2D DIGE, label free quantification, principal component and mass spectrometry analysis for biomarkers discovery in MCF-7/BOS cells exposed to 17β-estradiol and endocrine disruptors.

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Introduction

Endocrine system disruption has become a subject of great interest over the last few decades, since it has become evident that natural and also synthetic substances can mimic or reduce the activity of endogenous hormones. Compounds with estrogenic activity are an important family of potential endocrine disruptors that have to be monitored either in the food chain or in the environment. Estrogens are known to induce or promote hormonal dependent cancers, to reduce sperm counts and fertility in men and generate the feminization of exposed wildlife populations.
The rapid screening of unwanted chemicals in the food chain is beset by difficulties. The number of toxic compounds is very large and no universal method can cope with their diversity.
In this work, emergent differential proteomic techniques are used to discover a set of biomarkers for the development of a multiple estrogen contaminants screening test.

Materials and Methods

Cell culture and endocrine diruptors exposure

The MCF-7/BOS cells, an estrogen responsive mammary cancer cell line expressing both estrogen receptors alpha and beta. MCF-7/BOS cell are dependant on estrogenic stimulation for their proliferation and thus are used as a transducer of the exposure to xenoestrogenic compounds.
In order to do this, the cells were treated with 17β-estradiol (E2), a femalle endogenous hormone, considered as the more the potent estrogen and a reference compound. Cells were also treated with xenooestrogens such as phytoestrogens coming from pure commercialy available molecules or complex matrices such as food extracts.
Prior to the exposure, cells were stravated from estrogenic stimulations with a 48 Hrs culture in charcoal stripped medium. Exposure to E2 and other tested compounds lasted for 24 Hrs at different concentrations.

Differential proteomics and principal componant analysis

After the exposure, the proteome was fractionated into 2 sub-cellular fractions: the cytosolic fractionand a fraction containing the membranes and organelles.
2 diffenrential proteomic approaches were used to observe protein abundance changes. 2D DIGE (2 Dimension DIfferential Gel Electrophoresis) combined with time of flight mass spectrometry and 2D UPLC (Ultra Performance

Liquid Chromatography) MS^E label free quantification were carried out on the extracts to quantify and identify proteins with their abundance variations.
Each condition was run on biological triplicate.
Principal component analysis (PCA) is a statistical procedure that reduces all the variables of a data set into a smaller number of variables called principal components. PCA were performed to show up groups of proteins or samples that discriminate from each other either using DIGE or label free technique.

Results and Discussion

The differential proteomic analyses were realised with the objective to highlight biomarkers characterized with abundance variations in the sub-proteomes of the MCF-7/BOS cell line. PCAs performed on both differental proteomic technics show similar information. Principal component (PC)1 and PC2 tend to separate the biological replicates one from each other. Both 2D DIGE and label free plots seem to show with PC1 (respectively 51.1 % and 35 %) and PC2 (respectively 20.3 % and 21 %) the inter-replicate variation (figure 1). In both study, biological variability seem to be the first source of variability in the experiment, hiding the estrogenic action.
In both analyses, by merging identical condition, the biological background variability was removed and the significant variation of the remaining data was promoted. Now, in the 2D DIGE analysis PCA separates the conditions according their concentration in E2 and thus highlight a concentration dependence response of the cell line (figure 2).
The use of A gel based proteomic approche allowed the observation of post-translational modifications in response to estrogenic stimulation such as protein proteolisys or multiple phosphorilation (figure 3).
Considering the proteins identified and significantly regulated in both differential analyses, 6 proteins were retained and considered as potential biomarkers of estrogenic exposition.

Conclusions

The differential proteomic studies showed that results obtained using MCF-7/BOS cells demonstrate that a single day exposure clearly shows responses depending on the dose of exposure and differences in the estrogenic potency of the chemicals.

The aim of this work was the detection of potential biomarkers to 17-β estradiol exposure, with a long term perspective of being used in a rapid screening test for the presence of estrogens in environmental and food matrices. The 2D DIGE and label free results obtained using MCF-7/BOS cells as reporters demonstrate that one single day's exposure to pM concentrations of E2 show clear responses depending on the concentration and allows the identification of sensitive potential biomarkers, whereas the MTS metabolic assay only shows a significant effect of E2 after several days. 6 cytosolic proteins showing similar response profiles could be highlighted from the combination of 2D DIGE and UPLC label free proteomic techniques. In this study both gel based and gel free techniques are complementary. Label free seems to be quiet always more accurate in quantification than 2D DIGE giving access to more significantly regulated proteins. On other side, gel electrophoresis highlighted proteins post translational modifications as phosphorylation or proteolysis which could not be visualised using the other method. This study also showed the possibility of using independent 2D DIGE experiments even so they have not a common internal standard condition.
The renormalisation procedure allowed the analysis of 3 separately processed biological replicates, with the identification significantly regulated protein correlated with a label free analysis.

The potential selected biomarkers will be further investigated in order to determine precisely the threshold, the saturation concentration and the exposure duration that gives the maximal cellular response for the biomarkers. Potential biomarkers regulation will also be studied with other estrogenic compounds such as phytoestrogens. The normalisation processes conducted in these analyses will allow the process of the new data without the need of a common internal standard between E2 and new data.

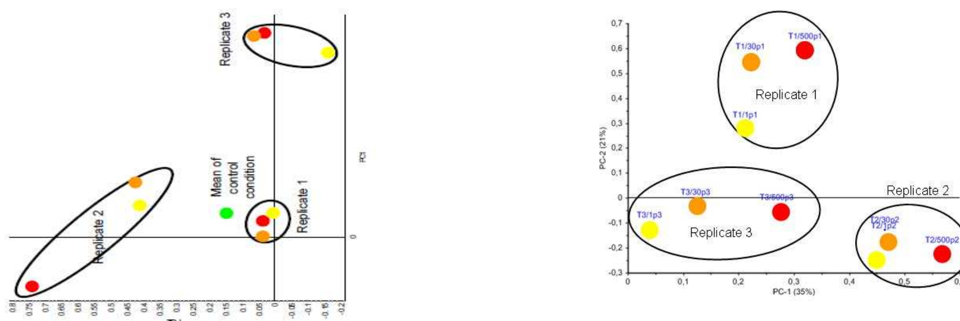

Figure 1: PCA plot representing inter-replicate variability within the 2D DIGE (rigth) or UPLC label free qauntification (left)

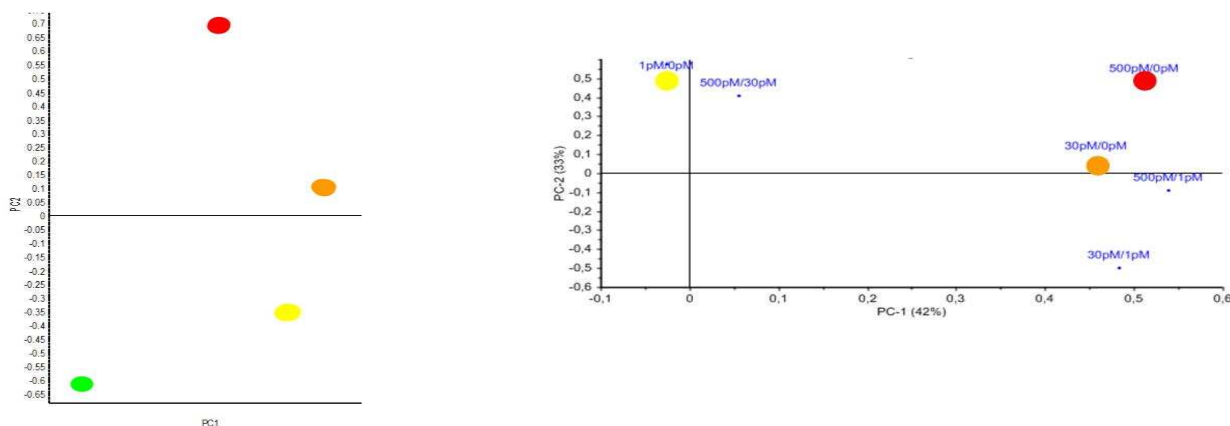

Figure 2: PCA plot representing dose depence response of MCF-7/BOs cells within the 2D DIGE (rigth) or UPLC label free qauntification (left). Increasing concentrations from green (control) to red.

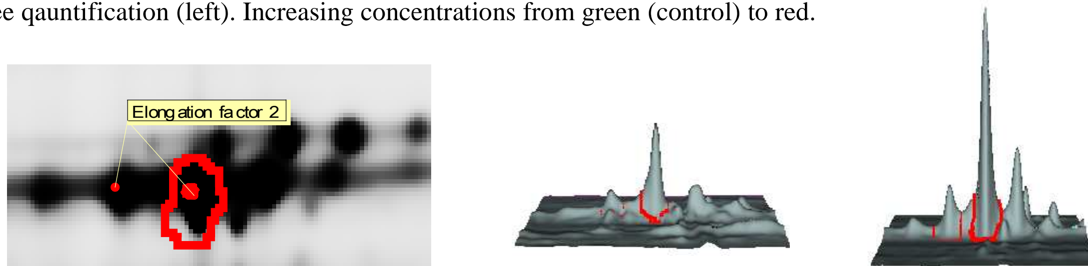

Figure 3: 2D gel images representing from left to righ : 4 phosphorilation states of elongation factor 2 (EF2), state of EF2 without estrogenic stimulation and EF2 after estrogenic stimulation.