testing in terms of the type of sample and the concentrations commonly encountered and have been validated with multiple laboratories. This material consists of hair fortified with controlled substances for use by forensic laboratories as controls and/or calibrators to identify and measure drugs of abuse. Two goals of this project were to

- Validate these HRMs to determine analyte concentrations with an uncertainty measurement using established hair testing procedures performed by several hair testing laboratories
- Improve the resolution and sensitivity of forensic analytical tools, as well as to enhance the productivity and portability of methods used in forensic laboratories by commercially offering HRMs to laboratories with proceeds funding future productions.

In the preparation and validation of these materials, RTI International's (RTI's) Center for Forensic Sciences used research designs and methods that were applicable to current forensic guidelines and practices. The research methods and reference range calculation were selected to realistically represent inter-laboratory variability for hair testing by subjecting randomly selected HRM samples to multiple forensic laboratories using different analytical techniques for replicate analyses using multiple calibrators on different days. A test's reference range can be defined as the values used to estimate the probability of finding an observed value within a population of measurements. Common reference ranges are set for 95% (or 2 standard deviations [SD]) of the population to fall into, and our reference range is expanded by using variance (2*SQRT[VAR]). In doing so, the random intra- and inter-laboratory variabilities could be estimated and incorporated into a realistic reference range of analyte concentrations. The study's results may ultimately affect policy implementation for many forensic applications. Finally, RTI will use the experience and the reference material products of this study to establish a self-sustaining commercial product of HRMs for forensic laboratories to purchase and implement into their analytical protocols.

Research Design and Methods

This study was designed to investigate the development and large-scale production of drugs of abuse in HRMs. RTI's general protocol and production scheme was divided into two stages. For Stage 1, three single drug reference materials were developed and produced to contain THCA targeted at 0.3 pg/mg, morphine (MOR) at 500 pg/mg, and cocaine (COC) at 1500 pg/mg. For Stage 2, we developed and produced one multiple drug analyte HRM containing amphetamine (AMP), methamphetamine (MAMP), and methylenedioxymethamphetamine (MDMA, Ecstasy); each analyte was added at 750 pg/mg. RTI chose a simple study design protocol for the first large-scale production of reference materials to minimize potential interferents. Fortification of a hair matrix with a drug is complicated and difficult to evaluate or control if too many variables are present. For these reasons, RTI chose to begin with one analyte production schemes of one multiple drug analyte. These target concentrations represent the selected drug analytes at two to three times the confirmatory cut-off concentrations recommended by government agencies and organizations affiliated with hair testing.

Each of the production stages has been grouped and discussed as a section in this report based on each stage's experimental design, sample type and preparation, analysis procedures, findings, and conclusions:

- Stage 1: Reference Material Development and Production—Fortification of Hair with Single Drug Analyte in Solution
- Stage 2: Reference Material Development and Production—Fortification of Hair with Multiple Drug Analytes in Solution.

As the contractor for the NLCP under the U.S. Department of Health and Human Services (HHS), RTI developed and currently administers a pilot PT program for forensic hair testing laboratories (HHS, 2004; Ropero-Miller, 2005). Our ongoing work includes the design and preparation of the hair materials used as PT samples. This experience has afforded us an extensive working knowledge of efficient and effective methods for preparing HRMs, and we have gained knowledge about the relevant analytes and concentrations for hair testing, both those proposed in the federal regulations for workplace drug testing and those that are pertinent to

other forensic analyses. These hair proficiency samples are produced on a much smaller scale than the production scheme used in this study.

For each production process, head hair strands (14–20 g) were purchased, determined to be drug-free for analytes of interest, and washed to remove potential surface contaminants (e.g., dirt, shampoos, and other hair products) using deionized water. For all HRMs, we obtained from one individual a medium brown hair sample that was not chemically treated and determined to be in good physical condition. Scanning electron microscopy (SEM) was used to qualitatively determine the condition of the cuticle (e.g., intact, damaged, or devoid) of multiple strands of this initial hair source before and after drug fortification. Fortification solutions were prepared with the appropriate analytes, and the intact whole head hair strands (longer than 10 cm) were completely submerged in the solutions at room temperature for a period of time dependent on the analyte in the fortifying solution. Aliquots were periodically removed during the fortification process to test for analyte concentration. At the completion of the fortification process, the hair was decontaminated with successive isopropanol (for 15 minutes, air dried overnight) and phosphate buffer washes (three at 30 minutes, air dried overnight), manually homogenized and divided into 100- to 110-mg aliquots, and placed in glass vials for storage and distribution. The identification of the numerical sequence in which each glass vial was filled with a 100- to 110-mg aliquot (fill order) was documented for the validation process and certificate of analysis. Using the fill order, RTI created a stratified, random sampling for aliquot selection and submittal to reference laboratories for analysis. By performing random sampling testing and allowing forensic laboratories to further sample testing aliquots from each vial, inter-laboratory testing results demonstrated the combination of the variability of the HRM product and the laboratory methods of analysis. For instance, the highest individual laboratory % coefficient of variation (CV) measured for this study was 14.8% for COC. The reported % CV for between run imprecision reported by the reference laboratories was between 4.8% and 10%. If the laboratory variation is subtracted from the individual laboratory % CV, then the estimated variability of the HRM produced in this study was assumed to be approximately 4% to 9%. Positive and negative controls were added to the specimens before shipment to laboratories for QC purposes. Laboratories were instructed to perform duplicate analyses of multiple vials using their in-house standard operating procedures. At least two theoretically distinguishable analytical techniques and two reference laboratories were used to achieve a realistic reference range for analyte concentrations. Primary methods were targeted for 72 replicate analyses, and the secondary and tertiary methods were targeted for 15 to 20 replicate analyses. **Table ES-1** provides the overall analytical testing scheme and total samples analyzed for each reference material.

Table ES-1. Number and Analysis of Specimens in RTI Study

Stage of Study	Reference Material Description	Number of Specimens and Type of Analysis
Stage 1	THCA	98 LC-MS/MS, GC-GC/MS
Stage 1	MOR	102 LC-MS/MS, LC-MS, GC-MS
Stage 1	COC	98 LC-MS/MS, GC-MS

Stage of Study	Reference Material Description	Number of Specimens and Type of Analysis
Stage 2	AMP MAMP MDMA	89 LC-MS/MS, LC-MS, GC-MS
All Stages	QC materials (10%)	80
TOTAL		**447**

To estimate the intra- and inter-laboratory and analytical method variability, SAS statistical software (SAS Version 9.1.3 [XP PRO procedure]) calculated the uncertainty or variability as two times the square root of the calculated variance. Analyte concentration reference ranges were defined as the mean concentration plus or minus the uncertainty measurement (Average ± 2*SQRT[VAR]).

Results

A total of 447 aliquots of the four reference materials were analyzed. All analytes of interest were successfully incorporated into hair at or above targeted concentrations. **Table ES-2** summarizes the target and reference range concentrations for all control materials. Determined reference ranges for the THCA and MOR reference materials were comparable to the targeted fortification concentrations. The THCA reference range was 0.25 ± 0.17 pg/mg, and the individual laboratory average % CV was 23%. Intra-laboratory % CVs ranged from 12.0 to 12.7%. MOR's range was 627 ± 320 pg/mg, and its intra- and inter-laboratory % CVs were 9.3 to 10.1%, and 28.6% respectively. COC's range was 2,212 ± 672 pg/mg; much higher than its targeted concentration of 1500 pg/mg. COC's intra- and inter-laboratory % CVs were 8.7 to 13.8% and 23.8%, respectively. Finally, AMP analytes were similar, but all were twice the targeted concentration of 750 pg/mg. As an example, AMP's reference range was 1352 ± 600 pg/mg, and AMP's inter-laboratory % CV was 13.4%. Individual laboratory % CVs ranged from 7.8 to 12.6%. The higher-than-expected amphetamine concentration is not unusual based on RTI's experience with prior hair fortification studies, especially for amphetamines because they tend to be sensitive to variations in hair structure. To determine if there was an observable structural reason for the higher than expected AMP concentration, the hair sample was visualized with SEM. The hair appeared to have some cuticle damage. An additional study was performed to further investigate if the fortification solution volume or the donor hair contributed to the increased incorporation of amphetamine into the reference material. This study indicated that unspecified characteristics of the donor hair were responsible for the increased AMPs incorporated in the original fortification study.

Table ES-2. Reference Material Results

Reference Material	Analyte	Target Concentration (pg/mg)	Reference Range (pg/mg)	% CV (Individual Laboratory % CV Range)
RM-RTI-CFS-2407-THCA-1	THCA	0.30	0.25 ± 0.17	23% (12.0%, 12.7%)
RM-RTI-CFS-2407-MOR-2	MOR	500	627 ± 320	28.6% (9.3%, 9.5%, 10.1%)
RM-RTI-CFS-2407-COC-3	COC	1500	2212 ± 672	23.8% (8.7%, 9.2%, 13.8%)
RM-RTI-CFS-2407-AMPS-4	AMP MAMP MDMA	750 (each analyte)	1352 ± 600 1507 ± 473 1294 ± 294	13.4% (7.8%, 9.1%, 12.6%) 11.2% (7.0%, 8.9%, 11.5%) 7.4% (5.2%, 6.2%, 7.6%)

Note: CV = coefficient of variation

Statistical evaluations were determined on 98 THCA measurements, 102 MOR measurements, 98 COC measurements, and 89 AMP measurements. **Table ES-3** summarizes the mean concentration, standard deviation of the mean concentration, total variance (e.g., intra- and inter-laboratory) and uncertainty measurement of the concentration for each of the reference materials. Most of the uncertainty measurements were large based on the variability between reference laboratory results.

Eighty control samples were submitted to the reference laboratory as randomized samples for analysis, and the laboratory was unaware that it was receiving positive and negative controls. Controls were analyzed at an 18% frequency rate in comparison to other samples within the study. All controls were correctly identified as positive or negative.

Table ES-3. Statistical Results of Reference Materials

All Measurements	THCA	MOR	COC	AMP	MAMP	MDMA
Mean (pg/mg)	0.25	627	2212	1352	1507	1294
SD (pg/mg)	0.06	179	527	181	168	95
% CV	23.0	28.6	23.8	13.4	11.2	7.4
n	98	102	98	89	89	89
Total variance	0.0080	25537	111489	90051	55999	21607
Uncertainty (pg/mg)	0.1788	320	672	600	473	294
Reference range (pg/mg)	0.246 ± 0.17	627 ± 320	2212 ± 672	1352 ± 600	1507 ± 473	1294 ± 294

Discussion and Conclusions

Because hair is a solid matrix, this makes it one of the most difficult to determine concentrations of drugs of abuse and to produce QC samples to be used by forensic laboratories. Several efforts have attempted to provide hair PT samples and reference materials using fortified and known drug-user hair. Efforts resulting in tight distributions of reported results have necessitated consistent methods of sample preparation such as conducting all the testing within the same laboratory (Welch et al., 2003; Lee et al., 2008). In studies, for which a QC sample was evaluated by a system of multiple hair testing laboratories, the variability between laboratory testing methods resulted in substantial variation in QC sample results (Welch et al., 2003; Ropero-Miller, 2005 and 2007b; Ventura, 2008; Jurado, 2003). The purpose of this project was to produce a reference material that reflects the current status of hair testing with established

ranges that are pertinent to the inter-laboratory variation currently inherent in hair testing and to be at concentrations relevant to cutoffs generally used.

HRMs are subject to many limitations because it is not an ideal matrix for easily reproducible QC samples. Each limitation must be carefully evaluated against the objectives and preferred characteristics of the QC samples being manufactured. RTI's study design for this project was faced with five primary limitations and for each a chosen approach was necessary. **Table ES-4** summarizes inherent limitations of hair QC samples and RTI study design approach to minimize the variability of HRM results due to these limitations and possible future investigations to improve variability of the HRMs.

Table ES-4. Inherent Limitations, RTI's Approach, and Improvement Plan

Limitations	RTI Approach	Improvement Plan
Physical composition of hair QC samples (*intact hair strands versus pulverization*)	RTI chose to use slightly longer strands (~1–3 cm) as opposed to pulverizing the hair. Prior RTI experience suggests that pulverized hair is not consistent with typical hair samples received and prepared by laboratories. RTI selected hair strands longer than 10 cm to provide a large quantity of material to produce all HRMs from the same hair source and incorporated homogenization steps during and after the drug fortification process (long-term soaking in drug-spiked solution). Variability in our reference ranges were larger than those reported by Welch and colleagues (2003), but those materials had 3 to 4000 times the concentration of drug present.	To promote homogeneity of the sample, the size of the hair strands will be reduced. Pulverization can be performed by the laboratory based on preference. A small subset of pulverized hair aliquots can be investigated when determining future HRMs reference ranges.
Source of HRM *(stock hair consisting of drug-user pool versus external fortification of drug-free hair)*	RTI chose to use an external fortification of drug-free hair to better control the drugs present and their concentration to provide a useful HRM that is reflective of the current state of testing. Drug user pools for HRMs represent endogenous routes of drug incorporation, but these deposition mechanisms are highly variable and difficult to predict drug concentrations. Results reported by HAIRVEQ, SOHT, and NLCP for PT programs indicate more variability with drug-user PT samples. Reference materials currently available for hair are prepared at high concentrations that cannot help a laboratory evaluate their calibration curves at lower concentrations.	No improvement plan

Limitations	RTI Approach	Improvement Plan
Determining homogeneity of the HRM	The ability to produce a homogenous reference material that will allow consistent results among laboratories is extremely difficult. RTI used several processes to ensure homogeneity between aliquots. First, HRMs were produced using hair from the same individual. Hair was homogenized by manual mixing, cutting it into smaller segments, and allowing the hair to flow freely in solution during the fortification process. Second, multiple aliquots taken from randomly selected bottles during the fill order process were sent to multiple laboratories to assess the homogeneity of the samples. Lack of a correlation between fill order and determined concentrations supports the aliquots being homogenous and an equal probability of any individual vial containing different portions of the hair shaft. Laboratories tested both within and between aliquots and results demonstrated some variability (% CV range 5.2–13.8, % CV average 9.6). Laboratories further selected random aliquots from each HRM bottle for analysis to provide a mixture of locations on the hair shaft as sampling between aliquots.	Implement methods to improve homogeneity, including the use of smaller segments of hair strands, adapting more rigorous homogeneity methods prior to preparing individual aliquots (e.g., prolonged manual or mechanical mixing), and investigating a subset of pulverized hair aliquots when establishing HRM reference ranges.
High variability of HRMs	The purpose of manufacturing this material was to attempt to produce a material that is representative of the current state of hair testing and be applicable to this situation. Variation between laboratories is substantial, particularly for the THCA and MOR materials. The material and laboratory methodology contribute to variability. Because it is more difficult to control the laboratory methodology, variability in the HRM material will need to be investigated.	Perform homogeneity studies.
Lack of metabolites in the HRMs *(inclusion of parent compounds without all metabolites)*	RTI chose to include the list of compounds in the study based on information from laboratories that currently provide hair testing services. For these first large-scale productions, RTI chose simpler fortification schemes to minimize potential interferents.	RTI will continue to survey the laboratories to determine what drugs are of use to the hair testing community as HRMs.

The high variability or uncertainty in the concentration range but reasonable individual laboratory % CV <15% (represents a combined variability of reference material variability and intra-laboratory variability), suggests that the reference laboratories performed well within their own system of protocols, but not as well as a system of laboratories analyzing the same samples by different protocols. When the performance of a system of laboratories is evaluated, many organizations report similar results (Welch et al., 2003; Ropero-Miller, 2005 and 2007b; Ventura, 2008; Jurado, 2003). Given the non-homogeneity of the hair matrix and demonstrated variability of drug hair testing, the reference ranges for these HRMs were calculated to represent all potential variation and thus, realistic for drug testing laboratories to use to evaluate the laboratories' performance.

This research has provided external HRMs with four drugs of abuse classes near the confirmatory cut-off/threshold concentrations currently used by hair testing laboratories. Approximately 500 vials of these four HRM products are available for distribution to forensic laboratories. RTI has received approval from the National Institute of Justice to sell these materials, and the proceeds will go toward funding future productions of materials. RTI intends

to produce more multiple drug analyte HRMs to better represent confirmatory methods of analyses (i.e., parent drug and metabolites) and/or improved applicability for drug screening methods of testing. Forensic laboratories can now benefit from relevant HRMs that have been validated, quality controlled, quality assured, determined to have realistic reference ranges with estimated uncertainty, and implemented for forensic use. These reference materials will add a layer of forensic reliability for the laboratory's data and the laboratory's clients, the court, and the subjects being tested.

Regardless of the forensic application, the testing results are only as good as the control and calibration upon which they are based. Having access to quality matrix-matched reference materials refereed in independent laboratories and independent from PT materials will allow laboratories to produce quality results through more routine verification of their procedures.

This work will directly affect the use of hair testing in drug-related criminal cases, workplace drug testing, and other legal arenas, such as child custody and abuse, parole, and probation hearings. HRMs can help regulate laboratory performance and improve the reliability of hair testing results to better withstand emerging and potentially harsher legal requirements and the laboratory's ability to assure quality and demonstrate forensically defensible analytical performance.

1. INTRODUCTION

1.1 Background

For more than 30 years, hair has been used as a biological matrix to detect controlled substances and to indicate drug use. Although matrices, such as blood, oral fluids, and urine, document an individual's drug exposure for a period ranging from minutes to days, hair can extend the detection period from months to years, depending on the hair sampled and the collection process. Some advantages of hair testing include its noninvasive and simple collection process, the stability of drug incorporated into its matrix, and the low probability of adulteration or substitution.

Although hair testing has many applications, including death investigations, workplace drug testing, drug-facilitated crimes, and violation of probation or parole, many of the issues limiting hair testing's widespread use have not been eliminated. These issues include the absence of standardized techniques between laboratories, consistent results within and between laboratories (intra- and inter-laboratory variability), consistent control and proficiency testing (PT) materials, whether there is a laboratory certification program, easily identifiable drug analytes that discriminate between environmental contamination and drug use, and a potential bias of drug incorporation into hair (i.e., color or ethnic differences) (Ropero-Miller, 2007a). This research investigated the production of hair reference materials (HRMs) for use in forensic hair testing laboratories. This study directly addresses the need for externally produced HRMs, and the availability of these HRMs can assist laboratories with evaluating their performance and reducing their variability in results by implementing improved methods.

1.2 Statement of the Problem

Drug testing programs are used worldwide to help detect drug abuse, monitor drug prevalence, and act as a deterrent of use. Drug abuse impacts society through decreased job productivity and earnings and increased crime, drug-related fatalities, health costs, prevention costs, and social welfare recipients. In fact, the number of current illicit drug users in the United States was approximately 20.4 million, or 8.3% of our nation's population, according to the 2006 National Survey on Drug Use and Health (NSDUH, 2006), which was conducted by the Substance Abuse and Mental Health Services Administration (SAMHSA).

Hair testing was introduced for testing drugs of abuse primarily because it offered a longer window of detection (months to years) compared to conventional matrices such as blood and urine (minutes to days). Among these issues is the lack of consistent matrix-matched control materials (Ropero-Miller, 2007a).

As with all analytical testing, the reliability and quality of the test results largely depend on the quality assurance/quality control (QA/QC) and calibration upon which the results are based. Control materials can be used to validate methods, calibrate an analytical procedure, and continuously verify a laboratory's performance for a given protocol. Ideally, these reference materials should be produced and validated outside of the laboratory's control to remove bias and improve authenticity of results. Moreover, the use of a matrix-matched control, which is sufficiently similar to the representative matrix of the samples being tested, helps to detect the

presence of matrix effects. Matrix-matched controls can help monitor inter-laboratory variability and intra-laboratory precision.

There are two types of hair control materials available to forensic laboratories to assist them with their QA/QC programs: PT hair samples and HRMs. Proficiency hair samples are provided to a laboratory for testing at a scheduled time as part of a program that evaluates laboratory performance among the system of laboratories. Three hair proficiency programs for drugs of abuse testing include the National Laboratory Certification Program (NLCP), the international Society of Hair Testing (SOHT), and the German Society of Toxicological and Forensic Chemistry (GFTCh). The NLCP uses drug-free hair soaked in drug analyte solutions and decontaminated to simulate drug concentrations within a drug-user's head hair, whereas SOHT use a large homogenized pool of drug-users' hair. The GFTCh uses both drug-user pooled hair samples and drug-fortified hair. Each type of proficiency sample has its strengths and weaknesses. Hair proficiency testing (PT) samples are provided in limited quantity and can only be used briefly by that laboratory to evaluate its sample preparation and analytical procedures. Although it is important for a laboratory to participate in a PT program for the matrices it routinely tests, it is not required, and these samples are generally not available to laboratories for troubleshooting, method validation, or other times that require a testing procedure evaluation. However, external HRMs can be purchased by a forensic laboratory for "at will" use for its QA/QC program. However, the fortification of drugs into hair allows for relevant target drug concentrations to be achieved and a larger product batch can be made from the same or similar drug-free hair. An authentic control material made from a pool of many drug users is difficult to homogenize, and the drug concentration in the hair cannot be controlled, but these control samples contain drug analytes that have been incorporated in vivo. PT hair samples are provided in limited quantity and can only be used briefly by that laboratory to evaluate its sample preparation and analytical procedures. Although it is important for a laboratory to participate in a PT program for the matrices it routinely tests, it is not required, and these samples are generally not available for troubleshooting, method validation, or other times that require a testing procedure evaluation. However, external HRMs can be purchased by a forensic laboratory for "at will" use for its QA/QC program.

There is a need for matrix-matched HRMs at relevant concentrations in forensic laboratories. Commercial availability of such HRMs will provide an external source of QC to forensic laboratories. Using these HRMs will improve the quality of laboratory results and will standardize quantitation between laboratories, thereby producing results that are more applicable to policy decisions and more defensible in judicial proceedings.

1.3 Review of the Literature

1.3.1 Drugs in Hair

Forensic laboratories use hair as a complementary and alternative matrix to blood and urine in testing for controlled substances. Hair attributes include its stability and durability, its ability to indicate long-term drug use (weeks to years depending on hair length), and its ease of collection and storage. Forensic applications for hair testing include death investigations, workplace drug testing, and crime scene analysis, and the results of hair tests have been used as evidence in civil, criminal, and military courts of law.

Hair is a unique matrix that is an invaluable forensic tool in cases of drug-facilitated sexual assault, child custody, theft, and drug-suspected fatalities (Ropero-Miller et al., 1997; Selavka et al., 1995; Cheze et al., 2004 and 2005; Ropero-Miller, 2007b; Kintz, 2007). For many years, the private sector has used hair testing in workplace drug testing programs, and the U.S. Department of Health and Human Services (HHS) proposed hair as an acceptable alternative matrix to urine for pre-employment, random, return-to-duty, and follow-up tests in federal workplace drug-testing programs (HHS, 2004).

After extensive research, several professional forensic organizations and governmental agencies have published initial, confirmatory, and threshold cut-off concentrations (pg/mg) for drugs of abuse in hair. Both parent drug and metabolites, which are the predominant analytes in the hair matrix, can be detected in hair. Factors such as the chemical nature of the drug analyte; pharmacokinetics, including metabolism; and drug analyte's stability play a role in the parent and metabolite concentrations of a drug. The study design of this research is limited to confirmatory concentrations for opiates, COC, cannabinoids, and AMPs. Not all analytes that can be detected in hair were included in the study design of these HRMs to minimize variables during the first productions. **Table 1-1** summarizes these concentrations and the country of origin for each agency or organization.

Table 1-1. Published Confirmatory and Lower Limit Cut-off Concentrations (pg/mg) for Drugs of Abuse in Hair

Agency or Organization	Testing Level	THCA	MOR	COC	AMPs
Substance Abuse and Mental Health Services Administration (United States of America proposed 2004)	Confirmatory	≥0.05	≥200	≥500	≥300
Society of Hair Testing	Confirmatory	≥0.2	≥200	≥500	≥200
Gesellschaft fur Forensische und Toxikologische Chemie (German Society of Toxicological and Forensic Chemistry)	Confirmatory	≥50	≥200	≥500	≥200
Societe Francaise de Toxicologie Analytique (French Society of Analytical Toxicology)	Lower limit	None (THC, CBD only)	200	200	200

Source: Table reprinted from Ropero-Miller, 2007a.

Note: AMP = amphetamine; CBD = cannabidiol; COC = cocaine; MOR = morphine; THC = tetrahydrocannabinol; THCA = 11-Nor-delta-9-THC-9-carboxylic acid

1.3.2 Reference Materials in Hair Drug Testing

Cone's (2001) global assessment of legal, workplace, and treatment testing with alternate matrices, such as hair, called for programs to consider a multiplicity of factors for establishing testing guidelines, including the standardization of processes within and across geographic boundaries, checks and balances, provisions for change, and evolution toward universal standards, among others. The implementation of external, matrix-matched reference materials is one way a forensic laboratory could simultaneously address many of the factors that Cone challenges the hair drug testing community to face.

Presently, HRMs for drugs of abuse testing are mostly available for blood and urine. The College of American Pathologists (CAP) provides urine drug screening and confirmation of PT samples, serum drugs, serum volatiles or alcohols, and whole blood forensic toxicology samples. CAP offers QC samples, referred to as toxicology samples (urine and serum), forensic sciences surveys (whole blood), as well as DNA and pathology PT samples (whole blood). CAP is not currently providing hair materials. There are also other commercial companies that provide blood- and urine-based HRMs.

Although hair testing for drugs of abuse has demonstrated potential in the fields of forensics and criminology, its use has been limited because of issues that could impact the defensibility of results in court. These issues include potential hair color bias, external contamination, high individual variability (i.e., factors such as age, gender, hygiene, drug biotransformation and excretion, and hair growth rate), and lack of appropriate control and calibration materials (see ABFT's Web site at www.abft.org/Documents.asp; ASCLD LAB's Web site at www.ascld-lab.org/legacy/indexlegacy.html). As with all analytical testing, the reliability and quality of the test results largely depend on the QC and calibration upon which the results are based. Laboratory-certifying organizations, such as the American Board of Forensic Toxicology (ABFT) and the American Society of Crime Laboratory Directors (ASCLD), support QA measures, such as matrix-matched calibrators and controls, to help laboratories provide better overall service to the death investigation and criminal justice system (AAFS/SOFT, 2006). In 2006, the Joint American Academy of Forensic Sciences (AAFS)/Society of Forensic Toxicology (SOFT) Forensic Toxicology Guidelines Committee discussed both Certified Reference Materials (CRMs) and controls. They defined a CRM as "a reference material, one or more of whose properties are certified by a valid procedure or accompanied by or traceable to a certificate or other documentation which is issued by a certifying body." This definition was taken from an early publication of the Association of Official Analytical Chemists' Official Methods of Analysis published in 1984. The AAFS/SOFT guidelines state that reference materials used to prepare calibrators and controls should be matrix-matched, where possible, to the specimens being analyzed. The guidelines further state that the reference materials should be certified by methods that have been approved by the scientific community to analyze for the analyte of interest (SOHT, 2004). Forensic laboratories cannot currently follow these guidelines because an appropriate HRM does not exist for testing for controlled substances in hair. At present, hair testing laboratories are obliged to produce their own calibrators and controls for which they have limited or no external validation. Results produced by even the most sensitive and advanced analytical techniques are only as defensible and appropriate as the control and calibration materials that are used for the test. Laboratories may put exceptional efforts into their analytical methods and procedures and still produce results that are potentially defeated in court challenges to the control and calibration.

The international organizations, SOHT and GTFCh, and the U.S. NLCP have maintained a long-term hair PT program, but these control materials are only offered to a few approved hair testing laboratories. Generally, these hair PT samples are offered, at the most, a few times a year and only a limited quantity of samples are shipped to each laboratory for analysis and reporting to the respective program. The laboratories cannot use these PT samples for repeated performance evaluations at their discretion. Although PT control materials can assist a laboratory in evaluating their performance generally against other participating laboratories, they cannot help with method validations, troubleshooting, and routine documentation of performance for its

QA/QC program. SOHT and GTFCh use reference materials prepared from homogenized drug-user pools (authentic drug use), and GTFCh and the NLCP use spiked drug-free hair.

The National Institute of Standards and Technology (NIST) is the only organization that provides Standard Reference Materials (SRMs) with certified concentrations of drugs of abuse in hair. NIST's mission promotes innovation and industrial competitiveness in the United States by advancing measurement science, standards, and technology. As part of its mission, NIST began investigating HRMs for drugs of abuse in hair in 1993 (Welch et al., 1993). Ten years later, NIST began offering two HRMs: Standard Reference Material (SRM) 2379, which contains cocaine (COC), benzoylecgonine (BE), cocaethylene (CE), phencyclidine (PCP), amphetamine (AMP), and methamphetamine (MAMP); and SRM 2380, which contains morphine (MOR), codeine (COD), 6-acetylmorphine (6-AM), and THC, the primary active constituent of marijuana. These samples are prepared using drug-free hair that is fortified (multiple days in a spiking solution) with the drug analytes. To achieve lower concentrations following their fortification design, NIST "back-extracted" the drug out of the hair matrix and continually monitored the analyte concentrations. Gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-mass spectrometry (LC-MS) were used for analysis of the HRMs. Both methods used 0.1 M HCl for extracting all the analytes from the hair, except for THC, which was extracted with 1 M NaOH. Measurements for the HRM concentrations with both analytical techniques ranged from 4% to 16% (Welch et al., 2003). These SRMs are not currently available but can be back-ordered according to NIST's Web site; however, these SRMs (i.e., SRM 2379 and SRM 2380) are at concentrations that are not applicable to most forensic purposes, limiting the use of these HRMs. For example, the amount of THC present in SRM 2380 is 4000 times higher than established confirmatory cut-off concentrations currently used by forensic hair testing laboratories for this drug analyte. Furthermore, the marijuana analyte included in the NIST reference material (SRM 2380) is parent THC. Although this is the most psychoactive constituent of marijuana (*Cannabis sativa*), 11-Nor-delta-9-THC-9-carboxylic acid (THCA) is the marijuana analyte commonly used in hair testing. Identification of THCA in hair documents marijuana use more effectively because the parent THC may be present as a result of environmental exposure (Welch et al., 2003; Baselt, 2004; Kim et al., 2005; Uhl and Sachs, 2004; Uhl, 1997; and NIST's Web site at http://ts.nist.gov/measurementservices/referencematerials/index.cfm).

Similarly, other drugs that are commonly abused are not included in NIST's SRMs. For example, the AMP analogues, such as methylene dioxyamphetamine (MDA), methylene dioxymethamphetamine (MDMA), and methylene dioxyethylamphetamine (MDEA), are not in the NIST SRMs, but are analytes in the Substance Abuse and Mental Health Services Administration's (SAMHSA's) proposed guidelines. **Tables 1-2** and **1-3** compare the drug of abuse analytes and their respective concentrations in the NIST SRMs to testing cutoffs proposed by HHS for federal workplace drug testing programs (SAMHSA, 2004). In most instances, the drug analytes in NIST's SRMs are at least 15 to 80 times the proposed confirmatory test cut-off concentrations. Ideally, QC standard concentrations should either be at low and high concentrations within the linear range of an assay or toward the median concentration of the calibration curve. These criteria are not fully met with NIST SRMs for many forensic laboratory analytical methods.

Table 1-2. Certified Concentrations of Drug Analytes in NIST SRM 2379 Compared to Confirmatory Test Cut-off Concentrations for Hair in the HHS Proposed Revisions to Mandatory Guidelines for Federal Workplace Drug Testing Programs

Analyte	NIST SRM 2379 (ng/mg)	Proposed Confirmatory Test Cutoffs (ng/mg)
AMP	6.00 ± 0.32	0.3
BE	4.01 ± 0.31	0.05
CE	2.67 ± 0.24	0.05
COC	7.45 ± 0.40	0.5
MAMP	5.20 ± 0.27	0.3
PCP	6.24 ± 0.42	0.3
MDA	Not applicable	0.3
MDMA	Not applicable	0.3
MDEA	Not applicable	0.3

Note: AMP = amphetamine; BE = benzoylecgonine; CE = cocaethylene; COC = cocaine; MAMP = methamphetamine; MDA = methylene dioxyamphetamine; MDEA = methylene dioxyethylamphetamine; MDMA = methylene dioxymethamphetamine; NIST = National Institute of Standards and Technology; ng/mg = nanogram per milligram; PCP = phencyclidine; SRM = standard reference material

Table 1-3. Certified Concentrations of Drug Analytes in NIST SRM 2380 Compared to Confirmatory Test Cutoffs for Hair in the HHS Proposed Revisions to Mandatory Guidelines for Federal Workplace Drug Testing Programs

Analyte	NIST SRM 2380 (ng/mg)	Proposed Confirmatory Test Cutoffs (ng/mg)
COD	9.82 ± 0.70	0.2
MOR	10.54 ± 0.68	0.2
6-acetylmorphine	2.71 ± 0.30	0.2
THC	0.99 ± 0.10	Not applicable
THCA	Not applicable	0.00005

Note: COD = codeine; MOR = morphine; NIST = National Institute of Standards and Technology; ng/mg = nanogram per milligram; SRM = standard reference material; THC = delta-9-tetrahydrocannabinol; THCA = 11-Nor-delta-9-THC-9-carboxylic acid

Lee and colleagues (2008) recently developed an HRM using authentic hair samples for the determination of MAMP and AMP at 7,640 ± 1,240 pg/mg and 540 ± 70 pg/mg, respectively. GC-MS analysis and two extraction procedures were used to evaluate hair specimen homogeneity and reference ranges with uncertainty values. These researchers chose a pool of drug abusers' hair as their HRM source, citing SOHT's recommendation (2004) that "For external QC, the laboratory should enroll in a PT program, where authentic standard hair specimens are sent for testing." Again, the concentration for MAMP was nearly 20 times the confirmatory concentration in the proposed mandatory guidelines and does not challenge the calibration at its lower limits (HHS, 2004).

1.4 Rationale for the Research (Statement of Hypothesis)

As the contractor for the NLCP under HHS, RTI developed and administered a pilot PT program for forensic hair testing laboratories (HHS, 2004; Ropero-Miller, 2005). Our ongoing work on

this contract included the design and preparation of the hair materials used as PT samples. This experience has afforded us an extensive working knowledge of efficient and effective methods for preparing HRMs. We have also gained valuable knowledge of the relevant analytes and concentrations for hair testing proposed in the federal regulations for workplace drug testing.

Although PT materials are a vital component of the NLCP, laboratories outside of this program also need HRMs for their analytical methods. These HRMs must be developed separately from PT materials so that appropriate checks and balances of a laboratory's analytical performance can be verified. Thus, although we have worked to develop PT materials for hair, completely separate HRMs also need to be developed for laboratories to use in their internal QC programs if they are to produce quality, defensible results. Forensic laboratories can benefit from appropriate HRMs that have been validated, quality controlled, quality assured, and implemented for forensic use, thereby extending these characteristics into the data they generate.

The purpose of this study was to investigate the development and production of external HRMs for drugs of abuse testing using RTI-developed drug fortification processes. RTI sought to validate HRMs outside of the laboratory's control to remove bias and improve authenticity of results. Common reference ranges are set for 95% (or 2 standard deviations [SD]) of the population to fall into, and our reference range is expanded by using variance (2*SQRT[VAR]). Following HRM production, a validation scheme in which representative, random aliquots of the final product were sent to multiple reference laboratories for analysis by at least two analytical techniques (e.g., GC-MS, GC-GC/MS, liquid chromatography-mass spectrometry [LC-MS], liquid chromatography-tandem mass spectrometry [LC-MS/MS]) to determine a realistic reference range for drug analyte concentrations fortified into hair. These reference materials will assist forensic laboratories to improve the defensibility of their analytical results by documenting their ability to accurately measure drugs of abuse in hair.

2. RESEARCH DESIGN AND METHODS

The research design for this project includes the following four primary steps:

- Step 1. Determination of analytes and target concentrations of HRMs
- Step 2. Production of HRMs
- Step 3. Analysis and validation of HRMs
- Step 4. Distribution of HRMs to forensic laboratories.

Steps 1 and 4 will be summarized in this section. Steps 2 and 3 will be combined and discussed in further detail as subsections (*Stage 1: Fortification of Hair with Single Drug Class or Analyte in Solution* and *Stage 2: Fortification of Hair with Multiple Drug Analytes in Solution*) to this section because these subsections have methods, results, and conclusions that require additional discussion.

Determination of analytes and target concentrations of reference materials: In the initial development stages of this project, RTI employed a laboratory survey, internal experience with the production of NLCP PT samples, and current hair testing literature to decide which drug analytes would be the most feasible HRMs for this research to produce. Although RTI's knowledge base for PT sample production includes major drugs of abuse (e.g., opiates, COC, AMPs, marijuana, and PCP), a survey of these laboratories provided information to help determine the final composition of the HRMs. Approximately 10 laboratories, including prospective reference laboratories, that perform hair testing were contacted by telephone received a follow-up e-mail. The survey determined each laboratory's current protocol and how the availability of an HRM might impact the laboratory (e.g., would the laboratory be interested in purchasing HRMs). In addition, specific forensic applications (drug-facilitated sexual assault, workplace drug testing) were discussed to determine which drugs were more prevalent, which had unique testing needs, and which had other characteristics that warranted inclusion of one drug analyte over another in an HRM. This information was considered in context with the practicality of having a specified drug in the HRM. The survey indicated that all analytes RTI has used in hair PT sample production were listed by laboratories as analytes for which they currently perform hair testing. These analytes include opiates, AMPs, COC analytes, PCP, and THCA. A final choice of drug analytes (i.e., THCA, COC, MOR, AMP, MAMP, and MDMA) was decided based on the number of laboratories with validated methods in place for analysis, familiarity, and RTI's current production experience with these drug analytes.

One example of a drug class of interest that was not ultimately chosen for several reasons was benzodiazepines. RTI did not have direct experience with fortifying hair with benzodiazepines, and including them in the study would have increased method development time and cost. Furthermore, none of the prospective reference laboratories routinely analyzed for benzodiazepines, which meant that analytical method development would increase costs and take as much as 6 to 12 months to validate. Finally, a literature review supported that the demand for a benzodiazepine HRM would be less than the demand for other analytes (e.g., AMPs, COC, MOR, and THCA). A PubMed Medline search found only 28 references for benzodiazepine concentrations in hair in humans. Of these 28 references, 23 were from European laboratories, with one European group responsible for 10 of these manuscripts. Furthermore, even the most cited benzodiazepine analytes determined in hair, diazepam (nine manuscript citations), is cited 9

to 25 times less than the analytes RTI ultimately selected (THCA and COC, respectively) for inclusion in this project.

Production of HRMs: Hair-drug fortification processes are used to prepare QC samples (e.g., matrix-matched calibrator or PT samples). In the fortification model, hair is placed in a solution that contains drug analyte(s) of interest, which leads to the incorporation of some of the drug into the hair. RTI's production process introduced drug-free human head hair into a fortification solution that contains target drug concentrations dependent on the analyte and the concentration of that analyte in the fortifying solution. After the hair was fortified with drug analytes to achieve a target concentration of at least twice the proposed confirmatory cut-off concentrations, an effective decontamination protocol previously examined by RTI and others was used to remove weakly associated compounds. These hair samples were then submitted in a blinded manner to a reference laboratory for analysis of drug analyte concentrations. HRMs can be prepared from powdered (i.e., pulverized) hair or remain as long or short intact strands (millimeter to centimeter segments) and the source can be a drug user, an authentic hair pool, or drug-free hair fortified (long-term spiking) with drug analytes. Each of these choices has its limitations. Powdered hair is much easier to produce; however, it does not allow assessment of all procedures in a hair testing laboratory and is inappropriate for laboratories that extract the drug from unpulverized hair. Intact hair strands will incorporate drugs differently from one location to another based on the health of the cuticle (e.g., damaged, intact, or devoid), and if it is taken from an authentic drug user, the frequency and dosing amount pattern also contribute to variations. Drug-user hair pools demonstrate actual drug incorporation into the hair matrix (in vivo source), but the drug analyte concentration is difficult to control. RTI chose to use intact, whole head hair samples because this type of HRM 1) allows the presence of a drug analyte to be controlled during incorporation into the hair and in the final concentration, 2) produces larger batches of HRMs, and 3) provides reproducible results as judged by the PT samples that RTI has previously produced for the NLCP.

Analysis and validation of HRMs: Analytical work was conducted by three independent forensic laboratories: Psychemedics Corp. of Culver City, CA; Immunalysis Corp. of Pomona, CA; and the University of Utah's Center for Human Toxicology (CHT) of Salt Lake City, UT. Analysis was defined as primary or secondary and tertiary based on the number of replicate analyses a laboratory was requested to complete. Primary analysis was targeted for the separate analysis of 72 replicates by one laboratory using analytical techniques chosen by the laboratory. Because RTI performed decontamination washes before aliquoting and shipping hair samples, reference laboratories were instructed to analyze samples using standard operating procedures for specimen preparation, excluding decontamination, and was performed using multiple calibrators with analyses conducted on multiple days (**Appendix A-2**, HRMs Overview and Instructions for Laboratory Use, informs laboratories not to decontaminate prior to performing extraction procedures). Fewer analyses (15 to 20 replicates) were performed with secondary and tertiary analyses conducted using an analytical technique theoretically distinct from the primary method and those used by other participating laboratories. All technologies provided ample sensitivity for these studies. In all, 447 samples were analyzed to determine the drug analyte concentration for the HRMs produced in this study.

Analytical results were statistically evaluated to determine sample homogeneity, accuracy, precision, and uncertainties for each analyte. To estimate the intra- and inter-laboratory, the

analytical method, and the sample variabilities, SAS statistical software (SAS Version 9.1.3 [XP PRO procedure]) calculated the uncertainty or variability as two times the square root of the calculated variance. Analyte concentration reference ranges were defined as the mean concentration plus or minus the uncertainty measurement (Average ± 2*SQRT[VAR]).

Distribution of HRMs to forensic laboratories: The final step in this project's research design involved the distribution of the HRMs to forensic laboratories for implementation into their QA/QC and analytical process. Identifying the numerical sequence in which each glass vial was filled with a 100- to 110-mg aliquot (fill order; +10% above weight to allow for sample loss to container and still provide 5 to 10 aliquots for most laboratory analysis) was documented for the validation process and certificate of analysis. Samples were randomly selected based on the fill order and submitted for reference testing. The remaining aliquots (100-110 mg) are available for purchase and shipment to interested forensic laboratories. A Certificate of Analysis (CoA) was prepared for each individual aliquot. The CoA (**Appendix A-1**) includes product information and characterization, QC and analysis data, signature of responsible person, and the date of the CoA submission, which was determined by the last date of analysis of the reference material during the verification testing. In addition, laboratories will be provided with an HRMs Overview and Instructions for Laboratory Use sheet (Appendix A-2), which provides the laboratory with basic instructions for using the HRMs and summarizes the purpose, production, analysis, and validation procedures. Nearly 500 vials (i.e., 145 AMPs, 149 COC, 91 MOR, and 92 THCA) of four HRM products are available for distribution to forensic laboratories. RTI has received approval from NIJ to sell these materials, with the proceeds going toward funding future production of materials. After careful evaluation of the production and shipping costs and future development costs, RTI established a purchase prices for each aliquot as shown in **Table 2-1**. These prices are comparable to quality control samples and reference materials currently offered by other organizations (e.g., CAP, NIST).

Table 2-1. Reference Material Pricing

Reference Material	Analyte	Price*/Quantity
RM-RTI-CFS-2407-THCA-1	THCA	$615/100–110 mg
RM-RTI-CFS-2407-MOR-2	MOR	$615/100–110 mg
RM-RTI-CFS-2407-COC-3	COC	$615/100–110 mg
RM-RTI-CFS-2407-AMPS-4	AMP MAMP MDMA	$725/100–110 mg

- All prices are listed in U.S. currency and do not include shipping and handling.

Note: AMP = amphetamine; COC = cocaine; MAMP = methamphetamine; MDMA = methylene dioxymethamphetamine; MOR = morphine; THCA = 11-Nor-delta-9-THC-9-carboxylic acid

RTI created a pricing and marketing flyer (**Appendix A-3**), which we began distributing at the annual AAFS meeting in Washington, DC, in February 2008. RTI continued to publicize these HRMs at SOHT, The International Association of Forensic Toxicologists (TIAFT), and SOFT meetings scheduled later in 2008. RTI also performed an extensive review of the hair testing literature to extract contact information for researchers and scientists worldwide who published manuscripts investigating drugs of abuse in hair. RTI used this contact information to disseminate information on the availability of these HRMs to individuals at nearly 400

potentially interested forensic laboratories. We also have information about the availability of HRMs on our Web site at www.rti.org.

The following research and production stages have been grouped and discussed as subsections in this report based on each stage's experimental design, sample type and preparation, analysis procedures, findings, and conclusions:

- Stage 1: Fortification of Hair with Single Drug Class or Analyte in Solution
- Stage 2: Fortification of Hair with Multiple Drug Analytes in Solution.

During Stage 1, hair was fortified with one specific drug class per production session to minimize the potential for interference. Although relevant metabolites are important for interpretation, their added complexity to the production scheme was not warranted. (RTI initially wanted to determine if we could reproducibly manufacture a large-scale reference material product of a much lower concentration than had previously been reported.) A total of three single-analyte reference materials were produced and validated. During Stage 2, RTI fortified one hair sample with one drug class (i.e., AMPs). This HRM investigated a multiple drug HRM similar to those prepared by NIST. An HRM for multiple drug analytes has a potential benefit for hair testing laboratories to improve throughput and be more cost effective. In addition, a multiple drug analyte is more practical for initial testing or screening performed by forensic laboratories where multiple drug classes are investigated simultaneously by immunoassays and other broadly based techniques.

2.1 Stage 1: Fortification of Hair with Single Drug Class or Analyte in Solution

2.1.1 Experimental Design

Stage 1 of this research project focused on developing and producing three single-analyte HRMs to include THCA, MOR, and COC. Drug-free hair that had not been chemically treated (e.g., straightened, permanent waved, and/or colored) was collected, as well as demographic information on gender, age, and ethnicity to submit to RTI with hair specimens. RTI recorded the information provided in such a manner that individuals cannot be identified directly or through identifiers linked to them.

2.1.2 Materials

Hair: Non-chemically treated hair was collected and purchased from a commercial source (in Raleigh-Durham, NC) from consenting volunteers. Head hair was shaved or cut as close to the root as possible (no ponytail cuts) using methanol-cleaned shavers or barber shears. Hair was collected in a sealed plastic bag at room temperature, and demographic information and visual characterization of the hair was documented. The donor hair was self-reported as non-chemically treated. The hair was determined to be healthy (e.g., not visually damaged, and cuticle intact) and straight. RTI collected information and recorded it in such a manner that individuals cannot be identified, directly or through identifiers linked to them. All drug-free hair specimens were analyzed for analytes of interest before inclusion in this study. All drug-free hair used as starting material was visually long and brown (Schwarzkopf 4.0, medium brown), measuring longer than 10 centimeters and obtained from one individual. To promote ease of handling, the hair remained as long, intact strands during the entire fortification process. During this process, large containers were used, and hair was allowed to move around freely in the drug-spiked solution to promote

homogeneity of the sample. Hair was homogenized by manual mixing and cutting it into smaller segments prior to scintillation vial packaging. With the sampling design, 10% to 20% of the total final product was no longer available for purchase during analysis to determine the concentration reference ranges.

11-Nor-delta-9-THC-9-carboxylic acid (THCA): Ampules of THCA in ethanol were purchased from RTI (Research Triangle Park, NC). The THCA was analyzed by RTI and found to be >99% pure by high-performance liquid chromatography (HPLC). The final spiking solution was 24.3 mg/L of THCA.

Morphine sulfate salt pentahydrate: Ampules were purchased for Sigma-Aldrich Inc. (St. Louis, MO). This compound was analyzed by the manufacturer and was documented to be between 98% to 100% pure. The manufacturer's documented purity was used for the fortification study. The final spiking solution was 94.3 mg/mL of MOR.

Cocaine hydrochloride (COC). This was purchased from the U.S. Pharmacopoeia. The sample used in the fortification study was characterized by the U.S. Drug Enforcement Administration (DEA) and was found to contain 98.9% COC and 1.1% CE. The final spiking solution was 32.2 mg/mL of COC.

Reagents and laboratory supplies: Sodium chloride (American Chemical Society grade, NaCl), sodium phosphate monobasic (analytical grade, NaH_2PO_4), sodium hydroxide (NaOH), and ethanol and isopropanol (HPLC grade) were purchased from VWR International. Deionized water from a Pure Water Solutions System was used. Bovine serum albumin (minimum 96%) was purchased from Sigma-Aldrich. The shaker (Eberbach Model 600), pH meter (Mettler Toledo), analytical balance (Mettler Toledo Model AX 105), amber storage jars (Qorpac), and other laboratory supplies were purchased from VWR. Prior to using the pH meter in the study, it was validated over a range from 2.00 to 12.45. The instrument was calibrated daily before use, and appropriate controls were analyzed concurrently with the fortification solutions.

2.1.3 Methods

2.1.3.1 Hair Characterization and Decontamination Procedures

Characterization of Hair Specimens: Upon receipt, the hair was received into inventory, weighed, and visually evaluated for color using the Schwartzkopf scale, which is used by professional cosmetologists for categorizing hair color (1 = black; up to 10 = light blonde and gray). One person visually and physically determined all hair specimen color types, and another individual independently confirmed these determinations (Schwartzkopf, 2001). A modified scale used in RTI's study is shown in **Table 2-2.** The donor hair for this study was determined to be brown by visual observation and have a Schwartzkopf color of 4.0. More than 100 g of hair from one individual donor was used to produce all reference materials (Stages 1 and 2) for this study.

Table 2-2. A Modified Scale Based on the Schwartzkopf Scale

Hair Color	Schwartzkopf Scale
Black	1
Medium to dark brown	2 through 4
Light to medium brown	5 and 6
Light brown	7
Medium blonde	8 and 9
Light blonde and gray	10

Decontamination of hair specimens: Before beginning the fortification protocol, hair samples were washed three times with deionized water for 15 minutes to remove hygienic residues (e.g., shampoo, conditioner, and/or styling products). The hair was thoroughly dried, and an aliquot was sent to the reference laboratory for analysis. Radioimmunoassay analysis indicated that the hair was negative for COC, opiates, PCP, AMP, and THCA analytes. One donor hair was divided into 14- to 20-g aliquots and used for each of the four HRMs produced. Each portion was placed in a plastic zippered bag to protect it from environmental drug exposure during the fortification study.

2.1.3.2 Hair Fortification

A total of four HRMs were produced. The HRMs were fortified at different times to prevent cross contamination. Target concentrations were determined to be 0.3 pg/mg for THCA, 500 pg/mg for MOR, 1500 pg/mg for COC, and 750 pg/mg for AMP, MAMP, and MDMA. The target concentrations for each analyte were selected based on previously discussed criteria proposed in the mandatory guidelines (SAMHSA, 2004) and commonly used for non-regulated testing as well. The COC HRM was targeted at three times (1500 pg/mg of COC) the proposed cutoff. Both the MOR and AMP, MAMP, MDMA HRMs were targeted at two and a half times the proposed cutoff. Additionally, the THCA HRM was targeted at six times the proposed cutoff.

Production of HRM RM-RTI-CFS-2407-THCA-1: A 0.76-mL portion of the THCA (concentration 1 mg/mL) was pipetted from the commercially produced, certified standard ampule. The THCA was first diluted in reagent-grade ethanol (0.5 L), and then was further diluted in an aqueous normal saline solution (pH 6.0) to the target concentrations of the final fortification solution. The buffered solution was prepared by adding 9 g of NaCl and 0.5 g of NaH_2PO_4 into 1 L deionized water and adjusting the pH with 10 N NaOH.

Hair aliquots (14–20 g) were placed in a clean, pre-labeled amber jar (1 L), and a fortification solution was added until the hair was covered (420–900 mL). To promote ease of handling, the hair remained as long, intact strands (longer than 10 cm) during the entire fortification process. The container was capped to prevent external contamination. The fortification solution was oscillated on a shaker for a period of time based on RTI protocols and target concentration (**Table 2-3**). The entire protocol was performed under ambient conditions. To monitor the progress of the hair incorporation, 100- to 200-mg portions of hair were removed at 24, 48, 72, and 96 hours and immediately washed with isopropanol for 15 minutes. Then the hair was washed with three aliquots (100–200 mL) of phosphate buffer (pH 6.0) prepared by adding 12 g of NaH_2PO_4 and 100 mg of BSA into 100 mL of deionized water, followed by a 1/100 further dilution in deionized water. The pH with adjusted with 10 N NaOH. Following the phosphate

buffer washes, the hair was placed between two sheets of filter paper and air dried overnight. At the same time an aliquot was pulled in the morning, the pH of the fortification solution was measured. These daily uptake samples were stored for analysis to determine the drug concentration. The solution's pH was monitored daily to ensure that the pH remained at 6.0 ± 0.2.

Each hair sample was wrapped in filter paper and dried at ambient temperature overnight. After the material was completely dried, the hair was cut into approximately 1–3 cm lengths and completely mixed before aliquoting it out into glass scintillation vials for storage and distribution. During fill order processing, glass scintillation vials were randomly collected for analysis by the reference laboratory, each 100- to 110-mg aliquot was used for three replicate analyses by the laboratory. The hair was weighed using a top-loading balance (1 mg ± 0.01 mg), placed into a pre-labeled scintillation vial as intact strands (no pulverization), capped with a foil-lined screw cap, and placed in an individual plastic zippered bag. The hair aliquots were then placed into a secondary bag and sent overnight to the reference laboratory for analysis.

Following 96–120 hours of exposure to the drug fortification solution (32–40 hours of exposure, samples were oscillated to move hair around in solution), the hair was filtered by a vacuum through a small funnel. The final hair preparation was washed first with 100 mL of isopropanol at room temperature and gently agitated for 15 minutes. Hair was filtered under a vacuum and air dried overnight. Next, final hair preparation was further washed with three sequential replicates of 100 mL of phosphate buffer at room temperature by gently agitating it for 30 minutes each. Following three successive phosphate buffer washes, the final hair preparation was filtered, spread out on filter paper, covered with another sheet of filter paper, and air dried overnight.

Production of HRM RM-RTI-CFS-2407-MOR-2: For MOR sulfate, a 31.98-mg portion was weighed and dissolved in deionized water to produce the first stock solution. The MOR stock solution was then further diluted in an aqueous solution (pH 6.0) to the target concentrations of the final fortification solution. The remainder of the fortification process followed the previously described methods for the THCA HRM.

Production of HRM RM-RTI-CFS-2407-COC-3: Using a similar procedure, a 37.75-mg portion of the COC was dissolved first in water, and then diluted in an aqueous solution to the target concentrations of the final fortification solution. The remainder of the fortification process followed the previously described methods for the THCA HRM.

2.1.3.3 Analytical Procedures

Hair specimens were sent to reference laboratories for analysis with their standard hair testing procedures. All specimens were submitted to Psychemedics Corp., CHT, or Immunalysis Corp. for Stage 2 analyses. These laboratories quantified COC, opiates (i.e., COD, MOR, and 6-acetylmorphine [6-AM]), and THCA analytes in the hair specimens and were compensated for the analytical work. When available, additional analyte concentrations (i.e., CE, norcocaine [NCOC], COD, 6-AM, MDA, MDEA) were measured for informational purposes. Appropriate digestion methods for hair were selected to maintain all COC analyte concentrations with minimal COC hydrolysis to BE, which can be more labile under basic digestion methods (pH >8.0). Matrix-matched QCs were included in the analysis to monitor for hydrolysis, with <5% considered acceptable.

Analysis by Psychemedics Corp.: LC-MS/MS was used for hair testing according to proprietary methods that have not been published. More complete details of analysis are considered proprietary by the laboratory; therefore they are omitted from this report. Instrumentation included a triple-quadrupole API 2000 PerkinElmer Sciex (Thornhill, Ontario, Canada) mass spectrometer that was equipped with an atmospheric pressure ionization source and a Model 200 binary micropump with a PerkinElmer Series 200 autosampler. The LC-MS/MS was operated in a positive chemical ionization mode. **Table 2-4** provide validation statistics for Psychemedics Corp.'s analyses.

Table 2-3. Timing of RTI Fortification Process

Reference Material–Single Analyte	Timing of Fortification Process
THCA	4 days, oscillated 8 hr/day
MOR	5 days, oscillated 8 hr/day
COC	5 days, oscillated 8 hr/day

Note: COC = cocaine; MOR = morphine; THCA = 11-Nor-delta-9-THC-9-carboxylic acid

Table 2-4. Validation Statistics for Psychemedics Corp.'s LC-MS/MS Methods Used for Hair Analysis and Reported to RTI for This Research

LC-MS/MS Operating in APCI Mode	THCA
Limit of detection (pg/mg)	0.02
Limit of quantification (pg/mg)	0.02
Limit of linearity (pg/mg)	10
Between run imprecision data; *n* value	NR
Target concentration (pg/mg)	NR
Coefficient of variation (% CV)	NR

Note: APCI = atmospheric pressure chemical ionization; LC-MS/MS = liquid chromatography-tandem mass spectrometer; NR = not reported; THCA = 11-Nor-delta-9-THC-9-carboxylic acid

Analysis by Immunalysis Corp.: Immunalysis Corp. used solid-phase extraction (CleanScreen, United Chemical Technologies) to isolate drug analytes from the hair. GC-MS, GC-GC/MS, and LC-MS/MS were used for hair testing according to previously published and peer-reviewed methods to detect THCA, MOR, and COC, respectively (Moore et al., 2006a and b and 2007). Quantitative analytical procedures for determining THCA and MOR in hair were performed on an Agilent Technologies 5973 Series GC-MS using electron capture chemical ionization (ECCI) and electron impact ionization, respectively. The limit of quantitation for THCA and MOR were 0.1 pg/mg and 50 pg/mg. The THCA method uses several small improvements in the extraction and GC and MS procedures to improve sensitivity to the sub-picogram concentrations. **Table 2-5** provides validation statistics for the GC-GC/MS method for THCA. The mass selective detector was operated in a selected ion monitoring mode with four ions in a single group. Ions *593.1* and 425.1 were monitored for THC-COOH-d3 and *590.1* and 422.1 for THC-COOH with a dwell time of 100 ms for each ion. The retention time of THC-COOH was 11 minutes. These two ions

and the enhanced separation of the GC-GC using cryfocusing to allow the analyte to be "cold-trapped" and rapidly remobilized at the prevailing column temperature, provided a high degree of confidence in the determinations. Ions *432.2* and 417.2 were monitored for MOR-d3 and *429.2*, 414.2, and 401.2 for THC-COOH with a dwell time of 100 ms for each ion. In each case, the quantitative ions are italicized and underlined. Representative GC-GC/MS and GC-MS chromatograms are shown for THCA and MOR in **Figures 2-1** and **2-2**.

Table 2-5. Validation Statistics for Immunalysis Corp.'s GC-MS Method Used to Detect THCA and MOR in Hair

GC-GC/MS Operating in ECCI Mode (1); GC-MS Operating in Electron Ionization Mode (2)	THCA (1)	MOR (2)
Limit of detection (pg/mg)	0.05	0.05
Limit of quantification (pg/mg)	0.1	50
Limit of linearity (pg/mg)	10	1000
Accuracy; *n* value	5	NR
0.05 pg/mg target	–0.0075	NR
0.1 pg/mg target	+ 0.014	NR
Within run imprecision data; *n* value	6	5
Target concentration (pg/mg) 0.05, 0.1, and 0.5	0.05	200
Coefficient of variation (% CV)	0.4	2.78
Target concentration (pg/mg)	0.1	NR
Coefficient of variation (% CV)	1.04	NR
Target concentration (pg/mg)	0.5	NR
Coefficient of variation (% CV)	1.21	NR
Between run imprecision data; *n* value	6	5
Target concentration (pg/mg)	1.0	200
Coefficient of variation (% CV)	1.61	2.97
% Recovery data; *n* value	3	6
% Recovery	50	96.1

Source: Moore et al., 2006a and b.

Note: ECCI = electron capture chemical ionization; GS- MS = gas chromatography-mass spectrometry; GC-GC/MS = two dimensional gas chromatography- mass spectrometer; MOR = morphine; THCA = 11-Nor-delta-9-THC-9-carboxylic acid; NR= not reported

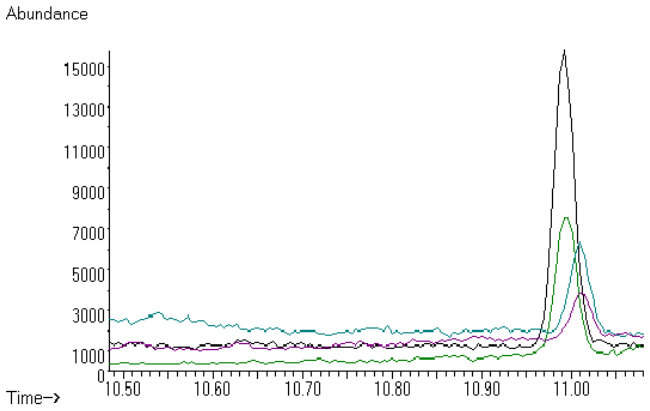

Figure 2-1. GC-GC/MS chromatogram of THCA and its deuterated internal standard (Immunalysis Corp.) in HRM (mean 0.27 pg/mg).

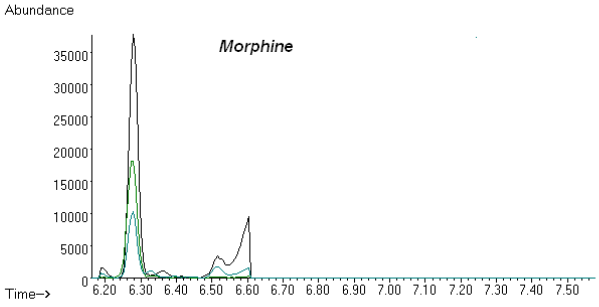

Figure 2-2. GC-MS chromatogram of morphine (Immunalysis Corp.) in HRM at 415 pg/mg. This was a hair sample taken during the fortification process to monitor concentration and time.

Quantitative analytical procedures for determining COC, BE, CE, NCOC, and MOR in hair were performed on an Agilent Technologies 1200 Series liquid chromatograph pump coupled to a 6410 triple quadrupole mass spectrometer, operated in positive Atmospheric Pressure Chemical Ionization (APCI) mode. For confirmation, two transitions were monitored, and, in some cases, one ion ratio was determined and found to be acceptable if it was within 20% of the ratio performance of known calibration standards. For the MOR method, the following multiple reaction monitoring (MRM) parameters were used: MOR-d3 289.2 to 211.2, fragmentor voltage 120 V, collision energy (CE) voltage 35 V, dwell time 50 ms and MRM1 for MOR 286.3 to 165.2; fragmentor voltage 160 V, CE voltage 35 V, dwell time 50 ms and MRM2 for MOR 286.3 to 155.2; fragmentor voltage 120 V, CE voltage 35 V, and dwell time 50 ms. Other parameters included gas temperature a350°C, vaporizer 400°C, gas flow 5 L/min, and capillary voltage 4500 V. The LC column (Zorbax Eclipse XDB C18 [4.6 x 50 mm x 1.8 mm]) was

maintained at 50°C, and the injection volume was 5 µL. The LC mobile phase flow rate was 0.9 mL/min 20 mM ammonium formate pH 6.4 (Solvent A): methanol (Solvent B) 75:25 v/v with solvent settings at 1.5 min ratio 70:30 (A/B); at 4.5 min 1 mL/min flow; 55% B; and at 5 min 60% B. Similar settings were used for the COC LC-MS/MS method. **Table 2-6** shows validation statistics for MOR and COC methods. **Figures 2-3** and **2-4** show representative LC-MS/MS chromatograms for MOR and COC, respectively.

Table 2-6. Validation Statistics for Immunalysis Corp.'s LC-MS/MS Methods Used to Detect Morphine and Cocaine in Hair

LC-MS/MS Operating in APCI Mode	MOR	COC	BE	CE	NCOC
Limit of detection (pg/mg)	50	25	25	25	25
Limit of quantification (pg/mg)	100	50	50	50	50
Limit of linearity (pg/mg)	1000	10000	10000	10000	10000
Accuracy; *n* value	5	5	5	5	5
50 pg/mg target; % accuracy	NR	99.9	101.7	99.3	108
100 pg/mg target; % accuracy	NR	101.4	93.7	92.5	88.4
200 pg/mg target; % accuracy	101.3	94.5	94.3	88.4	86.1
Within run imprecision data; *n* value	8	5	5	5	5
Target concentration (pg/mg)	200	100	100	100	100
Coefficient of variation (% CV)	8.9	1.3	8.1	0.8	0.4
Between run imprecision data; *n* value	6	10	10	10	10
Target concentration (pg/mg)	200	100	100	100	100
Coefficient of variation (% CV)	9.1	4.8	9.2	15.7	12.6
% Recovery data; *n* value	3	3	3	NR	NR
% Recovery	46.2	82.7	93.8	NR	NR

Source: Moore et al., 2006a and 2007.

Note: BE = benzoylecgonine; CE = cocaethylene; COC = cocaine; LC-MS/MS = liquid chromatography-tandem mass spectrometer; MOR = morphine; NCOC = norcocaine; NR = not reported

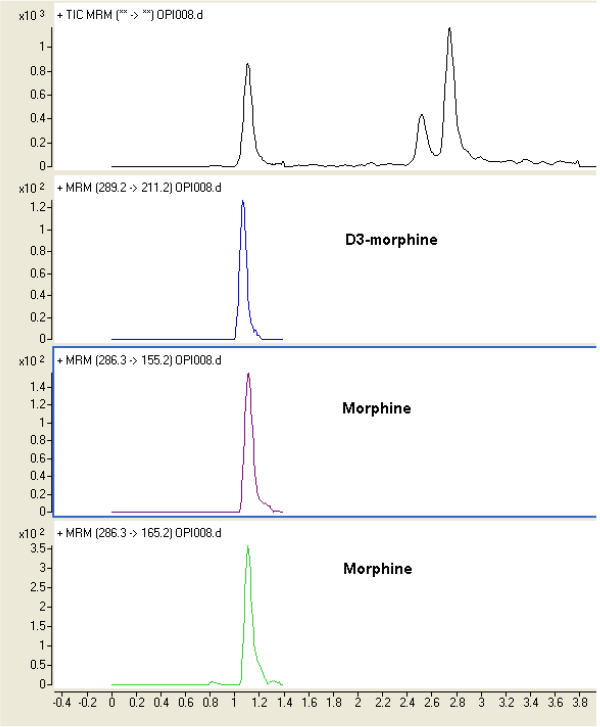

Figure 2-3. LC-MS/MS chromatogram of morphine (Immunalysis Corp.) in HRM (mean 716 pg/mg).

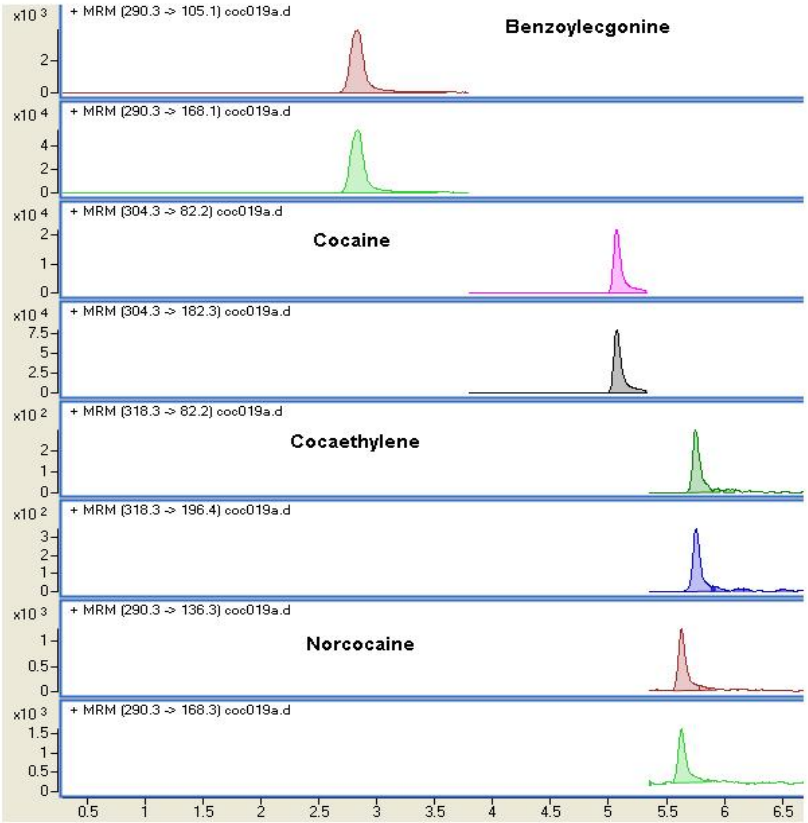

Figure 2-4. LC-MS/MS chromatogram of cocaine analytes (Immunalysis Corp.) in HRM (mean 2,212 pg/mg).

Analysis by CHT: CHT developed a compound-specific drug screening assay for controlled substances and major illicit drug groups to identify patterns of drug use through the analysis of human hair samples. The assay measures the presence of several drugs, selected major metabolites, and related compounds in human hair. Procedures used were based on those previously described (Borges et al., 2001 and 2003; Paulsen et al., 2001; Hubbard et al., 2000). For the purposes of this report, only analytes of interest are detailed herein.

Briefly, deuterated internal standards (i.e., COC-d3, BE-d3, NCOC-d3, and MOR-d3) were added to human hair as internal standards. Hair was partially digested overnight in 0.1 N of HCl, followed by solid phase extraction on Bond Elut (Varian, CA) columns to separate analytes of interest from other hair constituents. The extracted analytes and their internal standards were analyzed by LC atmospheric pressure ionization-electrospray (API-ES) MS. The LC system consists of an Agilent 1100 series in-line degasser, binary pump with solvent switching valve, autosampler, and temperature-controled column compartment. Chromatographic separation was achieved on a ZORBAX SB-C18 Narrow–Bore RR (2.1 x 100 mm x 3.5 µm; Agilent, Palo Alto, CA) with an Eclipse XDB-C8 Narrow–Bore Guard Column (2.1 x 12.5 mm x 5µ, Agilent, Palo Alto, CA). A Hewlett-Packard series 1100 LC–mass selective detector (MSD) (Hewlett-Packard Corp., Palo Alto, CA) was operated in the selected ion monitoring (SIM) mode. Monitored ions and their corresponding analyte of interest are summarized in **Table 2-7.**

Table 2-7. Monitored Ions of Morphine and Cocaine Analytes for CHT's LC-MS Method

Analyte	Quantifying Ion	Qualifying Ion
Cocaine	304	182
Cocaine-d3	307	185
Benzoylecgonine/norcocaine	290	168
Benzoylecgonine-d3/norcocaine-d3	293	171
Morphine	286	NR
Morphine-d3	289	NR

Note: NR = not reported

The concentration of each analyte was determined using Agilent's ChemStation software by comparing the ratio of the peak area (or peak height) of the drug to the peak area (or peak height) of its deuterated internal standard to the calibration curve that was generated from the analysis of human hair fortified with known concentrations of each compound. Chromatographic separation (1,000 pg/mg extracted matrix calibrator), assay precision, accuracy, linearity, limit of detection, and limit of quantitation are shown in **Table 2-8** and **Figure 2-5**.

Table 2-8. Cocaine-Related Validation Statistics for CHT (The Method That Was Used for Hair Analysis and Reported to RTI for This Research)

LC-MS Operating in ESI Mode	COC	BE	NCOC
Limit of detection (pg/mg)	20	20	20
Limit of quantification (pg/mg)	20	20	20
Between run accuracy and imprecision data at the limit of quantification; *n* value	NR	NR	NR
Mean concentration (target 20 pg/mg)	NR	NR	NR
Coefficient of variation (% CV)	13.0%	9.6%	11.7%
Limit of linearity (pg/mg)	10000	10000	10000
Within run accuracy and imprecision data; *n* value	5	5	5
Mean concentration (target 100 pg/mg)	100	110	100
Coefficient of variation (% CV)	2.5%	2.5%	3.3%
Mean concentration (target 1000 pg/mg)	1010	980	960
Coefficient of variation (% CV)	1.3%	1.0%	1.5%
Mean concentration (target 5000 pg/mg)	5140	5000	4980
Coefficient of variation (% CV)	2.8%	3.3%	2.8%
Between run accuracy and imprecision data; *n* value	127	128	125
Mean concentration (target 100 pg/mg)	90	100	90
Coefficient of variation (% CV)	6.4%	9.1%	7.5%
Mean concentration (target 1000 pg/mg)	960	1000	930
Coefficient of variation (% CV)	6.6%	7.5%	13.8%
Mean concentration (target 5000 pg/mg)	4700	5100	4600
Coefficient of variation (% CV)	7.9%	8.0%	14.5%

Note: COC = cocaine; BE = benzoylecgonine; NCOC = norcocaine; LC-MS = liquid chromatography-mass spectrometer; NR= not reported.

Acceptable QC criteria is $\pm$ 20% of target. Maximum number of data points for between-run calculations is 130. (Only data points within 20% of target are included in table).

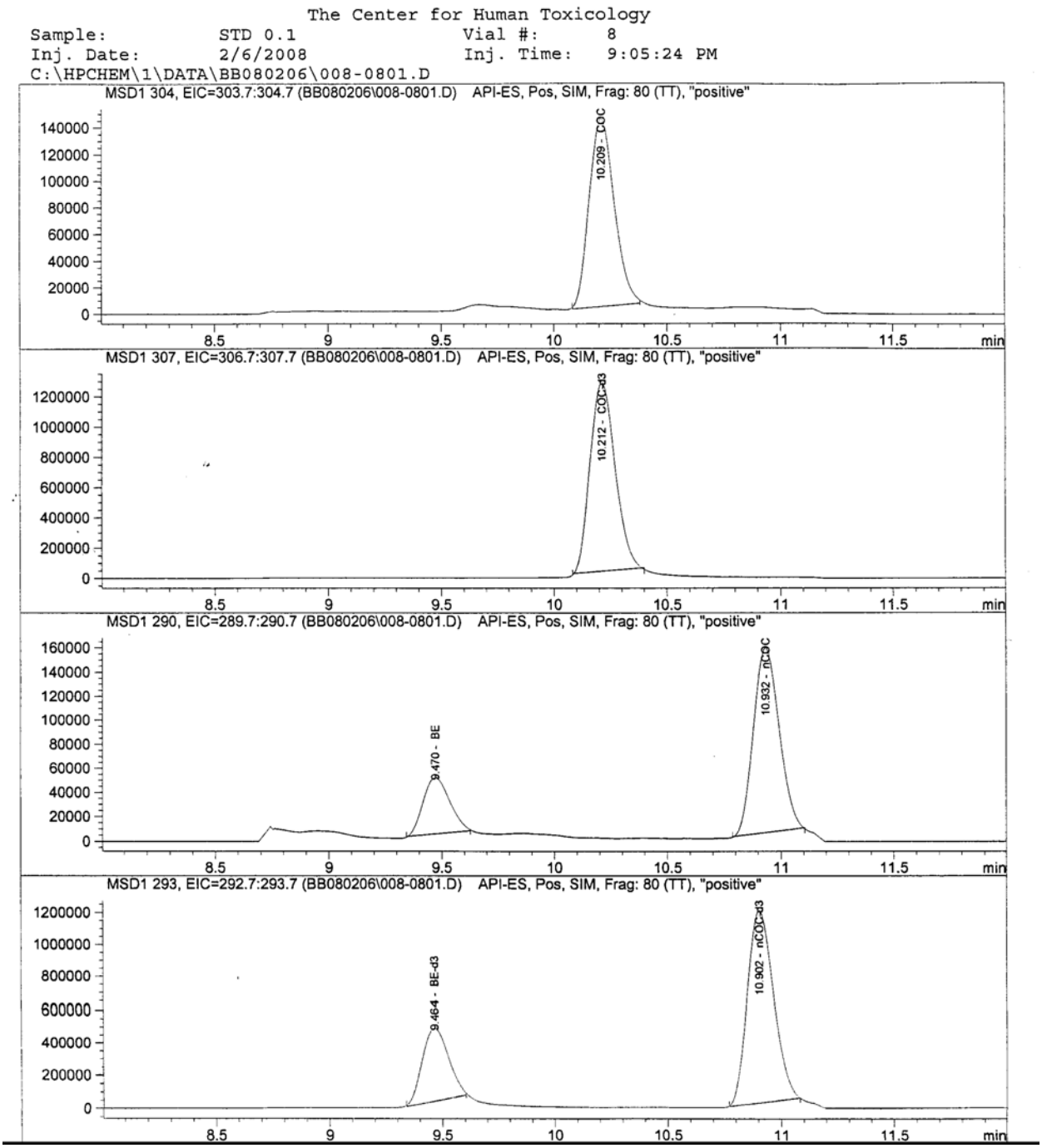

Figure 2-5. CHT chromatogram of COC, BE, and NCOC at 0.1 ng/mg (100 pg/mg) in extracted hair standards.

Statistical analysis to measure variability and uncertainty in analytical results: To estimate the intra- and inter-laboratory and analytical method variability, SAS statistical software (SAS Version 9.1.3 [XP PRO procedure]) was used for all statistical analysis. The Procedure Variance Components (PROC VARCOMP) was used to estimate the two sources of variability (intra- and inter-laboratory). The VARCOMP procedure handles general linear models that have random effects; the results of each laboratory are considered random effects because the results should be generalizable to all possible laboratories, not just the ones selected for this study. Random effects

are classification effects with levels that are assumed to be randomly selected from an infinite population of possible levels. PROC VARCOMP estimates the contribution of each of the random effects to the variance of the dependent variable.

In addition, the Restricted Maximum Likelihood Method was used to estimate variance. This method first separates the likelihood into two parts: one that contains the fixed effects and one that does not (Patterson and Thompson 1971; Searle et al., 1992). The procedure uses a Newton-Raphson algorithm.

After the variance was calculated, the uncertainty was determined by multiplying the square root of the calculated variance by two. From this calculation, analyte concentration reference ranges were defined as the mean concentration plus or minus the uncertainty measurement (Average ± 2*SQRT[VAR]).

2.1.3.4 Modifications to Research Design and Rationale

For these HRM productions, there were no modifications to the research design.

2.1.4 Findings

Primary methods were targeted for 72 replicate analyses and the secondary, and tertiary methods were targeted for 15 to 20 replicate analyses.

Seventy-nine measurements were made by the primary analytical method GC-tandem MS (GC-GC-MS), and 19 measurements were made by the secondary analytical method (LC-MS/MS), for a total of 98 THCA measurements. The average mean, SD, and CV were determined for the primary and secondary methods and for the combined results from all measurements. Comparison of the average mean and the SD indicated that there was a large disparity between the primary (0.27 ± 0.03 pg/mg) and secondary measurements (0.15 ± 0.02 pg/mg). Combined THCA results gave an average mean and SD of 0.25 ± 0.06 pg/mg. The estimated total variance (sum of intra- and inter-laboratory variance) for all THCA measurements was 0.008. The uncertainty measurement, defined as twice the square root of this estimate, was 0.17 pg/mg. The THCA reference range for reference material RM-RTI-CFS-2407-THCA-1, determined by this validation process and defined as the average mean plus or minus the uncertainty, was calculated to be 0.246 ± 0.17 pg/mg. The high analytical variability estimated for this HRM resulted in a large, but realistic reference range. Fourteen QC samples (i.e., 6 positive and 8 negative) were randomly placed in the samples sent to each reference laboratory and their identity was unknown to the laboratory. This was a frequency of 14% of the total THCA samples analyzed. All QC samples were correctly identified (**Appendix B-1-4**).

Seventy-seven measurements were made by the primary analytical method (LC-MS/MS), and 25 measurements were made by the secondary analytical method (LC-MS), for a total of 102 MOR measurements. The average mean and the SD for the primary method was 716 ± 96 pg/mg, and the secondary method was half that at 350 ± 33 pg/mg. Combined MOR results gave an average mean and SD of 627 ± 179 pg/mg. The estimated total variance for all MOR measurements was 25537, and the calculated uncertainty measurement using the total variance was 320 pg/mg. Thus, the MOR reference range for reference material RM-RTI-CFS-2407-MOR-2 was calculated to be 627 ± 320 pg/mg. Again, the high analytical variability estimated for this HRM

resulted in a large, but realistic reference range. Twenty-nine QC samples (i.e., 8 positive and 21 negative), or 28% of the total MOR samples analyzed, were correctly identified (**Appendix B-2-4**). COD and 6-AM were undetectable by both analytical methods.

For the COC reference material, only the primary analytical method (LC-MS/MS) was used in the calculations (see Section 2.1.3.4). A total of 98 COC measurements were performed. The average mean and the SD for the primary method was 2,212 ± 530 pg/mg. The estimated total variance for all COC measurements was 111489, and the calculated uncertainty measurement using the total variance was 672 pg/mg. The COC reference range for reference material RM-RTI-CFS-2407-COC-3 was calculated to be 2,212 ± 672 pg/mg. Again, the high analytical variability estimated for this HRM resulted in a large, but realistic reference range. Concentrations for benzoylecgonine (BE), cocaethylene (CE), and norcocaine (NCOC) were also determined. BE was present in all samples, CE in one-third and NCOC was not detected in any of the samples. A small amount of BE (<100 pg/mg) and CE (<50 pg/mg) was detected in a small portion of the aliquots. Twenty-one QC samples (i.e., 7 positive and 14 negative), or 21% of the total COC samples analyzed by both the primary and the secondary methods, were correctly identified (**Appendix B-3-5**).

Determined reference ranges for the THCA and MOR HRMs were comparable to the targeted fortification concentrations. The THCA reference range was 0.246 ± 0.17 pg/mg, and the individual laboratory average % CV was 23%. Intra-laboratory CVs ranged from 12.0% to 12.7%. MOR's range was 627 ± 320 pg/mg, and its inter- and intra-laboratory CVs were 28.6% and 9.3% to 10.1%, respectively. COC's range was 2,212 ± 672 pg/mg; much higher than its targeted concentration of 1,500 pg/mg. COC's inter- and intra-laboratory CVs were 23.8% and 8.7% to 14.8%, respectively. Tables in **Appendixes B-1** through **B3** contain all of the results for RM-RTI-CFS-2407-THCA-1, RM-RTI-CFS-2407-MOR-2, RM-RTI-CFS-2407-COC-3, respectively. The appendices tables include primary and secondary method replicate analysis results, overall statistical results, and blind QC results. A summary of the single analyte HRM results is provided in **Table 2-9**.

Table 2-9. Single Analyte Reference Material Results

Reference Material	Analyte	Target Concentration (pg/mg)	Reference Range (pg/mg)	% CV (Individual Laboratory % CV Range)
RM-RTI-CFS-2407-THCA-1	THCA	0.30	0.25 ± 0.17	23% (12.0%, 12.7%)
RM-RTI-CFS-2407-MOR-2	MOR	500	627 ± 320	28.6% (9.3%, 9.5%, 10.1%)
RM-RTI-CFS-2407-COC-3	COC	1500	2212 ± 672	23.8% (8.7%, 9.2%, 13.8%)

Note: COC = cocaine; MOR = morphine; THCA = 11-Nor-delta-9-THC-9-carboxylic acid

2.1.5 Conclusions

The HRMs developed and produced by RTI demonstrated high variability or uncertainty in the concentration range, but intra-laboratory CVs were below 15%. These findings suggest that the reference laboratories performed well within their own system of protocols, but that there is not good inter-laboratory agreement in a system of laboratories that analyze the same samples using different protocols. This is consistent with the lack of standard materials available for this type of

testing. RTI has observed similar findings while working with the NLCP pilot hair performance program over the past 7 years.

Approximately 350 vials of four HRM products are available for purchase and distribution to interested forensic laboratories. RTI has received approval from NIJ to sell these materials, with the proceeds going toward funding future production of materials. Forensic laboratories can use these HRMs to improve the defensibility of their analytical results by documenting their performance to accurately measure drugs of abuse in hair. Consequently, these HRMs will add a layer of forensic reliability for the laboratory's data to its clients, the court, and the individuals being tested. This research provides external HRMs with THCA, MOR, and COC analytes near the confirmatory cut-off/threshold concentrations currently used by hair testing laboratories.

2.2 Stage 2: Fortification of Hair with Multiple Drug Analytes in Solution

2.2.1 Experimental Design

In Stage 2, HRMs containing multiple drug analytes were produced. Three AMP analytes were included in this fortification process: AMP, MAMP, and MDMA. The same sampling and analysis schemes used in Stage 1 were also used for Stage 2. Thus, the primary laboratory analyzed 69 specimens, 10 measurements were made by the secondary analytical method (LC-MS/MS), and another 10 measurements were made by the tertiary analytical method (LC-MS), for a combined sample size of 89. An additional 16 blind controls were analyzed by the reference laboratories, for a total analysis of 105 analyses.

A statistical analysis was performed to determine measurements such as the analytical mean, SD for replicate analysis, relative standard deviation, and the uncertainty in each measurement. All statistical analyses were performed using SAS (version 9.1.3).

2.2.2 Materials

Hair: The same donor hair (Schwarzkopf 4.0, medium brown hair) was used for Stage 1 and Stage 2 of this study. All drug-free hair specimens were analyzed for analytes of interest before inclusion in this study.

D-amphetamine sulfate salt, (+)-MAMP hydrochloride, and (±)-3,4-methylenedioxymethamphetamine hydrochloride: All compounds were purchased from Sigma-Aldrich Inc. (St. Louis, MO). Each compound was analyzed by the manufacturer and was documented to be between 98–100% pure. The manufacturer's documented purity was used for the fortification study.

Reagents and laboratory supplies: The same donor hair was used for Stage 1 and Stage 2 of this study. Additionally, all reagents and laboratory supplies used for this part of the study were the same for Stage 1 and Stage 2 of this study and were previously described in Section 2.1.2.

2.2.3 Methods

2.2.3.1 Hair Characterization and Decontamination Procedures

Characterization of hair specimens: The same donor hair was used to produce all HRMs.

Decontamination of hair specimens: The same decontamination procedures were used for Stages 1 and 2 of this study.

2.2.3.2 Hair Fortification

Target concentrations for the multiple drug analyte HRMs were determined to be 750 pg/mg for AMP, MAMP, and MDMA. The target concentrations for each analyte were selected based on previously discussed criteria, such as the confirmatory cutoff (AMPs at 300 pg/mg) proposed in mandatory guidelines (SAMHSA, 2004). The multiple drug analyte HRM was targeted at two and a half times the proposed cutoff.

Production of HRM RM-RTI-CFS-2407-AMPS-4: A 57.16-mg portion of AMP, a 50.75-mg portion of MAMP, and a 21.81-mg portion of MDMA were dissolved first in deionized water, and then diluted in an aqueous solution to their target concentrations of the final fortification solution.

The fortifying solution was created by adding stock solutions of AMP, MAMP, and MDMA in ethanol to a normal saline solution that contained 9.0 g/L of NaCl and 0.5g/L of NaH_2PO_4. Stock solutions of AMP, MAMP, and MDMA were created by dissolving solid material in ethanol after correcting for salt form and purity. The fortifying solution contained 0.16 μg/mL AMP, 0.16 μg/mL MAMP and 0.07 μg/mL MDMA. Then, 20.0 g of hair from one donor collected as previously described was submerged in 800 mL of spiking solution.

The solution was gently agitated for 120 hours of agitation time (5 days at 8 hours per day). To monitor the progress of the hair incorporation, 100- to 200-mg portions of hair were removed at 24, 48, 72, and 96 hours and immediately washed with isopropanol for 15 minutes. Then, the hair was washed with three aliquots (100–200 mL) of phosphate buffer (pH 6.0) prepared by adding 12 g NaH_2PO_4 and 100 mg of BSA in 100 mL of deionized water, followed by a 1/100 further dilution in deionized water. The pH was adjusted with 10 N NaOH. Following the phosphate buffer washes, the hair was placed between two sheets of filter paper and air dried overnight. At the same time the aliquot was pulled in the morning, the pH of the fortification solution was measured. These daily uptake samples were stored for analysis to determine the drug concentration. The pH of the solution was monitored daily to ensure that the pH remained at 6.0 ± 0.2.

After 120 hours of agitation, the final product was removed from the solution, washed with 500 mL of isopropanol by shaking it for 15 minutes, filtered, and then dried overnight. The HRM product was then washed in the same manner previously described for the 100-mg portions.

After the material was completely dried, the hair was cut into approximately 1–3 cm lengths and completely mixed before aliquoting it out into glass scintillation vials for storage and distribution. During fill order processing, glass scintillation vials were randomly collected for analysis by the reference laboratory, and each 100- to 110-mg aliquot was used for three replicate analyses by the laboratory.

2.2.3.3 Analytical Procedures

The multiple drug analyte HRM was analyzed by three laboratories and by using three analytical techniques: LC-MS/MS, LC-MS, and GC-MS. Each reference laboratory has established

standard operating procedures for performing hair drug testing. All specimens were submitted to Psychemedics Corp., Immunalysis Corp., or CHT for Stage 2 analyses. These laboratories were compensated for performing the analytical work. Appropriate digestion methods for hair were selected to maintain all AMP analyte concentrations.

Analysis by Immunalysis Corp.: Immunalysis Corp. used an Agilent 6890 GC/MS 5973 MSD for the analysis of amphetamines. GC parameters included the following: Column DB-5 (or equivalent) 5% phenyl, 95% methyl silicone (15 m x 0.25 mm i.d. x 0.25 µm film thickness), injection volume 1 µL (splitless), injection temperature 150°C, gas flowrate 1.5 mL/min, oven program 60°C (1 min hold), and then ramp at 25°C/min to 140°C (4 min hold), then ramp at 20°C to 200°C (4 min hold), and then ramp at 80°C to 300°C. The run time was 14.45 minutes.

Mass spectrometer parameters include transfer line 280°C, MS source 230°C, and MS quadrupole 150°C. Ions 244.0 and 123.1 were monitored for AMP-d5 and 240.0, 118.1, and 91.1 for AMP; ions 258.1 and 213.0 were monitored for MAMP-d5 and 254.0, 210.0, and 118.1 for MAMP; ions 258.1 and 213.0 were monitored for MDMA-d5 and 254.0, 210.0, and 162.0 for MDMA. In each case, the dwell time was 50 ms; the quantitative ions are listed first and the qualifying ions are listed second.

Analysis by Psychemedics Corp.: All samples for AMP LC-MS/MS analysis were extracted using liquid–liquid extraction. Further details of the sample analysis methods are considered proprietary by the company; therefore, they are not reported. The same instrumental procedures described in Section 2.1.3.3 were used by Psychemedics Corp. for Stage 2 of this project. Monitored ions and validation parameters are provided in **Tables 2-10** and **2-11**.

Table 2-10. Monitored Ions of Amphetamines for Psychemedics LC-MS Method

Analyte	Quantitative Ion
AMP	91
AMP-d5	96
MAMP	91
MAMP-d11	96
MDMA	194

Note: AMP = amphetamine; MAMP = methamphetamine; MDMA = methylenedioxymethamphetamine

Table 2-11. Validation Statistics for Psychemedics Corp.'s LC-MS/MS Methods for the Analysis of Amphetamines in Hair

LC-MS/MS Operating in APCI Mode	AMP	MAMP	MDMA
Limit of detection (pg/mg)	25	100	10
Limit of quantification (pg/mg)	25	100	10
Limit of linearity (pg/mg)	15000	15000	20000

Source: Cairns et al., 2004.

Note: AMP = amphetamine; MAMP = methamphetamine; MDMA = methylenedioxymethamphetamine

Analysis by CHT: The same compound-specific drug screening assay described in Section 2.1.3.3 was used by CHT for Stage 2 of this project. Deuterated internal standards (i.e., AMP-d5, MAMP-d8, MDA-d5, and MDMA-d5) were added to human hair. The same sample preparation and instrumental analysis were used to analyze AMP, MAMP, and MDMA. Monitored ions and their corresponding analyte of interest are summarized in **Table 2-12**.

Table 2-12. Monitored Ions of Amphetamines for CHT LC-MS Method

Analyte	Quantitative Ion	Secondary Ion
AMP	136	119
AMP-d5	141	124
MAMP	150	119
MAMP-d8	158	124
MDMA	194	163
MDMA-d5	199	165

Note: AMP = amphetamine; MAMP = methamphetamine; MDMA = methylenedioxymethamphetamine

Chromatographic separation (1,000 pg/mg extracted matrix calibrator), assay precision, accuracy, linearity, limit of detection, and limit of quantitation are shown in **Table 2-13** and **Figure 2-6**.

Table 2-13. Amphetamine-Related Validation Statistics for CHT for the Analysis of Amphetamines in Hair

LC-MS Operating in ESI Mode	AMP	MAMP	MDMA
Limit of detection (pg/mg)	20	20	20
Limit of quantification (pg/mg)	20	20	20
Between run accuracy and imprecision data at the limit of quantification; *n* value	64	64	64
Mean concentration (pg/mg)	20	20	20
Coefficient of variation (% CV)	12.7	14.4	10.9
Limit of linearity	1000	1000	1000
Within run accuracy and imprecision data; *n* value	5	5	5
Mean concentration (target 100 pg/mg)	90	110	110
Coefficient of variation (% CV)	5.6	3.9	1.4
Mean concentration (target 1000 pg/mg)	1000	1180	1050
Coefficient of variation (% CV)	1.4	1.33	1.6
Mean concentration (target 5000 pg/mg)	5050	5430	5410
Coefficient of variation (% CV)	4.2	5.9	5.0

LC-MS Operating in ESI Mode	AMP	MAMP	MDMA
Between run accuracy and imprecision data; *n* value	124	114	124
Mean concentration (target 100 pg/mg)	90	100	100
Coefficient of variation (% CV)	10.8	12.0	8.2
Mean concentration (target 1000 pg/mg)	960	1020	990
Coefficient of variation (% CV)	8.3	13.9	7.8
Mean concentration (target 5000 pg/mg)	4720	4999	5000
Coefficient of variation (% CV)	9.1	12.5	8.8

Source: Borges et al., 2001.

Note: AMP = amphetamine; MAMP = methamphetamine; MDMA = methylenedioxymethamphetamine; LC-MS = liquid chromatography-mass spectrometer; ESI = electrospray ionization.

Acceptable QC criteria is ± 20% of target. The maximum number of data points for between run calculations is 125. (Only data points within 20% of target are included in the table.)

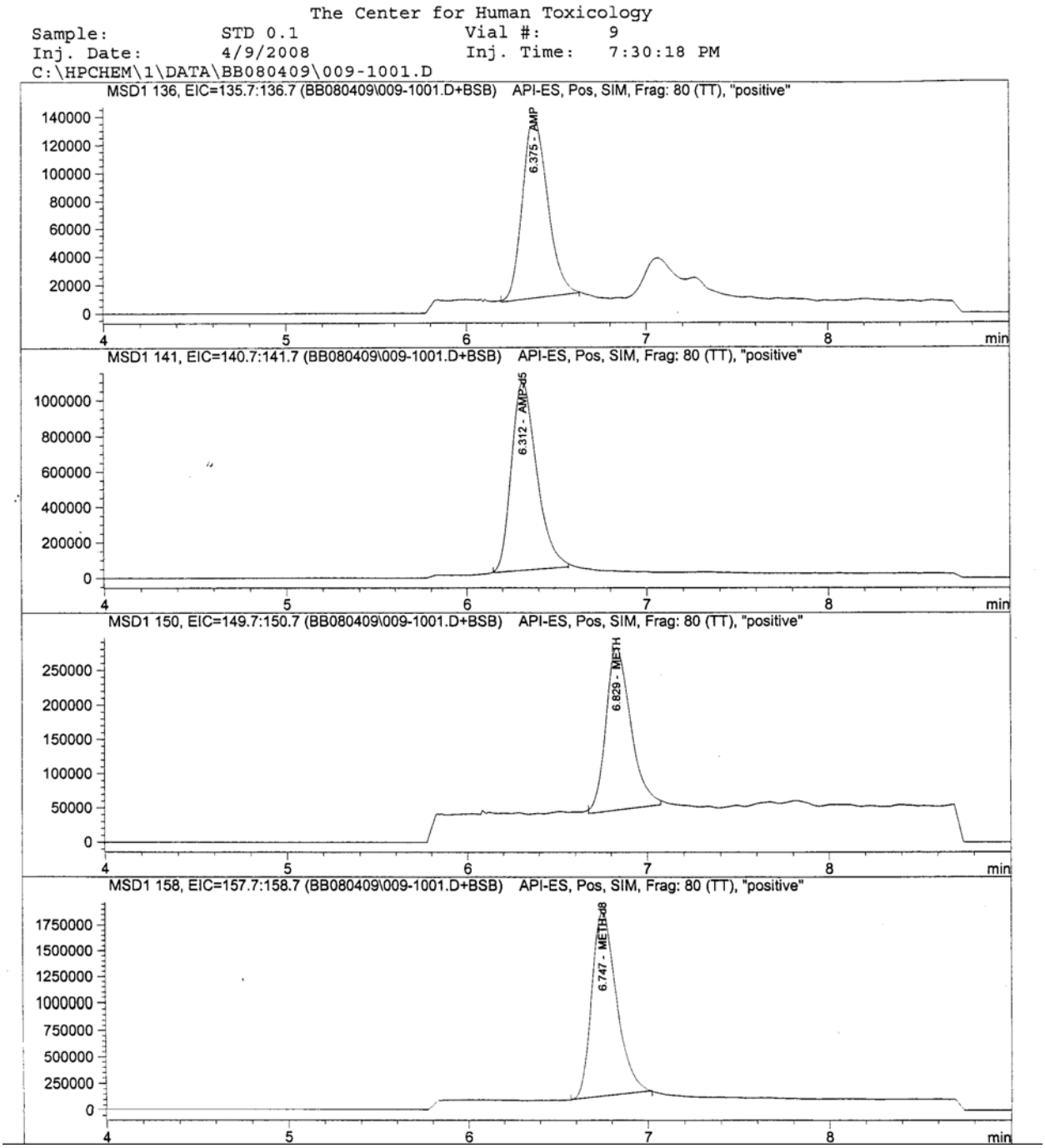

Figure 2-6. CHT chromatogram of AMP and MAMP at 0.1 ng/mg (100 pg/mg) in extracted hair standards.

Statistical analysis to measure variability and uncertainty in analytical results: The same SAS statistical software (SAS Version 9.1.3 [XP PRO procedure]) described in Section 2.2.3.3 was used for the variance and uncertainty estimates for the multiple drug analyte HRM containing AMPs.

2.2.3.4 Modifications to Research Design and Rationale

For this reference material, there were no modifications to the research design.

2.2.4 Findings

Sixty-nine measurements were made by the primary analytical method (GC-MS), 10 measurements were made by the secondary analytical method (LC-MS/MS), and another 10 measurements were made by the tertiary analytical method (LC-MS), for a total of 89 AMPS measurements. The average mean, SD, and total variance was determined for all three methods for each AMP analyte. These results were as follows: AMP 1352 ± 181 pg/mg; MAMP 1507 ± 168 pg/mg; MDMA 1294 ± 95 pg/mg. Total variances were 90051, 55999, and 21607, respectively. The uncertainty measurements ranged from 294 pg/mg for MDMA to 600 pg/mg for AMP. Calculated reference ranges were 1352 ± 600 pg/mg (AMP), 1507 ± 473 pg/mg (MAMP) and 1294 ± 294 pg/mg (MAMP). MDA and MDEA were not detected. Sixteen QC samples (i.e., 7 positive and 9 negative) were correctly identified as to whether AMPs were present (**Appendix B-4-5**). **Table 2-15** lists the target concentrations, reference ranges, and % CVs for the multiple drug analyte HRM results for AMP, MAMP, and MDMA.

Finally, AMP analytes were similar and more than twice the target concentration. As an example, reference range for AMPs was 1353 ± 600 pg/mg, and its intra-laboratory CV was 13.4%. Individual laboratory CVs ranged from 7.8% to 12.8%. All three AMP analytes were targeted at 750 pg/mg. This is not unusual based on RTI's experience with the NLCP pilot hair performance testing program, especially for AMPs because they tend to be sensitive to variations in hair. Because of this high fortification concentration of more than two times the target concentration for the AMP HRM, the hair sample was observed by SEM. The hair appeared to have some damage to the cuticle (**Figure 2-7**). Tables in **Appendix B-4** provide the results for all HRMs and statistical analysis.

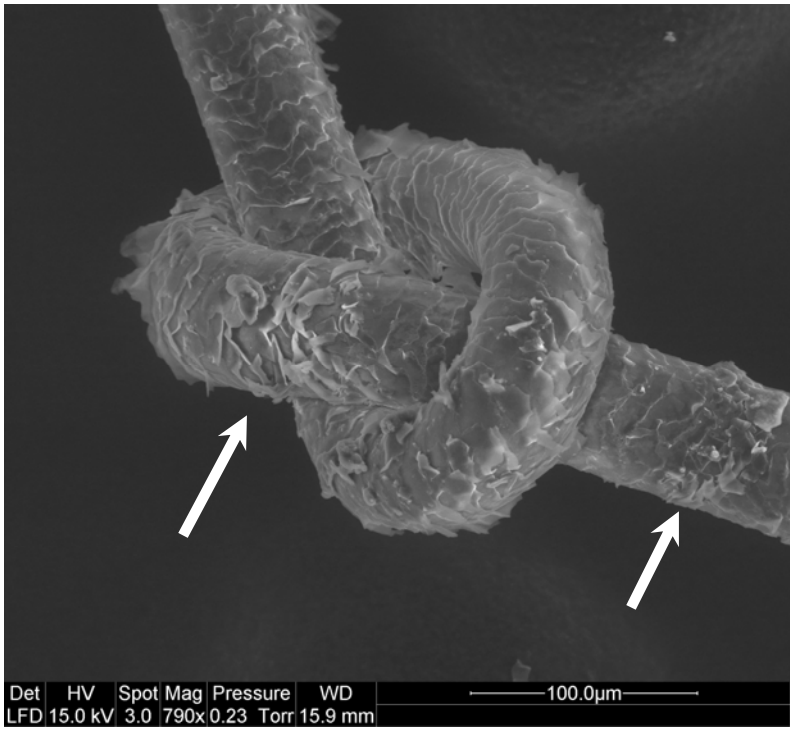

Figure 2-7. A SEM micrograph of representative donor hair strand used in the production of AMP HRM (RM-RTI-CFS-2407-AMPS-4). Arrows indicate damaged areas to the cuticle.

To investigate the higher-than-expected AMP results, RTI developed an additional study to determine if the solution volume or the donor hair contributed to this outcome. The same procedure as described for the original production was followed, with the exception of the volume and a comparison with an additional donor. The study involved aliquoting two volumes of a fortification solution (30 and 45 mL) and 1.0-g portions of hair from two individuals, for a total of four samples. The first donor hair sample (Schwarzkopf 4.0, medium brown hair) was from the same individual who was used to fortify the original AMP HRM. The second donor hair sample was from a 24-year-old white female who had medium-to-light brown hair with a Schwarzkopf color of 6.0. This donor's hair had been previously analyzed by a reference laboratory and was found to be negative for AMPs by radioimmunoassay analysis. Thirty and 45 mL of the original fortification solution, which was stored at room temperature for the 2-week time period between the two fortification studies, was added to each donor hair. After the fortification study was completed, hair aliquots from each sample were sent for replicate analysis, and results agreed within 10%. The average AMP concentrations are listed in **Table 2-14**.

Table 2-14. Comparison of Fortification of Two Donor Hairs with Amphetamine by LC-MS Method

Hair Type and Fortification Solution Volume (mL) Description	AMP (pg/mg)	MAMP (pg/mg)	MDMA (pg/mg)
Original hair donor (Schwartzkopf color of 4.0), 30 mL	1376	1358	1167
Original hair donor (Schwartzkopf color of 4.0), 45 mL	1441	1534	1382
Comparison hair donor (Schwartzkopf color of 6.0), 30 mL	706	620	736
Comparison hair donor (Schwartzkopf color of 6.0), 45 mL	732	687	769
Target concentration (pg/mg)	750	750	750
Original reference range (pg/mg)	1352 ± 600	1507 ± 473	1294 ± 294

Note: AMP = amphetamine; MAMP = methamphetamine; MDMA = methylenedioxymethamphetamine

The first donor hair had similar results to those originally obtained for the validated AMP HRM. However, the second donor hair produced results that were consistent with the target concentration that was established by RTI for the fortification protocol. Based on the data from the previously mentioned study, it appears that the first donor hair used to prepare the AMP HRM demonstrated an unexpected and greater affinity for AMPs than did the second donor hair of a slightly lighter color.

Table 2-15 provides some of the multiple drug analyte HRM results, and tables in Appendix B-4 contain all of the results for RM-RTI-CFS-2407-AMPS-4, including primary and secondary method replicate analysis results, overall statistical results, and QC results.

Table 2-15. Multiple Drug Analyte Hair Reference Material Results

Reference Material	Analyte	Target Concentration (pg/mg)	Reference Range (pg/mg)	% CV (Individual Laboratory % CV Range)
RM-RTI-CFS-2407-AMPS-4	AMP	750 (each analyte)	1352 ± 600	13.4% (7.8%, 9.1%, 12.6%)
	MAMP		1507 ± 473	11.2% (7.0%, 8.9%, 11.5%)
	MDMA		1294 ± 294	7.4% (5.2%, 6.2%, 7.6%)

Note: AMP = amphetamine; MAMP = methamphetamine; MDMA = methylenedioxymethamphetamine (Ecstasy)

2.2.5 Conclusions

There are currently 145 multiple drug analyte HRM aliquots containing AMPs, MAMP, and MDMA that are currently available for purchase and distribution to forensic laboratories. RTI has received approval from NIJ to sell these materials, with the proceeds going toward funding future productions of materials.

3. CONCLUSIONS

3.1. Discussion of Limitations of Produced Hair Reference Materials

Because hair is a solid matrix, this makes it one of the most difficult to determine concentrations of drugs of abuse and to produce QC samples to be used by forensic laboratories. Several efforts have attempted to provide PT samples and HRMs for hair using fortified and known drug-user hair. In studies (**Table 3-1**), for which a QC sample was evaluated by a system of multiple hair testing laboratories, the variability between laboratory testing methods resulted in substantial variation in quality control sample results (Welch et al., 2003; Ropero-Miller, 2005 and 2007b; Ventura, 2008; Jurado, 2003). Efforts that have resulted in tight distributions of reported results (**Table 3-2**) have necessitated consistent methods of sample preparation such as conducting all the testing within the same laboratory (Welch et al., 2003; Lee et al., 2008). The purpose of this project was to produce an HRM that reflects the current status of hair testing with ranges developed that are pertinent to the inter-laboratory variation currently inherent in hair testing and to be at concentrations relevant to the cutoffs generally used.

HRMs are subject to many limitations because they are not ideal matrices for easily reproducible QC samples. Each limitation must be carefully evaluated against the objectives and preferred characteristics of the QC samples being manufactured. RTI's study design for this project was faced with six primary limitations; therefore, a chosen approach was necessary for each limitation. RTI will continue to refine our methodologies to improve the variability observed in future HRMs.

Intact hair strands versus pulverization: The hair was not pulverized and was left as slightly longer strands of approximately 1–3 cm. This approach was used because our previous experience with NLCP pilot PT studies indicated that pulverized hair was not consistent with typical samples received and the preparation methods used by laboratories. Not all laboratories pulverize hair as part of sample preparation. With our study design, the hair is well homogenized in the fortification process, and then again before packaging. Because of the necessity of using hair strands longer than 10 cm to accommodate having a large quantity of material all produced from the same hair source, the hair may be variable in its physical condition from root end of the hair to tip. This is typical of hair that has weathered in the environments and hygienic treatments (e.g., shampooing, perming, and/or coloring). The physical conditions of the hair may account for some of the variability observed in the results, but the extent was not independently measured and is represented by the intra-laboratory variability, which also includes each laboratory's analytical variability; however, intra-laboratory variation was relatively tight (average % CV 9.6).

Inter-laboratory variation was largely due to differences in laboratory processes. Additionally, there was no correlation between fill order of the vials and concentration obtained, which also supports the aliquots being homogenous (see **Appendix B**). Our ranges were larger than those reported by Welch and colleagues (2003), but those materials had 3 to 4000 times the concentration of drug present, and they were all tested using the same sample preparation methods in the same laboratory. Again, our objective was to produce an HRM that is useful and reflective of the current state of testing which is highly variable between laboratories.

Stock hair consisting of drug-user pool versus external fortification of drug-free hair: The use of external fortification of drug-free hair may not be representative of the deposition from endogenous routes. Although this is likely because the deposition mechanisms are highly variable, the compromise of more controlled knowledge of the drugs present is warranted. Results reported by HAIRVEQ, SOHT, and NLCP for PT indicate that there is more variability with drug-user PT samples in comparison to external fortification of drug-free hair (**Table 3-1**). Moreover, HRMs currently available for hair are prepared at high concentrations that cannot help a laboratory evaluate their calibration curves at the low end. RTI wanted to produce HRMs that were two to three times as common as the confirmatory cut-off concentration and drug-user hair as an initial would be extremely difficult to obtain at the required volume of hair needed. The objective of this study was to produce an HRM pertinent to the current state of hair testing. These materials reflect the current state of highly variable between laboratory methodologies. Individual laboratories are able to perform consistently on materials, but different laboratories have highly variable results regardless of the material (Ropero-Miller, 2005 and 2007b; Juardo, 2003; Ventura, 2008).

Table 3-1. Comparison of Hair Proficiency Results for Analytes Included in This Study

Analyte	Proficiency Testing Organization	Type of PT	Number of Labs	Target or Reference Concentration (pg/mg)	Range (pg/mg)	Mean (pg/mg)	SD (pg/mg)	%CV
COC	HAIRVEQ	Drug-user hair (In vivo ingestion)	22-28	150800	NR	NR	NR	42.1-80.7
COC	SOHT	Drug-user hair (In vivo ingestion)	15	3190	600-3900	2160	900	41.7
COC	NLCP	Drug-user hair (In vivo ingestion)	5	5000	1686-16396	6271	2438	38.9
COC	NLCP	Fortification of Drug-free hair (In vitro spike)	5	1500	485-787	1224	216	17.6
MDMA	SOHT	Drug-user hair (In vivo ingestion)	7	750	300-1400	740	330	44.6
MDMA	NLCP	Fortification of Drug-free hair (In vitro spike)	5	900	325-1214	784	195	24.9
MDMA	NLCP	Fortification of Drug-free hair (In vitro spike)	5	450	90-603	380	98	25.8
MOR	HAIRVEQ	Drug-user hair (In vivo ingestion)	22-27	3600	NR	NR	NR	50.5-75.7
MOR	SOHT	Drug-user hair (In vivo ingestion)	15	2450	500-4000	1320	920	69.7
MOR	NLCP	Drug-user hair (In vivo ingestion)	5	630	124-1879	510	332	65.2
MOR	NLCP	Fortification of Drug-free hair (In vitro spike)	5	600	146-873	364	124	34.0
THC	SOHT	Drug-user hair (In vivo ingestion)	6	1540	200-1900	580	540	93.1
THCA	NLCP	Drug-user hair (In vivo ingestion)	3	0.82	0.36-1.37	0.71	0.24	34.3
THCA	NLCP	Fortification of Drug-free hair (In vitro spike)	3	0.15	0.09-0.29	0.15	0.05	31.8

Note: COC = cocaine; MDMA = methylene dioxymethamphetamine; MOR = morphine; NLCP = National Laboratory Certification Program; NR = not reported; SD = standard deviation; SOHT = Society of Hair Testing; THC = delta-9-tetrahydrocannabinol; THCA = 11-Nor-delta-9-THC-9-carboxylic acid

Determining homogeneity of the HRM: Hair is a solid matrix with varying physical and chemical attributes along each individual hair shaft. The ability to produce a homogenous reference material that will allow consistent results among laboratories is extremely difficult. Cuticle composition and porosity differences contribute to variation in a hair's homogeneity and subsequently hair drug testing results. Several steps in the study design were implemented to

minimize this non-homogeneity of samples. First, all of the HRMs were produced using hair from the same individual. At several points in the fortification process, the hair was homogenized by manual mixing, cutting it into smaller segments, and allowing the hair to flow freely in solution during the fortification process. Second, multiple aliquots taken from randomly selected bottles during the fill order process were sent to multiple laboratories to assess the homogeneity of the samples. Again, lack of a correlation between the determined fill order and concentrations supports the aliquots being homogenous and an equal probability of any individual vial containing different portions of the hair shaft.

Laboratories tested both within and between aliquots with consistent intra-laboratory results. Variability ranged from 5.2–13.8 % CV with an average of 9.6. This intra-laboratory variability for RTI produced HRMs was higher than those reported by Lee and colleagues (2008) and Welch and colleagues (2003). This suggests that RTI will need to improve our methods of obtaining sample homogeneity. One way to do this may be to cut the HRMs into smaller segments prior to fortifying the hair with drug analyte(s) and by adapting more rigorous homogeneity methods prior to preparing individual aliquots of 100 mg (e.g., prolonged manual or mechanical mixing). It is just as likely that a laboratory sampling within a sample aliquot will have a mixture of locations on the hair shaft as sampling between aliquots. Although pulverization may offer a more easily homogenized sample, RTI would like to continue producing HRM as hair strands to allow more laboratories to use as much of their standard protocols as possible. However, when evaluating the drug concentrations to establish the reference range of the HRMs, a small subset of pulverized hair aliquots may be investigated.

High variability of HRMs: Comparing the variability of RTI's HRMs to those previously produced shows that RTI's variability is higher. A primary objective of RTI's approach was to provide an HRM using results from multiple laboratories to determine the reference ranges. This approach goes one step beyond prior HRMs, where the stated objective was to measure the reproducibility of two instrumental methods (Welch et al., 2003) or two extraction methods (Lee et al., 2008) performed by one laboratory with other potential variables restricted (e.g., preparation of hair prior to extraction, technician proficiency). Moreover, the extraction methods used by Lee and colleagues were similar, and more distinct extraction methods (i.e., enzymatic digestion) were not reported. Variation between laboratories is substantial, particularly for the THCA and MOR materials. Components of this variability include the material, differences in laboratory methodology, and analytical differences. The purpose of manufacturing this material was to attempt to create a material that represents the current state of hair testing and one that would be applicable to the situation. Currently, the methods by which laboratories analyze hair is highly variable, and substantial impact on results would be expected from varying the use of decontaminations, the types of decontaminations, and variations in extraction preparation, including the use of pulverization, enzymatic hydrolysis, or no steps to break down the hair matrix. All of which are reported by laboratories that conduct hair testing (Welch et al., 2003; Ropero-Miller, 2005 and 2007b; Juardo, 2003, Ventura, 2008). As previously stated, RTI will continue studies to improve variation of our HRMs. Although the inter-laboratory variability is difficult to control, improvement in the homogeneity of the sample may improve this variability by 5% to 10% based on the estimated variability of the produced materials.

Table 3-2. Testing Variability of RTI HRMs to Variability of Previously Published HRMs

Reference Material Analyte	Type of Hair Reference Material	Manufacturer	Reference	Method 1 (M1)	M1 Mean (pg/mg)	M1 RSD (%)	Method 2 (M2)	M2 Mean (pg/mg)	M2 RSD (%)	Difference %	Certified Value Mean* (pg/mg)	Uncertainty (U)
COC	Fortify Drug-free hair	NIST	Welch, 2003	LC/MS	7280	1	GC/MS	7630	0.9	4.8	7450	400
COC	Fortify Drug-free hair	RTI	Current Study	GC/MS	2433	14.8	LC/MS	2418	9.2	-0.6	2212	672
AMP	Fortify Drug-free hair	NIST	Welch, 2003	LC/MS	5860	2.3	GC/MS	6140	2.7	4.6	6000	320
AMP	Fortify Drug-free hair	RTI	Current Study	LC/MS	1347	9.1	GC/MS	1648	7.8	22.3	1352	600
AMP	Fortify Drug-free hair	RTI	Current Study	LC/MS/MS	1096	12.6	GC/MS	1648	7.8	50.4	1352	600
AMP	Fortify Drug-free hair	RTI	Current Study	LC/MS/MS	1096	12.6	LC/MS	1347	9.1	22.9	1352	600
AMP	Authentic Drug-user pool	NISI	Lee et al, 2008	GC/MS; agitation in 1% HCl in methanol for 20 h at 38 ◦C	530	7.6	GC/MS; ultrasonication methanol/5M HCl (20:1) for 1 h followed overnight storage in 1% HCl in methanol for 20 h at 38◦C	540	9.2	1.9	540	70
MAMP	Fortify Drug-free hair	NIST	Welch, 2003	LC/MS	5310	0.8	GC/MS	5090	1.2	-4.2	5200	270
MAMP	Fortify Drug-free hair	RTI	Current Study	LC/MS	1535	8.9	GC/MS	1597	7.0	4.0	1507	473
MAMP	Fortify Drug-free hair	RTI	Current Study	LC/MS/MS	1224	11.5	GC/MS	1597	7.0	30.5	1507	473
MAMP	Fortify Drug-free hair	RTI	Current Study	LC/MS/MS	1224	11.5	LC/MS	1535	8.9	25.4	1507	473
MAMP	Authentic Drug-user pool	NISI	Lee et al, 2008	GC/MS; agitation in 1% HCl in methanol for 20 h at 38 ◦C	7630	7.1	GC/MS; ultrasonication methanol/5M HCl (20:1) for 1 h followed overnight storage in 1% HCl in methanol for 20 h at 38◦C	7650	7.4	0.3	7640	1240
MDMA	Fortify Drug-free hair	RTI	Current Study	LC/MS	1287	5.2	GC/MS	1445	6.2	12.3	1507	473
MDMA	Fortify Drug-free hair	RTI	Current Study	LC/MS/MS	1186	7.6	GC/MS	1445	6.2	21.8	1507	473
MDMA	Fortify Drug-free hair	RTI	Current Study	LC/MS/MS	1186	7.6	LC/MS	1287	5.2	8.5	1507	473
MOR	Fortify Drug-free hair	NIST	Welch, 2003	LC/MS	10960	1	GC/MS	10110	2.4	-8.0	105400	680
MOR	Fortify Drug-free hair	RTI	Current Study	GC/MS	750	9.3	LC/MS/MS	579	10.1	-22.8	627	320
MOR	Fortify Drug-free hair	RTI	Current Study	LC/MS	350	9.5	LC/MS/MS	579	10.1	65.4	627	320
MOR	Fortify Drug-free hair	RTI	Current Study	LC/MS	350	9.5	GC/MS	750	9.3	114.0	627	320
THCA	Fortify Drug-free hair	RTI	Current Study	LC/MS/MS	0.151	12.0	GC/GC/MS	0.27	12.7	80.0	0.25	0.17

Notes: NISI = National Institute of Scientific Investigation (Korea); NIST = National Institute of Standards and Technology (USA); LC/MS = liquid chromatography/mass spectrometry; GC/MS = mass chromatography/mass spectrometry; NR = not reported; RSD (%CV) = 100*(SD/mean)

NIST Uncertainty was calculated by taking the square root of the sum of the squares of the uncertainty from the “Bayesian BOB” calculation, an uncertainty component for the purity of the reference compound used, and an uncertainty component for the possibility of incomplete extraction. The standard uncertainty was multiplied by a coverage factor of two to get the expanded uncertainty.

RTI Uncertainty was calculated by taking the square root of variance calculation. The Procedure Variance Components [PROC VARCOMP; SAS Version 9.1.3 (XP PRO procedure)] was employed to estimate the two sources of variability (intra- and inter-laboratory) which handles general linear models that have “random effects” that can be generalizable to all possible laboratories, not just the ones chosen for this study. Random effects are classification effects with levels that are assumed to be randomly selected from an infinite population of possible levels. The standard uncertainty was multiplied by a coverage factor of two to get the expanded uncertainty.

NISI Uncertainty was calculated as URM = k*SQRT*sum of the squares [u2(habm) + u2(hubm) + u2(cabm) + u2(cubm)] where k is coverage factor (k = 2), u(habm) is the uncertainty in the homogeneity test by the agitation-based method, uhubm is the uncertainty in the homogeneity test by the ultrasonication-based method, u(cabm) is the uncertainty in the characterization by the agitation-basedmethod and u(cubm) is the uncertainty in the characterization by the ultrasonication-based method.

References: Welch 2003-NIST (Anal Bioanal Chem); Lee et al 2008-NISI (J Chrom B)

Stability studies of HRMs: RTI's previous experience with using hair manufactured in the same manner as hair from this study has demonstrated that the samples are stable to within 20% of original means over an 18-month period when the materials are stored dry, in the dark, and at room temperature (Ropero-Miller, 2005 and 2007b). For COC-fortified PT samples, BE appears to continue to form over time in situ in the hair, but the COC concentration was not impacted by this process. RTI will continue to monitor the stability of these HRMs.

Lack of metabolites in the HRMs: The materials do not contain all metabolites that may be pertinent to a particular drug. Although it is preferable to include all compounds, again highly variable testing procedures impacted the choices made in manufacturing these samples. Not all laboratories test for all of the potential metabolites of all drugs. Laboratories that currently provide hair testing services were surveyed to ensure that the compounds selected would be the most useful to the greatest number of laboratories. RTI continues to survey the laboratories to determine what drugs are of use to the community for use in an HRM.

3.2. Discussion of Findings

Hair-drug fortification processes are used by laboratories to prepare QC samples (e.g., matrix-matched calibrator or PT samples) that will allow them to assess analytical processes and instrumental performance. The HRMs developed and produced by RTI demonstrated high variability or uncertainty in the concentrations reported by all reference laboratories (% CV range 7.4–28.6, average 17.9), but each individual laboratory % CVs demonstrated much less variability (% CV range 5.2–14.8, % CV average 9.6). This intra-laboratory variability for RTI produced HRMs that were higher than those reported by Lee and colleagues (2008) and Welch and colleagues (2003). This suggests that RTI will need to improve our methods of obtaining sample homogeneity, most likely through reducing the size of the hair strands prior to drug fortification and adapting more rigorous homogeneity methods prior to the preparation of individual aliquots of 100-110 mg. These findings suggest that the system of laboratories and analytical procedures used in this study design represent the current state of hair testing laboratories, and laboratories performed well within their own system of protocols, but inter-laboratory agreement was not as good. Again, this was consistent with laboratories that perform analyses without an adequate reference material upon which to standardize the system's performance. RTI has observed similar findings while working with the NLCP pilot hair performance program over the past 7 years.

The purpose of this project was to produce an HRM that reflects the current status of hair testing with ranges developed that are pertinent to the inter-laboratory variation currently inherent in hair testing and to be at concentrations relevant to cutoffs generally used. Because of RTI's approach, it was anticipated that our concentration reference ranges for the produced HRMs would demonstrate greater variability than other previously produced HRMs. Efforts that have resulted in tight distributions of reported results have necessitated consistent methods of sample preparation, such as conducting all the testing within the same laboratory (Welch et al., 2003; Lee et al., 2008). In studies, for which a QC sample was evaluated by a system of multiple hair testing laboratories, the variability between laboratory testing methods resulted in substantial variations in QC sample results (Welch et al., 2003; Ropero-Miller, 2005 and 2007b; Ventura, 2008; Jurado, 2003).

Approximately 500 vials of four HRM products are available for distribution to forensic laboratories. RTI has received approval from NIJ to sell these materials, with the proceeds going toward funding future production of materials. Forensic laboratories will benefit from appropriate HRMs that have been validated, quality controlled, quality assured, and determined with realistic reference ranges with estimated uncertainty and implemented for forensic use, thereby promoting these characteristics into the data they generate. These HRMs will add a layer of forensic reliability for the laboratory's data to its clients, the court, and the individuals being tested. This research provides external HRMs with four drugs of abuse classes near the confirmatory cut-off/threshold concentrations currently used by hair testing laboratories.

3.2.1 Implications for Policy and Practice

Regardless of the forensic application, the results from hair tests, conducted in support of crime investigations, postmortem toxicology, or workplace drug testing, are only as good as the control and calibration upon which they are based. Having access to quality HRMs refereed in independent laboratories and independent from PT materials will allow laboratories to produce quality results that are standardized between laboratories and are comparable.

This work will directly affect the use of hair testing in drug-related criminal cases, workplace drug testing, and other legal arenas, such as child custody and abuse, parole, and probation hearings. The study results will affect how hair testing results are interpreted and may significantly impact whether governmental agencies and other employers use hair testing to drug test individuals in their programs. HRMs can help regulate laboratory performance and improve the reliability of hair testing results to better withstand emerging and potentially harsher legal requirements and the laboratory's ability to assure quality and demonstrate forensically defensible analytical performance.

3.2.2 Implications for Further Research

These results demonstrate that four distinct classes of drugs of abuse could be fortified into hair at concentrations currently used in forensic applications. There are many other drug analytes that have been successfully detected in hair, and HRMs fortified with these analytes could be produced if a demand is established in the forensic community.

RTI has received approval from NIJ to sell these HRMs to interested forensic laboratories so that the proceeds could be used to produce more HRMs, leading eventually to a self-sustaining commercial product. RTI will continue to monitor the most popular drugs of abuse determined in hair and will work to produce additional HRMs in the future. For instance, RTI is currently surveying all attendees of a Web-based, on-demand training course (see Section 5, *Dissemination of Research Findings*), which began mid-March 2008. Preliminary findings of the Web-based survey indicate that 25%, or 5 out of 20, recommends a benzodiazepines HRM, and 25%, or 5 out of 20, recommends another opiate HRM. Investigations that RTI would like to pursue include the following:

- Homogeneity studies to improve sample variability
- Production of additional analyte HRMs to include benzodiazepines
- Production of additional multi-analyte HRMs

- Production of multi-analyte/multi-drug class HRMs
- Production of HRMs targeted at initial testing or screening cut-off concentrations.

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5. DISSEMINATION OF RESEARCH FINDINGS

Portions of this final report will be used to prepare at least one manuscript for publication. Research findings of this project have been presented at the following annual meetings:

1) Ropero-Miller, J.D, P.R. Stout, E.J. Minden, M.A. Meaders, M.R. Baylor, and J.M. Mitchell. 2007. *Development and Production of Hair Reference Materials for Use as Control and Calibration for Hair Drug Testing*. Presented at the Annual TIAFT 2007, Seattle, WA. August 26–30.

2) Ropero-Miller, J.D., P.R. Stout, E.J. Minden, M.A. Meaders, M.R. Baylor, and J.M. Mitchell. 2007. *Development and Production of Hair Reference Materials for Use as Control and Calibration for Hair Drug Testing.* Presented at the Annual SOFT Meeting 2007, Raleigh, NC. October 14–19.

3) Ropero-Miller, J.D. 2008. NIJ Grantees' Meeting—RTI's Forensic Toxicology Research and Development Program. AAFS, Washington, DC. February 19.

In March 2008, the presentation given at the NIJ Grantees' Meeting was modified into one of a three-part Web-based continuing education module entitled, February 2008 AAFS Presentations on NIJ Projects (available at www.rti.org/forensiced). This training module, which further disseminates the research findings of this project, is now a part of an NIJ cooperative agreement (2007-DN-BX-K208), with a goal to develop and deliver online continuing education for forensic scientists.

In addition, RTI has publicized the availability of these reference materials to the forensic community by disseminating marketing flyers (see an example in Appendix A-3) at professional meetings of The International Association of Forensic Toxicologists (August 2007), SOFT (October 2007), and AAFS (February 2008). RTI also performed an extensive review of the hair testing literature to extract contact information for researchers and scientists involved in hair testing. Through this effort, we collected names, addresses, e-mail addresses, and telephone numbers for more than 300 forensic practitioners worldwide. This contact information will further allow RTI to disseminate information on the availability of these HRMs to more potentially interested forensic laboratories. RTI completed a mass mailing effort to send information to all of these contacts in June 2008.

APPENDIX A

DISSEMINATION OF RESEARCH FINDINGS AND MARKETING EFFORTS

A-1 CERTIFICATE OF ANALYSIS FOR THCA HRM

RTI Center for Forensic Sciences

CERTIFICATE OF ANALYSIS

I. Product Information

RTI Lot Number: RM-RTI-CFS-2407-THCA-1

RTI Specimen Number: 11367-38-1-4-THCA 94

Date Manufactured: March 2007
Container: 20-mL glass, wide-mouth, foil-seal, screw-top, RTI amber bottles

Fill Size: 100-130 milligrams

II. Quality Control (QC) Information

Analyte	Target (pg/mg)	Mean Concentration (pg/mg)	QC Data	
			Confirmation Testing	Reference Range (pg/mg)
THCA	0.30	0.269	Agilent GC/GC/ MS 5973 (n=79)	0.201-0.337
		0.151	Perkin Elmer SCIEX API 2000 PCI LC/MS/MS (n=19)	0.115-0.187
			OVERALL	**0.25 ± 0.17**

*Deviation acceptable to RTI: 10/01/07

Note: Sample screened for amphetamines (500 pg/mg), cocaine analytes (500 pg/mg), opiates (200 pg/mg), PCP (300 pg/mg), cannabinoids (1.0 pg/mg), MDMA (500 pg/mg), prior to drug fortification protocol using radioimmunoassay techniques

III. Product Characterization

Material Type: Human hair

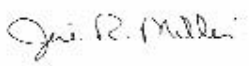
Physical description/visual appearance of specimen: Visual appearance of long brown hair strands-Schwarzkopf 4.0 medium brown

Stability :	Initial Stability Duration	Storage requirements:	Temperature:
	12 months	Keep in dark, dry environment (container provides this requirement if maintained closed)	Room temperature not to exceed 37°C

IV. Statement of Compliance

I attest that this product is in compliance with RTI manufacturing specifications.

VI. Signature of person responsible for release of product



VII. Date of COA Submission

August 17, 2007

coa.doc
2/13/2008

A-2 HRMs Overview and Instructions for Laboratory Use

RTI INTERNATIONAL | Center for Forensic Sciences
3040 Cornwallis Road ▪ PO Box 12194 ▪ Research Triangle Park, NC 27709-2194 ▪ USA
Telephone 919.541.7242 ▪ Fax 919.541.7042 ▪ www.rti.org

RTI International's Drugs of Abuse in Hair Reference Material

Instructions for Use: DO NOT decontaminate hair reference material prior to extraction procedures. These reference materials were washed by RTI as a post-production process prior to storage and shipment. A laboratory should use their standard operating procedure (SOP) and instrumental setup for the analyte(s) of interest contained in the hair reference material. An appropriate amount of hair should be weighed based on your laboratory's SOP. Confirmatory analytical procedures were used to quantitate the analyte of interest and establish the hair reference ranges. The reference range for the hair reference material is detailed in the *Certificate of Analysis*. No qualitative testing (e.g. immunoassay) has been performed on these samples by RTI and laboratories should evaluate their cut-off concentration and limit of detection prior to using these hair reference materials for initial testing purposes.

Purpose: External hair reference materials were developed and produced by RTI International. Once reference materials were produced, a validation scheme in which representative, random aliquots of the final product were sent to multiple reference laboratories for analysis by at least two analytical techniques [i.e., GC/MS, GC/GC/MS, LC/MS, LC/MS/MS] to determine a realistic reference range for drug analyte concentrations fortified into hair. These reference materials will assist forensic laboratories to improve the defensibility of their analytical results by documenting their ability to accurately measure drugs of abuse in hair.

Production of Reference Materials: In RTI's fortification model, hair is placed in a solution containing drug analyte(s) of interest, which leads to incorporation of some of the drug into the hair. RTI's production process introduced drug-free human head hair to a fortification solution containing target drug analytes. Once the hair was fortified with drug to achieve a target concentration at least twice the proposed confirmatory cutoff concentrations, an effective decontamination protocol previously examined by RTI and others was used to remove weakly associated compounds. These hair samples were then submitted in a blinded manner to reference laboratories for the determination of drug analyte concentrations.

Analysis and Validation of Reference Materials: Analytical work was conducted by three independent forensic laboratories. Analysis was defined as primary or secondary based on the number of replicate analyses a laboratory was requested to complete. Primary analysis was targeted for the separate analysis of 72 replicates by one laboratory using analytical techniques chosen by the laboratory. Since RTI performed decontamination washes before aliquoting and shipping hair samples, reference laboratories were instructed to analyze samples using standard operating procedures for specimen preparation, excluding decontamination, and to perform instrumental analysis using multiple calibrators and multiple analysis days. Secondary analysis was conducted using an analytical technique theoretically distinct from the primary method and fewer analyses (15 to 20 replicates) were performed. All technologies provided ample sensitivity for these studies. Blinded positive and negative controls were included for analysis at no less than a 10% frequency.

Analytical results were evaluated statistically to determine sample homogeneity, accuracy, precision, and uncertainties for each analyte. To estimate the within and between laboratory and analytical method variability, SAS statistical software [SAS Version 9.1.3 (XP PRO procedure)] calculated the uncertainty or variability as 2 times the square root of the calculated variance. Analyte concentration reference ranges were defined as the mean concentration plus or minus the uncertainty measurement [Average ± 2*SQRT(VAR)].

Revision Date: 6/1/2008

turning knowledge into practice

A-3 MARKETING FLYER TO PROMOTE AVAILABILITY OF HRMS

RTI INTERNATIONAL

Analytical Hair Reference Materials Are Now Available to Forensic Testing Laboratories

Limited quantities of hair reference materials are now available for purchase by forensic testing laboratories from the Center for Forensic Sciences at RTI International (RTI). Laboratories can benefit from four distinct hair reference materials that have been manufactured under strict quality control and validated for forensic use. These matrix-matched calibrators or quality controls will enhance the supportability of forensic data by allowing laboratories to include external reference materials with drugs of abuse at near cut-off/threshold concentrations in their analytical procedures.

The following reference materials are available, and a Certificate of Analysis will be provided.

Reference Material	Drug	Concentration Range	Price*/Quantity
RM-RTI-CFS-2407-THCA-1	THCA	0.25 ± 0.17 pg/mg	$615/100 mg
RM-RTI-CFS-2407-MOR-2	Morphine	627 ± 320 pg/mg	$615/100 mg
RM-RTI-CFS-2407-COC-3	Cocaine	2212 ± 672 pg/mg	$615/100 mg
RM-RTI-CFS-2407-AMPS-4	• Amphetamine • Methamphetamine • Methylenedioxymethamphetamine (MDMA, Ecstasy)	• 1352 ± 600 pg/mg • 1507 ± 473 pg/mg • 1294 ± 294 pg/mg	$725/100 mg

* All prices are listed in U.S. currency and do not include shipping and handling.

RTI has been producing urine performance testing materials for over 20 years. In 2000, RTI expanded its program to include alternate matrices such as hair and oral fluid. In 2007, as part of a research effort funded by the National Institute of Justice,* RTI began producing reference materials for hair to be used by forensic laboratories as calibrators and controls for quality assurance and quality control measures.

For purchase information, contact

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RTI-5607 2/2008

* NIJ Award No: 2006-DN-BX-K012

APPENDIX B

REFERENCE MATERIAL RESULTS

B-1 Results of RM-RTI-CFS-2407-THCA-1

Appendix B-1-1. Primary Analytical Method Results of RM-RTI-CFS-2407-THCA-1

Sample ID	Replicate	THCA (pg/mg)
THCA1-1	1	0.27
THCA1-1	2	0.30
THCA1-1	3	0.28
THCA1-2	1	0.32
THCA1-2	2	0.32
THCA1-2	3	0.29
THCA1-3	1	0.32
THCA1-3	2	0.30
THCA1-3	3	0.31
THCA1-4	1	0.32
THCA1-4	2	0.31
THCA1-4	3	0.29
THCA1-5	1	0.30
THCA1-5	2	0.32
THCA1-5	3	0.32
THCA2	1	0.32
THCA2	2	0.32
THCA2	3	0.17
THCA2	4	0.22
THCA7	1	0.28
THCA7	2	0.32
THCA7	3	0.24
THCA7	4	0.26
THCA8	1	0.24
THCA8	2	0.24
THCA8	3	0.28
THCA8	4	0.27
THCA8	5	0.28
THCA16	1	0.27
THCA16	2	0.28
THCA16	3	0.26
THCA16	4	0.20
THCA20	1	0.28
THCA20	2	0.28
THCA20	3	0.28
THCA20	4	0.23
THCA25	1	0.27
THCA25	2	0.28
THCA25	3	0.21

Sample ID	Replicate	THCA (pg/mg)
THCA25	4	0.23
THCA34	1	0.22
THCA34	2	0.26
THCA34	3	0.32
THCA34	4	0.27
THCA34	5	0.27
THCA40	1	0.28
THCA40	2	0.28
THCA40	3	0.26
THCA40	4	0.27
THCA45	1	0.28
THCA45	2	0.29
THCA45	3	0.21
THCA45	4	0.26
THCA59	1	0.27
THCA59	2	0.23
THCA59	3	0.25
THCA59	4	0.25
THCA68	1	0.28
THCA68	2	0.25
THCA68	3	0.27
THCA68	4	0.27
THCA73	1	0.26
THCA73	2	0.22
THCA88	1	0.28
THCA88	2	0.28
THCA88	3	0.20
THCA88	4	0.25
THCA92	1	0.28
THCA92	2	0.28
THCA92	3	0.28
THCA92	4	0.29
THCA94	1	0.26
THCA94	2	0.26
THCA94	3	0.27
THCA94	4	0.28
THCA107	1	0.22
THCA107	2	0.18
THCA107	3	0.30
THCA107	4	0.25
Mean		0.27
Standard deviation		0.03

Sample ID	Replicate	THCA (pg/mg)
% Coefficient of variation		12.7
n		79

Appendix B-1-2. Secondary Analytical Method Results of RM-RTI-CFS-2407-THCA-1

Sample ID	Replicate	THCA (pg/mg)
THCA3	1	0.16
THCA3	2	0.16
THCA3	3	0.17
THCA3	4	0.18
THCA3	5	0.13
THCA21	1	0.16
THCA21	2	0.16
THCA47	1	0.16
THCA47	2	0.15
THCA67	1	0.16
THCA67	2	0.16
THCA67	3	0.17
THCA67	4	0.12
THCA67	5	0.14
THCA101	1	0.15
THCA101	2	0.12
THCA101	3	0.15
THCA101	4	0.13
THCA101	5	0.15
Mean		0.15
Standard deviation		0.02
% Coefficient of variation		12.0
n		19

Appendix B-1-3. Overall Statistical Results of RM-RTI-CFS-2407-THCA-1

THCA Overall Results	All Methods
Mean (pg/mg)	0.25
Standard deviation (pg/mg)	0.06
% Coefficient of variation	23.0
n	98
Total variance	0.0080
Uncertainty (pg/mg)	0.1788
Reference range (pg/mg)	0.246 ± 0.17

Appendix B-1-4. Control Results of RM-RTI-CFS-2407-THCA-1

Control Results	Control Type	Reported THCA (pg/mg)
THCA128-1	NEG	ND
THCA128-2	NEG	ND
THCA125-1	NEG	ND
THCA125-2	NEG	ND
THCA125-3	NEG	ND
THCA125-4	NEG	ND
THCA126-1	POS	0.10
THCA126-2	POS	0.10
THCA126-3	POS	0.10
THCA126-4	POS	0.09
THCA131-1	NEG	ND
THCA131-2	NEG	ND
THCA129-1	POS	0.07
THCA129-2	POS	0.06

NEG = negative; POS = positive; ND = none detected

B-2 Results of RM-RTI-CFS-2407-MOR-2

Appendix B-2-1. Primary Analytical Method Results of RM-RTI-CFS-2407-MOR-2

Sample ID	REP	MOR (pg/mg)	COD (pg/mg)	6-AM (pg/mg)
5A	1	573	ND	ND
5A	2	567	ND	ND
5B	1	588	ND	ND
5B	2	564	ND	ND
5C	1	570	ND	ND
5C	2	580	ND	ND
MOR2	1	694	ND	ND
MOR2	2	725	ND	ND
MOR2	3	715	ND	ND
MOR2	4	706	ND	ND
MOR2	5	736	ND	ND
MOR2	6	725	ND	ND
MOR2	7	735	ND	ND
MOR2	8	751	ND	ND
MOR106	1	794	ND	ND
MOR106	2	730	ND	ND
MOR106	3	775	ND	ND
MOR106	4	751	ND	ND
MOR106	5	758	ND	ND
MOR106	6	764	ND	ND

Sample ID	REP	MOR (pg/mg)	COD (pg/mg)	6-AM (pg/mg)
MOR106	7	752	ND	ND
MOR106	8	740	ND	ND
MOR16	1	787	ND	ND
MOR16	2	761	ND	ND
MOR16	3	819	ND	ND
MOR16	4	830	ND	ND
MOR16	5	824	ND	ND
MOR16	6	788	ND	ND
MOR16	7	776	ND	ND
MOR16	8	762	ND	ND
MOR45	1	812	ND	ND
MOR45	2	814	ND	ND
MOR45	3	810	ND	ND
MOR45	4	810	ND	ND
MOR45	5	809	ND	ND
MOR45	6	802	ND	ND
MOR45	7	756	ND	ND
MOR45	8	760	ND	ND
MOR88	1	810	ND	ND
MOR88	2	823	ND	ND
MOR88	3	810	ND	ND
MOR88	4	815	ND	ND
MOR88	5	822	ND	ND
MOR88	6	757	ND	ND
MOR88	7	709	ND	ND
MOR88	8	648	ND	ND
MOR94	1	806	ND	ND
MOR94	2	808	ND	ND
MOR94	3	754	ND	ND
MOR94	4	782	ND	ND
MOR94	5	770	ND	ND
MOR94	6	719	ND	ND
MOR94	7	698	ND	ND
MOR94	8	721	ND	ND
MOR20	1	812	ND	ND
MOR20	2	806	ND	ND
MOR20	3	743	ND	ND
MOR20	4	747	ND	ND
MOR20	5	767	ND	ND
MOR20	6	770	ND	ND
MOR20	7	781	ND	ND
MOR20	8	784	ND	ND

Sample ID	REP	MOR (pg/mg)	COD (pg/mg)	6-AM (pg/mg)
Mean		750		
Standard deviation		70		
% Coefficient of variation		9.3		
n		62	0	0

Appendix B-2-2. Secondary Analytical Method Results of RM-RTI-CFS-2407-MOR-2

Sample ID	REP	MOR (pg/mg)	COD (pg/mg)	6-AM (pg/mg)
MOR101	1	575	ND	ND
MOR101	2	582	ND	ND
MOR101	3	501	ND	ND
MOR101	4	561	ND	ND
MOR101	5	563	ND	ND
MOR101	6	484	ND	ND
MOR101	7	558	ND	ND
MOR101	8	Bad injection	ND	ND
MOR67	1	596	ND	ND
MOR67	2	610	ND	ND
MOR67	3	544	ND	ND
MOR67	4	539	ND	ND
MOR67	5	554	ND	ND
MOR67	6	677	ND	ND
MOR67	7	661	ND	ND
MOR67	8	684	ND	ND
Mean		579	0	0
Standard deviation		59	0	0
% Coefficient of variation		10.1	0	0
n		15	0	0

Appendix B-2-3. Tertiary Analytical Method Results of RM-RTI-CFS-2407-MOR-2

Sample ID	REP	MOR (pg/mg)	COD (pg/mg)	6-AM (pg/mg)
MOR3	1	314	ND	ND
MOR3	2	296	ND	ND
MOR3	3	340	ND	ND
MOR3	4	339	ND	ND
MOR3	5	364	ND	ND
MOR21	1	321	ND	ND
MOR21	2	350	ND	ND
MOR21	3	348	ND	ND
MOR21	4	350	ND	ND
MOR21	5	381	ND	ND
MOR47	1	356	ND	ND

Sample ID	REP	MOR (pg/mg)	COD (pg/mg)	6-AM (pg/mg)
MOR47	2	389	ND	ND
MOR47	3	358	ND	ND
MOR47	4	374	ND	ND
MOR47	5	443	ND	ND
MOR59	1	337	ND	ND
MOR59	2	321	ND	ND
MOR59	3	352	ND	ND
MOR59	4	349	ND	ND
MOR59	5	399	ND	ND
MOR96	1	299	ND	ND
MOR96	2	305	ND	ND
MOR96	3	340	ND	ND
MOR96	4	351	ND	ND
MOR96	5	381	ND	ND
Mean		350	0	0
Standard deviation		33	0	0
% Coefficient of variation		9.5	0	0
n		25	0	0

Appendix B-2-4. Overall Statistical Results of RM-RTI-CFS-2407-MOR-2

Morphine Overall Results	All Methods MOR
Mean (pg/mg)	627
Standard deviation (pg/mg)	179
%R Standard Deviation	29
%Coefficient of variation	28.6
n	106
Total variance	25537
Uncertainty (pg/mg)	320
Reference range (pg/mg)	627 ± 320

Appendix B-2-5. Control Results of RM-RTI-CFS-2407-MOR-2

Control Results	Control Type	Reported MOR (pg/mg)	Reported COD (pg/mg)	Reported 6-AM (pg/mg)
MOR125	NEG	ND	ND	ND
MOR125	NEG	ND	ND	ND
MOR125	NEG	ND	ND	ND
MOR125	NEG	ND	ND	ND
MOR125	NEG	ND	ND	ND
MOR125	NEG	ND	ND	ND
MOR125	NEG	ND	ND	ND
MOR125	NEG	ND	ND	ND
MOR126	Pos	586	550	608

Control Results	Control Type	Reported MOR (pg/mg)	Reported COD (pg/mg)	Reported 6-AM (pg/mg)
MOR126	Pos	582	555	615
MOR126	Pos	558	556	605
MOR126	Pos	553	559	594
MOR126	Pos	541	534	583
MOR126	Pos	571	588	585
MOR126	Pos	589	592	576
MOR126	Pos	589	592	639
MOR127	NEG	ND	ND	ND
MOR127	NEG	ND	ND	ND
MOR127	NEG	ND	ND	ND
MOR127	NEG	ND	ND	ND
MOR127	NEG	ND	ND	ND
MOR127	NEG	ND	ND	ND
MOR127	NEG	ND	ND	ND
MOR127	NEG	ND	ND	ND
MOR129	NEG	ND	ND	ND
MOR129	NEG	ND	ND	ND
MOR129	NEG	ND	ND	ND
MOR129	NEG	ND	ND	ND
MOR129	NEG	ND	ND	ND

NEG = negative; POS = positive; ND = none detected

B-3 Results of RM-RTI-CFS-2407-COC-3

Appendix B-3-1. Primary Analytical Method Results of RM-RTI-CFS-2407-COC-3

Sample ID	REP	COC (pg/mg)	BE (pg/mg)	CE (pg/mg)	NCOC (pg/mg)
COC2	1	1969	100	78	ND
COC2	2	2715	78	46	ND
COC2	3	2489	79	39	ND
COC2	4	2135	91	43	ND
COC2	5	2959	84	55	ND
COC2	6	2742	100	55	ND
COC2	7	2823	80	54	ND
COC25	1	2354	91	88	ND
COC25	2	2536	86	51	ND
COC25	3	1958	88	38	ND
COC25	4	2746	93	53	ND
COC25	5	2957	92	55	ND
COC25	6	2987	90	58	ND
COC25	7	3071	86	52	ND
COC153	1	2223	82	77	ND
COC153	2	2098	80	ND	ND
COC153	3	2319	77	ND	ND
COC153	4	1892	91	ND	ND
COC153	5	2607	83	51	ND
COC153	6	2264	102	55	ND
COC153	7	2782	87	ND	ND
COC88	1	1765	99	79	ND
COC88	2	2233	76	ND	ND
COC88	3	2112	78	53	ND
COC88	4	2199	95	ND	ND
COC88	5	2731	86	55	ND
COC88	6	2780	78	51	ND
COC88	7	2817	88	ND	ND
COC40	1	2251	79	79	ND
COC40	2	2017	91	ND	ND
COC40	3	2058	85	ND	ND
COC40	4	2287	79	ND	ND
COC40	5	2361	83	51	ND
COC40	6	2890	88	57	ND
COC40	7	3098	87	53	ND
COC94	1	2089	84	79	ND
COC94	2	2201	96	ND	ND
COC94	3	2300	83	ND	ND
COC94	4	2365	79	ND	ND

Sample ID	REP	COC (pg/mg)	BE (pg/mg)	CE (pg/mg)	NCOC (pg/mg)
COC94	5	2822	83	54	ND
COC94	6	2603	81	65	ND
COC94	7	2924	96	51	ND
COC126	1	2260	70	ND	ND
COC126	2	2063	78	ND	ND
COC126	3	1860	89	ND	ND
COC126	4	2365	83	ND	ND
COC126	5	2734	79	51	ND
COC126	6	2921	86	51	ND
COC126	7	2784	87	ND	ND
COC114	1	1989	108	ND	ND
COC114	2	2611	76	ND	ND
COC114	3	2192	74	ND	ND
COC114	4	1882	82	ND	ND
COC114	5	2658	83	50	ND
COC114	6	2424	89	52	ND
COC114	7	2006	100	ND	ND
COC53	1	1984	95	ND	ND
COC53	2	2473	77	ND	ND
COC53	3	2209	88	ND	ND
COC53	4	2325	62	ND	ND
COC53	5	3089	69	ND	ND
COC53	6	2491	75	ND	ND
COC53	7	2458	76	ND	ND
Mean		2433	85	57	0
Standard deviation		359	9	12	0
% Coefficient of variation		14.8	10.1	21.4	0
n		63	63	33	0

FIR= failed ion ratio

Appendix B-3-2. Secondary Analytical Method Results of RM-RTI-CFS-2407-COC-3

Sample ID	REP	COC (pg/mg)	BE (pg/mg)	CE (pg/mg)	NCOC (pg/mg)
COC67	1	2674	76	ND	ND
COC67	2	2654	77	ND	ND
COC67	3	2686	52	ND	ND
COC67	4	2586	50	ND	ND
COC67	5	2587	52	ND	ND
COC67	6	2554	60	ND	ND
COC67	7	2537	60	ND	ND
COC67	8	FIR	FIR	ND	ND
COC101	1	2324	68	ND	ND

Sample ID	REP	COC (pg/mg)	BE (pg/mg)	CE (pg/mg)	NCOC (pg/mg)
COC101	2	2377	69	ND	ND
COC101	3	1883	53	ND	ND
COC101	4	2337	46	ND	ND
COC101	5	2348	47	ND	ND
COC101	6	2276	52	ND	ND
COC101	7	2287	57	ND	ND
COC101	8	2159	57	ND	ND
Mean		2418	58	0	0
Standard deviation		223	10	0	0
% Coefficient of variation		9.2	17.0	0	0
n		15	15	0	0

FIR = Failed ion ratio

Appendix B-3-3. Tertiary Analytical Method Results of RM-RTI-CFS-2407-COC-3

Sample ID	REP	COC (pg/mg)	BE (pg/mg)	CE (pg/mg)	NCOC (pg/mg)
COC21	1	1314	35	ND	ND
COC21	2	1329	35	ND	ND
COC21	3	1168	39	ND	ND
COC21	4	1460	48	ND	ND
COC21	5	1672	50	ND	ND
COC47	1	1331	36	ND	ND
COC47	2	1360	39	ND	ND
COC47	3	1233	41	ND	ND
COC47	4	1171	41	ND	ND
COC47	5	1552	44	ND	ND
COC81	1	1351	35	ND	ND
COC81	2	1297	35	ND	ND
COC81	3	1380	49	ND	ND
COC81	4	1403	47	ND	ND
COC81	5	1408	44	ND	ND
COC146	1	1272	33	ND	ND
COC146	2	1345	35	ND	ND
COC146	3	1319	43	ND	ND
COC146	4	1447	48	ND	ND
COC146	5	1386	44	ND	ND
Mean		1360	41	0	0
Standard deviation		118	6	0	0
% Coefficient of variation		8.7	13.5	0	0
n		20	20	0	0

Appendix B-3-4. Overall Statistical Results of RM-RTI-CFS-2407-COC-3

COC Overall Results	All Methods COC
Mean (pg/mg)	2212
Standard deviation (pg/mg)	527
% Coefficient of variation	23.8
n	98
Variance	111489
Uncertainty (pg/mg)	672
Reference range (pg/mg)	2212 ± 672

Appendix B-3-5. Control Results of RM-RTI-CFS-2407-COC-3

Control Results	Control Type	Reported COC (pg/mg)	Reported BE (pg/mg)	Reported CE (pg/mg)	Reported NCOC (pg/mg)
COC166	POS	917	64	113	79
COC166	POS	697	60	92	82
COC166	POS	677	59	87	64
COC166	POS	587	63	68	63
COC166	POS	838	65	71	95
COC166	POS	773	70	69	90
COC166	POS	640	68	61	77
COC167	NEG	ND	ND	ND	ND
COC167	NEG	ND	ND	ND	ND
COC167	NEG	ND	ND	ND	ND
COC167	NEG	ND	ND	ND	ND
COC167	NEG	ND	ND	ND	ND
COC167	NEG	ND	ND	ND	ND
COC167	NEG	ND	ND	ND	ND
COC170	NEG	ND	ND	ND	ND
COC170	NEG	ND	ND	ND	ND
COC172	NEG	ND	ND	ND	NA
COC172	NEG	ND	ND	ND	NA
COC172	NEG	ND	ND	ND	ND
COC172	NEG	ND	ND	ND	ND
COC172	NEG	ND	ND	ND	ND

NEG = negative; POS = positive; ND = none detected

B-4 Results of RM-RTI-CFS-2407-AMPS-4

Appendix B-4-1. Primary Analytical Method Results of RM-RTI-CFS-2407-AMPS-4

Sample ID	Replicate	AMP (pg/mg)	MAMP (pg/mg)	MDMA (pg/mg)	MDA (pg/mg)	MDEA (pg/mg)
AMP2	1	1327	1432	1252	ND	ND
AMP2	2	1343	1458	1262	ND	ND
AMP2	3	1408	1628	1246	ND	ND
AMP2	4	1459	1621	1290	ND	ND
AMP2	5	1238	1543	1279	ND	ND
AMP8	1	1531	1610	1422	ND	ND
AMP8	2	1474	1569	1351	ND	ND
AMP8	3	1492	1696	1335	ND	ND
AMP8	4	1497	1716	1318	ND	ND
AMP8	5	1263	1629	1336	ND	ND
AMP16	1	1524	1655	1410	ND	ND
AMP16	2	1468	1617	1333	ND	ND
AMP16	3	1535	1747	1369	ND	ND
AMP16	4	1538	1909	1366	ND	ND
AMP16	5	1242	1616	1355	ND	ND
AMP25	1	1485	1633	1396	ND	ND
AMP25	2	1535	1670	1415	ND	ND
AMP25	3	1472	1653	1346	ND	ND
AMP25	4	1506	1797	1369	ND	ND
AMP25	5	1280	1648	1375	ND	ND
AMP34	1	1355	1467	1261	ND	ND
AMP34	2	1391	1497	1300	ND	ND
AMP34	3	1530	1708	1341	ND	ND
AMP34	4	1451	1786	1293	ND	ND
AMP34	5	1276	1620	1331	ND	ND
AMP40	1	1385	1492	1258	ND	ND
AMP40	2	1494	1453	1458	ND	ND
AMP40	3	*Bad injection	*Bad injection	*Bad injection	*Bad injection	*Bad injection
AMP40	4	1426	1536	1267	ND	ND
AMP40	5	1204	1492	1288	ND	ND
AMP45	1	1275	1348	1285	ND	ND
AMP45	2	1234	1233	1277	ND	ND
AMP45	3	1206	1375	1175	ND	ND
AMP45	4	1195	1389	1183	ND	ND
AMP45	5	1059	1245	1157	ND	ND
AMP68	1	1075	1298	1203	ND	ND
AMP68	2	1275	1388	1277	ND	ND
AMP68	3	1287	1412	1227	ND	ND

Sample ID	Replicate	AMP (pg/mg)	MAMP (pg/mg)	MDMA (pg/mg)	MDA (pg/mg)	MDEA (pg/mg)
AMP68	4	1243	1477	1230	ND	ND
AMP68	5	1156	1391	1248	ND	ND
AMP88	1	1396	1483	1279	ND	ND
AMP88	2	1415	1503	1284	ND	ND
AMP88	3	1384	1552	1247	ND	ND
AMP88	4	1355	1559	1247	ND	ND
AMP88	5	1139	1459	1247	ND	ND
AMP92	1	1277	1370	1207	ND	ND
AMP92	2	1290	1412	1237	ND	ND
AMP92	3	1260	1420	1203	ND	ND
AMP92	4	1312	1421	1212	ND	ND
AMP92	5	1129	1348	1212	ND	ND
AMP107	1	1415	1577	1342	ND	ND
AMP107	2	1405	1567	1352	ND	ND
AMP107	3	1354	1516	1249	ND	ND
AMP107	4	1362	1539	1256	ND	ND
AMP107	5	1228	1503	1294	ND	ND
AMP115	1	1340	1465	1284	ND	ND
AMP115	2	1268	1406	1257	ND	ND
AMP115	3	1316	1484	1212	ND	ND
AMP115	4	1239	1391	1155	ND	ND
AMP115	5	1097	1342	1140	ND	ND
AMP133	1	1344	1522	1242	ND	ND
AMP133	2	1381	1544	1273	ND	ND
AMP133	3	1425	1595	1276	ND	ND
AMP133	4	1497	1666	1322	ND	ND
AMP133	5	1246	1591	1294	ND	ND
AMP150	1	1399	1554	1318	ND	ND
AMP150	2	1401	1594	1337	ND	ND
AMP150	3	1433	1722	1309	ND	ND
AMP150	4	1463	1790	1335	ND	ND
AMP150	5	1231	1582	1321	ND	ND
Mean		1347	1535	1287	0	0
Standard deviation		123	136	67	0	0
% Coefficient of variation		9.1	8.9	5.2	0	0
n		69	69	69	0	0

Bad injection = insufficient volume to reinject

Appendix B-4-2. Secondary Analytical Method Results of RM-RTI-CFS-2407-AMPS-4

Sample ID	Replicate	AMP (pg/mg)	MAMP (pg/mg)	MDMA (pg/mg)	MDA (pg/mg)	MDEA (pg/mg)
AMPS101	1	1650	1514	1327	ND	ND
AMPS101	2	1546	1506	1439	ND	ND
AMPS101	3	1682	1499	1419	ND	ND
AMPS101	4	1488	1474	1394	ND	ND
AMPS101	5	1431	1479	1355	ND	ND
AMPS147	1	1752	1671	1371	ND	ND
AMPS147	2	1813	1736	1468	ND	ND
AMPS147	3	1713	1649	1565	ND	ND
AMPS147	4	1606	1695	1577	ND	ND
AMPS147	5	1795	1742	1537	ND	ND
Mean		1648	1597	1445	0	0
Standard deviation		129	112	89	0	0
% Coefficient of variation		7.8	7.0	6.2	0	0
n		10	10	10	0	0

Appendix B-4-3. Tertiary Analytical Method Results of RM-RTI-CFS-2407-AMPS-4

Sample ID	Replicate	AMP (pg/mg)	MAMP (pg/mg)	MDMA (pg/mg)	MDA (pg/mg)	MDEA (pg/mg)
AMPS21	1	1165	1338	1213	ND	ND
AMPS21	2	1336	1351	1312	ND	ND
AMPS21	3	1050	1367	1283	ND	ND
AMPS21	4	1276	1396	1246	ND	ND
AMPS21	5	1152	1251	1179	ND	ND
AMPS109	1	1033	1091	1195	ND	ND
AMPS109	2	1040	1053	1062	ND	ND
AMPS109	3	1034	1224	1177	ND	ND
AMPS109	4	867	1002	1020	ND	ND
AMPS109	5	1005	1164	1173	ND	ND
Mean		1096	1224	1186	0	0
Standard deviation		138	141	90	0	0
% Coefficient of variation		12.6	11.5	7.6	0	0
n		10	10	10	0	0

Appendix B-4-4. Overall Statistical Results of RM-RTI-CFS-2407-AMPS-4

AMPS Overall Results	AMP	MAMP	MDMA
Mean (pg/mg)	1352	1507	1294
Standard deviation (pg/mg)	181	168	95
% Coefficient of variation	13.4	11.2	7.4
n	89	89	89
Total variance	90051	55999	21607
Uncertainty (pg/mg)	600	473	294
Reference range (pg/mg)	1352 ± 600	1507 ± 473	1294 ± 294

Appendix B-4-5. Control Results of RM-RTI-CFS-2407-AMPS-4

Control Results	Control Type	Reported AMP (pg/mg)	Reported MAMP (pg/mg)	Reported MDMA (pg/mg)	Reported MDMA (pg/mg)	Reported MDEA (pg/mg)
AMP165	NEG	ND	ND	ND	ND	ND
AMP165	NEG	ND	ND	ND	ND	ND
AMP165	NEG	ND	ND	ND	ND	ND
AMP165	NEG	ND	ND	ND	ND	ND
AMP165	NEG	ND	ND	ND	ND	ND
AMP166	POS	1178	1388	554	579	589
AMP166	POS	1101	1317	530	601	610
AMP166	POS	1216	1399	526	583	576
AMP166	POS	1190	1395	514	567	557
AMP166	POS	1068	1324	509	593	579
AMPS169	NEG	ND	ND	ND	ND	ND
AMPS169	NEG	ND	ND	ND	ND	ND
AMPS169	NEG	ND	ND	ND	ND	ND
AMPS169	NEG	ND	ND	ND	ND	ND
AMPS169	NEG	ND	ND	ND	ND	ND
AMPS171	NEG	ND	ND	ND	ND	ND

NEG = negative; POS = positive; ND = none detected