Analysis of Carbohydrate and Fatty Acid Marker Abundance in Ricin Toxin Preparations for Forensic Information

ARTICLE *in* ANALYTICAL CHEMISTRY · JULY 2010

Impact Factor: 5.64 · DOI: 10.1021/ac1006206 · Source: PubMed

6 AUTHORS, INCLUDING:

Heather Anne Colburn
PAML
20 PUBLICATIONS **136** CITATIONS
SEE PROFILE

David S Wunschel
Pacific Northwest National Laboratory
48 PUBLICATIONS **981** CITATIONS
SEE PROFILE

James J Moran
Pacific Northwest National Laboratory
26 PUBLICATIONS **296** CITATIONS
SEE PROFILE

Anal. Chem. **2010,** *82,* 6040–6047

Analysis of Carbohydrate and Fatty Acid Marker Abundance in Ricin Toxin Preparations for Forensic Information

Heather A. Colburn,* David S. Wunschel, Helen W. Kreuzer, James J. Moran, Kathryn C. Antolick, and Angela M. Melville

Chemical and Biological Signature Sciences, National Security Directorate, Pacific Northwest National Laboratory, P.O. Box 999, MSIN P7-50, Richland Washington 99352

One challenge in the forensic analysis of ricin samples is determining the method and extent of sample preparation. Ricin purification from the source castor seeds is essentially a protein purification through removal of the nonprotein fractions of the seed. Two major, nonprotein constituents in the seed are the castor oil and carbohydrates. We used derivatization of carbohydrate and fatty acid markers followed by identification and quantification using gas chromatography/mass spectrometry (GC/MS) to assess compositional changes in ricin samples purified by different methods. The loss of ricinoleic acid indicated steps for oil removal had occurred, and a large decrease of ricinoleic acid was observed between unextracted mash and solvent extracted and protein precipitate preparations. Changes to the carbohydrate content of the sample were also observed following protein precipitation. The differential loss of arabinose relative to mannose was observed indicating the removal of the major carbohydrate fraction of the seed and enrichment of the protein content. When the data is combined and multivariate principle component analysis is applied, these changes in fatty acid and carbohydrate abundance are discriminating enough to be indicative of the preparation method used for each sample.

The poison ricin is a protein that exerts its lethal effects by depurinating an adenine residue within eukaryotic rRNA, thereby eliminating a binding site for an elongation factor and inhibiting protein synthesis.[1] Ricin has a molecular mass of 66 kDa and is composed of two polypeptide chains, designated A and B, which are linked by a disulfide bond.[2] The holotoxin has a lethal dose of 5−10 μg/kg body weight and is classified as a Schedule 1 controlled substance under the Chemical Weapons Convention and a Category B (biological agents/toxin) substance under the Biological Toxins Weapons Convention.[3] It is also included as a Category B select agent set forth by the U.S. Department of Health and Human Services (42 CFR Part 73). In recent years, ricin-containing samples have been seized primarily in connection with biocriminal activity.[4]

Ricin is produced in the seeds of the castor plant *Ricinus communis*, which grows wild in tropical and subtropical climates. The oil present in the seeds is a valued industrial commodity,[5−7] and castor is widely cultivated as an oilseed crop, primarily in India, China, and Brazil;[5] the striking appearance of the plant has also led to its common use as an ornamental. Castor seeds are an unregulated agricultural product, but as soon as they are broken open with the intent of obtaining ricin, the resulting material is considered a ricin sample.[8] Thus, an important challenge in law enforcement is demonstrating whether castor seeds were intentionally processed.

Numerous methods by which ricin can potentially be purified from castor seeds have been published. Some of these methods are technical procedures for producing highly purified ricin and can be found in the scientific literature,[9−11] while others are "kitchen" procedures found in various booklets, pamphlets and Internet sites.[12−14] All of these preparation methods are generally variations on common themes for biochemical purification of proteins from a complex sample matrix, in this case the castor seed. Castor seeds are 40−60% oil by weight; approximately 25% of the seed weight is carbohydrate, primarily in the form of cellulose and starch, with the remainder being protein and inorganic material.[7] The seeds are encased in a hard protective coat, and many procedures begin with removal of the seed coat to access the seed's pulp and oil. Further purification steps can include removal of the oil through solvent extraction or pressing,

* Corresponding author. E-mail: Heather.Colburn@pnl.gov.

(1) Endo, Y.; Morishita, R.; Imashevich, K. M.; Yoshinari, S. *J. Toxicol.: Toxin Rev.* **1998**, *17*, 427–439.
(2) Lundger, S.; Melin, L.; Nilsson, C.; von Shoenberg, P. Swedish Defence Research Agency User Report No. FOI-R-1261-SE, Umea, SE, **2004**.
(3) Bradberry, S. M.; Dickers, K. J.; Rice, P.; Griffiths, G. D.; Vale, J. A. *Toxicol. Rev.* **2003**, *22*, 65–70.
(4) Schier, J. G.; Patel, M. M.; Belson, M. G.; Patel, A.; Schwartz, M.; Fitzpatrick, N.; Drociuk, D.; Deitchman, S.; Meyer, R.; Litovitz, T.; Watson, W. A.; Rubin, C. H.; Kiefer, M. *Am. J. Public Health* **2007**, *97* (Suppl. 1), S152–157.
(5) Ogunniyi, D. S. *Bioresour. Technol.* **2006**, *97*, 1086–1091.
(6) Naughton, F. C. In *Kirk-Othmer Encyclopedia of Chemical Technology*, 5th ed.; John Wiley & Sons, Inc.: New York, 1993; pp 1−17.
(7) Weiss, E. A. *Oilseed Crops*; Longman: London and New York, 1983.
(8) Department of Health and Human Services. 42 CFR Part 73; U.S. Government Printing Office: Washington, DC, 2002; Vol. 240, Part 1003.
(9) Craig, H.; Alderks, O. H.; Germin, A. H.; Dieke, S. H.; Karel, C. L. U.S. Patent 3,060,165, October 23,1962.
(10) Simmons, B.; Russell, J. H. *Anal. Biochem.* **1984**, *146*, 206–210.
(11) Despeyroux, D.; Walker, N.; Pearce, M.; Fisher, M.; McDonnell, M.; Bailey, S. C.; Griffiths, G. D.; Watts, P. *Anal. Biochem.* **2000**, *279*, 23–36.
(12) Preisler, S. In *Silent Death*; Festering Publications: Green Bay, WI, 1997; Chapter 10.
(13) Harber, D. In *Assorted Nasties*; Desert Publications: El Dorado, AR, 1993.
(14) Saxon, K. *The Poor Man's James Bond*; Desert Publications: El Dorado, AR, 1991.

10.1021/ac1006206
Published on Web 06/22/2010

solubilization of the toxin from the starch-rich mash, and precipitation of the toxin along with other proteins. Finally, an affinity purification step can be used to purify ricin from other proteins and toxic lectins.

Numerous previous reports describe the analysis of ricin for forensic purposes. Many of these efforts focused on either the protein toxin itself or associated proteins in the preparation.[11,15−19] Alternatively, several reports centered on identification of the unique castor alkaloid ricinine as a marker of ricin poisoning following exposure.[16,19] However, few published reports focus on characterizing a ricin-containing sample to determine if and how it was intentionally prepared.[20]

We speculated that various potential steps in toxin extraction would differentially remove fatty acid and carbohydrate markers and thus leave residual components characteristic of the preparation method. Approximately 90% of the fatty acid in castor oil is ricinoleic acid (RA), an unusual hydroxylated and unsaturated fatty acid that is the major constituent of castor oil and confers on it the heat resistance and chemical reactivity prized by industry.[5,6] Following derivatization to the fatty acid methyl ester (FAME) form, RA can be separated from other fatty acids by gas chromatography (GC) of methyl ester derivatives and identified by mass spectrometry (MS). Toxin preparation steps that decrease oil content should therefore decrease the weight percent of RA in the sample.

The majority of carbohydrate within a castor seed is found in the starch, cell wall polymers (cellulose and hemicellulose), and galactose-inositols present for desiccation resistance and protein glycosylation. A number of monosaccharide constituents may be present in different amounts in these plant structures. Examples of importance to this study are the *myo* and *chiro*-inositol derived from phospholipids and galactinols,[21] arabinose and xylose found within cell wall hemicellulose,[22] and mannose and *N*-acetyl glucosamine abundant in protein glycosylation.[23] Carbohydrate profiles of ricin preparations can be generated by hydrolyzing the samples to monomers, derivitizing the monomers to their alditol acetate forms, and subjecting the mixture to GC/MS analysis. This approach was previously used for profiling the relative abundance of monosaccharides derived from bacterial samples.[24−26] Toxin purification steps that enrich the protein fraction were anticipated to reduce the total carbohydrate content and differentially reduce the cell wall carbohydrates relative to those associated with protein glycosylation.

Here we present results demonstrating that carbohydrate and fatty acid abundances can be used to differentiate between types of toxin preparations. We show that removal of castor oil reduces resulting RA concentrations. Likewise, the removal of starch and cell wall polymers by protein precipitation changes the relative abundances of specific monosaccharides. These results support our hypothesis that carbohydrate and fatty acid abundance data reflect the purification steps used to prepare ricin samples.

EXPERIMENTAL SECTION

Castor Seeds. Ornamental castor seeds were obtained from four different sources: Onalee's Seeds (Brookesville, FL), Whatcom Seed Co. (Eugene, OR), Bouncing Bear Botanicals (Flagstaff, AZ), and locally cultivated (Richland, WA). Prior to preparation of toxin, the seeds were inactivated using dry 100 °C heat for at least 30 min. Some samples of toxin were prepared without inactivation to evaluate the effect that heat had on the analytical results. The heat inactivation did not impact the analytical results for carbohydrate content and methyl ricinoleate content.

Reagents. Sodium hydroxide solutions were prepared from sodium hydroxide pellets (Sigma-Aldrich, St. Louis, MO). Water was purified by a Milli-Q system (Millipore, Billerica, MA). Epsom salt (magnesium sulfate heptahydrate, Kroger brand) was purchased at a local grocery store. Reagent-grade acetone, methyl-*tert* butyl ether, boron trifluoride, methanol, hexane, sulfuric acid, $NaBD_4$, acetic acid, and chloroform were obtained from Sigma-Aldrich (St. Louis, MO). Acetic anhydride was purchased from Supelco, Inc. (Bellafonte, PA), C18 solid phase extraction cartridges were obtained from Phenomenex (Torrance CA), and Chem-Elut solid phase extraction cartridges were purchased from Perkin-Elmer (Waltham, MA). The carbohydrate and fatty acid standards indicated below were obtained in the highest purity available from Sigma-Aldrich (St. Louis, MO).

Toxin Preparation. Four relatively simple methods that differ in their approaches to oil removal and protein precipitation were used to prepare ricin-containing samples. The first three are kitchen recipes that can be found in clandestine publications often referred to as the "anarchist literature" while the fourth is a relatively simple laboratory procedure. (1) Castor seed mash (CM).[14] Castor seeds were placed in a beaker, covered with 3 M NaOH, and allowed to stand for 1−2 h. Afterward, the NaOH was decanted, and the seeds were thoroughly rinsed with water and either patted dry or allowed to air-dry. The softened husks of the seeds were removed and the white meat of the bean was ground into a mash. (2) Acetone extraction of oil from mash (AE).[12] Castor seed mash was prepared as described above, placed in an Erlenmeyer flask, and covered with 4 g of acetone for every gram of mash. A stir bar was added to the beaker, which was sealed with aluminum foil and Parafilm. The mixture was stirred on a stir plate for 72 h. The acetone-extracted bean mash was collected by pouring the mixture through a double layer of coffee filters, allowing the material captured on the filter to dry, and scraping it into a vial. (3) Epsom salt protein precipitation (ESP).[13] Five castor seeds were soaked in 3 M NaOH and peeled as described above. The peeled beans were crushed between sheets of wax paper and scraped into a line on layered paper towels. The crushed bean pulp was covered with more paper towels, placed under 4−5 kg of weight, and allowed to stand overnight to squeeze oil from the pulp. The next day, the bean pulp was transferred from the

(15) Fredriksson, S. A.; Hulst, A. G.; Artursson, E.; de Jong, A. L.; Nilsson, C.; van Baar, B. L. M. *Anal. Chem.* **2005**, *77*, 1545–1555.
(16) Johnson, R. C.; Lemire, S. W.; Woolfitt, A. R.; Ospina, M.; Preston, K. P.; Olson, C. T.; Barr, J. R. *J. Anal. Toxicol.* **2005**, *29*, 149–155.
(17) Kalb, S. R.; Barr, J. R. *Anal. Chem.* **2009**, *81*, 2037–2042.
(18) Ovenden, S. P. B.; Fredriksson, S. A.; Bagas, C. K.; Bergstrom, T.; Thomson, S. A.; Nilsson, C.; Bourne, D. J. *Anal. Chem.* **2009**, *81*, 3986–3996.
(19) Mouser, P.; Filigenzi, M. S.; Puschner, B.; Johnson, V.; Miller, M. A.; Hooser, S. B. *J. Vet. Diagn. Invest.* **2007**, *19*, 216–220.
(20) Kreuzer, H. W.; Wahl, J. H.; Metoyer, C. N.; Colburn, H. A.; Wahl, K. L. *J. Forensic Sci.* **2010** DOI: 10.1111/j.1556-4029.2010.01334.x.
(21) Horobowicz, M.; Obendorf, R. L. *Seed Sci. Res.* **1994**, *4*, 385–405.
(22) Esteves, B.; Graca, J.; Pereira, H. *Holzforschung* **2008**, *62*, 344–351.
(23) Browder, S. K.; Beevers, L. *Plant Physiol.* **1980**, *65*, 924–930.
(24) Fox, A.; Black, G. E.; Fox, K.; Rostovtseva, S. *J. Clin. Microbiol.* **1993**, *31*, 887–894.
(25) Wunschel, D.; Fox, K. F.; Black, G. E.; Fox, A. *Syst. Appl. Microbiol.* **1994**, *17*, 625–635.
(26) Fox, A., Gilbart, J., Morgan, S. L., Eds. *Analytical Microbiology Methods: Chromatography and Mass Spectrometry*; Plenum Press: New York, 1990; pp 71−88.

paper towels to a mortar and ground; the pulp was then suspended in 8.75 mL of 10% w/vol NaCl. The suspension was capped and allowed to stand for 48 h with occasional shaking. Next, it was filtered through a double layer of coffee filters. Residual liquid was squeezed from the filters and added to the filtrate. A solution of 1 g of epsom salt in 22.5 mL of water was added to the filtrate to precipitate the protein. The mixture was allowed to stand for 1 h, then the precipitate was collected by centrifugation for 5 min at 5 000 rpm and allowed to air-dry. (4) Acetone precipitation (AP) of proteins from seed mash.[27] A total of 10 g of castor seeds was soaked in 3 M NaOH and peeled as described above. The peeled seeds were placed in a beaker with 40 g of phosphate-buffered saline solution (per liter 8.5 g of NaCl, 1.24 g of Na_2HPO_4, 0.18 g of NaH_2PO_4, pH 7.2) and pulverized with an immersion blender. The mixture was centrifuged at 10 000 rcf for 20 min at 4 °C, which separated it into three layers: solid debris at the bottom, an aqueous, ricin-containing layer in the middle, and oil on top. The middle layer was withdrawn with a pipet and placed in a fresh container (volume approximately 30 mL). To this, 270 mL of acetone was added, and the mixture was placed in the freezer overnight. The following day, the majority of the acetone was decanted. The remaining mixture was centrifuged for 10 min at 5 000 rcf, the remaining acetone was decanted, and the precipitate was air-dried.

Fatty Acid Methyl Ester (FAME) Preparation. We used saponification in 0.5 M NaOH at 70 °C for 2 h to cleave the ester bonds and liberate fatty acids (in their ionic form) from lipids, lipoproteins, and lipopolysaccharide within a prepared sample. Since ricin can be inactivated by exposure to 0.06 N NaOH for 30 min at room temperature, this treatment also inactivated any ricin that might be present in the sample. Following saponification, the solutions were acidified to pH 3−6 with approximately 4 M HCl, and the reprotonated fatty acids were extracted into methyl-*tert*-butyl ether (MTBE). The MTBE was concentrated under nitrogen, and when the solution reached a small volume, it was transferred into a fresh vial for methylation.

The remaining MTBE was evaporated, and the samples were resuspended in approximately 3% BF_3 in methanol and incubated for 2 h at 100 °C to methylate the fatty acids. This step must be anhydrous, as water hydrolyzes the esters. Following methylation, the FAMEs were extracted into hexane and the final volume decreased by evaporation under nitrogen. The samples were reconstituted in methanol to a final volume of 100 μL for injection. Standard solutions of methyl ricinoleate were prepared for GC/MS analysis in order to generate a calibration curve. Methyl ricinoleate was diluted in HPLC-grade methanol (Sigma-Aldrich, St. Louis, MO) in concentrations ranging from 0.925 μg/mL up to 925 μg/mL. The standards were stored at −20 °C until analysis.

Carbohydrate Derivatization Methods. This method relies on first liberating sugar monomers (e.g., glucose, inositol, arabinose, mannose, etc.) from any oligomeric or polymeric structures using acid hydrolysis at elevated temperature under vacuum.[25] Examples of such compounds in castor beans are the glucose-rich starch, inositol of phospholipids, and desiccation-resistance disaccharides, xylose/arabinose-rich hemicellulose polymers within the cell wall and the mannose/*N*-acetyl glucosamine-rich protein glycosylations.

The alditol acetate method for derivatization of carbohydrate monomers has been described at length previously.[28] Briefly, following acid hydrolysis under vacuum in 2 N H_2SO_4, the samples were neutralized using an organic base and hydrophobic contaminants were removed by solid phase extraction. The aldose (C-1 carbonyl containing) sugars were reduced to their alditol form using sodium borodeuteride. The borodeuteride adds a deuterium to the C-1 carbon, allowing native aldoses to be distinguished from native alditols in the mass spectrum (e.g., glucose is distinguishable from glucitol) by virtue of C-1-containing fragment masses that are 1 m/z larger for aldoses (e.g., glucose) than those found for native alcohol sugars (e.g., glucitol). Following reduction, the residual borodeuteride was removed by addition of methanol-acetic acid to dry it away as tetramethyl borate gas. The reduced sugars were derivatized by addition of acetic anhydride to form acetate derivatives for GC/MS analysis.

An external standard mix containing 18 carbohydrates was prepared in triplicate along with each batch of castor samples. The neutral monosaccharides within the standard mixture were fucose, rhamnose, ribose, arabinose, deoxyglucose, xylose, pinitol, *chiro*-inositol, *myo*-inositol, *allo*-inositol, *muco*-inositol, *scyllo*-inositol, mannose, galactose, and glucose. The amino monosaccharides within the mixture were mannosamine, glucosamine, and galactosamine. Methylglucose was used as an internal standard for quantitation of neutral monosaccharides while *N*-methylglucamine was the internal standard for amino monosaccharides. Each internal standard was included within the external standard mix as well as in each sample.

Gas Chromatography/Mass Spectrometry (GC/MS). Analysis of the alditol acetate-derivatized carbohydrates was performed on an Agilent Technologies 7890A/5975C GC/MS instrument equipped with a CTC CombiPAL autosampler system. Chromatographic separation of the alditol acetate mixtures was carried out in helium carrier gas on an HP-5 column (Agilent Technologies, 250 μm × 0.25 μm × 30 m). A 1 μL volume of each sample was injected into a split/splitless injector operated in split mode using a 10:1 split ratio with 10.8 mL/min split flow, 1.08 mL/min column flow. The injector temperature was held at 250 °C and the transfer line at 250 °C. The GC oven was held at 100 °C for 1 min, ramped at 2.5 °C/min to 225 °C, then ramped at 25 °C/min to 250 °C with a hold for 5 min. The ion source temperature was 230 °C with an electron impact ionization energy of −70 V. Following a 10 min delay, data was collected from 35 m/z to 250 m/z using a detector voltage of 1059 V.

Analysis of the FAME samples was performed on the same Agilent Technologies 7890A/5975C GC/MS equipped with an HP-5 column (Agilent Technologies, 250 μm × 0.25 μm × 30 m). A 1 μL volume of each sample was injected into a split/splitless injector operated in pulsed splitless mode, with 1.08 mL/min column flow of He carrier gas. The injector temperature was held at 280 °C and the transfer line at 250 °C. The GC oven was held at 35 °C for 21 min, ramped at 25 °C/min to 150 °C, then ramped at 10 °C/min to 280 °C with a hold for 1.4 min. The ion source

(27) Ehleringer, J. R.; Kreuzer-Martin, H. W.; West, J. B.; Lowe, T. M.; Barnette, J. E.; Hoffman, J. FBI Report BAA-0034104, Salt Lake City UT, 2006.

(28) Fox, A., Morgan, S., Gilbart, J., Eds. *Preparation of Alditol Acetates and Their Analysis by Gas Chromatography and Mass Spectrometry*; CRC Press: Boca Raton, FL, 1989; pp 87−117.

temperature was 230 °C with an ionization energy of −70 V. Following a 5 min delay, data was collected from 50 m/z to 550 m/z using a detector voltage of 1059 V. Because of the wide range of methyl ricinoleate content in the samples, the samples were diluted 1:10 and 1:100 in HPLC-grade methanol. The undiluted and diluted samples were analyzed. The results that fell within the linear range of the calibration were used for calculation of methyl ricinoleate content within the samples.

Data Analysis. Carbohydrate data was analyzed using the Agilent Chem Station software to identify and integrate peaks for determination of the presence and abundance of individual carbohydrates. The response factor of each of the 18 monosaccharides in the external standard mixture was determined by dividing the peak area of each carbohydrate in the external standard mixture (extstd) by the peak area of the appropriate internal standard, eq 1. A response factor for each of the 18 carbohydrates was determined by averaging the response factors for that carbohydrate in each standard mix analyzed within a sample set.

$$\mathrm{RF_{std}} = \frac{\mathrm{area_{extstd}}}{\mathrm{area_{intstd}}} \quad (1)$$

Response factors for carbohydrates derived from the ricin samples were calculated by dividing the peak area of the carbohydrate peak (carbpeak) by the peak area of the internal standard, eq 2

$$\mathrm{RF_{sample}} = \frac{\mathrm{area_{carbpeak}}}{\mathrm{area_{intstd}}} \quad (2)$$

The mass of each carbohydrate in the sample was calculated based on the carbohydrate response factor in the sample as compared to the response factor for 60 ng/μL of carbohydrate in the external standard, multiplied by the sample volume to determine the total mass (in nanograms) of each known carbohydrate in the sample, eq 3.

$$\mathrm{mass_{carb}} = \frac{(\mathrm{concn_{std}})\,(\mathrm{vol_{std}})\,(\mathrm{RF_{sample}})\,(\mathrm{vol_{sample}})}{(\mathrm{RF_{std}})\,(\mathrm{vol_{inj}})} \quad (3)$$

where $\mathrm{concn_{std}}$ = the concentration of the known standard in the standard mix in nanogrms/microliter, $\mathrm{vol_{std}}$ = the volume of standard mix injected, $\mathrm{vol_{sample}}$ = the total sample volume of the final derivatized sample, and $\mathrm{mass_{carb}}$ = mass of the known carbohydrate in the final sample in nanograms.

Finally, the % weight of each carbohydrate in the sample can be calculated if needed by eq 4

$$\%\,\mathrm{wt} = \frac{\mathrm{mass_{carb}}}{\mathrm{mass_{sample}}} \times 100 \quad (4)$$

The abundance of methyl ricinoleate in each FAME sample was based on the peak area determined with Agilent Chem Station for the methyl ricinoleate fragment m/z 166.1. An external calibration curve was generated, and the sample peak areas were fit to this curve to determine the amount of methyl ricinoleate in the samples.

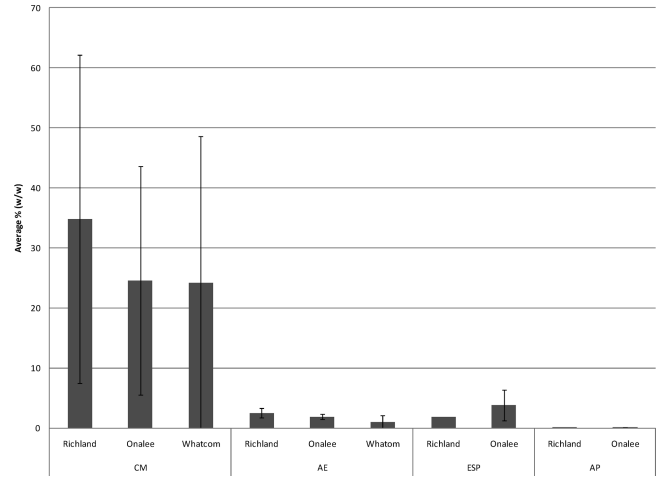

Figure 1. Average methyl ricinoleate content of castor preparation expressed as % (w/w) of the original sample. Because of the limited amounts of the precipitate preparations, not all were converted to FAMEs and analyzed. The error bars represent 1 standard deviation.

Safety Considerations. Caution must be used when preparing samples from castor seeds that have not undergone inactivation due to the toxicity of ricin. Proper protective equipment must be used when working with active toxin containing materials.[29] Furthermore, preparation or possession of 100 mg or more of active toxin requires select agent registration with the CDC in accordance with 42 CFR Part 73.[8]

RESULTS AND DISCUSSION

Toxin Preparation. The four preparation methods yielded different final sample masses. Both the CM and AE methods had high yields, with several hundred milligrams of sample derived from roughly 2 g of seeds. However the ESP procedure yielded significantly less material, with 50−100 mg of precipitated material obtained from roughly 2 g of seeds. The AP procedure typically yielded over 300 mg of material. The ricin content of these preparations was not determined.

Fatty Acid Analysis. In initial experiments, we observed significant variability in the methyl ricinoleate (MR) abundances measured from similar samples suspended in hexane. We therefore compared hexane, chloroform, and methanol as the final solvent for reconstituting samples. Of these solvents, methanol produced the greatest sample reproducibility (data not shown) and was used to resuspend all MR samples.

The MR content of the samples was determined by GC/MS as described above. In general, there was a clear difference in MR content among the different toxin preparation types. Figure 1 shows the MR content of the samples expressed as the % weight of the original sample, broken out by preparation type and castor seed source. As expected, the CM samples had the highest MR content, average % wt of 28.5, because no attempt had been made to remove the castor oil from the samples. The AE and ESP samples contained an average % wt 1.8 and 3.3, respectively, while the AP samples contained the least MR with the average % wt of 0.1. Overall, the data set included one data point that was deemed to be an outlier

(29) *Biosafety in Microbiological and Biomedical Laboratories Handbook*, 5th ed.; Millard, E. C. B., Ed.; Centers for Disease Control: Atlanta, GA, 2005; pp 1−14.

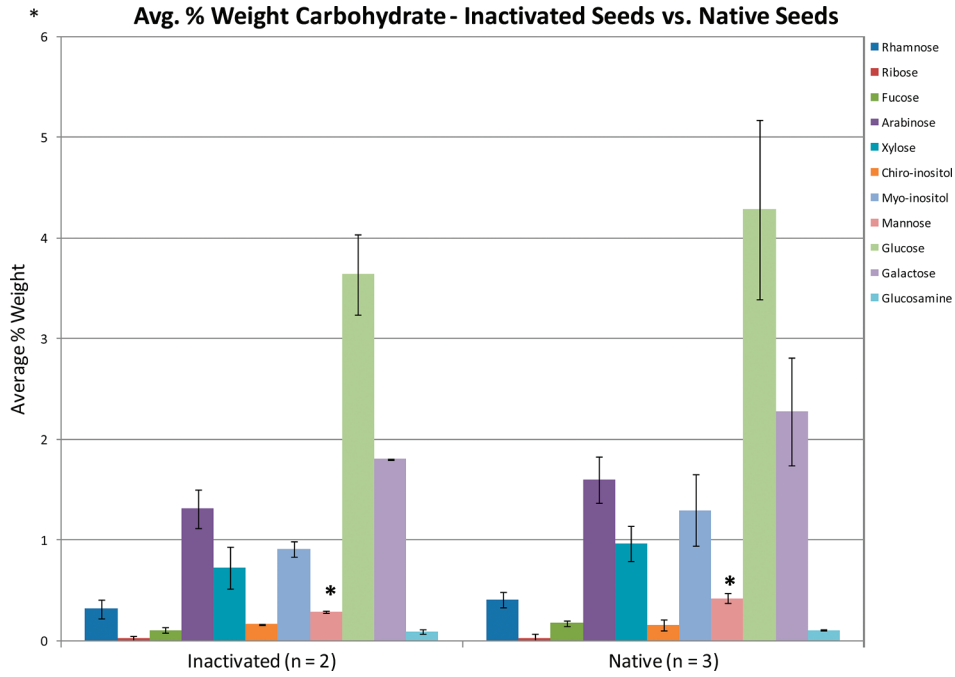

Figure 2. Comparison of castor seed mash prepared from heat inactivated and native castor seeds. Error bars represent 1 standard deviation. Asterisks indicate statistically significant differences between inactivated and native seeds.

using the Dixon's Q test at the 90% confidence level and was not included for the average and error calculations.[30] The CM samples had larger errors than the other preparation types. The large error bars associated with the CM sample analyses are in part due to the large peak size, sample heterogeneity, and the requirement of dilution to bring the analysis into the linear range of the methyl ricinoleate standard curve. Both a large peak area and additional dilutions can introduce error to the measurement, additional error could have been introduced in the methanol resuspension of the samples. In spite of the large errors, for a single prep type each seed type is undistinguishable from the other based on *t* tests at the 95% confidence interval. Each prep type (CM vs AE, etc.) is statistically significant from the other with the exception of the AE samples when compared to the ESP by *t* test at the 95% confidence interval ($p = 0.1067$).

Carbohydrate Analysis. The abundances of the 18 carbohydrates in the standard mix (derivatized into their alditol acetate form) were determined and their identities confirmed using GC/MS. The GC separation was sufficient to resolve all 18 carbohydrates. A total of 11 of the 18 were found in one or more type of castor preparation: rhamnose, ribose, fucose, arabinose, xylose, *chiro*-inositol, *myo*-inositol, mannose, glucose, galactose, and glucosamine. The percent weight of each was calculated using the method described above for each replicate toxin preparation.

In this study, we prepared and analyzed samples from heat-inactivated castor seeds to both reduce the hazard of working with the material and reduce inventory of active toxin. It was therefore necessary to examine the possible effects of heat inactivation of the seeds on measured carbohydrate abundance. Carbohydrate profiles of CM preparations from heat-inactivated and native seeds are shown in Figure 2. The native CM samples appeared to have slightly higher amounts of each carbohydrate than the inactivated CM samples; however, the abundances were within 1 standard deviation of each other and with the exception of mannose ($p = 0.0270$), *t* tests at the 95% confidence interval did not show the inactive vs native seeds to be statistically significant from one another. Castor oil is known to withstand high temperatures,[5] so this same test was not performed for the MR analysis.

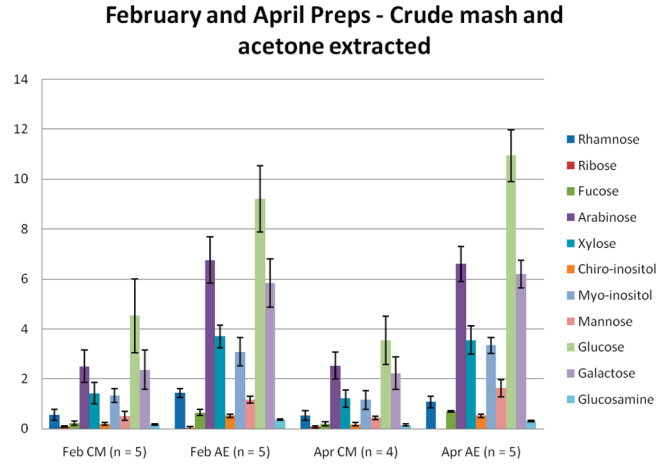

Figure 3. Comparison of data across two different preparation dates. Error bars represent 1 standard deviation.

To gain an estimate of the variability in carbohydrate abundance measurements, CM and AE samples created on two different dates were derivatized for GC/MS analysis. Five aliquots of each sample were derivatized for each recipe type and sample preparation date. The values for % dry weight and standard deviation for the carbohydrates found in each sample are shown in Figure 3. The standard deviation for carbohydrate measurements remained less than 30%. Importantly, we observed consistent abundance of each monosaccharide in the replicate derivitizations of samples prepared according to the same recipe regardless of the date of preparation with the exception of rhamnose ($p = 0.0297$), mannose ($p = 0.0220$), and

(30) Dean, R. B.; Dixon, W. J. *Anal. Chem.* **1951**, *23*, 636–638.

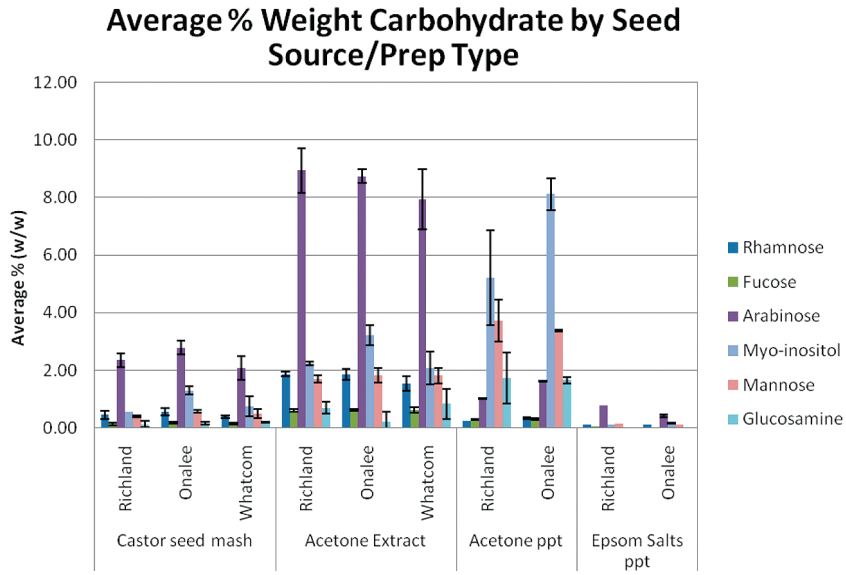

Figure 4. Average % wt carbohydrate comparison across preparation and seed source. Only 6 of the 18 carbohydrates are included. Error bars represent 1 standard deviation.

Table 1. Average % Weight of Carbohydrates Detected in Samples Prepared by Four Methods Using Seeds from Three Sources

	castor seed mash			acetone extract			acetone ppt		epsom salts ppt	
average % wt	Richland	Onalee	Whatcom	Richland	Onalee	Whatcom	Richland	Onalee	Richland	Onalee
rhamnose	0.45	0.55	0.39	1.86	1.84	1.54	0.24	0.34	0.12	0.12
ribose	0.01	0.10	0.05	0.08	0.08	0.16	0.09	0.20	0.00	0.09
fucose	0.13	0.18	0.15	0.60	0.62	0.62	0.30	0.31	0.05	0.00
arabinose	2.35	2.79	2.08	8.93	8.73	7.94	1.01	1.62	0.77	0.42
xylose	1.15	1.28	1.20	2.37	4.38	4.23	0.45	0.92	0.34	0.15
chiro-inositol	0.02	0.07	0.06	0.09	0.07	0.42	0.38	0.49	0.00	0.00
myo-inositol	0.57	1.30	0.74	2.24	3.21	2.08	5.21	8.11	0.13	0.17
mannose	0.39	0.57	0.50	1.70	1.83	1.81	3.73	3.37	0.14	0.12
glucose	3.29	7.89	4.22	13.68	21.11	11.90	8.33	12.86	2.15	1.28
galactose	1.43	2.38	1.52	6.18	6.37	5.32	7.76	10.53	0.00	0.36
glucosamine	0.12	0.16	0.19	0.70	0.20	0.83	1.73	1.65	0.03	0.00

glucosamine ($p = 0.0039$) for the acetone extract (AE) prepared samples which were found to be statistically different between the Feb and Apr preparations based on the *t* test at the 95% confidence interval. Additionally, for a given prep date, the preparation types were statistically different from one another with the exception of ribose ($p = 0.1074$) in the Feb preps of CM and AE.

The data in Figure 3 also show an approximate 2-fold increase in the abundance of most monosaccharides in the AE samples as compared to the CM samples. Further comparisons were made to distinguish between a broader set of toxin preparation recipes. Three additional sources of castor seeds (Onalee (O), Whatcom (W), and Richland (R)) were used to prepare samples using four toxin preparation recipes (CM, AE, ESP, and AP). Consistent with earlier observations, an increase in each monosaccharide was measured for the AE samples relative to the CM preparation. We also observed significant differences in the abundance of specific monosaccharides for the CM and AE samples versus the two precipitation methods (ESP and AP) (Figure 4; Table 1.). The Whatcom seeds were in limited supply so they were not used for the lower yield ESP procedure.

As observed previously, a given preparation type did not tend to be statistically significant from one seed source to another by *t* test at the 95% confidence interval, with the exception of the Richland CM compared to Onalee CM where fucose ($p = 0.0179$), *myo*-inositol ($p = 0.0006$), and mannose ($p = 0.0059$) were statistically significant from each other. In the case of the AE preparations, the Richland AE and Onalee AE were *myo*-inositol contents ($p = 0.0323$) statistically significant from one another as were the Onalee AE and Whatcom AE myo-inositol contents from each other ($p = 0.0436$). When the Richland AP and Onalee AP samples were compared to one another, the rhamnose ($p = 0.0045$) and arabinose ($p = 0.0001$) contents were statistically significant from one another. Because of the limited number of replicates of the ESP samples, the *t* test was not conducted on this data to look for significance across bean type.

For a given seed source, the differences between carbohydrate content of different preparation types was generally statistically significant from one another by *t* test at the 95% confidence interval with the following exceptions: Richland CM and Richland AP did not vary significantly in rhamnose ($p = 0.0728$), Richland AP and Richland AE did not vary significantly

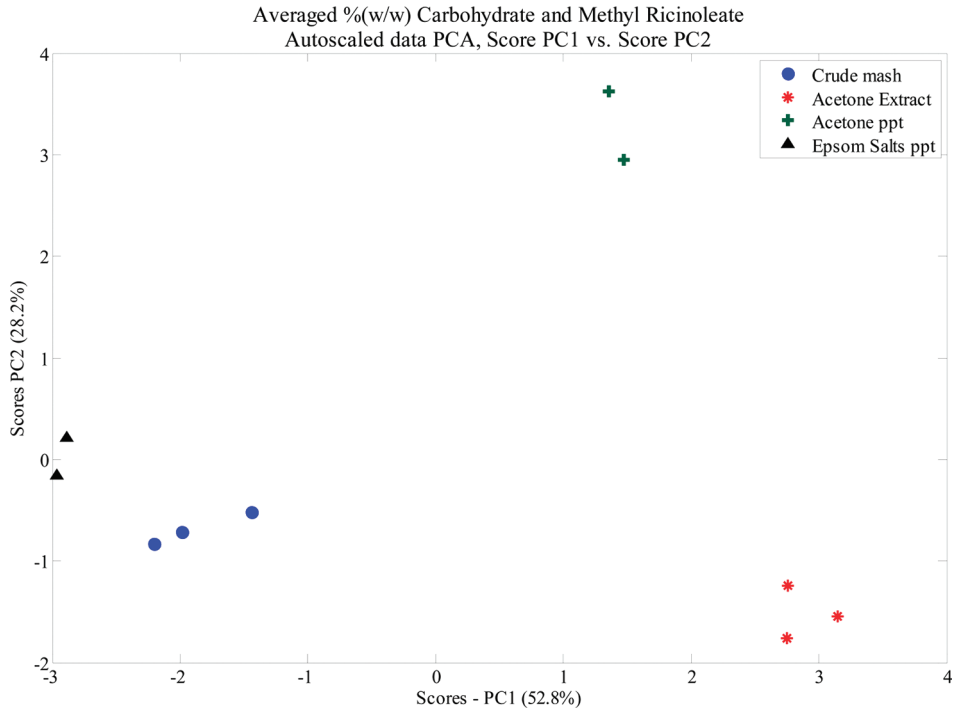

Figure 5. Scores vs scores plot of PC1 vs PC2 for the autoscaled, combined average data set for each seed source, represented once for each recipe indicated. Three replicate data sets are averaged for each symbol.

in *myo*-inositol ($p = 0.0947$) and glucosamine ($p = 0.2188$), Onalee CM and Onalee AE did not vary significantly in glucosamine ($p = 0.8537$), Onalee CM and Onalee AP did not vary significantly in rhamnose ($p = 0.1202$), Onalee AE and Onalee ESP did not vary significantly in glucosamine ($p = 0.4991$), and Whatcom CM and Whatcom AE did not vary significantly in glucosamine ($p = 0.1048$).

The above t test results are for very small sample size populations (i.e., $n = 2$ or $n = 3$). Given the fact that the samples are visually heterogeneous in nature, the above incongruities are indeed possible. When the data is combined across seed source for a given preparation recipe and t tests are conducted between individual carbohydrate contents by preparation recipe (p-values < 0.009), the only insignificant difference is glucosamine between the AE and ESP preparations ($p = 0.0765$). However, the overall carbohydrate profile is distinctly different between the two preparation types allowing for discrimination between preparation recipes, which is further demonstrated below.

The data show a significant reduction of arabinose between either the CM or AE (above 1.5%) and the protein precipitated samples ESP and AP (1% or less). In the case of the ESP precipitated method, the % wt of arabinose is <0.5. The ratio of arabinose to mannose is also a discriminating feature where it is >2 in all preparations except the AP samples where it is <1. The same trend is evident in the arabinose to *myo*-inositol ratio, which is >2 in all preparation methods except AP, where it is <0.5. The content of glucosamine is also significantly reduced in the ESP samples (less than 0.05% weight), and *chiro*-inositol is undetectable.

Combined Data Analysis. The average methyl ricinoleate and average carbohydrate content data for the four different castor preparation types and three different bean sources was autoscaled and subjected to principle component analysis (PCA). The variance in the combined data set could be described with a four PC model. Figure 5 shows the plot of PC1 scores vs PC2 scores. The four preparation types were distinctly grouped, independent of the source of castor seeds, demonstrating that the combination of the two data types can be combined for discrimination of ricin preparation samples. The discriminating variables include rhamnose, fucose, arabinose, xylose, and methyl ricinoleate.

CONCLUSIONS

Quantitative analysis of monosaccharides and ricinoleic acid revealed systematic variations between ricin samples prepared from castor seeds by different methods. This study included both preparatory methods accessible to individuals with limited experience and equipment and analytical laboratory methods designed to have a higher yield and purity.

The detected abundance of MR in a sample reflected its castor oil content. In all of the recipes we used, as well as others cited above, purification of toxin beyond CM includes an oil removal step. Therefore, a significant decrease in MR content compared to that of CM is an important indicator of toxin processing.

The carbohydrate abundance data showed significant differences between preparation types. Simple oil removal resulted in no significant changes in the relative abundances of individual monosaccharides but increased the relative carbohydrate content of the sample. Protein precipitation removed hemicellulose from the sample, as indicated by a decrease in the relative abundance of arabinose. Differences between the protein precipitation methods could also be seen. The ratios of arabinose to mannose and arabinose to glucosamine in AP preparations were less than 1, indicating enrichment in protein glycosylation monosaccharides relative to the residual hemi-

cellulose. In contrast, the ESP preparations showed a marked reduction of almost all carbohydrates.

Both the carbohydrate and fatty acid data provide stand-alone differentiating information. However the power of combining these data streams is clearly evident. PCA of the MR and carbohydrate data shows a clear discrimination between the four different preparation types regardless of the castor seed source.

ACKNOWLEDGMENT

Funding for this research was provided through Contracts AGRHSHQDC07X00207 and AGRHSHQDC08X00571/B1 to Pacific Northwest National Laboratory by the Department of Homeland Security, Science and Technology Directorate. Pacific Northwest National Laboratory is operated by Battelle Memorial Institute for the United States Department of Energy under Contract DE-AC06-76RLO.

Received for review March 8, 2010. Accepted June 4, 2010.

AC1006206