

## Serum Proteomic Analysis for the Identification of Biomarkers by Two-Dimensional Differential Gel Electrophoresis (2D-DIGE) after Exposure to the Food-Processed Contaminant Furan

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### Abstract

**Background:** Furan is a compound known to be present in cooked or thermally-processed foods by formation through traditional heat treatment processes and also known to be a potent hepatotoxin. To identify potential biomarkers of furan exposure, two-dimensional differential gel electrophoresis (2D-DIGE) was performed on individual serum samples to identify proteomic profiles that were differentially expressed in rats exposed to furan at 0, 0.03, 0.5 and 8.0 mg/kg bw/day.

**Results:** There were no differences in protein expression between control and the 0.03 and 0.5 mg/kg bw/day dose groups (one-way ANOVA  $P < 0.05$ , avg ratio +1.5). At the 8.0 mg/kg bw/day of furan exposure, there were 22 protein spots that showed a difference in expression when compared to the control animals (one-way ANOVA  $P < 0.05$ , avg ratio +1.5). After manual spot picking, four proteins were identified by standard in-gel tryptic digestion followed by mass spectrometry and bioinformatics.

**Conclusions:** Of these four proteins apolipoprotein C-III (isoform CRA\_b) and fetuin-B (precursor) were up-regulated, whereas  $\alpha$ -1-macroglobulin and pre-proapolipoprotein A-1 were down-regulated. These biomarkers could be of diagnostic relevance to identify furan exposure.

**Keywords:** Furan; Biomarkers; Serum protein profiling

### Introduction

Furan is a colorless, volatile, lipophilic compound that is formed during traditional heat treatment processes such as cooking, canning, and baking [1]. A recent survey performed by the United States Food and Drug Administration [2] on approximately 300 food samples showed that furan is present in a wide range of foods up to levels greater than 100 ng/g. For example, it is found in cooked and canned meats, roasted coffee, beer and wheat breads [1,3-5]. These data have been supported by similar findings by the European Food Safety Authority [6] and the Swiss Office of Public Health [7]. In humans, furan has been observed in breast milk samples and in the breath of both smokers and non-smokers [8-10]. Hence, based on the widespread industrial use and potential exposure to furan, the toxic properties of this compound are a public health concern. Furan had been previously found to be a potent hepatotoxin [11,12] although little toxicological information was available until recently [13]. Recent proteomic studies of human body fluids have resulted in identifying potential biomarker candidates for many conditions [14,15]. Of particular interest in preclinical and clinical safety assessments are biomarkers in the peripheral blood or serum that are quantitatively altered functionally or morphologically as an indication of the early exposure to chemicals or in the progression of diseases. This is particularly true for diseases that are either asymptomatic or where a late diagnosis generally results in a poor outcome. Early detection of

hazardous exposures of humans may significantly reduce adverse health effects through appropriate reductions [16]. Plasma or serum is a preferred specimen for the early diagnosis of malignant tumors or chemical exposure because samples are readily accessible by non-invasive methods. Protein profiling of serum offers opportunities to discover potentially new biomarkers for the early detection of chemical exposure or diseases and could also facilitate their prognosis [14,15].

Recent advances in proteomic technologies by two-dimensional differential gel electrophoresis (2D-DIGE) and improved mass spectrometry have provided new opportunities for identifying biomarkers and therapeutic targets [17]. 2D-DIGE is effective in separating and quantifying complex protein samples. A pool of all the samples within an experiment is used as a common internal standard on each gel with a dedicated fluorescent dye (Cy2TM). This allows all proteins to be included within the experiment to ultimately match and normalize protein patterns within the same gel and across different gels thus reducing the problems of intra- and inter-gel variations. Quantitative comparisons of proteins from individual samples randomized through additional fluorescent dyes (Cy3TM/ Cy5TM) are analysed by the relative changes between the protein spots and the internal standard (Cy2TM) thus allowing accurate quantification of induced biological change between samples with an associated statistical significance (Ettan DIGE System User Manual, 18-1173-17, Edition AB). Proteins of interest (POIs) can then be selected, excised and identified by tandem mass spectrometry (LC-MS/MS) and bioinformatics.

The object of this study was to use proteomic approaches (2D-DIGE/LC-MS/MS) to assess possible biomarkers of exposure in male rats that were administered furan at 0.0, 0.03, 0.5 and 8.0 mg/kg bw/day. Significant differences in protein expression (one-way ANOVA  $P < 0.05$ , avg ratio+1.5) between control and various dose groups was used to establish protein profiles that could be relevant for early discrimination between these groups. In addition, our objectives were to identify serum proteins in rats that might serve as sensitive indicators of hepatomegaly, hepatocellular necrosis or hepatobiliary injury as these were the histological observations from our previous study [13].

## Method and Material

### Test compound and dosing solutions

Furan doses were prepared by mixing the appropriate quantity of furan with Mazola<sup>®</sup> corn oil to deliver final concentrations at 0, 0.03, 0.12, 0.5, 2.0 and 8.0 mg/ml. Each dose was prepared separately on a volume-to-weight (v:w) ratio. Chilled corn oil was weighed to the nearest milligram in a conical flask. Chilled furan solution was drawn up in a Hamilton syringe, measured to the nearest microliter, injected into corn oil and mixed using a magnetic stir bar. Dosing solutions were dispensed into brown glass vials and capped with plastic closures adapted with silicon septa ensuring no remaining air space. Dosing solutions were stored at 4°C with fresh solutions were prepared every 14 days [18].

### Animal studies

Fischer-344 male and female rats were obtained from Charles River Laboratories Inc. (St.-Constant, QC) at 5-6 weeks of age and were acclimatized for a period of twelve days before studies began. Animals were handled and treated according to the Guidelines of the Canada Council of Animal Care (Ottawa, ON). Animals received treatment by gavage over a 90-day period, dosing 5 days a week final furan concentrations at 0, 0.03, 0.12, 0.5, 2.0 and 8.0 mg/kg bw/day. These animals were weighed daily on weekdays prior to gavage. Food consumption was measured on a weekly basis [13].

### Sample collection

At the end of the study, animals were sacrificed by exsanguination under isoflurane anaesthesia. Blood from the abdominal aorta was collected in SST Vacutainer<sup>®</sup> tubes (Becton-Dickinson, Franklin Lakes, NJ) and allowed to clot at room temperature. Serum was separated by centrifugation at 3,000x g, aliquoted and stored at -80°C for two-dimensional differential gel electrophoresis (2D-DIGE) and clinical chemistry analysis. Each sample was limited to a maximum of 3 freeze/thaws cycles [13].

### Immunodepletion of serum

Randomly selected male serum samples (n=5) at doses of 0, 0.03, 0.5 and 8.0 mg/kg bw/day were processed using the Albumin and IgG Depletion Spintrap Kit (GE Healthcare) following the manufacturer's instructions. This allowed the removal of high abundant proteins (Albumin/IgG) allowing a larger quantity of less abundant proteins to be analyzed by 2D-DIGE. In brief, each column was depleted of storage solution by centrifugation for 30 seconds at 100 xg. Columns were then equilibrated several times with 400 l binding buffer (20 mM sodium phosphate, 0.15 M sodium chloride pH 7.4) followed by centrifugation

for 30 sec at 800 xg and discarding the flow through. Each diluted serum sample (50 l serum: 50l binding buffer) was added to a column in a new tube and incubated at room temperature for 5 min. The depleted sample was collected by centrifugation for 30 seconds at 800x g. Binding buffer (100 l) was added twice to each column and centrifuged for 30 seconds at 800x g to give a final volume of 300 l of depleted serum. Immunodepleted serum was diluted (1:2) in binding buffer to determine protein concentrations by the Bradford assay (BioRad). Samples were stored at -80°C until further proteomic analysis.

### Preparation of samples for two-dimensional gel electrophoresis

Samples were additionally precipitated while removing interfering substances to improve the labelling efficiency by using the 2D Clean-up Kit (GE Healthcare) following the manufacturer's instructions. All other reagents were purchased from GE Healthcare unless otherwise specified. Pellets were resuspended in Standard Cell Lysis Buffer (pH 9.0) containing 7 M urea, 2 M thioruea, 30 mM Tris-HCL, 4% (w/v) 3-[3-cholamidopropyl] dimethylammonio]-1-propanesulfonate (CHAPS) to a final concentration of [10 g/l]. All samples were adjusted to a final pH of 9.0. Stock solution of CyDye DIGE Fluor Cy2TM, Cy3 TM and Cy5 TM minimal dyes were made by adding 5 l of fresh dimethylformamide (Sigma-Aldrich) to each vial containing 5nmol of dye. To take advantage of the multiplexing capabilities of 2D-DIGE, an internal standard representing a pool of all samples (n=20) was labelled with Cy2TM to be included on each gel to facilitate gel-to-gel matching and statistical analysis. The internal standard protein (Cy2TM) was labelled in a single tube for all gels in the study for improved consistency. For analytical gels, each sample containing 50 g of protein was labelled with 400 pmol of the appropriate dye. Each individual protein sample was labelled with either Cy3 TM or Cy5 TM alternating dyes between doses in a random fashion. Labelling was conducted in the dark for 30 minutes and the reaction was stopped with 0.2 µl of 10 mM Lysine as outlined for 2D-DIGE minimal labelling in the EttanTM DIGE Imager user manual (GE Healthcare).

### Two-dimensional gel electrophoresis

First dimensional separation was completed using 18 cm Immobiline Drystrips pH 4-7 (GE Healthcare) with the Ettan IPGphor3 Isoelectric Focusing System. The Cy2 TM, Cy3 TM and Cy5TM labelled samples for each analytical gel were combined and the total volume adjusted to 340 l by the addition of rehydration buffer containing 7 M Urea, 2 M Thioruea, 2% (w/v) 3-[3-cholamidopropyl] dimethylammonio]-1-propanesulfonate (CHAPS), 0.5% IPG Buffer pH 4-7, 0.2% (w/v) Dithiothreitol (DTT) and bromophenol blue. Proteins were separated in 18 cm ceramic strip holders (GE Healthcare) by active rehydration following the manufacturer's instructions (GE Healthcare). In brief, each labelled sample in rehydration buffer was placed into an 18 cm ceramic strip holder between the electrodes. Immobiline DryStrips 18 cm pH 4-7 were placed gel side down into the strip holder using forceps. DryStrip Cover Fluid was added to the top of each strip to prevent the evaporation of sample or urea crystallization. Ceramic strip holders were added to the Ettan IPGphor3 unit for first dimensional separation of proteins by isoelectric focusing (IEF) at 20°C and 75 A/strip using the outlined running protocol: 14 hrs at 30 V; 1 hr at 500 V; 1 hr gradient up to 1,000 V; 3 hr gradient up to 8,000 V and a final step of 8,000 V to a total of 33217 V hrs. The focused Immobiline Drystrips had the excess

cover fluid removed and were stored at  $-80^{\circ}\text{C}$  for several hours until the equilibration of strips and second dimensional separation was performed.

Second dimensional separation (SDS-PAGE) was completed by casting 15% polyacrylamide gels using the Ettan DALT Gel Caster and low fluorescent Ettan DALT plates (GE Healthcare). Gels were overlaid with water-saturated butanol for one hour followed by 1x SDS DALT electrophoresis running buffer and allowed to polymerize overnight. Focused Immobline Drystrips were removed from  $-80^{\circ}\text{C}$  and left in a solution containing Equilibration Buffer (6 M Urea, 30% glycerol, 2% (w/v) SDS, 50 mM Tris-HCl (1.5 M, pH 8.8) and 0.02% bromophenol blue) with the addition of 0.5% (w/v) of DTT for 15 minutes. This was followed by a second incubation of Equilibration Buffer but with the addition of 4.5% (w/v) Iodoacetamide for 15 minutes. The strip was added to the top of each gel and covered with 0.5% (w/v) agarose overlay solution containing 0.02% bromophenol blue. Electrophoresis was performed using the Ettan DALT six electrophoresis system with a Multitemp III cooling unit and the EPS601 Electrophoresis Power Supply (GE Healthcare) by applying 0.5 W/gel overnight followed by 17 W/gel until the bromophenol blue dye front reached the bottom of the gels.

### Image acquisition and statistical analysis

Analytical gels were scanned using the EttanTM DIGE Imager (GE Healthcare) at 100  $\mu\text{m}$  resolution using excitation/emission wavelengths specified by selecting the DIGE File Naming Format: Cy2 TM at 480 nm/530 nm, Cy3 TM at 540 nm/595 nm and Cy5 TM at 635 nm/680 nm which enabled the fluorescence proteins to be visualized. The exposure was set for each channel in that the maximum pixel value was achieved without saturation as outlined in the EttanTM DIGE Imager user manual (GE Healthcare). Images were analysed using the Decyder 2D software v7.0 (GE Healthcare) according to the manufacturer's recommendation. In brief, all gel images were viewed and cropped in the Image Loader. This allowed the images to be the same size and exclude areas (i.e., vertical streaks at extreme pH, Immobline Drystrip, dye front) to achieve relevant areas of interest and optimize spot matching across the individual gels. A single cropped "master" gel containing an internal standard (Cy2 TM) and two individual samples (Cy3 TM, Cy5 TM) was analyzed in the DIA (Differential In-Gel Analysis) module. Spot detection was performed using an estimation of 10,000 spots with an exclusion of background by selecting the volume of the first true spot. Cropped images (n=30) representing each channel (Cy2 TM, Cy3 TM and Cy5 TM) of all gels were processed using the Batch Processor module which performs automated matching of spot boundaries from all gels to the "master" gel using the parameters selected in the DIA module. Samples were specified into dose groups or as internal standards with the master gel identified. Individual samples (Cy3 TM, Cy5 TM) were compared to the corresponding protein spot of the internal standard (Cy2 TM) on the same gel to provide spot protein abundance ratios (i.e., Cy2: Cy3, Cy2: Cy5) used for normalization. In addition, the identical pooled internal standard (Cy2 TM) from each gel was matched and spot protein abundance ratios were compared from each gel to reduce the amount of gel-to-gel variability. Overall, the intra- and inter-gel variation is reduced allowing only the biological variation to be compared in future analysis. Images were then viewed in the BVA (Biological Variation Analysis) module to verify that all spots on the gel images were matched.

Statistical analysis was conducted using the EDA (Decyder Extended Data Analysis) module. A base set was created to filter both proteins and spot maps by selecting proteins spots present in 90% of the gel images and removing unassigned proteins. Differential Expression Analysis calculations for one-way ANOVA ( $P < 0.05$ ) with a multi-comparison test between all doses as well as the t-test and average ratio between each dose and the control were performed. An additional set was created by filtering only those proteins which had a one-way ANOVA ( $P < 0.05$ ) and a change in avg ratio expression of +1.5 to be used for future analysis. Principle Component Analysis (PCA) was conducted to analyse the protein distribution patterns for dose related changes and identify differentially expressed proteins. Pattern Analysis was completed by Hierarchical Clustering, Kmeans and Self-Organizing Maps to visualize the data and group similar data into subsets or clusters. Points of interest (POIs) for further analysis were identified based on results of the Differential Expression Analysis (one-way ANOVA  $P < 0.05$ , avg ratio +1.5) compared to the control dose. All the POIs identified as significant, were exported as a pick list into BVA where all spots were manually confirmed before proceeding with the preparative gels for spot picking.

### Preparative gels and spot picking

Preparative gels were conducted in duplicate using the same protocol as analytical gels with the following exceptions. A single sample containing 500 g of pooled unlabelled protein was used which represented all dose groups. This enabled more protein to be selected for mass spectrometry analysis. First dimensional separation was completed on the unlabelled sample and the total volume was adjusted to 340 l by the addition of rehydration buffer. For second dimensional separation, the glass plates were first washed in 1% Decon (v/v) overnight, 1% HCl for one hour and then treated with Bind Saline working solution (GE Healthcare) and left to dry for a minimum of 1.5 hours before casting the gels. Upon completion of the electrophoresis, the glass plates were separated with the gel remaining on the bind saline treated plate. Gels were notched for identification and placed in a tray covered with fixing solution (50% Methanol, 7% Acetic acid) for 30 minutes on an orbital shaker twice. Fixed gels were placed in a new tray covered with SYPRO Ruby staining solution in the dark until optimal staining was achieved. In order to minimize background, gels were transferred to a tray with wash solution (10% Methanol, 7% Acetic acid) for 30 minutes on an orbital shaker. This was followed by two washes in ultrapure water before imaging. Preparative gels were scanned using the EttanTM DIGE Imager (GE Healthcare) at 480 nm/595 nm (SR1-filter setting) which enabled the fluorescence proteins to be visualized. The exposure was set in that the maximum pixel value was achieved without saturation. Gels were scanned to identify POIs and improve manual spot picking. Gels were placed on a Dark Reader<sup>®</sup> Transilluminator (Clare Chemical Research, Dolores, CO, USA) and POI's were manually picked using the OneTouch Plus spot-picker pipette with disposable tips (Gel Company Inc., San Francisco, CA, USA). Selected gel plugs containing POIs were placed in a microcentrifuge tube containing 1% acetic acid and then stored at  $-20^{\circ}\text{C}$  until identification. Gels were scanned a second time to confirm spot picking.

### Tryptic digestion

Excised spots were placed in low-binding tubes (LoBind, Eppendorf) and washed with 200  $\mu\text{l}$  of water (Milli-Q 18  $\Omega$ ). The gel pieces were dehydrated with 200  $\mu\text{l}$  of 50% acetonitrile (ACN)/buffer

(50 mM  $\text{NH}_4\text{HCO}_3$ ) followed by another 200  $\mu\text{l}$  of 100% ACN. The ACN was removed and the gel pieces were dried in a vacuum centrifuge (Thermo Electron Corporation, Savant, DNA 120 SpeedVac Concentrator) to remove residual solvent. The gel pieces were rehydrated with 50  $\mu\text{l}$  DTT (500 mM in buffer) and the protein cysteine residues were allowed to reduce for 30 minutes at 37°C. The proteins were alkylated in the dark at room temperature for 30 minutes by the addition of 50  $\mu\text{l}$  of a freshly prepared iodoacetamide (1 M in buffer). The gel pieces were washed with 400  $\mu\text{l}$  Milli-Q water to remove any residual iodoacetamide and the digestion was initiated by the addition of trypsin (10  $\mu\text{l}$  of 20 ng/ $\mu\text{l}$  Promega Sequence Grade Modified in buffer) and a minimal amount of buffer (30  $\mu\text{l}$ ) to cover the gel pieces. The samples were incubated overnight at 37°C. The digested peptides were extracted from the gel plugs with 50  $\mu\text{l}$  of 1% formic acid (FA) and mixed for 10 minutes (max speed on bench top centrifuge) and the supernatant was removed and put into a new low binding tube. The gel pieces were then extracted with 80  $\mu\text{l}$  of 70% ACN/5% FA and mixed for 10 minute, adding the supernatant to the previous supernatant. The peptide solution was dried in a vacuum centrifuge and reconstituted in mass spectrometry buffer (30  $\mu\text{l}$  of 0.1% FA).

### LC-MS/MS analysis and protein identification

Liquid chromatography (LC) and mass spectrometry (MS) was performed on a hybrid MALDI Q-TOF Premier (Waters, Milford, MA) fitted with a nanolockspray source, which was coupled to a nanoAcquity UPLC system (Waters, Milford, MA). An auxiliary solvent manager was used to deliver a reference mass calibration standard [Glu1]-Fibrinopeptide B in 50% aqueous methanol (0.5  $\mu\text{M}$ ). The LC system consisted of a trap column (Symmetry C18, 5  $\mu\text{m}$  180  $\mu\text{m}$  x 20 mm, Waters) and an analytical column (BEH 130 C18, 1.7  $\mu\text{m}$  100  $\mu\text{m}$  x 100 mm, Waters). Solvent A consisted of  $\text{H}_2\text{O}$  with 0.1% FA and solvent B consisted of ACN with 0.1% FA. In a typical experiment, digests were injected (4  $\mu\text{L}$ ) onto the trap column for 3 minute at a flow rate of 3.0  $\mu\text{L}/\text{min}$  using 99% solvent A. The samples were then diverted to the analytical column and eluted at 300 nL/min. The elution program started with 99% A for 1 minute followed by a gradient to 50% B in 35 minutes.

The mass spectrometer was operated in positive ion mode in a V configuration (resolution >10000 FWHH) and the acquired data collected using MassLynx v4.1 software package. Data was collected in both MS full scan survey mode and an automated data directed analysis (DDA) mode. MS survey scan data was acquired in continuum mode from m/z 100 to 1500 and the collection of MS/MS information (m/z 100 to 1500) was triggered when the threshold rose above a minimum (5 counts/sec). Data was collected on up to three simultaneous masses and the system was returned to MS survey scan mode when the counts for each triggered mass returned to a minimum (10 counts/sec) or a time constraint was reached (4.8 sec). Data collection continued with mass- and charge-dependent collision energies for charge states of 2+, 3+ or 4+ until of the above set of specifications was reached.

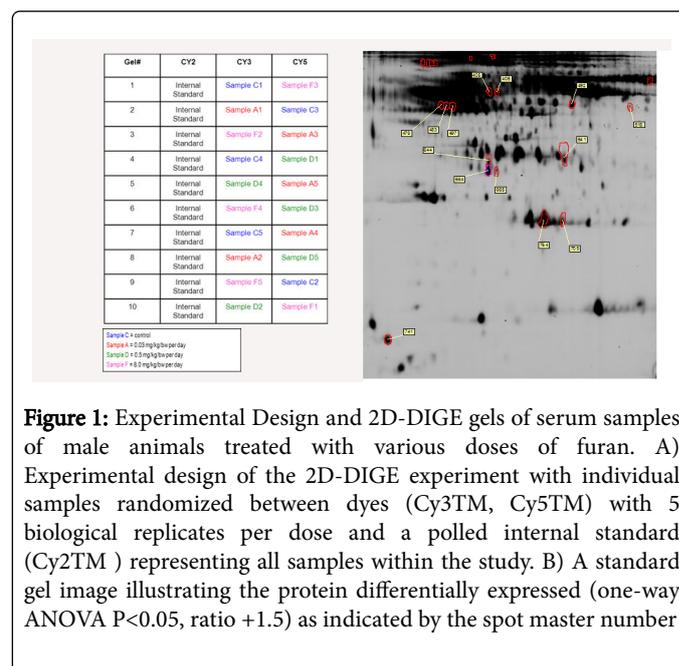
Peak lists were developed from the data collected in the LC-MS/MS analysis of each gel sample using MassLynx 4.1 and submitted directly to an in-house Mascot server with distiller software version 2.4.3.1. The tandem MS information was then searched against the NCBI database (version 20130503) with specific taxonomy (Rattus, 68, 381 sequences), 1 missed cleavage site, a fixed carbamidomethylation modification at cysteine, variable modifications deamidation (NQ), oxidation (M),

phosphorylation (ST), phosphorylation (Y), peptide tolerance  $\pm$  0.1 Da, and MS/MS fragment tolerance  $\pm$  0.1 Da. Proteins were identified and included if detected in all samples, a protein score above the confidence threshold and sequence coverage of at least 10% based on at least 2 identified peptides [19].

## Results

### Proteome differential expression in serum between treated and control animals

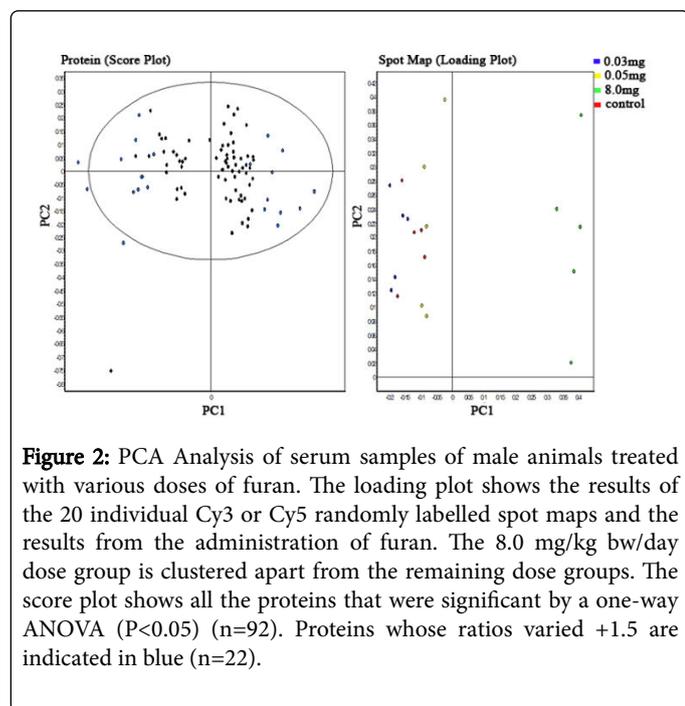
Statistical analysis was conducted using the EDA (Decyder Extended Data Analysis) module. The differential expression analysis (DEA) module was used to established the base set of proteins (n=238) by the filtering criteria by selecting proteins spots present in 90% of the gel images and removing unassigned proteins. Of these proteins, those with a one-way ANOVA with multiple comparison test ( $P < 0.05$ ) were further reduced (n=92). Comparisons between control and each dose group by the t-test and average ratio allowed the further filtering of a differential protein average ratio expression of +1.5 as a parameter. At both the 0.03 or 0.5 mg/kg bw/day dose groups, no proteins were identified as significantly changed (one-way ANOVA  $P < 0.05$ , avg ratio of +1.5). In comparison, there were 22 protein spots differentially expressed at the 8.0 mg/kg bw/day dose group. A gel image illustrating the proteins identified (one-way ANOVA  $P < 0.05$ , avg ratio +1.5) and the randomization of samples between dyes is shown (Figure 1).



**Figure 1:** Experimental Design and 2D-DIGE gels of serum samples of male animals treated with various doses of furan. A) Experimental design of the 2D-DIGE experiment with individual samples randomized between dyes (Cy3TM, Cy5TM) with 5 biological replicates per dose and a pooled internal standard (Cy2TM) representing all samples within the study. B) A standard gel image illustrating the protein differentially expressed (one-way ANOVA  $P < 0.05$ , ratio +1.5) as indicated by the spot master number

Principle Component Analysis was conducted to analyse the protein distribution patterns for dose related changes and identify those differentially expressed. The experimental groups are represented by each of the four dose groups of furan (0, 0.03, 0.5 and 8.0 mg/kg bw/day). The samples within each dose group (n=5) are grouped together thus illustrating that the biological replicates are responding in a similar way. The 8.0 mg/kg bw/day group are clearly separated from the remaining dose groups on the spot maps thus they are responding differently (Figure 2). The PCA analysis also displays several proteins which are found outside of the ellipse representing a 95% significance

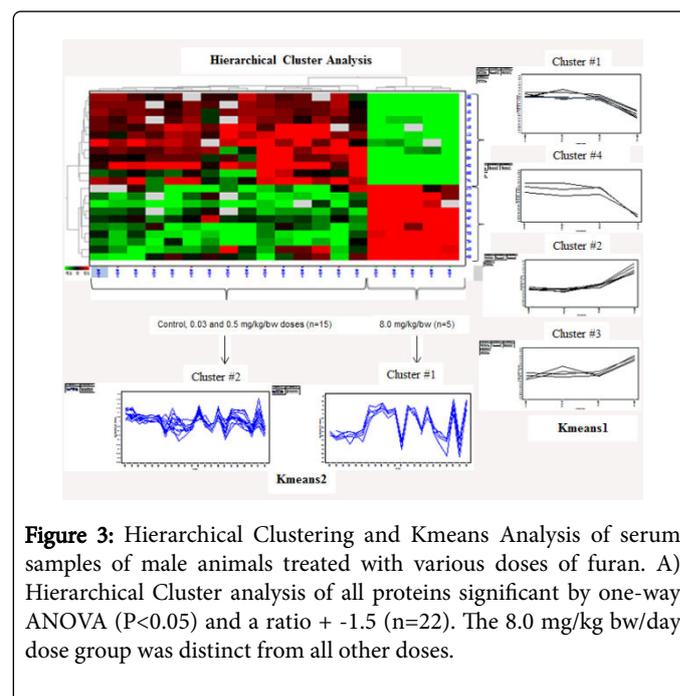
level (Figure 2). This represents proteins that have a strong differential expression.



**Figure 2:** PCA Analysis of serum samples of male animals treated with various doses of furan. The loading plot shows the results of the 20 individual Cy3 or Cy5 randomly labelled spot maps and the results from the administration of furan. The 8.0 mg/kg bw/day dose group is clustered apart from the remaining dose groups. The score plot shows all the proteins that were significant by a one-way ANOVA ( $P < 0.05$ ) ( $n = 92$ ). Proteins whose ratios varied  $+1.5$  are indicated in blue ( $n = 22$ ).

Pattern Analysis was completed by Hierarchical Clustering, Kmeans and SOM analyses to visualize the data and group similar data (proteins and treatment groups) into subsets or clusters (Figure 3). Hierarchical Clustering analysis of proteins (one-way ANOVA  $P < 0.05$ , avg ratio  $+1.5$ ) resulted in separating the 8.0 mg/kg bw/day dose group from all other dose groups. This also was confirmed by Kmeans2 analysis where Cluster #1 ( $q = 92.9$ ) ( $n = 5$ ) represents the highest dose group and further supports the previous results from the PCA analysis. Kmeans1 analysis of proteins (one-way ANOVA  $P < 0.05$ , avg ratio  $+1.5$ ) results in four clusters which were significant. Cluster #1

( $q = 81.2$ ) ( $n = 9$ ) and Cluster #4 ( $q = 81.3$ ) ( $n = 3$ ) were the results of proteins that were down-regulated as a result of an increase in furan exposure. Cluster #2 ( $q = 81.3$ ) ( $n = 6$ ) and Cluster #3 ( $q = 78.5$ ) ( $n = 4$ ) represented proteins that were up-regulated under the same conditions. At the 8.0 mg/kg bw/day dose group, 22 protein spots were significantly altered (one-way ANOVA  $P < 0.05$ , avg ratio  $+1.5$ ) but only 4 of these spots were able to be manually picked and identified with high confidence with the previously mentioned parameters [19]. Fetuin B (precursor) and apolipoprotein C-III, isoform CRA\_b, were up-regulated and  $\alpha$ -1-macroglobulin and pre-proapolipoprotein A-1 were down-regulated (Table 1).



**Figure 3:** Hierarchical Clustering and Kmeans Analysis of serum samples of male animals treated with various doses of furan. A) Hierarchical Cluster analysis of all proteins significant by one-way ANOVA ( $P < 0.05$ ) and a ratio  $+ -1.5$  ( $n = 22$ ). The 8.0 mg/kg bw/day dose group was distinct from all other doses.

Proteins	gi	Mass	pI	score	% coverage	#peptides	Biological Function	Fold change
Fetuin-B (precursor)	gi 17865327	42361	6.71	465	49	23	Immune system regulation and inflammation	-2.4
Apolipoprotein C-III, isoform CRA_b	gi 149041555	7847	4.65	150	27	3	Lipid metabolism	-2.2
Pre-proapolipoprotein A-1	gi 55747	30126	5.52	439	42	18	Anti-inflammatory and anti-oxidant	2.1
$\alpha$ -1-macroglobulin	gi 205384	168422	6.46	223	4	10	Immune system regulation and inflammation	1.6

**Table 1:** Identification of proteins that were differentially expressed in animals exposed to 8.0 mg/kg bw/day of furan. Expression levels were determined using 2 DIGE and proteins were identified by in-gel digestion, mass spectrometry (q-TOF MS/MS) and bioinformatics (MASCOT).

## Discussion

Serum and other body fluids are non-invasive resources to gain access to information for the clinical diagnosis and identification of novel biomarkers. Serum provides a rich sample for diagnostic analyses because of the expression and release of potential protein biomarkers into the bloodstream via various tissues or organs in response to specific physiological states. Potential protein biomarkers may be present at low concentrations and could be masked by high abundant proteins if not depleted since they possess similar biophysical characteristics [20]. In this study, a serum proteomic profiles were used for the exposure assessment of furan which is found through food processing. Principle Component Analysis, Hierarchical Clustering and the Kmeans Analysis of identified proteins (one-way ANOVA  $P < 0.05$ , avg ratio +1.5) confirm that the 8.0 mg/kg bw/day dose group of furan is distinct from all other doses as no proteins were identified as significant at the 0.03 or 0.5 mg/kg bw/day dose groups. At the the 8.0 mg/kg bw/day dose group, four proteins were identified having a significant differential expression and were identified by bioinformatic analysis of mass spectrometry data. Pre-proapolipoprotein A-1 (+2.1) and  $\alpha$ -1-macroglobulin (+1.6) were upregulated whereas apolipoprotein C-III- isoform CRA\_b (-2.2) and fetuin-B precursor (-2.4) were down-regulated. These proteins belong to the class of acute-phase protein (APPs) which are a large group of biochemically and functionally unrelated proteins whose plasma concentrations can decrease and/or increase following exposure to inflammatory conditions such as external trauma, hemorrhage/tissue injury, acute infections, burns and chronic inflammation [21]. The changes in APP plasma levels correlate with changes in the rate of hepatic synthesis and in the hepatic levels of the corresponding mRNA [22-24]. The serum concentrations of macroglobulins have been shown to more than double after injury [22]. This glycoprotein serves as the principal circulatory proteinase binder. Proteinases released in response to tissue injury, necrosis or inflammation would be bound and inactivated by  $\alpha$ -1-macroglobulin, and in turn the protein complex would stimulate the liver to synthesize a number of acute-phase proteins. Fetuin is a transporter protein in the serum and is an anti-inflammatory mediator that is critical to regulating the innate immune response following tissue injury in response to pro-inflammatory cytokines early in the inflammatory response. During inflammation, circulating fetuin levels substantially decrease as fetuin becomes associated with the membranes of macrophages. It is thought to mediate serum calcium homeostasis and mineralization and to potentially participate in the transport of bioactive molecules. The calcium levels were not altered in clinical biochemistry [13]. Pre-proapolipoprotein, in addition to being a marker of inflammation, is also a major precursor or component of plasma HDL which mediates the reverse transport of cholesterol from the tissues into the liver. The increased synthesis of pre-proapolipoprotein A-1 suggests an up-regulation of HDL synthesis and the subsequent increase in cholesterol catabolism in the liver. Apolipoprotein C-III, isoform CRA\_b is involved in lipid metabolism. A decrease of approximately two-fold in the amount of apolipoprotein C-III-containing HDL particles was found in rats 24hr after the induction of inflammation [24]. Its production rate is strongly correlated with plasma triglyceride levels [25-27]. Triglycerides were significantly reduced at the same dose [13] supporting the down- regulation of the apolipoprotein C-III, isoform CRA\_b by 2D-DIGE analysis.

The APPs results in a complex systemic reaction with the goal of re-establishing homeostasis and promoting healing. These proteins are generally referred to as the 'molecular thermometer' whereby

quantitation of individual APPs can provide an assessment of the response to the triggering event [28,29]. These APPs can be of diagnostic relevance and also of prognostic value. Within a few hours after exposure to toxicants, the pattern of protein synthesis by the liver is drastically altered resulting in an increase of some of these APPs [30,31]. The changes in the concentrations of APPs are due largely to changes in their production by hepatocytes. The magnitude of the increases varies from about 50 percent in the case of ceruloplasmin to as much as 1000-fold in the case of C-reactive protein and serum amyloid A, the plasma precursor of amyloid A. The maximal level could be seen after 8-10 days and it is maintained during each stage of disease [30]. Although, the acute phase response typically lasts only a few days, in cases of chronic or recurring inflammation, an aberrant continuation of some aspects of the acute phase response may contribute to the underlying tissue damage that accompanies the disease and may also lead to further complications. This was seen in the case of the subchronic study of furan for both the clinical biochemistry and histology [13] as well as being documented in other conditions such as cardiovascular or protein deposition diseases such as reactive amyloidosis.

Serum proteomic profiling as a tool to determine chemical exposure is still in its infancy stages. Humans are unavoidably exposed to a variety of environmental toxicants and combinations potentially contributing to an increased risk for a number of diseases. Detection of novel biomarkers could aid in the identification of an early-stage of chemical exposure and/or stages of disease. Biomarkers in accessible body fluids could greatly enhance the ability to identify exposures and could provide a warning sign prior to being symptomatic [17]. This is particularly important for asymptomatic conditions where a late diagnosis generally results in a poor outcome.

## Conclusions

In the present study, we used 2D-DIGE, mass spectrometry and bioinformatics for a translational approach using serum proteomic profiling for the identification of novel biomarkers related to the subchronic exposure to furan. Proteomic profiling may expand the repertoire of identified predictive biomarkers of toxicant exposures to not only provide critical tools in the evaluation of their safety for future health risk assessment but also the appropriate measures to minimize adverse effects. All the identified serum proteins were involved in the acute phase response pathway and lipid metabolism which also corroborates the histological and clinical biochemistry results of our earlier publication.

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