LIQUID CHROMATOGRAPHY – MASS SPECTROMETRY QUANTITATION: APPLICATIONS AND METHODS

By

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ABSTRACT

Accurate quantitation of analytes in simple or complex matrices is imperative for detailed understandings of biological or synthetic systems and is also necessary to ensure consumer safety with regards to food, pharmaceutical formulations, and environmental hazards. Liquid chromatography-mass spectrometry (LC-MS) is capable of completing such tasks using a variety of quantitative methods. In Chapter 1, these methods are presented with regards to chemical warfare agent studies.

External calibration, arguably the simplest of these techniques, quantifies an analyte by comparing the analyte's response in the sample matrix to the analyte's response in standard solutions. The success of this method hinges upon the consistency of the analyte's response between the blank and matrix samples. The work in Chapters 2 and 3 is an investigation into causes of inconsistency in an analyte's LC-MS response, namely matrix effects and nonspecific vial adsorption. Several case studies were performed highlighting the importance of incorporating vial adsorption studies into the method development stages of external calibration experiments.

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CHAPTER 1

INTRODUCTION TO LC-MS QUANTITATIVE METHODS

1.1 INTRODUCTION

The use of liquid chromatography-mass spectrometry (LC-MS) has skyrocketed since the invention of electrospray ionization (ESI)[1,2] and atmospheric pressure chemical ionization (APCI)[3]. These interfaces allow for the LC to be easily coupled to the MS, creating a powerful instrument capable of both separation and identification of a wide range of target compounds in an assortment of sample matrices. LC-MS can also be utilized to provide quantitative information using a variety of techniques. Quantitative applications of LC-MS are quite diverse including quality control for food[4], environmental[5], and pharmaceutical samples[6]; proteomic[7], metabolomics[8], and genomic[9] studies; biomedical diagnostic tests[10]; and forensic investigations such as toxicology[11]. A specific application falling under the previously listed categories is chemical warfare agent quantitation. The following encompasses a review of LC-MS quantitative analysis with respect to chemical warfare agents.

Chemical warfare is not a new phenomenon. It has been documented as early as 3000B.C.E. when the Egyptians investigated the lethal effects of plant poisons[12]. Large scale usage began during World War I with the development of several chemical warfare agents (CWAs)[12]. Chemical weapons used during this era had distinct odors or colors and could be detected by the human senses[13]; however, as new agents were created, this method of detection was deemed insufficient, and better ways to protect soldiers and civilians became imperative. Although detection of CWAs is the first goal when identifying a chemical warfare attack, quantitative data is also necessary to assess hazard levels, assist with containment and

decontamination, and determine the cause of death[14,15]. Consequently, improved techniques were developed to not only detect and identify chemical agents, but to provide quantitative information as well.

One of the first detection methods developed was treated paper that could indicate the presence of a specific chemical. Several types of detector paper were created; some of the most popular being M8 and M9[14]. These papers turned different colors in the presence of various chemicals including some CWAs[16]. There are several major downfalls to using indicator paper. The first being that the paper can give many false positives because the color change can take place in the presence of chemical interferents or due to prolonged exposure to heat[16]. In addition, indicator paper is not quantitative and only applies to liquid chemicals.

Due to the need for more accurate detection of CWAs, a new technique started gaining popularity; ion mobility spectrometry (IMS). IMS was first introduced in the 1960's with the first CWA application occurring in the 1970's[17]. IMS separates ions based on their drift times in a buffer gas operated in an electric field where ions are separated based on their mass and charge, temperature, and the properties of the buffer gas[18]. Hand held IMS instruments have been developed that offer many advantages such as on-site and real time monitoring, and IMS is inexpensive when compared to other CWA monitoring techniques[19]. Hand held IMS instruments, such as the chemical agent monitor (CAM) and improved chemical agent monitor (ICAM), sample the air for CWA vapors and can be used to provide relative quantitation levels for airborne CWAs [14]. However IMS instruments have limited selectivity due to similar drift times between ions, and response is affected by changes in humidity and temperature, making CWA quantitation difficult in extreme environments and complex matrices[20].

Gas chromatography- mass spectrometry (GC-MS) is the primary instrumental method

for detecting CWAs both in the Organization for the Prohibition of Chemical Weapons (OPCW) mobile laboratory and in off-site laboratories[21,22]. This technique offers both molecular mass information from chemical ionization (CI) and structural information from electron impact ionization (EI) making it the only approved stand-alone detection and quantitation technique by the Chemical Weapons Convention for on-site analysis of CWAs[23,24]. GC-MS is also the most widely adopted approach because it has high selectivity, low limits of detection (LOD), and can be used with spectral libraries[25]. Although many of the CWAs themselves are volatile, making them directly applicable to GC-MS, most readily degrade in aqueous matrices leading to the need for retrospective detection and quantitation in these instances[26]. The majority of the small molecule degradation products are not volatile making derivatization necessary[27]. Derivatization can cause loss of the analyte, giving rise to error in quantitative methods[27]. Many of the matrices commonly under investigation in CWA studies are aqueous which requires matrix exchange before analysis by GC-MS[28]. Additionally, GC-MS is not directly amenable to large CWAs, such as ricin, or to CWA adducts with large molecules such as proteins[28].

Using LC-MS for CWA analysis in off-site laboratories offers a solution to some of the problems associated with analysis by GC-MS. Analytes used in liquid chromatography do not have to be volatile, alleviating the need for derivatization and allowing for direct investigation of nonvolatile CWAs or their degradation products. Aqueous matrices also do not have to undergo exchange to a more suitable solvent. Additionally LC-MS is applicable to analytes with larger molecular masses which opens up the possibility to quantify large CWAs and CWA adducts that are not able to be analyzed directly with GC-MS. As previously mentioned, many CWAs rapidly degrade in aqueous environments which makes their direct analysis by LC-MS a challenge, but the ability to directly analyze their nonvolatile products helps circumvent this problem[29].

Consequently LC-MS is best suited for retrospective studies or studies involving large analytes.

The system suitability of LC-MS is comparable to other analytical techniques for CWA studies. Recent advances of LC-MS have achieved LODs similar to those of GC-MS[30]. Another concern is whether or not LC-MS is able to achieve accuracy and precision levels that fall within an acceptable ±15% range. Several quantitation studies for CWAs have already been successful in reaching that goal[31,32,33]. However, at this time LC-MS is not accepted as a stand-alone technique for detection and quantitation of CWAs and must be supported with a complimentary method[34]. This chapter gives an overview of degradation products, metabolites, and adducts formed by CWAs, the matrices these compounds are frequently encountered in, followed by a more detailed perspective on how available LC-MS quantitation methods are used with CWAs and their associated matrices.

1.2 CHEMICAL WARFARE AGENTS AND THEIR ASSOCIATED MATRICES

CWAs may be found in many different matrices, which can make detection and quantitation difficult. Furthermore, CWAs and the means to manufacture them are becoming more available to the general public[35]. In addition to chemical attacks, CWAs can also be released accidently from chemical stockpiles[36]. The North Atlantic Treaty Organization (NATO) has identified three time intervals in which CWAs need to be monitored: before, during, and after exposure[23]. Prior to CWA introduction, the analysis system serves as an early warning of toxic chemical release. Once a threat has been detected, the instrument can determine the concentration of the CWA which allows a responder to determine if the hazard poses an incapacitating or lethal effect. In addition to knowing the identity of the agent and exposure area, concentration information would also help responders determine what level of protection is

necessary, how long a worker could be safely in the environment, and what types of decontamination would need to be employed[14]. Additionally, the system should be capable of testing human samples to retrospectively confirm exposure to a particular agent. During these different time periods, CWAs would be encountered in an assortment of simple and complex matrices. LC-MS systems have been used to successfully quantify some CWAs, their degradation products, adducts, or metabolites in a variety of matrices as can be seen in Table 1.

1.2.1 Environmental Matrices

The environment is a principal source of matrices for CWA quantitation. CWAs can be released into the environment either intentionally or accidentally. Intentional release can occur by several pathways, including aerosol spraying, dispersion by an explosion, heat induced vaporization, surface contamination, or adulteration of food or water[37,38]. All of these release methods can result in the analyte being present in one or many different matrices, and every matrix presents a unique challenge to an analytical method. Recent studies have investigated CWA detection and quantification in a variety of matrices including snow[39], office media[40], beverages[33], battlefield terrain[41], clothing[42], toxic waste[43], fuel[44], and food[45]. In addition to the previously noted release mechanisms, accidental release can also occur when chemical agent stockpiles are spilled or they leach into the environment. In these cases, concentration levels would need to be determined in matrices such as soil, water, or munitions samples[46]. Even though LC-MS has not yet been used to quantify CWAs in all of these matrices, it is a viable option.

Table 1 LC-MS Quantitation Studies

Substance(s)	Matrix	Method	Reported LOQ[†]	Linear Range	Polarity	Mode	Ref
VX*	plasma	structural analogues for IS*	2 pg/mL	0.5-100 pg/mL	positive	MRM*	47
VX	plasma, blood	combination[‡]	0.5 pg/mL	0.5-300 pg/mL	positive	MRM	48
VX	blood	combination	750pg	0.5-2000 pg/mL	positive	MRM	49
VX	micro-dialysate	matrix-matched	0.002 ng/mL	0.002-1 ng/mL	positive	SRM	50
VX	exposure discs	SIL IS	-	5.0-1000 ng/mL	positive	MRM	51
i-BuMPA EMPA, IMPA, CMPA, PMPA	urine	combination	-	1-200 ng/mL	negative	SRM	32
EMPA, i-BuMPA, CMPA, IMPA, PMPA	urine	combination	0.5 ng/µL	1-200 ng/µL	negative	MRM	52
IMPA, CMPA	plasma	SIL IS	5 ng/mL	5 - 125 ng/mL	negative	MS[1]	53
IMPA	serum	external, SIL IS	-	-	both	MRM	54
MPA, IMPA, EMPA, PMPA, CMPA, TDG, TDGO, TDGO2	urine, saliva	IS	-	10-500 ng/mL	positive	MRM	55
MPA	water	SIL IS	0.25 mg/kg	-	negative	MS[1]	56
EMPA, IMPA, PMPA	water	combination	10, 5, 5 ng/mL	10-1000, 5-1000, 5-1000 ng/mL	positive	MS[2]	57

Substance(s)	Matrix	Method	Reported LOQ†	Linear Range	Polarity	Mode	Ref
IMPA, iBuMPA, PMPA	water	structural analogues for IS	-	5-30 µg/mL	positive	MS^n	58
EMPA, IMPA, PMPA	water	structural analogues for IS	0.2, 0.12, 0.03 µg/L	0.2-9, 0.12-12, 0.03-20 µg/L	negative	MS^1	59
MPA, EMPA, IMPA, CMPA, i-BuMPA, & PMPA	water, soil	external	-	0.1-10 µg/mL (except MPA 1-20 µg/mL)	negative	SIM	46
MPA, EMPA, IMPA, CMPA, & PMPA	juice, water, cola, & milk	matrix matched & SIL IS	0.25, 0.5, 0.25, 0.25, 0.05 ng	0.05-5 µg/mL	both	MRM	33
albumin adduct with GB, GD, and VX	plasma	combination	0.01, 0.01, 0.05 ng/mL	0.1-1000, 0.5-1000, 0.1-160 ng/mL	positive	MRM	31
BuChE adducts w GB, GF, VX, VR	serum	SIL IS	-	0.8-630 ng/mL	positive	MRM	60
BuChE adducts w GB, GF, VX, VR	plasma	SIL IS	-	1.0-510 ng/mL	positive	MRM	61
BuChE-VX	serum	SIL IS	-	4.0-510 ng/mL	positive	MRM	62
BuChE-VX	water, hamburger, soil	SIL IS	-	0.025 - 4.0 ng/mL	positive	MRM	63
SMO, TDG, TDGO, SBMTE, MSMTESE, SBMSE, SBSNAE	plasma	combination	0.05, 5, 0.5, 0.05, 0.01, 0.01, 1 µg/L	0.05-500, 5-500, 0.5-500, 0.05-500, 0.01-500, 0.01-500, 1-500 µg/L	positive	SRM	64

Substance(s)	Matrix	Method	Reported LOQ†	Linear Range	Polarity	Mode	Ref
SBMSE, MSMTESE	urine	combination	-	0.1-100 ng/mL	positive	SRM	65
SBMSE, MSMTESE	urine	combination	10, 11 ng/mL	5-200ng/mL	positive	SRM	66
SBMTE	urine	combination	0.08 ng/mL	0.1-100 ng/mL	positive	MRM	67
SBSNAE	urine, plasma	matrix-matched	-	0.5-5000 & 30-3000 ng/mL	negative	MRM	68
albumin adduct with HD	blood	combination	4.5 nM	0.1 to 10 μM	positive	MRM	69
albumin adduct with HD	blood	combination	4.5 nM	5 - 30nM & 100-1000 nM	positive	MRM	70
proteins affected by HD	cell cultures	SILAC	-	-	both	MS^2	71
EDEA, MDEA	urine	combination	30 pg	1-500 ng/mL	positive	MRM	72
EDEA, MDEA	urine	SIL IS	0.947, 2.23 ng/mL	1.6-270 ng/mL	positive	MRM	73
EDEA, MDEA, TEA	urine	combination	1, 3, 10ng/mL	1-500ng/mL	positive	MRM	74
TEA, EDEA, MDEA	wipes	external	73.2, 9.8, 39.3 ng/mL	10-500 ng/mL	positive	MRM	75
CVAA, CVAOA	urine	matrix-matched	-	0.5-1000, 5.5-1090 ng/mL	negative	MRM	76
CVAA, CVAOA	urine	combination	4.2, 4.6 μg/L	9.09–2895 μg/L	-	evacu-ated cell	77
CVAA, CVAOA	urine	external	-	1–100, 0.5–100 ppb	-	dynamic reaction cell	78

Substance(s)	Matrix	Method	Reported LOQ†	Linear Range	Polarity	Mode	Ref
STX	urine	SIL IS	-	4.8-145.2 ng/mL	positive	MRM	79
STX	water	external	634 ng/L	100ng/L to 10µg/L	positive	MRM	80
STX	algae	structural analogues for IS	11ng/mL	5-50 & 25-200ng/mL	positive	MS^2	81
STX	algae	standard addition	1789 pg	10-100 ng/mL	positive	MRM	82
STX	algae	external	-	1-50ng	positive	MID	83
STX	mussel extract	external	-	2.87–8.61 ng	positive	SRM	84
STX	seafood	external	-	0.3–64ng	positive	SIM	85
STX	seafood	matrix-matched	5.7 µg/kg	38.8-194 ng/mL	positive	MRM	86
ricin	protein digest	SIL IS	-	10-90 fmol/µL	positive	MRM	87
ricin	water, milk, juice	combination	-	10 – 10,000 fmol/mL	positive	PIM	88
ricinine	urine	combination	0.083 ng/mL	0.083-832 ng/mL	positive	MRM	89
ricinine	urine, blood, vitreous humour	structural analogue for IS	0.2 ng/mL	2-30ng/mL	positive	MRM	90
ricinine	serum, urine	combination	-	15-60, 14-60 ng/mL	positive	MS^1	91
ricinine	urine	combination	-	0.08-150 ng/mL	positive	MRM	92
ricinine	feed	external	15 pg	5-1000 ng/mL	positive	SIM	93

† The LOQ's reported here are identical to those published in each study. Various definitions were used to determine the LOQ, and in some cases, the method used to determine LOQ resulted in an LOQ that was higher than the reported linear range. For definitions please refer to the referenced articles.
‡ Combination indicates the use of both matrix-matched and an internal standard as explained in section 1.4.7.

* MPA, methylphosphonic acid; EMPA, ethyl methylphosphonic acid; IMPA, isopropyl methylphosphonic acid; CMPA, cyclohexyl methylphosphonic acid; PMPA, pinacolyl methylphosphonic acid; i-BuMPA, isobutyl methylphosphonic acid; EDEA, N-ethyldiethanolamine; MDEA, N-methyldiethanolamine; SBSNAE, 1,1 -sulfonylbis-[2-S-(N-acetylcysteinyl)ethane]; SBMSE, 1,1 -sulfonylbis-[2-(methylsulfinyl)ethane]; MSMTESE, 1-methylsulfinyl-2-[2-(methylthio)ethylsulfonyl]ethane; CVAA, 2-chlorovinylarsonous acid; CVAOA, 2-chlorovinylarsonic acid; TEA, N-triethanolamine; STX, saxitoxin; SMO, bis-β-chloroethyl sulfoxide; TDG, thiodiglycol; TDGO, thiodiglycol sulfoxide; SBMTE, 1,1 -sulfonylbis[2-(methylthio)ethane]; TDGO2, thiodiglycol sulfone; BuChE, butyrylcholinesterase; GA, tabun; GB, sarin; GD, soman; GF, cyclosarin; HD, bis(2-chloroethyl)sulfide; SIL, stable isotopically labeled; IS, internal standard; MRM, multiple reaction monitoring; SIM, single ion monitoring; SRM, single reaction monitoring; MID, multiple ion detection.

1.2.2 Pharmaceutical Matrices

Another target matrix is pharmaceuticals. Several cases of intentional contamination of pharmaceuticals with CWAs have already been documented[94]. Although analyses of pharmaceuticals tainted with CWAs have been limited, LC-MS has been used to evaluate other types of contaminants and impurities in pharmaceutical samples making it relevant to CWAs[95].

1.2.3 Biological Matrices

LC-MS quantitative analysis is particularly applicable to biological matrices. Although most quantitative studies in biological matrices to date focus on studying the toxicity of the agent, another goal of LC-MS analysis is the accurate identification and quantitation of CWAs or their metabolites to help confirm an individual's exposure to a CWA. To date the majority of the confirmations have taken place in post mortem samples[96]. The most common matrices to test CWA concentration levels after exposure are blood and urine due to their ease of collection[97]. Through a variety of analytical means, CWAs or their related products have been identified in tissue samples[98], saliva[55], skin[99], and blister fluids[100], however quantitation using these matrices may not provide an accurate reflection of an individual's exposure level. In many of these matrices, the CWA degrades, forms an adduct, or is metabolized, making direct quantitation of

the CWA unrealistic, and an alternative compound for quantification must be chosen.

1.3 CHEMICAL WARFARE AGENTS, DEGRADATION PRODUCTS, METABOLITES, AND ADDUCTS

A chemical warfare agent is defined by the OPCW as any toxic chemical or its precursor not intended to be used for peaceful purposes such as for completing industrial, agricultural, pharmaceutical, research, or protective goals[101]. The OPCW has divided these toxic agents into schedules associated with their hazard class[101]. These schedules can be further broken down into the types of toxicological effects the agents have on the body. Substances in schedule one are considered the most toxic. Schedule one contains chemicals that fall into the categories of nerve agents, blister agents, and toxins. Many of the chemicals found in these categories are very reactive, making detection of the substances themselves a challenge and accurate quantitation impractical. Consequently many LC-MS based quantitation methods do not focus on quantifying the analyte directly, but instead use a more long lived degradation product, metabolite or adduct to retrospectively determine the concentration of the CWA. It should be noted that the production of degradation products, metabolites, and adducts for a particular CWA vary according to several factors including duration and method of exposure, dosage, and sample collection time, presenting a challenge when selecting an analyte to retrospectively quantify a particular CWA. Several comprehensive reviews have been written outlining degradation products, metabolites, and adducts used to identify CWAs[102,103, 29]. As a result, this section will only give a brief overview of CWA related compounds that have been used in LC-MS quantitative studies.

1.3.1 Nerve Agents

One of the most frequently studied groups of chemical agents is nerve agents. Nerve agents are organophosphorus compounds that irreversibly bind to the enzyme acetylcholinesterase (AChE)[104]. AChE hydrolyses the neurotransmitter acetylcholine (ACh)[104]. Excess ACh can result in overstimulation of muscles and eventually lead to paralysis[32]. Nerve agents can be split into two separate classes; the G-series and V-series. Commonly studied schedule one substances in the G-series include tabun (GA), sarin (GB), soman (GD), and cyclosarin (GF). While the most frequently studied agents in the V-series are VX and Russian VX (VR).

Organophosphorus nerve agents are highly reactive compounds making their degradation products the main targets during quantitative studies. In both environmental and biological matrices, hydrolysis products of the nerve agents are key degradation products to determine the extent of nerve agent exposure[103]. As can be seen in Figure 1, GB, GD, GF, VX, and VR undergo hydrolysis resulting in their corresponding alkyl methylphosphonic acid (AMPA)[59]. When these degradation products undergo a second hydrolysis reaction, the result is methylphosphonic acid[59]. At this stage none of the nerve agents can be distinguished from one another, however the rate of the secondary hydrolysis reaction is slow[26]. Additionally, in solutions with an alkaline pH, VX can also form a secondary hydrolysis product resulting from the cleavage of the O-C bond instead of the S-C bond[48]. GA can also undergo hydrolysis, but unfortunately its initial hydrolysis products are unstable and have not been used in any LC-MS quantitation studies[102]. Unlike using a GC, an LC based system does not require derivatization of AMPAs prior to analysis[103].

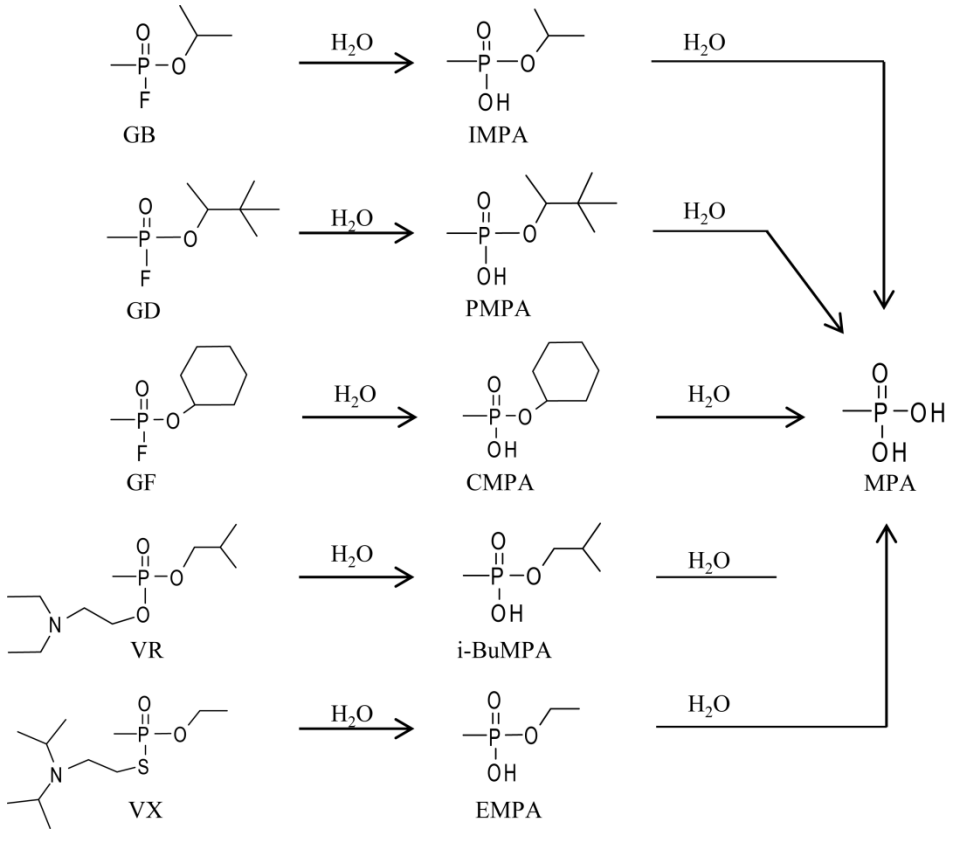

Figure 1 Primary hydrolysis products of GB, GD, GF, VX and VR are isopropyl methylphosphonic acid (IMPA), pinacolyl methylphosphonic acid (PMPA), cyclohexyl methylphosphonic acid (CPMA), isobutyl methylphosphonic acid (i-BuMPA), and ethyl methylphosphonic acid (EMPA) respectively. Secondary hydrolysis product is methylphosphonic acid (MPA) for each agent. Adapted from references 33 and 46.

Retrospective quantitation is useful in cases when samples cannot be collected right away. For retrospective detection in biological samples, the hydrolysis products are relatively short lived because they are excreted from the body after a few days[102]. In blood, nerve agents form adducts with select proteins that can be measured up to a few weeks after exposure, making them a better choice for retrospective detection[102]. Soman, sarin, cyclosarin, tabun, and VX covalently bond to a tyrosine residue in albumin[31,105,106]. After the albumin is digested, it can be analyzed directly by LC-MS, and nerve agent exposure can be evaluated. One such study has used the tyrosine adduct to accurately quantify nerve agent exposure in rats[31].

The central targets for retrospective studies in biological matrices for the nerve agents are

adducts with butyrylcholinesterase (BuChE). BuChE is an enzyme that is similar to AChE, but present in a higher abundance in human serum[107]. BuChE forms an adduct with the nerve agents by binding at serine-198 residue, inhibiting BuChE's activity[108]. A decrease in activity can be used to determine nerve agent exposure[60]. These adducts can persist for up to 16 days after exposure[108]. Measuring inhibited BuChE can take place by removing the phosphyl agent with fluoride ions and analyzing the fluorinated agent[109] or by digesting the adducted enzyme and analyzing the peptide containing the serine binding site [108]. However, the fluoride-reactivation method is impeded by spontaneous loss of alkyl groups from the attached agents making the reaction with fluoride unfavorable; a process known as aging[107]. Another problem for relating BuChE activity levels to nerve agent exposure is the starting levels may be highly variable in the general public[61]. However a few LC-MS quantitative studies have targeted BuChE for nerve agent exposure[60,63,61].

1.3.2 Blister Agents

The second group identified as schedule one chemical agents are blister agents. Blister agents, also known as vesicants, include a variety of substances that cause chemical burns to different extents[38]. Sulfur mustards, nitrogen mustards, and lewisites are blister agents that are categorized as schedule one agents. There are nine different types of sulfur mustards, three different nitrogen mustards, and three lewisites[101]. Although the exact biochemical processes behind sulfur and nitrogen mustard toxicity is unknown, these agents are believed to alkylate many cellular nucleophiles, including DNA, eventually causing cell death[110]. Lewisites bind to pyruvate dehydrogenase hindering the formation of acetyl coenzyme A[107]. Like nerve agents, blister agents are highly reactive making their degradation products targets for LC-MS studies.

Out of the nine sulfur mustards listed as schedule one agents, bis(2-chloroethyl)sulfide (HD), is the most commonly studied. Subsequently, over ten degradation products and metabolites for HD have been identified and used in LC-MS quantitative studies. Figure 2 shows a schematic of the common degradation products and metabolites of HD. In environmental and biological matrices, HD can undergo hydrolysis, oxidation, or a combination of both to produce bis-β-chloroethyl sulfoxide (SMO), thiodiglycol (TDG), thiodiglycol sulfoxide (TDGO), and thiodiglycol sulfone (TDGO2)[26]. However TDG and TDGO have been detected at relatively high concentrations in unexposed individuals which limits their ability to be unambiguous biomarkers[111,112,113]. In addition to those degradation products, in biological samples HD can be metabolized by glutathione conjugation and undergo further oxidation or hydrolysis reactions to form mono and bis N-acetylcysteine conjugates, four of which are illustrated in figure 2[26,114]. One of these products, 1,1 -sulfonylbis-[2-S-(N-acetylcysteinyl)ethane] (SBSNAE), has been isolated in urine and used for HD quantitation[68]. The bis-N-acetylcysteine conjugates can undergo further metabolism by β-lyase to form 1,1 -sulfonylbis[2-(methylthio)ethane] SBMTE, which is readily oxidized to 1,1 -sulfonylbis-[2-(methylsulfinyl)ethane] (SBMSE) and 1-methylsulfinyl-2-[2-(methylthio)ethylsulfonyl]ethane (MSMTSE)[67]. Usually SBMTE is analyzed as a result of SBMSE and MSMTSE being reduced with titanium trichloride ($TiCl_3$) to a single product[67]. However one study has quantified SBMTE directly at very low levels in rat plasma[64].

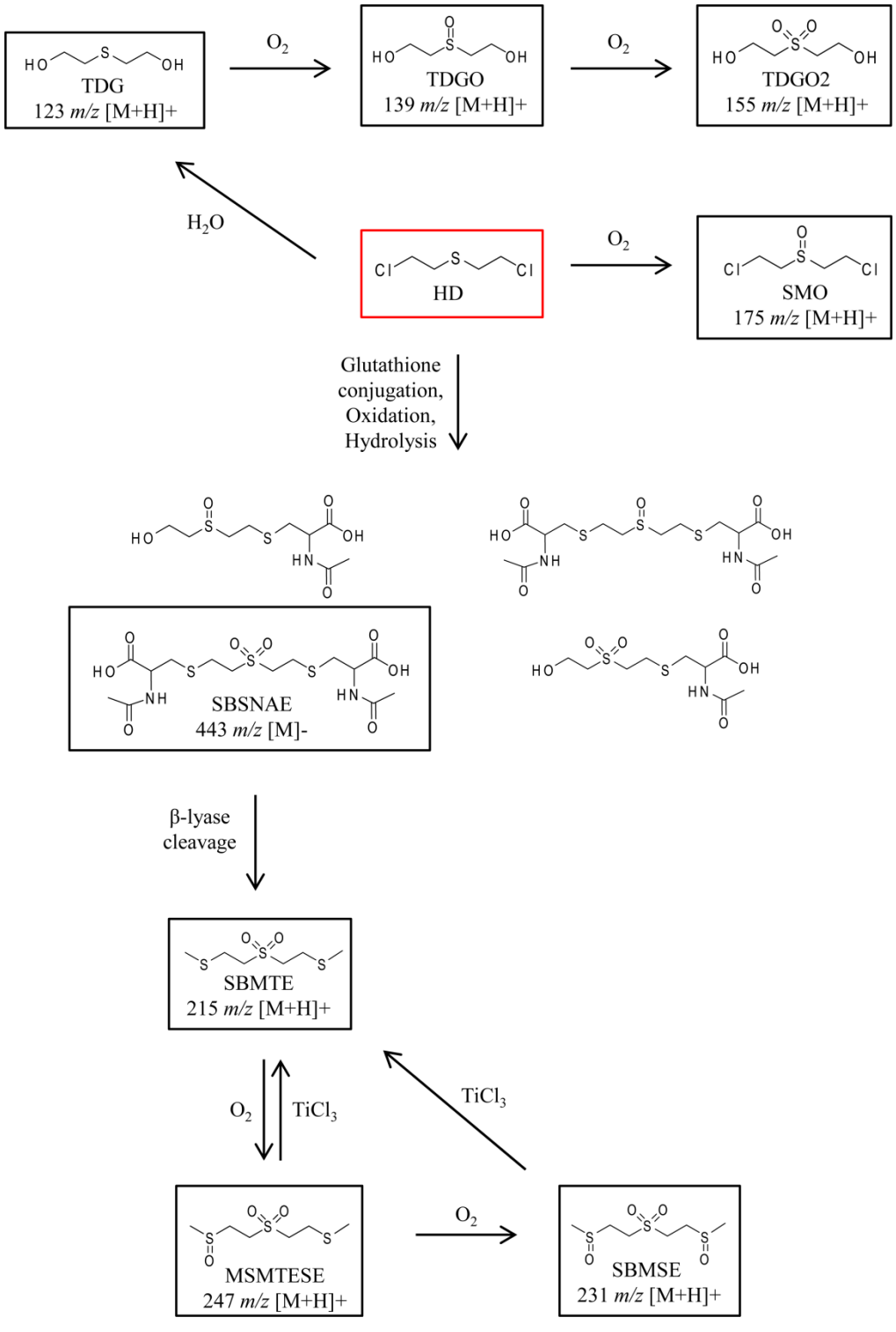

Figure 2 Schematic diagram for the degradation products and metabolites of HD, boxed in red. Analytes boxed in black have been used in LC-MS quantitation studies. Adapted from references 107 and 114.

As with the nerve agents, analytes with a longer lifetime are required to diagnose HD exposure. HD forms adducts with many nucleophiles in the body including proteins and DNA[103]. HD forms an adduct with both albumin and hemoglobin as well[115], however current LC-MS quantitation studies have only used the albumin adduct to retrospectively determine HD exposure [69].

The nitrogen mustards and lewisites are less commonly studied analytes because they pose less of a threat than sulfur mustard. A few quantitation studies have been completed on their hydrolysis products and oxidation products[76,75,72,77]. Figure 3 shows the hydrolysis products for the nitrogen mustards and figure 4 shows the hydrolysis and further oxidation products for lewisite one (L1). Nitrogen mustards hydrolyze to form their corresponding ethanolamines[102]. L1 hydrolyzes to form 2-chlorovinylarsonous acid (CVAA) and then undergoes oxidation to form 2-chlorovinylarsonic acid (CVAOA)[102]. Nitrogen mustards also form adducts with albumin, but this complex has not yet been a target of an LC-MS quantitative study[116].

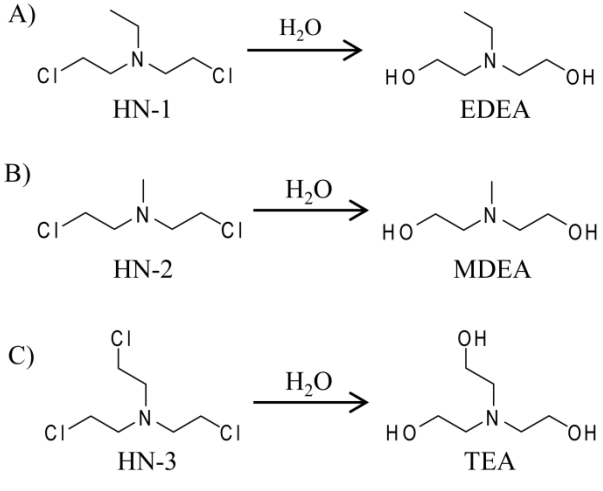

Figure 3 Primary hydrolysis products of the nitrogen mustards A) Bis(2-chloroethyl)ethylamine (HN1) hydrolyses to form N-ethyldiethanolamine (EDEA). B) Bis(2-chloroethyl)methylamine (HN2) hydrolyses to form N-methyldiethanolamine (MDEA). C) Tris(2-chloroethyl)amine (HN3) hydrolyses to form N-triethanolamine (TEA).

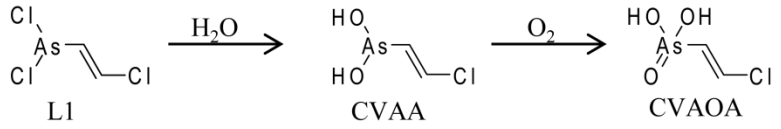

Figure 4 2-Chlorovinyldichloroarsine (L1) undergoes hydrolysis to form 2-chlorovinylarsonous acid (CVAA). CVAA is further oxidized to form 2-chlorovinylarsonic acid (CVAOA). Adapted from reference 76.

1.3.3 Toxins

Toxins, unlike the other compounds listed as schedule one CWAs, are harmful substances produced naturally in biological systems[38]. Two toxins are listed in schedule one as chemical warfare agents, the first being saxitoxin (STX). STX is a small molecule with a molecular weight of 299.29 g/mol (Figure 5). It is a potent neurotoxin that binds sodium channels, consequently blocking sodium ions from entering the cell, and eventually leading to paralysis[38]. STX falls into the category of paralytic shellfish toxins because it is secreted by marine organisms such as cyanobacteria, dinoflagellates and algae[117]. Subsequently seafood and aqueous matrices are particularly applicable for LC-MS quantitation of STX. STX does not degrade rapidly, so it can be quantified directly by LC-MS, in positive ion mode[79].

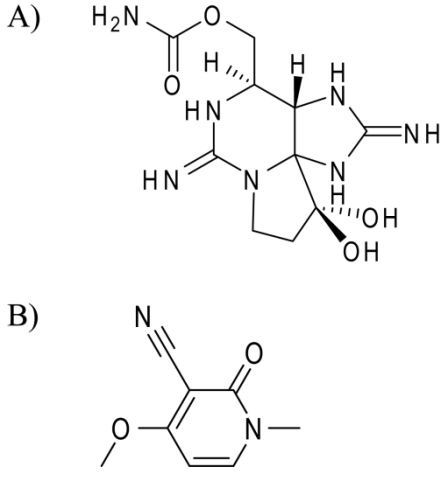

Figure 5 A) Chemical structure of saxitoxin B) Chemical structure of ricinine

The second toxin listed as a schedule one chemical warfare agent is ricin. Ricin is a glycoprotein composed of two amino acid chains held together by a disulfide bond[118]. It inhibits protein synthesis in cells eventually leading to cell death[118]. Ricin is also a stable compound; consequently, it can be analyzed directly by LC-MS after proteolytic digestion[87]. However, many quantitation studies use ricinine (Figure 5) as a biomarker for exposure to ricin[89]. Ricin and ricinine both originate in the castor bean plant and ricinine can be analyzed without the need for proteolytic digestion, so it is frequently employed as the analyte of choice over ricin. LC-MS plays a major role in studies involving STX and ricin because these analytes cannot be analyzed by GC[28].

1.4 LC-MS QUANTITATION METHODS

LC-MS (and LC-MS/MS) offers a variety of quantitation methods for CWAs. A universal technique for CWA quantitation does not exist; consequently, choosing a method can be a complicated task. Methodology choice is based on the desired application with regards to the target analyte, matrix, and available resources. Additionally the type of quantitation desired, either relative or absolute, can impact decision making in determining which method is best suited for the quantitation goal. The next section focuses on methods that have been successfully employed in quantifying CWAs. A brief description of the principles behind each technique along with the advantages, disadvantages, and successful implementations of each method is given.

1.4.1 External Calibration

LC-MS can use external standards to quantify analytes, in this approach, extracted ion

chromatograms (XIC's) are used to measure the analyte ions' abundance, and the concentration is determined using a standard curve. There are several situations and reasons where external calibration is particularly advantageous. The most important advantage to using external standards is that sample preparation can be minimized because incorporation of an internal standard is not necessary; making this technique favorable when a suitable internal standard cannot be found[119]. Additionally the sample is not made more complex by the addition of an internal standard. Since the sample is less complex, it allows for more MS/MS spectra to be gathered for the compound of interest[120]. Finally there is not a limit to the number of samples in which the amount of analyte can be quantified[120].

External calibration is considered by some to be the least accurate absolute quantitative method available which can sometimes be the case[120]. The inaccuracy is because external calibration operates on the assumption that the ion intensity for the analyte will remain constant between the standard solutions used to construct the calibration curve and the matrix samples. However, ion intensity can be suppressed or enhanced between different types of matrices affecting the accuracy and reproducibility of this technique; a phenomenon known as matrix effects[121]. Matrix effects vary between analytes, matrices, and even between different lots of the same matrices (such as blood from two different victims) making them hard to predict. Regardless of this problem, external calibration can still be used in simple or uniform matrices or when matrix effects have been eliminated, or accounted for.

Degradation products of CWAs have been quantified using the external calibration technique. An interesting external calibration study investigated different types of wipes to swab surfaces for CWA contamination[75]. Degradation products of nitrogen mustard were spiked onto various surfaces including glass, wood, drywall, and vinyl then wiped with five different types of

materials. An external calibration curve was prepared using the peak areas of the degradation products. The different wipe materials used on each surface were then compared to the curve to determine which wipe most accurately collected the degradation products and had the least amount of background interferences. Several of the wipes showed contamination with the degradation products, which could lead to false positives or inaccurate quantitation of nitrogen mustard[75]. Using external calibration, filter paper wipes accurately quantified the degradation products encouraging their use when responding to exposure incidents.

As can easily be predicted from the downfalls of external calibration, this technique is most commonly employed for CWA quantitation in simple environmental matrices; the primary matrix being water[80,46]. The majority of external calibration techniques for CWAs are targeting STX. These studies are generally geared toward finding naturally occurring STX contamination in marine based samples, such as in seafood[85] or algae[83], because this poses a more serious threat than STX's use a chemical weapon. However these methods could be adapted for chemical warfare testing.

1.4.2 Matrix-matched Calibration

Matrix-matched calibration is a branch of external calibration that seeks to account for matrix effects in the sample. Instead of measuring the analyte's response in a standard blank solution, the analyte's response is measured in the same type of matrix as the unknown sample. Ideally the analyte will experience the same matrix effects in the standards that are experienced in the unknown, compensating for any matrix effects. Consequently matrix-matched calibration offers the same advantages as external calibration, but is also able to compensate for matrix effects.

The matrix-matched calibration method has a few weaknesses. For this technique to be applicable, blank matrix has to be available, meaning matrix that does not contain the compound of interest and is the same type of matrix as the unknown sample[122]. Also this method works on the assumption that a relative matrix effect does not exist, in other words the matrix effect does not vary from lot to lot[123]. However relative matrix effects do exist, posing a potential problem with this method[123].

Matrix-matched calibration is particularly applicable for food and beverage matrices because they are easy to obtain without CWA contamination. One such study analyzed the AMPAs of sarin, soman, cyclosarin, and VX in five different beverages including bottled water, cola, whole milk, and juice[33]. Matrix-matched calibration standards were made in each of the different matrices and then prepared for LC-MS/MS analysis by using solid phase extraction (SPE). The AMPAs were analyzed with multiple reaction monitoring (MRM) in negative ion mode. The authors reported better alleviation of ion suppression using the matrix-matched calibration than using isotopically labeled internal standards[33]. Matrix-matched calibration was also used to alleviate matrix effects experienced by STX in seafood matrices[86].

Urine is easily obtained noninvasively and can be used for matrix-matched calibration in some CWA studies. Because CVAA and CVAOA are not naturally occurring in urine samples, good linearity and reproducibility can be obtained when performing matrix-matched calibration[76]. However in other instances urine is not suitable for use as a blank matrix. One example of when this occurs is with TDG and TDGO since they are present in significant and varying concentrations in urine from non-exposed individuals. To verify HD exposure using this quantitation technique in urine, it is necessary to choose a different biomarker. One possible choice is a glutathione conjugate. The concentration of SBSNAE has been successfully

determined in urine from rats exposed to sulfur mustard using matrix-matched calibration[68].

Nonvolatile compounds such as salt ions are particularly notorious for causing ion suppression in mass spectrometry[124]. Such ions are commonly present in microdialysate buffers leading to ion suppression in these samples[125]. Using a matrix-matched solution offers an alternative to compensate for ion suppression caused by a salty sample. Ringer's solution is a composite of sodium chloride, potassium chloride, and calcium chloride in water[50]. This solution was used to compensate for matrix effects in an investigation of free VX in microdialysate samples obtained using blood probes[50]. As shown by the authors, the ability to use microdialysis as a sampling technique can provide a picture of VX concentration levels in blood over a given time frame without taking multiple blood samples[50].

1.4.3 Standard Addition

The standard addition quantitation technique is a variation of matrix-matched calibration. Instead of using a blank matrix to create the calibration curve, the actual sample is used. The actual sample is divided into portions and known concentrations of the analyte are spiked into all the different portions except for one. LC-MS responses are then plotted against the added concentration, including the lot with nothing added. Linear regression is then used to extrapolate the best-fit line to zero[126]. The slope of the line and the y-intercept, the response for the unknown, can then be used to determine the concentration of the analyte in the original solution[126].

Standard addition offers many of the same benefits as matrix-matched calibration. It corrects for both relative and absolute matrix effects without the need for a suitable standard other than the analyte itself. However, unlike the matrix-matched technique, it does not require

obtaining blank matrix.

Unfortunately the standard addition technique is limited by the original sample's volume. A large starting volume is needed in order to be able to divide the sample into multiple lots. Consequently when the sample is in short supply, it might have to be diluted to obtain enough lots. In trace analysis, such as when working with CWAs, diluting the sample could cause the unknown's response to become negligible when compared to the added amounts. Subsequently standard addition works best when an ample amount of the original sample is available.

Using standard addition to quantitate CWAs is most applicable to environmental matrices because the sample volumes are not as limited as with biological matrices. One LC-MS/MS study used standard addition to quantify STX in algae samples[82]. The application of this study was intended for naturally occurring environmental contamination, however this technique could be applied to the same analyte and matrix if used as a CWA. Although quantitation of STX was not the main objective of the study, it was shown that matrix effects are present in algae samples and standard addition can be used to accurately account for them[82].

1.4.4. Internal Calibration

Quantitation via internal calibration uses the same approach as external calibration with one key difference: Internal calibration utilizes a standard added to the sampling solution in a known amount to create a response ratio. The ratio is a comparison of the mass spectrometric response between the analyte and the standard which can be determined via an XIC. Then a calibration curve is constructed by plotting the response ratio versus concentration. The curve and response ratio can then be used to determine the amount of analyte in an unknown sample. The goal of an internal standard is to account for the matrix effects, both relative and absolute,

variable recoveries, and instrumental fluctuations that might occur during trials by mimicking these changes that the analyte experiences[127]. Standards can either be structural analogues or stable isotopically labeled analogues of the compound of interest.

1.4.4.1 Structural Analogues

Structural analogues of an analyte are frequently used to establish a calibration ratio in LC-MS quantitation studies. Structural analogues are compounds that are similar to the analyte but have a different mass and are varied at one or more moieties[128]. The best internal standard is one that has the most similar ionization pattern, recovery, and retention time of the analyte[129]. Another factor to be considered when choosing a structural analogue to serve as an internal standard, is whether the analogue can be found naturally in the matrix. Since a known amount of standard is added to the sample, it should not be naturally present, or the results will be skewed. The major caveat to this technique is that all analytes do not have a structural analogue that can be adequately used to mimic matrix effects[128]. Consequently a different technique would have to be selected.

A major benefit of using a structural analogue for internal calibration is that one standard can be used for multiple analytes, if those analytes all have similar retention times and ionization efficiencies as the standard. This characteristic was recently exploited to determine the concentration of AMPAs in water samples[59]. Diethyl phosphate (DEP) was used as an internal standard for three CWA degradation products, EMPA, IMPA, and PMPA. DEP had a similar retention time and chemical characteristics to the AMPAs in this study, and offered a less expensive alternative than an isotopically labeled standard or a standard for each individual analyte making it a suitable choice. When comparing this study to a similar GC-MS method, it

can be noted that the LOD's along with the interday and intraday precision were comparable between the two methods, however using LC-MS avoids the derivatization step necessary for GC analysis[130].

Two recent CWA studies highlight the importance of selecting a structural analog that has a retention time similar to the analyte. In the first study, fentanyl-d_5 was selected as an internal standard for ricinine[90]. The chromatographic parameters used in this experiment, caused the standard and analyte to have retention times differing by over seven minutes[90]. In the blood matrix, this time difference caused the standard to experience more ion suppression than the ricinine which lead to inaccurate quantitation[90]. However in the less complex urine matrix, there was negligible ion suppression, so the large time difference was not a factor. The second study used VR as an internal standard to quantify VX in plasma[47]. Despite the complexity of the sample matrix, VR is able to be used successfully as a standard because of the structural similarity to the analyte leading to a retention time difference less than 30 seconds[47].

1.4.4.2 Stable Isotopically Labeled Standards

Stable isotopically labeled (SIL) analogues of the compound of interest can be used as internal standards (IS) for LC-MS quantitative methods. Commonly used isotopes include ^{2}H, ^{13}C, ^{15}N, and ^{17}O, although incorporating ^{2}H has been shown to alter the retention time of the standard[131]. The standard usually contains three to eight isotopes in order for the mass difference between the standard and analyte to be large enough to avoid overlap, but retain the chemical properties of the analyte[131]. Additionally the standard should have high isotopic purity to prevent overlap with the naturally occurring isotopes in the analyte[131].

Stable isotopically labeled analogues are favored over other compounds as internal

standards because of their chemical identity to the compound of interest. Chemical structure plays a role in the amount of ion suppression or enhancement a compound experiences making SIL compounds the best choice for an internal standard[132]. Since the standard and the analyte only differ by their masses, they elute from the LC at very similar times making both compounds susceptible to the same matrix components[128]. Ideally the SIL standard should experience the same ion suppression or enhancement the analyte encounters theoretically making it the most accurate method available[132].

Despite the many benefits of using a SIL standard for quantitation there are a few drawbacks. First a SIL standard is not always commercially available, and when it is, it can be expensive. Also a different standard should be used for each analyte to ensure the best results. Finally cases exist where a SIL standard has been used and it has not successfully combated the matrix effects lowering the accuracy of the method[133]. However several successful usages of this method to quantify CWAs have been completed[52,72,32].

Stable isotopically labeled standards used for internal calibration offers one of the most versatile techniques available to quantify CWAs. This method has been used in an assortment of matrices including plasma, urine, beverages, and water[53,79,33,56]. It is also applicable to a wide variety of analytes including schedule one CWAs and some of their degradation products and adducts. SIL standards are commercially available for many CWAs or their products, alleviating one of the problems associated with this method.

The SIL IS technique can be applied to determine exposure levels by monitoring the loss of an analyte. Prior knowledge of the analyte's original concentration is necessary for this application, limiting this type of study to controlled environments such as toxicological research. This application of SIL IS has been developed for observing exposure levels in monkeys[51]. The

monkeys were exposed to a known amount of VX on stainless steel discs bandaged to their skin and after exposure their skin was swabbed with a cotton swab[51]. Residual VX quantities were determined on both the discs and cotton swabs by the SIL IS method. This information was used to estimate the dose of VX absorbed and then related to the effects that were observed.

Another application of SIL IS quantitation is for bottom-up quantitation of proteins. Custom stable isotopically labeled peptides are commercially available, allowing for usage as SIL IS. Peptides resulting from protein digestion can be used to reflect the original protein's concentration. Generally, one or more peptides are selected that are unique to the protein of interest and present in a fairly high abundance. Choosing to use more than one peptide can result in a more accurate representation of the protein's concentration. One study quantified ricin by using this approach[87]. The authors' main goal was to test digestion parameters in order to ensure complete digestion[87]. Incomplete digestion changes the *m/z* of the naturally occurring peptide, which causes an underestimate of its actual concentration. This application of SIL IS is of great importance because GC-MS platforms are not able to analyze peptides.

BuChE and BuChE adducts with organophosphorus nerve agents can also undergo the digestion and subsequent quantitation using isotopically labeled peptides. One study attached antibutyrylcholinesterase antibodies to magnetic beads that captured BuChE in serum samples[60]. Both adducted and unadducted BuChE were captured and then digested. A peptide containing the binding site of the nerve agents was selected for use as a SIL internal standard because it could be used for both the adducted and unadducted compounds[60]. MRM mode was employed to allow for multiple analytes to be quantified, including adducts and the unadducted BuChE[60]. Being able to simultaneously quantify adducted and free BuChE is essential to determine the percent adducted BuChE without the need for prior knowledge of total BuChE levels[60]. This

quantitation approach effectively addresses the problem that the level of BuChE observed in different individuals is variable. Recently this process has been automated[61]. Additionally immobilized BuChE on magnetic beads can be used as a purification technique by binding to free nerve agents in an assortment of matrices; then the agents can be quantified using the previous process[63].

1.4.5 Echo Peak Calibration

A recently developed technique, known as the echo peak technique, is a branch of internal calibration, but uses the analyte itself as an internal standard. In this method, a standard is prepared using a known amount of the analyte. The standard and the unknown sample are injected consecutively within a short time frame giving rise to an echo peak[134]. Consequently the standard and analyte in the unknown sample will have close to the same retention time making both the standard and the sample's analyte susceptible to the same matrix effects[134]. To quantify the analyte, a calibration curve is constructed in the same way as the internal standard method; using a ratio of the analyte to standard's response versus concentration.

There are several assumptions made when using this method, which can lead to inaccurate quantitation. The first assumption is that the time frame where the matrix effects occur is large enough to encompass the peaks from the standard and the sample, so that both are experiencing the same level of matrix effects[135]. If the matrix effects occur only at the retention time of the one of the peaks, then ion suppression or enhancement will not be accurately accounted for. A second assumption is that the peaks do not exhibit tailing which could cause the signal of the second peak to be overestimated[134]. Sufficiently resolving the peaks from each other would solve that problem, but any increase in the time difference between the two peaks could

cause the matrix effects experienced to change.

Echo peak calibration has several benefits. Creating an echo peak does not require finding a suitable compound to use as a standard, but in most cases it is still able to compensate for matrix effects. Consequently, the method development phase for this technique is faster than using a structural analogue or SIL analogue as an internal standard[135]. Since this technique is relatively new, it has not yet been used to quantify CWAs. However, it has been used for pesticide quantitation including some organophosphate pesticides which are in the same chemical class as nerve agents[135].

1.4.6 Metabolic Labeling

If a protein or a peptide in a cell is being used for quantitation, then a branch of techniques called metabolic labeling can be employed. Metabolic labeling incorporates a stable heavy isotope to the protein/peptide of interest by growing it in a cell in isotopically enriched media[136]. A second set of samples is grown without the enriched media, and then the samples are combined and analyzed by LC-MS. Metabolic labeling techniques provide relative quantitative information meaning they do not provide the exact concentration of the analyte, but compare it's abundance between the control and experimental groups.

1.4.6.1 Stable Isotope Labeling by Amino Acids in Cell Culture

Stable isotope labeling by amino acids in cell culture (SILAC) by first described in 2002[137]. This method of metabolic labeling incorporates a stable heavy isotope to the experimental group by using media that only contains isotopically labeled essential amino acids. Since only isotopically labeled amino acids are available to the cell, the cell translates proteins

containing only isotopes. The control group is grown in media that contains natural amino acids leading to the development of heavy and light proteins. Heavy and light strains are mixed in equal ratios prior to being digested for LC-MS analysis. The heavy and light versions of the same peptide will co-elute from the LC because they have identical structures, but they can be easily distinguished by their difference in mass. By comparing the ion intensities between the labeled and unlabeled peptides, relative quantitative information can be gathered about the abundances of the two samples.

Due to the similarity between using stable isotopes for internal calibration and using them for SILAC, these techniques offer some of the same advantages. Once again the analyte and the labeled compound elute at the same time and have the same ionization efficiency essentially eliminating matrix effects as a problem. The isotopically enriched media used for SILAC is commercially available, alleviating the problem of finding an isotopically labeled standard. Additionally SILAC provides information about what is happening to the chemical agent *in vivo*.

SILAC is not applicable for many desired goals of CWA quantitation. For instance SILAC only provides relative quantitation information and frequently the absolute concentration is needed. Furthermore, SILAC can only be applied to biological samples and still those samples are limited to proteins in the cell. Also this technique is slow compared to other quantitation methods such as external and internal standard calibration because cells have to be grown. Consequently, it is not useful when rapid information is necessary such as before or during exposure to CWA. However, it is effective for toxicological studies to determine how CWAs react in the cell.

A recent study by Everley and Dillman used SILAC to investigate phosphorylation changes in cells exposed to sulfur mustard[71]. Using the light and heavy conditions, proteins were

monitored in the cell to identify which were up regulated, down regulated, or not affected by sulfur mustard exposure. Eighty-six proteins were identified that showed greater than 2-fold concentration changes, some of which had been identified in other studies, but numerous had not been previously characterized[71]. Using the relative quantitation information gathered during the experiment, Everley and Dillman were able to create a network map relating proteins with altered concentrations due to sulfur mustard exposure to the cellular processes the protein is involved in[71]. This map is useful because it provides evidence for sulfur mustard's toxicological mechanisms in cellular systems giving rise to potential therapeutic targets.

1.4.7 Combinational Methodology

Matrix effects, insufficient and variable recovery, instrumental fluctuations, and inconsistent analyte stability may be difficult to entirely account for using a single quantitation technique leading to low reproducibility, accuracy or precision. The difficulty encompassing the previously listed factors is most frequently encountered in extremely complex matrices such as blood or plasma. In instances where quantitation is deemed insufficient using a single method, two methods can be combined to reach the desired level of accuracy or precision. The most common combination of methods utilized in CWA studies is incorporating an internal standard into matrix-matched calibration. Either a structural analog or a SIL standard can be employed. The disadvantage of using a combination of methodologies is that both blank matrix and an appropriate internal standard have to be obtainable. However, as can be seen in Table 1, using a combination of techniques is the most widely utilized methodology for CWA studies. The following section highlights several examples where a combination of methods was used for quantifying CWA's.

Structural analogs have been used in conjunction with blank matrix to improve method few accuracy in a few CWA investigations[64,49,57,48]. One of these methods simultaneously monitored the levels of seven different metabolites of HD in plasma from an exposed rat using blank plasma and comparing each metabolite's response to a single structural analog[64]. 1,1-sulfonylbis[2-(ethylsulfinyl)ethane] (SBESE) was used for the structural analog, which was particularly advantageous because it eluted in the middle of the chromatographic run making it a good fit for both early and late eluting analytes[64]. Using the blank matrix helped to account for absolute matrix effects experienced in the plasma samples, lowering the need for the internal standard to have a very similar retention time to each analyte. The internal standard corrected for relative matrix effects, changes in recovery, and instrumental fluctuations between each run. This method was used for a time course study on the concentration of each metabolite after HD exposure[64]. The information gathered from this study can be used to select the best biomarker during a particular time interval after exposure.

As with individual quantitation techniques, stable isotopically labeled standards can be used in combination with blank matrix to provide additional precision and accuracy. Two recent studies have utilized a combination of blank urine and commercially available stable isotopically labeled ricinine to examine ricinine levels in human urine samples[91,92]. The first method used this combination to verify an individual had self-administered castor bean extract which resulted in death[91]. Another investigation using urine and commercially available standards determined ricinine's ability to be used as a biomarker for ricin poisoning by assessing background levels in urine samples of unexposed individuals[92]. After analyzing almost 1000 urine samples, the author's reported about 1.2% of the unexposed population has levels of ricinine of 4.15ng/mL or lower[92]. The individual who had been exposed to castor bean extract reported an initial urine

concentration of ricinine over 13 times as high (57.6 ng/mL) as the unexposed population[91]. Since such a dramatic difference in concentrations is present, ricinine can be used as a biomarker in urine for cases when concentration data can be compared with other information such as a patient's symptoms, but caution should be exercised when confirming ricin poisoning when low levels of ricinine are reported.

Blank urine and stable isotopically labeled standards were also used in combination to quantify AMPAs [32]. The study in reference 21 reached LODs more than 10-fold lower than the AMPA concentration levels reported in a victim's urine, highlighting the clinical relevance for using AMPAs as biomarkers for nerve agent exposure. The authors also reflected upon the importance of high throughput when detecting biomarkers for nerve agent exposure, due to the extreme number of samples that might be submitted to a lab after CWA release. Consequently, a goal of this analysis method was to have high-throughput, and analysis of almost 300 samples per day was achieved. Additionally when responding to an exposure incident, many different analysts would be handling samples. This study was completed by several different analysts, and no false positives or negatives were reported. The results were compared to a similar GC/MS/MS method and the LC/MS/MS method showed accuracy as good as or better than GC-MS/MS method.

Using a combination of SIL internal standards and blank matrix has also been employed when the internal standard, blank matrix, or both were not easily obtainable. Bao *et al* quantified the covalent adducts of nerve agents with albumin using SIL standards, but these standards had to be synthesized in house[31]. In this same study, the albumin fraction purified from plasma samples was used as a blank matrix[31]. This combination was successfully used to quantify the tyrosine adducts of sarin and VX using deuterated O-(O-isopropyl methylphosphonyl) tyrosine

and deuterated O-(O-ethyl methylphosphonyl) tyrosine with limits of quantitation (LOQ) reached 0.05 ng/mL and 0.1 n/mL for sarin and VX, respectively[31].

A combination of methods more frequently uses blank matrix and a SIL internal standard as opposed to using a structural analog. Generally this quantitation technique is employed when both blank matrix and stable isotopically labeled standard are readily available. The vast majority of CWA applications using the combination technique were conducted in biological matrices, with the most common application quantifying ricinine in urine. This application is likely popular due to the ease of collection of blank urine and the commercial availability of ricinine.

1.4.8 Method Comparison

The ideal quantitation method is one that produces instantaneous results with 100% accuracy at extremely low concentrations for free. Experience in quantitative chemistry quickly reveals that such a method does not exist. Therefore experimenters must choose which factors are the most important for their particular study and generate a suitable balance between the different methods' characteristics. Table 2 offers a comparison of LC-MS quantitation methods used for absolute concentration determination in CWA studies. Merits are highlighted in green while demerits are listed with red. In addition to the quantitation method's characteristics, evaluating the sample matrix and the analyte or analytes greatly influence the method choice.

The sample matrix has a large impact on which quantitation method is appropriate. The accessibility of blank matrix controls whether or not matrix-matched calibration can be used. In CWA studies, blank environmental matrices and biological matrices that are collected noninvasively are most easily obtained, lending themselves to matrix-matched calibration. However, the complexity of the matrix and its subsequent processing are the determining factors

Table 2 Quantitation Method Comparison

	External	Matrix-matched	Standard Addition	Structural Analog	SIL IS	Combination
Additional analyte required	✓	✓	✓	✓	✓	✓
Blank matrix required		✓				✓
Internal standard required				✓	✓	✓
Large sample volume necessary			✓			
Corrects for absolute matrix effects		✓	✓	✓	✓	✓
Corrects for relative matrix effects			✓	✓	✓	✓
Corrects for inter-run variations				✓	✓	✓
Unlimited number of analytes	✓	✓		✓		

for which quantitation method is necessary. For example, in very simple matrices, such as water samples, matrix-matched calibration is probably not necessary due to the low number of chemical interferences for CWAs even though blank matrix is readily available. In more complex samples, such as whole blood, the experimenter will have to decide if it is more advantageous to obtain blank matrix or use a standard to correct for matrix effects that may be experienced by the analyte.

The identity and number of analytes influence the choice of an internal standard. If the goal is to quantify two or more CWAs or their products, selecting a structural analog that reflects the retention time and ionization of all the compounds can be used effectively. However if a SIL internal standard is available for each of the analytes, this can save time in selecting a structural analog. Stable isotopically labeled standards are commercially available for all the AMPAs, TDG, and ricinine along with labeled peptides for protein adducts or ricin. For the analytes that

do not have commercially available SIL internal standards, a structural analog can be used or a SIL IS can be synthesized.

1.5 CONCLUSION

Exposure to chemical warfare agents is a possibility to both soldiers and civilians. Accurate quantitation information is necessary during an exposure incident to determine the hazard level which will assist responders with choosing the correct containment and decontamination procedures. In the future, quantitative levels *in vivo* may be used to supply medical personnel with the knowledge necessary to treat the victim and supply the adequate amount of antidote, if one is available. The high importance of quantitative information for CWAs in a variety of settings causes quantitative analytical strategies to continuously be improved.

Chemical warfare agent quantitation using LC-MS or LC-MS/MS offers a variety of opportunities not applicable when using other analytical techniques. LC-MS has the ability to quantify CWAs, their degradation products, and adducts without the need for derivatization. Many different methods to quantify these compounds are available, including use of external standards, with or without matrix-matched calibration, and stable isotopically labeled internal standards. Quantitation can take place in a variety of matrices including environmental and biological samples. Chromatographic retention time combined with the molecular weight information, along with diagnostic fragment ions gathered from tandem MS experiments, allow for irrefutable identification of the analytes being quantified. The diversity of quantitation procedures available combined with the low amount of sample preparation make LC-MS an unparalleled analytical tool for the quantitation of CWAs and their degradation products.

Consequently, LC-MS/MS provides enough information to become a stand-alone quantitation technique for CWAs.

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CHAPTER 2

SAMPLE / VIAL INTERACTIONS DURING QUANTITATIVE ANALYSIS

ABSTRACT

Sample/vial interactions are a well-known phenomenon, with many reports in the literature demonstrating that a variety of materials can absorb to vials of various types[1, 2, 3]. However, investigators will not detect such interactions unless they look for them specifically, and these interactions are rarely tested for in developing quantitative proteomic methods. By contrast, tests for matrix effects are quite a routine aspect of quantitative method development. This report is a case study demonstrating examples of how sample/vial interaction can result in quantitative data that appears to be hindered by matrix effects, when, in fact, no matrix effects are present. By demonstrating that vial interactions can produce outcomes consistent with a sample experiencing matrix effects, we provide analysts with information to help develop their quantitative methods when initial calibration results fail to provide acceptable data. Additionally the data herein provide an important reminder to those doing quantitative analysis that glass autosampler vials are not inert; they may interfere with some analyses.

2.1 INTRODUCTION

Mass spectrometry is quickly becoming the technique of choice for quantifying analytes in complex matrices. Absolute quantitation can be achieved using internal or external calibration. Internal calibration provides high accuracy, precision, and reproducibility by correcting for any changes experienced by the targeted compound in an internal standard[4]. However every analyte does not have an appropriate internal standard. In these and other instances, external calibration methods are used [5].

One of the most well-known barriers to surmount while developing an absolute quantitation method by LC-MS using external standards is the problem of signal suppression and signal enhancement, otherwise known as matrix effects. The causes and mechanisms behind matrix effects are not yet fully understood, making them difficult to encompass in a quantitative method. However, one known cause of matrix effects is from a component of the sample matrix eluting at the same time as the analyte of interest. Co-elution affects the ionization efficiency of the analyte by suppressing, enhancing, or having no effect on the analyte's signal. The degree of signal suppression or enhancement has been correlated to the concentration of the other components of the matrix, making complex biological samples especially prone to matrix effects[6].

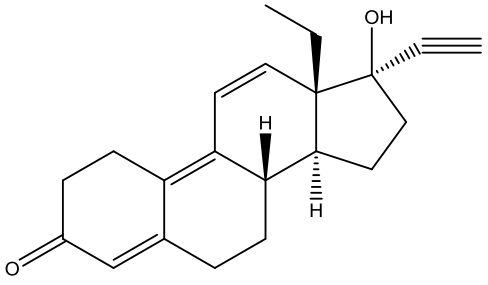
Post-column infusion is one commonly employed approach used to monitor ion suppression and enhancement prior to the development of a quantitative method[7, 8]. This approach, first described by Bonfiglio *et al*., utilizes a tee splitter to combine the flow from the LC gradient with a continuous flow of analyte; the combined flow is directed to the source of the mass spectrometer[9]. Using this technique, it is possible to predict whether or not an analyte is susceptible to matrix effects at a particular retention time.

When there are no matrix effects impacting an analyte, or the matrix effects have been avoided, removed, or accounted for, analysts often proceed with developing quantitative methods by generating a calibration curve and running quality control samples, to verify the quality of the calibration results. The external calibration approach has been successfully employed in a wide variety of applications including analysis of trace environmental contamination[10, 11], biomedical samples[12], food and beverage contamination[13, 14, 15], illicit drugs[16], and explosive residues[17].

Unfortunately, sometimes a successful calibration method cannot be developed, even after matrix effects have been deemed to be absent, because external calibration is susceptible to other sources of inaccuracy including variable extraction recoveries, instrumental fluctuations, and vial adsorption. Despite this known problem, many of these factors are often disregarded during method development. Optimizing recovery from sample containers is rarely assessed despite studies showing variable recovery from both long term storage containers[1, 2, 18] and injection vials[3, 19]. The FDA's guidelines for analytical method development and validation require extraction recovery and analyte stability to be assessed, but they do not require adsorption studies to be completed for the method to be fully validated[20]. Vial absorption or changes in adsorption levels throughout an experiment could lead to inaccurate quantitative results[21, 22].

The work described here is a quantitative investigation of leucine enkephalin, MRFA, bradykinin, and gestrinone in various matrices of tryptically digested proteins. Amino acid sequences or structures of the analytes along with select physical properties are shown in Table 1. Both vial adsorption and matrix effects were assessed. While variability in the quality of the quantitative results initially suggested that matrix effects were present, these effects had been ruled out experimentally. Vial adsorption was shown to be the major contributing factor in

Table 1 Physical Properties of Analyzed Compounds

Compound	Sequence/Structure	log P	pI*
Leucine Enkephalin	Tyr-Gly-Gly-Phe-Leu	-2.3	5.50
MRFA	Met-Arg-Phe-Ala	-3.8	10.00
Bradykinin	Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg	-4.8	11.97
Gestrinone		2.2	8.14

*pI values were estimated from the pKa values of isolated residues and could be altered from protein folding

obtaining accurate and precise quantitation. This case study highlights the importance of investigating adsorption, particularly when matrix effects are deemed to be absent, but quantitative data does not meet acceptable accuracy and precision requirements.

2.2 EXPERIMENTAL

2.2.1 Materials and Reagents

Leucine enkephalin (YGGFL), MRFA, bovine serum albumin (BSA), lysozyme from chicken egg white, dithiothreitol (DTT), iodoacetamide (IAM), Trizma hydrochloride, Trizma base, formic acid, and glacial acetic acid were purchased from Sigma-Aldrich (St Louis, MO).

Optima grade methanol and acetonitrile were obtained from Fisher scientific (Fair Lawn, NJ). Sequencing grade trypsin was purchased from Promega (Madison, WI). Bradykinin was purchased from Enzo Life Sciences (Farmingdale, NY). Gestrinone was purchased from Caymen Chemical (Ann Arbor, MI). Water was purified using a Millipore Direct-Q3 filtration system (Billerica, MA).

2.2.2 Trypic Digestion

Lysozyme and BSA were dissolved in pH 8.5 Tris buffer to give working solutions of approximately 6 mg/mL for each protein. The samples were reduced with 100mM DTT for 1 hour at room temperature and then alkylated with 500mM IAM for 1 hour at room temperature in the dark. Alkylation was quenched by adding an additional aliquot of DTT and incubating for another 30 minutes at room temperature. Samples were digested with trypsin at a protein to enzyme ratio of 30:1 for 18 hours at 37°C. The digestion was stopped with the addition of glacial acetic acid at a volume of 1µL for every 100µL of sample. The subsequent digest samples were stored at -20°C until they were used as matrices for the infusion and quantitation experiments.

2.2.3 Sample Preparation

Stock solutions of YGGFL, MRFA, and bradykinin were made in 50:50 methanol to water with 0.1% formic acid to give a final concentration of ~200uM. Gestrinone was prepared in methanol with 0.1% formic acid with a final concentration of 3.25 µM. Calibration standards were prepared daily by serial dilution from the stock solution in water. Five points were chosen to construct the calibration curve over the linear range for each analyte. A quality control (QC) set was prepared by spiking each analyte into four different matrices. The matrices were water

(control), ~4.3 µM BSA digest, ~18.5 µM lysozyme digest, and a mixture of the BSA and lysozyme digests. Samples were placed in glass vials for autosampler injection. A second set of standards and QC samples of MRFA and bradykinin were also placed in polypropylene vials for injection. Stock solutions were stored at -20°C until diluted for LC-MS analysis.

2.2.4 LC-ESI-MS/MS Analysis

The analytes were injected in the following order: One set of standards, followed by one trial of the QC set and then repeated (n=2). Each sample was injected with a volume of 5µL into an ultra-high performance liquid chromatograph (Waters Acquity, Milford, MA) and separated on a Hypersil GOLD column (C18, 100 mm X 1.0 mm i.d., 5 µM, 175 Å; Thermo Scientific, San Jose, CA). Mobile phase A was 99.9% water and 0.1% formic acid, while mobile phase B was 99.9% acetonitrile and 0.1% formic acid. The analytes were eluted using the following gradient operating at a flow rate of 50 µL/min. Initially the mobile phase was 98% A and 2% B for the peptides. The initial mobile phase for gestrinone was altered to 90% A and 10% B because the analyte is not soluble in aqueous solutions. Mobile phase B was increased linearly to 60% over 24 minutes and held there for 2 minutes. It was then returned to 2% B and maintained for 4 minutes to reequilibrate the column. A blank was run between each sample to minimize the analyte carryover.

Detection was carried out on a LTQ Velos dual linear ion trap mass spectrometer (Thermo Scientific, San Jose, CA) operating in positive ion mode. The eluent was infused into the mass spectrometer via an electrospray ionization source. The source used a spray voltage of 3 kV and had a capillary temperature of 250° C. Extracted ion chromatograms of the MS data were used to construct calibration curves.

2.2.5 Post-Column Infusion

Post-column infusion was set up as described by Bonfiglio *et al* (Figure 1)[9]. Approximately 5µL of matrix was injected into the LC-MS system and ran under the conditions described above. The eluent was directed toward a T-splitter using PEEK tubing where it was continuously infused with the analyte. Both were then directed to the ESI source. The analyte was pumped at 5 µL/min using a Harvard Apparatus Pump 11 (Holliston, MA) to give a concentration at the source of ~200nM for the peptides and ~3.5nM for gestrinone. The signal fluctuation for each analyte was monitored throughout the runs using MS^1 data.

2.2.6 Quantitative Analysis

LC-MS data was analyzed in Xcalibur 2.1 software (Thermo Fisher Scientific, San Jose, CA). The peptides and gestrinone were assigned by matching their theoretical masses with the actual masses acquired. Extracted ion chromatograms (XICs) were generated by plotting the signal of the analyte versus time, using a 0.5 Da mass range for each selected *m/z*. Table 2 shows

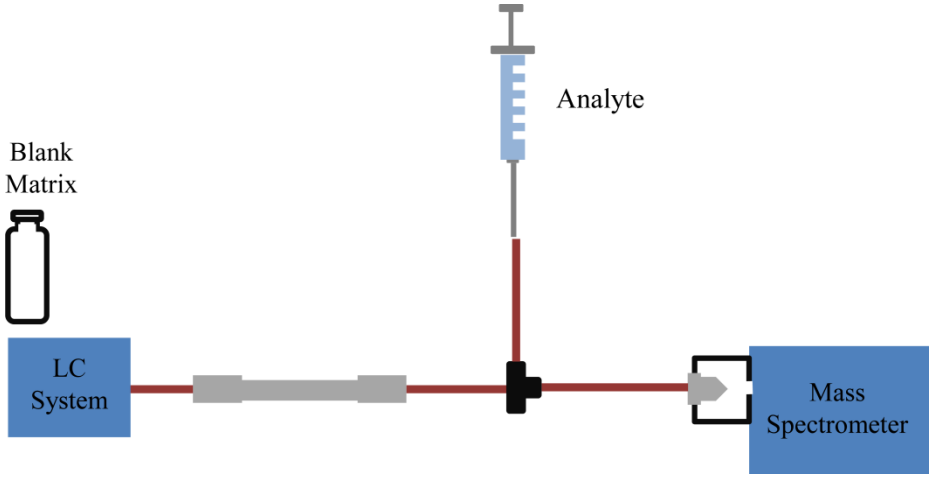


Figure 1 Schematic diagram of the post-column infusion experimental set-up used to screen for matrix effects, as adapted from reference 9

the *m/z* range used for integration. Peaks were automatically integrated by Xcalibur software and manually verified to ensure consistency in integration. A standard curve was then constructed in Microsoft Excel by plotting the peak area against the concentration. Linear regression analysis was completed to generate the best fit line. Using the best fit line, the concentrations for each of the samples in the QC set were experimentally determined, and the accuracy and precision of these measurements is reported herein.

2.3 RESULTS AND DISCUSSION

2.3.1 Overview

The original goal of this study was to monitor matrix effects experienced by a variety of analytes in simple matrices and then to quantify the analytes in these matrices using external calibration. While pursuing this goal, unexpectedly poor reproducibility and robustness was observed in both the calibration curves and validation sets for two of the four analytes. An investigation into these problems lead to new insights about the challenges in accurately predicting whether external calibration would be successful, even when matrix effects are verified to be absent. The data presented here are a case study showing how standard methods of measuring matrix effects can provide false hopes that quantitation using external standards could

Table 2 Analyte Characteristics Used in the Study

Analyte	Monoisotopic Mass	Charge State	*m/z* range monitored	Linear Range (nM)	Retention Time (min)
YGGFL	555.2766	1	556.0-556.5	100-1000	12.2
Gestrinone	308.1776	1	309.0-309.5	2-100	19.2
MRFA	523.2650	2	262.5-263.0	100-1000	8.3
Bradykinin	1059.5687	3	354.0-354.5	250-1250	9.8

be successful: In our case, accurate quantitation was thwarted because of the autosampler vials chosen.

2.3.2 Post-Column Infusion

Three peptides, YGGFL, MRFA, and bradykinin, and one steroid, gestrinone, were the target analytes in the study. To determine which of the analytes would experience matrix effects upon quantitation with external standards, the method reported by Bonfiglio *et al* was used[9]. Each of the analytes was infused separately with water, BSA digest, lysozyme digest, and a mixture of the two digests. By continuously monitoring the signal of the analyte during co-infusion of the matrix, matrix effects can be readily detected because they suppress or enhance the signal of the analyte. Matrix effects have been shown to occur most frequently when the analyte is at much lower concentrations than the matrix[23]. Consequently the analyte's concentration was chosen to be between $1/20^{th}$ and $1/100^{th}$ of the matrices' concentration in order to create optimal conditions for matrix effects to be easily observed.

Figure 2A shows the total ion chromatogram (TIC) for the post-column infusion of bradykinin with a mixture of lysozyme and BSA digests. Figure 2B compares the signal of bradykinin infused with the digest mixture to that when it was infused with just the mobile phase. The overall signal for both trials increased as the chromatographic run progressed. This was expected, due to the increase in organic content of the mobile phase throughout the run. Increasing the organic content allows for more of the analyte to become charged because the solvent evaporates faster and creates a more stable spray[24]. The signal is approximately the same between the two trials except for a dramatic increase slightly after five minutes in the trial with matrix infused. This increase was attributed to an interference from the alkylated peptide

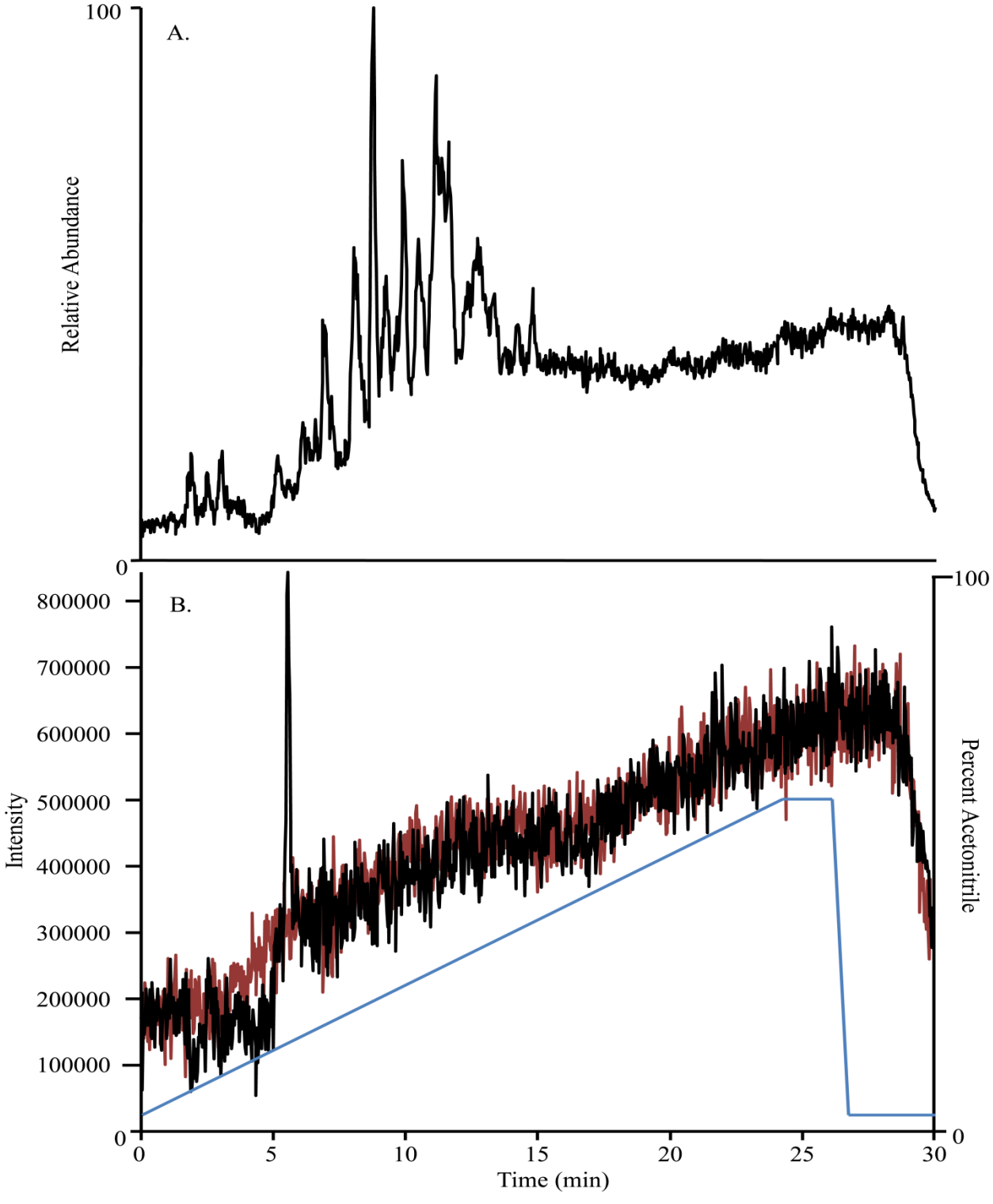


Figure 2 Post-column infusion chromatograms for bradykinin with tryptic digest mixture A) TIC B) XIC of +3 charge state for bradykinin. Infusion with the blank and digest mixture are indicated by the red and black lines respectively. Blue line indicates the changes in percent acetonitrile. These data demonstrate that, with the possible exception of the spike at 5 minutes, no matrix effect is observable.

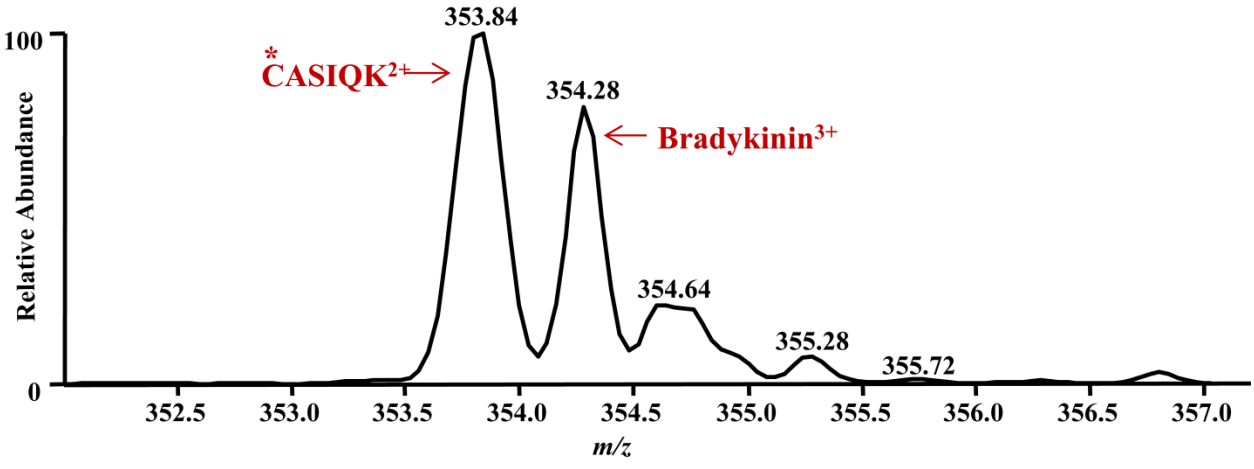


Figure 3 MS data for the +3 charge state of bradykinin when co-eluting with a peptide that has a similar *m/z*. These data were obtained for the peak eluting at ~5.5 minutes in the chromatogram in Figure 2B.

CASIQK^{2+} as can be seen in Figure 3. Since bradykinin does not co-elute with this peptide (see retention times listed in Table 2), no matrix effects were predicted. YGGFL, MRFA, and gestrinone produced similar results, which indicated that none of the analytes would experience significant matrix effects at their known retention times (data not shown).

2.3.3 Quantitation using Glass Vials

Five points were used to establish the calibration curve for each analyte over the selected linear range listed in Table 2. Since the post-column infusion experiments indicated that no co-eluting compounds or matrix effects were present at the retention time of the analytes, the MS response was used for quantitation instead of conducting MS/MS experiments and quantifying the analytes based on the intensity of product ions. XICs were generated using a 0.5 Dalton window around the desired *m/z* to ensure slight variations in the analyte's *m/z* did not negatively impact the calibration.

The calibration curves for YGGFL, gestrinone, and MRFA all exhibited good linearity ($R^2 \geq 0.992$) over the concentration range (Figure 4A, B and C). YGGFL also displayed

excellent precision with its %RSD ≤ 2.2 at each point. Although slightly less precise, gestrinone and MRFA both had moderately good precision with %RSD ≤ 11.85 and 7.85 respectively. Somewhat alarmingly, each analyte displayed a decrease in response between the first and second trials, and this trend was the main contributing factor in the lack of precision.

Bradykinin's calibration curve is shown in Figure 4D. Bradykinin provided poor linearity (R^2 = 0.9693) over the concentration range. In addition, the level of precision produced was extremely poor (%RSD ≥ 42.2). The low precision level is accounted for by a significant loss in signal between the first and second trials.

The linear equations generated from the calibration curves were used to predict the

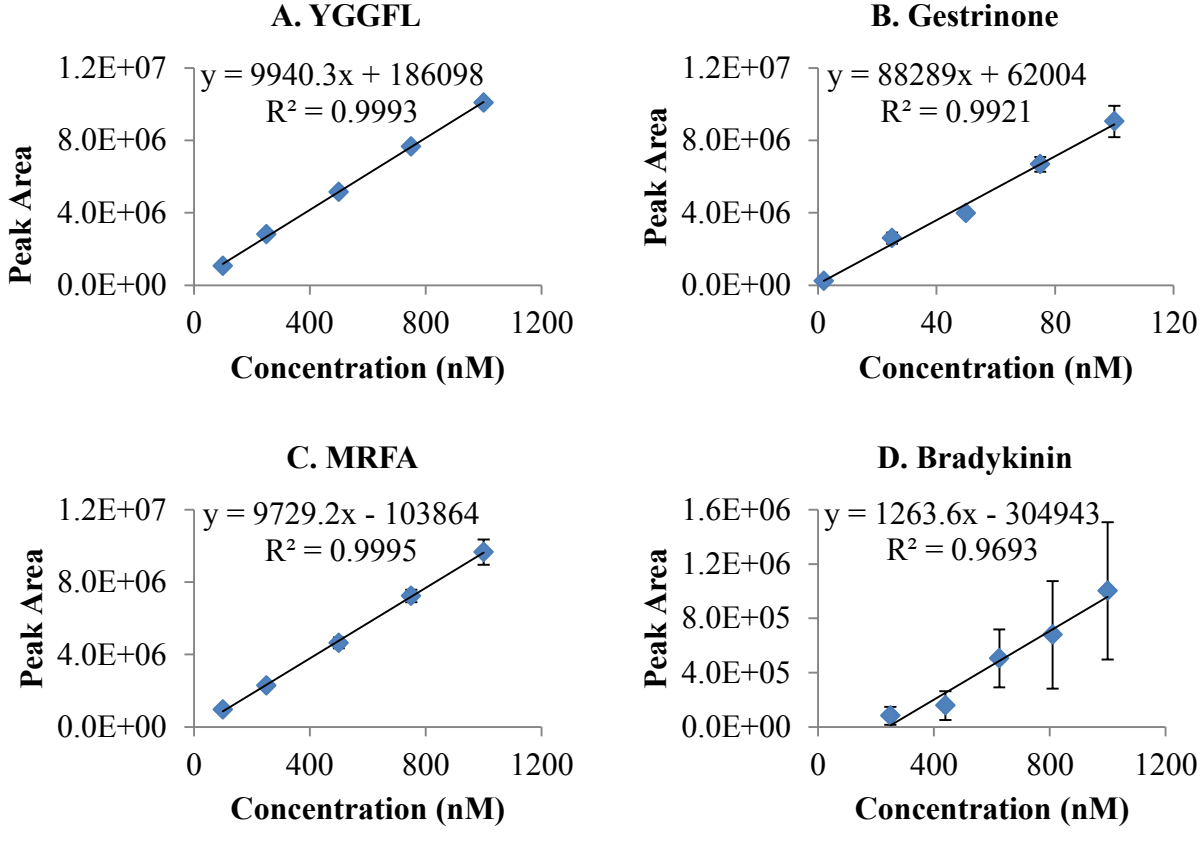


Figure 4 Calibration curves generated in glass vials

concentrations of spiked analytes in tryptic digests. The peptides were spiked to a final concentration of 600 nM, while gestrinone was spiked at 60 nM . Table 3 shows the results for the analytes in the four different matrices. YGGFL and gestrinone produced acceptable results; MRFA had slightly less than ideal results, and bradykinin's results were completely inaccurate.

Bradykinin, and to a lesser extent MRFA, exhibited a notable increase in response between the water blank and digest matrices. A change in signal between the analyte in a blank solution and in the sample matrix is characteristic of a matrix effect. Consequently, upon initial analysis of this issue, it appeared that a matrix effect was present, but this phenomenon was unobserved in the post-column infusion data. Further investigation led to a hypothesis that nonspecific adsorption to the glass injection vials, and not a matrix effect, was the cause of this discrepancy.

Adsorption can occur through numerous types of interactions including hydrophobic or electrostatic interactions and hydrogen bonding[25]. Adsorption between a peptide and a solid surface occurring through hydrophobic and electrostatic interactions is illustrated in figure 5. Since these interactions are specific to each analyte, variations in adsorption between each analyte would be expected. Therefore, it is possible that the analytes experienced differential degrees of adsorption, and this adsorption would explain the inconsistent calibration results.

Table 3 Experimentally Determined Concentrations for the Quality Control Sets using Glass Vials[a]

Analyte	Water	BSA digest	Lysozyme digest	Digest mixture
YGGFL	667 ± 14 nM	537 ± 42 nM	608 ± 16 nM	553 ± 26 nM
Gestrinone	67 ± 3 nM	57 ± 1 nM	63 ± 3 nM	43 ± 4 nM
MRFA	554 ± 47 nM	827 ± 22 nM	825 ± 21 nM	819 ± 39 nM
Bradykinin	972 ± 604 nM	2830 ± 440 nM	2090 ± 194 nM	19400 ± 16100 nM

[a]Two QC samples were run for each analyte/matrix pair. Peptide concentrations were 600 nM, while gestrinone's concentration was 60 nM.

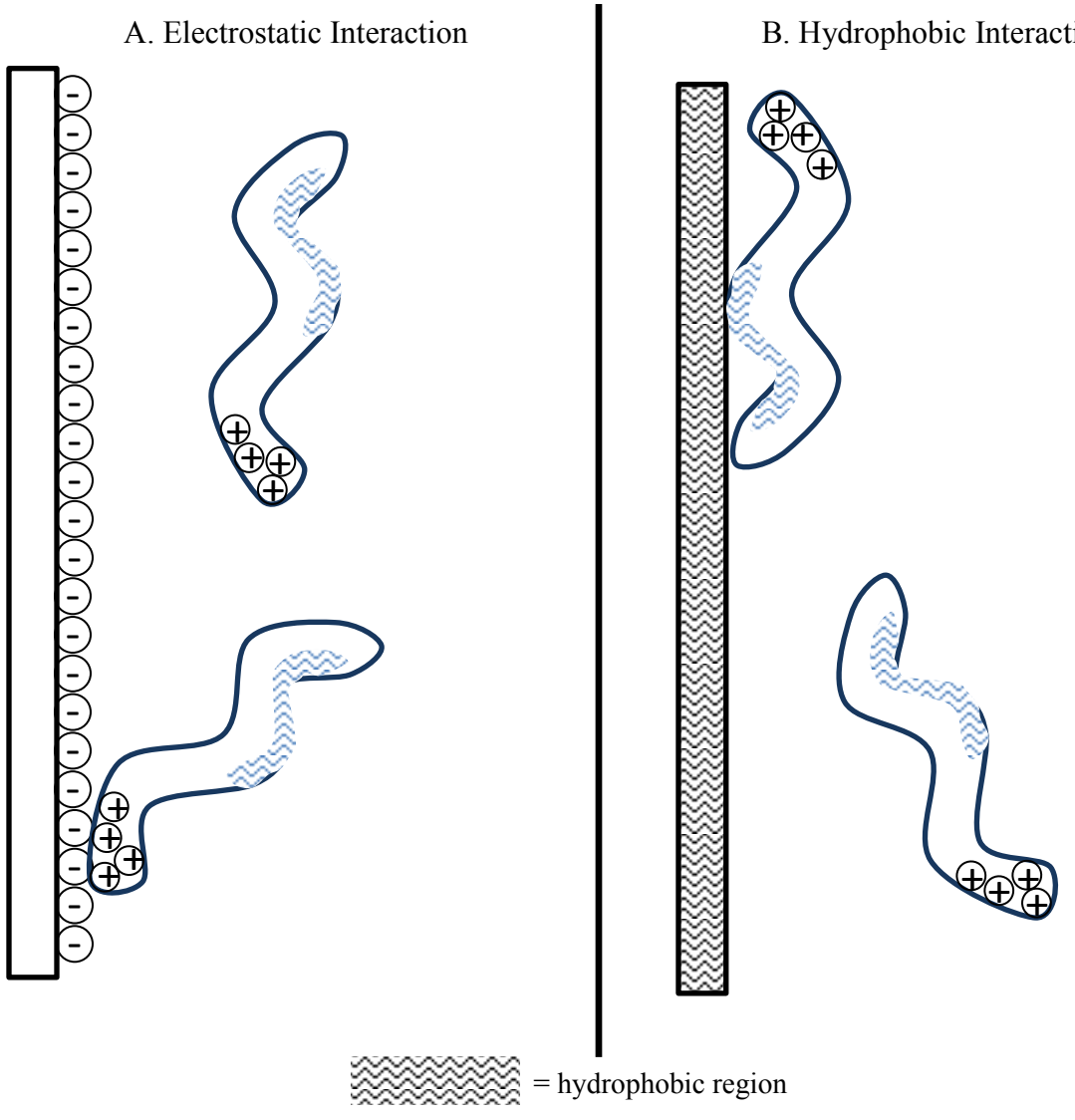


Figure 5 Interactions between a peptide with both hydrophobic and ionic regions and a solid surface. A. Adsorption with an ionic surface B. Adsorption with a hydrophobic surface. Adapted from reference 27.

The dramatic decrease in signal between trial one and trial two for bradykinin, and to a lesser extent, for the other three analytes further bolsters the hypothesis that adsorption was occurring. The second trial of samples sat in the autosampler for a longer time, because they were run after the entire initial round of samples and QC standards were run. The increased exposure time between the analytes and the vials' interior surface could have allowed more of the adsorptive interactions to take place prior to the re-analysis of the samples.

The vial adsorption hypothesis also offers an explanation for the overestimation of the concentration of bradykinin in the (blank) water sample, which would not be explained by matrix effects. Trial 1 of the control sample was predicted to have a concentration of over 2.5 times the theoretical amount, while trial 2 predicted slightly above half of the theoretical concentration. The trial 1 QC was almost immediately sampled while the trial 2 QC sample spent an additional 9 hours in the autosampler, leading to changes in adsorption levels as a factor of time, as previously explained.

To explain the increased response between the control samples and digest matrices for bradykinin and MRFA, a careful literature search revealed components of the matrix can cause changes in vial adsorption[2, 3, 19, 26]. Various compounds can disrupt the analyte's interactions with the container's surface or displace the analyte from the surface leading to an increase of the analyte in solution[25]. We hypothesized that these types of changes were occurring, accounting for the increased response experienced by bradykinin and MRFA in the samples containing matrix.

2.3.4 Quantitation using Polypropylene Vials

To test the vial adsorption hypothesis and locate the cause of the quantitation inaccuracy, bradykinin was injected from polypropylene vials. None of the other vials used in conjunction with bradykinin, such as the stock solution storage vial, were altered between the two studies.

Figure 6A illustrates the resulting calibration curve from analyzing bradykinin in polypropylene vials. Using polypropylene vials greatly improved both the linearity ($R^2 \geq 0.99$) and precision (%RSD ≤ 20.5) of the method. However the best fit line goes slightly below the origin, indicating that some adsorption may still be taking place.

The QC set in polypropylene vials exhibited vast improvements in both the accuracy and precision for bradykinin (Table 4). The concentration of the control sample was predicted within 6% of the theoretical concentration compared to previous overestimate which was greater than 62%. Additionally the precision was greatly enhanced from the previous RSD values of 62.1% to slightly less than 2%. Despite the improvement of accuracy and precision levels in all three of the digest matrices, the concentration of bradykinin was still slightly overestimated. Again this could be due to some small remaining adsorption interactions between bradykinin and the interior surface of the vial.

Due to the dramatic improvements seen for bradykinin, MRFA was also analyzed in polypropylene vials. The calibration curve (Figure 6B) generated in polypropylene vials offered slightly less linearity than in the glass vials, but the R^2 value was still greater than 0.99. Table 4 shows the QC set results for MRFA in polypropylene vials. Accuracy was improved using polypropylene vials in the water, BSA digest, and lysozyme digest matrices when compared to the glass vials. In the BSA digest matrix and the lysozyme digest matrix accuracy was improved from approximately a 38% overestimate to within 13% of the theoretical concentration. The concentration in the digest mixture still fell outside of an acceptable range of error, and an

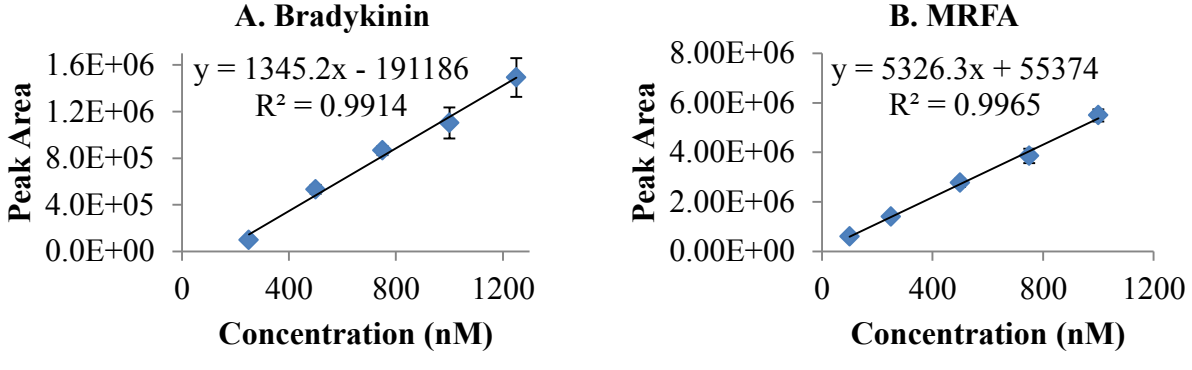


Figure 6 Calibration curves generated in polypropylene vials

Table 4 Experimentally Determined Concentrations for the Quality Control Sets in Polypropylene Vials[a]

Analyte	Water	BSA digest	Lysozyme digest	Digest mixture
Bradykinin	635 ± 9 nM	755 ± 28 nM	756 ± 10 nM	775 ± 65 nM
MRFA	650 ± 39 nM	506 ± 15 nM	572 ± 31 nM	331 ± 15 nM

[a]Two QC samples were run for at 600 nM for each analyte/matrix pair.

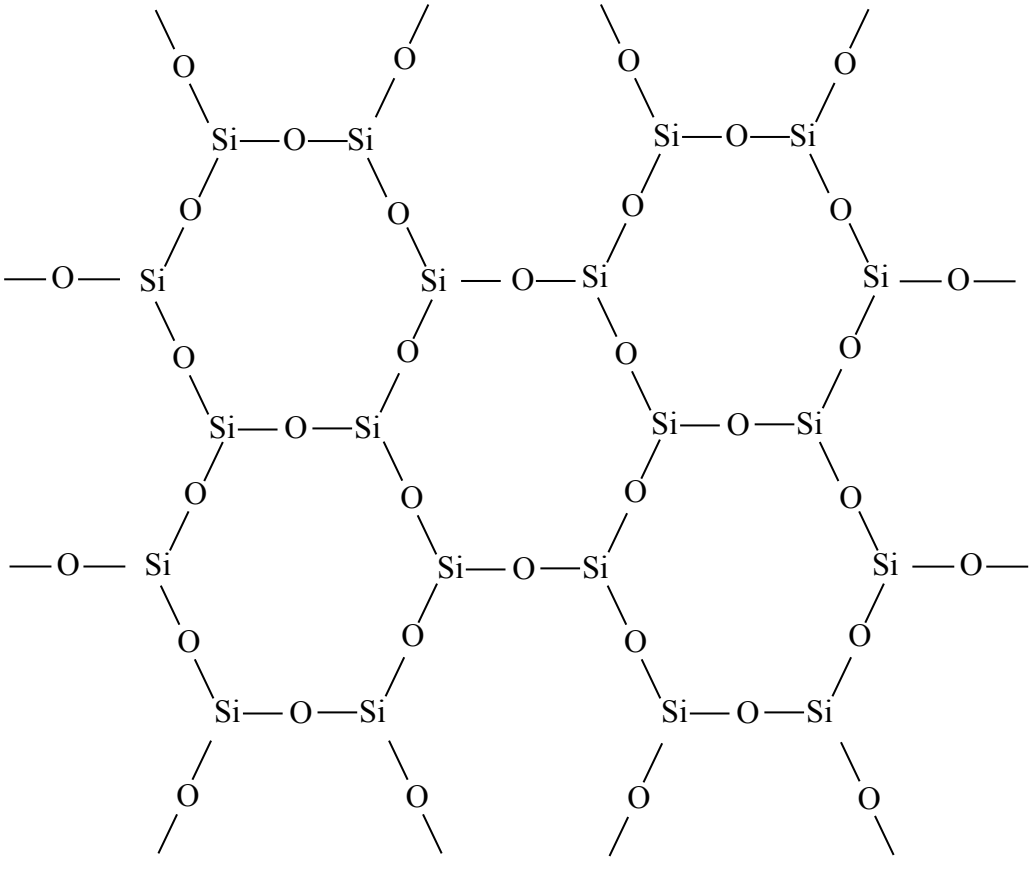
additional investigation would have to be completed to determine the source of this inaccuracy.

The improvements in accuracy observed from switching bradykinin and MRFA from glass injection vials to polypropylene injection vials demonstrated that adsorptive interactions were the main source of the errors during the external calibration method. Since adsorption interactions are unique between a particular analyte and vial material, testing bradykinin's quantitative results using other injection vial materials may offer further improvement, allowing the method to fall within the desired ±15% range. When nonspecific adsorption is masquerading as a matrix effect, it can be difficult to identify. However this study demonstrates the importance of testing for vial adsorption if a matrix effect appears to be skewing the quantitative results, even after one has experimentally determined matrix effects to be absent. Errors in quantitation due to vial adoption can be easily minimized by changing the material that is interacting with the analytes.

Predicting the vial material that is most suitable for each compound could also help alleviate quantitative inaccuracy. Since vial and analyte interactions are dominated by hydrophobic and electrostatic interactions, studying a compound's hydrophobicity using its partition coefficient (log P), and its isoelectric point (pI) could prove to be beneficial. These two properties for the compounds in this study are listed in Table 1. Bradykinin and MRFA have pI values of 11.97 and 10.00 respectively, meaning they will have a positive charge at the pH of all the matrices used in this experiment. The structure of glass and polypropylene are shown in

figure 7. Glass has a negative charge at the surface while polypropylene is neutral leading to Bradykinin and MRFA having a more favorable interaction with glass than with polypropylene. Although isoelectric points seem to explain the adsorption trend for the small set of analytes presented in this study, a larger assortment of compounds will most likely show that a combination of hydrophobicity and charge account for non-specific adsorption.

A.



B.

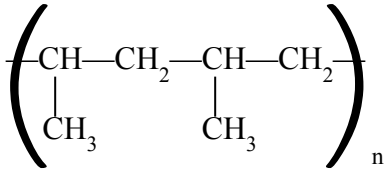


Figure 7 A. Crystalline structure of glass B. Structure of polypropylene. Adapted from reference 28.

2.4 CONCLUSION

Our investigation sought to understand the source of some inaccuracies experienced during external calibration methods. A pre-screen for matrix effects was accomplished using standard literature procedures and ion suppression and enhancement was not observed for any of the analytes in our study. Despite the lack of detected matrix effects, an apparent matrix effect was observed when calibration curves were generated and QC samples were analyzed. This apparent matrix effect for bradykinin and, to a lesser extent, MRFA, was eventually ascribed to vial absorption. The results from our study indicate that vial adsoption can be a significant factor in obtaining high quality quantitative analyses, and addressing this issue is straightforward, if investigators know to check for the problem. Although the analytes in our study only represent a very small sum of compounds analyzed by LC-MS, they adequately portray the variable success rate for different analytes using external calibration. The unique source of inaccuracy for bradykinin provides an important reminder to those doing quantitative analysis that glass autosampler vials are not inert, and they may interfere with some analyses.

2.5 ACKNOWLEDGEMENTS

The authors thank Dr. Susan Lunte, Distinguished Professor of Chemistry and Pharmaceutical Chemistry and Director of the Adams Institute for Bioanalytical Chemistry at the University of Kansas, for helpful discussion. This study was funded by NIH grant R01AI094797 (awarded to Heather Desaire).

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CHAPTER 3

FUTURE OUTLOOK

ABSTRACT

Every compound targeted during a quantitative analysis experiences distinct interactions with the sample matrix and the many surfaces encountered during experimentation. These interactions play a crucial role in determining the success of the analysis. Quantitative analysis via liquid chromatography-mass spectrometry (LC-MS) is specifically affected by interactions occurring during the ionization stages of analysis, known as matrix effects, and adsorption interactions that occur during sample handling. This study is a follow up to our previous investigation and includes case studies for additional peptides noting their differences with regards to matrix effects and vial adsorption interactions. A description of future experiments necessary to increase the success of LC-MS external calibration quantitative methods is also presented.

3.1 INTRODUCTION

Every quantitation application results in a unique blend of interactions between the target analyte, sample matrix, and surfaces encountered during the analysis. These interactions are crucial during multiple steps of method development including instrument selection, sample preparation, and optimization parameters. When liquid chromatography-mass spectrometry (LC-MS) is chosen as the analytical technique, two additional areas affected by analyte and matrix interactions are ionization efficiency and vial recovery.

There are many mechanistic theories attempting to describe the interactions between the analyte and matrix that can lead to changes in ionization efficiency. These mechanisms are dependent on the type of ionization source used. Electrospray ionization (ESI) couples the LC to the mass spectrometer and creates ions by applying an electrical charge to the liquid phase which produces charged droplets via Taylor cone formation (Figure 1)[1]. Subsequent solvent evaporation and droplet fission allows the analyte to reach the gas phase as an ion[2]. Interactions in both the liquid and gas phases have been theorized to change an analyte's ionization efficiency[3].

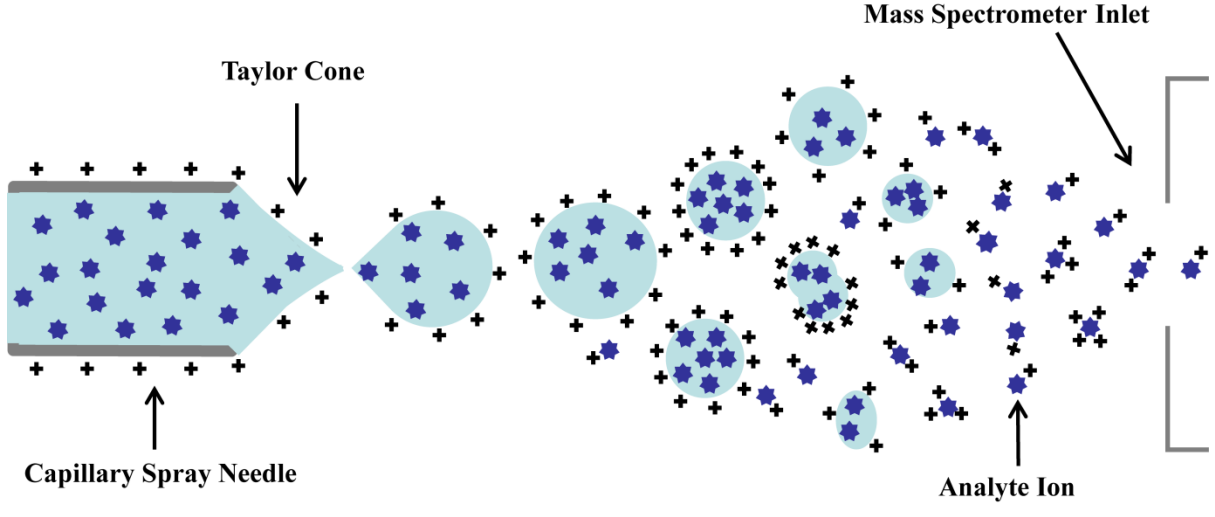

Figure 1 Electrospray ionization mechanism adapted from reference 1

The liquid phase is generally described as the primary phase where ionization suppression or enhancement occurs. Molecules on the outer surface of a droplet enter the gas phase as ions more readily than molecules on the interior of the droplet[4]. An analyte's placement within a droplet is determined by its surface affinity with respect to other droplet components[5]. Consequently when matrix compounds are present, they compete with the targeted compound for surface space, potentially altering the mass spectrometric response of the analyte[6]. King *et al* noted two additional mechanisms in the liquid phase that could cause changes in ionization[7]. Changes in droplet properties, such as surface tension or boiling point, could affect solvent evaporation lowering gas phase analyte ion production. Non-volatile matrix or mobile phase components could precipitate from the droplets, encompassing analyte molecules within the solid particles. Although the gas phase is not the principal source of adverse interactions, charge transfer or neutralization reactions could alter the amount of analyte ions that reach the detector[8].

Nonspecific adsorption is also another phenomenon that presents a challenge to quantitation using LC-MS. Adsorption occurs when it is either entropically or enthalpically favorable for the analyte to interact with the solid surface[9]. Electrostatic and hydrophobic interactions are generally cited as the primary factors governing adsorption[10]. Increasing exposure time incorporates unpredictability to adsorption. When a solution is first introduced to a surface, adsorption/desorption interactions are reversible causing a pseudo-equilibrium between the surface and analyte, but as time progresses adsorption interactions can cause the analyte to undergo conformational changes allowing for stronger bonds with the surface lowering the level of desorption[9]. Figure 2 illustrates how competition for surface space and surface displacement can be caused by compounds in the solution with a higher surface affinity. This phenomenon is known as the Vroman effect[11] and adds to the difficulty of evaluating adsorption interactions.

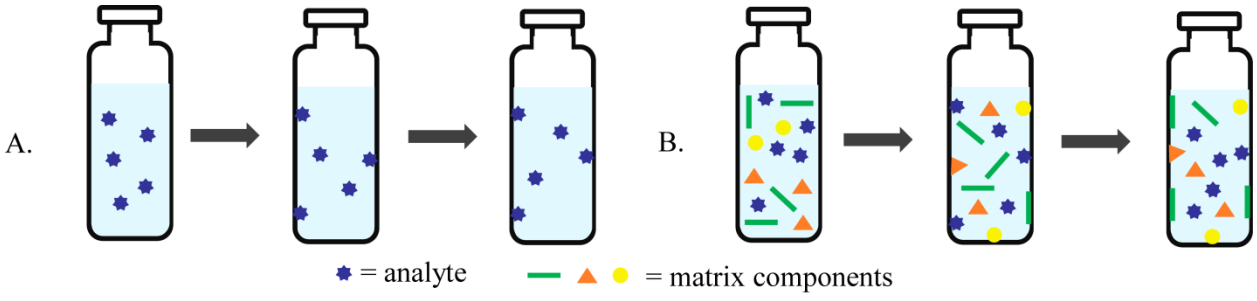

Figure 2 A. Analyte adsorption in a blank solution B. Analyte adsorption with competing matrix components

The lack of knowledge regarding both matrix effects and nonspecific adsorption makes incorporating them into a quantitative method a challenge. This investigation illustrates the variety of analyte and matrix interactions and their role in quantitation accuracy. It is meant as a brief follow-up to our previous study detailed in Chapter 2 and provides a reflection of future changes and experimentation necessary to improve external calibration quantitative results.

3.2 EXPERIMENTAL

3.2.1 Materials and Reagents

Angiotensin I human acetate salt hydrate and insulin from bovine pancreas were obtained from Sigma-Aldrich (St Louis, MO). Stock solutions of angiotensin I and insulin were prepared in 50:50 methanol to water with 0.1% formic acid with concentrations of 50µM and 200µM respectively. Stock solutions were stored at -20°C until use. Additional details regarding materials and reagents, tryptic digestion, and sample preparation can be found in the experimental section of chapter 2.

3.2.2 LC-MS Analysis

Multiple LC-MS instruments were used throughout this study due to the availability of each instrument.

3.2.2.1 Insulin

A nanoACQUITY UltraPerformance LC system (Waters Acquity, Milford, MA) equipped with a Hypersil GOLD column (C18, 100 mm X 1.0 mm i.d., 5 µM, 175 Å; Thermo Scientific, San Jose, CA) was used for the insulin experiments. The insulin calibration standards and quality control (QC) samples were injected with a volume of 5µL. Mobile phase A was 99.9% water and 0.1% formic acid and mobile phase B was 99.9% acetonitrile and 0.1% formic acid. Gradient elution at a flow rate of 15µL/min was used with mobile phase B starting at 2%, then increased to 60% over 24 minutes and held there for 2 minutes. At the end of each run, the column was reequilibrated for 4 minutes with a mobile phase B concentration of 2%. A water blank was included after each sample to eliminate potential analyte carryover.

An LTQ-FTICR hybrid mass spectrometer (Thermo Scientific, San Jose, CA) was coupled to the LC system via an electrospray ionization source. The source was operated in positive ion mode with a spray voltage of 2.80 kV and a capillary temperature of 200°C. MS^1 data was collected using the FT mass analyzer with the resolution set at 25000 for an *m/*z range of 400 to 2000. The three most intense ions in MS^1 were subjected to collision induced dissociation (CID) in the linear ion trap to generate MS/MS data. For CID, the precursor ion width was set at 2 Da with a normalized collision energy of 35%.

3.2.2.2 Angiotensin I and YGGFL

YGGFL was injected into the LC-MS system from polypropylene injection vials. Two trials were completed for angiotensin I; one from glass vials, and the second from polypropylene vials. Instrumentation parameters were used exactly as described in section 2.2.4.

3.2.3 Post-Column Infusion

The post-column infusion system was set-up as previously described in section 2.2.5 Briefly, 5µL of digested BSA or lysozyme was injected into the LC-MS system. Insulin and angiotensin I were consecutively infused with the LC eluent via a T splitter and the flow was directed toward the ESI source. Both peptides had a concentration at the source of 100nM. MS^1 data was analyzed to determine the peptide's signal fluctuation throughout the run.

3.2.4 Data Analysis

Data analysis was completed using Xcalibur 2.1 software (Thermo Fisher Scientific, San Jose, CA). Calibration curves were constructed by plotting concentration against peak area in Microsoft Excel. Peak areas were determined by integrating extracted ion chromatograms (XICs) created using the desired *m/z* in MS^1. The best fit line was generated using linear regression analysis. It was then used to predict experimental concentrations for the validation set.

Table 1. Analyte Characteristics

Analyte	Monoisotopic Mass	Charge State	*m/z* range monitored	Linear Range (nM)	Retention Time (min)
Insulin	5729.6009	5	1146.75-1147.25	100-1240	17.9
Angiotensin I	1296.6848	2	648.5-649	500-1500	11.4
YGGFL	555.2766	1	556.0-556.5	100-1000	12.2

3.3 RESULTS AND DISCUSSION

3.3.1 Overview

External calibration was completed for insulin, angiotensin I, and YGGFL using the *m/z* values and concentration ranges detailed in Table 1. The chemical characteristics and structures of insulin and angiotensin I are shown in Table 2. The external calibration method was evaluated with regards to each analyte and injection vial material. Future improvements and experiments are presented at the conclusion of each subsection.

3.3.2 Insulin

The +4, +5, and +6 charge states of insulin were observed in the full mass spectrum. In these studies, the +5 charge state was the most intense and was chosen for the quantitative analysis. Insulin was infused with BSA and lysozyme digests consecutively. As can be seen in Figure 3 and Figure 4, no ion suppression or enhancement was experienced by the +5 charge state of insulin throughout the entire chromatographic run.

Table 2. Properties of Anigotensin I and Insulin

Compound	Sequence	log P	pI
Angiotensin I	NRVYIHPFHL	-1.1	9.06
Insulin	GIVEQCCASVCSLYQLENYCN FVNQHLCGSHLVEALYVCGERGFFYTPKA	-12.8	5.30

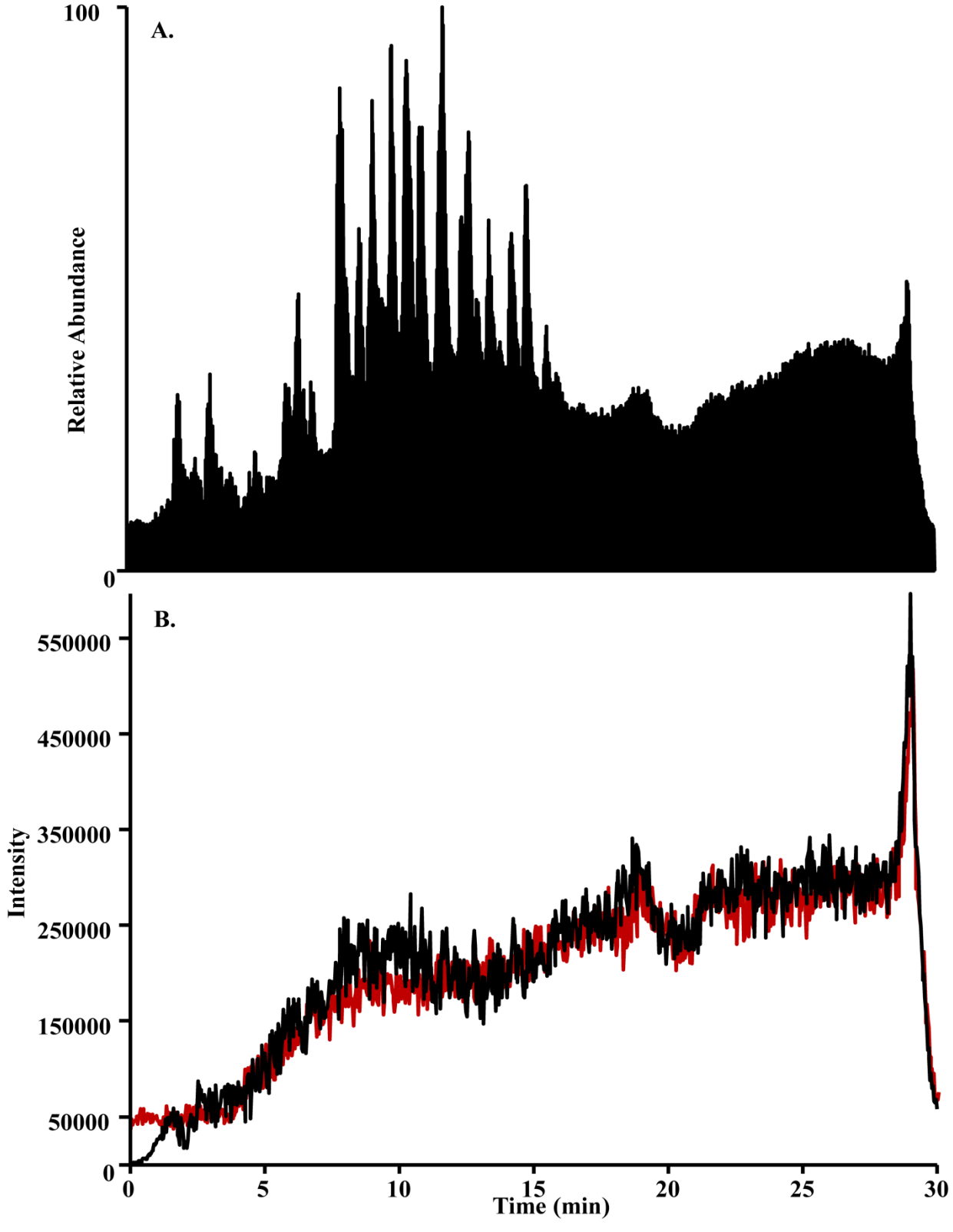

Figure 3 Post-column infusion chromatograms for BSA digest with insulin A) TIC B) XIC of +5 charge state of insulin. Infusion with the blank and BSA are indicated by the red and black lines respectively.

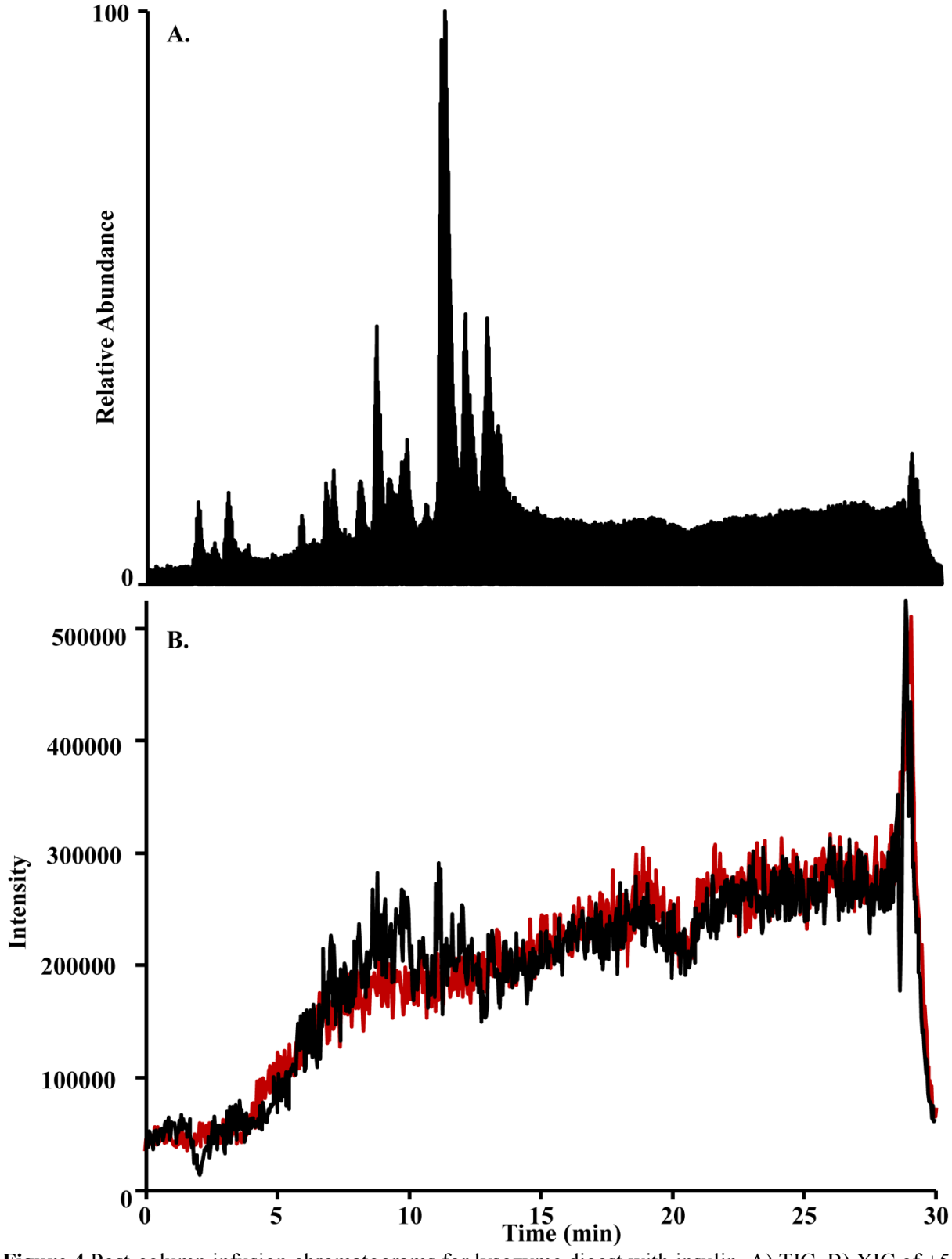

Figure 4 Post-column infusion chromatograms for lysozyme digest with insulin. A) TIC B) XIC of +5 charge state of insulin. Infusion with the blank and lysozyme are indicated by the red and black lines respectively.

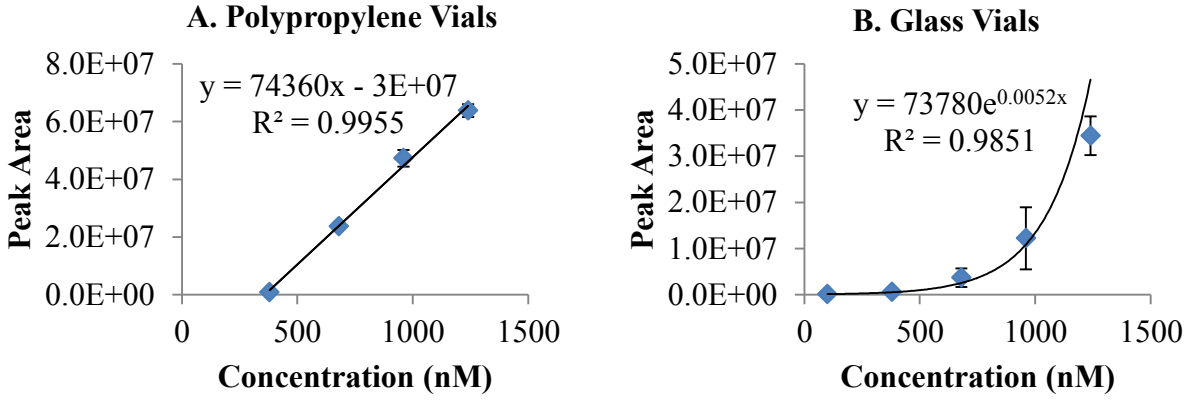

Figure 5 Insulin calibration curves in glass and polypropylene vials

The calibration curves for insulin are shown in Figure 5. There are many notable differences between the polypropylene and glass vials standard curves; the foremost being that an exponential equation instead of a linear equation represents the best-fit line using the glass vials. Upon closer examination of this issue, the overall response using the glass vials is significantly lower than the response using polypropylene vials. For example, the 680nM standard gives a response over 6 fold lower in glass vials than in polypropylene. The significant response difference is attributed to insulin adsorbing to the glass vial. This effect results in the linear range being slightly higher in the glass vials, which causes the data points to fall below the linear range leading to an exponential fit. Additionally the 100nM standard fell below the linear range when using the polypropylene vials, so it was not included in the standard curve.

The best fit equation generated by analyzing the samples in the polypropylene vials had good linearity and reproducibility. However switching to the glass vials caused the reproducibility to decrease due to more adsorption interactions taking place between trial 1 and trial 2. In the glass vials, the R^2 value was not ideal, but this can be accounted for by experimental error. The last point on the standard curve (1240nM), was lower than expected

because the nitrogen gas ran out on the initial trial of this sample and this concentration was repeated at a later time altering the exposure time to the vial.

The theoretical concentration of insulin was 750nM in each of the QC samples. As can be seen in Table 3, the accuracy and precision using polypropylene vials is very good, but using glass vials yields unacceptable results. Since 750nM in not within the linear range when using glass vials these results are not surprising. Additionally, while using the glass injection vials there is over a 45% difference for the concentrations predicted in water and the various digest matrices. However, when using the polypropylene vials the concentrations are all predicted within 15% of each other. This change is hypothesized to be from competition between the matrix and analyte for adsorption sites on the vial's interior, causing an increase in solution for insulin. These results are consistent with those experienced by bradykinin, described in chapter 2, however they do not follow the trend described with the isoelectric point.

The calibration standards and QC samples for the insulin experiments were completed using an LTQ-FTICR mass spectrometer. Since this mass spectrometer is a high resolution instrument, it allowed for easy determination of the various charge states from the MS^1 mode and incorporation of a specific number of isotope peaks during integration. However, this instrument was not able to achieve the low detection limits obtained by using the LTQ Velos mass spectrometer that was used for the other analytes. Preliminary data (not shown) using the LTQ Velos predicted the linear range in glass vials to start at 50nM, significantly lower than the linear range for polypropylene vials obtained using the LTQ-FTICR. Consequently additional experiments using an LTQ Velos instrument should result in an even lower linear range using the polypropylene injection vials

Table 3 Experimentally Determined Concentrations for the Quality Control Sets of 750 nM Insulin

Vial	Water	BSA digest	Lysozyme digest	Digest mixture
PP* (n=2)	731 ± 23 nM	696 ± 20 nM	806 ± 3 nM	707 ± 16 nM
Glass (n=2)	603 ± 81 nM	1100 ± 15 nM	944 ± 44 nM	1020 ± 2 nM

*Polypropylene

3.3.3 Angiotensin I

The +2 charge state of angiotensin I was selected as the target ion for data analysis. The results of the post-column infusion of angiotensin I with both the BSA digest (Figure 6) and the lysozyme digest (Figure 7) showed significant signal enhancement around the retention time of angiotensin I. This was due to co-eluting tryptic peptides from the digest matrices with overlapping *m/z* values and not from changes in ionization efficiency resulting from the matrix. Similar results were obtained when monitoring the other observed charge states of angiotensin I.

Before this problem was discovered, the calibration and validation samples in both glass and polypropylene injection vials were tested (data not shown). In the glass vials, there was a decrease in signal between the first and second trials although this was not experienced in the polypropylene vials. However neither set of standards provided a calibration curve with ideal linearity ($R^2 \geq 0.99$). When it came to the QC sets, peak integration was a challenge; it was often impossible to obtain an area representative only of angiotensin. Consequently the QC data did not give an accurate representation of the actual concentration of angiotensin I and is not reported. Figure 8 shows the poor chromatographic resolution between angiotensin and other co-eluting peptides with the same *m/z*. This data was obtained when angiotensin I was spiked in the BSA and lysozyme digestion mixture. Before matrix effects or adsorption studies can be completed, a quantitation method that has a higher likelihood for success needs to be generated. Resolution between angiotensin I and matrix components with similar *m/z* values and/or the

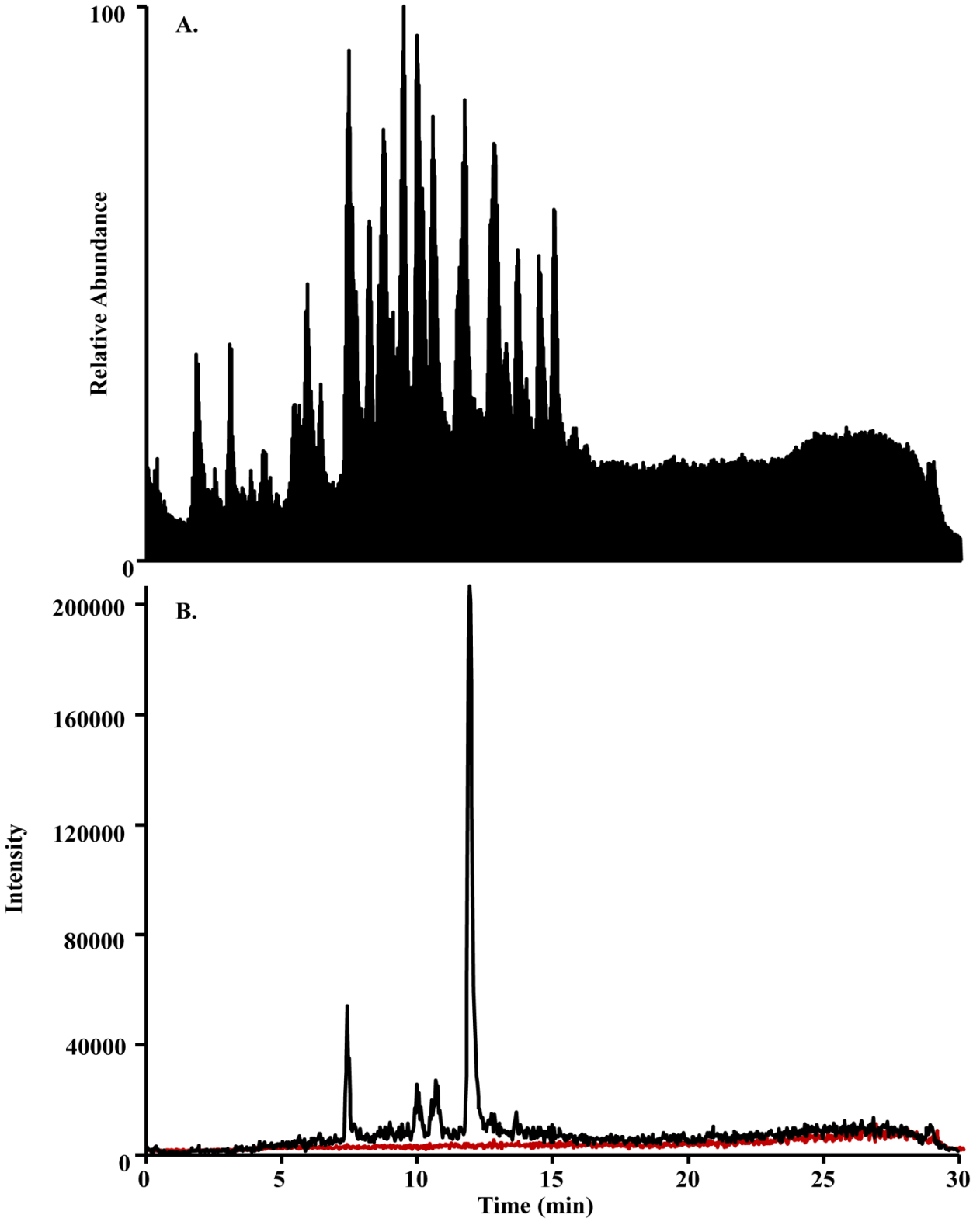

Figure 6 Post-column infusion chromatograms for BSA digest with Angiotensin I. A) TIC B) XIC of +2 charge state of Angiotensin I. Infusion with the blank and BSA are indicated by the red and black lines respectively.

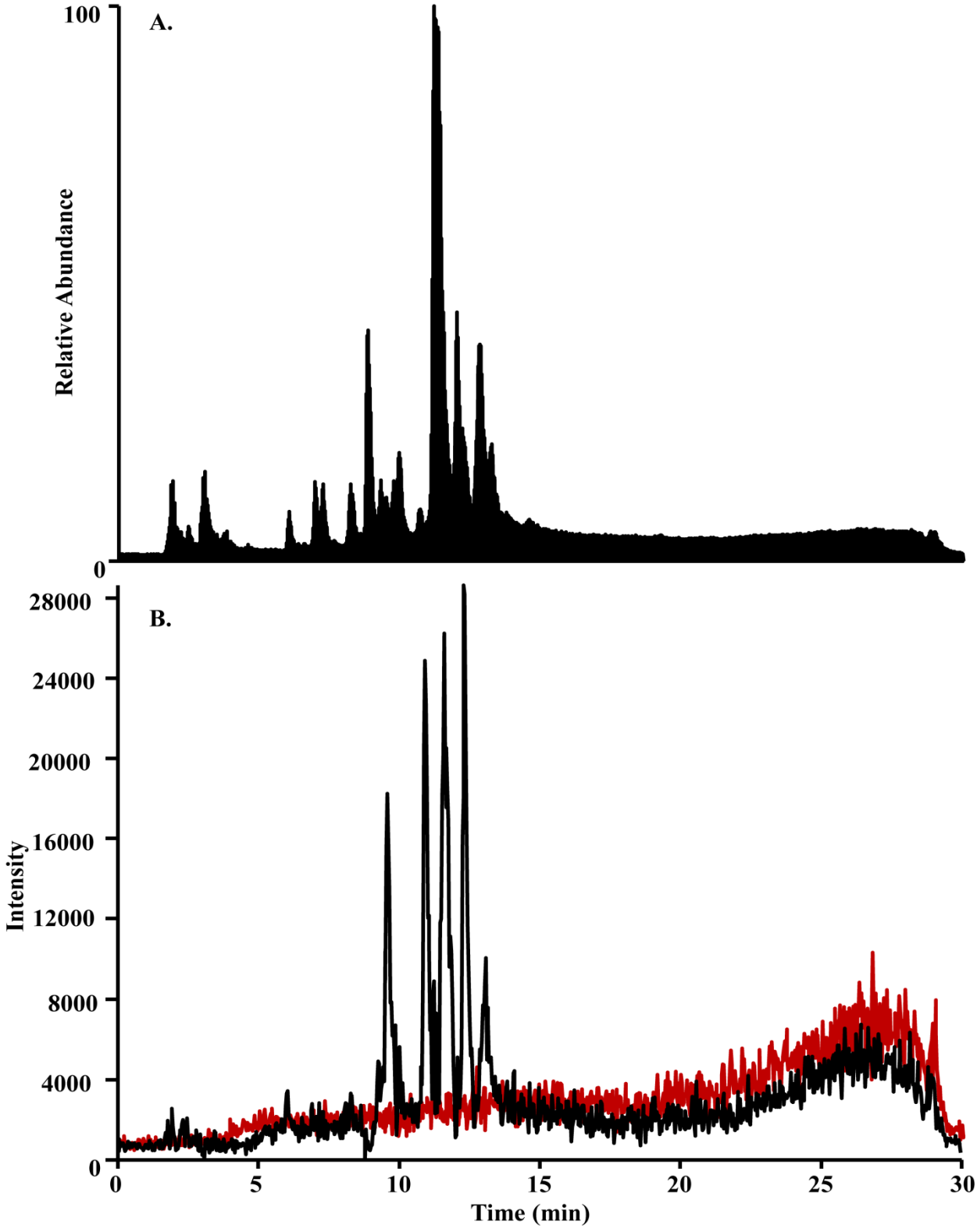

Figure 7 Post-column infusion chromatograms for lysozyme digest with Angiotensin I. A) TIC B) XIC of +2 charge state of Angiotensin I. Infusion with the blank and lysozyme are indicated by the red and black lines respectively.

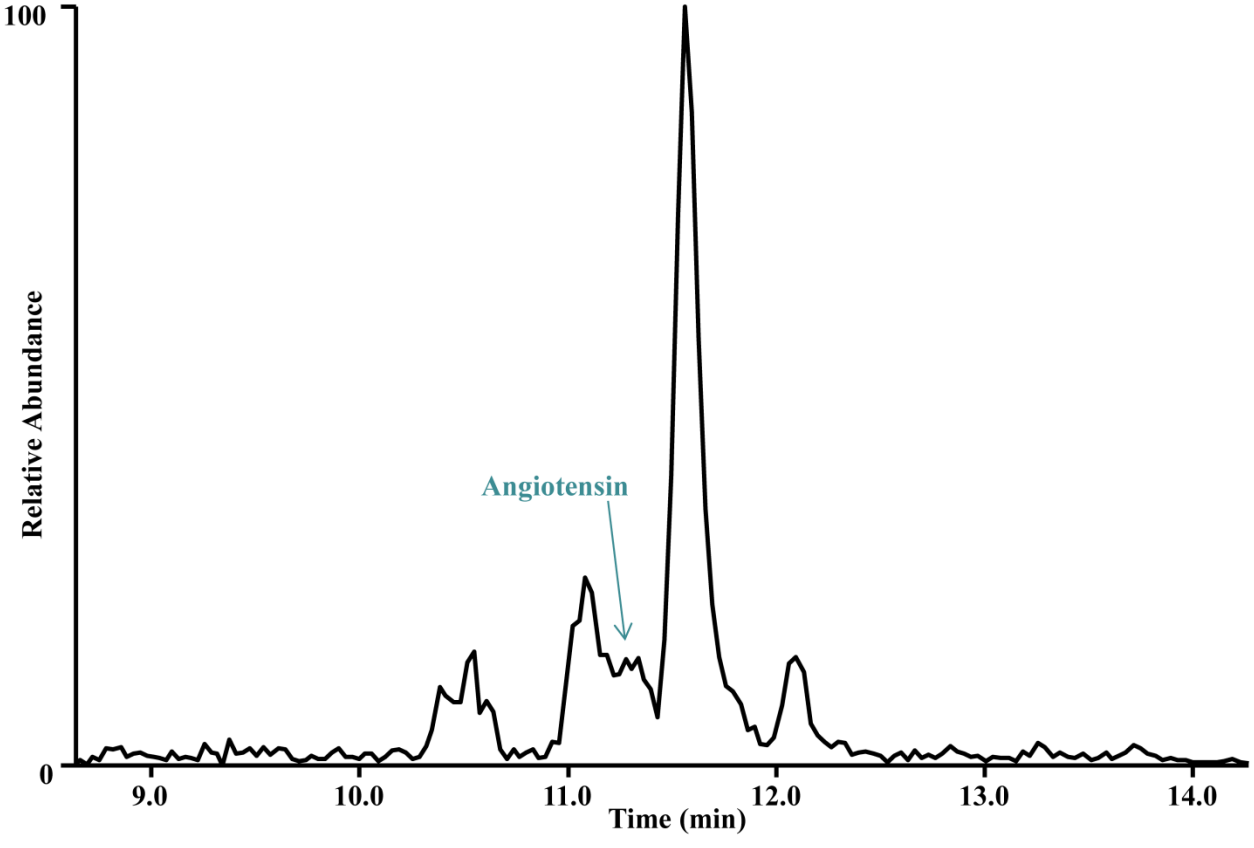

Figure 8 XIC of Angiotensin I with co-eluting peptides with similar *m/z* values

method's selectively need to be enhanced.

Resolution can be improved by altering the chromatographic conditions employed for the experiment. Although there are many parameters that can enhance resolution including lengthening the run time, increasing the flow rate, altering temperature, and lengthening the column, these factors only make minute changes in resolution. Decreasing the slope of the gradient is effective in some instances to resolve peaks, but other times it can cause additional overlap[12]. Testing various slopes and their effect on angiotensin's resolution could prove to be advantageous. Due to the extreme amount of peak overlap experienced by angiotensin I, utilizing a different stationary phase which can distinguish angiotensin I from the matrix components is the most likely way to enhance resolution. Phenyl, C_{18}, and C_4 columns have been shown to alter

the retention time of angiotensin I and could offer increased resolution for this experiment[12]. Selectivity can be improved by changing the mass spectrometric parameters. In this experiment, peaks were integrated from MS^1 data. Single reaction monitoring (SRM) monitors one or more ions resulting from fragmentation of a particular precursor *m/z* value[13]. Picking a precursor to product ion transition that is specific to angiotensin I would offer an increase in selectivity and potentially eliminate interferences from peptides with the same precursor *m/z*. Although such changes were not made, they would be beneficial in future studies involving angiotensin I.

3.3.4. YGGFL

The calibration and validation samples for YGGFL were analyzed from polypropylene injection vials. The calibration standards showed a slight decrease between trials one and two (Table 4) indicating that some adsorption may be occurring. Additionally the 750 nM and 1000 nM standards in trial one (highlighted in yellow in Table 4) were significantly lower than expected. The injection volume was determined for both the 750nM and 1000nM samples by measuring the volume of sample remaining after the injection was completed, and it was found they were injected at a much lower volume than desired. This could have been a result of an unobserved air bubble present in the sample or an instrument malfunction. Consequently those two values were not used to generate the calibration curve. The calibration curve was constructed by plotting peak area against concentration. An average of the peak area for trials one and two were used for the concentrations of 100 nM, 250 nM, and 500 nM, but only trial two was used for 750 nM and 1000 nM. As a result, the linearity is not ideal ($R^2 = 0.9209$) because the 750nM and 1000nM data points are slightly lower than expected, altering the best fit line's placement (Figure 9).

Table 4 Peak area for YGGFL's calibration standards

Concentration (nM)	Trial 1 Area	Trial 2 Area	Average Area
100	38672	12696	25684
250	1461817	1209704	1335761
500	4564226	2411847	3488037
750	730900*	3515206	2123053
1000	728423	4978353	2853388

* highlighting indicts an improper injection

The concentration predictions for the QC set exhibited good accuracy and precision (Table 5). Aside from trial 1 in the BSA digest, all of the trials were predicted within 8% of the theoretical value (600 nM) with % RSD values ≤ 8.5. Trial 1 in the BSA digest matrix experienced an improper injection therefore the results for the BSA digest are reported as trials 1 and 2 separately. Although the accuracy is comparable between glass (as reported in chapter 2) and polypropylene vials, the precision is better in all instances using the glass vials. The lack of precision is due to the decrease between trial 1 and trial 2 attributed to adsorptive interactions with the polypropylene vial. Interestingly, YGGFL is the only analyte in our study that produced

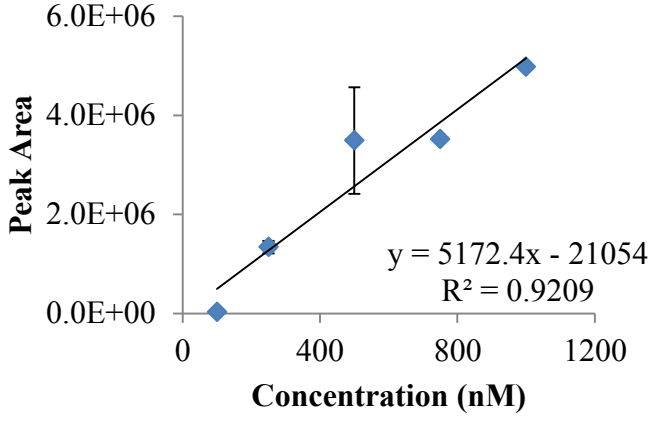

Figure 9 YGGFL Calibration Curve in Polypropylene Vials

better results using glass injection vials than polypropylene. This observation highlights the variability in analyte and vial interactions indicating the importance of optimizing sample containers for each analyte during a quantitation study.

An internal standard could be used to account for variable injection volumes such as the ones that were experienced during this experiment. However since this problem was rare with regards to the numerous trials completed for other analytes, it would be more time efficient to rerun the samples that were not injected properly than to develop a sufficient internal standard.

3.3.5 Future Analytes

There are many other classes of compounds that can be quantified using LC-MS besides the protein/peptide category focused on in this particular study. Many of these compounds exhibit ionic or hydrophobic characteristics that have been associated with adsorptive interactions. Small molecule drugs have been noted to experience both adsorption interactions[14] and matrix effects[15]. Gestrinone, a particularly hydrophobic steroid, could be quantified using polypropylene injection vials and the results compared to those obtained using glass vials as described in chapter 2. The information gathered could be used as a starting point to analyze the effects injection vials have on small molecules.

Table 5 Experimentally Determined Concentrations for the Quality Control Sets of 600 nM YGGFL

Vial	Water	BSA digest[‡]	Lysozyme digest	Digest mixture
PP* (n=2)	632 ± 33 nM	9.24/107 nM	612 ± 50 nM	604 ± 29 nM
Glass[†] (n=2)	667 ± 14 nM	537 ± 42 nM	608 ± 16 nM	553 ± 26 nM

*Polypropylene
[†] Study taken from Chapter 2
[‡] Accuracy is reported for trial 1 and 2 in polypropylene vials to show how trial 1 was effected by an improper injection. Accuracy reported for glass vials remains the average of trials 1 and 2.

3.4 CONCLUSION

Analyte and matrix interactions have been shown to alter LC-MS quantitation accuracy. These interactions are unique to a specific analyte and matrix pair as we have highlighted in this study. Although matrix effects with respect to ionization efficiency are frequently evaluated during method development, nonspecific adsorption is rarely considered. This aspect of analyte and matrix interactions needs to be incorporated into the method development stages of LC-MS external calibration quantitation studies.

Our study only compared analyte and matrix interactions with injection vials; however other surfaces encountered during quantitative analysis could also be affected by variable adsorption. Several surfaces that have been noted to experience adsorption and loss of analyte such as storage containers[16], connective tubing[17], and various parts of the injection system including the needle[18], value rotor seal[19], and injection loop[14]. A thorough investigation of adsorption to these surfaces would encompass not only an analyte's interactions with the material in question, but how that interaction is altered by the addition of the sample matrix.

Glass and polypropylene were the only materials assessed during this study. Injection vials are also made out of many other materials including deactivated glass, amber glass, polystyrene, polyethylene, polymethylpentene, and polytetrafluoroethylene. Utilizing injection vials made from these materials could provide additional quantitation accuracy and precision. Additives can also be applied to coat the vial's interior and reduce adsorption. Examining a variety of such compounds could also improve a method's accuracy and precision.

The accuracy of an external calibration method hinges upon consistent measurements obtained by the LC-MS system. Therefore, when measurements are hindered by one of the previously explained mechanisms, the accuracy of the method is expected to be low. However,

thoroughly investigating potential error sources and tweaking the quantitation method if necessary will create successful external calibration techniques capable of both high accuracy and precision.

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