# Separation methods in cancer research, real samples

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Cancer belongs to the most terrifying diseases. Therefore, diagnostic and therapeutic tools are desperately searched. To detect the disease at it early stage, reliable biomarkers have to be employed, however to identify these molecules and find their connection to the type and stage of the disease is a challenging task. To meet this goal, extremely powerful methods have to be used. Among such methods, mass spectrometry occupies the leading position. However, even such powerful technique needs to be coupled with some kind of separation, which simplifies the complex biological sample prior to the analysis.

**Keywords:** cancer; separation science; capillary electrophoresis; chromatography; mass spectrometry

## 1. Introduction

Cancer is one of the biggest threats of current population because it is the most common cause of death [1]. In total, about 200 cancer forms have been recognized, however lung cancer, prostate cancer, breast cancer, and colon cancer cause more than 50% of all deaths [1]. The high mortality is caused not only by effectivity of the treatment, which may be in numerous cases quite low, but also by the diagnostics of the disease in the late stage [2]. Therefore, it is obvious that an early diagnosis of cancer is playing a key role in the successful treatment and biomarkers are sought in genome, proteome and metabolome. The main disadvantage connected to modern gene arrays, which are nowadays one of the most commonly used tools for genomic analysis is the fact that they require mRNA as starting material. Therefore, even though one can survey the expression of all genes in cells or tissues, the sample preparation is a drawback.

In proteomic research there are in general two strategies used. One is the 'bottom-up' approach and the other is the 'top-down' method. The bottom-up technique is based on a tryptic digestion of the protein mixture followed by separation of the fragments and analysis by MS, which can be done either on-line by electrospray ionization of off-line by matrix-assisted laser desorption and ionization. The disadvantage of this type of analysis is that limited information about the intact protein is provided. On the other hand, during the "top-down" method, the intact proteins and protein complexes are separated first and then analyzed by MS. Therefore, this approach can be used to obtain molecular information about the intact protein and may be advantageous for the detection of proteins' post-translational modifications [3].

The main problem is the high complexity and wide dynamic range of peptides in body fluids (i.e. blood serum, saliva, sputum, cerebrospinal fluid, etc.). Too many peptides are present spread over a range of concentrations exceeding 1012 in the case of serum. To overcome this obstacle, it is crucial to simplify the complexity and remove the major components of the matrix, which are usually masking the signal

of components of interest by some kind of separation method, which offers high resolution and can cope with a wide dynamic range of peptide concentrations [4].

In both, top-down and bottom-up methods, a powerful separation technique is required to obtain the information of interest. Despite the development of improved analytical tools for analysis of clinical and biochemical samples, gel electrophoresis is still the gold standard used up-to-date. Its two-dimensional variant – 2D gel electrophoresis – is taking advantage of combination with isoelectric focusing. The 2D sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) approach to protein profiling are accessible and economical method that enables the detection of hundreds of proteins on a single gel plate. Resolution has been enhanced by the introduction of immobilized pH gradients, which enable the analyst to tailor the pH gradient for maximum resolution using ultrazoom gels with a narrow pH gradient range. With modern 2D-PAGE, it is not unusual to resolve two proteins that differ in pI by 0.001 U [5]. Another limitation of 2D-PAGE include the labor-intensive and time-consuming nature of the technique, poor reproducibility, limited dynamic range of detection (undetectable for the mass range <20,000 m/z), and under-representation of certain classes of proteins, so that truly comprehensive analysis is impossible. Furthermore, it cannot provide accurate Mr information and it still remains difficult to interface 2-DE directly to MS analysis [4,6].

## 2. Chromatography

Another separation, which has proven to be applicable for cancer analysis is gas chromatography (GC). GC-MS is reported to have many advantages in the area of clinical samples. It is applicable for variety of samples which include not only serum [7] and/or plasma [8] but also breath gas from the lung cancer patients [9,10]. GC-MS is characterized by minimal sample requirements, rapid analysis and reduced use of expensive labeled substrates. [1]. Besides GC-MS also liquid chromatography is a powerful method providing the valuable information in cancer research. Liquid chromatographic techniques are faster, quantitative, easier to automate, and couple more readily to mass spectrometry than two-dimensional gel electrophoresis [11]. The main area of recent advances in LC-MS technologies is the improvement linked to capillary LC instrumentation that provides improved peak capacities and dynamic range of detection needed to analyze biological samples [12]. The enhancements have been achieved mainly by use of very high pressure, very small porous particles, columns with smaller inner diameter, nanoelectrospray interfaces, and relatively long columns and long gradients for separations. By using smaller inner diameter columns, the sensitivity of the system continues to increase inversely as the mobile phase flow rates drop to as low as 20 nl/min, demonstrating the advantages of ESI-MS analyses at very low liquid flow rates [12]. Separation columns for capillary LC are usually prepared by packing of conventional beads into a fused silica capillary with internal diameter of 10-300 μm. However, the large void volume between the packed particles and the slow diffusional mass transfer of solutes are the major factors limiting the separation efficiency of porous packing materials, especially for proteins and peptides having low diffusivities [11]. Ultra-performance liquid chromatography (UPLC) operates with sub-2 μm chromatographic particles and a fluid system capable of operating at pressures in the 6000–15000 psi range, providing an increased chromatographic selectivity compared with conventional HPLC, which uses larger particles. Increased sensitivity of UPLC compared to conventional HPLC is caused by reduced peak width and therefore by increased S/N ratio [13]. To improve the stationary-phase properties with enhanced mass-transfer abilities even more, the monolithic stationary phases were introduced in which the separation medium consists of a continuous rod of a rigid, porous polymer that has no interstitial volume but only internal porosity consisting of micro and macropores. All of the mobile phase is forced to flow through the channels of the porous separation medium, resulting in enhanced mass transport and improved chromatographic efficiency [11].

## 3. Capillary electrophoresis (CE)

Compared to chromatography, CE separation is separating the sample based on a completely different principle – ion mobility in the electric field. Therefore, it is providing different sort of information, which may be complementary to the one provided by chromatographic analysis. Moreover, there is a whole group of the electrophoretic methods including capillary zone electrophoresis, capillary gel electrophoresis, capillary micellar chromatography, capillary electrochromatography, capillary isoelectric focusing and/or capillary isotachophoresis. This variability enables the researcher to select the method providing the required information of interest. Moreover, the potential of miniaturization broadens the application potential even further [2].

Whereas HPLC is more widely used in clinical analysis, CE offers several advantages over HPLC by these including stable constant flow, no gradient, which results in changes in the ideal ionization parameters; fairly robust and inexpensive capillaries; compatible with essentially all buffers and analytes; fast separation; and high resolution [4]. These advantages are especially beneficial when analyzing a large number of heterogeneous samples that contain interfering compounds, such as lipids, precipitates, etc.

From the technical point of view, main advantages are the robustness and the possibility to renew the capillary inner surface by hydroxide. On the other hand, the small volume and therefore the small loading capacity is the limitation. Whereas ml quantities can be loaded onto an LC column, a CE can be filled with a maximum of several hundreds of nanolitres. Although pH stacking and other types of sample preconcentration methods can be used very effectively, a maximum of 30–50% of the total capillary volume can be filled with sample to maintain the separation power, which corresponds to 0.5–2 µl when using 50 or 75 µm internal diameter capillaries with length of 80 –100 cm. The major limitation is due to the dynamic range of the mass spectrometer (4 orders of magnitude at best), and that more abundant peptides will obscure minor signals [14].

## 4. Data analysis

Commonly, data are presented in two ways: (A) comparison of peaks from normal and cancerous samples. The peaks are usually not identified and the evaluation is just on the fingerprinting level which is sufficient for medical diagnostics, (B) Selected peaks are at least partly identified by the use of tandem mass spectrometry. The important challenges for proteomic studies are to use the variety of protein databases and algorithms applied for compound identification. One difficulty for peptide identification is to verify the accuracy of the match. The scoring algorithm is usually used to rank the candidates and assign only the highest scoring of all. Another difficulty is that some post-translational modifications of amino acids residues influence the masses the peptides, for example, phosphoserine usually exhibits a neutral loss of 98 Da ($H_3PO_4$) because of the elimination of phosphoric acid during MS/MS of phosphopeptides, whereas acetylated lysine exhibits a diagnostic ion at m/z 126.0913 during MS/MS of peptides [15].

## 5. Conclusions

From the literature review can be clearly concluded that even though MS is extremely powerful, without application of the separation methods, the analysis of bodily fluids is insurmountable problem. Especially, the biomarker research requires high separation power, which is mainly provided by CE-MS and its variants. Even though CE-MS is not so commonly used in clinical practice, the undoubtable advantages provided by this hyphenated technique predispose it for a wide application.

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## Conflicts of Interest

The authors declare no conflict of interest.

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