Protein Fingerprinting Technology for Detecting Stored-product Insect Fragments in Wheat Flour

A Thesis

Submitted to the Faculty of Graduate Studies

The University of Manitoba

In partial fulfillment of the requirement for the degree of

Master of Science

by

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Dedicated to my grandparents and parents

ABSTRACT

Insect contamination of grain products is one of the main factors that causes serious decrease in quality of processed products. We have developed a rapid detection method for insect parts in wheat flour, based on special peptides of insects, as these peptides gave a consistent response for a very broad range of insects. Five of the most common insects of stored products, *Cryptolestes ferrugineus* (Stephens), *Rhyzopertha dominica* (F.), *Sitophilus oryzae* (Linnaeus), *Tribolium castaneum* (Herbst), and *Tribolium confusum* (Jacquelin du Val), were investigated in this experiment; *T. castaneum* is the predominant insect in flour. In this research, an Agilent 1100 reversed phase high performance liquid chromatograph (RP-HPLC) was used for peptide purification and fractionation. Matrix-assisted laser desorption/ionization (MALDI) was used to read the RP-HPLC fractions, and the spectra were analyzed by an in-house software developed by the Time-of-Flight Lab in the Department of Physics and Astronomy at the University of Manitoba. Initially, three protein purification buffers, 50% acetonitrile; sterilized water; and the buffer of 10 mM imidazole pH 7.0, 1 mM phenylthiourea, 1% Triton X, and protease inhibitor cocktail mixture (PTU buffer), were compared. Later, *T. castaneum* (red flour beetle) protein identification and peptide spectra mapping were done using the protein fingerprinting technique. Peptide spectra mapping method, which could set up biomarkers for each type of insect, was proven to

be a more effective and practical way to detect insect contamination. This peptide mapping technique could obtain 55 stable biomarkers of *T. castaneum*, when *T. castaneum* was 0.1% in wheat flour by mass. This level needs to be lowered further during future research.

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LIST OF ABBREVITIONS

RFB	**Red flour beetle**
FDA	**Food and Drug Administration**
C. ferrugineus	***Cryptolestes ferrugineus***
R. dominica	***Rhyzopertha dominica***
S. oryzae	***Sitophilus oryzae***
T. castaneum	***Tribolium castaneum***
T. confusum	***Tribolium confusum***
RP-HPLC	**Reversed phase high performance chromatography**
MALDI	**Matrix-assisted laser desorption/ionization**
TOF	**Time-of-flight**
NIRS	**Near-infrared spectrometry**
ELISA	**Enzyme-Linked Immunosorbent Assay**
m/z	**mass-to-charge ratio**
RCB	**Randomized complete block**
CV	**Coefficient of variation**
CBDs	**Chitin-binding domains**
AACC	**American Association of Cereal Chemists**
Si	**Silicon**

InGaAs	**Indium-gallium-arsenide**
FT-NIR	**Fourier transform near infrared**
PLS	**Partial least squares**
SECV	**Standard error o cross-validation**
CWRS	**Canada Western Red Spring**
PCR	**Polymerase chain reaction**
KCl	**Potassium chloride**
$MgCl_2$	**Magnesium Chloride**
$NaHCO_3$	**Sodium bicarbonate**
MS	**Mass spectrometry**
DTT	**Dithiothreitol**
NH_4HCO_3	**Ammonium bicarbonate**
TFA	**Trifluoroacetic acid**
ESI	**Electrospray ionization**
FAB	**Fast atom/ion bombardment**
DHB	**Dihydroxybenzoic acid**
PVDF	**Polyvinylidene fluoride**
NCBI	**National Center for Biotechnology Information**
SDS	**Sodium dodecyl sulfate**

RT	**Retention time**

INTRODUCTION

Stored-product insects cause considerable damage in stored grains and their products. The American Food and Drug Administration (FDA) (1988) regulates a defect action level of insect contamination of 75 insect fragments (> 0.2 mm) per 50 g of wheat flour. In Canada, this level has been set to less than 20 fragments in each of 3 test samples of 50 g wheat flour (Evaluation Division 1999). Most internal infestations (insects inside kernels) are invisible, and insect sizes, species, and life stages vary greatly. Therefore, it is important to develop a detection method that is not limited by insect properties, and is able to detect minute quantities of insect fragments within the stored products. Several detection methods have been studied or used in industry, such as the flotation method, Iodine method, near-infrared spectrometry (NIRS), soft X-ray method, DNA fingerprinting, and immunoassay method (Morris 1946; Quinn et al. 1992; Schatzki et al. 1993; Belay et al. 1997; Dowell 2000; Jayas et al. 2000; Paliwal et al. 2001; Karunakaran et al. 2003; Balasubramanian 2005; Perez-Mendoza et al. 2005; Atui et al. 2007). Nevertheless, these methods all have different disadvantages. The flotation method is less sensitive and more harmful to the environment (Balasubramanian 2005); the Iodine method is affected seriously by different iodine concentrations, and needs extra precautions to decide and control the iodine concentration (Morris 1946); the NIRS is less precise than other methods (Perez-Mendoza et al. 2005); the soft X-ray method requires large computational power, and has limitations in being operated with fast processors (Jayas et al. 2000; Paliwal et al. 2001); detection ranges of the DNA fingerprinting method are not as

sensitive as the FDA regulation (Balasubramanian 2005); and the immunoassay method is unstable depending on the myosin degradation (Atui et al. 2007). Therefore, we proposed to develop a protein fingerprinting technique to detect insect parts in wheat flour. This method involves in identifying protein characteristics of insects and wheat flour, various technologies of proteomics, especially for identifying insect proteins. The five most common insects in grain storage and processing are: *Cryptolestes ferrugineus* (Stephen), *Rhyzopertha dominica* (F.), *Sitophilus oryzae* (Linnaeus), *Tribolium castaneum* (Herbst), and *Tribolium confusum* (Jacquelin du Val). *Tribolium castaneum* and *T. confusum* are most common in flour.

Insect protein tends to be low in the amino acids methionine and cysteine. It contains high levels of lysine and threonine, one or both of which might be deficient in wheat, rice, cassava and maize-based flours. Consequently, insect detection methods based on special proteins could give a consistent response for a very broad range of insects. Therefore, the objective of this study was to assess the capability of a protein fingerprinting technique combined with mass spectrometry to detect insect proteins present in wheat flour, which indicates contamination by insects.

LITERATURE REVIEW

Choice of insect species

Flour beetles of the genus *Tribolium* belong to the family Tenebrionidae. *Tribolium* species are the most common pests in flour mills causing considerable financial losses (Hill 2002). Cranshaw and Peairs (2005) indicated that flour most

commonly was infested by either of two closely related beetles, the confused flour beetle, *T. confusum* and the red flour beetle, *T. castaneum*. A survey of 1019 flour mills from 1969-1981 in five cities of Western Canada namely Armstrong, Lethbridge, Medicine Hat, Saskatoon, and Winnipeg reported that 25% of flour mills were infested with *T. castaneum* and 20% with *T. confusum*. *Tribolium* sp. was followed by the occurrence of *S. oryzae* (rice weevil), *C. ferrugineus* (rusty grain beetle) and *C. pusillus* (Schönherr) (flat grain beetle). As well, *S. oryzae* and *R. dominica* (lesser grain borer) were described as two of the most destructive pests of stored grain in the world by Sinha and Watters (1985). In Canada, Smith and Barker (1987) found *C. ferrugineus* in the grain residues from 11.9% of 1,752 farm bins on 296 farms across Western Canada. They found *T. castaneum* in the grain residue from 2.3% of the grain bins. In samples of 1.0-1.5 L each, a mean of 0.1 *C. ferrugineus* adults and 0.02 *T. castaneum* adults were reported. Five common stored-product insects were chosen for the present study. They were *C. ferrugineus, R. dominica, S. oryzae, T. castaneum,* and *T. confusum.*

Wheat protein

Wheat is of great importance nutritionally to many people of the world. Wheat grain protein plays a fundamental part in food processing, for instance, in bread manufacture, biscuits, breakfast cereals and pasta products (Payne and Rhodes 1982). The protein content of cereal grain is low and for wheat is normally between 9% and 16% of the dry weight (Payne et al. 1984). Šimić et al. (2006) set up field trials to grow wheat cultivars on eutric cambisol soil at Osijek using a Randomized Complete Block (RCB) design in 4 repetitions during 7 growing

seasons. There were 10 wheat cultivars (Žitarka, S. Žitarka, Barbara, Ana, Demetra, Srpanjka, Golubica, Monika, Klara and Hana) from the Agricultural Institute, Osijek. The crude protein content on a dry matter basis was measured by NIT technology (Infratec 1241, Foss Tecator, France). According to their research, the mean total protein content in the wheat samples for a six year period (1997-2002) varied between 12.4% (cv. Ana) and 14.3% (cv. Golubica) (Table 1). In 2004, protein content varied from 13.8% (cv. Ana) to 15.9% (cv. Golubica) (Table 1).

Table I: Protein content of wheat cultivars (Šimić et al. 2006)

Wheat cultivars	Protein Content (%) Years 1997-2002	Protein Content (%) Year 2004
Žitarka	13.2	14.2
S. Žitarka	13.6	14.8
Barbara	12.4	13.8
Ana	12.7	14.2
Demetra	13.5	15.8
Srpanjka	14.3	15.9
Golubica	14.2	14.5
Monika	13.4	14.5
Klara	13.4	14.5
Hana	12.9	14.2
CV (%)	8.60 (n=60)	5.10 (n=10)

CV represents Coefficient of Variation.

There was a significant difference in the amount of protein among different cultivars from the same year. There was a significant difference in the means of the protein content among the 6 years as well. Therefore, the cultivars of wheat flour need to be reported in a study dealing with wheat protein identification.

Insect protein

Stored-product insects infest processed grain and processed cereal products (Cox 1991). Although insects possess relatively much lower proteins than stored-products, because of their small numbers in bulk grain, they have some distinctive proteins that cannot be found in grains.

Chitin is a homopolymer of b-1, 4-*N*-acetyl-Dglycosamine. It is an important component of the cell wall of fungi and of the exoskeleton of arthropods (Kramer and Koga 1986). Chitin-binding proteins can also be detected in higher plants, which exist most frequently as chitinases and lectins (Shen and Lorena 1999). From the observations of Shen and Lorena (1999), plant and invertebrate Chitin-binding domains (CBDs) shared a common core structure and chitin-binding mechanism while being unrelated in evolution; however, convergent evolution was highly suggested to cause their differences. Similarly, convergent evolution was also noted for trypsin and subtilisin (Rawlings and Barrett 1994). These proteases shared the same catalytic triad while bearing no other significant similarity in amino acid sequence or in three-dimensional structure.

Myosins are a large family of motor proteins found in eukaryotic tissues. They are responsible for actin-based motility. Moore et al. (2000) described that myosin, the molecule that interacted with actin to produce force and movement, consisted of two heavy chains (~220 kDa) and four light chains (~20 kDa). Flinn and Hagstrum (2001) said that insect myosin was a better indicator of insect contamination than insect fragments. Moreover, Belay et al. (1997) mentioned that

the insect fragment procedure had a number of drawbacks for grain and flour analysis. For example, dead adult grain insects can yield as many as sixty times the number of fragments as live larvae (Sachdeva 1978). Myosin has evolved very slowly and thus differs little from insect species to insect species (Emerson and Bernstein 1987). Consequently, the insect detection methods based on myosin give a consistent response from a very broad range of insects. Hammond and Goll (1975) obtained the result that the protein isolated from insect muscle could be identified with a-actins by its solubility properties, mobility on gels, amino acid composition and elution pattern from DEAE-cellulose columns, although the very small amounts of protein that could be obtained from insect fibril muscle (0.5-2 mg from 5-20 g of muscle) precluded its identification as a 6S peak in the analytical ultracentrifuge. The immunoassay method involves the sandwich Enzyme-Linked Immunosorbent Assay (ELISA), which also aims to bind to the protein myosin using antibodies. In conclusion, myosin is the most representative and practical protein for protein identification and quantization among insects and wheat flour, which should be employed primarily in the current research.

Protein contents comparison between wheat kernels and insects

Wheat protein is normally between 9% and 16% of the dry mass of a kernel (Payne et al. 1984). The acceptable 1000-kernel weight for durum wheat is 35-40 g (Abaye et al. 1997). Therefore, one wheat kernel is around 35-40 mg, and its protein content is around 3.15 mg to 6.4 mg. After sampling and measurement, we roughly gained the gross weight of each single insect of the 5 species used in this experiment (Table II). Water possesses 50%-90% of the insect body weight

(Feltwell 1981). DeFoliart (1992) investigated the insect protein contents in village markets of the developing world, and found that insects were mostly high in crude protein, with many species approaching above 60% of their dry mass. So that dry mass and protein contents of the 5 species can also be estimated (Table II)

Table II: Gross weights, dry mass and protein contents of *T. castaneum, T. confusum, C. ferrugineus, R. dominica and S. oryzae.*

Species	*T. castaneum*	*T. confusum*	*C. ferrugineus*	*R. dominica*	*S. oryzae*
Weight (per insect)	1.73 mg	2.32 mg	0.21 mg	1.35 mg	1.65 mg
Dry mass (mg)	0.173-0.865	0.232-1.16	0.021-0.105	0.135-0.675	0.165-0.825
Protein content(mg)	0.104-0.519	0.139-0.696	0.013-0.063	0.081-0.405	0.099-0.495

Comparing with the estimated protein contents of wheat kernel (3.15 – 6.4 mg/kernel), protein contents of insects are much lower. Thereby, the insect detection method based on protein identification needs to be highly sensitive.

Identification methods used to detect insect presence in wheat flour

1. Flotation method (AACC method 28-22A and 28-51A)

This method is to soak ground grain sample with chloroform, and examine the sample on a wide field microscope, after Buchner funnel filtration and

saturation procedures (AACC 1995). This method was used by Glaze and Bryce to extract light filth from whole-wheat flour (Glaze and Bryce 1994). Thind (2000) modified the flotation method and examined the mite and insect contamination in food and feedstuffs with it. The flotation method is considered to be efficient in dealing with large quantities of samples, very sensitive at the FDA action level and costs less than other methods. However, it has limitations in the analysis of finely powdered materials, and is considered to be harmful to the environment, because of evaporation of the chloroform (Thind 2000; Perez-Mendoza et al. 2002; Balasubramanian 2005).

2. Iodine method (AACC method 28-44)

The iodine method is to sieve wheat flour, digest the remaining portion that does not pass through the sieve with alcohols and hydrogen sulphate, and then use iodine to stain the filter paper, when the samples are going through it. The stained paper is washed and examined under 20 X magnification under a microscope. Insect eggs are not stained, but the amylase in the starch produces a blue violet colour with iodine; the insect eggs then could be counted under the microscope (AACC 2000). Leelaja et al. (2007) used this method to compare and develop stained materials for distinguishing eggs of stored-product insects and refined wheat flour particles easily. This method had the advantages of using relatively smaller amounts of solvents, which was safer to operators, and stable under various temperatures (Morris 1946; Davis and Diener 1979). However, the AACC (2000) method did not perform distinct color differences between insect eggs and the flour particles (Leelaja et al. 2007). The comparison results also varied widely,

according to different iodine concentrations. Extra precautions should be paid to decide the iodine concentration and prevent evaporation (Morris 1946).

3. Near-infrared spectrometry

Perez-Mendoza et al. (2005) studied the use of near-infrared spectrometry to detect insects. They mixed 200 unsexed lesser grain borer adults with 200 g of whole kernel, hard red winter wheat, *Triticum aestivum* L. (2002 crop), adjusted to 13.5% moisture content by adding distilled water as needed, in 800-ml glass jars capped with screened lids. Jars were held in a rearing chamber at 30 ±1°C and 70 ± 5% RH with a photoperiod of 12:12 h (L: D). All found adults were removed, and the remaining immature insects were killed by maintaining the temperature of the infested wheat at 130°C for 30 min. After cooling to room temperature, desired numbers of infested kernels with each life stage were added to batches of normal wheat to complete 100 g samples. The samples were milled in the laboratory with a milling efficiency of 60%, in order to produce 60 g flour. Three near-infrared spectrometers were used to collect spectral data from wheat flour samples containing varying levels of insect infestation: 1) Perten Diode Array (DA) 7000 (Perten Instruments Inc., Springfield, IL), 2) Cognis-QTA Bruker Optics FT-NIR (Cognis, Cincinnati, OH), and 3) Foss NIRSystems 6500 (Foss NIRSystems, Silver Spring, MD). The DA 7000 was able to collect absorbance spectra over a range of 400-1690 nm. It used both silicon (Si) and indium-gallium-arsenide (InGaAs) photodiodes with a high-intensity tungsten halogen lamp. The QTA system was based on Bruker Optics's MATRIX Fourier transform near infrared (FT-NIR) interferometer. A PbS detector was used to collect spectra in the 12,000-4,000 cm^{-1}

(830-2500 nm) wavelength range every 8 cm^{-1}. The Foss NIR Systems 6500 scanning monochromator was able to collect spectra from 400 to 1098 nm (visible to short-wave NIR regions) with a silicon detector and from 1,100 to 2,500 nm (NIR region) with a PbS detector. The results of NIR spectrometry technology were analyzed by partial least squares (PLS) regression (Martens and Naes 1989) by using PLSPlus/IQ software (Galactic Industries 2003). The potential of the NIRS technique was evaluated by the coefficient of determination (r^2), standard error of cross-validation (SECV), which was used to determine the "best" number of independent variables in building a calibration equation, and beta coefficients. Beta coefficients indicated the wavelengths (positive and negative peaks in the plot) that were more heavily weighted. Their plots could be compared with NIR absorption spectra of specific flour and insect fragment components, such as protein, starch, lipids, or chitin to decide which components cause unique NIR absorptions between flour samples with and without insect fragments (Dowell 2000). Perez-Mendoza et al. (2005) found that NIRS was a rapid and nondestructive method to detect and quantify the number of insect fragments. It could be operated easily for a sophisticated sampling protocol for large flour bulks, requiring less extensive sample preparation. However, NIRS was less precise in comparison with other methods (e.g., standard flotation method and visual inspection method).

4. Soft X-ray method

A soft X-ray method was employed to detect internal seed infestations by the rice weevil, *S. oryzae* in Canada Western Red Spring (CWRS) wheat

(Karunakaran et al. 2003). The infested kernels were identified by the presence of egg plugs and X-rayed every 5 to 7 d until the adults emerged from the kernels. A total of 57 features using histogram groups, histogram and shape moments, and textural features were extracted from the X-ray images. A four-layer back propagation neural network correctly identified 99% of sound kernels and classed 1% as infested. About 97% of wheat kernels infested by larvae and all kernels infested by pupa and adult stages were correctly identified as infested. The X-ray imaging system consisted of i) Lixi fluoroscope (Model: LX-85708, Lixi Inc., Downers Grove, IL), ii) CCD black and white camera (Sony XC-75/75CE), iii) black and white monitor, iv) image digitizer (Dazzle digital video creator, Dazzle Multimedia Inc., Fremont, CA), and v) personal computer (5300 series, Compaq Computer Corporation, Houston, TX). This was a 25 mm diameter detection system with a resolution of 62.5 μm. Grain kernels were placed manually on Saran Wrap on the sample platform and single kernels were X-rayed (15 kV and 65 μA) for 3 to 5 s at a time. The X-ray images were digitized as 8-bit images at a resolution of 60-pixels/ mm and saved as grey scale images. Soft X-ray method was advanced in detecting internal infestations in raw grain. It had the advantages of non-destruction, high accuracy, the abilities of detecting both internal and external insects, and detecting both live and dead insects inside grain kernels. However, it can not detect insect eggs, and it needed plenty of computational power to detect infestations, according to shape, size and colour variation of products and insect species. (Karunakaran et al. 2003; Neethirajan et al. 2007).

5. DNA fingerprinting

Deoxyribonucleic acid (DNA) fingerprinting technology was used to detect insect fragments in commercial and lab milled wheat flour (Balasubramanian et al. 2007). They used the high salt extraction method to extract DNA of pure insects and flour samples, and then insect nuclear primers were used to identify the specific primer for red flour beetle and confused flour beetle. The primers were verified for their specificity against other commonly occurring stored pests like *C. ferrugineus, C. pusillus, R. dominica and S. oryzae.* In addition, Promega wizard genomic DNA kit was found effective in extracting DNA from single red flour beetle. Polymerase chain reaction (PCR) of the extracted DNA using insect specific primers was successful in amplifying the elongated factor 1-alpha gene. The method was adopted to extract DNA from a mixture of flour and insects and they found that DNA fingerprinting technique was able to identify red flour and confused flour beetle contamination at a 1.0% level in both commercial and lab milled flour. However, it could not successfully identify a single insect leg added to the flour and the six different species crushed and mixed equally and added at different percentage (< 1.0 %) to the flour.

6. Immunoassay method

Quinn et al. (1992) worked on the immunoassay method. This method involved the sandwich Enzyme-Linked Immunosorbent Assay (ELISA), which used antibodies to bind to the protein myosin. The materials in their experiment were 50 g wheat flour and 75 mL extract buffer (0.1 M potassium phosphate pH

7.0, 0.5 M sodium chloride and 0.05% tween-20). Fifty millilitre of the supernatant was taken after centrifugation and then was placed into a microwell strip coated with 50 μL of antibody and was incubated for 18 min. During detection, myosin at various dilutions was used as the standard and the buffer was used as a blank, both of which were run along with the samples for comparison. The strip needed to be washed for 25 min and the colour would emerge in the wells. A plate reader (Multiscan Plus, Labsystems, and Needham Heights, MA) was employed to measure the absorbency of the colour. Quinn et al. (1992) found the sensitivity of ELISA was high in measuring low nanogram quantities of insect myosin, and to detect a wide variety of common insect pests of stored grain. Their quantification results showed that the assay can detect as little as one granary weevil per 50 g of grain and can detect adult, pupae, and larval life stages, but not eggs. Enzyme-Linked Immunosorbent Assay was considered to be highly specific, rapid, and less expensive than the insect fragment count test (Quinn et al. 1992; Schatzki et al. 1993; Belay et al. 1997). However, the myosin degradation made this method relatively unstable. It was advised to test wheat flour samples using this method immediately, or refrigerate them as soon as possible after milling (Atui et al. 2007).

Protein purification

Protein extraction is the initial step to do protein analysis for protein fingerprinting. The insects are needed to do protein purification separately for quantification, and the mixture of wheat and insect flour should follow a similar procedure. Ausubel and Frederick (1990) proposed the following factors to be considered when determining how a protein or protein fragments should be isolated:

(1) The amount of a protein in the available starting material.

(2) The cost of preparing the starting material (e.g., cell culture, fermentation, or organs) and the cost of labour.

(3) The molecular size of the protein.

(4) Physical properties of the protein.

Furthermore, protein purification procedures usually have the following stages:

1. Making a solution containing the desired protein.
2. Dealing with mixtures of proteins and other molecules in the original solution.
3. Generating a nearly homogeneous product.
4. Removing minor contaminants.

Gressent et al. (2003) described that they ground 1 g of insects with a mortar and pestle in liquid nitrogen, resuspended the resulting powder in 2 mL of extraction buffer (20 mM Tris-HCl pH 8; 0.25 M sucrose; 2 mM $MgCl_2$). The slurry was centrifuged for 2 min at 1000 *g*, and the supernatant was stored at -20 °C until required. All the operations were performed at 4°C. Campbell (2005) homogenized individual adult *Drosophila* with 50 μL of water in 1.5 mL microcentrifuge tubes, centrifuged the homogenates at 15 000g for 5 min, and stored the supernatant at –20°C. Dr. Y. W. Qian in the Time-of-Flight lab in the Department of Physics of the University of Manitoba, suggested the use of 50%

acetonitrile as the protein extraction buffer. Meinrenken (1969) resuspended the sediment myofibrils from 5-20 g of insect muscle in 2 volumes of the solution to desensitize fibres, with added dithiothreitol: 0.05 M KCl, 20 mM Tris-HCl (pH8.0) and 2 mM dithiothreitol. The suspension was then dialysed against the solution for 20 h, centrifuged at 10 000 g for 30 min and the residue was re-extracted for about 10 h with 1 volume of the solution. Hartshorne and Mueller (1968) used another method to deal with the myofibrils from 5-20 g of insect muscle. The extraction was done using 1 M KCl, 2 mM dithiothreitol, pH7.0, and then was dialysed for about 16 h against 0.2 M KCl, pH 6.5-7.0 and centrifuged at 50000 g for 30 min to remove paramyosin. Bullard et al. (1973) extracted insect myosin from washed myofibrils with Hasselbach-Schneider-Zebe solution, which included 1 M KCl, 0.01 M sodium pyrophosphate, 1 mM $MgCl_2$ and 0.02 M potassium phosphate buffer (pH 6.5). In their experiment, three volumes of the above solution were used to sediment myofibrils from 5-25 g of insect muscle for 10-15 min and the residue was removed by centrifugation at 10000 g for 30 min. The floating lipid needed to be removed by filtering through glass wool, and the supernatant was dialysed for about 16 h against 0.25 M KCl, adjusted to pH 6.5 with 10 mM $NaHCO_3$. Actomyosin could be gained by centrifuging at 50000 g for 30 min. Again the supernatant was dialysed for 16 h against 0.03 M KCl, adjusted to pH 6.5 with 10 mM $NaHCO_3$, and the precipitated myosin was spun at 1000 g for 30 min, resuspended in a small volume of 2 M KCl (adjusted to pH 7.0 with 10 mM $NaHCO_3$) and diluted to make the final KCl concentration 0.5 M. Two-millimolar dithiothreitol or 14 mM-2-mercaptoethanol was adopted in all the solutions used

for preparing myosin. Finally, myosin was purified by centrifuging at 50 000 g for 1 h. Then they found that the average yield of insect myosin from this method was 2.3 mg from 1 g of muscle. Insect actin could be purified by gel filtration through Sephadex G-200 (Rees and Young 1967). They loaded sample fractions on the G-200 column, and then used an Amicon apparatus with a PM-10 membrane to do ultrafiltration for concentrating pooled column fractions. The fractions could be concentrated about six times to approximately 2 mg/mL. Concentrated peak fractions were finally polymerized by dialysing against 0.05 M KCl, 1 mM $MgCl_2$, 0.3 mM $NaHCO_3$, pH 7.0. Professor S. Whyard, Department of Zoology in University of Manitoba, recommended using an extraction buffer of 10 mM imidazole pH 7.0, 1 mM phenylthiourea, 1% Triton X-100 and protease inhibitor cocktail.

Krause (2006) pointed out that the gel-based electrophoresis methods had some drawbacks, such as producing significant numbers of false-positive and false-negative interactions as well as being not or limitedly suitable for all kinds of biological samples and for detailed analysis of protein complexes. Therefore, electrophoresis methods for identification and quantification of the insect protein are not delicate enough to be used in the current project. Mass spectrometry has been considered to be a powerful tool for quickly and efficiently probing protein interactions in biological samples. Consequently, it is better to choose a protein extraction buffer that is helpful with skipping the gel electrophoresis step and detecting insect protein with MS directly. After considering the compatibility between each buffer and MS, three extraction buffers were studied in my research.

They were 50% acetonitrile; 100% sterilized water; and the one with 10 mM imidazole pH 7.0, 1 mM phenylthiourea, 1% Triton X-100 and protease inhibitor cocktail (PTU buffer). The most suitable buffer from these three was decided initially in my experiment for using MS to detect insect proteins from wheat flour.

Reduction, alkylation and dialysis

Dithiothreitol (DTT) is a strong reducing agent with a redox potential of -0.33 V at pH 7.0. It is frequently used to reduce the disulfide bonds of proteins and to prevent intramolecular and intermolecular disulfide bonds from forming between cysteine residues of proteins. After reduction, alkylation is generally used to stabilize SH groups with suitable alkylating re-agents such as iodoacetic acid, iodoacetamide, or N-ethylmaleimide (Anfinsen and Haber 1961). Sechi and Chait (1998) demonstrated that cysteine alkylation was crucial for improving the coverage of proteins by proteolysis and mass spectrometry. Following reduction and alkylation, unexpected reagents were removed by dialysis of the reduced-alkylated proteins (Isa 1969).

The protocol of dialysis was described by the University of Arizona, Center for Toxicology, Proteomics Core:

1. Make dialysis buffer, 0.2 M NH_4HCO_3, pH ~ 8.00.

2. Use 8000 M.W.C.O., cut a 3.5-inch piece of dialysis membrane and cut open along seam (for Mini-micro dialyzer) or use premade dialysis frames for Micro-dialyzer.

3. Wash dialysis tubing thoroughly with ddH_2O.

4. Fill the dialysis unit with dialysis buffer and secure the dialysis membrane in place.

5. Place the dialysis unit on stir plate, slowly stir.

6. Dispense protein solutions into wells and dialyze for a minimum of 3 h.

7. After dialysis, dispense equal amounts of the protein solutions into clean 1.5 mL microcentrifuge tubes.

8. These solutions can be frozen at -20°C, indefinitely for use as digestion controls.

Trypsin digest

Calbiochem (2003) described that tryptic digestion of unknown proteins was a standard tool in proteomics for subsequent protein identification or characterization, and it was a significant procedure for making the analysis quick, efficient, reproducible and easy, prior to mass spectrometry (MALDI-MS, ESI-MS) or protein arrays. Mihályi and Szent-györgyi (1952) studied the digestion of myosin in the centrifuge. They found that myosin incubated with trypsin showed a dramatic fall in viscosity; the high rate of the viscosity drop, compared with the very slow formation of non-protein nitrogen, suggested that trypsin digestion reaction formed heavy intermediate products.

Tryptic digestion can be achieved by following the protocols given by the

University of Arizona, Center for Toxicology, Proteomics Core:

1. Make a Trypsin Solution to a concentration of 0.1 μg/μL, 1 h before use and place on ice.

2. If proteins have disulfide bonds, a reduction/alkylation process will be necessary prior to trypsin addition.

3. Add 40 μL DTT solution and incubate at 56°C for 45 min.

4. Remove samples from heat and allow cooling to room temperature.

5. Add 40 μL of 55 mM iodoacetamide solution and incubate at room temperature for 30 min, in the dark.

6. Add trypsin to each tube at a 1:50, (protease: protein) ratio, close the tube and gently flick to mix.

7. Place the samples in 37°C water bath for 2 h.

8. Then add an additional amount of trypsin to each sample, equal to the first addition.

9. Continue to incubate at 37°C for 17-18 h.

10. Add 10 μL of Formic Acid to solution. Alternative: Add 10 μL of 10% TFA to solution

11. Store at -20°C.

High performance liquid chromatography (HPLC)

Rossomando and Deyl (1998) discussed chromatography and high performance liquid chromatography (HPLC) in detail in their book. They indicated that chromatography is the separation of classes or groups of molecules. It requires two phases in principle, one liquid, and the other a stationary phase, which is usually bonded to a solid. The emergent time (elution time), which could be expressed by emergent time = distance traveled / rate (velocity) of travel, is used to measure the property of the packing of the material. The molecules, whose emergent time is short, would be considered not to have interacted with the stationary phase; on the other hand, those having long emergent time do interact. The HPLC, using a pressurized solvent delivery system to pump the mobile phase through the packing and new packing materials, shortens the separation time and increases the detection sensitivity. An HPLC system could be described like this: the sample is introduced by an injector and then is pushed through the analytical column by the constant pumping of solvent (or mobile phase) from the reservoir through the system.

Libera (2001) said that the HPLC utilizing a support of small rigid particles of uniform size could provide a rapid method for separation of many types of molecules of biological interest. It has the potential to become an excellent alternative to conventional techniques of peptide fractionation. The HPLC is also recognized to be an alternative to more cumbersome approaches widely used for measurement of ATPase activity (Samizo et al. 2001), and its high sensitivity reduces the volume of reaction mixture needed. By this method, Libera (2001) was

able to demonstrate differences in the peptide maps of light chains characterized either by a high degree of homology or by the same apparent molecular weight and apparent isoelectric point. The use of the HPLC chromatographic column filled with hydroxyl apatite made possible the purification of the whole myosin molecule (molecular weight about 500000 Daltons) in non-denaturing conditions from relatively complex mixtures. Hydroxyl apatite offers unique selectivity and can be very valuable for separating proteins, which are not well resolved by other methods. Gorbunoff and Timasheff (1984) noted that protein- hydroxyl apatite interactions are a function of the net charge on the protein, whether acidic or basic. Libera (2001) had adopted this column to purify the whole myosin molecule (i.e., heavy chains + light chains) from a complex mixture of proteins under non-denaturing conditions and found that it could be utilized successfully for purifying myosin from those biological tissues in which classical methods were unsuitable to obtain a degree of purity sufficient for enzymatic and structural studies. The reversed-phase high performance liquid chromatography (RP-HPLC) with excellent resolving power is used as a predominant technique for peptide separations and many protein separations both for analytical purposes and for scale up for preparative purification (Neverova and van Eyk 2005; Mant and Hodges 1991). The crude protein extract from insects are full of interference including chemical reagents, insect particles and some unknown contamination. It is efficient to run HPLC purification first followed by HPLC protein separation. The RP-HPLC instrument I used in my research was the Agilent 1100 series (fig. 1).

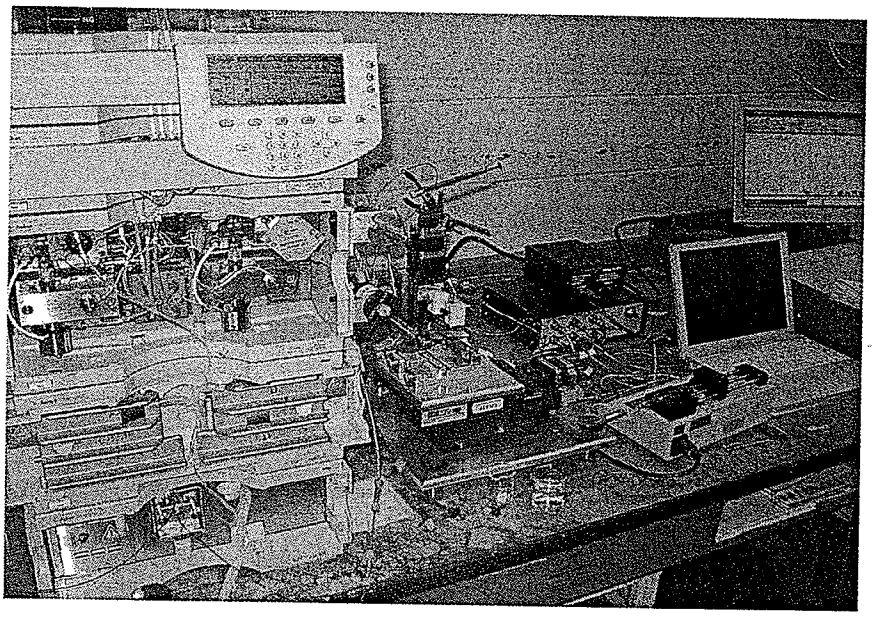

Fig. 1. LC and sample deposition. This picture is taken from the Time-of-Flight lab in the Department of Physics and Astronomy of the University of Manitoba.

Ausubel and Frederick (1990) gave the conditions and facilities for HPLC as follows:

1. Samples should be kept at 4°C for HPLC.

2. Instruments: pump module, mixing chamber, spectrophotometer with preparative flow cell, injection valve, preparative 500 μL sample loop, column oven, computer, printer, and software (e.g., Beckman, system Gold; Hewlett-Packard HP-2090A liquid chromatograph; or Waters 600/486 HPLC system) with attendant data management system automation controllers.

3. Chemicals: Acetonitrile (HPLC grade), methanol (HPLC grade), acetone (HPLC grade), thiourea, Milli-Q-purified water, helium, and nitrogen (for the auto sampler).

System preparation and operation procedures for Agilent 1100 series RP-HPLC instrument are as follows (Krokhin et al. 2004):

1. Five μL sample injected into 150 μm × 150 mm column
2. Linear gradient of 1-80% acetonitrile (0.1% TFA) in 120 min (0.66 % gradient)
3. Four μL/min effluent mixed with DHB matrix (0.5 μL/min)
4. Deposited in 30 s fractions onto movable MALDI target using a robot (fig. 2)
5. Fractions air dried for archiving and later analysis

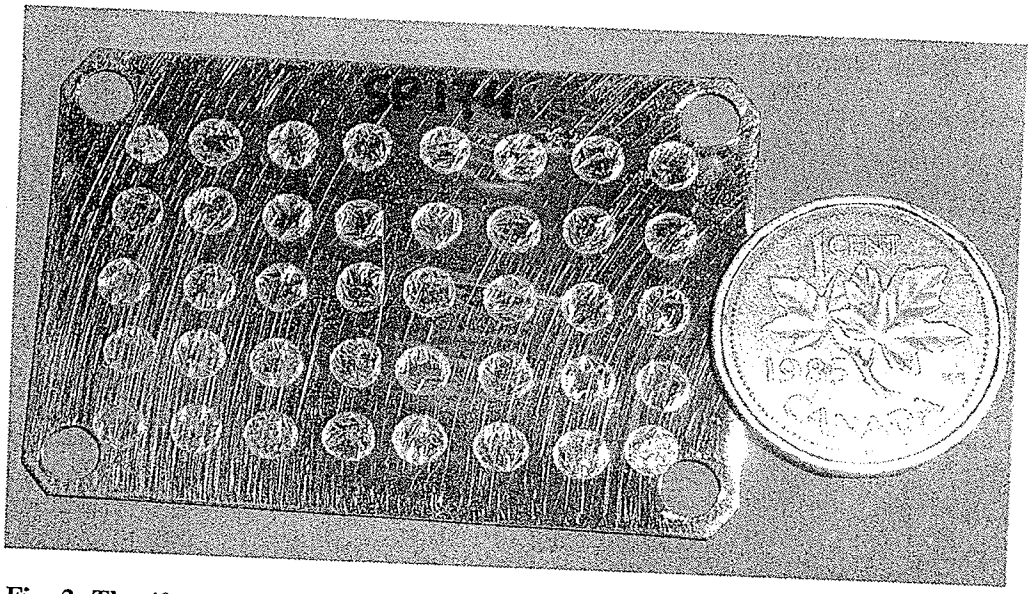

Fig. 2. The 40 spot MALDI target (1 min of HPLC elution per spot). This picture is taken from the Time-of-Flight lab in the Department of Physics and Astronomy of the University of Manitoba.

Mass spectrometry (MS)

Mass spectrometry has become an important tool in biochemical research. Siuzdak (1996) identified the mass spectrometer as an analytical device that determined the molecular weight of chemical compounds by separating molecular ions according to their mass-to-charge ratio (m/z). The most commonly employed methods for ionization of bioorganic compounds are matrix-assisted laser desorption/ionization (MALDI) and electrospray ionization (ESI) and fast atom/ion bombardment (FAB). Peptides are ionized easily through protonation, because of the presence of amide and amine groups. Acids in peptides could also determine that peptides would be observed in negative ion mode by deprotonation. Good solubility is the essential condition for all three methods.

The ESL was more sensitive than FAB, but it needed constant monitoring from sample to sample to ensure that the observation ions were not carried over from a previous sample. MALDI was useful when too many samples were used before, or the instrument was particularly sensitive to a previously analyzed compound, because the probes of MALDI were relatively easy to clean, and sensitivity was not sacrificed with MALDI. Electrospray had low tolerance to salt and detergents, with contamination less than millimolar. Its signal was more susceptible to the contaminations and would result in loss of sensitivity. However, MALDI was known for giving good signals even in the presence of salt and impurities. In modern studies, purification procedures such as reverse-phase chromatography, normal-phase chromatography, and dialysis were commonly used, where reverse-phase liquid chromatography was used for protein analysis.

Chaotropic agents such as urea and salt would reduce the sensitivity of the analysis with FAB, MALDI, or ESL. So that it was necessary to purify the compounds before mass analysis. Matrix-assisted laser desorption/ionization (MALDI) had the advantages of allowing rapid preparation and analysis, high sensitivity, good tolerance of heterogeneous samples, the ability to measure compounds in the presence of salt (~ 1.0 mM) and the ability to analyze complex mixtures. It was typically achieved with a time-of-flight analyzer which, with resolving capabilities in the order of 200-500 amu. For MALDI analysis of small molecules and peptides (200-1000 Da), the 2, 5 dihydroxybenzoic acid (DHB) matrix worked well, since it produced only a minimal amount of interference in the range of low molecular weight.

The protocol from Siuzdak (1996) using DHB produced good data for various compounds:

Matrix: Make 10 mg/mL solution of DHB in 0.1% trifluoroacetic acid in water/acetonitrile, 2:1.

Make a 10 pmol/μL solution of glycoprotein in 0.1% trifluoroacetic acid in water/acetonitrile 2:1.

Make a 3:1- 10:1 mixture of matrix and protein solution, depending on protein molecular weight.

Apply 0.5- 2 μL on to the sample target.

Use a gentle stream of cold air from a blower to assist in sample drying.

Martin de Llano et al. (1993) also developed a preparation protocol for proteins as follows:

Matrix: Prepare a saturated solution of CCA in 2-propanol/water/formic acid, 2: 3: 1 solution.

Proteins: Mix 5 μL protein solution (1- 10 pmol/ μL) in 0.1% TFA with 5 μL matrix solution. Apply 1 μL on to the sample target.

Use a gentle stream of cold air from a blower to assist in sample drying.

In my research, I detected m/z in single-MS mode by running MALDI-TOF (fig. 3). The MALDI-TOF can observe around 10,000 (FWHM) mass power and accuracy in the range of 10 ppm (Loboda et al. 2000). Since mass spectrometry will be the most complex and significant step during the whole experiment, the knowledge above was critical for my further study.

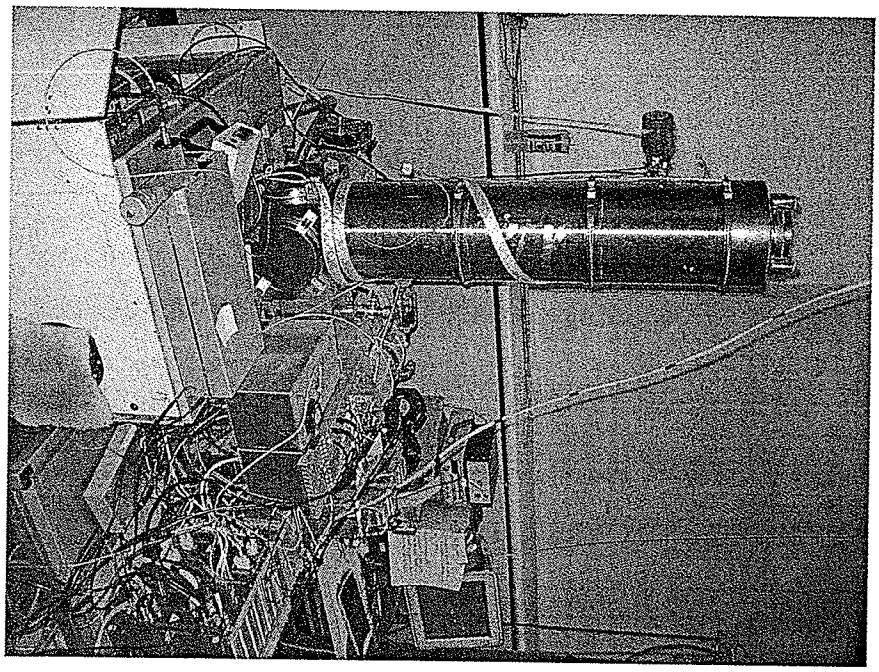

Fig. 3. MALDI QqTOF MS. This picture is taken from the Time-of-Flight lab in the Department of Physics and Astronomy of the University of Manitoba.

MATERIALS AND METHODS

Beetle strains

All insects were maintained at 30°C and 70% RH in controlled environmental chambers. *Sitophilus oryzae* was maintained on whole-wheat moisture conditioned to 15%. *Tribolium castaneum* and *Tribolium confusum* were maintained on enriched white flour mixed with brewer's yeast at a ratio of 20:1 by mass. *Cryptolestes ferrugineus* and *Rhyzopertha dominica* were maintained on cracked 15% MC whole wheat mixed with wheat germ at a ratio of approx. 40:1. All five species were dehydrated at –60°C for 24 h, ground into fine powder with a mortar and a pestle separately, and stored at –20°C until used.

Pure wheat flour was the 2006 hard red spring wheat flour without chemical additions.

Comparison of the protein extraction buffers

Technique of protein fingerprinting was assessed in the current study. The technique includes protein purification, trypsin digestion, High Performance Liquid Chromatography (HPLC), and mass spectrometry which will be the most crucial and complex step in this research. A mass spectrometer as an analytical device determines the molecular weight of chemical compounds by separating molecular ions according to their mass-to-charge ratio (m/z) (Siuzdak 1996). This technology has become an important tool in biochemical research.

1. Protein purification

Tribolium castaneum, red flour beetle, was chosen for its importance in grain storage, its rapid life cycle and ease of feeding. Two hundred milliliters of 50% acetonitrile; sterilized water; and the buffer of 10 mM imidazole pH 7.0, 1 mM phenylthiourea, 1% Triton X-100 and protease inhibitor cocktail (PTU buffer) were added to dissolve 40 mg of *T. castaneum* powder in three 1.5 mL centrifuge tubes separately. At the same time, 40 mg pure wheat flour as blank were measured and added into three 1.5 mL centrifuge tubes. Two hundred millilitres of 50% acetonitrile; sterilized water; and PTU buffer (10 mM imidazole pH 7.0, 1 mM phenylthiourea, 1% Triton X-100 and protease inhibitor cocktail) were injected into these three tubes separately. These 6 tubes were inverted gently several times, in order not to get foaming, and stayed at 4°C for an hour. After that, the tubes

were centrifuged at 14,000 rpm for 30 min, and the supernatants were taken as protein extraction.

2. Reduction, alkylation, dialysis and trypsin digest

Protein extractions were filtered with 0.45 μm PVDF and 0.2 μm PVDF syringe filters to remove large impurities. After filtration, each of the 6 samples was injected with 20 μL dithiothreitol (1 M), and incubated at 57°C for 45 min for reduction. Sixty micro litres iodoacetamide was then added to the solution, and kept in the dark area for 30 min for alkylation. Following these steps, the samples were dialyzed in 1 L 50 mM ammonium hydrogen carbonate at room temperature for 24 h. After dialysis, each sample was incubated at 37°C for 20 h with 6 μL 0.5 μg/μL trypsin enzymes. After 20 h trypsin digestion, the samples were centrifuged to dryness with liquid nitrogen.

3. Reversed-phase high-performance liquid chromatography (RP-HPLC) purification and fractionation

Protein extractions from *T. castaneum* had many impurities, which caused a high risk of blocked columns, if RP-HPLC fractionation was used directly. Therefore, RP-HPLC purification was used before the fractionation step. We used Agilent 1100 RP-HPLC for both sample purification and fractionation with different kinds of columns and operating programs. We injected 110 μL sterilized water into each of the 6 samples, vortexed them for 10 min, and centrifuged them at 14,000 rpm for 5 min. A hundred microliter supernatants of each extraction were injected into the HPLC instrument for purification. Then each purified sample was

collected with a 1.5 mL centrifuge tube, and centrifuged to dryness with liquid nitrogen. For preparing RP-HPLC fractionation, we injected 12 μL sterilized water with 0.1% trifluoroacetic acid and 3 μL standard peptides into each sample, vortexed samples for 10 min, and centrifuged them at 14,000 rpm for 5 min. Finally each sample was separated to 40 fractions by RP-HPLC (300A pore size column) with the flow rate of 46 μL/h, mixed with DHB matrix and loaded on a MALDI target plate.

4. Mass spectrometry (MS)

After offline RP-HPLC fractionation, one-microliter calibration and one microliter dihydroxybenzaldehyde (DHB) mixture were added in front of the first row on each target. All spots dried in several minutes at room temperature. Then mass spectra of the spots were obtained using an in-house MALDI-QqTOF spectrometer of the Time-of-flight lab in the Department of Physics and Astronomy at the University of Manitoba, Winnipeg, MB.

Mass spectra mapping of insects and wheat flour

Because of insufficient information of beetles' database from NCBI for sMART engine, quite a few proteins from our extraction matched this database, even though their spectra looked good. So that peptide mappings among different amounts of insects and wheat flour, depending on 2-dimentional (mass and hydrophobicity) peptide profiles in the mass range of 500 to 4300 Daltons, were easier for deciding insect protein markers to identify insect contaminations in wheat flour. Before RP-HPLC fractionation, we had spiked our standard six

custom tryptic peptides into purified samples. Krokhin et al. (2004) devised these six custom tryptic peptides to calibrate observed retention time into hydrobobicity for a given HPLC experimental run. Their mass range was 890-1002 amu, but they exibited a wide span of hydrophobicty (3-45 H-units). Figure 4 showed a linear regression against these observed peptides in their experimental results that provided the calibration constants A and B for RT=A*H+B, allowing observed retention times for unknown peptides to be mapped into hydrophobicity.

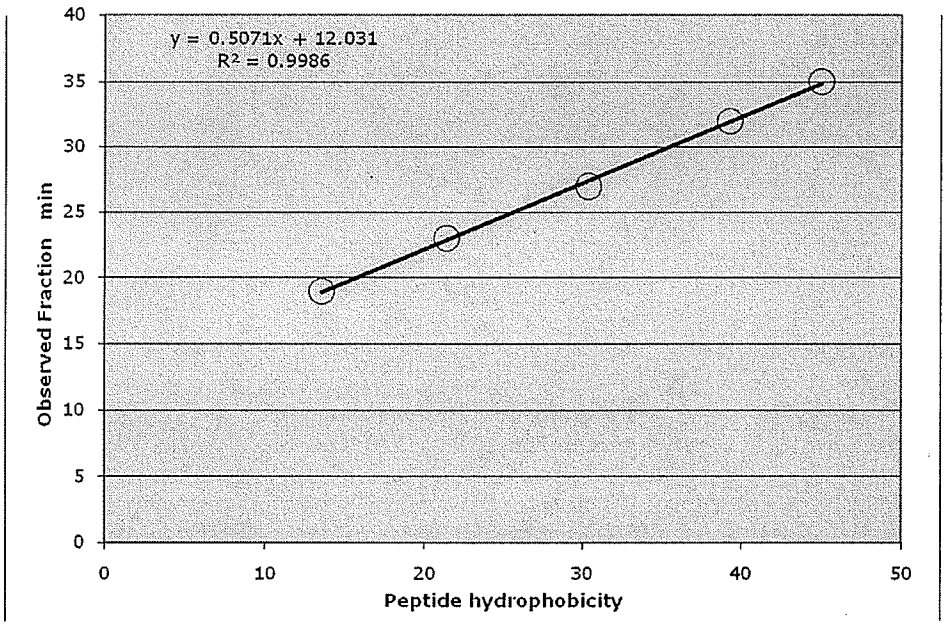

Fig. 4. Typical application of custom HPLC calibration peptides.

Usually, standard peptides were distinctive to be used by the in-house software to draw a standard slope for calculating peptide mass-hydrophobicity profiles of our samples, but the red flour beetle caused the most problems of all five species in looking for standard peptides. Considering industrial applications of

this detecting method, it was important to figure out how this technique worked on red flour beetles, in order not to confound detecting results of our insects and wheat flour mixtures. Another problem was that dialysis tubes were easily blocked by our samples, which caused overlaps by interferences. Therefore, reduction, alkylation, dialysis, and trypsin digestion steps were all abandoned for avoiding overlaps in later studies. This change was to detect original peptides without trypsin digestion, from our samples.

1. Pure wheat and *T. castaneum* peptide extraction

Tribolium castaneum was measured into two 1.5 μL centrifuge tubes, 40 mg each. As the same, 40 mg pure wheat flour were measured and added into one 1.5 μL centrifuge tube. Two hundred milliliters of 50% acetonitrile were added into each of these 3 tubes. Then the 3 tubes were inverted gently several times, in order not to get foaming, and held at 4°C for 1 h. After that, the mixture was centrifuged at 14,000 rpm for 10 min, and the supernatant was taken as the protein extraction.

2. Mixture peptide extraction

Pure wheat flour, 39.6 mg, was mixed with 0.4 mg of *T. castaneum* in a 1.5 mL centrifuge tube, in order to gain 1% *T. castaneum* by mass in the mixture. Similarly, *T. castaneum* and wheat flour mixture containing 0.1% *T. castaneum*, and the insect and wheat flour mixture with 1% of the five insect species (0.2% each of *C. ferrugineus, R. dominica, S. oryzae, T. castaneum*, and *T. confusum*) were measured. Two hundred milliliters of 50% acetonitrile were added into each

of these 3 tubes. Following the same protocol of pure flour peptide extraction, these 3 tubes were inverted gently several times, and held at 4°C for 1 h. Then mixtures were centrifuged at 14,000 rpm for 10 min. Their supernatants were transferred to 3 new tubes as peptide extractions.

Both the pure flour extractions and the extractions of mixtures were purified through 0.45 μm PVDF syringe filters, following with 0.2 μm PVDF syringe filters.

3. Reduction, alkylation, dialysis and trypsin digest

After filtration, one of the pure *T. castaneum* flour sample was injected, 20 μL dithiothreitol (1 M), incubated at 57°C for 45 min for reduction, and then was added to 60 μL iodoacetamide, and kept in dark area for 30 min for alkylation. After alkylation, the sample was dialyzed in 1 L 50 mM ammonium hydrogen carbonate at room temperature for 24 h. After alkylation, it was added 6 μL 0.5 μg/μL enzyme, and incubated at 37°C for 20 h. After that, this sample was centrifuged to dryness with liquid nitrogen.

4. Reversed-phase high-performance liquid chromatography (RP-HPLC) purification and fractionation

Same as in the buffer comparison protocol, extractions, with and without reduction, alkylation, dialysis, and trypsin digestion, were purified and then fractioned by offline RP-HPLC. Each of the extractions was added 110 μL sterilized water, vortexed for 10 min, and centrifuged at 14,000 rpm for 5 min.

Then a hundred-microliter supernatants of each sample was injected into the HPLC instrument. Purified peptides of each sample were collected with a 1.5 mL centrifuge tube, and the collections were centrifuged to dryness with liquid nitrogen. For preparing RP-HPLC separation, 40 μL sterilized water with 0.1% trifluoroacetic acid was added into each sample. Then the samples were vortexed for 10 min, and centrifuged at 14,000 rpm for 5 min. After that, 8 μL of each sample were transferred into a new tube, and 2 μL standard peptides were injected into each of them. These new tubes were votexed for 5 min. Finally each sample was separated 40 spots with DHB matrix, and loaded on a MALDI target plate by RP-HPLC (300A pore size column) with the flow rate of 46 μL/h.

5. Mass spectrometry (MS)

After RP-HPLC fractionation, one-microliter calibrations and one-microliter dihydroxybenzaldehyde (DHB) mixture were added in front of the first row on each target. All spots dried in several minutes at room temperature. Then MALDI-QqTOF in single MS mode was used to read the spots.

RESULTS AND DISCUSSION

Comparison of the protein extraction buffers

Reversed phase high performance liquid chromatography (RP-HPLC) and mass spectrometry (MS) were very sensitive to impurities and easy to be interfered with by other chemicals, when detecting proteins. Although some homogenized buffers had already been developed, they were mainly used for SDS-gel

electrophoresis. Since SDS-gel electrophoresis was not sophisticated enough for my research, buffer decision was important for supporting accurate MS identification. The following comparison results helped to choose a buffer for purifying and detecting insects in wheat flour more efficiently.

The sMART Engine, which was developed by V. Spicer in the Department of Physics and Astronomy at the University of Manitoba, Winnipeg, and MB, was used to analyze all mass spectra from our samples. Figure 5a, 5b and 5c (large size figures are given in Appendix A) show the mass spectra of the 19th spots from RP-HPLC fractionation of wheat flour samples extract by different buffers separately. The three graphs (Figs. 5a-5c, large size figures are given in Appendix A) of MS results of wheat flour protein extractions had a lot of sharp peaks. Many typical wheat flour proteins, like alpha-amylase/trypsin inhibitor CM16 precursor (chloroform/methanol-soluble protein CM 16), alpha-amylase/trypsin inhibitor CM 2 precursor (chloroform/methanol-soluble protein CM 2) and gamma-gliadin precursor and so on, could be found to match the database of wheat proteins. The results represented that the buffers of 50% acetonitrile; sterilized water; and PTU (10 mM imidazole pH 7.0, 1 mM phenylthiourea, 1% Triton X-100 and protease inhibitor cocktail) could all extract various wheat proteins, and the whole procedure worked well for identifying wheat proteins by MS. However, peaks of red flour beetle proteins were not as good as those of wheat flour. Figure 5d, 5e and 5f (large size figures are given in Appendix A) show the spectra of the 19th spots from RP-HPLC fractionation of *T. castaneum* protein extractions by different buffers separately. The extractions with the buffer of 50% acetonitrile had more

distinctive peaks from their background than the extractions with the other two buffers. The peaks of the PTU buffer extractions were better than the ones of sterilized water extractions. There are only 10 *T. castaneum* proteins in NCBI database. By the sMART Engine, the extractions with 50% acetonitrile had 3 matches with the proven *T. castaneum* proteins of the NCBI database and the most matches with the theoretical proteins in these three extractions. The real matches were polyubiquitin [*Tribolium castaneum* mass: 77.00 kDa], glass [*Tribolium castaneum* mass: 43.47 kDa] and ultrabithorax [*Tribolium castaneum* mass: 33.92 kDa]. There were 2 matches between the PTU buffer extractions and the proven *T. castaneum* proteins, which were polyubiquitin [*Tribolium castaneum* mass: 77.00 kDa] and even-skipped [*Tribolium castaneum* mass: 31.35 kDa]. No matches could be found between sterilized water extractions and the real *T. castaneum* proteins. Incomplete information on the *T. castaneum* protein database caused difficulties in comparing the protein extraction buffers. Nevertheless, 50% acetonitrile buffer gave the best results from both its figure (fig. 5a, large size figures are given in Appendix A) and data matches, analyzed by sMART Engine. Its spots had high resolutions (fig. 5d, large size figures are given in Appendix A). These results could be concluded as follows: Initially, the design of the whole experiment worked well, because of the good wheat flour protein identification. Secondly, since there were only 10 proven proteins of *T. castaneum* in the NCBI database, it was hard to tell whether the peptides were from the red flour beetles or not. Thirdly, 50% acetonitrile worked the best in these three buffers to extract *T. castaneum* protein. This buffer was also easy to prepare. Therefore, *T. castaneum* protein

extractions by 50% acetonitrile were the best choice and they were valuable in getting more proven beetle proteins or *T. castaneum* protein identification.

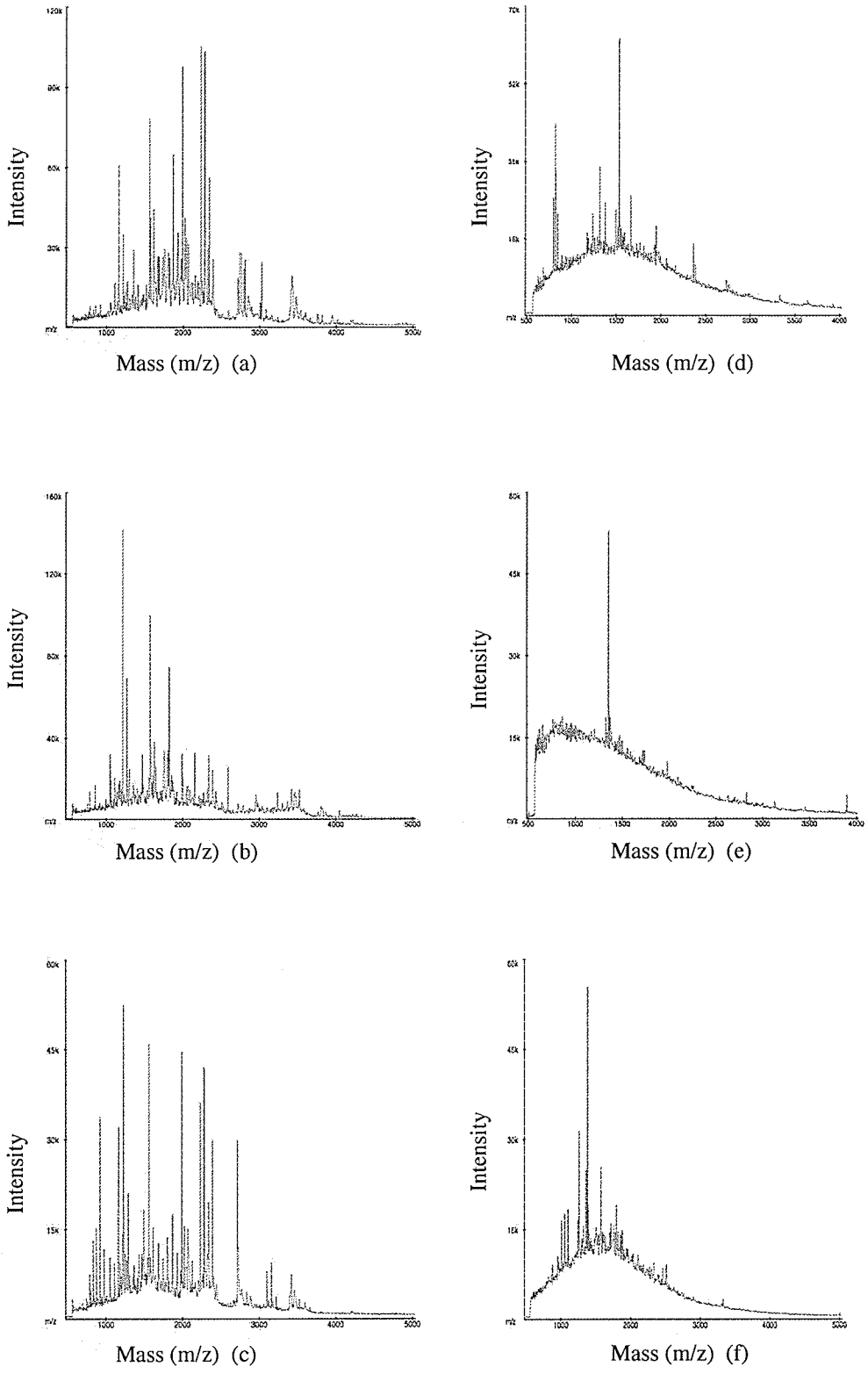

Fig. 5. See Appendix A for large size figures and details.

Mass spectra mapping of insects and wheat flour

Extractions without reduction, alkylation, dialysis, and trypsin digestion brought less chemical interference, and were easier to be spiked by six standard custom tryptic peptides than the ones with all the above procedures. Because it was still not certain what was exactly in the extractions, standard peptides came to be critical for unknown peptide mass mappings. The peptide distribution spectra of pure red flour beetle with this simplified protocol looked normal (fig. 6). Those peptide spectrum dots decreased gently, following the increasing of peptide mass, and no large gaps of peptide dots appeared on the spectra. This method also saved almost half the time, effort, and cost of the original one. Thus, the simplified protocol as follows was more suitable for mass spectrum mappings: 50% acetonitrile extraction – 0.45 μm PVDF syringe filters – 0.2μm PVDF syringe filters – RP-HPLC purification – RP-HPLC fractionation – MALDI-QqTOF. This protocol used the same amount of pure *C. ferrugineus, R. dominica, S. oryzae, T. castaneum,* and *T. confusum*. Our standard peptides had been spiked in all samples before RP- HPLC fractionation. The analytical result of *T. castaneum* found the least standard peptides (2 peaks) in all 5 species. In industrial applications, samples to be studied are mixtures of wheat flour and many insect species, so it was important to ensure that the peptide mass fingerprinting technique worked well on *T. castaneum*, and whether its interference would affect detections on mixtures. Therefore, we concentrated on studying *T. castaneum*. The mapping results I describe below were all obtained using the modified protocol. The spectra were drawn by peptide visual software, developed by V. Spicer in the Department of

Physics and Astronomy with reference numbers of PPM 30, I-RATIO 10 and H-DIST 4. This software not only showed results of spot distribution and mapping in pictures, but also listed all useful information of peptide peaks, following the order of sample names, mass, intensity, fractionation, and hydrophobicity (Appendix B, C, D and E).

Initially, we compared the spectra of 1% *T. castaneum* in wheat flour with the diluted pure *T. castaneum*, both of which had the same amount of *T. castaneum*. Surprisingly, the spectra of 1% *T. castaneum* in wheat flour (fig. 7) gave more information than that of 1% diluted pure *T. castaneum* (fig. 8). We found more standard peptides in the mixture with 1% *T. castaneum*. This may be caused by roles of relatively larger amount of wheat flour in the mixture. High intensities of wheat flour helped to reduce noises from *T. castaneum* and other contaminations. We also found 6 strong peaks of standard peptides in wheat and 5 insect species mixtures (0.2% each of *C. ferrugineus, R. dominica*, *S. oryzae*, *T. castaneum*, and *T. confusum*). Therefore, wheat and insect mixtures were more feasible to be analyzed and compared to get biomarkers rather than the pure *T. castaneum*. Samples we made and the comparison procedures were as follows:

1: 1% *T. castaneum* in wheat flour

2: 0.1% *T. castaneum* in wheat flour

3: Pure wheat flour

4: 1% 5 species (0.2% each of *C. ferrugineus, R. dominica, S. oryzae, T. castaneum*, and *T. confusum*) in wheat and insect mixtures

5: Common peptides between # 1 and # 3 (fig. 9, series 3), which are from wheat flour and system error

6: Common peptides between # 2 and # 3 (fig. 10, series 3), which are from wheat flour and system error

7: Common peptides between # 4 and # 3 (fig. 11, series 3), which are from wheat flour and system error

8: Common peptides between # 1 and # 2 (fig. 12, series 3), which are from red flour beetle, wheat flour and system error

9: Common peptides between # 1 and # 4 (fig. 13, series 3), which are from red flour beetle, wheat flour and system error

10: # 8 - # 5 - # 6 (Peptides comes from red flour beetle only, when there is 0.1% *T. castaneum* in wheat flour.)

11: # 9 - # 5 - # 7 (Peptides comes from red flour beetle only, when there is 0.2% *T. castaneum* in wheat flour and 5 insect species mixture.)

12: Common between # 6 and # 7

The triangle spots (Series 3) in Fig. 9, Fig. 10, Fig. 11, Fig. 12 and Fig. 13 represented # 5, # 6, # 7, # 8 and # 9, respectively. According to the Mass-hydrophobicity profiles of peptides, the software found 124 pairs of common

peptides in # 8 (Appendix B), and 116 pairs of common peptides in # 9 (Appendix C). Theoretically, # 5, # 6 and # 7 were peptides only from wheat flour. However, some contamination from instruments, operators and the environment could not be avoided. These contaminations would cause some common peptides as well. They were called system peptides. Similarly, # 8 and # 9 would be peptides from *T. castaneum*, wheat flour and systems. We subtracted # 5, # 6, and # 7 instead of # 3. Because we could have more typical system peptides in # 5, # 6, and # 7 than those in # 3. To find peptides of *T. castaneum*s, all possible interferences were deducted from # 8 and # 9, from which we got # 10 and # 11. There were still 90 pairs of common peptides in # 10 (Appendix D), and 84 pairs of common peptides in # 11 (Appendix E). The common peptides between # 10 and # 11, which was # 12, were the repeatable ones that only existed in *T. castaneum*. Finally we got 60 repeatable peptides from *T. castaneum* (table III).

In table III, similar pairs of peptide peaks between 1.0% *T. castaneum* in wheat flour and 0.1% *T. castaneum* in wheat flour were listed from small mass to large mass. The third column showed intensities of those peaks. Only intensities of 5 pairs in these 60 common peptides were lower than 1000, which were too weak to be used experientially, but intensities of the other 55 pairs were strong enough. The fraction shifts of peaks in each pair were also small, less than 2 fractions. Hydrophobicities of the peaks in each pair were close to each other. These all convinced us to consider them as peptides from *T. castaneum*. For example, in the 28th pair (highlighted in the table), there were only 0.011 differences in mass between the two peaks. These two peaks had intensities of 3234 and 3156,

respectively, and hydrophobicities of 38.42 and 38.43. Their intensities showed that even the concentration of *T. castaneum* in sample B was 10 times less than that in sample A, their intensities were still close to each other, under the same experimental operation. Therefore, detectable concentrations of *T. castaneum* have the potential to be lowered further. The peak of 1% *T. castaneum* in wheat and the beetle flour mixture was found in the 24^{th} fraction of the 40 fractionated spots of the sample. The peak from 0.1% red flour beetle in wheat and the beetle flour mixture was found in the 22^{nd} fraction of the 40 fractionated spots of the sample. This showed that the eluted time of these two peaks were very close to each other. All parameters of the two peaks proved that these two peaks represented the same *T. castaneum* peptide, but from different samples. In total, there were 60 peptides of *T. castaneum* as shown in table III, in which peaks of 55 ones had high intensities. These 55 peptides could be used to identify *T. castaneum* contamination in wheat flour and insect mixtures. The detection limit should be a minimum *T. castaneum* contamination of 0.1% of the total mass. Furthermore, there were 55 distinctive peptides for *T. castaneum*, when *T. castaneum* proportions reached 0.1% of the total mixture. Potentials of detecting lower percentages of this insect contamination were still high. Moreover, peptides of *T. castaneum* were almost the toughest ones to be extracted, cleaned and detected by RP-HPLC and single MS. In our research, RP-HPLC and single MS worked well on *T. castaneum*. Researches on other stored-product insects could follow the protocol of studying detection of *T. castaneum*, and this hopefully would lead to easier experiments.

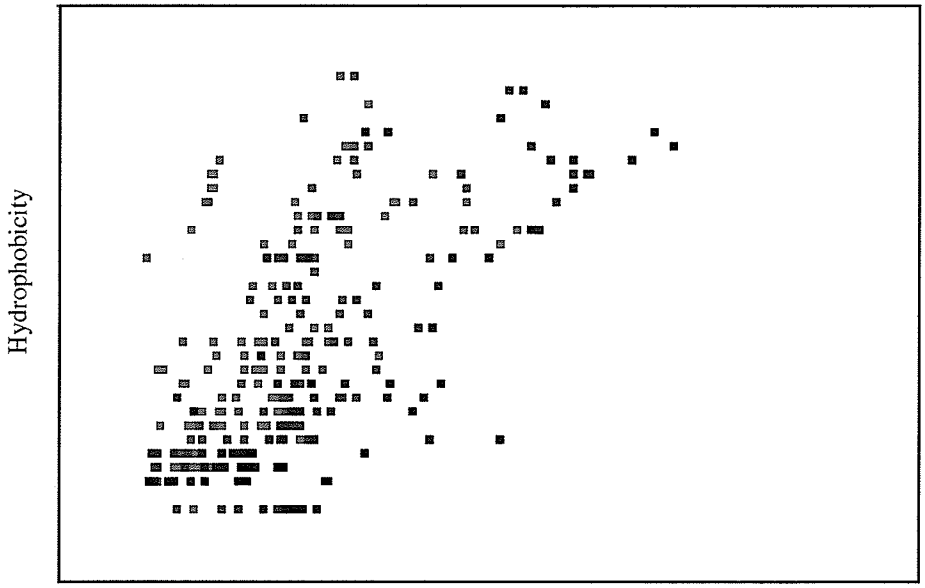

Fig. 6. Peptide distribution spectrum of the pure red flour beetle extracted by the 50% acetonitrile. Reduction, alkylation, dialysis and trypsin digestion were not operated on this sample. The spots are distributed according to mass to retention time (RT).

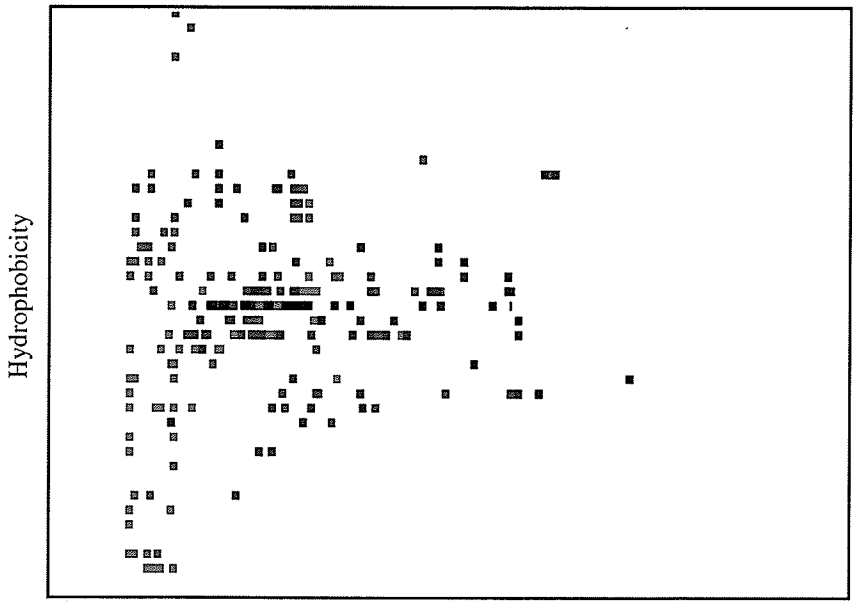

Fig. 7. Peptide distribution spectrum of wheat and red flour beetle mixture, including 1% red flour beetle, extracted by the 50% acetonitrile. Reduction, alkylation, dialysis and trypsin digestion were not operated on this sample. The spots are distributed according to mass to RT.

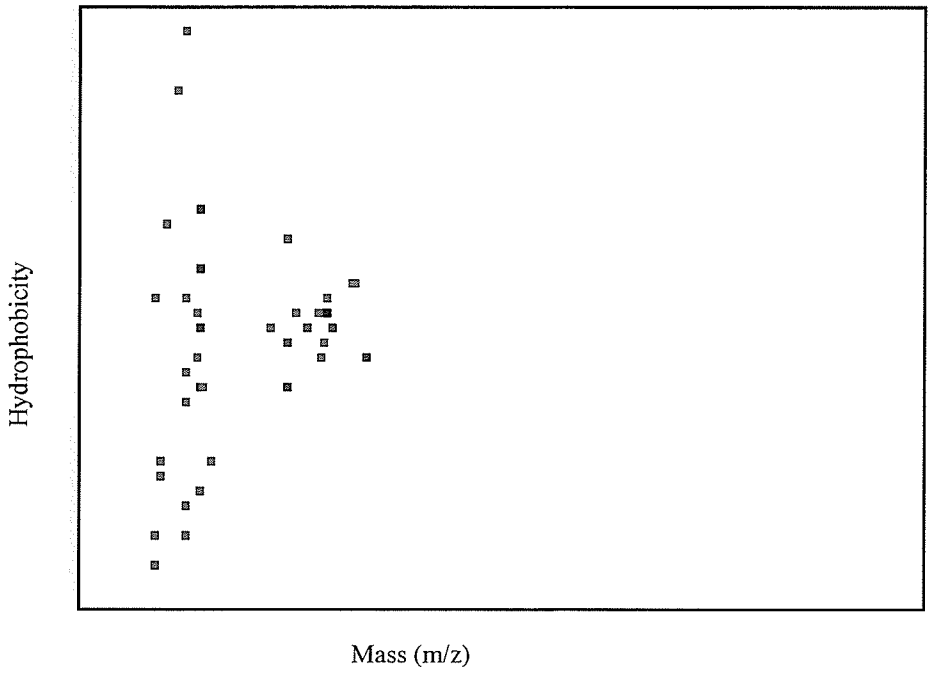

Fig. 8. Peptide distribution spectrum of pure red flour beetle mixture diluted to a concentration of 1% of the original one, extracted by the 50% acetonitrile. Reduction, alkylation, dialysis and trypsin digestion were not operated on this sample. The spots are distributed according to mass to RT.

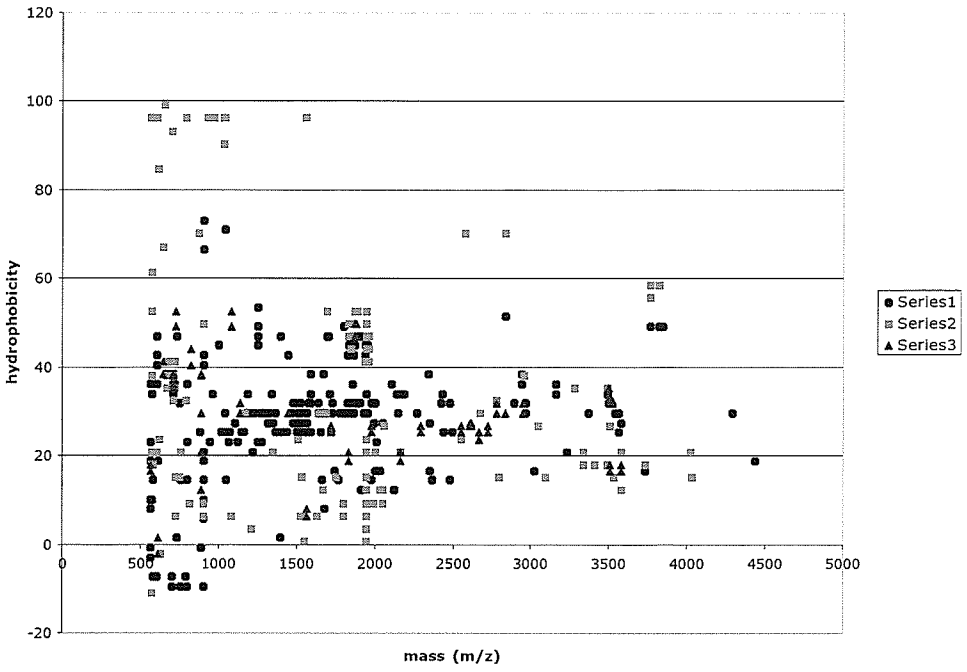

Fig. 9. Peptide distribution mapping between the mixture of wheat flour and 1% red flour beetle and the pure wheat flour.

Series 1 – peptide spectra from the mixture of wheat flour and 1% red flour beetle only

Series 2 – peptide spectra from the pure wheat flour only

Series 3 – common peptide spectra between the mixture of wheat flour and 1% red flour beetle and the pure wheat flour

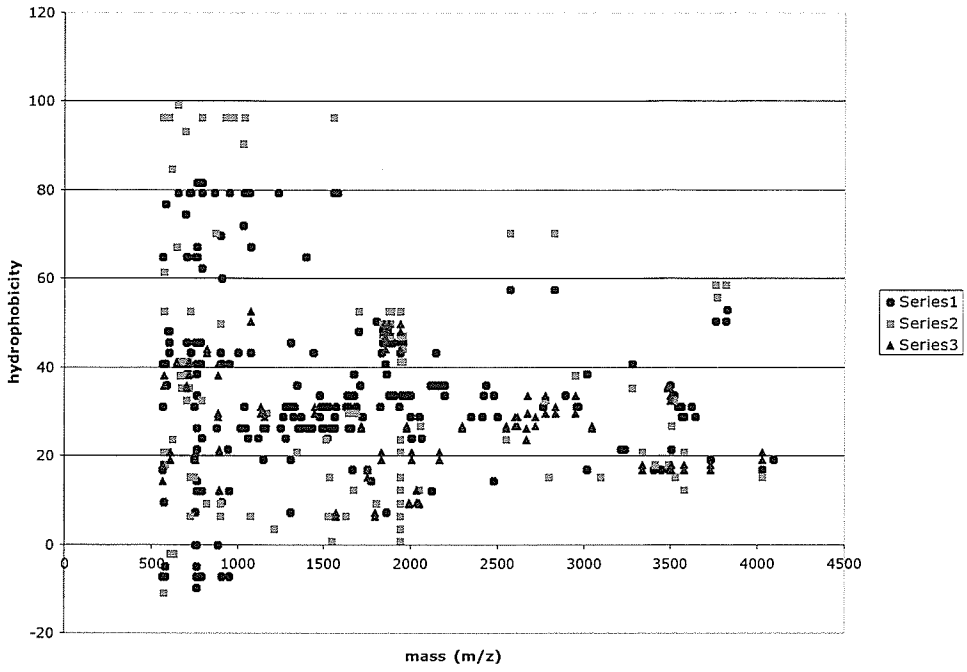

Fig. 10. Peptide distribution mapping between the mixture of wheat flour and 0.1% red flour beetle and the pure wheat flour.

Series 1 – peptide spectra of the mixture of wheat flour and 0.1% red flour beetle only

Series 2 – peptide spectra of the pure wheat flour only

Series 3 – common peptide spectra between the mixture of wheat flour and 0.1% red flour beetle and pure wheat flour

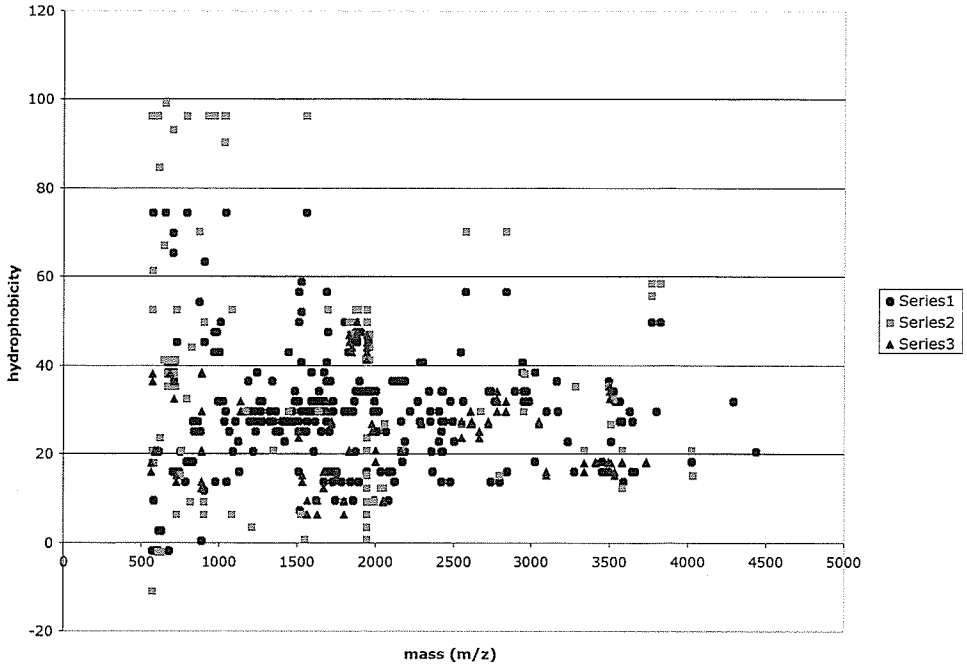

Fig. 11. Peptide distribution mapping between the mixture of wheat flour and 1% 5 species (0.2% each of *C. ferrugineus, R. dominica, S. oryzae, T. castaneum*, and *T. confusum*) and the pure wheat flour.

Series 1 – peptide spectra of the mixture of wheat flour and 1% 5 species (0.2% each of C. *ferrugineus, R. dominica, S. oryzae, T. castaneum*, and *T. confusum*) only

Series 2 – peptide spectra of the pure wheat flour only

Series 3 – common peptide spectra between the mixture of wheat flour and 1% 5 species (0.2% each of *C. ferrugineus, R. dominica, S. oryzae, T. castaneum*, and *T. confusum*) and the pure wheat flour

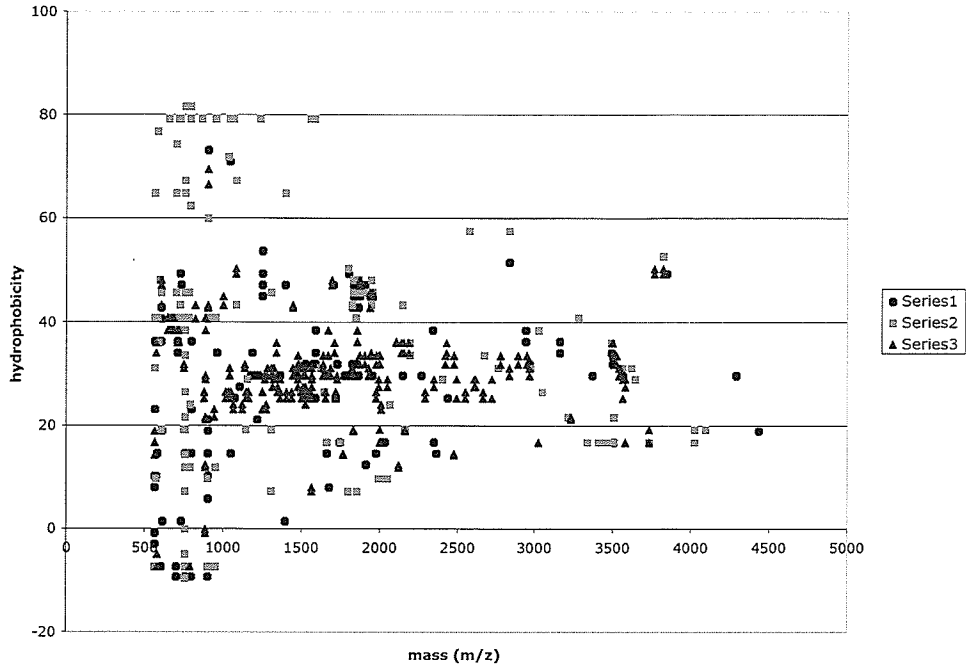

Fig. 12. Peptide distribution mapping between the mixture of wheat flour and 1% red flour beetle and the mixture of wheat flour and 0.1% red flour beetle.

Series 1 – peptide spectra of the mixture of wheat flour and 1% red flour beetle only

Series 2 – peptide spectra of the mixture of wheat flour and 0.1% red flour beetle only

Series 3 – common peptide spectra between the mixture of wheat flour and 1% red flour beetle and the mixture of wheat flour and 0.1% red flour beetle

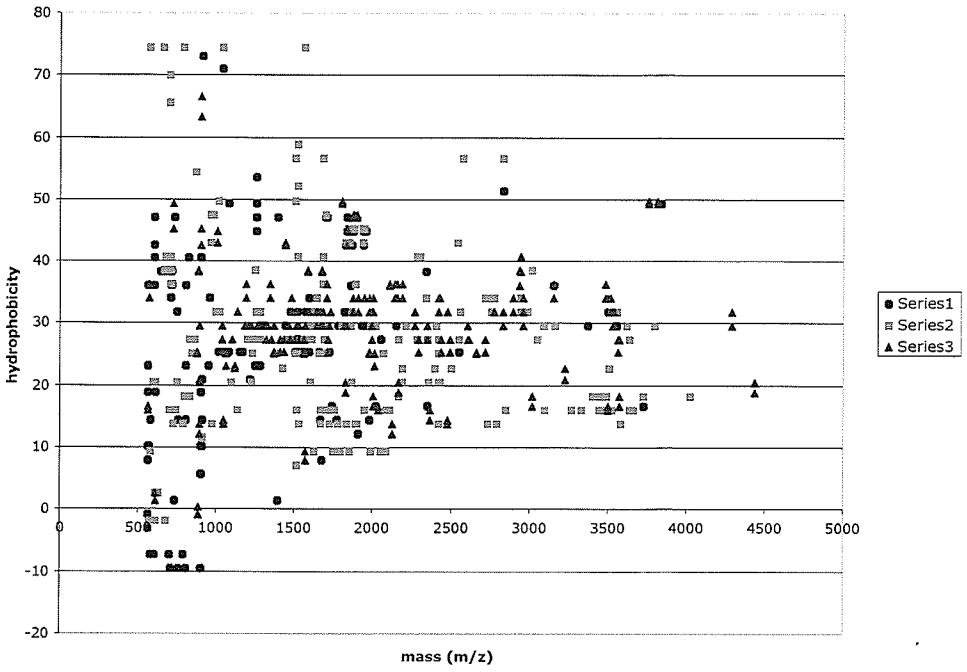

Fig. 13. Peptide distribution mapping between the mixture of wheat flour and 1% red flour beetle and the mixture of wheat flour and 1% 5 species (0.2% each of *C. ferrugineus*, *R. dominica*, *S. oryzae*, *T. castaneum*, and *T. confusum*).

Series 1 – peptide spectra of the mixture of wheat flour and 1% red flour beetle only

Series 2 – peptide spectra of the mixture of wheat flour and 1% 5 species (0.2% each of *C. ferrugineus*, *R. dominica*, *S. oryzae*, *T. castaneum*, and *T. confusum*) only

Series 3 – common peptide spectra between the mixture of wheat flour and 1% red flour beetle and the mixture of wheat flour and 1% 5 species (0.2% each of *C. ferrugineus*, *R. dominica*, *S. oryzae*, *T. castaneum*, and *T. confusum*)

Table III: Sixty pairs of common peptides which are considered to be from *T. castaneum*

Sample	Mass	Intensity	Fraction	Hydrophobicity	
A1%RFBmix	877.445	5637	18	25.38	
B0.1%RFBmix	877.449	1765	17	26.41	
A1%RFBmix	903.039	3572	37	66.68	
B0.1%RFBmix	903.04	3570	35	69.68	
A1%RFBmix	903.04	2486	26	42.77	
B0.1%RFBmix	903.038	2623	24	43.24	
A1%RFBmix	1035.626	3919	18	25.38	
B0.1%RFBmix	1035.628	2194	17	26.41	
A1%RFBmix	1040.502	1879	20	29.72	
B0.1%RFBmix	1040.504	1512	19	31.22	
A1%RFBmix	1063.544	6187	17	23.2	
B0.1%RFBmix	1063.537	5401	16	24.01	
A1%RFBmix	1121.591	20914	17	23.2	
B0.1%RFBmix	1121.583	12457	16	24.01	
A1%RFBmix	1265.668	3570	20	29.72	
B0.1%RFBmix	1265.669	8431	18	28.81	
A1%RFBmix	1320.673	2222	19	27.55	
B0.1%RFBmix	1320.681	2524	18	28.81	
A1%RFBmix	1327.648	3027	20	29.72	
B0.1%RFBmix	1327.655	2319	19	31.22	
A1%RFBmix	1343.748	2408	22	34.07	
B0.1%RFBmix	1343.746	2756	21	36.03	
A1%RFBmix	1350.688	2257	19	27.55	
B0.1%RFBmix	1350.695	1699	17	26.41	
A1%RFBmix	1366.683	14328	18	25.38	
B0.1%RFBmix	1366.688	4289	17	26.41	
B0.1%RFBmix	1366.712	3070	18	28.81	
A1%RFBmix	1405.74	37070	18	25.38	
B0.1%RFBmix	1405.744	22473	17	26.41	
A1%RFBmix	1435.755	48629	18	25.38	
B0.1%RFBmix	1435.757	22312	17	26.41	
A1%RFBmix	1441.871	1666	26	42.77	
B0.1%RFBmix	1441.871	2162	24	43.24	
A1%RFBmix	1476.777	38062	19	27.55	
B0.1%RFBmix	1476.784	36079	18	28.81	
A1%RFBmix	1476.795	2671	21	31.9	
B0.1%RFBmix	1476.804	1821	20	33.62	
A1%RFBmix	1483.746	3261	21	31.9	
B0.1%RFBmix	1483.75	1511	19	31.22	
A1%RFBmix	1506.784	29781	19	27.55	
B0.1%RFBmix	1506.791	31566	17	26.41	
A1%RFBmix	1513.753	1335	20	29.72	
B0.1%RFBmix	1513.755	1168	19	31.22	
A1%RFBmix	1529.746	5371	20	29.72	

B0.1%RFBmix	1529.753	3652	19	31.22	
A1%RFBmix	1561.849	5421	18	25.38	
B0.1%RFBmix	1561.855	2328	17	26.41	
A1%RFBmix	1564.8	4229	19	27.55	
B0.1%RFBmix	1564.807	5496	18	28.81	
A1%RFBmix	1568.813	4937	20	29.72	
B0.1%RFBmix	1568.819	5910	19	31.22	
A1%RFBmix	1639.841	19925	20	29.72	
B0.1%RFBmix	1639.849	25955	19	31.22	
A1%RFBmix	1669.858	14595	20	29.72	
B0.1%RFBmix	1669.87	1991	20	33.62	
A1%RFBmix	1674.813	3234	21	33.42	
B0.1%RFBmix	1674.824	3156	22	33.49	
A1%RFBmix	1685.847	67308	20	29.72	
B0.1%RFBmix	1685.854	57851	19	31.22	
A1%RFBmix	1708.868	3478	22	34.07	
B0.1%RFBmix	1708.865	3407	21	36.03	
A1%RFBmix	1724.909	13481	20	29.72	
B0.1%RFBmix	1724.907	15493	18	28.81	
A1%RFBmix	1821.864	1936	20	29.72	
B0.1%RFBmix	1821.873	1487	19	31.22	
A1%RFBmix	1878.014	4753	21	31.9	
B0.1%RFBmix	1878.019	4971	20	33.62	
A1%RFBmix	1908.033	5193	21	31.9	
B0.1%RFBmix	1908.034	4214	20	33.62	
A1%RFBmix	1949.051	9752	22	34.07	
B0.1%RFBmix	1949.053	9689	20	33.62	
A1%RFBmix	1979.059	6738	21	31.9	
B0.1%RFBmix	1979.066	7072	20	33.62	
A1%RFBmix	1995.057	22408	21	31.9	
B0.1%RFBmix	1995.063	14817	20	33.62	
A1%RFBmix	1997.077	7136	19	27.55	
B0.1%RFBmix	1997.084	11859	18	28.81	
A1%RFBmix	2001.053	13884	21	31.9	
B0.1%RFBmix	2001.059	15632	20	33.62	
A1%RFBmix	2009.057	2751	17	23.2	
B0.1%RFBmix	2009.038	3446	16	24.01	
A1%RFBmix	2112.12	5907	23	36.25	
B0.1%RFBmix	2112.116	7361	21	36.03	
A1%RFBmix	2125.014	2243	12	12.33	
B0.1%RFBmix	2125.008	2219	11	11.99	
A1%RFBmix	2142.139	2965	22	34.07	
B0.1%RFBmix	2142.132	3720	21	36.03	
A1%RFBmix	2158.134	18208	22	34.07	
B0.1%RFBmix	2158.132	16585	21	36.03	
A1%RFBmix	2187.16	4095	22	34.07	
B0.1%RFBmix	2187.151	5660	21	36.03	

A1%RFBmix	2351.23	1648	19	27.55	
B0.1%RFBmix	2351.248	2397	18	28.81	
A1%RFBmix	2420.281	2318	21	31.9	
B0.1%RFBmix	2420.292	1716	20	33.62	
A1%RFBmix	2434.29	2464	22	34.07	
B0.1%RFBmix	2434.291	1937	21	36.03	
A1%RFBmix	2478.218	2479	13	14.51	
B0.1%RFBmix	2478.22	2655	12	14.39	
A1%RFBmix	2479.323	3160	21	31.9	
B0.1%RFBmix	2479.322	2499	20	33.62	
A1%RFBmix	2497.322	3206	18	25.38	
B0.1%RFBmix	2497.328	2192	18	28.81	
A1%RFBmix	2895.481	1443	21	31.9	
B0.1%RFBmix	2895.492	1894	20	33.62	
A1%RFBmix	2960.652	1168	20	29.72	
B0.1%RFBmix	2960.664	1673	19	31.22	
A1%RFBmix	3017.556	2566	14	16.68	
B0.1%RFBmix	3017.565	2346	13	16.8	
A1%RFBmix	3231.666	737	16	21.03	
B0.1%RFBmix	3231.684	1582	15	21.6	
A1%RFBmix	3536.847	269	20	29.72	
B0.1%RFBmix	3536.84	435	19	31.22	
A1%RFBmix	3544.816	314	20	29.72	
B0.1%RFBmix	3544.841	493	19	31.22	
A1%RFBmix	3562.838	1008	18	25.38	
B0.1%RFBmix	3562.835	935	18	28.81	
A1%RFBmix	3761.693	936	29	49.29	
B0.1%RFBmix	3761.71	569	27	50.45	
A1%RFBmix	3818.7	3268	29	49.29	
B0.1%RFBmix	3818.701	1597	27	50.45	

A1%RFBmix – Wheat flour and *T. castaneum* mixture with 1% *T. castaneum* by mass

B0.1%RFBmix – Wheat flour and *T. castaneum* mixture with 0.1% *T. castaneum* by mass

CONCLUSIONS

For preparing rapid MALDI-QqTOF detection of insect contaminations from insect and wheat flour mixtures, the buffer of 50% acetonitrile was the most efficient of the three buffers of 50% acetonitrile; sterilized water; and PTU buffer (10 mM imidazole pH 7.0, 1 mM phenylthiourea, 1% Triton X-100 and protease inhibitor cocktail).

Since protein information on stored-product insects from NCBI database was incomplete for identifying the insects examined in this experiment, mass spectra mapping based on two-dimensional mass to hydrophobicity of the insect peptides, and looking for specific peptides of insects to differentiate various species from wheat flour were more feasible. It was better to abandon the steps of reduction, alkylation, dialysis and trypsin digestion to introduce less interference into MS. Because *T. castaneum* was the most difficult beetle to be purified and detected among all five species (*C. ferrugineus, R. dominica, S. oryzae, T. castaneum,* and *T. confusum*), this protocol could also be used on the other four species besides *T. castaneum*, and their mixtures with wheat flour.

In this research, when *T. castaneum* proportions by mass were down to 0.1% *T. castaneum* in wheat flour mixtures, we could still find 55 peptides of *T. castaneum* with high intensities. It will be useful to try lower levels of concentrations. It will also be useful to find more evidence to prove that the 55 peptides were exactly from *T. castaneum.*

This protein fingerprinting technique is easier to prepare, more rapid, and more sophisticated than most of the contamination detection methods of stored products. Since peptides were distinctive features of insects, this technique could be used in a wide range of insect identification in stored-products.

RERENCES

AACC. 1995. *Approved Methods of the American Association of Cereal Chemists.* AACC, Inc., St Paul, MN.

AACC. 2000. *Approved Methods of the American Association of Cereal Chemists.* AACC, Inc., MN, USA.

Anfinsen, C.B. and E. Haber. 1961. Studies on the reduction and re-formation of protein disulfide bonds. *The Journal of Biological Chemistry* 236: 1361-1363.

Atui, M.B., P.W. Flinn, S.M.N. Lazzari and F.A. Lazzari. 2007. Detection of *Rhyzopertha dominica* larvae in stored wheat using ELISA: The impact of myosin degradation following fumigation. *Journal of Stored Products Research* 43: 156-159.

Ausubel, I. and M. Frederick (eds.). 1990. *Current Protocols in Molecular Biology.* Chichester, UK: John Wiley & Sons. Inc.

Balasubramanian, A., D.S. Jayas, W.G.D. Fernando, V. Li and N.D.G. White. 2007. Sensitivity analysis of DNA fingerprinting technique for detecting insect fragments in wheat flour. *Canadian Biosystems Engineeering* 49: 41-45.

Balasubramanian, A. 2005. Sensitivity analysis of DNA fingerprinting technique for detecting insect fragments in wheat flour. Unpublished M. Sc. thesis. Department of Biosystems Engineering, University of Manitoba, Winnipeg, MB.

Belay, A., M. McCalmont and G.B. Kitto. 1997. Development of an immunoassay to detect insect contamination of microalgal products. *Journal of Applied Phycology* 9: 431-436.

Bullard, B., R. Dabrowska and L. Winkelman. 1973. The contractile and regulatory proteins of insect flight muscle. *Biochemical Journal* 135: 277-286.0

Calbiochem, 2003. ProteoExtract all-in-one trypsin digestion kit. Cat. No. 650212. User Protocol 650212 Rev. 04-November-03 CML Page 1 of 8.

Campbell, P.M. 2005. Species differentiation of insects and other multicellular organisms using matrix-assisted laser desorption/ionization time of flight mass spectrometry protein profiling. *Systematic Entomology* 30: 186-190.

Clauser, K., P. Baker and A. Burlingame. 1999. Role of accurate mass measurement (±10 ppm) in protein identification strategies employing MS or MS/MS and database searching. *Analytical Chemistry* 71: 2871-2882.

Cox, J. 1991. *Urban Integrated Pest Management, A Guide for Commercial Applicators.* Arlington, VA: DUAL & Associates, Inc.

Cranshaw, W.S. and F.B. Peairs. 2005. E:\insects\Insect pests of home-stored foods.htm.

Davis, N.D. and V.L. Diener. 1979. A fluorometric-iodine (FL-1) method for measuring aflatoxin in corn. *Journal of Applied Biochemistry* 1:115-122.

DeFoliart, G. 1992. Insects as human food. *Crop Protection* 11: 395-399.

Dowell, F.E. 2000. Differentiating vitreous and nonvitreous durum wheat kernels by using near-infrared spectroscopy. *Cereal Chemistry* 77: 155-158.

Emerson, C.P. and S.I. Bernstein. 1987. Molecular genetics of myosin. *Annual Review of Biochemistry* 56: 695-726.

Evaluation Division. 1999. Health Protection Branch Guidelines for the General Cleanliness of Food- an Overview. Extraneous Materials Overview. Ottawa, ON: Health Protection Branch, Sir Frederick G. Banting Research Centre, Health Canada.

Feltwell, J. (ed.). 1981. *Large White Butterfly: The Biology, Biochemistry and Physiology of Pieris Brassicae (Linnaeus).* Norwell, MA: Kluwer Academic Publishers.

Flinn, P.W. and D.W. Hagstrum. 2001. Augmentative releases of parasitoid wasps in stored wheat reduces insect fragments in flour. *Journal of Stored Products Research* 37: 179-186.

FDA, Food and Drug Admnistration. 1988. Compliance Policy Guides. Section 7104.06. Office of Enforcement, Division of Compliance Policy, Food and Drug Administration, Washington, DC.

Galactic Industries. 2003. Algorithms: Partial least squares. Available at: www.galactic.com/algorithms/pls.htm.

Glaze, L.E. and J.R. Bryce. 1994. Extraction of light filth from whole-wheat flour, flotation method: collaborative study. *Journal of AOAC International* 77: 1150–1152.

Gorbunoff, M.J. and S.N. Timasheff. 1984. The interaction of proteins with hydroxyapatite III mechanism. *Analytical Biochemistry* 136: 433-439.

Gressent, F., I. Rahioui and Y. Rahbe. 2003. Characterization of a high affinity binding site for the pea albumin 1b (PA1b) entomotoxin in the weevil *Sitophilus*. *European Journal of Biochemistry* 270: 2429-2435.

Hammond, K.S. and D.E. Goll. 1975. Purification of insect myosin and a-actinin. *Biochemical Journal* 151: 189-192.

Hartshorne, D.J. and H. Mueller. 1968. Fractionation of troponin into two distinct proteins. *Biochemical and Biophysical Research Communications* 31, 647-653.

Hill, D.S. (ed.). 2002. *Pests of Stored Foodstuffs and Their Control*. Norwell, MA: Kluwer Academic Publishers.

Isa, A.M. 1969. Susceptibility of anti-chlamydial IgG antibodies to reduction and alkylation 1. *The Journal of Immunology* 102: 1332-1335.

Ja¨ger, D., P.R. Jungblut and U.M. Werdan. 2002. Separation and identification of human heart proteins. *Journal of Chromatography* B 771: 131-153

Jayas, D.S., J. Paliwal and N.S. Visen. 2000. Multi-layer neural networks for image analysis of agricultural products. *Journal of Agricultural Engineering Research* 77 (2):119-128.

Karunakaran, C., D.S. Jayas and N.D.G. White. 2003. Soft X-ray inspection of wheat kernels infested by *Sitophilus oryzae*. *Transaction of the ASAE* 46(3): 739-745.

Kramer, K.J. and D. Koga. 1986. Insect chitin. *Insect Biochemistry* 16: 851-877.

Krause, F. 2006. Detection and analysis of protein–protein interactions in organellar and prokaryotic proteomes by native gel electrophoresis: (Membrane) protein complexes and supercomplexes. *Electrophoresis* 27: 2759-2781

Krokhin, O.V., R. Craig, V. Spicer, W. Ens, K. G. Standing, R. C. Beavis and J. A. Wilkins. 2004. An improved model for prediction of retention times of tryptic peptides in ion pair reversed-phase HPLC. *Molecular & Cellular Proteomics* 3: 908-919.

Leelaja, B.C., Y. Rajashekar, and S. Rajendran. 2007. Detection of eggs of stored-product insects in flour with staining techniques. *Journal of Stored Products Research* 43: 206-210.

Libera, L.D. 2001. High performance liquid chromatography in the analysis and separation of contractile proteins. *Basic and Applied Myology* 11 (3): 115-118.

Loboda, A.V., A. N. Krutchinsky, M. Bromirski, W. Ens and K. G. Standing. 2000. A tandem quadrupole/time-of-flight mass spectrometer with a matrix-assisted laser desorption/ionization source: design and performance. *Rapid Communications in Mass Spectrometry* 14: 1047–1057.

Mant, C.T. and R.S. Hodges (eds.). 1991. *High-Performance Liquid Chromatography of Peptides and Proteins: Separation, Analysis and Conformation.* Boca Raton, FL: CRC Press.

Martens, H. and T. Naes. 1989. *Multivariate Calibration.* Chichester, UK: John Wiley & Sons. Inc.

Martin de Llano, J.J., W. Jones, K. Schneider, B.T. Chait, J.M. Manning, G. Rodgers, L.J. Benjamin and B. Weksler. 1993. Biochemical and functional properties of recombinant human sickle hemoglobin expressed in yeast. *Journal of Biological Chemistry* 268: 27004-27011.

Meinrenken, W. 1969. Calciumionen-unabhängige kontraktion und ATPase bei glycerinierten muskelfasern nach alkalischer extraktion von troponin. *Pflügers Archiv European Journal of Physiology* 311: 243-255.

Mihályi, E. and A.G. Szent-györgyi. 1952. Trypsin digestion of muscle protein 1. ultracentrifugal analysis of the process. *The Journal of Biological Chemistry* 201(1): 189-196.

Moore, J.R., M.H. Dickinson, J.O. Vigoreaux and D.W. Maughan. 2000. The effect of removing the N-Terminal extension of the drosophila myosin regulatory

light chain upon flight ability and the contractile dynamics of indirect flight muscle. *Biophysical Journal* 78(3): 1431-1440.

Morris, D.L. 1946. Colorimetric determination of glycogen, disadvantages of the iodine method. *Journal of Biological Chemistry* 166(1): 199-203.

Neethirajan, S., C. Karunakaran, D.S. Jayas and N.D.G. White. 2007. Detection techniques for stored-product insects in grain. *Food Control* 18: 157-162

Neverova, I. and J.E. van Eyk. 2005. Role of chromatographic techniques in proteomic analysis. *Journal of Chromatography B* 815: 51–63.

Paliwal, J., N.S. Visen and D.S. Jayas. 2001. Evaluation of neural network architectures for cereal grain classification using morphological features. *Journal of Agricultural Engineering Research* 79(4): 361-370.

Payne, P.L., L.M. Holt, E.A. Jackson and C.N. Law. 1984. Wheat storage proteins: their genetics and their potential for manipulation by plant breeding. *Philosophical Transactions of the Royal Society Biological Sciences* 304: 359-371.

Payne, P.I. and A.P. Rhodes. 1982. Cereal storage proteins: structure and role in agriculture and food technology. *Encyclopedia of Plant Physiology* 14: 346-369.

Perez-Mendoza, J., J.E. Throne, F.E. Dowell and J.E. Baker. 2002. Detection of insect fragments in wheat flour by near-infrared spectroscopy. *Journal of Stored Products Research* 39: 305–312.

Perez-Mendoza J., J.E. Throne, E.B. Machirang, F.E. Dowell and J.E. Baker. 2005. Insect fragments in flour: relationship to lesser grain borer (Coleoptera: Bostrichidae) infestation level in wheat and rapid detection using near-infrared spectroscopy. *Journal of Economic Entomology* 98(6): 2282-2291.

Quinn, F., W. Burkholder and G.B. Kitto. 1992. Preliminary development of an immunological technique for measuring insect contamination of whole and milled grain. *Journal of Economic Entomology* 85: 1463-1470.

Rawlings, N.D. and A.J. Barrett. 1994. Families of serine peptidases. *Methods Enzymol* 244:19-60.

Rees, M.K. and M. Young. 1967. Studies on the isolation and molecular properties of homogeneous globular actin. Evidence for a single polypeptide chain structure. *Journal of Biological Chemistry* 242: 4449-4458.

Rossomando, E.F. and Z. Deyl (eds.). 1998. *HPLC in Enzymatic Analysis*. New York: John Wiley & Sons. Inc.

Sachdeva, A.S. 1978. Effect of infestation stage, form, and treatment on fragment count in flour. Unpublished M.S. thesis. Department of Grain Science and Industry, Kansas State University, Manhattan, Kansas.

Samizo, K., R. Ishikawa, A. Nakamura and K. Kohama. 2001. A highly sensitive method for measurement of myosin ATPase activity by reversed-phase high-performance liquid chromatography. *Analytical Biochemistry* 293: 212-215.

Schatzki, T.F., E.K. Wilson, G.B. Kitto, P. Behrens, I. Heller. 1993. Determination of hidden Sitophilus granarius (Coleoptera: Curculionidae) in wheat by myosin ELISA. *Journal of Economic Entomology* 86: 1584–1589.

Sechi, S. and B.T. Chait. 1998. Modification of cysteine residues by alkylation. A tool in peptide mapping and protein identification. *Analytical Chemistry* 70: 5150-5158

Shen, Z.C. and M.J. Lorena. 1999. Evolution of chitin-binding proteins in invertebrates. *Journal of Molecular Evolution* 48:341–347.

Šimić, G., D. Horvat, Z. Jurković, G. Drezner, D. Novoselović and K. Dvojković. 2006. The genotype effect on the ratio of wet gluten content to total wheat grain protein. *Journal of Central European Agriculture* 7: 13-18.

Sinha, R.N. and F.L. Watters. 1985. *Insect Pests of Flour Mills, Grain Elevators, and Feed Mills and Their Control.* Ottawa, ON: Agriculture Canada.

Siuzdak, G. (ed.). 1996. *Mass Spectrometry for Biotechnology.* San Diego, CA: Academic Press Limited.

Smith, L.B. and P.S. Barker. 1987. Distribution of insects found in granary residues in the canadian prairies. *The Canadian Entomologist* 119: 873-880.

Thind, B.B. 2000. Determination of low levels of mite and insect contaminants in food and feedstuffs by a modified flotation method. *Journal of AOAC International* 88: 113-119.

APPENDIX A – Spectra

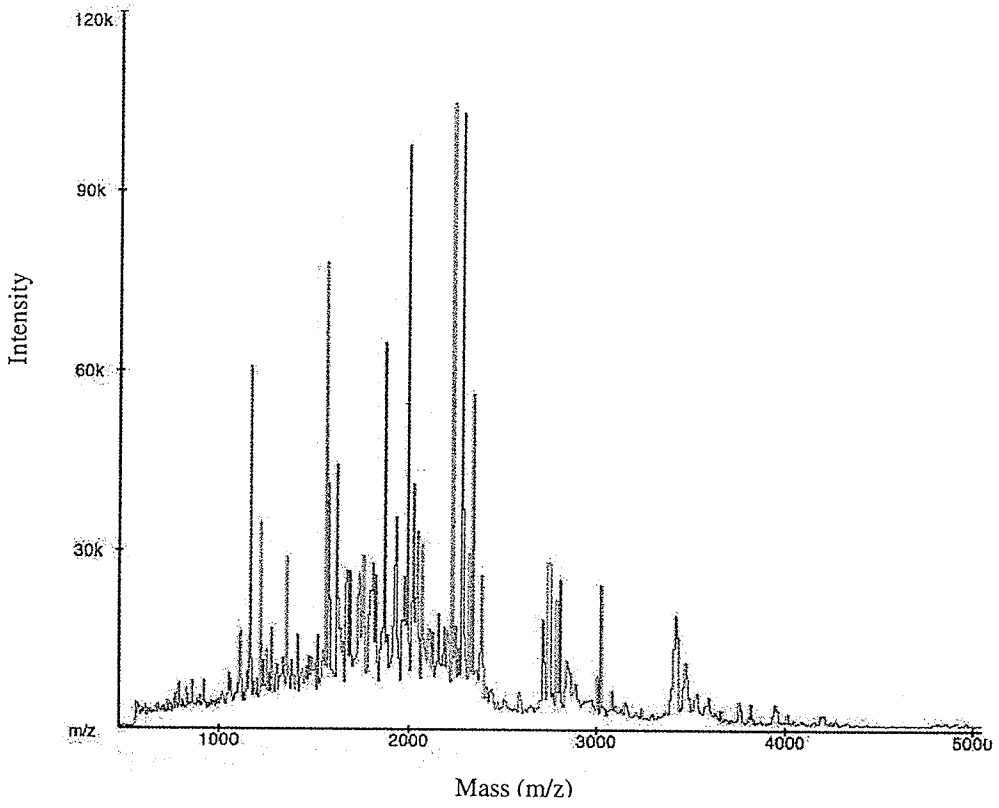

Fig. 5a. A typical mass spectrum of protein extractions of the pure wheat flour with the buffer of 50% acetonitrile. This is spectrum of the 19th spot, which is separated and loaded on a target by reversed-phase high performance liquid chromatography (RP-HPLC). The peaks in the spectra represent the separated peptides according to their mass-to-charge ratio (m/z), with different resolutions.

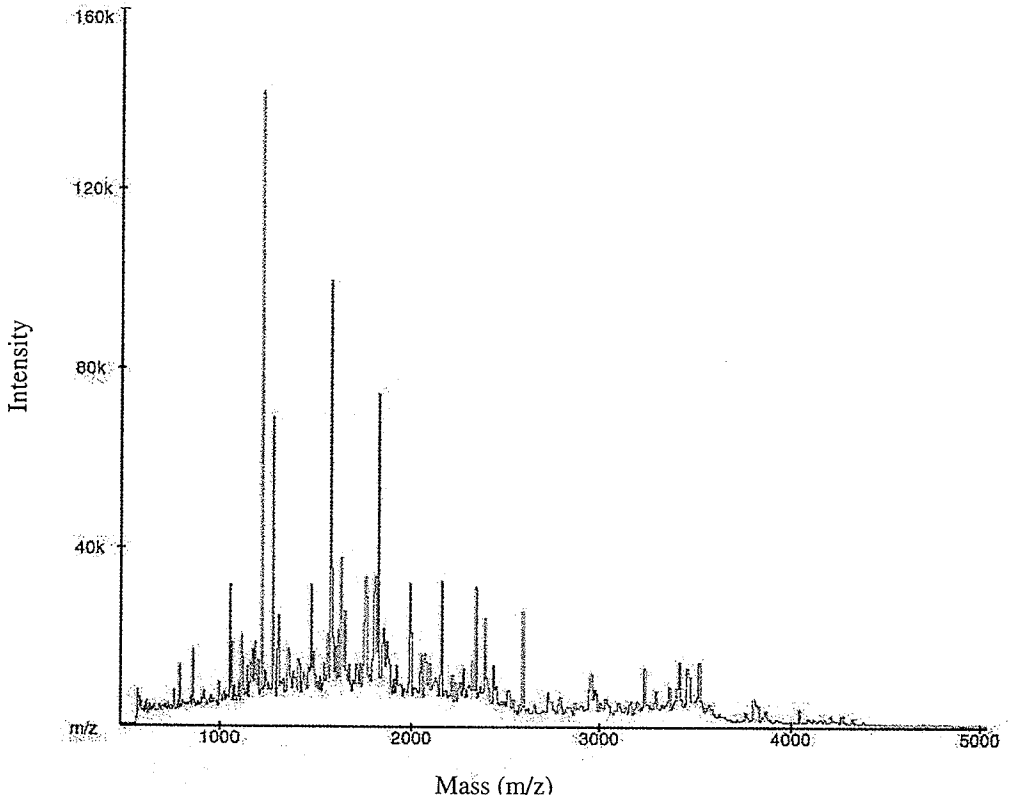

Fig. 5b. A typical mass spectrum of protein extractions of the pure wheat flour with the buffer of 100% sterilized water. This is spectrum of the 19th spot, which is separated and loaded on a target by reversed-phase high performance liquid chromatography (RP-HPLC). The peaks in the spectra represent the separated peptides according to their mass-to-charge ratio (m/z), with different resolutions.

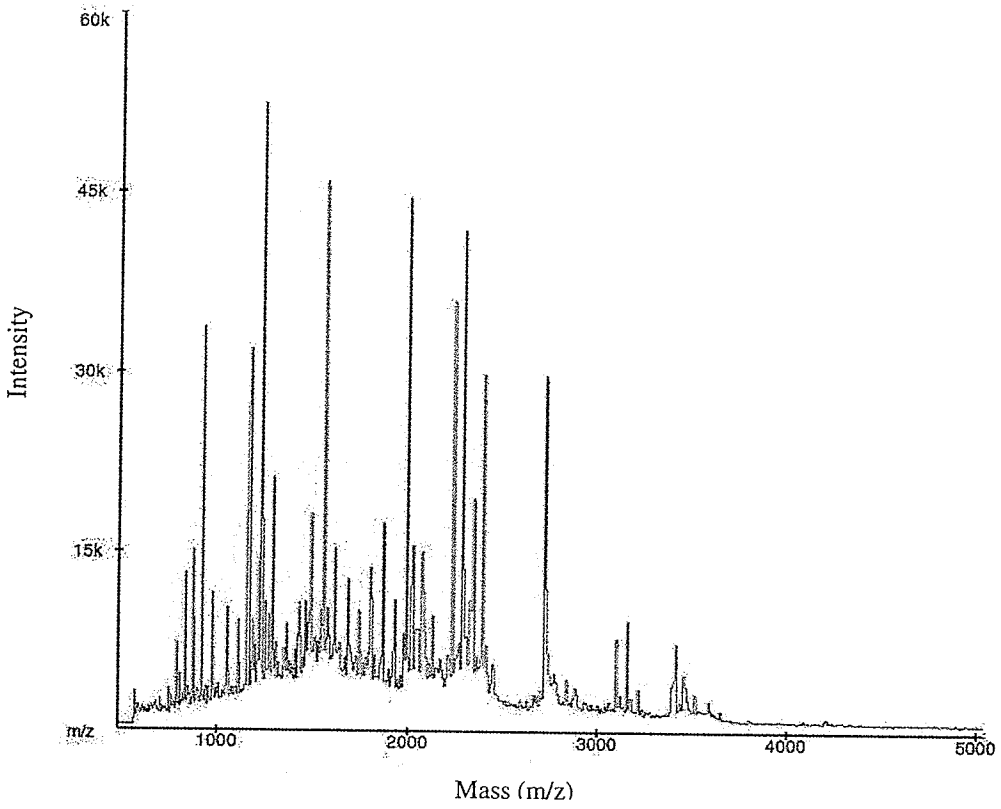

Fig. 5c. A typical mass spectrum of protein extractions of the pure wheat flour with the PTU buffer (10 mM imidazole pH 7, 1 mM phenylthiourea, 1% Triton X-100 and protease inhibitor cocktail). This is spectrum of the 19th spot, which is separated and loaded on a target by reversed-phase high performance liquid chromatography (RP-HPLC). The peaks in the spectra represent the separated peptides according to their mass-to-charge ratio (m/z), with different resolutions.

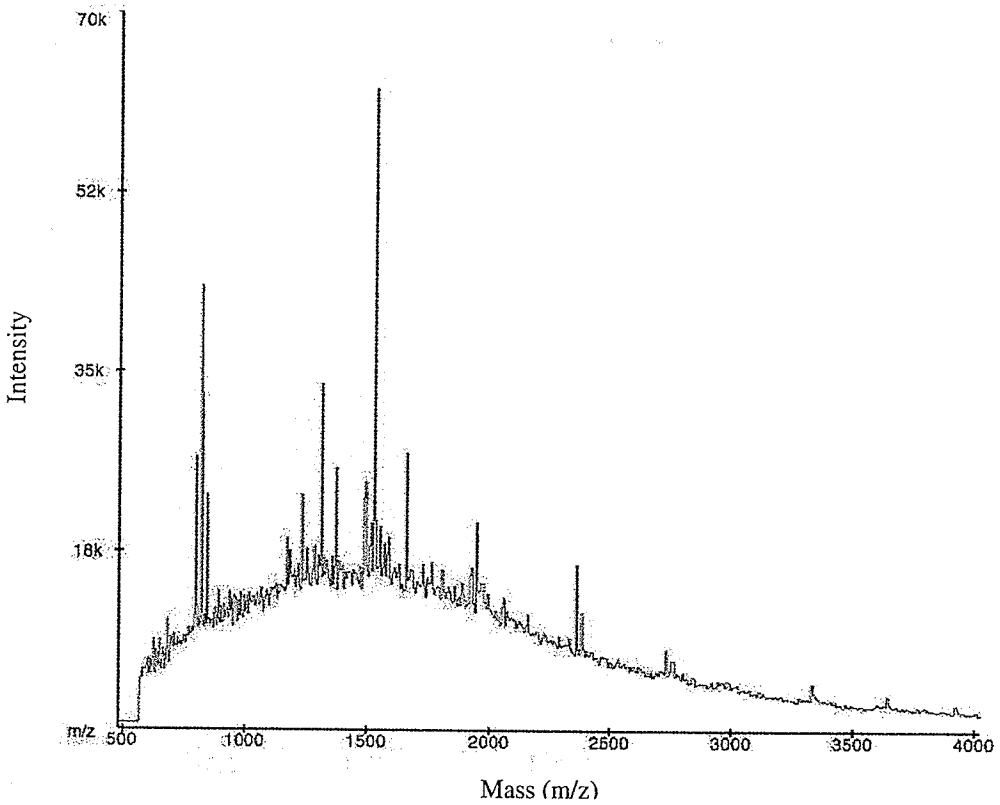

Fig. 5d. A typical mass spectrum of protein extractions of the pure *T. castaneum* with the buffer of 50% acetonitrile. This is spectrum of the 19th spot, which is separated and loaded on a target by reversed-phase high performance liquid chromatography (RP-HPLC). The peaks in the spectra represent the separated peptides according to their mass-to-charge ratio (m/z), with different resolutions.

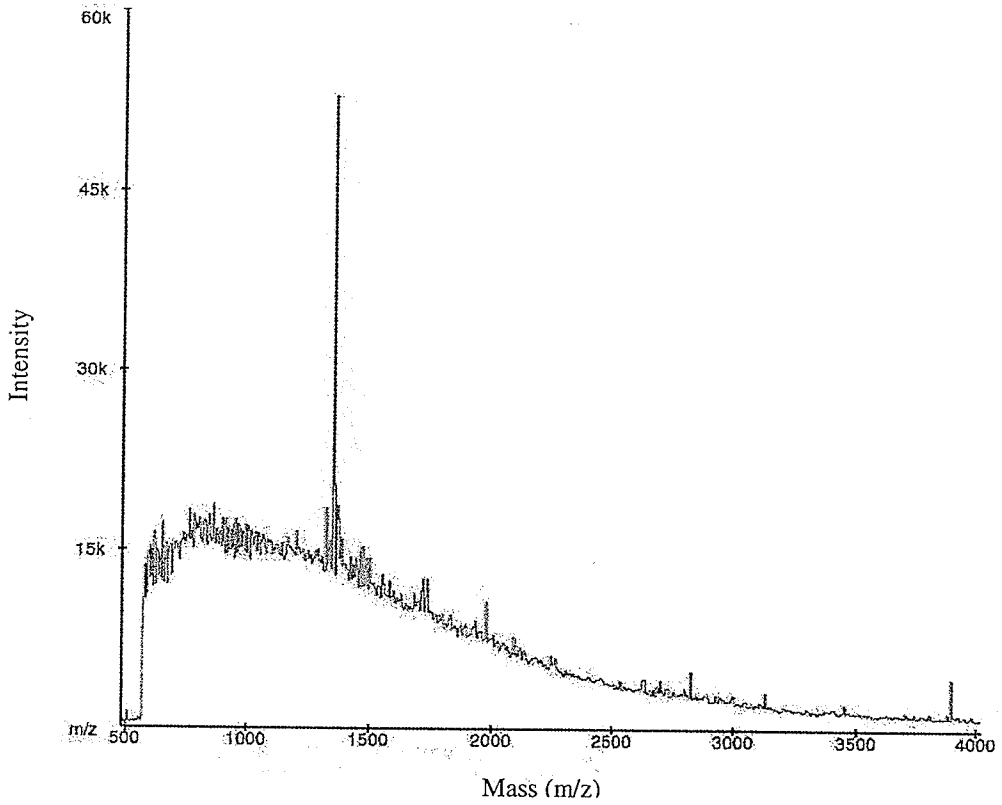

Fig. 5e. A typical mass spectrum of protein extractions of the pure *T. castaneum* with the buffer of 100% sterilized water. This is spectrum of the 19th spot, which is separated and loaded on a target by reversed-phase high performance liquid chromatography (RP-HPLC). The peaks in the spectra represent the separated peptides according to their mass-to-charge ratio (m/z), with different resolutions.

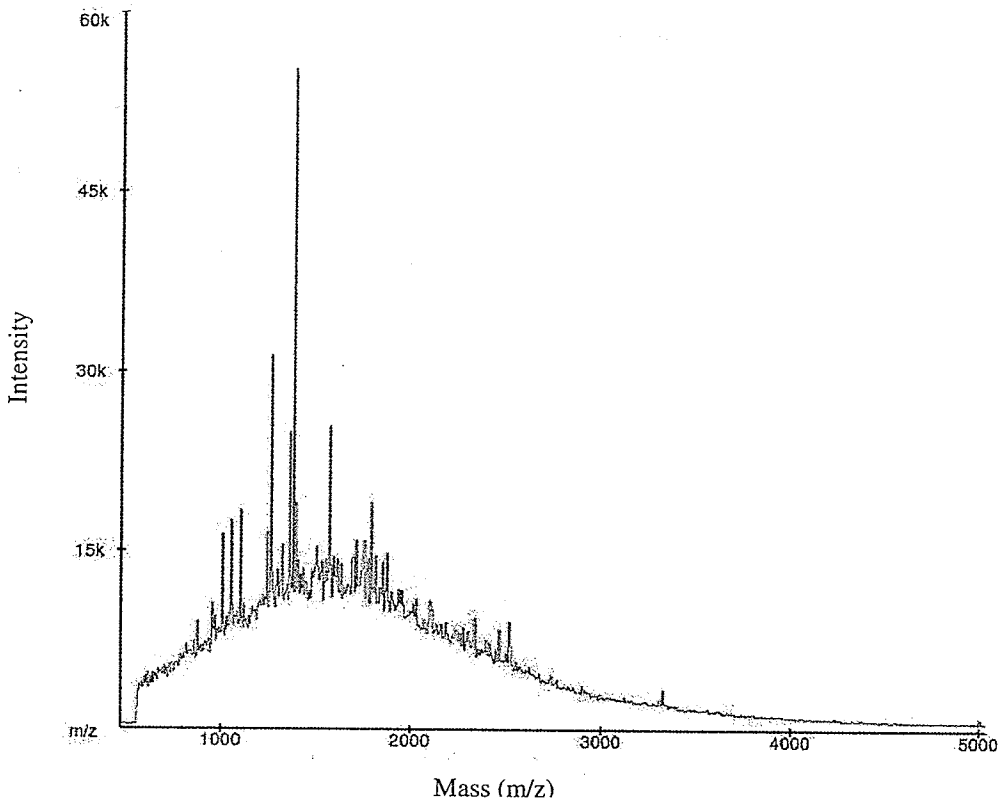

Fig. 5f. A typical mass spectrum of protein extractions of the pure *T. castaneum* with the PTU buffer (10 mM imidazole pH 7, 1 mM phenylthiourea, 1% Triton X-100 and protease inhibitor cocktail). This is spectrum of the 19th spot, which is separated and loaded on a target by reversed-phase high performance liquid chromatography (RP-HPLC). The peaks in the spectra represent the separated peptides according to their mass-to-charge ratio (m/z), with different resolutions.

APPENDIX B: One hundred and twenty-four pairs of similar peaks between the wheat flour and *T. castaneum* mixture with 1% *T. castaneum* and the one with 0.1% *T. castaneum*

Sample	Mass	Intensity	Fraction	Hydrophobicity	
A1%RFBmix	564.624	8121	15	18.85	
B0.1%RFBmix	564.629	4133	13	16.8	
A1%RFBmix	565.151	15201	14	16.68	
B0.1%RFBmix	565.15	17909	12	14.39	
A1%RFBmix	573.253	27958	22	34.07	
B0.1%RFBmix	573.251	41172	21	36.03	
A1%RFBmix	578.66	14893	3	-7.23	
B0.1%RFBmix	578.661	8848	3	-7.24	
B0.1%RFBmix	578.665	7776	4	-4.84	
A1%RFBmix	603.546	2267	28	47.12	
B0.1%RFBmix	603.535	1747	26	48.05	
B0.1%RFBmix	603.561	2510	24	43.24	
A1%RFBmix	645.273	4659	24	38.42	
B0.1%RFBmix	645.273	5285	23	40.83	
A1%RFBmix	668.625	4941	24	38.42	
B0.1%RFBmix	668.624	4136	23	40.83	
A1%RFBmix	682.664	8099	24	38.42	
B0.1%RFBmix	682.651	8115	23	40.83	
A1%RFBmix	708.635	6230	24	38.42	
B0.1%RFBmix	708.636	4448	21	36.03	
B0.1%RFBmix	708.645	9893	22	38.43	
A1%RFBmix	718.127	4217	23	36.25	
B0.1%RFBmix	718.107	5763	22	38.43	
A1%RFBmix	750.456	2881	21	31.9	
B0.1%RFBmix	750.459	2010	19	31.22	
A1%RFBmix	788.895	29824	3	-7.23	
B0.1%RFBmix	788.899	14496	3	-7.24	
A1%RFBmix	821.385	3327	25	40.59	
B0.1%RFBmix	821.385	2588	24	43.24	
A1%RFBmix	877.445	5637	18	25.38	
B0.1%RFBmix	877.449	1765	17	26.41	
A1%RFBmix	889.422	5437	6	-0.71	

B0.1%RFBmix	889.422	7375	6	-0.03	
A1%RFBmix	889.553	27432	24	38.42	
B0.1%RFBmix	889.555	17140	23	40.83	
A1%RFBmix	890.51	7768	20	29.72	
B0.1%RFBmix	890.512	9014	18	28.81	
A1%RFBmix	891.465	41954	16	21.03	
B0.1%RFBmix	891.468	26805	15	21.6	
A1%RFBmix	892.426	32436	12	12.33	
B0.1%RFBmix	892.424	28851	11	11.99	
A1%RFBmix	903.039	3572	37	66.68	
B0.1%RFBmix	903.04	3570	35	69.68	
A1%RFBmix	903.04	2486	26	42.77	
B0.1%RFBmix	903.038	2623	24	43.24	
A1%RFBmix	948.491	4755	17	23.2	
B0.1%RFBmix	948.482	5401	15	21.6	
A1%RFBmix	1002.636	1649	27	44.94	
B0.1%RFBmix	1002.639	2069	24	43.24	
A1%RFBmix	1017.532	2568	18	25.38	
B0.1%RFBmix	1017.533	1408	17	26.41	
A1%RFBmix	1035.626	3919	18	25.38	
B0.1%RFBmix	1035.628	2194	17	26.41	
A1%RFBmix	1040.502	1879	20	29.72	
B0.1%RFBmix	1040.504	1512	19	31.22	
A1%RFBmix	1047.548	4350	18	25.38	
B0.1%RFBmix	1047.548	2199	17	26.41	
A1%RFBmix	1063.544	6187	17	23.2	
B0.1%RFBmix	1063.537	5401	16	24.01	
A1%RFBmix	1079.049	2382	29	49.29	
B0.1%RFBmix	1079.042	1033	27	50.45	
A1%RFBmix	1121.591	20914	17	23.2	
B0.1%RFBmix	1121.583	12457	16	24.01	
A1%RFBmix	1137.634	4653	21	31.9	
B0.1%RFBmix	1137.636	1856	19	31.22	
A1%RFBmix	1148.594	2329	18	25.38	
B0.1%RFBmix	1148.602	1869	17	26.41	
A1%RFBmix	1164.592	7409	18	25.38	
B0.1%RFBmix	1164.592	2420	17	26.41	
A1%RFBmix	1249.654	5166	17	23.2	
B0.1%RFBmix	1249.652	1576	17	26.41	

A1%RFBmix	1265.668	3570	20	29.72	
B0.1%RFBmix	1265.669	8431	18	28.81	
A1%RFBmix	1279.664	8203	17	23.2	
B0.1%RFBmix	1279.653	5735	16	24.01	
A1%RFBmix	1281.646	997	20	29.72	
B0.1%RFBmix	1281.654	1054	19	31.22	
A1%RFBmix	1311.654	910	20	29.72	
B0.1%RFBmix	1311.657	1170	19	31.22	
A1%RFBmix	1320.673	2222	19	27.55	
B0.1%RFBmix	1320.681	2524	18	28.81	
A1%RFBmix	1327.648	3027	20	29.72	
B0.1%RFBmix	1327.655	2319	19	31.22	
A1%RFBmix	1343.748	2408	22	34.07	
B0.1%RFBmix	1343.746	2756	21	36.03	
A1%RFBmix	1350.688	2257	19	27.55	
B0.1%RFBmix	1350.695	1699	17	26.41	
A1%RFBmix	1366.683	14328	18	25.38	
B0.1%RFBmix	1366.688	4289	17	26.41	
B0.1%RFBmix	1366.712	3070	18	28.81	
A1%RFBmix	1405.74	37070	18	25.38	
B0.1%RFBmix	1405.744	22473	17	26.41	
A1%RFBmix	1435.755	48629	18	25.38	
B0.1%RFBmix	1435.757	22312	17	26.41	
A1%RFBmix	1441.871	1666	26	42.77	
B0.1%RFBmix	1441.871	2162	24	43.24	
A1%RFBmix	1444.496	702	20	29.72	
B0.1%RFBmix	1444.501	1350	19	31.22	
A1%RFBmix	1476.777	38062	19	27.55	
B0.1%RFBmix	1476.784	36079	18	28.81	
A1%RFBmix	1476.795	2671	21	31.9	
B0.1%RFBmix	1476.804	1821	20	33.62	
A1%RFBmix	1483.746	3261	21	31.9	
B0.1%RFBmix	1483.75	1511	19	31.22	
A1%RFBmix	1504.781	4247	18	25.38	
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A1%RFBmix	1513.753	1335	20	29.72	
B0.1%RFBmix	1513.755	1168	19	31.22	

A1%RFBmix	1522.77	3205	19	27.55	
B0.1%RFBmix	1522.772	11552	16	24.01	
B0.1%RFBmix	1522.789	954	19	31.22	
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A1%RFBmix	1568.813	4937	20	29.72	
B0.1%RFBmix	1568.819	5910	19	31.22	
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B0.1%RFBmix	1570.448	2554	9	7.18	
A1%RFBmix	1635.87	2923	21	31.9	
B0.1%RFBmix	1635.873	2019	20	33.62	
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B0.1%RFBmix	1708.865	3407	21	36.03	
A1%RFBmix	1716.763	3379	18	25.38	
B0.1%RFBmix	1716.762	2358	17	26.41	
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B0.1%RFBmix	1724.907	15493	18	28.81	
A1%RFBmix	1772.906	2945	13	14.51	
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A1%RFBmix	1941.883	3878	26	42.77	
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B0.1%RFBmix	1958.654	5127	25	45.64	
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B0.1%RFBmix	1976.953	1216	17	26.41	
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A1%RFBmix	1995.057	22408	21	31.9	
B0.1%RFBmix	1995.063	14817	20	33.62	
A1%RFBmix	1997.077	7136	19	27.55	
B0.1%RFBmix	1997.084	11859	18	28.81	
A1%RFBmix	2001.053	13884	21	31.9	
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A1%RFBmix	2004.742	5321	14	16.68	
B0.1%RFBmix	2004.74	7605	14	19.2	
A1%RFBmix	2009.057	2751	17	23.2	
B0.1%RFBmix	2009.038	3446	16	24.01	
A1%RFBmix	2050.083	1134	19	27.55	
B0.1%RFBmix	2050.098	2000	18	28.81	
A1%RFBmix	2112.12	5907	23	36.25	
B0.1%RFBmix	2112.116	7361	21	36.03	
A1%RFBmix	2125.014	2243	12	12.33	
B0.1%RFBmix	2125.008	2219	11	11.99	
A1%RFBmix	2142.139	2965	22	34.07	

B0.1%RFBmix	2142.132	3720	21	36.03	
A1%RFBmix	2158.134	18208	22	34.07	
B0.1%RFBmix	2158.132	16585	21	36.03	
A1%RFBmix	2167.806	16286	15	18.85	
B0.1%RFBmix	2167.806	29359	14	19.2	
A1%RFBmix	2187.16	4095	22	34.07	
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A1%RFBmix	2297.206	1579	18	25.38	
B0.1%RFBmix	2297.206	1013	17	26.41	
A1%RFBmix	2351.23	1648	19	27.55	
B0.1%RFBmix	2351.248	2397	18	28.81	
A1%RFBmix	2420.281	2318	21	31.9	
B0.1%RFBmix	2420.292	1716	20	33.62	
A1%RFBmix	2434.29	2464	22	34.07	
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A1%RFBmix	2478.218	2479	13	14.51	
B0.1%RFBmix	2478.22	2655	12	14.39	
A1%RFBmix	2479.323	3160	21	31.9	
B0.1%RFBmix	2479.322	2499	20	33.62	
A1%RFBmix	2497.322	3206	18	25.38	
B0.1%RFBmix	2497.328	2192	18	28.81	
A1%RFBmix	2551.085	1753	18	25.38	
B0.1%RFBmix	2551.099	1015	17	26.41	
A1%RFBmix	2605.074	1268	19	27.55	
B0.1%RFBmix	2605.136	2964	18	28.81	
A1%RFBmix	2617.93	4254	19	27.55	
B0.1%RFBmix	2617.942	5607	18	28.81	
A1%RFBmix	2665.123	6574	18	25.38	
B0.1%RFBmix	2665.128	3997	17	26.41	
A1%RFBmix	2719.174	1670	18	25.38	
B0.1%RFBmix	2719.195	1319	18	28.81	
A1%RFBmix	2778.207	3565	21	31.9	
B0.1%RFBmix	2778.223	4614	20	33.62	
A1%RFBmix	2835.229	652	20	29.72	
B0.1%RFBmix	2835.24	1888	19	31.22	
A1%RFBmix	2895.481	1443	21	31.9	
B0.1%RFBmix	2895.492	1894	20	33.62	
A1%RFBmix	2951.986	1709	21	31.9	
B0.1%RFBmix	2952.051	6120	20	33.62	

A1%RFBmix	2960.652	1168	20	29.72	
B0.1%RFBmix	2960.664	1673	19	31.22	
A1%RFBmix	3017.556	2566	14	16.68	
B0.1%RFBmix	3017.565	2346	13	16.8	
A1%RFBmix	3231.666	737	16	21.03	
B0.1%RFBmix	3231.684	1582	15	21.6	
A1%RFBmix	3498.704	1191	22	34.07	
B0.1%RFBmix	3498.647	860	20	33.62	
B0.1%RFBmix	3498.753	2185	21	36.03	
A1%RFBmix	3518.738	914	21	31.9	
B0.1%RFBmix	3518.799	927	20	33.62	
A1%RFBmix	3536.847	269	20	29.72	
B0.1%RFBmix	3536.84	435	19	31.22	
A1%RFBmix	3544.816	314	20	29.72	
B0.1%RFBmix	3544.841	493	19	31.22	
A1%RFBmix	3562.838	1008	18	25.38	
B0.1%RFBmix	3562.835	935	18	28.81	
A1%RFBmix	3576.257	1349	14	16.68	
B0.1%RFBmix	3576.262	8668	13	16.8	
A1%RFBmix	3576.853	571	19	27.55	
B0.1%RFBmix	3576.856	1077	18	28.81	
A1%RFBmix	3731.339	1060	14	16.68	
B0.1%RFBmix	3731.328	1288	14	19.2	
A1%RFBmix	3761.693	936	29	49.29	
B0.1%RFBmix	3761.71	569	27	50.45	
A1%RFBmix	3818.7	3268	29	49.29	
B0.1%RFBmix	3818.701	1597	27	50.45	

A1%RFBmix – Wheat flour and *T. castaneum* mixture including 1% *T. castaneum* by mass

B0.1%RFBmix – Wheat flour and *T. castaneum* mixture including 0.1% *T. castaneum* by mass

APPENDIX C: One hundred and sixteen pairs of similar peaks between the wheat flour and *T. castaneum* mixture with 1% *T. castaneum* and the wheat flour and 5 species (0.2% each of *C. ferrugineus, R. dominica, S. oryzae, T. castaneum*, and *T. confusum*) mixture

Sample	Mass	Intensity	Fraction	Hydrophobicity
A1%RFBmix	565.151	15201	14	16.68
B5species1%mix	565.154	17129	13	16.16
A1%RFBmix	573.253	27958	22	34.07
B5species1%mix	573.251	9390	22	36.38
A1%RFBmix	613.142	4517	7	1.46
B5species1%mix	613.144	13333	7	2.68
A1%RFBmix	727.028	3316	29	49.29
B5species1%mix	727.024	1954	26	45.37
A1%RFBmix	877.445	5637	18	25.38
B5species1%mix	877.442	3253	17	25.15
A1%RFBmix	889.422	5437	6	-0.71
B5species1%mix	889.418	6833	6	0.43
A1%RFBmix	889.553	27432	24	38.42
B5species1%mix	889.553	33366	23	38.63
A1%RFBmix	890.51	7768	20	29.72
B5species1%mix	890.508	10428	19	29.64
A1%RFBmix	891.465	41954	16	21.03
B5species1%mix	891.471	12146	15	20.65
A1%RFBmix	892.426	32436	12	12.33
B5species1%mix	892.429	20039	12	13.91
A1%RFBmix	903.039	3572	37	66.68
B5species1%mix	903.039	2173	34	63.35
A1%RFBmix	903.04	2486	26	42.77
B5species1%mix	903.033	1691	26	45.37
A1%RFBmix	1002.636	1649	27	44.94
B5species1%mix	1002.631	4598	25	43.13
A1%RFBmix	1035.626	3919	18	25.38
B5species1%mix	1035.63	6912	18	27.4
A1%RFBmix	1040.502	1879	20	29.72
B5species1%mix	1040.502	5161	19	29.64

A1%RFBmix	1049.568	5899	13	14.51
B5species1%mix	1049.569	7044	12	13.91
A1%RFBmix	1063.544	6187	17	23.2
B5species1%mix	1063.545	3720	17	25.15
A1%RFBmix	1103.582	2330	19	27.55
B5species1%mix	1103.582	5049	18	27.4
A1%RFBmix	1121.591	20914	17	23.2
B5species1%mix	1121.586	5685	16	22.9
A1%RFBmix	1137.634	4653	21	31.9
B5species1%mix	1137.633	1995	20	31.89
A1%RFBmix	1180.594	848	20	29.72
B5species1%mix	1180.604	1811	19	29.64
A1%RFBmix	1187.646	2536	22	34.07
B5species1%mix	1187.642	3696	22	36.38
A1%RFBmix	1210.599	726	20	29.72
B5species1%mix	1210.605	2095	19	29.64
A1%RFBmix	1226.605	2047	20	29.72
B5species1%mix	1226.601	6873	19	29.64
A1%RFBmix	1265.668	3570	20	29.72
B5species1%mix	1265.665	9238	19	29.64
A1%RFBmix	1320.673	2222	19	27.55
B5species1%mix	1320.679	4691	18	27.4
A1%RFBmix	1327.648	3027	20	29.72
B5species1%mix	1327.656	2868	19	29.64
A1%RFBmix	1343.748	2408	22	34.07
B5species1%mix	1343.749	6458	22	36.38
A1%RFBmix	1350.688	2257	19	27.55
B5species1%mix	1350.696	5178	18	27.4
A1%RFBmix	1366.683	14328	18	25.38
B5species1%mix	1366.691	6124	17	25.15
A1%RFBmix	1366.718	2544	20	29.72
B5species1%mix	1366.713	4386	19	29.64
A1%RFBmix	1405.74	37070	18	25.38
B5species1%mix	1405.742	22281	18	27.4
A1%RFBmix	1435.755	48629	18	25.38
B5species1%mix	1435.759	21882	18	27.4
A1%RFBmix	1441.871	1666	26	42.77
B5species1%mix	1441.873	2162	25	43.13
A1%RFBmix	1467.761	708	20	29.72

B5species1%mix	1467.761	5301	19	29.64
A1%RFBmix	1476.777	38062	19	27.55
B5species1%mix	1476.783	43985	18	27.4
A1%RFBmix	1483.746	3261	21	31.9
B5species1%mix	1483.743	3670	20	31.89
B5species1%mix	1483.758	2474	21	34.14
A1%RFBmix	1506.784	29781	19	27.55
B5species1%mix	1506.789	39094	18	27.4
A1%RFBmix	1513.753	1335	20	29.72
B5species1%mix	1513.752	2854	20	31.89
A1%RFBmix	1529.746	5371	20	29.72
B5species1%mix	1529.75	9580	19	29.64
A1%RFBmix	1561.849	5421	18	25.38
B5species1%mix	1561.855	4050	17	25.15
A1%RFBmix	1564.8	4229	19	27.55
B5species1%mix	1564.81	5319	18	27.4
A1%RFBmix	1568.813	4937	20	29.72
B5species1%mix	1568.811	24149	19	29.64
A1%RFBmix	1570.453	1624	10	7.98
B5species1%mix	1570.452	5066	10	9.42
A1%RFBmix	1587.773	1467	24	38.42
B5species1%mix	1587.778	3641	23	38.63
A1%RFBmix	1593.862	2400	21	31.9
B5species1%mix	1593.833	1228	20	31.89
A1%RFBmix	1639.841	19925	20	29.72
B5species1%mix	1639.841	24352	20	31.89
A1%RFBmix	1669.858	14595	20	29.72
B5species1%mix	1669.856	11322	20	31.89
A1%RFBmix	1674.813	3234	24	38.42
B5species1%mix	1674.815	5003	23	38.63
A1%RFBmix	1685.847	67308	20	29.72
B5species1%mix	1685.853	15108	20	31.89
A1%RFBmix	1708.868	3478	22	34.07
B5species1%mix	1708.867	5354	22	36.38
A1%RFBmix	1716.763	3379	18	25.38
B5species1%mix	1716.771	2715	18	27.4
A1%RFBmix	1724.909	13481	20	29.72
B5species1%mix	1724.906	59952	19	29.64
A1%RFBmix	1727.86	2844	21	31.9

B5species1%mix	1727.872	3474	20	31.89
A1%RFBmix	1800.902	1947	29	49.29
B5species1%mix	1800.925	2171	28	49.87
A1%RFBmix	1821.864	1936	20	29.72
B5species1%mix	1821.87	3141	19	29.64
A1%RFBmix	1829.89	1699	15	18.85
B5species1%mix	1829.89	3573	15	20.65
A1%RFBmix	1836.87	3059	26	42.77
B5species1%mix	1836.841	4143	26	45.37
A1%RFBmix	1837.954	711	20	29.72
B5species1%mix	1837.978	2010	19	29.64
A1%RFBmix	1872.806	2842	28	47.12
B5species1%mix	1872.766	3621	27	47.62
A1%RFBmix	1878.014	4753	21	31.9
B5species1%mix	1878.019	5270	21	34.14
A1%RFBmix	1881.813	3314	28	47.12
B5species1%mix	1881.867	3972	27	47.62
A1%RFBmix	1903.824	4345	28	47.12
B5species1%mix	1903.851	4329	27	47.62
A1%RFBmix	1908.033	5193	21	31.9
B5species1%mix	1908.031	4586	21	34.14
A1%RFBmix	1949.051	9752	22	34.07
B5species1%mix	1949.054	11009	21	34.14
A1%RFBmix	1976.957	2066	18	25.38
B5species1%mix	1976.971	2167	17	25.15
A1%RFBmix	1979.059	6738	21	31.9
B5species1%mix	1979.068	8559	21	34.14
A1%RFBmix	1995.057	22408	21	31.9
B5species1%mix	1995.051	5961	20	31.89
A1%RFBmix	1997.077	7136	19	27.55
B5species1%mix	1997.08	11477	19	29.64
A1%RFBmix	2001.053	13884	21	31.9
B5species1%mix	2001.062	6983	21	34.14
A1%RFBmix	2004.742	5321	14	16.68
B5species1%mix	2004.764	1670	14	18.41
A1%RFBmix	2009.057	2751	17	23.2
B5species1%mix	2009.063	17994	17	25.15
A1%RFBmix	2037.982	3541	14	16.68
B5species1%mix	2037.992	1007	13	16.16

A1%RFBmix	2112.12	5907	23	36.25
B5species1%mix	2112.12	9591	22	36.38
A1%RFBmix	2125.014	2243	12	12.33
B5species1%mix	2125.028	2407	12	13.91
A1%RFBmix	2142.139	2965	22	34.07
B5species1%mix	2142.144	4816	22	36.38
A1%RFBmix	2158.134	18208	22	34.07
B5species1%mix	2158.132	16801	22	36.38
A1%RFBmix	2167.806	16286	15	18.85
B5species1%mix	2167.821	13900	15	20.65
A1%RFBmix	2187.16	4095	22	34.07
B5species1%mix	2187.162	3385	22	36.38
A1%RFBmix	2270.077	507	20	29.72
B5species1%mix	2270.082	1493	20	31.89
A1%RFBmix	2297.206	1579	18	25.38
B5species1%mix	2297.198	2170	18	27.4
A1%RFBmix	2351.23	1648	19	27.55
B5species1%mix	2351.243	5752	19	29.64
A1%RFBmix	2368.016	1357	13	14.51
B5species1%mix	2368.017	970	13	16.16
A1%RFBmix	2420.281	2318	21	31.9
B5species1%mix	2420.291	2634	21	34.14
A1%RFBmix	2434.29	2464	22	34.07
B5species1%mix	2434.292	2391	21	34.14
A1%RFBmix	2438.28	1857	18	25.38
B5species1%mix	2438.285	3005	18	27.4
A1%RFBmix	2478.218	2479	13	14.51
B5species1%mix	2478.235	1832	12	13.91
A1%RFBmix	2479.323	3160	21	31.9
B5species1%mix	2479.313	1832	20	31.89
A1%RFBmix	2497.322	3206	18	25.38
B5species1%mix	2497.327	5821	18	27.4
A1%RFBmix	2605.074	1268	19	27.55
B5species1%mix	2605.135	1402	19	29.64
A1%RFBmix	2617.93	4254	19	27.55
B5species1%mix	2617.949	3167	18	27.4
A1%RFBmix	2665.123	6574	18	25.38
B5species1%mix	2665.131	4620	17	25.15
A1%RFBmix	2719.174	1670	18	25.38

B5species1%mix	2719.196	2189	18	27.4
A1%RFBmix	2778.207	3565	21	31.9
B5species1%mix	2778.21	2061	20	31.89
A1%RFBmix	2835.229	652	20	29.72
B5species1%mix	2835.24	1763	20	31.89
A1%RFBmix	2895.481	1443	21	31.9
B5species1%mix	2895.476	1766	21	34.14
A1%RFBmix	2942.637	1059	23	36.25
B5species1%mix	2942.551	1361	23	38.63
A1%RFBmix	2943.603	1116	24	38.42
B5species1%mix	2943.592	2968	24	40.88
A1%RFBmix	2960.652	1168	20	29.72
B5species1%mix	2960.636	3406	20	31.89
A1%RFBmix	2961.612	6302	21	31.9
B5species1%mix	2961.607	13710	21	34.14
A1%RFBmix	3017.556	2566	14	16.68
B5species1%mix	3017.569	1456	14	18.41
A1%RFBmix	3158.507	1275	22	34.07
B5species1%mix	3158.531	1990	22	36.38
A1%RFBmix	3231.666	737	16	21.03
B5species1%mix	3231.703	1942	16	22.9
A1%RFBmix	3485.713	1947	22	34.07
B5species1%mix	3485.715	1435	22	36.38
A1%RFBmix	3498.704	1191	22	34.07
B5species1%mix	3498.706	1180	21	34.14
A1%RFBmix	3505.225	2420	14	16.68
B5species1%mix	3505.231	10415	13	16.16
A1%RFBmix	3518.738	914	21	31.9
B5species1%mix	3518.815	960	21	34.14
A1%RFBmix	3536.847	269	20	29.72
B5species1%mix	3536.832	968	20	31.89
A1%RFBmix	3544.816	314	20	29.72
B5species1%mix	3544.828	615	20	31.89
A1%RFBmix	3562.838	1008	18	25.38
B5species1%mix	3562.841	1330	18	27.4
A1%RFBmix	3576.257	1349	14	16.68
B5species1%mix	3576.28	2866	14	18.41
A1%RFBmix	3576.853	571	19	27.55
B5species1%mix	3576.863	805	18	27.4

A1%RFBmix	3761.693	936	29	49.29
B5species1%mix	3761.702	1010	28	49.87
A1%RFBmix	3818.7	3268	29	49.29
B5species1%mix	3818.683	3101	28	49.87
A1%RFBmix	4288.262	149	20	29.72
B5species1%mix	4288.176	1321	20	31.89
A1%RFBmix	4434.657	665	15	18.85
B5species1%mix	4434.703	1149	15	20.65

A1%RFBmix – Wheat flour and *T. castaneum* mixture with 1% *T. castaneum* by mass

B5species1%mix – Wheat flour and 5 species (0.2% each of *C. ferrugineus, R. dominica, S. oryzae, T. castaneum*, and *T. confusum*) mixture by mass

APPENDIX D: One hundred and twenty-four pairs of similar peaks between the wheat flour and *T. castaneum* mixture with 1% *T. castaneum* and the one with 0.1% *T. castaneum* without peaks from wheat flour and systems

Sample	Mass	Intensity	Fraction	Hydrophobicity	
A1%RFBmix	564.624	8121	15	18.85	
B0.1%RFBmix	564.629	4133	13	16.8	
A1%RFBmix	578.66	14893	3	-7.23	
B0.1%RFBmix	578.661	8848	3	-7.24	
B0.1%RFBmix	578.665	7776	4	-4.84	
A1%RFBmix	603.546	2267	28	47.12	
B0.1%RFBmix	603.535	1747	26	48.05	
B0.1%RFBmix	603.561	2510	24	43.24	
A1%RFBmix	668.625	4941	24	38.42	
B0.1%RFBmix	668.624	4136	23	40.83	
A1%RFBmix	682.664	8099	24	38.42	
B0.1%RFBmix	682.651	8115	23	40.83	
A1%RFBmix	750.456	2881	21	31.9	
B0.1%RFBmix	750.459	2010	19	31.22	
A1%RFBmix	788.895	29824	3	-7.23	
B0.1%RFBmix	788.899	14496	3	-7.24	
A1%RFBmix	877.445	5637	18	25.38	
B0.1%RFBmix	877.449	1765	17	26.41	
A1%RFBmix	903.039	3572	37	66.68	
B0.1%RFBmix	903.04	3570	35	69.68	
A1%RFBmix	903.04	2486	26	42.77	
B0.1%RFBmix	903.038	2623	24	43.24	
A1%RFBmix	948.491	4755	17	23.2	
B0.1%RFBmix	948.482	5401	15	21.6	
A1%RFBmix	1002.636	1649	27	44.94	
B0.1%RFBmix	1002.639	2069	24	43.24	
A1%RFBmix	1017.532	2568	18	25.38	
B0.1%RFBmix	1017.533	1408	17	26.41	
A1%RFBmix	1035.626	3919	18	25.38	
B0.1%RFBmix	1035.628	2194	17	26.41	
A1%RFBmix	1040.502	1879	20	29.72	
B0.1%RFBmix	1040.504	1512	19	31.22	

A1%RFBmix	1047.548	4350	18	25.38	
B0.1%RFBmix	1047.548	2199	17	26.41	
A1%RFBmix	1063.544	6187	17	23.2	
B0.1%RFBmix	1063.537	5401	16	24.01	
A1%RFBmix	1121.591	20914	17	23.2	
B0.1%RFBmix	1121.583	12457	16	24.01	
A1%RFBmix	1148.594	2329	18	25.38	
B0.1%RFBmix	1148.602	1869	17	26.41	
A1%RFBmix	1164.592	7409	18	25.38	
B0.1%RFBmix	1164.592	2420	17	26.41	
A1%RFBmix	1249.654	5166	17	23.2	
B0.1%RFBmix	1249.652	1576	17	26.41	
A1%RFBmix	1265.668	3570	20	29.72	
B0.1%RFBmix	1265.669	8431	18	28.81	
A1%RFBmix	1279.664	8203	17	23.2	
B0.1%RFBmix	1279.653	5735	16	24.01	
A1%RFBmix	1281.646	997	20	29.72	
B0.1%RFBmix	1281.654	1054	19	31.22	
A1%RFBmix	1311.654	910	20	29.72	
B0.1%RFBmix	1311.657	1170	19	31.22	
A1%RFBmix	1320.673	2222	19	27.55	
B0.1%RFBmix	1320.681	2524	18	28.81	
A1%RFBmix	1327.648	3027	20	29.72	
B0.1%RFBmix	1327.655	2319	19	31.22	
A1%RFBmix	1343.748	2408	22	34.07	
B0.1%RFBmix	1343.746	2756	21	36.03	
A1%RFBmix	1350.688	2257	19	27.55	
B0.1%RFBmix	1350.695	1699	17	26.41	
A1%RFBmix	1366.683	14328	18	25.38	
B0.1%RFBmix	1366.688	4289	17	26.41	
B0.1%RFBmix	1366.712	3070	18	28.81	
A1%RFBmix	1405.74	37070	18	25.38	
B0.1%RFBmix	1405.744	22473	17	26.41	
A1%RFBmix	1435.755	48629	18	25.38	
B0.1%RFBmix	1435.757	22312	17	26.41	
A1%RFBmix	1441.871	1666	26	42.77	
B0.1%RFBmix	1441.871	2162	24	43.24	
A1%RFBmix	1476.777	38062	19	27.55	
B0.1%RFBmix	1476.784	36079	18	28.81	

A1%RFBmix	1476.795	2671	21	31.9	
B0.1%RFBmix	1476.804	1821	20	33.62	
A1%RFBmix	1483.746	3261	21	31.9	
B0.1%RFBmix	1483.75	1511	19	31.22	
A1%RFBmix	1504.781	4247	18	25.38	
B0.1%RFBmix	1504.785	1687	17	26.41	
A1%RFBmix	1506.784	29781	19	27.55	
B0.1%RFBmix	1506.791	31566	17	26.41	
A1%RFBmix	1513.753	1335	20	29.72	
B0.1%RFBmix	1513.755	1168	19	31.22	
A1%RFBmix	1522.77	3205	19	27.55	
B0.1%RFBmix	1522.772	11552	16	24.01	
B0.1%RFBmix	1522.789	954	19	31.22	
A1%RFBmix	1529.746	5371	20	29.72	
B0.1%RFBmix	1529.753	3652	19	31.22	
A1%RFBmix	1544.767	21884	18	25.38	
B0.1%RFBmix	1544.775	6475	17	26.41	
A1%RFBmix	1561.849	5421	18	25.38	
B0.1%RFBmix	1561.855	2328	17	26.41	
A1%RFBmix	1564.8	4229	19	27.55	
B0.1%RFBmix	1564.807	5496	18	28.81	
A1%RFBmix	1568.813	4937	20	29.72	
B0.1%RFBmix	1568.819	5910	19	31.22	
A1%RFBmix	1635.87	2923	21	31.9	
B0.1%RFBmix	1635.873	2019	20	33.62	
A1%RFBmix	1639.841	19925	20	29.72	
B0.1%RFBmix	1639.849	25955	19	31.22	
A1%RFBmix	1658.805	5397	18	25.38	
B0.1%RFBmix	1658.813	1731	17	26.41	
A1%RFBmix	1669.858	14595	20	29.72	
B0.1%RFBmix	1669.87	1991	20	33.62	
A1%RFBmix	1674.813	3234	24	38.42	
B0.1%RFBmix	1674.824	3156	22	38.43	
A1%RFBmix	1685.847	67308	20	29.72	
B0.1%RFBmix	1685.854	57851	19	31.22	
A1%RFBmix	1697.894	1517	28	47.12	
B0.1%RFBmix	1697.881	1237	26	48.05	
A1%RFBmix	1708.868	3478	22	34.07	
B0.1%RFBmix	1708.865	3407	21	36.03	

A1%RFBmix	1724.909	13481	20	29.72	
B0.1%RFBmix	1724.907	15493	18	28.81	
A1%RFBmix	1772.906	2945	13	14.51	
B0.1%RFBmix	1772.904	3232	12	14.39	
A1%RFBmix	1821.864	1936	20	29.72	
B0.1%RFBmix	1821.873	1487	19	31.22	
A1%RFBmix	1859.024	1862	23	36.25	
B0.1%RFBmix	1859.021	2450	22	38.43	
A1%RFBmix	1878.014	4753	21	31.9	
B0.1%RFBmix	1878.019	4971	20	33.62	
A1%RFBmix	1908.033	5193	21	31.9	
B0.1%RFBmix	1908.034	4214	20	33.62	
A1%RFBmix	1934.026	835	20	29.72	
B0.1%RFBmix	1934.04	1001	19	31.22	
A1%RFBmix	1941.883	3878	26	42.77	
B0.1%RFBmix	1941.939	5183	25	45.64	
A1%RFBmix	1949.051	9752	22	34.07	
B0.1%RFBmix	1949.053	9689	20	33.62	
A1%RFBmix	1958.609	5584	27	44.94	
B0.1%RFBmix	1958.654	5127	25	45.64	
A1%RFBmix	1979.059	6738	21	31.9	
B0.1%RFBmix	1979.066	7072	20	33.62	
A1%RFBmix	1995.057	22408	21	31.9	
B0.1%RFBmix	1995.063	14817	20	33.62	
A1%RFBmix	1997.077	7136	19	27.55	
B0.1%RFBmix	1997.084	11859	18	28.81	
A1%RFBmix	2001.053	13884	21	31.9	
B0.1%RFBmix	2001.059	15632	20	33.62	
A1%RFBmix	2009.057	2751	17	23.2	
B0.1%RFBmix	2009.038	3446	16	24.01	
A1%RFBmix	2050.083	1134	19	27.55	
B0.1%RFBmix	2050.098	2000	18	28.81	
A1%RFBmix	2112.12	5907	23	36.25	
B0.1%RFBmix	2112.116	7361	21	36.03	
A1%RFBmix	2125.014	2243	12	12.33	
B0.1%RFBmix	2125.008	2219	11	11.99	
A1%RFBmix	2142.139	2965	22	34.07	
B0.1%RFBmix	2142.132	3720	21	36.03	
A1%RFBmix	2158.134	18208	22	34.07	

B0.1%RFBmix	2158.132	16585	21	36.03	
A1%RFBmix	2187.16	4095	22	34.07	
B0.1%RFBmix	2187.151	5660	21	36.03	
A1%RFBmix	2351.23	1648	19	27.55	
B0.1%RFBmix	2351.248	2397	18	28.81	
A1%RFBmix	2420.281	2318	21	31.9	
B0.1%RFBmix	2420.292	1716	20	33.62	
A1%RFBmix	2434.29	2464	22	34.07	
B0.1%RFBmix	2434.291	1937	21	36.03	
A1%RFBmix	2478.218	2479	13	14.51	
B0.1%RFBmix	2478.22	2655	12	14.39	
A1%RFBmix	2479.323	3160	21	31.9	
B0.1%RFBmix	2479.322	2499	20	33.62	
A1%RFBmix	2497.322	3206	18	25.38	
B0.1%RFBmix	2497.328	2192	18	28.81	
A1%RFBmix	2895.481	1443	21	31.9	
B0.1%RFBmix	2895.492	1894	20	33.62	
A1%RFBmix	2960.652	1168	20	29.72	
B0.1%RFBmix	2960.664	1673	19	31.22	
A1%RFBmix	3017.556	2566	14	16.68	
B0.1%RFBmix	3017.565	2346	13	16.8	
A1%RFBmix	3231.666	737	16	21.03	
B0.1%RFBmix	3231.684	1582	15	21.6	
A1%RFBmix	3536.847	269	20	29.72	
B0.1%RFBmix	3536.84	435	19	31.22	
A1%RFBmix	3544.816	314	20	29.72	
B0.1%RFBmix	3544.841	493	19	31.22	
A1%RFBmix	3562.838	1008	18	25.38	
B0.1%RFBmix	3562.835	935	18	28.81	
A1%RFBmix	3731.339	1060	14	16.68	
B0.1%RFBmix	3731.328	1288	14	19.2	
A1%RFBmix	3761.693	936	29	49.29	
B0.1%RFBmix	3761.71	569	27	50.45	
A1%RFBmix	3818.7	3268	29	49.29	
B0.1%RFBmix	3818.701	1597	27	50.45	

A1%RFBmix – Wheat flour and *T. castaneum* mixture with 1% *T. castaneum* by mass

B0.1%RFBmix – Wheat flour and *T. castaneum* mixture with 0.1% *T. castaneum* by mass

APPENDIX E: One hundred and sixteen pairs of similar peaks between the wheat flour and *T. castaneum* mixture with 1% *T. castaneum* and the wheat flour and 5 species (0.2% each of *C. ferrugineus, R. dominica, S. oryzae, T. castaneum*, and *T. confusum*) mixture without peaks from wheat flour and systems

Sample	Mass	Intensity	Fraction	Hydrophobicity
A1%RFBmix	877.445	5637	18	25.38
B5species1%mix	877.442	3253	17	25.15
A1%RFBmix	903.039	3572	37	66.68
B5species1%mix	903.039	2173	34	63.35
A1%RFBmix	903.04	2486	26	42.77
B5species1%mix	903.033	1691	26	45.37
A1%RFBmix	1002.636	1649	27	44.94
B5species1%mix	1002.631	4598	25	43.13
A1%RFBmix	1035.626	3919	18	25.38
B5species1%mix	1035.63	6912	18	27.4
A1%RFBmix	1040.502	1879	20	29.72
B5species1%mix	1040.502	5161	19	29.64
A1%RFBmix	1049.568	5899	13	14.51
B5species1%mix	1049.569	7044	12	13.91
A1%RFBmix	1063.544	6187	17	23.2
B5species1%mix	1063.545	3720	17	25.15
A1%RFBmix	1103.582	2330	19	27.55
B5species1%mix	1103.582	5049	18	27.4
A1%RFBmix	1121.591	20914	17	23.2
B5species1%mix	1121.586	5685	16	22.9
A1%RFBmix	1180.594	848	20	29.72
B5species1%mix	1180.604	1811	19	29.64
A1%RFBmix	1187.646	2536	22	34.07
B5species1%mix	1187.642	3696	22	36.38
A1%RFBmix	1210.599	726	20	29.72
B5species1%mix	1210.605	2095	19	29.64
A1%RFBmix	1226.605	2047	20	29.72
B5species1%mix	1226.601	6873	19	29.64

A1%RFBmix	1265.668	3570	20	29.72
B5species1%mix	1265.665	9238	19	29.64
A1%RFBmix	1320.673	2222	19	27.55
B5species1%mix	1320.679	4691	18	27.4
A1%RFBmix	1327.648	3027	20	29.72
B5species1%mix	1327.656	2868	19	29.64
A1%RFBmix	1343.748	2408	22	34.07
B5species1%mix	1343.749	6458	22	36.38
A1%RFBmix	1350.688	2257	19	27.55
B5species1%mix	1350.696	5178	18	27.4
A1%RFBmix	1366.683	14328	18	25.38
B5species1%mix	1366.691	6124	17	25.15
A1%RFBmix	1366.718	2544	20	29.72
B5species1%mix	1366.713	4386	19	29.64
A1%RFBmix	1405.74	37070	18	25.38
B5species1%mix	1405.742	22281	18	27.4
A1%RFBmix	1435.755	48629	18	25.38
B5species1%mix	1435.759	21882	18	27.4
A1%RFBmix	1441.871	1666	26	42.77
B5species1%mix	1441.873	2162	25	43.13
A1%RFBmix	1467.761	708	20	29.72
B5species1%mix	1467.761	5301	19	29.64
A1%RFBmix	1476.777	38062	19	27.55
B5species1%mix	1476.783	43985	18	27.4
A1%RFBmix	1483.746	3261	21	31.9
B5species1%mix	1483.743	3670	20	31.89
B5species1%mix	1483.758	2474	21	34.14
A1%RFBmix	1506.784	29781	19	27.55
B5species1%mix	1506.789	39094	18	27.4
A1%RFBmix	1513.753	1335	20	29.72
B5species1%mix	1513.752	2854	20	31.89
A1%RFBmix	1529.746	5371	20	29.72
B5species1%mix	1529.75	9580	19	29.64
A1%RFBmix	1561.849	5421	18	25.38
B5species1%mix	1561.855	4050	17	25.15
A1%RFBmix	1564.8	4229	19	27.55
B5species1%mix	1564.81	5319	18	27.4
A1%RFBmix	1568.813	4937	20	29.72
B5species1%mix	1568.811	24149	19	29.64

A1%RFBmix	1587.773	1467	24	38.42
B5species1%mix	1587.778	3641	23	38.63
A1%RFBmix	1593.862	2400	21	31.9
B5species1%mix	1593.833	1228	20	31.89
A1%RFBmix	1639.841	19925	20	29.72
B5species1%mix	1639.841	24352	20	31.89
A1%RFBmix	1669.858	14595	20	29.72
B5species1%mix	1669.856	11322	20	31.89
A1%RFBmix	1674.813	3234	24	38.42
B5species1%mix	1674.815	5003	23	38.63
A1%RFBmix	1708.868	3478	22	34.07
B5species1%mix	1708.867	5354	22	36.38
A1%RFBmix	1724.909	13481	20	29.72
B5species1%mix	1724.906	59952	19	29.64
A1%RFBmix	1727.86	2844	21	31.9
B5species1%mix	1727.872	3474	20	31.89
A1%RFBmix	1800.902	1947	29	49.29
B5species1%mix	1800.925	2171	28	49.87
A1%RFBmix	1821.864	1936	20	29.72
B5species1%mix	1821.87	3141	19	29.64
A1%RFBmix	1837.954	711	20	29.72
B5species1%mix	1837.978	2010	19	29.64
A1%RFBmix	1878.014	4753	21	31.9
B5species1%mix	1878.019	5270	21	34.14
A1%RFBmix	1903.824	4345	28	47.12
B5species1%mix	1903.851	4329	27	47.62
A1%RFBmix	1908.033	5193	21	31.9
B5species1%mix	1908.031	4586	21	34.14
A1%RFBmix	1949.051	9752	22	34.07
B5species1%mix	1949.054	11009	21	34.14
A1%RFBmix	1979.059	6738	21	31.9
B5species1%mix	1979.068	8559	21	34.14
A1%RFBmix	1995.057	22408	21	31.9
B5species1%mix	1995.051	5961	20	31.89
A1%RFBmix	1997.077	7136	19	27.55
B5species1%mix	1997.08	11477	19	29.64
A1%RFBmix	2001.053	13884	21	31.9
B5species1%mix	2001.062	6983	21	34.14
A1%RFBmix	2009.057	2751	17	23.2

B5species1%mix	2009.063	17994	17	25.15
A1%RFBmix	2037.982	3541	14	16.68
B5species1%mix	2037.992	1007	13	16.16
A1%RFBmix	2112.12	5907	23	36.25
B5species1%mix	2112.12	9591	22	36.38
A1%RFBmix	2125.014	2243	12	12.33
B5species1%mix	2125.028	2407	12	13.91
A1%RFBmix	2142.139	2965	22	34.07
B5species1%mix	2142.144	4816	22	36.38
A1%RFBmix	2158.134	18208	22	34.07
B5species1%mix	2158.132	16801	22	36.38
A1%RFBmix	2187.16	4095	22	34.07
B5species1%mix	2187.162	3385	22	36.38
A1%RFBmix	2270.077	507	20	29.72
B5species1%mix	2270.082	1493	20	31.89
A1%RFBmix	2351.23	1648	19	27.55
B5species1%mix	2351.243	5752	19	29.64
A1%RFBmix	2368.016	1357	13	14.51
B5species1%mix	2368.017	970	13	16.16
A1%RFBmix	2420.281	2318	21	31.9
B5species1%mix	2420.291	2634	21	34.14
A1%RFBmix	2434.29	2464	22	34.07
B5species1%mix	2434.292	2391	21	34.14
A1%RFBmix	2438.28	1857	18	25.38
B5species1%mix	2438.285	3005	18	27.4
A1%RFBmix	2478.218	2479	13	14.51
B5species1%mix	2478.235	1832	12	13.91
A1%RFBmix	2479.323	3160	21	31.9
B5species1%mix	2479.313	1832	20	31.89
A1%RFBmix	2497.322	3206	18	25.38
B5species1%mix	2497.327	5821	18	27.4
A1%RFBmix	2895.481	1443	21	31.9
B5species1%mix	2895.476	1766	21	34.14
A1%RFBmix	2942.637	1059	23	36.25
B5species1%mix	2942.551	1361	23	38.63
A1%RFBmix	2943.603	1116	24	38.42
B5species1%mix	2943.592	2968	24	40.88
A1%RFBmix	2960.652	1168	20	29.72
B5species1%mix	2960.636	3406	20	31.89

A1%RFBmix	2961.612	6302	21	31.9
B5species1%mix	2961.607	13710	21	34.14
A1%RFBmix	3017.556	2566	14	16.68
B5species1%mix	3017.569	1456	14	18.41
A1%RFBmix	3158.507	1275	22	34.07
B5species1%mix	3158.531	1990	22	36.38
A1%RFBmix	3231.666	737	16	21.03
B5species1%mix	3231.703	1942	16	22.9
A1%RFBmix	3485.713	1947	22	34.07
B5species1%mix	3485.715	1435	22	36.38
A1%RFBmix	3536.847	269	20	29.72
B5species1%mix	3536.832	968	20	31.89
A1%RFBmix	3544.816	314	20	29.72
B5species1%mix	3544.828	615	20	31.89
A1%RFBmix	3562.838	1008	18	25.38
B5species1%mix	3562.841	1330	18	27.4
A1%RFBmix	3761.693	936	29	49.29
B5species1%mix	3761.702	1010	28	49.87
A1%RFBmix	3818.7	3268	29	49.29
B5species1%mix	3818.683	3101	28	49.87
A1%RFBmix	4288.262	149	20	29.72
B5species1%mix	4288.176	1321	20	31.89
A1%RFBmix	4434.657	665	15	18.85
B5species1%mix	4434.703	1149	15	20.65

A1%RFBmix – Wheat flour and *T. castaneum* mixture with 1% *T. castaneum* by mass

B5species1%mix – Wheat flour and 5 species (0.2% each of *C. ferrugineus, R. dominica, S. oryzae, T. castaneum*, and *T. confusum*) mixture by mass