Validation of an Ultra Performance Liquid Chromatography Tandem Mass Spectrometry (UPLC™/MS/MS) Method for Forensic Toxicological Analysis:

Confirmation and Quantitation of Lysergic Acid Diethylamide (LSD) and its Congeners in Forensic Samples

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Abstract

The Royal Canadian Mounted Police (RCMP) Forensic Laboratory Services (FLS) needed a method to confirm positive lysergic acid diethylamide (LSD) immunoassay screening results. As a result, an ultra performance liquid chromatography tandem mass spectrometry (UPLC™/MS/MS) method was validated for the confirmation and quantitation of LSD, iso-LSD, N-demethyl-LSD (nor-LSD), and 2-oxo-3-hydroxy-LSD (O-H-LSD). The method was validated in urine and whole blood, where linearity, accuracy, precision, sensitivity, stability, selectivity, recovery, matrix effects, and reproducibility were evaluated.

The method involved a liquid-liquid extraction (LLE) of the analytes and the deuterated internal standard from 1 mL of urine or whole blood with dichloromethane:isopropyl alcohol after being basified. The average recovery for all analytes was ≥ 62%, and the matrix effect was found to be insignificant. MS/MS analysis was conducted with a triple quadrupole mass spectrometer by positive electrospray ionization (ESI+) in multiple reaction monitoring (MRM) mode. The lowest limit of quantitation (LLOQ) was 20 pg/mL for LSD and iso-LSD, and 50 pg/mL for nor-LSD and O-H-LSD. The method was linear, accurate, precise, selective, and reproducible from 20 to 2000 pg/mL for LSD and iso-LSD, and from 50 to 2000 pg/mL for nor-LSD and O-H-LSD with an $r^2 \geq 0.99$.

The refrigerated and frozen long term stability was investigated for 90 days. LSD was stable at all temperatures for 90 days. Iso-LSD in blood was also stable at all temperatures for 90 days, but iso-LSD in urine showed an initial decrease followed by a gradual increase back to day 0 concentrations. Nor-LSD was stable at all temperatures up to day 14, with >43% decrease by day 30, with no additional decrease for the next 60 days. O-H-LSD in urine was stable at all temperatures for 90 days, but by day 90 O-H-LSD in whole blood stored refrigerated decreased in concentration by >37%. Additionally, a case sample that was stored at -50°C for ten years was found to still contain measurable amounts of each compound.

The method was applied to blind samples and a case that screened positive with immunoassay. Retention time, relative retention time, and ion ratios were used as identification parameters and found to correctly identify the analytes 100% of the time with no false positives. The case sample showed that the concentration of O-H-LSD was 4 times greater than LSD in urine. Furthermore, both the detection of O-H-LSD in a blood case sample, and LSD in a vitreous humor case sample were the first to be documented.

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List of Abbreviations

AAFS	American Academy of Forensic Sciences
ACN	Acetonitrile
APcI	Atmospheric pressure chemical ionization
API	Atmospheric pressure ionization
BEH	Bridged ethyl hybrid
BSTFA	N,O-bis(trimethylsilyl) trifluoroacetamide
CDSA	Canada's Controlled Drugs and Substance Act
CE	Capillary electrophoresis
CEDIA	Cloned enzyme donor immunoassay
CI	Chemical ionization
CID	Collision induced dissociation
%CV	Coefficient of variation
DC	Direct current
EI	Electron ionization
ELISA	Enzyme-linked immunosorbant assay
EMIT	Enzyme-multiplied immunoassay technique
ESI	Electrospray ionization
FDA	Food and Drug Administration
FLS	Forensic Laboratory Services
GC	Gas Chromatography
GC/MS	Gas Chromatography-Mass Spectrometry
GC/MS/MS	Gas Chromatography-Tandem Mass Spectrometry
HMDS	Hexamethyl-disilazane
HPLC	High Performance Liquid Chromatography
HPPD	Hallucinogen persisting perceptual disorder
IAE	Immunoaffinity extraction
ICH	International Conference on Harmonization
IOC	International Olympic Committee
iso-LSD	Lysergic acid diethylamide isomer
LAE	Lysergic acid ethylamide
LAMPA	Lysergic acid methyl propylamide
LC	Liquid Chromatography
LC/MS	Liquid Chromatography-Mass Spectrometry
LC/MS/MS	Liquid Chromatography-Tandem Mass Spectrometry
LD_{50}	Lethal dose
LEO	Lysergic acid ethyl-2-hydroxyethylamide
LIF	Laser-induced fluorescence
LLE	Liquid-liquid extraction
LLOQ	Lowest limit of quantitation
LOD	Limit of detection
LSD	Lysergic acid diethylamide
LSD-D3	Deuterated lysergic acid diethylamide
$[M+H]^+$	Protonated molecular ion
ME	Matrix Effect
MeOH	Methanol

MRM	Multiple reaction monitoring
MS	Mass Spectrometry
MS/MS	Tandem Mass Spectrometry
MSD	Mass selective detector
m/z	Mass to Charge Ratio
nBuCl	n-butylchloride
nor-LSD	N-demethyl lysergic acid diethylamide
O-H-LAMPA	2-oxo-3-hydroxy-lysergic acid methyl propylamide
O-H-LSD	2-oxo-3-hydroxy-lysergic acid diethylamide
PA	Peak area
PAR	Peak area ratio
PDA	Photodiode array
RCMP	Royal Canadian Mounted Police
RIA	Radioimmunoassay
RF	Radiofrequency
RRT	Relative retention time
RT	Retention time
SD	Standard deviation
SIM	Single ion monitoring
S/N	Signal to noise ratio
SOFT	Society of Forensic Toxicologists
SPE	Solid phase extraction
$S_{y.x}$	Standard deviation of the residuals
TEA	Triethylamine
TIC	Total ion chromatogram
TMS	Trimethylsilyl
TS	Toxicology Services
T-Wave™	Travelling Wave™
ULOQ	Upper limit of quantitation
USP	United States Pharmacopeia
UPLC™	Ultra Performance Liquid Chromatography
UPLC™/MS/MS	Ultra Performance Liquid Chromatography-Tandem Mass Spectrometry
WADA	World Anti-Doping Agency

Chapter 1

Introduction to Lysergic Acid Diethylamide (LSD) and its Forensic Significance

1.1 Introduction

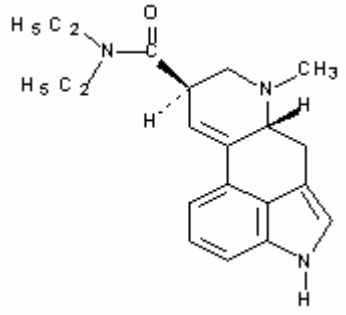


Figure 1.1: Structure of LSD, MW = 323.43 g/mol

LSD is considered the most potent hallucinogen (Figure 1.1). In 1938, Albert Hoffman of the Sandoz Laboratories in Basal, Switzerland began experimenting with lysergic acid derivatives hoping to find a new medicine (McKim 2003). In 1943, Albert Hoffman ingested 250 μg of LSD to test its effects, and felt pleasantly sleepy, and then experienced fantastic, vivid images (O'Shea and Fagan 2001). LSD's popularity was the highest during the 1960s and 1970s. Its use started to decline in the 1980s; however its popularity began to increase again in the 1990s (Ellenhorn 1997). In Canada, the lifetime use of LSD has gradually increased from 4.1% in 1989 to 13.2% in 2004 (Adalf et al. 2004). Initially, LSD was being used licitly in mental hospitals during the 1960s, and then it got out into the streets and was being used recreationally for so-called "spiritual purposes" (O'Shea and Fagan 2001). LSD is clear, odourless, and tasteless (Klein and Kramer 2004) and is self-administered through either inhalation, topical application, or more commonly by oral ingestion in the form of pills, blotter paper, or liquid (Blaho K et al. 1997; Klein and Kramer 2004; O'Shea and Fagan 2001). LSD, or "acid", is sold on the street illicitly as hits, with one hit typically consisting of 40 to 120 μg of LSD at $3 to $5 a hit (Clarkson et al. 1998).

In Canada LSD first became illegal to possess in 1962, and is now prohibited under Schedule III of Canada's Controlled Drugs and Substance Act (CDSA). A survey on 210 rave attendees in Montreal, Quebec conducted in 2002, found that LSD was ranked number 4 out of 11 as the drug used for first drug experience at an average age of 16.3, and 56.2% of the respondents had used LSD (Gross et al. 2002). The Canadian Centre on Substance Abuse conducted a telephone survey of 13909 Canadians aged 15 years or older between December 2003 and April 2004 and found that 11.4% of the participants had used hallucinogens, making hallucinogens the second most commonly used illicit drug during one's lifetime (Adalf et al. 2004). So, even today LSD is being used in Canada illicitly, making it a concern for forensic toxicologists.

1.2 Clinical Effects of LSD

The hallucinogenic effects of LSD are commonly referred to as "trips". LSD causes panic, anxiety, negative effects, and feelings of insanity, with the effects being unpredictable, varying with the amount ingested and the user's personality, mood, expectations, and surroundings (Nichols 2004). There has not been any proof that LSD is addictive, as most people do not continue to use LSD and other hallucinogens on a long-term basis after initial experimentation. However, chronic users can develop a tolerance known as tachyphylaxis (Nichols 2004). The tolerance to LSD's psychosocial effects can occur after just a few doses, probably due to the desensitization of 5-HT receptors (O'Shea and Fagan 2001). Since the 1960s four chronic reactions to LSD have been recognized: (1) prolonged psychotic reactions, (2) depression sufficiently severe so as to be life threatening, (3) flashbacks, and (4) exacerbations of a pre-existing psychiatric illness (Ellenhorn 1997). Chronic flashbacks are now recognized as hallucinogen persisting perceptual disorder (HPPD) (Ellenhorn 1997; Halpern and Jarrison 2003). The American Psychiatric Association require three criteria to be fulfilled to be diagnosed with HPPD; "(1) re-experiencing, following cessation of use of hallucinogen, of one or more of the perceptual symptoms that were experienced while intoxicated with the hallucinogen, (2) symptoms cause clinically significant distress or impairment, and (3) symptoms are not due to other mental or medical conditions" (Halpern and Jarrison 2003).

1.3 Mechanism of Action

The interaction between LSD and serotonin has been known since 1961 (Ellenhorn 1997). LSD is a potent agonist at 5-HT_{1A} receptors, and also has an affinity for postsynaptic 5-HT_2 receptors in the locus ceruleus and the cerebral cortex (Ellenhorn 1997). LSD has been shown in rat studies to activate the 5-HT_{2A} receptor causing an increase in the extracellular glutamate concentrations in the prefrontal cortex (Muschamp et al. 2004). The increased glutamate may play a significant role in the behavioural effects of LSD. The signalling of LSD at 5-HT_{2C} receptor has also been shown to differ from that of serotonin (Backstrom et al. 1999). More recently, LSD has been shown to cause an increase in the expression of a small set of genes in the mammalian brain that are involved in a wide array of cellular functions, such as synaptic plasticity, glutamatergic signalling, and cytoskeleton architecture (Nichols and Sanders-bush 2002). Despite all the research done on the mechanism of action of LSD, the exact signalling pathway to explain LSD's potency is still not fully known. Two possible explanations are that the interaction of LSD is synergistic with its 5-HT_{2A} receptor activation, and/or that there may be an undiscovered signalling pathway coupled to the 5-HT_{2A} receptor (Nichols 2004).

1.4 Pharmacokinetics

Lysergic acid is a constituent of the ergot alkaloids, and LSD is its semi-synthetic hydrolytic derivative (O'Shea and Fagan 2001). Lysergic acid is obtained by treating ergot alkaloids with strong alkali and then neutralizing the basic mixture with an acid. After purification of the lysergic acid it is coupled with diethylamine (Figure 1.2), resulting in the formation of LSD (Nichols 2001). During the production of illicit LSD an impurity diastereoisomer (iso-LSD – Figure 1.3) is formed during the synthesis from lysergic acid (Johansen and Jensen 2005). In theory, 4 optical isomers are possible from the 2 asymmetric carbon atoms in the LSD molecule (Clarkson et al. 1998). However, many of the ergot alkaloid derivatives have only been found to be isomeric at the C-8 carbon, but not at the C-5. In LSD, the C-5 hydrogen atom and the C-8 carboxylic amide are in cis configuration, and in iso-LSD they are in trans configuration. Iso-LSD can also form from LSD when exposed to basic aqueous solutions and increased

temperatures (Reuschel et al. 1999a; Li et al. 1998), ultimately reaching a LSD/iso-LSD ratio of 9:1 (Reuschel et al. 1999a; Salamone et al. 1997).

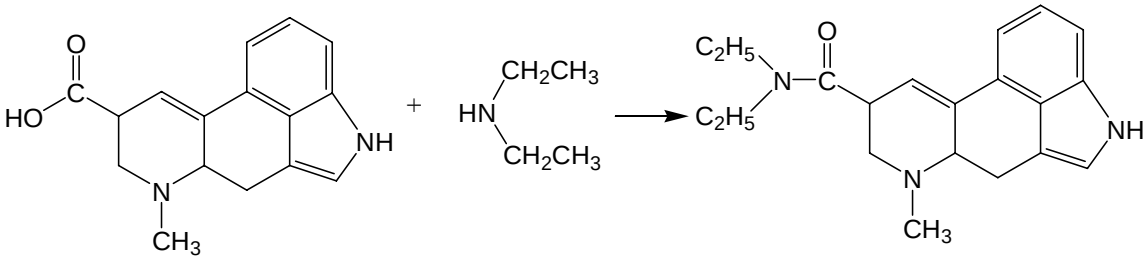


Figure 1.2: Synthesis of LSD.

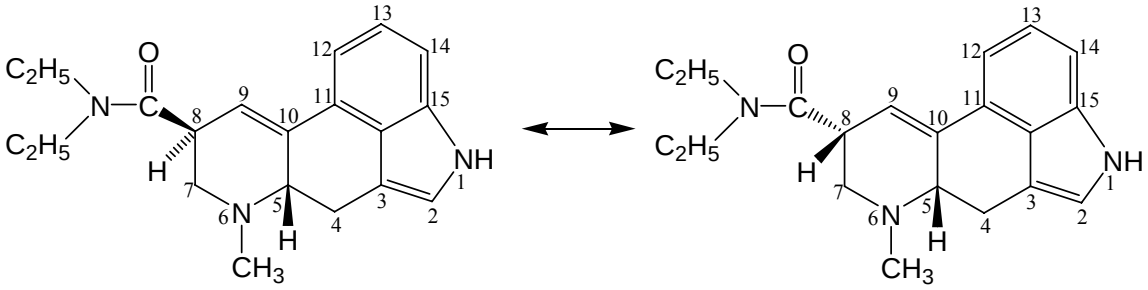


Figure 1.3: LSD isomerization to iso-LSD at the C-8 carbon.

LSD is the most potent known hallucinogen, with doses as small as 50 to 200 μg causing hallucinogenic effects lasting 6 to 12 hours (Nichols 2004). A dose of 0.2 mg/kg is considered to be a fatal dose (Ellenhorn 1997), with a lethal dose (LD_{50}) of 14 mg (Clarkson et al. 1998). There was one documented case of a 25 year old male who died 16 hours after being admitted to the hospital, whose cause of death was poisoning by LSD (Fysh et al. 1985). In this case antemortem plasma and stomach contents analyzed by high performance liquid chromatography (HPLC) contained 8 and 60 ng/mL LSD, respectively. However, there is no evidence that LSD causes damage to

any human body organs (Nichols 2004). Instead deaths associated with LSD use are usually due to injuries received under the influence of the drug (Fysh et al. 1985). LSD acute effects last from 0.7 to 8 hours, whereas the psychotropic effects may last for days (Ellenhorn 1997). The urinary elimination half-life and plasma half-life of LSD is 3.6 hours (Lim et al. 1988) and 5.1 hours (Papac and Foltz 1990), respectively, with an elimination rate (K_e) of 0.2/hour (Lim et al. 1988). After a 50 μg dose of LSD, the urinary LSD concentration normally drops to < 200 pg/mL within 12 to 24 hours (Reuschel et al. 1999a). The maximum rate of LSD excretion occurs 4 to 6 hours after an oral dose (Lim et al. 1988). The peak plasma concentration of LSD occurs approximately 3 hours after administration (Papac and Foltz 1990). In general the half-lives of the metabolites of LSD are longer and the rate of elimination is slower, for example the urinary elimination half-life of N-demethyl-LSD (nor-LSD) is 10 hours and K_e is 0.1/hour (Lim et al. 1988).

1.5 Metabolism of LSD

LSD is extensively and rapidly metabolized (Figure 1.4), with only 0.9% of the LSD dose being excreted in the urine (Lim et al. 1988). The metabolites of LSD do not have any psychoactive properties, but their presence is indicative of LSD use. Nor-LSD was the first metabolite of LSD confirmed in vivo in humans. LSD undergoes metabolic N-demethylation to produce nor-LSD in humans (Lim et al. 1988). By using neutral loss tandem mass spectrometry experiments, nor-LSD was detected and subsequently confirmed using authentic nor-LSD standards (Canezin et al. 2001). LSD also undergoes aromatic hydroxylation to form 13- or 14-hydroxy-LSD glucuronide (Lim et al. 1988; Canezin et al. 2001). Both 13- and 14-hydroxy-LSD have been tentatively identified in human urine. It was also determined that 13- and 14-hydroxy-LSD appear in the urine as glucuronide conjugates (Lim et al. 1988). Lysergic acid ethylamide (LAE) was determined to be another metabolite in human liver in vitro and also in vivo (Cai and Henion 1996). LAE is formed by the removal of one of the two N-ethyl groups on LSD. Lysergic acid ethyl-2-hydroxyethylamide (LEO) is formed by adding a hydroxyl group on the branched side chain of LSD. However, in vitro metabolism of LSD in human liver found that LEO was not a metabolite (Cai and Henion 1996).

Another possible metabolite of LSD in vitro is 2-oxo-LSD (Cai and Henion 1996). However, 2-oxo-LSD was not found in positive LSD urine samples (Cai and Henion 1996).

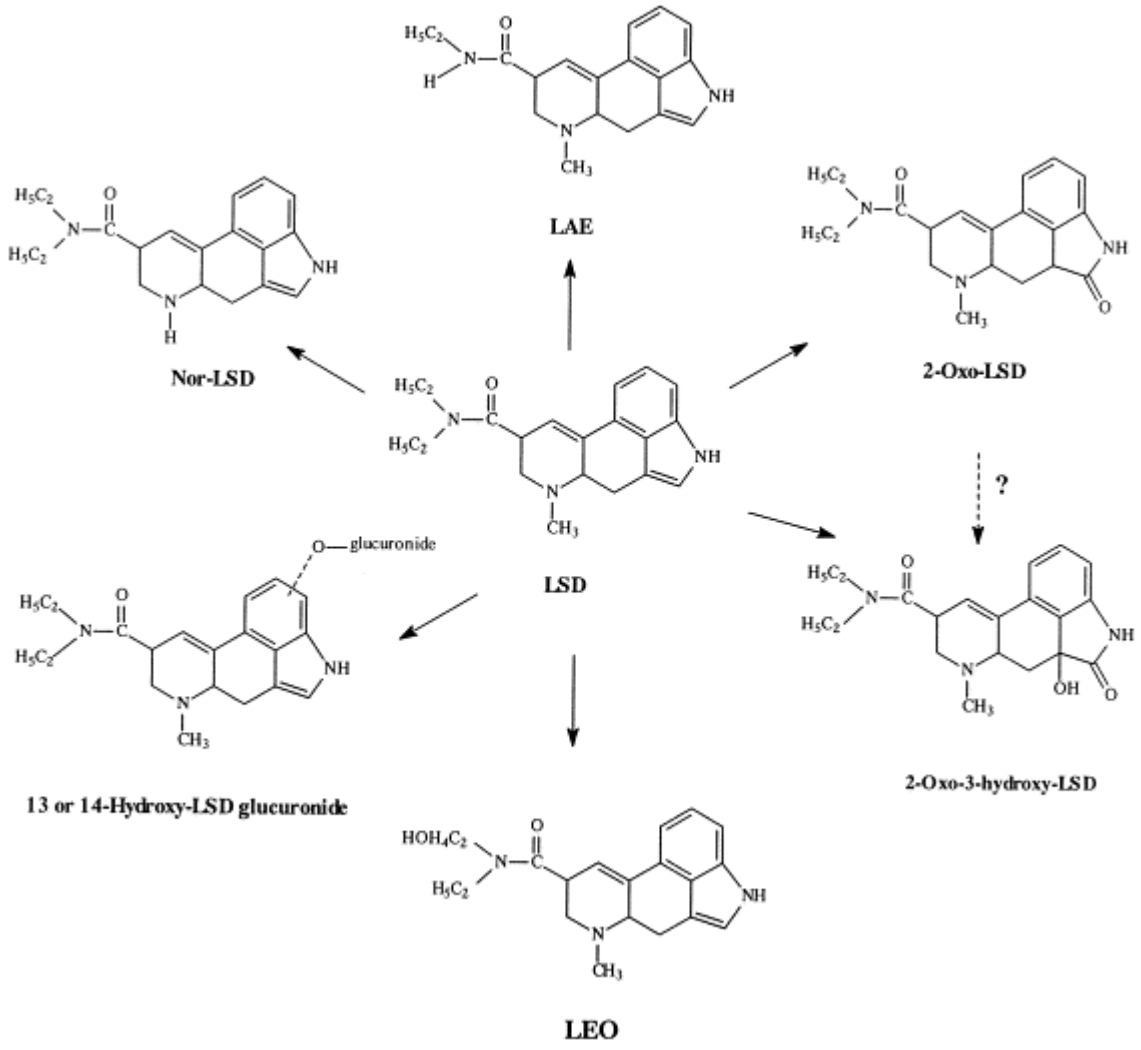


Figure 1.4: Metabolism of lysergic acid diethylamide (LSD) (LAE = lysergic acid ethylamide, LEO = lysergic acid ethyl-2-hydroxyethylamide, nor-LSD = N-demethyl-LSD) (Canezin et al. 2001, figure reproduced with permission from A. Cailleux).

More recently 2-oxo-3-hydroxy-LSD (O-H-LSD) was determined to be another major metabolite of LSD (Klette et al. 2000). The formation of O-H-LSD is still under investigation, but it is proposed that LSD is oxidized to 2-oxo-LSD which then undergoes hydroxylation to O-H-LSD. Also, when cytochrome P450 in human liver microsomes were inhibited, no O-H-LSD was formed (Klette et al. 2000). Therefore, O-H-LSD may also be produced via an epoxide intermediate or a dihydroxy-LSD intermediate.

Due to the ethical and legal restrictions of carrying out research on humans with hallucinogens, research on the metabolism of LSD may never be fully complete. However, it is known that the concentrations of the majority of the metabolites are greater than that of LSD. More recently, it has been found that the concentration of O-H-LSD is 16 (Poch et al. 2000) to 25 (Horn et al. 2003) times higher than that of LSD. However, this is only true in urine because O-H-LSD has not been detected in human plasma *in vivo* (Canezin et al. 2001; Sklerov et al. 2000). Also, O-H-LSD is not generated due to the extraction, analytical processes, or degradation of the sample (Klette et al. 2000; Klette et al. 2002). Therefore, within the last few years methods developed for detecting LSD have been directed at identifying O-H-LSD.

Recent method development and validation for LSD and its metabolites have only been done with 2 of the metabolites of LSD, because the research to date is incomplete for most of the other metabolites of LSD. For example, no reference compounds of 13- or 14-hydroxy-LSD are available (Clarkson et al. 1998) and their concentrations in vivo are too low (Lim et al. 1988), so they cannot be conclusively identified or quantified. LAE also has no available reference compounds. LEO has only been described in human urine by using specific tandem mass spectrometry (MS/MS) transitions (Canezin et al. 2001). And finally, 2-oxo-LSD was not found in positive LSD urine samples (Cai and Henion 1996). Either it is because 2-oxo-LSD concentrations decrease rapidly during storage (Cai and Henion 1996), or 2-oxo-LSD is really an intermediate compound in the formation of O-H-LSD (Klette et al. 2000).

1.6 Literature Review for the Laboratory Analysis of LSD

An extensive literature search was conducted to examine existing methods for the analysis of LSD and its metabolites from human biological specimens. Suitable methods were chosen, adapted, and further developed so that they could be used for the analysis of forensic samples using LC/MS/MS. The method was validated using accepted method validation procedures where linearity, sensitivity, accuracy, precision, stability, selectivity, recovery, and matrix effects were evaluated (Standards Council of Canada 2003; FDA 2001; ICH 1996; SOFT/AAFS 2006). The validated method was then used to analyze forensic samples suspected of containing LSD.

The analysis of LSD in biological fluids has been a challenge analytically. The challenges are due to the small doses taken (Clarkson et al. 1998), the rapid metabolism (Ellenhorn 1997), short half-life (Schneider et al. 1998), and the instability of LSD (Li et al. 1998). In the United States a urine sample must contain >200 pg/mL to be considered positive, however after one dose the LSD urine concentration drops to <200 pg/mL within 12 to 24 hours (Reuschel et al. 1999a). In order to increase the detection time window of LSD a highly sensitive method that detects LSD and its metabolites is necessary.

1.6.1 Sample Matrix

The analysis of LSD and its metabolites has been carried out on urine, blood and hair. Because LSD is rapidly metabolized, has such a short half life, and is not usually associated with death, the analysis of organs postmortem is not common. LSD and all its known metabolites have been detected in urine. In the blood, O-H-LSD, which is the most recent metabolite identified, has not been detected. The analysis of hair has been attempted using rat experiments (Nakahara et al. 1996). The rats were given LSD and it was found that LSD was detectable in hair even with low doses of LSD, but nor-LSD was present only with higher doses. However, when examining hair of humans known to be positive for LSD only 2 out of 17 had measurable LSD levels in the hair. The same results were observed when an immunoaffinity extraction (IAE) method using commercially available columns for LSD was performed (Röhrich et al. 2000). Using

this method only 1 out of 9 human hair samples tested positive. Overall, the specimens of choice are urine and blood for detecting LSD.

1.6.2 Stability

The instability of LSD has proven to be a problem in its analysis. LSD rapidly decomposes in urine samples exposed to increased temperatures, sunlight, or ultraviolet light (Li et al. 1998; Skopp et al. 2002; de Kanel et al. 1998; Webb et al. 1996). The optimum temperature where LSD is relatively stable is 4°C and 22°C when protected from light (Skopp et al. 2002). In one day LSD was shown to decrease to 3% of its initial concentration when exposed to sunlight, and after 3 days no LSD was detectable (Skopp et al. 2002). On the other hand, both nor-LSD and O-H-LSD are less susceptible to photodegradation (Klette et al. 2002; Skopp et al. 2002). Changes in pH resulted in significant loss of O-H-LSD. O-H-LSD is stable at pH 4.6 to 6.5 when maintained at temperatures of -20°C to 24°C (Klette et al. 2002). Trace amounts of metal ions in buffer or urine could also catalyze the decomposition of LSD (Li et al. 1998).

1.6.3 Extraction Procedures

Liquid-liquid extractions (LLE), solid phase extractions (SPE), a combination of both, and IAE have been utilized when preparing samples for analyzing LSD. Since forensic analysis is done on various matrices, an extraction procedure to obtain a clean extract is very important. More specifically for LSD, problems with co-eluting impurities are a major concern. Since LSD is in such small concentrations, even minor impurities can cause analytical problems as they can mask the LSD. The most common LLE involves basifying the sample with NH_4OH and then extracting with a methylene chloride/isopropanol mixture. LLE alone failed to provide the cleanest sample possible, as a result SPE was performed after LLE in some analysis. Since this involves two extractions, the percent recovery decreased to as low as 31%. The overall precision had a coefficient of variation (%CV) of 10.8% which is much higher than for LLE or SPE alone (Libong et al. 2003a) (Table 1.1).

Extractions using SPE have been shown to give better results than those obtained by LLE. In one study SPE was shown to have a 45% increase in extraction efficiency,

38% decrease of limit of detection (LOD) and lowest limit of quantitation (LLOQ), and 33% reduction of injection volume for O-H-LSD (Horn et al. 2003). This study used an automated SPE procedure, and gave a fast, simple extraction with a recovery as high as 92%.

Microgenic Corporation has developed an affinity resin for IAE that is specific for LSD (Röhrich et al. 2000). The LSD in the sample is bound to a monoclonal antibody that is immobilized on the surface of a solid support. After incubation the resin is washed to remove matrix components. Finally, a solvent like methanol is added to denature the antibodies which cause the release of LSD from the antibody (Röhrich et al. 2000; Kerrigan and Brooks 1999). However, IAE has been shown to have less recoveries which limits its use for quantitative analysis (Webb et al. 1996). Other disadvantages include its limited LSD binding capacity and the high cost of reagents (Kerrigan and Brooks 1999).

Table 1.1: Comparison of methods for analyzing LSD and O-H-LSD

Extraction	Chromatography	% Recovery		% CV	
		LSD	O-H-LSD	LSD	O-H-LSD
LLE	GC/MS(/MS) (Lim et al. 1988; Burnley and George 2003)	65-86	72	2.8-13	4.0-17.3
	LC/MS(/MS) (Sklerov et al. 2000; Canezin et al. 2001)	67	69-74	2-8.8	6-14.3
SPE	LC/MS(/MS) (Skopp et al. 2002; Horn et al. 2003)	68-76	48-54 (92*)	1.6-5.3	2.9-6.1 (2.0*)
LLE/SPE	GC/MS(/MS) (Libong et al. 2003a)	60	-	10.8	-
	LC/MS(/MS) (Sklerov et al. 2000)	31-61	32-42	2-8.8	6-14.3

*with automated SPE

1.6.4 Immunoassays

Immunoassays can be used as an initial screen for LSD. An immunoassay is simply a reaction between an antibody and an antigen. The most commonly used immunoassays include enzyme-multiplied immunoassay technique (EMIT), radioimmunoassay (RIA), enzyme-linked immunosorbant assay (ELISA), and cloned enzyme donor immunoassay (CEDIA) (see Table 1.2). Immunoassays are relatively sensitive, with the cutoff limit for a positive LSD being 500 pg/mL (Schneider et al. 1998; Acosta-Armas 2003). However, immunoassays lack selectivity resulting in high incidence of false positives (Acosta-Armas 2003). A few studies have shown that other prescription drugs were possibly cross reacting causing this false positive (Acosta-Armas 2003; Ritter et al. 1997). A high number of samples, positive for LSD by immunoassay methods, are not positive using confirmatory methods. EMIT has more cross reactivity and poorer precision than CEDIA and RIA (Ritter et al. 1997; Wiegand et al. 2002). RIA is not a popular method due to the increase cost and the handling and disposal of radioactive material. Despite the lack of selectivity immunoassays are still used due to the simplicity, short turnaround times, and low cost.

Table 1.2: Comparison of immunoassay methods for the detection of LSD (Wiegand et al. 2002; Moore et al. 1999; Immunalysis 2001)

		EMIT	CEDIA	RIA	ELISA
Positive cutoff (ng/mL)	LSD	0.5	0.5	0.5	0.5
Cross reactivity concentration (ng/mL)	nor-LSD	100	100	10	50
	iso-LSD	50	50	100	na
	O-H-LSD	100	100	100	na
False positive incidence		high	low	low	low
% CV at positive cutoff concentration		9.0	3.5	3.6	8.4

na = no data available

1.6.5 Capillary Electrophoresis (CE)

The use of CE with laser-induced fluorescence (LIF) detector has been proposed as a possible routine forensic method for detecting LSD (Frost and Kohler 1998). Using special sample preparation, electrokinetic injection, and bubble cell capillary, a LOD of 100 to 200 pg/mL was obtained. CE/LIF has an advantage over immunoassays, because

it does not have cross-reactivity problems. However, this method has not been shown to work using urine samples.

1.6.6 Gas Chromatography-Mass Spectrometry (GC/MS)

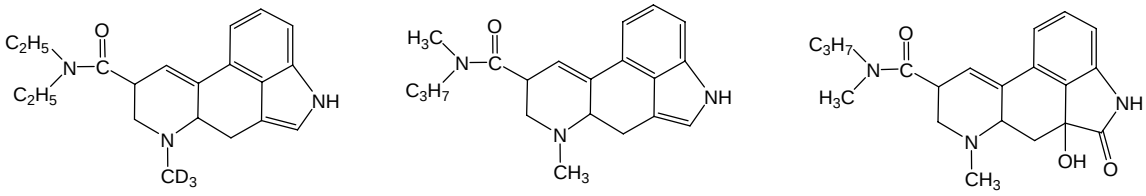
Most laboratories today use GC/MS as their confirmatory method for LSD because the instrumentation is available. A sensitive method for detecting LSD using GC/MS has been developed with a LLOQ of 20 pg/mL (Libong et al. 2003a). However, the analysis of LSD using GC/MS has its challenges. LSD undergoes irreversible adsorption during the chromatographic process, it is relatively non-volatile, and finally it is not stable at the GC temperatures (Libong et al. 2003a). Furthermore, sample preparation is laborious because the samples require derivatization via silylation of the indole nitrogen (Klette et al. 2002; Libong et al. 2003a).

Peak tailing is the consequence of an adsorption-desorption phenomenon which depends on the polarity of the analyte, the nature of the MS system components, and the temperature of analysis (Libong et al. 2003b). To reduce the irreversible adsorption of LSD to both the capillary column and the electrodes of the MS, the column requires regular conditioning and the MS requires regular cleaning. Conditioning the column with silylating agents N,O-bis(trimethylsilyl) trifluoroacetamide (BSTFA) and hexamethyl-disilazane (HMDS) resulted in increased background interferences, and conditioning with blank extracted matrix gave satisfactory results (Sklerov et al. 1999). Temperature programmed on-column injections have also been used to reduce the irreversible adsorption (Libong et al. 2003b; Sklerov et al. 1999).

Another problem with analyzing LSD is its short detection window. As a result, the development of GC/MS methods for analyzing iso-LSD and the metabolites of LSD has been attempted. Iso-LSD determination using GC/MS has been attempted by first isomerizing iso-LSD to LSD, because the instability of iso-LSD does not make it ideal for quantification. The LLOQ of iso-LSD was determined to be 50 pg/mL (Clarkson et al. 1998). However, this method is time consuming requiring two procedures to calculate iso-LSD concentrations.

More recently with the discovery of O-H-LSD, the use of GC/MS to detect it has been attempted (Reuschel et al. 1999b; Burnley and George 2003). Derivatization of

LSD attaches a trimethylsilyl (TMS) group to the indole nitrogen, however when O-H-LSD is derivatized a TMS group can also attach to the hydroxyl group which results in a mixture of mono-TMS and bis-TMS derivatives (Reuschel et al. 1999b). In order to accurately quantitate O-H-LSD, when using a GC/MS method requiring derivatization, an internal standard with similar properties, such as 2-oxo-3-hydroxy-lyergic acid methylpropylamide (O-H-LAMPA-Figure 1.5), must also be used. GC/MS has also been shown to have low sensitivity and irreproducible chromatography for O-H-LSD (Poch et al. 1999).



(A) LSD-D3
MW = 326.44

(B) LAMPA
MW = 323.44

(C) O-H-LAMPA
MW = 355.44

Figure 1.5: Internal standards for LSD analysis, (A)LSD-D3 = deuterated LSD, (B) LAMPA = lysergic acid methylpropylamide, and (C) O-H-LAMPA = 2-oxo-3-hydroxy-LAMPA.

1.6.7 Liquid Chromatography-Mass Spectrometry (LC/MS or LC/MS/MS)

The development and validation of LC/MS methods for analyzing LSD and its metabolites is an ongoing area of research. Due to the difficulties of analyzing LSD by GC/MS and the increase popularity of HPLC, recent methods developed for LSD analysis have used LC/MS (Table 1.3, page 15) or LC/MS/MS (Table 1.4, page 16). The sensitivity of the methods developed have a wide LOD range of 10 (Skopp et al. 2002) to 500 pg/mL (Webb et al. 1996; White et al. 1999) for LSD, 50 (Skopp et al. 2002) to 400 pg/mL (Poch et al. 2000; Sklerov et al. 2000) for O-H-LSD, and 25 (de Kanel et al. 1998) to 60 pg/mL (Skopp et al. 2002) for nor-LSD. Methods using tandem MS provided the most sensitive methods compared to single MS methods.

To the Author's knowledge, there are no known published validated methods for the simultaneous confirmatory and quantitative analysis of LSD, iso-LSD, nor-LSD, and O-H-LSD in both urine and blood. The most recent published method is a LC/MS/MS method determination of LSD, iso-LSD and O-H-LSD (Johansen and Jensen 2005). However, sample preparation and MS detection were not optimized for O-H-LSD and therefore not validated for quantitative analysis. Also a LLOQ of 10 pg/mL is reported for both LSD and iso-LSD in urine and blood, however the accuracy and precision at this level are not reported.

LC/MS/MS is not only sensitive, but is also highly specific. As a result, common over-the-counter products, prescription drugs and their metabolites, other illicit compounds, and compounds related to LSD, were found not to cause significant interference (Horn et al. 2003; Klette et al. 2002).

1.7 Hypothesis and Study Objectives

Currently a confirmatory method to analyze LSD and its metabolites in forensic laboratories in Canada is lacking in terms of a reliable validated method. The Royal Canadian Mounted Police (RCMP) Forensic Laboratory Services (FLS) in Winnipeg has acquired a new tandem mass spectrometer (MS/MS) with a liquid chromatography (LC) interface that makes it possible to explore the development and validation of a highly sensitive and selective method for the analysis of LSD and its metabolites. LC has been shown to be more sensitive and simpler to perform than GC/MS, especially when using MS/MS. It is hypothesized that LC/MS/MS will make it possible to analyze extracts of blood and urine for LSD and its metabolites with enhanced sensitivity and greater ease of analysis. More importantly, the method should be acceptable as a reliable method for analyzing forensic samples suspected of containing LSD because it will be fully validated. Furthermore, the project will impact the RCMP FLS in the general use of LC/MS/MS as a confirmatory technique. Consequently the knowledge gained in this project can be directly applied to other analyses where it is necessary to confirm the presence of other drugs in forensic samples.

Table 1.3: LC/MS (SIM) methods for analyzing LSD, metabolites, and impurity

	Sample	Extraction	Instrument	ISTD	HPLC Separation	LOQ (pg/mL)
Webb et al. 1996	Urine	SPE	LC (Waters 600S)/MS (Finnigan SSQ 7000) ESI+	methysergide	Isocratic, 0.25% TEA, 0.1M CH_3COONH_4 (pH 8)/ACN (75:25), 0.5mL/min Hypersil C18 ODS (3× 125mm, 3μm)	LSD = 500
White et al. 1997	Urine	SPE				
White et al. 1999	Urine	SPE	Isotope dilution MS	LSD-D3		
Hoja et al. 1997	Urine	SPE	HPLC/MS (API 100) ESI+	LSD-D3 (limits LSD qualifier ions)	Isocratic, 2mM $HCOONH_4$ (pH 3)/ACN (70:30), 0.04mL/min Nucleosil C18 (1×150mm)	LSD = 100 nor-LSD = 250
Sklerov et al. 2000	Urine	LLE	HPLC(Agilent 1100)/MSD ESI+	O-H-LAMPA	Gradient, 20mM $HCOONH_4$ (pH 4.3)/ MeOH, 0.7mL/min	LSD = 100 O-H-LSD = 400
	Blood	LLE/SPE			Zorbax Eclipse® XDB C18 (4.6×75mm, 3.5μm)	Not determined
Bodin and Svensson 2001	Urine	LLE	HPLC(Agilent 1100)/MS single quadrupole ESI+	LSD-D3	Isocratic, 20mM CH_3COOH, 5mM NH_3, 20% ACN (pH 4.5), 0.3mL/min Luna C18 (2×100mm, 3μm)	LSD = 500
Horn et al. 2003	Urine	Speedisk® with Cerex® PolyChrom™ CLIN II columns	HPLC (Agilent 1100)/MSD APcI+	O-H-LAMPA	Gradient, 50mM CH_3COONH_4, 0.02% TEA (pH 8)/ACN, 0.8mL/min Zorbax Eclipse® XDB C18 (4.6×150mm, 3.5μm)	O-H-LSD = 250
Poch et al. 2000	Urine	LLE/SPE				O-H-LSD = 400
Klette et al. 2002	Urine	LLE/SPE			Same as above except 10mM CH_3COONH_4 (pH 8)/ACN	O-H-LSD = 400

Table 1.4: LC/MS/MS (MRM) methods for analyzing LSD, metabolites, and impurity

	Sample	Extraction	Instrumentation	ISTD	HPLC Separation	LOQ (pg/mL)
de Kanel et al. 1998	Urine and Blood	Zymark RapidTrace™ automated SPE	HPLC (HP 1100)/Micromass Quattro II ESI+	LSD-D3	Isocratic, CH_3COONH_4:ACN/n-propanol (35:65), 0.25mL/min Zorbax SB-phenyl (4.6×75mm, 3.5µm)	LSD = 50 Did not quantitate nor-LSD (↑ CV)
Poch et al. 1999	Urine	LLE/SPE	HPLC (HP 1050) /LCQ ion trap MS-Finnigan APcI+	O-H-LAMPA	Isocratic, 0.01M CH_3COONH_4, 0.02% TEA (pH 8)/ACN (80:20), 0.8mL/min Eclipse® XDB-C18 (4.6×150mm, 3.5µm)	O-H-LSD = 400
Poch et al. 2000	Urine	LLE/SPE			Gradient, 50mM CH_3COONH_4, 0.02% TEA(pH 8)/ACN, 0.8mL/min Same column as above	
Canezin et al. 2001	Urine and Blood	LLE	HPLC (Perkin Elmer)/Triple quadropole API 300 Perkin-Elmer SCIEX MS ESI+	LSD-D3	Isocratic, water (40%) and ACN (60%) containing 0.1% CH_3COOH and 2mM CH_3COONH_4, 0.4mL/min Spherisorb 5 RP 8S (2.1×100mm, 5µm)	LSD = 20 Iso-LSD = 20
Skopp et al. 2002	Urine	SPE	HPLC (Perkin Elmer)/API 365 – Applied Biosystems MS ESI+	LAMPA	Isocratic, ACN:MeOH:20mM CH_3COONH_4 (pH 6.0) (33:33:34), 0.25mL/min Zorbax Eclipse® XDB C8 (2.1×150mm, 5µm)	LSD = 60 O-H-LSD = 190 nor-LSD = 240
Johansen and Jensen 2005	Urine and Blood	LLE	HPLC (Agilent 1100)/Quattro Micro tandem quadrupole MS ESI+	LSD-D3	Gradient, 5% ACN, 0.05% formic acid:100% ACN, 0.05% formic acid (5:95), 0.2mL/min Zorbax SB C18 (2.1×30mm, 3.5µm)	LSD = 10 Iso-LSD = 10 O-H-LSD = 500 (not validated)

Chapter 2

Introduction to the use of Liquid Chromatography Coupled to Tandem Mass Spectrometry (LC/MS/MS) in Forensic Toxicology

2.1 Introduction

Toxicology is "the study of the adverse effects of chemicals on living organisms" (Klaassen 2001), and forensic toxicology is "the study and practice of the application of toxicology to the purposes of the law" (Cravey and Baselt 1981). The main objectives of a forensic toxicologist are to detect the presence of drugs or poisons, followed by the subsequent confirmation and quantitation of any toxicologically relevant drugs or poisons, and finally the interpretation of the results. Since forensic toxicology pertains to the law, the most important objective of a forensic toxicologist after detecting the drug is its confirmation. To obtain a reliable analytical result, the confirmatory test should be more specific than the initial screening test, and mass spectrometry should be used where possible and practical (SOFT/AAFS 2006).

Mass spectrometry should be considered a separate and distinct instrumental technique, due to its complexity, expense, and capability, and also since it can be used either alone with direct injections or with either gas or liquid chromatography as a means of sample separation and injection (Cravey and Baselt 1981). Since forensic toxicology exhibits are normally complex biological matrices, methods such as chromatography are needed to separate the compound of interest from matrix interferences or from other drugs. Chromatography requires a flowing mobile phase that passes through a stationary phase. When the mobile phase is liquid it is referred to as LC, where separation is based on the relative solubility of the solutes in the mobile and stationary phase (Burtis and Ashwood 1996). At the RCMP FLS Winnipeg, LC is being used specifically for acidic and neutral drug screening using a photodiode array (PDA) detector (Lyttle, personal communication). LC with the PDA detector is not used as a confirmatory method due to the lack of specificity and selectivity of the PDA detector compared to MS. As a result, GC/MS has been the method of choice for confirmatory

analysis. The RCMP FLS Winnipeg has acquired a new MS/MS with a LC interface that now makes it possible to explore the development and validation of confirmatory methods for analytes more suited to LC analysis.

2.2 LC/MS versus GC/MS

In forensic and clinical laboratories GC/MS is the "gold standard" for toxicological screening and confirmation (Maurer 1998). Laboratories may rely on GC/MS for a few reasons; (1) laboratories already have extensive experience using GC/MS, (2) instrumentation is already available, (3) GC/MS electron ionization (EI) provides structural information, (4) a searchable spectral library exists, (5) technology to couple LC to MS is relatively new compared to GC/MS, and (6) few laboratories can actually afford a LC/MS. However, GC/MS is somewhat limited for the analysis of polar, thermolabile, and volatile analytes. Before the advancement of LC, laborious techniques such as derivatization were employed with GC to increase sample volatility. On the other hand, LC/MS has a number of advantages over GC; (1) no derivatization is necessary, allowing for simpler sample preparation, (2) extracts may be frozen and re-examined, and (3) the spectrum of analytes can now include polar active metabolites of numerous drugs, which are not suitable for GC/MS (Bogusz 2000). By assessing the polarity and molecular mass of an analyte, LC is capable of analyzing a significantly higher number of analytes compared to GC (Figure 2.1).

Not only is LC able to analyze many compounds that cannot be analyzed by GC, but very difficult separations can be achieved by LC versus GC (Cravey and Baselt 1981). This is due to the fact that the efficiency or the number of theoretical plates of LC (≥5000 plates/m) is better than GC (≥2000 plates/m). In chromatography the column efficiency is measured using the theoretical plate number (N), which is calculated from the retention volume of a solute (V_R) and the volume occupied by the solute or the peak width (w_B) (Equation 2.1), where N gets larger as the column becomes more efficient (Lindsay 1987).

$$\mathbf{N = 16\ (V_R/w_B)^2} \qquad \text{Equation 2.1}$$

Also along with the ability to vary the stationary phase, flow rate, and temperature, LC can also vary its mobile phase. And finally LC has a wide variety of column packings versus GC.

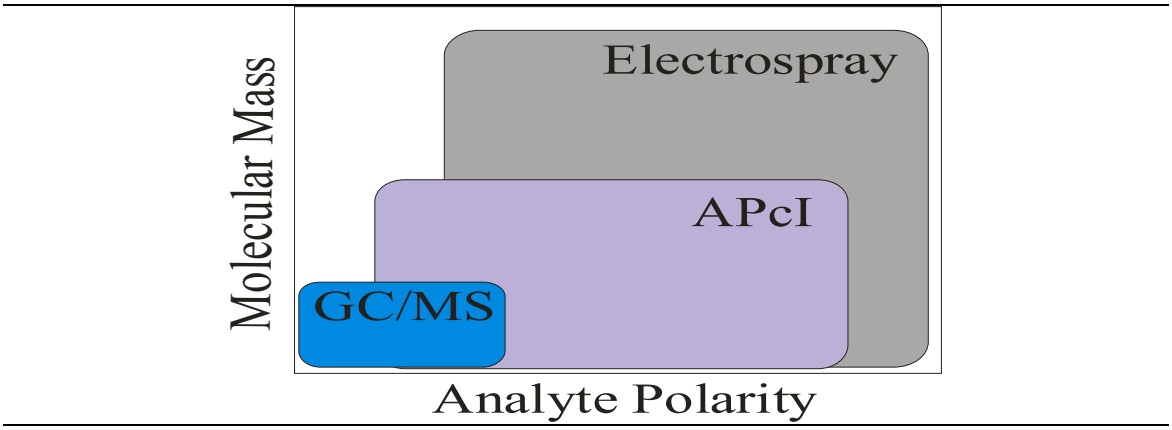


Figure 2.1: Determining optimal analysis based on molecular mass and analyte polarity (adapted from Mauer 1998).

2.3 MS with GC versus LC

In forensic toxicological analysis MS is recommended as the confirmatory technique when possible (SOFT/AAFS 2006). This is because MS is capable of determining both molecular weight and structural information (McLafferty and Tureček 1993). The basic principles of MS are the formation of ions via ionization, separation of these ions according to their mass to charge ratio (m/z), and final detection of these ions (Cravey and Baselt 1981). There are generally two types of ionization methods in MS, "hard" and "soft" ionization (McLafferty and Tureček 1993). "Hard" ionization creates ionized molecules that tend to undergo fragmentation, whereas "soft" ionization reduces the propensity for fragmentation. EI, a "hard" ionization method, is commonly used with GC/MS. The fragment ions produced in EI provide structural information because the masses of these fragment ions are characteristic of the original molecule. Chemical ionization (CI), a "soft" ionization method, is more useful for determining the molecular weight of a molecule. With CI, amines and ethers usually give abundant $[M+H]^+$ ions and saturated hydrocarbons normally yield $[M-H]^+$ ions. Both species are useful for determining the molecular weight (McLafferty and Tureček 1993). EI provides more

fragment ions with relative high abundances, but lacks the sensitivity, whereas CI provides a spectrum in which the base peak ($[M+H]^+$ or $[M-H]^+$) is more prominent making it ideal for highly sensitive quantitative analysis (Libong et al. 2003a).

Coupling MS to LC presents many challenges. A suitable interface for LC/MS must be able to; (1) eliminate a large volume of gases and vapours produced from the LC mobile phase, and (2) transform the molecules in solution in the mobile phase into ions in the gas phase without thermal degradation (Marquet and Lachâtre 1999). The most commonly used interfaces for LC/MS with the largest polarity range are Atmospheric Pressure chemical Ionization (APcI) and electrospray ionization (ESI), which are considered "soft" ionization methods. Combining both APcI and ESI on one instrument would theoretically be complementary to EI (Marquet and Lachâtre 1999). In ESI, liquid is sprayed out of a capillary tube to which a high voltage is applied to form a spray of charged droplets (Whitehouse et al. 1985). In APcI, liquid is passed through a heated tube and evaporated to produce gas phase molecules. The gas phase molecules are ionized by electrons produced from a corona discharge (Niessen 1998). When deciding on what form of ionization to use, both molecular weight and polarity should be considered (Figure 2.1, page 19). ESI is suitable for small to larger polar molecules up to several hundred thousand Daltons, whereas APcI is better suited to ionization of small, less polar molecules. Ionization can also be achieved in either polarity, producing either positively or negatively charged ions.

Since LC/MS utilizes "soft" ionization, the mass spectrum typically consists of only the protonated molecular ion $[M+H]^+$, which could provide a sensitive method but lacks specificity. It is possible to induce in-source fragmentation by varying the potential across the ion transfer lens between the ESI source and the quadrupole analyzer (Webb et al. 1996). Excessive fragmentation could reduce sensitivity through reduction of the $[M+H]^+$. It has also been found that with a given voltage the degree of fragmentation varied with different batches of mobile phase. As a result, fragmentation voltage must be monitored when changing batches of mobile phase (White et al. 1997).

Alternatively, fragmentation can be induced during tandem MS (MS/MS) experiments, for which triple quadrupole instruments are ideally suited. Ions in a quadrupole travel between four co-linear rods, and mass separation is achieved by

combining radio frequency (RF) and direct current (DC) fields applied to these rods (McLafferty and Tureček 1993). Depending on the exact potential applied to the quadrupoles, only ions with a specific mass to charge ratio (m/z) will pass through. When conducting MS/MS experiments with a triple quadrupole mass spectrometer the first quadrupole is used in a narrow band pass (Q) mode, allowing ions of a particular m/z to pass through. The second quadrupole is used in wide band pass (q) mode as a collision cell, in which ions collide with argon atoms and fragment into product ions. And finally the third quadrupole is used to resolve and transmit product ions of a particular m/z to the detector.

Triple quadrupole MS has the capability of acquiring data in a variety of modes, such as (1) product (daughter) ion scan, (2) precursor (parent) ion scan, (3) constant neutral loss, and (4) multiple reaction monitoring (MRM). In product ion scan the first quadrupole is fixed to allow only ions of a specific m/z (precursor ion) to pass through, and ions then undergo collision induced dissociation (CID) with argon atoms in the second quadrupole, and finally the third quadrupole is in full scanning mode to allow detection of all fragment product ions. Product ion scan is useful for determining the fragmentation pattern of a compound. In precursor ion scan the first quadrupole is in full scanning mode and the third quadrupole is fixed. Precursor ion scanning is beneficial for detecting compounds with similar structures that produce the same charged fragment. For constant neutral loss mode both first and third quadrupole are in full scanning mode. Constant neutral loss mode is used for compounds with similar structures that produce the same neutral fragment.

For MRM mode both the first and third quadrupole are fixed to allow only ions of a specific m/z to pass through. With complex matrices, MRM has the capability to eliminate the interference from the matrix (Figure 2.2). As a result, with MRM, the development of a highly sensitive method is possible. MRM is also useful for compounds that are difficult to resolve chromatographically, but have unique precursor to product ion transitions (Figure 2.3).

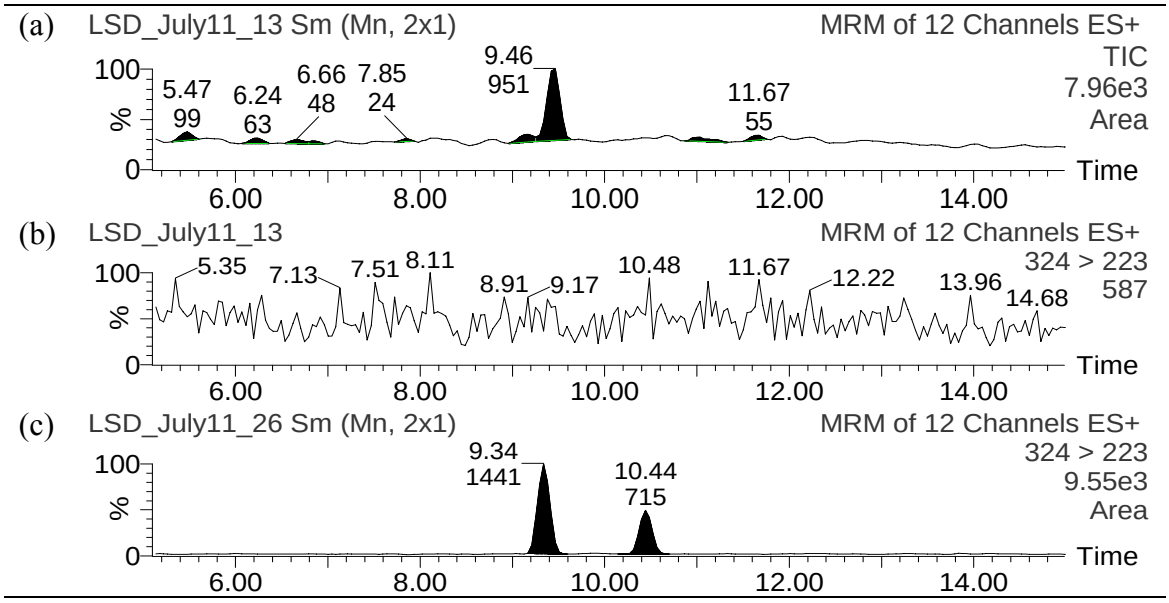


Figure 2.2: (a) Total ion chromatogram (TIC) of blank urine showed a peak close to the retention time of LSD ((c) MRM channel of LSD (RT = 9.34) standard), (b) but when monitoring the MRM 324>223 channel specific for LSD it showed the absence of this peak, suggesting that the peak was an impurity present in the matrix.

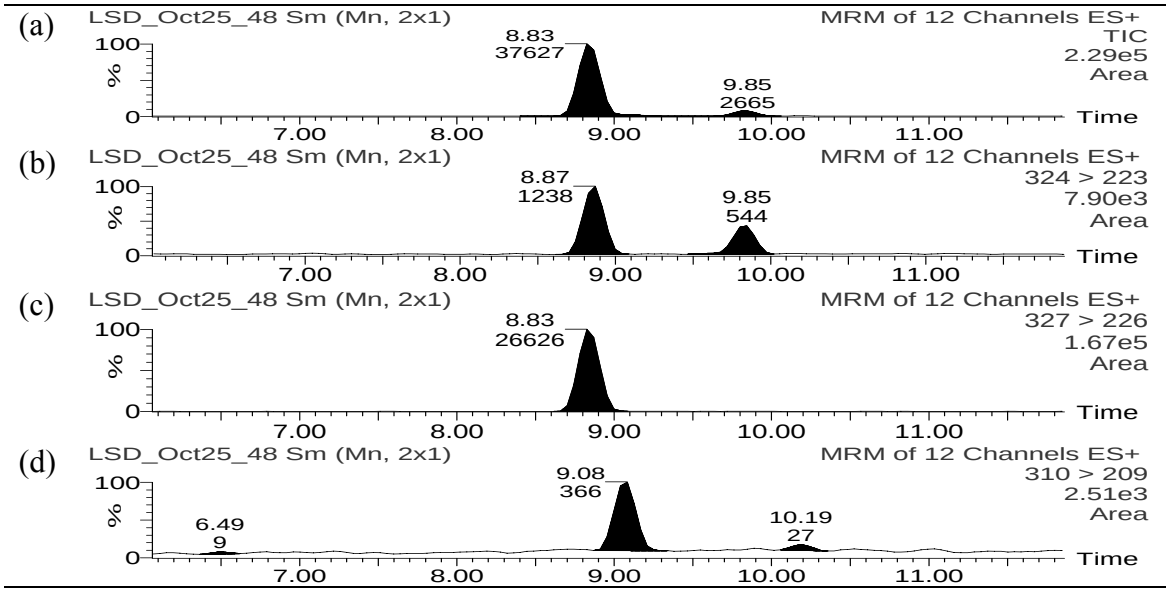


Figure 2.3: (a) TIC and MRM acquisition of (b) LSD, (c) LSD-D3, and (d) nor-LSD. Chromatographically LSD, LSD-D3, and nor-LSD are not totally resolved, but MRM provides unique channels for monitoring all three compounds.

2.4 HPLC versus Ultra Performance Liquid Chromatography (UPLC™)

Waters Corporation has taken the principles of HPLC and further adapted them to create Ultra Performance Liquid Chromatography (UPLC™), a new separation technique with increased speed, sensitivity and resolution (Swartz. 2005). The performance of a column can be measured in terms of the height equivalent to the theoretical plates (HETP or H) which is calculated from the column length (L) and the column efficiency, or number of theoretical plates (N). N is calculated from an analyte's retention time (t_R) and the standard deviation of the peak (σ).

$$\mathbf{H = L/N} \qquad \text{Equation 2.2}$$

$$\mathbf{N=(t_R/\sigma)^2} \qquad \text{Equation 2.3}$$

The van Deemter equation (Equation 2.4) is the empirical formula that describes the relationship between linear flow velocity (μ) and column efficiency, where A, B, and C are constants related to the mechanistic components of dispersion.

$$\mathbf{H = L/N = A + B/\mu + C\mu} \qquad \text{Equation 2.4}$$

According to the van Deemter plot (Figure 2.4), column efficiency is inversely proportional to the particle size (dp) (Equation 2.5), so by decreasing the particle size there is an increase in efficiency. Since resolution is proportional to the square root of N (Equation 2.6), decreasing particle size increases resolution. Also, by using smaller particles, analysis time can be decreased without sacrificing resolution, because as particle size decreases, column length can also be reduced proportionally to keep column efficiency constant. By using the same HPLC mobile phase and flow rate, UPLC™ reduces peak width and produced taller peaks which increased the S/N 1.8 to 8 fold, improving both sensitivity and resolution (Churchwell et al. 2005).

$$\mathbf{N \alpha 1/dp} \qquad \text{Equation 2.5}$$

$$\mathbf{R = \sqrt{N}/4(\alpha-1/\alpha)(k/k+1)} \qquad \text{Equation 2.6}$$

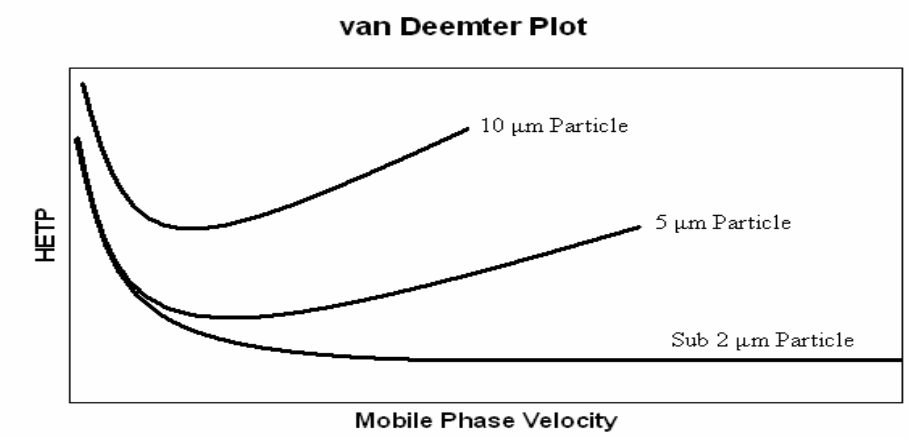


Figure 2.4: van Deemter plot (adapted from Waters 2004c).

Also according to the van Deemter plot, use of particles smaller than 2 µm produces no loss in column efficiency with increasing flow rates. However, by increasing flow rates to decrease analysis time, there is a corresponding increase in system pressure. As a result, a system capable of withstanding the proper pressures while still maintaining efficiency is required. As well, a mechanically stable column is needed. Waters Corporation has designed the ACQUITY system which can withstand pressures as high as 15000 psi and a column with enhanced mechanical stability utilizing a second generation bridged ethyl hybrid (BEH). The empirical formula of the BEH particle is $SiO_2(O_{1.5}SiCH_2CH_2SiO_{1.5})_{0.25}$ which is synthesized by the co-condensation of 1,2-bis(triethoxysilyl)ethane with tetraethoxysilane (Wyndham et al. 2003). Not only do BEH columns have enhanced mechanical and chemical stability, but also reduce peak tailing significantly for basic analytes compared to silica columns, due to the reduced acidity of the unreacted surface silanol groups (Wyndham et al. 2003).

2.5 Waters Quattro Premier

The ion source configuration, collision cell technology, and data acquisition speed of the Waters Quattro Premier instrument have all been designed to optimize the use of UPLC™. The Quattro Premier has a Z-Spray™ dual orthogonal ion source (Figure 2.5), which protects the source against contamination by non-ionic components in complex sample matrices (Waters 2004b). As well, an exhaust trap positioned

opposite the ionization probe allows for rapid removal of non-ionized materials. Since the orthogonal position protects the source from matrix contamination, the cone orifice can be increased in size, allowing more ions to be transferred to the MS (Waters LC/MS booklet). As a result, there is a decrease in noise which corresponds to an increase in sensitivity. Furthermore, an isolation valve allows for easy removal and cleaning of source elements without breaking vacuum (Waters 2004b). Compared to previous Waters models, the Z-Spray™ is more robust and provides better sensitivity (Niessen 1998).

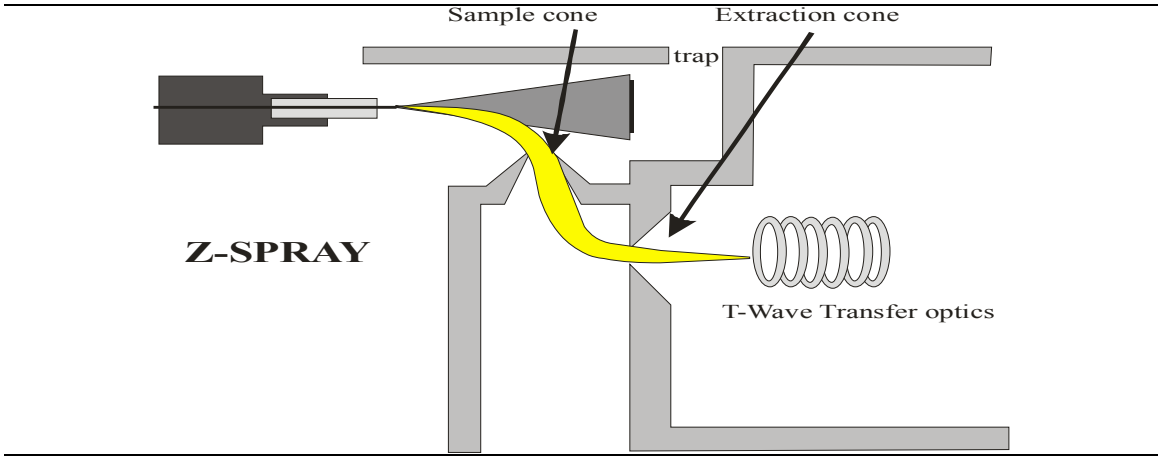


Figure 2.5: Z-Spray™ (adapted from Waters LC/MS booklet).

Previous Waters quadrupole MS utilized a hexapole assembly in the collision cell. However, the Quattro Premier utilizes T-Wave™ (Travelling Wave™) ion optics in the collision cell. The T-Wave™ collision cell is a stacked-ring with only RF (Figure 2.6) which propels ions by superimposing a voltage pulse on the RF of an electrode and moving the pulse to an adjacent electrode to provide a travelling voltage wave on which the ions are carried (Figure 2.7), reducing their residence time in the cell (Giles et al. 2004). With MRM the duty cycle depends upon two parameters; (1) dwell time, during which the ions are monitored, and (2) inter-channel delay between successive MRM transitions, during which ions are cleared from the collision cell. When operating in MRM mode with very short dwell times it is important that the time the ions reside in the collision cell is minimal to avoid cross-talk between successive transitions (Giles et

al. 2003). T-Wave™ reduces cross-talk to 0.01% without losing sensitivity (Giles et al. 2003).

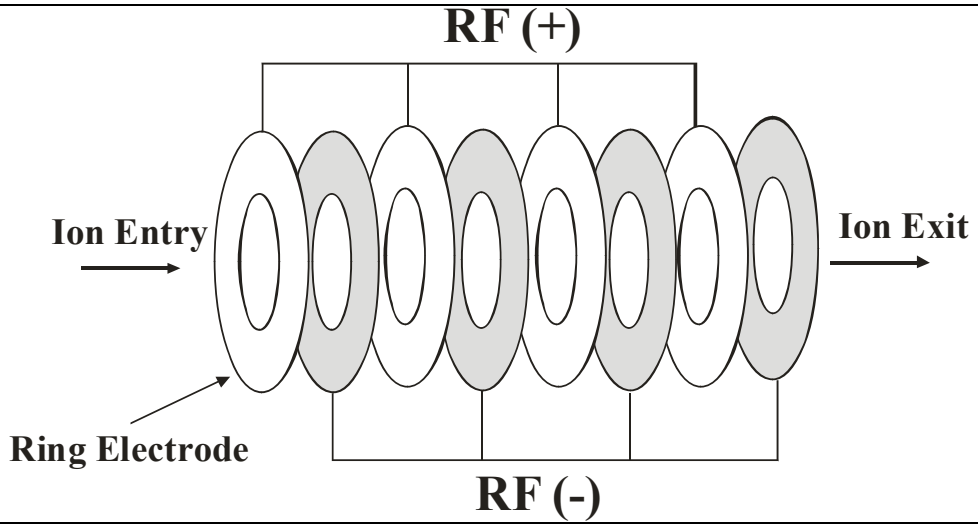


Figure 2.6: RF-only stacked ring ion guide (adapted from Giles et al. 2004).

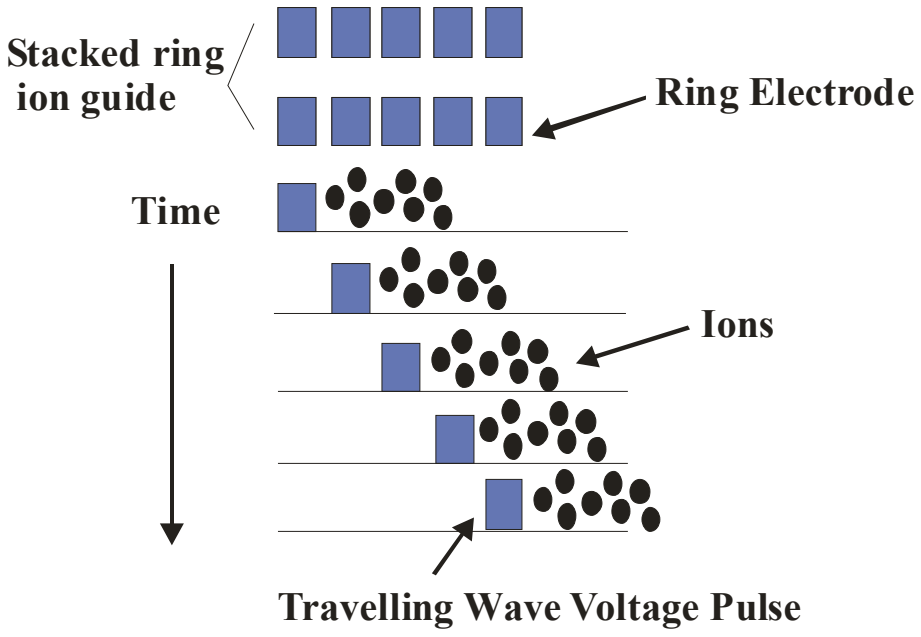


Figure 2.7: T-Wave™ on which the ions surf (adapted from Giles et al. 2004).

For reproducible peak area quantification, a chromatographic peak should have no less than 10 data points. As a result, in order to accurately quantify the narrow peaks produced with UPLC™, the MS needs to acquire data at a fast rate. The Quattro Premier has the capability of acquisition speeds of 100 data points per second (Waters 2004a). The fast acquisition speed is made possible with T-Wave™ as described earlier.

2.6 The Potential of LC/MS(/MS) in Forensic Toxicology

LC/MS(/MS) at the RCMP FLS Toxicology Services (TS) is a fairly new technology, and is currently in the developmental process being used only for confirmation of a small number of compounds more suited to LC/MS(/MS) versus GC/MS. Other forensic toxicology laboratories, such as the Institute of Forensic Medicine in Aachen, Germany, are using a LC with a single quadrupole MS routinely (Bogusz 2000). Attempts have also been made to create a spectral library using LC/MS/MS (Gergov et al. 2004). But to what extent can LC/MS/MS be used for systematic toxicological analysis? The existing searchable spectral library for GC/MS makes it ideal for toxicological screening. In order for a spectral library to be useful it should be reproducible long term and between different laboratories. By standardizing collision energy and gas pressure with a tuning compound, it was found that LC/MS/MS spectra using two different instruments from different manufacturers were similar (Gergov et al. 2004). Once standardization of fragmentation intensities and patterns between different laboratories is achieved, LC/MS(/MS) may one day even replace GC/MS as the “gold standard”.

Chapter 3

Validation Parameters in the Development of Qualitative and Quantitative LC/MS/MS Methods in Forensic Toxicology

3.1 Introduction

The investigation of drugs or chemicals can be divided into three steps: (1) obtaining the case history and suitable specimens, (2) the toxicological analyses, and (3) the interpretation of the analytical findings (Klaassen 2001). In most cases the suspected drug or poison is unknown. As a result, screening tests capable of detecting a wide variety of drugs are usually the first step in forensic toxicological analysis. If the screening methods detect the presence of a drug or chemical, it must be confirmed using a more specific method. While the sole presence of a poison may suffice for some cases in forensic toxicology, an actual quantity of the drug or poison may also be needed.

Methods used in forensic toxicology must be validated to ensure reliable results. Validation is a permanent process that starts from the beginning of the life of the method until its retirement, with the objective of achieving high-quality results by means of an analytical method (Boulanger et al. 2003). Validating a method provides an assurance of reliability during normal use, and can also provide documented evidence that the method works (Shabir 2003). There are many guidelines that have been written on how to validate a method; however, one must consider the intended purpose of an analytical procedure when deciding on what parameters to validate. For example, the significance of certain parameters when validating a qualitative versus quantitative analytical procedures, or a pharmaceutical versus forensic analytical procedure, will be different.

There are many different regulatory agencies, such as the US Food and Drug Administration (FDA), the United States Pharmacopeia (USP), and the International Conference on Harmonization (ICH), that have put forth validation requirements for analytical methods. However, the guidelines put forth by these three sources are specific for pharmaceutical analysis. The Society of Forensic Toxicologists (SOFT) and the American Academy of Forensic Sciences (AAFS) have written guidelines specifically

for forensic laboratories (SOFT/AAFS 2006). As well, the Standards Council of Canada has written guidelines for the accreditation of forensic testing laboratories, where it is stated that confirmation and quantitative analysis should use methods that have been documented and validated by the laboratory, where linearity, selectivity, LOD, accuracy, and precision should be assessed (Standards Council of Canada 2003). The FDA also requires the assessment of recovery, stability, and when using LC/MS/MS, matrix effects.

3.2 Linearity and Range

A calibration curve should be constructed from biological matrix standards which are spiked with a known amount of analyte. A calibration curve describes the relationship between the instrument response and known concentrations of analyte, with a linear relationship being the simplest. Assuming that a relationship is linear when developing a method must be avoided. Instead, linearity should be evaluated by visual inspection of a plot of signal (instrument response) as a function of analyte concentration, and by appropriate statistical methods (ICH 1996). The correlation coefficient (r), y-intercept, slope of the regression line, and residual sum of squares should also be submitted (ICH 1996).

Although it has been common practice to define linearity using r, it is not really a measurement of linearity. Linear regression finds the best fit line, whereas correlation quantifies how consistently two variables vary together (Motulsky and Christopoulos 2005). Another term related to r is the coefficient of determination (r^2), which quantifies the goodness of fit. When r^2 is 1, all points lie exactly on the curve with no scatter. However, using r or r^2 as the main criterion for determining the goodness of fit and linearity should be avoided.

Residuals and a runs test can further support the linearity of data. By visually inspecting the residuals, the assumption of linear regression may be verified if the residuals are randomly scattered above and below. Also, a runs test can determine if the straight line deviates from the data. A run is a series of consecutive points that are either all above or all below the regression line (Motulsky and Christopoulos 2005). A low P value from the runs test means that data deviates from a straight line.

The goal of linear regression is to find the best fit straight line through the points of the calibration standards (Miller 1991). Normally linear regression with unweighted least squares makes two assumptions: (1) the y-direction errors are normally distributed (Gaussian distribution), and (2) that the standard deviation of y-direction errors is the same for all x values (homoscedastic data) (Motulsky and Christopoulos 2005; Miller 1991). Therefore, when using unweighted least squares regression all points on the graph are of equal weight or importance in the calculation of the best fit line. In most cases of linear regression the first assumption is true, but the second assumption is usually not true. Instead the standard deviation of y-direction errors often increases as x increases (heteroscedastic data) (Miller 1991). As a result, for heteroscedastic data a weighted regression should be used instead.

Linearity should be established using at least three calibrators (calibration standards), which should bracket the anticipated concentration of the specimen (SOFT/AAFS 2006). However, according to the FDA a calibration curve should consist of six to eight non-zero samples covering the expected range (FDA 2001). For the purposes of this project, all qualitative analysis followed the SOFT/AAFS guideline using three calibrators, and all quantitative analysis used the FDA guideline of six to eight calibrators. Although r is not necessarily a good measurement of linearity, SOFT/AAFS suggests that r should be 0.99 for accepting a multi-point calibration. Back-calculating the value of each calibrator against the curve and then using a percent deviation of ±20% acceptance limit is also recommended by SOFT/AAFS.

3.3 Accuracy

Accuracy is the degree of closeness of the determined value to the nominal or known true value (FDA 2001). To assess accuracy the FDA suggest using a minimum of five determinations per concentration, with a minimum of three concentrations within the expected range. In order to determine if a method is accurate the measured values should be within 15% of the nominal values, and within 20% for the LLOQ.

3.4 Precision

Precision is the closeness of agreement between a series of measurements obtained from multiple sampling of the same homogenous sample under the prescribed condition (FDA 2001). The SOFT/AAFS mentions % CV, but not in the context of method validation. Instead it states that it is good practice to monitor the performance of assays by periodically calculating the % CV, and that a % CV greater than 15% indicates poor precision. The FDA provides a detailed method for determining precision. A minimum of five determinations per concentration using a minimum of three concentrations within the expected range should be measured. The % CV should be 15% (20% for LLOQ) for acceptable precision (FDA 2001). The FDA also further subdivides precision into three more categories; (1) within-run, (2) intra-batch, (3) and between-run.

3.5 Sensitivity (LOD and LLOQ)

The LOD is the lowest concentration of an analyte that the bioanalytical procedure can reliably differentiate from background noise, and the LLOQ is the lowest concentration that can be quantitatively determined with suitable precision and accuracy (FDA 2001). For simplification, the SOFT/AAFS states that for chromatographic assays, the LOD and LLOQ may be defined as the concentration of the lowest calibrator. If results need to be reported below the value of the lowest calibrator, the LOD and LLOQ would then need to be determined experimentally.

The FDA suggests that the LLOQ should be at least five times the response of the blank and that the analyte peak be identifiable, discrete, and reproducible with a precision of 20% CV and accuracy of 80 to 120% (FDA 2001). Another method of determining LOD and LLOQ is based on signal to noise ratio (S/N). S/N of 3:1 and 10:1 is considered acceptable for estimating the LOD and LLOQ, respectively (ICH 1996). A method utilizing a calculation based on standard deviation (σ) of the response and the slope (S) is also available (Equation 3.1 & 3.2, ICH 1996).

$$\mathbf{LOD = 3.3\sigma/S} \qquad \text{Equation 3.1}$$

$$\mathbf{LLOQ = 10\sigma/S} \qquad \text{Equation 3.2}$$

The σ can be determined by using the residual standard deviation of the regression line of a calibration curve in the LOD range. And S may be determined from the calibration curve in the LOD range. ICH guidelines do not favour one method of determining LOD and LLOQ over another. Instead they state that the S/N method only be used for analytical procedures that exhibit baseline noise. Regardless of which method is used, the only requirement is that the method chosen for determining LOD and LLOQ be presented and supported with an appropriate number of samples analyzed at the limit (ICH 1996; Shabir 2003).

3.6 Selectivity

Selectivity is defined as the ability of a method to differentiate an analyte in the presence of other components in the sample (FDA 2001). According to the FDA selectivity should be assessed by analyzing at least six sources of blank samples of appropriate matrix. Each blank should then be tested for interference and selectivity should be established at the LLOQ.

3.7 Recovery

Recovery is the extraction efficiency of an analytical process (FDA 2001). A high recovery is not essential unless the sensitivity of the method is poor. However, recovery experiments must be assessed at different concentration levels and recovery must be consistent, precise, and reproducible.

3.8 Matrix Effect (ME) (Ion Suppression and Ion Enhancement)

LC/MS/MS has been perceived as a highly selective technique. As a result, simple extraction procedures and chromatographic conditions that result in limited retention and separation of analytes from endogenous compounds are often employed. Consequently, matrix effects (ME) become more of a concern with LC/MS/MS procedure (Matuszewski et al. 2003). ME in LC/MS/MS is somewhat different from other detection techniques. Interferences from the sample matrix with detection techniques such as UV are visible as peaks, whereas in LC/MS/MS interferences are generally unseen, causing a suppression or enhancement of the signal (Larger et al.

2005). ME in LC/MS/MS occurs when co-eluting, undetected matrix components reduce or enhance the ion intensity of the analytes, and affect the reproducibility and accuracy of the assay (Matuszewski et al. 2003). Specifically with ESI, ME arise from the competition between matrix constituents (non-volatile solutes) and analyte molecules for access to the droplet surface and subsequent gas phase emission (King et al. 2000). Non-volatile matrix constituents can also change eluent properties such as boiling point, surface tension, and viscosity (King et al. 2000).

The FDA suggests that ME be investigated for LC/MS/MS procedures, but no specific method on how to accomplish this is given (FDA 2001). However, a number of methods for investigating ME have been proposed. The first is by using a post-column infusion system in which an infusion pump delivers a constant flow of an analyte post column, and then blank sample extracts are injected on-column (Bonfiglio et al. 1999). Any interference that elutes from the column and causes a variation in ESI response of the infused analyte is considered as ionization suppression. Although this method provides information on the chromatographic profile of the interference, it does not provide quantitative information (Larger et al. 2005).

Another proposed method involved injection of sets of samples (Matuszewski et al. 2003). Samples in set 1 are neat solutions made up in the mobile phase, and samples in set 2 are extracts of blank matrices spiked with analyte after the extraction. The absolute ME can then be determined by comparing the peak areas of the neat analyte standards (A) to the standards spiked after extraction (B) (Equation 3.3).

$$\textbf{ME \%} = \textbf{B/A} \times \textbf{100} \qquad \text{Equation 3.3}$$

A ME % greater than 100% suggests an ion enhancement and a ME % less than 100% suggest an ion suppression. Matuszewski et al. suggests using five different sources of matrices to provide more accurate bioanalytical data. A relative ME can be assessed as well by comparing the B values of different sources of matrices expressed as the % CV. Large differences between B values indicate that the MS/MS response of an analyte is different in different sources of matrix.

When analyzing multiple analytes in one method, the absence of ME for all individual analytes needs to be demonstrated. The presence of either an absolute or relative ME does not necessarily suggest that a method is not valid. As long as the

analyte and the internal standard (ISTD) exhibits the same relative ME, the drug to ISTD ratio used to calculate drug concentration should not be affected (Matusewski et al. 2003). However, if the presence of ME invalidates a method, the ME may be eliminated by: (1) changing sample extraction procedures, (2) changing chromatographic conditions to separate analytes of interest from undetected endogenous compounds, and (3) evaluating and changing the LC/MS interface and the mechanism of ionization (Matusewski et al. 2003).

3.9 Stability

Drug stability in biological fluids is a function of the storage conditions, the chemical properties of the drug, the matrix, and the container system (FDA 2001). The FDA suggests evaluating freeze and thaw, short term, long term, stock solution, and post preparative stability. All stability studies should evaluate a low and high concentration in at least replicates of three. All conditions should reflect situations likely to be encountered during actual sample handling and analysis.

For evaluating freeze and thaw stability of an analyte a minimum of three cycles should be assessed. Each cycle involves storing the sample at the intended storage temperature, then allowing the sample to thaw at room temperature. If the analyte is unstable at the intended storage temperature the sample should be frozen at -70°C.

For short term stability a sample should be thawed at room temperature and kept at room temperature for the expected duration that samples will be at room temperature during actual sample analysis. Whereas, for long term stability the storage time of the sample should exceed the time between the date of first sample collection and the date of last sample analysis.

The stock solutions also need to be shown to be stable in terms of both short term and long term stability. The stability of the stock solutions of drug and ISTD should be evaluated at room temperature for at least 6 hours. Then the storage temperature of the stock solutions should be assessed. And finally the post preparative stability involves the assessment of processed samples, such as the time in the autosampler.

3.10 Retention Times

Retention times (RT) should also be included in the acceptance criteria where a 1 to 2% deviation from the calibrators or controls is acceptable for GC based assays (SOFT/AAFS 2006). For LC based assays, especially when mobile phases are programmed via gradient elution, large deviations from the calibrators or controls may be acceptable. SOFT/AAFS does not state a deviation level specific for LC based assays. The International Olympic Committee (IOC) and the World Anti-Doping Agency (WADA) have suggested that for GC the RT and relative RT (RRT) of the analyte should not differ more than 1% from the calibrators, and 2% for HPLC (Rivier 2003).

3.11 Ion Ratios

For MS procedures used in selected ion monitoring modes for confirmation and/or quantitation, a qualifying ion in addition to the primary ion, is encouraged (SOFT/AAFS 2006). The acceptance criterion for ion ratios is ±20% relative to that of the corresponding control or calibrator. For LC/MS the acceptance criteria may be increased up to ±25 to 30%, because LC/MS assays may be more concentration and time dependent.

Chapter 4

Preliminary Development of a HPLC/MS/MS Method for Analyzing LSD, iso-LSD, nor-LSD, and O-H-LSD in Urine

4.1 Introduction

A preliminary method using an Agilent 1100 HPLC coupled to a Waters Quattro Ultima MS was developed for analyzing LSD, iso-LSD, nor-LSD, and O-H-LSD. The purposes of this method were; (1) to determine if HPLC/MS/MS could achieve the required sensitivity, and (2) to determine if simultaneous analysis of LSD, iso-LSD, nor-LSD, and O-H-LSD was possible.

4.2 Materials and Methods

4.2.1 Reference Standards

LSD, iso-LSD, nor-LSD, O-H-LSD, LSD-D3, LAMPA, and O-H-LAMPA were obtained from Cerilliant Corporation (Round Rock, TX). A stock solution of 5 ng/mL was prepared in methanol (MeOH). 10, 50, 100, 200, 400, 800, and 2000 pg/mL working standards were prepared by spiking blank urine with the 5 ng/mL stock solution. An internal standard test mix (LSD-D3, LAMPA, and O-H-LAMPA) stock solution of 200 ng/mL was prepared in MeOH.

4.2.2 Sample Preparation and Extraction

A simple SPE procedure for basic compounds was done using Waters Corporation (Milford, MA) Oasis extraction cartridges. To the 5 mL working standards, 20 µL of internal standard test mix (200 ng/mL) was added. First to condition the SPE cartridges, 1 mL of MeOH was added to and drawn through each cartridge. Next 1 mL of water was added to and drawn through each cartridge to equilibrate the column. After conditioning and equilibrating the column, 1 mL of the 5 mL working standard with ISTD was added to and drawn through each cartridge. To wash the cartridges, 1 mL of 5% MeOH in water (v/v) was added to and drawn through each cartridge. Finally to

elute off the compounds, 1 mL of MeOH was added and the eluate was collected. Then the eluate was evaporated to dryness under nitrogen at 55°C. The residue was reconstituted in 200 µL of 20:80 (v/v) acetonitrile (ACN): 20 mM ammonium acetate in 0.05% formic acid. Twenty five microlitres of sample were injected into the HPLC.

4.2.3 HPLC Conditions

An Agilent Technologies (Palo Alto, CA) HPLC 1100 with an autosampler was used. Chromatographic separation was done using a Zorbax SB C18 (2.1 × 50 mm, 5 µm particle size, Agilent Technologies, Palo Alto, CA) column with a gradient elution using 20 mM ammonium acetate, 0.05% formic acid and ACN at a flow rate of 0.2 mL/min. An initial solvent composition of 90:10 ACN:20 mM ammonium acetate in 0.05% formic acid was held for 1 minute, followed by a step gradient to 15:85 ACN:aqueous solvent for 7 minutes. Then another step gradient to 25:75 for 11 minutes was used and held for 3 minute. The column was then equilibrated for 6 minutes giving a total run of 27 minutes.

4.2.4 HPLC/MS/MS Conditions

A Waters Corporation (Milford, MA) Quattro Ultima™ tandem quadrupole mass spectrometer was used. API was done via positive ESI. The electrospray probe tip potential was set at 3.00 kV. The source and desolvation temperature were set at 120 and 350°C, respectively. The cone and desolvation gas flow were set at 113 and 748 L/Hr, respectively. The collision gas (Argon) pressure was set 3.54×10^{-3} mbar. The mass spectrometer was run in MRM mode. Four MRM channels in total were monitored (Table 4.1).

Table 4.1: MRM transitions with Water Quattro Ultima

Standards	MRM transition	Collision Energy	Cone Voltage
LSD, iso-LSD, LAMPA	324 > 223	21	37
nor-LSD, iso-nor-LSD	310 > 209	21	37
O-H-LSD, O-H-LAMPA	356 > 237	22	37
LSD-d_3	327 > 226	22	37

4.2.5 Assessment of Linearity and Accuracy

Linearity was assessed using standards prepared by spiking blank urine with analyte concentrations ranging from 10 to 2000 pg/mL and extracting with the SPE method described earlier (section 4.2.2). The calibration curve was plotted with nominal concentration to peak area ratios (PAR = peak area (PA) of standard/PA of ISTD). Both unweighted and weighted linear regression for the calibration curve were evaluated. Data collection, peak integration, % bias, and linear regression were performed using MassLynx™ version 4.0 software (Waters Corporation, Milford, MA). A suitable linear range was determined by evaluating the accuracy (% bias) of back calculated results.

4.3 Results and Discussion

4.3.1 Gradient versus Isocratic Elution

Various ratios of solvent A (20 mM Ammonium acetate, 0.05% formic acid) and solvent B (ACN) were investigated initially using isocratic elutions. However, isocratic elution was unable to adequately chromatographically separate all compounds with similar MRM transitions, and within an optimal time. Therefore, gradient elution was ultimately chosen. At the beginning of the run the mobile phase has a low organic content so that analytes are retained on the column and salts flow through the column. Then the mobile phase is ramped from low organic to high organic content so that analytes will begin to elute off the column and enter the MS. The mobile phase then has a high organic content to wash any hydrophobic contents off the column. Finally the column is equilibrated back to initial conditions. Using a neat standard mix, all compounds with similar MRM transitions were chromatographically separated using a gradient elution with a total run time of 27 minutes (Figure 4.1).

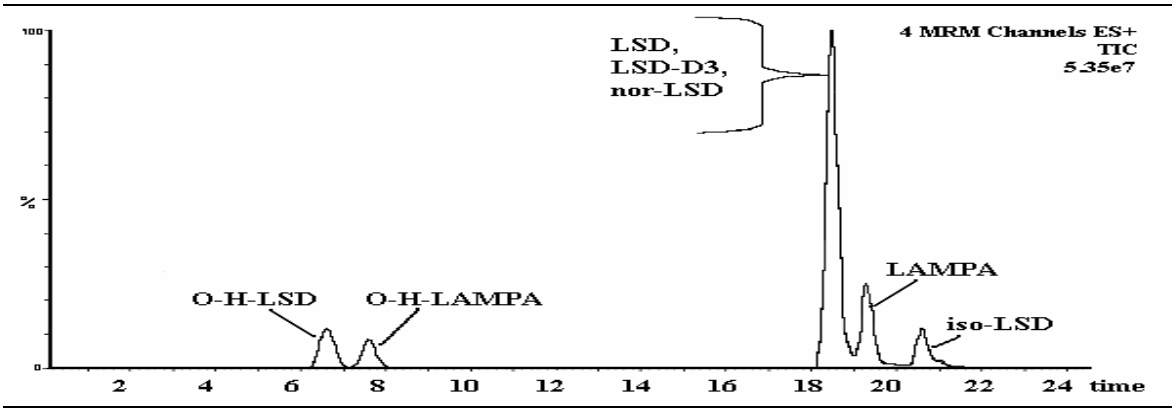


Figure 4.1: TIC of a 250 ng/mL neat solution of O-H-LSD, O-H-LAMPA, LSD, LSD-D3, nor-LSD, LAMPA, and iso-LSD.

4.3.2 Sample Extraction

Initially a very simple extraction method was employed in an attempt to extract all the compounds. However, this SPE method did not extract O-H-LSD or O-H-LAMPA (Figure 4.2). In order to perform simultaneous analysis of all compounds, the extraction method therefore needed to be modified or changed.

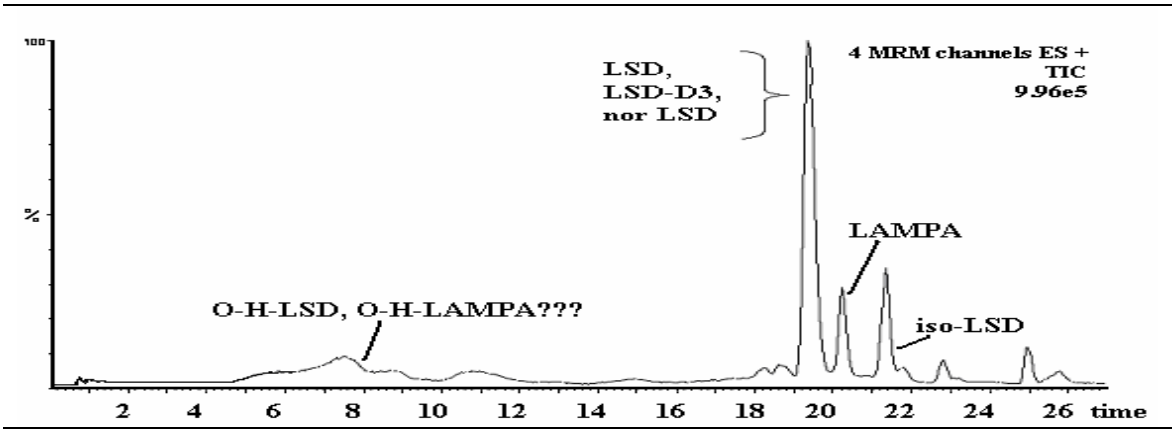


Figure 4.2: TIC of 2000 pg/mL O-H-LSD, O-H-LAMPA, LSD, LSD-D3, nor-LSD, LAMPA, and iso-LSD in a urine extract.

4.3.3 Unweighted versus Weighted Linear Regression

As previously mentioned in Chapter 3 (section 3.2, page 29), unweighted linear least squares assumes that the standard deviation of y-direction errors is the same for all

x values (homoscedastic data) (Motulsky and Christopoulos 2005; Miller 1991). In some cases the standard deviation of y-direction errors often increases as x increases (heteroscedastic data) (Miller 1991). As a result, for heteroscedastic data a weighted regression should be used instead. From experience it was assumed that since the range in the x-values was large (10 to 2000 pg/mL), that the variance at each point was expected to be different. So for the purposes of this preliminary method, weighted ($1/x^2$) linear regression was selected. During the preliminary development of this HPLC method, replicate analysis was not conducted, so the standard deviation of the y-direction errors could not be evaluated. As a result, evaluation of the proper weighting scheme for the calibration curve was not possible. The suitability of this weighting scheme will be further evaluated with replicate analysis in Chapter 5.

4.3.4 Linearity and Accuracy

The extracted urine standards were linear from 10 to 2000 pg/mL for LSD (Figure 4.3). The r, r^2, intercept, and slope, using weighted ($1/x^2$) linear regression are shown in Figure 4.3.

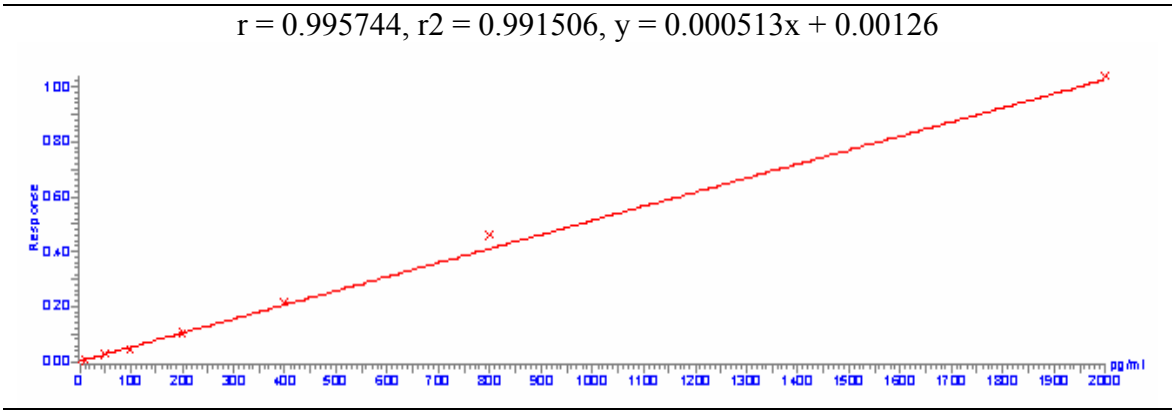


Figure 4.3: Calibration curve of LSD with $1/x^2$ weighting.

A quantitative method was deemed to have acceptable accuracy if the % bias of back calculated results from the nominal concentration did not exceed ±15%. The back calculated results were shown to have acceptable accuracy within the entire linear range (Table 4.2).

Table 4.2: Accuracy results of a neat LSD standard

Sample	STD (pg/mL)	RT (min)	PAR	Calculated (pg/mL)	% Bias
Blank + IS	-	-	-	-	-
LSD 10	10	19.37	0.0065	10.27	2.68
LSD 50	50	19.41	0.0248	45.95	-8.10
LSD 100	100	19.37	0.0457	86.68	-13.32
LSD 200	200	19.37	0.1033	198.98	-0.51
LSD 400	400	19.37	0.2171	421.05	5.26
LSD 800	800	19.41	0.4635	901.86	12.73
LSD 2000	2000	19.41	1.0393	2025.16	1.26

4.4 Conclusion

By using HPLC/MS/MS, LSD, iso-LSD, nor-LSD, and O-H-LSD were simultaneously analyzed. Compounds that shared similar MRM transitions were chromatographically resolved and compounds that could not be chromatographically resolved had unique MRM transitions. The method was capable of achieving a sensitive analysis of LSD at the low picogram concentration levels. However, this preliminary method requires further adaptation before it can be applied to UPLC™/MS/MS. Not only is the LC and MS instrumentation different, but the models are different as well (i.e. HPLC vs. UPLC™, and Waters Quattro Ultima vs. Waters Quattro Premier MS). Furthermore, the extraction method needs to be modified in order to extract O-H-LSD, and the weighting scheme for the calibration curve requires further evaluation.

Chapter 5

Preliminary Development of an UPLC™/MS/MS Method for Analyzing LSD, Iso-LSD, Nor-LSD, and O-H-LSD in Forensic Samples

5.1 Introduction

The method developed on the HPLC/MS/MS system needed to be transferred to the UPLC™/MS/MS system. Even if the systems were similar the method would still need to be validated on the specific instrument to be used for forensic analysis by the RCMP. Certain parameters were changed and optimized for the UPLC™/MS/MS system to further enhance the method sensitivity and robustness prior to beginning full method validation.

5.2 Column Change

The 1.7 μm particle size in the Acquity UPLC™ BEH column versus the 5 μm particle size in the Agilent Zorbax column decreased the total analysis time by half (Table 5.1). Resolution was not lost by decreasing total analysis time. The Acquity UPLC™ BEH column maintained the total peak area by producing narrower peak widths (~0.45 minute versus ~1 minute peak width at 5% height) and taller peak heights (e.g. 4.59×10^6 versus 5.37×10^5) (Figure 5.1).

It is important to note that accurate quantitation was still possible with the narrow peaks. Although it is generally accepted that a chromatographic peak should be made up of 15 to 20 points to accurately define it (Waters 2004a; Dyson 1999), other studies have conducted successful quantitative analysis using 6 to 8 points per peak (Dallüge et al. 2002; King et al. 2003). For the narrow (~27 second) peak widths obtained when monitoring 12 MRM channels, with a dwell time of 0.2 second and a 0.01 second inter-channel delay, the 2.52 second duty cycle corresponded to 0.4 data points/second, or 11 data points per ~27 second peak.

Table 5.1: Parameters for HPLC versus UPLC™

	HPLC	UPLC™
Column	Agilent Zorbax SB C18, 2.1 × 50 mm,5 µm particles	Acquity UPLC BEH C18, 2.5 × 50 mm,1.7 µm particles
Flow Rate	0.2 mL/min	0.2 mL/min
Injection Volume	25 µL	25 µL
Total Run Time	30 min	15 min

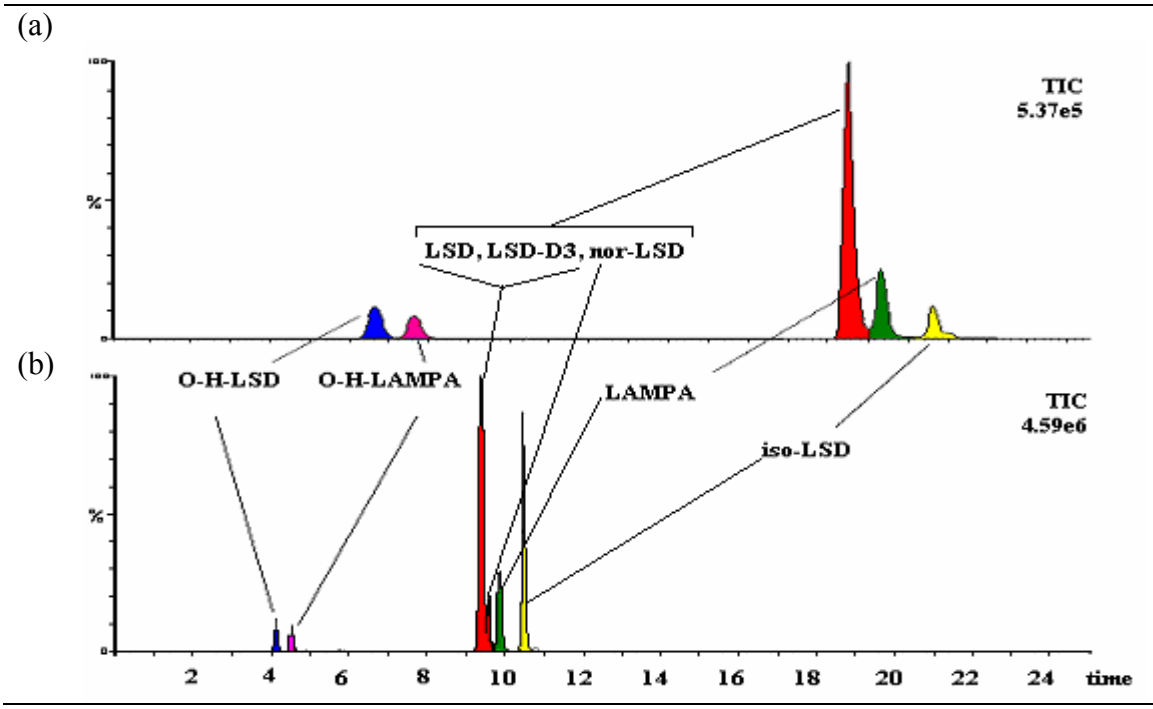


Figure 5.1: Comparison of TIC of LSD and congeners using (a) HPLC versus (b) UPLC™.

5.3 Mobile Phase Change

The mobile phase was changed from 20 mM Ammonium acetate with 0.05% formic acid to a 20 mM Ammonium acetate buffer (pH 4.0). A low concentration of 20 mM for the buffer was chosen to ensure that there was no potential competition between the analyte and the buffer ions for conversion to gas phase ions. If the buffer ion is in large excess it can inhibit charge separation and subsequent ion evaporation during ESI. It may also prevent the ionization of analyte molecules present at low concentrations

(Waters LC/MS booklet). Acetate buffer was chosen because it is volatile and therefore compatible with MS, and also because its pKa of 4.8 allows accurate pH control at pH 4 to 5. LSD has a pKa of 7.8 (Baselt 2000); consequently, a pH of 4.0 for the buffer was chosen because, according to the Henderson-Hasselback equation (Equation 5.1), LSD should be completely protonated if the mobile phase pH is 2 units below the pKa. For an analyte which is ionized under reversed phase conditions, optimal peak shape, detection limits, and consistent retention times will be obtained when using a properly buffered mobile phase (Heyrman and Henry 1999).

$$\mathbf{pH = pKa + \log([base\ conjugate]/[acid\ conjugate])} \qquad \text{Equation 5.1}$$

The ACQUITY columns offer greater efficiency and speed, but the minimum peak width attainable under isocratic conditions will be limited by extra column broadening. When using gradient elution this is less of a problem, because sample components will be concentrated onto the head of the column by the weak mobile phase starting conditions (Jerkovich et al. 2005). As a result, like the HPLC/MS/MS method the UPLC™/MS/MS method also utilized a gradient elution. The ACQUITY column also reduced the re-equilibration time by taking advantage of the low system dwell volume, which is 15% of that of the HPLC (Yang and Hodges 2005).

5.4 Optimizing MS/MS Conditions

Optimization of the MS conditions was done by direct infusion of reference standards (2 μg/mL) in mobile phase (20 mM Ammonium acetate buffer (pH 4.0):ACN (80:20)) at 20 μL/min by the syringe pump installed on the MS. MS parameters were adjusted to maximize sensitivity of product ions produced by CID of protonated ions. The full scan and product ion mass spectra of LSD, iso-LSD, nor-LSD, O-H-LSD, and LSD-D3 are shown in Figure 5.2. The product ion mass spectrum of LSD and iso-LSD were nearly the same as reported by Canezin et al. 2001 using another triple quadrupole instrument (API 300 Perkin-Elmer SCIEX, Thornhill, Canada). Similarly, the product ions of LSD, iso-LSD, nor-LSD, O-H-LSD, and LSD-D3 monitored with triple quadrupole MS were the same as reported by others (Canezin et al. 2001; de Kanel et al. 1998; Skopp et al. 2002). LSD and iso-LSD share common precursor and product ions, but were easily separated chromatographically.

The proposed fragmentation of LSD, iso-LSD, nor-LSD, O-H-LSD, and LSD-D3 are shown in Figure 5.3. The proposed mechanism for the fragmentation of the precursor ion to the most abundant fragment ion (LSD and iso-LSD m/z 223, nor-LSD m/z 209, LSD-D3 m/z 226) includes cleavage at the carbon-carbon bond alpha to the carbonyl group with elimination of the side chain at position eight (Cai and Henion 1996). The same is true for the m/z 237 product ion of O-H-LSD, but also includes the cleavage of the hydroxyl group (Canezin et al. 2001; Poch et al. 2000). The m/z 281 (LSD, iso-LSD, and LSD-D3) and m/z 313 (O-H-LSD) product ions are formed by the retro-Diels-Alder reaction induced by the 9-10 double bond (Cai and Henion 1996). The m/z 208 (LSD, iso-LSD, and LSD-D3) and m/z 222 (O-H-LSD) product ions are also formed by the retro-Diels-Alder reaction, but also includes a loss of the diethylamine group and an additional loss of the hydroxyl group on O-H-LSD (Canezin et al. 2001). The m/z 237 product ion of nor-LSD corresponds to a loss of the diethylamine group (Canezin et al. 2001), with the m/z 74 product ion being the protonated diethylamine group.

LSD and iso-LSD shared common precursor and product ions, but the fragmentation pattern ion intensities of LSD and iso-LSD were different. The equilibrium between LSD and iso-LSD has been shown to be six times slower when iso-LSD was used versus LSD, showing that the epimerizable proton on LSD is more easily removed than the same proton of iso-LSD (Salamone et al. 1997). The most abundant product ion of LSD (m/z 223) is due to cleavage at the epimerizable proton; however, since the same proton is more difficult to remove on iso-LSD, the m/z 281 product ion, whose fragmentation does not involve the C-8 chiral center, is the most abundant product ion for iso-LSD instead.

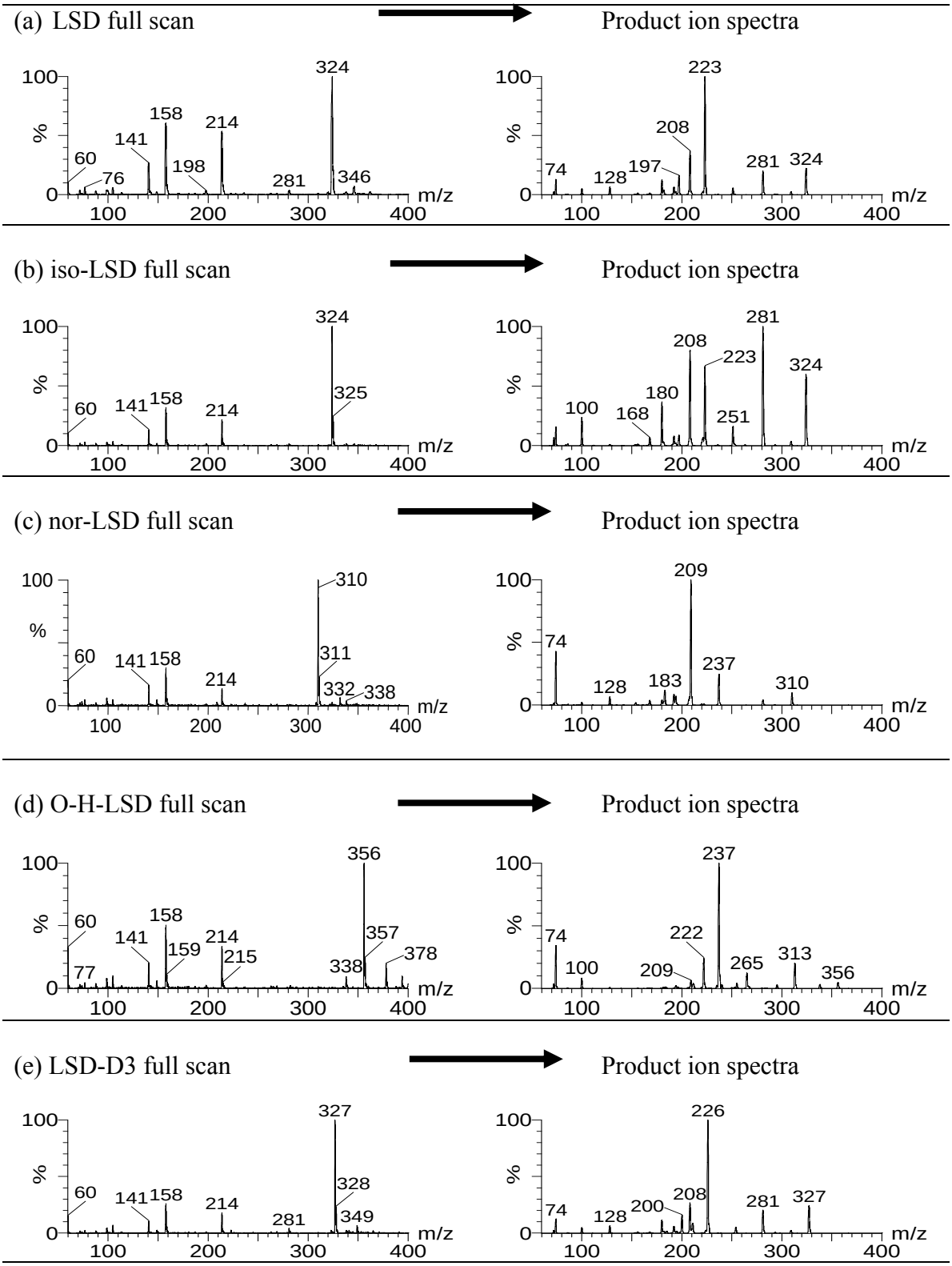


Figure 5.2: Infusion full scan and product ion spectras for (a) LSD, (b) iso-LSD, (c) nor-LSD, (d) O-H-LSD, and (e) LSD-D3.

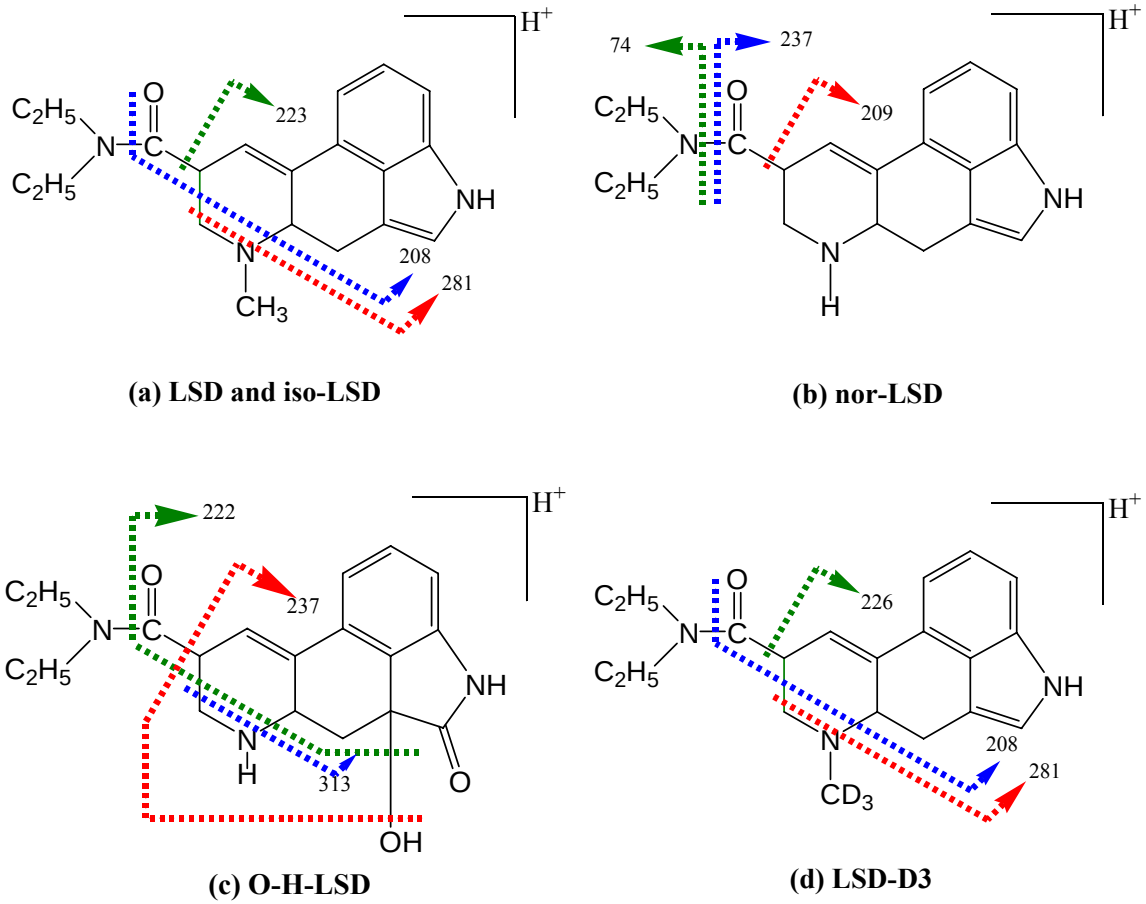


Figure 5.3: Proposed fragmentation of (a) LSD, iso-LSD, (b) nor-LSD, (c) O-H-LSD, and (d) LSD-D3 (Cai and Henion 1996; Canezin et al. 2001).

5.5 Effect of Increasing Flow Rates

In order to take full advantage of the UPLC™ technology, flow rate can be increased to further decrease analysis time. The difficulty with increasing flow rate was the increased back pressure, which caused leaking in connecting tubing. In order to decrease the back pressure larger diameter peek tubing could possibly help. Larger diameter PEEK tubing stopped the leaking; however, the larger diameter in the tubing caused band broadening in the peaks. By ensuring a tight seal, smaller diameter PEEK tubing could be used without leaking problems. By increasing the flow rate to 0.4

mL/min from 0.2 mL/min the pressure reading was ~7900 psi and the analysis time decreased from 15 to 8 minutes (Figure 5.4).

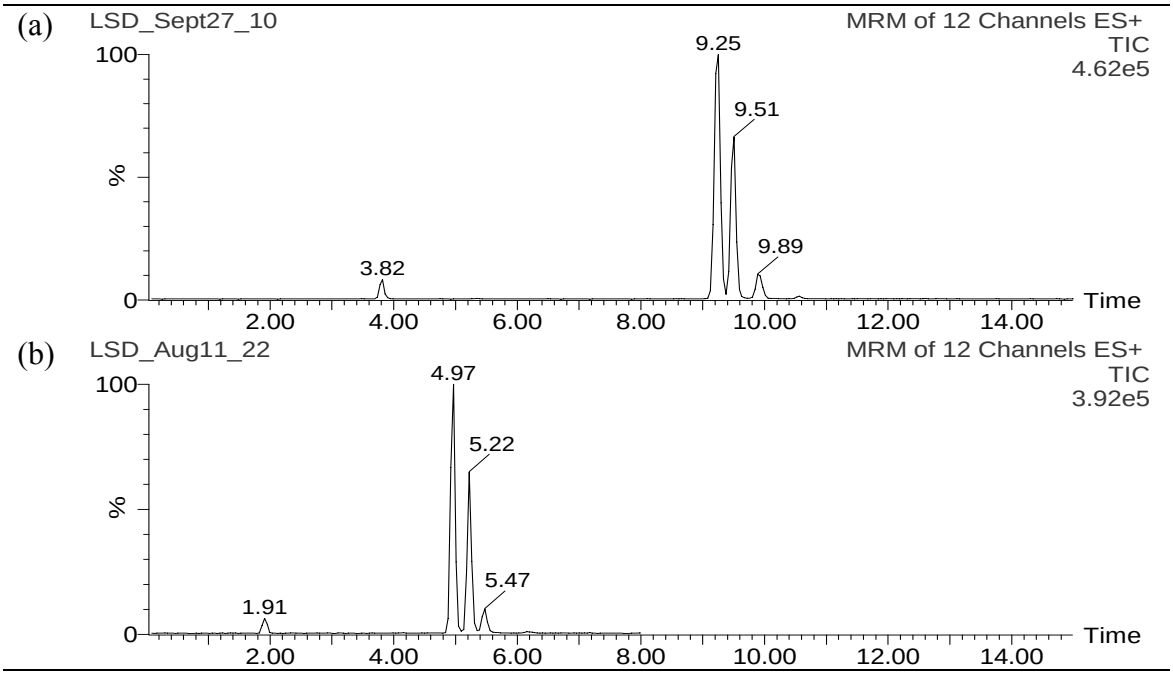


Figure 5.4: TIC of a neat 500 pg/mL LSD test mix showed a decrease in total run time from 15 minutes to 8 minutes by increasing flow rate from (a) 0.2 mL/min to (b) 0.4 mL/min.

Although analysis time was reduced, the peak response height was slightly lower (3.92×10^5 versus 4.62×10^5) when the flow rate was increased to 0.4 mL/min. Also the peak width at 5% height did not change (~0.45 minutes at both 0.2 mL/min and 0.4 mL/min). As a result, increasing flow rate from 0.2 mL/min to 0.4 mL/min did not enhance sensitivity. Although sensitivity was not enhanced, total analysis time was decreased without sacrificing resolution. Despite the decreased analysis time achieved for a neat LSD test mix standard, however, extracted samples run at 0.4 mL/min showed a decrease in both sensitivity and resolution. The peak shape was poor and there was a decrease in peak purity % (Figure 5.5). By decreasing total analysis time there seemed to be poor separation of the compounds of interest from endogenous materials present in the sample matrix. Therefore, for method validation, a 0.2 mL/min flow rate was chosen because resolution and sensitivity were more important than analysis time.

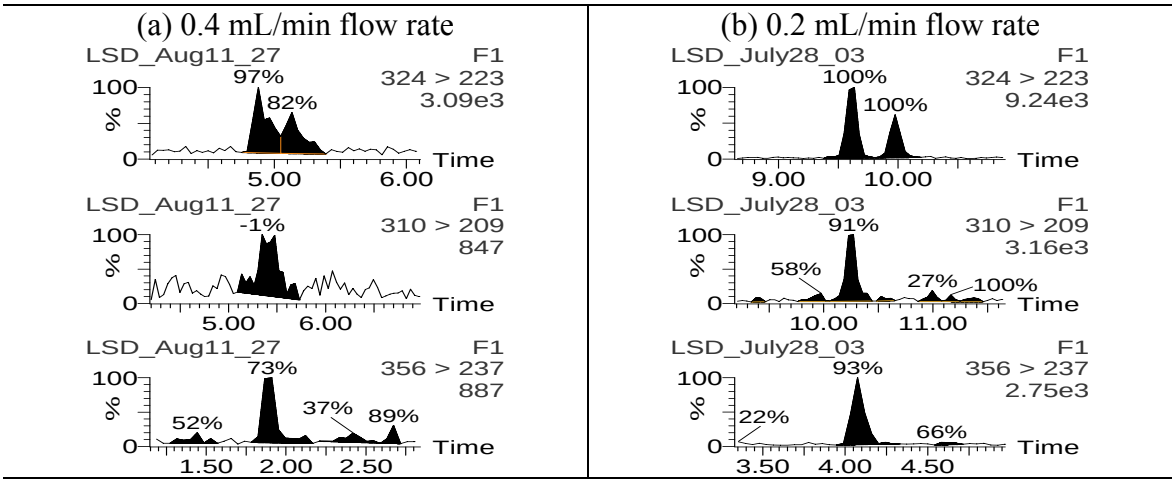


Figure 5.5: MRM channels for a spiked blood extract (50 pg/mL LSD, iso-LSD, nor-LSD, and O-H-LSD) run at (a) 0.4 mL/min flow rate showed poor peak shape and a decrease in peak purity % versus (b) 0.2 mL/min flow rate.

5.6 Effect of Changing Sample Injection Volumes

The ACQUITY system has the ability to use both partial and full loop-fill injections. Partial loop-filled injections are preferred to full loop-filled injections, with partial loop-filled precision being good at volumes up to 80% of the loop total volume (Yang and Hodges 2005). The UPLC™ uses air-gap sandwiching of the sample, thereby allowing for better utilization of the sample loop and higher injection precision (Yang and Hodges 2005). The ACQUITY system also incorporates a portion of a weak solvent wash to be co-injected with partial loop-filled samples. The weak solvent wash helps to enhance sample focusing onto the column. Partial loop-filled injections with needle overfill draws an excess of sample into the needle and through the valve while the loop remains in line with the pump. The valve is switched to bring the loop off-line and the syringe then draws the appropriate volume of sample into the loop. The valve is then switched back again to complete the injection (Jerkovich et al. 2005).

In order to realize the increased sensitivity benefits, low volume injections with minimal carryover are required (Swartz 2005). A 50 pg/mL neat standard was injected using different volumes. When 15 µL of a 50 pg/mL neat standard was injected the LSD S/N was 11.90. The LSD S/N increased two fold to 23.62 when the injection volume was increased to 25 µL (Figure 5.6). To see if injecting volumes smaller than

the loop volume would further enhance precision of repeated injections, one sample was injected 6 times using 15 and 25 µL volume injections. The precision (% CV) of the repeated injections using 15 and 25 µL were comparable (Table 5.2). In order to achieve a highly sensitive method for LSD, 20 µL injection volume (sample loop size) was chosen for method validation.

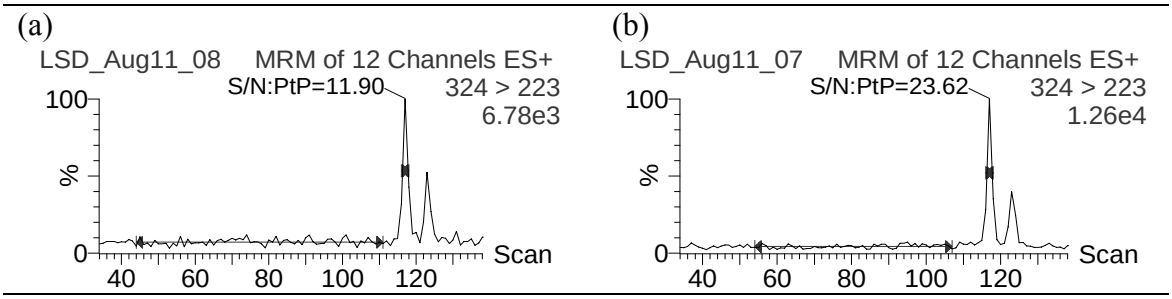


Figure 5.6: The effect of two different injection volumes, (a) 15 µL and (b) 25 µL on the S/N of LSD.

Table 5.2: Precision of repeated injections of a 500 pg/mL neat standard (n=6) using 15 and 25 µL volume injections

	15 µL injection %CV	**25 µL injection %CV**
LSD	3.6	2.3
Iso-LSD	3.5	1.0
Nor-LSD	5.9	2.9
O-H-LSD	1.9	2.2

5.7 Selection of Reference Standards

Certified reference standards were obtained from Cerilliant Corporation (Round Rock, TX). All standards came prepared in 1 mL ACN. All stock standard solutions were prepared by diluting volumes of standards to 5 mL. All stock standards prepared for the HPLC/MS/MS (Chapter 4) were prepared in MeOH, and for UPLC™/MS/MS all stock standards were prepared in ACN. It has been shown that iso-LSD rapidly isomerizes to LSD when dissolved in MeOH and stored at 2-6°C (Clarkson et al. 1998). To prevent isomerization of LSD, standards should not, therefore, be made up in MeOH.

5.8 Selection of Internal Standard

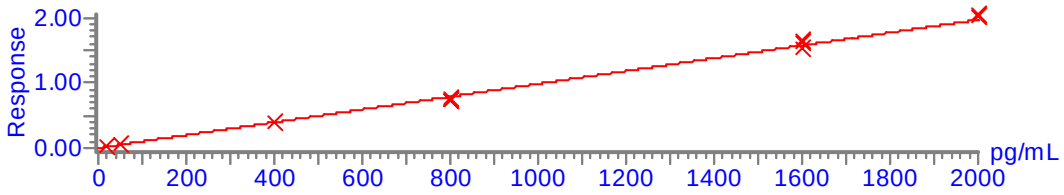
According to the SOFT/AAFS 2006 guidelines for forensic laboratories, the ISTD should have similar chemical and physical properties as the analyte. Stable isotope standards are recommended for LC/MS methods, as these normalize the responses of target drugs, thus compensating for variations in injections, sample preparation, instrumental parameter, and matrix effect (Liang et al. 2003). Although a non-deuterated ISTD may give equivalent or better performance, the use of an isotope standard may be the only way to compensate for ion suppression.

By comparing the slope of the lines, a greater slope value may indicate that better accuracy at higher concentrations may be expected. Methods for analyzing LSD have used LAMPA, LSD-D3, and O-H-LAMPA as the ISTD (See Chapter 1, Tables 1.3 and 1.4). A calibration curve using each ISTD was constructed (Figure 5.7). The slopes differed and O-H-LAMPA had the largest slope. The ion chromatogram of each ISTD (1000 pg/mL) showed that the PA of LSD-D3 and LAMPA were more than 7 times greater compared to O-H-LAMPA (Figure 5.8). The larger slope could be explained by the smaller PA of O-H-LAMPA. Since the PAR is the PA of the standard divided by PA of the ISTD, the PAR was larger for O-H-LAMPA compared to LSD-D3 and LAMPA. The slope will be larger if the ΔPAR is larger since the slope is essentially determined from the ΔPAR divided by Δnominal concentration. With a greater slope O-H-LAMPA may provide better accuracy compared to LSD-D3 and LAMPA. The % bias and % CV of LSD using each ISTD at 20, 50, 500, and 1500 pg/mL were all within acceptable range (Table 5.3).

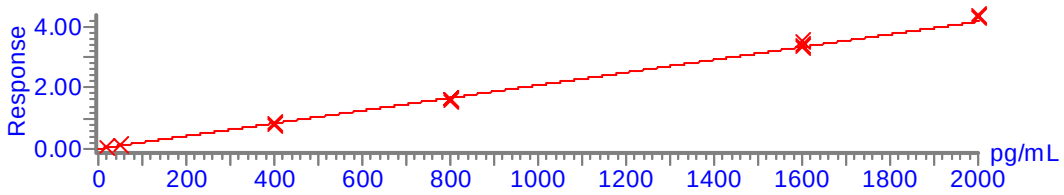
Table 5.3: Accuracy and precision of LSD using 3 different internal standards.

Nominal	LSD-D3		LAMPA		O-H-LAMPA	
(pg/mL)	% bias (n=6)	% CV (n=6)	% bias	% CV	% bias	% CV
20	3.82	10.91	3.97 (n=6)	6.83 (n=6)	-2.33 (n=5)	9.54 (n=5)
50	3.17	3.02	-2.54 (n=6)	6.79 (n=6)	-8.77 (n=5)	3.19 (n=5)
500	2.30	4.76	-2.84 (n=3)	3.11 (n=3)	3.59 (n=4)	4.86 (n=4)
1500	6.48	3.96	1.41 (n=3)	0.31 (n=3)	6.00 (n=4)	2.19 (n=4)

(a) ISTD = LSD-D3, R = 0.998921, R^2 = 0.997843, y = 0.000981812x + 0.00430119



(b) ISTD = LAMPA, R = 0.998399, R^2 = 0.996801, y = 0.00207504x + 0.00739736



(c) ISTD = O-H-LAMPA, R = 0.998527, R^2 = 0.997057, y = 0.0044249x + 0.0210852

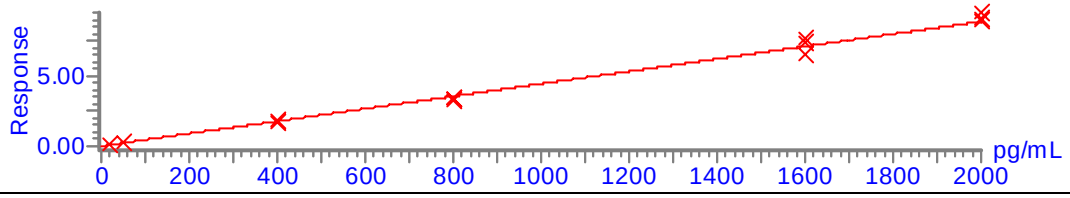


Figure 5.7: Calibration curves for LSD using (a) LSD-D3, (b) LAMPA, and (c) O-H-LAMPA as the ISTD.

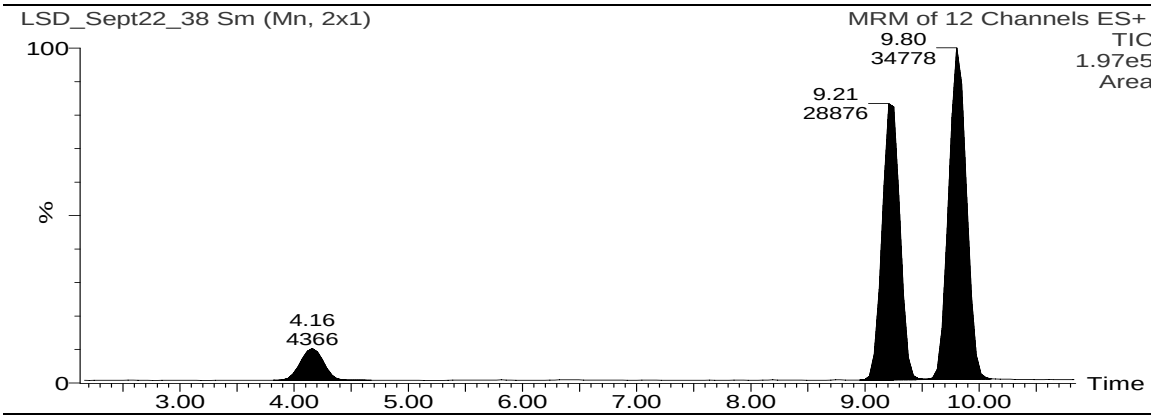


Figure 5.8: TIC of 1000 pg/mL neat standard mix of O-H-LAMPA (RT = 4.16 minutes), LSD-D3 (RT = 9.21 minutes), and LAMPA (RT = 9.80 minutes).

One-way ANOVA (analysis of variance) showed no significant difference ($p>0.05$) between the mean values of LSD at 20, 500, and 1500 pg/mL calculated using each ISTD. However, there was a significant difference ($p = 0.003$) in the mean value of LSD at 50 pg/mL. When performing a Bonferroni's multiple comparison test there was a significant difference ($p<0.01$) between the calculated mean value of LSD using LSD-D3 versus O-H-LAMPA as the ISTD. A one-sample t-test showed a significant difference ($p = 0.0025$) of the calculated mean value of LSD using O-H-LAMPA as the ISTD from the hypothetical value of 50 pg/mL. The same results were observed when comparing the accuracy and precision of calculated mean values of iso-LSD, nor-LSD, and O-H-LSD using the 3 different ISTD's. The slope of LSD-D3 and LAMPA could be increased by using a smaller concentration to decrease its PA, but since O-H-LAMPA did not enhance either the accuracy or precision of the assay, this was not necessary. As mentioned earlier in Chapter 1 (section 1.6.6) quantitating O-H-LSD via GC/MS requires an ISTD with similar properties because a mixture of mono-TMS and bis-TMS derivatives occurs when O-H-LSD is derivatized (Reuschel et al. 1999b). This was not as critical with LC/MS/MS since no derivatization was performed, so O-H-LAMPA was excluded as an ISTD, leaving LSD-D3 or LAMPA.

LAMPA is an isomeric compound of LSD, and is itself a controlled drug (White et al. 1999). As a result, using LAMPA as an ISTD may cause problems if LAMPA was also present in the sample to be analyzed. Previous LC/MS methods using single ion monitoring (SIM) were limited to using a structurally similar non-deuterated compound (i.e. LAMPA) versus an isotope as an ISTD, due to the fact that LSD and LSD-D3 were not chromatographically separated and also shared a common major product ion. With MS/MS this was not a problem because MRM could determine which precursor ion gave rise to a particular product ion. As a result, LSD-D3 was chosen as the ISTD for method validation.

5.9 Selection of Sample Matrix

A significant risk in bioanalytical method validation is that spiked matrix samples are a poor model for real samples (James et al. 2004). Despite this risk, it has been common practice to used spiked matrix samples for validating bioanalytical

methods. It is recognized that it is quite difficult to make spiked matrix samples exactly the same as real case samples. This is even more difficult with forensic analysis due to the variety of samples submitted. Forensic samples can either be ante- or postmortem, with the potential of also receiving decomposing samples. As well there are many different types of blood collection vials with or without preservatives. As a result, the only thing that can be done is to select a sample matrix that is as close as possible to real case samples.

The RCMP FLS Winnipeg uses synthetic drug free urine from Immunalysis Corporation (Pomona, CA) and porcine blood diluted 25% with water, containing 0.25% sodium fluoride and 0.20% potassium oxalate, as model matrices. The suitability of using porcine blood as the matrix for constructing calibration curves for quantitation was determined by comparing calibration curves constructed from the porcine versus blank human whole blood collected in grey top Vacutainers™ (sodium fluoride, potassium oxalate preservative) using GraphPad Prism version 4.03 for windows (GraphPad Software, San Diego, CA) (Table 5.4). The slope and intercept of the calibration curves were compared and the F-test was used to test whether one curve suffices for all data or if individual curves were statistically distinguishable. There was no significant difference ($p>0.05$) between the calibration curves of human versus porcine whole blood for LSD, iso-LSD, nor-LSD and O-H-LSD, which showed that porcine whole blood was an acceptable substitute for human whole blood.

Since urine analysis by the RCMP FLS is only qualitative, full calibration curves for quantitation are not done for urine analysis. As a result, the suitability of synthetic urine for constructing calibration curves was not tested. Instead, the suitability of the use of synthetic urine for qualitative analysis was tested for by assessing the selectivity and matrix effect compared to human urine. Blank synthetic urine and porcine blood were extracted as outlined in Section 6.2.5 and were determined to be suitable if no drugs were present and no interfering peaks were seen (Section 6.3.4). Selecting the appropriate sample matrix is even more critical when using LC/MS/MS due to the potential for ion suppression/enhancement (James et al. 2004). If the sample matrix chosen for method validation exhibits ion suppression/enhancement but the real case samples did not, the method would be invalid. As a result, it is essential to demonstrate

the absence of ME using different sources of matrix. ME is described in detail in Chapter 3 (section 3.9), with results of the ME study in Chapter 6 (section 6.3.5).

Table 5.4: Comparison of the slope and intercept (± 95% CI) of calibration curves constructed from human (H) versus porcine (P) whole blood.

		Separate curves		One curve		Comparison of intercepts	
		Slope ($\times 10^{-4}$)	Intercept ($\times 10^{-3}$)	Slope ($\times 10^{-4}$)	Intercept ($\times 10^{-3}$)	F ratio (DFn, DFd)	p value
LSD	H	8.12 ± 0.37	8.61 ± 1.68	8.37 ± 0.26	7.81 ± 1.19	2.413 (2,20)	0.115
	P	8.63 ± 0.38	7.00 ± 1.71				
Iso-LSD	H	6.01 ± 0.30	-2.20 ± 1.35	6.19 ± 0.28	2.50 ± 1.28	2.140 (2,14)	0.155
	P	6.54 ± 0.75	-3.12 ± 3.41				
Nor-LSD	H	2.15 ± 0.08	2.26 ± 0.88	2.28 ± 0.11	1.62 ± 1.23	3.610 (2,16)	0.051
	P	2.40 ± 0.20	0.98 ± 2.21				
O-H-LSD	H	2.31 ± 0.15	-0.09 ± 1.66	2.25 ± 0.10	-0.20 ± 1.13	2.844 (2,13)	0.095
	P	0.17 ± 0.07	-0.66 ± 0.96				

5.10 Selection of Extraction Method

Forensic toxicology specimens are normally complex matrices which require some sort of preparation prior to analysis. In a method that analyzes multiple types of matrices, it would be ideal to have a single sample preparation method applicable to all types of matrices. A thorough literature search was conducted to find extraction methods used for LSD. However, the majority of the methods in the literature are specific for and therefore optimized for only extracting LSD.

An automated SPE method was used to extract LSD and nor-LSD from blood, serum, plasma, and urine (de Kanel et al. 1998). In this method, Varian Bond Elut Certify SPE cartridges (Varian Sample Preparation Products, Harbor City, CA) were used. Since the sample batch volume at the RCMP FLS is not expected to be large, an automated method is not practical, so the same procedure was conducted manually (Figure 5.9a). This extraction method used a lot of solvents, so another SPE method was considered (Reuschel et al. 1999b) (Figure 5.9b). Overall, SPE was time consuming, utilized lots of solvents, yielded dirty extracts, and did a poor job at extracting O-H-LSD (Figure 5.10).

Due to the poor results with SPE, LLE was considered as an alternative. A LLE method adapted from Canezin et al. 2001 and Sklerov et al. 2000 was used (see Chapter

6). The only change was the volume and concentration of ISTD and the volume of dichloromethane:isopropyl alcohol used. The LLE was simple to perform and was able to extract all compounds with similar % recoveries, causing no significant ion suppression or enhancement (see Chapter 6).

A. SPE method # 1

1. Condition column
   a) 1.5 mL mix [dichloromethane: isopropanol:NH_4OH (78:20:2)]
   b) 3.0 mL MeOH
   c) 1.0 mL water
   d) 3.0 mL 0.1M Phosphate buffer
2. Add sample (1 mL sample + LSD-D3 + 1 mL water + 2 mL phosphate buffer)
3. Wash column
   a) 3.0 mL 0.1M Phosphate buffer
   b) 2.0 mL 1M Acetic acid
   c) 3.0 mL MeOH
   d) 0.5 mL mix
4. Collect eluate
   a) 2.0 mL mix
5. Dry eluate to dryness under N_2
6. Reconstitute

B. SPE method # 2

1. Condition column
   a) 2.0 mL MeOH
   b) 2.0 mL 0.1 M Phosphate buffer
2. Add sample (4 mL sample + LAMPA + O-H-LAMPA + 2 mL phosphate buffer)
3. Wash column
   a) 1.0 mL 1 M Acetic acid
   b) 5.0 mL MeOH
4. Collect eluate
   a) 2 mL mix [dichloromethane: isopropanol:NH_4OH (78:20:2)]
5. Dry eluate to dryness under N_2
6. Reconstitute

Figure 5.9: (A) SPE method for extracting LSD and nor-LSD from whole blood, serum, plasma, and urine (de Kanel et al. 1998). (B) SPE method extracting LSD and O-H-LSD in urine (Reuschel et al. 1999b).

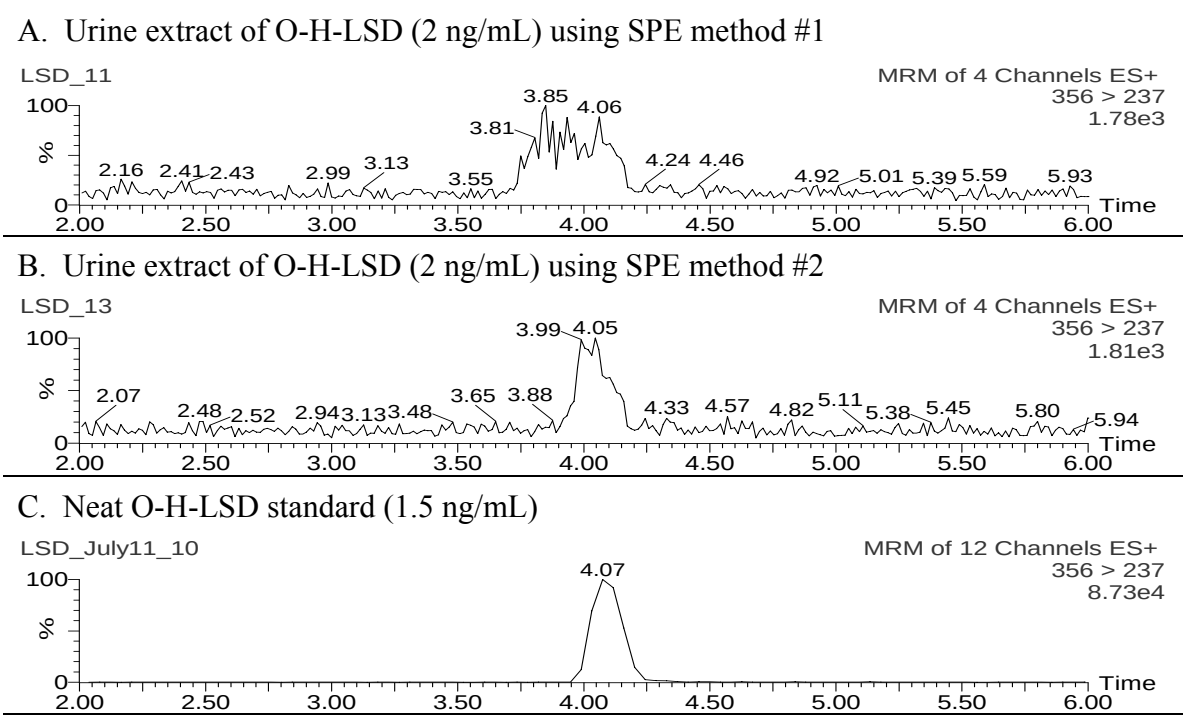


Figure 5.10: Comparison of two SPE methods for O-H-LSD in urine. Same results were seen in whole blood.

5.11 Selection of Weighting Scheme

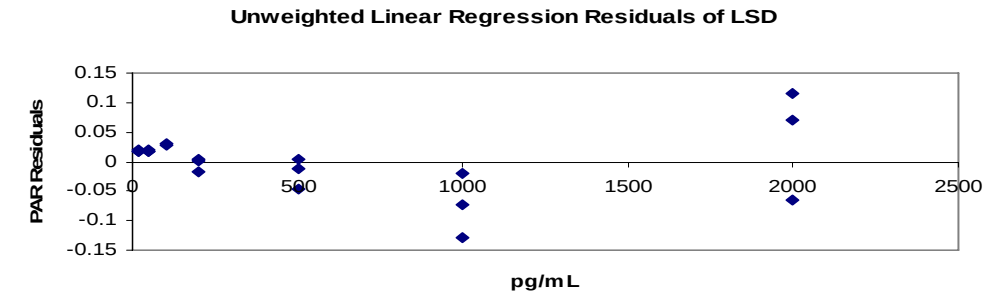
Prior to performing linear regression, the data should be tested for homoscedasticity versus heteroscedasticity by plotting residuals versus concentration (Mulholland and Hibbert 1997) then applying an F-test to compare the variances. The experimental F-value (F_{exp}) was expressed as the ratio between the variances obtained at the lowest (s_1^2) and at the highest (s_2^2) concentration level of the working range (Equation 5.2), and the tabled F-value (F_{tab}) was then obtained from the F-table (Almeida et al. 2002). For homoscedasticity the variance will be constant and the residuals will be scattered randomly around the x-axis and the $F_{exp} < F_{tab}$.

$$\mathbf{F_{exp} = (s_2)^2/(s_1)^2} \qquad \text{Equation 5.2}$$

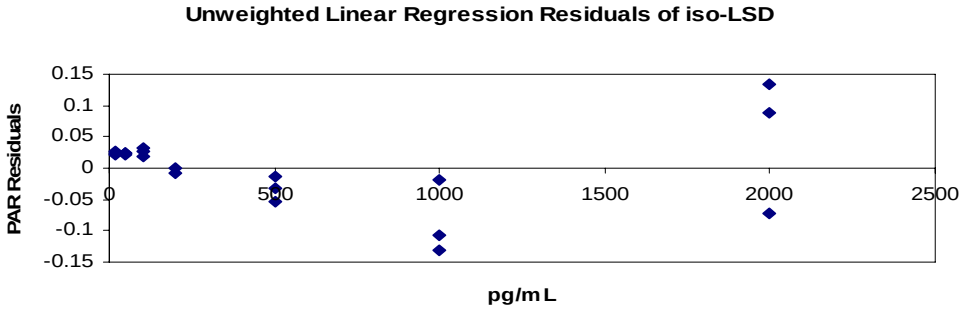
The residual plots and the F-test showed that the data was heteroscedastic. The residuals were increasing as concentration increased (Figure 5.11). As well, when tested with the F-test at the confidence level of 95% for $f_1 = f_2 =$ (n-1) degrees of freedom, there was a significant difference ($p<0.05$ and $F_{exp} > F_{tab}$) between the variances

obtained at the lowest and at the highest concentration level (Table 5.5). As a result, the calibration curve was constructed using weighted linear regression.

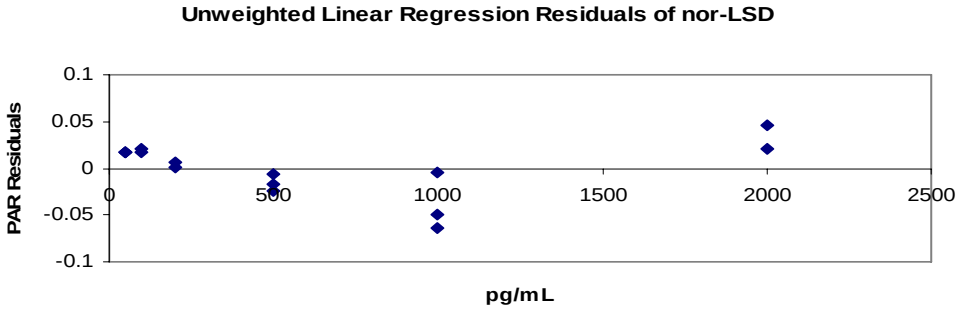
(a)



(b)



(c)



(d)

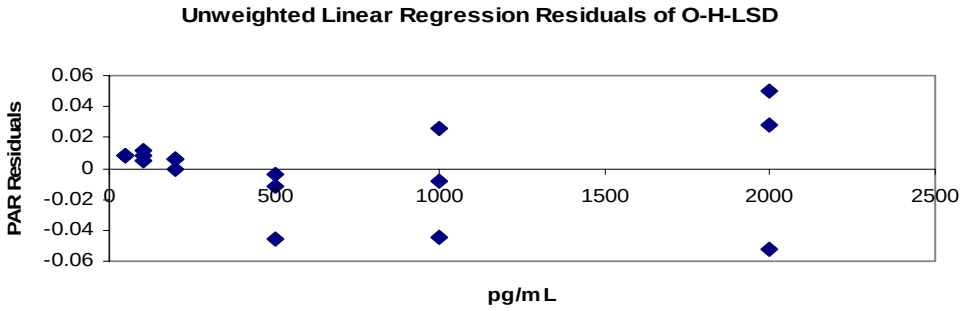


Figure 5.11: Residual plots of (a) LSD, (b) iso-LSD, (c) nor-LSD, and (d) O-H-LSD using unweighted linear regression.

Table 5.5: F-test to compare homogeneity of variances

	pg/mL	Mean PAR	s^2	F_{exp}	p value of variances
LSD	20	0.024	1.82×10^{-6}	142	0.0135
	2000	2.035	2.68×10^{-4}		
Iso-LSD	20	0.027	5.85×10^{-8}	11820	0.0002
	2000	2.075	6.91×10^{-4}		
Nor-LSD	50	0.031	1.10×10^{-6}	228	0.0087
	2000	1.279	2.51×10^{-4}		
O-H-LSD	50	0.021	2.06×10^{-6}	211	0.0094
	2000	0.991	4.34×10^{-4}		

$F_{tab}(f_1, f_2; 0.95) = F_{tab}(2, 2, 0.95) = 19.0$

Since the evidence showed that the data was not homoscedastic, the next step was to determine the appropriate weighting factor. The best weighting factor was chosen according to the % bias (Equation 5.3).

$$\% \text{ Bias} = \left(\frac{\text{calculated concentration - nominal concentration}}{\text{nominal concentration}} \right) \text{x } 100 \qquad \text{Equation 5.3}$$

The best weighting factor will give rise to a narrow horizontal band of randomly distributed % bias around the concentration axis and presents the least sum of the absolute % bias values across the whole range (Almeida et al. 2002). The sums of the absolute % bias values across the whole concentration range are shown in Table 5.6. The % bias plots of unweighted and different weighted (1/y, $1/y^2$, 1/x, and $1/x^2$) linear regressions of LSD, iso-LSD, nor-LSD and O-H-LSD are shown in Figures 5.12, 5.13, 5.14, and 5.15. The % bias plots showed that unweighted linear regression overestimated the concentrations at the LLOQ. The % bias plots of $1/y^2$ and $1/x^2$ showed the best distribution around the concentration axis, and $1/x^2$ had the least sum of the absolute % bias values. Based on both the % bias plots and the sum of the absolute % bias values, $1/x^2$ was chosen as the weighting scheme for the calibration curve.

Table 5.6: Sums of the absolute % bias

	Unweighted	1/x	$1/x^2$	1/y	$1/y^2$
LSD (n = 18)	308.9	60.3	58.5	60.8	58.7
Iso-LSD (n = 18)	377.9	58.1	54.8	63.1	57.8
Nor-LSD (n = 15)	182.3	58.0	54.6	59.4	55.8
O-H-LSD (n = 15)	233.3	73.3	66.1	72.4	68.2

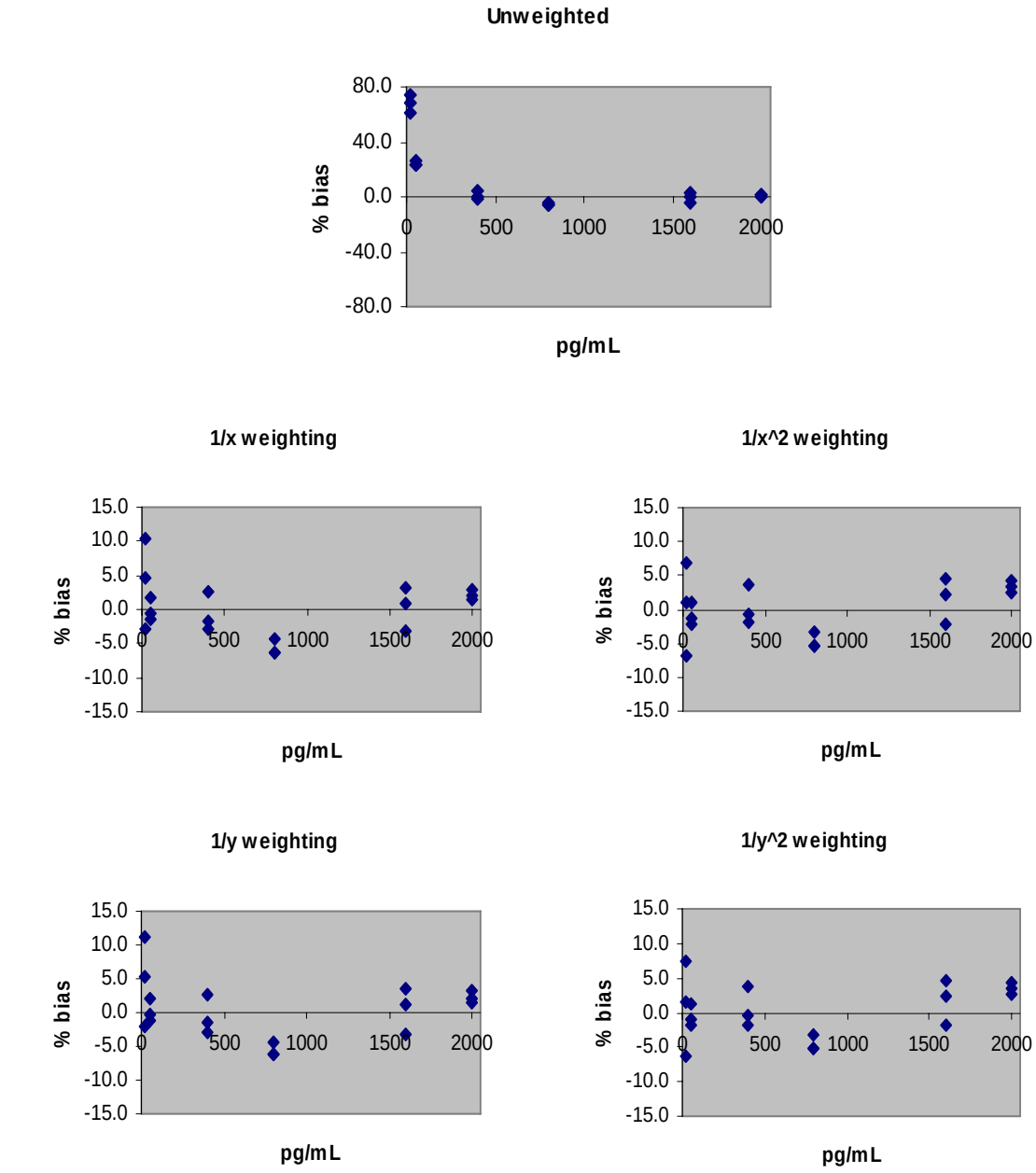


Figure 5.12: Percent bias versus concentration obtained for unweighted and different weighted linear regression models for LSD.

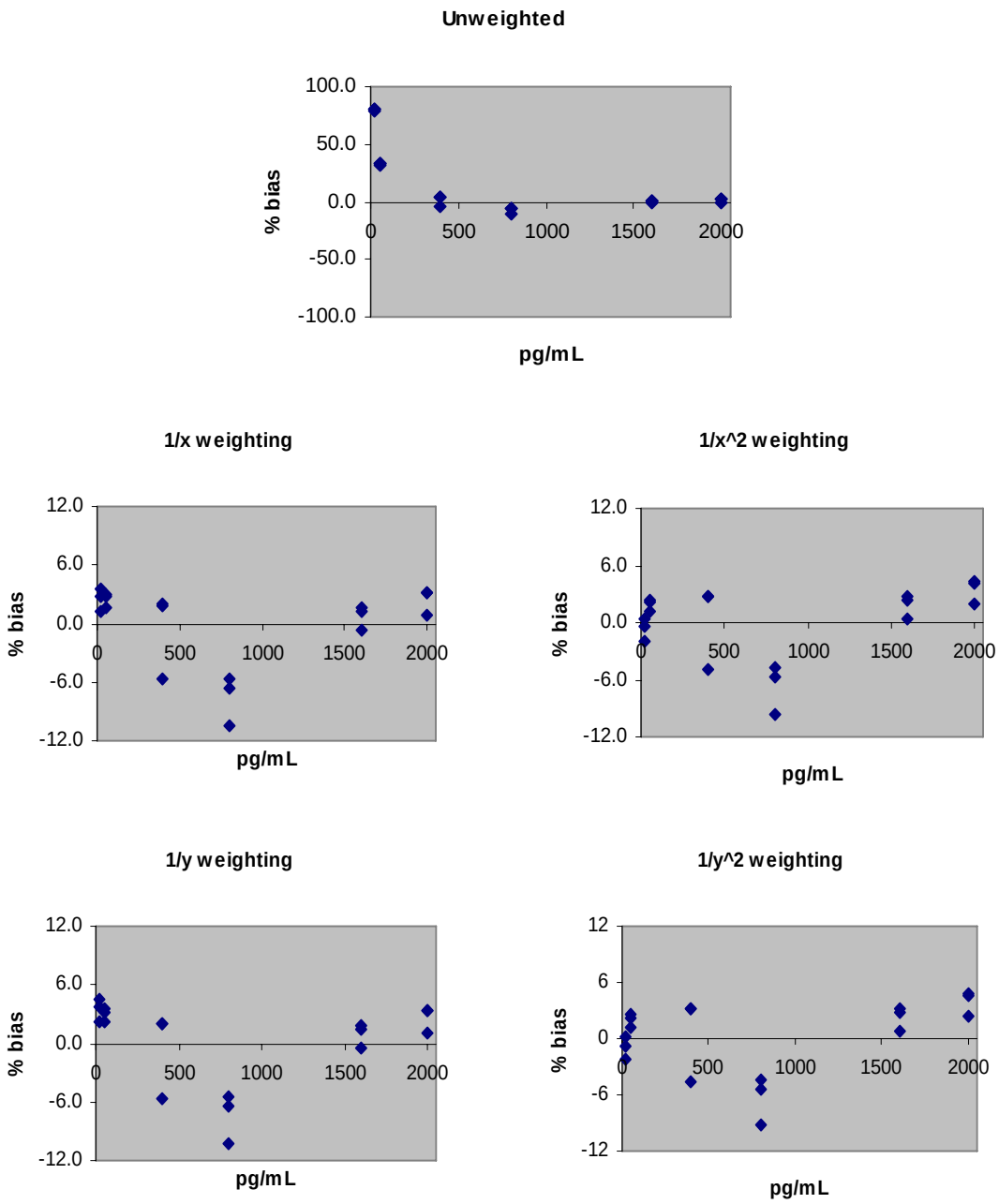


Figure 5.13: Percent bias versus concentration obtained for unweighted and different weighted linear regression models for iso-LSD.

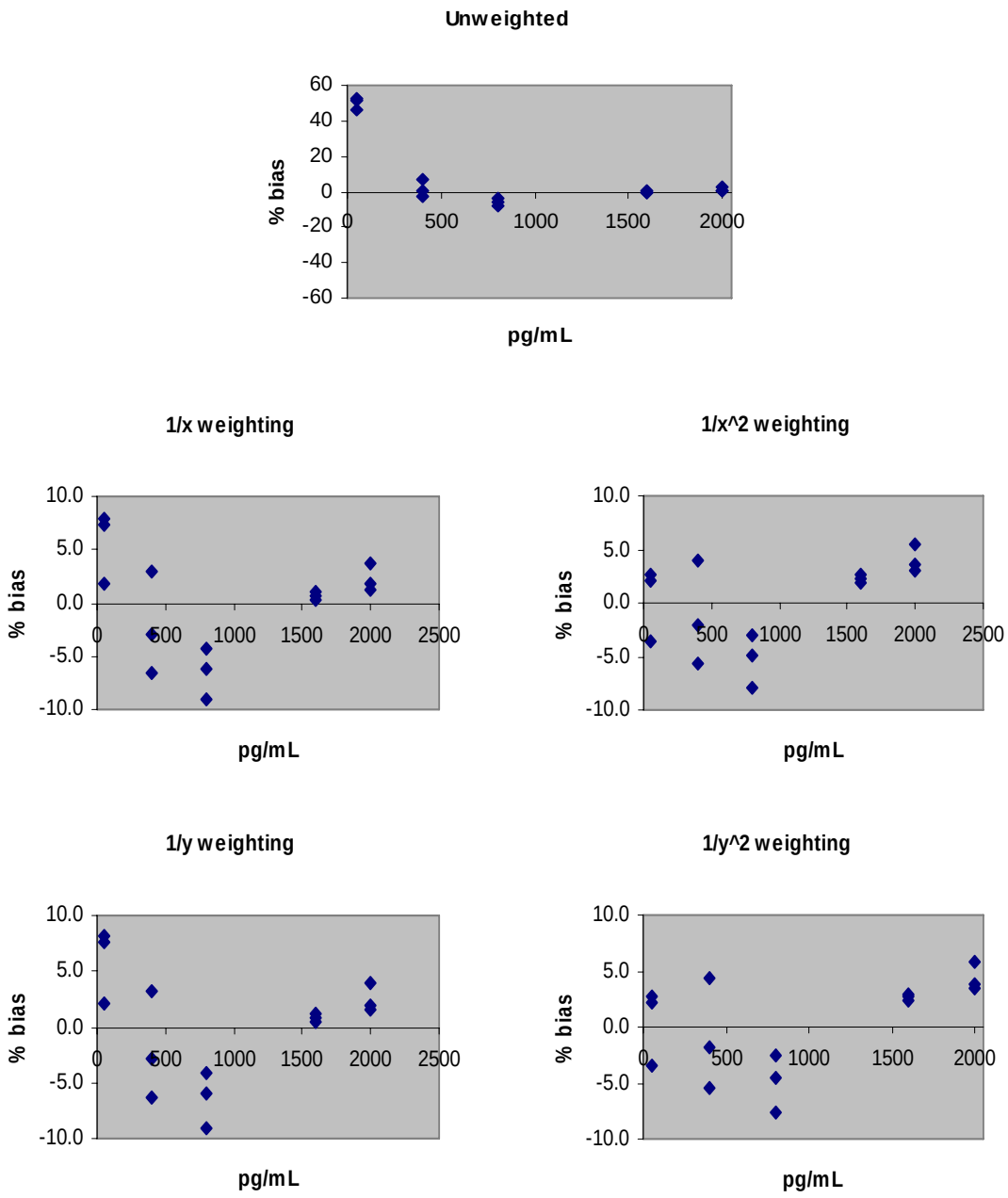


Figure 5.14: Percent bias versus concentration obtained for unweighted and different weighted linear regression models for nor-LSD.

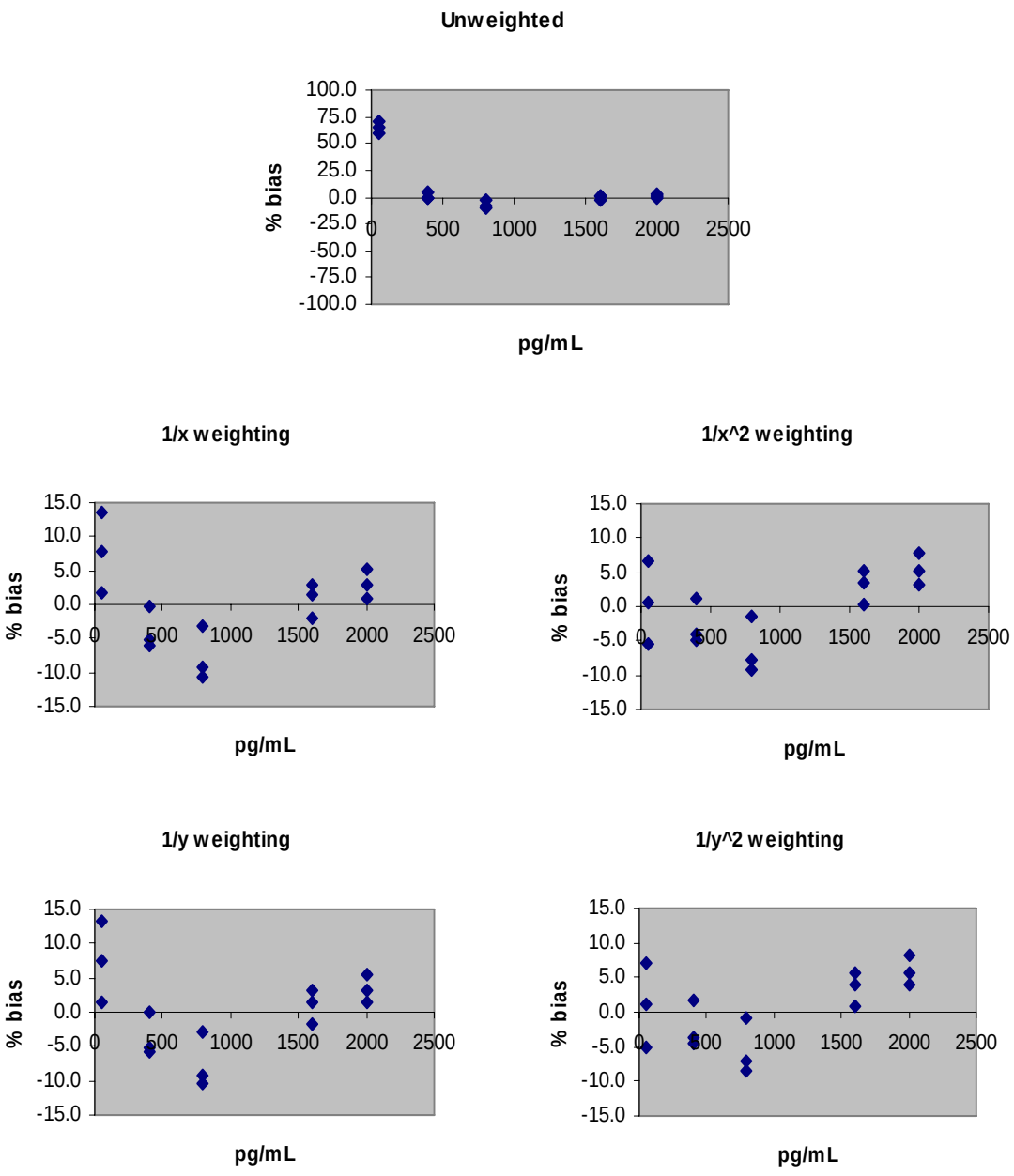


Figure 5.15: Percent bias versus concentration obtained for unweighted and different weighted linear regression models for O-H-LSD.

5.12 Conclusion

Before beginning the process of validating a method, a prevalidation should be completed to assess the suitability of the system (Shabir 2003). One of the main objectives was to develop a highly sensitive method for LSD. As a result, prior to beginning the full validation study it was important to show that the proposed method had the ability to achieve the required sensitivity. Once it was shown that the method would be suitable, other factors such as the mobile phase, injection volumes, flow rates, internal standards, and extraction methods, were changed or optimized to further increase the sensitivity of the method. In conclusion, careful planning prior to beginning a full validation study is essential in order to save time and ensure a successful validation.

Chapter 6

Validation of an UPLC™/MS/MS Method for Analyzing LSD, Iso-LSD, Nor-LSD, and O-H-LSD in Whole Blood and Urine

6.1 Introduction

The RCMP FLS has been involved in the analysis of LSD since about 1991 (Daplé-Scott et al. 1997). In 1992 the Abuscreen® RIA procedure was used by the RCMP FLS as the screening method but was discontinued in 1997 by the suppliers, Roche Diagnostic Systems. The Coat-A-Count® RIA and ELISA methods were tested and found to be suitable replacements (Daplé-Scott et al. 1997). Today the Immunalysis direct ELISA is used by the RCMP FLS as the LSD screening method (Towse and Easton 2004). In 1997 a confirmatory GC/MS/MS method using the Zymark Rapid Trace solid phase extractor was developed for the trimethylsilyl derivative of LSD and LAMPA, with a LLOQ of 100 pg/mL (Perrigo 2003; Garbutt 2001). The method was shown to work in real case samples (Daplé-Scott et al. 1997), but had its disadvantages. Firstly, no metabolites of LSD were included in the analysis. Secondly, high volumes of sample (2 mL blood and 5 mL urine) were required. Thirdly, samples required derivatization for analysis. Lastly, the GC required pre-conditioning with a minimum of six injections of unextracted derivatized LSD followed by several blank injections of the derivatizing agent to prevent adsorption.

This study was done to provide a sensitive, simple, and non-laborious method for analyzing LSD, iso-LSD, nor-LSD, and O-H-LSD. This study describes a UPLC™/MS/MS method for analyzing LSD, iso-LSD, nor-LSD, and O-H-LSD in whole blood and urine. Validation parameters including sensitivity, accuracy, precision, stability, selectivity, recovery, and matrix effects were evaluated.

6.2 Materials and Methods

6.2.1 Chemicals and Reagents

LSD, iso-LSD, nor-LSD, O-H-LSD and LSD-D3 were obtained from Cerilliant Corporation (Round Rock, TX). All reagents were HPLC or reagent grade. ACN, ammonium acetate, ammonium hydroxide, and methylene chloride were obtained from Fisher Scientific (Fairlawn, NJ). Isopropyl alcohol and glacial acetic acid were obtained from Caledon Laboratories Ltd (Georgetown, ON). All water was purified with the NANOpure II water purification system (Barnstead, a division of Apogent Technologies Inc, Dubuque, IA). Synthetic drug-free urine was obtained from Immunalysis Corporation (Pomona, CA). Porcine blood obtained from Maple Leaf Meats (Winnipeg, MB) was diluted 25% with water containing 0.25% sodium fluoride and 0.20% potassium oxalate. The mobile phase buffer, 20 mM ammonium acetate buffer (pH 4.0), was prepared by dissolving 1.54 g of ammonium acetate in ~800 mL of water. The pH was then adjusted to 4.0 (±0.1) with glacial acetic acid and then made up to 1 L with water. The mobile phase buffer was then filtered and sonicated before use. Ammonium acetate buffer (1 M, pH 9.0) was prepared by dissolving 19.27 g of ammonium acetate in ~150 mL of water. The pH was then adjusted to 9.0 (±0.1) with concentrated ammonium hydroxide and then made up to 250 mL with water.

6.2.2 Standard Solutions

A stock solution of each component at 500 ng/mL was prepared in ACN. A 1 and a 20 ng/mL calibrator mix containing LSD, iso-LSD, nor-LSD, and O-H-LSD were prepared from the stock solutions in ACN. An ISTD (LSD-D3) stock solution of 500 ng/mL was prepared in ACN and diluted to give a 20 ng/mL working ISTD. The calibrator mixes were used to prepare calibrators and quality control samples in either blank whole blood or urine (Appendix A, page 148).

6.2.3 LC

A Waters Corporation (Milford, MA) ACQUITY UPLC™ with an autosampler and an ACQUITY UPLC™ BEH C18 (2.5×50 mm, 1.7 µm, Waters Corporation, Milford, MA) column was used for chromatographic separation at 30°C. A gradient

elution was conducted using 20 mM ammonium acetate buffer (pH 4.0) and ACN at a flow rate of 0.2 mL/min. Buffer:ACN (90:10) was initially held for 1 minute, followed by a step gradient to 75:25 for 10 minutes and held for 1 minute. The column was then equilibrated for 2 minutes giving a total run time of 15 minutes.

6.2.4 LC/MS/MS

A Waters Corporation (Milford, MA) Quattro Premier™ tandem quadrupole mass spectrometer equipped with a Z-spray™ ion source was coupled to the UPLC™ system. API was done via positive ESI. The electrospray probe tip potential was set at 3.00 kV. The source and desolvation temperature were set at 120 and 350°C, respectively. The cone and desolvation gas flow were set at 110 and 747 L/Hr, respectively. The collision gas (argon) flow and pressure were set at 0.28 mL/min and 4.21×10^{-3} mbar, respectively. The mass spectrometer was run in MRM mode with a dwell time of 0.20 seconds (inter-channel delay of 0.01 seconds) (Table 6.1).

Table 6.1: UPLC™/MS/MS MRM parameters

Standards	Precursor ion $[M+H]^+$	Product ion	Collision	Cone Voltage
LSD	324	223*	24	35
		208	31	35
		281	19	35
iso-LSD	324	281*, 208, 223	24	35
nor-LSD	310	209*, 237, 74	24	37
O-H-LSD	356	237*, 222, 313	25	35
LSD-d_3	327	226*, 208, 281	24	35

*Product ions to be used for quantitation; other ions are qualifier ions.

6.2.5 Sample Preparation

Sample preparation was adapted from Canezin et al. 2001 and Sklerov et al. 2000. Blank drug free synthetic urine was spiked with either calibrator mix A (1 ng/mL) or B (20 ng/mL). To 1 mL of sample, 50 µL of LSD-D3 internal standard (20 ng/mL) was added. After vortexing, 500 µL of 1 M ammonium acetate buffer (pH 9.0) was added and vortexed again. Samples were then extracted with 5 mL of dichloromethane:isopropyl alcohol (85:15). The tubes were capped and manually shaken briefly, then placed on a shaker bed (Eberbach Corporation, Ann Arbor, MI) at

low speed for 20 minutes. The tubes were then centrifuged at 3500 rpm for 15 minutes. The aqueous (top) layer was pipetted off and discarded. The organic (bottom) layer was transferred to a clean tube and evaporated to dryness under nitrogen at room temperature. The residue was reconstituted in 100 µL 20 mM ammonium acetate buffer (pH 4.0):ACN (80:20), and 20 µL of sample was injected into the UPLC™ system.

Blank drug-free porcine whole blood was prepared the same as the urine matrix, except after centrifugation the aqueous (top) layer was decanted. Then the organic (bottom) layer was transferred to a clean test tube by passing a pipette through the solid layer of blood. Then the organic layer was repipetted into another clean test tube to obtain a clean layer free of blood particulates. Reconstituted blood sample extracts were also either centrifuged or filtered with a 0.2 µm PTFE 13mm syringe filter (Chromatographic Specialties Inc, Brockville, ON) to remove particulates before being injected.

6.2.6 Assessment for Linearity and Range

Calibrators (5, 10, 20, 50, 100, 400, 800, 1600, 2000 pg/mL) were prepared by spiking blank drug free whole blood or urine in triplicate. Samples were processed and extracted as described earlier (section 6.2.5). Nominal concentration versus PAR were plotted to define the calibration curve. Weighted ($1/x^2$) linear regression was used. Data collection, peak integration, and weighted linear regression were performed using MassLynx™ version 4.0 software (Waters Corporation, Milford, MA). Weighted linear regression was also performed using GraphPad Prism version 4.03 for windows (GraphPad Software, San Diego, CA) to further evaluate the linearity. A suitable linear range was determined by evaluating the accuracy (% bias) of calculated results (Equation 6.1).

$$\% \text{ Bias} = \left(\frac{\text{calculated concentration - nominal concentration}}{\text{nominal concentration}} \right) \text{x } 100 \qquad \text{Equation 6.1}$$

6.2.7 Assessment for Accuracy and Precision

After determining the linear range, a 3 point calibration curve using spiked urine (50, 500, 1500 pg/mL) in duplicate, and a 5 to 6 point calibration curve using spiked

blood (20, 50, 400, 800, 1600, 2000 pg/mL) in duplicate was used, plus one blank and one blank with ISTD. Three quality control concentrations (50, 500, 1500 pg/mL) using both spiked urine and blood were prepared in replicates of six over three days to assess inter- and intra-day accuracy and precision. The back calculated results were determined from the equation, as defined by the weighted linear regression of the calibration curve. The accuracy of the method was determined by % bias. The precision of the method was determined by the % CV (Equation 6.2).

% CV = (standard deviation/mean)×100% Equation 6.2

6.2.8 Assessment of LOD and LLOQ

Two methods for the determination of LOD and LLOQ were evaluated. Firstly, the calculated method (Equation 6.3 and 6.4) was evaluated. Weighted ($1/x^2$) linear regression was performed on the lower end of the calibration curve using GraphPad Prism version 4.03 for windows (GraphPad Software, San Diego, CA) in order to calculate the standard deviation of the residuals ($S_{y.x}$). Secondly, S/N ratios were evaluated. LOD and LLOQ were determined where S/N > 3:1 and 10:1, respectively. LLOQ must also demonstrate acceptable accuracy and precision.

$LOD = 3.3 \times (S_{y.x}/slope)$ Equation 6.3

$LLOQ = 10 \times (S_{y.x}/slope)$ Equation 6.4

6.2.9 Assessment of Selectivity

To assess the selectivity, 6 different sources of both whole blood and urine blank matrices were extracted and analyzed. Each blank was then tested for interference at the LLOQ.

6.2.10 Assessment of Recovery

Blood and urine at 50 and 1500 pg/mL were prepared for recovery assessment by preparing 6 replicates of extracted and unextracted samples at each concentration. The extracted samples were processed as described earlier (section 6.2.5), whereas the appropriate volume of calibrator mix A or B was added after extraction for the unextracted samples. The average PARs were used to assess recovery (Equation 6.5).

% Recovery = [PAR extracted/PAR unextracted] × 100% Equation 6.5

6.2.11 Assessment of Matrix Effect (ME)

Six different sources of matrix were assessed for both blood and urine. For urine, 5 different lot numbers of synthetic drug-free urine, and one human urine sample were evaluated. For blood, porcine blood and 5 human volunteer blood samples collected in grey top Vacutainers™ (XF947, 20 mg potassium oxalate, 100 mg sodium fluoride) were evaluated. Blank blood or urine was extracted and, after extraction, appropriate volumes of calibrator A or B and ISTD were added. Samples were then allowed to evaporate under N_2 to dryness and reconstituted with 100 µL 20 mM ammonium acetate buffer (pH 4.0):ACN (80:20). The PA of standard and ISTD in matrix was compared to PA of neat standard and neat ISTD in 20 mM ammonium acetate buffer (pH 4.0):ACN (80:20) (Equation 6.6).

% ME = [PA standard in matrix/PA neat standard] × 100% Equation 6.6

6.2.12 Assessment of Stability

At RCMP FLS TS after a sample exhibit is received, it is placed in a freezer until the start of analysis (Treacy, personal communication). Whole blood and urine are the most commonly encountered specimens. Whole blood is commonly collected in a Vacutainer™ with or without preservatives. The most common preservative used is a mixture of potassium oxalate and sodium fluoride at varying concentrations. Urine may also be received with or without preservatives. Urine may either be received in plastic screw top containers, or aliquoted into a Vacutainer™. Stability studies were assessed in whole blood and urine stored in Vacutainers™ with and without preservatives.

Whole blood and urine for the stability studies were collected from human volunteers. The whole blood samples collected in the Vacutainers™ with preservative (XF947, 20 mg potassium oxalate, 100 mg sodium fluoride) were pooled into a glass beaker and mixed with a magnetic stir bar. The volume of pooled whole blood was then measured using a graduated cylinder. The pooled whole blood was divided into 2 equal parts to make a 50 and 1500 pg/mL whole blood working stock. The whole blood working stock was then aliquoted into new Vacutainers™ with the preservative removed. Before the whole blood samples that were collected in the Vacutainers™ without preservative were pooled, mixed, spiked and redistributed to new Vacutainer™, they

were first mixed with a blender to break up the clots. Urine samples were handled the same way as the whole blood samples. Stability studies were conducted to avoid exposure to light by storing samples in the dark and by using amber vials for sample preparation, unless otherwise noted.

The stability of the drugs was assessed by comparing concentrations to the concentrations determined on day 0. A calibration curve constructed each day with freshly prepared standards was used to determine concentrations.

6.2.12.1 Short Term Stability

To assess short term stability three aliquots of each of the low (50 pg/mL) and high (1500 pg/mL) concentrations were kept at room temperature and analyzed at 0 and 24 hours. To assess the effects of light exposure in the RCMP FLS Winnipeg laboratory, the short term stability study also included one set exposed to normal laboratory light.

6.2.12.2 Long Term Stability

To assess long term stability three aliquots at low (50 pg/mL) and high (1500 pg/mL) concentrations were stored in the dark at room temperature (also one set exposed to normal laboratory light for 7 days), refrigerated (2 to 3°C) and frozen (-15 to -12°C) and analyzed at 0, 7, and 14 days. To further assess the typical storage temperatures and duration of case exhibits, the aliquots stored in the fridge and freezer were also analyzed at day 30, 60, and 90.

6.2.12.3 Freeze-Thaw Stability

Since exhibit samples are commonly received and then frozen, freeze-thaw stability was assessed where three aliquots at each of the low (50 pg/mL) and high (1500 pg/mL) concentrations were stored at -15 to -12°C for 24 hours and thawed unassisted at room temperature. When completely thawed, the samples were refrozen for 24 hours under the same conditions. The freeze-thaw cycle was repeated two more times and then analyzed on the third cycle.

6.2.12.4 Stock Solution Stability

To assess the short and long term stability of the stock solutions, stock solutions containing individual drug standards at 500 pg/mL were prepared in ACN, MeOH, water, pH 4.0 buffer, and pH 9.0 buffer. Stock solutions were stored at room temperature, refrigerated (2 to 3°C), and frozen (-15 to -12°C) and analyzed on day 0, 1, and 30. Stock solutions stored in the fridge and freezer were stored for another 30 days and analyzed on day 60.

6.2.12.5 Post Preparative Stability

To assess the short term post preparative stability, extracted samples at both low (50 pg/mL) and high (1500 pg/mL) concentrations in triplicate were stored at 10°C (autosampler temperature) and analyzed at 0, 4, and 24 hours.

6.2.13 Other Qualitative Parameters

For further confirmation, the RT and the ion ratios (Equation 6.7) were included. Since an ISTD was used, the RRT was also used (Equation 6.8). The RT, RRT, and ion ratio of calibration standards were compared to the quality control and test samples.

Ion Ratio = PA of base peak/PA of another product ion Equation 6.7

RRT = RT standard/RT ISTD Equation 6.8

6.2.14 Assessment of Existing Extraction Methods at the RCMP FLS for Basic Drugs

At the RCMP FLS Winnipeg an n-butylchloride (nBuCl) and Toxi-Lab A method are used to extract basic drugs. Whole blood samples are extracted using the nBuCl extraction method, where 1 mL blood with ISTD and 75 µl concentrated NH_4OH is extracted with 4 mL nBuCl. The nBuCl layer is then transferred to a new test tube and evaporated to dryness under N_2, then reconstituted in appropriate mobile phase (Mason-Daniel 2005). Urine samples are extracted using Toxi-Lab A tubes from Varian Inc (Lake Forest, CA), where 1 mL urine with ISTD is added to the tube and the tubes are capped and shaken. The upper organic layer is transferred to a new test tube and evaporated to dryness under N_2, then reconstituted in appropriate mobile phase (Pan and Yawney 2005). To assess the suitability of both the nBuCl and Toxi-Lab A extraction

method, ME and recovery were assessed. ME was assessed by analyzing each analyte at both a low (50 pg/mL) and high (1500 pg/mL) concentration in triplicate (section 6.2.11). Recovery was assessed by analyzing a low (50 pg/mL) and high (1500 pg/mL) concentration in replicates of six (section 6.2.10).

6.3 Results and Discussion

The MRM ion chromatograms of LSD, iso-LSD, nor-LSD, O-H-LSD, LSD-D3, and the corresponding blank in urine and whole blood are shown in Figures 6.1 and 6.2. The same MRM transitions were monitored by others using triple quadrupole MS (Canezin et al. 2001; de Kanel et al. 1998; Skopp et al. 2002). LSD and iso-LSD have the same MRM transitions and are baseline separated, but different product ions were used for quantitation for LSD versus iso-LSD due to each having a different base peak in the product ion spectrum. The m/z 281 product ion was the most abundant for iso-LSD, whereas m/z 223 was the most abundant product ion for LSD.

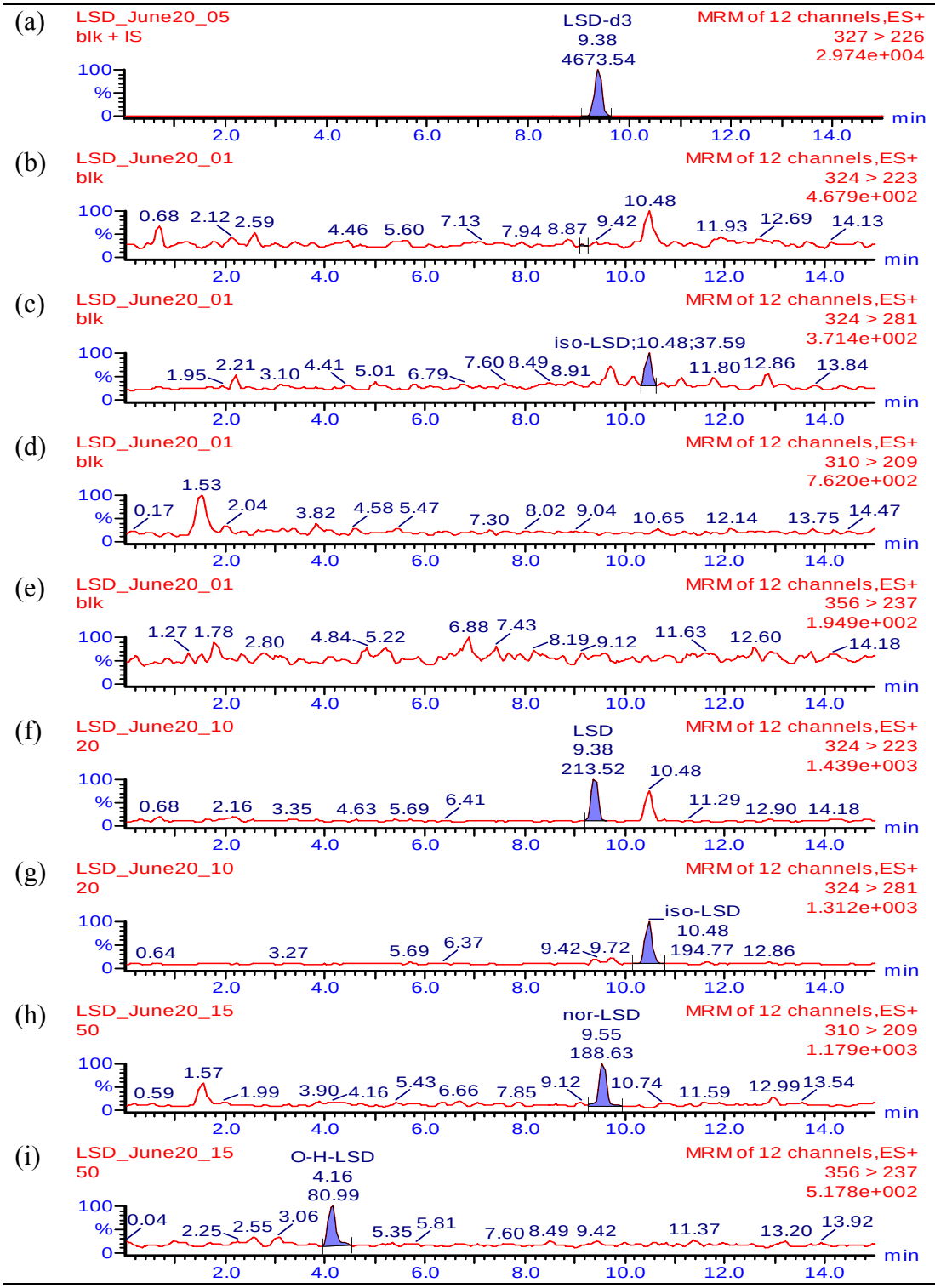


Figure 6.1: (a) MRM ion chromatograms of blank urine + ISTD, and (b-e) blank urine, and urine spiked with (f) 20 pg/mL LSD (RT = 9.38 min), (g) 20 pg/mL iso-LSD (RT = 10.48 min), (h) 50 pg/mL nor-LSD (RT = 9.55 min), and (i) 50 pg/mL O-H-LSD (RT = 4.16 min).

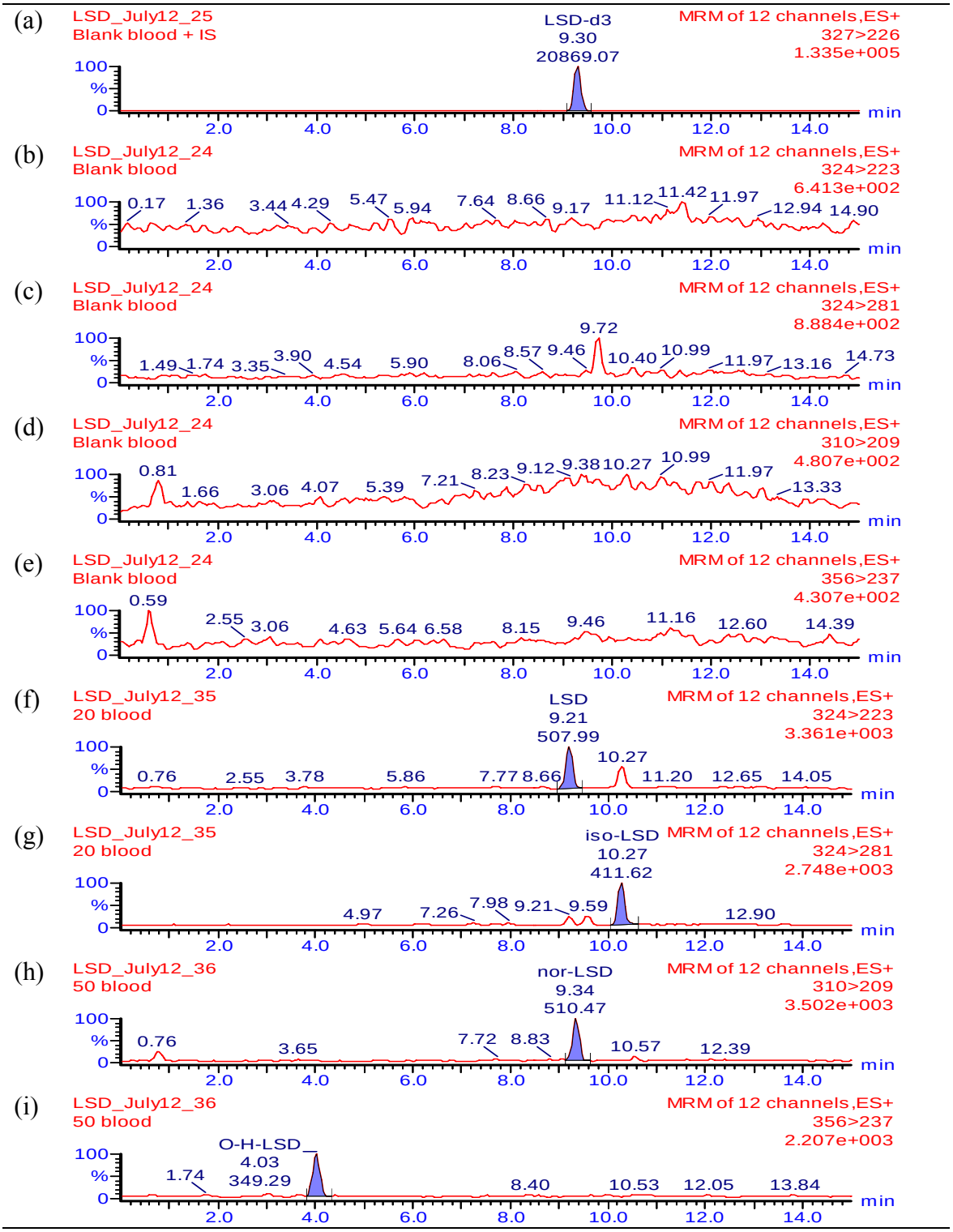


Figure 6.2: (a) MRM ion chromatograms of blank blood + LSD-D3 ISTD, (b-e) blank blood, and blood spiked with (f) 20 pg/mL LSD (RT = 9.21 min), (g) 20 pg/mL iso-LSD (RT = 10.27 min), (h) 50 pg/mL nor-LSD (RT = 9.34 min), and (i) 50 pg/mL O-H-LSD (RT = 4.03 min).

6.3.1 Linearity and Range

The linear range of extracted urine standards was 20 to 2000 pg/mL for LSD (Figure 6.3a) and iso-LSD (Figures 6.4a), and 50 to 2000 pg/mL for nor-LSD (Figure 6.5a) and O-H-LSD (Figures 6.6a). For extracted blood standards the linear range was 10 to 2000 pg/mL for LSD (Figure 6.3b), iso-LSD (Figures 6.4b), and nor-LSD (Figure 6.5b), and 20 to 2000 pg/mL for O-H-LSD (Figures 6.6b). The coefficient of determination (r^2) of all analytes, both in extracted urine and blood was greater than 0.99. The intercept, slope, and correlation coefficient (r) ± standard error (SE), standards deviation of the residuals (Sy.x) and the runs test p value using weighted ($1/x^2$) linear regression are shown in Table 6.2.

Table 6.2: Linearity parameters in both urine and whole blood

		Intercept ± SE	**Slope ± SE**	**r ± SE**	**Sy.x**	**runs p**
LSD	Urine	0.0049 ± 0.0009	0.00101 ± 0.00002	0.997 ± 0.005	0.068	0.30
	Blood	0.0035 ± 0.0006	0.00107 ± 0.00002	0.999 ± 0.001	0.048	0.16
iso-LSD	Urine	0.0082 ± 0.0010	0.00110 ± 0.00002	0.998 ± 0.004	0.063	0.60
	Blood	-0.0016 ± 0.0007	0.00089 ± 0.00001	0.999 ± 0.001	0.045	0.34
nor-LSD	Urine	0.036 ± 0.0008	0.00047 ± 0.00001	0.996 ± 0.008	0.036	0.87
	Blood	0.00086 ± 0.0003	0.00052 ± 0.00001	0.998 ± 0.003	0.027	0.11
O-H-LSD	Urine	0.0027 ± 0.0007	0.00026 ± 0.00001	0.993 ± 0.014	0.029	0.87
	Blood	-0.0003 ± 0.0003	0.00031 ± 0.00001	0.998 ± 0.003	0.017	0.71

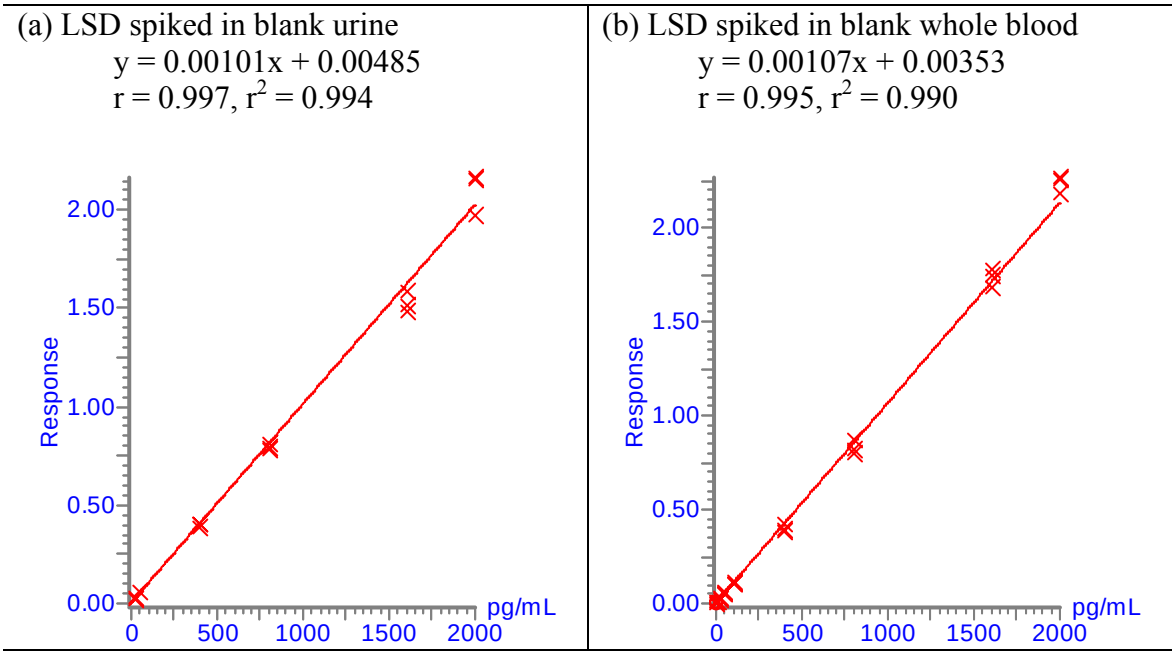


Figure 6.3: Calibration curves for LSD in (a) urine and (b) whole blood.

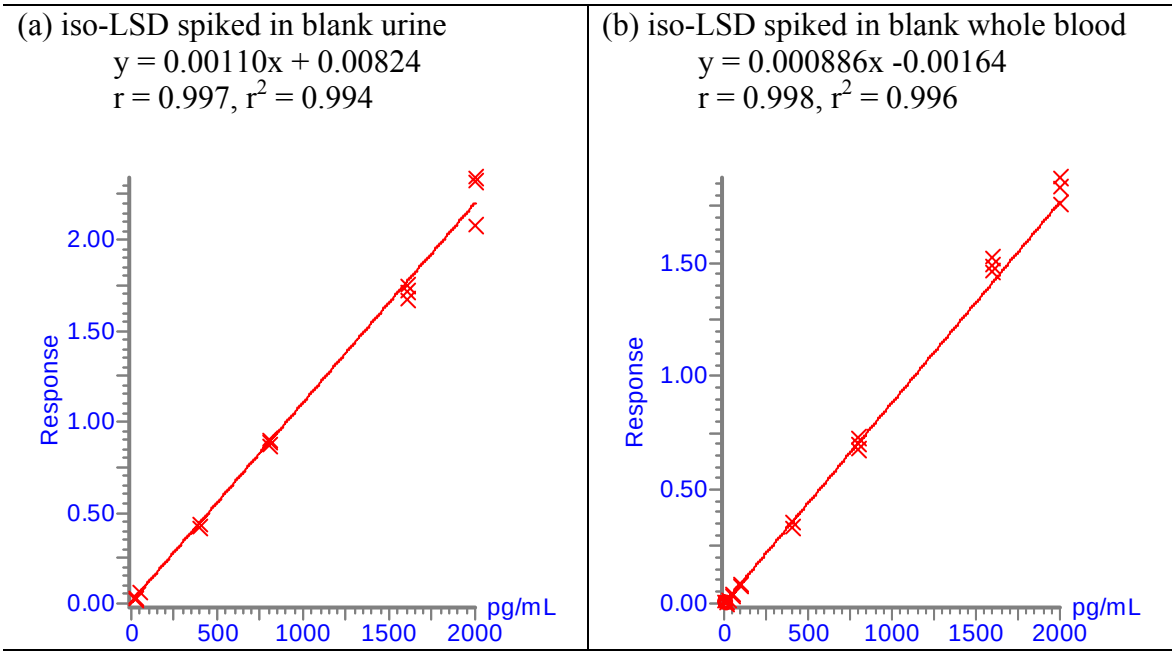


Figure 6.4: Calibration curves for iso-LSD in (a) urine and (b) whole blood.

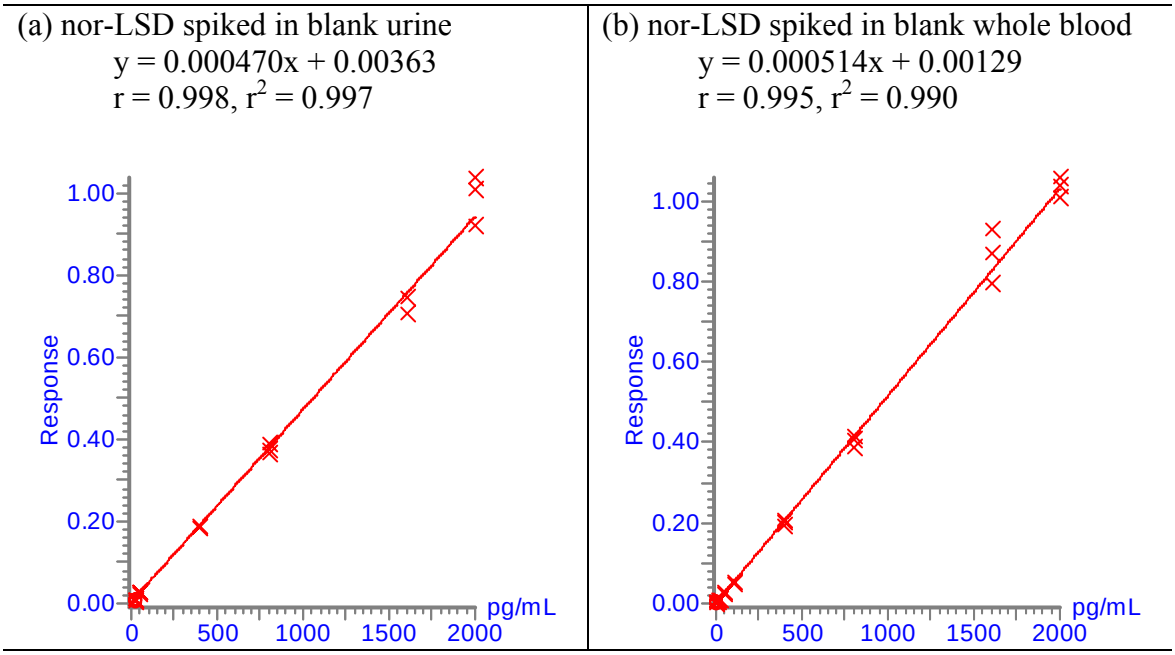


Figure 6.5: Calibration curves for nor-LSD in (a) urine and (b) whole blood.

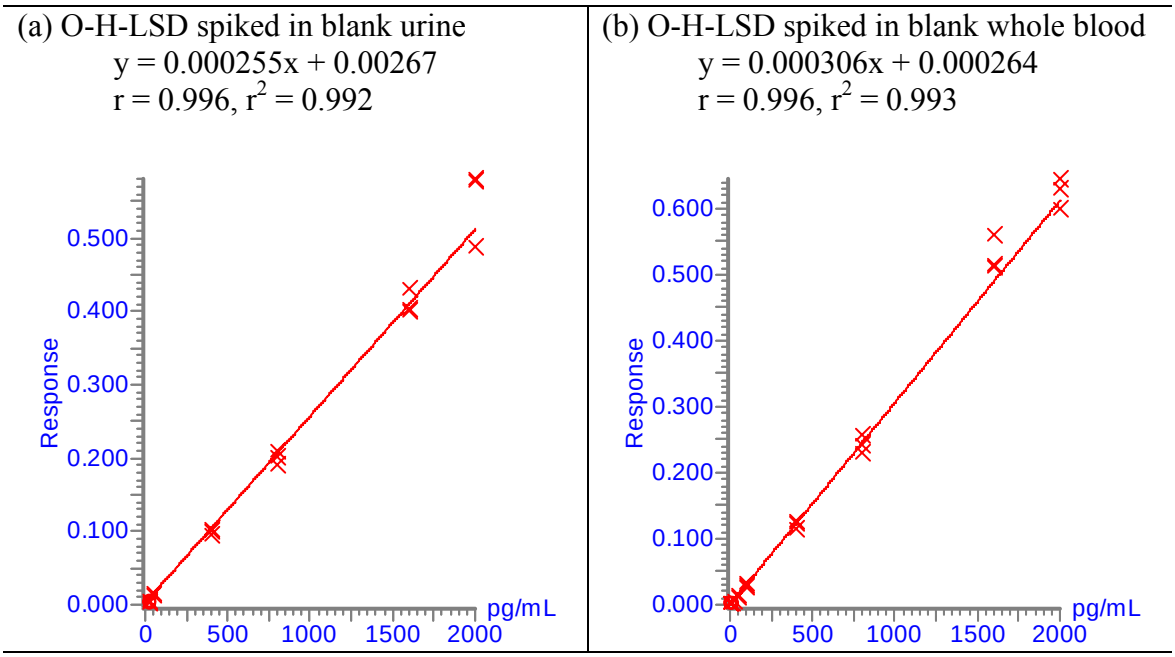


Figure 6.6: Calibration curves for O-H-LSD in (a) urine and (b) whole blood.

6.3.2 Accuracy and Precision

The accuracy was deemed acceptable if the % bias of the back calculated result was ±15%, and ±20% at LLOQ, of the nominal concentration. The precision was deemed acceptable if the % CV of back calculated results were ≤15% and ≤20% at LLOQ. Quantitations are normally only done on blood samples at FLS Winnipeg. This is because levels in blood give more information than levels in urine. Urine levels are difficult to interpret due to levels being influenced by urine pH, volume eliminated, and other factors (Cravey and Baselt 1981). Urine analysis is still conducted because it is very useful for detecting drugs of abuse, since metabolites are generally more polar than the parent compound. Drug metabolites will appear in higher concentrations in urine versus blood and persist for a much longer time than parent drugs. As a result, for the validation experiment, quantitative analysis for blood was conducted using a full calibration curve with 5 to 6 points, whereas semi-quantitative analysis was conducted for urine using a 3 point calibration curve. Both inter- and intra-day accuracy and precision for all compounds both in urine and blood were within acceptable limits (Table 6.3, 6.4, 6.5, and 6.6).

Table 6.3: Accuracy (% Bias) results over three days of analysis in urine

	Nominal (pg/mL)	Day 1		Day 2		Day 3		Average	
		%bias	n	%bias	n	%bias	n	%bias	n
LSD	50	-3.97	6	5.07	6	1.32	5	0.78	17
	500	-2.45	6	-5.66	6	0.06	6	-2.69	18
	1500	2.07	6	-0.39	5	1.42	6	1.12	18
iso-LSD	50	5.17	6	2.83	6	-6.84	5	0.81	17
	500	-3.07	6	0.59	6	0.78	6	-0.57	18
	1500	1.09	6	-1.47	5	2.00	6	0.66	18
nor-LSD	50	1.73	6	3.77	6	-9.52	5	-0.86	17
	500	-2.21	6	3.06	6	2.74	6	1.20	18
	1500	2.06	6	8.37	5	3.65	6	4.48	18
O-H-LSD	50	-0.70	6	-2.60	6	2.48	5	-0.44	17
	500	-3.77	6	-9.44	5	4.13	6	-2.65	17
	1500	2.80	6	-11.98	5	-0.58	5	-2.88	16

Table 6.4: Precision (% CV) results over three days of analysis in urine

	Nominal (pg/mL)	Day 1		Day 2		Day 3		Average	
		% CV	n	% CV	n	% CV	n	% CV	n
LSD	50	5.76	6	6.48	6	3.97	5	6.55	17
	500	4.77	6	7.46	6	4.83	6	5.96	18
	1500	3.51	6	3.15	5	2.56	6	3.08	18
iso-LSD	50	6.22	6	13.19	6	8.73	5	10.61	17
	500	4.06	6	10.68	6	5.30	6	7.13	18
	1500	4.77	6	2.14	5	4.05	6	3.96	18
nor-LSD	50	5.05	6	11.04	6	10.46	5	10.37	17
	500	5.28	6	4.09	6	5.92	6	5.42	18
	1500	5.17	6	6.65	5	4.43	6	5.70	18
O-H-LSD	50	4.67	6	12.01	6	17.64	5	11.70	17
	500	3.87	6	13.88	5	12.28	6	11.58	17
	1500	9.47	6	8.13	5	3.48	5	9.81	16

Table 6.5: Accuracy (% Bias) results over three days of analysis in whole blood

	Nominal (pg/mL)	Day 1		Day 2		Day 3		Average	
		%bias	n	%bias	n	%bias	n	%bias	n
LSD	50	-5.42	6	-8.03	6	-8.87	6	-7.44	18
	500	-3.37	6	-3.03	6	-12.44	6	-6.29	18
	1500	-2.44	5	-1.35	6	-6.46	6	-3.47	17
iso-LSD	50	-10.64	6	-7.78	6	-11.96	6	-10.12	18
	500	-3.09	6	4.34	6	-11.77	6	-3.51	18
	1500	-2.08	5	4.46	6	-5.06	6	-0.82	17
nor-LSD	50	-13.85	6	-16.59	6	7.13	6	-7.77	18
	500	-8.03	6	-13.17	6	-1.66	6	-7.63	18
	1500	-2.75	5	-8.11	6	1.38	6	-3.19	17
O-H-LSD	50	-14.49	6	-14.68	6	-10.50	6	-13.22	18
	500	-10.16	6	-10.96	6	-10.47	6	-10.53	18
	1500	-10.13	5	-9.43	6	-5.45	6	-8.23	17

Table 6.6: Precision (% CV) results over three days of analysis in whole blood

	Nominal (pg/mL)	Day 1		Day 2		Day 3		Average	
		% CV	n	% CV	n	% CV	n	% CV	n
LSD	50	5.00	6	1.54	6	7.22	6	5.09	18
	500	4.72	6	6.10	6	6.51	6	7.23	18
	1500	2.37	5	1.89	6	2.02	6	3.09	17
iso-LSD	50	4.63	6	4.15	6	3.83	6	4.44	18
	500	5.68	6	7.43	6	4.37	6	9.08	18
	1500	2.60	5	3.50	6	3.83	6	5.30	17
nor-LSD	50	7.15	6	7.95	6	7.53	6	13.80	18
	500	4.51	6	5.75	6	6.27	6	7.43	18
	1500	2.87	5	5.28	6	2.81	6	5.54	17
O-H-LSD	50	2.17	6	4.58	6	11.33	6	7.25	18
	500	5.11	6	5.11	6	6.53	6	5.30	18
	1500	3.70	5	4.91	6	3.93	6	4.60	17

6.3.3 LOD and LLOQ

LOD and LLOQ were determined by two different methods, where each method gave a slightly different value (Table 6.7). To calculate the LOD and LLOQ both the slope and Sy.x were derived from a calibration curve within the LOD range. A peak-to-peak S/N was determined using the MassLynx™ version 4.0 software (Waters Corporation, Milford, MA), where the greatest height of the signal range above the mean noise value was divided by the variance (Figure 6.7, 6.8, 6.9, & 6.10). Regardless of the LLOQ result obtained using the calculated or S/N method, LLOQ was ultimately defined as the lowest concentration that showed acceptable accuracy (±20% bias) and precision (≤20% CV) (Table 6.8 and 6.9).

When comparing the two methods used to determine the LOD and LLOQ, the S/N method resulted in lower values for both. This can be explained by two possible reasons; (1) possible overestimation errors using the calculated method, and (2) the highly efficient columns used with UPLC™. The calculated method of determining LOD and LLOQ could lead to overestimation of the limits, by using a concentration range far away from the LOD and LLOQ when creating the calibration curve to estimate

the slope and residual standard deviation (Peters and Maurer 2002). Also, chromatography can affect the LOD and LLOQ, where narrower and taller peaks with highly efficient columns will result in a higher S/N resulting in lower LOD and LLOQ (Shabir 2003). As a result, to take advantage of UPLC™, the S/N method was chosen as the method for determining both the LOD and LLOQ.

Table 6.7: LOD and LLOQ determined using two different methods in both urine and whole blood

		(pg/mL) Calculated		(pg/mL) S/N	
		LOD	**LLOQ**	**LOD**	**LLOQ**
LSD	Urine	11	33	10	20
	Blood	10	32	5	10
iso-LSD	Urine	10	29	10	20
	Blood	11	36	5	10
nor-LSD	Urine	21	64	10	50
	Blood	13	45	10	20
O-H-LSD	Urine	23	70	10	50
	Blood	20	66	10	20

Table 6.8: LLOQ in urine and whole blood with acceptable accuracy and precision

	Acceptable accuracy and precision LLOQ (pg/mL)
LSD	20
iso-LSD	20
nor-LSD	50
O-H-LSD	50

Table 6.9: Accuracy and precision at LLOQ in urine and whole blood

		% Bias at LLOQ	**CV% at LLOQ**
LSD	Urine	14.50	10.90
	Blood	2.08	9.79
iso-LSD	Urine	7.33	14.82
	Blood	1.92	8.01
nor-LSD	Urine	-1.03	17.19
	Blood	0.17	6.13
O-H-LSD	Urine	11.50	9.47
	Blood	0.03	4.66

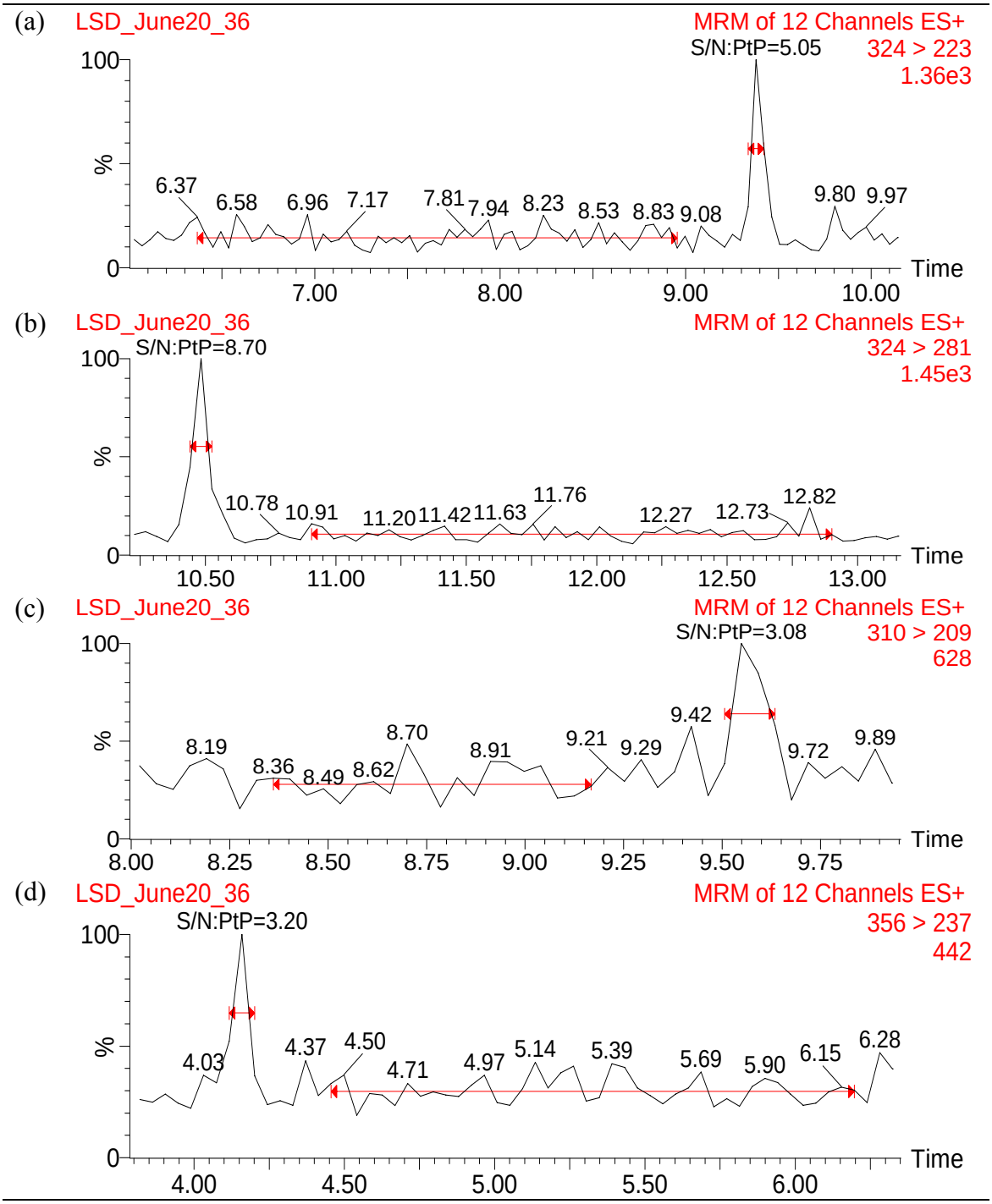


Figure 6.7: S/N at LOD of 10 pg/mL for (a) LSD (S/N = 5.05), (b) iso-LSD (S/N = 8.70), (c) nor-LSD (S/N = 3.08), and (d) O-H-LSD (S/N = 3.20) in urine.

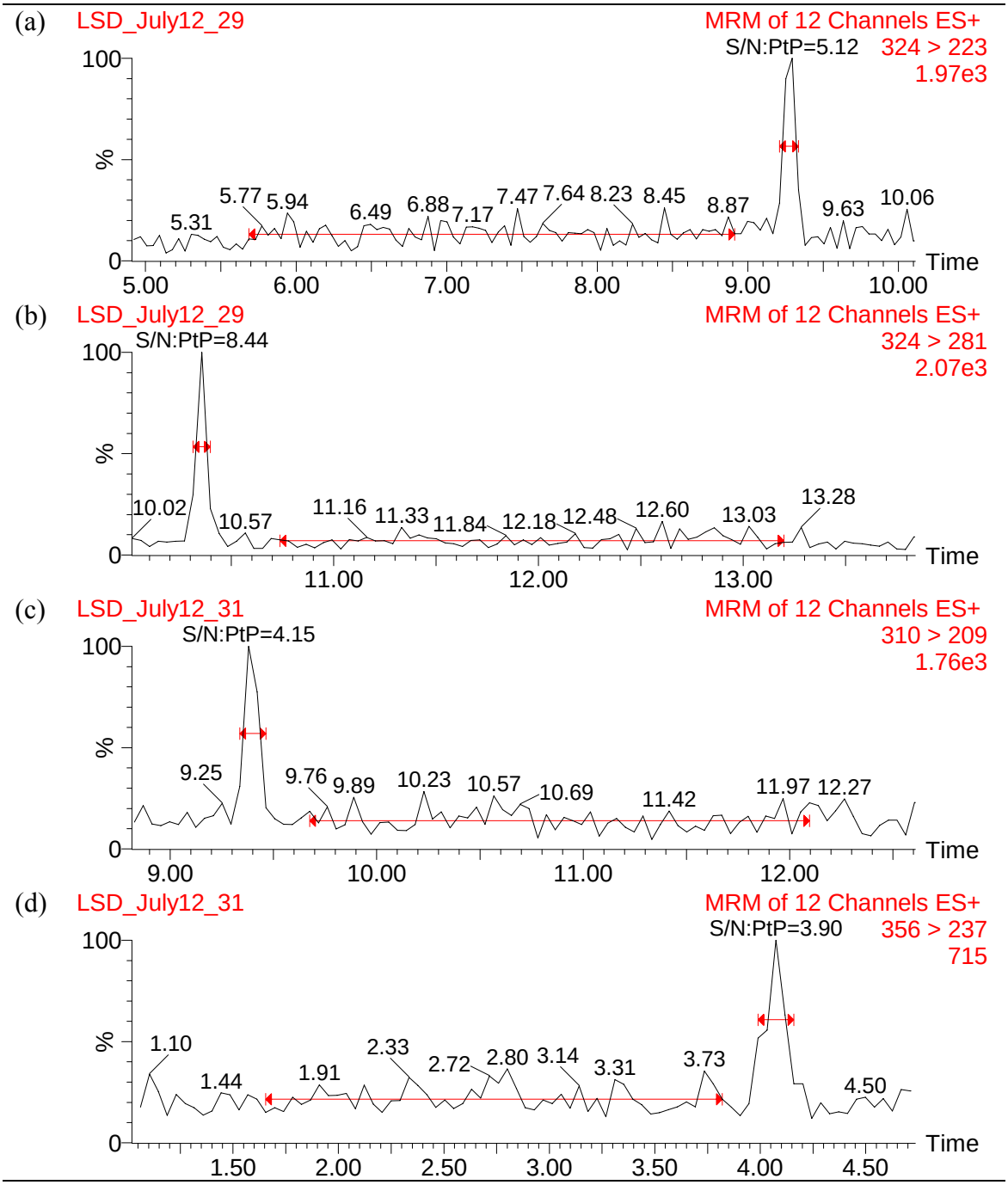


Figure 6.8: S/N at LOD of 5 pg/mL for (a) LSD (S/N = 5.12) and (b) iso-LSD (S/N = 8.44), and 10 pg/mL for (c) nor-LSD (S/N = 4.15) and (d) O-H-LSD (S/N = 3.90) in whole blood.

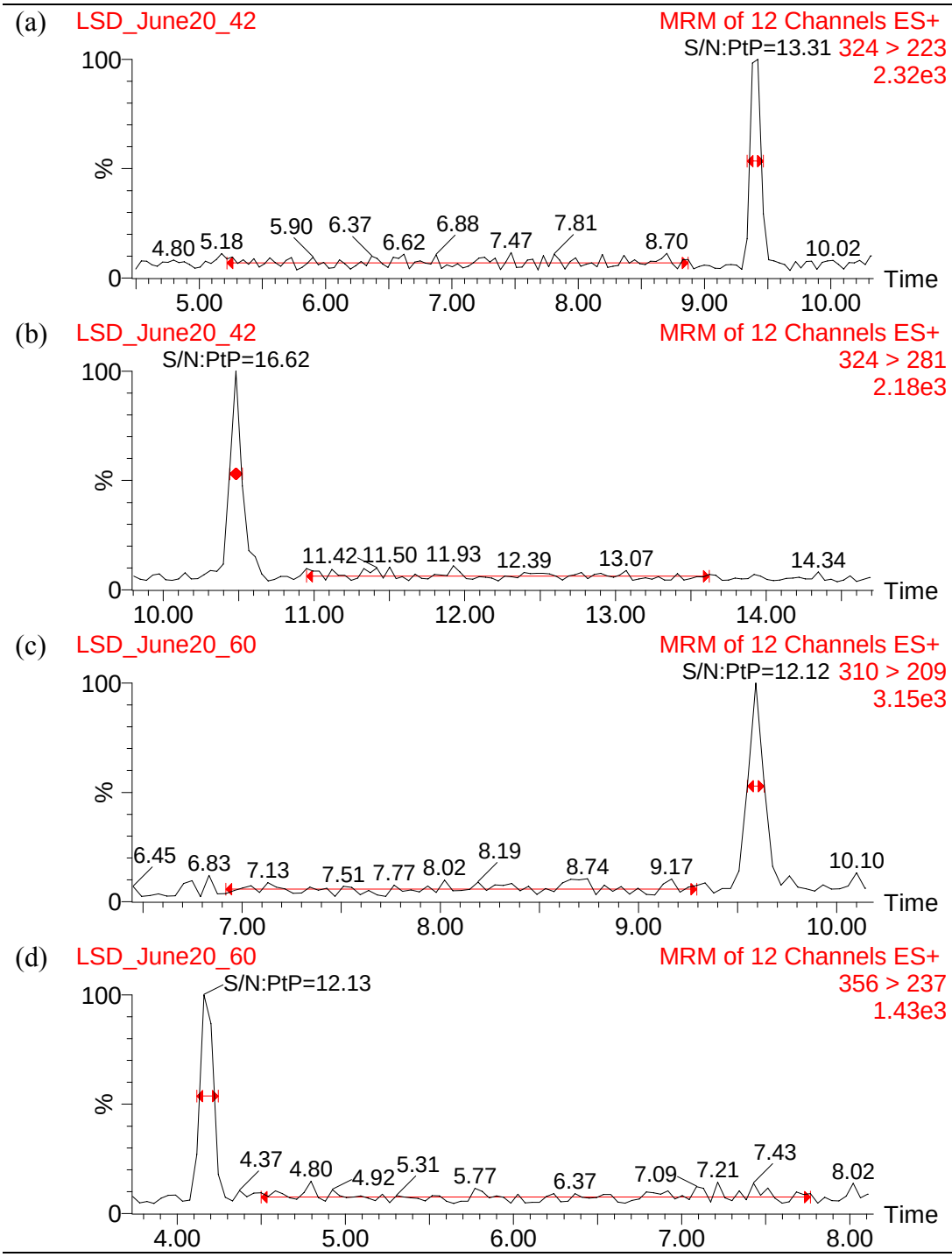


Figure 6.9: S/N at LLOQ of 20 pg/mL for (a) LSD (S/N = 13.31), (b) iso-LSD (S/N = 16.62), and 50 pg/mL for (c) nor-LSD (S/N = 12.12), and (d) O-H-LSD (S/N = 12.13) in urine.

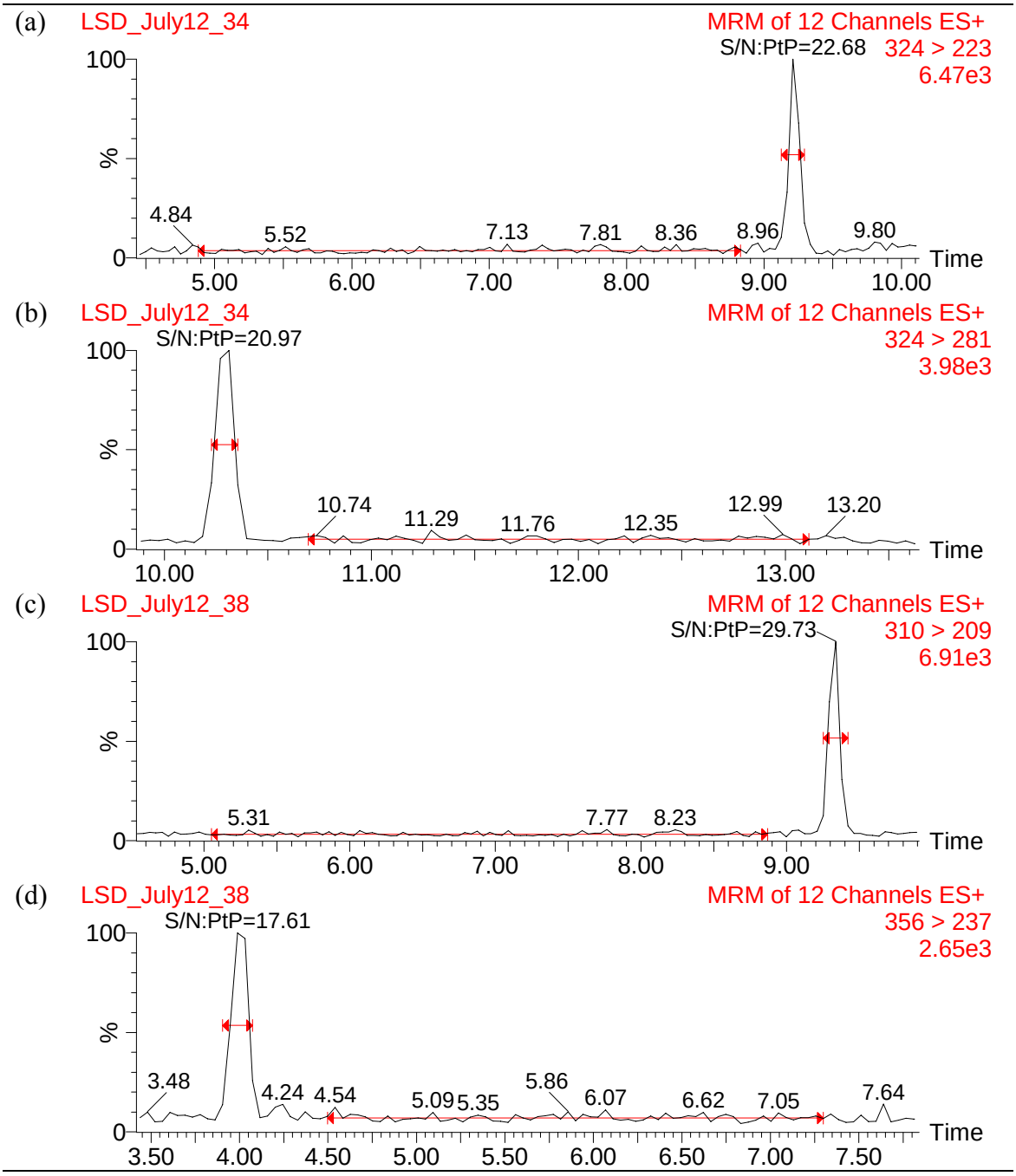


Figure 6.10: S/N at LLOQ of 20 pg/mL for (a) LSD (S/N = 22.68) and (b) iso-LSD (S/N = 20.97), and 50 pg/mL for (c) nor-LSD (S/N = 29.73) and (d) O-H-LSD (S/N 17.61) in whole blood.

The LLOQ of 20 pg/mL for LSD and iso-LSD in urine and whole blood were the same as obtained by others using LC/MS/MS with a different triple quadrupole MS (API 300 Perkin-Elmer SCIEX, Thornhill, Canada) (Canezin et al. 2001). Johansen and Jensen reported a LLOQ of 10 pg/mL for both LSD and iso-LSD in urine and whole blood using LC/MS/MS with a triple quadrupole MS (Quattro Micro, Waters); however, the accuracy and precision at this level was not reported. The LLOQ of 50 pg/mL for nor-LSD and O-H-LSD in urine and whole blood was a more sensitive result compared to others using LC/MS/MS. Skopp et al. reported a LLOQ of 240 pg/mL and 190 pg/mL for nor-LSD and O-H-LSD, respectively using LC/MS/MS (API 365, Applied Biosystems, Foster City, CA).

When GC/MS/MS with an ion trap MS was used with cold-on column injections, a detection threshold of 20 pg/mL was reported for LSD in whole blood (Libong et al. 2003a) and urine (Sklerov et al. 1999). When urine samples were derivatized and analyzed using GC/MS/MS with a MSD a LOD and LLOQ of 500 and 1000 pg/mL, respectively were reported for O-H-LSD (Burnley and George 2003). In contrast, a very sensitive GC/MS/MS method using positive CI and selected reaction monitoring has been reported for LSD and O-H-LSD in urine samples with a LLOQ of 10 pg/mL (Reuschel et al. 1999b). Compared to GC/MS/MS methods, the UPLC™/MS/MS method is simpler to perform with similar or better sensitivity.

The LSD-D3 standard obtained from Cerilliant Corporation (Round Rock, TX) was 99% pure as determined by chromatographic analysis (Cerilliant Certificate of Analysis). When analyzed via UPLC™/MS/MS there was a residual amount of LSD present. The S/N of the residual LSD in the ISTD was not greater than 3 (Figure 6.11), and the response at the LLOQ for LSD was five times greater than the response of residual LSD in LSD-D3 (Figure 6.12). It may be argued that the residual amount of LSD in the ISTD may cause an overestimation during quantitation. However, since the standards used to make up the calibration curve, the QC standards, and the unknowns all have the same amount of ISTD added there should be no effect seen on the quantitated value in the unknowns.

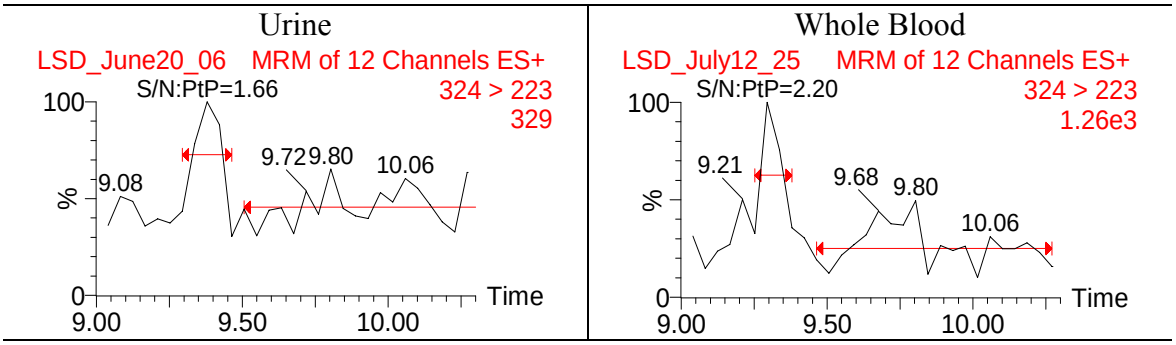


Figure 6.11: S/N of the residual LSD in the LSD-D3 ISTD.

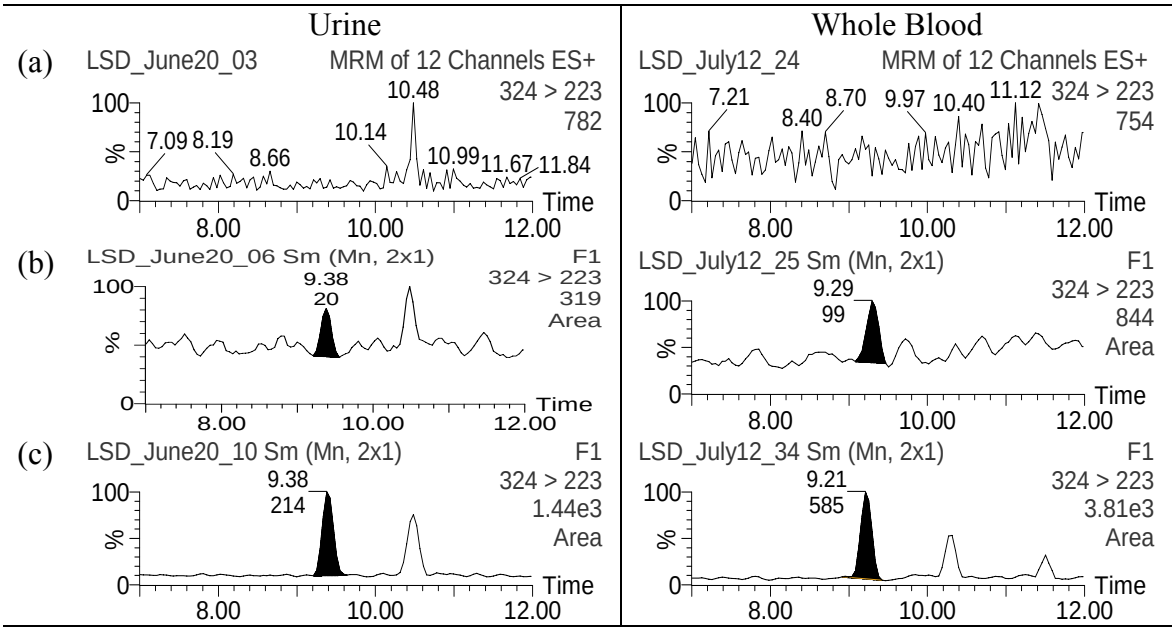


Figure 6.12: MRM acquisitions of a (a) blank showed the absence of a LSD peak, (b) blank + ISTD (LSD-D3) showed the presence of residual LSD (urine PA = 20, whole blood PA = 99), and (c) LSD PA at LLOQ of 20 pg/mL in urine and whole blood was 214 and 585, respectively.

6.3.4 Selectivity

The blank urine in Figure 6.1c (page 74) showed a response (PA = 37.59) at the same retention time as iso-LSD (RT = 10.48 minutes). However, since the PA response at the LLOQ was 194.77 it adhered to the recommendations of the FDA that the LLOQ should be at least five times the response compared to the blank (FDA 2001). Furthermore, 6 different sources of blank urine were extracted and analyzed with none

showing a PA response greater than ~40. When 5 different sources of blank human whole blood, and porcine whole blood were extracted and analyzed, no interfering peaks were detected.

6.3.5 Recovery

The percent recoveries are shown in Table 6.10. Percent recoveries were slightly lower in whole blood than in urine. This was acceptable since the sensitivity in whole blood was not affected. The percent recoveries are nearly the same as reported in the original LLE methods (Canezin et al. 2001; Sklerov et al. 2000). The percent recoveries were reproducible with a %CV range of 3.9 to 11.4% and 2.9 to 8.1% in urine and whole blood, respectively. Both high and low concentrations of each analyte showed similar percent recoveries. As well, percent recoveries were similar among different compounds.

The lower percent recovery of whole blood can be explained due to the whole blood samples being filtered prior to being injected. To prevent the loss of analyte through filtration, alternatively samples could be centrifuged at a high speed to separate the particulate. However, since the overall sensitivity was not affected this was not essential.

Table 6.10: Extraction percent recovery

		50 pg/mL	1500 pg/mL	Average
LSD	Urine	78	78	78
	Blood	62	61	62
iso-LSD	Urine	74	78	76
	Blood	62	66	64
nor-LSD	Urine	88	85	86
	Blood	69	67	68
O-H-LSD	Urine	76	74	75
	Blood	66	72	69

6.3.6 Matrix Effect (ME)

Both urine and whole blood demonstrated similar absolute ME at both the high and low concentrations. LSD, iso-LSD, O-H-LSD, and LSD-D3 showed slight ion suppression, whereas nor-LSD showed slight ion enhancement (Table 6.11). The

difference seen in nor-LSD may be explained by the chemistry of the analyte. It has been found that the chemical nature of the analyte may have more of an effect on the amount of suppression than the sample preparation method (Bonfiglio et al. 1999). Compared to LSD, iso-LSD, LSD-D3, and O-H-LSD, nor-LSD lacks a methyl group. The removal of the methyl group on nor-LSD may somehow cause an enhancement in ionization. On the other hand, the endogenous matrix constituents in the nor-LSD MRM transition of 309>210 may also account for the slight ion enhancement. To fully understand the different ME response of nor-LSD further investigation is required. Ion suppression studies have been reported for LSD and iso-LSD by injecting extracted spiked blank whole blood samples through a syringe pump into a T piece with mobile phase (Johansen and Jensen 2005). This study showed no ion suppression and states that the deuterated ISTD corrected for any possible suppression of samples.

Table 6.11: Matrix effect

		50 pg/mL	1500 pg/mL	Average
LSD	Urine	89	93	91
	Blood	99	90	95
iso-LSD	Urine	89	100	95
	Blood	96	97	96
nor-LSD	Urine	119	126	123
	Blood	122	114	118
O-H-LSD	Urine	93	101	97
	Blood	97	99	98
LSD-D3	Urine	90	95	93
	Blood	94	91	93

Even though the absolute ME of nor-LSD was different compared to the other compounds, this did not necessarily indicate that the bioanalytical method was invalid. If the relative ME exhibit the same pattern for the drug and the ISTD in all sources of matrix studied, the drug to ISTD ratio should not be affected (Matuszewski et al. 2003). The relative ME, which was defined by comparing the ME values between different sources of matrix, showed no significant difference ($p>0.05$) when tested with F test between the variances of the PAR of neat standard versus whole blood and urine (Figure 6.13). The relative ME of all compounds were not shown to differ significantly,

confirming that the ME had no effect on the quantitation of LSD, iso-LSD, nor-LSD, and O-H-LSD.

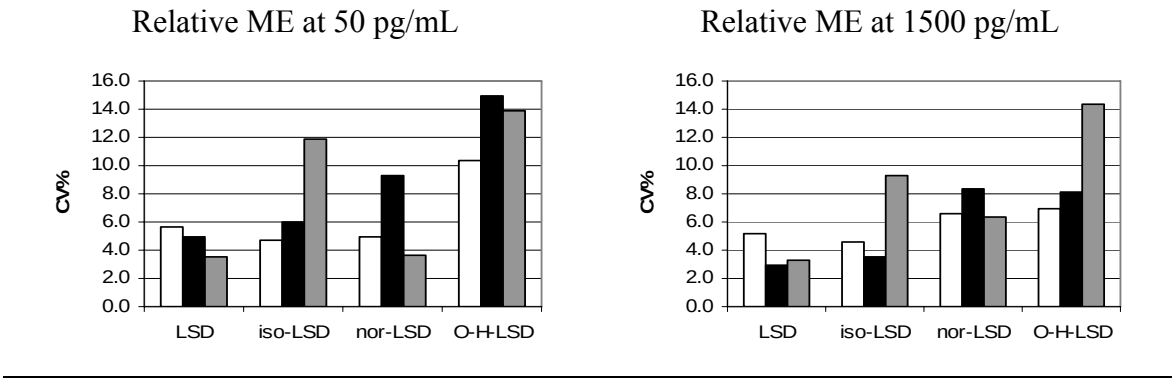


Figure 6.13: Precision (CV%) of the PAR of LSD, iso-LSD, nor-LSD, and O-H-LSD at both 50 and 1500 pg/mL in □ neat standard (n=6), and ■ 6 different sources of whole blood, and ■ 5 different sources of urine.

6.3.7 Stability

The stability was deemed acceptable if the back calculated result did not deviate by more than 20% from day 0 concentration. Nor-LSD and O-H-LSD stability were only assessed at the high concentration (1500 pg/mL) because the low concentration (50 pg/mL) is the LLOQ. As a result, any results below LLOQ cannot be accurately quantified. LSD and iso-LSD stability were assessed at both low and high concentration.

6.3.7.1 Short Term Stability

LSD, nor-LSD, and O-H-LSD were stable in both preserved and unpreserved urine and whole blood stored at room temperature for 24 hours when exposed or not exposed to normal laboratory lighting. Iso-LSD in preserved and unpreserved whole blood was also determined to be stable for 24 hours when exposed and not exposed to laboratory lighting. When iso-LSD in urine was stored for 24 hours only the unpreserved urine stored in the dark was stable for 24 hours at room temperature. Iso-LSD in unpreserved urine exposed to light and in preserved urine both stored in the dark

and exposed to the light decreased in concentration by 21 to 39% after 24 hours at room temperature.

6.3.7.2 Long Term Stability

LSD, iso-LSD, nor-LSD and O-H-LSD were all found to be stable for 7 days when exposed to normal laboratory lighting at room temperature. The same results were reported for O-H-LSD in urine samples at pH 4.6 and 6.5 kept at 24°C exposed to fluorescent light for 9 days (Klette et al. 2002). It has also been reported that LSD in urine samples can withstand normal room light conditions at 25°C for 1 week without noticeable structural change (Li et al. 1998). Another study found that LSD concentrations in urine, plasma, and whole blood decreased in concentration to about 50% when kept at room temperature exposed to fluorescent light over 14 days (de Kanel et al. 1998).

Results of the 30 day room temperature and the 90 day refrigerated (2 to 3°C) and frozen (-15 to 12°C) stability study of samples kept in the dark are shown in Figures 6.14 and 6.15. LSD was stable at room temperature in all sample types for 30 days. Other studies have also found that LSD in urine was stable at 25°C for 4 weeks in the dark (Li et al. 1998; Francom et al. 1988). LSD was stable both refrigerated and frozen in all sample types for 90 days. Similar results were reported for LSD in urine in another study (Francom et al. 1988).

Iso-LSD was stable at room temperature in both preserved and unpreserved whole blood for 30 days. Iso-LSD in urine was generally found to be unstable (22 to 30% decrease) at room temperature for the first 14 days, but then within stable concentrations by day 30. Iso-LSD was stable refrigerated and frozen in both preserved and unpreserved whole blood for 90 days. Like iso-LSD at room temperature, iso-LSD in urine refrigerated and frozen followed a general trend, where there was an initial decrease in concentration on day 7 and 14, followed by a gradual increase to day 0 concentrations by day 90. Stability studies for iso-LSD in biological matrices have not been previously reported; however, it has been found that iso-LSD rapidly isomerizes to LSD when dissolved in organic solvents and stored at 2-6°C (Clarkson et al. 1998).

Nor-LSD was only stable for 14 days at room temperature, decreasing by 23% in unpreserved urine, 37% in preserved urine, and >50% in both unpreserved and preserved blood at day 30. Nor-LSD was also only stable for 14 days refrigerated and frozen, decreasing by 43% in both unpreserved and preserved urine, and 47% in both unpreserved and preserved blood at day 30 and remained the same up to day 90. Another study evaluated the stability of nor-LSD in urine, plasma, and whole blood and found that nor-LSD was stable in all sample types for two weeks stored at 4 and -20°C in the dark (de Kanel et al. 1998).

O-H-LSD in preserved urine was stable for 30 days at room temperature, but in unpreserved urine O-H-LSD was unstable decreasing in concentration by 30% after 7 days. In both unpreserved and preserved whole blood, O-H-LSD was only stable for 7 days at room temperature decreasing in concentration by 30 to 45% after 14 days and greater than 68% after 30 days. O-H-LSD was stable both refrigerated and frozen in all sample types for 60 days. At day 90 only O-H-LSD in urine both refrigerated and frozen was stable. O-H-LSD in whole blood was unstable by day 90 deviating more than 38% and 22% refrigerated and frozen, respectively. Another study states that O-H-LSD is stable in specimens at pH 4.6 to 6.5 when maintained at -20 to 24°C for 60 days (Klette et al. 2002). Stability studies for O-H-LSD in whole blood have never been previously reported.

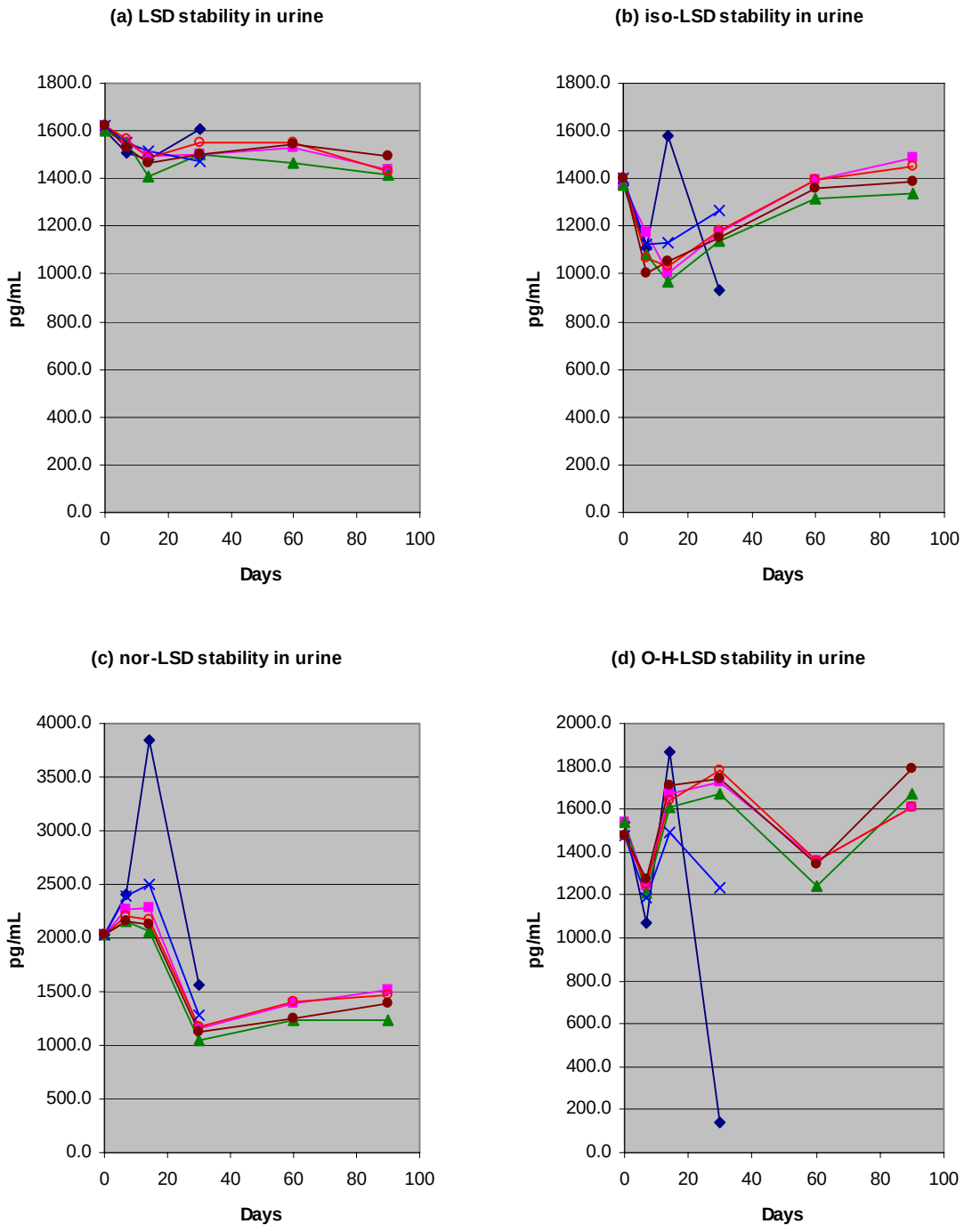


Figure 6.14: Long term stability of (a) LSD, (b) iso-LSD, (c) nor-LSD, and (d) O-H-LSD in urine. Samples (unpreserved ♦ room temperature, ■ refrigerated, ▲ frozen, and preserved × room temperature, ○ refrigerated, and ● frozen) were kept at room temperature for 30 days and in the fridge and freezer for 90 days and were analyzed on day 0, 1, 7, 14, 30, 60, and 90.

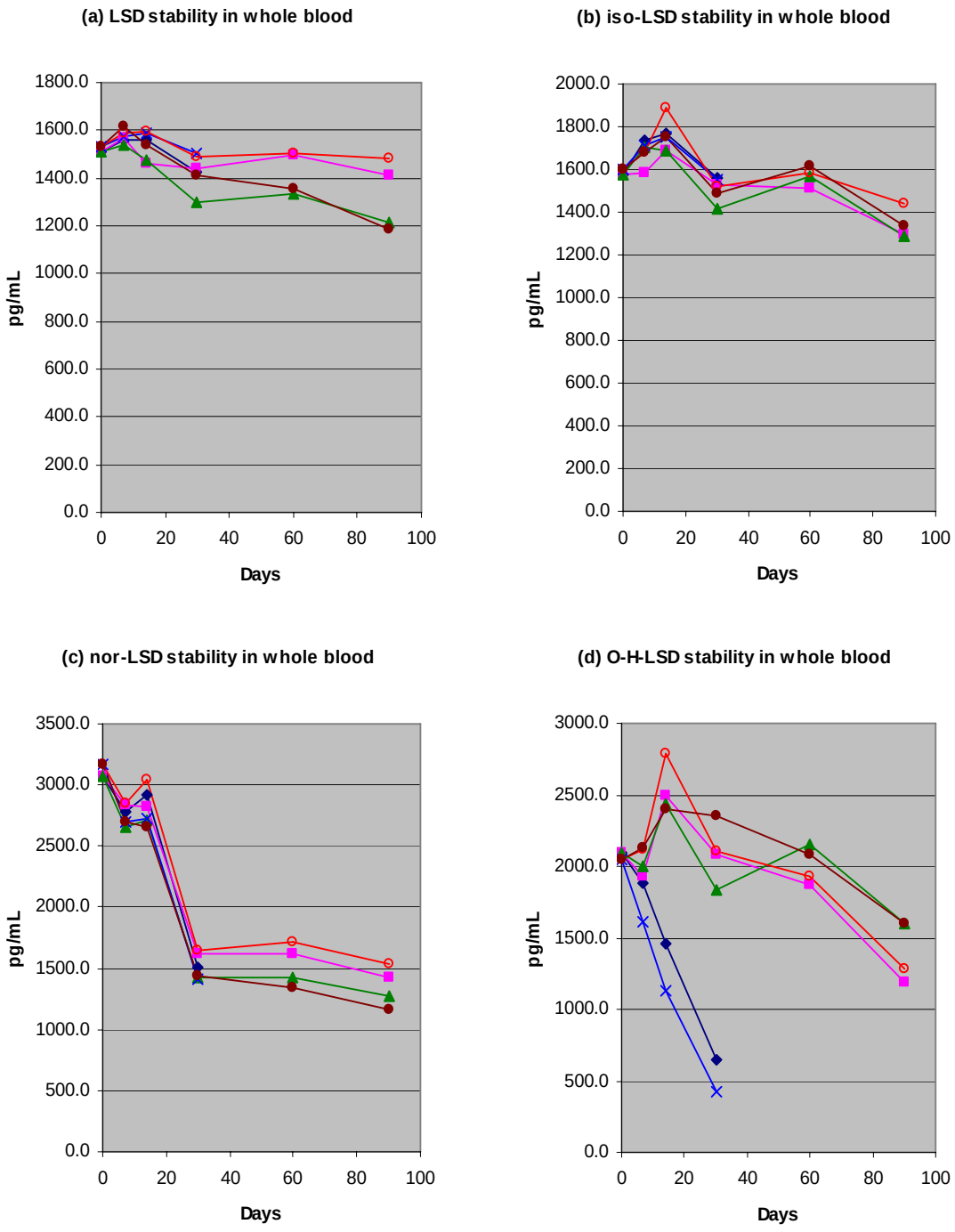


Figure 6.15: Long term stability of (a) LSD, (b) iso-LSD, (c) nor-LSD, and (d) O-H-LSD in whole blood. Samples (unpreserved ♦ room temperature, ■ refrigerated, ▲ frozen, and preserved × room temperature, ○ refrigerated, and ● frozen) were kept at room temperature for 30 days and in the fridge and freezer for 90 days and were analyzed on day 0, 1, 7, 14, 30, 60, and 90.

With respect to normal sample handling and conditions at the RCMP FLS the results presented here showed that urine and whole blood samples suspected of containing LSD, nor-LSD, and O-H-LSD would be stable during the normal sample preparation in the laboratory. Iso-LSD in whole blood was also found to be stable; in urine, however, accurate quantitation may not be possible. Based on the results of this study, it is recommended that samples be frozen for long term storage. LSD in urine and whole blood was stable when maintained at temperatures from -15 to 3°C for 90 days in the dark. Iso-LSD was stable in whole blood when maintained at temperatures from -15 to 3°C for 90 days in the dark. Iso-LSD was unstable in urine samples and therefore accurate quantitation is not possible. Nor-LSD was stable when maintained at temperature from -15°C to room temperature for 14 days in the dark. O-H-LSD was stable when maintained at temperatures from -15 to 3°C for 60 days in whole blood and 90 days in urine in the dark.

6.3.7.3 Freeze-Thaw Stability

LSD, iso-LSD, nor-LSD, and O-H-LSD were all stable after being frozen and thawed over three cycles. This is the first study to assess the freeze-thaw stability of LSD, iso-LSD, nor-LSD, and O-H-LSD. These results are important because it showed that samples submitted for LSD analysis could be shipped on ice and then frozen on receipt. Furthermore, case samples that were stored in the freezer would still be stable if the samples were thawed for LSD reanalysis.

6.3.7.4 Stock Solution Stability

The stability results of the stock solutions prepared in various solvents are shown in Figure 6.16. After 24 hours only LSD stock solution prepared in ACN and MeOH stored at all three temperatures were stable. For long term stability LSD prepared in ACN was most stable for 90 days refrigerated and only for 60 days frozen. After 24 hours only iso-LSD stock solution prepared in ACN stored at all three temperatures was stable. As seen in the urine and whole blood stability tests, a general trend was observed where there was an initial decrease followed by a gradual increase in iso-LSD stock solutions stored refrigerated and frozen. Nor-LSD stock solution prepared in ACN was

most unstable decreasing in concentration by more than 50% after only 24 hours stored at room temperature and refrigerated. This instability of nor-LSD in ACN may be due to possible evaporation, whereas the standard obtained from Cerilliant are sealed so to prevent any evaporation. O-H-LSD stock solution prepared in ACN, water, and pH 4.0 was stable after 24 hours stored at all temperatures. O-H-LSD stock solution prepared in MeOH was also stable after 24 hours, except at room temperature. O-H-LSD was unstable at a high pH of 9.0 decreasing by 23% after 24 hours and by as much as 87% after 30 days.

Since the Cerilliant standards come prepared in ACN, it made most sense to prepare stock standard solutions in ACN as well. For preparing stock standard solutions of LSD, iso-LSD, nor-LSD, and O-H-LSD in ACN, ideally all stock standard solutions should be prepared fresh. This is especially true for nor-LSD, which was found to be unstable in ACN stored refrigerated and at room temperature after 24 hours. LSD, iso-LSD, and O-H-LSD stock solutions prepared in ACN may be stored frozen or refrigerated for 30 days if required.

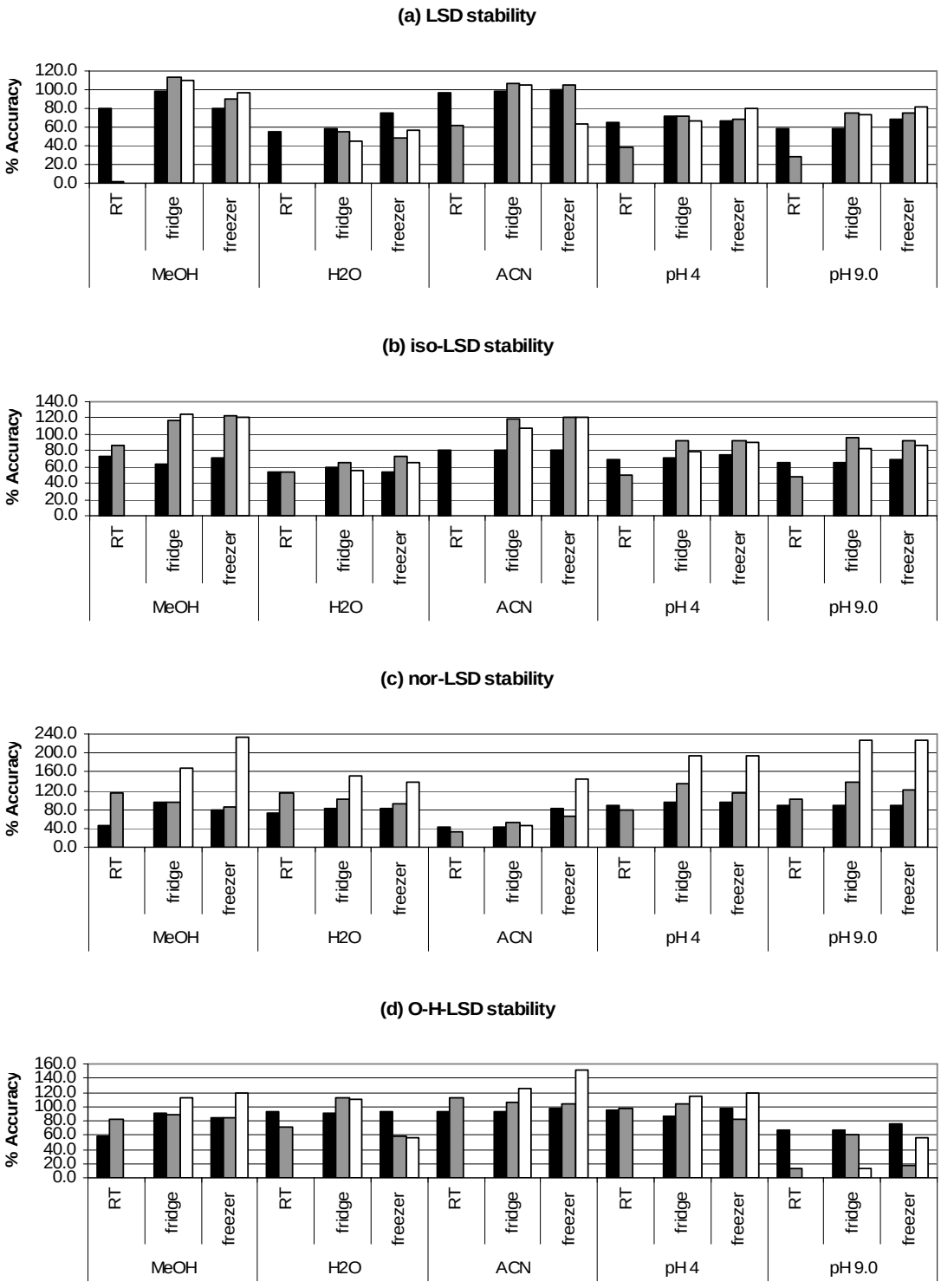


Figure 6.16: Stability of (a) LSD, (b) iso-LSD, (c) nor-LSD, and (d) O-H-LSD stock standards in MeOH, H_2O, ACN, pH 4.0 buffer, and pH 9.0 buffer at room temperature, in the fridge, and freezer analyzed on day ■1, ■ 30, and □ 60.

All stock standards were prepared to contain only that one compound to test for the production of degradation products. On day 0 there were no detectable degradation products identified in any of the standards. The same results were seen after 24 hours. By day 60, there was still no detectable degradation products identified in the nor-LSD and O-H-LSD standards. After 30 days, detectable levels of LSD were identified in the iso-LSD standards prepared in MeOH and pH 9.0 buffer stored at room temperature, and in pH 9.0 buffer stored in the fridge. After 60 days, there were still detectable levels of LSD identified in the iso-LSD standard prepared in MeOH at room temperature, and pH 9.0 buffer stored in the fridge. There were also detectable levels of iso-LSD identified in the LSD standard prepared in pH 9.0 buffer stored at both room temperature and refrigerated, and in MeOH stored refrigerated. There were still detectable levels of iso-LSD in the LSD standard prepared in pH 9.0 buffer stored in the fridge after 60 days. These results are in accordance with other studies that show that LSD will isomerize to iso-LSD and vice versa when exposed to basic aqueous solutions, increased temperatures (Reuschel et al. 1999a; Li et al. 1998), and when dissolved in MeOH (Clarkson et al. 1998).

6.3.7.5 Post Preparative Stability

LSD, iso-LSD, nor-LSD, and O-H-LSD in extracted samples were all stable after being stored at 10°C for 4 and 24 hours. These results showed that the extracted samples were stable while waiting to be analyzed in the autosampler.

6.3.8 Other Qualitative Parameters

6.3.8.1 RT and RRT

Both the RT and the RRT were precise (% CV $\leq$ 4.54%) over three days of analysis in both urine and whole blood (Table 6.12). However, over a period of 2 months of running on the same column the RT and RRT began to shift (Figure 6.17). This shift could be attributed to the cleanliness of the extracts. The RT and RRT precision for the urine samples were better compared to the blood samples. The extracts of the blood samples needed to be filtered to remove the particulate matter that was present, whereas the urine samples did not. The injection of "dirty" extracts may have

plugged the column over time to affect the RT. Others have found it difficult to remove the cellular components of blood sample using LLE alone, as a result others have used a combination of LLE and SPE to obtain "cleaner" blood extracts (Sklerov et al. 2000).

Table 6.12: RT and RRT over three days of analysis in urine and whole blood

		RT (minutes)		RRT (minutes)	
		Mean ± SD	**% CV**	**Mean ± SD**	**% CV**
LSD	Urine	9.38 ± 0.03	0.34	1.00 ± 0.00	1.00
	Blood	8.85 ± 0.26	2.97	1.00 ± 0.00	0.00
Iso-LSD	Urine	10.48 ± 0.04	0.37	1.12 ± 0.00	0.20
	Blood	9.81 ± 0.33	3.33	1.11 ± 0.01	0.50
Nor-LSD	Urine	9.54 ± 0.05	0.56	1.02 ± 0.00	0.20
	Blood	9.03 ± 0.23	2.51	1.02 ± 0.01	0.56
O-H-LSD	Urine	4.15 ± 0.01	0.35	0.44 ± 0.00	0.30
	Blood	3.77 ± 0.17	4.54	0.43 ± 0.01	1.70

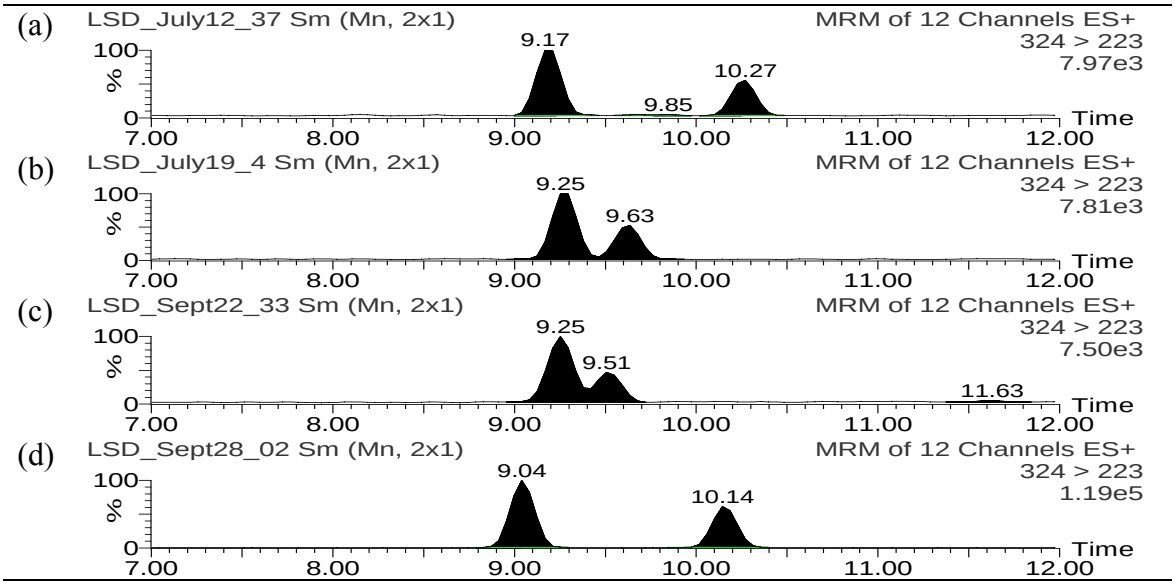


Figure 6.17: Over a 3 month time period the chromatographic separation of LSD (first one to elute) and iso-LSD run on the (a-c) same column shifted and ultimately affected the resolution. The initial RT of LSD and iso-LSD were restored when changing to a (d) new column.

Since the sample batch volume at the RCMP FLS is not expected to be large for LSD analysis, and the within day RT and RRT of quality control and test samples

relative to the calibration standards were accurate (Table 6.13, 6.14, 6.15, and 6.16), the need for "cleaner" extracts may not be necessary. The RT and RRT were deemed acceptable if the quality control and test samples did not differ by more than 2% (% bias) relative to the calibration standards (Rivier 2003). As well, the within day RT and RRT were precise with % CV less than 3.33% and 1.58% for RT and RRT, respectively.

Table 6.13: Within day LSD RT and RRT of calibration standards on 10 different days and the corresponding average accuracy (%bias) of the quality control and test samples

RT				RRT			
Calibration Standards			%bias of QC	Calibration Standards			%bias of QC
Mean	± SD	% CV	and test samples	Mean	± SD	% CV	and test samples
9.24	± 0.02	0.20	0.65	1.00	± 0.00	0.20	-0.04
9.29	± 0.05	0.50	0.05	1.00	± 0.00	0.18	0.12
8.79	± 0.00	0.00	0.28	1.01	± 0.00	0.00	-0.15
8.89	± 0.16	1.75	0.27	1.00	± 0.00	0.24	0.10
9.15	± 0.03	0.29	0.13	1.00	± 0.00	0.19	-0.08
9.12	± 0.08	0.87	-0.20	1.00	± 0.00	0.23	-0.09
9.17	± 0.00	0.00	0.43	1.00	± 0.00	0.00	0.39
8.98	± 0.14	1.51	-0.27	1.00	± 0.00	0.23	0.04
9.08	± 0.00	0.00	0.23	1.00	± 0.00	0.24	0.02
8.98	± 0.07	0.80	0.19	1.00	± 0.00	0.20	0.19

Table 6.14: Within day iso-LSD RT and RRT of calibration standards on 10 different days and the corresponding average accuracy (%bias) of the quality control and test samples

RT				RRT			
Calibration Standards			%bias of QC	Calibration Standards			%bias of QC
Mean	± SD	% CV	and test samples	Mean	± SD	% CV	and test samples
9.30	± 0.00	0.00	0.37	1.08	± 0.00	0.23	-0.32
10.00	± 0.04	0.41	-0.16	1.08	± 0.00	0.03	-0.09
9.63	± 0.00	0.00	0.55	1.10	± 0.00	0.00	0.12
9.81	± 0.17	1.73	0.05	1.11	± 0.00	0.19	-0.12
10.05	± 0.02	0.21	0.15	1.10	± 0.00	0.08	-0.07
10.11	± 0.09	0.89	-0.10	1.11	± 0.00	0.20	0.00
10.15	± 0.02	0.23	0.46	1.11	± 0.00	0.23	0.44
9.96	± 0.15	1.50	-0.31	1.11	± 0.00	0.18	0.01
10.04	± 0.02	0.21	0.04	1.11	± 0.00	0.02	-0.17
10.00	± 0.08	0.76	0.06	1.12	± 0.00	0.16	0.06

Table 6.15: Within day nor-LSD RT and RRT of calibration standards on 10 different days and the corresponding average accuracy (%bias) of the quality control and test samples

RT				RRT			
Calibration Standards			%bias of QC	Calibration Standards			%bias of QC
Mean	± SD	% CV	and test samples	Mean	± SD	% CV	and test samples
9.61	± 0.02	0.23	0.29	1.04	± 0.00	0.01	-0.35
9.65	± 0.04	0.39	-0.09	1.04	± 0.00	0.11	-0.00
9.04	± 0.00	0.00	0.51	1.03	± 0.00	0.00	0.11
9.13	± 0.16	1.74	0.05	1.03	± 0.00	0.22	-0.00
9.40	± 0.02	0.23	0.13	1.03	± 0.00	0.07	-0.12
9.31	± 0.07	0.79	-0.16	1.02	± 0.00	0.15	-0.02
9.38	± 0.00	0.00	0.32	1.02	± 0.00	0.00	0.32
9.17	± 0.12	1.35	-0.27	1.02	± 0.00	0.21	0.12
9.32	± 0.03	0.29	0.11	1.03	± 0.00	0.05	-0.09
9.18	± 0.06	0.67	-0.02	1.02	± 0.00	0.14	0.07

Table 6.16: Within day O-H-LSD RT and RRT of calibration standards on 10 different days and the corresponding average accuracy (%bias) of the quality control and test samples

RT				RRT			
Calibration Standards			%bias of QC	Calibration Standards			%bias of QC
Mean	± SD	% CV	and test samples	Mean	± SD	% CV	and test samples
3.91	± 0.02	0.52	0.70	0.42	± 0.00	0.47	0.06
3.98	± 0.02	0.56	-0.28	0.43	± 0.00	0.50	-0.20
3.68	± 0.02	0.44	0.10	0.42	± 0.00	0.44	-0.29
3.77	± 0.13	3.33	1.01	0.42	± 0.01	1.58	0.97
3.90	± 0.04	0.94	0.46	0.43	± 0.00	0.60	0.24
3.97	± 0.04	1.02	-0.18	0.44	± 0.00	0.63	0.00
3.95	± 0.00	0.00	1.78	0.43	± 0.00	0.00	1.77
3.83	± 0.07	1.93	0.20	0.43	± 0.00	0.76	0.53
3.89	± 0.02	0.42	1.97	0.43	± 0.00	0.38	1.76
4.04	± 0.03	0.86	0.03	0.42	± 0.00	0.33	-0.18

6.3.8.2 Ion Ratio

MRM ion ratios in urine were consistent within day, but when ion ratios between days were averaged the precision worsened (Table 6.17). The ion ratios were consistent within and between days for all compounds in whole blood (Table 6.18). Another study found that short term intra-laboratory reproducibility of LC/MS mass spectra was satisfactory, with small differences in ion abundances occurring sporadically (Bogusz et al. 1999).

Table 6.17: Intra- and inter-day ion ratio over three days of analysis in urine

	Day 1		Day 2		Day 3		Average	
	Mean ± SD	% CV	Mean ± SD	% CV	Mean ± SD	% CV	Mean ± SD	% CV
LSD	2.1±0.1	5.8	3.1±0.2	5.3	2.9±0.1	4.9	2.7±0.5	17.9
Iso-LSD	0.7±0.1	7.6	1.1±0.1	4.3	1.1±0.1	5.1	0.9±0.2	22.3
Nor-LSD	5.5±0.2	4.4	4.7±0.2	4.3	4.4±0.4	8.9	4.9±0.5	11.2
O-H-LSD	3.2±0.2	6.5	4.6±0.6	13.6	4.8±0.5	9.6	4.1±0.8	20.1

Table 6.18: Intra- and inter-day ion ratio over three days of analysis in whole blood

	Day 1		Day 2		Day 3		Average	
	Mean ± SD	% CV	Mean ± SD	% CV	Mean ± SD	% CV	Mean ± SD	% CV
LSD	3.3±0.2	6.3	3.3±0.2	4.8	3.4±0.2	7.0	3.2±0.2	6.2
Iso-LSD	1.1±0.1	4.6	1.1±0.1	5.3	1.1±0.1	5.7	1.1±0.1	5.5
Nor-LSD	4.6±0.4	7.8	4.7±0.3	6.9	4.5±0.6	12.2	4.6±0.4	9.2
O-H-LSD	4.6±0.4	9.6	4.8±0.4	7.2	4.4±0.6	14.1	4.6±0.5	11.0

The ion ratios were also found to be concentration dependent. The precision at the low concentration (50 pg/mL) was less compared to the higher concentration (1500 pg/mL) (Table 6.19), even more so for O-H-LSD. Since it was demonstrated that the ion ratios were concentration dependent, a ±30% bias was chosen for the acceptance criteria (SOFT/AAFS 2006). Another study using a ±20% bias acceptance criteria for the ion ratios of O-H-LSD in urine found that 45.3% of the samples tested were outside the range relative to the calibrators (Poch et al. 2000).

Table 6.19: Precision of ion ratios at different concentrations in urine and whole blood

		50 pg/mL % CV	500 pg/mL % CV	1500 pg/mL % CV
LSD	Urine	7.4	5.7	2.4
	Blood	9.3	3.1	2.2
Iso-LSD	Urine	4.8	5.2	0.0
	Blood	7.0	3.7	0.0
Nor-LSD	Urine	6.3	2.2	3.5
	Blood	12.8	3.7	2.4
O-H-LSD	Urine	20.4	6.9	6.0
	Blood	16.6	3.0	2.7

The variation in the between day ion ratios could be explained by the instrumentation. API sources generally consists of five parts; (1) the device where the liquid is introduced, (2) atmospheric pressure ion source region, (3) ion sampling aperture, (4) atmospheric pressure to vacuum region, and (5) an ion optical system (Niessen 1998), and any variability of these five parts could effect the ionization and fragmentation of the compound. If needed, a tuning compound could be used to standardize the instrumentation each day. However, since the within day ion ratios of the calibration standards were found to be precise, and the ion ratios of the quality control and test samples were found to be accurate (±30 %bias) relative to the mean ion ratio of the calibration standards (Table 6.20, 6.21, 6.22, and 6.23), a tuning compound was not necessary. The results presented here showed that ion ratios were a reliable parameter for supporting the confirmation of the compounds when using a ±30% bias for the ion ratios of quality control and test samples relative to the calibration standards.

Table 6.20: Within day LSD ion ratios of calibration standards on 10 different days and the corresponding average accuracy (%bias) of the quality control and test samples

Calibration Standards Ion ratio			**Average %bias of QC**
Mean	**± SD**	**% CV**	**and test samples**
1.95	± 0.10	4.98	-3.2
1.91	± 0.14	7.09	0.2
1.93	± 0.12	6.33	-0.6
1.93	± 0.17	8.67	-5.8
1.95	± 0.08	4.09	0.6
1.89	± 0.16	8.70	1.6
2.01	± 0.16	7.79	-2.3
1.90	± 0.11	5.63	-4.0
2.05	± 0.12	6.06	-2.8
2.08	± 0.18	8.83	-1.8

Table 6.21: Within day iso-LSD ion ratios of calibration standards on 10 different days and the corresponding average accuracy (%bias) of the quality control and test samples

Calibration Standards Ion ratio			Average %bias of QC and test samples
Mean	± SD	% CV	
0.79	± 0.03	3.81	-3.0
0.74	± 0.05	7.02	7.5
0.78	± 0.07	8.57	-4.7
0.76	± 0.05	7.06	0.7
0.78	± 0.06	8.02	-4.1
0.78	± 0.04	5.21	-1.6
0.79	± 0.05	6.50	-1.4
0.76	± 0.05	6.79	0.6
0.81	± 0.08	9.81	-1.0
0.83	± 0.05	5.61	-2.6

Table 6.22: Within day nor-LSD ion ratios of calibration standards on 10 different days and the corresponding average accuracy (%bias) of the quality control and test samples

Calibration Standards Ion ratio			Average %bias of QC and test samples
Mean	± SD	% CV	
4.16	± 0.32	7.70	8.4
4.58	± 0.44	9.70	-2.3
4.60	± 0.60	13.15	-4.5
4.57	± 0.51	11.14	-4.8
4.59	± 0.27	5.94	-3.5
4.45	± 0.55	12.37	0.9
4.48	± 0.39	8.79	-1.1
4.52	± 0.15	3.26	-2.6
4.24	± 0.54	12.73	-0.3
3.73	± 0.45	12.18	10.0

Table 6.23: Within day O-H-LSD ion ratios of calibration standards on 10 different days and the corresponding average accuracy (%bias) of the quality control and test samples

Calibration Standards Ion ratio Mean ± SD	% CV	Average %bias of QC and test samples
4.74 ± 0.38	8.05	-4.4
4.35 ± 0.56	12.82	-15.8
4.76 ± 0.58	12.08	-1.1
4.35 ± 0.56	12.82	-5.0
4.59 ± 0.34	7.43	2.8
4.10 ± 0.60	14.63	-4.4
4.30 ± 0.47	10.96	6.9
4.22 ± 0.71	16.93	-12.3
4.62 ± 0.44	9.62	1.7
4.82 ± 0.73	15.17	-10.7

6.3.9 Existing Extraction Methods at RCMP FLS (nBuCl and Toxi-Lab A)

The nBuCl extraction method for whole blood was not suitable for simultaneous extraction of LSD, iso-LSD, nor-LSD, and O-H-LSD, because it was unable to extract O-H-LSD (Figure 6.18).

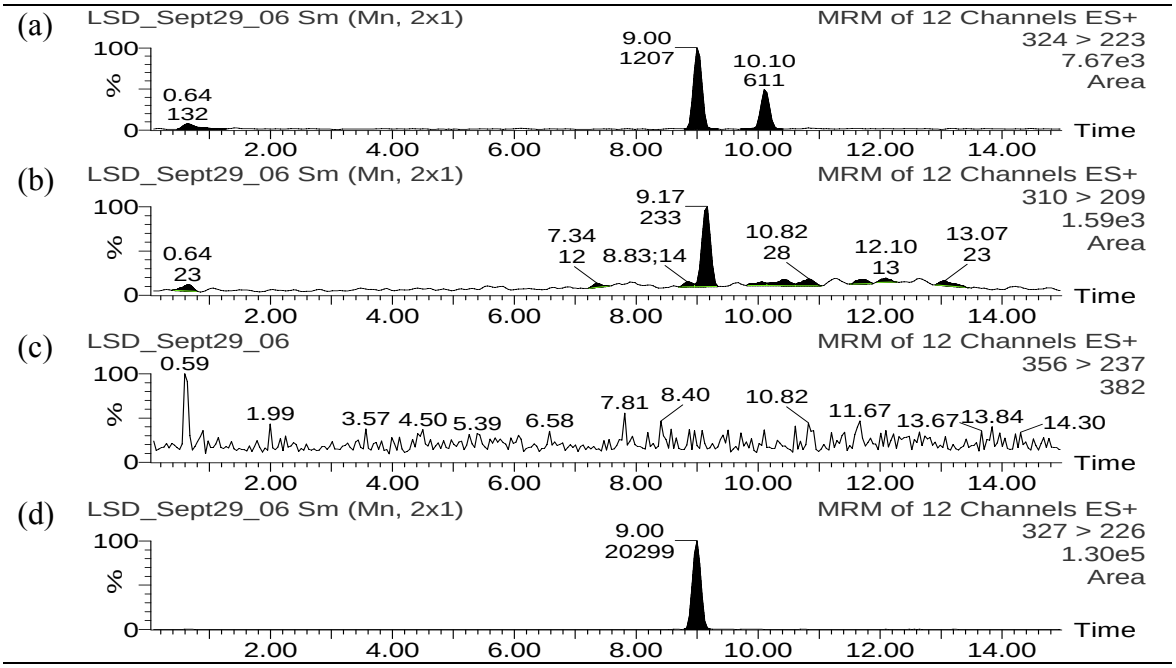


Figure 6.18: MRM channels for 50 pg/mL (a) LSD (RT = 9.00 minutes), iso-LSD (RT = 10.10 minutes), (b) nor-LSD (RT = 9.17 minutes) , (c) O-H-LSD (not present), and (d) LSD-D3 in spiked blood extracted using the nBuCl method.

As well, the percent recovery of the remaining compounds were inconsistent between the low (50 pg/mL) and high concentration (1500 pg/mL) (Table 6.24). Furthermore, nBuCl provided optimal extraction of LSD and iso-LSD with a % recovery of 86 and 80%, respectively, over nor-LSD with % recovery of 66%.

Table 6.24: Percent recovery in whole blood for LSD, iso-LSD, nor-LSD, and O-H-LSD using the nBuCl extraction method

	50 pg/mL	1500 pg/mL	Average
LSD	93	79	86
iso-LSD	84	77	80
nor-LSD	70	61	66
O-H-LSD	-	-	-

When urine was extracted using the Toxi-Lab A method, all compounds were extracted with similar % recoveries (Table 6.25 and Figure 6.19). Like the nBuCl extraction method, the Toxi-Lab A resulted in inconsistent % recoveries between the low (50 pg/mL) and high concentration (1500 pg/mL). The precision of the % recoveries of nor-LSD and O-H-LSD at 50 pg/mL were 22.9 and 21.8% CV, respectively, which was less precise than the dichloromethane/isopropyl alcohol extraction method with 8.7 and 11.4% CV for nor-LSD and O-H-LSD, respectively.

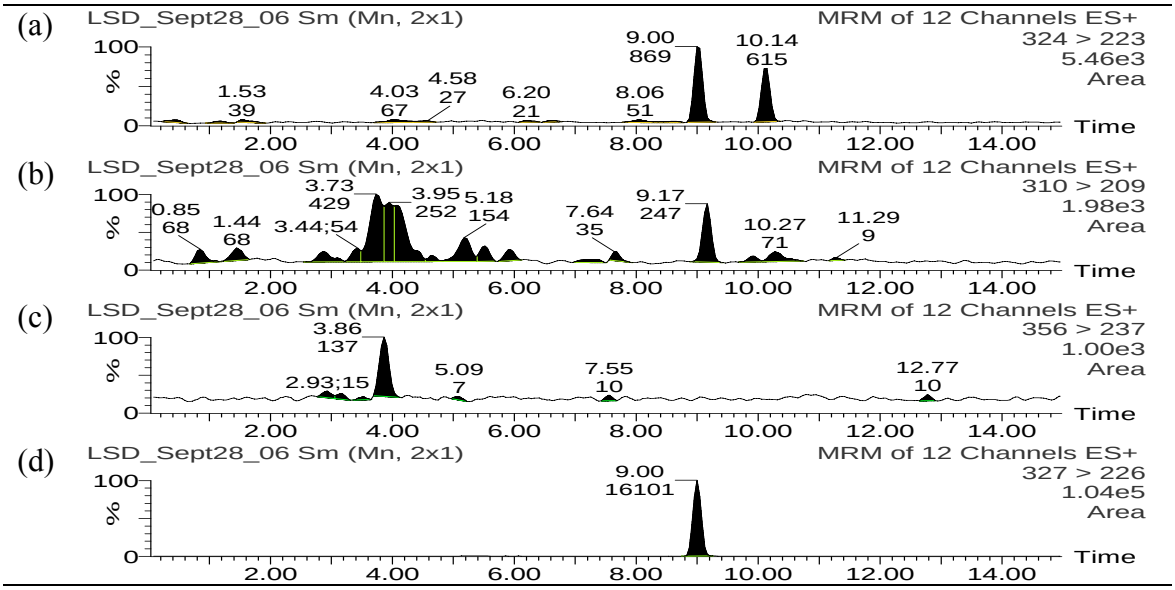


Figure 6.19: MRM channels for 50 pg/mL (a) LSD (RT = 9.00 minutes), iso-LSD (RT = 10.14 minutes), (b) nor-LSD (RT = 9.17 minutes) , (c) O-H-LSD (RT = 3.86 minutes), and (d) LSD-D3 in spiked urine extracted using the Toxi-Lab A method.

Table 6.25: Percent recovery in urine for LSD, iso-LSD, nor-LSD, and O-H-LSD using the Toxi-Lab A extraction method

	50 pg/mL	1500 pg/mL	Average
LSD	86	77	81
iso-LSD	75	83	79
nor-LSD	63	80	72
O-H-LSD	74	74	74

Both the nBuCl and Toxi-Lab A extraction methods resulted in slightly different ME (Table 6.26) compared to each other and to the dichloromethane/isopropyl alcohol extraction method. The nBuCl resulted in different ME at low and high concentrations for LSD and iso-LSD with ionization suppression at 50 pg/mL and ion enhancement at 1500 pg/mL. The Toxi-Lab A suppressed ionization in LSD 10% more compared to the dichloromethane/isopropyl alcohol method (91% versus 81% ME). Overall, the nBuCl method resulted in more ion enhancement, and the Toxi-Lab A resulted in more ion suppression compared to the dichloromethane/isopropyl alcohol extraction method.

Table 6.26: nBuCl and Toxi-Lab A matrix effect

		50 pg/mL	1500 pg/mL	Average
LSD	nBuCl	92	102	97
	Toxi-Lab A	79	83	81
iso-LSD	nBuCl	97	113	105
	Toxi-Lab A	90	102	96
nor-LSD	nBuCl	110	110	110
	Toxi-Lab A	107	103	105
O-H-LSD	nBuCl	92	107	99
	Toxi-Lab A	94	95	94
LSD-D3	nBuCl	104	105	104
	Toxi-Lab A	86	84	85

Since LSD is found in such small concentrations compared to other commonly encountered drugs of abuse, an extraction method specific for LSD and its metabolites is optimal. Using extraction methods that already exist may be beneficial for efficiency. With case samples there is also the possibility of encountering an insufficient volume of sample to analyze for LSD after completing screening tests, so to be able to use an already extracted sample used on other instruments may also be useful. During the 8 months of method validation a few positive LSD ELISA cases were found to be false

positives using the UPLC™/MS/MS method. However, in these cases the nBuCl and Toxi-Lab A extraction methods were used. The effect of these two extraction methods on the selectivity and sensitivity were not tested and therefore the extraction methods could have affected the results. However, in most cases of LSD use, the case history alone makes the investigators suspect LSD. In the cases tested to date, the case history gave no indication of behaviour related to hallucinogens. To summarize, use of the nBuCl extraction method for accurate quantitation is not recommended. For confirmation, the Toxi-Lab A and nBuCl could be used as alternative extraction methods, but they are not ideal and should be avoided if possible.

6.4 Conclusion

An accurate, precise, selective, and sensitive UPLC™/MS/MS method was validated to identify, confirm, and quantitate LSD, iso-LSD, nor-LSD, and O-H-LSD in urine and whole blood. Based on the stability results, accurate quantitation is only possible for all compounds in whole blood samples if stored frozen or refrigerated for up to 2 weeks. Accurate quantitation of iso-LSD in urine samples is not possible, but LSD, nor-LSD, and O-H-LSD in urine samples may be accurately quantitated if stored frozen or refrigerated for 2 weeks. For samples stored 14 to 60 days frozen or refrigerated only LSD and O-H-LSD can be accurately quantitated. Although quantitative analysis is commonly performed on whole blood specimens, for LSD the qualitative analysis may be more important. Instead the stability studies should serve more as a guideline to prevent loss of LSD due to improper storage and handling. The one-step LLE will allow simple confirmation of urine and whole blood samples testing positive with ELISA at the positive cutoff of 1 ng/mL for urine and 0.5 ng/mL for whole blood. Furthermore, the simultaneous analysis of LSD, iso-LSD, nor-LSD, and O-H-LSD provides a superior interpretation versus analyzing LSD alone.

To the Author's knowledge, there are no known published validated methods for the simultaneous confirmatory and quantitative analysis of LSD, iso-LSD, nor-LSD, and O-H-LSD in both urine and blood. Furthermore, compared to other published methods, the LOD and LLOQ obtained in this study for O-H-LSD and nor-LSD in both whole blood and urine are among the lowest.

Chapter 7

UPLC™/MS/MS Analysis of LSD, iso-LSD, nor-LSD, and O-H-LSD in Blind Samples and a Case Sample

7.1 Introduction

As previously mentioned, the purpose of validating a method is to provide an assurance of reliability during normal use, and to provide evidence that the method works (Shabir 2003). All the validation parameters tested in Chapter 6 were conducted using blanks spiked with known amounts of standard. To remove the bias associated with the spiked samples, eight blind samples prepared by another laboratory (McKay, Pharmalytics Inc, Saskatoon, SK), were tested. Additionally, the blind samples were analyzed by two different analysts to demonstrate the reproducibility of the method. To provide further evidence that the method works, a case which screened positive for LSD previously with RIA was tested.

7.2 Blind Samples

Proficiency testing involves analyzing simulated case samples, and evaluating the results to determine the quality of the performance of the method (Burtis and Ashwood 1997). Simulated case samples were provided in the form of blind samples, which were samples that the analysts had no knowledge of the identity of the compounds as well as their concentration levels. The blind sample results obtained by the analysts were then compared to the actual results to test the reliability of the method. Eight blind samples (4 urines and 4 whole bloods) prepared by another laboratory were supplied. Samples were shipped on ice and placed in the freezer on receipt. On the day of analysis, samples were thawed at room temperature. The samples were prepared and extracted in duplicate, with calibration standards (Appendix A, B, and C). The analysis was conducted by two different analysts on different days.

7.2.1 UPLC™/MS/MS Results

A 5 (O-H-LSD and nor-LSD) or 6 (LSD and iso-LSD) point calibration curve was constructed to quantitate the blood samples. A 3 (O-H-LSD and nor-LSD) or 4 (LSD and iso-LSD) point calibration curve was constructed to quantitate the urine sample. The r and r^2 of all calibration curves were greater than 0.99. Quality control samples (0.50 ng/mL) were run in duplicate and were within acceptable limits. The MRM ion chromatograms of the blind samples are shown in Figures 7.1 to 7.10.

RT, RRT, and ion ratios were used for confirmation. For confirmation of each compound, all ions for each compound (LSD = MRM 324>223, 208, 281, iso-LSD = MRM 324>223, 208, 281, nor-LSD = MRM 310>209, 237, 74, O-H-LSD = MRM 356>237, 222, 313) were present in the blinds and quality control sample at the same RT (±2%) as the calibration standards. The ion ratios were also within ±30% of the calibration standards (LSD = 223/208, 223/281, iso-LSD = 281/208, 281/223, nor-LSD = 209/237, 209/74, O-H-LSD = 237/222, 237/313). The ISTD was also found and the RT, ions (MRM 327>226, 208, 281), and ion ratios (226/208, 226/281) were within range. The measured results obtained with the UPLC™/MS/MS method are summarized in Table 7.1. In order for a compound to be confirmed, all three identification criteria (RT, RRT, and ion ratios) must be met. None detected means that the level was below the LOD or one or more of the identification criteria failed.

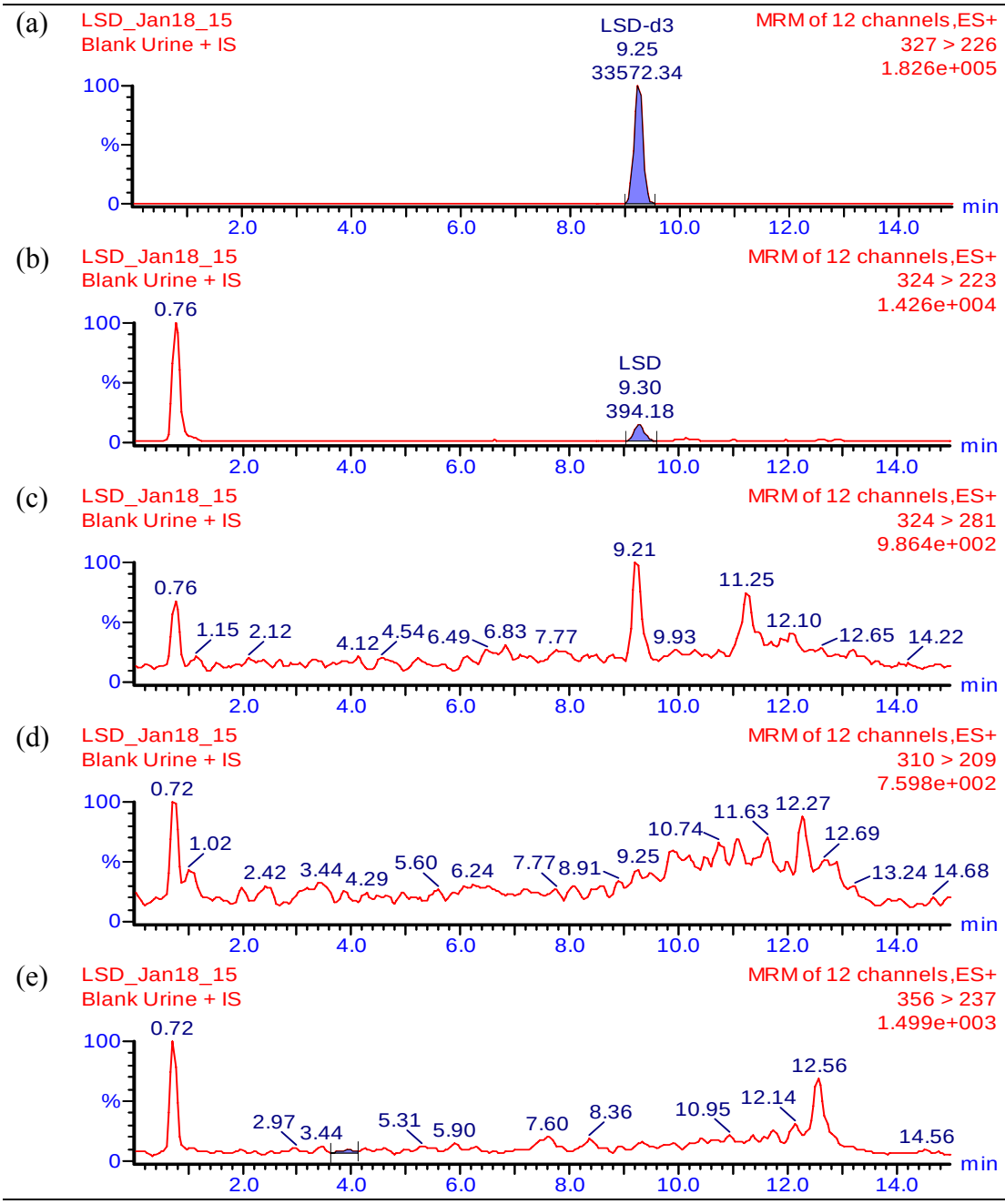


Figure 7.1: MRM acquisitions of blank urine + ISTD (LSD-D3) for (a) LSD-D3, (b) LSD, (c) iso-LSD, (d) nor-LSD, and (e) O-H-LSD. The response of the blank is acceptable since at the LLOQ was five times the response compared to the blank.

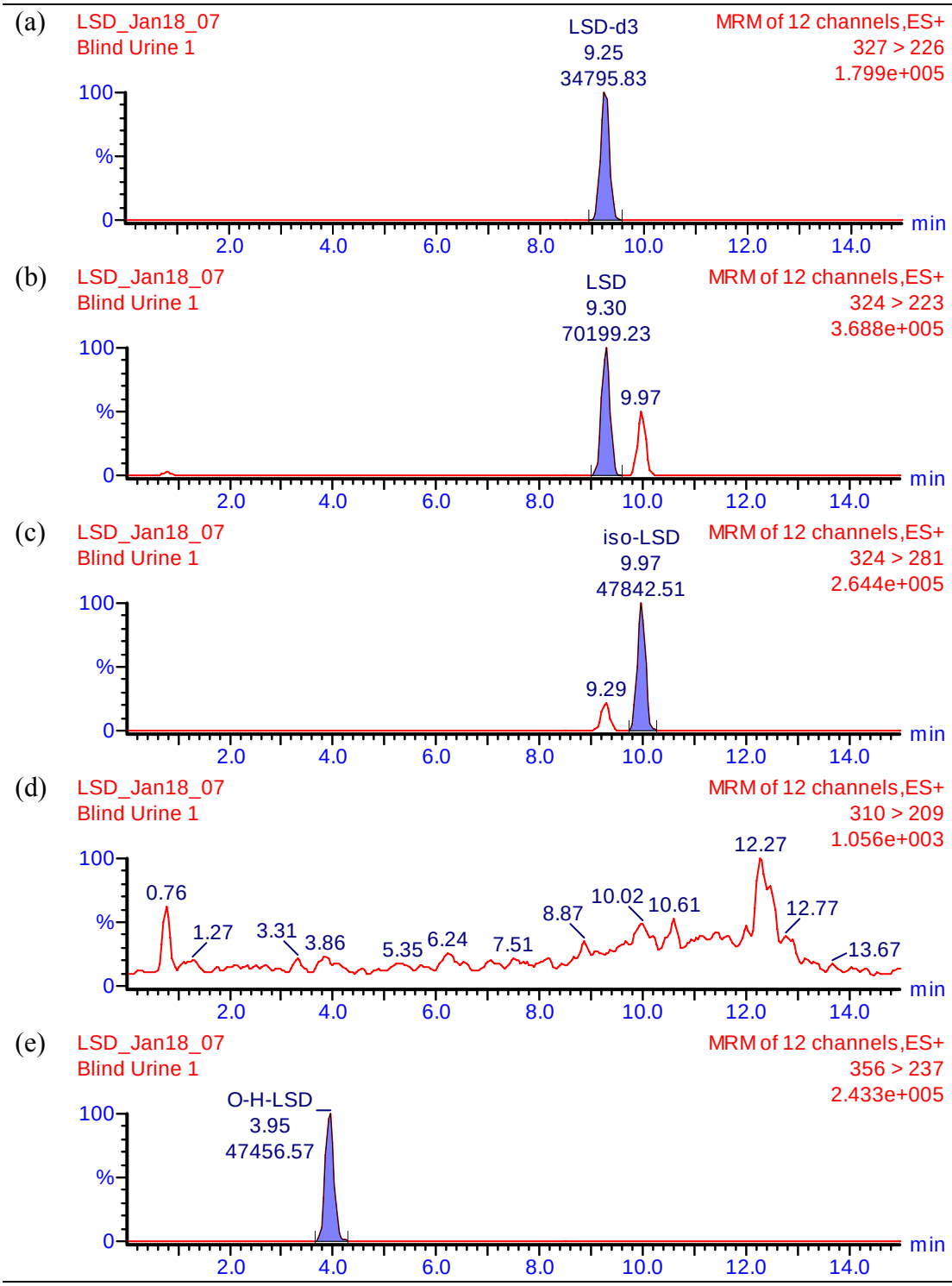


Figure 7.2: MRM acquisitions of extracted blind urine sample #1 for (a) LSD-D3, (b) LSD, (c) iso-LSD, (d) nor-LSD, and (e) O-H-LSD. Refer to Figure 7.1 for blank results.

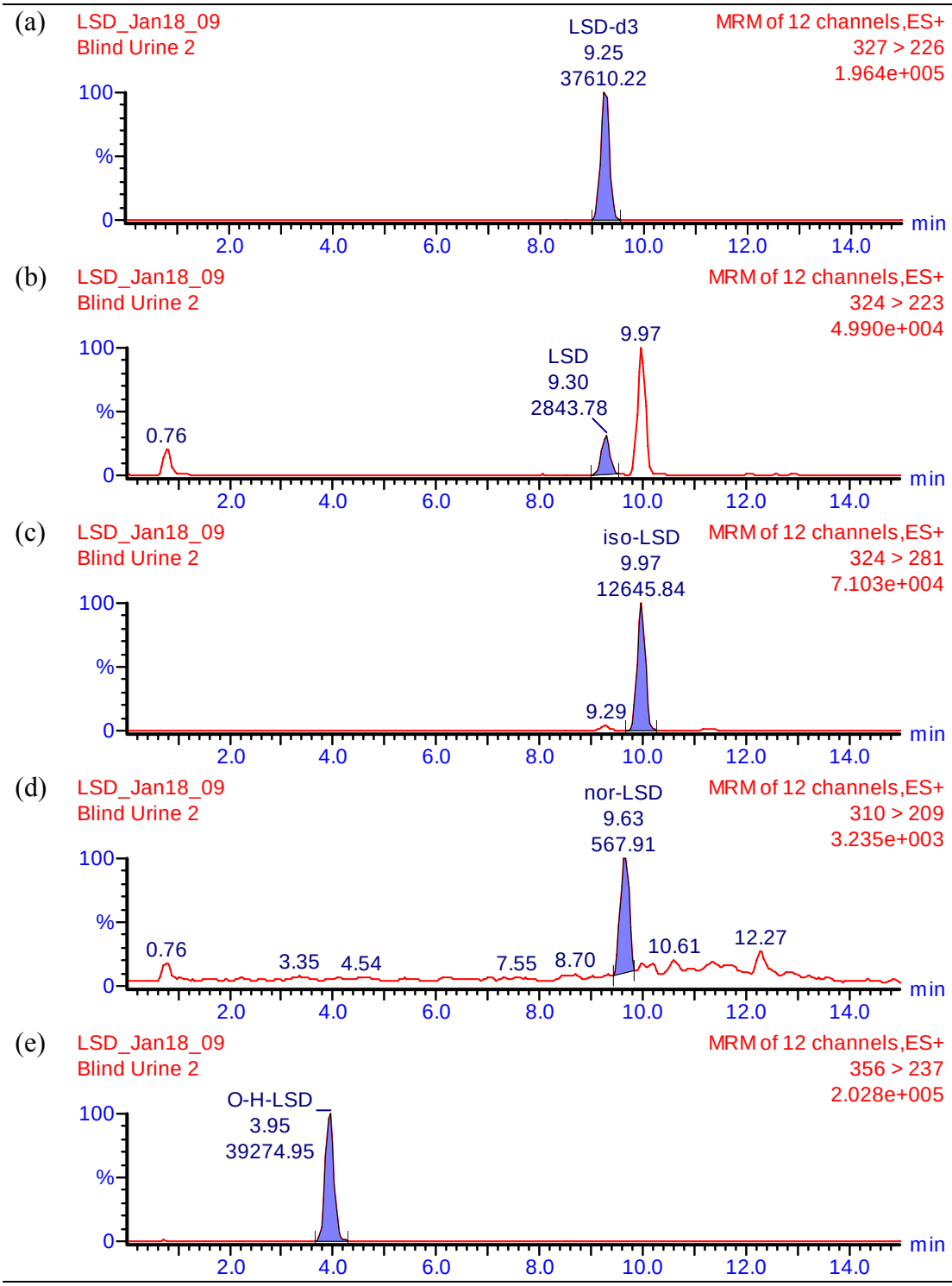


Figure 7.3: MRM acquisitions of extracted blind urine sample #2 for (a) LSD-D3, (b) LSD, (c) iso-LSD, (d) nor-LSD, and (e) O-H-LSD. Refer to Figure 7.1 for blank results.

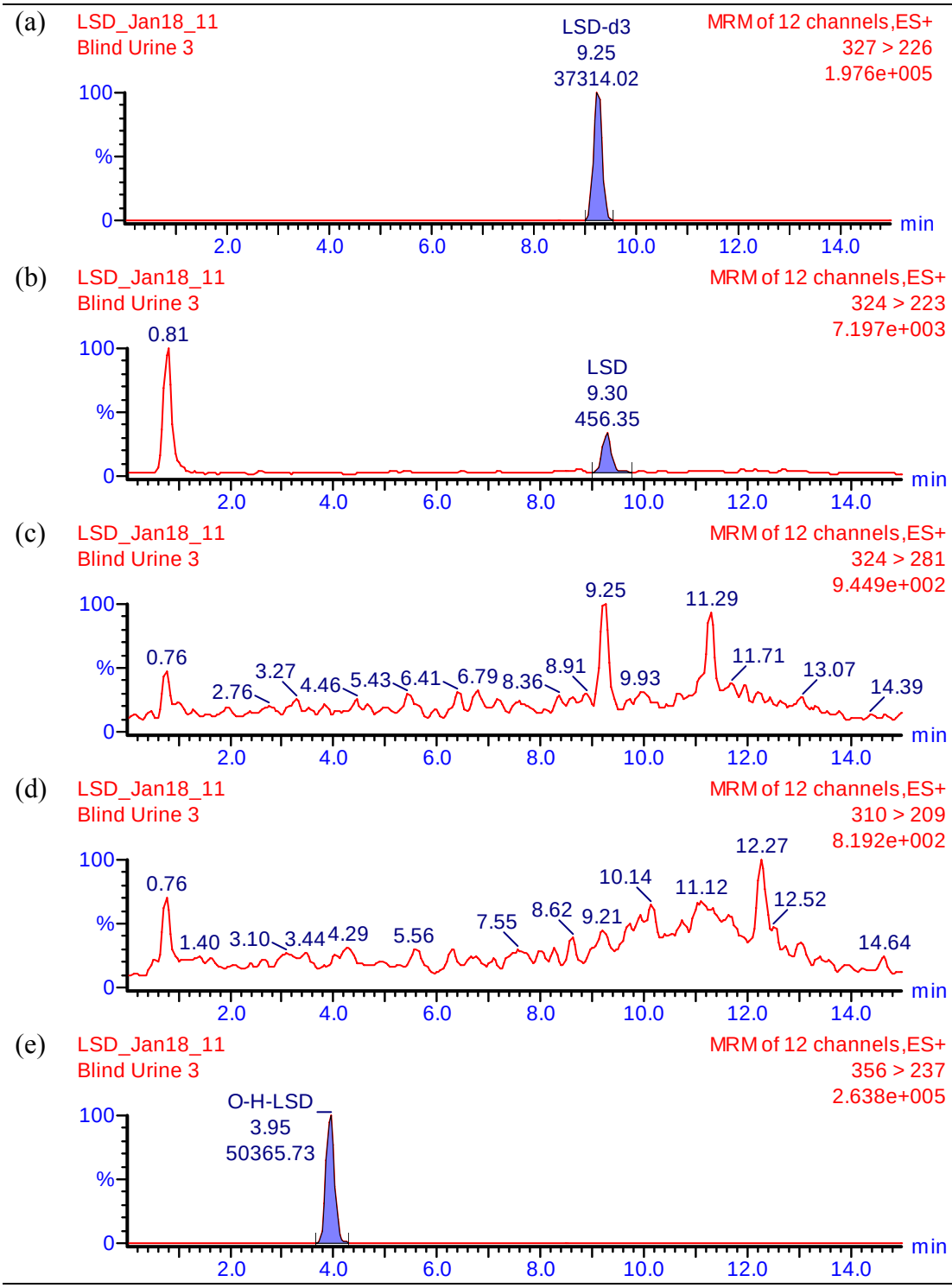


Figure 7.4: MRM acquisitions of extracted blind urine sample #3 for (a) LSD-D3, (b) LSD, (c) iso-LSD, (d) nor-LSD, and (e) O-H-LSD. Refer to Figure 7.1 for blank results.

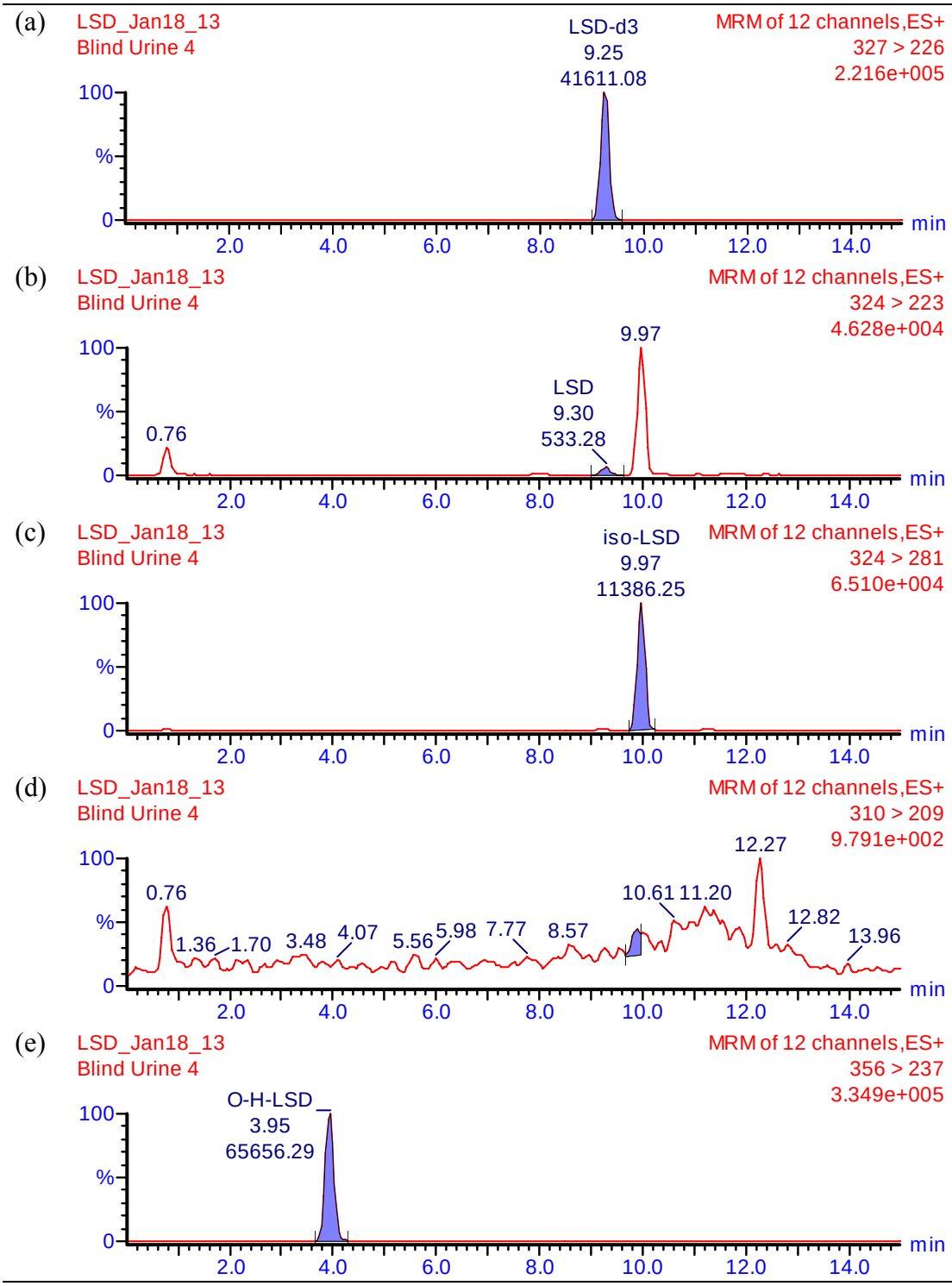


Figure 7.5: MRM acquisitions of extracted blind urine sample #4 for (a) LSD-D3, (b) LSD, (c) iso-LSD, (d) nor-LSD, and (e) O-H-LSD. Refer to Figure 7.1 for blank results.

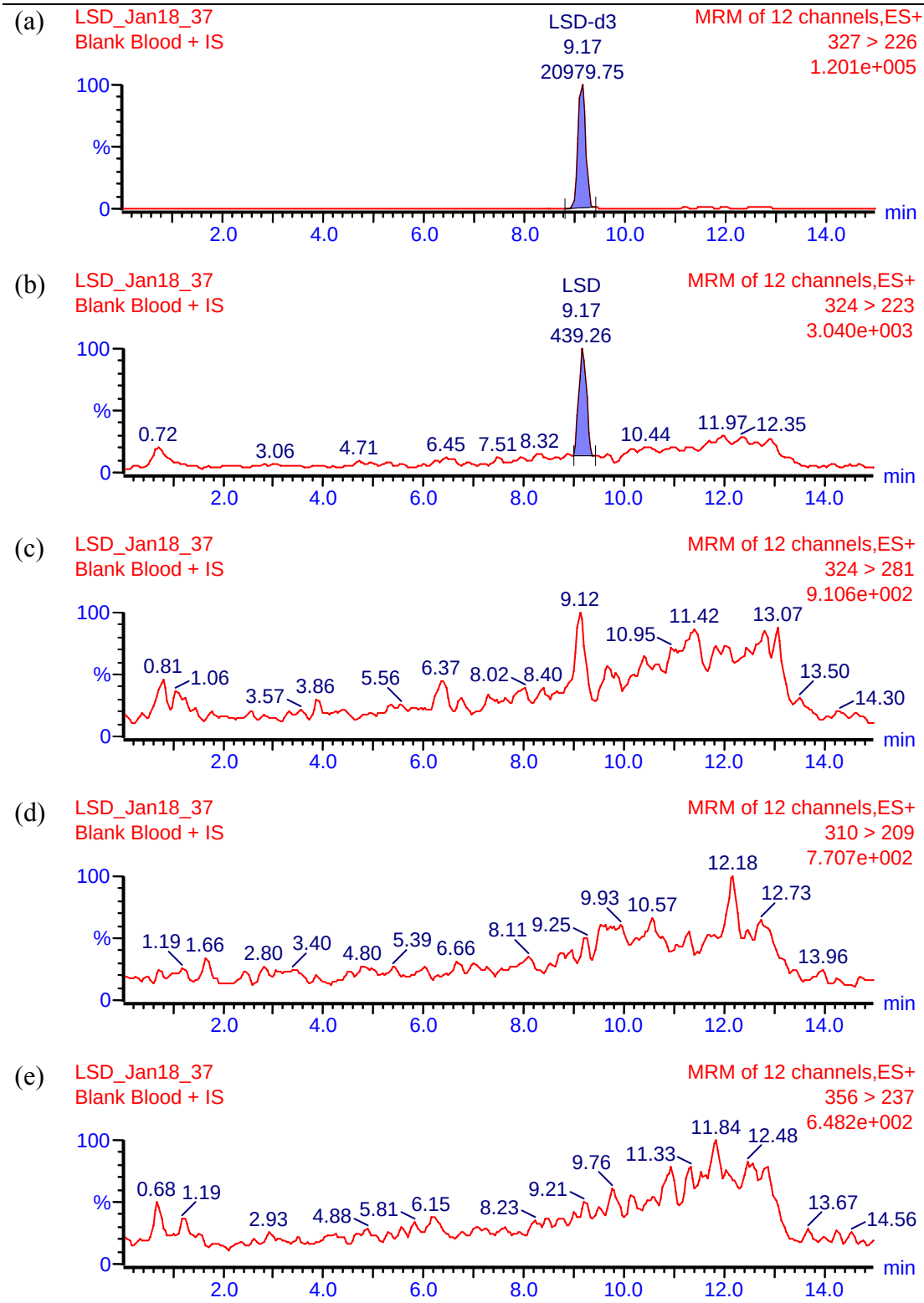


Figure 7.6: MRM acquisitions of blank whole blood + ISTD (LSD-D3) for (a) LSD-D3, (b) LSD, (c) iso-LSD, (d) nor-LSD, and (e) O-H-LSD. The response of the blank is acceptable since at the LLOQ was five times the response compared to the blank.

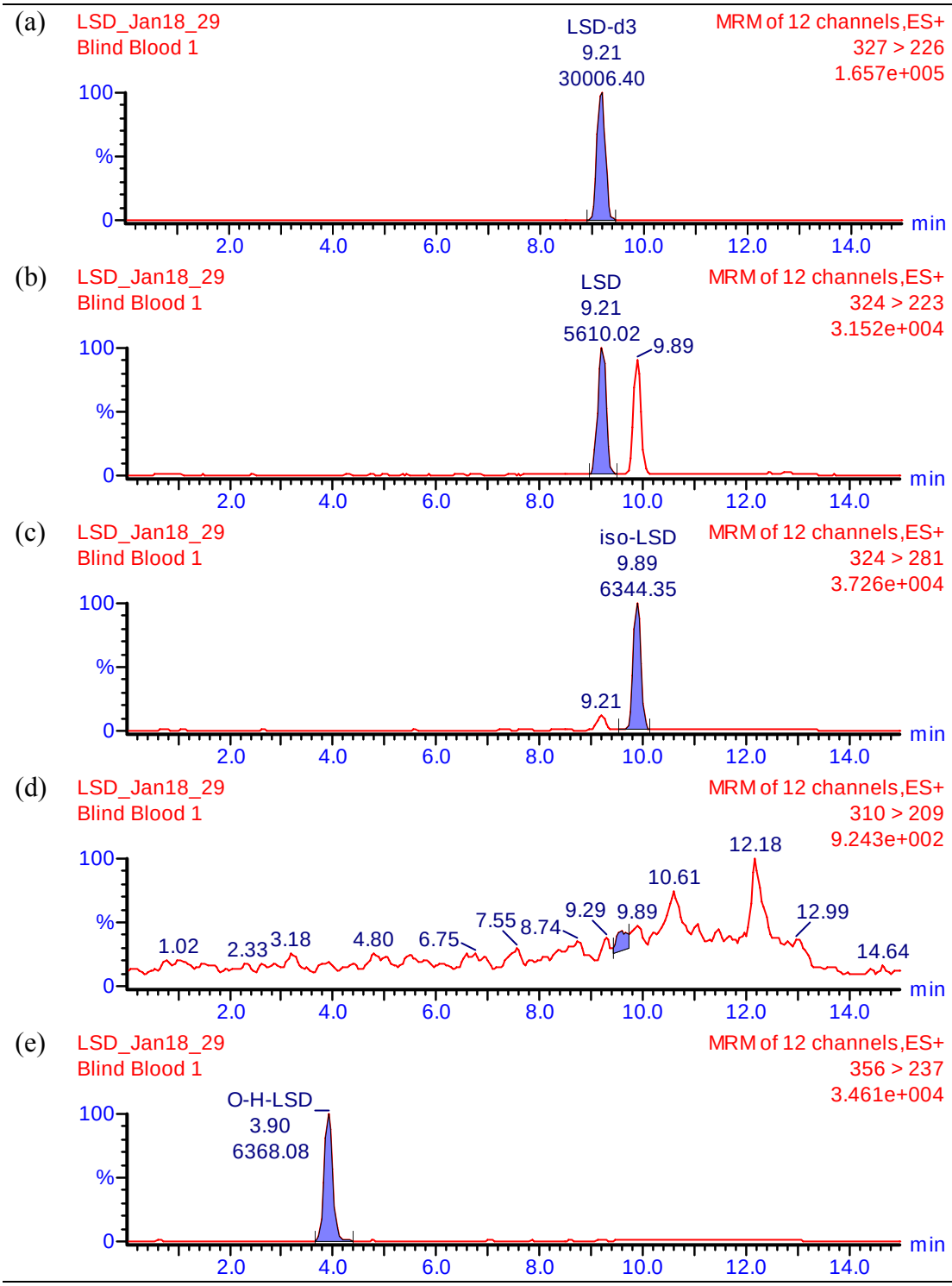


Figure 7.7: MRM acquisitions of extracted blind whole blood sample #1 for (a) LSD-D3, (b) LSD, (c) iso-LSD, (d) nor-LSD, and (e) O-H-LSD. Refer to Figure 7.6 for blank results.

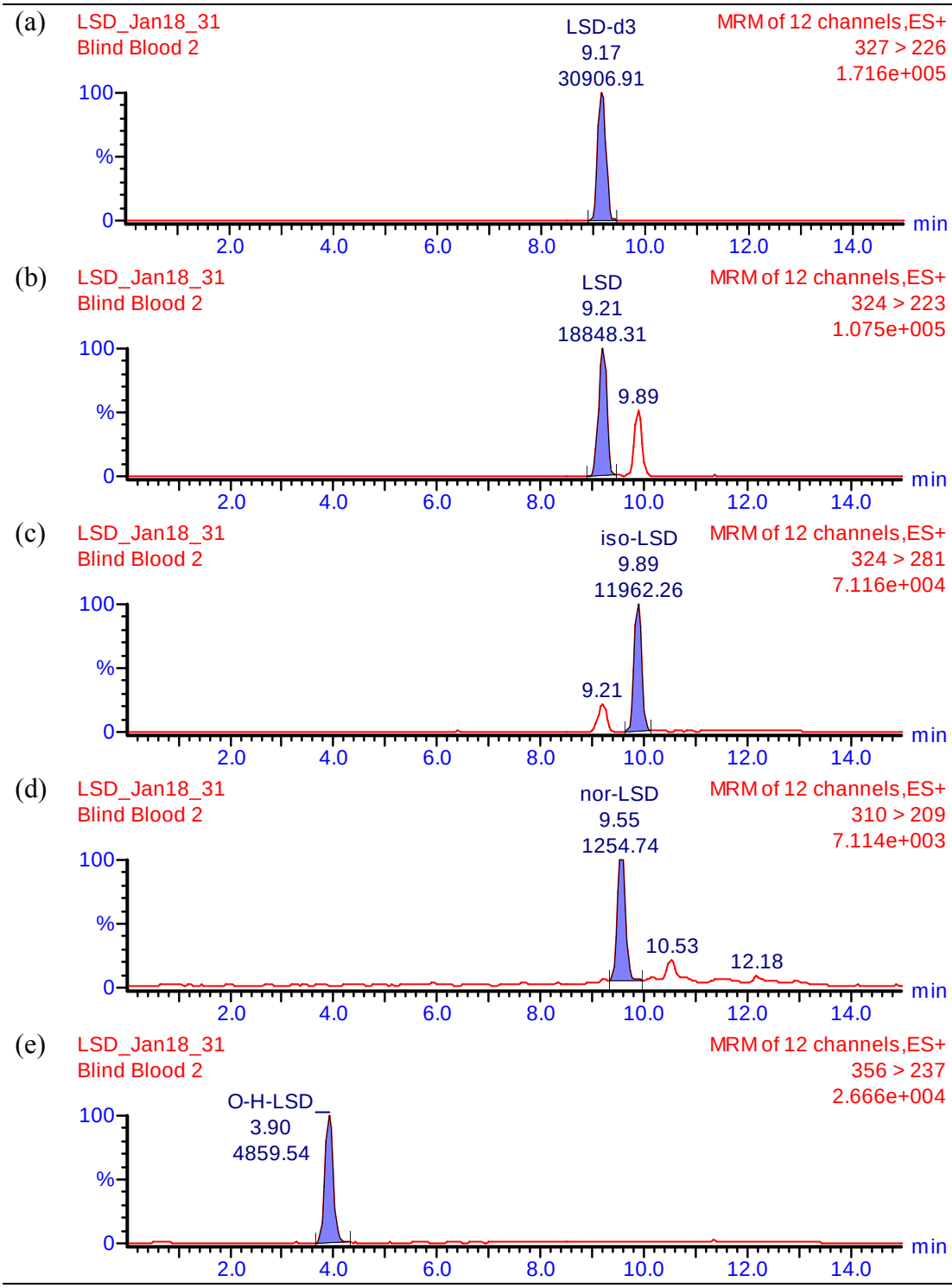


Figure 7.8: MRM acquisitions of extracted blind whole blood sample #2 for (a) LSD-D3, (b) LSD, (c) iso-LSD, (d) nor-LSD, and (e) O-H-LSD. Refer to Figure 7.6 for blank results.

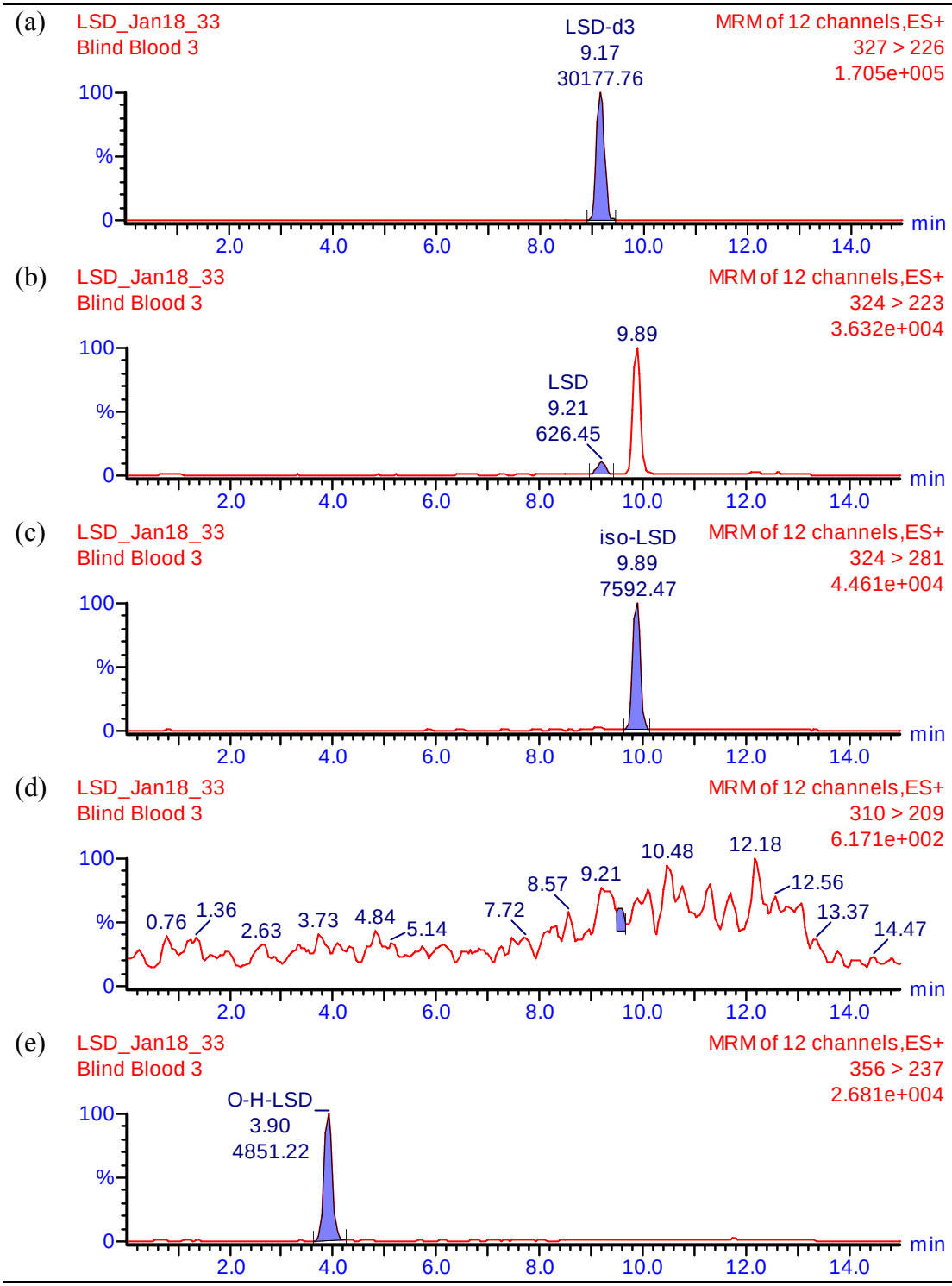


Figure 7.9: MRM acquisitions of extracted blind whole blood sample #3 for (a) LSD-D3, (b) LSD, (c) iso-LSD, (d) nor-LSD, and (e) O-H-LSD. Refer to Figure 7.6 for blank results.

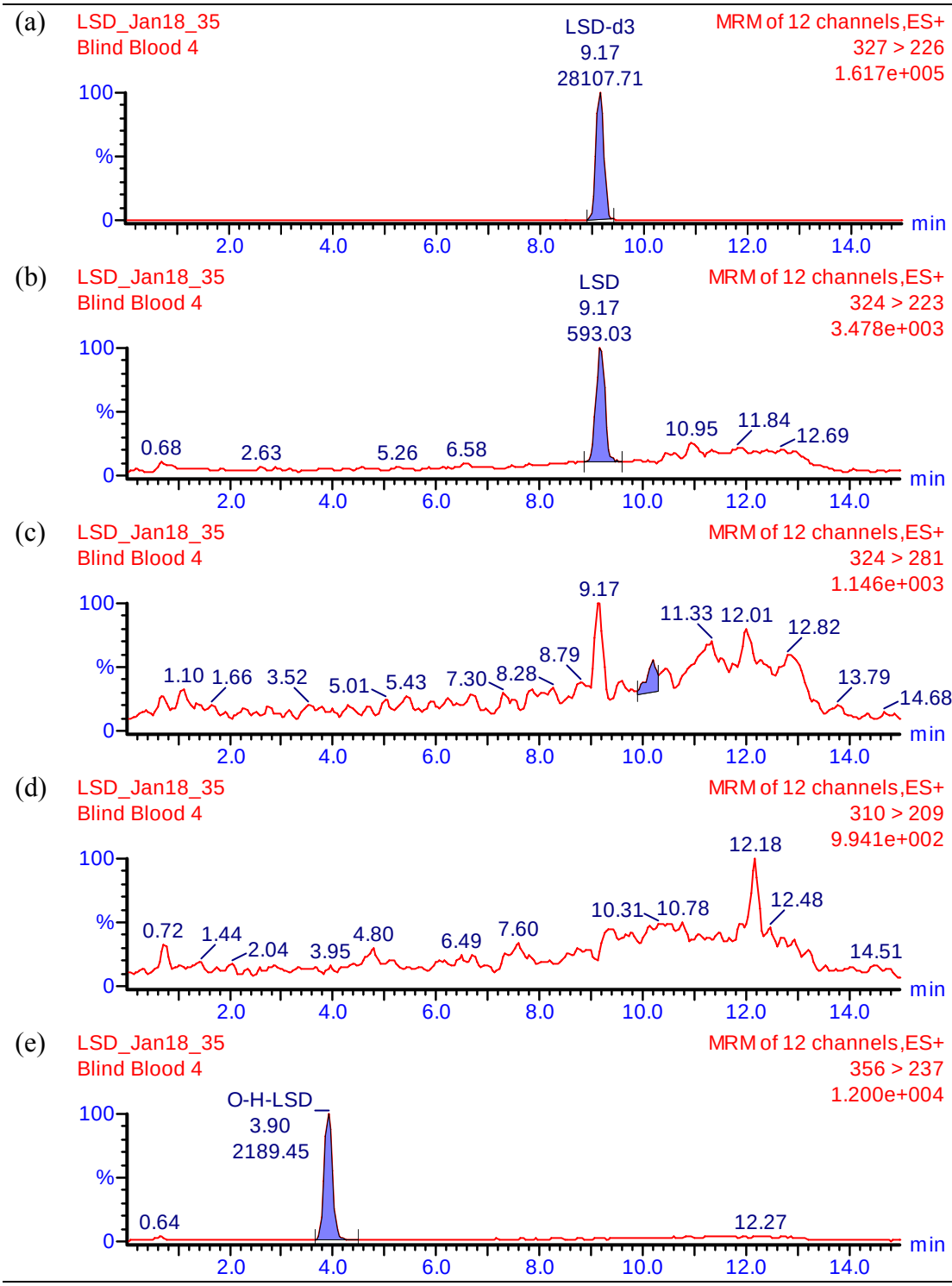


Figure 7.10: MRM acquisitions of extracted blind whole blood sample #4 for (a) LSD-D3, (b) LSD, (c) iso-LSD, (d) nor-LSD, and (e) O-H-LSD. Refer to Figure 7.6 for blank results.

Table 7.1: Blind trial results from two different analysts

		Spiked Level (ng/mL)	Measured Values (ng/mL) Analyst 1	Analyst 2
Urine #1	LSD	3.00	>1.50 (2.59)	>1.50 (2.48)
	iso-LSD	2.40	>1.50 (2.42)	>1.50 (2.28)
	nor-LSD	0.00	ND	ND
	O-H-LSD	8.80	>1.50 (9.09)	>1.50 (9.62)
Urine #2	LSD	0.10	0.09	0.09
	iso-LSD	0.60	0.58	0.57
	nor-LSD	0.10	0.09	0.08
	O-H-LSD	6.50	>1.50 (6.76)	>1.50 (6.95)
Urine #3	LSD	0.00	ND	ND
	iso-LSD	0.00	ND	ND
	nor-LSD	0.00	ND	ND
	O-H-LSD	8.00	>1.50 (9.01)	>1.50 (9.01)
Urine #4	LSD	0.00	ND	ND
	iso-LSD	0.50	0.49	0.48
	nor-LSD	0.00	ND	ND
	O-H-LSD	10.00	>1.50 (10.63)	>1.50 (10.53)
Blood #1	LSD	0.27	0.22	0.15
	iso-LSD	0.44	0.39	0.34
	nor-LSD	0.00	ND	ND
	O-H-LSD	1.50	1.29	1.30
Blood #2	LSD	1.00	0.77	0.56
	iso-LSD	0.75	0.69	0.62
	nor-LSD	0.20	0.14	0.12
	O-H-LSD	1.00	0.92	0.89
Blood #3	LSD	0.00	ND	ND
	iso-LSD	0.50	0.48	0.45
	nor-LSD	0.00	ND	ND
	O-H-LSD	1.00	0.92	0.99
Blood #4	LSD	0.00	ND	ND
	iso-LSD	0.00	ND	ND
	nor-LSD	0.00	ND	ND
	O-H-LSD	0.50	0.45	0.41

ND = none detected.
Bracketed result is from extrapolating the calibration curve above the highest calibrator.

To test both the reliability and reproducibility of the method, the results obtained from two different analysts were compared one another and to the spiked values. The results obtained from the two different analysts, showed that the method was qualitatively reproducible. Both analysts were able to correctly identify the analytes 100% of the time. When comparing the results from the two analysts to the spiked value, there was a good correlation of the expected value to the experimental quantitated results. As well, the analysis is reproducible between different analysts, because there was also a good correlation of the quantitated results of analyst 1 and 2.

7.2.2 Discussion

Some of the results were reported as >1.50 ng/mL because the levels were higher than the highest calibrator (1.50 ng/mL). The accuracy of extrapolating the calibration curve has not been tested. However, by extrapolating the calibration curve, the calculated concentrations were all ±15% bias from the spiked value, suggesting that the method was quite linear and not near saturation for detection at 3.0 ng/mL for LSD, 2.4 ng/mL for iso-LSD, and 10.0 ng/mL for O-H-LSD.

The higher % bias of the measured nor-LSD may be explained by the reported instability of the nor-LSD stock standard in ACN (Chapter 6, section 6.3.7.4). All the measured values from both analysts that were within the range of the calibration curves were lower than the spiked value. The standards used to prepare the blind samples were different than the standards used to prepare the calibration curve. Since the quality control samples were within acceptable limits, and the residuals were randomly scattered with no obvious trend, the calibration curves were deemed acceptable. Since the calibration curves were acceptable, the lower measured values may have been caused by the fact that different standards were used to prepare the calibration standards versus the blinds. It may also have been possible that during shipment of the blind samples that the compounds degraded slightly.

This was the first time that Analyst 2 performed the method by following the written standard operating procedures (SOPs) (Appendix A, B, and C), as a result, Analyst 2 lacked the experience of Analyst 1 (author). Analyst 2 had difficulties in obtaining an organic layer free of blood particulates, so resorted to using a syringe filter.

LSD may have been lost from using the syringe filter. From experience, the author had found that by practising and using the right technique that it is possible to obtain a "clean" organic layer without having to use syringe filters. The quantitative results should be reproducible when performed by experienced analysts.

The significance of the actual level of LSD, iso-LSD, nor-LSD, and O-H-LSD in blood and urine has yet to be discussed in the literature. Unlike prescription drugs or other medications, LSD does not have a therapeutic concentration range and has not been found to cause organ damage. Forensically, because LSD is an illicit drug the mere presence of it is significant. Most importantly, the blind trial results from the 2 analysts demonstrated that the incidence of false positives was absent. By following the identification criteria regarding the ion ratios, RT, and RRT the correct identification of LSD, iso-LSD, nor-LSD, and O-H-LSD were obtained 100% of the time. Furthermore, by ensuring that the LLOQ samples response for LSD was at least 5 times the blank sample containing the ISTD, the residual LSD present in the ISTD did not result in false positive results.

7.3 Case Sample

7.3.1 History

This case occurred in 1995. A 19 year old man leapt out of a 16th floor window and landed on the 5th floor of a parking garage. He died shortly after from "multiple blunt force injuries". When his friends were questioned, they said that he may have taken LSD. The man's father stated that he was not aware of any specific history of drug abuse, other than an occasional beer. There was also no history of depression or suicidal thoughts.

7.3.2 Initial 1995 Laboratory Analysis

Four samples were collected post-mortem; (1) femoral blood collected in grey top (potassium oxalate, sodium fluoride) Vacutainer™, (2) subclavian blood collected in plastic screw top container, (3) vitreous humor collected in Vacutainer™ with no additives, and (4) urine collected in grey top (potassium oxalate, sodium fluoride) Vacutainer™. An alcohol and routine toxicology screen (immunoassay and GC/MS)

was conducted. There was no alcohol or other common drugs in the routine screen except for 3 mg/L acetaminophen in the blood. Based on the history, a LSD RIA kit was obtained and run with appropriate controls. All samples "screened positive". Final 1995 report was "presumptive" LSD, a confirmatory method is not available.

7.3.3 UPLC™/MS/MS Confirmation Results

Since 1995, the samples were initially stored refrigerated for about 2 to 4 months, then stored at -50°C. Samples were shipped and received on ice and stored at 4°C for one day until analyzed. The samples were extracted in duplicate and analyzed (Appendix A, B, and C). A 5 to 6 point calibration curve was constructed to quantitate the blood samples. A 3 point calibration curve was constructed to quantitate the urine sample. A 3 point calibration curve using reference standards made in acetonitrile was constructed to quantitate the vitreous humor sample. The r and r^2 of all calibration curves were greater than 0.99. Quality control samples were run in duplicate and all were within acceptable limits. The MRM ion chromatograms of the case sample are shown in Figure 7.11 to 7.16.

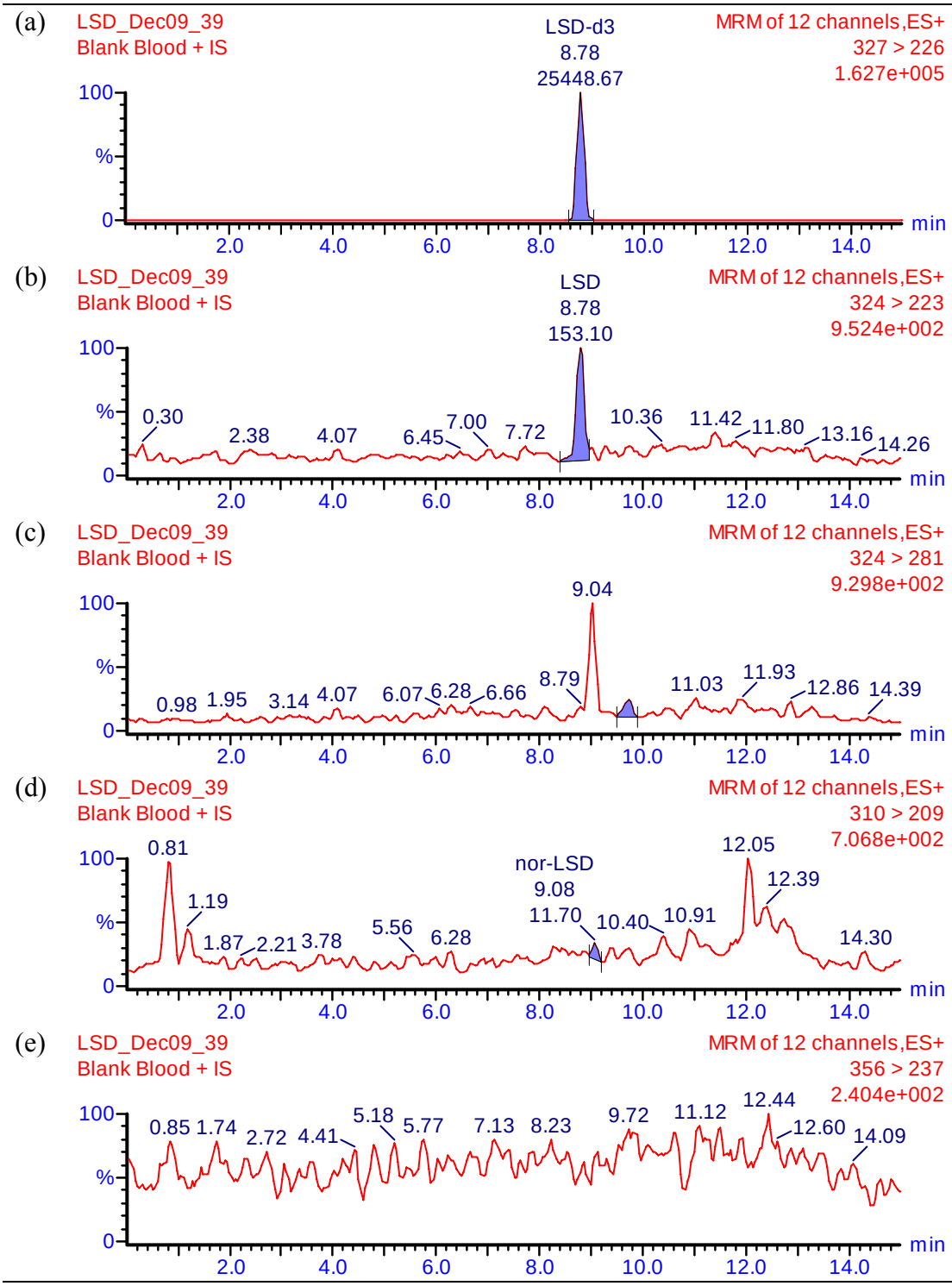


Figure 7.11: MRM acquisitions of blank whole blood + ISTD (LSD-D3) for (a) LSD-D3, (b) LSD, (c) iso-LSD, (d) nor-LSD, and (e) O-H-LSD. The response of the blank is acceptable since at the LLOQ was five times the response compared to the blank.

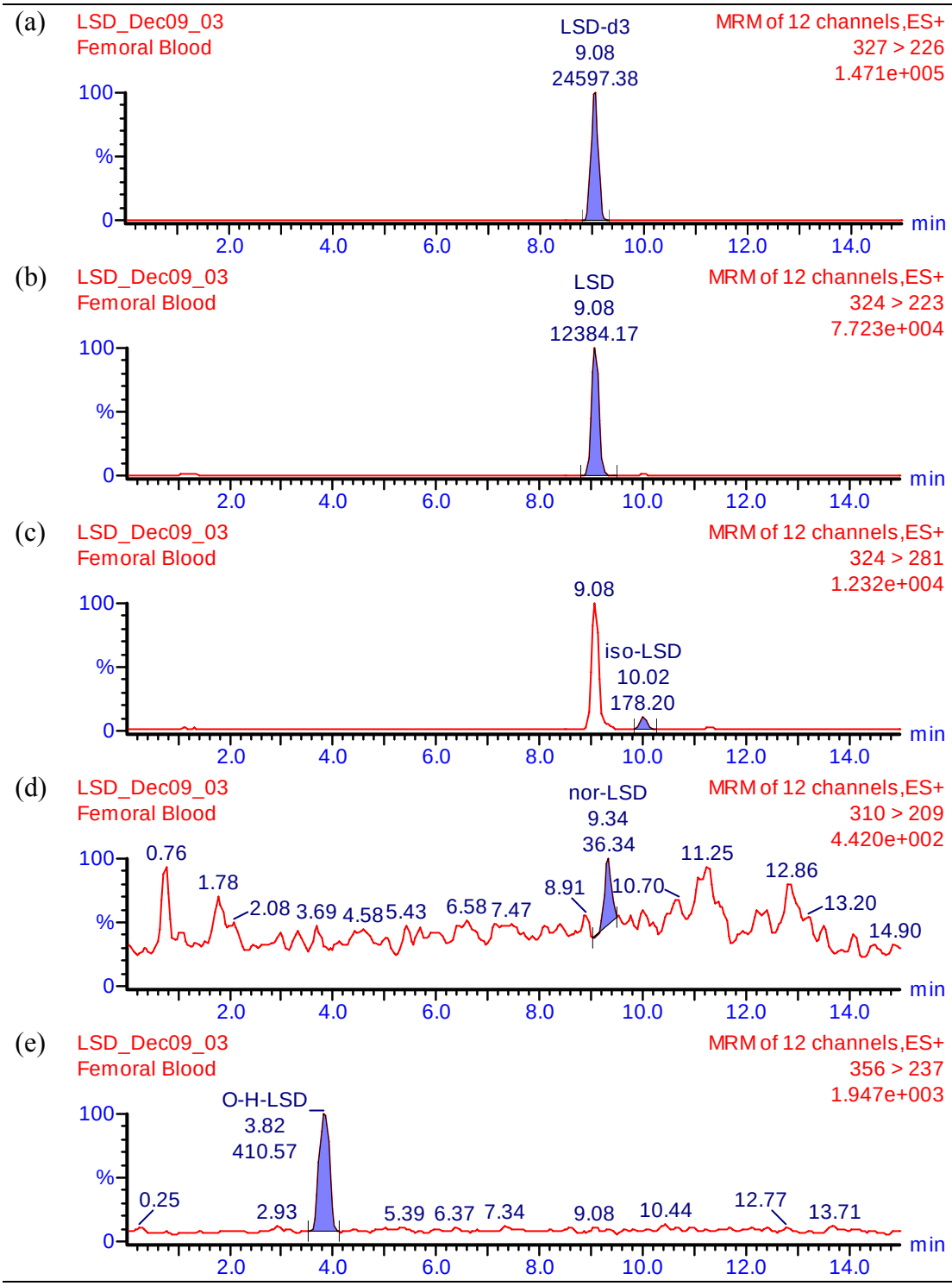


Figure 7.12: MRM acquisitions of extracted femoral blood case sample for (a) LSD-D3, (b) LSD, (c) iso-LSD, (d) nor-LSD, and (e) O-H-LSD. Refer to Figure 7.11 for blank results.

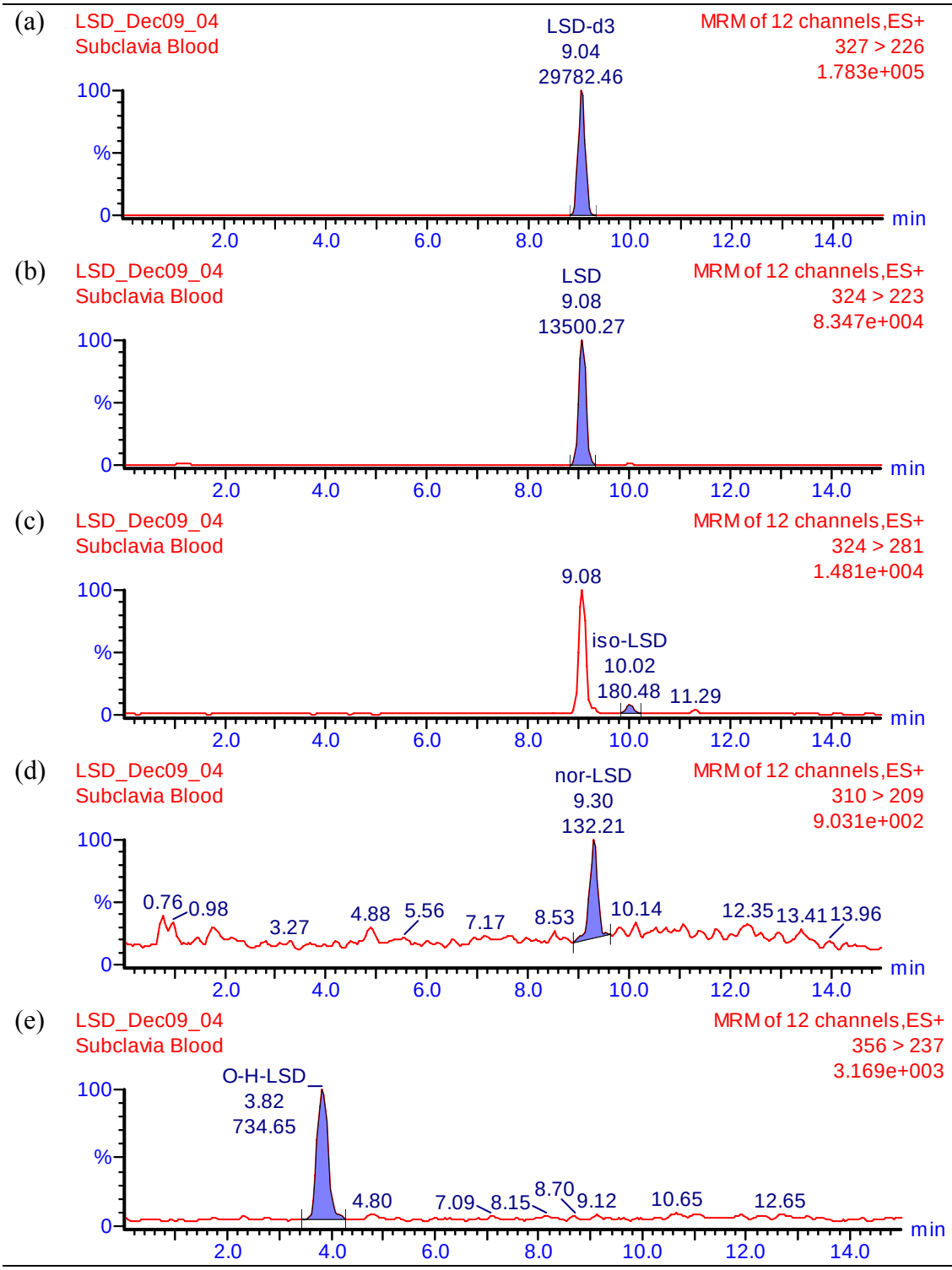


Figure 7.13: MRM acquisitions of extracted subcalvian blood case sample for (a) LSD-D3, (b) LSD, (c) iso-LSD, (d) nor-LSD, and (e) O-H-LSD. Refer to Figure 7.11 for blank results.

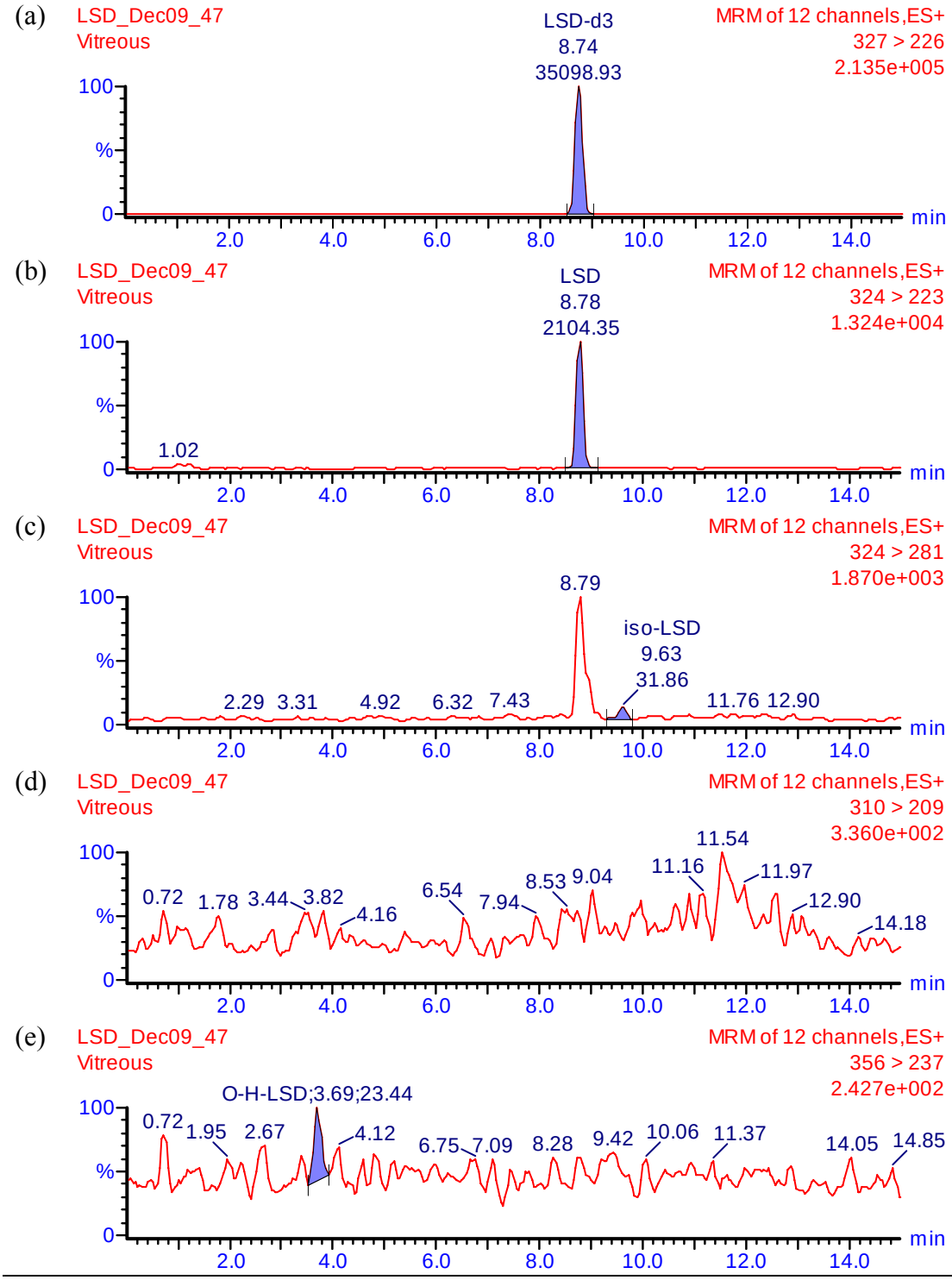


Figure 7.14: MRM acquisitions of extracted vitreous humor case sample for (a) LSD-D3, (b) LSD, (c) iso-LSD, (d) nor-LSD, and (e) O-H-LSD. Method has not been validated for vitreous humor matrix.

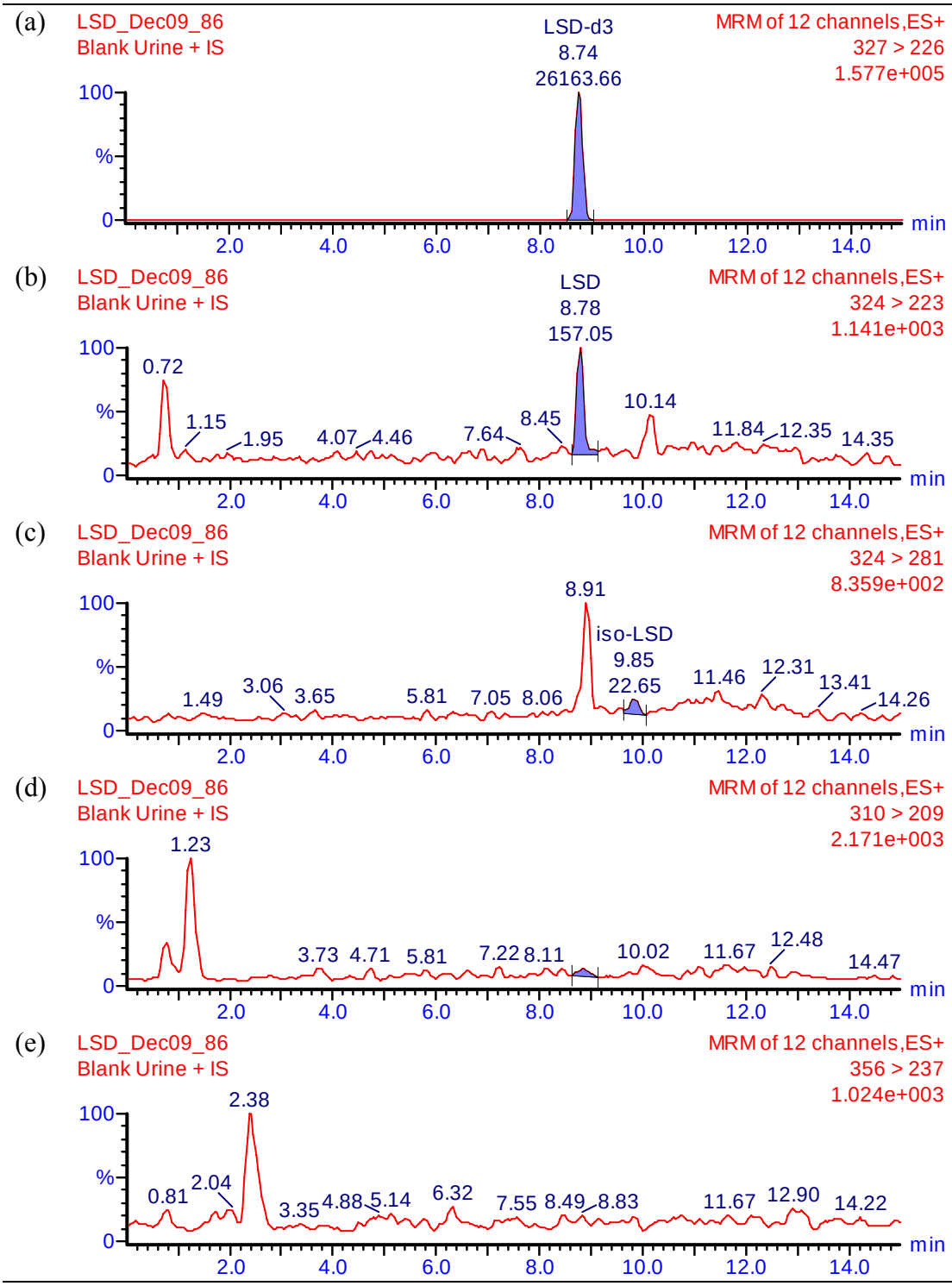


Figure 7.15: MRM acquisitions of blank urine + ISTD (LSD-D3) for (a) LSD-D3, (b) LSD, (c) iso-LSD, (d) nor-LSD, and (e) O-H-LSD. The response of the blank is acceptable since at the LLOQ was five times the response compared to the blank.

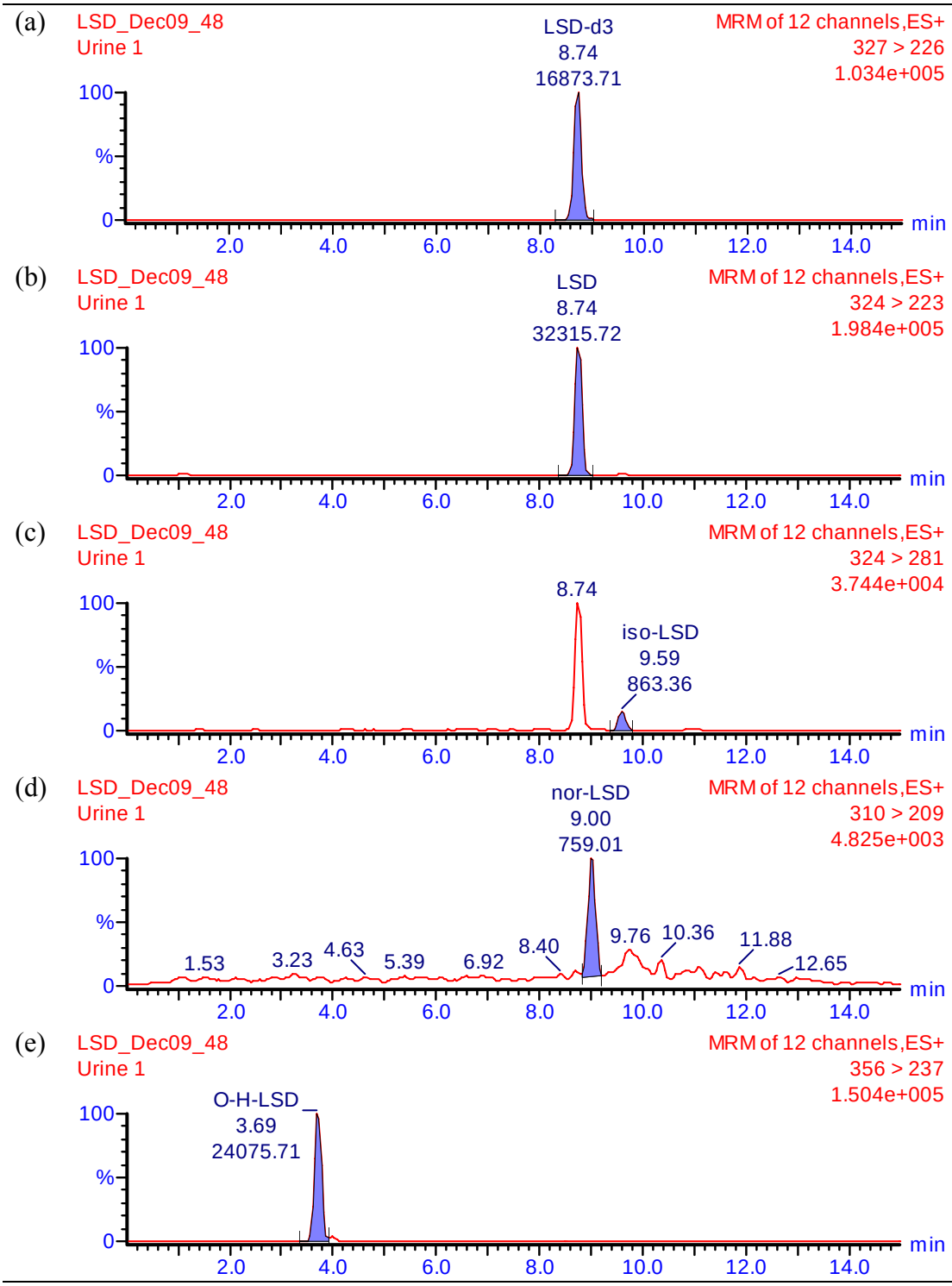


Figure 7.16: MRM acquisitions of extracted urine case sample for (a) LSD-D3, (b) LSD, (c) iso-LSD, (d) nor-LSD, and (e) O-H-LSD. Refer to Figure 7.15 for blank results.

Like the blind samples, RT, RRT, and ion ratios were used for confirmation, where all the ions were present for each compounds, the RT and RRT were within ±2% of the calibration standards, and the ion ratios were within ±30% of the calibration standards (Table 7.2). The results obtained with the UPLC™/MS/MS method are summarized in Table 7.3. Trace results means that the level was above the LOD, but lower than the LLOQ. None detected means that the level was below the LOD or one or all three identification criteria failed.

Table 7.2: The % bias of the confirmatory parameters of the case sample relative to the calibration standards

		RT	**RRT**	**Ion ratio 1**	**Ion ratio 2**
Femoral	LSD	0.8	-0.2	0.00	-9.4
Blood	iso-LSD	0.8	-0.2	11.4	7.2
	nor-LSD	0.9	-0.1	-49.3*	9.4-
	O-H-LSD	0.2	-0.8	-1.5	14.1
Subclavian	LSD	0.8	0.3	-7.3	-10.9
Blood	iso-LSD	0.6	0.1	5.2	3.9
	nor-LSD	0.7	0.1	-9.2	32.1*
	O-H-LSD	0.2	-0.3	4.0	-3.0
Vitreous	LSD	1.6	-0.3	4.2	-9.1
humor	iso-LSD	2.0*	0.1	160.8*	-96.7*
	nor-LSD	-48.1*	-50.1*	-91.6*	-100.0*
	O-H-LSD	10.4*	8.2*	-62.2*	-67.9*
Urine	LSD	-0.6	-0.6	-3.6	-11.1
	iso-LSD	-0.4	-0.4	9.1	5.1
	nor-LSD	-0.4	-0.4	3.1	-6.6
	O-H-LSD	0.2	0.2	-4.5	17.2

* Outside acceptable parameters for confirmation. Acceptable parameters for RT and RRT is ±2% bias, and for ion ratios ±30% bias.

Table 7.3: Measured concentrations of LSD, iso-LSD, nor-LSD, and O-H-LSD in whole blood, urine, and vitreous humor samples from a 19 year old male

	LSD (ng/mL)	Iso-LSD (ng/mL)	Nor-LSD (ng/mL)	O-H-LSD (ng/mL)
Femoral Blood	0.56	trace	ND	0.17
Subclavian Blood	0.54	trace	ND	0.17
Urine	>1.50*	0.07	0.19	>1.50[†]
Vitreous humor[§]	0.07	ND	ND	ND

ND = none detected.
Trace is when result is ≥ LOD, < LLOQ.
Extrapolating the calibration curve gives a result of *2.33 ng/mL, and [†]9.29 ng/mL.
[§] The method has not been validated for vitreous humor sample matrix.

7.3.4 Discussion

To my knowledge this is the first time that O-H-LSD has been detected and confirmed in whole blood case samples. The majority of the methods published for O-H-LSD have only attempted analysis in urine (Horn et al. 2003; Poch et al. 2000; Klette et al. 2002; Skopp et al. 2002), but methods that have attempted to analyze O-H-LSD in blood were unable to detect O-H-LSD in case blood samples positive for LSD (Sklerov et al. 2000; Canezin et al. 2001). It is still not confirmed that O-H-LSD is strictly a urinary metabolite since the research on the metabolism of LSD is incomplete. Despite publications stating that O-H-LSD was not detected in blood samples, O-H-LSD was still included during method validation in whole blood.

Previous LC/MS(/MS) methods for analyzing O-H-LSD in blood may have been unsuccessful in detecting O-H-LSD in real samples due to the lack in sensitivity compared to this UPLC™/MS/MS method. One method using an Agilent HPLC coupled to a MSD was unable to detect O-H-LSD in blood case samples (Sklerov et al. 2000). When spiked blood samples were extracted only 32 to 42% was recovered versus 69 to 73% in spiked urine. The low recovery is due to the SPE done after the initial LLE to obtain "cleaner" extracts. The LOD and LLOQ of 400 pg/mL was only determined in urine, but the low recovery in blood could ultimately affect the sensitivity of the method resulting in a higher LOD and LLOQ. Nonetheless, a LOD and LLOQ of 400 pg/mL for O-H-LSD would not have been able to detect O-H-LSD in this case sample.

Like the blind samples, a few results for LSD and O-H-LSD were reported as >1.50 ng/mL because levels were higher than the highest calibrator (1.50 ng/mL). If the calibration curve was extrapolated the concentration of LSD was approximately 2.33 ng/mL and O-H-LSD was approximately 9.29 ng/mL. The most important result was that urine O-H-LSD was in higher concentrations than LSD. Others have found that the concentration of O-H-LSD is 16 (Poch et al. 2000) to 25 (Horn et al. 2003) times higher than that of LSD. This study found that O-H-LSD was 4 times higher than LSD in urine.

The method has not been validated for vitreous humor sample matrix. Since it was shown that vitreous humor samples may also contain LSD, validating for the vitreous humor sample matrix could be done for future experiments. No other published methods have analyzed vitreous humor samples for LSD.

The most interesting result was that even after 10 years LSD and metabolites were still detectable. Since no quantitation was conducted on the samples 10 years ago, the stability cannot be assessed. These results show that LSD is relatively stable if kept frozen. More importantly, these results showed that 10 year old cases screening positive for LSD during a time when no confirmatory analysis was available, could now be analyzed for confirmation if required.

7.4 Conclusion

The results of the blind trial and the case sample further support the reliability of this method. By following the identification criteria regarding the ion ratios, RT, and RRT the correct identification of LSD, iso-LSD, nor-LSD, and O-H-LSD should be obtained 100% of the time. The interpretation of the results to confirm the use of LSD was further supported by also analyzing metabolites and the isomer of LSD.

Chapter 8
Summary, Future Experiments, and Conclusion

8.1 Introduction

Selective and sensitive methods for forensic analysis must be developed to withstand the scrutiny of the law. The detection and confirmation of highly potent drugs and their metabolites from complex matrices need techniques that employ mass spectrometry as the detector in order to achieve the necessary selectivity and sensitivity. One such forensic example is in analyzing biological samples for the presence of LSD. In order for a method to be used routinely at the RCMP FLS TS, the method must be validated. Validating a method ensures that the method would work during normal use. Full validation as recommended by the FDA, USP, ICH, SOFT, and AAFS suggest the evaluation of linearity, selectivity, LOD, accuracy, precision, recovery, stability, and ME. These parameters were evaluated to validate an UPLC™/MS/MS method that will be used by the RCMP FLS TS for routine confirmatory analysis of samples screening positive for LSD with ELISA.

8.2 Summary

The intended study objectives and hypothesis (Chapter 1, section 1.7) were successfully achieved. Compared to the GC/MS method previously utilized by the RCMP in 1997 (section 6.1), the UPLC™/MS/MS method was able to analyze extracts of blood and urine for LSD with better sensitivity and greater ease of analysis (Table 8.1). The UPLC™/MS/MS method is more efficient than the previously used GC/MS method, decreasing total analysis time and cost. The preparation of samples using the GC/MS method first requires mixing the sample with buffer then adjusting the pH for urine samples and sonicating blood samples. Next an automated SPE is used which takes approximately 18 minutes for one sample, followed by evaporation of the eluent and 30 minutes for derivatization. Additionally, 6 injections of blanks and several injections of derivatizating agents to prevent adsorption, requires a total of 15 minutes

for each injection. So a full run with an unknown, 6 calibration standards, a quality control sample, and a blank each run in duplicate would take about 20 hours. In contrast, the UPLC™/MS/MS method only takes about 10 hours. The GC/MS method is more costly because it requires SPE cartridges and more solvents compared to the LLE method. The interpretation of confirming LSD use is also further enhanced by simultaneously analyzing LSD with iso-LSD, nor-LSD, and O-H-LSD. Furthermore, the linearity, accuracy, precision, LOD, LLOQ, selectivity, recovery, ME, stability, RT and RRT, and ion ratio were all found to be acceptable, to validate the method.

Table 8.1: GC/MS/MS method used in 1997 by RCMP versus the UPLC™/MS/MS method validated in this study

	GC/MS/MS	**UPLC™/MS/MS**
Analytes analyzed	LSD only	LSD iso-LSD nor-LSD O-H-LSD
LLOQ (pg/mL)	100	20 to 50
Volume of Sample	2 mL blood 5 mL urine	1 mL blood 1 mL urine
Derivatization	yes	no
Adsorption onto column	yes*	no

*pre-conditioning required

The validation parameters were all conducted using samples spiked with known amounts of standard. To remove the bias of using spiked standards prepared by the analyst, known positive case samples confirmed with another method or another laboratory should ideally be analyzed and compared. Before this method was validated no other forensic laboratories in Canada had a method that was being used to analyze LSD. So comparing results between different laboratories was not possible. To provide further proof of the reliability of this method, a case sample screening positive with RIA and blind samples prepared by another laboratory were analyzed (Chapter 7). Validating the method, and analyzing both the case sample and blind samples, showed that the method was reliable for analyzing forensic samples suspected of containing LSD.

Finally the knowledge gained from this project, impacted the RCMP FLS in the general use of LC/MS/MS as a confirmatory technique. By showing that a compound that was difficult to analyze via GC/MS/MS, such as LSD, was more amenable to LC/MS/MS analysis, demonstrated that other "problem" compounds could also be more suitable for LC/MS/MS. Furthermore, the steps involved in conducting the validation study for the detection, confirmation, and quantitation of LSD could be used as a model for validating other compounds using LC/MS/MS.

8.3 Future Experiments

It was previously mentioned that validation is a permanent process that starts from the beginning of the life of the method until its retirement, where the objective should be to build results of quality by means of an analytical method (Boulanger et al. 2003). So although the intended purpose of this study was achieved, if time permitted there are a few experiments that may be considered for the future to further increase the quality of the method.

8.3.1 Upper Limit of Quantitation (ULOQ)

In Chapter 7, the case urine sample resulted in LSD and O-H-LSD values above the highest calibrator. Another study found that the average concentration of O-H-LSD in human urine samples to be 3.47 ng/mL (Reuschel et al. 1999b). The ULOQ could be increased or the accuracy of diluted standards could be tested. This may be important to show if the method is linear at the higher concentrations with no saturation effect. Certain validation parameters may also need to be shown to be valid at the higher concentrations, particularly the ion ratios. Ion ratios are a qualitative confirmatory parameter that would be used for urine samples. Based on the results obtained for the ion ratios precision, it would be expected that the precision would increase with increasing concentration, because results showed that the precision at the low concentration was less compared to the higher concentration (Table 6.19).

8.3.2 Specificity

The specificity of the method can also be evaluated by testing to see if other drugs and their metabolites, other illicit compounds, and compounds related to LSD cause interference. According to the recommendations by the FDA, specificity is not included in the parameters included for validation. However, since in forensic analysis the identities of compounds in the samples are unknown it may be possible that other drugs are present in the samples.

8.3.3 Extraction Method

The extraction method used was less laborious than the GC/MS/MS method used in 1997 by the RCMP, but compared to the nBuCl method used routinely for basic drug screens in blood it was more laborious. This was because the organic layer was at the bottom below a layer of solid blood. As a result, trying to transfer the organic layer without transferring blood particulate was difficult. If possible, other extraction methods with the organic layer on top may be considered. Additional cleanup after the initial LLE of the blood extract may be considered as well. However, this may result in lower recoveries and subsequently affect the sensitivity of the method. To compensate for the possible loss of sensitivity from performing additional cleanup, the final extract may be reconstituted in a smaller volume (<100 μL) so that the final extract is more concentrated. Yet, reconstituting in a smaller volume could limit the ability to re-inject (20 μL injected) a sample if needed.

8.3.4 Other Matrices

In Chapter 7, the case tested included a vitreous humor sample. The original validation method was conducted for urine and blood samples only. The case vitreous humor sample confirmed the presence of LSD, so validating this method for vitreous humor matrix could be done as well. Furthermore, other sample matrices may also be tested for, such as tissue samples.

8.3.5 ELISA Cross Reactivity

Since May 2005, none of the samples screening positive for LSD with ELISA were not confirmed to be LSD when tested with LC/MS/MS. The cross-reactivity of iso-LSD, nor-LSD, and O-H-LSD with the LSD ELISA test was tested and found to be insignificant (Appendix D). As a result, a cross-reactivity study of other commonly encountered drugs or compounds structurally related to LSD could be conducted to try and determine the compounds that are causing the false positives.

8.3.6 Case Samples

The most important work to do in the future would be to run more cases, because there is a need to publish more information on the levels of LSD in blood. At the RCMP FLS actual case samples will only be run by LC/MS/MS for confirmation if the LSD ELISA screening method tests positive. In order to report a positive result with the LSD ELISA screening method, the levels must be greater than 0.50 ng/mL in blood and 1.00 ng/mL in urine. To take full advantage of the highly sensitive UPLC™/MS/MS method, with a LLOQ of 0.02 ng/mL for LSD, the LSD ELISA cutoff level for positive LSD may need to be reconsidered. As an example, the case blood samples analyzed by the UPLC™/MS/MS method in Chapter 7 were quantitated to have concentrations of 0.56 and 0.54 ng/mL in the femoral blood and subclavian blood, respectively. Concentrations of 0.56 and 0.54 ng/mL are close to the 0.50 ng/mL LSD ELISA cutoff level for blood, so those samples may or may not have tested positive for LSD via ELISA. As a result, under normal circumstances the blood samples may have been reported as negative by ELISA and not of been carried on for confirmatory analysis by LC/MS/MS. This example demonstrates that there is a possibility of reporting a sample that actually contains LSD, as being negative with the current ELISA cutoff concentrations.

8.4 Conclusion

A majority of forensic toxicology laboratories lack a reliable validated method for detecting LSD. Without an available method it is not known if the illicit use of LSD actually exists or if it is just simply missed. Without a method for confirming LSD, the diagnosis of suspected overdose situations can only be done by clinical signs. In

conclusion, a validated UPLC™/MS/MS is now available to the RCMP FLS for confirming the illicit use of LSD in Canada. This method will be applied to real cases screening positive for LSD with ELISA. Furthermore, other agencies in Canada can also rely on the RCMP FLS for confirmatory analysis of LSD if needed.

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Appendix A

Title: Quantitation and Confirmation of LSD, iso-LSD, nor-LSD, and O-H-LSD in whole blood

Identification/Filename of this SOP: *LSD Quant Blood.wpd*

Scope: This SOP describes the method that is used to confirm the use of LSD and to quantitate LSD, iso-LSD, nor-LSD, and O-H-LSD in whole blood.

Written/Revised by:	Authorized by:	Bench copies
Name: A. Chung	**Name:**	**How many?_____**
Position:	**Position:**	**Located?_____**
Date	**Date:**	

1. Associated SOPs -

1.1 LSD LC_MS_MS...
1.2 quant...
1.3 LCMS_confirm…

2. Definitions -

2.1 LSD - lysergic acid diethylamide
2.2 iso-LSD - lysergic acid diethylamide isomer
2.3 nor-LSD - N-demethyl lysergic acid diethylamide
2.4 O-H-LSD - 2-oxo-3-hydroxy lysergic acid diethylamide
2.5 LSD-D3 - deuterated lysergic acid diethylamide
2.6 ISTD - internal standard
2.7 LLOQ - lowest limit of quantitation
2.8 UPLC™/MS/MS - ultra performance liquid chromatography tandem mass spectrometry
2.9 BEH - bridged ethyl hybrid

3. Safety -

3.1 Routine laboratory safety guidelines for handling biohazardous materials are followed.

4. Equipment Used -

4.1 Waters Corporation (Milford, MA) Acquity UPLC™ with an autosampler.
4.2 Waters Corporation (Milford, MA) Quattro Premier™ tandem quadrupole mass spectrometer.
4.3 Acquity UPLC™ BEH C18 (2.5×50 mm, 1.7 μm, Waters Corporation, Milford, MA)
4.4 pH meter
4.5 Mechanical Shaker
4.6 Centrifuge

5. Reagents Needed -

5.1 **20 mM Ammonium acetate buffer (pH 4.0)**
Dissolve 1.5416 g ammonium acetate and dissolve in 800 mL NANOpure water, then adjust pH to 4.0 (± 0.1) using glacial acetic acid. Make up to 1 L with NANOpure water. Thoroughly mix then filter and sonicate.

5.2 **1 M Ammonium acetate buffer (pH 9.0)**
Dissolve 19.270 g ammonium acetate in 150 mL NANOpure water. Adjust pH to 9.0 (± 0.1) with ammonium hydroxide. Make up to 250 mL with NANOpure water.

5.3 methylene chloride:isopropyl alcohol (85:15 (v:v))

5.4 Acetonitrile (HPLC grade)

5.5 **Calibrator solutions** in acetonitrile are prepared as follows: (Calibrators are purchased from Cerilliant)

5.5.1 **LSD -** prepare a 500 ng/mL stock.

25 µg/mL Cerilliant standard -	100 µL diluted to 5 mL with acetonitrile giving a 500 ng/mL stock
100 µg/mL Cerilliant standard -	25 µL diluted to 5 mL with acetonitrile giving a 500 ng/mL stock
1 mg/mL Cerilliant standard -	First prepare a 100 µg/mL stock by diluting 100 µL of the 1 mg/mL standard to 1 mL with acetonitrile. Then follow 100 µg/mL standard directions.

5.5.2 **iso-LSD -** same as for LSD (5.5.1)

5.5.3 **nor-LSD -** same as for LSD (5.5.1)

5.5.4 **O-H-LSD** - same as for LSD (5.5.1)

5.5.5 **Working Spiking mix #1**
Pipette 10 µL of each 500 ng/mL stock standards in a 5 mL volumetric flask and make up to volume with acetonitrile, giving 1 ng/mL of LSD, iso-LSD, nor-LSD, and O-H-LSD.

5.5.6 **Working Spiking mix #2**
Pipette 200 µL of each 500 ng/mL stock standards in a 5 mL volumetric flask and make up to volume with acetonitrile, giving 20 ng/mL of LSD, iso-LSD, nor-LSD, and O-H-LSD.

Working spiking mix's are prepared fresh before each quantitation.

5.6 **Internal Standard** in acetonitrile is prepared as follows: (Internal standard purchased from Cerilliant)

5.6.1 **LSD-D3** 500 ng/mL Stock - 100 µL of the 25 µg/mL Cerilliant standard diluted to 5 ml with acetonitrile giving 500 ng/mL LSD-D3.

5.6.2 **Working ISTD** -
200 µL of the 500 ng/mL LSD-D3 stock diluted to 5 mL with acetonitrile giving 20 ng/mL LSD-D3.

Working internal standard is prepared fresh before each quantitation.

6. Procedure -

6.1 Carry appropriate calibrators through the analytical procedure in parallel with samples to be analyzed. Set up a calibration curve selecting similar concentrations as illustrated in the table below.

Sample (1 mL Blood)	µL of spiking mix #1 (1 ng/mL)	µL of spiking mix #1 (20 ng/mL)	µL of working LSD-D3 ISTD (20 ng/mL)
Blank	0	0	0
Blank + ISTD	0	0	50
0.02 ng/mL	20	0	50
0.05 ng/mL	50	0	50
0.40 ng/mL	0	20	50
0.80 ng/mL	0	40	50
1.60 ng/mL	0	80	50
2.00 ng/mL	0	100	50
Control 1: 0.05 ng/mL	50	0	50
Control 2: 0.50 ng/mL	0	25	50
Control 3: 1.50 ng/mL	0	75	50

6.2 Add 1 mL blood and 50 µL working ISTD to a test tube and mix.

6.3 Basify the samples by adding 500 µL 1 M Ammonium acetate buffer (pH 9.0) to each tube and mix thoroughly.

6.4 Extract with 5 mL methylene chloride:isopropyl alcohol (85:15) to each tube and cap. Vortex tube to break up blood clot then shake the tube manually to ensure tube is capped tightly. Then put on a mechanical shaker for 20 minutes.

6.5 Centrifuge at 3500 rpm for 15 minutes.

6.6 Decant off aqueous (top) layer and discard.

6.7 Using a glass pipette, pass through the solid blood layer and pipette the organic (bottom) layer to a new test tube.

6.8 Repipette organic layer into another new test tube and ensure no transfer of blood particulate and evaporate to dryness under nitrogen with no heat.

6.9 Reconstitute residue in 100 µL 20 mM ammonium acetate buffer (pH 4.0):ACN (80:20).

6.10 Centrifuge reconstituted extracts or filter to remove particulates before being injected.

6.11 Transfer supernatant to a Waters ACQUITY UPLC™ using a Waters 150 µL insert.

6.12 Analyze by UPLC™/MS/MS (See LSD LC_MS_MS SOP).

7. QC and Analytical Results-

7.1 Data collection, peak integration, and weighted ($1/x^2$) linear regression of the calibration curve is performed using the MassLynx™ version 4.0 software (Waters Corporation, Milford, MA).

7.2 Exclude the 20 pg/mL calibrator from nor-LSD and O-H-LSD calibration curve. LLOQ of nor-LSD and O-H-LSD is 50 pg/mL.

7.3 Calculate the concentration and ion ratios of LSD, iso-LSD, nor-LSD, and O-H-LSD using the MassLynx™ version 4.0 software (Waters Corporation, Milford, MA). The use of internal standards and at least four calibrators bracketing the unknown samples, as recommended by SOFT guidelines are adhered to. A calculated correlation coefficient of 0.98 or better is considered acceptable.

7.4 The results of control measurements are considered acceptable if they are within ±15% of target values. The 50 pg/mL (LLOQ) quality control sample for nor-LSD and O-H-LSD is permitted to be within ±20% of the target value.

7.5 The lowest calibrator must be at least 5 times the response of the blank.

7.6 For positive confirmation, the ion ratios of the quality control samples and the unknown should be within ±30% for the calibrators.

Ion ratios: LSD = 223/208, 223/281
Iso-LSD = 281/208, 281/223
Nor-LSD = 209/237, 209/74
O-H-LSD = 237/222, 237/313
LSD-D3 = 226/208, 226/281

7.7 Relative retention times of unknowns should be within 2% of the calibrators.

8. Method Validation Including References -

8.1 [FDA] Food and Drug Administration: Guidance for Industry: Bioanalytical Method Validation, 2001.

8.2 [SOFT/AAFS] Society of Forensic Toxicologist and American Academy of Forensic Sciences: Forensic toxicology laboratory guidelines, draft 2006 version.

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8.4 Sklerov, J. H., Magluilo, J., Jr., Shannon, K. K., and Smith, M. L.: Liquid chromatography-electrospray ionization mass spectrometry for the detection of lysergide and a major metabolite, 2-oxo-3-hydroxy-LSD, in urine and blood. J Anal Toxicol 24 (7)**:** 543-9, 2000.

9. Appendices -

9.1 Extraction worksheet

9.2 Calibration review

LSD, iso-LSD, nor-LSD, and O-H-LSD Extraction & Confirmation

Requisition for Sample Extraction of LSD, iso-LSD, nor-LSD, and O-H-LSD and LC/MS/MS confirmation

Case #:_____ **Date Submitted:**_____
Exhibit #:_____ **Submitted by:**_____
Case Type:_____ **Common Locker: YES NO**

Exhibit Type: Blood / Urine / Other _____

Special Instructions

Extraction:

Date of sample extraction and submission for LC/MS/MS analysis:_____By:_____

- ☐ 1mL blood, 50µL ISTD (20ng/mL) LSD-D3 and mix.
- ☐ Basify sample with 500µL 1M Ammonium acetate buffer (pH 9.0) and mix thourougly.
- ☐ Extract with 5mL dichloromethane:isopropyl alcohol (85:15). Shake for 20 minutes. Centrifuge at 3500 rpm for 15 minutes. Remove organic (bottom) layer and evaporate to dryness under nitrogen.
- ☐ Reconstitute residue in 100µL 20mM Ammonium acetate buffer (pH 4.0):Acetonitrile (80:20)
- ☐ Transfer to LC (Waters Amber screw top) vials using a 150µL insert (Waters). If delay in analysis, store at -20^{B}C until ready for analysis.
- ☐ Inject 20µL on LC/MS/MS

Comments & Results:

☐ Positive Case: One or more analytes are present.
All ions:
LSD = MRM 324>223, 208, 281
iso-LSD = MRM 324>223, 208, 281
Nor-LSD = MRM 310>209, 237, 74
O-H-LSD = MRM 356>237, 222, 313
are present in the unknown and control at the same retention time (±2%) as the calibrator.

Ion ratios must be within ±30% of the calibrator
LSD = 223/208, 223/281
Iso-LSD = 281/208, 281/223
Nor-LSD = 209/237, 209/74
O-H-LSD = 237/222, 237/313

Similarly, the internal standard must be found and the retention time, ions (MRM 327>226, 208, 281) and ion ratios (226/208, 226/281) must be within range.

☐ Negative Case

Calibration Review: LC/MS/MS (ESI+) LSD, iso-LSD, nor-LSD, and O-H-LSD

File Number: _____ Date:_____

□ Residual Plot included and doesn't show any unusual trends

□ Regression line has a minimum of four valid calibrators (within 15% of target). It is permitted for the LLOQ calibrator to be within 20% of target.

□ Controls are within 15% of the target and bracketed by valid calibrators. It is permitted for the controls at the LLOQ level to be within 20% of target.

□ A valid blank was run (Lowest calibrator is at least 5 times the response of the blank)

□ No significant changes in RRT (<2% deviation from the mid calibrator(s))

□ Calculations for the slope, intercept, and coefficient of determination (r^2>0.98) are correct.

□ Calculations for the unknowns are correct. The unknowns are within 20% of their mean. The correct number of significant figures are expressed.

□ Comments:

The calibration has been reviewed.

Reviewer:_____

Appendix B

Title: LC/MS/MS confirmation of LSD, iso-LSD, nor-LSD, and O-H-LSD in urine and whole blood

Identification/Filename of this SOP: *LSD Urine SOP.wpd*

Scope: This SOP describes the method that is used to identify LSD, iso-LSD, nor-LSD, and O-H-LSD in urine and whole blood.

Written/Revised by:	Authorized by:	Bench copies
Name: A. Chung	**Name:**	**How many?_____**
Position:	**Position:**	**Located?_____**
Date	**Date:**	

1. Associated SOPs -

1.1 LSD LC_MS_MS…

1.2 LCMS_confirm…

2. Definitions -

2.1 LSD - lysergic acid diethylamide

2.2 iso-LSD - lysergic acid diethylamide isomer

2.3 nor-LSD - N-demethyl lysergic acid diethylamide

2.4 O-H-LSD - 2-oxo-3-hydroxy lysergic acid diethylamide

2.5 LSD-D3 - deuterated lysergic acid diethylamide

2.6 ISTD - internal standard

2.7 MS - mass spectrometer

2.8 UPLC™/MS/MS - ultra performance liquid chromatography tandem mass spectrometry

2.9 BEH - bridged ethyl hybrid

3. Safety -

3.1 Routine laboratory safety guidelines for handling biohazardous materials are followed.

4. Equipment Used -

4.1 Waters Corporation (Milford, MA) Acquity UPLC™ with an autosampler.

4.2 Waters Corporation (Milford, MA) Quattro Premier™ tandem quadrupole mass spectrometer

4.3 Acquity UPLC™ BEH C18 (2.5×50mm, 1.7µm, Waters Corporation, Milford, MA) column

4.4 pH meter

4.5 Mechanical Shaker4.6Centrifuge

5. Reagents Needed -

5.1 **20mM Ammonium acetate buffer (pH 4.0)**

Dissolve 1.5416 g ammonium acetate and dissolve in ~800 mL NANOpure water, then adjust pH to 4.0 (± 0.1) using glacial acetic acid. Make up to 1 L with NANOpure water. Thoroughly mix then filter and sonicate

5.2 **1M Ammonium acetate buffer (pH 9.0)**

Dissolve 19.270 g ammonium acetate in ~150 mL NANOpure water. Adjust pH to 9.0 (± 0.1) with ammonium hydroxide. Make up to 250 mL with NANOpure water.

5.3 Methylene chloride:isopropyl alcohol (85:15 (v:v))

5.4 Acetonitrile (HPLC grade)

5.5 **Calibrator solutions** in acetonitrile are prepared as follows: (Calibrators are purchased from Cerilliant)

5.5.1 **LSD -** Prepare a 500 ng/mL stock (follow instructions based on Cerilliant standard concentration).

25 µg/mL Cerilliant standard -	100 µL diluted to 5 mL with acetonitrile giving a 500 ng/mL stock
100 µg/mL Cerilliant standard -	25 µL diluted to 5 mL with acetonitrile giving a 500 ng/mL stock
1 mg/mL Cerilliant standard -	First prepare a 100 µg/mL stock by diluting 100 µL of the 1 mg/mL standard to 1 mL with acetonitrile. Then follow 100 µg/mL standard directions.

5.5.2 **iso-LSD -** same as for LSD (5.5.1)

5.5.3 **nor-LSD -** same as for LSD (5.5.1)

5.5.4 **O-H-LSD** - same as for LSD (5.5.1)

5.5.5 **Working Spiking mix**
Pipette 200 µL of each 500 ng/mL stock standards in a 5 mL volumetric flask and make up to volume with acetonitrile, giving 20 ng/mL of LSD, iso-LSD, nor-LSD, and O-H-LSD.

Working spiking mix is prepared fresh before each analysis.

5.6 **Internal Standard** in acetonitrile is prepared as follows: (Internal standard purchased from Cerilliant)

5.6.1 **LSD-D3** 500 ng/mL Stock - 100 µL of the 25 µg/mL Cerilliant standard diluted to 5 ml with acetonitrile giving 500 ng/mL LSD-D3.

5.6.2 **Working ISTD** -
200 µL of the 500 ng/mL LSD-D3 stock diluted to 5 mL with acetonitrile giving 20 ng/mL LSD-D3.

Working internal standard is prepared fresh before each quantitation.

6. Procedure -

6.1 Carry a blank and 1.5 ng/mL quality control sample (refer to table below) through the analytical procedure in parallel with samples to be analyzed.

Sample (1 mL Urine)	µL of spiking mix (20 ng/mL)	µL of working LSD-D3 ISTD (20 ng/mL)
Blank	0	0
Blank + ISTD	0	50
Control: 1.5 ng/mL	75	50

6.2 Add 1 mL urine and 50 µL working ISTD to a test tube and mix.

6.3 Basify the samples by adding 500 µL 1 M Ammonium acetate buffer (pH 9.0) to each tube and mix thoroughly.

6.4 Extract with 5 mL methylene chloride:isopropyl alcohol (85:15) to each tube and cap. Shake the tube manually to ensure tube is capped tightly. Then put on a mechanical shaker for 20 minutes.

6.5 Centrifuge at 3500 rpm for 15 minutes.

6.6 Pipette off aqueous (top) layer and discard.

6.7 Transfer organic (bottom) layer to a new test tube and evaporate to dryness under nitrogen (no heat).

6.8 Reconstitute residue in 100 µL 20 mM Ammonium acetate buffer (pH 4.0):ACN (80:20).

6.9 Transfer reconstituted residue to a Waters ACQUITY UPLC™ using a Waters 150 µL insert.

6.10 Analyze by UPLC™/MS/MS (See LSD LC_MS_MS SOP).

7. QC and Analytical Results-

7.1 Data collection and peak integration is performed using the MassLynx™ version 4.0 software (Waters Corporation, Milford, MA).

7.2 Calculate the ion ratios using the MassLynx™ version 4.0 software (Waters Corporation, Milford, MA).

7.3 For positive confirmation the ion ratios of the case should be within ±30% for the calibrators.

Ion ratios: LSD = 223/208, 223/281
Iso-LSD = 281/208, 281/223
Nor-LSD = 209/237, 209/74
O-H-LSD = 237/222, 237/313
LSD-D3 = 226/208, 226/281

7.4 Relative retention times of unknowns should be within 2% of the calibrators.

7.5 The lowest calibrator must be at least 5 times the response of the blank.

7.6 This is a qualitative method only. Quantitative results are not given for urine.

8. Method Validation Including References -

8.1 [FDA] Food and Drug Administration: Guidance for Industry: Bioanalytical Method Validation, 2001.

8.2 [SOFT/AAFS] Society of Forensic Toxicologist and American Academy of Forensic Sciences: Forensic toxicology laboratory guidelines, draft 2006 version.

8.3 Canezin, J., Cailleux, A., Turcant, A., Le Bouil, A., Harry, P., and Allain, P.: Determination of LSD and its metabolites in human biological fluids by high-performance liquid chromatography with electrospray tandem mass spectrometry. J Chromatogr B 765: 15-27, 2001.

8.5 Sklerov, J. H., Magluilo, J., Jr., Shannon, K. K., and Smith, M. L.: Liquid chromatography-electrospray ionization mass spectrometry for the detection of lysergide and a major metabolite, 2-oxo-3-hydroxy-LSD, in urine and blood. J Anal Toxicol 24 (7)**:** 543-9, 2000.

9. Appendices - N/A

Appendix C

Title: LSD, iso-LSD, nor-LSD, and O-H-LSD UPLC/MS/MS Analysis

Identification/Filename of this SOP: LSD LC_MS_MS SOP.wpd

Scope: This SOP describes conditions used for UPLC/MS/MS analysis of LSD, iso-LSD, nor-LSD, and O-H-LSD extracts.

Written/Revised by:	Authorized by:	Bench copies
Name: A. Chung **Position:** **Date**	**Name:** **Position:** **Date:**	**How many?_____** **Located?_____**

1. Associated SOPs -

1.1 LSD Quant Blood…
1.2 LSD Urine SOP...
1.3 LCMS_confirm…

2. Definitions -

2.1 LSD - lysergic acid diethylamide
2.2 iso-LSD - lysergic acid diethylamide isomer
2.3 nor-LSD - N-demethyl lysergic acid diethylamide
2.4 O-H-LSD - 2-oxo-3-hydroxy lysergic acid diethylamide
2.5 ESI+ - electrospray ionization positive
2.6 MS - mass spectrometer
2.7 MRM - multiple reaction monitoring
2.8 UPLC™/MS/MS - ultra performance liquid chromatography tandem mass spectrometry
2.9 BEH - bridged ethyl hybrid

3. Safety -

3.1 Routine laboratory safety guidelines for handling biohazardous materials are followed.

4. Equipment Used -

4.1 Waters Corporation (Milford, MA) Acquity UPLC™ with an autosampler.
4.2 Waters Corporation (Milford, MA) Quattro Premier™ tandem quadrupole mass spectrometer.
4.3 Acquity UPLC™ BEH C18 (2.5×50 mm, 1.7 µm, Waters Corporation, Milford, MA) column.

5. Reagents Needed -

5.1 **Mobile Phase:**

Solvent A: 20 mM Ammonium acetate buffer (pH 4.0)
Dissolve 1.54 g ammonium acetate and dissolve in 800 mL NANOpure water, then adjust pH to 4.0 (± 0.1) using glacial acetic acid. Make up to 1 L with NANOpure water. Thoroughly mix then filter and sonicate

Solvent B: Acetonitrile (HPLC grade)

5.2 **Seal Wash** - Acetonitrile:water (10:90)

5.3 **Weak Needle Wash** - Acetonitrile:water (10:90)

5.4 **Strong Needle Wash** - Acetonitrile:water (75:25)

6. Procedure –

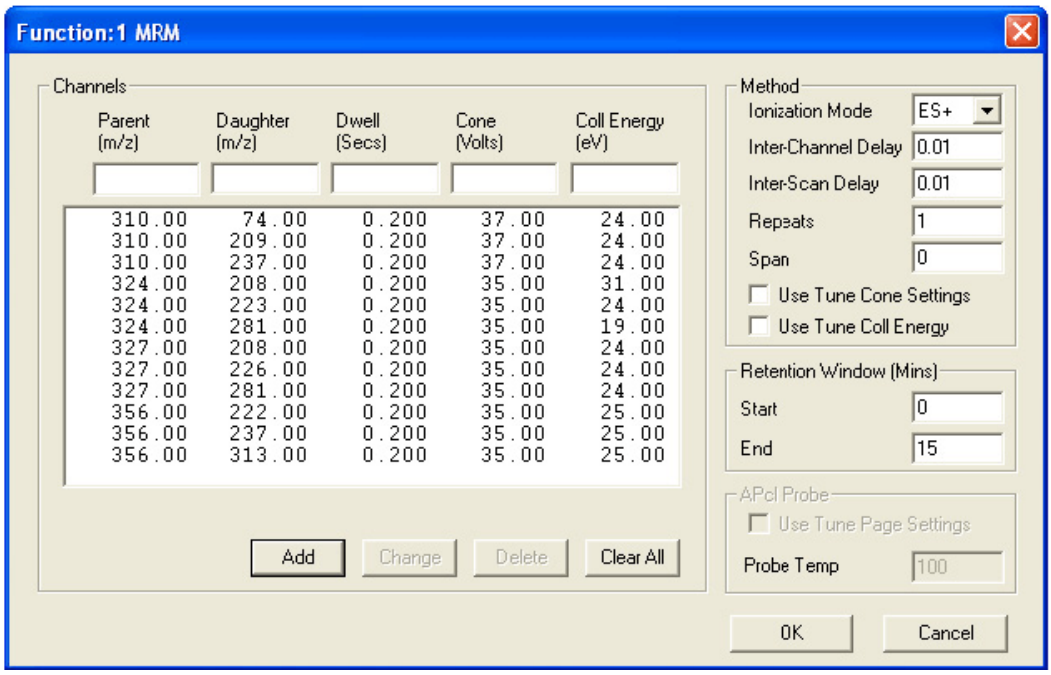
6.1 ESI Source Conditions:

Ion Mode -	ESI+
Capillary (kV) -	3.0
Cone (V) -	MRM Specific
Extractor (V) -	6.0
RF Lens (V) -	0.2
Source Temperature (^{B}C) -	120
Desolvation Temperature (^{B}C) -	350
Cone Gas Flow (L/Hr) -	110
Desolvation Gas Flow (L/Hr) -	750

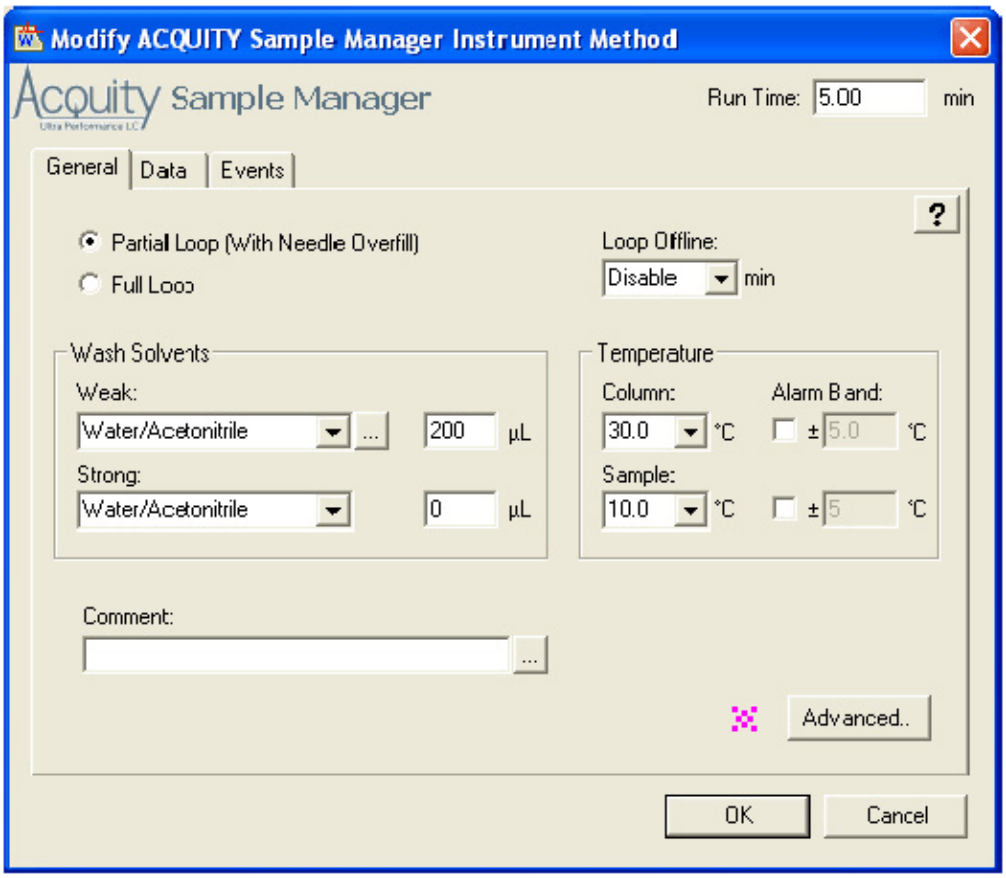
6.2 MS Analyzer Conditions:

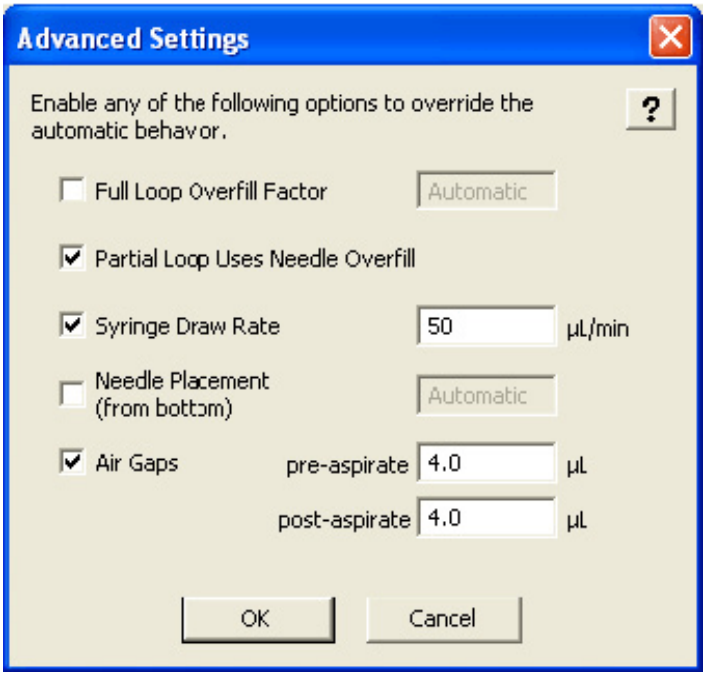
LM Resolution 1 -	11.0
HM Resolution 1 -	11.0
Ion Energy 1 -	1.0
Entrance -	5.0
Collision -	MRM Specific
LM Resolution 2 -	11.0
HM Resolution 2 -	11.0
Ion Energy 2 -	2.1
Multiplier -	650
Collision Gas Flow (mL/min) -	0.28
Collision Gas Pressure (mbar) -	4.21×10^{-3}
Source T-Wave Parameters -	Automated
Collision Cell T-Wave Parameters -	Automated

6.3 MS Tune Parameters:



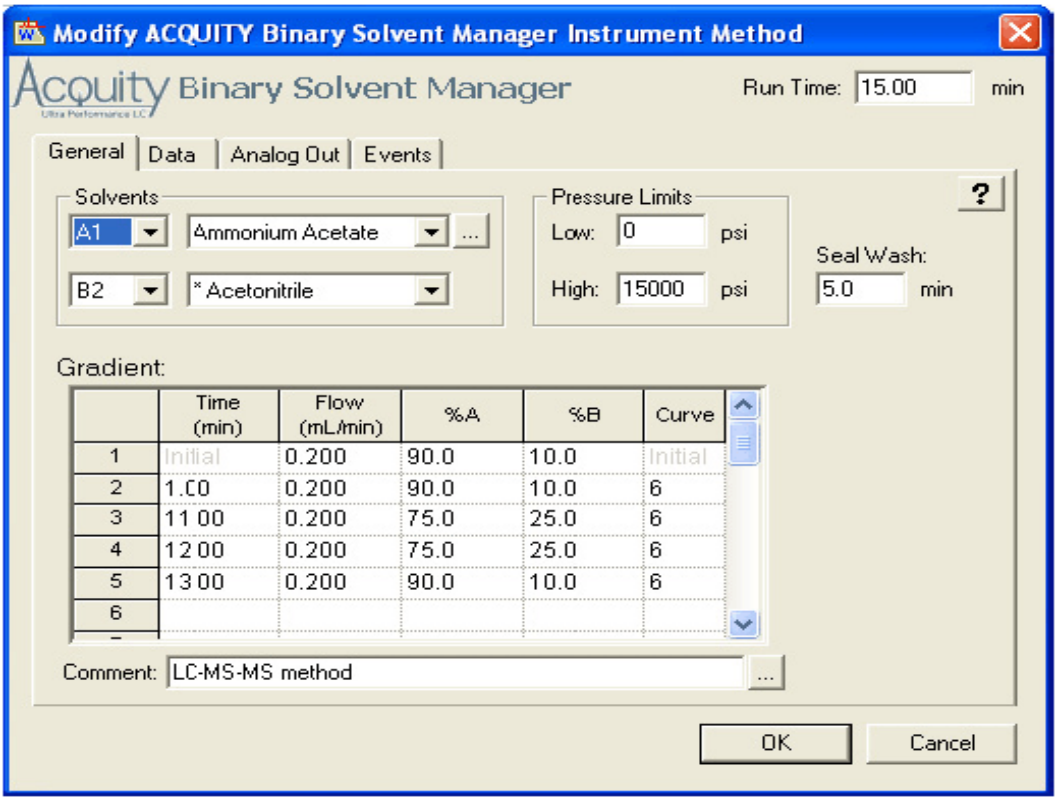
6.4 Sample Conditions:





6.5 Solvent Manager Conditions:

Injection Volume (µL): 20



7. QC and Analytical Results-

7.1 Before any analytical work is attempted, do a signal to noise check (LCMS_confirm SOP) to ensure LC/MS/MS is properly working.

7.2 When switching over solvent systems, perform seal wash, syringe wash, and prime all lines with appropriate solvents.

8. Method Validation Including References -

8.1 N/A

9. Appendices - N/A

Appendix D

LSD ELISA Cross Reactivity

Since it has been found that O-H-LSD, nor-LSD, and iso-LSD are found along with LSD in the samples of people taking LSD, their reactivity with the LSD direct ELISA kit was tested. The RCMP FLS uses the Immunalysis LSD direct ELISA kit (Pomona, CA) to screen for LSD in both urine and whole blood. The Immunalysis LSD direct ELISA kit is based on the competitive binding to antibody of enzyme labelled antigen and unlabeled antigen. The procedure in outlined in Figure D1.

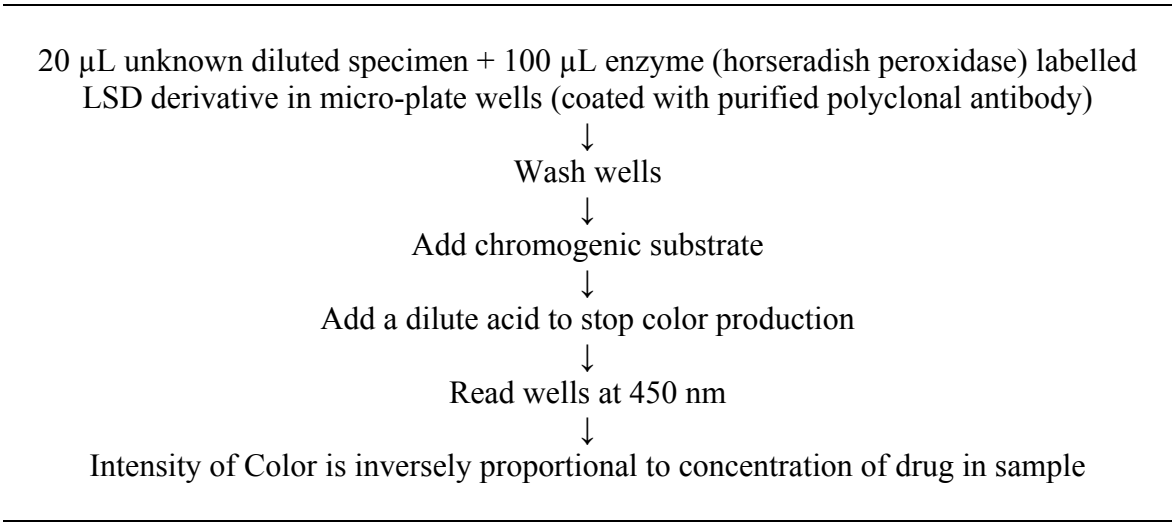


Figure D1: Immunalysis LSD direct ELISA kit procedure.

Varying concentrations of LSD (0.02, 0.05, 0.1, 0.4, 0.8, 1.6 ng/mL), a 2 ng/mL LSD test mix (containing LSD, iso-LSD, nor-LSD, and O-H-LSD), and 2 ng/mL of iso-LSD, nor-LSD, and O-H-LSD each alone in both blood and urine matrix were analyzed via ELISA. Results are summarized in Table D1. The averaged absorbance must be equal to or less than the averaged absorbance of the cutoff level (0.05 ng/mL in whole blood, and 1 ng/mL in urine) to be reported as positive for LSD.

O-H-LSD and iso-LSD in both whole blood and urine at 2 ng/mL did not cross react. The method is reported to be sensitive to 0.05 ng/mL (Immunalysis 2001), and the absorbance of O-H-LSD and iso-LSD in both whole blood and urine were above the

0.05 ng/mL LSD absorbance. Nor-LSD at 2 ng/mL in whole blood resulted in a positive result with an absorbance close to the 1 ng/mL positive control. In urine, the absorbance of nor-LSD at 2 ng/mL was above the 1 ng/mL cutoff, but below the 0.05 ng/mL LSD absorbance. The LSD test mix tested positive and the presence of iso-LSD, nor-LSD, and O-H-LSD did not suppress the reactivity of LSD. The results showed that LSD needed to be present to test positive, with the exception of nor-LSD. The concentration of nor-LSD has been found to be inconsistent, so although nor-LSD cross reacted its presence without LSD present is uncommon (Poch et al. 1999).

Table D1: ELISA results of LSD, iso-LSD, nor-LSD, and O-H-LSD in whole blood and urine.

ng/mL (n = 2)	Whole Blood		Urine	
	Absorbance	Result	Absorbance	Result
LSD negative control	2.509		2.231	
LSD cut off level (0.05 – whole blood, 1.00 – urine)	0.981		0.822	
LSD positive control (1.00 – whole blood, 2.00 – urine)	0.723		0.367	
0.02 LSD	2.089	-	2.039	-
0.05 LSD	1.922	-	1.665	-
0.10 LSD	1.761	-	1.513	-
0.40 LSD	1.086	-	1.375	-
0.80 LSD	0.690	+	0.837	-
1.60 LSD	0.543	+	0.612	+
2.00 LSD test mix	0.382	+	0.489	+
2.00 iso-LSD	2.166	-	2.086	-
2.00 nor-LSD	0.745	+	0.932	-
2.00 O-H-LSD	2.046	-	1.987	-

(-) negative, (+) positive