On-Line Post-Column Reactions for the Analysis of Enzyme Inhibitors and Antioxidants

Bram Miserez

Promotor: Prof. dr. Frédéric Lynen

Co-promotor: Em. Prof. dr. Pat Sandra

Academic year 2012-2013

Thesis submitted in partial fulfilment of the requirements for the degree of

Doctor of Science: Chemistry

Board of Examiners

Reading Committee

Prof. dr. F. Lynen, promotor	Ghent University, Belgium
Prof. dr. F. David	Ghent University, Belgium
Prof. dr. D. Cabooter	Catholic University of Leuven, Belgium
Prof. dr. A. de Villiers	University of Stellenbosch, SouthAfrica

Other Members of the Board of Examiners

Prof. dr. A. Adrieans, chairman	Ghent University, Belgium
Em. Prof. dr. P. Sandra, co-promotor	Ghent University, Belgium
Prof. dr. J. Martins	Ghent University, Belgium
Prof. dr. F. Vanhaecke	Ghent University, Belgium

TABLE OF CONTENTS

TABLE OF CONTENTS.....1

INTRODUCTION 7

CHAPTER 1

LIQUID CHROMATOGRAPHY AND POST-COLUMN REACTIONS 9

1. BASIC PRINCIPLES OF LIQUID CHROMATOGRAPHY..... 9
2. SEPARATION MODES IN HPLC 20
3. INSTRUMENTAL ASPECTS OF HPLC..... 22

3.1 The Injector 22

3.2 The Pump 22

3.3 The Column 23

3.4 The Detector..... 23

3.4.1 UV-Vis Detection 24

3.4.2 Mass Spectrometry 26

3.4.3 Specific Detection..... 31

4. POST-COLUMN DERIVATIZATION IN HPLC 32

4.1 Open Tubular Reactors..... 32

4.2 Packed Bed Reactors..... 35

4.3 Segmented Flow Reactors..... 36

4.4 Advantages and Disadvantages of the Various Reactors Combined to HPLC 39

4.5. Mixing 39

4.6 Typical Applications of Post-Column Derivatization 41

5. CONCLUSION..... 44

REFERENCES 45

CHAPTER 2

RATIONALE OF COMBINING TEMPERATURE RESPONSIVE LC TO ENZYMATIC POST-COLUMN REACTIONS 47

REFERENCES 53

CHAPTER 3

TEMPERATURE AS A TOOL TO TUNE RETENTION, SELECTIVITY AND RESOLUTION IN GREEN CHROMATOGRAPHY 55

1. THE INFLUENCE OF TEMPERATURE ON HPLC ANALYSIS 56

2. THE USE OF TEMPERATURE RESPONSIVE STATIONARY PHASES IN HPLC 60

2.1 Temperature responsive Polymers 60

2.2 Temperature responsive Liquid Chromatography 61

2.2.1 PNIPAA for RPLC-like Separations on Silica Particles 62

2.2.2 PNIPAA for IEX-like Separations 65

2.2.3 The Use of Temperature Gradients in TRLC 66

2.2.3 Other Carriers for PNIPAA 67

2.2.4 Other Applications of PNIPAA Based Phases in Separation Science 69

2.2.5 Other Stimuli Responsive Polymers in HPLC 70

3. CONCLUSION 71

REFERENCES 73

CHAPTER 4

TEMPERATURE RESPONSIVE POLY-(N-VINYLCAPROLACTAM) AS STATIONARY PHASE FOR AQUEOUS AND GREEN LIQUID CHROMATOGRAPHY 77

1. INTRODUCTION 78

2. EXPERIMENTAL 79

2.1 Chemicals 79

2.2 Final Synthesis 79

2.3 Analytical Conditions 80

3. RESULTS AND DISCUSSION 80

3.1 Synthesis of a PVCL-based Stationary Phase *80*

3.2 Chromatographic Evaluation of PVCL *83*

4. CONCLUSION 87

REFERENCES 88

CHAPTER 5

DETECTION OF ENZYME INHIBITORS BY HPLC - POST - COLUMN REACTION 89

1. INTRODUCTION 90

2. MATERIALS AND METHODS 98

2.1 Chemicals and Instrumentation *98*

2.2 Experimental *99*

3. RESULTS AND DISCUSSION 101

3.1 Experiments with Cathepsin B using RPLC *101*

3.2 Experiments with Trypsin using TRLC *106*

4. CONCLUSION 112

REFERENCES 114

CHAPTER 6

ANTIOXIDANT ANALYSIS 117

1. INTRODUCTION 118

2. OFF-LINE RADICAL SCAVENGING ANTIOXIDANT ASSAYS 119

3. ON-LINE RADICAL SCAVENGING ANTIOXIDANT ASSAYS 121

4. OTHER ON-LINE ANTIOXIDANT ASSAYS 127

5. ANTIOXIDANT ASSAYS VS. ENZYME-INHIBITOR ASSAY 129

6. CONCLUSION 130

REFERENCES 132

CHAPTER 7

OPTIMIZATION OF AN ABTS RADICAL SCAVENGING ASSAY COUPLED ON-LINE TO HPLC FOR MAXIMIZING PEAK CAPACITY 135

1. INTRODUCTION 136
2. MATERIAL AND METHODS 139
   2.1. Chemicals 139
   2.2. Analytical System 139
   2.3. Optimization 140
   2.4. Analysis of Red Wine 140
   2.5. Analysis of Food Additives and Deep Frying Oil 141
3. RESULTS AND DISCUSSION 142
   3.1. ABTS Assay Optimization 142
   3.2. Antioxidant Capacity 147
   3.3. Analysis of Red Wine 148
   3.4. Analysis of Food Additives and Deep Frying Oil 149
4. CONCLUSION 151

REFERENCES 152

CHAPTER 8

ON-LINE COMBINATION OF ABTS AND DPPH RADICAL SCAVENGING ASSAYS WITH HYDROPHILIC INTERACTION CHROMATOGRAPHY 155

1. INTRODUCTION 156
2. EXPERIMENTAL 157
   2.1 Materials 157
   2.2 Instrumentation 158
   2.3 Analytical Conditions 158
3. RESULTS AND DISCUSSION 160
   3.1 Radical Scavenging Assays Coupled to RPLC 160
   3.2 Radical Scavenging Assays Coupled to HILIC 163

4. CONCLUSION 166
REFERENCES 168

CONCLUSION 169

SCIENTIFIC CONTRIBUTIONS 171

SUMMARY 173

SAMENVATTING 177

LIST OF ABBREVIATIONS 181

DANKWOORD 185

Table of Contents

INTRODUCTION

Detection has been a problem since the introduction of liquid chromatography and despite the number of modern detectors available to chromatographers, new detector systems continue to raise interest. UV-Vis detection has been introduced decades ago and remains the most popular system, but analytes lacking chromophores cannot be detected. For sensitive detection of these analytes, they were often converted by pre- or post-column reactions into colored analytes. With the introduction of mass spectrometry, the use of these reactions has diminished, but remains of importance in, for example, amino acid analysis.

In the last decade, several detectors for universal detection, i.e. capable of detecting all analytes, have been introduced, such as the evaporative light scattering detector. In complex samples, it can however be beneficial to detect not all compounds, but rather scan the sample for the presence of compounds having a certain property of interest. In biochemistry for example, phosphorylated peptides are specifically detected because of the extra biological information they offer. Using post-column reactions, one can target the analytes that can react or influence a reaction, as is the case in antioxidants and enzyme inhibitors, respectively.

Post-column reactions have one major drawback: the introduction of extra dead volume between the column and the detector. This extra volume allows extra diffusion of the analytes and results in peak broadening. The longer the reaction time needed, the more peak broadening is allowed, leading to sensitivity problems when using long reaction times. Even when using short reaction times, optimization of the reaction parameters are needed to preserve peak capacity.

In this work, enzyme inhibitors and antioxidants are specifically detected in complex mixtures. Enzyme inhibitors are of interest in pharmaceutical research, as they are drug candidates. However, enzymes are sensitive to organic solvents, making the commonly used solvents in liquid chromatography incompatible with enzymatic

post-column reactions. This problem was addressed by the use of temperature responsive liquid chromatography, using stimuli responsive polymers coupled to silica as stationary phase and allowing the use of a purely aqueous mobile phase. A novel stationary phase was developed based on poly(N-vinylcaprolactam), as only one polymer, poly(N-isopropylacrylamide) has been used extensively in literature. Despite the purely aqueous reaction conditions, the analysis of enzyme inhibitors by post-column reactions was found to suffer from insensitivity and lack of robustness, due to the long reaction times needed. This is very different for the analysis of antioxidants, where reaction times are much shorter.

Antioxidants are receiving a lot of attention in scientific literature and thus so does their analysis. Targeted antioxidant analysis is possible by the reaction with stable colored radicals. These radical reactions can occur in all solvents and are very fast, making them ideal for coupling with liquid chromatography. For sensitive analysis however, optimization is necessary, as shown by the optimization of the 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) assay. Antioxidant assays have so far been used in combination with reversed phase and normal phase liquid chromatography, but both techniques lack the ability to analyze hydrophilic compounds, which is why in this work, hydrophilic interaction chromatography has been coupled to antioxidant assays.

Chapter 1.

LIQUID CHROMATOGRAPHY AND POST-COLUMN REACTIONS

In this introduction, the basics of both liquid chromatography and post-column reactions will be discussed, as the combination of both techniques was used in this work for the analysis of enzyme inhibitors and antioxidants. As some terms and definitions will be used later in this work and to emphasize some theoretical aspects later needed, the fundamentals of liquid chromatography are discussed first. Secondly, some instrumental aspects are discussed to complete the part on liquid chromatography. For further reading on liquid chromatography, we refer to [1-5]. As post-column reactions and the instrumental aspects thereof are much less known, these will be discussed in depth.

1. BASIC PRINCIPLES OF LIQUID CHROMATOGRAPHY

Chromatography is a physical separation technique based on the partitioning of chemical substances between two immiscible phases of which one is mobile and the other stationary. Chromatographic techniques are divided according to the aggregation state of the mobile phase, i.e. gaseous, liquid or supercritical phases can be used. The corresponding chromatographic techniques are Gas Chromatography (GC), Liquid Chromatography (LC) and Supercritical Fluid Chromatography (SFC), respectively. LC in itself can be subdivided by the format of the stationary phase, which can be positioned in a cylindrical column or on a planar carrier, as in Thin Layer Chromatography (TLC). In a cylindrical column, the stationary phase can be placed on the wall of the column (Open Tubular LC) or on porous particles which are packed in the column. This is called High Performance Liquid Chromatography (HPLC)

and it is today by far the most used LC technique. As only HPLC was used in this work, the further discussion will focus on the characteristics of this technique.

Separation is achieved by moving the mobile phase through the stationary phase. Analytes will migrate at a velocity dependent on their interactions with both phases and the speed of the mobile phase. The difference in interactions of the various solutes is the reason that a separation can take place.

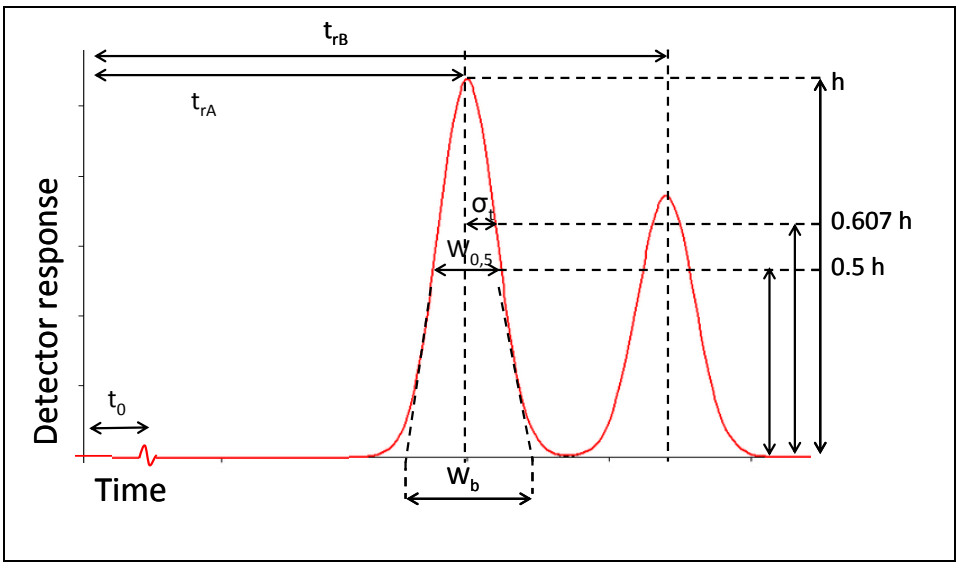


Fig. 1.1. Schematic chromatogram of two separated peaks, representing the dead time (t_0), retention time of peaks A (t_{rA}) and B (t_{rB}), the peak height of peak A (h) and the different variables used to express the width of peak A: the peak width at the base (W_b), peak width at half height ($W_{0.5}$) and the total standard deviation (σ_t).

Under ideal conditions, compounds elute as peaks with a Gaussian shape and they are characterized by a solute dependent retention time (t_r), a height, an area and a width, expressed as the standard deviation (σ) or the corresponding variance (σ^2) (figure 1.1). The standard deviation of the Gaussian peak is equal to half the peak width at 60.7% of the peak height. For further discussion, this will be called σ_t or the

total standard deviation, as this is the sum of a number of factors. The term W_b corresponds to the peak width at the base and is therefore equal to $4\sigma_t$.

Peak broadening is commonly measured at half the maximum peak height ($W_{0,5}$). In chromatography, it is expressed as efficiency or plate number (N) and is calculated as

$$N = \left[\frac{t_r}{\sigma_t}\right]^2 = 16\left[\frac{t_r}{W_b}\right]^2 = 5.54\left[\frac{t_r}{W_{0.5}}\right]^2 \qquad \text{(eq. 1.1)}$$

From the plate number, the plate height (H) can be calculated as H=L/N (L being the length of the column). In a column packed with fully porous particles, the achievable minimum for H (H_{min}) is two times the mean particle diameter of the stationary phase (d_p). The reduced plate (h) height is calculated as H/d_p. Accordingly, a good column thus has a reduced plate height of 2, a parameter which is in theory independent of the column dimensions.

Another way of describing a chromatogram uses the maximum amount of peaks that fit in a chromatogram, a number called the peak capacity. It can be calculated as the average peak width divided by the analysis time plus 1. The peak width is taken at a height where the peaks are $4\sigma_t$ wide.

Two other important chromatographic parameters can be determined experimentally, the retention factor (k) and the selectivity factor (α). The retention factor is related to the retention time and is calculated as

$$k = \frac{t_r - t_0}{t_0} = \frac{t_r'}{t_0} = \frac{K}{\beta} \qquad \text{(eq. 1.2)}$$

where t_0 is the void or dead time (the elution time of a non-retained peak) and t_r' is the corrected retention time. The retention factor is linked to the partition coefficient (K) by the phase ratio (β). K is the ratio between the concentration of the analyte in the stationary and in the mobile phase. The phase ratio β is the ratio between the volume of mobile and stationary phase.

The selectivity factor (α) is a measure for the separation between two peaks and is calculated as

$$\alpha = \frac{k_B}{k_A} = \frac{t'_{rB}}{t'_{rA}} \qquad \text{(eq. 1.3)}$$

where t'_{rA} and t'_{rB} are the corrected retention times for compound A and compound B, respectively. As compound A is thereby eluting before compound B, the selectivity factor is always larger than 1.

The most important chromatographic parameter is the resolution (R_s), a measure for the separation between two peaks. The resolution is chromatographically defined as

$$R_s = \frac{2(t_{rB} - t_{rA})}{W_{bB} + W_{bA}} \qquad \text{(eq. 1.4)}$$

From equation 1.1, 1.2, 1.3 and 1.4, the master equation of chromatography can be derived.

$$R_s = \frac{\sqrt{N}}{4}\frac{\alpha - 1}{\alpha}\frac{k}{k+1} \qquad \text{(eq. 1.5)}$$

The influences of the three governing parameters on the resolution are shown in figure 1.2. The retention factor appears to be of little influence once it is larger than 3. The main contributor to the optimization of resolution is the selectivity factor. This is easily apparent from the number of HPLC phases available on the market compared to the limited number of phases which are used in GC, where very high efficiency is mostly responsible for the quality of the separation. However, selectivity is the most difficult parameter to optimize as it depends on the chemical properties of the mobile and stationary phase and of the analytes. The LC modes to choose from to optimize the selectivity, are discussed further in this chapter.

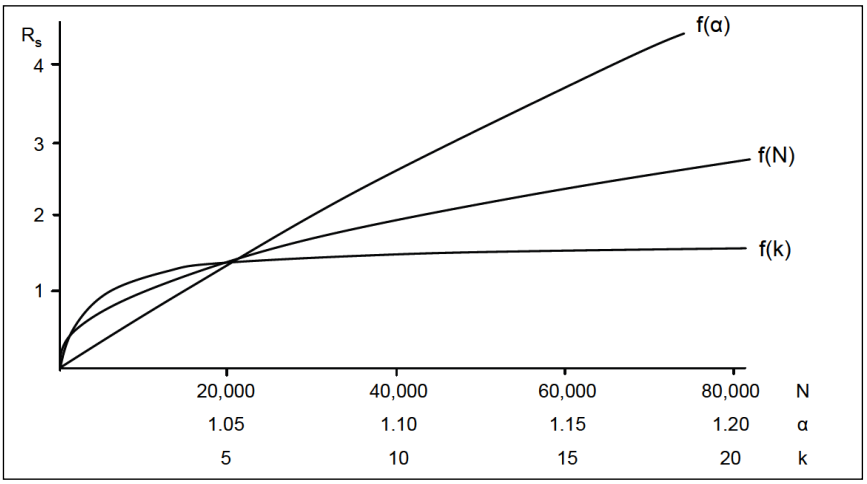


Figure 1.2. Influence of the plate number (N), the selectivity factor (α) and the retention factor (k) on the resolution (R_s).

In the last few years, there has been much renewed interest in efficiency enhancement in HPLC. For example sub-2 µm particles [6] (requiring high pressure instruments) and superficially porous particles [7] have been introduced to provide high efficiency in short analysis times. The use of extended column lengths in combination with elevated temperatures [8] or low viscosity solvents has also proven successful in significantly increased plate numbers, but this will be shown in chapter 3.

The efficiency of an analysis can be negatively influenced by a variety of factors. Band broadening mainly occurs during the chromatographic process, known as intra column band broadening, but it can also arise in any other part of the LC system (extra column band broadening), if this has not been properly optimized. This can be expressed as a sum of variances.

$$\sigma_t^2 = \sigma_c^2 + \sigma_o^2 = \sigma_c^2 + \sigma_i^2 + \sigma_d^2 + \sigma_f^2 \qquad \text{(eq. 1.6)}$$

where σ_t^2 is the total variance, σ_c^2 is the chromatographic variance, σ_o^2 is the variance introduced by processes outside the column, σ_i^2 is the variance introduced by the injector, σ_d^2 is the variance introduced by the detector and σ_f^2 is the variance caused by the fluidic path between the injector and detector, i.e. the tubing and connectors. Extra column band broadening can be caused by many factors. Injection of large volumes or improper mixing of the sample plug and mobile phase can lead to pre-column band broadening. Using large connecting tubes, faulty connections or a too large detector cell can lead to stagnant zones and cause large dead volumes, giving the analyte bands time to disperse and thus to broaden, as is the case for post-column reactors. In an HPLC system where all extra column band broadening has been minimized, σ_c is responsible for most of the measured band broadening. As will be shown later, this is not the case when post-column reactors are applied.

Note that, the peak width in chromatography is usually measured in time units. If spatial units are required, the standard deviation in length units (σ_L) is used. The link between σ_L and $\sigma_{t'}$ (the standard deviation in time units, t' being applied to distinguish it from the total standard deviation in a chromatographic process (σ_t)), is the linear velocity of the mobile phase (u_0), calculated as the column length (L) divided by the dead time (t_0).

$$\frac{\sigma_L}{u_0} = \sigma_t' \qquad \text{(eq. 1.7)}$$

The van Deemter equation is the most widely used model to describe intra column band broadening [9].

$$H = A + \frac{B}{u} + Cu \qquad \text{(eq. 1.8)}$$

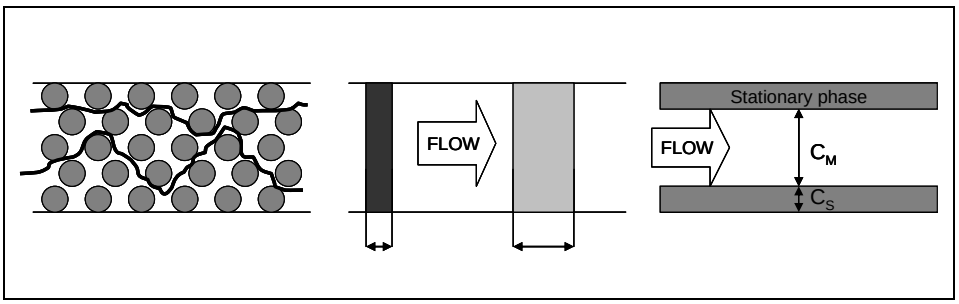


Fig. 1.3. Terms of the van Deemter equation (eq. 1.8). Left: The A-term (eddy dispersion). Two random paths through a packed column are shown. Difference in path length accounts for peak broadening. Middle: B-term (longitudinal diffusion). As an analyte band travel through the column, it broadens because of diffusion. Right: The C-term (resistance to mass transfer). C_M and C_S are respectively the resistance in the mobile and stationary phase. For ease of representation, this is shown in a capillary column.

The terms of this simple model are the A-term or eddy dispersion, the B-term or longitudinal diffusion and the C-term or the resistance to mass transfer. They are graphically represented in figure 1.3.

The eddy dispersion term, the A-term in equation 1.8, is a factor contributing to band broadening due to the differences in path length as experienced by the different analyte molecules when traveling through a packed column. The difference in path lengths of the solutes is dependent on the quality of the packing material and the packing itself. As this is a random process, the resulting peak shows a Gaussian shape. The A-term can be minimized by using materials that can be orderly packed (perfect spheres) with a very small size distribution. Accordingly, the A term is 0 for open tubular columns, as used in GC and Open Tubular Liquid Chromatography (OTLC). The A-term can be mathematically described as

$$A = \lambda' d_p \qquad \text{(eq. 1.9)}$$

λ' is a factor expressing the quality of the packing. It is normally between 1.5 and 2 and if it exceeds 2, the packing quality is not as good as it could be. In very well packed columns, values lower than 1.5 can be observed.

The B-term represents the longitudinal diffusion occurring in the column. As a band of analytes moves through the column, analyte molecules will diffuse in every direction, according to Ficks laws of diffusion. Although diffusion occurs in both the radial and axial direction, only the latter is affecting the eventual peak width. The B-term can be calculated as

$$B = 2 \gamma' D_M \qquad \text{(eq. 1.10)}$$

γ' is obstruction factor and depends an the amount and nature of the obstruction against the free movement of a molecule. It is normally between 0.5 and 1. D_M is the diffusion coefficient in the mobile phase.

The C-term expresses the resistance to mass transfer and is the sum of C_M, the resistance to mass transfer from the mobile to the boundary of the mobile and stationary phase, and C_S, the resistance to mass transfer in the stationary phase. The resulting van Deemter curve (or H-u plot) and the three factors contributing to it are shown figure 1.4.

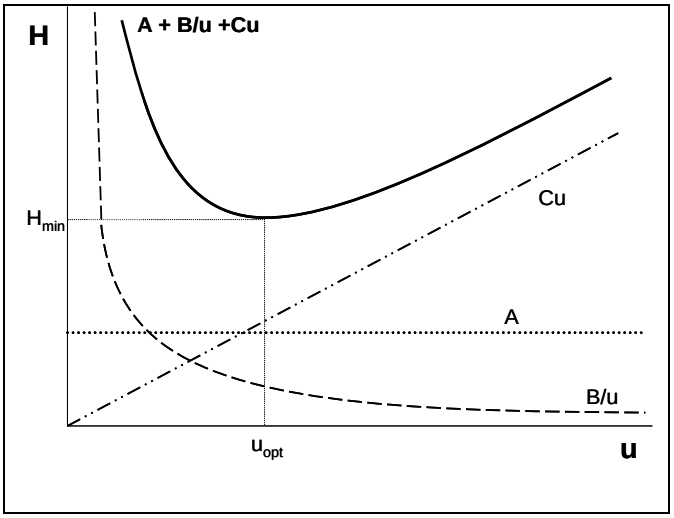


Fig. 1.4. The van Deemter curve, with contributing factors. H_{min} is the minimal plate heigth, corresponding to u_{opt} or the optimal mobile phase velocity.

The peak variance component related to the A-term is independent of mobile phase velocity. However, the influence of the combined effect of the longitudinal diffusion and of the resistance to mass transfer on peak broadening shows an optimal linear velocity where the dispersion is minimal. This optimum depends on the choice of

column (chemistry of the stationary phase and particle size), the type of solute analyzed, the mobile phase which is used and the column temperature.

More models on peak broadening in chromatography, such as the Knox [10] and Giddings equations [11], have been described, which have been described in literature, for example.

From equation 1.1, σ_c can be calculated for conventional and state-of-the-art column dimensions (see table 1.1), using the following equation.

$$\sigma_c = \sqrt{\frac{t_r^2}{N}} \qquad \text{(eq. 1.11)}$$

Table 1.1. Comparison of different column types by σ_c. Note the calculated efficiency is only valid for ideal conditions and very well packed columns with *h*=2.

column	L (mm)	d_p (µm)	N	t_r (min)	σ_c (s)
Column A	250	5	25,000	20	7.6
Column B	150	3	25,000	12	4.6
Column C	75	1.5	25,000	6	2.3

All the columns have 25,000 plates under ideal circumstances, but the peak width of an eluting peak can be very different depending on the column dimensions. Because of this, extra-column band broadening factors will affect the peak width of a narrow peak in a much more dramatic way. This is the case when using shorter columns with smaller particles where retention time is reduced.

In practice, this effect is further enlarged by the shape of the van Deemter curves, as shown in figure 1.5. The van Deemter curves for different particle sizes show that

columns with smaller particles can be operated at higher flow rates without loss in efficiency. This means the retention time can be further decreased, resulting in an even smaller standard deviation. For example, if the flow rate is 3 times higher on column C, this leads to a retention time of 2 min without loss in efficiency, resulting in a σ_c of 0.8 s.

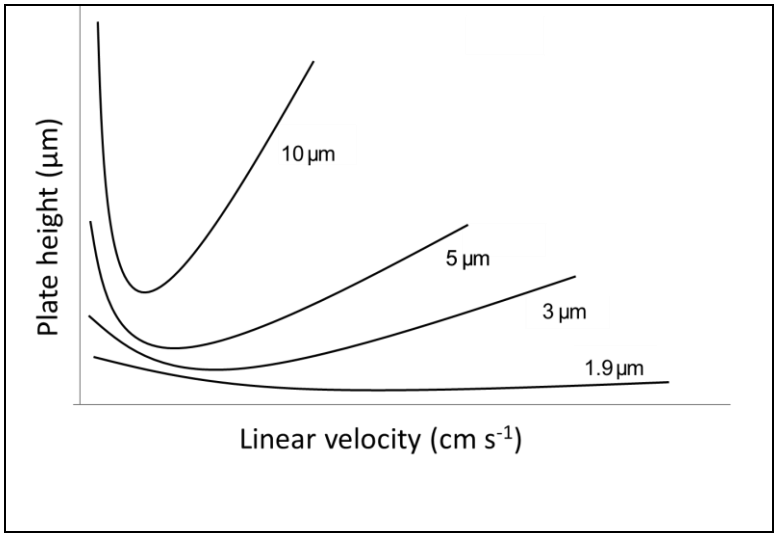


Fig. 1.5. van Deemter curves for columns packed with different particle sizes.

In this work in which post-column reactions are the research subject, conventional 250 mm columns packed with 5 µm particles are used. Since the peak broadening introduced by the detector system described further would be very large, the application of columns with smaller particles and smaller lengths makes no sense.

2. SEPARATION MODES IN HPLC

As for the optimization of the selectivity, there are a number of LC modes to choose from, depending on the analyte. Nowadays, there is a wide variety of columns commercially available, but all of them belong to one of the modes described below. The discussion is kept brief, as only two modes are used in this work.

The oldest form of liquid chromatography and the mode used by Tswett in the original publication [8], is known as normal phase LC (NPLC). It uses a polar stationary phase, such as pure silica or bonded phases such as aminopropyl, cyanopropyl and diol, and an apolar mobile phase, such as n-pentane, iso-octane, ethyl acetate, etc. NPLC is very useful for the separation of analytes with polar functional groups, which are problematic to separate using other LC modes. The wide variety of solvents to choose from allows fine tuning of the analysis, but polar contaminants in the solvents can cause irreproducibility and long equilibration times. Care must be taken not to contaminate the organic phases with water, which can be very difficult for hygroscopic solvents. Forthese reasons, NPLC has been surpassed in popularity by reversed phase LC.

Reversed phase LC (RPLC) is by far the most used LC mode in modern HPLC. Compared to NPLC, the polarity of the phases is switched, hence its name, so RPLC uses an apolar stationary phase and a more polar mobile phase. RPLC is capable of separating a wide variety of analytes, both in molecular weight and polarity and it is able to produce very reproducible analyses. A wide variety of bonded phases is available, such as alkyl (C4, C8, C18 and C30), cyanopropyl and phenyl phases. The C18 RPLC column especially is extremely popular and versatile. RPLC is used for 90% of all LC analysis of small molecules. Addition of counter ions, as used in ion pairing LC, has further expanded the working field of RPLC.

Ion exchange chromatography (IEX) uses a stationary phase with ionic groups, which can be weak or strong acids or bases, to retain ionic compounds. The mobile phase contains counter ions, to push the analytes from the column or a shift in pH, to

change the ionic interactions between the analytes and the stationary phase. This form of chromatography is highly useful for the analysis of very polar molecules, including various biomolecules such as peptides and amino acids. The phases available for IEX can be divided by the ion type of the stationary phase and whether these are strong or weak acids or bases. The column types are thus strong cation exchange (SCX), weak cation exchange (WCX), strong anion exchange (SAX) and weak anion exchange (WAX).

Recently, hydrophilic interaction chromatography has gained a lot of attention. It uses similar stationary phases to NPLC, such a pure silica, but combined with a mobile phase containing up to 20% water. The separation is based on the formation of a pseudo-stationary water layer on the silica surface, serving as the stationary phase. It can be used to separate more polar, ionizable or ionic compounds with low or no retention on RPLC columns.

Affinity chromatography is a mode of chromatography using affinity ligands bound to the stationary phase to capture and retain the compounds of interest. They can be eluted by a stronger binding agent or by changing the binding properties for example by a change in pH.

A separation based on size can be achieved using size exclusion chromatography. The pores in the particles then serve as a filter and smaller solutes will travel a longer route through the column as they can enter more pores.

In this work, RPLC was mostly selected when working with enzymes, because of the better compatibility of the largely aqueous mobile phase with the post-column reactions used. HILIC and NPLC both use large concentrations of organic solvents and IEX uses strong acids and/or bases, making these techniques less suitable for work with enzymes. In the antioxidant activity studies, both RPLC and HILIC were applied.

3. Instrumental Aspects of HPLC

All HPLC systems are composed of four major components: mobile phase pumps, an injector to introduce the sample, a column and a detection system. Although additional parts can be included, such as a degasser, column oven, fraction collector, etc. the discussion below is limited to the four essential components.

3.1 The Injector

The injector introduces the sample onto the column, under conditions where the mobile phase is percolated at constant velocity through the column. Essential hereby is that a small plug of sample is introduced in the system without affecting the flow rate and pressure in a significant way. In analytical HPLC systems, the injector requires a valve system to deliver the sample into the flow of the mobile phase (The biggest challenge for the injector is the reproducible delivery of the sample and the need to minimize extra column band broadening.

3.2 The Pump

The pump delivers the mobile phase at constant flow rate, but often at variable mobile phase composition. This is needed as modern HPLC often uses gradient elution. The strict necessity of a constant flow rate and reproducible composition is best met with binary pumps, nowadays often able of delivering pressures up to or over 1,000 bar. A constant flow rate is needed to minimize peak broadening, both in and out of the column, but also for reproducible retention times and peak areas, as the response of most HPLC detectors is sensitive to the flow rate.

3.3 The Column

The column is the heart of each chromatographic system, as it actually separates the analytes. Today, HPLC separations are almost exclusively performed with columns packed with micron-sized spherical silica particles.

The internal column diameter of an analytical HPLC column varies from 4.6 to 1 mm for routine applications, with 4.6 and 2.1 mm as the most used column diameters. Preparative LC uses larger diameters as this allows loading larger volumes and concentration of samples. Capillary LC uses very small diameter packed or open tubular columns, and these columns are most used in biochemical research where small sample volumes and very complex samples are often used and analysis time is of less consequence. The flow rate, and thus solvent consumption, is proportional to the square of the column internal diameter. This relationship allows changing the column internal diameter and recalculating the flow without the need to redo the optimization as described by the van Deemter curve (equation 1.8).

The quality of the packing and the type of packing material are closely related. Efficient packing materials are spherical with a narrow particle size distribution, as this decreases the A-term in the van Deemter equation and thus peak broadening (see equation 1.8). Ideally the material should be packed closely in an orderly manner to increase the efficiency, but this is not always the case (see equation 1.9). A well packed column has an external porosity of approximately 40%. As stated earlier, very small particles (sub 2 µm) and solid core particles can be used to increase efficiency.

3.4 The Detector

In its earliest form, chromatography introduced by Tswett allowed only the visual determination of colored analytes [12]. Subsequently, fractions of the effluent were collected and analyzed spectrophotometrically and the resulting signals were plotted versus fraction number and a chromatogram was obtained. As many compounds

amenable to HPLC analysis do not absorb in the visible or UV range, a large variety of alternative detectors and pre- and post-column derivatization techniques have been developed over the years, including refractive index, evaporative light scattering, charged aerosol detectors and many more. This discussion is limited to UV-Vis and mass spectrometry (MS) as those are the detectors applied in this work.

3.4.1 UV-Vis Detection

In the UV-Vis detector (figure 1.6), a beam of light (mono- or polychromatic) is passed through the column effluent and the absorption is measured. Several types of UV-Vis detectors have been developed. In a conventional single wavelength UV-Vis detector monochromatic light is sent through the column effluent. Variable wavelength detectors allow the use of 2 or more distinct wavelengths. Monochromatic light is obtained from a light source emitting polychromatic light which is filtered by a monochromator (a prism or a diffraction grating). In earlier models, monochromatic light sources, such as mercury lamps (emitting at 254 nm), were routinely used. The amount of light absorbed by a compound is proportional to the concentration of the analyte and the path length, as expressed by the law of Lambert-Beer.

$$A = \log\frac{I_0}{I} = \varepsilon\lambda c \qquad \text{(eq. 1.12)}$$

where I_0 is the intensity of the incident light, I is the intensity of the absorbed light, A is the absorption, c is the concentration of the analyte, λ is the path length. ε is called the extinction coefficient which is constant for a specific compound at a given wavelength.

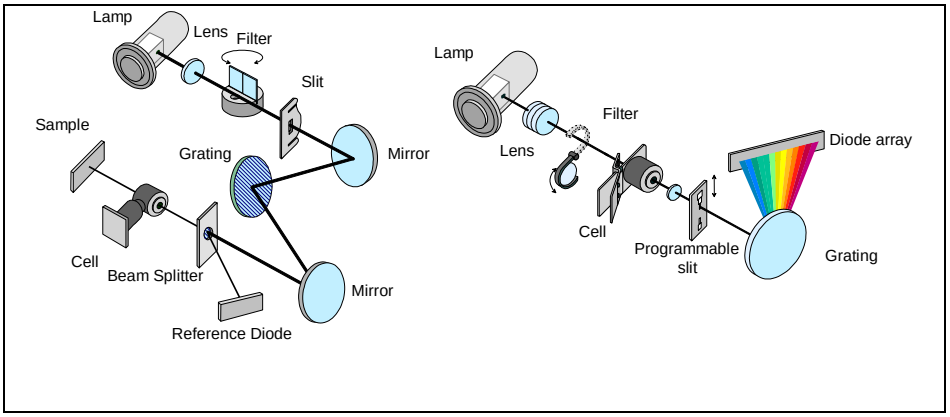


Fig. 1.6. Schematical drawing of the variable wavelength UV detector (left) and DAD detector (right).

In the diode array detector (DAD see figure 1.6), polychromatic light is beamed through the flow cell and subsequently split into the composing wavelengths using a prism or grating device. Detection of the beams is performed by means of an array of diodes, allowing construction of the UV spectra of the signals eluting from the HPLC column. This allows for easy identification when comparing with a library and extraction of the absorbance at a specific wavelength by using the data obtained from specific diodes.

Because of their simplicity, sensitivity and ruggedness, UV-Vis and DAD detectors are nowadays the most popular detection systems in LC.

Before the widespread use of mass spectrometry, compounds lacking chromophores were often converted into UV-active solutes. For example, amino acids, peptides, sugars and carbamate pesticides show naturally poor absorbance in UV detection (typically between 200 and 700 nm). In table 1.2 some popular derivatization reagents allowing subsequent UV detection are summarized [13]. Derivatization can be done before or after separation, called pre- and post-column derivatization respectively. The benefit of the former is that it can both change the chromatographic properties of the analytes, allowing for better separation, and the detectability of the analytes. Furthermore, the reaction kinetics are of less

importance, as the sample preparation can be performed off-line. Post-column derivatization does not require adaptation of the separation, which can be an advantage or a disadvantage, and it requires fast reaction kinetics, except when fraction collection is used.

Table 1.2. Derivatization reagents for common functional groups

Functional group	Derivatization reagent
$-NH_2$	o-phthalaldehyde ninhydrin
-NHR	ninhydrin
-COOH	p-bromophenylacylbromide 2-naphtacylbromide
-OH	phenylisocyanate
-CHO and –CO-	2,4-dinitrophenylhydrazine

3.4.2 Mass Spectrometry

The coupling of MS to LC has been a main breakthrough in separation sciences. LC-MS has evolved into a stable technique and is now being used extensively in different fields. In MS, the analytes are converted into gas-phase ions in the ion source, which are then separated according to their mass to charge ratio (m/z) in the mass analyzer and finally detected often by an electron multiplier. This allows elucidation of the molecular weight of the analyte and if further fragmented, to obtain structural information. This is often performed by tandem mass spectrometry (MS/MS): the serial coupling of two mass analyzers, via an intermediate fragmentation.

Many ionization sources have been developed over the last decades, but the real breakthrough has come with the appearance of atmospheric pressure ionization sources; electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI) and atmospheric pressure photon ionization (APPI). ESI is by far the most used ion source and the one used in this work. It was introduced by Fenn who was awarded the Nobel prize for this work [14].

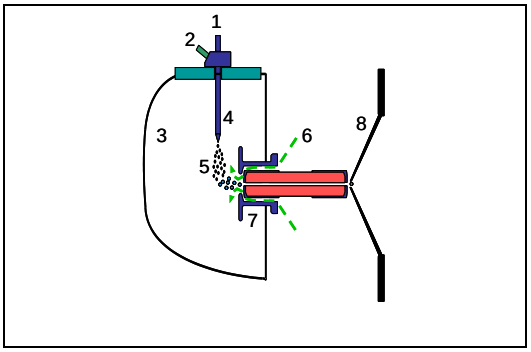


Fig. 1.7. Electrospray ionization. 1. LC effluent, 2. N_2 nebulizing gas, 3. ionization chamber, 4. ESI needle with Taylor cone at the tip, 5. solvent droplets, 6. heated N_2 drying gas, 7. MS inlet, 8. skimmer (first MS lens).

In ESI, the column effluent is channeled through a needle which is exposed to a potential drop of several kV at its tip, causing the formation of the Taylor cone at the tip of the needle. A fine mist of charged droplets, known as a spray, escapes from this tip carrying the analyte molecules. During desolvation, aided by a counterflow of heated nitrogen gas, the droplets shrink up to a point where the surface tension of the droplet equals the electrostatic repulsion of the charges in the droplet. This point is known as the Rayleigh limit. Beyond this point, the droplet disintegrates into smaller droplets and the process is repeated, until the analyte molecule is left as a gas phase ion. One disadvantage of ESI is that it often produces multiple charged analyte molecules, and as they are separated on their mass to charge ratio, one

analyte can give multiple signals. For small molecules this problem is minor, but when analyzing polymers (synthetic or biological), spectrum deconvolution is often necessary. ESI is also prone to adduct formation: other than protons, Na^+, K^+, NH_4^+, etc. adducts can be observed, dependent on experimental conditions.

Various mass analyzers have been developed, differing in sensitivity and mass resolution. Mass resolution is defined as the smallest difference between two mass signals divided by the mass of the analytes (Δm/m). Δm can be measured as the width of a peak at half its height, what is known as full width at half maximum (FWHM). The most used and cost effective mass analyzer is the quadrupole, which is capable of unit resolution, meaning it can distinguish between two peaks at a difference of 1 Da or a resolution of 2,000. High resolution mass spectrometers have resolution of 20,000 or more.

The quadrupole is made out of four parallel rods, as shown in figure 1.8. It functions as a mass filter, as only ions with a defined mass to charge ratio will have a stable trajectory through the analyzer. Opposing rods are pairwise connected and a radio frequency voltage and a direct current are applied. Because of the complex and changing fields in the analyzer, most ions will collide with the rods and only ions with a specific mass to charge ratio travel through the quadrupole. An inherent disadvantage of the quadrupole is its lack of sensitivity when used in scanning mode. Due to the fact that it is a filter, rather than a separation device, only a small amount of ions will reach the detector. If one is only interested in one or a few specific molecules, the quadrupole can be used to detect only a small number of mass to charge ratio's, allowing for much higher sensitivity. The main advantage of the quadrupole is its price, as it is relatively cheap compared to other instruments, such as the time of flight (TOF) mass spectrometer. The TOF has a much higher sensitivity and mass resolution (20,000 up to 40,000), but is more costly.

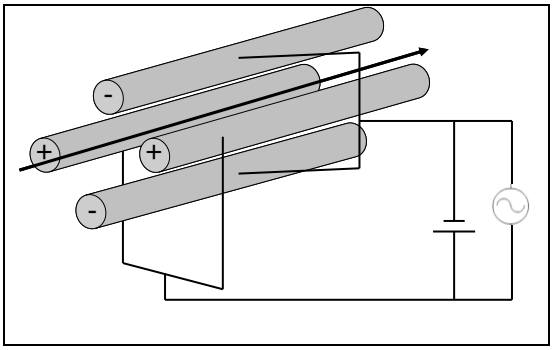


Fig. 1.8. Quadrupole mass analyzer with the trajectory of an ion that will reach the detector.

In a time-of-flight, ions are accelerated by an electric field and travel through a vacuum tube, which has the detector at the end (detailed in figure 1.9). The time an ion needs to fly through the tube is used to calculate its mass to charge ratio, using the potential energy given by the electric field and the length of the tube. The resolution of a time-of-flight mass spectrometer is further enhanced by the use of a reflectron, a device that reflects the ions in the flight tube using a constant electric field. This has two advantages: it lengthens the flight distance, creating a bigger time gap between ions of different mass to charge ratio, and it allows focusing of ions with the same mass to charge ratio but a slightly different initial speed (because of the position in the source or the initial velocity before acceleration). Higher energetic ions will penetrate the electric field further and ions with the same mass to charge ratio will reach the detector simultaneously. Another technique to enhance the resolution of the time-of-flight mass analyzer is the use of an orthogonal acceleration, meaning the ions are accelerated by the electric field in a direction orthogonal to the direction they arrive in the field. Using this technique, the speed of the ions in the direction of the flight tube is almost zero for all ions. As all ions reach the detector in time-of-flight, it has a significantly higher sensitivity than the quadrupole.

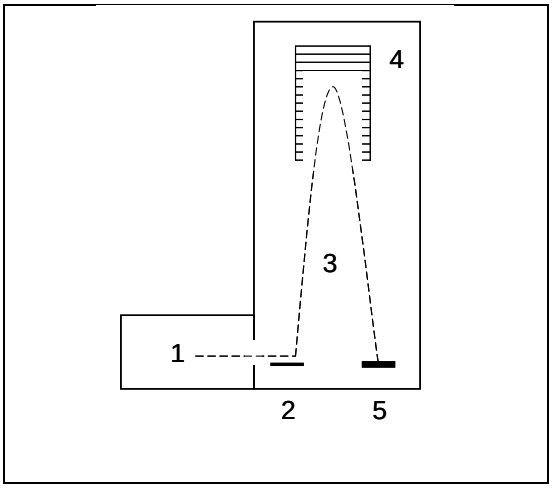


Fig. 1.9. Time-of-flight mass analyzer with orthogonal acceleration and reflectron. Dashed line: path of an ion. 1. ion source, 2. repeller plate (generating the electric field for the acceleration), 3. flight tube, 4. reflectron, 5. detector.

Other mass analyzers have even higher resolution, with the orbitrap going up to 240,000 and the Fourier Transfrom Ion Cyclotron Resonance MS up to 500,000 as the instrument with the highest mass resolution.

Despite the extra information quadrupole and TOF mass spectrometers deliver, there is still the need for more details, certainly when analytes have closely resembling structures or masses. This problem can be solved by the introduction of tandem mass spectrometry, using two mass analyzers, coupled by a collision cell. In this cell, the analytes collide with gas atoms, fragmenting the analyte ions. This serves two purposes: 1. extra structural information can be derived from the mass spectra (allowing the identification of for example certain functional groups or the amino acid sequence of a peptide) and 2. more selective detection, as only if the selected ion produces the correct fragment(s), it is considered the compound of interest.

The two most used instruments for LC-MS/MS are the triple quadrupole or triple quad (QQQ) and quadrupole-time-of-flight (QTOF) instruments. In the triple quad, the second quadrupole serves as the collision cell, while the others serve as mass analyzers. Again, the QTOF provides higher sensitivity and higher mass resolution, but at a higher price, making the triple quad the most used instrument for routine qualitative and quantitative analyses. Other mass spectrometers, such as the ion trap, orbitrap and Fourier transform ion cyclotron resonance MS also allow fragmentation, but without the need for a second mass analyzer. In this study, quadrupole MS was chosen for the monitoring of known analytes and TOF MS was chosen for identification in complex samples.

3.4.3 Specific Detection

Since a number of detectors have been developed allowing the detection of analytes that cannot be detected by UV-detection, the use of derivatization reactions with HPLC has decreased. However, there are still some disadvantages of for instance MS compared to post-column reactions: possible smaller linear range, more expensive instrumentation, lab technicians less familiar with MS, etc. In those cases, specific detection by means of post-column reactions can offer a solution.

The term “specific detection” is used here as the opposite for universal detection. In universal detection, the aim is to detect all compounds in a mixture. Specific detection aims at detecting only compounds with a well-defined, specific property. These properties can be diverse: a specific chemical property or structure or a specific chemical or biochemical activity, such as enzymatic inhibitors or antioxidants. Not-universal detectors or selective detectors do not always provide information on a certain property of the analyte, especially in GC.

Post-column reactions can sometimes be used for specific detection because they allow the use of a reaction resulting in a detectable product if a suitable functional group is present on the molecule or if the molecule has a desired activity. An

important note here is that post-column reactions for specific detection do not necessarily need coupling to UV-Vis or fluoresence detection. The reaction product can for example also be detected by MS. MS is an ideal detector as it can be used to detect only the reaction product, by following that particular molecular weight.

4. Post-column Derivatization in HPLC

In this work, post-column reactions for the specific detection of enzyme inhibitors and antioxidants were studied as described further. Several reactor types have been developed for the use in post-column derivatization: open tubular reactors, packed bed reactors and segmented flow reactors. Despite all research invested in post-column reactions, there is one serious disadvantage of all post-column reactions. The additional "dead volume" the reactor introduces between the column and the detector, causes peak broadening (see equation 1.6). The most interesting reactors are therefore the ones where this is minimized, without affecting reaction kinetics and permeability.

4.1 Open Tubular Reactors

Open tubular reactors are the most simple reactor type as they simply consist of a piece of tubing that can be made from different materials: stainless steel, polyether ether ketone (PEEK), polytetrafluoroethylene (PTFE), etc. Their advantages are the simple instrumentation and good reproducibility [15]. However, the analytes can diffuse freely leading to peak dispersion, linearly increasing with the reactor length [16]. This is described by the Einstein-Smoluchowski equation [17]:

$$\sigma_L^2 = 2D_M t \qquad \text{(eq. 1.13)}$$

where D_M is the diffusion coefficient of the analyte in the mobile phase, t is the reaction time and σ_L represents the standard deviation of the Gaussian peak in length units. The spatial peak variance (σ_L^2) can be recalculated into variance in time units using equation 1.7. The linear velocity (u) is the reactor length (L) divided by the reaction time (t). Note the linear velocity here is slightly different from the one used in chromatographic equations. The variance introduced by this type of reactor can be written as

$$\sigma_t^2, = \frac{2D_M t}{u^2} = \frac{2D_M L}{u^3} \qquad \text{(eq. 1.14)}$$

Equation 1.14 shows the influences of different parameters on the peak broadening in open tubular reactors. Faster diffusion and longer reactors are disadvantageous to the peak width, while an increased flow rate is beneficial. The flow rate is hereby the most influential influence, as it is linked to the variance in by a third power. The linear velocity u used here differs slightly from the one used in chromatographic equations. It can still be seen as the speed at which a non-retained peak moves through the reactor, instead of through the column, and all peaks should be non-retained in the reactor.

The variance introduced by the reactor is a factor contributing to σ_f^2, as it introduces extra tubing and thus extra dead volume to the system. Because of this peak broadening, the regular open tubular reactor is limited to applications with short reaction times.

The chromatographic efficiency and sensitivity of the open tubular reactor could be increased by knitting the tubing: a series of knots is made in the tube, disturbing the regular, parabolic flow profile [18]. There are many types of knitting possible for the construction of reactors, an example for PTFE is shown in figure 1.10. However,

there are also reports in literature that the influence of knotting the tubing is negligible [19].

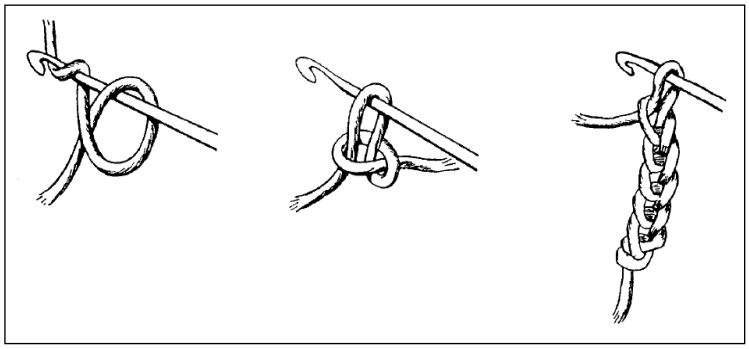


Fig. 1.10. Example of knitting a reactor [20].

Because of the knots, the flow pattern is also disturbed and circular flow patterns perpendicular to the primary flow (along the length of the tubing) are created. These secondary flows ensure better mixing and thus decrease reaction times as they are no longer dependent on diffusion alone [17].

Open tubular reactors generally do not cause extensive back pressure, unless very long reactors or very small internal diameters are used. PTFE tubing can only be used at low pressure (< 50 bar), but PEEK and conventional stainless steel tubing can be used at high pressures (<300 and <1,000 bar respectively). The back pressure for an open tubular reactor can be calculated using the Hagen-Poisseuille equation, after adaption for knitted coils [17]:

$$\Delta P = \beta' \frac{8FL\eta}{\pi r^4} \qquad \text{(eq. 1.16)}$$

where ΔP is the pressure drop, η is the viscosity, F is the flow rate, L is the length of the tube, r is the internal diameter of the tubing, β' is the Dean factor, increasing with smaller diameter of the knots (β' is by definition 1 for straight capillaries). This is only valid for laminar flows. At higher flow rates, the flow will become turbulent and the pressure drop over the reactor will increase drastically. As the flow (F) can be written as a function of the reactor volume (V) and the reaction time (t), it follows that

$$F = \frac{V}{t} = \frac{\pi r^2 L}{t} \qquad \text{(eq. 1.16)}$$

and since the linear velocity (u) corresponds to the reactor length (L) divided by the reaction time (t), the pressure differential over the reactor can be written as

$$\Delta P = \beta' \frac{8uL\eta}{r^2} \qquad \text{(eq. 1.17)}$$

Equation 1.17 demonstrates that the pressure drop is dependent on the linear flow, but the influence is smaller compared to the influence on the peak broadening. In other words, the back pressure increases much slower than the peak broadening decreases when higher flow rates are used, provided laminar flows are used.

4.2 Packed Bed Reactors

Packed bed reactors are reactors made from a tube with large internal diameters and packed with beads. The beads should be made of inert material that does not retain the analytes or the post-column reagents, as peaks will be distorted severely

by interaction with the beads. Non porous particles give the smallest amount of peak broadening. As in LC, the smaller the particles are, the better they perform, but they provide the highest back pressure. Reaction times (t) can be calculated as the extra particle volume of the reactor (V_e) divided by the flow (F).

$$t = \frac{V_e}{F} \qquad \text{(eq. 1.18)}$$

4.3 Segmented Flow Reactors

Peak broadening occurs when analytes diffuse into the solvent adjacent to the peak. If the effluent is fractionated, the broadening stops. On-line fractionation can be done using a segmented flow, as introduced by for clinical analysis in 1957 [21,22]. A gas flow or a flow of immiscible solvent is added and bubbles form at regular intervals, fractionating the effluent. Before detection, both phases are separated using a phase separator, to allow the use of conventional detectors. Gas segmented reactors, as shown in figure 1.11, will be reviewed first.

Diffusion of the analytes into the adjacent segments is not stopped entirely because of two reasons: a thin film of solvent forms on the reactor wall, allowing diffusion from one segment into another and small dead volumes in imperfect connectors [23]. Two equations are important [17]:

$$\sigma_{t'}^2 = \frac{2\pi^2 d_f L r^3 L_s}{F} \qquad \text{(eq. 1.19)}$$

$$d_f = \pi r \left(\frac{u\eta}{\gamma}\right)^{2/3} \quad \text{(eq. 1.20)}$$

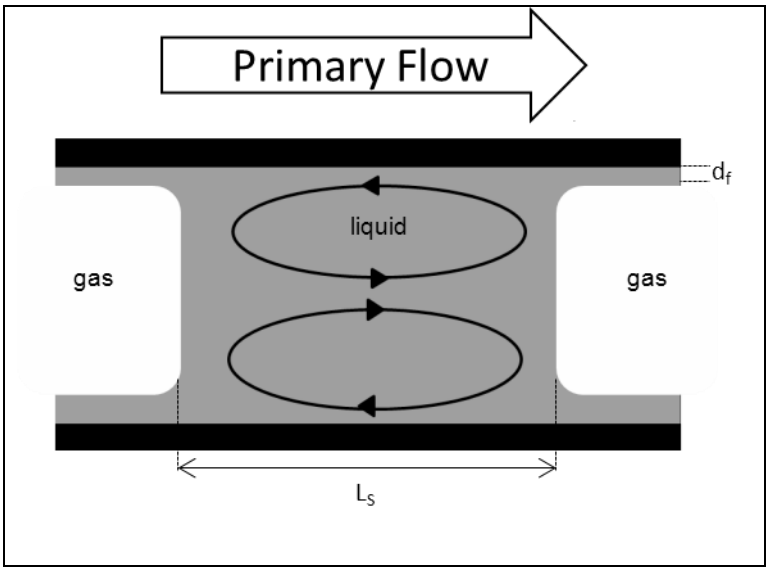


Fig. 1.11. Secondary flow in gas segmented reactors. Grey area: liquid, black area: tube, white area: gas bubbles. Shown are a liquid segment and a part of the proceeding and preceeding gas bubble. The primary flow is directed left to right. The black arrows indicate the direction of the secondary flow.

where σ'_t is the standard deviation of the Gaussian peak in time units, d_f is the film thickness, L is the length of the reactor, r is the radius of the capillary, L_s is the length of a solvents segment, F is the flow rate, u is the linear velocity and γ is the surface tension of the liquid. The experimental parameters that can be optimized are thus the flow rate, choice of the solvent, choice of reactor material, reactor dimensions, temperature (influencing the viscosity) and length of the solvent sections, controlled by the gas flow. By optimizing these parameters, long reaction times can be achieved without creating extensive band broadening when working with low efficiency HPLC. Because of the large amount of parameters to be optimized and the expected interference between some of them, optimization of these six parameters requires a

large number of experiments. Using experimental design, even in the simplest full factorial design (i.e. no repetitions and only two levels per parameter) 64 experiments are needed.

In gas segmented reactors, circular mixing in each liquid segment is observed [17,23]. The outer layers of the solvent are slowed by contact with the wall, causing the secondary flows as shown in figure 1.11. The solvent adjacent to the reactor wall (black line) will be slowed down compared to the liquid in the center of the tube, causing a secondary flow. In a normal capillary, this leads to a parabolic flow pattern. Because of the segments, a circular pattern forms: the outer layers go slower, while the inner layers move faster. This repeats itself in every segment, increasing mixing drastically.

Liquid segmented reactors are comparable, but extra care should be taken. The film on the capillary wall can be formed by either the effluent or the immiscible solvent, depending on the solvents and the reactor material. The reagents can be in the immiscible solvent, but then the reaction occurs only on the contact surfaces of both solvents. Reagents, analytes or products can also be introduced in one solvent, but diffuse to the other phase. If the product is dissolved in both phases, sensitivity will be influenced as only one phase reaches the detector.

This type of reactor is quite complicated, as several extra parts need to be introduced. Older phase separators, based on the difference in density between the two phases, introduced severe band broadening [17], but recently introduced microfluidic devices have been developed to address this problem [24,25]. They are being used for example in microbiology [26], immunoassays [27] and coupled to HPLC [19].

4.4 Advantages and Disadvantages of the Various Reactors Combined to HPLC

The three reactor types discussed are being used today for post-column derivatization. There are contradictory reports in literature of the use of open tubular reactors; some reports claim the use with enzymatic reactions (see chapter 5) and others claim their application with long reaction times is detrimental to sensitivity [15,19]. However, these reactors can be made in house very easily and are quite robust.

Two problems appear when combining packed bed columns with HPLC; extensive back-pressure and the packing of the reactor. The former is almost unavoidable when using this type of reactor and can only be alleviated by increasing the particle size of the reactor or decreasing the reactor length. Both solutions are however detrimental as bigger particles will diminish the chromatographic efficiency and the effective mixing of the reagents, while a shorter length will decrease the reaction time. Additionally, packed bed reactors are not commercially available, leading to the necessity to pack these reactors in house and to reproducibility problems.

As for segmented flow reactors, they have been reported to be used with long reaction times [15,17], but remain complex. Because of the pulsating nature of the flow and the complexity of the system, it seems very unlikely to couple these reactors to LC-MS. Their use is restricted to microfluidic devices and UV-detection in literature, despite their excellent capability to counter diffusion and thus peak broadening.

4.5. Mixing

An important aspect of post-column reactions is the mixing of the reagent and substrates [17]. Proper mixing ensures good contact between the reactants, which are otherwise dependent on diffusion alone to encounter each other. Improperly

mixed reagents would need more time to react, causing a higher loss in chromatographic efficiency.

Mixing occurs in two phases; when the reagents are added to the effluent stream and in the reactor itself. For the first type, ideally identical flow rates are mixed using a conventional T-piece with 90° angles. However, if both flow rates differ, they rather form two layers and other mixing devices should be used. Y-shaped mixers have been shown to allow mixing of flows with flow rate ratios up to 3:1. For higher ratios, more complicated mixers have been developed [13]. Mixing in reactor coils has been studied in, for example, microfluidic devices and the lack of turbulescence in laminar flows can be problem for proper mixing of reagents [28].

In LC, mixing is also important when using gradient elution and more and more complicated mixing devices are used in chromatography to ensure as low as possible delay times with as high as possible robustness. The latest system of Agilent Technologies, for example, uses a system called the Jet Weaver, a mulitilayer microfluidic system (see figure 1.12), to mix the mobile phase, where older systems used to rely on a simple mixing chamber.

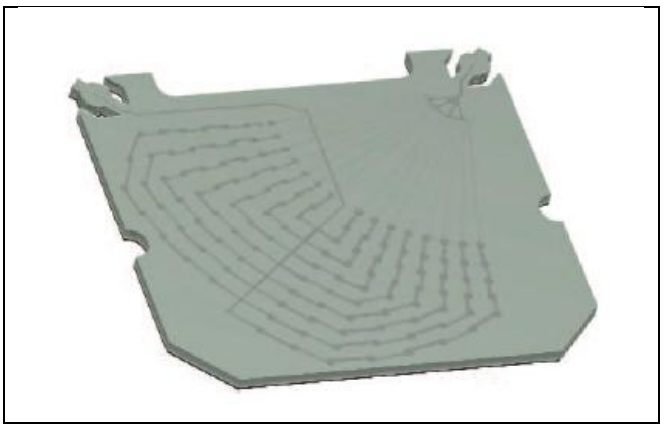


Fig. 1.12. T Jet Weaver mixing device as used in the latest Agilent HPLC instruments. This is a multilayered microfluidic device to mix solvents coming from the LC pumps.

In the reactor, secondary flow is needed for good mixing. For a gas segmented reactor this was shown in figure 1.10. Packed bed reactors also show secondary flow patterns as the beads disturb a laminar flow pattern. In a knotted open tubular reactor, secondary flow is formed because of the bends in the reactor. An unknotted reactor has no secondary flows, unless it is run at high enough flow rates to create turbulescence, causing high back-pressure.

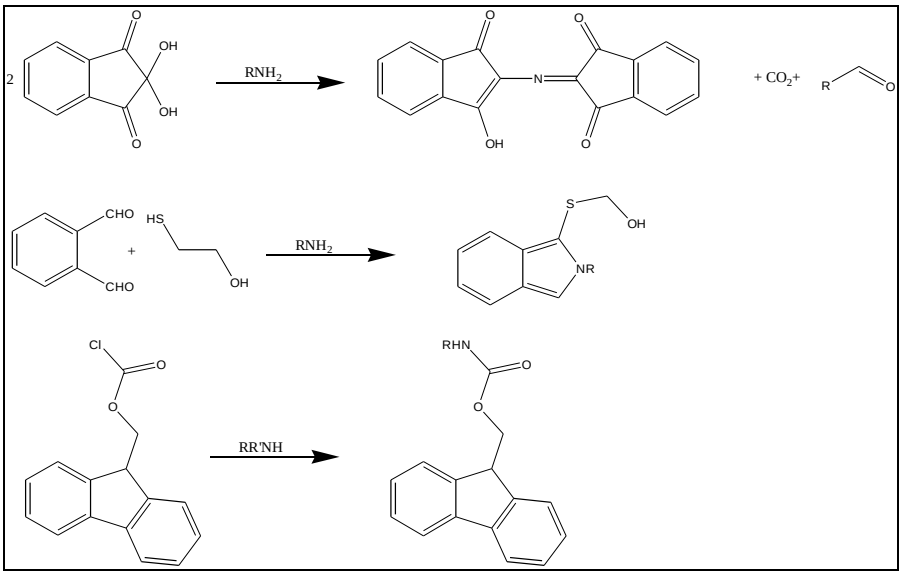


Figure 1.13. Reaction of amines. Top: with nynhidrin, as used in post-column reactions, Middle: with o-phtaldehyde (OPA), as used in pre-column derivatization, Bottom: with fluorenylmethylchloroformate (FMOC) for secondary amines as in proline, as used in pre-column derivatization.

4.6 Typical Applications of Post-Column Derivatization

Examples of post-column reaction are numerous, even though routine application has diminished. For example, peptides by fluorescence detection after reaction with fluorescamine [29], organic acids by pH indicators [30], formaldehyde by reaction

with lutidine [31], sugars by reaction with thymol in 96% sulphuric acid [32], etc. Two well known examples, pre- and post-column reactions for the detection of amino acids and post-column derivatization of carbamates, are detailed below.

Amino acids can be derivatized using fluorenylmethylchloroformate (FMOC), o-phtaldehyde (OPA) and ninhydrin (figure 1.13). OPA and FMOC are generally used in pre-column derivatization, derivatizing the primary amino groups and the secondary amino group of proline, respectively. Ninhydrin is applied in post-column derivatization.

In 1948, the off-line post-column reaction of amino acids with ninhydrin was reported by Moore and Stein [33,34]. After automated fraction collection, the fractions were treated with ninhydrin and measured by UV-detection. Analysis of a sample took about 4 days. The on-line post-column approach was reported in 1958 [35], decreasing the analysis time to 1 day. In the last 50 years, analysis times have dropped further. For example, in 1969 analysis took only 1 h [36] and in 2010, amino acid analysis in less than 10 min was shown using a column with sub-2 µm particles [37].

Originally, it was thought that pre-column derivatization of amino acids would be a problem for the separation, as a bulky side chain, identical for all amino acids, was coupled to a small analyte. The analytes thus become almost identical molecules, making them harder to separate. However, derivatization with OPA-FMOC allowed the use of RPLC, a much more robust technique than IEX, used in combination with ninhydrin. Despite this, both methods are still being used today in routine analyses.

Another often investigated reaction is the post-column detection of carbamate pesticides, developed in the 1970's [38,39]. After separation, NaOH is added and the effluent is passed through a heated reactor loop resulting in the hydrolysis of the carbamates yielding methylamine (see figure 1.14). OPA and 2-mercaptoethanol react with methylamine with the formation of (1-hydroxyethylthio)-2-methylindole a strongly fluorescent molecule (see figure 1.11, middle with R as methyl). After the

original articles by Sparacino et al. [38] and Moye et al. [39], the assay was further extensively optimized and studied by Krause [40,41]. Krause optimized most reactor parameters, such as reagent concentrations, temperatures and reaction times and concluded that the optimum for these parameters differs for the different carbamates. This is an important conclusion as one can never choose the optimal conditions for all analytes and the setup or a post-column reaction is always a compromise, as will also be shown in chapter 7. The research on this topic has however continued after the 1970's and in the 1980's, further optimization, using autosamplers and newer column types lowered the sensitivity to the ng mL^{-1} level [42,43]. Even in the last decade, the method continues to be used and further optimized (for example [44]), although alternative detection techniques such as LC-MS/MS are being used more and more.

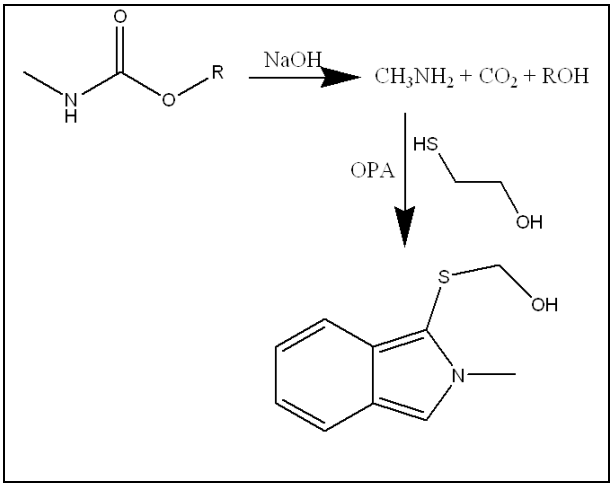


Figure 1.14. Hydrolysis of a carbamate insecticide with NaOH and subsequent reaction with ninhydrin.

5. CONCLUSION

In this chapter, post-column reactions for the specific detection of enzyme inhibitors and antioxidants were studied. Post-column reactions were originally developed for the detection of analytes with UV-Vis detection, introducing chromophores into otherwise undetectable compounds. Since the introduction of LC-MS, post-column reactions were no longer needed for this goal. However, they exhibit unique characteristics for specific detection of certain “activities”, such as enzymatic inhibition or antioxidant activity. Both can be coupled with UV- and MS-detection, as used in this work.

Because of the extensive peak-broadening introduced by post-column reactors, conventional sized LC-columns are used in combination with knitted open tubular reactors. Post-column reactors introduce dead volume between the column and the detector and while traveling through this extra-column volume, a peak will broaden because of diffusion. This peak broadening diminishes the overall efficiency. The very high efficiency possible with state-of-the-art LC-columns, would be destroyed by the post-column reactors.

REFERENCES

[1] Neue, U.D., HPLC Columns, Wiley VCH, New York, USA (1997)
[2] Deelder, R.S., De Jong, G.J., Van Den Berg, J.H.M., Chromatografie, Bohn Stafleu Van Loghum, Houten/Zaventem, Nederland/België(1994)
[3] Snyder, L.R., Kirkland, J.J., Dolan, J.W., Introduction to Modern Liquid Chromatography, John Wiley & Sons, New York, USA (2009)
[4] Snyder, L.R., Kirkland, J.J., Glajch, J.L., Practical HPLC Method Development, John Wiley & Sons, New York, USA (1997)
[5] Dong, M.W., Modern HPLC for Practicing Scientists, Wiley, New York, USA (2006)
[6] Mazzeo, J.R., Neue, U.D., Kele, M., Plumb, R.S., Anal. Chem. (2005),77, 460A-467A
[7] Guiochon, G., Gritti, F., J. Chromatogr. A (2011), 1218, 1915-1938
[8] Lestremau, F., Cooper, A., Scuzs, R., David, F., Sandra, P., J. Chromatogr. A (2006), 1109, 191-196
[9] van Deemter, J.J., Zuiderweg, F.J., Klinkenberg, A., Chem. Eng. Sc. (1956), 5, 271-289
[10] Knox, J.H., Laird, G.R., Raven, P.A., J. Chromatogr. (1976), 122, 129-145
[11] Giggings, J.C., Dynamics of chromatography, part I: principles and theory, Marcel Dekker, New York/Basel (1965)
[12] Tswett, M., Berichte der Deutschen Botanischen Gesellschaft (1906), 24, 316-323
[13] Lawrence, J.F., Frei, R.W., Chemical Derivatization in Liquid Chromatography, J. Chromatogr. Library, volume 7, Elsevier, Amsterdam (1976)
[14] Fenn, J., Angew. Chem. Int. Ed. Eng. (2003), 42, 3871-3894
[15] Edwards, J.L., Bauman, R., Spence, D.M., Anal. Chim. Acta (1999), 401, 209-214
[16] Frei, R.W., Michel, L., Santi, W., J. Chromatogr. (1977), 142, 261-270
[17] Lillig, B., Engelhardt, H. in Reaction Detection in Liquid Chromatography, Ed. Krull, I.S., Marcel Dekker, New York/Basel (1986)
[18] Haswell, S.J., Skelton, V., Trends in Anal. Chem. (2000), 19, 389-395
[19] Fabel, S., Niessner, R., Weller, M.G., J. Chromatogr. A (2005), 1099, 103-110
[20] Kuhlmann, O., Krauss, G.J., J. Pharm. Biomed. Anal. (1997), 16, 553-559
[21] Skeggs, L.T., Am. J. Clin. Pathol. (1957), 28, 311-322
[22] Skeggs, L.T., Clin. Chem. (2000), 46, 1425-1436
[23] Bartsch, J.W., Tran, H.D., Waller, A., Mammoli, A.A., Buranda, T., Sklar, L.A., Edwards, B.S., Anal. Chem. (2004), 76, 3810-3817
[24] Tsai, J.-H., Lin, L., Sensors Actuat. A (2002), 97-98, 665-671
[25] Song, H., Tice, J.D., Ismagilov, R.F., Angew. Chem. Int. Ed. (2003), 42, 768-772
[26] Godrian, A., Metze, J., Henkel, T., Martin, K., Roth, M., Köhler, J.M., Biosensors Bioelectron (2004), 19, 1421-1428
[27] Linder, V., Sia, S.K., Whitesides, G.M., Anal. Chem. (2005), 77, 64-71

[28] Stone, H.A., Kim, S., AlChE J. (2001), 47, 1250-1254
[29] Frei, R.W., Michel, L., Santi, W., J. Chromatogr. (1976), 126, 665-677
[30] Wada, A., Bonoshita, M., Tanaka, Y., Hibi, K., J. Chromatogr. (1984), 291, 111-118
[31] Engelhardt, H., Klinkner, R., Chromatographia (1985), 20, 729-731
[32] Engelhardt, H., Ohs, P., Chromatographia (1987), 23, 657-662
[33] Stein, W.H., Moore, S., J. Biol. Chem. (1948), 176, 337-365
[34] Stein, W.H., Moore, S., J. Biol. Chem. (1948), 176, 367-388
[35] Spackman, D.H., Stein; W.H., Moore, S. Anal. Chem. (1958), 30, 1190-1206
[36] Ertinghausen, G., Adler, H.J., Reichler, A.S., J. Chromatogr. (1969), 42, 355-366
[37] Henderson, J.W., Brooks, A., Agilent Application Note (2010) 5990-4547EN
[38] Sparacino, C.M., Hines, J.W., J. Chromotogr. (1976), 14, 549-555
[39] Moye, H.A., Scherer, S.J., St. John, P.A., Anal. Lett. (1977), 10, 1049-1073
[40] Krausse, R.T., J. Chromatogr. Sci. (1978), 16, 281-288
[41] Krausse, R.T., J. Chromatogr. (1979), 185, 615-624
[42] Hill, K.M., Hollowel, R.H., Dal Cortivo, L.A., Anal. Chem. (1984), 56, 2465-2468
[43] Engelhardt, H. Lillig, B., Chromatographia (1986), 21, 136-142
[44] Bassett, M.V., Wendelkes, S.C., Pepich, B.V., Munch, D.J., J. Chromatogr. Sci. (2003), 41, 100-106

Chapter 2.

RATIONALE OF COMBINING TEMPERATURE RESPONSIVE LC TO ENZYMATIC POST-COLUMN REACTIONS

Enzymatically catalyzed reactions are key reactions in any living organism and therefore enzymes are targets for many drug compounds. In modern pharmaceutical research, screening of large numbers of potential drug candidates is an early step in drug discovery. As synthesis of a large number of molecules is possible in a very small time scale e.g. by combinatorial and solid-phase synthesis, the speed and cost of High Throughput Screening (HTS) methods should be optimized. Screening of natural extracts for pharmaceutical usefulness is also performed by similar screening techniques. Presently, most of this screening is done by multi-well plate assays: each sample or eventually a fraction after a separation is tested in batch on the desired activity. HTS can be automated by the use of robotics but this is an expensive and labor intensive method.

Bioactivity screening for toxic components by Thin Layer Chromatography (TLC), where the stationary phase is coated on a thin carrier such as a glass plate, and High Performance Liquid Chromatography (HPLC), using a packed column at high pressure, followed by bioluminescence detection via luminescent microorganisms was first described by G. Eberz et al. [1]. In 2003, a new approach was introduced to identify new possible enzymatic inhibitors [2]. An enzymatic post-column reaction scheme was proposed and experimentally tested. The system combines the separation power of HPLC, the selectivity of enzymatic reactions and the power of MS to follow the reaction and identify the drug candidate. The system contains three parts: a conventional HPLC system, a post-column reactor and an MS (figure 2.1). In this arrangement, the post-column reactor scheme is more complex than a simple addition of a reagent flow, as two separate flows need to be added: one containing

the enzyme and one containing the substrate for the enzymatic reaction (see figure 2.2).

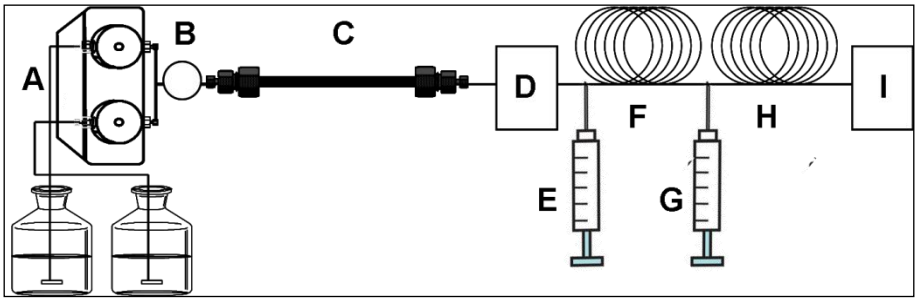


Fig. 2.1. Setup of the system for detection of enzyme inhibitors by post-column detection. A. HPLC pump and solvents, B. Injector, C. Column, D. Optional flow through UV-detector, E. Syringe delivering the enzyme, F. First reactor loop, G. Syringe delivering the substrate, H. Second reaction loop, I. MS-detector.

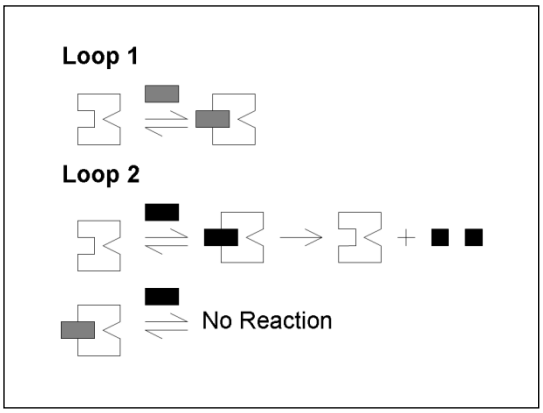


Fig. 2.2. Simplified scheme of the reactions in both loops of the post-column reactor for detection of enzyme inhibitors. White: enzyme, grey: inhibitor, black: substrate (big) and products (small). In loop 1 (F in figure 2.1), the enzyme reacts with the inhibitor when present. In loop 2 (H in figure 2.1), the enzyme reacts with the substrate to form products, if no inhibitor is present.

When a compound elutes from the LC column, it can react with the enzyme in a first reactor loop. After this loop, the substrate is added and the enzyme will normally convert this substrate into the product. However, if the eluting compound inhibits the enzyme's normal reaction, product formation will be reduced (see figure 2.2), as can directly be assessed by monitoring the reaction products with MS. This will also result in an increase in substrate concentration and the mass to charge ratio of the inhibitor causing the decreased enzyme activity can directly be obtained from the total ion chromatogram. It is possible to replace the MS by a UV- or fluorescence detector if a substrate is used where the enzymatic reaction changes its UV or fluorescence spectrum. Furthermore, a first detector can be added, mostly UV, between the column and the reactor loops to record a chromatogram (D in figure 2.1).

Ideally, a synthetic or natural mixture can be injected on the system and enzyme inhibitors are detected through the described procedure. A schematic drawing of the chromatograms and mass spectra is shown in figure 2.3. A detailed description and experimental results will be presented in Chapter 5.

A drawback of this approach is solvent incompatibility between the HPLC effluent and the enzymes in the bioreactor. Today, most LC separations are performed by RPLC, because it allows the analysis of hydrophobic and moderately hydrophilic compounds with excellent reproducibility. Ion Pairing RPLC can be applied to analyze more hydrophilic compounds, such as acids and bases. RPLC, however, uses a combination of water and organic solvents as mobile phase and commonly in gradient elution, i.e. more organic solvent is added as the analysis proceeds. As enzymes are proteins, their activity is highly dependent on their structure and this structure is only stable in aqueous buffers, resembling in vivo situations. For example, the active site of trypsin is the catalytic triad formed by the side chains of histidine, aspartate and serine. They are far apart in the amino acid sequence (respectively the 57^{th}, the 102^{nd} and the 195^{th} amino acid), but the three-dimensional protein structure folds them closely together. It is therefore essential to

keep this structure and avoid any denaturation. The amount of organic solvent used in an analysis using post-column enzymatic reactions, should therefore be kept to a minimum and developments in "green chromatography", whereby the use of less organic solvents is intended, should be exploited.

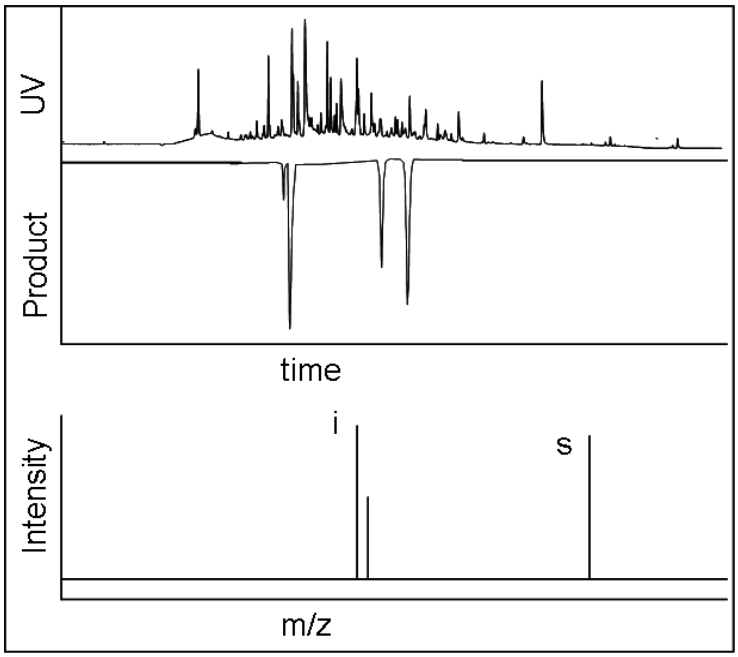


Fig. 2.3. Idealized result of an analysis of enzymatic inhibitors. Top: UV chromatogram taken by detector D in figure 2.1, Middle: Product signal taken by detector I in figure 2.1, Bottom: MS scan of an inhibitor peak, showing the substrate (s) and the inhibitor (i).

"Green chemistry" has been defined by Lawrence as "the use of chemistry techniques and methodologies that reduce or eliminate the use or generation of feedstocks, products, byproducts, solvents, reagents, etc. that are hazardous to human health or the environment" [3]. Anastas and Warner published "the twelve principles of green chemistry" [4], giving a brief list of how to create an as green as possible chemistry. The main advantages of a greener analytical approach are the

reduction of health risks for the analyst, a reduction in hazardous waste and a reduction of the cost of analysis, as the main green solvent, water, is cheaper than most other solvents and waste treatment is reduced. A number of possibilities exist to make RPLC greener and they have been reviewed recently [5-7]. Using a column with smaller internal diameter is often the easiest solution, as this will reduce the flow and thus the amount of organic solvent needed, by a factor equal to the square of the column internal diameter ratio's, but this is not useful for enzymatic assays as the concentration of organic modifier stays the same. Using GC or SFC is also out of the question, considering the polarity of the solutes. High Temperature LC (HTLC) is a possible solution, as at higher temperatures lower concentrations of organic modifier are needed as the dielectric constant of water decreases at high temperatures. This technique has been coupled to enzymatic assays in 2005 [8], but has limited applicability because of the thermal stability of enzyme inhibitors. Another possibility is the use of Temperature Responsive Liquid Chromatography (TRLC).

TRLC uses a very different approach compared to conventional LC techniques in which the mobile phase strength is varied to achieve a separation. TRLC uses a stationary phase that changes its properties in water when the temperature is changed. This allows the use of a single aqueous mobile phase. The technique was introduced in 1996 as an alternative to RPLC [9]. In TRLC, the stationary phase is in -almost- all cases made of porous silica particles to which temperature responsive polymers are attached. These intelligent polymers respond to a change in environmental temperature with a reversible change in hydrophobicity when dissolved in water. Above a certain temperature, they are insoluble in water, while they become water soluble below a certain temperature. The temperature where this change occurs is known as the Lower Critical Solution Temperature (LCST) and depends on the polymer type, length and concentration. When used as a stationary phase, the polymers are stretched out in the mobile phase below the LCST, resulting in a hydrophilic stationary phase. Above the LCST, however, the polymers collapse and a hydrophobic stationary phase is formed, as shown in figure 2.4.

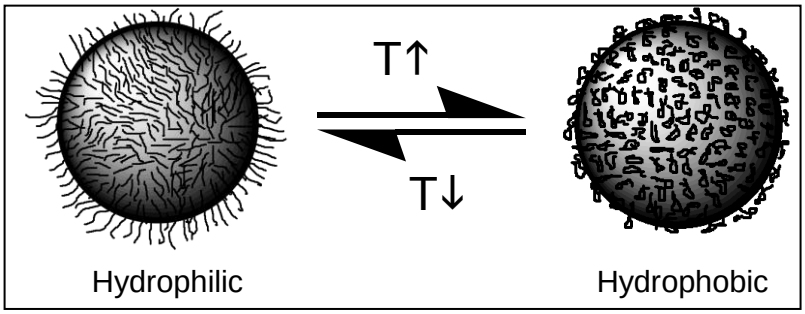


Fig. 2.4. Schematic representation of the stationary phase used for Temperature Responsive Liquid Chromatography. Left: Hydrophilic at temperatures below the Lower Critical Solution Temperature, Right: Hydrophobic at temperatures above the Lower Critical Solution Temperature.

As a result for such a stationary phase, a mobile phase gradient as used in RPLC, can be replaced by a downward temperature gradient in TRLC using pure water as the only mobile phase ingredient. The most common temperature responsive stationary phase is based on poly-(N-isopropylacrylamide) (PNIPAA), but as shown in Chapter 3 and 4, other polymers can also be used. In Chapter 3, a review on intelligent polymers as stationary phases developed for HPLC is presented. According to the literature, TRLC can be used as alternative to RPLC, Ion Exchange Chromatography and Affinity LC. In Chapter 4, the development of a new temperature responsive stationary phase is presented.

TRLC with a purely aqueous mobile phase is therefore ideally suited for the coupling to an enzymatic post-column reactor. This idea was the main drive to investigate TRLC in this work.

REFERENCES

[1] Eberz, G., Rast, H.-G., Burger, K., Kreiss, W., Wiesemann, C. (1996) Chromatographia 43 (1/2), 5-9
[2] Derks, R.J.E., Hogenboom, A.C., van der Zwan, G., Irth, H. (2003) Anal. Chem. 75, 3376-3384
[3] Lawrence, D.H., on www.chemistshelpingchemists.org, retrieved on October 21, 2010
[4] Anastas, P., Warner, J.C., Green Chemistry: Theory and Practice, Oxford University Press, New York, USA, 1998, p.30
[5] Sandra, P. (2010) LC-GC Europe 23, 240
[6] Sandra, P., Sandra, K., Pereira, A., Vanhoenacker, G., David, F. (2010) LC-GC Europe 23, 241-259
[7] Sandra, P., Pereira, A., Dunkle, M., Brunelli, C., David, F. (2010) LC-GC Europe 23, 396-405
[8] de Boer, A.R., Alcaide-Hidalgo, J.M., Krabbe, J.G., Kolkman, J., Boas, C.N.V., Niessen, W.M.A., Lingeman, H., Irth, H. (2005) Anal. Chem. 77, 7894-7900
[9] Kanazawa, H., Yamamoto, K., Matsushima, Y., Takai, N., Kikuchi, A., Sakurai, Y., Okano, T. (1996) Anal. Chem. 68, 100-105

Chapter 3.

TEMPERATURE AS A TOOL TO TUNE RETENTION, SELECTIVITY AND RESOLUTION IN GREEN CHROMATOGRAPHY

In this chapter, the influence of temperature on an LC separation is described, with an emphasis on the use of temperature to minimize the use of organic solvents in HPLC. The dielectric constant of water changes with temperature and thus, so do its dissolving properties. In a pressurized system such as an HPLC column, water can replace organic solvents, as used in high temperature LC (HTLC). But temperature also has an influence on many other factors, such as detector response, selectivity, efficiency and retention.

Selectivity can be influenced by a change in interactions, for example by a change in ionization. Efficiency is influenced by a change in the van Deemter curve and the drop in viscosity of the mobile phase, allowing the use of longer columns or smaller particles without high back-pressures. The retention of most compounds drops when a higher temperature is used, which is due to the thermodynamics of LC. This again favors using a longer column.

In temperature responsive LC (TRLC) temperature is used to tune the hydrophobicity of the stationary phase. In contrast to conventional HPLC, retention increases with increasing temperature in RPLC-like separations. An overview of the relevant literature on TRLC is presented, as this technique is important in the experimental work of chapter 4 and 5.

1. The Influence of Temperature on HPLC Analysis

High temperature LC (HTLC) uses elevated temperatures to obtain an optimized separation in LC. The influence of temperature on an equilibrium process can be thermodynamically expressed by the following equation [1].

$$\Delta G^0 = \Delta H^0 - T\Delta S^0 = -RT \ln K \qquad \text{(eq. 3.1)}$$

where ΔG^0 is the change in Gibbs free energy, ΔH^0 is the change in enthalpy, ΔS^0 in the change in entropy and R is the gas constant. As already stated in chapter 1 (equation 1.2), the retention factor k relates to the equilibrium constant K by the phase ratio β. Thus from equation 1.2 and 3.1, the van't Hoff equation can be derived [2].

$$\ln k = -\frac{\Delta H^0}{RT} + \frac{\Delta S^0}{R} - \ln \beta \qquad \text{(eq. 3.2)}$$

The van't Hoff equation allows to construct a plot of ln k versus 1/T, as seen in figure 3.1. The slope of the curve allows to calculate the enthalpy of interaction of an analyte with the stationary phase. The entropic contribution is obtained from the intercept with the y-axis. RPLC is an enthalpy driven process, in which the greater entropy of the free analyte is smaller than the enthalpy gained by the formation of the stationary phase-analyte complex. This explains the decrease in retention when temperature is raised. Therefore it is often interesting to increase the temperature to decrease the retention of highly retained solutes. An additional interesting consequence of this is that it allows to decrease the amount of organic modifier when temperature is increased.

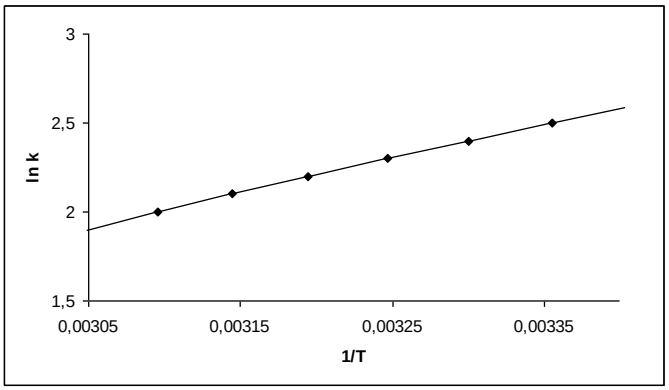


Fig. 3.1. Example of a van't Hoff plot.

Retention is also influenced by temperature through another factor. The dielectric constant of water changes when changing the temperature and thus, less organic solvent is needed at elevated temperatures. It has been shown that 3.75°C is equivalent to 1% of methanol [3] and 5°C is comparable to 1% acetonitrile [4]. This suggests that a solvent gradient can be replaced by a temperature gradient, but it can be calculated that for a 5 to 95 % organic solvent gradient the temperature would have to go from 20°C to 360°C for methanol and from 20°C to 470°C for acetonitrile. These temperatures cannot be used in practice and thus, a small amount of organic modifier is often needed to replace these broad gradients, even when working with specialized columns and column ovens. However, smaller gradient differences can be replaced by temperature gradients and this makes HTLC a possibility for green chromatography. Applying superheated water in HPLC allows the use of a purely aqueous mobile phase and thereby makes it possible to use special detectors, that would otherwise by unusable because of the organic solvent present, such as flame ionization detection or inductive coupled plasma MS. Another advantage of the replacement of a solvent gradient with a temperature gradient lies in the use of detectors that are sensitive to changes in mobile phase composition, such as the charged aerosol detector and the evaporative light scattering detector.

In electrospray MS, the ionization efficiency of a compound is solvent dependent, making it impossible to quantatively compare peaks in a gradient analysis. However, if the solvent gradient is replaced by a temperature gradient, peak areas of similar analytes can be compared. As will be shown in chapter 5 and 7, the change in solvent is also the cause for a difference in response when working with post-column reactors.

Elevated temperature also speeds up the analysis. Because of the lower solvent viscosity and hence higher analyte diffusivity at elevated temperature, the C-term of the van Deemter equation becomes smaller, leading to a flatter curve at high velocities, allowing the use of higher flow rates without loss in chromatographic efficiency. Elevated temperature and the faster diffusion it causes, also lead to an increased B-term. The combined effect of temperature on the B- and C-term leads to an increase in optimal velocity. The result on a van Deemter curve is a flatter curve, with the same minimal plate height, but higher optimal velocity, allowing the use of higher flow rates, decreasing the analysis time.

To increase efficiency, longer columns and/or smaller particles can be used (see chapter 1), but both create more back pressure and thus, the efficiency is often limited by the pressure limit of the system. At elevated temperatures however, the viscosity drops and so does the back pressure, allowing much more efficient separations and reaching up to 100,000 plates in LC at 400 bar [5]. To obtain these plate numbers, however, very long columns have to be used (1000 mm for 5 µm particles and 750 mm for 3.5 µm particles) and thus long analysis times are needed. Temperature can further enhance the efficiency by improving the peak shape for positively charged analytes. These compounds often interact with the silica support, causing tailing and these interactions are reduced at higher temperatures [1].

Temperature is an often overlooked parameter for the optimization of a separation, as it can also have an influence on the selectivity. By changing the temperature, the interactions between the analyte molecules and the stationary and mobile phases can change. Depending on the chemistry involved, this can result in major or minor

shifts. Especially polar and ionizable compounds are sensitive to temperature as the pKa values can shift with a change in temperature [1]. Another good reason to choose to optimize the temperature rather than start to change mobile phase composition (buffer, salt concentration, etc.) is the fact that temperature is a relatively simple instrument parameter and thus highly reproducible.

The most common type of stationary phases in HPLC, silica particles, are not stable in water at elevated temperatures, causing the need for special stationary phases that can be used in HTLC. Some more recently developed types of silica are more stable due to the inclusion of organic bonds in the silica backbone. However, for temperatures well above 100°C, silica phases are no longer usable and graphitized carbon, zirconium oxide or polystyrene-divinylbenzene phases need to be used.

When using HTLC, it is often feared the analytes might decompose. Some analytes are truly thermolabile, but it has been shown most analytes are amendable for HTLC [6]. The time an analyte spends on the column is a very important factor in its breakdown, as it is by interaction with the stationary phase most analytes degrade.

HTLC thus has the advantage of speed, a more constant detector response, higher efficiency and a greener separation, but there are also some problems, such as the hydrothermal instability of most stationary phases, thermolability of analytes and the need for dedicated instrumentation. Temperature can, however, also be used to affect the stationary phase rather than the mobile phase when using stimuli responsive polymers.

2. The Use of Temperature Responsive Stationary Phases in HPLC

2.1 Temperature responsive Polymers

Temperature responsive polymers are stimuli responsive polymers illustrating a change in water solubility when the environmental temperature is varied. Various phase transition diagrams are obtained depending on the type of polymer and the chain length. Three main types have been identified and are represented in figure 3.2. The lowest point in this curve is known as the lower critical solution temperature (LCST).

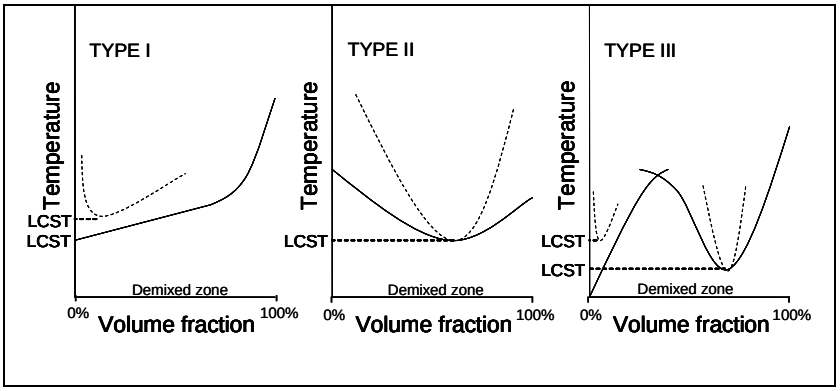


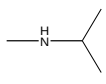
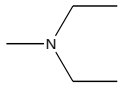
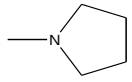
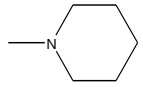
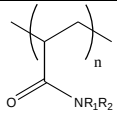
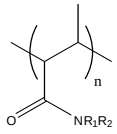
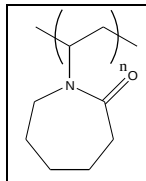
Fig. 3.2. Described phase diagrams for temperature responsive polymers showing the demixing behavior as a function of volume fraction of polymer and temperature. Black line: infinitely long polymer, dashed line: polymer with finite molecular weight. Below the curve, two phases are present (polymer and water), above it the only phase is present (dissolved polymer).

Type I demixing shows a minimum for a zero concentration of polymer for infinitely large molecules. Molecules with a finite molecular weight have higher LCST, increasing with a decreasing length of the polymer. Poly-(N-vinylcaprolactam (PVCL), the polymer studied in chapter 4, shows a type I demixing behavior. Type II demixing shows an LCST at a certain concentration, independent of the polymer length, but

longer polymers have flatter curves. PNIPAA has a type II demixing behavior. Type III demixing shows both a type I and type II minimum.

In the literature, a variety of temperature responsive polymers has been described, but the most studied temperature responsive polymers are, however, the polyacrylamides with an N-alkyl group. In table 3.1 a series of LCST's of temperature responsive polymers are given. The core structure is therein often the same and the length and shape of the alkyl side chain determines the LCST. Note that co-polymerization with non-temperature responsive materials can further alter the demixing behaviour, as shown in the next part.

Table 3.1. Structure and LCST of several temperature responsive polymers, with PNIPAA in the top left corner.

NR_1R_2=					
	**32°C**	**36°C**	**86°C**	**56°C**	**5°C**
	**45°C**			 **34°C** **(PVCL)**	

2.2 Temperature responsive Liquid Chromatography

In this section, an overview of the relevant literature concerning the use of temperature responsive polymers in separation sciences is provided. Reviews on the subject have been published in the last decade [7-13]. Here, the relevant papers have been divided by the chemistry of the stationary phase used.

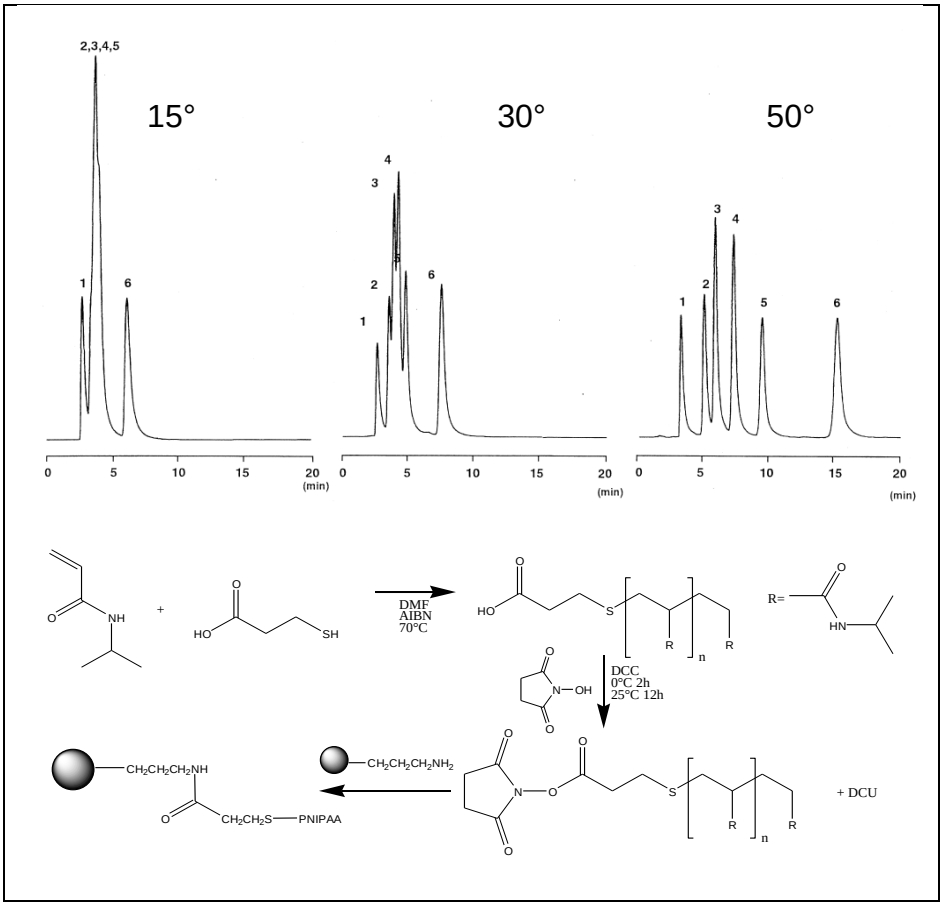


Fig. 3.3. Top: Separation of a mixture of 5 steroids and benzene on a PNIPAA column at various temperatures with pure water as mobile phase. Peaks: 1. benzene, 2. hydrocortisone, 3. prednisolone, 4. dexamethasone, 5. hydrocortisone acetate, 6. testosterone. Reprinted from [15]. Bottom: Synthesis of a PNIPAA stationary phase.

2.2.1 PNIPAA FOR RPLC-LIKE SEPARATIONS ON SILICA PARTICLES

The idea of using PNIPAA as a stationary phase was first described in 1995 and the possibility to separate drugs and steroids by purely aqueous chromatography was demonstrated [14,15], using various coupling strategies [16]. An example of a separation on a column packed with silica modified by PNIPAA polymers and its synthesis is shown in figure 3.3. As can be seen, the separation improves greatly

when heating the column up to 50°C and retention times increase with increasing column temperature.

The influence of organic modifiers such as methanol has been investigated [17]. The temperature responsive effect of PNIPAA was shown to disappear above certain concentrations of methanol as the retention actually decreased with increasing temperature. The use of a methanol/water gradient is however not of real interest in TRLC, as using green LC is the main focus.

The use of PNIPAA polymers in green LC has been evaluated for a series of compounds, showing large shifts in retention when varying the temperature [18]. LC-MS was also performed with a PNIPAA based column to analyze carbamate pesticides, and LC-UV was used for the separation of steroids, parabens, sulfonamide drugs, carbamates, benzoic acids, aniline derivatives, phenols, phenones and other benzene derivatives. This versatility has also been used in a small number of applications using TRLC, analyzing bisphenol A [19], cathechins [20], herbicides [21] and barbiturates [22]. Modification of the PNIPAA stationary phase in purely aqueous chromatography by copolymerization has also been investigated to increase applicability.

Copolymerization with more hydrophobic monomers, such as butylmethacrylate, leads to a lower LCST of the polymer as a whole [23]. Some structures of the described copolymers can be found in figure 3.4. The LCST for a mole fraction of 0, 0.6, 1.9 and 3.2 mol % of butylmethacrylate were 32°C, 30°C, 23°C and 20°C respectively. The copolymer columns showed greater retention for steroids at all temperatures and the columns could also be used for the separation of peptides [24] and derivatized amino acids [25] and for the quantitative analysis of propofol, an anaesthetic drug, in serum [26]. Recently, a dense layer of PNIPAA-t-butylacrylamide was developed to enhance the stability of the stationary phase [27]. The stationary phase was more stable, even in a high pH, but the efficiency of the columns was much lower, probably due to the slow diffusion in the large volume of polymer.

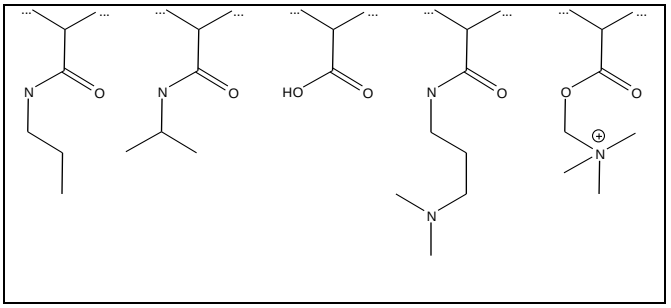


Fig. 3.4. Structures of the copolymers often used with PNIPAA. From left to right: butyl acrylamide, t-butylacryl amide, acrylic acid, N,N-dimethylaminopropylacrylamide and (2-(methacryoloyloxy)-ethyl)trimethylammonium.

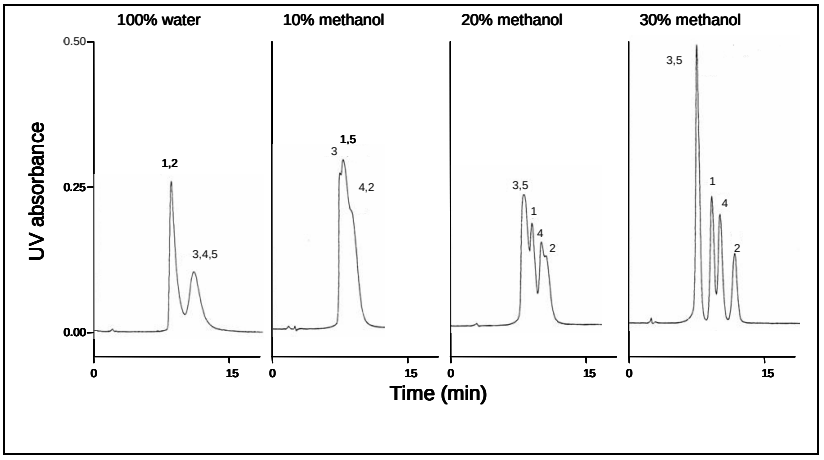


Fig. 3.5. Separation of benzene derivates in various percentages of methanol on a PNIPAA-hydrogel column. Peak identification: benzene-R with R= 1. OCH_3, 2. CH_3, 3. CHO, 4. NO_2, 5. $COCH_3$. Data from [28].

Another method for the improvement of hydrophobic interactions, was the development of temperature responsive hydrogels, cross-linked PNIPAA structures, rather than linear PNIPAA. In pure PNIPAA hydrogels, it was shown temperature can increase retention, but also an increased concentration of methanol (up to 30%), as shown in figure 3.5 [28]. This strange finding can be explained by the swelling behavior of the hydrogel, which is swollen in water, collapsed at 30-40% methanol and swells again in over 50% of methanol. The hydrogel concept was further

investigated by copolymerization with butylmethacrylate [29]. Furthermore, it was proven that the hydrogel protected the silica from hydrolysis in alkaline solutions [30].

Other copolymers have been tested to introduce a different selectivity, such as acryloyl-L-proline methyl ester, a proline derivate [31] and N-acryloyl-3-(2-naphtyl)-L-alanine methyl ester [32]. The proline derivatized column was shown to separate a mixture of steroids and a mixture of derivatized amino acids, much as the PNIPAA-butylmethacrylate column, while the naphtyl groups of the other phase seem to change the selectivity by introducing π-π-interactions. Despite these small changes in selectivity, PNIPAA and its neutral copolymers showed limited applicability for the separation of more hydrophilic analytes. Introduction of charged monomers into the copolymer made the retention behavior both temperature and pH dependable, increasing the retention for highly polar analytes.

2.2.2 PNIPAA FOR IEX-LIKE SEPARATIONS

Anionic groups have been introduced by means of copolymerization with acrylic acid [33-37]. Proof of principle was provided by a separation of four dopamine derivatives [33,34]. A copolymer of PNIPAA, acrylic acid and t-butylacrylamide was coupled to silica and the separation was influenced by temperature as for any PNIPAA-based phase and an ion exchange mechanism was present. This stationary phase was also used to separate peptides [35] and amino acid derivatives [36]. The stationary phase also proved useful in the analysis of melatonin and its precursors [37]. It is worth mentioning that another derivatization technique used to couple temperature responsive polymers, such as the PNIPAA-acrylic acid copolymer, has been studied, but the chromatographic gains using this technique are yet to be proven [38].

Positively charged groups were introduced by the copolymerization of nitrogen containing groups, such as N,N-dimethylaminopropylacrylamide. This phase was

shown to be able to separate some important biomolecules, such as small nucleotide sequences [39,40], short peptides [41] and nucleosides [42], next to the more studied separation of steroids [43]. Another interesting phase has been developed with a quaternary ammonium group, thus introducing permanent positive charges, using (2-(methacryloyloxy)-ethyl)-trimethylammonium polymer [44]. PNIPAA and the charged polymer were both coupled to silica independently in a ratio of 4 to 1. The phase was used successfully to separate lactic acid and creatinine phosphate, two charged molecules, but the buffer pH was shown to be the most influential factor rather than temperature.

2.2.3 The Use of Temperature Gradients in TRLC

While it seems a logical choice to make, the use of temperature gradients in TRLC has been relatively little applied thus far [44-48]. A stepwise gradient applying two temperatures was used to separate β-hydroxytestosterone and testosterone [44] and the oral contraceptives levonergestrel and ethinylestradiol [45]. In both occasions, the last eluting peak showed much retention at elevated temperature (40°C) and the first analyte eluted fast. Changing the temperature after elution of the first compound to a much lower temperature (10°C) allowed a faster separation with higher sensitivity.

The separation of the two oral contraceptives was also performed by a linear and a near linear temperature gradient [46,47]. In the linear temperature gradient, the column was cooled down from 40 to 10°C in 35 min, improving the speed and sensitivity of the analysis in urine greatly, as can be seen in figure 3.6. The analysis was repeated with a slightly modified stationary phase, using “looped” PNIPAA, that is attached to the silica surface on multiple points of the chain, rather than only one end, shortening the analysis time [46].

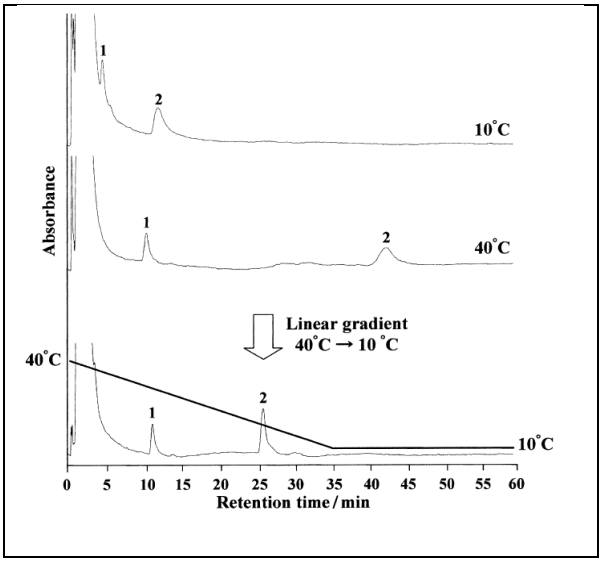


Fig. 3.6. Separation of the oral contraceptives levonorgestrel (1) and ethinylestradiol (2) by a linear temperature gradient on a PNIPAA phase. Reprinted from [45].

The same principle has been applied to the separation of peptides, with a temperature gradient from 40 to 10°C, speeding up the analysis and increasing efficiency [48]. The steeper the temperature gradient, the better the results, but this is limited, as peaks would start co-eluting if lower temperatures or a steeper gradient is used.

2.2.3 Other Carriers for PNIPAA

So far, the described stationary phases use silica particles as a carrier for the temperature responsive polymers. However, some problems exist using silica particles. Firstly, silica is hydrolyzed by water at elevated temperatures and secondly, the efficiency of the phase is not very high. Both problems have been addressed by the use of other stationary phases, using more hydrolysis free material

or using monoliths, which in theory offer a very high plate number, as longer can be used.

For stability, two other particle materials have been tested: poly(hydroxyl methacrylate) [48] and polystyrene-divinylbenzene [49]. The poly(hydroxyl methacrylate) phase was used for the successful separation of peptides using a temperature gradient, while the polystyrene-divinylbenzene phase resulted in low efficiency chromatograms.

The low efficiency of most TRLC phases was countered with the study of monolithic columns using PNIPAA [50-53]. A PNIPAA monolithic column was prepared much like a packed column by Roohi et al. [50], coupling PNIPAA to an existing silica backbone, which was here a silica monolith. The column showed clear temperature responsive properties and successfully separated a steroid mixture under purely aqueous conditions. However, only 1,000 plates were reached on a 100 mm column, making this actually worse than packed PNIPAA columns. The in-column polymerization probably led to the closing of many of the original monoliths pores, effectively lowering the efficiency.

Columns with higher efficiency were achieved by the synthesis of a pure PNIPAA monolith [51] and a PNIPAA-N,N-methylenebisacrylamide monolith [52], as shown in figure 3.8. The polymerization of these phases was performed at -12°C, hence the name cryogel, rather than hydrogel. The main advantage is the slower deswelling, which would otherwise have an adverse effect on efficiency. A plate height of 18 µm was achieved on a 100 mm column from pure PNIPAA [51] and 25 µm on the copolymer column [52].

The same group synthesized a polystyrene-divinylbenzene monolith with added PNIPAA, but the temperature effect was not described [53]. It was used for the separation of intact proteins by hydrophobic interaction chromatography, using a salt gradient to elute the analytes.

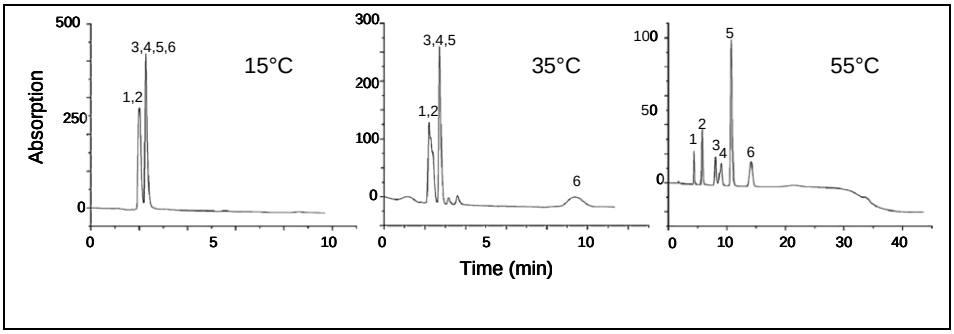


Fig. 3.7. Separation of steroids on a PNIPAA monolith at different temperatures. Peaks: 1. hydrocortisone, 2. cortisone acetate, 3. prednisolone acetate, 4. fluocinolone acetonide, 5. betamethasone-21-acetate, 6. beclomethasone dipropionate. Reprinted from [50].

One last phase has been developed explicitly for TRLC using PNIPAA, using magnetite (Fe_3O_4), which can be heated by induction [54,55]. The particles were not spherical but randomly shaped, and therefore the chromatographic analysis showed very low efficiency. Furthermore, it is doubtful if there is any demand for this type of stationary, as most modern HPLC system contain a column oven, but no induction coil.

2.2.4 Other Applications of PNIPAA Based Phases in Separation Science

The temperature response of PNIPAA can also be used in other chromatographic applications. In affinity LC, it has been used to hide or show the affinity sites on the stationary phase, hiding the affinity site when the material is heated. This has been described for the selection of serum albumines [56] and of asialotransferrin [57]. However, it has also been used in combination with molecular imprinted polymers (MIPs), in such a way that the MIP was built on a PNIPAA chain and when temperature was increased, the PNIPAA collapsed, hiding the binding sites [58]. The protein lysozyme was successfully selected from egg white using this technique.

An affinity technique using PNIPAA modified nano-beads (100 nm diameter) in a microfluidic separation device was developed with the well-known streptavidin-

biotin affinity interaction [59]. Beads were modified with PNIPAA and biotin and above the LCST, they adhered to the walls of a poly-ethyleneterephtalate microchannel. Streptavidin was injected into the channel and retained onto the beads. Below the LCST, both the beads and the streptavidin eluted from the channel.

Chiral LCST was studied in one paper, where PNIPAA was copolymerized in multiple ways with L-valine diamide to obtain a chiral stationary phase [60]. The separation of enatiomeric leucine derivatives in 1% methanol was shown, but the phase lacks further applications.

A last application of PNIPAA in separation science can be found in sample preparation, where PNIPAA has been used in extraction techniques [61,62]. Solid phase extraction (SPE) has been performed using MIPs with some success, as the extraction of dopamine from urine was temperature dependent and to some extent selective, but dopamine derivatives were also retained on the SPE cartridge [61]. More success was obtained by applying PNIPAA in solid-phase microextraction (SPME), using PNIPAA for a preconcentration step in the analysis of estrogens in milk [62].

2.2.5 Other Stimuli Responsive Polymers in HPLC

In temperature responsive chromatography, three other phases have also been described for HPLC applications. Poly-(N,N'-alkylcarbamido)-propyl methacrylamide was studied for the separation of proteins and showed larger retention factors than PNIPAA [63]. The hydrophobicity of the phase could be tuned further by choosing which alkyl-group is present. Another phase much like PNIPAA is poly-(N-vinylcaprolactam) (PVCL) which will be detailed in the next chapter [64]. But also one polymer that does not resemble PNIPAA has been used in TRLC. Poly-ethylene glycol (PEG) has been used for the separation of proteins and steroids, but very little data has been published on this stationary phase [65].

A very different approach using intelligent polymers, is the use of an intelligent polymer not responding to temperature, but to pH. A pH-sensitive copolymer of

sulfonamide and N,N-dimethylacrylamide, switching from hydrophobic to hydrophilic has been described [66]. The phase allows the tuning of a separation of proteins by pH, but as pH is very important in the electrostatic forces between a protein and a charged stationary phase, the retention mechanism is quite complex.

3. CONCLUSION

Temperature can be used to optimize a separation and to diminish the consumption of toxic solvents in HPLC, as it influences efficiency, selectivity, retention and detector response. The main advantages of HTLC are the possibility for greener LC and high efficiency HPLC. However, when very high temperatures are used, column and analyte stability may cause problems and some specialized instrumentation is needed.

Since its introduction in 1996, a significant amount of research has been conducted in developing temperature responsive liquid chromatography, although it should be noted that this work has been done by a limited number of research groups. Poly(N-isopropylacrylamide) (PNIPAA) was almost exclusively used for this application. Several types of chromatography have been performed on these phases: reversed phase, ion exchange (both anion and cation exchange) and affinity chromatography.

Purely aqueous liquid chromatography can be achieved by TRLC, however, several disadvantages remain. The main disadvantage is the low efficiency, even in high efficient column formats such as monolithic columns. As for selectivity, temperature can be used to some extent to tune selectivity, but very few applications have proven the use of a temperature gradient. Up till now, higher efficiency seems the main gain of applying a higher temperature, rather than a way to change the selectivity of a TRLC column. Selectivity can be tuned by copolymerizing PNIPAA and thus changing the stationary phase. This hampers the application of TRLC, as it is much easier to change the mobile phase, as can be done in for example RPLC.

Applications of TRLC remain limited both because of the lack of selectivity and efficiency and because the columns are not commercially available. For routine applications to be possible, these disadvantages over commercially available columns have to be solved.

There must be a special reason to select TRLC. In this study, TRLC was evaluated because of the feature of using only water as mobile phase, making TRLC ideally suited for coupling to enzymatic post-column reactors. However, because of some drawbacks experienced with PNIPAA based phases, mostly related to low efficiency, a new temperature responsive phase namely poly-(N-vinylcaprolactam) (PVCL) was synthesized and chromatographically evaluated.

REFERENCES

[1] Vanhoenacker, G., Sandra, P., J. Sep. Sci. (2006), 29, 1822-1835
[2] Chester, T.L., Coym, J.W., J. Chromatogr. A (2003), 1003, 101-111
[3] Bowermaster, J., McNair, H.M., J. Chromatogr. Sci. (1984), 22, 165-170
[4] Chen, M.H., Horvarth, C., J. Chromatogr. A (1997), 788, 51-61
[5] Lestremau, F., Cooper, A., Szucs, R., David, F., Sandra, P., J. Chromatogr. A (2006), 1109, 191-196
[6] Thompson, J.D., Carr, P.W., Anal. Chem. (2002), 74, 1017-1023
[7] Kanazawa, H., Matsushima, Y., Okano, T., in Brown, P.R., Grushka, E. (Eds.) Advances in Chromatography, vol. 41, 8, Marcel Dekker, New York (U.S.A.), 2001, 311-336
[8] Kikuchi, A., Okano, T., Prog. Polym. Sci. (2002), 27, 1165-1193
[9] Kanazawa, H., Anal. Bioanal. Chem. (2004), 378, 46-48
[10] Ayano, E., Kanazawa, H., J. Sep. Sci. (2006), 29, 738-749
[11] Kanazawa, H., J. Sep. Sci. (2007), 30, 1646-1656
[12] Kanazawa, H., Matsushima, Y., Okano, T., Trends Anal. Chem. (2008), 17, 435-440
[13] Kanazawa, H., Okano, T., J. Chromatogr. A (2011), 1218, 8738-8747
[14] Hosoya, K., Kimata, K., Araki, T., Tanaka, N., Fréchet, J.M.J., Anal. Chem. (1995), 67, 1907-1911
[15] Kanazawa, H., Yamamoto, K., Matsushima, Y., Takai, N., Kikuchi, A., Sakurai, Y., Okano, T. Anal. Chem. (1996), 68, 100-105
[16] Yamamoto, K., Kanazawa, H., Matsushima, Y., Takai, N., Kikuci, A., Okano, T., Chromatography (2000), 21, 209-215
[17] Song, Y.X., Wang, J.Q., Su, Z.X., Chen, D.Y., Chromatographia (2001), 54, 208-212
[18] Lynen, F., Heijl, J.M.D., Du Prez, F.E., Brown, R., Szucs, R., Sandra, P., Chromatographia (2007), 66, 143-150
[19] Yamamoto, K., Kanazawa, H., Matsushima, Y., Oikawa, K., Kikuchi, A., Okano, T., Environ. Sci. (2000), 7, 47-56
[20] Sakamoto, C., Okada, Y., Kanazawa, H., Kikuchi, A., Okano, T., Bunseki Kagaku (2003), 52, 903-906
[21] Ayano, E., Okada, Y., Sakamoto, C., Kanazawa, H., Okano, T., Ando, M., Nihimura, T., J. Chromatogr. A (2005), 1069, 281-285
[22] Kanno, S., Watanabe, K., Hirano, S., Yamagischi, I., Gonmori, K., Minakata, K., Suzuki, O., Forensic Toxicol. (2009), 27, 103-106
[23] Kanazawa, H., Kashiwase, Y., Yamamoto, K., Matsushima, Y., Kikuchi, A., Sakurai, Y., Okano, T., Anal. Chem. (1997), 69, 823-830
[24] Kanazawa, H., Yamamoto, K., Kashiwase, Y., Matsushima, Y., Takai, N., Kikuchi, A., Sakurai, Y., Okano, T., J. Pharm. Biomed. Anal. (1997), 15, 1545-1550
[25] Kanazawa, H., Sunamoto, T., Matsushima, Y., Kikuchi, A., Okano, T., Anal.

Chem. (2000), 72, 5916-5966
[26] Nishio, T., Suzuki, R., Tsukada, Y., Kanazawa, H., Okano, T., Miyabe-Nishiwaki, T., J. Chromatogr. A (2009), 1216, 7427-7432
[27] Nagase, K., Kobayashi, J., Kikuchi, A., Akiyama, Y., Kanazawa, H., Okano, T., Appl. Mater. Interfaces (2012), 4, 1998-2008
[28] Go, H., Sudo, Y., Hosoya, K., Ikegami, T., Tanaka, N., Anal. Chem. (1998), 70-4068-4093
[29] Yakushiji, T., Sakai, K., Kikuchi, A., Aoyagi, T., Sakurai, Y. Okano, T., Anal. Chem. (1999), 71, 1125-1130
[30] Kanazawa, H., Sunamoto, T., Ayano, E., Matsushima, Y., Kikuchi, A., Okano, T., Anal. Sci. (2002), 18, 45-48
[31] Kanazawa, H., Ayano, E., Sakamoto, C., Yoda, R., Kikuchi, A., Okano, T., J. Chromatogr. A (2006), 1106, 152-158
[32] Nishio, T., Kanazashi, R., Nojima, A., Kanazawa, H., Okano, T., J. Chromatogr. A (2012), 1228, 148-154
[33] Kobayashi, J., Kikuchi, A., Sakai, K., Okano, T., Anal. Chem. (2001), 73, 2027-2033
[34] Kobayashi, J., Kikuchi, A., Sakai, K., Okano, T.,J., Chromatogr. A (2002), 958, 109-119
[35] Kobayashi, J., Kikuchi, A., Sakai, K., Okano, T., Anal. Chem. (2003), 75, 3244-3249
[36] Sakamoto, C., Okada, Y., Kanazawa, H., Ayano, E., Nishimura, T., Ando, M., Kikuchi, A., Okano, T., J. Chromatogr. A (2004), 1030, 247-253
[37] Ayano, E., Suzuki, Y., Kanezewa, M., Sakamoto, C., Morita-Murase, Y., Nagata, Y., Kanazawa, H., Kikuchi, A., Okano, T., J. Chromatogr. A (2007), 1156, 213-219
[38] Hosoya, K., Watabe, Y., Kubo, T., Hoshino, N., Tanaka, N., Sano, T., Kaya, K., J. Chromatogr. A (2004), 1030, 237-246
[39] Ayano, E., Sakamoto, C., Kanazawa, H., Kikuchi, A., Okano, T., Anal. Sci. (2006), 22, 539-543
[40] Nishio, T., Ayano, E., Suzuki, Y., Kanazawa, H., Okano, T., J. Chromatogr. A (2011), 1218, 2079-2084
[41] Kanazawa, H., Nishikawa, M., Mizutani, A., Sakamoto, C., Moria-Murase, Y., Nagata, Y., Kikuchi, A., Okano, T., J. Chromatogr. A (2008), 1191, 157-161
[42] Kikuchi, A., Kobayashi, J., Okano, T., Iwasa, T., Sakai, K., J. Bioact. Comp. Polymers (2007), 22, 575-588
[43] Ayano, E., Nambu, K., Sakamoto, C., Kanazawa, H., Kikuchi, A., Okano, T., J. Chromatogr. A (2006), 1119, 58-65
[44] Kanazawa, H., Ayano, E., Chiba, A., Kikuchi, A., Okano, T., Anal. Sci. (2001), 17, i875-i876
[45] Ayano, E., Okada, Y., Sakamoto, C., Kanazawa, H., Kikuchi, A., Okano, T., (J. Chromatogr. A (2006), 1119, 51-57
[46] Ayano, E., Kanazawa, H., Bunseki Kagaku (2005), 54, 593-603
[47] Kanezawa, M., Nambu, K., Suzuki, Y., Nishikawa, M., Kanazawa, H., Bunseki Kagaku (2007), 56, 397-407

[48] Mizutani, A., Nagase, K., Kikuchi, A., Kanazawa, H., Akiyama, Y., Kobayashi, J., Annaka, M., Okano, T., J. Chromatogr. A (2010), 1217, 5978-5985
[49] Mizutani, A., Nagase, K., Kikuchi, A., Kanazawa, H., Akiyama, Y., Kobayashi, J., Annaka, M., Okano, T., J. Chromatogr. B (2010), 878, 2191-2198
[50] Roohi, F., Antonietti, M., Titirici, M.-M., J. Chromatogr. A (2008), 1203, 160-167
[51] Liu, H., Liu, M., Bai, L., Sun, S., Liu, Y., Yang, G., Talanta (2011), 85, 1113-1198
[52] Liu, M., Liu, H., Liu, Y., Bai, L., Yang, G., Yang, C., Cheng, J., J. Chromatogr. A (2011), 1218, 286-292
[53] Zhang, R., Yang, G., Xin, P., Qi, L., Chen, Y., J. Chromatogr. A (2009), 1216, 2404-2411
[54] Yagi, H., Yamamoto, K., Aoyagi, T., J. Chromatogr. B (2008), 876, 97-102
[55] Yagi, H., Ebara, M., Yamamoto, K., Aoyagi, T., Mater. Sci. Eng., C (2011), 31, 1681-1687
[56] Yoshizako, K., Akiyama, Y., Yamanaka, H., Shinohara, Y., Hasegawa, Y., Kikuchi, A., Okano, T., Anal. Chem. (2002), 74, 4160-4166
[57] Yamanaka, H., Yoshizako, K., Akiyama, Y., Sota, H., Hasegawa, Y., Shinohara, Y., Kikuchi, A., Okano, T., Anal. Chem. (2003), 75, 1658-1663
[58] Qin, L., He, X.-W., Jia, M., Li, W.-Y., Zhang, Y.-K., Chem. Eur. J. (2011), 17, 1696-1704
[59] Malmstadt, N., Yager, P., Hoffman, A.S., Stayton, P.S., Anal. Chem. (2003), 75, 2943-2949
[60] Kurata, K., Shimoyama, T., Dobahi, A., J. Chromatogr. A (2003), 1012, 47-56
[61] Suedee, R., Seechamnanturakit, V., Canyuk, B., Ovatlarnporn, C., Martin, G.P., J. Chromatogr. A (2006), 1114, 239-249
[62] Yu, Q.-W., Ma, Q., Feng, Y.-Q., Talanta (2011), 84, 1019-1025
[63] Akiyama, Y., Shinohara, Y., Hasegawa, Y., Kikuchi, A., Okano, T., J. Polym. Sci. A (2008), 46, 5471-5482
[64] Miserez, B., Lynen, F., Wright, A., Euerby, M., Sandra, P., Chromatographia (2010), 71, 1-6
[65] Tan, I., Zarafshani, Z., Lutz, J.-F., Titrici, M.-M., Appl. Mater. Interaces (2009), 1, 1869-1872
[66] Kang, S.I., Bae, Y.H., J. Biomater. Sci. Polym. Ed. (2004), 15, 879-894

Chapter 4.

TEMPERATURE RESPONSIVE POLY-(N-VINYLCAPROLACTAM) AS STATIONARY PHASE FOR AQUEOUS AND GREEN LIQUID CHROMATOGRAPHY

Poly-(N-vinylcaprolactam) (PVCL) connected to aminopropyl silica is a new stationary phase for temperature responsive liquid chromatography (TRLC). PVCL shows a transition from hydrophilic to hydrophobic interaction between 30 and 40°C. The synthesis is described in detail. The temperature responsive characteristic of the phase is illustrated with a mixture of steroids using pure water as mobile phase. An increase in retention is observed when raising the temperature. H-u-plots at different temperatures were constructed. Below the lower critical solution temperature (LCST), no optimal velocity could be measured because of substantial resistance to mass transfer indicating an adsorption mechanism. Above the LCST, partitioning controls the separation resulting in higher efficiencies and an u_{opt} *of ca. 0.3 mm* s^{-1}*. Reduced plate heights decreased from 4 at 45°C to 3 at 65°C. The temperature responsive nature of the polymer is lost in green chromatography with ethanol as modifier in concentrations above 5%.*

This chapter was published as Miserez, B., Lynen, F., Wright, A., Euerby, M., Sandra, P. (2010) Chromatographia, 71: 1-6.

1. INTRODUCTION

Because of environmental concerns much effort is being put into the reduction of the consumption of harmful organic solvents in HPLC. To achieve this goal, miniaturization of the column dimensions or the use of elevated temperatures have been advanced [1]. As described in the previous chapters, purely aqueous temperature responsive liquid chromatography (TRLC) can also be used to achieve this goal.

So far, polyacrylamides have been favored in temperature responsive applications because of their rapid change in hydrophobicity over a very narrow temperature range. The chromatographic application of temperature responsive polymers, however, does not necessarily require a fast transition between the hydrophilic and hydrophobic state as a function of temperature. Poly(N-vinylcaprolactam) (PVCL) is a polymer showing a more gradual change from the hydrophilic to the hydrophobic state upon raising the temperature. The polymer has an LCST between 32 and 37°C and demixing behavior, dependent on chain length and polymer concentration, of PVCL differs when compared to PNIPAA [2,3]. This is, however, not expected to influence the chromatography much as, just for the latter, an increase in temperature will still lead to an increase in retention. Note that PVCL has a syndiotactic structure (ie its side chains are positioned alternatively on other sides of the polymer chain) in this way also differing from PNIPAA which has an atactic structure (ie the stereochemical orientation of its side chain is random) . PVCL has been studied in solution [3], as a copolymer [4], in hydrogels (hydrophilic network) [5,6] and bound to carriers [7]. It has been applied for cell and enzyme entrapment [5], as a catalyst support [8] and, combined with peptides, for wound healing [9]. It has also been used to control pore openings in track etched membranes [10]. PVCL has been coupled to silica and the use of this material for chromatography has been hinted [11], but no reports have been published.

In this chapter, the preparation of a silica stationary phase containing poly(N-vinylcaprolactam) is described. Columns packed with PVCL were evaluated in the temperature range 15 to 65°C with water and with water/ethanol as mobile phase.

2. EXPERIMENTAL

2.1 CHEMICALS

Aminopropyl silica (Nucleosil 100-5 NH_2, 5 µm particles having 100 nm pore size) was purchased from Macherey-Nagel (Düren, Germany). The following chemicals were used for the synthesis and obtained from Sigma-Aldrich (Bornem, Belgium): 4,4'-azobis(4-cyanovaleric acid), mercaptopropionic acid, N-hydroxysuccinimide, N,N'-dicyclohexylcarbodiimide, 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline (EEDQ), and N-vinylcaprolactam (VCL). HPLC grade water, methanol (MeOH), acetonitrile (ACN), isopropanol and N,N-dimethylformamide (DMF) were obtained from Sigma-Aldrich. DMF and mercaptopropionic acid were distilled under reduced pressure before use. VCL was re-crystallized from benzene and dried under vacuum. The steroid mixture was composed of hydrocortisone, prednisolone, cortisone, cortexolone, hydrocortisone acetate and methylprednisolone; the paraben mixture included methylparaben, ethylparaben, n-propylparaben and n-butylparaben; and the phenone mixture acetophenone, propiophenone, butyrophenone, valerophenone, benzophenone, hexanophenone, heptanophenone and octanophenone. All standards were from Sigma-Aldrich.

2.2 FINAL SYNTHESIS

10 g aminopropyl silica, 15.5 g EEDQ (62.5 mmol) and 8.7 g 4,4'-azobis(4-cyanovaleric acid) (31.0 mmol) were reacted in 125 mL DMF. After 16 h gentle swirling under nitrogen, the silica was filtered and washed with 250 mL water and 250 mL MeOH and dried under vacuum. 10 g of this silica and 15 g of VCL (107.8

mmol) were added to 125 mL of DMF and heated to 90°C for 16 h under nitrogen and gentle shaking. The silica was filtered, washed with 200 mL MeOH and dried under vacuum. The UV absorbance of the hydrolysed solutions was measured at 500 nm with a Uvikon XL UV spectrophotometer (BioTek instruments, Winooski, Vermont, USA).

2.3 Analytical Conditions

150 mm x 2.1 mm ID columns were slurry packed with a Haskel air driven pump (Burbank, California, USA). 0.8 g of the derivatized silica was slurried in 7 mL MeOH/isopropanol (1/1). Packing was done with MeOH. Columns were conditioned with water at 250 µL min^{-1} until a stable baseline in UV-detection and a stable back pressure were obtained. 150 mm x 3 mm ID columns were packed at Hichrom (Berkshire, England). Analyses were performed on an Agilent 1100 LC system equipped with a binary pump, autosampler, degasser and diode array detector (Agilent Technologies, Santa Clara, California, USA). The system was operated with Chemstation software. Detection was performed at 254 nm with a sample rate of 80 Hz. The temperature of the chromatographic column was accurately controlled with a Polaratherm 9000 series oven (SandraSelerity Technologies, Kortrijk, Belgium). The standard samples were dissolved at 1 mg mL^{-1} in ACN and diluted with water to 50 µg mL^{-1}. The injection volume was 5 µL.

3. Results and discussion

3.1 Synthesis of a PVCL-based Stationary Phase

Initially, the same synthesis route as previously described for PNIPAA derivatized aminopropyl silica was investigated [12]. In brief, this route consisted of the polymerisation of VCL in the presence of mercaptopropionic acid, which acts as a chain transfer agent to end the growth of the polymer chain and to provide it with a

carboxylic function for anchoring it to aminopropyl silica. The synthesis of PVCL was successful as could be ascertained by precipitation of the polymer in water at temperatures exceeding 35°C. Subsequently the carboxylic group at the end of the chain was activated with N-hydroxysuccinimide and N,N'-dicyclohexylcarbodiimide followed by reaction with aminopropyl silica. The coupling to aminopropyl silica was, however, not successful. This is most probably due to steric hindrance of the large VCL side groups complicating the coupling reaction.

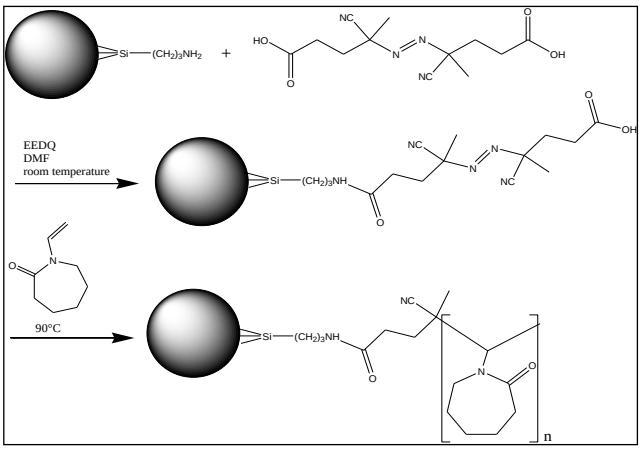


Fig. 4.1. Synthesis route of poly(N-vinylcaprolactam) on aminopropyl silica.

Therefore a strategy whereby the polymer is formed directly onto the aminopropyl silica was studied. This strategy has previously been described by Kanazawa et al. [13] with PNIPAA in the presence of a cross-linking agent for making an on-silica hydrogel. The synthesis route is presented in figure 4.1. 4,4'-azobis(4-cyanovaleric acid) was coupled to aminopropyl silica using 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline (EEDQ) as coupling agent. Subsequently the 4,4'-azobis(4-cyanovaleric acid) modified aminopropyl silica was heated to 90°C in the presence of

VCL starting the radical polymerization. Linear VCL chains coupled to aminopropyl silica were obtained in this way.

The resulting silica was analyzed by thermal gravimetric analysis. A weight loss of 23% was measured upon gradually heating the material up to 900°C under inert atmosphere. This carbon load exceeds the ones routinely obtained with common reversed phase materials or with PNIPAA [12] as a stationary phase. In order to ascertain that the temperature responsive PVCL was formed on the silica, 50 mg of the latter was hydrolyzed in 3 mL of a 5% NaOH solution overnight at room temperature. The UV-absorbance at 500 nm of the obtained transparent solution was measured as a function of temperature and compared to the results obtained for a solution of 3 mg mL^{-1} PVCL in water. The absorbance curves shown in figure 4.2 demonstrate the temperature responsive characteristics of the polymer by an increase in absorbance when reaching the cloud point. Although both solutions show the temperature responsive characteristics, the hydrolyzed silica solution shows the conversion from hydrophilic to hydrophobic at a much lower cloud point compared to the PVCL solution in water. This is due to the high ionic strength of the former solution which is a known parameter shifting the conversion temperature to lower values [14].

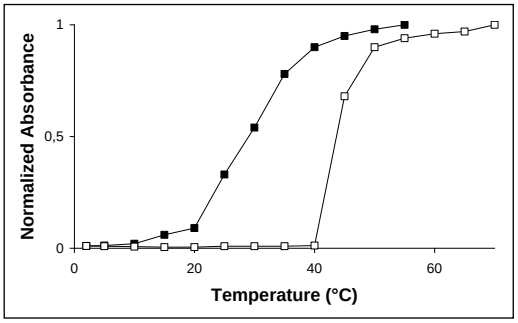


Fig. 4.2. UV-absorbance of two PVCL solutions as a function of temperature. Open symbols: PVCL in pure water. Closed symbols: PVCL in 5 % NaOH solution and overnight hydrolysis.

3.2 Chromatographic Evaluation of PVCL

The temperature responsive character of PVCL was, in first instance, evaluated by analysis of the steroid test mixture with pure water as mobile phase at 15, 25, 35 and 45°C. The column ID was 2 mm and the flow rate arbitrary set at 150 µL min^{-1}. The chromatograms are shown in figure 4.3.

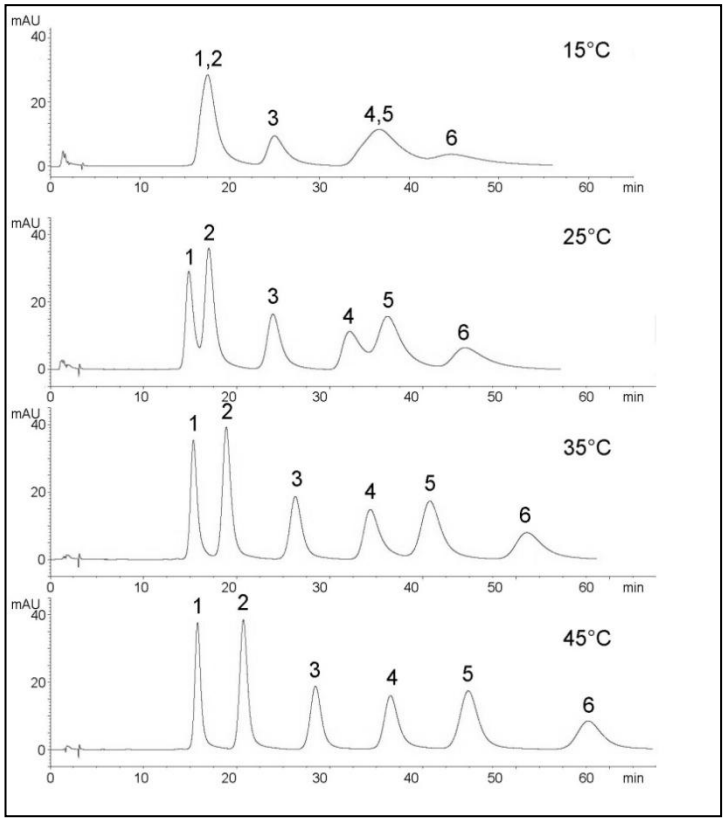


Fig. 4.3. Analysis of a mixture of six steroids with pure water at four different temperatures. Flow rate 150 µL min^{-1}, detection at 254 nm. Column: 150 mm x 2.1 mm ID PVCL. Peaks: 1. cortisone, 2. hydrocortisone, 3. prednisolone, 4. cortexalone, 5. methylprednisolone, 6. hydrocortisone acetate.

Contrary to conventional LC where retention decreases as a function of temperature (positive Van 't Hoff plots) [15], on temperature responsive columns, retention increases as function of temperature and negative Van 't Hoff plots are recorded. This is ascribed to an increase in hydrophobicity of the PVCL attached to the silica demonstrating the temperature responsive characteristics of the phase. Concerning resolution and efficiency, at 15°C the steroids are not separated because of the very low efficiency (1,250 plates for prednisolone) pointing out an adsorption rather than a partitioning mechanism. On the other hand, at 45°C the steroids are baseline separated and the plate number for prednisolone increased to 5,000. A similar behavior was observed for PNIPAA [12].

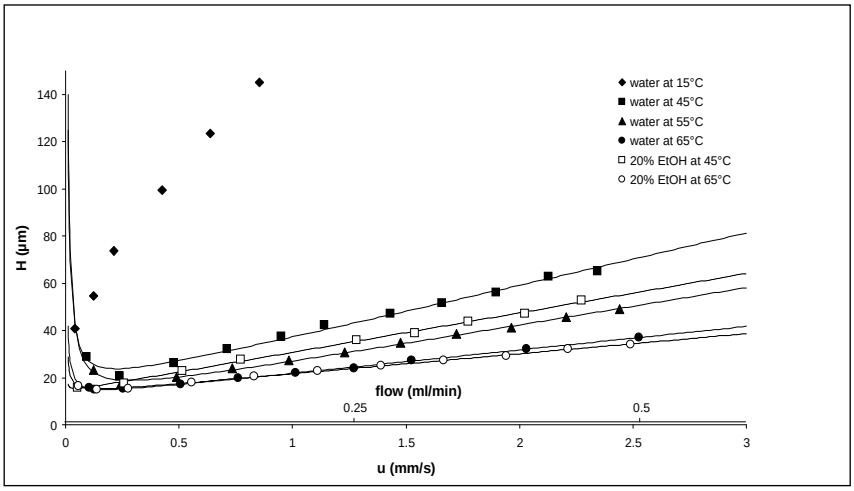


Fig. 4.4. van Deemter plots at different temperatures for prednisolone. Column: 150 mm x 2.1 mm ID PVCL. Full lines: pure water. Dotted lines: 20% ethanol.

A noticeable difference between PNIPAA [12] and PVCL is the much stronger retention of the solutes. On the one hand, this is due to the high loading of the stationary phase with PVCL, and, on the other hand, to the increased carbon number (including the propyl from aminopropyl and the remaining part of the initiator

between the PVCL chain and the silica) of the latter. The linker contains 9 carbon atoms, is not temperature responsive and will therefore remain hydrophobic at all temperatures causing severe retention for hydrophobic solutes.

As efficiencies in the temperature range 15-45°C were rather disappointing, van Deemter plots were recorded for prednisolone at 15, 45, 55 and 65°C. The H versus u plots are shown in figure 4.4.At 15°C, no minimum in the curve was noted because of the very slow mass transfer (C-term). At 45°C, i.e. above the LCST value where the separation mechanism is supposed to be partitioning, a normal H-u plot was obtained but H_{min} and u_{opt} are rather low, 25 µm and 0.25 mm s^{-1}, respectively. Efficiency increased in function of temperature H_{min} 20 at 55°C and 15 at 65°C but u_{opt} remained nearly constant. This is an unusual behaviour as for silica-based columns u_{opt} increases in function of temperature keeping the efficiency constant while for polymeric columns, both efficiency and optimal velocity increase in function of temperature [16, 17]. Compared to octadecyl silica columns, a reduced plate height h (H_{min}/d_p) of 3 at 65°C is still high but not exceptional considering the low viscosity of pure water. Operating the column at 65°C and a velocity of 2.5 mm s^{-1} (flow 0.5 mL min^{-1}) gives the same efficiency as at 45°C and a velocity of 0.7 mm s^{-1} (flow 0.15 mL min^{-1}). The retention time of prednisolone in figure 4.3 at 45°C of 30 min is thus reduced to less than 10 min.

Another alternative to reduce analysis times is the addition of an organic modifier. Ethanol was selected not only because it is biodegradable (green chromatography) but mainly because some of the target enzymes (see further in Chapter 5) tolerated low concentrations of ethanol. Figure 4.5A shows the chromatograms for the steroid mixture at 15 and 45°C with 5% ethanol. The temperature responsive character is still maintained while at 10% ethanol (figure 4.5B) the transition from hydrophilic to hydrophobic is no longer observed. Increasing the ethanol concentration to 20% (figure 4.5C) results in linear Van 't Hoff plots. Apparently, PVCL is fully soluble in this mobile phase at all temperatures and under these conditions the material acts as a conventional stationary phase. The influence of 20% ethanol on the H-u plots is

given in figure 5.4 dotted lines and at 45°C the efficiency drastically increases while this is no longer the case at 65°C.

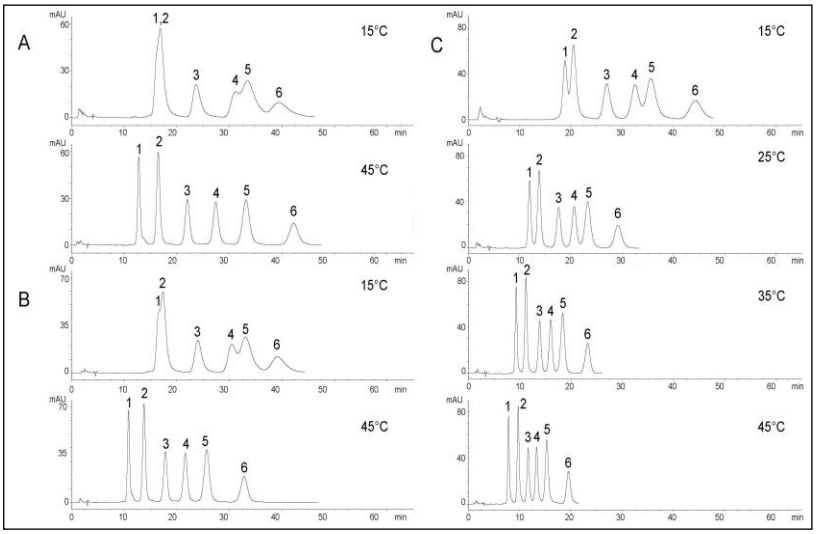


Fig. 4.5. Analysis of a mixture of six steroids with water/ethanol at different temperatures. A. 5% ethanol at 15 and 45°C, B. 10% ethanol at 15 and 45°C, C. 20% ethanol at 15, 25, 35 and 45°C. Peak identification as in figure 4.3.

Because reproducibility in column manufacturing is of utmost importance to any application, it was decided to outsource column packing to Hichrom that operates under ISO 9001. Selected column dimensions were 15 cm x 3 mm ID. Figure 4.6 shows performance chromatograms for the analysis of phenones (Top) and of parabens (Bottom) at a flow rate of 0.4 mL min^{-1} of the mobile phase water/20% ethanol at 65°C. The columns showed higher efficiency compared to the in-house packed columns, as the methylparaben peak showed a plate count of 10,000.

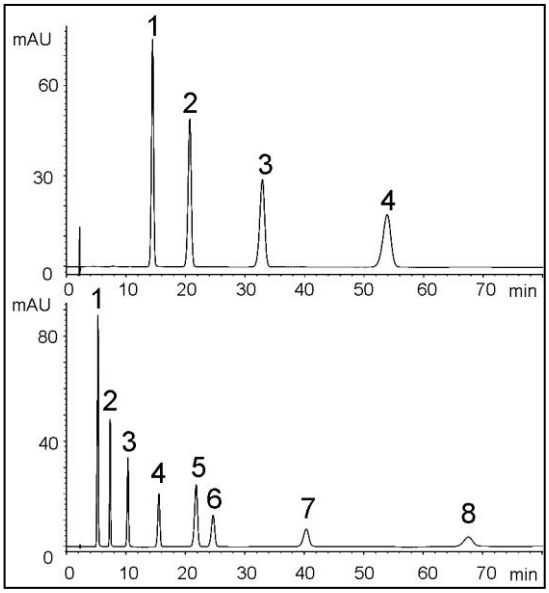


Fig. 4.6. Analysis of parabens (Top) and phenones (Bottom). Column: 150 mm x 3 mm ID PVCL. Flow rate 0.4 mL min^{-1} water/20% ethanol at 65°C. Peaks Top. 1. methylparaben, 2. ethylparaben, 3. n-propylparaben, 4. n-butylparaben. Peaks Bottom. 1. acetophenone, 2. propiophenone, 3. butyrophenone, 4. valerophenone, 5. benzophenone, 6. hexanophenone, 7. heptanophenone, 8. octanophenone.

4. Conclusion

A PVCL-based stationary phase has been successfully synthesized and can be used for aqueous temperature responsive LC. The expected increase in retention as a function of temperature is illustrated with a steroid standard mixture using water as mobile phase. An increase in efficiency and reduction of analysis times is noted at elevated temperatures. When adding ethanol at percentages above 10%, the temperature responsive characteristics of the phase are lost.

REFERENCES

[1] Vanhoenacker, G., Sandra, P., Anal. Bioanal. Chem. (2008), 390, 245-248

[2] Kirsh, Y., Yanul, N., Kalninsh, K., Eur. Polymer J. (1999), 35, 305-316

[3] Meeussen, F., Nies, E., Berghmans, H., Verbrugghe, S., Goethals, E., Du Prez, F.E., Polymer (2000), 41, 8597-8602

[4] Verbrugghe, S., Laukkanen, A., Aseyev, V., Tenhu, H., Winnik, F.M., Du Prez, F.E., Polymer (2003), 44, 6807-6814

[5] Markvicheva, E., Kuskina, I., Pashkin, I., Plechko, T., Kirsh, Y., Zubov, V., Biotechnol. Tech. (1991), 5, 223-226

[6] Markvivhva, E., Kuptsova, S., Mareeva, T., Vikhrov, A., Dugina, T., Strukova, S., Belokon, Y., Kochetkov, K., Baranova, E., Zubov, V., Poncelet, D., Parmar, V., Kumar, R., Rumsh, L., Appl. Biochem. Biotech. (2000), 88, 145-157

[7] Agrawal, M., Pich, A., Gupta, S., Zafeiropoulos, N., Rubio-Retama, J., Simon, F., Stamm, M., J. Mater. Chem. (2008), 18, 2581-2586

[8] Okhapkin, I., Bronstein, L., Makhaeva, E., Matveeva, V., Sulman, E., Sulman, M., Khoklov, A., Macromolecules (2004), 37, 7879-7883

[9] Strukova, S., Dugina, T., Chistov, I., Lange, M., Markvicheva, E., Kuptsova, S., Zubov, V., Glusa, E., Clin. Appl. Thromb.-Hemost. (2001), 7, 325-329

[10] Lequieu, W., Shtanko, N., Du Prez, F., J. Membr. Sci. (2005), 256, 64-71

[11] Ivanov, A., Pure Appl. Chem. (1999), A36, 1981-1989

[12] Lynen, F., Heijl, J.M.D., Du Prez, F.E., Brown, R., Szucs, R., Sandra, P., Chromatographia (2007), 66, 143-150

[13] Kanazawa, H., Sunamoto, T., Ayano, E., Matsushima, Y., Kikuchi, A., Okano, T., Anal. Sci. (2002), 18, 45-48

[14] Liu, X.M., Wang, L.S., Wang, L., Huang, J.C., He, C.B., Biomaterials (2004), 25, 5659-5666

[15] Melander, W., Campbell, D.E., Horváth, C., J. Chromatogr. (1978), 158, 215-225

[16] Vanhoenacker, G., Sandra, P., Anal. Bioanal. Chem. (2008), 390, 245-248

[17] Vanhoenacker, G., Pereira, A.D.S., Kotsuka, T., Cabooter, D., Desmet, G., Sandra, P., J. Chromatogr. A (2009), 1217, 3217-3222

Chapter 5.

Detection of Enzyme Inhibitors by HPLC - Post - Column Reaction

On-line coupling of HPLC with a post-column reaction to detect enzyme inhibitors appears a promising approach to identify, for example, new drug candidates from complex mixtures in a simple and fast way. Because of the sensitivity of enzymes to organic solvents, pure water is preferably used as mobile phase. The goal of this section of the work was therefore to study the use of the combination of temperature responsive liquid chromatography (TRLC) and an enzymatic post-column reaction for detection and on-line identification of enzyme inhibitors. Two model enzymes, namely cathepsin B and trypsin were used for this purpose.

Cathepsin B, having been studied intensively in the literature, and trypsin were chosen as model substrates. Cathepsin B was used to test the experimental setup, while trypsin was tested as it is a more sensitive enzyme, degrading in organic solvents. Despite initial success with cathepsin B, experiments with trypsin showed the problems of the approach. Practical problems were encountered with the system and they are mainly related to lack of robustness, sensitivity and universality. Moreover, optimization is needed for every enzyme. For some enzymes, the system works well, but the long reaction times needed for most enzymatic reactions, make the system impractical for routine application.

1. INTRODUCTION

As stated in chapter 2, an important topic in pharmaceutical research is finding new enzyme inhibitors as these molecules are potential drug candidates. For example, the well-known non-steroidal anti-inflammatory drug (NSAID) ibuprofen is a cyclooxygenase (I and II) inhibitor. In the search for new inhibitors nowadays, natural extracts, artificial libraries, etc. are screened on a high-throughput basis by separation, fractionation and off-line testing, making it time-consuming and labor intensive. Combining the separation power of HPLC, the specificity of enzymatic reactions and the sensitivity of mass spectrometry would be the ideal tool to separate, detect and identify inhibitors in one automated step. In the first part of this chapter an overview of the different on-line strategies using enzyme specificity in on-line combination with HPLC is provided, followed by a discussion of the inhibition assays performed for the purpose of this work.

Enzyme inhibitors are compounds that slow down the reaction catalyzed by an enzyme and they can work by different mechanisms. Some common inhibitor types are presented in figure 5.1. The normal enzymatic reaction consists of two steps: the reversible formation of the enzyme-substrate complex and the irreversible formation of the products. This is a generalization as not all enzymes use this two-step mechanism, but this mechanism is often used to explain and calculate enzyme kinetics, such as the Michaelis-Menten equation. Competitive inhibitors (part A) bind reversibly to the active site of the enzyme, thus competing with the substrate for the enzyme. Uncompetitive and non-competitive inhibitors (part B) bind at another site of the enzyme, changing the enzyme structure and the reaction rate. In non-competitive inhibition (part B1) the inhibitor can bind at any moment, but the final reaction cannot proceed with the inhibitor bound. In uncompetitive inhibition (part B2) the inhibitor can only bind the enzyme-substrate complex and has the same effect. Suicide inhibitors (part C), bind irreversibly at the active site of the enzyme, destroying the catalytic activity.

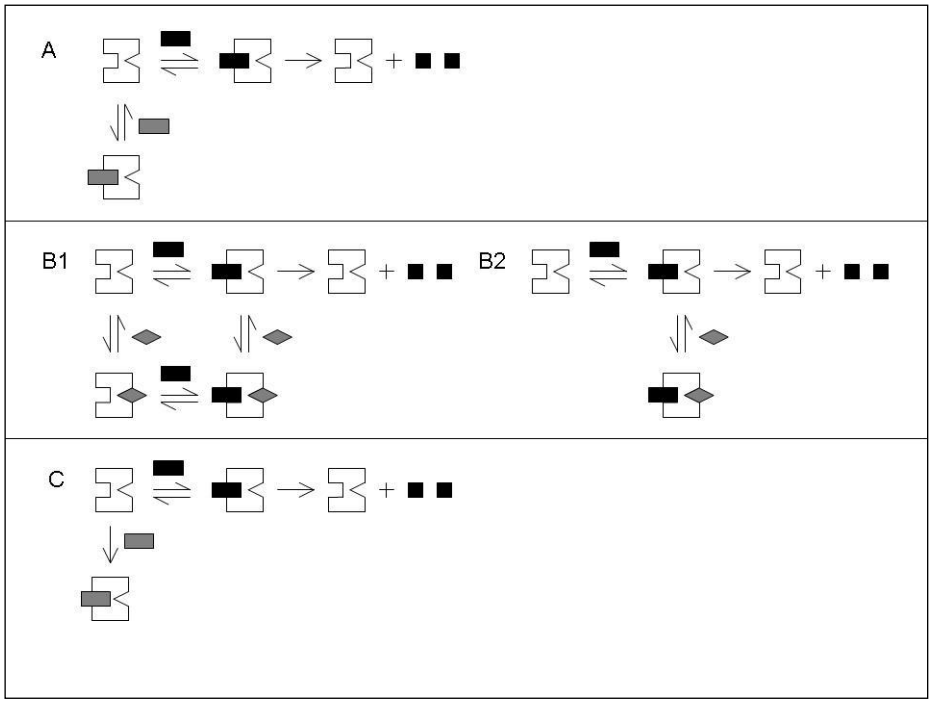


Fig. 5.1. Inhibitor types. White: enzyme, black: substrate and products, grey: inhibitor. A. Competitive inhibition: the inhibitor binds at the active site of the enzyme. B1. Non-competitive inhibition: the inhibitor binds at another part of the enzyme or the enzyme-substrate complex and the reaction cannot proceed when the inhibitor is bound. B2. Uncompetitive inhibition: the inhibitor binds at another part of the enzyme-substrate complex and the reaction cannot proceed when the inhibitor is bound. C: Suicide inhibition: the inhibitor binds irreversibly at the active site of the enzyme.

One of the first contributions on coupling a bioactivity assay to HPLC used fluorescence detection for the elucidation of ligands for the human estrogen receptor [1]. After reversed phase LC separation, the steroid binding domain of the human estrogen receptor was added, followed in a second phase by coumestrol, a fluorescent estrogenic ligand. As unbound coumestrol is more fluorescence than bound coumestrol, compounds eluting from the column that bind to the estrogen receptor increase the fluorescence. The setup for this reaction is similar to the one described in chapter 2, figure 2.1.. The small volumes of the reactor loops namely 270 and 570 µL, respectively, combined with high flow rates (HPLC: 0.5 mL min^{-1},

receptor: 1.4 mL min^{-1}, coumestrol: 0.5 mL min^{-1}) allowed a total reaction time of only 0.4 min. In a spiked urine sample, 17β-estradiol was detected at a concentration of 10^{-5} M (2.5 µg mL^{-1}), but no real samples were analyzed during this study. This work pioneered the possibility of an enzymatic post-column reaction. The main drawback of the system is the lack of mass spectrometric detection for identification of new ligands. This problem was addressed by van Liempd et al. who published a method in which the above described assay was combined with screening of the analytes by MS [2].

Another approach employing an enzyme affinity complex, was reported by Hogenboom et al., using a system to detect avidine and digoxin affinity in flow injection experiments. A sample was injected into a flow, avidine or digoxin was added and after a first loop, biotine or antidigoxin, respectively, was added. After the second loop, the free biotine or antidigoxin was detected by MS [3]. Both interactions were also used by Derks et al. [4] and Schenk et al. [5].

The first publication combining an enzyme inhibition assay with MS is the acetylcholinesterase inhibition assay published by Ingkaninan et al. in 2000 [6]. A colorimetric method for inhibitor detection was used and the flow was split before addition of the enzyme. One part was diverted to the MS and one part to the assay. The colorimetric method used the product of the enzymatic reaction, thiocholine, to react with 5,5'-dithiobis-(2-nitro-benzoic acid) to produce a colored compound. Thus inhibitors will be detected as negative peaks. A total reaction time of 1.9 min was obtained. The modifications to the system shown in figure 2.1 are (i) the addition of a splitter to MS detection before addition of the enzyme, (ii) the removal of loop 1 and (iii) the addition of an extra flow of 5,5'-dithiobis-(2-nitro-benzoic acid), positioned before the loop. The fluorescence detector was changed to a UV-Vis detector. The known inhibitor galanthamine was detected in *Narcissus* bulbs, as seen in figure 5.2.

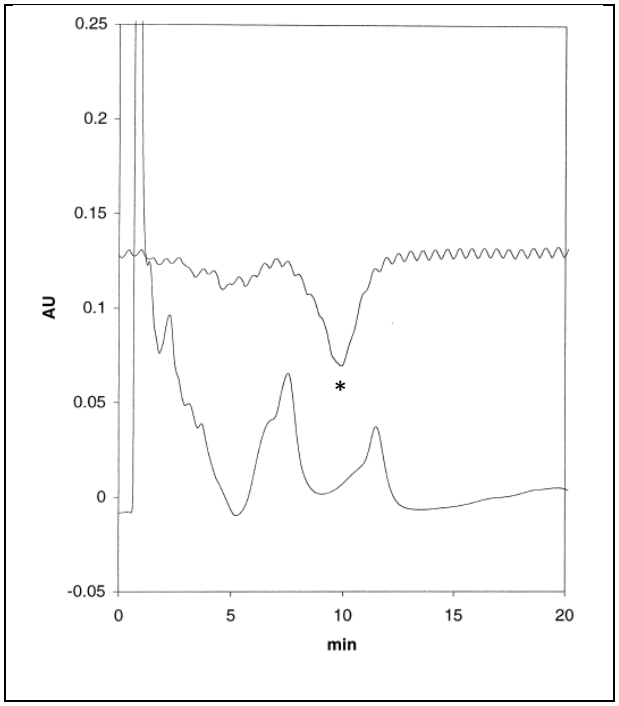


Fig. 5.2. Analysis of an extract of *Narcissus* by the acetylcholinesterase inhition assay. Top line: inhibition signal with * being the galanthamine peak. Bottom line: UV chromatogram. Galanthamine peak not identified. Data from [6].

Acetylcholinesterase is known to be a very fast enzyme, each molecule converting up to 25,000 molecules of acetylcholine per second [7], making it an ideal enzyme for post-column assays. However, for a post-column reaction, the solvent also plays a vital role. The ideal solvent for acetylcholinesterase was tested in two publications on post-column reactions [6,8]. Both compare the effect of various modifiers often used in chromatography: methanol, acetonitrile and acetic acid, but they differ in their reference solvent, as one uses water [6] and the other a 50 mM phosphate buffer at pH 7 [8]. In both cases, the aqueous solvent (water or phosphate buffer) proved to be the best solvent and 10% acetonitrile and 0.25% acetic acid were the worst solvents. However, the publications contradict each other as to the extent of the effect of the solvents. In the first publication, 10% methanol leaves only 60% of

the activity intact, whereas in the second publication, it leaves 90% of the activity intact. The same is true for acetonitrille, which leaves 20% and 50% of the activity intact respectively. This seems strange, unless the phosphate buffer is actually a far worse solvent than pure water for the reaction, which is very unlikely as the buffer is a better mimic of the in vivo situation. No comment on this discrepancy is given by the authors.

Plant extracts were analyzed by flow injection and compared to the method performed by a microplate assay. Extracts showing 0 to 39% inhibition on microplates were not detected as inhibitors by the flow injection assay, whereas extracts with 71 to 96% inhibition by microplate assay could be detected. Other percentages were not discussed. This illustrates the insensitivity of the flow injection assay, as some extracts showing inhibition in the microplate assay were not detected as inhibitors and can therefore be labeled as false negatives.

For the identification of peptides inhibiting angiotensin converting enzyme (ACE), a system with fluorescence detection and MS was developed [9]. The product of the reaction could be followed by fluorescence, while the inhibitor was identified by MS. Milk samples were analyzed and inhibition of ACE by several milk peptides was shown and the peptides were identified. This is one of the few papers describing the identification of new inhibitors by this technique. Other papers detected only known or added inhibitors. For example, the assay for inhibitors for phosphate consuming enzymes was tested with a plant extract spiked with 500 µM tetramisole and only tetramisole was detected as enzyme inhibitor [10]. In 2006, the ACE inhibitor assay was further optimized using only MS and not fluorescence for enzyme activity detection [11]. The substrate for fluorescence detection was not stable in solution and therefore a non-fluorescent substrate was used, explaining the need for MS detection. The assay was used for analysis of a *Narcissus* extract and three inhibitors were found but not identified.

In a publication concerning cathepsin B, de Boer et al. detected inhibitors in fungi extracts, but could not identify them [12]. Furthermore, because of an unstable

baseline, solutes were not detectable before a retention time of 10 min and after a retention time of 50 min. The analysis of a natural extract by this method is shown in figure 5.3. In 2005, the authors described a capillary setup for this assay in which the reactor was etched on a chip and a capillary column (I.D. 180 µm) was used for separation [13]. The performance of the assay was comparable to the conventional setup. Flow rates were decreased 25 times and thus so did enzyme consumption.

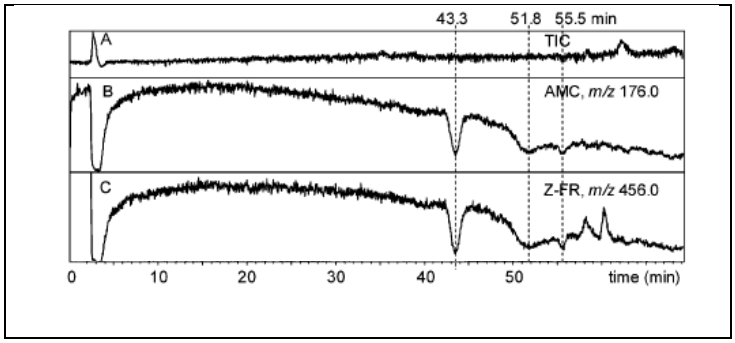


Fig. 5.3. Analysis of a natural extract by the cathepsin B inhibition assay. The marked times are retention times for known inhibitors. A. Total ion chromatogram, B. and C. m/z for the two products. Data from [12].

The problem of the identification of inhibitors from natural samples can be illustrated with a report detecting three phytoestrogens in pomegrate (*Punica granatum*) [14]. Two negative peaks were detected when analyzing a pomegrate extract by an assay with an estrogen receptor and coumestrol. The peaks were broad and spanned multiple signals in a parallel MS analysis. The only way the peaks could be identified was by injecting the pure inhibitors identified in an off-line bio-assay. In this way, luteolin, quercitin and kaempferol were identified as the phytoestrogens, but obviously this is useless to identify unknown substances or when standards are absent.

So far, two main problems with these assays remain: the lack of identification for newly found active analytes and solvent incompatibility between the LC separation and the enzymatic post-column reaction. A solution to the problem of solvent incompatibility between the RPLC analysis and the enzymatic assay, was proposed in 2005 by de Boer et al., by using High Temperature LC (HTLC) for the previously optimized cathepsin B reaction [15]. Using HTLC with column temperatures up to 208°C, the separation could be performed with only 10% methanol in the mobile phase, a great improvement for the enzyme activity. However, in this publication the only inhibitors that could be detected were the known cathepsin B inhibitors CA-074, E64 and leupeptin spiked to a tea extract. The question whether this technique is as powerful as the conventional RPLC method [12] remains open. On the other hand, temperature stability at 208°C of target solutes was not addressed at all and is questionable. In recent publications, the idea of using HTLC was not picked up, despite its claimed advantages and the progress made in HTLC over the last years. Conventional RPLC was used by Kool et al. [16] and Azevedo Marques et al. [17] for the detection of glutathione S-transferase inhibitors and acetylcholinesterase inhibitors in degradation products of tacrine, respectively.

A new approach to deal with solvent incompatibility was described by Schebb et al. [18]: the use of a countergradient. Because the gradient in RPLC contains organic solvents and the enzyme activity is changed during the analysis, a counter gradient was introduced after the analytical column so that a solvent with constant composition reached the bioreactor. The organic modifier was constant at 50% and although the baseline was indeed flat, the enzyme activity was severely diminished compared to lower concentrations as organic solvents.

In the area of affinity testing with enzymes, little progress has been made since the first publications. Kool et al. reported an on-line affinity test for cytochrome P450, but due to a very noisy baseline results were disappointing [19]. Falck et al. reported an affinity test for the mitogen activated protein kinase p38 (MAPK p38) [20]. The optimization procedure was described in detail. The most interesting parts deal with

solvent compatibility and choice of material for the reactor. To assess the effect of gradient elution, the system was operated under different isocratic conditions, ranging from 0 to 90% methanol. The resulting fluorescence signal was about 50% lower in 90% methanol. However, inhibitor peaks showed a bigger reduction in enzyme activity and thus, despite a decreasing baseline, the inhibitors could still be detected. Because hydrophobic compounds were included in the study, the influence of the tubing material was tested. Fused silica coated with polyethyleneglycol (PEG) was performing significantly better compared to polytetrafluoroethylene (PTFE), polyether ether ketone (PEEK) or fused silica with apolar coatings. This was due to adsorption of the hydrophobic compounds. However, most pharmaceuticals and biomolecules are hydrophilic, so the general use of this tubing is probably not relevant. Using the estrogen receptor, de Vlieger et al. coupled high resolution MS and NMR to this setup to identify derivatives of norethisterone, a known estrogen like molecule [21], leading to reliable identification of 7 derivatives.

Other post-column reactions with enzymes, not screening for ligands or inhibitors, have also been reported. As an example Schebb et al. described a screening method to detect proteolytic enzymes in mixtures [22].

For further reading, we refer to two recent reviews on these topics [23, 24]. From literature data, several problems can be seen. Newly discovered active analytes are hard to identify and indeed, very few publications have shown this. Furthermore, many of the cited articles are not true inhibition reactions, but show enzyme affinity, a much less interesting topic for pharmaceutical applications. A last comment comes from the lack of any real applications, as these assays have not yet proven themselves despite being around for over 10 years. This might be due to the experimental difficulties encountered with them, as will be discussed further in this chapter.

To broaden the applicability of bioreactors in HPLC and to avoid activity loss of the enzyme, our aim was to couple pure aqueous chromatography to enzymatic post-

column reactions. TRLC was chosen because it is the only LC technique theoretically allowing the adaptation of a separation while water as the only mobile phase constituent. Two model enzymes were selected namely cathepsin B and trypsin to develop a fast, universal and rugged method to detect enzyme inhibitors on-line with HPLC.

2. Materials and Methods

2.1 Chemicals and Instrumentation

Trypsin, N_{α}-benzoyl-L-arginine ethyl ester hydrochloride, water, methanol, acetonitrile, cathepsin B, dithioerythritol, Z-Phe-Arg-7-amido-4-methylcoumarin hydrochloride (Z-FR-AMC), acetic acid, ammonium formate, 4-(2-aminoethyl)benzenesulfonyl fluoride and E-64 ([1-[*N*-[(L-3-trans-carboxyoxirane-2-carbonyl)-L-leucyl]amino]-4-guanidinobutane]) were purchased from Sigma-Aldrich (Bornem, Belgium). The solvents were LC-MS grade. For the enzymes, the 1 mg stock diluted in 1 mL water was further diluted to 20 ng mL^{-1} and placed in separate vials of 500 µL. The vials were kept frozen at -20°C.

The experiments were conducted on a Waters Alliance 2695 with diode array detector and a Platform LC mass spectrometer controlled by Masslynx 4.0 (Waters, Milford, Massachusetts, USA). An ESI-ion source was used in the positive mode for all measurements. For flow injection experiments, a manual six way valve (Rheodyne, IDEX, Westheim-Monfeld, Germany) was used to introduce the sample. To deliver the enzyme and substrate, a Chemyx Fusion 100 dual syringe pump was used (Chemyx, Stafford, UK) equipped with two Hamilton 1 mL gas tight syringes (Hamilton, Bonaduz, Switzerland) for the experiments with trypsin and with two plastic 25 ml syringes for the experiments with cathepsin B. Set-up for these experiments can be seen in figure 2.1. For UV measurements, a Uvikon XL UV detector (BioTek Instruments, Winooski, Vermont, USA) was used.

Reactor loops were made from PTFE tubing with an internal diameter of 0.25 mm (VICI, Houston, Texas, USA). The reactors were knitted and heated in a water bath (Julabo, Seelbach, Germany). Connections were made with regular stainless steel unions, steel T-pieces and plastic syringe fittings.

2.2 EXPERIMENTAL

For the experiments with cathepsin B, a Zorbax C-8 column (3.5 µm particles, 2.1 mm I.D., 200 mm length, Agilent Technologies, Santa Clara, California, USA) was used. The LC flow was 200 µL min^{-1} of pure water. The reactor loops were 40 and 200 µL in volume and the flows of both the enzyme and substrate solution were set at 60 µL min^{-1}. The enzyme solution was made with 1.3 mg mL^{-1} ammonium formate set at pH 7 and contained 1.1 µg mL^{-1} catepsin B and 7.7 µg mL^{-1} dithioerythritol, to reduce the sulfide bridges in cathepsin B and thus activate the enzyme. The substrate solution was 32.5 µg mL^{-1} Z-FR-AMC in the same ammonium formate buffer. The inhibitor, E-64, was dissolved in water and used at a concentration of 0.1 ng $µL^{-1}$. All experimental conditions are detailed and explained further under the section results and discussion.

For the experiments with trypsin, the influence of organic solvents on the reaction rate was investigated. In 2 mL, 1 µg mL^{-1} trypsin was allowed to react at 37°C with 50 µg mL^{-1} N_{α}-benzoyl-L-arginine ethyl ester hydrochloride in pure water and 5, 10 and 25% methanol or acetonitrile. An aliquot of 5 µL of this solution was analyzed by MS using injection with a flow of 100 µL min^{-1} methanol/water 50/50 and scanning from m/z 200 to 400 in positive mode. The peak for the resulting product (at m/z 280) was integrated. The experiment was done in triplicate. To study the kinetics of the trypsin reaction, a mixture of 0.2 µg mL^{-1} tryspin and 10 µg mL^{-1} substrate was made and several aliquots of 5 µL were injected during 17 min in fow injection MS. The same mixture was made but with 10 µg mL^{-1} inhibitor added and subjected to the same analysis.

An in-house made PVCL column (150 mm L x 2.1 mm ID x 5 µm d_p, details can be found in Chapter 4), was used for an HPLC experiment with trypsin. The flow rate was 100 µL min^{-1} water and the coumn was kept at 35°C. The loop volumes were 100 and 800 µL and 2 µg mL^{-1} trypsin and 100 µg mL^{-1} N_α-benzoyl-L-arginine ethyl ester hydrochloride in water were added at 10 µL min^{-1} each. The loop size of the second loop was decided by testing different loop volumes (100 to 1000 µL). The loop volume with the smallest volume resulting in a detectable reaction was chosen. A make-up flow of 25 µL min^{-1} of 0,2% acetic acid in methanol was added before the MS. Detection was performed with DAD from 200 to 260 nm and with MS in positive mode and scanning from m/z 200 to 400. 5 µL of a 100 µg mL^{-1} 4-(2-aminoethyl)benzenesulfonyl fluoride solution in water was injected.

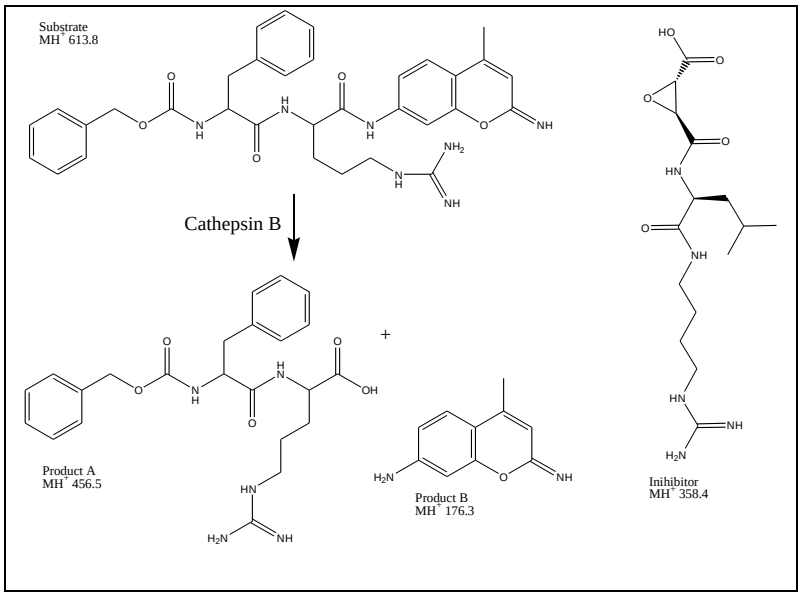


Fig. 5.4. The reaction of Z-FR-AMC catalyzed by cathepsin B. On the left is the inhibitor E-64.

3. Results and Discussion

3.1 Experiments with Cathepsin B using RPLC

To study the instrumental setup and evaluate the possibility of purely aqueous chromatography in an enzymatic post-column assay, the reaction setup described by de Boer et al. was used [12]. In this paper, the detection of enzyme-inhibitors of cathepsin B was described. The reaction catalyzed by cathepsin B is shown in figure 5.4. In figure 5.5, the mass spectra of the substrate and products are presented.

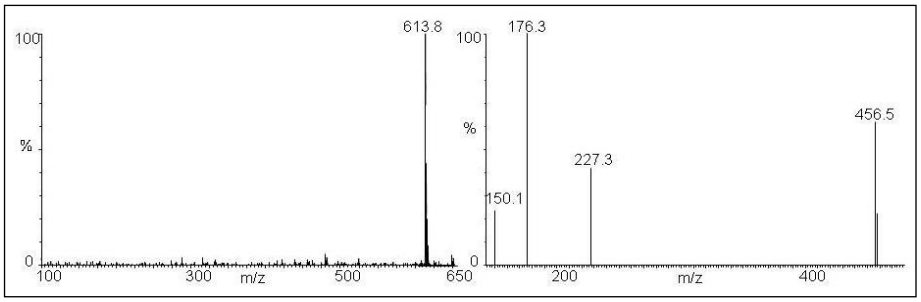


Fig. 5.5. Mass spectra showing the cathepsin B substrate Z-FR-AMC at m/z 613.8 (left) and the products after the reaction at m/z 176.3 and m/z 456.5 (right).

Initially, the experimental setup described in literature was adjusted to allow purely aqueous liquid chromatography. While in the literature a 45/55 mixture of methanol and water was used, the enzymatic reaction would benefit from the use of pure water as explained in chapter 2. Water was therefore selected as mobile phase. Because of the extensive retention of the inhibitor on a C-18 column when using pure water, a C-8 column was used. Furthermore, in literature the eluent was split before the enzymatic assay and to simplify the setup, the LC was miniaturized, using a 2.1 mm column, and the splitter was deleted. This changed the flow rate as well, as the full 200 µL min^{-1} now reached the reactor. To allow the inhibitor to react with the enzyme, the second reactor loop was increased in volume from 60 to 200 µL and

the enzyme and substrate flow were increased from 25 to 60 µL min^{-1}. In the original reactor, a total flow of 100 µL min^{-1} (50 µl min^{-1} from the LC, 25 µL min^{-1} enzyme and 25 µL min^{-1} substrate) reached the second loop of 60 µL, resulting in a reaction time of 0.6 min. In the improved setup, 320 µL min^{-1} of total flow (200 µL min^{-1} from the LC, 60 µL min^{-1} enzyme and 60 µL min^{-1} substrate) reached the 200 µL reactor, with a reaction time of 0.6 min, exactly the same as in literature. If the reactor was left at 60 µL, the reaction time in the second loop would have been 0.2 min, what would have led to a loss in sensitivity. Because of the small reaction time, very little substrate would be formed, resulting in a low baseline and low sensitivity. The result can be seen in figure 5.6. The inhibitor E-64 can be successfully detected by this method, proving that pure water can be used as the only solvent for the on-line detection of enzyme inhibitors by post-column reaction.

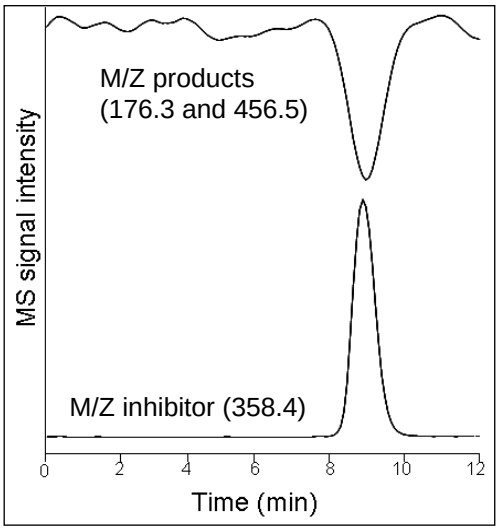


Fig. 5.6. Analysis of the cathepsin B inhibitor E-64 by post-column reactor using RPLC and pure water. Top: MS signal for the reaction products at m/z 176.3 and 456.5. Bottom: MS signal for the inhibitor E-64 at m/z 358.4.

As it was shown, the detection of a cathepsin B inhibitor by post-column reaction was feasible in pure water as the only solvent. To assess the influence of various amounts of organic solvents on the system, several concentrations of methanol and acetonitrile were tested. Because of the influence on the LC separation, the retention times shifted for the various concentrations. In figure 5.7, the results when using 100, 50, 25 and 0% methanol and acetonitrile are shown. It is very clear from this experiment that the presence of organic solvent, except for 100% acetonitrile, does not affect the system in any way. This is contrary to intuition and to literature [25], as organic solvent destabilizes cathepsin B. Cathepsin B denaturates in organic solvents and as it loses its three dimensional structure, it loses its ability to catalyze the reaction. Thus, introducing organic solvents as reaction solvents should decrease or even completely destroy the enzyme activity.

However, the reaction time is short and the concentration of water in the reactor is higher than in the LC effluent, as both the enzyme and substrate are added dissolved in water. As 120 µL min^{-1} of water is thus added to 200 µL min^{-1} of LC effluent, the water concentration for 25, 50 and 100% organic solvent in the mobile phase is 84, 69 and 38% in the reactor. Furthermore, the enzyme is in contact with the organic solvent for only 0.8 min in total. It is possible that the water molecules, bound to the outer hydrophilic groups of the enzyme, form a protective layer around it, so the enzyme actually does not come into contact with the organic solvent in the reactor. In its normal conformation, a water soluble protein will have a conformation to maximize the amount of polar groups at the surface and direct the apolar side chains of the amino acids towards its centre, thus forming a stable structure in water [26]. Water molecules bind to the polar groups by hydrogen bonds, ionic interactions and Van der Waals interactions. It is possible the water molecules form a protective layer around the enzyme and this layer of water is not penetrated by the organic solvent in the short reaction time needed.

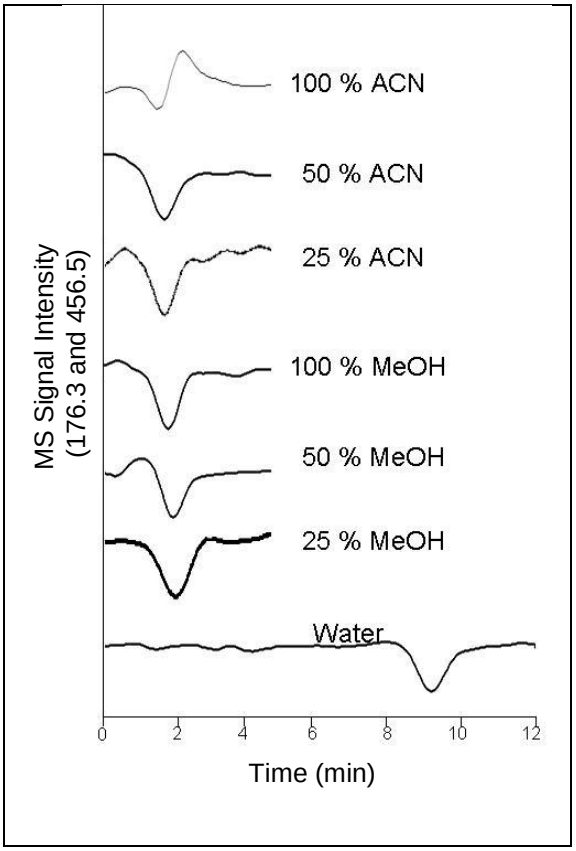


Fig. 5.7. Effect on the peak size of organic solvents as LC solvent. All other parameters were kept constant. Differences in retention time are due to the change in LC solvent.

To assess the sensitivity and the possibility of quantification, varying injection volumes were used. Therefore sample plugs of 10, 25 and 50 µL, corresponding to 10, 25 and 50 ng of inhibitor, were injected on the column. Lower sample amounts gave no discernible response. The obtained responses are shown in figure 5.8. It can be seen that the system responds to the amount of inhibitor injected. However, the observed response is not changing linearly with the injected volume, making it harder to use this setup for quantitative analysis.

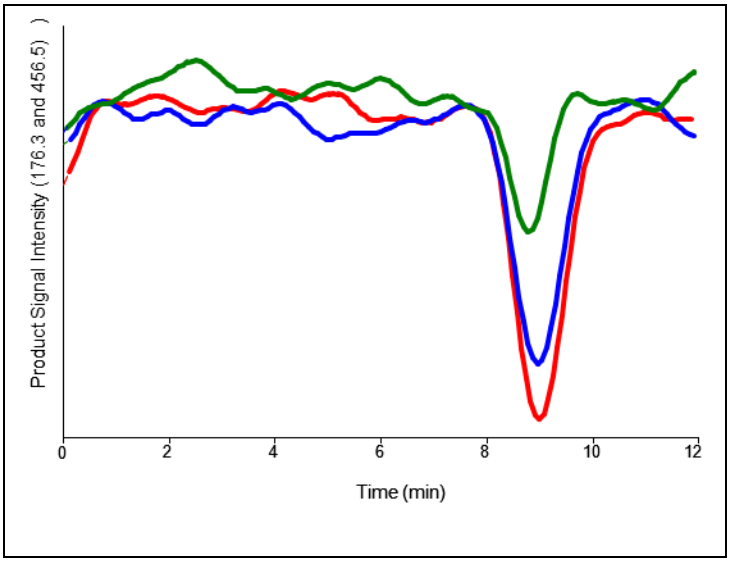


Fig. 5.8. Difference in response for different amounts of injected inhibitor. Green: 10 ng, blue: 25 ng, red: 50 ng.

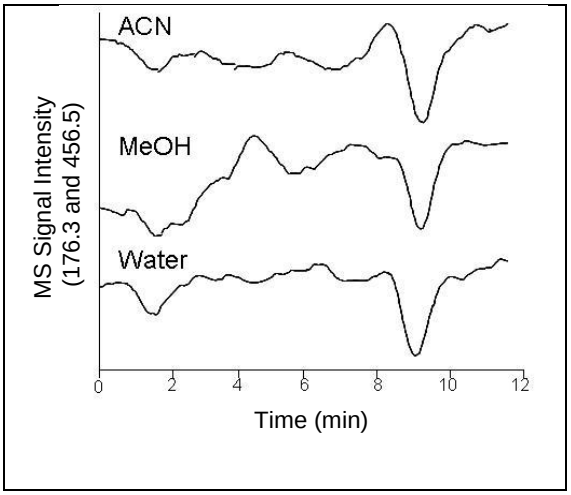


Fig. 5.9. Effect on the peak size of the post-reactor addition of different solvents between the reactor and the detector. Top: acetonitrile, middle: methanol, bottom: water.

As the only solvent in this assay, is pure water, the ionization efficiency in ESI-MS can be questioned. To solve this problem, a T-piece introducing a flow of 30 µL min^{-1} methanol or acetonitrile was built in between the reactor and the detector, resulting in 16 % organic solvent in the fluid when reaching the ESI-source. The result of this experiment can be seen in figure 5.9. The total sensitivity of the system is not increased and the introduction of the extra organic solvent was discarded.

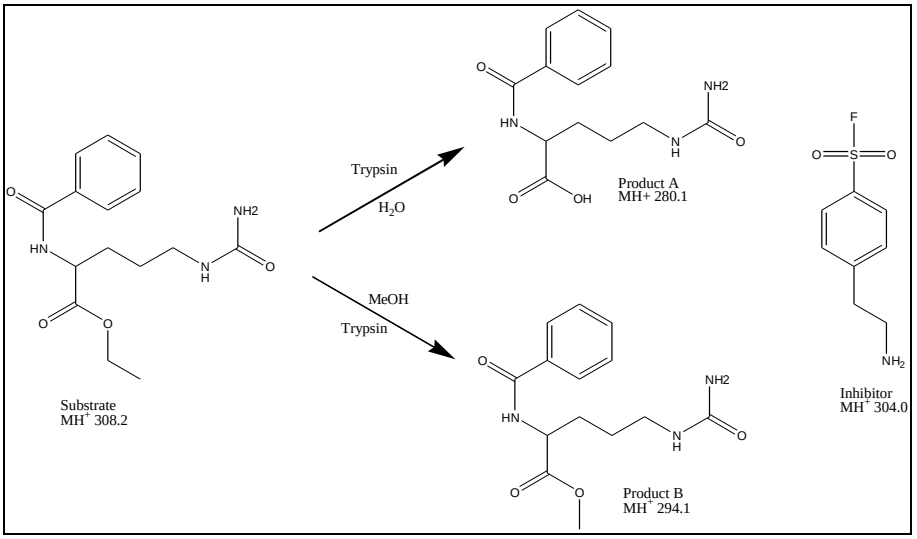


Fig. 5.10. Reaction catalyzed by trypsin, using water (product A) and methanol (product B). The inhibitor is shown on the right.

3.2 Experiments with Trypsin using TRLC

When working with an enzyme that is more sensitive to organic solvents and/or requires a longer reaction time, it becomes necessary to use purely aqueous chromatography. Here, TRLC was chosen with a PVCL phase, as described in chapter 4. As seen in the experiments using cathepsin B, the presence of organic solvents does not always imply the destabilization of the enzyme and resulting loss of activity. Since it was our aim to make a more universal detection system for enzyme

inhibitors, a second enzyme was used and to start, the stability of the enzyme in various organic solvents was tested.

To assess this, the trypsin catalyzed hydrolysis of N_α-benzoyl-L-arginine ethyl ester was used. The reaction is shown in figure 5.10. If the reaction solvent contains methanol, trypsin can also use methanol instead of water for the reaction, resulting in the second product shown. In vivo, trypsin hydrolyses the amide bond at the C-terminal side of the basic amino acids lysine and arginine, except when followed by proline. The ester hydrolysis of this substrate follows the exact same mechanism as the natural substrate for trypsin, but the amino-group at the C-terminal end of the basic amino acid is replaced by an oxygen atom. The comparison of both substrates, natural and synthetic, are shown in figure 5.11.

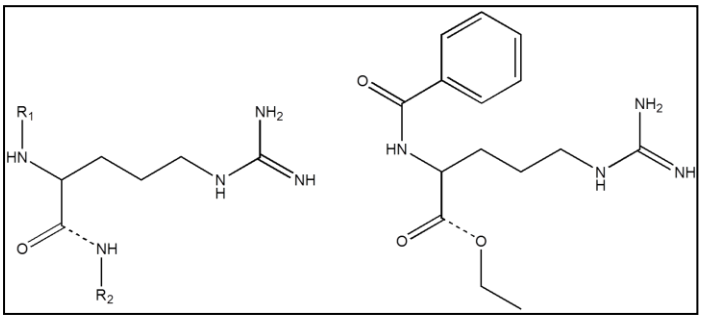


Fig. 5.11. Natural (right) and used (left) substrate of trypsin. The enzyme hydrolyses the bond shown as a dashed line. R_1 and R_2 are adjacent amino acids in the peptide.

The reaction mixture (1 µg mL^{-1} trypsin and 50 µg mL^{-1} N_α-benzoyl-L-arginine ethyl ester) was prepared in different concentrations of methanol and acetonitrile, two frequently used solvents in liquid chromatography. After 5 min reaction time, an aliquot of 5 µL was analyzed by mass spectrometry and the peaks at m/z 308.2 (the substrate) and m/z 280.4 (the product) were integrated. In figure 5.12, the resulting areas are plotted for each different solvent tested. In the case of reaction in

methanol, the peak at 294.1, was also integrated and the sum of the peak at 280.4 and 294.1 is shown. The reaction in pure water has the highest reaction rate. As the error bars in figure 5.12 represent the standard deviation, the differences between the various solvents are small compared to the errors, suggesting no statistical difference. However, a trend is clearly visible and even a small change in reaction rate could be a serious setback in an on-line system. An on-line system has low sensitivity and a small loss of sensitivity could have a devastating effect on the response. This is the reason why TRLC and more precisely PVCL-based columns were chosen for the coupling with enzymatic assays. Preliminary tests were conducted without a column, to test the reactor.

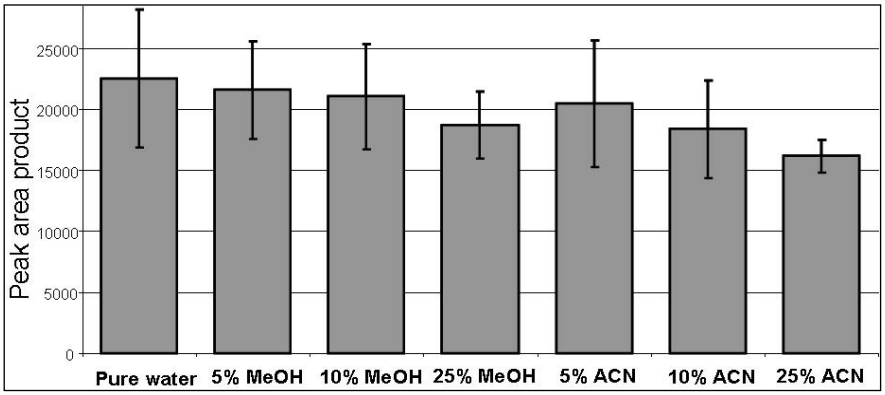


Fig. 5.12. Trypsin activity in various solvents. Peak areas of the product formed by reaction with trypsin in different solvent mixtures over 5 min. Error bars represent standard deviations on 3 measurements

Acetonitrile has a worse effect than methanol. This can be expected as acetonitrile is more hydrophobic, resulting in a more detrimental effect on the three-dimensional structure of the protein. As small as 5% methanol or acetonitrile already causes less product to be formed. This result seems contradictory to the experiment with varying organic solvents using the cathepsin B reaction (see figure 5.7). However, the

reaction times used here are far greater than the reaction times used for the post-column reaction with cathepsin B. This experiment strengthens our hypothesis that when reaction times are short, the water molecules bound to the enzymes outer polar groups protect the enzyme from denaturation by organic solvents. However, when reaction times are longer, as in this experiment, the water layer dissolves in the organic solvent and denaturation occurs.

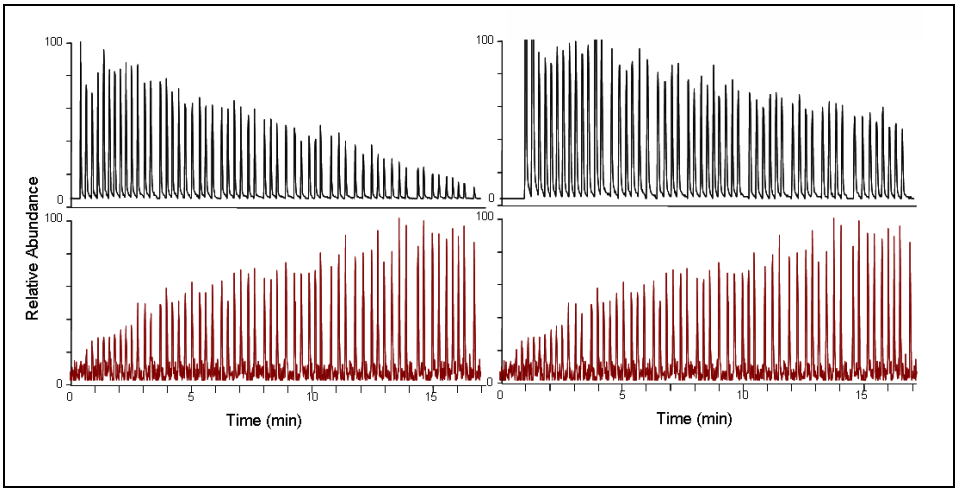


Fig. 5.13. Speed of the trypsin catalyzed hydrolysis of the model substrate. Left: no inhibitor present, right: inhibitor present. Top: amount of substrate present, bottom: Amount of product formed. Note all scales are different as the highest peak is 100% (outliers excluded).

The kinetics of the inhibitor reaction were studied by following the trypsin catalyzed hydrolysis of the model substrate over time. Trypsin (0.2 µg mL^{-1}) and its substrate (10 µg mL^{-1}) were allowed to react in water and aliquots were injected in flow injection MS over a time span of 20 min. In figure 5.13 the amount of substrate formed and product hydrolyzed can be seen for a mixture without and with the inhibitor added at 10 µg mL^{-1}. Without the inhibitor, substrate is being hydrolyzed to product, whereas when inhibitor is added, the reaction is visibly slower. As can be

seen, the concentration of substrate drops to about 15% when no inhibitor is present, while with the inhibitor, it only drops to about 45%.

Trypsin showed a slower reaction than cathepsin B and the reactor loops needed to be adjusted accordingly. A system was built using loops of 100 and 800 µL, resulting in a total theoretical reaction time of 7.5 min. The volume of the loops was based on tests done with various loop volumes. The 800 µL loop resulted in the shortest reaction time allowing the formation of a clearly detectable product. The 100 µL loop as a first loop was needed to see any inhibition by the inhibitor 4-(2-aminoethyl)benzenesulfonyl fluoride. This suicide inhibitor actually destroys the enzymes active site and if present before the substrate is introduced, the effect of the inhibitor will be bigger.

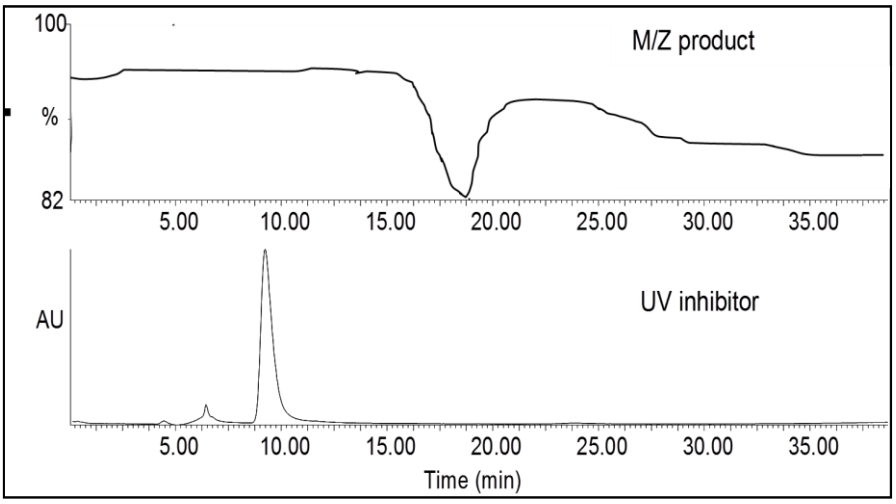


Fig 5.14. Result of 4-(2-aminoethyl)benzenesulfonyl fluoride injection on a PVCL-enzyme system. Chromatographic conditions: in-house made PVCL-column (150 mm long, 2.1 mm I.D., 5 µm particles) at 35°C, flow rate 100 µL min^{-1}.

Another modification to the setup was the use of an in-house made PVCL-column, as described in chapter 4. This should allow tuning the separation by temperature and

thus avoiding the use of organic solvents, used to control the separation in RPLC. The result of the experiment can be seen in figure 5.14. A clear inhibitor signal can be seen. However, the peak is quite broad and the baseline clearly drops during the analysis. The difference in elution time between the top and bottom chromatogram can be explained by the position of the detectors. The bottom chromatogram is detected by the UV-detector positioned before the reactor loops, between the column and the first T-piece adding the enzyme. The top chromatogram is positioned after both reactor loops. The time difference between both chromatograms is thus approximately the reaction time, i.e. about 8.5 min in this setup. The slightly bigger offset compared to the calculated value (7.5 min) is probably due to the extra volume of the connectors.

From this result, the peak variance introduced by the post-column reactor can be calculated. A chromatographic peak on this column under these circumstances should have an efficiency of approximately 5,000 plates or a σ_t^2 of 0.02 min^2 (calculated using a retention time of 10 min). The peak for the inhibitor has a peak width at half height of 0.9 min, or a σ_t^2 of 0.15 min^2 (see chapter 1). This means the post-column reactor is responsible for a σ_d^2 of 0.13 min^2. This is 87% of the total variance when working with a conventional sized TRLC column. If the column would have been a conventional RPLC column, the estimated σ_c^2 is 0.004 min^2 (calculated using a retention time of 10 min and an efficiency of 25,000). Working with state-of-the-art columns with even higher efficiencies is thus a waste of chromatographic efficiency, as the reactor would destroy it entirely. The peak broadening makes the system very insensitive, as analyte bands become less concentrated. This causes “false negatives”: analytes will not be detected as enzyme inhibitors, even if they are. The lack of robustness and the very nature of enzymes are the other problems.

Despite this successful experiment, several problems were encountered. The first drawback of this system is the fact that it is widely applicable. For the reaction with trypsin, major modification had to be made to ensure a longer reaction time in the second reactor loop. The first loop also had to be enlarged to see the inhibition of

trypsin. The optimization needed for every enzyme, is a drawback and makes the system cumbersome in routine use. This is, however, not the only problem.

The lack of robustness might by the biggest problem with this system, especially for introduction in the pharmaceutical industry. It is caused by various factors. One problem is the contamination of the MS source, leading to irreproducible ionization. The delivery system of enzyme and substrate can also have a large influence. The reactor itself was temperature controlled, but the temperature for the storage of the enzyme during the analysis might be of influence as well. The concentrations of inhibitor, substrate and enzyme can also have an influence. Especially the enzyme concentration seems crucial as it is kept very low and minor deviations can have a big impact. The enzyme itself can hydrolyze and trypsin actually catalyzes its own hydrolysis as it is a protease, resulting in further changes in reaction speed.

Enzymatic reactions are very sensitive to a number of influences; salt concentration, temperature, pH, etc. But a lack of sensitivity already arises from the nature of inhibitory reactions. Suicide inhibitors are more easily detected when using a large first reactor loop, while competitive, non-competitive and uncompetitive inhibitors benefit from a short first reactor loop. This is due to the peak broadening in this first loop: competitive, non-competitive and uncompetitive inhibitors have little effect when no substrate is present. The detection of suicide inhibitors benefits from a reaction without substrate present, as they can already destroy the enzyme without the substrate being converted into product. Thus sensitivity for suicide inhibitors can be enhanced by lengthening the first loop, while that for competitive and uncompetitive inhibitors decreases because of extra peak broadening.

4. Conclusion

The objective, the design of a universal system to detect enzyme inhibitors using HPLC and on-line post-column reactions, could not be realized. The main problem

with an on-line setup for the detection of enzymatic inhibitors is the extensive peak broadening caused by the large reaction times. Examples have been published in the literature, but little new inhibitors have been identified by this approach and no applications have been reported. Most publications are written by a small group of researchers and only a limited number of enzymes have been studied. Furthermore, few data are available from literature on repeatability or robustness.

The system is very insensitive and highly irreproducible, leading to an analytical device that is very hard to use. In pharmaceutical research, the introduction of this system is not an option. According to our research, off-line high throughput screening is vastly more reliable than the on-line system.

REFERENCES

[1] Oosterkamp, A.J., Vilaverde Herraiz, M.T., Irth, H., Tjaden, U.R., van der Greef, J., Anal. Chem. (1996), 68, 1201-1206

[2] van Liempd, S.M., Kool, J., Niessen, W.M.A., van Elswijk, D.E., Irth, H., Vermeulen, N.P.E., Drug Metabolism Disposition (2006), 34, 1640-1649

[3] Hogenboom, A.C., de Boer, A.R., Derks, R.J.E., Irth, H., Anal. Chem. (2001), 73, 3816-3823

[4] Derks, R.J.E., Hogenboom, A.C., van der Zwan, G., Irth, H., Anal. Chem. (2003), 75, 3376-3384

[5] Schenk, T., Molendijk, A., Irth, H., Tjaden, U.R., van der Greef, J., Anal. Chem. (2003), 75, 4272-4278

[6] Ingkaninan, K., de Best, C.M., van der Heijden, R., Hofte, A.J.P., Karabatak, B., Irth, H., Tjaden, U.R., van der Greef, J., Verpoorte, R., J. Chromatogr.. A (2000), 872, 61-73

[7] Purves, D., Augustine, G.J., Fitzpatrick, D., Hall, W.C., LaMantia, A.S., McNamara, J.O., White, L.E. in Neuroscience, 4th edition (2008). Sinauer Associates. Sunderland, MA, USA

[8] Rhee, I.K., Appels, N., Luijendijk, T., Irth, H., Verpoorte, R., Phytochem. Anal. (2003), 14, 145-159

[9] van Elswijk, D.A., Diefenbach, O., van der Berg, S., Irth, H., Tjaden, U.R., van der Greef, J., J. Chromatogr. A (2003), 1020, 45-58

[10] Schenk, T., Appels, N.M.G.M., van Elswijk, D.A., Irth, H., Tjaden, U.R., van der Greef, J., Anal. Biochem. (2003), 316, 118-126

[11] de Jong, C.F., Derks, R.J.E., Bruyneel, B., Niessen, W., Irth, H., J. Chromatogr. A (2006), 1112, 303-310

[12] de Boer, A.R., Letzel, T., van Elswijk, D.A., Lingeman, H., Niessen, W.M.A., Irth, H., Anal. Chem. (2004), 76, 3155-3161

[13] de Boer, A.R., Bruyneel, B., Krabbe, J.G., Lingeman, H., Niessen, W.M.A., Irth, H., Lab on a Chip (2005), 5, 1286-1292

[14] van Elswijk, D.A., Schobel; U.P., Lansky, E.P., Irth, H., van der Greef, J., Phytochem. (2004), 65, 233-241

[15] de Boer, A.R., Alcaide-Hidalgo, J.M., Krabbe, J.G., Kolkman, J., van Emde Boas, C.N., Niessen, W.M.A., Lingeman, H., Irth, H., Anal. Chem. (2005) 77, 7894-7900

[16] Kool, J., Eggink, M., Van Rossum, H., Van Liempd, S.M., van Elswijk, D.A., Irth, H., Commandeur, J.N.M., Meerman, J.H.N., Vermeulen, N.P.E., J. Biomol. Screen. (2007), 12, 396-405

[17] Azevedo Marques, L., Kool, J., de Kanter, F., Lingeman, H., Niessen, W., Irth, H., J. Pharm. Biomed. Anal. (2010), 53, 609-616

[18] Schebb, N.H., Heus, F., Saenger, T., Karst, U., Irth, H., Kool, J., Anal. Chem. (2008), 80, 6764-6772

[19] Kool, J., van Liempd, S.M., van Rossum, H., van Elswijk, D.A., Irth, H.,

Commandeur, J.N.M., Vermeulen, N.P.E., Drug Metabolism Disposition (2007), 35, 640-648

[20] Falck, D., de Vlieger, J.S.B., Niessen, W.M.A., Kool, J., Honing, M., Giera, M., Irth, H., Anal. Bioanal. Chem. (2010), 389, 1771-1780

[21] de Vlieger, J.S.B., Kolkman, A.J., Ampt, K.A.M., Commandeur, J.N.M., Vermeulen, N.P.E., Kool, J., Wijmenga, S.S., Niessen, W.M.A., Irth, H., Honing, M., J. Chromatogr. B. (2010), 878, 667-674

[22] Schebb, N.H., Vielhaber, T., Jousset, A., Karst, U., J. Chromatogr. A (2009), 1216, 4407-4415

[23] Shi, S.Y., Zhou, H.H., Zhang, Y.P., Jiang, X.Y., Chen, X.Q., Huang, K.L., Trends in Anal. Chem. (2009), 28, 865-877

[24] Kool, J., Giera, M., Irth, H., Niessen, W.M.A., Anal. Bioanal. Chem. (2011), 399, 2655-2668

[25] Turk, B., Dolenc, I., Zerovnik, E., Turk, D., Gubensek, F., Turk, V., Biochemistry (1994), 33, 14800-14806

[26] Branden, C., Tooze, J., Introduction to Protein Structure, sec. ed. (1999), Garland publishing, New York, USA, p. 14

Chapter 6.

ANTIOXIDANT ANALYSIS

In the previous chapter, it was shown that the analysis of enzyme inhibitors by post-column reaction lacks robustness and sensitivity, not to say the idea is not feasible, because of the slow kinetics of enzymatic reactions. In the following chapters, a fast reaction was chosen to evaluate if reactions with faster kinetics can be coupled to HPLC more successfully. The selected reaction exhibits a radical mechanism to detect antioxidants.

As an introduction to the experimental work on antioxidant analysis, an overview of methods for antioxidant analysis is presented. The main emphasis is on radical scavenging assays using the stable radicals 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) and 2,2-diphenyl-1-picrylhydrazyl (DPPH). These assays can relatively easily be coupled to LC using conventional instrumentation; only one extra LC-pump and UV-detector are needed. As reactions with stable radicals and antioxidants are fast, post-column peak broadening in the reactor can be minimal.

Despite many applications, only the DPPH assay has been optimized recently in the literature. Furthermore, the detrimental effect of these post-column assays on the chromatographic efficiency has never been studied. Another lack in the current technique is the inability to study highly polar antioxidants, as they are not retained on RPLC or NPLC, the only two techniques used so far in combination with radical scavenging assays.

1. INTRODUCTION

Antioxidant analysis is of great interest to a wide range of scientific fields: dietary intake of antioxidants in food research, antioxidant levels in biological fluids as biomarkers, food additives to prevent oxidation or to obtain a higher nutritional value, additives in fossil fuels and polymers to protect against oxidative damage, etc. The rising interest is shown in the number of publications, an evolution seen in figure 6.1.

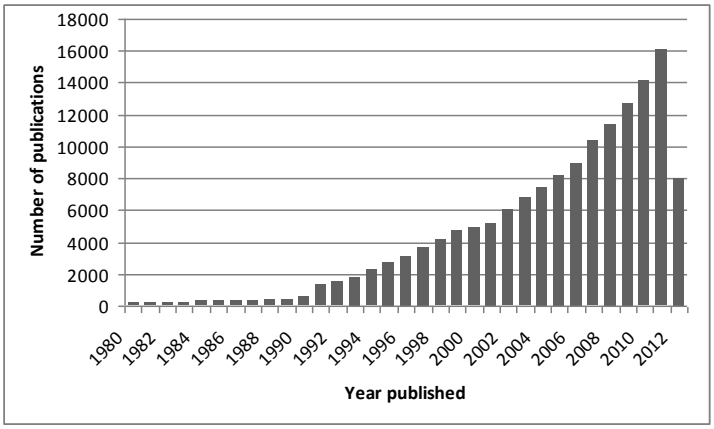


Fig 6.1. Number of publications per year on antioxidants in Web of Science from 1980 to July 2012 with the search term "antioxidant* or anti-oxidant*".

Antioxidants can be defined as compounds that, when present in lower quantities compared to the target molecules, protect them from oxidative damage. The targets can be present in food, polymers, fuels, etc. but also in in vivo molecules such as proteins, DNA and lipids, whose oxidation can lead to severe illnesses such as cardiovascular diseases and cancer. Protection can be performed at multiple levels e.g. stopping the formation of Reactive Oxygen Species (ROS), such as singlet oxygen or peroxides, complexation of metals whose presence might lead to oxidizing

compounds, or the formation of stable radicals, thus preventing the radical from further reaction.

Depending on the information needed, a number of methods can be applied for antioxidant analysis. Quantification of a target antioxidant or assessing the antioxidant capacity of a mixture is the primary goal of antioxidant determinations. Identifying new antioxidants in complex mixtures is, by far, the hardest task. Solutions can be found in coupling an antioxidant assay on-line with HPLC using a post-column reaction. The compounds in a complex mixture are first separated in the HPLC column and selectively detected by the antioxidant reactor. Upon coupling with MS, the antioxidants can be identified.

2. OFF-LINE RADICAL SCAVENGING ANTIOXIDANT ASSAYS

Radical scavenging antioxidant assays use a radical such as 2,2-diphenyl-1-picrylhydrazyl (DPPH) or a radical cation like 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS). The structures are shown in figure 6.2. Both molecules are colorless in the non-radical form and colored in the radical form.

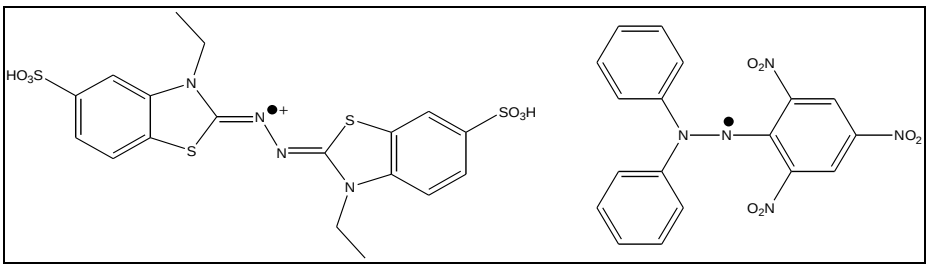


Fig. 6.2. Structure of the radical cationic form of ABTS (left) and radical form of DPPH (right).

The currently used ABTS assay was first published under its present form as an off-line method to screen the antioxidant activity in 1999 [1]. The novelty of this method

was the generation of the ABTS cationic radical before the assay, instead of in situ enzymatic generation as previously used. The advantage of this was its applicability in both hydrophobic and hydrophilic environments. The fact that the assay no longer needed a hydrophilic environment made it ideal to be coupled to RPLC (see further). This off-line method and a comparable method using DPPH are often used to determine the antioxidant activity of an individual solute or a mixture.

The off-line ABTS and DPPH methods have been compared, concluding that there is only a weak correlation between the results from the ABTS and DPPH assays [2]. For the DPPH assay, a sample is added to a DPPH radical solution and the difference in absorbance before and after addition is measured and referenced to the difference caused by addition of 1 mM α-tocopherol. For the ABTS assay, the procedure is similar but Trolox (commercial name for 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) is used instead of α-tocopherol. The weak correlation between both values could be attributed to the different reference solutes used. In our experience, α-tocopherol shows a lower response in both the DPPH and ABTS assays when compared to Trolox (see chapter 7). However, for unknown solutes, DPPH and ABTS could also react differently with the analytes. This should be a warning when comparing data from different techniques.

The weak correlation between different antioxidant assays was also illustrated by the comparison of the ABTS and DPPH assays to qualify the antioxidant activity of plant extracts [3] and of several wines [4]. Dilution and time have a great influence on the ABTS and DPPH assays. Wine samples, and by extrapolation all samples, should be diluted sufficiently to minimize the influence of matrix compounds that could absorb at the wavelengths used in the assay. The time of the reaction should be carefully set as the longer the reaction time is, the higher the ABTS and DPPH values are [4]. Several reports using off-line ABTS and/or DPPH assays to assess the antioxidant capacity of various fruit, tea's, wines, etc. have been published [5-14].

3. ON-LINE RADICAL SCAVENGING ANTIOXIDANT ASSAYS

Radical scavenging assays coupled to HPLC were introduced in 2000 by Koleva et al. [15]. The instrumental setup is quite simple: a conventional HPLC system is used and the effluent from the column is mixed via a T-piece with the reagent flow, pumped by a second conventional HPLC pump. The T-piece is coupled to a reactor loop and the loop to a second UV-detector. The system is shown in figure 6.3. A result of plant extracts can be seen in figure 6.3. DPPH was used as stable colored radical and coupled to RPLC. It was shown that the DPPH radical exhibited different absorption at 517 nm in function of the composition of water/methanol mixtures. Absorption was stable between 10 to 90% methanol but was higher in pure methanol and lower in pure water. At a pH lower than 3, the absorbance of the DPPH solution decreased significantly.

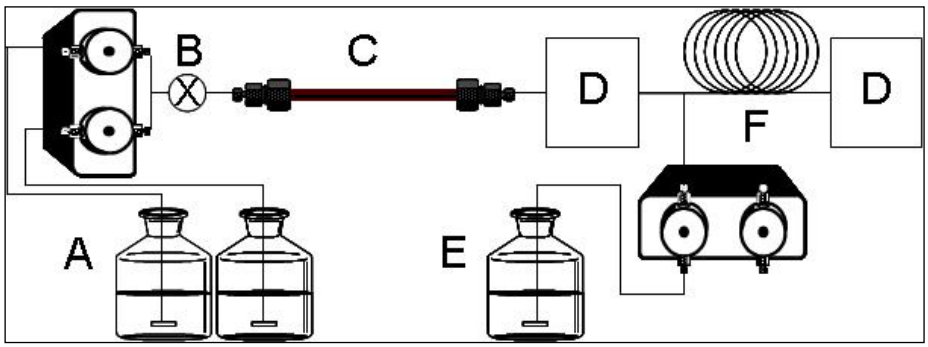


Figure 6.3. Setup for antioxidant analysis using either DPPH or ABTS. A. HPLC solvents and pump, B. Injector, C. Column, D. UV-detectors, E. Stable radical solution and pump, F. Reactor coil.

The DPPH method was used with Trolox as standard for the analysis of crude extracts of *Sideritis scardica*. An important loss in efficiency and in sensitivity compared to the first UV-chromatogram was noted. In 2001, Koleva et al. published a paper using a similar setup but with the ABTS radical [16]. A result of plant extracts from *S. syriaca* is shown in figure 6.4. The method was compared to the DPPH

method and proved to be more sensitive for 8 out of 10 tested compounds when using identical chromatographic conditions, by a factor 1,6 up to 300. The efficiency was slightly increased by applying a slightly higher flow rate for the ABTS solution (0.8 mL min^{-1}) compared to the DPPH solution previously described (0.7 mL min^{-1}). As pointed out in chapter 1, a higher flow rate in an open tubular reactor should theoretically lead to higher efficiency and this could explain the efficiency gain for ABTS compared to DPPH.

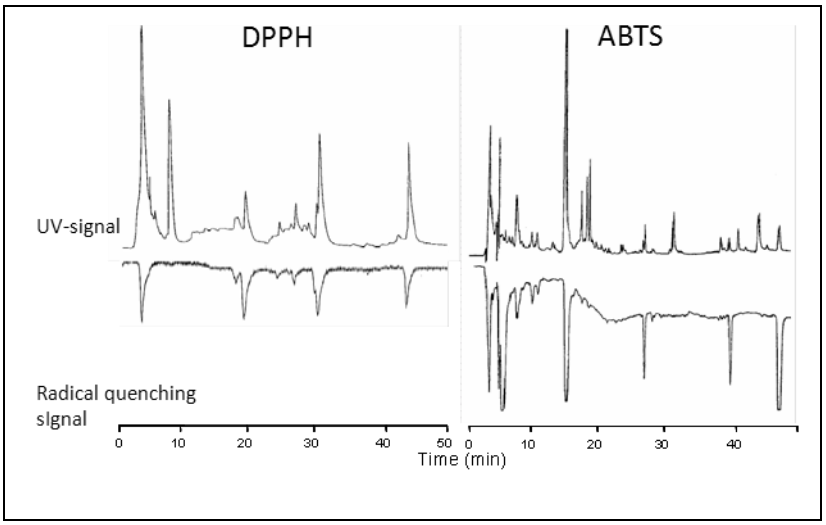


Fig. 6.4. Analysis of plant extracts using radical scavenging assays. Left: analysis of an extract from *Sideritis scardica* using the DPPH radical scavenging assay. Data from [15]. Right: analysis of an extract from *Sideritis syriaca* using the ABTS radical scavenging assay. Data from [16]. Note the absence of the absorbance scale, as it is not given in the original publications.

In applications, the DPPH assay seems to be favored over the ABTS assay. Dapkevicius et al. used the DPPH method for the isolation and identification of antioxidants in *Thymus vulgaris* leaves [17]. The DPPH signal was used to guide fractionation. Eight compounds were isolated and successfully identified by NMR. One new antioxidant was identified. Bandoniene et al. used the method to study the antioxidant activity of extracts of several species of the genus *Salvia* [18] and *Borago*

officinalis L. [19]. Kosar et al. described the identification of antioxidants in various herbs using DPPH [20-22]. In the first publication [20], the detection limits and minimal detectable concentrations of several standards were measured and were 7 times higher than in the original papers by Koleva et al. [15,16]. In two follow-up publications, the same method was used to study water extracts from herbs [21] and a complex series of extracts from *Salvia officinalis* L. [22]. In comparison to the work of Bandoniene [18], more antioxidants were detected and this is due to the complex extraction procedures, resulting in a prefractionation of the analytes.

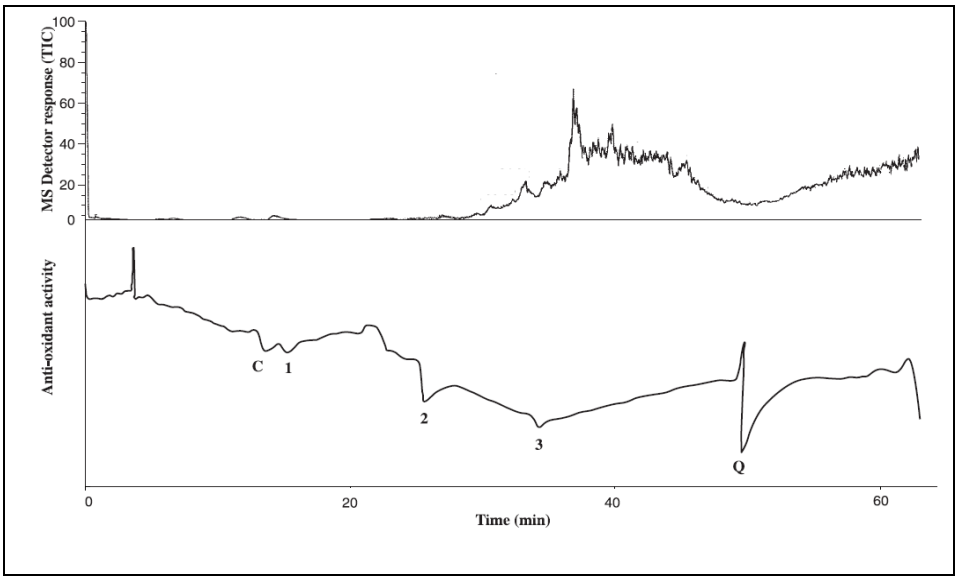


Figure 6.5. Analysis of an extract of *Butea superba* by LC-MS-DPPH assay. Peak labels: 1-3: antioxidants present in the extract, C: spiked catechin, Q: spiked quercetin. Data from [28].

An interesting paper from 2004 describes the use of flow injection coupled to the DPPH radical scavenging assay [23]. This gives the same information as the older off-line assay, but can be done at relatively high speed and in an automated way. If high throughput is needed, this system has a clear advantage over the off-line method. The system can analyze ca. 1 sample per min in a fully automated way. Other papers

on these assays include the analysis of *Potentilla fruticosa* [24] and *Germanium macrorrhizum* [25] by Milliauskas et al. In trying to identify new antioxidants, radical scavenger assays and NMR were hyphenated [26,27]. Only a few compounds could be identified in plant extracts by this approach. A better way to identify antioxidants is coupling the system to MS/MS. However, the first paper describing the use of on-line HPLC-post-column antioxidant reaction combined to MS/MS showed very bad peak shapes and low efficiency in the DPPH quenched chromatogram [28]. The reactor size, flow rate of the LC and DPPH radical solution and the DPPH concentration were changed compared to the original article by Koleva et al. [15] and this setup was obviously far from optimal. A high concentration of DPPH radical and low flow rate caused insensitivity and broad peaks, as shown in figure 6.5.

From 2006 onwards, the DPPH radical scavenging assay was used with varying results for the study of antioxidants in various plant extracts [29-31] and in coffee [32]. Low sensitivity and high noise levels were observed in the study by Gioti et al. [29]. Compared to the original publication [15], shorter reaction loops, lower flows and smaller concentrations were used. In a second report of coupling DPPH to MS, the DPPH quenching chromatogram showed low sensitivity, due to a large baseline drift [30]. Tung et al. used DPPH for the analysis of antioxidants in coffee, but used a low efficiency LC separation [32]. A nice application of the DPPH assay was shown by Oki et al. Changes in radical scavenging activity during the maturation of mulberry fruit were studied [31].

The first applications of the ABTS radical scavenging assay were published in 2005, four years after the original paper [33-36]: the study of olive tree wood [34], of phenolics in coffee [35], and of a biochemical pathway in tomato leading to novel flavonoids [36]. The first paper by Stewart et al. shows very high efficiency, with very little loss in efficiency [33] between the UV-chromatogram recorded after the column and the ABTS quenching chromatogram, recorded after the reactor. A flow is split to an MS and this work is beyond doubt better than the coupling of the DPPH assay to MS detection [28]. However, no remarks are made on the efficiency in the

paper itself. An example of this work can be seen in figure 6.6. A 1 mL min^{-1} HPLC flow was combined with a 0.5 mL min^{-1} 2 mM ABTS flow and reacted in a 1.5 m by 0.4 mm internal diameter loop, resulting in the shortest described reaction time of 7.5 s.

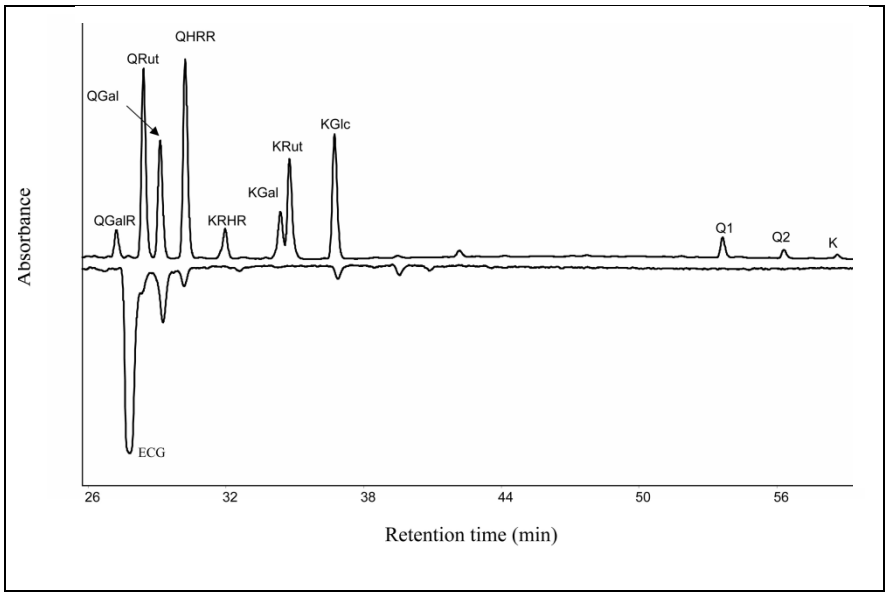


Figure 6.6. Analysis of a green tea extract by the ABTS radical scavenging assay. Top: UV chromatogram 365 nm. Bottom: ABTS quenching signal. Peak identification: QGalR, quercetin rhamnosyl galactoside; QRut, quercetin-3-rutinoside; QGal, quercetin-3-galactoside; QHRR, quercetin hexose rhamnose rhamnoside; KRHR, kaempferol rhamnose hexose rhamnoside, KGal, kaempferol galactoside; KRut, kaempferol-3-rutinoside, KGlc, kaempferol-3-glucoside; ECG, (–)-epicatechin gallate; Q1 and Q2: unidentified quercetin conjugates; K: unidentified kaempferol conjugate. Data from [33].

Other applications of the ABTS assay include the identification of antioxidants in essential oils [37] and in licorice (extract of the root of *Glycorrhiza glabra)* [38]. Over 40 peaks were detected and the ABTS quenching chromatogram showed no loss in efficiency compared to the UV chromatogram, but no reaction details were given. He et al. illustrated the use of a DAD as the only detector placed after the reactor [39].

The chromatogram was recorded at 280 nm while the ABTS quenching chromatogram was recorded at 350 nm. This simplified the setup from two UV-Vis detectors to one DAD detector.

A number of publications of importance for the goals set in this part of this work were published. These papers describe the optimization of the DPPH assay, a comparison of the ABTS and DPPH assays by applying them to the same sample and the coupling of the DPPH assay to NPLC. Exarchou et al. used both the ABTS and DPPH assays and showed that the ABTS assay is more sensitive than the DPPH assay [40]. This explains why the ABTS assay was chosen for optimization in the next chapter. Methanolic extracts of various plants were studied and antioxidants were identified by NMR. In all shown chromatograms, the ABTS assay was able to detect more antioxidants than the DPPH assay. Moreover, it was clearly stated that the ABTS assay is more sensitive, as already stated in the original paper introducing the ABTS assay [16]. Despite this, McDermott et al. optimized the DPPH assay [41] to study coffee [42]. The optimization study was performed in a univariate way. Better results were obtained with the optimized setup compared to the original setup. A smaller reactor (2 m L x 0.25 mm ID vs. 15 m L x 0.25 mm ID previously used) and higher DPPH concentration were the main changes in the new setup. In a study of coffee, the optimized DPPH radical scavenging assay was shown to be less sensitive than a chemiluminiscence assay based on a reaction with permanganate [42].

All previously described on-line methods used RPLC. Zhang et al. reported the application of Normal Phase LC (NPLC) with the DPPH radical scavenging assay for the analysis of non-polar antioxidants [43]. Good sensitivity was obtained. The ABTS method was used to study both hydrophilic and lipophilic antioxidant standards in respectively polar and apolar solvents, showing that the assay is applicable in both polar and apolar environments [44].

Recently, the ABTS assay has also been used to identify antioxidants, by calculating the ratio between their UV and ABTS quenching signal and comparing this to

previously analyzed standards[45]. Problematic in this setup is the fact one need to use the same gradient, a change in mobile phase composition changes this ratio.

From these many examples from literature, it is clear that these assays are quite popular, but a study of the efficiency loss caused by these post-column reactors or an optimization in this light has not been conducted. Another lack is a study of the applicability, determining linear range and sensitivity for both assays and broadening the applicability to polar analytes, by coupling with other chromatographic techniques. These issues will be discussed in the following chapters.

4. OTHER ON-LINE ANTIOXIDANT ASSAYS

A number of other methods for detection of antioxidants coupled to HPLC have been described and some of them are be briefly discussed. They can be subdivided according to detection: electrochemical detection, chemiluminescence detection or UV detection. Furthermore, a method to determine anti- and pro-oxidants will be discussed.

Using electrochemical detection, researchers from the University of Milan analyzed polyphenols in wine [46] and used this system to evaluate antioxidant activity in different wines [47]. The system is based on electrochemical oxidation of polyphenols, the main antioxidants in wine, by a dual amperometric detector. This allowed the use of two voltages and immediately gave information on the chemical structure of the analyte. Whether this method is a true antioxidant assay is questionable, as it might not detect antioxidants whose electrochemical properties are different from the polyphenols analyzed here.

An enzymatic method using cytochrome c coupled to a gold electrode was developed for antioxidants analysis [48]. Superoxide ($O_2^{-\bullet}$) is generated by xanthine oxidase, catalyzing the oxidation of xanthine to uric acid and releasing hydrogen peroxide and superoxide. When an antioxidant is added, the superoxide

concentration drops, resulting in activity of cytochrome c and thus a lower electrochemical signal. This method was only used in flow injection analysis.

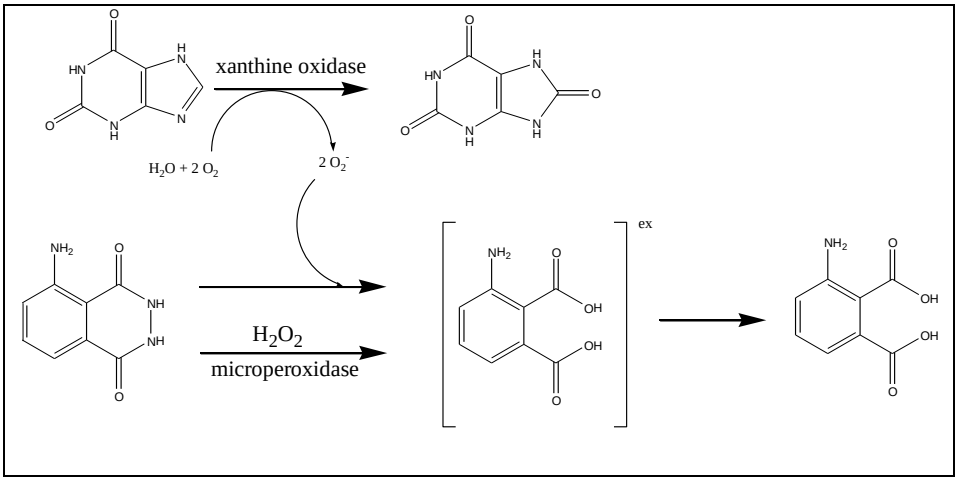


Figure 6.7. Reaction scheme used for luminol chemiluminiscence. Two possibilities are shown. The last reaction in the step is the exited 3-aminophthalate going to its ground state and thus emitting light.

Chemiluminiscence has been used for the detection of antioxidants in combination with luminol. Two reaction schemes can be used, as shown in figure 6.7. In the first, luminol, microperoxidase and hydrogen peroxide are added to the LC effluent [49]. If no antioxidant is present, luminol is oxidized by hydrogen peroxide (a reaction catalyzed by microperoxidase) to 3-aminophtalate that emits light as it relaxes into its ground state. Good results were obtained but the sensitivity was rather low. The DPPH assay was compared to this assay [50] and sensitivity of the luminol based assay was lower, due to a high noise level. The main problem is however the lack of compatibility with water/methanol mixtures, making it far less suited to be coupled to RPLC. A Japanese group used luminol chemiluminiscence [51, 52] but with a different reaction scheme. The reaction of xanthine with xanthine oxidase was used to generate superoxide and hydrogen peroxide while no catalyst was used for the oxidation of luminol. This resulted in a noisier baseline and a drop in efficiency

between the regular chromatogram and the luminol signal. Both reaction schemes are shown in figure 6.6.

In 2010, a new method was coupled to HPLC using UV detection: the Cupric Reducing Antioxidant Capacity (CUPRAC) assay [53,54]. It uses copper(II)-neocuproine which is reduced by antioxidants to copper(I)-neocuproine, resulting in less absorbance at 450 nm. The sensitivity of the technique was not determined in the publication, nor compared to other techniques. From the articles, some noise and a sloping baseline are visible and this could be bad for sensitivity.

A system has recently been described allowing the detection of both anti- and pro-oxidants [55]. The experimental setup is rather complicated. After the LC, a make-up flow is needed so that a constant concentration of water and organic modifier reaches the post-column assay. In the assay two flows are added: one containing cytochrome P450, cytochrome P450 reductase, horseradish peroxidase and superoxide dismutase and one containing nicotinamide adenine dinucleotide phosphate (NADPH) and 4-hydroxyphenylacetic acid. As a result of this complicated reaction scheme, long reaction times are used and the peaks are extremely broad (up to 5 min) leading to very low sensitivity. Another disadvantage of this method is the high cost per analysis, as this assay uses several enzymes.

5. ANTIOXIDANT ASSAYS VS. ENZYME-INHIBITOR ASSAY

The identification of enzyme inhibitors by post-column reaction was proven to be very difficult and, in fact, unrealistic. The long reaction times and complex setup were the biggest problems. The analysis of antioxidants by post-column reaction was selected because of the more favorable experimental conditions: shorter reaction times, simple setup and higher flow rate of reagents. The shorter reaction times ensure less peak broadening, while the reagent flow rate is closer to the chromatographic flow rate and a simple T-piece can be used for adequate mixing of

the reagent flow and the effluent. This results in a more robust system. The main differences between the enzyme inhibitor and the antioxidant assay are summarized in table 6.1.

Table 6.1. Comparison between the post-column assays for enzyme inhibitors and antioxidants. The numbers are based on experimental conditions used in this work and data obtained.

	Enzyme inhibitor assay	**Antioxidant assay**
reaction time	0.1 to 15 min	< 0.5 min
reagent flows	2	1
reagent flow rate	20-60 µL min^{-1}	800 µL min^{-1}
reagent concentration	20 µg mL^{-1} enzyme 100 µg mL^{-1} substrate	10 µg mL^{-1} radical
pump used	syringe pump	HPLC pump
solvent	water is the optimal solvent	any solvent can be used
reaction type	enzymatic	Radical

6. CONCLUSION

Antioxidant analysis coupled on-line to HPLC has been introduced successfully a decade ago and is based on older off-line assays. Two assays have become very popular: the radical scavenging assays based on 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) and 2,2-diphenyl-1-picrylhydrazyl (DDPH). Care should be taken when using these assays and optimization is needed. The reactor size, reagent concentration, reagent flows, etc. should not be chosen arbitrarily as this will have a drastic effect on sensitivity and on chromatographic efficiency. Chromatographic efficiency should be kept as high as possible and is easily affected by the post-column reactor dimensions. When coupling these reactors to other detectors than UV, optimization becomes critical, as often sensitivity or efficiency is lost.

A number of other on-line assays have been reported with the CUPRAC assay as most promising. However, no advantages over the ABTS assay have been reported in the current literature. Other assays often use complicated equipment and/or expensive reagents.

As stated before, the following chapters will focus on the study of efficiency preservation using radical scavenging assays, as this would increase the applicability to complex mixtures. The final chapter will focus on the applicability of the techniques, comparing the sensitivity of the DPPH and ABTS radical scavenging assays and studying the use of HILIC in combination with these assays, making it possible to analyze highly polar antioxidants.

REFERENCES

[1] Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M., Rice-Evans, C., Free Rad. Biol. Med. (1999), 26, 1231-1237

[2] Fernandez-Pachon, M.S., Villano, D., Garcia-Parilla, M.C., Troncoso, A.M., Anal. Chimica Acta (2004), 513, 113-118

[3] Lissi, E.A., Modak, B., Torres, R., Escobar, J., Urzua, A., Free Rad. Res. (1999), 30, 471-477

[4] Villano, D., Fernandez-Pachon, M.S., Troncoo, A.M., Garcia-Parilla, M.C. Talanta (2004), 64, 501-509

[5] Shui, G., Peng, L.L., J. Chromatogr. A (2004), 1048, 17-24

[6] Chandrasekar, D., Madhusudhana, K., Ramakrishna, S., Diwan, P.V., J. Pharm. Biomed. Anal. (2006), 40, 460-464

[7] Kulisic-Bilusic, T., Katalinic, V., Dragovic-Uzelac, V., Ljubenkov, I., Krisko, A., Dejanovic, B., Jukic, M., Politeo, O., Pifat, G., Milos, M., Food. Technol. Biotechnol. (2008), 46, 368-375

[8] Cote, J., Caillet, S., Doyon, G., Sylvain, J.F., Lacroix, M., Crit. Rev. Food Sci. Nutr. (2010), 50, 666-679

[9] Ghazghazi, H., Miguel, M.G., Hasnaoui, B., Sebai, H., Ksontini, M., Figueiredo, A.C., Pedro, L.G., Barroso, J.G., Afr. J. Biotechnol. (2010), 9, 2709-2716

[10] Poovarodom, S., Haruenkit, R., Vearesilp, S., Namiesnik, J., Cvikrova, M., Martincova, O., Ezra, A., Suhaj, M., Ruamsuke, P., Gorinstein, S., Int. J. Food Sci. Technol. (2010), 45, 921-929

[11] Breska, A.P., Takeoka, G.R., Hidalgo, M.B., Vilches, A., Vasse, J., Ramming, D.W., Food Chem. (2010), 121, 740-745

[12] Lucena, A.P.S., Nascimento, R.J.B., Mcial, J.A.C., Tavares, J.X., Barbosa-Filho, J.M., Oliveira, E.J., J. Food Composit. Anal. (2010), 23, 30-36

[13] Komes, D., Horzic, D., Belscak, A., Kovacevic Ganic, K., Vulic, I., Food Res. Int. (2010), 43, 167-176

[14] Neo, Y.P., Ariffin, A., Tan, C.P., Tan, Y.A., Food Chem. (2010), 122, 353-359

[15] Koleva, I.I., Niederlander, H.A.G., van Beek, T.A., Anal. Chem. (2000), 72, 2323-2328

[16] Koleva, I.I., Niederlander, H.A.G., van Beek, T.A., Anal. Chem. (2001), 73, 3373-3381

[17] Dapkevicius, A., van Beek, T.A., Lelyveld, G.P., van Veldhuizen, A., de Groot, A., Linssen, J.P., Venukutonis, R., J. Nat. Prod. (2002), 65, 892-896

[18] Bandoniene, D., Murkovic, M., Pfannhauser, W., Venukutonis, R., Gruzdiene, Eur. Food Res. Technol. (2002), 214, 143-147

[19] Bandoniene, D., Murkovic, M., J. Biochem. Biophys. Methods (2002), 53, 45-49

[20] Kosar, M., Dorman, H.J.D., Bachmayer, O., Baser, K.H.C., Hiltunen, R., Chem. Nat. Compounds (2003), 39, 161-166

[21] Kosar, M. Dorman, H.J.D., Baser, K.H.C., Hiltunen, R., J. Agricult. Food Chem.

(2004), 52, 5004-5010
[22] Kosar, M. Dorman, D., Baser, K., Hiltunen, R., Chromatographia (2004), 60, 635-638
[23] Pollasek, M., Skala, P., Opletal, L., Jahodar, L., Anal. Bioanl. Chem. (2004), 379, 754-758
[24] Milliauskas, G., van Beek, T.A., Venskutonis, P.R., Linssen, J.P.H., de Waard, P., Sudholter, E.J.R., J. Science Food Agricult. (2004), 84, 1997-2009
[25] Milliauskas, G., van Beek, T.A., Venskutonis, P.R., Linssen, J.P.H., de Waard, P., Eur. Food Res. Technol. (2004), 218, 253-261
[26] Milliauskas, G., van Beek, T.A., de Waard, P., Venskutonis, R.P., Sudholter, E.J., J. Nat. Prod. (2005), 68, 168-172
[27] Pukalskas, A., van Beek, T.A., de Waard, P., J. Chromatogr. A (2005), 1074, 81-88
[28] Nuengchamnong, N., de Jong, C.F., Bruyneel, B., Niessen, W.M.A., Irth, H., Ingkanian, K., Phytochem. Anal. (2005), 16, 422-328
[29] Gioti, E.M., Fiamegos, Y.C., Skalkos, D.C., Stalikas, C.D., Food Chem. (2009), 117, 398-404
[30] Nuengchammong, N., Ingkaninan, K., Food Chem. (2010), 118, 147-152
[31] Oki, T., Kobayashi, M., Nakamura, T., Okuyama, A., Masuda, M., Shiratsuchi, H., Suda, I., Food Chem. Toxicol. (2006), 71, C18-C22
[32] Tung, Y.T., Wu, J.H., Hsieh, C.Y., Chen, P.S., Chang, S.T., Food Chem. (2009), 115, 1019-1024
[33] Stewart, A.J., Mullen, W., Crozier, A., Mol. Nutr. Food Res. (2005), 49, 52-60
[34] Perez-Bonilla, M., Salido, S., van Beek, T.A., Linares-Palomino, P.J., Altarejos, J., Nogueras, M., Sanchez, A., J. Chromatogr. A (2006), 1112, 311-318
[35] Stalmach, A., Mullen, W., Nagai, C., Crozier, A., Braz. J. Plant Physiol. (2006), 18, 253-262
[36] Schijlen, E., de Vos, C.H.R., Jonker, H., van den Broeck, H., Molthoff, J., van Tunen, A., Martens, S., Bovy, A., Plant Biotechnol. J. (2006), 4, 433-444
[37] Li, S.Y., Yu, Y., Li, S.P., J. Agricult. Food Chem. (2007), 55, 3358-3362
[38] Lee, Y.S., Kim, S.H., Kim, J.K., Shin, H.K., Kang, Y.H., Park, J.H.P., Lim, S.S., J. Sep. Sci. (2010), 33, 664-671
[39] He, L.H., Xu, H., Liu, X., He, W., Yuan, F., Hou, Z., Gao, Y., Food Res. Int. (2011), 44, 1161-1167
[40] Exarchou, V., Fiamegos, Y.C., van Beek, T.A., Nanos, C., Vervoort, J., J. Chromatogr. A (2006), 1112, 293-302
[41] McDermott, G.P., Noonan, L.K., Mnatsakanyan, M., Shalliker, R.A., Conlan, X.A., Barnett, N.W., Francis, P.S., Anal. Chem. Acta (2010), 675, 76-82
[42] Mnatsakanyan, M., Goodie, T.A., Conlan, X.A., Francis, P.S., McDermott, G.P., Barnett, N.W., Shock, D., Gritti, F., Guiochon, G., Shalliker, R.A., Talanta (2010), 81, 837-842
[43] Zhang, Q., van der Klift, E.J.C., Janssen, H.G., van Beek, T.A., J. Chromatogr. A (2009), 1216, 7268-7274
[44] Cano, A., Alcaraz, O., Acosta, M., Arnao, M.B., Redox Report (2002), 7, 103-109

[45] Liao, C.-I, Ku, K.-L., Anal. Chem. (2012), 84, 7440-7448

[46] Brenna, O., Buratti, S., Cosio, M.S., Mannino, S., Electroanalysis (1998), 10, 1204-1207
[47] Mannino, S., Brenna, O., Buratti, S., Cosio, M.S., Electroanalysis (1998), 10, 908-912
[48] Beissenhirtz, M., Scheller, F., Lisdat, F., Electroanalysis (2003), 15, 1425-1435
[49] Dapkevicius, A., van Beek, T.A., Niederlander, H.A.G., de Groot, A., Anal. Chem. (1999), 71, 736-740
[50] Dapkevicius, A., van Beek, T.A., Niederlander, H.A.G., J. Chromatogr. A (2001), 912, 73-82
[51] Ogawa, A., Arai, H., Tanizawa, H., Miyahara, T., Toyo'oka, T., Anal. Chem. Acta (1999), 383, 221-230
[52] Toyo'oka, T., Kashiwazaki, T., Kato, M., Talanta (2003), 60, 467-475
[53] Celik, S.E., Ozyurek, M., Guclu, K., Apak, R., Anal. Chem. Acta (2010), 674, 79-88
[54] Karaman, S., Tutem, E., Baskan, K.S., Apak, R., Food Chem. (2010), 120, 1201-1209
[55] Kool, J., Van Liempd, S.M., Harmsen, S., Schenk, T., Irth, H., Commandeur, J.N.M., Vermeulen, N.P.E., Anal. Bioanal. Chem. (2007), 388, 871-879

Chapter 7.

Optimization of an ABTS Radical Scavenging Assay Coupled on-line to HPLC for maximizing peak capacity

The fast decolorization of the stable radical cationic form of 2,2'-azino-bis(3-ethylbenzothiazoline)-6 sulfonic acid (ABTS), when in contact with solutes which act as radical scavengers and therefore show potential antioxidant activity, is increasingly used in on-line assays which are coupled to HPLC. Although the performance of the latter in terms of speed of analysis and separation performance evolved significantly in the last decade, little emphasis has thus far been set on the development of on-line radical scavenging assays allowing maximal preservation of the peak capacity.

In this chapter the key figures in the design of this type of on-line assay are re-evaluated in that light to allow for the analysis of more complex mixtures of solutes. A methodology is proposed, involving minimal peak broadening in the post column reactor and in this way allowing preservation of peak capacity up to 95%.

This chapter has been submitted for publication as de Smet, S., Miserez, B., Rambla-Allegre, M., de Villiers, A., Lynen, F., Sandra, P., Talanta

1. INTRODUCTION

Antioxidants are receiving a rising amount of interest, both from the general public and from the scientific community, and thus the search for methods to analyze them has also intensified in the last decade. A number of different assays have been described to measure "antioxidant capacity" of food samples. Most of them estimate the "total antioxidant capacity" of a sample without providing information on the molecules responsible for this activity. Therefore, there has been an increasing trend to couple antioxidant assays on-line post-column to HPLC. A typical setup for such an experiment can be seen in figure 6.2. The HPLC-coupled assays have been reviewed recently [1] and can be divided in three main categories: assays involving interactions between reactive oxygen species (ROS) and a substrate [2], assays involving a stable single electron oxidizing reagent (using this reagent as a ROS mimic) and electrochemical assays (using an electrode as a ROS mimic) [3], using a setup requiring coupling of a standard HPLC-UV to an electrochemical cell. ROS are oxygen containing chemicals that easily oxidize lipids, nucleic acids or proteins in vivo, such as hydrogen peroxide (H_2O_2), superoxide anion (O_2^-), singlet oxygen ($^1O_2$), etc. They can form radicals and therefore are extremely reactive.

In 1999, Dapkevicius et al. published the first method to analyze antioxidants coupled on-line to HPLC based on the chemiluminescence of luminol [4]. However this method demonstrated a high noise and a low reproducibility. Koleva et al. introduced the first on-line method using the stable free radical of 2,2-diphenyl-1-picrylhydrazyl (DPPH), of which the UV absorbance changes drastically upon interaction with an antioxidant [5]. The same authors introduced the 2,2'-azino-bis(3-ethylbenzothiazoline)-6 sulfonic acid (ABTS) radical scavenging method as an improvement of the DPPH method [6]. Today, these two assays are almost exclusively used for this type of analyses. The ABTS assay is based on the Trolox equivalent antioxidant capacity (TEAC) assay, first developed by Miller et al. as an inhibition assay. An antioxidant thereby inhibits the loss of color of ABTS by an in situ generated ROS [7]. Re et al. introduced the decolorization variant, whereby the ABTS

radical is generated before the assay and the amount of ABTS radical being scavenged by the antioxidant is measured [8].

Since its introduction a decade ago, the ABTS assay coupled to HPLC has been used to analyze, for example, various plant extracts [9-13] and food sources [14-16]. A system combining HPLC, the ABTS assay and NMR was described by Miliauskas et al. [17] and Exarchou et al. [18] and the assay was used to provide evidence for the engineering of a new pathway for flavonoids in tomatoes [19]. Although the original ABTS assay setup has been subjected to various modifications, supporting evidence justifying these changes and therefore a complete optimization has been lacking so far. By contrast, the setup for the DPPH assay has been optimized by McDermott et al. in a univariate way [20]. An important benefit of the use of ABTS over DPPH is that its enhanced water solubility makes it applicable in both hydrophobic and hydrophilic environments and therefore more generically usable in reversed phase HPLC. The main advantage of these assays in this setup is, however, the simplicity. By producing the radical beforehand, no complex reaction schemes or flows are needed; a normal HPLC-pump to deliver the radical is sufficient. The ABTS radical covers a broad absorbance spectrum with maxima at 414 nm, 645 nm, 734 nm and 815 nm allowing detection in the UV and visible range. Less matrix or sample interference at higher wavelengths have been mentioned due to the lower prevalence of these extensively conjugated systems in natural products [8], however, as is shown in this chapter UV detection at 414 nm is often applicable as well.

The relevance of antioxidant assays has been the topic of some discussion, because the experimental conditions of these assays are quite different from an in vivo environment, due to the difficult quantification of the results [21] or because antioxidants are not necessarily radical scavengers. Strube et al. have published some critical remarks on the TEAC assay in 1997, stating correctly that the in vivo antioxidant capacity cannot be derived directly from a TEAC assay and that all data should be reviewed critically [22]. Prior and Cao pointed out that the ABTS assay is

useful for the analysis of phytochemicals, but when used in plasma to asses in vivo total antioxidant capacity, the results are influenced by the protein content [23]. This is not a surprising find since there are number of reactive groups in proteins that can react with radicals, as they also do with ROS. It is because of these misunderstandings that Prior et al. proposed in 2005 to change the terminology and to use, for example, "ABTS radical scavenging capacity" or "peroxyl radical scavenging capacity" instead of "total antioxidant capacity" [23]. It should also be noted that the ABTS assay does not detect antioxidants as such but rather radical scavengers. However, antioxidants could also work by inhibiting the formation of ROS, as is for example the case with some metal chelators or enzyme cofactors [24]. This kind of antioxidants will not be detected by an assay measuring radical scavenging. As for assays measuring the radical scavenging of a biological important radical, such as the peroxyl radical, various experimental parameters do not resemble the in vivo situation: concentration is often too high, various side reactions are not taken into account, etc. [25]. Hence, although any test raises some questions, the effectiveness of the ABTS assay to detect various radical scavengers has extensively been demonstrated by now, which justifies their use.

An essential requirement for all post-column reactions, derivatizations, interaction or inhibition assays is the need to minimize peak broadening, in order not to affect the peak capacity, and therefore the information obtained in the original chromatographic separation. This is dependent on the reactor dimensions, reaction kinetics and the residence time in the reactor. A too small reactor will not allow enough time for the reaction, while extra dead volume will introduce unnecessary peak broadening [26]. In this work, these aspects are investigated in relation to the ABTS assay in order to maximize the peak capacity and the overall performance of the system. The effectiveness and usefulness of the setup is tested on various natural and synthetic mixtures of antioxidants under various experimental conditions.

2. Material and Methods

2.1. Chemicals

Methanol was bought from Fiers (Kuurne, Belgium). MilliQ Water was prepared in house by a water purification instrument from Millipore (Bedford, New Hampshire, USA). Ethyl acetate, formic acid, HCl, H_2SO_4, NaCl, KCl, Na_2HPO_4, KH_2PO_4, MnO_2, ABTS, ascorbic acid, uracil, tyrosine, gallic acid, catechin, sulfamethoxazole, 2,3-dihydroxybenzoic acid, t-resveratrol, acetophenone, ethylparaben, prednisolone, testosterone, Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), butylparaben, phenanthrene, isoascorbic acid, ascorbyl palmitate, propyl, octyl and dodecyl gallate, α-, δ- and γ-tocopherol, tert-butyl-hydroquinone (THBQ), butylhydroxyanisole (BHA) and butylhydroxytoluene (BHT) were supplied by Sigma Aldrich (St Louis, Missouri, USA). α-, δ- and γ-tocotrienol were supplied by Bio-connect BV (Huissen, The Netherlands). Standards were prepared at 1000 µg mL^{-1} in water or methanol, depending on solubility and further diluted with water.

2.2. Analytical System

All analyses were performed on a modular HPLC 10A system (Shimadzu, Kyoto, Japan), with an Agilent 1050 autosampler (Agilent, Santa Clara, California, USA) and a 7600 Solvent Degasser (Jones Chromatography, Cardiff, UK) and two 10A VP UV-detectors (one post-column, set at 280 nm and one after the reactor, set at 414 nm). Luna C18 columns (250 mm length, 4.6 mm I.D., 5 µm particles, Phenomenex, Torrance, California, USA) were used for all analyses. The flow rate for all analyses was 0.5 mL min^{-1}. Solvent A was water with 0.1% of formic acid, solvent B was methanol with 0.1% of formic acid. The ABTS solution was delivered by a 420 HPLC pump (Kroton Instruments, Watford, UK). The reactor was made from PTFE tubing (VICI, Houston, Texas, USA). UV measurements were done on a Uvikon XL UV spectrophotometer (BioTek Instruments, Winooski, Vermont, USA).

2.3. Optimization

Preliminary testing of the reaction speed and influence of the solvent by UV absorbance was done by mixing 5 mL of mobile phase A or B and 8 mL of ABTS solution. Cuvettes were filled with 3 mL of this solution and used as a blank or 20 µL of 1000 µg mL^{-1} 2,3-dihydroxybenzoic acid was added. The UV absorbance was measured at 414 nm for 15 min.

The initial ABTS assay was setup as described by Koleva et al. [6], with a reactor knotted as described by Kuhlman et al. [27] without being filled when knotted. All parameters were optimized as described in Results and Discussion. The optimized conditions are as follows. The reactor was a PTFE tube covered with aluminium foil with a 2.8 m length and 0.25 mm internal diameter kept at ambient temperature. The ABTS flow was set at 0.8 mL min^{-1}. The ABTS solution was made by adding 2 g MnO_2 to 1 L 0.02 mM ABTS in a PBS buffer (137 mM NaCl, 10 mM Na_2HPO_4, 2 mM KCl, 2 mM KH_2PO_4), stirred for 10 min at room temperature and filtered. The ABTS solution was kept at 0°C and protected from light and was changed every 12 h. Detection was performed at 414 nm. The standard used for the optimization sample was a mixture containing 20 µg mL^{-1} ascorbic acid, uracil, tyrosine, gallic acid, catechin, sulfamethoxazole, 2,3-dihydroxybenzoic acid, t-resveratrol, acetophenone, ethylparaben, prednisolone, testosterone, Trolox, butylparaben and phenanthrene in water. All analyses were done in triplicate and the results are an average of the three measurements. 10 µL of the sample was injected on the system described in 2.2 and a gradient analysis was used (0-10 min: 96% A- 4% B, 10-50 min: linear gradient to 4% A- 96% B, 50-60 min: 4% A-96% B).

2.4. Analysis of Red Wine

1.5 mL of red wine (Bordeaux blend, France, 2008) was treated with SPE to extract the polyphenols using a Strata PSDVB cartridge of 500 mg (Agilent, Santa Clara, California, USA) as previously described [28]. The cartridge was preconditioned with 3 mL of ethyl acetate and 3 mL methanol and equilibrated with 3 mL of water at pH

2.5 (pH adjusted with 1 M HCl). The wine sample, set at pH 2.5 using 6 M HCl, was loaded. Washing was done using 4 mL 20 mM H_2SO_4. Compounds of interest (polyphenols and phenolic acids) were eluted using 10 mL ethylacetate and evaporated under nitrogen flow. The sample was redissolved in 1 mL water:methanol (1:1). An aliquot of 10 µL was injected on the analytical system. A precolumn (Security Guard, Phenomenex, Torrance, California, USA) was used to protect the analytical columns as two Luna C18 columns (250 mm length, 4.6 mm I.D., 5 µm particles, Phenomenex, Torrance, California, USA) were used for this analysis. The gradient was 0-40 min: 95% A-5% B, 40-200 min: gradient to 5% A- 95% B, 200-240 min: 5% A- 95%B.

2.5. Analysis of Food Additives and Deep Frying Oil

The food additives mixture was a mixture of ascorbic acid (E 300, E 301, E 302), isoascorbic acid (E 315, E 316), ascorbyl palmitate (E 304), propyl, octyl and lauryl gallate (respectively E 310, E 311, E 312), α-, δ- and γ-tocopherol (respectively E 307, E 308, E 309), tert-butyl-hydroquinone (THBQ) (E319), butylhydroxyanisole (BHA) (E 320) and butylhydroxytoluene (BHT) (E 321). Calibration curves were made for these compounds from 0.5 to 50 µg mL^{-1} by running seven dilutions in water in triplicate.

The deep frying oil was pretreated by SPE, using C18 SPE cartridges (Agilent SampliQ 500 mg, Agilent, Santa Clara, California, USA). The cartridge was preconditioned using 3 mL methanol and 15 mg of oil dissolved in 500 µL of methanol was loaded. Then, 10 mL of methanol was used for the elution of the compounds of interest and evaporated using a rotavapor (Büchi Rotavapor R-200, Büchi, Flawil, Switserland). The sample was diluted with 50 µL of methanol and 10 µL was injected on the system described in 2.2. The gradient profile was 0-5 min: 96% A- 4% B, 5-12 min: linear gradient to 94% A, 6% B, 12-25 min: linear gradient to 8% A- 92% B, 25-60 min: linear gradient to 100% B, 60-80 min: 100% B. A precolumn (Security Guard, Phenomenex, Torrance, California, USA) was used to protect the analytical column.

Identification was done by rerunning the sample on LC-MS and comparing with standards.

3. Results and Discussion

3.1. ABTS Assay Optimization

To optimize reactor dimensions and thus reaction time, an insight into the reaction kinetics of the radical quenching of ABTS was required. Comparable studies have been conducted [29-32], but vary in conclusion. These suggest a fast initial phase (less than 10 s) for most, if not all, antioxidants, possibly followed by a slower second phase.

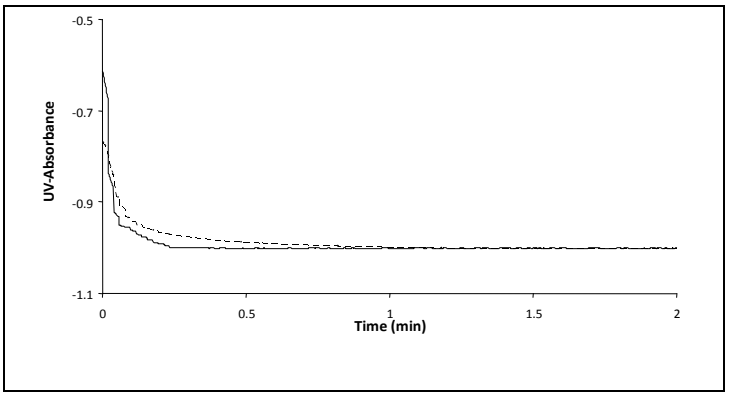


Fig. 7.1. Monitored UV absorbance at 414 nm of an ABTS solution after administration of radical scavengers for establishment of necessary residence time in the on-line reactor. Evolution of the absorbance after addition of 2,3-dihydroxybenzoic acid. Full line: experiment performed in methanol, dashed line: performed in water.

Therefore the UV absorbance of the ABTS free radical as a function of time after administration of a 2,3-dihydroxybenzoic acid was measured in water and in methanol and compared with blank analyses. As can be seen in the results shown in

figure 7.1, 2,3-dihydroxybenzoic acid reacts sufficiently fast in both solvents to allow the development of a small volume reaction reservoir providing the residence time therein exceeds a few seconds. Radical scavenging proceeded thereby somewhat faster in methanol compared to water.

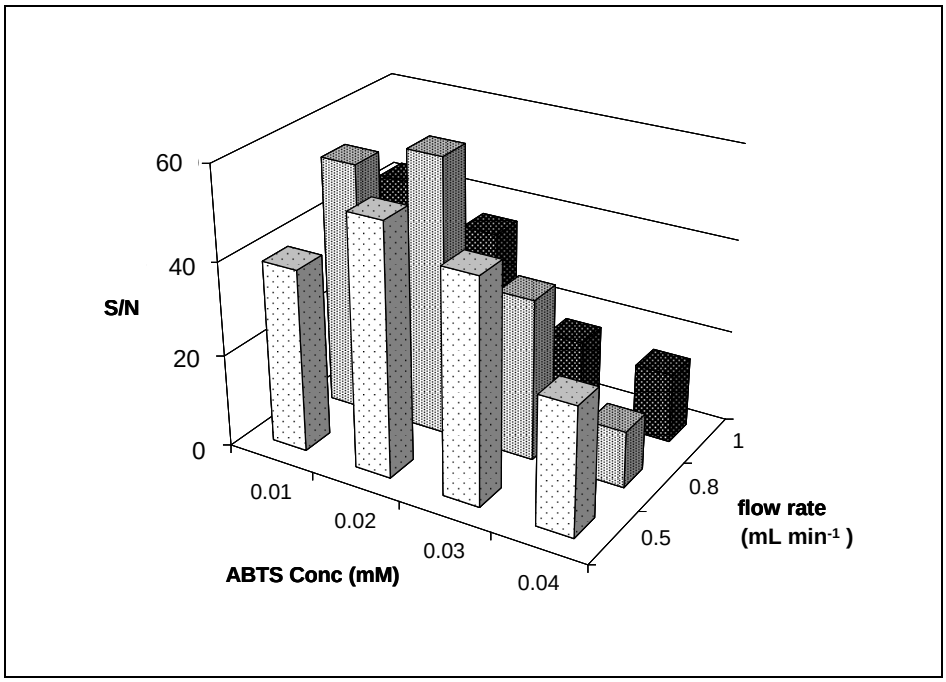


Fig. 7.2. Signal to noise ratio for 2,3-dihydroxybenzoic acid as a function of varying ABTS concentrations and reagent flow rates.

Subsequently the influence of the ABTS concentration and the flow rate of the added reagent solutions were studied to maximize assay sensitivity as measured through the signal to noise ratio of the negative signals. This is shown in figure 7.2 for the assay sensitivity for 2,3-dihydroxybenzoic acid (10 µL was injected using a 10 µg mL^{-1} concentration). Although a broad optimum was obtained and as the apex therefore tended to vary depending on the solute which was analyzed, when ABTS concentrations exceeding 30 µM were used loss of sensitivity was observed in all

cases through excessive noise production. Optimal conditions were therefore established between 0.8 to 1 mL min^{-1} added ABTS flow rate and 0.01 or 0.02 mM ABTS concentration.

The next parameters to be optimized were the length and internal diameter of the reactor. Together with the ABTS flow, these parameters govern the reaction time. However, the back pressure from the reactor is also of some importance, since a first UV detector is coupled directly after the column and before the reactor and a too high back pressure from the reactor would destroy the flow cell of the detector.

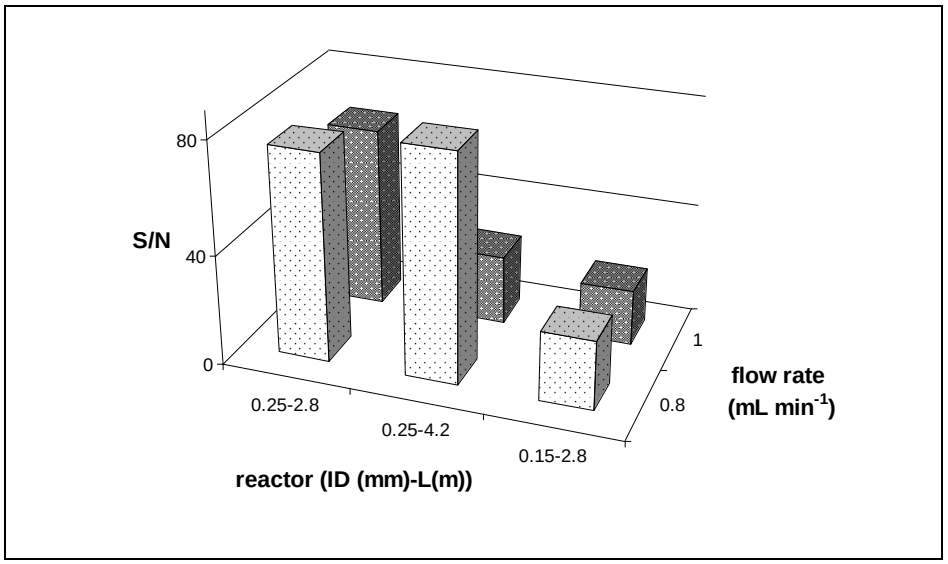


Fig. 7.3. Optimization of the assay sensitivity as a function of reactor dimensions and reagent flow rate for gallic acid.

Reactors with internal diameters of 150, 180, 250 and 380 µm and lengths of 1.4, 2.8 and 4.2 m were tested, in all these cases the back pressure was below 5 MPa (50 bar). Very small internal diameters gave rise to high noise levels, as did long reactors. The optimum was determined at 2.8 m length and 250 µm internal diameter as this gave for most compounds the highest peak areas. As these were the previously used

conditions, this effect could have been due to the univariate way of the optimization. ABTS flow rate was therefore varied on the three most promising reactors (see figure 7.3) and the optimal conditions were chosen to be a 0.8 mL min^{-1} flow rate of ABTS and a reactor with 2.8 m length and 0.25 mm internal diameter. The chosen conditions correspond to a mean optimum for all analytes, as most have a broader optimum. This is shown for gallic acid in figure 7.3.

Three remaining reaction parameters, buffer concentration, reactor temperature and reactor geometry did not show any significant variation in the signal to noise ratio or peak area. The reactor coil was heated in a water bath and the temperature was set at 20°C, 40°C and 60°C, without any significant changes in the response. The original phosphate buffer was used as such or at double, half and one quarter this concentration, but again without changes in the response. As for the reactor geometry, the coil was either knotted or coiled with a radius of 20 cm (i.e. almost straight). Although this was expected to be visible in the peak height, it gave no significant differences. This could be due to the very small reaction time.

In figure 7.4, an analysis of a standard mixture using the optimized conditions is shown. The optimal conditions were an ABTS concentration of 0.02 mM in a PBS buffer containing 137 mM NaCl, 10 mM Na_2HPO_4, 2 mM KCl and 2 mM KH_2PO_4. The ABTS flow was 0.8 mL min^{-1} and the reactor 2.8 m length by 250 µm internal diameter of knotted PTFE tubing at room temperature, giving a residence time in the reactor of around 6.5 s. The retention time added by the post-column reactor is constant for all peaks. The ABTS signal responds only to antioxidants and to all antioxidants used here, as all of them are radical scavengers. Phenanthrene might not be seen as an antioxidant, but the extensive π-system can react with the radical ABTS and thus be detected as an antioxidant.

The chromatographic efficiency is slightly affected by the addition of post-column dead volume. The peak capacity for the gradient run in figure 7.5, calculated as described by Gilar et al. [33], showed a capacity of 181 in the UV chromatogram (top signal) and a peak capacity of 162 in the ABTS quenching signal (bottom signal). Thus

only 10% of the original peak capacity is lost using this experimental setup. This shows clearly the capability of this optimized assay to preserve the chromatographic resolution and peak capacity. The reason for this is the short reaction time and optimized reactor. In Table 7.1, the developed method is compared with other published setups for the ABTS radical scavenging assay. The main change in this method is the short reaction time and high concentration of ABTS, leading to a limited loss of chromatographic efficiency and low detection limits.

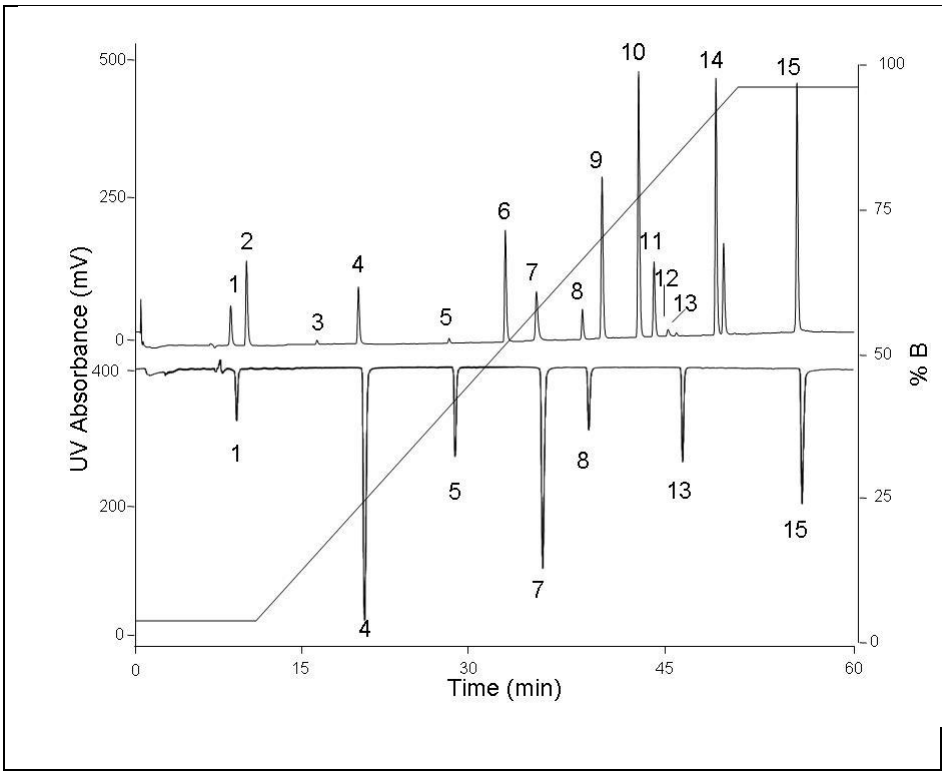


Fig. 7.4. Analysis of a standard mixture under optimized conditions. Top: UV chromatogram at 280 nm, Bottom: ABTS quenching signal at 414 nm. Peak identification: 1. ascorbic acid, 2. uracil, 3. tyrosine, 4. gallic acid, 5. catechin, 6. sulfamethoxazole, 7. 2,3-dihydroxybenzoic acid, 8. t-resveratrol, 9. acetophenone, 10. ethylparaben, 11. prednisolone, 12. testosterone, 13. Trolox, 14. butylparaben, 15. phenanthrene.

Table 7.1. Comparison of the parameters used in literature and in the optimized assay for the ABTS radical scavenging assay. N.A.: values not available.

ref.	LC flow (mL min^{-1})	ABTS flow (mL min^{-1})	ABTS conc (µM)	Length (m)	I.D. (mm)	Vol (µL)	Reaction Time (s)	UV (nm)
[6]	0.8	0.5	5.5	4.4	0.25	216	10.0	734
[9]	0.8	0.5	5.5	4.4	0.25	216	10.0	734
[10]	0.8	0.5	5.5	4.4	0.25	216	10.0	734
[13]	1.0	0.7	5.5	15	0.25	736	26.0	734
[14]	1.0	0.5	25.0	1.5	0.40	188	7.5	720
[15]	0.7	0.8	N.G.	15	0.25	736	29.5	734
[16]	0.8	0.3	66.7	N.A.	N.A.	N.A.	N.A.	N.A.
[19]	0.6	0.6	45.0	4.4	0.25	216	10.8	N.A.
New method	0.5	0.8	20.0	2.8	0.25	142	6.5	414

3.2. Antioxidant Capacity

Because of the solvent dependence of the reaction speed, assessing of antioxidant capacity is problematic. In off-line analyses the antioxidant capacity is measured by TEAC-value. This value is the extent of decolorization of the assay calculated relatively to Trolox as a standard. In on-line analyses the ratio of the peak areas could be used (antioxidant versus Trolox) for the TEAC value. To investigate the solvent dependence of the peak areas isocratic runs at different mobile phase compositions were developed using the standard optimized conditions of the ABTS assay. The resulting peak areas show a variation for Trolox and 2,3-dihydroxybenzoic acid. In Table 7.2, the TEAC value (Trolox eluting at 40% B) is calculated for a 20 µg mL^{-1} solution of 2,3-dihydroxybenzoic acid at different mobile phase strengths. As expected the TEAC value changes when the mobile phase composition is different for Trolox and 2,3-dihydroxybenzoic acid. This shows that two compounds with a different retention time in gradient analysis but the same antioxidant capacity will

show a different TEAC value. The results indicate that TEAC values obtained from gradient analyses will inevitably involve error margins of about 25% RSD if the solvent composition prior to the radical scavenging assay is not compensated for or addressed.

Table 7.2. TEAC value for 20 µg mL^{-1} 2,3-dihydoxybenzoic acid (relative to the Trolox signal eluting at 40% B).

% B	Peak Area 2,3-dihydroxybenzoic acid	*TEAC value (Trolox area= 1.9 10^6 at 40%B)*
40	*5.8 10^6*	**3.1**
50	*6.2 10^6*	**3.3**
75	*6.6 10^6*	**3.5**
90	*7.3 10^6*	**3.8**

3.3. Analysis of Red Wine

Red wine is a well-known source of antioxidants and was therefore chosen to demonstrate the possibilities of the optimized ABTS assay. The resulting chromatograms are shown in figure 7.5. In this complex mixture, a wide variety of antioxidants is present and some were identified by comparison to standards. The peak capacity was calculated on both the UV and ABTS signals. The peak capacity of the UV chromatogram was 286, while the peak capacity for the ABTS quenching chromatogram was 275. This means a drop in peak capacity of only 4%, which seems very acceptable in a post-column reactor.

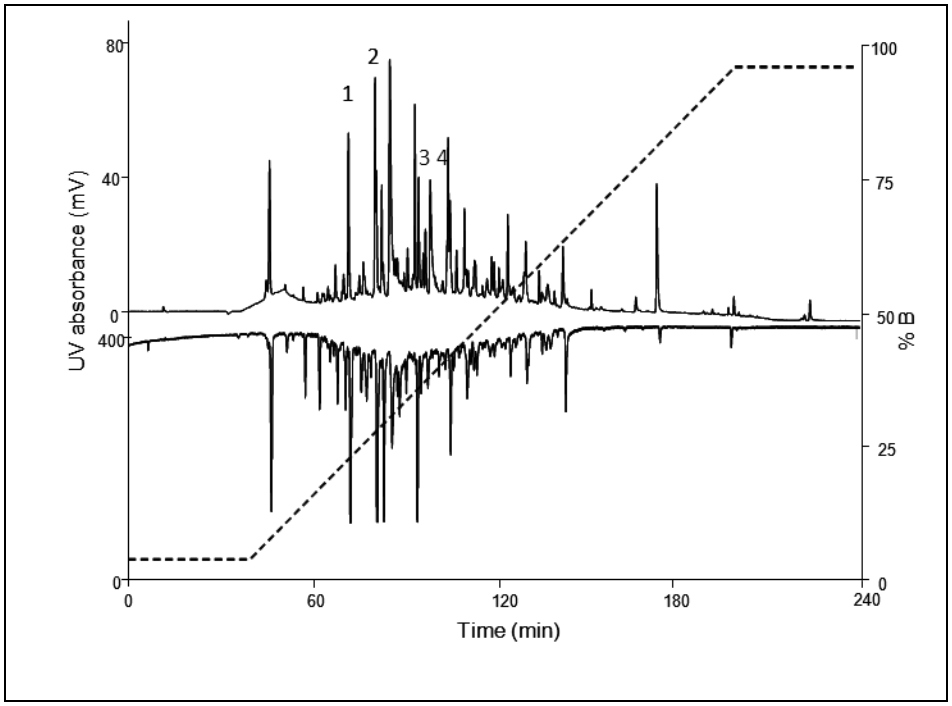


Fig. 7.5. Analysis of red wine showing the chromatogram at 280 nm (top) and the ABTS quenching signal at 414 nm (bottom). Peak identification was done by standards: 1. catechin, 2. para-hydroxybenzoic acid, 3. caffeic acid, 4. vanillic acid.

3.4. Analysis of Food Additives and Deep Frying Oil

The developed setup was used for the analysis of the approved hydrophobic antioxidants as additives to food and beverages (E 304, E 307 to E 312 and E 319 to E 321) together with the widely used ascorbic (E 300 to E 302) and isoascorbic acid (E 315 and E 316). A separation of this sample was achieved in RPLC and the analysis was coupled to the optimized ABTS assay. The result is shown in figure 7.6.

A somewhat increased noise level was thereby observed for the beginning of the chromatogram when RPLC conditions were still purely aqueous. This initial mobile phase composition was required to retain and separate ascorbic from isoascorbic acid.

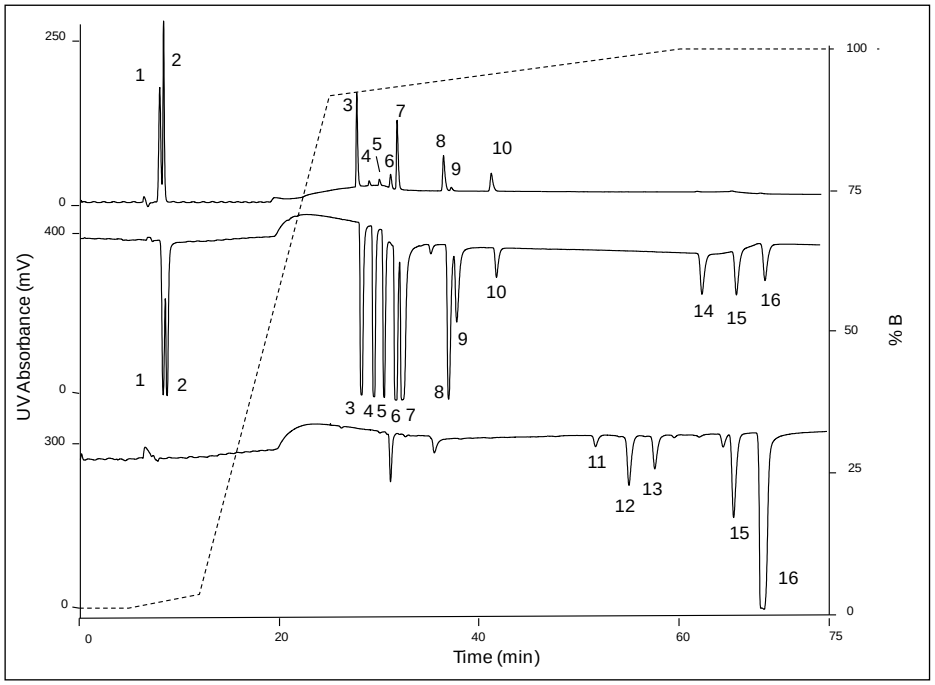


Fig. 7.6. Separation and antioxidant assay for a mixture of food additives and deep frying oil. Details can be found in materials and methods. Top: Food additives standards at 280 nm, Middle: ABTS quenching signal of the food additives standards at 414 nm, Bottom: ABTS quenching signal of fresh deep frying oil at 414 nm. Peak identification: 1. ascorbic acid (E300-E301-E302), 2. isoascorbic acid (E315-E316), 3. propyl gallate (E310), 4. TBHQ (E319), 5. Trolox, 6. BHA (E320), 7. octyl gallate (E311), 8. lauryl gallate (E312), 9. BHT (E321), 10. ascorbyl palmitate (E304), 11. δ-tocotrienol, 12. γ-tocotrienol, 13. α-tocotrienol, 14. δ-tocopherol (E309), 15. γ-tocopherol (E308), 16. α-tocopherol (E307).

Calibration curves for all of the compounds in the mixture were constructed ranging from 0.5 μg mL^{-1} to 50 μg mL^{-1}, including 7 calibration levels (10 μL injected volume). The highest level resulted in saturated signals illustrating a narrower linear range compared to e.g. direct UV detection. In between linearity was very acceptable with R^2 values exceeding 0.98 in all cases. Ascorbic and isoascorbic acid, however, showed a higher limit of quantification due to the high noise level at high water content. BHT, ascorbyl palmitate and tocopherols showed lower peak heights, due to longer retention times under almost isocratic conditions. For these peaks, the limit of quantification was 30 ng, injecting 10 μL at 3 μg mL^{-1}.

The method was also used to analyse deep frying oil, a mixture of sunflower and rapeseed oil. This mixture contains α-, δ- and γ-tocotrienol and α- and γ-tocopherol and the amount of these antioxidants can be used to determine when the oil needs to be changed. The result of a fresh oil analysis can be seen in figure 7.7. It is clear this method easily detects the compounds and they can be quantified by integration. The peaks have been identified by standard addition and by rerunning the sample on an LC-MS system.

4. CONCLUSION

The combination of ABTS radical scavenging assays coupled on-line with HPLC was further optimized, whereby particular attention was set on preservation of the peak capacity. Parameters of influence such as the buffer and ABTS concentrations, flow rate of the reagent solution, temperature and reactor dimensions were thereby studied. Optimal conditions in terms of minimal peak broadening and maximal sensitivity were found for an ABTS concentration of 0.02 mM in a PBS buffer containing 137 mM NaCl, 10 mM Na_2HPO_4, 2 mM KCl and 2 mM KH_2PO_4, HPLC and reagent flow rates of 0.5 and 0.8 mL min^{-1}, respectively, reactor dimensions of 2.8 m and 250 µm internal diameter knotted PTFE tubing and operation at room temperature. The optimized method resulted in minimal reduction in chromatographic efficiency with only 10% of the peak capacity being lost through inevitable peak broadening phenomena in the reaction coil and 6.5 s offset between direct UV detection and the radical scavenging assay signal. The RPLC mobile phase composition was observed to influence TEAC values, whereby up to 25% differences were measured between a 40 and 90% methanol content in aqueous mobile phases, an aspect of relevance in gradient analysis. The applicability of the optimized method was demonstrated through analysis of a red wine sample with high efficiency HPLC making use of 2x25 cm columns to maximize peak capacity. Subsequently common food additives were analyzed in this way and the methodology was used for monitoring the quality of deep frying oil.

References

[1] Niederländer, H.A.G., van Beek, T.A., Bartasiute, A., Koleva, I.I., J. Chromatogr. A (2008), 1210, 121-134

[2] Toyo'oka, T, Kashiwazaki, T., Kato, M., Talanta (2003), 60, 467-475

[3] Mannino, S. ,Brenna, O., Buratti, S., Cosio, M.S., Electroanalysis (1998), 10, 908-912

[4] Dapkevicius, A., van Beek, T.A., Niederländer, H.A.G., de Groot, A., Anal. Chem. (1999) 71, 736-740

[5] Koleva, I.I., Niederländer, H.A.G., van Beek, T.A., Anal. Chem. (2000), 72, 2323-2328

[6] Koleva, I.I., Niederländer, H.A.G., van Beek, T.A., Anal. Chem. (2001) 73, 3373-3381

[7] Miller, N.J., Rice-Evans, C., Davies, M.J., Gopinathan, V., Milner, A., Clin. Sci. (1993), 84, 407-412

[8] Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M., Rice-Evans, C., Free Radical Biol. Med. (1999), 26, 1231-1237

[9] Miliauskas, G., van Beek, T.A., Venskutonis, P.R., Linssen, J.P.H., de Waard, P., Sudhölter, E.J., J. Sci. Food Agric. (2004), 84,1997-2009

[10] Miliauskas, G., van Beek, T.A., Venskutonis, P.R., Linssen, J.P.H., de Waard, P., Eur. Food Res. Technol. (2004), 218, 253-261

[11] Beekwilder, J., Jonker, H., Meesters, P., Hall, R.D., van der Meer, I.M., de Vos, C.H.R., J. Agric. Food Chem. (2005), 53, 3313-3320

[12] Li, S.Y., Yue, Y., Li, S.P., J. Agr. Food Chem. (2007), 55, 3358-3362

[13] Pérez-Bonilla, M., Salido, S. van Beek, T.A., Linares-Palomino, P.J., Altarejos, J., Nogueras, M., Sánchez, A., J. Chromatogr. A (2006), 1112, 311-318

[14] Stewart, A.J., Mullen, W., Crozier, A., Mol. Nutr. Food Res. (2005) 49, 52-60

[15] He, L., Xu, H., Liu, X., He, W., Yuan, F., Hou, Z., Gao, Y., Food Res. Int. (2011), 44, 1161-1167

[16] Stalmach, A., Mullen, W., Nagai, C., Crozier, A., Braz. J. Plant Physiol. (2006), 18, 253-262

[17] Miliauskas, G., van Beek, T.A., de Waard, P., Venskutonis, R.P., Sudhölter, E.J.R., J. Nat. Prod. (2005), 68, 168-172

[18] Exarchou, V., Fiamegos, Y.C., van Beek, T.A., Nanos, C., Vervoort, J., J. Chromatogr. A (2006), 1112, 293-302

[19] Schijlen, E., de Vos, C.H.R., Jonker, H., van den Broeck, H., Molthoff, J., van Tunen, A., Martens, S., Bovy, A., J. Plant Biotechnol. (2006), 4, 433-444

[20] McDermott, G.P., Noonan, L.K., Mnatsakanyan, M., Shalliker, R.A., Conlan, X.A., Barnett, N.W., Francis, P.S., Anal. Chim. Acta (2010) 675, 76-82

[21] Schofield, D., Braganza, J.M., Clin. Chem. (1996) 42, 1712-1714

[22] Strube, M., Haenen, G.R.M.M., Van Den Berg, H., Bast, A., Free Radical Res. (1997), 26, 515-521

[23] Prior, R.L., Cao, G., Free Radical Biol. Med. (1999) 27, 1173-1181

[24] Huang, D., Ou, B., Prior, R.L., J. Agric. Food Chem. (2005) 53, 1841-1856
[25] Magalhaes, L.M., Segundo, M.A., Reis, S., Lima, J.L.F.C., Anal. Chim. Acta (2008), 613, 1-19
[26] Kucera, P., Umagat, H., J. Chromatogr. (1983), 255, 563-579
[27] Kuhlmann, O., Krauss, G.J., J. Pharm. Biomed. Anal. (1997), 16, 553-559
[28] de Villiers, A., Lynen, F., Crouch, A., Sandra, P., Chromatographia (2004), 59, 403-409
[29] Campos, A.M., Lissi, E.A., Int. J. Chem. Kinet. (1997) 29, 219-224
[30] Pannala, A.S., Chan, T.S., O'Brien, P.J., Rice-Evans, C.A., Biochem. Biophys. Res. Commun. (2001) 282, 1161-1168
[31] Henriquez, C., Alliaga, C., Lissi, E., J. Chil. Chem. Soc. (2004) 49, 65-67
[32] Villano, D., Fernandez-Pachon, M.S., Troncoso, A.M., Garcia-Parrilla, M.C., Talanta (2004), 64, 501-509
[33] Gilar, M., Daly, A.E., Kele, M., Neue, U.D., Gebler, J.C., J. Chromatogr. A (2004), 1061, 183-192

Chapter 7. Optimization of an ABTS Radical Scavenging Assay Coupled on-line to HPLC for maximizing peak capacity

Chapter 8.

On-line Combination of ABTS and DPPH Radical Scavenging Assays with Hydrophilic Interaction Chromatography

Radical scavenging assays coupled on-line to HPLC are increasingly used for the detection of compounds showing potential antioxidant activity. Stable free radical solutions, containing 2,2-diphenyl-1-picrylhydrazyl (DPPH) or 2,2'-azino-bis(3-ethylbenzothiazoline)-6 sulfonic acid (ABTS) are thereby mixed post-column with HPLC effluents and changes in UV absorbance are followed as a function of the eluting chromatogram to display radical scavenging activity. So far limited information was available about relative assay sensitivity and as the latter often appeared to be lower for the early eluting polar solutes when combining the assay with reversed phase LC, the combination of the assays with hydrophilic interaction chromatography (HILIC) was studied. The sensitivity and linearity of both approaches is compared and applied for the analysis of ascorbic acid in citrus fruits. Under conventional RPLC conditions (except for the very early eluting solutes) and in combination with HILIC the ABTS assay was more sensitive compared to the DPPH approach and comparable linear ranges of about two orders of magnitude were observed.

This chapter has been submitted for publication as Miserez, B., Rambla Alegre, M., De Smet, S., Lynen, F., Sandra, P., J. Sep. Sci.

1. INTRODUCTION

The search for antioxidants, molecules inhibiting the oxidation of other molecules, and assessment of their relative activity is an ongoing process of importance to various application areas such as in medicinal, food and polymer chemistry. Several off-line tests have been developed allowing measurement of the total antioxidant activity of a sample, such as the Ferric Reducing Ability of Plasma (FRAP) [1] or the Trolox Equivalent of Antioxidant Capacity (TEAC) assay [2]. However, on-line combination with separation techniques such as HPLC, allows obtaining more insight in the solutes responsible for the detected antioxidant activity. Initial successful attempts combining luminol based chemiluminescence with HPLC are now only rarely used due to limited assay sensitivities and relatively poor reproducibilities which can be obtained in this way [3]. Enzymatic systems able to detect both anti- and pro-oxidants [4] and a cupric reducing antioxidant capacity (CUPRAC) assay have proven sufficiently sensitive for hyphenation with HPLC [5], however, fast reaction kinetics are preferable to minimize peak broadening phenomena and ensuing loss of separation. Therefore the antioxidant assays which are nowadays most often coupled to HPLC are radical scavenging assays based on 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) [6,7]. In these assays, a flow containing a colored stable free radical solution is mixed post-column with the HPLC effluent generating a constant background signal in the second UV detector. When a radical scavenger (i.e. a potential antioxidant) elutes from the column, the radical is oxidized and a dramatic change in UV absorbance is observed as a negative peak in the chromatogram recorded by the second detector. A typical setup for the combination of HPLC with such on-line radical scavenging assays is shown in figure 6.2. Both reactions are fast and require residence times in the reactor of only a few seconds, which allows minimizing losses in chromatographic efficiency [6-8].

The stable free radical assays can also be used in the conventional off-line approach to assess total antioxidant activity. When comparing the DPPH and ABTS based assays in this way only a relatively weak correlation between the antioxidant capacity of polyphenols measured using each assay was observed. This has been related to the varying reaction chemistry between both approaches [9]. The reaction with DPPH involves several reversible reactions, while the reaction with ABTS is almost unrelated to the expected reactivity of a polyphenol towards a free radical. This makes data interpretation difficult and care should be taken when comparing antioxidant data from different studies or results obtained from different techniques [9,10]. However, the ABTS assay appears significantly more sensitive compared to the DPPH based assay as has been reported for both on- and off-line assays [7,9]. The solvent dependency of the ABTS response has been mentioned, influencing gradient analysis results [7].

Despite the large range of antioxidants and their respective polarity or even their ionic nature, radical scavenging assays are today almost exclusively coupled to reversed or occasionally normal phase LC [11]. As none of those HPLC modes is suitable for the analysis of very polar solutes because of the poor retention or solubility problems which are respectively involved, the possibilities of combining both radical scavenging assays with Hydrophilic Interaction Chromatography (HILIC) are investigated in this work. Ascorbic and isoascorbic acid are thereby used as test solutes.

2. EXPERIMENTAL

2.1 MATERIALS

MilliQ Water was prepared in house by a water purification instrument from Millipore (Bedford, New Hampshire, USA). Benzoic acid (E210), ascorbic acid (vitamin C, E300), iso-ascorbic acid (E315), gallic acid, catechin, 2,3-dihydroxybenzoic acid, propyl gallate (E310), tert-butylhydroxyquinone (TBHQ, E319), butylated

hydroxyanisole (BHA, E320), octyl gallate (E311), NaCl, KCl, Na_2HPO_4, KH_2PO_4, MnO_2, ABTS, formic acid, ammonium formate, DPPH, citric acid, potassium citrate, NaOH, acetonitrile and methanol were supplied by Sigma Aldrich (Beernem, Belgium). All stock solutions (1000 µg mL^{-1}) were prepared in methanol, except ascorbic and isoascorbic acid which were dissolved in water and further diluted with water for RPLC analysis or methanol for HILIC analysis.

2.2 Instrumentation

All analyses were performed on a modular HPLC 10A system (Shimadzu, Kyoto, Japan) equipped with two LC-10AD mobile phase pumps, a SCL-10A system controller, 7600 Solvent Degasser (Jones Chromatography, Cardiff, UK) and two SPD-10A UV-VIS detectors. ABTS or DPPH solutions were delivered by an 1100 binary pump (Agilent, Santa Clara, California, USA). Data was collected and processed with LC solution software (version 1.21 SP, Shimadzu). 2.8 m x 0.25 mm ID coiled PTFE tubing (137 µL reactor volume), was used as a radical scavenging reactor (VICI, Houston, Texas, USA).

2.3 Analytical Conditions

RPLC analysis was performed on a 250 mm x 4.6 mm x 5 µm Luna C18 column (Phenomenex, Torrance, California, USA). The mobile phase was composed of A) water with 0.1% formic acid and B) methanol with 0.1% formic acid. The gradient composition profile was: 0-5 min: 2% B; 5-55 min: gradient to 98% B, 55-60 min: 98% B. HILIC analyses were performed on a 250 mm length x 4.6 mm x 5 µm Zorbax RXSil column (Agilent, Santa Clara, California, USA). Isocratic conditions were used with a mobile phase composed of 90% acetonitrile and 10% 100 mM ammonium formate pH 4 (adjusted with formic acid). The mobile phases and the reagent solutions, with flow rates of 0.5 and 0.8 mL min^{-1} respectively were mixed by means of a zero dead volume PEEK tee piece (Upchurch, Oak Harbor, Washington, USA).

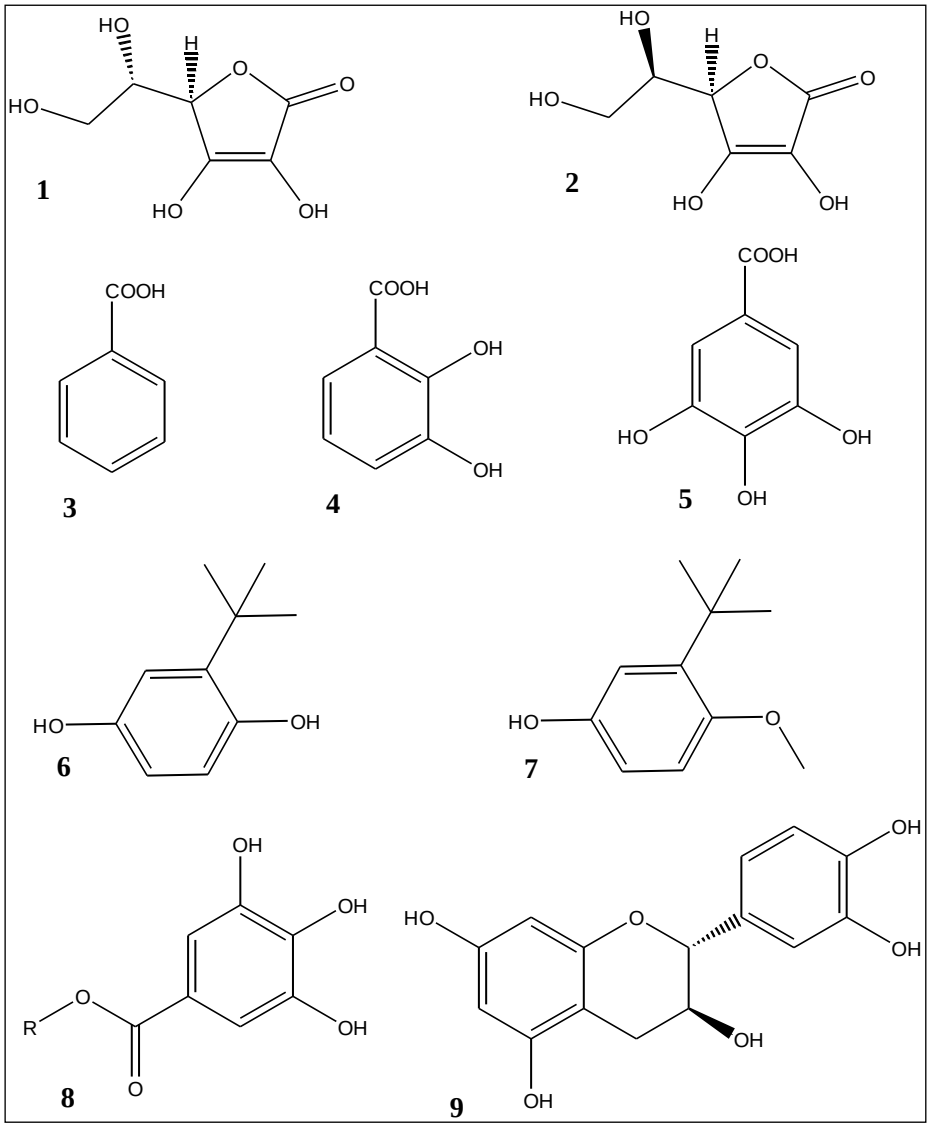


Fig. 8.1. Structures of all used standards. 1. ascorbic acid, 2. isoascorbic acid, 3. benzoic acid, 4. 2,3-dihydroxy benzoic acid, 5. gallic acid, 6. tert-butylhydroxyquinone (TBHQ), 7. butylated hydroxyanisole (BHA), 8A. propyl gallate (R= C_3H_7), 8B. octyl gallate (R= C_8H_{17}), 9. catechin.

The ABTS solution was composed of 23 mM MnO_2 and 20 µM ABTS dissolved in a PBS buffer (137 mM NaCl, 10 mM Na_2HPO_4, 2 mM KCl, 2 mM KH_2PO_4), which was stirred for 10 min at room temperature and filtered over 0.45 µm nylon solvent filter (Grace, Columbia, Maryland, USA). The DPPH solution was prepared by dissolving 50 µM of the reagent in a 75% methanol/25% 40 mM citrate buffer of pH 6 [8]. All reagent solutions were prepared daily, filtered over a 0.45 µm nylon solvent filter (Grace, Columbia, Maryland, USA), degassed, cooled at 4°C and shielded from light before and during use. During analysis, the ATBS and DPPH solutions were kept in an ice bath. UV detection was performed at 254 nm and 414 nm (for ABTS) or 521 nm (for DPPH) for the collection of the UV absorbance chromatograms and of the radical scavenging data, respectively. The RPLC and HILIC samples (figure 8.1) were further diluted from the stock solutions in methanol or water to a concentration of 20 µg mL^{-1} with water/methanol (1/9) or with the HILIC mobile phase, respectively. Orange juice was filtered and diluted in acetonitrile (1/1 and 1/10). The limits of quantification (LOQ) were determined according to the EPA recommended procedure [12]. A standard solution containing each analyte at a 2 µg mL^{-1} level was thereby injected seven times, and the standard deviations of the results were calculated.

3. Results and Discussion

3.1 Radical Scavenging Assays Coupled to RPLC

In figure 8.2 a generic RPLC analysis of a number of typical antioxidants (see figure 8.1) is shown together with the corresponding DPPH and ABTS radical scavenging assays. Note that benzoic acid is not detected in these assays as it is, although a widely used antioxidant, not capable of scavenging the unpaired electron in any of the assays. Although the DPPH solution is two and a half times more concentrated compared to the ABTS reagent, it is immediately clear that the latter is the more sensitive assay.

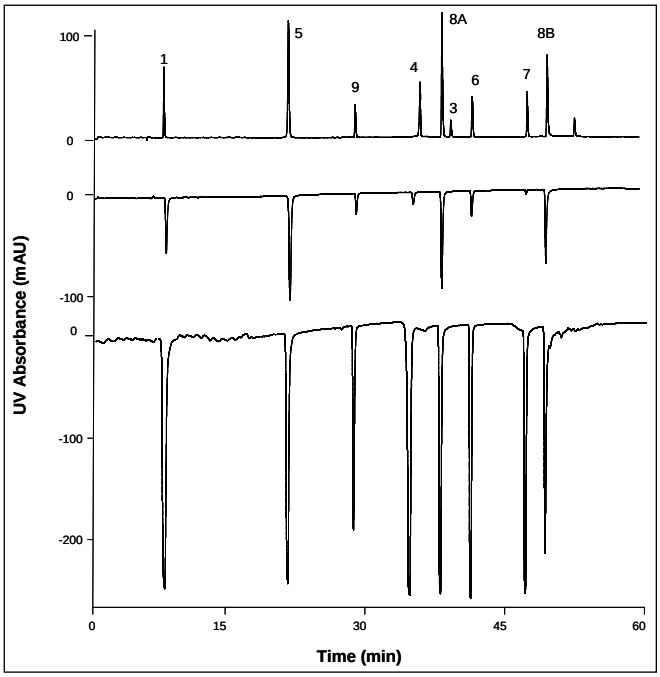


Fig. 8.2. Analysis of the RPLC standard mixture. Top: UV-chromatogram at 254 nm, middle: DPPH quenching chromatogram at 521 nm, bottom: ABTS quenching chromatogram at 414 nm. Peak identification see figure 8.2.

This was confirmed in the LOQ determinations as shown in Table 8.1. Next to the increased sensitivity assay interspecies response homogeneity is also improved when comparing the ABTS with the DPPH assay, respectively. The combined effect of the short residence time in the reactor and the multiple step reaction mechanism involved could lead to incomplete reactions between several of the analyzed solutes and the DPPH reagent and therefore to larger variation in LOQ's. The sensitivity for gallic acid was 3.5 times higher for the ABTS assay compared to the DPPH approach, while this ratio increased to 91 for butylated hydroxyanisole (BHA). However, for the early eluting polar ascorbic acid the sensitivity of the DPPH assay was double compared to what could be obtained with the ABTS test. This was related to the noisy background signal in the latter case. Contrary to the DPPH assay in which the

reagent flow contains 75% organic modifier, the use of the ABTS assay in combination with the RPLC conditions at the beginning of the gradient leads to almost purely aqueous conditions and a noisy background signal in the second UV detector. The underlying reason for the noise generation is most probably associated to the reduced solubility of the ABTS free radical or of its reduced form under the altered pH conditions once mixed with the RPLC mobile phase. As the more hydrophobic DPPH requires dissolution in less polar solvent combinations (75% methanol), this type of noise is not observed with the latter. As the ABTS solution rapidly degrades in methanol/water solutions the use of ABTS for the analysis of the polar solutes in RPLC would require either the addition of a ternary methanol flow or of alternative organic modifiers. Additionally the retention and separation with RPLC is generally poor for this type of solute and thus the use of alternative LC modes for combination with radical scavenging assays should be explored.

Table 8.1. Limits of quantification measured for the DPPH and ABTS assays for the standards analyzed in figure 8.3.

	LOQ (μg mL^{-1})	
	ABTS	*DPPH*
ascorbic acid	5.0	2.5
gallic acid	0.2	0.7
catechin	0.2	3.8
2,3-dihydroxy benzoic acid	0.2	6.7
propyl gallate	0.2	0.8
TBHQ	0.2	3.1
BHA	0.2	18.2
octyl gallate	0.2	1.8

3.2 Radical Scavenging Assays Coupled to HILIC

The possibility of coupling these assays with the Hydrophilic Interaction LC mode (HILIC) was investigated. HILIC allows for the analysis of polar solutes which are difficult to dissolve in the organic solvents typically used in normal phase LC. Although this mode has been used for several decades for saccharide analyses the term HILIC was coined in 1990 by Alpert [13] and since then applications thereof have much increased [14-16]. A benefit of HILIC compared to NPLC, is that it routinely offers to reach the theoretically expected column efficiencies as the mode is characterized by a fast partitioning mechanism in between a water rich aqueous phase close to the polar particle surface and the organic rich mobile phase [17]. The correlation between organic solvent content in the mobile phase and the corresponding intensity of the ABTS and DPPH signals observed in the RPLC analyses, leads one to expect high assay sensitivity when combining those with HILIC which typically applies about 90% acetonitrile in the mobile phase.

In figure 8.3, the analysis of five of the solutes used above is shown under HILIC conditions. An inversion of the elution order compared to the reversed phase LC analysis in figure 8.2 and baseline resolution of ascorbic and iso-ascorbic acid are thereby visible, demonstrating the suitability of HILIC for the analysis of this type of polar solute.

As the three benzoic acid variants are only partially deprotonated at pH 4.0 only low retention is obtained for those compounds in HILIC. The corresponding DPPH and ABTS quenching chromatograms demonstrate stable sensitive radical scavenger signals. As was the case in the RPLC based assays, benzoic acid does not generate a signal in any of the assays and the response obtained with 2,3-dihydroxybenzoic acid is 40 times lower with the DPPH compared to ABTS (table 8.2). This trend is to a lesser extent confirmed with gallic acid, where a fivefold sensitivity difference is measured between both assays. The sensitivity for ascorbic and isoascorbic acid is comparable to gallic and 2,3-dihydroxy benzoic acid when the ABTS assay was used. These results and the ones given in table 8.1, demonstrate that the ABTS based

assay allows to obtain sub-µg mL^{-1} sensitivity for all radical scavengers provided the organic modifier percentage in the mobile phase exceeds 10%-15%.

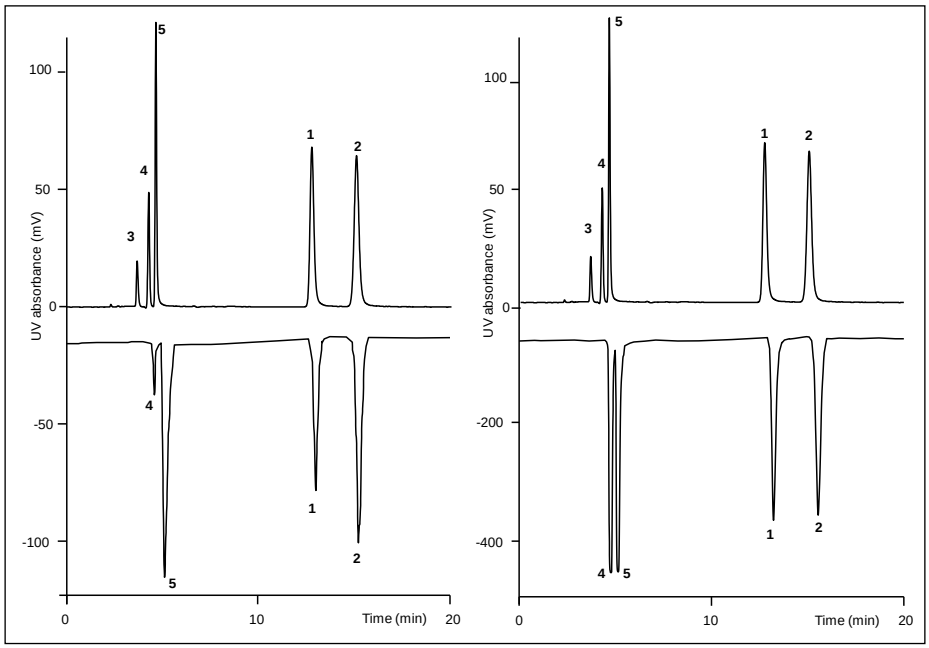


Fig. 8.3. Analysis of 5 antioxidants by HILIC coupled to the ABTS or DPPH radical scavenging assay. Top: UV chromatogram at 254 nm, bottom: radical scavenging assay. Left: DPPH quenching signal at 521 nm, right: ABTS quenching signal at 414 nm. Identification as in figure 8.2. Note the difference in axis when comparing the ABTS and DPPH quenching signals.

As ascorbic acid (vitamin C, E300) is found in high concentration in citrus fruits, the setup was used to measure the radical scavenging activity in orange juice (figure 8.4). The ascorbic acid peak is the dominant peak in both the UV chromatogram and in the corresponding ABTS and DPPH traces. It can be seen that the responses of the antioxidant assays are saturated when the 1/1 juice/ACN dilutions were analysed, corresponding to a local complete utilization of the available DPPH or ABTS. Note that the ABTS assay also indicates the presence of a number of additional

antioxidants, which are not well visible by UV at the measured wavelengths or with the DPPH based assay. By diluting the samples 1/10 in ACN a response for ascorbic acid in the linear range of the assay could again be obtained but the smaller antioxidant peaks where no longer visible.

Table 8.2. Limits of quantification for the DPPH and ABTS assays for the standards, analyzed by HILIC in figure 8.4.

	LOQ (µg mL^{-1})	
	ABTS	*DPPH*
2,3-dihydroxy benzoic acid	0.5	20
gallic acid	0.5	2.5
ascorbic acid	0.6	3.3
iso-ascorbic acid	0.7	2.9

Although it was not surprising that radical scavenging assays saturate relatively easily compared to the broad linear range of UV detection, it was interesting to estimate the range in which a linear response could be obtained with the proposed methodology. Note that a trade-off is necessarily made between assay sensitivity which is increasing by reducing the ABTS or DPPH concentration and response linearity which is diminished when too low reagent concentrations are used. Therefore calibration curves from 1 to 500 µg mL^{-1} were constructed for ascorbic acid for both assays. The DPPH and ABTS assays thereby showed a linear response from 5 tot 100 µg mL^{-1} and from 1 to 50 µg mL^{-1} respectively, above which saturation occurred. Correlation coefficients of 0.88 and 0.94 were obtained for the DPPH and ABTS assays respectively. The ABTS approach opens up the possibility to apply radical scavenging assays in quantitative analysis.

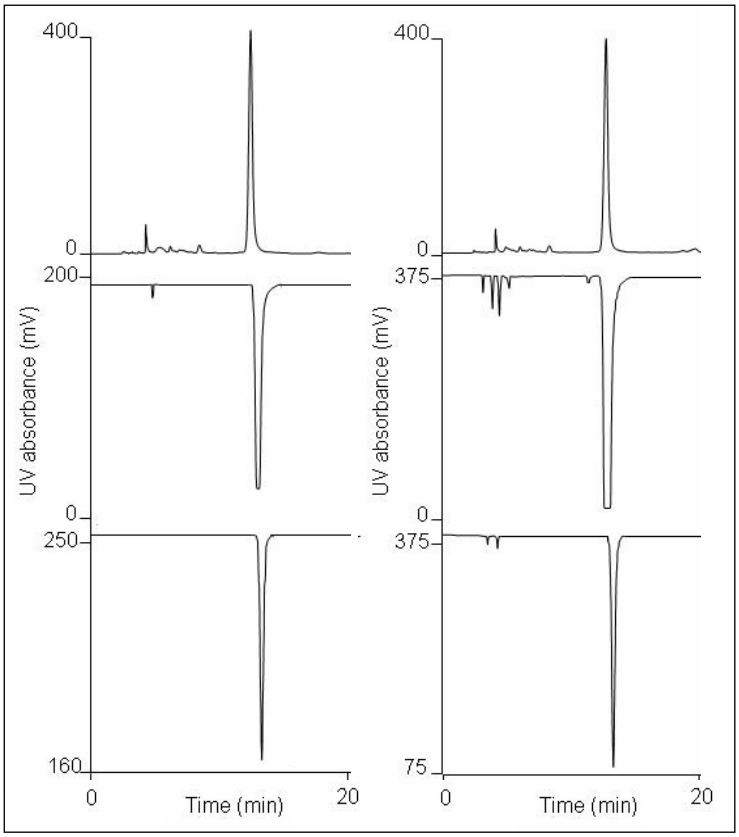


Fig. 8.4. Analysis of orange juice (1/1 and 1/10 diutions) by HILIC coupled to the ABTS or DPPH radical scavenging assay. Left: using DPPH, Right: using ABTS. Top: UV chromatogram at 254 nm, Middle: quenching chromatogram at 521 nm (DPPH) or 414 nm (ATBS), Bottom: quenching chromatograms of the 1/10 dilution.

4. CONCLUSION

DPPH and ABTS radical scavenging assays were successfully combined to HILIC, whereby the latter was the more sensitive approach. The methodology is ideally suitable for the analysis of polar solutes having radical scavenging and thus potential antioxidant activity. The high organic content of the HILIC mobile phase thereby ensured stable assay background signals. When RPLC analyses with high aqueous

content (>85%) were combined with the ABTS assay noisy assay background signals were obtained. Because of the trade-off between reagent concentration and assay sensitivity two orders of magnitude were observed in terms of linearity before reagent depletion occurred.

REFERENCES

[1] Benzie, I.F.F., Strain, J.J., Anal. Biochem. (1996) 239, 70-76
[2] Miller, N.J., Rice-Evans, C., Davies, M.J., Gopinathan, V., Milner, A., Clin. Sci. (1993), 84, 407-412
[3] Dapkevicius, A., van Beek, T.A., Niederländer, H.A.G., de Groot, A., Anal. Chem. (1999) 71, 736-740
[4] Kool, J., Van Liempd, S.M., Harmsen, S., Schenk, T., Irth, H., Commandeur, J.N.M., Vermeulen, N.P.E., Anal. Bioanal. Chem. (2007), 388, 871-879
[5] Celik, S.E., Özyürek, M., Güçlü, K., Apak, R., Anal. Chim. Acta (2010), 674, 79-88
[6] Koleva, I.I., Niederländer, H.A.G., van Beek, T.A., Anal. Chem. (2000), 72, 2323-2328
[7] Koleva, I.I., Niederländer, H.A.G., van Beek, T.A., Anal. Chem. (2001) 73, 3373-3381
[8] McDermott, G.P., Noonan, L.K., Mnatsakanyan, M., Shalliker, R.A., Conlan, X.A., Barnett, N.W., Francis, P.S., Anal. Chem. Acta (2010), 675, 76-82
[9] Lissi, E.A., Modak, B., Torres, R., Escobar, J., Urzua, A., Free Rad. Res. (1999), 30, 471-477
[10] Strube, M., Haenen, G.R.M.M., Van Den Berg, H., Bast, A., Free Radic. Res. (1997), 26, 515-521
[11] Zhang, Q., van der Klift, E.J.C., Janssen, H.G., van Beek, T.A., J. Chromatogr. A (2009), 1216, 7268-7274
[12] Longbottom, J. E., Lichtenberg, J. J., Eds. Methods for Organic Chemical, Analysis in Industrial Wastewater, EPA-600/4-82-057; U.S. EPA, Environmental, Monitoring and Support Laboratory: Cincinnati, OH, 1982
[13] Alpert, A.J., J. Chromatogr. (1990), 499, 177-196
[14] Rabel, F.M., Caputo, A.G., Butts, E.T., J. Chromatogr. (1976) 126, 731-740
[15] Bajad, S.U., Lu, W., Kimball, E.H., Yuan, J., Peterson, C., Rabinowitz, J.D., J. Chromatogr. A (2006), 1125, 76-88
[16] Louw, S., Pereira, A.S., Lynen, F., Hanna-Brown, M., Sandra, P., J. Chromatogr. A (2008), 1208, 90-94
[17] Louw, S. Lynen, F., Hanna-Brown, M., Sandra, P., J. Chromatogr. A (2010), 1217, 514-521

CONCLUSION

Two main parts can be identified in this work: the analysis of enzyme inhibitors by the combination of TRLC and post-column reactions and the study of antioxidant assays.

Due to the solvent incompatibility of enzymatic reactions with most HPLC techniques, TRLC was studied and a novel phase based on PVCL was developed. As literature up to now has been almost exclusively devoted to PNIPAA based stationary phases, PVCL offers a new selectivity. PVCL was successfully coupled onto silica and used in TRLC, where retention was tunable by temperature and a purely aqueous phase could successfully be used. Retention times increased with increasing temperature, showing a clear temperature responsive effect. However, efficiency was not very high, probably due to the thick layer of polymer on the silica, slowing down diffusion and thus increasing the C-term in the van Deemter equation. In literature, TRLC has made a significant amount of improvement since its first introduction, but it remains a technique which is only used when the need for purely aqueous LC arises. The lack of efficiency and selectivity hamper the widespread use of TRLC and there are no columns commercially available.

The development of an analytical system for enzyme inhibitors based on the coupling of TRLC with an enzymatic reaction has not been successful. Two main problems with the setup remain: the long reaction times needed and the complexity of the system. The long reaction times cause peak broadening in the reactor, leading to insensitivity. The complex analytical setup is the main reason for the lack of robustness. The combination of both makes the whole system irreproducible and cumbersome to use. In literature, no robustness data has been published, nor has any real application. Few new enzyme inhibitors were identified by this system and

off-line testing of fractions is a more sensitive, more robust and more reliable method for enzyme inhibitor analysis.

Radical scavenging assays for antioxidant analysis, such as the ABTS and DPPH assays, don't have the disadvantages of the enzymatic post-reaction, as they are both fast and simple. However, even fast post-column reactions cause losses in efficiency and optimization of the ABTS assay for the preservation of chromatographic peak capacity showed an improved sensitivity and analytical capability. Further study of the ABTS assay also showed that the response of an analyte is dependent on the solvent composition, making it impossible to compare the antioxidant capacity of analytes in gradient analysis, other than a rough estimation. This and the fact that radical scavenging of ABTS and DPPH is already a questionable technique to compare antioxidants, lead to the conclusion that a detailed study of a compound is needed to understand its antioxidant properties. The analysis by ABTS or DPPH assay should be used to search and identify antioxidants in mixtures.

To further improve the usefulness of these assays, coupling with HILIC was performed, allowing the analysis of highly polar antioxidants such as ascorbic acid (vitamin C). When comparing the ABTS and DPPH assays coupled to both RPLC and HILIC, the ABTS assay is most sensitive and is able to detect more antioxidants. It is therefore recommended to use the ABTS assay when sensitivity is needed.

As a general conclusion, optimization of any post-column reaction is needed, but if the reaction is too slow, fractionation is most likely a better option.

SCIENTIFIC CONTRIBUTIONS

PUBLICATIONS

Miserez, B., Lynen, F., Wright, A., Euerby, M., Sandra, P. *Thermoresponsive Poly(N-vinylcaprolactam) as Stationary Phase for Aqueous and Green Liquid Chromatography*, Chromatographia, 71:1-6 (2010)

de Smet, S., Miserez, B., Rambla Alegra, M., de Villiers, A., Lynen, F., Sandra, P. *Optimization of the ABTS Radical Scavenging Assay and Applications in Food Analysis*, submitted to Talanta

Miserez, B.,Rambla Alegra, M., de Smet, S., Lynen, F., Sandra, P., *Online combination of the ABTS and DPPH radical scavenging assays with hydrophilic interaction chromatography*, submitted to J. Separation Sci.

POSTERS

Miserez, B., Lynen, F., Sandra, P., *Study of Poly(N-Vinylcaprolactam) as a Thermoresponsive Phase for HPLC*, 31st International Symposium on Liquid Chromatography and Related Techniques (HPLC 2007), in Ghent, Belgium (June 2007)

Malanchin, V., Miserez, B., Lynen, F., Sandra, P., *Screening of Retention Behaviour of Different Sorbents by Matrix- Solid Phase Dispersion* 30th International Symposium on Capillary Chromatography, in Riva Del Garda, Italy (May 2008)

Miserez, B., de Smet, S., Rambla Allegre, M., Lynen, F., Sandra, P., *Elucidation of the antioxidant activity in complex mixtures: Optimization of the On-Line ABTS-Based Radical Scavenging Assay and Application in Food Analysis,* 33rd International Symposium on Liquid Chromatography and Related Techniques (HPLC 2011), in Budapest, Hungary (June 2011) (selected as top 25 poster)

Miserez, B., de Smet, S., Rambla Allegre, M., Lynen, F., Sandra, P., *Elucidation of the antioxidant activity in complex mixtures: DPPH and ABTS radical scavenging coupled to RPLC and HILIC*, 33rd International Symposium on Liquid Chromatography and Related Techniques (HPLC 2011), in Budapest, Hungary (June 2011)

Miserez, B., de Smet, S., Rambla Allegre, M., Lynen, F., Sandra, P., *Optimization of the On-Line ABTS-Based Radical Scavenging Assay and Application in Food Analysis,*

12th International Symposium for Hyphenated Techniques in Chromatography (HTC-12), in Bruges, Belgium (February 2012)

Miserez, B., de Smet, S., Rambla Allegre, M., Lynen, F., Sandra, P., *Coupling the ABTS and DPPH radical scavenging assays to RPLC and HILIC, a comparison*, 12th International Symposium for Hyphenated Techniques in Chromatography (HTC-12), in Bruges, Belgium (February 2012)

LECTURES

Miserez, B., Lynen, F., Sandra, P., *New stationary phases for temperature responsive liquid chromatography*, 9th Youth Congress of the Royal Chemical Society of Flandres, Blankenberge, Belgium (April 2009)

de Smet, S., Miserez, B., Lynen, F., Sandra, P., *Development of an HPLC-MS method for the determination of antioxidants in natural products*, UDIAS award lecture, Brussels, Belgium (October 2011)

SUMMARY

The analysis of enzyme inhibitors and antioxidants by post-column reactions was studied. To minimize solvent incompatibility when combining HPLC with enzymatic reactions, temperature responsive liquid chromatography (TRLC) was used and a new stationary phase, based on poly(N-vinylcaprolactam) (PVCL) was synthesized.

An introduction on HPLC and post-column reactions is presented in chapter 1. Both fundamental and instrumental aspects of HPLC are discussed. An overview of the most important parameters of post-column reactions and reactor types is given.

In chapter 2, the reason to study TRLC in the context of this work is given. As enzymes are used in a post-column reactor and enzymes are unstable in organic solvents, care must be taken to match the solvents of the coupled techniques. As common HPLC modes are nearly impossible without the use of organic modifiers, an alternative mode needed to be used. In TRLC, a purely aqueous mobile phase is combined with a stationary phase of silica modified with temperature responsive polymers. These stimuli responsive or smart polymers show a change in hydrophobicity when the environmental temperature is varied: they are water soluble above and water insoluble under a specific temperature, the lower critical solution temperature (LCST). Thus, the hydrophobicity of the stationary phase is tunable by a change in temperature and a mobile phase gradient can be replaced by a temperature gradient. The purely aqueous mobile phase makes TRLC ideally suited for the coupling with enzymatic post-column reactions.

The influence of temperature on chromatography is discussed in chapter 3. Temperature is not only an important parameter in TRLC, as it influences efficiency, selectivity, retention and detector response of all LC separations. At a higher temperature, retention and back-pressure decrease, while the van Deemter curve becomes flatter. This allows the use higher flow rates, making it possible to achieve higher efficiencies and/or shorter analysis times. With higher temperature, the dielectric constant of water also shifts towards that of apolar solvents, making it

possible to use less organic solvents. In high temperature LC (HTLC), this is exploited to minimize organic solvent use and obtain high efficiency separations. As the detector response is often influenced by the mobile phase composition, temperature gradients to replace solvent gradients make the detector response more constant. In TRLC, temperature is used to change the hydrophobicity of the stationary phase, which contains temperature responsive or temperature responsive polymers. In the literature, poly(N-isopropylacrylamide) (PNIPAA) has been used almost exclusively for this purpose. Originally, TRLC was introduced as an alternative for RPLC, but the copolymerization of PNIPAA with other monomers has made it possible to perform IEX as well. Other apolar monomers have been used, but these affect mostly the LCST of the polymer, rather than the selectivity of the column. Despite these successes, little use has been made of temperature gradients in TRLC and almost no applications have been described. Renewed interest in these phases may come when they become more efficient. A major problem with TRLC remains the lack of selectivity and difficulty to optimize a separation, as well as the need for commercial TRLC columns.

To further enhance the possibilities of TRLC, a novel stationary phase, based on the temperature responsive polymer PVCL was developed and evaluated, as described in chapter 4. The phase showed a clear temperature responsive effect, as retention and efficiency for steroids increased with increasing temperature. Below the LCST, the phase showed very low efficiency, probably due to extended polymer on the surface, making diffusion in and out of the stationary phase very slow. Addition of ethanol, a green organic solvent, decreased the analysis time, but destroyed the temperature responsive effect. The separation of phenones and parabens was also shown, proving the wide applicability of the PVCL phase.

The application of TRLC in the analysis of enzyme inhibitors by post-column reaction was studied in chapter 5. First, the possibility of an enzymatic post-column reaction coupled to HPLC with water as the only solvent was tested. The recently described cathepsin B inhibition assay was selected, but water was used as the only solvent.

The column was changed from a C18 to a C8 column to diminish retention when only using water and reactor dimensions were altered to allow the entire column flow to enter the reaction. The system was shown to capable of detecting a model inhibitor. 25, 50 and 100% methanol and acetonitrile were used as LC mobile phase to assess the influence of the solvent, but no change in the inhibition assay was visible. The short reaction time (0.6 min) combined with the presence of water from the addition of flows of enzyme and substrate solutions (lowering the concentration of organic solvent to 62, 31 and 16%, respectively) causes little or no enzyme deactivation. In the tests with temperature responsive columns, trypsin was selected as model enzyme. Trypsin was shown to be deactivated by organic solvents using a reaction time of 5 min. The inhibition assay was shown to be successful, but lacked reproducibility and sensitivity, due the large reaction time needed for most enzymatic reactions. The absence of robustness of the system makes it very hard to use and the off-line approach is more user friendly, despite the need for larger amounts of reagents, the longer analysis times and the lack of automation.

A post-column reaction with a much shorter reaction time was selected to study the feasibility of high efficiency HPLC coupled to post-column reactors. Radical scavenging assays, used to analyze antioxidants, were selected, as these radical reactions have fast kinetics. Two radicals have been described for this purpose: 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) and 2,2-diphenyl-1-picrylhydrazyl (DPPH). In chapter 6, an overview of relevant literature is presented. Antioxidant capacity is often used to describe the strength of an antioxidant, but it is assay dependent. It is advised to interpret the results of a radical scavenging assay with care. Not all antioxidants are radical scavengers and the in vivo situation and radicals are very different from the circumstances in a post-column reaction and the radicals used.

The literature suggests superior sensitivity for the ABTS assay and this assay was optimized in chapter 7. Several parameters were optimized such as reactor length, internal diameter and geometry, as well as reagent flow rates. The optimized

reaction conditions showed a loss in peak capacity by maximally 10%, even when coupled to high efficiency HPLC (coupled columns of 50 cm L with 5 µm particles, resulting in 50,000 plates). The analyses of wine, food additives and plant oil are shown. Furthermore, the solvent was shown to influence the radical reaction, making it impossible to compare antioxidant capacity of the various analytes in a gradient run.

In the work presented in chapter 8, the ABTS and DPPH assays are coupled to HILIC. RPLC and NPLC have previously been coupled to these assays, but the analysis of polar analytes remained problematic. As HILIC is very capable of separating polar analytes, it was chosen for the analysis of orange juice. Furthermore, the ABTS and DPPH assays were compared in sensitivity, using both RPLC and HILIC. ABTS showed a higher noise level in the first few minutes of an RPLC analysis, but this disappeared when the organic modifier concentration reach 15%. When comparing limits of quantification, ABTS is more sensitive, except for analytes eluting in less than 15% organic solvent in RPLC. However, these compounds are better separated by HILIC, as they are mostly polar analytes

SAMENVATTING

In dit werk werd de analyse van enzyme-inhibitoren en antioxidantia met behulp van postkolomreacties bestudeerd. Omwille van de solventincompabiliteit bij de koppeling van HPLC en enzymatische reacties werd temperatuursresponsieve LC (TRLC) gebruikt en werd een nieuw kolomtype, gebaseerd op poly(N-vinycaprolactam) (PVCL), aangemaakt.

In hoofdstuk 1 wordt een inleiding gegeven over HPLC en postkolomreacties. Zowel fundamentele als instrumentele aspecten van HPLC worden besproken. Daarnaast worden de belangrijkste eigenschappen van postkolomreacties en de meest gebruikte reactortypes beschreven.

Het belang van temperatuursresponsieve LC wordt besproken in hoofdstuk 2. Enzymen zijn onstabiel in organische solventen en deze worden vaak gebruikt in HPLC. De koppeling van beide technieken verondersteld dan ook dat het gebruik van deze eluenten wordt geminimaliseerd, maar alle HPLC modes maken gebruik van dergelijke oplosmiddelen. Bij TRLC wordt gebruik gemaakt van water als mobiele fase, terwijl de stationaire fase bestaat uit op silica gekoppelde temperatuursresponsieve polymeren. Deze “intelligente” polymeren reageren op een temperatuursverandering: beneden een bepaalde temperatuur zijn ze wateroplosbaar en erboven wateronoplosbaar. In TRLC kan dus de hydrofobiciteit van de stationaire fase worden veranderd door een temperatuurswijziging en kan een solventgradiënt worden vervangen door een temperatuursgradiënt. De mobiele fase van zuiver water maakt TRLC ideaal voor de koppeling met enzymatische reacties.

De invloed van temperatuur op een vloeistofchromatografische scheiding wordt besproken in hoofdstuk 3. Temperatuur is immers niet enkel in TRLC een belangrijke parameter, maar beïnvloedt alle HPLC-scheidingen op vlak van efficiëntie, retentie, selectiviteit en detectorrespons. Bij een hogere temperatuur wordt de mobiele fase minder visceus, zodat de tegendruk daalt. Doordat ook de retentie vermindert en de

van Deemter-curve vlakker wordt, wordt het gebruik van langere kolommen, kleinere partikels en/of hogere debieten bruikbaar, zodat de efficiëntie stijgt en/of de analysetijd verminderd. Verder verandert de diëlectrische constante van water, zodat bij hogere temperaturen minder organische solventen moeten worden gebruikt of een (deel van een) solventgradiënt kan worden vervangen door een temperatuursgradiënt. Aangezien de detectorrespons vaak beïnvloed wordt door het eluens, is het mogelijk door het gebruik van verhoogde temperaturen deze respons meer constant te maken. In TRLC wordt de temperatuur gebruikt voor het veranderen van de hydrofobiciteit van de stationaire fase, die bijvoorbeeld poly(N-iopropylacrylamide) (PNIPAA) bevat. PNIPAA is bijna het enige polymeer dat voor deze toepassing werd beschreven. Eerst werd TRLC vooral als alternatief voor RPLC gebruikt, maar door de introductie van copolymeren met geladen groepen is ook IEX mogelijk. Gebruik van apolaire copolymeren heeft geleid tot veranderingen in de kritische temperatuur van de polymeren, maar niet tot veranderde selectiviteit. Temperatuursgradiënten blijven weinig gebruikt en toepassingen van de ontwikkelde kolommen zijn schaars. De lage efficiëntie, het gebrek aan selectiviteit en de afwezigheid van commerciële kolommen zijn de belangrijkste oorzaken hiervan. Misschien kunnen de recent ontwikkelingen hier verandering in brengen.

Om de mogelijkheden van TRLC verder te verkennen, werd een nieuwe stationaire fase, gebaseerd op PVCL, geïntroduceerd, zoals beschreven in hoofdstuk 4. De fase vertoonde een duidelijk temperatuursresponsief effect, waarbij de retentie en de efficiëntie steeg bij stijgende temperatuur. Wanneer een temperatuur lager dan de kritische temperatuur van PVCL werd gebruikt, werd een lage efficiëntie opgemerkt, die waarschijnlijk te wijten is aan de dikke laag van uitgestrekt polymeer wat voor trage diffusie zorgt. Toevoegen van ethanol, een groen organisch solvent, verminderde de analysetijd en deed het temperatuurseffect teniet. De scheiding van parabenen en fenonen werd ook getoond, wat de toepasbaarheid van deze kolommen aantoont.

De toepassing van TRLC bij de analyse van enzyme-inhibitoren met gebruik van postkolomreacties is het onderwerp van hoofdstuk 5. Eerst werd de mogelijkheid om enkel water te gebruiken bij dit soort analyses getest door gebruik te maken van de cathepsine B assay. De C18 kolom werd vervangen door een C8 kolom om de retentie te verminderen en het volledige eluens van de kolom werd naar de reactor gestuurd. De werkbaarheid van het systeem werd aangetoond met een modelinhibitor. De invloed van organische solventen werd getest door 25, 50 en 100 % methanol of acetonitrille te gebruiken als mobiele fase, maar er werd geen verschil waargenomen in de inhibitorassay. Dit kan misschien verklaard worden door de aanwezigheid van water in toegevoegde stromen van enzyme en substraat en de korte reactortijd van 0,6 min, zodat het enzyme zelf stabiel blijft. Om TRLC te testen werd geopteerd om te werken met trypsine, wat in een reactie van 5 min een duidelijk negatief effect ondervind van de aanwezigheid van organische solventen. De inhibitorassay werd succesvol uitgevoerd, maar een duidelijk gebrek aan reproduceerbaarheid en robuustheid werden waargenomen. Bovendien is de setup ook niet erg gevoelig. Dit wordt vooral veroorzaakt door de lange reactietijden. Ter conclusie kan worden gezegd dat het testen van enzyme-inhibitoren beter offline gebeurd, ondanks de noodzaak voor langere analysetijden, groter verbruik van reagentia en het gebrek aan automatisering.

Om de haalbaarheid van postkolomreacties in combinatie met moderne HPLC te bestuderen, werd besloten reacties met een snellere kinetiek te selecteren. Radicaalvangende assays, gebruikt voor de analyse van antioxidanten, werden gekozen omwille van hun zeer snelle kinetiek. Twee radicalen worden in dit soort assays gebruikt: 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonzuur) (ABTS) en 2,2-diphenyl-1-picrylhydrazyl (DPPH). In hoofdstuk 6 wordt een overzicht gegeven van de relevante literatuur. Antioxidantcapaciteit wordt vaak gebruikt om de kracht van een antioxidant te beschrijven, maar dit verandert wanneer een andere assay wordt gebruikt. Bovendien reageren niet alle antioxidanten met radicalen en zijn de omstandigheden van de postkolomreactie niet de in vivo situatie. Daarom moeten

de resultaten van een antioxidantassay steeds met de nodige zorg worden geïnterpreteerd.

Uit de literatuur blijkt dat de ABST-assay de meest gevoelige is en daarom werd deze assay geoptimaliseerd in hoofdstuk 7. De te optimaliseren parameters waren onder andere de reactordimensies en –geometrie, en de vloeistofdebieten. De geoptimaliseerde reactor vertoonde een piekcapaciteit die minder dan 10% lager lag dan die van de originele scheiding, ook wanneer deze werd gekoppeld met een hoog-efficiënte HPLC scheiding (50 cm kolomlengte, 5 µm partikels, 50.000 platen). De analyse van wijn, voedseladditieven en plantaardige olie wordt beschreven. Bovendien werd aangetoond dat de respons verandert bij veranderd solvent, zodat vergelijking van componenten in gradiëntanalyses onmogelijk is.

In hoofdstuk 8 werden de ABTS- en DPPH-assay gekoppeld met HILIC. De koppeling met RPLC en NPLC werd reeds beschreven in de literatuur, maar de analyse van hydrofiele antioxidantia bleef problematisch. Aangezien HILIC de aangewezen techniek is voor de scheiding van polaire moleculen, werd deze techniek gebruikt voor de analyse van sinaasappelsap. Verder werden de ABTS- en DPPH-assay met elkaar vergeleken op basis van kwantificatielimiet, waarbij werd bewezen dat de ABTS-assay gevoeliger is. Enkel wanneer in RPLC minder dan 15% organische fase aanwezig is, is de DPPH-assay gevoeliger, omwille van een hoog ruissignaal in de ABTS-assay. Componenten die echter dermate vroeg van de RPLC-kolom elueren, worden meestal gescheiden in HILIC, waar dit probleem niet optreedt.

LIST OF ABBREVIATIONS

A	absorption
ABTS	2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid)
ACE	angiotensin converting enzyme
ACN	acetonitrile
AIBN	azobisisobutyronitrile
APCI	atmospheric pressure chemical ionization
APPI	atmospheric pressure photon ionization
Arg	Arginine
ATRP	atom transfer radical polymerization
BHA	butylated hydroxyanisole
BMA	Butylmethacrylate
c	Concentration
CUPRAC	cupric reducing antioxidant capacity
DAD	diode array detector
DCC	N,N'-dicyclohexylcarbodiimide
DCU	N,N'-dicyclohexylurea
d_f	film thickness
D_M	diffusion coefficient in the mobile phase
DMF	N,N-dimethylformamide
d_p	particle size
DPPH	2,2-diphenyl-1-picryhydrazyl)
EEDQ	2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline
ESI	electrospray ionization
EtOH	Ethanol
F	flow rate (in equations)
F	Phenylalanine (in structures and names)
FMOC	Flourenylmethylchloroformate
FRAP	ferric reducing ability of plasma
FWHM	full width at half maximum
GC	gas chromatography
h	peak height
H	plate height
h	reduced plate height
HILIC	hydrophilic interaction chromatography
H_{min}	minimal plate height
HPLC	high performance liquid chromatography
HRP	horseradish peroxidase
HTLC	high temperature liquid chromatography
HTS	high throughput screening
I_0	intensity of incident light
I	intensity of absorbed light
ID	internal diameter

IEX	ion exchange liquid chromatography
k	retention factor
K	partition coefficient
kV	Kilovolt
L	Length
LC	liquid chromatography
LC-MS	liquid chromatography mass spectrometry (hyphenated technique)
LC-MS/MS	liquid chromatography tandem mass spectrometry
LCST	lower critical solution temperature
LOQ	limit of quantification
L_s	length of a solvent segment in a gas-segmented reactor
M	Mass
MAPK	mitogen activated protein kinase
MeOH	methanol
min	Minute
MIP	molecular imprinted polymer
MS	mass spectrometry
MS/MS	tandem mass spectrometry
N	plate number or efficiency
NADPH	nicotinamide adenine dinucleotide phosphate (neutral form)
NMR	nuclear magnetic resonance spectroscopy
NPLC	normal phase liquid chromatography
NSAID	non-steroidal anti-inflammatory drug
OPA	o-phtaldehyde
OTLC	open tubular liquid chromatography
P	Pressure
PBS	phosphate buffered saline
PEEK	polyether ether ketone
PEEK	polyether ether ketone
PEG	polyethylene glycol
Phe	phenylalanine
PNIPAA	poly-(N-isopropylacrylamide)
PSDVB	polystyrene-divinylbenzene
PTFE	polytetrafluoroethylene
PTH	phenylthiodantoin
PVCL	poly-(N-vinylcaprolactam)
QQQ	triple quadrupole tandem mass spectrometer
QTOF	quadrupole-time-of-flight tandem mass spectrometer
r	(internal) radius
R	arginine (in structures and names)
R	gas constant (in equations)
ROS	reactive oxygen species
RPLC	reversed phase liquid chromatography
R_s	resolution
SAX	strong anion exchange chromatography
SCX	strong cation exchange chromatography
SFC	supercritical fluid chromatography
SOP	superoxide dismutase

SPE	solid phase extraction
SPME	solid phase micro extraction
t	time
T	temperature
t_0	dead time
TBHQ	tert-butylhydroxyquinone
TEAC	trolox equivalent antioxidant capacity
TLC	thin layer chromatography
TOF	time-of-flight
t_r	retention time
t_r'	corrected retention time
TRLC	temperature responsive liquid chromatography
u_0	linear velocity, calculated by using t_0
u_{opt}	optimal linear velocity
UV	ultraviolet
V	volume
VCL	N-vinylcaprolactam
V_e	extra-particle volume
Vis	Visual
$W_{0.5}$	peak width at half the height of the peak
W_b	peak width at the base of the peak
WAX	weak anion exchange chromatography
WCX	weak cation exchange chromatography
z	charge
Z-FR-AMC	N-carbobenzoxy-phenylananyl-argininyl-7-amido-4-methylcoumarin
α	selectivity factor
β	phase ratio
β'	Dean factor
γ	surface tension
γ'	obstruction factor
ΔP	pressure difference
ΔG^0	change in Gibss free energy
ΔH^0	change in enthalpy
ΔS^0	change in entropy
ε	molar extinction coefficient
η	viscosity
λ	path length
λ'	packing factor
σ	standard deviation
σ^2	variance
σ_c^2	chromatographic variance
σ_d^2	variance caused by the detector
σ_i^2	variance caused by the injector
σ_i^2	variance caused by the fluidic path between detector and injector
σ_L	standard deviation of a peak in length units
σ_o^2	variance caused by processes outside the chromatographic column
σ_t	total standard deviation
σ_t'	standard deviation of a peak in time units

DANKWOORD

In de eerste plaats dank ik mijn co-promotor, Em. Prof. Dr. Pat Sandra, die me de mogelijkheid gaf dit onderzoek uit te voeren in zijn onderzoeksgroep, toen nog het Pfizer Analytical Research Centre, nu terug het Laboratorium voor Scheidingstechnieken. Prof. Sandra gaf me de kans in een modern laboratorium met bekwame medewerkers te werken. Bij deze wens ik ook Pfizer en voornamelijk het IWT te bedanken voor hun financiële ondersteuning.

Ongetwijfeld van zeer groot belang voor mijn onderzoek was mijn promotor, Prof. Dr. Frédéric Lynen. Zijn begeleiding en hulp bij allerlei problemen stel ik erg op prijs. Als zijn eerste doctoraatstudent kan ik enkel hopen dat er nog vele volgen. Naast deze beide professoren, volgde ook dr. Adrian Wright vanuit Pfizer U.K. mijn werk. Zijn interesse en frisse ideeën motiveerden me na elke “teleconferentie”.

Naast de begeleiding in mijn onderzoek, moet ik ook enkele mensen bedanken voor hun bereidwilligheid bij praktische problemen en instrumentele moeilijkheden. Op de eerste plaats vermeld ik Marc Schelfhaut, met wie ik uren aan massaspectrometers heb gesleuteld. Voor alle hulp in LC, vermeld ik ook Isabelle Francois, Alberto Dos Santos Pereira en Stefan Louw. Voor alle werkjes waarmee ik steeds bij hen terecht kon, wil ik trouwens ook uitdrukkelijk de personeelsleden van de centrale werkplaats bedanken. Vooral bij het pakken van kolommen ben ik geregeld bij hen langsgegaan en werd steeds snel en met een glimlach geholpen.

Twee collega’s uit het labo verdienen een heel groot dankjewel, Seppe de Smet en Maria Rambla Alegre. Ze hebben samen met mij het onderzoek naar antioxidanten gevoerd en het is dan ook dankzij hen dat dit deel zo uitgebreid is geworden. Naast het vele werk, wil ik hen ook bedanken voor de excellente sfeer tijdens deze samenwerking.

Daarnaast verdienen natuurlijk heel wat collega’s een woord van dank, voor hulp in het labo, afleiding, plezier op congressen,... Hierbij vermeld ik onder andere Maarten

De Beer, Sander Delahaye, Thomas Van Damme, Mike De Vrieze, Nathalie De Coensel, Els Van Hoeck, Isabelle Francois, Vivienne Malanchin, Michaël Pollet, Yente Boellaert, Barbara D'hoop, Engdawork Admasu, Kai Chen, Dieter Verzele, Alberto dos Santos, Beatriz Bicalho, Hamed Eghbali, Deirdre Cabooter, Luis Saveedra en andere collega's die ik hier misschien over het hoofd heb gezien.

Ook buiten het laboratorium hebben veel mensen me gesteund en geholpen. In de eerste plaats gaat het om mijn ouders, die mij al mijn jaren aan de universiteit hebben ondersteund, moreel en financieel. Zonder hen zou ik niet staan waar ik vandaag sta en niet zijn wie ik vandaag ben. Zij hebben zich de nodige (en overbodige) zorgen gemaakt over mijn studies en over deze scriptie en hebben me in de moeilijke momenten er steeds terug zin in doen krijgen. Ik ben ontzettend dankbaar voor alles wat zij voor mij hebben gedaan.

Daarnaast zijn er natuurlijk ook tal van vrienden zonder wiens enthousiasme, blik van buitenaf en afleiding, deze klus een ware opdracht was geweest. Ik wil dan ook speciaal al mijn huisgenoten van de Halvemaanstraat bedanken, Bart, Els, Benjamin en Jan, met jullie allemaal werd het thuis nooit een saaie boel. Maar ook Wouter verdient extra aandacht, voor de ontelbare fietsritjes en caféavonden, maar vooral voor zijn opbeurende speeches. En in dit rijtje past ook mijn broer Tim, voor de gezellige zondagavonden en de motoweekends.

En dan is natuurlijk nog één heel speciaal iemand die ik hier wens te vermelden en dat is Valérie. Vooral de laatste maanden is dit werk ook voor haar een hele opgave geweest. Zij brengt de rust die ik af en toe nodig heb, maar ook de bemoedigende woorden om me weer achter de computer te krijgen. Ze is waarschijnlijk even opgelucht als ik dat deze scriptie af is en ik hoop dat ik haar bij haar doctoraat even goed kan helpen als zij mij geholpen heeft.