THESIS ON NATURAL AND EXACT SCIENCES B47

Capillary Electrophoretic Monitoring of Biochemical Reaction Kinetics

MARIA KULP

Department of Chemistry
Faculty of Science
TALLINN UNIVERSITY OF TECHNOLOGY

Dissertation was accepted for the commencement of the degree of Doctor of Philisophy in Natural and Exact Sciences on 10 February 2006.

Supervisor: Prof. Mihkel Kaljurand, Department of Chemistry, Faculty of Science, Tallinn University of Technology

Opponents: Dr. Alejandro Cifuentes, Institute of Industrial Fermentations (CSIC), Madrid, Spain
Dr. Matti Elomaa, Laboratory of Polymer Chemistry, Department of Chemistry, Faculty of Science, University of Helsinki, Finland

Commencement: 18 May 2006

Declaration: Hereby I declare that this doctoral thesis is my original investigation and achievement, submitted for the doctoral degree at Tallinn University of Technology, has not been submitted for any degree or examination.

Maria Kulp _____

ISSN 1406-4723
ISBN 9985-59-610-2

LOODUS- JA TÄPPISTEADUSED B47

Biokeemiliste reaktsioonide kineetika kapillaarelektroforeetiline seire

MARIA KULP

Моим родителям посвящается...

CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals.

I. **M. Kulp**, I. Vassiljeva, R. Vilu, M. Kaljurand, Monitoring of the degradation of phenols by *Rhodococcus* bacteria by using micellar electrokinetic chromatography. *J. Sep. Science*, *25,* 2002, 1129-1135.

II. **M. Kulp**, M. Kaljurand, On-line monitoring of enzymatic conversion of adenosine triphosphate to adenosine diphosphate by micellar electrokinetic chromatography. *J. Chromatography A*, *1032*, 2004, 305-312.

III. **M. Kulp**, M. Kaljurand, T.Käämbre, P.Sikk, V.Saks, *In situ* monitoring of kinetics of metabolic conversion of ATP to ADP catalyzed by MgATPases of muscle Gastrocnemius skinned fibers using micellar electrokinetic chromatography. *Electrophoresis*, *25*, 2004, 2996 – 3002.

IV. A. Ebber, S. Ehala, M. Kudrjashova, **M. Kulp**, M. Vaher, M. Kaljurand, Flow sampling in capillary electrophoresis with a pneumatically driven computerized sampler. Proc. Estonian Acad., *Sci. Chemistry*, *53*, 2004, 6-20.

V. **M. Kulp**, M.Vaher, M. Kaljurand, Miniaturization of sampling for chemical reaction monitoring by capillary electrophoresis. *J. Chromatography A*, *1100,* 2005, 126-129.

INTRODUCTION

Recent rapid developments in biotechnology have promoted the adaptation and improvement of bioprocess monitoring capabilities both in physiological studies and in bioprocess development. More efficient process monitoring, which allows better process modelling and closer process control, is becoming a necessity today. In particular, quick analysis methods are needed, which allow immediate response to the changes in a process under study.

In modern separation science, a variety of analytical techniques have been developed for monitoring of bioprocesses. Flow injection analysis combined with a sufficiently selective detection system, e.g., biosensors, is typically used for single analyte monitoring. High-performance liquid chromatography (HPLC) based assays are widely used in multi-analyte studies. Different spectroscopic methods (mass, infrared and NMR spectroscopy) are also applied for monitoring purposes. However, none of the contemporary methods are ideal for the direct monitoring of changes in the composition of the reaction medium. Some of them require the use of radioactive materials, which is neither economical nor environment-friendly. Others require the reaction to be stopped by adding reagents into the system, making it inconvenient to establish a rapid kinetic analysis. For example, in most HPLC approaches, sample preparation procedure is off-line, which adds a significant amount of work and leads to errors in analytical results. This requires a need for automated on-line monitoring systems with minimum/no sample preparation, especially if the rapid response is required due to, for example, process speed.

Capillary electrophoresis (CE) is a relatively new highly efficient separation technique, which has gained enormous popularity among separation scientists all over the world. It has been established as a promising analytical separation technique due to its phenomenal efficiency and selectivity, ease of operation, low eluent consumption and moderate cost of equipment. Hitherto CE has not been used extensively for bioprocess monitoring and there are only a few reports of the on-line monitoring of bioprocesses in the bioreactor by CE. The main restriction to the wide application of CE to bioprocesses monitoring seems to be the shortage of convenient on-line sampling/sample preparation devices. Therefore, the development of samplers, realized mainly due to automation and computerization of experimental sampling devices, is becoming an issue if the reproducible and reliable monitoring data are required. As a result, in addition to numerous commercially available instruments, the last decade has seen many “non-conventional” devices developed to overcome the disadvantages/limitations of the available commercial devices.

Apart from the need in reproducible and exact sample aliquot introduction, the sample preparation procedure is also very important in the case of complicated bacterial or physiological mixtures. Ideally, sample preparation step should be minimized or eliminated to allow fast and reliable analysis of reaction media components. Different types of processes require different approaches for sample preparation. In the case of bacterial processes, the simplest solution could be the combining of an automated sampling system with a convenient membrane unit, positioned on the sample line. Sample preparation could be completely eliminated for the monitoring of enzyme kinetics, if the proper CE separation protocol is applied. For example, the addition of cationic detergents to the CE buffer provides an efficient separation of analytes in the presence of enzymes and proteins, without a need of their precipitation and centrifugation as in the case of HPLC. Thus, the sample could be directly delivered from the bioreactor and injected to the separation capillary by means of an automated sampler.

Another important feature of non-conventional computerized samplers is the possibility of their miniaturization. In biochemical assays, sample amount is frequently limited to microliters, which means that for on-line analysis a sampler should be able to operate with tiny amounts of liquids. Samplers in most commercially available instruments need a mililiter amount of chemicals, which is an order of magnitude higher. Therefore, the development and application of new approaches for the introduction of microvolumes of the sample become highly desirable.

AIMS OF THE PRESENT STUDY

The main goals of the present work are related to the development and application of the capillary electrophoresis method to monitor various biochemical reactions taking place in a srtictly controlled environment. For that purpose, the following aims were set:

1. To develope a reliable CE analysis system for on-line monitoring it is necessary to

    - search for sampling methods, which allow on-line sample multiple introduction according to a fixed time function, taking advantage of the flexibility provided by the computer-controlled sampling devices;
    - develop CE separation conditions to ensure an effective and selective analysis of key compounds in complex biological mixtures, avoiding the need for a sample preparation procedure;
    - develop a membrane-based interface for on-line sample preparation in case of bacterial processes.

2. To apply the developed CE system for the monitoring of various biological processes kinetics, it is nesessary to study

    - the performance and possibilities of the developed pneumatic flow samplers to provide reliable kinetic analysis of test bacterial and enzymatic processes;
    - the possibilities of flow sampler miniaturization to perform kinetic analysis on microscale;
    - the application of the developed CE separation protocol for *in situ* kinetic analysis of the tissue metabolites associated with the cellular energetic state.

1 LITERATURE SURVEY: ANALYTICAL STRATEGIES FOR BIOMONITORING BY CE

1.1 Introduction: off-line or real-time bioprocess monitoring?

As a result of the advances in microbiology, molecular biology, genetic engineering, genomics and proteomics in the last 30 years, biotechnology and biotechnological processes have exploded from a traditional craft into a dynamic cutting edge technology. Today biotechnology is seen as a prestigious technology of the future, with countries striving to claim their positions in future markets. The number of biotechnological products and applications is increasing constantly. The food, pharmaceutical and orthopedic industry, environment, energy production, new materials, and chemical and biochemical analysis are some examples. To take a full advantage of the possibilities of these new emerging technologies, a full understanding of the processes occuring in bioreactors is necessary. This increases the role of analytical methods used to control and monitor bioreactions significantly. Analytical systems have been developed in a huge variety for different applications in the area of biomonitoring. Several applications for use in bioprocess monitoring are described and reviewed in the literature [1-6]. However, additionally, new analytical systems or procedures must be developed to meet special demands of modern or future bioprocessing or additional efforts are nesessary to adapt the already existing systems for an efficient bioprocess monitoring.

A pre-requisite for good bioprocess control is to use an adequate monitoring methodology. Generally, monitoring technologies could be divided into two types, depending on the sampling method applied (Figure 1). *Off-line* monitoring involves manual sampling of part of the bioreactor medium, followed by transport of the sample to the laboratory for analysis. This laborious and rather time-consuming procedure will easily give rise to infrequent and delayed data and is not very attractive for monitoring purposes. Off-line monitoring is normally used to develop mathematical models that are used for preprogrammed control of the future processes. In contrast, *real-time* bioprocess monitoring techniques have the advantage of providing information during the process. This approach gives direct insight to the bioprocess states and is essential for early problem detection (i.e., detection of the drift of process parameters from the desired values) and allows an immediate action to be taken in order to resolve the situation while the process is being carried out.

Real-time monitoring methods that are routinely applied on an industrial or laboratory scale are still relatively scarce. In the most typical cases, comparatively simple real-time analyses have been implemented to measure physical variables, such as temperature, pressure, agitation or fluorescence, or chemical variables, such as pH, dissolved oxygen or carbon dioxide. However, these measurements generally provide information of little value for an understanding of a bioprocess. What is really needed is the measurements of biological variables, such as extracellular concentrations of substrates and products, activity of enzymes or intracellular concentrations of certain metabolites. The lack of analytical methods suitable for real-time monitoring of biological variables can be attributed to difficulties related to the complexity of the sample composition and the specificity of the monitored analytes. A specific compound (e.g., a nutrient, hormone, metabolite, antibiotic, gene expression inductor) can have an important influence on cell metabolism even at very low concentrations. Measurement of these very specific analytes in low concentrations and in the presence of normally a very complex nutritive medium is another difficulty for bioprocess monitoring. Generally, isolation, purification, concentration and determination of these compounds are time-consuming processes that are therefore carried out using off-line analytical methods. On the other hand, as the demand increases for time-efficient ways of extracting more information from an experiment, the development of effective and exact analytical techniques and their adaptation for real-time monitoring becomes highly desirable.

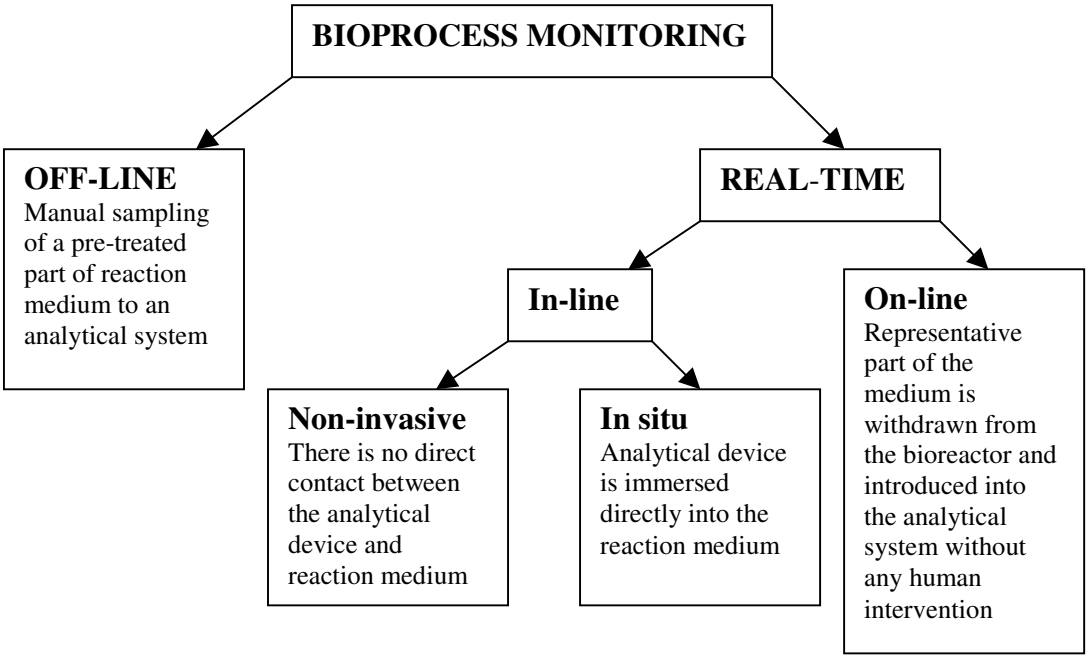

Figure 1. Bioprocess monitoring methodologies

1.2 Contemporary analytical techniques applied for biomonitoring

The rapid development in biotechnology during the last few years has enhanced the need in efficient process monitoring and control. This fact entails the development and application of a great variety of analytical methods for the monitoring of biological processes. The simplest way of the determination of dissolved organic molecules during the monitoring is to perform analysis *off-line*. Chromatographic techniques, in particular high performance liquid chromatography (HPLC), are widely used for off-line monitoring of bioprocesses [7,8], which implies that the pre-treatment of the complex sample has to be performed to ensure a proper protection of the analytical system. Obviously, these laborious and rather time-consuming procedures add a significant amount of work and lead to errors in analytical results, therefore the present tendency is to replace off-line analyses to more sophisticated methods.

One of the methods for obtaining real-time information about the process is optical spectroscopy [4,6], which includes all the analytical methods based on the interactions of analytes with light. Usually it is applied for *in-line* analysis, which can be subdivided into two approaches: *non-invasive* and *in situ* measurements (Figure 1). A major advantage of the in-line analysis is that there is no need for sample withdrawal and pre-treatment, which means that measurements can be performed without time delay and, in principle, continuously. In the case of *non invasive* analysis, spectroscopic probes are placed in the bioreactor wall in such a way that there is no direct contact between the analyzer and the fermentation broth. In spite of this attractive advantage, very few organic molecules have, however, been monitored in this way; this is due to difficulties arising from a number of complex interactions inside the reaction medium, for example, turbidity, which can change drastically during the process and bubbles that occur in the stirred reaction medium. Similar difficulties limit the wide application of *in situ* optical sensors for monitoring purposes. Optical density probes, fiberoptic sensors or fluorescence sensors do not consume analyte, do not interfere with the process. Also, they can be connected to or removed from the bioreactor at any time for sterilization purposes or in the case of a defect. But multiphase systems with a medium, cells, bubbles and other liquid or even solid phases cause difficulties, because the optical

measurement cannot define the distribution of a component in different phases. Optical sensors are also sensitive to fouling; the signal-to-noise ratio is often higher than in non-optical monitoring techniques. In addition, single-analyte measurements mostly provided by spectroscopy are not sufficient if the kinetics of a complex process is of interest. The application of two-dimensional fluorescence spectroscopy [9], capable of spectra analysis of several fluorescent compounds simultaneously, leads to difficulties of the interpretation of large amount of data due to spectral overlap and complex interferences. In this case, multi-component analysis of substrates and products by separation techniques, such as HPLC or capillary electrophoresis (CE), are preferable. Spectroscopic sensors, in turn, could be joined with the separation method. One interesting example of a separations-based fiber optic sensor for bioanalysis was recently published [10].

Near- [11] and mid-infrared spectra [12] can also provide a wealth of information. Glucose, fructose, glutamine, glutamate, proline, ammonia, carbon dioxide and phosphate are among the components that can be measured, however, the high cost and difficulty of calibration prevent widespread use of these methods.

In the recent years, the application of biosensors for *in situ* measurements [13,14] has increased dramatically. A biosensor can be defined as an analytical device that combines a biological component with a transducer. The biological component can be an enzyme, an organelle, a microoganism or an antibody and it confers specificity on the system. A transducer converts the biological signal into an electrical signal, which is proportional to the analyte concentration. Biosensors provide close to a real-time, continuous and very specific measurement of the given analyte. However, the use of sensors for the monitoring of organic compounds during bioprocesses has been rather limited so far, mainly because of problems associated with the fouling of the sensors by broth components, their inability to withstand sterilization and the unfeasibility of the recalibration of the sensor. Consequently, biosensors are currently used mostly for the analysis of cell-free samples obtained from automatic sampling systems. In this function, biosensors can also work as detectors in flow-injection analysis [15,16] or they can be placed as detectors following a chromatographic column [17].

It is generally assumed that in the near future the application of *on-line* methods for the determination of organics in biotechnological samples will be most attractive for monitoring purposes. In this case, a representative part of the medium is withdrawn from the bioreactor and after pretreatment, if any, introduced directly into an analytical system without any human intervention (Figure 1). Two techniques are employed for the on-line analysis of biochemical processes: flow injection analysis (FIA) and high performance liquid chromatography (HPLC). The choice between these techniques depends on several factors, i.e., the number of analytes, sample complexity, and the required analysis frequency. FIA is a way of implementing analysis in a flow system [18,19]. It is based on the injection of a well-defined volume of a liquid sample into a moving continuous carrier stream, typically a buffer, which is directly introduced into a detection device. The most highly valued virtues of FIA are the low response time, the high sampling frequency and its versatility and flexibility, however the lack of robustness and single analyte nature of the analysis makes it impossible to apply FIA if several analytes must be determined in the complex sample, since each analyte will require a separate manifold.

The major advantage of separation techniques over single-analyte analysis is, naturally, that many components can be determined in one run. Consequently, chromatographic techniques have a high information density, allowing multi-component analysis. In addition, these systems are very stable and robust since they are based on physical principles. The relatively high cost and long time of analysis, which typically is in the order of 10 min, but can be as long as an hour for complex separations, are the major drawbacks of chromatography. Also, chromatographic columns require a thorough sample pre-treatment to remove macromolecular sample constituents which easily slog the columns and ruin the separation efficiency. All of these facts attribute to non-extensive use of HPLC for on-line monitoring, only few papers report on monitoring of low molecular medium components during bioprocesses by on-line HPLC [20-22]. Here, sampling systems use a

membrane for the straightforward and efficient removal of macromolecular material from the sample. Coupling of chromatographic analysis to mass spectrometry (MS) has a great potential in bioprocess monitoring [22], especially as the price of MS detection has decreased significantly over the recent years. The MS detection based on the structural elucidation analysis by fragmentation experiments provides a sensitive and selective multi-component analysis of reaction medium. However, the problems related to sampling and sample preparation, optimization of chromatographic conditions for the following MS analysis and the complexity of the analytical system set-up obstruct on-line LC-MS analysis of biochemical reactions [23].

The major drawback of on-line analysis compared with non-invasive and in-line techniques is its relatively long response time (typically several minutes), particularly when chromatographic methods are involved. However, in many cases such response time is not the most important parameter, but rather the response time relative to the time constant of the bioprocess. On the other hand, as compared to single-analyte analysis, separation techniques designed for on-line analysis are much advantageous allowing the determination of all analytes of interest in one run and providing favourable long-term stability of the total system.

The use of capillary electrophoresis (CE) for kinetic studies of bioprocesses has aroused considerable interest. Due to its high separation efficiency, short analysis time and low eluent consumption CE has gained enormous popularity among separation scientists. In particular, the low eluent consumption makes CE a real "green" analytical method, which suits well to the requirements and recommendations of the contemporary, environmentally friendly approach to chemistry [24]. The moderate cost of CE equipment, the possibility of assembling CE apparatus in-house and the ease of operation makes the capillary electrophoresis technique very attractive for contemporary research laboratories. CE has remarkable advantages over single-analyte methods, providing fast and effective multi-component separations, where the migration times of analytes are considerably shorter than in HPLC. The high detection limit – a disadvantage that has often been pointed out when comparing CE with HPLC in general – is hardly a serious restriction to monitoring bioprocesses because the analytes concentration in the reactor is usually high. Still, surprisingly few reports on process monitoring by CE have been published. In most of the present applications, the CE method is used in off-line mode, either for the monitoring of prodrugs in cell extracts [25], in the studies of enzymatic reaction kinetics [26,27] or for the monitoring of the level of inorganic ions in pulp and paper mills [28]. In rare examples of the real-time monitoring of pharmacokinetics in living animals by CE, a home-made ingenious "animal/CE" interface based on micro dialysis probes is used [29-31]. In similar experiments Kennedy's group is using a flow-gated interface [32].

1.3 Introduction to CZE and MEKC

Capillary electrophoresis (CE) ia a powerful analytical technique which has received marked attention after its introduction more than two decades ago. The term 'capillary electrophoresis' describes a family of related techniques in which separations are carried out in narrow bore capillaries under the influence of an electric field. The separations obtained by capillary electrophoresis are highly efficient, rapid, and may be applied to both charged and neutral species, ranging from small inorganic anions to large molecules, such as proteins and even cells and particles. The theory of capillary electrophoresis has been described in many papers and in a book [33], therefore only a short overview will be given here.

In its simplest form, capillary electrophoresis involves the separation of charged analytes, based on the difference in their electrophoretic mobilities, resulting in different migration velocities. These separations are carried out in fused silica capillaries, typically 25–75 μm i.d. and 50–100 cm in length, filled with a background electrolyte [34]. If high voltage is applied, electro-osmotic flow (EOF) ensures that both negatively and positively charged species migrate towards the same end of

the capillary, where under typical conditions, towards the cathode end, with neutral species not being separated and migrating with the electro-osmotic flow. This mode is also termed capillary zone electrophoresis (CZE). The key factor affecting selectivity, when using CZE for separations, is charge to size ratio and pH. The latter parameter will determine the degree of ionization for moderate and weakly basic, or moderate and weakly acidic analytes. The background electrolyte requires a good buffering capacity at a chosen pH for reproducible separations, and low conductivity not to generate a high current, which leads to excessive Joule heating. The most commonly employed background electrolytes have been derived from the large body of work with gel electrophoresis, and include phosphate, borate, phosphate/borate, and citrate buffers [35]. As compared to HPLC, CE analyses are generally faster and more flexible, the solvents used are inexpensive buffer salts and smaller (up to microliter range) quantities of both buffer and sample are required.

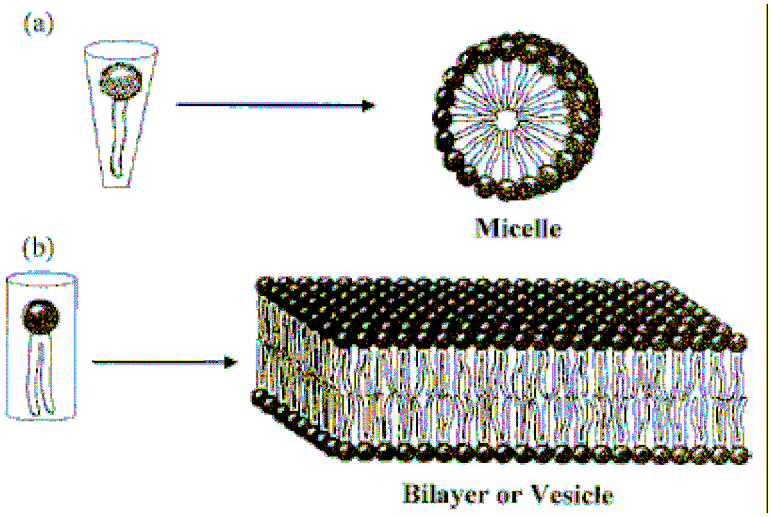

Figure 2. Aggregate structures of (a) single-chained surfactants and (b) double-chained surfactants [41]

The versatility of capillary electrophoresis is derived from its additional separation modes. The ability to perform these separations on the same capillary and instrument makes this technique very attractive in the laboratory. With CE, a commonly encountered mode of capillary electrophoresis is micellar electrokinetic chromatography (MEKC). This combination of electrophoresis and chromatography allows for the separation of both neutral and charged solutes [36]. It is achieved by the addition of surfactants to the background electrolyte at concentrations greater than the critical micelle concentration (CMC) [37], which allows the surfactant molecules group together to form micelles (Figure 2(a)). Neutral solutes partition with these micelles in a chromatographic fashion and are separated based on their retention factors similar to reversed-phase HPLC. Generally, anionic (negativity charged) micelles are formed using solutions of sodium dodecyl sulphate (SDS) above 10 m*M* concentration. However, different selectivities can be obtained [38] if different surfactants are used. Alternative surfactants include anionic surfactants such as bile salts, cationic surfactants such as cetyltrimethylammonium bromide (CTAB) and neutral surfactants such as Tween. Alternatively a charged additive such as an anionic cyclodextrin can be used instead of a surfactant to separate neutral solutes. Selectivity can also be manipulated [36] by factors such as surfactant concentration, addition of urea, cyclodextrins, ion-pair reagents, temperature, pH, buffer type and concentration. Several of these factors may be optimised for a complex separation and experimental design schemes have been employed in certain instances.

Manipulation of the surface charge of the capillary can lead to the control of the electroosmotic flow (EOF) as well as prevent wall adsorption of analytes such as proteins, which is crucial in the case of complex biological samples [39]. The most commonly used wall coatings for modifying the

charge on the capillary surface are the following: covalently bonded/cross-linked polymers, adsorbed cationic polymers (noncovalent), and adsorbed surfactants (dynamic). Each of these techniques has achieved excellent results in EOF control and/or prevention of protein adsorption [40,41]. Covalently modified capillaries generally employ polymers, such as polyacrylamide or polyethyleneimine, to permanently shield the silanol groups on the capillary wall from the bulk solution. Such coatings are used for both EOF suppression and prevention of protein adsorption.

For noncovalent capillary coatings, cationic polymers, such as polybrene and polyethyleneimine (PEI) have been used. These polymers adsorb strongly onto the capillary surface due to the strong electrostatic attraction between these polycations and the anionic silanols. However, both covalent and noncovalent adsorptive coatings can be lengthy, the column lifetime may be short, the coatings are unstable outside a limited pH range, and the reproducibility from capillary to capillary may be poor.

Alternatively, in dynamic coatings, a buffer additive equilibrates with the capillary surface and in doing so alters the surface. Conventional surfactants used for wall coatings in capillary electrophoresis consist of a polar headgroup and a single (CTAB) or double (DDAB) hydrocarbon chain. Figure 2(b) shows schematically a bilayer of double chained surfactant, which forms on the capillary wall during the coating procedure. At concentrations above the critical micelle concentration (cmc), cationic surfactants adsorb reversibly onto the negatively charged wall, reversing the surface charge and thus the EOF [41].

Due to all its positive features and marked advantages over other separation techniques, capillary electrophoresis ideally suites for monitoring of biochemical reaction kinetics. First, the combination of such characteristics as high-resolution separations, short analysis periods and low sample load allows CE to monitor the biological reaction progress without disturbing the reaction. Second, capillary electrophoresis provides an efficient separation of the reaction products from the substrates in a short time, therefore it is possible to monitor both the loss of substrate and the formation of products simultaneously. Third, a proper CE separation protocol allows the suppression of possible interfering components from the complicated sample matrix where the bioprocess occurred, thus it becomes possible to take aqueous samples directly from the reaction mixture without the need for stopping the reaction prior to analysis, which is very important for kinetic studies of rapid processes. Also, several relatively sensitive detection methods, such as UV-Vis spectrophotometry, laser-induced fluorescence (LIF) and mass spectrometry, are available for CE. Therefore, detection can be accomplished without the use of radiolabeled materials.

The real restriction to the wide application of CE to bioprocesses monitoring seems to be the shortage of convenient on-line sampling/sample preparation devices. Commonly commercially available CE instruments are equipped with autosamplers, and they are principally capable of performing on-line measurements. But commercial autosamplers, which have a rigid operating program are inflexible and they do not permit multiple injections and monitoring without interruption of the high voltage. The latter disadvantage was reported by the authors [42]. Thus, as an alternative to commercial instruments, the development of flexible automatic samplers for on-line monitoring of reactions becomes highly desirable.

1.4 Non-conventional samplers in CE

Monitoring requires multiple computer-controlled samplings from the same reaction vessel, especially when the time resolution between the experiments is an issue. The possibility of applying sophisticated sampling sequences of the buffer, sample, and washing liquids at the capillary inlet could be advantageous in many cases of rapid kinetics measurements. For the sake of reproducibility, ampling should be carried out without high voltage (HV) interruption between consecutive injections. The sampler should also be able to operate with both small and large sample amounts. Samplers in commercially available instruments are suitable for introducing a large number of stationary samples, but are inconvenient for the introduction of samples, whose composition changes with time.

Alternatively, several ingenious approaches for sample introduction were proposed in the literature during the last years. One route to precise, fast, small volume injections is *optical gating* [43]. In this system, the sample is fluorescently tagged and placed in the upper electrode vessel. When voltage is applied, the sample is continuously drawn into the capillary by the electroosmotic flow and electrophoretic mobility. Near the entrance to the capillary, an intense laser beam (gating beam) is used to photodegrade the fluorescent tag rendering it undetectable at the detector downstream. Injection is accomplished by blocking the gating beam for a brief period of time with the shutter, which allows a narrow plug of the labeled analyte into the separation zone, which then separates by electrophoresis and is detected by LIF with the detection beam.

Another system suitable for rapid injection and fast separation is the *flow gate interface* [44]. In the system, a capillary containing a sample is positioned across from the separation capillary with a small gap between the capillaries. Gating is accomplished by pumping an electrophoresis buffer between the inlet and separation capillaries. While the gating flow is applied, the sample that exits the inlet capillary is swept away by the gating flow and is not injected onto the separation capillary. When the gating flow is stopped, the sample is drawn into the separation capillary by electrokinetic effects. Flow-gated CE, like optically gated CE, has been used as a second dimension in multi-dimensional separations [44] and for in vivo monitoring using microdialysis probes [45].

Dasgupta and his co-workers have described several innovative samplers for CE, capable of performing multiple injections of tiny amounts of a sample from the same sample vessel [46]. In this approach, the inlet of a capillary is joined to an open stream of the buffer formed on an inclined narrow surface, and a constant voltage is applied to the capillary. A drop of the sample falls directly to the capillary inlet and then is washed away quickly. Thus, a short sample plug with limited dilution by the buffer stream and limited fronting or tailing is injected in an electrokinetic mode. This approach was later implemented for hyphenating flow injection analysis (FIA) to CE.

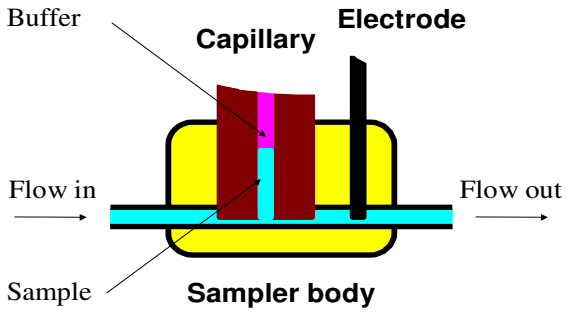

Figure 3. Principle of flow sampling

Continuous flow systems are powerful tools for the implementation of the preliminary operations of the analytical process. The combination of a flow system with the separation techniques enhances the analytical potential, providing more robust and reliable methods. Recent developments in the coupling of flow injection to CE (Figure 3) demonstrated the favorable potentials in achieving efficient continuous sample introduction for CE, including enhanced sampling frequencies, improved reproducibility, as compared to conventional sample introduction and possibilities of readily incorporating various pre-column on-line sample pretreatments. Fang [47], Kuban [48-50] and Chen [51] have provided possible prototypes for samplers for on-line monitoring applications, hyphenating the flow-injection analysis and CE. The advantages of such input devices are: the absence of a voltage rise/drop time during sampling, ease of operation (no manipulation with sample vials is involved) and ease of automation and computerization. Recent developments in micro fluidics also offer some ingenious solutions for automatic fluid manipulation [52-54] without interrupting high voltage.

1.5 Dialysis sampling systems for on-line monitoring

One of the most widespread approaches to facilitate sample pretreatment for any of analytical techniques is the usage of different kinds of membranes. Membrane interfaces are capable of interface transfer, matrix isolation and preconcentration. During recent years dialysis membranes have been applied to different separation methods using both off-line and on-line sample preparation for monitoring of biochemical reactions *in situ* and *in vivo*. However, if on-line monitoring of biochemical processes is of interest, then usually sample on-line preparation is required.

Several investigators have developed samplers that hyphenate dialysis with different separation methods to perform sample on-line preparation and monitoring. For example, the microdialysis sampling system has been coupled on-line with HPLC [55] and atomic absorption spectrometry (AAS) [56] for continuous *in vivo* monitoring of inorganics in the blood of living animals. Also, Yao et al. have shown the coupling of microdialysis sampling with a flow-injection biosensor system for simultaneous *in vivo* monitoring of glucose and other species in rat brain [57]. The possibility of on-line coupling of microdialysis with CE has been described by Kennedy's group [58]. Kuban and Karlberg described a FIA-CE interface for on-line coupling of a dialysis unit which allowed consecutive injections in one electrophoretic run [48].

Dialysis membrane interfaces are not very demanding in construction sophistication terms and can be easily manufactured in house. As an example of the dialysis membrane interface for the CE, in the present work a CE system was developed, where a dialysis unit for sample clean-up was connected to the pneumatic sampler (will be described below in the Experimental section).

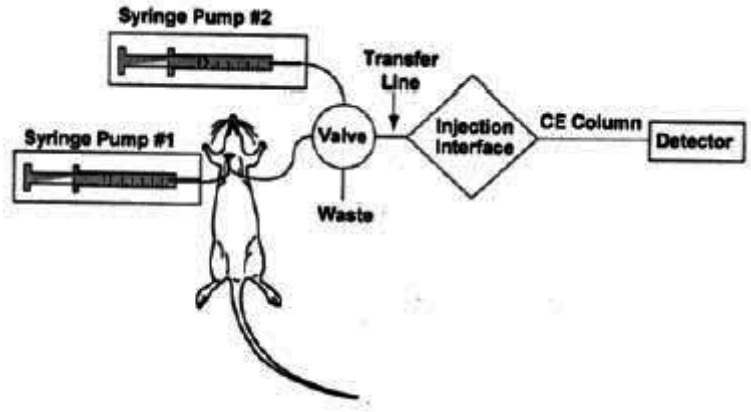

Figure 4. Schematic of the in-line microdialysis/capillary electrophoresis system [59]

With the ongoing miniaturization of analytical systems, the application of microdialysis for sample pretreatment has become more popular in recent years. Microdialysis sampling has proved to be an

important means of continuously monitoring reactions *in vivo*. It is accomplished by implanting a small semipermeable fibre at the site of interest. This fiber is slowly perfused with a sampling solution. Small molecules in the extracellular space diffuse into the fiber and are swept away to be collected for analysis. Microdialysis probes can be implanted in many tissues with minimal discomfort to the experimental animal. The introduction of microdialysis sampling has provided a technique which can continuously monitor chemical reactions in vivo. By the use of an appropriate analytical method, several compounds can be determined simultaneously. Microdialysis therefore can provide both the temporal and chemical information needed to fully elucidate biochemical processes.

Microdialysis samples have traditionally been analyzed by liquid chromatographic (LC) methods to gain resolution and quantification of the molecules of interest. However, LC separations have a relatively large injection volume requirement which, as a consequence, increases microdialysis sampling times. Capillary electrophoresis (CE), with its very small sample volume requirements and high resolving power, has therefore gained popularity as an alternative to LC. Studies of Lunte and Kennedy groups have described the on-line coupling of microdialysis with capillary electrophoresis (Figure 4) to analyze many endogenous molecules, such as neurotransmitters, as well as exogenous species such as drug substances [59,60].

2 EXPERIMENTAL

2.1 Pneumatic samplers developed and applied in the present work

To adapt the capillary electrophoresis technique for the purpose of on-line monitoring of biochemical processes, several different approaches for sample injection were developed and applied in the present work. The construction of the first, **pneumatic autosampler**, specially designed for multiple injection applications without HV interruptions, was first reported in 1995 [61] by researchers at the Department of Chemistry of Tallinn University of Technology. The application of this sampler for on-line monitoring of biochemical reactions is described in articles I, II and IV. Two other approaches, **cross sampler** and **falling droplet interface**, intended for computerized on-line sampling of the small amounts of reaction media, were recently described in [V].

2.1.1 Pneumatic autosampler

The schematic of pneumatically-driven computerized autosampler is shown in Figure 6. The dimensions of the particular autosampler made of polyether ether ketone (PEEK) material are 4.7 x 1.6 x 1.6 cm. The sample and buffer reservoirs are connected to the sampler body with a 100 x 0.7 mm Teflon tubing. The work of the sampler is based on the principle of rapid exchange of the buffer to the sample (and *vice versa*) in a narrow input channel (25 x 1.5 mm, 44 µL volume) into which the capillary and high voltage electrodes are inserted. The flow of liquids is controlled by PC that activates the pair of solenoid valves (not shown in Figure 5.) connected to the compressed air tank, thus providing pressure pulses necessary for activating the liquid flow. The applied pressure ranged from 0.3 to 0.8 10^5 Pa. The sampling logic is presented in Table 1. Channels are opened by Teflon films by liquid pressure and closed by air delivered through solenoid valves. Correct balancing of the liquid/air pressure is important and can be performed easily. It is evident from Table 1 that to execute the sampling process, pressures must satisfy the following relationship P1=P2 < P3=P4.

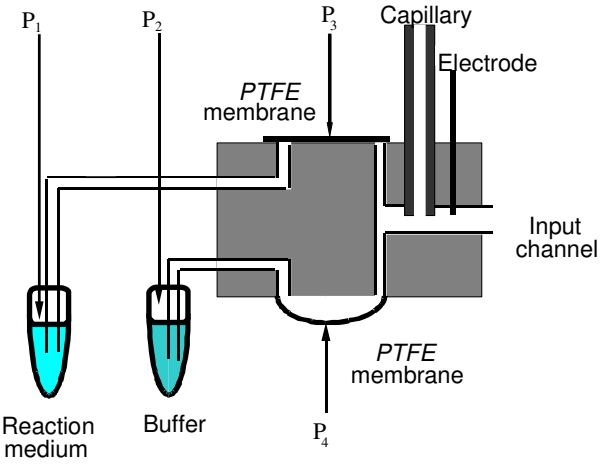

Figure 6. Schematic of pneumatic autosampler
P1-P4 stand for pressures, while P1=P2<P3=P4. During sample and buffer flow, either P3 or P4 is made equal to atmospheric pressure and the respective channel opens.

Table 1. Sampling sequence logic

Step #	Action	P1	P2	P3	P4
1	Sample rinse	On	Off	Off	On
2	Electrokinetic sampling	Off	Off	On	On
3	Buffer rinse	Off	On	On	Off

4	Pherogram run	Off	Off	On	On

The sampling mechanism is believed to be a mixed mode of both electrokinetics and hydrodynamics [62]. The contribution of a particular mode depends on the timing parameters: if the buffer to the sample exchange is performed extremely rapidly, then the sample is introduced entirely electrokinetically and the sample amount depends only on the time during which the sample is kept still in the input channel. With an increase in the time period for the sample flowing through the inlet channel, the hydrodynamic component of the sample introduction increases. The higher the pressure applied to the sample reservoir, the larger the amount of the sample introduced, and *vice versa*. The flexibility of the sampler construction allows operation with tiny amounts of sample (sub mL-range). This could be important if the available sample amounts are decreased. Another important feature of the sampler is that it always provides a fresh portion of the background electrolyte for the next injection, therefore eliminating changes of the buffer composition due to the electrolysis process.

The flow sampler has several properties, which can be considered advantageous as compared to the common samplers that appear in commercial instruments. First, the HV should not necessarily be interrupted during the sampling process. Although no special investigations have been performed with this particular sampler, this should improve the sampling process reproducibility since the voltage rise/fall during the sampling has been recognized to be the least reproducible part of the sampling procedure [63]. The relative standard deviation of sampling was estimated to be less than 1% [61]. No sample leakage or carry-over has been noticed. Second, flow samplers can be very easily computerized. Indeed, as demonstrated by us, sophisticated sample sequences can be generated easily [64]. Third, contrary to samplers included into commercial instruments, flow samplers are very useful for process monitoring. On-line sample preparation devices (such as membrane separators) can easily be implemented into the flow line (see Article I). Fourth, flow-sampling devices are very cheap and can be manufactured in a lab by researchers themselves from the materials and parts available in every lab. This feature differentiates flow samplers advantageously from the CE devices manufactured on the silicone or polymeric substrate.

2.1.1.1 Dialysis unit

The membrane-based dialysis unit (Figure 7) is connected to the pneumatic autosampler, thoroughly described in the previous section and positioned in the sample line. It consists of a glass cylinder (ID 2 cm, volume 30 mL) that forms the donor compartment of the dialysis unit. The threaded bottom of the cylinder is screwed into the Plexiglass block that forms the acceptor compartment. The volume of the acceptor compartment was estimated to have a volume of about 1.6 mL. The donor and acceptor compartments are separated by a Polypore® membrane that is fixed airtight between them. The molecular weight cut-off of the membrane is 12,000 daltons. The donor compartment is covered by a bacterial filter and stirred continuously by the air flow (30 mL/min) purged through the cylinder. The membrane-based dialysis unit was placed in the Plexiglas box together with the pneumatic autosampler and the buffer/reagent vessels and thermostated at 20 ^{o}C by the forced air flow. The acceptor compartment was introduced into the sample line of the pneumatic autosampler. This design enables analysis of complex sample matrices without any other sample preparation.

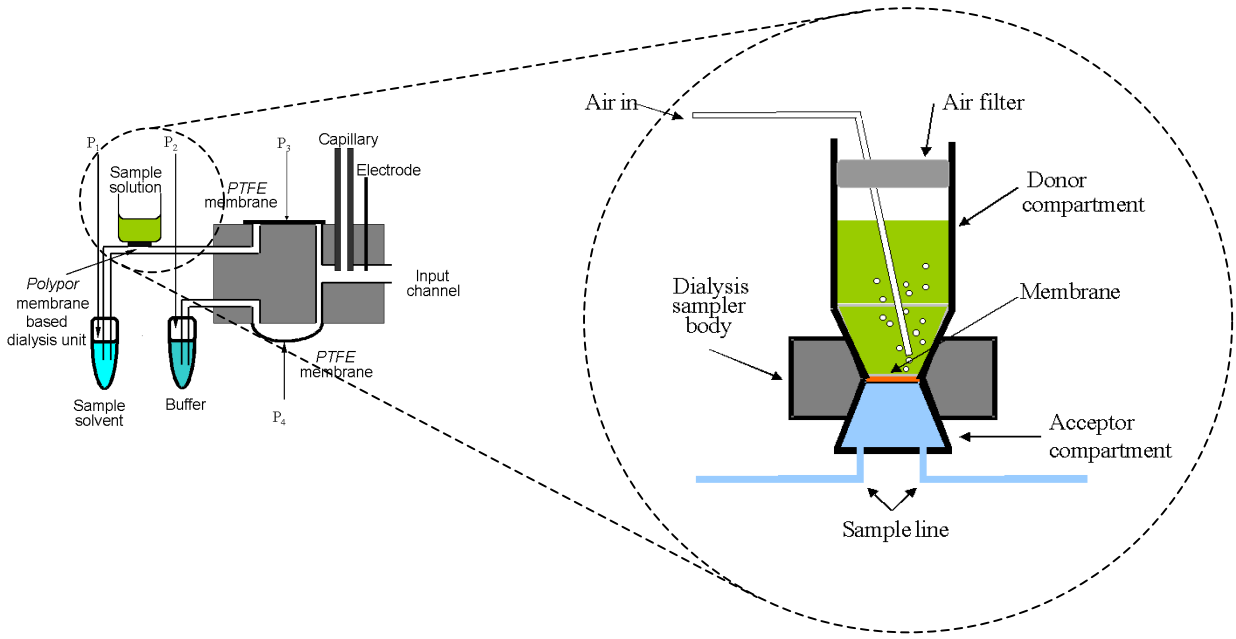

Figure 7. Dialysis unit

2.1.2 Cross sampler

The design of a cross sampler is similar to a "cross" injection device commonly used in lab-on-chip devices where the sample and separation channels are located perpendicularly on a chip. However, instead of electrokinetic loading, here the sample is loaded into a capillary by a pressure pulse. For microfabricated systems, this sampling technique has been used by Lin *et al*. [65]. The aim of the design developed in the present work was to achieve computerized on-line sampling of small volume reaction media without incurring the complications of microfabrication. The sample body is just a cross connector of separation and (wide) sample channels. With this sampler, a single power supply could be held at a constant voltage over the separation channel, while rapid short duration (a few seconds) programmed pressurizing of the sample vessel forces an aliquot of the reaction mixture to flow through the cross and fill the gap between separation capillaries with the sample. This portion is later carried to the separation capillary by the electroosmosis action.

The design of the cross sampler patterned from a single "cross" microchip design and is illustrated in Figure 8. A capillary connector made from Plexiglas and fabricated in the mechanical workshop of the Department of Chemistry was designed to accommodate rapid injections of sample and buffer plugs. A 0.4-mm diameter channel was drilled through a 23 x 23-mm area and by a 10-mm thickness Plexiglas sampler body. Another 1.5-mm diameter channel was drilled perpendicularly through the sampler body to form a cross. At all four ends, threads for 1/8-in. fittings were cut.

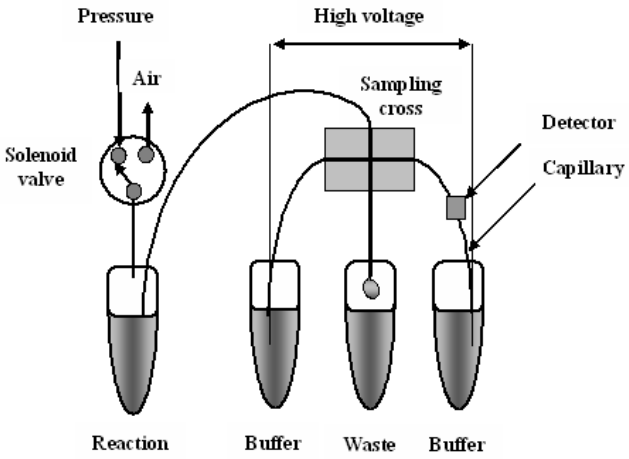

Figure 8. Schematic of a cross sampler

Two fused silica capillaries (365 µm o.d, 75 µm i.d capillaries, Polymicro Technologies, Phoenix, AZ, USA) with lengths of 18 cm and 50 cm, correspondingly, were inserted into both 0.4-mm

channels until their ends were approximately 30 μm apart (estimated visually under a microscope). The longer fused silica capillary passed through the UV detector and served as the separation channel. Both of the free ends of fused silica capillaries were placed into reservoirs filled with separation buffer. A separation voltage of 13 kV was used throughout all experiments. Two 1.5-mm o.d. and 0.3-mm i.d. Teflon capillaries with lengths of 9 cm and 20 cm were inserted perpendicularly to the fused silica capillaries. The Teflon capillaries served as sampling channels. All of the capillaries at the channel entrances were fixed from outside by 1/8 PEEK nuts to prevent leakage.

One free end of the longer arm of the sample channel was placed into the reaction vessel and the other free end was lead to waste. The reaction vessel situated in the gas displacement pump manufactured from 100 mL DURAN ISO laboratory bottle (Schott UK) with bottleneck attachment (Metrohm AB, Herisau, Switzerland part #6.1602.150, (Bottleneck attachment / GL 45 -3x10/32)). The bottleneck attachment had three threaded connector openings. One of the connectors was used for pressurizing of the bottle via a solenoid valve, the second for passing sample tubing and the third opening was closed.

2.1.3 Falling droplet interface

In the second approach, a sample is delivered as droplets (10 μL volume) into a buffer situated in a pipette tip. The falling droplet sampler was first proposed by Liu and Dasgupta [46], later implemented for hyphenating flow injection analysis (FIA) to CE [66-68]. Recently, falling droplet sampling was adopted in the FIA-CE system to avoid the deterioration of separation due to hydrodynamic pressure created in the inlet, especially with higher flow rates in the FI system [69]. In our design, the construction of the falling droplet sampler is simplified further by rejecting a specially designed FI-CE interface body. Computer-controlled delivery of droplets of a sample or a buffer is performed by gas displacement pumps into a common pipette tip where the separation capillary and electrode are also placed. Liquid is kept in the tip by surface tension, and if a new portion of liquid is delivered, it displaces the old portion of it, which flows into a waste.

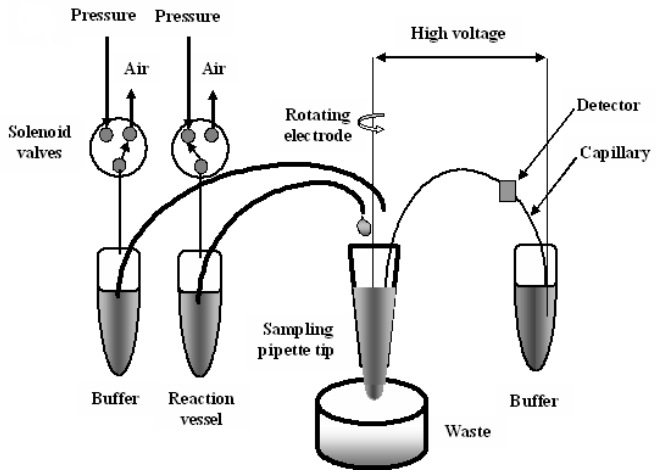

Figure 9. Schematic of falling droplet interface

The falling droplet interface is shown in Figure 9. The CE column input vessel was achieved by positioning the inlet of the separation capillary into a 1 mL pipette tip where at the inside a sample/buffer liquid stays by surface tension (ca. 50 μL). A sample/buffer inside the tip can be replaced to a new portion just by delivering liquid droplets into the pipette tip. This action forces the old sample/buffer portion to flow out of the tip and to be displaced by the new sample/buffer portion. A platinum electrode (0.3 mm diameter), serving as the anode/cathode, was inserted through a hole on a 30mm × 130mm plastic rod installed above the pipette tip. The electrode was rotated (2 rps) by an isolated DC micro motor (type L149, Elfa, Järfälla, Sweden) to facilitate stirring of the liquid inside the pipette tip. Also, a separation capillary (365-μm o.d 75 i.d Polymicro Technologies, Phoenix, AZ, USA) with a length of 70 cm (39 cm to detector) was inserted into the pipette tip. A separation voltage of 25 kV was used throughout all the experiments.

Two glass capillaries (5 cm long, 1.6 mm o.d, 0.5mm i.d) for the delivery of sample and buffer droplets were inserted through holes on the upper rod. The outlets of the capillaries were coaxially positioned 24 mm above the liquid surface inside the pipette tip. The capillaries were connected via PTFE tubing (20 cm long, 0.7 mm i.d.) to the sample and buffer vessels located inside gas displacement pumps the construction of which was described above.

2.2 CE apparatus

Most of the experiments were performed using a Spellmann 2000 High Voltage Power Supply (Spellmann, Hauppaugue, NY, USA) and UV detection (Lambda 1000, Prince Technologies, the Netherlands) at various wavelengths (214, 256 nm). For analyte separation, fused silica capillaries (Polymicro Technologies, Phoenix, AZ USA) with various dimensions were used throughout the study. Experiments were controlled by a PC, using software written in house in LabView (National Instruments, Austin, TX, USA) environment via Adam ADC/DAC interface (Advantech, Taipei, Taiwan). Electroperograms were edited by Matlab (MathWorks, Natick, MA, USA) routines written in house. Computer-controlled sampling was accomplished by controlling the corresponding solenoid valves (Type 6012 Miniature Solenoid Valve, Brükert, Helsinki, Finland) which delivered pressure pulses to the gas displacement pumps.

2.3 Chemicals and reagents

1. **CE buffer components:** Sodium dihydrogenphosphate, cetyltrimethylammonium bromide, and EDTA were purchased from Sigma; phosphoric acid and SDS were obtained from YA-Kemia (Helsinki, Finland); sodium tetraborate and sodium hydroxide from Merck (Darmstadt, Germany).
2. **Analytes and reagents:** Nucleotides adenosine-5'-diphosphate and adenosine-5'-triphosphate were obtained from Sigma (Taufkirchen, Germany); hexokinase was from Calbiochem (Darmstadt, Germany); *p*-Cresol, *3,4*-dimethyl phenol, resorcinol, *5*-methyl resorcinol and 4-methyl catechol were obtained from YA-Kemia; L-ascorbic acid, hydrogen peroxide were purchased from Merck (Darmstadt, Germany).
3. **Reaction media components:** Sodium hydrogencarbonate, anhydrous D-glucose and magnesium sulfate were obtained from YA-Kemia (Helsinki, Finland).

   Solution A contained, in mM: CaK_2EGTA 2.77, K_2EGTA 7.23, $MgCl_2$ 6.56, dithiothreitol 0.5, Mes 50, imidazole 20, taurine 20, Na_2ATP 5.3, phosphocreatine 15, pH 7.1 adjusted at 25°C.
   Solution B contained, in mM: CaK_2EGTA 2.77, K_2EGTA 7.23, $MgCl_2$ 1.38, dithiothreitol 0.5, Mes 100, imidazole 20, taurine 20 and K_2HPO_4 3 and pH 7.1 adjusted at 25°C. Solution A and B reagents were purchased from Sigma (USA).

   Minimum salt medium: M9 - 21%, microelements – 0.125%, $MgSO_4$ – 0.09%, $FeSO_4$ – 0.65%

   Solution M9: NH_4Cl – 0.4%, NaCl – 0.2%, KH_2PO_4 – 1.2%, Na_2HPO_4 – 2.8%, $Na_2HPO_4 \cdot 12H_2O$ – 7.03%
4. **Micro-organism and culture conditions:** For experiments with the biodegradation of phenols, a pure culture of *Rhodococcus pyridinivorans* was used. The inoculum for the experiment was pre-grown for 24 hours in a sterilized 250 mL conic flask containing 100 mL of 25% Plate count Agar medium (Pepton from casein – 0.5%, yeast extract – 0.25%, D(+) glucose – 0.1%). The flask was incubated at 20 ºC and shaken on a rotary shaker, the complete mixing and necessary transfer of oxygen were ensured. Then the inocculum was placed in the bioreactior with an initial biomass 7.6×10^6 CFU/ml (CFU - Colony Forming Units).

Standard solutions were prepared daily. Water used for the standard and buffer solution preparation was obtained from Milli-Q-water system (Millipore corporation, Bedford, MS, USA). All electrolytes and rinsing solutions were filtered before use through 0.45 μm nitrocellulose Millipore filters.

2.4 Data processing procedures

Electropherograms were transferred from Labview format to Matlab (Mathworks, MA, Natics, USA) and edited for spike removal and baseline subtractions using the subroutines written in house. Also, peak areas were calculated and saved as Excel worksheets (Microsoft, Seattle, WA, USA).

Calibration was performed by an external standard method throughout the study using five calibration standard values covering a concentration range of about two orders. The calibration lines for key compounds appeared to be linear with the correlation coefficient close to unity.

Initial rates of the reactions (Articles II and III) were calculated by linear least squares fitting of concentrations as time functions to straight lines. Further, the collection of concentrations (Articles I and V) or initial rate data (Articles II and III), as a function of time or substrate concentrations correspondingly, was fitted to the kinetic model functions. To minimize the summed squares of differences between the experimental data and the model function the "Excel Solver" minimization procedure was implemented, which resulted in the desired parameters of the kinetic model functions. Parameter uncertainties were estimated according to the procedure proposed in [70]. First, the "ksisquare" function was calculated according to the formula

$$\chi^2 = SS / s^2, \qquad (1)$$

where *SS* is a squared sum of the differences between the model and the experimental data and s^2 is a variance of measured initial rates of the reaction. The χ^2-function can be approximated with the sum of square function of sought parameters as follows:

$$\chi^2 = Const + (a_i^{(\min)} - a_i)^2 / s_i^2 + \ldots, \qquad (2)$$

where $a_i, a_i^{(\min)}, s_i^2$ are the *i*-th parameter arbitrary value, its value at the minimum and its variance, correspondingly. From this, it can easily be deduced that the variation of each of the parameters around the minimum, which results in the change of χ^2-function by a unit value, is equal to that parameter variance. Thus, after finding $\chi^2_{\min}$ by the Excel Solver, the model parameters were varied individually by the amount that resulted in the desired increase of χ^2-function (i.e. by $\chi^2 = \chi^2_{\min} + 1$).

3 RESULTS AND DISCUSSION

3.1 On-line CE monitoring with automated sampling

3.1.1 Bacterial degradation of phenolic compounds (Article I)

Phenolic compounds mainly emitted to the nature from the anthropogenic sources are widely distributed organic pollutants. The degradation of organic compounds in the environment can occur due to different chemical, physical, biological, or photochemical processes. Biological decomposition of organic pollutants by microorganisms is one of the most important and effective ways to remove these compounds from the environment. The monitoring and control of this kind of biological degradation in the reactor in a strictly controlled environment provides a better understanding of bioremediation of polluted sites by bacteria, taking place in the natural environment.

In this part of work, the process of bacterial degradation of selected phenolic compounds by bacteria *Rhodococcus pyridinivorans sp.* (Article I) was monitored on-line and quantitatively studied in a miniaturized bioreactor in strictly controlled environment. The adaptation of capillary electrophoresis for direct on-line analysis of bacterial degradation of phenols was achieved by combining the pneumatic autosampler (see section 2.1.1) specially designed for multiple sample injections with a membrane-based dialysis unit (see section 2.1.1.1), which allows us to eliminate the preparation step of a complex macromolecular sample.

The on-line experiments were performed as follows: 30 mL of salt medium containing phenolic compounds (resorcinol (R), 5-methylresorcinol (5-MR), p-cresol (p-Cr), 3,4-dimethyl-phenol (3,4-DMPh)) was introduced to the donor compartment of the dialysis unit and the reaction media components were allowed to penetrate through the membrane for two hours. After that, 3 mL of inoculum was placed in the reactor and the reference electropherogram of the media was recorded. Then the inoculate was kept in the reactor overnight and monitoring was started after 14 hours. Monitoring was performed at two-hour intervals. At the moment of sampling, the content of the acceptor compartment of the dialysis unit was taken into the inlet channel of the pneumatic autosampler and the acceptor compartment was filled with a fresh amount of pure water (for details see Article I).

3.1.1.1 System performance

Straightforward considerations suggest that phenols are removed from the bioreactor via three different processes: 1) extraction via the membrane from the donor compartment to the acceptor compartment which is then periodically sampled and discarded, 2) evaporation due to an intensive purging of the bioreactor by air and 3) consumption of phenols by bacteria. To determine the contribution of biodegradation to the total concentration change, each of these factors has to be studied and quatified. For that, several tasks must be solved beforehand, viz. a suitable protocol for the separation of phenols must be chosen first, which takes into account the possible appearance of new products in the reactor. Next, to determine of the membrane recovery and extraction kinetics for different compounds, it is necessary to find the contribution of the membrane extraction to an overall decrease of analytes in the reactor to be taken into account and separated from those resulting from evaporation and biodegradation only.

In Figure 10 a typical set of pherograms obtained for one of the biodegradation experiments is presented. For the separation of four phenols, 100 mM phosphate buffer (pH 7.4) with an addition of 50 mM sodium dodecyl sulphate (SDS) was used. As this figure shows, the migration time of the peaks decreases in the course of the experiment. The exact reason for this change was not investigated but it may be accounted for by the temperature change in the lab during the working day, because the thermostating of the equipment was performed by ventilating the equipment by

lab air. Nevertheless, since the patterns of the peaks can be easily recognized, no attempts to improve equipment thermostating were made.

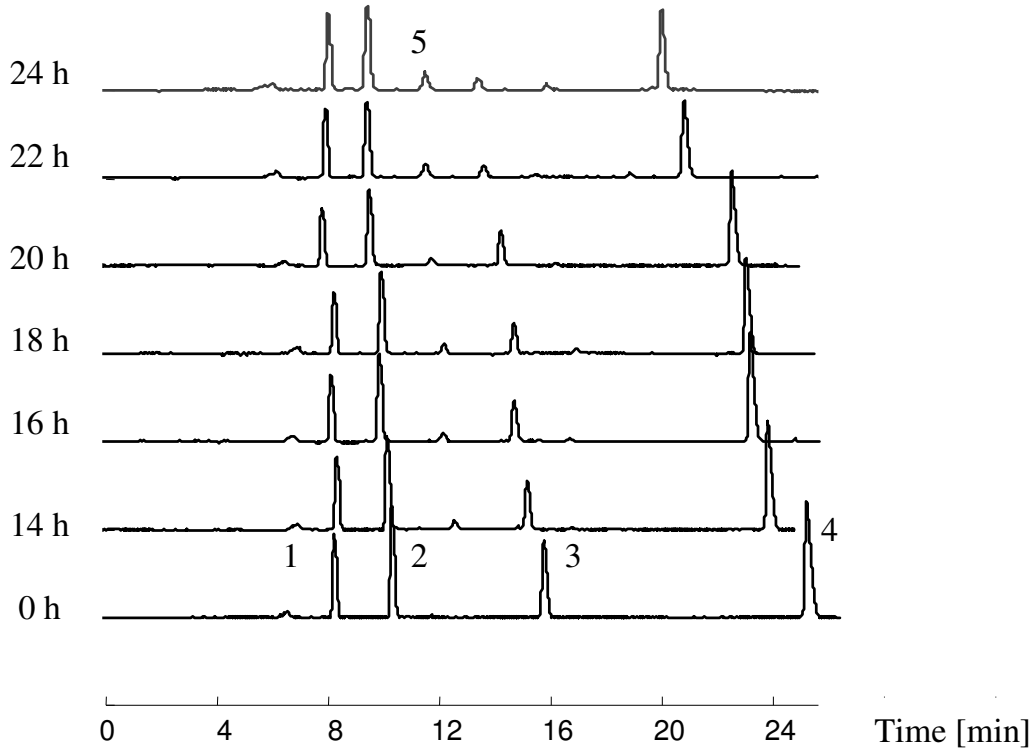

Figure 10. Typical set of electropherograms recorded during the monitoring. Experimental conditions: phosphate buffer 100mmol, pH=7.5, 50mM SDS; sample, a pure culture of *Rhodococcus pyridinivorans* isolated from industrially polluted sites of Estonia, four phenols with concentration about 0.3 g/L, λ=214nm, potential +17kV. Peaks: (1) resorcinol, (2) 5-methylresorcinol, (3) p-cresol, (4) 3,4-dimethyl-phenol, (5) product of biodegradation process (4-metylcatechol).

The calibration was performed by the external standard method using five calibration standard values covering a concentration range of about two orders. The calibration line for the identified compounds appeared to be linear with the correlation coefficients close to unity (see Table 2). All the concentrations measured were well above the LOD of the assay used. That is despite the decreased recovery caused by the membrane.

In the context of this study the decrease, of the analytes in the reactor due to the sampling occurs via a membrane extraction. The estimation of the analyte decrease due to the membrane extraction can be described by the following equation:

$$C_k^{(d)} = C_0^{(d)}(1 - R\frac{V_a}{V_d})^k, \qquad (3)$$

where V_d and V_a are volumes of the donor and acceptor compartments, correspondingly, R – membrane recovery coefficient, $C_0^{(d)}$ - initial concentration of the analyte in the donor compartment, $C_k^{(d)}$ - concentration of the analyte in the donor compartment after k sample injestions, k – number of experiments (sample injections) during the monitoring.

Table 2. Performance data of monitoring with membrane interface

Compd.	R(%) (Eq. (1))	R [a] (%) (direct)	CC [b]	LOD [c] (μg mL^{-1})	Reproducibility (%; at monitoring instant), n = 5					
					14h	16h	18h	20h	22h	24h
R	60	79	0.985	8.91	22	32	37	34	35	28
5-MR	94	94	0.993	6.45	7	10	7	5	9	15
p-Cr	60	70	0.998	7.52	19	29	24	18	67	24
3,4-DMPh	79	79	0.993	3.75	25	33	25	14	17	18

a) Recovery;
b) Linear regression correlation coefficient of calibration curve;
c) Limit of detection, determined as intersept of the horizontal line (drawn from the upper confidence limit at zero concentration) to the calibration line [71].

Taking, for example, R=0.8, V_d=30 mL and V_a=1.6 mL one obtains that, for example, if k=*10* a decrease of the analytes due to the membrane extraction is about 35% which means that it can not be neglected.

The R values were determined by the least squares fit of the measured concentrations to Eq.(3). The corresponding values are given in Table 2. Also, the validity of Eq.(3) was tested in a monitoring experiment where a stirred reactor contained analytes only and was not purged by air. For that, the key compounds dissolved in water with a known concentration were first delivered directly to the sampling valve. The electropherogram was recorded and the peak areas were measured. Then the same aqueous solution of the key compounds was placed in a stirred donor compartment of the reactor. The key compounds were allowed to penetrate into the acceptor compartment for two hours and then the content of the acceptor phase was delivered to the sample injection device and the electropherogram was recorded. Assuming that the peak area on the pherogram is proportional to the sample amount in the sampler, the straightforward reasoning allows the calculation of the recovery coefficient, R, for the key compounds as follows:

$$R = \frac{A_R}{A_{direct}}, \qquad (4)$$

where A_{direct} and A_R are the peak areas for the direct electropherogam and the electropherogam when the analytes were situated at the donor compartment, respectively. The results are presented in Table 2. The data shown indicate that there is a good agreement between the two measurements, the recovery coefficient varies between 70 and 94% and the equilibrium (100% recovery) between the donor/acceptor compartments during two hours was not established.

3.1.1.2 Kinetics of biodegradation of phenols

From the pherograms recorded at different time moments in the course of the experiment (see Figure 9), the normalized concentration curves were calculated for the membrane extraction only, for membrane extraction with purging the reactor with air and for membrane extraction with purging the reactor with air in the presence of microorganisms. The contribution of bacterial degradation of the total removal of phenols from the reactor was found by subtracting the results of experiments with evaporation only from those of experiments with bacteria in the reactor. According to the literature data, degradation reactions are typically described by first order equations [72]. Indeed, degradation data obtained fit well with the first-order kinetics:

$$\frac{dC}{dt} = -k_1 C\,, \tag{5}$$

where C represents the concentration of a degraded compound at the time t; k_1 is the first-order rate constant. In practice, the first-order rate constant often is replaced by a half-life H, and the degradation rate is expressed as

$$\frac{dC}{dt} = -\frac{\ln 2}{H} C\,, \tag{6}$$

where $H = ln2/k_1$. If half-life, H remains constant in a degradation process, the residual concentration $C(t)$ may be expressed as an exponential function of time. That is

$$C(t) = C_0 e^{-\frac{\ln 2}{H} t}\,, \tag{7}$$

where C_0 is the initial concentration. Rate constants here were calculated by a least squares fitting of phenol concentrations to this exponential dependence. Uncertainties in rate constants were calculated according to the procedure described in Section 2.4. The data on the degradation rate constants and half-lives for four different phenols are presented in Table 3. The correlation coefficients (r) indicate the goodness of the first order fit of phenol degradation. The results show that the degradation rate of *p*-Cresol was higher than that of other phenols. A possible explanation for this is that the phenols with higher molecular weights are more resistant to biotransformation than the lower molecular weights of *p*-Cresol. The toxicity of phenolic compounds is also different, for example, 5-methyl resorcinol is known to be more toxic as compared to other phenols reported here, therefore, consumption of this compound is supposed to go slower.

Table 3. Degradation rate constants and half-lives for four phenols.

Substrate	k_1*10^{-3} (hour^{-1})	S_k*10^{-3} (hour^{-1})	RSD, %	H (hour)	r
5-MR	9.3	0.57	6.50	79.7	0.941
R	16.4	0.63	3.98	44.7	0.978
p-Cr	19.8	1.09	3.04	35.7	0.961
3,4-DMPh	16.3	0.92	5.98	45.6	0.949

The appearance of at least one intermediate product peak can be detected during the monitoring (Figure 9). According to the model of the cleavage of the aromatic ring by *Rhodococcus sp*., the most probable product is *4*-methyl catechol. Indeed, the product peak was identified as *4*-methyl catechol by spiking.

The contribution of different factors of the removal of phenols from the bioreactor during the source of monitoring (24 hours) is summarized in Figure 11. The contribution of the evaporation of phenols due to an intensive purging of the bioreactor by air was estimated by simply subtracting the membrane extraction results from those of evaporation without bacteria. The diagram demonstrates that the most volatile phenol is p-Cresol. 5-methyl resorcinol has the greatest concentration decrease caused by sampling, it can be explaned by its recovery coefficient, which was 94%.

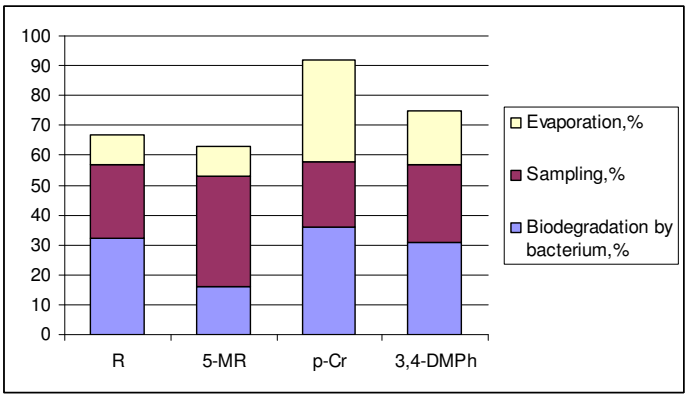

Figure 11. Contribution of different processes of the removal of phenols from the reactor

The overall reproducibility of the assay was measured by performing monitoring experiments on different days (sometimes at an interval of a couple of weeks). Thus, the reproducibility in Table 2 accounts for the reproducibility of inoculum preparation as well as for the day-to-day reproducibility of performing CE measurements (buffer preparation and lab environment temperature, humidity, etc.). The reproducibility is around 20 % for each phenol, and taking into account considerations above, this should be considered acceptable for the monitoring purposes to make quantitative conclusions about the process.

3.1.2 Enzymatic ATP hydrolysis (Articles II and IV)

For further studies of the overall potential of capillary electrophoresis in the field of biomonitoring, the possibilites of the pneumatic autosampler were also explored for on-line monitoring of enzymatic reaction kinetics (Article II). The model reaction was chosen to be a well-known enzymatic conversion of adenosine triphosphate (ATP) to adenosine diphosphate (ADP) by hexokinase. Hexokinase phosphorylates glucose to glucose-6-phosphate, using ATP as a substrate. During this reaction, ATP is converted to ADP. This catalytic process is thoroughly described in the literature and has been investigated by many methods. Thus, it was chosen as a good reference to estimate the characteristics of our monitoring equipment and procedure. This reaction was tested in various conditions, which allowed a general estimation of the suitability of the developed CE method for the monitoring of enzymatic reactions in general. First, the electrophoretic separation conditions were studied for an optimal monitoring of ATP hydrolysis. Furthermore, a kinetic study of the hydrolysis of ATP was carried out. Substrate and enzyme concentrations were varied and the Michaelis-Menten constant was calculated.

3.1.2.1 CE method development

The hexokinase enzyme hydrolyzes ATP to ADP, as shown in Figure 12. For nucleotide separation (see Figure 13, *a*), the method of micellar electrokinetic chromatography was applied. For that, the cationic surfactant CTAB was added to the background electrolyte, which causes the capillary wall to be positively charged and reversing the EOF toward the anode. Also the voltage polarity was reversed to reestablish the electroosmotic flow toward the detector. Buffer pH was reduced up to 4.5 for a partial neutralization of nucleotides, which provided more complicated separation mechanism, when the nucleotide mobility depends on the charge to the radius ratio as well as on hydrophobic interactions with micelles. Despite the fact that the buffering capacity of the phosphate buffer is limited at that pH, electroferograms demonstrate that the method of MEKC that

employs reversed EOF, provided a good resolution of ATP and ADP and a short analysis time of less than 3 minutes.

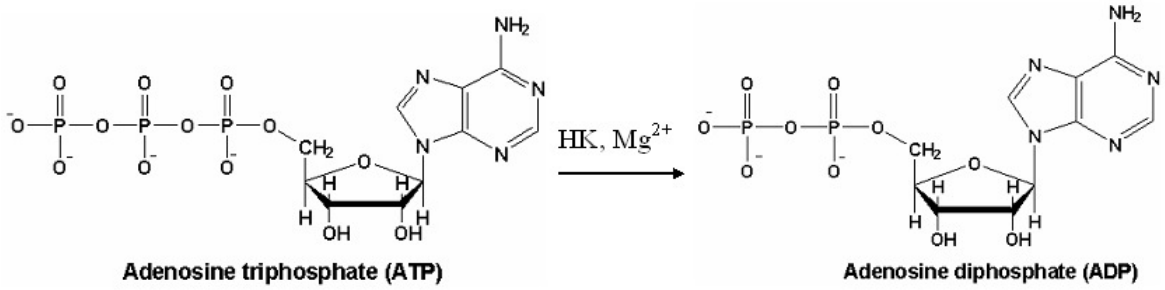

Figure 12. Enzymatic hydrolysis of ATP to ADP by hexokinase (HK)

The determination of nucleotides by CE was accomplished with UV detection at 254 nm. The other components of the reaction mixture (glucose, glucose-6-phosphate, inorganic ions) have a low UV absorbance at this wave-length, thus allowing the detection of key compounds without a significant disturbance.

To evaluate the precision of the CE system, an experiment was made, using replicate (n=3) injections of ATP standard solution. The precision of migration times and peak areas was determined by calculating the relative standard deviation (RSD) of the measured parameters. The migration times were precise with RSD values within 1%, while the peak area reproducibility varied from 2.51 to 5.5% over the measured concentration range. The system gave a linear response to ATP concentration from 0.05 to 0.25mM with a correlation coefficient of 0.977.

3.1.2.2 Enzyme assay and Michaelis-Menten kinetics

In the second step hexokinase activity was determined. For that, adenosine triphosphate was incubated with hexokinase in the presence of glucose and magnesium ions. The reaction was initiated by adding hexokinase. An automated sample injection device allowed us to monitor enzymatic activity directly by using sample vial as a catalysis reactor, so ATP enzymatic conversion kinetics was determined without any reaction medium perturbation and sample preparation. We suppose that hexokinase separates within the first few seconds from the injection of the reaction mixture from the analytes band and reaction stops. The separation of ATP and ADP is shown in Figure 13(a). Figure 13(b) shows a typical pherogram obtained by a repeated injection of a reaction mixture containing of 0.037 U/mL hexokinase, 0.1mM ATP and buffer components. Each group of peaks represents a new injection of the reaction mixture.

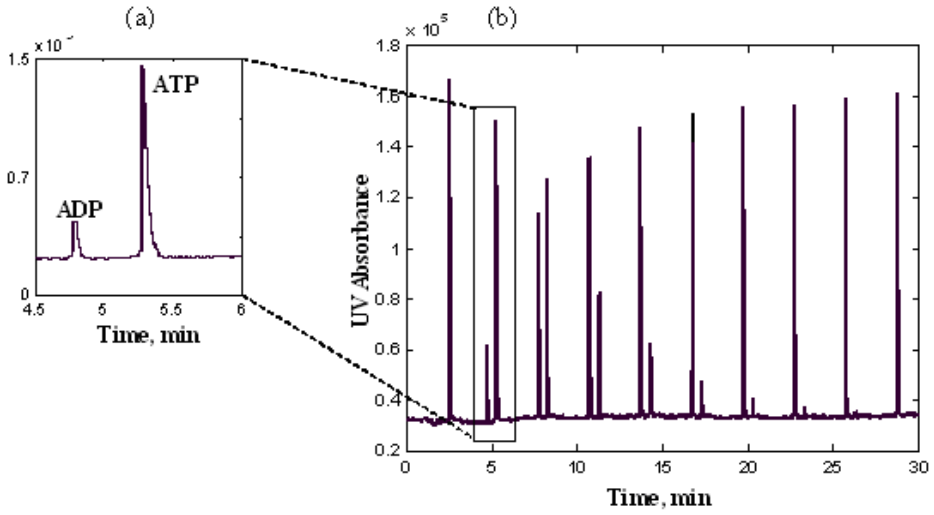

Figure 13. Electropherogram of the reaction mixture recorded during the monitoring. Experimental conditions: phosphate buffer 25mM, 25mM CTAB, pH 4.5, fused silica capillary L_{eff}: 25cm×50μm i.d., UV detection at 254 nm, potential –25kV. Sample-medium: $NaH_2PO_4 \cdot 2H_2O$ 12mM, $NaHCO_3$ 4.2mM, D-glucose 22mM, $MgSO_4$ 2.7mM, hexokinase 0.037 U/mL, ATP 100μM, sample pH=7.

Figure 13(b) shows that both the formation of the product and the disappearance of the substrate can be monitored simultaneously and quantified according to their respective peak areas. During the course of the reaction, a continuous decrease in substrate concentration was observed with an increase in product formation, correspondingly, as one should expect.

The mechanism for an enzyme-catalyzed reaction can be summarized in a generalized Michaelis-Menten scheme [73]

$$E + S \underset{k_{-1}}{\overset{k_1}{\longleftrightarrow}} ES \xrightarrow{k_2} E + P \ , \qquad (8)$$

where k_1 is the rate constant for the formation of the enzyme-substrate complex *ES*, from the enzyme *E* and the substrate *S*; k_{-1} is the rate constant for the reverse reaction, and k_2 is the rate constant of the conversion of the *ES* complex to product *P*. The kinetics of the enzymatic reaction was analyzed according to the Michaelis-Menten model, which describes the relation between the initial reaction velocity *V* and the substrate concentration [S]:

$$V = \frac{[S]V_{\max}}{[S] + K_m} , \qquad (9)$$

where $V_{\max}$ is maximum reaction velocity, K_m is Michaelis constant, the substrate concentration at half the maximum velocity. It follows from Eq.(8) that the Michaelis constant is

$$K_m = \frac{k_{-1} + k_2}{k_1} . \qquad (10)$$

The Michaelis constant is a unique parameter of a specific enzyme. It reflects the binding affinity of the enzyme for a specific substrate. The determination of K_m requires the measurement of initial velocity at several initial substrate concentrations. For that, the reaction mixture containing 0.04 U/mL of HK was used and the concentration of ATP varied between 0.05 and 0.25 mM. Typical reaction progression curves at different ATP concentrations, monitored by measuring the appearance of the product ADP, are illustrated in Figure 14, where the product peak areas are plotted versus time. The initial reaction velocities were deduced from the slopes of the linear part (first 2-6 minutes) of these curves. To estimate the precision of the initial velocity determination, the initial reaction rate was determined five times for 0.1 mM concentration of ATP. RSD value of 5% was obtained, which indicated a good repeatability of this CE method.

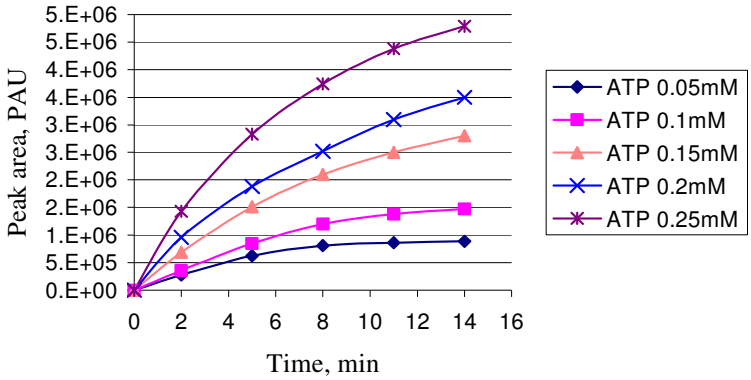

Figure 14. Formation of ADP during the hydrolysis of ATP

By the linearization of the Michaelis-Menten equation, the Lineweaver-Burk plot is obtained, which describes a linear relation between 1/V and 1/[S]

$$\frac{1}{V} = \frac{K_m}{V_{\max}} \cdot \frac{1}{[S]} + \frac{1}{V_{\max}} \qquad (11)$$

The Michaelis constant K_m for ATP hydrolysis was estimated by the linear least squares regression from Lineweaver-Burk plots. The described procedure for K_m determinations was repeated for two more HK concentrations (Figure 15). The K_m values determined for three different enzyme concentrations (0.037, 0.05 and 0.068 U/mL) were 0.2 , 0.18 and 0.22 mM, representing an average K_m 0.201 ± 0.015 mM (i.e., n = 3, RSD 7.4%). The correlation coefficients (r) were 0.993, 0.993 and 0.995, correspondingly. The experimentally obtained K_m value is within the previously reported data measured in different laboratories by different methods from K_m = 0.2 mM [74,75] to K_m = 0.4 mM [76,77].

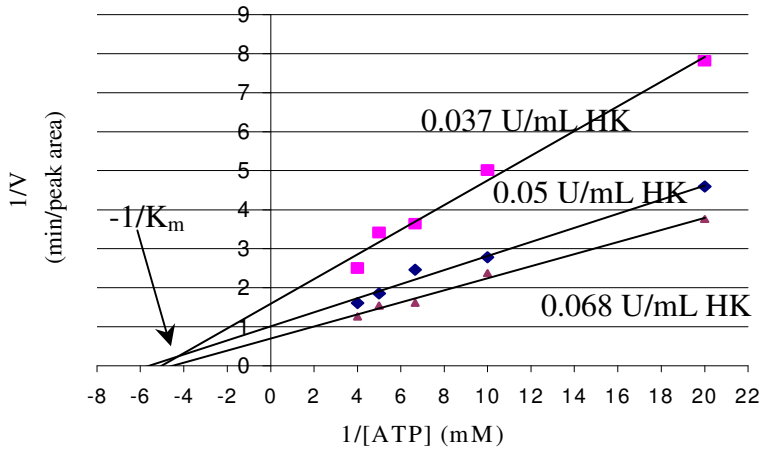

Figure 15. Lineweaver-Burk plots for three different HK concentrations (0.037, 0.05 and 0.068 U/mL); The concentration of ATP varied between 0.05 and 0.25mM. Each concentration was analyzed in triplicate.

Clearly, the reaction studied here, ATP conversion to ADP by hexokinase, is a well-known textbook example reaction and as such is not of much interest. It has served here as a good reference point for the estimation of capabilities of on-line monitoring equipment, measuring the characteristics of the sampling system and estimating the overall potential of CE in such

experiments. The study carried out demonstrated that CE indeed could and should be developed into a routine method for an on-line monitoring and control of complex enzymatic processes.

3.1.3 L-ascorbic acid oxidation by hydrogen peroxide (Article V)

In capillary electrophoresis a sharp contrast exists between the nanoliter amount of sample, necessary for the separation/detection process inside the capillary and the milliliter amount of chemicals needed for operation in samplers in most of commercially available instruments. In biochemical assays sample amount is frequently limited to microliters. Such amount can be usually sampled to a CE instrument manually. However, if the monitoring of biochemical reactions by CE is of interest, then manual sampling is inconvenient, not economical and imprecise, therefore the development of automatic samplers capable of operating with tiny amounts of samples becomes highly desirable.

In this chapter, two simple and economical techniques intended for computerized on-line sampling of the small amount (couple of hundreds of microliters) of reacting media into CE capillary are described (see also Article V). The model reaction was chosen to be an oxidation of L-ascorbic acid by hydrogen peroxide. During this simple chemical reaction, L-ascorbic acid (reduced form) is converted to dehydroascorbic acid (oxidized form). The kinetics of this reaction was tested with two different CE samplers: cross sampler and falling droplet interface, the designs of which are described in detail in Experimental sections 2.1.2 and 2.1.3, correspondingly.

3.1.3.1 Experimental parameter optimization

For the cross sampler the alignment of the separation channel capillaries was critical for proper operation and reproducibility of the sampler. The alignment procedure, however, appeared to be a relatively robust action and could be proceeded routinely (e.g., if the replacement of new capillaries was necessary). In the falling droplet case the sample and buffer tubing must be centered properly to avoid droplets falling on the pipette tip wall above the liquid surface stored in the tip. Also, this adjustment could easily be performed.

The optimal pressure and sampling time was worked out for both samplers. Obviously it is determined by the dimensions of the sample tubing. It was found for a cross sampler that the minimal pressure necessary for the proper functioning was about 98 kPa. By applying the pressure pulse for 1 s it was possible to deliver a minimum of ca. 25 μL of the sample (two droplets). This means that to record five to ten concentration values during the reaction course, about 150-300 μL of the initial amount of the reacting mixture would be necessary.

For a falling droplet, a 1.0 s long 50 kPa pressure pulse delivered one 25 μL droplet into the pipette tip. Usually, up to five droplets were necessary to increase the weight of the liquid inside the pipette tip and to force sample/buffer from the previous analysis cycle to flow off the pipette tip. Commonly, this was used for the replacement of the sample from the previous cycle by the buffer for the new analysis cycle. Also, it was possible to deliver fewer (one/two) droplets of the sample into the (e.g. separation) buffer located in the pipette tip. Then a newly delivered sample portion is diluted in the separation buffer. This fact can be advantageously used for matching sample concentration (dictated, e.g., by the reaction under study) to the level needed for the separation process.

In summary, the lower limit of reacting mixture volume necessary for monitoring kinetics could approximately be between 200-500 μL, depending on the number of samplings (measured concentration points) necessary for reaction rate measurements. However, in this work, in most cases, the volume of the reacting media was larger (1mL and above) for the convenience of performing several kinetic experiments in sequence.

3.1.3.2 Kinetic analysis and system performance

The monitoring of oxidation of L-ascorbic acid by hydrogen peroxide was performed in two media: in water and in a separation buffer solution. In the latter case, the reaction rate was considerably faster. Initial solutions were prepared by different persons and reactions were repeated for several days (sometimes with an interval of weeks between experiments) to estimate interday reproducibility. Typical results are presented in Figure 16, where the peak with the constant intensity is hydrogen peroxide and the peak with exponentially decreasing intensity – L-ascorbic acid. In this experiment no product peaks were observed due to the fact that the oxidized form of L-ascorbic acid - dehydroascorbic acid is very unstable in an aqueous solution and may be transformed and degraded into unstable compounds, which have a low UV absorbance at 254 nm.

Notable difference in the L-ascorbic acid migration times of both samplers is due to the different separation voltages applied. It follows from Figure 16 that both samplers perform well and indeed on-line computerized monitoring of concentrations in small reaction volumes is possible.

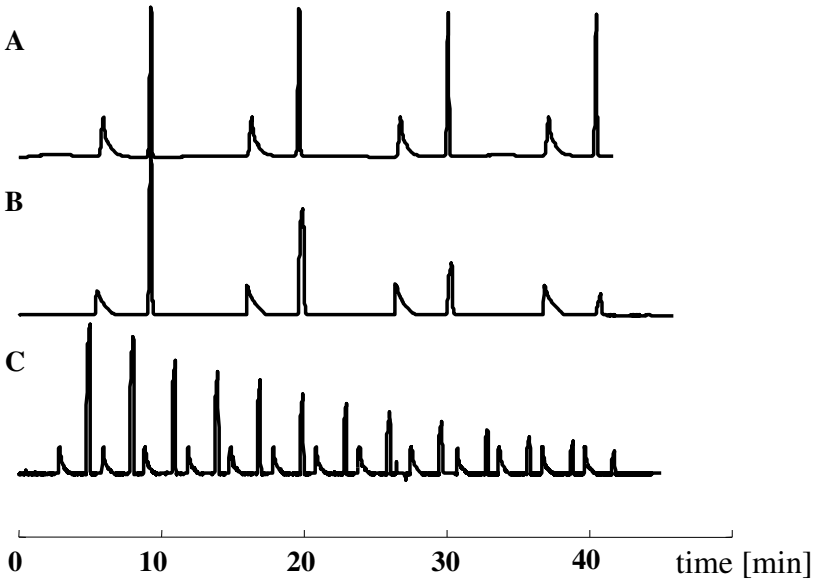

Figure 16. Electropherograms of the reaction mixture recorded during the monitoring. Experimental conditions: 25mM sodium tetraborate buffer, pH 9.4, UV detection at 254 nm. (A) - cross sampler, reaction in water, (B) cross sampler reaction in buffer and (C) falling droplet sampler, reaction in buffer. Peak with constant intensity - H_2O_2, peak with exponentially decreasing intensity – L-ascorbic acid.

Based on the collection of kinetic experiments those data for both samplers are represented in Table 4. It follows from Table 4 that the reproducibility of peak migration times can be considered as good. However, the efficiency is not very high (except the reaction in water obviously because of the stacking of L-ascorbic acid peak). The low efficiency is probably due to the fact that both samplers contribute much to initial extracolumn band broadening which is not surprising taking into account the construction of both samplers. For the cross sampler, the peak half width ratio to the migration time is about 3%, which means that the initial band occupies the same ratio of the column length when the separation starts. Similar reasoning for the falling droplet sampler results in a 4% value. Those numbers are much larger than accepted in common capillary electrophoresis. However, since both samplers are intended to be used for reaction monitoring in which case the sample is a relatively simple solution of a few components, it is possible to separate them anyway despite the small efficiency of the separation. Definitely, improvements are possible, however further studies are required.

Table 4. Performance data and their relative standard deviations (RSD) of samplers

Parameter	Cross	Falling droplet
H_2O_2 peak migration time, min	5.5, RSD = 0.5 % (n=5)	2.8, RSD = 0.2 % (n=7)
RSD of H_2O_2 peak area, %	4.4±1.6 (n=3)	7.5±0.5 (n=3)
L-ascorbic acid peak migration time, min (in water)	8.9, RSD = 0.1 % (n=9)	5.8, RSD = 2 % (n=9)
L-ascorbic acid peak efficiency# (in water)	23756, RSD = 5.51 % (n=9)	18802, RSD = 11.74 % (n=7)
L-ascorbic acid peak half width, s (in water)	8.1, RSD = 2.6 % (n=9)	9.3, RSD = 6 % (n=9)
L-ascorbic acid peak migration time, min (in buffer)	9.5, RSD = 0.8 % (n=4)	5.1, RSD = 1 % (n=7)
L-ascorbic acid peak half width, s (in buffer)	17.1, RSD = 3.4 % (n=4)	13.5, RSD = 4 % (n=7)
L-ascorbic acid peak efficiency# in buffer	6242, RSD = 7.92 % (n=4)	2857, RSD = 7.43 % (n=7)

From the L-ascorbic acid peak areas it is possible to estimate reaction rate constants. Assuming a simple case of a pseudo first order irreversible A+B→C type reaction, one can consider that the peak shape of either of the reactants, is an exponential decay with a rate, say k as follows, e.g. for A

$$S_A(t) = S_{A0}e^{-kt} \tag{12}$$

Here index A stands for L-ascorbic acid and B is in excess; t is the running time and S_{A0} is a constant proportional to reactant concentration at the moment of sampling of the reacting mixture. Rate constants were calculated by a least squares fitting of L-ascorbic acid peak areas to Eq.(12). Uncertainties in rate constants were calculated according to the procedure described in Section 2.4.

Rate constants are presented in Table 5. It follows that the gross average reaction rate is approximately 10^{-3} s^{-1}, which is in the same order as in published data ($3.7\ 10^{-3}$ s^{-1} [78]). The relative precision for a particular single experiment is excellent against the corresponding RSD values of 1-3 %. Interday reproducibility is larger than can be expected. Evidently, with this precision, there is a slight disagreement in the rate constant values between two samplers, as the calculated Student t-value is $t = 3.24$ when the critical two-sided t value is $t_{(95\%,\ 4)} = 2.77$. This disagreement and large interday standard deviation values can probably be associated with the 1% variations in the temperature control of the reactors. Since the application here is intended as a proof of the principle, no further actions for improvements in temperature control were undertaken.

Table 5. Rate constants

Cross sampler				Falling droplet interface			
Run	$k*10^{-4}$ (s^{-1})	s_k*10^{-4} (s^{-1})	RSD, %	Run	$k*10^{-4}$ (s^{-1})	s_k*10^{-4} (s^{-1})	RSD, %
1	9.6	0.2	1.7	1	6.6	0.3	5.2
2	14.1	0.6	4.2	2	7.0	0.2	2.7
3	13.9	0.3	1.9	3	11.1	0.2	1.6
4	15.7	0.3	2.2	4	11.4	0.5	4.1
5	17.7	0.7	0.4	5	7.2	0.3	4.4
Average	14.2	0.4	2.7	Average	8.7	0.3	3.6
Strd.dev	3.0	0.2	1.1	Strd.dev	2.4	0.1	1.5

For correct estimation of the rate constant, the reaction must be stopped rapidly which means that the reactants must be rapidly separated after the injection. Thus, contact time of reactants must be short compared to the reaction characteristic duration. Mathematically, this statement can be written as follows:

$$t_c = \frac{d}{v_B - v_A} = \frac{w_A t_B}{t_A - t_B} << \frac{1}{k}, \qquad (13)$$

where t_c is the contact time of the reactants; d is a sample zone initial width; v_A and v_B are migration velocities of the reactants, say, for L-ascorbic acid and H_2O_2, correspondingly; t_A and t_B are migration times of L-ascorbic acid and H_2O_2 and w_A is L-ascorbic acid peak base, which can be estimated approximately taken as being 1.7 times wider of the corresponding peak half width; k is the reaction rate constant. Velocity values in the denominator are subtracted, since the H_2O_2 zone moves with a greater velocity than L-ascorbic acid zone.

As it follows from the data in Table 5, the reaction characteristic time has a value of $1/k \approx 10^3$ s. On the other hand, using values for migration times and L-ascorbic acid peak half width, the contact times are for cross and falling droplet samplers 40 s and 20 s, correspondingly. Thus, indeed, a brief contact of reactants at the beginning of the separation can be neglected when calculating reaction rates.

3.2 In situ monitoring by CE

In situ measurements of the kinetics of any cellular process from the biological point of view means the monitoring of the reaction going on in the intact cells. This kind of kinetic analysis of the tissue metabolites associated with the cellular energetic state has special significance in biology. Therefore, the development of new methods for investigation of enzymatic reactions *in situ* is becoming a necessity. Since capillary electrophoresis has proved to be an extremely suitable technique for an *in vitro* monitoring of the hydrolysis of adenosine 5'-triphosphate (ATP) by Hexokinase (Article II), in the present work, an attempt for further development of CE for specific *in situ* monitoring of metabolic transformation of adenosine 5'-triphosphate (ATP) by MgATPases of muscles was made.

3.2.1 Metabolic conversion of ATP to ADP by MgATPases of muscles (Article III)

The adenine nucleotide adenosine 5'-triphosphate (ATP) is the primary source of cellular energy and is essential for the maintenance of cellular energy homeostasis. In order to demonstrate the potential of the capillary electrophoresis, we have measured the kinetics of the hydrolysis of ATP by saponin-skinned fibers of muscle *Gastrocnemius* white (G_w) in a solution with an ionic composition resembling that of the cytoplasm of the muscle cells. In the present chapter, a CE method developed in Article II was further applied for the *in situ* measurement of the kinetics of ATP metabolic transformation by using saponin-permeabilized muscle fibers.

3.2.1.1 CE separation conditions

For the nucleotide analysis, the method of micellar electrophoretic chromatography (MEKC) described in Section 3.1.2.1. was applied. It was also found that the addition of 10 mM EDTA to 25 mM phosphate buffer in order to strongly bind Mg^{2+} enhanced the separation of ATP from ADP since the distribution of ATP and ADP species between the free acid and magnesium salt forms

could be avoided. Typical electropherograms are shown in Figure 17 for different reaction runtimes and initial substrate concentrations. Despite the fact that the buffering capacity of the phosphate buffer is limited at that pH, electropherograms demonstrate that the method of MEKC employing reversed EOF provided a good resolution of ATP and ADP and a short analysis time of less than 6 minutes.

Proposed separation protocol allowed the quantification of ADP peaks in a complex matrix even for very large substrate/ product concentration ratio (more than two orders in this work), which has not been possible so far (e.g. using reverse phase HPLC). Also, the CE protocol enabled us to suppress possible interfering components from the complicated sample matrix where the bioprocess occurred.

The precision of the CE system was evaluated by an experiment using replicate (n = 5) injections of the sample solution. The precision of migration times and peak areas was determined by calculating the relative standard deviation (RSD) of the measured parameters. The migration time reproducibility had RSD values within 1%, while the peak area reproducibility varied from 2 to 6.5% over the measured concentration range. The system gave a linear response to ATP concentration from 0.1 to 35 mM with a correlation coefficient of 0.996 (not shown).

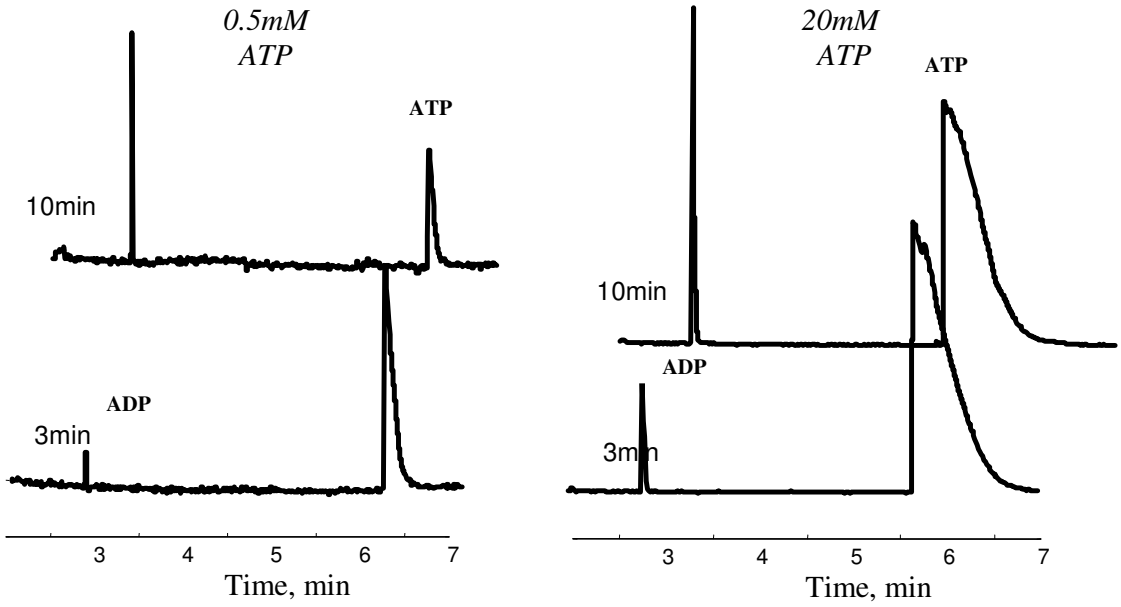

Figure 17. Electropherograms of the reaction mixture at different reaction runtime and different initial substrate concentrations. Reaction medium: 5mg/mL BSA in solution B and ATP with various concentrations. Samples taken after 3 and 10 minutes after reaction initiation. CE conditions: phosphate buffer 25 mM, 25 mM CTAB, 10 mM EDTA, pH 4.5, fused silica capillary L_{eff}: 25 cm×50 μm i.d., UV detection at 254 nm, electrokinetic injection and separation at -25 kV.

3.2.1.2 Comparison of possible kinetic schemes

The measured initial reaction rates as a function of substrate concentration are presented in Figure 17 as circles. The curve has an inflection point and cannot be described by a simple Michaelis-Menten type hyperbola. Evidently, the muscle skinned fibres used in this work, are a complex system, which contains a number of proteins whose function is related to their ATPase activity. Briefly, a major amount of ATP, about 70%, is consumed by myosin filaments responsible for the muscle contraction due to their sliding along actin filaments. The contraction is initiated by the

efflux of Ca^{2+} from the sarcoplasmic reticulum through the ryanodine receptor proteins and terminated by the ATP-dependent pumping of Ca^{2+} back into sarcoplasmatic reticulum at the expense of the consumption of up to 30% (at high Ca^{2+} concentrations) of the total ATP present in the muscle [79]. Other ATP-dependent proteins, e.g., ions channels, signalling protein kinases, consume lower amounts of ATP.

The regulation of the myosin ATPase reaction via binding to a regulatory site is commonly analyzed in the framework of the classical Botts-Morales formalism for the action of a modifier on the properties of an enzyme [80]. The simplest kinetic scheme that could account for this phenomenon includes a high-affinity substrate binding site and low-affinity regulatory site (K_{m1} and K_{m2}, respectively) and results in Eq.(14)

$$v = \frac{V_{\max}([S] + \alpha[S]^2 / K_{m_2})}{K_{m_1} + [S] + [S]^2 / K_{m_2}} \tag{14}$$

where v is the initial reaction rate; $V_{\max}$ is the maximum reaction velocity; K_m is the Michaelis constant; [S] is substrate concentration; α is the proportionality coefficient. The collection of the initial rate data as a function of substrate (i.e. ATP) concentrations was fitted to this function, and then $\chi^2_{\min}$ was calculated as described in Section 2.4. However, Eq.(4) (dashed line in Figure 18) gave a poor fit of the data, especially at lower ATP concentrations ($\chi^2_{\min} = 30$).

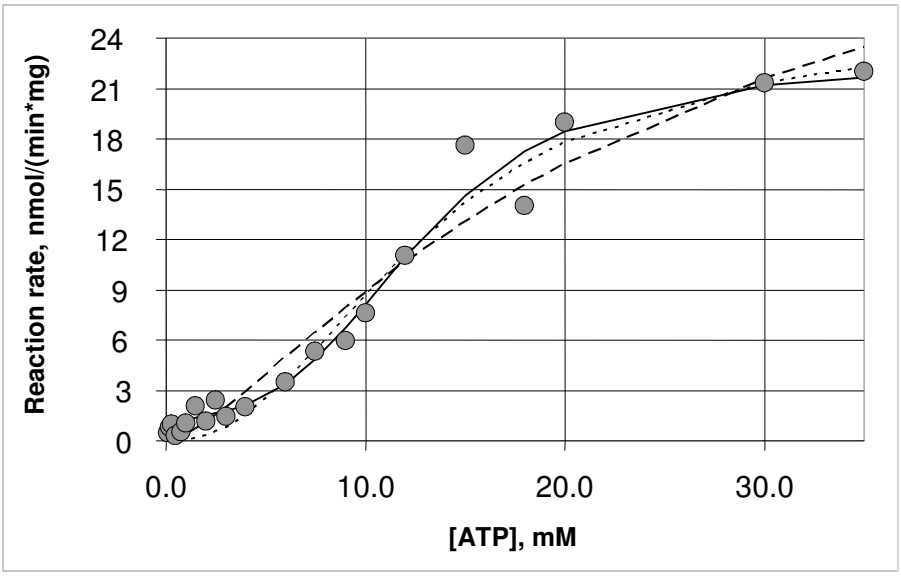

Figure 18. Reaction rates as functions of initial substrate concentrations. Circles – experimental data, solid line – composite Michaelis-Menten and Hill model, dashed line – Botts-Morales model and dotted line – Hill model.

The curves with the inflection point are frequently fitted by the Hill equation, which assumes the involvement of several equivalent, allosterically interacting binding sites, but it is difficult to fit experimental data to this model in the low substrate concentration region (dotted line in Figure 17, $\chi^2_{\min} = 22$).

The best fit of the data (solid line in Figure 17, $\chi^2_{\min} = 15$) was achieved by the equation that involves the sum of the Michaelis-Menten and Hill equations

$$v = \frac{V_{\max_1}[S]}{K_{m_1} + [S]} + \frac{V_{\max_2}[S]^n}{K_{m_2}^n + [S]^n}, \qquad (15)$$

where n is the number of enzyme active cites. Solution of this equation provides a set of constants with corresponding standard deviations: K_{m1} = 0.67 ± 0.21 mM, K_{m2} = 12.85 ± 0.21 mM, n = 3.24 ± 0.16, V_{max1} = 1.99 ± 0.15 and V_{max2} = 21.75 ± 0.32 nmol/min·mg WW. Thus, the system includes a relatively high-affinity catalytic binding site and an allosteric regulatory site(s), which become essential at higher substrate concentrations. Additional cooperative binding of (apparently three) ATP molecules to these sites results in an increase in ATPase activity of the fibres by an order of magnitude, as it follows from the ratio of V_{max2} and V_{max1}.

In previous studies [81,82], the determination of ATPase activity at a single, supposedly high enough, ATP concentration (typically up to 5 mM) is a routine procedure used to characterize the fibre behaviour. The present study has shown (Figure 17) that at least in the relaxed G_w muscle, these activities are far below those that operate in intact muscle. Thanks to the use of the efficient separation technique, MEKC, the present study provides kinetic data over much extended substrate concentration range than it has been done before. The provided range 0.1 - 35 mM of $[ATP]_0$ covers such concentrations that correspond to realistic, physiological situations met in the muscle cells.

4 CONCLUSIONS

The aim of the present work has been to develop a capillary electrophoresis method, which would provide fast and reliable data for kinetic studies of various biochemical reactions. The goal has further been to apply this technique for on-line monitoring of bacterial and enzymatic processes using specially designed non-conventional automated samplers based on the flow injection principle. The research described in this thesis also includes studies of CE application for specific *in situ* monitoring of metabolic transformations.

The principal results of the present study can be summarized as follows:

1. The on-line monitoring of bioprocesses by using capillary electrophoresis is easy to perform, inexpensive and could be introduced in most of biotechnology laboratories. The use of specially designed CE samplers provides an opportunity for on-line monitoring of the biochemical reactions without any reaction medium perturbation and sample preparation, thus avoiding potential errors resulting from a failure to stop the reaction prior to sample injection, faced in some other assay methods.

2. Three simple and economical techniques for automatic and on-line monitoring of the reaction media by CE have been described and two samplers of relatively simple construction, which can be manufactured easily in house, were proposed. Neither of the approaches requires advantaged technologies for microsystems fabrication. Moreover, an important advantage of the developed autosamplers is that their construction allows the operation with small amounts of the sample for each analysis (μL range) so that the amount of the sample injected for each analysis does not change the reaction volume significantly. Also, the sample can be introduced into column without HV interruptions, a factor essential for good CE reproducibility.

3. The CE methods developed provide on-line kinetic analysis of several different biochemical reactions, including bacterial and enzymatic processes. The sample preparation procedure has been simplified and performed on-line to determine the analytes in complex bacterial mixtures by using a membrane-based sample preparation unit connected to the automatic sampler. Sample preparation step has been eliminated for enzymatic assays by using appropriate CE separation protocols, which enabled us to suppress possible interfering components from the complicated sample matrix where the bioprocess occurred.

4. The separation of analytes by capillary electrophoresis proved to be especially advantageous over single component monitoring in research and development where a better monitoring and understanding of bioprocesses is crucial, as it provides details of the consumption and production of many compounds simultaneously in a single run. The separation time by using CE is usually shorter and efficiency higher than in HPLC, which may be essential in some applications. Thus, the separation of the key compounds in this work was completed within several minutes, but can be reduced, if necessary. The measuring equipment was stable during a long run, and the reproducibility of the results was acceptable for monitoring purposes.

5. The CE separation method specially developed for quick analysis of nucleotides allowed in situ monitoring of ATP metabolic transformation in a complex biological sample matrix without a need for a time-consuming sample preparation procedure prior to analysis. Moreover, the described CE method provided quantification of ADP peaks even for a very high substrate/ product concentration ratio (more than two orders in this work), enabling kinetic analysis of the reaction to be performed in a large range of nucleotide concentrations that approaches physiological concentrations of ATP in the muscle cells.

REFERENCES

[1] N. C. van de Merbel, H. Lingeman, U. A. Th. Brinkman. Sampling and analytical strategies in on-line bioprocess monitoring and control. – *J. Chromatogr. A* 1996, 725, 13-27.

[2] L. Olsson, U. Schule, J. Nielsen. On-line bioprocess monitoring – an academic discipline or an industrial tool? – *Trends in Anal. Chem.* 1998, 17, 88-95.

[3] T. Scheper, B. Hitzmann, E. Stärk, R. Ulber, R. Faurie, P. Sosnitza, K. F. Reardon. Bioanalytics: detailed insight into bioprocesses. – *Anal. Chim. Acta* 1999, 400, 121-134.

[4] S. Marose, C. Lindemann, R. Ulber, T. Scheper. Optical sensor systems for bioprocess monitoring. – *Trends in Biotechnology* 1999, 17, 30-34.

[5] K. Schügerl. Progress in monitoring, modeling and control of bioprocesses during the last 20 years. – *J. Biotechnol.* 2001, 85, 149-173.

[6] V. Vojinović, J.M.S. Cabral, L.P. Fonseca. Real-time bioprocess monitoring: Part I: In situ sensors. – *Sensors and Actuators B*: Chemical, In Press, Available online 3 October 2005.

[7] L. H. Chen, C. E. Price, A. Goerke, A. L. Lee, P. A. DePhillips. A reversed phase HPLC assay for the simultaneous quantitation of non-ionic and ionic surfactants in bioprocess intermediates. – *J. Pharm. Biomedic. Anal.*, In Press, Available online 19 October 2005.

[8] B. Merelli, S. Hamm, A. Carlin-Sinclair, J.-C. Cherton. *N*-acylaziridines as potential proinsecticides of carboxylic acids: Part VI. Direct HPLC monitoring of the metabolism in insect tissues. – *J. Chromatogr. B* 2005, 826, 129-138.

[9] D. Simon, R. H. Müller, H. J. Gobe, T. H. Bley, W. Babel. Response of *Ralstonia eutropha* JMP 134 to long – term exposure to toxic substrates in nutristat cultivation as indicated by on-line fluorescence measurements. – *Bioprocess Eng.* 2000, 23, 1-10.

[10] D. L. Stokes, M. J. Sepaniak, T. Vo-Dinh. Demonstration of a separations-based fiberoptic sensor for bioanalysis. – *Anal. Chim. Acta* 1999, 399, 201-212.

[11] S. Vaidyanathan, L. M. Harvey, B. McNeil. Deconvolution of near-infrared spectral information for monitoring mycelial biomass and other key analytes in a submerged fungal bioprocess. – *Anal. Chim. Acta* 2001, 428, 41-59.

[12] C. Ruckebusch, L. Duponchel, J.-P. Huvenne. Degree of hydrolysis from mid-infrared spectra. – *Anal. Chim. Acta* 2001, 446, 255-266.

[13] A. Amine, H. Mohammadi, I. Bourais, G. Palleschi. Enzyme inhibition-based biosensors for food safety and environmental monitoring. – *Biosensors and Bioelectronics*, In Press, Available online 25 August 2005.

[14] P. Wang, G. Xu, L. Qin, Y. Xu, Y. Li, R. Li. Cell-based biosensors and its application in biomedicine. – *Sensors and Actuators B*: Chemical 2005, 108, 576-584.

[15] S. Varma, B. Mattiasson. Amperometric biosensor for the detection of hydrogen peroxide using catalase modified electrodes in polyacrylamide. – *J. Biotechnol.* 2005, 119, 172-180.

[16] J. Lammertyn, P. Verboven, E. A. Veraverbeke, S. Vermeir, J. Irudayaraj, B. M. Nicolaï. Analysis of fluid flow and reaction kinetics in a flow injection analysis biosensor. – *Sensors and Actuators B*: Chemical, In Press, Available online 10 August 2005.

[17] M. Trojanowicz, M. Szewczyńska. Biosensing in high-performance chemical separations. – *Trends in Anal. Chem.* 2005, 24, 92-106.

[18] V. Vojinović, C. R. Calado, A. I. Silva, M. Mateus, J. M. S. Cabral, L. P. Fonseca. Micro-analytical GO/HRP bioreactor for glucose determination and bioprocess monitoring. – *Biosensors and Bioelectronics* 2005, 20, 1955-1961.

[19] O.-J. Sohn, K.-A. Han, J. I. Rhee. Flow injection analysis system for monitoring of succinic acid in biotechnological processes. – *Talanta* 2005, 65, 185-191.

[20] X. Wang, D. Kou, S. Mitra. Continuous, on-line monitoring of haloacetic acids via membrane extraction. – *J. Chromatog. A* 2005, 1089, 39-44.

[21] A. Plum, G. Braun, A. Rehorek. Process monitoring of anaerobic azo dye degradation by high-performance liquid chromatography–diode array detection continuously coupled to membrane filtration sampling modules. – *J. Chromatog. A* 2003, 987, 395-402.

[22] E. Dumont, K. De Cremer, M. Van Hulle, C. C. Chéry, F. Vanhaecke, R. Cornelis. Identification of the major selenium compound, Se-Methionine, in three yeast (*Saccharomyces*

cerevisiae) dietary supplements by on-line narrowbore liquid chromatography–electrospray tandem mass spectrometry. – *J. Chromatogr. A* 2005, 1071, 191-196.
[23] A. Plum, A. Rehorek. Strategies for continuous on-line high performance liquid chromatography coupled with diode array detection and electrospray tandem mass spectrometry for process monitoring of sulphonated azo dyes and their intermediates in anaerobic–aerobic bioreactors. – *J. Chromatogr. A* 2005, 1084, 119-133.
[24] J. Namiesnik. Green analytical chemistry - Some remarks. – *J. Sep. Sci.* 2001, 24, 151-153.
[25] C. Perrin, G. Coussot, I. Lefebvre, C. Périgaud, H. Fabre. Separation of 3′-azido-2′,3′-dideoxythymidine pronucleotide diastereoisomers in biological samples by CZE with cyclodextrin addition. – *J. Chromatog. A*, In Press, Available online 12 July 2005.
[26] X. Xing, Y. Cao, L. Wang. Determination of rate constants and activation energy of 3-chloro-1,2-propanediol hydrolysis by capillary electrophoresis with electrochemical detection. – *J. Chromatogr. A* 2005, 1072, 267-272.
[27] J. Jitkova, C. N. Carrigan, C. D. Poulter, S. N. Krylov. Monitoring the three enzymatic activities involved in posttranslational modifications of Ras proteins. – *Anal. Chim. Acta* 2004, 521, 1-7.
[28] H. Siren, R. Kokkonen, T. Hiissa, T. Särme, O. Rimpinen, R. Latinen. Determination of soluble anions and cations from waters of pulp and paper mills with on-line coupled capillary electrophoresis. – *J. Chromatogr. A* 2000, 895, 189-196.
[29] B. L. Hogan, S. M. Lunte, J. F. Stobaugh, C. E. Lunte. Online coupling of in vivo microdialysis sampling with capillary electrophoresis. – *Anal. Chem.* 1994, 66, 596-602.
[30] S. L. Zhou, H. Zou, J. F.Stobaugh, S. M. Lunte, C. E. Lunte. Continuous in vivo monitoring of amino acid neurotransmitters by microdialysis sampling with online derivatization and capillary electrophoresis separation**. –** *Anal. Chem.* 1995, 67, 594-599.
[31] S. L. Zhou, D. Heckert, H. Zou, S. M. Lunte, C. E. Lunte. On-line coupling of in vivo microdialysis with capillary electrophoresis/electrochemistry. – *Anal. Chim. Acta* 1999, 379, 307-317.
[32] M. Shou, A. D. Smith, J. G. Shackman, J. Peris, R. T. Kennedy. In vivo monitoring of amino acids by microdialysis sampling with on-line derivatization by naphthalene-2,3-dicarboxyaldehyde and rapid micellar electrokinetic capillary chromatography. – *J. Neurosci. Methods* 2004, 138, 189-197.
[33] R. Kuhn, S. Hoffstetter-Kuhn, Capillary Electrophoresis: Principles and Practice, Springer-Verlag, Berlin, Heidelberg, 1993.
[34] D. N. Heiger, High-Performance Capillary Electrophoresis—An Introduction (second ed.), Hewlett Packard Company, France, 1992.
[35] F. Tagliaro , S. Turrina, F. P. Smith. Capillary electrophoresis: principles and applications in illicit drug analysis. – *Forensic Science International* 1996, 77, 211-229.
[36] W. K. Goetzinger, H. Cai. Buffer system for the separation of neutral and charged small molecules using micellar electrokinetic chromatography with mass spectrometric detection. – *J. Chromatogr. A* 2005, 1079, 372-381.
[37] C.-E. Lin. Determination of critical micelle concentration of surfactants by capillary electrophoresis. – *J. Chromatogr. A* 2004, 1037, 467 - 478.
[38] H. Watzig, M. Degenhardt, A. Kunkel. Strategies for capillary electrophoresis: Method development and validation for pharmaceutical and biological applications. – *Electrophoresis* 1998, 19, 2695-2752.
[39] J. E. Melanson, N. E. Baryla, C. A. Lucy. Double-chained surfactants for semipermanent wall coatings in capillary electrophoresis. – *Anal. Chem*. 2000, 72, 4110 – 4114.
[40] J. Preisler, E. S. Yeung. Characterization of nonbonded poly(ethylene oxide) coating for capillary electrophoresis via continuous monitoring of electroosmotic flow. *Anal. Chem.* 1996, 68, 2885-2889.
[41] C. A. Lucy, R. S. Underhill. Characterization of the cationic surfactant induced reversal of electroosmotic flow in capillary electrophoresis. – *Anal. Chem.* 1996, 68, 300-305.
[42] H. J. Dai, C. N. Parker, J. J. Bao. Characterization and inhibition study of MurA enzyme by capillary electrophoresis. – *J. Chromatogr. B* 2002, 766, 123- 132.

[43] C. A. Monnig, J. W. Jorgenson. On-column sample gating for high-speed capillary zone electrophoresis. – *Anal. Chem.* 1991, 63, 802-807.
[44] A. Lemmo, J. Jorgenson. Transverse flow gating interface for the coupling of microcolumn LC with CZE in a comprehensive two-dimensional system. – *Anal. Chem.* 1993, 65, 1576-1581.
[45] M.W. Lada, T. Vickroy, R.T. Kennedy. Quantitative in vivo monitoring of primary amines in rat caudate nucleus using microdialysis coupled by a flow-gated interface to capillary electrophoresis with laser-induced fluorescence detection. – *Anal. Chem.* 1996, 68, 2790-2797.
[46] H. Liu, P. K. Dasgupta. A falling drop for sample injection in capillary zone electrophoresis. – *Anal. Chem.* 1997, 69, 1211- 1216.
[47] Z-L. Fang, Z-S. Liu, Q. Shen. Combination of flow injection with capillary electrophoresis. Part I. The basic system. – *Anal. Chim. Acta* 1997, 346, 135-143.
[48] P. Kuban, B. Karlberg. On-line dialysis coupled to a capillary electrophoresis system for determination of small anions. – *Anal. Chem.* 1997, 69, 1169-1173.
[49] P. Kuban, B. Karlberg. On-line monitoring of kraft pulping liquors with a valveless flow injection–capillary electrophoresis system. – *Anal. Chim. Acta* 2000, 404, 19- 28.
[50] P. Kuban, A. Engström, J. C. Olsson, G. Thorsén, R. Tryzell, B. Karlberg. New interface for coupling flow-injection and capillary electrophoresis. – *Anal. Chim. Acta* 1997, 337, 117- 124.
[51] H. L. Chen, K. T. Wang, Q. S. Pu, X. G. Chen, Z. D. Hu. On-line conversion and determination of artemisinin using a flow-injection capillary electrophoresis system. – *Electrophoresis* 2002, 23, 2865- 2871.
[52] W-B. Du, Q. Fang, Q-H. He, Z-L. Fang. High-throughput nanoliter sample introduction microfluidic chip-based flow injection analysis system with gravity-driven flows. – *Anal. Chem.* 2005, 77, 1330- 1337.
[53] J. Lorieau, G. K. Shoemaker, M. M. Palcic. Quantitative nanopipettor for miniaturized chemical and biochemical reaction sampling. – *Anal. Chem.* 2003, 75, 6351-6354.
[54] A. Emmer, J. Roeraade. Enzymatic protein digest in chip-based nanovials with immobilized proteolytic enzymes. – *Anal. Chim. Acta* 2005, 542, 137-143.
[55] W.-C. Tseng, G.-W. Cheng, C.-F. Lee, H.-L. Wu, Y.-L. Huang. On-line coupling of microdialysis sampling with high performance liquid chromatography and hydride generation atomic absorption spectrometry for continuous in vivo monitoring of arsenic species in the blood of living rabbits. – *Anal. Chim. Acta* 2005, 543, 38-45.
[56] W.-C. Tseng, Y.-C. Sun, C.-F. Lee, B.-H. Chen, M.-H. Yang, Y.-L. Huang. On-line microdialysis sampling coupled with flame atomic absorption spectrometry for continuous in vivo monitoring of diffusible magnesium in the blood of living animals. – *Talanta* 2005, 66, 740-745.
[57] T. Yao, T. Yano, H. Nishino. Simultaneous in vivo monitoring of glucose, L-lactate, and pyruvate concentrations in rat brain by a flow-injection biosensor system with an on-line microdialysis sampling. – *Anal. Chim. Acta* 2004, 510, 53-59.
[58] M. Shou, A. D. Smith, J. G. Shackman, J. Peris, R. T. Kennedy. In vivo monitoring of amino acids by microdialysis sampling with on-line derivatization by naphthalene-2,3-dicarboxyaldehyde and rapid micellar electrokinetic capillary chromatography. – *J. Neurosci. Methods* 2004, 138, 189-197.
[59] B. L. Hogan, S. M. Lunte, J. F. Stobaugh, C. E. Lunte. Online coupling of in vivo microdialysis sampling with capillary electrophoresis. – *Anal. Chem.* 1994, 56, 596-602.
[60] R. T. Kennedy, C. J. Watson, W. E. Haskins, D. H. Powell, R. E. Strecker. In vivo neurochemical monitoring by microdialysis and capillary separations. – *Current Opinion in Chem. Biol.* 2002, 6, 659-665.
[61] M. Kaljurand, A. Ebber, T. Sõmer. An automatic sampling device for capillary zone electrophoresis. – *J. High Resol. Chromatogr.* 1995, 18, 263-265.
[62] R. Kuldvee, M. Kaljurand. Stacking from the sample stream in CZE using a pneumatically driven computerized sampler. – *Anal. Chem.* 1998, 70, 3695-3698.
[63] J. N.van der Molen, Correlation Techniques and Micromachinery in CZE; Improving Precision and Accuracy, Dissertation, University of Amsterdam, 1998.

[64] R. Kuldvee, M. Kaljurand, H. C. Smit. Improvement of signal-to-noise ratio of electropherograms and analysis reproducibility with digital signal processing and multiple injections. – *J. High Resol. Chromatogr.* 1998, 21, 169-174.
[65] Y-H. Lin, G-B. Lee, C-W. Li, G-R. Huang, S-H. Chen. Flow-through sampling for electrophoresis-based microfluidic chips using hydrodynamic pumping. – *J. Chromatogr. A* 2001, 937, 115-125.
[66] C.-G. Fu, Z.-L. Fang. Combination of flow injection with capillary electrophoresis: Part 7. Microchip capillary electrophoresis system with flow injection sample introduction and amperometric detection. – *Anal. Chim. Acta* 2000, 422, 71-79.
[67] X.-J. Huang, Q.-S. Pu, Z.-L. Fang. Capillary electrophoresis system with flow injection sample introduction and chemiluminescence detection on a chip platform. – *Analyst* 2001, 126, 281-284.
[68] X-D. Cao, Q. Fang, Z-L. Fang. Miniaturized capillary electrophoresis system with ultraviolet photometric detection combined with flow injection sample introduction using a modified falling-drop interface. – *Anal. Chim. Acta* 2004, 513, 473-479.
[69] S-L. Wang, X-J. Huang, Z-L. Fang, P. K. Dasgupta. A miniaturized liquid core waveguide-capillary electrophoresis system with flow injection sample introduction and fluorometric detection using light-emitting diodes. – *Anal. Chem.* 2001, 73, 4545-4549.
[70] P. R.Bewington, D.K. Robinson, Data Reduction and Error analysis for the Physical Sciences, 2nd ed., McGraw-Hill Inc., New York, 1992, p. 147.
[71] P.C Meier, R. E. Zünd, *Statistical Methods in Analytical Chemistry*, 1993, J. Wiley & Sons, N-Y. Chichester, Brisbane, Toronto, Singapore, p. 104.
[72] D. Dabrowska, A. Kot-Wasik, J. Namieśnik. Pathways and analytical tools in degradation studies of organic pollutants. – *Crit. Rew. in Anal. Chem.* 2005, 35, 155-176.
[73] M.K. Campbell, Biochemistry. Sounders College Publishing, USA, 1996, p. 657
[74] D. Balinsky, H.J. Fromm. Hexokinase III from *Rana catesbeiana*. – *Comparative Biochem. Physiol.* 1978, 60, 71-75.
[75] S.C. Supowit, B.G. Harris. *Ascaris suum* hexokinase: Purification and possible function in compartmentation of glucose 6-phosphate in muscle. – *Biochim. Biophys. Acta* 1976, 422, 48-59.
[76] K.B. Storey. Regulatory properties of hexokinase from flight muscle of *Schistocerca americana gregaria*. Role of the enzyme in control of glycolysis during the rest-to-flight transition. – *Insect Biochem.* 1980, 10, 637-645.
[77] D.L. Nelson, A.L. Lehninger, M.M. Cox, Lehninger Principles of Biochemistry. Worth Publishing, New York, USA, 2000, p.1255
[78] M.Vaher, S. Ehala, M. Kaljurand. On-column capillary electrophoretic monitoring of rapid reaction kinetics for determination of the antioxidative potential of various bioactive phenols. – *Electrophoresis* 2005, 26, 990-1000.
[79] A. M. Gordon, E. Homsher, M. Regnier. Regulation of contraction in striated muscle. – *Physiol. Revs*. 2000, 80, 853-924.
[80] J. Botts, M. Morales, *Trans Faraday Society* 1953, 49, 696-707.
[81] I. Amitani, T. Sakamoto, T. Ando. Link between the enzymatic kinetics and mechanical behavior in an actomyosin motor. – *Biophys. J.* 2001, 80, 379-397.
[82] S. Highsmith. Myosin regulatory light chain and nucleotide modulation of actin binding site electric charge. – *Biochemistry* 1997, 36, 2010-2016.