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 α-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 (Cy2TM). 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® 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 Canadia Council of Animal Care (Ottawa, ON). Animals received treatment by gavage over a 90-day period, dosing 5 days a week. Dosing solutions were added to 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].

Sample collection

At the end of the study, animals were sacrificed by exsanguination under isofluorane anaesthesia. Blood from the abdominal aorta was collected in SST Vacutainer® tubes (Becton-Dickinson, Franklin Lakes, NJ) and allowed to clot at room temperature. Serum was separated by centrifugation at 3,000g, 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: 50 l 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 thiourrea, 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 5 mol 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 ml of 10 M 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 Immobile Drystrips pH 4-7 (GE Healthcare) with the Ettan IPGphor3 Isoelectric Focusing System. The Cy2 TM, Cy3 TM and Cy5 TM 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 Thioura, 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 rehybridization 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. Immobile DryStrips 18 cm pH 4-7 were placed gel side down into the stripholder 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 Immobile Drystrips had the excess
cover fluid removed and were stored at -80°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 Immobiline Drystrips were removed from -80°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 µm 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, Immobiline 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 Blind 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 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 twice. 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 (SRI-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® 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 low-binding tubes (LoBind, Eppendorf) and washed with 200 µl of water (Milli-Q 18 Ω). The gel pieces were dehydrated with 200 µl of 50% acetonitrile (ACN)/buffer.

Tryptic digestion

Excised spots were placed in low-binding tubes (LoBind, Eppendorf) and washed with 200 µl of water (Milli-Q 18 Ω). The gel pieces were dehydrated with 200 µl of 50% acetonitrile (ACN)/buffer.
(50 mM NH₄HCO₃) followed by another 200 µl 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 µl 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 µl of a freshly prepared iodoacetamide (1 M in buffer). The gel pieces were washed with 400 µl Milli-Q water to remove any residual iodoacetamide and the digestion was initiated by the addition of trypsin (10 µl of 20 ng/µl Promega Sequence Grade Modified in buffer) and a minimal amount of buffer (30 µl) to cover the gel pieces. The samples were incubated overnight at 37°C. The digested peptides were extracted from the gel plugs with 50 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 µl 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 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 µM)). The LC system consisted of a trap column (Symmetry C18, 5 µm x 20 mm, Waters) and an analytical column (BEH 130 C18, 1.7 µm 100 µm x 100 mm, Waters). Solvent A consisted of H₂O with 0.1% FA and solvent B consisted of ACN with 0.1% FA. In a typical experiment, digests were injected (4 µl) onto the trap column for 3 minute at a flow rate of 3.0 µl/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 deamination (NQ), oxidation (M), phosphorylation (ST), phosphorylation (Y), peptide tolerance ± 0.1 Da, and MS/MS fragment tolerance ± 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 establish 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).

![Image](image1.png)

**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.

Principal 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...
This represents proteins that have a strong differential expression.

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 α-1-macroglobulin and pre-proapolipoprotein A-1 were down-regulated (Table 1).

![Figure 2](image1.png)

**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).

![Figure 3](image2.png)

**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)            |    | 42361  | 6.71 | 465   | 49         | 23        | Immune system regulation and inflammation               | -2.4        |
| Apolipoprotein C-III, isoform CRA_b |    | 7847   | 4.66 | 150   | 27         | 3         | Lipid metabolism                                         | -2.2        |
| Pre-proapolipoprotein A-1       |    | 30126  | 5.52 | 439   | 42         | 18        | Anti-inflammatory and anti-oxidant                       | 2.1         |
| α-1-macroglobulin               |    | 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 and p-value <0.05) from 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 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-I (+2.1) and α-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 circulating proteinase binder. Proteinases released in response to tissue injury, necrosis or inflammation would be bound and inactivated by α-1-macroglobulin [-1.5] eliciting that the proteinase 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-I 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 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 changes 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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References

1. Morehouse KM, Nyman PJ, McNeal TP, Dinovi MJ, Perfetti GA (2008) Survey of furan in heat processed foods by headspace gas chromatography/mass spectrometry and estimated adult exposure. Food Addit Contam Part A Chem Anal Control Expo Risk Assess 25: 259-264.
2. US FDA (US Food and Drug Administration) (2004) Exploratory data on furan in food.
3. Bolger PM, Tao SS, Dinovi M (2008) Hazards of dietary furan. In Process-induced Toxicants: Occurrence Formation, Mitigation and Health Risks, R. H. Stadler and D. R. Lineback, (2nd eds.) John Wiley, Hoboken, NJ pp. 111-32.
4. Crews C, Castle L (2007) A review of the occurrence, formation and analysis of furan in heat processed foods. Trends Food Sci Tech 18: 365-72.

5. Maga JA (1979) Furans in foods. CRC Crit Rev Food Sci Nutr 11: 355-400.

6. EFSA (European Food Safety Authority) (2004) Report on the scientific panel on contaminants in the food chain on provisional findings on furan in food. EFSA J 137: 1-20.

7. Reinhard H, Sager F, Zimmermann H, Zoller O (2004) Furan in foods on the Swiss markets method and results. Mitt Lebensmittelunters Hyg 95: 532-35.

8. Butterworth BE, Sprankle CS, Goldsworthy SM, Wilson DM, Goldsworthy TL (1994) Expression of myc, fos, and Ha-ras in the livers of furan-treated F344 rats and B6C3F1 mice. Mol Carcinog 9: 24-32.

9. International Agency for Research on Cancer (1995) IARC Monographs on the Evaluation of Carcinogenic Risks to Humans 63: 3194-407.

10. EFSA (European Food Safety Authority) (2004) Report on the carcinogenicity of furan (CAS No. 110-00-9) in F344/N rats and B6C3F1 Mice (Gavage Studies). Natl Toxicol Program Tech Rep Ser 402: 1-286.

11. Mally A, Graff C, Schmal O, Moro S, Hamberger C, et al. (2010) Functional and proliferative effects of repeated low-dose oral administration of furan in rat liver. Mol Nutr Food Res 54: 1556-1567.

12. Liu B, Qiu FH, Voss C, Xu Y, Zhao MZ, et al. (2011) Evaluation of three high abundance protein depletion kits for umbilical cord serum proteomics. Proteome Sci 9: 24.

13. Ruminy P, Gangeux C, Claeyssens S, Scotte M, Daveau M, et al. (2001) Gene transcription in hepatocytes during the acute phase of a systemic inflammation: from transcription factors to target genes. Inflamm Res 50: 383-390.

14. Zorin NA, Zhabin SG, Belogorlova TI, Chirikova TS, Krayushkina NA, et al. (1994) Changes in tissue distribution of rat alpha 1-macroglobulin and pregnancy-associated alpha 1-glycoprotein after inflammatory injury. Int J Exp Pathol 75: 425-431.

15. Liu B, Qiu FH, Voss C, Xu Y, Zhao MZ, et al. (2011) Evaluation of three high abundance protein depletion kits for umbilical cord serum proteomics. Proteome Sci 9: 24.

16. Shen P, Howlett G (1993) Alteration in rat apolipoprotein C-III gene expression and lipoprotein composition during inflammation. Inflamm Res 17: 153-166.

17. Rahman SM, Choudhury M, Jansen RC, Baquero KC, Miyazaki M, et al. (2013) CCAAT/enhancer binding protein ß deletion increases mitochondrial function and protects mice from LXR-induced hepatic steatosis. Biochem Biophys Res Commun 430: 336-339.

18. Gill S, Kavanagh M, Poirier C, Weber D, Koerner T (2016) Serum proteomic analysis for the identification of biomarkers by two-dimensional differential gel electrophoresis (2D-DIGE) after exposure to the food-processed contaminant furan. J Clin Toxicol 6: 301. doi:10.4172/2161-0495.1000301