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A ricin forensic profiling approach based on a complex set of biomarkers

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ABSTRACT

A forensic method for the retrospective determination of preparation methods used for illicit ricin toxin production was developed. The method was based on a complex set of biomarkers, including carbohydrates, fatty acids, seed storage proteins, in combination with data on ricin and *Ricinus communis* agglutinin. The analyses were performed on samples prepared from four castor bean plant (*R. communis)* cultivars by four different sample preparation methods (PM1–PM4) ranging from simple disintegration of the castor beans to multi-step preparation methods including different protein precipitation methods. Comprehensive analytical data was collected by use of a range of analytical methods and robust orthogonal partial least squares-discriminant analysis- models (OPLS-DA) were constructed based on the calibration set. By the use of a decision tree and two OPLS-DA models, the sample preparation methods of test set samples were determined. The model statistics of the two models were good and a 100% rate of correct predictions of the test set was achieved.

1. Introduction

Ricin is a potent Chem-Bio threat agent, frequently relevant in cases of intoxications and “white powder letter” incidents worldwide [1,2]. The toxin is present in the seeds of the castor bean plant, *Ricinus communis*, and active toxin can be produced from seed material by simple extraction methods. With the toxicity of ricin and the high availability of castor beans, ricin has become a threat of concern for the society. The castor plant grows wild in tropical and subtropical climates and the plant is cultivated in large scale for the commercial production of castor oil, a major component of the castor bean, with almost two million tons of seeds harvested annually [3]. Castor oil has a high resistance to thermal degradation which makes it valuable in various industrial applications. Also, the striking appearance of the plant makes it popular as an ornamental, and the seeds are commercially available at garden shops. During the cold war, ricin was included in the chembio-weapons programs of several states. Therefore, ricin was classified as a Schedule 1 controlled substance under the Chemical Weapons Convention, and the implementation of the Convention in the national legislation of the 192 signatory states (June 2017) makes undeclared ricin purification a crime globally [4]. Thus, castor beans are an unregulated agricultural product but the intentional purification of the toxin, present in the seed's pulp, is regulated in U.S. federal law and in the national law of most other countries. The production of ricin from castor beans has been described both in scientific and anarchist literature, and on the internet numerous hits related to the topic can be found. The technical level of the methods varies from simple processes where the seeds are mashed with or without extraction of residual oil [5–7] to multi-step procedures including protein precipitation and chromatographic purification techniques [8–12]. The major components of castor beans include lipids, proteins and carbohydrates, whereas ricin constitutes about 1% of the seed dry weight. Ricin preparation procedures involve processes to enrich the toxin by removal of the bulk components. This induces dramatic changes in the composition of a ricin sample compared to an unpurified seed mash [13]. The seeds have a hard protective coat and many purification protocols starts with removal of the seed coat to access the pulp and castor oil. Castor oil, rich in ricinolein, the triglyceride of ricinoleic acid, is a dominating component of the pulp and toxin purification usually starts with the removal of the oil by solvent extraction. Further steps may involve aqueous extraction of the toxin from the mashed seed pulp followed by protein precipitation. Ricin and the closely homologous lectin *R. communis* agglutinin (RCA) can be separated from other highly abundant proteins in the precipitate by affinity purification [12].

Forensic analysis of samples suspected to contain ricin has mostly been focused on screening, detection and verification of the presence of ricin (*e.g.* in “white powder samples”) [14,15]. The methods often involve trypsin digestion and mass spectrometric analysis of the resulting

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peptides. Unambiguous identification of ricin requires MS/MS-based methods, giving detailed amino acid sequence information of ricin-specific peptides, strictly distinguishing them from other proteins including RCA [10]. In addition, methods for chemical analysis of forensic attribution markers related to the purification of ricin, have been the focus of a number of studies. The markers studied include residual solvents [16], carbohydrates and ricinoleic acid since they are linked to the production process of ricin [17]. The residual solvent analysis focuses on traces of the reagents (*i.e.* solvents) used for ricin preparation, while the latter methods use the level of ricinolein and polysaccharides in the sample as markers for purification method efficacy. The alkaloid ricinine [18] and a set of highly crosslinked peptides, the *R. communis* biomarkers (RCB's), are also potential attribution markers for ricin [19].

Acquisition of comprehensive analytical data by multiple techniques is a common approach to monitor changes in the properties or composition of complex samples. Integrated analytical platforms including GC-MS, LC-MS and NMR offer sensitivity and reproducible detection to monitor biological processes such as disease development and plant responses [20,21]. The multi-omic approach has been applied to research questions across the fields of medicine, molecular biology and plant physiology [22–24]. Integrating analytical information of a broad array of attribution markers; low molecular weight compounds as well as metabolite and protein marker profiles, provides for improved tools to detect intentional toxin production and to differentiate between production techniques. Such methods would also open up for association of different ricin samples, *e.g.* samples produced by the same method and relating a site or illegal toxin production method to a threat or use of ricin [25].

This study, a collaboration between FOI CBRN Defence and Security (FOI) and Pacific Northwest National Laboratory (PNNL), sought to develop a comprehensive strategy for forensic ricin analysis. The approach was to acquire data on the composition of ricin samples produced by four different preparation methods using a number of different analytical techniques, and to apply multivariate statistical methods to evaluate the complex chemical analytical information. The statistical methods would support the conclusion whether a crime was committed, *i.e.* if the toxin was enriched and purified. In addition, the analysis would reveal details of forensic interest regarding preparation methods as well as information on the seed material that could be linked to a specific case.

2. Experimental

2.1. Plant material

Castor seeds from four selected cultivars of *R. communis* were exchanged between FOI and PNNL (Table 1).

2.2. Preparation of "illicit samples"

Ricin extracts of the cultivars were prepared in duplicate using four preparation methods, PM1–PM4, selected from the scientific literature, clandestine publications and internet sites (Table 2) in accordance with Wunschel et al. [13]. They range from simpler methods using normal household equipment to more technical procedures for extracting ricin.

Table 1
The *R. communis* cultivars used for comparative analysis on extraction methods.

Cultivar #	Cultivar	Type	Source
#1	Sanguineus	Ornamental	Rara växter, Ornö, Sweden
#2	Impala	Ornamental	Sandeman Seeds, UK
#3	TMVCH 1	Oil seed	Asian Green Fields, Tamil Nadu, India
#4	GCH4	Oil seed	Asian Green Fields, Tamil Nadu, India

The performance of the preparation methods, in terms of ricin purity, varies from the simple PM1 to a more technically demanding PM4. However the purification efficiency of a method depends on a number of factors. The selected procedures were adapted for laboratory use and are briefly described below.

Subsamples from the different preparations were analyzed for proteins, protein markers as well as the content of carbohydrates, fatty acids and solvent residues. In Fig. 1a flowchart of the sample preparation procedures and adopted analytical techniques is presented. Compound classes associated with each of the sample fractions are indicated.

2.3. Chemical analysis

2.3.1. Derivatization reagents and materials

Sodium hydroxide solutions were prepared from sodium hydroxide pellets (Sigma-Aldrich, St. Louis, MO, US). For carbohydrate and fatty acid derivatization, acetone, methyl-tert butyl ether (MTBE), boron trifluoride, methanol, hexane, sulfuric acid, sodium borodeuteride, acetic acid, ammonium sulfate and chloroform were obtained from Sigma-Aldrich at ACS reagent grade (St. Louis, MO, US). Acetic anhydride was obtained from Supelco, Inc. (Bellafonte, PA, US). The solid-phase extraction (SPE) was performed using C-18 SPE cartridges obtained from Phenomenex (Torrance CA, US), and Chem-Elut SPE cartridges purchased from Perkin-Elmer (Waltham, MA, US). The carbohydrate and fatty acid standards listed below were obtained in the highest purity available from Sigma-Aldrich (St. Louis, MO, US).

2.3.2. Carbohydrate determination

The analysis of monosaccharides was performed using the alditol acetate method for hydrolysis, reduction and acetylation of the liberated sugars as used previously [17,26]. Briefly, triplicate 10 mg samples of each PM were prepared by first performing hydrolysis under a nitrogen atmosphere in 1 M sulfuric acid at 100 °C for 12–16 h. Following hydrolysis, samples were neutralized in N,N-dioctylmethylamine:-chloroform solvent and the aqueous extract reduced using sodium borodeuteride. Following evaporation of borodeuteride after addition of methanol and acetic acid, the reduced monosaccharides were acetylated with acetic anhydride and suspended in chloroform for GC-MS analysis. Nineteen monosaccharide standards were derivatized with each sample batch in known concentrations to use in quantifying the corresponding monosaccharides in each sample. Those included the neutral sugars fucose, rhamnose, ribose, arabinose, deoxyglucose, xylose, pinitol, *chiro*-inositol, *myo*-inositol, *allo*-inositol, *muco*-inositol, *scyllo*-inositol, mannose, D-glycero mannoheptose, galactose, and glucose as well as the amino sugars N-acetyl glucosamine, N-acetyl galactosamine and N-acetyl mannosamine. Due to the reduced and acetylated form of the final derivatives, amino sugars were all reported as their acetylated form. Two internal standards were also added to each sample and standard mixture to aid quantification, methyl glucose was added as a quantification standard for neutral sugars and N-methyl glucamine as an internal standard for amino sugars. Data was collected using an Agilent Technologies (Santa Clara, USA) 7890A Gas Chromatography Mass Spectrometry (GC-MS) equipped with an Rtx-5 MS column (Restek, Bellefonte, USA). A 1 µL sample injection was used in a 10:1 split mode.

2.3.3. Lipids determination

Analysis of fatty acid content focused on the measurement of ricinoleic acid following derivatization and GC-MS as described earlier [13,17]. Briefly, a 10 mg sample was saponified in sodium hydroxide at 70 °C for 2 h. The liberated acid monomers were acidified and extracted into MTBE. The methylation reaction was performed in methanol and 3% boron trifluoride for 2 h at 100 °C followed by extraction into hexane and evaporation. Samples were reconstituted in methanol for analysis. Two 50 µg aliquots of a ricinoleic standard were also