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CRANBERRY METABOLITES WITH

URINARY ANTI-ADHESION ACTIVITY

A Dissertation
presented in partial fulfillment of the requirements
for the degree of Doctorate of Philosophy
in the Department of BioMolecular Sciences
School of Pharmacy
The University of Mississippi

by

CHRISTINA M. COLEMAN

August 2014

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ABSTRACT

The fruit of the American cranberry (*Vaccinium macrocarpon* Aiton, Ericaceae) is a common food and a top selling dietary supplement in the U.S. Cranberry juice is traditionally used for the prevention of urinary tract infections (UTIs), many of which are caused by P-fimbriated *Escherichia coli*. Human urine produced after cranberry juice consumption can prevent *E. coli* adhesion, but the urinary metabolites responsible for this activity are currently not known. High-molecular weight components of cranberry juice, specifically proanthocyanidin oligomers (PACs), are currently believed to be responsible for the ability of cranberry juice to prevent UTIs. Cranberry PACs can prevent the adherence of P-fimbriated *E. coli* to uroepithelial cells in vitro and are hypothesized to act by a similar mechanism of action in vivo. Many questions exist, however, regarding the potential for PACs to be absorbed, metabolized, and excreted into urine. Adult female sows were therefore fed spray-dried cranberry juice powder (5 g/kg/d) and urine was collected via catheter. Urine fractions were tested for anti-adhesion activity using a human red blood cell (HRBC) anti-agglutination assay with uropathogenic P-fimbriated *E. coli*. Active urine fractions but not control urine or inactive fractions were found to contain complex mixtures of oligosaccharides but not PACs. An octasaccharide, consisting of a tetrameric, β-(1→4)-linked D-glucose backbone and possessing two D-xylose-L-arabinose side chains, was purified from the oligosaccharide mixture of active urine fractions in sufficient quantity to assign the full structure. Oligosaccharide mixtures of similar character were subsequently identified in cranberry products using methods adapted from those applied to urine samples. Cranberry oligosaccharides were enriched in large scale

from three sources of cranberry material and were shown to have activity in both the anti-agglutination assay and a second anti-adhesion assay that used uropathogenic *E. coli* and uroepithelial cells. The results of these studies indicate that the compounds responsible for the anti-adhesion properties of urine after cranberry juice consumption are oligosaccharides that are structurally related to those found in cranberry. These findings overturn the current paradigm regarding the active components of cranberry juice that prevent UTIs and provide many new directions for future research.

DEDICATION

To everyone in my life

who believed I could do it,

even when I doubted.

LIST OF ABBREVIATIONS

AFM	Atomic Force Microscopy
Ara	Arabinose
BuOH	Butanol
CCRC	Complex Carbohydrate Research Center
CC	Cranberry Juice Concentrate
CID	Collision-Induced Dissociation
CJ	Cranberry Juice Powder
Conc	Concentration
DCM	Dichloromethane/Methylene Chloride
DMSO	Dimethyl Sulfoxide
EI	Electron Impact (Ionization)
ELS	Evaporative Light-Scattering
ELSD	Evaporative Light-Scattering Detector
ESI	Electrospray Ionization
EtOAc	Ethyl Acetate
EtOH	Ethanol
FDA	Food and Drug Administration
Gal	Galactose
GC-MS	Gas Chromatography Mass Spectrometry

Glc	Glucose
GI	Gastrointenstinal
HCl	Hydrochloric Acid
HILIC	Hydrophilic Interaction Chromatography
HPLC	High-Performance Liquid Chromatography
HRBC	Human Red Blood Cell
LC	Liquid Chromatography
LC-MS	Liquid Chromatography Mass Spectrometry
LPS	Lipopolysaccharide
MALDI	Matrix-Assisted Laser Desorption Ionization
MeCN	Acetonitrile
MeOH	Methanol
MS	Mass Spectrometry
MSD	Mass Selective Detector
NCCAM	National Center for Complementary and Alternative Medicine
NCNPR	National Center for Natural Products Research
NDM	Non-Dialyzable Material
NIH	National Institutes of Health
NMR	Nuclear Magnetic Resonance
NSI-MS	Nanospray Ionization-Linear Ion Trap Mass Spectrometry
PAC	Proanthocyanidin
PBS	Phosphate Buffered Saline
PDA	Photodiode Array

PMAA	Partially Methylated Alditol Acetate
R_f	Retention Factor
RU	Rutgers University, NJ
SLM	Standard Liters per Minute
SPE	Solid Phase Extraction
temp	temperature
TFA	Trifluoroacetic Acid
TLC	Thin-Layer Chromatography
TMS	Trimethylsilane/Trimethylsilyl (protecting group)
TOF-MS	Time-of-Flight Mass Spectrometry
UEC	Uroepithelial Cell
UM	University of Mississippi
UPEC	Uropathogenic P-fimbriated *Eshcerichia coli*
UTI	Urinary Tract Infection
UV	Ultraviolet
UWM	University of Wisconsin, Madison, WI
VAS	Visual Agglutination Score
Vol	Volume
WSU	Washington State University
Xyl	Xylose

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Thanks are also due to the collaborators involved in these studies. Dr. Amy B. Howell at Rutgers University, NJ, obtained the original NIH/NCCAM funding for the project and performed the anti-agglutination assay. Dr. Christian G. Krueger and Dr. Jess D. Reed at UWM managed all aspects of the swine feedings and urine collection. Dr. Boon Chew, Lindsey Kimble, and Bridget Mathison at WSU developed and performed both versions of the epithelial cell anti-adhesion assay with the assistance of Kerrie Kaspar and financial support from Ocean Spray Cranberries, Inc. I also would like to thank Brian Killday of Bruker, Biospin for assisting with the acquisition of 700 MHz data for the urine octasaccharide, and Dr. Jannie P. J. Marais for providing me with samples of synthetic PAC oligomers and for training me when I first started my graduate studies.

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CO-AUTHOR CONTRIBUTIONS

CHAPTER 2

Amy B. Howell and coworkers tested swine urine and cranberry samples in the UPEC-HRBC anti-agglutination assay developed and performed at Rutgers University, NJ, and reported the assay results to UM. Christian G. Krueger, under the supervision of Jess D. Reed, maintained the study animals at the University of Wisconsin, Madison, WI, and was responsible for feeding cranberry juice powder to swine, and collecting, freezing, and shipping urine samples to UM. Brian Killday assisted with the collection of high resolution NMR data for compound **1** using a 700 MHz instrument. Kimberly M. Auker assisted with the NMR-based comparisons made between cranberry and urinary oligosaccharides used to facilitate compound **1** structural assignments.

CHAPTER 3

Kimberly M. Auker managed the CJA2 Sephadex LH-20 column and assisted with analytical HPLC analysis of Sephadex LH-20 fractions from this column and with the analysis of commercial cranberry products. Lindsey Kimble and Bridget Mathison, under the supervision of Boon P. Chew, tested cranberry samples in the UPEC-UEC anti-adhesion assay developed and performed at Washington State University, and reported assay results to UM. Yurdanur Akgul, a research professor at Ege University, Bornova-Izmir, Turkey, managed the CCA1 and CCA2 Sephadex LH-20 columns while she was a visiting scientist at UM under the supervision of Daneel Ferreira.

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CHAPTER 1: CRANBERRY JUICE & URINARY TRACT INFECTIONS

A. The American Cranberry

The American cranberry (*Vaccinium macrocarpon* Ait., Ericaceae) is a perennial, evergreen, low-growing, shrub-like plant that is native to North America (Roper & Vorsa 1997, McCaleb *et al*. 2000, Neto & Vinson 2011) (Appendix A color plates: Figure 94). The American cranberry is closely related to the European cranberry, or lingonberry (*V. vitis-idaea*), to blueberries (*V. corymbosum*), and to myrtlberries, or bilberries (*V. myrtillus*). Many *Vaccinium* species have received attention regarding their chemistry and biological properties and all are known for their flavonoid composition, acidic or tannic taste, and distinctive flavors. As with other plants in the *Vaccinium* genus, cranberry prefers acidic, bog-type soils. It is cultivated in northern areas of the U. S., with different agricultural varieties suited to different geographic regions. The oldest cranberry cultivars include 'Early Black', 'Howes,' 'McFarlin,' and 'Searles', and many other varieties are actively being developed to improve disease resistance, fruit production, and suitability to different growing environments. Fruits are hollow with a waxy cuticle, ripen in the fall, and are typically harvested through a unique method that involves flooding bogs and knocking the fruit from plants with specialized mechanical beaters.

Cranberry fruit has long been part of the American diet, and cranberry products are familiar to most people as common food items including cranberry juice, sweetened-dried cranberries, and cranberry sauce (McCaleb *et al*. 2000, Neto & Vinson 2011). Cranberry juice

is industrially prepared by pressing fresh berries, and the resulting liquid is filtered and concentrated via spray drying or reverse osmosis to yield juice powder or a concentrated syrup, respectively. Powder and syrup are used as ingredients for the preparation of many different cranberry-based products, especially juices and sweetened juice blends. The pressed berries may be re-infused with a sugar solution and dried to yield sweetened-dried cranberries which may be sold and consumed directly or used as an ingredient in other commercial food products.

Cranberry fruit products are prominent in the U. S. dietary supplement market due to their purported medicinal properties, and cranberry was the number one top selling herbal dietary supplement in 2011, with sales of just over $40 million (Blumenthal *et al*. 2012). Cranberry dietary supplements are typically composed of fruit or juice powder in capsule form with additional inert ingredients that act as binders (gelatin), fillers (cellulose), or stabilizing agents (ascorbic acid). Fruit and juice materials may be concentrated or extracted using methods that are not disclosed by manufacturers to yield products that are marketed as “extra-strength” or “concentrates.”

Cranberry fruit and juice products have been the focus of many studies regarding chemical composition and biological properties and these materials will also be a focus of this report.

B. Cranberry Chemical Composition

The majority of compound isolation and characterization has been done with the ‘Early Black’ cultivar, in part because of its prominence in the food industry, and studies that compare the known chemistry of multiple varieties of cranberry typically do so with a food industry perspective (Justesen *et al.* 1998, Vvedenskaya & Vorsa 2004). The known compounds of

cranberry have been reviewed (Hong & Wrolstad 1986, Pappas & Schaich 2009, Neto & Vinson 2011) and highlights relevant to the present studies are included below. Investigation of cranberries using metabolomics-type methods have indicated that many thousands of compounds are additionally present that remain uncharacterized (Brown *et al*. 2012a,b).

Flavonoids were the first compounds isolated and identified from cranberry (Grove & Robinson 1931) and are consequentially the best studied group of compounds. Flavonoids such as anthocyanins are the main pigments in the berries, and the first of these identified included cyanidin-3-arabinoside, peonidin-3-arabinoside, cyanidin-3-galactoside, and peonidin-3-galactoside (Sakamura & Francis 1960, Zapsalis & Francis 1965). Many early studies involving cranberry flavonoid pigments followed common themes of improving color quality or color stability in commercial cranberry products (Fuleki & Francis 1968a–d, Fuleki & Francis 1967). Work on cranberry anthocyanins has also involved quantification using Matrix-Assisted Laser-Desorption Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF-MS) and other methods (Wang & Sporns 1999, Krueger *et al*. 2004).

Carotenoids and volatile aroma constituents were the next two groups of compounds investigated in cranberry. Compound identifications were based on comparisons of retentions times, fragmentation patterns by GC-MS, and UV spectra to those of standard compounds, and led to the identification of 23 carotenoids and about 45 volatile constituents (Curl *et al.* 1963, Anjou & Von Sydow 1967, Anjou & Von Sydow 1968, Croteau & Fagerson 1968).

Studies have also examined varietal differences in relative quantities of glucose, fructose, sucrose, pectins, ascorbic acid, benzoic acid, and 6-benzoyl-D-glucose (Schmid 1977, Marwan & Nagle 1986a, Marwan & Nagle 1982), and of the flavonoid monomers catechin, epicatechin, myricetin, and quercetin (Wang *et al.* 1978, Justesen *et al.* 1998).

The identification of various cranberry bioactivities and the availability of improved technology and resources led additional researchers to investigate cranberry chemistry. These researchers examined differences in the profiles of known aromatic acids and flavonoids present in fresh and canned cranberry juice before and after acid hydrolysis (Chen *et al.* 2001), identified two new iridoid glucosides and several organic acids (Jensen *et al.* 2002), and reported several additional volatile constituents as part of a controlled atmosphere study examining the stability of berries during storage (Gunes *et al.* 2002). The presence of both *cis*- and *trans*-resveratrol in fresh cranberry juice was initially reported as part of a study to determine the presence and relative quantity of resveratrol in fruit juices and wines (Wang *et al.* 2002), and was later confirmed independently (Rimando *et al.* 2004). Resveratrol was also later identified from other *Vaccinium* species (Rimando & Barney 2005, Rimando & Cody 2005). A series of flavonoid glycosides has been isolated from spray dried cranberry juice powder (Vvedenskaya *et al.* 2004), and several complex organic and aromatic acids, additional glycosylated flavonoids, plant sterols, terpenoids, a peroxypyran, two additional iridoid glucosides, and a depside have also been reported (Turner *et al.* 2007).

Interest in the anti-adhesive properties of cranberry led to the identification of proanthocyanidins (PACs) as compounds of interest. Plant PACs are complex, oligomeric flavonoids, commonly referred to as condensed tannins. They typically occur as a complex, heterogeneous pools of polymers, with both A- and B-type linkages between units, and decoration with galloyl groups and sugars (Foo & Porter 1980, Foo & Porter 1981, Foo *et al*. 2000b, Porter *et al*. 2001). Proanthocyanidin-enriched fractions of cranberry are typically prepared via extraction with EtOAc and the use of dialysis membranes and gel filtration resins such as Sephadex LH-20 or Sephadex G-10 (Foo & Porter 1980, Portor 1988). More soluble,

dialyzable, lower molecular weight (MW) polymers are typically fractionated by gel filtration into groups containing dimers and trimers or tetramers through octamers, while higher MW, non-dialyzable material (NDM) can be retained by dialysis membranes of different MW cut-offs (Delehanty *et al*. 2007). Enriched fractions may then be further purified using high-performance liquid chromatography (HPLC) combined with ultraviolet (UV) spectroscopy and various types of mass spectrometry (MS) to detect and isolate pure compounds (Mabry *et al*. 1970, Prior *et al*. 2001, Berhow 2002). Using methods such as these, epicatechin dimers and trimers with A- and B-type linkages (Figure 1) have been isolated from fresh-pressed cranberries (Foo *et al.* 2000a,b) and from fresh cranberry juice (Vorsa *et al.* 2003). The NDM fraction of cranberry juice has been partially characterized, is reported to lack sugars, proteins, and other low MW compounds, and is believed to consist of PACs with MWs of greater than 14,000 or 6,000 depending on the dialysis membrane and method used (Bodet *et al.* 2007b).

The structural characteristics and complexities of purified cranberry PACs have been investigated using MALDI-TOF-MS with the aid of calculations to determine the number of polymeric units based on mass fragments (Neto *et al.* 2006, Howell *et al.* 2005, Krueger *et al.* 2004, Reed *et al*. 2005), and MALDI-TOF-MS has been combined with ESI-MS, ^{13}C NMR spectroscopy, and acid catalyzed degradation with phloroglucinol for comprehensive structural assignments (Foo *et al.* 2000b). Additional chemical analysis methods may also be used to aid in structural characterization (Mabry *et al*. 1970, Foo & Porter 1981, Berhow 2002, Gu *et al*. 2003). Proanthocyanidin bioactivities are typically reported for crude PAC subfractions due to the structural complexity of these polymeric compounds and the difficulties of isolating pure entities. Proanthocyanidin-enriched extracts are typically prepared by the research group conducting a given biological study and are often presumed to be free of lower MW, non-PAC

molecules. Proanthocyanidin-enriched fractions are typically characterized by MS alone or in conjunction with UV spectroscopy, TLC, and other methods (Mabry *et al*. 1970, Porter *et al.* 2001, Howell *et al.* 2005). Methods typically used cannot, however, assess sample purity or provide information about all other compounds that may be present, and the use of MS-based methods alone for the characterization of PAC-enriched fractions may cause minor constituents to be overlooked or masses to be incorrectly assigned to isobaric compounds. Proanthocyanidin-enriched fractions that are known to contain other flavonoid molecules with potential bioactivity have even been used in some assays (Kandil *et al.* 2002), making it difficult to determine if PACs or other compounds are responsible for a reported bioactivity.

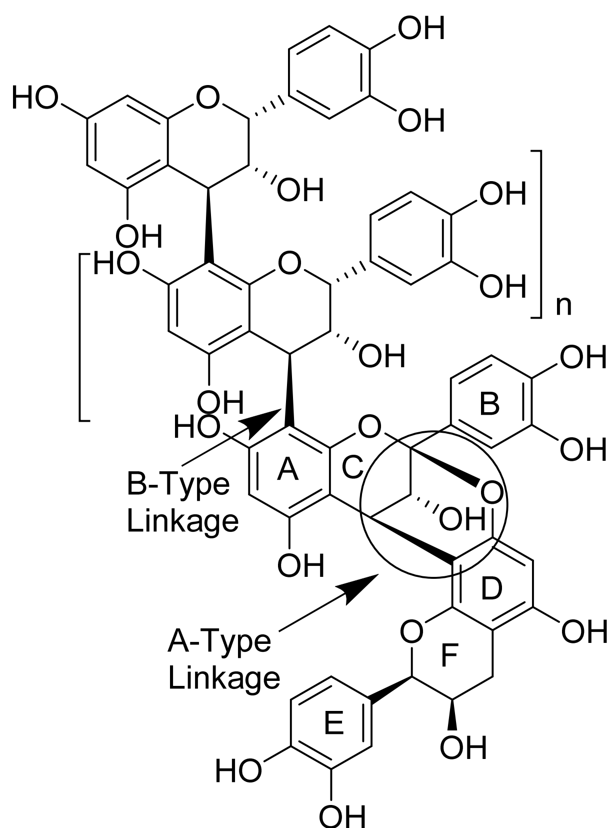


Figure 1. Structure of an oligomeric PAC with indicated A- and B-type linkages.

C. Cranberry Bioactivity

Cranberry juice has been reported to possess numerous bioactivities, many of which have been attributed to PACs, other flavonoids, and other compounds known to be present (Cook & Samman 1996, Beecher 2004, Pappas & Schaich 2009, Neto & Vinson 2011). For many studies assessing biological properties, purified compounds with confirmed biological activity are not obtained.

1. *Anti-oxidant Activity*

Anti-oxidant properties of cranberry have been recognized in numerous studies and are typically attributed primarily to flavonoids such as PACs, flavonols, flavonol glycosides, and anthocyanins. Other compounds also likely contribute to the anti-oxidant properties of cranberry materials, may of which still have yet to be identified. Anti-oxidant assays examining cranberry materials have assessed the oxidation of low density lipoproteins (Porter *et al.* 2001, Wilson *et al.* 1998), free radical scavenging ability (Neto *et al.* 2003), and TPA-induced ornithine decarboxylase activity (Kandil *et al.* 2002), among others (Gunes *et al*. 2002). The anti-oxidant properties of cranberry phytochemicals are thought to be a mechanism by which cranberry possesses antitumor activity (Gao *et al.* 1994, Yan *et al.* 2002), and promotes cardiovascular health (McKay & Blumberg 2007, Porter *et al.* 2001, Wilson *et al.* 1998).

2. *Antitumor Activity*

The potential antitumor activity of cranberry has been assessed primarily using in vitro antiproliferation assays with various tumor cell lines (Neto 2007, Neto *et al.* 2008, Singh *et al.* 2012, Vu *et al.* 2012, Kresty *et al.* 2008). Fractions and individual compounds from cranberry

have been found to inhibit the growth of a range of tumor cell lines and are proposed to act by a variety of mechanisms (Kandil *et al.* 2002; Neto *et al.* 2003; Neto *et al.* 2006). Studies examining the antiproliferative properties of PACs have typically used crude PAC-enriched materials with minimal, if any, structural elucidation of the presumed bioactive components (Ferguson *et al.* 2004, Ferguson *et al.* 2006; Bomser *et al.* 1996; Seeram *et al.* 2004, Seeram *et al.* 2006). Cranberry PACs may be able to prevent the proliferation of several tumor cell lines, but further studies are needed to identify and confirm the structures of the components responsible and to determine the in vivo relevance of cranberry as a cancer chemopreventative.

3. *Antimicrobial & Anti-adhesion Activity*

The reported antimicrobial properties of cranberry juice may be due, in part, to numerous mechanisms that work synergistically to prevent microbial growth (Leitão *et al.* 2005, Shmuely *et al.* 2012, Lee *et al.* 2000). Historically, cranberry juice antimicrobial properties were attributed to the high benzoic acid content (Marwan & Nagel 1986). More recent studies examining cranberry anthocyanins and PAC-enriched fractions (Leitão *et al.* 2005, Hui *et al.* 2004) have suggested that PACs may possess antimicrobial properties against certain select organisms under specific growth conditions. Decreased growth rates were observed for *Staphylococcus aureus*, while P-fimbriated *Escherichia coli* growth was unaffected in the presence of cranberry PACs (Hui *et al.* 2004). Cranberry juice and high-MW cranberry PACs are also reported to possess antiviral activity, hypothesized to be the result of the inhibition of viral particle binding to target cell surface receptors (Weiss *et al.* 2005, Lipson *et al.* 2007, Su *et al.* 2010).

Observed antimicrobial effects of cranberry juice may be due in part to indirect effects resulting from the inhibition of microbial adhesion to substrates. The prevention of adhesion can slow growth rates by preventing the binding of microbes to food sources and can prevent biofilm formation (Hui *et al*. 2004). High-MW cranberry PACs have been reported to prevent the formation of biofilms and subsequent oral disease and tooth decay caused by human mouth bacteria, including *Streptococcus* sp. (Duarte *et al*. 2006, Steinberg *et al.* 2004, Gregori *et al.* 2007, Yamanaka *et al.* 2004), and *Porphyromonas* sp. (Labrecque *et al.* 2006). Cranberry juice has also been found to interfere with the co-aggregation of different species of oral plaque bacteria (Weiss *et al.* 1998). Cranberry juice and high-MW PACs have been shown to interfere with the adhesion of *Helicobacter pylori* to gastric mucous (Shmuely *et al.* 2004, Burger *et al.* 2000). Studies have also shown that high-MW NDM cranberry PACs bind to the Lipid A moiety of lipopolysaccharides (LPS) from Gram negative bacteria, preventing the association of LPS with epithelial cell surface receptors and the subsequent release of signal-transduction components involved in inflammation (Delehanty *et al.* 2007, Bodet *et al.* 2007a,b, Johnson *et al.* 2008). Lipopolysaccharide-induced inflammation plays a role in periodontitis and inhibition of this process may contribute to the ability of cranberry to promote oral health (Bodet *et al.* 2007a,b). Many of these studies have established that the ability of cranberry juice to prevent the adhesion of various bacteria is not due to cytotoxic or antimicrobial effects (Allison *et al*. 2000, Steinberg *et al*. 2004, Yamanaka *et al*. 2004) or to direct interference with host cell surface receptors such as those on epithelial cells or mucous (Burger *et al*. 2000), but rather to direct interactions between cranberry components and microbial cells (Ofek *et al*. 1996, Liu *et al*. 2006, Liu *et al*. 2008).

Cranberry juice has been shown in vitro to prevent the adhesion of P-fimbriated uropathogenic *E. coli* to the receptors found on epithelial cells. Atomic force microscopy (AFM) studies indicate that this inhibition of *E. coli* adhesion is due to molecular-scale changes in the conformation of *E. coli* surface macromolecules resulting from contact with cranberry juice (Liu *et al*. 2006, Liu *et al*. 2008). Bioassay-guided fractionation using an in vitro human red blood cell (HRBC) anti-agglutination assay has led to the identification of A-type PACs with a degree of polymerization of 3–6 as compounds that could be responsible for the *E. coli* anti-adhesion properties of cranberry juice (Foo *et al.* 2000a,b). Anti-adhesion studies comparing PAC-enriched fractions to whole cranberry juice indicate, however, that additional anti-adhesion compounds may be present in the juice (Turner *et al.* 2005, Pinzón-Arango *et al.* 2009, Holguin *et al.* 2008, Tao *et al*. 2011, Ermel *et al*. 2012, Kimble *et al*. 2014).

Bacteria are known to bind to carbohydrate receptors on the surfaces of human epithelial cells using protein adhesins. These adhesins, such as the P-type and Type-1 fimbriae of *E. coli*, are differentially expressed by various strains of a given pathogen (Ofek & Beachey 1978, Enerbäck *et al*. 1987, Dodson *et al*. 2001, Hull *et al*. 2002, Lane & Mobley 2007), and many pathogens have the ability to simultaneously express multiple types of adhesins (De Man *et al.* 1987, Sharon 2006, Pieters 2007). Different adhesins selectively bind to carbohydrate ligands with different structures, thereby resulting in cell and tissue tropism (Abraham *et al*. 2002, Pieters 2007, Dodson *et al.* 2001, Strömberg *et al*. 1991). Compounds that mimic the natural binding ligands of microbial adhesins or that interfere with adhesin-ligand binding would be able to interfere with microbial adhesion to cell surfaces, thereby preventing subsequent infection (Beachey 1981, Sharon & Ofek 2000, Sharon 2006, Pieters 2007). The

identification of the anti-adhesive compounds present in cranberry is therefore of great medical interest (Sobota 1984, Ofek *et al*. 1991, Howell *et al*. 1998, Shoaf-Sweeney & Hutkins 2008).

Several types of assays have been used to assess the *E. coli* anti-adhesion properties of cranberry. These typically measure the ability of the study organism to bind to (a) a purified receptor bound to an artificial substrate, (b) human red blood cells (HRBCs), or to (c) the target epithelial cells. Purified receptor assays are highly specific and are typically used to help identify the binding epitope of specific microbial adhesins (De Man *et al.* 1987, Zafriri *et al*. 1989). Results of these assays are informative regarding adhesin binding sites and the mechanisms of microbial binding, but cannot be reliably extrapolated to in vivo systems. The PapG adhesin of P-fimbriated *E. coli* binds to the α-Gal-(1→4)-β-Gal disaccharide epitope found on the surfaces of (A_1, Rh+) HRBCs causing agglutination (Leffler & Svanborg-Edén 1980, Kallenius *et al*. 1980, Leffler & Svanborg-Edén 1981, Winberg 1984, Enerbäck *et al*. 1987, Lane & Mobley 2007). In vitro assays that measure HRBC agglutination have been generally accepted as a suitable, indirect measure for bacterial adherence to eukaryotic epithelial cells. They typically measure hemagglutination visually, however, and may therefore be susceptible to inter-experiment and inter-observer variations that can make it difficult to quantify results (Johnson *et al*. 1997). Bacterial-epithelial cell adhesion assays are superior to bacterial-HRBC anti-agglutination assays as they more closely simulate in vivo systems. These assays are logistically more difficult to perform and maintain, but can provide quantifiable, direct measures of bacterial-epithelial cell adhesion (Turner *et al.* 2005, Mathison *et al*. 2013, Kimble *et al*. 2014). The studies discussed in this report used both bacterial-HRBC anti-agglutination and bacterial-epithelial cell anti-adhesion assays (Howell *et al*. 2010, Mathison *et al*. 2013, Kimble *et al*. 2014) to assess fraction anti-adhesion properties.

D. Urinary Tract Infections & Cranberry

Cranberry juice is commonly used as a traditional American folk remedy for the prevention of urinary tract infections (UTIs) and there is increasing use of cranberry dietary supplements for the same application (Zafriri *et al*. 1989, Ofek *et al*. 1991, Blumenthal *et al*. 2012, Smith 2013).

1. *Urinary Tract Infections*

Types of UTIs include cystitis (bladder infection) and the more severe pyelonephritis (kidney infection) which can lead to permanent kidney damage, bloodstream infections, sepsis and death (Winberg 1984, Scholes *et al*. 2005). Nearly half of all women are believed to experience a UTI at some point in their lives, and recurrent UTIs are common (Kunin 1994b, Foxman 2010). Uropathogenic P-fimbriated *E. coli* (UPEC) cause up to 80% of uncomplicated, community-acquired UTIs (Kunin 1994b, Dowling *et al*. 1987, Enerbäck *et al*. 1987). Bacteria are typically inoculated into the urinary tract from the gastrointestinal (GI) tract or are introduced by medical devices during hospitalization (Scholes *et al*. 2005, Foxman 2010). Bacteria adhere to and then infect the cells lining the urinary tract, known as uroepithelial cells (UECs), causing inflammation and pain associated with uroepithelial cell shedding (Beachey 1981, Justice *et al*. 2004, Mulvey *et al*. 1998, Hull *et al*. 2002). A variety of antimicrobial agents are used to treat UTIs, but drug resistant infections are a common problem (Kunin 1994a, Foxman 2010). This is partly due to the formation of dormant *E. coli* reservoirs that are protected from antimicrobial agents and partly due to the presence of resistant *E. coli* strains in the GI tract that serves as the infection source (Mulvey *et al*. 2001, Justice *et al*. 2004, Howell & Foxman 2002).

The high incidence of UTIs results in an estimated $2.3 billion in annual (2010) direct health care costs and other societal costs (Foxman 2010). As the initiation of a UTI requires bacterial adhesion to uroepithelial membranes, compounds or products that interfere with such adhesion are of great clinical interest (Sobota 1984, Sharon & Ofek 2000, Howell & Foxman 2002). Women who have recurrent UTIs may be given low doses of antimicrobial agents as prophylaxis, but such prophylactic treatments can eventually lead to increased drug resistance for the strains of uropathogenic *E. coli* that infect these women (Gupta *et al*. 2001, Kunin 1994a, Howell & Foxman 2002). Prevention of UTIs is generally preferable to treatment with antimicrobial agents, as the use of preventive strategies would not provide a selective pressure for the development of antibacterial drug resistance (Kunin 1994a, Signoretto *et al*. 2012, Reid *et al*. 2003). Preventative therapies are also generally equally effective against both drug-susceptible and drug-resistant strains of bacteria (Howell & Foxman 2002, Shoaf-Sweeney & Hutkins 2008).

2. *Clinical Use of Cranberry for UTIs*

Anecdotal and clinical evidence supports the ability of cranberry juice to help prevent UTIs (Zafriri *et al*. 1989, Avorn *et al*. 1994, Ofek *et al*. 1991, Ofek *et al*. 1996, Reid *et al*. 2003, Howell *et al*. 2010). These reports often employ cranberry juice, cranberry juice concentrate, or cranberry juice powder, and the use of native cranberry juice material rather than extracts or "enriched" fractions may have contributed to the success of these studies (Reid *et al*. 2001, Howell *et al*. 2010, Wang *et al*. 2012a, Jepson *et al*. 2012). In contrast, other reports are inconclusive or do not directly support the effectiveness of cranberry as a preventative agent, and these studies often used extracts or cranberry dietary supplements with

unknown chemical composition (Beerepoot *et al*. 2011, Jepson *et al*. 2012). Studies that have used meta-analyses to evaluate available clinical trials concerning the use of cranberry for the prevention of UTIs have reached differing conclusions as to whether cranberry should be recommended as a UTI preventative (Wang *et al*. 2012a, Jepson *et al*. 2012, Smith 2013). These conflicting reports highlight the current mix of results that exists regarding the efficacy of cranberry products and prompt questions regarding which cranberry products are the most effective. Many of these studies may be assessing the efficacy of specific preparations or products rather than the efficacy of actual cranberry constituents, and a better understanding of cranberry bioactive constituents is necessary to resolve these questions and conflicts.

3. ***Cranberry Mechanism of Action Hypotheses for UTI Prevention***

It was once hypothesized that cranberry juice acidified the urine, dropping the pH through the action of hippuric acid and thereby creating an unfavorable environment for bacterial growth in the bladder and the rest of the urinary tract (Bodel *et al*. 1959). This hypothesis was later disproven (Papas *et al*. 1968, Ofek *et al*. 1991) and has since been replaced by the hypothesis that certain high-MW constituents of cranberry prevent infections by inhibiting the adherence of uropathogenic bacteria to UECs (Sobota 1984, Zafriri *et al*. 1989, Avorn *et al.* 1994, Ofek *et al*. 1996, Howell *et al*. 1998). Studies using AFM have shown that cranberry juice constituents have direct effects on the conformation *E. coli* P-fimbriae (Liu *et al*. 2006, Liu *et al*. 2008). The results of UPEC-UEC anti-adhesion assays validated with galabiose and globotriose provide strong evidence that cranberry constituents bind to microbial cells rather than to epithelial cells (Turner *et al* 2005). Cranberry juice and human urine produced after cranberry consumption both possess anti-adhesion activity (Howell *et al*. 2010,

Tao *et al*. 2011) and it has therefore been assumed that the compounds responsible for the activity of both materials are structurally related and present in both juice and urine. The active compounds present in urine are currently believed to be PACs or PAC-derived metabolites due to the reported in vitro anti-adhesion properties of A-type PACs isolated from cranberry (Howell *et al*. 1998, Foo *et al*. 2000a,b), and to the anti-adhesion activity of human urine produced after the consumption of cranberry powder standardized for PAC content (Howell *et al*. 2010), but PAC-related anti-adhesive metabolites have yet to be identified in mammalian urine.

E. Anti-adhesive Urinary Metabolites of Cranberry Juice

Many different digestive processes may apply to generate the anti-adhesive urinary metabolites of cranberry. Urinary metabolites are water soluble and chemical components are typically of medium to low MW. Many types of compounds are excreted into human urine, and urine metabolomics studies indicate that thousands of individual compounds are present in a given urine sample, many of which are unknown (Saude *et al*. 2007, Bouatra *et al*. 2013).

Compounds with high polarity may be absorbed directly into the bloodstream and filtered through the kidneys into the urine within a relatively short period of time without metabolic modification (Brunton 2011, Rowland & Tozer 2011). Compounds with low polarity may be modified via enzymatic and nonenzymatic metabolic and catabolic pathways to yield more polar or lower MW metabolites, and may therefore take a longer period of time to reach the urine. Processes may include oxidation, hydroxylation, hydrolysis, and the addition of sulfate, glucose, glucuronic acid, or other polar groups via Phase I and Phase II metabolic pathways (Brunton 2011, Rowland & Tozer 2011). Phase I enzymes involved in these

processes typically include cytochrome P450 oxidases and reductases, and various esterases and hydrolases, among others. Phase II enzymes that conjugate low-polarity ingested compounds to more polar groups include glutathione *S*-transferases, glycosyl transferases, and acetyl transferases, among others. In general, lower-polarity and higher-MW compounds are more likely to be excreted through the fecal route. In the lower digestive tract, microbial enzymatic biotransformations can then occur to modify ingested compounds, yielding higher-polarity and lower-MW metabolites that are then absorbed into the bloodstream and possibly excreted via urine.

Little is currently known regarding in vivo PAC absorption and metabolism, but the chemical nature of PACs suggests that these compounds are unlikely to be directly excreted into urine. Many flavonoids are excreted into the feces, and may be derivatized or degraded by gut microflora before re-entering the bloodstream to be later excreted in the urine (Schneider & Blaut 2000, Braune *et al.* 2001, Rios *et al.* 2003, Blaut *et al.* 2003, Goldin 1986). A PAC-type drug has recently been approved by the U.S. FDA for the treatment of non-infectious diarrhea in HIV patients (Frampton 2013), further suggesting fecal elimination of PACs and similar compounds. It is also likely that ingested PACs bind to other food molecules and protein receptors along the digestive tract due to the tendency of PACs to form non-specific, covalent bonds with proteins (Zhu *et al*. 1997). Such protein binding may prohibit or delay absorption into the bloodstream.

Flavonoids that have been identified in urine are typically glucuronated, sulfated, or glycosylated derivatives of ingested compounds with relatively low concentrations of free phenolic forms (Hollman *et al*. 1995, Hollman *et al*. 1999, Harada *et al.* 1999, Li *et al.* 2001, McGhie *et al*. 2003, Shimoi *et al*. 2003, Daykin *et al.* 2005, Kumazawa *et al*. 2004). If PACs

are excreted into urine, it would therefore likely be as either conjugated or derivatized forms of the known anti-adhesive PAC oligomers (Rechner *et al*. 2002). However, analytical studies that have specifically searched for the presence of flavonoids and related metabolites in urine after cranberry consumption have been unsuccessful at identifying PACs (Wang *et al.* 2012b, Iswaldi *et al.* 2013). Many urinary metabolite studies depend upon comparisons of unknown metabolites to reference compounds to confirm compound identifications, and the relative lack of PAC standards makes the identification of these structurally complex, high-MW compounds in urine impractical using typical, standards-based methods.

F. Objectives

The primary objectives of these studies were therefore (1) to isolate and identify the urinary metabolites of cranberry juice that are responsible for the prevention of UPEC adherence, and (2) to identify the compounds present in cranberry that are related to the anti-adhesive urinary metabolites.

CHAPTER 2: PORCINE URINARY METABOLITES OF CRANBERRY

A. Introduction

Human volunteers who consume cranberry juice produce urine that has anti-adhesive properties (Howell *et al.* 2005, Howell *et al.* 2010, Tao *et al.* 2011). Anti-adhesion bioactivity appears shortly after consumption of the juice until about eight hours later, and maximum anti-adhesion effects are observed within four to six hours after ingestion (Howell *et al.* 2010, Tao *et al*. 2011). This suggests rapid absorption and excretion of anti-adhesive cranberry metabolites into the urine.

The identification of the anti-adhesive urinary compounds produced after cranberry consumption is essential for further studies regarding the efficacy and bioactive constituents of cranberry, but questions exist regarding whether PACs are the rapidly excreted urinary metabolites with anti-adhesion properties (Chapter 1, Section E). The primary objective of this study was therefore to isolate and identify the compounds responsible for the anti-adhesion properties of urine after the consumption of cranberry products. This objective was based on the hypothesis that the anti-adhesive urinary metabolites of interest were either PACs or metabolically derived from PACs (Howell *et al*. 1998, Howell *et al*. 2005). These hypothesized anti-adhesive metabolites were expected to be structurally related to the PACs previously identified from cranberry that have been shown to have in vitro UPEC-HRBC anti-agglutination activity (Foo *et al*. 2000a,b).

Methods used for this study included the collection of urine after cranberry consumption, bioassay testing using a UPEC-HRBC anti-agglutination assay, and bioassay-guided isolation of active fractions and metabolites, followed by structural characterization of putative active compounds using various methods. Sows were chosen to generate cranberry-derived urinary metabolites due to their similar physiology and dietary adaptations to those of humans (Pond 1986, Tumbleson 1986, Tumbleson & Schook 1986), and because they can be fed a consistent diet supplemented with relatively large quantities of cranberry powder per day. Sows also generate the volumes of urine (~1–2 L/d) necessary to provide sufficient material for bioassay-guided fractionation and metabolite purification.

B. Materials & Methods

1. *General Experimental Equipment*

Lyophilization was performed using a variety of Labconco FreeZone Systems housed in the NCNPR and Faser Hall at the University of Mississippi. All sample materials were stored at –20 or –60 ºC. The centrifuge used was a Thermo IEC Centra CL3R instrument with exchangeable holders for different sizes of Falcon tubes. Early analytical and preparative HPLC separations of FA urine fractions were performed on a Waters Delta Prep 4000 system with #5 pump heads and a Prep LC Controller connected to a 2487 dual-wavelength UV detector. Analytical HPLC separations were later primarily performed on a Waters Alliance 2695 Separations Module with a 996 photodiode array (PDA) UV detector connected in series to a Polymer Laboratories PL-ELS2100 analytical scale evaporative light scattering detector

(ELSD). Preparative HPLC separations for H and I urine fractions were performed on a Waters Delta Prep 4000 system with #5 pump heads and a Prep LC Controller connected in series to a 2487 dual-wavelength UV detector and a Polymer Laboratories PL-ELS1000 ELSD. A Bruker MicroTOF-Agilent LC 1100 series instrument was used to acquire LC-MS data with a Phenomenex Luna C_8 column (4.6 x 150 mm) at 27 ^{o}C, with a flow rate of 0.4 mL/min and a 20 min gradient from 20–100% MeCN or MeOH with 0.1% formic acid. Additional equipment used by the CCRC is discussed in each respective section.

Regular solvents, TLC plates, HPLC solvents, and most reagent chemicals were obtained from Fisher Scientific, Inc. Additional reagents, Sephadex LH-20, and Diaion and XAD resins were obtained from Sigma-Aldrich, Inc., and SPE sorbents were obtained from Phenomenex Inc. and Waters, Inc. Columns for HPLC were obtained from Waters, Inc., and HPLC sample filters were obtained from Millipore, Inc. Water used for all experiments was obtained from ultrafiltration systems located in Faser Hall or NCNPR. Water for HPLC was additionally filtered within two days prior to use.

2. *NMR Spectroscopy*

Two different Bruker Avance III 400 MHz NMR instruments were used to acquire NMR data. These instruments were housed in Faser Hall at the University of Mississippi, School of Pharmacy, and were equipped with Ultrashield^{TM} and Ultrashield^{TM} Plus magnets and 5 mm probes. Additional spectra were recorded on a Bruker Avance 700 MHz instrument equipped with a 5 mm CPTCI cryoprobe, housed in the Bruker facilities in Billerica, MD.

Samples for NMR analysis were prepared by dissolving 2–10 mg of material in a minimal amount of high purity DMSO-d_6 or D_2O (Cambridge Isotope Laboratories, Inc.).

Selected samples were repeatedly lyophilized with D_2O to avoid interference from the water resonance. Either DMSO-d_6 (99.99%) or D_2O (99.99%) was used for most sample preparations, and selected samples in NMR tubes were topped with argon gas to delay the absorption of atmospheric water prior to data acquisition. The use of pulse programs for water suppression during data acquisition was minimized to avoid resonance loss in this region. Spectra for most fractions were recorded at 400 MHz and additional high-resolution spectra were obtained at 700 MHz for use in assigning the structure of **1**.

3. *Source Material*

Research-grade, spray-dried cranberry (*Vaccinium macrocarpon* Ait., Ericaceae) juice powder (Ocean Spray Cranberries, Inc.) was supplied through NIH grant no. 1R21AT002076-01 to both the University of Wisconsin, Madison, WI, (UWM) for the feeding of swine, and to the University of Mississippi, Oxford, MS, (UM) for preliminary separations and analytical studies.

Five separate feedings produced the urine samples used for these studies, four of which (Table 1) are discussed in this chapter. Animal protocols were approved by the UWM Review Committee (approval number A3368-01, 04/25/2002). Adult female sows (~160 kg each, 1–2 yrs of age) used in this study were housed in gestation crates at the Livestock Laboratory on the UWM campus and were offered a maintenance amount of a basal diet consisting of corn and soybean supplemented with tryptophan, methionine, lysine, and various vitamins and minerals as necessary to meet the recommended maintenance requirements of the National Research Council (National Research Council 1998).

Research-grade cranberry juice powder as described above was mixed with basal diet components at an administration rate of 800 g powder/d/sow (~5 g/kg body weight) for 3–5 d prior to collection of treatment urine. Feeding was continued through a collection period of 3–4 d and urine was collected via Foley catheters (2–6.5 L per sow). Control urine (~2 L) was also collected via catheter prior to initiating the third feeding of cranberry powder. Urine was collected in containers placed over ice, containers were emptied at 4 h intervals, and collected urine was frozen. Animals were monitored two times per day and Foley catheters checked regularly by research staff during collection periods.

The D and E urine materials were lyophilized as whole urine by UWM prior to shipment to UM for chemical analysis. The F, H and I urine samples were frozen at –20 ^{o}C immediately after collection, and were shipped to UM as frozen liquid on dry ice. Aliquots of each fresh urine (~5 mL each) were frozen and sent to the laboratory of Dr. Amy B. Howell at the Philip E. Marucci Center for Blueberry and Cranberry Research, Rutgers University, Chatsworth, NJ, (RU) for preliminary testing in the UPEC-HRBC anti-agglutination assay to confirm anti-adhesion activity (not shown) prior to materials being shipped to UM.

Table 1. Details of swine urine collections used as source materials.

Feeding Period	Urine Collection Dates	Received by UM	Animal Number	Urine Vol.[a] (L)	Solute Conc.[a] (g/L)	UM Sample Code
1	2005/12/19[b]	2006/02	NA[b]	2.5	11	D
3[c]	2006/10/27 (control, 1 day)	2006/12	5081	2.0	18	FC
3	2006/10/31 – 2006/11/02 (3 days treatment)	2006/12	5081	7.0	20	F
4	2008/06/24 – 2008/06/27 (4 days treatment)	2008/07	5023	3.5	17	H
5	2008/06/24 – 2008/06/27 (4 days treatment)	2008/07	8401	2.5	18	I

[a] Approximate values. [b] Incomplete information was provided for this material. [c] The second feeding, designated sample E, was used for numerous studies, but this parent material was ultimately found to have questionable UPEC-HRBC anti-agglutination activity.

4. *Anti-agglutination Assay*

The anti-agglutination assay used for this study was performed at RU using methods described previously with minor modifications (Foo *et al.* 2000a,b). This assay has been used to identify PACs as anti-adhesive constituents of cranberry samples (Foo *et al.* 2000a,b) and to detect anti-adhesion properties of human urine samples after cranberry product consumption (Howell *et al.* 2005, Howell *et al*. 2010). The results of this assay have been accepted as as representative of the ability of UPEC to adhere to epithelial cells (see Chapter 1, Section C3).

a. *Bacterial Strains*

Clinical strains of uropathogenic P-fimbriated *E. coli* (UPEC) were isolated and cultured as described previously (Foo *et al.* 2000a,b) with the modifications that P-fimbriated bacteria were not washed to prevent fimbriae from breaking off and were suspended directly in phosphate buffered saline (PBS, pH 7.0) at a concentration of 5 x 10^8 bacteria/mL PBS for testing. Cultures were kept on agar slants at 4 ^oC for short-term use over several months and strains were kept frozen at –70 ^oC in tryptose broth (30% glycerol) for long-term storage.

b. *Hemagglutination Assessment*

Test samples were dissolved in different volumes of PBS based on the amount of sample provided (*e.g*. 150 µL for 10–30 mg, 200 µL for 40–70 mg, 400 µL for > 80 mg), and adjusted to neutral, physiologic pH (between 5.5 and 7.0) with 1 N NaOH. Serial two-fold dilutions of each test sample were prepared in PBS and were tested as described previously (Foo *et al.* 2000a,b). For ease of reference these methods are reproduced as follows:

> "A 30 µL drop of each dilution was incubated with 10 µL of bacterial suspension on the lid of a 24-well polystyrene plate for 10 min at room temperature on a rotary shaker. Freshly-drawn HRBCs (A_1, Rh +) [donated

by human volunteers] were suspended (3%) in PBS and added separately (10 µL drops) to test suspensions. Suspensions were incubated for 20 min on a rotary shaker at room temperature and evaluated microscopically for the ability to prevent agglutination. The final dilution was recorded at which agglutination suppression by the test fraction occurred. Controls included wells containing bacteria + PBS, HRBC + PBS, bacteria + test fraction, HRBC + test fraction, and bacteria + HRBC." (Foo *et al*. 2000b)

This assay has been reported to detect PAC anti-adhesion activity at 25–50 µg/mL when performing two-fold serial dilutions with a starting amount of 1 mg purified PAC material (A. B. Howell 2006, personal communication).

The microscopic evaluation for agglutination involved a visual assessment using a 0–4 scale as established by RU (Table 2). For example, a visual agglutination score (VAS) of '0' represented 90–100% HRBC agglutination (Figure 2a) and therefore no inhibition of *E. coli* adhesion by the test sample, while a score of '4' represented 0–5% HRBC agglutination (Figure 2b) and total inhibition of *E. coli* adhesion by the test sample. Dilution endpoints were reported as the lowest dilution concentration at which the sample received a VAS of '1' or '2' with the next dilution receiving a VAS of '0' and having no anti-agglutination activity. Lower concentration dilution endpoints therefore indicated higher relative anti-agglutination activity.

Table 2. Assignment criteria for visual agglutination score (VAS) values associated with UPEC-HRBC agglutination.

VAS	HRBC Agglutination (%)	Inhibition of *E. coli* Adhesion by Test Sample (%)
0	90–100	0
1	60–90	10–40
2	30–60	40–70
3	5–30	70–95
4	0–5	95–100

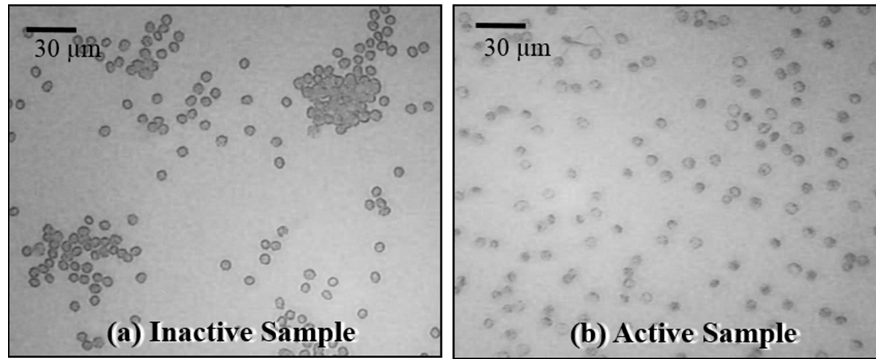


Figure 2. Light microscope image of HRBC agglutination by *E. coli* in the presence of inactive (a) and active (b) samples.
The VAS value assigned to panel (a) would be '0' as clumps of HRBCs have formed, while the VAS value assigned to panel (b) would be '4' as no HRBC agglutination has occurred. Photographs provided by A. B. Howell.

c. *Test Samples*

Samples were submitted to RU as dry powders (~10–400 mg per sample) and were shipped overnight on cold packs or dry ice. Sample weights were provided, but sample identities were coded and samples were randomized within a set to avoid possible evaluation and test sequence biases. Duplicate or triplicate samples were submitted for parent materials and some fractions, but sample quantity limitations and the labor-intensive nature of the assay made it impractical to submit triplicate samples for every sample tested in every sample set. To account for assay variations over time, selected urine fractions were repeatedly tested with each set of samples submitted. These included control urine samples and fractions and the parent fractions for each separation step.

d. *Interpretations of Assay Raw Data*

Assay results were interpreted by UM using a qualitative scoring system similar to one previously published in association with this assay (Foo *et al*. 2000a). The scoring system used for this study assigned one of five scores (UM Scores) to each sample tested, based on four

different criteria (Table 3). The majority of UM Score assignments were made based primarily on dilution endpoint values, with secondary consideration given to the number of two-fold dilutions applied to each sample. The third and fourth criteria, involving the comparisons of fractions to parent materials or replicates, respectively, were considered but were given lower priority as they did not apply for all samples. The UM scores were used to determine whether a sample was or was not considered to be active and possibly able to prevent UPEC-epithelial cell adhesion.

Table 3. Description of the qualitative scoring system developed by UM to interpret the results provided by the UPEC-HRBC anti-agglutination assay.
The dilution endpoint values and number of two-fold dilutions for individual samples can be found in the tables of Appendix B-3. Samples were assessed within an individual sample set test date and then across test dates. Scoring criteria were applied in the order shown to determine the probable activity of individual samples and assign a UM Score. Replicates were not available for most samples.

Scoring Criteria:		First	Second	Third	Fourth
UM Score	UM Assessment of Sample Activity	Dilution Endpoint Range	No. of Two-fold Dilutions	Sample Compared to Parent Fraction:	Replicates (if available):
+ +	active	< 10 mg/mL	5+	more active	show activity
+	probably active	10–30 mg/mL	4	more active	show activity
+/–	may or may not be active	30–60 mg/mL	3	about the same	may or may not show activity
–	probably not active	60–100 mg/mL	2	less active	show no activity
– –	not active	> 100 mg/mL	0, 1	less active	show no activity

5. ***Initial Characterization & Method Development***

The premise that the anti-adhesive metabolites of cranberry would be polyphenolic compounds such as PACs or related flavonoids was used to guide initial method development (Mabry *et al*. 1970, Porter 1988, Berhow 2002). Numerous methods were therefore applied in attempts to enrich for putative PAC metabolites. These included solvent-solvent extraction, gel

filtration with Sephadex LH-20 and G-10, column chromatography with silica or C_{18} packing, hydrophobic extraction resins (Diaion, XAD-8 and XAD-16), and many types of solid phase extraction (SPE) cartridges (Waters Oasis® and Phenomenx®) with sorbents including C_8, C_{18}, WAX, WCX, MAX, MCX, HLB, phenyl and others. Numerous TLC sorbents and solvent systems were also employed in efforts to visualize and characterize fraction components. The details of these methods have been omitted because they did not yield usable results.

6. *Enrichment for Active Urinary Components*

a. <u>*Urine Processing & Extractions*</u>

Frozen liquid urine (F, H, & I) was thawed overnight at room temperature. The general processing and purification protocols shown in Figure 3 were applied to four batches of urine (FC, F, H, and I). Crude urine was centrifuged (5 min at 2500 RPM) or gravity filtered through filter paper (Whatman #1) to remove casts and solids. A portion of each clarified urine was removed and lyophilized to serve as pre-extraction control samples. Clarified urine aliquots (1 L each) were partitioned with EtOAc (2–4 x 250 mL) or EtOAc:MeOH (2–4 x 50:250 MeOH:EtOAc). After solvent partitioning, EtOAc and aqueous urine fractions were evaporated under reduced pressure with a water bath temperature of ~40 ^{o}C to remove organic solvents and decrease the total volume of aqueous samples. EtOAc fractions were re-dissolved in water, aqueous samples were diluted as necessary, and all samples were frozen and lyophilized. Repeated dilution and lyophilization was often necessary for highly hygroscopic samples. All samples were stored as dry powders or concentrated syrups (hygroscopic samples) in airtight containers at –20 ^{o}C. Selected samples were submitted to the anti-agglutination assay as dry powders.

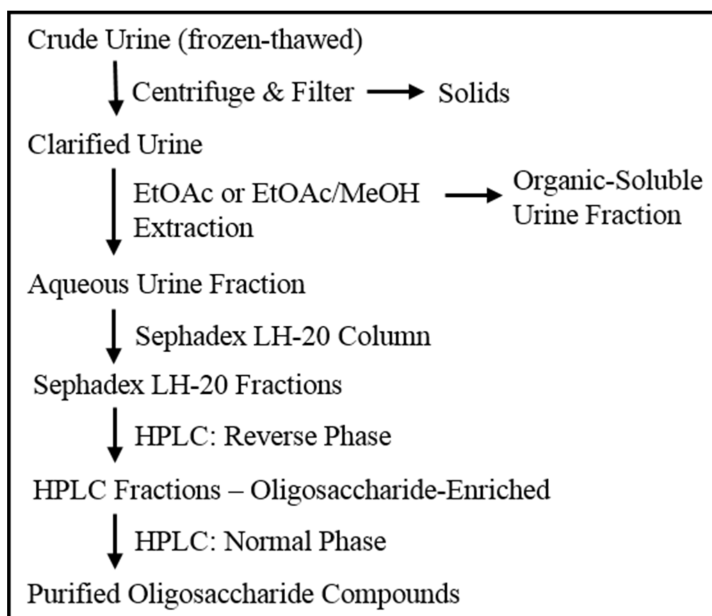


Figure 3. General purification protocol used for isolation of active urine metabolites.

Control urine (FC) was partitioned using the procedures developed for the active urine samples, but was processed only through to the stage of analytical reverse-phase HPLC analysis due to the absence of oligosaccharide components. Approximately 1.5 L raw urine was centrifuged and filtered and the clarified urine (1.5 L) was extracted with EtOAc (2 x 375 mL). Both fractions were concentrated, diluted, and lyophilized as before. The aqueous fraction could not be successfully dried by lyophilization and instead produced a concentrated, viscous, gooey material with about 90–95% of the original water removed. A portion of this material (FC1, 15–20 mL, resulting from 250 mL of the concentrated aqueous fraction) was separated on a Sephadex LH-20 column (4 x 33 cm) using an isocratic 75% EtOH solvent system, a flow rate of 0.8–1.2 mL/min, and a collection time of 5 min/tube (Appendix A color plates: Figure 95). Fraction composition was compared by TLC on silica (MeOH:EtOAc:water, 8:1:1, sprayed with ninhydrin) and fractions were combined to give three fractions (FC1-1, 3.539 g; FC1-2, 3.065 g; and FC1-3, 0.233 g). Even with enrichment, control urine fraction FC1-1

lacked characteristic oligosaccharide components detectable by HPLC-ELSD or NMR spectroscopy, and further HPLC separation was not pursued. Control urine fractions were submitted to the anti-agglutination assay as negative controls.

A portion of the raw I urine sample (~250 mL) was used for a rudimentary form of stability testing. This material was mixed with 60 mL MeOH and left covered in a 1 L Erlenmeyer flask at room temperature with ambient lighting for a period of three months. After three months, this solution (IZ) was centrifuged, filtered, extracted with EtOAc (150 x 4), and the resulting fractions lyophilized to yield IZ-A (3.573 g) and IZ-E (0.048 g). A portion of the IZ-A sample was submitted to the anti-agglutination assay to determine if activity remained in the aqueous fraction after the stability treatment described.

b. *Sephadex LH-20 Chromatography*

Aqueous urine fractions (10–50 g per column; various loading concentrations in eluting solvent) were loaded onto Sephadex LH-20 columns (various dimensions) and eluted with 50 or 70% EtOH (isocratic) with gravity fed solvent and various flow rates (Tables 4–6; Appendix A color plates: Figures 96, 97). Fractions (5 to 25 mL each) were collected using an automated fraction collector and tubes were combined based on appearance and relative elution volume. Selected preliminary fractions and Sephadex LH-20 fractions were submitted to the anti-agglutination assay for testing. Active and putatively active Sephadex LH-20 fractions were recombined and further purified as described below using additional Sephadex LH-20 columns (for the FA sample) and reverse and normal phase HPLC with UV and ELS detection.

Table 4. Details of multiple Sephadex LH-20 separations for the F aqueous urine sample. The total amount of active material (FA, 1.747 g) recovered from all five columns accounted for 1.3% of the total loaded samples (133 g) and contained both active and inactive components.

Column Designation	F1	F2	F3	F4	F5
Amount of Loaded Material (g)	10.0	14.0	10.0	50.4	48.5
Loaded Volume & Concentration	45 mL 222 mg/mL	36 mL 388 mg/mL	31.5 mL 317 mg/mL	120 mL 417 mg/mL	100 mL 485 mg/mL
Column Dimensions (cm; width x height)	5.5 x 32	7.5 x 5	5.5 x 34.5	10 x 24	10 x 24
Solvent Composition	50% EtOH	70% EtOH	50% EtOH	70% EtOH	70% EtOH
Solvent Flow Rate (mL/min)	1.8–2.0	1.0–1.2	1.2–1.4	2.0–4.0	3.0–5.0
Active Material Total Elution Volume (mL)	150	50	114	456	572
Active Fractions	F1-1 F1-2 F1-7[a]	F2-1 F2-2[b]	F3-1 F3-2	F4-1 F4-2	F5-1 F5-2[b]
Total Active Material per Column (g)	0.239	0.287	0.138	0.356	0.727
Active % of Loaded Material	2.39%	2.05%	1.38%	0.71%	1.50%

[a] Activity in this sample was the result of contamination with part of fraction F1-2 during material transfers.

[b] These samples did not show activity but were included in recombined FA sample to ensure full recovery of potentially active metabolites.

Table 5. Details of Sephadex LH-20 separation for the recombined, activity-enriched FA sample.
The total amount of active material recovered from this column (0.917 g) accounted for 54.5% of the loaded FA sample contains primarily active components, and is equivalent to 0.7% of the total amount of separated F samples (133 g).

Column Designation	FA
Amount of Loaded Material [a]	1.683 g
Loaded Volume & Concentration	5 mL; 337 mg/mL
Column Dimensions (width x height)	2.5 x 40.5 cm
Solvent Composition	75% EtOH
Solvent Flow Rate	0.5–1.0 mL/min
Active Material Total Elution Volume	200 mL
Active Fraction	FA1 (0.030 g) FA2 (0.887 g)
Total Active Material per Column	0.917 g
Active % of Loaded Material	54.5%

[a] Approximately 0.06 g of FA was reserved as a bioassay testing control.

Table 6. Details of Sephadex LH-20 separations for the H and I urine samples.
Combined oligosaccharide-containing fractions (1.334 g) comprised approximately 1–2% of the total amount of separated material (69 g) and contained oligosaccharides with putative activity as well as other, unrelated compounds.

Column Designation	HF1	HF2	IF1	IF2
Amount of Loaded Material (g)	10	40	30	14
Loaded Volume & Concentration	30 mL 333 mg/mL	125 mL 320 mg/mL	50 mL 300 mg/mL	50 mL 280 mg/mL
Column Dimensions (cm; width x height)	7 x 38	10 x 25	7 x 38	7 x 38
Solvent Composition	70% EtOH	70% EtOH	70% EtOH	70% EtOH
Solvent Flow Rate (mL/min)	1.0–1.5	1.5–2.0	1.0–1.5	1.0–1.5
Total Elution Volume for Oligosaccharide-containing Fractions[a] (mL)	350	450	380	200
Oligosaccharide-containing Fractions[a]	HF1-1 HF1-2	HF2-1 HF2-2	IF1-1 IF1-2 IF1-3	IF2-1[b] IF2-2[b]
Oligosaccharide Material per Column (g)	0.148	0.607	0.425	0.154
Active[c] % of Loaded Material	1.48%	1.52%	1.42%	1.10%

[a] HF1 fractions were tested in the Rutgers assay. HF2, IF1, and IF2 fractions were compared by HPLC to HF1, FA1, and FA2 fractions to direct isolation efforts while conserving material.
[b] The IF2 fractions were combined with the respective IF1 fractions prior to later separations.
[c] Putative activity based on composition comparisons using HPLC and NMR.

7. *Isolation of Oligosaccharides by HPLC*

Analytical scale HPLC was performed on a Waters 2695 separations module equipped with a Waters 996 PDA detector connected in series to a Polymer Laboratories PL-ELS2100 ELSD. Preparative HPLC separations were performed using a Waters Delta Prep 4000 chromatographic system equipped with an LC controller and a 2487 dual-wavelength UV detector connected in series to a Polymer Laboratories PL-ELS1000 ELSD. Preparative scale fractions were collected in parallel with ELSD monitoring through the use of a flow splitter installed in the output line after the UV flow cell. This device directed approximately 0.5–3 mL/min of eluent to the ELSD, allowing the rest to be collected. For Polyamine semi-preparative separations, the HPLC instrument was configured with analytical scale tubing, the analytical-scale UV detector flow cell, and split detection-collection with ~0.5–1 mL/min of eluent delivered to the ELSD and the remaining ~2–2.5 mL/min delivered to a collection vessel. For AtldC18 preparative separations, the HPLC instrument was configured with preparative scale tubing, the preparative-scale UV detector flow cell, and split detection-collection with ~1–3 mL/min of eluent delivered to the ELSD and the remaining ~22–24 mL/min to a collection vessel. System pressure data was acquired for all separations to monitor system and column performance.

Analytical columns used included a Waters Atlantis dC_{18} (AtldC18) column (4.6 x 150 mm, 5 µm particle size), a YMC Polyamine II (Polyamine) column (10 x 150 mm, 5 µm particle size, 12 nm pore size), and a Waters XTerra MS C_{18} (XTMSC18) column (4.6 x 150 mm, 5 µm particle size). The AtldC18 and XTMSC18 analytical columns were both equipped with appropriate guard columns (4.6 x 20 mm). Analytical flow rates of 1–3 mL/min were typically used. The AtldC18 column was used with either water:MeCN or water:MeOH

gradients and a flow rate of 1 mL/min with a commonly used solvent systems of 100% water for 5 min, 100% water to 30% MeOH over 30 min, and 30 to 100% MeOH over 20 min. A commonly used solvent system for the Polyamine column consisted of 5 min at 65% MeCN, followed by a gradient from 65 to 45% MeCN over 25 min, with a flow rate of 2–3 mL/min for analytical separations. All columns were equilibrated in a minimum of 10 column volumes of the initial solvent for each separation. Reverse phase samples were dissolved in a minimum volume of water, while normal phase samples were dissolved in a minimum amount of either DMSO, water, or, at most, 60% MeOH. Samples were syringe filtered with 0.2 μm filters (PTFE or Nylon, 13 or 33 mm diameter) unless otherwise indicated, and various amounts of material were injected per separation depending on the nature of the sample, the column, and chromatographic conditions used. Typical ELSD settings for analytical AtldC18 and XTMSC18 separations were a gas flow rate of 0.8–1.2 SLM, an evaporator temperature of 100 ^{o}C, and a nebulizer temperature of 50 ^{o}C, and those for the Polyamine column were a gas flow rate of 1.2–1.4 SLM, an evaporator temperature of 90 ^{o}C, and a nebulizer temperature of 50 ^{o}C. These values remained constant throughout each separation and the ELSD was allowed to equilibrate for at least 30 min at the desired settings prior to initial data acquisition.

Columns used for preparative and semi-preparative separations included an AtldC18 column (19 x 250 mm, 10 μm particle size), and a YMC Polyamine II column (10 x 150 mm, 5 μm particle size, 12 nm pore size), respectively. Guard columns were not used. Instrument parameters, solvent systems, and sample preparation methods used for semi-preparative and preparative separations were based on analytical methods and were scaled appropriately to maintain chromatographic resolution. Commonly used solvent flow rates for the preparative AtldC18 and semi-preparative Polyamine columns were 20–25 and 4–6 mL/min, respectively,

with ~0.5–1 mL/min directed to the ELSD. Various amounts of material were injected per separation, depending on the nature of the sample, the column, and chromatographic conditions used (Tables 16, 20). An example method for the preparative AtldC18 column used MeOH:water, a flow rate of 25 mL/min, and 100% water for 5 min, 100% water to 20% MeOH over 30 min, 20% MeOH to 100% MeOH over 10 min, 3 min at 100% MeOH, followed by a 17 min gradient from MeOH to 100% water with an equilibration of 10 min prior to the next separation. An example method for the Polyamine column used MeCN:water, a flow rate 4 mL/min, and 65% MeCN for 5 min, 65–45% MeCN over 25 min, and a 3 min gradient to 65% MeCN, followed by equilibration in 65% MeCN.

Typical ELSD settings for the AtldC18 preparative separations were a gas flow rate of 0.8–1.2 SLM, an evaporator temperature of 100 ^{o}C, and a nebulizer temperature of 50 ^{o}C, while those for the Polyamine semi-preparative separations were a gas flow rate of 0.8–1.2 SLM, an evaporator temperature of 90 ^{o}C, and a nebulizer temperature of 45 ^{o}C. These values remained constant throughout each separation and the ELSD was allowed to equilibrate for at least 30 min at the appropriate settings prior to initial data acquisition.

Fractions collected by preparative or semi-preparative HPLC were recombined based on retention time and chromatographic profiles and were concentrated under reduced pressure with a water bath temperature of ~40 ^{o}C to remove solvent and decrease water content. Resulting samples were dissolved in water, frozen, and lyophilized. Selected samples were analyzed by NMR and LC-MS.

Residual material recovered after HPLC separations from putatively active H and I Sephadex LH-20 fractions was recombined based on HPLC profiles to give three fractions,

HI1, HI2 and HI3. These fractions were submitted to the anti-agglutination assay testing and examined by ^{1}H NMR spectroscopy.

8. *Carbohydrate Derivatization Analyses*

The methods described above yielded a single sample, compound **1**, in sufficient purity and quantity (~10 mg) for further structural characterization. This sample was determined to be a complex carbohydrate using preliminary NMR data and was therefore submitted to the Complex Carbohydrate Research Center (CCRC) at the University of Georgia, for specialized, professional, fee-for-service analysis. The majority of the structural characteristics for **1** were determined using a series of analyses that required derivatization of the sample and were therefore destructive. The results of these analyses provided information on (a) glycosyl composition, (b) glycosyl linkage positions, (c) high-resolution mass by MALDI-MS, (d) monomer configuration, and (e) glycosyl sequence. Details of the methods used by the CCRC are included below.

a. Glycosyl Composition

Glycosyl composition was determined using gas chromatography/mass spectrometry (GC-MS) of per-*O*-trimethylsilyl (TMS) derivatives of the sample. Monosaccharide methyl glycosides were produced by acidic methanolysis (1 M HCl in MeOH at 80 °C, 18–22 h), followed by re-*N*-acetylation with pyridine and acetic anhydride in MeOH for detection of amino sugars. The sample was then per-*O*-trimethylsilylated by treatment with Tri-Sil (Pierce) at 80 °C (30 min) as previously described (Merkle & Poppe 1994, York *et al.* 1986). Inositol was added to the sample before derivatization as an internal standard (20 μg per sample). GC-MS analysis of the TMS methyl glycosides was performed on an HP 6890 GC interfaced to a

5975b MSD, using an All Tech EC-1 fused silica capillary column (30 m × 0.25 mm ID). Monosaccharides were identified by comparisons of retention times to those of standards, and mass spectra were used to authenticate the carbohydrate nature of the derivatives.

b. *Glycosyl Linkage (NaOH method)*

For glycosyl linkage analysis, the sample was permethylated, depolymerized, reduced, and acetylated. The resulting partially methylated alditol acetates (PMAAs) were analyzed by GC-MS (York *et al.* 1986). Initially, an aliquot of the sample after dialysis was suspended in about 200 μL of DMSO. The sample was then permethylated using treatment with NaOH and CH_3I in dry DMSO (Ciucanu & Kerek 1984). The sample was subjected to NaOH base for 10 min and CH_3I was added and left for 20 min. This process was repeated to ensure complete methylation. Following sample workup, the permethylated material was reduced by superdeuteride to the methyl ester of uronic acid, hydrolyzed using 2 M TFA (2 h in a sealed tube at 121 °C), reduced with $NaBD_4$, and acetylated using Ac_2O/TFA. The resulting PMAAs were analyzed on a Hewlett Packard 5890 GC interfaced to a 5970 MSD (mass selective detector, electron impact ionization mode). Separation was performed on a Supelco 2330 bonded phase fused silica capillary column (30 m).

c. *MALDI-MS*

The sample was dissolved in deionized water (1 mg/mL) and 1 μL of the solution was deposited on a spot of 2,5-dihydroxybenzoic acid matrix dried from MeCN:water (1:1). This material was subjected to MALDI-MS on a Bruker MicroFlex Mass Spectrometer (+ mode). All masses were calibrated to malto-oligosaccharide controls separated immediately before the sample.

d. *Monomer Configuration*

The absolute configuration of monomeric glycosyl units was determined by comparisons between derivatized compound substituents and derivatized standards. The sample was hydrolyzed in 2M TFA (500 µL) at 120 ºC for 1.5 h, the hydrolysate was dissolved in 200 µL (*S*)-(+)-2-BuOH (Fluka 19025), 15 µL acetyl chloride (Aldrich 236691) was added, and N_2 was bubbled through the solution for 30 s. The mixture was capped tightly and incubated at 80 ºC for 16 h. The mixture was dried under N_2, and absolute MeOH was added to ensure sample dryness. To the dry sample, 250 µL TMS was added and derivatization was carried out at 121 ºC for 20 min. The same procedure was applied to authentic standards D-Glc, L-Ara, and D-Xyl; two sets of standards were derivatized to which either (*S*)-(+)-2-BuOH or (*R*)-(-)-2-BuOH was added separately. The sample and standard derivatives were analyzed on an Agilent 5975C GC interfaced with a 7890A MS detector.

e. *Glycosyl Sequence*

Glycosyl sequence was determined using per-*O*-methylated glycans analyzed by Nanospray Ionization-Linear Ion Trap Mass Spectrometry (NSI-MS^n) (Anumula & Taylor 1992). The sample was dissolved in DMSO, permethylated as described above (Anumula & Taylor 1992), extracted with DCM, and dried under N_2. The resulting per-*O*-methylated glycan was dissolved in 1 mM NaOH in 50% MeOH and directly infused with a syringe flow rate of 0.5 µL/min into an LTQ Orbitrap Discoverer mass spectrometer (Thermo Scientific) equipped with a nanospray ion source. The capillary temperature was set to 210 °C, and MS analysis was performed in positive ion mode with fragmentation by CID (25% collision energy) in MS/MS and MS^n modes. Indicative partial-fragmentation patterns from NSI-MS^n analysis of the per-*O*-

methylated derivative of **1** were used to confirm side chain linkage positions and establish monomer connectivity.

Compound 1: β-D-glucopyranosyl-(1→4)-[α-L-arabinofuranosyl-(1→2)-α-D-xylopyranosyl-(1→6)]-β-D-glucopyranosyl-(1→4)-[α-L-arabinofuranosyl-(1→2)-α-D-xylopyranosyl-(1→6)]-β-D-glucopyranosyl-(1→4)-β-D-glucopyranose (**1**); White amorphous powder; ^{1}H NMR and ^{13}C NMR data: Figure 4, Figures 59–68, Table 28; MALDI-MS (Figure 42) (positive ion mode) $[M+Na]^+$ at *m/z* 1218.117, to give a calculated molecular formula of $C_{44}H_{74}NaO_{37}^+$ for **1**.

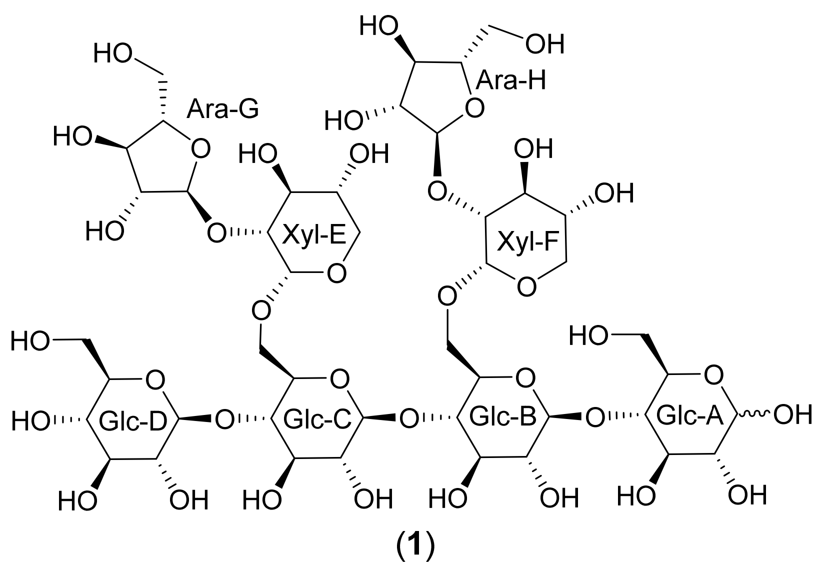


Figure 4. Structure of the urinary octasaccharide (**1**) showing monosaccharide identities and absolute configuration.

C. Results & Discussion

1. *Anti-agglutination Properties of Urine Fractions*

a. <u>Overview of Anti-agglutination Assay Results</u>

Cumulative results across test dates for selected urine samples and fractions are shown below (Tables 7–15). The raw assay data provided by RU (dilution endpoints) were interpreted by UM using a qualitative scoring system (Table 3) as discussed above (Section B4d). The raw data for these samples, organized by test date, are included in Appendix B-3 with some additional discussion points regarding assay limitations in Appendix B-2. Raw data include the same sample descriptions as shown below, the dilution endpoint values (mg/mL) provided by RU, the number of two-fold dilutions applied to each sample before loss of detectable anti-agglutination activity, the amount of sample submitted by UM, the amount of PBS used to dilute each sample (dilution volume), and the starting concentration at which anti-agglutination properties were first assessed. The VAS values were reported by RU to UM for only some of the samples submitted and these scores are included in the raw data where available (Appendix B-3: Tables 46, 48, 50, 52).

Although numerical dilution endpoint values were provided as the result for each sample, these values were, at best, qualitative assessments of sample activity due to the qualitative nature of the visual agglutination scoring system used. False negative results were also likely for many samples, as the reported dilution endpoints may have been affected by numerous sample, environmental, and methodological variables (See Appendix B-2 discussion). In spite of the limitations of the UPEC-HRBC anti-agglutination assay, the results were determined to provide sufficient information to guide preliminary isolation efforts by

identifying which samples were or were not active. The results of these studies indicated, however, that this assay is not suitable for future quantitative assessments of the ability of test samples to prevent UPEC adhesion, especially for small quantities (< 10 mg) of material with unknown activity. Sample limitations therefore dictated that priority be given to the isolation and structure elucidation of putative active components in preference to further testing of urine fractions using this assay.

b. *Results for Urine Fractions*

The D urine fractions and DA Sephadex LH-20 fractions were tested across five different test dates and all samples were not tested for each test date (Table 7). Retesting of fractions DA1–DA8 yielded results with questionable reproducibility as some of samples that showed activity in the 05/2006 test set were inactive in the 03/2007 test set and vice versa. Retesting of fractions DA13–DA20 also yielded some results with questionable reproducibility, but some of these samples maintained activity. Samples within this set that were considered active included DA13, DA18, and DA19. A third retesting of fraction DA19 in the 07/2011 test set showed that this sample retained activity as a dry powder stored at –20 ºC for at least five years. Insufficient amount of all DA materials were available for further isolation or structural characterization.

The F urine fractions were tested across six different test dates (Table 8). The FF sample (the aqueous fraction of the F urine crude material) was tested repeatedly as a positive control for other urine samples. Although it was considered active, this sample did not always result in numerical dilution endpoints that fell within the defined ranges for activity, and dilution endpoints for replicate samples within a single test date were inconsistent. The variations in UM Scores reflect this variation in dilution endpoints. The observed variation in

sample activity may have been partly due to the complexity and heterogeneity of the sample and the hygroscopic nature of the material, as well as to other reasons (see Appendix B-2 discussion). The FF sample was later submitted to preliminary versions of the UPEC-UEC assay used for cranberry studies (see Chapter 3) and it also showed anti-adhesion activity in this assay (data not shown). The FF sample retained activity for at least four to six years.

The FF fraction was separated using five different Sephadex LH-20 columns (Table 4), and all fractions from these separations were submitted to the anti-agglutination assay (Table 9, 10). Fractions that showed consistent activity were those that eluted early from each column, including F1-1, F1-2, F2-1, FF3-1, FF3-2, FF4-1, FF4-2, and FF5-1. These active FF fractions were combined to give the FA fraction which was also found to be active (

Table 11). The FA fraction was further separated by Sephadex LH-20 (Table 5) and the resulting fractions were tested on two different test dates (

Table 11). As before, early-eluting Sephadex LH-20 fractions (FA1 and FA2) showed highest activity (09/2007 test date). Retesting of the FA fractions in the 06/2008 sample set yielded negative activity for the FA1 and FA2 fraction. This apparent lack of activity was most likely due to the submission of less than 10 mg of each sample (Appendix B-3: Tables 51, 54).

Control urine fractions were primarily inactive, as expected (Table 12), and the FC1 fractions were tested repeatedly as negative controls for other urine samples. Similar separation methods were applied to the FC material as were used for active urine materials (Section B5a); the fraction FC1 was analogous to fractions FF, HF and IF, and FC1-1 was analogous to FA1 and HF1-1. Fraction FCE showed possible activity and this activity may indicate the presence of innate or other dietary-derived urine components that can interfere with HRBC agglutination by *E. coli*. Limited FCE material was available for further analyses.

The H urine material and H fractions were tested across three different test dates (Table 13). The HF fraction showed activity and fractionation of HF by Sephadex LH-20 (Table 6) yielded early-eluting fractions with highest activity. This pattern of activity was similar to that observed with the F urine material, with active components being retained in the aqueous fraction after organic solvent extraction and eluting early from a Sephadex LH-20 column. The HCE fraction also showed possible activity but a limited amount of this material was available and attempts at isolating the constituents present were unsuccessful. Combination of selected HF and IF fractions (see below) produced the three HI fractions shown, and the combined fraction derived from early-eluting Sephadex LH-20 fractions (HI1) had the best activity.

The I urine material was tested across two different test dates and I fractions showed a pattern of activity similar to that observed with H and F samples (Table 14). The IF fraction was separated using Sephadex LH-20 (Table 6) but the resulting fractions were not tested. Combinations of selected HF and IF Sephadex LH-20 fractions were, however, tested as HI samples, confirming the activity of early eluting IF fractions (Table 13). The ICE fraction also showed activity but a limited amount of this material was available. The I urine material was subjected to a stability test followed by solvent-solvent extraction to yield the IZ and IZA materials. Both of these IZ samples showed activity, indicating that the active constituents were stable in 20% MeOH solution with other crude urine components for up to three months.

c. <u>*Results for Standards*</u>

Carbohydrate standards were submitted with the last two sets of test samples (Table 15) with the objective of assessing assay specificity for different monosaccharides. Of the submitted carbohydrate standards, D-glucose showed reproducible activity, suggesting that the presence of this compound in urine fractions may be a cause for false-positive results. While

glucose may be present in crude urine materials, it would have been removed from later, enriched fractions (such as FA1 and HF1-1) by the chromatographic methods used (See Appendix B-5: Figure 105). It also could not be detected in cranberry fractions that were later collected and shown to be active in a different anti-adhesion assay (See Chapter 3, Sections B5b, C2d, and C4b). A possible reason for the positive result obtained with glucose relates to the fact that this compound can serve as a growth substrate for *E. coli*. The presence of this compound in test media may cause *E. coli* to alter gene expression profiles associated with adhesion (A. B. Howell personal communication). Glucose may therefore inhibit HRBC agglutination by *E. coli*, but this inhibition may be due to an indirect mechanism of action that is not clinically relevant. Additional studies would be necessary to fully investigate this phenomenon.

Table 7. Anti-agglutination assay results for D urine fractions and DA Sephadex LH-20 fractions tested in three different test date sets.
Samples within this set that were considered active included DA13, DA18, and DA19. Fraction DA19 retained activity as a dry powder stored at –20 °C for at least five years.

Sample Description	UM Score[a]	Test Date[b]	UM Score	Test Date	UM Score	Test Date
D (crude urine)	– –	03/2006				
DE (D EtOAc fraction	– –	03/2006				
DS (D *sec*-BuOH fraction)	– –	03/2006				
DA (D aqueous fraction)	+/–	03/2006				
DA1 (Sep LH-20 fraction)	–	05/2006	+ +	03/2007		
DA2 (Sep LH-20 fraction)	–	05/2006	+	03/2007		
DA3 (Sep LH-20 fraction)	+/–	05/2006	+	03/2007		
DA4 (Sep LH-20 fraction)	+/–	05/2006	– –	03/2007		
DA5 (Sep LH-20 fraction)	– –	05/2006	– –	03/2007		
DA6 (Sep LH-20 fraction)	– –	05/2006	– –	03/2007		
DA7 (Sep LH-20 fraction)	– –	05/2006	– –	03/2007		
DA8 (Sep LH-20 fraction)	– –	05/2006	+ +	03/2007		
DA9 (Sep LH-20 fraction)	– –	05/2006				
DA10 (Sep LH-20 fraction)	– –	05/2006				
DA11 (Sep LH-20 fraction)	– –	05/2006				
DA12 (Sep LH-20 fraction)	– –	05/2006				
DA13 (Sep LH-20 fraction)	+ +	05/2006	+ +	08/2006		
DA14 (Sep LH-20 fraction)	+ +	05/2006	–	08/2006		
DA15 (Sep LH-20 fraction)	+ +	05/2006	–	08/2006		
DA16 (Sep LH-20 fraction)	+ +	05/2006	+/–	08/2006		
DA17 (Sep LH-20 fraction)	– –	05/2006	+ +	08/2006		
DA18 (Sep LH-20 fraction)	+ +	05/2006	+ +	08/2006		
DA19 (Sep LH-20 fraction)	+ +	05/2006	+ +	08/2006	+ +	07/2011
DA20 (Sep LH-20 fraction)	– –	05/2006	– –	08/2006		

[a] The evaluation criteria for assigning the qualitative UM Scores are described in Table 3.
[b] Samples with the same test date were submitted as part of the same shipment to RU but may not have been tested on the same day or in the same batch. See Appendix B-2 for additional discussion.

Table 8. Anti-agglutination assay results for F urine fractions.
The FF sample (the aqueous fraction of the F urine sample) was tested repeatedly as a positive control for other urine samples. The variations in UM Scores reflect the variation in dilution endpoints both within and across test dates. These data indicate that the FF sample retained activity for at least four years. Later testing in preliminary versions of the UPEC-UEC indicated that anti-adhesion activity was still retained in the FF sample for at least six years.

Sample Description	UM Score	Test Date
FB (F EtOAc fraction)	–	09/2007
FG (F solids)	– –	03/2007 x3[a]
FF (aqueous fraction)	+/–	03/2007
FF (aqueous fraction)	+/–	03/2007
FF (aqueous fraction)	+ +	05/2007
FF (aqueous fraction)	+	05/2007
FF (aqueous fraction)	–	05/2007
FF (aqueous fraction)	+/–	06/2007
FF (aqueous fraction)	+/–	06/2007
FF (aqueous fraction)	–	06/2007
FF (aqueous fraction)	–	09/2007
FF (aqueous fraction)	–	09/2007
FF (aqueous fraction)	+	07/2011
FF (aqueous fraction)	+	07/2011

[a] This sample was submitted and tested in triplicate for this test date.

Table 9. Anti-agglutination assay results for FF Sephadex LH-20 fractions, columns 1 and 2. The FF Sephadex LH-20 fractions that showed consistent activity were those that eluted early from the columns: F1-1, F1-2, and F2-1. F2-2 – F2-4 may also have been active. Fraction FF1-7 was contaminated with a small amount of FF1-1 during sample transfers.

Sample Description	UM Score	Test Date
FF1-1 (Sep LH-20 fraction)	+ +	03/2007
FF1-2 (Sep LH-20 fraction)	+ +	03/2007
FF1-3 (Sep LH-20 fraction)	– –	03/2007
FF1-4 (Sep LH-20 fraction)	– –	03/2007
FF1-5 (Sep LH-20 fraction)	–	03/2007
FF1-6 (Sep LH-20 fraction)	+/–	03/2007
FF1-7 (Sep LH-20 fraction)	+ +	03/2007
FF1-8 (Sep LH-20 fraction)	– –	03/2007
FF1-9 (Sep LH-20 fraction)	– –	03/2007
FF2-1 (Sep LH-20 fraction)	+ +	05/2007
FF2-2 (Sep LH-20 fraction)	+	05/2007
FF2-3 (Sep LH-20 fraction)	+	05/2007
FF2-4 (Sep LH-20 fraction)	+	05/2007
FF2-5 (Sep LH-20 fraction)	– –	05/2007
FF2-6 (Sep LH-20 fraction)	+/–	05/2007
FF2-7 (Sep LH-20 fraction)	+/–	05/2007
FF2-8 (Sep LH-20 fraction)	– –	05/2007

Table 10. Anti-agglutination assay results for FF Sephadex LH-20 fractions, columns 3, 4, and 5.
The FF Sephadex LH-20 fractions that showed consistent activity were those that eluted early from the columns: FF3-1, FF3-2, FF4-1, FF4-2, and FF5-1.

Sample Description	UM Score	Test Date
FF3-1 (Sep LH-20 fraction)	+ +	05/2007
FF3-2 (Sep LH-20 fraction)	+ +	05/2007
FF3-3 (Sep LH-20 fraction)	– –	05/2007
FF3-4 (Sep LH-20 fraction)	– –	05/2007
FF3-5 (Sep LH-20 fraction)	– –	05/2007
FF3-6 (Sep LH-20 fraction)	+/–	05/2007
FF4-1 (Sep LH-20 fraction)	+	06/2007
FF4-2 (Sep LH-20 fraction)	+	06/2007
FF4-3 (Sep LH-20 fraction)	–	06/2007
FF4-4 (Sep LH-20 fraction)	– –	06/2007
FF4-5 (Sep LH-20 fraction)	– –	06/2007
FF4-6 (Sep LH-20 fraction)	– –	06/2007
FF4-7 (Sep LH-20 fraction)	– –	06/2007
FF4-8 (Sep LH-20 fraction)	– –	06/2007
FF5-1 (Sep LH-20 fraction)	+ +	06/2007
FF5-2 (Sep LH-20 fraction)	– –	06/2007
FF5-3 (Sep LH-20 fraction)	– –	06/2007
FF5-4 (Sep LH-20 fraction)	– –	06/2007
FF5-5 (Sep LH-20 fraction)	– –	06/2007
FF5-6 (Sep LH-20 fraction)	– –	06/2007
FF5-7 (Sep LH-20 fraction)	– –	06/2007
FF5-8 (Sep LH-20 fraction)	–	06/2007

Table 11. Anti-agglutination assay results for FA urine fractions.
In the 09/2007 sample set, the FA sample showed probable activity and the early-eluting Sephadex LH-20 fractions (FA1 and FA2) showed highest activity, as before. When all FA samples were retested in the 06/2008 sample set, however, FA1 and FA2 showed no activity. This result is probably a false negative due to the submission of less than 10 mg of each sample (see Appendix B-3: Tables 50, 53).

Sample Description	UM Score	Test Date	UM Score	Test Date
FA (combined active FF samples)	+/–	09/2007	+/–	06/2008
FA1 (Sep LH-20 fraction)	+ +	09/2007	– –	06/2008
FA2 (Sep LH-20 fraction)	+	09/2007	– –	06/2008
FA3 (Sep LH-20 fraction)	– –	09/2007	– –	06/2008
FA4 (Sep LH-20 fraction)	+/–	09/2007	+/–	06/2008
FA5 (Sep LH-20 fraction)	– –	09/2007	+/–	06/2008

Table 12. Anti-agglutination assay results for FC urine fractions.
The FC1 fractions were tested repeatedly and were inactive each time, as expected. Fraction FCE showed possible activity, but limited material was available for further analyses. The presence of possible activity in the FCE fraction indicated that other innate or dietary-derived urinary components may be present in this fraction that can inhibit agglutination.

Sample Description	UM Score	Test Date
FC (control urine)	– –	09/2007
FCE (FC EtOAc fraction)	+/–	09/2007
FC1 (aqueous fraction)	–	07/2011 x2, 09/2007 x3, 12/2008
FC1-1 (Sep LH-20 fraction)	– –	06/2008, 12/2008, 07/2011 x2
FC1-2 (Sep LH-20 fraction)	– –	06/2008, 07/2011
FC1-3 (Sep LH-20 fraction)	– –	06/2008

Table 13. Anti-agglutination assay results for H urine fractions.
The H crude urine material and the HF aqueous fraction both showed activity. The HCE fraction also showed possible activity. Fractionation of HF by Sephadex LH-20 yielded early-eluting fractions with activity: HF1-1 and HF1-2. Combination of HF and IF Sephadex LH-20 fractions produced the HI fractions shown, and the fraction derived from early-eluting H and I Sephadex LH-20 fractions (HI1) had the best activity of the HI fractions.

Sample Description	UM Score	Test Date
H (crude urine)	+/–	08/2008, 12/2008, 07/2011
HG (H solids)	– –	08/2008
HCE (H EtOAc extract, water soluble)	+	08/2008
HCM (H EtOAc fraction, MeOH soluble)	– –	08/2008
HD (H EtOAc:MeOH fraction, water soluble)	– –	08/2008, 12/2008
HF (H aqueous fraction)	+	08/2008, 12/2008
HF1-1 (Sep LH-20 fraction)	+ +	12/2008
HF1-2 (Sep LH-20 fraction)	+/–	12/2008
HF1-3 (Sep LH-20 fraction)	–	12/2008
HF1-4 (Sep LH-20 fraction)	–	12/2008
HF1-5 (Sep LH-20 fraction)	– –	12/2008
HF1-6 (Sep LH-20 fraction)	–	12/2008
HF1-7 (Sep LH-20 fraction)	– –	12/2008
HI1 (combined H & I active fractions, HF2-1 + IF1-1)	+ +	07/2011
HI2 (combined H & I active fractions, HF1-1 + HF2-2 + IF1-2)	+	07/2011
HI3 (combined H & I active fractions, HF1-2 + IF1-3 + HF2-3)	– –	07/2011

Table 14. Anti-agglutination assay results for I urine fractions.
The I urine material and the IF fraction both showed activity. The ICE fraction also showed activity but a limited amount of this material was available. The Sephadex LH-20 fractions of IF were not tested. The IZ and IZA materials derived from the stability test both showed activity, indicating that active components of the urine are not degraded under the conditions used.

Sample Description	UM Score	Test Date
I (crude urine)	+/–	12/2008
ICE (I EtOAc:MeOH fraction, water soluble)	+ +	12/2008
ICM (I EtOAc:MeOH fraction, EtOAc soluble)	– –	12/2008
ID (EtOAc:MeOH fraction, water soluble)	– –	12/2008
IF (I aqueous fraction)	+/–	12/2008
IZ (I stability test)	+	12/2008
IZA (IZ aqueous fraction after stability test)	+ +	07/2011

Table 15. Anti-agglutination assay results for submitted reference compounds.
Of the submitted carbohydrate standards, D-glucose showed reproducible activity, suggesting that the presence of this compound in test samples may cause false-positive results. Queries directed at RU regarding the activity of glucose in the assay led to the proposal that glucose may inhibit agglutination of HRBCs by *E. coli* via an indirect mechanism of action that is not clinically relevant for UTI prevention.

Sample Description	UM Score	Test Date
D-Glucose (Sigma Aldrich 99.5%)	+/–	12/2008
D-Glucose (Sigma Aldrich 99.5%)	+ +	07/2011 x2
Glucose Tabs (ReliOn Glucose Tablets commercial product, powdered)	+ +	07/2011 x2
D-Glucuronic Acid	– –	12/2008
D-Galacturonic Acid	– –	12/2008
D-Sorbitol	– –	12/2008
D-Fructose (CalBioChem)	–	07/2011 x2
Sucrose (Great Value Brand)	– –	12/2008, 07/2011

2. *Initial Results with Early (D, E, & F) Source Materials*

The first shipment of active urine material (D) provided information relevant to method development but did not provide sufficient material from which to purify the active components. This material was handled under the premise that the target metabolites were PACs or PAC-derived. Methods that would normally be used for PAC isolation, such as those previously applied to cranberry materials (Foo *et al*. 2000a), were therefore attempted, including EtOAc and *sec*-BuOH extraction and Sephadex LH-20 chromatography with 100% EtOH. The use of SPE cartridges (Oasis® HLB) and hydrophobic extraction resins (Diaion, XAD-8 and XAD-16) was also attempted based on the premise that PACs would be retained by such sorbents.

The fractions from solvent-solvent extraction using EtOAc and *sec*-BuOH were sent to the anti-agglutination assay, and the aqueous fraction (DA) yielded the best activity (Table 7). The DA material was therefore the focus of additional work. Of the methods attempted, the Sephadex LH-20 column yielded the most useful information for further method development. This column was packed in 100% EtOH but the sample would only partially dissolve in 75% EtOH and fell out of solution upon loading. The eluting solvent subsequently used was a gradient, with gradual 10% decreases in EtOH concentration in 1–2 L increments so as to redissolve the sample without overexpanding the Sephadex and breaking the glass column. This gradient method resulted in elution of anti-adhesive constituents at ~70–75% EtOH, as fractions DA13-DA19. Low amounts of material were recovered and half of the material for most fractions collected was sent for initial testing in the anti-agglutination assay (Table 7, Appendix B-3: Tables 42–44). Later retesting of the DA column fractions confirmed activity for fractions DA13, DA18 and DA19. Unexpectedly, D fractions that showed activity gave

negative results for an anthocyanidin test, indicating that PACs were not present in these fractions (Ofek *et al*. 1996). The active D fractions from this separation were analyzed by ^{1}H NMR spectroscopy and LC-MicroTOF-MS, but data quality was poor and the spectra obtained could not be reliably interpreted. The low-quality ^{1}H NMR spectra for fractions DA18 and DA19 (Appendix B-5: Figures 106, 107) were later determined to have similar features as spectra obtained for other active urine fractions (FA1 and HF1-1).

Early studies also indicated that active fractions of unknown composition were soluble in water, dimethyl sulfoxide, and water-alcohol mixtures (EtOH or MeOH) of up to 80% alcohol with diluted material. Active metabolites were found to be stable after exposure to moderate heat (up to 60 °C), light, alcohols (EtOH, MeOH, *sec*-BuOH), EtOAc, DMSO, ether, and acid at room temperature (titrated to pH 2.5 using 1 M HCl). Activity also remained after freezing in powder or liquid form for long periods of time (up to 5 years or more) in both complex mixtures and in more purified active fractions.

The second shipment of active urine material (E) was used for further attempts at method development using numerous approaches. Many fractions of this material were sent for assay testing and the results indicated that the material supplied to UM may have lost activity. Issues with this apparent loss of activity prompted consideration of mechanisms by which the unknown (at that time) metabolites could be degraded or inactivated. The E urine samples had been lyophilized as crude urine by the UWM group prior shipment to UM and issues during this stage of processing could have contributed to the degradation of active components. Raw urine material is difficult to lyophilize due to relatively high solute concentrations, the presence of fats and surfactants, and the logistics of processing large volumes of liquid via lyophilization. Of the possible scenarios, crude urine material could have been subjected to

extended periods of time in untreated aqueous solution and to multiple freeze-thaw cycles. During such times, active metabolites could have been degraded by native enzymes present in crude active urine or could have chemically reacted with other crude urine components (Saude *et al*. 2007). Enzymatic degradation can be prevented by mixing crude urine samples with MeOH or other alcohols to denature proteins, but alcohol addition would have interfered with lyophilization, freezing, and shipping. The alternative addition of common urine stabilizing agents, such as sodium azide, may have interfered with the unknown active metabolites and the assay results (Saude *et al*. 2007). Degradation can also be avoided, and lyophilization efficiency improved, by filtering and extracting urine samples with organic solvents prior to an initial lyophilization step. Later urine samples were therefore kept frozen for the entire time between collection and extraction, including during shipping, and up to 20% of MeOH was added to urine samples immediately upon thawing.

The stability test later performed using the crude I urine sample confirmed that the active constituents were stable at room temperature in a 20% MeOH solution in the presence of crude urine constituents. During the three month incubation time, a precipitate formed, the color of the material changed from amber yellow to dark chocolate brown, and the smell of the material changed. All of these observations indicated the presence of oxidation and other chemical changes that were not prevented by the presence of MeOH. When tested in the anti-agglutination assay, the IZ-A material showed higher activity than the analogous aqueous material (IF) prepared without the room temperature incubation. These data suggested that additional enrichment for the active constituents may have occurred, possibly through degradation of non-active urinary components or assay-confounding materials. As MeOH denatures proteins, it is likely that enzymatic mechanisms may have been responsible for the

loss of activity in the E urine material. Additional testing and analyses would be required to profile the chemical and biological changes that could affect the stability of active urinary components, but such studies were not feasible during the present project.

3. *Enrichment for Active Components*

Active urinary components were consistently present in aqueous fractions after extraction with organic solvents (using EtOAc, EtOAc-MeOH mixtures, *sec*-BuOH, or ether) or with the use of solid phase extraction (SPE) cartridges, or XAD resins. Low anti-agglutination activity was present in some EtOAc fractions (HCE, ICE; Tables 13, 14) but not others (FB; Table 8). Attempts were made to isolate components from HCE, but insufficient amounts of this and similar materials were available to pursue further purification. The control urine EtOAc fraction (FCE; Table 12) also displayed low anti-agglutination activity, indicating that constitutive urinary metabolites or dietary sources other than cranberry may be responsible for the activity observed in some EtOAc fractions. Efforts were therefore focused on the isolation of possible active components from aqueous fractions (FF, HF, IF).

The anti-agglutination assay data for the FF sample Sephadex LH-20 fractions (Tables 9, 10) provided certainty as to methods that allowed for satisfactory enrichment of the active 1–2% of total urine material. Active fractions consistently eluted within the first 1–3 fractions of each Sephadex LH-20 column regardless of variations in column dimensions or EtOH percentages (50–70%). The total amount of active material enriched from the F urine samples accounted for ~0.7% of the starting material by weight, giving an estimated starting urine concentration for active metabolites of less than 0.14 mg/mL. Some of this material was used for method development and fraction characterization without success. Remaining anti-

adhesive urine fractions from the five separate Sephadex LH-20 columns of the FF material were combined to give F Actives (FA; 1.75 g). This material was separated on Sephadex LH-20 (Table 5), to give two anti-adhesive fractions (FA1 and FA2). Half of each FA1 and FA2 sample was submitted to the anti-agglutination assay (

Table 11), and fraction FA1 was used primarily for analytical HPLC separations and comparative analyses due to its limited quantity. Method development with FA1 and FA2 used HPLC-UV with HILIC and XCMSC18 sorbents and various solvent systems (see analytical HPLC example in Appendix B-5: Figure 108). All fractions isolated from FA2 using UV detection, however, gave inconclusive or negative assay results, and LC-MicroTOF-MS of both FA1 and FA2 gave inconclusive results (not shown) regarding possible fraction composition.

4. *Characterization of Active Components*

Comparisons between the ^{1}H NMR data of active urine fractions FA1 and FA2 (Figure 5), commercial fruit pectin (Figure 6), and galacturonic acid (Figure 7), a monomeric component of pectin, indicated that the active fractions could contain complex carbohydrates. While the ^{1}H NMR spectra of active urine fractions and pectin appeared similar, the urine fractions had significantly different characteristics in aqueous solution than pectin. The composition of the FA1 sample could also not be sufficiently distinguished from that of other, inactive, FA fractions by NMR (Figure 5).

Analysis of FA1 via analytical HPLC-ELSD/UV indicated that the major components of this fraction lacked chromophores and therefore required the use of ELSD or other chromophore-independent detection methods to monitor and guide HPLC separations (see Appendix B-4: overview of ELSD principles). Purification and structure elucidation of the

compounds present in the FA1 fraction was impractical, however, due to material limitations. The observation that active fractions with the highest anti-agglutination activity lacked UV-detectable components was later supported by HPLC-ELSD/UV analysis of HF1-1 (Figure 8).

Further guidance regarding the possible composition of the FA and HF1-1 active mixtures was obtained from additional ^{1}H NMR data. Comparisons between the ^{1}H NMR spectra of FA1 and HF1-1 (Figure 9) indicated a similar composition. Comparisons of these spectra with the ^{1}H NMR spectra of synthetic trimeric and tetrameric PACs (Figure 10), however, indicated that the active urine fractions did not contain these compounds. This was established by the lack of characteristic overlapping aromatic resonances from the A- & B-rings of different PAC monomer units that would correspond to PACs or possible PAC-related metabolites. Although NMR spectra of crude active urine fractions (HF; Figure 11) did contain resonances in the aromatic region (Figure 12), these resonances were also present in the inactive enriched control urine fraction (FC1-1), and in inactive fraction HI3 (Figures 13, 14). Aromatic resonances were present only at trace levels in enriched fractions with higher anti-agglutination activity (FA, HF1-1, HI1; Figures 11–14).These aromatic region resonances were therefore likely due to the presence of simple aromatic acids commonly found in mammalian urine (Saude *et al*. 2007, Bouatra *et al*. 2013).

The dominant ^{1}H NMR resonances present in all active fractions were indicative of carbohydrates even though the possible presence of monosaccharides within these fractions had been eliminated through chromatography (see Appendix B-5: Figure 105) and comparisons to various carbohydrates and other standards (galacturonic acid, apple pectin, D-glucose, sucrose, fructose, corn starch, sodium citrate, and epicatechin) using multiple TLC spray reagents (*e.g.* ninhydrin, *p*-anisidine, bromocresol green) (not shown). The use of TLC spray reagents that

produced color reactions indicative of flavonoids (*e.g.* vanillin, p-anisaldehyde) further indicated that PACs and other flavonoids were not present in the active fractions (not shown). Inactive Sephadex LH-20 fractions also lacked the characteristic profile present in the HPLC-ELSD spectra of active urine fractions (Figures 15, 16). It was therefore concluded that the active components of interest in enriched samples were complex oligosaccharides.

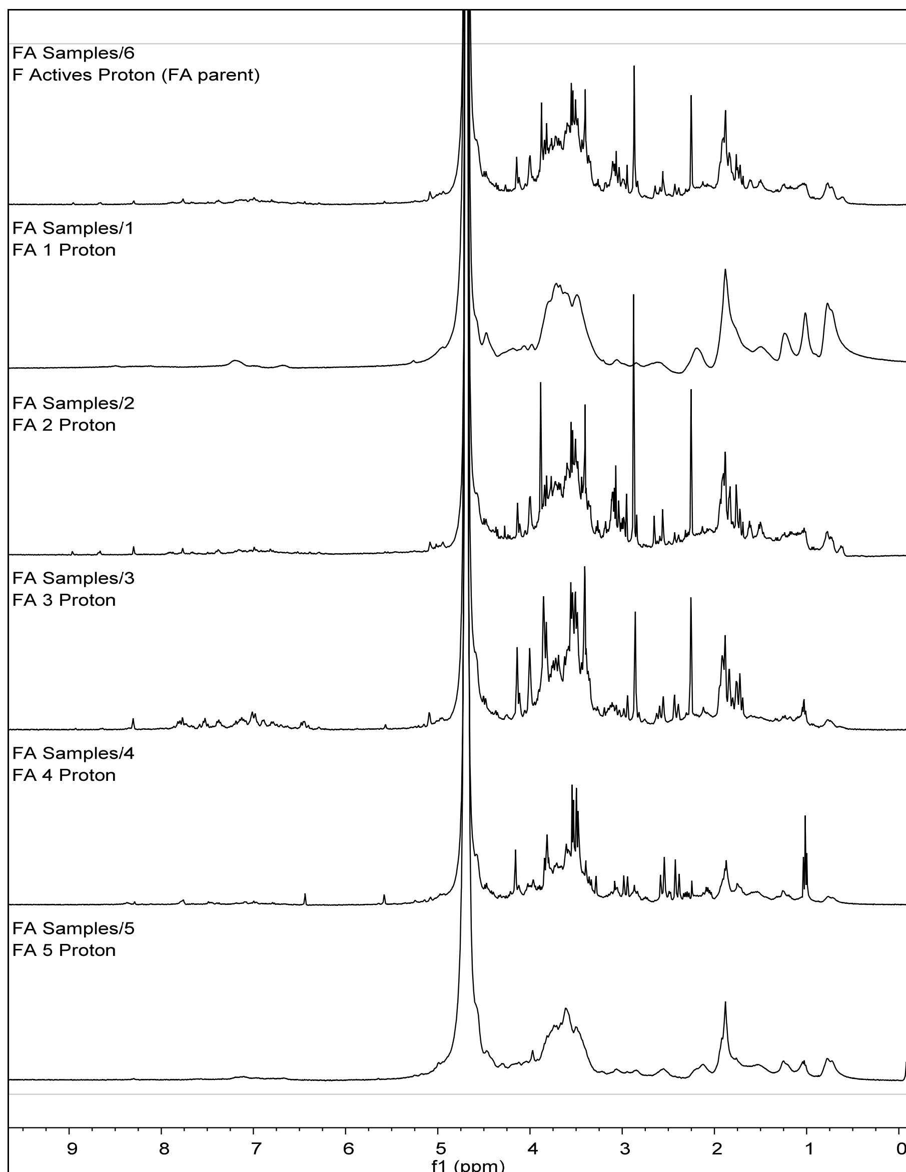


Figure 5. Comparison of the ^{1}H NMR spectra for FA and fractions FA1–FA5, 400 MHz, D_2O. Fraction FA1 and FA2 exhibited the best anti-agglutination activity but could not be significantly distinguished from the other FA fractions using NMR data alone. The FA1 fraction also resulted in a low resolution NMR spectra.

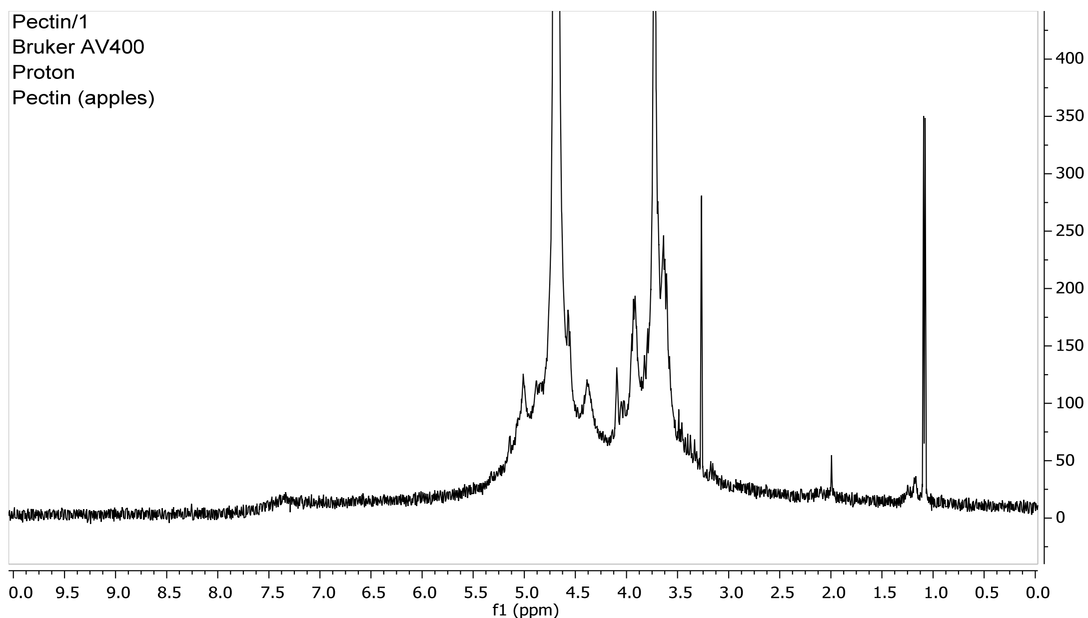


Figure 6. The ^{1}H NMR spectrum of pectin (from apples) reference sample, 400 MHz, D_2O. The overlapping resonances between 3–5.5 ppm are indicative of numerous monomeric carbohydrate substituents with similar chemical shift values.

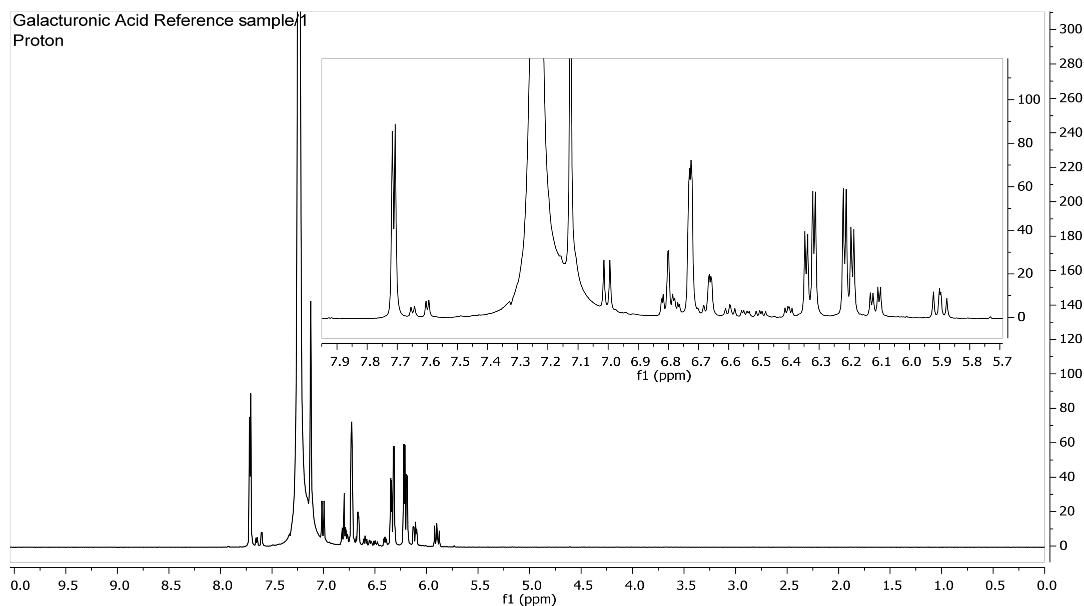
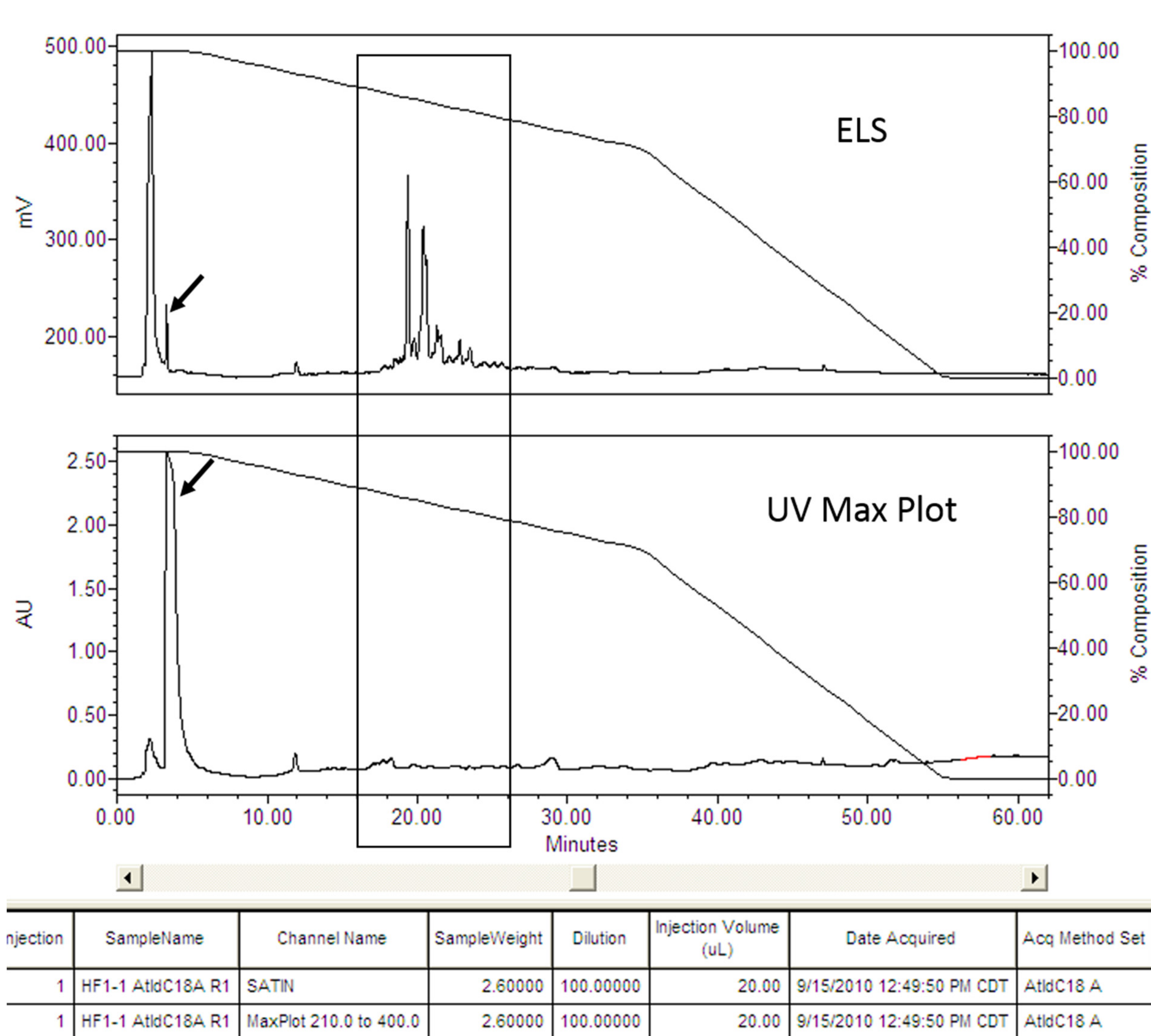


Figure 7. The ^{1}H NMR spectrum of galacturonic acid reference sample, 400 MHz, $CDCl_3$. Pure galacturonic acid, one of the monomer components of pectin yields an NMR spectrum with distinct resonances. This spectrum has little similarity to that of pectin or urine samples of interest.



njection	SampleName	Channel Name	SampleWeight	Dilution	Injection Volume (uL)	Date Acquired	Acq Method Set
1	HF1-1 AtldC18A R1	SATIN	2.60000	100.00000	20.00	9/15/2010 12:49:50 PM CDT	AtldC18 A
1	HF1-1 AtldC18A R1	MaxPlot 210.0 to 400.0	2.60000	100.00000	20.00	9/15/2010 12:49:50 PM CDT	AtldC18 A

Figure 8. HPLC of HF1-1 visualized by ELS and UV (max plot).
Comparison of the ELS and UV chromatograms for the active urine fraction HF1-1 indicates that a set of major fraction components (box) is not visible by UV detection methods. Similar UV-transparent components were present in the active FA1 fraction without the presence of additional UV-absorbing components (see Appendix B-5: Figure 108). The semi-quantitative nature of ELSD makes it possible to determine that the UV-visible component of HF1-1 is of relatively low concentration (arrows) (see Appendix B-4: ELSD overview).

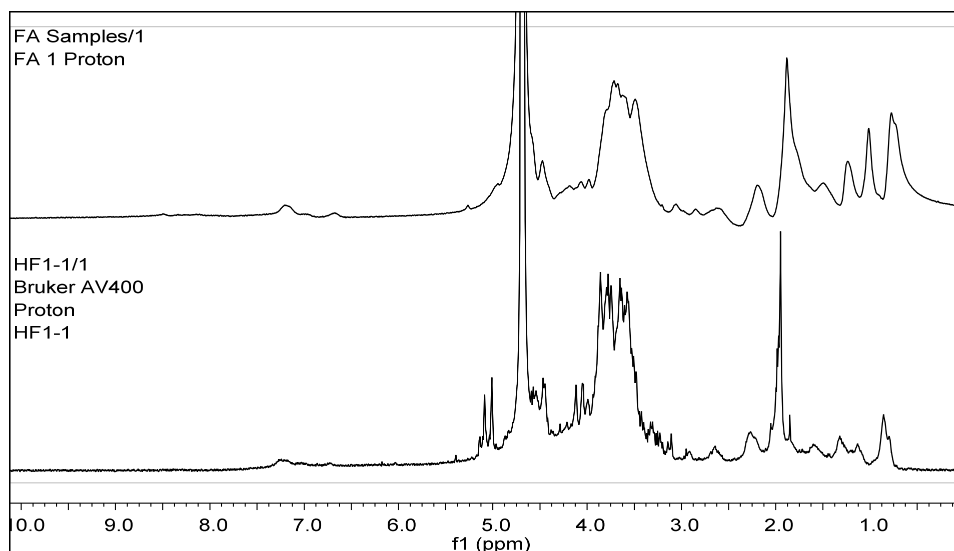


Figure 9. Comparison of the ^{1}H NMR spectra for active fractions FA1 and HF1-1, 400 MHz, D_2O.

The FA1 and HF1-1 materials both had activity in the anti-agglutination assay and produced ^{1}H NMR spectra with similar profiles even though the parent material was collected from different source animals. The HF1-1 spectrum showed improved resolution over that of FA1 but still provided insufficient information for structural assignments.

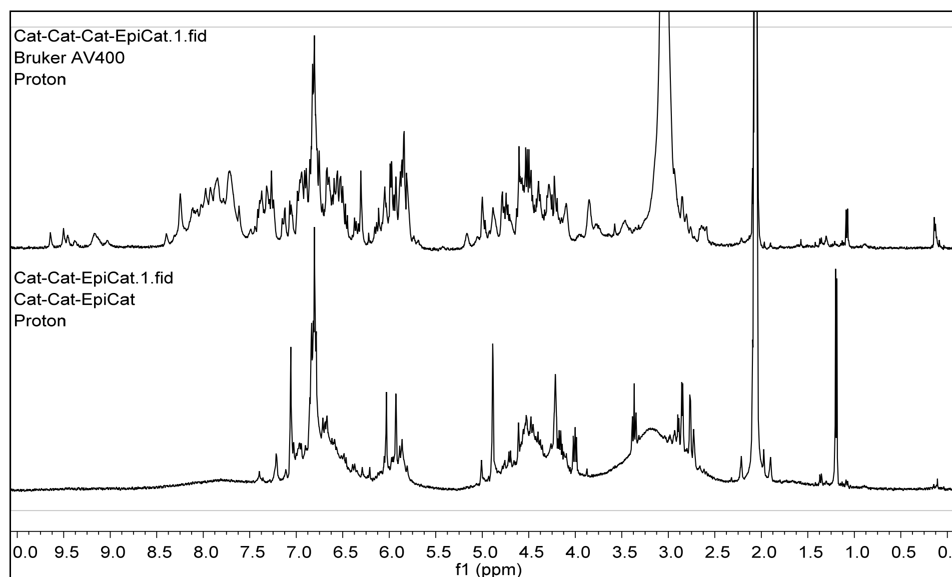


Figure 10. The ^{1}H NMR spectra of synthetic PAC oligomers, 400 MHz, Acetone-d_6.

Spectra for the proanthocyanidin trimer and pentamer both contained sets of complex multiplets in the 6.2 – 8.5 ppm region corresponding to the multiple aromatic protons present in these compounds.

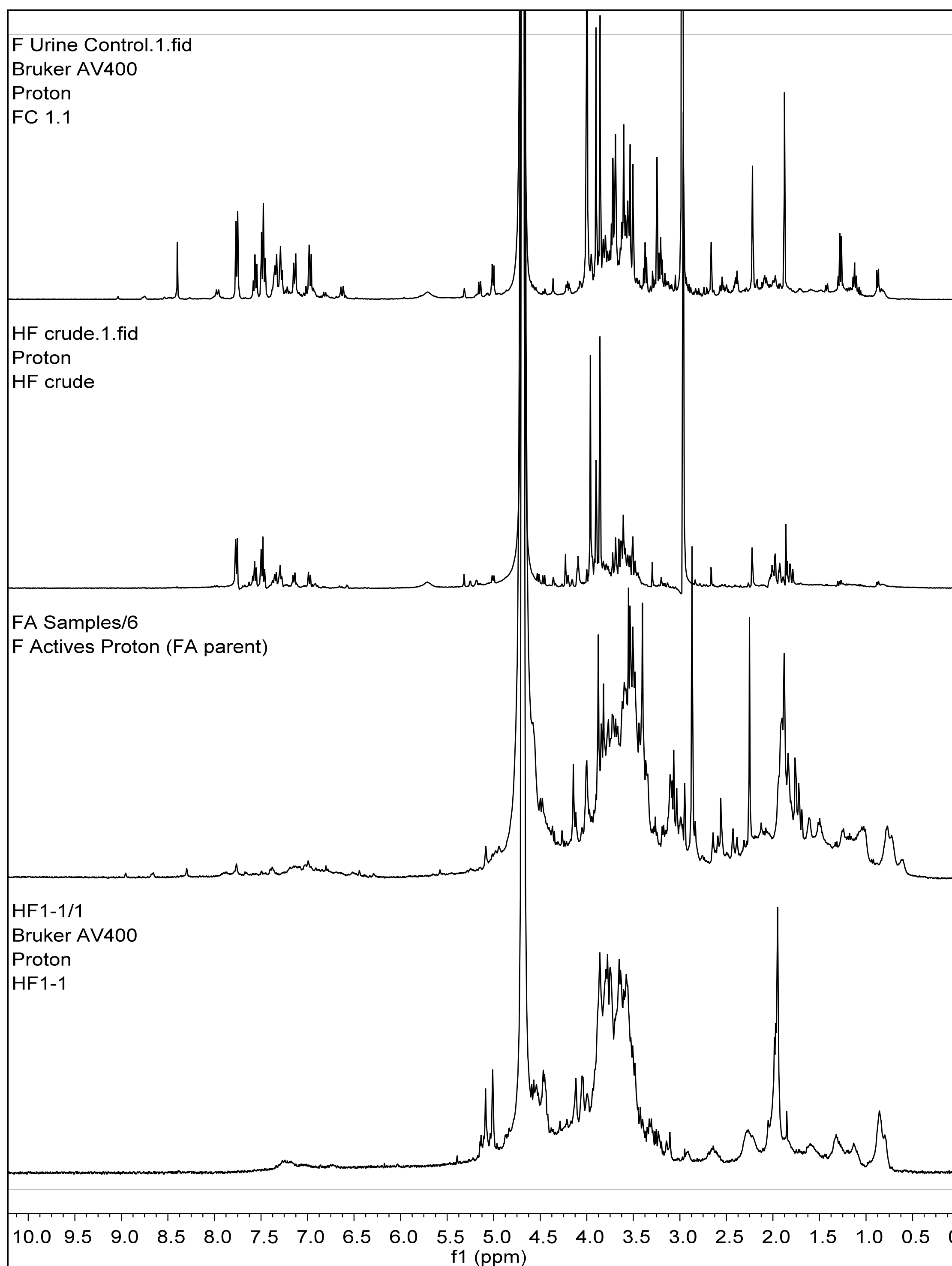


Figure 11. Comparison of the ^{1}H NMR spectra for FC1-1, HF, FA and HF1-1 samples, 400 MHz, D_2O.
Fraction ^{1}H NMR profiles changed as samples increased in anti-agglutination activity, from inactive (FC1-1), to probably active (HF), to active (FA), to highly active (HF1-1).

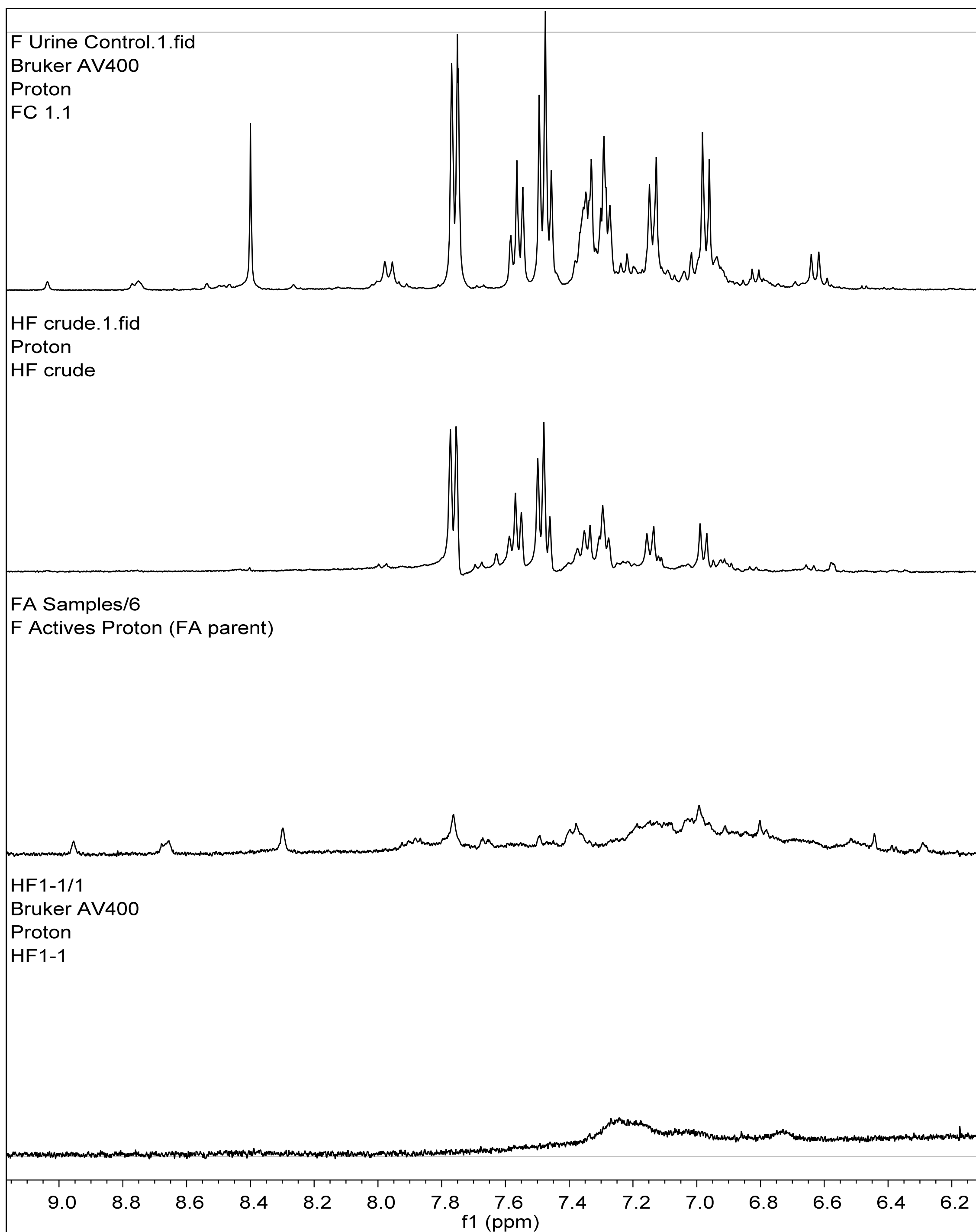


Figure 12. Comparison of the ^{1}H NMR spectra for FC1-1, HF, FA and HF1-1 samples, expansion of the aromatic region, 400 MHz, D_2O.
Samples with a higher degree of activity (FA1 and HF1-1), and, presumably, a corresponding higher degree of enrichment for active components, lacked aromatic resonances, while inactive samples and samples with lower or no activity (HF and FC1-1, respectively) contained these resonances.

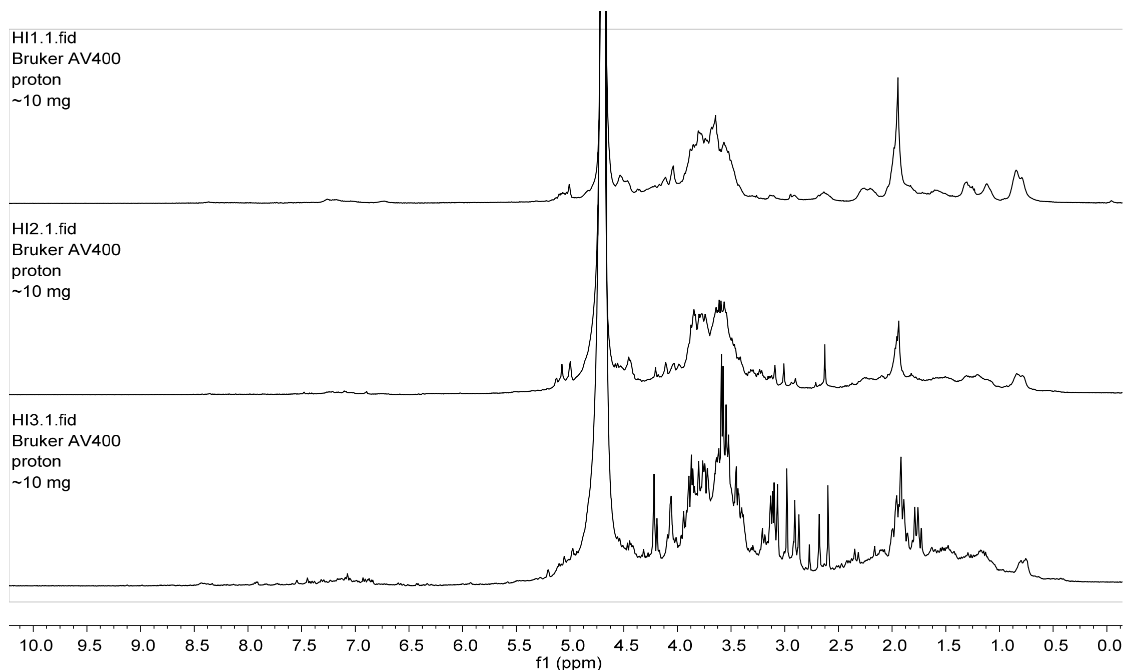


Figure 13. Comparison of the ^{1}H NMR spectra for HI1, HI2, and HI3, D_2O, 400 MHz. Fraction HI1 had the highest activity, fraction HI2 had moderate activity and fraction HI3 lacked activity.

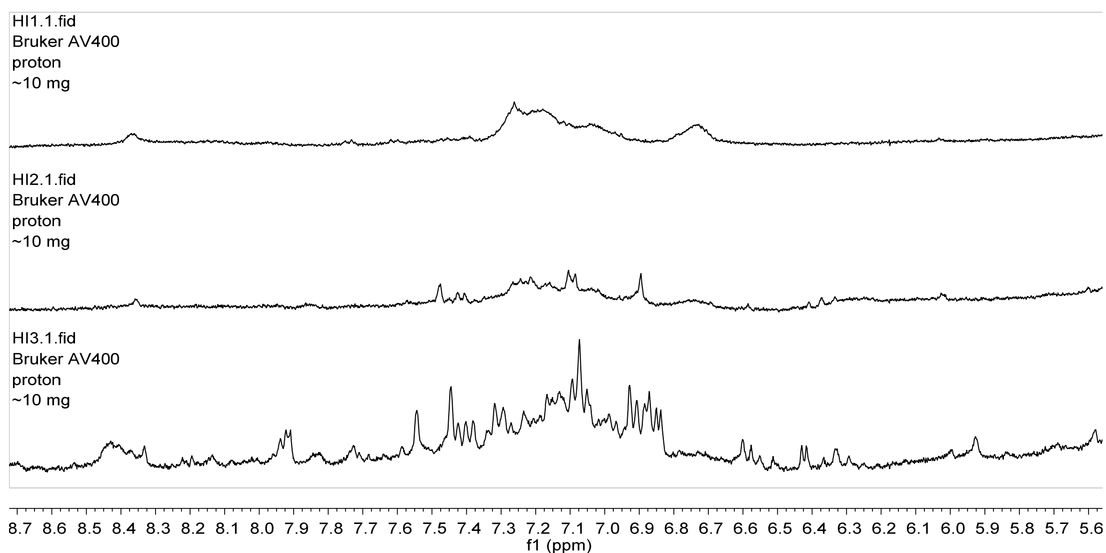
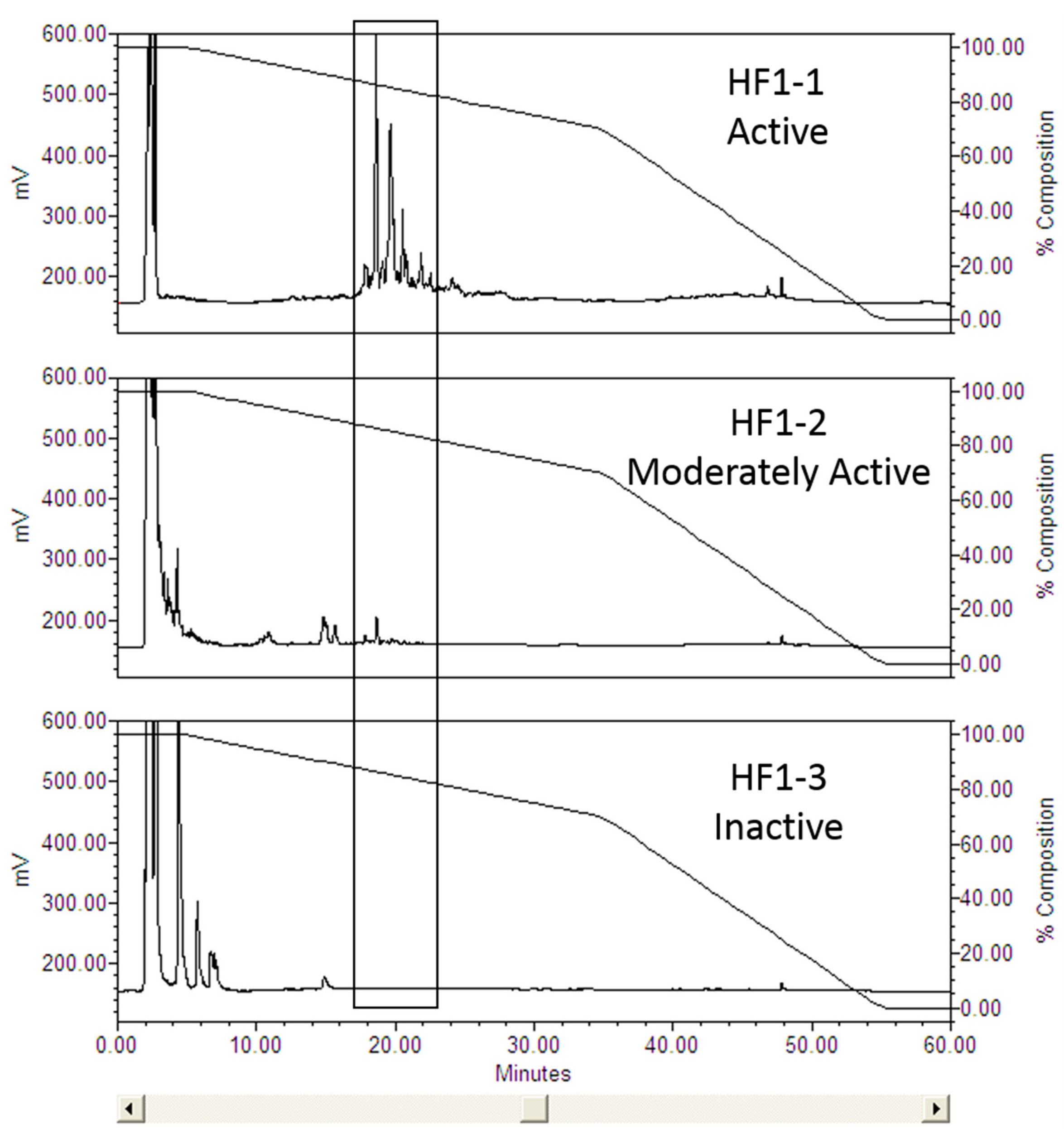
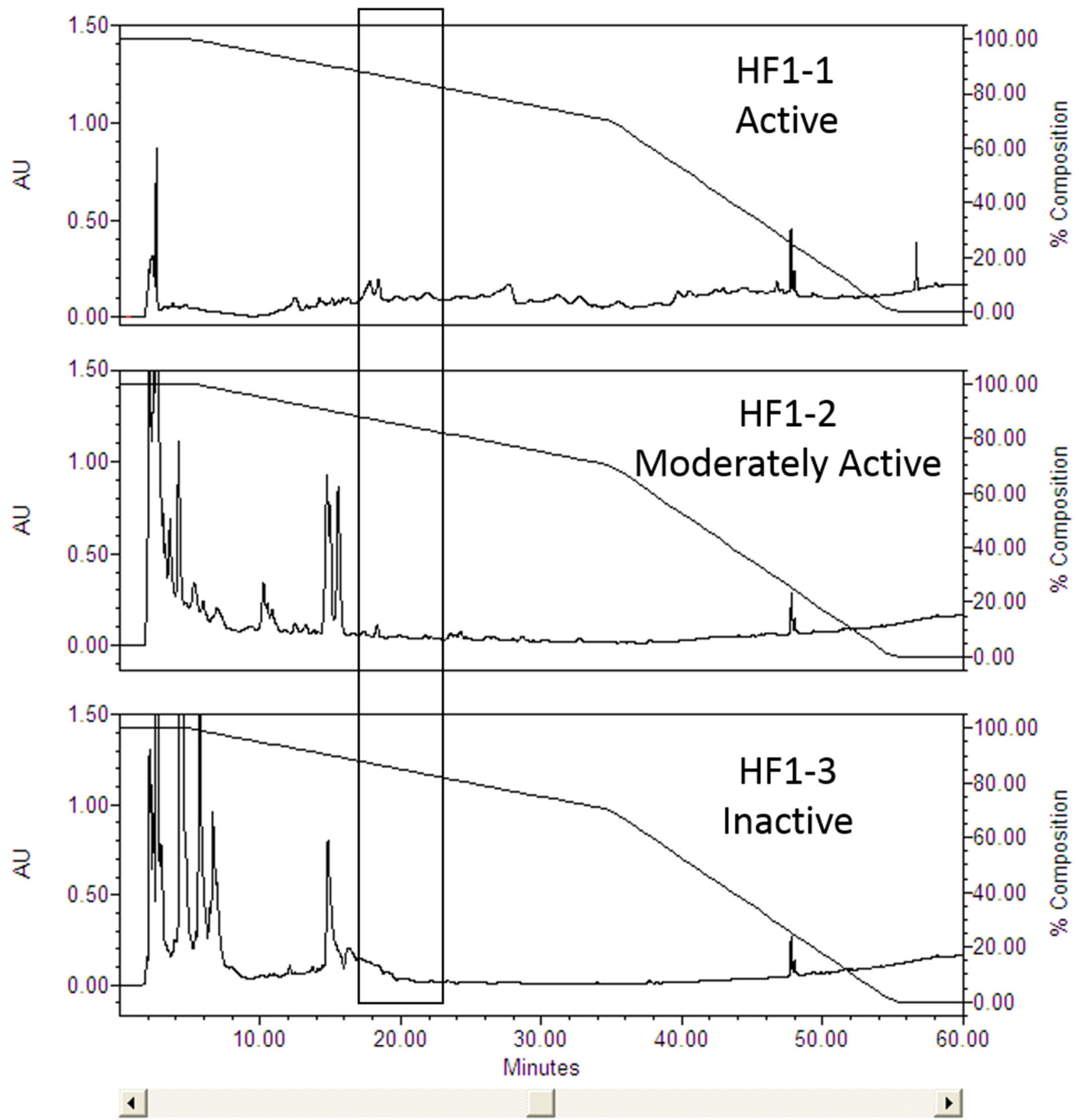


Figure 14. Comparison of the ^{1}H NMR spectra for HI1, HI2, and HI3, expansion of the aromatic region, D_2O, 400 MHz.
The fraction with highest activity, HI1, lacked distinct resonances in the aromatic region.



Visible	Vial	SampleName	SampleWeight	Dilution	Injection Volume (uL)	Date Acquired	Acq Method Set	
☑	18	HF1-1 AtldC18-A R1	2.00000	200.00000	50.00	7/10/2009 1:08:44 AM CDT	AtldC18 A	7/
☑	19	HF1-2 AtldC18-A R1	3.00000	300.00000	50.00	7/10/2009 2:45:02 AM CDT	AtldC18 A	7/
☑	20	HF1-3 AtldC18-A R1	2.60000	260.00000	50.00	7/10/2009 4:21:20 AM CDT	AtldC18 A	7/

Figure 15. Analytical HPLC-ELSD of Sephadex LH-20 fractions HF1-1, HF1-2, and HF1-3 (AtldC18).
The UV transparent components of interest (box) identified in the active fraction HF1-1 are detectable at low concentrations in the moderately active fraction HF1-2 and absent from the inactive fraction HF1-3. All three of these samples were injected at the same concentration.



Visible	Vial	SampleName	Date Acquired	SampleWeight	Dilution	Injection Volume (uL)	Channel Name
☑	18	HF1-1 AtldC18-A R1	7/10/2009 1:08:44 AM CDT	2.00000	200.00000	50.00	MaxPlot 210.0 to 40
☑	19	HF1-2 AtldC18-A R1	7/10/2009 2:45:02 AM CDT	3.00000	300.00000	50.00	MaxPlot 210.0 to 40
☑	20	HF1-3 AtldC18-A R1	7/10/2009 4:21:20 AM CDT	2.60000	260.00000	50.00	MaxPlot 210.0 to 40

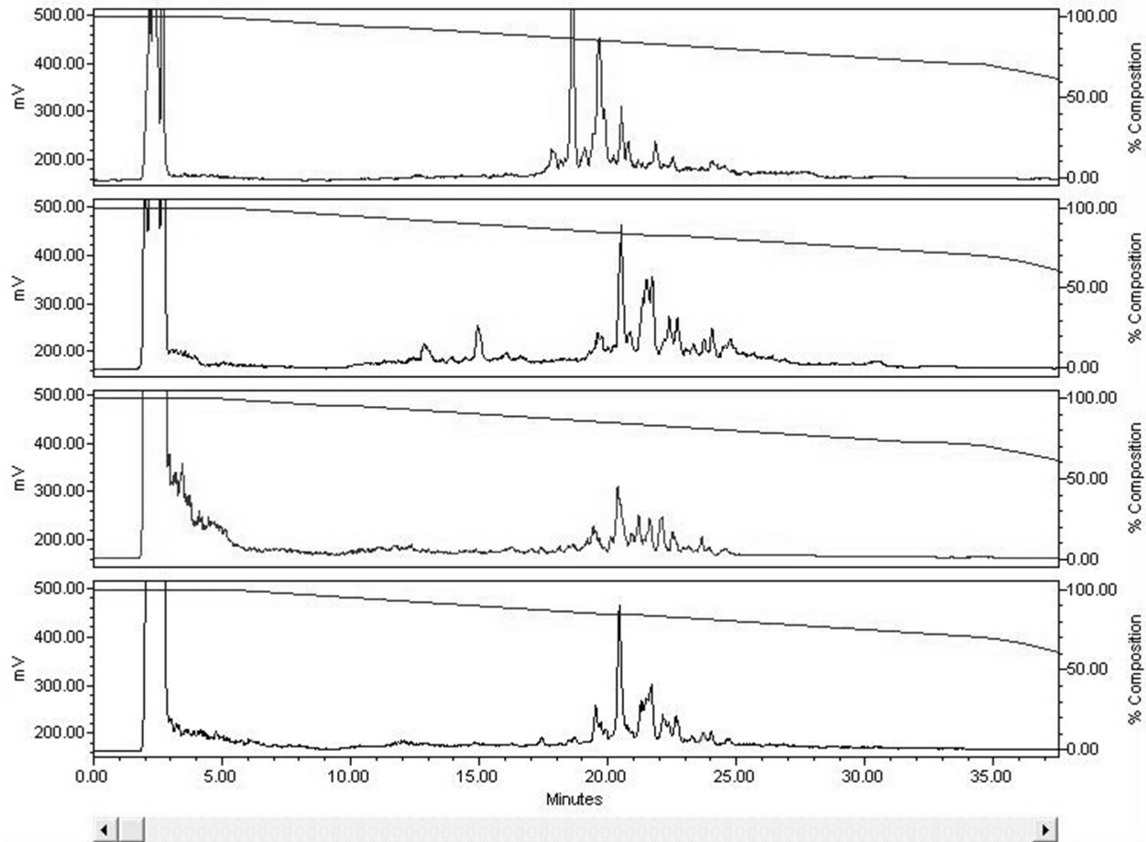
Figure 16. Analytical HPLC-UV (Max Plot) of Sephadex LH-20 fractions HF1-1, HF1-2, and HF1-3 (AtldC18).
The ELS-detectable components present at 18-22 min (box) in Figure 15 are not detectable by UV spectroscopy using wavelengths of 210-400 nm (Max Plot).

5. *Isolation of Urine Oligosaccharides from H & I Source Materials*

a. <u>*Sephadex LH-20 Fractionation*</u>

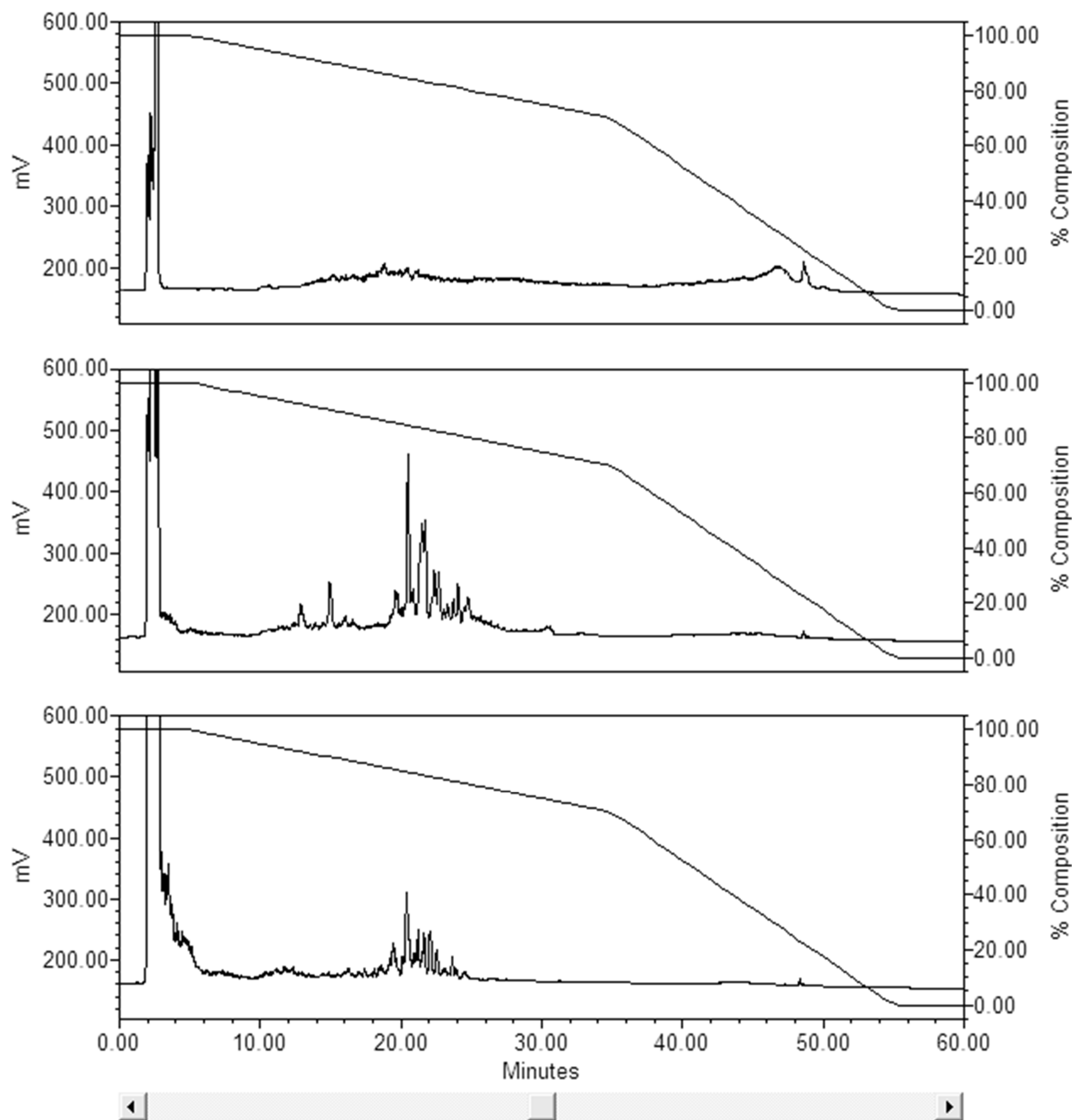
The H and I urine samples were separately extracted with EtOAc and fractionated following the Sephadex LH-20 protocols developed for F urine samples (Figure 3; Tables 4–6). As with the F urine fractions, the major active constituents remained in the aqueous layer after EtOAc extraction and eluted early from a Sephadex LH-20 column, with fractions HF1-1, HI1, and HI2 giving the best anti-agglutination assay results of the enriched samples tested (Tables 13, 14). All fractions were not tested to conserve material, and comparisons of the HPLC-ELSD profiles of active and inactive fractions were used to guide isolation efforts (Figures 17–21). Comparative analysis via HPLC-ELSD indicated that a series of UV-transparent compounds with a distinct profile, eluting between 17–25 min (Figure 17) with optimized HPLC methods, was consistently present in all active samples and absent from inactive samples. The HF2-2 fraction series (Figures 19–21) is representative of the HPLC-ELSD fraction profiles observed for the HF and IF Sephadex LH-20 column fractions.

Method development for HPLC separations was pursued under the new paradigm that the target compounds were carbohydrates. Purification for all components of the UV-transparent mixture was attempted, but these efforts were complicated by many factors related to the structural nature of the compounds, the complexity of the mixture, and the chromatographic systems used.



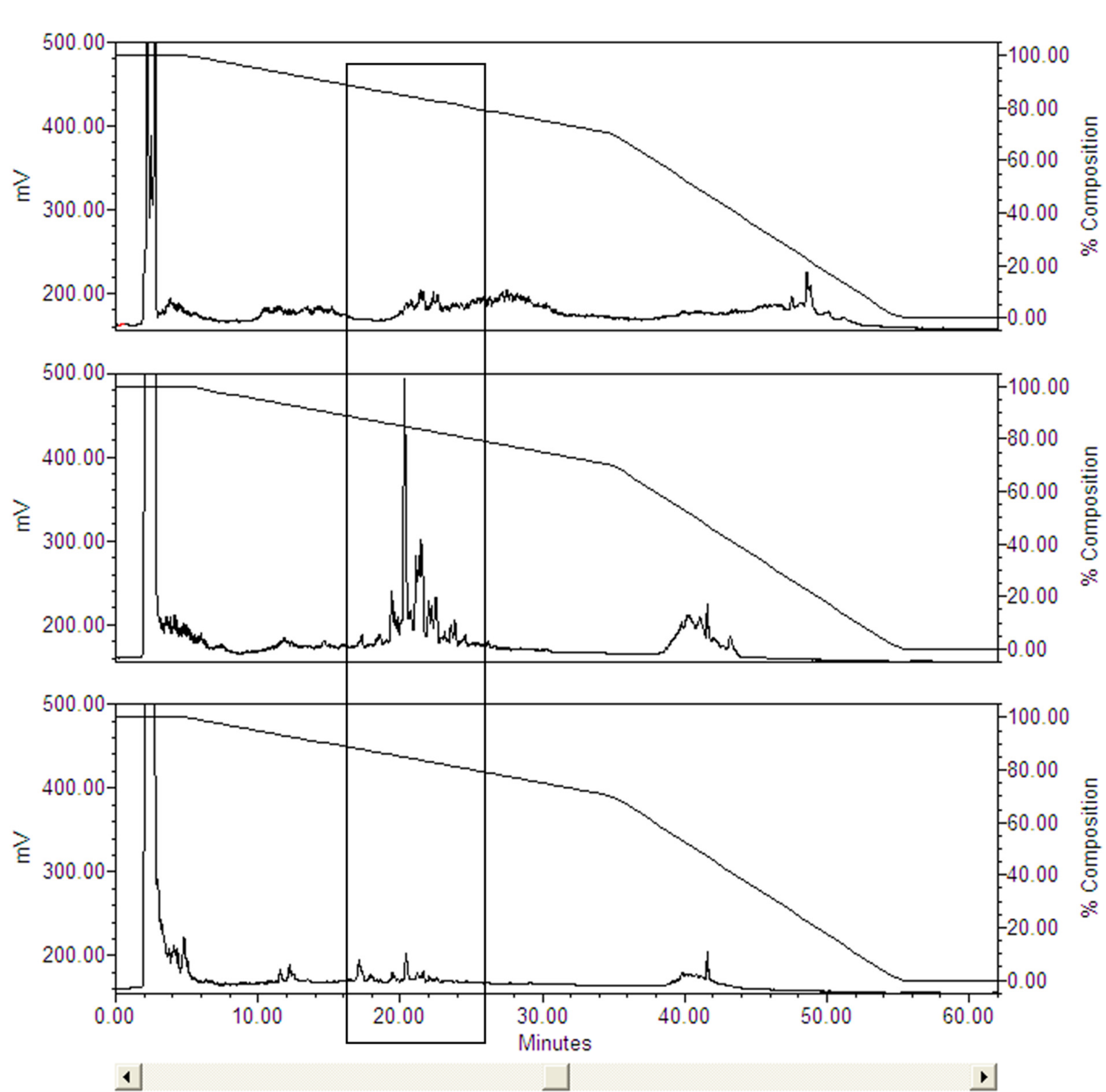
/isible	Date Acquired	SampleName	Channel Name	Total Area	Injection Volume (ul)	Vial	Dilution	SampleWeight	Acq Method Set	
☑	7/10/2009 1:08:44 AM	HF1-1 AtldC18-A R1	SATIN	52439423	50.00	18	200.00000	2.00000	AtldC18 A	T
☑	7/9/2009 6:47:22 AM	IF1-2 AtldC18-A R3	SATIN	69275845	50.00	16	270.00000	2.70000	AtldC18 A	T
☑	7/9/2009 8:23:36 AM	IF1-3 AtldC18-A R1	SATIN	90016796	50.00	17	250.00000	2.50000	AtldC18 A	T
☑	7/9/2009 1:58:41 AM	HF2-2 AtldC18-A R4	SATIN	68681872	50.00	5	240.00000	2.40000	AtldC18 A	T

Figure 17. Comparison of the HPLC-ELSD data for active fraction HF1-1 and untested samples IF1-2, IF1-3, HF2-2, showing a similar profile of ELS detectable peaks.
The profile of peaks eluting in the region of interest for each sample was similar. The gradient used for the HF1-1 sample was slightly different than that used for the other samples, resulting in shifts of retention times but a similar overall profile.



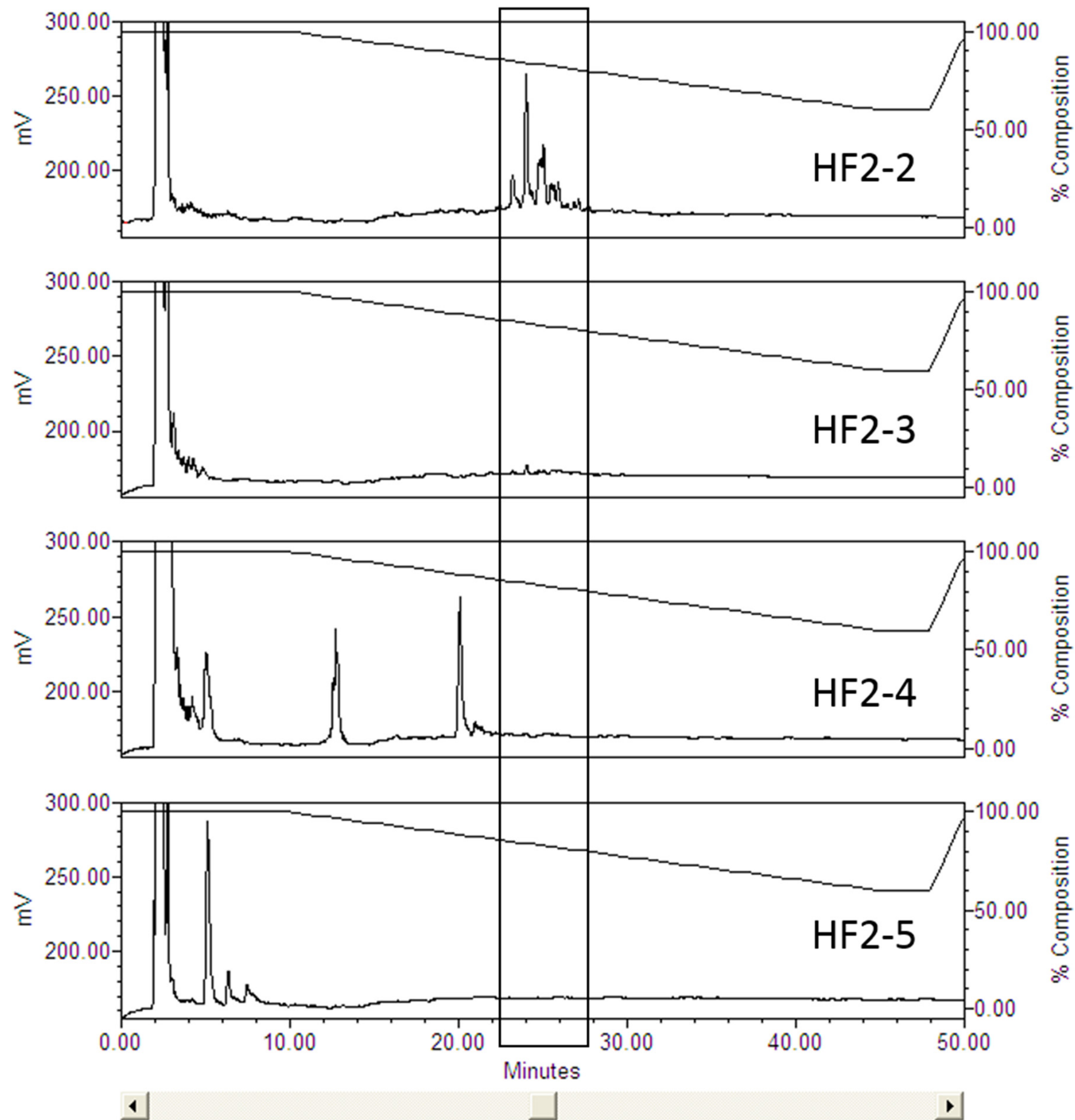
Visible	SampleName	SampleWeight	Dilution	Injection Volume (uL)	Date Acquired	Acq Method Set	Channel N
☑	IF1-1 AtldC18-A R3	2.80000	280.00000	50.00	7/9/2009 5:11:09 AM CDT	AtldC18 A	SATIN
☑	IF1-2 AtldC18-A R3	2.70000	270.00000	50.00	7/9/2009 6:47:22 AM CDT	AtldC18 A	SATIN
☑	IF1-3 AtldC18-A R1	2.50000	250.00000	50.00	7/9/2009 8:23:36 AM CDT	AtldC18 A	SATIN

Figure 18. Analytical HPLC-ELSD of IF1-1, IF1-2, and IF1-3 (AtldC18).
The components of interest (18–24 min) were readily detectable in Sephadex LH-20 fractions IF1-2 and IF1-3 but could only be detected at trace levels in fraction IF1-1.



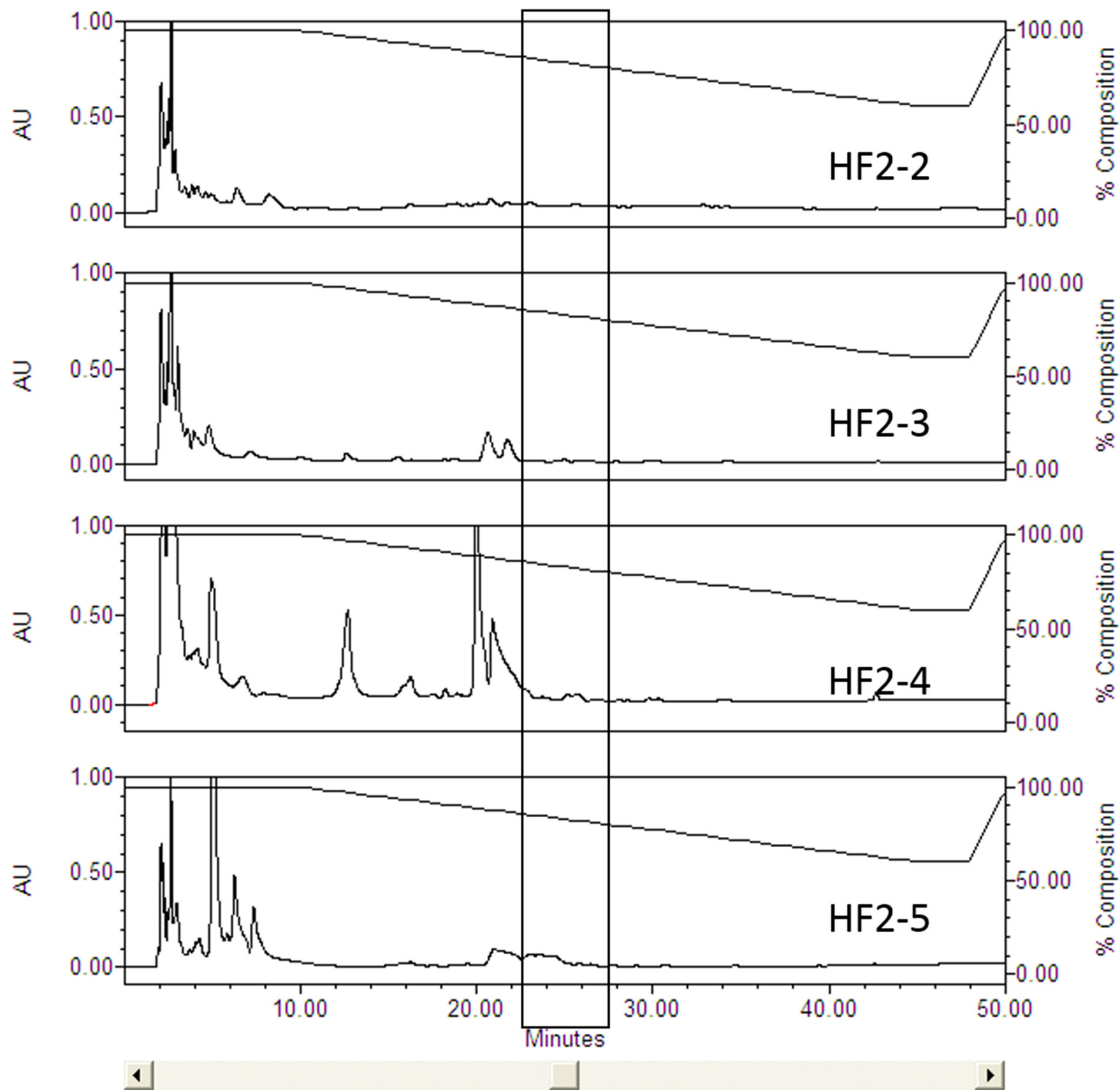
/isible	SampleName	SampleWeight	Dilution	Injection Volume (uL)	Channel Name	Date Acquired	Acq Method Set	P
☑	HF2-1 AtldC18-A R2	2.40000	240.00000	50.00	SATIN	7/9/2009 12:22:28 AM CDT	AtldC18 A	
☑	HF2-2 AtldC18-A R3	2.40000	240.00000	50.00	SATIN	7/8/2009 9:45:57 PM CDT	AtldC18 A	
☑	HF2-3 AtldC18-A R3	2.40000	240.00000	50.00	SATIN	7/8/2009 11:02:11 PM CDT	AtldC18 A	

Figure 19. Analytical HPLC-ELSD of HF2-1, HF2-2, and HF2-3 (AtldC18).
The components of interest (18–24 min, box) were predominantly present in Sephadex LH-20 fraction HF2-2 with trace amounts present in fractions HF2-1 and HF2-3.



Visible	Vial	SampleName	Date Acquired	SampleWeight	Dilution	Injection Volume (uL)	Acq Method Set	
☑	5	HF2-2 AtldC18-A R2	7/5/2009 6:51:24 AM CDT	2.40000	240.00000	20.00	AtldC18 A	7/
☑	6	HF2-3 AtldC18-A R2	7/5/2009 7:57:37 AM CDT	2.40000	240.00000	20.00	AtldC18 A	7/
☑	9	HF2-4 AtldC18-A R2	7/5/2009 11:16:22 AM CDT	2.00000	200.00000	50.00	AtldC18 A	7/
☑	10	HF2-5 AtldC18-A R2	7/5/2009 12:22:27 PM CDT	4.00000	200.00000	10.00	AtldC18 A	7/

Figure 20. Analytical HPLC-ELSD of HF2-2, HF2-3, HF2-4 and HF2-5 (AtldC18).
The components of interest (22–28 min) present in Sephadex LH-20 fraction HF2-2 were detected at trace levels in fraction HF2-3, and were not detected in fractions HF2-4, and HF2-5.



Visible	Vial	SampleName	Date Acquired	Acq Method Set	Injection Volume (uL)	Channel Name	Manua
☑	5	HF2-2 AtldC18-A R2	7/5/2009 6:51:24 AM CDT	AtldC18 A	20.00	MaxPlot 210.0 to 400.0	☐
☑	6	HF2-3 AtldC18-A R2	7/5/2009 7:57:37 AM CDT	AtldC18 A	20.00	MaxPlot 210.0 to 400.0	☐
☑	9	HF2-4 AtldC18-A R2	7/5/2009 11:16:22 AM CDT	AtldC18 A	50.00	MaxPlot 210.0 to 400.0	☐
☑	10	HF2-5 AtldC18-A R2	7/5/2009 12:22:27 PM CDT	AtldC18 A	10.00	MaxPlot 210.0 to 400.0	☐

Figure 21. Analytical HPLC-UV (Max Plot) of HF2-2, HF2-3, HF2-4 and HF2-5 (AtldC18). The components of interest (22–28 min) present in Sephadex LH-20 fraction HF2-2 were not detectable by UV and the HF2-2 fraction could not be readily distinguished from the other fractions using only UV detection.

b. *Preparative AtldC18 HPLC-ELSD*

Reverse phase HPLC separations were performed with H and I Sephadex LH-20 fractions that contained UV-transparent oligosaccharides by ELSD and ^{1}H NMR spectroscopy, starting with those fractions that were available in the highest quantity (Table 16). These included HF1-1, HF1-2, HF1-3, HF2-1, HF2-2, IF1-1, IF1-2 and IF1-3 (Table 6).

The majority of the HF1-1, HF1-2, and HF1-3 samples were used for method development. Attempts were made to resolve the components of these fractions using direct injection onto both XTMSC18 and Polyamine semi-preparative columns with both PDA and/or ELS detection, but were unsuccessful at yielding pure compounds. The fractions collected from these separations were not combined with later materials due to the differences in the chromatographic methods used. Efforts were also made to isolate compounds from the inactive fraction HF2-6 and the compounds isolated were found to be known urinary metabolites (not shown).

Once an acceptable method was obtained, the same or highly similar methods were used for all subsequent separations and the results are included below (Tables 18, 19; Figures 22–24). H and I fractions were initially kept separate with the goal of identifying and comparing the components from both source animals. As isolation efforts proceeded, chromatographic profiles indicated that the components of both samples were similar and selected fractions from both materials were therefore combined. Aliquots of fractions that were available in lower quantities were co-injected with samples from more abundant fractions to aid in the alignment of peaks, to account for instrument variations between separations, and to account for possible sample matrix effects.

Attempts at method optimization during preparative separations led to several method variations. The method for HF2-2 R1 used 5 min at 100% water, a 30 min gradient to 30% MeOH, and a 20 min gradient to 100% MeOH. The ELSD conditions were adjusted for HF2-2 R1. Initial conditions were an evaporator temperature of 100 °C, a nebulizer temperature of 50 °C, and a gas flow rate of 1.0 SML. At 40 min into the separation, corresponding to 50% MeOH, the evaporator temperature was changed to 90 °C to account for the increase in the percentage of organic solvent. This modification did not improve detector resolution and changes to the evaporator temperature were not made in subsequent separations. The method for HF2-2 R2–R6, IF1-2 R1, IF1-3 R1–R3, and HF2-1+IF1-1 R1 used 5 min at 100% water, a 30 min gradient to 20% MeOH, and a 10 min gradient to 100% MeOH. The method for HF2-1+IF1-1 R2 used modified gradient times with 5 min at 100% water, a 10 min gradient to 20% MeOH, and a 30 min gradient to 100% MeOH. The ELSD conditions were maintained at an evaporator temperature of 100 °C, a nebulizer temperature of 50 °C, and a gas flow rate of 1.0 SML.

Selected fractions of the AtldC18 preparative separations were combine across different parent materials: HF2-2P1t20, IF1-3P1t20, H/IF2/1-1P1t20 were combined into fraction HF2-2P1t20, and HF2-2P1t22 + IF1-3P1t22 were combined into fraction HF2-2P1t22 (17.1 mg). Both 1D and 2D NMR data were acquired for HF2-2P1t20 and HF2-2P1t22 combined samples (Figures 25–27).

Table 16. Details of preparative-scale sample preparations for AtldC18-based separations of oligosaccharide-containing Sephadex LH-20 fractions.
Samples were assessed by analytical HPLC-ELSD to determine their composition prior to preparative separation. All of the fractions show below were determined to contain the components of interest.

Parent Fraction	Amount (mg)	Sample Dissolved In Water (μL)	Filter Wash (μL)	Sample Vol. (μL)	Injected per Run (mg/μL)	Separation Dates
IF1-1	12	200	200	400	R1: 6 mg/200 μL	2009/06/24
HF2-2	122	675	500[a]	775	R1: 50/250 R2: 50/275 25/150[a] + IF1-2R1	2009/07/09
HF2-2	208	800	200	1000	R3: 50/250 R4: 50/250 R5: 50/250 R6: 50/250	2009/07/10
IF1-2	53.3	250 (HF2-2 wash)[b]	100	250	R1: 53.3+/250 (contained small amount of HF2-2)	2009/07/10
IF1-3	182	600 (+ IF1-2 wash)[b]	150 (IF1-2 wash)[b]	750	R1: 60/250 R2: 60/250 R3: 60/250	2009/07/10
HF2-1 + IF1-1[c]	67.5 + 59.7	300	200	500	R1: 60/250 R2: 60/250	2009/07/14

[a] This material was combined with the sample shown.
[b] The filter wash collected from the fraction shown was used as the dilution solvent for this material
[c] These fractions were determined to be sufficiently similar by analytical HPLC-ELSD to justify combining them.

Table 17. Combined preparative HPLC fractions resulting from HF2-2 and IF1-2 separations, designated HF2-2P1.
Fractions t20 and t22 were analyzed by NMR spectroscopy and the results are discussed below.

Retention Time (min)[a]	Fraction	mg	Notes
1.7–3.7	03	92.9	
3.7–4.57	04	14.4	
4.57–5.52	05	10.4	
5.52–9	06-09	13.1	
18.3–19.4	19	10.6	
19.4–20.0	20 *	11.9	Major fraction of interest
20.0–20.8	21	8.0	
20.8–22.0	22 *	17.1	Major fraction of interest
22.0–24.0	24	14.7	
24.0–25.0	25	-	
25.0–26.0	26	5.1	
Baseline[b]	B	-	

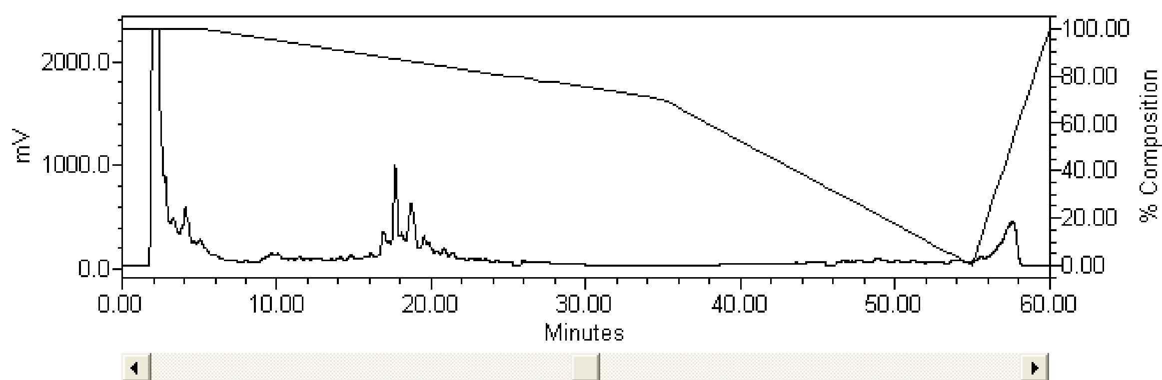
[a] Time points for fraction numbers were based on HF2-2 R4 and R5.
[b] Baseline contains all peaks not accounted for by other time points.

Table 18. Combined preparative HPLC fractions resulting from IF1-3 separations, designated IF1-3P1.
Fractions t20 and t22 from these separations appeared to have the same composition as analogous fractions from the HF2-2 and IF1-2 parent materials.

Retention Time (min)	Fraction	mg	Notes
1.7–3.7	03	77.6	
3.7–5.0	04	10.1	
5.0–8.3	05-08	14.2	
8.3–11	08-11	8.0	
18.5–19.4	19	4.3	
19.4–20.0	19.5	2.4	
20.0–20.5	20 *	4.3	Major fraction of interest
20.5–21.4	21	16.4	
21.4–22.0	22 *	3.5	Major fraction of interest
22.0–22.8	22.5	2.7	
22.8–23.5	23	2.1	
23.5–24.1	24	2.3	
24.1–25.0	25	1.7	
25.0–60	B	25.4	

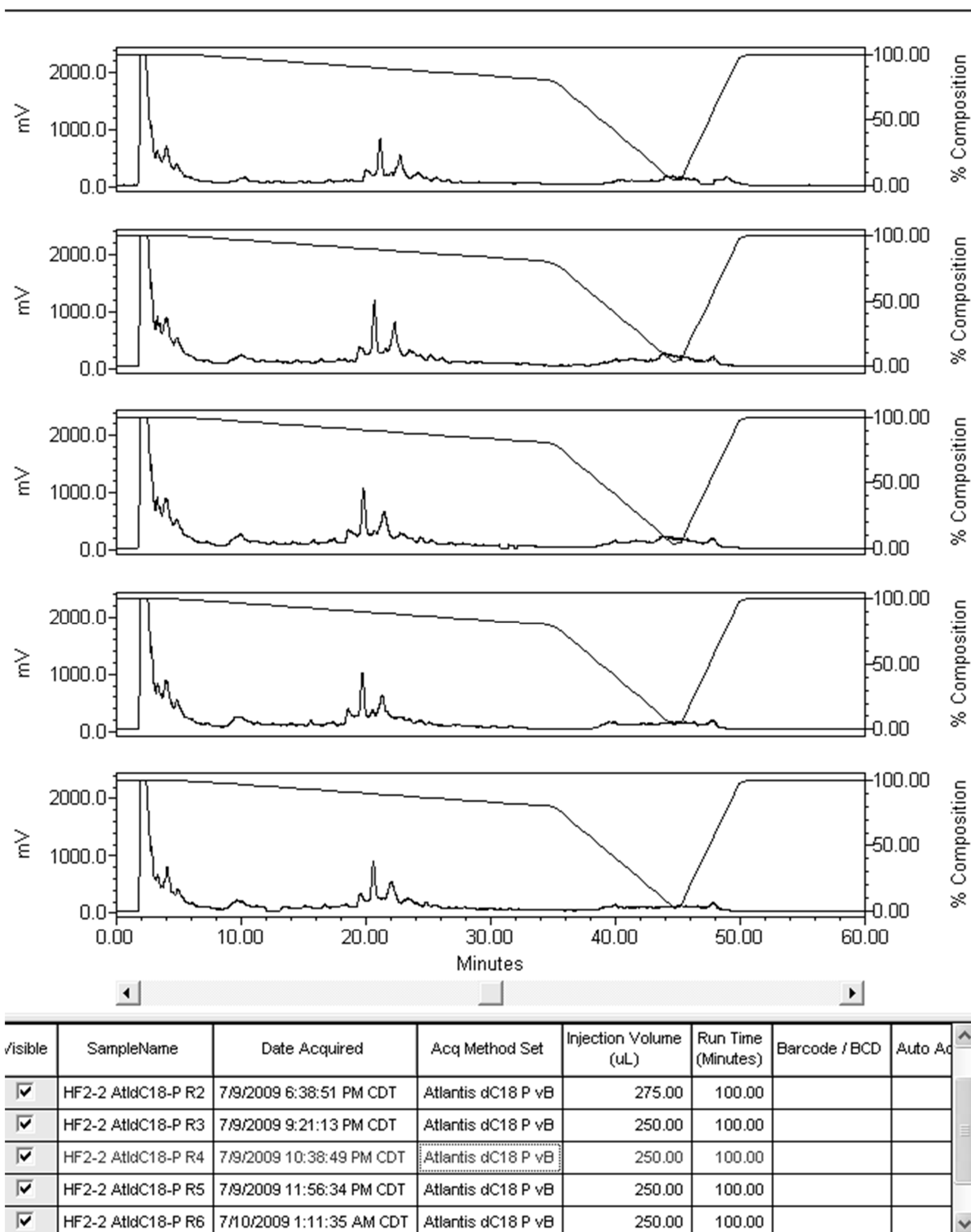
Table 19. Combined preparative HPLC fractions resulting from HF2-1 and IF1-1 separations, designated H/IF2/1-1P1.
The HF2-1 and IF1-1 fractions were determined to be of similar composition and the subfractions from these separations were therefore combined.

Retention Time (min)		Fraction	mg	Notes
R1	R2	R1 + R2		
1.5–7.0	1.5–8.5	02	24.2	
7.0–14	8.5–11.5	B	-	Part of baseline
14–22.6	11.5–15.0	20	12.6	Major fraction of interest
22.6–37.7	15.0–17.5	25	17.0	
37.7–48.0	17.5–30.0	40	32.9	
48.0–70.0	30.0–60.0	B	-	Part of baseline



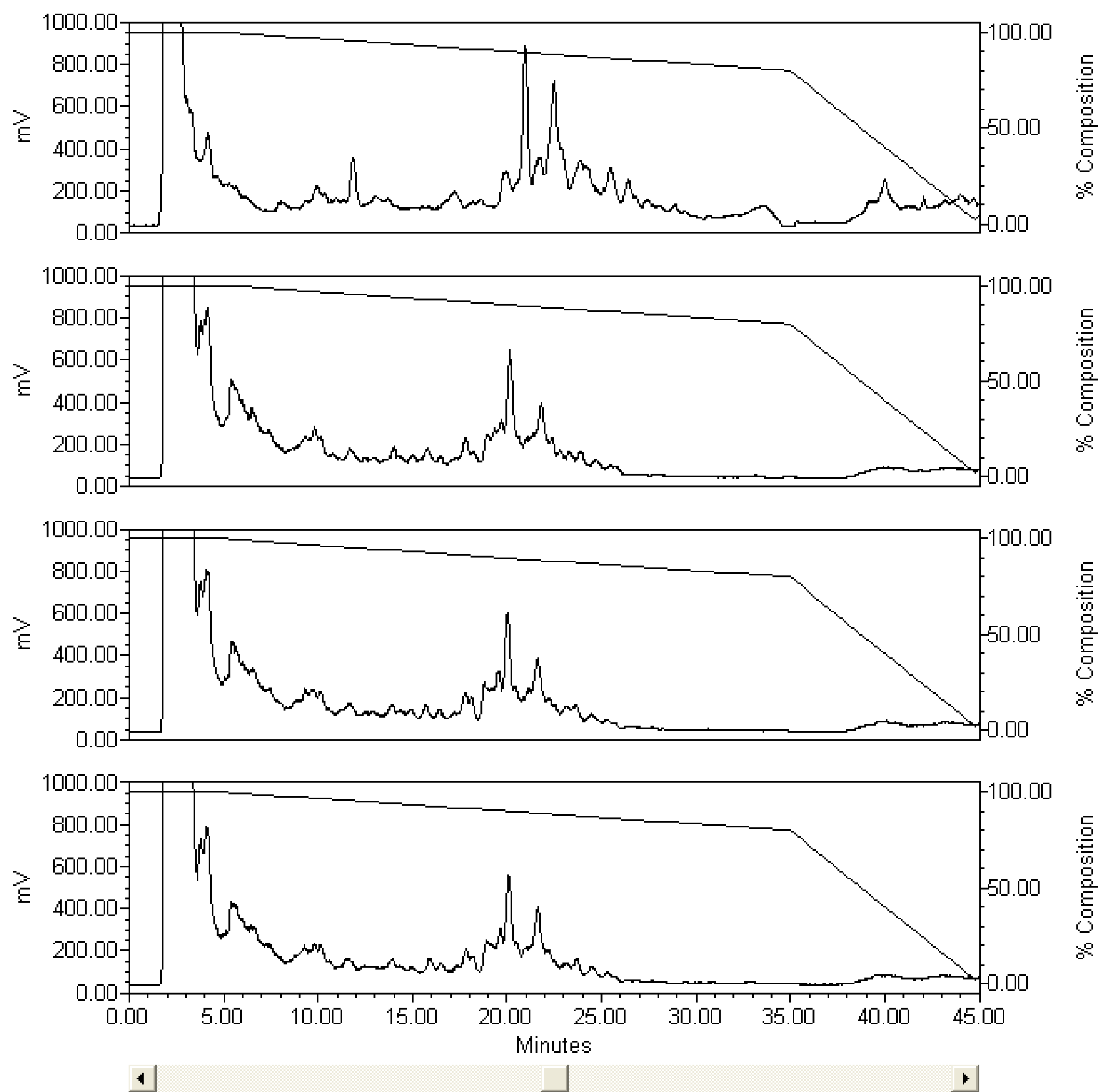
Visible	SampleName	Date Acquired	Acq Method Set	Injection Volume (uL)	Run Time (Minutes)	Barcode / BCD	Auto Addition
☑	HF2-2 AtldC18-P R1	7/9/2009 5:08:07 PM CDT	Atlantis dC18 P vB	250.00	100.00		

Figure 22. Preparative HPLC-ELSD of HF2-2, R1 (AtldC18).
The gradient used for this separation varied from that used for subsequent separations. ELSD parameters were also changed during this separation in an effort to improve ELSD resolution, but resulted in no apparent improvement. The parameters used for subsequent separations (below) were preferred for later separations.



Visible	SampleName	Date Acquired	Acq Method Set	Injection Volume (uL)	Run Time (Minutes)	Barcode / BCD	Auto Ad
☑	HF2-2 AtldC18-P R2	7/9/2009 6:38:51 PM CDT	Atlantis dC18 P vB	275.00	100.00		
☑	HF2-2 AtldC18-P R3	7/9/2009 9:21:13 PM CDT	Atlantis dC18 P vB	250.00	100.00		
☑	HF2-2 AtldC18-P R4	7/9/2009 10:38:49 PM CDT	Atlantis dC18 P vB	250.00	100.00		
☑	HF2-2 AtldC18-P R5	7/9/2009 11:56:34 PM CDT	Atlantis dC18 P vB	250.00	100.00		
☑	HF2-2 AtldC18-P R6	7/10/2009 1:11:35 AM CDT	Atlantis dC18 P vB	250.00	100.00		

Figure 23. Preparative HPLC-ELSD of HF2-2, R2-6 (AtldC18).
Retention times were similar across preparative separations and chromatographic resolution was not significantly affected by slight variations in injection volumes or sample amounts.



/isible	SampleName	Injection Volume (uL)	Det. Units	Date Acquired	Acq Method Set
☑	IF1-2 AtldC18-P R1	275.00	mV	7/9/2009 8:01:07 PM CDT	Atlantis dC18 P vB
☑	IF1-3 AtldC18-P R1	250.00	mV	7/10/2009 4:22:03 AM CDT	Atlantis dC18 P vB
☑	IF1-3 AtldC18-P R2	250.00	mV	7/10/2009 5:44:05 AM CDT	Atlantis dC18 P vB
☑	IF1-3 AtldC18-P R3	250.00	mV	7/10/2009 7:04:42 AM CDT	Atlantis dC18 P vB

Figure 24. Preparative HPLC-ELSD of IF1-2 and IF1-3 R1–3 (AtldC18).
Retention times were similar across preparative separations and chromatographic resolution was not significantly affected by slight variations in injection volumes or sample amounts. Fractions from the IF1-2 separation (top pane) were combined with those of HF2-2 to give HF2-2P1 subfractions, and fractions from the IF1-3 separations were combined to give IF1-3P1 subfractions.

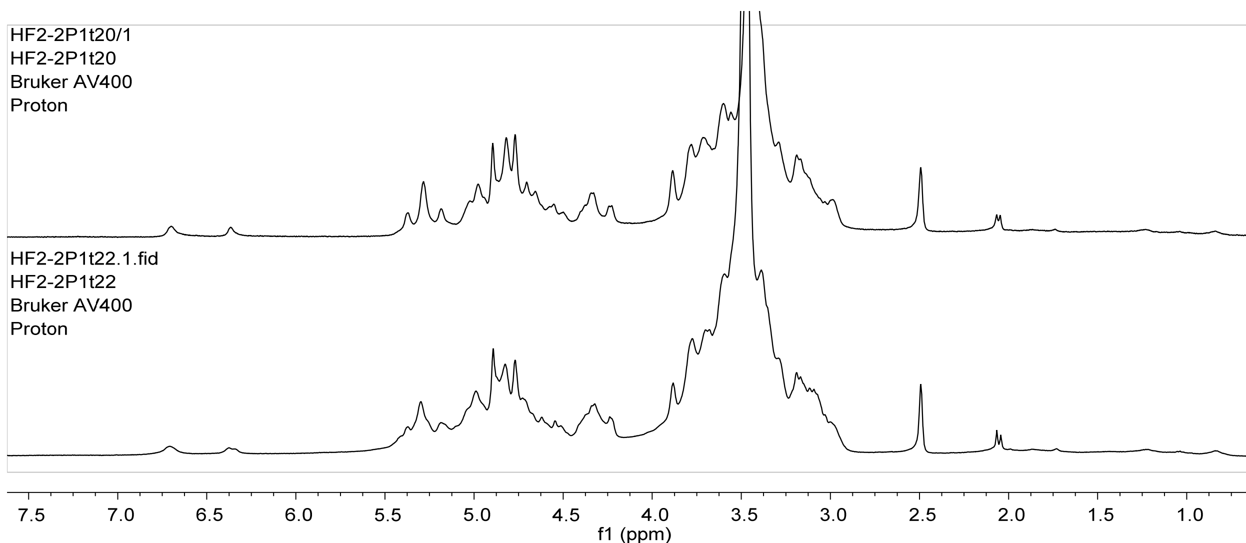


Figure 25. Comparison of the ^{1}H NMR spectra for HF2-2P1t20 and HF2-2P1t22 showing highly similar composition, DMSO-d_6, 400 MHz.
The HF2-2P1t20 sample was later determined to be relatively pure while HF2-2P1t22 was determined to be a mixture of three major components in approximately equal amounts. Resonances were not present in the regions of the spectra that are not displayed.

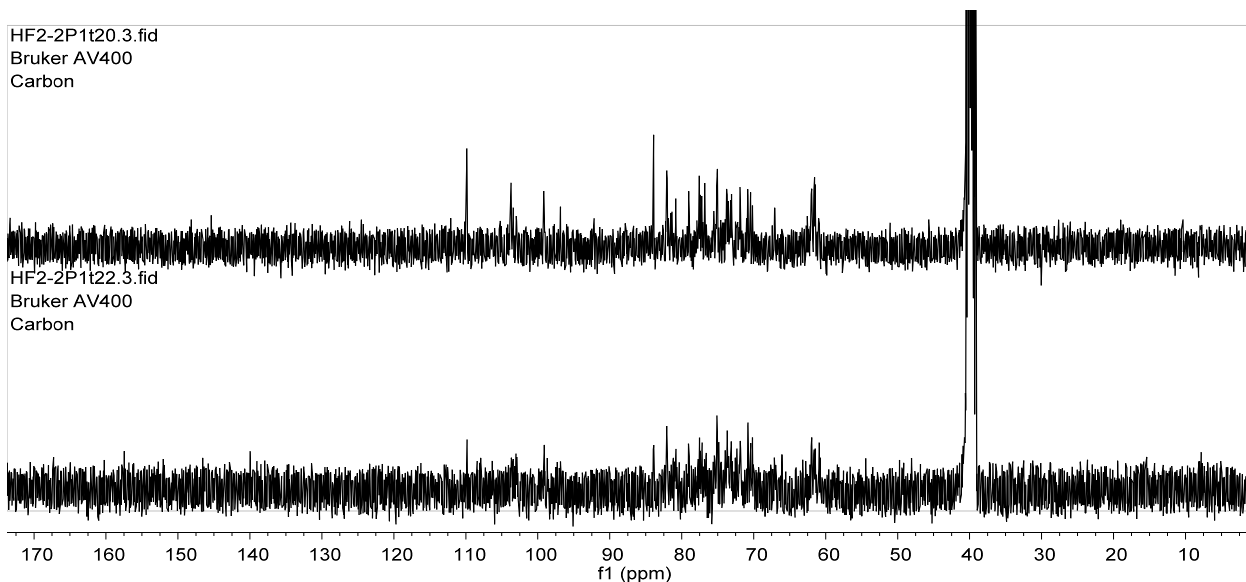


Figure 26. Comparison of the ^{13}C NMR spectra for HF2-2P1t20 and HF2-2P1t22 showing highly similar composition, DMSO-d_6, 400 MHz.

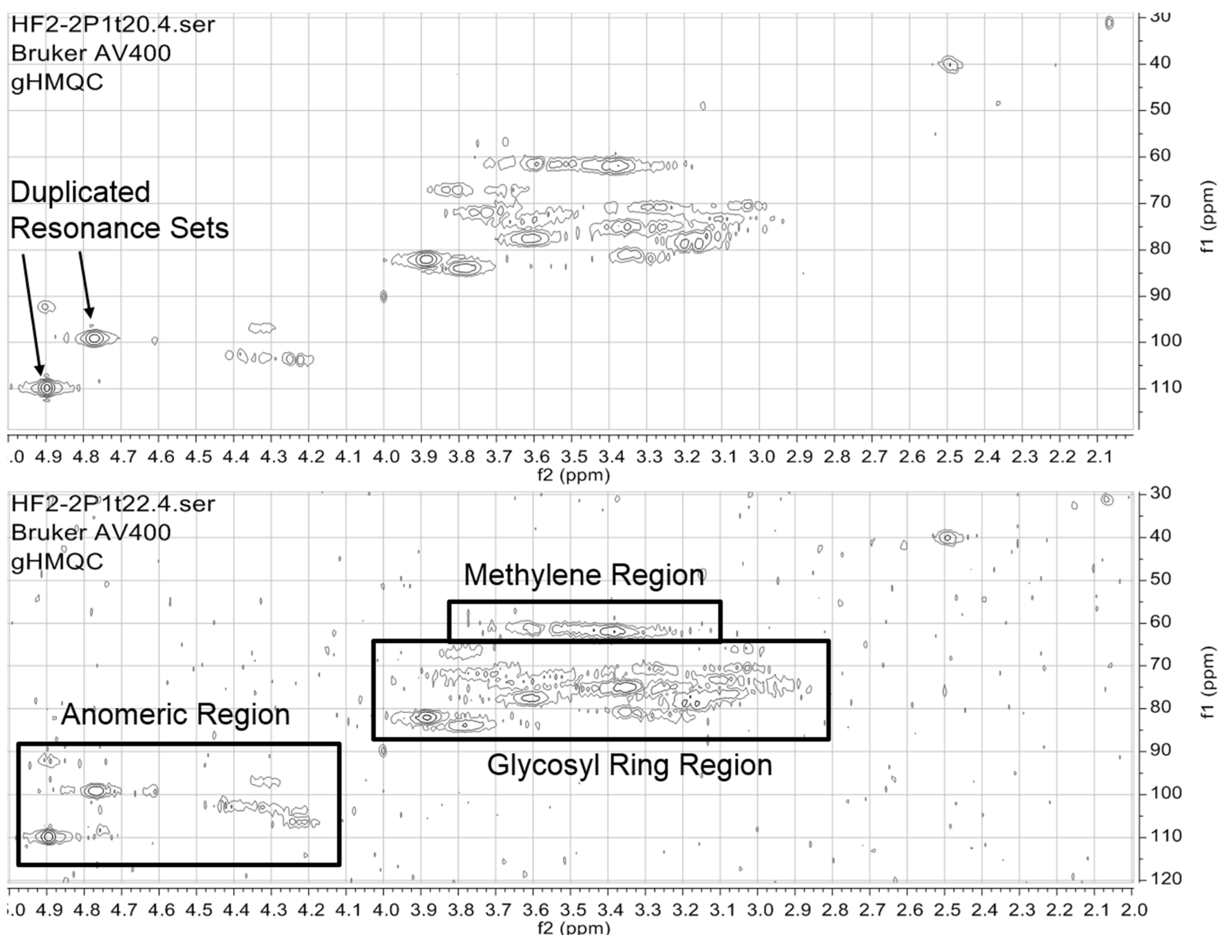


Figure 27. Comparison of the HMQC spectra for HF2-2P1t20 and HF2-2P1t22 showing the similarity of composition for these fractions, DMSO-d_6, 400 MHz.
The anomeric, methylene, and glycosyl ring regions are indicated for the HF2-2P1t22 spectrum. Integration of the anomeric region of the ^{1}H NMR spectrum and consideration of the data shown here led to the refined working hypothesis that the carbohydrates of interest were oligosaccharides with 7-9 monomeric units. It was later determined that a reducing glucosyl unit was responsible for two of the anomeric resonances shown and that the two high intensity resonances indicated for the HF2-2P1t20 spectrum were duplicated anomeric resonance sets corresponding to two Xyl and two Ara residues.

c. *Semi-Preparative Polyamine HPLC-ELSD*

Methods for semi-preparative separations were developed using the refined working hypothesis that the compounds of interest were oligosaccharides. Fractions eluting from the AtldC18 column were combined based on retention time and profile and were further analyzed by normal-phase analytical HPLC-ELSD (Figures 28–30). Selected fractions were further purified using the Polyamine column (Figures 31– 34, Table 20). Resulting Polyamine fractions were cautiously combined across parent materials in an effort to obtain sufficient material for detailed structural analysis. Analytical scale HPLC-ELSD data (Figures 28–30) and considerations of the results of sample co-injections (Table 20) were both used to aid in comparisons and sample fraction combinations (Tables 21–26).

Fraction HF2-2P1t24 was used to evaluate the effects of changes in starting solvent percentages and gradient length on the resolution of semi-preparative separations. A consistent flow rate of 4 mL/min was used for all separations. The method for HF2-2P1t24 R1 used MeCN:water, with 10 min at 70% MeCN, a 30 min gradient to 40% MeCN, and a 3 min gradient to 70% MeCN. The method for HF2-2P1t24 R2 used MeCN:water, with 5 min at 65% MeCN, a 30 min gradient to 40% MeCN, and a 3 min gradient to 65% MeCN. The method for HF2-2P1t24 R3–R5 (Figure 31) used MeCN:water, with 5 min 65% MeCN, a 25 min gradient to 45% MeCN, and a 3 min gradient to 65% MeCN. This last method was also used for HF2-2P1t21 R1–R5 and later separations.

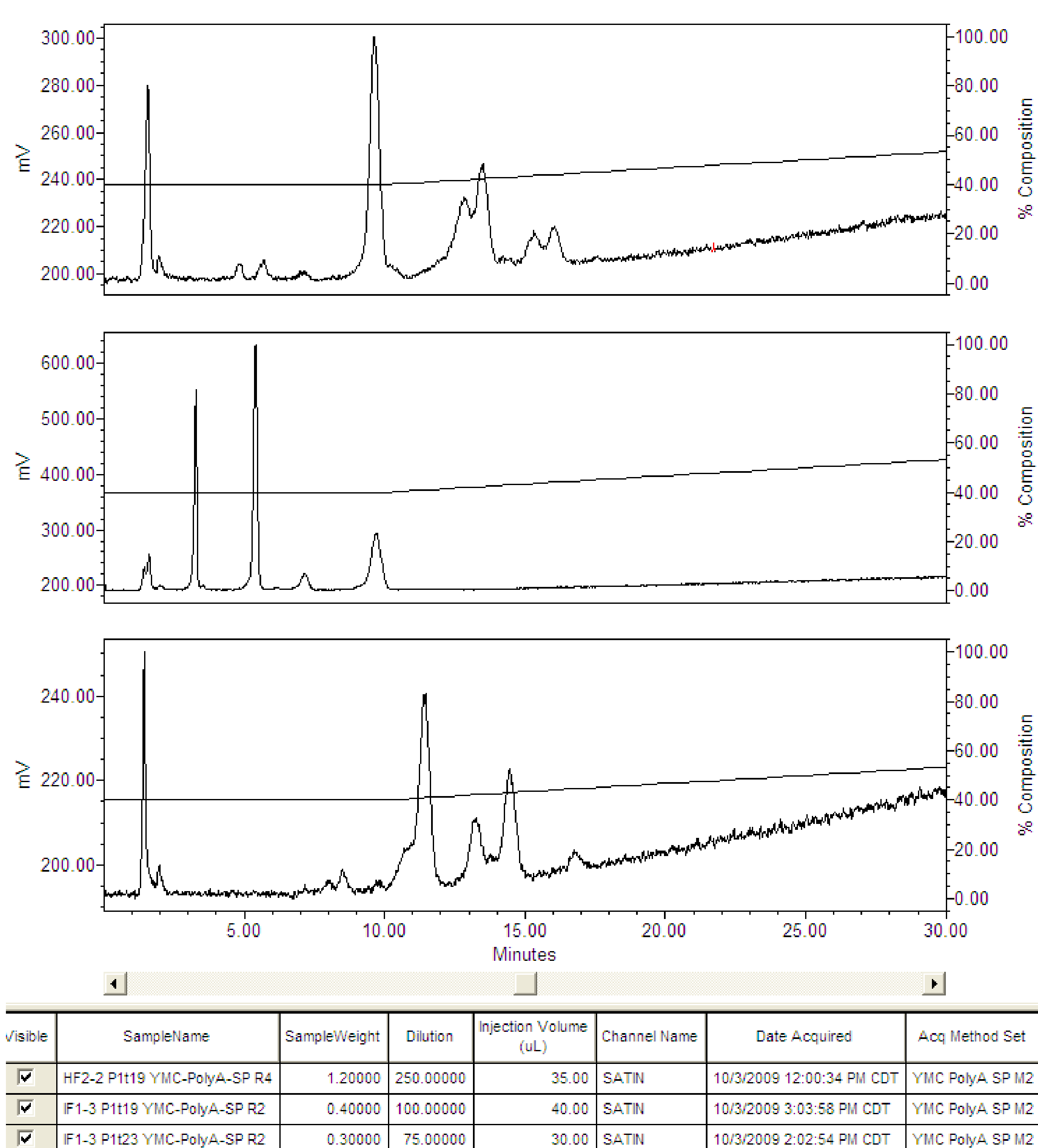
Initial ELSD conditions used for HF2-2P1t24 were an evaporator temperature of 90 ^{o}C, a nebulizer temperature of 45 ^{o}C, and a gas flow rate of 1.0 SLM. The instrument configuration and ELSD conditions were modified for HF2-2P1t21 and later separations with changes in the ELSD inlet tubing to give 3.9 mL/min to collection and 0.1 mL/min to ELS detection. These

changes dictated that the ELSD gas flow rate be decreased to 0.8 SLM to maintain sensitivity; other temperature conditions remained the same.

Fractions from Polyamine separations were minimally combined due to minor shifts in retention times between samples and the difficulties of obtaining NMR data on small quantities of material. The three major components of the HF2-2P1t22 fraction (A17, A18 and A19; Figure 33; Table 25) were of interest, but the ^{1}H NMR (Figure 35) and LC-MS data for these fractions could not be reliably interpreted and insufficient material was available for further analyses at the time.

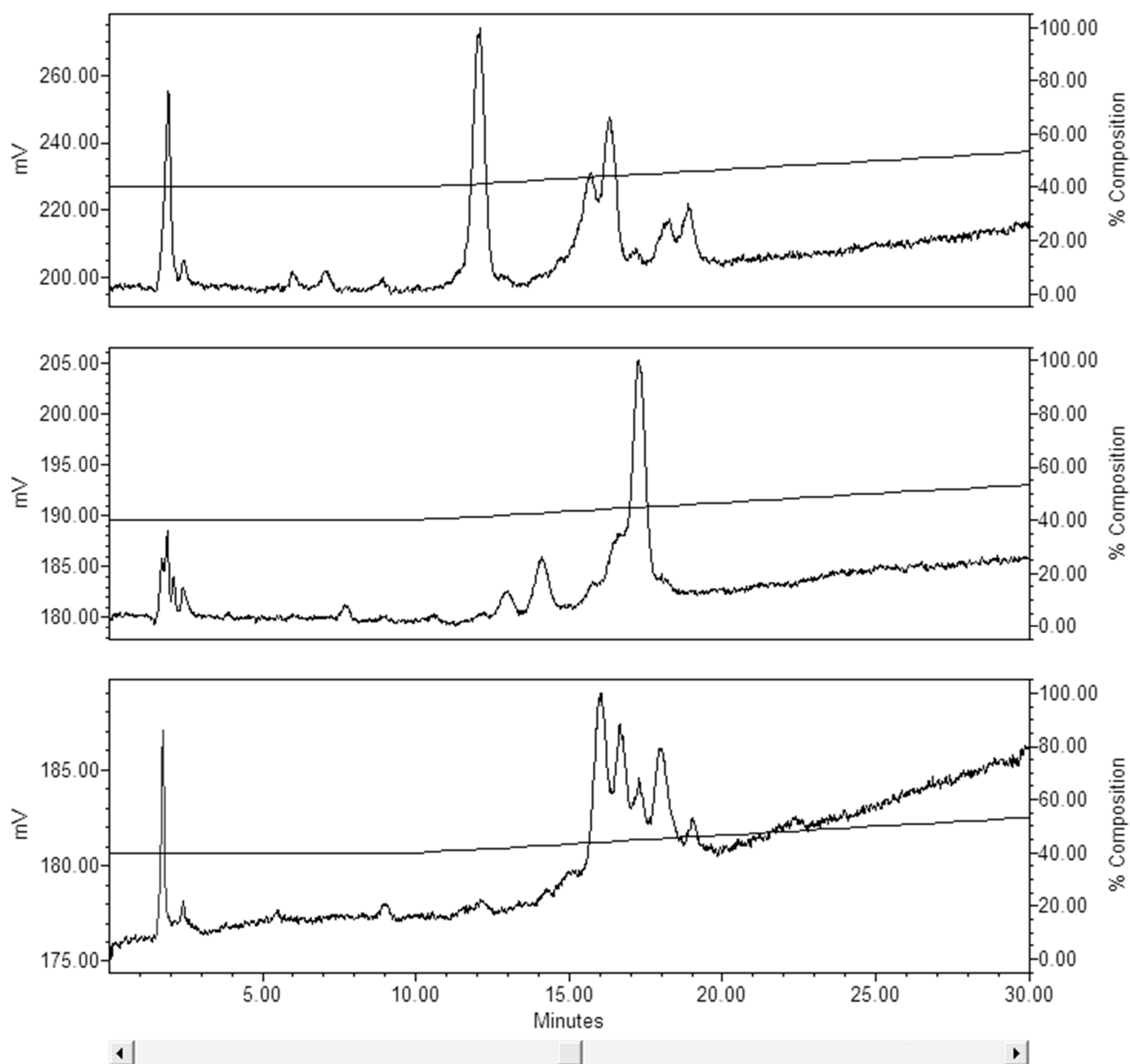
All fractions that corresponded to HF2-2P1t20A17 (Table 26) were combined and the resulting material was further purified using the same Polyamine method as before to yield a single major component. This material was submitted for CCRC derivatization analyses and for high resolution NMR data at 700 MHz.

Fraction 02 and baseline elutions (B) were combined across all HF and IF separations. These materials had low solubility in either DMSO or water, yielding precipitate and cloudy solutions. Preliminary ^{1}H NMR data was acquired for these samples in an attempt to determine the nature of these fractions but provided little useful information.



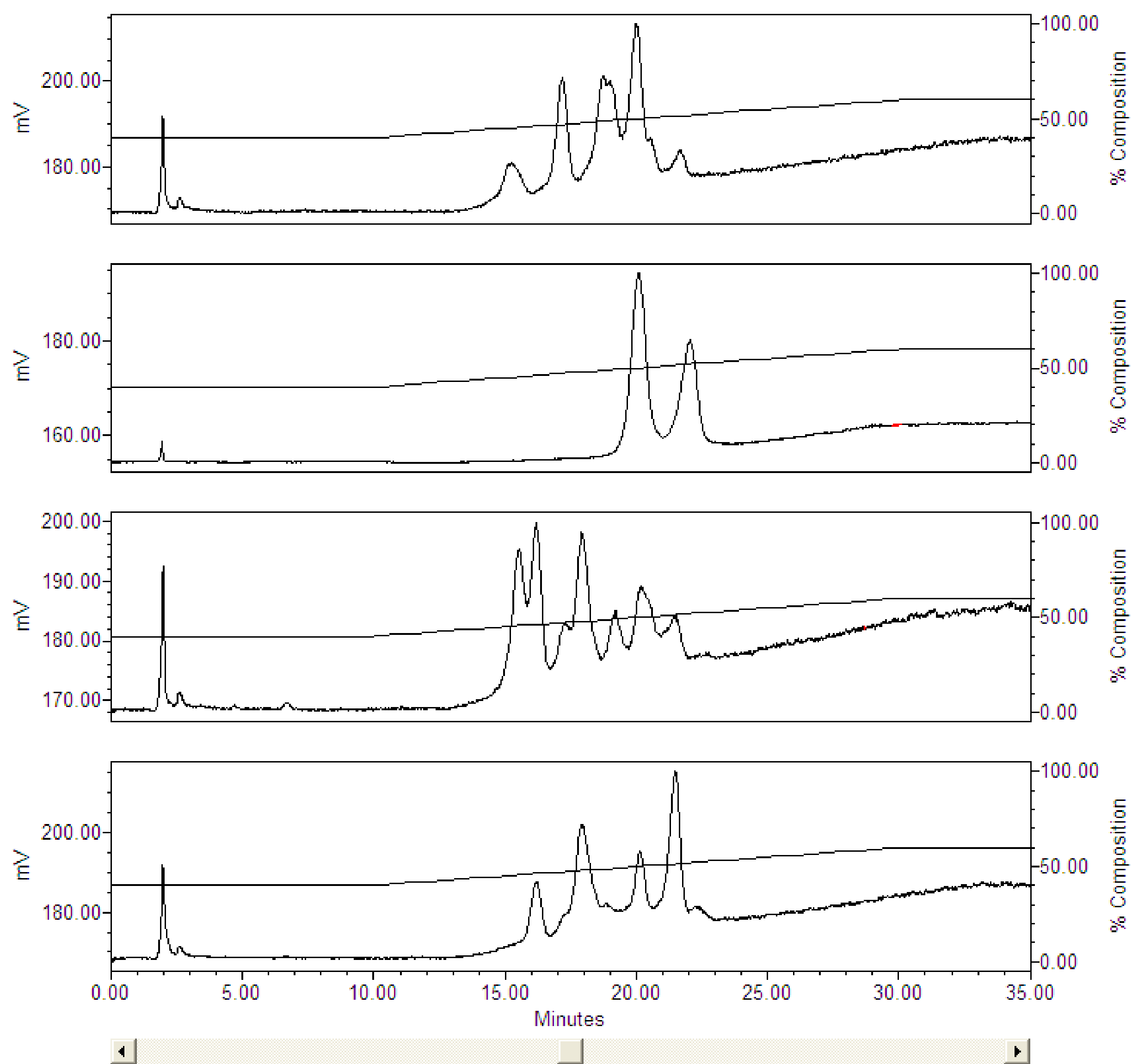
Visible	SampleName	SampleWeight	Dilution	Injection Volume (uL)	Channel Name	Date Acquired	Acq Method Set
☑	HF2-2 P1t19 YMC-PolyA-SP R4	1.20000	250.00000	35.00	SATIN	10/3/2009 12:00:34 PM CDT	YMC PolyA SP M2
☑	IF1-3 P1t19 YMC-PolyA-SP R2	0.40000	100.00000	40.00	SATIN	10/3/2009 3:03:58 PM CDT	YMC PolyA SP M2
☑	IF1-3 P1t23 YMC-PolyA-SP R2	0.30000	75.00000	30.00	SATIN	10/3/2009 2:02:54 PM CDT	YMC PolyA SP M2

Figure 28. Analytical HPLC-ELSD comparisons of AtldC18 fractions HF2-2P1t19, IF1-3P1t19, and IF1-3P1t23 showing the composition of these fractions in support of preparative combinations (Polyamine).
Fractions HF2-2P1t19 and IF1-3P1t19 were combined for semi-preparative separations while IF-3P1t23 was combined with IF1-3P1t22.5 (Figure 30) based on the results of additional analytical analyses (not shown).



Visible	SampleName	SampleWeight	Dilution	Injection Volume (uL)	Channel Name	Date Acquired	Acq Method Set
☑	HF2-2 P1t19 YMC-PolyA-SP R3	1.20000	250.00000	35.00	SATIN	10/3/2009 2:11:08 AM CDT	YMC PolyA SP M2
☑	IF1-3 P1t20 YMC-PolyA-SP R3	0.50000	100.00000	30.00	SATIN	10/3/2009 4:13:26 AM CDT	YMC PolyA SP M2
☑	IF1-3 P1t25 YMC-PolyA-SP R2	0.20000	50.00000	20.00	SATIN	10/3/2009 6:15:44 AM CDT	YMC PolyA SP M2

Figure 29. Analytical HPLC-ELSD comparisons of AtldC18 fractions HF2-2P1t19, IF1-3P1t20, and IF1-3P1t25, and showing the composition of these fractions in support of preparative combinations (Polyamine).
The parent fractions shown here were determined to be distinct enough to warrant independent separations and fraction recombinations. Fraction IF1-3P1t20 appears to contain the same major component as HF2-2P1t20.



Visible	SampleName	SampleWeight	Dilution	Injection Volume (uL)	Channel Name	Date Acquired	Acq Method Set
☑	HF2-2 P1t21 YMC-PolyA-SP R2	0.40000	100.00000	30.00	SATIN	9/5/2009 2:05:42 AM CDT	YMC PolyA SP M1
☑	IF1-3 P1t22 YMC-PolyA-SP R2	0.30000	75.00000	20.00	SATIN	9/5/2009 8:02:56 AM CDT	YMC PolyA SP 2
☑	IF1-3 P1t22.5 YMC-PolyA-SP R2	0.40000	100.00000	30.00	SATIN	9/5/2009 3:11:43 AM CDT	YMC PolyA SP M1
☑	HF2-2 P1t24 YMC-PolyA-SP R2	0.40000	100.00000	30.00	SATIN	9/5/2009 4:17:46 AM CDT	YMC PolyA SP M1

Figure 30. Analytical HPLC-ELSD comparisons of AtldC18 fractions HF2-2P1t21, IF1-3P1t22, IF1-3P1t22.5 and HF2-2P1t24, and showing the composition of these fractions in support of preparative combinations (Polyamine).
The parent fractions shown here were determined to be distinct enough to warrant independent separations and fraction recombinations.

Table 20. Details of semi-preparative-scale sample preparations used for Polyamine-based separations of HF and IF AtldC18 fractions.
Samples were separated in the sequential order shown (Separation Date) and chromatographic separations with the same date were often performed back-to-back within a single session.

Parent Fraction	Amount (mg)	Sample Dissolved In (µL)	Filter Wash (µL)[a]	Sample Vol. (µL)	Injected per Run (mg/µL)	Separation Date
HF2-2P1t24	14.7	150	0	150	R1: 2/20 R2: 4/40 R3: 4.5/45 R4, R5: 3.5/35[b]	2009/10/04
IF1-2P1t24 + HF2-2P1t24[c]	6.8	45[b]	25	70	Added to HF2-2P1t24 R4 & R5	2009/10/04
HF2-2P1t21 + IF1-3P1t21[c]	8.0 16.4	200	0	200	R1: 4.3/35 R2: 5.5/45 R3: 4.9/40	2009/10/04
HF2-2P1t21 + IF1-3P1t21[c]	(9.76)[d]	80 (prev. sample)	40	120	R4: 5/60 R5: 5/60	2009/10/11
HF2-2P1t19	10.6	75	0	80	R1: 4.0/30 6.6/50 added to MixR1–R2	2009/10/11
IF1-3P1t19 + IF1-3P1t19.5[c]	4.3 + 2.4	60	20	80	R1: 3.3/40 3.4/40 added to MixR1–R2	2009/10/11
HF2-2P1t19 IF1-3P1t19[c]	(6.6)[d] (3.4)[d]	50 + 45	25	120	MixR1: 4.2/50 MixR2: 5.8/70	2009/10/12
IF1-3P1t20	4.3	30	0	30	R1: 4.3/30	2009/10/12
IF1-3P1t22	3.5	25	45	70	R1: 2.5/50 1.0/20 added to IF1-3P1t23 R2	2009/10/12
IF1-3P1t23 + IF1-3P1t22.5[c]	2.1 + 2.7 + (1.0)[d]	50	40	90	R1: 3.2/60 R2: 2.6/50[a]	2009/10/12
HF2-2P1t22	17.1	100	50	130	R1: 5.3/40 R2: 4/30 R3[e]: 5.3/40 R3: 5.0/40[b]	2009/10/14
HF2-2P1t20	11.9	50	90	140	R1: 4/50 R2: 2/25 R3, R4: 2.4/30 1.1/10 added to HF2-2P1t22 R3	2009/10/14

[a] Samples for which the filter wash amount is 0 µL were not filtered. [b] Contained material from an additional parent fraction. [c] These fractions were determined to be sufficiently similar by analytical HPLC-ELSD to justify combining them during separations. [d] Material from a previously prepared sample. [e] Sample and data were lost due to instrument/operator malfunction.

Table 21. Combined semi-preparative HPLC fractions resulting from HF2-2P1t24 separations, designated HF2-2P1t24A.
Fractions eluting at t16, t18, t20 and t22 were assessed by ^{1}H NMR spectroscopy and were found to contain-closely related oligosaccharides, but full structural assignments could not be made.

Retention Time (min)[a]	Fraction	mg	Notes
1.2–15	02	1.2	few compounds between 0–6, low % of sample
15.0–16.0	15	0.5	
16–16.8	16 *	0.8	Major component of interest
16.8–17.6	17	0.6	
17.6–18.7	18 *	1.6	Major component of interest
18.7–19.8	19	0.9	
19.8–20.8	20 *	1.7	Major component of interest
20.8–21.5	21	0.3	
21.5–22.5	22 *	0.8	Major component of interest
22.5–24.0	23	0.4	

[a] Time points for fraction numbers were based on R2, as R1 had an extended gradient time.

Table 22. Combined semi-preparative HPLC fractions resulting from HF2-2P1t21 and IF1-3P1t21 separations, designated HF2-2P1t21A.
Fractions eluting at t15, t17, and t21 were assessed by ^{1}H NMR spectroscopy and were found to contain closely-related oligosaccharides, but full structural assignments could not be made.

Retention Time (min)[a]	Fraction	mg	Notes
0–2.5	02	-	large peak, inconsistent profile, large % of sample
2.5–14	04	2.9	multiple peaks between start and 8 min – inconsistent retention times
14–15	14	1.2	
15–16	15 *	1.1	Major component of interest
16–17	16	0.9	
17–18	17 *	1.2	Major component of interest
18–18.5	18	0.5	
18.5–19.2	19	0.9	first of 19–20 double peak
19.2–20	20	1.0	second of 19–20 double peak
20–21	21 *	1.2	Major component of interest
21–21.5	21.5	0.4	shoulder of 21
21.5–23	22	0.6	

Table 23. Combined semi-preparative HPLC fractions resulting from HF2-2P1t19, IF1-3P1t19 and IF1-3P1t19.5 separations, designated HF2-2P1t19A.

Retention Time (min)[a]	Fraction	mg	Notes
0–3	2	4.6	
3–4	4	0.5	
4–13	B	3.0	flat line before eluting peak set
13–14.5	14	0.2	first minor peak
14.5–16.5	15	0.6	baseline peaks between 14 and 17
16.5–17.5	17 *	1.9	major peak for t19 fractions, possibly same as HF2-2P1t20A17
17.5–18.5	18	0.5	side peak of 17 (minor component)
18.5–20	19	0.4	last minor peak of set
20–22	21	0.3	baseline peaks tailing off end of separation

[a] Time points for fraction numbers were based on HF2-2t19R1 & IF1-3t19R1.

Table 24. Combined semi-preparative HPLC fractions resulting from IF1-3P1t22 and IF1-3P1t23 separations, designated IF1-3P1t23A.
Fractions eluting at t15, t16, and t18 were assessed by ^{1}H NMR spectroscopy and were found to contain closely-related oligosaccharides, but full structural assignments could not be made.

Retention Time (min)[a]	Fraction	mg	Notes
1.3–4.0	2	-	
4.0–13.5	B	-	baseline between early eluting impurity and major peak set
14.5–16	15 *	0.6	Major component of interest
16–17	16 *	0.5	Major component of interest
17–18	17	0.5	
18–19	18 *	1.0	Major component of interest
19–20	19	0.9	
20–21.5	21	0.9	mix of baseline minor components
21.5–22.5	22	0.8	mix of baseline minor components

[a] Times points for fraction numbers were based on IF1-3P1t23 R1.

Table 25. Combined semi-preparative HPLC fractions resulting from HF2-2P1t22 separations, designated HF2-2P1t22A.
Fractions eluting at t17, t18, and t19 were assessed by ^{1}H NMR spectroscopy and were found to contain closely-related oligosaccharides, but full structural assignments could not be made. Fraction t17 from this separation appears to be the same compound as HF2-2P1t20A17 based on co-injection of the HF2-2P1t20 and HF2-2P1t22 samples.

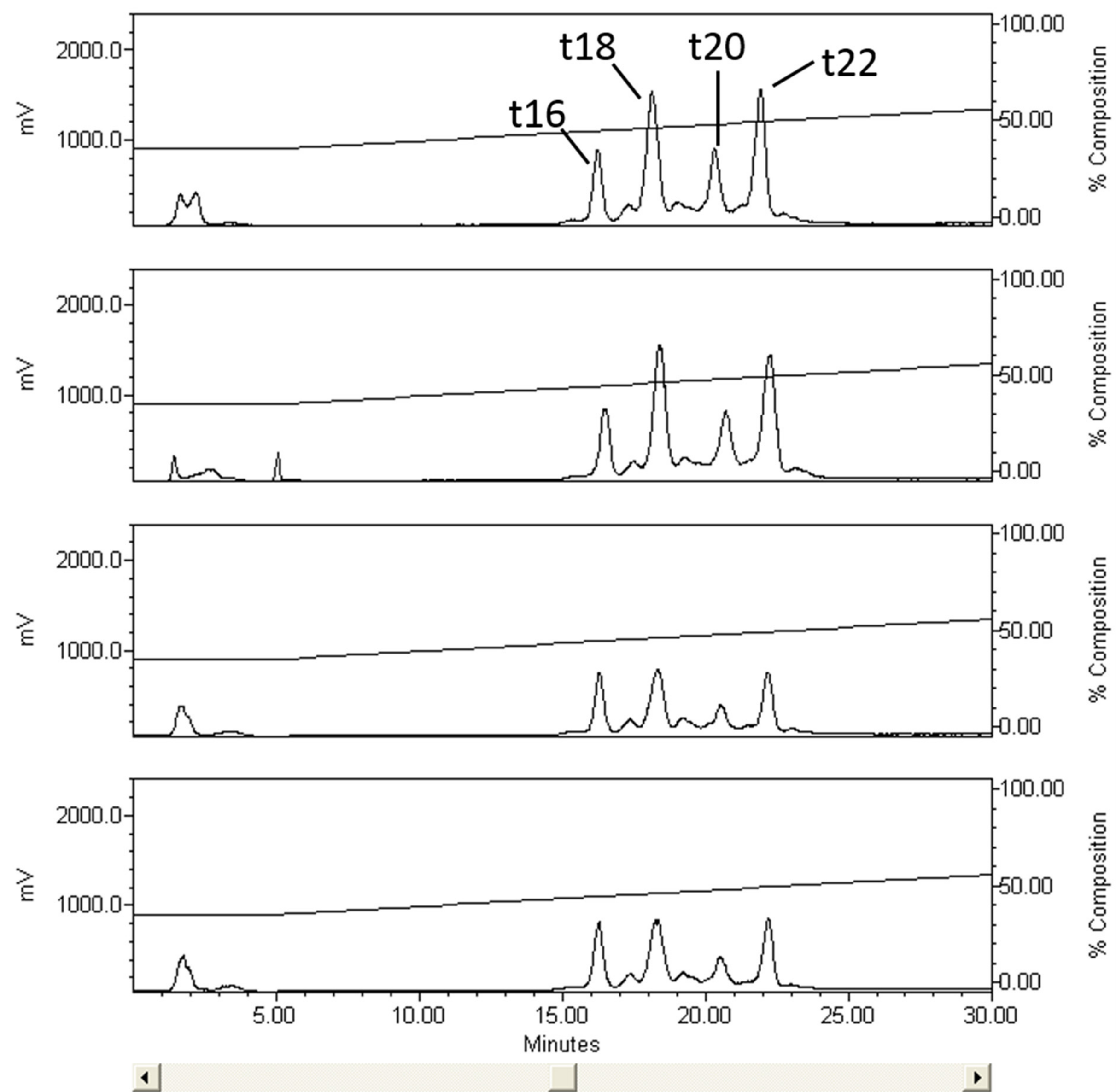
Retention Time (min)[a]	Fraction	mg	Notes
1.3–4.0	2	-	
4.0–13.5	B	-	baseline between early eluting impurity and major peak set
13.5–14.5	14	0.9	
14.5–15.5	15	0.4	minor – almost baseline relative percent
15.5–16.5	16	0.5	
16.5–17.5	17 *	1.9	same compound as major component of HF2-2t20
17.5–18.5	18 *	1.8	first major peak of set found in HF2-2t22 sample
18.5–20.0	19 *	1.9	second major peak of set found in HF2-2t22 sample
20.0–22	21	0.4	minor baseline peak set

[a] Times points for fraction numbers were based on HF2-2P1t22 R2 as this sample contained a portion of the HF2-2Pt120 sample.

Table 26. Combined semi-preparative HPLC fractions resulting from HF2-2P1t20 and IF1-3P1t20 separations, designated HF2-2P1t20A.
Fraction t17 was the primary source of compound **1**; other fractions with similar retention times were combined with this sample and the resulting material was re-purified to yield the sample of **1** that was submitted to the CCRC for analysis and analyzed by NMR spectroscopy at 700 MHz.

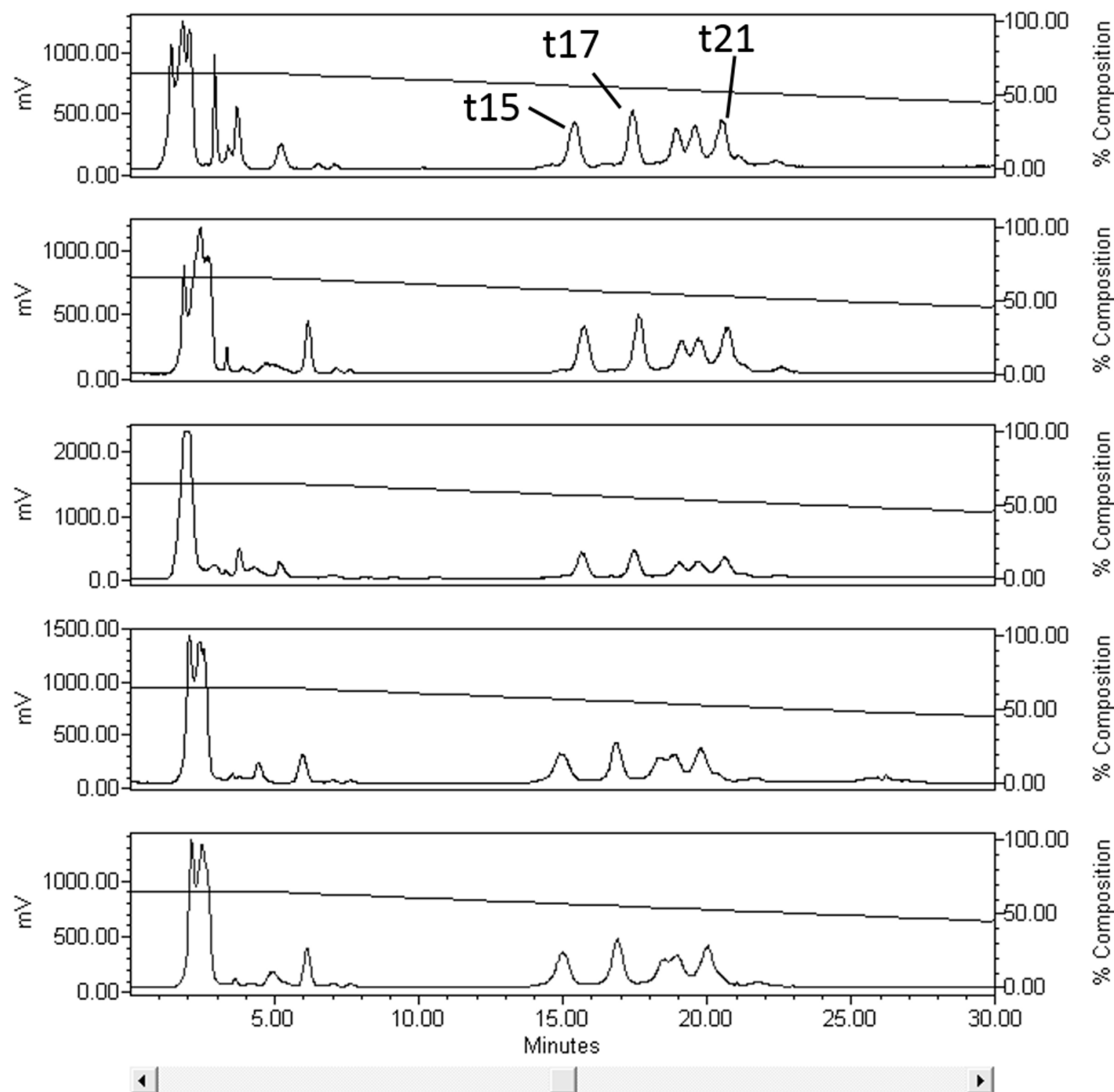
Retention Time (min)[a]	Fraction	mg	Notes
1.3–4.0	02	3.2	
4.0–12.5	B	1.1	baseline between early eluting impurity and major peak set
12.5–13.5	13	1.0	
13.5–14.5	14	1.6	
14.5–15.8	B	-	baseline peaks just before major component – perhaps one primary component
15.8–17.8	17 *	8.8	major peak/major component of sample mass
17.8–19	18	0.8	baseline minor peak (mixture)
19–21	20	0.9	baseline minor peak set (mixture)

[a] Time points for fraction numbers were based on HF2-2P1t20 R2 & R3.



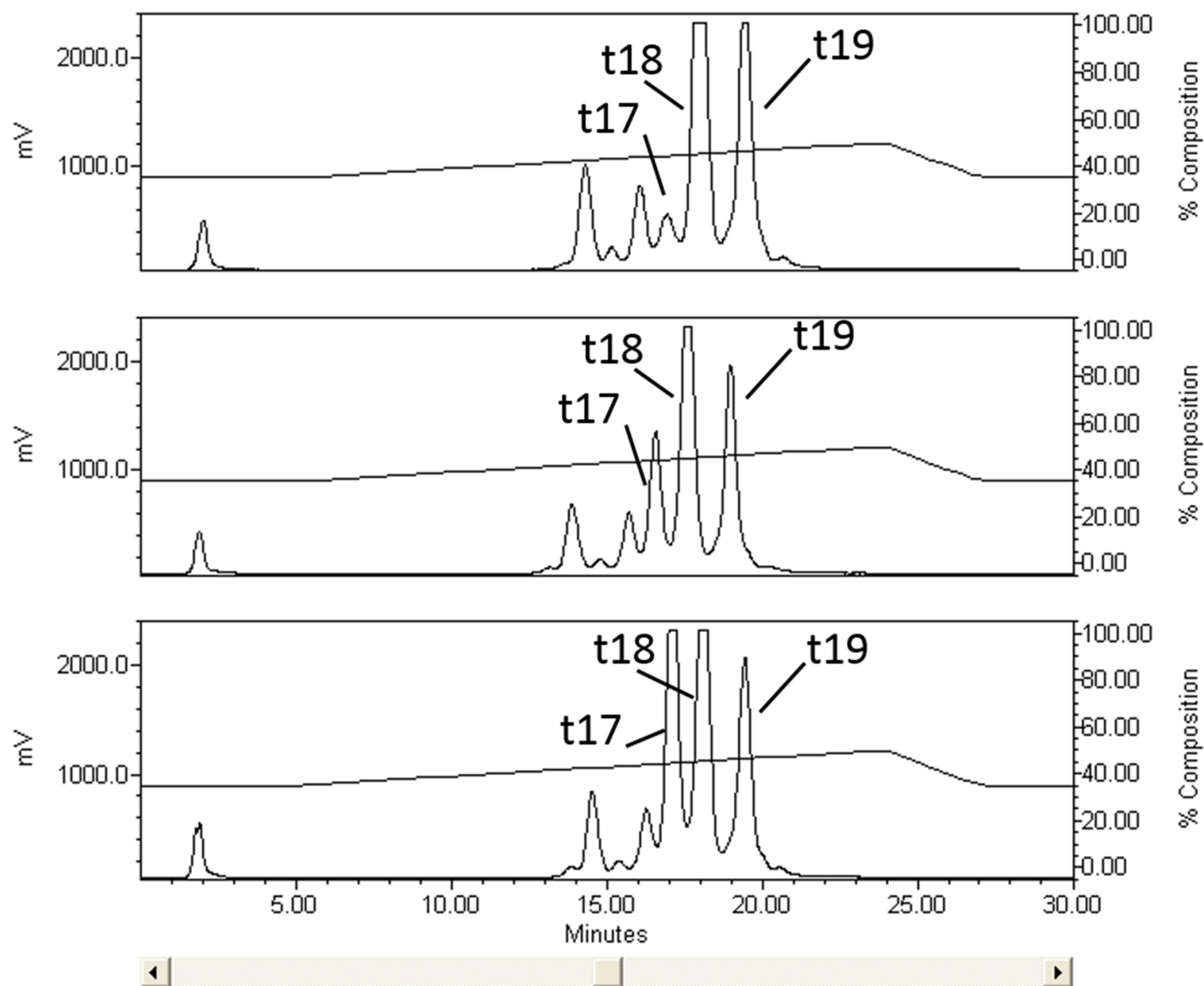
Visible	SampleName	Injection Volume (uL)	Det. Units	Date Acquired	Acq Method Set
☑	HF2-2P1t24 YMC-PolyA-SP R2	40.00	mV	10/4/2009 6:27:46 PM CDT	YMC PolyAmine SP vB
☑	HF2-2P1t24 YMC-PolyA-SP R3	45.00	mV	10/4/2009 7:36:07 PM CDT	YMC PolyAmine SP vB
☑	HF2-2P1t24 YMC-PolyA-SP R4	35.00	mV	10/4/2009 8:35:26 PM CDT	YMC PolyAmine SP vB
☑	HF2-2P1t24 YMC-PolyA-SP R5	35.00	mV	10/4/2009 11:15:08 PM CDT	YMC PolyAmine SP vB

Figure 31. Semi-preparative HPLC-ELSD of HF2-2P1t24 R2–5 (Polyamine).
Fractions eluting at t16, t18, t20 and t22 were assessed by ^{1}H NMR spectroscopy and were found to contain closely-related oligosaccharides.



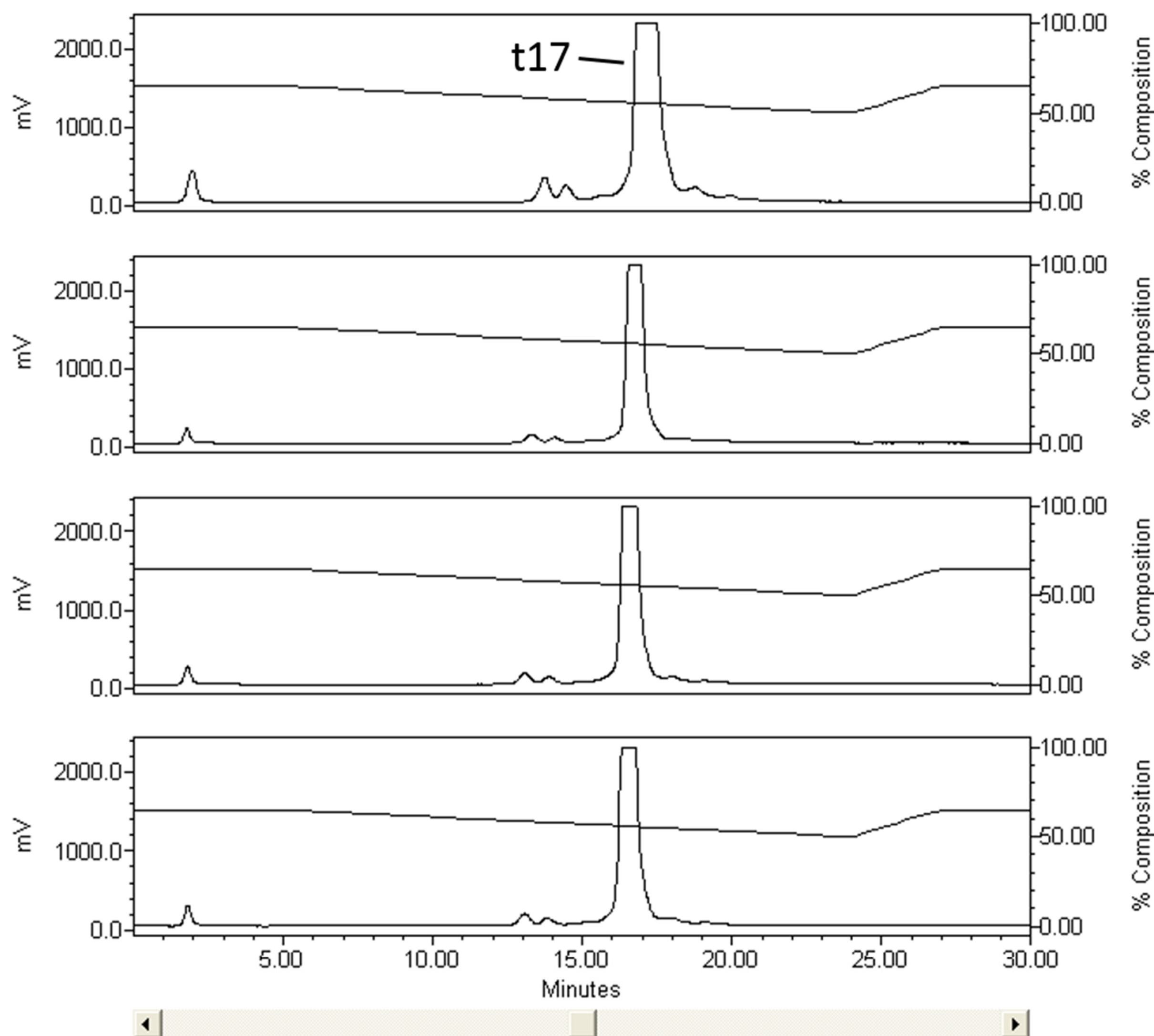
Visible	SampleName	Date Acquired	Injection Volume (uL)	Run Time (Minutes)	Instrument Method Name	Acq Me
☑	HF2-2P1t21 YMC-PolyA-SP R1	10/5/2009 12:08:54 AM CDT	35.00	100.00	YMC PolyAmine SP vB	YMC PolyA
☑	HF2-2 P1t21 YMC-PolyA-SP R2	10/5/2009 2:23:08 PM CDT	45.00	100.00	YMC PolyAmine SP vB	YMC PolyA
☑	HF2-2 P1t21 YMC-PolyA-SP R3	10/5/2009 4:36:34 PM CDT	45.00	80.00	YMC PolyAmine SP vB	YMC PolyA
☑	HF2-2 P1t21 YMC-PolyA-SP R4	10/11/2009 1:40:29 PM CDT	60.00	90.00	YMC PolyAmine SP vB	YMC PolyA
☑	HF2-2 P1t21 YMC-PolyA-SP R5	10/11/2009 2:51:11 PM CDT	60.00	90.00	YMC PolyAmine SP vB	YMC PolyA

Figure 32. Semi-preparative HPLC-ELSD of HF2-2P1t21 R1–5 (Polyamine).
Fractions eluting at t15, t17, and t21 were assessed by ^{1}H NMR spectroscopy and were found to contain closely-related oligosaccharides. (% MeCN shown)



/isible	SampleName	Injection Volume (uL)	Det. Units	Date Acquired	Acq Method Set
☑	HF2-2 P1t22 YMC-PolyA-SP R1	40.00	mV	10/13/2009 10:07:35 PM CDT	YMC PolyAmine SP vB
☑	HF2-2 P1t22 YMC-PolyA-SP R2	30.00	mV	10/14/2009 2:02:20 AM CDT	YMC PolyAmine SP vB
☑	HF2-2 P1t22 YMC-PolyA-SP R3	40.00	mV	10/14/2009 2:51:17 AM CDT	YMC PolyAmine SP vB

Figure 33. Semi-preparative HPLC-ELSD of HF2-2P1t22 R1–3 (Polyamine).
Fractions eluting at t17, t18, and t19 were assessed by ^{1}H NMR spectroscopy and these data are discussed further below. The t17 peak for the third separation (R3) is of increased intensity presumably due to co-injection of a small amount of HF2-2P1t20. If the same compound is indeed present in the HF2-2P1t22 sample, this data indicates the low resolution obtained with the AtldC18 separations. The increased intensity of t17 may also indicate, however, similar retention times for two different components as resulted with many of these fractions (see Section C5d). Although fraction t17 from this separation appears to be the same compound as HF2-2P1t20A17 these materials were kept separate.



Visible	SampleName	Date Acquired	Injection	Injection Volume (uL)	Run Time (Minutes)	Instrument Method Name
☑	HF2-2 P1t20 YMC-PolyA-SP R1	10/13/2009 10:54:15 PM CDT	2	50.00	80.00	YMC PolyAmine SP vB
☑	HF2-2 P1t20 YMC-PolyA-SP R2	10/13/2009 11:38:42 PM CDT	3	25.00	60.00	YMC PolyAmine SP vB
☑	HF2-2 P1t20 YMC-PolyA-SP R3	10/14/2009 12:25:13 AM CDT	4	30.00	60.00	YMC PolyAmine SP vB
☑	HF2-2 P1t20 YMC-PolyA-SP R4	10/14/2009 1:14:02 AM CDT	5	30.00	60.00	YMC PolyAmine SP vB

Figure 34. Semi-preparative HPLC-ELSD of HF2-2P1t20 R1–4 (Polyamine).
Fraction t17 was the primary source of compound **1**. The fraction of IF1-3Pt20 with similar retention time was combined with this sample. The resulting material was re-purified to yield the sample of **1** that was submitted to the CCRC for analysis and analyzed by NMR spectroscopy at 700 MHz. (% MeCN shown)

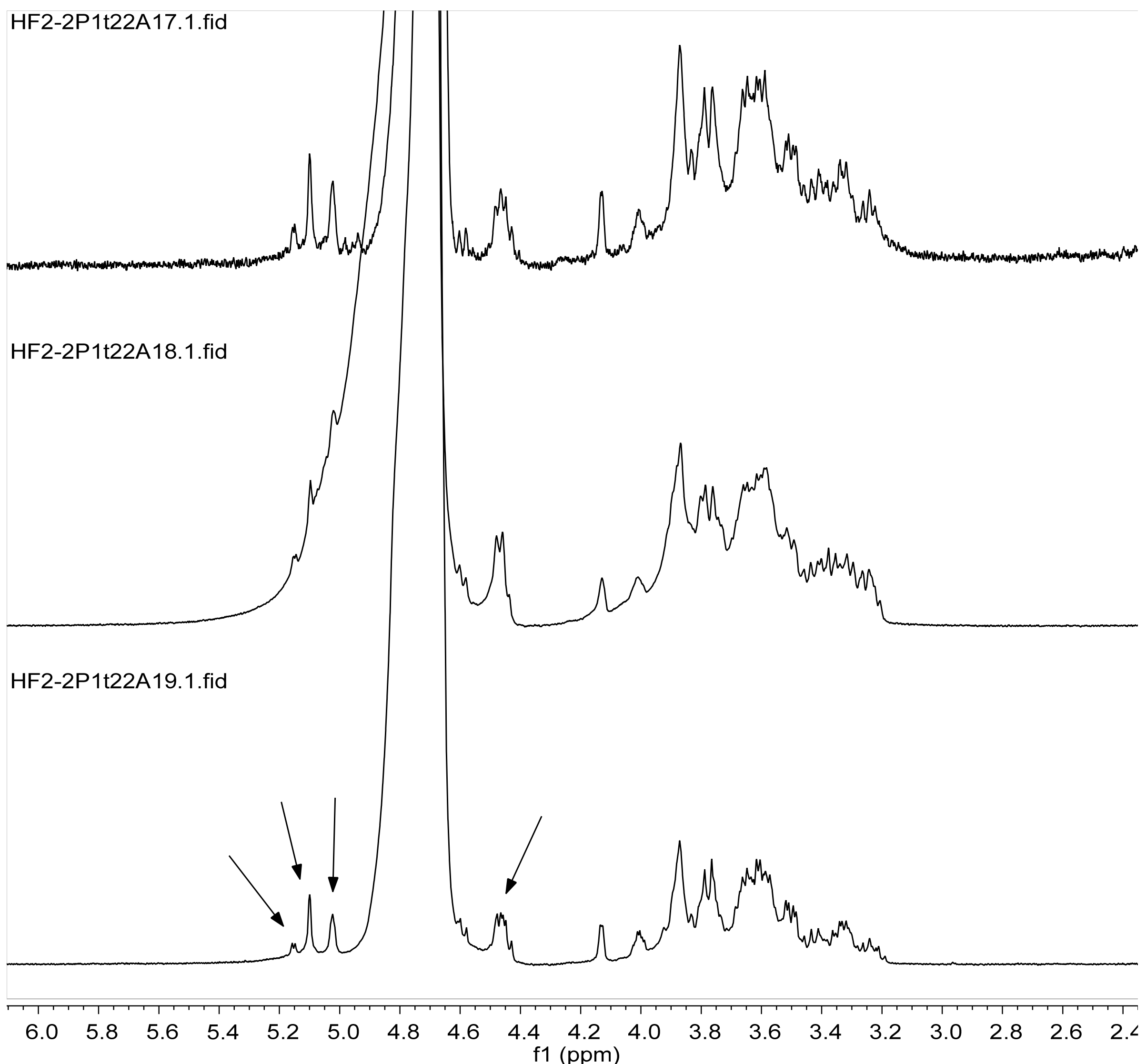
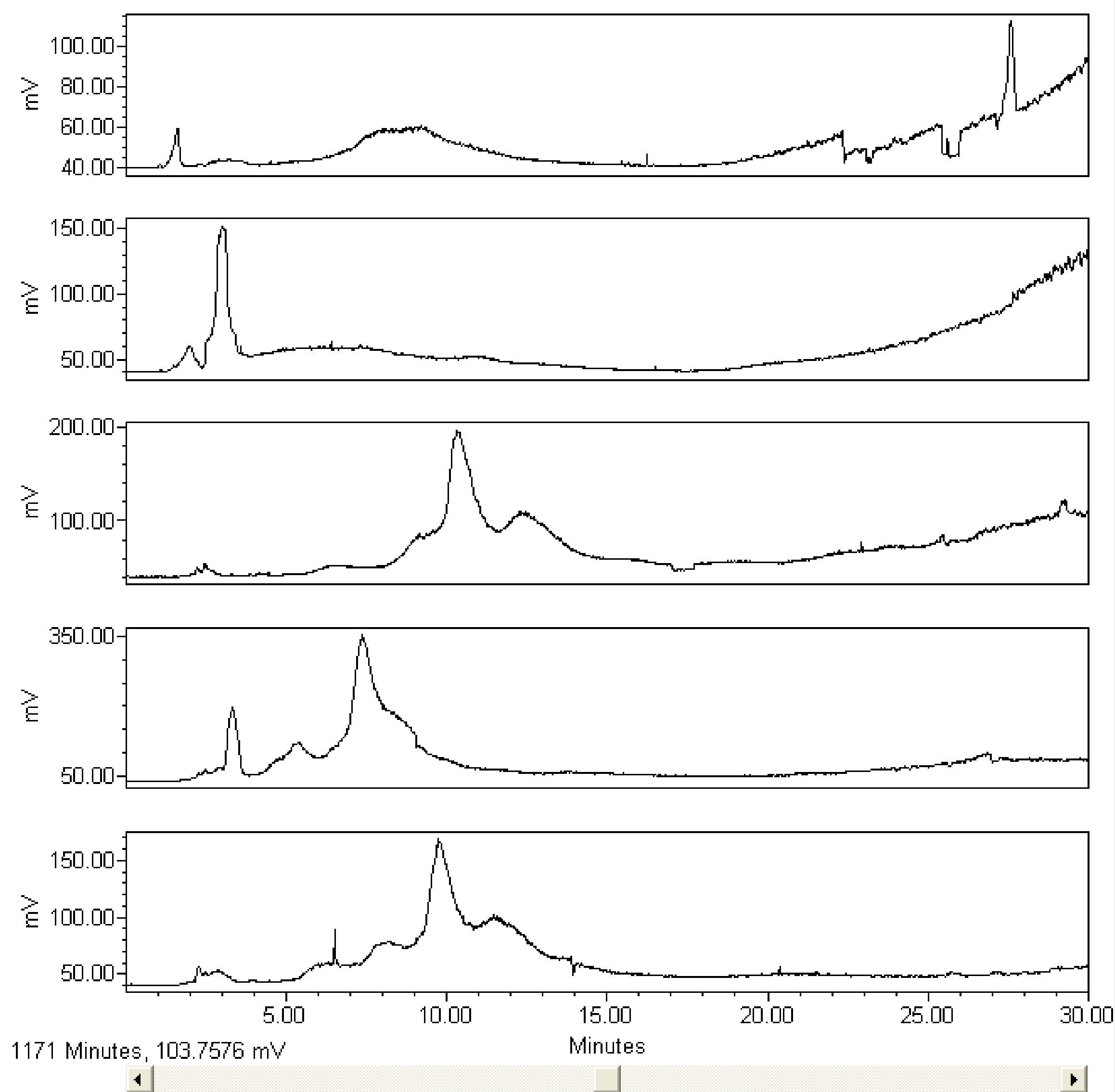


Figure 35. Comparison of the ^{1}H NMR spectra for HF2-2P1t22A fractions eluting at 17, 18, and 19 min, D_2O, 400 MHz.
No resonances were present in the spectra regions not displayed. Characteristic resonances were present at 4.5, 5.0, 5.1 and 5.15 ppm (arrows) in all three spectra shown. These resonances could not be interpreted at the time these spectra were collected but were later determined to potentially correspond to the anomeric protons for arabinoxyloglucan type oligosaccharides. All three spectra are similar to that of **1**. HF2-2P1t22A17 may be the same compound as **1** based on the results of co-injection during preparative separations.

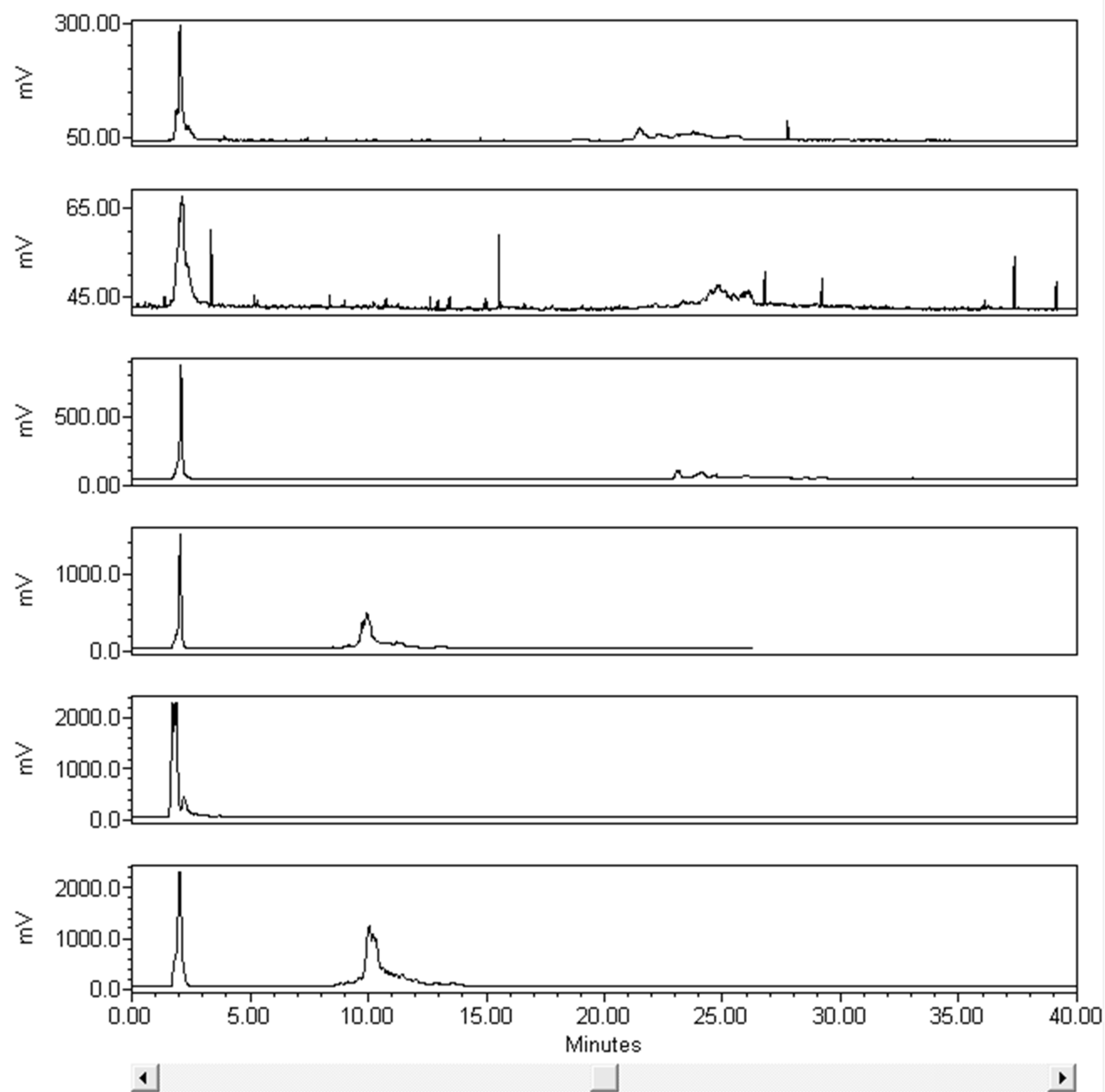
d. *Challenges of HPLC-ELSD-Based Oligosaccharide Isolation*

Carbohydrates such as oligosaccharides are often purified using amino or polyamine stationary phases. Multiple attempts were therefore made to directly separate the components of HF1-1 using amino, polyamine, and other normal phase sorbents with various solvent systems and conditions. These efforts did not yield sufficient resolution for the compounds of interest (Figure 36). Attempts at reverse phase separations with more nonpolar sorbents, such as the XTMSC18 column, and with sorbent appropriate starting solvents of 10% or more MeOH or MeCN, were also unsuccessful for retaining and resolving the oligosaccharide components (Figure 37). The method that yielded the best results employed a reverse phase AtldC18 column but still did not yield baseline resolution of the components of interest. The AtldC18 sorbent has polar groups embedded into the silica-bound C_{18} stationary phase, allowing complete wetting with water without the need for an organic modifier such as MeOH or MeCN. The ability to equilibrate and use the column in 100% water appears to have contributed to the resolution that was achieved with this sorbent.



Visible	SampleName	Injection Volume (uL)	Date Acquired	Acq Method Set	Instrument Method Name	System Name
☑	HF 1-1 YMC-NH2 R1	50.00	3/6/2009 2:57:35 PM CST	YMC NH2 250	YMC NH2 250	Bru_UV_ELS
☑	HF 1-1 YMC-NH2 R2	150.00	3/6/2009 4:18:03 PM CST	YMC NH2 250	YMC NH2 250	Bru_UV_ELS
☑	HF 1-1 YMC-NH2 R3	100.00	3/6/2009 5:28:36 PM CST	YMC NH2 250	YMC NH2 250	Bru_UV_ELS
☑	HF1-1 YMC-NH2 R4	50.00	3/10/2009 2:18:51 AM CDT	YMC NH2 250	YMC NH2 250	Bru_UV_ELS
☑	HF1-1 YMC-NH2 R5	25.00	3/10/2009 3:35:50 AM CDT	YMC NH2 250	YMC NH2 250	Bru_UV_ELS

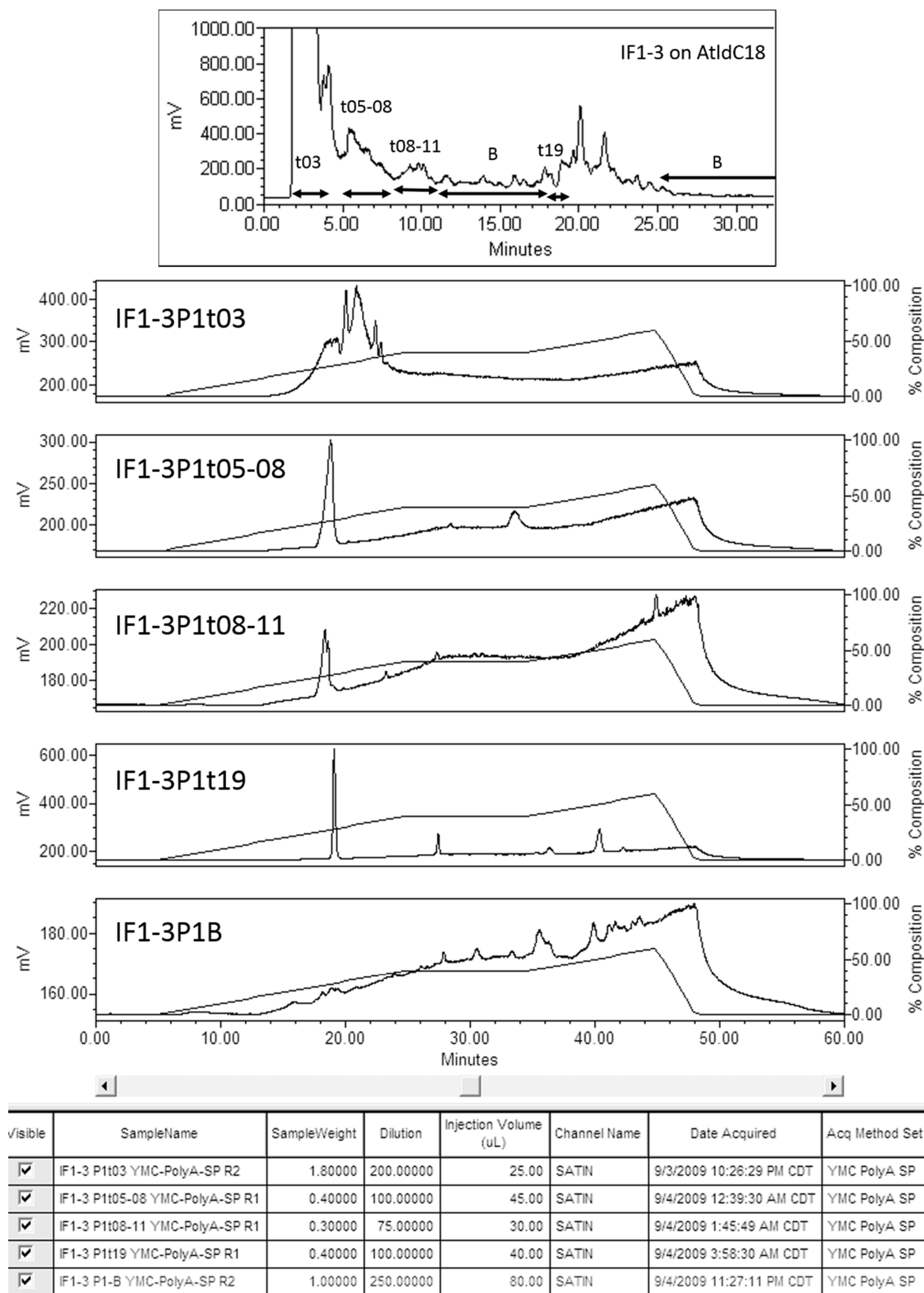
Figure 36. Examples of the analytical HPLC-ELSD chromatograms produced during HF1-1 method development with an amino HPLC sorbent.
These HPLC chromatograms correspond to variations in solvent systems (changes in solvent %, use of MeOH or MeCN) using a YMC NH_2 column (4.6 x 150 mm, 5 µm) and a flow rate of 1 mL/min.



Visible	SampleName	Injection Volume (uL)	Date Acquired	Acq Method Set	Instrument Method Name	System Name
☑	HF 1-1 XTMSC18 R1	10.00	3/2/2009 7:25:19 PM CST	XTerra MS C18	XTerra MS C18	Bru_UV_ELS
☑	HF 1-1 XTMSC18 R2	10.00	3/2/2009 8:41:50 PM CST	XTerra MS C18	XTerra MS C18	Bru_UV_ELS
☑	HF 1-1 XTMSC18 R3	20.00	3/2/2009 10:00:08 PM CST	XTerra MS C18	XTerra MS C18	Bru_UV_ELS
☑	HF 1-1 XTMSC18 R4	20.00	3/2/2009 11:21:58 PM CST	XTerra MS C18	XTerra MS C18	Bru_UV_ELS
☑	HF 1-1 XTMSC18 R5	15.00	3/3/2009 12:11:16 AM CST	XTerra MS C18	XTerra MS C18	Bru_UV_ELS
☑	HF 1-1 XTMSC18 R6	25.00	3/3/2009 1:34:49 AM CST	XTerra MS C18	XTerra MS C18	Bru_UV_ELS

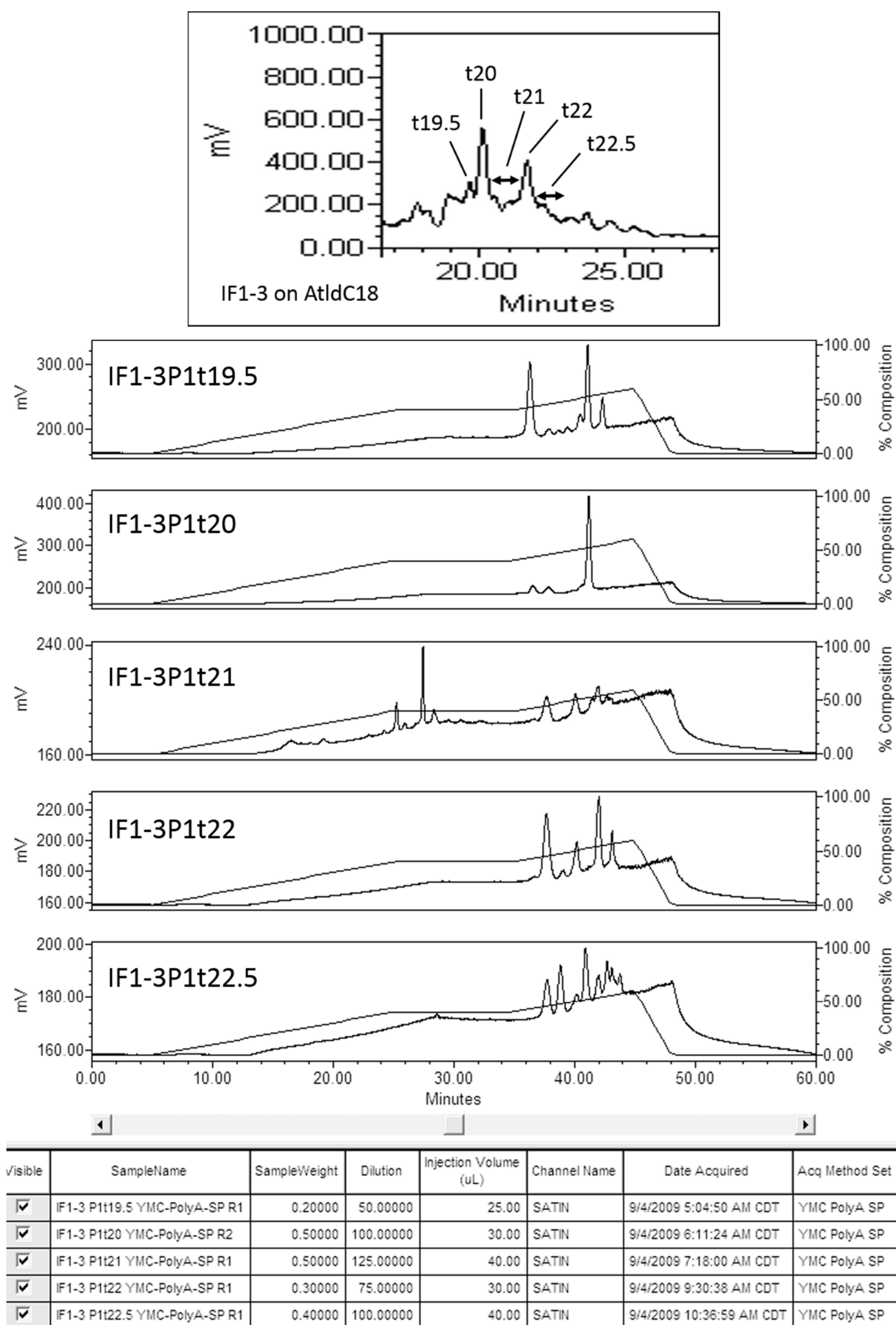
Figure 37. Examples of the analytical HPLC-ELSD chromatograms produced during HF1-1 method development attempts using a C_{18} HPLC sorbent.
These HPLC chromatograms correspond to variations in solvent systems (changes in solvent %, use of MeOH or MeCN) using an XTMSC18 column (4.6 x 150 mm, 5 µm) and a flow rate of 1 mL/min.

The enriched fractions obtained from the AtldC18 separations could be further resolved by amino, polyamine, or other normal phase sorbents. Multiple sorbents and solvent systems were tested and the polyamine sorbent yielded the best resolution with MeCN as the solvent. The lack of resolution previously observed for attempts at direct, normal phase separation of oligosaccharide components could be explained by observations made with AtldC18 fractions. Fractions collected from a single AtldC18 separation (*e.g.* IF1-3; Figure 24) were separated sequentially using the polyamine sorbent with an optimized analytical instrument and controlled, repeated conditions. Comparisons of the resulting analytical HPLC-ELSD spectra showed that fractions eluting with different AtldC18 retention times, and therefore different major components, contained compounds that had similar retention times on the polyamine sorbent (Figures 38–40). For example, during semi-preparative separation, the IF1-3P1t20 subfraction collected from the AtldC18 column contains a major compound that appears at 16–17 min on the Polyamine column (Figure 41). This component overlaps in retention time with compounds in the t22 and t23 subfractions even though they are derived from different parent AtldC18 fractions and are unlikely to contain the same components. Peaks that appear to contain single or major components in the AtldC18 chromatogram (*e.g*. the peak at 22 min; Figure 24) were also found to contain multiple components when examined on the Polyamine stationary phase (Figure 39). In non-enriched parent fractions, all such compounds would have overlapped and led to the observed poor resolution. These phenomena also made the recombination of fractions from multiple separations especially challenging, as there was little guarantee, based solely on retention time data, that any two peaks from related fractions represented the same compounds.



Visible	SampleName	SampleWeight	Dilution	Injection Volume (uL)	Channel Name	Date Acquired	Acq Method Set
☑	IF1-3 P1t03 YMC-PolyA-SP R2	1.80000	200.00000	25.00	SATIN	9/3/2009 10:26:29 PM CDT	YMC PolyA SP
☑	IF1-3 P1t05-08 YMC-PolyA-SP R1	0.40000	100.00000	45.00	SATIN	9/4/2009 12:39:30 AM CDT	YMC PolyA SP
☑	IF1-3 P1t08-11 YMC-PolyA-SP R1	0.30000	75.00000	30.00	SATIN	9/4/2009 1:45:49 AM CDT	YMC PolyA SP
☑	IF1-3 P1t19 YMC-PolyA-SP R1	0.40000	100.00000	40.00	SATIN	9/4/2009 3:58:30 AM CDT	YMC PolyA SP
☑	IF1-3 P1-B YMC-PolyA-SP R2	1.00000	250.00000	80.00	SATIN	9/4/2009 11:27:11 PM CDT	YMC PolyA SP

Figure 38. Analytical Polyamine HPLC-ELSD comparisons of IF1-3 AtldC18 fractions t03, t05-08, 08-11, 19, and B, showing the changes in elution profile associated with the change in sorbent.
The fractions shown here eluted at distinct times on the AtldC18 sorbent, but the major components of each all had similar retention times on the Polyamine sorbent (~18 min). The lack of distinct components in the IF1-3P1B fraction indicates that the major components of the IF1-3 parent material have been collected as parts of other fractions.



Visible	SampleName	SampleWeight	Dilution	Injection Volume (uL)	Channel Name	Date Acquired	Acq Method Set
☑	IF1-3 P1t19.5 YMC-PolyA-SP R1	0.20000	50.00000	25.00	SATIN	9/4/2009 5:04:50 AM CDT	YMC PolyA SP
☑	IF1-3 P1t20 YMC-PolyA-SP R2	0.50000	100.00000	30.00	SATIN	9/4/2009 6:11:24 AM CDT	YMC PolyA SP
☑	IF1-3 P1t21 YMC-PolyA-SP R1	0.50000	125.00000	40.00	SATIN	9/4/2009 7:18:00 AM CDT	YMC PolyA SP
☑	IF1-3 P1t22 YMC-PolyA-SP R1	0.30000	75.00000	30.00	SATIN	9/4/2009 9:30:38 AM CDT	YMC PolyA SP
☑	IF1-3 P1t22.5 YMC-PolyA-SP R1	0.40000	100.00000	40.00	SATIN	9/4/2009 10:36:59 AM CDT	YMC PolyA SP

Figure 39. Analytical Polyamine HPLC-ELSD comparisons of IF1-3 AtldC18 fractions t19.5, t20, t21, t22, and t22.5, showing the changes in elution profile associated with the change in sorbent.
The fractions shown here eluted at distinct times on the AtldC18 sorbent, but the major components of each all had similar retention times on the Polyamine sorbent.

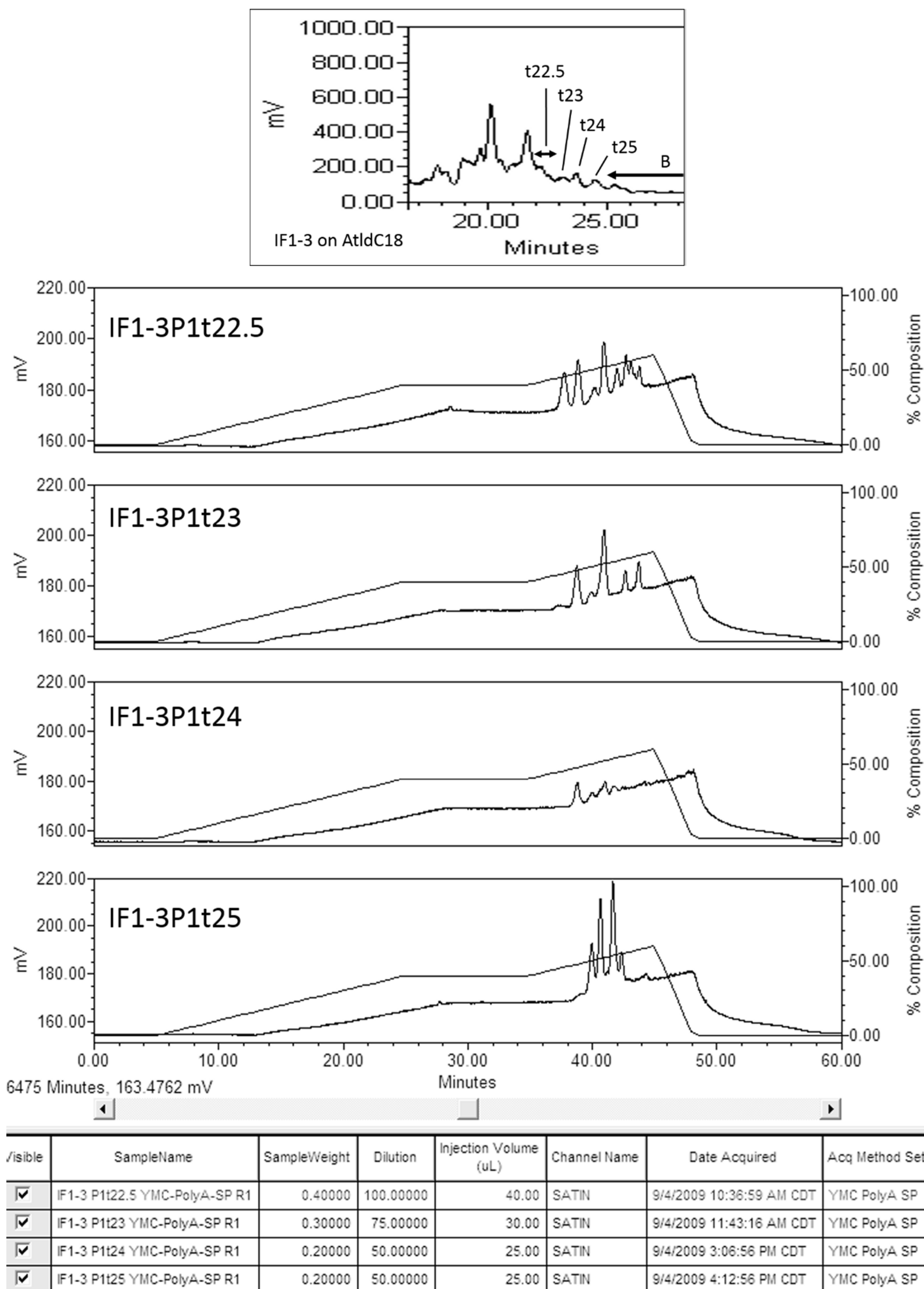
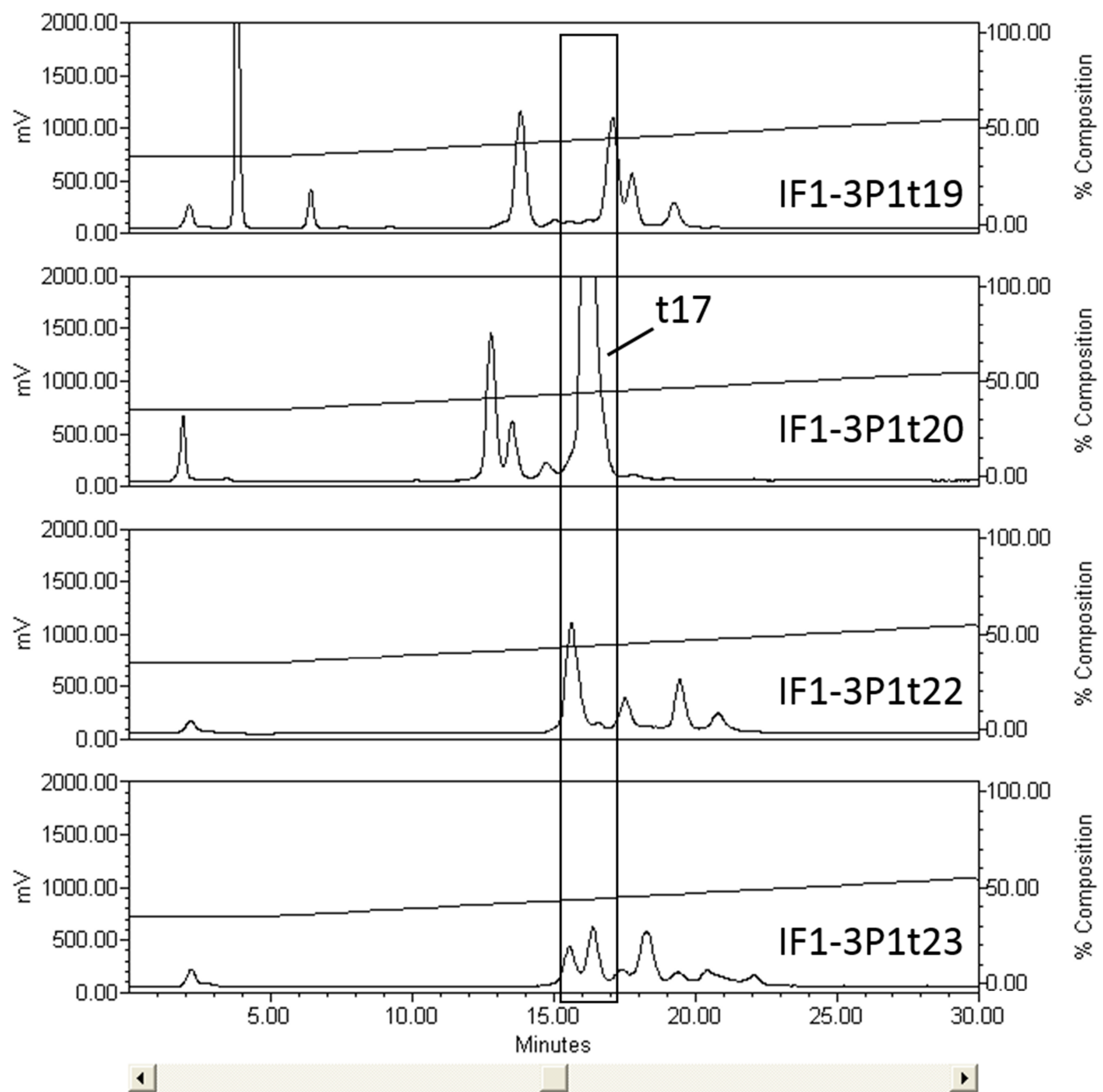


Figure 40. Analytical Polyamine HPLC-ELSD comparisons of IF1-3 AtldC18 fractions t122.5, t23, t24, and t25, showing the changes in elution profile associated with the change in sorbent. The fractions shown here eluted at distinct times on the AtldC18 sorbent, but the major components of each all had similar retention times on the Polyamine sorbent.



Visible	SampleName	Injection Volume (uL)	Det. Units	Date Acquired	Acq Method Set
☑	IF1-3 P1t19 YMC-PolyA-SP R1	40.00	mV	10/11/2009 4:43:24 PM CDT	YMC PolyAmine SP vB
☑	IF1-3 P1t20 YMC-PolyA-SP R1	30.00	mV	10/12/2009 11:03:51 PM CDT	YMC PolyAmine SP vB
☑	IF1-3 P1t22 YMC-PolyA-SP R1	50.00	mV	10/13/2009 12:38:13 PM CDT	YMC PolyAmine SP vB
☑	IF1-3 P1t23 YMC-PolyA-SP R1	60.00	mV	10/13/2009 1:34:33 PM CDT	YMC PolyAmine SP vB

Figure 41. Semi-preparative HPLC-ELSD separations for IF1-3P1 subfractions (Polyamine). The IF1-3P1t20 subfraction was similar in ELSD profile to the HF2-2P1t20 subfraction and both were collected at 20 min from the AtldC18 column (Figure 24). This fraction contains a major compound that appears at 16-17 min (indicated as t17) on the Polyamine column. This component overlaps in retention time with compounds in the t22 and t23 subfractions (box) even though these fractions are derived from different parent AtldC18 fractions and are unlikely to contain the same components.

A factor that further detracted from efforts to isolate sufficient quantities of pure oligosaccharides was the requirement for destructive, ELSD-based detection and collection. The preparative HPLC configuration involved the placement of a flow splitter after the UV detector but before the ELSD. This splitter directed the majority of the flow to collection vessels, with a smaller portion directed to the detector. Typically, 2–4% of the eluting solvent entered the detector for the flow rates of 20–25 mL/min used with the preparative AtldC18 column, but this percentage may have been as high as 10% for the slower flow rates (5–10 mL/min) used with the semi-preparative Polyamine column. At the time, a larger, preparative-scale, polyamine column was not available. This arrangement meant that as much as 10% of a given sample would be lost to the detector with every injection and separation. All attempts were made with available resources to decrease this percentage of sample loss but such efforts were limited by the sensitivity and configuration of the detector and by the flow dynamics of the system.

The issue of poor baseline resolution combined with the need for split detection and collection further hindered isolation efforts. Steps were taken to design the system in such a way as to ensure that the sample would reach the detector and the collection vessel at the same time, but as little as a ten second delay or shift could change the composition of the collected fraction from that observed on the chromatogram. This phenomenon further contributed to the need for multi-step purification protocols involving more than one chromatographic sorbent.

Multiple, instrument-related issues could also interfere with the re-combination of fractions from different samples based on retention times. These were typically due to (a) alterations in HPLC instrument configuration due to clogged tubing or leaks, (b) changes in HPLC instrument performance due to inappropriate methods of switching between normal and

reverse solvent systems, and (c) decreased detector sensitivity or response due to the deposition of unknown, insoluble sample materials on the ELSD nebulizer, within the evaporator tube, or on the detection optics. Most of these issues were due to abuse of the instruments by other individuals.

Numerous efforts were therefore made to ensure the stability of the chromatographic system to achieve repeatable retention times and to maintain the highest possible detector sensitivity. For the majority of samples, material was divided into portions that were intensively separated over a series of back-to-back preparative separations. Instrument performance was checked with known samples prior to batches of separations and instrument back-pressure data was acquired with every separation to monitor instrument and column performance. Sets of separations were also typically performed over a period of several days when the instrument was not accessible to other users.

The amounts of material obtained for AtldC18-Polyamine purified fractions (0.5–5 mg) were typically insufficient to obtain quality ^{1}H NMR data. This was likely due partly to the high-MW of the compounds in question and partly to the high resonance overlap inherent to oligosaccharides. The oligosaccharide nature of many purified compounds could be established by ^{1}H NMR, but the data obtained could often not reliably distinguish two components that were known to have different retention times and originated from different parent fractions. These issues further contributed to the difficulty of recombining samples to obtain higher quantities and additional data. For most samples, sufficient purity could not be assured for the pursuit of chemically based structure elucidation approaches, and the limited quantities available made additional ELSD-based separations impractical due to the losses inherent to the system.

Many of these factors did provide useful information even though they made isolation and structural characterization challenging. The difficulty of obtaining baseline resolution between the components of the series indicated that the compounds present possessed similar chromatographic properties, most likely due to similar structures. Similarities in the NMR data that could be obtained indicated that many of the distinct compounds present have structures closely related to that subsequently established for **1**.

6. *Structure Elucidation*

The primary and secondary structure of **1** was established using standard carbohydrate derivatization and analysis procedures, MS^n fragmentation data, and 1D and 2D NMR experiments as discussed below.

a. *Results of Carbohydrate Derivatization Analyses*

Glycosyl composition analysis by GC-MS of the TMS methyl glycosides of **1** indicated it was composed of arabinose, xylose and glucose with a molar ratio of 1:1:2 (Table 27). Mass fragments of the TMS methyl glycosides at *m/z* 204 and 217 (not shown) indicated that **1** contained neutral sugars. MALDI-TOF-MS (Figure 42) analysis showed an $[M+Na]^+$ ion of 1218.117 for the highest intensity molecular ion, consistent with an octasaccharide composed of two arabinosyl, two xylosyl and four glucosyl residues.

GC-MS analysis of the (*S*)-(+)-butyl glycoside TMS derivatives of **1** as compared to standards (Figure 43) indicated that the absolute configurations of these residues were D-Glc, D-Xyl, and L-Ara, consistent with the natural abundances of these monosaccharides.

The linkage positions on each monomer were determined using partially methylated alditol acetate derivatives of **1** (Jahfar & Azadi 2004). These data, when evaluated in

conjunction with the molar ratio and mass results, indicated that **1** contained two terminally-linked arabinofuranosyl residues, two 2-linked xylopyranosyl residues, one terminally-linked glucopyranosyl residue, one 4-linked glucopyranosyl residue, and two 4,6-linked glucopyranosyl residues. The 2-linked xylopyranosyl residues, the 4-linked glucopyranosyl residue, and the 4,6-linked glucopyranosyl residues could also be linked through the anomeric position.

Further MS and NMR analyses were used to confirm glycosyl linkages, to confirm connectivity through anomeric positions, and to assign anomeric configurations.

Table 27. Results of the glycosyl composition analysis for **1**.
Arabinose, xylose, and glucose were determined to be the monosaccharides present in **1** in a ratio of 1:1:2.

Residue	Weight (μg)	Mole%
Arabinose (Ara)	26.5	22.0
Ribose (Rib)	0.0	0.0
Rhamnose (Rha)	0.0	0.0
Fucose (Fuc)	0.0	0.0
Xylose (Xyl)	30.4	25.1
Glucuronic Acid (GlcUA)	0.0	0.0
Galacturonic Acid (GalUA)	0.0	0.0
Mannose (Man)	0.0	0.0
Galactose (Gal)	0.0	0.0
Glucose (Glc)	76.7	52.9
N-Acetyl Galactosamine (GalNAc)	0.0	0.0
N-Acetyl Glucosamine (GlcNAc)	0.0	0.0
Heptose (Hep)	0.0	0.0
3 Deoxy-2-Manno-2-Octulsonic Acid (KDO)	0.0	0.0
Sum		100

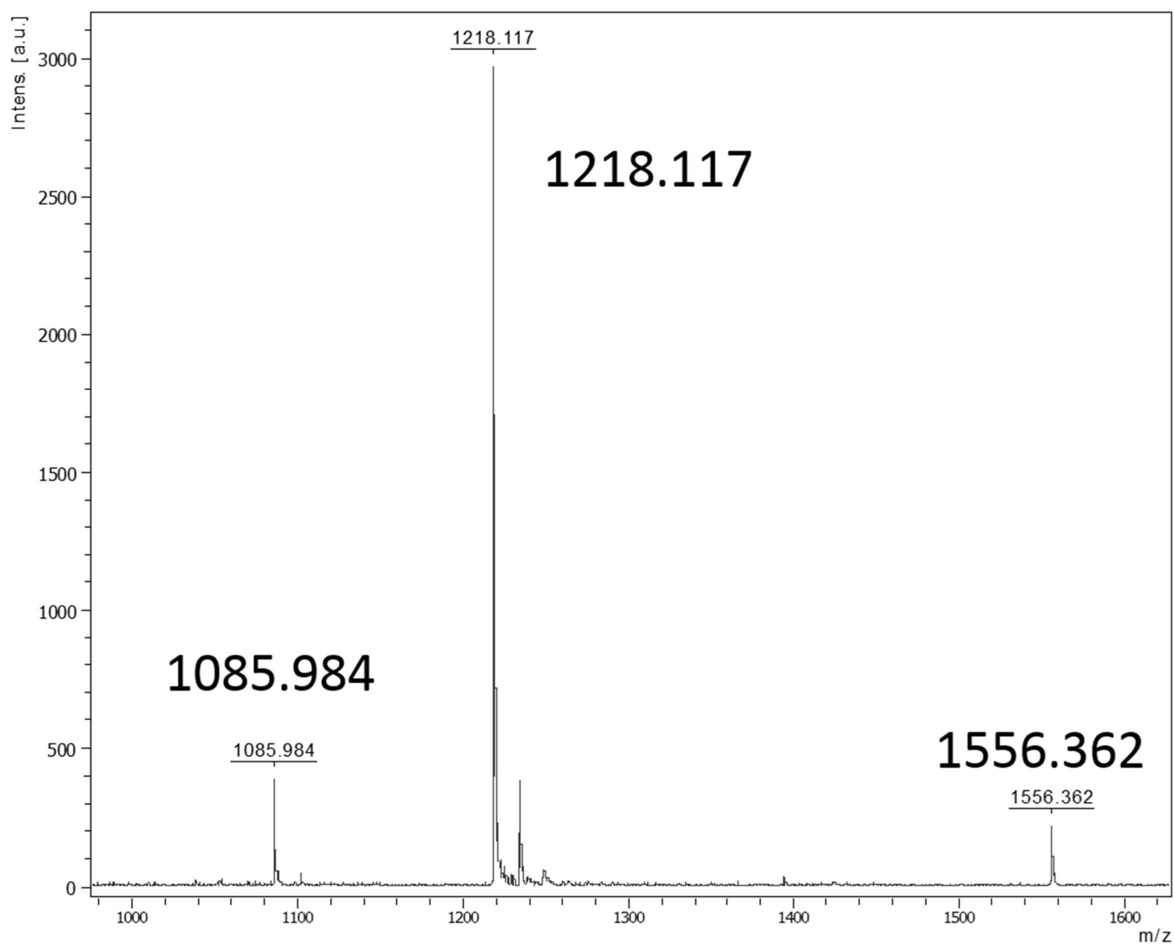


Figure 42. Results of the MALDI-MS analysis for **1** showing an *m/z* of 1218.117 corresponding to the sodium adduct of the compound in the (+) ionization mode.

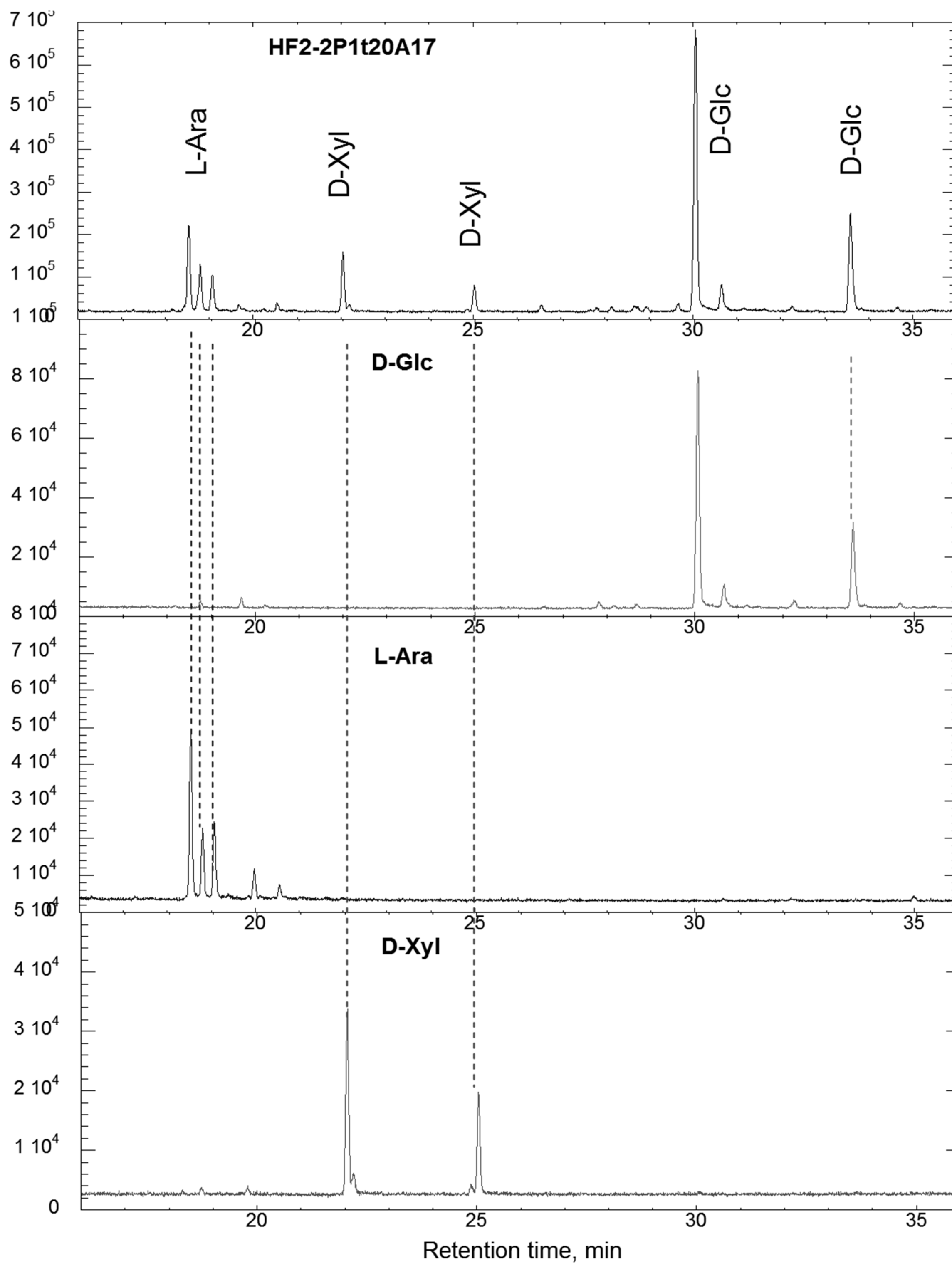


Figure 43. Results of the GC-MS analysis of the TMS derivatives of (*S*)-(+)-butyl glycosides of authentic standards D-Glc, L-Ara, D-Xyl and **1**.

b. *Partial Fragmentation Pattern Analysis for Linkage Positions*

A pure sample of **1** was derivatized via per-*O*-methylation and then subjected to NSI-MS with fragmentation by CID in MS/MS and MS^n modes using direct infusion of the sample. As the only material in the sample was the compound of interest, resulting fragment ions were presumed to have been generated from this material. The derivative produced from **1** was detected as singly and doubly (2+) charged species (Figure 44) and selected ions were subjected to additional fragmentation (Figures 44–58).

Two possible structures for **1** were consistent with the data from previous analyses (Figure 45). These could be distinguished using the results of the fragmentation pattern analysis, even though mass fragments were only generated at unit resolution due to the nature of the method used. Methylation patterns and knowledge of typical fragmentation sites (glycosidic bonds) and fragmentation sequences for oligosaccharides made it possible to distinguish between different mass fragments and associate the resulting mass losses with the two putative structures for **1** (Domon *et al*. 1990, Costello & Vath 1990, Hisamatsu *et al*. 1992, York *et al*. 1996). Terminal, internal, and reducing glucopyranosyl residues could be distinguished based on unit mass differences of 218 (terminal), 190 (internal), and 236 (reducing) amu. Arabinofuranosyl and xylopyranosyl residues could also be distinguished based on mass losses of 174 and 160 amu, respectively, or 334 amu for an Ara-Xyl side chain.

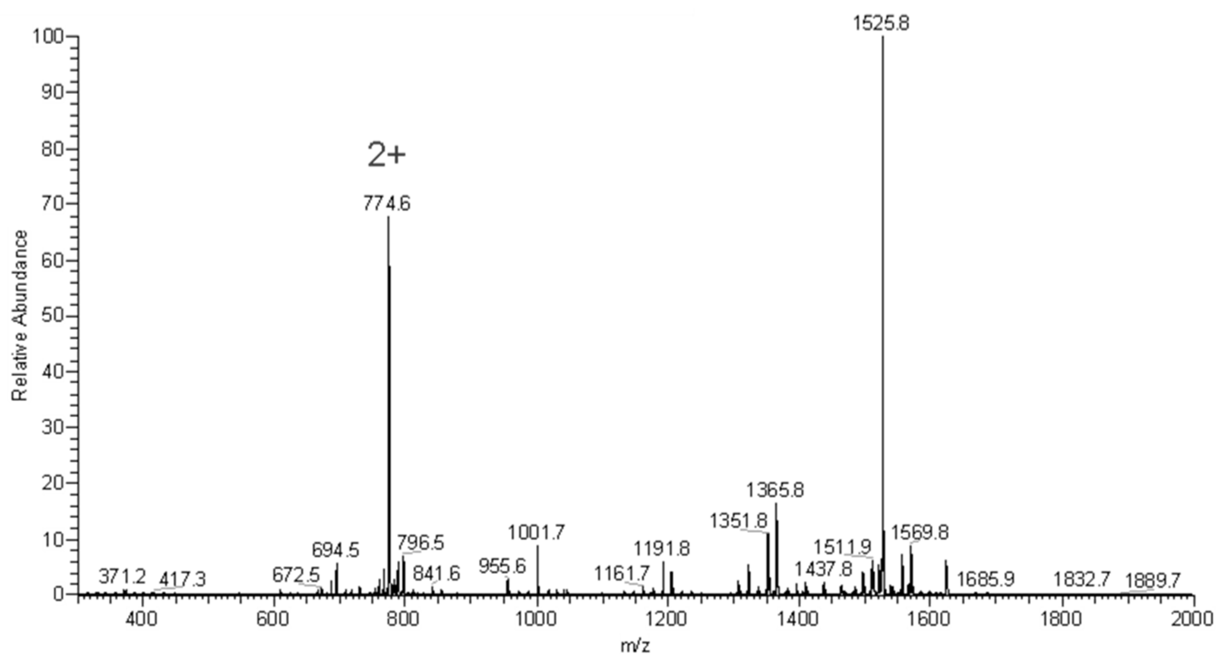
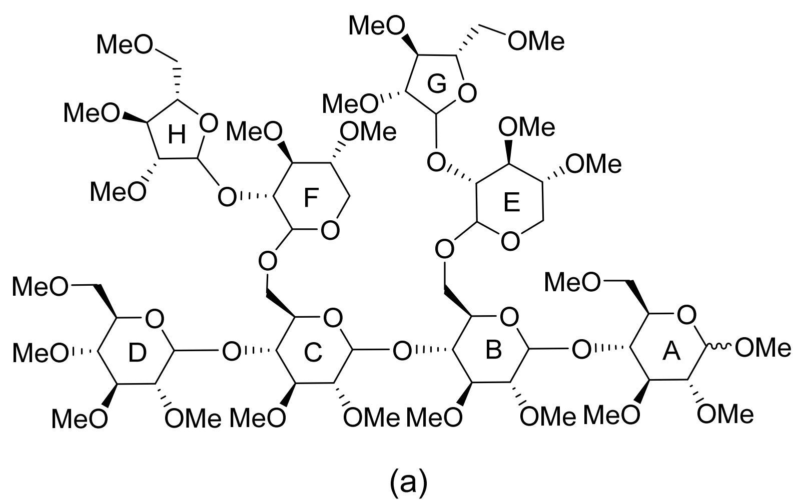
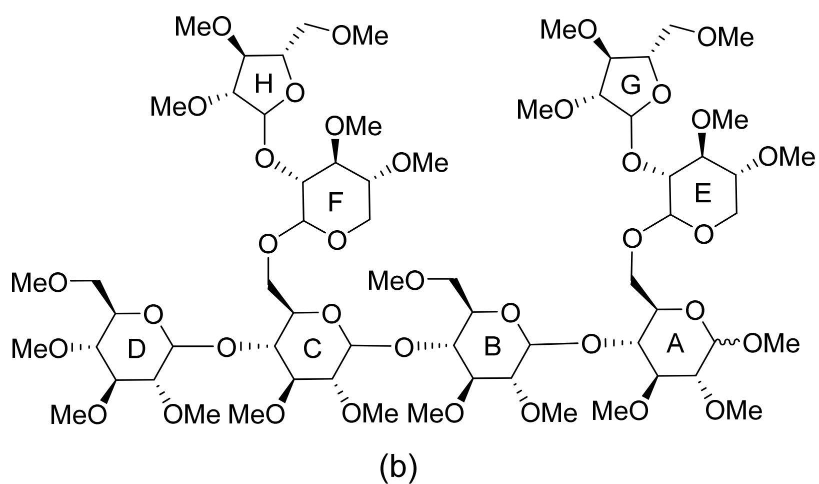


Figure 44. Results of the NSI-MS-based glycosyl linkage analysis showing mass fragments for the per-*O*-methylated derivative of **1**.
The per-*O*-methylated derivative of **1** was detected as singly and doubly (2+) charged species. The parent ion at *m/z* 1526 was selected for further fragmentation.



(a)



(b)

Figure 45. Possible structures for **1** showing alternate placements of a Xyl-Ara side chain on either Glc*p*-B (a) or Glc*p*-A (b).

The NSI-MS^2 fragmentation at *m/z* = 1526 for the per-*O*-methylated derivative of **1** (Figure 46) gave indicative mass fragments of *m/z* 1352, 1308, 1290, 1192, and 784. The mass fragment of *m/z* 1352 is consistent with the loss of a terminal arabinose (Δ*m/z* = 174), *m/z* 1308 is consistent with the loss of terminal glucose (Δ*m/z* = 218), *m/z* 1290 is consistent with the loss of reducing end glucose (Δ*m/z* = 236), *m/z* 1192 is consistent with the loss of a terminal arabinose and an internal xylose (Δ*m/z* = 334), and *m/z* 784 is consistent with the loss of a terminal arabinose, an internal xylose, an internal glucose and a terminal glucose (Δ*m/z* = 742). These mass fragments were consistent with structure (a) (Figure 47), as structure (b) (Figure 48) did not result in the production of an *m/z* 1290 fragment corresponding to the loss of a reducing end glucose (Δ*m/z* = 236).

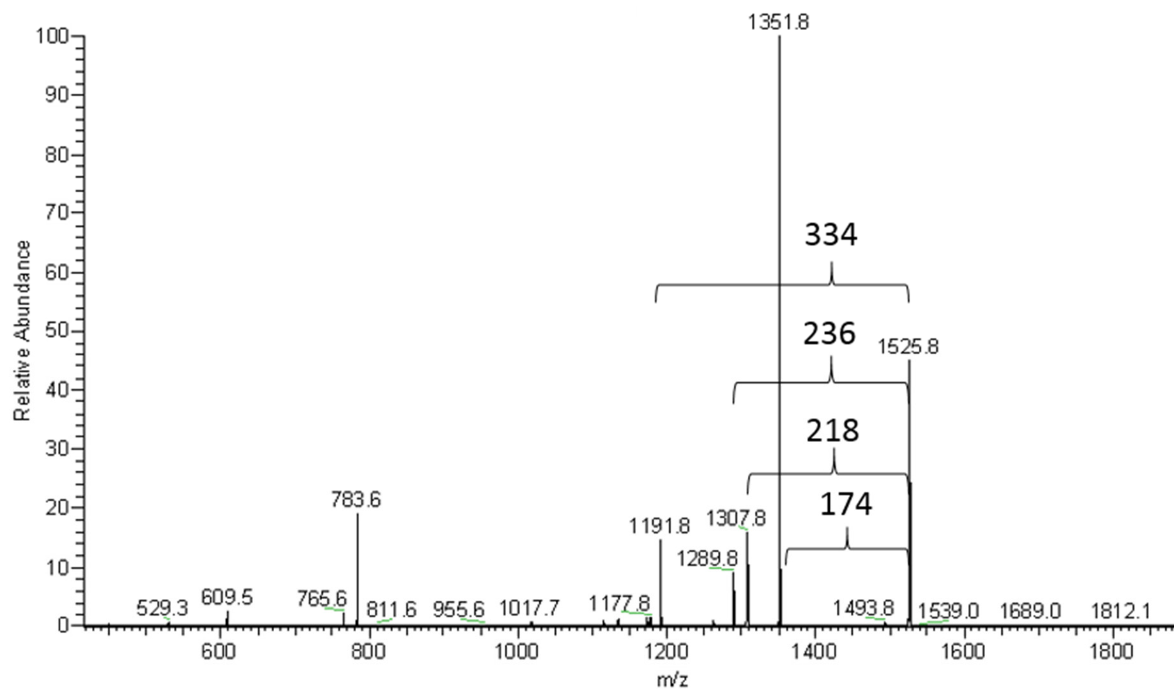


Figure 46. The NSI-MS^2 fragmentation at *m/z* = 1526 for the per-*O*-methylated derivative of **1**. Mass losses of Δ*m/z* 174, 218, 236, and 334 were indicative of key putative fragments consistent with structure (a). Ions at *m/z* 1308 and 1352 were selected for further fragmentation.

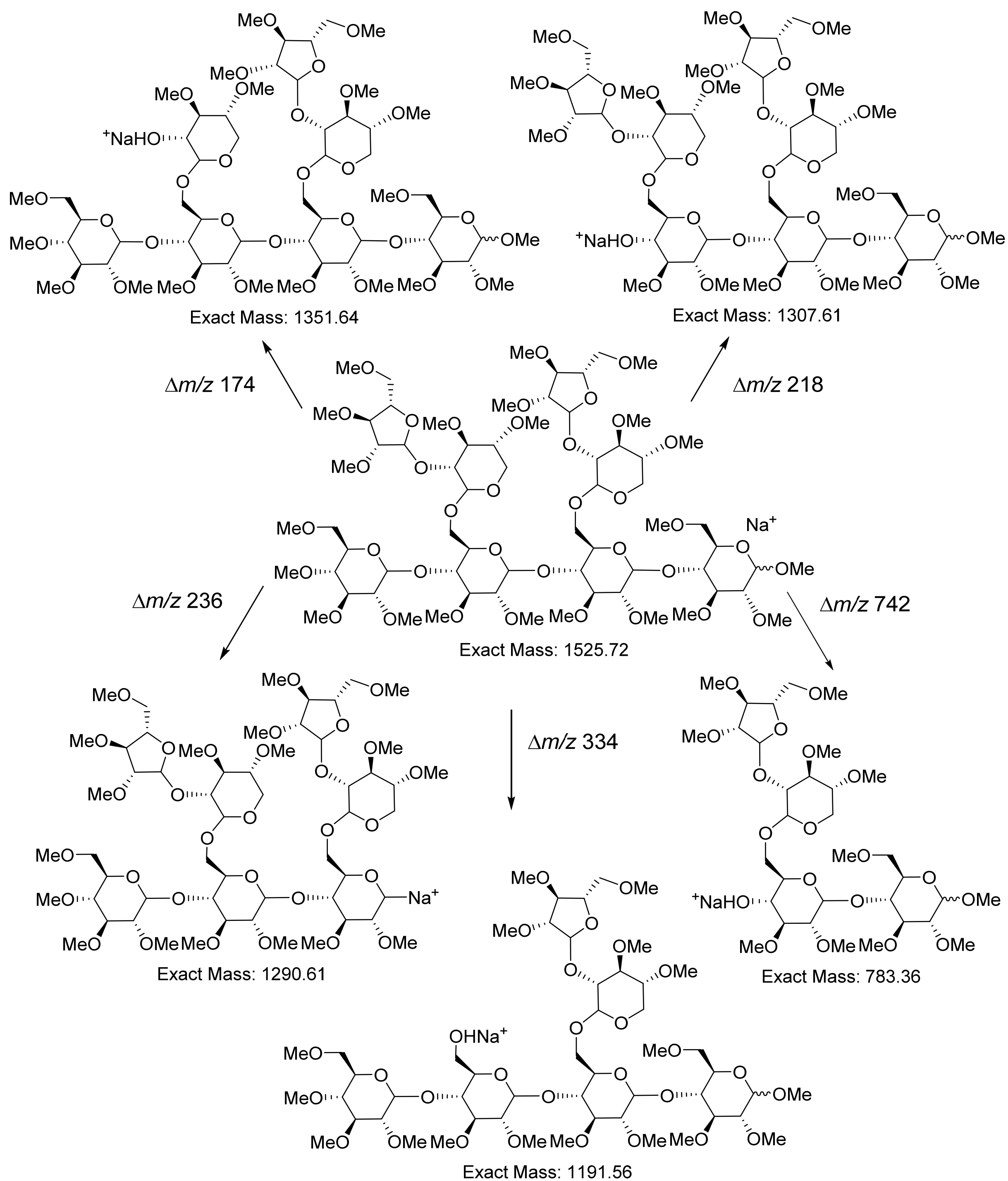


Figure 47. Structure (a) putative mass fragments produced from the *m/z* = 1526 parent ion of per-*O*-methylated **1**.

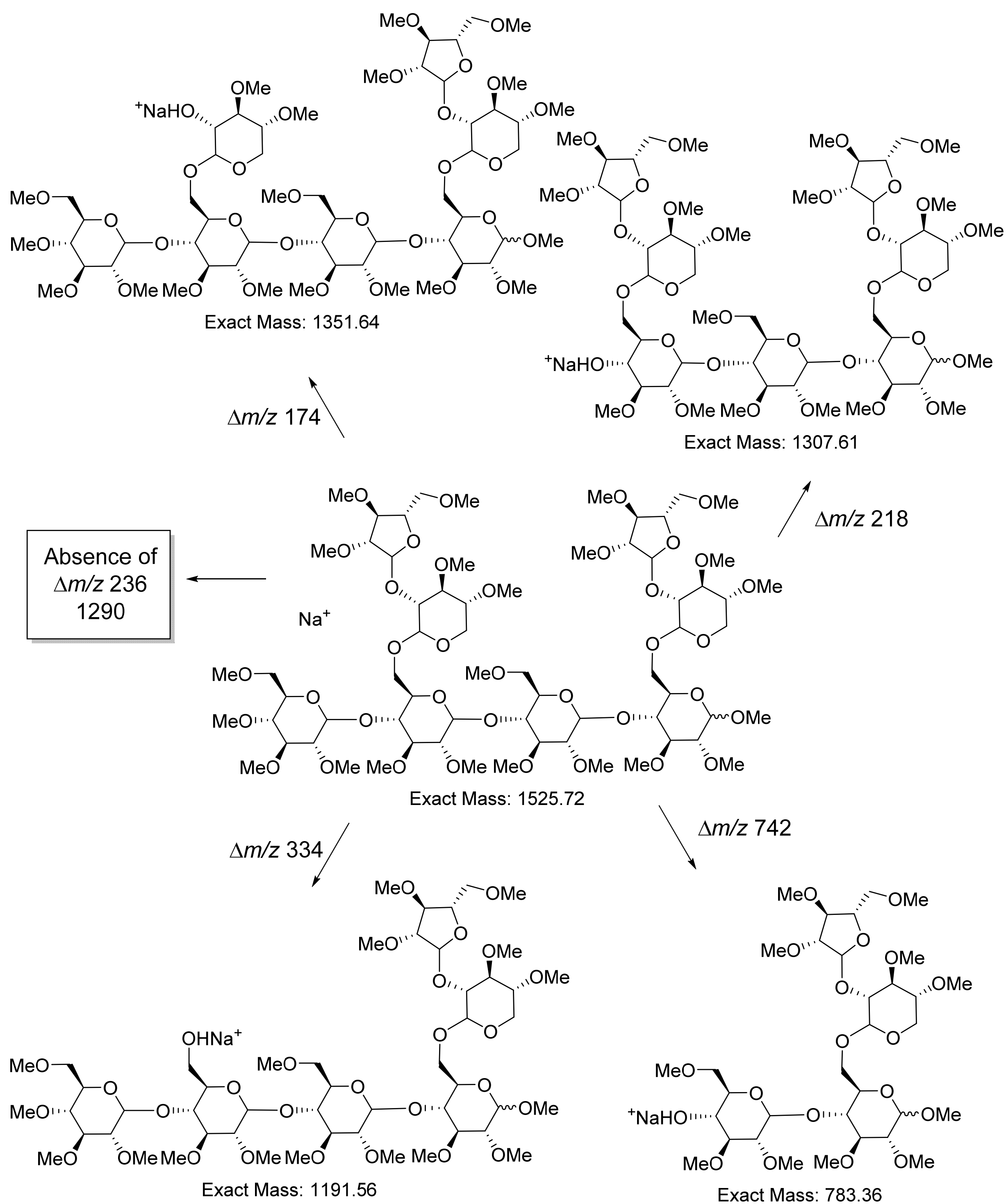


Figure 48. Structure (b) putative mass fragments produced from the *m/z* = 1526 parent ion of per-*O*-methylated **1**.

The NSI-MS^3 fragmentation at *m/z* = 1526 → 1308 for the per-*O*-methylated derivative of **1** (Figure 49) gave indicative mass fragments of *m/z* 1134, 1072, 974, and 784. The mass fragment of *m/z* 1134 is consistent with the loss of a terminal arabinose (Δ*m/z* = 174), *m/z* 1072 is consistent with the loss of a reducing end glucose (Δ*m/z* = 236), *m/z* 974 is consistent with the loss of a terminal arabinose and an internal xylose (Δ*m/z* = 334), and *m/z* 784 is consistent with the loss of a terminal arabinose, an internal xylose, and an internal glucose (Δ*m/z* = 524). These mass fragments were consistent with structure (a) (Figure 50), as the putative *m/z* 1308 fragment of structure (b) (Figure 51) did not produce a fragment at *m/z* 1072 corresponding to the loss of a reducing end glucose (Δ*m/z* = 236).

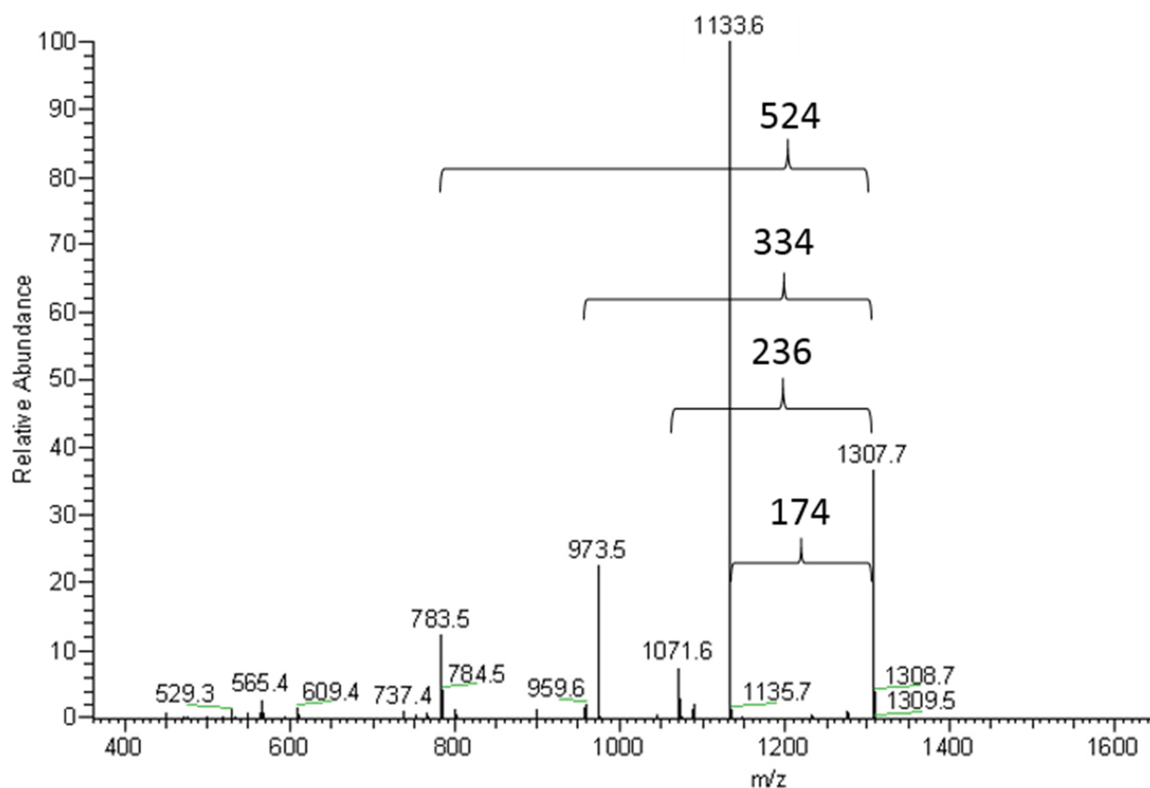


Figure 49. The NSI-MS^3 fragmentation at *m/z* = 1526 → 1308 for the per-*O*-methylated derivative of **1**.
This spectrum was generated using CID = 30%. Mass losses of Δ*m/z* 174, 236, 334, and 524 were indicative of key putative fragments consistent with structure (a).

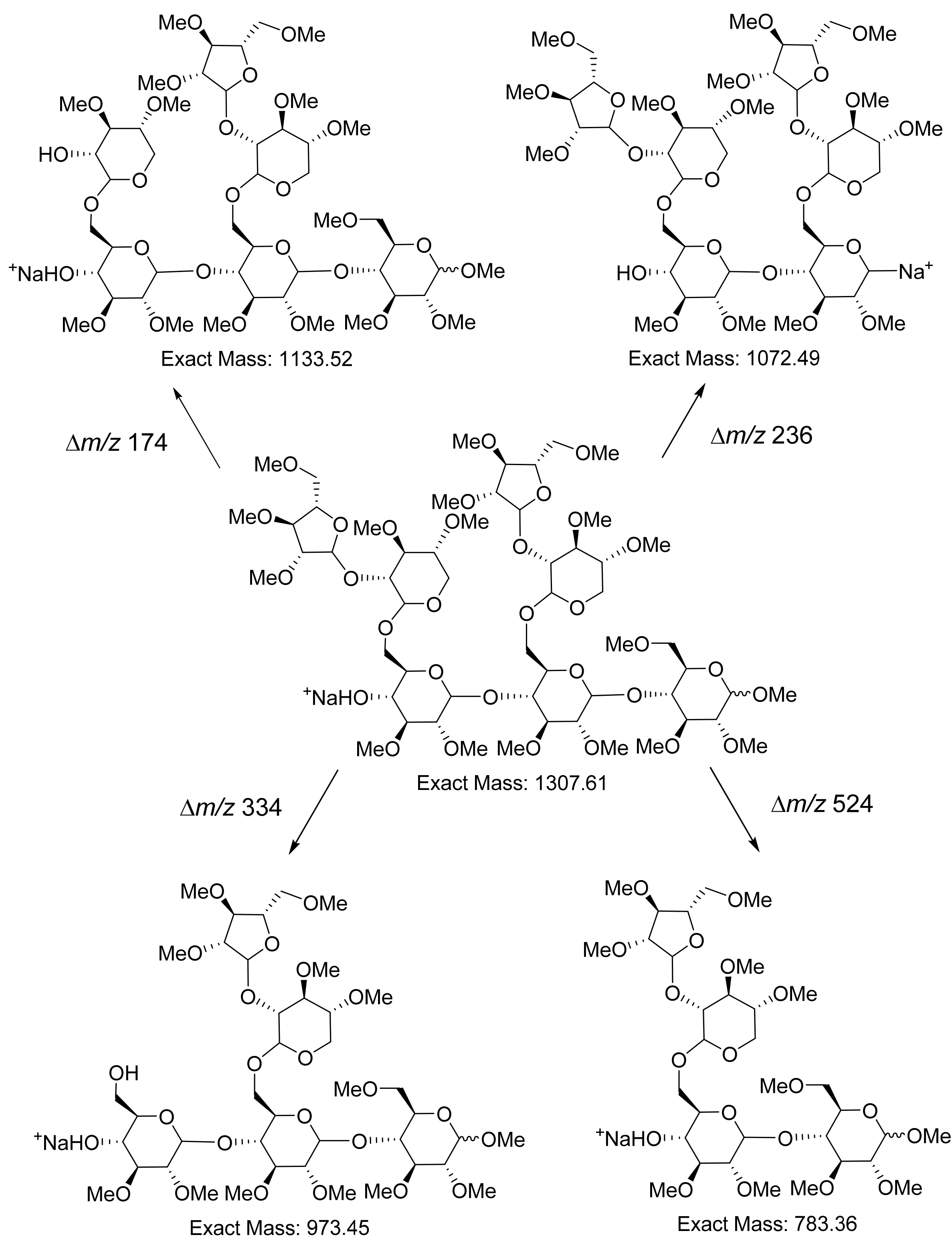


Figure 50. Structure (a) putative mass fragments produced from the *m/z* = 1308 fragment of per-*O*-methylated **1**.

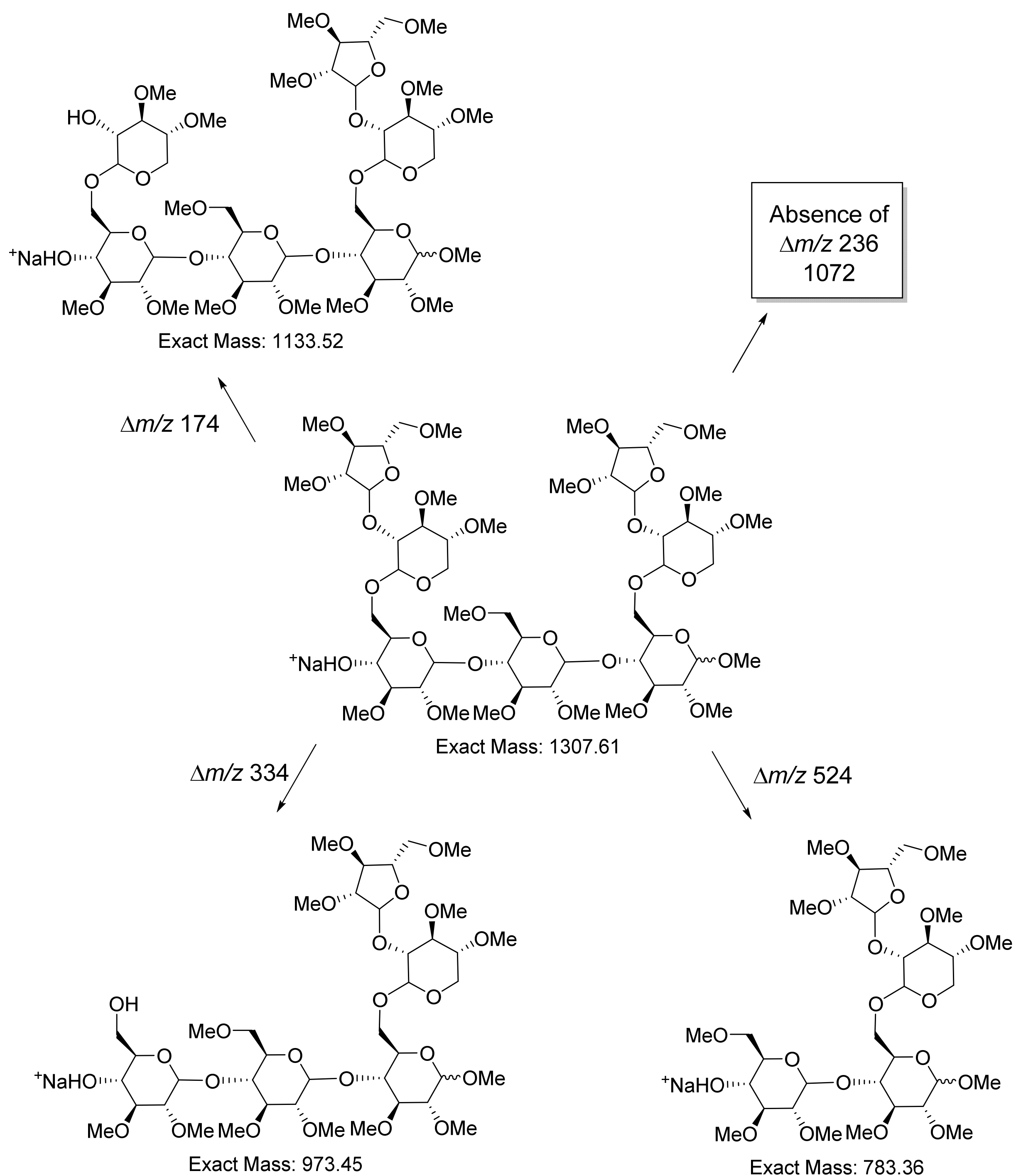


Figure 51. Structure (b) putative mass fragments produced from the *m/z* = 1308 fragment of per-*O*-methylated **1**.

The NSI-MS^3 fragmentation at *m/z* = 1526 → 1352 for the per-*O*-methylated derivative of **1** (not shown; see Figures 47, 48 for putative structures of the *m/z* 1352 ion) yielded a high intensity, indicative fragment at *m/z* 1192, with additional low intensity mass fragments of *m/z* 1178, 1017, 955, 857, and 784. The *m/z* 1178 fragment was consistent with the loss of a terminal arabinose ($\Delta m/z$ = 174), and the *m/z* 1192 fragment was consistent with the loss of an internal xylose ($\Delta m/z$ = 160). The fragmentation pattern for the *m/z* 1352 ion was of minimal utility in distinguishing between the two putative structures for **1**, and the highest intensity ion at *m/z* 1192 was therefore selected for further fragmentation.

The NSI-MS^4 fragmentation at *m/z* = 1526 → 1352 → 1192 for the per-*O*-methylated derivative of **1** (Figure 52) gave indicative mass fragments of *m/z* 1017, 974, 955, 857, and 784. The mass fragment of *m/z* 1017 is consistent with the loss of a terminal arabinose ($\Delta m/z$ = 174), *m/z* 974 is consistent with the loss of a terminal glucose ($\Delta m/z$ = 218), *m/z* 955 is consistent with the loss of a reducing end glucose ($\Delta m/z$ = 236), *m/z* 857 is consistent with the loss of an internal xylose and a terminal arabinose ($\Delta m/z$ = 334), and *m/z* 784 is consistent with the loss of a terminal and an internal glucose ($\Delta m/z$ = 408). These mass fragments were consistent with structure (a) (Figure 53), as the putative *m/z* 1192 fragment of structure (b) (Figure 54) did not produce a fragment at *m/z* 955 corresponding to the loss of a reducing end glucose. The *m/z* 1192 fragment of structure (b) would also have been expected to result in a fragment at *m/z* 579 corresponding to the loss of a terminal glucose and two internal glucose units ($\Delta m/z$ = 612). This fragment was not present in the NSI-MS^4 data at *m/z* 1192.

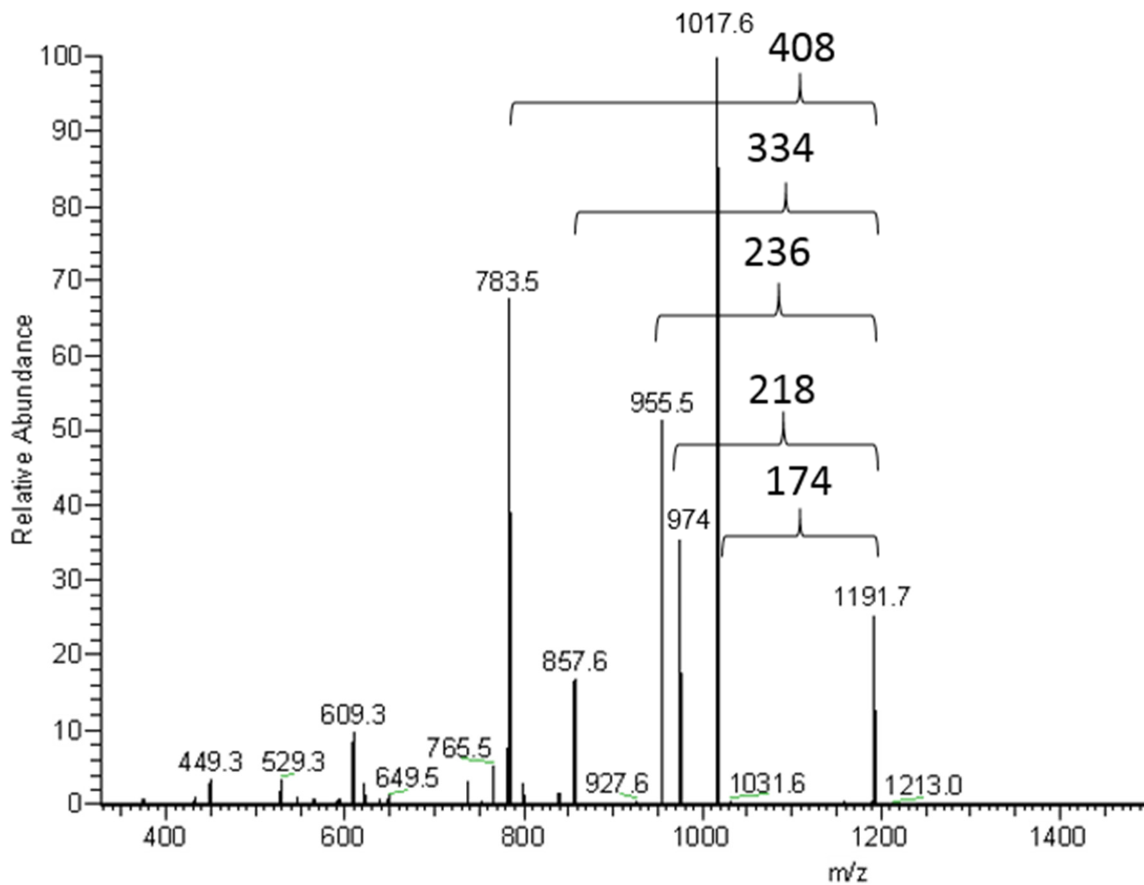


Figure 52. The NSI-MS[4] fragmentation at *m/z* = 1526 → 1352 → 1192 for the per-*O*-methylated derivative of **1**.
Mass losses of Δ*m/z* 174, 218, 236, 334, and 408 were indicative of key putative fragments consistent with structure (a). The ion at *m/z* 1192 was selected for further fragmentation.

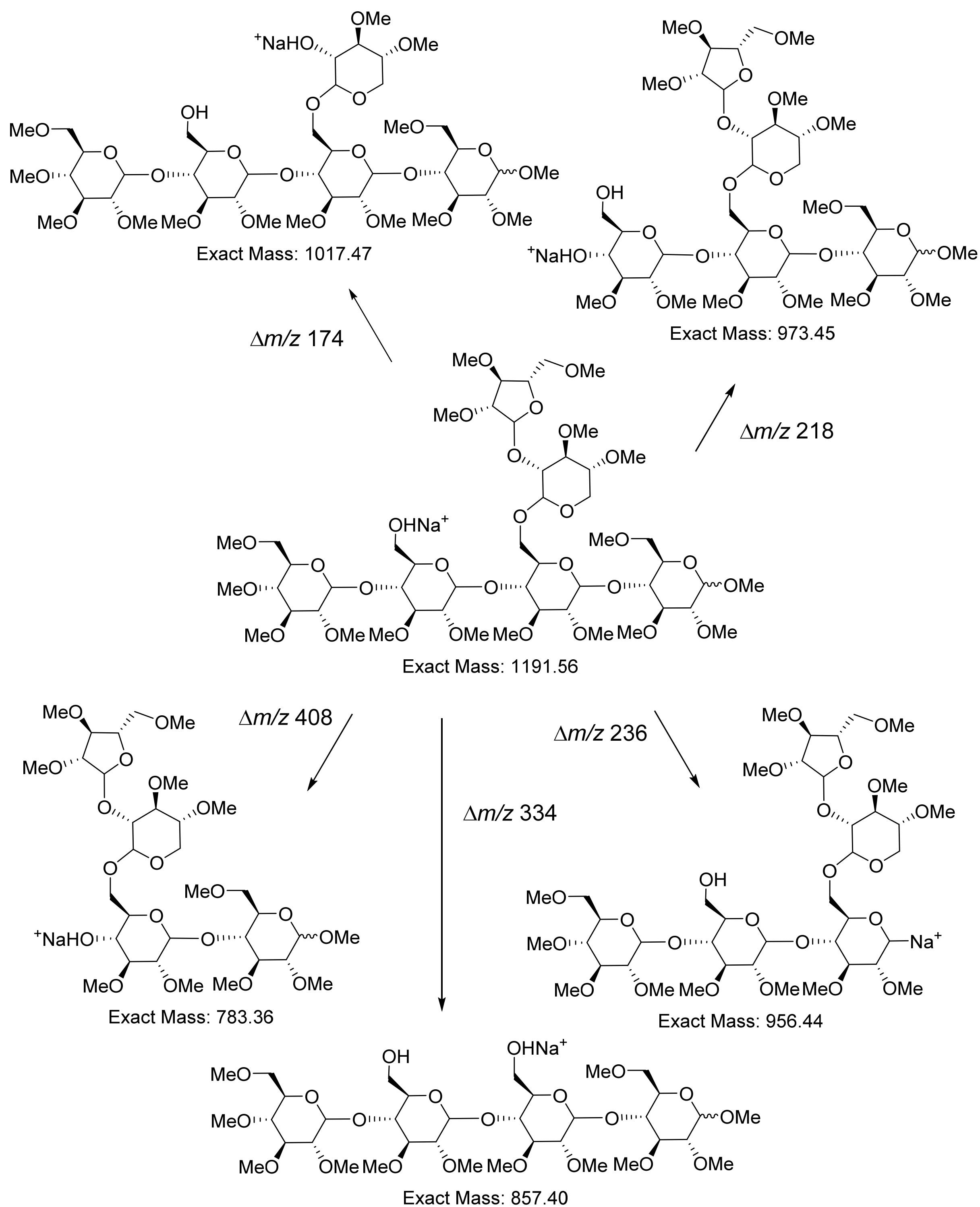


Figure 53. Structure (a) putative mass fragments produced from the m/z = 1192 fragment of per-*O*-methylated **1**.

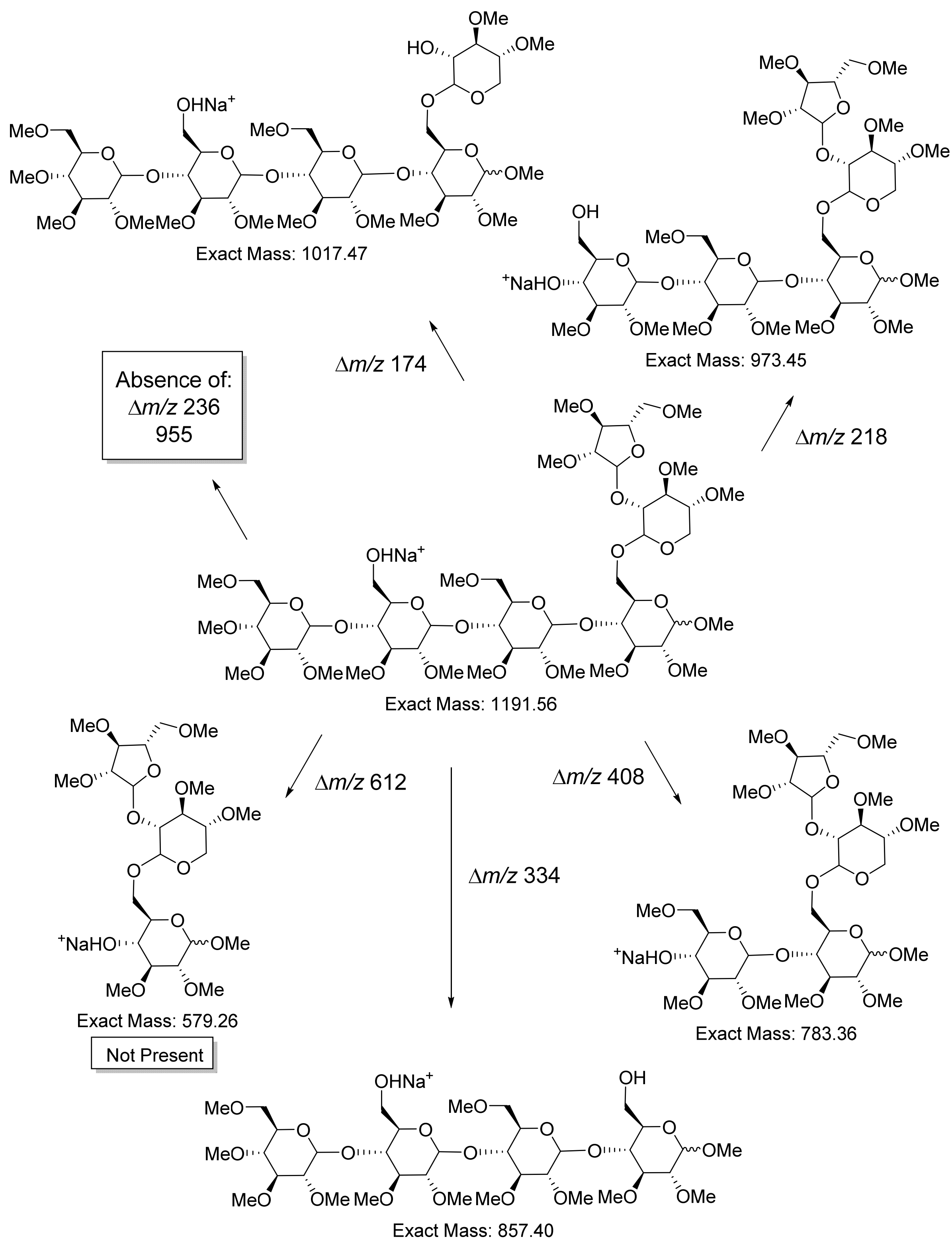


Figure 54. Structure (b) putative mass fragments produced from the $m/z = 1192$ fragment of per-*O*-methylated **1**.

The NSI-MS^5 fragmentation at *m/z* = 1526 → 1352 → 1192 → 784 for the per-*O*-methylated derivative of **1** (Figure 55) gave indicative mass fragments of *m/z* 609, 565, 547, and 449. The mass fragment of *m/z* 609 is consistent with the loss of a terminal arabinose ($\Delta m/z$ = 174), *m/z* 565 is consistent with the loss of a reducing end glucose minus an oxygen ($\Delta m/z$ = 219), *m/z* 547 is consistent with the loss of a reducing end glucose ($\Delta m/z$ = 236), and *m/z* 449 is consistent with the loss of an internal xylose and a terminal arabinose ($\Delta m/z$ = 334). These mass fragments were consistent with structure (a) (Figure 56), as the putative *m/z* 784 fragment of structure (b) (Figure 57) did not produce fragments at *m/z* 547 or 565 corresponding to the loss of a reducing end glucose. The *m/z* 784 fragment of structure (b) would also have been expected to result in fragments at *m/z* 579, corresponding to the loss of an internal glucose ($\Delta m/z$ = 204), and at *m/z* 563, corresponding to the loss of an internal glucose minus an oxygen ($\Delta m/z$ = 221). These fragments were not present in the NSI-MS^5 data.

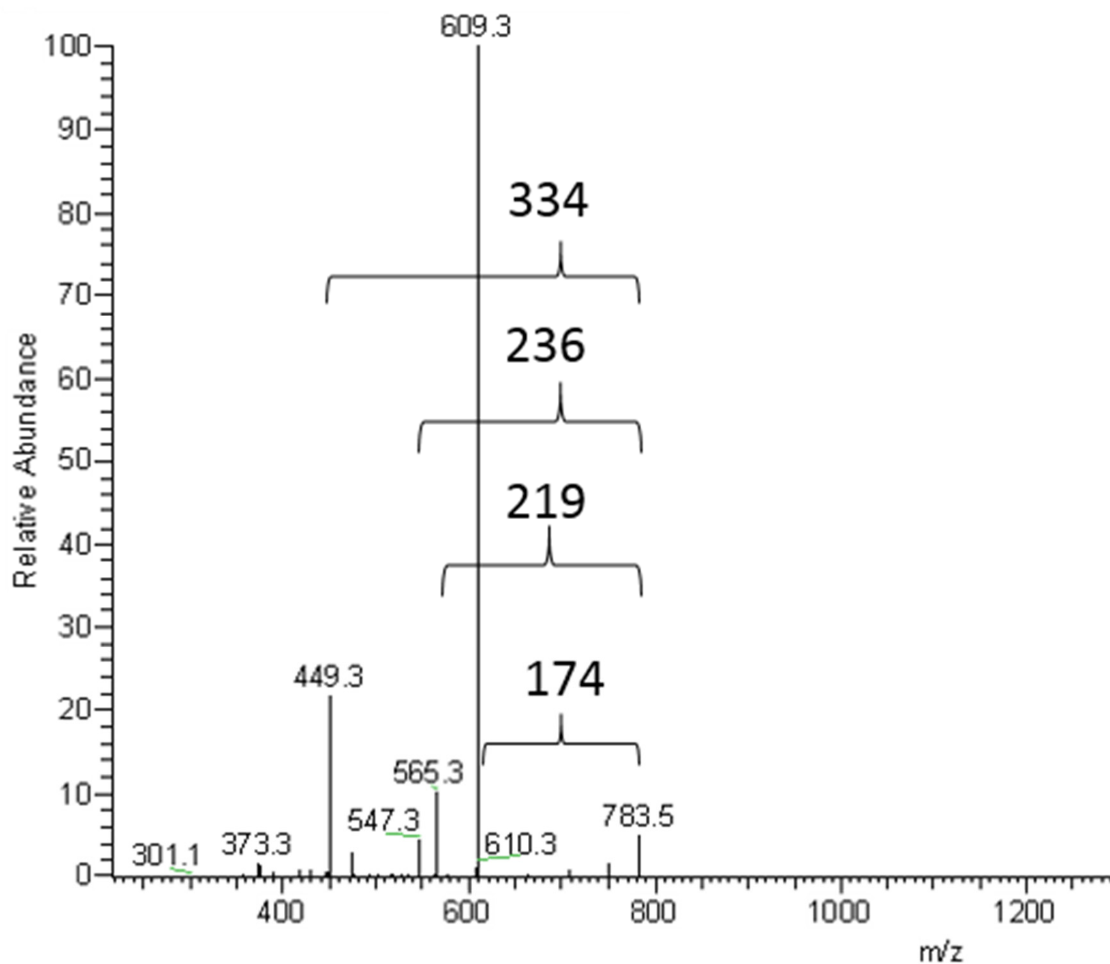


Figure 55. NSI-MS[5] fragmentation at m/z = 1526 → 1352 → 1192 → 784 for the per-*O*-methylated derivative of **1**.
Mass losses of $\Delta m/z$ 174, 219, 236, and 334 were indicative of key putative fragments consistent with structure (a).

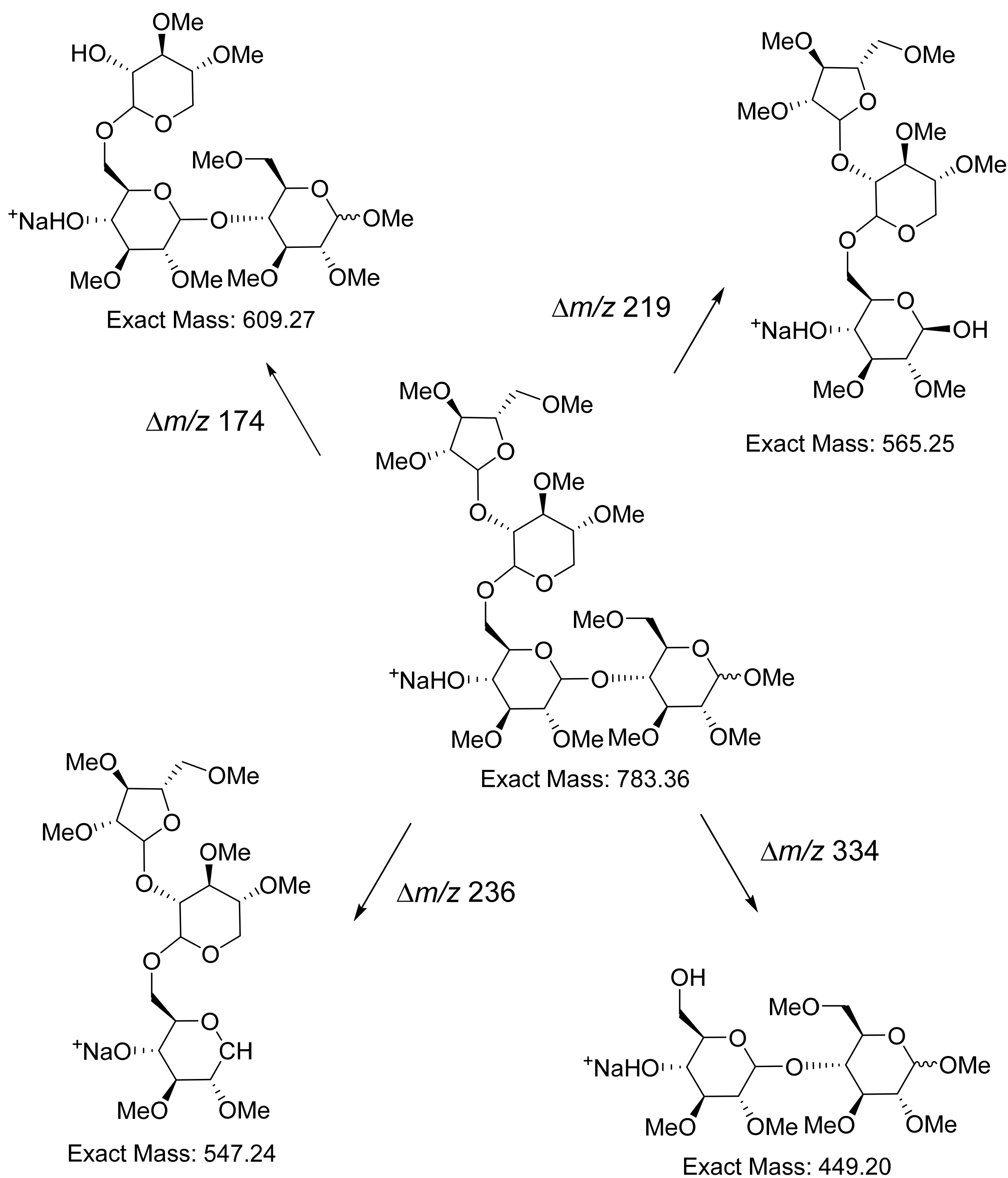


Figure 56. Structure (a) putative mass fragments produced from the m/z = 784 fragment of per-*O*-methylated **1**.

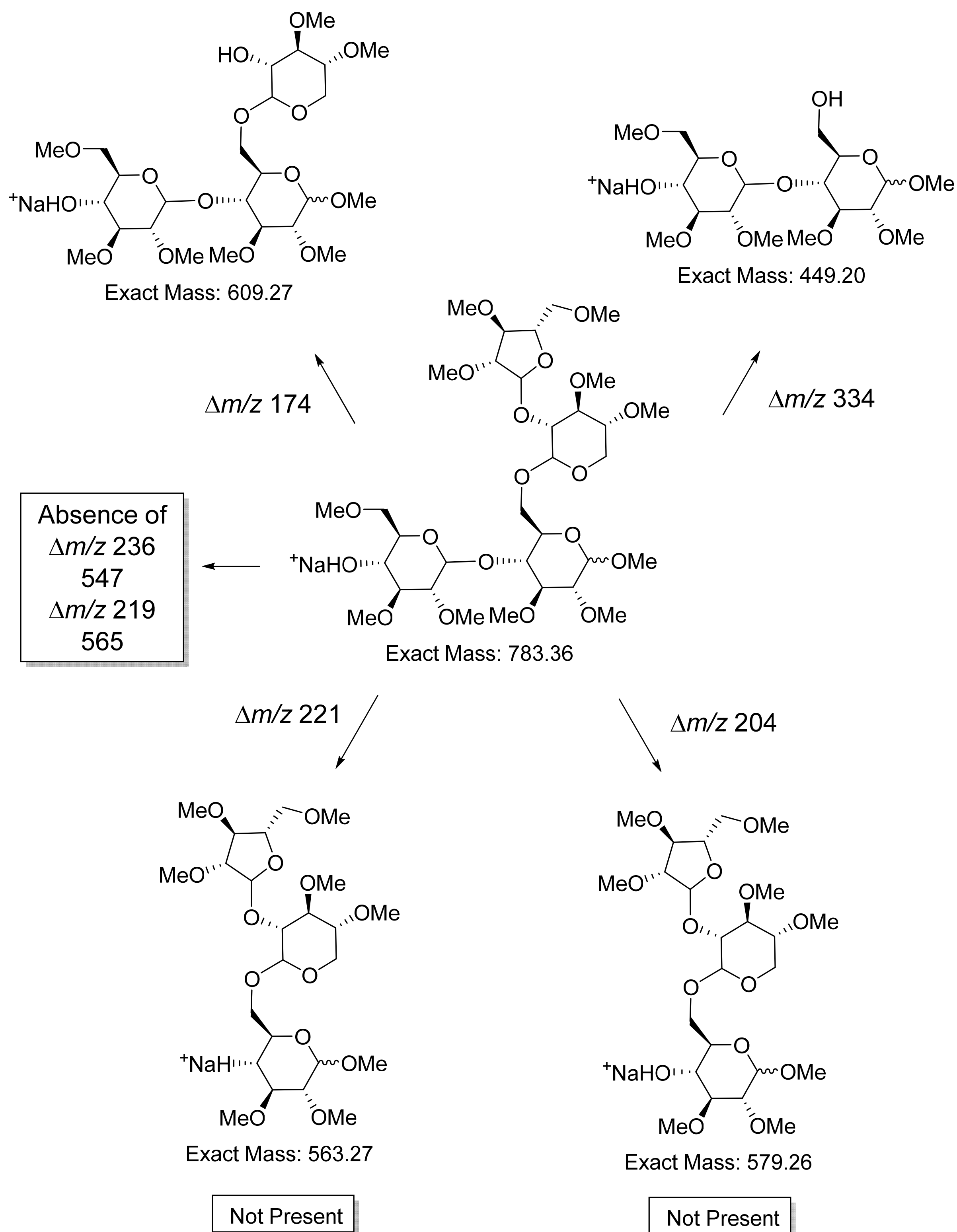


Figure 57. Structure (b) putative mass fragments produced from the m/z = 784 fragment of per-*O*-methylated **1**.

Structure (a) was therefore confirmed as the structure of **1** through the presence of key putative fragments produced from partial and sequential fragmentation of the *m/z* 1526 parent ion. These data supported the presence of a reducing, unsubstituted glucosyl moiety connected via a single glycosidic bond to the remainder of the compound, and allowed placement of the two Xyl-Ara side chains on the two internal glucosyl residues of the tetrameric backbone. Alternative placement of one of the side chains on the reducing glucosyl unit (structure b) would have resulted in a different pattern of MS^n fragments. A key indicator of the presence of the 4-linked glucosyl unit was a mass loss of $\Delta m/z$ = 236 from all fragment ions analyzed (*m/z* 1526, 1308, 1192, and 784). If the reducing glucosyl unit had instead been substituted with a side chain on C-6, the mass loss of $\Delta m/z$ = 236 would not have been detected. Additional fragments indicative of C-6 substitution on the reducing glucosyl unit would instead have been detected. Placement of the Xyl-Ara side chains was further confirmed using NMR analyses.

All data obtained from the CCRC analyses were used to assign the structure of **1** as shown (Figure 58), with a D-glucopyranosyl-(1→4)-D-glucopyranosyl backbone and two L-arabinofuranosyl-(1→2)-D-xylopyranosyl side chains connected to the internal glucosyl residues via D-xylopyranosyl-(1→6)-D-glucopyranosyl linkages. Configurations at the C-1 positions were determined using NMR analyses.

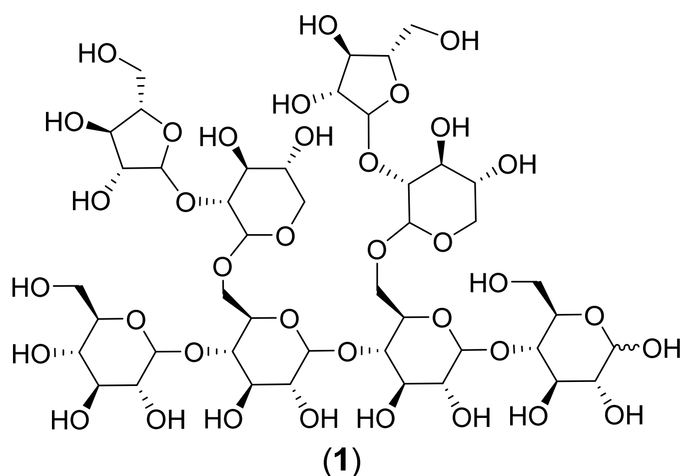


Figure 58. The structure of **1** as determined by the CCRC analyses.
Anomeric configurations could not be assigned using the data collected from the CCRC analyses, but all other structural features shown could be conclusively determined.

c. <u>*NMR Spectroscopy*</u>

The complexity of the compound and the extensive degree of overlap for monomer ring resonances made it necessary to assign putative NMR values based on comparisons with reference compounds and previously determined knowledge of compound structure. This approach is considered appropriate as the majority of the structure had been previously determined using chemical derivatization and associated analyses. The NMR data was primarily used to confirm or refute previously made assignments and to assign anomeric configuration (York *et al*. 1990, Hisamatsu *et al*. 1992, York *et al*. 1996). Spectra were therefore compared to those of purified cranberry oligosaccharides (Auker 2013, Auker *et al.* 2014) as the oligosaccharides isolated from both cranberry and urine sources were determined to be similar.

The NMR spectra indicated the presence of minor impurities, but sufficient resolution could be obtained to assign putative ^{1}H and ^{13}C values for **1** (Figure 59–Figure 62, Table 28).

These assignments were supported by HMQC (Figure 64), COSY (Figure 65), and TOCSY (Figure 66) data, and by comparisons to reference values for monomer units, cellotetraose, and cranberry oligosaccharides (Auker 2013, Auker *et al.* 2014). Duplicate resonance sets were detectable for the two pairs of Ara and Xyl units, but could not be assigned independently. These duplicated resonance sets indicated a high degree of structural symmetry for the local environment of each side chain. These observations were consistent with the structural assignments made using the partial-fragmentation pattern analyses.

The anomeric positions of Glc-B, Glc-C, and Glc-D were assigned β-orientation based on their downfield (δ_H < 4.70) chemical shifts and coupling constants of $J_{H1,H2}$ > 5 Hz, while Xyl-E, and Xyl-F were assigned α-orientation due to their upfield (δ_H > 4.70) chemical shifts and coupling constants of $J_{H1,H2}$ < 5 Hz (Duus *et al*. 2000). The anomeric positions of the Ara-G and Ara-H residues were assigned α-orientation due to their $J_{H1,H2} \approx$ 1–2 Hz, although the exact *J*-value for these residues could not be assigned as a result of resonance overlap (Duus *et al*. 2000, Islam *et al*. 2011). The reducing glucosyl residue indicated by MS^n-fragmentation patterns was assigned to Glc-A and its presence confirmed by duplicate resonances for the α- and β-anomers of this monomer (Table 28).

Numerous COSY, TOCSY, and HMBC correlations further confirmed the identities and connectivity of the units indicated by the CCRC analyses and the MS^n fragmentation patterns (Figures 63–66). Key HMBC correlations between H-1 of Ara*f*-G and H (δ_H 4.93) and C-2 of Xyl*p*-E and F (δ_C 78.9), and between H-2 of Xyl*p*-E and F (δ_H 3.21) and C-1 of Ara*f*-G and H (δ_C 109.9), supported the assignment of α-L-arabinofuranosyl-(1→2)-α-D-xylopyranosyl side chains. These side chains were linked to C-6 of the two internal glucose residues Glc*p*-B and C through HMBC correlations between H-1 of Xyl*p*-E and F (δ_H 4.79), and δ_C 67.1. Key HMBC

correlations also further confirmed the connectivity of the glucose backbone with a correlation between H-1 of Glc*p*-C (δ_H 4.27) and C-4 of Glc*p*-B (δ_C 81.0). The structure of the resulting octasaccharide (**1**) was assigned as β-D-glucopyranosyl-(1→4)-[α-L-arabinofuranosyl-(1→2)-α-D-xylopyranosyl-(1→6)]-β-D-glucopyranosyl-(1→4)-[α-L-arabinofuranosyl-(1→2)-α-D-xylopyranosyl-(1→6)]-β-D-glucopyranosyl-(1→4)-β-D-glucopyranose.

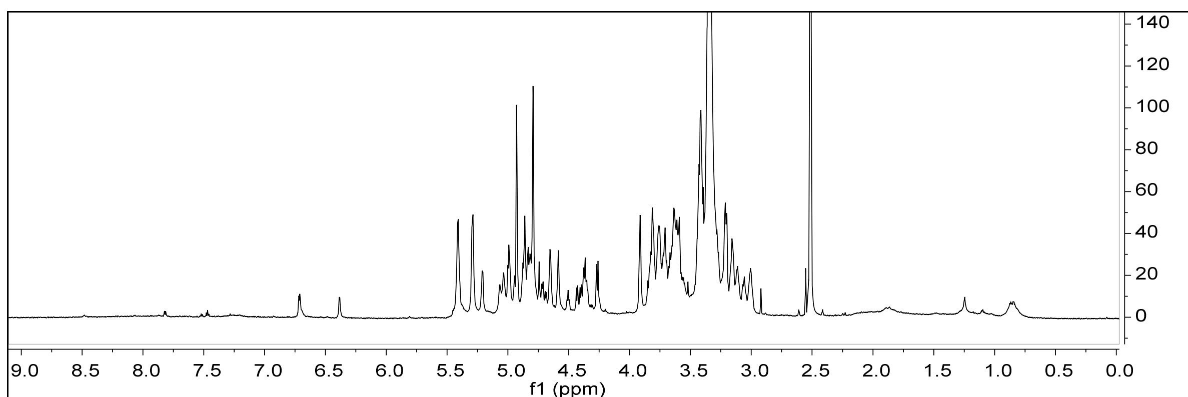


Figure 59. The ^{1}H NMR spectrum of **1** in DMSO-d_6, 700 MHz, showing sufficient resolution to obtain structural information.

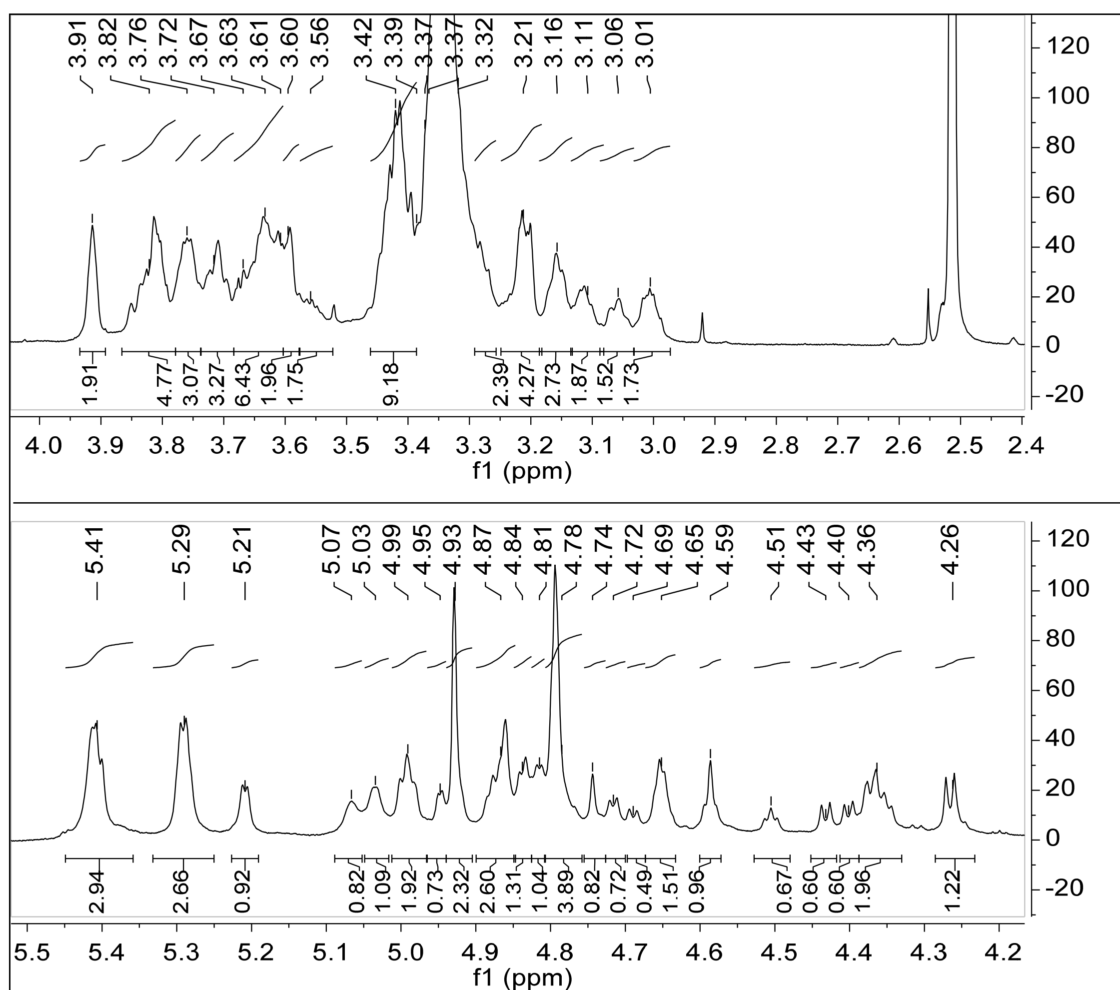


Figure 60. Expansions of the ^{1}H NMR spectrum of **1**, DMSO-d_6, 700 MHz, showing integration and chemical shift assignments.

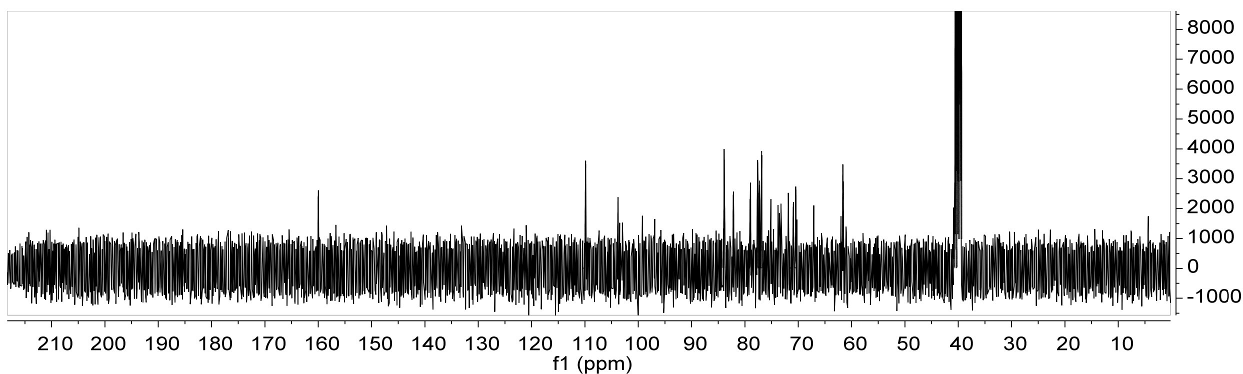


Figure 61. The ^{13}C NMR spectrum of **1** in DMSO-d_6, 175 MHz, showing sufficient resolution to obtain structural information.

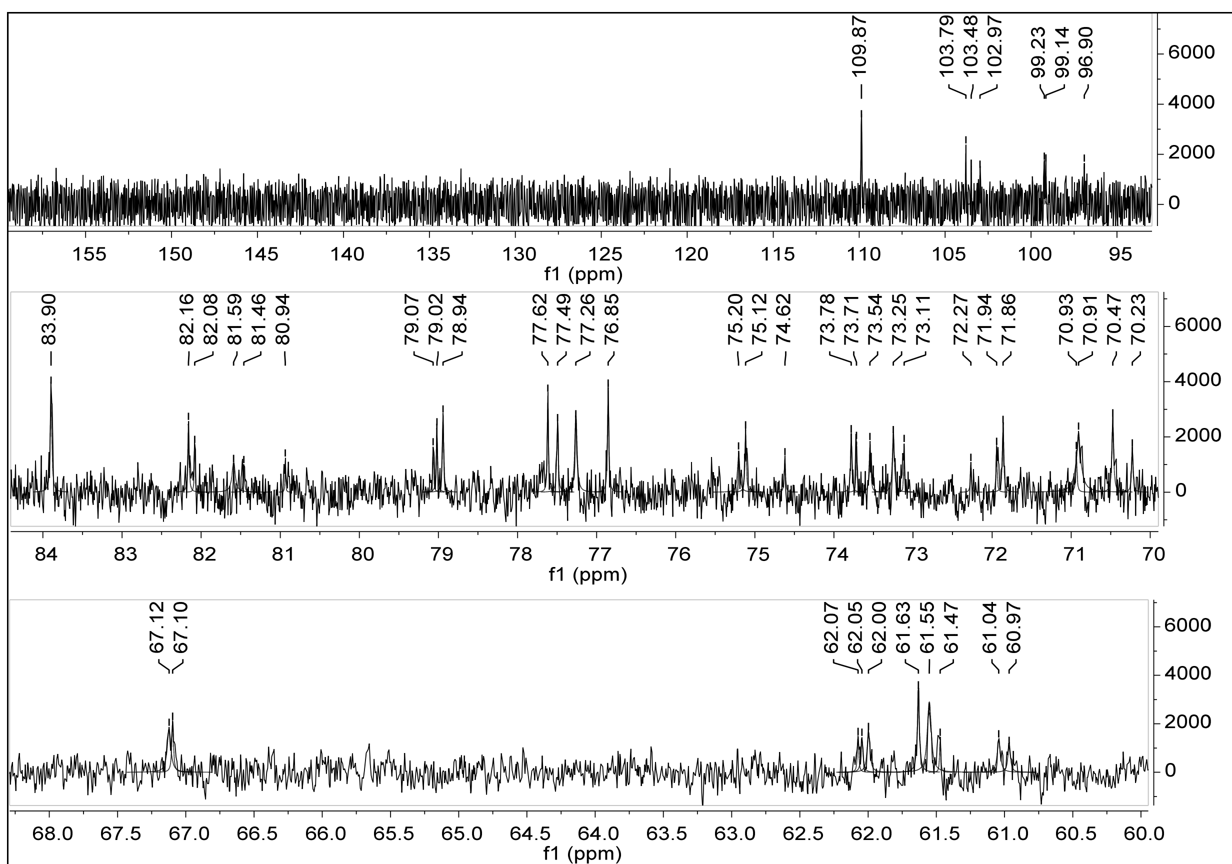


Figure 62. Expansions of the ^{13}C NMR spectrum of **1**, DMSO-d_6, 175 MHz, showing chemical shift assignments.

Table 28. Proposed ^{1}H and ^{13}C NMR assignments for **1**, DMSO-d_6, 700/175 MHz. Duplicate resonance sets were observed for the two sets of Xyl and Ara residues. The same chemical shift values were therefore assigned to the two sets of Xyl and Ara residues. Values for the Glc residues were assigned using the aid of reference compounds including glucose, cellotetraose, and the two oligosaccharides of similar structure isolated from cranberry materials. The majority of these assignments were supported by 2D correlations. Numerical assignments for the 2- and 3-positions of the glucosyl residues are tentative.

Monomer	Position	δ_C	δ_H (mult[a]; J_{HH})		Monomer	Position	δ_C	δ_H (mult[a]; J_{HH})
Glc*p*-A	1	92.2	4.92 (3.9)		Glc*p*-D	1	103.5	4.36 (8.0)
α-anomer	2	72.0	3.59			2	73.2	3.12
	3	72.3	3.24			3	75.1	3.37
	4	81.7	3.32			4	73.7	3.63
	5	77.3	3.21			5	81.5	3.38
	6	61.5	3.61, 3.71			6	61.0	3.55, 3.75
Glc*p*-A	1	96.9	4.35 (6.8)		Xyl*p*-E	1	99.1	4.79 (3.3[b])
β-anomer	2	73.7	3.01			2	78.9	3.21[b]
	3	70.9	3.29			3	71.9	3.76
	4	81.6	3.28			4	70.9	3.29
	5	76.9	3.16			5	62.0	3.42
	6	61.1	3.44, 3.72		Xyl*p*-F	1	99.1	4.79 (3.3[b])
Glc*p*-B	1	102.9	4.43 (8.1)			2	78.9	3.21
	2	73.1	3.15			3	71.9	3.76
	3	74.7	3.01			4	70.9	3.29
	4	81.0	3.36			5	62.0	3.42
	5	79.0	3.20[b]		Ara*f*-G	1	109.9	4.93[c]
	6	67.1	3.70, 3.85			2	82.1	3.91
Glc*p*-C	1	103.8	4.27 (7.8)			3	77.6	3.63
	2	73.5	3.66			4	83.9	3.81
	3	75.2	3.40			5	61.7	3.42, 3.59
	4	82.1	3.91		Ara*f*-H	1	109.9	4.93[c]
	5	79.0	3.20			2	82.1	3.91
	6	67.1	3.75, 3.83			3	77.6	3.63
						4	83.9	3.81
						5	61.6	3.42, 3.59

[a] All protons appeared as overlapping multiplets. [b] Approximate *J* value
[c] A reliable numerical *J* value assignment could not be made due to resonance overlap.

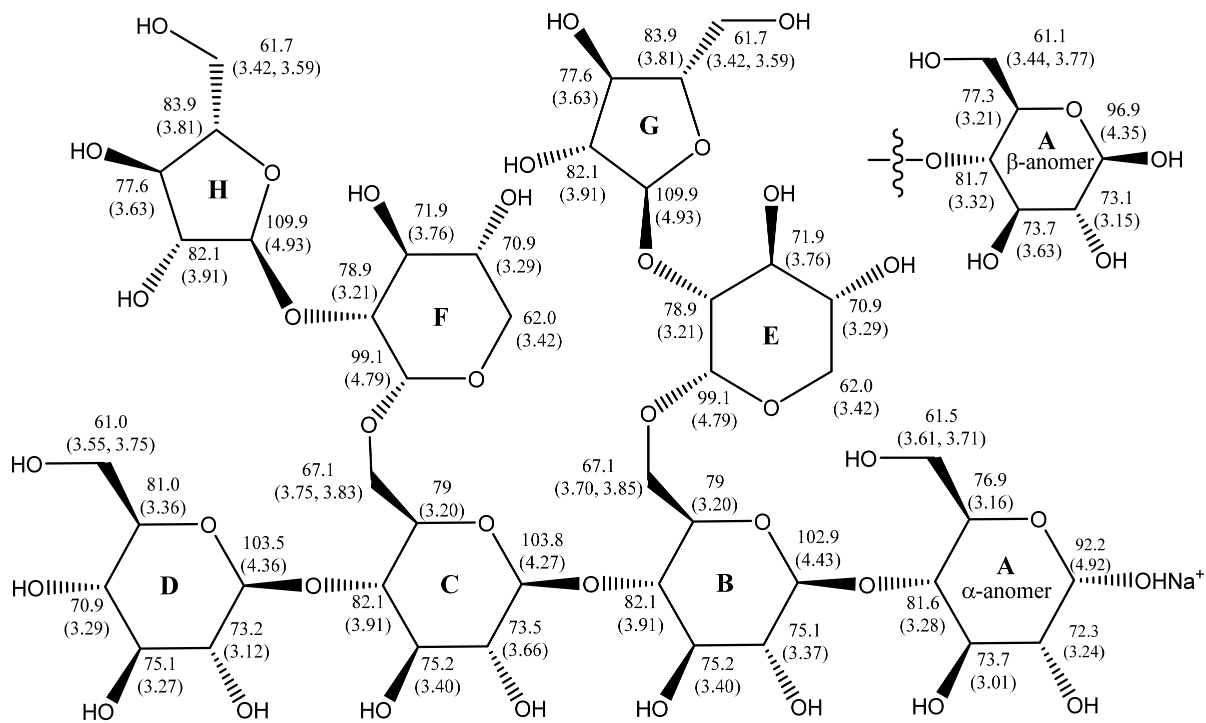


Figure 63. The structure of **1** with putative 1D NMR numerical assignments shown.

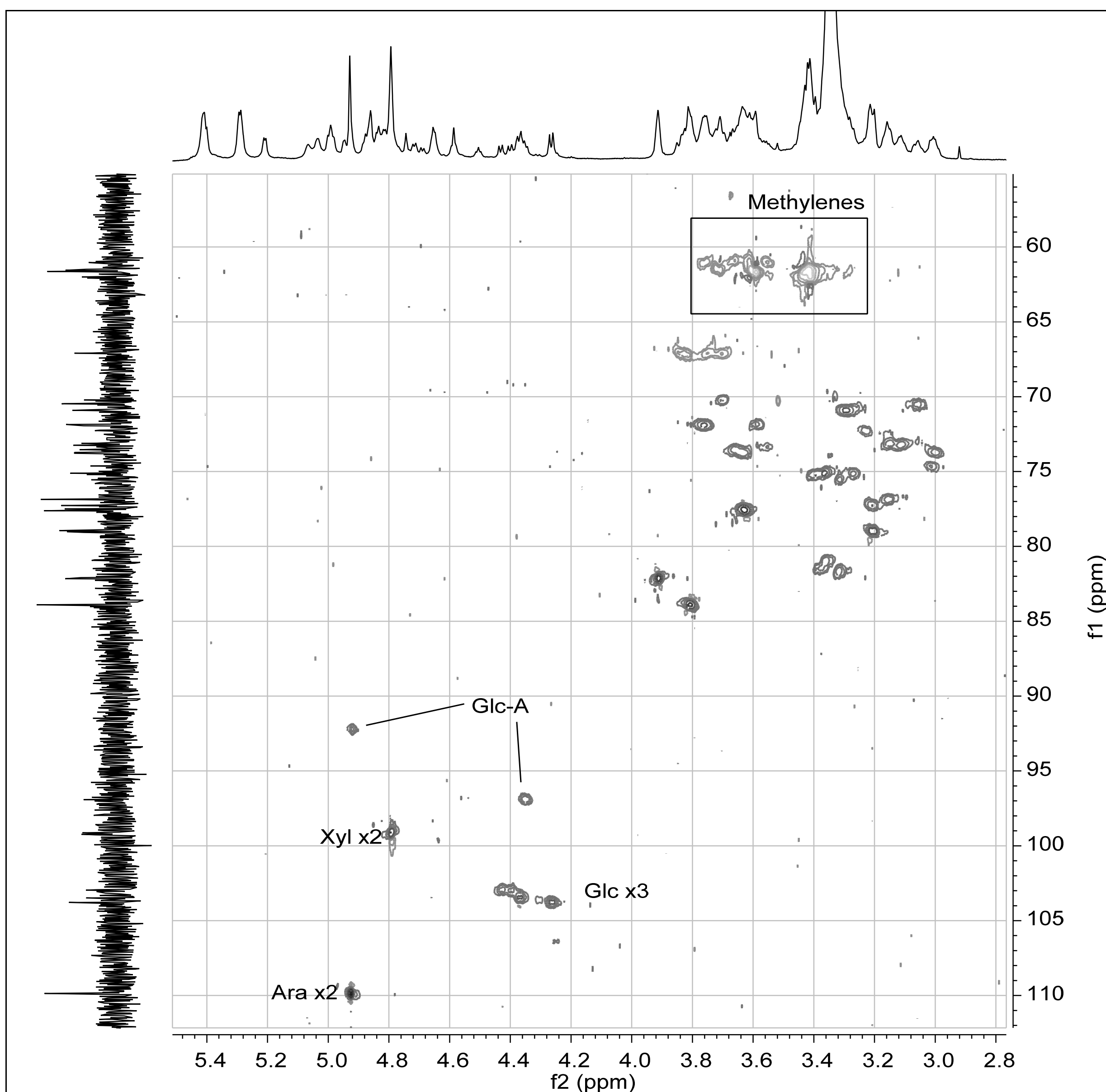


Figure 64. The HMQC spectrum for **1**, DMSO-d_6, 700/175 MHz.
Anomeric correlations and methylene correlations have been indicated on the spectrum. Remaining correlations were assigned to ring carbons and protons. Two resonances corresponding to C-1/H-1 of the Glc-A moiety were present due to the reducing nature of this moiety.

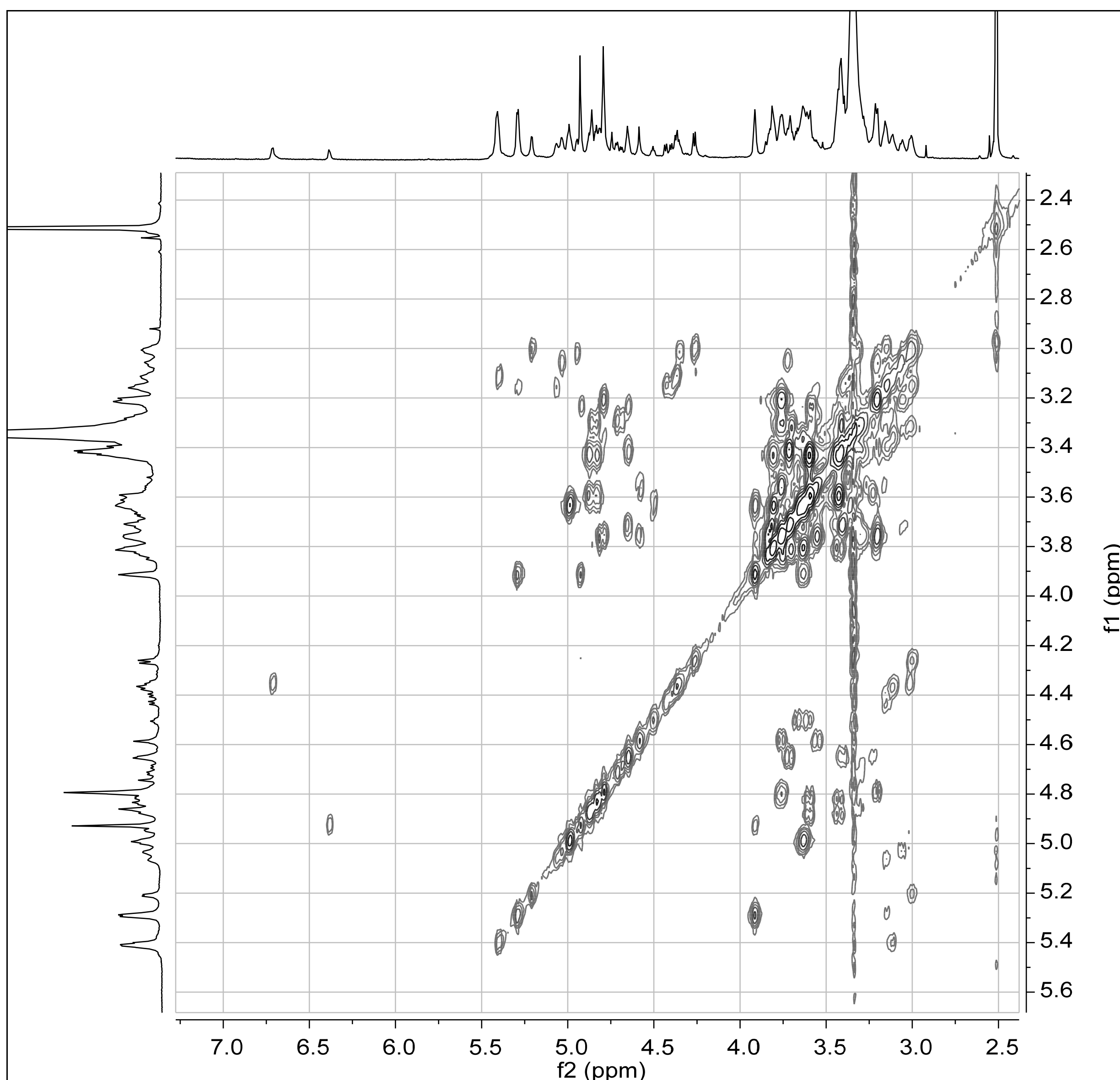


Figure 65. The COSY spectrum for **1**, DMSO-d_6, 700 MHz.
These correlations were used to confirm HMQC assignments for ring protons and are shown below on the structure of **1** (Figure 68).

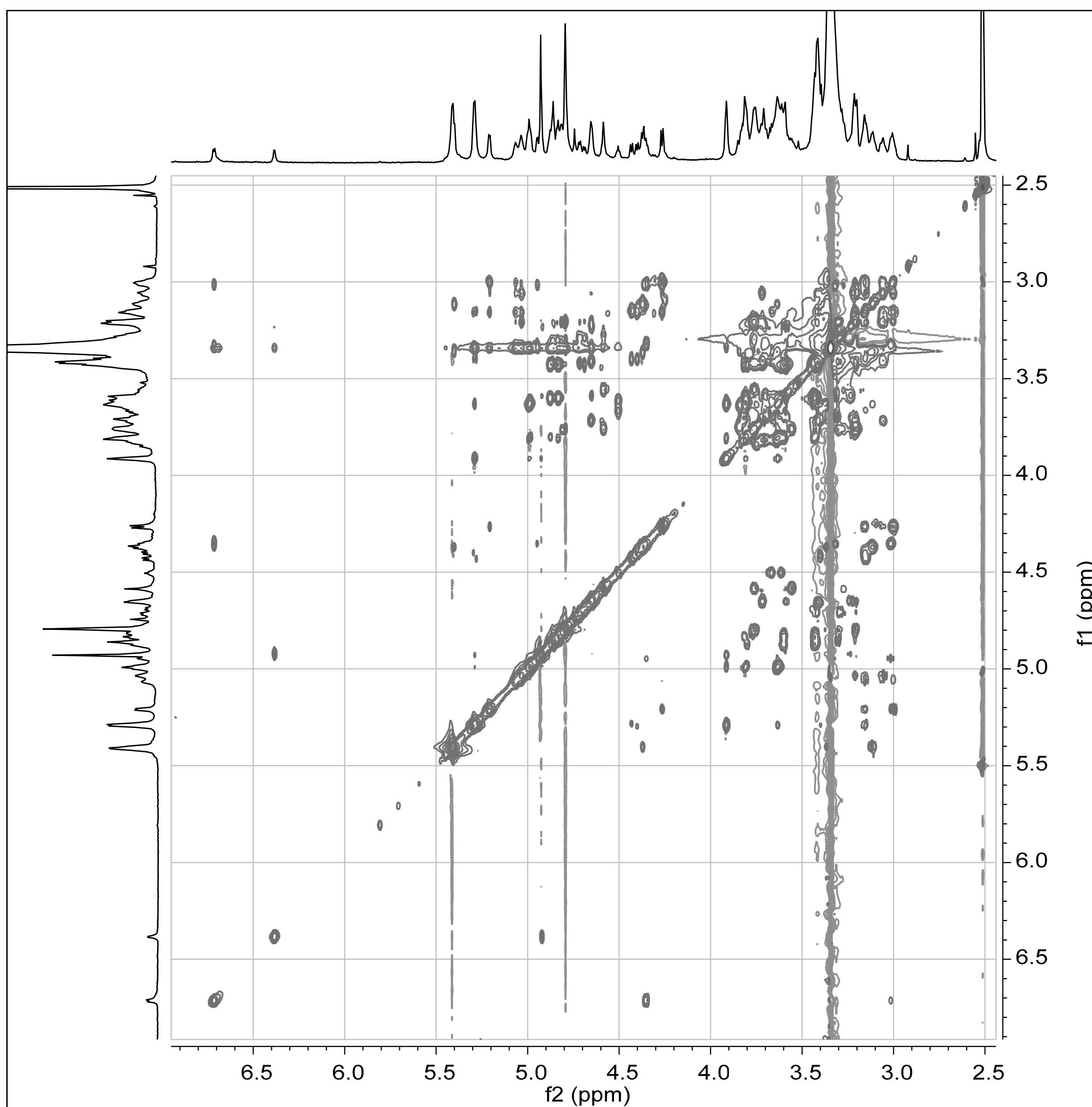


Figure 66. The TOCSY spectrum for **1**, DMSO-d_6, 700 MHz.
These correlations were used to provide further support for the assignment of ring protons and associated carbons.

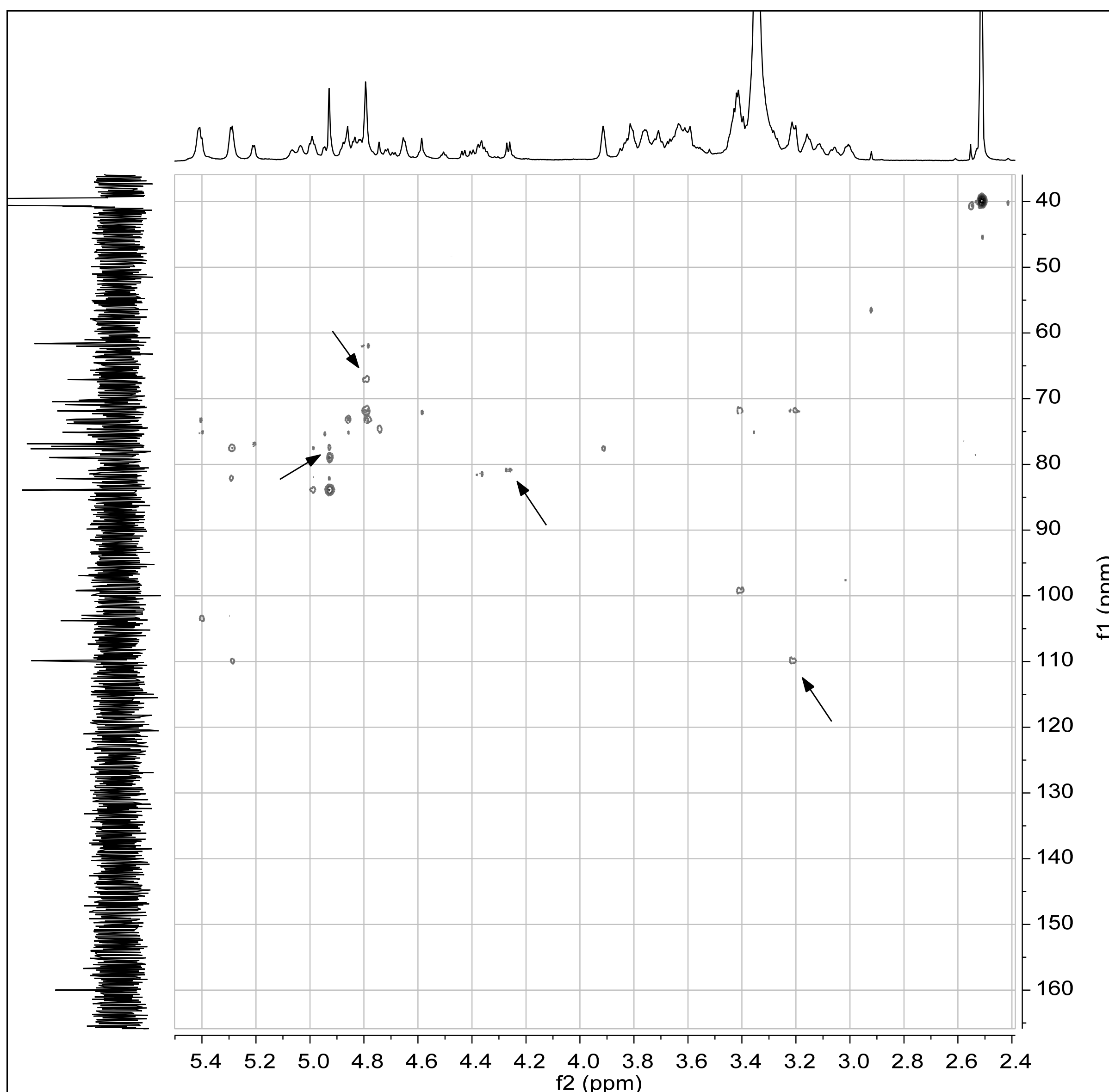


Figure 67. The HMBC spectrum for **1**, DMSO-d_6, 700 MHz.
Key HMBC correlations (arrows) supported the assignments of connectivity between monomer units and were consistent with the structure determined by the CCRC analyses.

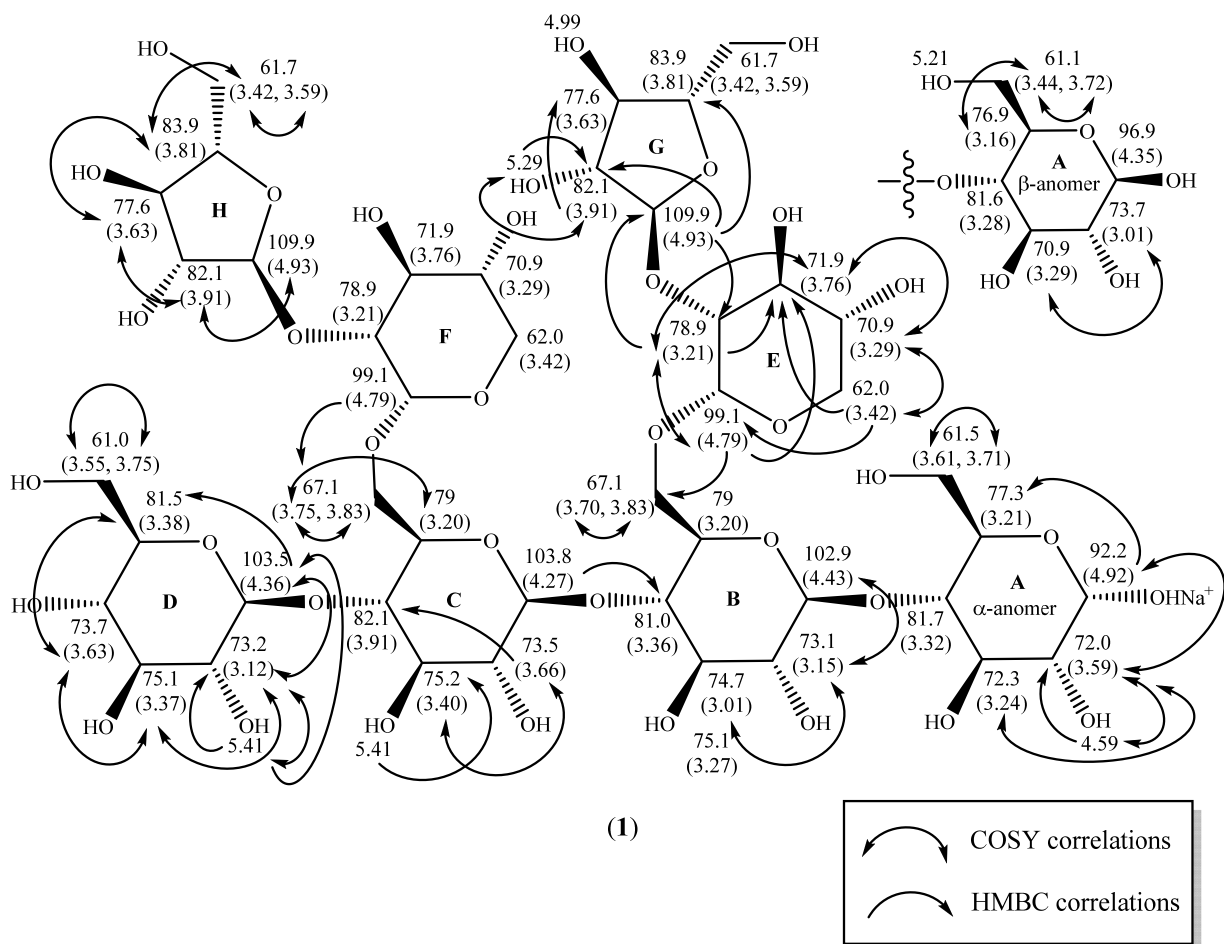


Figure 68. Structure of the urinary octasaccharide (**1**) showing putative COSY and HMBC correlations.

D. Conclusions

This project was initiated on the premise that the active compounds present in treated swine urine would be A-type PACs or metabolically-transformed PACs (Chapter 1). Such compounds would be expected to retain aromatic protons detectable by ^{1}H NMR spectroscopy, even in complex mixtures and regardless of metabolic transformations such as polymer hydrolysis or glycosidic conjugation. As the project progressed, it became evident that active fractions contained no indication of PACs, flavonoids, or related compounds. These results did

not support the original PAC-based hypothesis. The isolation and purification approach was therefore changed to focus on the isolation and characterization of unknown compounds from active aqueous fractions. During the subsequent method development it was determined that the major components of the active fractions lacked chromophores, making them undetectable by conventional UV-based methods.

The use of ELSD made it possible to detect and characterize a group of urinary compounds from active fractions that were not PAC-derived and were instead determined to be complex carbohydrates, specifically oligosaccharides. A single compound from the series of oligosaccharides detected by HPLC-ELSD was purified in sufficient quantity for full structural elucidation. This compound (**1**) was found to be an octasaccharide composed of a tetrameric, β-(1→4)-linked glucose backbone with two xylose-arabinose side chains. Preliminary NMR data and chromatographic characteristics of the other purified compounds indicates that **1** is representative of the complex pool of oligosaccharides that is present.

The structure of **1** is similar to that of arabinoxyloglucans isolated from solanaceous plants (York *et al.* 1996). Such compounds are believed to be derived from the hemicellulose polymers that serve as structural components of plant primary cell walls. These hemicellulose polymers are enzymatically cleaved and solubilized during the process of fruit ripening (York *et al.* 1996, Brummell *et al.* 2004, Goulão & Oliveira 2008). The presence of similar compounds in cranberry would support the hypothesis that the oligosaccharides present in swine urine originate from cranberry hemicelluloses, but little is known about the composition of cranberry hemicelluloses or cranberry oligosaccharides.

Oligosaccharide urinary metabolites were consistently present in active urine collected from three different swine after cranberry consumption and were present in active fractions but

not in inactive fractions, control urine, or control urine fractions. Retrospective examination of NMR data from the first active D fractions also confirmed the evidence of oligosaccharides, but this evidence was discounted at the time it was collected as it did not indicate the presence of PACs. The concentration range estimates for the urinary metabolites isolated during this study are within a reasonable range for bioactivity, with single components being present at ~1–5 µg/mL urine (Saude *et al.* 2007, Bouatra *et al*. 2013). These data strongly indicated that arabinoxyloglucan oligosaccharides are a component that may be responsible for the anti-adhesion properties of urine and urine fractions following cranberry consumption, but *E. coli* anti-adhesion properties had not been previously reported for this class of compounds.

At the conclusion of this study, insufficient amounts of urinary oligosaccharides were available for testing in the UPEC-HRBC anti-agglutination assay and the reproducibility of the assay had been questioned. The source of the urinary oligosaccharides was also unknown, as arabinoxyloglucan oligosaccharides had not been previously reported from cranberry or other *Vaccinium* species. The information available suggested a major paradigm transition regarding the mechanism by which cranberry juice prevents UTIs, but, alone, provided insufficient direct evidence to support such a proposition with certainty. Additional studies using cranberry materials were therefore pursued (Chapter 3).

CHAPTER 3: CRANBERRY COMPOSITION

A. Introduction

The majority of previous cranberry juice chemical composition studies have focused on the low-MW, relatively nonpolar compounds of cranberry which are responsible for the aroma, flavor, color, and anti-oxidant properties of the juice (Pappas & Schaich 2009, Singh *et al*. 2012, Vu *et al*. 2012, Su *et al*. 2010). Compounds previously investigated include terpenoids, aromatic acids, monosaccharides, simple phenolics, polyphenols, and a wide variety of flavonoids (Chapter 1, Section B).

The compounds believed to be responsible for the anti-adhesion properties of cranberry juice are PAC oligomers of epicatechin units containing at least one A-type linkage (Foo *et al*. 2000a,b). Cranberry PACs are typically isolated from the EtOAc fraction of acetone-extracted whole cranberry fruit (Howell *et al*. 1998, Foo *et al*. 2000a,b) and make up a relatively small percentage of whole cranberry starting material (Gu *et al*. 2004, Santos-Buelga & Scalbert 2000, Gu *et al*. 2003). Studies examining the anti-adhesion properties of cranberry-derived PACs and whole cranberry juice have indicated that additional compounds may be present that could contribute to the anti-adhesion properties of the juice (Turner *et al*. 2007, Pinzón-Arango *et al.* 2009, Holguin *et al.* 2008, Mathison *et al*. 2013, Kimble *et al*. 2014).

Studies investigating the anti-adhesive components of urine after cranberry consumption (Chapter 2) have indicated that arabinoxyloglucan oligosaccharides with anti-

adhesion properties may be present in cranberry materials, but relatively little is known regarding the oligosaccharide components of cranberry fruit or juice products. The gel-forming characteristics of cranberries, as exhibited by the classic cranberry sauce, led to early structural investigations of complex carbohydrates including pectins and other cell wall-associated insoluble polysaccharides (Holmes & Rha 1978), but other complex carbohydrates including oligosaccharides have received limited attention. In addition, studies on soluble cranberry carbohydrates are typically limited to known mono- and di-saccharide components using comparisons to standards (Marlett & Vollendorf 1994).

The objectives of these studies were therefore to characterize additional components of cranberry that could contribute to anti-adhesion activity and to determine if cranberry is potentially the source of the oligosaccharides found in active urine fractions. This objective was based on the outcome of the studies discussed in Chapter 2 and on the newly formed hypothesis that complex carbohydrates with anti-adhesion properties are present in cranberry materials and are related to the identified urinary metabolites.

These studies used primarily chemically-guided fractionation methods to focus on the isolation of compounds that could be similar to putative active urinary metabolites. Cranberry fractions were submitted to the previously used UPEC-HRBC anti-agglutination assay and to a newly-developed assay that assessed the ability of uropathogenic P-fimbriated *E. coli* (UPEC) to adhere to uroepithelial cells (UECs).

B. Materials & Methods

1. *General Experimental Equipment*

Lyophilization was performed using a variety of Labconco FreeZone Systems housed in the NCNPR and Faser Hall at the University of Mississippi. All sample materials were stored at –20 or –60 ºC. The centrifuge used was a Thermo IEC Centra CL3R instrument with exchangeable holders for different sizes of Falcon tubes. Preparative and analytical HPLC separations for CJE fractions were performed on a Waters Delta Prep 4000 system with #5 pump heads and a Prep LC Controller connected to a 2487 dual-wavelength UV detector. Analytical HPLC separations for most samples were performed on a Waters Alliance 2695 Separations Module with a 996 PDA connected in series to a Polymer Laboratories PL-ELS2100 analytical scale ELSD. Preparative HPLC separations for CJ-P1 samples were performed on a Waters Delta Prep 4000 system with #5 pump heads and a Prep LC Controller connected in series to a 2487 dual-wavelength UV detector and a Polymer Laboratories PL-ELS1000 ELSD. Additional analytical and preparative HPLC separations were performed on a Waters Delta Prep 600 with a Waters 600 Controller and a Waters 996 PDA detector connected in series to a Softa 300S ELSD. Two different Bruker Avance III 400 MHz NMR instruments were used to record NMR spectra and both were housed in Faser Hall at the University of Mississippi, School of Pharmacy. They were equipped with Ultrashield^{TM} and Ultrashield^{TM} Plus magnets and 5 mm probes.

Regular solvents, TLC plates, HPLC solvents, and most reagents were obtained from Fisher Scientific, Inc., Sephadex LH-20 and additional reagents and were obtained from Sigma-Aldrich, Inc., and HPLC sample filters and Amicon Ultra MW cut-off filters were obtained

from Millipore, Inc. Water used for all experiments was obtained from ultrafiltration systems located in Faser Hall or the NCNPR. Water for HPLC was additionally filtered within two days prior to use.

2. *Materials*

Spray-dried cranberry juice powder (CJ) was supplied by Ocean Spray Cranberries, Inc. through NIH grant no. 1R21AT002076-01. This same material was used for swine feeding to generate the urine samples discussed in Chapter 2. Cranberry juice concentrate (CC) was also supplied by Ocean Spray Cranberries, Inc. This material is used in the cranberry industry for preparation of various cranberry juice products. Commercially produced cranberry dietary supplements (Table 29) and juices (Table 30) were purchased at local grocery stores. Color images and additional label and product information for these materials can be found in Appendices A & C (color plates: Figures 98–101; Appendices C-2 – C4).

Table 29. Descriptions of cranberry dietary supplements obtained from local grocery stores. The information included in this table was derived from the label information provided with each product. Examples of these labels and associated dietary supplement product literature can be found in Appendix C. Images of these products can be found in Appendix A color plates: Figure 98.

Brand Name	Form	Cranberry Ingredient	Label Description	Observations
Finest Natural (FN)	softgel capsule	fruit powder concentrate	140 mg/capsule from 12:1 concentrate: equivalent to 1680 mg fruit powder/capsule	Dark purple-mauve goo
Nature's Bounty (NB)	softgel capsule	fruit powder concentrate	40 mg/capsule from 50:1 concentrate: equivalent to 2000 mg fruit powder/capsule	Dark pink goo
Sundown Naturals (SN)	powder capsule	fruit powder	475 mg/capsule	Dry, bright pink powder
Nature Made (NM)	softgel capsule	fruit extract	450 mg/capsule from 15:1 concentrate of a fruit extract	Undescribed extraction method; pinkish-mauve goo

Table 30. Descriptions of cranberry juices obtained from local grocery stores.
The information shown below was derived from the label of each product. These labels and images of the juice material can be found in Appendix A color plates: Figures 100, 101.

Brand Name	Label Name	Total Juice Content (%)	Ingredients Listed[a]
Best Choice[b] (BCB)	"Cranberry Blend"	100	grape juice, cranberry juice, apple juice, natural flavors, ascorbic acid, citric acid
Great Value[b] (GVC)	"Cranberry Juice Cocktail"	30	cranberry juice, sugar, ascorbic acid
Great Value[b] (GVNS)	"No Sugar Added Cranberry Juice Blend"	100	grape juice, cranberry juice, apple juice, natural flavors, ascorbic acid
Ocean Spray (OSC)	"Cranberry Juice Cocktail"	27	cranberry juice, cane or beet sugar, ascorbic acid
Langers (LCP)	"Cranberry Plus" (with vitamins A, C & E and Calcium	100	apple juice, cranberry juice, calcium gluconate, calcium lactate, natural flavors, citric acid, fruit and vegetable juice for color, ascorbic acid, vitamin E acetate, vitamin A palmitate
Northland (NL)	"Cranberry Flavored Blend"	100	apple juice, cranberry juice, pear juice, grape juice, citric acid, natural flavor, vegetable color, fruit extracts (grape skin, blueberry, pomegranate, cranberry, red grapes and apple), ascorbic acid, vitamin A palmitate, vitamin E acetate

[a] Water was an ingredient for all products, and all juices were listed as juice concentrates. Ingredients are listed here in the order they are shown on product labels.
[b] These products were store brands specific to the grocery store company.

3. *Characterization of Cranberry Material*

a. *Molecular Weight Cut-Off Filtration*

Cranberry juice powder (CJ) was dissolved in water (5 mg/mL) and subjected to centrifugal filtration using Amicon Ultra regenerated cellulose centrifugal filters with MW cut-off sizes of 3, 10 and 30 kDa. Samples (4 mL each; 20 mg/filter tube insert) were centrifuged (4000 x g, 20 min), filtrate and retentate were transferred to separate vials, and filter inserts and centrifuge tubes were washed with water (3–4 x 0.5 mL, combined with their respective

fractions). Samples were lyophilized, weighed, analyzed by ^{1}H NMR, and submitted to the UPEC-HRBC anti-agglutination assay for testing.

b. *Acid/Base Incubation*

Aliquots (98–104 mg) of cranberry juice powder (CJ) were dissolved in water (7–10 mL) in 4 dram glass vials and titrated to pH 2, 6, or 8 using 10–100 mM NaOH, 100 mM HCl, and an electronic microscale pH meter. The final volume per sample was adjusted after titration to give final concentrations of 10 mg/mL. Samples were incubated at 37 °C with shaking for 2, 4, or 8 h. After incubation, samples were directly frozen, lyophilized, weighed, and analyzed by ^{1}H NMR spectroscopy and C_{18} HPLC-PDA. Aliquots (20–25 mg) of each sample were submitted to the UPEC-HRBC anti-agglutination assay for testing.

4. *Detection & Isolation of Cranberry Oligosaccharides*

a. *Analytical HPLC-ELSD of Cranberry Material*

Cranberry juice powder (CJ) and the aqueous fraction of this material (CJA) were examined by analytical HPLC using the same column and a similar method as used for detection of oligosaccharides in active urine fractions (AtldC18, 4.6 x 150 mm, 5µm). The method was as follows: 100% water for 5 min, 100% water to 30% MeOH over 30 min, 30% MeOH to 100% MeOH over 20 min, and 100% MeOH for 5 min, followed by a 10 min gradient from MeOH to 100% water with 20 min of re-equilibration with 100% water before injection of the next sample. Chromatograms were collected using ELS/PDA detection, with ELS conditions of a gas flow rate of 1.0 SLM, an evaporator temp of 100 °C, and a nebulizer temp of 50 °C. Max plot chromatograms (200–400 nm) were extracted from PDA data for

detection of UV-active metabolites. Samples were prepared in a single session, were not filtered due to low sample volumes, and were sequentially separated using a single method and without flow interruptions between injections. Different sample concentrations and column load volumes were used (Table 31) to preserve enriched reference materials (HF1-1 and HF2-2) and to ensure the detection of putative oligosaccharides in crude cranberry materials (CJ and CJA).

Table 31. Sample details for analytical HPLC-ELSD comparisons between CJ and HF materials.

Sample Code	Amount (mg)	Water (µL)	Conc. (mg/mL)	Injection Amount (µL; mg/inj)
CJ	6.5	300	21.7	50; 1.09
CJA	6.0	200	30.0	40; 1.20
HF2-2	1.6	160	10.0	25; 0.25
HF1-1	2.6	100	26.0	20; 0.52

b. *Preparative HPLC-ELSD of Cranberry Juice Powder*

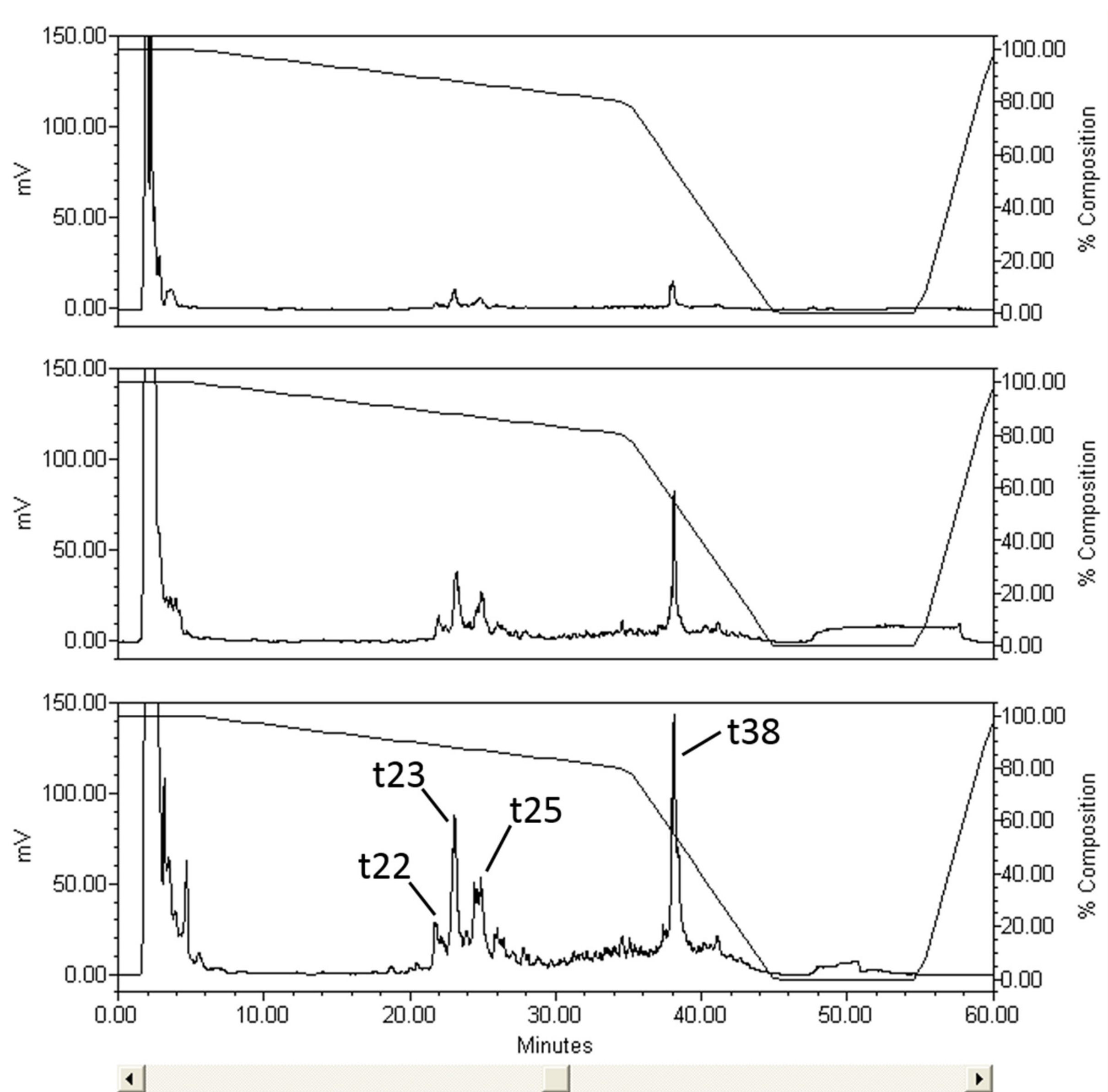
Cranberry juice powder (CJ) was directly separated using the same methods and preparative instrument configuration as used previously for urinary fraction HF2-2 separations (Chapter 2, Section B7). These methods used a preparative AtldC18 column (19 x 250 mm, 10 µm particle size) with a flow rate of 25 mL/min as follows: 100% water for 5 min, 100% water to 20% MeOH over 30 min, 20% MeOH to 100% MeOH over 10 min, 3 min at 100% MeOH, followed by a 17 min gradient from MeOH to 100% water with an equilibration of 10 min at 100% water prior to the next separation. The ELSD settings for this separation were a gas flow rate of 0.8 SLM, an evaporator temperature of 100 ^{o}C, and a nebulizer temperature of 50 ^{o}C. These ELSD values remained constant throughout the course of each separation, and the ELSD was allowed to equilibrate for at least 30 min at the appropriate settings prior to initial data acquisition.

Two batches of CJ (99.2 mg for R1 and R2, and 250 mg for R3) were dissolved in water (800 µL and 1 mL), filtered (0.2 µm nylon filter), and separated over three separations with different amounts of material injected per separation (R1: 200 µL, R2: 500 µL and R3: 1 mL). Fractions from these separations (Table 32) were combined based on chromatographic profile and time points (Figures 69–71), and were concentrated under reduced pressure with a water bath temperature of ~40 ^{o}C to remove solvent and decrease water content. Resulting samples were dissolved in water, frozen, and lyophilized. Fractions CJ-P1t22, CJ-P1t23, CJ-P1t25, and CJ-P1t38 were analyzed by ^{1}H NMR.

Table 32. Fractions collected from direct HPLC separation of cranberry juice powder (CJ-P1). Time points for fraction recombination were compared to those of urinary fractions and materials were combined across three separate injections.

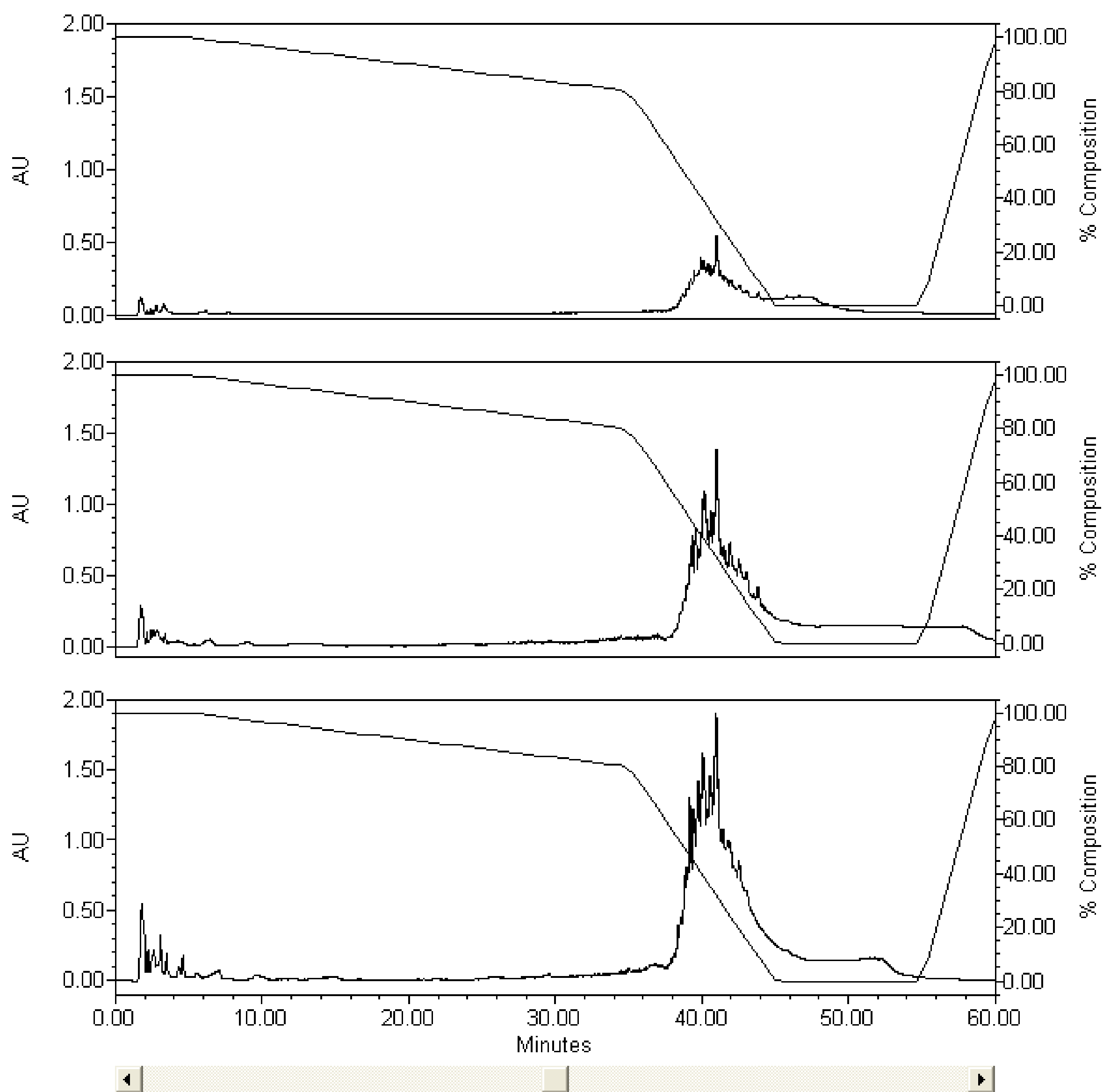
Retention Time (min)[a]	Fraction	mg	Notes
0–6	01	82.7	early eluting mass majority – mixture, UV active
6–21.5	16	9.5	no peaks – blank area before next set – some minor peaks by UV
21.5–22.7	22[b]	3.3	first short peak set in area of interest – no UV
22.8–23.7	23[b]	4.0	tallest peak of set in area of interest – no UV
23.8–25.8	25[b]	7.3	low peaks before and second tallest peak in area of interest – no UV
25.8–30	28	7.6	low peaks after three primary eluting peaks of set – mixture – no UV
30–37.7	35	12.8	wash between peak set of interest and final gradient peak
37.7–39.1	38[b]	6.5	final peak – elutes with gradient to MeOH – no or low UV – mixture
39.1–47	40	6.1	end wash – UV chromophores (pink)

[a] Time points for fraction numbers were based on CJP-crude R1.
[b] These fractions were analyzed by ^{1}H NMR spectroscopy.



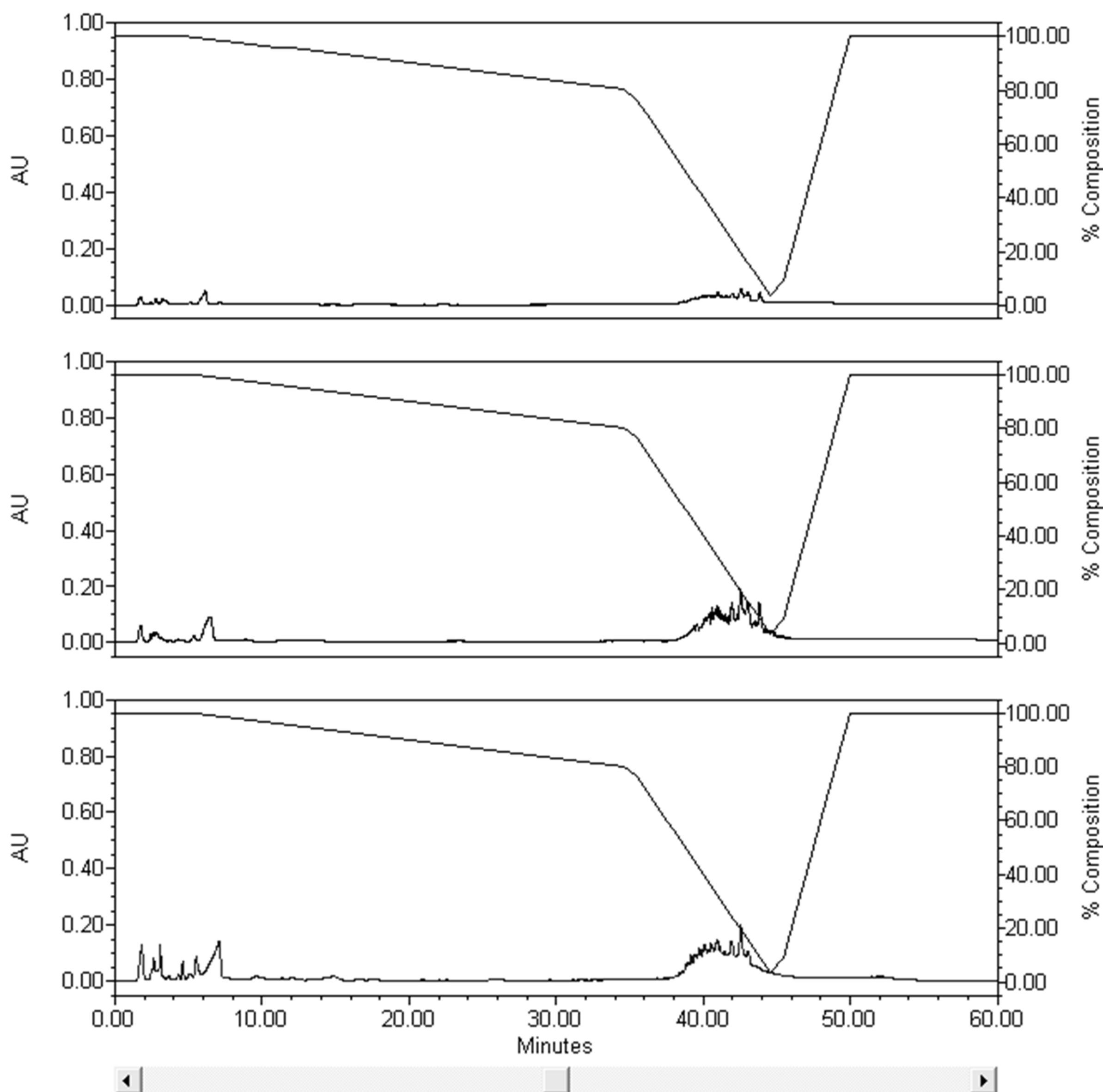
Visible	SampleName	Injection Volume (uL)	Det. Units	Date Acquired	Acq Method Set
☑	CJP-Crude AtldC18-P R1	200.00	mV	9/20/2010 3:29:28 PM CDT	Atlantis dC18 P vB
☑	CJP-Crude AtldC18-P R2	500.00	mV	9/20/2010 6:11:37 PM CDT	Atlantis dC18 P vB
☑	CJP-Crude AtldC18-P R3	1000.00	mV	9/20/2010 7:55:07 PM CDT	Atlantis dC18 P vB

Figure 69. Preparative HPLC-ELSD chromatograms of CJ-P1 separations, R1–3.
Different amounts of material were injected over the three separations, resulting in the differing intensities of the ELSD chromatograms shown. All eluting material was collected as fractions and fractions t22, t23, t25 and t38 were analyzed by NMR spectroscopy.



Visible	SampleName	Date Acquired	Injection Volume (uL)	Run Time (Minutes)	Instrument Method Name	Acq Method Set
☑	CJP-Crude AtldC18-P R1	9/20/2010 3:29:28 PM CDT	200.00	90.00	Atlantis dC18 P vB	Atlantis dC18 P vB
☑	CJP-Crude AtldC18-P R2	9/20/2010 6:11:37 PM CDT	500.00	90.00	Atlantis dC18 P vB	Atlantis dC18 P vB
☑	CJP-Crude AtldC18-P R3	9/20/2010 7:55:07 PM CDT	1000.00	90.00	Atlantis dC18 P vB	Atlantis dC18 P vB

Figure 70. Preparative HPLC-UV (215 nm) chromatograms of CJ-P1 separations, R1–3. Comparison of these chromatograms to those in Figure 69 shows the absence of chromophores for the compounds eluting from 18–38 min and the presence of weak chromophores for compounds eluting from 0–8 min. The profile of compound chromophores visible between 38 and 46 min does not appear to correlate to the profile of the fraction eluting at 38 min, suggesting that fraction CJP1t38 lacks a chromophore. The components eluting from 39–47 min showed a visible pink color in solution suggesting the presence of known anthocyanins.



Visible	SampleName	Date Acquired	Injection Volume (uL)	Run Time (Minutes)	Instrument Method Name	Acq Method Set
☑	CJP-Crude AtldC18-P R1	9/20/2010 3:29:28 PM CDT	200.00	90.00	Atlantis dC18 P vB	Atlantis dC18 P vB
☑	CJP-Crude AtldC18-P R2	9/20/2010 6:11:37 PM CDT	500.00	90.00	Atlantis dC18 P vB	Atlantis dC18 P vB
☑	CJP-Crude AtldC18-P R3	9/20/2010 7:55:07 PM CDT	1000.00	90.00	Atlantis dC18 P vB	Atlantis dC18 P vB

Figure 71. Preparative HPLC-UV (254 nm) chromatograms of CJ-P1 separations, R1–3.
The characteristics for the chromatographic profile of the CJ-P1 separations at 254 nm were similar to the chromatographic profile visible at 210 nm (Figure 70) and further supported the lack of chromophores for the compounds eluting from 18–38 min. The low intensity absorbance for the components eluting from 39–47 min further supported the conclusion that this fraction contained anthocyanins.

5. *Enrichment for Cranberry Oligosaccharides*

a. Cranberry Juice Powder Separations

Cranberry juice powder (CJ) (1.24 kg, in 80–120 g aliquots) was dissolved in water (450 mL/100 g) and extracted with EtOAc (10 x 1:3 v/v EtOAc:water). The EtOAc extracts (CJE) were combined (12.706 g; 1.02% w/w of CJ), solvent removed under reduced pressure and the residual amorphous burgundy solid was resuspended in a minimum amount of water and lyophilized. Aqueous material (CJA) was combined, residual solvent removed under reduced pressure, and the resulting burgundy liquid was diluted, frozen and lyophilized in 1–2 L batches.

The CJA and CJE fractions were both assessed using an anthocyanidin test: 1 mg of material was combined with 5 mL of isopropanol: 3 N HCl (4:1) (test samples) or isopropanol:water (4:1) (control samples) and heated for 30 min at 96 ^{o}C under pressure. Both CJE and CJA materials were used for further separations. The CJE fraction was investigated by TLC, PLC, Sephadex LH-20, HPLC with reverse phase sorbents, and derivatization via methylation and acetylation. Purified components were characterized using UV, LC-MS, and NMR. The further characterization of CJ and CJA materials will be discussed below.

The CJ aqueous residue (CJA) was fractionated on Sephadex LH-20 using protocols similar to those developed for urine samples (Table 33). The CJA samples were dissolved in water and EtOH (100%) was added gradually with intermittent sonication to give a final concentration of 70% EtOH (v/v). The sample was briefly centrifuged to remove precipitate and samples in 70% EtOH were loaded onto Sephadex LH-20 and eluted with 70% EtOH (isocratic) (Appendix A color plates: Figure 102). Columns were washed with 80% EtOH and 60–100% acetone after the majority of the fractions eluted. Fractions were combined based on

appearance and elution volume, concentrated under reduced pressure to remove EtOH, dissolved or diluted in water, frozen, and lyophilized. Selected CJA fractions were submitted for bioassay testing and further characterized by ^{1}H NMR and HPLC-ELSD/UV.

Table 33. Details of Sephadex LH-20 separations for CJA samples 1 & 2.
The recombination of fractions collected from columns CJA1 and CJA2 differed slightly and fractions were labeled with different numbers, but both columns yielded similar percentages of oligosaccharide-containing material.

Column Designation	CJA1	CJA2[a]
Amount of Loaded Material (g)	25.6	25.3
Loaded Volume & Sample Concentration	100 mL 256 mg/mL	100 mL 253 mg/mL
Column Dimensions (cm; width x height)	7 x 37	7 x 37
Solvent Composition	70% EtOH	70% EtOH
Solvent Flow Rate (mL/min)	1.5–1.7	1.5–1.7
Total Elution Volume for Oligosaccharide Fractions (mL)	300	450
Oligosaccharide-containing Fractions (g)	CJA1-02 (3.619) CJA1-02B (1.280) CJA1-03B (1.669)	CJA2-03 (2.090) CJA2-04 (3.980) CJA2-05 (1.740)
Total Oligosaccharide-containing Material per Column (g)	6.568	7.810
Oligosaccharide % of Loaded Material	25.6%	30.8%

[a] Some information for this separation is also reported in Auker 2013 and Auker *et al*. 2014.

b. *Cranberry Juice Concentrate Separations*

Cranberry juice concentrate (CC) syrup was also used as starting material for the isolation of cranberry oligosaccharides via protocols similar to those used for urine and CJ samples. Syrup (500 mL) was diluted with water (300 mL) and exhaustively extracted with EtOAc (8 x 200 mL; CCE). Aqueous residue (CCA) was lyophilized and portions were separated by Sephadex LH-20 (Appendix A color plates: Figures 103, 104). As before, CCA

samples were dissolved in water and 100% EtOH was added gradually, with sonication, to yield concentrated material in column solvent (70% EtOH). This material was briefly centrifuged to remove precipitate and separated on two different Sephadex LH-20 columns (Table 34). The columns were washed with 80% EtOH and 60–100% acetone after the majority of the fractions eluted. Fractions were concentrated under reduced pressure, dissolved or diluted with water, frozen, and lyophilized. Selected CCA fractions were submitted for bioassay testing and further characterized by ^{1}H NMR and HPLC-ELSD.

Table 34. Details of Sephadex LH-20 separations for CCA samples 1 & 2.
The recombination of fractions collected from columns CCA1 and CCA2 differed and fractions were labeled with different numbers, but both columns yielded similar percentages of oligosaccharide-containing material. The elution flow rate for column CCA2 was higher than that of column CCA1 to account for the larger diameter of this column. The CCA2 fractions yielded higher resolution between different oligosaccharide components than the CCA1 fractions.

Column Designation	CCA1	CCA2
Amount of Loaded Material (g)	50.53	151.33
Loaded Volume & Sample Concentration	200 mL 250 mg/mL	500 mL 303 mg/mL
Column Dimensions (cm; width x height)	7 x 37	10 x 49
Solvent Composition	70% EtOH	70% EtOH
Solvent Flow Rate (mL/min)	1.2–1.5	1.5–2.0
Total Elution Volume for Oligosaccharide Fractions (mL)	520	1500
Oligosaccharide-containing Fractions (g)	CCA1-01 (0.377) CCA1-02 (0.913) CCA1-03 (0.733) CCA1-03 (0.026)	CCA2-01 (0.032) CCA2-02 (0.215) CCA2-03 (1.686) CCA2-04 (3.158) CCA2-05 (1.739)
Total Oligosaccharide-containing Material per Column (g)	2.05	6.83
Oligosaccharide % of Loaded Material	4.06%	4.51%

6. *Preliminary Characterization of Commercial Cranberry Products*

Aliquots of commercial cranberry dietary supplements (Table 29) were removed from their gel capsules (one capsule per product) dissolved in water, sonicated, and left to sit for about 4 h to yield aqueous solutions (Appendix A color plates: Figure 98). Dissolved material was removed from insoluble matter with a pipette, filtered with glass wool in a glass pipette and then filtered with a 20 µm syringe filter (30 mm). Samples of CJ and CJA were prepared in a similar manner (0.2 µm filter) and analyzed at the same time for comparison. Filtered samples (Table 35) were injected as part of a single analysis set onto an analytical HPLC column (AtldC18, 4.6 x 150 mm, 5 µm, with 4.6 x 20 mm guard cartridge) and separated using a method similar to that used for enriching and detecting oligosaccharides in active urine samples. This method was as follows: 5 min 100% water, 30 min gradient to 30% MeOH, 10 min gradient to 100% MeOH, 10 min at 100% MeOH, 10 min gradient to 100% water, with 20 min of re-equilibration with 100% water before injection of the next sample. Chromatograms were collected using ELS/PDA detection with ELS conditions of a gas flow rate of 1.0 SLM, an evaporator temp of 100 ^{o}C, and a nebulizer temp of 50 ^{o}C. Max plot chromatograms (200–400 nm) were extracted from PDA data for detection of UV-active metabolites.

Samples of each commercially obtained juice were directly injected onto the HPLC-ELSD instrument with injection volumes of 50 µL each. The dry weights of each juice material corresponding to the 50 µL injection volumes are not known. All juice samples were analyzed within a single sample set using automated sample handling to avoid changes in instrument performance and retention times between injections. The same HPLC-ELSD method was used for the analysis of juice samples as was used for the dietary supplement samples and urine samples (see above).

Table 35. Sample details for analytical HPLC-ELSD comparisons of cranberry dietary supplement products.
Multiple injection amounts were used for the commercial product samples in an attempt to detect minor components. Data was not available to assess the "fruit equivalents" of the CJ materials.

Sample Code	Amount (mg)	Fruit Powder Equivalent[a] (mg)	Water (mL)	Sample Conc. (mg/mL)	Injection Amount (µL; mg/inj)	Injected Fruit Equivalent (mg)
Commercial Products						
FN	140	1680	10	14.0	25; 0.35 & 50; 0.70	4.2–8.4
NB	40	2000	5	8.0	50; 0.40 & 100; 0.8	20.0–40.0
SN	475	475	10	47.5	25; 1.20 & 50; 2.40	1.2–2.4
NM	450	6750	10	45.0	25; 1.13 & 50; 2.25	16.9–33.8
Reference Cranberry Juice Powder						
CJ	2.9	N/A	0.2	14.5	50; 0.73	N/A
CJ	6.5	N/A	0.3	21.7	50; 1.08	N/A
CJA	6.1	N/A	0.3	20.3	50; 1.02	N/A
CJA	6.0	N/A	0.2	30.0	50; 1.50	N/A

[a] Based on manufacturer labeling.

7. ***Bioassay Testing***

a. *Anti-agglutination Assay*

The UPEC-HRBC anti-agglutination assay used for cranberry-derived samples was the same as that used for urine samples (Chapter 2: Section B4). Several CJ-derived samples (Sections B3) and selected CJA1 and CJA2 fractions (Section B5) were submitted to this assay.

b. *Anti-adhesion Assay*

Selected CJA1 and CJA2 fractions were submitted to an early version of the uroepithelial cell-uropathogenic *E. coli* (UPEC-UEC) anti-adhesion assay developed at Washington State Univ. (WSU). This early version of the assay used radiolabeled *E. coli*, and the number of *E. coli* that adhered to uroepithelial cells were quantified using scintillation

counts (Mathison *et al*. 2013). Samples were tested in duplicate at different dilutions (Auker 2013).

The CCA1 fractions (~10 mg each) were submitted to a later version of the UPEC-UEC anti-adhesion assay that used fluorescent-labeled *E. coli* and uroepithelial cells according to previously established protocols (Kimble *et al*. 2014). Samples were tested using serial dilution and each sample was tested in duplicate. Duplicate results were corrected for baseline fluorescence and averaged to give fluorescence values for each dilution tested. Samples were tested using five to eight serial dilutions and IC_{50} values were calculated.

c. *Antimicrobial Assays*

Selected cranberry samples (CJ: 35.6 mg, CJE: 12 mg, CJA: 24.7 mg) were submitted to the antibacterial, antifungal, and antiprotozoal screening programs available at the UM National Center for Natural Products Research (NCNPR). Samples were tested according to previously reported methods (Zhang *et al.* 2012, Rahman *et al.* 2011) modified from the methods of the Clinical and Laboratory Standards Institute (CLSI), formerly the National Committee for Clinical and Laboratory Standards (NCCLS 2002a,b, NCCLS 2003, NCCLS 2006). Samples were tested against a panel of pathogens: five fungi (*Candida albicans* ATCC 90028, *C. glabrata* ATCC 90030, *C. krusei* ATCC 6258, *Cryptococcus neoformans* ATCC 90113, and *Aspergillus fumigatus* ATCC 204305), five bacteria (*Staphylococcus aureus* ATCC 29213, methicillin-resistant *Staph. aureus* ATCC 33591, *E. coli* ATCC 35218, *Pseudomonas aeruginosa* ATCC 27853, and *Mycobacterium intracellulare* ATCC 23068), and two protozoa (*Leishmania donovani* and *Plasmodium falciparum* D6 Clone)

In the antibacterial and antifungal assays, samples were tested at concentrations of 50 μg/mL. In the antiprotozoal assays, samples were tested at a concentration of 15.9 μg/mL

against *Plasmodium falciparum* and at 80 µg/mL against *Leishmania donovani*. Amphotericin B served as the positive control for the *Leishmania donovani* assay and resulted in 100% inhibition under the parameters used. Percent growth inhibition for each organism was calculated relative to positive and negative controls.

C. Results & Discussion

1. *Characterization of Cranberry Material*

The EtOAc soluble CJE fraction composed 1% w/w of the starting CJ material and the results of the anthocyanidin test indicated that PACs were present primarily in this fraction. The CJE test sample, but not the control sample or the CJA samples, produced an intense fuchsia color. This color indicated the formation of anthocyanins resulting from acid-catalyzed cleavage of leucoanthocyanidins and PACs (Berhow 2002, Fletcher *et al*. 1977). The CJE fraction also showed similar TLC R_f values when compared to PAC reference dimers, procyanidins A2 and B1 (benzene:acetone, 4:6, v/v), indicating the presence of these compounds. Sephadex LH-20 of CJE yielded 20 fractions that were further separated by PLC and HPLC and derivatized via methylation and acetylation. Purified components were characterized using UV, LC-MS, and NMR. These separations yielded a series of known flavonoids and phenolic compounds (not shown).

The CJ and CJA materials were the focus of the remaining studies discussed below. Selected samples from these studies were submitted to the UPEC-HRBC anti-agglutination assay in conjunction with the urine samples discussed in Chapter 2. The results of this assay for

the cranberry samples were evaluated using the same methods described in Chapter 2, Section B4d, to provide relative activity scores (UM Scores; Table 3). The results of the MW cut-off filtration and acid/base incubation assessments are shown below (Table 36). Additional anti-agglutination assay results for CJA Sephadex LH-20 fractions are discussed below (Section C4a). The raw data for these evaluations can be found in Appendix B-3: Tables 52, 58.

Table 36. Anti-agglutination assay results for experiments characterizing cranberry (CJ) material.
The CJ material consistently showed UPEC-HRBC anti-agglutination activity. Use of MW cut-off filters indicated that low-MW components of CJ were unlikely to contribute to the observed anti-agglutination activity.

Sample Description	UM Score[a]	Test Date
CJ (cranberry juice powder)	+ +	06/2008 x2[b]
CJ-MA3a (3 kDa retentate)	+ +	06/2008
CJ-MB3a (10 kDa retentate)	+ +	06/2008
CJ-MC3a (30 kDa retentate)	+ +	06/2008
CJ-MA3b (3 kDa filtrate)	– –	06/2008
CJ-MB3b (10 kDa filtrate)	– –	06/2008
CJ-MC3b (30 kDa filtrate)	– –	06/2008
CJ pH 2.0, 2 h	+	06/2008
CJ pH 2.0, 4 h	+	06/2008
CJ pH 2.1, 8 h	+	06/2008
CJ pH 5.9, 2 h	+ +	06/2008
CJ pH 6.0, 4 h	+ +	06/2008
CJ pH 6.1, 8 h	+ +	06/2008
CJ pH 7.9, 2 h	+ +	06/2008
CJ pH 8.0, 4 h	+ +	06/2008
CJ pH 8.2, 8 h	+ +	06/2008
CJ Native pH (2.7), 2 h	+ +	06/2008
CJ Native pH (2.7), 4 h	+ +	06/2008
CJ Native pH (2.7), 8 h	+ +	06/2008

[a] The criteria used to assign the UM Score can be found in Table 3. Raw data for these samples can be found in Appendix B-3: Table 52.
[b] Sample was tested in duplicate for this test date.

a. *Molecular Weight Cut-off Filtration*

CJ material partitioned in similar ratios across the three sizes of centrifugal filters used, with 3.25–3.5 mL of filtrate recovered from each separation. After lyophilization, the filtrate accounted for 70–75% of the total recovered sample mass. Both retentate and filtrate fractions did not lyophilized to powders, instead producing viscous goo. Filtrate samples were bright pink in color, similar to the starting material, while retentate samples were tan in color. Filter inserts also retained a slight pinkish tinge after washing. The anti-agglutination assay results indicated that the active constituents from the cranberry juice powder were concentrated into the retentate portion of the filtered samples (Table 36).

The active components of cranberry juice powder were retained and enriched by 3, 10 and 30 kDa regenerated cellulose centrifuge filters under the conditions used. Retention could have been the result of molecular size, sample concentration and filtration time, or repellant interactions between the active compounds and the filter material. The physics of centrifugal filtration dictates that the smallest molecules will pass through the filter matrix before larger molecules in solution, and the filter matrix of regenerated cellulose can bind to certain compounds (pink color) while repelling others. Hydrogen bonding between compounds in solution, yielding larger apparent MW complexes, may also have contributed to the retention of the active constituents.

b. *Acid/Base Incubation*

Cranberry juice powder in solution has a native pH of about 2.7. The adjusted pH of each sample remained the same from the beginning to the end of the incubation periods. All samples had anti-agglutination activity (Table 36). The pH 6 and pH 8 samples showed slightly higher activity than the non-incubated reference material, while the pH 2 samples showed

slightly lower activity. Incubation time did not appear to have any effects on the anti-agglutination activity profile.

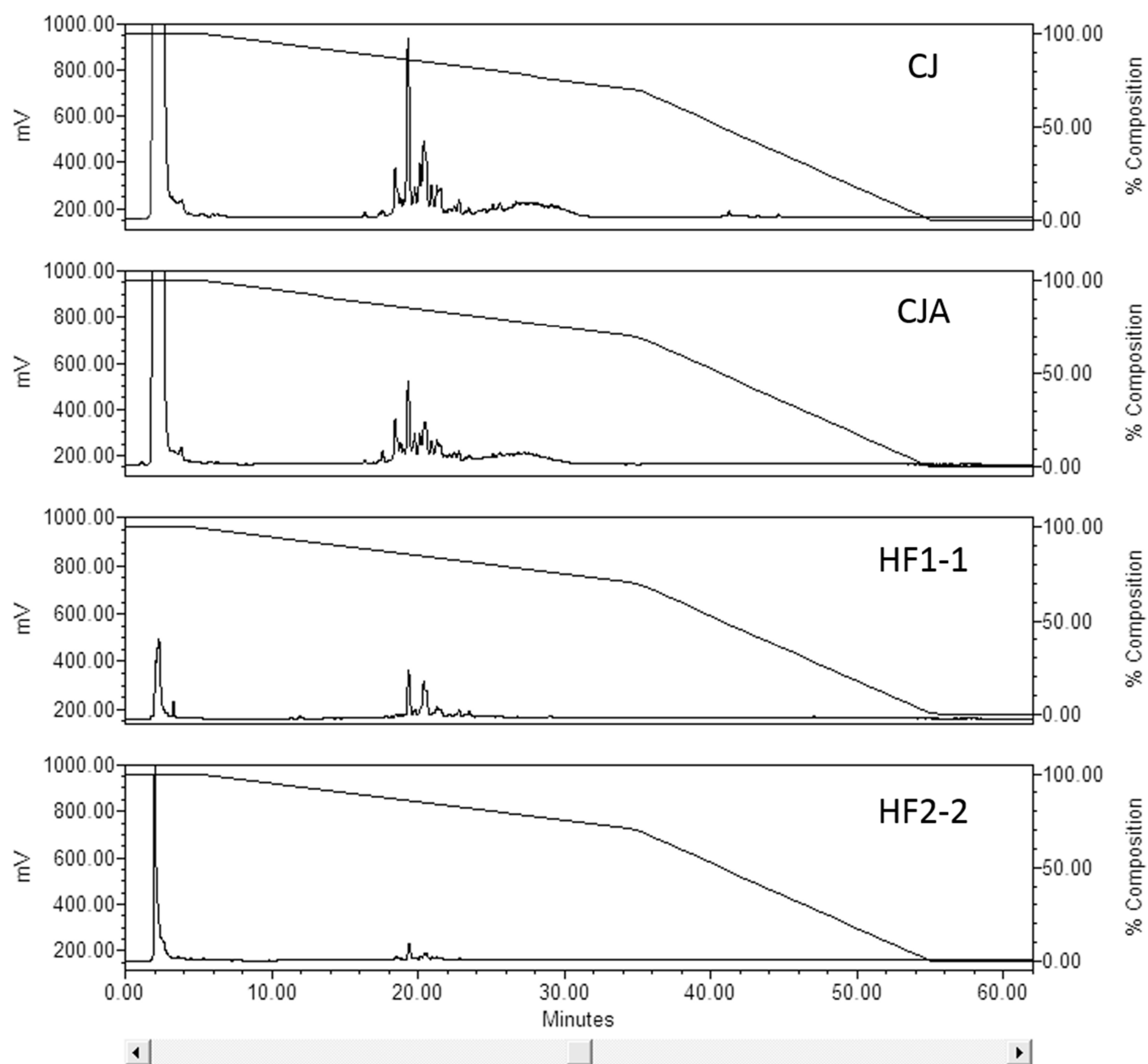
Cranberry juice powder samples were incubated at human body temperature for three time periods and three different pH values similar to those that could be experienced by cranberry juice components during digestive processes (Evans *et al*. 1988). The presence of anti-agglutination activity after incubation under these conditions is consistent with the presence of active metabolites in both cranberry and urine materials. These data indicate that the active constituents are relatively stable to moderate acid/base chemical degradation and to moderate heat and would be likely to remain intact when subjected to non-enzymatic digestive conditions.

2. *Detection & Isolation of Cranberry Oligosaccharides*

a. Analytical HPLC of Cranberry Materials

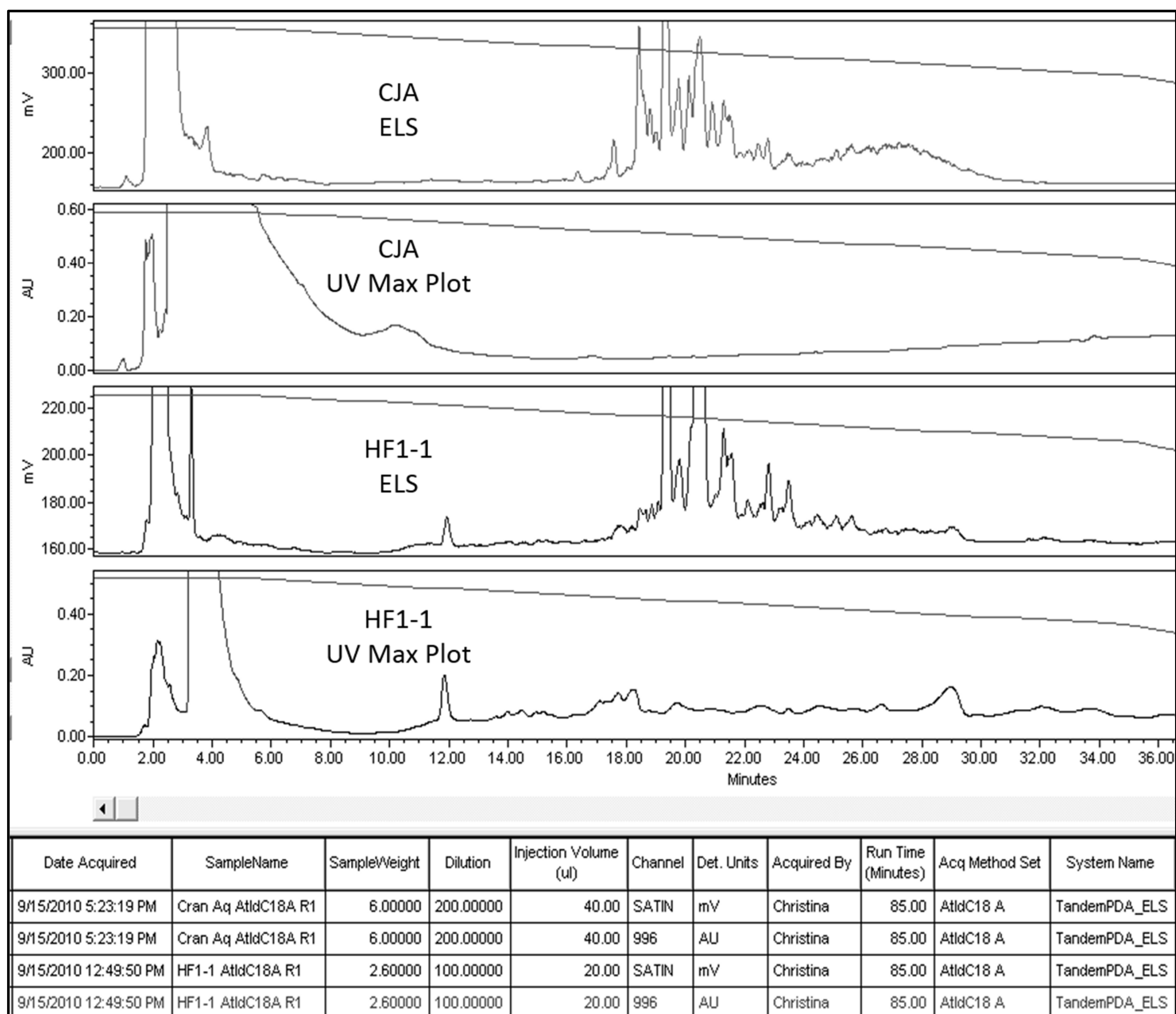
Analytical scale HPLC-ELSD analysis of cranberry starting material in comparison to active urine fractions showed that cranberry contains the same HPLC-ELSD/UV profile in the region of interest as active urine fractions (18–24 min; Figure 74). The chromatographic behavior of the cranberry material suggested that these compounds were also oligosaccharide in nature with chromophores absent for all cranberry peaks, as was seen with urine fractions (Figure 73). The elution times for the cranberry profile could be directly overlaid with those of the urine fractions and the relative ratios of the components of the mixture were similar (Figure 74). As ELSD is a semi-quantative, mass-sensitive method (see Appendix B-4: ELSD overview), this observation further supports the hypothesis that similar components are present. Although the cranberry material was injected at 2–4 x higher concentrations than the urine

fraction material (Figure 74) retention times and relative peak ratios remained similar further suggesting that the compounds present in cranberry material and urine were similar, and that the compounds present were unaffected by the cranberry sample matrix. The cranberry profile appears to be more complex than that found in urine samples, but this could be due to higher concentrations of individual metabolites present in the cranberry sample. Overall, it appears that many of the oligosaccharides present in the urine sample are directly derived from oligosaccharides present in the cranberry material, even at the same approximate concentration ratios.



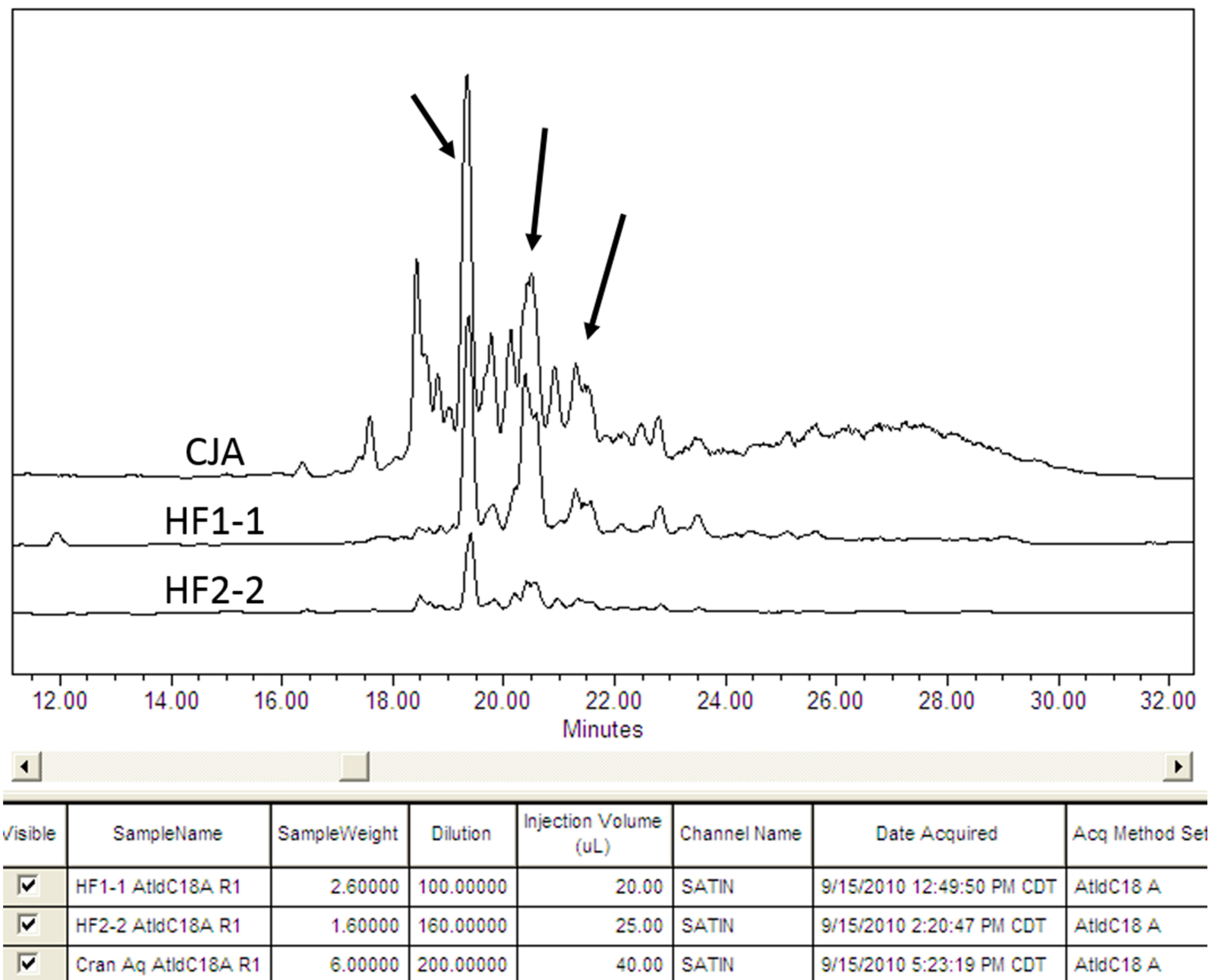
Visible	SampleName	SampleWeight	Dilution	Injection Volume (uL)	Channel Name	Date Acquired	Acq Method Set
☑	Cran Ref Powder AtldC18A R1	6.50000	300.00000	50.00	SATIN	9/15/2010 3:52:15 PM CDT	AtldC18 A
☑	Cran Aq AtldC18A R1	6.00000	200.00000	40.00	SATIN	9/15/2010 5:23:19 PM CDT	AtldC18 A
☑	HF1-1 AtldC18A R1	2.60000	100.00000	20.00	SATIN	9/15/2010 12:49:50 PM CDT	AtldC18 A
☑	HF2-2 AtldC18A R1	1.60000	160.00000	25.00	SATIN	9/15/2010 2:20:47 PM CDT	AtldC18 A

Figure 72. Analytical HPLC-ELSD comparison of cranberry material (CJ and CJA) and urine fractions (HF2-2 and HF1-1).
The same profile of oligosaccharide components was present in both urine- and cranberry-derived materials. Additional complexity was apparent in the CJ and CJA materials, possibly due to higher sample concentrations. The higher intensities of the peaks for the cranberry material also likely reflect higher sample concentrations and higher injection amounts.



Date Acquired	SampleName	SampleWeight	Dilution	Injection Volume (ul)	Channel	Det. Units	Acquired By	Run Time (Minutes)	Acq Method Set	System Name
9/15/2010 5:23:19 PM	Cran Aq AtldC18A R1	6.00000	200.00000	40.00	SATIN	mV	Christina	85.00	AtldC18 A	TandemPDA_ELS
9/15/2010 5:23:19 PM	Cran Aq AtldC18A R1	6.00000	200.00000	40.00	996	AU	Christina	85.00	AtldC18 A	TandemPDA_ELS
9/15/2010 12:49:50 PM	HF1-1 AtldC18A R1	2.60000	100.00000	20.00	SATIN	mV	Christina	85.00	AtldC18 A	TandemPDA_ELS
9/15/2010 12:49:50 PM	HF1-1 AtldC18A R1	2.60000	100.00000	20.00	996	AU	Christina	85.00	AtldC18 A	TandemPDA_ELS

Figure 73. Analytical HPLC-ELSD/UV of CJA compared to HF1-1 confirming the absence of chromophores for cranberry oligosaccharides.



Visible	SampleName	SampleWeight	Dilution	Injection Volume (uL)	Channel Name	Date Acquired	Acq Method Set
☑	HF1-1 AtldC18A R1	2.60000	100.00000	20.00	SATIN	9/15/2010 12:49:50 PM CDT	AtldC18 A
☑	HF2-2 AtldC18A R1	1.60000	160.00000	25.00	SATIN	9/15/2010 2:20:47 PM CDT	AtldC18 A
☑	Cran Aq AtldC18A R1	6.00000	200.00000	40.00	SATIN	9/15/2010 5:23:19 PM CDT	AtldC18 A

Figure 74. Analytical HPLC-ELSD of CJA compared to HF1-1 and HF2-2 (overlay). This comparative profile indicated that urine and cranberry fractions have similar relative quantities of what appear to be the same set of components. Several of these components have been highlighted (arrows) and other components present also appear to vary in similar ratios.

b. *Preparative HPLC of Cranberry Juice Powder*

Direct preparative HPLC separation of 349.2 mg of crude cranberry juice powder (CJ) resulted in a total fraction weight of 139.8 mg from the starting amount to give 40% recovery (w/w). It is possible that most of this loss was due to split-ELSD detection-collection, with additional material being retained by the sample filter, lost during transfers, lost due to loop overflow during injection, or lost due to irreversible retention on the HPLC column. From the

fractions collected, 22.2 mg of material eluted in the oligosaccharide region of interest (21.5–30 min, Figure 69) to give 15.9% of the total recovered CJ material (139.8 g) as being composed of oligosaccharides. Most of the CJ-P1 fractions contained mixtures of compounds, but the fraction at 22.8–23.7 min, designated CJ-P1t23 (4.0 mg), had similar HPLC-ELSD characteristics as **1** isolated from urine samples (Chapter 2). Comparison of this sample to that of the enriched fraction HF2-2P1t20, containing primarily **1**, via ^{1}H NMR spectroscopy confirmed the similar chemical composition of the two materials (Figure 75). Insufficient pure material of CJ-P1t23 was available for full structural characterization, but resonances in the anomeric region indicated the probable presence of an arabinoxyloglucan with eight monomer units. The presence of a series of oligosaccharides with structural composition similar to each other and to the previously isolated urinary compounds was further supported by ^{1}H NMR data of the other enriched cranberry oligosaccharide fractions (Figure 76). In particular, fractions CJ-P1t22 and CJ-P1t25 also contained resonances in the anomeric region indicative of arabinoxyloglucans. Fraction CJ-P1t38, however, yielded a ^{1}H NMR spectrum unique from those of the other CJ-P1 fractions. This spectrum indicated that CJ-P1t38 is also probably a complex carbohydrate but not an arabinoxyloglucan. Larger quantities of cranberry oligosaccharides were needed for further structural studies and bioactivity assessments, and direct preparative HPLC separations were not necessarily an optimum method for obtaining this material.

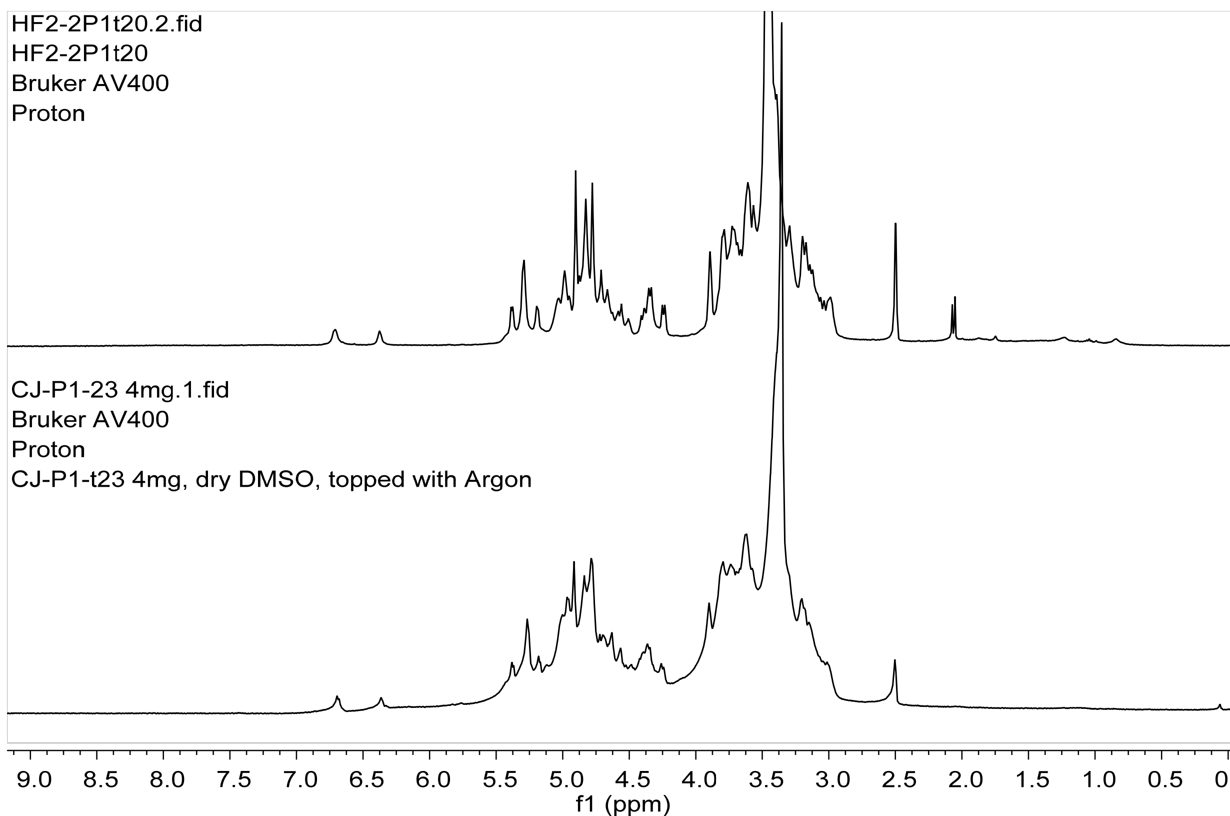


Figure 75. Comparison of the ^{1}H NMR spectra for the enriched cranberry oligosaccharide fraction CJ-P1t23 and the urinary fraction HF2-2P1t20, enriched for compound **1**, DMSO-d_6, 400 MHz.
The spectra for both fractions were highly similar indicating similar chemical composition. These data suggested that the cranberry fraction was also an arabinoxyloglucan oligosaccharide.

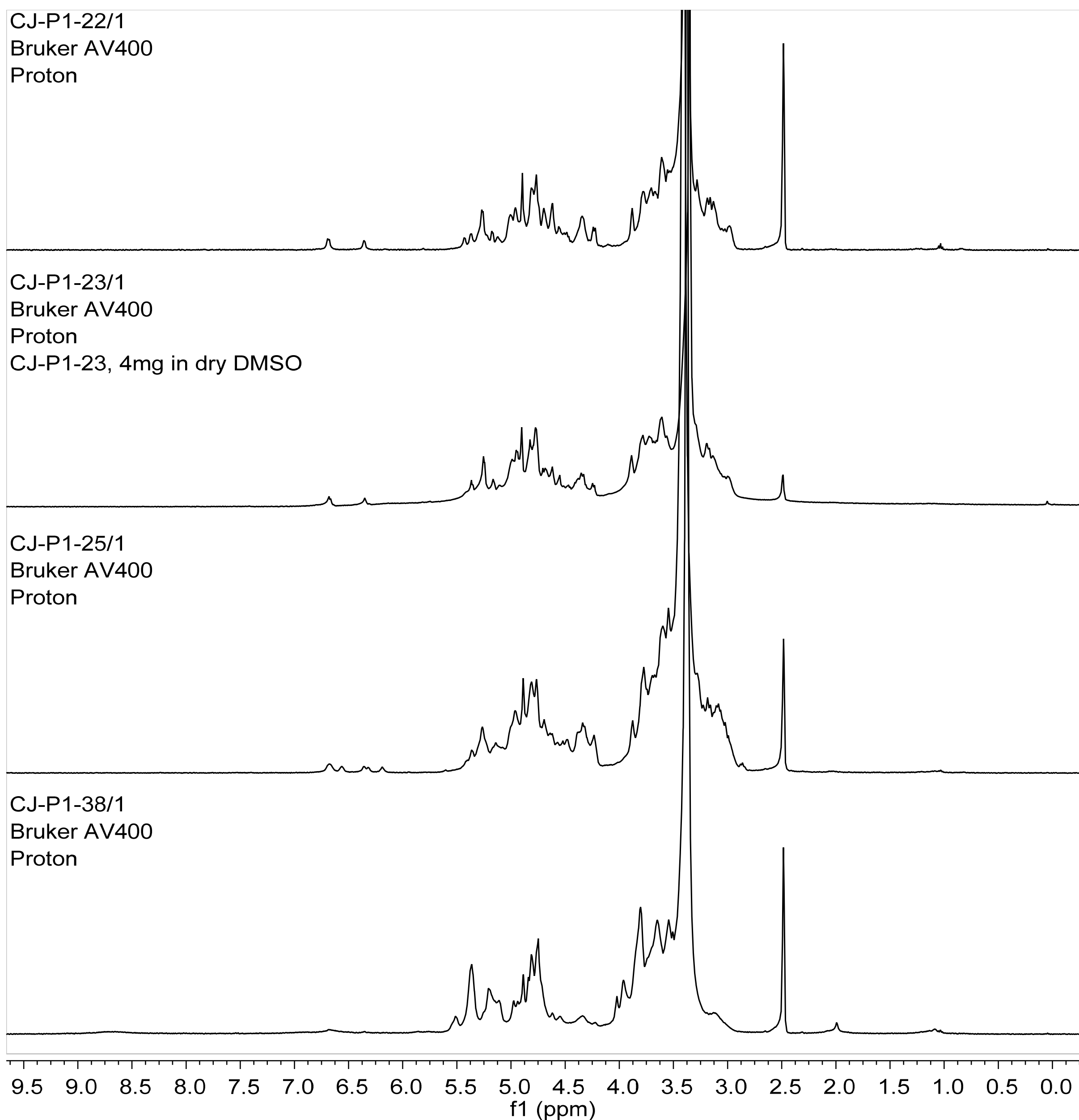
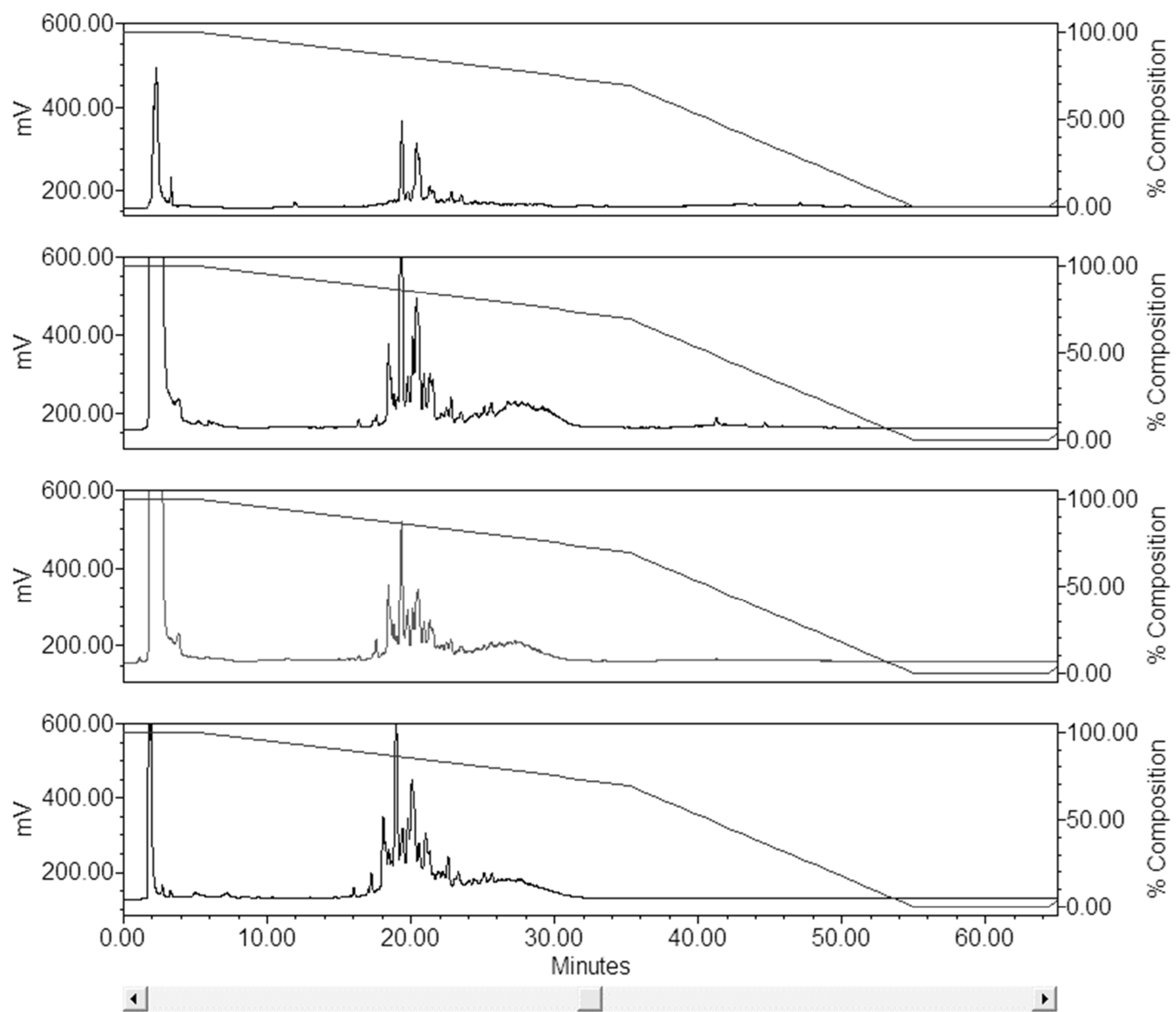


Figure 76. Comparison of the ^{1}H NMR spectra for major CJ-P1 oligosaccharide fractions, DMSO-d_6, 400 MHz.
Fractions CJ-P1-22, CJ-P1-23, and CJ-P1-25 all contained resonances similar to those of arabinoxyloglucan oligosaccharides. The spectrum of CJ-P1-38 was distinct and is probably not an arabinoxyloglucan, but is likely to be another type of oligosaccharide.

c. *Cranberry Juice Powder Separations*

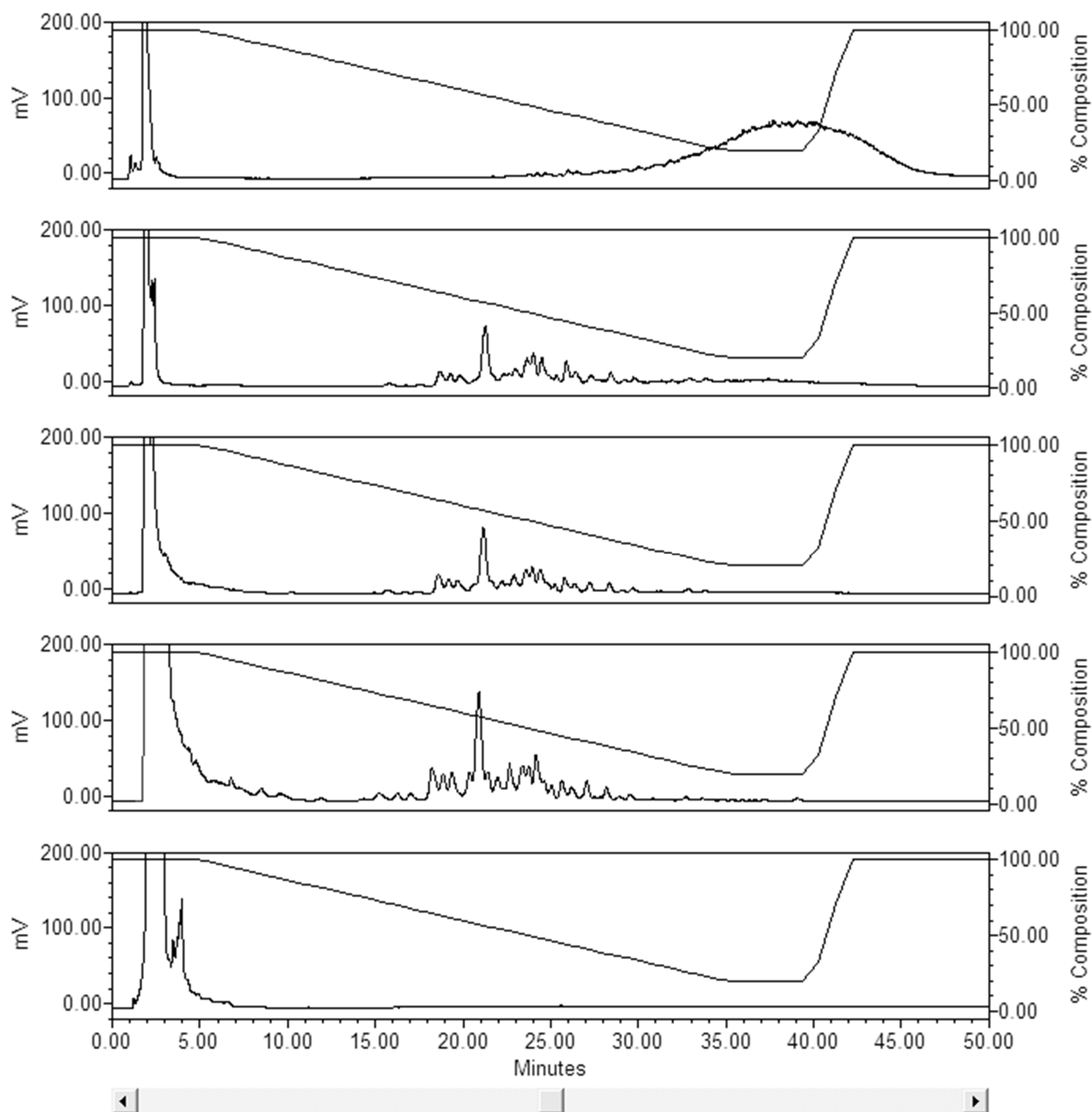
The CJ material was processed and separated using Sephadex LH-20 via methods similar to those applied to urine samples. The color of fractions can be used to track the progressive elution of compounds and shows that the majority of the pigmented components of the juice elute at later retention times (Appendix A color plates: Figure 102). Later fractions (CJA1-04 – CJA1-06) are also more hygroscopic, probably due to the presence of high concentrations of simple sugars such as fructose. Fractions of CJA that eluted early, at similar retention times as active urine fractions (*e.g*. CJA2-03), were found to contain oligosaccharide mixtures of similar composition as active urine fractions by HPLC-ELSD (Figures 77–79) and NMR spectroscopy (Figure 80). The appearance of CJA oligosaccharide fractions was cloudy white to pale lavender in appearance in 70% EtOH, and these fractions eluted just before or in conjunction with the visible lavender band that appeared on the column (CJA1 column; Appendix A color plates: Figure 102). Changes in both the HPLC system and the ELS detector resulted in lower-resolution chromatography and different retention times for some fractions than obtained previously, but UV-transparent oligosaccharides could still be detected (20–30 min; Figure 79).

Fraction CJA2-03 served as part of the source material for the further isolation of two oligosaccharides in sufficient purity and quantity to assign structures (Auker 2013) (Figure 81). Methods used to make these assignments included NMR and carbohydrate derivatization methods similar to those discussed in Chapter 2 Methods.



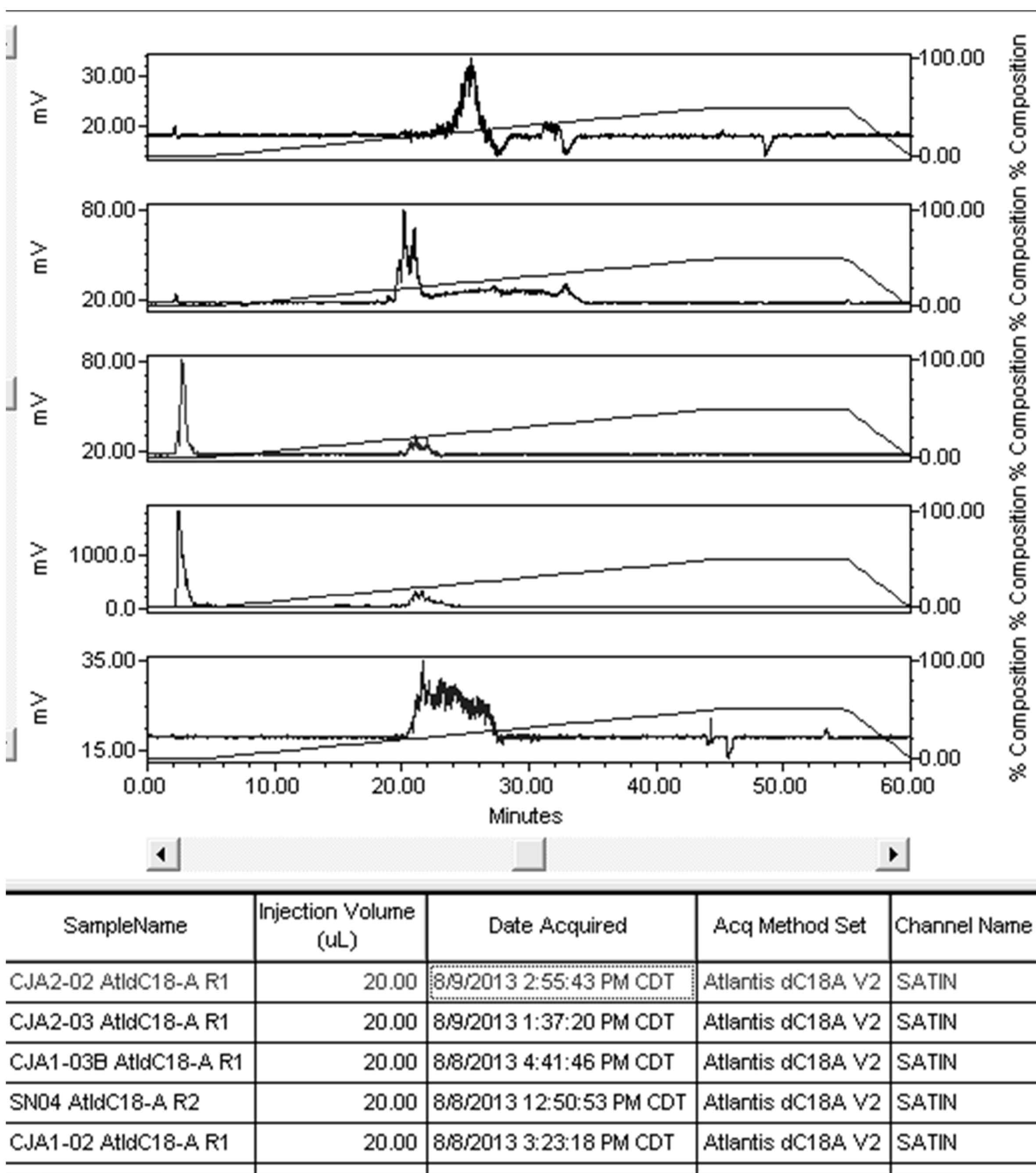
isible	SampleName	Date Acquired	Acq Method Set	SampleWeight	Dilution	Injection Volume (uL)	Channel Name	Processed E
☑	HF1-1 AtldC18A R1	9/15/2010 12:49:50 PM CDT	AtldC18 A	2.60000	100.00000	20.00	SATIN	Christina
☑	Cran Ref Powder AtldC18A R1	9/15/2010 3:52:15 PM CDT	AtldC18 A	6.50000	300.00000	50.00	SATIN	Christina
☑	Cran Aq AtldC18A R1	9/15/2010 5:23:19 PM CDT	AtldC18 A	6.00000	200.00000	40.00	SATIN	Christina
☑	CJA2-03 AtldC18 R1	5/4/2011 2:28:16 AM CDT	AtldC18 A	2.20000	100.00000	30.00	SATIN	Mohamed

Figure 77. Analytical HPLC-ELSD comparison HF1-1, CJ, CJA, and CJA2-03 (AtldC18) showing the similarity of oligosaccharide profiles present in all four samples.



Visible	SampleName	SampleWeight	Dilution	Injection Volume (uL)	Channel Name	Date Acquired	Acq Method Set
☑	CJA2-02W AtldC18-A R2	2.90000	145.00000	25.00	SATIN	3/8/2011 5:30:53 PM CST	AtldC18 A v4
☑	CJA2-03W AtldC18-A R2	2.30000	115.00000	25.00	SATIN	3/8/2011 7:07:12 PM CST	AtldC18 A v4
☑	CJA2-04W AtldC18-A R2	3.80000	190.00000	25.00	SATIN	3/8/2011 8:43:31 PM CST	AtldC18 A v4
☑	CJA2-05W AtldC18-A R1	1.00000	150.00000	25.00	SATIN	3/9/2011 3:08:55 AM CST	AtldC18 A v4
☑	CJA2-06W AtldC18-A R1	2.60000	130.00000	25.00	SATIN	3/7/2011 9:39:47 PM CST	AtldC18 A v4

Figure 78. Analytical HPLC-ELSD of CJA2 fractions 02, 03, 04, 05, and 06 (AtldC18) showing the resolution and composition of these Sephadex LH-20 fractions. Oligosaccharide components of interest are present in fractions 03, 04, and 05.



SampleName	Injection Volume (uL)	Date Acquired	Acq Method Set	Channel Name
CJA2-02 AtldC18-A R1	20.00	8/9/2013 2:55:43 PM CDT	Atlantis dC18A V2	SATIN
CJA2-03 AtldC18-A R1	20.00	8/9/2013 1:37:20 PM CDT	Atlantis dC18A V2	SATIN
CJA1-03B AtldC18-A R1	20.00	8/8/2013 4:41:46 PM CDT	Atlantis dC18A V2	SATIN
SN04 AtldC18-A R2	20.00	8/8/2013 12:50:53 PM CDT	Atlantis dC18A V2	SATIN
CJA1-02 AtldC18-A R1	20.00	8/8/2013 3:23:18 PM CDT	Atlantis dC18A V2	SATIN

Figure 79. Analytical HPLC-ELSD of CJA1 and CJA2 oligosaccharide-containing fractions in comparison to SN-04 (% MeOH shown).
These fractions were analyzed using an HPLC instrument that produced lower chromatographic resolution and the Softa ELS detector. This instrument configuration was sufficient for detecting oligosaccharide content by comparisons to fractions with previously known oligosaccharide content, but instrument parameters and the chromatographic system shown here would need to be further optimized for preparative separations.

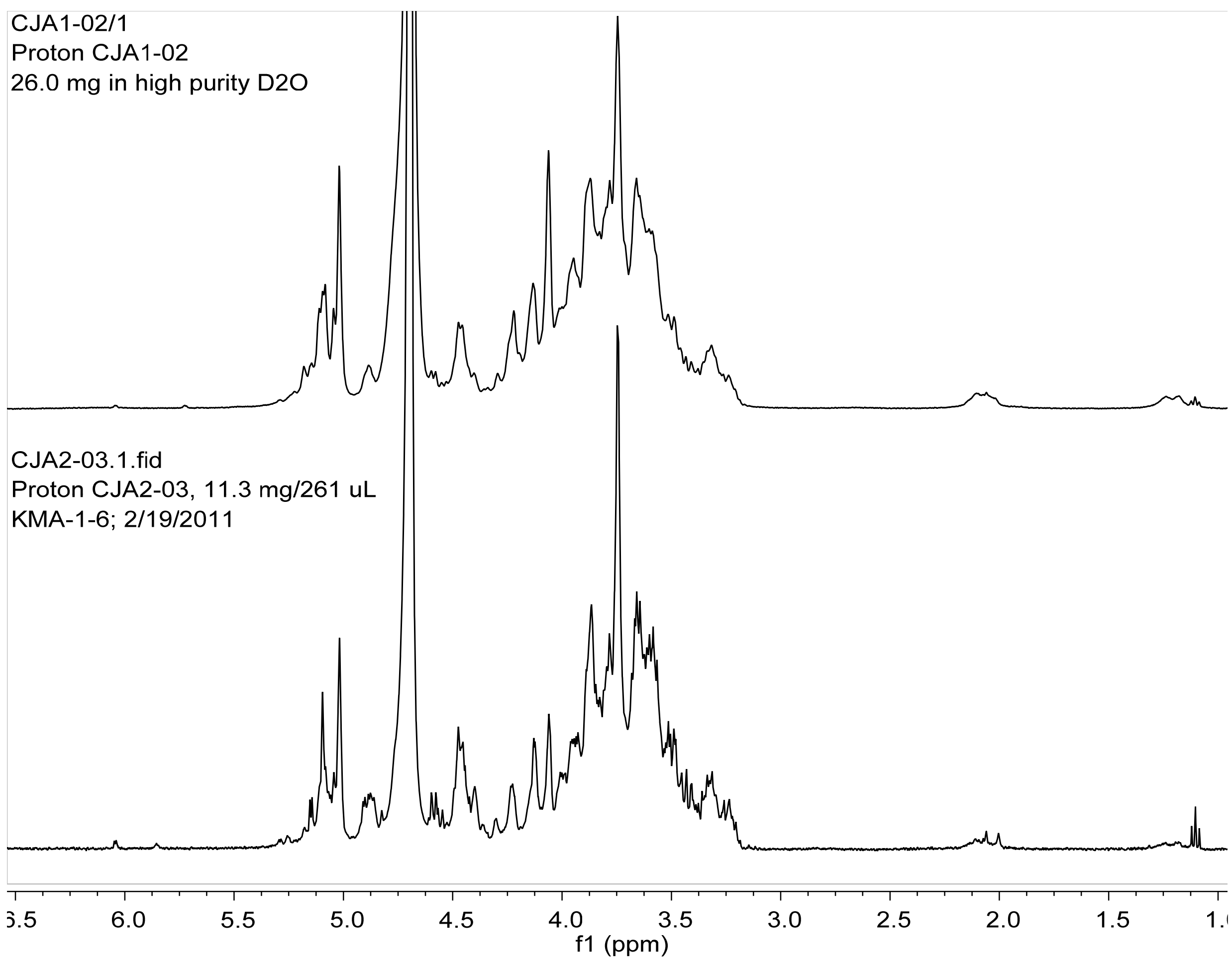
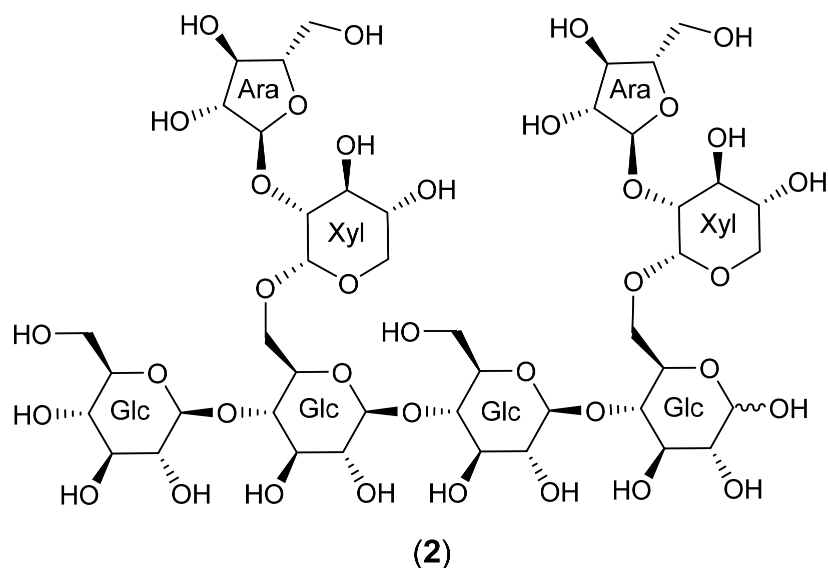


Figure 80. Comparison of the ^{1}H NMR spectra for fractions CJA1-02 and CJA2-03, D_2O, 400 MHz.
These spectra show the similarity of composition for the oligosaccharide-enriched fractions from both CJA1 and CJA2 columns. These spectra do not contain resonances in the regions that are not displayed.



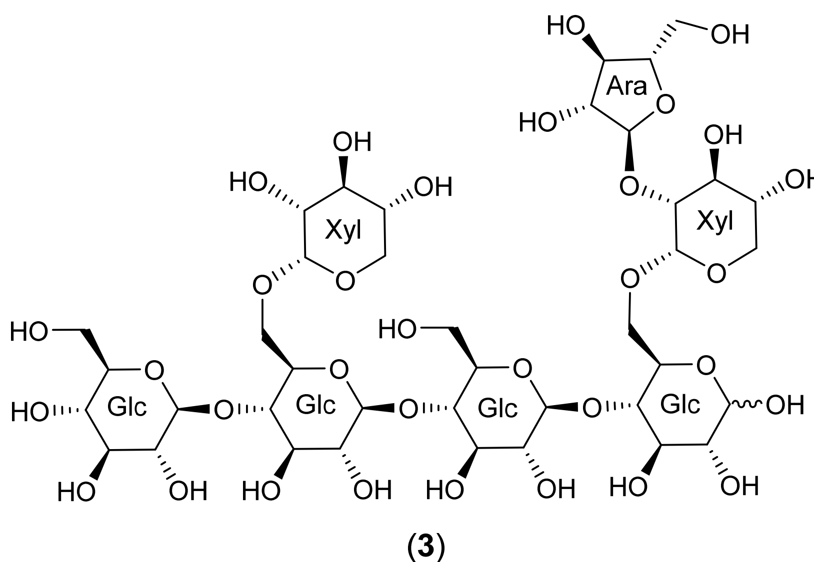


Figure 81. Structures of the two oligosaccharides isolated from fraction CJA2-03 (Auker 2013). These compounds are highly similar in structure to that of **1**, discussed in Chapter 2.

d. *Cranberry Juice Concentrate Separations*

The aqueous fraction of CC material (CCA) yielded early eluting sub-fractions from both CCA1 and CCA2 Sephadex LH-20 columns that were composed of oligosaccharides. As with the CJA separations, these fractions were cloudy white to pale lavender in appearance in 70% EtOH, and eluted just before the visible lavender band that appears on the column (CCA2

column; Appendix A color plates: Figure 103). Fractions 1–3 from column CCA1 (CCA1-01, CCA1-02, CCA1-03) contained mixtures of penta- to octameric oligosaccharides as determined by the number and integration of anomeric resonances observed in ^{1}H NMR spectra (Figure 84). The components of these three fractions are similar in structure and have similar HPLC retention times and profiles as those isolated previously from CJ cranberry materials (CJ and CJA) and CJ-derived urine samples (Figure 82). Fraction CCA1-04 also contained oligosaccharides, but ^{1}H NMR and HPLC-ELSD data suggest differences in monosaccharide composition and branching patterns as compared to fractions CCA1-01 – CCA1-03. Fractions CCA1-05 – CCA1-12 contained a variety of other classes of compounds and lacked the oligosaccharide components of interest. Fractions CCA1-01 – CCA1-03 accounted for a total of ~2 g of material and ~4% w/w of the loaded aqueous cranberry extract. Column 2 (CCA2) yielded 19 fractions with improved resolution of oligosaccharide components across fractions CCA2-01 – CCA2-05 as determined by ^{1}H NMR spectroscopy. The oligosaccharide fractions of interest from CCA2 separations accounted for ~7 g of material and ~5% w/w of the loaded CCA sample.

Changes in both the HPLC system and the ELS detector resulted in lower resolution chromatography than obtained previously for the CCA1 fractions (Figures 82, 83). Use of the same HPLC column and method, and comparisons to other fractions with previously established oligosaccharide composition still made it possible, however, to detect the presence of the UV-transparent oligosaccharides of interest, eluting between 12–18 min, in CCA fractions (Figures 82, 83).

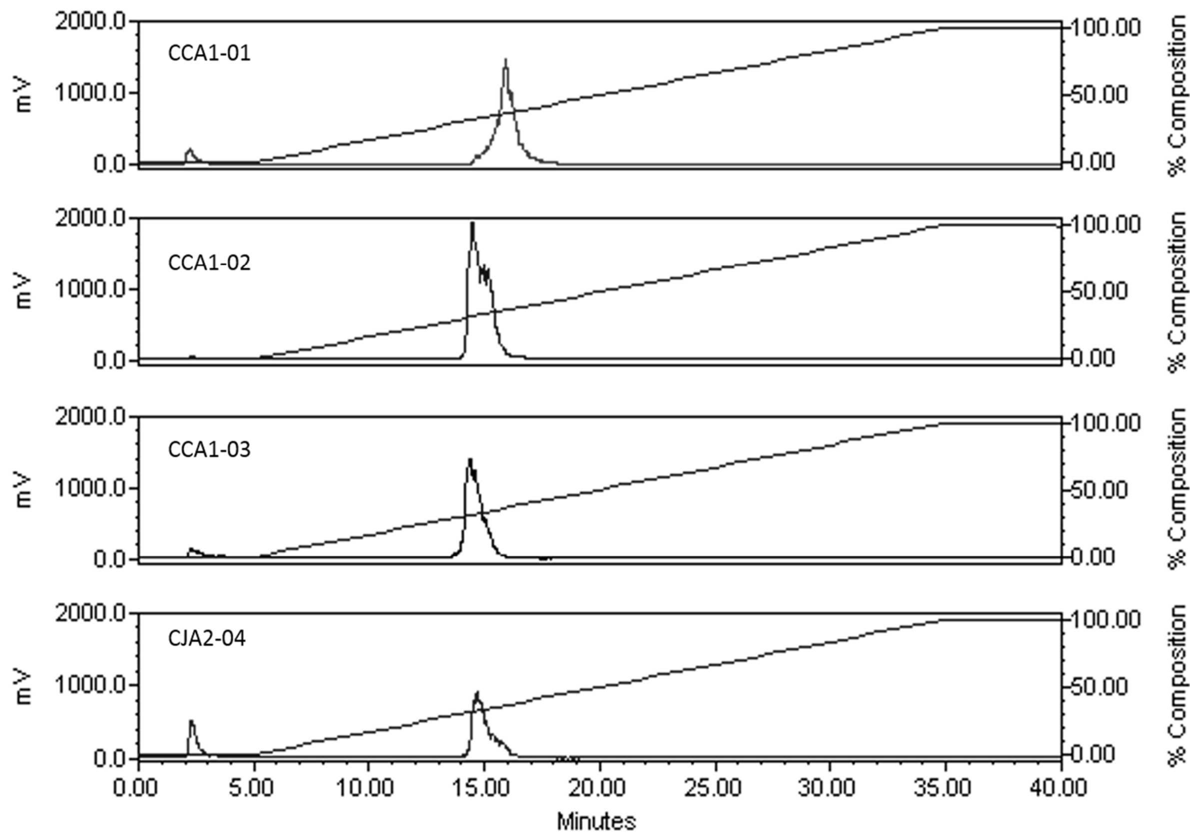


Figure 82. Analytical HPLC-ELSD of CCA1 fractions 01, 02, and 03, compared to CJA2-04 showing a similar elution profile (AtldC18, % MeOH shown).
The chromatographic resolution for these separations is lower than that shown previously due to changes in the instruments used. These materials were highly enriched for oligosaccharides as evidenced by the lack of other compounds that eluted during the full gradient. From previous chromatographic analyses, glucose, fructose and sucrose were determined to elute at < 5 min with the conditions used (See Appendix B-5: Figure 105). The minimal quantity of compounds eluting at this retention time for fractions CCA1-01 – CCA1-03 indicates that these samples are unlikely to contain monosaccharide contaminants such as glucose.

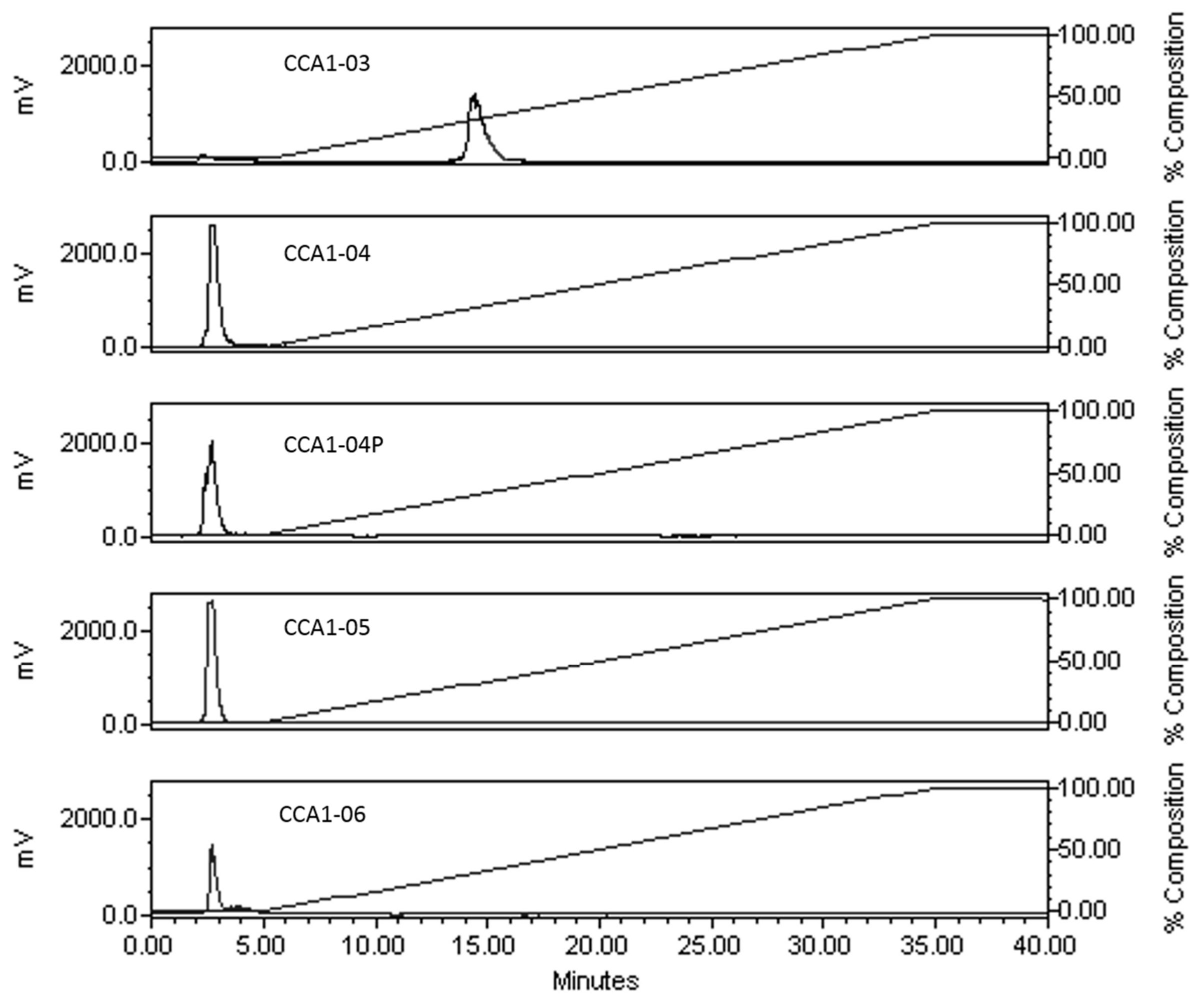


Figure 83. Analytical HPLC-ELSD of CCA1-03, 04, 04P, 05, and 06 showing the absence of oligosaccharide components in the CCA1-04 and later Sephadex LH-20 fractions (AtldC18, % MeOH shown).

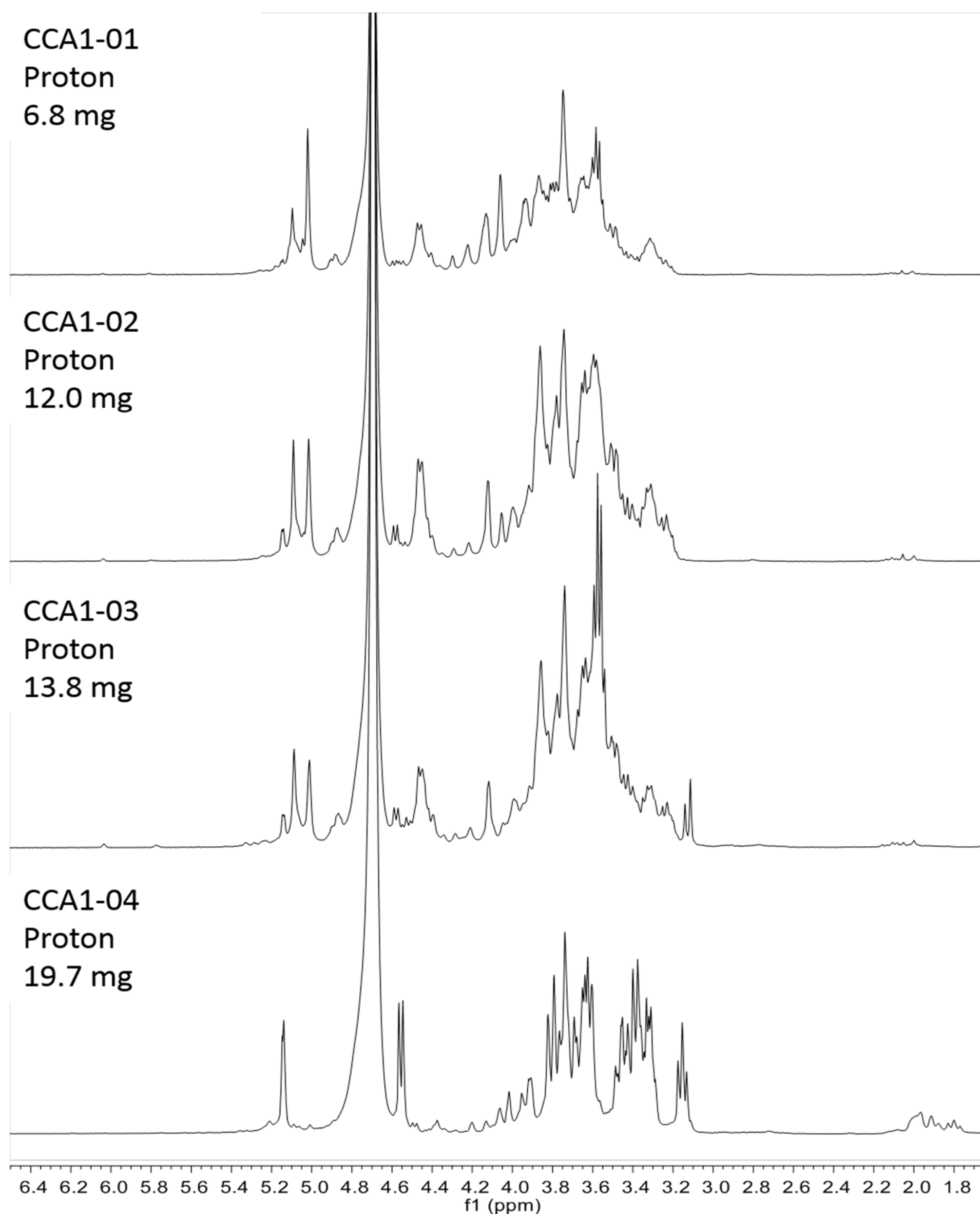


Figure 84. Comparison of the ^{1}H NMR spectra for fractions CCA1-01, CCA1-02, CCA1-03, and CCA1-04, D_2O, 400 MHz.

Fractions CCA1-01, CCA1-02 and CCA1-03 were determined to probably contain arabinoxyloglucan oligomers based on the presence of indicative resonances at 4.4, 5.0, 5.1, and 5.2 ppm. These arabinoxyloglucan mixtures are likely composed of penta- to octameric oligosaccharides as determined by the number and integration of these observed anomeric resonances. Fraction CCA1-04 does not contain the indicative anomeric resonances and instead appears to contain only a reducing glucose moiety based on the presence of α- and β-anomeric resonances at 5.15 and 4.55 ppm, respectively. Additional analyses would be required to determine the full composition and structural assignments for each of these fractions.

3. *Preliminary Characterization of Commercial Cranberry Products*

An optimized HPLC-ELSD method was used to detect oligosaccharides in commercial cranberry products. The semi-quantitative nature of ELSD (see Appendix B-4: ELSD overview) makes it possible to assess the relative ratios and concentrations of components across and within samples, regardless of the nature of the compounds. Direct quantification of oligosaccharides was not possible during these studies due to the lack of available standards, the complexity of the mixture, and instrument and detector limitations. Previous studies with urine samples (Chapter 2) using the same equipment and method indicated, however, that this system could be used to detect oligosaccharides in 20 µL samples at concentrations of less than 10 mg/mL (Table 35; Figure 72). The use of a full gradient from 100% water to 100% MeOH allows for the elution of the majority of compounds present, with polar compounds eluting earlier (< 10 min) and nonpolar compounds eluting later (35–55 min) within a chromatogram.

Possible evidence of oligosaccharides was detected in one of the four commercial cranberry dietary supplement products analyzed, the SN fruit powder (Figure 85). Of the samples tested, the SN product appeared to have the least amount of processing. Based on labeling (Appendix C-2), SN was a powder obtained through direct lyophilization of cranberries. It was therefore expected that it would contain components seen in the cranberry juice powder (18–30 min) as well as additional compounds (eluting between 4–18 min). When in solution, the SN material was different in appearance than the CJ material (Appendix A color plates: Figure 99), but this is likely due to the presence of fruit-derived components that had been removed during juice manufacturing. Further work with 100 g of the SN material in conjunction with CJA2 material resulted in the identification of two arabinoxyloglucan oligosaccharides from both sources (Master's Thesis of Kimberly Auker 2013).

Evidence of oligosaccharides was not apparent in three of the four dietary supplement products analyzed: FN, NB, and NM (Figure 86). Based on the label claims of these products (Table 29, Appendices C-3, C-4), the amounts injected of these commercial "concentrates" should have been equivalent to much higher concentrations of cranberry metabolites (Table 35), but there was no detectable evidence of such a concentration effect. Additionally, the HPLC-ELSD profiles of the dietary supplement fruit powder concentrates were markedly different from those of the SN cranberry fruit powder and the cranberry juice powder (CJ). This was consistent with the appearance of these materials as observed during preparation for HPLC (Table 29, Appendix A color plates: Figure 98). These data suggest that manufacturer processing methods for producing "concentrates" significantly alter the composition of the final product and probably remove the oligosaccharides of interest (see Appendix C-1 discussion).

The HPLC-ELSD profile characteristic of oligosaccharides (18–24 min) was readily detected in four of the juice products analyzed: OSC, LCP, GVC, and GVNS (Figures 87–90). The other two juice products, BCB and NL also appeared to contain the profile of interest but these components were present at much lower concentrations and were almost undetectable (Figures 87, 89). All putative oligosaccharide components were minor constituents relative to the rest of the material present in the samples, and they could not be detected without baseline expansions (Figures 88, 90). The putative oligosaccharide components of LCP and OSC materials were similar to each other in appearance and relative concentrations (box; Figure 90). The GVC and GVNS profiles were also similar to each other, with lower concentrations of metabolites in the region of interest (18–24 min) for the GVNS material (box; Figure 88). The juice chromatograms for GVC, GVNS, OSC, and LCP were similar to each other from 18-48 min with differences in relative concentrations.

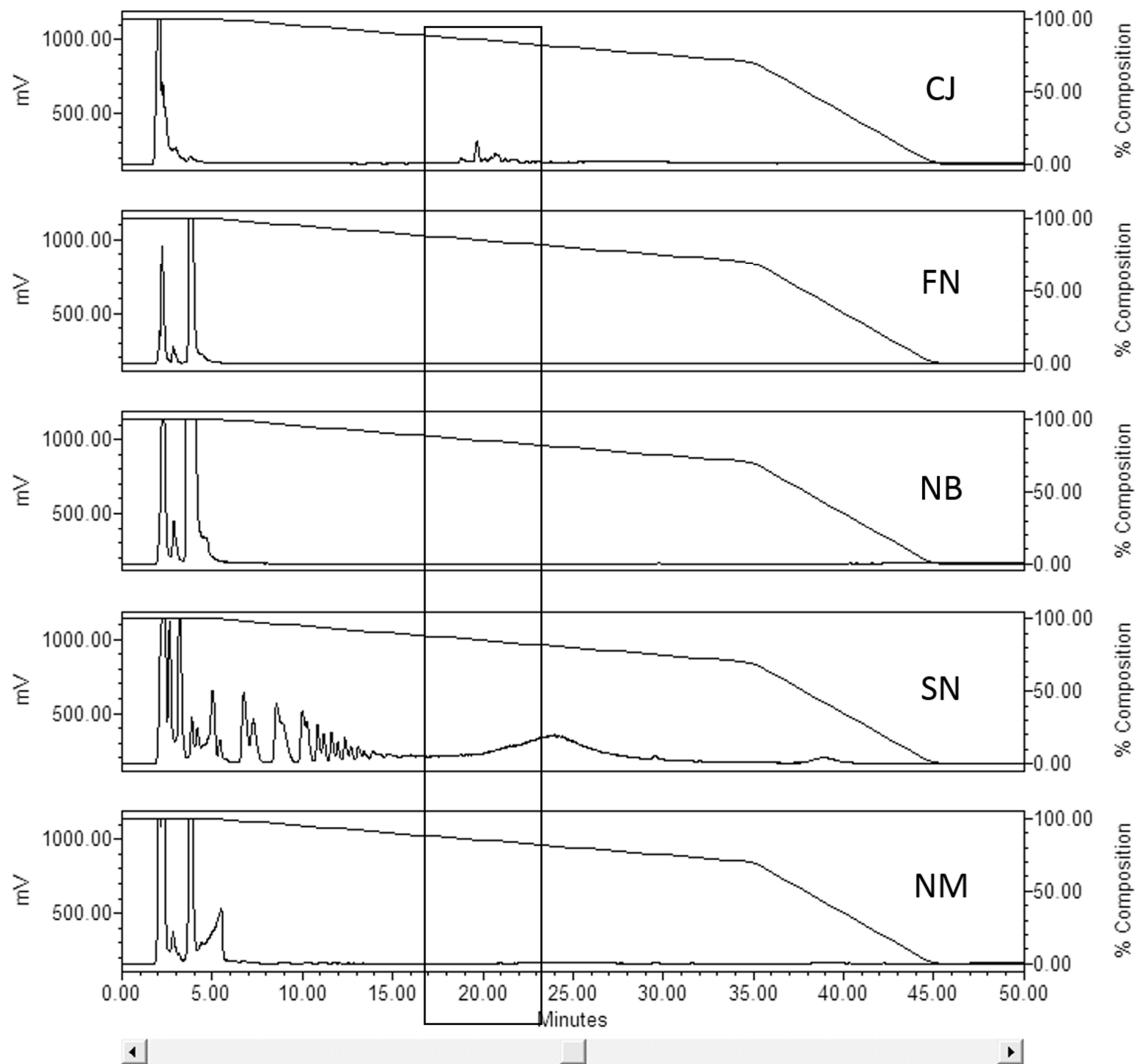
The similarity of oligosaccharide profiles across juice brands indicates the presence of similar constituents, presumably due to the presence of cranberry juice concentrate in each product. As the same amount of each juice sample was analyzed for each product, the differences in relative concentration of putative oligosaccharides may reflect different percentages of cranberry juice in the different products. Only two of the six products analyzed indicated the percent content of cranberry juice (OSC: 27% and GVC: 30%), and both of these contained only sugar (presumably sucrose) and ascorbic acid as the other two added ingredients. These two products also had the highest detectable concentrations of oligosaccharide components. Of the other four products analyzed, cranberry juice is the second ingredient listed on the labels, indicating that it is at lower relative concentration than the first ingredient listed (either grape or apple juice concentrate). These other juice concentrates are added as a way to naturally sweeten and balance the flavors of cranberry juice, and are relatively less expensive to manufacture. Higher relative percentages of grape or apple juice therefore decrease the overall cost of the cranberry juice product for the manufacturer and provide the sweetness many consumers expect with the marketing benefit of a label claim of 100% juice. Based on the semi-quantitative results of the preliminary HLPC-ELSD analyses in these studies, it is therefore likely that the products with lower apparent oligosaccharide concentrations (GVNS and LCP) have corresponding lower overall percentages of cranberry juice as an ingredient (probably less than 27%).

All six juice products had a similar color (Appendix A color plates: Figures 100, 101), even though they differed in apparent cranberry oligosaccharide content, and therefore cranberry content, as detected by HPLC-ELSD. Products that contained grape or apple juice as the first ingredient still appeared to be the same color as cranberry-only products even though

both apple and grape juices are known to have distinct color profiles. This observation may reflect the efforts of manufacturers to ensure that their products meet consumer expectations of appearance for a juice product advertised as “cranberry juice.” The addition of “vegetable color” to products that have apple juice as the first ingredient (LCP and NL) may therefore further indicate lower relative concentrations of cranberry juice, as the resulting products without added “vegetable color” may not necessarily satisfy consumer expectations.

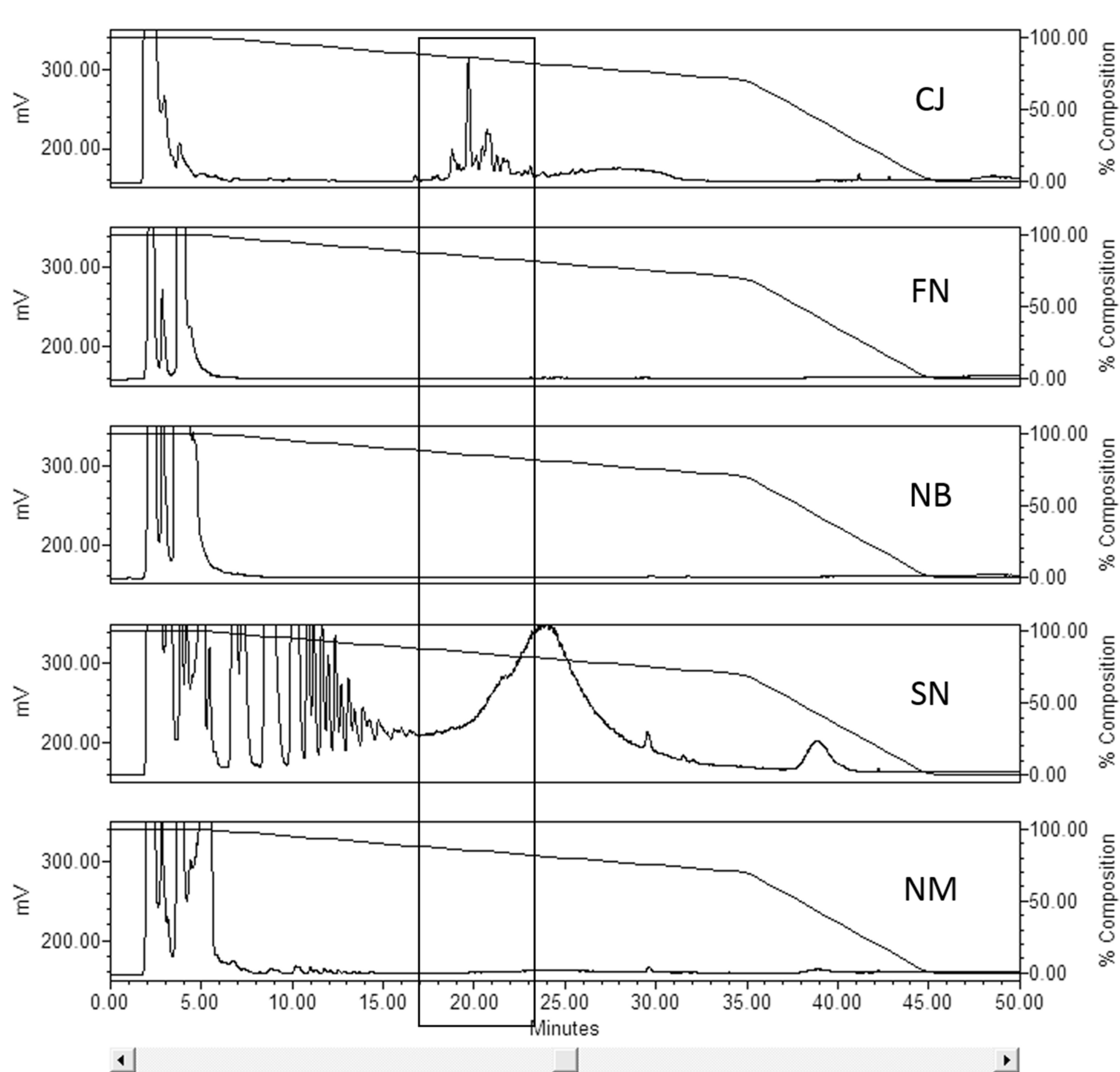
The results of the preliminary studies of commercial juice products also indicate that grape and apple juices probably lack the oligosaccharide components of interest that are found in cranberry. The primary ingredient for the BCB product is grape juice, while the primary ingredient for the NL product is apple juice. Both of these products also contain cranberry juice as a reported ingredient, but have trace (NL) or undetectable (BCB) levels of the characteristic cranberry oligosaccharide profile. These observations would support previous reports that the consumption of apple or grape juice does not lead to the production of urine with anti-adhesion properties by human volunteers (Howell *et al*. 2010).

Overall, HPLC-ELSD profiles of cranberry juice products, including those with relatively lower percentages of cranberry, were similar in elution profile (8–48 min) to the cranberry juice powder (CJ) used for swine feeding studies (Chapter 2). The juice products profiles differed greatly from those of dietary supplement products, with juices containing many more detectable constituents in both polar (early eluting) and nonpolar (later eluting) regions of the chromatograms. These results further support the hypothesis that dietary supplement products lack the oligosaccharide components of interest, while juice products retain them. Additional studies will be necessary to fully quantify and characterize the occurrence of cranberry oligosaccharides in various commercial products.



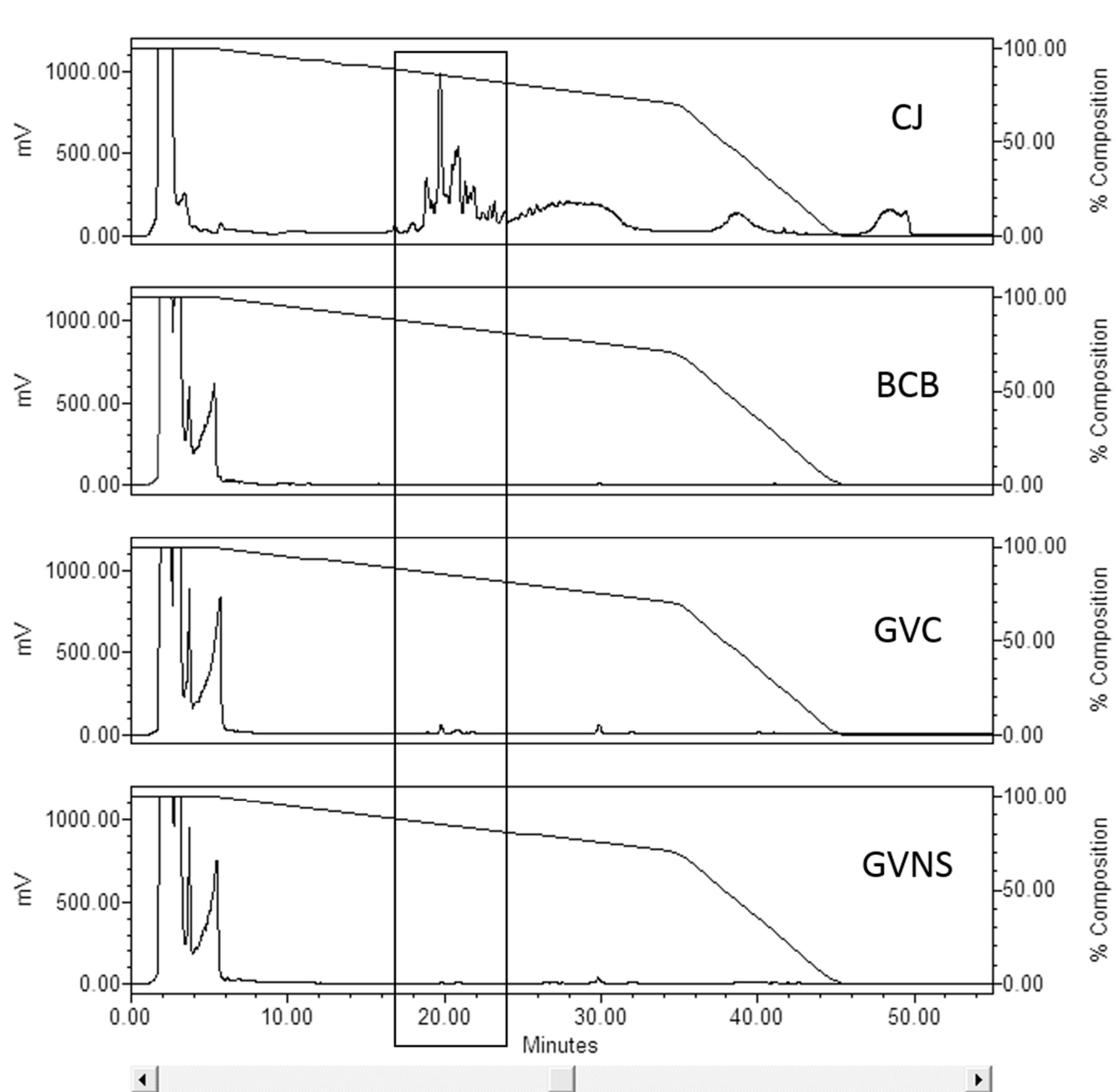
Visible	SampleName	SampleWeight	Dilution	Injection Volume (uL)	Channel Name	Date Acquired	Acq Method Set
☑	CranRef-filtered AtldC18A R3	2.90000	200.00000	50.00	SATIN	3/25/2011 7:12:12 PM CDT	AtldC18 A
☑	FNFruitConc AtldC18A R1	14.00000	1000.00000	50.00	SATIN	3/24/2011 10:46:07 PM CDT	AtldC18 A
☑	NBFruitConc AtldC18A R1	8.00000	1000.00000	100.00	SATIN	3/25/2011 5:58:52 AM CDT	AtldC18 A
☑	SNFruitConc AtldC18A R3	47.50000	1000.00000	50.00	SATIN	3/24/2011 7:51:34 PM CDT	AtldC18 A
☑	NMFruitConc AtldC18A R1	45.00000	1000.00000	25.00	SATIN	3/25/2011 8:51:34 AM CDT	AtldC18 A

Figure 85. Analytical HPLC-ELSD comparisons of commercial cranberry dietary supplements (AtldC18).
Components of interest (box), indicative of possible oligosaccharide content, could not be detected in FN, NM, or NB materials. Based on this analysis it was determined that compounds of interest may have been present in SN material and further studies confirmed the presence of oligosaccharides in this product.



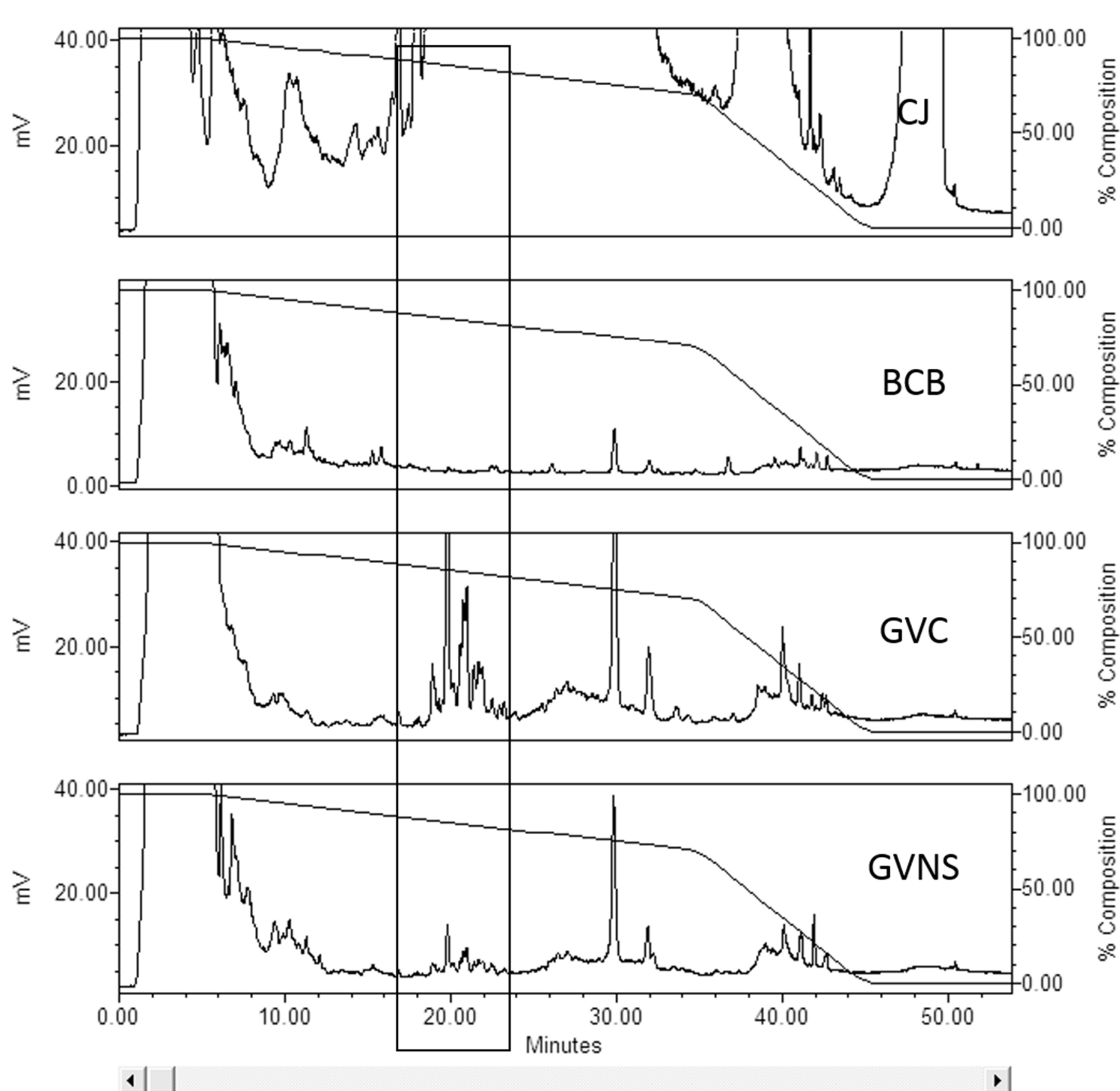
Visible	SampleName	SampleWeight	Dilution	Injection Volume (uL)	Channel Name	Date Acquired	Acq Method Set
☑	CranRef-filtered AtldC18A R3	2.90000	200.00000	50.00	SATIN	3/25/2011 7:12:12 PM CDT	AtldC18 A
☑	FNFruitConc AtldC18A R1	14.00000	1000.00000	50.00	SATIN	3/24/2011 10:46:07 PM CDT	AtldC18 A
☑	NBFruitConc AtldC18A R1	8.00000	1000.00000	100.00	SATIN	3/25/2011 5:58:52 AM CDT	AtldC18 A
☑	SNFruitConc AtldC18A R3	47.50000	1000.00000	50.00	SATIN	3/24/2011 7:51:34 PM CDT	AtldC18 A
☑	NMFruitConc AtldC18A R1	45.00000	1000.00000	25.00	SATIN	3/25/2011 8:51:34 AM CDT	AtldC18 A

Figure 86. Analytical HPLC-ELSD comparisons of commercial cranberry dietary supplements, baseline expansion (AtldC19).
Even with baseline expansions and multiple analytical separations, components indicative of oligosaccharides could not be detected in FN, NM, or NB materials.



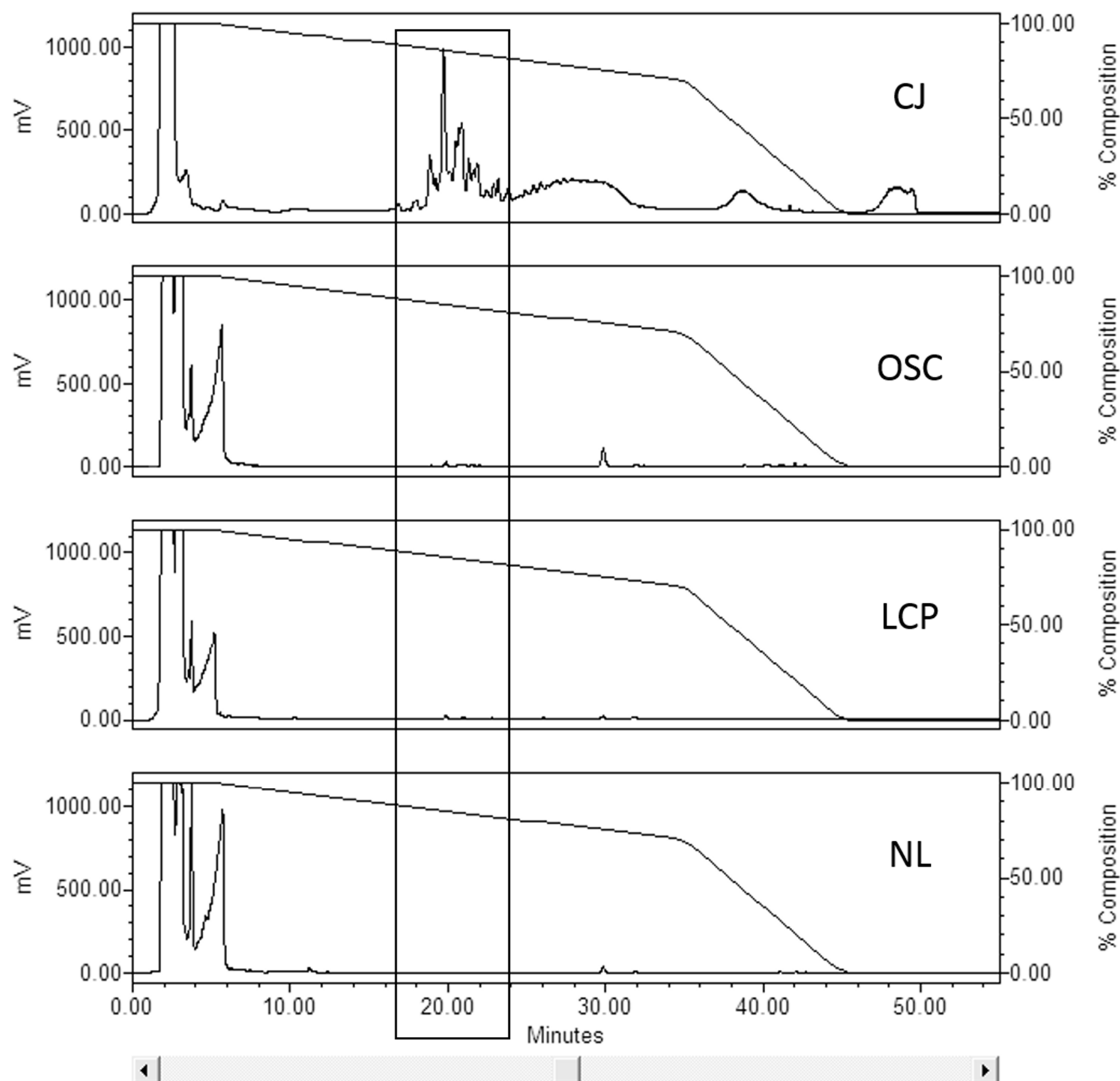
Visible	SampleName	SampleWeight	Dilution	Injection Volume (uL)	Channel Name	Date Acquired	Acq Method Set
☑	Cran Ref 9-2010	6.50000	300.00000	50.00	SATIN	4/20/2011 8:11:19 PM CDT	AtldC18 A
☑	BCB AtdC18A R1	1.00000	1.00000	50.00	SATIN	4/21/2011 1:57:02 AM CDT	AtldC18 A
☑	GVC AtdC18A R1	1.00000	1.00000	50.00	SATIN	4/20/2011 9:37:44 PM CDT	AtldC18 A
☑	GVNS AtdC18A R1	1.00000	1.00000	50.00	SATIN	4/20/2011 11:04:10 PM CDT	AtldC18 A

Figure 87. Analytical HPLC-ELSD comparisons of commercial juice products (BCB, GVS, and GVNS) to CJ material.
Early-eluting polar components constituted the majority of the BCB, GVS and GVNS juice samples. The presence of the putative oligosaccharide components (box) was not readily apparent.



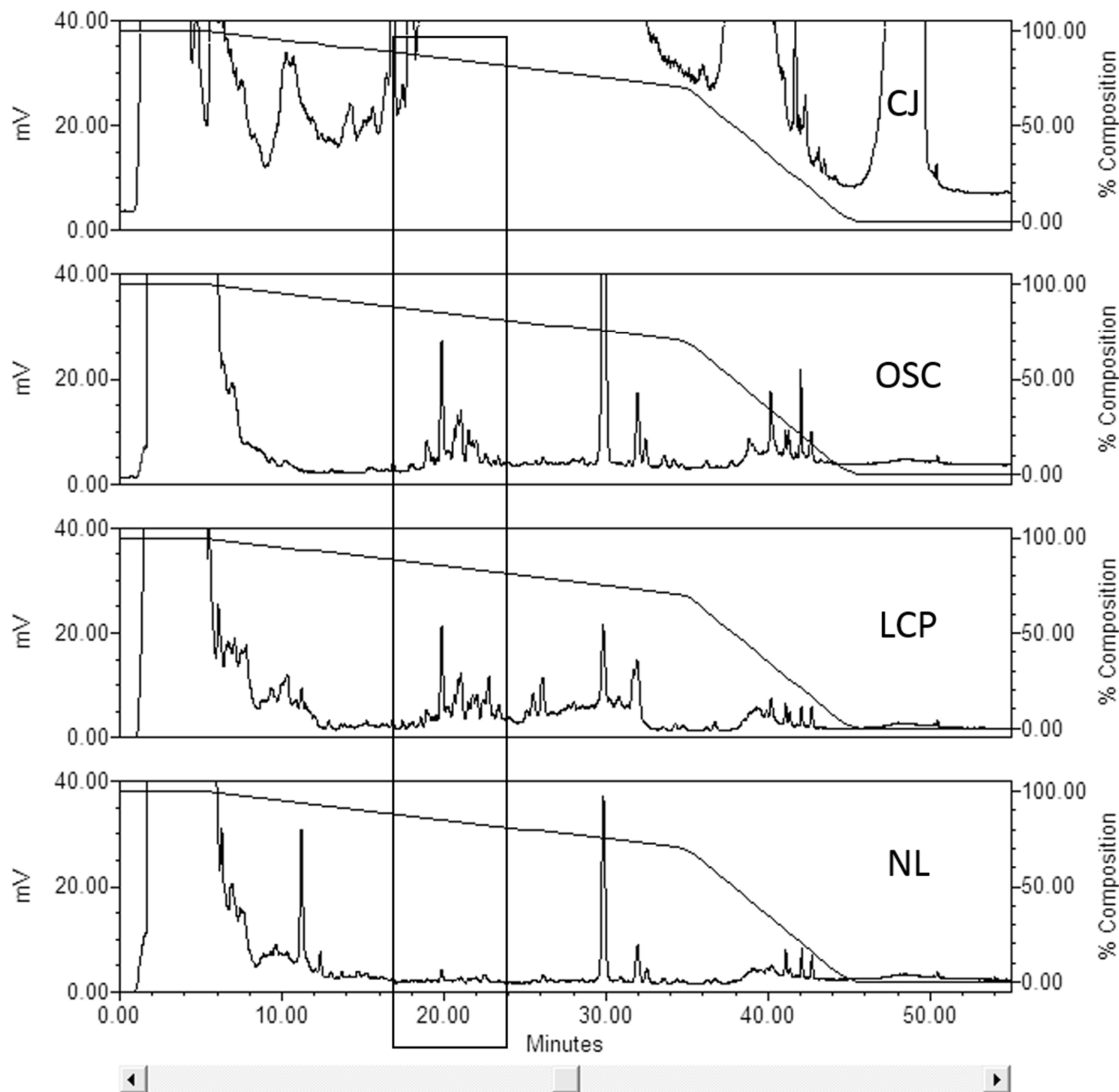
/isible	SampleName	SampleWeight	Dilution	Injection Volume (uL)	Channel Name	Date Acquired	Acq Method Set
☑	Cran Ref 9-2010	6.50000	300.00000	50.00	SATIN	4/20/2011 8:11:19 PM CDT	AtldC18 A
☑	BCB AtdC18A R1	1.00000	1.00000	50.00	SATIN	4/21/2011 1:57:02 AM CDT	AtldC18 A
☑	GVC AtdC18A R1	1.00000	1.00000	50.00	SATIN	4/20/2011 9:37:44 PM CDT	AtldC18 A
☑	GVNS AtdC18A R1	1.00000	1.00000	50.00	SATIN	4/20/2011 11:04:10 PM CDT	AtldC18 A

Figure 88. Analytical HPLC-ELSD comparisons of commercial juice products (BCB, GVS, and GVNS) to CJ material with baseline expansions.
Putative oligosaccharide components (box) in the GVS and GVNS juice samples could be detected with baseline expansion. The GVS and GVNS materials were similar in overall composition (18-45 min) with differences in the relative concentrations of the detected compounds. The BCB material contained lower concentrations of all compounds present and the oligosaccharide profile of interest was not detectable in this product at the sample concentration used.



Visible	SampleName	SampleWeight	Dilution	Injection Volume (uL)	Channel Name	Date Acquired	Acq Method Set
☑	Cran Ref 9-2010	6.50000	300.00000	50.00	SATIN	4/20/2011 8:11:19 PM CDT	AtldC18 A
☑	OSC AtdC18A R1	1.00000	1.00000	50.00	SATIN	4/21/2011 12:30:36 AM CDT	AtldC18 A
☑	LCP AtdC18A R1	1.00000	1.00000	50.00	SATIN	4/21/2011 4:49:54 AM CDT	AtldC18 A
☑	NL AtdC18A R1	1.00000	1.00000	50.00	SATIN	4/21/2011 3:23:28 AM CDT	AtldC18 A

Figure 89. Analytical HPLC-ELSD comparisons of commercial juice products (OSC, LCP, and NL) to CJ material.
Early-eluting, polar components constituted the majority of the OSC, LCP and NL juice samples. The presence of the putative oligosaccharide components (box) was suggested for the OSC and LCP but was not readily apparent.



/isible	SampleName	SampleWeight	Dilution	Injection Volume (uL)	Channel Name	Date Acquired	Acq Method Set
☑	Cran Ref 9-2010	6.50000	300.00000	50.00	SATIN	4/20/2011 8:11:19 PM CDT	AtldC18 A
☑	OSC AtdC18A R1	1.00000	1.00000	50.00	SATIN	4/21/2011 12:30:36 AM CDT	AtldC18 A
☑	LCP AtdC18A R1	1.00000	1.00000	50.00	SATIN	4/21/2011 4:49:54 AM CDT	AtldC18 A
☑	NL AtdC18A R1	1.00000	1.00000	50.00	SATIN	4/21/2011 3:23:28 AM CDT	AtldC18 A

Figure 90. Analytical HPLC-ELSD comparisons of commercial juice products (OSC, LCP, and NL) to CJ material with baseline expansions.
Putative oligosaccharide components (box) in the OSC and LCP juice samples could be detected with baseline expansion. The OSC and LCP materials were similar in overall composition (18-45 min), with differences in the relative concentrations of the detected compounds. The NL material contained lower concentrations of most compounds present and the presence of the oligosaccharide profile of interest is suggested but could not be confirmed in this product at the sample concentration used.

4. ***Bioactivity of CJ and CC Cranberry Components***

Two different anti-adhesion assays were available at different points in time. Selected samples were submitted to both assays based on the availability of the assay and logistical considerations.

a. Anti-agglutination Assay

Samples of CJ, CJA and selected CJA fractions were submitted for preliminary testing in the UPEC-HRBC anti-agglutination assay (Table 37). Samples that contained the oligosaccharides of interest appeared to be active while fractions that lacked such oligosaccharides (CJA2-05) were not active. The apparent low activity of fractions CJA2-03 and CJA2-04 may have been due to issues with the repeatability of the assay rather than to an actual lack of activity, as exhibited by the differing results for the duplicated CJA samples. All fractions from CJA separations were therefore not exhaustively tested because of the questionable reproducibility of the assay.

Table 37. Anti-agglutination assay results for cranberry fractions (CJ, CJA and CJA Sephadex LH-20 fractions).
The aqueous fraction of CJ and early-eluting fractions from CJA Sephadex LH-20 columns showed anti-agglutination activity.

Sample Description	UM Score[a]	Test Date
CJ (cranberry juice powder)	+	07/2011
CJA (aqueous fraction)	+/–	07/2011
CJA[b] (aqueous fraction)	+ +	07/2011
CJA1-02B (Sep LH-20 fraction)	+ +	07/2011
CJA2-02 (Sep LH-20 fraction)	+ +	07/2011 x2[b]
CJA2-03 (Sep LH-20 fraction)	+/–	07/2011
CJA2-04 (Sep LH-20 fraction)	+/–	07/2011
CJA2-05 (Sep LH-20 fraction)	–	07/2011 x2[b]

[a] The criteria used to assign the UM Score can be found in Table 3. Raw data for these samples can be found in Appendix B-3: Table 58. [b] Sample was tested in duplicate for this test date.

b. *Anti-adhesion Assay*

Selected CJA2 and SN fractions were tested in the radiolabeled version of the UPEC-UEC anti-adhesion assay using methods described previously (Auker 2013, Mathison *et al*. 2013).

The fluorescence-based version of the UPEC-UEC anti-adhesion assay (Kimble *et al*. 2014) was used for the CCA1 fractions and IC_{50} values from these analyses are shown below (Table 38). Fraction CCA1-01 had the highest activity ($IC_{50} = 0.25$ mg/mL), and fractions CCA1-01 – CCA1-03 appear to account for the majority of the anti-adhesion activity observed in the CCA material. Fractions CCA1-04 – CCA1-12 are either not active or have relatively low activity. The differences in structure and composition as determined by NMR spectroscopy and HPLC-ELSD for fraction 04 versus fractions CCA1-01 – CCA1-03 appear to correlate to activity, with fraction 04 having lower activity than CCA1-01 – CCA1-03. Fractions CCA1-01 to CCA1-03 lack components that elute early in HPLC-ELSD chromatograms (Figure 82), further supporting the assignment of anti-adhesion activity to the components of the complex oligosaccharide mixtures present in these fractions.

Table 38. UPEC-UEC anti-adhesion assay results for CCA1 fractions.
The aqueous fraction of the CC material (CCA) displayed higher anti-adhesion activity than the EtOAc fraction (CCE). This anti-adhesion activity could be attributed to Sephadex LH-20 fractions CCA1-01 – CCA1-03P.

Sample	IC_{50} (mg/mL)
CCE (EtOAc fraction of CC material)	2.88
CCE (EtOAc fraction of CC material)	2.87
CCA1 (aqueous fraction of CC material)	1.21
CCA1 (aqueous fraction of CC material)	1.10
CCA1-01 (Sep LH-20 fraction)	0.25
CCA1-02 (Sep LH-20 fraction)	1.55
CCA1-03 (Sep LH-20 fraction)	1.22
CCA1-03P (precipitate from Sep LH-20 fraction CCA1-03 in EtOH)	1.50
CCA1-04 (Sep LH-20 fraction)	3.81
CCA1-04P (precipitate from Sep LH-20 fraction CCA1-04 in EtOH)	2.06
CCA1-05 (Sep LH-20 fraction)	3.05
CCA1-06 (Sep LH-20 fraction)	>10
CCA1-07 (Sep LH-20 fraction)	NA[a]
CCA1-08 (Sep LH-20 fraction)	>10
CCA1-09 (Sep LH-20 fraction)	4.03
CCA1-10 (Sep LH-20 fraction)	>10
CCA1-11 (Sep LH-20 fraction)	>10
CCA1-12 (Sep LH-20 fraction)	>10

[a] This fraction was not available for testing when samples were submitted.

c. *Antimicrobial Assays*

For the NCNPR antimicrobial assays, compounds or fractions that show greater than 50% growth inhibition at the test concentration were considered to be active and would be tested in secondary assays. All of the cranberry materials submitted to these assays had less than 50% growth inhibition and were therefore considered inactive (Table 39). These results indicate that cranberry materials do not possess cidal or growth-inhibitory activity against the microbes tested at the concentrations used. Cranberry oligosaccharide fractions prepared from SN and CJA2 cranberry material by K. Auker (2013) were also submitted to the antimicrobial

assays offered by NCNPR at UM, as well as to two separate breast cancer cell viability assays. These assays produced similar negative results, further confirming the lack of antimicrobial activity and additionally indicating a lack of cytotoxicity.

The negative results for cranberry materials in the antimicrobial assays and cytotoxicity assays of NCNPR supports the relative safety of cranberry in general, as would be expected for a common food product. These results also support the hypothesis that cranberry components exhibit anti-adhesion-specific bioactivity without concurrent antimicrobial effects.

Table 39. Results of the NCNPR antimicrobial assays for CJ material and fractions. The CJ material and the water (CJA) and EtOAc (CJE) fractions of this material showed no apparent antimicrobial activity in the NCNPR assays.

↓ Organism	Conc. Tested (μg/mL)	Percent Growth Inhibition by Sample (%)		
→ Sample Code		CJ	CJE	CJA
Plasmodium falciparum (D6 Clone)	15.9	29	6	7
Leishmania donovani	80	4.37	7.89	5.07
Candida albicans	50	0	0	0
Candida glabrata	50	0	8	0
Candida krusei	50	0	9	1
Candida neoformans	50	8	12	8
Aspergillus fumigatus	50	0	1	0
Staphylococcus aureus	50	0	0	0
Methicillin-Resistant *Staphylococcus aureus*	50	0	14	1
Escherichia coli	50	0	10	0
Pseudomonas aeruginosa	50	0	5	0
Mycobacterium intracellulare	50	16	0	24

D. Conclusions

It is likely that a number of compounds are present in cranberry that contribute to the anti-adhesion properties of the juice. Cranberry PACs and NDM are already known to have anti-adhesion activity, and these compounds were likely to be retained by MW cut-off centrifugal filters under the conditions used in these studies. Additional compounds, such as oligosaccharides could also have been retained through reversible associations with high-MW components. All three classes of compounds would also likely be stable under the acid/base incubation conditions used.

The use of methods similar to those developed for the isolation of oligosaccharides from urine enabled the identification of similar oligosaccharide components in cranberry. Analyses using HPLC-ELSD/UV and NMR spectroscopy provide strong support for the presence of an entire pool of such structurally related carbohydrate compounds in spray-dried cranberry juice powder, cranberry juice concentrate and certain commercial products. Two cranberry oligosaccharides were isolated and fully elucidated from CJA material (Auker Thesis 2013) and found to have similar structures as the fully elucidated urinary octasaccharide (**1**). These studies are the first reports of arabinoxyloglucan oligosaccharides from cranberry.

The similarities in structures for the oligosaccharides from both cranberry and urine sources, combined with the similar HPLC-ELSD profiles of oligosaccharides from both sources (Figure 74), strongly supports the hypothesis that cranberry oligosaccharides are the source of urinary oligosaccharides. In foods, hemicellulose oligomers such as arabinoxyloglucans are considered to be a type of dietary fiber due to their lack of human metabolic biotransformation (Haard & Chism 1996). The presence of β-(1→4)-linked glucose backbones in oligosaccharides from both sources therefore further supports the hypothesis that these

compounds are stable to normal digestive processes and may be directly excreted without metabolic modification.

Numerous clinical trials that have tested the effectiveness of cranberry products have used cranberry dietary supplements rather than cranberry juice or cranberry juice concentrate. A possible reason for the failure of some of these trials is therefore the absence of anti-adhesive cranberry metabolites from the products used. This hypothesis is supported by the observation that oligosaccharides were absent from three of the four cranberry fruit dietary supplement products analyzed. In contrast, the characteristic oligosaccharide HPLC-ELSD profile was readily detectable in four of the six juice products analyzed at concentrations corresponding to the marketed juice preparations. This observation supports the traditional use of cranberry juice for preventing UTIs, and provides a possible reason for why cranberry dietary supplements manufactured using current processes may not be as effective for this same application. Knowledge of the active metabolites of cranberry will help to resolve many of the existing issues with regards to clinical trial testing and cranberry dietary supplement composition.

In these studies, oligosaccharide mixtures with similar chemical profiles were isolated in large scale from two sources, and comprised 2–6% of the CJ and CCA starting materials. The oligosaccharide-containing fractions from the CJA separations constituted a relatively high percentage of this starting material (25-30%), but this may reflect a lower degree of enrichment rather than a higher actual yield of oligosaccharides. In contrast, only 1% of the total CJ material was extractable by EtOAc. This EtOAc fraction was found to contain PACs and as well as other known flavonoids. These data indicate that PACs and other flavonoids are present at lower relative concentrations than oligosaccharides (Santos-Buelga & Scalbert 2000).

The presence of a similar complex profile of oligosaccharide components in the CJ, CC, and SN (Auker 2013) materials, as well as in commercial juice products from multiple manufacturing sources, supports the hypothesis that these compounds are a native component of cranberry that is naturally enriched in cranberry juice, rather than a processing or extraction artifact. The structures of the two arabinoxyloglucan oligosaccharides isolated from cranberry material and the ^{1}H NMR data of the oligosaccharide-enriched Sephadex LH-20 fractions indicate that the series of oligosaccharide compounds present are all structurally related and are all likely to be arabinoxyloglucans derived from hemicellulose. Plant hemicellulose polymers are components of plant primary cell walls that are enzymatically cleaved and solubilized during fruit ripening (Redgewell & Fry 1993, York *et al.* 1996, Brummell *et al.* 2004, Goulão & Oliveira 2008). Complex mixtures of such compounds with arabinoxyloglucan structures unique to cranberry could therefore reasonably end up in the juice of pressed ripe cranberries at relatively high concentrations. The relatively high yield of oligosaccharide mixtures from cranberry materials indicates that cranberry could therefore be a valuable source from which to obtain additional oligosaccharides.

The results from two independent anti-adhesion assays using fractions from three cranberry source materials (CJ, CC, and SN) confirmed the anti-adhesion activity of cranberry arabinoxyloglucan mixtures. These studies are the first report of the *E. coli* anti-adhesion properties of arabinoxyloglucan oligosaccharides. Active cranberry samples, both crude and enriched fractions, contained similar HPLC-ELSD and ^{1}H NMR profiles as active urine fractions, with the presence of a series of UV-transparent oligosaccharides. These results, combined with the high degree of structural similarity between urinary and cranberry oligosaccharides, further support the anti-adhesion properties of urinary oligosaccharides.

Future studies are required to conclusively establish the bioactivity profiles of individual arabinoxyloglucans oligosaccharides using additional bioassay resources and methods. The large scale isolation of cranberry oligosaccharides during these studies has resulted in the collection of sufficient amounts of material (~20 g) to facilitate future studies involving the isolation and detailed structure elucidation of individual oligosaccharide components. It also provides sufficient material for future bioassay testing and analytical method development. The potential use of cranberry anti-adhesive oligosaccharides for the prevention of infections, especially UTIs, warrants continued investigation into the structural characteristics and biological properties of these compounds.

CHAPTER 4: CHANGING THE CRANBERRY PARADIGM

A. Introduction: The Story of a Paradigm Transition

1. *Swine Urine Studies*

The primary objectives of these studies were (1) to isolate and identify the urinary metabolites of cranberry juice that are responsible for the prevention of UPEC adherence, and (2) to identify the compounds present in cranberry that are related to the anti-adhesive urinary metabolites. This work started under the paradigm and guiding hypothesis that the compounds of interest would be PACs or PAC-derived (Howell *et al*. 1998, Howell *et al*. 2010, Ofek *et al*. 1996). Efforts to isolate such compounds from active urine fractions failed, however, and delayed the identification of the actual, active components of interest.

The chance observation of a ^{1}H NMR spectrum for a pectin sample during a conference presentation led to the realization that the ^{1}H NMR spectra of active fractions FA1 and FA2 were similar to those of complex carbohydrates. Subsequent comparisons of active samples to the ^{1}H NMR spectra of commercial monosaccharides, pectin, synthetic PACs, and other samples, combined with chemical analyses using TLC spray reagents, confirmed the observation that the compounds of interest were probably UV-transparent carbohydrates. The hypothesis that PACs were the target urinary metabolites was discarded based on data collected during initial studies, and investigations were continued under the newly formulated hypothesis

that complex carbohydrates were the compounds of interest. These observations and this newly formed hypothesis prompted the use of ELSD, a mass-sensitive, semi-quantitative detection method, to monitor subsequent HPLC separations.

Multiple swine feedings were required to obtain sufficient material and suitable methods for the isolation of the compounds of interest from active fractions. Ultimately, a sufficient quantity (12 mg) of a single pure compound (**1**), low quantities of other pure compounds (1-2 mg), and many other partially purified compounds were obtained from the complex carbohydrate mixture. The structure of **1** was elucidated using multiple carbohydrate-specific analyses and NMR spectroscopic techniques. This compound was highly similar in structure to the other purified and partially purified compounds present in the mixture of interest, and all were determined to be arabinoxyloglucan oligosaccharides. Data indicated that these urinary oligosaccharides contained cellulose-type β-(1 → 4)-linked glucose backbones, supporting the newly formed hypothesis that these compounds were plant-derived. Circumstantial evidence suggested that components of this arabinoxyloglucan oligosaccharide mixture were responsible for the UPEC-HRBC anti-agglutination activity of active fractions: oligosaccharides were the only compounds detectable in some active fractions, were present in all active fractions, and were absent from all inactive fractions. Material limitations and issues with assay reproducibility made it impossible, however, to directly confirm the anti-adhesion properties of the isolated urinary arabinoxyloglucan oligosaccharides. Additionally, arabinoxyloglucans from cranberry had not been previously identified, and there were no previous reports of anti-adhesion properties for arabinoxyloglucan oligosaccharides. The conclusions of the urinary metabolites study led to the formulation of the new hypothesis that

the anti-adhesive compounds present in urine after cranberry consumption were arabinoxyloglucan oligosaccharides derived from cranberry.

2. *Cranberry Studies*

Cranberry materials were investigated using the same methods developed for urine samples to determine if similar oligosaccharides were present in the starting material fed to swine. Analytical HPLC-ELSD data showed evidence of a highly similar elution profile for the compounds of interest from cranberry material. Direct separation of cranberry juice powder on preparative HPLC, with no prior extractions or enrichments, led to the isolation of sufficient quantities (2-4 mg) of several cranberry oligosaccharides, confirming that the same class of compounds was present and was of highly similar chemical composition. More material was required, however, for full structural elucidation of the cranberry oligosaccharides of interest.

Large scale isolation of cranberry oligosaccharide mixtures was pursued using the Sephadex LH-20 methods similar to those developed for urine separations. Cranberry source materials used included cranberry juice powder, cranberry juice concentrate, and cranberry fruit powder. These isolations led to the total collection of approximately 20 g of enriched oligosaccharide material from all three sources. Enriched cranberry oligosaccharide mixtures were used for further analyses and bioactivity testing. The purification and structure elucidation of two compounds from the cranberry oligosaccharide series was the subject of the Master's Thesis for Kimberly M. Auker (2013). The two purified and fully characterized cranberry oligosaccharides were also found to be arabinoxyloglucan oligosaccharides that were highly similar in structure to the urinary octasaccharide and the associated series of urinary oligosaccharides. The similar structures of urine- and cranberry-derived oligosaccharides

supports the hypothesis that swine urine oligosaccharides are derived from ingested cranberry products and provided support for the new hypothesis that cranberry arabinoxyloglucan oligosaccharides are absorbed and excreted unchanged into mammalian urine.

Oligosaccharide-enriched fractions from cranberry materials were submitted to two different anti-adhesion assays and showed activity in both. These assays both used uropathogenic P-fimbriated *E. coli* (UPEC) and included the original, qualitative, anti-agglutination assay and a newly-developed, quantitative, uroepithelial cell (UEC) anti-adhesion assay. The results of the newly-developed assay confirmed those of the original anti-agglutination assay. The results from testing cranberry fractions in antimicrobial assays provided additional support for the published hypothesis that cranberry-derived compounds prevent adhesion without direct antimicrobial effects. The anti-adhesion assay results for the cranberry oligosaccharides confirmed, by association, the previously putative anti-adhesion activity of urinary oligosaccharides. The conclusions of these studies supported the hypotheses that urinary and cranberry arabinoxyloglucan oligosaccharides can both prevent the adhesion of UPEC to uroepithelial cells.

Analysis of commercial cranberry products (dietary supplements and juices) by an oligosaccharide-optimized HPLC-ELSD method showed that some products lack detectable levels of oligosaccharides while others contain readily detectable amounts of the oligosaccharide mixture of interest. Many clinical studies testing the effectiveness of cranberry for UTI prevention have used cranberry products enriched for PACs or standardized to PAC content. Such products may therefore lack detectable levels of carbohydrates such as arabinoxyloglucan oligosaccharides. These observations and the results of these studies therefore support the hypothesis that the inconclusive results of certain clinical studies may be

due, in part, to the use of cranberry products that lack cranberry oligosaccharide compounds with anti-adhesion activity.

3. *Overview of Results*

As reported here, cranberry arabinoxyloglucan oligosaccharide mixtures prevent the adhesion of uropathogenic P-fimbriated *E. coli* in two different anti-adhesion assays, are present in relatively high percentages in renewable source material, and are present in mammalian urine after the consumption of cranberry products. These observations and the availability of enriched cranberry oligosaccharide fractions lay the groundwork for future studies based on many new hypotheses concerning oligosaccharides.

B. Significance of Cranberry-Derived Urinary Oligosaccharides

1. *Cranberry Oligosaccharides as Anti-adhesion Compounds*

Carbohydrates have many roles with regards to host-pathogen interactions (Beachey 1981, Pieters 2007). Eukaryotic cell surface carbohydrates are the natural ligands for many types of microbial adhesins, including the P fimbriae of *E. coli* (Enerbäck *et al*. 1987). Free oligosaccharides are recognized to be natural interference ligands, and various sources of such compounds with anti-adhesion properties are of significant medical interest for the prevention of infections (Zopf & Roth 1996, Sharon 2006, Pieters 2007, Shoaf-Sweeney & Hutkins 2008). Carbohydrates that mimic the natural carbohydrate ligands of microbial adhesins may be able to directly interfere with microbial adhesion to cell surfaces by competitive binding.

Alternatively, carbohydrates that bind to microbial fimbriae in other locations may prevent fimbrial adhesion to target ligands by indirect mechanisms such as by altering fimbrial conformations.

Carbohydrates are well recognized as compounds that can prevent P-fimbriated *E. coli* adhesion. For example, globotriose [α-D-Gal-(1→4)-β-D-Gal-(1→4)-β-D-Gal], galabiose, multivalent galabiose, and tetrasaccharides containing an α-Gal-(1→4)-β-Gal moiety are able to inhibit *E. coli* adhesion both in vitro and in vivo by binding to P fimbriae in place of cell surface receptors (Svanborg-Edén *et al*. 1984, Korhonen *et al*. 1986, Leach *et al*. 2005, Turner *et al*. 2005, Salminen *et al*. 2007). The binding of the PapG adhesin of *E. coli* P fimbriae can also be inhibited by dipentasaccharide fragments derived from the globoseries of glycolipids that contain the minimum α-Gal-(1→4)-β-Gal binding epitope (Larsson *et al*. 2003). Studies have shown that mixtures of neutral oligosaccharides isolated from breast milk and the urine of breast-feeding babies are able to interfere with *E. coli* adhesion (Rosenstein *et al*. 1988, Coppa *et al*. 1990). While these and other studies show proof-of-principle for the ability of carbohydrates to prevent *E. coli* adhesion, currently known compounds are not clinically viable agents due to non-specific lectin binding, limited material availability, and high manufacturing costs, among other reasons (Larsson *et al*. 2003, Turner *et al*. 2005, Sharon 2006, Pieters 2007). Additional carbohydrate-type compounds that can prevent the adhesion of P-fimbriated *E. coli* are of great medical interest but suitable compounds have not yet been identified and developed into therapies.

Studies investigating the effects of cranberry juice on the P fimbriae of individual *E. coli* using the nanoscale tool of AFM microscopy indicate that cranberry components directly interact with fimbriae and prevent adhesion by affecting the conformations of *E. coli* surface

macromolecules (Liu *et al*. 2006). Studies of *E. coli* strains with variations in Pap-G adhesin types indicate that the conformation and overall structure of cell surface carbohydrate chains influences bacterial binding (Strömberg *et al*. 1991). Specifically, the Pap-G adhesin isotype that is most often found in UPEC preferentially binds to neutral, 4- and 5-sugar containing, cell-surface glycosphingolipid isoreceptors containing the minimum Gal-(α1→4)-Gal binding epitope, the major isoreceptor of uroepithelial cells (Strömberg *et al*. 1991, Strömberg *et al*. 1990). The UPEC-associated Pap-G adhesin isotype also preferentially binds to compounds containing multiple versions of the target galabiose disaccharide (multivalent compounds), showing higher binding affinities for these compounds than for compounds with a single version of the galabiose disaccharide (monovalent compounds) (Figure 91) (Salminen *et al*. 2007).

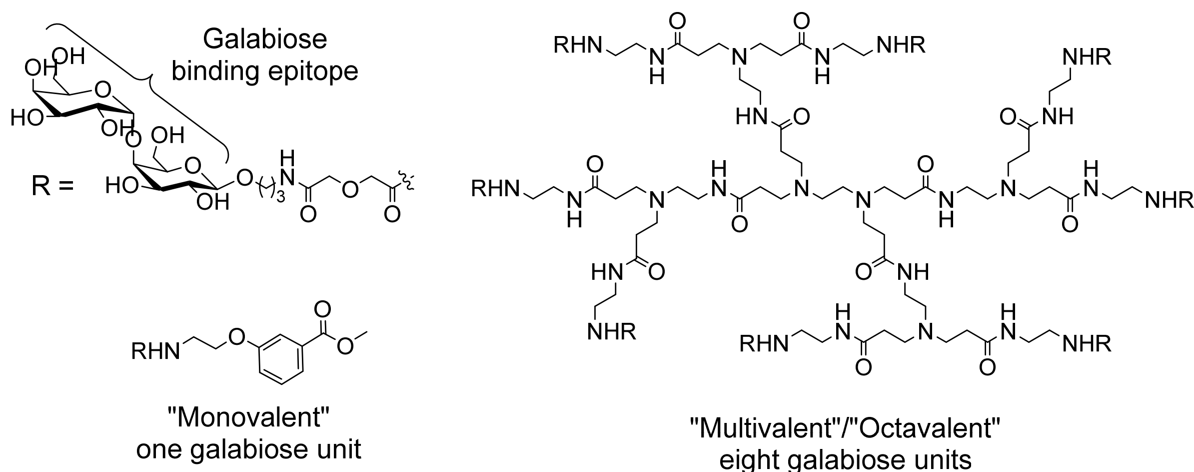


Figure 91. Examples of monovalent and multivalent carbohydrate derivatives containing the galabiose binding epitope (Salminen *et al*. 2007).
The octavalent compound shown inhibited the adhesion of UPEC with the $PapG_{J96}$ adhesin to epithelial cells at an IC_{50} value of 22 μM and inhibited HRBC agglutination at an MIC of 7 μM. In contrast, the monovalent compound shown inhibited cell adhesion at an IC_{50} value of 170 μM and inhibited HRBC agglutination at an MIC of 320 μM in the same assays (Salminen *et al*. 2007).

Together, these data support the proposed hypothesis that the neutral cranberry oligosaccharides identified in the present studies bind to the Pap-G adhesin of UPEC P fimbriae, changing its conformation and thereby preventing the adhesion of UPEC to uroepithelial cells. Numerous further studies are necessary, however, to fully characterize the mechanisms by which cranberry oligosaccharides alter interactions between bacterial and epithelial cells to prevent adhesion. Studies using AFM could be used to confirm that cranberry oligosaccharides are able to cause the changes in fimbrial conformation observed with cranberry juice (Liu *et al*. 2008). Modeling, crystal structures, and various types of labeling could be used to investigate putative binding sites for oligosaccharides on P fimbriae and to characterize how different PapG adhesin classes and isotypes interact with oligosaccharides of different structures (Strömberg *et al*. 1991, Larsson *et al.* 2003, Salminen *et al*. 2007). The effectiveness of oligosaccharides for preventing the adhesion of *E. coli* during different stages of infection, of different clinical strains of *E. coli*, or of clinical *E. coli* strains with different fimbrial variants could also be examined (Marrs *et al*. 2002, Howell & Foxman 2002, Justice *et al*. 2004). Many additional types of studies are also likely to be proposed by other researchers in relation to their chosen specialties.

Various clinical approaches that aim to interfere with adhesin-ligand binding between pathogen and host cells have met with mixed success, due partly to the complexities of in vivo systems and partly to the structural complexities of carbohydrates (Zopf & Roth 1996, Sharon & Ofek 2000, Sharon 2006). The approaches that currently show the greatest potential are those that use multivalent carbohydrates or mixtures of oligosaccharides (Sharon & Ofek 2000, Pieters 2007, Salminen *et al*. 2007). Neutral oligosaccharides composed of four to six monosaccharide units, or compounds containing multiple versions of the known carbohydrate

binding epitope of *E. coli* (oligovalent or multivalent compounds; see Figure 92) have also shown increased binding affinities and higher anti-adhesion activity against P-fimbriated *E. coli* (Svanborg-Edén *et al*. 1984, Coppa *et al*. 1990, Larsson *et al*. 2003, Salminen *et al*. 2007, Pieters 2007). Complex carbohydrates or oligosaccharide mixtures possess superior anti-adhesion abilities as compared to mono- or disaccharides, and are believed to be more effective at preventing adhesion due to their ability to mimic host cell carbohydrate receptors and their simultaneous interactions with multiple bacterial adhesions (Pieters 2007, Salminen *et al*. 2007). Microbes typically express multiple adhesion mechanisms during infection, and mixtures of compounds may be required to prevent all types of adhesion and obtain clinical efficacy. These observations support the hypothesis that the anti-adhesion properties of cranberry and urinary oligosaccharides may be enhanced due to the presence of mixtures of complex, neutral oligosaccharides. Numerous studies would be necessary to investigate this hypothesis, and all would require the availability of sufficient quantities of purified and fully characterized individual oligosaccharides for the controlled testing of known ratios of compounds.

2. *Implications for Cranberry Bioactivity*

The identification of oligosaccharides as anti-adhesive components of cranberry products provides a new direction for all researchers interested in cranberry bioactivity. Data from the present studies indicate that oligosaccharides are likely to be partly responsible for previously observed cranberry product anti-adhesion effects (Ofek *et al*. 1996, Howell *et al*. 1998, Howell *et al*. 2010, Tao *et al*. 2011, Turner *et al*. 2005). The structural and chromatographic characteristics of oligosaccharides also indicate that these compounds may be

present, but undetected, in many other "enriched" bioactive fractions derived from cranberry. Unlike many typical small molecule natural products, oligosaccharides are of relatively high MW, are UV transparent and are not readily ionized or detected by generalized MS-based methods (Table 40).

Table 40. Overview of reasons for the difficulty of detection, isolation, and structural characterization of oligosaccharides using classical bioassay-guided fractionation.

Compound Type	**Typical Natural Product**	**Oligosaccharides**
Extraction	Organic extracts	Water soluble, not organic soluble
Isolation	Relatively few components with structural similarity in a series	Complex series of many structurally related components
Detection	UV detection possible	No chromophore, ELSD or other chromatographic detection method required
Ionization	Readily ionized for detection by (+) or (-) LC-MS	Poor ionization for LC-MS, requires derivatization, specialized MS methods and/or custom calibration
NMR Spectra	Simple NMR spectra, <30 carbons	Complex NMR spectra, >30 carbons
Structural Elucidation	Full characterization possible using spectroscopic/ spectrometric methods	Full characterization requires multiple, complementary methods that are typically destructive
MW	200-800 Da, < 10 mg yields usable NMR data at 400 MHz	800-2000+ Da, requires 15 + mg to yield usable NMR data at 400 MHz, MW > typical LC-MS detection threshold
Bioassay Types	Established, quantitative assays, or identified by high-throughput screening	Qualitative or new assays, specific to an individual organism or research question
Bioassay Endpoints	Based on clear endpoints such as the death of the target organism or growth inhibition	Based on a biological response of a living organism that can be difficult to quantify and correlate to in vivo effects. *e.g.* anti-adhesion

As PAC-enriched fractions are typically characterized through the use of MS or UV data (Porter *et al.* 2001, Kandil *et al*. 2002, Howell *et al.* 2005, Ermel *et al*. 2012), oligosaccharide constituents would not have been detected. Many methods for enrichment or partial purification would also not necessarily have eliminated oligosaccharides as possible components of the "enriched" final product. Oligosaccharides may also form reversible hydrogen bonds with PAC oligomers or other oligosaccharide components in solution to form complexes with high apparent MWs. It is therefore possible that oligosaccharides may be present in the PAC-enriched or high-MW fractions used by many researchers and may contribute to the anti-adhesion effects and other biological properties observed for these fractions (Delehanty *et al*. 2007, Shmuely *et al*. 2004, Steinberg *et al*. 2004). Future studies will be necessary to investigate the contributions of cranberry oligosaccharides to the various biological properties attributed to cranberry.

Multiple compounds with various mechanisms of action are likely to be involved in the reported bioactivities of cranberry products (Shmuely *et al*. 2012). With regards to the anti-adhesion properties of cranberry, multiple compounds have been identified that have been shown to prevent microbial adhesion. These include fructose (Zafriri *et al*. 1989, Ofek *et al*. 1996), mannose (Ofek & Beachey 1978), PACs (Howell *et al*. 1998, Foo *et al*. 2000a,b), myricetin (Mathison *et al*. 2013, Kimble *et al*. 2014), and now also oligosaccharides, as shown by the present studies. All of these compounds, and possibly others that have yet to be identified, are likely to act together by various mechanisms to prevent bacterial-mammalian cell adhesion in different parts of the human body after cranberry consumption. All may participate in the prevention of biofilm formation within the human mouth, and all may affect gut microbes by altering species composition or gene expression profiles, including that of *E.*

coli (Ofek *et al*. 1996, Weiss *et al*. 1998, Rajan *et al*. 1999, Yamanaka *et al*. 2004, Howell *et al*. 2005, Duarte *et al*. 2006, Labrecque *et al*. 2006). Current data, however, give the strongest support to the hypothesis that carbohydrate-type compounds including mannose, fructose, and now oligosaccharides have the most significant roles for the prevention of microbial adhesion in the urinary tract, and therefore for the in vivo prevention of urinary tract infections. Many types of additional studies of cranberry products and cranberry-derived compounds will be necessary to fully elucidate all of the ways in which cranberry components may work together to prevent microbial adhesion to epithelial cells.

3. *Cranberry Oligosaccharides as Urinary Components*

Data from the present studies support the hypothesis that cranberry oligosaccharides are absorbed intact and excreted into urine without modification by mammalian enzymes or digestive processes. The urinary octasaccharide (**1**) and the octa- and hepta-saccharides (**2** & **3**) purified from cranberry oligosaccharide mixtures (Auker 2013) are arabinoxyloglucans with highly similar structures, all have cellulose-type β-(1 → 4)-linked glucose backbones, and all are relatively stable over time. In general, hemicelluloses such as these are considered to be a type of dietary fiber, are water soluble, acid stable, and resistant to degradation during mammalian digestion, as mammals lack β-glucosidase digestive enzymes (Haard & Chism 1996, Robyt 1998). Crude cranberry materials containing oligosaccharides were also found to retain anti-adhesion activity after incubation at human body temperature at pH 2, 4, and 8. Comparison of analytical HPLC-ELSD profiles for urinary and cranberry oligosaccharide mixtures indicated similar ratios for the different components of the mixture in both materials

(Figure 74), further supporting the hypothesis that many of the compounds present in the cranberry mixture are absorbed and excreted intact into the urine in similar ratios.

The primary objective of the present studies was to identify active urinary constituents, but attempts were also made to monitor sample recovery for relative quantification of the active constituents. Such quantification proved difficult, however, due to numerous, uncontrollable sources of sample loss (ELSD-based separations) and to the need for pooling starting materials across collection periods and study animals to provide sufficient cumulative amounts for the isolation of active components. Conservative estimates can be made for oligosaccharide urinary concentrations in relation to cranberry consumption, but additional, detailed studies will be necessary to fully quantify cranberry oligosaccharide absorption, distribution, and excretion profiles. Currently available data suggests a minimum urinary oligosaccharide mixture concentration of approximately 0.15 mg/mL, corresponding to a minimum excretion of 0.04% w/w oligosaccharides per gram of ingested cranberry powder. This concentration is expected to vary based on the urinary solute concentrations of individual organisms, but is within a reasonable range for compounds that are excreted into the urine (Saude *et al*. 2007, Bouatra *et al*. 2013). Currently available data also indicates that the cranberry powder used for swine feedings contained a maximum oligosaccharide content of 16-30% w/w, with the wide range based on the method used for enrichment. Together, these data indicate that a minimum of 0.13-0.25% of ingested cranberry oligosaccharides are excreted into urine. While the values presented here are rough estimates, these data support the hypothesis that orally ingested cranberry oligosaccharides are also excreted through feces or retained in body compartments and excreted into urine over time.

Direct absorption and rapid elimination of unmodified oligosaccharides into mammalian urine would be consistent with the reported rapid appearance of anti-adhesion activity in human urine after the consumption of cranberry juice (Howell *et al*. 2005). Oligosaccharides have been previously isolated from normal human urine (Parkkinen & Finne 1984), and reports have shown that mixtures of complex oligosaccharides ranging in size from trimers to heptamers and larger can be ingested and subsequently found in adult and infant human urine (Rosenstein *et al.* 1988, Coppa *et al.* 1990). These studies have reported 500-800 mg/day of uncharacterized oligosaccharide mixtures in the urine of adult women (Coppa *et al*. 1990). Mice gavaged with high doses of globotriose [Gal-(α1→4)-Gal-(β1→4)-Glc] (Figure 92) were found to rapidly clear this trisaccharide from plasma ($t_{1/2}$ = 6 min) and excrete it primarily into urine without metabolic biotransformation at levels of > 5 mg/mL for 4-12 hours after administration, indicating that this compound is orally bioavailable and retaind for later excretion over time (Leach *et al*. 2005).

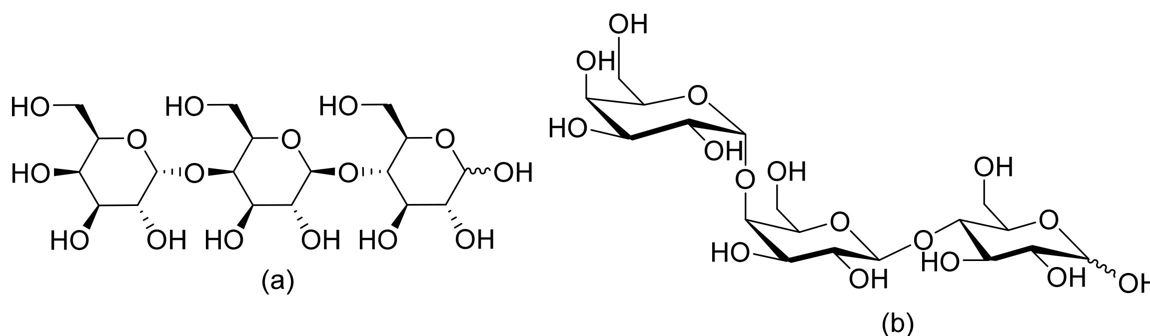


Figure 92. Structure of globotriose [Gal-(α1→4)-Gal-(β1→4)-Glc] depicted with IUPAC stereodescriptors (a) and chair conformers (b).

Together these observations further support the hypothesis that cranberry-derived oligosaccharides are orally bioavailable and excreted relatively rapidly into urine without metabolic biotransformation. These data also support the feasibility of oral administration of

cranberry juice for the prevention of UTIs. Future detailed studies regarding cranberry oligosaccharides will be needed to quantitatively examine the absorption, distribution, metabolism, and excretion (ADME) profiles of these compounds, individually and as mixtures. Such studies will be facilitated by the availability of cranberry compounds as analytical standards, and the apparent lack of biotransformation, but will require the development of targeted, sensitive methods for oligosaccharide detection and quantification. High-throughput analytical platforms optimized for oligosaccharide detection and quantification will also be required for effective evaluation of clinical samples and dietary source materials that may contain oligosaccharides.

4. ***Implications for Human Clinical Trials***

Studies are currently in progress at UM to identify the anti-adhesive components of human urine collected after cranberry juice consumption using isolation and detection methods similar to those applied to swine urine samples and cranberry oligosaccharides. Human urine fractions with anti-adhesion activity are hypothesized to contain cranberry-derived oligosaccharides and preliminary evidence suggests that these compounds are indeed present. The identification and characterization of cranberry-derived oligosaccharides from human urine is the minimum piece of information necessary to directly link the current report to probable in vivo efficacy. Additional studies will be necessary to assess the clinical relevance of oligosaccharides for the prevention of UTIs in various patient populations. Knowledge of these active compounds will make it possible to quantify oligosaccharide consumption and excretion for individual study subjects. These data could then be correlated to long and short term clinical outcomes, including an absence of UTIs and the oligosaccharide content and anti-adhesion

properties of patient urine. Improved knowledge regarding the anti-adhesive components of cranberry that are excreted into urine will also help to resolve many of the current uncertainties regarding the efficacy of cranberry for preventing UTIs (Jepson *et al*. 2012).

The variation in procedures for commercial cranberry product preparation and the probable lack of oligosaccharide constituents in certain products is a possible explanation for some of the heterogeneity of results obtained from cranberry-focused clinical trials (Jepson *et al*. 2012). Preliminary profiling of cranberry dietary supplements with regards to oligosaccharide content indicates that "concentrated" cranberry dietary supplements may not contain anti-adhesive oligosaccharides. Most dietary supplement manufacturers do not disclose the full product manufacturing processes. It is therefore difficult to determine whether certain products may contain oligosaccharides without subjecting product samples to HPLC-ELSD, NMR spectroscopy, and other analyses. It is likely, however, that many manufacturers are enriching for flavonoid content based on the hypothesis that PACs are the target anti-adhesive constituents, and the methods used for flavonoid enrichment may or may not also enrich for oligosaccharides. Additional studies are needed to independently quantify the relative amounts of cranberry oligosaccharides in different dietary supplements prior to the use or marketing of such products for UTI prevention.

One survey of cranberry clinical trials compared the reported efficacy of cranberry juice to that of dietary supplements and found that juice was more effective (Wang *et al*. 2012a). The authors hypothesized that this may be due to improved patient hydration (Wang *et al*. 2012a), but this observed result may also have been due to the presence of cranberry oligosaccharides in juice products but not in dietary supplement products, as observed in the present studies.

It is also possible that clinical studies using various cranberry products are showing that certain cranberry preparations do or do not contain the active ingredients, rather than that cranberry itself isn't active. For example, in one of the clinical trials that reported a lack of cranberry efficacy (Beerepoot *et al*. 2011), the cranberry product used was a dietary supplement called Cran-Max® (Proprietary Nutritionals, Inc., Kearny, NJ) that contained a standardized dose of A-type PACs. Based on published product literature, it appears that the Cran-Max® extract had been enriched for anthocyanins and flavonoids and likely lacked oligosaccharides (Proprietary Nutritionals 2007, Appendix C-5). A different study that supported the effectiveness of cranberry products (Howell *et al*. 2010) used a cranberry dietary supplement known as Ellura™ (Ellura, Trōphikōs Nutritive Health, Atlanta, GA) that reportedly contained "cranberry powder." The limited product literature that could be found on the internet regarding Ellura™ (Appendix C-6) indicated that this material was derived from cranberry juice and standardized with regards to PAC content, but provided minimal information regarding other possible constituents. Another study that used whole cranberry powder with standardized PAC content also showed efficacy for preventing UTIs (Sengupta *et al*. 2011). Additional cranberry compounds, such as oligosaccharides, may have therefore been present in cranberry products that have shown efficacy (Howell *et al*. 2010, Sengupta *et al*. 2011).

The most recent survey of cranberry clinical trials (Jepson *et al*. 2012) has concluded that "cranberry products cannot be recommended for preventing UTIs" and that more studies are needed that quantify the amount of active ingredient in source material. The results of the studies presented in this report indicate that such quantification may now be possible for both source material and patient samples.

C. Occurrence & Potential Uses of Arabinoxyloglucan Oligosaccharides

1. *Natural Sources of Arabinoxyloglucan Oligosaccharides*

The oligosaccharides identified in both urine and cranberry are structurally related to known hemicellulose polymers, specifically xyloglucans and arabinoxyloglucans. Hemicelluloses are heterogeneous, branching polymers with a high degree of variety in structural components and arrangements (Scheller & Ulvskov 2010). They typically contain backbones composed of β-(1 → 4)-linked glucose units, but backbones may also be composed of xylose, mannose, or galactose units. Branching patterns and side chain compositions vary across plant families and species, and any given plant may produce a wide range of hemicelluloses with common structural motifs. Monosaccharides commonly found in side chains include galactose, mannose, arabinose, glucuronic acid, xylose, fucose, and rhamnose. The xyloglucans, composed of cellulose-type β-(1 → 4)-linked glucose backbones and xylose-containing side chains, are one of the most common classes of hemicelluloses (Figure 93). Hemicelluloses are an essential component of plant primary cell walls, crosslinking cellulose fibers to provide structure and strength (York *et al*. 1986, Scheller & Ulvskov 2010). Native hemicellulose polymers are typically studied using enzymatic cleavage via fungal enzymes, and it has been shown that hemicellulose polymers are naturally cleaved during fruit ripening (Hisamatsu *et al*. 1992, York *et al*. 1996, Brummell *et al*. 2004, Goulão & Oliveira 2008).

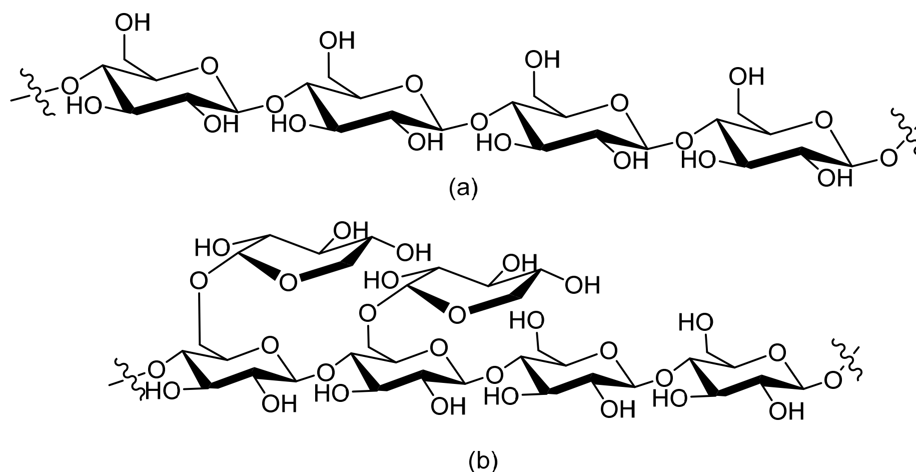


Figure 93. Structures of cellulose (a) and xyloglucan (b) segments showing the cellulose-type backbone of xyloglucans and the preferred equatorial and boat conformations of the glucosyl and xylosyl units, respectively.

Current research supports the potential for complex oligosaccharides to play a clinical role in the prevention of UTIs, but the availability of such compounds has been limited, delaying progress in this area. As oligosaccharides are relatively difficult and expensive to synthesize de novo, there is a need for renewable natural sources of oligosaccharides that possess anti-adhesion properties (Sharon & Ofek 2000, Larsson *et al*. 2003, Turner *et al*. 2005). Hemicellulose-derived oligosaccharides may be able to meet this need, and cranberry oligosaccharides could be used to guide investigations of similar compounds from other possible human dietary sources.

Investigations to identify anti-adhesive hemicellulose-derived oligosaccharides could begin with examinations of variations in oligosaccharide content for juice produced from cranberry at different stages of fruit ripeness (Vvedenskaya & Vorsa 2004) or from different cranberry cultivars. Additional studies may investigate other plants of the *Vaccinium* genus, especially the closely related blueberry (*V. corymbosum*), using quantitative survey approaches similar to those applied to characterize resveratrol and other stilbenes in *Vaccinium* species

(Rimando *et al*. 2004, Rimando & Barney 2005, Rimando & Cody 2005). Plant species within the same genus often share biosynthetic pathways, especially for the biosynthesis of "primary metabolites" such as hemicellulose, and it is likely that other *Vaccinium* species produce oligosaccharides with similar structures during fruit ripening.

Blueberry juice has been reported to possess an ability similar to that of cranberry juice to prevent P-fimbriated *E. coli* adhesion (Ofek *et al*. 1991, Ofek *et al*. 1996). Similar, partially characterized, high-MW inhibitors were reported to be present in both cranberry and blueberry juices and the active fractions were initially characterized to contain a type of PAC. While PACs were subsequently purified from cranberry and shown to have in vitro anti-adhesion properties (Foo *et al*. 2000a,b), the active fractions described in the initial reports of cranberry and blueberry anti-adhesion activity could also have contained oligosaccharides (Ofek *et al*. 1991, Ofek *et al*. 1996). The presence of oligosaccharide-type compounds in blueberry has, however, yet to be confirmed. Additional studies involving the detection, quantification, isolation and structural elucidation of oligosaccharides from multiple *Vaccinium* species will be necessary to support or refute the hypothesis that the anti-adhesive oligosaccharides identified in cranberry are present in other *Vaccinium* species.

Plants other than cranberry, such as tobacco, tomato, and flax, are known to produce xyloglucan and arabinoxyloglucan containing polymers, but the structures of free, soluble oligosaccharides derived from these polymers are unknown (York *et al*. 1990, York *et al*. 1996, Naran *et al*. 2008). Free arabinoxyloglucan oligosaccharides produced by non-*Vaccinium* plants may also possess anti-adhesive properties, but such compounds have yet to be identified. Many studies regarding hemicellulose-derived oligosaccharides have focused on characterizing biosynthesis pathways and structural variation and on genetic modifications to improve animal

feedstock or biofuel production (Scheller & Ulvskov 2010). These studies often employ fungal hemicellulases that cleave polymers to produce lower-MW compounds that can be more easily studied, and studies that do exist typically have a botanical or agricultural focus rather than a human medicine focus (Scheller & Ulvskov 2010). Neutral oligosaccharides are inherently difficult to detect, isolate, and characterize structurally (York *et al*. 1996, Chai *et al*. 2002, Yamagaki *et al*. 2006, LaMotte 2008). Studies of the biological properties of naturally-occurring, plant-derived oligosaccharides as pure compounds or as native mixtures have therefore been limited, and additional work is needed in this area.

2. *Cranberry Oligosaccharides as Analytical Standards*

To date, two cranberry oligosaccharides have been purified to the degree necessary for full structural elucidation and identification as arabinoxyloglucans (Auker 2013). Evidence from NMR and HPLC-ELSD data indicates the presence of a series of structurally related compounds in cranberry, all of which are hypothesized to be arabinoxyloglucans based on the presence of anomeric ^{1}H NMR resonances characteristic of this chemical class. Current data also supports the hypothesis that the absolute configurations of the primary monosaccharide units will be D-Glc, D-Xyl, and L-Ara, consistent with the natural abundances of these monosaccharides. An additional monosaccharide that may be present is galactose based on preliminary chemical analysis of cranberry oligosaccharide fractions (CJA2, Auker 2013).

Further work to isolate individual cranberry oligosaccharides is still necessary and full structural elucidation for each compound will require the application of multiple complimentary techniques (Hisamatsu *et al*. 1992, York *et al*. 1996, Weiskopf *et al*. 1997, Chai *et al*. 2002, Yamagaki *et al*. 2006, LaMotte 2008). Multiple structural features for each

oligosaccharide must be determined before complete structures can be assigned, including (a) molecular weight, (b) glycosyl composition, (c) glycosyl linkage positions, (d) glycosyl configuration (D vs. L), (e) glycosyl anomeric configuration (α vs. β), and (f) glycosyl sequence. Individual oligosaccharides within the series may differ by single structural elements, such as a single change in linkage position or anomeric configuration between any two compounds (York *et al*. 1990, Hisamatsu *et al*. 1992, York *et al*. 1996, Scheller & Ulvskov 2010). Any analytical methods used to detect individual compounds within the series must therefore be appropriate for discriminating between such closely related compounds or must consider this limitation when assigning putative structures (York *et al*. 1996, Weiskopf *et al*. 1997, Chai *et al*. 2002, Yamagaki *et al*. 2006, LaMotte 2008).

The present studies have yielded a large quantity (~20 g) of enriched cranberry oligosaccharide fractions that can be used for further studies, and the methods presented here provide simple, reproducible, low cost procedures for obtaining relatively large amounts of oligosaccharides from renewable and readily available starting material. These cranberry oligosaccharide mixtures will primarily be useful as starting material for further purification of individual components. Once obtained, purified compounds will be useful for detailed structural assignments, biological mechanism of action studies, and as analytical standards for many applications. Individual cranberry oligosaccharides and oligosaccharide mixtures with known composition have the potential to be used as analytical standards for (a) the development of optimized methods suited to rapid detection of oligosaccharides, (b) the detection of oligosaccharides in clinical biofluids, (c) the detection of oligosaccharides in various plants and dietary sources, and (d) the standardization of cranberry-containing dietary supplements. Additional applications will also likely be found by future researchers.

3. *Commercial Product Development*

Preparations of cranberry-derived oligosaccharides, as mixtures or as pure compounds, could potentially be developed into numerous products with commercial applications. These include dietary supplements, botanical drugs (FDA CDER 2004), and anti-adhesion coatings for medical devices (Nickel *et al*. 1994), among others. Cranberry is a common food item, and cranberry-derived oligosaccharides would therefore be generally regarded as safe (GRAS) for human consumption and medical use (McCaleb *et al*. 2000). As a botanical drug, or as oligosaccharide-enriched dietary supplements, cranberry-derived oligosaccharides could be used in place of whole cranberry products or cranberry juice for the prevention of UTIs.

Enrichments for anti-adhesive oligosaccharides and the removal of other cranberry compounds may increase the potential marketability of cranberry-derived materials, providing the benefits of cranberry anti-adhesion properties while avoiding the inclusion of unwanted components. Common reasons given for patient withdrawal from clinical trials concerning cranberry were the "unpalatable or intolerable nature of the cranberry product" (Jepson *et al*. 2012). The objectives of avoiding caloric intake (simple sugars) or cranberry flavor (tartness) and the convenience of a pill form are common reasons consumers may choose cranberry dietary supplements instead of the traditionally recommended cranberry juice (Jepson *et al*. 2012, Proprietary Nutritionals 2007). Cranberry compounds can also cause herbal-drug interactions such as those reported for warfarin (Aston *et al*. 2006). Enriched preparations of oligosaccharides could be prepared from cranberry materials to exclude simple sugars, flavor components, and other compounds that cause interactions, while providing consumers with a convenient product form.

As the active anti-adhesive metabolites of cranberry that are excreted into urine have remained unidentified prior to this report, the "concentrated" versions of cranberry currently sold as dietary supplements are not necessarily enriched for the appropriate cranberry components. The need for standardization of dietary supplement materials to appropriate concentrations of active constituents is a well-recognized problem, but requires that the constituents responsible for a given bioactivity have been suitably characterized. The identification of oligosaccharides as anti-adhesive components of cranberry in this report will enable researchers to identify, quantify, and standardize to target these active components in various commercial products. The preliminary analyses presented here regarding the oligosaccharide composition of commercially obtained cranberry products suggests that juice products with a sufficient percentage of cranberry juice already contain the active components. These studies also suggest that dietary supplement manufacturers may need to redesign their cranberry processing protocols and implement new standardization procedures to ensure that their products contain sufficient oligosaccharide content to provide the target biological activity.

D. Summary of Future Directions

The above discussion addresses the results of the current study within the context of existing research and highlights the need for additional research regarding both the chemistry and biological properties of cranberry oligosaccharides. Future research will need to address many different areas, especially with regards to clinical, agricultural, and basic science applications.

The anti-adhesion properties of cranberry oligosaccharides, as both pure compounds and as mixtures, need further study, especially with regards to the mechanism of action for the prevention of UPEC adhesion and possible synergistic effects of oligosaccharide mixtures. Further anti-adhesion studies will also be needed to determine if oligosaccharides contribute to the reported abilities of cranberry to prevent the adhesion or biofilm formation of bacteria other than *E. coli*. The inherent difficulties of oligosaccharide detection without specialized methods suggests that these compounds may be present in many cranberry materials that have been attributed with different biological activities. The possible bioactivities of cranberry oligosaccharides will therefore need to be assessed in many different types of bioassays.

Additional cranberry oligosaccharides need to be purified and fully elucidated, and the natural occurrence of these or similar compounds in cranberry, in related *Vaccinium* species, and in other plants needs to be assessed. Purified oligosaccharides and oligosaccharide mixtures can be used as analytical reference standards for any future study that aims to investigate the presence of these compounds. Such studies may include investigations of cranberry oligosaccharide content as a function of fruit ripeness, investigations of possible dietary sources other than cranberry, mammalian ADME studies, and studies that aim to establish clinical correlations between oligosaccharide intake, urinary excretion and UTI occurrence, among others.

Cranberry oligosaccharides, as pure compounds or as mixtures, have potential for development into a variety of commercial products, including anti-adhesive coatings for medical devices, oligosaccharide-enriched dietary supplements, and even possibly a botanical drug product. Such products would be useful as clinical therapies for the prevention of UTIs

caused by P-fimbriated *E. coli* and may have other applications that have not yet been identified.

E. Final Conclusions

These studies have identified mixtures of arabinoxyloglucan oligosaccharides as the anti-adhesive components of both cranberry products and the urine of swine after the consumption of cranberry products. The anti-adhesion activity of oligosaccharide mixtures has been confirmed using two separate assays. The structure of a representative arabinoxyloglucan octasaccharide from swine urine was fully elucidated and found to be similar in structure to two arabinoxyloglucan oligosaccharides isolated from cranberry products. Similar HPLC-ELSD profiles are present in both cranberry and urine materials, indicating direct absorption and excretion of a complex mixture of structurally related oligosaccharides. The results of these studies support the new hypothesis that cranberry-derived oligosaccharides are a major constituent responsible for the anti-adhesion effects of urine produced after cranberry product consumption. Overall, the hypotheses presented in this report provide a new guiding paradigm for future research regarding the bioactive constituents of cranberry fruit and juice products. The information presented here regarding the anti-adhesion properties of cranberry oligosaccharides and their hypothesized role as urinary anti-adhesion metabolites will significantly change how researchers and the general public view cranberry for the prevention of UTIs.

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LIST OF APPENDICES

APPENDIX A: COLOR PLATES

1. ***Overview of Appendix A***

The images shown below are included here to show color characteristics in support of the methods and materials described in Chapters 2 and 3. All photographs included as color plates were taken by C. M. Coleman during the studies discussed in this report.

Figure 94. A cranberry bog located in northeastern U.S. with an inset that shows the cranberry plant. This photograph was taken by C. M. Coleman on 2013-05-20.

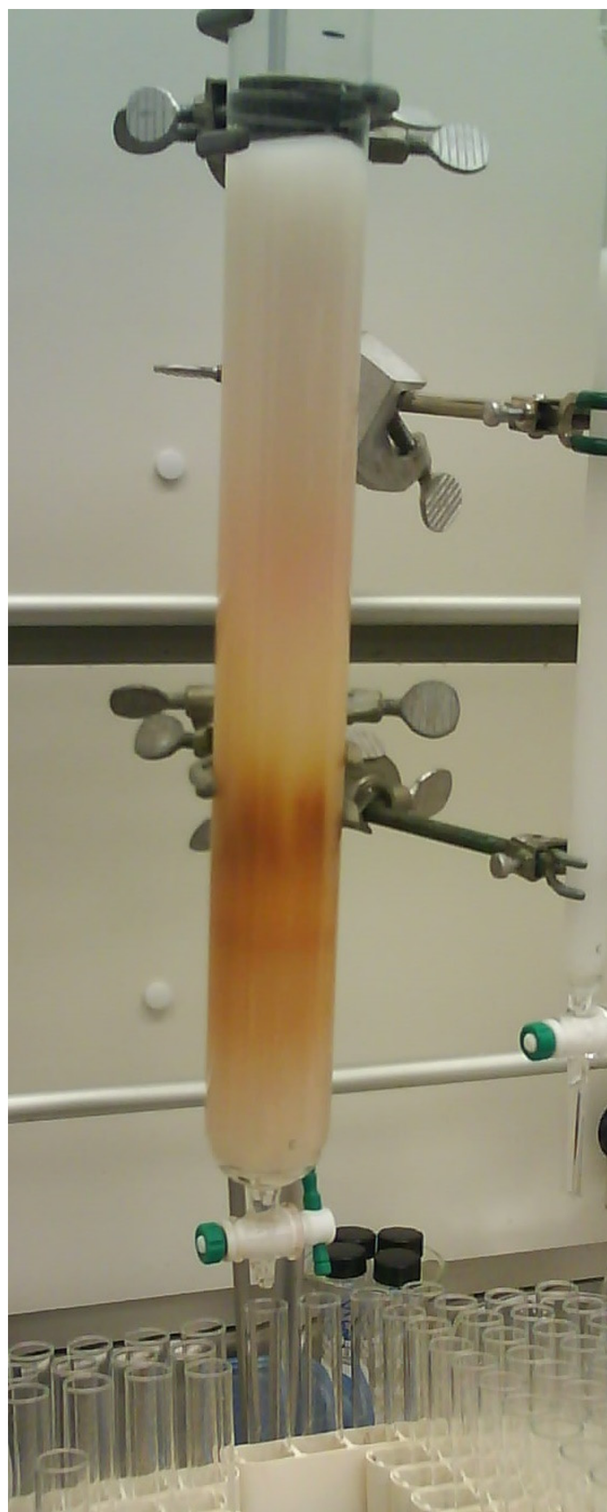


Figure 95. Sephadex LH-20 column for the FC urine separation.
The control urine material was similar in appearance to the active urine study materials, with an apparent lack of pink color. This photograph was taken by C. M. Coleman on 2008-05-07.

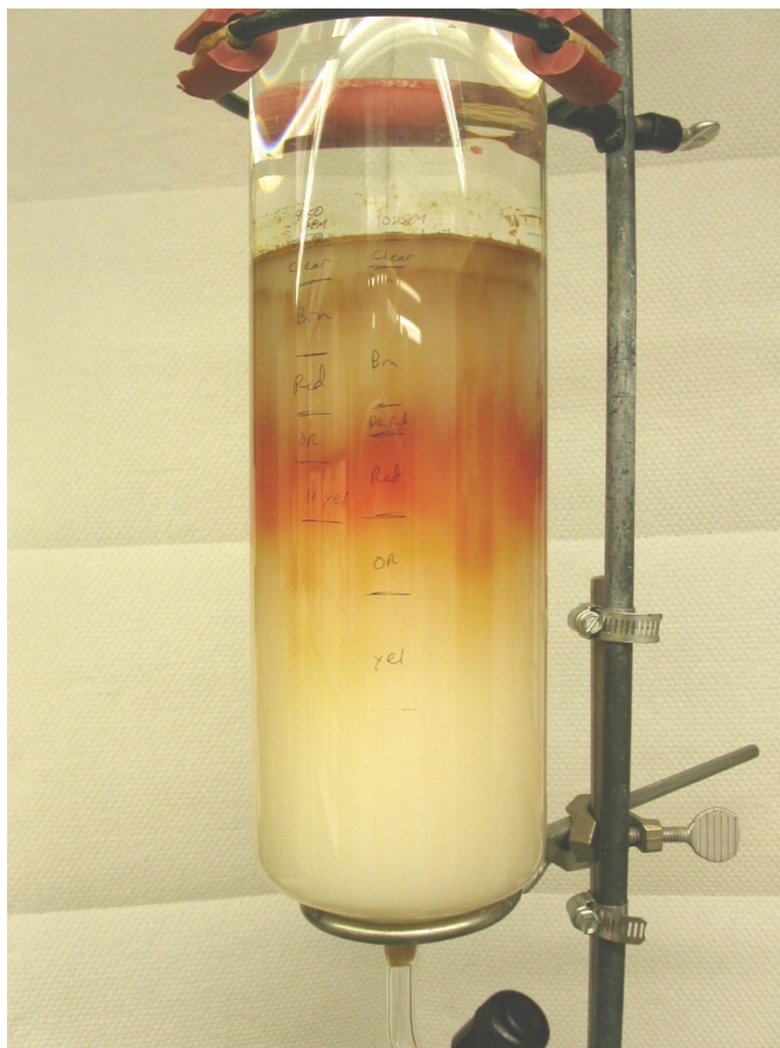


Figure 96. Sephadex LH-20 column for the F5 urine separation.
Compounds of interest eluted as the first fraction below the lowest visible band of yellow. The darker bands visible on this column (redish, orange, and brown) constituted the bulk of the collected material, and the dried fractions from these bands were hygroscopic. This photograph was taken by C. M. Coleman on 2007-07-20.

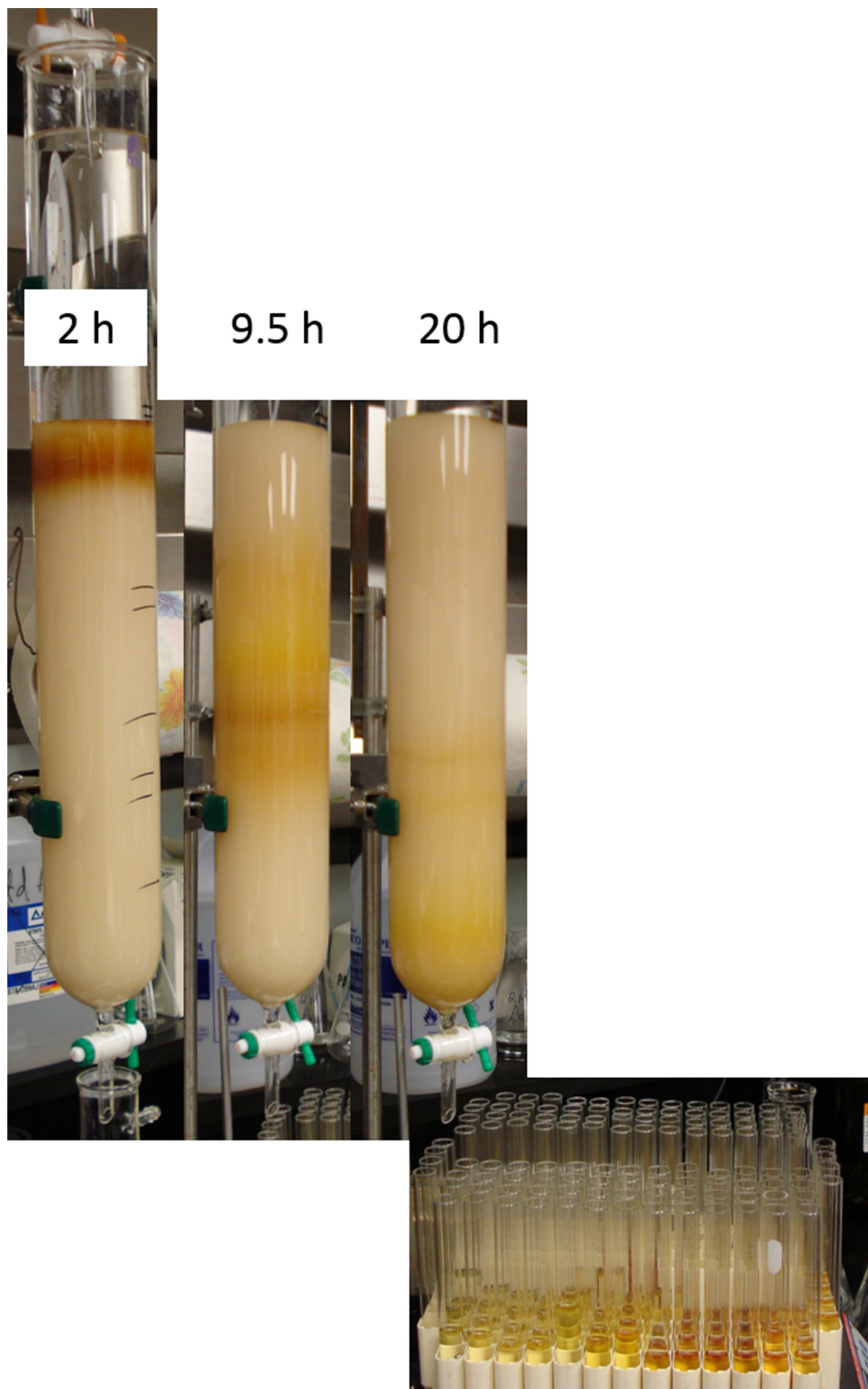


Figure 97. Sephadex LH-20 column for the IF2 urine separation showing elution progression (2, 9.5 and 20 h) and the appearance of the resulting fractions in test tubes.
These photographs were taken by C. M. Coleman on 2009-06-23 and 2009-06-24.

Figure 98. Cranberry dietary supplement products analyzed by HPLC-ELSC, showing packaging, labeling, capsules, and samples in aqueous solution.
All four dietary supplement products resulted in aqueous solutions that had high amounts of insoluble material. These solutions were not similar to that obtained from cranberry juice powder (see Figure 99). These photographs were taken by C. M. Coleman on 2011-03-24.

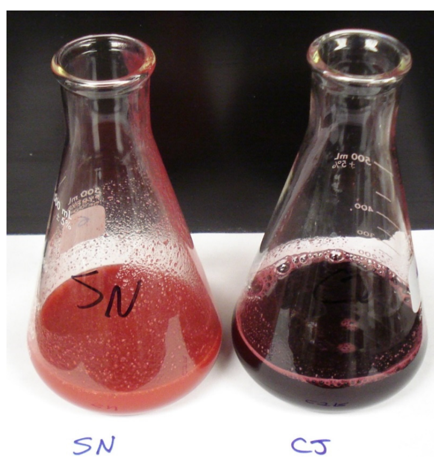


Figure 99. Cranberry fruit (SN) and juice (CJ) powders in aqueous solution, showing the differences in appearance. This photograph was taken by C. M. Coleman on 2011-03-24.

Figure 100. Cranberry juice products analyzed by HPLC-ELSD (BCB, GVC, and GVNS), showing packaging, label information, and juice appearance.
All juice products analyzed appeared similar in color in spite of the differences in primary ingredient. These photographs were taken by C. M. Coleman on 2011-04-20.

Figure 101. Cranberry juice products analyzed by HPLC-ELSD (OSC, LCP, and NL), showing packaging, label information, and juice appearance.
All juice products analyzed appeared similar in color in spite of the differences in primary ingredient. These photographs were taken by C. M. Coleman on 2011-04-20.

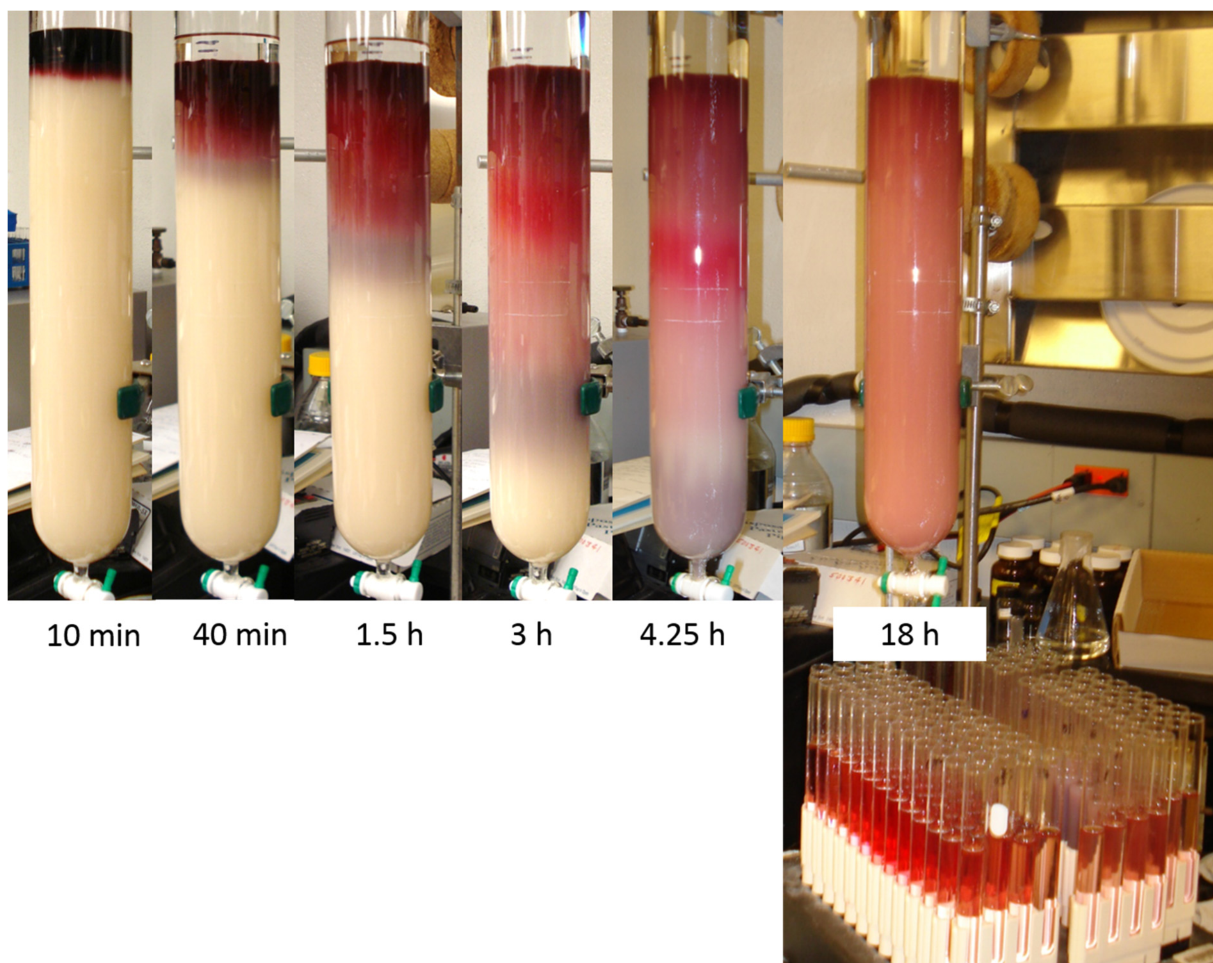


Figure 102. Sephadex LH-20 column for the CJA1 cranberry separation showing elution progression (10 min – 18 h) and the appearance of resulting fractions in test tubes. Oligosaccharide components eluted early from this column, below the visible lavender band. The pink/red material remaining on the column at 18 h produced a relatively low quantity of material in spite of the visible color. These photographs were taken by C. M. Coleman on 2010-09-24 and 2010-09-25.

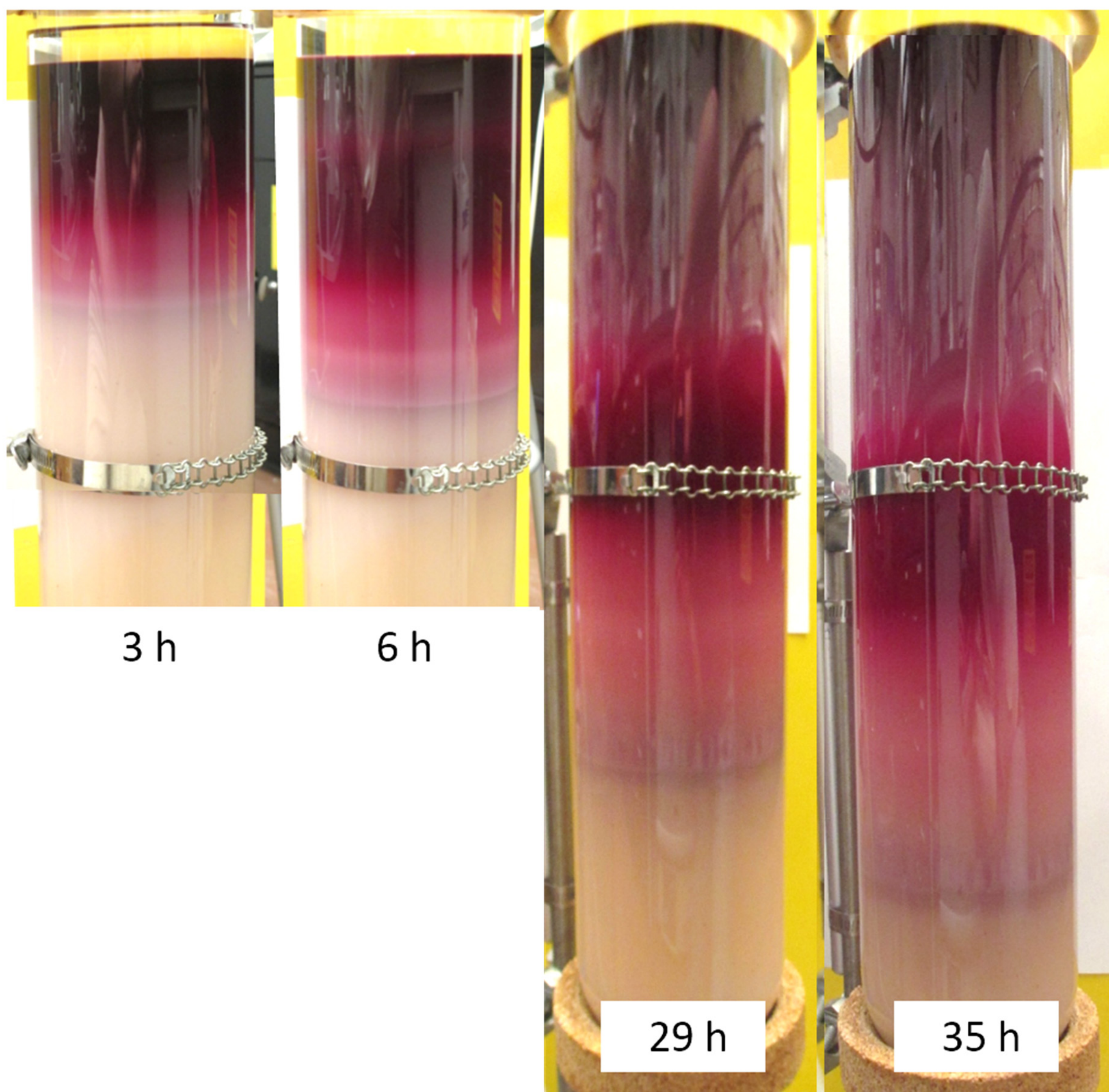


Figure 103. Sephadex LH-20 column for the CCA2 cranberry separation showing elution progression (3–35 h).
Distinct visible bands are apparent with this column (*e.g.* 6 h), corresponding to the higher resolution of components into discrete fractions. These photographs were taken by C. M. Coleman on 2013-09-03 and 2013-09-04.

Figure 104. Overview of the configuration and equipment set-up for the CCA2 Sephadex LH-20 column.
This photograph was taken by C. M. Coleman on 2010-09-25.

APPENDIX B: RAW DATA SUPPLEMENTS

1. *Overview of Appendix B*

The data in this appendix is provided in support of the discussion points presented in the main text. This appendix contains additional discussion points in relation to the UPEC-HRBC anti-agglutination assay and the raw data provided by this assay as discussed in Chapter 2; Section C1, and Chapter 3; Sections C1 and C4a. This appendix also contains figures with NMR spectra and chromatograms in support of the efforts made to isolate active urine components, as discussed in Chapter 2; Sections C2 – C4.

2. *Additional Discussion of the UPEC-HRBC Anti-agglutination Assay*

Results were provided by RU to UM in the form of Excel spreadsheets that contained dilution endpoint values (mg/mL) assigned by RU corresponding to the sample codes provided by UM. These data were decoded at UM and interpreted using qualitative activity scores assigned by UM (UM Scores) as discussed above (Chapter 2, Section B3, Table 3: reproduced below). The results of a given data set were compared to those of previously obtained assay data sets to evaluate relative data quality and interpretations. For some data sets (Tables 45, 47, 49, 51), the visual agglutination scores (VAS) determined for each dilution of each sample were included by RU. Most result files did not include VAS values and the number of two-fold dilutions used to obtain the reported dilution endpoint value was calculated based on the methods reported by RU. The reported number of two-fold dilutions does not include the starting concentration.

The initial dilution volume used for a given sample was arbitrarily determined by RU based on the weight of the sample within a range. An example of a sample weight range to dilution volume is as follows: sample mg > 80: 400 µL, 79 > mg > 40: 200 µL, 39 > mg > 10:

150 µL, 9 > mg > 7: 100 µL. The dilution volumes used per sample weight range varied between sample sets over time. An example of this variation is the use of 150 µL PBS for 24.5 mg (08/2006 sample; Table 43), 200 µL for 24.5 mg (06/2008 sample; Table 52), and 100 µL for 27.0 mg (12/2008 sample; Table 56). Changes in sample initial dilution volumes combined with the variety of submitted sample weights resulted in the testing of a range of sample starting concentrations at any one time; *e.g*. 74–420 mg/mL; Table 44, and 26–740 mg/mL; Table 54. Two-fold dilutions performed on these starting concentrations yielded yet another set of varied concentrations and ultimately yielded the reported dilution endpoint values. These variations in sample concentration throughout the performance of the assay may have affected the overall assay sensitivity and contributed to issues with assay reproducibility.

Table 3. Description of the qualitative scoring system developed by UM to interpret the results provided by the UPEC-HRBC anti-agglutination assay.
Samples were assessed within an individual sample set test date and then across test dates. Scoring criteria were applied in the order shown to determine the probable activity of individual samples and assign a UM Score. Replicates were not available for most samples.

Scoring Criteria:		First	Second	Third	Fourth
UM Score	UM Assessment of Sample Activity	Dilution Endpoint Range	No. of Two-fold Dilutions	Sample Compared to Parent Fraction:	Replicates (if available):
+ +	active	< 10 mg/mL	5+	more active	show activity
+	probably active	10–30 mg/mL	4	more active	show activity
+/–	may or may not be active	30–60 mg/mL	3	about the same	may or may not show activity
–	probably not active	60–100 mg/mL	2	less active	show no activity
– –	not active	> 100 mg/mL	0, 1	less active	show no activity

Samples for which the dilution endpoint was equal to the starting concentration may have had visual agglutination scores of ‘1’ or ‘2’ but the apparent activity for these samples was not retained (VAS = ‘0’) after the first two-fold dilution. Single-concentration sample

activity cannot be accurately assessed without VAS values, but most of these samples were tested at starting concentrations of > 100 mg/mL and were therefore assigned as inactive. Samples that were assigned a dilution endpoint of "Neg" (= negative) were found to have no ability to inhibit HRBC agglutination at the highest (first) concentration tested.

Samples that were assigned a dilution endpoint of "Solv" (= solvent) were associated with the bursting of HRBCs at the highest concentration tested. Although RU attributed this bursting of HRBCs to the presence of solvent in the samples, most samples that could have contained solvent had been dried completely, re-dissolved in water, lyophilized, and then provided to RU as dry powders. These samples were therefore unlikely to have contained sufficient solvent to have caused this effect. While samples may have contained other components that could have contributed to the bursting of HRBCs, no sample-related pattern could be discerned regarding the assignment of the "Solv" dilution endpoint. Replicates of some samples that showed the "Solv" effect (*e.g*. FG; Table 44) were found to lack activity and all samples for which this was the reported dilution endpoint were assigned a negative UM score ('– –' Table 3). The "Solv" effect may have been related to issues with the HRBCs themselves or other methodological factors unknown to UM.

When provided, the VAS values assigned to samples were predominantly '1' or '2'. The assay methods suggested that higher VAS values of '3' or '4' could be obtained, indicating the inhibition of agglutination for 70-95 and 95-100% of HRBCs, respectively. Published evidence could not be found in support of the ability of the assay to yield these VAS values with positive controls, and VAS values or other information for positive control samples tested with the samples from this study were not provided to UM.

False negative results were more likely than false positive results for most samples, as the reported dilution endpoints may have been higher than actual sample concentrations. Many urine fractions were hygroscopic and the absorption of water from the atmosphere would have resulted in higher than actual apparent weights for these samples, yielding lower actual solute concentrations. Crude urine materials often had significant sample heterogeneity, containing insoluble and soluble components within any give aliquot. Efforts were made to homogenize these materials prior to assay submission, but only part of a given sample may have been soluble in the PBS used for the assay. The low-volume drops used for the assay were also reported to be affected by ambient humidity, absorbing water from the atmosphere on days when it rained and further diluting test samples (A. B. Howell, personal communication). Cumulative results indicate that samples did not lose activity when stored at –20 ^{o}C as dry powders, and the apparent changes in sample activity (*e.g*. the FF aqueous fraction; Chapter 2, Table 8) are likely to be false negative results due to some of the reasons presented here.

Additional assay variation can be attributed to the subjective nature of human judgment and visual assessments, variations between collections of HRBCs, and variations in the characteristics of the clinical strains of *E. coli* used. While *E. coli* were subcultured on colonization factor agar to enhance the expression of P fimbriae (Evans *et al.* 1977) prior the use of the bacteria for sample testing, the expression of P fimbriae was not quantified for each sample set. Changes in P fimbriae expression or strain characteristics over time may therefore have affected the assay results. Other unknown factors may also have contributed to the inconsistencies that were observed in sample data.

3. *Supporting Data Tables for the UPEC-HRBC Anti-agglutination Assay*

The raw data results for the UPEC-HRBC anti-agglutination assay are included in the following tables (Tables 41–59). These tables are arranged in date order by test date. Samples within a given test date were sent from UM to RU within a single shipment but were not necessarily tested on the same day at RU. Individual samples within a test date were coded, randomized, and not necessarily tested in the order shown. Sample descriptions contain sample codes as well as brief descriptions of sample origins. Urine samples and fractions are discussed in more detail in Chapter 2 while cranberry samples and fractions are discussed in Chapter 3.

Table 41. Urine fractions (D) tested in the anti-agglutination assay, 03/2006.

Sample Description	Test Date	UM Score	Dilution Endpoint (mg/mL)	No. of Two-fold Dilutions	Amount Submitted (mg)	Dilution Volume (µL)	Starting Conc. (mg/mL)
D (crude urine)	03/2006	– –	111.0	1	110.6	500	221.2
DE (D EtOAc fraction	03/2006	– –	Neg	0	16.1	150	107.3
DS (D *sec*-BuOH fraction)	03/2006	– –	Solv	1	120.2	500	240.4
DA (D aqueous fraction)	03/2006	+/–	87.4	1	87.4	500	174.8

Table 42. Urine fractions (DA Sephadex LH-20 fractions) tested in the anti-agglutination assay, 05/2006.

Sample Description	Test Date	UM Score	Dilution Endpoint (mg/mL)	No. of Two-fold Dilutions	Amount Submitted (mg)	Dilution Volume (µL)	Starting Conc. (mg/mL)
DA1	05/2006	–	52.2	2	52.2	250	208.8
DA2	05/2006	–	112.0	1	56.0	250	224.0
DA3	05/2006	+/–	43.1	2	86.1	500	172.2
DA4	05/2006	+/–	43.2	2	172.9	1000	172.9
DA5	05/2006	– –	226.4	1	226.4	500	452.8
DA6	05/2006	– –	Neg	2	57.6	250	230.4
DA7	05/2006	– –	Neg	2	77.7	250	310.8
DA8	05/2006	– –	Neg	1	91.7	500	183.4
DA9	05/2006	– –	Neg	2	59.1	250	236.4
DA10	05/2006	– –	202.4	0	50.6	250	202.4
DA11	05/2006	– –	328.4	0	82.1	250	328.4
DA12	05/2006	– –	106.0	0	53.0	250	212.0
DA13	05/2006	+ +	1.4	7	44.9	250	179.6
DA14	05/2006	+ +	5.3	4	21.3	250	85.2
DA15	05/2006	+ +	1.4	7	46.3	250	185.2
DA16	05/2006	+ +	1.6	7	51.4	250	205.6
DA17	05/2006	– –	191.6	0	47.9	250	191.6
DA18	05/2006	+ +	7.7	4	30.9	250	123.6
DA19	05/2006	+ +	0.8	8	48.0	250	192.0
DA20	05/2006	– –	Neg	0	50.6	250	202.4

Table 43. Urine fractions (DA Sephadex LH-20 fractions) tested in the anti-agglutination assay, 08/2006.

Sample Description	Test Date	UM Score	Dilution Endpoint (mg/mL)	No. of Two-fold Dilutions	Amount Submitted (mg)	Dilution Volume (µL)	Starting Conc. (mg/mL)
DA13	08/2006	+ +	2.5	5	14.4	150	96.0
DA14	08/2006	–	76.0	1	11.4	150	76.0
DA15	08/2006	–	74.6	2	37.3	250	149.2
DA16	08/2006	+/–	48.9	2	48.9	250	195.6
DA17	08/2006	+ +	5.0	5	24.5	150	163.3
DA18	08/2006	+ +	2.5	5	13.4	150	89.3
DA19	08/2006	+ +	2.5	6	19.1	150	127.3
DA20	08/2006	– –	Neg	2	35.3	250	141.2

Table 44. Urine fractions (F and Sephadex LH-20 fractions for DA and FF columns 1 & 2) tested in the anti-agglutination assay, 03/2007.

Sample Description	Test Date	UM Score	Dilution Endpoint (mg/mL)	No. of Two-fold Dilutions	Amount Submitted (mg)	Dilution Volume (µL)	Starting Conc. (mg/mL)
DA1	03/2007	+ +	2.6	5	12.2	150	81.3
DA2	03/2007	+	23.2	2	13.9	150	92.7
DA3	03/2007	+	10.0	3	8.0	100	80.0
DA4	03/2007	– –	74.6	0	11.2	150	74.7
DA5	03/2007	– –	Solv	0	14.6	150	97.3
DA6	03/2007	– –	Solv	0	9.3	100	93.0
DA7	03/2007	– –	186.0	0	37.2	200	186.0
DA8	03/2007	+ +	5.1	5	32.5	200	162.5
FF (aqueous fraction)	03/2007	+/–	35.8	3	114.5	400	286.3
FF (aqueous fraction)	03/2007	+/–	52.5	3	168.1	400	420.3
FG (F solids)	03/2007	– –	Neg	0	62.7	200	313.5
FG (F solids)	03/2007	– –	Solv	0	47.7	200	238.5
FG (F solids)	03/2007	– –	Neg	0	42.1	200	210.5
FF1-1	03/2007	+ +	9.7	3	11.6	150	77.3
FF1-2	03/2007	+ +	6.2	4	9.9	100	99.0
FF1-3	03/2007	– –	Neg	0	42.5	200	212.5
FF1-4	03/2007	– –	Solv	0	53.5	200	267.5
FF1-5	03/2007	–	84.6	2	135.4	400	338.5
FF1-6	03/2007	+/–	31.4	3	50.3	200	251.5
FF1-7	03/2007	+ +	2.6	5	12.5	150	83.3
FF1-8	03/2007	– –	Neg	0	11.6	150	77.3
FF1-9	03/2007	– –	Neg	0	16.5	150	110.0

Table 45. Urine fractions (F and Sephadex LH-20 fractions for DA and FF columns 1 & 2) tested in the anti-agglutination assay, 03/2007, showing dilution concentrations tested and visual agglutination scores (VAS).

Sample	UM Score	Dilution Endpoint (mg/mL)	No. Dils.	Start Conc. (mg/mL)	VAS (start)	Dil. 1 Conc. (mg/mL)	VAS (dil 1)	Dil. 2 Conc. (mg/mL)	VAS (dil 2)	Dil. 3 Conc. (mg/mL)	VAS (dil 3)	Dil. 4 Conc. (mg/mL)	VAS (dil 4)	Dil. 5 Conc. (mg/mL)	VAS (dil 5)	Dil. 6 Conc. (mg/mL)	VAS (dil 6)
DA1	+ +	2.6	5	81.3	2	40.7	2	20.3	2	10.2	2	5.1	2	2.5	2	1.3	0
DA2	+	23.2	2	92.7	2	46.3	2	23.2	1	11.6	0						
DA3	+	10.0	3	80.0	2	40.0	2	20.0	1	10.0	1	5.0	0				
DA4	– –	74.6	0	74.7	1	37.3	0										
DA5	– –	Solv	0	97.3	X												
DA6	– –	Solv	0	93.0	X												
DA7	– –	186.0	0	186.0	1	93.0	0										
DA8	+ +	5.1	5	162.5	2	81.3	2	40.6	2	20.3	2	10.2	2	5.1	2	2.5	0
FF (water fraction)	+/–	35.8	3	286.3	2	143.1	2	71.6	2	35.8	2	17.9	0				
FF (water fraction)	+/–	52.5	3	420.3	2	210.1	2	105.1	2	52.5	2	26.3	0				
FG (F solids)	– –	Neg	0	313.5	0												
FG (F solids)	– –	Solv	0	238.5	X												
FG (F solids)	– –	Neg	0	210.5	0												
FF1-1	+ +	9.7	3	77.3	2	38.7	2	19.3	2	9.7	1	4.8	0				
FF1-2	+ +	6.2	4	99.0	2	49.5	2	24.8	2	12.4	2	6.2	1	3.1	0		
FF1-3	– –	Neg	0	212.5	0												
FF1-4	– –	Solv	0	267.5	X												
FF1-5	–	84.6	2	338.5	1	169.3	1	84.6	1	42.3	0						
FF1-6	+/–	31.4	3	251.5	2	125.8	2	62.9	2	31.4	1	15.7	0				
FF1-7	+ +	2.6	5	83.3	2	41.7	2	20.8	2	10.4	2	5.2	2	2.6	1	1.3	0
FF1-8	– –	Neg	0	77.3	0												
FF1-9	– –	Neg	0	110.0	0												

Table 46. Urine fractions (FF Sephadex LH-20 fractions, columns 2 & 3) tested in the anti-agglutination assay, 05/2007.

Sample Description	Test Date	UM Score	Dilution Endpoint (mg/mL)	No. of Two-fold Dilutions	Amount Submitted (mg)	Dilution Volume (µL)	Starting Conc. (mg/mL)
FF (aqueous fraction)	05/2007	+ +	11.8	5	75.2	200	376.0
FF (aqueous fraction)	05/2007	+	19.9	3	79.9	500	159.8
FF (aqueous fraction)	05/2007	–	105.8	2	84.7	200	423.5
FF2-1	05/2007	+ +	2.5	5	11.9	150	79.3
FF2-2	05/2007	+	12.8	4	30.8	150	205.3
FF2-3	05/2007	+	14.3	4	135.8	600	226.3
FF2-4	05/2007	+	13.6	4	32.8	150	218.7
FF2-5	05/2007	– –	Neg	0	55.6	200	278.0
FF2-6	05/2007	+/–	42.5	2	25.5	150	170.0
FF2-7	05/2007	+/–	34.3	2	20.6	150	137.3
FF2-8	05/2007	– –	222.5	0	44.5	200	222.5
FF3-1	05/2007	+ +	9.3	3	11.2	150	74.7
FF3-2	05/2007	+ +	3.7	5	17.9	150	119.3
FF3-3	05/2007	– –	119.6	1	35.9	150	239.3
FF3-4	05/2007	– –	Neg	0	56.8	200	284.0
FF3-5	05/2007	– –	Neg	0	23.3	150	155.3
FF3-6	05/2007	+/–	38.8	2	38.1	250	152.4

Table 47. Urine fractions (FF Sephadex LH-20 fractions, columns 2 & 3) tested in the anti-agglutination assay, 05/2007, showing dilution concentrations tested and visual agglutination scores (VAS).

Sample	UM Score	Dil. Endpt (mg/mL)	No. Dils.	Start Conc. (mg/mL)	VAS (start)	Dil. 1 Conc. (mg/mL)	VAS (dil 1)	Dil. 2 Conc. (mg/mL)	VAS (dil 2)	Dil. 3 Conc. (mg/mL)	VAS (dil 3)	Dil. 4 Conc. (mg/mL)	VAS (dil 4)	Dil. 5 Conc. (mg/mL)	VAS (dil 5)	Dil. 6 Conc. (mg/mL)	VAS (dil 6)
FF2-1	+ +	2.5	5	79.3	2	39.7	2	19.8	2	9.9	2	5.0	2	2.5	1	1.2	0
FF2-2	+	12.8	4	205.3	2	102.7	2	51.3	2	25.7	1	12.8	0				
FF2-3	+	14.3	4	226.3	2	113.2	1	56.6	1	28.3	1	14.1	0				
FF2-4	+	13.6	4	218.7	1	109.3	1	54.7	1	27.3	1	13.7	1	6.8	0		
FF2-5	– –	Neg	0	278.0	0												
FF2-6	+/–	42.5	2	170.0	2	85.0	2	42.5	2	21.3	0						
FF2-7	+/–	34.3	2	137.3	2	68.7	2	34.3	1	17.2	0						
FF2-8	– –	222.5	0	222.5	1	111.3	0										
FF3-1	+ +	9.3	3	74.7	2	37.3	2	18.7	2	9.3	2	4.7	0				
FF3-2	+ +	3.7	5	119.3	2	59.7	2	29.8	1	14.9	0						
FF3-3	– –	119.6	1	239.3	2	119.7	1	59.8	0								
FF3-4	– –	Neg	0	284.0	0												
FF3-5	– –	Neg	0	155.3	0												
FF3-6	+/–	38.8	2	152.4	2	76.2	2	38.1	1	19.1	0						
FF (water fraction)	+ +	11.8	5	376.0	2	188.0	2	94.0	2	47.0	2	23.5	1	11.8	1	5.9	0
FF (water fraction)	+	19.9	3	159.8	2	79.9	2	40.0	1	20.0	1	10.0	0				
FF (water fraction)	–	105.8	2	423.5	2	211.8	2	105.9	1	52.9	0						

Table 48. Urine fractions (FF Sephadex LH-20 fractions, columns 4 & 5) tested in the anti-agglutination assay, 06/2007.

Sample Description	Test Date	UM Score	Dilution Endpoint (mg/mL)	No. of Two-fold Dilutions	Amount Submitted (mg)	Dilution Volume (µL)	Starting Conc. (mg/mL)
FF (aqueous fraction)	06/2007	+/–	54.6	2	43.7	200	218.5
FF (aqueous fraction)	06/2007	+/–	53.0	3	84.8	200	424.0
FF (aqueous fraction)	06/2007	–	73.4	2	117.4	400	293.5
FF4-1	06/2007	+	12.4	4	29.7	150	198.0
FF4-2	06/2007	+	25.5	2	15.3	150	102.0
FF4-3	06/2007	–	105.0	2	168.2	400	420.5
FF4-4	06/2007	– –	120.6	2	193.0	400	482.5
FF4-5	06/2007	– –	120.0	0	98.5	820	120.1
FF4-6	06/2007	– –	300.0	0	60.1	200	300.5
FF4-7	06/2007	– –	323.0	0	129.3	400	323.3
FF4-8	06/2007	– –	116.0	0	17.5	150	116.7
FF5-1	06/2007	+ +	9.8	3	7.9	100	79.0
FF5-2	06/2007	– –	204.6	0	30.7	150	204.7
FF5-3	06/2007	– –	240.6	0	36.1	150	240.7
FF5-4	06/2007	– –	371.0	0	148.4	400	371.0
FF5-5	06/2007	– –	120.0	0	18.1	150	120.7
FF5-6	06/2007	– –	374.0	0	149.6	400	374.0
FF5-7	06/2007	– –	208.0	0	31.3	150	208.7
FF5-8	06/2007	–	62.3	2	49.9	200	249.5

Table 49. Urine fractions (FF Sephadex LH-20 fractions, columns 4 & 5) tested in the anti-agglutination assay, 06/2007, showing dilution concentrations tested and visual agglutination scores (VAS).

Sample	UM Score	Dilution Endpoint (mg/mL)	No. Dils.	Start Conc. (mg/mL)	VAS (start)	Dil. 1 Conc. (mg/mL)	VAS (dil 1)	Dil. 2 Conc. (mg/mL)	VAS (dil 2)	Dil. 3 Conc. (mg/mL)	VAS (dil 3)	Dil. 4 Conc. (mg/mL)	VAS (dil 4)	Dil. 5 Conc. (mg/mL)	VAS (dil 5)
FF4-1	+	12.4	4	198.0	2	99.0	2	49.5	2	24.8	2	12.4	2	6.2	0
FF4-2	+	25.5	2	102.0	2	51.0	2	25.5	1	12.8	0				
FF4-3	–	105.0	2	420.5	2	210.3	2	105.1	1	52.6	0				
FF4-4	– –	120.6	2	482.5	2	241.3	1	120.6	1	60.3	0				
FF4-5	– –	Neg	0	120.1	0										
FF4-6	– –	Neg	0	300.5	0										
FF4-7	– –	Neg	0	323.3	0										
FF4-8	– –	Neg	0	116.7	0										
FF5-1	+ +	9.8	3	79.0	2	39.5	2	19.8	2	9.9	2	4.9	0		
FF5-2	– –	204.6	0	204.7	1	102.3	0								
FF5-3	– –	240.6	0	240.7	1	120.3	0								
FF5-4	– –	371.0	0	371.0	2	185.5	0								
FF5-5	– –	Neg	0	120.7	0										
FF5-6	– –	374.0	0	374.0	1	187.0	0								
FF5-7	– –	Neg	0	208.7	0										
FF5-8	–	62.3	2	249.5	2	124.8	2	62.4	1	31.2	0				
FF (water fraction)	+/–	54.6	2	218.5	2	109.3	2	54.6	2	27.3	0				
FF (water fraction)	+/–	53.0	3	424.0	2	212.0	2	106.0	2	53.0	2	26.5	0		
FF (water fraction)	–	73.4	2	293.5	2	146.8	1	73.4	1	36.7	0				

Table 50. Urine fractions (FF and FC fractions, and FA Sephadex LH-20 fractions) tested in the anti-agglutination assay, 09/2007.

Sample Description	Test Date	UM Score	Dilution Endpoint (mg/mL)	No. of Two-fold Dilutions	Amount Submitted (mg)	Dilution Volume (µL)	Starting Conc. (mg/mL)
FF (aqueous fraction)	09/2007	–	92.5	2	74.0	200	370.0
FF (aqueous fraction)	09/2007	–	95.3	2	152.5	400	381.3
FB (F EtOAc fraction)	09/2007	–	Neg	0	6.3	100	63.0
FA (combined active F fractions)	09/2007	+/–	35.2	2	21.1	150	140.7
FA1 (Sep LH-20 fraction)	09/2007	+ +	5.8	4	14.0	150	93.3
FA2 (Sep LH-20 fraction)	09/2007	+	13.9	4	33.3	150	222.0
FA3 (Sep LH-20 fraction)	09/2007	– –	204.0	0	30.6	150	204.0
FA4 (Sep LH-20 fraction)	09/2007	+/–	55.7	1	16.7	150	111.3
FA5 (Sep LH-20 fraction)	09/2007	– –	Neg	0	27.5	150	183.3
FC (control urine)	09/2007	– –	305.5	0	61.1	200	305.5
FCE (FC EtOAc fraction)	09/2007	+/–	35.0	2	21.0	150	140.0
FC1 (FC aqueous fraction)	09/2007	–	106.8	2	170.8	400	427.0
FC1 (FC aqueous fraction)	09/2007	–	99.9	2	159.8	400	399.5
FC1 (FC aqueous fraction)	09/2007	–	82.6	2	132.1	400	330.3

Table 51. Urine fractions (FF and FC fractions, and FA Sephadex LH-20 fractions) tested in the anti-agglutination assay, 09/2007, showing dilution concentrations tested and visual agglutination scores (VAS).

Sample	UM Score	Dilution Endpoint (mg/mL)	No. Dils.	Start Conc. (mg/mL)	VAS (start)	Dil. 1 Conc. (mg/mL)	VAS (dil 1)	Dil. 2 Conc. (mg/mL)	VAS (dil 2)	Dil. 3 Conc. (mg/mL)	VAS (dil 3)	Dil. 4 Conc. (mg/mL)	VAS (dil 4)	Dil. 5 Conc. (mg/mL)	VAS (dil 5)
FF (water fraction)	–	92.5	2	370	2	185.0	2	92.5	2	46.3	0				
FF (water fraction)	–	95.3	2	381.3	2	190.7	2	95.3	2	47.7	0				
FB (F EtOAc fraction)	–	Neg	0	63	0										
FA (combined active F samples)	+/–	35.2	2	140.7	2	70.4	2	35.2	1	17.6	0				
FA1 (Sep LH-20 fraction)	+ +	5.8	4	93.3	2	46.7	2	23.3	2	11.7	1	5.8	1	2.9	0
FA2 (Sep LH-20 fraction)	+	13.9	4	222	2	111.0	2	55.5	2	27.8	1	13.9	1	6.9	0
FA3 (Sep LH-20 fraction)	– –	204.0	0	204	1	102.0	0								
FA4 (Sep LH-20 fraction)	+/–	55.7	1	111.3	2	55.7	2	27.8	0						
FA5 (Sep LH-20 fraction)	– –	Neg	0	183.3	0										
FC (control urine)	– –	305.5	0	305.5	1	305.5	0								
FCE (FC EtOAc fraction)	+/–	35.0	2	140	2	70.0	2	35.0	1	17.5	0				
FC1 (FC water fraction)	–	106.8	2	427	2	213.5	2	106.8	1	53.4	0				
FC1 (FC water fraction)	–	99.9	2	399.5	2	199.8	2	99.9	1	49.9	0				
FC1 (FC water fraction)	–	82.6	2	330.3	2	165.2	2	82.6	1	41.3	0				

Table 52. Cranberry juice powder fractions (CJ, and MW-filter and Acid/Base experiments) tested in the anti-agglutination assay, 06/2008.

Sample Description	Test Date	UM Score	Dilution Endpoint (mg/mL)	No. of Two-fold Dilutions	Amount Submitted (mg)	Dilution Volume (µL)	Starting Conc. (mg/mL)
CJ (cranberry juice powder)	06/2008	+ +	3.8	6	49.2	200	246.0
CJ (cranberry juice powder)	06/2008	+ +	3.4	6	44.3	200	221.5
CJ-MA3a (3 kDa retentate)	06/2008	+ +	0.7	7	9.5	100	95.0
CJ-MB3a (10 kDa retentate)	06/2008	+ +	0.7	7	8.7	100	87.0
CJ-MC3a (30 kDa retentate)	06/2008	+ +	0.5	7	6.3	100	63.0
CJ-MA3b (3 kDa filtrate)	06/2008	– –	Neg	0	21.4	200	107.0
CJ-MB3b (10 kDa filtrate)	06/2008	– –	Neg	0	23.7	200	118.5
CJ-MC3b (30 kDa filtrate)	06/2008	– –	Neg	0	19.1	200	95.5
CJ pH 2.0, 2 h	06/2008	+	13.4	3	21.4	200	107.0
CJ pH 2.0, 4 h	06/2008	+	13.6	3	21.8	200	109.0
CJ pH 2.1, 8 h	06/2008	+	12.3	3	19.7	200	98.5
CJ pH 5.9, 2 h	06/2008	+ +	1.8	6	23.0	200	115.0
CJ pH 6.0, 4 h	06/2008	+ +	1.9	6	24.5	200	122.5
CJ pH 6.1, 8 h	06/2008	+ +	1.8	6	23.2	200	116.0
CJ pH 7.9, 2 h	06/2008	+ +	1.7	6	22.2	200	111.0
CJ pH 8.0, 4 h	06/2008	+ +	2.0	6	25.3	200	126.5
CJ pH 8.2, 8 h	06/2008	+ +	1.9	6	24.6	200	123.0
CJ Native pH (2.7), 2 h	06/2008	+ +	1.6	6	20.1	200	100.5
CJ Native pH (2.7), 4 h	06/2008	+ +	1.8	6	23.6	200	118.0
CJ Native pH (2.7), 8 h	06/2008	+ +	3.6	5	23.1	200	115.5

Table 53. Urine fractions (FA and FC Sephadex LH-20 column fractions) tested in the anti-agglutination assay, 06/2008.

Sample Description	Test Date	UM Score	Dilution Endpoint (mg/mL)	No. of Two-fold Dilutions	Amount Submitted (mg)	Dilution Volume (µL)	Starting Conc. (mg/mL)
FA (combined active F fractions)	06/2008	+/–	45.2	1	18.1	200	90.5
FA1 (Sep LH-20 fraction)	06/2008	– –	Neg	0	3.7	100	37.0
FA2 (Sep LH-20 fraction)	06/2008	– –	Neg	0	3.3	100	33.0
FA3 (Sep LH-20 fraction)	06/2008	– –	Neg	0	6.9	100	69.0
FA4 (Sep LH-20 fraction)	06/2008	+/–	47.2	1	18.9	200	94.5
FA5 (Sep LH-20 fraction)	06/2008	+/–	34.0	1	8.5	125	68.0
FC1-1 (Sep LH-20 fraction)	06/2008	– –	182.5	1	73.0	200	365.0
FC1-2 (Sep LH-20 fraction)	06/2008	– –	Neg	0	46.5	200	232.5
FC1-3 (Sep LH-20 fraction)	06/2008	– –	118.2	1	47.3	200	236.5

Table 54. Urine fractions (H) tested in the anti-agglutination assay, 08/2008.

Sample Description	Test Date	UM Score	Dilution Endpoint (mg/mL)	No. of Two-fold Dilutions	Amount Submitted (mg)	Dilution Volume (µL)	Starting Conc. (mg/mL)
H (crude urine)	08/2008	+/–	40.8	4	653.7	1000	653.7
HCE (H EtOAc extract, water soluble)	08/2008	+	13.1	3	20.9	200	104.5
HCM (H EtOAc fraction, MeOH soluble)	08/2008	– –	Neg	0	1.3	50	26.0
HD (H EtOAc: MeOH fraction, water soluble)	08/2008	– –	157.5	1	94.5	300	315.0
HF (H aqueous fraction)	08/2008	+	23.1	5	369.8	500	739.6
HG (H solids)	08/2008	– –	Neg	0	71.8	1000	71.8

Table 55. Urine fractions (FC and I) tested in the anti-agglutination assay, 12/2008.

Sample Description	Test Date	UM Score	Dilution Endpoint (mg/mL)	No. of Two-fold Dilutions	Amount Submitted (mg)	Dilution Volume (µL)	Starting Conc. (mg/mL)
FC1 (FC aqueous fraction)	12/2008	–	73.3	3	146.6	250	586.4
FC1-1 (Sep LH-20 fraction)	12/2008	–	102.8	2	82.2	200	411.0
I (crude urine)	12/2008	+/–	45.1	3	72.2	200	361.0
ICE (I EtOAc: MeOH fraction, water soluble)	12/2008	+ +	9.5	2	5.7	150	38.0
ICM (I EtOAc: MeOH fraction, EtOAc soluble)	12/2008	– –	Neg	0	38.7	100	387.0
ID (EtOAc: MeOH fraction, water soluble)	12/2008	– –	Neg	0	100.7	200	503.5
IF (I aqueous fraction)	12/2008	+/–	57.4	3	68.9	150	459.3
IZ (I stability test)	12/2008	+	14.1	3	16.9	150	112.7

Table 56. Urine fractions (H and HF Sephadex LH-20 fractions) and reference compounds tested in the anti-agglutination assay, 12/2008.

Sample Description	Test Date	UM Score	Dilution Endpoint (mg/mL)	No. of Two-fold Dilutions	Amount Submitted (mg)	Dilution Volume (µL)	Starting Conc. (mg/mL)
H (crude urine)	12/2008	+/–	61.7	3	123.4	250	493.6
HD (H EtOAc-MeOH fraction, water soluble)	12/2008	– –	120.8	2	96.7	200	483.5
HF (H aqueous fraction)	12/2008	– –	169.5	2	203.4	300	678.0
HF1-1 (Sep LH-20 fraction)	12/2008	+ +	8.5	5	27.3	100	273.0
HF1-2 (Sep LH-20 fraction)	12/2008	+/–	55.3	3	66.3	150	442.0
HF1-3 (Sep LH-20 fraction)	12/2008	–	108.6	2	86.9	200	434.5
HF1-4 (Sep LH-20 fraction)	12/2008	–	102.7	2	82.2	200	411.0
HF1-5 (Sep LH-20 fraction)	12/2008	– –	269.0	1	107.6	200	538.0
HF1-6 (Sep LH-20 fraction)	12/2008	–	109.6	2	65.8	150	438.7
HF1-7 (Sep LH-20 fraction)	12/2008	– –	416.7	0	62.5	150	416.7
D-Glucose	12/2008	+/–	35.8	3	28.7	100	287.0
D-Glucuronic Acid	12/2008	– –	388.0	0	38.8	100	388.0
D-Galacturonic Acid	12/2008	– –	270.0	0	27.0	100	270.0
D-Sorbitol	12/2008	– –	226.0	0	22.6	100	226.0
Sucrose (Great Value Brand)	12/2008	– –	224.0	0	22.4	100	224.0

Table 57. Urine fractions (DA19, FC, FF, I, H and HI) tested in the anti-agglutination assay, 07/2011.

Sample Description	Test Date	UM Score	Dilution Endpoint (mg/mL)	No. of Two-fold Dilutions	Amount Submitted (mg)	Dilution Volume (µL)	Starting Conc. (mg/mL)
DA19	07/2011	+ +	3.4	6	21.8	100	218.0
FC1 (aqueous fraction)	07/2011	–	87.5	2	52.5	150	350.0
FC1 (aqueous fraction)	07/2011	– –	Neg	0	49.8	150	332.0
FC1-1 (Sep LH-20 fraction)	07/2011	– –	199.0	1	59.7	150	398.0
FC1-1 (Sep LH-20 fraction)	07/2011	– –	353-176	1	53.0	150	353.3
FC1-2 (Sep LH-20 fraction)	07/2011	– –	338.0	0	50.7	150	338.0
FF (aqueous fraction)	07/2011	+	11.9	5	57.1	150	380.7
FF (aqueous fraction)	07/2011	+	28.6	4	68.6	150	457.3
IZA (IZ aqueous fraction after stability test)	07/2011	+ +	10.8	5	51.9	150	346.0
H (crude urine)	07/2011	+/–	47.7	3	57.3	150	382.0
HI1 (Combined H & I Active fractions, HF2-1 + IF1-1)	07/2011	+ +	6.3	5	20.1	100	201.0
HI2 (Combined H & I Active fractions, HF1-1 + HF2-2 + IF1-2)	07/2011	+	12.7	4	20.4	100	204.0
HI3 (Combined H & I Active fractions, HF1-2 + IF1-3 + HF2-3)	07/2011	– –	101.0	1	20.2	100	202.0

Table 58. Cranberry fractions (CJ, CJA, and CJA Sephadex LH-20 fractions) tested in the anti-agglutination assay, 07/2011.

Sample Description	Test Date	UM Score	Dilution Endpoint (mg/mL)	No. of Two-fold Dilutions	Amount Submitted (mg)	Dilution Volume (µL)	Starting Conc. (mg/mL)
CJ (cranberry juice powder)	07/2011	+	21.3	4	51.2	150	341.3
CJA (aqueous fraction)	07/2011	+ +	7.1	6	51.2	150	341.3
CJA (aqueous fraction)	07/2011	+/–	43.3	3	52.0	150	346.7
CJA1-02B (Sep LH-20 fraction)	07/2011	+ +	5.1	6	49.0	150	326.7
CJA2-02 (Sep LH-20 fraction)	07/2011	+ +	10.5	5	50.7	150	338.0
CJA2-02 (Sep LH-20 fraction)	07/2011	+	21.2	4	50.9	150	339.3
CJA2-03 (Sep LH-20 fraction)	07/2011	+/–	42.7	3	51.3	150	342.0
CJA2-04 (Sep LH-20 fraction)	07/2011	+/–	42.9	3	51.5	150	343.3
CJA2-05 (Sep LH-20 fraction)	07/2011	+/–	41.2	3	49.5	150	330.0
CJA2-05 (Sep LH-20 fraction)	07/2011	– –	168.0	1	50.4	150	336.0

Table 59. Reference compounds tested in the anti-agglutination assay, 07/2011.

Sample Description	Test Date	UM Score	Dilution Endpoint (mg/mL)	No. of Two-fold Dilutions	Amount Submitted (mg)	Dilution Volume (µL)	Starting Conc. (mg/mL)
Glucose Tabs (ReliOn Glucose Tablets commercial product, powdered)	07/2011	+ +	5.2	6	50.2	150	334.7
Glucose Tabs (ReliOn Glucose Tablets commercial product, powdered)	07/2011	+ +	10.3	5	49.3	150	328.7
D-Glucose (Sigma Aldrich 99.5%)	07/2011	+ +	10.7	5	51.4	150	342.7
D-Glucose (Sigma Aldrich 99.5%)	07/2011	+ +	10.8	5	52.1	150	347.3
D-Fructose (CalBioChem)	07/2011	+/–	41.4	3	49.7	150	331.3
D-Fructose (CalBioChem)	07/2011	–	81.6	2	49.0	150	326.7
Sucrose (Great Value Brand)	07/2011	–	82.6	2	49.6	150	330.7

4. ***Overview of Principles for Evaporative Light-Scattering Detection***

Evaporative light-scattering detection (ELSD) is a semi-quantitative, mass-sensitive method (Mourey & Oppenheimer 1984). Solvent is introduced to the detector and nebulized using a gas such as nitrogen to form a cloud of sample droplets. The resulting cloud of droplets is carried into a flight/evaporator tube by the stream of nebulizing gas where heat is used to evaporate solvent and produce dry solute particles. This cloud of dried particles moves past a light source and scatters the incident light. The scattered light is collected by a photomultiplier tube detector to generate an electronic signal (mV) that corresponds to the concentration of sample present (more particles of solute = more signal). Adjustable parameters that affect detector sensitivity and function include the nebulizor temp, the evaporator tube temp, and the gas flow rate. The inlet solvent flow rate and the solvent used for a given separation dictate the settings for these parameters. This type of detector is moderately compatible with gradient separations as long as the solvents used for the gradient can be removed by relatively similar evaporator temperatures.

Non-volatile and semi-volatile compounds can be detected by ELSD regardless of the presence or absence of specific structural features such as chromophores. In contrast, UV detection methods require that compounds contain chromophores with absorbances typically above 210 nm (for MeOH as an eluting solvent).

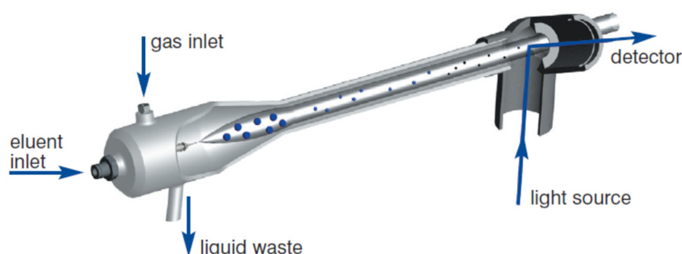
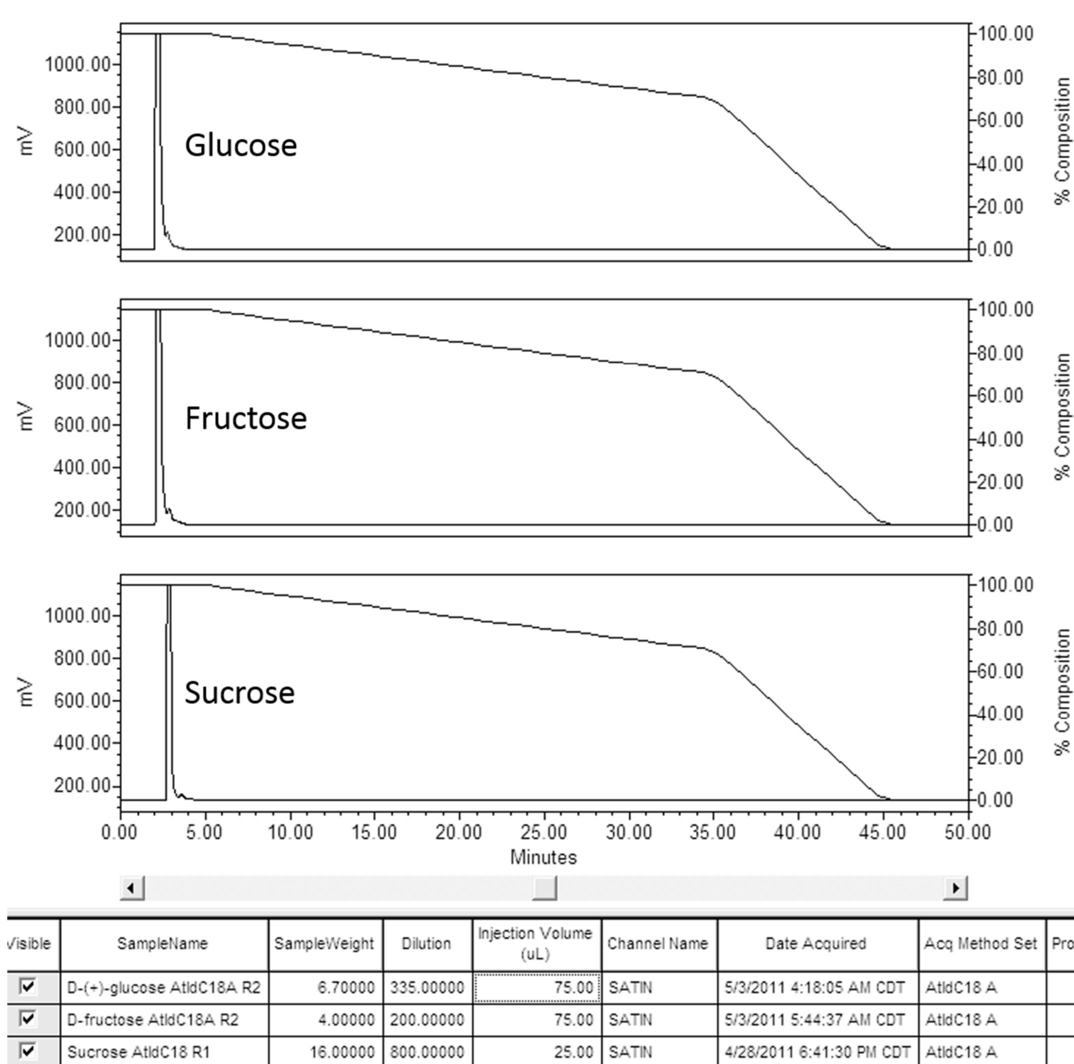


Image reproduced from Polymer Laboratories PL-ELS 2100 Operator's Manual, Version 1.01, Revision August 2003, Document # 6/26069B

5. ***Additional Chromatographic & Spectroscopic Data***

The additional figures included below contain supporting information relevant to the text of Chapters 2 and 3.



Visible	SampleName	SampleWeight	Dilution	Injection Volume (uL)	Channel Name	Date Acquired	Acq Method Set	Pro
☑	D-(+)-glucose AtldC18A R2	6.70000	335.00000	75.00	SATIN	5/3/2011 4:18:05 AM CDT	AtldC18 A	
☑	D-fructose AtldC18A R2	4.00000	200.00000	75.00	SATIN	5/3/2011 5:44:37 AM CDT	AtldC18 A	
☑	Sucrose AtldC18 R1	16.00000	800.00000	25.00	SATIN	4/28/2011 6:41:30 PM CDT	AtldC18 A	

Figure 105. Analytical HPLC-ELSD of glucose, fructose, and sucrose standards (AtldC18) showing the early elution (< 5 min) of these compounds.
Standards of the monosaccharides glucose and fructose, and the disaccharide, sucrose, were analyzed via analytical HPLC-ELSD using the optimized method used for detecting the oligosaccharides discussed in Chapters 2 and 3. All three standards eluted with 100% water at < 5 min, and were therefore not significantly retained by the C_{18} sorbent of the AtldC18 column. Compounds such as these low-MW carbohydrates also elute at later retention times from gel-filtration chromatography sorbents such as Sephadex LH-20. In contrast, the oligosaccharide mixtures of interest from both cranberry and urine samples eluted with the void volume from the Sephadex LH-20 sorbent, indicating larger MW, and at ~20 min on the AtldC18 analytical column with the method shown here.

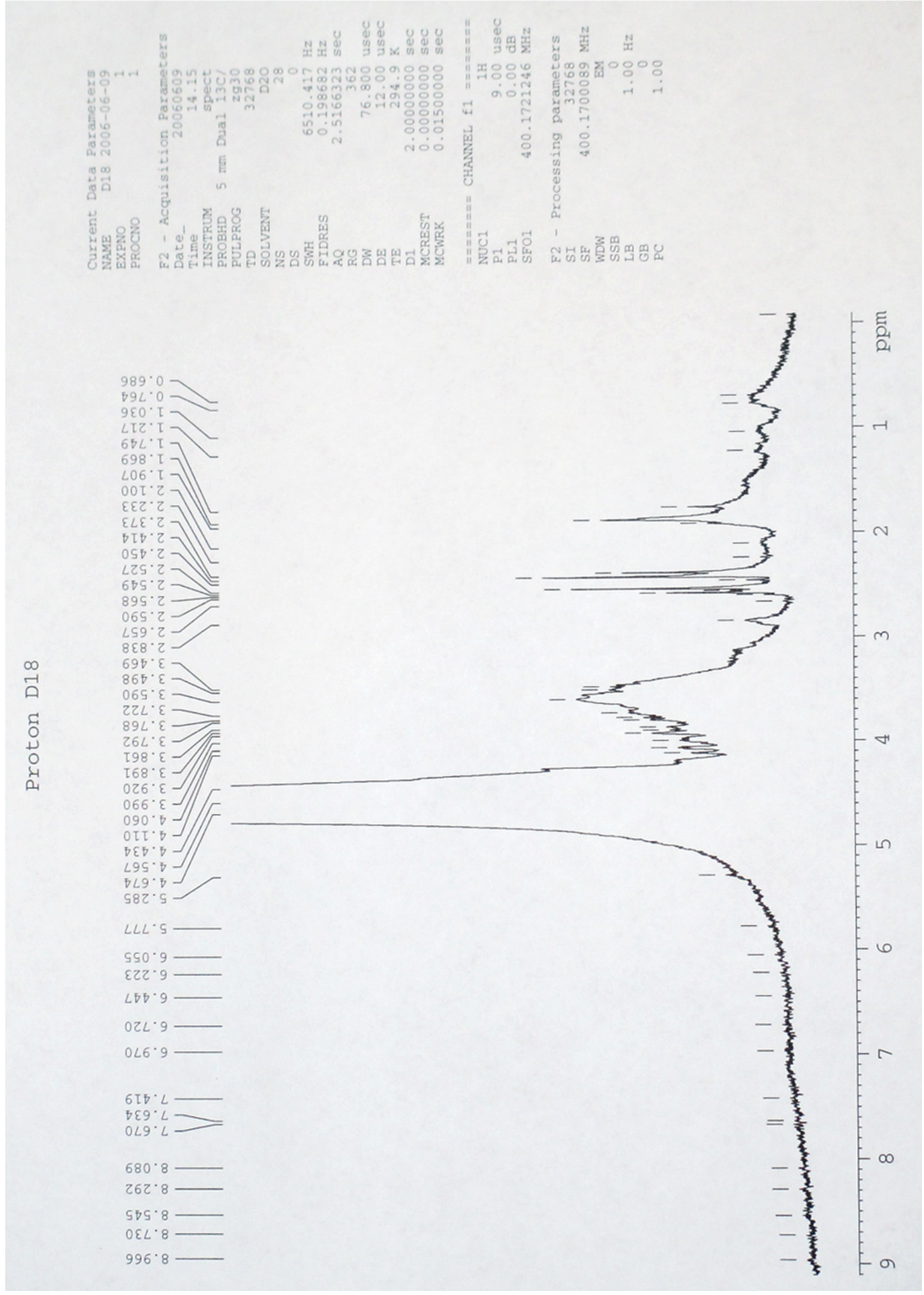


Figure 106. The 1H NMR spectrum of urine fraction DA18 showing the low quality of the data, 400 MHz, D2O.

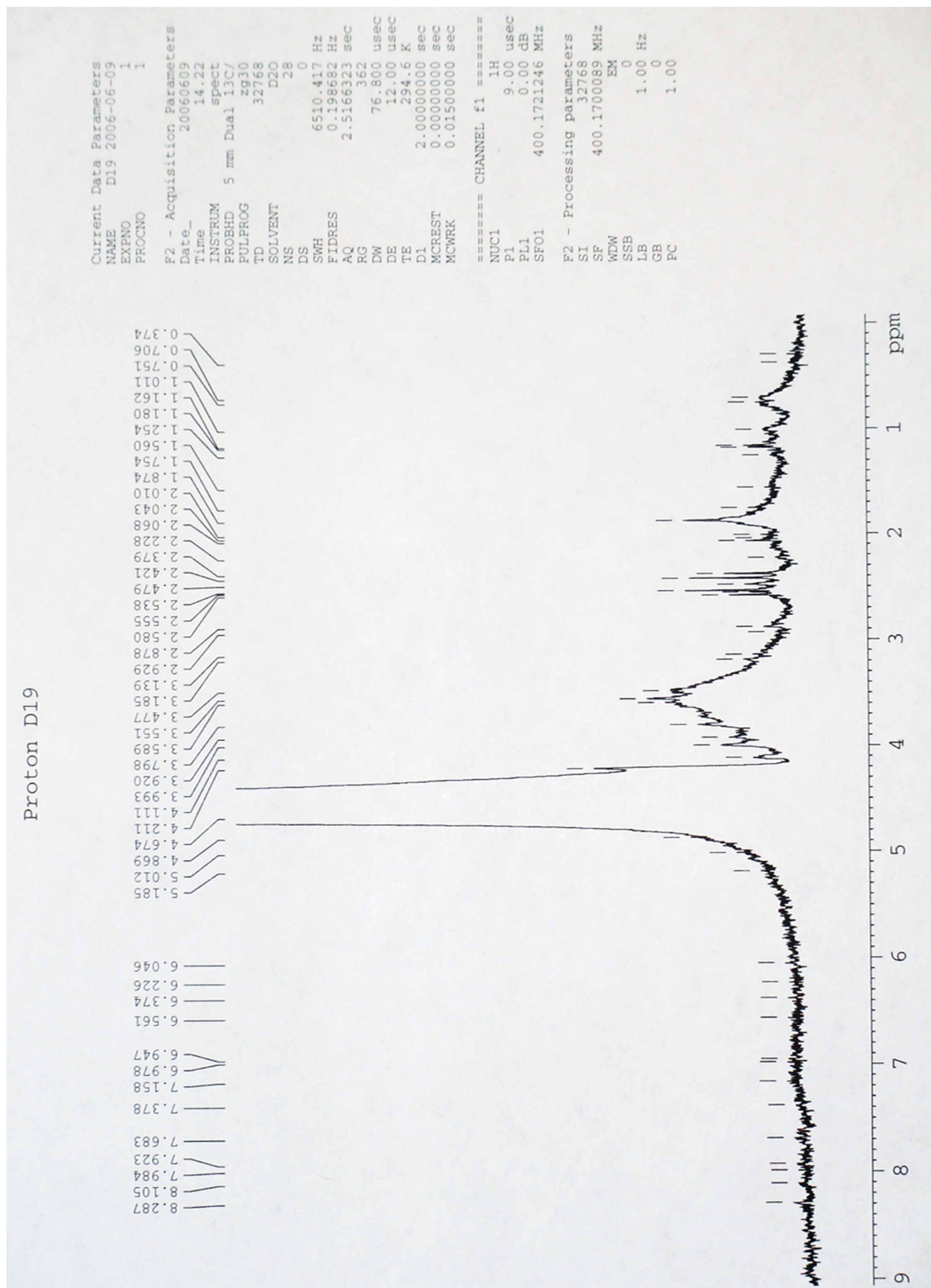
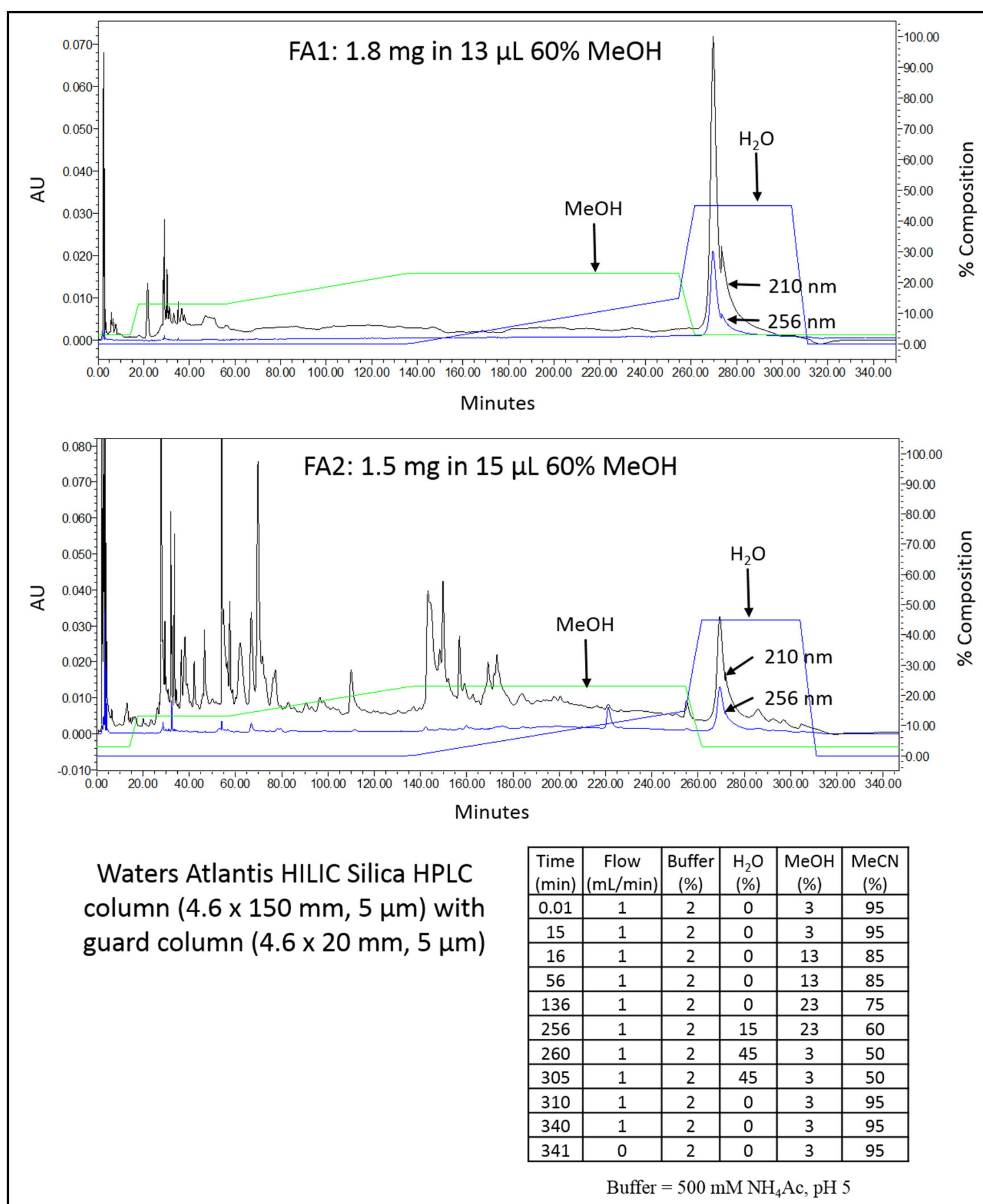


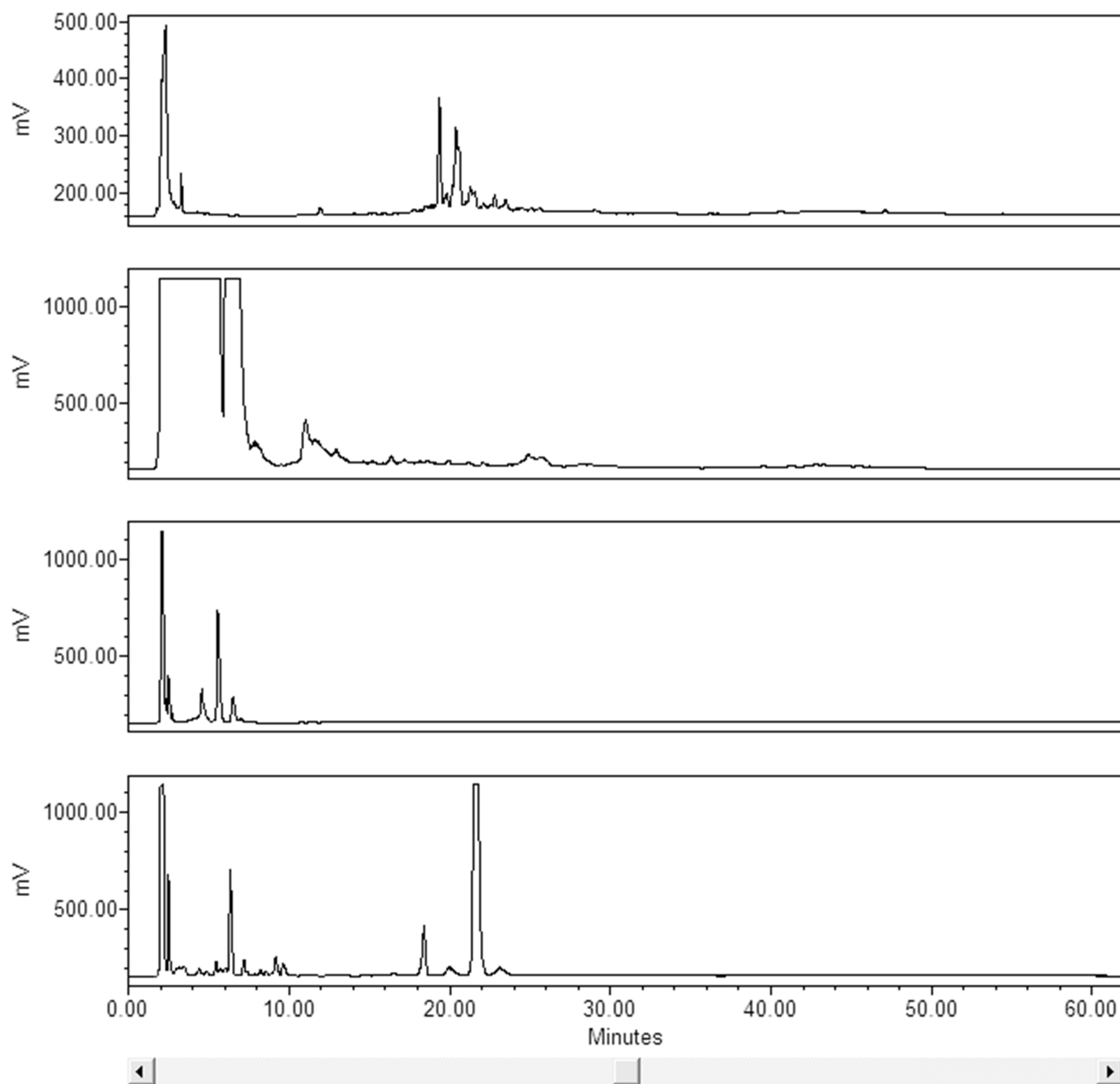
Figure 107. The ^{1}H NMR spectrum of urine fraction DA19 showing the low quality of the data, 400 MHz, D2O.



Time (min)	Flow (mL/min)	Buffer (%)	H_2O (%)	MeOH (%)	MeCN (%)
0.01	1	2	0	3	95
15	1	2	0	3	95
16	1	2	0	13	85
56	1	2	0	13	85
136	1	2	0	23	75
256	1	2	15	23	60
260	1	2	45	3	50
305	1	2	45	3	50
310	1	2	0	3	95
340	1	2	0	3	95
341	0	2	0	3	95

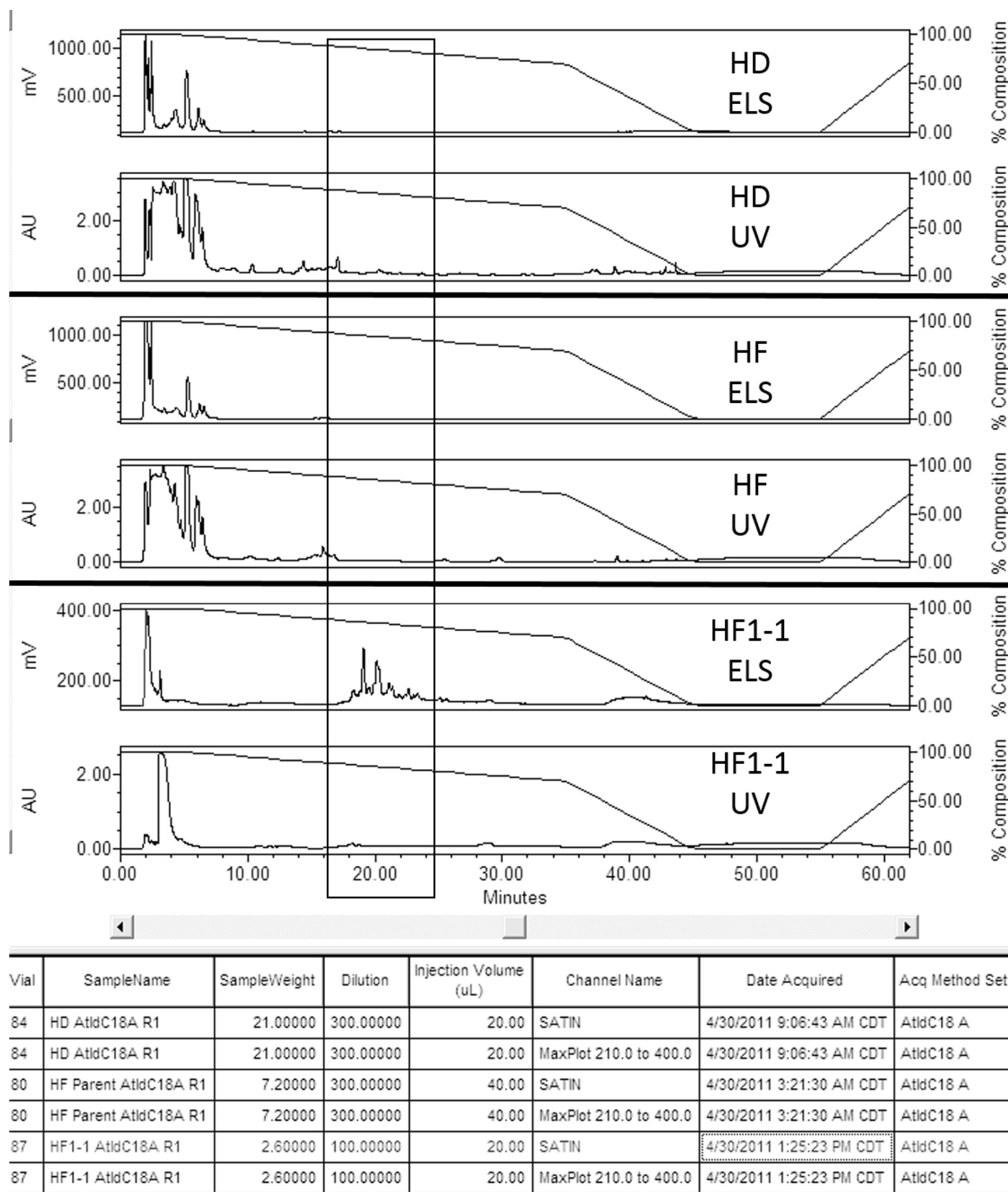
Figure 108. Analytical HPLC-UV (210 and 256 nm) chromatograms of fractions FA1 and FA2 (HILIC).

The material eluting at ~270 min appears to be a major component of the FA1 sample with the scale used, but actually has a relatively low absorbance (0.07 AU), and would normally be considered part of a baseline. A HILIC method similar to the one shows for these analytical separations was used to isolate the detectable components from the FA2 sample, especially those corresponding to the FA1 sample. The resulting fractions were tested in the anti-agglutination assay but showed no detectable activity, possibly due to the low amounts of sample submitted (< 10 mg each).



/isible	SampleName	SampleWeight	Dilution	Injection Volume (uL)	Channel Name	Date Acquired	Acq Method Set
☑	HF1-1 AtldC18A R1	2.60000	100.00000	20.00	SATIN	9/15/2010 12:49:50 PM CDT	AtldC18 A
☑	FC1-1-Liq AtldC18A R1	1.00000	300.00000	50.00	SATIN	9/15/2010 12:41:55 AM CDT	AtldC18 A
☑	FC1-2 AtldC18A R1	14.70000	300.00000	20.00	SATIN	9/15/2010 8:16:53 AM CDT	AtldC18 A
☑	FC1-3 AtldC18A R1	12.10000	300.00000	25.00	SATIN	9/15/2010 9:47:49 AM CDT	AtldC18 A

Figure 109. Comparisons of the analytical HPLC-ELSD spectra for active urine fraction HF1-1 and control urine fractions (FC1-11, FC1-2, and FC1-3) (AtldC19).
These spectra were collected using the same HPLC-ELSD method as applied to HF1-1 previously. Additional baseline expansions of the region of interest (18-24 min) for Fraction FC1-1 show no evidence of possible oligosaccharide components. The components visible in this region for FC1-1 and FC1-3 both contain chromophores detectable by UV.



Vial	SampleName	SampleWeight	Dilution	Injection Volume (uL)	Channel Name	Date Acquired	Acq Method Set
84	HD AtldC18A R1	21.00000	300.00000	20.00	SATIN	4/30/2011 9:06:43 AM CDT	AtldC18 A
84	HD AtldC18A R1	21.00000	300.00000	20.00	MaxPlot 210.0 to 400.0	4/30/2011 9:06:43 AM CDT	AtldC18 A
80	HF Parent AtldC18A R1	7.20000	300.00000	40.00	SATIN	4/30/2011 3:21:30 AM CDT	AtldC18 A
80	HF Parent AtldC18A R1	7.20000	300.00000	40.00	MaxPlot 210.0 to 400.0	4/30/2011 3:21:30 AM CDT	AtldC18 A
87	HF1-1 AtldC18A R1	2.60000	100.00000	20.00	SATIN	4/30/2011 1:25:23 PM CDT	AtldC18 A
87	HF1-1 AtldC18A R1	2.60000	100.00000	20.00	MaxPlot 210.0 to 400.0	4/30/2011 1:25:23 PM CDT	AtldC18 A

Figure 110. Comparisons of the analytical HPLC-ELSD/UV spectra for active urine fraction HF1-1 and crude urine fractions (HF and HD) (AtldC19).
The HF material was the parent fraction for the HF1-1 material and both showed activity in the anti-agglutination assay. The oligosaccharide components of HF1-1 were not readily apparent in the HF material via HPLC-ELSD at the concentration of sample used above, indicating that enrichments or methods with increased sensitivity are necessary to detect oligosaccharide components in mammalian urine samples. The HD fraction (the EtOAc fraction of H urine) did now show anti-agglutination activity, did not contain detectable oligosaccharide components, and the majority of this fraction eluted in the polar region between 0-10 min. The composition of HF and HD appeared similar when examined with ELSD/UV, indicating the predominantly polar nature of the urine material.

APPENDIX C: EXAMPLES OF CRANBERRY DIETARY SUPPLEMENT PRODUCT LITERATURE

1. ***Overview of Appendix C***

Many consumers make decisions regarding their use of dietary supplements based on the product literature that is most easily accessible to them. Most of this literature is, unfortunately, inaccurate and often contains pseudo-scientific content that can be misleading to consumers. The actual composition and method of preparation for many dietary supplement products is often incompletely reported, making it impossible to propose possible compounds that may be present in any given material. For example, a product label may specify that the material contains an “extract” or “concentrate” but no information is given as to the method or solvents used, thereby making it impossible to determine the types of compounds that may have been enriched in the resulting material. Claims of equivalency (concentrate equivalent to X g of fruit) are also specious as no information is given as to the basis for comparison between the original material and the final dietary supplement product.

Many ‘supplement facts’ labels on products in the U.S. include an entry for ‘total carbohydrate’ with sub-entries for ‘dietary fiber’ and ‘sugars.’ Current nutrition science considers compounds with cellulose-type backbones to be a type of dietary fiber as such compounds are not digested by mammalian enzymes (Haard & Chism 1996). The oligosaccharides identified as active components of cranberry in these studies would therefore most likely be represented as a component of the carbohydrate label fields currently on products. Although detection and accurate measurement of oligosaccharide-specific content would depend on the method used for assessment, products that currently claim to have no sugars or carbohydrates are probably less likely to contain the active oligosaccharides identified in these studies. Sensitive methods for the detection of soluble oligosaccharides would need to be used to determine the presence or absence of these compounds in products.

2. *Sundown Naturals® Product Literature*

Below is a reproduction of the label from the SN product used in Chapter 3, analyzed in Section B6, and discussed in Section C3. No additional information was available on the internet for this product during these studies.

DIRECTIONS: FOR ADULTS, TAKE FOUR (4) CAPSULES DAILY, PREFERABLY WITH MEALS. As a reminder, discuss the supplements and medications you take with your health care providers.

Supplement Facts

Serving Size 4 Capsules
Servings Per Container 50

Amount Per Serving		%Daily Value
Calories	10	
Total Carbohydrate	2 g	1%**
Dietary Fiber	<1 g	1%**
Sugars	<1 g	***
Cranberry (*Vaccinium macrocarpon*) (fruit)	1,900 mg (1.9 g)	***

**Percent Daily Values are based on a 2,000 calorie diet.
***Daily Value not established.

Other Ingredients: Gelatin, Silica.

PLEASE RECYCLE

No Artificial Color, Flavor or Sweetener, **No** Preservatives, **No** Starch, **No** Milk, **No** Lactose, **No** Soy, **No** Gluten, **No** Wheat, **No** Yeast, **No** Fish. Sodium Free.

Manufactured by REXALL SUNDOWN, INC.
Boca Raton, FL 33487 USA

Sundown Naturals

Cranberry Fruit

475 MG

Natural Whole Herb

Promotes Healthy Urinary Tract Function*

200 CAPSULES HERBAL SUPPLEMENT

Smart Facts

Cranberries are well known for their capacity to support urinary health, both in women and in men.* Use every day for healthy urinary tract function.*

*These statements have not been evaluated by the Food and Drug Administration. This product is not intended to diagnose, treat, cure or prevent any disease.

WARNING: If you are pregnant, nursing, taking any medications or have any medical condition, consult your doctor before use. Discontinue use and consult your doctor if any adverse reactions occur.

◊Sundown Naturals' Natural Whole Herb products utilize ground plant parts to provide the natural components in the amounts found in nature.

KEEP OUT OF REACH OF CHILDREN. STORE AT ROOM TEMPERATURE AND AVOID EXCESSIVE HEAT. TAMPER RESISTANT: DO NOT USE IF SEAL UNDER CAP IS BROKEN OR MISSING.

Questions? Call toll free 1-888-VITAHELP (848-2435) or visit us at www.sundownnaturals.com

FREE OF Gluten & Wheat

Prod. No. 44694

GUARANTEED QUALITY

08F B11900
AAB ©2012

0 30768 03308 8

3. *Nature's Bounty™ Product Literature*

Below is a reproduction of the label from the NB product used in Chapter 3, analyzed in Section B6 and discussed in Section C3. The information available for this product on the internet during these studies is also included on the following page.

Our unique Triple Strength Cranberry Concentrate contains the equivalent of 25,200 mg per serving of fresh Cranberry fruit, so two softgels daily are all you need! Cranberries are known for their ability to help maintain a healthy urinary tract. This product also contains Vitamin C to support antioxidant and immune health, plus Vitamin E, an essential nutrient crucial for good health.**

WARNING: If you are pregnant, nursing, taking any medications, planning any medical procedure or have any medical condition, consult your doctor before use. Discontinue use and consult your doctor if any adverse reactions occur. Keep out of reach of children. Store at room temperature. Do not use if seal under cap is broken or missing.

‡Based on Nielsen data Total FDMX 52 weeks ending 03.30.13

Nutrition Questions or Comments?
Call 1-800-433-2990
Mon. - Sat. 9 AM - 7 PM ET

For educational health tips and to join our money saving Healthy Rewards™ program, visit www.NaturesBounty.com

*These statements have not been evaluated by the Food and Drug Administration. This product is not intended to diagnose, treat, cure or prevent any disease.

0 74312 13794 5

Prod. No. 13794 B36117 15G

NATURE'S BOUNTY

TRIPLE STRENGTH

Cranberry

Fruit Concentrate
25,200 mg
equivalent per serving**

with Vitamin C

Helps Maintain a Healthy Urinary Tract*

Supports Immune Health*

RAPID RELEASE LIQUID SOFTGEL

#1 Selling Cranberry Brand In The U.S.‡

60 softgels

DIETARY SUPPLEMENT

DIRECTIONS: For adults, take two (2) softgels daily, preferably with meals.

Supplement Facts

Serving Size 2 Softgels
Servings Per Container 30

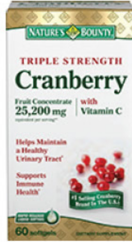
Amount Per Serving		%Daily Value
Vitamin C (as Ascorbic Acid)	40 mg	67%
Vitamin E (as dl-Alpha Tocopheryl Acetate)	6 IU	20%
Cranberry Concentrate *(Vaccinium macrocarpon)* (fruit) **(a 50:1 concentrate, equivalent to 25,200 mg fresh cranberries)	504 mg	***

***Daily Value not established.

Other Ingredients: Soybean Oil, Gelatin, Vegetable Glycerin. **Contains <2% of:** Soy Lecithin, White Beeswax.

No Artificial Color, Flavor or Sweetener, **No** Preservatives, **No** Sugar, **No** Starch, **No** Milk, **No** Lactose, **No** Gluten, **No** Wheat, **No** Yeast, **No** Fish. Sodium Free.

Carefully Manufactured by **NATURE'S BOUNTY, INC.**
Bohemia, NY 11716 U.S.A. ©2013 Nature's Bounty, Inc.



Product Label

Triple Strength Natural Cranberry with Vitamin C

60 Softgels | Product No. 013794

Our unique Triple Strength Cranberry Concentrate contains the equivalent of 25,200 mg per serving of fresh Cranberry fruit, so two softgels daily are all you need! Cranberries are known for their ability to help maintain a healthy urinary tract.* This product also contains Vitamin C to support antioxidant and immune health, plus Vitamin E, an essential nutrient crucial for good health.*

- We're the #1 selling Cranberry brand in the U.S.!◊
- Helps maintain a healthy urinary tract*
- Enhanced with Vitamin C to support antioxidant and immune health*

◊Based on Nielsen data Total FDMX 52 weeks ending 3/30/13.

Related Products

Cranberry Plus Vitamin C (100 Softgels)

Dual Spectrum Cranberry With Hibiscus Softgels (60 Softgels)

© 2014 Nature's Bounty, Inc.

*These statements have not been evaluated by the Food and Drug Administration. These products are not intended to diagnose, treat, cure or prevent any disease.

4. *Nature Made™ Product Literature*

A reproduction of the product label was not available for the NM product used in Chapter 3, analyzed in Section B6, and discussed in Section C3. The limited information available for this product on the internet during these studies is included in the following five pages and includes a reproduction of the product label. The product shown here differs slightly from that used by the added presence of vitamin C; the study product lacked vitamin C but was otherwise the same in labeling and apparent composition.

English

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Nature Made Super Strength Cranberry + Vitamin C helps support a healthy immune system.

Nature Made Super Strength Cranberry + Vitamin C supports urinary tract and immune health. Cranberry comes from small evergreen shrubs that produce the tart berries. Cranberry contains compounds called proanthocyanidins (phytonutrients) that prevent bacteria from adhering to the wall of the urinary tract. Vitamin C is a potent antioxidant that helps neutralize free radicals that can damage healthy cells and supports a healthy immune system.

- Supports urinary tract health
- Supports immune health

Nature Made Super Strength Cranberry Plus Vitamin C is guaranteed to meet our high quality standards – it is made from carefully selected ingredients under strict manufacturing processes.

- Nature Made is the #1 Pharmacist Recommended Brand*
- No Artificial Flavors
- No Preservatives
- No Yeast, Starch or Gluten

*Based on US News & World Report - Pharmacy Times Survey, Nature Made is the #1 Pharmacist Recommended Brand in Eight Segments - Letter Vitamins, Omega-3/Fish Oil, Coenzyme Q10, Flax Seed Oil, , Herbal supplements, Cholesterol Management-Natural, Garlic (tie) and Diabetic Multivitamins (tie).

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Dosage:
SUGGESTED USE: As an addition to your diet, take two softgels daily with water at mealtimes. We recommend drinking a full 8-ounce glass of water when taking cranberry softgels and drinking 6-8 glasses of water or other liquids each day.

Interactions:
Cranberry supplements may interact with the following:

- Antacids
- Anticoagulants/Antiplatelets
- Aspirin

Vitamin C supplements may interact with the following:

- Aspirin
- Anticoagulants (Coumadin)
- Antipsychotics
- Oral Contraceptives
- Acetaminophen (Tylenol)

Given that no two people are alike, if you are taking any medications you should be aware that potential drug-nutrient interactions may occur and are encouraged to consult a health care professional before using this product. Combining certain prescription drugs and dietary supplements can lead to undesirable effects such as:

- Diminished drug effectiveness
- Reduced supplement effectiveness
- Impaired drug and/or supplement absorption

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Multi for Him

Fish Oil & Omegas
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Super Strength Cranberry Plus Vitamin C

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Supplement Facts

Serving Size 2 softgels
Servings Per Container TBD

Amount Per Serving	%Daily Value
Calories 20	
Calories from Fat 10	
Total Fat 1 g	2%**
Total Carbohydrate Less than 1 g	Less than 1%**
Protein Less than 1 g	
Vitamin C (as Ascorbic Acid) 250 mg	417%
Cranberry fruit extract *(Vaccinium macrocarpon)* (15:1 concentration) 900 mg	*

* Daily Value not established.
** Percent Daily Values are based on a 2,000 calorie diet.

OTHER INGREDIENTS: Soybean Oil, Gelatin, Glycerin, Maltodextrin, Rice Bran Oil, Water, Yellow Beeswax, Colors Added.

Contains: Soy

Dietary Facts: Nature Made Super Strength Cranberry + Vitamin C provides Cranberry fruit extract and Vitamin C. Dietary sources of cranberry are the raw berries and cranberry juice. Cranberry is also a good source of vitamin C. Other dietary sources of vitamin C are fruits, particularly citrus fruits, and vegetables, including green and red peppers, tomatoes, potatoes, and green, leafy varieties like spinach and collard greens.

As ingredients may change from time to time, please check the label on the bottle.

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Super Strength Cranberry Plus Vitamin C

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Do Nature Made® Herbs products contain artificial flavors/colors?

Nature Made® and Nature Made® Herbs product lines are made to strict quality standards of purity, potency and release. Most all products are produced without artificial colors or preservatives. The few products that do contain artificial colors will state this in the ingredient listing on the label.

How many glasses of juice does one capsule of Cranberry equal?

Our capsules are in a concentrated juice form, therefore we cannot tell you how many capsules are equivalent to one cup of juice, or vice versa. Many cranberry juice products are not 100% pure cranberry juice and so the concentrated amount of cranberry may vary from product to product.

How Much Sugar is in Each Cranberry Extract with Vitamin C Softgel?

There is less than 0.1 gram of sugar in each cranberry extract plus vitamin C softgel.

What is Cranberry Used For?

Cranberry fruit is a potent source of proanthocyanidins, antioxidant compounds that have been found to help prevent bacteria from adhering to the urinary tract wall. As a result, cranberry fruit is taken by many people to promote urinary tract health. Many people take a cranberry supplement or drink pure cranberry juice to promote healthy urinary tract function.

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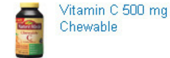

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Nature Made Adult Gummies & VitaMelts:

We're so sure you'll love the taste, **we guarantee it.**

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† These statements have not been evaluated by the Food and Drug Administration. These products are not intended to diagnose, treat, cure or prevent disease.

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5. *CranMax® Product Literature*

A reproduction of the product label was not available for the CranMax® product discussed in Chapter 4, Section B4, and used in the studies of Beerepoot and coworkers (2011). Selected information for this product excerpted from the Proprietary Nutritionals 2007 White Paper found on the internet during the writing of this report is included below for ease of future reference. This literature is an excellent example of the pseudo-scientific content that ends up in the advertisements for many dietary supplement products. Note the relatively large number of nonsensical and contradictory statements.

P R O P R I E T A R Y   N U T R I T I O N A L S ,   I N C .

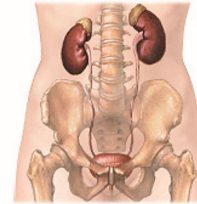
WHITE PAPER

THE "JUST IN TIME" EVOLUTION OF NUTRACEUTICAL CRANBERRY

2007

Cranberry bares fruit for Good Urinary Tract Health:

The strong growth in cranberry nutraceutical sales is directly related to a high prevalence of urinary tract infections (UTI's) predominantly effecting women. The following are key statistics: (*Source: National Kidney Foundation and the NIH*).

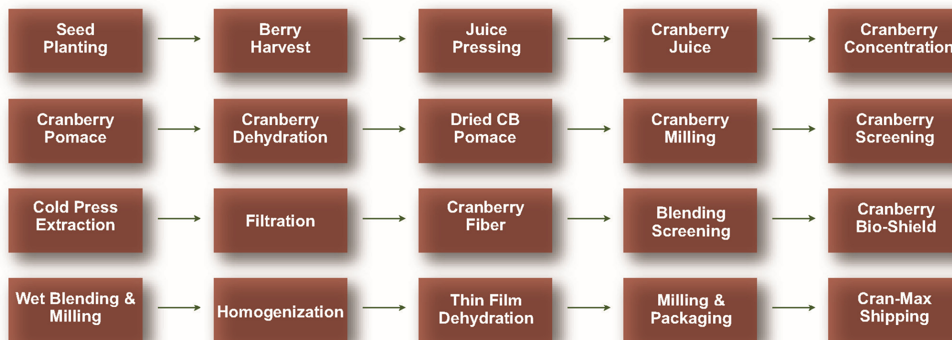


- *Urinary tract infections are responsible for nearly 10 million doctor visits each year*
- *Over $1 billion dollars are spent per year on UTI's.*
- *One in five women will have at least one UTI in her lifetime.*
- *Nearly 20 percent of women who have a UTI will have another, and 30 percent of those will have yet another. Of this last group, 80 percent will have recurrences.*
- *Total number of cases involving UTI's are approximately 40 million per year, of which 30 million are unreported and involve self medication.*
- *UTI's are the 2nd leading cause of lost work days for women.*
- *More than 1.5 million hospitalizations are related to UTI's*

Indigenous peoples have used cranberry preparations to treat urinary tract infections and other illness for centuries. Modern medical research has revealed the chemical and physiological effects cranberries have on the urinary tract and in its natural ability to inhibit the growth of E. Coli bacteria responsible for urinary tract infections (UTI's). It's important to understand the natural mechanism of action of cranberry. The E. Coli bacteria are never "destroyed" by the cranberries, but "physiologically changed", thereby disallowing their attachment within the urinary tract. In contrast to the use of pharmaceutical medication, these harmful bacteria are essentially whisked away, all within the flow of natural bodily processes.

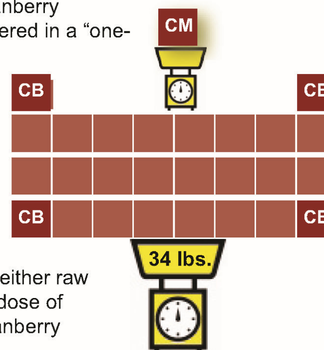
Cran-Max® and the Evolution of Nutraceutical Cranberry:

Nutraceutical supplements are in fact medicines of the natural kind. As with any medicine, they must deliver an end-benefit as promised. The process by which a phyto-medicine is developed and prepared during manufacture is crucial to its efficacy. The innate goodness of plant medicines must be hermetically sealed and delivered with optimum efficiency in the body. If a phyto-remedy is compromised by natural bodily process it will not deliver on its consumer promise. In the case of cranberry, it's imperative that its synergistic healing elements remain intact and in greatest abundance while being delivered most efficiently to their targets. Cran-Max has been developed to deliver on both counts. Nothing within the cranberry fruit is ever tampered with or wasted. The "whole fruit and nothing but the fruit" is utilized within a unique 20-step manufacturing process outlined below.



The Benefits of Cranberry Magnified:

Cran-Max is a highly concentrated herbal supplement. A high multiple of whole fresh cranberries are required to produce finished product for sale. It takes 34 pounds of raw cranberry material to produce a single pound of Cran-Max® natural cranberry. While most other cranberry products on the market require a daily "multi-dose" regimen, Cran-Max is delivered in a "one-per-day" dose.



It's highly concentrated content is significant when addressing the issue of quality. The natural synergistic elements of cranberry are untampered and condensed in Cran-Max resulting in a highly active and magnified dosage making it extremely convenient for the consumer to use and reap its benefits.

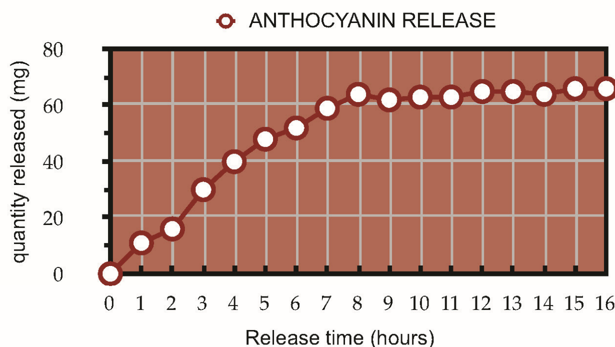
Testing has shown that its highly concentrated character makes it appealing to those who want the benefits of cranberry without the excessive ingestion of either raw fruit or juice. A recent study has shown that the anthocyanin content in a daily dose of Cran-Max (500/mg) is equivalent to 7 eight-ounce glasses of Ocean Spray Cranberry Juice Cocktail.

Nothing wasted. Everything Gained:

The efficacy of any nutraceutical remedy is dependent on its ability to survive the intrinsic acidic conditions of the GI tract. It's common for the innate goodness inherent in any natural medicine to be neutralized during this process and its potential health related benefits lost.

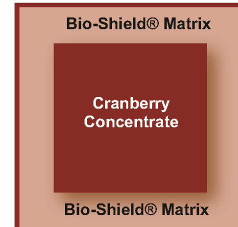
Cran-Max contains Bio-Shield® Technology which serves to "protect" the active healing elements found in cranberry, allowing them free and unhindered passage through the digestive tract to specific target receptor sites throughout the body. Bio-Shield is analogous to a protective vehicle, enveloping and safeguarding its important cargo within an impenetrable casing, delivering its payload intact and with optimum efficacy to its final site of action. Cran-Max® uses the patented Bio-Shield system to deliver its bioactives through the digestive tract's acidic medium without deterioration.

Cran-Max is prepared as part of this unique, manufacturing process where "the whole fruit and nothing but the fruit", (solids, seeds, skins and concentrated juices) are first highly concentrated, leaving the fruit's natural and synergistic elements intact, then infused into the patented Bio-Shield carrier where these actives (anthocyanins) are escorted through the stomach acids to the lower intestines where they are released in controlled fashion, absorbed by the body over a 12-16 hour time period.



5

More specifically, the key benefit of Bio-Shield technology is to maintain the integrity of the anthocyanin to glucose bond which allows anthocyanins to be passed into the lower gastrointestinal tract intact and readily absorbed into the body from a non-acidic aqueous medium. It's the role of the Bio-Shield "envelope", comprised of natural plant fibers, derivatives of the natural fruit itself, that is credited with this activity. The genius of Bio-Shield and what makes Cran-Max truly unique is that nothing is wasted and everything gained in the process. Nothing is ever introduced into the manufacturing process that interferes with the intrinsic natural value or goodness of what nature has intended. Given the guarantee of a high concentration of fruit actives coupled with Bio-Shield protection, consumers are assured an optimum delivery of the nutritional benefits related to the cranberry fruit, even greater then ingesting such foods in their raw form.



Clinical Affirmation of Branded Cranberry:

The developers of Cran-Max have made strong efforts to clinically affirm their product. Several studies have been conducted and others are underway to prove product efficacy. An overview of these studies follow:

Results of Completed Cran-Max Studies*:

- Results of a randomized, double-blind study on the prevention of recurrent cystitis with GynDelta. Dr Jean-Marc BOHBOT.
- A randomized trial to evaluate effectiveness and cost effectiveness of naturopathic cranberry products as prophylaxis against urinary tract infection in women. L. Strothers. June 2002. Can J Urol 2002 June: 9(3): 1558-62.
- The Effect of Prophylactic Administration of Cranberry Extract (Swiss Cran-Max™ 7500mg) on the Occurrence if Recurring Infections on the Urinary Tract. L.Hejzlar, Orology Clinic of PPCHC, SANUS in Hradec Kralove: J. Poduska, Urology Clinic of UNV, Prague.
- Initial Pilot Study. Jan Poduska, MD, Department of Urology, Central Military Hospital, Prague, Czech Republic, 1999.
- Dr. Ronald Wheeler Cran-Max Study. Outcome Study, Dr. Ronald Wheeler.

Preliminary Results and Abstracts of Cran-Max® Studies Under Way*:

- Preventive Treatment With Cranberry Extract Of Vesico-Ureteral Reflux (VUR) 14 In Children - Preliminary Report. Yosef Binyamini, Yuval Bar-Yosef, Mario Sofer, Haim Matzkin and Yaakov Ben Haim.; Children's Urology Unit, Urologic Department, Tel Aviv Medical Center
- Effectiveness of cranberry extracts as primary prevention of urinary tract 15 infections and evaluation of cost-effectiveness, amongst female soldiers.; Abstract of the trial procedure
- Evaluation of Cran-Max for the Prevention of UTI in Spinal Cord Injured 17 Patients with Neurogenic Bladders. Veteran Affairs, Boston Healthcare System.
- Can Cran-Max® Be Used To Treat Current Urinary Tract Infections. 18 McMaster University. Dr. Anil Kapoor. Treatment of urinary tract infection.

Independent Laboratory Results for BioShield®*:

- Proposed Mechanism of BioShield bound Anthocyanins, Integrated Biomolecule 22 Corporation. Tucson, Arizona
- Controlled Delivery of Components from CranMax-an In vitro Study, 25 Doddabele Madhavi, Ph.D,
- BioActives, Inc., Worcester, MA

****Clinical Study abstracts available upon request***

This White Paper has been written to provide an overview of the cranberry category, and highlights Cran-Max as one of its unique entries. It seeks to shine light on the importance of cranberry within a rapidly growing herbal marketplace, and to make a case for Cran-Max as a solution to a problem as old as the supplement industry itself: product consistency, quality and efficacy. The question all nutraceutical manufacturers are responsible to answer is whether their products are safe and efficacious and whether there is sufficient clinical evidence to confirm these claims.

In summation, the development of Cran-Max parallels important trends unfolding within the nutraceutical industry, and has come into existence due to a strong desire on the part of its developers to elevate cranberry to a higher level within the world of nutraceuticals. Cran-Max delivers the essential elements of cranberry in a natural, consistent and untampered fashion to consumers at large.

- Contact Information -

Mr. Dean Mosca
President
519.647.2071
dean@pnibrands.com

Mr. Skip Hammock
Key Account Executive
321.984.1742
skiphammock@earthlink.net

6. *Ellura^{TM} Product Literature*

A reproduction of the product label was not available for the Ellura^{TM} product discussed in Chapter 4, Section B4, and used in the studies of Howell and coworkers (2010). An excerpt of the information for this product found on the internet during the writing of this report is included below for ease of reference. This literature is an excellent example of the pseudo-scientific content that ends up in the advertisements for many dietary supplement products.

general FAQs

Q: What is ellura?

A: ellura is a natural cranberry supplement made from the purest cranberry juice and designed to promote and maintain urinary tract health. Because ellura is extracted from pure cranberry juice concentrate, it has high bioactivity to begin protecting you from bacteria faster than other products. Each capsule of ellura delivers 36mg proanthocyanidin (PAC) as measured by BL-DMAC. PAC is the active component in cranberries that inhibits bacteria from sticking to the inside of the urinary tract. This is the required daily PAC dosing that has been clinically proven to effectively promote urinary tract health. ellura is superior to other cranberry extract products which usually contain only 2mg to 8mg PAC as measured by the BL-DMAC method.

Q: Is there a difference in quality among cranberry supplements?

A: Yes. ellura is extracted from pure cranberry juice, which has the highest levels of PACs, and are the most bioactive. Most other cranberry supplements are extracted from presscake, which is what's left of the cranberry after the juice has been squeezed out. Presscake also contains seeds, stems and skins which the PACs attach to so they are not free to work in your body. PACs taken from presscake show little or no bioactivity, and can't be accurately measured.

Q: What are proanthocyanidins (PACs)?

A: PACs, with unique molecular structures, have been identified in cranberry and exhibit powerful bacterial anti-adhesion activity. In 1998, Dr. Amy Howell of the Marucci Center of Blueberry and Cranberry Research at Rutgers University discovered that the A-type PAC in cranberries is responsible for the bacterial anti-adhesion in the urinary tract.PACs are defense compounds produced by plants in response to environmental stress and microbial infection. The high levels of astringency of PACs protect fruits from animal and insect predators. Large quantities of PACs are found in apples, grapes, prunes, peanuts, coffee, tea, cocoa and many other fruits. Through our diet, some of these beneficial compounds are transferred into the human body. Most PACs are B-type (single bonds) that enable them to demonstrate antioxidant, anti-inflammatory and other health characteristics that are essential to our daily diet.

It is important to note that PACs can have very diverse structures and characteristics, which lead to extremely varied biological properties.The American cranberry, Vaccinium macrocarpon, is unique in that it contains A-type PACs. These A-type PACs are the only compound with proven bacterial anti-adhesion activity that promotes a healthy urinary tract. The PACs of other fruits or plants (with a B-type single bond) have not been shown to have this ability. A-type PACs help keep bacteria away from adhering to the bladder walls, allowing the bacteria to be eliminated with the urine. This anti-adhesion mechanism has been validated on three occasions by AFSSA (Agence Française de Sécurité Sanitaire des Aliments – the French approval authority for food and drugs) at an effective dosage of 36 mg once daily as measured by BL-DMAC.

Q: Is PAC bioactivity important?

A: Yes. Bioactivity means that the PACs and their metabolites are able to interact with the bacteria. If the PACs are less bioactive, their effect can be strongly reduced. Without bioactive PACs the anti-adhesion and flushing out of bacteria will not occur efficaciously. Unlike some products that show limited and delayed bioactivity like after 24 to 36 hours, ellura's strong anti-adhesion activity can be measured by a bioactivity test after 3 to 4 hours. The results of systematic testing over the past 5 years show that ellura delivers the highest bioactivity ever measured among commercially available cranberry products.

Q: Are all PACs created equal?

A: No, it is critical to measure the quantity of PACs in cranberry products accurately in order to guarantee that you, the consumer, get what you need and what you expect. 36mg PAC as measured by

buy now

testimonials

ellura seems to be working really well, I'm taking it every day without fail. - ***Chelly***

still have questions?

enter your email

ask us a question

SUBMIT

the BL-DMAC method is not equivalent to 36 mg PAC using other methods of measurement such as HPLC or Bate-Smith. An additional ruling related to the original cranberry health claim reinforces that PACs must be measured using the standardized method of BL-DMAC to show an accurate reading.

Q: What is BL-DMAC?

A: BL-DMAC is the only validated, standardized and published method to accurately measure the PAC content of cranberry extract. The BL-MAC method was developed by Brunswick Laboratories (BL), Southborough, MA. It replaced the 10-year-old methodology, DMAC/PAC003 and produces statistically the same results as the operating procedures it replaces. With the endorsement of the Cranberry Institute and the Cranberry Marketing Committee of the United States, the BL-DMAC method was internationally validated in April 2010. It is also the only method that has been validated by French authorities for use in urinary health claims on products containing 36 mg proanthocyanidins per day. Other methods are available, but the quantities they measure do not accurately reflect the quantity of "UTI-PACs" needed for the anti-adhesion against the bacteria in the bladder.

Q: I'm pregnant. Can I take ellura?

A: Yes. ellura is made from a pure cranberry juice extract, which like all fruit, is permitted during pregnancy. Plus, women have a higher risk of getting UTIs while pregnant, which can cause risks during pregnancy. Make sure to follow the dosage instructions on the label, and talk to your doctor before you start taking ellura.

Q: I'm a nursing mother, can I take ellura?

A: ellura is derived from cranberries, and no negative effects are known at the recommended dosing indicated on the label. As with all dietary supplements considered for use during nursing, consult your physician prior to use.

Q: Can I give ellura to my child?

A: Yes, ellura has been tested for safety and efficacy for use with children. Children under 50 pounds can take half a capsule of ellura once daily. Children 50 pounds and up can take the entire capsule. If the child is unable to swallow the capsule, the contents of the capsule can be mixed into a beverage or soft food, preferably sweet due to the somewhat bitter taste of the cranberry juice extract.

Q: I'm a diabetic, can I take ellura?

A: Yes. ellura has a very low glycemic index. There is no added sugar in ellura and only a trace amount exists from the fruit as the majority is removed during the extraction process.

Q: Can I take ellura continuously?

A: Yes, ellura is all natural and derived from a fruit. ellura may be taken continuously throughout the year if necessary.

Q: Does ellura color urine?

A: No. The pigments in ellura break down during the digestive process, leaving no discoloration to urine.

Q: Will ellura modify my CBEU (cytobacteriological examination of urine)?

A: Yes. If you do a CBEU at regular intervals, say every 2 weeks, you should see a systematic decrease in the presence of uropathogenic E.coli in the results of the tests, on condition that you take your daily capsule of ellura.

Q: I have a known allergy to red berries. Can I take ellura?

A: If you are allergic to cranberries, you should not take ellura. If you are allergic to other red fruits such as strawberries, consult with your doctor before taking ellura.

Q: Are there any side effects?

A: No serious side effects have been reported when taking ellura at the recommended amount of one or two capsules per day.

Q: What about interactions with other medications or dietary supplements?

A: Cranberry juice cocktail has been suspected of interfering with the metabolism of warfarin (Coumadin)

causing increasing levels of warfarin in the blood. However this interaction has not been scientifically proven. No incidents of interaction with blood thinners have been reported involving ellura. If you are taking these types of medications, we recommend you talk to your physician before you start taking ellura.

Q: How much ellura should I take?

A: To maintain a clean urinary tract: Take 1 capsule daily with water.

At onset of known triggers: Kick-start ellura's benefit by taking 2 capsules daily with water for 2 days. Continue to take 1 capsule daily for 26 days.

With sexual intercourse: Take 2 capsules with water 30 minutes before or after sexual intercourse. Then take 1 capsule daily for the following 3 days.

ellura may be taken with or without food at any time during the day, but preferably at approximately the same time each day.

As with all supplements and medications, we advise you to discuss the use of ellura (a cranberry juice extract supplement) with your health care provider. Discontinue use and consult your doctor if any adverse reactions occur.

Q: Does ellura acidify urine?

A: Only to a slight degree. The lower a pH level of a substance, the higher its acidity. ellura has pH of 4.44 which is similar to a cup of coffee or a cola. Water has a pH of 7. Cranberry juice is much more acidic with a pH of 2.7. If you can tolerate coffee or soda, you should be able to tolerate ellura.

Q: Do urinary infections affect only women?

A: No. While it is true that women of all ages are more prone to UTIs because of the short distance between the urethra and the colon, men also report to be affected by urinary tract infections. In most cases, men will face bacterial prostatitis caused by E. coli. A small percentage of children from birth to 5 years are systematically infected with E. coli for various reasons and show recurrent UTI.

Q: Can I take ellura with an antibiotic?

A: Yes. There is no restriction in taking ellura along with antibiotics. It is even recommended to do so during an active urinary tract infection. One study showed that the anti-adhesion action of ellura promotes the flushing of certain bacteria that are resistant to antibiotics.

Q: Can ellura serve as a substitute for antibiotics?

A: No. Antibiotics are essential because they kill the bacteria. Unfortunately, we have seen the resistance of uropathogenic E. coli grow steadily over the past years, reaching levels of up to 25 to 30 percent for Ciprofloxacin (a fluoroquinolon antibiotic). There will be no new families of efficacious antibiotics against UTIs available in the foreseeable future. The bioactive cranberry PACs contained in ellura interfere with the P-fimbriae (arms that attach to the bladder wall) of E. coli bacteria. This keeps the bacteria from sticking to the walls of the urinary tract, thus avoiding the first step in the development of an infection. As a dietary supplement, ellura promotes and helps to maintain a clean urinary tract.

Q: Will ellura cause or aggravate kidney stones?

A: No. Unlike cranberry juice, ellura is very low in oxalates.The majority of the naturally occurring oxalates are removed during the extraction process of the PACs. ellura has .104 mg oxalic acid per capsule—a negligible amount not capable of causing or aggravating kidney stones. Comparatively, an 8 ounce glass of cranberry juice cocktail contains 0.98 mg. Almost 10 times more oxalates!

Reducing antibiotic resistance is a top priority for both the CDC and WHO

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how does ellura work?
how is ellura different?
how do I take ellura?
the science behind ellura
general FAQs
events
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about recurring UTIs
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are my UTIs recurrent?
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UTI management

UTI prevention
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myth vs. fact
tips for urinary tract health

how we can help you
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testimonials

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**These statements have not been evaluated by the FDA. This product is not intended to diagnose, treat, cure or prevent any disease.*

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TRŌPHIKŌS
nutritive health

VITA

EDUCATION

Bachelor of Science in Botany, Magna Cum Laude (3.78), University Honors Scholar, Aug 2000 – May 2004. Auburn University (AU), Auburn, AL, Honors College, College of Sciences & Mathematics, Dept. of Biological Sciences. *Undergraduate Honors Thesis*: "Extending the Elemental Defense Hypothesis: Metals May Protect Plants from Herbivores at Accumulator and Hyperaccumulator Concentrations."

RESEARCH EXPERIENCE

Graduate Research Assistant — **Aug 2004 – Aug 2014**

UM, Dept. of Pharmacognosy, Dr. Daneel Ferreira

- Isolation and identification of cranberry juice metabolites from porcine urine
- Isolation and identification of cranberry juice chemical constituents
- Electronic circular dichroism (ECD) of flavonoids and other small molecule natural products
- Training & supervision of post-doctoral research associates, visiting scientists, and graduate students
- General lab and equipment maintenance and supply ordering

Research Assistant/Personal Assistant — **Dec 2006 – May 2008**

UM, Office of Research & Sponsored Programs (Vice Chancellor), Dr. Alice Clark

- Literature searches, document summaries & reviews, PowerPoint slide preparation (see below), fact finding, source verification
- Collaborated with the preparation of the following invited oral PowerPoint presentations given by Dr. Alice Clark:
  - "The Role of Natural Products in Drug Discovery and Development," National Institutes of Health Staff Training in Extramural Programs Symposium, Bethesda, MD, January 17, 2008.
  - "The Evolution of Antimicrobial Drug Discovery & Development," University of GA, Athens, GA, April 2007.
  - "The Evolution of Antifungal Drug Discovery," First Tennessee Chair of Excellence Symposium, University of Tennessee Health Science Center, Memphis, TN, January 25, 2007.

Undergraduate Research Assistant — **Oct 2001 – Dec 2003**

AU, Dept. of Biological Sciences, Dr. Robert Boyd & AU, Dept. of Entomology & Plant Pathology, Dr. Micky Eubanks

- Conducted experiments to determine the toxicity of heavy metals hyperaccumulated by plants, using the diamondback moth, *Plutella xylostella*, as a bioassay herbivore
- Optimized colony maintenance protocols for maximal production of quality diamondback moth eggs

Undergraduate Research Assistant **Jan 2001 – Dec 2003**
AU, Dept. of Biological Sciences, Dr. Robert Locy & José M. Barbosa
- Aseptic growth of *Arabidopsis thaliana* on modified agar media to determine the growth effects of β-alanine and other amino acids
- Feeding of ^{13}C-labeled L-alanine to *A. thaliana* and subsequent tissue preparation for GC-MS and NMR analysis of metabolic pathway components
- Cultivation and selection of *A. thaliana* mutants for subsequent gene screening

Undergraduate Laboratory Assistant **May – Aug 2001**
AU, USDA-ARS National Soil Dynamics Lab, Dr. Hugo Rogers & Dr. Michael Davis
Global Change Research Project (study of the effects of elevated CO_2)
- Assisted with the collection and preparation of plant samples for TEM and SEM analysis
- Collection of Redheaded Pine Sawfly life cycle data
- Assisted with field sample plot harvesting and sample sorting

TEACHING EXPERIENCE

Instructor for PHCG 422: Natural Product Derived Pharmaceuticals **Jan – May 2013**
UM, Dept. of Pharmacognosy, School of Pharmacy, Professional Curriculum Course
Full responsibility for all course material related to antimicrobial agents (70% of the course). Dr. Jordan Zjawiony as the instructor of record.

Laboratory Technical Training Instructor **2007 – Present**
UM, Dept. of Pharmacognosy & the UM School of Pharmacy, Research Institute of Pharmaceutical Sciences
Instructed research scientists, postdoctoral scientists and graduate students regarding background theory and correct equipment use and maintenance for the following instruments:
- OLIS DSM 20 Circular Dichroism Spectrophotometer
- JASCO 815 Circular Dichroism Spectrometer
- Various HPLC systems: Waters Prep 400, 600, and 2695 Separations Modules with PDA, UV, and ELS Detectors
- 400 MHz Bruker NMR instrument with Topspin 2.1 software

Graduate Teaching Assistant **Aug 2005 – Dec 2010**
UM, Dept. of Pharmacognosy

Courses Assisted: Pharmacognosy (PHCG):

- PHCG 321 – Dr. Marc Slattery: Pathogenesis of Infectious Diseases, Spring 2009, Fall 2009, Fall 2010
- PHCG 421 – Dr. Marc Slattery: Pathogenesis and Etiology of Infectious Diseases, Fall 2005 – 2008
- PHCG 422 – Dr. Dale G. Nagle & Dr. Jordan Zjawiony: Natural Product Derived Pharmaceuticals, Spring 2006 – 2008, 2010
- PHCG 544 – Dr. Daneel Ferreira: Departmental Seminar Series, Spring 2007
- PHCG 627 – Dr. Daneel Ferreira: Natural Products Chemistry I, Fall 2005 – 2007
- PHCG 628 – Dr. Daneel Ferreira: Natural Products Chemistry II, Spring 2006, 2007
- PHCG 634 – Dr. Daneel Ferreira: Natural Product Biosynthesis, Spring 2006, 2007

Duties:

PHCG 321/421/422: Exam preparation, proctoring & grading; student counseling, tutoring & group study sessions; preparation and presentation of selected lectures for 20-40% of course material (see below); management of Blackboard online student interface; preparation of study guides and question sets for each lecture to aid students in learning course material

PHCG 627/628, 634: guest lecturer; typing, formatting & copying of exams and homework problem sets; preparation of lecture slides from book figures; hosting of student problem solving sessions; exam proctor

Selected Lectures Given:

- PHCG 321: Pulmonary Infections; CNS Infections; Cardiovascular Infections; Gastrointestinal Infections; HIV/AIDS, Group Book Exercises
- PHCG 421: Normal Flora and Gram (+) Bacteria Intro; Respiratory Acquired Gram (-) Rods; STD/Genitourinary Tract Infections; Retroviridae & HIV/AIDS
- PHCG 422: Introduction to Antibiotics, Drug Selection Criteria & Management of Antimicrobial Therapy; Broad Spectrum Agents & Principles of Combination Therapy; Antimicrobial Drug Resistance – Types, Mechanisms, & Prevention; Tetracyclines; Ribosomes Review & Antibiotics that Inhibit Protein Synthesis; Vitamins
- PHCG 627: Biosynthetic Construction Mechanisms; The Acetate Pathway: Prostaglandins, Aromatic Polyketides
- PHCG 634: Guest lecturer & problem solving sessions

Undergraduate Teaching Assistant **Jan 2003 – May 2003**

AU, Dept. of Biological Sciences

Biology (BIOL) 3101 – Dr. Joe Cherry: Plant Biology Laboratory

- Solely responsible for laboratory lecture preparation, student instruction and assessment, laboratory set-up, and protocol revisions for assigned student experiments.

GENERAL WORK EXPERIENCE

Head Greenhouse Technician **Feb 2002 – Sept 2002**

Flower Zone Nursery, Opelika, AL

- Transplanting, watering, fertilizing, pruning, general greenhouse maintenance

Assistant Manager, Designer, Decorator & Baker **Feb 1997 – June 2000**

Ellen's Creative Cakes, Huntsville, AL

- Custom client sales, managed and trained employees, coordinated deliveries
- Baking of cakes, pastries, and appetizers
- Assembly, sculpting, and decorating of cakes
- Catering of wedding receptions and other events
- Training & supervision of regular empolyees and temporary event employees

AWARDS & HONORS

Scholarships & Awards

- NIH Common Fund Travel Fellowship to Attend the 2nd Annual Metabolomics Workshop at the University of Alabama, Birmingham, AL, June 2014
- UM Graduate School Dissertation Fellowship, Spring 2012
- UM Dept. of Pharmacognosy, Outstanding Service Award, Dec 16, 2010
- UM Dept. of Pharmacognosy Retreat, Outstanding Podium Presentation Award, Dec 16, 2010
- UM Graduate School, Graduate Research Assistant Scholarship (full tuition), Fall 2004 – Summer 2014
- Entomological Society of America and the Entomological Foundation, BioQuip Scholarship (1 of 1 awarded), Fall 2003 – Spring 2004
- National Botanical Society of America Award for Best Student Poster in the Ecological Section, July 31, 2003
- AU College of Sciences and Mathematics, Dean's Undergraduate Research Fellowship (1 of 4 awarded), Fall 2002 – Summer 2003
- AU Dept. of Biological Sciences, Fund For Excellence Undergraduate Research Award (1 of 9 awarded), Fall 2002 & Spring 2003
- AU Honors College, Drummond Company Honors Scholarship, Fall 2002 & Fall 2003
- AU Freshman Academic Scholarship (full tuition), Fall 2000 – Spring 2004
- AU College of Sciences and Mathematics, Academic Scholarship, Fall 2000
- Padgett Business Services, Book Scholarship, Huntsville, AL, Fall 2000

Honors

- Graduate Achievement Award in Pharmacognosy, April 2012
- Who's Who Among Students in American Universities and Colleges, Jan 2010
- Teaching Assistant of the Year, 2008–2009, Selected by the P4 Class (4th Year Pharmacy Students), May 2009
- Young Botanist of the Year, American Botanical Society, 2004
- Student Spotlight in AU College of Sciences & Mathematics, *Journey* Newsletter (p. 38), 2004
- Dean's List, AU College of Sciences & Mathematics, Fall 2000, Spring 2002 – Fall 2004
- Junior Honors Certificate, AU Honors College, Spring 2002
- Harry M. Rhett, Jr., Community Service Book Award, Randolph School, Huntsville, AL, May 26, 2000
- National Merit Scholarship Program Letter of Commendation, Spring 2000

Honor Societies

Rho Chi (Spring 2009), Phi Kappa Phi (Spring 2004), Beta Beta Beta (Spring 2003), Alpha Lambda Delta (Spring 2001), Phi Eta Sigma (Spring 2001), National Society of Collegiate Scholars (Spring 2001)

PROFESSIONAL SERVICE & INVOLVEMENT

Peer Review

- NIH-NCRR COBRE CORE-NPN Predoctoral Fellowship *ad hoc* Study Section 2009, Oxford, MS, Oct – Nov 2009
  - Mentored review of scientific research proposals submitted by UM graduate students. Mentor: Dr. David Pasco, UM National Center for Natural Products Research.
- Peer Reviewer for Scientific Journals, May 2012 – Present
  - Journal of Natural Products
  - Molecular Nutrition and Food Research

Professional Committee Positions

- Department of Pharmacognosy Graduate Student Committee for Graduate Program Revision, May – Dec 2011
  - Revised Departmental published degree requirements for MS and PhD students
- MALTO-PharmForum Joint Meeting, Executive Organizing Committee, Jan – May 2010
  - Participated in all aspects of planning necessary for meeting organization
  - Focused on recreational event planning, design and purchase of give-away items, volunteer coordination, and on-site operations
- American Society of Pharmacognosy, Young Members Committee, July 2007 – July 2010
  - Contributed two biographies of previous ASP presidents for the 50th anniversary commemorative book
- Phytochemical Society of North America, Young Members Committee, July 2007 – July 2009
  - Organized & implemented conference activities, wrote newsletter articles, membership drive participation
  - Hosted: "Ask the Editor, A Panel Discussion on Common Manuscript Errors and Ways to Avoid Them." Panel Members: Dr. Daneel Ferreira, Dr. John Pezzuto, Dr. John Romeo, and Dr. Norman Lewis. Hosts: **Coleman, Christina M.**; Kutrzeba, Lukasz; Mazourek, Michael. Phytochemical Soc. N. America Annual Meeting, Pullman, WA, June 25–30, 2008.
- Historian – AU Beta Beta Beta Biological Honor Society — Fall 2003 – Spring 2004
- Social Committee Planner – AU Honors Congress — Fall 2000 – Fall 2002
- Assistant Director, Outdoor Recreation – AU Program Council — Spring 2001 – Spring 2002
- President & Founder – AU Hiking Club — Spring 2001 – Summer 2002
- Committee Member, Outdoor Recreation – AU Program Council — Fall 2000 – Spring 2001
- Secretary and Assistant Event Organizer – Residence Hall Council — Fall 2000 – Spring 2001

Professional Organizations

American Society for Microbiology
American Society of Pharmacognosy
American Chemical Society
Phytochemical Society of North America
American Botanical Society

Volunteer Education & Outreach

- Department of Pharmacognosy Representative, Fall 2007 – Spring 2014
  - Host and tour guide for prospective graduate students.
  - Poster: Ferreira, Daneel; **Coleman, Christina M**. "The Department of Pharmacognosy: An Overview" UM School of Pharmacy, National Center for Natural Products Research Annual Poster Session, Oxford, MS, Nov 8, 2012 & October 11, 2013
- Science Fair Judge – North Mississippi Regional Science Fair; Grades 4–12, Botany Division, UM Tad Smith Coliseum, Oxford, MS, March of 2007, 2009 – 2012, 2014
- Science Fair Judge – Local Science Fair; Grades 4–7, Oxford University School, Oxford, MS, Feb 5, 2008; Feb 5, 2009
- Chemistry lab tour & career counseling for high school students attending a BISC 102 summer course with the UM Department of Biology, June 2009
- Adult GED Tutor – Algebra, vocabulary, reading skills; Lafayette County Literacy Coalition, Susan Nicholas, Managing Director, Oxford, MS, July 2005 – Mar 2006
- Adult GED Tutor – Algebra, grammar, reading skills; Lee County Literacy Coalition, Opelika, AL, Aug 2003 – July 2004

PROFESSIONAL DEVELOPMENT & ADVANCED TRAINING

Professional Development

- "Grants Workshop Series: (1) Proposal & Award Lifecycle, (2) Budgeting for Proposals, (3) Data Security, (4) Obtaining a Patent." UM, Office of Research and Sponsored Programs, Oxford, MS, Sept 2013
- UM, School of Pharmacy, Interdepartmental Faculty Retreat, June 6–7, 2013
- UM, Dept. of Pharmacognosy Annual Retreat, Oxford, MS, Dec 16, 2010; Aug 26, 2011
- Graduate Student/Post-doc Summer Institute, American Chemical Society Publications Division, Organizer: David Martinsen. ACS Headquarters Building, Washington, DC, Aug 8–12, 2011
  - Objective of the Institute: "To bring together creative graduate students and postdocs to help [the ACS Publications Division] redefine how science is published, distributed, and incorporated into the research and learning workflow."
  - Competitively selected from a pool of applicants nominated by ACS Journal Editors.
- UM Women in STEM Dinner Program, Follow Up Round Table Discussion of recent attendees to the National Conference for College Women Student Leaders, Oxford, MS, Oct 26, 2010
- UM Women in STEM Roundtable Series: "What Women Need to Know to Survive and Succeed in STEM Careers," Hosted by the Sarah Isom Center for Women and Gender Studies, Spring 2010

Advanced Technical Training

- University of Alabama at Birmingham, 2nd Annual Workshop on Metabolomics, Sponsored by the National Institute of General Medical Sciences as part of the NIH Common Fund Metabolomics Initiative, and by the Departments of Chemistry and Pharmacology and Toxicology at UAB, Birmingham, AL, June 2–5, 2014
- University of Florida Metabolomics Workshop & Symposium, Sponsored by the Southeast Center for Integrated Metabolomics (SECIM), and the Clinical and Translational Science Institute, University of Florida, Gainesville, FL, May 20–23, 2014
- Metabolomics Afternoon Workshop. Presenters: Dr. Paul Shiply, Dr. Susan Murch, & Dr. Christina Turi from the University of British Columbia. American Society of Pharmacognosy Annual Meeting, St. Louis, MO, July 13, 2013
- Electronic and Vibrational Circular Dichroism Afternoon Workshop. Presenters: Dr. Nina Berova, Professor & Senior Research Scientist, Columbia University, and Dr. Ana Petrovic, Assistant Professor, New York Institute of Technology. American Society of Pharmacognosy Annual Meeting, New York, NY, July 28, 2012
- X-Ray Crystallography Introductory Short Course, Dr. Frank Fronczek, Director of X-Ray Crystallography Facility, Dept. of Chemistry, Louisiana State University, Held at UM, June 1–5, 2009
- Bruker NMR Training Seminar Series. Mike Brown, Application Scientist for the Southern Region, Bruker South Training Center, Held at UM, Jan 2006

Communications & Education Training

- "Best Practices in Teaching Online" Workshop Series, Mark Yacovone, UM Graduate School, Oxford, MS, Spring 2013
- "Introduction and Overview of the Wimba Classroom" Workshop, Randall Uncapher, IT Designer, UM Division of Outreach & Continuing Education, UM Faculty Technology Development Center, Oxford, MS, Sept 16, 2011
- "Science: Becoming the Messenger" Full Day Workshop. Presenters: Dan Agan, President, Panthera Group LLC; Chris Mooney, Science Journalist; Joe Schreiber, President, Mattmar Productions. National Science Foundation EPSCoR Workshop, Mississippi State University, Starkville, MS, Aug 29, 2011
- "The Use of Technology for Teaching," iLearning Event Workshop for UM Faculty and TAs, UM Faculty Technology Development Center, Oxford, MS, Nov 18, 2010
- "Elevator Talks," Bringing Scientists to the People Workshop, Carolyn Gayle, Clear Communication Group, UM Graduate School, Oxford, MS, Nov 25, 2008
- "School of Pharmacy Graduate Teaching Assistant Seminar Series" (Monthly meetings each fall). Dr. Alicia Bouldin, Professor & Teaching Assistant Mentor for the School of Pharmacy, UM, Fall 2006 – 2010

- “Using PRS RF-Clickers in Classrooms Workshop” (beginning & advanced sessions). Julie Weiss, InterWrite Inc., and Penny Rice, UM Faculty Technology Development Center, Oxford, MS, Jan 14, 2008
- “Teaching Assistant Orientation & Training” (3 day workshop). Dr. Johnny Lott, UM Center for Excellence in Teaching and Learning, Oxford, MS, Aug 2007, 2008

University of Mississippi: Selected Elective Courses

- BISC 502: Mycology – Instructor: Dr. Jason Hoeksema, Fall 2008; Included laboratory work studying gross anatomy and microscopy of macro-fungi of the southeast U.S.
- PHAD 692: Drug Development & Marketing – Instructor: Ms. Allyson Best, Spring 2006; Participated in the research and design of a development and marketing plan for a compound possessing antitumor activity. Acted as the group director with eight people in the group.
- BISC 522: Microbial Ecology – Instructor: Dr. Clifford Ochs, Fall 2005
- PHCL 381: Introduction to Toxicology (Audit) – Instructor: Dr. Kristie Willett, Fall 2005

Auburn University: Selected Elective Courses

- NUFS 643: Food Chemistry – Instructor: Dr. Leonard Bell, Spring 2004
- HORT 622: Greenhouse Management Science – Instructor: Dr. Raymond Kessler, Fall 2003; Developed a business plan for the foundation and management of a greenhouse business
- BIOL 497 Special Topics: Plant Anatomy & Development – Instructor: Dr. Roland Dute, Spring 2003; Special Project: TEM sample preparation and TEM & confocal microscopy of wood tissue segments
- ENTM 304: General Entomology – Instructor: Dr. Wayne Clark, Fall 2002; Special Project: Insect collection and preservation

RESEARCH SUPPORT

Ongoing Research Support

Ocean Spray Cranberries, Inc. Ferreira (PI) 10/01/2011 – 03/15/2014
Human Urinary Metabolites Produced as a Result of Cranberry Juice Consumption
The goal of this study was to isolate and identify the anti-adhesive metabolites of human urine produced as a result of cranberry consumption. Role: Investigator & proposal co-author

Completed Research Support

1R21AT002076-01 NIH/NCCAM Howell (PI) 04/05/2004 – 04/04/2009
Identification of Bioactive Cranberry Metabolites
The goal of this study was to isolate and identify the anti-adhesive metabolites of swine urine produced as a result of cranberry consumption. Role: Investigator

PUBLICATIONS

Refereed Original Research and Scholarly Review Articles

1. **Coleman, Christina M.**; Boyd, Robert S.; Eubanks, Micky D. Extending the Elemental Defense Hypothesis: Dietary Metal Concentrations Below Hyperaccumulator Levels Could Harm Herbivores. *Journal of Chemical Ecology,* 2005, *31* (8), 1669-1681. PMID: 16222801.
2. **Coleman, Christina M.**; Prather, Brian L.; Valente, Matthew J.; Dute, Roland R.; Miller, Michael E.; Torus Lignification in Hardwoods. *International Association of Wood Anatomists (IAWA) Journal,* 2004, *25* (4), 435-447.
3. Ferreira, Daneel; Marais, Jannie P. J.; **Coleman, Christina M.**; Slade, Desmond. "Proanthocyanidins: Chemistry and Biology" In: *Comprehensive Natural Products Chemistry II*, Pergamon Press (Elsevier), New York, 2009, Chapter 6.15.
4. Ferreira, Daneel; **Coleman, Christina M.** Towards the Synthesis of Proanthocyanidins: Half a Century of Innovation, Invited Review Publication, *Planta Medica,* 2011, *77* (11), 1071-85. doi: 10.1055/s-0030-1270908. PMID: 21412691.
5. Xu, Yong-Jiang; Foubert, Kenn; Dhooghe, Liene; Lemière, Filip; Maregesi, Sheila; **Coleman**, **Christina M.**; Zou, Yike; Ferreira, Daneel; Apers, Sandra; Pieters, Luc. Rapid Isolation and Identification of Minor Natural Products by LC-MS, LC-SPE-NMR, and ECD: Isoflavanones, Biflavanones, and Bisdihydrocoumarins from *Ormocarpum kirkii*, *Phytochemistry,* 2012, *79*, 121-128. doi: 10.1016/j.phytochem.2012.04.004. PMID: 22575670.
6. Belofsky, Gil; Kolaczkowski, Marcin; Adams, Earle; Schreiber, John; Eisenberg, Victoria; **Coleman, Christina M.**; Zou, Yike; Ferreira, Daneel. Fungal ABC Transporter-Associated Activity of Isoflavonoids from the Root Extract of *Dalea formosa* (Fabaceae). *Journal of Natural Products,* 2013, *76* (5), 915-925. doi: 10.1021/np4000763. PMID: 23631483. Accepted without change by two independent reviewers.
7. Li, Jun; **Coleman, Christina M.**; Wu, Hankui; Burandt, Charles L.; Ferreira, Daneel; Zjawiony, Jordan K., Triterpenoids and Flavonoids from *Cecropia schreberiana* Miq. (Urticaceae). *Biochemical Systematics and Ecology*, 2013, *48* (June), 96-99. PMID: 23459662.
8. Campana, Priscilla R. V.; **Coleman, Christina M.**; Teixeira, Mauro M.; Ferreira, Daneel; Braga Fernão C., TNF-α Inhibition Elicited by Mansoins A and B, Heterotrimeric Flavonoids Isolated from *Mansoa hirsuta*. *Journal of Natural Products,* 2014, *77* (4), 824-830. PMID: 24576254.
9. Canuto, Kirley M.; Leal, Luzia K. A. M.; Lopes, Amanda A.; **Coleman, Christina M.**; Ferreira, D.; Silveira, Edilberto R. Amburanins A and B from *Amburana cearensis*: Daphnodorin-Type Biflavonoids That Modulate Human Neutrophil Degranulation. *Journal of the Brazilian Chemical Society*, 2014, 25 (4), 639-647. doi: 10.5935/0103-5053.20140011.

10. Villinski, Jacquelyn R.; Bergeron, Chantal; Cannistra, Joseph C.; Gloer, James B.; **Coleman, Christina M.**; Ferreira, Daneel; Azelmat, Jabrane; Grenier, Daniel; Gafner, Stefan. Pyrano-Isoflavans from *Glycyrrhiza uralensis* with Antibacterial Activity Against *Streptococcus mutans* and *Porphyromonas gingivalis*. *Journal of Natural Products*, 2014, *77* (3), 521-526. PMID: 24479468.

11. Belofsky, Gil; Aronica, Mario; Foss, Eric; Diamond, Jane; Santana, Felipe; Darley, Jacob; Dowd, Patrick; **Coleman, Christina M.**; Ferreira, Daneel. Antimicrobial and Antiinsectan Phenolic Metabolites of *Dalea searlsiae* (Fabaceae). *Journal of Natural Products,* 2014, *77* (5), 1140–1149.

12. Du, Kun; **Coleman, Christina M.**; Van Vuuren, Sandy F.; Van Zyl, Robyn L.; Zietsman, Pieter C.; Ferreira, Daneel; Marston, Andrew; Van Der Westhuizen, Jan H. Flavonol Acyl Glucosides from the Aril of *Schotia brachypetala* Sond. and Their Antioxidant, Antibacterial and Antimalarial Activities. *Phytochemistry Letters*, Available online, June 2014.

In Preparation for Refereed Journals

Coleman, Christina M.; Howell, Amy B.; Krueger, Christian G.; Auker, Kimberly M.; Reed, Jess D.; Ferreira, Daneel. Porcine Anti-Adhesive Urinary Metabolites Produced as a Result of Cranberry Consumption. *Journal of Agricultural and Food Chemistry,* 2014, In Preparation.

Auker, Kimberly M.; **Coleman, Christina M.**; Wang, Mei; Avula, Bharathi; Khan, Ikhlas; Ferreira, Daneel. Structural Characterization of Cranberry Oligosaccharides. *Journal of the American Chemical Society*, 2014, In Preparation.

Auker, Kimberly M.; **Coleman, Christina M.**; Kimble, Lindsey; Mathison, Bridget; Chew, Boon P.; Howell, Amy B.; Ferreira, Daneel. Human Anti-Adhesive Urinary Metabolites Produced as a Result of Cranberry Juice Consumption. In Preparation.

Coleman, Christina M.; Akgul, Yurdanur; Auker, Kimberly M.; Kimble, Lindsey; Mathison, Bridget; Wang, Mei; Avula, Bharathi; Khan, Ikhlas; Chew, Boon P.; Ferreira, Daneel. Anti-Adhesive Oligosaccharides from Cranberry Products. In Preparation.

Coleman, Christina M.; Nael, Manal A.; Zou, Yike; Eisenberg, Victoria; Belofsky, Gil; Doerksen, Robert J.; Ferreira, Daneel. The Absolute Configuration of 3-Hydroxyisoflavanones Through Experimental and Calculated Electronic Circular Dichroism. In Preparation.

Coleman, Christina M.; Zou, Yike; Ferreira, Daneel. How to Study Small Molecule Natural Products by Electronic Circular Dichroism. In Preparation.

Peddikotla, Prabhakar; **Coleman, Christina M.**; Adelli, Vijender R.; Marais, Johannes P. J.; Ferreira, Daneel, Synthesis of A-Type Proanthocyanidins. In Preparation.

Radhakrishnan, Sri Vedavyasa Sri; Sharma, Vimal K.; Ankisetty, Sridevi; **Coleman, Christina M.**; Ferreira, Daneel; Zjawiony, Jordan K. 12a-Hydroxyrotenoids from *Mirabilis multiflora*. In Preparation.

Professional Newsletter Publications

Clark, Alice M. & **Coleman, Christina M.** "The Changing Face of ASP: The Role of Women." *American Society of Pharmacognosy Newsletter*, December 2007, 43(4), p 4, 9.

Coleman, **Christina M.;** Mazourek, Michael; Biedrzycki, Meredith; Kutrzeba, Lukasz. "Potential Conference Activities for Young Members for PSNA 2008 General Scientific Meeting." *Phytochemical Society of North America Newsletter*, December 2007, 46(2), p 18-21.

Technical Writing & Reports

Coleman, Christina M. "Operational Instructions for the JASCO J-815 Circular Dichroism Spectrometer," May 2013, Instrument Operation Manual.

Coleman, Christina M. "Operational Instructions for the OLIS DSM-20 Circular Dichroism Spectrophotometer," May 2012, Instrument Operation Manual.

Coleman, Christina M., "Toosendanin – Structure, Sourcing, Uses & General Notes." Technical Report prepared for Dr. Alice Clark, March 03, 2007.

PROFESSIONAL MEETINGS ATTENDED

- American Society of Pharmacognosy Annual Meeting, St. Louis, MO, July 13–17, 2013
- International Congress on Natural Products Research (Joint with ASP), New York, NY, July 28 – Aug 1, 2012
- Joint Meeting of the American Society of Pharmacognosy and the Phytochemical Society of North America, St. Petersburg, FL, July 10–14, 2010
- Joint Meeting of MALTO and PharmForum, Oxford, MS, May 23–26, 2010
- American Society for Microbiology National Meeting, Philadelphia, PA, May 17–21, 2009
- Phytochemical Society of North America Annual Meeting, Pullman, WA, June 25–30, 2008
- Phytochemical Society of North America Annual Meeting, St. Louis, MO, July 21–25, 2007
- American Society of Pharmacognosy Annual Meeting, Portland, ME, July 14–18, 2007
- MALTO Medicinal Chemistry & Pharmacognosy Annual Meeting, Monroe, LA, May 20–22, 2007
- American Chemical Society National Meeting (Fifth Tannin Conference), San Francisco, CA, Sept 10–14, 2006
- Phytochemical Society of North America Annual Meeting, Oxford, MS, July 8–12, 2006
- MALTO Medicinal Chemistry & Pharmacognosy Annual Meeting, Houston, TX, May 21–23, 2006
- MALTO Medicinal Chemistry & Pharmacognosy Annual Meeting, Oxford, MS, May 22–24, 2005
- Botanical Society of America National Meeting, Mobile, AL, July 26–31, 2003

PRESENTATIONS

Scientific Meetings – Presenting Author

Coleman, Christina M.; Auker, Kimberly M.; Ferreira, Daneel. "Anti-adhesion Properties of Cranberry Products: Current Status and New Directions." American Society of Pharmacognosy Annual Meeting, St. Louis, MO, July 17, 2013. Oral.

Auker, Kimberly M.; **Coleman, Christina M.**; Wang, Mei; Avula, Bharathi; Kimble, Lindsey; Mathison, Bridget; Khan, Ikhlas; Chew, Boon P.; Ferreira, Daneel. "Structural Characterization and Bioactivity of Cranberry Oligosaccharides." American Society of Pharmacognosy Annual Meeting, St. Louis, MO, July 15, 2013. Poster.

Coleman, Christina M.; Zou, Yike; Eisenberg, Victoria; Belofsky, Gil; Ferreira, Daneel. "Absolute configuration of 3-hydroxyisoflavanones and conjugated 2-(5*H*)-furanones through electronic circular dichroism." ICNPR/ASP Joint Meeting, New York, NY, July 28 – Aug 1, 2012. Poster.

Coleman, Christina M.; Ferreira, Daneel; Howell, Amy B.; Reed, Jess D.; Krueger, Christian G.; Marais, Jannie P. J. "Anti-Adhesive Urinary Metabolites Produced as a Result of Cranberry Juice Consumption." UM Department of Pharmacognosy Annual Retreat, Oxford, MS, Dec 16, 2010. Oral.

Coleman, Christina M.; Ferreira, Daneel; Howell, Amy B.; Reed, Jess D.; Krueger, Christian G.; Marais, Jannie P. J. "Anti-Adhesive Urinary Metabolites Produced as a Result of Cranberry Juice Consumption." ASP/PSNA Joint Meeting, St. Petersburg, FL, July 13, 2010. Oral.

Coleman, Christina M.; Ferreira, Daneel; Howell, Amy B.; Reed, Jess D.; Krueger, Christian G.; Marais, Jannie P. J. "Isolation and Identification of Anti-Adhesive Urinary Metabolites from Cranberry Juice." Phytochemical Society of North America Annual Meeting, Pullman, WA, June 25–30, 2008. Poster.

Coleman, Christina M.; Ferreira, Daneel; Marais, Jannie P. J.; Howell, Amy B.; Cihlar, Ronald; Cohen, Jesse. "Cranberry Juice Compounds with Anti-Adhesive Properties in Urine." Phytochemical Society of North America Annual Meeting, Pullman, WA, June 25–30, 2008. Poster.

Coleman, Christina M.; Ferreira, Daneel; Howell, Amy B.; Reed, Jess D.; Krueger, Christian G.; Marais, Jannie P.J. "Isolation and Identification of Anti-Adhesive Urinary Metabolites from Cranberry Juice." Phytochemical Society of North America Annual Meeting, St. Louis, MO, July 21–25, 2007. Poster.

Coleman, Christina M.; Ferreira, Daneel; Howell, Amy B.; Reed, Jess D.; Krueger, Christian G.; Marais, Jannie P. J. "Bioactive Metabolites of Cranberry Juice." American Society of Pharmacognosy Annual Meeting, Portland, ME, July 14–18, 2007. Poster

Coleman, Christina M.; Ferreira, Daneel; Howell, Amy B.; Reed, Jess D.; Krueger, Christian G.; Marais, Jannie P. J. "Bioactive Metabolites of Cranberry Juice." MALTO Medicinal Chemistry & Pharmacognosy Annual Meeting, Monroe, LA, May 20–22, 2007. Poster.

Coleman, Christina M.; Ferreira, Daneel; Howell, Amy B.; Reed, Jess D.; Krueger, Christian G.; Marais, Jannie P. J. "Bioactive Metabolites of Cranberry Juice." American Chemical Society National Meeting, CELL Division – Fifth Tannin Conference, San Francisco, CA, Sept 10–14, 2006. Poster.

Coleman, Christina M.; Ferreira, Daneel; Howell, Amy B.; Reed, Jess D.; Krueger, Christian G.; Marais, Jannie P. J. "Bioactive Metabolites of Cranberry Juice." Phytochemical Society of North America Annual Meeting, Oxford, MS, July 8–12, 2006. Oral.

Coleman, Christina M.; Ferreira, Daneel; Howell, Amy B.; Reed, Jess D.; Krueger, Christian G.; Marais, Jannie P.J. "Bioactive Metabolites of Cranberry Juice." MALTO Medicinal Chemistry & Pharmacognosy Annual Meeting, Houston, TX, May 21–23, 2006. Oral

Coleman, Christina M.; Prather, Brian L.; Valente, Matthew J.; Dute, Roland R.; and Miller, Michael E.; "Torus Lignification in Hardwoods." AU Undergraduate Research Forum, Auburn, AL, October 9–10, 2003. Poster.

Coleman, Christina M.; Boyd, Robert S.; Eubanks, Micky D. "Herbivore Defense as an Explanation for Hyperaccumulation: Heavy Metal Toxicity to Diamondback Moth (*Plutella xylostella*)" Botanical Society of America National Meeting, Ecological Section, Mobile, AL, July 26–31, 2003. Poster.

Invited Presentations – Presenting Author

Coleman, Christina M. "Department of Pharmacognosy Innovative Teaching Strategies: PHCG 422." UM, School of Pharmacy, Interdepartmental Faculty Retreat, June 6, 2013. Oral.

Coleman, Christina M. "An Overview of PHCG 422: Natural Product Derived Pharmaceuticals, Spring 2013." UM, School of Pharmacy, Interdepartmental Faculty Retreat, June 6, 2013. Poster.

Coleman, Christina M.; Howell, Amy B.; Krueger, Christian G.; Auker, Kimberly M.; Reed, Jess D.; Ferreira, Daneel. "Anti-Adhesive Urinary Metabolites Produced as a Result of Cranberry Consumption." Ocean Spray Cranberries, Inc., Corporate Headquarters Site Visit, Lakeville-Middleboro, MA, May 20, 2013.

Coleman, Christina M. "Communications Technology and the 'Scientist of the Future.'" Department of Pharmacognosy Annual Retreat, Oxford, MS, Aug 26, 2011. Oral.

Coleman, Christina M. "Why Plants and Science are Both Awesome!" A visit to the 3rd grade class of Mrs. Judy Caldwell, Lafayette County Upper Elementary School, Oxford, MS, Aug 18, 2011. Interactive presentation & demonstration.

Aiyar, Avishek; **Coleman, Christina M.**; Havanki, Kathy; McCrary, Parker; Reilly, Maureen. "The Dynamic Scientist." American Chemical Society Publications Division, Graduate Student/Postdoc Summer Institute, ACS Headquarters Building, Washington, DC, Aug 12, 2011. Oral.

Coleman, Christina M. "Cranberry Juice: What do we really know?" Invited presentation to UM Course PHCG 329, Herbal Dietary Supplements, Instructor: Dr. Charles Hufford, Feb 24, 2011. Oral.

Coleman, Christina M. "What Makes a Good PowerPoint Presentation?" Invited Seminar for UM Course PHCG 544, Instructor: Dr. Daneel Ferreira, Feb 2007. Oral.

Scientific Meetings & Invited Presentations – Collaborating Author

Auker, Kimberly M.; **Coleman, Christina M.**; Wang, Mei; Avula, Bharathi; Kimble, Lindsey; Mathison, Bridget; Khan, Ikhlas; Chew, Boon P.; Ferreira, Daneel. "Structural Characterization and Bioactivity of Cranberry Oligosaccharides." Ocean Spray Cranberries, Inc., Corporate Headquarters Site Visit, Lakeville-Middleboro, MA, May 20, 2013.

Ferreira, Daneel; **Coleman**, **Christina M.** "Absolute Configuration of Secondary Metabolites via Electronic Circular Dichroism." ICNPR/ASP Joint Meeting, New York, NY, July 28 – Aug 1, 2012.

Auker, Kimberly M.; **Coleman, Christina M.**; Avula, Bharathi; Ferreira, Daneel. "Structural Elucidation of Complex Carbohydrates from Cranberry." ICNPR/ASP Joint Meeting, New York, NY, July 28 – Aug 1, 2012.

Ferreira, Daneel; **Coleman**, **Christina M.** "Absolute Configuration of Secondary Metabolites via Electronic Circular Dichroism." Frank Warren 2012 Conference hosted by the South African Chemical Institute, Bloemfontein, South Africa, April 15–18, 2012.

Ferreira, Daneel; **Coleman**, **Christina M.** "Absolute Configuration of Secondary Metabolites via Electronic Circular Dichroism." PSNA 2011, Kohala Coast, HI, Dec 12, 2011.

Ferreira, Daneel; **Coleman**, **Christina M.** "Half a Century of Proanthocyanidin/Polyphenol Research." PSNA 2011, Kohala Coast, HI, Dec 13, 2011.

Ferreira, Daneel; **Coleman, Christina M.**; Marais, Jannie P. J.; Howell, Amy B. "Cranberry Polyphenols and Urinary Tract Infections." International Conference on Polyphenols & Health, Kyoto, Japan, Nov 2007.

Ferreira, Daneel; **Coleman, Christina M.**; Marais, Jannie P. J.; Howell, Amy B. "Chemistry of Condensed Tannin & Effect of Cranberry Tannin." Invited Presentation, Nippon Shinyaku Corporate Meeting Presentation, Kyoto, Japan, Nov 27, 2007.

Ferreira, Daneel; Li, Xing-Cong; Ding, Yuanxing; Marais, Jannie P. J.; **Coleman, Christina M.** "Absolute Configuration of Proanthocyanidins via Circular Dichroism." Barton Conference, St. Lucia, May 2007.

Ferreira, Daneel; Marais, Jannie P. J.; Li, Xing-Cong; **Coleman, Christina M.** "Circular Dichroism: A Powerful Tool for the Definition of Absolute Configuration of Proanthocyanidins." American Chemical Society National Meeting, Division of Agricultural and Food Chemistry, Chicago, IL, March 29, 2007.

Ferreira, Daneel; Marais, Jannie P. J.; Li, Xing-Cong; **Coleman, Christina M.** "Absolute Configuration of Proanthocyanidins via Circular Dichroism." American Chemical Society National Meeting, CELL Division – Fifth Tannin Conference, San Francisco, CA, Sept 10–14, 2006.

Other Presentations – Presenting Author

Coleman, Christina M. "Chemical Profiles of Microbial Interactions for Antimicrobial Drug Discovery." Original Research Proposal, Oral Defense Seminar. Degree requirement. March 27, 2014

Coleman, Christina M. "Bioprospecting for Novel Natural Products: Where can we look next?" Oral Seminar – Chosen Topic. Degree requirement. May 1, 2007

Coleman, Christina M. "Viral Infections & HIV/AIDS," Invited Lab Group Presentation, January 31, 2007

Coleman, Christina M. "Saturation Transfer Difference (STD) NMR: Theory, Methods, and Applications." Oral Seminar – Assigned Topic. Degree requirement. March 9, 2006

Coleman, Christina M. "Urinary Tract Infections: An Overview." Invited Lab Group Presentation, September 30, 2005

Coleman, Christina M. "Genistein as a Molecular Probe: Tyrosine kinase inhibition & the role of tyrosine kinases in the cell." Oral Seminar – Assigned Topic. Degree requirement. March 30, 2005

Coleman, Christina M. Graduate Level Graded Coursework Presentations

- PHCG 632: Structure Elucidation – "Brief Introduction to the Fundamentals of ORD & CD," Spring 2005
- PHCG 628: Natural Products Chemistry – "Porphyrins" and "Saponins," Spring 2005
- PHCG 627: Natural Products Chemistry – "Lignins & Lignans" and "Carbohydrates: Polysaccharides – Starch, Cellulose, and Fructans," Fall 2004