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Development of automated methods using  
syringe based flow analysis techniques  
and capillary electrophoresis for  
biotechnological process monitoring  
and environmental analysis

Memoria presentada para optar al grado de Doctor en Ciencias  
Experimentales y Tecnología (Química) con mención europea

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CERTIFICAN:

Que el presente trabajo titulado: "*Development of automated methods using syringe based flow analysis techniques and capillary electrophoresis for biotechnological process monitoring and environmental análisis*" ha sido realizado por Burkhard Horstkotte en el Área de Química Analítica del Departamento de Química de la Universidad de las Islas Baleares con una estancia en la mencionada Universidad de Ciencias Aplicadas Hamburgo. Constituye la memoria para optar al título de Doctor en Ciencias Experimentales y Tecnología, mención Química y con mención europea, cuya presentación autorizan.

Y para que así conste a los efectos oportunos, firman la presente en  
Palma de Mallorca, 04 de Septiembre de 2008.

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*"La verdadera grandeza de la ciencia acaba valorándose por su utilidad."*

*"The true greatness of science ends up valuing itself by its utility"*

*Gregorio Marañón y Posadillo*

*Spanish physician, scientist, historian, writer, and philosopher (1887-1960)*



Meinen Eltern  
(To my parents)



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# ABSTRACT

Since the appearance of flow analyzer systems, the monitoring of environmental or industrial processes and the automation of sample treatment prior to analysis have become stronghold domains for the application of flow techniques. Thus, they present important analytical tools for environmental and process vigilance enabling system studies, risk control, optimization, and higher process sustainability. They are further characterized by high time resolution, cost and consumable efficiency, and reproducibility.

In this work, five automated analytical systems using the syringe pump-based flow techniques – Sequential Injection Analysis (SIA) and Multisyringe Flow Injection Analysis (MSFIA) – have been developed and applied with satisfactory analytical performance.

A SI-analyzer for formaldehyde was developed automating the Hantzsch reaction with acetylacetone. Formaldehyde is the first intermediate in the metabolic degradation of the carbon source methanol by the yeast *Pichia pastoris*, one of the most frequently used microorganism in biotechnological productions. The analyzer was successfully applied to the formaldehyde monitoring in a continuous medium filtrate flow of a semi-industrial cultivation of *P. pastoris*. By this, an analytical tool for the study of the relation between process conditions and formaldehyde concentration was made available for the first time.

A second SI-analyzer was developed for the determination of glycerol and sorbitol, two frequently used carbon sources in yeast cultivations. Periodate was used as an additional reagent to carry out the Malaprade reaction. Formaldehyde, generated by the oxidation of the polyalcohol using periodate was quantified by Hantzsch reaction. The system included automated sample dilution and procedures for two complementing working ranges, which were selected by smart software decision (glycerol) or by software-guided user-input (sorbitol), respectively. By this, analyte quantification within an amplified concentration range was possible. The system was applied to the monitoring of both analytes in *P. pastoris* cultivation processes.

A capillary electrophoresis (CE) system was developed and coupled to a SIA system for fully automated maintenance, sampling, and sample injection. The system was applied to the separation and on-capillary photometric detection of mono-nitrophenols used as model substances of high environmental impact. In a second work, the system was modified and MSFIA and CE were coupled for the first time. The fully automation of analyte, solid phase pre-concentration, eluate injection, separation, and quantification of mono-nitrophenols was achieved. Both systems were applied to leaching, ground, and surface waters showing highly satisfying analyte recoveries.

Besides, a review of MSFIA applications was presented. It includes a comprehensive overview of the applications and potentials of the technique and the different possibilities of sample injection, manifold configuration, and use of the control software AutoAnalysis.

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# ZUSAMMENFASSUNG

Seit der Einführung von Fließanalyse-Systemen war eine ihrer wichtigsten Anwendungsbereiche das Monitoring von sowohl Umwelt- als auch Industrie-Prozessen sowie die Automatisierung verschiedener Arten der Probenvorbehandlung. Fließtechniken stellen damit wichtige analytische Instrumente für die Umwelt- und Prozessüberwachung dar. Sie erlauben Systemstudien, Risikokontrolle, Optimierung und eine bessere Nachhaltigkeit der überwachten Prozesse, und sind gekennzeichnet durch eine hohe Zeitauflösung, Kosten- und Material-Effizienz sowie durch eine hohe Reproduzierbarkeit.

In dieser Arbeit wurden fünf automatische Analysensysteme unter Verwendung der Spritzenpumpen-basierenden Fließtechniken Sequentielle Injektionsanalyse (SIA) und Multisyringe Fließinjektionsanalyse (MSFIA) entwickelt.

Ein SI-Analyzer für Formaldehyd wurde durch die Automatisierung der Hantzsch-Reaktion mit Acetylaceton entwickelt. Formaldehyd stellt das erste Zwischenprodukt in der Stoffwechselkette der Kohlenstoffquelle Methanol der Hefe *Pichia pastoris* dar, eines der am meistverwendeten Mikroorganismen in der Biotechnologie. Der Analyzer wurde erfolgreich für das Monitoring von Formaldehyd in einem kontinuierlichen Medium-Filtratstrom einer Kultivierung von *P. pastoris* angewendet. Hiermit wurde erstmalig ein analytisches Instrument für die Untersuchung des Zusammenhangs von der Formaldehydkonzentration und anderer Prozessgrößen verfügbar.

Ein zweiter SI-Analyzer wurde entwickelt für die Bestimmung von Glycerin und Sorbitol, zweier häufig verwendeter Kohlenstoffquellen in Hefe-Kultivierungen. Periodate wurde verwendet als zusätzliches Reagenz zur Durchführung der Malaprade Reaktion. Das Formaldehyd, das bei der Periodat-bedingten Oxidation des Polyalkohols entstand, wurde über die Hantzsch-Reaktion quantifiziert. Das System beinhaltete die automatisierte Probenverdünnung und Prozeduren für zwei, sich ergänzender Arbeitsbereiche, die entweder über eine intelligente Softwareentscheidung aktiviert wurden (Glycerin) bzw. entsprechend Software-geführter Anwender-Eingabe (Sorbitol). Hierdurch wurde die Quantifizierung in einem erweiterten Konzentrationsbereich möglich. Das System wurde für das Monitoring beider Analyten in *P. pastoris* Kultivierungen angewendet.

Ein Kapillarelektrophorese (CE) System wurde entwickelt und an ein SIA System angebunden für die voll-automatisierte Wartung, Probenübergabe und Injektion. Das System wurde angewendet zur Trennung und photometrischen *on-capillary* Detektierung von den als Modellsubstanzen mit hoher Umwelteinwirkung verwendeten mono-Nitrophenolen. Im zweiten Schritt wurde das System modifiziert durch die erstmalige Kupplung von MSFIA und CE zur vollständig automatisierten Festphasen-Aufkonzentrierung der Nitrophenole, Injektion des Eluats, sowie die Trennung und Quantifizierung der Analyten. Beide Systeme zeigten gute Wiederfindungen für Bodenlaugen, Grund- und Oberflächenwässer.

Desweiteren wurde ein Review über die Anwendungen der MSFIA Technik angefertigt. Es beinhaltet einen umfassenden Überblick der Anwendungen, Beschreibungen der Potentiale der Technik sowie die Möglichkeiten der Probeninjektion, der *Manifold* Konfiguration und der Verwendung der Kontrollsoftware AutoAnalysis.

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# RESUMEN

Desde su comienzo, la aplicación de técnicas en flujo se ha enfocado a la monitorización del medio ambiente o de procesos industriales y a la automatización del tratamiento de muestras previo al análisis. Son herramientas poderosas de análisis para la vigilancia del medioambiente y de procesos, facilitando estudios de sistemas, el control de riesgos y la optimización para obtención de un mayor rendimiento. Se caracterizan por una alta resolución temporal, bajo coste, bajo consumo de reactivos y una alta reproducibilidad de análisis.

En este trabajo se han desarrollado cinco sistemas analíticos automatizados utilizando las técnicas en flujo basadas en el uso de jeringas, el análisis por inyección secuencial (SIA) y el análisis por inyección en flujo multijeringa (MSFIA).

Se ha desarrollado un analizador SIA para la determinación de formaldehído utilizando la reacción de Hantzsch con acetilacetona. El formaldehído es el primer metabolito en la degradación de la fuente de carbono metanol por la levadura *Pichia pastoris*, uno de los microorganismos más frecuentemente usados en biotecnología. El analizador fue aplicado con éxito a la monitorización del analito en un flujo de filtrado de un medio de cultivo semi-industrial de *P. pastoris*. Así, se ha desarrollado por primera vez una herramienta para el estudio de la relación entre las condiciones del proceso y la concentración del formaldehído.

Se ha desarrollado un segundo analizador SIA para la determinación de glicerol y sorbitol, dos fuentes de carbono frecuentemente usadas para cultivos de levaduras. Se ha utilizado periodato como reactivo adicional para llevar a cabo la reacción de Malaprade. El formaldehído, generado por la oxidación del polialcohol por el periodato ha sido cuantificado con la reacción de Hantzsch. El sistema incluye la dilución automatizada de la muestra y procedimientos para dos rangos consecutivos de trabajo, seleccionado bien por decisión inteligente del programa (glicerol) o por el control del usuario guiado por el programa (sorbitol). Así es posible la cuantificación en un rango amplio de concentraciones. El sistema se aplicó a la monitorización de ambos analitos en cultivos de *P. pastoris*.

Se ha desarrollado un sistema de electroforesis capilar (CE) acoplado a un sistema SIA para la automatización del mantenimiento, provisión e inyección de la muestra. Se ha aplicado el sistema a la separación y detección fotométrica *on-capillary* de mono-nitrofenoles usados como sustancias modelo por su alto impacto ambiental. A continuación se ha modificado el sistema para acoplar por primera vez el MSFIA con la CE para la automatización de la pre-concentración en fase sólida, inyección del eluato, separación y cuantificación de mono-nitrofenoles. Ambos sistemas muestran recuperaciones satisfactorias para aguas subterráneas y superficiales y lixiviados.

Además se presenta una revisión de las aplicaciones de la técnica MSFIA. La misma incluye una visión general de las aplicaciones, descripción de las potencialidades de esta técnica y de las diferentes posibilidades de introducir la muestra, configuración del *manifold* y del uso del programa de control AutoAnalysis.

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# STRUCTURE OF THE THESIS

This thesis has been structured in ten chapters:

- Chapter 1 gives a general introduction to the applied and related analytical techniques, and describes the objectives and difficulties of process analytical chemistry, bioprocess strategies, and the analytes of interest for this work.
- Chapter 2 overviews the objectives of this work.
- Chapter 3 describes the general materials applied throughout in this work such as the flow system instrumentation components and sampling equipments, detection devices, and the software used for data acquisition and statistics.

Chapters 4 to 8 contain the experimental part of this work including descriptions of the developed automated systems and analytical methodologies, their applications and discussions of the obtained results.

- Chapter 4 deals with the development of a sequential injection analyzer for formaldehyde monitoring in cultivation medium and its application to a cultivation of the yeast *Pichia pastoris*.
- Chapter 5 deals with the development of a sequential injection analyzer for glycerol monitoring in cultivation medium and its application to a cultivation of *P. pastoris*.
- Chapter 6 deals with the development of a sequential injection analyzer for sorbitol monitoring in cultivation medium and its application to a cultivation of *P. pastoris*.
- Chapter 7 deals with the development of a sequential injection analyzer coupled to capillary electrophoresis for the monitoring of mono-nitrophenols in waters.
- Chapter 8 deals with the development of a multisyringe flow injection analyzer coupled to capillary electrophoresis for the in-line concentration and monitoring of mono-nitrophenols in waters.
- Chapter 9 contains the general conclusions of this work.
- Chapter 10 gives a short outlook for perspectives of this work.

Indices of figures, tables, graphical and written symbols, and formula signs can be found prior to the introduction. The references cited in this work are listed in a separate chapter at the end of this work. Journal names are abbreviated as done in the FI Science Citation Index published by the Spanish Ministry of Education and Science [1].

All publications or manuscripts accepted for publication, respectively, accomplished during this work and dealing with the investigation carried out are further included. Finally, the actual curriculum vitae of the author is added after the reference chapter.

The appendix contains data tables, spectra, program protocols, instrument serial numbers and characteristics, technical drawings, chemical data of the applied reagents, and a photo documentation of the used commercial and developed devices in this work.

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# INDEX OF ABBREVIATIONS

AcAc	acetylacetone (2,4-pentanedione)
Add	(standard) addition
AOX	alcohol oxidase
BI	blank
BTB	bromothymol blue
C18	octadecylsilylated silica
CCD	Coupled Charge Device
CE	capillary electrophoresis
CTFE	chlorotrifluoroethylene
DDL	2,4-diacetyl-1,4-dihydrolutidine
DLL	dynamic link library
DOC	dissolved organic compounds
EL	eluent
EOF	electro-osmotic flow
ESIP	eppendorf sterilizable inline probe
ETFE	ethylene tetrafluorethylene
FEP	fluorinated ethylene propylene
FIA	flow injection analysis
GC	gas chromatography
HC	holding coil
H <sub>2</sub> CO	formaldehyde
HPLC	high performance liquid chromatography
HUAS	Hamburg University of Applied Sciences
HV	high voltage
i.d.	inner diameter
I-TAC	interferon inducible T-cell alpha chemoattractant
IR	infrared
IV	injection valve
LED	light-emitting diode
LLE	liquid-liquid extraction
LOD	limit of detection
LOQ	limit of quantification
LOV	lab-on-the-valve
MCSV	multicommutation solenoid valve
MCFIA	multicommutation flow injection analysis
MeCN	acetonitrile
MeOH	methanol
MSFIA	multisyringe flow injection analysis
n.t.	not tested

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NH <sub>4</sub> Ac	ammonium acetate
NP	(o- ortho-, m- meta-, p- para-) nitrophenol
o.d.	outer diameter
PEEK	polyetheretherketone
pH	negative decadic logarithm of hydronium ions concentration
pK <sub>A</sub>	negative decadic logarithm of the dissociation constant K <sub>A</sub> of an acid
PMMA	polymethylmethacrylate
P <sub>AOX1</sub>	promoter of the AOX1 gene
Pos	position (position of valve port)
PPS	polyphenylsulfide
PTFE	polytetrafluorethylene
PVC	polyvinyl chloride
PVDF	polyvinylidene fluoride
R	reagent
S	sample / syringe
SFA	(air) segmented flow analysis
SIA	sequential injection analysis
SPE	solid phase extraction
Std	standard
SV	selection valve
UAB	Autonomous University of Barcelona
UIB	University of the Balearic Islands
UNF	unified fine (American thread series)
UNS	unified special (American thread series)
USB	universal serial bus
UV	ultraviolet
V	(solenoid) valve
W	waste

# INDEX OF FORMULA SIGNS

## Latin characters

Character	Meaning	SI unit *
$a_i$	polynomial coefficients	-
A	absorbance / signal	-
c	molar concentration	mol·l <sup>-1</sup>
D	dispersion coefficient	-
e	elementary charge	approx 1.6022·10 <sup>-19</sup> C
E	(electrical) field strength	V·m <sup>-1</sup>
F	force	kg·m·s <sup>-2</sup>
h	height	m
l	length	m
m	mass	kg
M	molar mass	kg·mol <sup>-1</sup>
n	number of data sets	-
N	plate number	-
pO <sub>2</sub>	oxygen partial pressure	% of saturation
p	pressure	kg·m <sup>-1</sup> ·s <sup>-2</sup>
r	regression coefficient / radius	- / m
RSD	relative standard deviation	%
R	recovery	%
R <sub>s</sub>	(peak) resolution	-
SD	standard deviation	-
t	time	s
t <sub>D</sub>	delay or dead time	s
t <sub>P</sub>	process execution time	s
t <sub>R</sub>	retention time	s
U	voltage	V
V	volume	l
$\dot{V}$	flow rate	m <sup>3</sup> ·s <sup>-1</sup>
w	width	m
w <sub>B</sub>	base line peak width	s
z	number of charge	-
$\bar{x}$	mean of values of magnitude x	-

\* Units used in the text can differ from the SI unit, applying mostly magnitude prefixes (...k, m,  $\mu$ , n,...) and rather minutes (min) and hours (h) than seconds (s) for time scales, bar instead of Pascal (Pa) for pressure specification, and liters (l) instead of cubic meters for volumes, respectively.

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## Greece characters

Character	Meaning	SI unit *
$\beta$	mass concentration	$\text{g}\cdot\text{l}^{-1}$
$\varepsilon$	molar absorptivity / relative dielectrical constant	$\text{l}\cdot\text{mol}^{-1}\cdot\text{m}^{-1}$ / -
$\zeta$	zeta potential	V
$\eta$	dynamic viscosity	$\text{kg}\cdot\text{m}^{-1}\cdot\text{s}^{-1}$
$\lambda_{\text{Det}}$	detection wavelength	m
$\lambda_{\text{Ref}}$	reference wavelength	m
$\mu$	electrophoretic mobility	$\text{m}^2\cdot\text{V}^{-1}\cdot\text{s}^{-1}$

\* Units used in the text can differ from the SI unit, applying mostly magnitude prefixes (...k, m,  $\mu$ , n,...) and rather minutes (min) and hours (h) than seconds (s) for time scales and bar instead of Pascal (Pa) for pressure specification, respectively.

# 1. GENERAL INTRODUCTION

## 1.1. Analytical flow techniques

### 1.1.1. Introduction and principles

Analytical flow techniques are techniques used for the automation of the analyst work. The common principle is given by the carrying and treating of a sample in flow within a tube assembly, following denoted as "manifold". The basis of automation in flow distinguishes these techniques from batch-wise laboratory automation techniques such as using beakers on a conveyer belt or robots [2,3].

Commonly, peristaltic, syringe, or solenoid membrane pumps are used for liquid driving. Alternative propulsion techniques such as gravimetric flow [4], electro(end)osmotic flow (EOF) [5] or the use of miniature piston [6] or piezoelectric pumps [7] have been reported further. Though liquid chromatography shows the basic characteristics of flow techniques, it is generally considered as an individual analytical technique.

The simplest conceivable system is represented by the sample transport to a detection flow cell. Mostly, in-line addition of an analyte-selective, e.g. chromogenic reagent is carried out in order to obtain a reaction product of enhanced detection sensitivity. More complex systems are used to automate laboratory preparative and analytical procedures in order to eliminate matrix interferences and to improve the method selectivity.

Among others, sample filtering, titration, mixing, leaching [8], dilution, microwave- [9], ultrasonic- [10], UV-irradiation-, and agent-assisted digestion or photo-degradation [11,12], analyte pre-concentration, or analyte separation from the matrix by distillation, membrane techniques (gas diffusion [13], pervaporation [14-16], dialysis [17],...), extraction (liquid-liquid [18], solid-phase ,...), or co-precipitation [19] have been performed so far in flow systems. For these tasks, a multitude of devices have been used in flow manifolds such as phase separators, leaching cells, columns for resins or solid samples [8], membrane cells, dilution chambers, ultrasonic reactors, gas-sampling units, optical and electrochemical detection flow cells of ingenious designs, or serpentine, knotted or coiled tubes [20].

The analytical information is obtained by the continuous registration of the physical or chemical property of interest in the liquid flow. The signals are either rectangular or peak-shaped, whose characteristics area, height, or width are correlated with the analyte concentration. Apart from most frequently used detection techniques i.e. potentiometry, amperometry, spectrophotometry, and fluorescence spectrometry, also applications of conductimetry [21], reflectometry [22], infrared (IR) spectroscopy [23], turbidimetry [24], chemiluminescence detection [25], flame (FAAS) and electrothermal atomic absorption spectrometry (ETAAS) [8,26-28], atomic fluorescence spectrometry [29,30], inductive-coupled-plasma atomic-emission-spectrometry (ICP-AES), and mass spectrometry (ICP-MS) [31,32] can be found.

## 1 General introduction

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Among the benefits of automation of analytical procedures, the increase of sample frequency, miniaturization of the analytical system, and lower reagent and sample consumption, implying lower personal and consumable costs need to be highlighted. In consequence, the analysis can be carried out faster, more economically, and in absence of trained personal e.g. overnight and with a reduced environmental impact.

As a further advantage, all procedures are carried out in a closed manifold made of resistant polymer or glass tubes. This prevents sample contamination or alteration by oxidation, evaporation, or radiation and decreases further the risk for laboratory personal and working area of contamination with applied hazardous substances. Generally, automation using analytical flow techniques involves a considerable gain of the overall repeatability of the analytical procedure due to the control of the volumes, volume ratios, and flow rates whereas the much vaunted robustness and flexibility of analytical flow techniques depend highly on the individually assembled instrumentation and the user's skills. Due to all former described characteristics, analytical flow techniques are widespread used where fast, economic, reliable, and automated analysis of a large number of similar samples is demanded such as in routine clinical analysis, industrial or biotechnological process control, and environmental vigilance as discussed in detail in chapter 1.3.6.

The different modes of propulsion and sample introduction, operation schemes and flow characteristics have led to the proposal of various distinct analytical flow techniques. In the following sections, the most frequently used analytical flow techniques and their characteristics are described. Extensive comparisons and introductions into the different techniques can be found in specialist books [2,33-38] and in numerous reviews and treatises about the different flow techniques and their fundamental aspects [3,20,39-50]. It should be highlighted, that some of the analytical characteristics of the automated method such as the sample frequency depend more on e.g. the used detector than on the applied flow technique and a comparison between these is only feasible for similar applications.

Finally, the IUPAC technical report prepared by ZAGATTO ET AL. (2002) [51] stated the existence of only four basic flow-based analytical techniques with various distinct modes, likewise to be characterized by applied flow pattern, stream parameters, sample introduction, manifold configurations, sample processing, detection and its performance parameters. In contrast, the number of proposed flow techniques is much higher. However, to the author's opinion, novel benefits of a technique variant are much more relevant than claiming the novelty of a technique with variant benefits. It is even more the inventive utilization of ideas from distinct techniques and their intelligent combination for the fulfilling of the analytical task.



## 1 General introduction

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### 1.1.2. Pump types

The used liquid driving devices, either peristaltic, syringe, or solenoid membrane pumps, have a considerable influence on the robustness, precision, flow characteristics, and chemical resistance of the analytical system and applied technique. It is noteworthy, that one pump type is characteristically assigned to each flow technique.

Peristaltic pumping is carried out by the positive displacement of the liquid contained within elastic pumping tubes fitted inside the circular pump casing. The tube is closed on various points under the compression exerted by cylinders mounted onto the pump rotor. By rotor turning, the fluid is forced to move through the tube as the points of compression move. The flow rates are modified by using pumping tubes with different inner diameters and by the rotation speed. The tube interior walls are the only part in liquid contact, which allows sterilization. The pumps are relatively inexpensive in maintenance since no moving parts are in contact with the liquid and neither valves nor seals are required. Most models can be equipped with several parallel channels. Computer interfacing enables the control of flow rate and direction but is not required for operation. The main disadvantages are the peristalsis-caused flow pulsation, flow rate drift, and progressive material abrasion caused by the mechanical stress and flattening of the pumping tubes, thus required re-calibration, and the moderate volumetric precision in sequential operation or under pressure. Finally, the typical tube materials PVC (Tygon®) is not resistant to common chemical agents in even moderate concentration such as various acids, bases, and organic solvents. Here, water-immiscible solvents can to be propelled using displacement bottles [36,52].

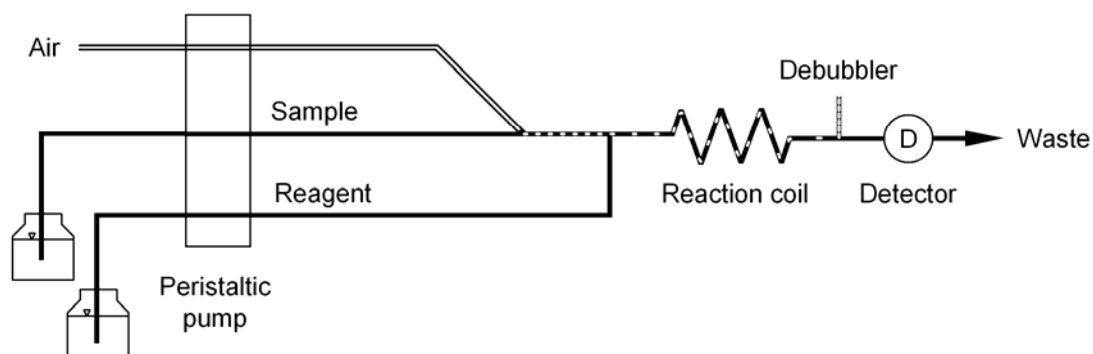
In syringe pumps, the liquid is dispensed or aspirated into a syringe by the movement of a piston, mostly driven by a step-motor-turned screw shaft. Important advantages are high-pressure stability, high volumetric precision, hardly pulsation, adaptable flow rate by computer control over a wide range, and suitability for solvents and acids due to the use of normally glass and highly resistant polymers. On the other side, syringe pumps require necessarily computer control. Most devices are equipped with only one syringe, which complicates the construction and increases the price of multichannel manifolds. Besides, the maximal volume is limited by syringe size and the time of analysis is prolonged by the required syringe refilling. Finally, gas bubbles can arise due to the negative pressure applied on the liquid during rapid refilling and affect the operational precision.

Solenoid diaphragm micropumps operate by means of strokes generated by an electro-mechanical solenoid, which actuates over a liquid contacting diaphragm. Due to the incorporation of a dual check valve, a unidirectional, intermittently turbulent flow results from the line-up of several flow pulses. The high advantage of these devices are the small dimensions and low power consumption allowing the construction of miniature systems, the low purchase costs and simplicity of the required control, and the advantageous arising from the turbulent flow conditions. On the other hand, the reliability of the pumps is highly affected by the presence of gas bubbles, the practicable flow pressure is low, and bi-directional flow is not possible using only one pumping device.

### 1.1.3. Segmented flow analysis

Segmented Flow Analysis (SFA) technique was firstly introduced by SKEGGS in 1957 [53,54]. The "AutoAnalyzer" from Technicon Corporation became the first commercially available instrument based on this technique. SFA was the first widespread implemented continuous flow technique and applied especially to clinical and industrial analysis. The production of the AutoAnalyzer was continued by Bran & Luebbe Company since 1987.

A typical structure of a SFA manifold is shown in figure 1. Sample and reagents are propelled by a multichannel peristaltic pump towards the detector, mixed prior in a static manifold. Air is introduced by one channel generating short liquid segments containing sample and reagents in ideally reproducible proportions, separated from each other by air bubbles. This segmentation prevents sample dispersion and lowers carry-over between two segments, which allows sample change in after less than one minute. However, the sample flow is unsegmented on the pump aspiration side and due to resulting dispersion, intermediate cleaning with water is required and various segments have to be generated for each sample. The combination of air segmentation and tube wall friction causes the homogenization of each segment by convection leading to rectangular-shaped detection signals. In order to avoid air bubble stacking in the manifold, hydrophilic materials such as glass are typically used for coils. The detection has to be done after reaching steady-state of the reaction, since the residence time of the sample in the manifold varies due to the compressibility of the air bubbles and a reasonable, earlier quantification is not possible.



**Figure 1: Typical structure of a SFA manifold.**

**The concept consists in the air-segmentation of the liquid flow for the effective separation and homogenization of each sample.**

Thus, the time of analysis is typically long (2-30 min), whereas simultaneously, high sample throughputs can be achieved. Due to the completed reaction, the detection sensitivity can surpass the ones of other techniques, where detection is carried out before the steady-state conditions of the applied reaction are reached. On the other side, observation of reaction kinetics and instable intermediate products is not possible. Further shortcomings are the high consumption of reagents and the requirement of air bubble elimination prior to certain manifold elements (e.g. the detection cell) or the compensation of the bubble disturbance of the detection signal by an adequate software tool [36,55].

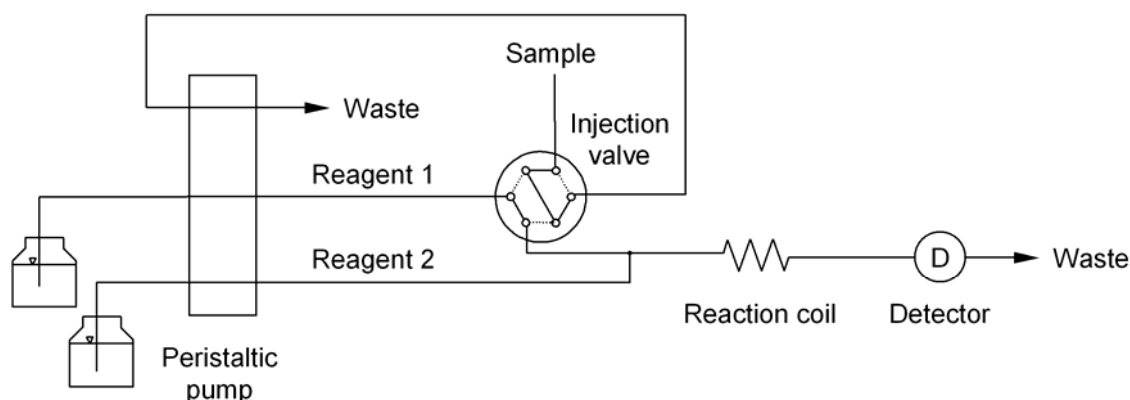
## 1 General introduction

### 1.1.4. Flow injection analysis

Flow Injection Analysis (FIA) was firstly proposed by RUZICKA AND HANSEN (1975) [55]. In contrast to SFA, a sample aliquot is inserted into a non-segmented carrier flow by the use of an injection valve. By the radial and axial forces in the laminar flow, the sample disperses in the carrier flow in function of physical parameters such as sample volume and viscosity, flow rate, inner diameter, and length of the transport line. FIA signals are therefore peaks with a characteristic tailing and, compared to an equivalent SFA method, of reduced signal height. The manifold and the resulting signal decrease is described by the dispersion coefficient  $D$ . Given by formula 1,  $D$  is calculated as the ratio of the signal  $A_0$  that would result from sample plug flow and the actually obtained peak maximum  $A_{\max}$ .

$$D = \frac{A_0}{A_{\max}} \quad (1)$$

Apart from confluent mixing with required reagents, one reagent itself can be used as carrier. In this case, the reaction product is formed in the penetration zones of sample and reagent resulting from dispersion. By not requiring air and one reagent line less, simpler manifolds as in SFA are possible. Peristaltic pumps are typically applied in FIA implying the former named disadvantages. In SFA, coils of the hydrophilic but fragile material glass of typically 2 mm i.d. are used to avoid air bubble stacking. In contrast, flexible tubes of chemically resistant polymers of a reduced diameter of 0.5 and 0.8 mm i.d. are typically used in FIA and later flow techniques. A typical FIA manifold structure is shown in figure 2.



**Figure 2: Typical structure of a FIA manifold.**

**The concept consists in the reproducible insertion of the sample into a non-segmented continuous flow. The reagent product is formed at the penetration zones of sample and reagent or by confluent mixing with the reagent.**

Controlled dispersion and reproducible timing and injection are the key principles and conditions of FIA technique [35]. In first works, the sample was introduced via a septum-interface by injection whereas later, sample insertion was done via injection valves. This and a constant flow rate due to the absence of air bubbles ensure a constant sample residence time in the manifold and a very high signal reproducibility of 1-2 % RSD [56].

## 1 General introduction

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This enables one of the most important innovations of FIA: the possibility of data reading prior to reaching reaction steady-state, at each peak position and using optionally its width, area, or height. In consequence, the analysis time can be considerably reduced, intermediate reaction products can be quantified, and the signal contribution of interfering side-products of lower reaction kinetics can be decreased by early quantification. Important advantages of FIA are its simplicity, the economic equipment, the reduction of sample consumption, and continuous flushing of the manifold with carrier. Last one makes cleaning circles unnecessary and prolongs the lifetime of incorporated enzyme cartridges, electrodes, optodes, etc. Though the sample frequency is mainly limited by the residence time of each sample in the manifold, values passing 100 injections per hour have been reported [56]. Due to these advantages, FIA is today the most widespread flow technique in monitoring, investigation, and routine analysis with over 15.000 publications [57].

Several different modes and techniques have been derived in order to enhance the potential of FIA or to overcome the disadvantage of the monoparametric character and elevated consumption of the continuously propelled reagents. One alternative is the injection of the reagent instead of the sample or both solutions ("double injection") [13,58] allowing the economic application of high-cost reagents such as enzyme solutions. Progressive miniaturization by microconduits [6,35,36], capillary flow injection [59], and lab-on-a-chip systems [60] is a trend in flow technique development allowing the analysis of smaller sample volumes even of single cell analysis, reduction of reagents, and rigid, pocket-sized chemistry equipment. To combine the benefits of FIA and SFA technique, hybrid techniques such as mono-segmented flow analysis [61] and air-segmented flow injection [62] have been developed allowing both the reduction of liquid consumption and dispersion, which leads to a higher detection sensitivity.

Further possibilities for kinetics studies have been enabled by the reverse-flow and stop-flow modes. In the first variant, the flow is reversed after detection of the reaction product peak in a way, that the product passes the detection flow cell a second time [63]. In the second mode, the flow is stopped when the peak maximum has just reached the detection cell and the change of the signal height is monitored [64]. Both techniques allow the evaluation of the signal change after a second period under simultaneous reduction of reagent consumption, e.g. to quantify quenching effects, to eliminate the sample blank, to quantify a concentration or efficiency of a catalyst [58,65] or to improve gas diffusion [13].

An approach to overcome the need of manual reconfiguration of the manifold was recently reported as the All-Injection-Analysis technique (AIA) by ITABASHI ET AL. (2001) [66]. Here, all used solutions are injected into one loop, in which the solutions are circulated for a defined time. During this circulation, sample and reagents are mixed by zone penetration while the chemical reaction takes place. The advantages are the possibility of reaction time optimization without loss of sensitivity due to dispersion, reduction of the consumption of all solutions and the applicability to kinetics monitoring of the reaction by carrying out the detection within the circulation loop.

### 1.1.5. Sequential injection analysis

#### 1.1.5.1. Classical sequential injection analysis

Sequential Injection Analysis (SIA) was firstly proposed by RUZICKA AND MARSHALL (1990) [67] as an alternative technique to FIA overcoming some of the basic problems of its predecessor. A typical SIA system (see figure 3) consists of a bi-directional pump connected via a tube denoted holding coil (HC) to the common port of a multiposition selection valve. One lateral port of the valve is connected to the manifold, generally consisting of a reaction coil (RC) and the detection flow cell. Sample, standards, and reagents are situated at other lateral ports of the selection valve.

In contrast to the former described continuous flow techniques, an intermittent flow scheme is carried out by obligatory computer control. The sample and reagents are aspirated in succession from the selection valve into the holding coil. By flow direction reversal, the stacked solutions are propelled through the respective selection valve port and reaction coil towards the detection flow cell giving a peak shaped signal.

The reaction product is formed in the overlapping zones of reagents and sample penetrating each other by dispersion. As in FIA, the analytical performance depends on the control and reproducibility of dispersion, timing, and sample injection, i.e. sample aspiration. However, in SIA, influencing volumes, flow rates, and optionally holding time (to prolong the time of reaction) can be adjusted via the control software. The influences of volumes, flow rates, geometry of manifold and holding coil, ionic strength, and repeated flow reversal on the degree of zone penetration and thus on the achievable sensitivity by minimal consumption of reagent and sample were studied in fundamental works [67-70] and overviewed in a comprehensive review by TALJAARD AND VAN STADEN (1998) [71]. Further reviews overview the analytical applications of SIA and its potential for process monitoring [20,43,72], gradient and stop-flow techniques [64], and mono-segmentation [73] as former described for FIA.

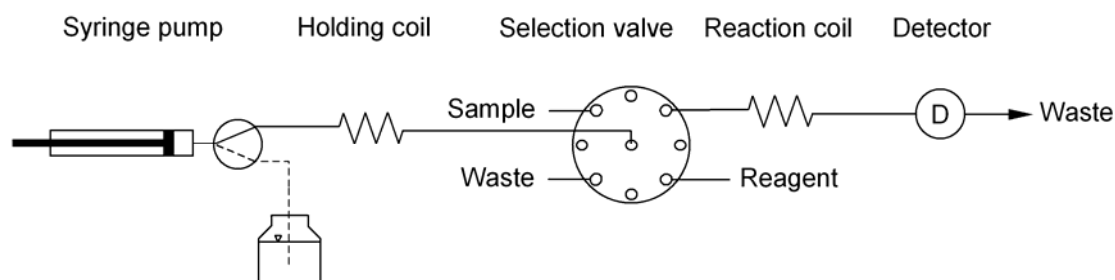
In the beginning, sinusoidal flow piston pumps were applied as a more economic alternative to linear syringe pumps refilled from one lateral port of the selection valve. Further, the detection flow cell was integrated into the holding coil. The later utilization of linear syringe pumps [74] and the placement of the detection cell to the opposite side of the selection valve enhanced the versatility of the SIA technique considerably, among others because the flow rate became independent from the actual piston position.

The important advantages of the SIA technique are a lower consumption of sample and reagent, the benefits arising from the use of pulsation-free and robust syringe pump (see chapter 1.1.2), and the methodic flexibility by computer control of all operational parameters without required manifold reconfiguration. Therefore and in contrast to FIA, SIA enables multi-parametric analysis by the simple change of the operational computer protocol and placing the respectively required reagents on the valve ports. If required, the use of an alternative manifold path via another valve port is also possible [75].

## 1 General introduction

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The disadvantages of SIA compared to FIA are a worse zone penetration and the impossibility of confluence mixing. This results in a higher signal contribution of the refraction index, higher dispersion due to wider tube diameters (0.8-1.5 mm i.d.), and in consequence, lower detection sensitivity. The syringe refilling and sequential aspiration of the solutions leads also to a longer time of analysis and lower reproducibility compared to FIA. The required computer control and reliable software are further in spite of all named benefits the main obstacles considered for the development of SIA applications [40,76].



**Figure 3: Typical structure of a SIA manifold.**

**The concept consists in the performance of an intermittent flow scheme, during which sample and reagents are aspirated from a multiposition valve into a holding coil and afterwards propelled towards the detection cell passing a reaction coil by flow reversal.**

### 1.1.5.2. Lab-on-valve and bead injection technique

The SIA variation Lab-On-Valve (LOV) was firstly proposed by RUZICKA (2000) [77] using on the rotary multiposition valve a stator with integrated microconduit including a multipurpose detector flow cell and a flow-through port as shown in figure 4. This concept allows downscaling of the classical SIA in a rigid and unmodified manifold gaining injection throughput, robustness, simplicity, and minimal consumption of solution. By this, low-cost automation e.g. of enzymatic assays is possible [78].

One of the most noteworthy advantages is the easy application of LOV to the handling of sorbent materials in flow with the derived technique Bead Injection (BI), reviewed by RUZICKA AND SCAMPAVIA in 1999 [49]. In BI, first an aliquot of a bead suspension is aspirated, e.g. a solid phase resin modified by immobilized enzymes, optionally circulated at the flow-through port. Afterwards, the suspension is propelled to waste through the detection channel, in which the beads are trapped due to the integration of a frit or another obstacle. By this, a column reactor with renewable filling or a renewable electrode surface can be generated. The implementation of the miniature column into one valve port allows fast pre-concentration, clean-up, and e.g. effective enzyme utilization. Besides, elution can be done directly into the detection cell achieving a very low dispersion and thus high sensitivity [79]. After usage, the beads can be re-aspirated and discharged.

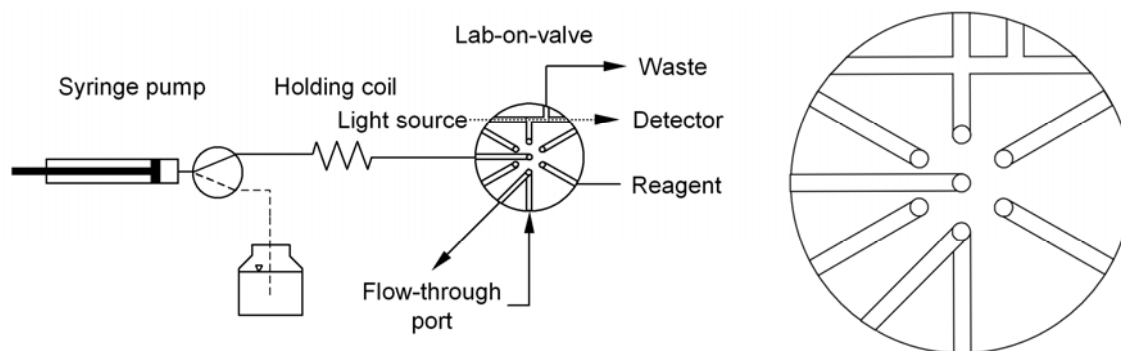
Several reviews describe the characteristics, potentials, and applications of LOV and BI technique with the main focus each on different aspects [46,49,80,81]. Among others, the advantageous use of BI-LOV for analyte pre-concentration for coupled well-established

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analytical techniques ICP-MS [31], atomic fluorescence spectrometry [29], ETAAS [27,82] and HPLC [83] have been described. For the later works, a multisyringe pump was used.

The concept of integration of a flow-through port allows also the direct coupling of the analyzer instrument to a continuous sample flow as discussed in chapter 4.3.2.2. Shortcomings of LOV and classical SIA are quite similar. Using an integrated conduit, e.g. the robustness of LOV is higher, whereas the benefits of the prefixed design are lost when further manifold components are required, e.g. a debubbler device. Here, the use of the LOV-conduit with a multisyringe pump offers higher versatility as shown in chapter 1.1.6.3.



**Figure 4: Typical structure of a LOV manifold (left) and LOV conduit (right).**

**In contrast to SIA, a special selection valve design with integrated detection cell and flow-through port is used. This facilitates downscaling of the analyzer system and handling of active beads for in-situ preparation of columns with renewable fillings.**

### 1.1.6. Multicommutated flow techniques

#### 1.1.6.1. Preliminary remarks to multicommutation

The term multicommutation refers to the possibility of repeated activation or deactivation of a solution supplying line or manifold path as a function of time [84]. According the former named IUPAC technical report [51], this concept can be implemented into each analytical flow technique. Generally, the term multicommutation is assigned only to the three following described techniques, compared elsewhere [85].

#### 1.1.6.2. Multicommutated flow injection analysis

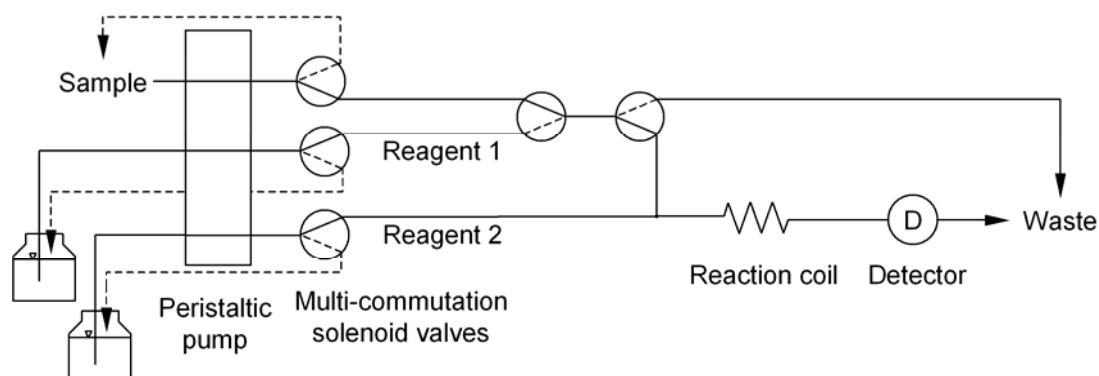
The Multicommutated Flow Injection Analysis technique (MCFIA) was firstly proposed by REIS ET AL. (1994) [84]. Their characteristics, potentials, and applications have been reviewed in 2002 by CATALÁ ET AL. and by ROCHA ET AL. [39,47]. It is based on the use of 3-way multicommutation solenoid valves (MCSV) as exemplarily shown in the annex. Alternatively, clamp valves have been successfully used as an economic alternative [86]. MCSV act as commutators with two states, ON (activated, normally closed, NC) and OFF (inactive, normally open, NO) and are powered via software-controlled relay-cards.

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MCSV are used to substitute the injection valve of classical FIA, to enable the return of actually not required solutions to their reservoir (recycling) and by this, deactivation of the respective pumping line, or to choose between distinct manifold paths, e.g. to circumvent a catalytic column reactor. A typical structure of a MCFIA manifold is shown in figure 5. Apart from loop based injection, confluent or binary sampling schemes are possible, i.e. the simultaneous or sequential loading of sample and reagents aliquots directly into the analytical flow path. Binary sampling refers to zone stacking techniques (sample slicing, trapping, or tandem-streams) in order to improve sample dilution or mixture with the used reagents [87]. MCSV operate within 30 - 100 ms and with high precision. By precise time control, the provision of very small volumes either by sample splitting or by zone sampling [88,89] and the repeated sample re-circulation [90] was successfully demonstrated.

Recycling of the carrier allows prolonging the sample residence time in the manifold and stop-flow detection mode. Recycling of reagents as long as they are not required and volume alteration as a function of MCSV activation time lead to a considerable reduction of the solutions consumption. MCFIA allows the construction of software-adaptable "flow networks" of high flexibility where physical changes of the manifold are not required but only the alteration of the commutation sequence. Therefore, as in SIA, multi-parameter determinations on one system are possible. The substitution of the rotary injection valve by the smaller, faster, and more economic MCSV allows further the miniaturization of the flow assembly and high injection frequencies with up to 220 per hour reported [84]. The benefits of MCSV have led to their implementation also in sequential flow techniques.

The disadvantages of MCFIA are basically the use of peristaltic pumps, the moderate pressure robustness of the MCSV, the required software control, and the considerable dead volume and stroke pulsation generated by particular MCSV devices. The permanent activation of the valve solenoid generates further considerable heat, which can lead to the destruction of the valve. This effect can be diminished by commercial protection circuits such as shown in the annex, where the effective voltage power is decreased shortly after initiation to a third, which is sufficient for holding the solenoid in the activated position. However, the nominal pressure stability is diminished in this mode as well.



**Figure 5: Typical structure of a MCFIA manifold.**

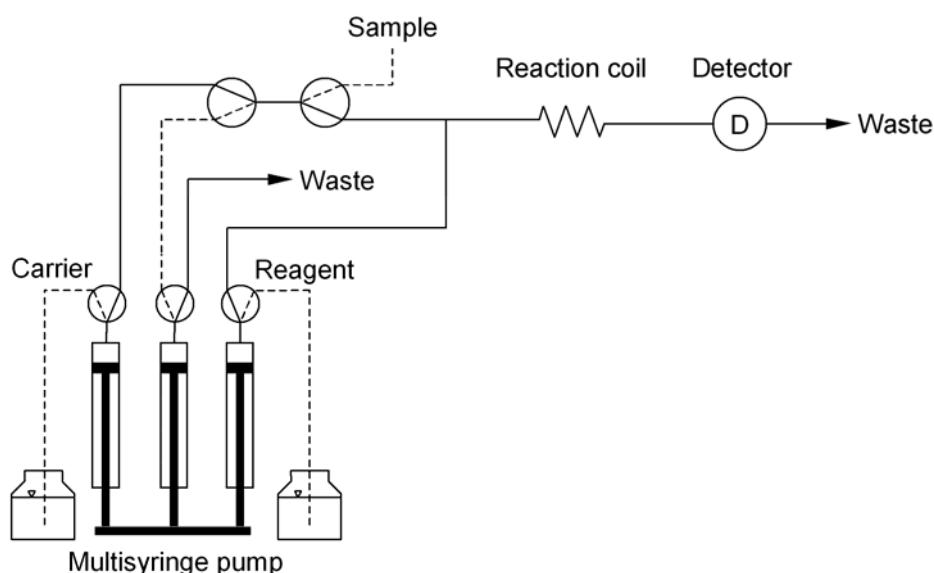
**A FIA manifold is modified by the introduction of multicommutation solenoid valves (MCSV) allowing the change of flow direction e.g. to reduce reagent and sample consumption.**



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### 1.1.6.3. Multisyringe injection analysis

Multisyringe flow injection analysis (MSFIA) has been firstly proposed by CERDÀ ET AL. (1999) [91] and was characterized in a first application by ALBERTÚS ET AL. (1999) [92]. Parallel moving syringes incorporated into one single pump device are used as liquid drivers. By this, advantages from FIA and SIA are combined: benefits of a multichannel flow technique such as confluent mixing and high injection frequencies up to  $180 \text{ h}^{-1}$  but as well precise and bi-directional liquid handling, flow rate stability, pressure robustness, and chemical resistance. By implementation of MCSV at each syringe head and optional valves, the multi-commutation concept can be applied with the possibility of carrying out the former described binary flow schemes, low solution consumption, advanced flow networks with alternative manifold paths, and multi-parameter analysis. Software control allows the easy adjustment of solenoid valve positions, flow rate, direction, volumes, and manifold configuration. The flow rate ratio between different channels is configured by the use of different syringe sizes. A typical structure of a MSFIA manifold is shown in figure 6.



**Figure 6: Typical structure of a MSFIA manifold.**

**A concept similar to MCFIA is applied but sequential and bi-directional operations are fundamental of the multisyringe pump.**

The characteristics, potentials, and applications of the MSFIA technique have been reviewed in 2002 [45], 2003 [93], 2006 [50], 2007 [94], and in 2005 with special focus on the use in combination with chemiluminescence detection [76]. The reader is also referred to a comprehensive review in 2005 by the author, which is attached to this work. It includes an overview of the possible injection modes and network configurations [41].

MSFIA can be considered as an ideal multichannel technique for challenging analytical procedures, which require high and precise flow rates such as chemiluminescence, high pressure stability such as for the implementation of sorbent columns, and handling of aggressive or volatile solutions such as for sample pre-treatment. However, the periodical

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syringe refilling causes a lower injection frequency as in an equivalent FIA system. Recent trends in the development of MSFIA are the combination with LOV [29,83] and low-pressure chromatography using monolithic columns [95,96]. In this work, the coupling with capillary electrophoresis was accomplished for the first time. A detailed description of the most frequently used module is given in chapter 3.1. A photo is shown in the Annex.

### 1.1.6.4. Multipump flow systems

Multipump flow systems (MPFS) have been firstly proposed by LAPA ET AL. (2002) [42]. Their characteristics, potentials, and applications have been reviewed in 2004 by LIMA ET AL. [44] and in 2007 by SANTOS ET AL. [97]. Self-priming solenoid diaphragm micro-pumps of a discrete displacement volume (3 – 50  $\mu\text{l}$ ) are used as mono-channel liquid drivers for each applied solution. Likewise MCSV, the micropumps are powered via software-controlled relay-cards. On each voltage pulse the solenoid attracts the pump diaphragm. It returns subsequently to its initial position due to an inner spring mechanism. Due to an integrated dual check valve, at each diaphragm stroke a small, reproducible liquid volume is aspirated from the pump inlet. At release, this volume is driven through the outlet. Repeated activation generates a unidirectional and pronounced pulsed flow.

The pulsed flow character was considered in the prior works from WEEKS AND JOHNSON (1996) and HANRAHAN ET AL. (2001) as disadvantage [98,99]. Meanwhile, the advantages of the intermediate turbulent flow conditions in the manifold tubes have been repeatedly demonstrated. Zone-penetration and mixing are improved and considerably higher signals were reported for MPFS compared to equivalent FIA systems. Due to the individual control of each pump, micropumps act also as liquid commutators allowing binary sampling and flow schemes likewise in MCFIA. In contrast to the combination of MCSV and a peristaltic pump, the flow rates can be altered moreover independently for each pump by changing the activation frequency (up to 250  $\text{min}^{-1}$ ).

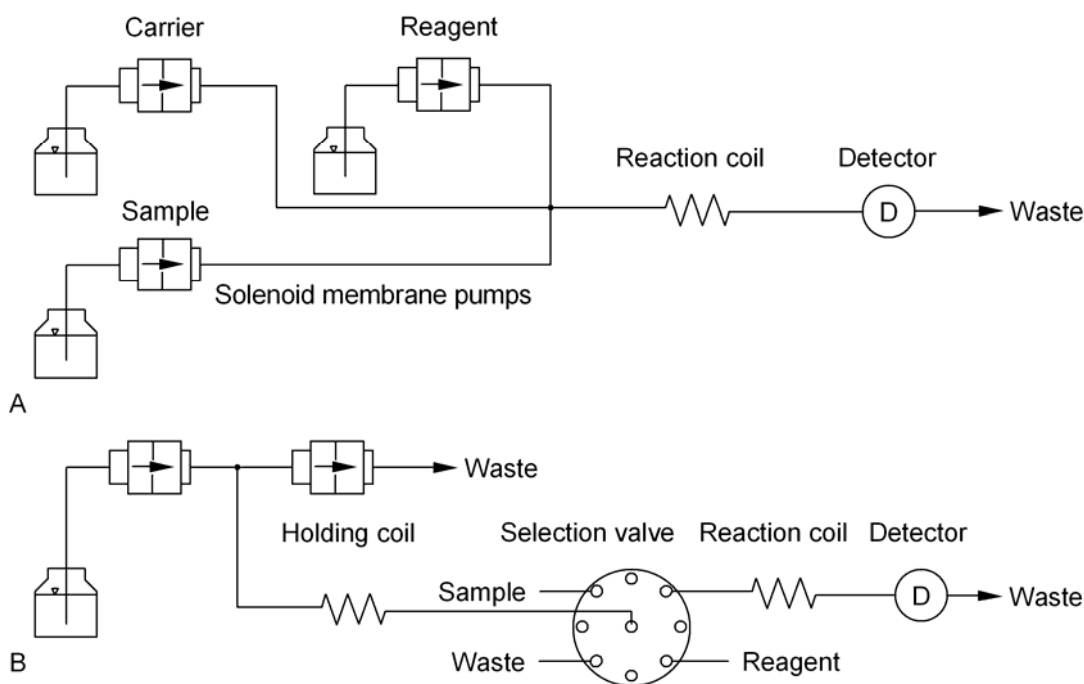
Since all parts in liquid contact are of resistant polymers, there is no limitation in respect of used chemicals. Further advantages of MPFS are a high versatility and flexibility of the flow system networking, especial in combination with MCSV. Further advantages are the simplicity of the system and the low price, easy maintenance, and small dimensions of the micropumps allowing integrated and transportable field analyzers.

Among the disadvantages of micropumps are the susceptibility to blockage by particles and the operation breakdown in the presence of air bubbles and backpressure. The low pressure stability affects already when tubes of 0.5 mm i.d. are used [98]. Nevertheless, the successful implementation of columns or membranes into MPFS has been reported [100,101]. A further problem is the required re-calibration of the displacement volume [85]. Finally, due to the error of the stroke volume, the quantity, which can be injected with high reproducibility into the manifold, is several times higher than one stroke volume. Although

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the integral flow rate is continuously adjustable, the real one is un-continuous and varying within each pulse. Thus, estimation of the resulting backpressure is difficult.

Typical structures of MPFS are shown in figure 7. Two configurations are possible, following either a FIA manifold by the parallel operation of the micropumps or following a SIA manifold by two oppositely operating micropumps, forming one bi-directional pumping unit [102]. Finally, the use of piezoelectric pumps has been reported recently with similar characteristics as the solenoid micropumps by RIBEIRA ET AL. (2007) [7].



**Figure 7: Typical structure of MPFS manifolds.**

**A: FIA configuration, parallel operating micropumps or B: SIA configuration, two micropumps form one bi-directional pumping unit.**

### 1.1.7. Limitations and future trends of analytical flow techniques

The manifold components listed in chapter 1.1.1 were designed for the separation of analytes from the sample matrix by non-chromatographic techniques taking advantage of differences regarding chemical and physical properties of both such as vapor pressure, dissociation constant values, or reactivity [40]. In contrast to chromatographic or electrophoretic techniques, a noteworthy shortcoming of all analytical flow techniques is the low capacity to discriminate or separate compounds of similar properties [37,103]. On the other hand, flow techniques are successfully applied for the determination of sum indices of organic species such as "total phenolic compounds" [104,105] or "sugars" [11].

More specific methods automated by the use of flow techniques are found by the majority for inorganic analytes in consequence of the availability of highly selective reagents or existence of specific RedOx potentials. So, discriminating detection

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techniques are preferably applied, enabling e.g. multi-variant analysis to compensate the low potential of physical analyte separation [18,106,107]. Selective analyte quantification can also be carried out by kinetic discrimination using the stop-flow technique [58,64,65].

Applications for the specific determination of organic analytes can be found for compounds of high reactivity such as formaldehyde discussed in detail in chapter 4, for well-defined sample matrices, where the presence of similar compounds to the analyte is improbable such as in pharmaceutical formulations [108] or spirits [109] or after required elimination, masking, or additional quantification of interfering species. Alternatively, selective enzymes can be used, which either generate an analyte specific, detectable product [110] or cause a signal change on enzyme-modified electrode surfaces [111,112]. However, the sample matrix influences on the enzyme efficiency such as the pH value, salinity, or the presence of denaturants such as certain metal ions, urea, or solvents, an insufficient selectivity, limited lifetime, efficiency loss by immobilization, and costs for enzyme itself or required cofactors often limit the application of enzymes [113-117].

A natural trend in the development and investigation area of analytical flow techniques is therefore the coupling with separation techniques such as liquid chromatography and capillary electrophoresis. Here, the flow system can be used for the automation of sample pre-treatment such as analyte pre-concentration, pre-column derivatization, separation from the sample matrix, reproducible sample injection, and required maintenance of the separation part. A higher sensitivity, better reproducibility, and prolonged stability of the separation instrumentation can be achieved by the simultaneous overcome of the lack of discrimination of flow techniques [118,119].

Coupling with a classical liquid chromatography apparatus is generally accomplished via a high-pressure injection valve, which is loaded with sample provided from the flow system [83]. With availability of commercial, low-pressure monolithic columns bearing flow stop and flow reversal [120,121], the direct connection of separation columns to syringe pumps is possible. Apart from automated pre-treatment, low-price purchase and running, also the miniaturization, portability, and exploitation of different flow modes is possible.

The first applications of monolithic columns in SIA, denoted as SIC, were described by ŠATÍNSKY ET AL. (2003) [122,123] and surveyed firstly in 2007 by CHOCHOLOUS ET AL. [103]. The application of a monolithic column in MSFIA system offered an even higher versatility as reported by GONZALEZ-SAN MIGUEL ET AL. (2007) [95,96]. The coupling of flow techniques with capillary electrophoresis is surveyed in detail in chapter 4.2.3 since the development and employment of such system is an essential part of the present work.

### 1.1.8. Selection for the proposed applications

*„I hate peristaltic pumps“ J. Ruzicka, Flow Analysis 2006 in Porto, Portugal*

For the developed automated systems proposed in this work, the syringe-pump based flow techniques, SIA and MSFIA, were applied since their characteristics meet best the requirements for the considered monitoring applications described in chapter 1.3.4 and

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1.3.5 and likewise considered in literature [20,71,124]. These characteristics are a high robustness regarding pressure, chemical agents of different character, and air bubbles, a high precision and stability of flow rates and volumes over a long period and independent from liquid viscosity without the need of frequent re-calibration, system simplicity, low maintenance effort, minute consumption of sample and reagents, and a high flexibility enabled by computer control without the need of manual manifold reconfiguration. The typically moderate injection frequency of the SIA technique was no exclusion criterion, since a time resolution of about 5 min was considered as sufficient in the most cases in advance by the collaboration groups at the Hamburg University for Applied Sciences (HUAS) and at the Autonomous University of Barcelona (UAB). Three of the proposed analyzers were tested on cultivation processes run by the respective group members.

### 1.1.9. Software control for modern flow techniques

One of the main out-standings of modern flow techniques is surely the computer control of the respective instrumentation (detection devices, syringe- or micro-pumps, valves, etc.) by specialized software [125]. The potential of fully automation, discontinuous operations, precise synchronization of the instruments, and electronic data acquisition and processing are important benefits of software control. Besides, automated data evaluation can enable feedback operations such as a decision about an actual need of modifications of the analytical procedure without required user intervention. On the other hand, the requirement of software control was considered as the most limiting factor in further development of these techniques [76]. Therefore, this can be considered as a crucial reason for the considerably lower numbers of publications applying these techniques [40].

Most research groups use in-house developed and purpose-made software solutions written in programming languages Basic, QuickBasic, Visual Basic, LabVIEW, or C++ [43]. Apart from the demand of a profound user skill, re-programming might become necessary if the instrumentation assembly is modified. Frequently, software control is only accomplished for the flow instrumentation, whereas data acquisition and processing have to be carried out with the accompanying software of the respective detector device.

Only few comprehensive programs for flow technique applications are commercially available but often limited to the combination with a specific instrumentation [71], among these the software packages FlowTEK (Mintek, Randburg, South Africa), AnalySIA (Centre for Biotechnology, Åbo Akademi University, Turku, Finland), CAFCA (Anasyscon GmbH, Hannover, Germany), FIALab (FIALab Instruments Inc., Bellevue, WA, USA) [57], or DARRAY (Sciware SL, Palma de Mallorca, Spain). For this work, the software package AutoAnalysis (Sciware SL) was used for the overall instrumental control, data acquisition, and on-line data evaluation, described in detail in chapter 3.4.1.

### 1.2. Capillary electrophoresis

#### 1.2.1. History

Electrophoresis means the attraction or repulsion of ions in an electrical field causing a migration in function of their charge and mobility (Greek *phoresis*: "carrying"). It was firstly described by TISELIUS (1930) for proteins in a buffer solution [126]. Since then, anti-convective media such as polyacrilamide or agarose gels have been used to suppress thermal diffusion of the charged species and liquid convection in order to improve the separation efficiency. For heat dissipation, the gels are either submerged in the separation buffer or are polymerized between two thermostated glass plates with both gel ends and respective electrodes placed in separation buffer reservoirs. Today, electrophoresis is one of the most frequently applied separation techniques for macromolecules such as proteins and nucleic acids. It is simple, economic, allows parallel separation of multiple samples, bi-dimensional separations and gradient techniques under native or denaturing conditions. However, the separations take long times and are of low reproducibility and efficiency.

Capillary electrophoresis (CE) was initiated by HJERTEN (1967) [127] using open, millimeter-bore and buffer filled glass capillaries rotated along their longitudinal axis in order to suppress convection. By the reduction of the inner capillary diameter to 75  $\mu\text{m}$ , rotation was not longer required since due to the enhanced efficiency of heat dissipation <sup>1</sup> and reduced radial temperature gradient the capillary becomes anti-convective itself [128].

KARGER (1989) [129] stated "High performance capillary electrophoresis is expected to be the fastest-growing analytical technique since HPLC". Indeed, CE is today a major instrumental separation technique like HPLC and GC due to the variety of operation and detection modes described extensively in specialist books [130-133] and reviews [118,119,134-142]. Fused silica capillary of reduced diameters (10-75  $\mu\text{m}$ ) enable the application of high electrical fields (100-500  $\text{V cm}^{-1}$ ) with consequently fast separations (< 1 min), high separation efficiencies <sup>2</sup> ( $N > 10^6$ ) and the required minute sample volume (1-50 nl) allows even single-cell analysis [143]. CE has been applied to a wide range of analytes including carbohydrates, carboxylic acids, inorganic anions, phenols, metal ions, pharmaceuticals, drugs, explosives, pigments, vitamins, toxins, chiral separations, DNA, pesticides, peptides and proteins in forensic, clinical, and food analysis.

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<sup>1</sup> It is noteworthy that the liquid volume in the capillary is proportional to the square of inner radius  $r_i^2$  whereas the wall surface is directly proportional to  $r_i$ . Thus, the radial temperature gradient is proportional to  $r_i^2$ . Also other wall effects become important in micrometer-bore capillaries due to the large surface-area-to-volume ratio such as adsorption of sample matrix components.

<sup>2</sup> The separation efficiency is proportional to the electrical field. However, the applicable voltage is limited by the resulting current, being inverse-proportional to the cross area or the square of  $r_i$ .

### 1.2.2. Theory and operation modes

Migration with a constant velocity  $v$  of an ion in an electrical field  $E$  results from the interplay of the exerted electric attraction force  $F_E$  and the friction force  $F_F$  between the ion and the given medium, expressed by the electrophoretic mobility  $\mu_e$  according formula 2.

$$v = \mu_e \cdot E \quad (2)$$

In steady-state, both forces are balanced and by approximation the ion as a spherical particle,  $\mu_e$  is given by formula 3. It follows that species of small ionic radius  $r$  and multiple charges ( $z \cdot e$ ) show the highest mobility favored by a medium of low dynamic viscosity ( $\eta$ ).

$$1 = \frac{F_E}{F_F} = \frac{z \cdot e \cdot E}{6 \cdot \pi \cdot \eta \cdot r \cdot v} = \frac{z \cdot e}{6 \cdot \pi \cdot \eta \cdot r \cdot \mu_e} \Rightarrow \mu_e = \frac{z \cdot e}{6 \cdot \pi \cdot \eta \cdot r} \quad (3)$$

The observed or "effective" mobility  $\mu$  differs generally from the theoretical value e.g. due to incomplete analyte dissociation and is further highly influenced by the composition and pH of the separation buffer. The best separation is achieved under conditions where the difference between the effective mobilities of the analytes is maximal.

The second component affecting the migration and fundamental constituent of CE operation is the electro(endo)osmotic flow (EOF). The EOF is a plug flow of the bulk liquid column in the capillary. The partly deprotonation of the silanol groups on the fused silica capillary inner wall led to a net negative charge of the surface. A double-layer is formed by attraction of dissolved cations, expressed by the zeta potential  $\zeta$ . The EOF results from the electric attraction force on the outside apparently positively charged liquid bulk moving to the cathode (lower potential). Magnitude and direction of the EOF is given by formula 4 (Smoluchovski equation) with  $\varepsilon$  being the dielectric constant of the electrolyte.

$$v = \frac{\varepsilon \cdot \zeta \cdot E}{\eta} \quad (4)$$

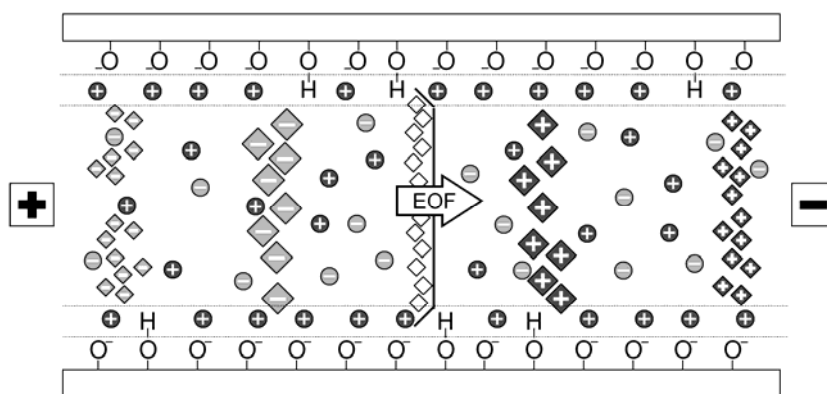
The EOF superimposes the analyte migration (figure 8), so, also uncharged species are transported towards the detector although they are not separated in this operation mode. The effective mobility  $\mu$  can be calculated from formula 5 with  $l$  being the capillary length,  $l_{\text{eff}}$  the effective length (to the detector),  $t$  the migration time, and  $U$  the applied separation voltage. The EOF can be calculated from the running time of an uncharged species added to the sample as marker (e.g. methanol). In the normal mode (negative wall charges), cations migrate in co-direction to the EOF, whereas anions migrate in counter-direction. For small anions (high mobility), reversal of the separation voltage polarity might be required to achieve an effectively positive migration towards the detector.

$$\mu = \mu_e + \mu_{\text{EOF}} = \frac{l_{\text{eff}}}{t \cdot E} = \frac{l \cdot l_{\text{eff}}}{t \cdot U} \quad (5)$$

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In contrast to the laminar flow typical in flow techniques or liquid chromatography, there is no pressure drop along the capillary, the EOF profile is flat and it does not contribute to the sample dispersion. This allows reaching high separation efficiencies in CE. On the other hand, control of the EOF by the maintenance of the inner capillary wall condition is crucial for successful and reproducible separations. A low-ripple separation voltage and a constant temperature, last one affects otherwise the buffer viscosity, are further required.

The main optimization work in CE is given by the design of an appropriate separation buffer since the zeta potential, and in consequence the EOF, depends highly on the ionic strength, pH, and the presence of surfactants and organic modifiers. As deprotonation of the silanol groups depends on the pH, the EOF is insignificant below pH 4. The EOF is highly reduced in polymer capillaries or when the capillary inner walls are coated with a nonionic polymer or at a high ionic strength or at a high content of nonpolar solvents.



**Figure 8: Capillary with double-layer and EOF plug profile superimposing the differential migration of the analytes (diamonds) and buffer components (bullets).**

The addition of a cationic surfactant leads to the adsorption of a nonpolar single layer on the inner walls and suppression of the EOF. Increasing the concentration of the surfactant, a double-layer is formed, which reverses the apparent charge of the inner wall and, in consequence, the EOF direction. The same effect is achieved by wall coating with a polymer exceeding positive charges. So there are four basic operation modes by applying either analyte counter- or co-migration to EOF of either normal or reversed flow direction.

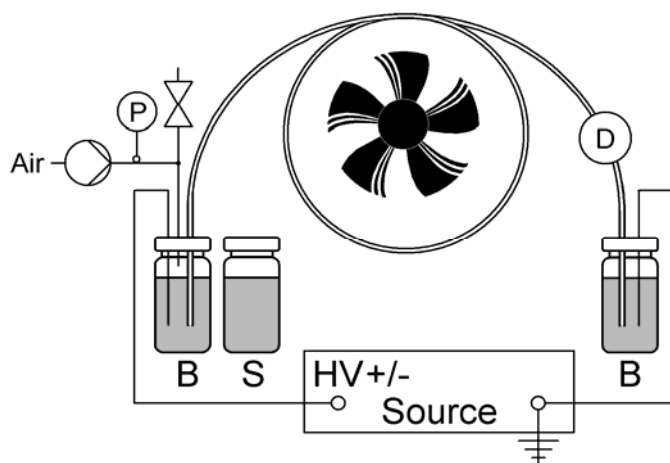
The so-far described separation of the analytes by their free-solution mobility is denoted as capillary zone electrophoresis (CZE). The generation of a pH or ion mobility gradients in the capillary allows analyte separation and focusing either according their isoelectric point (CIEF) or their electrophoretic mobilities (Isotachopheresis). Separation of macromolecules in a gel filled capillary is denoted capillary gel electrophoresis (CGE). Separation of uncharged or nonpolar compounds is achieved by micellar electrokinetic chromatography (MEKC). Here, cationic (anionic) surfactants are used in the separation buffer above their critical aggregation concentration. In consequence, they form positively (negatively) charged micelles, which act as a migrating separation phase. Higher analyte hydrophobicity and thus higher affinity to the micelle results in a longer transport time of the analytes in the micelle and by this a higher effective electrophoretic mobility.



### 1.2.3. Instrumentation and analytical parameters

#### 1.2.3.1. Fundamental instrumentation

The fundamental design of a CE instrumentation is shown in figure 9, consisting in the separation capillary, buffer reservoirs, high voltage (HV) source, detector, equipment for ventilation or thermostatzation and, in general, a unit for controlled pressure application and release on the injection side. In this chapter the operational requirements and influencing factors of injection, separation, and detection are described. So it reflects the fundamental considerations for the development of a CE system capable for the hyphenation with SIA and MSFIA as done in this work and described in chapter 7 and 8.



**Figure 9: Basic capillary electrophoresis system.**

**The system consists of buffer (B) and sample (S) reservoirs, high voltage source with optionally reversible polarity, capillary, ventilation / thermostatzation, detector, and controlled pressure application and release on the injection side.**

#### 1.2.3.2. Injection

Injection volumes in CE are generally in the order of nanoliters equivalent to a sample plug length of a few millimeters. The reproducible injection of such small volumes is done with the sample at the capillary entrance either hydrodynamically or electrokinetically. On-capillary injection via nanoliter injection valves or membrane interfaces is also possible, but hardly applied in commercial instruments.

Hydrodynamic injection is the most frequently used method. With the sample placed at the capillary entrance (injection side), it is done either by pressure application at this side, application of negative pressure at the capillary outlet (detection side), or by siphonation (elevation of the injection side for a given time). The injected volume can be estimated by the Poiseuille equation (see chapter 3.6.2). For siphonation, the pressure is equal to the product of applied height difference, the gravitational constant, and the buffer density. Hydrodynamic injection is nearly independent from the sample matrix. On the other hand, the extension of the sample plug affects the separation efficiency (see chapter 1.2.3.3).

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In electrokinetical injection, the analytes enter the capillary during appropriate voltage application by migration and the exerted pumping action of the EOF. By this, the analytes can be separated from non- or counter-charged components and be pre-concentrated at the capillary entrance. However, the introduced analyte mixture is enriched in respect of the original sample with the analytes, which show higher electrophoretic mobilities. The sample conductivity further affects the migration of the analytes into the capillary, which can be compensated by an internal standard [144,145]. Though not required for injection, the possibility of pressure application is nevertheless fundamental to enable in-situ flushing of the capillary for re-conditioning. The reversal of the source polarity is required if the respective analytes migrate in opposite direction of the EOF during later separation.

### 1.2.3.3. Separation

Diffusion along the capillary (longitudinal) is the main factor lowering the separation efficiency in CE, whereas radial diffusion does not affect the separation due to the EOF plug profile. Diffusion depends on the diffusion constant  $D$  of each analyte, being a function of buffer viscosity, temperature, and the separation time, during which diffusion can take place (formula 5). Formula 6 giving the number of theoretical plates  $N$  or height equivalent  $h$  states clearly, that a higher electrical field improves separation efficiency<sup>3</sup>.

$$N = \frac{\mu_e \cdot I_{\text{eff}} \cdot E}{2 \cdot D} = \frac{l_{\text{eff}}}{h} \quad (6)$$

Zone broadening results further from the injection plug length, wall interactions, electrodispersion, laminar flow, joule heating, and detector characteristics (chapter 1.2.3.4). The injection plug length should not exceed the diffusion caused zone broadening since it affects otherwise the separation efficiency. On the other hand, the increase of the injection plug length allows a moderate enhancement of the detection sensitivity. Analyte wall interactions are favored by the large surface-area-to-volume ratio but are mainly observed for macromolecules. They can be suppressed by buffer additives such as zwitterionic components, by wall coating, or by alteration of the buffer ionic strength or pH. Likewise, the adsorption of matrix components can affect the EOF. Therefore, cleaning and re-conditioning of the capillary e.g. by flushing with diluted base and separation buffer is highly important to achieve a good reproducibility and recommended after each analysis.

Electrodispersion means zone broadening due to a distinct conductivity of the sample compared to the one of the separation buffer, which causes either peak tailing or fronting (lower or higher sample conductivity, respectively). This effect results from the change of the electrical resistance and by this the electric field in the respective liquid segment. Thus, the migration accelerates when the conductivity decreases and slows down when the conductivity increases. Consequently, the analytes are stacked in their migration

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<sup>3</sup> This has led to the trend of reduction of the inner diameter of the capillary to few micrometers in order to enable the high separation voltages of up to 120 kV [146].

## 1 General introduction

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direction at the boundary of lower to higher conductivity. There are various on-capillary concentration techniques, which take advantage of this effect.

Laminar flow is caused by a pressure difference between capillary entrance and outlet, e.g. by a difference in height. The parabolic flow profile lead to zone dispersion but the contribution of this effect is relatively low due to the small capillary inner diameter. However, the laminar flow changes the analyte transport and separation time. So, leveling of the capillary ends is important in order to achieve high separation reproducibility.

Joule heating means the rise of the temperature in the capillary due to its actuation as electrical resistance. The resulting temperature gradient between the capillary center and walls causes differences in the buffer viscosity, an inhomogeneous EOF profile, and zone deformation. A lower buffer viscosity favors this diffusion process. A positive feedback results from the increase of the ion mobilities at higher temperature, which again lowers the electrical resistance of the buffer. Capillary thermostatization or appropriate heat dissipation is therefore fundamental for high reproducibility and separation efficiency.

### 1.2.3.4. Detection

The migrating analytes zones are only few millimeters in length. The detection cell length has therefore to be far below this extension in order to avoid an apparent loss of resolution. Likewise, the measuring frequency has to be high enough in order to allow a good peak representation. Peak area data are rather used than peak heights as they are less affected by migration time, injection plug length, and analyte stacking after injection.

Among the detection techniques applied in CE, amperometry, conductivity, mass spectrometry, fluorescence, chemiluminescence, and, most frequently, (spectro)photometry are found. The fused silica capillary is transparent down to deep UV. Thus, after removal of the polyimide protection layer, optical techniques can be used for on-capillary detection without affecting the electrophoretic separation. Electrochemical detection has to be done end-of-capillary and grounding of the detection side is mandatory. The sensitivity and baseline noise of most detection techniques depend on the detection cell volume or optical path length, which are in CE in the order of few nanoliters or tens of micrometers, respectively. Consequently, low sensitivity is characteristic for CE. It can be improved by applying long-path detection cells, techniques less affected by the detection volume such as contactless conductivity [141] or pre-concentration techniques such as stacking [136], sweeping [137], electro dialysis [147], or SPE pre-concentration [118,119,138,140].

Applying photometric detection as done in the present work, the maximal linear range and sensitivity is achieved only, when the detection window is smaller than the capillary inner diameter. In consequence, only photons count, which have passed the liquid volume ideally at its maximal extension and not only the bulk capillary walls. Reflection at the phase boundaries of air, silica, and liquid leads to the loss of illumination intensity and thus a higher baseline noise. So, ideally the light is focused to the center of the capillary and recollected by adequate focusing optics.

### 1.3. Process analytical chemistry

#### 1.3.1. Introduction to process monitoring

Process monitoring can be considered as the application of analytical chemistry to an operating system with the objective to obtain information about its condition such as the composition of the reaction phase, concentration data of components, or the alteration of these parameters. The analytical information is generally provided by methods based on the quantification of extensive (reaction volume dependent) or intensive (reaction volume independent) properties of the reaction phase or a concrete analyte. Extensive properties are e.g. mass changes and flow rates. Intensive properties are the reaction medium density, temperature, conductivity, or the signal height of optical techniques such as Raman-, fluorescence-, X-ray-, IR-, or UV-VIS-spectrometry. Optionally, they are applied after carrying out a chemical or enzymatic reaction in order to obtain a higher detection selectivity and sensitivity. Finally, two-dimensional analytical methods i.e. simultaneous analyte quantification and discrimination according a particular analyte quality are applied, e.g. chromatography, electrophoresis, polarography, or mass spectrometry.

An operation system might be an organism (yeast cell, patient...), an environmental compartment (air, surface water...), a composting or wastewater treatment plant, a landfill site, or an industrial process (biotechnological, pharmaceutical...) [148]. Most aspects and problems discussed in this section considering biotechnological processes are valid as well for other, e.g. natural, processes involving microorganisms' activity.

Analytical techniques and instrumentation applied for process monitoring are denoted in literature generally as "Process Analytical Chemistry" (PAC) [148,149]. Applications published since 1993 have been reviewed by WORKMAN ET AL. (1999, 2001) [150,151].

#### 1.3.2. Objectives of process analytical chemistry

The driving forces of PAC development and the objectives of the application of analyzers to a process or a representative effluent flow are manifold and based on economic, management, legislative, and investigation interests [71,148-151]. In the industry guidance "Process Analytical Technology" (PAT) [152] published in 2003 by the US Food and Drug Administration, PAC was considered as main tool for quality assurance in pharmaceutical manufacturing. In concrete, PAC is directed among others to

- improvement of manufacturing efficiency and process sustainability
- assurance and proof of product quality and optimal process conditions
- identification of process end-points
- fulfilling of restrictive regulations and obligated documentation
- safety assurance and risk prevention

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- minimization of by-products, costs, waste, raw materials, energy, and resulting environmental impact
- constant information of process status and problems
- rapid and automated corrective action based on analytical results and decision
- better process understanding e.g. for mathematical prediction models.

The former objectives and resulting benefits explain the increasing number of applied process analyzer systems. However, the costs for purchase, implementation, operation, and maintenance are in opposition to the gain of additional information [153]. Therefore, the decision for a particular process analyzer depends on the characteristics and demands discussed in chapter 1.3.3 and 1.3.4.

### 1.3.3. Modes of process monitoring

Analytical procedures and instrumentation used for monitoring can be distinguished according to locality and manner of measurement and the time delay between measurement initiation and the finally obtained analytical result. The four modes "in-line", "on-line", "at-line", and "off-line" are generally distinguished [148,149,152]. Sometimes and particularly in respect to flow techniques, the terms are inconsistently or imprecisely used [150]. Thus, the characteristics of the observed process and applied analytical technique give leeway to discussions [154]. Depending on the respective mode and applied detection technique, quantification data can be quasi-continuously with only the characteristic time delay of the analytical procedure or time-discrete including an additional and varying dead time. The four modes are exemplarily shown in figure 10 for a biotechnological process.

The term "in-line", or often denoted as "in-situ" refers to a measurement accomplished directly in the reaction phase (in biotechnology the cell-containing medium), consequently without sampling, sample pre-conditioning, or sample transport. Measurements performed in living organisms are designated "in-vivo" [155]. Invasive and non-invasive techniques are distinguished depending on whether the sensor, e.g. an electrode, is in physical contact with the reaction phase (pH, O<sub>2</sub>, ...) or not and the analytical information is transmitted beyond the reaction phase boundary. Non-invasive techniques are limited to physical parameters and techniques such as calorimetry, optical, or acoustical methods, e.g. fluorescence or near-IR spectrometry through a window in the reaction vessel [156]. In-line monitoring provides continuous data with a minimal time delay.

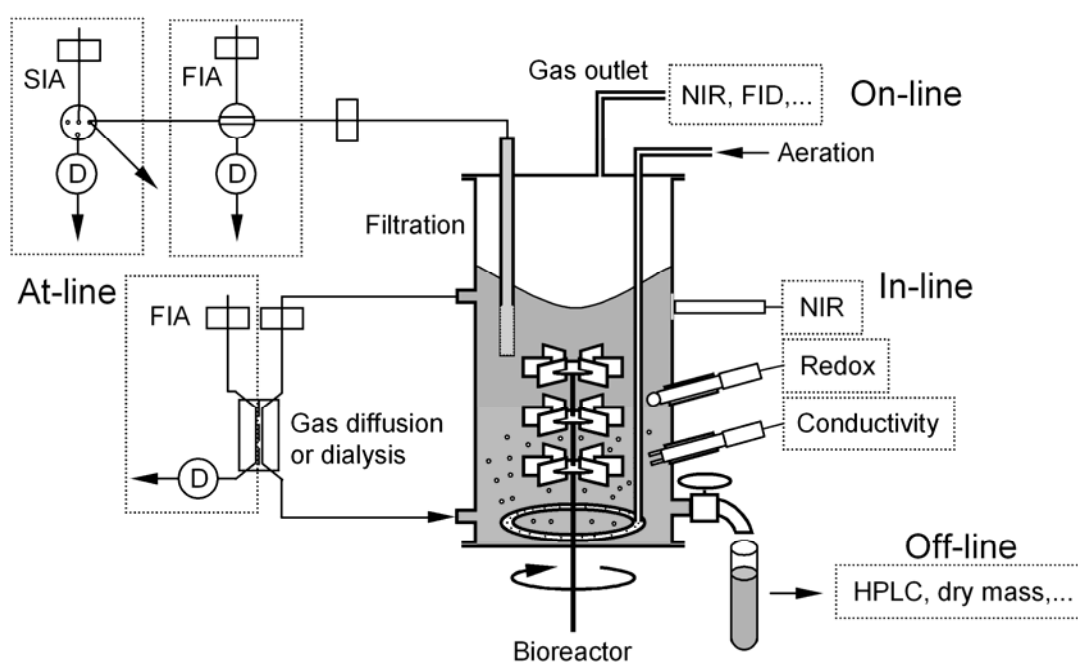
The term "on-line" refers to measurements accomplished outside of the reaction phase but in a peripheral process compartment or a diverted process stream e.g. recording of the agitation speed, the aeration rate, or gas sensor based analysis of the exhaust outlet of a biotechnological process. As in in-line mode, continuous data with comparable short time delay are obtained, however notable dead times can arise if distances become extended. Data provided from in-line or on-line operating process analyzer systems are

## 1 General introduction

denoted as "real-time" due to the short time delay. This allows a direct and automated feedback to an operation unit by closed control loops. On the basis of the analytical information and prior-defined criteria, noted deviations from the ideal conditions can be compensated automatically by the appropriate change of process parameters [157,158] and by this, lead to so-called expert systems [159,160].

The term "at-line" refers to measurements accomplished in a process-separated analytical system located "at-side" including sampling, sample transport, and if required, sample conditioning prior to the measurement itself. It follows, that only time-discrete data with a considerable delay time in the order of minutes can be obtained. The last result is therefore kept up to the next quantification result denoted "sample and hold". The effect of a conducted adjustment of process parameters will not be noted until the completion of the following analysis. Therefore, the possibility of feed-back control, i.e. the use of the analytical information to decide about required actuation is limited and might require a prediction model of the process behavior. The terms "near real-time" or "close-time" are typically used [148,157]. A limitation of the at-line mode is the possible change of the sample composition by sampling, transport, and required additives.

The term "off-line" refers to measurements accomplished "off-side" of the process e.g. remote from the production area in an analytical laboratory after sampling, sample conditioning, transport, and often, sample storage (e.g. refreezing) prior to the analysis. Consequently, the analysis is carried out independently from observed process regarding time and location and only time-discrete data with a typical time delay in the order of hours to days are obtained [148]. As in at-line mode, the problematic aspect is the possible change of the sample composition before the analysis is accomplished.



**Figure 10: In-line, on-line, at-line, and off-line monitoring at a biotechnological process with different sampling techniques and interfaces for ex-situ measurements.**

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The generally applied criterion to distinguish between "on-line" and "at-line" analyzers operation is whether automated and manual sampling is accomplished [148,152]. Hence, process monitoring using flow technique analyzers and HPLC is mostly denoted "on-line". Considering a differentiation according important characteristics of a process analyzer systems based on flow techniques such as their typical time resolution, limited operation feedback, modifications of the sample composition by the sampling procedure and device, transport, and preservation, the term "semi-on-line" [154,161] or less confusing "at-line" was suggested to be more appropriate in spite of performing automated sampling [150]. Further, it is arguable whether the terms "real-time" and "on-line" are correctly used only if continuous monitoring is accomplished or also if the response time of the process is so long that even a considerable time of analysis still permits appropriate countermeasures.

### 1.3.4. General requirements on process analyzer systems

A process analyzer system consists generally of a sampling point, a pre-conditioning system, a sample transport line, a conditioning system, and the analyzing instrument or "analyzer" itself with belonging control and output equipment [148,152,157]. Applying in-line monitoring, some of the listed components might coincide or be lacking. Therefore, mostly automated ex-situ flow techniques are considered in this section.

The sampling procedures and equipment (or sampling probe) have to allow taking a sample regularly, with minimal dead volume, and at each time fully representative for the monitored process without affecting it significantly, e.g. change of the reaction phase volume, the cell density, process contamination, etc.

Sample pre-conditioning means the treatment directly after sampling in order to ensure the consistency of the sample composition and to prevent possible alteration during the transport such as cooling, filtering, drying, or the inhibition of sample transformation due to adsorption, fouling, precipitation, oxidation or enzymatic or cellular activity.

The transport-line must provide the sample to the analyzer or an upstream conditioning system with a minimal time delay and without affecting its composition. The conditioning system enables the analysis, i.e. adapts the sample to the analyzer. The analyzer should provide information of high quantitative precision of the analyte of interest or qualitative significance of the monitored process, respectively.

Further demands can be deduced from the objectives from chapter 1.3.2 such as:

- low purchase costs and low maintenance effort
- no effect on other assembled process analytical chemistry
- minimal use of sample, consumables, and reagents
- avoidance of harmful reagents or risks for the operator
- rapid response time i.e. time required for analysis and report
- conforming to the demands on selectivity, sensitivity and concentration range

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- high robustness, precision, and reliability of instrumentation and procedure
- long-term stability and low frequency of re-calibration
- simplicity and ease of operation such as software based user assistance
- automation of the entire analytical procedure, data processing and transfer

Ideally, the analyzer or a supervision system is able to evaluate autonomously the plausibility of the obtained data and to detect failures occurred during the execution of the analytical procedure [159,160,162] as well as to evaluate the correct operation of the monitored process and to initiate appropriate countermeasures [160,163].

### 1.3.5. Particular aspects of biotechnological process monitoring

Apart from the former requirements, carrying out monitoring of biotechnological processes implies in general (not problematic in wastewater treatment) the avoidance of (biological) contamination of the process. In these cases, retrieval of medium samples has imperatively to be carried out under aseptic conditions. Consequently, the sampling probe or interface has to be in-situ sterilizable. The demand on a sensor of in-line sterilizability is one of the fundamental reasons why up to now in-line monitoring of biotechnological processes is restricted to few analytical parameters such as pH, turbidity, cell concentration, dissolved gases, temperature, RedOx potential, and using optical probes [154,164]. Besides, the operational and analytical reliability and the design of process analyzers used for the analysis of cultivation media is considerably effected by typical characteristics of the medium sample [124,154,158,165], among others:

- a complex matrix containing cells and its fragments, proteins, carbohydrates, lipids, volatile compounds (amines, organic acids,...) and elevated concentration of salts
- excellent conditions for microorganism growth in sections of the process analyzer system, e.g. biofouling and clogging of tubes, membranes, or columns
- a high viscosity and optical density
- high amount of dissolved gases such as carbon dioxide and oxygen
- presence of ions from various transition metals (Mn, Fe, Co, Ni, Co, Zn, Mo,...), which might catalyze or inhibit a chemical or enzymatic reaction in the analyzer
- change of medium composition, oxygen content, and the concentrations of most compounds including the analyte and interfering substances in a wide concentration range (in the order of  $10^{-3}$  to  $10^{+2}$  g l<sup>-1</sup>) during the process time
- decrease of the process response time due to increasing cell density and consequently higher conversion rates requiring a higher measuring frequency
- modification of the sample in the analyzer system, e.g. the sample's pH, temperature, RedOx potential, addition of solvents, etc. might cause precipitation of proteins or metal hydroxides and clogging of tubes and membranes



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Most of the listed characteristics and difficulties are also valid for the monitoring of wastewater and other biological media. The conversion of the originally, well-defined cultivation medium into a complex mixture of accumulating and unknown metabolites of unpredictable interfering potential demands for robust and selective PAC techniques.

### 1.3.6. Applications for biotechnological process monitoring

Only few parameters such as acidity, oxygen saturation, and cell density quantified via the optical density of the medium at 600 nm might be required to run a cultivation process. Further, analytical methods of moderate selectivity can be applied whenever the signal contribution or the concentration of interfering substances is low [166]. On the other hand, for research, risk prevention, and optimization of the process efficiency, the demand on reliable and comprehensive analytical information is high.

Since most components are present in the cultivation medium in high concentrations, the sensitivity of an applied analytical method is often of secondary importance as long as no dilution is required to suppress interferences of likewise highly concentrated matrix components. In consequence, high selectivity is required and methods using specific enzymes [14,43,78,115,167], fluorescence probes [156] or gas sensors [168] or liquid chromatography [167,169-171] are often applied.

Monitoring systems based on flow techniques can be found in various fields of analytical chemistry, such as for the control of biotechnological processes [33,164,172], food quality [11,173], environmental systems [75,99,174], or pharmacokinetic studies [108,175,176], overviewed in the cited reviews. This is due to their potential for economic and reproducible accomplishment of a chemical or enzymatic reaction and provision to a detector including required sample conditioning and separation procedures. They are further characterized by fast analytical response, high automation degree, and high robustness allowing portable systems for field studies [177].

Among others, flow technique based analyzers, have been applied to biotechnological process monitoring for carbon sources e.g. ethanol [163,178,179], methanol [180], glycerol [78], glucose, lactose, maltose, and sucrose [78,161,167,178,180-182], metabolites such as acetate [165,181,182] and aldehydes [14,183], nitrogen sources such as ammonia [78,161,181] and glutamine [167], further medium components such as phosphate [165,184] and iron [78], total acidity [163] or products such as L-amino acids [167], total protein [58], and penicillin [185]. Further applications e.g. for fructose, galactose, lactate, nitrate, nitrite, urea, xanthine, and xylose are reported in reviews [33,164,167,172].

Less-selective optical methods such as near-IR spectrometry are gaining importance since they permit in-situ, non-invasive, and multi-component monitoring [149]. However, an advanced mathematical model is required and to be validated by extensive calibration work and near-IR sensitivity is moderate [153,186]. Later reviews give comprehensive overviews about techniques applied for biotechnological process monitoring [158,172].

### 1.3.7. Sampling and conditioning at bio(techno)logical processes

Various devices and interfaces for medium sampling from the monitored process and for injection into the analyzer can be found in literature and are applied in commercial systems. Direct sampling is problematic due to the risk of contamination and required inhibition of metabolic activity directly after sampling, which otherwise would leave time for sample composition changes or biofouling of the sample transport line [154,167]. Nevertheless, the successful application of a coaxial catheter for direct in-line sampling from biotechnological process and human vein was reported, where an appropriated inhibitor was added directly at the tip of the sampling probe [187].

Generally, membrane based sampling techniques are used for sampling where the membrane serves as an aseptic, sterilizable barrier. As main advantage, a particle- and cell-free, non-turbid sample or sample fraction can be obtained. Cross-flow microfiltration or ultrafiltration membrane modules located in external circulation loops [165], tubular in-situ filtration, or dialysis probes [154,157,180] are mostly found [164,188,189]. Membrane deposits leading to clogging, decrease of the provided flow, and the selective absorption of medium components are the main drawbacks of these interfaces [157]. Besides, the process condition can differ in an external circulation loops from the ones found in bulk reaction volume e.g. due to temporary oxygen starvation [154]. To prevent biofilm formation on the membrane, a sufficiently high cross-flow rate has to be ensured.

Different sampling techniques applied in biotechnological process monitoring are shown in figure 10. For the monitoring of formaldehyde and glycerol accomplished during this work, a commercially available, in-situ sterilizable, in-line filtration probe was used [154], which is shortly described in chapter 4.3.1.1.

### 1.4. Biotechnological background

#### 1.4.1. Biotechnological processes

Biotechnological processes are controlled processes utilizing living cells or their components such as enzymes in order to obtain a desired product or to change the composition of the substrates. Biotechnology as the corresponding discipline combines molecular biology, biochemistry, genetics, chemical engineering, analytical chemistry, and cell biology. Applications of biotechnology can be found in manifold industry branches such as food processing, agriculture, pharmaceutical production, mining (bioleaching), chemical production (e.g. fuels, detergents...), environmental technology such as waste treatment and land area clean up (bioremediation), and, unfortunately, military sciences.

Frequently, biotechnological processes are carried out in a "fermenter" or "bioreactor", generally a closed vessel, in which controlled and optimal conditions for microorganism growth regarding structural supports, flow rate, temperature, pH, absence of undesired microorganisms, and supply with nutrients and dissolved gases (e.g. oxygen, carbon dioxide...) can be maintained. As main operation modes of biotechnological or industrial processes, batch, fed-batch, and continuous operation can be distinguished.

#### 1.4.2. Process operation modes

A batch production means a closed process, which is started with initial parameters and substrates and stopped or led to a following phase after the finalization of the production procedure e.g. at the depletion of the substrates or at reaching the maximal product concentration (e.g. wine fermentation). The composition of the process medium is therefore changing with time. Batch operation is simple regarding the required control and applied in most industrial productions. The important disadvantage is the "down time" for harvest, reactor cleaning, and system re-configuration before re-initialization.

Continuous processes represent the opposite of batch production. They are open processes, where the substrates are fed and the products are extracted both continuously, optionally including the circulation of a fraction of the extract (e.g. waste water treatment plants). Ideally, the process parameters such as concentration, volume, or production rate are maintained constant, which require an advanced process control.

A fed-batch process includes feeding of the limiting substrate but also time-discrete harvest. In biotechnological processes, fed-batch processes allow to reach high cell densities after an initial batch phase. Here, the limited feed of mostly the carbon source allows the controlled growth of the microorganisms including the diminishment of side metabolite production or limitation of other growth factors, e.g. oxygen supply and transfer. Harvest is done either at reaching a desired product concentration or regularly during the process including a medium refresh (quasi-continuous).

### 1.4.3. *Pichia pastoris* and promoter system P<sub>AOX1</sub>

*Pichia pastoris* is a methylotrophic yeast and one of the most frequently used expression systems for the production of recombinant proteins [190,191]. It was used in all biotechnological processes being monitored with the proposed SIA analyzers. Among the beneficial properties, *P. pastoris* has a high growth rate, is able to reach very high cell densities, and can grow on a simple, inexpensive medium requiring only one carbon and one nitrogen source. Another advantage is the possibility of secretion of the expressed protein into the medium due to the fusion of the expression sequence cassette with an appropriate secretion factor, which facilitates harvest and product purification. In contrast to bacteria expression systems, yeasts such as *P. pastoris* are further able to perform post-expression modifications such as disulfide bonding and glycosylation [190,191].

An important beneficial property of *P. pastoris* is the existence of one of the most strong and tightly regulated promoters today known, the one from the alcohol oxidase 1 gene P<sub>AOX1</sub> [191-194]. Alcohol oxidase (AOX) is the first enzyme of the assimilation pathway of methanol and catalyses its oxidation to formaldehyde [195,196]. There are two genes in *P. pastoris* coding this enzyme. The AOX1 gene is responsible for more than 85 % of the enzyme in the cells, the AOX2 gene for less than 15 % [197,198]. Growing on methanol, the amount of AOX can reach 30 % of the total cell protein [191,196].

The P<sub>AOX1</sub> is mostly used for recombinant protein production in *P. pastoris* by insertion of the target protein coding DNA sequence into the AOX1 gene. Insertion of the expression cassette into both AOX genes leads to an even higher expression rate. In both cases, the target protein is expressed in the presence of methanol [198]. However, P<sub>AOX1</sub> activation is tightly repressed by most other carbon sources such as glucose, glycerol, acetate, or ethanol [198]. Consequently, there are three types of *P. pastoris* host strains available varying with regard to their ability to use methanol in function of the active AOX genes: the wild type or methanol utilization plus phenotype (Mut<sup>+</sup>) and those resulting from deletion of the AOX1 gene (methanol utilization slow (Mut<sup>S</sup>)), or both AOX genes (methanol utilization minus (Mut<sup>-</sup>)) [194]. A Mut<sup>+</sup> phenotype with target protein expression under P<sub>AOX1</sub> control results from the insertion of the expression cassette into an adjoining gene of the used shuttle vector. Mut<sup>+</sup> and Mut<sup>S</sup> phenotype strains of *P. pastoris* can be cultivated on sole methanol at reasonably high concentrations beyond the tolerant limit of most other microorganisms, enabling an economic system set-up and maintenance. In contrast, Mut<sup>-</sup> phenotype strains strictly require alternative carbon sources since they cannot metabolize methanol, which is applied only as inductor for product expression.

Alternative promoter systems from *P. pastoris* have also been reported [191,199,200]. The resulting advantages are the avoidance of storage of large quantities of the fire hazard methanol and possible application of alternative carbon sources being less problematic in food protein production. The rapid metabolism of methanol and high oxygen consumption by Mut<sup>+</sup> strains bears further the risk of oxygen limitation [198-201] and formaldehyde accumulation with possible harm of the target protein and cell prickling [194,202,203].

### 1.5. The analytes

#### 1.5.1. Formaldehyde

##### 1.5.1.1. Properties and use of formaldehyde

Formaldehyde or methanal (IUPAC) is a pungent smelling, at ambient temperature gaseous compound. It irritates eyes and the respiratory tract and has denaturing effects as it reacts with primary amino groups of proteins and nearby nitrogen atoms in proteins and DNA under irreversibly cross-linking. Therefore, it is considered as potential genotoxic but presents also an excellent disinfectant, biocide, and preservative for tissues and biological preparates [204]. In Germany, a maximum admissible concentration in air of  $0.6 \text{ mg m}^{-3}$  was established. Formaldehyde is also assigned to category IIIB of potential carcinogenic compounds. Concentrated solutions have to be signed as very toxic (T+).

Formaldehyde shows similar chemical properties as other aldehydes but is more reactive. In concentrated solution it shows tendency to polymerize and has to be stabilized with methanol. In aqueous solutions of less than 2 % it is dissolved as its hydrate methanediol [204] and shows a low vapor pressure [205]. As an electrophile, formaldehyde undergoes electrophilic aromatic substitution and electrophilic addition with alkenes as well as condensation and RedOx reactions. It is an important initial component for the production of dyes, fertilizers, plastics, and adhesives such as urea-, melamine-, and phenol-formaldehyde resins. It is used as adjuvant in textile processing, cosmetics, and for conservation purposes [206]. Nevertheless, in September 2007, the European Union has decided to ban the use of formaldehyde due to its carcinogenic property.

Formaldehyde is formed by incomplete combustion, in digestion processes, and by photochemical oxidation of hydrocarbons in the atmosphere [207]. It can be found in food products such as juices, smoked, brewery, and distillation products. In the last decades, the symptoms caused by formaldehyde degassed from particle boards or plastic products have attracted public attention known as sick house syndrome [208,209].

##### 1.5.1.2. Significance of formaldehyde in yeast cultivation

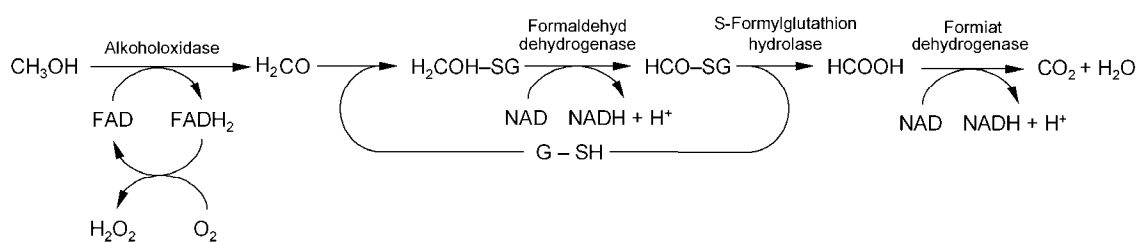
Formaldehyde is the first intermediate metabolite in the methanol utilization pathway of methylotrophic yeasts. The oxidation of methanol to formaldehyde is catalyzed by the enzyme AOX [191,195,196]. Therefore, it is formed by *P. pastoris* Mut<sup>+</sup> and Mut<sup>S</sup> phenotypes during methanol feed. In the cultivation processes of interest for this work, the promoter system P<sub>AOX1</sub> in Mut<sup>S</sup> *P. pastoris* phenotype strains was used for heterologous protein expression. In consequence, formaldehyde was formed during the production phase induced with methanol [188].

A fraction of the produced formaldehyde enters the cell biosynthetic pathway, i.e. is used for the synthesis of cellular components. The non-assimilated fraction is further

## 1 General introduction

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oxidized to carbon dioxide by an enzyme cascade shown in figure 11, which involves two dehydrogenases yielding chemical energy for cell growing [196,200].



**Figure 11: Enzymatic decomposition of methanol in methylotrophic yeast *P. pastoris*.**

**G-SH: glutathione, CH<sub>3</sub>OH: methanol, H<sub>2</sub>CO: formaldehyde, HCOOH: formic acid [195,196].**

Due to the high expression of the AOX enzyme in Mut<sup>S</sup> phenotype *P. pastoris*, formaldehyde can accumulate and rise to toxic levels in the presence of excess oxygen and elevated methanol concentration after prior oxygen limitation. A similar behavior was also considered for Mut<sup>S</sup> phenotype strains [194,202,203].

Proceeded considerations stated the interest of studying the formaldehyde concentration during methanol feed. The objective was to examine if formaldehyde monitoring presents a usable tool for the estimation of the target protein expression kinetics since methanol induction of both P<sub>AOX1</sub> and P<sub>AOX2</sub> led to their parallel formation. Information of formaldehyde can also contribute to a better understanding of the process and the expression system and provide data for mathematical models and process strategy optimization [210,211].

The knowledge about formaldehyde formation and detoxification via formaldehyde dehydrogenase in *P. pastoris*, including influencing factors and effects on the process is quite low. A rough estimation about the average concentration of formaldehyde in the cultivation medium is possible by a prior study where formaldehyde was monitored by off-line analysis in medium of yeast *Candida boidinii* culture of less than 15 mg l<sup>-1</sup> [202]. One important reason for the former fact is the lack of an adequate analyzer on the market for formaldehyde monitoring.

The objective of the first experimental work was the development and testing of an analyzer for formaldehyde study in *P. pastoris* cultures requested by the working group of the Research Center of Bioprocess Engineering and Analytical Techniques at the Hamburg University of Applied Sciences (HUAS). The observed process was carried out by the group members.

## 1 General introduction

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### 1.5.2. Glycerol

#### 1.5.2.1. Properties and use of glycerol

Glycerol or propane-1,2,3-triol (IUPAC) is a colorless, odorless, and viscous liquid with sweet taste. Glycerol has a low toxicity, and swallow of small quantities is harmless. Due to its hygroscopic character, undiluted glycerol can cause irritation of skin or intestine. Glycerol is a polyalcohol showing three hydroxyl groups. It is mixable with water and alcohol in every ratio and is applied among others as a hydrophilic solvent. It is an essential part of lipids and has originally been a by-product from saponification. Today, it is further synthesized due to the high market demand.

Glycerol is widely used in chemical industry for the production of plastics, dyes, fuels, explosives, pharmaceutical formulations, and cosmetics as precursor compound or additive like softener, protective, conservant, lubricant, humectant, stabilizer, viscosity controller, or also as sweetener in food products (E422) [204,206].

#### 1.5.2.2. Significance of glycerol in yeast cultivation

Production of heterologous proteins in high cell density cultivation of yeast *P. pastoris* is generally done according a well-established three-stage fermentation scheme as shown in figure 12A [191,194]. The first one is a batch phase on a medium generally containing glycerol as sole carbon source. After depletion of glycerol first with unlimited and later regulated oxygen supply, the second phase is carried out as fed-batch with growth limiting feed of glycerol. This stage acts as transition or priming phase for the yeast cells by consumption of accumulated by-products formed during the prior batch phase [212]. It also allows reaching a requested high cell density. The third stage is the production phase, in which the inductor, generally methanol for  $P_{AOX1}$  activation, is fed in appropriate amount in order to maintain an optimal medium concentration [189].

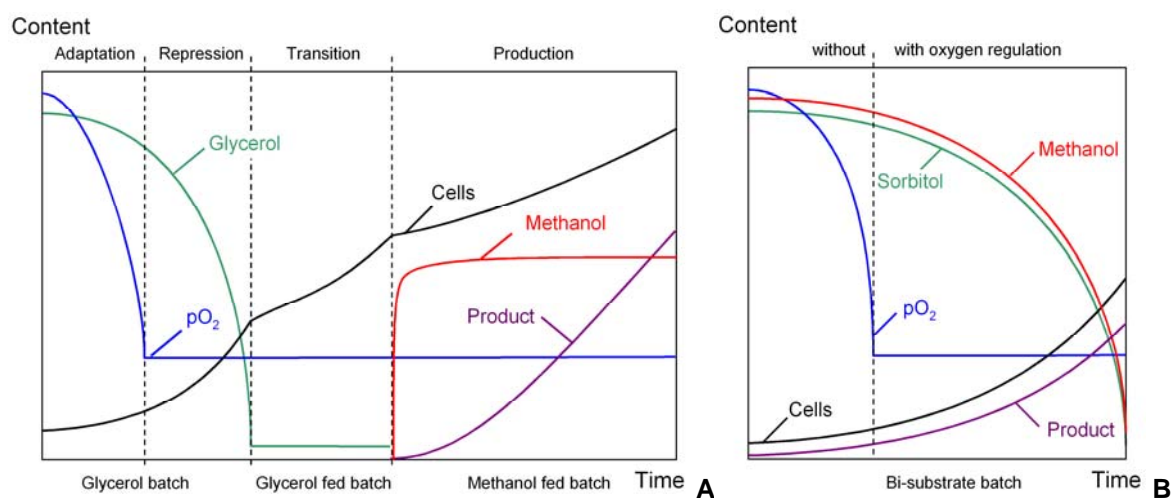
Glycerol and most other multicarbon sources repress the expression of the target protein under  $P_{AOX1}$  control tightly and even at low concentrations [170,198,213]. However, mostly used  $Mut^S$  phenotype strains of *P. pastoris* show a about third-time lower growing and assimilation rate on methanol as only carbon source and  $Mut^-$  are not able to metabolize methanol at all [214]. Nevertheless, the productivity of these strains can be improved by using a multicarbon substrate such as glycerol in addition to methanol (bi-substrate feed) [215,216]. However in this case, the feeding rate of glycerol and its medium concentration has to be controlled strictly in order to avoid the accumulation of glycerol in the medium and in consequence the repression of target protein expression.

The concentration of a single metabolizable carbon source can be controlled indirectly and uncomplicatedly by monitoring the yeast oxygen demand [188,215]. However, the discriminating quantification of two substrates can be only accomplished by PAC

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[168,180,186] and reliable mathematical models [215]. Alternatively to bi-substrate feed strategy, the advantageous intermediate feeding of glycerol during the induction phase in order to eliminate accumulated AOX enzyme in the cells was reported [217].

The objective of the second experimental work was the development of an analyzer for glycerol in Mut<sup>S</sup> *P. pastoris* cultivations requested by the group of the Research Center of Bioprocess Engineering and Analytical Techniques (HUAS) for the monitoring of its concentration in the cultivation medium with high time resolution. The observed process was carried out by the group members.



**Figure 12: Process phases and concentration courses of Mut<sup>S</sup> *P. pastoris* cultivations.**

**Scheme A presents a typical feed strategy using glycerol as multicarbon source whereas scheme B is only feasible with a non-repressive carbon source such as sorbitol. The process schemes were carried out similar by the members of the both collaboration groups where the proposed polyalcohol analyzers were tested at real cultivation processes.**

### 1.5.3. Sorbitol

#### 1.5.3.1. Properties and use of sorbitol

Sorbitol or hexane-1,2,3,4,5,6-hexaol (IUPAC) is like glycerol a polyalcohol but showing six hydroxyl groups. It has colorless, odorless, needle-shaped crystals showing optical activity, sweet taste, moderately hygroscopic, and a good solubility in water. Swallowing of large quantities can lead to diarrhea. Sorbitol is mainly produced by catalytic hydration of glucose or its condensates. It is mainly used in food production as emulsifier, humectant, and sugar substitute (E420) since it is not metabolized by caries bacteria. It is also used as synthesis precursor compound, in cosmetics, and as a cryoprotectant additive [206].



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### 1.5.3.2. Significance of sorbitol in yeast cultivation

As explained in chapter 1.5.2.2, the productivity of *P. pastoris* for heterologous protein expression can be improved by feeding another substrate in addition to methanol. Bi-substrate feed is generally reported for Mut<sup>S</sup> *P. pastoris* cultivations due to the reduced growth on methanol achieving higher cell densities and volumetric productivity [170,218]. The strategy was also successfully used for Mut<sup>+</sup> strains, where the reduction of methanol consumption and of the risk of oxygen limitation was achieved [199,213].

The P<sub>AOX1</sub> controlled expression of the target protein is tightly repressed in the presents of common conventional carbon sources such as glucose, glycerol, acetate, or ethanol [198]. Therefore, a limited and strictly controlled concentration of the second carbon source is necessary to avoid its accumulation during bi-substrate feed [194,198,213].

Among others, sorbitol has been reported as an excellent, non-repressing co-substrate on the PAOX1 system the AOX1 promoter by methanol [197,213]. Sorbitol accumulation did not affect the expression level of recombinant product during the induction phase with methanol [170] but presented lower heat and oxygen consumption rate comparing with glycerol and methanol [219]. Besides, induction can be done at any time of the process.

Bi-substrate feed with sorbitol was carried out successfully for *P. pastoris* phenotypes Mut<sup>+</sup> [219,220], Mut<sup>S</sup> [170,197,213,214,220] and Mut<sup>-</sup> [198]. Up to twofold increases of the volumetric and specific productivities were obtained using sorbitol as co-substrate compared to the sole feed of methanol [220]. Using the promoter system of formaldehyde dehydrogenase, sorbitol feed allowed even the substitution of methanol [200,221].

The study of an optimal sorbitol feeding strategy in order to maximize heterologous protein expression such as limitation [220] or accumulation of sorbitol [219] is a current task in biotechnology, but hindered by the lack of adequate PAC for sorbitol monitoring.

The objective of the third experimental work was the development of an SIA analyzer for sorbitol in *P. pastoris* cultivations requested by the group of Bioprocess Engineering and Applied Biocatalysis at the Autonomous University of Barcelona (UAB). The observed process was carried out by the group members as shown in figure 12B.

### 1.5.4. Mono-nitrophenols

Mono-nitrophenols are phenols with a nitro-group either in ortho-, meta-, or para-position to the hydroxyl group (o-NP, m-NP, or p-NP, respectively). All NP are slightly yellow, crystalline solids at ambient temperature with a characteristic, sweet smell. The nitro-group enhances the acidic character of the phenolic group. Therefore, they are soluble in most organic solvents but poorly in water in their acid form whereas in alkaline medium they are well soluble with dissociation of the hydroxyl group. They can be used as pH indicator with a change from colorless (acid form, neutral) to yellow (basic form, anion). The UV-VIS spectra of the compounds are given in the annex. The different molecule configurations lead to distinct physical properties of the three isomers, such as the dissociation constants, which were of main important for this work: o-NP:  $pK_A$  7.21, m-NP:  $pK_A$  8.39, p-NP:  $pK_A$  7.15 [206,222].

All mono-NP are toxic at inhalation, swallowing, and skin contact. They can cause methemoglobin formation, irritation of eyes, intestinal and respiratory tract, and damage of liver and nervous system [206,223,224]. They are considered to act as carcinogens and sensitizers and show mutagenic, cytotoxic, and phytotoxic effects. O-NP and p-NP are regarded as priority pollutant by the US-Environmental Protection Agency (EPA) [224].

Nitrophenols enter the environment during processing from the former named industry branches or from contaminated areas, landfills, and waste deposits. They are former emitted by pesticide degradation or combustion processes such as car exhausts [206,223,225]. Finally, they are formed as secondary pollutants by nitration of phenolic compounds in the atmosphere [226,227]. In consequence, nitrophenols can be found in air as well as in precipitation, surface, ground, or leaching waters [223,228].

Nitrophenols present ideal model substances for capillary electrophoresis. In the first, they show considerable absorption at visible and deep UV wavelength range at already moderately alkaline condition. In the second, they show negative charges at moderately alkaline conditions but clearly distinct dissociation constants. Third, they have moderate electrophoretic migration velocities. Due to these characteristics, the separation in a simple separation buffer with on-capillary photometric detection is possible. Buffer additives such as for EOF reversal (cationic detergent), for indirect photometric detection (absorbing buffer anion), or for micellar separation (anionic detergent) are not required.

# 2. OBJECTIVES

The general objectives of this work were the development of new automated analytical systems for monitoring applications on biotechnological processes and environmental water samples using the syringe pump based flow techniques, sequential injection analysis and multisyringe flow injection analysis as well as the separation technique capillary electrophoresis. This work is aimed to contribute to the development progress in process analytical chemistry used for the observation of industrial and environmental systems to improve the level of knowledge, sustainability, and to control product quality, efficiency, and legislative regulations.

The target analytes for process monitoring applications on biotechnological processes were formaldehyde, an intermediate degradation product of the carbon source methanol, and two polyalcohol carbon sources, glycerol and sorbitol. The target analytes for application in the environmental field were ortho-, meta-, and para-nitrophenol.

The systems were designed with the objectives of maximal robustness, simplicity, and stability, minimal consumption of reagents, waste generation, and time of analysis as well as the automation of required sample pre-treatment and applicability to real processes and autonomous, stand-alone operation.

Applied chemical reactions were selected under consideration of their practicability for the designated sample matrices. Optimization of physical and chemical parameters such as volumes, flow rates, instrumentation, or reagent composition had to be carried out with the intention to obtain a maximal selectivity, appropriate sensitivity for the aimed application, and a high sample throughput.

The experimental work was done also with the intent to explore the different beneficial features of the applied software in order to demonstrate its usefulness for monitoring applications and to identify possible improvements for future works.

By means of an adequate software control, the developed analyzers for biotechnological process monitoring were aimed either to operate autonomously by enabling smart decisions of actual required changes of the applied analytical procedures independently of the analyst or to allow the control of the running analytical procedure by decisions introduced by the respective user in order to offer a high flexibility of the system.

The specific milestones listed in continuation were scheduled for the realization of the formulated general objectives:

- Development of an automated system and analytical methodology based on the SIA technique for the monitoring of formaldehyde in medium of cultivation processes of

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the yeast *Pichia pastoris*. Evaluation by the application to a real process including continuous, automated medium blank evaluation, and in-line standard addition.

- Development of an automated system and analytical methodology based on the SIA technique for the monitoring of glycerol in medium of cultivation processes of the yeast *Pichia pastoris*. Enabling of smart decisions about the selection of an adequate analytical procedure including in-line sample dilution in order to obtain an amplified working range. Evaluation by the application of the system to a real cultivation process.
- Adaptation of the former system and methodology to the monitoring of sorbitol in medium of cultivation processes of the yeast *Pichia pastoris*. Evaluation by the application of the system to a real cultivation process.
- Development of new techniques and devices for sampling from a continuous sample transport line and improvement of instrumental robustness and reliability.
- Development and montage of a capillary electrophoresis system coupled to and controlled by a SIA system for capillary maintenance and injection for monitoring of compounds of high environmental interest and application to mono-nitrophenols as model analytes in waters.
- Development of a coupled system of capillary electrophoresis and MSFIA for the fully automated solid-phase pre-concentration and separation of mono-nitrophenols from water samples including capillary maintenance and injection control.

This work was incorporated into the project CTQ2004-01201, "Development of automatic flow methods for the monitoring and control of bioreactors and waste water plants" supported by the Spanish Ministry of Science and Technology. It contributed especially to the part of monitoring and control of bioreactors.

## 3. COMMON MATERIALS AND METHODS

### 3.1. Flow instrumentation

A multisyringe pump module Bu4S, a valve module VA1+1, and a rotary autosampler (Crison Instruments S.A.<sup>4</sup>, Alella, Spain) were used for all laboratory and monitoring experiments, shown in the annex with photos. The instruments were connected in series to a personal computer for remote operational control via the serial RS232C interface accomplished by the software AutoAnalysis as described in chapter 3.4.1.

The multisyringe module can be equipped with up to four syringes. The syringe tubes are fixed by direct Luer bayonet connection to 3-ways multicommutation solenoid poppet head valves (NRResearch, Caldwell, NJ, USA) integrated into the instrument front. The pistons are mounted on a common steel bar driven by a single step motor. Thus, all pistons are moved simultaneously and unidirectional for either liquid delivering (dispense) or aspirating (pickup). For each syringe (S1-S4) both operations are feasible either with the head valves activated (ON) or deactivated (OFF). Generally, the position ON is connected to the system manifold and the position OFF to a solution reservoir. This either allows syringe refilling or re-cycling of unneeded solution during dispense operations. The short time of only 35 ms required for switching allows change of the valve position even during flow operation. The valves withstand a backpressure of nominal 2 bars.

The step motor showed a speed range of 1024 – 20 s for total piston displacement corresponding to a flow rate of 0.14 to 7.5 ml min<sup>-1</sup> for a 2.5 ml syringe. The total displacement corresponded to 16.000 steps; thus, the module allowed the precise handling of microliters and minute pulsation down to low flow rates. Syringes of 0.5, 1, 2.5, 5, 10 and 25 ml are available, enabling a wide flow rate range and great combination variety. High chemical robustness is given by the use of the resistant polymers ETFE (head valves) and PTFE (piston heads, poppet flaps). Glass syringes of 2.5 ml (Hamilton Bonaduz AG, Bonaduz, Switzerland) and PE syringes of 10 ml (Sciware SL, Palma de Mallorca, Spain) were used throughout.

Four backside ports (heads 5 – 8, see annex) enable the powering of additional MCSV or other instruments either directly or via a relay allowing remote software control. This amplifies the possibilities to construct sophisticated flow networks. Each port provides 12 V with a maximal current of 0.5 A, which is sufficient for supplying up to three MCSV.

The valve module was equipped with an 8-port rotary selection valve and a 6-port injection valve, both of low pressure design and made of the chemical resistant polymers PEEK (stator) and PTFE (rotor). The dead volumes of the port flow channels are less than 7 µl and 4 µl, respectively. The time for position change of 60° was less than one second.

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<sup>4</sup> Only the company FIALab Instruments Inc. provides recently another pumping device considered likewise for MSFIA

### 3 Common materials and methods

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The autosampler was either equipped with a 45-position turntable for 15 ml tubes or a 15-position turntable for 120 ml beakers. The first one was used for interferences studies or calibrations using more than three standards for the SIA analyzers. The second one was applied for the optimization of the separation buffer required in the capillary electrophoresis systems and samples considered for analyte pre-concentration.

## 3.2. Manifold components

### 3.2.1. Tubing

The flow manifold was made of flexible tubes of chemical resistant polymers. PTFE tubes of 0.2, 0.5, 0.8, and 1.5 mm i.d. were used. For the holding coil of the SIA analyzers and wherever possible, tubes of FEP polymer were used since stacking of air bubbles was reduced and a higher transparency allowed a better visible control. Particular tube lengths are given individually at each manifold representation. Reagent supplying tubes at the lateral ports of the selection valve were 25-30 cm in length and of 0.8 mm i.d. Cellulose plugs were used to cap the liquid reservoirs and to fix entering tubes. Mixing coils were generally knitted as shown in figure 13 in order to promote the mixing process. The sharp changes of the flow direction lead to an enhanced radial dispersion at a simultaneously lower axial dispersion and in consequence to narrower and higher peaks [33,34]. Knot diameters were 7-9 mm. By this, the tubing lengths were reduced by an order of four. Junctions were homemade of PMMA without exception. Homemade tube fittings were made of polymers PVC, PPS, PVDF, or Deldrine®. For sealing, they require trumpet-shaped flanges obtained by the bead-over of the tube ends after heat-softening. Commercial fittings of PVDF or PPS with ferrules of PVDF or CTFE were used further.

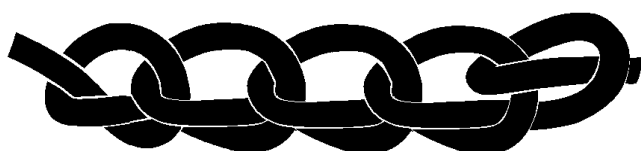


Figure 13: Tube knitting.

### 3.2.2. Heating water bath

A miniature water bath was constructed for the incubation of the reaction coil of the analyzers for formaldehyde and polyalcohols determination. It allowed minimizing the connecting tubes to the selection valve and detection flow cell. The bath was made from a plastic box of 8 cm x 8 cm side length and 6 cm in height, filled with about 300 ml of water. Heat provision was accomplished via a copper tube (20 cm, 6 mm i.d.) used as heat exchanger and connected via silicon tubes (1.5 m, 8 mm i.d.) to a commercial heating bath. Homogeneous temperature was achieved by agitation of the miniature bath water with a magnetic stir bar. A photo of the miniature bath is shown in the annex.

#### 3.2.3. Debubblers devices

Debubblers devices were used in the flow analyzers for formaldehyde and polyalcohols to get rid of gas bubbles. These were formed in the heated reaction coil due to degassing of the solution prior to detection. The first device was homemade and consisted of two pieces of PMMA. One part had a flow channel (l: 20 mm, w: 3 mm, h: 1 mm), while the counterpart had four outlet-bores for air. A folded strip of PTFE sealing tape (DIN DVGW) of about 0.1 mm strength was used as membrane, placed between both cell parts. The device was used in first experiments with the formaldehyde analyzer.

The second device was a commercial device denoted "Trace trap" from Trace Analytics (Braunschweig, Germany) with a circular flow channel (r: 25 mm, w: 3 mm, h: 1 mm) [229], equipped with a PTFE membrane of 1.5 mm thickness [230]. Engineering drawing of the first device and photos of both devices are given in the annex.

### 3.3. Detection instrumentation

UV-VIS photometric detection was carried out throughout using a miniature optical fiber spectrophotometer type USB 2000 from Ocean Optics Inc. (Dunedin, USA). It has a 2048 element linear CCD (*Coupled Charge Device*) array sensor. Light dispersion is done by a fixed grating. The spectral working range is specified by individual wavelength calibration coefficients. The USB 2000 was connected via interface USB 2.0 running on an IBM compatible PC. Configuration and data acquisition was carried out via software AutoAnalysis described in chapter 3.4.1. A photo of the USB 2000 is shown in the annex.

The light source DH-2000-DUV from Ocean Optics Inc. was used for the formaldehyde analyzer. It includes a deuterium bulb emitting a short wavelength spectrum (> 215 nm) and a tungsten halogen bulb emitting from 500 nm to 1700 nm with adjustable intensity. A flow cuvette made of special optical glass, type 75.15 SOG, with 1 cm light path, and 18  $\mu$ l inner volume (Starna, Essex, UK) was used in combination with the cuvette holder CUV-UV (Ocean Optics Inc.) for all SIA analyzers proposed in this work. The CUV-UV included focusing lenses to achieve higher intensity in the flow channel of the cuvette. It was connected via optical fibers (Ocean Optics Inc.) to the light source and the USB 2000, respectively. Fiber core diameters are given in the respective experimental chapters. For biotechnological process monitoring at the HUAS, a homemade spacer had to be placed between the light source and the optical fiber to avoid spectrometer saturation, since the source showed a considerably higher intensity than its homologue used for the optimization work at the UIB. A photo and an engineering drawing are given in the annex.

Absorbance spectra acquisition was done using the photodiode array spectrometer HP8453 (Hewlett Packard, Palo Alto, USA) and a half-micro quartz cuvette from Starna. Instrumental control and data acquisition were carried out with the software ChemStation running on an IBM compatible PC and connected via serial interface IEE 488.

### 3 Common materials and methods

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For fluorescence spectra acquisition, the luminescence photomultiplier spectrometer LD50B (Perkin Elmer, Boston, USA) and a quartz cuvette from Starna were used. Instrumental control and data treatment were carried out with the software FL WINLAB running on an IBM compatible PC and connected via serial interface type RS 232.

For the polyalcohol SIA analyzers, a blue LED<sup>5</sup> (380UBC) from Roithner Lasertechnik GmbH (Vienna, Austria) was used as an alternative light source. It emitted between 375 nm and 575 nm with a maximum at 440 nm. It was mounted directly onto the focusing lens on the illumination side of the cuvette holder by use of a homemade LED support and was powered by a stabilized constant current source (Sciware SL). Benefits were a reduced baseline noise, an adjustable illumination intensity, and system miniaturization.

Dual wavelength detection was carried out to minimize the contributions of schlieren<sup>6</sup>, gas bubbles, or turbidity to the analytical signal. Here, the signal was calculated as the difference of the absorbance at the detection wavelength  $\lambda_{\text{Det}}$  and the reference wavelength  $\lambda_{\text{Ref}}$ . NASH (1993) [265] recommended  $\lambda_{\text{Det}} = 412$  nm and  $\lambda_{\text{Ref}} = 520$  nm for the reaction product DDL. Nevertheless,  $\lambda_{\text{Det}} = 420$  nm and  $\lambda_{\text{Ref}} = 500$  nm were applied during formaldehyde monitoring since a smaller difference between both wavelengths allowed the minimization of signal contributions of the observed iron interference. This difference was further diminished for the glycerol ( $\lambda_{\text{Ref}} = 460$  nm) and sorbitol ( $\lambda_{\text{Ref}} = 470$  nm) analyzers due to the small emission spectrum of the used LED and in order to obtain similar emission intensities at both wavelengths and by this, best schlieren compensation.

A blue LED (Cu6SMA1) from Sciware SL (Palma de Mallorca, Spain) was used for the kinetic study of interferences for the formaldehyde analyzer. The LED was used as the former described one. It emitted on a wavelength interval between 380 nm and 450 nm. Detection was done at the emission maximum of 401 nm; dual-wavelength detection was not possible. The LED was further used as light source for the capillary electrophoresis systems. Likewise before, the benefits were a very low baseline noise and high illumination intensity as the LED was mounted directly on the detector above the capillary.

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<sup>5</sup> A LED (Light-Emitting Diode) is a diode, which emits light on a generally small spectrum interval with high efficiency. Advantages of LEDs are their long lifetime, economic price, small dimensions, low required power, hardly temperature increase, and highly stable, adjustable emission intensity. Utilization of LED as light source for optical detectors allows miniaturization of the analytical system, diminution of baseline noise, and in consequence a lower detection limit. [177,231,232]

<sup>6</sup> The schlieren effect means the disturbances of the baseline due to a change of the refraction index of the liquid flow. The resulting light ray deviation is registered as an apparent de- or increase of the transparency. The signal contribution of this deviation can be lowered by subtracting the registered absorbance on a reference wavelength, on which the analyte absorbance is neglectable. Since light deviation increase with shorter wavelengths, the fully compensation is not possible. The schlieren effect can also be used for quantification, if the influencing compound, i.e. the analyte, is present in a high concentration such as reported for alcohol in beverages and distillates [233].



### 3 Common materials and methods

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The current source circuitry, engineering drawings of the LED support, the LED spectra, and photos of the used detection instrumentation are shown in the annex. The LED emission intensities were adjusted with the constant current source to a working voltage about 70 % of the nominal maximum. The integration times for the USB-2000 were adjusted aiming to achieve 80 % to 90 % of intensity saturation. Acquisition at a frequency of 2 Hz and averaging over a maximum of data was generally accomplished. Further minimization of the baseline noise was achieved by integration over 8 elements of the CCD-array, which is equivalent to a slit width of 4 nm. Smoothing of the acquired data was done by the Savitsky-Golay method provided by the applied software AutoAnalysis with a polynomial order of 4 for all measurements using the flow cuvette. A polynomial order of 2 was applied for measurements on the capillary electrophoresis system due to the smaller peaks and in consequence less data points within a given time interval.

## 3.4. Instrumental control

### 3.4.1. AutoAnalysis, structure and principles

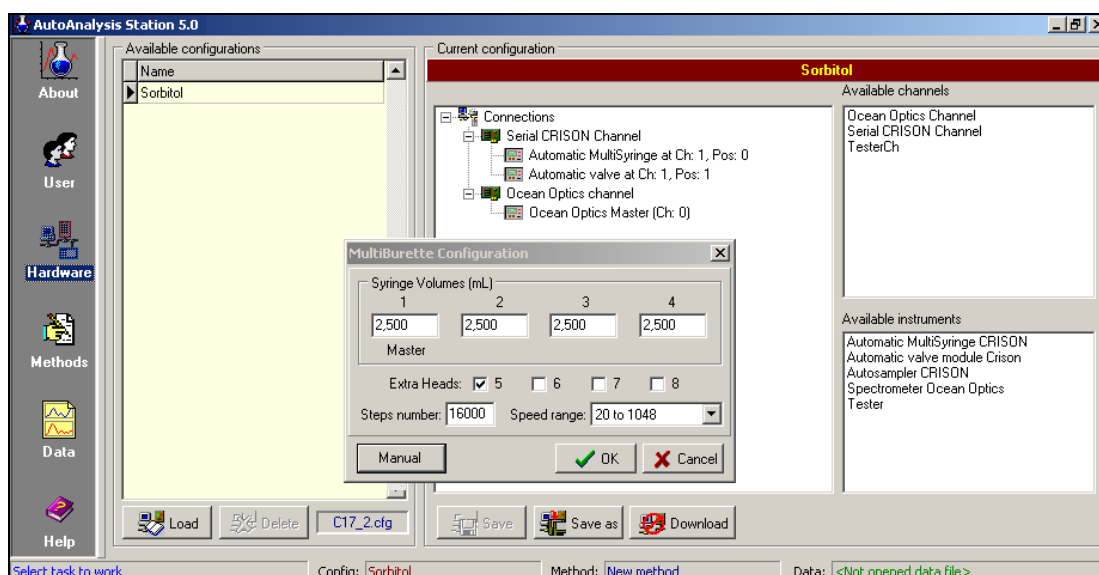
Data acquisition and processing as well as the control of the entire instrumentation were carried out with the software package AutoAnalysis 5.0. It was fundamentally described by BECERRA ET AL. (1999) [125]. It was developed in the author's working group of "Analytical Chemistry, Automation, and Environment" and is commercialized by Sciware S.L. The software is written in Delphi and Visual C++ and offers a window-based graphical surface. It runs on all 32-bit operation systems from Microsoft cooperation. For this work, the operating systems MS-Windows 2000 and MS-Windows XP were applied.

The basic program allows the creation of instruction lists following denoted as "methods". It provides a variability of programming functions for method creation including procedures, loops, indexing, basic calculations, variables, user input, waiting steps, event marking, comments, conditional inquiries, and on-line data processing. Further functions enable method test execution, definition of detection thresholds, manual data processing, such as calibration, data smoothing, spectral information processing, peak adjustment, and data export. Taking advantage of these features allows the creation and optimization of highly versatile applications [41]. Based on 32-bit technology, AutoAnalysis allows multitasking operations such as simultaneous method execution and data processing, multiple loading, and working with other programs running at the same time.

The establishment of a communication protocol or "channel" (e.g. RS232, RS485, USB...) and incorporation and configuration of the connected hardware (e.g. syringe-pump, spectrometer...) are carried out via individual dynamic link libraries (DLL). These are installed and loaded as required for each user and purpose (see figure 14) forming an individual configuration. By this, modification of the instrumentation assembly is possible with a minimum of adaptation effort. Due to the hardware independency of the basic program, the program versatility is limited only by the availability of the required DLL. Up-

### 3 Common materials and methods

to-now, DLL for seven communication channels and 30 devices are available including atomic fluorescence, fluorescence, spectrometric, and electrochemical detectors, autosampler, syringe-, peristaltic-, and solenoid-micropumps, valve modules, and I/O, A/D, or D/A PC digital cards for the connection and communication with other devices [40]. In this work, DLL for the communication channels *Ocean Optics* (USB) and *Serial Crison* (RS232), and for the instruments *Automatic Multisyringe Crison*, *Automatic Valve Module Crison*, *Autosampler Crison*, and *Spectrometer Ocean Optics* were used.



**Figure 14: Hardware configuration window of AutoAnalysis.**

The active user selects one of the available configurations (left), shown in the center window. It can be completed with the available channels or instruments (right) and configured element-wise (small window).

#### 3.4.2. AutoAnalysis, functions and programming

After loading the required configuration for the connected instruments, they can be addressed in each method and corresponding command forms became available in the editor window (see figure 15) in addition to the basic and former described functions. The analytical method is created by sequencing functions (wait, variable...) or instructions for the instruments (syringe pump, spectrometer...) by their selection from a pull-down menu or via icons and assigning with a specified command (waiting time, variable value or increment, flow rate, volume, detection wavelengths, frequency, integration time...).

The use of procedures was of high importance for this work. Procedures are prior-created instruction protocols, which can be loaded by all methods within the same hardware configuration. The main advantages are higher method clearness by apparent shorting, module-like programming, and repeated application in the same method.

Applying procedures, modification of a repeated operation has to be done only once, which lowers the risk of errors (missing of required corrections). Procedures can also be

### 3 Common materials and methods

called within another one, i.e. procedure interlock is possible. Frequently in this work, procedures were used repeatedly with at least one parameter varying during the method. This was accomplished by the definition and modification of general variables in the method, which indicate an autosampler or valve position, volumes, or waiting times, which were applied within the procedure. Therefore, one procedure could be used for the processing of multiple calibrations each carried out with another parameter set (e.g. reagent volume, incubation time, standard vial...) enabling overnight and multidimensional optimization obtaining data of sensitivity and calibration linearity for each parameter set.

Variables were changed either by active input during method execution allowing user inventions or by applying a simple calculation as done for loop counters (indexing). They were also used to store sampling process time or peak data obtained by on-line data processing optionally be used in following conditional inquiries. Conditional inquiries were an essential feature for autonomous decisions of the system about an actual requirement of change of method proceeding. They enabled the creation of smart analyzers or self-configuration of the system as described elsewhere [234] or they enabled user demanded execution of procedures as done for the sorbitol analyzer described in chapter 6.

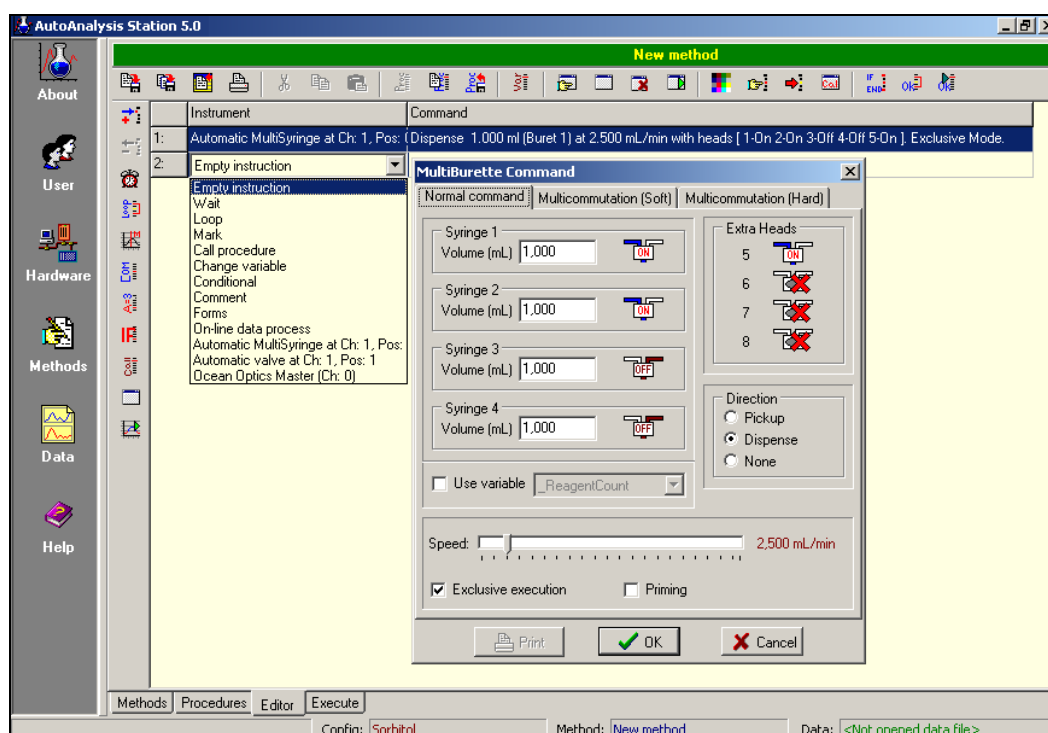


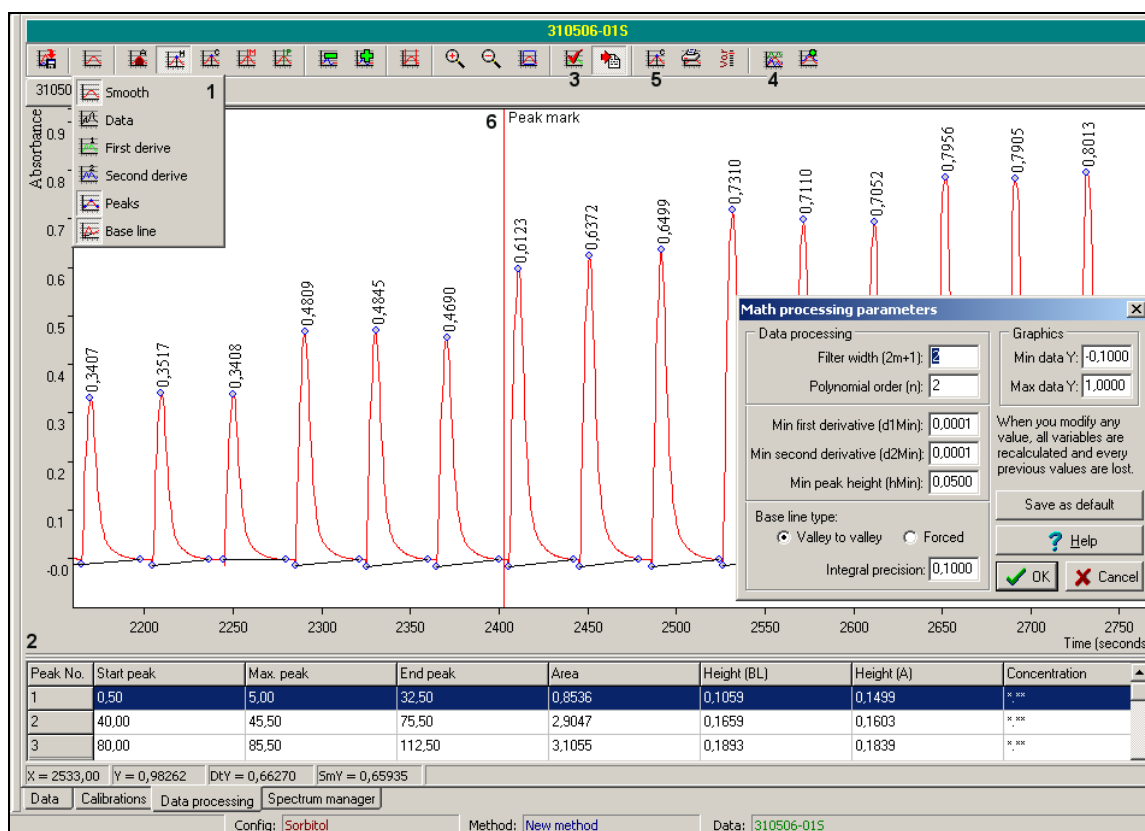
Figure 15: Editor window of AutoAnalysis.

A method is created by selecting an instrument or function from a pull-down menu (1<sup>st</sup> column) and assigning a specified command (2<sup>nd</sup> column). Here, the command window of a multisyringe pump is shown, allowing the specification of volume, direction of piston movement, flow rate, and valve positions.

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It is important to note that pump operations could be carried out either exclusively, i.e. the next instruction is not processed until the pump stops, or no-exclusively, i.e., the next instruction is processed already after initialization of the pump operation, which is then executed in background. This feature allowed simultaneous syringe refilling while passing a required waiting time. Data acquisition and processing instructions are principally no-exclusively, which enables measurement and simultaneous operations on the manifold.

The window "Execute" (figure 16) enables method initiation, pause, stop, as well as the control of the execution and continuously acquired data. Zoom, scale, and shift functions are also available. AutoAnalysis offers tools for data processing after method termination such as zoom functions, visualization of the original acquired data as well as of a smoothed function, baseline, and peak data. It allows peak height and peak area calibration, data export and saving, basic calculations, and overlay with prior acquired data. For peak maximum, start-, and end-point identification, thresholds for the numerical first and second derivative and a peak height minimum can be defined. Manual, mouse-drag peak correction, adding of non-identified peaks, or elimination of error-peaks is further possible.



**Figure 16: Data window of AutoAnalysis with shown smoothed data. Visualization of peak height data, baseline, maximum-, start- and end-points (1), and peak information (2) were activated. Peak identification is done applying given threshold values (3, small window). Later manipulation is feasible by dragging the peak characterizing blue bullets to an aimed position. The overlay function is used for data comparison (4), peak area or height calibration can be carried out (5). Marks are used for peak identification (6).**

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The following important facts were considered for the creation of an analytical method using the multisyringe pump device:

- In order to avoid degassing of unavoidable dissolved air from the applied solutions due to arising pressure decrease, the flow rate for aspiration was always reduced.
- A waiting step of 1-3 s was always integrated between flow operations and valve re-positing whenever a high precision was required such as for the aspiration of sample and in order to avoid carry-over at the movement of the selection valve rotor.
- At changing the operation direction (pickup or dispense), the thread backlash of the syringe-piston-driving shaft of a few tenth millimeters has to be overcome. To aspire or dispense a precise volume after a required change of the operation direction, first, an equal-directional operation was carried out with the respective syringe head valve in position OFF (reservoir). Not performing this step led to a considerable volumetric error and a notably lower reproducibility.

All basic methods and procedures are given in the annex including explications. Derived variants are described in the respective experimental chapters if required.

## 3.5. Reagents

All reagents used in this work were of analytical grade if not stated otherwise. Distilled or Millipore® cartridge (Billerica, USA) filtered water was used. Information about physical properties and handling of the used substances are given in the annex.

### 3.5.1. Standard and reagent preparation

#### 3.5.1.1. Formaldehyde analyzer

A 1 g l<sup>-1</sup> formaldehyde stock solution was prepared by dilution of commercial, methanol stabilized 30 % formaldehyde solution and was validated by titration. For this, 90 ml water, 10 ml of 1 mol l<sup>-1</sup> sodium sulfite solution, and few drops of a thymolphthalein solution were titrated to colorless with hydrochloric acid (verified concentration 0.1 mol l<sup>-1</sup>). After filling up of the burette, 10 ml of formaldehyde stock solution were added and the re-appeared blue color was titrated to against to colorless. The concentration  $\beta_{\text{CH}_2\text{O}}$  was calculated by formula 7 from the mean value of three titration repetitions.

$$\beta_{\text{CH}_2\text{O}} = M_{\text{CH}_2\text{O}} \cdot \frac{V_{\text{HCl}} \cdot C_{\text{HCl}}}{V_{\text{H}_2\text{CO}}} \quad (7)$$

The method is based on the quantitative reaction of formaldehyde and hydrogen sulfite ion to hydroxide ion and formaldehydesulfonate. The indicated increase of alkalinity is valuated with hydrochloric acid. As proposed in the official method [235], the volumes were properly adjusted for the present formaldehyde concentration and excess of the sulfite ion was assured. Standards were daily prepared by adequate dilution of the stock.

A 400 mmol l<sup>-1</sup> phosphate buffer stock was prepared with potassium di-hydrogen phosphate and adjusted to pH 6.0 with potassium hydroxide. A reagent stock solution of 2 mol l<sup>-1</sup> ammonium acetate (NH<sub>4</sub>Ac), a tenth part of the prior named phosphate buffer, and 0.3 v/v% of glacial acetic acid was prepared. The Hantzsch reagent was prepared daily by adding 0.2 v/v% of acetylacetone (AcAc) corresponding to 19.4 mmol l<sup>-1</sup> to the reagent stock solution. The reagent changed to a slight yellow within the first 35 min after preparation. For optimization, reagents of higher AcAc content were further prepared.

#### 3.5.1.2. Polyalcohol analyzers

Stock solutions and standards for sorbitol and glycerol of 100 g l<sup>-1</sup> or 200 g l<sup>-1</sup> were prepared by accurate weighting and appropriate dilution. Standards were daily prepared by adequate dilution with water or artificial medium. For long-term experiments, 0.5 v/v% of chloroform was added to the standards in order to prevent microorganism activity. Additionally to the former described Hantzsch reagent (R1), a 100 mmol l<sup>-1</sup> sodium meta-periodate solution (R2) was used for the Malaprade reaction (see chapter 5 and 6).

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#### 3.5.1.3. Nitrophenol CE analyzers

Stock solutions of o-NP, m-NP, and p-NP were prepared by accurate weighting and dilution in 1 mmol l<sup>-1</sup> NaOH. The stock solutions were stored unfiltered in the dark. Working solutions were prepared by serial dilution with Millipore purified or distilled water. Methanol (1 v/v%) was added as an EOF marker.

Water used for dilution and all stock solutions was filtered through 0.45 µm nylon membrane filters. Filtered sodium hydroxide solution 1 mol l<sup>-1</sup> was used for pH adjustment. Separation buffers of different compositions were prepared from a 1 mol l<sup>-1</sup> borate stock solution by dilution, appropriate addition of methanol as organic modifier, and by adjustment with sodium hydroxide solution to the requested apparent pH value as described in chapter 7.3.3. Sodium hydroxide 10 mmol l<sup>-1</sup> and hydrochloric acid 0.1 mol l<sup>-1</sup> were used for cleaning and conditioning of the separation capillary. Different solutions of sodium hydroxide and methanol or acetonitrile were used for the conditioning of the used solid phase microcolumn and for analyte elution as described in detail in chapter 8.3.4.

#### 3.5.2. Other solutions

##### 3.5.2.1. Indicators

Indicator solutions were used for validation, flow observance in the manifolds, and determination of dispersion factors. Preparations were done according WEST (1983) [236]. A solution of thymolphthalein (5',5''-Diisopropyl-2',2''-dimethylphenolphthalein) was prepared by dissolving 40 mg of the reagent in 100 ml of 50 v/v% ethanol. The solution indicates pH change from pH 9.3 to 10.5 (colorless to blue).

A solution of bromothymol blue (3,3'-Dibromthymolsulfonphthalein) was prepared by dissolving 100 mg of reagent in 16 ml of 10 mmol l<sup>-1</sup> sodium hydroxide and filling up to final volume of 250 ml. A 1:10 dilution of bromothymol blue solution was used for the determination of sample and reagent dispersion. Here, a 40 mmol l<sup>-1</sup> sodium borate carrier, pH 9 was used and detection was carried out at  $\lambda_{\text{Det}} = 620 \text{ nm}$  and  $\lambda_{\text{Ref}} = 800 \text{ nm}$ . For the same purpose, an aqueous solution of 40 µmol l<sup>-1</sup> fluorescein was used for tracing. Detection was carried out at  $\lambda_{\text{Det}} = 450 \text{ nm}$  and  $\lambda_{\text{Ref}} = 520 \text{ nm}$ .

##### 3.5.2.2. Cultivation medium and solutions for interference studies

For the development of the SIA analyzers for formaldehyde and polyalcohols, solutions of the different cultivation medium components, metabolites, and the basal salt medium itself were tested to their possible interference of analysis in concentrations equal or higher than present in the medium. Besides, solutions of different aldehydes, sugars, polyalcohols, and organic acids were tested.

### 3 Common materials and methods

The compositions of the synthetic basal salt batch media as used for the cultivations of *Pichia pastoris* carried out by the members of the collaboration investigation groups at the HUAS and the UAB are given in table 1. The so-called trace elements were added as an aliquot of a prior prepared stock solution as noted. Biotin (approx. 0.3 g l<sup>-1</sup> final medium concentration) and the antifoam agent were the only medium components, which have not been tested to its possible interference. The media were used for interference study either with or without added carbon source, being glycerol, methanol, or sorbitol, respectively.

**Table 1: Composition of basal salt batch media used for *P. pastoris* cultivations.**

Medium preparation		at HUAS (Hamburg)		at UAB (Barcelona)	
Carbon source	M [g mol <sup>-1</sup> ]	m [g] for 1l	c [mmol l <sup>-1</sup> ]	m [g] for 1 l	c [mmol l <sup>-1</sup> ]
Glycerol	92.09	30 / 40	280.5 / 374	8.0	43.9
Sorbitol	182.17			10.0	250
Methanol	32.04	2 / 5	62 / 156		
Basal salts	M [g mol <sup>-1</sup> ]	m [g] for 1l	c [mmol l <sup>-1</sup> ]	m [g] for 1 l	c [mmol l <sup>-1</sup> ]
H <sub>3</sub> PO <sub>4</sub> (85%)	98.00	44.5	385.6	45.7	396.0
K <sub>2</sub> SO <sub>4</sub>	174.27	18.0	103.3	18.3	105.0
KOH	56.11	4.0	71.3	4.1	73.6
CaSO <sub>4</sub> * 2 H <sub>2</sub> O	172.17	0.9	5.2	1.2	6.8
MgSO <sub>4</sub> * 7 H <sub>2</sub> O	246.48	14.0	56.8	7.3	29.5
Trace element solution added		80 ml		5 ml	
Trace element solution	M [g mol <sup>-1</sup> ]	m [g] for 1l	c <sub>Final</sub> [mmol l <sup>-1</sup> ]	m [g] for 1 l	c <sub>Final</sub> [mmol l <sup>-1</sup> ]
CoNO <sub>3</sub> * 6 H <sub>2</sub> O	291.04	0.044	0.012	1.39	0.024
ZnSO <sub>4</sub> * 7 H <sub>2</sub> O	136.29	2.006	1.177	20.00	0.734
FeSO <sub>4</sub> * 7 H <sub>2</sub> O	278.02	6.502	1.871	65.00	1.169
CuSO <sub>4</sub> * 5 H <sub>2</sub> O	249.69	0.603	0.193	6.00	0.120
MnSO <sub>4</sub> * H <sub>2</sub> O	169.02	0.300	0.142	3.00	0.089
KI	166.01	0.012	0.006	0.09	0.003
H <sub>2</sub> SO <sub>4</sub>	98.08	0.920	0.750	9.20	0.469
H <sub>3</sub> BO <sub>4</sub>	61.83	0.002	0.003	0.02	0.002
Na <sub>2</sub> MoO <sub>4</sub> * 2 H <sub>2</sub> O	241.95	0.021	0.007	0.20	0.004



## 3.6. Data evaluation and calculations

### 3.6.1. General statistics

All measurements on the SIA analyzers were performed at least threefold. The only exceptions were ten-point calibrations made for the characterization of the formaldehyde analyzer with double measurement of each standard. Most measurements on the coupled systems of SIA or MSFIA with CE were performed fourfold. The arithmetic mean  $\bar{x}$ , the standard deviation SD, and the relative standard deviation RSD were used for the description of data distribution (formula 8-10). Results and the square regression coefficient  $r^2$  of calibration functions were mostly round to three significant digits.

$$\bar{x} = \frac{1}{n} \cdot \sum_{i=1}^n x_i \quad (8)$$

$$SD = \sqrt{\frac{\sum_{i=1}^n (\bar{x} - x_i)^2}{n-1}} \quad (9)$$

$$RSD = \frac{SD}{\bar{x}} \cdot 100\% \quad (10)$$

Values differing more than the threefold standard deviation from the mean of the entire data set were considered as outliers. To prove the significance of difference between two results 1 and 2 characterized by mean and standard deviation of  $n_1$  and  $n_2$  values, respectively, the Students t-test according formula 11 with a level of 95 % was applied.

$$t = \frac{\bar{x}_1 - \bar{x}_2}{\sqrt{(n_1 - 1) \cdot s.d._1^2 + (n_2 - 1) \cdot s.d._2^2}} \cdot \sqrt{\frac{n_1 \cdot n_2 \cdot (n_1 + n_2 - 2)}{n_1 + n_2}} \quad (11)$$

### 3.6.2. Analysis specific statistics

Photometric detection was carried out throughout in the experimental works. The analytical signal was therefore the sample absorbance A defined as the logarithm of the quotient of the initial light intensity  $I_0$  of the radiation source at a given wavelength and the intensity I attenuated due to the interaction of photons with sample components, ideally only with the analyte of interest. The absorbance is then equivalent to the product of the molar concentration c of the analyte, the molar absorptivity  $\epsilon$  of the analyte at the given wavelength, and the light path length l as expressed by formula 12 [237]. Where sample matrix and applied chromogenic reagents contributed to the analytical signal, this effect was compensated by the subtraction of the respective blank absorbance values.

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$$A = \log\left(\frac{I_0}{I}\right) = c \cdot \varepsilon \cdot l \quad (12)$$

Slope ( $a_1$ ), y-axis intercept ( $a_0$ ), and linearity of obtained linear calibration regression functions were the generally applied optimization criteria. Here, the slope represents the method sensitivity or signal gain per addition of the analyte while the y-axis intercept represents the blank signal. Their ratio was used to characterize the gain of sensitivity in comparison to the lower limit of the usable analytical signal scale.

For the evaluation of the linearity of the analytical response of the analyzer system for glycerol, the absolute residue of the calculated values for the center standard of the calibration range for both linear and quadratic regression was used. A value of zero indicated therefore an ideal linear fit whereas a higher value corresponded to a curvature.

The limit of detection (LOD) and the limit of quantification (LOQ) for the single SIA systems were calculated as the concentration yielding a signal of the mean blank ( $n=10$ ) plus the threefold or tenfold resulting standard deviation, respectively, expressed by formula 13 and 14. The LOD applying capillary electrophoresis were calculated as the concentration yielding a signal of threefold the maximal amplitude of the baseline noise in the time interval of one minute.

$$\text{LOD} = \frac{((\bar{x}_{\text{Bl}} + 3 \cdot \text{SD}_{\text{Bl}}) - a_0)}{a_1} \quad (13)$$

$$\text{LOQ} = \frac{((\bar{x}_{\text{Bl}} + 10 \cdot \text{SD}_{\text{Bl}}) - a_0)}{a_1} \quad (14)$$

The analyte recovery  $R$  was calculated as the difference of the analyte concentration  $\beta_S$  of the sample and of the sample spiked with a known amount of analyte divided by the spiked analyte concentration  $\beta_{S+\text{Add}}$  as expressed by the first term of formula 15. During at-line monitoring of formaldehyde in biotechnological processes, automated standard addition and alternating photometric measurements of sample and sample plus analyte addition were accomplished. As a matter of fact, the analyte concentration in the cultivation medium changes during the delay time of analysis  $t_D$ . So the concentration of the unspiked sample at the process time  $t_p$ , at which the spiked sample is measured, was approximated by the mean of the unspiked sample concentrations measured prior ( $t_p - t_D$ ) and afterwards ( $t_p + t_D$ ), leading to the second term of formula 15.

$$R_{t_p} = \frac{\beta_{S+\text{Add},t_p} - \beta_{S,t_p}}{\beta_{\text{Add},t_p}} \approx \frac{\beta_{S+\text{Add},t_p} - \frac{(\beta_{S,t_p-t_D} + \beta_{S,t_p+t_D})}{2}}{\beta_{\text{Add},t_p}} \quad (15)$$

Likewise, the blank value of the cultivation medium was monitored during formaldehyde monitoring, since it contributed notably to the analytical signal and increased with time. The reagent blank also contributed to the analytical signal but was proven to be constant. Therefore, the effective blank value was calculated as the sum of the medium blank, i.e.

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measurement of medium substituting the reagent by water during monitoring and the reagent blank, i.e. measurement of water with reagent during calibration. The medium blank was interpolated from the medium blank values measured before and after sample measurement according formula 16.

$$A_{Bl, t=tp} = A_{Bl, R} + \frac{A_{Bl, M, t-t_D} + A_{Bl, M, t+t_D}}{2} \quad (16)$$

To describe the quality of the electrophoretic separation, the resolution  $R_S$  of the peaks of two analyte 1 and 2 was determined as the quotient of the difference of the retention times  $t_{R2} - t_{R1}$  and the mean base-line peak width  $\bar{w}_B$  given by formula 17. Fully baseline resolution corresponds to a value of 1.5.

$$R_S = (t_{R2} - t_{R1}) \cdot \frac{2}{w_{B1} + w_{B2}} \quad (17)$$

The number of theoretical plates  $N$  was determined as done in liquid chromatography as the square quotient of the peak retention time and baseline peak width multiplied with 16 given by formula 18.

$$N = 16 \cdot \left( \frac{t_R}{w_B} \right)^2 \quad (18)$$

The backpressure resulting from a given flow rate was calculated using an Excel datasheet shown in the annex applying the Poiseuille equation given in formula 19.

$$p = \frac{8 \cdot \eta \cdot l \cdot \dot{V}}{r^4} = \frac{8 \cdot \eta \cdot l \cdot V}{r^4 \cdot t} \quad (19)$$

#### 3.6.3. Error discussion

The error of an analytical procedure is generally given only for the measurement but does not include errors from sampling or sample pre-treatment. In this section some of the commonly observed errors on the proposed flow systems are shortly discussed.

Systematic or stochastic errors can arise from calibration of the analytical method. Systematic errors by pipetting, weighting, or pH measurement were minimized by adequate calibration protocols of the corresponding instruments (calibration of the used micropipettes and balance, use of standard buffers, correction of temperature...).

The analytical procedures and instruments proposed in this work were tested according their analytical performance by testing of the method long-term stability, determination of the analyte recovery in aqueous standard solutions and basal salt medium by spiking, and by testing of possible interferences. Monitoring data obtained by the SIA analyzers for glycerol and sorbitol were compared with well-established reference HPLC methods. Since no reference method was available for formaldehyde, in-line standard addition was carried out and the analyte recovery was calculated afterwards.

Problems of standard and real sample solution stability affected by light, oxygen, and, in the case of polyalcohols, by microorganism activity, were solved by adequate storage, daily preparation of the solutions, and additives as described in chapter 3.5 and 6.5.1.2. Air bubbles arising from degassing of solutions due to negative pressure during aspiration, heating, or introduced by direct aspiration caused considerable volumetric errors, deviations of sample and reagent dispersion and consequently mixing and false peak signals. Thus, flow protocols for system's cleaning were carried out during monitoring. A method for elimination of stacked air bubbles was further proposed in chapter 6.

In order to avoid biofouling of the manifold lines, regular cleaning of the analyzers with HCl and NaOH solutions was done during formaldehyde monitoring. Re-calibration, use of batch medium for standard preparation, in-line standard addition, and measurement of control standards were carried out to compensate errors due to variations of ambient temperature and changing medium blank and viscosity.

During biotechnological process monitoring at the HUAS, sampling was done from a continuous sample transport line. One error source of measurement was given by the time delay from the sampling line, varying over the process time in order to maintain the culture volume constant. Further error sources were analytical instruments connected upstream of the proposed SIA systems to the sample transport line via six-port injection valves. As described in chapter 4.2.3, at each injection event of these instruments, the respective injection loop volume of the applied carrier was injected into the sample transport line leading to intermediate dilution. In consequence, lower peaks of lower height were obtained with the same frequency as the injection events on these instruments. These error peaks had to be eliminated during manual data evaluation afterwards.

## 4. EXPERIMENTAL WORK 1:

### At-line determination of formaldehyde in bioprocesses by sequential injection analysis

Published equally entitled: HORSTKOTTE B., WERNER E., WIEDEMEIER S., ELSHOLZ O., CERDÀ V., LUTTMANN R. (2006) *Analytica Chimica Acta* 559, 248-256 [238].

#### 4.1. Task description for experimental work 1

The objective of this work was the development of an automated analyzer for the quantification of formaldehyde in the medium of a cultivation process of *Pichia pastoris*. After the optimization of physical and chemical parameters, the system was aimed to be applied to a real cultivation process and to be characterized. The method was further aimed to carry out automated in-line standard addition in order to evaluate the analyte recovery since an established reference method was not available for the actual matrix.

The analyzer had to work reliable during the monitoring time of the process, which was estimated to reach 300 hours. The applied methodology had further to be highly selective. A working range of up to 20 mg l<sup>-1</sup> was aimed based on experimental results from a former study [202]. SIA technique was chosen for the automation of the analytical methodology as a robust, simple, and easily adaptable technique. Instrumental aspects such as the coupling of the analyzer to the sample transport line were part of this work.

#### 4.2. Introduction

##### 4.2.1. Spectrophotometric detection of formaldehyde

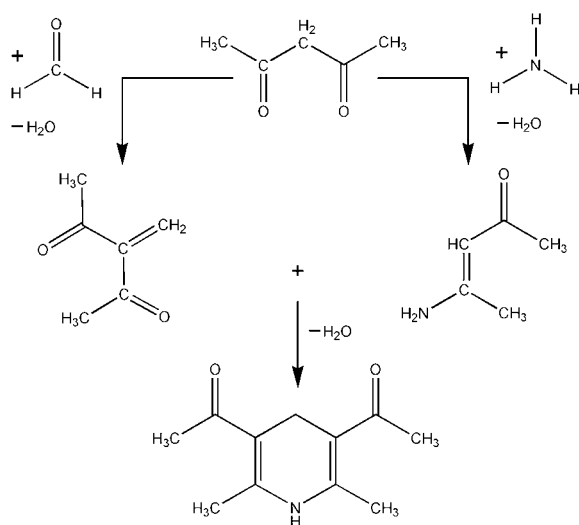
As prior stated in chapter 1.5.1, formaldehyde is a highly reactive compound. Therefore, carrying out a selective chromogenic reaction followed by spectrophotometric detection is feasible, easily practicable, and the most frequently applied way to quantify formaldehyde. An extensive overview of detection techniques for formaldehyde is given in reviews from PICKARD AND CLARK (1984) [239] and LI ET AL. (2005) [240]. The selectivity of the applied reactions is based on the considerably higher reactivity of formaldehyde compared to its homologues [204]. In contrast to the present task, the highest attention is generally directed to the maximization of the detection sensitivity whereas an already moderate selectivity is typically considered to be sufficient. This is, because most applications deal with the quantification of formaldehyde traces in air, i.e. are confronted with relatively low interferences from the matrix [207,232].

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An alternative to chromogenic reagents is the use of aldehyde dehydrogenase [14,241]. However, the limited enzyme stability [116,242] and the required coenzyme  $\text{NAD}^+$  as a cost and stability-affecting factor [243] make this method unattractive.

The overall chromogenic reactions are based either on the reduction potential of formaldehyde [244,245], the competitive reaction with sulfite used for the bleaching of a triarylmethane dye [246,247], the enlargement of the conjugated electron system of a molecule by Schiff-base formation with amines [248-251], with hydrazine-derivates [251-258], or with triarylmethane dyes [247,259-263], or the condensation with naphthylsulfonic acids giving xanthilium dyes [239,264] or with  $\beta$ -diketones and ammonia giving dihydropyridine derivates. The last reaction scheme is known as Hantzsch reaction [265]. Most of the former reactions show an insufficient selectivity for the present task since either medium components or metabolites interfere at their typical concentration level of the cultivation medium. Various reactions were rejected as they use instable reagents such as sulfite or highly toxic or carcinogenic reagents. Likewise, reactions requiring denaturing agents, extreme heating, concentrated acids, or alkaline reaction conditions were rejected as incompatible with the cultivation medium as stated in chapter 1.3.5 [177].

As the most promising method, the Hantzsch reaction was chosen. It is a multi-stage condensation reaction in the first of an amine and an aldehyde with each one molecule of a  $\beta$ -diketone. In the second, the intermediates react to give a dihydropyridine derivate [206,265]. The first application for the quantification of formaldehyde using acetylacetone (2,4-pentanedione) in an  $\text{NH}_4\text{Ac}$  buffer was described by NASH (1953) [265]. The intensive yellow cyclization product 2,4-diacetyl-1,4-dihydrolutidine (DDL) formed according the reaction scheme in figure 17 is quantified photometrically. Outstanding advantages of this reaction are that it uses only one simple but highly selective reagent of low toxicity and takes place at mild reaction conditions. Another advantage is given by the fact that the optimal pH 6 for the reaction is quite similar to the one of the cultivation medium of pH 5.



**Figure 17: Hantzsch condensation with AcAc, formaldehyde, and ammonia. Two intermediates resulting from the reaction of formaldehyde and ammonia with each one molecule of AcAc form the final product (DDL) 2,4-diacetyl-1,4-dihydrolutidine [206].**

Due to the enrichment of metabolites during the process time, the originally colorless cultivation medium changes to a yellow color with increasing absorbance in the short

#### 4 SIA analyzer for formaldehyde in *P. pastoris* cultivation process

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visible wavelength spectrum. The short absorption maximum wavelength of DDL of 412 nm presents therefore a considerable disadvantage since the medium blank value has to be monitored additionally in order to compensate its contribution to the analytical signal. An UV-VIS spectrum of the Hantzsch reaction product DDL is given in the annex.

The reaction kinetic is further slow but can be enhanced considerably by heating [266,267]. The sensitivity given by the molar absorptivity of DDL of  $8000 \text{ l mol}^{-1} \text{ cm}^{-1}$  is moderate compared to other chromogenic methods but sufficient for the estimated formaldehyde concentration level in cultivation medium in the order of  $\text{mg l}^{-1}$  [202]. Since DDL shows an intense fluorescence emission with a maximum at 510 nm, enhancement of both sensitivity and selectivity was former achieved using fluorescence detection on HPLC [268] and FIA [232,266]. A fourfold reduction of the LOD was further achieved by micelle generation with the detergent Triton X-100 using FIA [269]. Another approach for sensitivity enhancement was done by on-detector solid phase concentration of DDL [270].

Several method modifications have been reported using substitutes for acetylacetone (AcAc) reviewed lately [240]. TSUCHIYA ET AL. (1994) used 4-amino-3-penten-2-one (Fluoral P) instead of AcAc for the fluorescence determination of formaldehyde via DDL in beverages with FIA [271]. A phosphate carrier of pH 2 was used to obtain a fast reaction; however, the reagent had to be dissolved in acetonitrile since it hydrolyzes in water into AcAc and ammonia. A high reactivity of AcAc and no considerable advantages of Fluoral P over AcAc were found in a later work [267]. BURINI AND COLI (2004) achieved lowering the blank value while simultaneously increasing the selectivity for formaldehyde by using ethyl-3-oxobutanoate and an ammonium citrate buffer with photometric detection at 365 nm after chromatographic separation [272]. Highly sensitive fluorimetric quantification by was achieved by the use of the AcAc substitutes 1-3-cyclohexandione [243] and 5,5-dimethylcyclohexane-1,3-dione [273] with detection limits of a few nanomol per liter. Due to the slower reaction kinetics compared to AcAc, extreme reaction temperatures of 95°C and 130°C, respectively, were applied, for which they could not be used for the present task. Slight interference of  $\text{H}_2\text{O}_2$  on Hantzsch reaction is discussed elsewhere [207,240].

Fluorescence detection offer the possibility of an amplified linear working range and higher sensitivity compared to photometric detection. However, the lower robustness in respect of bubbles, particles, possibility of quenching, and the fluorescence of the observed cultivation medium itself probably due to the enrichment of metabolites during the process time ruin most of the possible benefits of this technique. Therefore, photometry was finally applied as the more simple and robust technique.

#### 4.2.2. Standard addition in flow systems

One objective of this work was the integration of in-line standard addition into the automated method for the estimation of the analyte recovery. The principle consists in the addition (spiking) of an exactly known analyte quantity to the sample. Ideally, the sample

#### 4 SIA analyzer for formaldehyde in *P. pastoris* cultivation process

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matrix does not affect the analytical performance, e.g. the detection sensitivity or the efficiency of required sample pre-treatment procedures. In this case, the analyte recovery is 100 %, i.e. the signal increase gained by the addition is equal to the one calculated from an obtained calibration using standard solutions. A distinct recovery value than 100 % does not enable to draw a conclusion about the cause, e.g. the binding of the added analyte molecules to sample matrix components might be a function of time and the determined recovery values might not be representative. Thus, recovery constancy and reliability might be of higher importance than obtaining a value near to 100 % [131,274].

Carrying out standard addition in flow systems is basically a mixing problem with the requirement, that the sample dispersion and volume are not altered by the addition of the standard solution. The common principle in FIA is the confluent mixing of the sample with either carrier or standard [275,276]. Though the sample is diluted by this procedure, the dispersion is not affected since ideally, the sample disperses equally in both solutions and the sample concentration is "overlaid" by the analyte concentration of the standard. Another possibility is the injection of the standard and the sample into a circulation loop where homogeneous mixing of both solutions is achieved similar as in AIA [66,277].

For the single channel technique SIA, neither confluent mixing nor flow circulation is practicable. Applying a mono-air-segmented flow scheme, the homogeneous mixing of standard and sample can be achieved due to the turbulent condition in the liquid segment [73,278]. Another possibility is the accomplishment of binary sampling, i.e. the repeated and alternated aspiration of small volumes of sample and standard. On flow reversal and propelling the stacked solutions towards the detector, these are homogeneously mixed. An advantage is the avoidance of air bubbles in the manifold; however, the liquid slugs have to be sufficient small since otherwise homogenous mixing cannot be achieved [174].

Standard addition was done in this work by binary sampling since this procedure also enabled a fully mixture of sample with the applied high-salty reagent. By this, the signal distortion due to the schlieren effect could be simultaneously and considerably reduced.

#### 4.2.3. Interfacing of sample transport line and analyzers

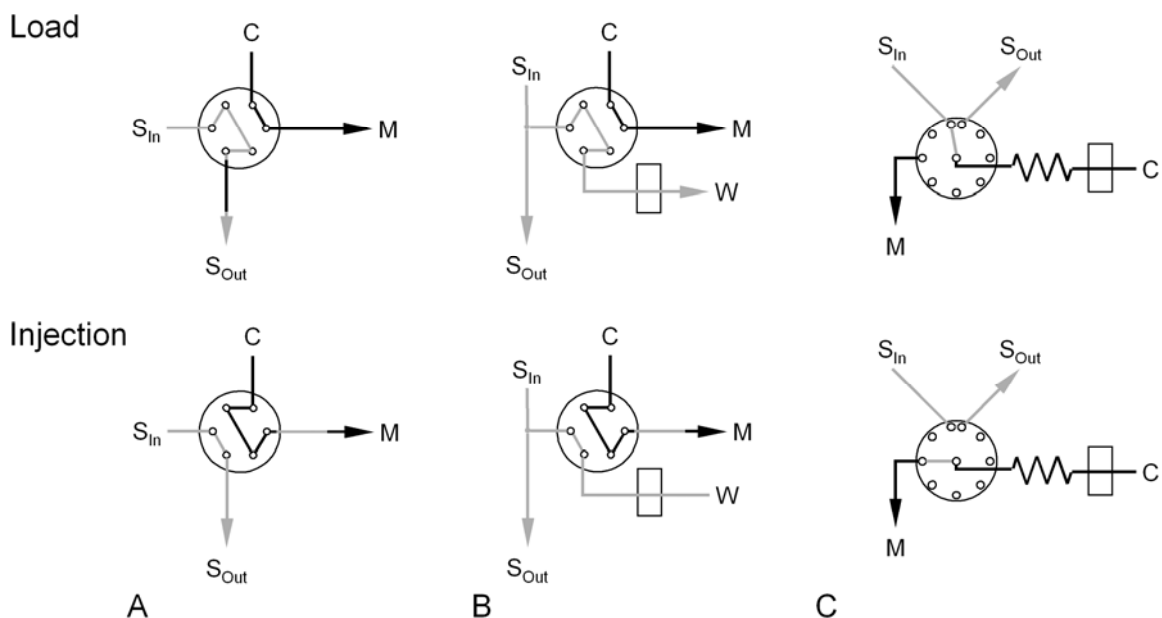
In the present work, interfacing of the sample transport line and the proposed analyzer had to be carried out with the objective of obtaining a reproducible sample volume without adulteration of the composition of the process medium flow and consequently without affecting the analytical results obtained by other, downstream connected analyzers.

Membrane modules as described in chapter 1.3.7 are frequently applied in PAC both for sampling by simultaneous elimination of same sample matrix components. In this case, the volume of the flow channel on the acceptor side acts as the injection volume. By variation of the sample volume passing the flow channel on the membrane donator side, enrichment or dilution of the analyte on the acceptor side can be achieved, which permits the advantageous amplification of the dynamic working range of the analyzer [71].



#### 4 SIA analyzer for formaldehyde in *P. pastoris* cultivation process

To avoid membrane clogging and biofouling, a pervaporation unit was proposed as an alternative interface between the sample transport line and the carrier of the respective analyzer system. Here, the membrane is not in direct contact with the sample and membrane biofouling becomes improbable [14,15]. However, pervaporation is reasonably applicable only for volatile substances being present in the sample in high concentrations.



**Figure 18: Connection of a continuous process medium flow to an analyzer.**

**A: Via injection valve with downstream adulterating of the sample flow by carrier injection;**  
**B: Via injection valve using an additional pump for injection loop filling;** **C: Via a flow-through-port [77,158]. C: carrier, M: manifold, S<sub>IN</sub>: sample inlet, S<sub>OUT</sub>: sample outlet.**

Three different possibilities of direct sampling from a continuous process medium flow are presented in figure 18. Six-port injection valves are generally used where the sample is provided to the analyzer by a continuous diverted process medium flow. Initially, the injection loop is flushed by the process medium in position "Load". Changing the position of the valve to position "Inject", the volume of the process medium in the injection loop is inserted into the carrier of the analyzer. Afterwards, the injection valve is returned to position "Load" and an equal volume of carrier is inserted into the process medium flow adulterating its composition intermediately. In consequence, the reliability of the analytical information obtained by downstream-assembled instruments is affected. This problem can be circumvented by the aspiration of sample from the continuous process medium flow through the injection valve loop whenever required by the use of an additional pump. By leading the process medium flow through the corresponding valve port, the dead volume, which has to be rinsed, is minimized. This concept denoted "flow-through port" was successfully applied in LOV by RUZICKA (2000) [77] and SIA by VAN STADEN (2002) [157] on selection valves. During this work an adaptor was developed described in chapter 4.3.2.2, whose implementation transforms a normal valve port into a flow-through port with the same function and without the requirement of a permanent modification of the valve.

### 4.3. Materials and methods

#### 4.3.1. Cultivation process

The proposed formaldehyde analyzer was tested by at-line monitoring at a cultivation process performed by the members of the Research Center of Bioprocess Engineering and Analytical Techniques (HUAS). For a detailed description of the process, the reader is therefore referred to specific references [188,218].

The *P. pastoris* strain SMD 1168 (pPIC9K+alphaF-(1-3del)I-TAC) of phenotype Mut<sup>S</sup> was used, purchased from the Institut für Pharmakologie und Toxikologie of the Pharmazentrum Frankfurt, Germany. The strain designation encodes the genetic modification of the yeast: use of the vector pPIC9K, enclosure of the alpha factor for control of product secretion, and the target 1-3del I-TAC (interferon inducible T-cell alpha chemoattractant).

The high cell density processes was started with a batch phase with unlimited growth on carbon source glycerol with an initial concentration of 40 g l<sup>-1</sup>. The composition of the batch medium is given in chapter 3.5.2.2. At reaching of glycerol limitation, the process was continued automatically as fed-batch with growth-limited feed of glycerol medium. At reaching a prior-specified cell density, product expression was initiated by feed change to pure methanol as inducer. The methanol concentration was maintained at a constant level during the production phase exceeding carbon source limitation of the yeast growth. Air was used for gasification. The oxygen content was regulated to 20 % saturation by increase of, in order, gasification rate, agitation, and the oxygen content of the feed gas.

The process was accomplished in a Biostat® fermenter ED5 from B. Braun Biotech International GmbH (Melsungen, Germany). The main component is an in-situ sterilizable, stainless steel vessel of a total volume of 7.1 l. Medium tempering is done by water circulation in the vessel jacket. Three blade impellers and four baffles allow homogeneous mixing of the medium. Aeration is done via a circular sparger. Probes for pH, pressure, temperature, oxygen, foam, and cell density as well as an ESIP filtration probe (chapter 4.3.1.1) for provision of sample to the proposed analyzer were placed at lateral ports and ports in the vessel lid. Further lid ports were for gas exhaust and feeding solutions.

Among other peripheral analytical instrumentation, photoacoustic analysis of methanol was accomplished in the exhaust gas with monitor systems from INNOVA Air Tech (Ballerup, Denmark). Control of the process magnitudes and mass flows was carried out with the associated digital control unit DCU 2 and supervisory software MFCS/win.

##### 4.3.1.1. Sampling of cultivation medium

For the continuous sampling of the cultivation medium from the bioreactor vessel, the in-line filtration probe ESIP (Eppendorf sterilizable inline probe) [154] from Trace Analytics GmbH (Braunschweig, Germany) was used. The probe was installed into a 19 mm union

## 4 SIA analyzer for formaldehyde in *P. pastoris* cultivation process

of the bioreactor vessel lid and was sterilized in-situ. The probe was equipped with a tubular polypropylene filtration membrane with a pore size of 0.2  $\mu\text{m}$ .

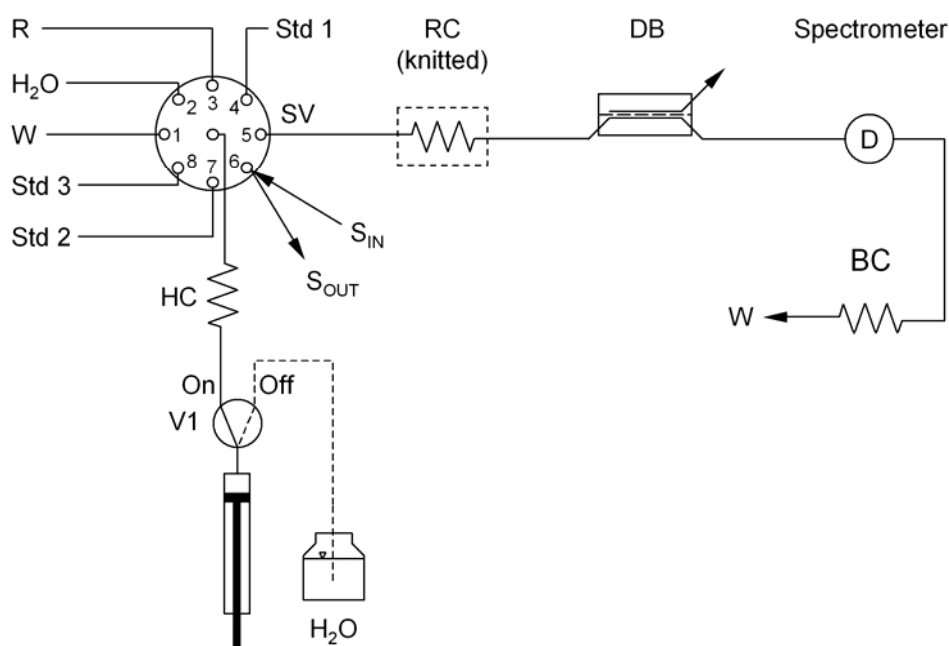
The probe was connected to a peristaltic pump operating in aspiration at a manually adjusted flow rate of 0.6 to 1.5  $\text{ml min}^{-1}$ . The dead volume of probe was 3 ml, the transport line from the filtration probe to the SIA system was about 3 m, 0.8 mm i.d., corresponding to a dead volume of 1.5 ml or an effective time delay of 8 min to 20 min, respectively.

The probe allows a representative and sterile sampling and provided a cell- and particle-free, continuous cultivation medium flow to a sample transport line. The ESIP flow compensated the addition of feeding and auxiliary solutions and allowed by this maintaining a working volume of 5 l cultivation medium.

### 4.3.2. Analyzer instrumentation

#### 4.3.2.1. SIA apparatus and manifold

The SIA instrumentation and manifold are shown in figure 19. All components have been former described in detail in chapters 3.1, 3.2, and 3.3. Instrumental control and data acquisition were accomplished with software AutoAnalysis as described chapter 3.4.



**Figure 19: Manifold of the SIA analyzer for formaldehyde.**

**V1:** solenoid valve with normally open position (OFF, deactivated) dotted, **SV:** selection valve with sample (S) via flow-through adapter, standards (Std), waste (W) and Hantzsch reagent (R), **HC:** holding coil (150 cm, 1.5 mm i.d.), **RC:** heated reaction coil (175 cm, 0.8 mm i.d., knitted), **DB:** debubbler, **BC:** backpressure coil (185 cm, 0.8 mm i.d.), tubes between SV, RC, DB and detector were 20 cm, 0.8 mm i.d. each.

#### 4 SIA analyzer for formaldehyde in *P. pastoris* cultivation process

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The SIA instrumentation consisted of one valve module VA 1 + 1 equipped with an 8-port rotary selection valve and a syringe module Bu 4 S equipped with a single 2.5 ml glass syringe from Hamilton Bonaduz AG. The position ON of the syringe head MCSV was connected to the central port of the rotary selection valve via a PTFE holding coil (150 cm, 1.5 mm i.d.). At position OFF the carrier (water) reservoir was placed.

The lateral ports of the selection valve were used for waste (Pos 1), water (Pos 2), Hantzsch reagent (Pos 3), standards (Pos 4, 7, and 8), and sample (Pos 6). In Pos 5, a knitted reaction coil (175 cm, 0.8 mm i.d.) placed into a miniature water bath, a "Trace trap" debubbler device, the cuvette holder CUV-UV with flow cuvette 75.15 SOG, and a backpressure coil (185 cm, 0.8 mm i.d.) were connected in series by PTFE tubes of each 20 cm and 0.8 mm i.d. The backpressure coil was used to increase the pressure in the manifold (restrictor) to favor the gas bubbles' passage through the debubbler membrane.

The spectrophotometer USB-2000 (USB2F043) and the light source DH-2000 were connected to the cuvette holder CUV-UV via optical fibers (Ocean Optics Inc.) with core diameters of 100  $\mu\text{m}$  and 200  $\mu\text{m}$ , respectively. The deuterium bulb was used for illumination. Dual-wavelength detection was done for schlieren compensation using the absorbance difference as analytical signal (see chapter 3.3). For optimization,  $\lambda_{\text{Det}} = 412 \text{ nm}$  and  $\lambda_{\text{Ref}} = 520 \text{ nm}$  were used, whereas  $\lambda_{\text{Det}} = 420 \text{ nm}$  and  $\lambda_{\text{Ref}} = 500 \text{ nm}$  were used during monitoring in order to reduce iron interference (see chapter 4.4.5.2).

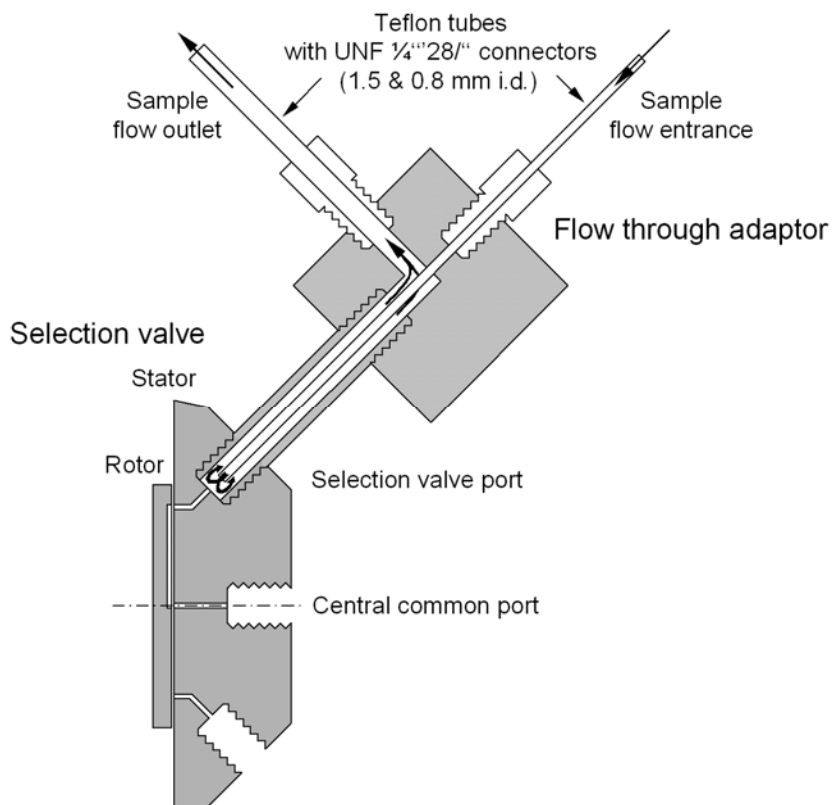
##### 4.3.2.2. Flow-through adapter

For at-line process monitoring, the connection of the SIA analyzers to the continuous medium filtrate flow provided by the ESIP filtration probe was required. During this work, a flow-through adapter was developed, which enabled the function of a flow-through port as described in chapter 4.2.3 on a lateral port of the rotary selection valve. By this, the dead volume to be rinsed before sampling was reduced to a few microliters.

The function principle of the adapter is shown in figure 20. The final design of the flow-through adapter is documented with engineering drawing and photo of the mounted device in the annex. A rigid threaded tube of PVC was screwed into a 3-way connector made of PMMA. The flow channels of tube and connector were 3 mm i.d. The opposite side of the PVC tube was screwed into the port 5 of the selection valve. A PTFE tube (0.8 mm i.d., 1.5 mm o.d.) was inserted into the PVC tube from the opposite side of the 3-way connector nearly down to port inlet. So, in operation, the medium was conducted in the PTFE tube directly to the port inlet and flowed out through the annular clearance between the telescoped tubes. A dead volume of approximately 7  $\mu\text{l}$  remained given by the short flow channel of the connected port in the selection valve stator.

Bubbles were observed to pass the adaptor easily. The flow-through adapter enabled sampling without alteration of the composition of the continuous medium filtrate flow and thus, without affection of the analysis carried out by downstream process analyzers.

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**Figure 20: Operation scheme of the flow-through adaptor.**

**The process medium flow is led to the inlet of the selection valve port entering by the PTFE tube on the right side and flows out through the clearance of the telescoped tubes.**

#### 4.3.3. Reagents

The reagents were prepared as prior described in chapter 3.5.1.1. All reagents were of analytical grade and distilled or Millipore® filtered water was used throughout for the preparation of solutions and applied also as carrier. The Hantzsch reagent denoted "R" was prepared half an hour before use, since during this time an increase of the reagent blank was observed. Satisfying stability for about two weeks was reported [265], whereas an increase of the reagent fluorescence blank with time was observed by LI ET AL. (2005) [240]. In the present case, no significant change of the reagent background absorbance was observed during a period of 24 h. Aqueous formaldehyde standards of 5, 10, and 20 mg l<sup>-1</sup> were used for at-line monitoring. The 20 mg l<sup>-1</sup> standard was further used for standard addition, leading in the actual method to the addition of 5 mg l<sup>-1</sup> of the analyte.

#### 4.3.4. Analytical procedure

Automated standard addition and improved mixing of reagent, sample, and addition standard was achieved by carrying out a zone-stacking procedure. In concrete, 50 µl of reagent, 40 µl of sample, and 10 µl of the highest standard or water, corresponding to

#### 4 SIA analyzer for formaldehyde in *P. pastoris* cultivation process

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sample quantification with or without standard addition, respectively, were aspirated by turns four times before the stacked solutions were propelled towards the thermostated reaction coil and incubated for 120 s at 45°C. Within this time, the syringe was refilled with water from the reservoir in position OFF. Finally, the mixture with the formed reaction product DDL was propelled towards the detection flow cell. In order to achieve a high reproducibility, the solutions were aspirated at a reduced flow rate. Further, a wait step of 2 seconds was implemented after each aspiration step in order to avoid carry-over of solutions at the movement of the selection valve rotor.

For the determination of the sample blank, water was used instead of the reagent. In order to ensure the absence of the reagent solution, 25 µl of water were prior dispensed into the reagent supplying tube. By this, unintentional introduction of the reagent by carry-over at the movement of the selection valve rotor was avoided. As a consequence, the reagent tube had to be rinsed afterwards using a particular cleaning procedure (see chapter 4.3.5). The reagent blank was determined only during calibration. The effective blank value was then calculated as the sum of both blank values. The method used for at-line monitoring consisted of three phases:

1. Initialization (Manifold cleaning and calibration).
2. Monitoring with standard addition with recurring measurement of sample (F), sample with addition (A), addition standard (S), and medium blank (B).
3. Monitoring without standard addition (only F and B).

Standard addition was reasonable only during the first 8 hours after calibration due to the limited stability of the standard. Therefore, standard addition was omitted in phase 3.

#### 4.3.5. Software and program methods

Instrumental control, data acquisition, and data processing were carried out using the software package AutoAnalysis 5.0 from Sciware S.L. described in detail in chapter 3.4 running on a personal computer with operating system MS-Windows 2000. The methods and procedures carried out for analysis and for system cleaning are given in the annex.

Four procedures, repeated within loops, were used within the method for monitoring. Two procedures were used for the rinsing of entire supplying tubes ("Clean channel") or the valves nearest section ("Clean reagent channel") at a variable-defined lateral port of the selection valve with fresh solution. Two further procedures were used for the analytical measurement itself ("Measurement with reagent" and "Measurement without reagent") including solutions aspiration, mixing, incubation, and reaction product quantification.

Variables were used to assign the positions of the reagent, sample, and the solution used for standard addition to lateral ports on the selection valve. Table 2 gives the values of these variables as defined prior to the activation of the respective procedure in order to carry out the different analytical measurement tasks.

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**Table 2: Combinations of variables used to perform different functions with one procedure.**

Variable * / Measurement task	"Reagent position"	"Sample position"	"Addition position"
Sample	3	6	2
Standard	3	4, 7, or 8	2
Reagent blank	3	2	2
Sample blank	2	6	2
Sample with addition	3	6	8

\* Definition of the selection valve positions: 2: water, 3: reagent, 6: sample, 4, 7, 8: standards

### 4.4. Experiments and results

#### 4.4.1. Optimization detection parameters

The intensity of the light source led to saturation of the spectrometer diode array using the instrumentation at the HUAS during formaldehyde monitoring. Optical fibers of smaller core diameter were not available and the emission intensity of the deuterium bulb was not adjustable. Therefore, a spacer was made, which was placed between the light source outlet and the optical fiber. A photo and a technical drawing of the spacer are given in the annex. The obtained eight-fold lower light intensity allowed the use of the detection equipment as given in chapter 4.3.2.1. Signal contribution of Schlieren as well as of light scattering on bubbles or dispersed solids increases with shorter wavelength exponentially. So the complete compensation of these effects by using one reference wavelength is not possible. Therefore, the reference wavelength was chosen as close as possible to the detection wavelength and the averaging time for one single data point was maximized.

#### 4.4.2. Sampling procedure

The Hantzsch reagent was prepared as former proposed by Nash et al. (1953) [265] using ammonium acetate ( $\text{NH}_4\text{Ac}$ ) in a concentration of  $2 \text{ mol l}^{-1}$ . In consequence, the reagent had a high refraction index of 1.3545 and of 1.3430 for a 1:1 dilution with water, both measured at  $22^\circ\text{C}$ . Considerable baseline disturbances were observed when volumes of  $200 \mu\text{l}$  for both reagent and sample were aspirated and propelled towards the detector, so called Schlieren effect. To reduce this effect, the fivefold diminution of the  $\text{NH}_4\text{Ac}$  concentration was tested, however, the peak heights for a formaldehyde standard of  $20 \text{ mg l}^{-1}$  were likewise reduced by the same factor. In order to adapt the carrier to the reagent in respect of its refraction index and salt content, solutions of sodium chloride were tested for the carrier instead of water. Solutions of  $0.5 \text{ mol l}^{-1}$  and  $1 \text{ mol l}^{-1}$  sodium chloride giving refraction indices of 1.3545 and 1.3420 at  $22^\circ\text{C}$  were used; however, the observed reduction of the Schlieren effect was poor for tested blank measurements.

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Zone stacking of reagent and sample as described in chapter 1.1.6 was tested. For this, reagent and sample were aspirated alternately four-times with each 50  $\mu\text{l}$  into the holding coil. By this intercalation, the mixing of both solutions at flow reversal was evidently improved and therefore further applied. A better reproducibility of the peak heights was obtained and baseline disturbances were considerably reduced. A further improvement was achieved by the knitting of the reaction coil as given in chapter 3.2.1.

### 4.4.3. Standard addition

An appropriate reference method for formaldehyde quantification in cultivation medium was not available when the SIA analyzer was tested by at-line monitoring at the HUAS. Therefore, standard addition was aimed in order to evaluate the recovery of formaldehyde applying the method to the complex matrix of the cultivation medium. The zone stacking of sample and reagent allowed the simple integration of in-line standard addition into the method as former described as "sequential injection standard addition" [174]. It was done by slicing the 50  $\mu\text{l}$  sample volume into two reduced volumes of 40  $\mu\text{l}$  of sample and 10  $\mu\text{l}$  of the addition standard. With the 2.5 ml syringe on the used syringe module, 10  $\mu\text{l}$  corresponded to 64 steps of the step motor, which resulted to be performable with satisfying reproducibility. The reduction of the sample volume and by this the sensitivity by 20 % seemed acceptable considering the sensitivity of the applied reaction and the expected formaldehyde concentration in the medium based on a former study [202].

The finally applied sampling procedure consisted therefore in the fourfold repeated pickup of 50  $\mu\text{l}$  reagent, 40  $\mu\text{l}$  sample, and 10  $\mu\text{l}$  water or the highest formaldehyde standard, respectively. A similar reproducibility as before was obtained, but as expected, 20 % lower peak heights were obtained. Using a fourfold higher volume of sample than of the addition standard, a 20  $\text{mg l}^{-1}$  corresponded to an addition to the sample concentration of 5  $\text{mg l}^{-1}$ . Formula 15 (see chapter 3.6.2) is therefore modified giving formula 16.

$$R = \frac{\beta_{S+Add} - \beta_S}{\beta_{Add}} = \frac{A_{S+Add} - A_S}{A_{standard}} \cdot 4 \quad (16)$$

The sampling procedure was also tested with threefold repeated zone stacking. It resulted in about 13 % lower sensitivity and was therefore not further applied. Penetration of the stacked zones by dispersion is shown in figure 21. The visualization was done by overlaying runs using 40  $\text{mg l}^{-1}$  bromothymol blue for one zone and 40  $\text{mmol l}^{-1}$  sodium borate buffer, pH 9 for the all others. Spectrophotometric detection was done with  $\lambda_{Det} = 620 \text{ nm}$  and  $\lambda_{Ref} = 800 \text{ nm}$ . The data correspond to mean values of three repetitions. Aspiration of 20  $\mu\text{l}$  additional reagent after the sampling procedure did not lead to any significant signal increase using a 10  $\text{mg l}^{-1}$  formaldehyde standard in procedure variants either with or without standard addition. This observation confirmed the visual impression of the fully penetration of the stacked addition standard and sample by the reagent.



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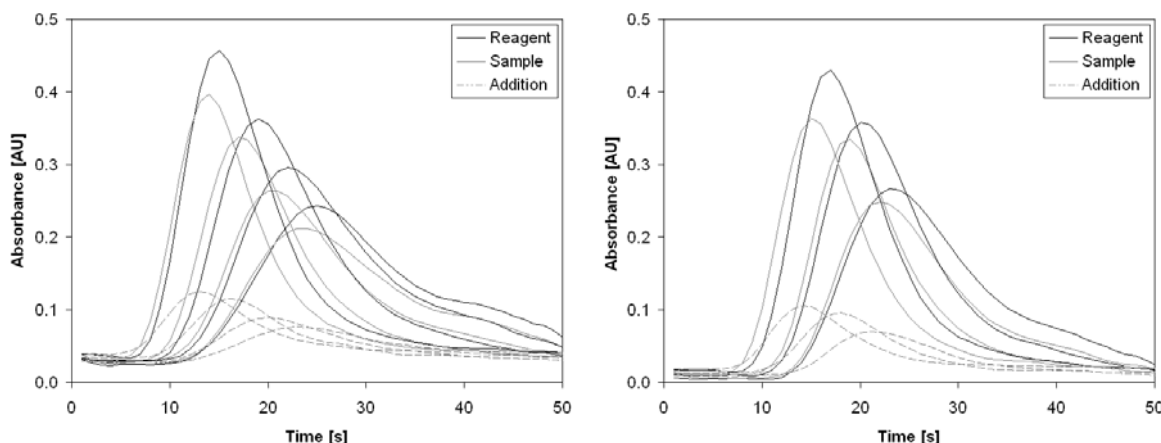


Figure 21: Representation of zone stacked volumes of reagent (50  $\mu\text{l}$ ), sample (40  $\mu\text{l}$ ), and addition (10  $\mu\text{l}$ ) with fourfold (left) and threefold (right) repetition of stacking. Incubation: 45°C, 120 s. BTB indicator 40 mg l<sup>-1</sup> in 40 mmol l<sup>-1</sup> sodium borate carrier, pH 9. Spectrophotometric detection was done with  $\lambda_{\text{Det}} = 620 \text{ nm}$  and  $\lambda_{\text{Ref}} = 800 \text{ nm}$ .

### 4.4.4. Optimization of the analytical method

#### 4.4.4.1. Optimization of reagent pH value

For the Hantzsch reaction, a pH optimum at 6.0 had been described earlier [265]. On the other hand, the cultivation medium as the supposed sample matrix is adjusted to pH 5 in order to obtain optimal growing conditions for the yeast *Pichia pastoris*. The medium had further a considerable buffering capacity showing among others (see chapter 3.5.2.2) phosphoric acid, ammonia (used for pH adjustment), and metabolites formed during the cultivation process such as organic acids.

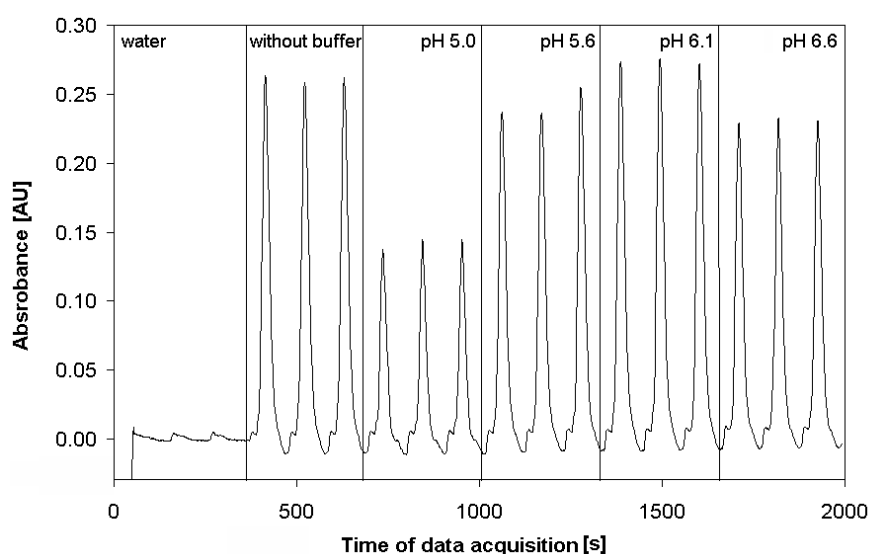


Figure 22: Peak representation of the optimization study of the reagent pH. Conditions are given in table 3.

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**Table 3: Influence of reagent pH on peak heights. \***

Hantzsch reagent	Peak heights [mAU] $\pm$ RSD (n=3)
without addition of phosphate buffer	259 $\pm$ 1.2 %
with addition of phosphate buffer of pH 5.0	139 $\pm$ 2.8 %
with addition of phosphate buffer of pH 5.6	237 $\pm$ 4.1 %
with addition of phosphate buffer of pH 6.1	270 $\pm$ 0.6 %
with addition of phosphate buffer of pH 6.6	223 $\pm$ 0.9 %

\* Conditions: Reagent with 19.4 mmol l<sup>-1</sup> AcAc, 2 mol l<sup>-1</sup> NH<sub>4</sub>Ac, 50 mmol l<sup>-1</sup> acetic acid, and addition of 40 mmol l<sup>-1</sup> phosphate buffer pH 6.0. Experiments were done with 5 mg l<sup>-1</sup> formaldehyde standard and zone stacking sample volumes of 50  $\mu$ l. Incubation was done for 120 s at 45°C.

In order to increase the buffering capacity of Hantzsch reagent prepared as reported [265], phosphate buffer was added to a final concentration of 40 mmol l<sup>-1</sup>. Phosphate was used as it was also a component of the medium and its interference was further known to be low. The influence of the reagent pH adjusted with the phosphate buffer was studied in the range of pH 5.0–6.6. The results are given in table 3 and are represented in figure 22. The highest peaks were obtained for pH 6.1. In comparison with Hantzsch reagent without addition of phosphate buffer, even 4 % higher peaks were obtained at this pH. Therefore, the work was continued using the Hantzsch reagent with 40 mmol l<sup>-1</sup> phosphate buffer adjusted to pH 6.0 in order to ensure the optimal reaction pH value.

#### 4.4.4.2. Optimization of incubation temperature

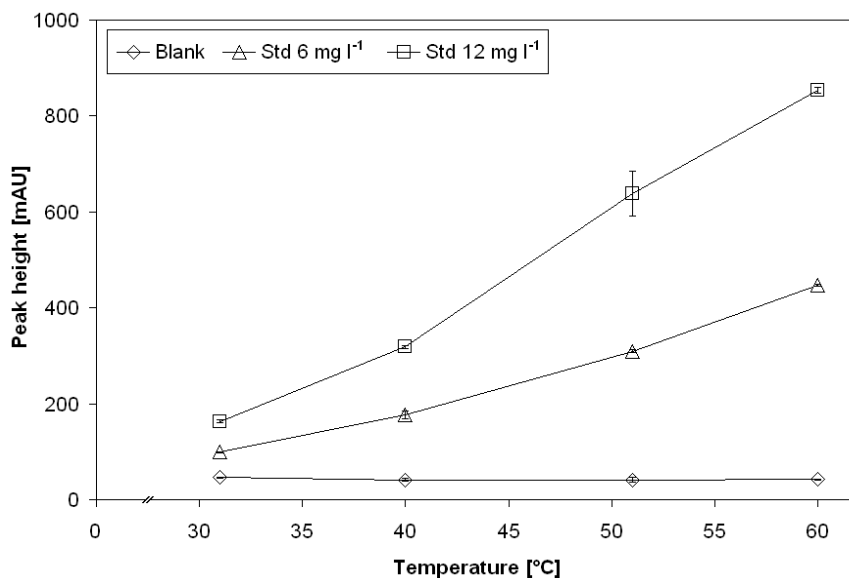
The influence of the incubation temperature was studied in the range of 30°C to 60°C for an incubation time of 120 s. Results are given in table 4 and are represented in figure 23. The standard peak heights increased nearly linear with the incubation temperature while the blank value did not vary significantly. A temperature of 45°C was chosen as a compromise of gas bubble formation observed for higher temperatures and the required sensitivity for the aimed working range up to 20 mg l<sup>-1</sup> reached at 45°C.

**Table 4: Influence of incubation temperature on peak heights. \***

Temperature [°C]	Peak heights [mAU] $\pm$ RSD (n=3)		
	Blank	Standard 6 mg l <sup>-1</sup>	Standard 12 mg l <sup>-1</sup>
31	46.1 $\pm$ 1.5 %	98.8 $\pm$ 1.4 %	163.2 $\pm$ 1.7 %
40	41.4 $\pm$ 7.3 %	177.8 $\pm$ 5.0 %	318.4 $\pm$ 1.1 %
51	41.5 $\pm$ 12.4 %	309.6 $\pm$ 1.1 %	637.9 $\pm$ 7.3 %
60	41.8 $\pm$ 3.6 %	447.3 $\pm$ 0.3 %	854.5 $\pm$ 0.7 %

\* Conditions: Reagent with 19.4 mmol l<sup>-1</sup> AcAc, 2 mol l<sup>-1</sup> NH<sub>4</sub>Ac, 50 mmol l<sup>-1</sup> acetic acid, and addition of 40 mmol l<sup>-1</sup> phosphate buffer pH 6.0, incubation for 120 s.

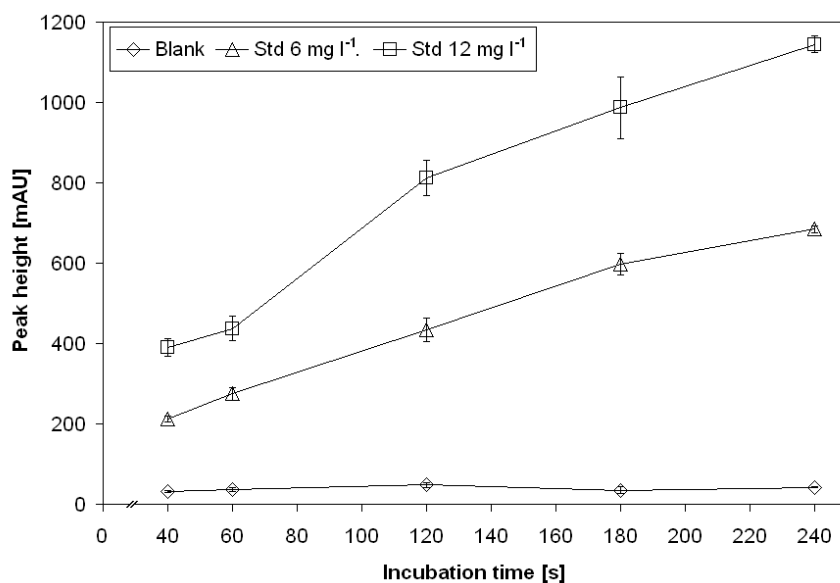
#### 4 SIA analyzer for formaldehyde in *P. pastoris* cultivation process



**Figure 23: Influence of the incubation temperature on peak heights.**  
Conditions are given in table 4.

#### 4.4.4.3. Optimization of incubation time

The influence of the incubation time was studied in the range of 40 s to 240 s for an incubation temperature of 45°C. The shortest time for required syringe refilling performed during incubation was 40 s, so shorter times were not studied, while longer times were not tested due to an unacceptable extension of the analysis duration. The results are given in table 5 and are represented in figure 24. The peak heights of standards increased with the incubation time with decelerating slope. Since the required sensitivity was reached for 120 s and linearity was lost for longer incubation times, 120 s were finally chosen.



**Figure 24: Influence of incubation time on Hantzsch reaction.**  
Conditions are given in table 5.

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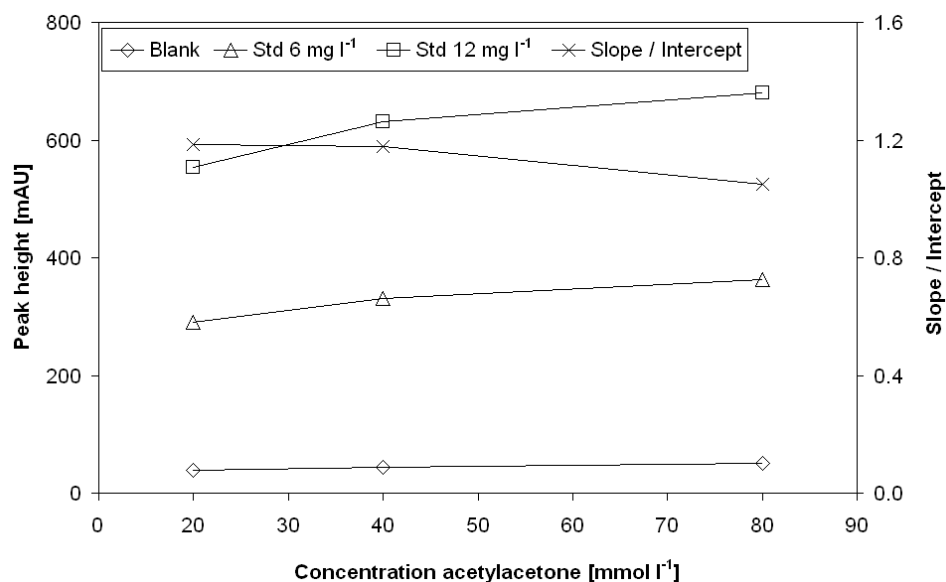
**Table 5: Influence of incubation time on peak heights. \***

Time [s]	Peak heights [mAU] $\pm$ RSD (n=3)		
	Blank	Standard 6 mg l <sup>-1</sup>	Standard 12 mg l <sup>-1</sup>
40	31.3 $\pm$ 6.7 %	212.9 $\pm$ 3.5 %	390.3 $\pm$ 5.4 %
60	36.0 $\pm$ 12.6 %	274.8 $\pm$ 6.1 %	437.8 $\pm$ 6.9 %
120	48.2 $\pm$ 13.2 %	433.9 $\pm$ 6.9 %	812.8 $\pm$ 5.5 %
180	34.6 $\pm$ 25.9 %	598.1 $\pm$ 4.4 %	987.2 $\pm$ 7.8 %
240	41.8 $\pm$ 2.5 %	684.8 $\pm$ 1.3 %	1144.9 $\pm$ 1.8 %

\* Conditions: Reagent with 19.4 mmol l<sup>-1</sup> AcAc, 2 mol l<sup>-1</sup> NH<sub>4</sub>Ac, 50 mmol l<sup>-1</sup> acetic acid, and addition of 40 mmol l<sup>-1</sup> phosphate buffer pH 6.0, incubation at 45°C.

#### 4.4.4.4. Optimization of acetylacetone concentration

The influence of the acetylacetone concentration of the Hantzsch reagent was studied for 19.4, 38.8, and 77.6 mmol l<sup>-1</sup>, corresponding to 0.05 v/v%, 0.1 v/v%, and 0.2 v/v%. Results are given in table 6 and are represented in figure 25. Blank as well as standard peak heights increased about 14 % and 25 % using acetylacetone in a two- and fourfold higher concentration, respectively. The ratio of slope and y-axis intercept of a linear calibration curve was calculated in addition and the highest value was found for 19.4 mmol l<sup>-1</sup>. Since a higher stability was reported for a lower AcAc concentration [265] and the ratio of slope to y-axis intercept was maximal, 19.4 mmol l<sup>-1</sup> were further applied.



**Figure 25: Effect of molarity of acetylacetone in reagent on Hantzsch reaction. Conditions are given in table 6.**

#### 4 SIA analyzer for formaldehyde in *P. pastoris* cultivation process

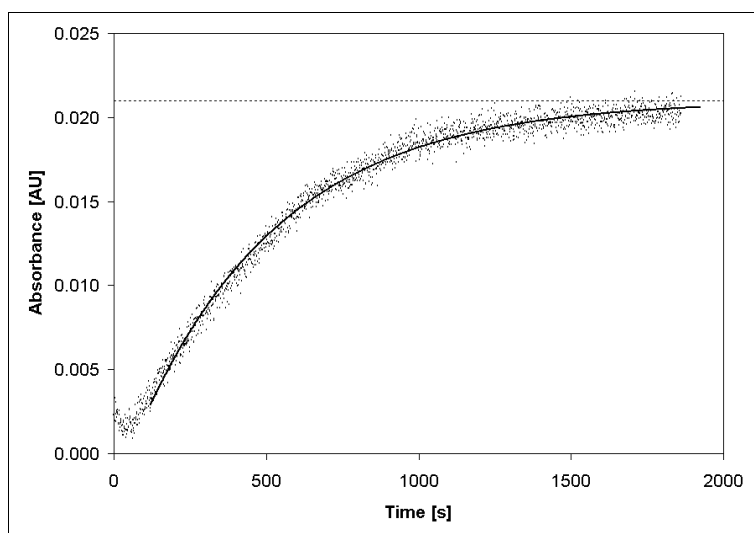
**Table 6: Influence of acetylacetone concentration on peak heights. \***

Acetylacetone [mmol l <sup>-1</sup> ]	Peak heights [mAU] ± RSD (n=3)			Slope / Intercept
	Blank	Standard 6 mg l <sup>-1</sup>	Standard 12 mg l <sup>-1</sup>	
19.4	38.1 ± 6.0 %	289.8 ± 0.9 %	552.8 ± 0.6 %	1.183
38.8	43.7 ± 2.4 %	331.3 ± 1.9 %	631.7 ± 0.3 %	1.179
77.6	50.9 ± 2.3 %	363.0 ± 0.2 %	680.7 ± 0.5 %	1.050

\* Conditions: Reagent with 2 mol l<sup>-1</sup> NH<sub>4</sub>Ac, 50 mmol l<sup>-1</sup> acetic acid, and addition of 40 mmol l<sup>-1</sup> phosphate buffer pH 6.0, incubation at 45°C for 120 s.

##### 4.4.4.5. Reagent yellowing

The Hantzsch reagent showed a slight yellowing directly after preparation. The effect was studied at ambient temperature by spectrophotometry observing the freshly prepared reagent in a 1 cm glass half micro cuvette at 420 nm and reference wavelength 500 nm. The experimental data represented in figure 26 satisfied a lag-behavior of first degree with a lag time of 470 s, corresponding to the stabilization of the yellowing process within 35 min (99.3 % of the final value). The yellowing is probable due to the condensation of AcAc and ammonia. In consequence, the reagent was not used within the first half hour after preparation. Ultrasonic degassing of the reagent showed to intensify the yellowing of the reagent irreversibly and was therefore omitted. Replacement of ammonium acetate by ammonium citrate has been reported to lower the blank value [272]. On the other hand, a significant blank peak, which can be easily detected by software, was considered as an advantage since it facilitated the manual data treatment after each experiment.



**Figure 26: Yellowing of Hantzsch reagent after preparation at room temperature. Incubation: 45°C, 120 s. Spectrophotometric detection with  $\lambda_{\text{Det}} = 420$  nm and  $\lambda_{\text{Ref}} = 500$  nm.**

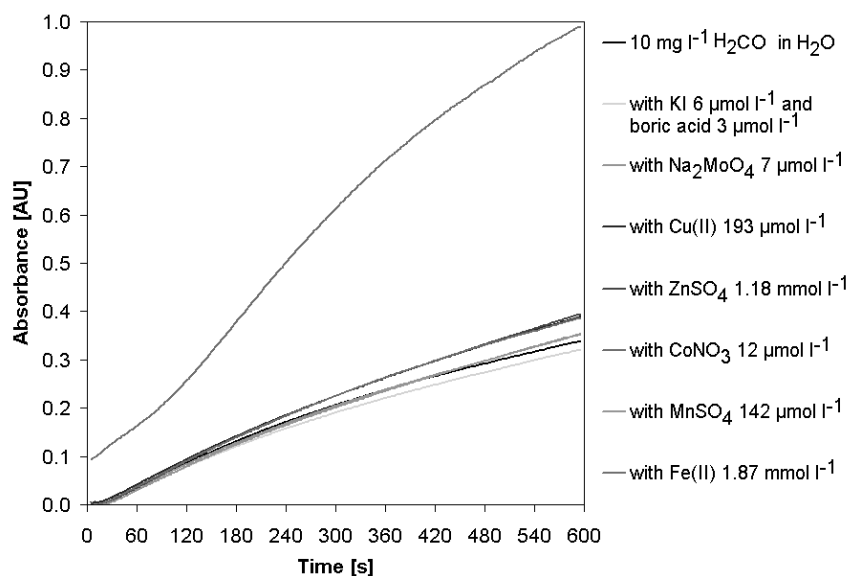
## 4 SIA analyzer for formaldehyde in *P. pastoris* cultivation process

### 4.4.5. Method selectivity

#### 4.4.5.1. Interference studies

In total, 25 compounds were tested regarding to their interference on the Hantzsch reaction and developed method, among these medium components, likely metabolites, and other aliphatic aldehydes, tested as aqueous solutions. A kinetic study was further performed in order to screen interferences from the trace element solution components. Here, aqueous  $10 \text{ mg l}^{-1}$  formaldehyde standards and the compound of interest were mixed 1:1 with the Hantzsch reagent and monitored during 10 min at  $25^\circ\text{C}$  ambient temperature. A blue LED was used as light source as described in chapter 3.3. The experimental data obtained for selected trace element are represented in figure 27.

As it can be seen, iron(II) ions caused a considerable signal increase, which was due to the instantaneous formation of a yellow acetylacetonate complex and the gradual formation of an insoluble precipitate starting at about 120 s. This observation can be explained by the pH of the Hantzsch reagent causing a hydroxide complex as well as by the presents of phosphate, which from a poor soluble compound with iron. Similar results were obtained by experiments on the SIA analyzer.



**Figure 27: Kinetic study with trace element solution components.**

**Solution with  $10 \text{ mg l}^{-1}$  of formaldehyde and detection at  $400 \text{ nm}$  emitted from a UV-LED were used. Each curve represents a mean of two repetitions done at  $25^\circ\text{C}$ .**

No significant interferences or blank signals above the LOQ were found for inorganic ions used for the basal salt medium and trace element solution (see chapter 3.5.2.2) finally containing borate ( $3 \text{ } \mu\text{mol l}^{-1}$ ), calcium ( $5 \text{ mmol l}^{-1}$ ), cobalt ( $14 \text{ } \mu\text{mol l}^{-1}$ ), copper ( $190 \text{ } \mu\text{mol l}^{-1}$ ), iodide ( $5 \text{ } \mu\text{mol l}^{-1}$ ), magnesium ( $57 \text{ mmol l}^{-1}$ ), manganese ( $140 \text{ } \mu\text{mol l}^{-1}$ ), molibdate ( $7 \text{ } \mu\text{mol l}^{-1}$ ), phosphate ( $385 \text{ mmol l}^{-1}$ ), potassium ( $270 \text{ mmol l}^{-1}$ ), sodium ( $100 \text{ mmol l}^{-1}$ ), sulfate ( $165 \text{ mmol l}^{-1}$ ), and zinc ( $1.17 \text{ mmol l}^{-1}$ ).

#### 4 SIA analyzer for formaldehyde in *P. pastoris* cultivation process

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No significant interferences or blank signals above the LOQ were further found for the following organic compounds at the given concentrations: methanol (24 g l<sup>-1</sup>), ethanol (16 g l<sup>-1</sup>), acetone (8 g l<sup>-1</sup>), acetic acid (5 g l<sup>-1</sup>), and formic acid (6 g l<sup>-1</sup>). An analytical signal equivalent to 0.5 mg l<sup>-1</sup> formaldehyde was observed for 40 g l<sup>-1</sup> glycerol. However, glycerol was tested using the concentration at the beginning of the batch phase, whereas the glycerol concentration during induction is below 1 g l<sup>-1</sup>. Likewise, 80 mg l<sup>-1</sup> of glyoxal, acetaldehyde, and glyceraldehyde gave responses like as 2 mg l<sup>-1</sup>, 1.9 mg l<sup>-1</sup>, and 2 mg l<sup>-1</sup> formaldehyde, respectively. In conclusion, high method selectivity was found as reported before [266]. Sulfite was not tested although it had been described as interference on Hantzsch reaction [265]. However, as a 20 % saturation of oxygen was maintained during the entire cultivation process, its presence in the medium was highly unlikely.

##### 4.4.5.2. Reduction of iron interference

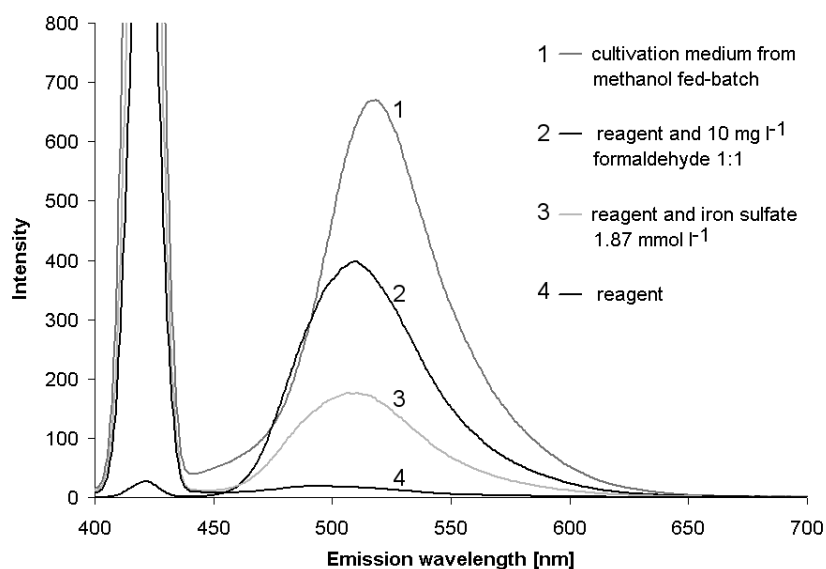
In order to suppress the observed interference from iron ions, EDTA, tartaric acid, and sodium fluoride were tested as masking compounds, used separately as additive to the Hantzsch reagent in concentrations of each 100 mmol l<sup>-1</sup>. The high concentrations of the additives were required since competing, chelate-forming ions such as Ca<sup>2+</sup> and Mg<sup>2+</sup> are present in the medium in high concentrations. In test tube experiments, aqueous solutions of sodium fluoride showed precipitation after the addition of FeSO<sub>4</sub> whereas solutions with tartaric acid and EDTA remained clear, but nevertheless ferrous acetylacetonate was formed. Although addition of ferrous ions did not lead to color formation in the presence of sodium fluoride, the analytical reaction itself was apparently inhibited. Applying the SIA analyzer, a maximal reduction of 25 % of the iron interference was found with addition of EDTA applying batch medium as sample. Neither tartaric acid nor sodium fluoride did lead to a significant reduction of iron interference. In conclusion, none of the tested substances was applicable for an effective suppression of the iron interference.

The use of the iminodiacetate chelating resin Chelex-100 (50-100 dry mesh, Sigma Aldrich, St. Louis, USA) was tested as a possibility to eliminate iron ions from the sample. In test tube experiments, no reaction product was formed using aqueous standards. Apparently, formaldehyde reacted with the amino-groups of the resin. Similar results were obtained with the SIA analyzer using a Chelex-100 filled column (3 cm length, 4 mm i.d.), which was placed between the holding coil and the central port of the selection valve. As before, formaldehyde peaks were considerably reduced and the use of chelating resins was therefore turned down. Cationic exchangers were considered to be impracticable due to the elevated cation concentration of the cultivation medium and the Hantzsch reagent.

As an alternative to eliminate the signal influence of iron acetylacetonate, fluorescence detection was tested downstream to spectrophotometric detection using a second and independent PC for data acquisition and the instrumentation described in chapter 3.3. The fluorescence detection cell was connected with the outlet of the spectrophotometric detection cell via a PTFE tube (1 m, 0.8 mm i.d.). Excellent repeatability and low baseline noise were found. However, the fluorescence signal of the medium corresponded to an

#### 4 SIA analyzer for formaldehyde in *P. pastoris* cultivation process

about 10-times higher formaldehyde concentration as when spectrophotometric detection was applied for quantification. As shown in figure 28, each, the cultivation medium, iron acetylacetonate, and the blank reagent showed a considerable fluorescence at the emission maximum of the reaction product DDL and similar emission spectra. Therefore, fluorescence detection was considered as not suitable to improve the method selectivity nor did it contribute to a simpler methodology since likewise the signal contributions of reagent and medium had to be taken into account.



**Figure 28: Fluorescence spectra from reagent, DDL, medium, and iron acetylacetonate. Excitation: 420 nm, slit: 10 nm, scan velocity for emission: 1000 nm min<sup>-1</sup>.**

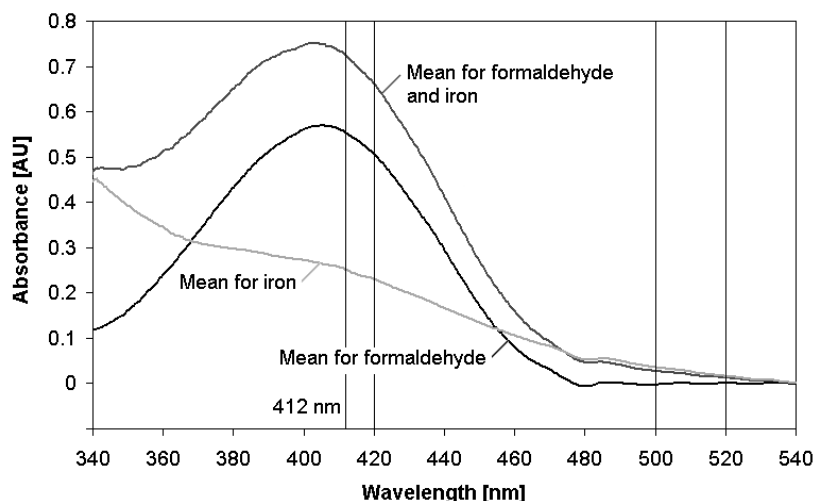
Suppression of iron interference by the separation of formaldehyde from the medium matrix by gas diffusion was further tested. A homemade gas diffusion cell equipped with a Durapore® membrane given with photo and engineering drawing in the annex was used. First the acceptor side channel was filled with the reagent. Afterwards, a sample volume of 500  $\mu\text{l}$  was propelled through the donator side channel to waste at reduced flow rate of 1 ml min<sup>-1</sup>. Finally, the reagent volume in the acceptor channel was pushed towards the reaction coil, incubated for 120 s at 45°C, and finally propelled towards the detector. No significant analytical response was obtained for standards up to 20 mg l<sup>-1</sup>. To improve the diffusion, the cell was heated to 45°C omitting the reaction coil, however, no significant improvement was found. Only exception was the analytical signal of a 100 mg l<sup>-1</sup> standard equaling 3 mg l<sup>-1</sup> applying with the normal method. Similar results were expected since the vapor pressure of formaldehyde in diluted aqueous solutions is low [204]. As further disadvantages, a high sample volume and prolonged time for analysis were required.

As a final possibility, the diminution of the iron interference by the variation of the applied detection wavelengths was tested. Absorbance spectra obtained with the SIA analyzer for a 12 mg l<sup>-1</sup> formaldehyde standard, 1.1 mmol l<sup>-1</sup> iron sulfate, and 1.1 mmol l<sup>-1</sup> iron sulfate with 12 mg l<sup>-1</sup> formaldehyde are given in figure 29. It can be seen, that the iron interference is not related only to the formation of the yellow iron acetylacetonate complex



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but also to the turbidity indicated by the continuous baseline increment with shorter wavelengths. Therefore, minimization of this effect is principally possible by the diminution of the distance between the detection and the reference wavelength. By increase of  $\lambda_{\text{Det}}$  from 412 nm to 420 nm and decrease of  $\lambda_{\text{Ref}}$  from 520 nm to 500 nm, the peak height of the iron sulfate solution decreased about 22 %, whereas the peak height of the formaldehyde standard only about 3 %. In consequence, the relative signal contribution of DDL increased as the signal contribution of iron was reduced and detection was further done at 420 nm versus 500 nm for formaldehyde monitoring at the cultivation process.



**Figure 29: Spectra for formaldehyde, iron, and mixture after reaction with acetylacetone. Determination was done at peak maxima applying Hantzsch reaction automated by SIA.**

#### 4.4.6. Cultivation process monitoring

##### 4.4.6.1. Preliminary experiments

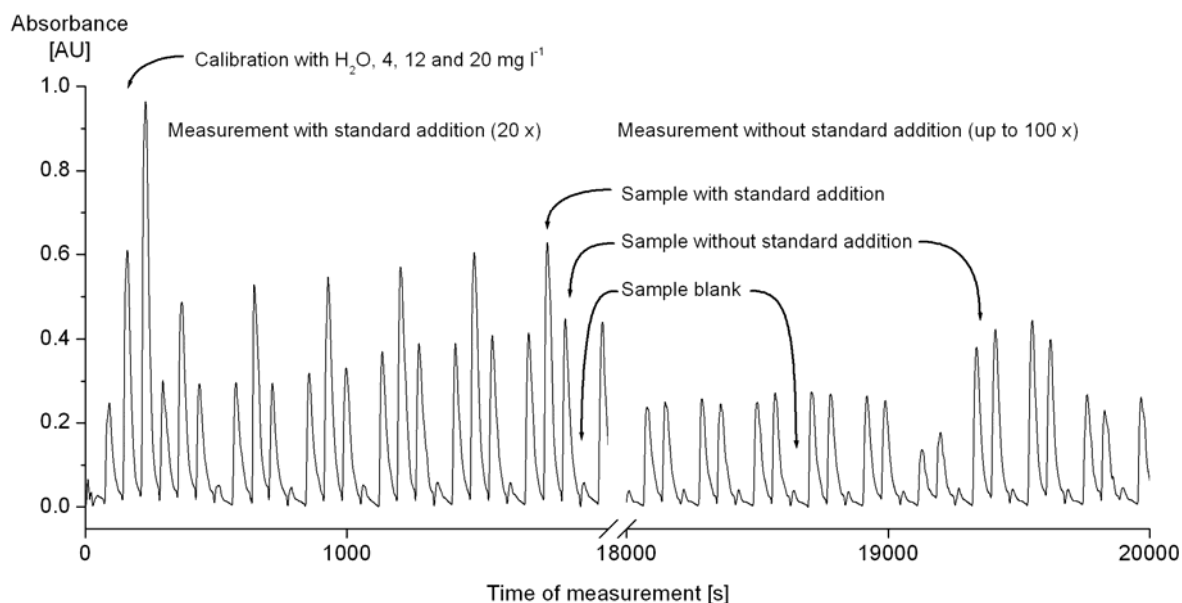
The flow analyzer and the method applied for monitoring were tested in laboratory experiments with formaldehyde standard, fresh basal salt medium with 5 g l<sup>-1</sup> methanol instead of glycerol, and processed medium from a former cultivation. The samples were circulated as given in chapter 4.5.1.5 while standards were placed the lateral valve ports.

The mean recoveries using formaldehyde standard, batch medium with methanol, and a medium sample were 94.9 % ± 5.0 %, 95.4 % ± 5.1 %, and 97.3 ± 3.2 %, respectively. The found values did not differ significantly. So, neither batch medium nor medium sample interfered significantly the determination of formaldehyde. The peak height of the 20 mg l<sup>-1</sup> formaldehyde standard decreased slowly indicating a poor stability. For the calculation of the recovery values, this decrease had to be taken into account. The effect of the medium matrix on the analytical signal was tested further by the comparison with medium diluted 1:4 with water. Peak heights were found to be 3.4 % higher for the undiluted medium sample.

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### 4.4.6.2. Cultivation process progress

The observed cultivation process was carried out over 292.5 h at the Research Center of Bioprocess Engineering and Analytical Techniques (HUAS). Formaldehyde monitoring was done during 251.4 h corresponding to 86 % of the entire process time.



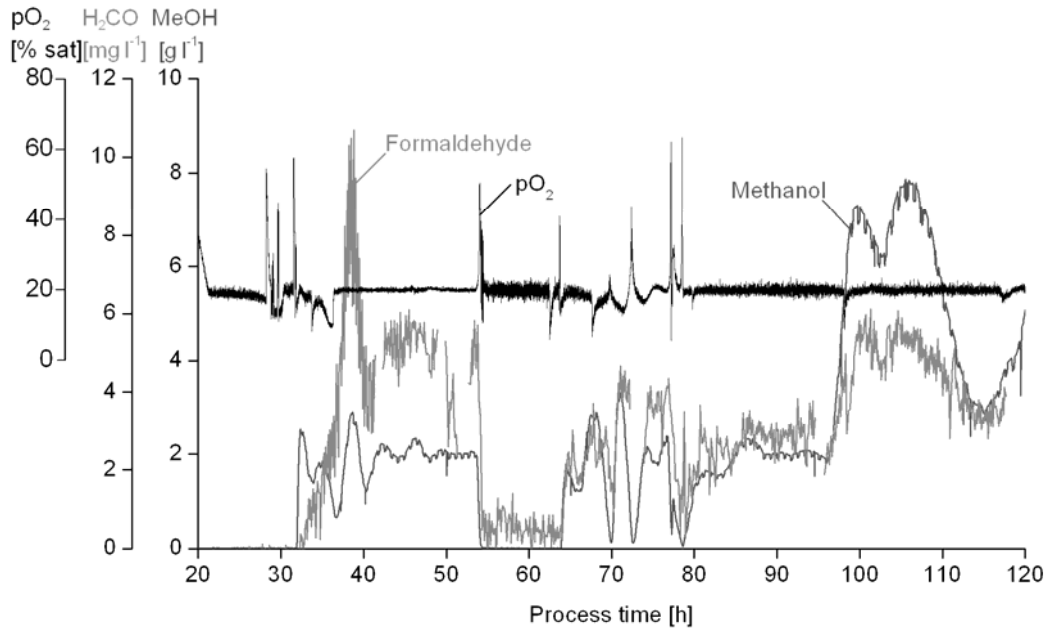
**Figure 30: Representative peak sequence for the monitored cultivation process. Calibration and a 20-fold repetition of the peak sequence including standard addition were followed by a 100-fold repetition of peak sequence without standard addition.**

Due to data saving and personal maintenance, the maximal continuous monitoring time was 26.9 h. The SIA analyzer was coupled via the flow-through adapter to the medium filtrate flow sampled continuously from the process as described in chapter 4.3.1.1. Formaldehyde standards of 4, 12, and 20 mg l<sup>-1</sup> were used throughout. The highest standard was used for standard addition during the first 8 hours of monitoring. The applied peak sequences with and without standard addition are shown in figure 30.

Concentration courses of formaldehyde, methanol, and oxygen saturation of the cultivation medium are shown for the process periods 20 h – 120 h and 140 h – 250 h in figure 31 and figure 32. Process events affecting the formaldehyde concentration are described only shortly since process performance and formaldehyde modeling were not part of this work, but only the development of the formaldehyde SIA analyzer.

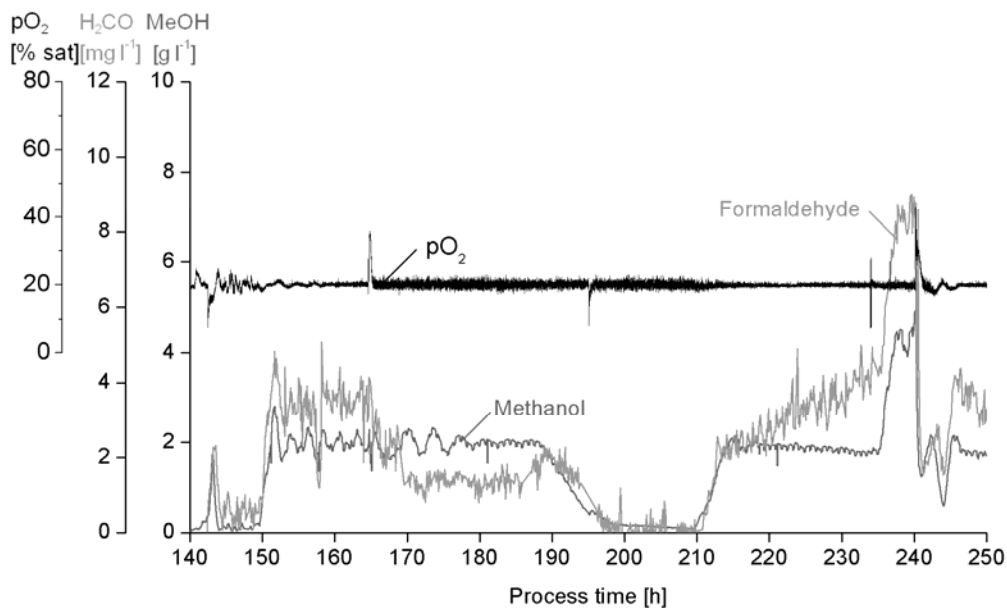
Glycerol batch was performed until 28.2 h. The process continued as glycerol fed-batch until a cell density of 40 g l<sup>-1</sup> was reached at 31.5 h. Methanol induction was initiated at 31.7 h. Formaldehyde was detected directly after induction. Its concentration followed the oscillation of the methanol content in the first hours. After passing a maximum at 38.5 h of 10.6 mg l<sup>-1</sup> formaldehyde, a stable concentration of 5-6 mg l<sup>-1</sup> was obtained. The high deflection in the beginning might be related to the fast increase of oxygen and methanol.

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**Figure 31: Concentration courses of formaldehyde, methanol, and oxygen saturation between process time 20 h to 120 h of the observed cultivation process.**

Bisubstrate feed with glycerol was started at 43.8 h but the formaldehyde concentration was not notably affected. However, it decreased immediately when the methanol feed was stopped at 53.5 h. Renewed methanol induction was done at 64.3 h. The formaldehyde concentration increased to a lower level but considerably faster than before, probably due to a higher cell density compared to the first induction with methanol. During the next 55 h, the formaldehyde concentration curve followed strongly the one of methanol until formaldehyde monitoring was stopped at 119 h.



**Figure 32: Concentration courses of formaldehyde, methanol, and oxygen saturation between process time 140 h to 250 h of the observed cultivation process.**

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Formaldehyde monitoring was started again at 142.1 h and its concentration was observed to follow the methanol course strictly. At 164.1, a cell harvest and medium refresh was done. During and after medium refresh, the formaldehyde concentration decreased to about the half of its former level. The formaldehyde concentration was reduced simultaneously, when methanol concentration was reduced at 188.1 h and increased simultaneously, when the methanol concentration was heightened again at 209.6 h. At 215 h, the former methanol concentration was reached again but formaldehyde increased in spite of constant methanol content. At 235.2 h, the methanol content was set to 5 g l<sup>-1</sup> and set to 2 g l<sup>-1</sup> at 240 h, both reflected by the formaldehyde concentration course.

## 4.5. Discussion

### 4.5.1. Analytical characterization

#### 4.5.1.1. Dispersion coefficient

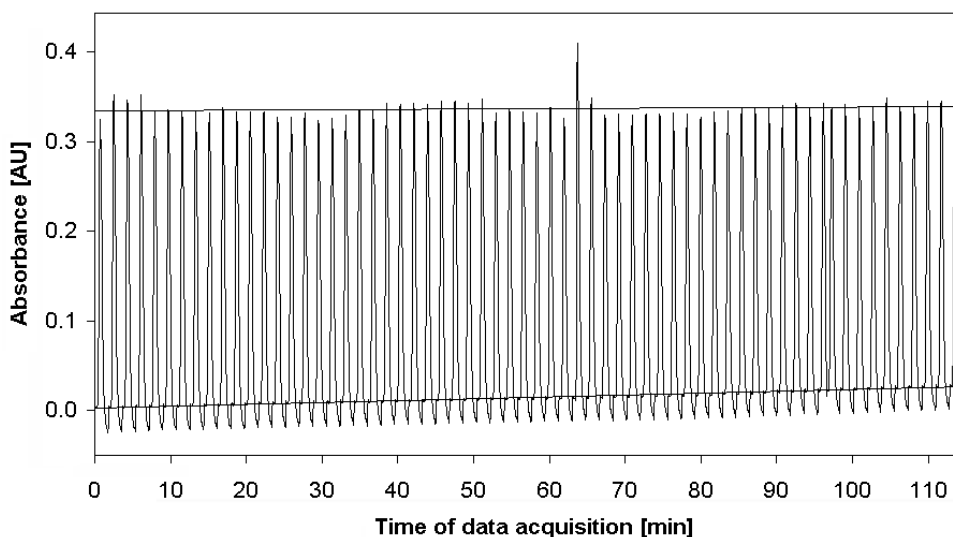
The dispersion coefficient *D* for the zone stacking volumes of sample, sample plus addition standard, and sample plus addition standard plus reagent was determined. Bromothymol blue (BTB) 40 mg l<sup>-1</sup> was used for the respectively tested volumes and 40 mmol l<sup>-1</sup> sodium borate buffer, pH 9 was used for any further solution and carrier. Spectrophotometric detection was done with  $\lambda_{\text{Det}} = 620 \text{ nm}$  and  $\lambda_{\text{Ref}} = 800 \text{ nm}$ . For the steady-state absorbance of BTB, the syringe and the holding coil were rinsed five times with the dye solution before propelling two times 2.5 ml of the solution towards the detector. The dispersion coefficients were 3.7 for the sample volume, 3.0 for sample plus the addition volume and 1.6 for the entire stack including reagent for fourfold zone stacking. For threefold zone stacking, the dispersion coefficient values were about 11% higher.

#### 4.5.1.2. Stability of the method

The stability of the method was studied during 21 h. Every 20 min, one determination of a 5 mg l<sup>-1</sup> formaldehyde standard was done. Reagent and standard flasks were protected from light with aluminum paper and plugged with compressed cellulose plugs. Results are shown in figure 33. The baseline increased nearly linear about 0.16 mAU per hour, which was probably due to the light source stability. The peak heights decreased nearly linear about 0.32 % per hour. Repeatability was satisfying with a RSD value of 5.4 %. The 36<sup>th</sup> peak was identified as outlier and caused probably by an air bubble and altered dispersion.

The decrease of peak heights was rather related to the limited stability of the standard than the stability of the reagent since up to two weeks stability of the reagent had been reported about 50 years before [265]. During monitoring at the yeast cultivation process, a 50 % signal decrease within one day was observed for a 5 mg l<sup>-1</sup> formaldehyde standard.

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**Figure 33: Stability of the method tested during 21 h.**  
One peak corresponded to 20 min of experiment. A sample volume of 50  $\mu\text{l}$  was applied.

##### 4.5.1.3. Linear working range, reproducibility, and detection limit

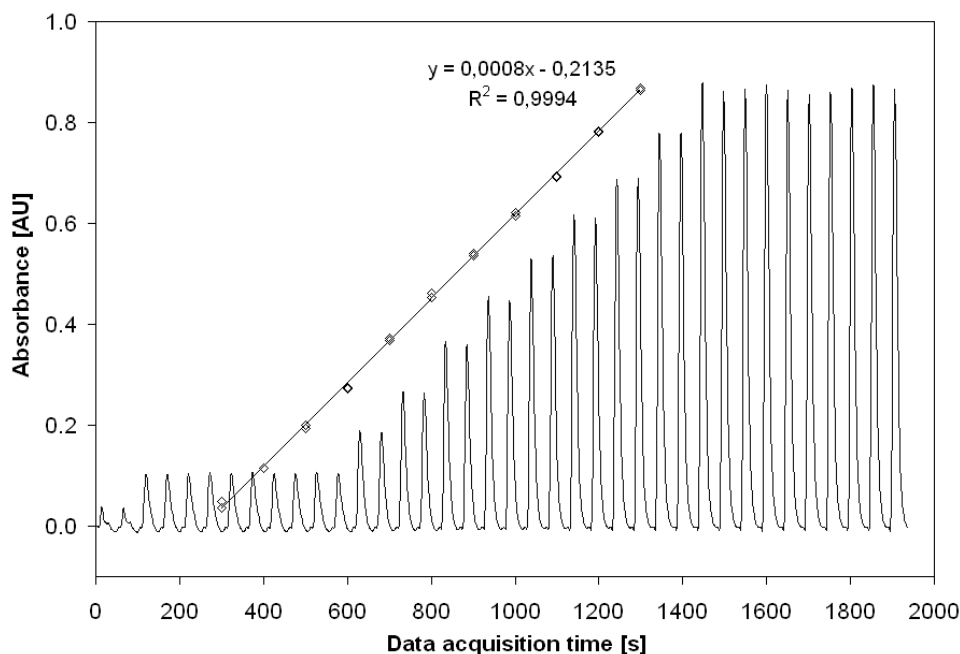
Limits of detection (LOD) and quantification (LOQ) were determined by ten-point calibration according to German Industry Norm DIN 32645 [279] and norm ISO 11843-2 [280] for the optimized method. The optimized parameters of the method are summarized in table 7. Water and standards of 4, 6, 8, 10, 12, 14, 16, and 18  $\text{mg l}^{-1}$  were measured two-times and standards of 2 and 20  $\text{mg l}^{-1}$  formaldehyde ten-times. Linearity was always achieved up to 20  $\text{mg l}^{-1}$ , in some cases even up to 25  $\text{mg l}^{-1}$ . Results were represented in figure 34. Peak height repeatability (RSD) was calculated from the tenfold repetition of highest and lowest standards to be 0.85 % and 1.32 %, respectively.

**Table 7: Parameters of optimized method for formaldehyde determination.**

Reagent	2 $\text{mol l}^{-1}$ $\text{NH}_4\text{Ac}$ , 19.4 $\text{mol l}^{-1}$ $\text{AcAc}$ , 50 $\text{mmol l}^{-1}$ acetic acid, 40 $\text{mmol l}^{-1}$ potassium phosphate buffer pH 6.0
Incubation	120 s at 45°C
Detection	420 nm with reference wavelength 500 nm
Sampling	fourfold zone stacking of 50 $\mu\text{l}$ reagent, 40 $\mu\text{l}$ sample, 10 $\mu\text{l}$ of water or standard for standard addition

Using the entire calibration data set, the variation of the method resulted to be 1.2 % and the LOD and LOQ were 0.4  $\text{mg l}^{-1}$  and 1.8  $\text{mg l}^{-1}$ , respectively. Due to the applied method, the LOD and LOQ were relatively high whereas the variation of the method was satisfyingly low. The calculated deviation of 1.32 % for the 2  $\text{mg l}^{-1}$  permitted a student test with significance level of 95 % for a concentration difference of 0.1  $\text{mg l}^{-1}$ .

#### 4 SIA analyzer for formaldehyde in *P. pastoris* cultivation process



**Figure 34: Ten-point calibration performed with automated Hantzsch reaction**  
Calibration data are given in table 8, conditions in table 7.

**Table 8: Ten-point calibration performed with automated Hantzsch reaction.**

Formaldehyde [mg l <sup>-1</sup> ]	Peak height [AU]	Formaldehyde [mg l <sup>-1</sup> ]	Peak height [AU]
0	0.043	12	0.539
2 (n=10)	0.115	14	0.618
4	0.197	16	0.692
6	0.274	18	0.781
8	0.370	20 (n=10)	0.870
10	0.458		

##### 4.5.1.4. Solution consumption and sampling frequency

The consumption of solutions was low with 160 µl of sample, 200 µl of reagent, and 2.75 ml of water used as carrier for each determination. One liter of water and 50 ml of reagent were sufficient for about 24 hours of monitoring. A sampling frequency of 12 peaks per hour was achieved for repeated measurement of the same sample solution or by sampling from the flow-through adapter. During at-line monitoring, the sampling frequency was reduced to 10.5 peaks per hour since rinsing of standard or reagent tubes in order to measure the medium blank and to perform standard addition was required. Due to the requirement of measuring blank and standard addition during the at-line monitoring, the effective sample frequency decreased to about 6 h<sup>-1</sup>.

## 4 SIA analyzer for formaldehyde in *P. pastoris* cultivation process

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### 4.5.1.5. Performance of flow-through adapter

The flow-through adapter described in chapter 4.3.2.2 was used for sampling with a dead volume to be rinsed of less than 10  $\mu\text{l}$ , given by the flow channel of the selection valve port. The tip of the adapter acted like a miniature mixing chamber and increased by this the dispersion of the sample. The dead volume was estimated to be about 40  $\mu\text{l}$ . So an additional response delay of a few seconds had therefore to be taken into account.

The flow through adapter was placed on the uppers port of the selection valve in order to facilitate the outflow of air bubbles. To study the effect of the flow-adapter on the analytical performance, a minipuls 3 peristaltic pump from Gilson (Middleton, USA) used to circulate water and standards of 5, 10, and 20  $\text{mg l}^{-1}$  formaldehyde at a flow rate of 1  $\text{ml min}^{-1}$  at the valve port. No significant differences were obtained in comparison with measurements where the solutions were aspirated from the normally used PTFE provision tubes. Likewise, the repeatability was not affected from the flow-through adapter. Performing standard addition, recovery and peak height values did not differ significantly. At no time, stacking of gas bubbles in the flow-through adaptor was observed.

### 4.5.1.6. Performance of debubbler devices

The homemade debubbler cell described in chapter 3.2.3 showed a high efficiency in elimination of gas bubbles. However, few hours after insertion of a new membrane, liquid leaking was observed. It turned out, that the membrane became permeable due to the mechanical stress from pressure application every time when the cell was flushed.

Thus, the device was replaced by the "Trace trap" debubbler. It was proven, that the placement of a "backpressure coil" downstream of the flow cuvette increased the efficiency of gas bubble elimination. This was due higher pressure in the manifold during flow application, so bubbles were enforced to pass the relatively thick membrane. The device worked without failure during the cultivation process monitoring campaign.

### 4.5.1.7. Performance of the method during monitoring

Personal maintenance was required 1-2 times per day for data saving, refilling of carrier reservoir, emptying the waste recipient, and method re-start including calibration with freshly prepared formaldehyde standards and reagent. Peak height repeatability for the standard solutions was generally high. Satisfying reproducibilities were found for slope ( $0.033 \text{ AU mg l}^{-1} \pm 4.5\%$ ) and x-axis intercept ( $0.045 \text{ AU} \pm 44.1\%$ ) of calibration functions.

A mean recovery of  $97.3 \% \pm 13.5\%$  was obtained, which did not differ from the values obtained in preliminary laboratory experiments significantly. The high deviation of the recovery values was mainly due to the impossibility to perform measurements with and without standard addition on the same sample but the requirement of the interpolation of the formaldehyde concentration at each time in the medium filtrate sample. The distribution of the recovery values calculated during the process is given in table 9.

#### 4 SIA analyzer for formaldehyde in *P. pastoris* cultivation process

**Table 9: Distribution of calculated recoveries of monitoring data of the cultivation process.**

Upper class limit [%]	Accumulative sum [%] (n = 189)	Upper class limit [%]	Accumulative sum [%] (n = 189)
50	0.0	110	92.1
60	0.5	120	96.8
70	1.6	130	97.9
80	5.8	140	98.4
90	29.6	150	99.5
100	56.1	170	100.0

The stability of formaldehyde in the medium filtrate flow during the provision time from the ESIP to the SIA analyzer could not be studied. The reaction of the analyte with organic compounds as amines might have led to a slight decrease of its concentration. On the other side, diffusion of formaldehyde through the PTFE tube walls was considered to be negligible as the vapor pressure of formaldehyde in water-methanol mixtures is low [204].

During monitoring of process phases where glycerol was fed as the only carbon source (e.g. 20 – 30 h), the analytical signal was in the order of the blank signal. Consequently, no interference of iron was observed, although the iron concentration of the batch-medium of  $1.87 \text{ mmol l}^{-1}$  was former evaluated to correspond to a formaldehyde concentration about  $5.4 \text{ mg l}^{-1}$ . During a preliminary monitoring (data not shown), an analytical signal corresponding to  $5 \text{ mg l}^{-1}$  formaldehyde was found in the very first beginning of the process in spite of the absence of methanol in the medium decreasing exponentially to zero within the first ten hours of the process.

In order to explain this observation, the formation of aldehydes during in-situ sterilization was tested. For this, batch medium, with and without addition of trace element stock solution, was autoclaved at  $120^\circ\text{C}$  for 0.5 h and compared with untreated media. For both media without trace element addition, no significant analytical signal was observed whereas the analytical signals of both media with trace element addition were similar corresponding to  $6.6 \text{ mg l}^{-1}$  (autoclaved) and  $5.7 \text{ mg l}^{-1}$  (untreated) formaldehyde.

At the cultivation medium pH of 5 and aerobic conditions, Fe(II) is oxidized to Fe(III). The former observations lead therefore to the consideration, that the ferrous ions originated from the trace element stock solution can pass the filtration membrane of the ESIP probe. Due to the proceeding oxidation, iron is retained as ferric hydroxide complex or bound as ferric phosphate. Both compounds are poor soluble at the cultivation medium pH and form precipitates. So the passage through the filtration membrane is hindered. The biological uptake by the yeast cells or the formation of complexes with organic compounds or cell fragments contributed probably to the retention of iron in the process.

In test tube experiments, no significant concentrations of ferric or ferrous ions were found in cultivation medium filtrate sampled during the methanol fed-batch using thiocyanate and phenantroline solutions, respectively. Possible inhibition by the medium matrix was excluded by a positive control (addition of the respective ferric or ferrous salts).



## 4 SIA analyzer for formaldehyde in *P. pastoris* cultivation process

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### 4.5.2. Possible improvements and outlook

The further reduction of the iron interference and the medium blank should be main objectives for future works. The probably simplest way would be to increase the method sensitivity in order to allow the dilution of the sample and by this, to reduce the former named affections of the method. Such sensitivity increase could be possible by a higher incubation temperature or the retention of the formed DDL on a C18 resin. However, both measures would probably diminish the method robustness. Raise of temperature had led in this work to enhanced air bubble formation and error peaks. Use of a pre-concentration column or membrane would higher the system pressure and the retention of signal contributing or resin blocking components of the cultivation medium is unknown but highly probable. A further increase of the method sensitivity is possible by using acetylacetone in a higher concentration. However, the stability of the reagent would be probably affected as former observed and described [265].

Since the cultivation medium matrix did not show any negative affect on the analyte recovery during the performed process monitoring, the analytical protocol could be simplified by omitting standard addition and so the sample frequency could be increased.

The validation of the proposed analyzer was difficult due to the lack of certified medium samples or a suitable alternative methodology for the quantification of formaldehyde in the medium. On the other hand, analysis of formaldehyde by GC in the presence of a 500-fold higher methanol concentration is sophisticated. The separation of DDL on reverse phase as former described [268] could be an adequate way to improve the method reliability.

A further improvement can probably be achieved by applying merging flow mixing of sample and reagent, i.e. application of a multichannel flow technique such as MSFIA or micropumps, in order to increase the sample frequency and sensitivity of the method.

## 4.6. Conclusion

In the present work, an automated analytical system was developed for the quantitative determination of formaldehyde in yeast cultivation medium. The Hantzsch reaction with acetylacetone was automated using sequential injection analysis and spectrophotometric detection. Reagent composition, flow rates, volumes, and the sampling procedure were optimized and the robustness, selectivity, sensitivity, and reagent stability were studied. The linear working range was up to 20 mg l<sup>-1</sup>, which fitted the aimed concentration range reported in the literature. Satisfying performance with a limit of detection of 0.4 mg l<sup>-1</sup>, deviation of method of 1.2 %, and high robustness were achieved.

Influences of batch medium components and other aldehydes on the reaction were studied in detail. Apart from a considerable interference of iron, excellent selectivity of the method was proven. In order to repress the interference of iron, reagent additives, gas

#### 4 SIA analyzer for formaldehyde in *P. pastoris* cultivation process

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diffusion, fluorescence detection, and use of a chelate resin were tested. The best effect was achieved with a minimal distance between the detection and reference wavelengths.

The system was successfully applied to the at-line monitoring of formaldehyde in the medium of cultivation process of about 250 h of the methylotrophic yeast *Pichia pastoris* during methanol feed. For this task, the analyzer was coupled by a flow-through valve port adapter developed during this work to a continuous medium filtrate line. The adapter allowed the direct and near-to-zero dead volume sampling from the filtrate flow without being affected by gas bubbles or affecting the downstream located analyzers.

During monitoring, formaldehyde concentration and medium blank were alternately measured reaching a sampling frequency of 12 per hour. Minimization of Schlieren and in-line standard addition was performed by zone stacking of reagent, sample, and water or the standard used for addition, respectively. The average recovery during process monitoring was 97.3 %. Recoveries determined in laboratory experiments did not differ significantly. A high RSD value of 13.5 % was obtained for the analyte recovery during monitoring, which is mainly due to the fact, that the analyzer was applied to a continuous filtrate flow with changing composition over time. Thus, quantification of the exactly same sample with and without addition was not possible to apply but the sample concentration had to be interpolated for the calculation of the analyte recovery.

The monitoring data clearly indicated that formaldehyde was generated directly after methanol introduction and that its concentration rapidly decreased whenever the methanol feed was stopped. At constant methanol medium content, a stabile formaldehyde concentration was observed. The formaldehyde concentration followed the changes of the methanol medium content immediately. A functional dependency could not be proven but influences of the cell density and oxygen content were observed. The highest measured formaldehyde concentration during normal oxygen supply was about 11 mg l<sup>-1</sup>. It had become clear that formaldehyde did not affect the cultivation process significantly at the found concentration level. The monitoring data were considered to contribute to a kinetic model and to a better understanding of the methanol-induced product expression.

No interference of iron was observed after process duration of 10 h. Iron is supposed to be retained in the fermenter as ferric ion by formation of complexes with hydroxide or phosphate anions or as complexes with organic chelate-forming compounds.

By this work, an analytic tool was provided for the further investigation of formaldehyde formation and concentration in yeast cultures and interactions with other process factors.

## 5. EXPERIMENTAL WORK 2

### Sequential injection analyzer for glycerol monitoring in yeast cultivation medium

Published equally entitled: HORSTKOTTE B., WERNER E., KAZEMI A., CORNELISSEN G., ELSHOLZ O., CERDÀ V., LUTTMANN R. (2007) *Talanta* 71, 941-947 [166].

#### 5.1. Task description for experimental work 2

The objective of this work was the development of an automated analyzer for the quantification of glycerol in medium of cultivation processes of *P. pastoris* as described in chapter 4.3.1. After the optimization of physical and chemical parameters, the system was aimed to be applied to a real cultivation process and to be characterized.

The concentration range of interest was given by the initial concentration of the batch medium of up to 40 g l<sup>-1</sup> and the concentration during the fed-batch phase with growth-limited feed of glycerol medium being in the range of few tens mg l<sup>-1</sup>. By this, it covered about three orders of magnitude. Since photometrical detection as a technique of choice can be generally applied only to 1 to 2 orders of magnitude, the analyzer had to enable distinct procedures corresponding to different concentration ranges. Thus, on-line sample dilution, optimization of the analytical method for different concentration ranges, and smart activation of the appropriate working range procedure based on on-line evaluated analytical data were required. Requests in respect of method reliability and stability were similar to the ones described in chapter 4. Benefit was taken of the high versatility and flexibility of the SIA technique by using the manifold described in chapter 4.3.2.

#### 5.2. Determination of glycerol

Glycerol neither does absorb in the visible or near UV spectrum nor does it show any specific reactions or RedOx potential. Thus, lasting HPLC separation at acidic conditions with refraction index or deep UV spectrometric detection is therefore the method of choice for its determination in cultivation media [188,197,200,214,281]. Sensitivity improvement can be achieved by laborious derivatization prior to separation [282]. The dissociation constant of glycerol is about 13, which also enables HPLC separation at highly alkaline conditions using anion exchanger columns [283]. At this conditions, glycerol oxidation is favored and amperometric quantification is possible [284]. In-situ quantification of glycerol in a *P. pastoris* cultivation using near-IR was further reported. However, the required extensive calibration and the low detection sensitivity are considerable limitations [186].

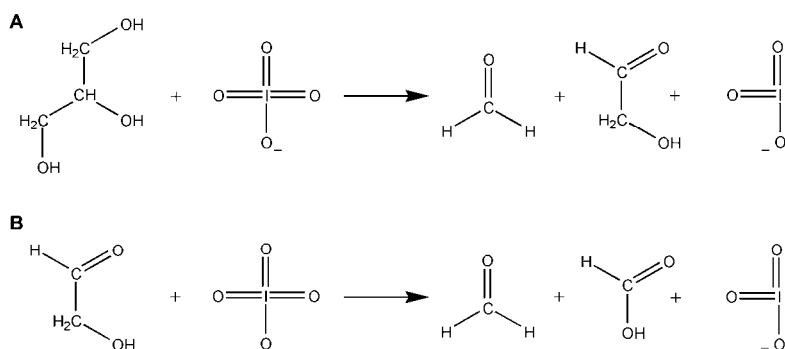
## 5 SIA analyzer for glycerol in *Pichia pastoris* culture medium

Alternatively, enzymes such as glycerol dehydrogenase or glycerol kinase enable methods for glycerol quantification [167,172]. The dehydrogenase reduces the coenzyme  $\text{NAD}^+$  to  $\text{NADH} + \text{H}^+$  under simultaneous dehydrogenation of glycerol. This reaction can be followed either by optical detection such as spectrophotometry [285-288], IR [289], or chemiluminometry [25] or by electrochemical detection such as cyclic voltametry [112] and amperometry [290,291]. Glycerol kinase catalyzes glycerol phosphatation and was applied to glycerol analysis successfully; however, adenosine-5 triphosphate, glycerol phosphate oxidase, peroxidase, and a chromogenetic reagent were additionally required to generate a detectable dye [78]. As main disadvantage, the later enzymes require expensive cofactors.

Glycerol oxidases such as isolated from fungi [292,293] require only dissolved oxygen but are not commercially available yet. Finally, the relatively unspecific galactose oxidase was used for the determination of glycerol [115], but the conversion rate was low compared to other substrates and interference from other medium components was highly probable. Enzymatic methods all suffer from the required sample dilution prior to analysis due to the high sensitivity typically obtained and affection of sample matrix components. Further, the well-known loss of the enzyme efficiency in the case of immobilization and the limited stability of enzymes in solutions as well as cartridges are important drawbacks.

An alternative to enzymatic methods is the oxidation of polyols with periodate, known as the Malaprade reaction. With glycerol, the reaction proceeds in two steps as shown in figure 35, where the second step had been proven to be the faster one. Two mol of formaldehyde and one mol of formic acid [294] result from each mol of glycerol. The reaction was applied to the indirect determination of polyols by quantification of periodate consumption using either optical techniques [295,296] or selective electrodes [109,297,298].

On the other hand, the oxidation product formaldehyde, generated in the Malaprade reaction, can be quantified e.g. by using the Purpald reagent [299]. The determination of glycerol by the selective quantification of the oxidation product was considered to show a considerably lower interference from other periodate reducing compounds compared to the quantification of the periodate consumption, especially, if other oxidation products than formaldehyde are generated [290]. Applicability and improvement of Hantzsch reaction for the determination of formaldehyde by fluorescence coupled to Malaprade reaction for the quantification of glycerol and triglycerides was studied in two former works [294,300].



**Figure 35: Malaprade reaction of periodate with glycerol. The oxidation proceeds in two steps giving formic acid and twice formaldehyde [294].**

## 5 SIA analyzer for glycerol in *Pichia pastoris* culture medium

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As discussed in detail in chapter 4, the Hantzsch reaction is in contrast to other chromogenic methods highly selective for formaldehyde [265], takes place at moderate acidity and temperature, does not require harmful reagents, and has further proven to be compatible with the cultivation medium. By modification of the proposed SIA formaldehyde analyzer [238], the methodology of the coupled Malaprade and Hantzsch reaction was automated using sodium meta-periodate as an additional reagent in the actual work.

### 5.3. Dilution in flow systems

In the aimed system, on-system sample dilution had to be carried out due to the high concentration of the analyte in the batch medium. A multitude of distinct techniques for sample dilution in flow systems have been described so far. Generally the enhancement of the sample dispersion is intended by increase of the tubing inner diameter and length. Besides, continuously flushed mixing chambers have been integrated into the flow manifold, which led to the effective enhancement of the sample dispersion, but required also a high volume of carrier for manifold cleaning after each analysis [36,55,63].

In a former work of the author, an atmospherically open and continuous agitated mixing chamber was used for dilution [92]. Here, the chamber was filled with the diluent and a small sample volume. After reaching homogenization, the dilution was used for analysis. Likewise, additional carrier and time were required for cleaning of the mixing chamber. An alternative is the confluent mixing of sample and diluent; however, the achievable dilution factor is quite low and the diluent consumption is likewise high. Another way to enhance the sample dispersion is the reduction of the injected sample volume such as by splitting, i.e. the partly sample drawing-off from the manifold line after injection. This either requires high precision and timing of the used pump and valves [88,89,301-303], an additional and independently operating pump [26,92,304,305], or a multichannel peristaltic pump in order to enable simultaneous aspiration and dispense [26]. By alteration of confluent mixing and splitting, dilution factors up to 300.000 were achieved in cascade systems [26,301,304].

While confluent mixing is not possible using the single channel technique SIA, sequential operation and flow reversal enables another fast and simple dilution technique: Firstly, the sample is aspirated and propelled into a dilution coil. By this, a dispersion profile is formed with high reproducibility but influenced by the sample viscosity and so by the ambient temperature. Secondly, an aliquot of the dispersed and thus diluted sample is aspirated from the dilution coil and used for the analytical procedure [306,307]. An alternative is the intercalation of water between sample and reagent [24,308]. By this, the zone penetration of reagent and samples is diminished in function of the extension of the intercalated water volume. No cleaning steps, no additional selection valve port, and only one pumping device are required and the dilution factor is adjustable without manifold reconfiguration. In this work, this highly versatile and fast technique was applied. Sample splitting was further accomplished using a technique, which to the best of our knowledge has not been described before.

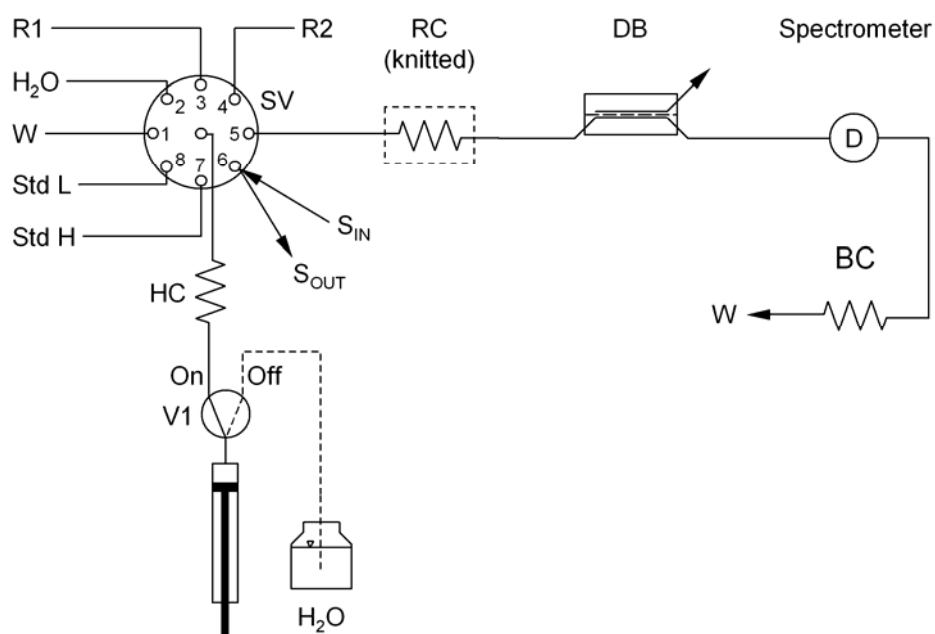
## 5.4. Materials and methods

### 5.4.1. Cultivation process and sampling

The proposed analyzer was tested by at-line monitoring during the batch and glycerol fed-batch phase of a cultivation process similar as described in chapter 4.3.1 using also the same yeast strain. As modifications, the initial batch medium glycerol concentration was lower with  $30 \text{ g l}^{-1}$  and the oxygen content was regulated to 25 % saturation. Growth rate limited feed was accomplished during the glycerol fed-batch phase. As in the former work, the analyzer was connected to the sample transport line from the ESIP probe used for sterile and continuous provision of a cell-free filtrate flow. At an applied ESIP flow rate of  $0.6 \text{ ml min}^{-1}$ , so the effective dead volume caused a calculative delay time of 22.5 min.

### 5.4.2. Analyzer instrumentation

The SIA instrumentation and manifold are shown in figure 36. All components were described in detail in the chapters 3.1, 3.2, and 3.3. Instrumental control and data acquisition were carried out as described chapter 3.4. The same SIA instrumentation and configuration was used as for the formaldehyde SIA analyzer described in chapter 4.3.2.



**Figure 36: Manifold of the SIA analyzer for glycerol.**

**V1: solenoid valve with normally open position (OFF, deactivated) dotted, SV: selection valve with sample (S) via flow-through adapter, standards (Std), waste (W), Hantzsch reagent (R1), and Malaprade reagent (R2), HC: holding coil (150 cm, 1.5 mm i.d.), RC: heated reaction coil (175 cm, 0.8 mm i.d., knitted), DB: debubbler, BC: backpressure coil (185 cm, 0.8 mm i.d.), tubes between SV, RC, DB and detector were 20 cm, 0.8 mm i.d. each.**

## 5 SIA analyzer for glycerol in *Pichia pastoris* culture medium

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Required modifications on the analyzer were the change of the analytical software protocol and used solutions. The lateral ports on the rotary multiposition selection valve during at-line monitoring were used for waste disposal (Pos 1), water (Pos 2), Hantzsch reagent (Pos 3), Malaprade reagent (Pos 4), control standards (Pos 7 and 8), and sample (Pos 6), last one provided via the flow-through adaptor described in chapter 4.3.2.2.

Likewise for the formaldehyde SIA analyzer, the spectrophotometer USB-2000 was used, connected to the support CUV-UV with flow cuvette 75.15 SOG via an optical fiber of 400 nm core diameter. In this work, a blue LED 380UBC (Roithner Lasertechnik GmbH) was used as light source. It was powered by a constant current source (Sciware S.L.), which allowed the adjustment of the light intensity. The LED emission spectrum and the electrical circuitry of the constant current source are given in the annex.

Dual-wavelength detection was done for Schlieren effect compensation. The detection wavelength of  $\lambda_{\text{Det}} = 420$  nm was maintained, however, the reference wavelength had to be changed to  $\lambda_{\text{Ref}} = 460$  nm due to the small emission spectrum of the LED and in order to obtain similar emission intensities on both wavelengths. The absorbance difference was used as analytical signal (see chapter 3.3). The LED was mounted directly onto the focus lens of the cuvette holder using a homemade support as shown with photo in the annex.

### 5.4.3. Reagents

The reagents were prepared as prior described in chapter 3.5.1.2. All reagents were of analytical grade and distilled or Millipore® filtered water was used throughout for the preparation of solutions and used further as carrier. In addition to the Hantzsch reagent with the same composition as used for the formaldehyde analyzer, denoted "reagent 1" (R1), 100 mmol l<sup>-1</sup> sodium meta-periodate solution was used as "reagent 2" (R2) to carry out the Malaprade reaction. Both reagents were proven to be stable for at least five days without any notable increase of the background absorbance or decrease of the signal of a control standard. For a high concentration range (HCR), aqueous standards of 5, 10, 20, 30, and 40 g l<sup>-1</sup> glycerol were generally used for optimization experiments. The 20 g l<sup>-1</sup> standard was used as control standard during at-line monitoring. For a low concentration range (LCR), aqueous standards of ten-times lower concentrations were used.

### 5.4.4. Analytical procedures

#### 5.4.4.1. Sample splitting

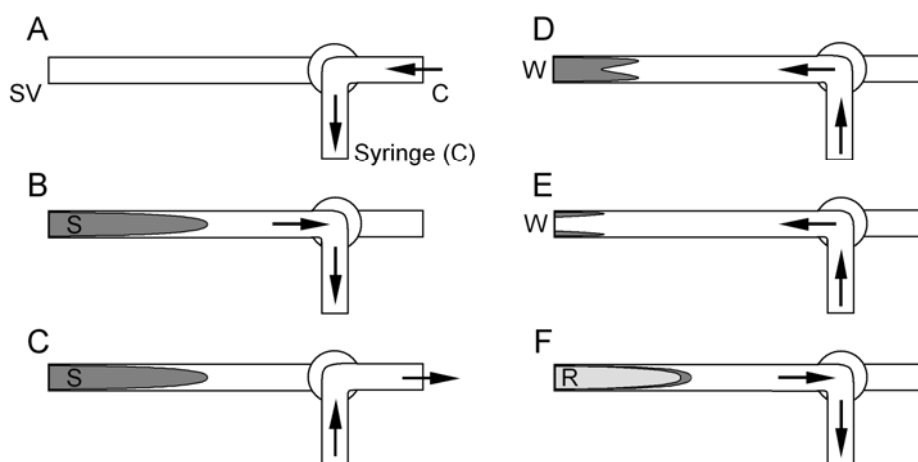
For the high concentration range procedure, a very small sample volume of 8.5 µl had to be used in order to achieve a linear signal response. The reproducible aspiration of such small volumes is sophisticated and is best accomplished by using an injection valve or applying sample splitting, thus by using a second independent pump or a precise

## 5 SIA analyzer for glycerol in *Pichia pastoris* culture medium

MCSV with a very low dead volume. In this work, a fast and simple splitting technique for SIA was developed and successfully applied. It consisted of the following four steps:

1. Aspiration of a small carrier volume in head valve position OFF
2. Aspiration of a small but still precisely manageable volume of sample (ON)
3. Dispense of a small carrier volume in head valve position OFF
4. Dispense of a small but still precisely manageable volume to waste (ON)

Using aspiration and discharge volumes of each 30  $\mu\text{l}$ , due to the sample dispersion in the holding coil during step 2 and 4, a fraction of the sample remains in the holding coil and can be used for the actual analytical procedure. This procedure allowed sampling of very small volumes with a demonstrable higher reproducibility than obtained by the direct aspiration. Steps 1 and 3 were required to minimize the volumetric error due to the thread backlash of a few tenth millimeter of the axle-drive shaft of the syringe module, which otherwise affected at the reversal of the piston movement direction. To the best of our knowledge, this splitting technique has not been described before. The procedure is shown schematically in figure 37 and a respective study is described in chapter 5.5.2.



**Figure 37: Scheme of the applied procedure for sample splitting.**

**A:** Carrier (C) is aspirated in from the reservoir in order to overcome the syringe backlash, **B:** Sample (S) is aspirated from the selection valve (SV) into the syringe, **C:** Carrier (C) is dispensed to the reservoir to overcome the syringe backlash, **D-E:** The mayor part of the sample is dispensed to Waste (W); due to the dispersion, a fraction of the sample remains in the holding coil, **F:** The procedure is continued by the aspiration of water or reagent (R).

### 5.4.4.2. Zone stacking and in-line dilution

Due to the high initial batch medium concentration of glycerol and the comparably high sensitivity of the accomplished Hantzsch reaction, aspiration and in-line dilution of a small sample had to be automated. It was observed that both requests were imperative in order to achieve a linear response over the aimed glycerol concentration ranges.



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Two procedures were optimized, which instructed two distinct analytical protocols, both applying the prior described intercalation technique: water intercalated between the sample and the both reagents. By this, two dynamic working ranges - complementing each other - were enabled. To enhance the dispersion of the sample segment and by this, the dilution factor, the solutions were aspirated in the order: sample, water, Hantzsch reagent (R1), and Malaprade reagent (R2). So the penetration of the sample with the Hantzsch reagent was higher than with the Malaprade reagent. Consequently, oxidation of only a part of the glycerol in the sample to formaldehyde by the Malaprade reagent was promoted equivalent to a "chemical" sample dilution. The Hantzsch reaction was the slower one of both applied reactions. Consequently, the reversion of the aspiration order, i.e. first R2 followed by R1, would have favored the formation of formaldehyde but not the formation of the signal-giving product DDL. Since dilution of glycerol and thus, limitation of formaldehyde formation was aimed but not the partial quantification of the formed formaldehyde, the chosen aspiration order was the best option.

The concentration ranges resulted from the maximal initial glycerol concentration of the batch medium of  $40 \text{ g l}^{-1}$ . Consequently, the aimed sensitivity for the high concentration range (HCR) procedure was  $0.025 \text{ AU l g}^{-1}$ . Considering a working range of 1.5 orders of magnitude applying spectrophotometric detection, an about ten-fold higher sensitivity was required for the low concentration range (LCR) procedure. Applying the LCR procedure ( $0.2 - 4 \text{ g l}^{-1}$ ),  $25 \mu\text{l}$  of sample,  $40 \mu\text{l}$  of water,  $100 \mu\text{l}$  of reagent 1, and  $40 \mu\text{l}$  of reagent 2 were aspirated in succession into the holding coil. Applying the HCR procedure ( $2 - 40 \text{ g l}^{-1}$ ),  $8.5 \mu\text{l}$  of sample (see chapter 5.4.4.1),  $300 \mu\text{l}$  of water,  $100 \mu\text{l}$  of reagent 1 and  $80 \mu\text{l}$  of reagent 2 were aspirated in succession into the holding coil. In both procedures, the stacked solutions were afterwards propelled towards the thermostated reaction coil and incubated for 80 s at  $45^\circ\text{C}$ . During incubation, the syringe was refilled with carrier. In a final step, the mixture with the formed reaction product DDL was propelled towards the detection flow cell for quantification. The applied sample volumes were small in both procedures. Therefore, the dead volume of the selection valve rotor and carry-over by rotor movement had to be taken into account and at the beginning of each cycle, first,  $20 \mu\text{l}$  of the sample were discharged to clean the dead volume of the valve port. Solution carry-over at the valve rotor movement was diminished by waiting 2 s after each aspiration.

### 5.4.5. Software and program structure

Instrumental control, data acquisition, and data processing were carried out using the software package AutoAnalysis 5.0 described in detail in chapter 3.4 running on a PC with operating system MS-Windows 2000. Methods and procedures used for analysis and for system cleaning are given in the annex. To apply the SIA analyzer for automated glycerol monitoring, autonomous decision at each process time about the application of the adequate procedure (high or concentration range, respectively) had to be accomplished in the analytical method. So, conditional inquiries about the on-line acquired peak maximum

## 5 SIA analyzer for glycerol in Pichia pastoris culture medium

were integrated, which enabled the “self-configuration” of the system [309]. To lower the probability that an error peak e.g. caused by an air bubble could lead to the change of the working range, average values of two successive peaks were used as decision criteria.

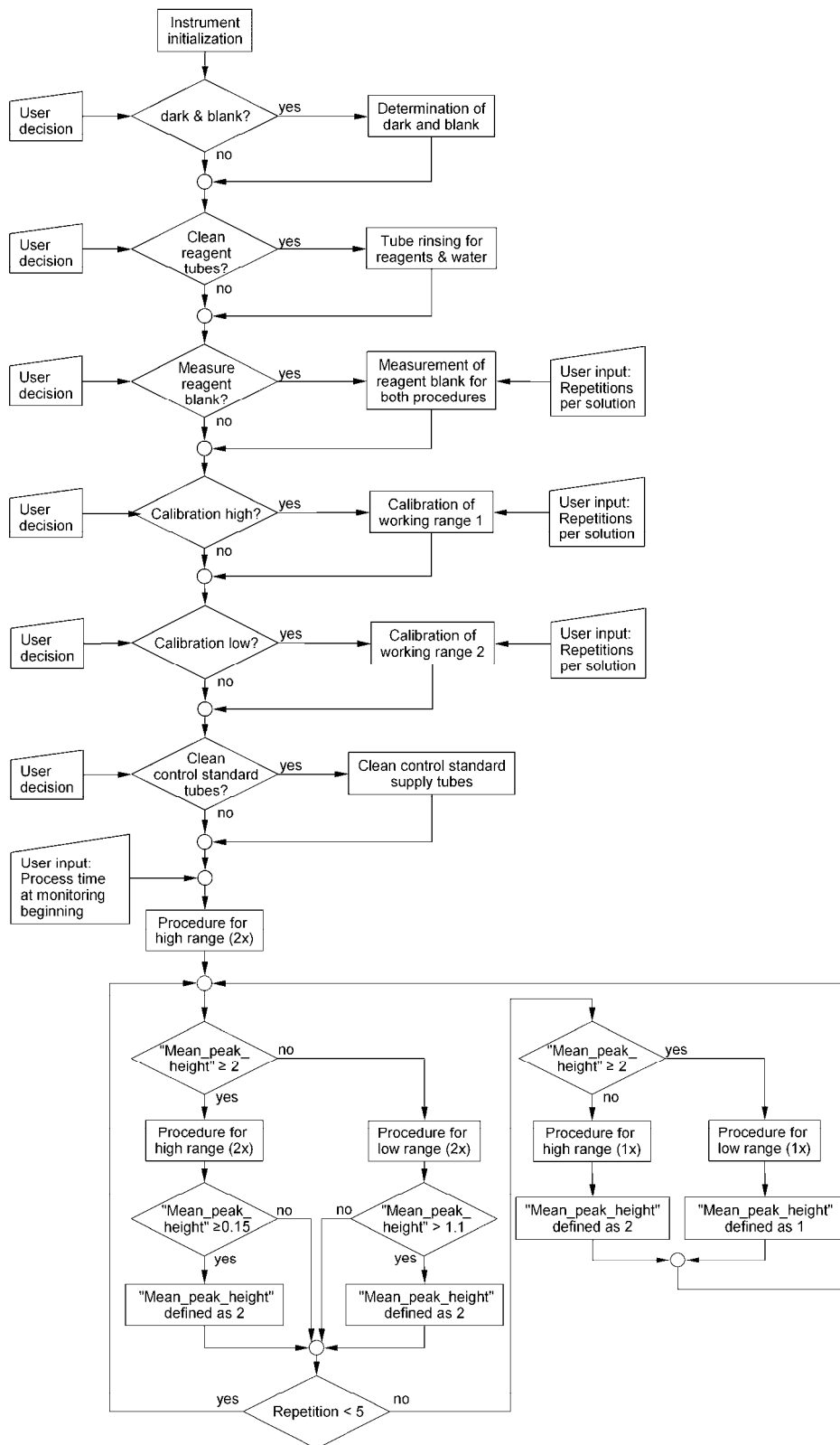


Figure 38: Program scheme of the method used for at-line monitoring of glycerol.

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The evaluation of the method's stability and robustness during at-line monitoring was aimed. For this, an adequate control standard for the active working range was measured once of after each tenth sample measurement. Calibration and cleaning procedures were done software-guided. Here, conditional inquiries enabled skipping certain sections of the methods or modifying the number of repetitions as requested by the user.

The full method scheme is given in figure 38. The first part instructs the initialization and calibration as well as cleaning of supplying tubes. The second part instructs the activation of either the HCR or the LCR procedure for both the sample and the control standard measurement depending on the variable "Mean\_peak\_height", being the mean value of the maxima of two successive peaks. If this variable falls short of 0.15 using the HCR procedure, the more sensitive LCR procedure is used for the next sample or control standard. On the other hand, if the variable exceeds 1.1 using the LCR procedure, the less sensitive HCR procedure is used for the next sample or control standard. The control standards were located on the lateral ports 7 and 8 on the rotary selection valve.

### 5.4.6. Reference method

Monitoring data obtained with the glycerol SIA analyzer were compared with the analytical results from reference samples. The samples were taken during the glycerol batch and the fed-batch phase from the continuous filtrate flow. Besides, medium samples were also taken directly from the bioreactor vessel via a sampling valve outlet and immediately centrifuged for 5 min at 4°C at 20.500 g. The samples were stored at -20°C.

Quantification of the reference samples was done offline by HPLC using a protocol, which was established and carried out by members of the collaborating working group. Prior to analysis, the samples were filtered through 0.2 µm syringe filters Minisart RC 4 (Sartorius BBI Systems, Göttingen, Germany). A HPLC system (Merck Hitachi, Hannover, Germany) with refraction index detector LaChrom L7490 equipped with a Monosaccharide RHM column and guard column (Phenomenex, Aschaffenburg, Germany) was used. The column was thermostated at 50°C. Water was used as eluent at a flow rate of 0.6 ml min<sup>-1</sup>. Separation was carried out within 22 min. Calibration was done with aqueous glycerol standards. Using an injection volume of 20 µl, the linear working range was 0.5 - 30 g l<sup>-1</sup>.

## 5.5. Experiments and results

### 5.5.1. Optimization detection parameters

The detection parameters such as wavelengths, integration time, or spectral smoothing were optimized in order to obtain a smooth baseline. This task was an important requirement to allow the correct on-line peak detection for the evaluation and smart software decision about the required working range or analytical procedure, respectively.

## 5 SIA analyzer for glycerol in *Pichia pastoris* culture medium

Due to the high emission stability of the LED, the baseline noise was considerably reduced compared to the experimental work 1 (chapter 4) where a commercial deuterium lamp had been used. Likewise, an improved compensation of the schlieren effect was achieved due to the small difference of detection and reference wavelength.

### 5.5.2. Sample splitting

The influence of the sample aspiration volume and discharge volume used for the splitting procedure described in chapter 5.4.4.1 on the remaining sample volume in the holding coil was studied. Firstly, a  $40 \mu\text{mol l}^{-1}$  fluorescein solution was used as sample. Different volumes were applied for the aspiration and discharge step and afterwards, the remaining sample volume was quantified by dispense towards the detection cell. The signals obtained for a zero-discharge volume were used as calibration. Secondly, a  $40 \text{ g l}^{-1}$  glycerol aqueous standard was used and the remaining sample volume was collected into plastic vials of 1.5 ml applying a dispense volume of 250  $\mu\text{l}$ . After mixing, the collected samples were quantified using the LCR procedure in comparison with aqueous standards. The results are given in table 10 and are represented in figure 39.

It resulted, that the volumes obtained for the fluorescein solution were significantly smaller and allowed only a rough estimation of the final volume. It was observed in preliminary experiments, that a volume of 10  $\mu\text{l}$  already resulted in saturation for glycerol concentrations higher than  $30 \text{ g l}^{-1}$ . In order to apply volumes, which can be handled with high precision but also to obtain a highly reproducible sample volume of less than 10  $\mu\text{l}$ , the combination of 30  $\mu\text{l}$  sample aspiration and 30  $\mu\text{l}$  sample discharge was chosen. This combination led to an effective sample volume of 8.6  $\mu\text{l}$ .

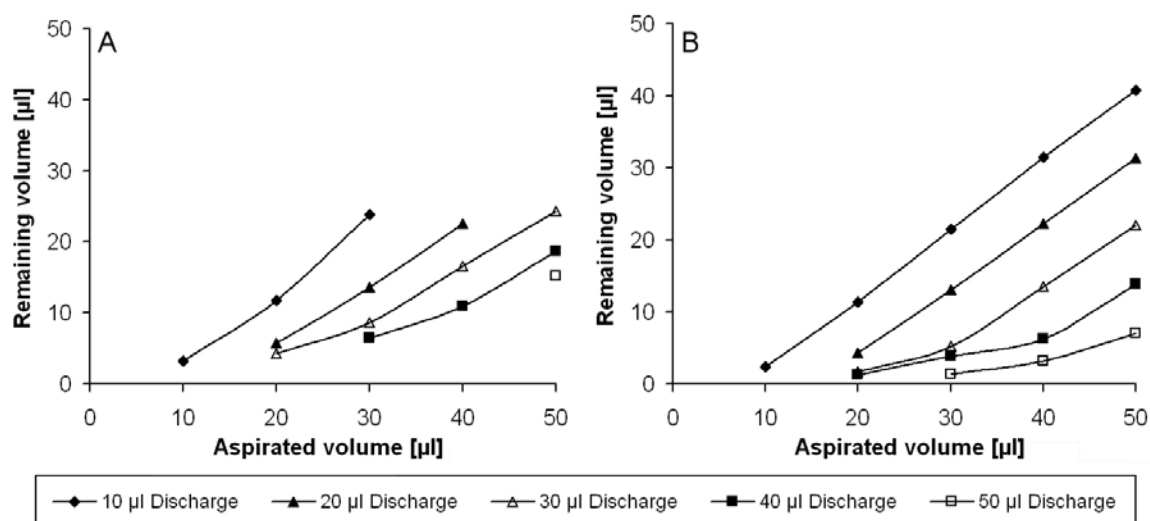


Figure 39: Study of the splitting procedure.

Either  $40 \text{ g l}^{-1}$  glycerol standard (A, quantification of the remaining sample volume with the LCR procedure) or  $40 \mu\text{mol l}^{-1}$  fluorescein solution (B, quantification of the remaining volume over peak height) was used.

## 5 SIA analyzer for glycerol in *Pichia pastoris* culture medium

**Table 10: Remaining sample volume in the holding coil after sample aspiration and partial discharge in dependency on the applied volumes.**

Aspirated volume [ $\mu\text{l}$ ]	10	20	30	40	50
Discharged volume [ $\mu\text{l}$ ]	Remaining sample volumes [ $\mu\text{l}$ ] (n=3) *				
10	3.2	11.8	23.9	-	-
20	-	5.8	13.6	22.6	-
30	-	4.3	8.6	16.6	24.3
40	-	-	6.4	10.9	18.7
50	-	-	-	-	15.2

Discharged volume [ $\mu\text{l}$ ]	Remaining sample volumes [ $\mu\text{l}$ ] (n=3) **				
10	2.4	11.4	21.5	31.5	40.8
20	-	4.3	13.1	22.3	31.4
30	-	1.7	5.2	13.5	22.1
40	-	1.2	3.9	6.3	13.9
50	-	-	1.4	3.2	7.1

\* Use of a  $40 \text{ g l}^{-1}$  glycerol standard. After execution of the splitting procedure,  $250 \mu\text{l}$  including the remaining sample volume were dispensed and collected for quantification with the LCR procedure.

\*\* Use of a  $40 \mu\text{mol l}^{-1}$  fluorescein solution. After execution of the splitting procedure, the remaining volume was dispensed towards the detection cell.  $\lambda_{\text{Det}} = 450 \text{ nm}$  and  $\lambda_{\text{Ref}} = 520 \text{ nm}$ .

The repeatability of measurements (n=3) applying either a sample volume of  $10 \mu\text{l}$  obtained by direct aspiration or a sample volume of  $8.6 \mu\text{l}$  obtained with the splitting procedure and chosen volume combination was compared using five aqueous standards. The mean RSD value was  $4.8 \% \pm 1.7 \%$  for the direct aspiration and  $3.6 \% \pm 1.3 \%$  for the splitting procedure. At the same time, the slope of the calibration function was  $17 \%$  reduced applying the splitting procedure. In conclusion, the splitting procedure allowed the provision of a smaller sample volume with higher reproducibility.

### 5.5.3. Zone stacking and in-line dilution

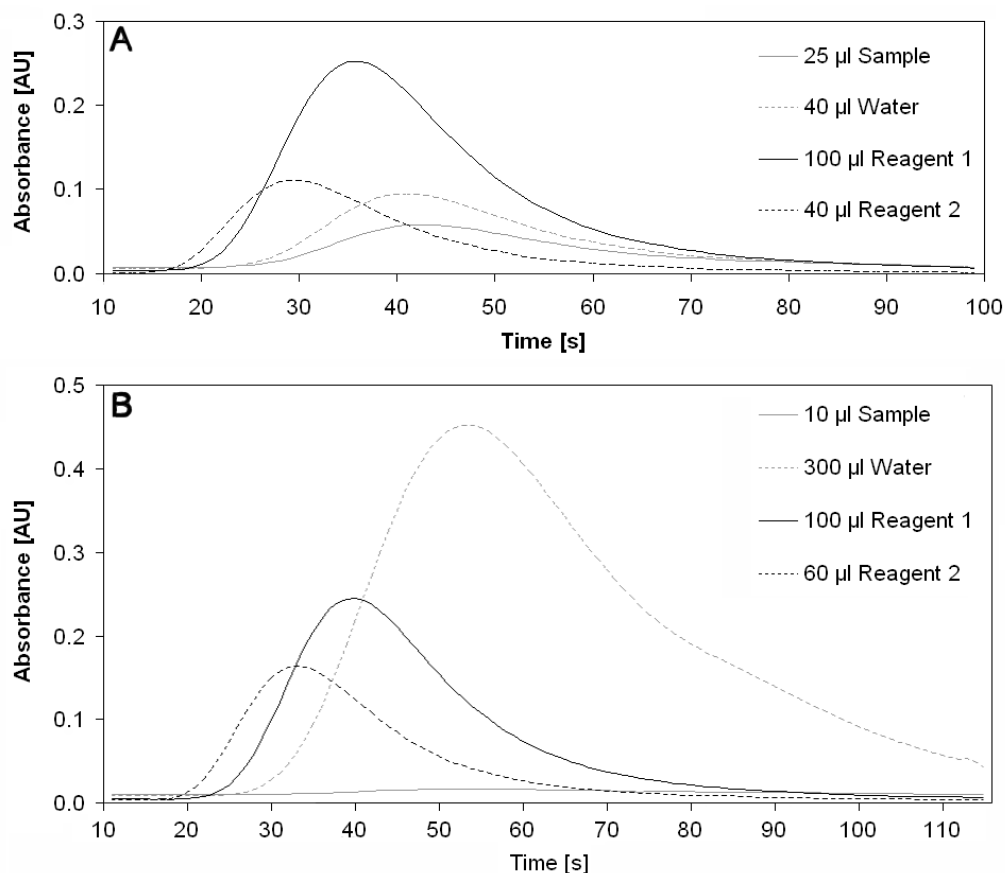
The efficient and fast dilution of the sample in the system was required for both procedures due to the considerably high concentration of glycerol in the cultivation medium in the order of part per mil. In the second, it was aimed to diminish matrix effects including the blank caused by the yellow medium color and the medium viscosity.

The final procedure and penetration of the stacked solutions by dispersion is shown in figure 40 with peak data and dispersion coefficient given in table 11. The visualization was done by averaging three runs using  $40 \mu\text{mol l}^{-1}$  fluorescein for always one zone and water for the others applying spectrophotometric detection at  $\lambda_{\text{Det}} = 450 \text{ nm}$  and  $\lambda_{\text{Ref}} = 520 \text{ nm}$ .

From the dispersion coefficients it can be seen that the sample segments were diluted in respect of the reagent 1 about 5- and 31-fold, respectively and the penetration of the

## 5 SIA analyzer for glycerol in *Pichia pastoris* culture medium

solution zone segments is not complete. So as aimed, only a fragment of the glycerol in the sample is oxidized to formaldehyde and can lead to the final product DDL.



**Figure 40: Zone stacked sample, water, R1, and R2 for the LCR (A) and HCR procedure (B). Fluorescein  $40 \mu\text{mol l}^{-1}$  with spectrophotometric detection at  $\lambda_{\text{Det}} = 450 \text{ nm}$  and  $\lambda_{\text{Ref}} = 520 \text{ nm}$ .**

**Table 11: Determination of dispersion coefficients for the analytical glycerol procedures.\***

LCR procedure	Volume [ $\mu\text{l}$ ]	Peak height (n = 3)	D
Sample	25	$0.051 \pm 0.002$	22.1
Water	40	$0.089 \pm 0.001$	12.7
R1	100	$0.249 \pm 0.001$	4.5
R2	40	$0.110 \pm 0.001$	10.2
HCR procedure	Volume [ $\mu\text{l}$ ]	Peak height (n = 3)	D
Sample	8.6	$0.008 \pm 0.001$	145
Water	300	$0.443 \pm 0.001$	2.5
R1	100	$0.240 \pm 0.002$	4.7
R2	60	$0.160 \pm 0.001$	7.1

\* A  $40 \mu\text{M}$  fluorescein solution was used for the experiment. The steady-state signal was 1.125

### 5.5.4. Optimization of the analytical method

#### 5.5.4.1. Preliminary remarks

In preliminary experiments it was observed that achievement of linearity over the aimed working ranges was a considerable problem due to the elevated analyte concentration in the cultivation medium. Further, degradation of AcAc and DDL in the presence of an excess of periodate was observed. Finally, the slow formation kinetic of the final product DDL prolonged the analysis time and limited notably the injection frequency. Main optimization criteria were linearity and the requested sensitivity over the aimed working ranges from 2 to 40 g l<sup>-1</sup> and 0.2 to 4 g l<sup>-1</sup> requiring sensitivity values of 0.025 AU l g<sup>-1</sup> and 0.25 AU l g<sup>-1</sup>, respectively, as former described in chapter 5.1.

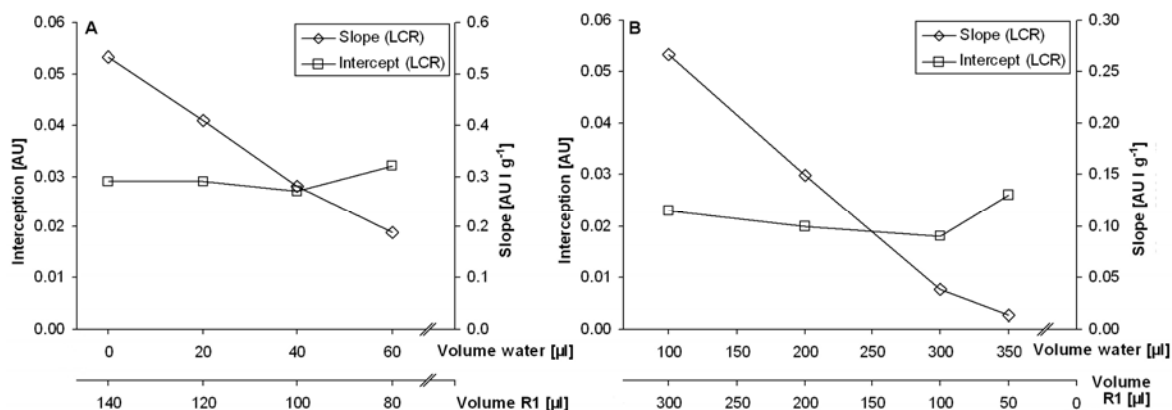
The quasi-simultaneous monitoring of glycerol and formaldehyde or at least the possibility to switch rapidly between both parameters using the same analyzer system was aimed. Therefore, the composition of R1 was not re-optimized and the manifold and incubation temperature of the reaction coil were maintained from experimental work 1. In the second, optimization in experimental work 1 showed that incubation temperatures higher than 45 °C lead to enhanced gas bubble formation in the reaction coil. On the other hand, the composition of R1 was a compromise between satisfying reagent stability, a low blank value, and resulting detection sensitivity.

#### 5.5.4.2. Optimization of volumes of Hantzsch reagent (R1) and water

The influences of the volumes of reagent 1 and water were studied by increasing one and decreasing the other while maintaining the total volume constant. For the LCR procedure, the volume of water was varied between zero to 60 µl and of reagent 1 between 140 µl to 80 µl giving a total volume of 140 µl. For the HCR procedure, the volume of water was varied between 100 µl to 350 µl and of reagent 1 between 300 µl to 50 µl giving a total volume of 400 µl. Slope and intercept of a linear calibration using aqueous standards were evaluated. Experimental conditions and results are given in table 12 and are represented in figure 41.

In order to achieve the aimed sensitivity values, the combination of 40 µl water and 100 µl reagent 1 was chosen for the LCR procedure applying a sample volume of 25 µl. for the HCR procedure, the combination of 300 µl water and 100 µl reagent 1 was chosen applying a sample volume of 10 µl. The calibration functions showed for both procedures a notable curvature increasing the volume of reagent 1 whereas for smaller volumes whereas the required sensitivity values were not achieved for the aimed working ranges.

## 5 SIA analyzer for glycerol in *Pichia pastoris* culture medium



**Figure 41: Influence of the volumes of reagent 1 and water on the sensitivity and blank value for the LCR procedure (A) and the HCR procedure (B).**

**Table 12: Influence of the volume of water and reagent 1 on the slope and intercept values of the linear calibration functions obtained for both procedures.**

Procedure LCR *	Volume R1 [ $\mu\text{l}$ ]	Volume H <sub>2</sub> O [ $\mu\text{l}$ ]	Slope	Intercept	r <sup>2</sup>
	140	0	0.533	0.029	0.975
	120	20	0.409	0.029	0.959
	100	40	0.281	0.027	0.960
	80	60	0.189	0.032	0.978
Procedure HCR **	Volume R1 [ $\mu\text{l}$ ]	Volume H <sub>2</sub> O [ $\mu\text{l}$ ]	Slope	Intercept	r <sup>2</sup>
	300	100	0.267	0.023	0.959
	200	200	0.149	0.020	0.999
	100	300	0.038	0.018	0.966
	50	350	0.013	0.026	0.879

\* Conditions LCR procedure: Incubation at 45°C for 60 s, 25  $\mu\text{l}$  sample, 40  $\mu\text{l}$  reagent 2, aqueous standards: 0, 1, 2, and 4 g l<sup>-1</sup>

\*\* Conditions HCR procedure: Incubation at 45°C for 80 s, 10  $\mu\text{l}$  sample, 60  $\mu\text{l}$  reagent 2, aqueous standards: 0, 10, 20, and 40 g l<sup>-1</sup>

### 5.5.4.3. Optimization of volume of Malaprade reagent (R2) and incubation time

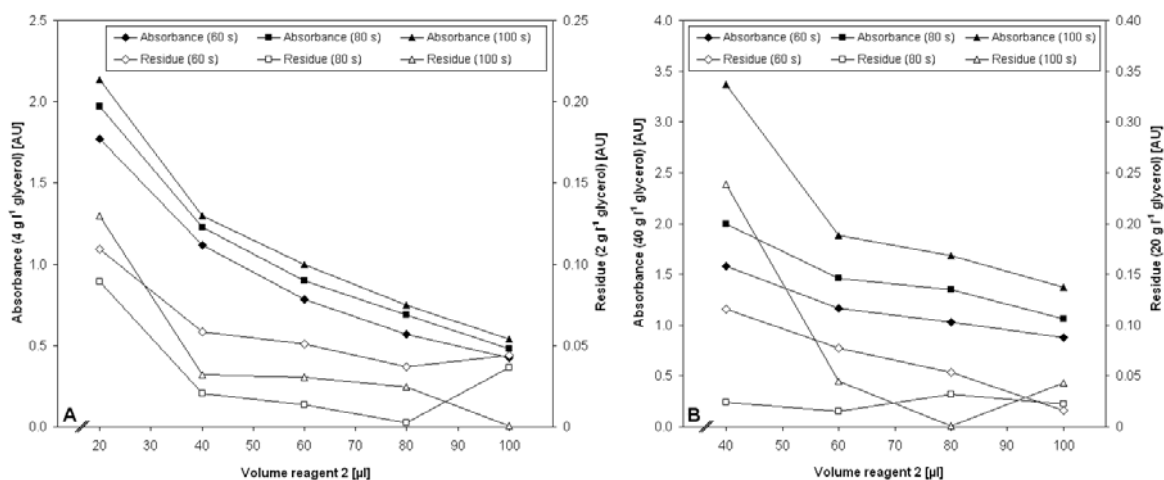
The influences of the volume of R2 and the incubation time were studied conjointly. For both procedures, the volume of R2 was varied between 20  $\mu\text{l}$  to 100  $\mu\text{l}$  for the incubation times 60 s, 80 s, and 100 s. Calibrations were performed using aqueous standards. The peak height of the highest standard and linearity as described in chapter 3.6.2 were evaluated. The results are given in table 13 and are represented in figure 42.

It was observed for both procedures that increase of volume of R2 led to a decrease of the calibration function curvature but simultaneously to a considerable decrease of the method sensitivity evaluated by the signal yielding the highest standard. Applying a volume of 20  $\mu\text{l}$  R2 for the HCR procedure, the peak heights for the highest standard were out of range and were therefore not represented. These observances could be related to



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the oxidation of the reaction product by periodate. In test tube experiments, discoloration of a DDL solution was observed in the presence of periodate. So matrix components, which are oxidized by periodate could lead to the increase of the method sensitivity.



**Figure 42: Influence of the volume of reagent 2 and incubation time on sensitivity and linearity for the LCR procedure (A) and the HCR procedure (B).**

**Table 13: Influence of the volume of reagent 2 and incubation time on sensitivity and linearity for both procedures.**

Procedure LCR *	Incubation time	60 s	80 s	100 s	60 s	80 s	100 s
	Volume	Peak height [AU] (40 g l <sup>-1</sup> )			Center regression residue		
	20 μl	1.77	1.97	2.14	0.109	0.089	0.130
	40 μl	1.12	1.23	1.30	0.058	0.021	0.032
	60 μl	0.78	0.90	1.00	0.051	0.013	0.030
	80 μl	0.57	0.69	0.75	0.037	0.002	0.025
	100 μl	0.43	0.48	0.54	0.044	0.037	0.000
Procedure HCR **	Incubation time	60 s	80 s	100 s	60 s	80 s	100 s
	Volume	Peak height [AU] (4 g l <sup>-1</sup> )			Center regression residue		
	40 μl	1.58	2.00	3.37	0.564	0.278	1.000
	60 μl	1.17	1.46	1.88	0.116	0.024	0.239
	80 μl	1.03	1.35	1.68	0.077	0.015	0.045
	100 μl	0.88	1.06	1.37	0.054	0.032	0.001

\* Conditions: Incubation 45°C, 25 μl standard, 40 μl water, 100 μl reagent 1, aqueous standards: 0, 1, 2, 3, and 4 g l<sup>-1</sup>, regression residue for standard 2 g l<sup>-1</sup>

\*\* Conditions: Incubation at 45°C, 8.6 μl standard, 300 μl water, 100 μl reagent 1, aqueous standards: 0, 10, 20, 30, and 40 g l<sup>-1</sup>, regression residue for standard 20 g l<sup>-1</sup>

The sensitivity of the HCR procedure increased considerably with the incubation time, whereas the influence was only moderately for the LCR procedure indicating a better mixture of the stacked solutions in the LCR solution and lower formaldehyde generation. The curvatures of the calibration functions were minimal and less affected by the volume

## 5 SIA analyzer for glycerol in *Pichia pastoris* culture medium

of reagent 2 for an incubation time of 80 s in both procedures. As a compromise between linearity, sensitivity, and time of analysis, 40  $\mu\text{l}$  and 80  $\mu\text{l}$  of reagent 2 were selected for the LCR and HCR procedure, respectively, applying for both an incubation time of 80 s.

### 5.5.5. Interference studies

Single components of the basal salt medium, metabolites, polyols, and saccharides were tested regarding to their interference on both analytical procedures. The compounds were tested as aqueous solutions in similar or higher concentrations than found in the cultivation medium. Likewise, the basal salt medium containing glycerol and the trace element solution composed as given in table 1 was tested.

No significant interferences or blank signals above the LOQ were found for the following organic compounds at the given concentrations: methanol, ethanol, and propanol (each 5  $\text{g l}^{-1}$ ), acetic, tartaric, and oxalic acid (each 1  $\text{g l}^{-1}$ ), and sodium pyruvate (1.25  $\text{g l}^{-1}$ ). No significant signal was further observed using the basal salt medium without glycerol. The analyte recoveries for glycerol in real cultivation medium spiked with glycerol were between 99.5 % and 105.8 % for the LCR procedure and between 114 % and 139 % for the HCR. Omitting the trace element solution caused a signal decrease of about 10 %. The high recovery values found for the HCR procedure are discussed in chapter 5.6.2.

Several compounds were found to interfere the analysis and are listed in table 14 with their relative sensitivity compared to an equal glycerol concentration. The relative sensitivity was generally higher for the HCR procedure while the effective interference was lower for the HCR procedure as it showed an about ten-fold lower sensitivity.

**Table 14: Sensitivity values relative to glycerol for both used analytical procedures \*.**

Species	Concentration [ $\text{g l}^{-1}$ ]	Relative sensitivity HCR procedure	Relative sensitivity LCR procedure
Formaldehyde	0.02	76 x (0.04 AU)	27 x (0.22 AU)
Glycerinaldehyde	4	63.9 %	52.3 %
Glyoxal	4	0.4 %	3.7 %
Glycol	5	97.3 %	39.1 %
Sorbitol	Calibration standards*	90.9 %	104 %
Mannitol	5	67.9 %	76.4 %
Galactose	10	23.4 %	11.5 %
Glucose	10	19.6 %	9.0 %
Lactose	10	6.6 %	0.2 %
Maltose	10	8.2 %	1.1 %

\* Standards of 5, 10, 15, 20, and 30  $\text{g l}^{-1}$  and 0.5, 1, 2, and 4  $\text{g l}^{-1}$ , respectively

Similar sensitivity as for glycerol was found for polyols. Glycerolaldehyde showed an about 50 % lower response since formaldehyde is generated equimolar whereas two

## 5 SIA analyzer for glycerol in *Pichia pastoris* culture medium

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molecules of formaldehyde are per each molecule of glycerol. The found sensitivities were considerably reduced for the tested monosaccharides and even more, disaccharides. The relative sensitivity for formaldehyde used in a concentration of 20 mg l<sup>-1</sup> was the highest one observed. However, it is formed only during at methanol feed and the concentration level was former observed to be generally less than 6 mg l<sup>-1</sup> (see chapter 4).

It was found that the method could be adapted to the quantification of sorbitol as another polyol used in cultivation processes of *Pichia pastoris* (see chapter 6). Linear responses were obtained for the concentration ranges of 0.5-2 g l<sup>-1</sup> and 5-20 g l<sup>-1</sup>. The sensitivities obtained for sorbitol and glycerol differed less than 10 % for both procedures. Although the applicability to other polyols might be advantageous, it is noteworthy that reliable monitoring of glycerol is only possible if the concentrations of these compounds in the cultivation medium are low or can be compensated by additional quantification.

### 5.5.6. Cultivation process monitoring

Glycerol monitoring was carried out in a real cultivation process of the yeast *Pichia pastoris* during glycerol batch and fed-batch phase over about 30 h at the Research Center of Bioprocess Engineering and Analytical Techniques (see chapter 5.4.1).

The SIA analyzer was coupled via the flow-through adapter to a medium filtrate flow sampled continuously from the process as described in chapter 4.3.1.1. After six hours of monitoring, a second Trace trap debubbler was incorporated into the medium filtrate line upstream of the SIA analyzer in order to eliminate observed air bubbles. These bubbles originated from the cultivation medium passing through the filtration probe membrane.

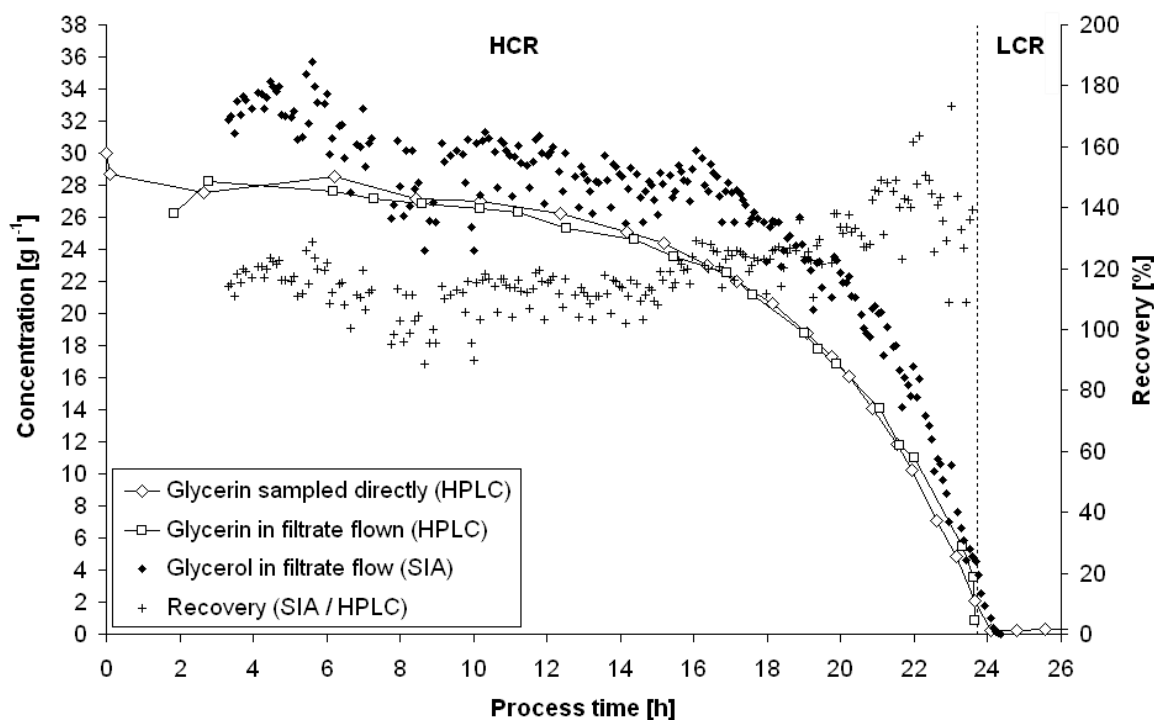
The cultivation process was started with an initial glycerol concentration of 30 g l<sup>-1</sup>. The decrease of the glycerol concentration was low during the first 10 h of monitoring but accelerated afterwards with increasing cell density of the medium following the typical depletion curve for exponential yeasts growth. After 23.5 h, the process was continued as fed-batch with cell growth limited feed of glycerol medium.

Change from the HCR procedure to the more sensitive LCR procedure was done automatically when the average height of two following peaks fall below 0.15 AU at 23.7 h. It was noted, that a higher threshold level should have been chosen since the glycerol concentration in the medium decreased so fast that only a few measurements could be carried out until the glycerol concentration was below the LOD of the procedure (24.3 h).

The concentration course of glycerol measured with the proposed SIA analyzer system as well as the concentration values obtained with HPLC are given in figure 43 including the analyte recovery. It was calculated by dividing the concentration values obtained with SIA analyzer by the interpolated concentration values obtained with HPLC using the filtrate flow samples. Comparing the glycerol concentrations measured with HPLC in the cultivation medium sampled directly from the bioreactor vessel and in the medium filtrate flow, the differences were below 5 %. This small difference can be attributed to the time

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delay of the continuous medium filtrate line as well as to the time, during which metabolism could still take place in the directly cultivation medium until the cells were removed centrifugation. In conclusion, sampling of the cultivation medium using the inline filtration probe proved to be representative with respect to glycerol.



**Figure 43: Concentration of glycerol sampled directly or from the filtrate flow and measured off-line with HPLC as well as at-line in the filtrate flow with the SIA analyzer.**

The recovery values were calculated by dividing the concentration values obtained with the SIA analyzer by the interpolated concentration values obtained with HPLC (Filtrate flow).

## 5.6. Discussion

### 5.6.1. Analytical characterization

#### 5.6.1.1. Linear working range, reproducibility, and detection limit

The repeatability of both procedures was calculated as the relative standard deviation from 12 consecutive injections of aqueous glycerol standards of  $0.2 \text{ g l}^{-1}$  ( $< 3 \%$ ) and  $4 \text{ g l}^{-1}$  ( $< 1 \%$ ) for the LCR and  $2 \text{ g l}^{-1}$  ( $< 4 \%$ ) and  $40 \text{ g l}^{-1}$  ( $< 2 \%$ ) for the HCR procedure.

Using the optimized experimental conditions, linear dynamic ranges were obtained for  $0.1\text{-}3 \text{ g l}^{-1}$  (LCR) and for  $1\text{-}30 \text{ g l}^{-1}$  glycerol (HCR), respectively. At higher concentrations up to  $4 \text{ g l}^{-1}$  (LCR) and  $40 \text{ g l}^{-1}$  (HCR), the calibration graphs for both procedures showed a notable curvature. The limits of detection, calculated as the concentration yielding a signal to the blank average plus three times the standard deviation of the blank ( $n=10$ ) were

## 5 SIA analyzer for glycerol in *Pichia pastoris* culture medium

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30 mg l<sup>-1</sup> (LCR) and 80 mg l<sup>-1</sup> (HCR), respectively. Using the blank value of the basal salt medium for calculation, the LOD values were two-fold higher.

### 5.6.1.2. Stability of the method

The stability of the method stability was tested over a period of 120 h. For this, a 2 g l<sup>-1</sup> glycerol standard was determined every 15 min in triplicate applying both the LCR and the HCR procedure. During the first 80 h, peak heights did not trend to increase or decrease significantly, showing a RSD values of 7.4 % (HCR) and 9.4 (LCR) over the entire observation time and a mean RSD values of 3.1 % (HCR) and 3.5 % (LCR) for each quantification triplicate, respectively. Afterwards, a decrease of the peak heights was observed probably due to microbiological degradation of the glycerol standard.

### 5.6.1.3. Solution consumption and sampling frequency

The consumption of sample for one determination was 50 µl for the HCR procedure and 45 µl for the LCR procedure. A volume of 100 µl of reagent 1 was required for both procedures. The consumption of reagent 2 was 80 µl and 40 µl, respectively. The volume of water used for dilution and as carrier was 3.72 ml and 3.46 ml respectively. One liter of water and 50 ml of reagent 1 and 25 ml of reagent 2 lasted for 20 hours of monitoring. A sampling frequency of 12 peaks per hour for the HCR procedure and 14 per hour for the LCR procedure were achieved for repeated measurement of the same sample solution or applying sampling from the flow-through adapter, i.e. without cleaning of supplying tubes.

### 5.6.1.4. Influence of ambient temperature

The analyzer system was considered to operate with high reliability and stability. Testing the system over a period of five days by measuring a control standard repeatedly, day-to-night oscillations of the peak height were observed probably related to ambient temperature fluctuations. In order to estimate the effect of ambient temperature on the peak height, quantification of glycerol standards (2 g l<sup>-1</sup> and 20 g l<sup>-1</sup>, respectively) was done with both procedures while the entire SIA system was ventilated with air of 23°C, 27°C, and 30°C. The LCR procedure was not affected significantly by the change of the temperature. In contrast, a temperature coefficient was found for the HCR procedure with a peak height increase of 2 % /°K<sup>-1</sup>. This was reduced to the decreasing sample viscosity with increasing temperature, which leads to a higher dispersion and thus to a higher sample volume remaining in the holding coil after the sample splitting procedure.

### 5.6.1.5. Quasi-simultaneous monitoring of formaldehyde and glycerol

Quasi-simultaneous monitoring of glycerol and formaldehyde was tested by alternated carrying out both analytical methods. In order to avoid carry-over of periodate during formaldehyde quantification, cleaning of the manifold and valve ports was done. Besides, a small volume of water was pushed into the supplying tube of reagent 2 prior to

## 5 SIA analyzer for glycerol in *Pichia pastoris* culture medium

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formaldehyde quantification. By these measurements, the carry-over of periodate was diminished notably. However, even the dispense of 100  $\mu\text{l}$  of water into the supplying tube of reagent 2 was not sufficient for the total exclusion of periodate. Considering the concentration ratio of glycerol to formaldehyde in the order of 1000, even traces of periodate in the manifold generated formaldehyde in an unacceptable quantity. Quasi-simultaneous monitoring was therefore not feasible without re-designing of the manifold by either incorporation of a solenoid valve in the supplying tube of reagent 2 or by carrying out both methods on two separated SIA systems using distinct selection valves.

### 5.6.2. Performance of the method during monitoring

In contrast to the former work, personal maintenance such as refilling of the carrier reservoir or data saving was not required during the 30 h of glycerol monitoring. The required solutions were prepared in sufficient quantity for the entire monitoring time.

Stacking of air bubbles in the holding coil was frequently observed. Since these bubbles were not eliminated during dispense operations due to the moderate applied flow rate, the expulsion was supported by knocking the holding coil manually from time to time during dispense operations. The debubbler device worked satisfactory and false peaks caused by air bubbles passing the detection cell were hardly observed. However, stacked air bubbles in the holding coil diminish the inner diameter of the holding coil locally, change the flow pattern and by this, the dispersion of the aspirated solutions. Besides, the volumetric precision is diminished since due to the air bubble, the liquid column in the holding coil is no longer incompressible. So, especially the sample splitting procedure was affected by stacked air bubbles, by the alteration of the sample dispersion and by the volumetric error during sample aspiration and partly discharge afterwards.

The repeatability of the method was satisfactory calculating the RSD value for each ten successive peaks during the first 20 hours with a mean of 4.5 % using a polynomial fourth-order regression over the entire data set. Nevertheless, the method reproducibility was significantly lower during the bioprocess monitoring compared to laboratory experiments due to the aspiration of gas bubbles from the medium filtrate flow or due to air bubbles generated by carrier degassing. The incorporation of a debubbler into the sampling line after 6 hours of process duration reduced the presence of bubbles in the transport line. Air bubble stacking was further reduced using FEP tubing instead of PTFE tubing for the holding coil, which can be reduced to a smoother inner surface of the tubing walls.

The analyte recovery was higher than 100 % throughout the entire monitoring time with an average value of 120 %  $\pm$  11 %. In detail, the recovery values increased during the monitoring time. Mean values were 112 %  $\pm$  7 % for the first 15 h, 122 %  $\pm$  6 % for 15 h-20 h, and 138 %  $\pm$  11 % for 20 h-23.6 h. The high and increasing recovery values for the HCR procedure were assumed to be related to the high salt content changing the dispersion of the sample during the splitting procedure.

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In consequence, although the pH in the reaction mixture was lowered by the cultivation medium showing pH 5, the reaction was apparently faster compared to pH 6, which had been proven before to be the optimal pH for the Hantzsch reaction [265]. The enrichment of metabolites can further lead to the increase of the analytical signal by the reaction with periodate either by the consumption of periodate, which has proven during optimization (see chapter 5.5.4.3) to enhance the analytical response or by the oxidative formation of additional formaldehyde, which would lead to a higher yield of DDL.

Nevertheless, the method was turned out to be acceptable for the fast monitoring of the kinetics of the glycerol consumption taking into consideration the continuous change of the cultivation medium. On the other side, the sample throughput during monitoring is not sufficient to allow feed-back control of the feeding rate of glycerol. For this task, a response time less than two minutes would probably be required. However, the time delay due to the medium filtrate flow contributed already with about 20 min. Therefore, the proposed SIA analyzer system is limited to the sole use as a monitoring tool.

### 5.6.3. Possible improvements and outlook

The proposed analyzer system showed mainly two aspects, which were aimed to be improved in a similar approach for the monitoring of sorbitol described in chapter 6.

For the first, the analyte concentration during monitoring was overestimated and the analyte recovery increased during the process. As later discussed in chapter 6, this was partly attributed to the high salt content of the cultivation medium. The recovery values obtained from preliminary experiments and analyte addition to batch cultivation medium were throughout higher than 100 % for the HCR procedure. It was not clarified, if this effect was due to a catalic mechanism or due to the high viscosity of the cultivation medium. However, the adaptation of the carrier or standard composition to the cultivation medium matrix was aimed for the following experimental work.

For the second, the standard deviation of peak heights during monitoring was relatively high, which could be attributed partly to air bubbles in the medium filtrate flow or to air bubbles, which were generated by carrier or reagent degassing. On the other hand, bubbles formed during incubation were eliminated effectively by the incorporated debubbler and did not affect the performance of the method considerably. Air bubbles in the holding coil can alter the dispersion of the aspirated solutions and lower the precision of the aspirated volume. In consequence, mainly air bubbles originated from the carrier and keeping stacked in the holding coil had to be eliminated by an improved analytical system in the following experimental work.

### 5.7. Conclusion

In the present work, an automated analytical system was developed for the quantitative determination of glycerol in yeast cultivation medium. Two coupled reactions were carried out in parallel and automatically using sequential injection analysis (SIA). Malaprade reaction with periodate was used to generate formaldehyde by the oxidation of glycerol. Formaldehyde was then quantified by spectrophotometry applying Hantzsch reaction with acetylacetone in order to obtain the detectable product diacetyldihydrolutidine.

Physical parameters such as flow rates, volumes, and reaction time were optimized in order to achieve linearity and adequate sensitivity for two working ranges in order to apply the method to monitoring of glycerol in the batch medium of yeast cultivations. Robustness, selectivity, sensitivity, and stability of the analytical approach were studied. The two established flow protocols corresponding to a low and a high concentration range of 0.1-3 g l<sup>-1</sup> (LCR) and 1-30 g l<sup>-1</sup> (HCR), respectively, were successfully applied to at-line monitoring and self-configuration of the system was achieved by the automated activation of the procedures based on on-line peak height evaluation and smart software decisions.

A new concept for sample splitting was developed, studied, and successfully applied for the provision of small sample volumes. Further in-line dilution was carried out by the zone intercalation of water between the sample and both reagents. By this, the analyte concentration is diminished in the penetration zone.

The analytical performance of both procedures was highly satisfying with sample frequencies of 12 and 14 per hour, limits of detection of 30 mg l<sup>-1</sup> and 80 mg l<sup>-1</sup>, and repeatabilities of < 1 % and < 2 % (n = 12) for the highest standards, respectively.

As only interferences, other polyols and about ten-times reduced, saccharides give an analytical response whereas simple alcohols, organic acids, and the components of the basal salt cultivation medium did not show any analytical response.

The system was successfully applied to at-line monitoring of glycerol in the medium of a cultivation process of the yeast *Pichia pastoris* during glycerol batch and fed-batch. The recovery of glycerol in the cultivation medium was determined after cultivation process monitoring using a HPLC protocol for validation. The average recovery over the entire monitoring time was 120 % ± 11 %. The recovery values were found to increase during the cultivation process. This effect was reduced to the change of the cultivation medium with time in respect of salt concentration, viscosity, and buffering capacity as well as to the enrichment of metabolites in the medium, which could cause a signal increase by the direct consumption of periodate or by being oxidized by periodate to formaldehyde.



## 6. EXPERIMENTAL WORK 3

### Monitoring of sorbitol in *Pichia pastoris* cultivation applying sequential injection analysis

In press equally entitled: HORSTKOTTE B., ARNAU C., VALERO F., ELSHOLZ O., CERDÀ V. (2008) *Biochem. Eng. J.* [310].

#### 6.1. Task description for experimental work 3

The objective of this work was the adaptation of the SIA analyzer used prior for glycerol quantification to the substrate sorbitol in medium of a *Pichia pastoris* cultivation process. After the optimization of physical and chemical parameters, the system was aimed to be applied to a real cultivation process and to be characterized. The upper limit of the concentration range of interest was given by the initial concentration of the batch medium of up to 20 g l<sup>-1</sup> sorbitol. Since coupling of the SIA analyzer to the fermenter was not feasible due to the small working volume, a software-guided protocol was made, which enabled method adaptation, execution of analysis, and cleaning protocols on request.

#### 6.2. Determination of sorbitol

Due to the similar chemical properties of sorbitol and glycerol, quantification of sorbitol in cultivation media is – likewise of glycerol - carried out mostly by HPLC with refraction index or UV spectrometric detection [170,197,200,213,214,220,283]. However, the advantage of multi-component analysis is opponent to by an extended time of analysis, high maintenance effort, and expenses for consumables such as filters and columns.

Alternatively, CE has been successfully applied to the analysis of sorbitol but as in HPLC; the sample frequency and detection sensitivity are quite low [311,312]. Enzymatic reactions used for quantification are problematic due to the limited stability of the applied enzyme and a decreasing efficiency over time was reported [111]. Recently, the application of an imprinted polymer for a sorbitol sensor was demonstrated [313]; however, the sensor did not exhibit a sufficient stability for process monitoring purposes.

The application of the Malaprade reaction for the quantitative oxidation of glycerol to formaldehyde was described in chapter 5. Here, formaldehyde as an intermediate product of this reaction was quantified by the Hantzsch reaction. Likewise glycerol also other polyalcohols such as sorbitol undergo the Malaprade reaction. Sorbitol was the only multi-carbon source used in the cultivation process of interest as a non-repressing substrate of the P<sub>AOX</sub> controlled expression in *P. pastoris* (see chapter 1.5.3.2). Consequently, the same reaction scheme could be applied for sorbitol quantification after adaptation.

### 6.3. Materials and methods

#### 6.3.1. Cultivation process and reference method

The proposed sorbitol analyzer was tested at a cultivation process performed by the members of the Bioprocess Engineering and Applied Biocatalysis from the Autonomous University of Barcelona (UAB). The *Pichia pastoris* strain KM71 (*arg4 his4 aox1Δ::SARG4 AOX2*) of phenotype Mut<sup>s</sup> was used. Extracellularly expressed target protein was the *Rhizopus oryzae* lipase [199,314,315]. A pre-culture was grown for 40 h in 1 l baffled shake flasks at 30°C, 200 rpm in a medium containing 1 w/v% yeast extract, 2 w/v% peptone, 100 mM potassium phosphate, pH 6.0, 4·10<sup>-5</sup> w/v% biotin and 1 w/v% glycerol. Before inoculation, the pre-culture was centrifuged at 4000 g and the cells were re-suspended in a sterilized 0.9 % NaCl isotonic solution.

The cultivation process was performed as batch with a bi-substrate medium containing methanol and sorbitol in the initial concentrations of 10 g l<sup>-1</sup> and 8 g l<sup>-1</sup>, respectively. The composition of the medium is given in chapter 3.5.2.2. Further component was 0.1 ml of antifoam Mazu DF 7960 (Mazer Chemicals, PPG Industries Inc. USA).

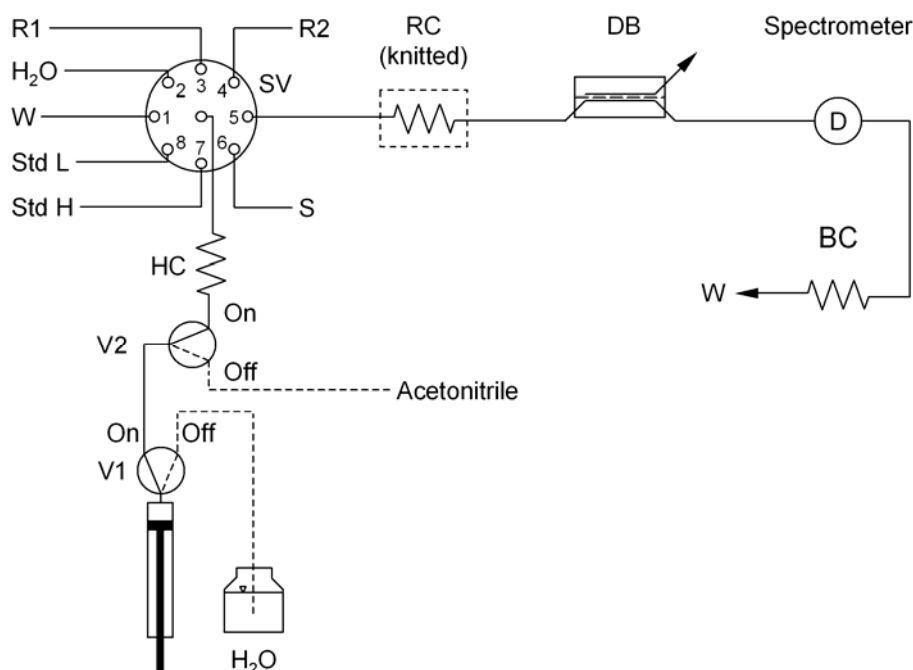
The duration of the cultivation process exceeded 100 h. Before inoculation, the pH was adjusted to a value of 5.5 and maintained stable along the process by automated addition of 30 % ammonia. The agitation speed was set to 500 rpm. The dissolved oxygen content was controlled to be 30 %. The exhaust gas of the bioreactor was cooled in a condenser at 4°C. Inoculation was done with pre-culture reaching an initial cell density of 0.95 g l<sup>-1</sup>. The monitored cultivation process was accomplished in a 2 l bench-top Biostat B Plus batch bioreactor (1.5 liter working volume) from B. Braun Biotech International GmbH.

Manual sampling was done about every 80 min. The samples were immediately centrifuged (10.000 g, 4°C) and supernatants used for ex-situ analysis. The cell biomass expressed as dry cell weight and the concentration of the target product expressed as lipolytic activity were determined as reported by RESINA ET AL. (2004) [200].

For off-line analysis of methanol and sorbitol, the samples were filtered through 0.45 µm membrane filters (Millipore, Ireland, Cat.No. HAWP01300). Analysis of organic acids, carbohydrates, and sorbitol was done with HPLC on a HP series 1050 system (Hewlett Packard) separated on a ICSEp ICE Coregel 87H3 column and quantified with refraction index detector HP1047A from Transgenomic (Elancourt, France). Water, adjusted with sulfuric acid to pH 3.0 was used for isocratic separation at a flow rate of 0.6 ml min<sup>-1</sup>. Sorbitol concentration data obtained with the former method were compared with the concentration data obtained with the proposed SIA analyzer. Sorbitol eluted at 12 min, the entire separation procedure required 30 min. Using an injection volume of 20 µl, the linear working range for sorbitol was 0.1-20 g l<sup>-1</sup> sorbitol [199].

## 6.3.2. Analyzer instrumentation

The SIA instrumentation and manifold of the sorbitol analyzer are shown in figure 44. All components were described in detail in the chapters 3.1, 3.2, and 3.3. Data acquisition and instrumental control were done as described chapter 3.4. The same instrumentation and configuration was used as for the glycerol SIA analyzer described in chapter 5.4.2.



**Figure 44: Manifold of the SIA analyzer for sorbitol.**

**V1, V2: solenoid valves with normally open position (OFF, deactivated) dotted, SV: selection valve with sample (S), standards (Std), waste (W), Hantzsch reagent (R1), and Malaprade reagent (R2), HC: holding coil (150 cm, 1.5 mm i.d.), RC: heated reaction coil (175 cm, 0.8 mm i.d., knitted), DB: debubbler, BC: backpressure coil (185 cm, 0.8 mm i.d.), tubes between SV, RC, DB, and detector were 20 cm, 0.8 mm i.d. each, tube between V1 and V2 was 6 cm, 1.5 mm i.d., sample tube was 6 cm, 0.5 mm i.d.**

An additional three-way MCSV was incorporated between the syringe head valve and the holding coil. This enabled the aspiration of a small volume of acetonitrile, which allowed the effective elimination of stacked air bubbles from the holding coil as described in chapter 6.4.1. The sample was aspirated over a short tube of diminished diameter (6 cm, 0.5 mm i.d.) in order to reduce the required sample volume for rinsing. An adapted cleaning procedure was carried out applying consequently reduced volumes.

The same detection assembly as for the glycerol SIA analyzer was used. However, during monitoring, another USB 2000 device (serial number USB2E6039) was used, differing in the wavelength calibration coefficients. Dual-wavelength detection was done with a reference wavelength of 470 nm. Here, the illumination intensity was the similar to the one at the detection wavelength and improved schlieren compensation was achieved.

### 6.3.3. Reagents

The same reagents were used as described in chapter 5.4.3. All reagents were analytical grade and distilled or Millipore® filtered water was used throughout for preparation of solutions and as carrier. Acetonitrile was used as an additional reagent for the rinsing of the holding coil in order to eliminate stacked air bubbles. Aqueous standards were used for most optimization experiments. For comparative experiments and sorbitol monitoring, standards were prepared with the basal salt medium in order to compensate the medium matrix effects such as high ion strength and viscosity. For this, the medium was adjusted to pH 5.5 with 25 % ammonium and filtered afterwards since a considerable quantity of salt, most likely ammonium magnesium phosphate, was found to precipitate.

Standards of 5, 10, 15, and 20 g l<sup>-1</sup> sorbitol were used for a high concentration range (HCR) and standards of ten-fold lower concentrations were used for a low concentration range (LCR) during optimization and monitoring. Chloroform was added to the standards to a final concentration of 0.5 v/v% in order to disable biodegradation of the analyte by microorganisms' activity. Stock solutions of sorbitol were stored in aliquots at -18°C.

### 6.3.4. Analytical procedures

The same splitting and in-line dilution techniques achieved by zone stacking as prior described in chapters 5.4.4.1 and 5.4.4.2 were applied. Volumes were modified in order to adapt the method to the analyte sorbitol and to the different working ranges. Likewise before, two procedures were used to carry out the analytical protocols for two, each other complementing dynamic working ranges. Optimized volumes applied for a high concentration range (HCR) procedure were 8.6 µl of sample, 300 µl of water, 140 µl of reagent 1, and 70 µl of reagent 2 and 25 µl of sample, 25 µl of water, 160 µl of reagent 1 and 45 µl of reagent 2 for a low concentration range (LCR) procedure. Only the HCR procedure was used during process monitoring due to the high analyte concentrations.

The aspiration was followed by the propelling of the stacked solutions into the knitted reaction coil and incubation of the mixture at 45 °C for during 60 s (HCR) or 65 s (LCR), respectively. Afterwards, quantification was done by propelling of the mixture including the reaction product DDL to the detection flow cell. For the removal of stacked air bubbles in the holding coil, 200 µl of acetonitrile were aspirated from the second MCSV and pushed forward through the holding coil to waste. Pre-considerations and the physical background of this procedure are explained in chapter 6.4.1.

### 6.3.5. Software and program structure

Instrumental control, data acquisition, and data processing were carried out using the software package AutoAnalysis 5.0 from Sciware S.L., described in detail in chapter 3.4,

## 6 SIA analyzer for sorbitol in Pichia pastoris culture medium

running on a PC with operating system MS-Windows 2000. The methods and procedures accomplished for analysis and for system cleaning are given in the annex.

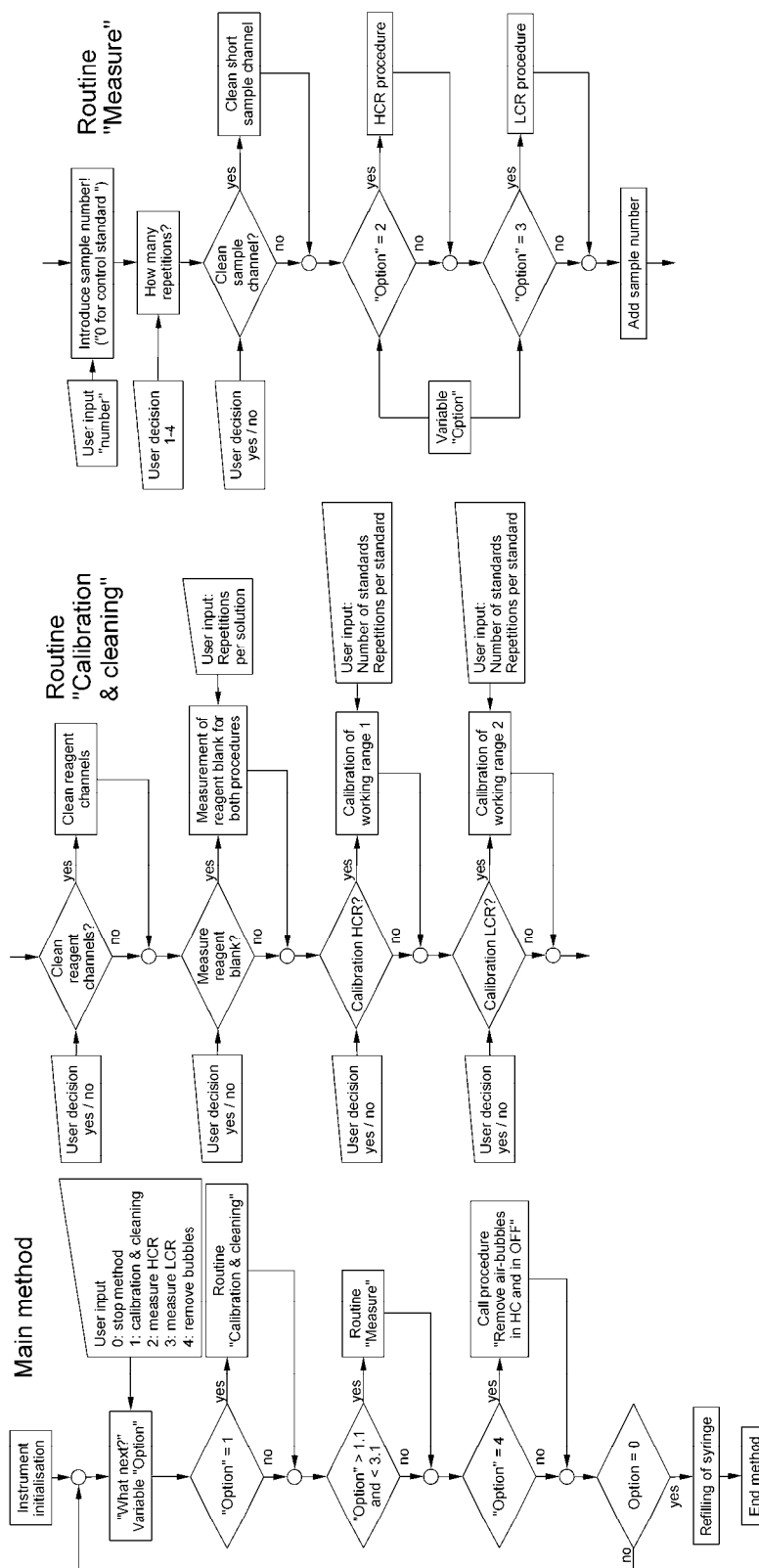


Figure 45: Program scheme of the method used for at-line monitoring of sorbitol.

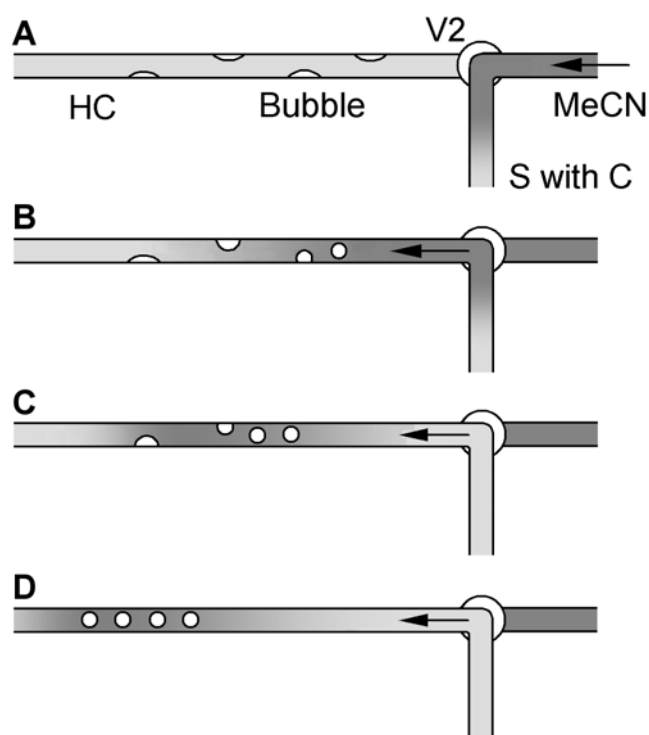
## 6 SIA analyzer for sorbitol in *Pichia pastoris* culture medium

Due to the limited medium volume of the cultivation process, provision of a continuous medium filtrate flow and in consequence at-line monitoring was not possible. Therefore, in this work, advantage of the versatility of the applied software AutoAnalysis was taken by creating a software-guided analytical method shown schematically in figure 45. Decisions about the required working range were taken by the user following software requests. Likewise, calibration and cleaning protocols were activated on user demand applying conditional inquiries. The user further decided about the number and repetition of measurements and rising of supplying tubes after the change of sample or reagent solutions. For peak marking, a sample identification number could be introduced. This facilitated the assignment of the peak appearing in the data window for data evaluation.

### 6.4. Experiments and results

#### 6.4.1. Elimination of stacked air bubbles

One of the main problems observed during the former works with the glycerol and the formaldehyde SIA analyzer and the belonging process monitoring campaigns were due to air bubbles keeping stacked in the holding coil. These air bubbles were formed during low-pressure exertion during aspiration steps or by degassing of the carrier and reagent solution over a period of several hours.



**Figure 46: Scheme of the applied procedure for gas bubble elimination.**

**A:** A small volume of acetonitrile (MeCN) is aspirated from V2 into the syringe (S). **B-D:** The acetonitrile slug followed by the carrier (C, water) is dispensed through the holding coil (HC) to the selection valve port 1 (waste). By this procedure, stacked gas bubbles are carried out.

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By stacking in the holding coil, the air bubbles could alter the dispersion of the aspirated solutions considerably and further led to error peaks if they are carried out to the detector at flow reversal. Commercial degasification devices working by the application of vacuum on the carrier or carrier degassing with helium are highly costly. Ultrasonic degasification is effective but has to be applied repeatedly during the time of process monitoring in order to avoid the re-saturation of the solutions with air.

In order to remove air bubbles from the holding coil, 200  $\mu\text{l}$  of acetonitrile were aspirated from position ON of the solenoid valve 2 and discharged to waste, passing the holding coil. As the holding coil was flushed with acetonitrile followed by the carrier, stacked air bubbles were expelled with high efficiency at an already moderate flow rate of  $7.5 \text{ ml min}^{-1}$ . This effect can be explained by the decrease of the hydrophilic character of the solvent zone passing the holding coil. Consequently, a lower adhesion force has to be overcome in order to remove the stacked air bubbles. The accomplished procedure is schematically shown in figure 46. It required two minutes and can therefore be applied during continuous monitoring without a significant loss of process observation time.

### 6.4.2. Bubbler separator

In this work, a bubble separator was constructed, which should eliminate air bubbler from a continuous sample flow with a higher efficiency than membrane based debubbler cells. Air bubbles in a continuous sample flow can affect the aspirated volume as well as sample dispersion and mixing with the reagents in the holding coil. On the other hand, membranes used in debubbler devices can be blocked by organic matter or biofouling.

In the proposed unit, the sample enters the separator from below and flows out at a lateral port located below the entrance. By this, bubbles entering the cell are separated and keep stacked in the head space of the cell. From here, they can than be drawn off from time to time by the use of an additional syringe pump or lateral valve port. In preliminary experiments, the separator worked highly effective but was not applied during process monitoring since un-continuous monitoring was carried out. A photo and engineering drawing of the device are given in the annex.

### 6.4.3. Optimization of the analytical method

#### 6.4.3.1. Preliminary remarks

Due to the high initial sorbitol concentration of the batch and fed-batch *P. pastoris* culture, the aim of optimization was not the achievement of the highest possible sensitivity but to achieve the effective and rapid in-line dilution of the sample and linearity over two concentration ranges from  $1\text{-}20 \text{ g l}^{-1}$  (HCR) and  $0.1\text{-}2.5 \text{ g l}^{-1}$  (LCR). Therefore, the aimed sensitivity values were  $0.05 \text{ AU l g}^{-1}$  (HCR) and  $0.4 \text{ AU l g}^{-1}$  (LCR). As in the experimental

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work 2 (chapter 5), sample dilution was done by zone-penetration, i.e. by intercalating water between the sample and the reagents. In consequence, the analyte concentration at the penetration zone with both reagents is diminished. Likewise, the same sample splitting procedure and the same parameters (30  $\mu\text{l}$  sample aspiration and 30  $\mu\text{l}$  sample discharge) as used for glycerol monitoring described in chapter 5.5 were applied in order to provide a sample volume of about 8.6  $\mu\text{l}$  for the HCR procedure.

Sample volumes of 10  $\mu\text{l}$  or more caused a nonlinear response and saturation for concentrations above 10  $\text{g l}^{-1}$ . Likewise, the same compositions of reagent 1 and reagent 2, manifold, aspiration order of solution, and incubation temperature were used as for the glycerol analyzer system as a consequence of the satisfying analytical performance achieved in the both former experimental works. By this, the high versatility of the SIA method and the analyzer system was demonstrated. In consequence, basic optimization parameters were the applied solution volumes and the incubation time starting from the former optimized values for the glycerol analyzer system. Generally, five aqueous standards were used for each procedure (5, 10, 15, 20, and 25  $\text{g l}^{-1}$  and 0.5, 1, 1.5, 2, and 2.5  $\text{g l}^{-1}$  of sorbitol, respectively) and quantified in triplicate.

### 6.4.3.2. Optimization of volume of Hantzsch reagent (R1)

The influences of the volume of reagent 1 on slope (sensitivity), intercept (blank), and calibration linearity were studied in the range of 60  $\mu\text{l}$  to 160  $\mu\text{l}$  for both procedures using aqueous standards (0-2.5  $\text{g l}^{-1}$  and 0-25  $\text{g l}^{-1}$ , respectively). Experimental conditions and results are given in table 15 and represented in figure 47. For the LCR procedure, linearity was achieved throughout, whereas linearity was obtained only for volumes above 140  $\mu\text{l}$  for the HCR procedure. For both procedures, the calibration function slope increased with the volume of reagent 1. Since the slope values met best the aimed sensitivity values, 160  $\mu\text{l}$  of reagent 1 was chosen for the LCR procedure and 140  $\mu\text{l}$  for the HCR procedure.

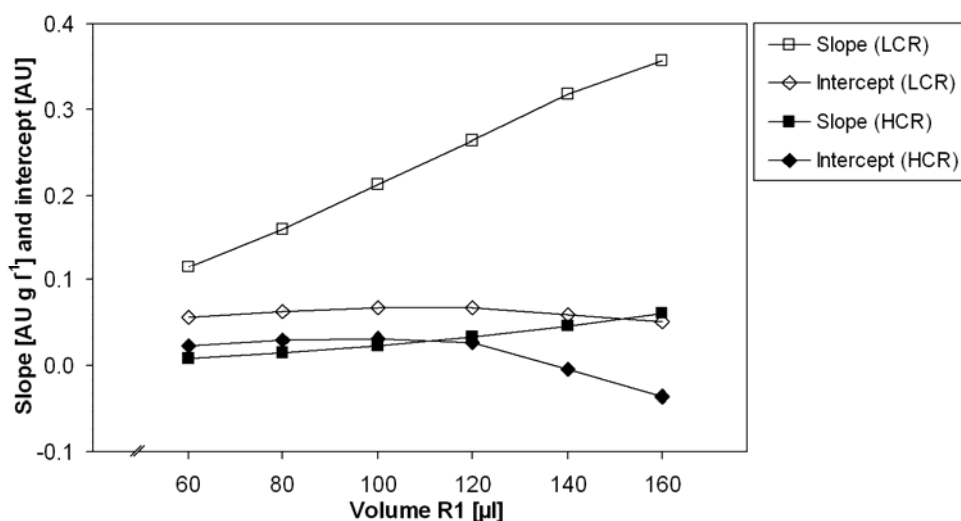


Figure 47: Optimization results for volume of reagent 1 for both procedures for sorbitol.



## 6 SIA analyzer for sorbitol in Pichia pastoris culture medium

**Table 15: Optimization results for volume of reagent 1 using the slope, intercept and coefficient of linear regressions ( $r^2$ )\* as criteria for both sorbitol procedures.**

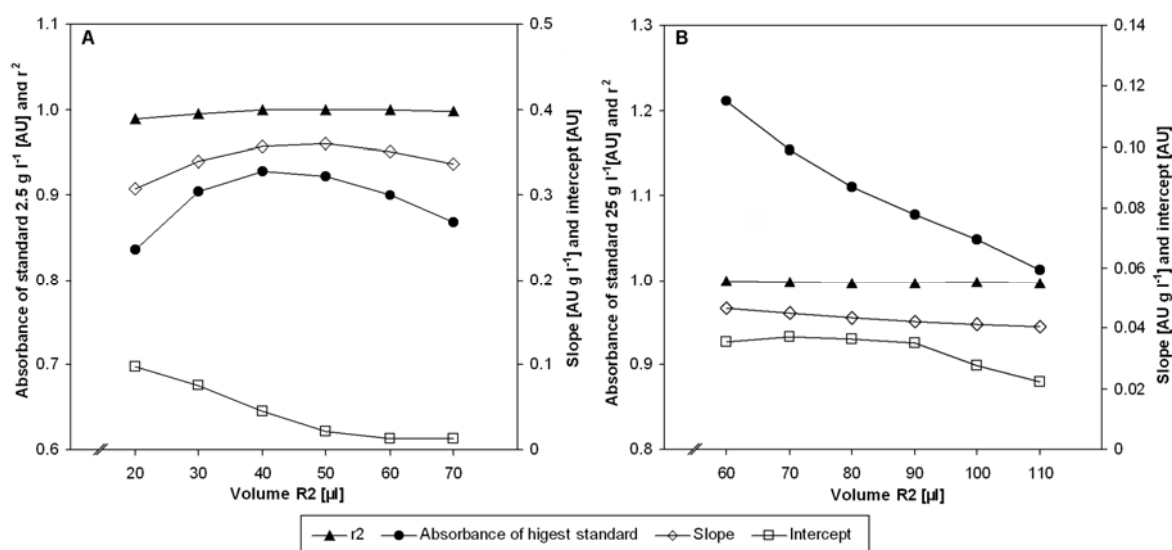
LCR procedure *	Reagent 1 [ $\mu$ l]	Slope [ $\text{AU l g}^{-1}$ ]	Intercept [AU]	$r^2$
	60	0.115	0.056	1.000
	80	0.160	0.063	0.999
	100	0.212	0.067	0.999
	120	0.263	0.067	1.000
	140	0.317	0.059	1.000
	160	0.358	0.051	1.000
HCR procedure **	Reagent 1 [ $\mu$ l]	Slope [ $\text{AU l g}^{-1}$ ]	Intercept [AU]	$r^2$
	60	0.009	0.023	0.989
	80	0.015	0.030	0.994
	100	0.023	0.032	0.998
	120	0.033	0.026	0.998
	140	0.047	-0.004	0.998
	160	0.062	-0.036	0.999

\* 40  $\mu$ l of reagent 2, 25  $\mu$ l water, 25  $\mu$ l sample, and incubation at 45°C for 80 s

\*\* 80  $\mu$ l of reagent 2, 300  $\mu$ l water, 8.6  $\mu$ l sample, and incubation at 45°C for 80 s

### 6.4.3.3. Optimization of volume of Malaprade reagent (R2)

The influences of the volume of reagent 2 being 100  $\text{mmol l}^{-1}$  meta-periodate on the slope (sensitivity), intercept (blank), and calibration linearity were studied in the ranges of 20  $\mu$ l to 70  $\mu$ l for the LCR procedure and 60  $\mu$ l to 110  $\mu$ l for the HCR procedure using aqueous standards (0-2.5  $\text{g l}^{-1}$  and 0-25  $\text{g l}^{-1}$ , respectively). The experimental conditions and results are given in table 16 and are represented in figure 48.



**Figure 48: Optimization results for volume of reagent 2 for the LCR procedure (A) and HCR procedure (B) for sorbitol.**

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**Table 16: Optimization results for volume of reagent 2 using the slope, intercept, and coefficient of linear regressions ( $r^2$ )\* as criteria for both procedures for sorbitol.**

LCR procedure *	Reagent 2 [ $\mu$ l]	Slope [AU l g <sup>-1</sup> ]	Intercept [AU]	$r^2$
	20	0.307	0.098	0.989
	30	0.339	0.076	0.995
	40	0.357	0.045	0.999
	50	0.360	0.021	1.000
	60	0.351	0.012	0.999
	70	0.337	0.013	0.998
HCR procedure **	Reagent 2 [ $\mu$ l]	Slope [AU l g <sup>-1</sup> ]	Intercept [AU]	$r^2$
	60	0.047	0.036	1.000
	70	0.045	0.037	0.999
	80	0.044	0.037	0.996
	90	0.042	0.035	0.997
	100	0.042	0.028	0.998
	110	0.040	0.022	0.997

\* 160  $\mu$ l of reagent 1, 25  $\mu$ l water, 25  $\mu$ l sample, and incubation at 45°C for 80 s

\*\* 140  $\mu$ l of reagent 1, 300  $\mu$ l water, 8.6  $\mu$ l sample, and incubation at 45°C for 80 s

Linearity up to the highest standard was achieved for volumes of R2 above 30  $\mu$ l for the LCR procedure and throughout for the HCR procedure. The intercept values decreased with higher volumes of R2 for both procedures. For the LCR procedure, a maximal sensitivity was found for 50  $\mu$ l of R2, whereas sensitivity decreased nearly linear with increasing volume of R2 for the HCR procedure. Since the slope values met best the aimed sensitivity values, 45  $\mu$ l of R2 were chosen for the LCR procedure and 70  $\mu$ l were chosen for the HCR procedure.

### 6.4.3.4. Optimization of volume of water and incubation time

The influences of the volume of water and the incubation time on the slope, intercept, and calibration linearity were studied in the range of 20  $\mu$ l to 50  $\mu$ l (LCR) and 200  $\mu$ l to 350  $\mu$ l (HCR) and 50 s to 80 s for both procedures, respectively. The experimental conditions and results are given in table 17. A parabolic regression for the slope values with coefficients given in table 18 was calculated and represented in figure 49.

The best compromise in respect of time of analysis, slope, intercept, and linearity was obtained for the LCR procedure for 25  $\mu$ l of water and an incubation time of 65 s. For a shorter incubation time, the aimed sensitivity was not achieved. For the HCR procedure, the best compromise was obtained applying 300  $\mu$ l of water and an incubation time of 60 s. Applying 350  $\mu$ l of water and 80 s of incubation, a lower intercept at an equal slope was obtained but the time of analysis was prolonged considerably. For smaller volumes of water, the calibration function was found to be curved.

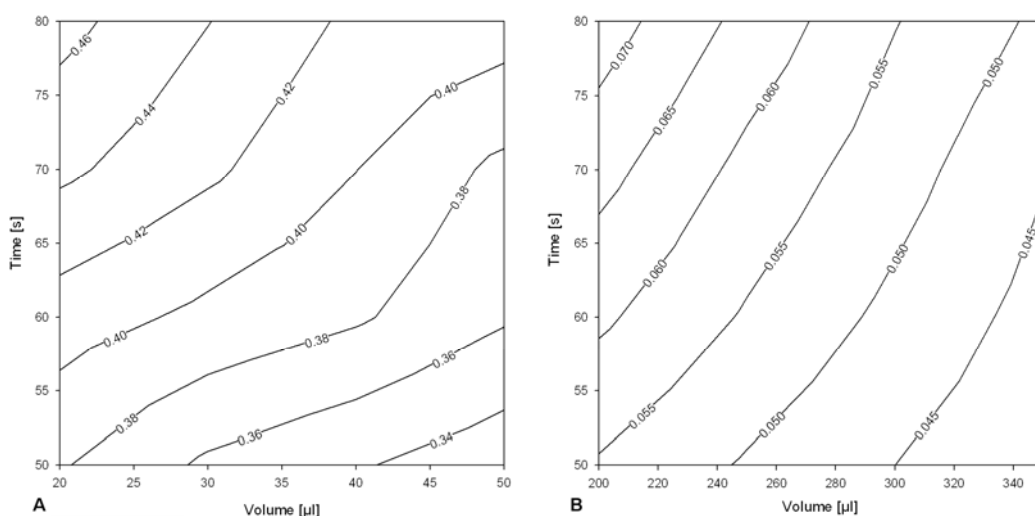
## 6 SIA analyzer for sorbitol in Pichia pastoris culture medium

**Table 17: Slope [AU l g<sup>-1</sup>], intercept [AU], and coefficient of linear regressions (r<sup>2</sup>) in dependence of the applied volume of water and incubation time for sorbitol.**

Procedure	Water [μl]	Incubation time [s]				
		Parameter	50	60	70	80
LCR *	20	Slope	0.382	0.410	0.444	0.467
		Intercept	0.013	0.009	-0.006	-0.015
		r <sup>2</sup>	1.000	0.999	0.999	0.998
	30	Slope	0.356	0.395	0.424	0.441
		Intercept	0.023	0.006	-0.002	-0.005
		r <sup>2</sup>	0.998	0.999	0.999	0.999
	40	Slope	0.342	0.383	0.400	0.416
		Intercept	0.014	-0.005	-0.004	-0.002
		r <sup>2</sup>	0.999	0.999	0.998	0.999
	50	Slope	0.327	0.362	0.375	0.410
		Intercept	0.009	-0.008	-0.002	-0.020
		r <sup>2</sup>	0.999	0.998	0.999	0.997
HCR **	200	Slope	0.055	0.061	0.067	0.073
		Intercept	0.117	0.096	0.069	0.033
		r <sup>2</sup>	0.996	0.997	0.995	0.992
	250	Slope	0.050	0.054	0.059	0.063
		Intercept	0.099	0.081	0.067	0.045
		r <sup>2</sup>	0.996	0.997	0.997	0.996
	300	Slope	0.045	0.049	0.052	0.055
		Intercept	0.081	0.069	0.060	0.049
		r <sup>2</sup>	0.996	0.998	0.998	0.998
	350	Slope	0.041	0.043	0.045	0.049
		Intercept	0.063	0.060	0.055	0.038
		r <sup>2</sup>	0.996	0.998	0.998	1.000

\* 140 μl of reagent 1, 70 μl of reagent 2, 8.6 μl sample, and incubation at 45°C

\*\* 160 μl of reagent 1, 45 μl of reagent 2, 25 μl sample, and incubation at 45°C



**Figure 49: Parabolic regression of calibration slope given as [AU l g<sup>-1</sup>] versus volume of water and incubation time for the LCR (A) and HCR (B) procedure for sorbitol.**

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**Table 18: Coefficients of parabolic regression of calibration slope versus volume of water and incubation time for both procedures.**

Coefficients	Values LCR procedure	Standard error	Values HCR procedure	Standard error
$a_0$	1.660E-01	5.795E-2	7.32E-02	2.200E-2
$a_1(\text{H}_2\text{O})$	-2.812E-03	9.517E-4	-2.13E-04	9.560E-5
$a_2(\text{time})$	7.019E-03	1.757E-3	4.09E-04	5.644E-4
$a_2(\text{H}_2\text{O})$	1.295E-05	1.349E-5	1.57E-07	1.733E-7
$a_2(\text{time})$	-3.344E-05	1.349E-5	6.54E-08	4.331E-6

### 6.4.4. Interference studies

Single components of the basal salt medium, metabolites, polyols, and saccharides were tested regarding to their interference on both analytical procedures. The compounds were tested as aqueous solutions in similar or higher concentrations than found in the cultivation medium were applied.

No significant interferences or blank signals above the LOQ were found from the following organic compounds at the given concentrations: formaldehyde (20 mg l<sup>-1</sup>), formic acid (6 g l<sup>-1</sup>), acetic acid (5 g l<sup>-1</sup>), tartaric acid (10 g l<sup>-1</sup>), oxalic acid (10 g l<sup>-1</sup>), pyruvic acid (2 g l<sup>-1</sup>), glucose (1 g l<sup>-1</sup>), galactose (1 g l<sup>-1</sup>), lactose (10 g l<sup>-1</sup>), saccharose (10 g l<sup>-1</sup>), and methanol (10 g l<sup>-1</sup>). Consequently, a higher selectivity was obtained than in experimental work 2 done for the quantification of glycerol (compare with table 14). For the present work, the insensitivity of the method for methanol was of special interest since it was fed as co-substrate and inductor in *P. pastoris* heterologous protein production jointly with sorbitol and the highest concentrated organic component in the medium.

**Table 19: Sensitivity values relative to sorbitol for the applied HCR procedure.**

Substance	Real concentration [g l <sup>-1</sup> ]	Relative sensitivity HCR procedure
Ethylene glycol	1	250 %
Ethylene glycol	10	119 %
Glycerin	1	22 %
Glycerin	10	168%
Maltose	10	7 %
Mannitol	1	13 %
Mannitol	10	127 %
Galactose	10	21 %
Glucose	10	17 %

## 6 SIA analyzer for sorbitol in *Pichia pastoris* culture medium

In higher concentrations - given in table 19 - monosaccharides were found to give a reduced response with relative sensitivity values compared to sorbitol in the range of 7 %-21 %. Likewise indicated, other polyalcohols gave a similar or even higher response than sorbitol. Therefore, the applicability of the analyzer system is limited to cultivation processes, in which no other polyalcohol is used as co-substrate.

When the standards were prepared with basal salt medium (composition given in chapter 6.3.3), the slope of the linear calibration function resulted to be 58 % higher for the LCR procedure and 45 % higher for the HCR procedure. This was in contrast to the SIA analyzer for the determination of glycerol where a significantly higher sensitivity applying cultivation medium was only observed for the HCR procedure.

The single components of the basal salt medium were tested in solutions containing 1 g l<sup>-1</sup> sorbitol for the LCR procedure or 10 g l<sup>-1</sup> sorbitol for the HCR procedure, respectively. The results are given in table 20. Calcium sulfate (0.5 g l<sup>-1</sup>) did not cause a significant increase of the peak height. Ammonium acetate (9 g l<sup>-1</sup>) caused a peak height increase of 10 % but only for the HCR procedure. High signal influences were observed for potassium and magnesium sulfate and the phosphate buffer as the most concentrated compounds in the medium. It seems therefore, that the reaction is favored in by a higher salt concentration. The observed sensitivity increase was further considered to be attributed to the higher viscosity and affected dispersion by the high salt content.

**Table 20: Effects of basal salt medium components on the peak height for sorbitol.**

Compound	Concentration	Peak height increase LCR procedure	Peak height increase HCR procedure
K <sub>2</sub> SO <sub>4</sub>	10 g l <sup>-1</sup>	25%	22%
	20 g l <sup>-1</sup>	31%	31%
MgSO <sub>4</sub>	2 g l <sup>-1</sup>	6%	27%
	5 g l <sup>-1</sup>	22%	43%
	10 g l <sup>-1</sup>	14%	52%
CaSO <sub>4</sub>	0.5 g l <sup>-1</sup>	2 %	3 %
H <sub>2</sub> NaPO <sub>4</sub>	0.2 mol l <sup>-1</sup> , pH 5.5	23%	50%
NH <sub>4</sub> Ac	9 g l <sup>-1</sup>	4%	11%

In order to compensate the sample matrix, the use of basal salt medium as spacer between sample and the reagents instead of water was tested as a method variant. Likewise, the aspiration of up to 150 µl of the medium or its main components, potassium sulfate or potassium phosphate solution in the same concentration as used in the medium prior the sample was tested. It was not possible to compensate the higher sensitivity observed for standards prepared with basal salt medium, however the reproducibility decreased notably. Therefore, standards prepared with basal salt medium were finally used for the calibration of the sorbitol analyzer for the monitoring application.

### 6.5. Discussion

#### 6.5.1. Analytical characterization

##### 6.5.1.1. Linear working range, reproducibility, and detection limit

Linear response was found in the range of 0.1 g l<sup>-1</sup> to 2.4 g l<sup>-1</sup> for the LCR procedure and 0.5 g l<sup>-1</sup> to 20 g l<sup>-1</sup> for the HCR procedure. Using samples or standards based on the basal salt medium an about 50 % higher sensitivity values were obtained and in consequence linear responses were found up 1.6 g l<sup>-1</sup> and 14 g l<sup>-1</sup>, respectively.

The repeatability of both procedures was calculated as the relative standard deviation from 12 consecutive injections of aqueous sorbitol standards of 0.2 g l<sup>-1</sup> (1.2 %) and 2 g l<sup>-1</sup> (0.7 %) for the LCR and 2 g l<sup>-1</sup> (1 %) and 20 g l<sup>-1</sup> (0.9 %) for the HCR procedure. The RSD value of three consecutive injections was typically < 1 % for the LCR procedure and < 2 % for the HCR procedure. During monitoring, the repeatability of the sole applied HCR procedure was typically < 3 % using basal salt medium standards and medium samples.

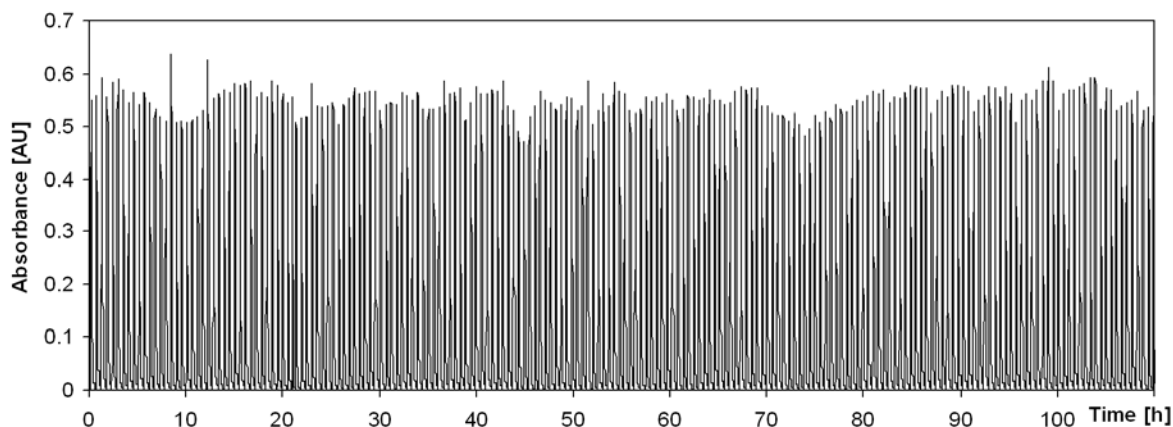
The limits of detection and quantification, calculated as the concentration yielding the triple and tenfold standard deviation of the blank were 0.01 g l<sup>-1</sup> and 0.03 g l<sup>-1</sup> for the LCR procedure and 0.16 g l<sup>-1</sup> and 0.22 g l<sup>-1</sup> for the HCR procedure using aqueous standards.

##### 6.5.1.2. Conservation of standard solutions and stability of method

An insufficient stability of the sorbitol standards over time was observed. This effect was clearly due to the degradation of the analyte by microorganism activity. Over a period of three days, turbidity and black moulds were observed in the higher concentrated standards. Addition of silver sulfate (30-600 µg l<sup>-1</sup>) and chloroform (0.5 v/v%) were tested as conservants. For lower concentrations of silver sulfate (<150 µg l<sup>-1</sup>) in the standards, the inhibition of mould formation was insufficient. For higher concentration, the standards showed to be stable, however, it was observed, that the reaction was catalyzed by the presents of silver ions since 20 % higher peaks were obtained. In constrast, the addition of chloroform did not influence the reaction significantly but showed to inhibit microorganism activity effectively. Therefore, standards were further stabilized by the addition of 0.5 v/v% of chloroform. The stability of the so-prepared sorbitol standards using basal salt medium was successfully demonstrated described in chapter 6.5.1.3.

##### 6.5.1.3. Stability of the method

The stability of the HCR procedure was tested by measuring a 10 g l<sup>-1</sup> sorbitol standard prepared with basal salt medium repeatedly over a period of 109 hours. The standard was stabilized by the addition of 0.5 v/v% chloroform as described above. The peak course is represented in figure 50. Neither significant decrease of the peak height nor increase of the baseline was observed. Deviation of the peak height was most likely related to stacked air bubbles and fluctuations of the ambient temperature.



**Figure 50: Stability test of the HCR procedure of sorbitol using a 10 g l<sup>-1</sup> aqueous standard.**

### 6.5.1.4. Solution consumption and sampling frequency

The volumes of water used for dilution and carrier in the LCR and HCR procedures were 3.44 ml and 3.72 ml respectively. The consumption of sample for one determination was 45  $\mu$ l for the LCR procedure and 50  $\mu$ l for the HCR procedure plus the volume required for rinsing the dead volume of the sample supplying tube (25  $\mu$ l). The consumption of reagent 1 and reagent 2 were 160  $\mu$ l and 45  $\mu$ l for the LCR procedure and 140  $\mu$ l and 70  $\mu$ l for the HCR procedure, respectively. Thus, one liter of water and 80 ml of reagent 1 and 30  $\mu$ l of reagent 2 were sufficient for 24 hours of monitoring.

A sampling frequency of 17.5 per hour was achieved for the HCR procedure and 18 per hour for the LCR procedure during the optimization experiments. During bioprocess monitoring, the injection frequency was reduced to 15 per hour due to the required time for the cleaning of the sample supplying tube.

### 6.5.2. Cultivation process monitoring

Sorbitol monitoring was carried out in a real bisubstrate (methanol and sorbitol) batch cultivation process of the yeast *Pichia pastoris* over about 60 h at the Autonomous University of Barcelona (UAB), Group of Bioprocess Engineering and Applied Biocatalysis (see chapter 6.3.1). Due to the limited working medium volume of 1.5 l, only time-discrete and manual sampling was carried out about every 80 min on average to avoid a significant reduction of the medium volume. Aliquots of the centrifuged and membrane filtered samples were analyzed by both HPLC and the proposed SIA analyzer. The method required the aspiration of the sample via a small and short supplying tube (6 cm, 0.5 mm i.d.) and the prior rinsing of this tube with the new sample. The required reagents were prepared in sufficient quantity for the entire monitoring time.

Elimination of stacked air bubbles from the holding coil using the procedure described in chapter 6.4.1 was required frequently, which was related to the relatively high ambient temperature at the cultivation process area. The procedure showed to work efficiently.

## 6 SIA analyzer for sorbitol in *Pichia pastoris* culture medium

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During the monitoring campaign, the procedure was activated on user request. For monitoring of sorbitol in a continuous sample flow, repeated and automated activation of the procedure, e.g. every hour, would have to be accomplished. The concentration of the substrate sorbitol decreased continuously from the initial concentration of  $8 \text{ g l}^{-1}$  following the typical depletion curve for exponential yeasts growth until 60 h where the sorbitol concentration was increased by an addition to  $2 \text{ g l}^{-1}$  sorbitol. Since the process was carried out as batch cultivation and stopped at the fully depletion of both substrates, only the HCR procedure was applied to the process. Another reason was that the HCR procedure was less affected by the sample matrix probably due to a high sample dilution.

The concentration courses of sorbitol obtained with the SIA analyzer and HPLC as well as methanol and biomass concentrations and heterologous ROL production expressed as lipolytic activity were quantified and are represented in figure 51. The number of samples quantified with the SIA analyzer exceeded the number of samples quantified with also the HPLC reference method about two-fold. Correlation of the sorbitol concentration data from samples analyzed with both methods is given in table 21 and represented in figure 52.

A satisfying correlation of the concentration data from the SIA analyzer and the HPLC was found. It satisfied the linear regression:  $[\text{SIA}] = 0.919 \cdot [\text{HPLC}] + 0.641$  ( $r^2 = 0.989$ ). As it can be seen from the equation and recovery data in table 21, the SIA showed generally a slightly higher concentration value. An acceptable difference of generally below  $0.2 \text{ g l}^{-1}$  was found during the first 51 h with a mean recovery  $103 \% \pm 7 \%$ . Afterwards, for medium contents of medium lower than  $2 \text{ g l}^{-1}$ , the found deviations between the concentration values exceeded 38 % and would not be sufficient for fed-back control of sorbitol addition to the medium in order to carry out an growth-limited sorbitol feed.

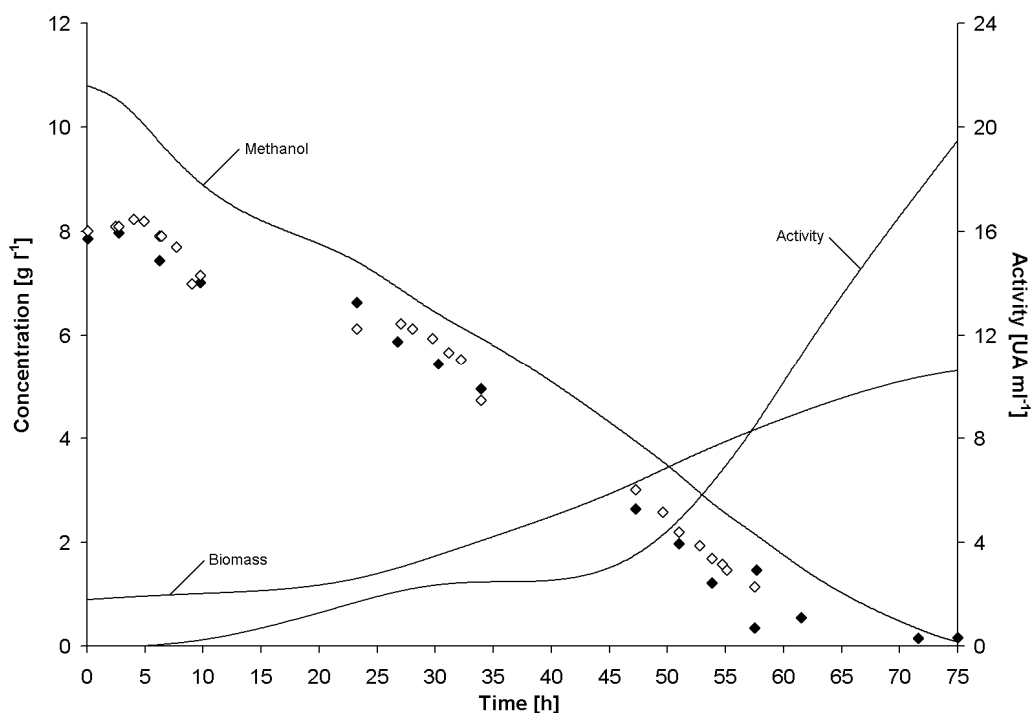
The low derivation between both methods in the first period of the process was in contrast to the recovery values found in the former work on the monitoring of glycerol in batch cultivation discussed in chapter 5.6.2. As main difference, the standards used for calibration prior to the present monitoring were prepared with basal salt medium, which showed to compensate mostly the former found overestimation of the polyalcohol. Besides, the initial deviation of the concentration values of about  $0.2 \text{ g l}^{-1}$  was in the order of the variation of both quantification methods including standard preparations.

The analytical performance and the results obtained during the batch cultivation were satisfying. The SIA analyzer showed to be applicable for the monitoring of sorbitol concentration in a mixed substrate fed-batch fermentation strategy with methanol. The SIA analyzer could be used for sorbitol concentration closed-loop control strategies at sorbitol accumulation, i.e. not at limited feed conditions and to study the effect of sorbitol concentration in heterologous protein productivity.

The considerable overestimation below  $2 \text{ g l}^{-1}$  sorbitol and over time was reduced to the changing properties of the cultivation medium with time, the concentration of metabolites and cell-components in the cell-free medium. As discussed in chapter 6.4.4, the high salt content showed to favor the analytical procedure and reaction.



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**Figure 51: Discrete concentrations of sorbitol determined by HPLC (black diamonds) and the proposed SIA analyzer (white diamonds). Lipolytic activity, methanol and biomass concentrations are shown as splint charts.**

**Table 21: Correlation data of sorbitol concentration data obtained with the proposed SIA analyzer and HPLC reference method.**

Process time [h]	Concentration HPLC [g l <sup>-1</sup> ]	Concentration SIA [g l <sup>-1</sup> ]	Recovery *
0.08	7.98	8.09	101%
2.75	7.85	8.01	102%
6.25	7.44	7.91	106%
9.75	7.02	7.15	102%
23.25	6.64	6.13	92%
26.75	5.88	6.22	106%
33.92	4.96	4.73	95%
47.25	2.64	3.01	114%
51.00	1.97	2.19	111%
53.80	1.22	1.68	138%
55.08	1.01	1.46	145%
56.17	0.60	1.48	247%
57.50	0.34	1.14	331%
57.68	1.47	2.18	148%

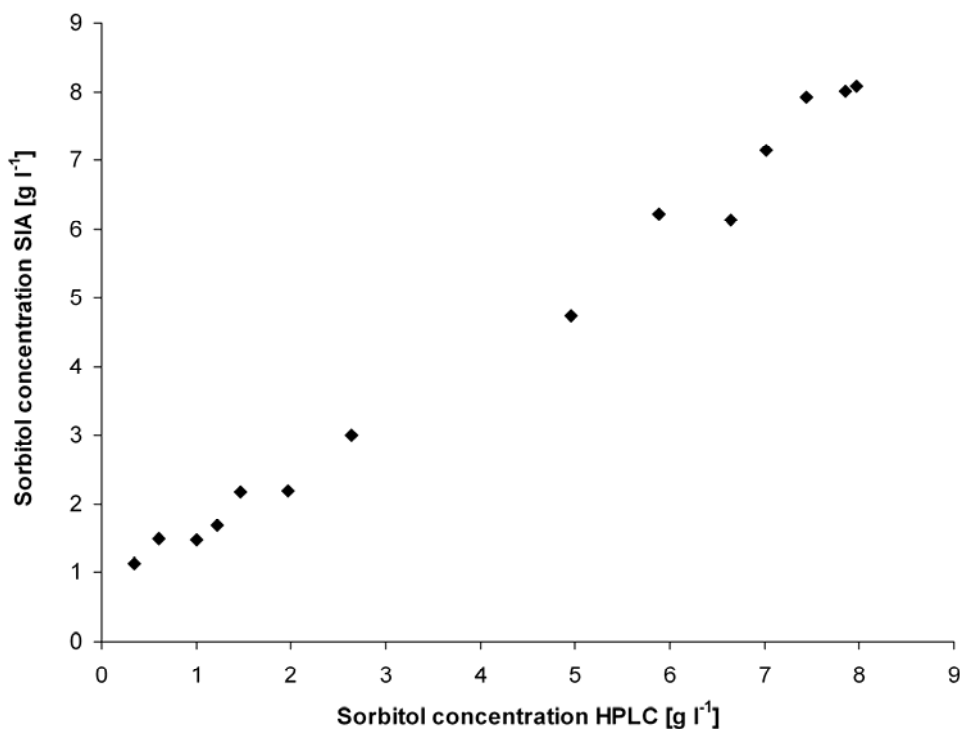
\* Calculated as concentration SIA divided by concentration HPLC

The addition of ammonia prior to inoculation causes the precipitation of a considerable amount of salt, most-likely ammonium magnesium phosphate. While this share was eliminated by filtration for the preparation of standard preparation, the same amount remains in the bioreactor. As no salt precipitate could be observed in the pellet of the centrifuged medium sampled in the second period of the cultivation process, it is most

## 6 SIA analyzer for sorbitol in *Pichia pastoris* culture medium

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likely, that this share dissolves as the content of metabolites increases or that the solubility increases by the uptake of e.g. magnesium by the yeast cells over the process time. As former discussed in chapter 5.6.2, the presence of other organic substances, which could be oxidized to formaldehyde by periodate or would consume periodate and thus decrease its concentration in the reaction mixture could further lead to a higher analytical response.



**Figure 52: Correlation chart of sorbitol concentration data obtained with the proposed SIA analyzer and HPLC reference method.**

### 6.5.3. Possible improvements and outlook

The system was improved compared to the former analyzer system for the at-line determination and monitoring of glycerol in respect of the analytical performance such as the sample frequency, selectively, the obtained analyte recovery, and system robustness. Nevertheless, improvements are possible in respect of these characteristics by changing the flow technique, such as by using multisyringe flow injection analysis (MSFIA).

This technique allows merging flow schemes, which could allow a higher sample frequency, though limited by the required incubation time of the reaction mixture. Applying merging flow schemes, viscosity effects could be further reduced. Higher reproducibility of the sample volume could be achieved by using an injection valve equipped with a small injection volume. However, MSFIA would also lead to a higher consumption of the required solutions. As a further improvement regarding robustness, batch-wise sample dilution in a stirred and thermostated mixing and detection chamber could be carried out.

### Conclusion

In the present work, an automated analytical system was developed for the quantification of sorbitol in yeast cultivation medium. The work presents an adaptation and continuation of the experimental work 2 dealing with the determination of glycerol. Likewise, Malaprade reaction and Hantzsch reaction with acetylacetone were carried out simultaneously and were automated using sequential injection analysis (SIA).

The physical parameters of the method, volumes and reaction time, were optimized regarding linearity and sensitivity for two working ranges and adaptation of the former protocol to the analyte sorbitol. Further, parameters of the analytical performance of the method were studied such as its robustness, selectivity, and detection sensitivity.

Software guided user-selection of the required analytical tasks was enabled, such as cleaning, calibration, and sample quantification applying one of two flow protocols corresponding to concentration ranges of 0.1-2.5 g l<sup>-1</sup> (LCR) and 1-20 g l<sup>-1</sup> (HCR). Further possibilities of user interventions in the analytical protocol were accomplished by using the software AutoAnalysis. By this, high flexibility and versatility of the method were achieved.

A new approach for the elimination of stacked air bubbles from the holding coil was developed and successfully applied. It was based on the flushing of the holding coil with acetonitrile, which was aspirated at the syringe head via an additional solenoid valve.

The analyzer was characterized by a low consumption of reagents and sample, an injection frequency of 17 per hour, and high repeatability for both established flow protocols. Possible interference from other medium components and possible metabolites were studied in detail. Apart from polyalcohols, only sugars were found to interfere the analysis but with at most an about ten-fold reduced analytical response.

The analyzer was successfully applied to the analysis of sorbitol in medium sample of a *Pichia pastoris* batch cultivation process. It was characterized by high robustness and reproducibility. Using standards prepared with the basal salt medium, the analytical results coincide well with concentration data obtained by off-line analysis with HPLC in the range of 8 to 2 g l<sup>-1</sup>. Lower concentrations were overestimated, which was probably due to the interferences of metabolites, which accumulated in the medium over the process duration. The analyzer enables on-line measurement of sorbitol as an important tool for the study, optimization, and control of the bi-substrate feeding strategy using sorbitol and methanol.



## 7. EXPERIMENTAL WORK 4

### Development of a capillary electrophoresis system coupled to sequential injection analysis and evaluation by the analysis of nitrophenols

Published equally entitled: HORSTKOTTE B., ELSHOLZ O., CERDÀ V. (2007) International Journal of Environmental Analytical Chemistry 87, 797-811 [316].

#### 7.1. Task description for experimental work 4

The objective of this work was the development of an analytical system based on the coupling of SIA and CE for automated analysis of environmental samples with suitability for monitoring purposes. The CE part was aimed to be totally maintained and controlled by the SIA part. Mono-nitrophenols were used as model analytes for the characterization of the system being at the same time substances of considerable environmental impact and consequently of high interest for environmental vigilance. Analytical objectives were high detection sensitivity and separation reproducibility. Constructive objectives were simplicity, robustness, high versatility, and easy maintenance of the coupled system.

#### 7.2. Introduction

##### 7.2.1. Determination of mono-nitrophenols

Mono-nitrophenols (NP) and other phenolic compounds are mostly analyzed using liquid chromatography in combination with highly sensitive detection techniques such as mass-spectrometry [226], deep-UV spectrometric detection [228,317], or electrochemical detectors [318,319]. Gas chromatography (GC) is another frequently applied technique for the analysis of phenolic compounds. However, the disadvantage of GC is that analyte derivatization is generally required to achieve high separation efficiency [320,321]. Using non-separative flow techniques, multivariant least square regression spectrophotometric analysis is mostly applied. This is possible due to the distinct UV-VIS absorbance spectra of the NP, which are given in the annex.

Due to the low concentrations of NP found in the environment, all former named techniques require sample clean-up and analyte enrichment procedures in order to achieved sufficient detection sensitivity. Generally, liquid-liquid extraction (LLE) [18,107] and solid phase extraction (SPE) for pre- [322] or on-detector [106,323] concentration are used. By combination of these techniques, e.g. solid phase extraction (SPE) followed by derivatization and LLE of the reaction product, highly sensitive quantification of total

## 7 SIA-CE and application to the determination of nitrophenols

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phenols was possible in an automated system [324]. Analysis of phenolic compounds in waters was overviewed in a comprehensive review of PUIG AND BARCELO (1996) [320].

CE is a less applied separation technique for the determination of NP, which can be contributed to the typically lower detection sensitivity [320]. On the other hand, NP have been used as model substances for the characterization of new CE separation techniques such as accomplishment of step-wise change of the separation buffer composition and pH [325], use of calixarenes [326] or cyclodextrines and polyvinylpyrrolidone [327] as additives to the separation buffer, or micellar separation [328]. In the present experimental work, separation of NP was done using a simple borate separation buffer with methanol as an organic modifier as prior described [329].

### 7.2.2. Coupling of flow techniques with capillary electrophoresis

Flow techniques are ideal tools for the automation of laboratory sample pre-treatment such as sample clean-up, analyte concentration, or automation of derivatization reactions carried out prior to injection in order to enhance detection sensitivity or specificity. By mechanization, these procedures generally yield higher reproducibility and diminished processing times and reagent consumption. To solve multi-analyte problems (see chapter 1.1.7), coupling with a separation technique such as CE is an elegant way to amplify the applicability of flow techniques. Besides, hyphenation with a flow system for automation of required sample pre-treatment allows the improvement of sensitivity and reproducibility of CE and the application to incompatible matrices such as samples of high salt or protein contents. Standard preparation for CE using a flow system was further reported [330].

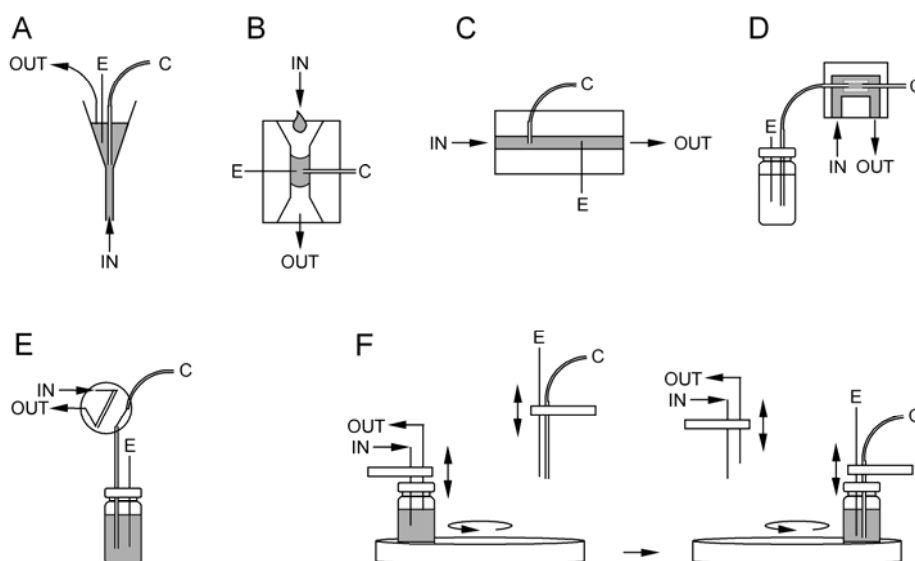
Instrumental solutions, applications, and resulting benefits of such hyphenated CE flow technique systems are found in various comprehensive reviews [118,119,138-140,331]. Different possibilities of interfacing flow techniques and CE are shown in figure 53 (A–F). They are explained shortly in the following with the applied interface type indicated by the respective letter (A–F). A synopsis about used instrumentation and operation versatility of hyphenated systems including the proposed systems is further given in table 33.

Pioneer work was done by BAO AND DASGUPTA (1992) [332], who used a membrane interface (D) for either on-capillary dialysis or gas diffusion for sample clean-up and analyte enrichment. On-capillary interfacing can further be done via nanoliter injection valves (E) [333-335]. Both types enable the use of commercial CE instruments, which are then used to carry out capillary re-conditioning and renewal of the separation buffer in the respective reservoirs. Alternatively, batch-wise injection using the autosampler of a commercial CE instruments was reported (F). Here, firstly, a vial is flushed intermediately with sample on an inactive sampler position and secondly, the vial is transported to the injection position [52,336,337]. HOGAN AND LUNTE (1994) [338] presented a hyphenated CE system as a monitoring tool for in vivo pharmacokinetic studies for the first time using an atmospherically open interface for EOF-traction injection (D). FANG ET AL. (1997) [339]

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proposed a miniaturized, conical-shaped, open flow interface (A). It enabled electrokinetic injection and was used for drug analysis [340-342]. Using an additional on-capillary membrane interface (D) injection via EOF-traction was further demonstrated [343]. In all these systems, automated buffer replacement and injection on the grounded capillary was done whereas application of pressure and thus, in-situ capillary flushing was not possible.

A tubular split-flow interface (C) was proposed by KUBAN ET AL. (1997) [344]. It allowed the easy integration into a flow manifold. High robustness originates from the fitting connection and tight fixation of the capillary to the interface. Multiple electrokinetic injections during running separations were possible since orthogonal positioning of the capillary to the flow direction led to minimal affection of the separation efficiency by the continuous flow [345-348]. A miniaturized chip-system applying the split-flow principle but controlled by SIA was proposed by FANG ET AL. (1999) [349]. It allowed quasi-continuous injections within 1 min. As considerable advantage, hydrodynamic injection (and capillary flushing) is possible due to the tight connections of capillary, electrode, and flow lines to the split-flow interface, either by positive pressure build-up by the intermediate closure of the interface outlet [350] or by the application of negative pressure at the capillary outlet [351]. Similar designs of split-flow interfaces have been used so far in flow systems based on FIA [4,352,353], SIA [354,355], or LOV technique [356,357].



**Figure 53: Principles and interface designs for the connection of flow techniques and CE.** Electrodes (E), capillary (C), flow entrance (IN), and outlet (OUT) are indicated. A: atmospherically open flow interface, B: falling-drop interface, C: tubular split-flow interface, D: membrane based on-capillary interface, E: micro-injection valve, F: batch-wise interfacing by the use of the autosampler of a commercial CE apparatus.

As overviewed in table 33 and in contrast to commercial systems, injection is generally done at the grounded side of the capillary in CE flow technique hyphenated systems, while the galvanic connection to high voltage (HV) is avoided in order to lower the risk of instrumental damage. Therefore, detection is done at the HV side and consequently,

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electrochemical detection techniques and in-situ cleaning of the outlet buffer reservoir are not possible. Nevertheless, connection of both buffer reservoirs to the flow manifold using a second flow interface for the HV side has been reported. The described problem was either overcome by using a resistant peristaltic pump and manifold grounding [353] or by applying gravimetric flow i.e. by avoiding an electrical pumping device [4], respectively. In another work, injection on the HV capillary side was required in order to enable the connection of the capillary outlet to a mass spectrometry interface. Here, the galvanic separation of manifold and HV electrode was achieved by filling the connection line either with air or with distilled water in order to enhance the exposed electrical resistance [352].

An alternative is given by falling-drop interfacing of the HV side, firstly proposed by FU AND FANG (2000) (B) [358]. Here, the solutions (separation buffer, sample...) are dropped from above into the tubular interface and flow out by gravity. An aliquot of the liquid remains stuck between the capillary tip and the HV electrode due to cohesion and acts as buffer reservoir. This configuration also allowed electrochemical and chemiluminescence end-of-capillary detection and the miniaturization of the FIA system as a chip [359-361].

### 7.3. Materials and methods

#### 7.3.1. SIA apparatus and manifold

The SIA instrumentation, manifold, and CE system are shown in figure 54. All components are described in detail in chapter 3.1, 3.2, and 3.3. Instrumental control, data acquisition, and data evaluation were carried out using the software AutoAnalysis as described chapter 3.4. The SIA instrumentation consisted of a valve module VA 1 + 1 equipped with an 8-port rotary selection valve and a syringe module Bu 4 S equipped with one 2.5 ml glass syringe purchased from Hamilton Bonaduz AG.

The original head MCSV of the syringe module did not show sufficient pressure stability for the performance of in-situ rinsing of the capillary. Consequently, the MCSV was used only for its function as connector for the syringe. For this, the front poppet of the MCSV was replaced by a commercial rubber septum and position ON was permanently closed with a blind connector. The head valve position OFF was connected via a short PTFE tube (8 cm, 1.5 mm i.d.) to a MCSV (V1) of enhanced pressure stability of nominal 600 kPa (type MTV-3-1/4UKGH) from Takasago Electric Inc. (Nagoya, Japan). The MCSV devices and undertaken modification are shown in the annex.

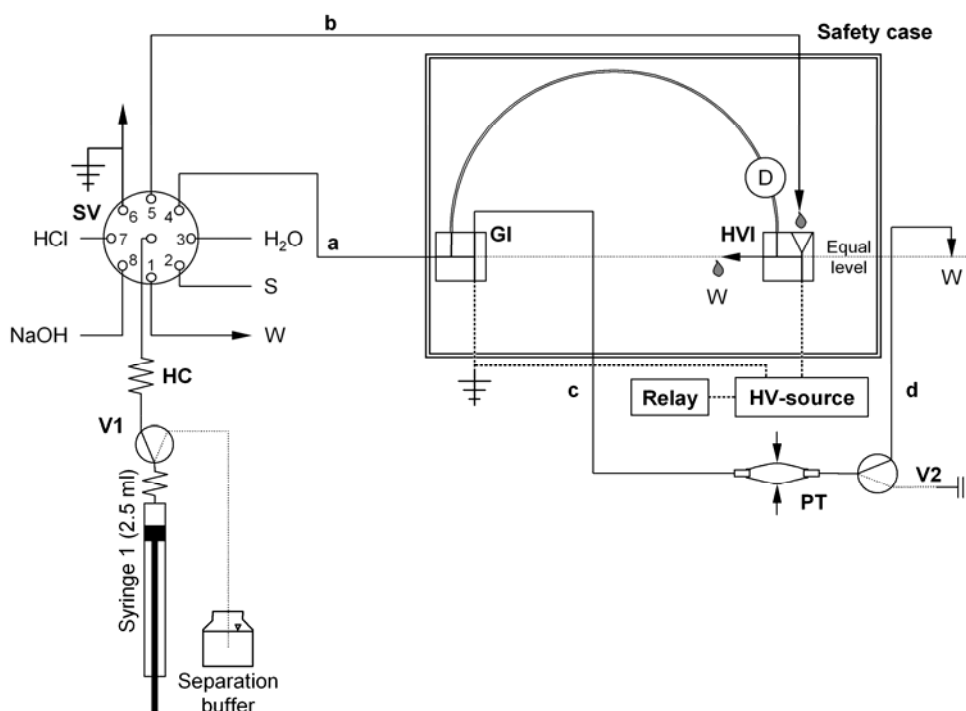
A second MCSV (V2) of 600 kPa nominal pressure stability was used for the intermediate closure of the grounded capillary flow interface outlet as described in chapter 7.3.2.4 and 7.3.4. It was powered by the supplying port 7 of the syringe module. Excessive valve heating was prevented using a protection circuit from Sciware S.L. Position ON of the MCSV V1 was connected to the central port of the selection valve via a PTFE holding coil (175 cm, 0.8 mm i.d.), position OFF was connected to the reservoir of



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the applied separation buffer used also as carrier. For optimization of the separation buffer, instead of the carrier reservoir, position OFF was connected to the supplying line of an autosampler. By this, automated exchange of the separation buffer was possible. The lateral selection valve ports of the selection valve were used for waste disposal (Pos 1), sample (Pos 2), water (Pos 3), connection to the grounded and the HV capillary flow interfaces (Pos 4 and Pos 5, respectively), air (Pos 6), 100 mmol l<sup>-1</sup> HCl (Pos 7), and 10 mmol l<sup>-1</sup> NaOH (Pos 8). Connection to the interfaces was done via PTFE tubes of reduced diameter (0.5 mm i.d.) in order to reduce the sample dispersion in the injection line (grounded side) and to heighten the electrical resistance of the tubing line to the HV interface for safety reasons, respectively. The interfaces are described in chapter 7.3.2.3.

The spectrophotometer USB 2000 (USB2E6039) was connected via an optical fiber of 400 µm core diameter to the on-capillary detection cell (D) described in chapter 7.3.2.1. The LED Cu6SMA1 (Roithner Lasertechnik GmbH) was used as light source, powered by a constant current source (Sciware S.L.). Single wavelength detection at  $\lambda_{\text{Det}} = 401 \text{ nm}$  was done. Due to the EOF plug flow character, the solvent of the injected sample (water) gave a change of the refraction index observed as baseline distortion. This allowed EOF identification. Data acquisition was done with 2 Hz applying an integration time of 20 ms, a wavelength interval of 4 nm, and averaging over 22 measurements.



**Figure 54: Manifold of the coupled SIA-CE system.**

**V1, V2: solenoid valves, SV: selection valve with sample (S), waste (W), water, and cleaning solutions (HCl, NaOH), HC: holding coil (175 cm, 0.8 mm i.d.), GI: grounded interface, HVI: high-voltage interface, D: detector, PT: pressure tube reservoir, tube a: 40 cm, 0.5 mm i.d., tube b: 50 cm, 0.5 mm i.d., tube c: 40 cm, 0.8 mm i.d., tube d: 20 cm, 0.8 mm i.d. Normally closed valve positions (OFF, deactivated) and electrical connections are given as dotted.**

### 7.3.2. CE apparatus and interfaces

#### 7.3.2.1. Detector

The detection cell used for on-capillary photometric detection was developed during this work. As stated in chapter 1.2.3.4, three main challenges have led to the constructive considerations. The first one was the fabrication of a small aperture smaller than the inner diameter of the separation capillary since the maximal sensitivity is only achieved when the detector visual field is limited to the liquid filled part of the capillary. In other words, only light passing the liquid volume in the capillary but not only the wall material is detected. Focusing lenses were avoided in order to avoid the complication of their optical alignment. In consequence, the optical window had to be equal or less than 75  $\mu\text{m}$ .

The second challenge was the alignment of the optical fiber connected to the USB 2000 spectrometer, focus of the illuminating light source, and the capillary. Here, the main objective was the maximization of the illumination intensity in order to minimize the required integration time of the spectrometer. Consequently, higher illumination intensity allows also the maximization of data averaging at a given measuring frequency and so the minimization of baseline noise and lower signal contributions of bubbles or particles.

The third challenge was the need of a stress-free fixation of the fused-silica capillary. This was due to the high fragility of the capillary where the polyimide protection coating had to be removed in order to obtain a transparent window. Besides, the capillary had to be securely fixed in order to assure its alignment with the aperture of the detection cell. A displacement of a few  $\mu\text{m}$  would already affect the detector performance considerably.

The engineering drawings and photo documentation of the final detection cell are given in the annex. It consisted of two parts made of aluminium. The parts were fabricated by turning and in one cycle with a four-jaw chuck to achieve highest manufacturing precision and to assure the perpendicularity of light path and contact area of the cell parts. On the outer face, both parts showed UNS  $\frac{1}{4}$ " 36" threaded holes for screwing-in short segments of threaded pipes as SMA 905 junctions for the connection of an optical light fiber (detection part) and a homemade LED support (illumination part), respectively. In both parts, the threaded holes ended about a half millimeter from the inner face in an aperture made with a 0.4 mm drill. The illumination cell part showed further a milled, trapezoid, and centered groove on the inner face of 0.4 mm depth and width to align the capillary.

For stress-free fixation, the capillary was placed into the groove, aligned with the prior produced transparent window to the opening, fixed with glue (UHU, Bühl, Germany), and finally adhered with black nail polish. The transparent window on the capillary was former prepared by removing the polyimide coating from about 5 mm by burning and cleaning the zone with methanol afterwards. The diameter of the drilled aperture had to be reduced to the inner diameter of the capillary, which was done by depositing a layer of black nail polish onto the hole walls and, if necessary, manipulation with an entomologic preparative needle under a stereo-microscope (magnification 1:20).

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This labor-intensive and patience taxing procedure done in this work was substituted in the second work using CE described in chapter 8. Here, two small pieces of aluminum foil were adjusted in order to cover the illuminated part of the capillary after its montage leaving open only a slit of about 50  $\mu\text{m}$  adhered with black nail polish. The aluminum foil pieces were protected against mechanical stress with two strips of adhesive film. Finally, the cell was assembled with the LED support and the optical fiber connection to the spectrometer. Both cell parts were carefully aligned in order to achieve maximal illumination intensity and finally securely screwed.

### 7.3.2.2. Capillary

The separations were carried out in a fused-silica capillary of 75  $\mu\text{m}$  i.d. from Polymicro technologies LLC. (Phoenix, USA). The total capillary length was 59 cm, the effective length, i.e. the length between capillary entrance and the detection window, was 53.5 cm. The capillary showed a polyimide protection layer against mechanical stress at the outside. The inner surfaces were uncoated.

### 7.3.2.3. Capillary flow system interfaces

The objective of the system autonomy and applicability for monitoring purposes demanded for the control of the entire system including the buffer reservoirs at both sides of the capillary. A second requirement was the possibility to perform both hydrodynamic and electrokinetic sample injection as well as capillary flushing. This required the ability of pressure application at least at one side of the capillary. Interfaces between the flow system manifold and both sides of the capillary acting also as buffer reservoirs were developed. The interfaces are shown with photos and engineering drawing in the annex. The different principles and designs of interfaces were already discussed in chapter 7.2.2.

In most flow systems coupled with CE, the manifold is galvanically connected to the injection interface. This is in contrast to commercial systems generally the grounded side in order to avoid instrumental damage by HV and to omit the otherwise required integration of adequate prevention measures [119]. For these reasons, the same concept was also applied in this work. The grounded interface followed a tubular split-flow design depicted in figure 53 C such as applied several times in coupled CE flow systems.

In order to enable pressure application, the outlet of the split flow interface was connected to the former named MCSV "V2". Its position OFF was permanently closed using a blind connector; the position ON was used as the atmospherically open interface outlet. The intermediate closure of the interface outlet allowed forcing liquid through the capillary (see chapter 7.3.2.4). A similar functionality was achieved using pinch valves [350,351-357]. Permanently open or membrane interface designs were rejected as less versatile since they do not enable pressure application at the capillary entrance as required for in-situ capillary re-conditioning or for hydrodynamic injection.

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For the development of a second interface the principle of falling-drop interfacing was adopted in order to connect also the HV-side to the flow manifold [358]. In the proposed system, HV interface cleaning and separation buffer exchange was done by dropping the required solutions from 3 cm above into a miniature funnel integrated in the HV interface. By this, galvanic separation of the flow manifold and simultaneously the exchange of the contact liquid between the capillary were maintained. The hydrostatic pressure of the introduced liquid forced flushing of the interface and flow-out at the sideward opening. By this, stacked gas bubbles originated from electrolysis could be further removed.

The flow channel of the grounded interface was made small with 0.7 mm i.d. in order to reduce the sample dispersion and the dead volume. On the other hand, it still allowed the visual positioning of the capillary tip in the center of the flow channel. A wider inner diameter was applied for the flow channel of the HV interface in order to facilitate the gravimetric buffer outflow and removal of stacked gas bubbles during the cleaning procedure. In both interfaces, the maintenance of the galvanic, i.e. liquid, contact between the capillary and the respective electrode was imperative. Thus, the electrode containing sections of the interfaces were made with wider diameter than the flow channel in order to avoid the interruption of the galvanic contact by gas bubbles formed by electrolysis.

The application of pressure at the grounded interface for capillary flushing as well as the height adjustment of both capillary ends required a tight connection of the capillary in the interfaces. Therefore, the capillary ends were inserted into short pieces of PTFE tube (ca. 2 cm, 0.5 mm i.d.), whose ends were squeezed into the conical capillary inlets of the interfaces by screwing-in the respective tube fittings. The electrodes were prepared from pieces of platinum wire (ca. 1.5 cm, 0.5 mm o.d.), soldered to copper contact cables, cemented into commercial fittings of polyphenylene sulfide with commercial adhesive, and sealed into the interface using slices of silicon tube of 1 mm thickness as washers.

### 7.3.2.4. Pressure reservoir

The objective of construction of an autonomously operating and long-term stable CE system implied the need of regular and in-situ rinsing of the capillary for re-conditioning of the capillary inner walls. The minimal performable flow rate with the multisyringe device using the smallest available syringe (1 ml dispense volume) was  $57 \mu\text{l min}^{-1}$ . The resulting backpressure estimated with formula 19 (see chapter 3.6.2) for the used capillary exceeds 25 bar and by this the pressure stability of the used MCSV several-fold. Besides, direct coupling enabled in former works by using instrumentation of higher stability and lower practicable flow rate [356,357], requires continuous pumping operation for capillary rising.

In consequence, direct capillary flushing had to be substituted by a pressure forced flow-rate in this work. So the objective was to build-up a stable and sufficiently high pressure for capillary flushing on command without affecting the integrity and functionality of the syringe pump or MCSV. Using an external air compressor as former described [351], the systems price and complexity would have increased. The use of backpressure

## 7 SIA-CE and application to the determination of nitrophenols

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coils in order to generate pressure as a function of the applied flow rate was rejected as it also required continuous pump operation and led to enhanced reagent consumption [352].

In this work, a silicon rubber tube (length 3.5 cm, 5.5 mm o.d., 1.5 mm i.d.), following denoted "pressure reservoir", was incorporated between the outlet of the grounded interface and the downstream located V2. It was fixed with nylon tie wraps to commercial tube fittings. Forcing liquid towards the grounded interface with V2 in position OFF (interface outlet closed) led to the expansion of the silicone tube and pressure build-up in consequence of the increasing tube wall tension (see photo documentation in the annex). With the interface outlet kept closed, the pressure could only be released via the capillary. Activation of V2 led to instantaneous pressure release by opening of the interface outlet.

The resulting flow rate through the capillary was in the order of microliters per minute. In consequence, the pressure decreased slowly and capillary flushing was feasible over 10 minutes. Since pumping was required only for pressure build-up, other operations were practicable during capillary flushing. In other words, background operations were feasible on a coupled flow technique CE system for the first time. The pressure increased with increasing volume applied for the inflation. Tube bursting became probable for volumes above 400  $\mu\text{l}$ . Measurement of the Flow rate through the capillary allowed the estimation of the resulting pressure. The finally applied volume of 300  $\mu\text{l}$  resulted from a pressure of ca. 3 bar. In-situ pressure measurement was not possible for want of a miniature sensor. Small volumes of a few microliters were applied to generate a low, reproducible pressure pulse to perform hydrodynamic injection. A similar construction was found to be used by KULKA ET AL. (2006) [354] but was used only in order to improve the injection precision.

### 7.3.2.5. Protection case

For reasons of convenient working, labor safety, minimization of external influences, transportability, and robustness, the components of the CE system (interfaces, detector, and capillary) were assembled in a protection case designed for this work. Constructive considerations were about the required electrical connections, dimensions, arrangement of the components, compactness, and requirements resulting from instrumental and operational aspects of the housed analytical system. Engineering drawings and photos of the entire protection case and details are given in the annex.

The case was made of polymers of excellent isolation properties, PMMA and PVC (electrical resistivities  $> 10^{15} \Omega\text{m}$ ). The back panel was mounted securely onto a support panel. It held an eight-pin coil support for the separation capillary and adaptors for both interfaces allowing an easy installation or removal of each one. Apertures in the back panel were made for the outlet tube of the grounded interface, a falling-drop outlet above the HV interface, for the montage of the detection cell, the connected optical fiber, the electrical supply cable of the LED used as light source and sockets for the both-way removable electrical connections to the capillary interfaces (male, inner side) and the HV power source (female, outer side).

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On the opposite side of the back panel, the MCSV “V2” showing an elevated pressure stability was mounted and connected with the common port to the outlet of the grounded interface by a short PTFE tube (10 cm, 0.8 mm id.) and the silicon rubber tube acting as pressure reservoir (see chapter 7.3.2.4). The valve position OFF was closed with a blind connector, in position ON, a rigid curved PEEK tube (15 cm, 0.8 mm id.) was used to adjust the atmospheric outlet of the interface to the same level as the outlet of the high-voltage interface in order to avoid laminar flow in the separation capillary by siphoning.

Two drain-groves were milled into the support panel to avoid the unhindered spread-out of eventually leaking separation buffer from the interfaces and by this to reduce the risk of galvanic connection. By four plastic screws at each corner of the support panel, declination of the surface could be balanced by appropriate height adjustment.

The side, front, and top panels of the protection case were made-up as a single unit, which could be removed completely. The only component included on the front part was a common computer fan located oppositely to the capillary coil support. This allowed the efficient dissipation of joule heat from the capillary generated by HV application and by this the reduction of thermal diffusion and current increase as explained in chapter 1.2.3.3. Besides, rise of air humidity in the protection case and consequently the risk of sparking were avoided by the continuous air exchange. The clearances remaining between front and the back part allowed the passage for the inlet tube of the grounded interface.

### 7.3.2.6. Power source and remote safety control

All experiments were performed by using a 2127 Tachophor HV supply (LKB, Bromma, Sweden). The separation voltage and maximal current were manually adjusted on the front panel of the source. Remote control of HV application was accomplished using a relay, which was powered by the supplying port 6 of the syringe module. On activation of the relay by software instruction [6-ON], the electrical contacts of the analog safety circuit of the HV source were connected, which initiated the HV application. On deactivation of the relay [6-OFF], fully HV shutdown happened within less than one second. HV shutdown was further enabled on the opening of the protection case by the integration of a safety key into the safety circuit, which was mounted in the right top-corner of the protection case back panel. Removing the front part of the protection case during HV application led to the interruption of the safety circuit and deactivation of the HV-source.

### 7.3.3. Reagents

The reagents were prepared as prior described in chapter 3.5.1.3. All reagents were of analytical grade and Millipore purified water was used throughout. Stock solutions of mono-NP of  $360 \text{ mg l}^{-1}$  were prepared by accurately weighting and dissolving in  $10 \text{ mmol l}^{-1}$  NaOH. Working solutions were prepared by dilution of the stock solutions.

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For optimization experiments, solutions containing o-NP, m-NP, and p-NP in a ratio of 2:7:1 in 1 mmol l<sup>-1</sup> NaOH were used achieving by this a partly compensation of the highly different molar absorptivity values of the analytes at the applied detection wavelength of 401 nm and of the detection sensitivity. Methanol (1 v/v%) was added for EOF marking.

Separation buffers of different compositions were prepared using a 0.5 mol l<sup>-1</sup> borate stock solution of pH 8.4 and methanol as organic modifier. The requested pH value was adjusted with filtered 1 mol l<sup>-1</sup> NaOH. Since the concept of pH is only defined for water, the designation "apparent pH" is used in the following. NaOH 10 mmol l<sup>-1</sup> and HCl, 0.1 mol l<sup>-1</sup> were further used for cleaning and re-conditioning of the capillary.

Water and stock solutions were filtered through 0.45 µm membrane filters. To study the analyte recovery in real samples, filtered water samples from a local urban wastewater treatment plant were used. The respective samples were from the plant entrance (E), the outlet of the mechanical treatment (S1), and the final outlet after the biological treatment and sedimentation (S3). Spiking was done with aqueous standards to final concentrations of 103.5, 362, and 51.8 µmol l<sup>-1</sup> for o-NP, m-NP, and p-NP, respectively.

### 7.3.4. Software and analytical procedures

Instrumental control, data acquisition, and data processing were carried out using the software package AutoAnalysis 5.0 from Sciware S.L. described in detail in chapter 3.4 running on a PC with operating system MS-Windows 2000. The methods and procedures accomplished for analysis and for system cleaning are given in the annex.

All experiments were performed using the same separation capillary. After installation, the capillary was first treated with 1 mol l<sup>-1</sup> hydrochloric acid, 1 mol l<sup>-1</sup> NaOH, and Millipore water, each for 30 min and flushed afterwards with separation buffer for 2 hours. Each day, the capillary was first flushed with 10 mmol l<sup>-1</sup> NaOH, water, and separation buffer for at least 5 min. After finalization of the experiments and for storage, the capillary was flushed with 10 mmol l<sup>-1</sup> NaOH for 5 min and with water for at least 10 min.

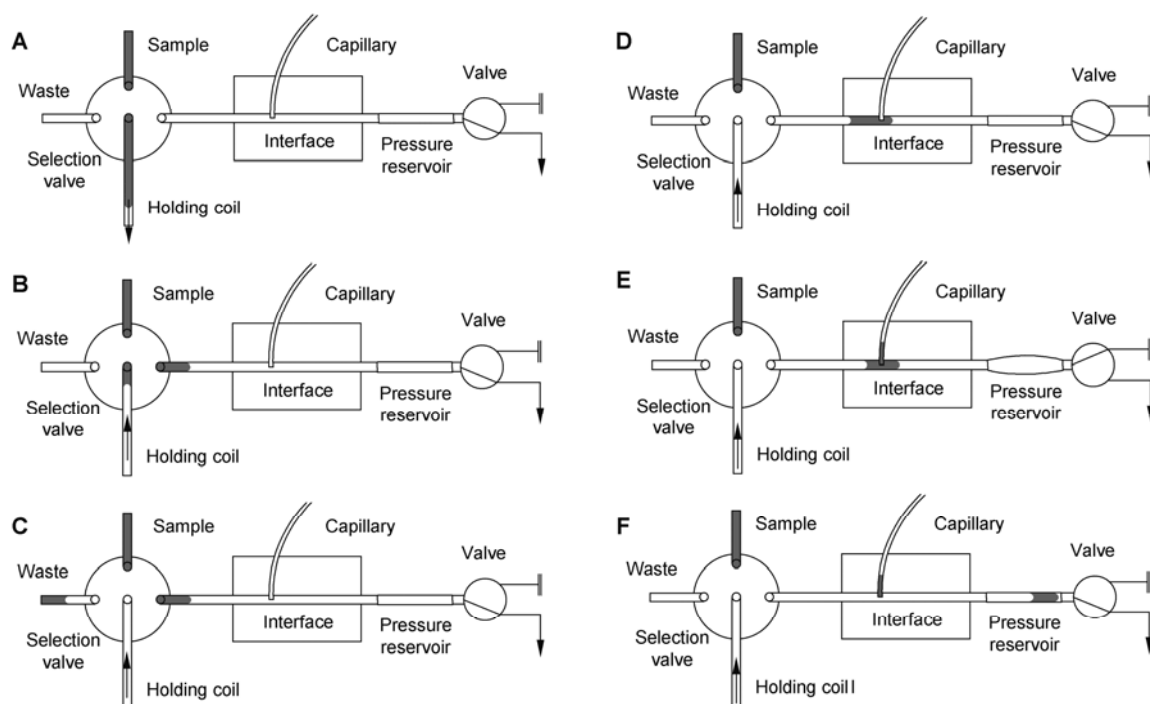
Flushing was done by the aspiration of 300 µl of the respective solution and propelling 50 µl towards the grounded interface with the interface outlet open (V2 activated, ON), then 300 µl with the outlet closed (V2 deactivated, OFF) leading to the inflation of the pressure reservoir and pressure build-up, and finally holding for 5 minutes. The pressure was relieved by activation of V2, i.e. by opening the grounded interface outlet. By this procedure, the respective solution was forced through the capillary resulting in the slow decrease of the pressure. Flushing over a longer period was done by the repetition of the cleaning procedure. For flushing with the separation buffer, i.e. the used carrier, 300 µl were directly propelled towards the interface with closed outlet.

A second cleaning procedure was accomplished for separation buffer replacement at the HV interface. Here, the buffer was dropped into the interface from above followed by the re-aspiration of the remaining liquid in the tube and its discharge to waste. So, the

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connecting tube remained empty during voltage application for safety precautions. Thanks to the pressure reservoir, this procedure could be accomplished in parallel to capillary flushing with separation buffer. A third cleaning procedure was used to remove stacked air bubbles from the holding coil and the syringe head and respective MCSV.

The procedure for sampling and injection is given schematically in figure 55. For injection, 100  $\mu\text{l}$  of sample were aspirated and an aliquot of 50  $\mu\text{l}$  was propelled towards the grounded interface. The rest of the sample was discharged to waste. By this, the dispersion of the sample prior to injection was reduced. Afterwards, the sample was further propelled towards the capillary entrance with atmospherically open interface outlet. Hydrodynamic injection was done by the dispense of 10  $\mu\text{l}$  with the interface outlet closed and holding for 800 ms. Finally, the outlet was opened and the grounded interface was flushed with 200  $\mu\text{l}$  of the separation buffer. Finally, the HV was turned on by software commanded switch of the control relay. During separation, the interface outlet remained open, i.e. V2 was continuously activated to ensure atmospheric pressure on both interface outlets. Before each analysis, the capillary was flushed for 2 min with separation buffer for re-conditioning and the buffer in the HV interface was exchanged.



**Figure 55: Scheme of the sampling and injection procedure on the CE-SIA analyzer.**

**A: Aspiration of sample, B: Propelling of a sample aliquot towards the grounded interface, C: Discharge of abundant sample, D: Positioning of the sample aliquot at the capillary tip, E: Hydrodynamic injection, F: Flushing of grounded interface with separation buffer.**



### 7.4. Experiments and results

#### 7.4.1. Optimization of instrumentation

##### 7.4.1.1. Manifold and interfaces

In order to improve the pressure robustness required in order to flush the capillary with separation buffer or cleaning solutions, MCSV of enhanced pressure stability (up to 6 bar) were used for the flow system, one replacing the original head valve of the syringe pump.

The dispersion of the sample aliquot used for injection was diminished in order to avoid its dilution and consequently a sensitivity loss by using small inner diameters for the connecting tube to the grounded interface and the respective flow channel. By this, the separation buffer volume for post-injectional flushing of the interface was also diminished.

It was observed, that gas bubbles were generated by electrolysis in the HV interface during the separation, which entered occasionally the flow channel. Testing different interface designs it was observed, that for small flow channel diameters these bubbles were able to interrupt the liquid contact between the electrode and the capillary outlet and consequently the electrical contact effectively. Therefore, the final design showed a flow channel of 2 mm i.d. in order to assure the electrical contact in each case and to facilitate the gravimetric flow through the interface for buffer replacement and bubble discharge.

Flushing of the interfaces with separation buffer was done before each analysis with 600  $\mu$ l on the injection (grounded) side and 500  $\mu$ l on the detection (HV) side. Due to the repeated conditioning and bubble removal, part of the optimization work could be carried out autonomously and overnight. For this, separation buffer solutions varying in their composition in respect of the parameter to be optimized were placed on the autosampler. By use of a software instructed protocol, the optimization experiments were carried out by:

1. placement of one autosampler vial (100 ml buffer) to be used as carrier reservoir
2. exchange of the separation buffer by four-fold rinsing of the syringe
3. conditioning of the capillary with the actual separation buffer for 15 min
4. separation in triplicate of a standard solution containing all three analytes
5. repetition of step 1 - 4 using the separation buffer on the next autosampler position.

During the experimental work, sparking or short circuits were never observed, which was reduced to the integration of the former listed safety measures. The reliability of the proposed analyzer is considered as one noteworthy achievement of this work.

Levelling of both capillary ends is a general requirement in CE in order to avoid laminar to siphoning and by this peak disruption, modification of the separation time, and decrease of the separation efficiency. It was observed, that a level difference of both interface outlets of about 1 cm did not show any significant influence on the peak resolutions, the peak heights, or the migration times.

### 7.4.1.2. Detection and data evaluation parameters

Due to the physical separation of the analytes from the sample matrix and the plug EOF characteristic, dual wavelength detection for the compensation of schlieren effects was not required. Therefore, photometric detection on a single wavelength was done. In a former work, detection was done at 191 nm due to the presence of a common maximum of the molar absorptivity values showing a similar height for the three NP [329]. A second common but considerably lower maximum of the NP in their basic i.e. dissociated form is located at about 400 nm, while other absorptivity maxima of the analytes do not match.

As a disadvantage, the analytes have clearly different molar absorptivity values at 400 nm. The values calculated from the spectrum data given in the annex for o-NP, m-NP, and p-NP were 3270, 1480, and 18350 l mol<sup>-1</sup> cm<sup>-1</sup>, respectively. For want of a deep-UV illumination source of sufficient intensity and due to the diminished ultraviolet sensitivity of the USB 2000 spectrometer, 401 nm were applied for visible photometric detection. The advantage of visual wavelength detection was the possibility to use a blue LED as a highly stable light source of adaptable illumination intensity. The LED was placed directly onto the on-capillary detection cell. Consequently, a second optical fiber was not required and higher illumination intensity was obtained. In order to obtain similar peak heights for the analytes in spite of the different absorptivity values and by this, different sensitivities, o-NP and m-NP were used in higher concentrations as p-NP in the standards (chapter 7.3.3).

Peak widths in CE are typically in the order of few seconds. To assure good sufficient data representation, an acquisition time of 0.5 s was applied. A higher data acquisition frequency was not required since observed peak widths were between 20 s and 30 s. The high emission intensity of the LED allowed nevertheless a small integration time of 20 ms and consequently averaging over 22 single measurements. Smoothing over a wavelength interval of 4 nm was done. The baseline noise was low with amplitude of 0.2 mAU. The diminution of one of the former variables led to an increase of the baseline noise. A four-order polynomial was used for data smoothing. Peak height data were used throughout since repeatability was better compared to peak area data (see chapter 7.5.1.2).

### 7.4.2. Optimization of physical variables

The method variables sample volume, sample placement, applied flow rate, volume for injection pressure, and respective holding time were all software controlled. Optimization was done by automated univariable modification of the respective parameter, whereas optimization of the separation voltage required manual adjustment<sup>7</sup>.

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<sup>7</sup> Thought described first in this work, the optimization of the separation voltage was done chronologically after the optimization of the separation buffer composition.

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### 7.4.2.1. Influence of flow parameters

The influence of the flow rate for the sample aliquot positioning and interface flushing with separation buffer after the injection on the peaks heights, widths, and resolution was studied in the range of 1.0-3.75 ml min<sup>-1</sup>. Experimental results and separation conditions are given in table 22 and are partly represented in figure 56. A slight decrease of the peak heights with flow rate and slightly wider peaks at a flow rate of 1 ml min<sup>-1</sup> were observed. This was reduced to the distortion of the injected sample volume by the enhanced syringe pump pulsation at flow rate. Therefore, a flow rate of 1.5 ml min<sup>-1</sup> was finally chosen.

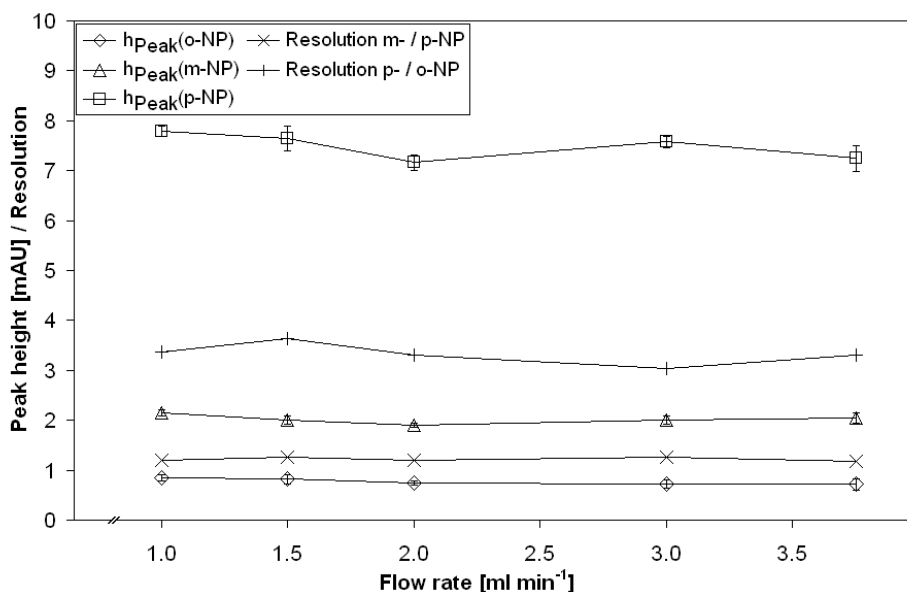


Figure 56: Data representation of the optimization of flow rate for the injection procedure.

Table 22: Influence of the flow rate applied for sample positioning and interface flushing. \*

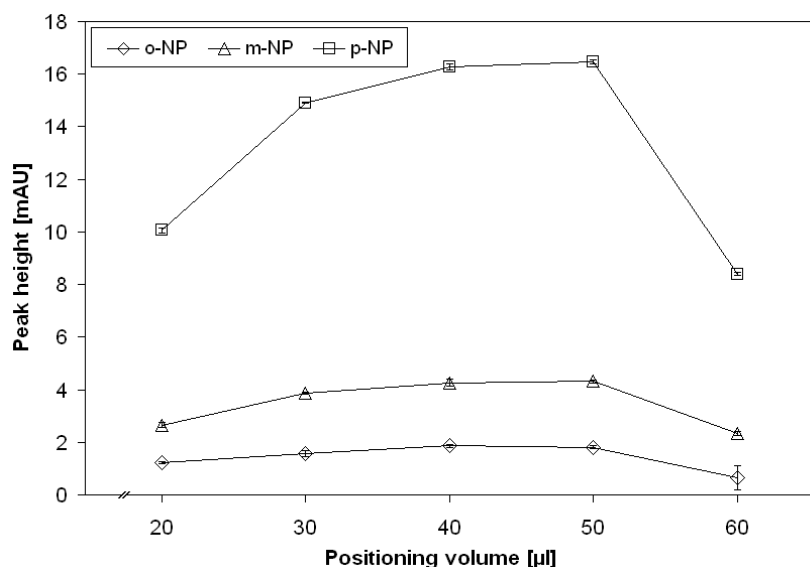
Flow rate [ $\mu$ l] $\pm$ SD (n = 3)	1.0	1.5	2.0	3.0	3.75
Peak height o-NP	0.84 $\pm$ 0.11	0.82 $\pm$ 0.06	0.74 $\pm$ 0.07	0.72 $\pm$ 0.07	0.72 $\pm$ 0.06
Peak height m-NP	2.15 $\pm$ 0.06	2.01 $\pm$ 0.09	1.90 $\pm$ 0.05	2.01 $\pm$ 0.08	2.05 $\pm$ 0.11
Peak height p-NP	7.80 $\pm$ 0.10	7.67 $\pm$ 0.25	7.18 $\pm$ 0.15	7.59 $\pm$ 0.11	7.26 $\pm$ 0.26
Peak width o-NP	29.5	23.2	28.0	24.7	26.2
Peak width m-NP	27.8	22.0	28.3	31.7	29.3
Peak width p-NP	40.8	30.3	37.0	41.7	35.7
Resolution m- / p-NP	1.20	1.26	1.21	1.27	1.19
Resolution p- / o-NP	3.38	3.64	3.32	3.05	3.32

\* Conditions: 40 mmol l<sup>-1</sup> borate, 25 kV, apparent pH 9.7, 10 v/v% methanol, sample: 14.4 mg l<sup>-1</sup> o-NP, 50.2 mg l<sup>-1</sup> m-NP, and 7.2 mg l<sup>-1</sup> p-NP

The positioning volume of the sample aliquot (50  $\mu$ l) at the capillary entrance prior to the application of the injection pressure pulse was optimized in the range of 20  $\mu$ l to 60  $\mu$ l. Experimental results and applied separation conditions are given in table 23 and are further represented in figure 57. The maximal peak heights were obtained for 40  $\mu$ l and

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50  $\mu\text{l}$ . A volume of 40  $\mu\text{l}$  was applied since the peak height dependency on the volume deviation (tangent slope) was low at this point promising higher robustness.



**Figure 57: Data representation of the optimization of the volume for sample positioning. Conditions of separation and analyte concentrations are given in table 23.**

**Table 23: Influence of volume for sample positioning of the applied 50  $\mu\text{l}$  sample plug. \***

Positioning volume [ $\mu\text{l}$ ] $\pm$ SD (n = 3)	Peak heights [mAU]		
	o-NP	m-NP	p-NP
20	1.22 $\pm$ 0.04	2.66 $\pm$ 0.09	10.06 $\pm$ 0.10
30	1.56 $\pm$ 0.11	3.87 $\pm$ 0.05	14.91 $\pm$ 0.03
40	1.86 $\pm$ 0.05	4.27 $\pm$ 0.12	16.29 $\pm$ 0.11
50	1.81 $\pm$ 0.05	4.31 $\pm$ 0.07	16.47 $\pm$ 0.09
60	0.64 $\pm$ 0.46	2.33 $\pm$ 0.07	8.39 $\pm$ 0.06

\* Conditions: 40 mmol  $\text{l}^{-1}$  borate, 25 kV, apparent pH 9.7, 10 v/v% methanol, sample: 28.8 mg  $\text{l}^{-1}$  o-NP, 100.4 mg  $\text{l}^{-1}$  m-NP, and 14.4 mg  $\text{l}^{-1}$  p-NP

**Table 24: Influence of the volume applied for interface flushing after injection. \***

Flushing volume [ $\mu\text{l}$ ] (n = 3)	120	160	200	240
Peak height o-NP	0.72	0.85	0.85	0.80
Peak height m-NP	2.00	2.06	1.96	1.93
Peak height p-NP	7.26	7.56	7.27	6.95
Peak width o-NP	23.7	29.5	28.5	27.0
Peak width m-NP	24.8	26.0	29.2	28.3
Peak width p-NP	35.0	36.7	38.7	37.0
Resolution m- / p-NP	1.51	1.43	1.46	1.47
Resolution p- / o-NP	4.11	3.78	4.03	4.08

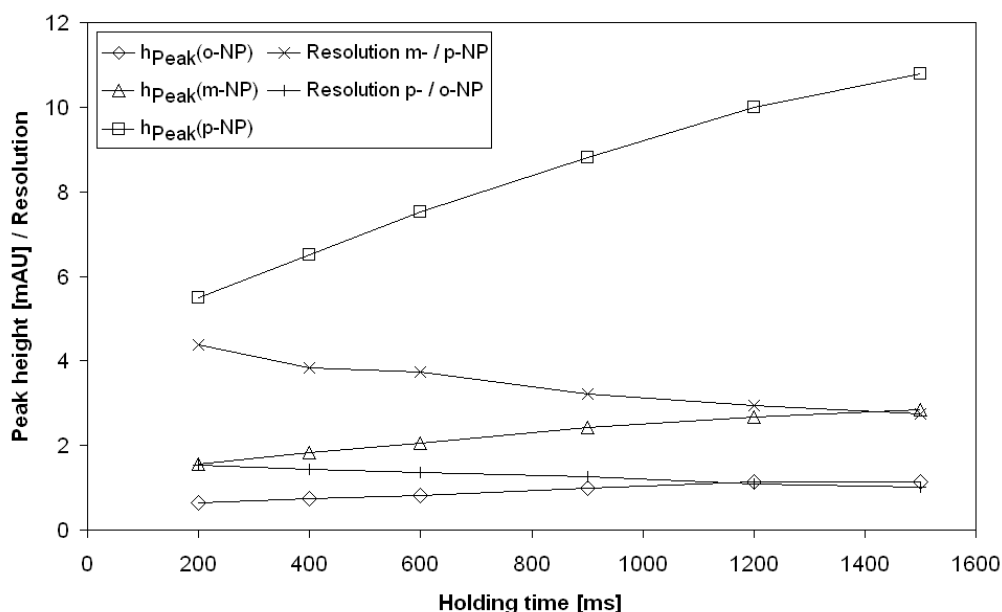
Separation conditions and sample as given in table 22

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The required volume for interface rinsing after injection was minimized. It was studied in the range of 120  $\mu\text{l}$ -240  $\mu\text{l}$ . Experimental results and separation conditions are given in table 24. No significant influences of the rinsing volume on peak heights or peak width were observed. However, for volumes below 200  $\mu\text{l}$ , the baseline increased notably after the detection of the slowest-migrating analyte o-NP. This was probably due to traces of the analytes remaining in the interface, which were introduced into the capillary by EOF traction. Therefore, a rising volume of 200  $\mu\text{l}$  was finally chosen.

### 7.4.2.2. Influence of hydrodynamic injection time

After sample aliquot positioning, hydrodynamic injection was performed with a pressure pulse volume of 10  $\mu\text{l}$  followed by a short, well-defined pressure holding time or injection time, respectively. Its influence was studied in the range of 200 ms-1500 ms. Results and separation conditions are given in table 25 and are represented in figure 58.



**Figure 58: Data representation of the optimization of the injection pulse holding time. Conditions of separation and analyte concentrations are given in table 25.**

**Table 25: Influence of holding time after injection pulse on peak heights and resolution. \***

Peak height [mAU] (n = 3)	Holding time [ms]					
	200	400	600	900	1200	1500
o-NP	0.64	0.74	0.82	0.99	1.13	1.13
m-NP	1.57	1.84	2.05	2.42	2.66	2.85
p-NP	5.50	6.50	7.52	8.80	9.99	10.78
Resolution m- / p-NP	4.37	3.84	3.73	3.21	2.95	2.75
Resolution p- / o-NP	1.53	1.44	1.35	1.26	1.08	1.01

\* Conditions: 40 mmol  $\text{l}^{-1}$  borate, 25 kV, apparent pH 9.7, 10 v/v% methanol, sample: 14.4 mg  $\text{l}^{-1}$  o-NP, 50.4 mg  $\text{l}^{-1}$  m-NP, and 7.2 mg  $\text{l}^{-1}$  p-NP

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Peak resolutions decreased with longer holding times whereas peak heights increased about 180 % over the tested range. A holding time of 800 ms was finally chosen to ensure baseline resolution of the peaks of p-NP and o-NP at a simultaneous high sensitivity. A volume for pressure build-up higher than 10  $\mu\text{l}$  would have required a very short holding time in order to achieve still baseline peak separation. So, the relative error caused by the deviation of the MCSV switching time would have been high. A lower pressure pulse volume than 10  $\mu\text{l}$  was considered not to be performable with satisfying reproducibility.

### 7.4.2.3. Influence of separation voltage

The influence of the separation voltage was studied applying negative voltage on the detection side of 15, 20, 25, and 30 kV. A standard solution containing o-NP 14.4 mg l<sup>-1</sup>, m-NP 50.4 mg l<sup>-1</sup>, and p-NP 7.2 mg l<sup>-1</sup> and a separation buffer of 40 mmol l<sup>-1</sup> borate with 10 v/v% methanol and an apparent pH of 9.7, were used. Experimental results are given in table 26 and are further represented in figure 59.

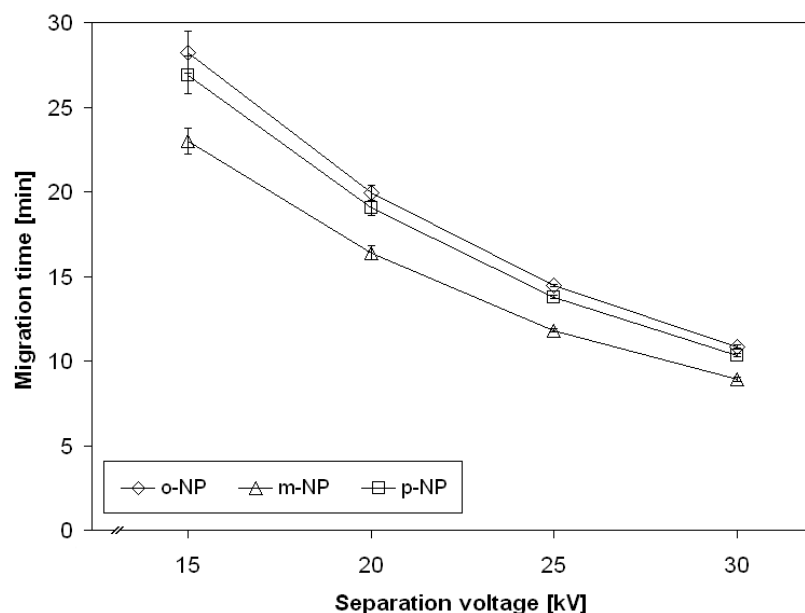


Figure 59: Data representation of the optimization of the separation voltage.

Table 26: Influence of the separation voltage on the migration times and resolution.

Migration time [min] $\pm$ SD (n = 3)	Separation voltage [kV]			
	15	20	25	30
EOF	8.76 $\pm$ 0.09	6.26 $\pm$ 0.10	4.52 $\pm$ 0.03	3.41 $\pm$ 0.02
o-NP	28.24 $\pm$ 1.24	19.94 $\pm$ 0.46	14.46 $\pm$ 0.07	10.85 $\pm$ 0.10
m-NP	22.98 $\pm$ 0.76	16.40 $\pm$ 0.40	11.80 $\pm$ 0.11	8.92 $\pm$ 0.10
p-NP	26.90 $\pm$ 1.10	19.05 $\pm$ 0.45	13.77 $\pm$ 0.10	10.35 $\pm$ 0.09
Resolution m- / p-NP	4.89	4.82	4.25	3.62
Resolution p- / o-NP	1.67	1.60	1.48	1.33

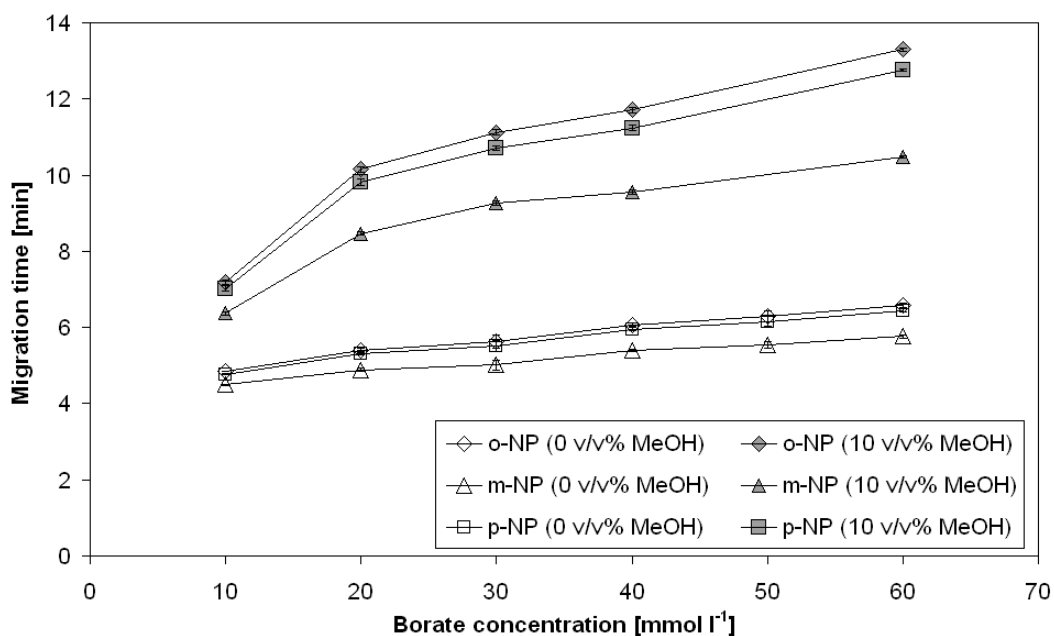
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Peak resolution and migration times decreased considerably with increasing voltage. A voltage of 25 kV was chosen as a compromise between migration time and the obtained peak resolution of p-NP and o-NP. An increase of the baseline noise with the applied voltage as former reported was not observed [329].

### 7.4.3. Optimization of separation buffer composition

#### 7.4.3.1. Influence of BGE borate concentration

The influence of the borate concentration in the separation buffer was studied for a methanol-free buffer and with a methanol content of 10 v/v%, both of pH 9.4 in the range of 10-60 mmol l<sup>-1</sup>. Experimental results are given in table 27 and represented in figure 60.



**Figure 60: Data representation of the optimization of the borate concentration of the separation buffer for buffer without methanol and with 10 v/v% of methanol. Conditions of separation and analyte concentrations are given in table 27.**

Migration times and the peak resolutions increased with higher borate concentrations. Baseline separation of the p-NP and o-NP peaks was not achieved using a separation buffer without methanol addition while a satisfying separation was achieved throughout with addition of 10 v/v% of methanol to the separation buffer. The peak heights showed to be slightly higher for the medium concentrations. Borate-carbonate buffer was tested as a former reported alternative separation buffer [329]. However, it was rejected since the electrical current was considerably higher and resolution did not significantly improve. A concentration of 40 mmol l<sup>-1</sup> borate with addition of 10 %v/v methanol was applied for further optimization experiments as a compromise of peak resolution, separation time, and a low separation current. Last one allowed preventing excessive joule heating.

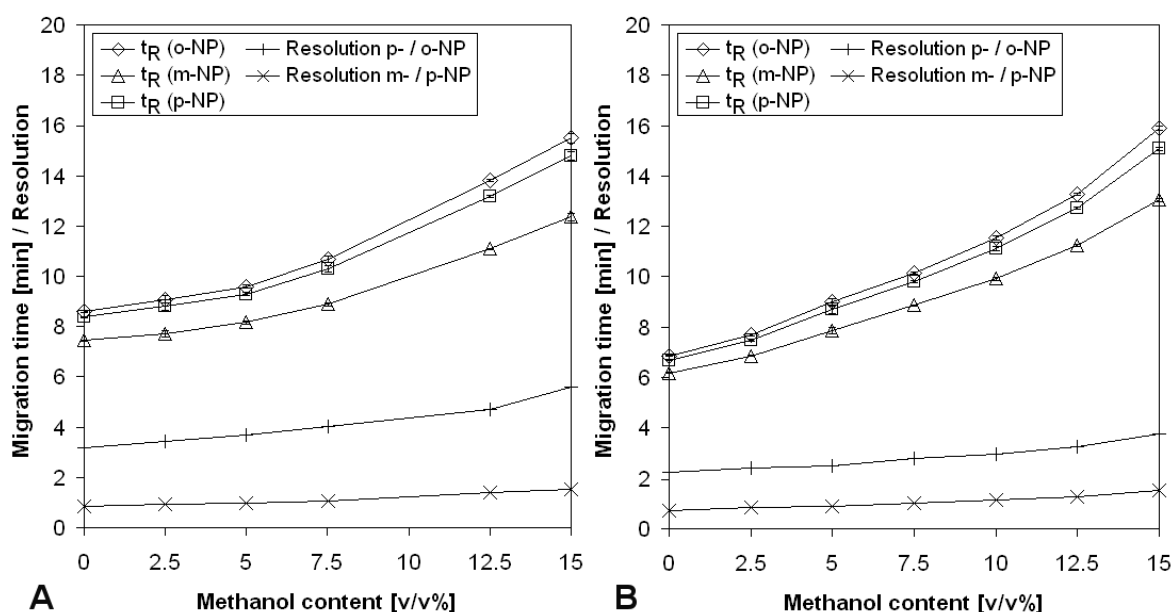
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**Table 27: Influence of the borate concentration of the separation buffer. \***

$t_R$ [min] $\pm$ SD (n=3)	without methanol			with 10 v/v% methanol		
Borate [mmol l <sup>-1</sup> ]	o-NP	m-NP	p-NP	o-NP	m-NP	p-NP
10	4.85 $\pm$ 0.01	4.49 $\pm$ 0.01	4.77 $\pm$ 0.02	7.19 $\pm$ 0.06	6.37 $\pm$ 0.05	7.02 $\pm$ 0.07
20	5.41 $\pm$ 0.04	4.89 $\pm$ 0.04	5.31 $\pm$ 0.04	10.15 $\pm$ 0.08	8.47 $\pm$ 0.05	9.81 $\pm$ 0.08
30	5.63 $\pm$ 0.16	5.01 $\pm$ 0.14	5.51 $\pm$ 0.15	11.12 $\pm$ 0.07	9.26 $\pm$ 0.05	10.71 $\pm$ 0.07
40	6.07 $\pm$ 0.04	5.39 $\pm$ 0.03	5.95 $\pm$ 0.04	11.71 $\pm$ 0.07	9.56 $\pm$ 0.06	11.24 $\pm$ 0.07
50	6.29 $\pm$ 0.14	5.54 $\pm$ 0.09	6.16 $\pm$ 0.12	11.66 $\pm$ 0.17	9.56 $\pm$ 0.16	11.24 $\pm$ 0.18
60	6.59 $\pm$ 0.06	5.76 $\pm$ 0.04	6.45 $\pm$ 0.05	13.30 $\pm$ 0.05	10.47 $\pm$ 0.03	12.76 $\pm$ 0.03

\* Conditions: apparent pH 9.4, 25 kV, sample: 14.4 mg l<sup>-1</sup> o-NP, 50.4 mg l<sup>-1</sup> m-NP, 7.2 mg l<sup>-1</sup> p-NP, current intensities for 0 and 10 v/v% MeOH: 15, 21, 30, 38, 45, 55  $\mu$ A and 8, 15, 25, 33, 40, 46  $\mu$ A

### 7.4.3.2. Influence of methanol content



**Figure 61: Data representation of the optimization of the methanol content of the separation buffer for an apparent pH of 9.4 (A) and of 9.7 (B).**

Conditions of separation and analyte concentrations are given in table 28.

The influence of the content of the organic modifier methanol was studied in the range of 0-15 v/v% for apparent pH of 9.4 and 9.7. Experimental results are given in table 28 and represented in figure 61. Addition of methanol was required to decrease the EOF rate, consequently to increase the separation time, in order to achieve baseline analyte separation. The peak resolution, migration times, and peak widths increased considerably with higher methanol contents and baseline separation was achieved for a content of methanol higher than 10 v/v%. Therefore, a concentration of methanol of 10 v/v% was finally chosen as a compromise between peak resolution and required separation time.



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**Table 28: Influence of separation buffer methanol content on analytes migration times. \***

MeOH [v/v%]	$t_R$ [min] $\pm$ SD (n=3)	o-NP	m-NP	p-NP	$R_S$ m- / p-NP	$R_S$ p- / o-NP
0.0	apparent pH 9.4	8.60 $\pm$ 0.03	7.44 $\pm$ 0.03	8.37 $\pm$ 0.04	0.86	3.17
2.5		9.08 $\pm$ 0.16	7.71 $\pm$ 0.12	8.81 $\pm$ 0.15	0.93	3.42
5.0		9.58 $\pm$ 0.06	8.16 $\pm$ 0.05	9.29 $\pm$ 0.06	1.00	3.69
7.5		10.68 $\pm$ 0.12	8.89 $\pm$ 0.07	10.29 $\pm$ 0.12	1.04	4.02
12.5		13.83 $\pm$ 0.04	11.09 $\pm$ 0.03	13.19 $\pm$ 0.05	1.39	4.69
15.0		15.49 $\pm$ 0.19	12.37 $\pm$ 0.15	14.78 $\pm$ 0.18	1.54	5.61
MeOH [v/v%]	$t_R$ [min] $\pm$ SD (n=3)	o-NP	m-NP	p-NP	$R_S$ m- / p-NP	$R_S$ p- / o-NP
0.0	apparent pH 9.7	6.86 $\pm$ 0.03	6.19 $\pm$ 0.03	6.70 $\pm$ 0.03	0.77	2.26
2.5		7.69 $\pm$ 0.05	6.87 $\pm$ 0.04	7.49 $\pm$ 0.04	0.89	2.46
5.0		8.99 $\pm$ 0.16	7.86 $\pm$ 0.13	8.71 $\pm$ 0.15	0.92	2.52
7.5		10.15 $\pm$ 0.05	8.86 $\pm$ 0.04	9.81 $\pm$ 0.05	1.04	2.83
10.0		11.55 $\pm$ 0.08	9.92 $\pm$ 0.08	11.11 $\pm$ 0.08	1.19	2.98
12.5		13.29 $\pm$ 0.04	11.26 $\pm$ 0.04	12.72 $\pm$ 0.04	1.32	3.30
15.0		15.90 $\pm$ 0.08	13.05 $\pm$ 0.07	15.08 $\pm$ 0.07	1.55	3.80

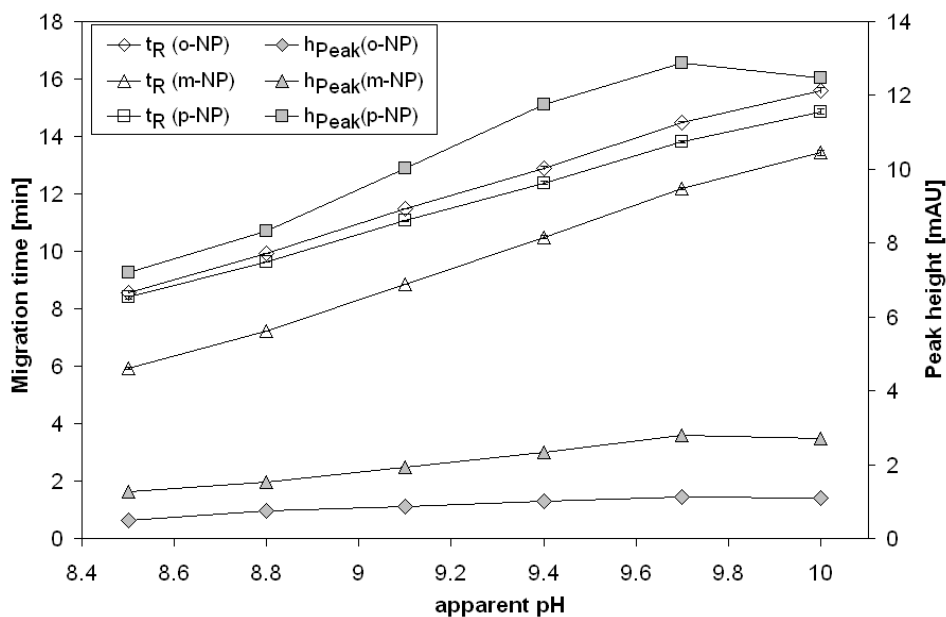
\* Condition: 40 mmol l<sup>-1</sup> borate, 25 kV, sample: 14.4 mg l<sup>-1</sup> o-NP, 50.4 mg l<sup>-1</sup> m-NP, 7.2 mg l<sup>-1</sup> p-NP

### 7.4.3.3. Influence of buffer pH

The EOF rate as well as the dissociation and mobility of the analytes are influenced strongly by the pH. Therefore, the influence of the apparent pH was studied in the range of pH 8.5-10. The denotation "apparent pH" was used since the concept of pH is not defined for methanol-water mixtures as used in this work. An apparent pH lower than 8.5 was not of interest, since the NP show absorption on the selected detection wavelength (400 nm) only in the dissociated form. The applicable pH range is therefore limited by the less acidic analyte m-NP (pK<sub>A</sub> 8.39). For the same reasons, the pH has also a significant effect on the detection sensitivity. Experimental results and separation conditions are given in table 29 and are represented in figure 62. A methanol content of 12.5 v/v% was used in order to assure baseline separation of p-NP and o-NP for each pH value.

Migration times and peak resolution of p-NP and o-NP increased highly with the apparent pH, while peak resolution of m-NP and p-NP decreased. Likewise, peak heights and separation current increased due to a higher concentration of dissociated analyte and borate as well as hydroxide ions. The highest sensitivity was obtained at an apparent pH of 9.7. At tested pH 10, the peak heights were lower than at pH 9.7 decreased probably due to enhanced thermal diffusion. Therefore, an apparent pH value 9.7 was chosen.

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**Figure 62: Data representation of the optimization of separation buffer apparent pH. Conditions of separation and analyte concentrations are given in table 29.**

**Table 29: Influence of the apparent pH value on the migration time of the solutes. \***

App. pH	8.5	8.8	9.1	9.4	9.7	10
Migration time [min] ± SD (n=3)						
o-NP	8.57±0.05	9.91±0.01	11.47±0.02	12.89±0.06	14.47±0.04	15.59±0.11
m-NP	5.92±0.04	7.23±0.01	8.85±0.01	10.49±0.05	12.19±0.04	13.43±0.08
p-NP	8.40±0.05	9.63±0.01	11.06±0.02	12.38±0.06	13.83±0.04	14.87±0.10
Peak height [mAU]						
o-NP	0.50±0.06	0.76±0.04	0.86±0.08	1.00±0.09	1.11±0.04	1.11±0.09
m-NP	1.28±0.03	1.54±0.11	1.93±0.08	2.34±0.27	2.79±0.04	2.70±0.35
p-NP	7.21±0.16	8.33±0.80	10.01±0.41	11.76±0.86	12.87±0.25	12.49±1.21
Resolution						
m-/p-NP	12.18	9.27	6.99	4.97	3.57	3.06
p-/o-NP	0.81	1.02	1.20	1.31	1.47	1.54

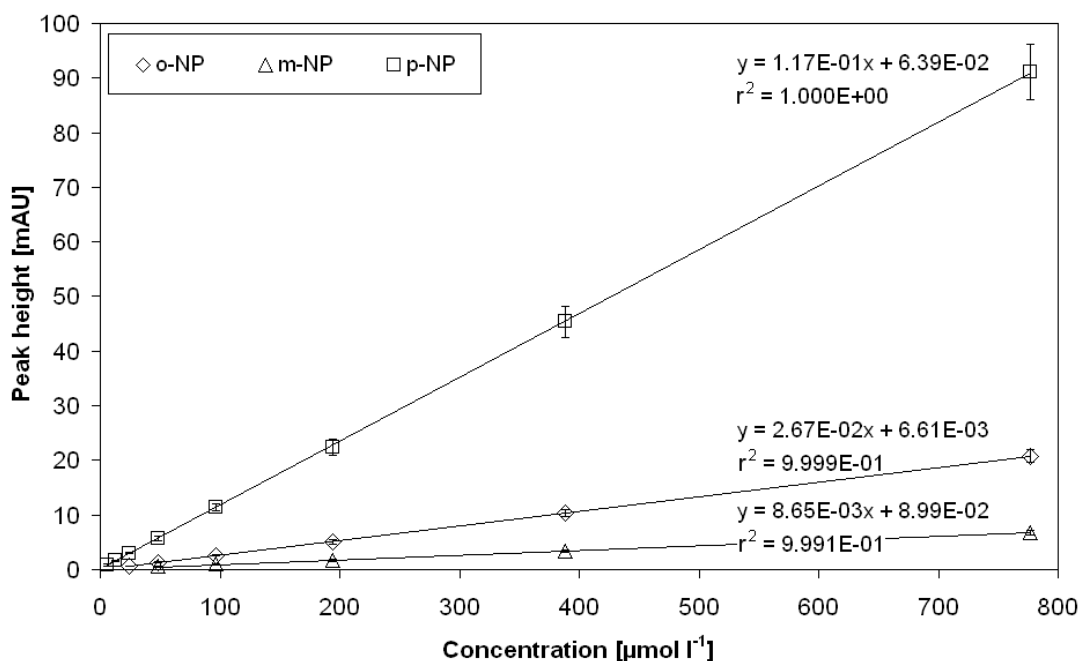
\* Conditions: 40 mmol l<sup>-1</sup> borate, 12.5 v/v% methanol, 25 kV, sample: 28.8 mg l<sup>-1</sup> o-NP, 100.8 mg l<sup>-1</sup> m-NP, and 14.4 mg l<sup>-1</sup> p-NP

## 7.5. Discussion

### 7.5.1. Analytical characterization

#### 7.5.1.1. Linear working range and detection limit

For the determination of the linear working range of the method, standards of the three NP of increasing concentrations were measured in triplicate. A linear response of the peak heights was found up to 776  $\mu\text{mol l}^{-1}$  for all NP. Higher concentrations were not tested. Calibration data are given in table 30 and calibration curves are represented in figure 63.



**Figure 63: Peak height calibration curves for the three NP using the SIA-CE analyzer. Conditions of separation and analyte concentrations are given in table 30.**

**Table 30: Data of calibration performed for the estimation of the linear working range.**

Concentration [ $\mu\text{mol l}^{-1}$ ]	Peak height [mAU] $\pm$ SD (n = 3)		
	o-NP	m-NP	p-NP
6.1	< LOD	< LOD	0.84 $\pm$ 0.11
12.1	< LOD	< LOD	1.63 $\pm$ 0.15
24.3	0.72 $\pm$ 0.02	< LOD	3.03 $\pm$ 0.15
48.5	1.35 $\pm$ 0.10	0.55 $\pm$ 0.06	5.72 $\pm$ 0.37
97.0	2.60 $\pm$ 0.14	1.00 $\pm$ 0.09	11.43 $\pm$ 0.57
194.1	5.10 $\pm$ 0.37	1.65 $\pm$ 0.16	22.44 $\pm$ 1.48
388.2	10.28 $\pm$ 0.64	3.42 $\pm$ 0.21	45.36 $\pm$ 2.78
776.4	20.80 $\pm$ 1.15	6.84 $\pm$ 0.40	91.09 $\pm$ 5.13

\* Conditions: 40  $\text{mmol l}^{-1}$  borate, 10 v/v% methanol, apparent pH 9.7, 25 kV

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A very low baseline noise was obtained due to the use of the stabilized-powered LED as light source with typical amplitude of 0.2 mAU. The LOD values were calculated as described in chapter 3.6.2 to be  $22.5 \mu\text{mol l}^{-1}$  for o-NP,  $68 \mu\text{mol l}^{-1}$  for m-NP, and  $5.1 \mu\text{mol l}^{-1}$  for p-NP. Comparing these values to the ones of a former work in, which deep UV detection at 191 nm had been applied [329], the obtained high signal-to-noise ratio due to the use of the LED over-compensated the considerably lower detection sensitivity at 400 nm. In spite of the about 4-, 26-, and 44-times lower molar absorptivity values of o-NP, m-NP, and p-NP, respectively, the calculated LOD values were only 1.25, 2- and 7-times lower, respectively. Nevertheless, both compared methods do not show sufficient sensitivities for their application to the direct analysis of environmental water samples without prior analyte pre-concentration. However, the comparison demonstrates that the proposed analyzer is applicable instead of a commercial instrument.

### 7.5.1.2. Reproducibility

The method showed highly satisfying reproducibilities regarding migration time, peak height, peak area, and peak width with RSD values given in table 31 of  $< 0.5 \%$ ,  $< 5 \%$ ,  $< 6.7 \%$  and  $< 8 \%$ , respectively ( $n = 15$ ). It can be seen that using peak area resulted in about 30 % higher RSD values for all analytes compared to peak heights. This can be partly reduced to the relatively broad peaks obtained and in consequence the difficulty of automated and exact software identification of the peak start and peak end points. In consequence, data evaluation was done using peak heights throughout.

The obtained RSD values for peak heights were comparable with values obtained with former CE flow analyzers applying hydrodynamic injection, either combining CE with SIA (3.8 %  $n=8$  [351], 5.7 %  $n=6$  [352], 5.4 %  $n=5$  [354], and 1.6 %  $n=5$  [355]) or with the more reproducible FIA technique (4.5 %,  $n=4$  [362], 1 %,  $n=10$  [4], and 3 %,  $n=8$  [350]).

Gas bubbles formed by electrolysis during the separation or originated from buffer degassing were considered as a principal factor affecting the method reproducibility. Bubbles stacked in the grounded interface were removed by aspiration of air from port 6 of the selection valve and propelling it through the grounded interface. Gas bubbles were removed from the HV interface by buffer exchange after each analysis.

**Table 31: Relative standard deviation of 15-fold repetition of analysis.**

RSD [%] ( $n = 15$ )	o-NP	m-NP	p-NP
Migration time	0.5	0.5	0.5
Peak height	6.3	3.1	2.8
Peak area	10.0	5.3	4.6
Peak width	8.1	6.6	4.8

\* Conditions:  $40 \text{ mmol l}^{-1}$  borate, apparent pH 9.7, 10 v/v% methanol, 25 kV, sample:  $14.4 \text{ mg l}^{-1}$  o-NP,  $50.4 \text{ mg l}^{-1}$  m-NP,  $7.2 \text{ mg l}^{-1}$  p-NP

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### 7.5.1.3. Recovery and time of analysis

The proposed method and the analytical system were tested with spiked water samples from a local wastewater treatment plant for the evaluation of the influence of different sample matrices on the analytical performance and analyte recovery. The samples were from the plant entrance (E), the outlet of the mechanical treatment (S1), and the outlet after the biological treatment and sedimentation (S3). They were filtered through nylon membrane filters of 0.45  $\mu\text{m}$  pore size prior to analysis. For all samples, the concentrations of all NP were below their respective LOD. Therefore, the samples were spiked with aqueous NP stock solutions obtaining final concentrations of 103.5  $\mu\text{mol l}^{-1}$  (o-NP), 362  $\mu\text{mol l}^{-1}$  (m-NP), and 51.8  $\mu\text{mol l}^{-1}$  (p-NP). The found recovery values calculated by applying peak height calibration with aqueous standards are given in table 32.

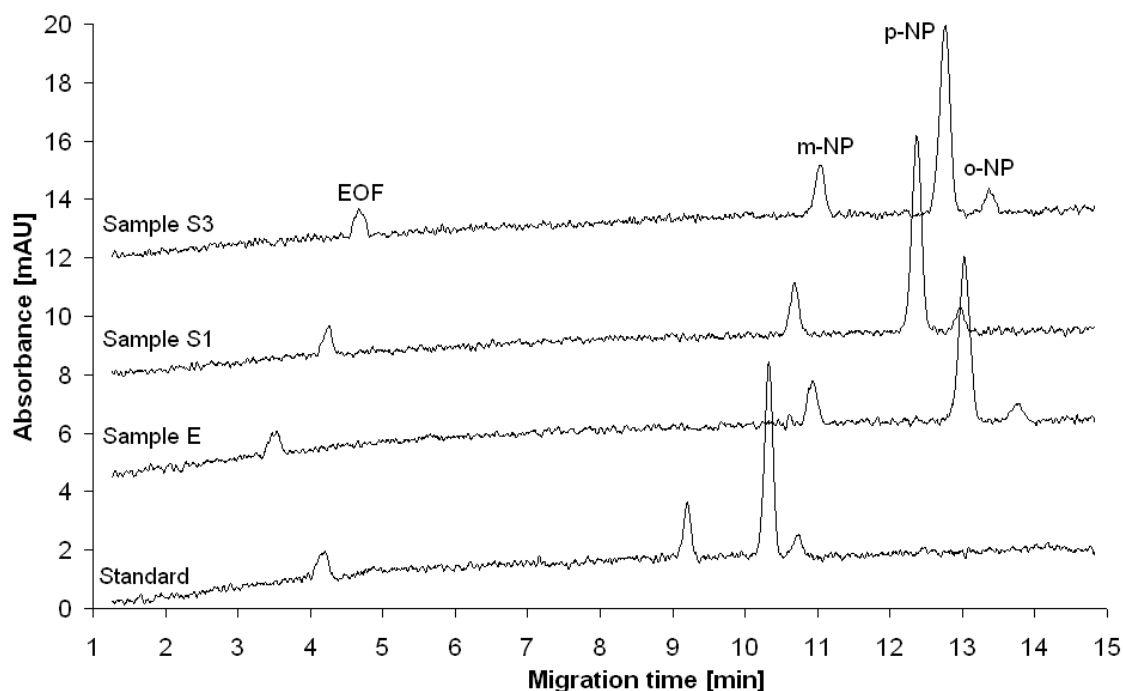
**Table 32: Determination of nitrophenols in spiked wastewater samples by SIA-CE. \***

Sample	Found concentrations [ $\mu\text{mol l}^{-1}$ ]			Resulting recovery [%]		
	o-NP	m-NP	p-NP	o-NP	m-NP	p-NP
E	98.6 $\pm$ 9.3	311.0 $\pm$ 15.5	45.5 $\pm$ 4.1	95.3 %	85.9 %	87.8 %
S1	98.2 $\pm$ 9.7	336.2 $\pm$ 46.1	48.1 $\pm$ 5.3	94.9 %	92.9 %	92.9 %
S3	103.4 $\pm$ 7.3	352.7 $\pm$ 24.0	50.2 $\pm$ 1.2	99.9 %	97.4 %	96.9 %

\* Results are expressed as the mean of three determinations given with standard deviation. Spiked NP concentrations were 103.5  $\mu\text{mol l}^{-1}$  of o-NP, 362  $\mu\text{mol l}^{-1}$  of m-NP, and 51.8  $\mu\text{mol l}^{-1}$  of p-NP for each sample. E: Plant entrance, S1: Mechanical treatment outlet, S3: Final plant outlet

The EOF and the migration times of the analyte were observed to be prolonged about 2-3 min for all waste water samples in comparison with aqueous standard solutions as it can be seen in figure 64. Flushing of the capillary with 0.1 mol  $\text{l}^{-1}$  NaOH and water was required after each waste water analysis prior to re-conditioning with the separation buffer in order to maintain a satisfying reproducibility of analyte migration times. Nevertheless, this procedure also led to a slightly shortened separation time for the standard solutions. The prolonged analyte migration times for the waste water samples can be explained by the typically high content of dissolved organic compounds (DOC). DOC adsorption at the capillary inner walls leads to a lower zeta potential and in consequence to a reduced EOF. Adsorbed DOC at the capillary walls could further have led to interaction with the NP.

The recovery values were below the ideal value of 100 % throughout, which was reduced to the prolonged migration times, leading to prolonged time for diffusion, peak broadening, and consequently, lower peak heights. Nevertheless, the obtained recovery values for o-NP were satisfying. In contrast, the recovery values for m-NP and p-NP were reduced for S1 and E compared to S3. This observation confirms the conjecture that the low recovery values were mainly attributed to the DOC content, since the waste water treatment mainly reduces this parameters and consequently, S3 could be considered as the sample of the lowest DOC content.



**Figure 64: Analysis of spiked waste water and standard with the proposed CE method. Concentrations were  $103.5 \mu\text{mol l}^{-1}$  of o-NP,  $362 \mu\text{mol l}^{-1}$  of m-NP, and  $51.8 \mu\text{mol l}^{-1}$  of p-NP. Baselines of S1 and S3 were shifted 4 mAU and 8 mAU, respectively.**

The former described observations led to the conclusion that automated and in-situ flushing of the separation capillary with cleaning agents and separation buffer for re-conditioning as well as the control of both buffer reservoirs is imperative for the analysis of untreated environmental water samples with CE. So the automated accomplishment of this task is one important characteristic of the proposed analyzer and analytical method.

The time of analysis was given by the migration time of the latest-detected analyte (o-NP) of 14 min plus the time required for the injection procedure, cleaning of the sample channel and both interfaces, and capillary flushing for re-conditioning with separation buffer. In total, 17 min are required for the analysis of one sample. This time is reduced if the sample is provided continuously or when one sample is measured in repetition.

#### 7.5.1.4. Limitations of the proposed system

With the proposed system, considerably wider peaks were obtained as former reported with commercial instruments [329] and independently from the analyte concentrations. In consequence, the separation efficiency was relatively low with theoretical plate numbers in the order of  $10^4$ . Various attempts were undertaken to ascertain the cause of peak broadening since the reduction of the peak width would have enabled an equivalent increase of the peak heights and, in consequence, of the detection sensitivity.

Several aspects and possible causes could be excluded by experiments, among these: laminar flow in the capillary by unbalanced flow interface outlets, flow pulsation caused by

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the syringe pump at specific flow rates, and the pressure pulse generated by the switch of the MCSV at the outlet of the grounded interface. Changing the respective conditions, (outlet heights, flow rate, valve type...etc.), no significant improvements were achieved.

It was further proven that joule heating was effectively dissipated by the ventilation of the protection case. Omitting the ventilation, the separation current increased immediately about 50 % leading to broader peaks, whereas during ventilation, an ambient temperature of 25 °C was maintained in the protection case with a separation current of about 40  $\mu$ A. Therefore, joule heating was excluded as possible reason for peak broadening. A possible explication for peak broadening was found when for the following work described in chapter 8 the HV source had to be exchanged (see 8.4.1) and smaller peak widths and shorter separation times were obtained. So the affection of the separation performance observed in the present work could be reduced at least partly to the used HV source.

Sample stacking was tested as a possibility to improve detection sensitivity and peak resolution by the injection of a small water plug prior to the actual sample injection. It was observed, that peak heights increased about 30 % but that this effect was not sufficiently reproducible and depends further highly on the ionic strength of each sample.

### 7.5.2. Comparison of versatility

An instrumental comparison of the proposed SIA-CE analyzer with former analytical systems combining flow techniques and CE is given in table 33. The overviewed aspects were the kind of interfacing the flow manifold and the separation capillary, the ability of buffer reservoir control, and versatility of operation, i.e. the possibility of performing in-situ conditioning of the capillary, electrokinetic and hydrostatic injection, and pressure control. It should be pointed out that considerations about the performance of separation or detection were not possible due to the application of the systems to different analytes and the use of different detection techniques in the respective works.

Highest versatility can be generally stated for the hyphenated systems of CE and syringe-pump based flow techniques SIA and LOV as well as for flow systems, which take benefit of commercial CE instrumentations. In contrast, most of the reported FIA-based systems did not allow pressure application for in-situ flushing or for the performance of hydrodynamic injection.

In comparison, the highest instrumental versatility using a non-commercial instrumentation was achieved in the present work: Both buffer reservoirs were accessible by the flow manifold, both hydrodynamic and electrokinetic injection mode were principally applicable, and the entire maintenance of the capillary could be carried out by the flow system. On the other hand, with the electrode configuration (HV on the detection side) of the proposed analyzer, electrochemical detection was not possible.

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**Table 33: Instrumental comparison of coupling SIA or FIA via flow interfaces with CE.**

Flow-technique	Type of interface *	Buffer reservoirs connected to manifold **	Injection location **	Electrokinetic injection	Hydrodynamic injection	In-situ conditioning	Pressure adaptation	Comments	References
FIA	A,D	G	OC	+	-	-	-	EOF-traction injection via membrane interface	[343]
FIA	A	G	G	+	-	-	-		[339-342]
FIA	C	G	G	+	-	-	-		[344-348]
FIA	A	G	G	+	+	-	-	chip design	[362]
FIA	B	H	H	+	-	-	-	chip design	[358-361]
FIA	C,C	G,H	G,H	+	+	-	-	gravimetric flow and dual side injection	[4]
FIA	C,C	G,H	G	+	-	+	+	directed connection of pump and HV interface	[353]
FIA	C	G	G	+	+	-	+		[350]
SIA	C	G	G	+	-	-	-	chip design	[349]
SIA	C,C	G	G	+	+	+	+	chip design, neg. pressure	[351]
SIA	C	G	G	+	+	+	-		[354]
SIA	C	G	G	+	+	+	+		[355]
LOV	C	G	G	+	+	+	-		[356,357]
SIA	C,B	G,H	G	+	+	+	+	see chapter 7	proposed work 1 [316]
MS-FIA	C,B	G,H	G	+	+	+	+	see chapter 8	proposed work 2 [363]
FIA	D	***	OC		***			***	[332]
FIA	F	***	H		***			***	[52,336,337]
SIA	A	G	G	+	-	-	-	c.i., EOF traction injection	[338]
SIA	E	***	OC		***			***	[334,335]
SIA	C	H	H	+	+	+	+	***	[352]

\* A: atmospherically open flow interface, B: falling drop interface, C: split flow interface, D: membrane interface, E: micro-injection valve, F: flushed vial, beaker-like automation

\*\* G: on grounded side, H: on high voltage side, OC: on capillary

\*\*\* via commercial apparatus



### 7.6. Conclusion

In the present work, an analytical system was proposed, which combined sequential injection analysis (SIA) and capillary electrophoresis (CE). The CE system part was homemade. Construction consideration and instrumental aspects were explained in detail.

The system was successfully applied to the separation and quantification of mono-nitrophenols. Photometric on-capillary detection on the visible wavelength 400 nm using a blue LED as a light source of low baseline noise was performed. The entire maintenance of the capillary including the control of both buffer reservoirs, constructed as individually designed flow interfaces, was carried out automatically and in-situ.

In comparison with prior described hyphenated systems of analytical flow techniques and capillary electrophoresis, a maximal instrumental versatility was obtained. All parameters of the performed hydrodynamic injection were software controlled and easily adaptable. The system showed a satisfying reliability and high robustness, which allowed autonomous operation. It is therefore potentially applicable for monitoring purposes and allowed overnight and autonomous optimization.

The analytical method was optimized in respect of physical and chemical parameters and highly satisfying reproducibility in respect of peak heights and retention times as well as satisfying analyte recovery values were obtained. The visible detection and the very low baseline noise by using the highly stable LED showed to over-compensate the lower detection sensitivity at the applied detection wavelength. These are given by the lower molar absorptivity values of the analytes compared to deep ultraviolet detection. In consequence, LOD values of similar order as former reported were nevertheless obtained using an even simpler, transportable, and field applicable flow analyzer system.



## 8. EXPERIMENTAL WORK 5

### Multisyringe Flow Injection Analysis coupled to Capillary Electrophoresis (MSFIA-CE) as a novel analytical tool applied to the pre-concentration, separation, and determination of nitrophenols

Published equally entitled: HORSTKOTTE B., ELSHOLZ O., CERDÀ V. (2008) *Talanta* 76, 72-79 [363].

#### 8.1. Task description for experimental work 5

The objective of this work was the development of an analytical system based on the coupling of MSFIA and CE for the automated analysis of environmental water samples. In addition to the system and respective requirements prior described in chapter 7, solid phase extraction was aimed to be integrated by taking benefit of the multichannel technique MSFIA described in chapter 1.1.6.3. Like in the experimental work 4, mono-nitrophenols were used as model analytes for system characterization. The capability and advantages of coupling MSFIA with CE should further be demonstrated in this work.

#### 8.2. Introduction

##### 8.2.1. Concentration techniques in capillary electrophoresis

Detection sensitivity in capillary electrophoresis is typically poor in comparison with liquid or gas chromatography. This is due to the fact, that sample volumes are in the order of nanoliters and detection cell diameters in the order of a few tens micrometers. On the other hand, CE often exceeds the former named techniques in respect to separation efficiency and injection frequency. Concentration techniques are therefore of high interest and probably the most important key factor for the successful implementation of CE.

Analyte enrichment can either be accomplished by manual sample treatment (off-line), directly on-capillary, or using an automated system coupled to the CE apparatus. Off-line pre-concentration is surely the simplest, most flexible, and most frequently found approach, but requires typically more sample, reagents, consumables, and personal time and yields generally a lower reproducibility than automated pre-concentration protocols.

On-capillary concentration techniques allow analyte concentration from a relatively low sample volume during injection or separation. One possibility is the integration of a miniature SPE column into the capillary [364]. After loading of the column with sample, the

eluent is injected and transported by the EOF through the column. By this, the sensitivity can be improved highly since all eluted analyte molecules are quantified [119]. On the other hand both migration time and separation efficiency are affected, the flushing of the capillary requires high pressure, and disturbing sample matrix components are not eliminated but enter the capillary during column loading [365].

On-capillary concentration refers to a sample stacking or analyte sweeping technique. Sample stacking is done by modifying the sample in a way that the conductivity in the sample zone is lowered. So the analyte molecules migrate faster in the sample zone than in the bulk separation buffer. They slow down and concentrate at the boundary of sample and separation buffer. Another way is the generation of a pH instead of a conductivity gradient between sample and buffer [136]. Sweeping means that the sample zone is penetrated by a component that acts as a quasi-stationary phase, e.g. micelles, which is able to carry and focus the analyte molecules effectively in a small band (sweeping) [137]. A focusing effect on the capillary entrance can further be achieved by electrokinetic injection, which allowed the 2000-fold concentration of NP applying EOF reversal [346].

CE coupled flow systems overviewed in detailed reviews [118,119,138-140,331] allow the automation of manual analyte pre-concentration procedures such as SPE [52,140,335,337,340,348]. Additionally, they can be used to carry out clean-up and pre-concentration techniques, which are difficult to perform manually such as gas diffusion [347], dialysis [345], gas extraction [336], or electrodialysis [147]. Due to the variety of commercially available sorbents, high selectivity, and easy integration into a flow system, SPE is the most frequently found concentration technique used in automated systems and is described in the following for phenolic compounds as the analytical group of interest.

### 8.2.2. Solid phase concentration of nitrophenols using flow techniques

Solid phase extraction of phenols was reviewed in general by RODRÍGUEZ ET AL. (2000) [321]. Among other aspects, applications using silica, polymeric, functionalized sorbents, and carbon were overviewed. SPE is continuously displacing the LLE technique, since it offers lower detection limits and better reproducibilities, manual work and preparation time are reduced, analyte loss by evaporation during extract concentration is avoided, and the amounts of required solvents is minimized [105,317,320,321]. Automation of LLE on flow systems, reviewed lately [366], is more sophisticated than SPE automation, since at least one organic solvent is required, two liquid phases have to be handled in high precision, and segmentators and phase separators have to be integrated into the manifold [18,107].

The mostly applied sorbents used in analytical flow technique systems for the enrichment of nitrophenols are anion exchanger and C18 reverse phase resins, bound in columns or in active membranes. Anion exchangers retain phenols in their anionic, basic form by electrostatic interaction with positively charged groups like quaternary ammonium salts displacing an less affinitive counterions such as Cl<sup>-</sup> or OH<sup>-</sup> [206,273]. LOD of few

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micromol per liter were achieved applying spectrophotometry [104,322,323,367,368] or even 3 nmol l<sup>-1</sup> by reflectometry on a membrane surface [106]. Their high disadvantage for the application with CE is that the eluent has either to show a high salt content or had to be an apolar solvent in combination with an acid. In the first case, the high ionic strength of the buffer could cause a high separation current and diminished separation efficiency whereas at acidic conditions the analytes are non-dissociated, i.e. are not charged.

Reverse phases like C18 functionalized silica or poly(styrenedivinyl-benzene) (PS-DVB) retain phenolic compounds effectively in their acid, non-dissociated form. In C18 resins, retention is based on apolar Van-der-Waals interactions whereas  $\pi$ - $\pi$  interactions of the sorbent aromatic ring structure and the analyte determine mainly the retention of phenols on polymeric resins. Polymeric sorbents show a better affinity to the nitrophenols and, in contrast to silica supports, a high stability over the entire pH range [320,321]. Besides, the apolar phases retain also effectively the reaction product of phenols with 4-aminoantipyrene used for the determination of total phenol index [105,369]. Extraction is generally done with a low-polar solvent such as methanol, optionally in alkaline condition. It was reported, that elution was improved using a PS-DVB resin with benzenesulfonic moieties, which repelled the phenols under alkaline condition by the presence of equal charges [322]. LOD values of less than a half microgram per liter were achieved using a polymeric SPE column integrated into the flow manifold and GC-MS detection [370].

The newer technique solid-phase micro-extraction using the coating of a fiber as sorbent shows the advantage that filtering of the sample is not required [320,371]. However, the applied extraction volumes are very small and difficult to handle in a flow system. Alternatively, liquid-liquid-liquid micro-extraction was applied for the concentration of NP for the determination with HPLC [317] but required quite long extraction times.

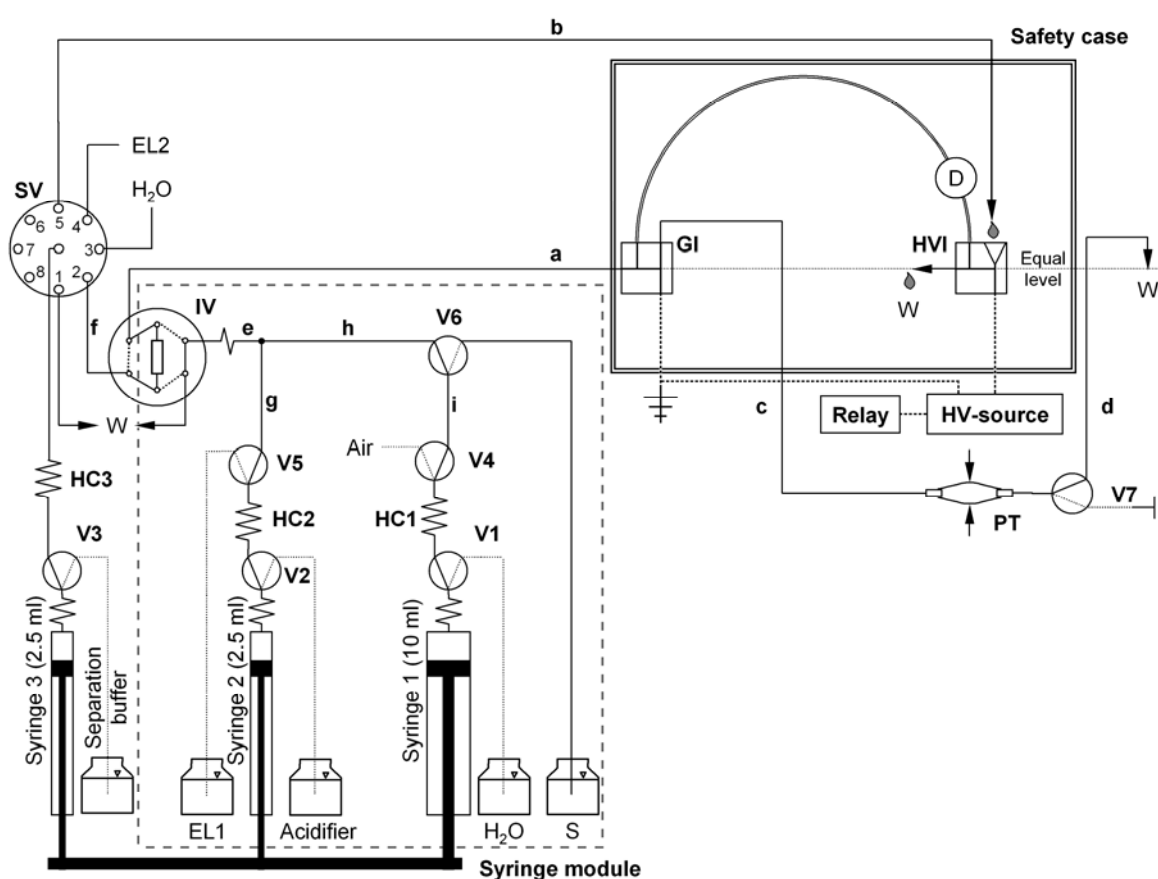
### 8.3. Materials and methods

#### 8.3.1. MSFIA apparatus and manifold

The MSFIA instrumentation, manifold, and CE system are shown in figure 65. All components have been former described in detail in chapter 3.1, 3.2, and 3.3. Instrumental control was accomplished with the software AutoAnalysis as described in chapter 3.4. The used instrumentation was basically the same as used for the SIA-CE system described in chapter 7.3.1 and 7.3.2 but extended by the integration of the injection valve (IV) of the valve module VA 1 + 1, applying two further syringes corresponding to two additional pumping lines on the syringe module Bu 4 S, and three additional MCSV. The CE part of the system was unmodified in respect of the former work [316]. It was controlled with a 2.5 ml glass syringe (S3) from Hamilton Bonaduz AG via the 8-port rotary selection valve (SV). The only modification was the integration of the injection valve into the supplying line to the grounded flow interface, which was de-placed on the lateral selection valve port 2. A second 2.5 ml glass syringe (S2) and a 10 ml polypropylene syringe (S1) from Sciware

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S.L. were used for the pre-concentration part of the system, circled in figure 65 with a broken gray line. Additional MCSV were used for the aspiration of air (V4) and sample (V6) with syringe 1 and an auxiliary eluent EL1 (V5) with syringe 2. The MCSV used for the intermediate closure of the grounded interface outlet was denoted V7. V6 and V5 were powered by the same supply port of the syringe module. All MCSV were purchased from Takasago Electric Inc. (Nagoya Japan). V3 and V7 were of an enhanced nominal pressure stability of 600 kPa (MTV-3-1/4UKGH), the V5 and V6 of 200 kPa (MTV-3-1/4UKG). Excessive valve heating was prevented by using protection circuits from Sciware S.L. Syringe 1 was used for sample aspiration and loading of a solid phase extraction column mounted on the injection valve (accessible in "LOAD"). Syringe 2 was used for in-line acidification at the respective confluence and column cleaning with EL1.



**Figure 65: Manifold of the coupled MSFIA-CE system.**

SV: selection valve (Pos1: Waste, Pos2: IV, Pos3: EL2, Pos4: water, Pos5: HVI, Pos6: HCl  $10 \text{ mmol l}^{-1}$ , Pos7: NaOH  $10 \text{ mmol l}^{-1}$ , Pos8: Sample), V1 – V7 solenoid valves with normally open position (OFF, deactivated) dotted (V3 and V7 of enhanced pressure stability), IV: Injection valve with SPE column, D: detector, HC: holding coils (1: 500 cm, 1.5 mm i.d., 2: 150 cm, 0.8 mm i.d., 3: 175 cm, 0.8 mm i.d., tube a: 40 cm, 0.5 mm i.d., tube b: 50 cm, 0.5 mm i.d., tube c: 40 cm, 0.8 mm i.d., tube d: 20 cm, 0.8 mm i.d., e: 20 cm, 0.8 mm i.d., knitted, tubes f & g: 10 cm, 0.8 mm i.d., tubes h & i: rigid PVC tubes 4 cm, 0.8 mm i.d., BGE: separation buffer, S: sample, W: waste, EL1:  $10 \text{ mmol l}^{-1}$  NaOH with 40 v/v% MeOH, EL2:  $10 \text{ mmol l}^{-1}$  NaOH with 10 v/v% MeCN. The pre-concentration part is circled by broken grey line.

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In order to minimize the volume of the connection lines between the confluence, V6, and V4, rigid PVC tubes (4 cm, 0.8 mm i.d.) were used instead of flexible PTFE tubes (see photo in the annex). The tube connecting the confluence and the injection valve was knitted to achieve an optimal mixture of acid (S2) and sample (S1). Air could be aspirated from V4 to minimize sample dispersion and by this to maximize the loadable sample volume using the given holding coil 1 (500 cm, 1.5 mm i.d.). All liquid contacted parts of the instruments and the tubing manifold were made of the resistant polymers ETFE, PEEK, PTFE, PVC, and PMMA.

The same detection equipment as described for the SIA-CE system in chapter 7.3.1 was used for all CE experiments. However, an optical fiber of enhanced UV transparency (Premium Grade Xtreme XSR Solarization-resistant Optical Fiber, Ocean Optics Inc.) with a core diameter of 455  $\mu\text{m}$  was used. This allowed reducing the integration time for data acquisition to 12 ms and performing an average over 35 measurements. In consequence, a reduced baseline noise was achieved. In contrast, for the optimization of analyte loading and elution, the CE part of the system was replaced by the detection equipment used for the polyalcohol SIA analyzers, described in the chapters 5.4.2 and 6.3.2. The only modification was the application of the LED Cu6SMA1 as light source mounted onto the cuvette holder CUV-UV with the flow cuvette 75.15 SOG. The connecting tube was about 25 cm in length and 0.8 mm i.d. By this configuration, the time-lasting separation by CE was not required for elution experiments and the analyte concentration could be highly reduced since the cuvette pathlength was 20-times the one of the separation capillary.

### 8.3.2. Solid phase extraction column

The used solid phase extraction (SPE) column was turned of a 25 mm long PMMA cylinder of 20 mm diameter. The inner cavity was 7.5 mm in length and 4.5 mm in diameter. The column showed bore threads for  $\frac{1}{4}$ "-28-UNF connectors on each side. It was filled with an octadecyl (C18) modified polymer resin obtained from commercial SPE cartridges "strata-X" from Phenomenex (Aschaffenburg, Germany). The reason for using a relatively short column for wide inner diameter was the maximization of the flow rate and by this of the sample throughput within a given time. A relative high backpressure had to be taken into account since the average particle diameter of the used resin was only 33  $\mu\text{m}$ .

The porous polypropylene frits of the SPE cartridges were used to tap both sides of the column filling. In order to lower the backpressure, the frits were cleaved into half and die-cut afterwards to a diameter of 5.5 mm. The resin was suspended in 50 v/v% ethanol obtaining by this a sufficient particle wetting. For filling, first, a polypropylene frit was inserted at one side of the column and a plastic syringe with an UNF-connector was connected afterwards. Then, small volumes of the particle slurry were transferred into the column with an automated micropipette and the abundant liquid was extracted with the syringe through the frit. By repeating this procedure several times, a homogeneous

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particle package was obtained. The column filling was capped by insertion of the second frit. The column was mounted with PTFE tubes (8 cm, 0.8 mm i.d.) onto the injection valve (see annex). A slice of a silicon rubber tube (5 mm o.d., 3 mm i.d.) at each column side allowed a finger tight sealing of the column against the respective ¼"-28-UNF-connectors.

The use of the injection valve allowed the insertion of the column either into the pre-concentration part for cleaning, conditioning, and loading or into the CE part for analyte elution. Column loading and elution were carried out in counter direction. By this, sharper profiles of the elution peaks and in consequence, higher maximal analyte concentrations were obtained and permanent compaction of the polymeric resin was avoided.

### 8.3.3. CE apparatus and interfaces

The same CE equipment was used as for the CE-SIA system described in chapter 7. The few and following described modifications were undertaken.

#### 8.3.3.1. Capillary

The separations were carried out in a fused-silica capillary of 75 µm i.d. from Polymicro technologies LLC. (Phoenix, USA). The total capillary length was 65 cm; the effective length was 55 cm. The same capillary batch was used as for the work described in chapter 7. The capillary had to be changed with slightly modified dimensions due to modifications of the back panel design of the protection case to facilitate the independent montage of the HV interface and the on-capillary detection cell (longer distance).

#### 8.3.3.2. Power source and remote safety control

Due to a breakdown of the former used source 2127 Tachophor (see chapter 7.3.2.6), the present experimental work was done with a HCP 35-35000 HV source, purchased from F.u.G. Elektronik GmbH (Rosenheim, Germany). The source had an analog interface for remote control. A photo of the device and the interface configuration are given in the annex<sup>8</sup>. As in the former work, a safety circuit connecting the respective analog interface pins 6 and 12 was made (see chapter 7.3.2.6). The circuit was controlled by a relay, which was powered by the additional supply port 6 of the syringe module. Likewise before, the safety key of the protection case was integrated into the safety circuit (see annex).

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<sup>8</sup> For a future work, an alternative wiring is further given in the annex, which enables electrokinetic injection with reversed polarity. However, this configuration was not feasible in this work due to the lack of an additional available control port on the syringe module. By the integration of a second relay and two external potentiometers, the adjustment of two voltage levels, one for each polarity, is possible. The maximal current is adjusted via the front panel slider of the source (pin 5-15). On activation of the second relay, the polarity is switched (connection or disconnection of pin 6 and 7) and one of the potentiometers become determining for the external analog voltage adjustment.



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On activation of supply port 6 by a software instruction [6-ON], the relay attracted and the safety circuit closed, which initiated the HV application. When the front part of the protection case was removed during voltage application, the safety circuit was interrupted and the HV source was deactivated. A zero potential was reached within 2 s. The maximal applied voltage and current were both adjusted using the sliders on the front panel of the HV-source, which was enabled by the connection of pin 4 to 8 and 5 to 15, respectively.

### 8.3.4. Reagents

The reagents were prepared as prior described in chapter 3.5.1.3. All reagents were of analytical grade and Millipore filtered water was used throughout. Stock solutions of o-NP, m-NP, and p-NP of each 600 mg l<sup>-1</sup> were prepared by accurate weighting and dilution in 1 mmol l<sup>-1</sup> sodium hydroxide (NaOH). An intermediate stock solution containing o-NP, m-NP, and p-NP in the concentrations of 60, 120, and 30 mg l<sup>-1</sup>, respectively, was used for the most experiments applying CE. This concentration ratio was applied in order to compensate the different molar absorbance values of the three NP. All stock solutions were stored in the dark. Standard working solutions were daily prepared by dilution. For optimization experiments on the pre-concentration and elution procedures applying a flow cuvette instead of the CE apparatus, a solution of p-NP of 0.3 mg L<sup>-1</sup> was used.

All electrophoretic separations were carried out using a 40 mmol l<sup>-1</sup> borate buffer of pH 9.7 containing 10 v/v% methanol (MeOH) as an organic modifier. The separation buffer was prepared daily from a sodium borate stock solution of 1 mol l<sup>-1</sup> and MeOH. The optimization of the separation buffer has been done in a prior work described in chapter 7.

Sodium hydroxide 10 mmol l<sup>-1</sup> containing 40 v/v% MeOH was used as a first eluent (EL1) for column cleaning, i.e. to remove all remaining absorbed acidic components after the elution for CE analysis. Hydrochloric acid of 0.1 mol l<sup>-1</sup> was applied by syringe 2 for in-line acidification of the sample during SPE column loading and for conditioning of the SPE column. Sodium hydroxide 10 mmol l<sup>-1</sup> containing 10 v/v% acetonitrile (MeCN) was used as a second eluent (EL2) for the elution of the concentrated analytes for the CE analysis.

To study the analyte recovery in real samples, lixivate waters, tap water, surface water from a biological waste water treatment plant, and ground water were used. The blank concentrations of all NP were below their respective LOD throughout. Therefore, the samples were spiked with the intermediate standard to final concentrations of 2.17, 4.35, and 1.09 µmol l<sup>-1</sup> for o-NP, m-NP, and p-NP, respectively. Samples, stock solutions, and water used for dilutions were filtered through 0.45 µm nylon membrane filters before use.

### 8.3.5. Software and analytical procedures

Instrumental control, data acquisition, and data processing were carried out using the software package AutoAnalysis 5.0 from Sciware S.L. (see chapter 3.4), running on a

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personal computer with the operating system MS-Windows 2000. The methods and procedures accomplished for analysis and for system cleaning are given in the annex. All experiments were performed with the same separation capillary. It was treated as prior described in chapter 7.3.4 and the same procedures for flushing and cleaning were applied. However, due to the integration of the injection valve into the tube connecting the selection valve and the grounded interface, the applied volumes had to be adjusted.

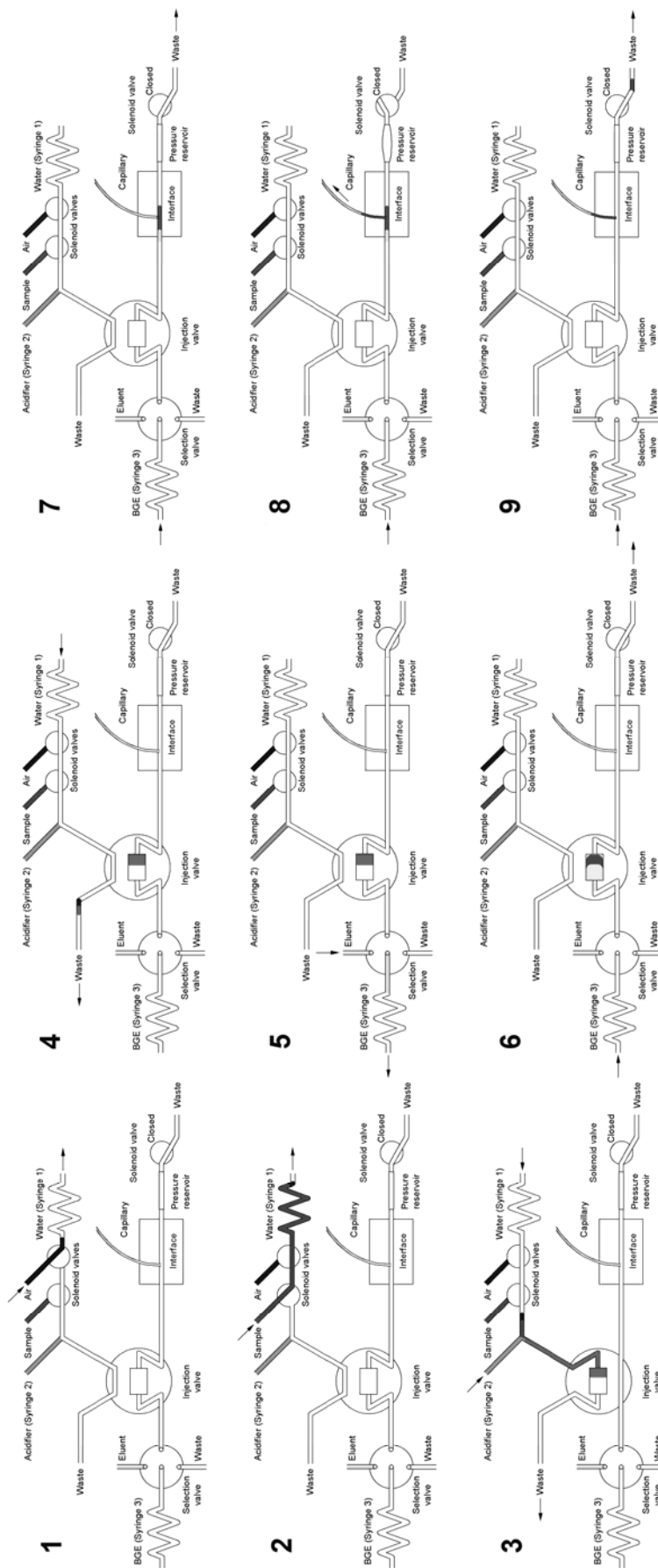
The analytical method included procedures for column conditioning, sample loading and washing, analyte elution and injection, and interface and column conditioning. A scheme is given in figure 66. Cleaning of the sample channel was done applying the mono-segmentation principle to reduce sample dispersion. First, air (100  $\mu\text{l}$ ) was aspirated from V4 followed by the aspiration of sample (500  $\mu\text{l}$ ) from V6. Then, a total volume of 2.5 ml was discharged to waste (IV in "INJECT") and the syringe was refilled with carrier.

For preparation, first, the SPE column was cleaned and conditioned by the aspiration of 200  $\mu\text{l}$  of EL1 by S2 from V5 and propulsion of a total volume of 600  $\mu\text{l}$  through the column (IV in "LOAD"). By this, the column was first washed with EL1, which eliminated all still absorbed organic, acid compounds and second with the carrier of S2 (0.1 mol  $\text{l}^{-1}$  HCl). Then, the column was conditioned with HCl 20 mmol  $\text{l}^{-1}$ , which resulted from the simultaneous dispense of S1 and S2, to the same acidity as obtained by the in-line acidification during loading. Finally, the syringes were refilled from the reservoirs in "OFF".

For pre-concentration, first, an air segment of 100  $\mu\text{l}$  was aspirated from V4 to reduce sample dispersion of the sample. Afterwards, 6 ml of sample were aspirated at a reduced flow rate in order to avoid the disruption of the segmentation air bubble. Afterwards, about 6.1 ml were dispensed through the SPE column from S1 with in-line acidification at the confluence point by the continuous addition of hydrochloric acid from S2. After loading, the rest of the sample and the segmentation air bubble were discharged to waste (IV in "INJECT") and the column was finally washed with 800  $\mu\text{l}$  of water from S1 (IV in "LOAD").

Elution was done by aspiration of 200  $\mu\text{l}$  of the auxiliary eluent EL2 with syringe 3 and propulsion through the SPE column (IV in "INJECT") towards the grounded interface. When the maximum of the elution peak had left the injection valve it was turned to position "LOAD". Hydrodynamic injection was then carried out by first positioning of the maximum of the elution peak at the capillary entrance, closure of the interface outlet, and propulsion of 5  $\mu\text{l}$  at a reduced flow rate (pressure increase). After a holding time of 800 ms, 5  $\mu\text{l}$  were aspirated at a reduced flow rate (pressure release) and the interface was flushed with separation buffer to eliminate the rest of the sample with the interface outlet open. Finally, the high voltage was turned on by activation of supply port 6. During separation, V7 remained activated to ensure atmospheric pressure on both sides of the capillary.

Separations were done in three or four repetitions. SPE column re-conditioning and the second pre-concentration could be started already during the CE separation of the first injected sample in background. For this, only the supply port 6 (safety circuit control) had to remain in position "ON". By this, the required analysis time was reduced considerably.

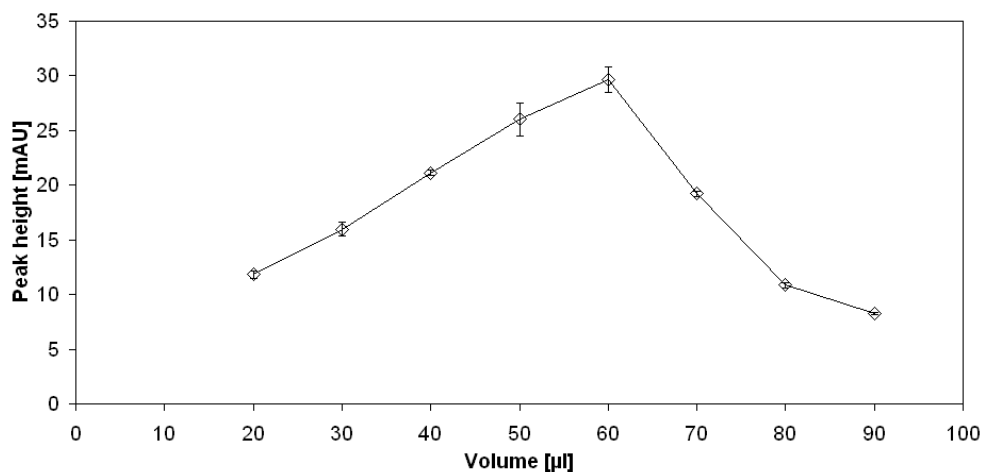


**Figure 66:** Scheme of the sampling, pre-concentration, elution, and injection procedure on the CE-MSFIA analyzer.  
**1:** Aspiration of segmentation air bubble, **2:** Aspiration of sample, **3:** In-line acidification and loading of SPE column,  
**4:** Discharge of segmentation air bubble, **5:** Aspiration of eluent EL2, **6:** Elution from SPE column,  
**7:** Positioning of sample, **8:** Hydrodynamic injection, **9:** Flushing of interface with separation buffer.

## 8.4. Experiments and results

### 8.4.1. Adaptation of the CE procedure

Electrophoretic separations were done throughout with the same buffer composition as optimized in the experimental work 4. Likewise, the same injection procedure was used with few modifications described below. Due to a modification of the backpanel of the safety box, a new capillary had to be used with a 6 % decreased ratio of effective and total length and 9 % lower electrical field strength (compare with chapters 7.3.2.2 and 8.3.3.1). Principally, this would have caused a decrease of the peak resolution. However, improved resolution and simultaneously, slight reduction of peak widths and migration times were found. This was reduced to the use of a different HV source of mostlikely higher stability and precision. So, renewed optimization of the separation conditions was not required.



**Figure 67: Data representation of optimization the sample positioning volume. Conditions of separation and analyte concentrations are given in table 34.**

**Table 34: Influence of the volume for sample positioning of the applied 50 μl sample plug. \***

Positioning volume [μl] ± SD (n=4)	Peak height [mAU]	Positioning volume [μl] ± SD (n=3)	Peak height [mAU]
20	11.83 ± 0.35	60	29.66 ± 1.17
30	15.97 ± 0.62	70	19.22 ± 0.22
40	21.14 ± 0.22	80	10.85 ± 0.37
50	26.02 ± 1.52	90	8.26 ± 0.10

\* Direct injection of aqueous standard of 30 mg l<sup>-1</sup> p-NP, separation conditions as in chapter 7

The procedure for the direct injection of the sample according the protocol described in chapter 7.3.4 had to be modified in respect of the positioning volume for the sample aliquot due to the incorporation of the injection valve. It was re-optimized in the range of 20 μl – 90 μl using a p-NP standard (compare with chapter 7.4.2.1). Experimental results and conditions are given in table 34 and are represented in figure 67. Peak heights were maximal for a volume of 60 μl, which were consequently applied in this work. Accordingly,

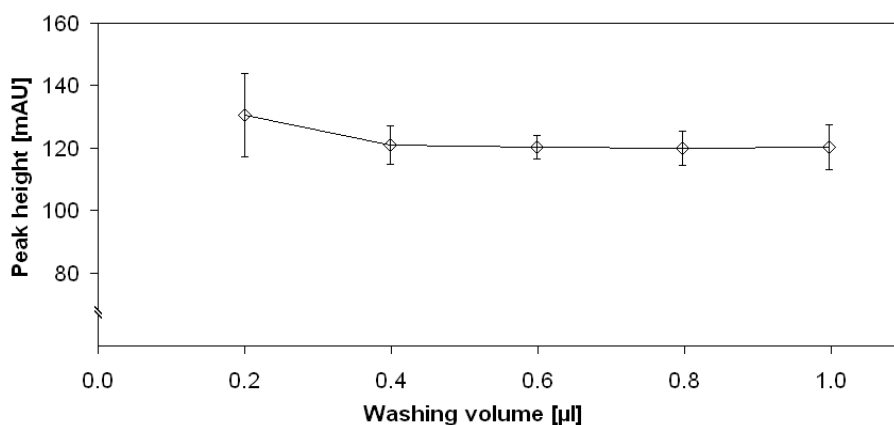
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the volumes applied in the former described cleaning procedures were adapted. Another modification was the reduction of the injection pulse volume from 10  $\mu\text{l}$  to 5  $\mu\text{l}$ . Maintaining the former applied holding time of 800 ms, a slightly better reproducibility was obtained.

### 8.4.2. Optimization of physical variables

#### 8.4.2.1. Water volume for washing of SPE column

The SPE column was washed after pre-concentration with the carrier from syringe 1 (water) to eliminate the rests of acid and sample. The influence of the water volume used for washing after the pre-concentration step on peak height and reproducibility was studied in the range of nominal 50-250  $\mu\text{l}$  (200-1000  $\mu\text{l}$  effective volume). Experimental results and conditions are given in table 35 and are represented in figure 68.



**Figure 68: Data representation of the water volume for SPE volume washing. Data, conditions, and analyte concentration are given in Table 35.**

**Table 35: Influence of water volume for SPE column washing. \***

Washing volume [ml] $\pm$ SD (n=4)	0.2	0.4	0.6	0.8	1.0
Peak height [mAU]	130.6 $\pm$ 13.5	121.0 $\pm$ 6.1	120.3 $\pm$ 3.8	119.9 $\pm$ 5.3	120.4 $\pm$ 7.2

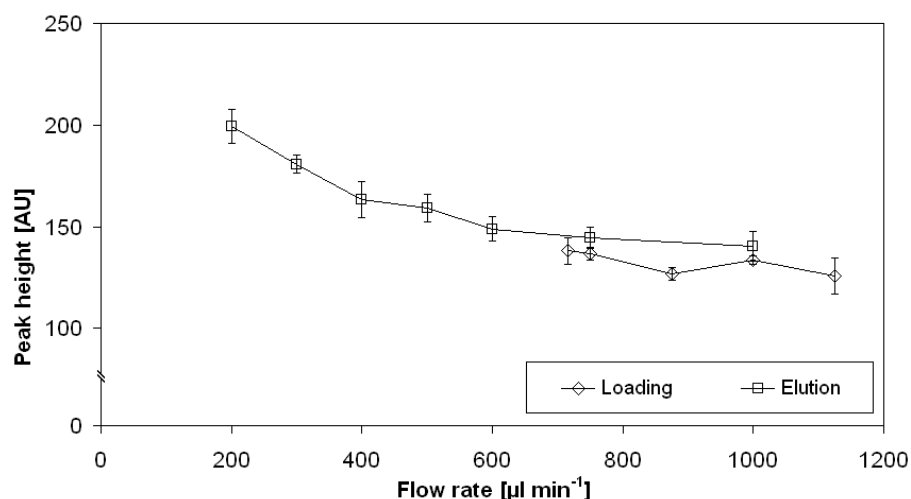
\* Concentration of 2 ml sample (5.5  $\mu\text{mol l}^{-1}$  p-NP, 1  $\text{mmol l}^{-1}$  NaOH), Elution with separation buffer

The height of the p-NP elution peak showed to decrease with increasing volume of water whereas the reproducibility was considerably improved using a volume of at least 100  $\mu\text{l}$  nominal (400  $\mu\text{l}$  effective). The decrease was probably due to the use of a relative small volume of sample for pre-concentration. So, the elimination of not adsorbed analyte by the washing step caused a notable signal decrease. The main improvement was the elimination of gas bubbles appearing during the elution, which otherwise led to baseline disturbances. Bubbles had to be avoided imperatively in the elution flow since they could interrupt the galvanic (liquid) contact in the interface or in the capillary. This would lead to current break down, cause sparking, and inhibit the electrophoretic separation. Thus, a volume of 200  $\mu\text{l}$  (800  $\mu\text{l}$  effectively) was applied for washing at a flow rate of 1  $\text{ml min}^{-1}$ .

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### 8.4.2.2. Flow rates for loading and elution

The influences of the flow rates used for column loading and later analyte elution on the height of the elution peak were studied with p-NP between 715-1125  $\mu\text{l min}^{-1}$  and 300-1000  $\mu\text{l min}^{-1}$ , respectively. Results and experimental conditions are given in table 36 and are represented in figure 69. The experiments were done with separation buffer as eluent.



**Figure 69: Data representation of the optimization study on loading and elution flow rates. Data, conditions, and analyte concentration are given in table 36.**

**Table 36: Influence of flow rates for SPE column loading and analyte. \***

Loading flow rate [ $\mu\text{l min}^{-1}$ ] $\pm$ SD (n=4)	Peak height [mAU]	Elution Flow Rate [ $\mu\text{l min}^{-1}$ ] $\pm$ SD (n=3)	Peak height [mAU]
715	138.2 $\pm$ 6.4	200	199.2 $\pm$ 8.4
750	136.6 $\pm$ 3.2	300	180.8 $\pm$ 4.3
875	126.8 $\pm$ 2.9	400	163.3 $\pm$ 8.8
1000	133.6 $\pm$ 2.0	500	159.2 $\pm$ 6.8
1125	126.0 $\pm$ 8.9	600	148.9 $\pm$ 6.0
		750	144.4 $\pm$ 5.2
		1000	140.4 $\pm$ 7.3

\* Concentration of 2 ml sample (5.5  $\mu\text{mol l}^{-1}$  p-NP, 1  $\text{mmol l}^{-1}$  NaOH), elution with separation buffer

For loading, 715  $\text{ml min}^{-1}$  was the lowest performable flow rate (nominal 143  $\mu\text{l min}^{-1}$ ) with the MSFIA module (20-1024 s for total stroke) since two syringes, S1 (sample) and S2 (HCl), were used. Peak heights decreased slightly (9 %) with increasing flow rate due to a shorter residence time in the column and analyte diffusion length in the sorbent pores. Due to the small diameter of the sorbent particles, the backpressure increased notably. As a compromise of loading time to backpressure and sensitivity, a flow rate of 750  $\mu\text{l min}^{-1}$  was chosen. For elution, peak heights decreased considerably (40 %) over the studied flow rate range. Since the elution volume was smaller and less time was required than for loading, 300  $\mu\text{l min}^{-1}$  was an adequate compromise between elution time and sensitivity.

### 8.4.2.3. Column length

The sample volume was fixed to 6 ml, which allowed to perform the pre-concentration during the electrophoretic separation with a minimum of additional time and within one dispense step. The objective was a concentration factor of at least 30. The upper limit of the working range was aimed to be at least the LOD of m-NP as the analyte of the lowest absorptivity value obtained by direct injection ( $68 \mu\text{mol l}^{-1}$ , see chapter 7.5.1.1). So, the required loading column capacity was calculated to be  $6 \text{ ml} \cdot 68 \text{ nmol ml}^{-1} = 408 \text{ nmol}$ . Reducing the sample volume by the aimed concentration factor, a capacity of 13.6 nmol would have been acceptable whereas an even higher capacity was aimed considering the retention of the other NP and adsorption of sample matrix components.

Two columns of 7.5 mm and 15 mm in length, respectively (both 4.5 mm i.d.) were tested. The wide inner diameter was chosen in order to minimize the flow resistance and so, to maximize the possible loading flow rate. A short column was aimed to minimize the resin amount, flow resistance, and analyte dispersion during elution. The loading capacity was tested by the pre-concentration of solutions of m-NP. Loading capacities of at least 258 nmol were found. Since the height of the elution peak was not significantly altered by the column length but higher backpressure and enhanced gas bubble formation were observed using the longer column, the shorter one was finally chosen for this work.

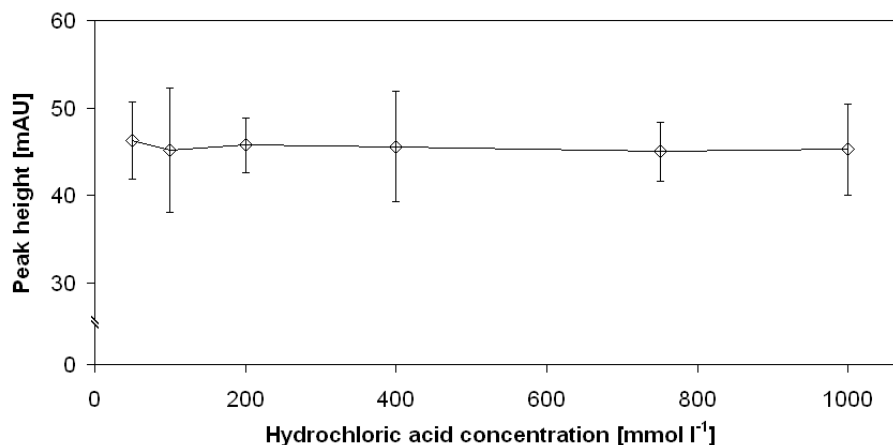
### 8.4.3. Optimization of chemical variables

#### 8.4.3.1. Sorbent

Apart from the later applied octadecyl (C18) modified polymer sorbent (chapter 8.3.2), two further reverse silica phases modified with phenyl-groups (DSC-Ph) or C18 (DSC-18), respectively, from Sigma Aldrich (St. Louis Agilent, MO, USA) were tested. The first sorbent showed a very low retention capacity for NP. The C18 resin worked very well but was not applicable over more than one day as it was not resistant to the acidic conditions applied for column loading. For the polymeric sorbent, no loss of loading capacity was observed. However, filtering of samples was imperative due to the fine particle diameter.

#### 8.4.3.2. Concentration of hydrochloric acid

The influence of the hydrochloric acid concentration used for in-line acidification of the sample during the loading procedure was studied for the following concentration 0.05, 0.1, 0.2, 0.4, 0.75, and  $1 \text{ mol l}^{-1}$ , corresponding to final acid concentrations in the sample of  $0.01 \text{ mol l}^{-1}$  to  $0.2 \text{ mol l}^{-1}$ . Experimental results are given in table 37 and are represented figure 70. No significant effect was observed using 2 ml of a p-NP aqueous standard. A concentration of  $0.1 \text{ mol l}^{-1}$  was chosen in order to maintain the reagent consumption low.



**Figure 70: Data representation of the optimization study of HCl concentration.**  
Data, conditions, and analyte concentration are given in table 37.

**Table 37: Influence of hydrochloric acid concentration for in-line sample acidification. \***

Hydrochloric acid [mmol l <sup>-1</sup> ] ± SD (n=3)	50	100	200	400	750	1000
Peak height [mAU]	46.2±4.4	45.2±7.1	45.7±3.1	45.6±6.3	45.0±3.4	45.3±5.2

\* Pre-concentration of 2 ml sample (5.5 μmol l<sup>-1</sup> p-NP in 1 mmol l<sup>-1</sup> NaOH). Elution was done towards a flow cuvette for detection described in chapter 8.3.1 instead of the CE instrumentation.

#### 8.4.3.3. Eluent composition

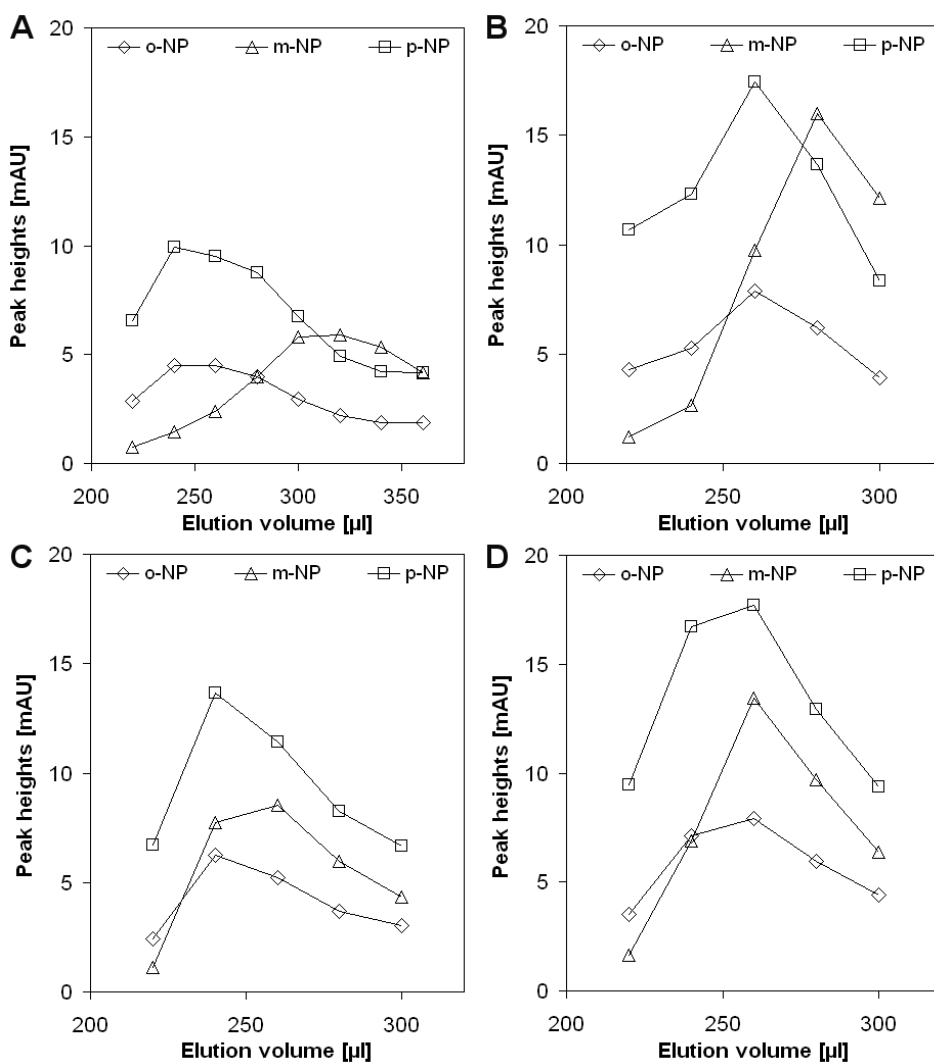
Due to the alkalinity of the used separation buffer (apparent pH 9.7) and the content of 10 v/v% methanol as an organic modifier, the direct elution of NP, prior retained under acidic conditions, was possible. Hydrodynamic injection was done at the maximum of the elution peak. So the objective was to obtain a narrow and high elution peak profile for all NP. In order to increase the elution strength, 100 μl to 300 μl of an additional eluent (EL2) was used, which was aspirated prior to elution and passed therefore the SPE column first.

Different compositions and volumes for EL2 were tested using a p-NP standard and either borate buffer or NaOH for alkalization and MeOH or acetonitrile (MeCN) as organic modifiers. MeCN was superior to MeOH in several aspects, 1.: lower polarity, thus higher elution strength, 2.: lower viscosity, thus lower flow resistance, and 3.: lower hydration heat and vapor pressure, thus lower risk of gas bubble formation at mixing with water or at the sudden pressure release at the column outlet. Separation buffer prepared with 10 v/v% MeCN led to a peak height increase of 50 % by a simultaneous reduction of bubble formation. Separation buffer with a content of 20 v/v% MeOH for EL2 led to a considerable increase of bubble formation in the eluent flow even after thorough ultrasonic degasification. Bubble formation using 20 v/v% MeCN was less compared to an equal content of MeOH but was nevertheless unusable since suppression of bubble formation was imperative for CE. Bubble formation increased further with the volume of EL2. Elution using 40 mmol l<sup>-1</sup> borate buffer (apparent pH 9.7) was similar to NaOH 10 mmol l<sup>-1</sup>.



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Testing the elution procedure with a NP standard mixture with coupled electrophoretic separation, the use of borate buffer with 10 v/v% MeCN (100  $\mu$ l) as EL2 resulted in an apparent loss of 71 % of m-NP. Studying the elution with the flow cuvette assembly it was observed that m-NP eluted about 80  $\mu$ l delayed compared to o-NP and p-NP, which eluted simultaneously (see figure 71). Since the elution volume had been optimized using p-NP, injection was done at the maxima of the elution peaks of o-NP and p-NP at the capillary entrance whereas the maximum of m-NP elution peak had not reached the interface yet.



**Figure 71: Data representation of optimization of eluent composition and volume.**

**A:** Borate 40 mmol  $l^{-1}$  pH9.7 with 10 v/v% MeCN, **B:** 100  $\mu$ l NaOH 10 mmol  $l^{-1}$  with 10 v/v% MeCN, **C:** 100  $\mu$ l NaOH 20 mmol  $l^{-1}$  with 10 v/v% MeCN, **D:** 200  $\mu$ l NaOH 10 mmol  $l^{-1}$  with 10 v/v% MeCN. Data, conditions, and analyte concentration are given in table 38.

Further experiments were made to achieve the synchronous elution of all NP. The modification of the apparent pH and borate concentration of EL2 and elution flow rate did not improve the overlap of the elution profiles of the three analytes significantly. Likewise, co-elution did not improve the elution but led to a higher dispersion and lower elution peaks. The application of a waiting step during elution did improve the elution neither.

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**Table 38: Influence of volume and composition of EL2 on the NP elution profiles. \***

Eluent	Volume [μl]	o-NP	m-NP	p-NP
A 100 μl borate 40 mmol l <sup>-1</sup> pH9.7 with 10 v/v% MeCN	220	2.87±0.22	0.76±0.07	6.55±0.54
	240	4.52±0.23	1.46±0.05	9.91±0.33
	260	4.51±0.23	2.37±0.15	9.51±0.29
	280	4.00±0.51	4.00±0.48	8.77±0.95
	300	2.94±0.33	5.83±0.56	6.75±0.70
	320	2.20±0.08	5.90±0.28	4.93±0.21
	340	1.89±0.05	5.36±0.37	4.23±0.09
	360	1.88±0.01	4.18±0.06	4.18±0.07
B 100 μl NaOH 10 mmol l <sup>-1</sup> with 10 v/v% MeCN	220	4.28±1.03	1.22±0.38	10.67±2.34
	240	5.27±0.23	2.67±0.03	12.32±0.45
	260	7.86±2.39	9.74±2.12	17.43±4.49
	280	6.21±0.80	15.97±1.9	13.65±1.90
	300	3.92±0.11	12.14±2.7	8.33±0.34
C 100 μl NaOH 20 mmol l <sup>-1</sup> with 10 v/v% MeCN	220	2.43±0.58	1.12±0.67	6.71±1.53
	240	6.22±1.20	7.75±1.75	13.64±2.43
	260	5.22±1.13	8.52±2.52	11.43±2.31
	280	3.68±0.33	5.97±0.70	8.27±0.83
	300	3.02±0.18	4.33±0.17	6.65±0.34
D 200 μl NaOH 10 mmol l <sup>-1</sup> with 10 v/v% MeCN	220	3.53±0.35	1.65±0.06	9.48±0.75
	240	7.12±0.30	6.91±0.24	16.70±0.34
	260	7.92±0.89	13.43±2.0	17.72±1.81
	280	5.93±0.51	9.69±1.44	12.92±1.30
	300	4.41±0.52	6.37±0.73	9.35±0.91

\* pre-concentration of 6 ml sample (0.3, 1.2, and 0.15 mg l<sup>-1</sup> o-, m-, and p-NP respectively), separation by CE using 40 mmol l<sup>-1</sup> borate, apparent pH 9.7, 10 v/v% MeOH, 25 kV

Finally, the exchange of borate by NaOH in EL2 was tested with the obtained elution profiles for the three NP represented in figure 71 and experimental results and conditions given in table 38. The use of NaOH with MeCN for EL2 instead of the modified borate buffer led to a considerably better overlapping of the three elution peaks. The best results were achieved using 200 μl EL2 being 10 mmol l<sup>-1</sup> NaOH with 10 v/v% MeCN. The former observations suggest that the difference in the elution characteristics was due to interaction of borate and m-NP leading to the formation of a more effectively retained complex. Similar effects had been reported earlier [372,373]. The complex seemed to be favored by the molecular conformation of the phenolic and the nitro-group of m-NP. The highest peaks were obtained for an elution volume of 260 μl using EL2 of the prior-given composition before performing hydrodynamic injection and were therefore adopted.

## 8 MSFIA-CE and application to the determination of nitrophenols

It was further observed, that peak heights were up to 80 % higher using NaOH for EL2 compared to borate and that peak heights were increased for a lower sodium hydroxide content of EL2. This was reduced to sample stacking at HV application occurring due to the lower conductivity of the injected sample compared to the separation buffer. This leads to the enrichment of the analyte molecules at the transition zone of sample and separation buffer on the ground-directed side of the sample segment. Besides, the high mobility of the hydroxide ion compared to the borate ion leads probably to a hydroxide ion "draining" of the sample by hydroxide enrichment also at the ground-directed side of the sample segment, which leads to an even lower conductivity of the sample zone and enhanced stacking effect. To shorten the elution procedure, the injection valve was turned into position "LOAD" after dispense of the elution volume and the column was flushed with an alkaline eluent EL1 (10 mmol l<sup>-1</sup> NaOH, 40 v/v% MeOH) prior to the next conditioning.

### 8.5. Discussion

#### 8.5.1. Analytical characterization

##### 8.5.1.1. Linear working range and detection limit

For the determination of the linear response of the method, standards of the three analytes of increasing concentrations were measured four-times. Concentrations between 0.11 and 86.33 µmol l<sup>-1</sup> were tested. The relation between for o-NP, m-NP, and p-NP was 2:4:1, respectively. Calibration data are given in table 39, calibration curves in figure 72.

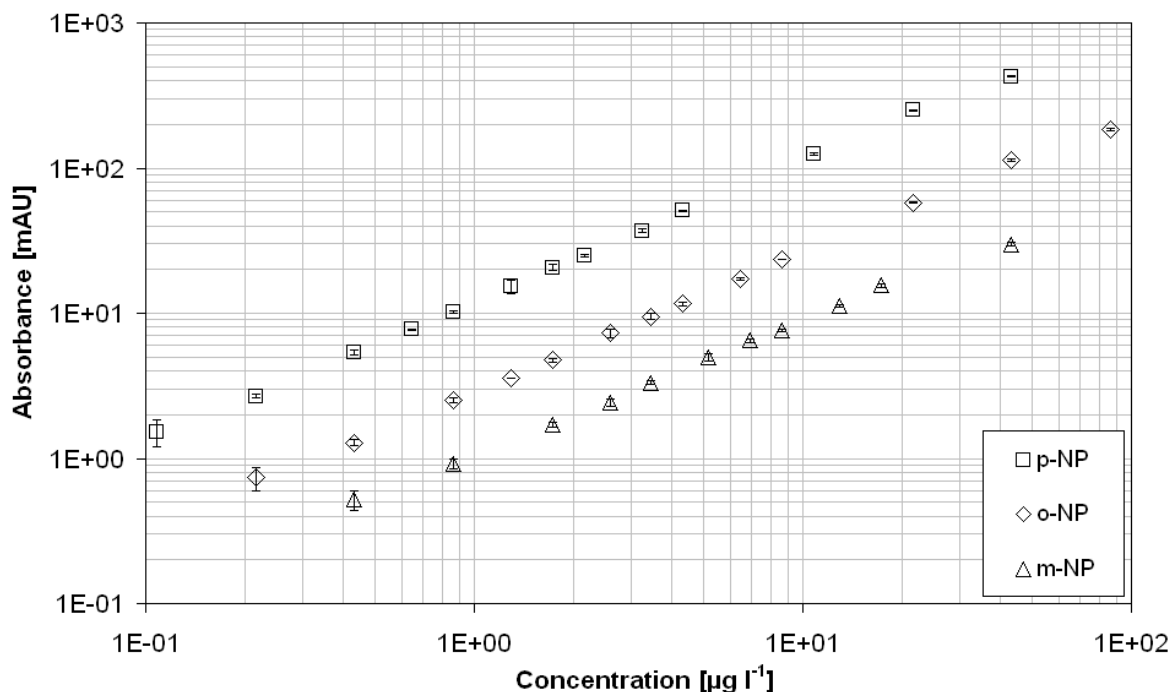


Figure 72: Peak height calibration curves for the three NP using the MSFIA-CE analyzer.

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**Table 39: Data of calibration performed for the estimation of the linear working range.**

Concentration		Peak height [mAU] $\pm$ SD (n=4)		
[ $\mu\text{g l}^{-1}$ ]	[ $\mu\text{mol l}^{-1}$ ]	o-NP	m-NP	p-NP
15	0.11	n.t.	n.t.	1.53 $\pm$ 0.32
30	0.22	0.73 $\pm$ 0.14	n.t.	2.68 $\pm$ 0.07
60	0.43	1.28 $\pm$ 0.06	0.52 $\pm$ 0.08	5.39 $\pm$ 0.18
90	0.65	n.t.	n.t.	7.73 $\pm$ 0.10
120	0.86	2.51 $\pm$ 0.11	0.92 $\pm$ 0.06	10.19 $\pm$ 0.23
180	1.29	3.56 $\pm$ 0.02	n.t.	15.28 $\pm$ 1.60
240	1.73	4.75 $\pm$ 0.15	1.71 $\pm$ 0.07	20.69 $\pm$ 0.91
300	2.16	n.t.	n.t.	24.91 $\pm$ 0.65
360	2.59	7.28 $\pm$ 0.46	2.43 $\pm$ 0.13	n.t.
450	3.24	n.t.	n.t.	37.10 $\pm$ 0.81
480	3.45	9.49 $\pm$ 0.47	3.33 $\pm$ 0.07	n.t.
600	4.32	11.59 $\pm$ 0.25	n.t.	50.88 $\pm$ 0.62
720	5.18	n.t.	4.98 $\pm$ 0.31	n.t.
900	6.47	17.16 $\pm$ 0.26	n.t.	n.t.
960	6.90	n.t.	6.48 $\pm$ 0.22	n.t.
1200	8.63	23.52 $\pm$ 0.18	7.60 $\pm$ 0.17	n.t.
1500	10.79	n.t.	n.t.	125.13 $\pm$ 1.63
1800	12.94	n.t.	11.31 $\pm$ 0.26	n.t.
2400	17.25	n.t.	15.50 $\pm$ 0.37	n.t.
3000	21.58	57.57 $\pm$ 0.64	n.t.	249.60 $\pm$ 4.03
6000	43.13	114.05 $\pm$ 2.59	29.98 $\pm$ 0.71	428.91 $\pm$ 7.32
12000	86.26	183.89 $\pm$ 2.76	28.71 $\pm$ 2.78	n.t.
Calibration function [AU   $\mu\text{mol l}^{-1}$ ]		2.641·c + 0.275	0.877·c + 0.215	11.556·c + 0.306
Range [ $\mu\text{mol l}^{-1}$ ]		0.22-43.17	0.43-17.258	0.11-21.58
Calibration curve $r^2$		$r^2 = 1.000$	$r^2 = 0.999$	$r^2 = 1.000$
Deviation of highest standard		-24.1 % (86.33)	-26.9 % (43.17)	-16.4 % (43.17)

\* Conditions: 6 ml sample volume, separation buffer 40 mmol l<sup>-1</sup> borate, 10 v/v% methanol, apparent pH 9.7, 25 kV, EL2 10 mmol l<sup>-1</sup> NaOH with 10 v/v% MeCN, n.t.: not tested.

Linear responses of the peak heights were found up to at least 43.13  $\mu\text{mol l}^{-1}$  (o-NP), 15.50  $\mu\text{mol l}^{-1}$  (m-NP), and 21.58  $\mu\text{mol l}^{-1}$  (p-NP). The highest standards showed negative deviations from the estimated values applying linear calibration functions of 16.4 % to 26.9 %. This was reduced to the limited loading capacity of the SPE column. The linear range can be modified by the appropriate increase or decrease of the sample volume used for pre-concentration. Due to the very low baseline noise showing with an amplitude of 0.1 mAU, the LOD as described in chapter 3.6.2 were 0.11  $\mu\text{mol l}^{-1}$  (o-NP), 0.35  $\mu\text{mol l}^{-1}$  (m-NP), and 0.03  $\mu\text{mol l}^{-1}$  (p-NP). Direct measurement of the highest standard (12 mg l<sup>-1</sup> o-NP, 24 mg l<sup>-1</sup> m-NP, 6 mg l<sup>-1</sup> p-NP), i.e. omitting pre-concentration, gave peak heights of

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1.86±0.15 mAU, 1.22±0.07 mAU and 4.27±0.17 mAU, respectively. Comparison of these data with the slopes of the calibration functions in table 39 resulted in pre-concentration factors for the three analytes of 123, 124, and 117, respectively.

The achieved LOD values are in the same order as achieved using other analytical methods designed for the analysis of nitrophenols given in table 40 and reviewed by PUIG AND BARCELÓ (1996) [320]. In contrast to the mostly automated techniques, the actual work allowed the physical separation of the analytes prior to quantification. Besides, the technique can be adapted to other nitrophenols, which show satisfying absorptivity values at the applied detection wavelength of 400 nm. Further lowering of the obtained LOD values can be achieved either by repeated execution of the pre-concentration procedure, as done so for the reported determination of total phenolic compounds using MSFIA [104]. However, repeated pre-concentration was rejected since the time of analysis would have increased unacceptably. Applying electrokinetic on-capillary enrichment, concentration factors of up to 2000 were achieved [346]. Electrokinetic injection of the anionic NP would have required the intermediate change of the separation voltage polarity. A circuit for remote control of polarity was developed (see annex). However, it could not be used in the actual work for want of a free additional supply port on the MSFIA module. Reversal of the EOF by the use of a cationic detergent in the separation buffer was further required.

**Table 40: Comparison of LOD values of the actual work with other analytical methods.**

Technique	LOD [mol l <sup>-1</sup> ]			Ref.
	o-NP	m-NP	p-NP	
SIA-LLE	2.6·10 <sup>-7</sup>	3.3·10 <sup>-7</sup>	3.5·10 <sup>-8</sup>	[107]
MSFIA-LLE	1.1·10 <sup>-7</sup>	4.6·10 <sup>-7</sup>	7.0·10 <sup>-8</sup>	[18]
LLE+HPLC	4.0·10 <sup>-9</sup>	4.0·10 <sup>-9</sup>	7.0·10 <sup>-9</sup>	[317]
MSFIA-SPE	1.2·10 <sup>-6</sup>	3.2·10 <sup>-6</sup>	3.0·10 <sup>-7</sup>	[106]
MSFIA-optosensing	5.0·10 <sup>-9</sup>	-	3.0·10 <sup>-9</sup>	[322]
MSFIA-SPE+CE	1.1·10 <sup>-7</sup>	3.5·10 <sup>-7</sup>	3.0·10 <sup>-8</sup>	actual work
SPE-HPLC	1.4·10 <sup>-7</sup>	-	4.3·10 <sup>-7</sup>	[366]
CE	4.0·10 <sup>-7</sup>	19·10 <sup>-7</sup>	4.0·10 <sup>-7</sup>	[327]
CE	1.0·10 <sup>-5</sup>	1.0·10 <sup>-5</sup>	4.1·10 <sup>-5</sup>	[329]

### 8.5.1.2. Reproducibility and column capacity

The entire method including pre-concentration, elution, and electrophoretic separation showed high reproducibilities regarding migration time, peak heights, peak areas, and peak widths with RSD values given in table 41. Migration times of o-NP, m-NP, and p-NP and were 508 s ± 11 s, 453 s ± 9 s, and 494 s ± 11 s with a theoretical plate number of up to 12.000. Peak height data were used for quantification since the respective RSD values were lower than the ones obtained using peak areas as stated before in chapter 7.5.1.2.

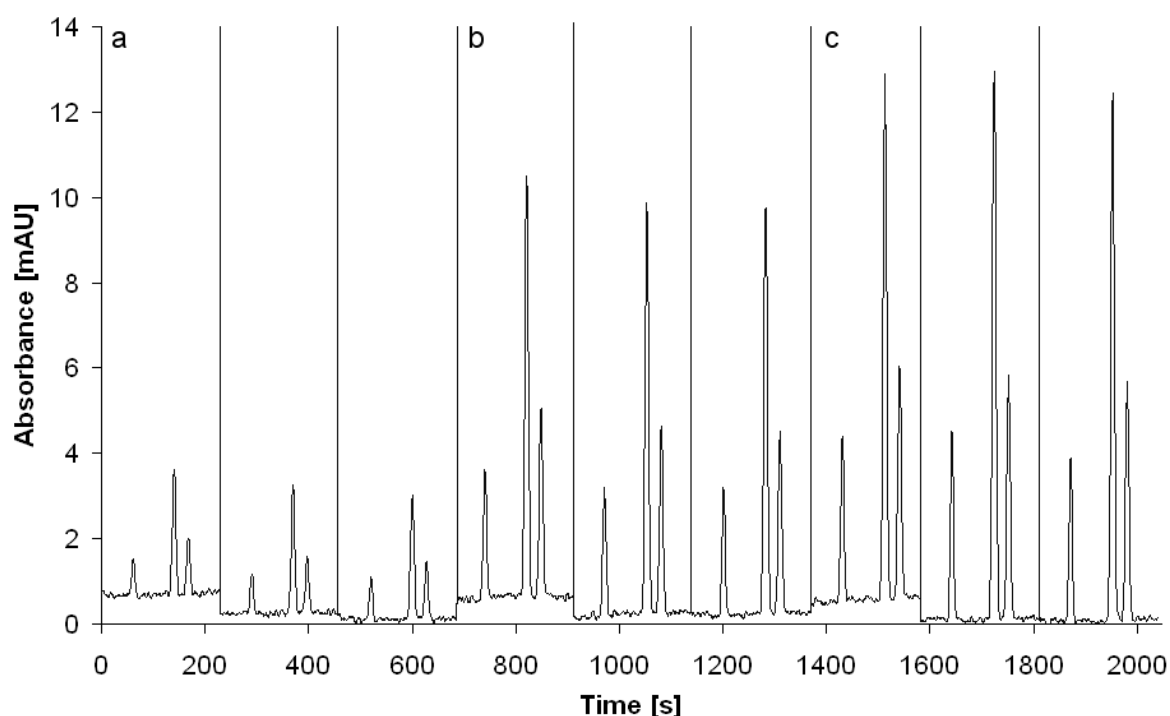
## 8 MSFIA-CE and application to the determination of nitrophenols

Separation runs of on-line pre-concentrated aqueous standards of different concentrations are shown in figure 73. The results were highly satisfying considering the sequence of various procedures automated by the analyzer system including analyte pre-concentration and the generally low reproducibility of the CE technique. Due to the elimination of the sample matrix, i.e. sample clean-up, by analyte pre-concentration, no significant differences of the migration times or peak widths were found between aqueous standards and different real samples.

**Table 41: Mean relative standard deviation of 10 measurements (samples and standards). \***

Mean RSD [%]	for one solution (n=3)			for 10 different solutions (n=30)		
	o-NP	m-NP	p-NP	o-NP	m-NP	p-NP
Migration time	0.42	0.42	0.42	2.0	2.2	2.1
Peak height	2.6	5.7	2.9	-	-	-
Peak area	4.3	5.3	4.1	-	-	-
Peak width	-	-	-	13.8	17.9	10.7

Conditions: separation buffer as described in chapter 7, pre-concentration volume of 6 ml



**Figure 73: Separations in triplicate of aqueous NP standards.**

Pre-concentrated with 6 ml, concentrations of o-NP, m-NP and p-NP in  $\mu\text{mol l}^{-1}$ : a: 0.43, 0.87, 0.22; b: 1.74, 3.48, 0.87; c: 2.17, 4.35, 1.09. Time scale refers to time of data acquisition. Separation conditions: borate buffer  $40 \text{ mmol l}^{-1}$ , apparent pH 9.7, 10 v/v% MeOH, 25 kV.

## 8.5.1.3. Recovery and time of analysis

The proposed analytical method was tested with spiked water samples for the evaluation of the influence of different sample matrices on the analytical performance and analyte recovery. Different soil leachates, tap water, and groundwater were analyzed without and with the addition of using aqueous NP standards of  $2.17 \mu\text{mol l}^{-1}$  of o-NP,  $4.35 \mu\text{mol l}^{-1}$  of m-NP, and  $1.09 \mu\text{mol l}^{-1}$  of p-NP. Prior to analysis, the samples were filtered through  $0.45 \mu\text{m}$  nylon membrane filters. The blank concentrations of all NP were below their respective LOD for all samples. The found recovery values calculated applying peak height calibration with aqueous standards are given in table 42.

**Table 42: Determination of nitrophenols in spiked wastewater samples by MSFIA-CE. \***

Sample	Found nitrophenols [ $\mu\text{mol l}^{-1}$ ]			Resulting recovery [%]		
	o-NP	m-NP	p-NP	o-NP	m-NP	p-NP
Leachate 1	$2.38 \pm 0.06$	$4.49 \pm 0.39$	$1.19 \pm 0.05$	109.3 %	103.3 %	109.8 %
Leachate 2	$2.04 \pm 0.06$	$4.01 \pm 0.40$	$1.01 \pm 0.02$	103.4 %	91.2 %	104.4 %
Leachate 3	$2.25 \pm 0.11$	$3.96 \pm 0.36$	$1.13 \pm 0.07$	110.9 %	111.0 %	108.4 %
Ground water	$2.41 \pm 0.08$	$4.82 \pm 0.29$	$1.18 \pm 0.04$	93.9 %	91.1 %	91.2 %
Tap Water	$2.04 \pm 0.07$	$3.96 \pm 0.07$	$0.99 \pm 0.05$	93.7 %	92.2 %	92.6 %

\* Results are expressed as the mean of three determinations given with standard deviation. Spiked NP concentrations were  $2.07 \mu\text{mol l}^{-1}$  of o-NP,  $4.35 \mu\text{mol l}^{-1}$  of m-NP, and  $1.09 \mu\text{mol l}^{-1}$  of p-NP.

All recovery values were between 91 % and 111 % with mean values of 102 % for o-NP, 97.7 % for m-NP, and 101 % for p-NP. Mean RSD values for one sample measured in triplicate were 3.4 % (o-NP), 7.1 % (m-NP), and 4.2 % (p-NP), respectively. The found recoveries and their low deviation were highly satisfying considering the accomplished analytical procedures. The analyte pre-concentration on the SPE column presented also a sample clean-up and better recovery values were obtained than in the former work (see chapter 7.5.1.3). By application of the pre-concentration procedure, inorganic and small organic ions as well as protonizable basic components are widely eliminated. So, the influence of the ionic strength is considerably lowered, which principally allows performing electrokinetic concentration techniques with high reproducibility.

However, other acidic components can be retained on the sorbent and might lower the loading capacity of the column. Macromolecules such as tannins, chlorophyll, humus acids, etc. might be retained permanently or even block the column. Therefore, the SPE column filling might have to be renewed from time to time measuring samples, which contain these components in high concentrations. The same column filling was used for several days applying the method to the former given water samples. All optimization experiments had been accomplished with the one filling without any observed problems.

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The sample ionic strength, buffering capacity, and a considerable content of solvents might alter the retention and the elution profile of the analytes. It was considered as main reason for the significant difference of the found recovery values for the distinct sample types. So the use of an acidic buffer instead of hydrochloric acid for sample in-line acidification might bring some advantage regarding lower deviation of the recovery values.

The entire pre-concentration procedure including SPE column conditioning required about 16 min, the flushing of the capillary and both interfaces 70 s, the elution and injection procedure 70 s, and syringe refilling 40 s. The electrophoretic separation of the three NP was completed after 500 s. By carrying out the SPE column conditioning and the pre-concentration procedure during the electrophoretic separation of the former injected sample, the duration of the entire analytical method given by the time between two injections was reduced considerably from about 27 min to 19 min. The flushing of the HV interface with separation buffer was already carried out in parallel of the flushing of the grounded interface for capillary reconditioning. The resulting operation order of the different analytical procedures is schematically represented in figure 74.

In conclusion, one main advantage of the present system was the physical separation of the pre-concentration and the CE part of the system and parallel operations. This would not have been feasible using the SIA or LOV techniques. On the other hand, the main advantages of the syringe based techniques over most FIA systems (compare with chapter 7.5.2), i.e. pressure robustness and sequential and bi-directional flow operations, allowed the in-line maintenance of the capillary and the use of a sorbent material of very small particle diameter of consequently large specific surface and loading capacity.

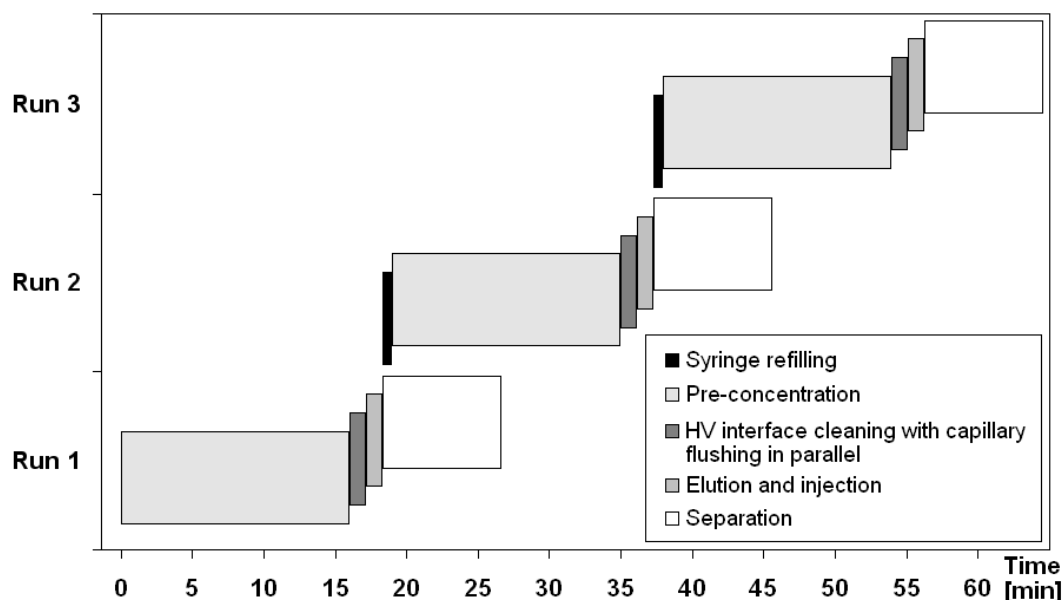


Figure 74: Schematic representation of operation order of the different analytical procedures carried out three times on the MSFIA-CE analyzer.



### 8.6. Conclusion

In the present work, an analytical system was proposed, in which for the first time the hyphenation of the multi-syringe flow injection technique and capillary electrophoresis was accomplished (MSFIA-CE). The system was successfully applied to the in-line solid phase pre-concentration of mono-nitrophenols with following elution and separation by CE.

The system showed to be robust, reliable, and applicable to different water matrices evaluated by real sample analysis. Due to the used control and data acquisition software, a versatile system was obtained, which is potentially applicable for monitoring purposes as it worked autonomously. It allowed the automation of the entire analytical procedure including in-line sample modification (acidification), pre-concentration, and elution as well as the entire CE apparatus maintenance and control of all injection and flow parameters. By using alternative sorbent materials for pre-concentration and clean-up and adaptation of the modifying agent, the systems can be applied to a variety of analytical applications.

By use of the multisyringe technique and of an additional injection valve, physical separation of the pre-concentration and the CE part of the system and in consequence parallel operations were possible. By performing the pre-concentration procedure in parallel to the electrophoretic separation of the previous sample, a higher sample frequency was achieved. The advantageous pressure robustness of the apparatus allowed the use of a very fine sorbent material and in-line capillary maintenance. Thus, a similar analytical performance would not have been achievable with a single syringe pump (SIA) or multichannel peristaltic pump (FIA). Highly satisfying repeatability, LOD, and recovery were achieved with concentration factors of about 120 for all three nitrophenols.



## 9. GENERAL CONCLUSIONS

In the presented work entitled "*Development of automated methods using syringe based flow analysis techniques and capillary electrophoresis for biotechnological process monitoring and environmental analysis*", five analyzer systems using syringe-based, sequential, multicommutation flow techniques for the determination of six different analytes were developed and successfully applied to real samples.

The used flow techniques Sequential Injection Analysis (SIA) and Multisyringe Flow Injection Analysis (MSFIA) allow the automation of laboratory analytical and preparative procedures. General improvements are a lower consumption of solutions and higher sample frequency, reliability, robustness, and reproducibility compared to continuous flow systems or even more, classical laboratory procedures.

The continuous or quasi-continuous control and observation of operation systems by monitoring analyzers is of high importance in order to understand and describe these systems. It is further of high interest for the improvement of sustainability, quality, risk prevention, or prevision of possible problems and to study the impact of distinct effective factors, independently if the operation system is an environmental or industrial process.

Analytical flow techniques are powerful tools for these monitoring tasks and the development of new analytical methods and exploration of better-suited instrumentation and flow techniques are therefore demanded by scientists, authorities, and industry. The suitability of the applied flow techniques SIA and MSFIA for monitoring tasks and automation of environmental analysis was successfully demonstrated in this work.

In three experimental works based on SIA technique, monitoring of the metabolite formaldehyde and the carbon sources glycerol and sorbitol in biotechnological processes was performed after the thorough optimization of the respective analytical systems. The high versatility of the SIA technique, used instrumentation, and software was demonstrated using the same manifold and instrumental assembly for the proposed analyzer systems varying mainly the analytical protocols. These included the instructions for the application of different reagents, volumes, and incubation times.

To achieve the aimed analytical characteristics, inline standard addition, zone stacking, and dilution by zone intercalation were applied in these systems. A novel splitting technique was developed allowing the provision of a very small sample volume with higher reproducibility than achieved by direct pickup.

Contributions for higher robustness and versatility of the instrumentation were made by the development of a novel flow-through adapter, which allowed using one normal lateral port on a selection valve as a flow-through port and sampling with a minor dead volume. Further, a procedure for the elimination of stacked air bubbles from the holding coil was developed by the flushing of the entire coil unidirectionally with acetonitrile and successfully tested during at-line monitoring of sorbitol.

## 9 General conclusions

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All SIA analyzer systems were successfully applied for biotechnological process monitoring with high injection frequency, repeatability, and reliability. High advantage of the versatility of the used software AutoAnalysis was taken using either by incorporation of smart decisions about the requirement of working range changing at each time of the process during at-line monitoring of glycerol (system self-configuration) or by the creation of a software-guided analytical protocol allowing user-interaction via requests for the sorbitol system. By this, both analytes could be determined in a wide concentration range.

For two further experimental works, a capillary electrophoresis (CE) system was built-up and coupled in a first step to a SIA analyzer system. The SIA-CE system enabled the software control of all injection parameters, connection of the flow system to both buffer reservoirs (detection and injection side of the capillary), and the entire maintenance of the capillary. By this, long-term operation without need of user intervention is possible.

Hydrodynamic injection with variable pressure and capillary flushing for conditioning was enabled by to the incorporation of a pressure reservoir allowing highly reproducible injection and background operations during the capillary conditioning procedure.

On-capillary photometric detection was done using a homemade detection cell and a blue LED as a highly stable light source. The high operation versatility and analytical performance of the homemade SIA-CE system was demonstrated by the analysis of nitrophenols in wastewaters. Buffer composition and all injection parameters were studied by thorough optimization work. The presented system was compared with former flow technique systems coupled to CE showing the highly satisfying operational performance of the proposed hyphenated system.

In the second work, the coupling of MSFIA to CE was accomplished for the first time. In addition with the former work, solid phase pre-concentration of mono-nitrophenols including in-line sample acidification, analyte elution, and separation by CE was carried out by the MSFIA-CE system. By parallel performance of electrophoretic separation and pre-concentration of the following sample, the sample frequency could be considerably enhanced. Concentration factors of about 120 were achieved for all three analytes with recovery values near to 100 % for different environmental water samples. High robustness, reliability, and versatility of the apparatus were demonstrated together with an excellent analytical performance. Both CE analyzers were characterized as suitable for monitoring purposes. Consequently they could contribute to a better knowledge of the environmental presence of nitrophenols used for this work as model substances of high environmental pollution impact.

# 10.OUTLOOK

Several considerations were already made in the respective sections of each chapter in respect of the continuation of the presented experimental works. For the SIA analyzers systems used for the monitoring of biotechnological processes, the use of multichannel flow techniques could allow an improvement in respect of sample frequency, robustness, and miniaturization. Another consideration was the implementation of continuously mixed, thermostated detection cells or applying the stop-flow technique in order to minimize effects caused by the sample matrix, background signal, or gas bubbles and in order to obtain a homogeneous dilution of the sample with the reagents.

In respect of the coupled systems of capillary electrophoresis with SIA and MSFIA, the implementation of remote polarity and voltage intensity change for electrokinetic injection and enrichment techniques is an important objective. For this, the required wiring of the analogous interface was already proposed in the annex. Likewise, the improvement of the detection cell, implementation of focusing optics, and other detection techniques such as fluorescence, chemiluminescence, or contactless conductivity detection are aimed, which in combination with the proposed system would lead to a universal MSFIA-CE system.

The experience and knowledge obtained during the presented experimental works are currently influencing project work on monitoring analytical parameters in seawater.

It has to be pointed out, that the confidence in flow techniques by industry is capable of improvement. There are also general doubts about the suitability and robustness of flow techniques and respective investigation outcomes. On the other hand, the advances in research and development in this area have led to commercial analyzer systems of high reliability and well acceptance. This is, because research works surely present an important pool of ideas for improvements of commercial problem solutions. Therefore, flow techniques will always be a stimulating and complementing technique entering other fields of analytical chemistry.



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# Annex



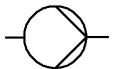
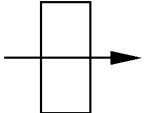
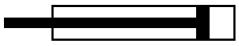
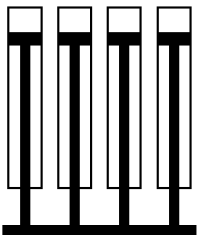
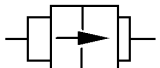
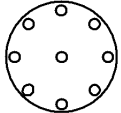
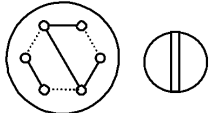






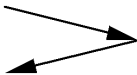
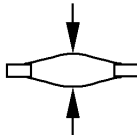


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
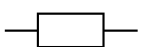
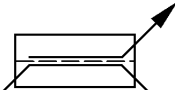


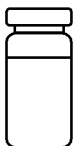



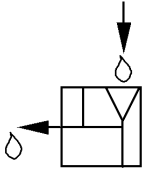

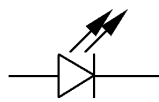

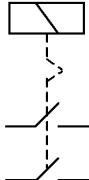


## I MANIFOLD SYMBOLS

	Pump general symbol
	Peristaltic pump
	Syringe pump
	Multisyringe pump
	Solenoid membrane pump
	Rotary selection valve with central common port and lateral ports
	Rotary six-port injection valve, scheme with and without drawing connectors
	Multicommutation solenoid 3-way valve
	Tube connector
	Tube crossing without connection
	Tube coil
	Tube coil tempered
	Polymeric tube
	Flow through adaptor (in- and outgoing flow)
	Inflatable tube

## I Manifold symbols

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	Closed tube or connection
	Solid phase extraction column
	Debubbler with gas permeable membrane
	Flow cell for absorbance detection
	Storage flask
	Vial
	Ventilator
	Fused silica capillary
	Split flow interface
	Dropping flow interface
	Electrical connection or control
	LED
	Electrical ground
	Relay with two switch-keys

## II PHOTOS OF APPLIED DEVICES



Figure 1: A: Multisyringe module equipped with glass syringes of 10, 5, 2.5, and 1 ml, the 5 ml syringe with loose Luer connection. B: Valve module with eight-port rotary selection valve (above) and low pressure six-port injection valve (below). C: Connections on the backside of the multisyringe device for RS232 control chain, power input and four additional external devices as solenoid valves (12V).

II Photos of applied devices

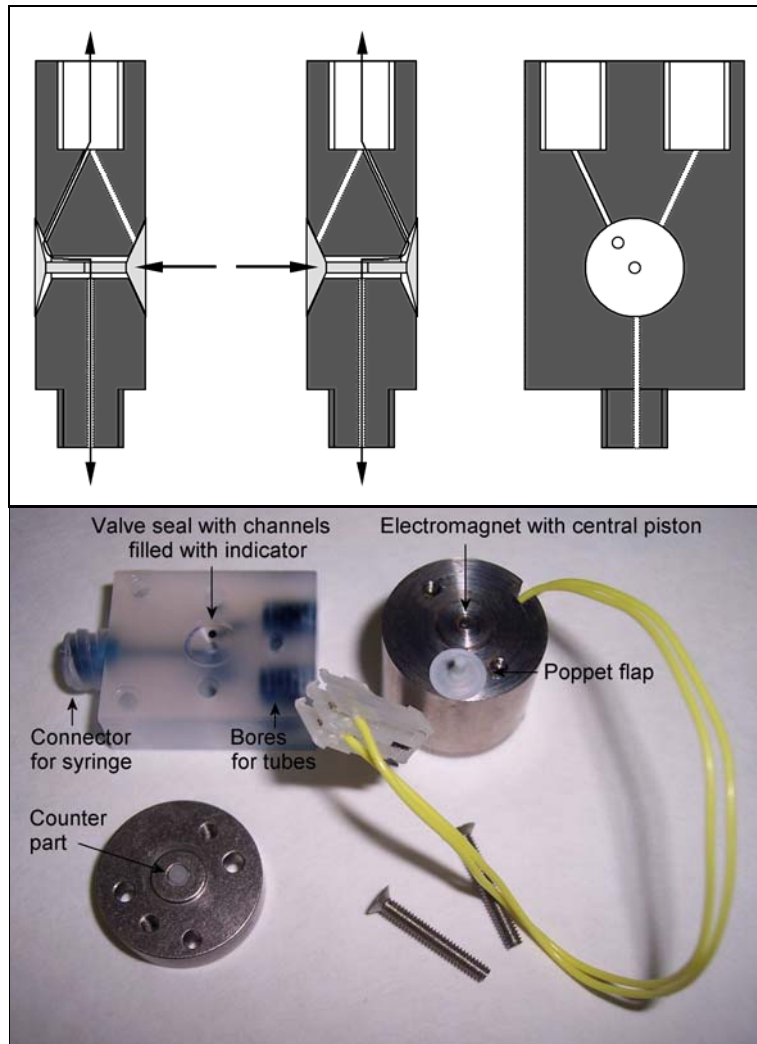


Figure 2: Assembly of the solenoid multisyringe head valves. Above: Flow scheme and poppet flap position. Below: Demounted solenoid valve with indicated components.

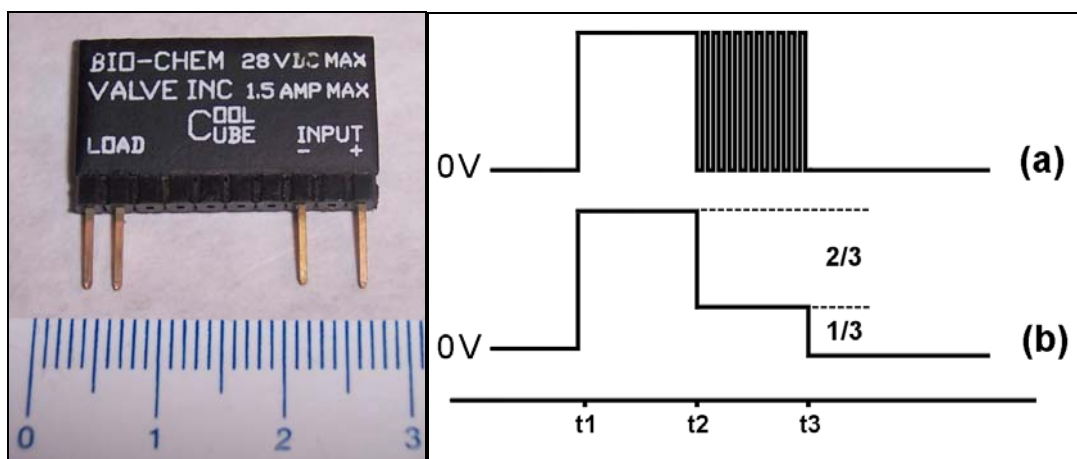


Figure 3: Cool cube hit and hold protection circuit for solenoid valves (left) and resulting output voltage (right). The device is inserted between valve and corresponding supply. Heat generation and risk of valve destruction at continuous operation is lowered by the change to a pulsating voltage (a) corresponding to a 66 % decrease of supplied power (b).

## II Photos of applied devices

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Figure 4: External multi-commutation solenoid valves (Takasago Electronic Inc.) used for flow manifolds. From left to right: STV-3-1/4UKG, MTV-3-N1/4UKG and MTV-3-1/4UKGH.

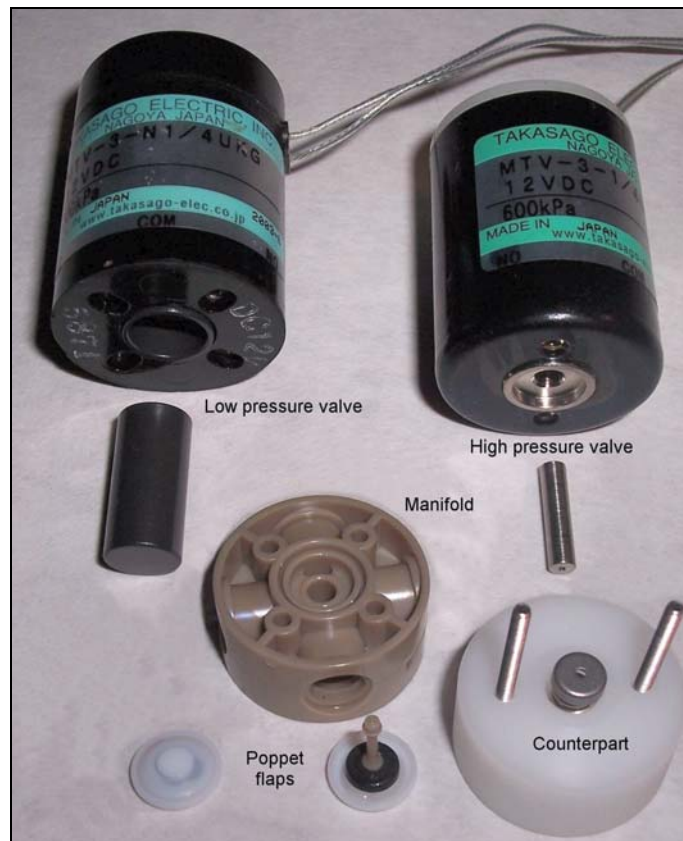
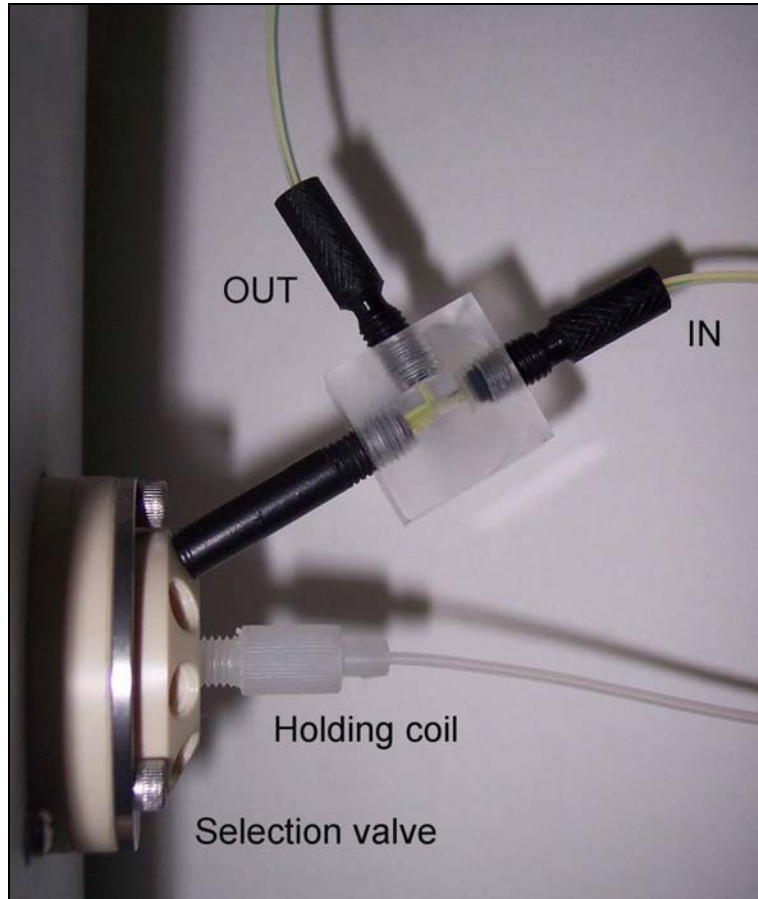


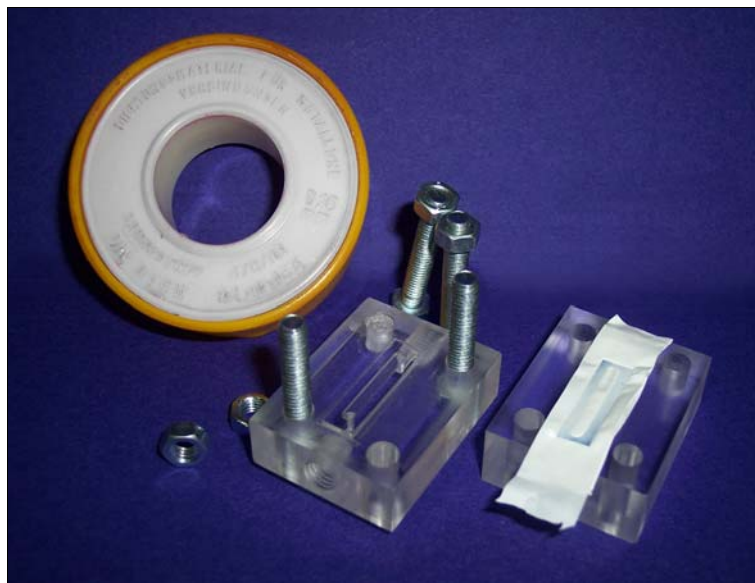
Figure 5: Demounted solenoid valves. In contrast to the head valves of the multisyringe device, the spacer assuring the permanent distance between the poppet flaps is not of the easily deformable material PTFE but of the more rigid polymer PEEK.

The difference between the solenoid valves of low (up to 2 bar) and high (up to 6 bar) pressure stability were the dimensions and material of bobbin and the core driven by the electromagnet, thus acting with different forces over the poppet flaps.





**Figure 6: Flow-through adapter developed in this work mounted on an 8-port rotary selection valve. The adapter enabled the function of a flow-through port. The containing liquid was tinted with fluorescein for better visibility.**



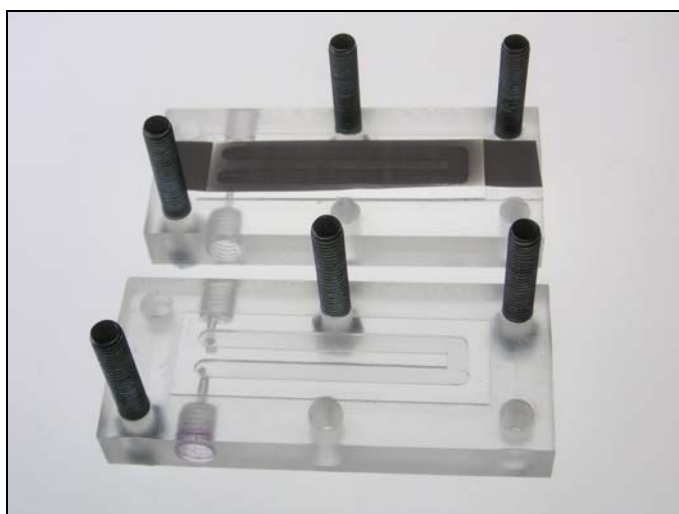
**Figure 7: Laboratory made debubbler cell manufactured of two PMMA blocks with flow channel. The used PTFE sealing tape used as membrane is shown.**

## II Photos of applied devices

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**Figure 8: Trace-Trap debubbler cell with 1.5 mm thick PTFE membrane. The cell is equipped with two channels of different length.**



**Figure 9: Home-made gas diffusion cell with Durapore® membrane.**



**Figure 10: Bubble separator to be used in the sample transport line for monitoring. Bubbles entering from below keep stacked in the head space of the cell and are drawn off from time to time by an additional pump. The sample stream flows out on the left side.**



**Figure 11: Miniature spectrometer USB 2000 used in this work. Connections for USB plug (left) and optical fiber (front) are visible.**



Figure 12: Cuvette holder CUV-UV with connections for fiber optics and special optical glass flow cell (1 cm, 14  $\mu$ l dead volume).

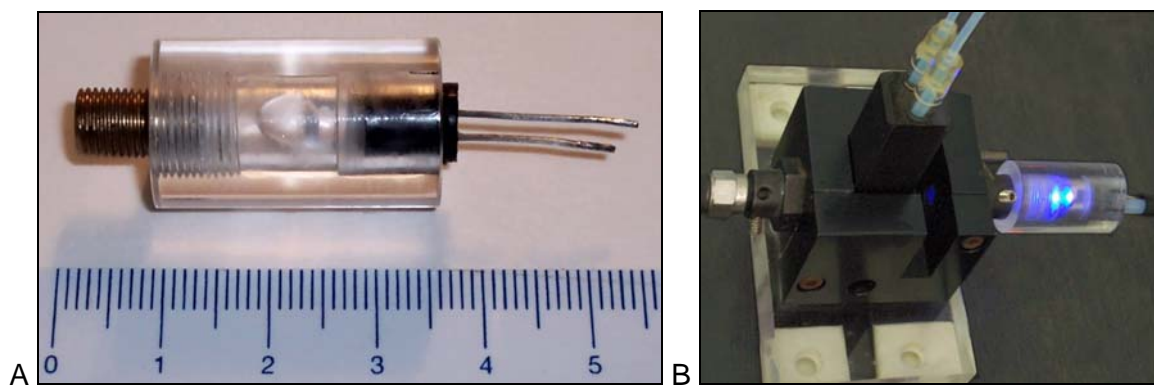


Figure 13: Laboratory made support (A) used for the connection of a light emission diode (LED) to an optical fiber, cuvette holder (B) or the capillary electrophoresis detection cell.

II Photos of applied devices

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Figure 14: Light source DH-2000-DUV with front side connection for the optical fiber (open safety lid) and power buttons for halogen and deuterium bulbs.

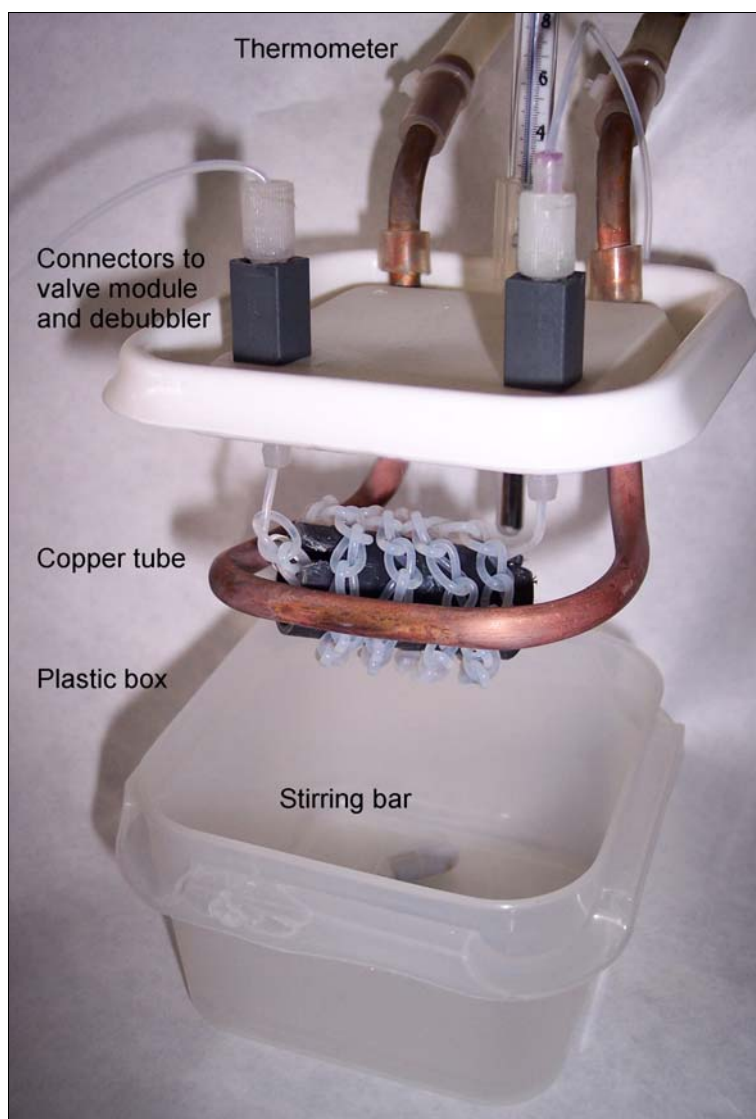


Figure 15: Spacer to be placed between optical fiber and light source to reduce the light intensity at the fiber tip by displacement of 2 cm. The spacer was made of bronze



Figure 16: Tubing weight made of stainless steel used to assure the submersion of liquid reservoir tubing.





**Figure 17: Miniaturized water bath including stirring bar, copper tube used as heat exchanger, knitted reaction coil and commercial thermometer for control.**

## II Photos of applied devices



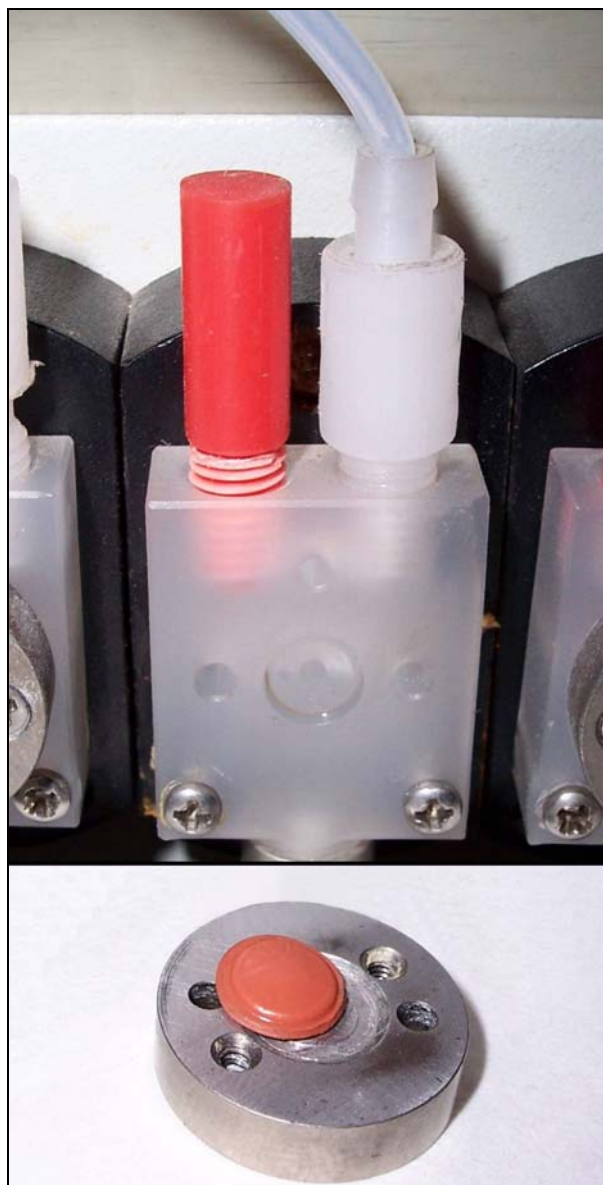
Figure 18: Front view of the high voltage power source HCP35-35000 (FuG Elektronik GmbH) with digital voltage, current and polarity indicators and controls.



Figure 19: Back view of the high voltage power source HCP35-35000 with high voltage cable socket (left), ground socket (center), and analog control interface and power socket (right).

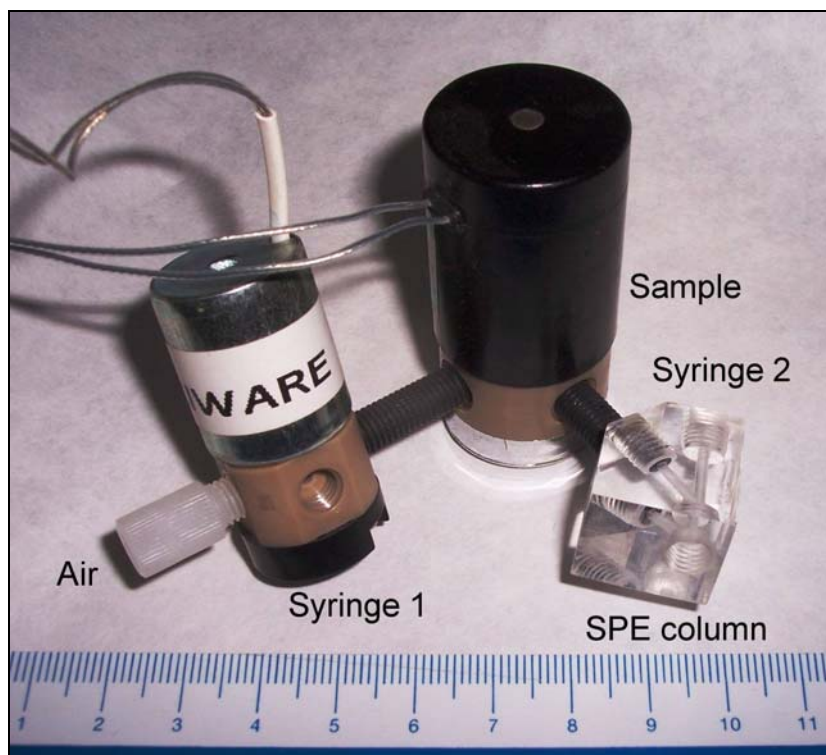


Figure 20: Cable and connectors for supply of high voltage from the power source HCP35-35000 (commercial connector) to the protection case (laboratory-made connector).

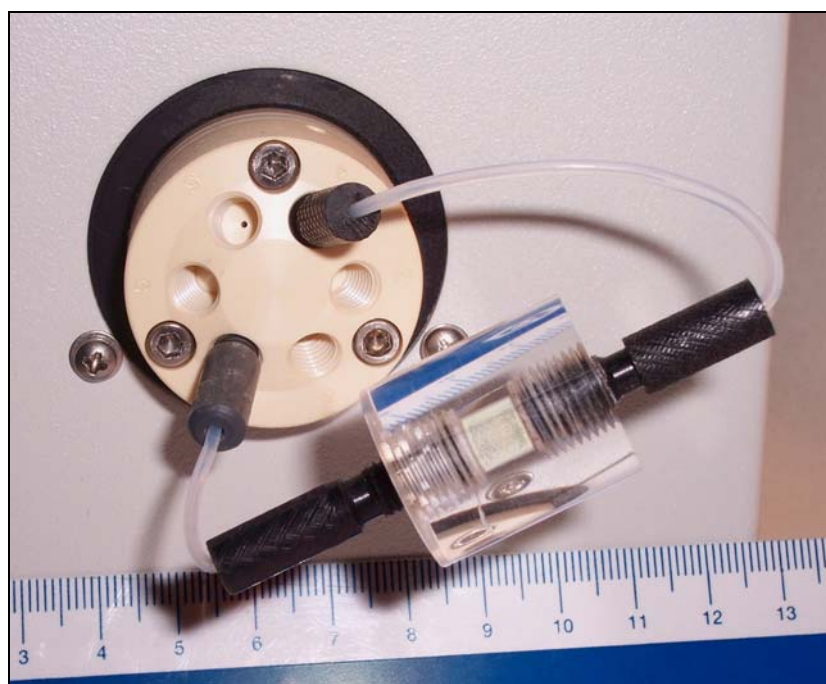


**Figure 21: Modified head valve of the multisyringe device (dismounted). The modification was done in order to achieve a higher robustness. The function of connecting the syringe either to a liquid reservoir or the manifold was assumed by pressure stable solenoid valve apart, connected to the original head valve via a short PTFE tubing of 1.5 id. The position ON of the original head valve was closed by a blind fitting; the pressure sensible PTFE poppets were replaced by commercial rubber septa. The electrical power supply of the original head valve was used for the operation of the pressure stable valve.**



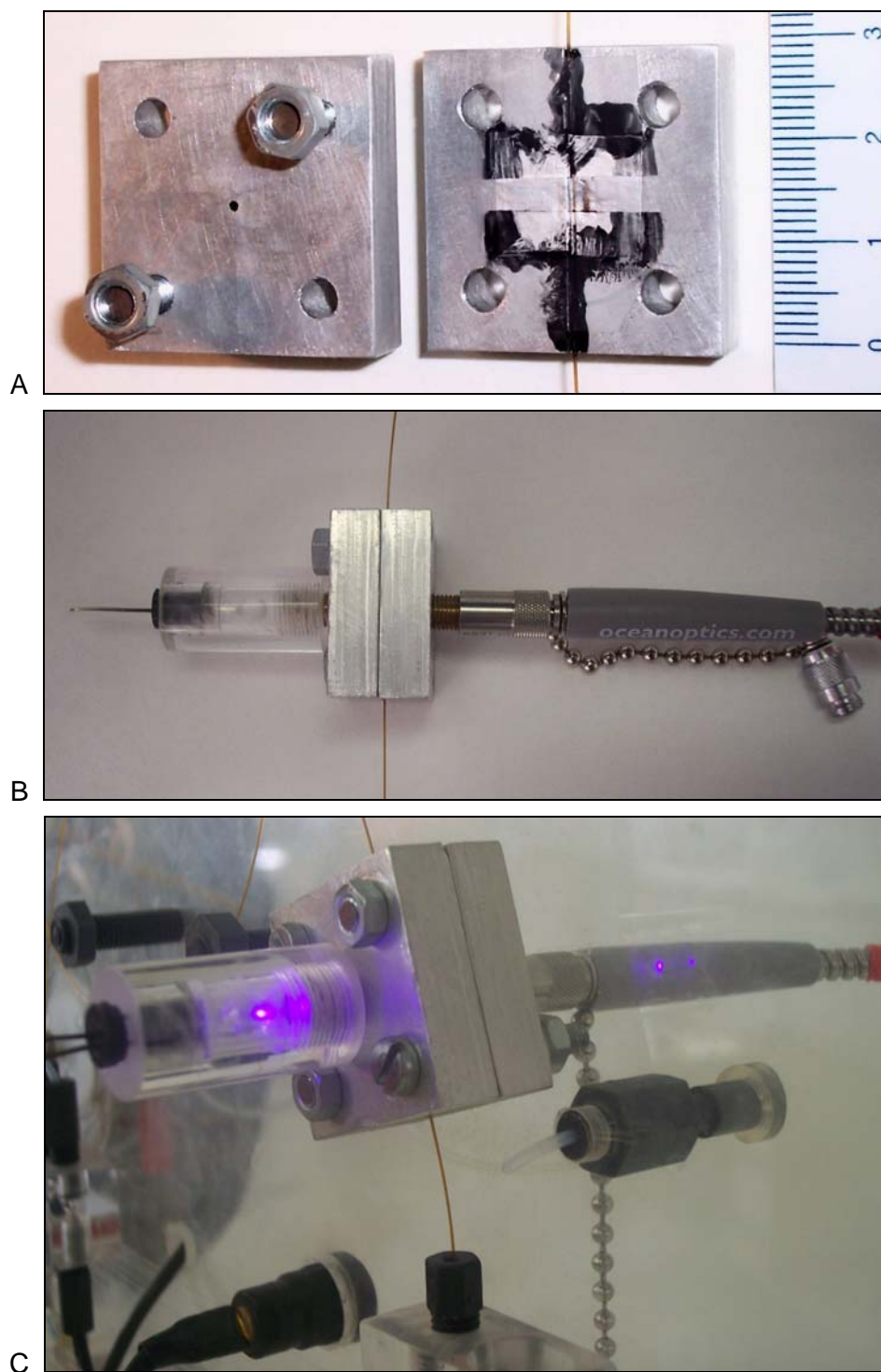


**Figure 22:** Valve assembly used in the proposed system for the pre-concentration of mononitrophenols. In order to minimize the dead volume, deldrine tubes with outside screw thread were used for the connection of both valves and the y-connector. The assembly was used for the aspiration of air and sample with syringe 1 and inline acidification by propelling hydrochloric acid from syringe 2 at loading of the SPE column with the aspirated sample.



**Figure 23:** Solid phase extraction column used for the pre-concentration of nitrophenols mounted on a six-port injection valve. The column length was 7 mm and 4.5 mm id.

## II Photos of applied devices



**Figure 24: Laboratory-made absorbance detection cell used for capillary electrophoresis. A: Demounted: the capillary is cemented into the linear groove with black fingernail polish. Two small pieces of aluminum foil are used to cover the capillary leaving a small slit of the inner-diameter corresponding width open. The aluminum foil pieces are fixed with black nail polish and covered for mechanical protection with adhesive film. B: Mounted and connected with laboratory-made LED support (left) and optical fiber (right). C: Mounted in the protection case with LED in operation. The falling drop outlet for the high voltage interface is shown as well.**

## II Photos of applied devices

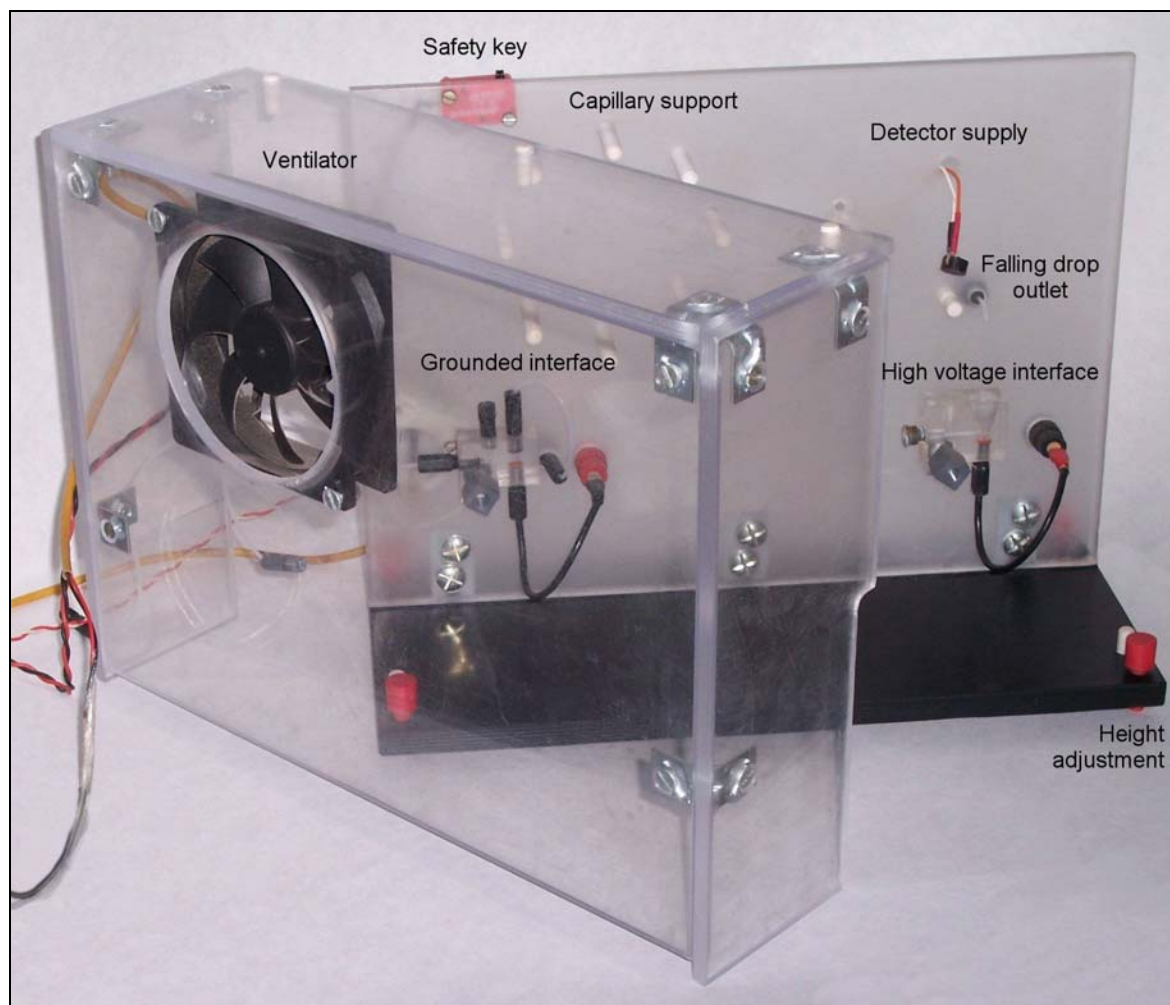


Figure 25: Front view of the open protection case for housing of the capillary electrophoresis system proposed in this work.

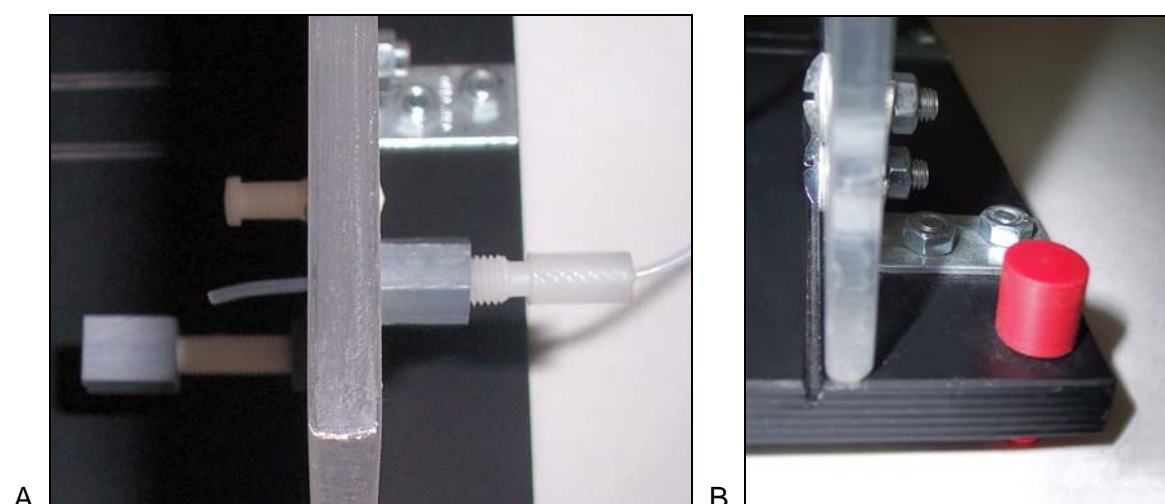


Figure 26: Detail view of A: falling-drop liquid outlet and B: height adjustment of the protection case used for capillary electrophoresis.



## II Photos of applied devices

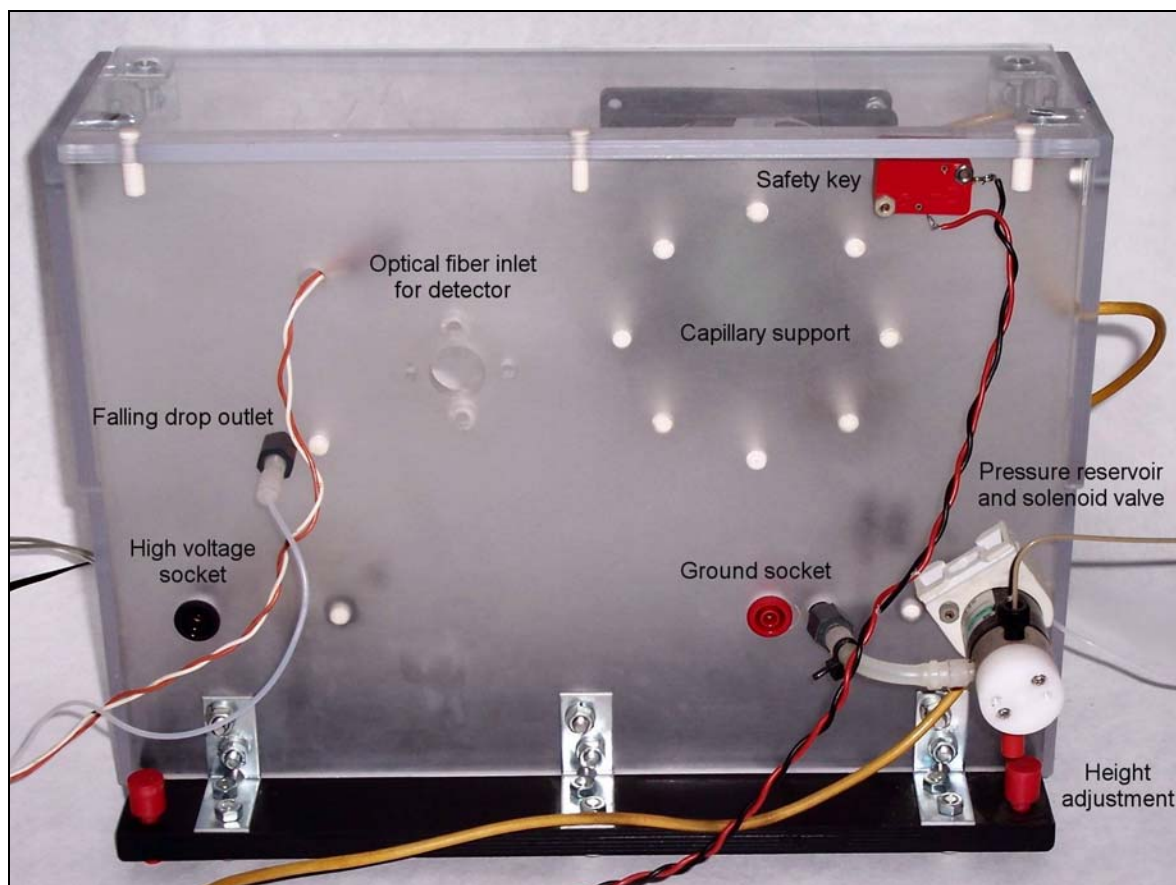


Figure 27: Back view of the closed protection case for housing of the capillary electrophoresis system proposed in this work.

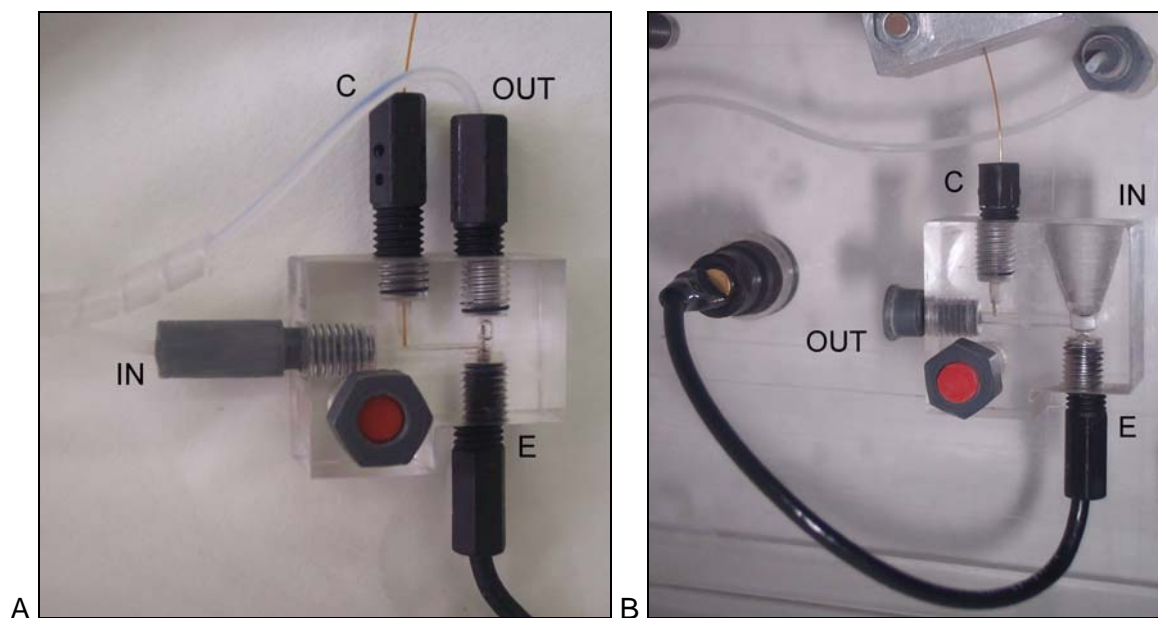
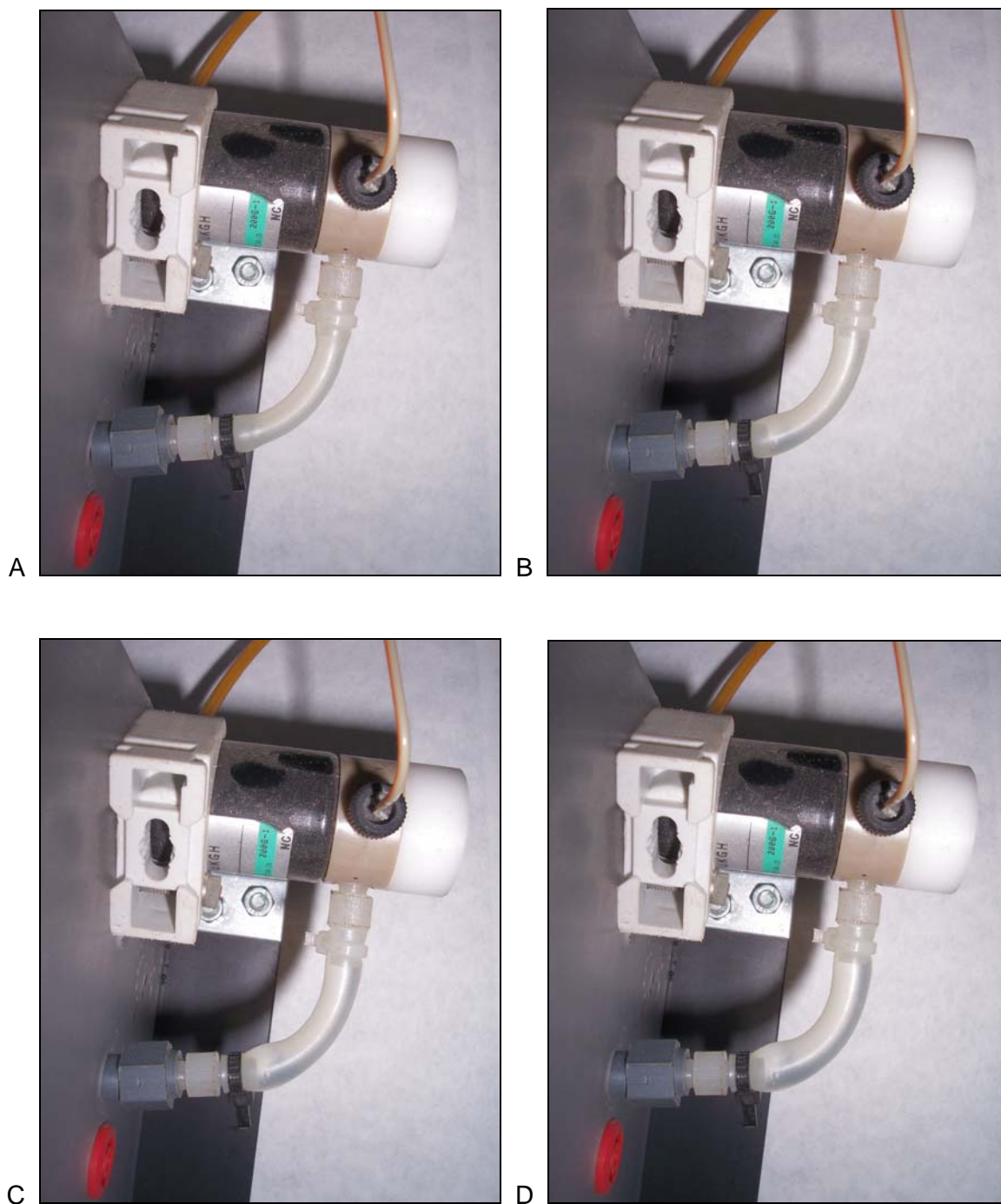


Figure 28: Grounded split-flow (A) and high voltage falling drop (B) interface. Electrodes (E), capillary (C), flow entrance (IN) and outlet (OUT) are indicated.

## II Photos of applied devices



**Figure 29: Silicone tubing used as pressure reservoir for the capillary electrophoresis system placed between the grounded interface outlet and the solenoid valve. A: solenoid valve open (not inflated), B: inflated with 100 µl C: inflated with 200 µl D: inflated with 300 µl.**

## III OPTICAL FIBERS

### Premium Grade Xtreme XSR Solarization-resistant Optical Fiber

Wavelength range:	180-900 nm
Assembly jacketing:	Stainless-steel BX
Used assembly	455 $\mu\text{m}$ core diameter, 1 m in length
Numerical aperture	0.22 $\pm$ 0.02
Half acceptance angle	12.4° in air
Core	Pure fused-silica core, fluorine-doped silica cladding and polyimide coating, step-index multimode
Jacket material	Stainless steel

### Laboratory-grade Patch Cord Optical Fiber

Wavelength range:	250-800 nm
Assembly jacketing:	Polymer
Used assemblies	100 $\mu\text{m}$ , 200 $\mu\text{m}$ and 400 $\mu\text{m}$ core diameter, 2 m in length
Numerical aperture	0.22 $\pm$ 0.02
Half acceptance angle	12.4° in air
Core	Pure fused-silica core, fluorine-doped silica cladding and polyimide coating, step-index multimode
Jacket material	PVDF

### Coding of optical fibers from Ocean Optics

Code: First ring	Core diameter
Purple	8 $\mu\text{m}$
Blue	50 $\mu\text{m}$
Green	100 $\mu\text{m}$
Yellow	200 $\mu\text{m}$
Green	300 $\mu\text{m}$
Red	400 $\mu\text{m}$
Orange	500 $\mu\text{m}$
Brown	600 $\mu\text{m}$
Clear	1.000 $\mu\text{m}$



## IV SPECTRA DATA

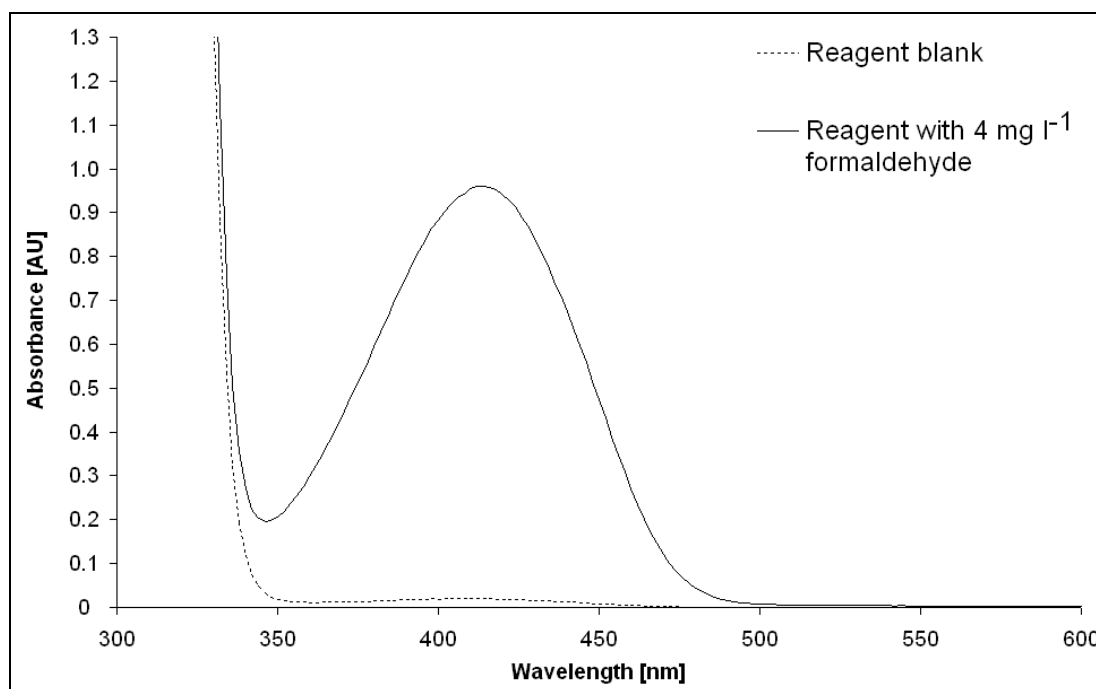


Figure 30: Steady-state absorbance spectra from Nash reagent (1 mol l<sup>-1</sup> NH<sub>4</sub>Ac, 0.3%v/v acetic acid, 0.2%v/v AcAc) with 40 mM potassium phosphate buffer, pH 6.0 and of the reaction product diacetyl-1,4-dihydrolutidine formed after addition of 4 mg l<sup>-1</sup> formaldehyde.

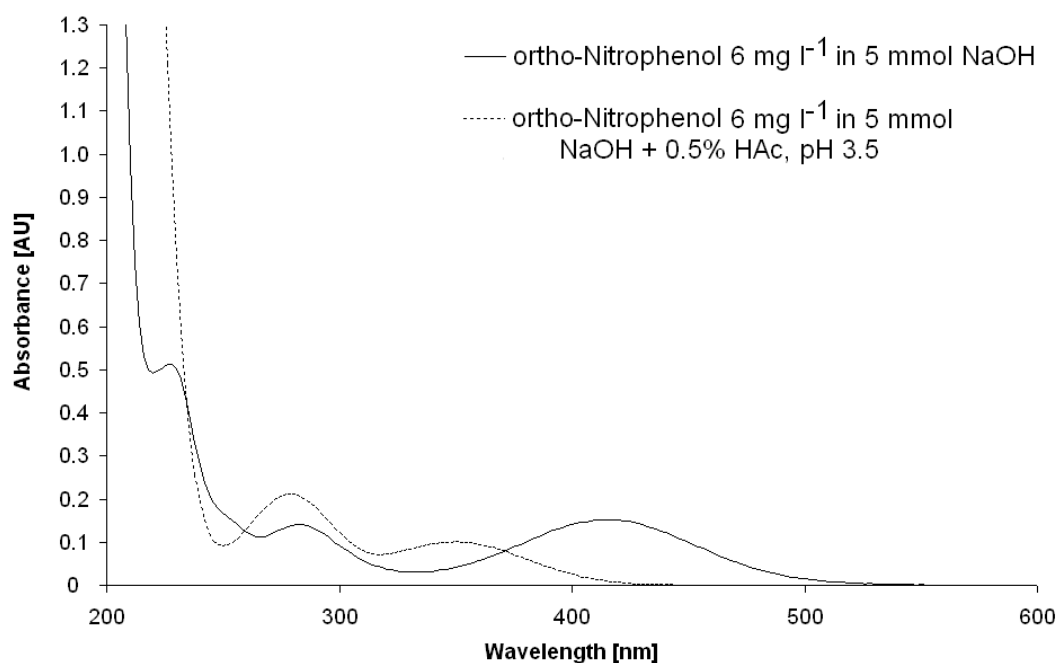
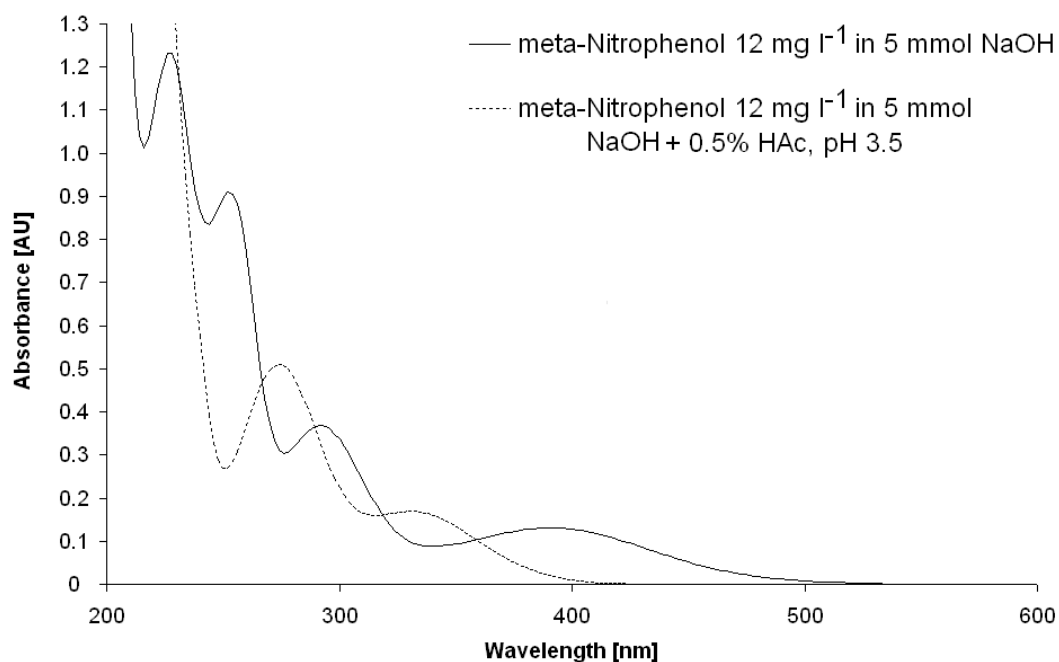
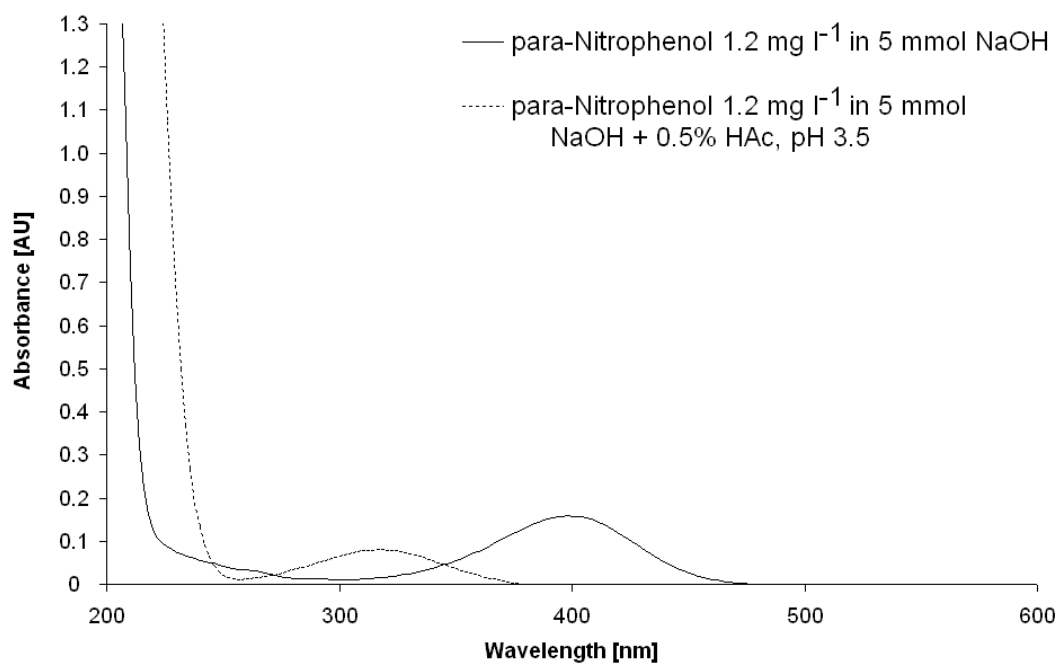


Figure 31: Absorbance spectra of ortho-nitrophenol in basic and moderate acid medium (concentration of ortho-nitrophenol approximately 6 mg l<sup>-1</sup>, it results a molar absorbance of approximately 3270 l mol<sup>-1</sup> cm<sup>-1</sup>).





**Figure 32: Absorbance spectra of meta-nitrophenol in basic and moderate acid medium (concentration of meta-nitrophenol approximately 12 mg l<sup>-1</sup>, it results a molar absorbance of approximately 1480 l mol<sup>-1</sup> cm<sup>-1</sup>).**



**Figure 33: Absorbance spectra of para-nitrophenol in basic and moderate acid medium (concentration of para-nitrophenol approximately 1.2 mg l<sup>-1</sup>, it results a molar absorbance of approximately 18350 l mol<sup>-1</sup> cm<sup>-1</sup>).**

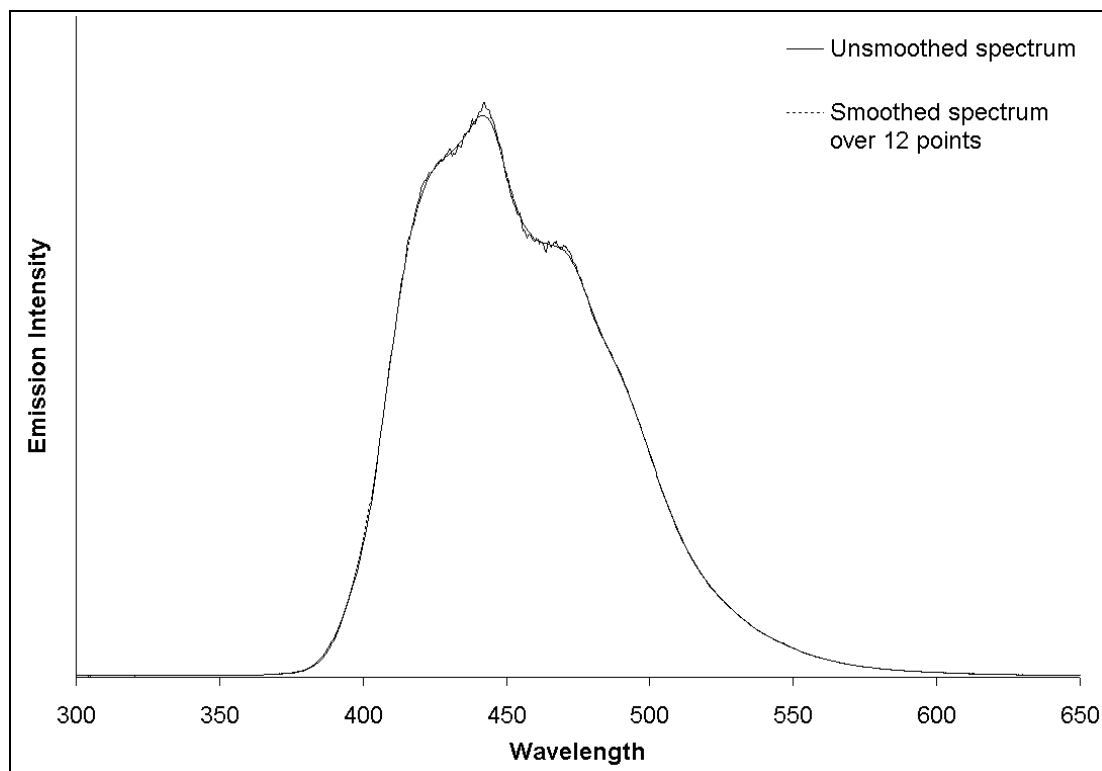


Figure 34: Emission spectrum of blue LED 380 UBC used for polyalcohol analyzer systems.

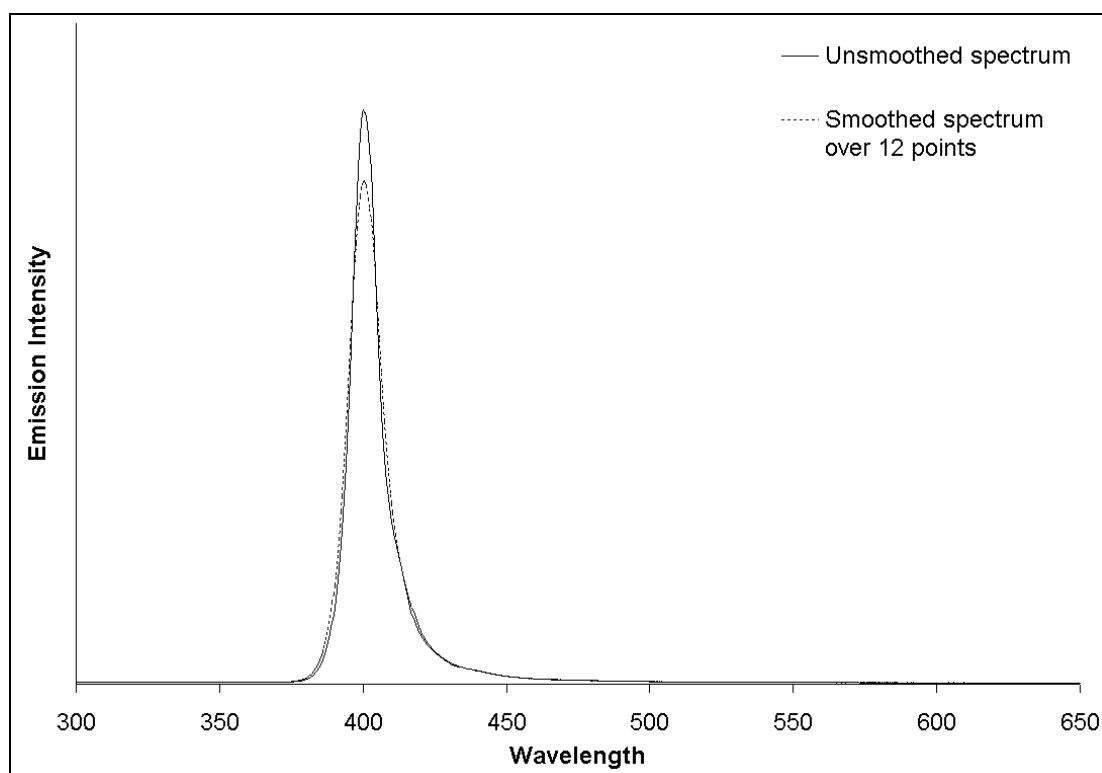


Figure 35: Emission spectrum of blue LED Cu6SMA1 used for CE on-capillary detector



## V ELECTRICAL CIRCUITRIES

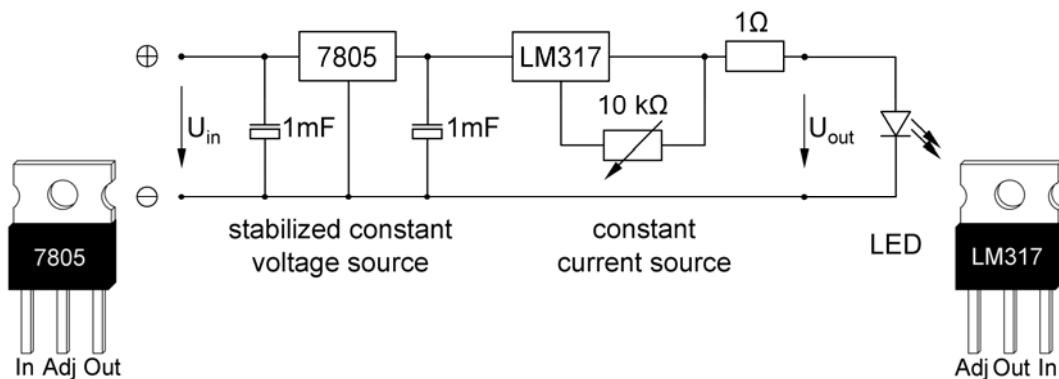



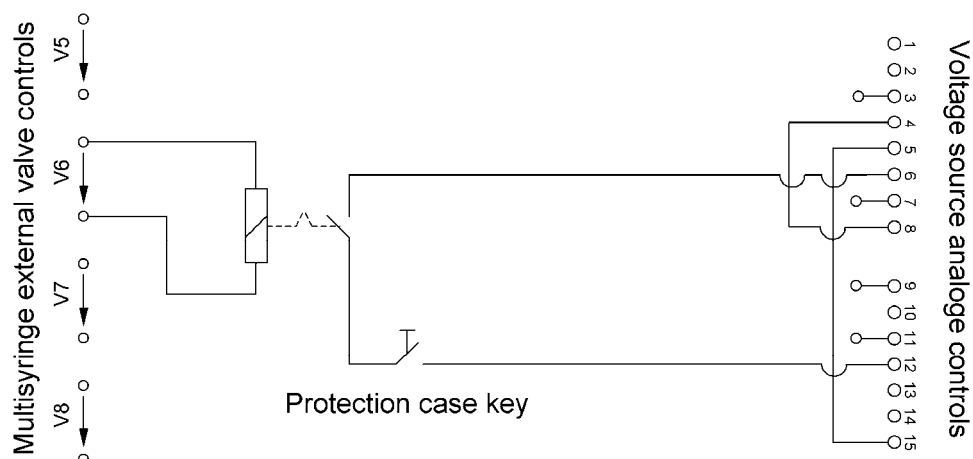
Figure 36: Circuitry of the constant current supply for light emission diode (LED) powering. The output intensity is adjusted by the potentiometer. The input voltage is 9 V (DC).


 View on solder side of connector

Pin Assignment

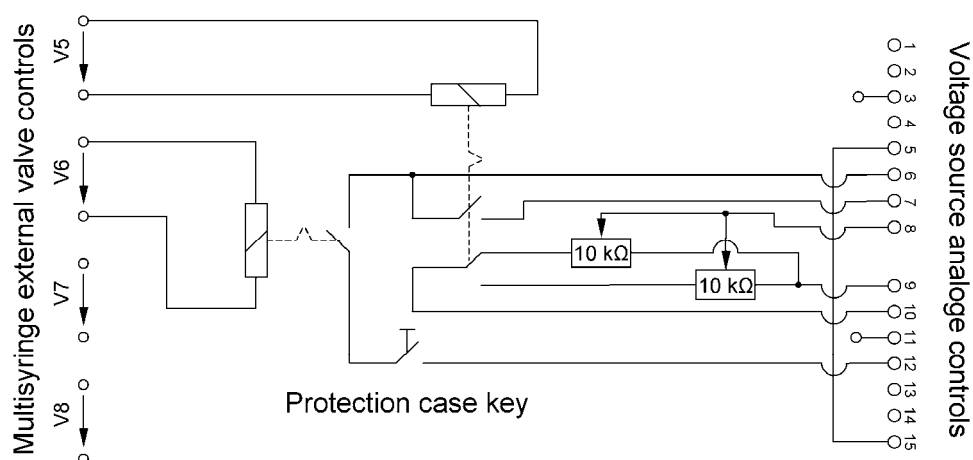
Pin-No.	Name	Type	Function
1	CC	Digital Output	supplies appr. +15V if power supply is in constant current mode. Same as LED CC on front panel.
2	CV	Digital Output	supplies appr. +15V if power supply is in constant voltage mode. Same as LED CV on front panel.
3	I-MON	Analog Output	Actual output current monitor signal. 0..+10V represents 0..nominal current Source impedance appr. 10kOhm at the directly connected analog programming and appr. 2kOhm at the floating analog programming
4	VPS	Analog Output (not connected at option floating analog programming)	Slider of voltage potentiometer on front panel 0..+10V represents 0..nominal voltage Source impedance appr. 10kOhm
5	IPS	Analog Output (not connected at option floating analog programming)	Slider of current potentiometer on front panel 0..+10V represents 0..nominal current Source impedance appr. 10kOhm
6	OVD	Digital Ground	Ground for digital signals
7	POL-SET	digital input	(option) control input for motor driven polarity reversal switch. pin 7 open: positive polarity pin 7 connected to OVD (pin 6) : negative polarity
8	V-SET	Analog Input	0..+10V controls 0..nominal voltage Input Resistance to 0V appr. 10MOhm
9	0V	Analog Ground	Ground for analog signals, must not carry any current
10	+10VREF	Analog Output	+10V precise reference voltage, max. load 3mA
11	V-MON	Analog Output	Actual output voltage monitor signal. 0..+10V represents 0..nominal voltage Source impedance appr. 10kOhm at the directly connected analog programming and appr. 2kOhm at the floating analog programming
12	UNIT-ON	digital input	A connection to Pin 6 (OVD) releases the output voltage Same function as switch OUTPUT on front panel.
13	POL	Digital Output	(option) actual position of the voltage reversal switch appr. +12V : positive polarity 0V: negative polarity:
14	NC	not connected	
15	I-SET	Analog Input	0..+10V controls 0..nominal current Input Resistance to 0V appr. 10MOhm

Figure 37: Extract from the technical data sheet of the HCP 35-35000 high voltage power source: Configuration of the analog interface for remote control.



**Figure 38: Connection scheme for control of the high voltage power source HCP35-35000 by the use of the analogous interface and an external supply of the multisyringe device applied in this work. When the safety contacts 6 and 12 of the analogous source interface are connected, high voltage is applied.**

Software instructed activation of the external supply (12 V) cause the relay to operate and allows remote initiation of the high voltage application. On opening the protection case and interrupting the safety circuit by the corresponding key, high voltage cannot be applied. Connection of pins 5 and 15 and pins 4 and 8 enable the adjustment of the maximal current and maximal voltage by use of the front panel potentiometers, respectively.



**Figure 39: Connection scheme for control of the high voltage power source HCP35-35000 by the use of the analogous interface and external supplies of the multisyringe device allowing electrokinetic on-capillary pre-concentration. The former control scheme is expanded using a second relay for remote polarity reversal switch.**

By a first circuit it either connects or disconnects pin 6 and 7 (negative or positive polarity, respectively). By a second circuit it connects pin 8 (external voltage control) to the sliders of one of two potentiometers between pin 9 (analog ground) and 10 (+10 V reference voltage). By this, different voltage levels can be pre-adjusted for both polarity states.

Likewise before, remote high voltage application is initiated by the safety circuit of pin 6 and pin 12. Connection of pins 5 and enable the adjustment of the maximal current by use of the front panel potentiometer.

## Comandos RS CRISON para BURETTE 1-4S

### 1. Especificaciones

- Frecuencia de transmisión: 2400 bauds.
- Paridad: Par.
- Sincronización: 1 bit Start y 2 bits Stop.
- Longitud palabra: 7 bits.

### 2. Conexión

Conector base CONTROLLER (Telefónico hembra 4 pins):



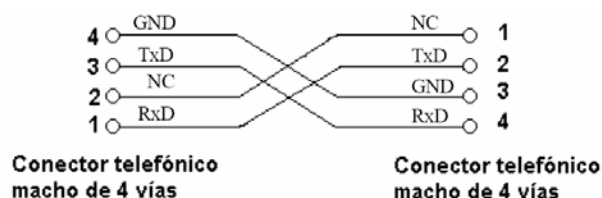
- Pin 1: No se usa (NC).
- Pin 2: Dato de salida (TxD).
- Pin 3: 0 V (GND).
- Pin 4: Dato de entrada (RxD).

Conector base PERIPHERAL (Telefónico hembra 4 pins):

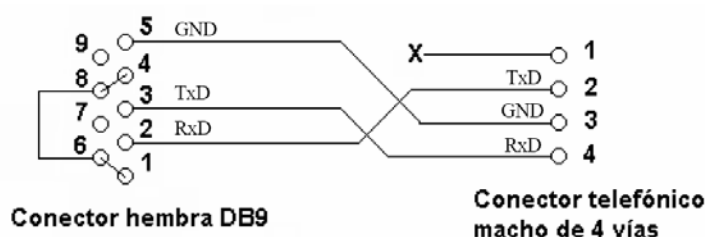


- Pin 1: Dato de entrada (RxD).
- Pin 2: No se usa (NC).
- Pin 3: Dato de salida (TxD).
- Pin 4: 0 V (GND).

Cable de módulo CRISON canal PERIPHERAL a otro módulo CRISON canal CONTROLLER:



Cable de PC(Ordenador) a módulo CRISON canal CONTROLLER:



### 3. Descripción de funcionamiento

La BURETTE 1-4S puede encadenarse a otros 9 módulos en una misma red CRISON, por tanto los comandos que se envíen a través de esta red deberán contener la dirección de destino. El primer carácter que se recibe del comando indica el módulo al cual va dirigido y puede tomar valores entre 0 y 9 siendo cada uno de estos una posible dirección de un módulo dentro de la red.

**Figure 40: Extract from the technical data sheet of the used multisyringe module (Crison instrument S.A.) for remote control by the RS232 interface.**



## VI ENGINEERING DRAWINGS

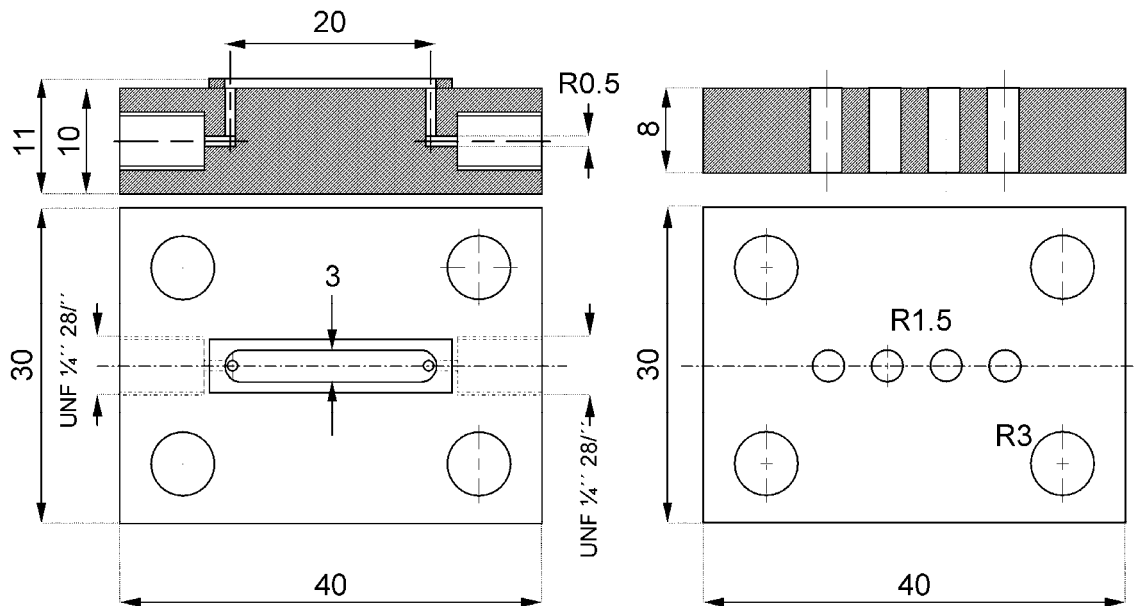


Figure 41: Debubblers cell used during formaldehyde monitoring. The cell was made of PMMA and equipped with a PTFE membrane. For mounting, the both parts of the cell were fixed with screws M6, 40 mm.

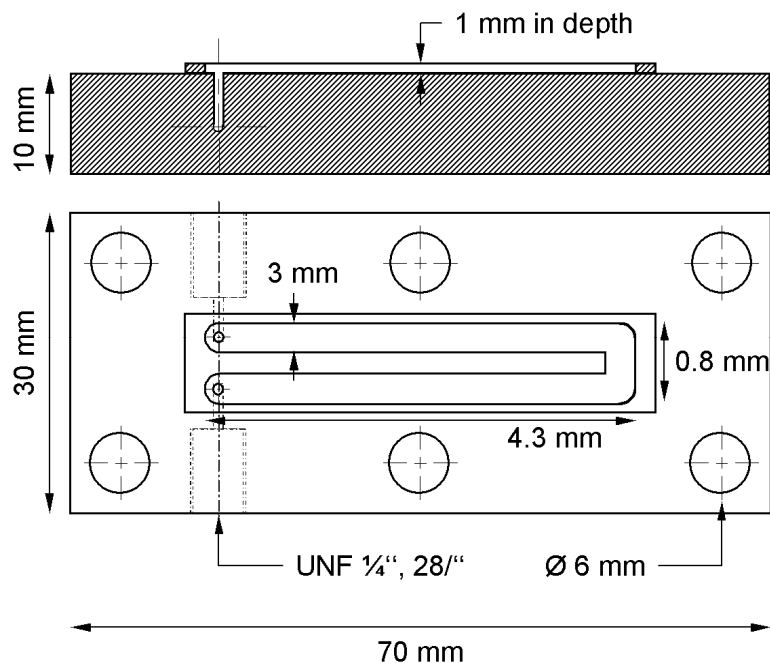


Figure 42: Diffusion cell used for testing gas diffusion of formaldehyde in order to eliminate iron interference. The cell was made of PMMA and equipped with a Durapore® membrane.



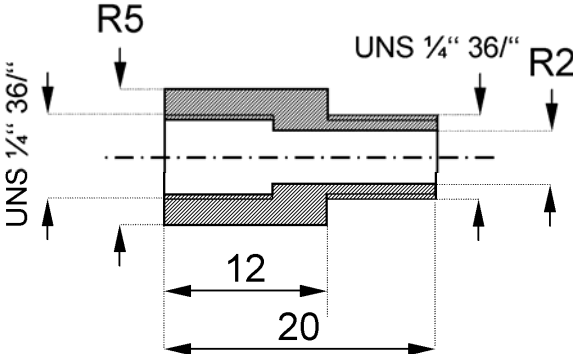


Figure 43: Spacer to be placed between optical fiber and light source to reduce the light intensity at the fiber tip by displacement of 2 cm. The spacer was made of bronze.

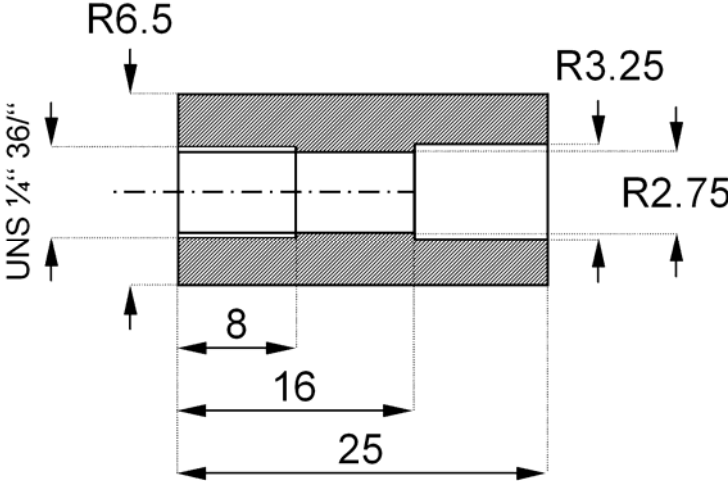


Figure 44: Laboratory-made LED support made of PMMA. The LED is placed into the right side opening, the thread-hole on the left side enables the screwing-in of a thread-tube

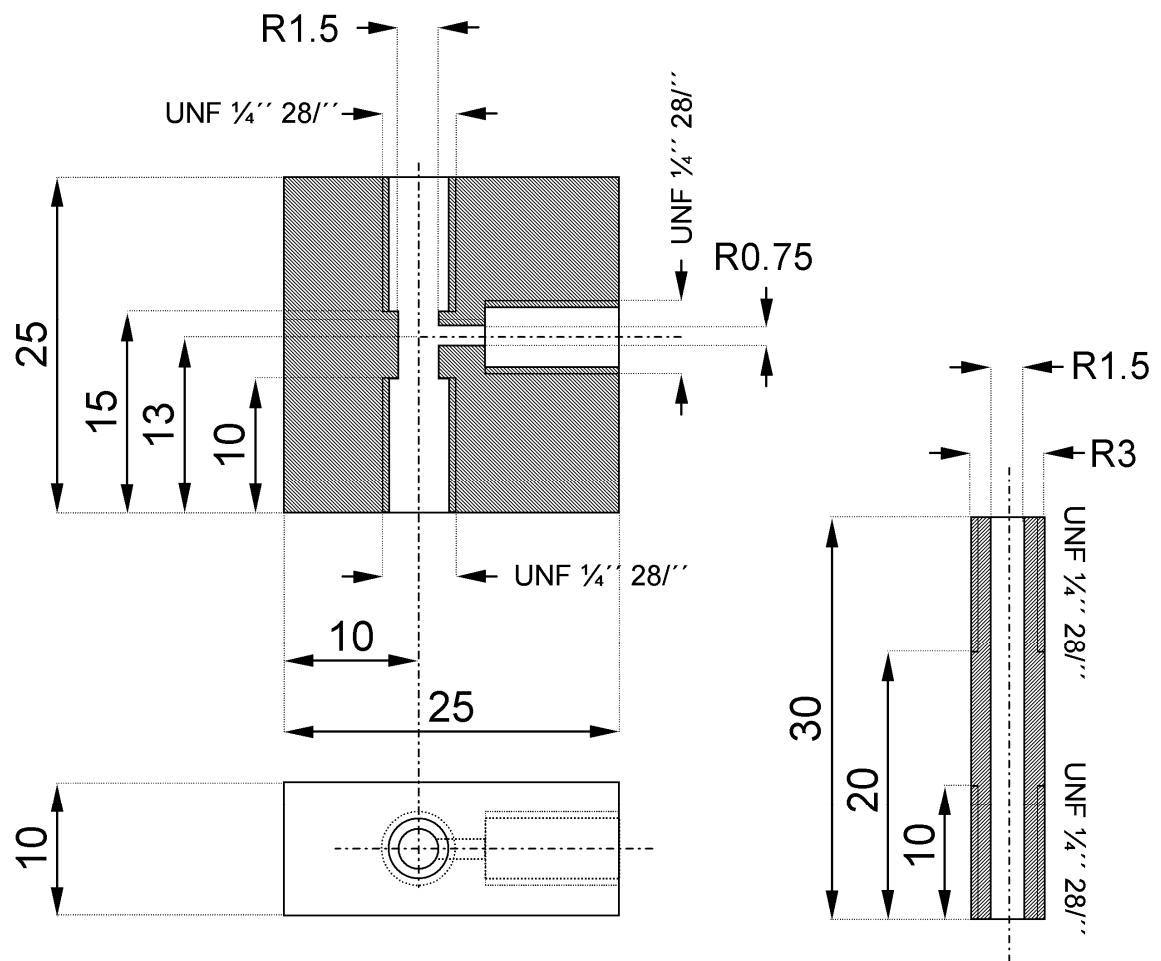


Figure 45: Flow through adapter, made of PMMA (head) and PVC (tube).

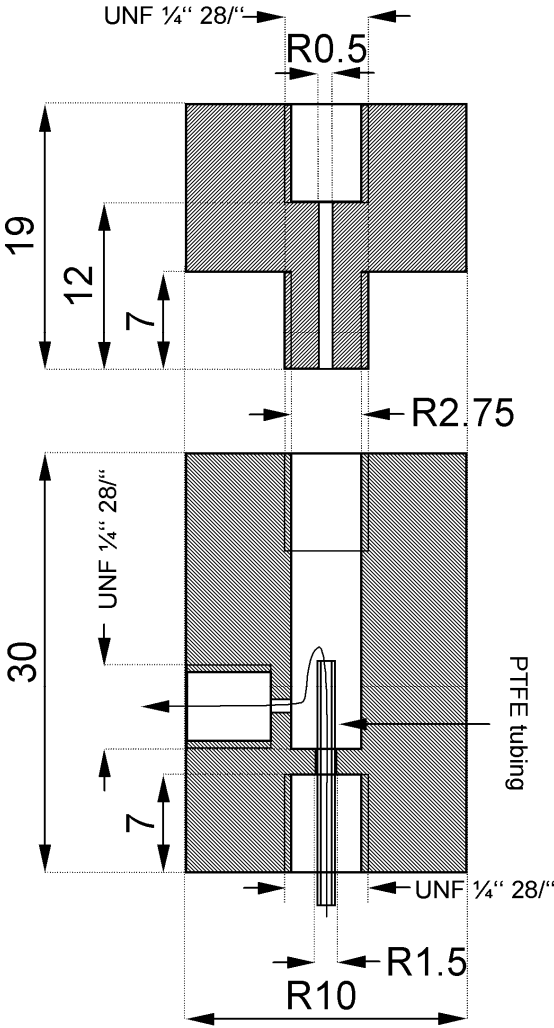
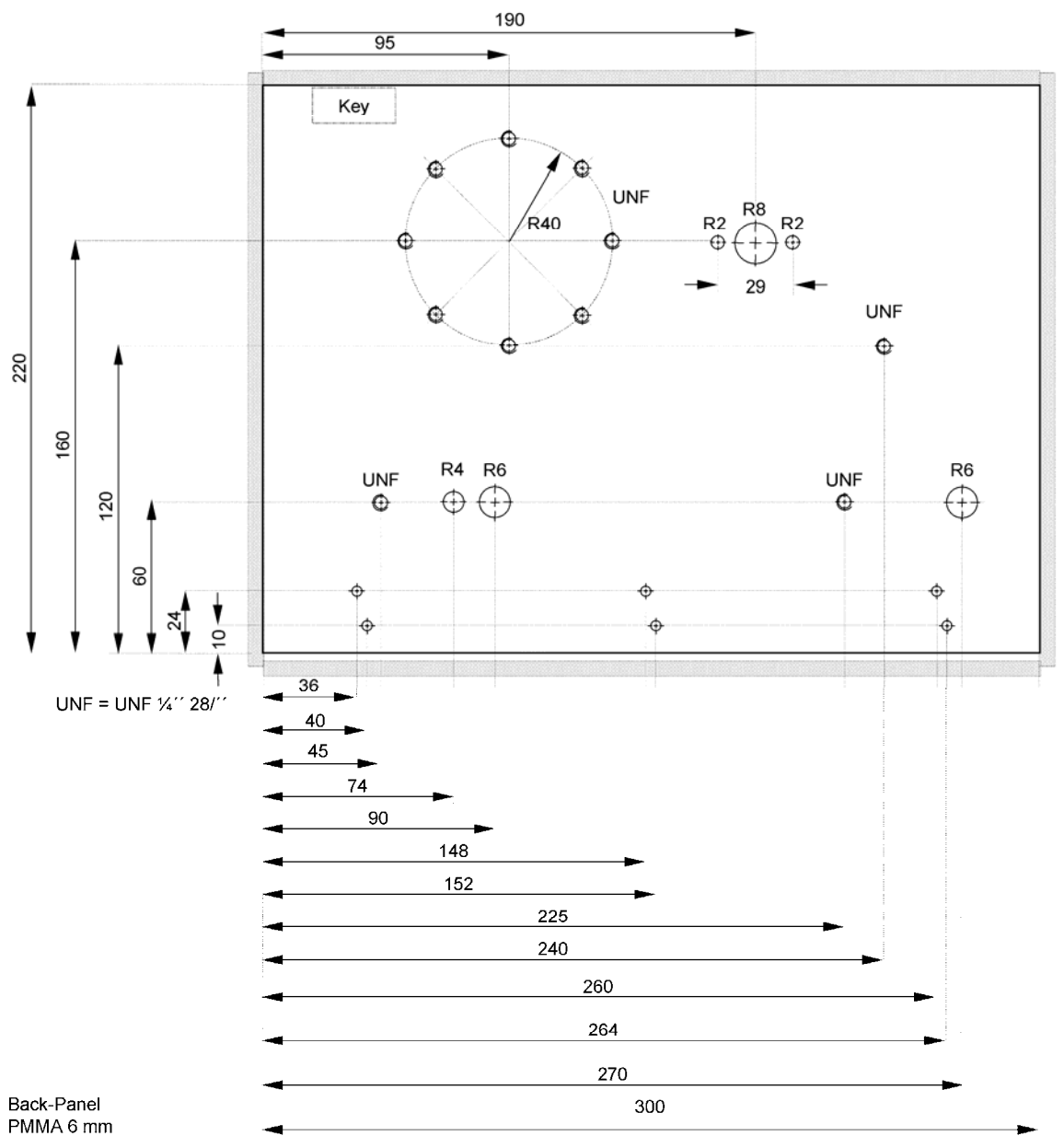


Figure 46: Bubble separator to be used in the sample transport line for monitoring. The bubblers entering from below keep stacked in the head space of the cell and are drawn off from time to time by an additional pump. The sample stream flows out on the left side. For sealing both parts, a rubber O-ring was used.



**Figure 47: Back-panel of the protection case constructed for the housing of the capillary electrophoresis system proposed in this work. The panel was made of PMMA slab (6 mm).**

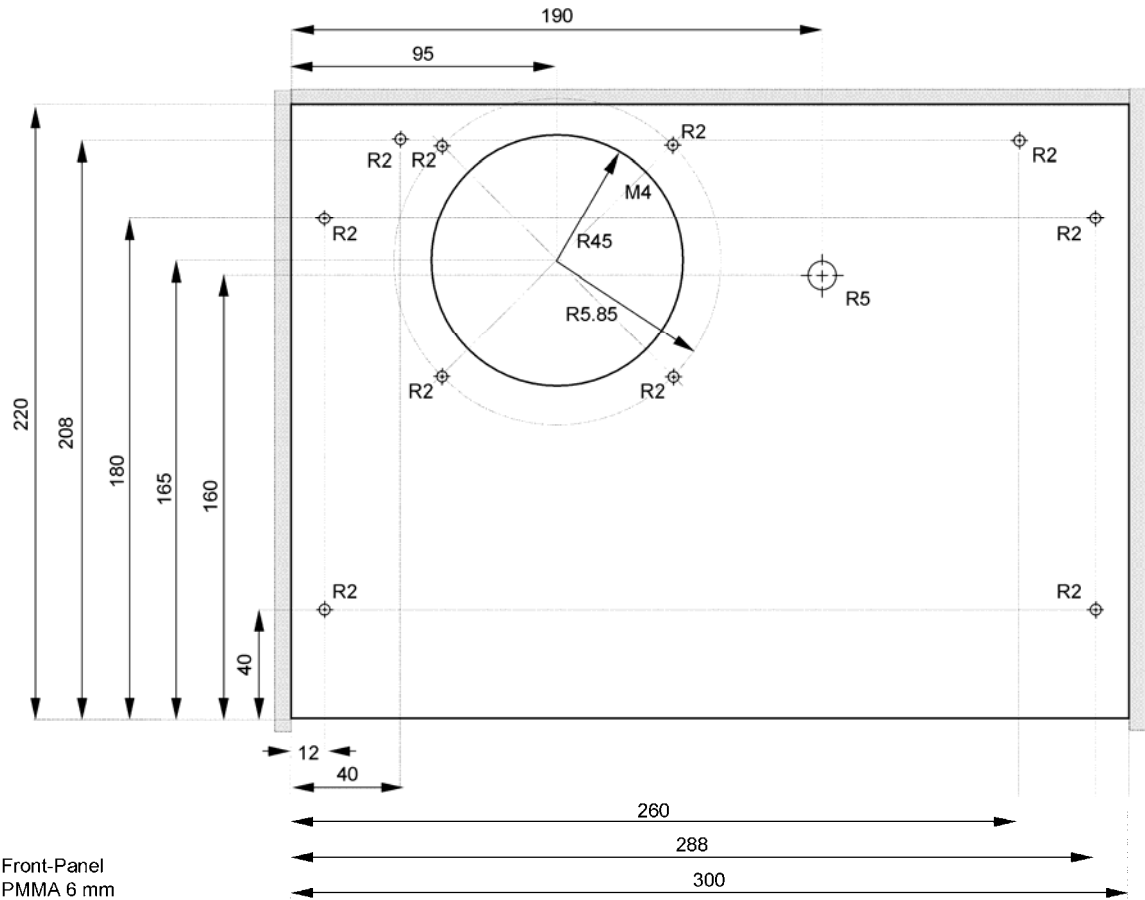


Figure 48: Front-panel of the protection case constructed for the housing of the capillary electrophoresis system proposed in this work. The panel was made of PMMA slab (6 mm).

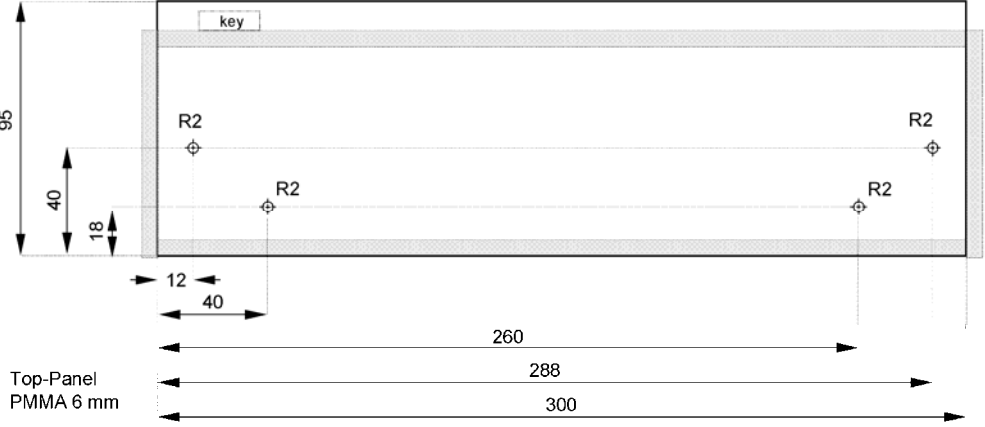
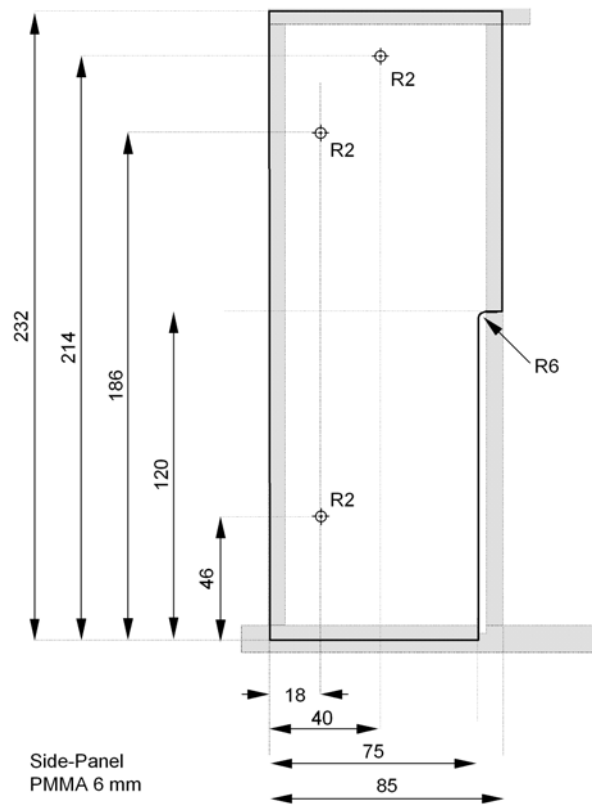
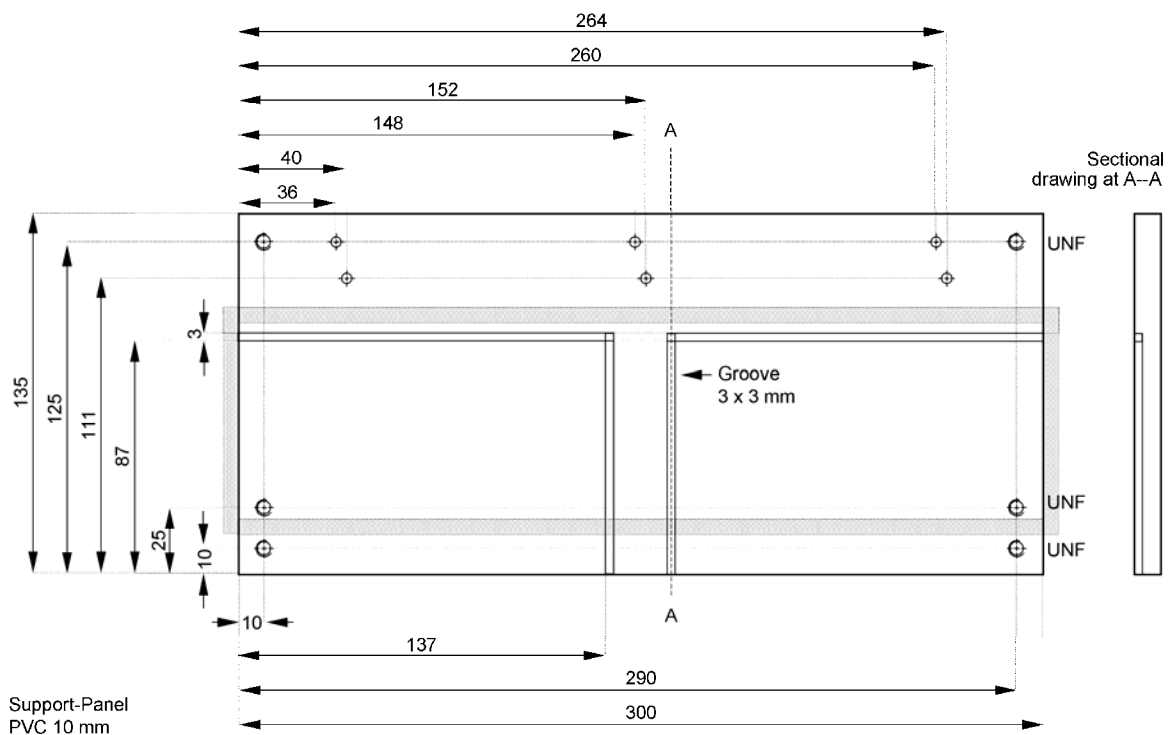


Figure 49: Top-panel of the protection case constructed for the housing of the capillary electrophoresis system proposed in this work. The panel was made of PMMA slab (6 mm).



**Figure 50: Side-panel of the protection case constructed for the housing of the capillary electrophoresis system proposed in this work. The panels were made of PMMA slab (6 mm).**



**Figure 51: Support-panel of the protection case constructed for the housing of the capillary electrophoresis system proposed in this work. The panel was made of PVC slab (10 mm).**

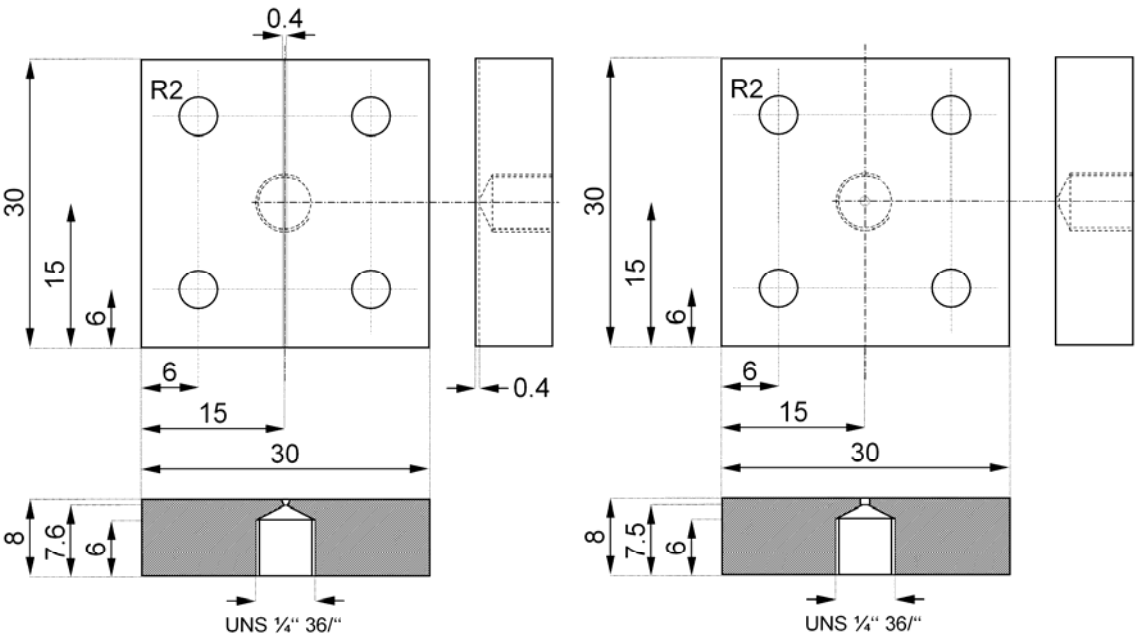


Figure 52: Final design of the laboratory made detector cell for on-capillary photometric detection, right: illumination side, left: detection side. Both parts were made of aluminum.

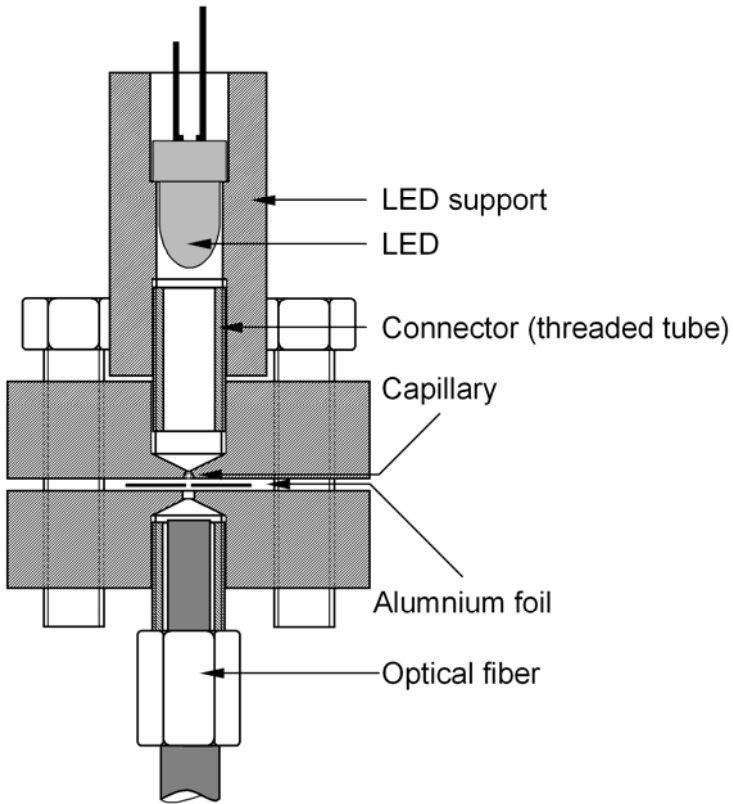


Figure 53: Assembly of the different parts of the on-capillary detection cell.







## VII EXCEL SHEET FOR TUBE CALCULATIONS

A		B		C		D	
1	I.D. [mm]	L. [cm]	T [°C]	dV/dt [ml/min]			
2	0,075	80	20	0,010			
3	0,8	20	25	2,000			
4							
5	V. [µl]	A. [mm <sup>2</sup> ]	p [bar]				
6	3,534	4,42E-03	5,39E+00				
7	100,5	5,03E-01	1,85E-02				
8	=A4*B8*10	=(A4/2)^2*PI()	=(8*C12*B4/100*D4/60*10^-6)/(A4/2*10^-3)^4/10^5				
9	ρ [kg/m <sup>3</sup> ]	Re	η [kg*s/m <sup>2</sup> ]				
10	9,98E+02	1,70E+02	9,99E-04				
11	9,97E+02	3,57E+03	8,89E-04				
12	=0,001*(b3*C4^3+b2*C4^2+b1*C4+b0)	=2*A4/2*10^-3*A13*D4/B8/C12	=0,001*(a3*C4^3+a2*C4^2+a1*C4+a0)				
13	b3=+0,000000004		a3=-0,0000079132999872				
14	b2=-0,0000008		a2=+0,0010340405650525				
15	b1=+0,000006		a1=-0,0565047179318015				
16	b0=+0,9999		a0=+1,77920738745666				

The present Excel sheet was used for the calculation of tube dimensions (cross section area and volume), water density and viscosity at a given temperature (approximated by third order polynomials) and resulting Reynolds number and back-pressure resulting from a given flow rate. The sheet could also be used to estimate the injected volume or resulting flow rate at a given pressure.



## VIII AUTOANALYSIS METHODS AND PROCEDURES

### I.I. General remarks

Here, the main procedures and methods applied in this work are given with comments. Further methods and procedures were made for optimization experiments but were mostly similar to the following printed ones. The volumes of the used syringes are given for each analyzer. For SIA methods only one syringe was employed, thus, only one head valve position is stated. The given volumes and flow rates for the in the multisyringe system are given as calculated for the reference syringe 3. Variables and loop designations are given in quotation marks. Comment lines and abundant information were eliminated and the original designations used by AutoAnalysis were modified for better clearness. Names of user-defined variables are given in quotation marks. Information, which could not be exported by AutoAnalysis were completed, e.g. flow rates used for total dispense or pickup operations of the multisyringe device. In the following, a few examples are given:

Original	Simplified
Valve A move to position 2. Valve B not move.	Valve A move to position 2
Automatic MultiSyringe at Ch: 1, Pos: 0	Multisyringe
Priming in pickup with heads [...]. Exclusive mode.	Priming in pickup at ... ml/min [...] E
Dispense ... ml (Buret 1) at ... mL/min with heads [...]. No exclusive.	Dispense ... ml at ... ml/min [...] NE

### I.II. Formaldehyde analyzer

Syringe sizes were: syringe 1: 2.5 ml, syringe 2 to 4 were not used. Selection valve positions were: 1: waste; 2: water; 3: reagent 1; 4: standard 1; 5: reaction coil and detector; 6: flow-through adaptor (sample); 7: standard 2; 8: standard 3, used for standard addition.

#### Procedure "Clean channel"

Instruction	Specification	Comment
Valve module	Valve A move to "Position"	Rising position defined by variable
Multisyringe	Pickup 0.3 ml, 2 ml/min [1-On] E	Rinsing of tube
Valve module	Valve A move to position 1	Position of waste
Multisyringe	Dispense 1.5 ml, 3.0 ml/min [1-On] E	Discharge of rinse volume
Multisyringe	Pickup 1.2 ml, 5 ml/min [1-Off] E	Filling up Multisyringe

The same procedure was applied similar on all systems in this work.

#### Procedure "Clean reagent channel"

Instruction	Specification	Comment
Valve module	Valve A move to "Reagent position"	Reagent position defined by variable
Multisyringe	Pickup 0.1 ml, 2.0 ml/min [1-On] E	Rinsing of tube
Valve module	Valve A move to position 1	Position of waste
Multisyringe	Dispense 0.3 ml, 3.0 ml/min [1-On] E	Discharge of rinse volume
Multisyringe	Pickup 0.2 ml, 5.0 ml/min [1-Off] E	Refilling of syringes

## VIII Autoanalysis methods and procedures

### Method "Clean system with solutions"

Instruction	Specification	Comment
Valve	Valve A move to position 1.	Clean syringe
Multisyringe	Priming in dispense at 3.000 ml/min [1-On] E	
Multisyringe	Priming in pickup at 5.000 ml/min [1-Off] E	
Multisyringe	Priming in dispense at 3.000 ml/min [1-Off] E	
Multisyringe	Priming in pickup at 5.000 ml/min [1-Off] E	
Multisyringe	Dispense 1.000 ml at 3.000 ml/min [1-On] E	
Variable	Position = 2	Clean all channels (2 – 8)
Procedure	Call "Clean channel"	
▪	▪	
▪	▪	
▪	▪	
Variable	Position = 8	
Procedure	Call "Clean channel"	Filling up of syringe
Multisyringe	Priming in pickup at 5.000 ml/min [1-Off] E	
Valve	Valve A move to position 5.	Flush reaction coil and detector
Multisyringe	Priming in dispense at 3.000 ml/min [1-On] E	
Multisyringe	Priming in pickup at 5.000 ml/min [1-Off] E	Filling up of syringe

The same procedure was applied for both polyalcohol analyzers.

### Procedure "Clean Autosampler channel"

Instruction	Specification	Comment
Valve	Valve A move to position 8.	Autosampler position *
Autosampler	Sampler move to 1 position, needle up. E	Pickup of air segment
Multisyringe	Pickup 0.025 ml at 1.500 ml/min [1-On] E	
Autosampler	Sampler move to 1 position, needle down. E	Clean outside of sampling tip
Multisyringe	Pickup 0.025 ml at 1.500 ml/min [1-On] E	
Autosampler	Sampler move to 2 position, needle up. E	Pickup of air segment
Multisyringe	Pickup 0.025 ml at 1.500 ml/min [1-On] E	
Autosampler	Sampler move to 2 position, needle down. E	Pickup of water
Multisyringe	Pickup 0.025 ml at 1.500 ml/min [1-On] E	
Autosampler	Sampler move to "PosAuto", needle up. E	Pickup of air segment
Multisyringe	Pickup 0.025 ml at 1.500 ml/min [1-On] E	
Autosampler	Sampler move to "PosAuto", needle down. E	Pickup of new sample
Multisyringe	Pickup 0.275 ml at 0.500 ml/min [1-On] E	
Wait	Wait 2 seconds	
Valve	Valve A move to position 1.	Dispense in waste
Multisyringe	Dispense 1.200 ml at 3.000 ml/min [1-On] E	
Valve	Valve A move to position 8.	Pickup of new sample
Multisyringe	Pickup 0.400 ml at 0.500 ml/min [1-On] E	
Wait	Wait 2 seconds	
Valve	Valve A move to position 1.	Dispense in waste
Multisyringe	Pickup 1.600 ml at 3.750 ml/min [1-Off] E	
Multisyringe	Priming in dispense at 3.000 ml/min [1-On] E	
Multisyringe	Pickup 1.300 ml at 3.750 ml/min [1-Off] E	Filling up of syringe

The same procedure was applied similar on all systems either for interference studies, calibration over a wide range or for the optimization of the BGE, beakers on autosampler position 1 and 2 contained water throughout.

\* For optimization of the BGE, the autosampler was connected to syringe head valve position OFF.

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### Procedure "Measurement with reagent"

Instruction	Specification	Comment
Valve module	Valve A move to "Reagent position"	Reagent position defined by variable
Multisyringe	Dispense 0.025 ml, 2.0 ml/min [1-Off] E *	Compare with procedure "Measurement without reagent" *
Multisyringe	Pickup 0.1 ml, 1.5 ml/min [1-Off] E *	To overcome backlash of Multisyringe
Wait	Wait 2 s	
Loop	START: "Sampling"	
Valve module	Valve A move to "Reagent position "	Reagent position defined by variable
Multisyringe	Pickup 0.050 ml, 1.5 ml/min [1-On] E	Pickup of reagent / water for medium blank
Wait	Wait 3 s	
Valve module	Valve A move to "Position "	Valve port position defined by variable
Multisyringe	Pickup 0.040 ml, 1.5 ml/min [1-On] E	Pickup of sample / standard for calibration
Wait	Wait 3 s	
Valve module	Valve A move to "Addition position"	Standard position defined by variable
Multisyringe	Pickup 0.010 ml, 1.5 ml/min [1-On] E	Pickup of water / standard for addition
Wait	Wait 3 s	
Loop	END: Repeat 4 times from "Sampling"	Repetition of sampling sequence for stacking to obtain a better mixture
Variable	"Time1" = Get method execution time	Store method execution time of sampling
Valve module	Valve A move to position 6	
Multisyringe	Dispense 0.9 ml, 2.0 ml/min [1-On] E	Dispense to reaction coil
Multisyringe	Priming in pickup 3.0 ml/min [1-Off] NE	Filling up syringe in background
Wait	Wait 120 s	Incubation time of reaction
Wait	Wait for instrument: "Multisyringe "	Last step was in background
Spectrometer	Measure on range 350 – 550 nm, 1 Hz (A:420 nm, R:500 nm)	
Multisyringe	Dispense 1.550 ml, 2.0 ml/min [1-On] E	Dispense to detector
Spectrometer	Stop measure	
Multisyringe	Pickup 0.250 ml, 3.0 ml/min [1-Off] E	Filling up syringe
Variable	"Time1" = "Time1"/3600000+"Time0"	Calculation of process time in [h] from method execution time at sampling and method starting time
Mark	Mark "Type" on "Spectrometer". Add variable "Time1"	Marks as sample, sample with standard addition, standard, or blank (F, A, S, B) and add the sampling process time value
Mark	Mark " _ ". Add variable "Peak_height"	Write peak height for later data processing

\* The procedure "Measurement without reagent" was identical with the former one "Measurement with reagent" apart from the given second and the third line. Due to the dispense of water into the reagent channel to assure its exclusion, later cleaning of the reagent channel was required. Thus is resulted:

Multisyringe	Dispense 0.025 ml, 2.0 ml/min [1-On] E	Water plug to avoid reagent entrance into the manifold
Valve module	Valve A move to 3. Valve B not move	Position of reagent solution

### Method "Monitoring of formaldehyde"

Instruction	Specification	Comment
Valve module	Valve A move to position 1	Initialize instrument by syringe positioning and cleaning of the manifold line
Valve module	Valve A move to position 5	
Multisyringe	Dispense 1.500 ml (Buret 1) at 3.000 mL/min with heads [1-On]. Exclusive Mode.	Measurement of dark and blank if variable "Temp" was prior set as 1
Variable	"Temp" = 1	
Conditional	Dark and Blank: IF ["Temp" = 1] THEN	

## VIII Autoanalysis methods and procedures

Spectrometer	Get dark current spectrum	
Spectrometer	Get blank spectrum	
Conditional	Dark and Blank: ENDIF	
Variable	"Temp" = 1	
Conditional	Clean of reagent: IF ["Temp" = 1] THEN	
Variable	"Position" = 3	
Comment	Call "Clean channel"	Cleaning of channels of reagent 1 and water if variable "Temp" was prior set as 1
Variable	"Position" = 2	
Comment	Call "Clean channel"	
Conditional	Clean of reagent: ENDIF	
Comment	Blank	
Variable	"Temp" = 1	
Conditional	Blank: IF ["Temp" = 1] THEN	
Variable	"Type" = "S"	
Variable	"Reagent position" = 3	
Variable	"Addition position" = 2	Measurement of blank value of the reagent (water instead of sample) if variable "Temp" was prior set as 1
Variable	"Repetition" = 2	
Variable	"Position" = 2	
Comment	Call "Clean channel"	
Comment	Call "Measurement with reagent"	
Mark	Mark " 0" on Spectrometer.	
Conditional	Blank: ENDIF	
Variable	"Temp" = 1	
Conditional	Calibration: IF ["Temp" = 1] THEN	
Variable	"Type" = "S"	Calibration of with three standards if variable "Temp" was prior set as 1 with a given number of repetitions, marking in the procedure as "S"
Variable	"Repetition" = 3	
Variable	"Position" = 4	
Comment	Call "Clean channel"	
Comment	Call "Measurement with reagent" Measurement with reagent	
Mark	Mark " 1" on Spectrometer.	
Variable	"Position" = 7	
Comment	Call "Measurement with reagent"	
Mark	Mark " 2" on Spectrometer.	
Variable	"Position" = 8	
Comment	Call "Clean channel"	
Comment	Call "Measurement with reagent"	
Mark	Mark " 3" on Spectrometer.	
Conditional	Calibration: ENDIF	
Change variable	"Time0" = User input: "Enter actual process time in hours"	Definition of start-time
Loop	START: Measure with standard addition	
Variable	"Type" = "F"	Measurement of formaldehyde in medium three-times without standard addition (water), marking in the procedure as "F"
Variable	"Reagent position" = 3	
Variable	"Addition position" = 2	
Variable	"Position" = 6	
Variable	"Repetition" = 3	
Procedure	Call "Measurement with reagent"	
Variable	"Position" = 8	Cleaning of channel of the addition standard
Procedure	Call "Clean channel"	

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Variable	Type = "A"	Measurement of formaldehyde in medium one-time with standard addition, marking in the procedure as "A"
Variable	"Addition position" = 8	
Variable	"Repetition" = 1	
Variable	"Position" = 8	
Procedure	Call "Measurement with reagent"	
Variable	Type = "F"	Measurement of formaldehyde in medium three-times without standard addition (water), marking in the procedure as "F"
Variable	"Addition position" = 2	
Variable	"Position" = 6	
Variable	"Repetition" = 3	
Procedure	Call "Measurement with reagent"	
Variable	Type = "S"	Measurement of standard used for addition one-time, marking in the procedure as "S"
Variable	"Position" = 8	
Variable	"Repetition" = 1	
Procedure	Call "Measurement with reagent"	
Variable	Type = "F"	Measurement of formaldehyde in medium three-times without standard addition, marking in the procedure as "F"
Variable	"Position" = 6	
Variable	"Repetition" = 3	
Procedure	Call "Measurement with reagent"	
Variable	Type = "B"	Measurement of medium blank value one-time, marking in the procedure as "B"
Variable	"Reagent position" = 2	
Variable	"Repetition" = 1	
Procedure	Call "Measurement without reagent"	
Procedure	"Clean reagent channel"	Cleaning of reagent channel from the introduced water slug
Loop	END: Repeat 10 times from "Measure with standard addition"	
Loop	START: Measure without standard addition	
Variable	Type = "F"	Measurement of formaldehyde in medium two-times without standard addition (water), marking in the procedure as "F"
Variable	"Position" = 6	
Variable	"Repetition" = 2	
Variable	"Reagent position" = 3	
Variable	"Addition position" = 2	
Procedure	Call "Measurement with reagent"	
Variable	Type = "B"	Measurement of medium blank value one-time, marking in the procedure as "B"
Variable	"Repetition" = 1	
Variable	"Reagent position" = 2	
Procedure	Call "Measurement without reagent"	
Procedure	Call "Clean reagent channel"	Cleaning of reagent channel from the introduced water slug
Loop	END: Repeat 200 times from "Measure without standard addition"	



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### I.III. Glycerol analyzer

Syringe sizes were: syringe 1: 2.5 ml, syringe 2 to 4 were not used. Selection valve positions were: 1: waste; 2: water; 3: reagent 1; 4: reagent 2; 5: reaction coil and detector; 6: flow-through adaptor (sample); 7: high concentration control standard; 8: low concentration control standard.

#### Procedure "Monitoring low glycerol concentrations"

Instruction	Specification	Comment
Variable	"Mean_peak_height" = 0	Reset of variable for "Mean_peak_height"
Loop	START: Measure	
Valve	Valve A move to "Position".	Valve port position defined by variable
Multisyringe	Pickup 0.020 ml at 2.000 ml/min [1-On] E	Clean first section of the sample channel
Valve	Valve A move to position 1.	
Multisyringe	Dispense 0.120 ml at 3.000 ml/min [1-On] E	
Wait	Wait 2 seconds	
Multisyringe	Pickup 0.100 ml at 3.000 ml/min [1-Off] E	
Wait	Wait 2 seconds	
Valve	Valve A move to "Position".	Valve port position defined by variable
Multisyringe	Pickup 0.025 ml at 1.200 ml/min [1-On] E	Pickup of sample / standard for calibration
Variable	"Time1" = Get method execution time	Store method execution time of sampling
Wait	Wait 2 seconds	
Valve	Valve A move to position 2.	Position of water
Multisyringe	Pickup 0.040 ml at 1.200 ml/min [1-On] E	Pickup of water for dilution
Wait	Wait 2 seconds	
Valve	Valve A move to position 3.	Position of reagent 1
Multisyringe	Pickup 0.100 ml at 1.200 ml/min [1-On] E	Pickup of reagent 1
Wait	Wait 2 seconds	
Valve	Valve A move to position 4.	Position of reagent 2
Multisyringe	Pickup 0.040 ml at 1.200 ml/min [1-On] E	Pickup of reagent 2
Wait	Wait 2 seconds	
Valve	Valve A move to position 5.	Position of reaction coil
Multisyringe	Dispense 0.800 ml at 2.000 ml/min [1-On] E	Dispense to reaction coil
Multisyringe	Priming in pickup at 3.750 ml/min [1-Off] NE	Filling up syringe in background
Wait	Wait 80 seconds	Incubation time of reaction
Wait	Wait for instrument: "Multisyringe"	Last step was in background
Spectrometer	Absorbance: Spectral range 400 nm – 550 nm every 0.5 seconds (A:420 nm, R:460 nm)	Initiate measurement
Data process	Start data processing on "Spectrometer"	Incubation data processing
Multisyringe	Priming in dispense at 2.000 ml/min [1-On] E	Dispense to detector
Data process	Finish data processing on "Spectrometer". "Peak_height" = Peak maximum	Stop of data processing, highest acquired value is stored in variable "Peak_height"
Spectrometer	Stop measure	Stop of measurement
Variable	"Mean_peak_height" = "Mean_peak_height"+"Peak_height"	Summation for mean calculation
Variable	"Time1" = "Time1"/3600000+"Time0"	Calculation of process time in [h] from method execution time at sampling and method starting time
Mark	Mark "L" on "Spectrometer". Add variable "Time1"	Marks as "carried out with procedure low"
Mark	Mark " _ ". Add variable "Peak_height"	Write peak height for later data processing
Multisyringe	Pickup 1.300 ml at 3.5 ml/min [1-Off] E	Filling up syringe in background
Loop	END: Repeat "Repetition" times from Measure	Repetition
Variable	"Mean_peak_height" = "Mean_peak_height"/"Repetition"	Mean calculation for smart decisions

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### Procedure "Monitoring high glycerol concentrations"

Instruction	Specification	Comment
Variable	Mean_peak_height = 0	Reset of variable for "Mean_peak_height"
Loop	START: Measure	
Valve	Valve A move to "Position".	Valve port position defined by variable
Multisyringe	Pickup 0.020 ml at 2.000 ml/min [1-On] E	Clean first section of the sample channel
Valve	Valve A move to position 1.	
Multisyringe	Dispense 0.120 ml at 3.000 ml/min [1-On] E	
Wait	Wait 2 seconds	
Multisyringe	Pickup 0.100 ml at 3.000 ml/min [1-Off] E	
Wait	Wait 2 seconds	
Valve	Valve A move to "Position".	Valve port position defined by variable
Multisyringe	Pickup 0.030 ml at 1.200 ml/min [1-On] E	Pickup of sample / standard for calibration
Variable	Time1 = Get method execution time	Store method execution time of sampling
Wait	Wait 2 seconds	
Multisyringe	Dispense 0.030 ml at 1.200 ml/min [1-Off] E	To overcome backlash of the multisyringe
Wait	Wait 2 seconds	
Valve	Valve A move to position 1.	Position of waste
Multisyringe	Dispense 0.030 ml at 1.200 ml/min [1-On] E	Dispense of most prior aspirated sample to waste
Wait	Wait 2 seconds	
Valve	Valve A move to position 2.	Position of water
Multisyringe	Pickup 0.300 ml at 1.200 ml/min [1-On] E	Pickup of water for dilution
Wait	Wait 2 seconds	
Valve	Valve A move to position 3.	Position of reagent 1
Multisyringe	Pickup 0.100 ml at 1.200 ml/min [1-On] E	Pickup of reagent 1
Wait	Wait 2 seconds	
Valve	Valve A move to position 4.	Position of reagent 2
Multisyringe	Pickup 0.080 ml at 1.200 ml/min [1-On] E	Pickup of reagent 2
Wait	Wait 2 seconds	
Valve	Valve A move to position 5.	Position of reaction coil
Multisyringe	Dispense 0.850 ml at 2.000 ml/min [1-On] E	Dispense to reaction coil
Multisyringe	Priming in pickup at 3.750 ml/min [1-Off] NE	Filling up syringe in background
Wait	Wait 80 seconds	Incubation time of reaction
Wait	Wait for instrument: "Multisyringe"	Last step was in background
Spectrometer	Absorbance: Spectral range 400 nm – 550 nm every 0.5 seconds (A:420 nm, R:460 nm)	Initiate measurement
Data process	Start data processing on "Spectrometer"	Incubation data processing
Multisyringe	Priming in dispense at 2.000 ml/min [1-On] E	Dispense to detector
Data process	Finish data processing on "Spectrometer". "Peak_height" = Peak maximum	Stop of data processing, highest acquired value is stored in variable "Peak_height"
Spectrometer	Stop measure	Stop of measurement
Variable	"Mean_peak_height" = "Mean_peak_height"+"Peak_height"	Summation for mean calculation
Variable	"Time1" = "Time1"/3600000+"Time0"	Calculation of process time in [h] from method execution time at sampling and method starting time
Mark	Mark "H" on Spectrometer. Add variable "Time1"	Marks as "carried out with procedure high"
Mark	Mark " _". Add variable "Peak_height"	Write peak height for later data processing
Multisyringe	Pickup 1.300 ml at 3.5 ml/min [1-Off] E	Filling up syringe in background
Loop	END: Repeat "Repetition" times from Measure	Repetition
Variable	"Mean_peak_height" = "Mean_peak_height"/"Repetition"	Mean calculation for smart decisions

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### Method "Monitoring of glycerol"

Instruction	Specification	Comment
Valve	Valve A move to position 1.	Initialize instrument
Valve	Valve A move to position 5.	
Multisyringe	Dispense 1.500 ml at 2.000 ml/min [1-On] E	
Wait	Wait for user input: "Measure dark and blank?" Button Yes & No. Store in "Temp"	Measurement of dark and blank on user request
Conditional	Measure dark and blank: IF ["Temp" = 1] THEN	
Spectrometer	Get dark current spectrum	
Spectrometer	Get blank spectrum	
Conditional	Measure dark and blank: ENDIF	
Wait	Wait for user input: "Clean reagent channels?" Button Yes & No. Store in "Temp"	Cleaning of channels of reagent 1, reagent 2 and water on user request
Conditional	Clean reagent channels: IF ["Temp" = 1] THEN	
Variable	"Position" = 3	
Procedure	Call "Clean channel"	
Variable	"Position" = 4	
Procedure	Call "Clean channel"	
Variable	"Position" = 2	
Procedure	Call "Clean channel"	
Conditional	Clean reagent channels: ENDIF	
Wait	Wait for user input: "Measure reagent blank?" Button Yes & No. Store in "Temp"	Measurement of blank value of the reagent (water instead of sample) on user request and with the requested repetition of determination
Conditional	Measure reagent blank: IF ["Temp" = 1] THEN	
Variable	"Repetition" = User input: "How many repetitions (1, 2 or 3)?"	
Variable	"Position" = 2	
Procedure	Call "Clean channel"	
Procedure	Call "Monitoring high glycerol concentrations "	
Mark	Mark " _B" on Spectrometer	
Procedure	Call "Monitoring low glycerol concentrations "	
Mark	Mark " _B" on Spectrometer	
Conditional	Measure reagent blank: ENDIF	
Wait	Wait for user input: "Calibration for working range 2 - 40 g/l? (4 Standards)" Button Yes & No. Store in "Temp"	Calibration of the high working range with four standards on user request and with the demanded repetition of determination. Mark of each peak group with "St" and number. Process time and procedure type is marked within the corresponding procedure
Conditional	Calibration for high working range: IF ["Temp" = 1] THEN	
Variable	Repetition = User input: "How many repetitions (1, 2 or 3)?"	
Variable	"Position" = 7	
Wait	Wait for user input: "Place standard 1 at position 7" Normal button	
Procedure	Call "Clean channel"	
Procedure	Call "Monitoring high glycerol concentrations"	
Mark	Mark " _St1" on Spectrometer	
.	.	
.	.	
.	.	
Wait	Wait for user input: "Place standard 4 at position 7" Normal button	
Procedure	Call "Clean channel"	
Procedure	Call "Monitoring high glycerol concentrations"	
Mark	Mark " _St4" on Spectrometer	
Conditional	Calibration for high working range: ENDIF	

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Wait	Wait for user input: " Calibration for working range 0.2 - 4 g/l? (4 Standards)" Button Yes & No. Store in "Temp"	
Conditional	Calibration for low working range: IF ["Temp" = 1] THEN	
Variable	Repetition = User input: "How many repetitions (1, 2 or 3)?"	
Variable	Position = 8	
Wait	Wait for user input: "Place standard 1 at position 8" Normal button	
Procedure	Call "Clean channel"	
Procedure	Call "Monitoring low glycerol concentrations"	
Mark	Mark " _St1" on Spectrometer	
	<ul style="list-style-type: none"> <li>▪</li> <li>▪</li> <li>▪</li> </ul>	<ul style="list-style-type: none"> <li>▪</li> <li>▪</li> <li>▪</li> </ul>
Wait	Wait for user input: "Place standard 4 at position 8" Normal button	
Procedure	Call "Clean channel"	
Procedure	Call "Monitoring low glycerol concentrations"	
Mark	Mark " _St4" on Spectrometer	
Conditional	Calibration for low working range: ENDIF	
Wait	Wait for user input: "Place control standards at position 7 and position 8" Button OK	
Wait	Wait for user input: "Clean control standards?" Button Yes & No. Store in "Temp"	
Conditional	Clean control standards: IF ["Temp" = 1] THEN	
Variable	"Position" = 7	
Procedure	Call "Clean channel"	
Variable	"Position" = 8	
Procedure	Call "Clean channel"	
Conditional	Clean control standards: ENDIF	
Variable	"Time0" = User input: "Enter actual process time in hours"	Store measure execution time of monitoring start
Variable	"Mean_peak_height" = 2	Reset of variable for mean peak height
Loop	START: Monitoring	Monitoring start
Loop	START: Filtrate	
Variable	"Repetition" = 2	
Variable	"Position" = 6	
Conditional	Condition 1: IF ["Mean_peak_height" >= 2.00] THEN	
Procedure	Call "Monitoring high glycerol concentrations"	
Conditional	Condition 2: IF ["Mean_peak_height" >= 0.15] THEN	
Variable	"Mean_peak_height" = 2	
Conditional	Condition 2: ENDIF	
Conditional	Condition 1: ELSE	
Procedure	Call "Monitoring low glycerol concentrations"	
Conditional	Condition 3: IF ["Mean_peak_height" >= 1.10] THEN	
Variable	"Mean_peak_height" = 2	
Conditional	Condition 3: ENDIF	
Conditional	Condition 1: ENDIF	
Loop	END: Repeat 5 times from Filtrate	
Variable	"Repetition" = 1	
Conditional	Condition 4: IF ["Mean_peak_height" >= 2.00] THEN	
Variable	"Position" = 7	
Procedure	Call "Clean channel"	
Procedure	Call "Monitoring high glycerol concentrations"	
Variable	"Mean_peak_height" = 2	

Calibration of the low working range with four standards on user request and with the demanded repetition of determination.  
Mark of each peak group with "St" and number.  
Process time and procedure type is marked within the corresponding procedure

Cleaning of control standards for monitoring on user request

Store measure execution time of monitoring start

Reset of variable for mean peak height

Monitoring start

Monitoring of sample on valve position 6 (medium filtrate flow through flow-through adaptor)

The mean peak height is used to decide, which procedure corresponding to one working range is to be carried out.

Process time and procedure type is marked within the corresponding procedure

Single measuring of the control standard after each 10<sup>th</sup> sample measurement of the actual working range.  
Mark of each peak with "St". Process time and

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Mark	Mark " _St" on Spectrometer	procedure type is marked within the corresponding procedure
Conditional	Condition 4: ELSE	
Variable	"Position" = 8	
Procedure	Call "Clean channel"	
Procedure	Call "Monitoring low glycerol concentrations"	
Variable	"Mean_peak_height" = 0.1	
Mark	Mark " _St" on Spectrometer	
Conditional	Condition 4: ENDIF	
Loop	END: Repeat 800 times from Monitoring	Repetition of monitoring

### I.IV. Sorbitol analyzer

Syringe sizes were: syringe 1: 2.5 ml, syringe 2 to 4 were not used. Selection valve positions were: 1: waste; 2: water; 3: reagent 1; 4: reagent 2; 5: reaction coil and detector; 6: flow-through adaptor (sample); 7: high concentration control standard; 8: low concentration control standard.

For sorbitol monitoring, an additional solenoid valve for the syringe-head-close aspiration of acetylacetone was incorporated, controlled with output "5".

#### Procedure "Remove air-bubbles in HC and in OFF"

Instruction	Specification	Comment
Multisyringe	Pickup 2.100 ml at 5.000 ml/min [1-Off 5-Off] E	Filling up syringe
Multisyringe	Pickup 0.300 ml at 1.500 ml/min [1-On 5-On] E	Aspiration of acetonitrile at syringe head
Multisyringe	Priming in dispense [1-On 5-Off] E	Dispense the acetonitrile slug to waste to remove stacked air-bubbles
Multisyringe	Priming in pickup [1-Off 5-Off] E	Filling up syringe
Multisyringe	Priming in dispense [1-On 5-Off] E	Dispense to waste

## VIII Autoanalysis methods and procedures

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### Procedure "Monitoring of low sorbitol concentrations"

All steps were principally the same as in the corresponding "Monitoring of low glycerol concentrations". Therefore, the comment column is omitted. The particular flow rates, times, wavelengths, and volumes were modified in order to adapt the procedure to the analyte sorbitol and different working and to achieve higher frequency of determination.

Instruction	Specification
Loop	START: Measure
Valve	Valve A move to position 8.
Multisyringe	Pickup 0.020 ml at 2.000 ml/min [1-On 5-Off] E
Valve	Valve A move to position 1.
Multisyringe	Dispense 0.120 ml at 3.000 ml/min [1-On 5-Off] E
Multisyringe	Pickup 0.100 ml at 3.000 ml/min [1-Off 5-Off] E
Wait	Wait 1 seconds
Valve	Valve A move to "Position".
Multisyringe	Pickup 0.025 ml at 1.200 ml/min [1-On 5-Off] E
Wait	Wait 1 seconds
Valve	Valve A move to position 2.
Multisyringe	Pickup 0.025 ml at 1.500 ml/min [1-On 5-Off] E
Wait	Wait 1 seconds
Valve	Valve A move to position 3.
Multisyringe	Pickup 0.160 ml at 1.500 ml/min [1-On 5-Off] E
Wait	Wait 1 seconds
Valve	Valve A move to position 4.
Multisyringe	Pickup 0.045 ml at 1.500 ml/min [1-On 5-Off] E
Wait	Wait 1 seconds
Valve	Valve A move to position 5.
Multisyringe	Dispense 0.800 ml at 2.000 ml/min [1-On 5-Off] E
Multisyringe	Priming in pickup at 3.750 ml/min [1-Off 5-Off] NE
Wait	Wait 65 seconds
Wait	Wait for instrument: "Multisyringe"
Multisyringe	Dispense 1.400 ml at 2.000 ml/min [1-On 5-Off] NE
Wait	Wait 10 seconds
Spectrometer	Absorbance: Spectral range 400 nm – 600 nm every 0.5 seconds (A:420 nm, R:470 nm)
Data process	Start data processing on "Spectrometer "
Wait	Wait for instrument: "Multisyringe"
Data process	Finish data processing on "Spectrometer ". "Peak_height" = Peak maximum
Spectrometer	Stop measure
Multisyringe	Pickup 0.200 ml at 5.000 ml/min [1-Off 5-Off] E
Mark	Mark "L" on "Spectrometer". Add variable "Peak_height"
Loop	END: Repeat "Repetition" times from Measure

## VIII Autoanalysis methods and procedures

### Procedure "Monitoring high sorbitol concentrations"

All steps were principally the same as in the corresponding "Monitoring of high glycerol concentrations". Therefore, the comment column is omitted. The particular flow rates, times, wavelengths, and volumes were modified in order to adapt the procedure to the analyte sorbitol and different working and to achieve higher frequency of determination.

Instruction	Specification
Loop	START: Measure
Valve	Valve A move to "Position".
Multisyringe	Pickup 0.020 ml at 2.000 ml/min [1-On 5-Off] E
Valve	Valve A move to position 1.
Multisyringe	Dispense 0.120 ml at 3.000 ml/min [1-On 5-Off] E
Multisyringe	Pickup 0.100 ml at 3.000 ml/min [1-Off 5-Off] E
Wait	Wait 1 seconds
Valve	Valve A move to "Position".
Multisyringe	Pickup 0.030 ml at 1.200 ml/min [1-On 5-Off] E
Wait	Wait 1 seconds
Multisyringe	Dispense 0.030 ml at 1.200 ml/min [1-Off 5-Off] E
Wait	Wait 1 seconds
Valve	Valve A move to position 1.
Multisyringe	Dispense 0.030 ml at 1.200 ml/min [1-On 5-Off] E
Wait	Wait 1 seconds
Valve	Valve A move to position 2.
Multisyringe	Pickup 0.050 ml at 1.500 ml/min [1-Off 5-Off] E
Wait	Wait 1 seconds
Multisyringe	Pickup 0.300 ml at 1.500 ml/min [1-On 5-Off] E
Wait	Wait 1 seconds
Valve	Valve A move to position 3.
Multisyringe	Pickup 0.140 ml at 1.500 ml/min [1-On 5-Off] E
Wait	Wait 1 seconds
Valve	Valve A move to position 4.
Multisyringe	Pickup 0.070 ml at 1.500 ml/min [1-On 5-Off] E
Wait	Wait 1 seconds
Valve	Valve A move to position 5.
Multisyringe	Dispense 0.850 ml at 2.000 ml/min [1-On 5-Off] E
Multisyringe	Priming in pickup at 3.750 ml/min [1-Off 5-Off] NE
Wait	Wait 60 seconds
Wait	Wait for instrument: "Multisyringe"
Multisyringe	Dispense 1.400 ml at 2.000 ml/min [1-On 5-Off] NE
Wait	Wait 9 seconds
Spectrometer	Absorbance: Spectral range 400 nm - 600 nm every 0.5 seconds (A:420 nm, R:470 nm)
Data process	Start data processing on "Spectrometer"
Wait	Wait for instrument: "Multisyringe"
Data process	Finish data processing on "Spectrometer " . "Peak_height" = "Peak maximum"
Spectrometer	Stop measure
Multisyringe	Pickup 0.200 ml at 5.000 ml/min [1-Off 5-Off] E
Mark	Mark "H". Add variable "Peak_height"
Loop	END: Repeat "Repetition" times from Measure

## VIII Autoanalysis methods and procedures

### Method "Monitoring of sorbitol"

Within this method, the procedure "Clean short sample channel" was used, which presented a cleaning a similar procedure as "Clean channel" but with reduced volumes since the sample supply tube was of reduced length and diameter in order to diminish the required volume

Instruction	Specification	Comment
Valve	Valve A move to position 1.	Initialize instrument
Valve	Valve A move to position 5.	
Multisyringe	Priming in dispense at 2.000 ml/min [1-On 5-Off] E	
Wait	Wait for user input: "Measure blank and dark?" Button Yes & No. Store in "Temp"	
Conditional	Dark and Blank: IF ["Temp" = 1] THEN	Measurement of dark and blank on user request
Spectrometer	Get dark current spectrum	
Spectrometer	Get blank spectrum	
Conditional	Dark and Blank: ENDIF	
Spectrometer	Absorbance: Spectral range 400 nm - 600 nm every 0.5 seconds (A:420 nm, R:470 nm)	Perform baseline start
Multisyringe	Priming in pickup [1-Off 5-Off] E	
Spectrometer	Stop measure	
Multisyringe	Dispense 1.200 ml at 3.750 ml/min [1-On 5-Off] E	
Loop	START: Running Method	User request for next procedure
Variable	"Option" = User input: 0:stop method 1:calibration and cleaning procedures 2:measure high concentration 3:measure low concentration 4:remove stacked bubbles	
Conditional	Option1: IF ["Option" = 1] THEN	
Wait	Wait for user input: "Clean reagent channels?" Button Yes & No. Store in "Temp"	
Conditional	Clean reagent channels: IF ["Temp" = 1] THEN	Cleaning of channels of reagent 1, reagent 2 and water on user request
Variable	Position = 3	
Procedure	Call "Clean channel"	
Variable	Position = 4	
Procedure	Call "Clean channel"	
Variable	Position = 2	
Procedure	Call "Clean channel"	
Conditional	Clean reagent channels: ENDIF	
Wait	Wait for user input: "Measure reagent blank (both procedures)?" Button Yes & No. Store in "Temp"	Measurement of blank value of the reagent (water instead of sample) on user request and with the requested repetition of determination
Conditional	Blank: IF ["Temp" = 1] THEN	
Variable	"Repetition" = User input: "How many repetitions (1, 2 or 3)?"	
Variable	Position = 2	
Procedure	Call "Clean channel"	
Procedure	Call "Monitoring high sorbitol concentrations "	
Mark	Mark " _B" on Spectrometer	
Procedure	Call " Monitoring low sorbitol concentrations"	
Mark	Mark " _B" on Spectrometer	
Conditional	Blank: ENDIF	
Wait	Wait for user input: "Calibration for 1 - 20 g/l?" Button Yes & No. Store in "Temp"	Calibration of the high working range on user request and with the demanded repetition and number of sample of determination.
Conditional	Calibration sorbitol high: IF ["Temp" = 1] THEN	
Variable	"Std_Number" = User input: " How many standards? (1 - 6)"	
Variable	"Repetition" = User input: "How many repetitions (1 - 3)?"	
Variable	"Position" = 6	
		Mark of each peak group with



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Loop	START: Calibration for high working range	"CSH". Process time and procedure type is marked within the corresponding procedure
Wait	Wait for user input: "Place next standard at valve position 6!"	
Procedure	Call "Clean Short Sample Channel"	
Procedure	Call "Monitoring high sorbitol concentrations"	
Loop	END: Repeat "Std_Number" times from Calibration sorbitol high	
Mark	Mark " _CSH" on Spectrometer	
Conditional	Calibration for high working range: ENDIF	Calibration of the low working range on user request and with the demanded repetition and number of sample of determination. Mark of each peak group with "CSL". Process time and procedure type is marked within the corresponding procedure
Wait	Wait for user input: "Calibration for 0.1 – 2.5 g/l?" Button Yes & No. Store in "Temp"	
Conditional	Calibration for low working range: IF ["Temp" = 1] THEN	
Variable	"Std_Number" = User input: " How many standards? (1 - 6)"	
Variable	"Repetition" = User input: "How many repetitions (1 - 3)?"	
Variable	"Position" = 6	
Loop	START: Calibration for low working range	
Wait	Wait for user input: "Place standard at valve position 6!"	
Procedure	Call "Clean Short Sample Channel"	
Procedure	Call "Monitoring low sorbitol concentrations"	
Loop	END: Repeat "Std_Number" times from Calibration sorbitol low	
Mark	Mark " _CSL" on Spectrometer	
Conditional	Calibration for low working range: ENDIF	Carrying out option "Remove stacked bubbles"
Conditional	Option1: ENDIF	
Conditional	Option5: IF ["Option" = 4] THEN	
Valve	Valve A move to position 1.	
Multisyringe	Priming in dispense [1-Off 5-Off] E	
Procedure	Call "Remove air-bubbles in HC and in OFF"	
Multisyringe	Pickup 1.300 ml at 7.500 ml/min [1-Off 5-Off] E	Option "Measurement"
Conditional	Option5: ENDIF	
Conditional	Option0: IF ["Option" between 1.9 and 3.1] THEN	Introduction of sample identification number
Variable	"Sample_Number" = User input: "Introduce sample number!" ("0" for control standard)"	
Variable	"Repetition" = User input: "How many repetitions? (1 - 4)"	Request for repetitions
Variable	Position = 6	Sample channel cleaning on user request
Wait	Wait for user input: "Clean channel?" Button Yes & No. Store in "Temp"	
Conditional	Option4: IF ["Temp" = 1] THEN	
Procedure	Call "Clean Short Sample Channel"	
Conditional	Option4: ENDIF	Sample measurement with either high or low concentration range procedure as requested before and with demanded repetitions. All sample peak groups are marked with "S" and given sample identification number.
Conditional	Option2: IF ["Option" = 2] THEN	
Procedure	Call "Monitoring high sorbitol concentrations"	
Conditional	Option2: ENDIF	
Conditional	Option3: IF ["Option" = 3] THEN	
Procedure	Call "Monitoring low sorbitol concentrations"	
Conditional	Option3: ENDIF	Option "Finalize method"
Mark	Mark " S" on Spectrometer . Add variable "Sample_Number"	
Conditional	Option0: ENDIF	Add baseline
Loop	END: Repeat while "Option" <> 0 repeat from "Running Method"	
Valve	Valve A move to position 5.	Cleaning and resetting of instrument
Spectrometer	Absorbance: Spectral range 400 nm - 600 nm every 0.5 seconds (A:420 nm, R:470 nm)	
Multisyringe	Priming in dispense [1-On 5-Off] E	
Spectrometer	Stop measure	
Multisyringe	Priming in pickup [1-Off 5-Off] NE	

## I.V. SIA-CE system

Syringe sizes were: syringe 3: 2.5 ml, syringe 1, 2 to 4 were not used. Selection valve positions were: 1: waste; 2: water; 3: sample; 4: grounded interface; 5: high voltage falling drop interface; 6: water; 7: HCl; 8: NaOH. For automation, the background electrolyte (BGE) was aspirated in OFF from an autosampler beaker of 100 ml.

An additional solenoid valve was used to close the outlet of the grounded interface, controlled with output "7". Output "5" and "6" controlled the ventilator and high-voltage application, respectively.

**Procedure "Clean HV interface"**

Instruction	Specification	Comment
Valve	Valve A move to position 5. Valve B move to "Load"	Dropping BGE into the high voltage interface
Multisyringe	Dispense 0.600 ml at 2.500 ml/min [3-On 5-Off 6-Off 7-Off] E	
Wait	Wait 1 s	
Multisyringe	Pickup 0.120 ml at 2.000 ml/min [3-On 5-Off 6-Off 7-Off] E	Emptying of the falling-drop-tube
Valve	Valve A move to position 1. Valve B move to "Load"	Discharge of air segment
Multisyringe	Dispense 0.120 ml at 3.000 ml/min [3-On 5-Off 6-Off 7-Off] E	
Wait	Wait 1 s	
Multisyringe	Pickup 0.600 ml at 3.750 ml/min [3-Off 5-Off 6-Off 7-Off] E	Filling up of syringe

The same procedure was used in the MSFIA-CE system with heads 1, 2, 4, and 8 in position "Off".

**Procedure "Head space discharge"**

Instruction	Specification	Comment
Valve	Valve A move to position 2	Position of water
Multisyringe	Dispense 0.200 ml at 3.750 ml/min [3-Off 7-Off] E	Dispense solution to reservoir
Multisyringe	Pickup 0.100 ml at 3.750 ml/min [3-Off 7-Off] E	Pickup from holding coil
Multisyringe	Dispense 1.800 ml at 3.750 ml/min [3-Off 7-Off] E	Flush channel to reservoir
Multisyringe	Priming in pickup [3-Off 7-Off] E	Filling up syringe

The same procedure was used in the MSFIA-CE system with heads 1, 2, 4, and 8 in position "Off".

**Procedure "Cleaning grounded interface and capillary"**

Instruction	Specification	Comment
Valve	Valve A move to position 4. Valve B move to "Load"	Position of grounded interface
Multisyringe	Dispense 0.300 ml at 3.000 ml/min [3-On 7-On] E	Flush interface with BGE
Wait	Wait 1 seconds	
Multisyringe	Dispense 0.300 ml at 3.000 ml/min [3-On 7-Off] E	Pressure build up in interface
Wait	Wait 1 seconds	
Multisyringe	Pickup 0.600 ml at 3.000 ml/min [3-Off 7-Off] NE	Filling up syringe
Wait	Wait 300 seconds	Time for hydrodynamic rinsing of the capillary
Wait	Wait for instrument: " Multisyringe"	
Multisyringe	Heads: 3-Off 7-On	Pressure release
Wait	Wait 5 seconds	
Multisyringe	Heads: 3-Off 7-Off	Deactivation of valve 7

The same procedure was used in the MSFIA-CE system with heads 1, 2, 4, and 8 in position "Off".

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### Procedure "Rinsing of capillary with solution"

Instruction	Specification	Comment
Valve	Valve A move to "Position".	Pickup of cleaning solution
Multisyringe	Pickup 0.300 ml at 2.000 ml/min [3-On 7-Off] E	
Wait	Wait 2 seconds	
Valve	Valve A move to position 4.	Position of grounded interface
Multisyringe	Dispense 0.050 ml at 2.000 ml/min [3-On 7-On] E	Propelling solution to interface
Multisyringe	Dispense 0.300 ml at 2.000 ml/min [3-On 7-Off] E	Pressure build up in interface
Multisyringe	Pickup 0.650 ml at 2.000 ml/min [3-Off 7-Off] NE	Filling up syringe
Wait	Wait 300 seconds	Time for hydrodynamic rinsing of the capillary
Wait	Wait for instrument: "Multisyringe"	
Valve	Valve A move to position 1.	Clean holding coil (discharge to waste) and pressure release
Multisyringe	Dispense 0.600 ml at 2.000 ml/min [3-On 7-On] E	

The same procedure was used in the MSFIA-CE system with heads 1, 2, 4, and 8 in position "Off".

### Method "Conditioning of capillary"

Instruction	Specification	Comment
Autosampler	Sampler move to 1 position. E	Full dispense to waste
Valve	Valve A move to position 1.	
Multisyringe	Priming in dispense [3-On 7-Off] E	
Variable	Option = 1	Change of syringe content with solution from the autosampler
Conditional	Syringe cleaning: IF [Option = 1] THEN	
Loop	START: Loop	
Multisyringe	Pickup 1.000 ml at 3.750 ml/min [3-Off 7-Off] E	
Multisyringe	Dispense 1.000 ml at 3.750 ml/min [3-On 7-Off] E	
Loop	END: Repeat 4 times from Loop	
Conditional	Syringe cleaning: ENDIF	
Multisyringe	Pickup 1.000 ml at 3.750 ml/min [3-Off 7-Off] E	Option of cleaning or not
Variable	Option = 1	
Conditional	Cleaning: IF [Option = 1] THEN	Flushing capillary with HCl
Variable	"Position" = 7	
Procedure	Call "Rinsing of capillary with solution"	Flushing capillary with NaOH
Variable	"Position" = 8	
Procedure	Call "Rinsing of capillary with solution"	Flushing capillary with water
Conditional	Cleaning: ENDIF	
Variable	"Position" = 6	Flushing capillary with BGE if Option is preset to 1
Procedure	Call "Rinsing of capillary with solution"	
Variable	Option = 1	
Conditional	Capillary rinsing with BGE: IF [Option = 1] THEN	Flushing capillary with BGE if Option is preset to 1
Procedure	Call "Clean grounded interface and capillary"	
Conditional	Capillary rinsing with BGE: ENDIF	
Multisyringe	Priming in pickup [3-Off 7-Off] E	

The first part of this method was used besides as a procedure for exchange of the syringe containing solution by a new solution from a autosampler beaker for BGE optimization.

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### Procedure "Injection"

Instruction	Specification	Comment	
Multisyringe	Pickup 0.050 ml at 2.000 ml/min [ 3-Off 7-On ] E	To overcome backlash of the multisyringe	
Valve module	Valve A move to "Position"	Aspiration of sample	
Multisyringe	Pickup 0.100 ml at 2.000 ml/min [ 3-On 7-On ] E		
Wait	Wait 2 seconds		
Multisyringe	Dispense 0.050 ml at 2.000 ml/min [ 3-Off 7-On ] E	To overcome backlash of the multisyringe	
Valve module	Valve A move to position 4	Propelling aliquot of sample towards the grounded interface	
Multisyringe	Dispense 0.050 ml at 2.000 ml/min [ 3-On 7-On ] E		
Wait	Wait 2 seconds		
Valve module	Valve A move to position 1	Discharge rest of sample	
Multisyringe	Dispense 0.200 ml at 2.000 ml/min [ 3-On 7-On ] E		
Wait	Wait 2 seconds		
Valve module	Valve A move to position 4	See below	
Multisyringe	Multicommutation command. E		
<b>* Steps of multicommutation command</b>		<b>Wait</b>	<b>Comment</b>
Dispense 0.040 ml at 1.500 ml/min [3-On 7-On] E		200 ms	Positioning of sample at capillary tip
Dispense 0.010 ml at 0.500 ml/min [3-On 7-On] E		800 ms	Pressure build-up (hydrodynamic injection)
Pickup 0.005 ml at 0.500 ml/min [3-On 7-Off] E		-	Pressure reduction
Heads: 3-Off 7-Off E		200 ms	Pressure release (opening of valve 7)
Dispense 0.20 ml at 1.500 ml/min [3-On 7-On] E		-	Flushing of interface

Heads 5 and 6 were in positions OFF during the entire procedure. The procedure was used similar for the MSFIA-CE system but with modified volumes.

### Method: "Separation"

Instruction	Specification	Comment
Valve module	Valve A move to position 2. Valve B not move.	Flushing of capillary for cleaning and reconditioning
Multisyringe	Dispense 0.300 ml at 2.000 ml/min [ 3-On 5-On 6-Off 7-On ] E	
Wait	Wait 1 seconds	
Multisyringe	Dispense 0.300 ml at 2.000 ml/min [ 3-On 5-On 6-Off 7-Off ] E	
Procedure	Call "Clean HV interface"	
Variable	"Position" = 3	Injection procedure with sample from selection port 3
Procedure	Call "Injection"	
Multisyringe	Heads: 3-Off 5-On 6-On 7-On 8-On	High voltage activation
Wait	Wait 58 seconds	
Spectrometer	Get blank spectrum	Two times blank measurement suppressed baseline jump
Spectrometer	Get blank spectrum	
Spectrometer	Transmission: Spectral range 350 nm - 450 nm every 0.5 seconds (A:400 nm)	Start measure
Wait	Wait 2 seconds	
Mark	Mark "+60s" on "Spectrometer"	Mark time fore later calculations
Multisyringe	Priming in pickup at 3.75 ml/min [3-Off 5-On 6-On 7-On] NE	Filling up syringe
Wait	Wait 700 seconds	Waiting for separation
Spectrometer	Stop measure	
Multisyringe	Heads: 3-Off 5-Off 6-Off 7-Off 8-Off	High voltage deactivation
Wait	Wait for instrument: "Multisyringe"	Last pump step was no exclusive

In optimization experiments of the BGE composition, in a first part of this method, for each optimization point, another BGE mixture was aspirated from the autosampler, the syringe was washed and the capillary was conditioned three-times (15 min) with the new BGE and afterwards, the protocol above was carried out three-times for three separations.

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### I.VI. MSFIA-CE system

Syringe 1 10 ml (Water), syringe 2: 2.5 ml (HCl), syringe 3: 2.5 ml (BGE). Syringe 4 was not used. Exceptionally, the given volumes and flow rates correspond to a syringe total volume of 2.5 ml, i.e. all values for syringe 1 (10 ml) have to be multiplied with a factor of 4. Selection valve positions were: 1: waste; 2: grounded interface; 3: Auxiliary eluent 2; 2: water; 5: high voltage falling drop interface; 6: HCl; 7: NaOH; 8: sample.

Additional solenoid valves were used to close the outlet of the grounded interface (valve 7, control output "7"), aspiration of air (valve 4, output "5") and aspiration of the auxiliary eluent 1 and sample (valve 5 and 6, respectively, both controlled via output "8").

#### Method "Separation with pre-concentration in background" \*

Instruction	Specification	Comment
Valve	Valve A move to position 2. Valve B move to "Load"	
Multisyringe	Dispense 1.600 ml at 2.000 ml/min [1-Off 2-Off 3-On 5-Off 6-Off 7-On 8-Off] E	Initialize of multisyringe
Procedure	Call "Clean channel"	
Procedure	Call "Conditioning of column"	First conditioning of SPE column
Procedure	Call "Pre-concentration"	First pre-concentration of sample
Loop	START: Repeat	
Valve	Valve A move to position 2. Valve B move to "Load"	
Multisyringe	Dispense 0.300 ml at 2.000 ml/min [1-Off 2-Off 3-On 5-Off 6-Off 7-Off 8-Off] E	Cleaning of grounded interface and conditioning of capillary with BGE In parallel cleaning of high voltage interface
Procedure	Call "Clean HV interface"	
Procedure	Call "Elution and Injection"	First elution and injection
Multisyringe	Ports: 1-Off 2-Off 3-Off 5-Off 6-On 7-On 8-Off	High voltage activation
Wait	Wait 10 s	
Multisyringe	Priming in dispense at 3.750 ml/min [1-Off 2-Off 3-Off 5-Off 6-On 7-On 8-Off] NE	Positioning of multisyringe for next pre-concentration
Wait	Wait 30 s	
Wait	Wait for instrument: "Multisyringe"	
Multisyringe	Pickup 0.900 ml at 3.750 ml/min [1-Off 2-Off 3-Off 5-Off 6-On 7-On 8-Off] NE	
Wait	Wait 28 s	
Wait	Wait for instrument: "Multisyringe"	
Spectrometer	Get blank spectrum	
Spectrometer	Measure absorbance at 401 nm every 0.5 s	
Wait	Wait 2 s	
Mark	Mark "+70s" on Ocean Optics	
Procedure	Call "Conditioning of column in background"	Following conditionings of SPE column
Procedure	Call "Pre-concentration in background"	Following pre-concentrations of sample
Spectrometer	Stop measure	
Multisyringe	Heads: 1-Off 2-Off 3-Off 5-Off 6-Off 7-Off 8-Off	High voltage deactivation
Loop	END: Repeat 2 times from Repeat	
Valve	Valve A move to position 2. Valve B move to "Load"	
Multisyringe	Dispense 0.300 ml at 2.000 ml/min [1-Off 2-Off 3-On 5-Off 6-Off 7-Off 8-Off] E	Cleaning of grounded interface and conditioning of capillary with BGE In parallel cleaning of high voltage interface
Procedure	Call "Clean HV interface"	
Procedure	Call "Elution and Injection"	Following elution and injection
Multisyringe	Heads: 1-Off 2-Off 3-Off 5-Off 6-On 7-On 8-Off	High voltage activation
Wait	Wait 10 s	

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Multisyringe	Priming in dispense at 3.750 ml/min [1-Off 2-Off 3-Off 5-Off 6-On 7-On 8-Off] NE	Positioning of multisyringe for next pre-concentration
Wait	Wait 30 s	
Wait	Wait for instrument: "Multisyringe"	
Multisyringe	Pickup 0.900 ml at 3.750 ml/min [1-Off 2-Off 3-Off 5-Off 6-On 7-On 8-Off] NE	
Wait	Wait 28 s	
Wait	Wait for instrument: "Multisyringe"	
Spectrometer	Get blank spectrum	
Spectrometer	Measure absorbance at 401 nm every 0.5 s	
Wait	Wait 2 s	
Mark	Mark "+70s" on Ocean Optics	
Wait	Wait 500 s	Wait for last separation
Spectrometer	Stop measure	
Multisyringe	Heads: 1-Off 2-Off 3-Off 5-Off 6-Off 7-Off 8-Off	High voltage deactivation

### Procedure "Clean channel"

Instruction	Specification	Comment
Multisyringe	Pickup 0.025 ml at 1.000 ml/min [1-On 2-Off 3-Off 5-On 6-Off 7-Off 8-Off] E	Aspiration of air
Multisyringe	Pickup 0.100 ml at 1.000 ml/min [1-On 2-Off 3-Off 5-Off 6-Off 7-Off 8-On] E	Aspiration of sample
Valve	Valve A move to position 1. Valve B move to "Inject"	Dispense to waste
Multisyringe	Dispense 0.500 ml at 2.000 ml/min [1-On 2-Off 3-Off 5-Off 6-Off 7-Off 8-Off] E	
Wait	Wait 1 s	
Multisyringe	Pickup 0.375 ml at 3.750 ml/min [1-Off 2-Off 3-Off 5-Off 6-Off 7-Off 8-Off] E	Filling up syringes

### Procedure "Conditioning of column (in background)"\*

Instruction	Specification	Comment
Valve	Valve A move to position 1. Valve B move to "Load"	
Multisyringe	Pickup 0.200 ml at 2.500 ml/min [1-Off 2-On 3-Off 5-Off 6-Off 7-Off 8-On] E	Pickup of auxiliary eluent 1
Wait	Wait 2 s	
Multisyringe	Dispense 0.600 ml at 0.750 ml/min [1-Off 2-On 3-Off 5-Off 6-Off 7-Off 8-Off] E	Dispense of auxiliary eluent 1 followed by carrier of syringe 2 (100 mmol l <sup>-1</sup> HCl) through the SPE column
Wait	Wait 2 s	
Multisyringe	Dispense 0.200 ml at 0.150 ml/min [1-On 2-Off 3-Off 5-Off 6-Off 7-Off 8-Off] E	Dispense from syringe 1 and 2, giving 20 mmol l <sup>-1</sup> HCl through the SPE column
Wait	Wait 2 s	
Multisyringe	Pickup 0.500 ml at 3.750 ml/min [1-Off 2-Off 3-Off 5-Off 6-Off 7-Off 8-Off] E	Filling up syringes

\* for operation in background, the power supply was permanently activated (output 6 in On)

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### Procedure "Pre-concentration / Pre-concentration in background" \*

Instruction	Specification	Comment
Multisyringe	Pickup 0.025 ml at 1.000 ml/min [1-Off 2-Off 3-Off 5-Off 6-Off 7-Off 8-Off] E	To overcome backlash of multisyringe
Multisyringe	Pickup 0.025 ml at 0.500 ml/min 1-On 2-On 3-Off 5-On 6-Off 7-Off 8-Off] E	Aspiration of air for segmentation
Wait	Wait 1 s	
Multisyringe	Heads: 1-Off 2-Off 5-Off 6-Off 7-Off 8-Off	Change of valve position
Wait	Wait 1 s	
Multisyringe	Pickup 1.500 ml at 1.200 ml/min 1-On 2-Off 3-Off 5-Off 6-Off 7-Off 8-On] E	Pickup of sample (6 ml)
Wait	Wait 4 s	
Valve	Valve A move to position 1. Valve B move to "Load"	To overcome backlash of multisyringe
Multisyringe	Dispense 0.025 ml at 1.000 ml/min 1-Off 2-Off 3-Off 5-Off 6-Off 7-Off 8-Off] E	
Multisyringe	Dispense 1.525 ml at 0.150 ml/min 1-On 2-Off 3-Off 5-Off 6-Off 7-Off 8-Off] E	Load of SPE column with sample and in-line acidification by dispense of 100 mmol l <sup>-1</sup> HCl from syringe 2
Wait	Wait 4 s	
Multisyringe	Pickup 0.400 ml at 3.750 ml/min 1-Off 2-Off 3-Off 5-Off 6-Off 7-Off 8-Off] E	Filling up syringes
Valve	Valve A move to position 1. Valve B move to "Inject"	Discharge of air and rest of sample
Multisyringe	Dispense 0.200 ml at 3.000 ml/min 1-On 2-Off 3-Off 5-Off 6-Off 7-Off 8-Off] E	
Wait	Wait 2 s	
Valve	Valve A move to position 2. Valve B move to "Load"	
Multisyringe	Dispense 0.200 ml at 0.250 ml/min 1-On 2-Off 3-Off 5-Off 6-Off 7-On 8-Off] E	Flush SPE column with water

\* for operation in background, the power supply was permanently activated (output 6 in On).

### Procedure "Elution and Injection"

Instruction	Specification	Comment
Multisyringe	Pickup 0.010 ml at 2.000 ml/min [3-Off 7-On] E	To overcome backlash of multisyringe
Valve	Valve A move to position 3. Valve B move to "Load"	Aspiration of auxiliary eluent 2
Multisyringe	Pickup 0.200 ml at 2.000 ml/min [3-On 7-On] E	
Wait	Wait 2 s	
Multisyringe	Dispense 0.010 ml at 2.000 ml/min [3-Off 7-On] E	To overcome backlash of multisyringe
Valve	Valve A move to position 2. Valve B move to "Inject"	Elution of analytes from SPE column
Multisyringe	Dispense 0.260 ml at 0.300 ml/min [3-On 7-On] E	
Wait	Wait 1 seconds	
Valve module	Valve A move to position 2. Valve B move to "Load"	Taking out of the SPE column from the injection tube line
Multisyringe	Multicommutation command. E	See below
<b>* Steps of multicommutation command</b>		<b>Comment</b>
Dispense 0.005 ml at 0.500 ml/min [3-On 7-On] E	800 ms	Pressure build-up (hydrodynamic injection)
Pickup 0.005 ml at 0.500 ml/min [3-On 7-Off] E	-	Pressure reduction
Heads: 3-Off 7-Off E	200 ms	Pressure release (opening of valve 7)
Dispense 0.250 ml at 1.500 ml/min [3-On 7-On] E	-	Flushing of interface

Heads 1, 2, 5, 6 and 8 were in positions Off during the entire procedure. The procedure was used similar for the MSFIA-CE system but with modified volumes.

## IX PROPERTIES OF USED SUBSTANCES

Analyte	Formula	M [g mol <sup>-1</sup> ]*	P at 20°C [g cm <sup>-3</sup> ]**	Solubility [g l <sup>-1</sup> ]**	Toxicity **	R sentences **	S sentences **
Acetaldehyde	C2H4O	44.05	0.78	soluble	F+, Xn	12-36/37-40	16-33-36/37
Acetic acid	C2H4O2	60.05	1.05	soluble	C	10-35, 23.2-26-45	23.2-26-45
Acetone	C3H6O	58.08	0.79	soluble	F, Xi	11-36-66-67	9-16-26
Acetonitrile	C2H3N	41.05	0.79	soluble	F, Xn	11-20/21/22-36	16-36/37
Acetylacetone	C5H8O2	102.12	0.97	200	Xn	10-22	21-23.2-24/25
Ammonia (25%)	NH3	17.03	0.91	soluble	C	34-50	26-36/37/39-45-61
Boric acid	H3BO3	61.83	1.52	50	-	-	-
Bromothymolblue	C27H28Br2O5S	624.39	1.25	insoluble	-	-	-
Calcium sulfate dihydrate	CaSO4 · 2 H2O	172.17	2.32	2	-	-	-
Cobalt nitrate hexahydrate	CoNO3 · 6 H2O	291.04	1.87	1330	Xn	22-40-43	36/37
Copper(II)sulfate pentahydrate	CuSO4 · 5 H2O	249.69	2.28	317	Xn, N	22-36/38-50/53	22-60-61
Ethanol	C2H6O	46.07	0.79	soluble	F	11	7-16
Formaldehyde	CH2O	30.03	0.82	soluble	T	20/21/22-36/37/38-39/23/24/25-40-43	26-36/37-45-51
Formic acid	CH2O2	46.02	1.22	soluble	C	35	23.2 - 26 - 5
Glyceraldehyde	C3H6O3	90.08	1.46	soluble	-	-	-
Glycerol	C3H8O3	92.09	1.26	soluble	-	-	-
Glyoxal	C2H2O2	58.04	1.14	soluble	-	-	-
Hydrochloric acid (37%)	HCl	36.46	1.19	soluble	C	34-37	26-36/37/39-45
Hydrogen peroxide (30%)	H2O2	34.02	1.11	soluble	C	34	3-26-36/37/39-45
Iron(II)sulfate heptahydrate	FeSO4 · 7 H2O	278.02	1.89	400	Xn	22	24/25
Magnesium sulfate heptahydrate	MgSO4 · 7 H2O	246.48	1.68	710	-	-	-
Manganese(II) sulfate monohydrate	MnSO4 · H2O	169.02	2.95	762	Xn, N	48/20/22-51/53	22-61
Methanol	CH4O	32.04	0.79	soluble	F, T	11-23/24/25-39/23/24/25	7-16-36/37-45
Methylorange	C14H14N3NaO3S	327.34	1.28	5	T	25	37-45
ortho-Nitrophenol	C6H5NO3	139.11	1.48	2.1	Xn	52/53	24-61
meta-Nitrophenol	C6H5NO3	139.11	1.49	insoluble	Xn	22-36/38	26
para-Nitrophenol	C6H5NO3	139.11	1.48	14.8	Xn	20/21/22-33	28.1
Phenolphthalein	C20H14O4	318.31	1.30	0.092	-	-	-
Phosphoric acid (85%)	H3PO4	98.00	1.71	soluble	C	34	26-36/37/39-45
Potassium dihydrogen phosphate	NaH2PO4	136.09	2.34	222	-	-	-
Potassium hydroxide (min 85%)	KOH	56.11	2.04	1130	C	22-35	26-32/37/39-45
Potassium iodide	KI	166.01	3.12	1430	-	-	-
Potassium meta-periodate	KIO4	230.00	3.98	92	O, Xi	8-41	17-26-39
Potassium sulfate	K2SO4	174.27	2.67	111	-	-	-
Sodium carbonate anhydrate	Na2CO3	105.99	2.53	220	Xi	36	22-26
Sodium hydroxide	NaOH	40.00	2.13	1090	C	35	26-36/37/39-45
Sodium meta-periodate	NaIO4	213.89	3.87	91	O	8	-
Sodium molybdate dihydrate	Na2MoO4 · 2 H2O	241.95	2.71	840	-	-	-
Sorbitol	C6H14O6	182.17	1.59	-	-	-	-
Sulfurous acid	H2SO4	98.08	1.85	soluble	C	35	26-30-45
Thymolphthalein	C28H30O4	430.55	-	-	-	-	-
Zinc sulfate heptahydrate	ZnSO4 · 7 H2O	136.29	1.97	965	Xn, N	22-41-50/53	22-26-39-46-60-61

\* FALBE AND REGITZ (1995) Römpp Chemie Lexikon

\*\* URL: <http://chemdat.merck.de/> (05.11.2007) Chemdat Merck





## X TECHNICAL DATA OF USED INSTRUMENTS

Instrument Deuterium-Halogen Light Source DH-2000-DUV  
Serial-No. 005450000 (UIB), 990051 (HUAC)  
Producer Ocean Optics Inc. (Dunedin, USA)  
Power consumption 78 W in total, deuterium bulb 25 W tungsten halogen 20 W  
Aperture 0.5 mm, numerical aperture 26° (13°); focused  
Wavelength range 215 nm – 1100 nm

Instrument USB2000 Miniature Fiber Optic Spectrometer  
Producer Ocean Optics Inc. (Dunedin, USA)  
Detector 2048-element linear CCD-array detector  
Wavelength range 200-1100 nm, light dispersion via fixed grating  
Remote control Universal Serial Bus (USB)  
Serial-No. USB2E6039 (UIB)  
Wavelength calibration  
Intercept 190.306701660156  
First coefficient 0.359490007162094  
Second coefficient -1.10080000013113E-5  
Serial-No. USB2F043 (HUAS)  
Wavelength calibration coefficients  
Intercept 339.3014315  
First coefficient 0.375967724  
Second coefficient -1.57215E-5

Instrument Spectrometer HP8453  
Serial-No. DE60400779  
Producer Hewlett Packard (Palo Alto, USA)  
Detector Photodiode array  
Wavelength range 190-1100 nm  
Software Hewlett Packard ChemStation

Instrument Luminescence spectrometer LS50 B  
Serial-No. 30698, Part-No. L225-0105  
Producer Perkin Elmer (Boston, USA)  
Detector Photomultiplier  
Wavelength range 200 – 800 nm (excitation) 200 – 900 nm (emission)  
scan function using monochromator grating  
Light source Xenon flash tube, 50 Hz  
Detection cell Flow cell or alternatively cuvette support  
Software FL WINLAB

Instrument Cuvette Holder CUV-UV for 1 cm cuvettes  
Producer Ocean Optics Inc. (Dunedin, USA)  
Optical specifications 200 nm – 2 µm, two adjustable 5 mm diameter f/2 quartz collimator lenses

## IX Properties of used substances

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Instrument                    Multisyringe module BU 4 S  
Producer                    Crison S.A. (Alella, Spain)  
Serial number                22303 (UIB), 14801 (HUAC)  
Specifications                16.000 steps, up to 4 syringes and 4 additional power supplies  
Time range                    20 s – 1048 s for total dispense  
Remote control                RS232

Instrument                    Valve module VA 1 + 1  
Producer                    Crison S.A. (Alella, Spain)  
Serial number                22301 (UIB), 21202 (HUAC)  
Valves                        Eight-port rotary selection valve with central commune port  
                                  Six-port injection valve  
Remote control                RS232

Instrument                    Rotary Autosampler Micro Sampler  
Producer                    Crison S.A. (Alella, Spain)  
Serial number                11601  
Number of positions         15 x 120 ml or 45 x 15 ml  
Remote control                RS232

Instrument                    Minipuls 3 peristaltic pump  
Producer                    Gilcon (Middleton, USA)  
Serial number                111059  
Speeds                        0 to 48 rounds per minute  
Head                          10 stainless steel rollers, 8 channels

Instrument                    Solenoid valves STV-3-1/4UKG  
Producer                    Takasago Electronic Inc. (Nagoya, Japan)  
Type                          3-way, multi-commutation, 12 V  
Sealing material              PTFE  
Maximal pressure             200 kPa

Instrument                    Solenoid valves MTV-3-N1/4UKG  
Producer                    Takasago Electronic Inc. (Nagoya, Japan)  
Type                          3-way, multi-commutation, 12 V  
Sealing material              PTFE  
Maximal pressure             200 kPa

Instrument                    Solenoid valves MTV-3-N1/U4KGH  
Producer                    Takasago Electronic Inc. (Nagoya, Japan)  
Type                          3-way, multi-commutation, 12 V  
Sealing material              Perfluoroelastomer  
Maximal pressure             600 kPa

## IX Properties of used substances

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Instrument	CoolCube™ solenoid control module
Producer	Bio-Chem Valve Inc. (Boonton, New Jersey, USA)
Operating power	12 VDC or a 24 VDC, max. 28 VDC, max. 1.5 A
Response time	Automatic drop of voltage after 110 ms to 1/3
Instrument	Magnetic stirrer Microfix
Serial-No.	66451
Producer	JP Selecta (Abrera, Barcelona, Spain)
Speed	500 rpm (fixed)
Instrument	Magnetic stirrer Mini-MR
Inv.-No.	M162/9.91/30
Producer	Jahnke & Kunkel (Staufen, Germany)
Speed	0 – 2000 min <sup>-1</sup>
Instrument	Thermostat Tectron 3473100
Producer	Selecta (Barcelona, Spain)
Power consumption	1050 W
Serial No.	236478
Instrument	Thermostat and Waterbath Haake F423 (Hamburg)
Producer	Haake, (Berlin & Karlsruhe, Germany)
Power consumption	1100 W
Serial No.	74386
Instrument	pH-meter Micro pH 2002
Serial-No.	0022569
Producer	Crison (Alella, Spain)
Specifications	Temperature compensation via additional pt100 probe
Instrument	HCP 35-35000 High voltage power source
Producer	F.u.G. Elektronik GmbH (Rosenheim, Germany)
Maximal output	35.000 V, 1 mA
Remote control	Analogous interface
Instrument	2127 Tachophor high voltage power source
Producer	LKB (Bromma, Sweden)
Maximal output	30.000 V, 500 µA
Remote control	Interrupt circuit
Instrument	Filtration probe ESIP-Lab 5442 (Eppendorf Sterile Inline Probe)
Membrane	tubular polypropylene in-situ sterilizable, pore diameter 0.6 µm - 0.2 µm
Maximal flow	2 ml min <sup>-1</sup>
Delay time	8 minutes (99 % recuperation)
Distributor	Trace Analytics GmbH (Braunschweig, Germany)