FACULTEIT WETENSCHAPPEN

# Novel chromatographic and mass spectrometric approaches for quantitation of cyclic nucleotides in biological matrices

**Van Damme Thomas**

Thesis submitted to the Faculty of Sciences in fulfillment of the requirements for the degree of Doctor in Science, Chemistry

**Promotor:** Prof. Dr. Frederic Lynen

**Co-promotor:** Em. Prof. Dr. Pat Sandra

2018

Faculty of Sciences

Department of Organic and Macromolecular Chemistry

Separation Science Group

UNIVERSITEIT GENT

FACULTEIT WETENSCHAPPEN

# Novel chromatographic and mass spectrometric approaches for quantitation of cyclic nucleotides in biological matrices

**Van Damme Thomas**

**Promotor**: Prof. Dr. Frederic Lynen

**Co-promotor**: Em. Prof. Dr. Pat Sandra

**Members of the Evaluation Committee**:

| | |
|---|---|
| Prof. Annemieke Madder | Ghent University |
| Prof. Roman Szucs | Ghent University |
| Prof. John Langley | University of Southampton |
| Prof Dominique Van Der Straeten | Ghent University |
| Prof. Deirdre Cabooter | KU Leuven |

UNIVERSITEIT GENT

# List of abbreviations

| | |
|---|---|
| A | absorbance |
| α | selectivity factor |
| APCI | atmospheric pressure chemical ionization |
| API | atmospheric pressure ionization |
| APPI | atmospheric pressure photo ionization |
| ATP | adenosine triphosphate |
| c | concentration |
| cADP-ribose | cyclic adenosine diphosphate ribose |
| cAMP | cyclic adenosine monophosphate |
| cCMP | cyclic cytidine monophosphate |
| cGMP | cyclic guanosine monophosphate |
| CNC | cyclic nucleotide-gated channel |
| CNGC | cyclic nucleotide-gated channel |
| cNMPs | cyclic nucleotide monophosphates |
| CO | carbon monoxide |
| CRE | cAMP response element |
| CREB | cAMP response element-binding protein |
| CRM | charge residue model |
| CSF | cerebrospinal fluid |
| cUMP | cyclic uridine monophosphate |
| d | path length |
| DAD | photodiode array detector |
| DAG | diacylglycerol |
| $D_c$ | direct current |
| $d_f$ | thickness of stationary phase film |
| $D_m$ | diffusion coefficient in mobile phase |

| | |
|---|---|
| $d_p$ | particle diameter |
| $D_s$ | diffusion coefficient in stationary phase |
| ε | molar absorptivity |
| EIA | enzyme immunoassay |
| ELISA | enzyme-linked immunosorbent assay |
| EMA | European Medicines Agency |
| EPA | Environmental Protection Agency |
| ESI | electrospray ionization |
| FAB | fast atom bombardment |
| FL | fluorescence detector |
| FW | fresh weight |
| GC | gas chromatography |
| GTP | guanosine triphosphate |
| HETP | height equivalent to a theoretical plate |
| HILIC | hydrophilic interaction liquid chromatography |
| HPLC | high-performance liquid chromatography |
| HQC | high quality control |
| I | emergent light intensity |
| i.d. | internal diameter |
| IBMX | 3-isobutyl-1-methylxanthine |
| IEC | ion exchange chromatography |
| IEX | ion exchange chromatography |
| $I_O$ | incident light intensity |
| $IP_3$ | inositol triphosphate |
| IS | internal standard |
| k | retention factor |
| L | length |
| λ | constant depending on particle size |

| | |
|---|---|
| $\lambda_{ex}$ | excitation wavelength |
| $\lambda_{ex}$ | emitting wavelength |
| LC-MS | liquid chromatography – mass spectrometry |
| LLE | liquid-liquid extraction |
| LLQC | lower limit quality control |
| LOD | limit of detection |
| LOQ | limit of quantitation |
| LQC | lower quality control |
| *m/z* | mass to charge ratio |
| MALDI | matrix-assisted laser desorption/ionization |
| MQC | medium quality control |
| MRM | multiple reaction monitoring |
| $\eta$ | viscosity |
| N | efficiency |
| NMR | nuclear magnetic resonance |
| NO | nitrogen oxide |
| NPLC | normal phase liquid chromatography |
| PDE | phosphodiesterase |
| $PIP_2$ | phosphatidylinositol 4,5-bisphosphate |
| PKA | protein kinase A |
| PKC | protein kinase C |
| PKG | protein kinase G |
| PPFD | photosynthetic photon flux density |
| pRIX | porcine relaxin |
| PVDF | polyvinylidene fluoride |
| Q1 | first mass filter |
| Q2 | collision cell |
| Q3 | second mass filter |

| | |
|---|---|
| QC | quality control |
| $r_c$ | column radius |
| $R_f$ | radio frequency |
| RIA | radio immunoassay |
| RNase | ribonuclease |
| RPLC | reversed phase liquid chromatography |
| $R_s$ | resolution |
| RSD | relative standard deviation |
| $\sigma^2$ | band variance |
| SD | standard deviation |
| SEC | size exclusion chromatography |
| SIL | stable isotopic labeled |
| SPE | solid phase extraction |
| SPPs | superficially porous particles |
| TOF | time-of-flight |
| $t_R$ | retention time |
| u | mobile phase velocity |
| UHPLC | ultra-high performance liquid chromatography |
| UV | ultraviolet |
| VIS | visible light |
| $W_b$ | peak width at the base |
| $W_h$ | peak with at half height |

# Table of contents

# General introduction and goals

## General introduction

Analysis of traces of small polar analytes in complex biological samples is a challenging task with about all hyphenated chromatographic separation techniques. Due to their lack of volatility or incompatibility with current supercritical fluids they are only effectively amenable to efficient analysis by liquid chromatography [1]. Among the aqueous separation modes in HPLC, allowing dissolution and therefore analysis of highly polar solutes, only ion exchange, hydrophilic interaction and if possible (ion pairing) reversed phase liquid chromatography can be suitable for analysis of such solutes. Ion exchange chromatography allows for retention and separation but is problematic in terms of robust coupling to, and ionization in, electrospray mass spectrometry. Effective analysis of such polar solutes by reversed phase LC can only be possible if retention can be achieved [2]. Hydrophilic interaction liquid chromatography is then again less generic compared to reversed phase LC, while it can depict column equilibration issues making it less suitable for high throughput analysis [3]. In this thesis this problem of the analysis of polar solutes is analyzed through the development of quantitative and qualitative analytical methods for the enhanced analysis of secondary metabolites in complex biological extracts.

Next to the separation issues related to the analysis of polar solutes, their often problematic ionization in mass spectrometry is exacerbated through the occurrence of an ubiquitous amount of competing solutes in the ionization source, leading to so called *matrix effects*, affecting the ionization efficiency and hence the quality of quantitative methods [4-7]. Preliminary extraction of the molecules of interest in an environment comprising such large quantities of competing and interfering solutes, *e.g.* salts and proteins, is therefore a key factor in the successful analysis of these molecules in biological samples.

Additionally phospholipids, such as phosphatidylcholines and lyso-phosphatidylcholines, are more challenging to extract, are also easily interfering and very detrimental for the quantitative analysis of low levels of (polar) metabolites in complex matrices [8,9]. In RPLC these phospholipids accumulate on-column and often elute only during later analyses, affecting MS sensitivity in an uncontrolled way. Driven by a strong demand by the pharmaceutical industry to fundamentally tackle the presence of phospholipids during quantitative analyses, and by the necessity for alternative approaches for the commercially available sample preparation methods for isolating endogenous components from biological samples, there was at the onset of this research much interest in the development of approaches allowing phospholipid removal from complex matrices comprising highly polar secondary metabolites analytes.

Also pre-concentration of these polar metabolites is essential to allow for quantitation in the sub ppb-range [10]. Enrichment of such target solutes together with fractionation of the possible interferences is thereby further complicated by the increasingly smaller volumes available in biomedical and pharmaceutical analysis obtained from *e.g.* live animal models (*i.e.* due to ethical issues). Because today the bulk of the operator's time is spent on sample preparation, the development of faster, more reliable and more automatable approaches, such as novel solid phase extraction (SPE) methods [11] are particularly relevant for the analysis of "heavy" samples such as blood, animal tissue [12,13] or plant material [14]. The vast majority of the SPE methods comprise reversed phase retention mechanisms to the detriment of the thus far lesser controlled and understood HILIC SPE approaches.

The cyclic nucleotides cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) were selected to be the targeted molecules of interest because they are crucial polar secondary metabolites. These signaling molecules play a central role in signal transduction, the process of transferring information from outside the cell to inside, in eukaryotes [15]. These messengers are important elements in diseases like certain types of cancer [16], uncontrolled cell growth [17], and physiological functions like apoptosis and neurotransmission [18]. The hypothesis has been proposed that quantitative measurement of levels of second messengers could serve as mechanism biomarkers for the pharmacological modulation by 'specific' inhibitors. For example in the cGMP mediated smooth muscle relaxation process, one of the numerous processes affected by cGMP, the drug sildenafil

(better known as Viagra) is a selective inhibitor of cGMP-specific enzyme phosphodiesterase (PDE), that degrades the cyclic nucleotide [19]. An increase in cGMP concentration and smooth muscle relaxation (vasodilation) and erection is the result, a topic of relevance in the pharmaceutical research. Also at the start of this work, limited information was available regarding the occurrence and kinetics of two less know 2',3'-isomers of cAMP and cGMP in plants [20].

Presently the enzyme-linked immunosorbent assays (ELISA) are the most commonly used quantitative method for analysis of such cyclic nucleotides [21,22]. However the precision and accuracy of ELISA can be problematic in applications were nucleotide concentrations are low. Moreover, this biochemical strategy is considered as expensive and limited by its single solute approach. When analyzing these secondary metabolites in biological matrices, the necessity for multi residue methods with sufficient selectivity is high. Therefore there is a trend towards the development of alternative sample preparation and hyphenated mass spectrometric and chromatographic techniques as a substitute for available immunosorbent based assays.

## Goals and outline

Additional solutions are therefore necessary for polar analysis in biological matrices which are of particular pharmaceutical and biochemical importance. The following problems and challenges are thereby identified which translate into the goals of this thesis:

Because the analysis of small, polar organic molecules in biological matrices with chromatographic strategies like LC-MS/MS is challenging due the matrix effects, tailor made sample preparation techniques need to be developed. Extra focus needs to be set on the detrimental impact of phospholipids present in heavy matrices during method development. Hence effective removal of the phospholipids in RPLC-MS/MS is of great relevance for pharmaceutical analysis.

With most samples of biological origin, especially when dealing with samples obtained through animal testing, the available sample quantities of volumes are often small. Therefore pre-accumulation of the target molecules (together with a fractionation from the interferences) is mandatory in the development of suitable sample preparation techniques. Solid phase

extraction can thereby offer the required robustness to address such issues. The possibilities of the less frequently used HILIC SPE on silica as an alternative sample preparation for analysis of polar solutes in biological matrices should thereby be investigated in depth [23].

The power of tandem mass spectrometry (MS/MS) needs to be further assessed and applied for more selective, more sensitive and simultaneous high throughput detection and quantitation of multiple molecules of interest in the sub-ppb range. This while the developed LC-MS/MS methods must be directly compatible with the tailor made sample preparation technique as well.

Considering the importance of second metabolites in eukaryotes, and the demand in pharmaceutical drug development for quantitative information, the development of more suitable analytical methods for such analytes is necessary. Therefore these secondary metabolites, in particular cyclic nucleotides, will be used as target solutes of interest throughout this thesis. However, the developed methods offer prospects for improved analysis of a broader range of polar organic molecules.

In chapter 1 the importance of messenger molecules in organisms is explained. An overview of the most relevant types of secondary metabolites is presented, with extra focus on the cyclic nucleotides cAMP and cGMP and their biochemical role in eukaryotes.

In chapter 2 an overview of *in vivo* metabolite quantification approaches for secondary metabolite analysis is described. Chromatographic and biochemical strategies for analysis of these secondary messengers in biological samples are explained, with extra focus on the techniques applied in this thesis. A literature study is included, describing the quantitation of these secondary messengers through various techniques.

A new tailor made sample preparation method, based on the HILIC separation mode, together with an LC-MS/MS method is developed for the determination and quantification of 3',5'-cGMP and 3',5'-cAMP and its naturally occurring 2',3' isomers in human plasma and animal tissue extracts in chapter 3. SPE on silica was used as sample preparation method for the enrichment of the targets and for the removal of interferences leading to MS ion suppression. Basal plasma concentrations for fifteen healthy human patients in the low ppb range were thereby also determined .

In chapter 4 the presence of 3',5' isomers of cyclic second messengers cAMP and cGMP is investigated in plant tissues, together with thus far unstudied 2',3'-isomers of cGMP and cAMP in these biological samples. A correlation between plant stress and a rapid increase in both 2',3-cAMP and 2',3-cGMP concentration was thereby quantitatively observed for the first time.

The detrimental matrix effects from the ubiquitous presence of a high number of competing molecules in biological matrices on LC-MS/MS methods when using soft ionization sources are investigated in chapter 5. These ion suppressing phenomena were addressed by the development of a novel phospholipid removal approach via SPE. The approach based on acetone based hydrophilic interaction liquid chromatography thereby proved particularly effective. This method was tested for a broad polarity range of selected pharmaceuticals. Post column infusion experiments confirmed the removal of the ion suppression phenomena.

## References


[1] W. Lu, B. D. Bennett, and J. D. Rabinowitz, "Analytical strategies for LC-MS-based targeted metabolomics," *Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences*, vol. 871, pp. 236-242, Aug 2008.

[2] M. Euerby and P. Petersson, "Chromatographic classification and comparison of commercially available reversed-phase liquid chromatographic columns using principal component analysis," *Journal of Chromatography A*, vol. 994, pp. 13-36, Apr 2003.

[3] P. Hemstrom and K. Irgum, "Hydrophilic interaction chromatography," *Journal of Separation Science*, vol. 29, pp. 1784-1821, Aug 2006.

[4] P. J. Taylor, "Matrix effects: The Achilles heel of quantitative high-performance liquid chromatography-electrospray-tandem mass spectrometry," *Clinical Biochemistry*, vol. 38, pp. 328-334, Apr 2005.

[5] E. Chambers, D. M. Wagrowski-Diehl, Z. L. Lu, and J. R. Mazzeo, "Systematic and comprehensive strategy for reducing matrix effects in LC/MS/MS analyses," *Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences*, vol. 852, pp. 22-34, Jun 2007.

[6] R. King, R. Bonfiglio, C. Fernandez-Metzler, C. Miller-Stein, and T. Olah, "Mechanistic investigation of ionization suppression in electrospray ionization," *Journal of the American Society for Mass Spectrometry*, vol. 11, pp. 942-950, Nov 2000.

[7] H. Trufelli, P. Palma, G. Famiglini, and A. Cappiello, "An overview of matrix effects in liquid chromatography-mass spectrometry," *Mass Spectrometry Reviews*, vol. 30, pp. 491-509, May 2011.

[8] Y. Q. Xia and M. Jemal, "Phospholipids in liquid chromatography/mass spectrometry bioanalysis: comparison of three tandem mass spectrometric techniques for monitoring plasma phospholipids, the effect of mobile phase composition on phospholipids elution and the association of phospholipids with matrix effects," *Rapid Communications in Mass Spectrometry*, vol. 23, pp. 2125-2138, Jul 2009.

[9] S. A. Gustavsson, J. Samskog, K. E. Markides, and B. Langstrom, "Studies of signal suppression in liquid chromatography-electrospray ionization mass spectrometry using volatile ion-pairing reagents," *Journal of Chromatography A*, vol. 937, pp. 41-47, Dec 2001.

[10] N. Masque, R. M. Marce, and F. Borrull, "New polymeric and other types of sorbents for solid-phase extraction of polar organic micropollutants from environmental water," *TrAC-Trends in Analytical Chemistry*, vol. 17, pp. 384-394, Jun 1998.

[11] R. Bonfiglio, R. C. King, T. V. Olah, and K. Merkle, "The effects of sample preparation methods on the variability of the electrospray ionization response for model drug compounds," *Rapid Communications in Mass Spectrometry*, vol. 13, pp. 1175-1185, 1999.

[12] K. M. Karlsson, L. E. M. Spoof, and J. A. O. Meriluoto, "Quantitative LC-ESI-MS analyses of microcystins and nodularin-R in animal tissue - Matrix effects and method validation," *Environmental Toxicology*, vol. 20, pp. 381-389, Jun 2005.

[13] S. Lee, B. Kim, and J. Kim, "Development of isotope dilution-liquid chromatography tandem mass spectrometry for the accurate determination of fluoroquinolones in animal meat products: Optimization of chromatographic separation for eliminating matrix effects on isotope ratio measurements," *Journal of Chromatography A*, vol. 1277, pp. 35-41, Feb 2013.

[14] Y. Izumi, A. Okazawa, T. Bamba, A. Kobayashi, and E. Fukusaki, "Development of a method for comprehensive and quantitative analysis of plant hormones by highly sensitive nanoflow liquid chromatography-electrospray ionization-ion trap mass spectrometry," *Analytica Chimica Acta*, vol. 648, pp. 215-225, Feb 2013.

[15] A. T. Bender and J. A. Beavo, "Cyclic nucleotide phosphodiesterases: Molecular regulation to clinical use," *Pharmacological Reviews*, vol. 58, pp. 488-520, Sep 2006.

[16] A. M. Fajardo, G. A. Piazza, and H. N. Tinsley, "The Role of Cyclic Nucleotide Signaling Pathways in Cancer: Targets for Prevention and Treatment," *Cancers*, vol. 6, pp. 436-458, Aug 2014.

[17] S. L. C. Jin, F. J. Richard, W. P. Kuo, A. J. D'Ercole, and M. Conti, "Impaired growth and fertility of cAMP-specific phosphodiesterase PDE4D-deficient mice," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 96, pp. 11998-12003, Oct 1999.

[18] S. H. Soderling and J. A. Beavo, "Regulation of cAMP and cGMP signaling: new phosphodiesterases and new functions," *Current Opinion in Cell Biology*, vol. 12, pp. 174-179, Apr 2000.

[19] N. K. Terrett, A. S. Bell, D. Brown, and P. Ellis, "Sildenafil (VIAGRA(TM)), a potent and selective inhibitor of type 5 cGMP phosphodiesterase with utility for the treatment of male erectile dysfunction," *Bioorganic & Medicinal Chemistry Letters*, vol. 6, pp. 1819-1824, Aug 1996.

[20] H. Richards, S. Das, C. J. Smith, L. Pereira, A. Geisbrecht, N. J. Devitt, J. van Geyschem, A. G. Brenton, and R. P. Newton, "Cyclic nucleotide content of tobacco BY-2 cells," *Phytochemistry*, vol. 61, pp. 531-537, Nov 2002.

[21] S. Doseyici, I. Mehmetoglu, A. Toker, F. H. Yerlikaya, and E. Erbay, "The effects of forskolin and rolipram on cAMP, cGMP and free fatty acid levels in diet induced obesity," *Biotechnic & Histochemistry*, vol. 89, pp. 388-392, Jul 2014.

[22] M. G. Ferrini, E. Garcia, A. Abraham, J. N. Artaza, S. Nguyen, and J. Rajfer, "Effect of ginger, Paullinia cupana, muira puama and L-citrulline, singly or in combination, on modulation of the inducible nitric oxide- NO-cGMP pathway in rat penile smooth muscle cells," *Nitric oxide-Biology and Chemistry*, vol. 76, pp. 81-86, Jun 2018.

[23] F. Tang, Q. W. Yu, B. F. Yuan, and Y. Q. Feng, "Hydrophilic materials in sample pretreatment," *TrAC-Trends in Analytical Chemistry*, vol. 86, pp. 172-184, Jan 2017.

# Chapter 1. The importance of messenger molecules in organisms

## 1.1 Introduction

Eukaryotes, such as humans, are cells or organisms that possess a clearly defined nucleus within a membrane. Signal transduction is consequently important to moderate information from the outside of the cells to the inside (Figure 1.1). The ensuing cascades are molecular circuits that detect, amplify, and integrate diverse external signals to generate responses such as changes in enzyme activity, gene expression, or ion-channel activity. A cell is highly responsive to specific chemicals in its environment as chemical signals tell a cell to respond to a change in conditions. This is part of the information metabolism: how cells receive, process, and respond to information from the environment. Messengers molecules play an important role in signal transduction within cells of eukaryotes, as explained in the next paragraphs.

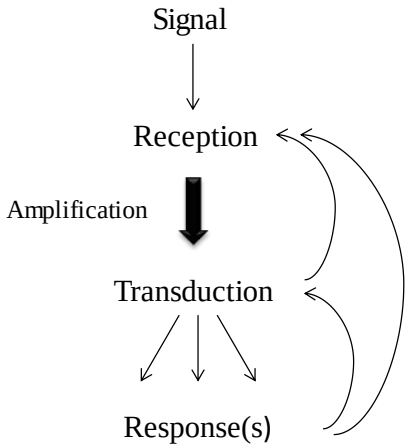


*Figure 1.1. Scheme of signal transduction.*

## 1.2 Receptor proteins, first messengers and G proteins

First messengers are very diverse, ranging from environmental factors such as light or sound, to small molecules and peptides, up to large multivalent proteins. When the cell detects extracellular signals from *e.g.* a signaling first messenger molecule, they activate a specific receptor on the cell membrane.

As most signal molecules are too large and/or too polar to pass through the membrane, the information from the signal molecules must be transmitted across the cell membrane by receptor proteins without the first messengers entering the cell. There are thousands of first messengers, there are, however, relatively few classes of receptors that can receive these signals and elicit a response within the cell. The latter can be, for example, ion channels, intracellular receptors, G-protein coupled receptors, single-pass transmembrane receptors and enzyme-linked membrane receptors [1] (Figure 1.2). These enzyme-linked membrane receptors, also called catalytic receptors, are transmembrane proteins possessing both receptor functions and enzymatic functions.

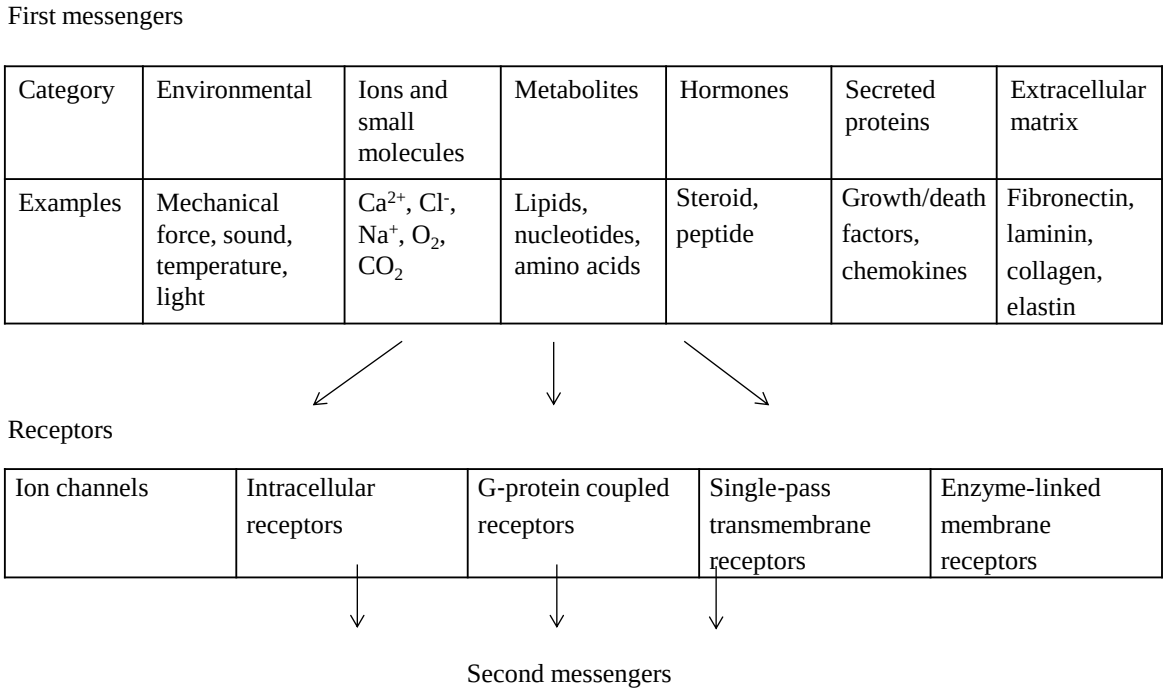


*Figure 1.2. Schematic representation of the messaging cascade involving typical first messengers and receptor.*

These receptors are transmembrane proteins that span the plasma membrane of the cell. They depict extracellular and intracellular domains. Again, the signaling molecule does not cross the membrane. The receptor protein will activate a group of proteins that are involved in transmitting chemical signals from outside the cell to the inside of the cell, known as transducers or G protein. The latter will bind to the activated receptor and will activate a cascade of molecules that causes a change in the cell. A primary effector or amplifier, like the

enzyme adenylyl cyclase will be activated by these G proteins. These primary effectors will generate a large number of signaling molecules that can subsequently diffuse in the cell.

## 1.3 Importance of secondary messengers in signal transduction

### 1.3.1 Introduction

Small, signaling molecules, also called secondary messengers, constitute the next step in the molecular information circuit. As each activated macromolecule (outside the cell) can lead to the generation of many second messengers within the cell, even a low concentration of signal in the environment, which can be as little as a single molecule, can yield a large intracellular signal and response. The second messenger will subsequently bind to and activate intracellular targets, the so called second effector (like protein kinase enzymes) whose effects depend on the particular second messenger system. An example of such a signaling pathway is the 3',5'-cyclic adenosine monophosphate (cAMP) signaling pathway, in which the second messenger cAMP activates a protein kinase A, which is the second effector (Figure 1.3).

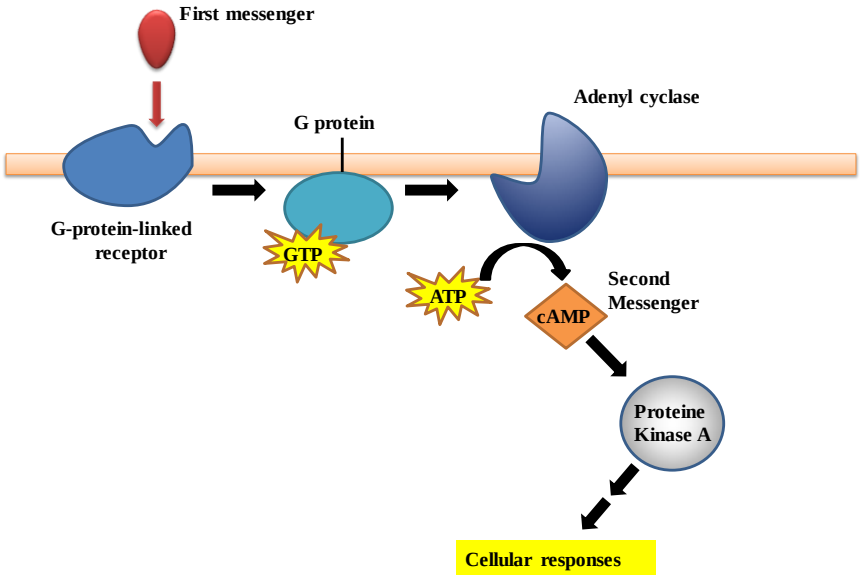


*Figure 1.3. Schematic representation of the cAMP signaling pathway.*

After a signaling process has been initiated and the information has been transduced to affect other cellular processes, the signaling processes must be terminated. Without such termination, cells lose their responsiveness to new signals. Moreover, signaling processes that

fail to be terminated properly may lead to uncontrolled cell growth and the possibility of cancer [2].

### 1.3.2 Overview of the most relevant types of secondary messengers

A large number of secondary messengers have been identified, like the hydrophilic cyclic nucleotides (cAMP, cGMP), inositol triphosphate ($IP_3$), diacylglycerol (DAG), $Ca^{2+}$ ions, NO and CO gas and many more.

#### 1.3.2.1 Messengers in the $PIP_2$ secondary system

One of the most widespread pathways of intracellular signaling is based on the use of second messengers derived from the membrane phospholipid phosphatidylinositol 4,5-bisphosphate ($PIP_2$) [3]. A variety of hormones or growth factors activates a G-protein coupled receptor. The activated $G_\alpha$ protein will activate the enzyme phospholipase C (PLC), located inside the membrane. This enzyme will hydrolyze (cut) the phospholipid $PIP_2$ into two molecules, *i.e.* two second messengers. The first is diacylglycerol (DAG), located inside the membrane, the other is inositol 1,4,5-trisphosphate ($IP_3$), soluble into the cytoplasma (Figure 1.4).

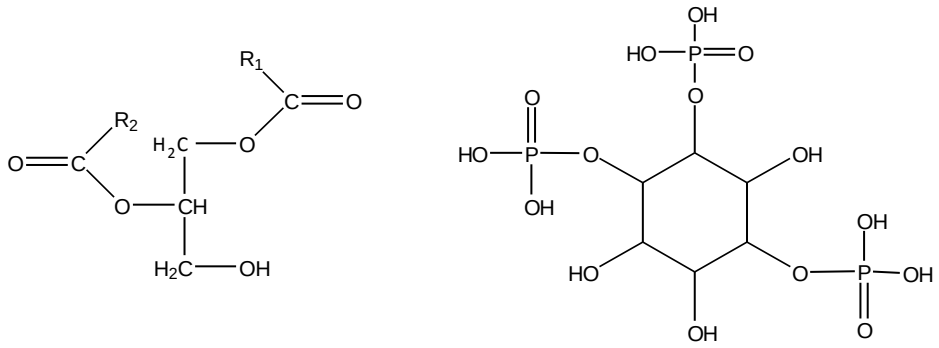


*Figure 1.4. Structures of DAG and $IP_3$.*

DAG will then activate a group of kinases called protein kinase C (PKC), which are playing an important role in the control of cell growth and differentiation [4].

$IP_3$ is a small polar molecule in the cytosol that will bind to ligand gated $Ca^{2+}$ receptors that will signal the release of $Ca^{2+}$ from endoplasmic reticulum (ER), an intracellular store of $Ca^{2+}$. A cytosolic concentration of $Ca^{2+}$ is maintained at an extremely low level (0.1 μM) as a result

of $Ca^{2+}$ pumps that actively export $Ca^{2+}$ from the cell. $Ca^{2+}$ is pumped not only across the plasma membrane, but also into the endoplasmic reticulum, which therefore serves as an intracellular $Ca^{2+}$ store. There is evidence that $Ca^{2+}$ fluxes occur during most forms of cell death (apoptosis), and that inhibiting such fluxes protects cells from death. $IP_3$ receptors are therefore emerging as key sites for regulation by pro- and anti-apoptotic factors [5].

$Ca^{2+}$ is a very common second messenger. Hence cells utilize a variety of mechanisms to regulate intracellular $Ca^{2+}$ levels, making $Ca^{2+}$ a versatile second messenger that controls a wide range of cellular processes [6]. $IP_3$-mediated release of $Ca^{2+}$ from the endoplasmic reticulum as discussed above is not the only mechanism by which the intracellular concentration of $Ca^{2+}$ can be increased. One alternative pathway involves the entry of extracellular $Ca^{2+}$ through $Ca^{2+}$ channels in the plasma membrane. The latter is important in the electrically excitable cells of nerve and muscle, in which voltage-gated $Ca^{2+}$ channels in the plasma membrane are opened by membrane depolarization. The resulting increase in intracellular $Ca^{2+}$ then triggers further release of $Ca^{2+}$ from the intracellular store endoplasmic reticulum by activating distinct $Ca^{2+}$ channels known as ryanodine receptors [7]. Also an increase in intracellular $Ca^{2+}$ in neurons triggers the release of neurotransmitters. Therefore $Ca^{2+}$ plays a critical role in converting electric to chemical signals in the nervous system. Cyclic adenosine diphosphate ribose, cADP-ribose, is also playing an important role in regulation of $Ca^{2+}$ signaling both in animal and plant cells.

An increase in cytosolic $Ca^{2+}$ levels affects the activities of a variety of target proteins, including protein kinases and phosphatases. Some members of the protein kinase C family require $Ca^{2+}$ as well as DAG for their activation, so through two second messengers generated during the $PIP_2$ signaling pathway.

#### 1.3.2.2 The importance of cyclic nucleotides

Two of the most relevant secondary messenger molecules are cyclic guanosine monophosphate (cGMP) and cyclic adenosine monophosphate (cAMP) (Figure 1.5).

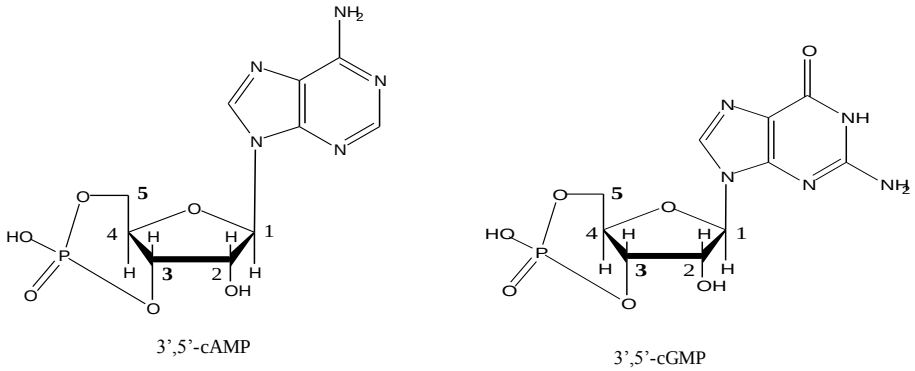


*Figure 1.5. Structure of 3',5'-cAMP and 3',5'-cGMP.*

One of the isomers thereof, 3',5'-cyclic guanosine monophosphate (3',5'-cGMP), is a cyclic nucleotide that acts as a secondary messenger for signal transduction within cells and diverse physiological functions such as neurotransmission [8]. It also directly mediates what is known to be an ever increasing number of plant responses [9-11].

3',5'-cyclic adenosine monophosphate (3',5'-cAMP) has also proved to be an important naturally occurring second messenger which modulates intra-cellular signal transduction, cell growth and differentiation in organisms [12]. It is one of the most prominent nucleotides in eukaryotic cells and is therefore present in almost all cell types of mammals.

cAMP was first identified and characterized by Sutherland and Rall in 1957 [13]. They were studying the action of glycogen phosphorylase in liver cells. Their results confirmed the hypothesis that a soluble cytoplasmic messenger (=second messenger) must transmit the message in response to epinephrine (hormone) binding to its receptor, at the membrane, to glycogen phosphorylase, located in the cytoplasm. This confirmed that the soluble secondary messenger diffused into the cytoplasm to activate the enzyme phosphorylase [14]. One year later this molecule was identified as cAMP [15,16]. Further research identified cAMP as the second messenger involved in the mechanism of action of ephinephrine as well as in many other hormones. Sutherland was awarded the Nobel Prize in Physiology or Medicine in 1971 for his 'discoveries concerning the mechanisms of the action of hormones'.

Hence the purine nucleotides cAMP and cGMP are now well established as second messengers. However, the existence of the cyclic pyrimidine nucleotides, cytidine 3',5'-cyclic monophosphate (cCMP) and uridine 3',5'-cyclic monophosphate (cUMP) (Figure 1.6), has

been controversial for decades. Antibodies against cCMP showed cross-reactivities with other nucleotide 3',5'-cyclic monophosphates (cNMPs) [17] and early mass spectrometric (MS) methods were insufficiently sensitive to unequivocally quantitate and identify cCMP and cUMP [18]. So non reproducible publications and methodological difficulties created skepticism in the cNMP research community concerning the relevance of these cyclic nucleotide molecules. Little research activity occurred for subsequent decades until the recent development of highly sensitive MS methods allowed precise quantitation and identification of these 'new' second messengers in cells [19,20]. Their role as second messengers and the emerging therapeutic implications of cCMP and cUMP signaling are described in a recent review by R. Seifert [19]. Similar generators, effectors and biological functions compared to cAMP and cGMP can thereby be found.

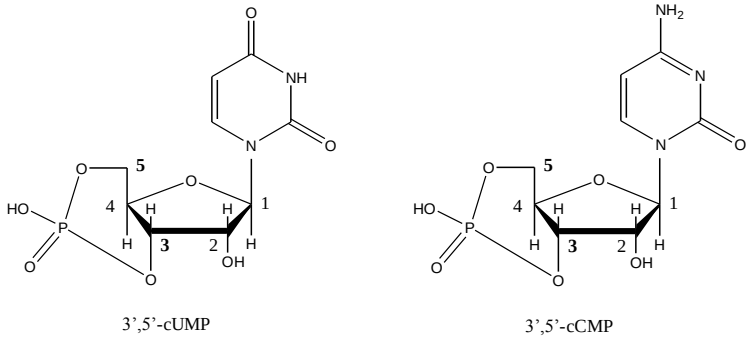


*Figure 1.6. Structure of 3',5'-cUMP and 3',5'-cCMP.*

## 1.4 Focus on the widely spread cyclic nucleotides cAMP and cGMP

This research focuses on the two most widely spread second messengers, *i.e.* 3',5'-cyclic guanosine monophosphate and 3',5'-cyclic adenosine monophosphate. Their formation and degradation will be discussed, as well as some of their biological functions.

### 1.4.1 Formation mechanisms of cAMP and cGMP

cAMP is formed upon stimulation of adenylyl cyclase (which is the primary effector) by a G protein. This enzyme, adenylyl cyclase, produces cAMP from ATP (adenosine triphosphate) in the cell by removing two phosphate groups of the latter (Figure 1.7).

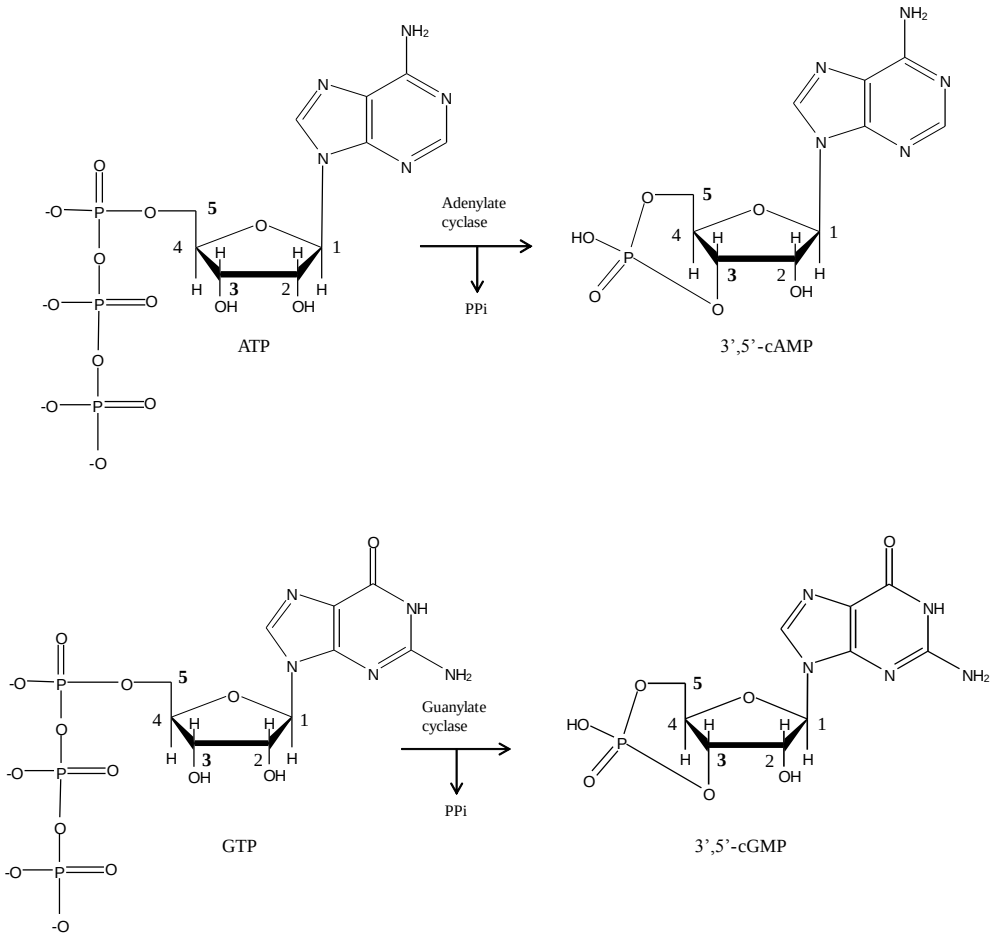


*Figure 1.7. Formation mechanisms of 3',5'-cAMP and 3',5'- cGMP.*

Adenylyl cyclase (AC) is located on the inner side of the membrane and is a transmembrane enzyme that also catalyzes the transformation of ATP to cyclic AMP. To control the level of cAMP in the cell, cyclic nucleotide phospdiesterases (PDEs) degrade the latter molecule which is converted to adenosine 5' monophosphate (AMP), as shown in the next section.

cGMP is formed in a similar way by stimulation of guanylate cyclase to produce the secondary messenger from GTP (guanosine triphosphate).

Note that the presence of naturally occurring 2',3'-isomers of these cyclic nucleotides (see Figure 1.8) in animal tissues has also been described [21,22]. *In vivo*, 2',3'-cAMP is converted to 2'-AMP/3'-AMP, and these AMPs are metabolized to adenosine. This pathway exists endogenously in both mice and human.

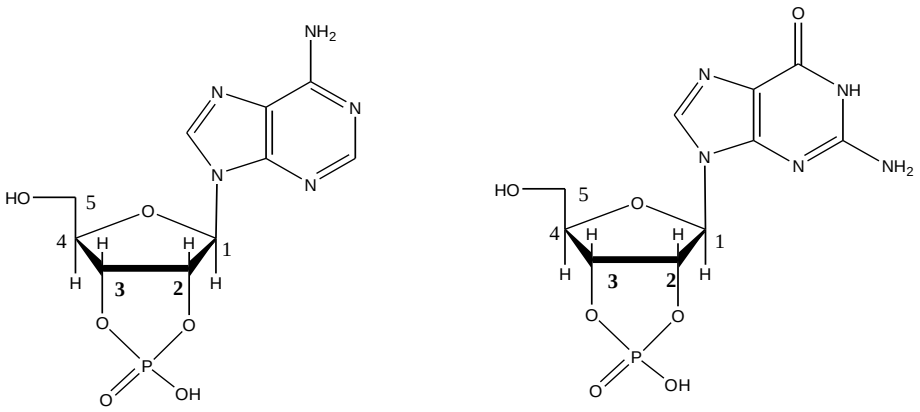


*Figure 1.8. Structure of the isomers of the 3',5' cyclic nucleotides, i.e. 2',3'-cAMP and 2',3'-cGMP.*

### 1.4.2 Degradation mechanisms of cAMP and cGMP

Both cyclic nucleotides degrade by cyclic nucleotide phosphodiesterases (PDEs) that break the phosphodiester bonds in these secondary messenger molecules (Figure 1.9).

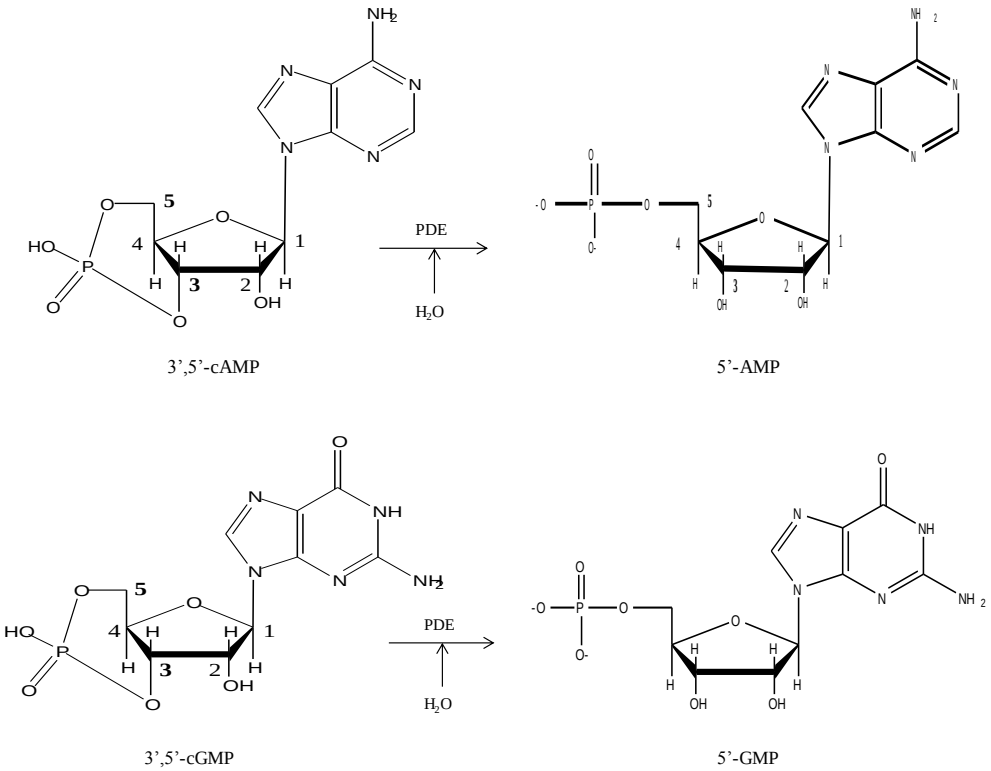


*Figure 1.9. Degradation mechanism of 3',5'-cAMP and 3',5'- cGMP.*

### 1.4.3 Biological functions of cAMP & cGMP dependent PDEs

These PDEs regulate the presence of cGMP and cAMP and are therefore important regulators of signal transduction mediated by these second messenger molecules [23]. PDEs are regarded as potential targets for new drug development opportunities spanning multiple indications. Inhibitors of PDE can prolong or enhance the effects of physiological processes mediated by cGMP or cAMP by inhibition of their degradation by PDE. Thus inhibition of the PDE isoenzymes will cause an increase in intracellular cGMP concentration, which could be potentially linked to therapeutic effects. Hence, cGMP could serve as a mechanistic biomarker for the pharmacological inhibition of PDEs by specific chemical entities [24,25] and the accurate measurement of its concentration can help to understand the underlying mechanisms and possibility of therapeutic interventions. For example the drug sildenafil (Viagra) is a selective and potent inhibitor of cGMP-specific phosphodiesterase type 5 (PDE5) that degrades cGMP in the corpus cavernosum (Figure 1.10). Consequently, an increase in cGMP concentration and smooth muscle relaxation (vasodilation) is the result, leading to an increase of blood flow in the tissues of the penis, causing an erection [26-29].

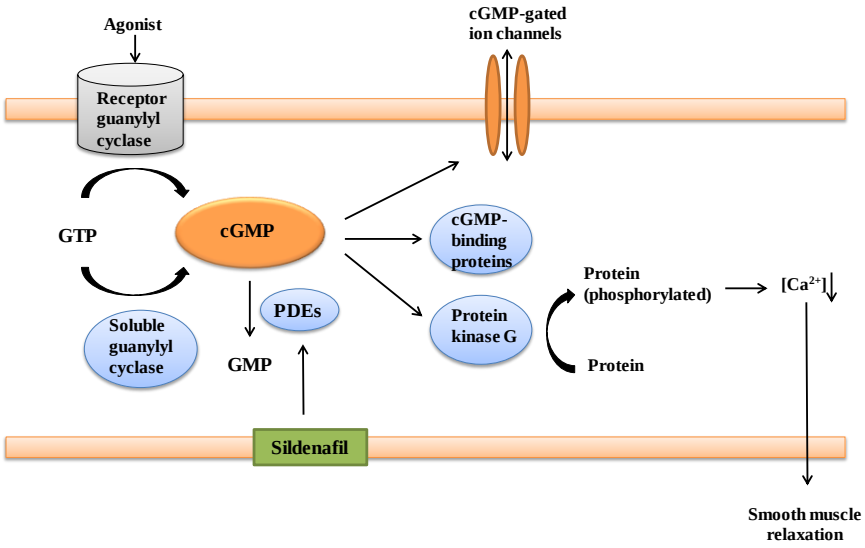


*Figure 1.10. Effect of sildenafil, an inhibitor of cGMP-specific phosphodiesterase type 5 (PDE5), on the smooth muscle relaxation process.*

### 1.4.4 Role further in the signal transduction system

Both cyclic nucleotides can bind to intracellular targets such as cyclic nucleotide-gated ion channels and target proteins like protein kinases. These target proteins are there for acting as secondary effectors.

#### 1.4.4.1 Activation of cyclic nucleotide-gated ion channels by cAMP and cGMP

Cyclic nucleotide-gated channels (CNCs) are nonselective ligand-gated cation channels and are opened by the direct binding of cyclic nucleotides. The main function of these channels is to convert alterations of cellular cAMP and cGMP into changes in the membrane potential. Cyclic nucleotide-gated channels play an important role in cellular development and chemotaxis [30,31]. They are also important in phototransduction and other sensory transduction processes like olfaction and taste, as will be described in the next paragraphs.

Cyclic GMP-gated channels in the outer segment membrane of rods and cones in the retina of the eye are responsible for the light-induced changes in the electrical activity of photoreceptors. In the absence of light, cGMP levels are high and bind to the cGMP-gated channels in photoreceptors. Consequently the channels are opened which permit $Na^+$ and $Ca^{2+}$ ions to flow into the cell. The outer segment of the photoreceptor will be depolarized by this resulting inward current. The resulting flow of ions is known as the dark current. Therefore in the dark, the number of open channels is high and the rate of neurotransmitter release, for example glutamate, is correspondingly high. When light is detected by the retina, the photo-isomerized rhodopsin will activate phosphodiesterases, enzymes that degrade cGMP. cGMP levels drop and consequently cGMP-gated channels in the membrane close, leading to hyperpolarization of the outer segment of the membrane. The release of neurotransmitters is lowered or even prevented (Figure 1.11) [32-34]. Studies have shown that over activation of cGMP-dependent cyclic nucleotide gated ion channels in photoreceptors can lead to their degeneration [35]. Retinitis pigmentosa (RP) is a genetic disease in which patient suffer from degeneration of rod and cone photoreceptors [36].

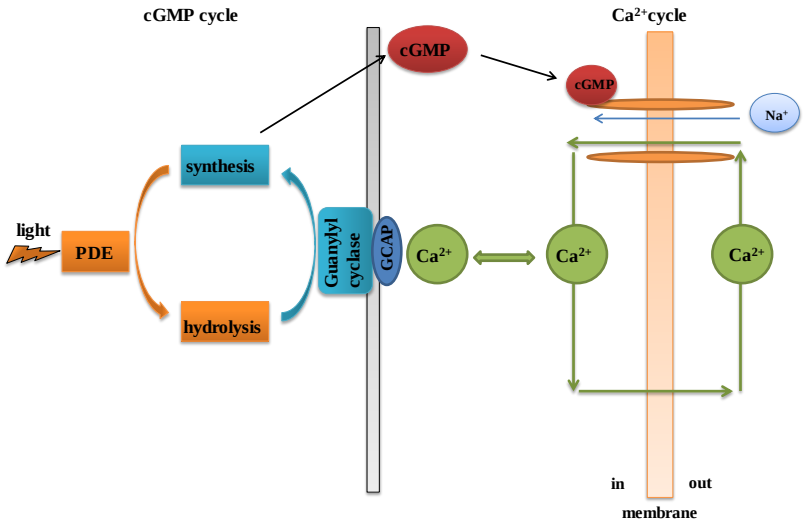


*Figure 1.11. $Ca^{2+}$ feedback mechanism in rod and cone photoreceptors involving cyclic nucleotide-gated channels.*

Another sensory transduction process where the cyclic nucleotides cGMP and cAMP play a role is the olfactory system (smell, odour). Odorant transduction begins with an odorant molecule binding to specific receptor proteins on the external surface of cilia. A cilium is an hair-like organelle that extends from cells found in eukaryotic cells. The olfactory receptor neurons contain an olfactory-specific G-protein ($G_{olf}$), which activates an olfactory-specific adenylyl cyclase. This results in an increase of cAMP that opens channels, cyclic nucleotide-gated ion channels, permitting $Na^+$ and $Ca^{2+}$ to enter, thus depolarizing the neuron and consequently neurotransmitter release is triggered. Also a small subset of olfactory sensory neurons have cGMP-selective cyclic nucleotide-gated ion channels [37-40].

In the gustatory system (taste), the transduction of the taste signals occurs in generic taste cells, containing apical and basolateral surfaces. The apical surface of the taste cell contains both ion channels and G-protein-coupled receptors that are activated by chemical stimuli. When stimulated, each of these channels or receptors changes neurotransmitter release by depolarization in the cell. The larger the tastant concentration, the larger the depolarization of the taste cell and concentration of $Ca^{2+}$ and the larger the release of neurotransmitter will be [41-43].

Note that cyclic nucleotide-gated channels in plants are similar in structure and amino acid sequence to comparative channels in animals. They play an important role in plant immunity

and apoptosis, *i.e.* process of programmed cell death [44,45]. Chapter IV is dedicated to the analysis of the cyclic nucleotides cAMP and cGMP in plant extracts. Information regarding these secondary metabolites in plants is presented and further discussed in chapter IV. Two reviews on cAMP and cGMP in plant signaling are added in this chapter [46,47].

#### 1.4.4.2 Activation of protein kinase by cAMP and cGMP

Protein kinases, the secondary effector, are enzymes that modify other proteins by adding phosphate groups (phosphorylation). The phosphate groups can be removed by another group of enzymes, the protein phosphates. The substrates of protein kinases and phosphates include enzymes, structural proteins, neurotransmitter receptors and ion channels. Phosphorylation results in a change of structure and functionality of the target protein by changing the enzyme activity. The connection between the second messenger cAMP and the second effector protein kinase A (PKA) is often referred to as the cAMP/PKA signaling pathway.

Protein kinase G (cGMP-dependent protein kinase or PKG) plays a role in smooth muscle relaxation/vasodilation [48] and cell division [49]. Protein kinases A (cAMP-dependent protein kinase or PKA) are present in a variety of cells and act on different substrates. PKA and cAMP regulation are involved in many different pathways, including regulation of the lipid metabolism [50].

One of the key downstream activation pathways regulated by cAMP-PKA signaling is the activation of the transcription factor cAMP response element-binding proteins (CREB). These proteins bind to a specific DNA sequence, called the cAMP response element (CRE), and regulate gene expression by promoting or suppressing transcription. CREB regulates a multitude of genes and can be overexpressed and constitutively activated in many cancer types [51]. CREB regulates the expression of several genes involved in metabolism, signaling, proliferation, differentiation and survival [52].

## 1.5 Conclusion

There is an increasing interest in the biological functions of second messengers and in improved understanding of these phenomena through more thorough analytical measurements. This thesis focuses on the second messengers cAMP and cGMP, two of the

most widely spread messenger molecules. Crucial in this research is the quantitation of these second messengers in representative samples like blood and plant material. Therefore developing new sample preparation techniques combined with sensitive and specific analytical separation methods for these solutes is important and discussed in depth in the subsequent chapters.

## 1.6 References

[1] T. D. Pollard, W. C. Earnshaw, J. Lippincott-Schwartz and G. Johnson, Cell Biology (Third Edition): Chapter 26 – Second Messengers, pp. 443-462, Nov 2016.

[2] S. Aznar and J. C. Lacal, "Rho signals to cell growth and apoptosis," *Cancer Letters*, vol. 165, pp. 1-10, Apr 2001.

[3] D. Raucher, T. Stauffer, W. Chen, K. Shen, S. L. Guo, J. D. York, M. P. Sheetz, and T. Meyer, "Phosphatidylinositol 4,5-bisphoshate functions as a second messenger that regulates cytoskeleton-plasma membrane adhesion," *Cell*, vol. 100, pp. 221-228, Jan 2000.

[4] J. Ohanian and V. Ohanian, "Lipid second messenger regulation: the role of diacylglycerol kinases and their relevance to hypertension," *Journal of Human Hypertension*, vol. 15, pp. 93-98, Feb 2001.

[5] C. J. Hanson, M. D. Bootman, and H. L. Roderick, "Cell signalling: IP3 receptors channel calcium into cell death," *Current Biology*, vol. 14, pp. 933-935, Nov 2004.

[6] E. W. McCleskey, A. P. Fox, D. Feldman, and R. W. Tsien, "Different types of calcium channels," *Journal of Experimental Biology*, vol. 124, pp. 177-190, Sep 1986.

[7] J. T. Lanner, D. K. Georgiou, A. D. Joshi, and S. L. Hamilton, "Ryanodine Receptors: Structure, Expression, Molecular Details, and Function in Calcium Release," *Cold Spring Harbor Perspectives in Biology*, vol. 2, pp. 21, Nov 2010.

[8] S. H. Soderling and J. A. Beavo, "Regulation of cAMP and cGMP signaling: new phosphodiesterases and new functions," *Current Opinion in Cell Biology*, vol. 12, pp. 174-179, Apr 2000.

[9] R. P. Newton, E. E. Kingston, D. E. Evans, L. M. Younis, and E. G. Brown, "Occurrence of guanosine 3',5'-cyclic-monophosphate (cyclic-GMP) and associated enzyme-systems in phaseolus-vulgaris," *Phytochemistry*, vol. 23, pp. 1367-1372, 1984.

[10] R. P. Newton, L. Roef, E. Witters, and H. Van Onckelen, "Tansley Review No. 106 - Cyclic nucleotides in higher plants: the enduring paradox," *New Phytologist*, vol. 143, pp. 427-455, Sep 1999.

[11] E. Witters, K. Vanhoutte, W. Dewitte, I. Machackova, E. Benkova, W. Van Dongen, E. L. Esmans, and H. A. Van Onckelen, "Analysis of cyclic nucleotides and cytokinins in minute plant samples using phase-system switching capillary electrospray-liquid

chromatography-tandem mass spectrometry," *Phytochemical Analysis,* vol. 10, pp. 143-151, May-Jun 1999.

[12] S. L. C. Jin, F. J. Richard, W. P. Kuo, A. J. D'Ercole, and M. Conti, "Impaired growth and fertility of cAMP-specific phosphodiesterase PDE4D-deficient mice," *Proceedings of the National Academy of Sciences of the United States of America,* vol. 96, pp. 11998-12003, Oct 1999.

[13] T. W. Rall, E. W. Sutherland, and J. Berthet, "The relationship of epinephrine and glucagon to liver phosphorylase: Effect of epinephrine and glucagon on the reactivation of phosphorylase in liver homogenates," *Journal of Biological Chemistry,* vol. 224, no. 1, pp. 463-475, 1957.

[14] E. W. Sutherland and T. W. Rall, "The properties of an adenine ribonucleotide produced with cellular particles, ATP, $Mg^{++}$, and epinephrine or glucagon," *Journal of the American Chemical Society,* vol. 79, pp. 3608-3608, 1957.

[15] T. W. Rall and E. W. Sutherland, "Formation of a cyclic adenine ribonucleotide by tissue particles," *Journal of Biological Chemistry,* vol. 232, pp. 1065-1076, 1958.

[16] E. W. Sutherland and T. W. Rall, "Fractionation and characterization of a cyclic adenine ribonucleotide formed by tissue particles," *Journal of Biological Chemistry,* vol. 232, pp. 1077-1091, 1958.

[17] T. R. Anderson, "Cyclic cytidine 3',5'-monophosphate (cCMP) in cell regulation," *Molecular and Cellular Endocrinology,* vol. 28, pp. 373-385, 1982.

[18] R. P. Newton, S. G. Salih, B. J. Salvage, and E. E. Kingston, "Extraction, purification and identification of cytidine 3',5'-cyclic-monophosphate from rat-tissues," *Biochemical Journal,* vol. 221, pp. 665-673, 1984.

[19] R. Seifert, "cCMP and cUMP: emerging second messengers," *Trends in Biochemical Sciences,* vol. 40, pp. 8-15, Jan 2015.

[20] H. Bahre, C. Hartwig, A. Munder, S. Wolter, T. Stelzer, B. Schirmer, U. Beckert, D. W. Frank, B. Tuemmler, V. Kaever, and R. Seifert, "cCMP and cUMP occur in vivo," *Biochemical and Biophysical Research Communications,* vol. 460, pp. 909-914, May 2015.

[21] J. Ren, Z. C. Mi, N. A. Stewart, and E. K. Jackson, "Identification and Quantification of 2 ',3 '-cAMP Release by the Kidney," *Journal of Pharmacology and Experimental Therapeutics,* vol. 328, pp. 855-865, Mar 2009.

[22] E. K. Jackson, J. Ren, and Z. C. Mi, "Extracellular 2 ',3 '-cAMP Is a Source of Adenosine," *Journal of Biological Chemistry,* vol. 284, pp. 33097-33106, Nov 2009.

[23] C. Mehats, C. B. Andersen, M. Filopanti, S. L. C. Jin, and M. Conti, "Cyclic nucleotide phosphodiesterases and their role in endocrine cell signaling," *Trends in Endocrinology and Metabolism,* vol. 13, pp. 29-35, Jan-Feb 2002.

[24] D. T. Manallack, R. A. Hughes, and P. E. Thompson, "The next generation of phosphodiesterase inhibitors: Structural clues to ligand and substrate selectivity of phosphodiesterases," *Journal of Medicinal Chemistry,* vol. 48, pp. 3449-3462, May 2005.

[25] S. Uckert, P. Hedlund, K. E. Andersson, M. C. Truss, U. Jonas, and C. G. Stief, "Update on phosphodiesterase (PDE) isoenzymes as pharmacologic targets in urology: Present and future," *European Urology,* vol. 50, pp. 1194-1207, Dec 2006.

[26] N. K. Terrett, A. S. Bell, D. Brown, and P. Ellis, "Sildenafil (VIAGRA(TM)), a potent and selective inhibitor of type 5 cGMP phosphodiesterase with utility for the treatment of male erectile dysfunction," *Bioorganic & Medicinal Chemistry Letters,* vol. 6, pp. 1819-1824, Aug 1996.

[27] S. A. Ballard, C. J. Gingell, K. Tang, L. A. Turner, M. E. Price, and A. M. Naylor, "Effects of sildenafil on the relaxation of human corpus cavernosum tissue in vitro and on the activities of cyclic nucleotide phosphodiesterase isozymes," *Journal of Urology,* vol. 159, pp. 2164-2171, Jun 1998.

[28] H. A. Ghofrani, I. H. Osterloh, and F. Grimminger, "Sildenafil: from angina to erectile dysfunction to pulmonary hypertension and beyond," *Nature Reviews Drug Discovery,* vol. 5, pp. 689-702, Aug 2006.

[29] A. T. Bender and J. A. Beavo, "Cyclic nucleotide phosphodiesterases: Molecular regulation to clinical use," *Pharmacological Reviews,* vol. 58, pp. 488-520, Sep 2006.

[30] I. Weyand, M. Godde, S. Frings, J. Weiner, F. Muller, W. Altenhofen, H. Hatt, and U. B. Kaupp, "Cloning and functional expression of cyclic-nucleotide-gated channel from mammalian sperm," *Nature,* vol. 368, pp. 859-863, Apr 1994.

[31] B. Wiesner, J. Weiner, R. Middendorff, V. Hagen, U. B. Kaupp, and I. Weyand, "Cyclic nucleotide-gated channels on the flagellum control $Ca^{2+}$ entry into sperm," *Journal of Cell Biology,* vol. 142, pp. 473-484, Jul 1998.

[32] A. Gerstner, X. G. Zong, F. Hofmann, and M. Biel, "Molecular cloning and functional characterization of a new modulatory cyclic nucleotide-gated channel subunit from mouse retina," *Journal of Neuroscience,* vol. 20, pp. 1324-1332, Feb 2000.

[33] M. S. Pankey, H. Sunada, and M. Sakakibara, "Dermal photoreception in the pond snail Lymnaea," *Integrative and Comparative Biology,* vol. 50, pp. E133-E133, Jul 2010.

[34] N. Vardi, D. F. Matesic, D. R. Manning, P. A. Liebman, and P. Sterling, "Identification of a G-protein in depolarizing rod bipolar cells," *Visual Neuroscience,* vol. 10, pp. 473-478, May-Jun 1993.

[35] G. Vallazza-Deschamps, D. Cia, J. Gong, A. Jellali, A. Duboc, V. Forster, J. A. Sahel, L. H. Tessier, and S. Picaud, "Excessive activation of cyclic nucleotide-gated channels contributes to neuronal degeneration of photoreceptors," *European Journal of Neuroscience,* vol. 22, pp. 1013-1022, Sep 2005.

[36] F. Paquet-Durand, S. Beck, S. Michalakis, T. Goldmann, G. Huber, R. Muhlfriedel, D. Trifunovic, M. D. Fischer, E. Fahl, G. Duetsch, E. Becirovic, U. Wolfrum, T. van Veen, M. Biel, N. Tanimoto, and M. W. Seeliger, "A key role for cyclic nucleotide gated (CNG) channels in cGMP-related retinitis pigmentosa," *Human Molecular Genetics,* vol. 20, pp. 941-947, Mar 2011.

[37] U. B. Kaupp and R. Seifert, "Cyclic nucleotide-gated ion channels," *Physiological Reviews,* vol. 82, pp. 769-824, Jul 2002.

[38] M. R. Meyer, A. Angele, E. Kremmer, U. B. Kaupp, and F. Muller, "A cGMP-signaling pathway in a subset of olfactory sensory neurons," *Proceedings of the National Academy of Sciences of the United States of America,* vol. 97, pp. 10595-10600, Sep 2000.

[39] U. B. Kaupp, "The cyclic nucleotide-gated channels of vertebrate photoreceptors and olfactory epithelium," *Trends in Neurosciences,* vol. 14, pp. 150-157, Apr 1991.

[40] S. Krannich and M. Stengl, "Cyclic Nucleotide-Activated Currents in Cultured Olfactory Receptor Neurons of the Hawkmoth Manduca sexta," *Journal of Neurophysiology,* vol. 100, pp. 2866-2877, Nov 2008.

[41] A. I. Spielman, W. Yan, V. Krizhanovsky, S. Rosenzweig, T. Yamamoto, and M. Naim, "Modulation of two second messengers in bitter taste transduction of agriculturally relevant compounds," in *Chemistry of Taste: Mechanisms, Behaviors, and Mimics*. vol. 825, pp. 18-31, July 2002.

[42] T. Misaka, Y. Kusakabe, Y. Emori, T. Gonoi, S. Arai, and K. Abe, "Taste buds have a cyclic nucleotide-activated channel, CNGgust," *Journal of Biological Chemistry,* vol. 272, pp. 22623-22629, Sep 5 1997.

[43] T. A. Cummings, C. Daniels, and S. C. Kinnamon, "Sweet taste transduction in hamster: Sweeteners and cyclic nucleotides depolarize taste cells by reducing a K+ current," *Journal of Neurophysiology,* vol. 75, pp. 1256-1263, Mar 1996.

[44] B. Kaplan, T. Sherman, and H. Fromm, "Cyclic nucleotide-gated channels in plants," *Febs Letters,* vol. 581, pp. 2237-2246, May 2007.

[45] R. Ali, W. Ma, F. Lemtiri-Chlieh, D. Tsaltas, Q. Leng, S. von Bodman, and G. A. Berkowitz, "Death don't have no mercy and neither does calcium: *Arabidopsis* cyclic nucleotide gated channel2 and innate immunity," *Plant Cell*, vol. 19, pp. 1081-1095, Mar 2007.

[46] C. Gehring, "Adenyl cyclases and cAMP in plant signaling - past and present," *Cell Communication and Signaling*, vol. 8, pp. 1-5, Jun 2010.

[47] J. C. Isner and F. J. M. Maathuis, ¨cGMP signalling in plants: from enigma to main stream," *Functional Plant Biology*, vol. 45, pp. 93-101, 2018.

[48] H. K. Surks, "cGMP-dependent protein kinase I and smooth muscle relaxation - A tale of two isoforms," *Circulation Research*, vol. 101, pp. 1078-1080, Nov 2007.

[49] S. Hindley, B. H. J. Juurlink, J. W. Gysbers, P. J. Middlemiss, M. A. R. Herman, and M. P. Rathbone, "Nitric oxide donors enhance neurotrophin-induced neurite outgrowth through a cGMP-dependent mechanism," *Journal of Neuroscience Research*, vol. 47, pp. 427-439, Feb 1997.

[50] N. Gerits, S. Kostenko, A. Shiryaev, M. Johannessen, and U. Moens, "Relations between the mitogen-activated protein kinase and the cAMP-dependent protein kinase pathways: Comradeship and hostility," *Cellular Signalling*, vol. 20, pp. 1592-1607, Sep 2008.

[51] K. M. Sakamoto and D. A. Frank, "CREB in the pathophysiology of cancer: Implications for targeting transcription factors for cancer therapy," *Clinical Cancer Research*, vol. 15, pp. 2583–2587, 2009.

[52] B. Mayr and M. Montminy, "Transcriptional regulation by the phosphorylation-dependent factor CREB," *Nature Reviews Molecular Cell Biology*, vol. 2, pp. 599–609, 2001.

# Chapter 2. Instrumental approaches for qualitative and quantitative analysis of secondary metabolites

## 2.1 Introduction

A variety of analytical techniques can be used to detect and quantify secondary messengers and more specifically cyclic nucleotides in biological fluids. The keys for successful quantitation of solutes in biological samples are, however, selectivity and sensitivity. Analytical approaches allowing these types of analyses are described in this chapter. A subdivision is thereby made between chromatographic (and mass spectrometric) strategies and biochemical strategies. In the last part of this chapter a literature study is presented, describing the quantitation of these secondary messengers in a chronological order.

## 2.2 Chromatographic strategies for secondary metabolite analysis

Although technically gas chromatography (GC) could be used to analyze these metabolites, the main disadvantage is the need to make thermal stable, volatile derivatives, which can be injected and separated by GC. As this is challenging or impossible for many of the too polar solutes, like these cyclic nucleotides, the most suitable technique for secondary metabolite analysis in pharmaceutical and biochemical studies is liquid chromatography (LC).

The latter was first described, at the beginning of the 20$^{th}$ century, by the Russian botanist Tswett, as a technique to analyze complex mixtures for the first time, separating plant pigments on glass lined columns with $CaCO_3$ as the solid phase and benzene as the liquid phase [1].

Development of the technique stagnated in the first half of the 20$^{th}$ century but in the late 60s led to the development of high-performance liquid chromatography (HPLC), which is the cornerstone of modern bio analysis. Small particles immobilized in columns in well-packed beds, have thereby proven to increase both efficiency and separation speed. The mobile phase migrates through the packed bed by using high pressure (with a pump). The term HPLC was first introduced by Horvath, originally meaning high-pressure LC, because high pressures

were necessary to reach an optimal flow [2]. Nowadays the term high-performance liquid chromatography is preferred. Continuous technological improvements made in particle chemistry performance, system optimization and detector design allowed reaching even higher resolution, sensitivity, peak capacity and speed of analysis. Separation methods with elevated temperatures contributed to this optimization as well.

HPLC is nowadays the most powerful and widely used separation technique. The technique allows to separate, detect and quantitate analytes of interest in complex mixtures and of different polarity and molecular size. Therefore HPLC is routinely applied in pharmaceutical and biochemical studies and applications, as well as in this research.

Below the principles of HPLC, including some theoretical background, separation mechanisms, instrumental information and detectors are explained to allow the reader to understand better the potential, diversity and success of this technique.

### 2.2.1 Underlying principles of HPLC

A chromatographic separation is controlled by three fundamental parameters, *i.e.* retention, selectivity and efficiency [3].

The retention time $t_r$ is the time it takes for a retained analyte to migrate through the column. It is the time that the analyte spends in both the mobile phase and stationary phase. The retention time for any type of component is characteristic for a certain stationary and mobile phase. The void time $t_o$ is the time it takes for a non-retained analyte to elute from the column, so the time the analyte spends in the mobile phase. The time the analyte spends in the stationary phase is called the adjusted retention time $t'_r$ (Figure 2.1)

$$t_r = t_o + t'_r \quad (1)$$

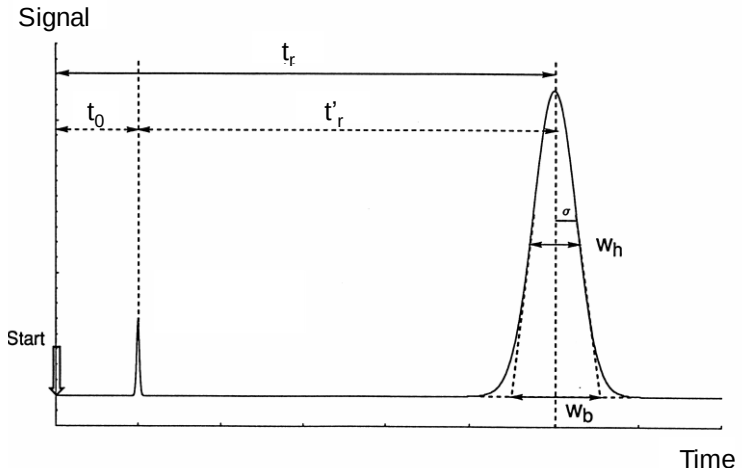


*Figure 2.1. Retention time and peak width.*

The retention factor (k) is the ratio of the time an analyte spends in the stationary phase compared to the time it spends in the mobile phase. The retention factor describes the retention independent from the column dimensions. It is also a way to normalize the retention time with respect to the flow rate.

$$k = \frac{t_r - t_o}{t_o} = \frac{t'_r}{t_o} \quad (2)$$

A component that has no affinity for the stationary phase, eluting from the column at time $t_o$, will have a retention factor of zero. The higher the affinity of the analyte for the stationary phase, the more time the analyte will spend in the stationary phase, leading to a higher $t_r$ and k value. Typically, chromatography is conducted at retention factors varying from 3 to 15. For values below 3, the interaction solute-stationary phase is not sufficient enough to achieve satisfactory resolution. For values larger than 15, the total analysis time will become long, making the analysis very time-consuming. Commonly, this is bypassed by applying a gradient.

The relative retention of two retained analytes is described by the selectivity factor α (equation 3). $k_1$ represents the retention factor of the less retained analyte (= first eluting peak) and $k_2$ is the retention factor of the more retained solute (= second eluting peak). Therefore the selectivity α is always larger than or equal to 1.

$$\alpha = \frac{k_2}{k_1} \tag{3}$$

Optimizing the selectivity is the most effective way to improve the separation of analytes, and this depends on the properties of the analytes, of the stationary phase and of the mobile phase.

A peak, which has ideally a Gaussian shape, is also characterized by the peak width ($w_b$ measured at the base or $w_h$ measured at half height). When the extra column band broadening mechanisms are not considered and assuming that the measured standard deviation σ is due only to chromatographic band broadening, the column efficiency N can be expressed as indicated in equation 4. It is thereby assumed that the injected sample plug yields a Gaussian distribution of the analyte molecules as it passes through the column.

$$N = \left(\frac{t_r}{\sigma}\right)^2 = 5.54\left(\frac{t_r}{w_h}\right)^2 = 16\left(\frac{t_r}{w_b}\right)^2 \tag{4}$$

The resolution $R_s$ is a measure for the separation of two components on a specific column under well-defined conditions. The resolution is component dependent and can experimentally be obtained from the chromatogram for a critical pair:

$$R_s = \frac{t_{r2} - t_{r1}}{(w_{b1} + w_{b2})/2} \tag{5}$$

The column efficiency N, retention factor k and the selectivity factor α are the fundamental parameters contributing to the chromatographic resolution. By incorporating these parameters in equation 5, the resolution of the separation of two components can be described according to equation 6, the master equation of chromatography.

$$R_s = \frac{1}{4}\sqrt{N}\left(\frac{\alpha - 1}{\alpha}\right)\left(\frac{k}{1+k}\right) \tag{6}$$

The two main parameters that influence the resolution that can be achieved with a packed HPLC column are, respectively, the efficiency N, depending on the column length, particle size and uniformity of the packed bed, and the selectivity α, based on the residence times in

the stationary phase and the mobile phase. The retention factor k has a smaller influence on the resolution for values larger than 3.

Based on the plate number N and the length of an analytical column L, the height equivalent to a theoretical plate HETP or plate height H can be calculated, characterizing the peak spreading in a column. H indicates the length of a column segment in which a perfect equilibrium of a component between mobile phase and stationary phases takes place.

$$H = \frac{L}{N} \tag{7}$$

The plate height H is a measure of the band broadening that takes place during the elution process. A plate height model can be defined, based on three different and independent contributions to band broadening in a chromatographic separation, as a function of the mobile phase velocity u. This relation can be depicted by the van Deemter equation (equation 8) [4]. It also provides information on the mobile phase optimal linear velocity ($u_{opt}$) at which an optimal resolution, hence minimal plate height H, can be achieved.

$$H = A + \frac{B}{u} + Cu \tag{8}$$

In this van Deemter model, the A-term, called the eddy diffusion, quantifies the total contribution to dispersion due to different path lengths through the packing of the column. It is influenced by the particle diameter and the particle size distribution. The quality of the packing will thus have a large influence on this A-term .

$$A = 2d_p\lambda \tag{9}$$

$d_p$ is the particle diameter and $\lambda$ is a constant depending on the packing quality of the column. A value of 1-1.5 is considered a good value for $\lambda$, when the columns are not well-packed this value will be higher. This band broadening contribution can be reduced by reducing the particle size. The column counter pressure will then, however, increase rapidly. Note that this factor is independent from the mobile phase linear velocity u.

The B-term or longitudinal diffusion describes the dispersion caused by the molecular diffusion of the analyte in the mobile phase, along the column length. This band broadening is expressed by:

$$B = 2\gamma D_m \quad (10)$$

in which γ is the obstruction factor and $D_m$ is the molecular diffusion coefficient in the mobile phase. The higher the linear velocity, the lower the impact on this band broadening.

The C-term of the van Deemter equation describes the resistance to mass transfer. It quantifies the time needed to achieve an equilibrium between both phases. This C-term consists of two contributions, $C_m$ and $C_s$, respectively the mass transfer in the mobile phase and the mass transfer in the stationary phase.

$$C = C_m + C_s \quad (11)$$

$C_m$ and $C_s$ are a function of the diffusion coefficients in the stationary phase ($D_s$) and in the mobile phase ($D_M$), respectively. Because at higher flow rates there is less time to reach equilibrium, peaks will be broader. Smaller particles are beneficial because the equilibrium will be reached more quickly, hence more narrow peaks will be generated.

Plotting the plate height H as a function of the mobile phase linear velocity u gives the well-known van Deemter plot. A curved relationship with a hyperbolic function is obtained (Figure 2.2). This curve is the sum of the three described independent dispersion phenomena that are taking place. At low velocities, left side of the figure, the overall plate height is determined by longitudinal diffusion, whereas at high velocities, right hand side, the plate height is determined by the limited rate of mass transfer between the two phases.

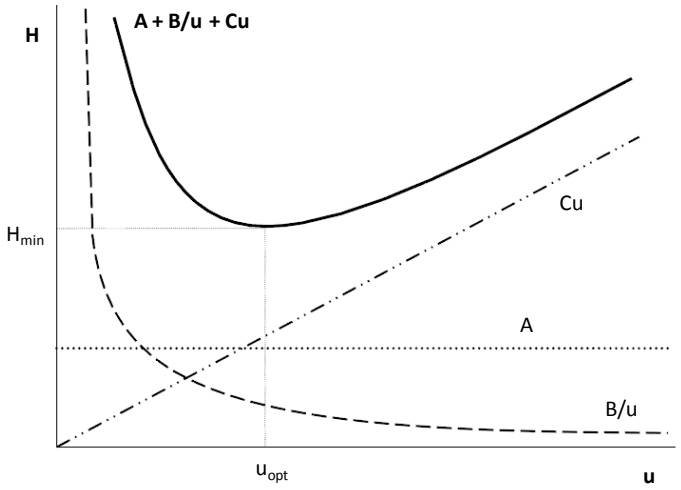


*Figure 2.2. Graphic representation of the van Deemter model describing the relation between HETP and the mobile phase velocity (u).*

The minimal plate heights $H_{min}$ for columns packed with spherical porous silica particles (1.5 to 20 µm), can be as low as twice the size of the particles $d_p$.

$$H_{min} \sim 2\, d_p \tag{12}$$

$$N \sim \frac{L}{2d_p} \tag{13}$$

To increase the column efficiency N, either the column length L can be increased or the particle size $d_p$ can be reduced. However, in liquid chromatography the plate number is limited by the pressure drop ΔP over the column, and can be calculated by the Darcy equation:

$$\Delta \mathrm{P} = \frac{K_O \eta L}{d_p^2} u = \frac{K_O \eta N H}{d_p^2} u \tag{14}$$

$K_O$ is a dimensionless constant (~ 1.000) and η is the viscosity of the mobile phase. For small porous particles (sub-2 µm), the elution speed can be increased without loss of efficiency (flat C-term in van Deemter plot) compared to the large fully porous particles. The small porous particles cause, however, large back pressures on the LC system (see equation 14) by which a conventional HPLC instrument (400 to 600 bar) cannot be used anymore for analysis. Therefore when using sub-2µm particles, Ultra High Performance Liquid Chromatography (UHPLC) becomes mandatory. There is no difference between UHPLC and HPLC, except

that smaller particles are used. The typical particle size in UHPLC is ~ 1.8 μm. Modern hardware systems can deliver pump pressures of 1500 bar coping with the optimal flow rates for such particles. Shorter columns are used in UHPLC as more plates per unit of length are produced when the column is packed with smaller particles. Shorter columns together with a flatter van Deemter curve in the right region, allow to obtain UHPLC analyses up to 10 times faster compared to HPLC analyses. MS detection (UHPLC-MS) is also significantly enhanced by the use of UHPLC because increased peak concentrations with reduced chromatographic dispersion at lower flow rates compared to HPLC, promotes increased source ionization efficiencies. A disadvantage is the reduced column life time because of the high pressures applied.

In recent years, also superficially porous particles (SPPs), called core-shell or fused-core particles, have been introduced. These particles contain a solid, nonporous silica core surrounded by a thin porous shell layer with similar properties compared to the fully porous materials used in conventional HPLC columns. Kirkland and his team investigated this type of particles and introduced the name 'Fused-Core' particles [5-7]. The high efficiency which can be achieved on columns packed with sub-3-μm SPPs, combined with convenient operating conditions like low back pressures and the ability to use conventional HPLC instruments has generated significant interest in the chromatographic community in such columns and to widespread applications in many fields [8,9]. Columns packed with sub-3-μm SPPs rival the efficiency of columns packed with sub-2-μm fully porous particles, but the former generate only half the back pressure. Moreover, as evidenced from van Deemter plots, the diffusion distance is shorter compared to fully porous particles, lowering the C-term and flattening the right part of the van Deemter plot. Also the A-term is lower as the particles are very homogeneous in size. The B-term is lower as well, compared to full porous particles, because more obstruction in a core-shell particle through its solid core. Faster separations and analyses can therefore be achieved with the same separation efficiency. Further performance improvement is possible with very fine SPPs (1.3–1.7 μm), though the use of ultrahigh-pressure liquid chromatography (UHPLC) systems is again mandatory. Faster analysis and higher efficiency of complex samples is particularly desirable in bioanalysis and pharmaceutical applications. Elevated temperatures also allow for higher flow rates by

lowering the viscosity of the mobile phase with significant reduction of the column backpressure as a consequence.

Extra column band broadening can be caused by more practical aspects of the HPLC separation. When too much sample volume is injected onto the column, band broadening occurs, expressed by $\sigma^2_{\text{inj}}$. The tubing between injector-column and column-detector also contributes to possible extra column band broadening $\sigma^2_{tub}$. If they are too long or too broad in diameter, molecules that were separated in the column can re-blend in the tubes, causing broader peaks and worse separation. Also the detection volume and the response time of a detector can cause band broadening, $\sigma^2_{det}$.

### 2.2.2 Separation modes in HPLC relevant for the analysis of secondary metabolites

The separation mechanisms in HPLC largely depend on the type of interaction of the solutes with the stationary phase and the mobile phases used. The most commonly used techniques in HPLC are normal phase liquid chromatography (NPLC), reversed phase liquid chromatography (RPLC), hydrophilic interaction liquid chromatography (HILIC) and ion exchange chromatography (IEC).

In normal phase liquid chromatography, a polar stationary phase and a non-polar mobile phase are applied. The retention mechanism is based on interaction of polar functional groups of the analytes with polar sites present on the stationary phase, *e.g.* pure silica or pure alumina, or -OH, -$NH_2$, -CN bonded to silica [10]. Affinity of molecules for the polar surface is determined by their polarity. Polar analytes interact more with the stationary phase and will elute later (more retained) compared to non-polar analytes. The strength of the eluents (expressed as eluting strength), is a representation of the adsorption energy of the eluent per unit of area of a specific adsorbent. Increasing the polarity of the mobile phase during analysis results in a decrease in the retention time of an analyte. Hexane is therefore considered a weak eluent and methanol a strong one. Other typical mobile phases in NPLC are pentane, ethyl acetate, isopropanol and chloroform. This separation mechanism is not used as often as reversed phase LC because of poorer robustness, asymmetric peak shapes and longer column regeneration times needed. The water content in the eluent is a serious problem. Water is

adsorbed strongly at the surface and controls the retention of the analytes. NPLC was not applied in this research, the polar second messenger molecules are not soluble in the apolar solvents used in NPLC.

The most commonly used separation mechanism in HPLC, and also the mechanism of choice for this research, is based on the interaction between a non-polar (hydrophobic) stationary phase and a polar mobile phase, called reversed phase liquid chromatography [11]. The stationary phase is apolar in nature and this is *e.g.* obtained by derivatizing silica with a long chain of alkyl groups. Typical hydrocarbon chains are octadecyl ($C_{18}$) and octyl ($C_8$). The polar eluent in RPLC is water, mixed with a water miscible organic solvent like acetonitrile and methanol. The weakest eluent with the lowest eluotropic power is water, and retention decreases with increasing concentration of organic modifier. The separation mechanism is related to hydrophobicity. Optimal results can be obtained by adapting the pH of the mobile phase to control the protonation of weak acids and bases, applied in this research. RPLC is a versatile mechanism, neutral and charged or ionizable compounds can be analyzed. It is also considered as a green technique compared to the other modes discussed in this section because of the use of water and environmentally less challenging solvents such as methanol and acetonitrile.

An alternative mechanism of separation, closely related to NPLC, is hydrophilic interaction liquid chromatography (HILIC). This mode has gained a lot of interest in the last decades and is also applied in this research. It is a technique for separating polar compounds on polar stationary phases with an organic mobile phase containing a limited amount of water. Retention is primarily caused by partitioning of the analytes between a water-enriched layer of immobilized eluent on a hydrophilic phase and a relatively hydrophobic bulk eluent. The composition of the mobile phase is typically 5-20% water in acetonitrile [12-14]. The combination with mass spectrometry, especially using electrospray ionization, applied in this thesis, is beneficial since ionization is not easily achieved with the apolar eluents in NPLC. During this thesis, and the research on cyclic nucleotides, HILIC technology is used extensively. Also in HILIC, column equilibration is of utmost importance to avoid repeatability issues [15,16]. Full equilibration was found to depend on the nature of the stationary phase, the pre-equilibrium (*e.g.* storage) solvent and the flow rate. The selectivity of the separation changed with equilibration time, suggesting that full equilibration is always

necessary. This topic was investigated and confirmed in chapter 4. An example of a HILIC separation is shown in Figure 2.3 [17].

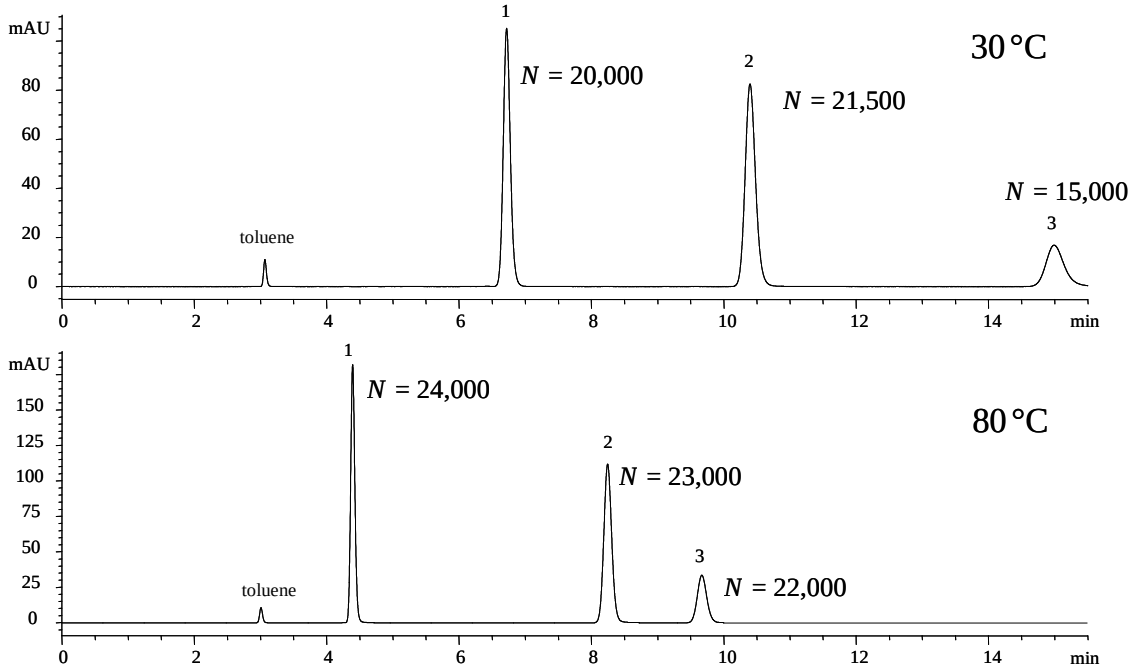


*Figure 2.3. HILIC separation and analysis of a test mixture containing adenine, ketoprofen and an active pharmaceutical ingredient using a 250 mm x 4.6 mm i.d. x 5 micron silica column at (A) 30°C and (B) 80°C. Mobile phase flow rate 1mL/min of 5mM ammonium formate pH 5 in 90% acetonitrile. Detection: UV at 254 nm. Peaks: 1 = ketoprofen; 2 = adenine; 3 = active pharmaceutical ingredient.*

Another separation mode is ion exchange chromatography (IEC), based on the exchange of analytes with the counter ions on the surface of the stationary phase. An ion exchanger commonly consists of a macromolecular matrix on which ionogenic groups are bonded. IEC is commonly used for the separation of water-soluble ionogenic compounds such as amino acids, peptides and proteins [18-20]. The counter ions can be positively (cation exchanger) or negatively charged (anion exchanger). The cation or anion from the sample can displace the counter-ion from the exchanger. Gradient elution by means of a change of the eluent during the separation is commonly applied in practice. This technique was not applied in this research.

Based on the mobile phase composition, a distinction between isocratic, gradient analysis or a combination of both elution modes is performed [21]. In isocratic elution, the composition of the mobile phase is held constant while in gradient elution the eluent composition is varied

throughout the separation window. Isocratic elution depicts some advantages such as the possibility of consecutive injections without the necessity to re-equilibrate the column. This mode, however, is limited to a relatively small polarity range of compounds. Gradient elution provides a solution as the eluotropic strength is increased as function of time. The elution is thus accelerated, resulting in acceptable analysis times.

### 2.2.3 Instrumental aspects of HPLC

Contemporary HPLC instrumentation allows for effective analysis of many small organic molecules like second messengers. The basic structural elements are briefly discussed. A liquid chromatograph consists of a liquid supply system, an injection system, a separation column, a detector and a recording system (Figure 2.4).

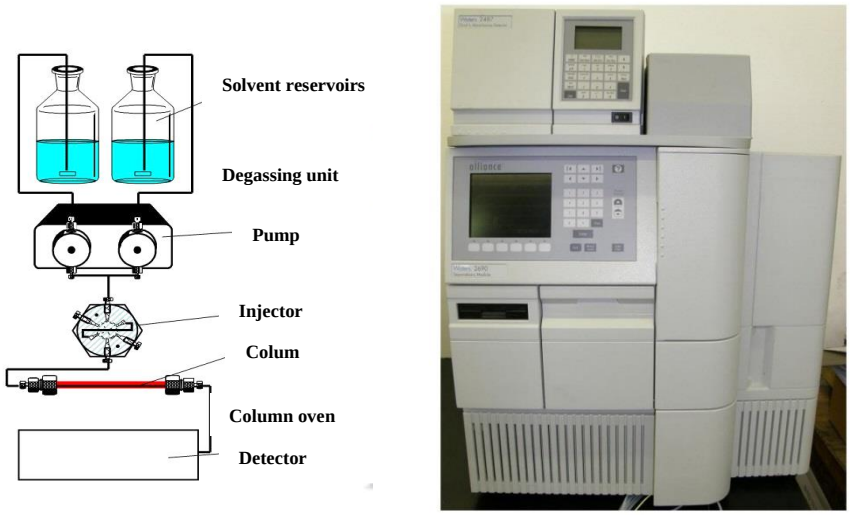


*Figure 2.4. Basic structural elements of a liquid chromatograph.*

A high pressure pump provides a strictly controlled and accurate flow from a solvent reservoir to the stationary phase (packed bed in the column). In most cases, this flow is generated by a reciprocating piston pump. High pressures need to be generated, *i.e.* 400-600 bar for HPLC systems and up to 1500 bar for UHPLC systems. An Alliance 2690 HPLC from Waters (Asse, Belgium) was routinely used during this research (Figure 2.4).

Subsequently the sample is injected into the liquid stream by means of a six-way valve. The sample loop has a specific volume, ranging from 1 to 100 µL. Furthermore all capillary tubes

must be kept short and with an as narrow as possible internal diameter to minimize extra peak broadening effects, detrimental for the resolution, as explained earlier and observed during this research. The Alliance 2690 was equipped with an autosampler, to allow samples to be injected in a fast and reproducible way.

Stainless steel columns comprise the vast majority of the HPLC columns because these columns are easy to handle and their inherent pressure resistance. Column dimensions vary from 5 to 50 cm in length (L), and 0.1 to 5 cm internal diameter (i.d.). The workhorse in analytical liquid chromatography for HPLC systems limited to 400 bar, like the system used in this research, is a 250 mm x 4.6 mm i.d. column, packed with 5 µm silica porous particles. As described earlier the L, $d_p$ and i.d. have a direct impact on the resolution of the separation. Efficiency can be increased by increasing column length L or by decreasing particle diameter $d_p$, limited by the pressure drop over the column as explained earlier.

Nowadays, one of the goals in analytical work is miniaturization of the internal diameters of the columns. The possible advantages of miniaturization were tested in this thesis and implemented, including less solvent consumption (green chemistry), improved compatibility with mass spectrometry (MS), and increased sensitivity in particular with concentration sensitive detectors. The sensitivity on a 2 mm i.d. column increases by a factor 4 in comparison with a 4 mm i.d. column because less dilution for concentration sensitive detectors. The different stationary phases were discussed above.

When the analytes of interest are separated, adequate detection with sufficient sensitivity and specificity is necessary. Only the detectors applied in this research are explained and discussed. The UV-detector is broadly used to measure components depicting an absorption spectrum in the ultraviolet region (typically between 190 to 400 nm). Note that not all compounds show UV-absorption, and that extinction coefficients differ greatly, resulting in an often too low sensitivity towards some compounds. The sensitivity and linear dynamic range are thus strongly component-dependent. The wavelength can be expanded to the region of visible light (VIS) for the detection of colored analytes. During the detection process, the UV transparent mobile phase is pumped through a microcuvette (flow-cell) with a volume of 1 to 10 µL. UV spectrometry is based on the comparison of incident light intensity ($I_O$) and emergent light intensity (I) after passing through the effluent. The absorbance (A) is directly

proportional to c, the concentration of the absorbing analyte in solution, if the path length (d) of the cell is held constant. This relationship is expressed by the Lambert-Beer law: $A = \varepsilon.c.d$ with $\varepsilon$ the molar absorptivity (depending on the wavelength) and $A = \log I_O/I$.

The UV detectors can be subdivided into three types: fixed wavelength, variable wavelength and photodiode-array. Fixed wavelength detectors have a specific discharge lamp that generates (nearly) monochromatic light. Typical examples are the mercury vapor lamp (254 nm) and the cadmium lamp (229 nm). These detectors are only rarely used today and have been replaced by variable wavelength and diode array detectors. In a variable wavelength detector (Figure 2.5), a deuterium lamp which emits a broad spectrum ranging from 180 to 750 nm, is used. The grating is in a rotational position and can be set to obtain the desired wavelength. The intensity of the light beam that passes through the flow cell is then measured with a sampling diode. This detector allows collecting the UV absorbance signal at several wavelengths simultaneously. In the early stages of this research, UV detection was applied to test the different standards available on the market and the performance of several HPLC columns. Sensitivity was however insufficient for the cyclic nucleotides, other detection systems had to be used.

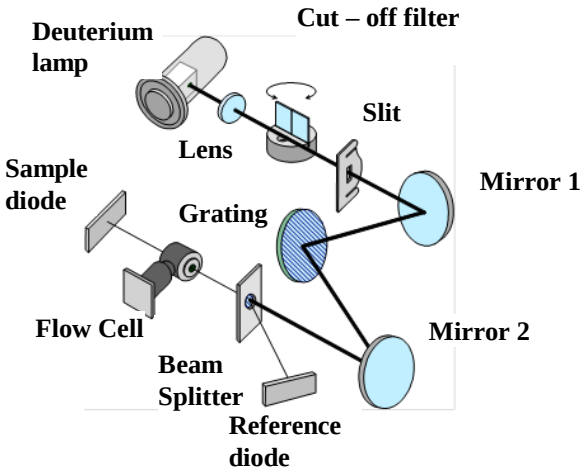


*Figure 2.5. Schematic drawing of a variable wavelength detector.*

When a photodiode array detector (DAD) is used, the light emitted by a deuterium lamp (full range of wavelength) is sent directly through the flow cell and only then the light is diffracted by a grating into the composing wavelengths which are all individually monitored via an array of diodes. The detector allows collecting complete UV absorbance spectra for each peak

eluting from the column. This information can be used for spectral identification of a compound or for comparison with UV spectral libraries. The detector also allows for fast selection of the most suitable wavelength for selective detection of a molecule.

Another detector briefly used in this work is the fluorescence detector (FL). In this detection technique a substance absorbs light to reach a high-energy level and then emits light, called fluorescence, to return to its original level. Light with a wavelength of $\lambda_{ex}$ (excitation wavelength) is beamed into the detection cell and the light emitted by the component with a longer wavelength, $\lambda_{em}$, is recorded. Components emitting fluorescence can be detected with higher sensitivity and selectivity compared to UV-VIS and DAD.

### 2.2.4 Integrating LC and MS

Mass spectrometry was extensively used in this work and is therefore explained more in detail in the section below.

#### 2.2.4.1 Historical background

The basic principles of mass spectrometry (MS) were first described by Thomson during a lecture for the Cambridge Philosophical Society in 1897, more than hundred years ago. The first experimental validation followed in 1913 when he applied a magnetic and an electrical field to a stream of ionized neon gas and separation into two isotopic components with masses of 20 and 22 was taking place. Rapid technical development followed in the next decades, with the coupling of gas chromatography to MS (GC-MS) in the 1950s. The coupling of MS with LC (LC-MS) was the next logical step, but suffered from the incompatibility of existing MS ion sources with a continuous liquid stream, like applied in direct liquid introduction and the moving belt interface [22-24]. If the LC is simply connected directly to the MS, the liquid mobile phase would vaporize, resulting in large amounts of gas being introduced into the MS unit. This would decrease the vacuum level and prevent the target ions from reaching the detector. Therefore a key issue in LC-MS was the removal of the mobile phase. This situation changed radically in the late 1980s when John Fenn developed the electrospray ionization source (ESI) [25] and some key technologies that allowed easy sample introduction into the mass spectrometer. He was awarded the Nobel Prize in 2002 for this pioneering research, together with Koichi Tanaka who developed matrix-assisted laser desorption/ionization,

MALDI, in 1987 [26]. ESI, is extensively used in this thesis and will be explained more in detail in this chapter. This development had great impact on protein and peptide biochemistry. Manufactures rapidly developed instruments equipped with electrospray sources, and in the early 1990s the performance of LC-MS instruments greatly improved at a substantial reduced cost. LC-MS is now a routine and robust technique that can be applied to a wide range of biological molecules.

#### 2.2.4.2 Introduction to mass spectrometry

The reason to choose LC-MS over LC with conventional detectors includes the high specificity and sensitivity that can be obtained and the ability to identify and quantify a broad variety of solutes. Acceptable volumes can be used and complex biological matrices can be investigated, necessary for this research. This technique proved its power in pharmaceutical and biochemical studies. Over the years, a lot of validated methods were developed and published. Even higher selectivity and sensitivity is obtained when tandem LC-MS (LC-MS/MS) is used.

In mass spectrometric techniques, gas phase ions are separated according to their mass-to-charge ratio *m/z* (under high vacuum) and subsequently detected. A mass spectrometer consists essentially of three zones:

(1) ***an ion production zone***, a sample inlet device (ionization source) that ionizes the solutes of interest and transfer these ions from solution into the gaseous phase, before they are subjected to mass spectrometric differentiation.
(2) ***a mass analyzer***, an ion path from atmospheric pressure of the source into the high vacuum of the actual mass analyser, where ions are separated according to their mass to charge ratio *m/z*.
(3) ***ion detection***, to detect and quantify the separated ions.

Several combinations of these components result in many different types of mass spectrometers. Only the most relevant applied in this research will be discussed below.

#### 2.2.4.3 Ionization mechanisms

A key issue in LC-MS is the removal of the liquid mobile phase and the ionization of the solutes of interest. Various interfaces have been developed. The most successful are based on

atmospheric pressure ionization and include electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI).

ESI uses an electrical potential difference to assist the transfer of ions from solution into the gaseous phase before they are subjected to mass spectrometric analysis. The process can be divided into three stages: dispersal of a fine spray of charged droplets, droplet shrinkage and gaseous ion formation (Figure 2.6). A continuous stream of sample solution is passed through a capillary tube, which is maintained at a high voltage (3-5 kV) relative to a counter electrode. The capillary is most often placed orthogonally to the entrance of the mass spectrometer (charged face plate) in order to minimize contamination. Evaporation of the solvent from the initially formed droplet, by applying heat and dry nitrogen gas, called pneumatically assisted ESI, leads to a reduction in diameter, and an increase in surface electric field, until the Rayleigh limit is reached. A coulomb explosion occurs, as the magnitude of the charge is sufficient to overcome the surface tension holding the droplet together. The resulting instability disperses the droplet into a collection of smaller droplets, that continues to evaporate until they reach the Rayleigh limit and disintegrate. Several mechanisms have been proposed by which solute ions are formed from charged droplets [27,28]. Dole and his research group proposed the 'charge residue model' (CRM), a continuation of the process explained above, that will result in the formation of an ion containing a single analyte molecule. The molecule retains some of its droplets charge to become a free ion as the last of the solvent vaporizes [29]. Another mechanism of ion formation, the ion desorption model (IDM) is based on the work of Iribarne and Thomson. This model assumes that before a droplet reaches the ultimate stage its surface electric field becomes sufficiently large to lift an analyte ion at the surface of the droplet over the energy barrier that prevents its escape [30,31].

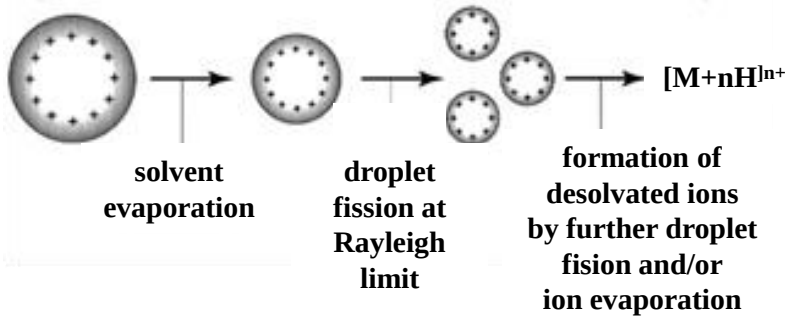


*Figure 2.6. Ion formation from the liquid phase into the gaseous phase in the electrospray interface.*

The individual solute ions then pass through on oppositely charged orifice in the high vacuum zone of the mass spectrometer. ESI is a soft ionization source, because relatively low energy is imparted to the analytes, and hence little fragmentation occurs. ESI is used for the ionization of medium to highly polar compounds, so the ionization source of choice for the analysis of polar organic second messengers in this thesis. Since ESI sometimes produces multiple charged ions, it is a powerful technique for the analysis of biopolymers, such as peptides and proteins. When the ion source is operated in the positive mode the addition of a proton to the analyte $[M + H]^+$ will occur, or the loss of a proton $[M - H]^-$ when operated in negative mode. Note that not only proton transfer reactions occur, but adduct ion formation is commonly observed. Cation adducts such as $[M + NH_4]^+$, $[M + Na]^+$ and $[M + K]^+$ in positive mode and anions such as $[M + CH_3COO]^-$, $[M + HCOO]^-$, $[M + Cl]^-$, can be observed, even though these modifiers may not have been deliberately added to the solution containing the analytes. A representation of an ESI-MS interface is shown in Figure 2.7 and the MS instrument that was used during this thesis, an API 3000 from Applied Biosystems, is shown in Figure 2.8. Note that the setup of an ESI-MS interface differs for different vendors (*e.g.* Applied Biosystems, Agilent, Waters). Each of these vendors have specific patented technology incorporated in their ESI interface. Also the inlet voltages relative to a counter electrode and the position of the spray is different.

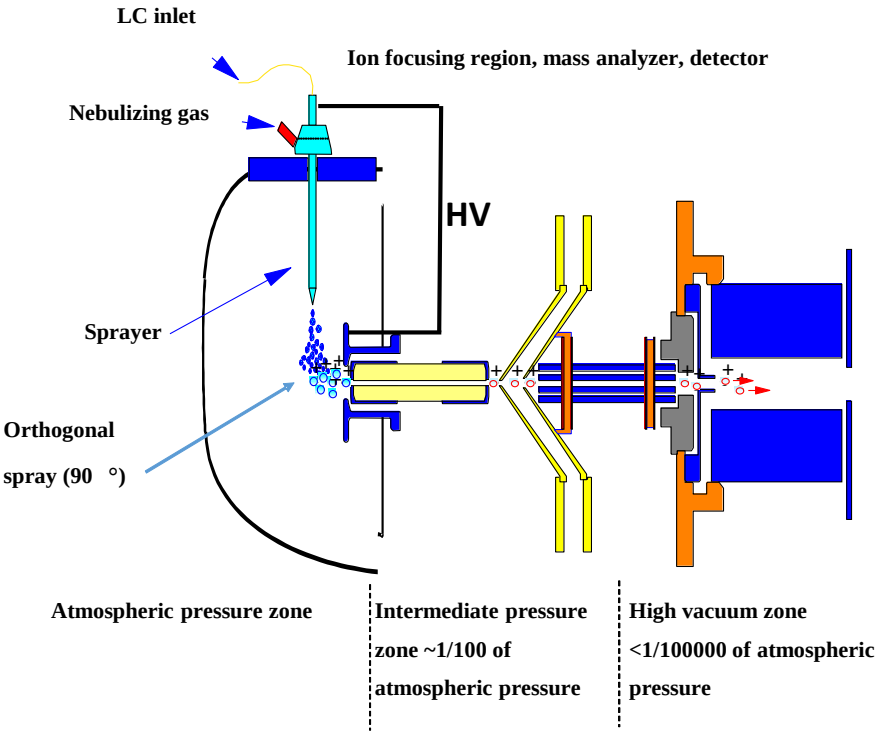


*Figure 2.7. Schematic drawing of an ESI-MS interface, indicating the ion production zone, with a potential difference between the sprayer and orifice, a specific spray needle and orthogonal spray position, and the mass analyzer, with the different pressure zones.*


*Figure 2.8. An illustration of an API 3000 mass spectrometer from Applied Biosystems, the MS/MS system applied during this thesis.*

Another atmospheric ionization technique is APCI, in which the liquid sample stream requires vaporization first, which is achieved by nebulization ($N_2$) and heating (350-550°C). The mechanism of APCI is based upon a high-current discharge of a corona needle, producing a high density of charges, which interact with surrounding molecules. As the solvent molecules are highly abundant, it is the primary charge carrier and results in charge transfer to the analyte molecules. APCI is a mass-sensitive technique, it is less flow rate depended compared to ESI. Best sensitivity is achieved at high flow rates, *i.e.* 200 µL – 1 ml/min, and a MS with APCI source is therefore easily interfaced to conventional HPLC. Analytes have to be thermally stable and volatile. APCI is effective for compounds of intermediate molecular weight and polarity, typically exhibiting a poor electrospray response. APCI can therefore be considered to be complimentary to ESI. Note that the cooling mechanism of the expanding solvent by adiabatic expansion occurring in the APCI source could counteract the effect of the (heated) sheath gas used in the source, opening up the possibility to perform APCI-MS on the polar solutes as well [32].

Phenomena like ion suppression and ion pairing effect the analyte ionization and so the analyte sensitivity. They have a detrimental impact on the analysis of molecules in biological matrices with MS, in particular with soft ionization sources like ESI. In this ion suppression process, matrix components, ubiquitously present in biological samples, tend to out-compete the solute of interest for ionization. This results in a decreased number of solute ions, characteristic for the analyte, leading to a lower sensitivity. This phenomena is explained and studied more in detail in chapter 5. Also ion pairing reagents can ionize and create a high background. Strong ion pairing with the analyte can thereby prevent the analyte from ionizing effectively.

#### 2.2.4.4 Mass separators

Differentiation according to mass-to-charge ratios can be performed by various mass separators including a quadrupole, a time of flight and an ion trap. The gaseous solute ions are thereby accelerated in electrical fields and subsequently reach the mass analyzer. The quadrupole analyser is used most often because of its simplicity and robustness, and is the mass separator of choice for the analysis of cyclic nucleotides in biological matrices.

A quadrupole consists of a set of four parallel metal rods. By continually changing the electrical field of the quadrupole, determined by constant (direct current $D_c$) and varying (radio frequency $R_f$) voltages, the fragments are registered one by one in order to their *m/z* ratios by an electron multiplier. This continuous change of mass is called a scan. The scanning of ions for a certain molecule (*m/z* 20-400) can occur within 1s to 10 ms, and the spectrum is stored in the memory of a computer. A mass spectrum on the x-axis is characterized by the specific ions and on the y-axis by their intensity. The spectrum of the peak can then subsequently be called upon for identification. In this full scan, the ions are formed continuously but are not continuously detected because of the limited dwell time and the necessary cycle time. The dwell time is the time the instrument measures one transition (ion). The cycle time is the time it takes to acquire all transitions. Data points are created, which are connected by a line, creating a peak in the MS-based chromatogram. An optimal amount of data points over one peak needs to be selected. The more transitions selected to be measured, the less data points can be generated because of this limited dwell and cycle time, causing a lower sensitivity of analysis. Full scan operation is however a necessity for identification.

To increase the sensitivity of detection, the quadrupoles can be set to monitor a specific *m/z* value, then set to monitor another *m/z* value, and so on, by stepping the voltages. This technique, called selected ion monitoring (SIM), is useful in improving the sensitivity of targeted analytes because a specific mass (ion) can be measured longer during a scan.

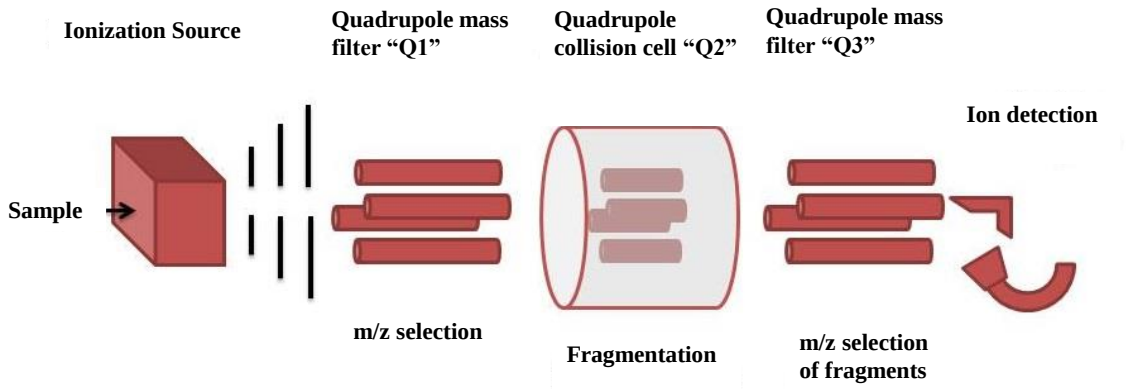


*Figure 2.9. Triple quadrupole mass spectrometer, with the three quadrupole analyser in series.*

A common configuration of a quadrupole analyser for selective and sensitive quantitative analysis is the triple quadrupole mass spectrometer, consisting of three quadrupoles in series, allowing fragmentation of the analyte ions of interest, also called precursor ions (Figure 2.9). The precursor ions are thereby mass selected by the first quadrupole (Q1) and allowed to collide with a collision gas (usually argon or nitrogen) in a second, $R_f$ only, quadrupole collision cell (Q2), where the precursor ions are activated by repeated collisions and undergo further fragmentation. This process is known as collision-induced dissociation (CID). In CID, a strong electric field accelerates ions into a neutral collision gas (typically He, $N_2$, or Ar), and the precursor ions are activated by repeated collisions with the collision gas. The high kinetic energy of the fast-moving precursor ion is converted to internal energy, thereby increasing the Boltzmann temperature and eventually breaking the weakest covalent chemical bonds. The resulting fragment ions are extracted from the collision chamber and injected into a mass analyzer. The product ions, resulting from CID, are related to the molecular structure of the ions and can be monitored by a third quadrupole mass analyser (Q3), providing structural elemental information of the molecular ions. As this can be done very selective, this type of LC-MS/MS detection allows for reliable and sensitive analysis and quantification of very specific molecules in complex matrices like blood or animal tissue.

Triple quadrupole (QQQ) mass spectrometers can be operated in several operational modes. The first one is called ‘full scan’. A scan is performed across the mass range of both mass analyzers Q1 and Q3, while Q2 contains no collision gas. All generated ions can be detected. A second mode is the ‘Product Ion Scan’, in which one specific *m/z* is selected in Q1 (= precursor ion), Q2 is filled with collision gas to fragment the selected *m/z*, and a scan is done across the mass range of Q3. All fragments (= product ions) of the selected precursor ion are monitored. In another mode, a scan is performed in Q1, while in Q2 all ions in the scanned range are fragmented, and one specific *m/z* is selected in Q3, determining which *m/z* precursor ion(s) generated the selected product ion. This mode is called ‘Precursor Ion Scan’. In the ‘Neutral Loss Scan’, a scan is performed in Q1, Q2 is again used to fragment all ions in the scan range, and Q3 is scanned across a predetermined range that corresponds to a fragmentation-induced loss of one specific mass having occurred for every potential ion in the precursor scan range. This experiment allows identification of all precursors that have lost a selected common chemical group. The most sensitive mode, and routinely applied in this

thesis, is the 'Multiple Reaction Monitoring (MRM)' mode. One specific *m/z* is selected in Q1 and is fragmented in Q2. In Q3 one specific *m/z* of the generated fragments is selected. This operation mode indicates the real strength of tandem MS, allowing highly selective and sensitive detection of an analyte, by analyzing one specific precursor ion and one specific product ion, generated by fragmentation. The first three of these modes are routinely used during method development, (i) to identify the precursor ion *m/z* of a molecule of interest in Full Scan, (ii) to determine the product ion mass to charge ratio of it fragments in the Product Ion Scan, and (iii) to confirm that in a biological matrix only the molecule of interest gives rise to the *m/z* product ion with a Precursor Ion Scan. The final method will be a Multiple Reaction Monitoring (MRM) in most situations, combining high analytical specificity with high analytical sensitivity as discussed earlier. This will be the operational mode of choice in this thesis, necessary for the study of cyclic nucleotides in representative biological matrices.

Another analyser operates by accelerating ions through a high voltage, called a time-of-flight (TOF) analyser. The velocity of the ions, and hence the time taken to travel down a flight tube to reach the detector, depends on their *m/z* values. If the initial accelerating voltage is pulsed, the output of the detector as a function of time can be converted into a mass spectrum. The TOF analyser can acquire spectra extremely fast with high sensitivity. It also allows for high mass accuracy, allowing elemental molecular formulas to be determined for small molecules.

Some modification of the LC separation may be desirable to improve MS assay performance. The flow rate, mobile phase composition and injection method are some of these parameters that can (and must) be modified for optimal LC-MS results.

## 2.3 Biochemical strategies for secondary metabolite analysis

### 2.3.1 Historical background

Immunoassays developed in the late 1950s by Yalow and Berson, incorporate the binding reaction of a target analyte, the antigen, with an antibody [33-35]. An antibody, also known as an immunoglobulin, is a large Y-shaped protein. The antibody recognizes a unique part of the antigen. The core concept of immunoassay is based on the specific 'antigen-antibody' binding reaction, like a 'lock and key', in a complex mixture of antigens (= analytes). A detectable

label is linked to the antibody to quantify the amount of antigen present in a sample. These labels can be radioactive isotopes or enzymes.

When radioactive isotopes are used as detectable labels, like beta and gamma radioactive isotopes of iodine-131 and later gamma radioactive isotopes of iodine (125-I), the term radio immunoassay is used. Radio immunoassays (RIA) were first described by Yalow and Berson in 1960 [36], comprising an in vitro assay that measures the presence of an antigen with very high sensitivity and specificity. Basically any biological substance for which a specific antibody exists can be measured, even in low concentrations. The target antigen is radioactively labeled and bound to a specific, known amount of antibodies. A (biological) sample is added to initiate a competitive reaction of the labeled antigens, and the unlabeled antigens in the sample with the specific antibodies. The competition for the antibodies will release a certain amount of labeled antigen. This amount is proportional to the ratio of labeled to unlabeled antigen. So as the concentration of unlabeled antigen is increased, more of it binds to the antibody, displacing the labeled variant. The bound antigens are then separated from the unbound ones, and the radioactivity of the bound or free antigens remaining in the supernatant is measured. RIAs were some of the earlier developed immunoassays and have been the first immunoassay techniques developed to analyze nanomolar and picomolar concentrations of hormones in biological fluids.

The unique ability of RIA to measure small molecules can be achieved in many cases by non-radioactive methods. Current assays and studies make use of enzymes as labels, where the antigen-antibody reaction is measured using colorimetric methods, such as absorbance (UV) or fluorescence (FLU) intensity. These biochemical strategies, in particular enzyme-linked immunosorbent assay (ELISA), are commonly used for secondary metabolite analysis and therefore the principles and some frequently used ELISA techniques are explained.

### 2.3.2 ELISA: enzyme-linked immunosorbent assay

Immunoassays that use enzymes as detectable labels are called enzyme-linked immunosorbent assay (ELISA), also known as an enzyme immunoassay (EIA). These assays were developed independently and simultaneously by Engvall and Perlmann [37], and by van Weemen and Schuurs [38] in 1971. Both techniques differed in assay design but are based on the same principle, immunoassay with an enzyme rather than radioactivity as reporter label.

The immunoassays were developed as a replacement of radio immunoassays, presenting great concerns towards the safety of laboratory personnel, the radioactive waste, expensive counting equipment and the necessity of building special laboratory facilities. ELISA is a biochemical, plate based assay that uses the basic immunology concept of an antigen binding to its specific antibody and an enzyme-mediated color change to detect the biological molecules. Different types of ELISA exist. They are typically based on the immobilization, mostly on 96 wells microtiter plates, of an antigen in fluid phase to a solid phase, specific connection with an antibody and subsequent detection by a secondary, enzyme-coupled antibody (Figure 2.11). A visible color change or fluorescence will allow to quantify the analytes of interest. A number of commercialized EIA/ELISA test kits are available that can be fully automated nowadays [39-44]. In this thesis a Thermo Scientific Cyclic GMP Competitive ELISA Kit, a 96-well plate from Thermo Fischer Scientific (Erembodegem, Belgium), was used to analyze 3',5'-cGMP in human plasma samples. Theoretical sensitivity of the method was ~1 pmol/mL. This test allowed quantifying 3',5'-cGMP independently of other cyclic nucleotides such as 3',5'-cAMP, 2',3'-cGMP and 2',3'-cAMP. Other, similar ELISA kits have to be purchased to obtain info on these other second messenger molecules, because it is a single solute approach.

The terms direct and indirect are commonly used to describe various ELISA methodologies and refer to the differences in the way antigens are captured or immobilized on the well of a microplate and how the captured antigen is detected.

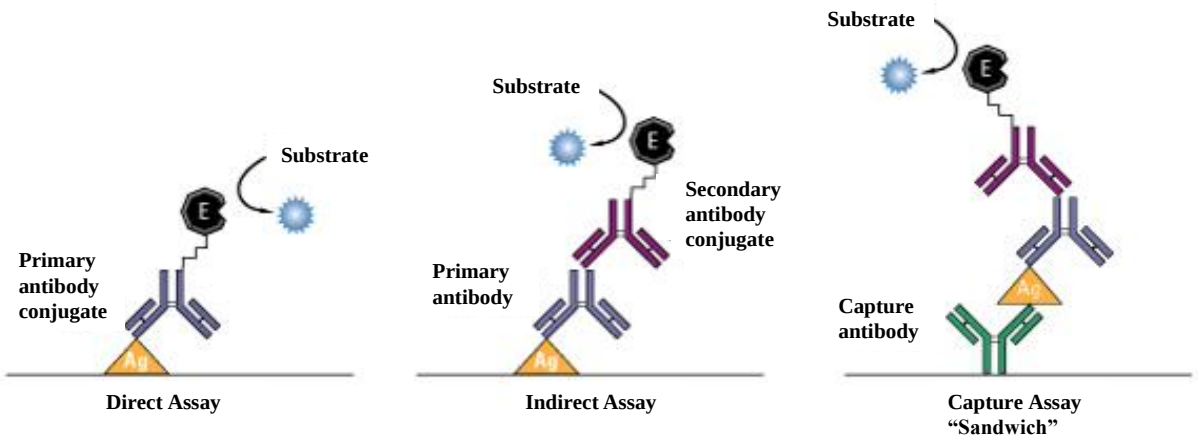


*Figure 2.10. Common ELISA formats, including direct assay, indirect assay and capture assay 'sandwich'.*

The key step, immobilization of the antigen of interest, can be accomplished by direct- or indirect absorption. In direct absorption, the antigen of interest is immobilized directly to the assay plate. Detection of the specific antigen can be performed using an enzyme-conjugated primary antibody, called direct detection, or a matched set of unlabeled primary antibody and conjugated secondary antibodies, called indirect detection, see Figure 2.10.

'Sandwich' ELISA is commonly used to refer to assays involving indirect absorption and indirect detection, since the antigen is sandwiched in between two specific antibodies (Figure 2.10). Most commercial ELISA kits are 'Sandwich' ELISAs. A known amount of antibody, that will couple to the specific antigen of interest, is added to the well surface. After the nonspecific binding sites are blocked, *i.e.* the remaining areas of the wells not coated with the antigen, the antigen-containing sample (*e.g.* blood, serum, urine) is applied to the plate. A specific primary antibody is then introduced that "sandwiches" the antigen, followed by enzyme-conjugated secondary antibodies that bind to the primary antibody. Unbound antibody–enzyme conjugates are washed away. A substrate for the enzyme is added to quantify the primary antibody through a color change. The concentration of primary antibody present in the serum directly correlates with the intensity of the color.

Another technique is *competitive ELISA*, based on the competitive reaction of the sample antigen and add-in antigen, with the specific primary antibody. Different procedures for competitive ELISA have been developed. In one variation, the primary antibody is incubated to the antigen containing sample. The resulting specific antibody–antigen complexes are

added to wells that have been pre-coated with the same antigen (Figure 2.11). The unbound antibody is removed by washing the plate. Because competitive binding is concentration depended, the signal output is inversely correlated with the amount of antigen in the sample. The more antigen in the sample, the more primary antibody will be bound to the sample antigen and thus a smaller amount of primary antibody is available to bind to the antigen coated on the well. Secondary antibody conjugated to an enzyme is added, followed by a substrate that creates a color change.

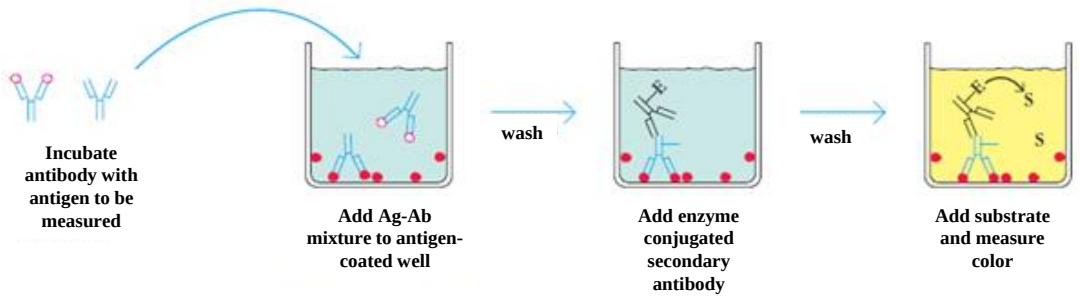


*Figure 2.11. Schematic overview of the different steps of competitive ELISA.*

In the last section of this chapter, a short overview of the published chromatographic and biochemical strategies for secondary metabolite analysis is described.

## 2.4 Overview of organic analytical approaches used and developed for the quantitation of secondary metabolites in biological samples

The first analyses on cAMP were performed by Sutherland *et al.* in the late 50s, early 60s [45-48]. The methods used to measure cyclic nucleotide concentrations in biological samples were based on the colorimetric estimation of pyruvate [49] or a double-isotope dilution and derivative analysis [50]. Also a two-step radioisotopic approach by Brooker *et al.* [51] was used to separate 5' nucleotides and cyclic nucleotides (cAMP and cGMP). The 5' nucleotide product of phosphodiesterase (PDE) action on the cyclic nucleotides was thereby dephosphorylated to the corresponding nucleosides using a 5' nucleotidase. The resulting reaction mixture was then separated on an appropriate anion exchange resin, with the cyclic nucleotides retained on the column and the nucleosides eluted. Quantification was done by

scintillation counting. This approach gives good precision and sensitivity but requires significant sample preparation and is therefore not particularly robust. White and Zenser described a method of purifying 3',5'-cyclic nucleotides by chromatography on columns of aluminum oxide [52]. The 5' nucleotides can be strongly retained by the alumina whereas the cyclic nucleotides are eluted at neutral pH. The method was refined by Alvarez and Daniels in 1990 by using another eluting buffer, *i.e.* ammonium acetate [53]. Also boronate chromatography was used to separate cAMP and AMP as described by Davis and Daly [54]. It is a form of cis-diol affinity chromatography, with its ability to bind 5'nucleotides that are cis-diols but not cyclic nucleotides.

Radioimmunoassay replaced these earlier approaches with a significant decrease in detection limit [55,56]. Health risks associated with the use of radioactively labeled compounds remained, as discussed earlier, an important issue. Other techniques for analyzing these cyclic nucleotides were therefore developed, including HPLC with fluorometric [57] and diode array detection (DAD) [58]. Also ion-pairing HPLC approaches with DAD detection and anion exchange column purification strategies were applied [59]. However relatively high detection limits and time consuming purification procedures of these methods remained an issue.

Therefore the enzyme-linked immunosorbent assay (ELISA) has been the most commonly used quantitative method for this type of nucleotides [60-63]. These assays provide high sensitivity and throughput capacity and have been used extensively for the quantification of cellular antigens and numerous molecules in biological fluids. However, these assays are single solute approaches. Also matrix interference in ELISA can occur, leading to lower precision and accuracy when measuring cGMP and cAMP in several biological samples.

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) is therefore a strong alternative due to its high sensitivity, selectivity and ruggedness. The application of LC-MS/MS for the determination of cyclic nucleotides has been well documented [64-69]. Individual cyclic nucleotides have been measured by LC-MS/MS in either human blood, animal tissues or plant extracts. Martens-Lobenhoffer *et al.* described the development of a weak anion exchange solid phase extraction (IEX SPE) method in combination with positive electrospray LC-MS/MS for the quantification of cGMP in human plasma [70]. Oeckl and

Ferger described an LC-MS/MS method for the analysis of both cGMP and cAMP [71]. The sample preparation included protein precipitation. However, from a pharmaceutical development perspective, the use of lower sample volumes is required, *e.g.* to allow quantification of cGMP and cAMP in animal samples as a function of the action of a given active pharmaceutical compound. Baehre *et al.* described a liquid chromatography-tandem mass spectrometry (LC-MS/MS) method for the measurement of four 2',3'-cyclic nucleotides in cells [72]. This method was proposed as an extension to the new developed method, described in chapter 3. Analytes were, however, only extracted after protein precipitation, with a mixture of acetonitrile, methanol and water. More LC-MS methods were developed to analyze these cyclic nucleotides in biological samples, revealing similar results [73-75]. Piec *et al.* developed in 2017 an LC-MS/MS method for the diagnostic measurement of cAMP in plasma and urine [76]. The assay performance was investigated in pharmacokinetic studies investigating the response to an oral dose of PTH (1-34) in rats and humans. Analytes were extracted from EDTA plasma using a weak anion exchange solid phase extraction. Positive electrospray ionization was used, in the MRM mode. Mean plasma cAMP concentrations of 36.5 ± 3.7 nmol/L were thereby measured, which is comparable to the 4.66 to 9.20 ng/mL concentration measured for 3',5'-cAMP in human plasma in chapter 3 [77].

## 2.5 References

[1] M. Tswett, " Adsorptionsanalyse und chromatographische Methode. Anwendung auf die Chemie des Chlorophylls," Berichte der Deutschen Botanischen Gesellschaft, vol. 24, pp. 384-393, 1906.

[2] C. Horvath, W. Melander, and I. Molnar, "Solvophobic interactions in liquid chromatography with nonpolar stationary phases," *Journal of Chromatography*, vol. 125, pp. 129-156, 1976.

[3] L. S. Ettre, "Nomenclature for chromatography," *Pure and Applied Chemistry*, vol. 65, pp. 819-872, Apr 1993.

[4] J. J. Vandeemter, F. J. Zuiderweg, and A. Klinkenberg, "Longitudinal diffusion and resistance to mass transfer as causes of nonideality in chromatography," *Chemical Engineering Science*, vol. 5, pp. 271-289, 1956.

[5] J. J. Kirkland, "Superficially porous silica microspheres for the fast high-performance liquid-chromatography of macromolecules, *Analytical Chemistry*, Article vol. 64, no. 11, pp. 1239-1245, Jun 1992.

[6] J. J. Zheng, D. Patel, Q. L. Tang, R. J. Markovich, and A. M. Rustum, "Comparison study of porous, fused-core, and monolithic silica-based C-18 HPLC columns for Celestoderm-V Ointment (R) analysis," *Journal of Pharmaceutical and Biomedical Analysis*, Article vol. 50, no. 5, pp. 815-822, Dec 2009.

[7] S. Fekete, J. Fekete, and K. Ganzler, "Shell and small particles; Evaluation of new column technology," *Journal of Pharmaceutical and Biomedical Analysis*, Article vol. 49, no. 1, pp. 64-71, Jan 2009.

[8] A. de Villiers, F. Lestremau, R. Szucs, S. Gelebart, F. David, and P. Sandra, "Evaluation of ultra performance liquid chromatography - Part I. Possibilities and limitations," *Journal of Chromatography A*, Article vol. 1127, no. 1-2, pp. 60-69, Sep 2006.

[9] A. Abrahim, M. Al-Sayah, P. Skrdla, Y. Bereznitski, Y. D. Chen, and N. J. Wu, "Practical comparison of 2.7 mu m fused-core silica particles and porous sub-2 mu m particles for fast separations in pharmaceutical process development," *Journal of Pharmaceutical and Biomedical Analysis*, Article vol. 51, no. 1, pp. 131-137, Jan 2010.

[10] M. Borowko and B. Oscik-Mendyk, "Adsorption model for retention in normal-phase liquid chromatography with ternary mobile phases," *Advances in Colloid and Interface Science*, vol. 118, pp. 113-124, Dec 2005.

[11] K. K. Unger, R. Ditz, E. Machtejevas, and R. Skudas, "Liquid Chromatography-Its Development and Key Role in Life Science Applications," *Angewandte Chemie-International Edition,* vol. 49, pp. 2300-2312, 2010.

[12] A. J. Alpert, "Hydrophilic interaction chromatography for the separation of peptides, nucleic acids and other polar compounds," *Journal of Chromatography*, vol. 499, pp. 177-196, Jan 1990.

[13] A. L. N. van Nuijs, I. Tarcomnicu, and A. Covaci, "Application of hydrophilic interaction chromatography for the analysis of polar contaminants in food and environmental samples," *Journal of Chromatography A,* vol. 1218, pp. 5964-5974, Sep 2011.

[14] P. Hemstrom and K. Irgum, "Hydrophilic interaction chromatography," *Journal of Separation Science*, vol. 29, pp. 1784-1821, Aug 2006.

[15] D. V. McCalley, "A study of column equilibration time in hydrophilic interactionchromatography," *Journal of Chromatography A,* vol. 1554, pp. 61-70, April 2018.

[16] D. L. Shollenberger and D. S. Bell, "Investigation of reequilibration in hydrophilicinteraction chromatography," *LC-GC (Europe)*, vol. 29, pp. 687–692, 2016.

[17] S. Louw, F. Lynen, M. Hanna-Brown, and P. Sandra, "High-efficiency hydrophilic interaction chromatography by coupling 25 cm x 4.6 mm ID x 5 microm silica columns and operation at 80 degrees C," *Journal of Chromatography A,* vol. 1217, pp. 514-521, Jan 2010.

[18] E. A. Peterson and H. A. Sober, "Chromatography of proteins. I. cellulose ion-exchange adsorbents," *Journal of the American Chemical Society,* vol. 78, pp. 751-755, 1956.

[19] P. B. Hamilton, "Ion exchange chromatography of amino acids. A single column, high resolving, fully automatic procedure," *Analytical Chemistry,* vol. 35, pp. 2055-2061, 1963.

[20] W. Ardelt and M. Laskowski, "Analytical ion-exchange chromatography of proteins", *Analytical Biochemistry,* vol. 120, pp. 198-203, 1982.

[21] U. D. Neue, HPLC Columns: Theory, Technology, and Practice, Wiley-VCH Weinheim, 1997.

[22] M. A. Baldwin and F. W. McLafferty, "Liquid chromatography-mass spectrometry interface–I: The direct introduction of liquid solutions into a chemical ionization mass spectrometer", *Organic Mass Spectrometry*, vol. 7, pp. 1111-1112, 1973.

[23] M. A. Baldwin and F. W. McLafferty, "Direct chemical ionization of relatively involatile samples: Application to underivatized oligopeptides", *Organic Mass Spectrometry,* vol. 7, pp. 1353-1354, 1973.

[24] W. H. McFadden, H. L. Schwartz, and S. Evans, "Direct analysis of liquid chromatographic effluents", *Journal of Chromatography A,* vol. 122, pp. 389-396, 1976.

[25] J. B. Fenn, M. Mann, C. K. Meng, S. F. Wong, and C. M. Whitehouse, "Electrospray ionization for mass spectrometry of large biomolecules," *Science,* vol. 246, pp. 64-71, Oct 1989.

[26] K. Tanaka, H. Waki, Y. Ido, S. Akita, Y. Yoshida, T. Yoshida, and T. Matsuo, "Protein and polymer analysis up to *m/z* 100000 by laser ionization time-of-flight", *Rapid communications in mass spectrometry*, vol. 2, pp. 151-158, 1988.

[27] A. Wortmann, A. Kistler-Momotova, R. Zenobi, M. C. Heine, O. Wilhelm, and S. E. Pratsinis, "Shrinking droplets in electrospray ionization and their influence on chemical equilibria," *Journal of the American Society for Mass Spectrometry,* Article vol. 18, no. 3, pp. 385-393, Mar 2007.

[28] E. de Hoffmann and V. Stroobant, Mass Spectrometry: Principles and Applications, Wiley-VCH Weinheim, 2007.

[29] M. Dole, L. L. Mack, and R. L. Hines, "Molecular beams of macroions", *Journal of Chemical Physics*, vol. 49, pp. 2240-2249, 1968.

[30] J. V. Iribarne and B. A. Thomson, "On the evaporation of small ions from charged droplets", *Journal of Chemical Physics*, vol. 64, pp. 2287-2294, 1976.

[31] B. A. Thomson and J. V. Iribarne, "Field-induced ion evaporation from liquid surface at atmospheric-pressure", *Journal of Chemical Physics*, vol. 71, pp. 4451-4463, 1979.

[32] M. Tsuchiya, "Development, principle and application of liquid ionization mass spectrometry," *Analytical Sciences,* vol. 14, pp. 661-676, Aug 1998.

[33] S. A. Berson and R. S. Yalow, "Quantitative aspects of the reaction between insulin and insulin-binding antibody," *Journal of Clinical Investigation,* vol. 38, pp. 1996-2016, 1959.

[34] R. S. Yalow and S. A. Berson, "Assay of plasma insulin in human subjects by immunological methods," *Nature*, vol. 184, pp. 1648-1649, 1959.

[35] S. A. Berson and R. S. Yalow, "Species-specificity of human anti-beef, pork insulin serum," *Journal of Clinical Investigation*, vol. 38, pp. 2017-2025, 1959.

[36] R. S. Yalow and S. A. Berson, "Immunoassay of endogenous plasma insulin in man," *Journal of Clinical Investigation*, vol. 39, pp. 1157-1175, 1960.

[37] E. Engvall and P. Perlmann, "Enzyme-linked immunosorbent assay (ELISA). Quantitative assay of immunoglobulin-G," *Immunochemistry*, vol. 8, pp. 871-874, 1971.

[38] B. K. Van Weeme and A. H. W. Schuurs, "Immunoassay using antigen-enzyme conjugates," *Febs Letters*, vol. 15, pp. 232-236, 1971.

[39] R. I. Henkin, I. Velicu, and A. Papathanassiu, "cAMP and cGMP in human parotid saliva: Relationships to taste and smell dysfunction, gender, and age," *American Journal of the Medical Sciences*, Review vol. 334, no. 6, pp. 431-440, Dec 2007.

[40] A. Keilbach, P. Ruth, and F. Hofmann, "Detection of cGMP dependent protein-kinase isozymes by specific antibodies," *European Journal of Biochemistry*, vol. 208, no. 2, pp. 467-473, Sep 1992.

[41] A. Voller, A. Bartlett, and D. E. Bidwell, "Enzyme immunoassays with special reference to ELISA techniques," *Journal of Clinical Pathology*, Review vol. 31, no. 6, pp. 507-520, 1978.

[42] X. Y. Jiang, D. Fuller, Y. H. P. Hsieh, and Q. C. Rao, "Monoclonal antibody-based ELISA for the quantification of porcine hemoglobin in meat products," *Food Chemistry*, vol. 250, pp. 170-179, Jun 2018.

[43] Y. Zhang, D. Dufield, J. Klover, W. L. Li, G. Szekely-Klepser, C. Lepsy, and N. Sadagopan, "Cross-validation of LC-MS/MS and ELISA techniques for the quantification of plamsa cyclic guanosine 3',5'-monophosphate (cGMP) for clinical applications," *Drug Metabolism Reviews*, Meeting Abstract vol. 40, pp. 40-40, 2008.

[44] A. G. Remacle, J. Dolkas, M. Angert, S. K. Hullugundi, A. V. Chernov, R. C. W. Jones, V. I. Shubayev, and A. Y. Strongin, "A sensitive and selective ELISA methodology quantifies a demyelination marker in experimental and clinical samples," Journal of Immunological Methods, vol. 455, pp. 80-87, Apr 2018.

[45] T. W. Rall, E. W. Sutherland, and J. Berthet, "The relationship of epinephrine and glucagon to liver phosphorylase: Effect of epinephrine and glucagon on the reactivation of phosphorylase in liver homogenates," *Journal of Biological Chemistry*, vol. 224, no. 1, pp. 463-475, 1957.

[46] E. W. Sutherland and T. W. Rall, "The properties of an adenine ribonucleotide produced with cellular particles, ATP, $Mg^{++}$, and epinephrine or glucagon," *Journal of the American Chemical Society,* vol. 79, pp. 3608-3608, 1957.

[47] T. W. Rall and E. W. Sutherland, "Formation of a cyclic adenine ribonucleotide by tissue particles," *Journal of Biological Chemistry,* vol. 232, pp. 1065-1076, 1958.

[48] E. W. Sutherland and T. W. Rall, "Fractionation and characterization of a cyclic adenine ribonucleotide formed by tissue particles," *Journal of Biological Chemistry,* vol. 232, pp. 1077-1091, 1958.

[49] S. Grisolia, L. C. Mokrasch, and V. D. Hospelhorn, "Adenosine mono-, di- and triphosphate, pyruvic kinase, hexokinase and polynucleotide phosphorylase assay," *Biochimica Et Biophysica Acta,* vol. 28, pp. 350-354, 1958.

[50] G. L. Pauk and W. J. Reddy, "Measurement of adenosine 3',5'-monophosphate," *Analytical Biochemistry,* vol. 21, pp. 298-307, 1967.

[51] G. Brooker, L. J. Thomas, and M. M. Appleman, "Assay of adenosine 3',5'-cyclic monophosphate and quanosine 3',5'-cyclic monophosphate in biological materials by enzymatic radioisotopic displacement," *Biochemistry,* vol. 7, pp. 4177-4181, 1968.

[52] A. A. White and T. V. Zenser, "Separation of cyclic 3',5'-nucleoside monophosphates from other nucleotides on aluminum oxide columns - Application to assay of adenyl cyclase and guanyl cyclase," *Analytical Biochemistry,* vol. 41, pp. 372-396, 1971.

[53] R. Alvarez and D. V. Daniels, "A single column method for the assay of adenylate-cyclase," *Analytical Biochemistry,* vol. 187, pp. 98-103, May 1990.

[54] C. W. Davis and J. W. Daly, "Simple direct assay of 3',5'-cyclic nucleotide phosphodiesterase activity based on the use of polyacrylamide-boronate affinity gel chromatography," *Journal of Cyclic Nucleotide Research,* vol. 5, pp. 65-74, 1979.

[55] A. L. Steiner, D. M. Kipnis, R. Utiger, and C. Parker, "Radioimmunoassay for measurement of adenosine 3',5'-cyclic phosphate," *Proceedings of the National Academy of Sciences of the United States of America,* vol. 64, pp. 367-373, 1969.

[56] A. L. Steiner, R. E. Wehmann, C. W. Parker, and D. M. Kipnis, "Radioimmunoassay for measurement of cyclic nucleotides," *Advances in Cyclic Nucleotide Research,* vol. 2, pp. 51-61, 1972.

[57] W. J. Wojcik and N. H. Neff, "Adenosine measurement by a rapid HPLC-fluorometric method - induced changes of adenosine content in regions of rat brain," *Journal of Neurochemistry,* vol. 39, pp. 280-282, 1982.

[58] L. Roef, E. Witters, J. Gadeyne, J. Marcussen, R. P. Newton, and H. A. Van Onckelen, "Analysis of 3',5'-cAMP and adenylyl cyclase activity in higher plants using polyclonal chicken egg yolk antibodies," *Analytical Biochemistry,* vol. 233, pp. 188-196, Jan 1996.

[59] M. J. D. Enrich, J. A. Villamarin, J. I. R. Martinez, and I. Ibarguren, "Measurement of adenosine 3 ',5 '-cyclic monophosphate and guanosine 3 ',5 '-cyclic monophosphate in mussel (Mytilus galloprovincialis Lmk.) by high-performance liquid chromatography with diode array detection," *Analytical Biochemistry,* vol. 285, pp. 105-112, Oct 2000.

[60] M. Tsugawa, K. Moriwaki, S. Iida, H. Fujii, M. Gomi, S. Tarui, R. Yamane, and M. Fujimoto "An enzyme-linked-immunosorbent-assay (ELISA) for guanosine 3', 5'-cyclic monophosphate (cGMP) in human plasma and urine using monoclonal-antibody," *Journal of Immunoassay,* vol. 12, pp. 263-276, 1991.

[61] R. Z. Abraham, L. Kobzik, M. R. Moody, M. B. Reid, and J. S. Stamler, "Cyclic GMP is a second messenger by which nitric oxide inhibits diaphragm contraction," *Comparative Biochemistry and Physiology a-Molecular and Integrative Physiology,* vol. 119, pp. 177-183, Jan 1998.

[62] J. Wellard, B. Blind, and B. Hamprecht, "An enzyme-linked immunosorbent assay for the rapid quantification of intracellular and extracellular guanosine 3 ',5 '-cyclic monophosphate in cultured glial cells," *Neurochemical Research,* vol. 29, pp. 2177-2187, Nov 2004.

[63] R. D. D. Bianca, E. Mitidieri, V. Mirone, F. Fusco, C. Imbimbo, G. Cirino, and R. Sorrentino, "An *ex vivo* standardized assay to measure human platelet cGMP," *Journal of Pharmacological and Toxicological Methods,* vol. 64, pp. 164-167, Sep-Oct 2011.

[64] R. P. Newton, "Qualitative and quantitative MS analysis of cyclic nucleotides and related enzymes," *Biochemical Society Transactions,* vol. 24, pp. 938-943, Aug 1996.

[65] E. Witters, L. Roef, R. P. Newton, W. VanDongen, E. L. Esmans, and H. A. Van Onckelen, "Quantitation of cyclic nucleotides in biological samples by negative electrospray tandem mass spectrometry coupled to ion suppression liquid chromatography," *Rapid Communications in Mass Spectrometry,* vol. 10, pp. 225-231, 1996.

[66] Y. H. Zhang, D. Dufield, J. Klover, W. L. Li, G. Szekely-Klepser, C. Lepsy, and N. Sadagopan, "Development and validation of an LC-MS/MS method for quantification of cyclic guanosine 3 ',5 '-monophosphate (cGMP) in clinical applications: A comparison with a EIA method," *Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences,* vol. 877, pp. 513-520, Feb 2009.

[67] G. Liu, C. Butler, K. Long, R. Sarno, R. Solinga, K. Tang, Y. T. Chien, K. Carlson, and J. Segal, "Evaluation of methodologies to assess intracellular cGMP levels induced by soluble guanylate cyclase (sGC) stimulators in whole cells," *Nitric Oxide-Biology and Chemistry,* vol. 42, pp. 131-132, Nov 2014.

[68] R. Seifert, "cCMP and cUMP: emerging second messengers," *Trends in Biochemical Sciences,* vol. 40, pp. 8-15, Jan 2015.

[69] X. H. Ren, C. F. Wang, L. Wu, H. Y. Li, L. D. Chi, L. X. Sun, X. Fu, and J. Zhang, "Bioactivity assay of porcine relaxin based on cAMP accumulation in THP-1 cells quantified by LC-MS/MS," *Journal of Pharmaceutical and Biomedical Analysis,* vol. 111, pp. 320-323, Jul 2015.

[70] J. Martens-Lobenhoffer, C. Dautz, and S. M. Bode-Boger, "Improved method for the determination of cyclic guanosine monophosphate (cGMP) in human plasma by LC-MS/MS," *Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences,* vol. 878, pp. 487-491, Feb 2010.

[71] P. Oeckl and B. Ferger, "Simultaneous LC-MS/MS analysis of the biomarkers cAMP and cGMP in plasma, CSF and brain tissue," *Journal of Neuroscience Methods,* vol. 203, pp. 338-343, Jan 2012.

[72] H. Bahre and V. Kdever, "Measurement of 2 ',3 '-cyclic nucleotides by liquid chromatography-tandem mass spectrometry in cells," *Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences,* vol. 964, pp. 208-211, Aug 2014.

[73] S. Bustamante, R. B. Gilchrist, and D. Richani, "A sensitive method for the separation and quantification of low-level adenine nucleotides using porous graphitic carbon-based liquid chromatography and tandem mass spectrometry," *Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences,* vol. 1061, pp. 445-451, Sep 2017.

[74] J. Chen, A. Tabatabaei, D. Zook, Y. Wang, A. Danks, and K. Stauber, "A surrogate analyte-based liquid chromatography-tandem mass spectrometry method for the determination of endogenous cyclic nucleotides in rat brain," *Journal of Pharmaceutical and Biomedical Analysis,* vol. 146, pp. 361-368, Nov 2017.

[75] G. Bajraktari, J. Burhenne, P. Bugert, W. E. Haefeli, and J. Weiss, "Cyclic guanosine monophosphate modulates accumulation of phosphodiesterase 5 inhibitors in human platelets," *Biochemical Pharmacology,* vol. 145, pp. 54-63, Dec 2017.

[76] I. Piec, J. Dutton, S. Al-Riyami, C. Washbourne, I. Schoenmakers, H. Galitzer, G. Burshtein, P. Schwartz, J. Tang, and W. Fraser, "A LC-MS/MS method for the

diagnostic measurement of cAMP in plasma and urine," *Journal of Bone and Mineral Research*, vol. 32, pp. S172-S172, Dec 2017.

[77] T. Van Damme, Y. H. Zhang, F. Lynen, and P. Sandra, "Determination of cyclic guanosine- and cyclic adenosine monophosphate (cGMP and cAMP) in human plasma and animal tissues by solid phase extraction on silica and liquid chromatography-triple quadrupole mass spectrometry," *Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences*, vol. 909, pp. 14-21, Nov 2012.

# Chapter 3. Determination of cyclic guanosine- and cyclic adenosine monophosphate (cGMP and cAMP) in human plasma and animal tissues by solid phase extraction on silica and liquid chromatography – triple quadrupole mass spectrometry

Adapted from T. Van Damme, Y. H. Zhang, F. Lynen, and P. Sandra, "Determination of cyclic guanosine- and cyclic adenosine monophosphate (cGMP and cAMP) in human plasma and animal tissues by solid phase extraction on silica and liquid chromatography-triple quadrupole mass spectrometry," *Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences,* vol. 909, pp. 14-21, Nov 2012. The practical work related to this chapter has been entirely performed by Thomas Van Damme.

## *Summary*

In this chapter the analysis of essential second messenger molecules 3',5'-cyclic guanosine monophosphate (cGMP) and 3',5'-cyclic adenosine monophosphate (cAMP) in human plasma and animal tissue is described. They are involved in signal transduction within cells, in physiological functions such as neurotransmission and in the modulation of cell growth and differentiation of organisms, respectively. A quantitative solid phase extraction method (SPE) based on hydrophilic interaction on silica was developed and applied to both plasma and tissue samples. The stable isotope-labeled internal standards $^{2}D_{1}{}^{15}N_{3}$-3',5'-cGMP and $^{13}C_{10},{}^{15}N_{5}$-3',5'-cAMP were added prior to the sample preparation to ensure high precision and accuracy. The samples were analyzed using reversed phase liquid chromatography (RPLC). Negative ion electrospray (ESI)-MS/MS was used to selectively monitor several transitions of each metabolite. The method for the analysis of 3',5'-cAMP and 3',5'-cGMP in plasma was validated in the range of 0.15-20 ng/mL ($R^2$= 0.9996 and 0.9994 for 3',5'-cAMP and 3',5'-cGMP, respectively). Basal plasma concentrations for fifteen healthy human patients determined with this method varied between 4.66 – 9.20 ng/mL for 3',5'-cAMP and between 0.30 – 1.20 ng/mL for 3',5'-cGMP, with precisions better than 9.1%. 3',5'-cGMP and 3',5'-cAMP together with their 2',3'-isomers were also determined in animal tissues and estimated concentrations were included. The structures of the isomers were confirmed by

analysis with LC-high resolution time-of-flight MS and subsequently by comparison of retention times with standards.

## 3.1 Introduction

As mentioned in chapter 2, until 1970, concentrations of cyclic nucleotides in biological samples were measured by rather complex experimental approaches [1]. Radioimmunoassay subsequently replaced these approaches with significant increases in detection limit [2-4]. Other techniques for analyzing these cyclic nucleotides were developed, including liquid chromatography (LC) with fluorescence [5] and photo diode array detection (DAD) [6]. Also ion-pair LC with DAD detection after fractionation of the sample on an anion exchange column was reported [7]. The low sensitivity and time-consuming purification procedures of these methods, limited the use of LC. Presently the enzyme-linked immunosorbent assays (ELISA) are still the most commonly used quantitative method for this type of nucleotides [8-11]. ELISA has been used extensively for the quantification of cellular antigens and numerous molecules in biological fluids. Although the specificity and the cost of ELISA analysis thereby involved are adequate, the method is increasingly hindered by its single solute approach. As more and more metabolites require monitoring, sensitive, specific and rugged LC-MS/MS methods are developed. LC-MS/MS has been applied for the determination of cyclic nucleotides in biological samples [12-14]. Martens-Lobenhoffer *et al.* described the development of a weak anion exchange solid phase extraction (IEX SPE) method in combination with positive electrospray LC-MS/MS for the quantification of 3’,5’-cGMP in human plasma [15]. Oeckl *et al.* described an LC-MS/MS method for the analysis of both 3’,5’-cAMP and 3’,5’-cGMP in plasma, cerebrospinal fluid (CSF) and brain tissue [16, 17]. 3’,5’-cAMP and 3’,5’-cGMP were in this approach chromatographically not resolved. Another contribution describes a bioactivity assay of porcine relaxin (pRIX) based on cyclic adenosine 3',5'-monophosphate (cAMP) accumulation in the human monocyte cell-line (THP-1 cells) quantified by liquid chromatography-tandem mass spectrometry [18]. The developed method indicated higher precision and selectivity than the commercial enzyme linked immunosorbent assay kits, however with a lower sensitivity compared to the work described in this chapter.

In order to determine cAMP and cGMP in hundreds of blood and tissue samples, in this chapter a method was therefore developed for relative small sample quantities *i.e.* 200 µL plasma and 150 mg of tissue and, moreover, that did not suffer from ion suppression phenomena in MS. In this respect, sample preparation proved to be of utmost importance. Solid phase extraction on silica, exploiting the mechanism of hydrophilic interaction liquid chromatography (HILIC), was very effective in enrichment of the polar target solutes and in fractionation of interferences. To detect the molecules of interest the power of a triple quadrupole mass spectrometer (MS/MS) had to be used to develop a robust and sensitive method. Reaching low limit (sub-ppb) of detection was necessary to make this a relevant functional method for pharmaceutical purposes.

## 3.2 Experimental

### 3.2.1 Chemicals, reagents and materials

3',5'-cGMP, 3',5'-cAMP, 2',3'-cAMP and IBMX (3-isobutyl-1-methylxanthine) were purchased from Sigma-Aldrich (Bornem, Belgium). 2',3'-cGMP was obtained from BIOLOG Life Science Institute (Bremen, Germany). The stable isotope-labeled $^{2}D_{1}{}^{15}N_{3}$-3',5'-cGMP and $^{13}C_{10},{}^{15}N_{5}$-3',5'-cAMP were synthesized in house (Pfizer Global Research & Development, Groton, CT, USA). Stock solutions of all standards (1 mg/mL) were prepared in 0.25% acetic acid in water in glass vials and stored at -20 °C. Working solutions were prepared each day by dilution in LC-MS water. LC-MS-grade water, acetonitrile, methanol, acetic acid and formic acid were obtained from Biosolve (Valkenswaard, the Netherlands). HyperSep Silica SPE cartridges (500 mg/3 mL) were purchased from Thermo Fisher Scientific (Erembodegem, Belgium). Sample grinding kits (1.5 mL microcentrifuge tubes, grinding resin, and disposable pestles) were obtained from GE Healthcare Europe GmbH (Diegem, Belgium). PVDF 0.45 µm micro filters from Grace Division (Lokeren, Belgium) were used.

### 3.2.2 Human plasma and animal tissues

Heparinized human blood was taken from fifteen volunteers in the laboratory. IBMX (phosphodiesterase inhibitor) was immediately added (10 µL of 100 mM IBMX solution to each mL of blood; 1 mM final concentration) to stabilize cAMP and cGMP that quickly degrade in human blood at room temperature through enzymatic processes. Plasma was obtained by cold centrifugation for 10 min at 2500 rpm and stored at -80°C until analysis. Animal tissues were kindly donated by the Faculty of Veterinary Medicine of the Ghent University, Belgium. These tissues were collected during a practical session, together with students of the Faculty of Veterinary Medicine. These exercises are regulated by strict ethical norms. The samples were stored at -80°C.

### 3.2.3 Sample preparation

Plasma samples were thawed to room temperature and 200 µL of plasma was spiked with 5 ng/mL of the labeled internal standards in 0.25% acetic acid in water. 600 µL of 2% acetic acid in water, 200 µL of 5/95 100 mM ammonium formate/acetonitrile and 3.2 mL of acetonitrile was added. The samples were then vortexed for 2 min and centrifuged for 5 min at 3000 rpm. The complete supernatant was transferred to the silica SPE cartridge that was previously conditioned with 5 mL of water under vacuum and equilibrated with 5 mL of 5/95 100mM ammoniumformate/acetonitrile. During loading the supernatant was slowly drawn through the SPE cartridge by vacuum. Subsequently the cartridge was washed with 1 mL of 10/90 water/acetonitrile and the analytes were eluted with 2 mL of water in eppendorf tubes and filtered. Samples were dried under nitrogen, reconstituted in 60 µL of water and vortexed for 2 min. 50 µL was injected into the LC-MS/MS system for analysis.

Frozen animal tissues were thawed to room temperature and cut into smaller pieces. 150 mg of animal tissue was transferred to a sample grinding tube and 600 µL of 2% acetic acid in water was added together with 6 µL of 100 mM IBMX stabilizer. The sample was grinded manually with a pestle and centrifuged for 5 min at 7000 rpm. The supernatant was transferred in a vial and 200 µL water, 200 µL 5/95 100mM ammoniumformate/acetonitrile

and 3.2 mL acetonitrile was added. Extracts were then further treated as described for the plasma samples.

### 3.2.4 Preparation of calibration standards and quality control (QC) samples

Calibration curve standards were prepared by dilution of 3',5'-cAMP and 3',5'-cGMP stock solutions in water down to 0.15, 0.5, 1, 2, 5, 10 and 20 ng/mL concentrations. These samples for calibration were also spiked with 5 ng/mL $^{13}C_{10}$,$^{15}N_5$-3',5'-cAMP and $^{2}D_1$$^{15}N_3$-3',5'-cGMP. Quality control (QC) samples at four concentrations were prepared by spiking the pooled cAMP and cGMP free plasma with the appropriate amounts of cyclic nucleotide standard solutions. cAMP and cGMP free plasma was obtained by aging the plasma at room temperature and regular LC-MS/MS monitoring of residue concentrations. 10 µL of 100 mM IBMX was subsequently added therein together with the 3',5'-cAMP and 3',5'-cGMP standards. These QC samples covered the full range of the intended calibration range: lower limit quality control (LLQC), low quality control (LQC), medium quality control (MQC), and high quality control (HQC). The corresponding concentrations were respectively matrix + 0.15 ng/mL (LLQC), matrix + 0.5 ng/mL (LQC), matrix + 5 ng/mL (MQC), and matrix + 15 ng/mL (HQC). In case of the LLQC sample, the 0.15 ng/mL added to the matrix was close to the LOQ of 0.125 ng/mL (for cGMP), measurable with the used triple quadropole instrument. Note that four QC samples at four levels in the calibration range have been prepared and tested. These experiments emulate therefor closely to a matrix matched calibration line.

### 3.2.5 Instrumentation

For LC-MS/MS analysis an Alliance 2690 (Waters, Milford, MA, USA) equipped with autosampler, column oven and binary pump was coupled to an API 3000 triple quadrupole mass spectrometer (Applied Biosystems, Toronto, Canada) with Turbo Ion Spray (electrospray) source. The MS was operated in the negative-ion multiple reaction monitoring mode (MRM). Data acquisition was performed with Analyst 1.4.1 software.

Separations were undertaken using a Zorbax SB-C18 column 150 mm × 3.0 mm i.d. × 3.5 µm particle size (Agilent Technologies, Brussels, Belgium), coupled to a C18 Security Guard column 4 × 3.0 mm i.d. (Phenomenex, Torrance, CA, USA). Solvent A was 0.1% formic acid

and solvent B acetonitrile/methanol/water in volume ratio 1/2/4. The flow rate was 0.35 mL/min. A linear gradient was used with initial conditions 100% A and increasing solvent B from 0% to 50% in 10 min. Solvent B then went to 100% B in 1 min to clean the column and maintained for 1 min, followed by column regeneration for 10 min. Total analysis time was 22 min. The column temperature was 25°C and the autosampler temperature was set at 10°C.

MS instrument parameters were optimized for maximum sensitivity in MRM mode. Negative voltage and temperature were respectively set on -4200 V and 470 °C. Nebulizer gas was set at 15, curtain gas at 6 and collision gas at 3. The dwell time was 150 ms. The Q1 resolution was set on unit, the Q3 resolution was set on low. The precursor to product ion transition was measured through direct infusion of each compound into the ion source and the most abundant product ion was selected. Solute identity was confirmed in all cases by various transitions. An Infinity 1290 LC coupled to a 6230 TOF LC/MS (Agilent Technologies, Waldbronn, Germany) with Jet Stream technology was used to record high resolution spectra of 2',3'-cAMP, 2',3'-cGMP and the 3',5'-analogues.

## 3.3 Results and discussion

Most important considerations in developing a method that can be routinely applied for the determination of the four cyclic nucleotides in biological samples were: (i) method should be highly selective and specific, (ii) sample sizes should be small and (iii) matrix effects resulting in ion suppression should be absent. To realize this, both the analytical (LC-MS/MS) and sample preparation steps were optimized.

### 3.3.1 Optimization of LC-MS/MS

The cyclic nucleotides can, in principle, be analyzed by different LC modes *e.g.* RPLC, HILIC, and ion-pair LC. RPLC is preferred in the pharmaceutical industry because of its high robustness compared to the other LC modes and has also been applied for the determination of 3',5'-cAMP and 3',5'-cGMP by LC-MS in biological samples [12-15]. Several RPLC columns were tested in this study. Zorbax SB-C18 was chosen for its long-term stability and reproducibility and because of the excellent retention, peak symmetry and efficiency which

was measured for the cGMP and cAMP standards. A 3 mm i.d. column was the best compromise for MS (flow rate of 0.35 mL/min, no flow splitting) and volume loadability (50 µL water injection volume). Concerning the mobile phase composition, high amounts of water are required to give the very polar cyclic nucleotides sufficient retention. Therefore the gradient started at 100% aqueous and increased to only 20% organic at the end of the gradient. Moreover, methanol was added in excess to the organic phase (2 compared to 1 for acetonitrile) to increase retention further [14].

Both positive and negative ESI voltage could be applied for sensitive detection of the cyclic nucleotides. Although positive ion ESI detection yielded somewhat higher signal-to-noise ratios for the protonated molecules of both compounds, it also introduced interferences from human plasma extracts in the MRM channel for several ion transitions used in this study as well as the formation of unstable adduct ratios. In contrast, MRM transitions in negative ion ESI were free of interfering components. Consequently the negative ion mode was selected. Optimization of the MRM conditions was established through direct infusion of the neat standards of each compound solubilized in the initial mobile phase. MS parameters were tuned and selected for the individual compounds to achieve maximum signal intensity. The MRM transitions for the individual compounds were investigated and the most abundant product ion was selected as quantifier ion (Table 3.1).

*Table 3.1. MRM transitions and compound dependent parameters. Quantifier ions are marked in bold.*

| Nr. | Compound | Precursor ion (m/z) | Parent ion (m/z) | Declustering potential (V) | Focusing potential (V) | Collision energy (V) | Collision cell exit potential (V) |
|---|---|---|---|---|---|---|---|
| I | 2',3'-cAMP | **328.0** | **134.0** | -61 | -50 | -22 | -7 |
| | | | 107.2 | | | -62 | -7 |
| | | | 92.2 | | | -70 | -5 |
| II | 2',3'-cGMP | 343.9 | 66.0 | -96 | -270 | -88 | -5 |
| III | 3',5'-cAMP | **328.0** | **134.2** | -81 | -230 | -36 | -7 |
| IV | 3',5'-cGMP | **344.0** | **149.9** | -91 | -215 | -34 | -7 |
| | | | 133.1 | | | -52 | -7 |
| | | | 79.0 | | | -74 | -5 |
| V | IS 3',5'-cAMP | 342.9 | 144.1 | -61 | -190 | -34 | -7 |
| VI | IS 3',5'-cGMP | 348.0 | 154.1 | -86 | -290 | -32 | -7 |

One quantifier product ion (corresponding to the nucleotide base and the most intense product ion) and two validation product ions for 3',5'-cGMP were added to ensure the identification and quantification of this cyclic nucleotide because of its lower signal intensity and limit of detection, compared to 3',5'-cAMP. An MRM chromatogram with the specific ion transitions of the four described cyclic molecules is shown in Figure 3.1.

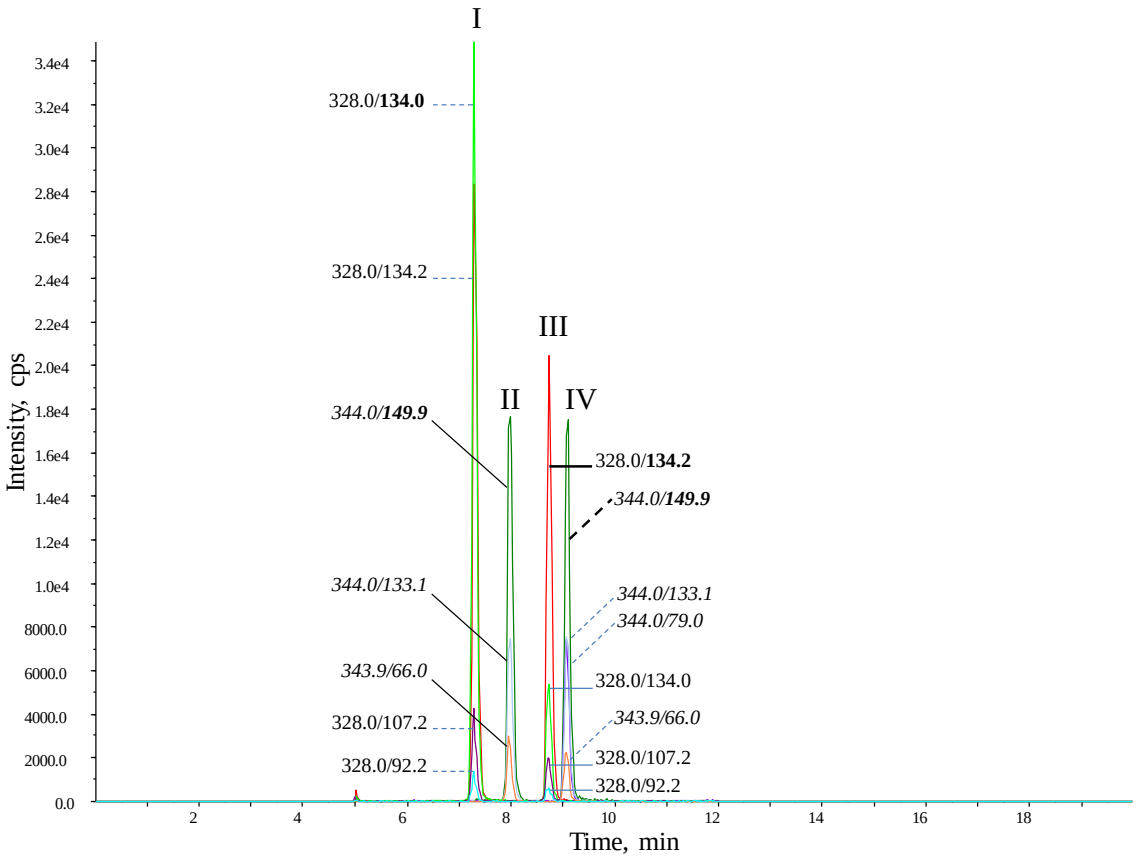


*Figure 3.1. MRM chromatogram of a mixture of: I. 2',3'-cAMP, II. 2',3'-cGMP, III. 3',5'-cAMP and IV. 3',5'-cGMP at a concentration of 10 ng/mL for each analyte using the validated LC-MS/MS method. Multiple MRM transitions for the same molecule (quantification- and validation product ions) are shown. Quantification product ions are marked in bold.*

Isotope dilution MS is by far the best method to provide accurate and precise data for the cyclic nucleotides. Stable isotope-labeled internal standards, with identical chemical and physical properties as the analytes, were used. Isotope-labeled standards of 3',5'-cGMP and 3',5'-cAMP are not commercially available but the synthesis using an isotopically labeled precursor has been described [14]. The precision of the LC-MS/MS assay was significantly improved by using labeled internal standards.

### 3.3.2 Sample preparation

In bio-analytical LC-MS/MS, ion suppression is a well-known phenomenon. Matrix effects from endogenous solutes in plasma can be linked to three main contributors: salts, proteins and phospholipids [19,20]. Ion suppression due to these solutes can be addressed by adequate sample preparation methods that include de-proteination and de-lipidation. In this work various innovative approaches are proposed regarding the development of high throughput sample preparation techniques for pharmacokinetic studies of biological samples by LC-MS/MS (see also chapter 5) [21]. HILIC SPE is, in this respect, a relatively unexploited but very powerful tool that was also applied in this chapter. In the sample preparation procedures described in 3.2.3, the vast majority of the proteins are removed by precipitation (and centrifugation) in the 90% acetonitrile solution. The supernatant is subsequently transferred to the HILIC SPE cartridge, which acts as an additional trap for residual protein content. Salts elute early in the RPLC analysis and are therefore of no concern as the solutes of interest are well retained. Note that phospholipids could experience some partial breakthrough during the SPE process (elution with 100% water), but they would, in this case, subsequently be completely retained on the (C18) guard column (which is replaced after 50 analyses) under the highly aqueous mobile conditions used. In this way, ion suppression was effectively avoided as ascertained by a constant response of the labeled standards during the complete study.

### 3.3.3 Figures of merit

Although a full validation was considered beyond the scope of this work, the figures of merit for analysis of cAMP and cGMP in biological matrices are discussed below. In order to investigate the figures of merit of the methodology for quantitative analysis of 3',5'-cGMP and 3',5'-cAMP in plasma, recovery, selectivity, sensitivity, linearity and precision were determined by using blank and spiked plasma. The recovery of the sample preparation method was evaluated by comparing the MS response of analytes in plasma samples spiked before and after SPE on silica. Three samples of the same plasma were spiked with 10 ng/mL of 3',5'-cGMP and 3',5'-cAMP and with 20 ng/mL of IS $^{2}D_{1}$,$^{15}N_{3}$-3',5'-cGMP and IS $^{13}C_{10}$,$^{15}N_{5}$-3',5'-cAMP. Each sample was then analyzed in triplicate. Recovery values of

103.2% (RSD 6.4%) and 105.6% (RSD 5.7%) for respectively 3',5'-cAMP and 3',5'-cGMP demonstrated the effectiveness of the sample preparation method while the instrumental RSD was below 2%. Selectivity is excellent due to precise retention times (± 0.1 min), specific MRM transitions and the ratio of the different MRM transitions.

The limit of detection (LOD) for the total assay was 0.023 ng/mL for 3',5'-cAMP and 0.038 ng/mL for 3',5'-cGMP (signal to noise ratio 3). The limit of quantification (LOQ) was 0.076 ng/mL for 3',5'-cAMP and 0.125 ng/mL for 3',5'-cGMP (signal to noise ratio 10). Linearity of the method was studied in the range of 0.15–20 ng/mL for both cyclic nucleotides and correlation coefficients ($R^2$) of 0.9996 and 0.9994 for, respectively, 3',5'-cAMP and 3',5'-cGMP were calculated. This broad calibration range was chosen to anticipate for possible unexpectedly low or high concentrations in the biological samples. Note that the lowest point in the calibration curve (0.15 ng/mL) is close (but above) the lowest LOQ of this method, *i.e.* 0.125 ng/mL for 3',5'-cGMP. 3',5'-cGMP and the tenfold higher concentrated 3',5'-cAMP were calibrated separately.

Precision and accuracy data, represented in Table 3.2, was validated using the European Medicines Agency (EMA) guidelines for bioanalytical method validation (21 July 2011) while also being in line with representative manuscripts following these procedures [22]. The intra-day precision and accuracy were determined by evaluating matrix-based QC samples prepared with six replicates at four different concentration levels (LLQC, LQC, MQC, HQC), while the inter-day precision and accuracy were evaluated over three different days. Precision was expressed as the relative standard deviation of the determined concentrations. Accuracy was calculated by dividing the mean measured concentration by the mean nominal concentration times 100. The results of the analysis of the plasma-based QC samples demonstrated acceptable precision and accuracy based on validation criteria (precision with RSD less than 20% and accuracy within 80-120% for LLQC and precision with RSD less than 15% and accuracy within 85-115% for the rest of the QCs) [22].

*Table 3.2. Precision and accuracy data for the 3',5'-cGMP and 3',5'-cAMP measurement in human plasma by LC-MS/MS.*

| | Spiked | Intra-run precision and accuracy | | | Inter-run precision and accuracy | | |
|---|---|---|---|---|---|---|---|
| | (ng/mL) | Measured (mean ± SD) | Precision (RSD in %) | Accuracy (%) | Measured (mean ± SD) | Precision (RSD in %) | Accuracy (%) |
| 3',5'-cGMP | 0.15 | 0.16 ± 0.02 | 10.1 | 106.1 | 0.16 ± 0.02 | 12.1 | 107.6 |
| | 0.50 | 0.50 ± 0.03 | 6.5 | 98.3 | 0.50 ± 0.05 | 10.3 | 99.1 |
| | 5.0 | 5.2 ± 0.3 | 5.7 | 104.2 | 5.2 ± 0.3 | 5.9 | 103.4 |
| | 15.0 | 14.9 ± 0.5 | 3.1 | 99.2 | 14.8 ± 0.7 | 4.6 | 98.9 |
| 3',5'-cAMP | 0.15 | 0.16 ± 0.02 | 11.3 | 106.7 | 0.16 ± 0.02 | 12.4 | 108.2 |
| | 0.50 | 0.52 ± 0.03 | 5.2 | 103.4 | 0.50 ± 0.03 | 6.8 | 100.2 |
| | 5.0 | 5.2 ± 0.3 | 6.1 | 103.3 | 5.2 ± 0.3 | 5.2 | 104.6 |
| | 15.0 | 14.8 ± 0.6 | 3.7 | 98.9 | 14.9 ± 0.5 | 3.4 | 99.3 |

The 3',5'-cGMP, 3',5'-cAMP and IS stock solutions (IS $^{2}D_{1}$,$^{15}N_{3}$-3',5'-cGMP and IS $^{13}C_{10}$,$^{15}N_{5}$-3',5'-cAMP) were determined to be stable for a least 24 h and for one month at room temperature and at -20 °C, respectively. When 1mM IBMX was added to the plasma samples prior to analysis, these solutes (including IS) after sample processing were found stable for up to 152 hours at room temperature. Long term stability of 3',5'-cGMP and 3',5'-cAMP in IBMX quenched human plasma was confirmed at -80°C for at least 6 months [14].

### 3.3.4 Applications

The concentration of 3',5'-cGMP and 3',5'-cAMP in human plasma was measured for fifteen healthy volunteers. A representative MRM chromatogram is shown in Figure 3.2.

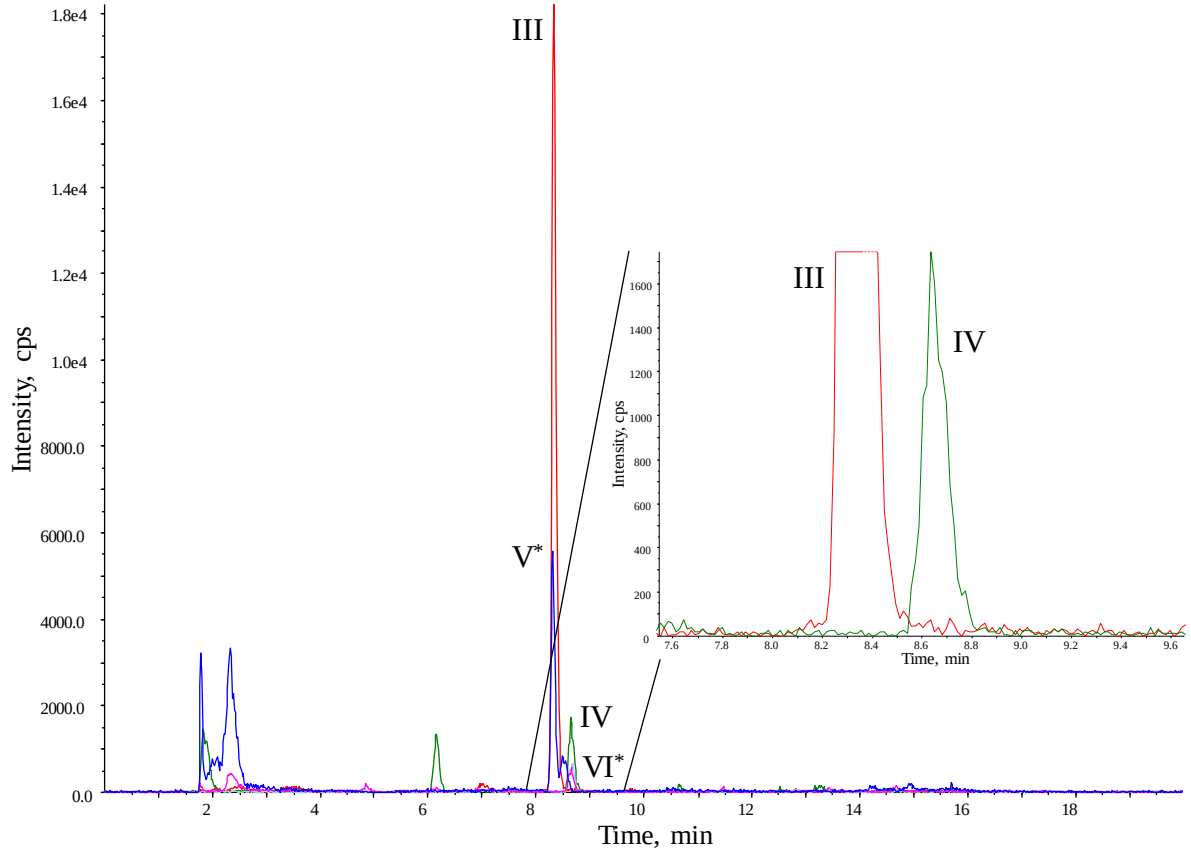


*Figure 3.2. Representative MRM chromatogram of a human plasma sample (200 µL) after SPE on silica and analysis by LC-MS/MS. III. 3',5'-cAMP and IV. 3',5'-cGMP. Spiked with 5 ng/mL internal (isotopically labeled) standard of 3',5'-cAMP (V*) and 3',5'-cGMP (VI*). Measured concentrations are 6.4 ng/mL and 0.6 ng/mL for respectively 3',5'-cAMP and 3',5'-cGMP. Also a zoom of the two quantifier product ions for 3',5'-cAMP and 3',5'-cGMP is displayed.*

Samples were measured in triplicate and standard deviations (SD) and relative standard deviations (RSD) were calculated. Results are shown in Table 3.3. 3',5'-cAMP concentrations varied between 4.66 – 9.20 ng/mL and 3',5'-cGMP between 0.30 – 1.20 ng/mL that is in excellent agreement with literature data [14-16]. Plasma sample number 14 was a surprisingly high concentration for 3',5'-cGMP and needs further biological investigation. It was not taken into account to calculate the mean value.

*Table 3.3. Measured concentrations of 3',5'-cAMP and 3',5'-cGMP in human plasma of fifteen healthy patients.*

| Plasma sample | Conc 3',5'-**cAMP** (ng/mL) | | SD (n=3) | Precision (RSD %) | Conc 3',5'-**cGMP** (ng/mL) | | SD (n=3) | Precision (RSD %) |
|---|---|---|---|---|---|---|---|---|
| 1 | 5.34 | ± | 0.18 | 3.36 | 0.30 | ± | 0.02 | 5.67 |
| 2 | 4.83 | ± | 0.10 | 2.07 | 0.40 | ± | 0.03 | 6.39 |
| 3 | 4.80 | ± | 0.16 | 3.43 | 0.74 | ± | 0.02 | 2.44 |
| 4 | 6.81 | ± | 0.24 | 3.53 | 0.96 | ± | 0.05 | 4.82 |
| 5 | 4.66 | ± | 0.29 | 6.12 | 0.39 | ± | 0.02 | 4.46 |
| 6 | 7.17 | ± | 0.49 | 6.79 | 1.06 | ± | 0.10 | 9.06 |
| 7 | 6.35 | ± | 0.13 | 1.98 | 0.68 | ± | 0.03 | 4.12 |
| 8 | 6.14 | ± | 0.32 | 5.17 | 0.43 | ± | 0.03 | 5.79 |
| 9 | 9.20 | ± | 0.39 | 4.27 | 1.20 | ± | 0.02 | 1.51 |
| 10 | 5.42 | ± | 0.11 | 2.07 | 1.03 | ± | 0.03 | 2.83 |
| 11 | 7.12 | ± | 0.14 | 1.96 | 0.87 | ± | 0.03 | 3.17 |
| 12 | 5.73 | ± | 0.11 | 1.85 | 0.80 | ± | 0.01 | 1.78 |
| 13 | 6.46 | ± | 0.17 | 2.70 | 0.85 | ± | 0.07 | 8.12 |
| 14 | 8.09 | ± | 0.18 | 2.17 | 5.39 | ± | 0.16 | 2.94 |
| 15 | 6.80 | ± | 0.28 | 4.18 | 0.80 | ± | 0.04 | 4.47 |

Quantifying cGMP and cAMP in animal tissues is becoming more and more relevant in drug discovery and several samples were investigated: the pancreas, the kidney and heart of rabbits and the pancreas of rats. Two representative samples are discussed, *i.e.* the pancreas and kidney of rabbits. Analysis of the pancreas of a rabbit with the SPE on silica LC-MS/MS method using the same MRMs showed four peaks (Figure 3.3).

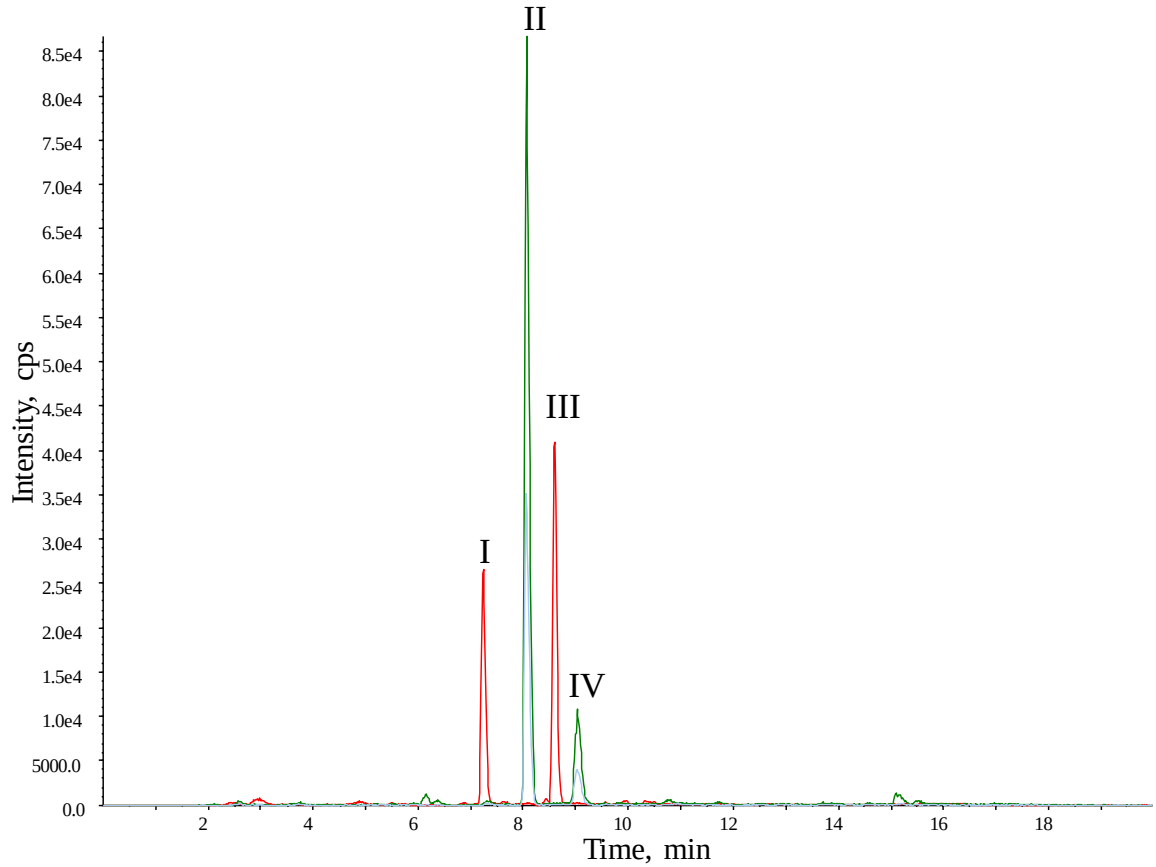


*Figure 3.3. MRM chromatogram of an extract of rabbit pancreas (150 mg) after SPE on silica LC-MS/MS: I. 2',3'-cAMP, II. 2',3'-cGMP, III. 3',5'-cAMP and IV. 3',5'-cGMP.*

Two peaks could be elucidated to 3',5'-cGMP and 3',5'-cAMP by their retention times and specific MRM transitions. However, two additional signals depicting the same MRM transitions as the 3',5'-cyclic nucleotides were detected at 1.5 min and 1 min earlier. Response on the same MRMs suggested isomers and indeed 2',3'-isomers of the 3',5'-cyclic nucleotides have been reported to occur in animal tissues [23-27]. High resolution MS was used to confirm the elemental composition of the "at that time" unknown structures (Table 3.4). The identity and retention times corresponding to the 2',3'-isomers was subsequently confirmed by purchasing these naturally occurring isomers of the 3',5'- cyclic nucleotides.

Corresponding quantitative data are included in Table 3.5. It should be mentioned that the method for the analysis of the tissue samples could not yet be fully validated because of limited sample availability. Therefore estimated concentrations were included to inform the

reader about the interesting possibilities that HILIC-SPE HPLC-MS/MS can offer for animal tissue analyses.

*Table 3.4. Difference between the accurate mass (measured) and the exact mass (calculated) from the correct formula (molecule) for a TOF-MS experiment with pancreas of a rabbit.*

| Compound | Formula | Accurate Mass (Dalton) | Exact Mass (Dalton) | Error (ppm) |
|---|---|---|---|---|
| 3',5'-cGMP | $C_{10}H_{12}N_5O_7P$ | 345.04715 | 345.04743 | 0.81 |
| 3',5'-cAMP | $C_{10}H_{12}N_5O_6P$ | 329.05321 | 329.05252 | 2.09 |
| 2',3'-cGMP | $C_{10}H_{12}N_5O_7P$ | 345.04685 | 345.04743 | 1.68 |
| 2',3'-cAMP | $C_{10}H_{12}N_5O_6P$ | 329.05221 | 329.05252 | 1.25 |

*Table 3.5. Measured concentrations of cAMP and cGMP in animal tissue samples by LC-MS/MS.*

| Animal tissue sample | Conc **2',3'**-c**A**MP (pg/mg) | Conc **2',3'**-c**G**MP (pg/mg) | Conc **3',5'**-c**A**MP (pg/mg) | Conc **3',5'**-c**G**MP (pg/mg) |
|---|---|---|---|---|
| Pancreas of rabbit | 7.6 | 30.7 | 11.8 | 5.2 |
| Kidney of rabbit | 4.5 | 19.4 | 1.3 | / |

## 3.4 Conclusion

An accurate and sensitive method was developed for the determination of the highly relevant second messenger molecules 3',5'-cAMP and 3',5'-cGMP and its naturally occurring 2',3'-isomers in human plasma and animal tissues. SPE on silica was used as sample preparation method for the enrichment of the targets and the removal of interferences leading to MS ion suppression. Samples were then analyzed by RPLC combined with MS/MS operated in the negative ESI mode. 3',5'-cAMP and 3',5'-cGMP concentrations in human plasma are 4.66 –

9.20 ng/mL and 0.30 – 1.20 ng/mL. Two isomers of 3',5'-cAMP and 3',5'-cGMP namely 2',3'-cAMP and 2',3'-cGMP were detected in tissue samples.

In the next chapter emphasis is set on the detection and quantitation of the 2',3' isomers of the cyclic nucleotides in lower plant samples.

## 3.5 References

[1] G. L. Pauk and W. J. Reddy, "Measurement of adenosine 3',5'-monophosphate," *Analytical Biochemistry*, vol. 21, pp. 298-307, 1967.

[2] A. L. Steiner, D. M. Kipnis, R. Utiger, and C. Parker, "Radioimmunoassay for measurement of adenosine 3',5'-cyclic phosphate," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 64, pp. 367-373, 1969.

[3] A. L. Steiner, D. M. Kipnis, and C. W. Parker, "Radioimmunoassay for cyclic nucleotides .1. Preparation of antibodies and iodinated cyclic nucleotides," *Journal of Biological Chemistry*, vol. 247, pp. 1106-1113, 1972.

[4] A. L. Steiner, A. S. Pagliara, D. M. Kipnis, and L. R. Chase, "Radioimmunoassay for cyclic nucleotides .2. Adenosine 3',5'-monophosphate and guanosine 3',5'-monophosphate in mammalian tissues and body fluids," *Journal of Biological Chemistry*, vol. 247, pp. 1114-1120, 1972.

[5] W. J. Wojcik and N. H. Neff, "Adenosine measurement by a rapid HPLC-Fluorometric method - Induced changes of adenosine content in regions of rat brain," *Journal of Neurochemistry*, vol. 39, pp. 280-282, 1982.

[6] L. Roef, E. Witters, J. Gadeyne, J. Marcussen, R. P. Newton, and H. A. Van Onckelen, "Analysis of 3',5'-cAMP and adenylyl cyclase activity in higher plants using polyclonal chicken egg yolk antibodies," *Analytical Biochemistry*, vol. 233, pp. 188-196, Jan 1996.

[7] M. J. D. Enrich, J. A. Villamarin, J. I. R. Martinez, and I. Ibarguren, "Measurement of adenosine 3 ',5 '-cyclic monophosphate and guanosine 3 ',5 '-cyclic monophosphate in mussel (Mytilus galloprovincialis Lmk.) by high-performance liquid chromatography with diode array detection," *Analytical Biochemistry*, vol. 285, pp. 105-112, Oct 2000.

[8] M. Tsugawa, K. Moriwaki, S. Iida, H. Fujii, M. Gomi, S. Tarui, and M. Fujimoto "An enzyme-linked-immunosorbent-assay (ELISA) for guanosine 3', 5'-cyclic monophosphate (cGMP) in human plasma and urine using monoclonal-antibody," *Journal of Immunoassay*, vol. 12, pp. 263-276, 1991.

[9] R. Z. Abraham, L. Kobzik, M. R. Moody, M. B. Reid, and J. S. Stamler, "Cyclic GMP is a second messenger by which nitric oxide inhibits diaphragm contraction," *Comparative Biochemistry and Physiology a-Molecular and Integrative Physiology*, vol. 119, pp. 177-183, Jan 1998.

[10] J. Wellard, B. Blind, and B. Hamprecht, "An enzyme-linked immunosorbent assay for the rapid quantification of intracellular and extracellular guanosine 3 ',5 '-cyclic monophosphate in cultured glial cells," *Neurochemical Research,* vol. 29, pp. 2177-2187, Nov 2004.

[11] R. D. D. Bianca, E. Mitidieri, V. Mirone, F. Fusco, C. Imbimbo, G. Cirino, and R. Sorrentino, "An *ex vivo* standardized assay to measure human platelet cGMP," *Journal of Pharmacological and Toxicological Methods,* vol. 64, pp. 164-167, Sep-Oct 2011.

[12] R. P. Newton, "Qualitative and quantitative MS analysis of cyclic nucleotides and related enzymes," *Biochemical Society Transactions,* vol. 24, pp. 938-943, Aug 1996.

[13] E. Witters, L. Roef, R. P. Newton, W. VanDongen, E. L. Esmans, and H. A. Van Onckelen, "Quantitation of cyclic nucleotides in biological samples by negative electrospray tandem mass spectrometry coupled to ion suppression liquid chromatography," *Rapid Communications in Mass Spectrometry,* vol. 10, pp. 225-231, 1996.

[14] Y. H. Zhang, D. Dufield, J. Klover, W. L. Li, G. Szekely-Klepser, C. Lepsy, and N. Sadagopan, "Development and validation of an LC-MS/MS method for quantification of cyclic guanosine 3 ',5 '-monophosphate (cGMP) in clinical applications: A comparison with a EIA method," *Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences,* vol. 877, pp. 513-520, Feb 2009.

[15] J. Martens-Lobenhoffer, C. Dautz, and S. M. Bode-Boger, "Improved method for the determination of cyclic guanosine monophosphate (cGMP) in human plasma by LC-MS/MS," *Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences,* vol. 878, pp. 487-491, Feb 2010.

[16] P. Oeckl and B. Ferger, "Simultaneous LC-MS/MS analysis of the biomarkers cAMP and cGMP in plasma, CSF and brain tissue," *Journal of Neuroscience Methods,* vol. 203, pp. 338-343, Jan 2012.

[17] P. Oeckl, P. Steinacker, S. Lehnert, S. Jesse, H. A. Kretzschmar, and A. C. Ludolph, "CSF Concentrations of cAMP and cGMP Are Lower in Patients with Creutzfeldt-Jakob Disease but Not Parkinson's Disease and Amyotrophic Lateral Sclerosis," *Plos One,* vol. 7, p. 7, Mar 2012.

[18] X. H. Ren, C. F. Wang, L. Wu, H. Y. Li, L. D. Chi, L. X. Sun, X. Fu, and J. Zhang, "Bioactivity assay of porcine relaxin based on cAMP accumulation in THP-1 cells quantified by LC-MS/MS," *Journal of Pharmaceutical and Biomedical Analysis,* vol. 111, pp. 320-323, Jul 2015.

[19] R. King, R. Bonfiglio, C. Fernandez-Metzler, C. Miller-Stein, and T. Olah, "Mechanistic investigation of ionization suppression in electrospray ionization," *Journal of the American Society for Mass Spectrometry,* vol. 11, pp. 942-950, Nov 2000.

[20] H. Trufelli, P. Palma, G. Famiglini, and A. Cappiello, "An overview of matrix effects in liquid chromatography-mass spectrometry," *Mass Spectrometry Reviews,* vol. 30, pp. 491-509, May-Jun 2011.

[21] T. Van Damme, M. Lachova, F. Lynen, R. Szucs, and P. Sandra, "Solid-phase extraction based on hydrophilic interaction liquid chromatography with acetone as eluent for eliminating matrix effects in the analysis of biological fluids by LC-MS," *Analytical and Bioanalytical Chemistry,* vol. 406, pp. 401-407, Jan 2014.

[22] J. F. Hou, F. Qu, C. S. Wu, Q. Ren, and J. L. Zhang, "Quantitative determination and pharmacokinetic study of the novel anti-Parkinson's disease candidate drug FLZ in rat brain by high performance liquid chromatography-tandem mass spectrometry," *Journal of Pharmaceutical and Biomedical Analysis,* vol. 66, pp. 232-239, Jul 2012.

[23] J. Ren, Z. C. Mi, N. A. Stewart, and E. K. Jackson, "Identification and Quantification of 2 ',3 '-cAMP Release by the Kidney," *Journal of Pharmacology and Experimental Therapeutics,* vol. 328, pp. 855-865, Mar 2009.

[24] E. K. Jackson, J. Ren, and Z. C. Mi, "Extracellular 2 ',3 '-cAMP Is a Source of Adenosine," *Journal of Biological Chemistry,* vol. 284, pp. 33097-33106, Nov 2009.

[25] E. K. Jackson, "The 2 ',3 '-cAMP-adenosine pathway," *American Journal of Physiology-Renal Physiology,* vol. 301, pp. F1160-F1167, Dec 2011.

[26] E. Boadu, S. Vaskinn, E. Sundkvist, R. Jaeger, and G. Sager, "Inhibition by guanosine cyclic monophosphate (cGMP) analogues of uptake of H-3 3',5'-cGMP without stimulation of ATPase activity in human erythrocyte inside-out vesicles," *Biochemical Pharmacology,* vol. 62, pp. 425-429, Aug 2001.

[27] J. D. Verrier, T. C. Jackson, R. Bansal, P. M. Kochanek, A. M. Puccio, D. O. Okonkwo, and E. K. Jackson, "The brain in vivo expresses the 2 ',3 '-cAMP-adenosine pathway," *Journal of Neurochemistry,* vol. 122, pp. 115-125, Jul 2012.

# Chapter 4. Study of 2',3' cyclic nucleotide metabolites cAMP and cGMP in plants under stress conditions.

Adapted from T. Van Damme, D. Blancquaert, P. Couturon, D. Van Der Straeten, P. Sandra, and F. Lynen, "Wounding stress causes rapid increase in concentration of the naturally occurring 2 ',3 '-isomers of cyclic guanosine- and cyclic adenosine monophosphate (cGMP and cAMP) in plant tissues," *Phytochemistry*, vol. 103, pp. 59-66, Jul 2014. The practical work related to this chapter has been entirely performed by Thomas Van Damme.


## *Summary*

3',5'-Cyclic guanosine monophosphate (cGMP) and 3',5'-cyclic adenosine monophosphate (cAMP) are well reported second messenger molecules involved in cellular signal transduction, in physiological functions such as neurotransmission in animals and in the modulation of cell growth and differentiation. In plants, 3',5'-cyclic nucleotides have been implicated in the regulation of ion homeostasis, hormone and stress responses. The behavior of the 2',3'-cyclic nucleotide variants is also known in animal tissue but no quantitative information is available about 2',3'-cAMP and 2',3'-cGMP in plant material. A recently developed HILIC-SPE LC-MS/MS method for the analysis of cyclic nucleotides in blood and animal tissue was therefore adapted to measure 2',3'-cAMP and 2',3'-cGMP concentrations in plant material. Cyclic nucleotide concentrations were measured in *Arabidopsis thaliana* (Col-0) leaves before and after the application of wounding stress. A significant (~5-fold) up-regulation of 2',3'-cAMP and 2',3'-cGMP was measured in *Arabidopsis* leaves compared to the control samples. The results indicate a thus far unreported strong correlation between plant stress and both 2',3'-cAMP and 2',3'-cGMP levels in plant material, and may open new avenues towards understanding the role of cyclic nucleotides in plants.


## 4.1 Introduction

Second messengers cyclic nucleotides, such as 3',5'-cAMP and 3',5'-cGMP, play an essential role in signal transduction in eukaryotic cells [1-4]. Since the discovery of 3',5'-cAMP in 1956 by Sutherland [5] (who was awarded the Nobel Prize in Physiology of Medicine in

1971), the biological functions of this and of other 3',5'- cyclic nucleotides have been extensively studied in microorganisms, animal tissues and in bio-fluids and are now associated with a broad number of diverse cellular and physiological processes [6-10]. They transfer signals from hormones and neurotransmitters to target cells and in this way affect biological processes such as phototransduction [11], vasodilation [12], lipid metabolism [13,14], and a range of physiological responses including sensory transduction [15,16] and cellular proliferation [8]. In plants, 3',5'-cyclic nucleotides have been implicated in the control of homeostasis of cations, such as $K^+$, $Ca^{2+}$ and $Na^+$. Cyclic nucleotide gated channels (CNGCs) have been known to be key targets for these cNMPs. More than 20 cyclic nucleotide gated channels (CNGCs) have been identified and several of these were functionally characterized [17]. Some CNGCs have been suggested to function in biotic and abiotic stress responses [18-21], for example heat stresses and drought [22,23], while also voltage independent channels can be under cAMP control, particularly upon NaCl stress [24]. Finally, 3',5'-cGMP plays a role in transduction of the gibberellin signal in aleurone [25]. Note that no biochemical or bioinformatic evidence has been found for a plant cyclic nucleotide regulated protein kinase, suggesting cyclic nucleotide functions in plants have evolved differently than in mammals [26].

Early reports on the occurrence of cyclic nucleotides in the tissues of higher plants were criticized as being based either upon indirect physiological evidence [27] or upon equivocal chromatographic identification data casting some doubt on the solute identification [28-30]. The occurrence of 3',5'-cAMP, including the 2',3' isomer, was distinctively demonstrated by Newton in 1973 using a rigorous multistage chromatographic and electrophoretic sequence employing ion-exchange, paper and thin layer chromatography and high voltage electrophoresis [31] with spectrophotometric detection. Subsequently, this was confirmed with radioimmunoassay [32,33] and protein binding assays [29], off-line mass spectrometry [30] and on-line LC-MS [34]. 3',5'-cAMP regulation in plants is characterized by broad dynamic ranges spanning from pico- up to micromolar concentration ranges per gram dry weight [27,32]. The fresh weight (FW) levels in plants are <20 pmol/g FW for 3',5'-cAMP and <1 pmol/g FW for 3',5'-cGMP [17]. Usually, however, endogenous concentrations and concentration fluxes of 3',5'-cAMP in plants are lower than in animal and microbial cells

requiring pmol detection limits to allow, for example, the elucidation of the kinetics of the metabolic cyclic NMP enzymes [34,35].

The presence of 3',5'-cGMP in higher plants (*i.e.* in bean seedlings) was reported early as well [36,37], although ambiguity in solute identity was also only removed when this was confirmed by Fast Atom Bombardment (FAB) mass spectrometry and Nuclear Magnetic Resonance (NMR) spectroscopy [38]. With the emergence of electrospray ionization and concomitant hyphenation to HPLC, quantitative and qualitative study of the regulation of 3',5'-cAMP and 3',5'-cGMP in plant material has significantly expanded [34, 35, 39-41]. Several pathways and signaling cascades have now been identified in which these secondary messengers are involved [21,42,43]. In addition, with the emergence of this technology, the relevance of cGMP and of other second messengers in plant material has been increasingly studied [44,45]. In the recent years much progress has been made in understanding the roles of cAMP and cGMP in plants, concerning cAMP and pollen tube growth [46], abiotic and biotic stresses [46-49] and also by developing new methods of analysis [50]. Although the 2',3 isomer of 3',5'-cAMP was already reported in 1973, little attention was paid to the quantitative study of the regulation of the 2',3' analogues in plant material. However, 2',3'-cGMP, 2',3'-cAMP and 2',3'-cCMP have been reported in a qualitative study on cyclic nucleotide content of tobacco BY-2 cells [51]. In 2009, Ren *et al.* reported the identification and quantification of 2',3'-cyclic AMP in rat kidney [52], detected by chance while investigating the release of 3',5'-cAMP. This isomer is derived from mRNA turnover, by transphosphorylation reactions catalyzed by RNases (ribonucleases). Jackson and colleagues conducted work on the occurrence of 2',3'-cyclic AMP in animals allowing them to identify it in rat vascular smooth muscle cells and perfused kidneys, and to propose a biochemical pathway [53]. Mostly high-performance liquid chromatography (HPLC), in combination with mass spectrometry was used to analyze these molecules in biological samples, mainly in animal tissues [9,54-56]. It was concluded that renal injury in rat kidneys activates the extracellular 2',3'-cAMP-adenosine pathway that converts 2',3'-cAMP to 2'-AMP and 3'-AMP, which are subsequently metabolized to adenosine. This conversion would protect tissues by reducing a pro-death factor (2',3'-cAMP) and by increasing the levels of adenosine [9]. Another assumption is that energy depletion activates the 2',3'-cAMP-adenosine, but not the 3',5'-cAMP-adenosine, pathway in mouse kidneys [54]. Also *in vivo* cerebral 2',3'-cAMP

is converted to 2'-AMP and 3'AMP, and subsequently metabolized to adenosine. This pathway exists endogenously in both mice and humans [56].

In this chapter, the occurrence of 2',3'-cAMP and 2',3'-cCMP in *Arabidopsis* samples is unambiguously demonstrated and quantified by HPLC-MS/MS on multiple small quantities of plant samples (25 mg) by employing a recently developed SPE (solid phase extraction) based sample preparation procedure. Concentrations of these cyclic nucleotides as a function of applied wounding stresses are reported.

## 4.2 Experimental

### 4.2.1 Chemicals, reagents and materials

3',5'-cGMP, 3',5'-cAMP, 2',3'-cAMP and IBMX (3-isobutyl-1-methylxanthine) were purchased from Sigma-Aldrich (Bornem, Belgium). 2',3'-cGMP was obtained from BIOLOG Life Science Institute (Bremen, Germany). The stable isotope-labeled $^{2}D_{1}{}^{15}N_{3}$-cGMP and $^{13}C_{10}$,$^{15}N_{5}$ -cAMP were synthesized in house (Pfizer Global Research & Development, Groton, CT, USA). Stock solutions of all standards (1 mg/mL) were prepared in 0.25% acetic acid (dissolved in water) and stored in glass vials at -20 °C. Working solutions were freshly prepared by dilution in LC-MS-grade water. LC-MS-grade water, acetonitrile, methanol, acetic acid and formic acid, used for sample preparation and chromatography, were obtained from Biosolve (Valkenswaard, the Netherlands). HyperSep Silica SPE cartridges (500 mg/3 mL) were purchased from Thermo Fisher Scientific (Erembodegem, Belgium). PVDF 0.45 µm micro filters from Grace Division (Lokeren, Belgium) were used. LC-MS grade water was used for mobile phase preparation, Milli-Q grade water was used for sample treatment and SPE.

### 4.2.2 Plant extracts

Soil-grown *Arabidopsis thaliana* Columbia-0 (Col-0) were used in all experiments. *Arabidopsis* plants were grown, in beds of 8x8 plants, in long-day light conditions (16 hours of light, 8 hours of darkness; 60 µmol/m²s, unit of Photosynthetic Photon Flux Density PPFD) at 21°C. Three quarter of the plants (8x6) were used for wounding, while the remaining quarter (8x2) were used as control samples in each bed. For all experiments, rosette leaves of

5-week-old *Arabidopsis* plants were used [57,58]. Upon harvesting, leaves were flash-frozen in liquid nitrogen (-196 °C) to instantly stop all physiological processes. The samples were stored at -80°C upon further analyses. 1 gram of plant material (~ 25 leaves) was harvested for each individual sample, characterized by the type of applied stress and duration of the stress treatment. To that end, approximately 4 to 6 leaves were randomly sampled from 4-6 different plants. Plants were stressed by scalpel or forceps. A scalpel was used to uniformly wound the leaves by making small, non-dissecting transversal cuts every 1-2 cm of the leaf blades [59,60]. A similar procedure was applied for stressing the plants with a forceps, every 1-2 cm the leaves were damaged by firmly pinching the forceps on these leaves without breaking/disconnecting the leaves from the plant.

### 4.2.3 Sample preparation

Frozen plant extracts (-80°C) were homogenized manually with a precooled mortar and pestle with liquid nitrogen. All the leaves harvested for each sample were homogenized to obtain optimal sample representativeness and to avoid outliners. A 25 mg tissue sample aliquot was transferred to a 2 mL Eppendorf tube and spiked with 30 µL of a 100 ng/mL solution of the labeled internal standards in 0.25% acetic acid in water, and with 10 µL IBMX (1 mM solution in water). IBMX is an phosphodiesterase inhibitor and was immediately added to stabilize 3',5'-cAMP and 3',5'-cGMP that degrade in the plants at room temperature through enzymatic processes. 600 µL of 4% acetic acid in water was added and centrifuged for 5 min at 4400x*g. The supernatant was collected and 1200 µL of acetonitrile was added. The sample was again centrifuged for 5 min at 4400x*g and the supernatant was collected. 200 µL of 5/95 100 mM ammonium formate/acetonitrile buffer, 200 µL water and 2 mL of acetonitrile were added, the samples were vortexed for 2 min and subsequently centrifuged for 5 min at 1400x*g. The supernatant was transferred to the silica SPE cartridge that was first conditioned with 5 mL of water under vacuum conditions and equilibrated with 5 mL of 5/95 100mM ammoniumformate/acetonitrile. During loading, the supernatant was slowly drawn through the SPE cartridge by vacuum conditions. Subsequently, the cartridge was washed with 1 mL of 10/90 water/acetonitrile and the analytes were eluted with 2 mL of water in eppendorf tubes and filtered. Samples were dried under nitrogen, reconstituted in 60 µL of water and vortexed for 2 min. 50 µL was injected on the LC-MS/MS system for analysis. All analyses were performed in triplicate.

### 4.2.4 Preparation of the calibration standards

Stock solutions of all standards (1 mg/mL) were prepared in 0.25% acetic acid (dissolved in water). Calibration curve standards were prepared by dilution of 2',3'-cAMP and 2',3'-cGMP stock solutions in water down to 20, 50, 100, 200, 500, 1000 and 2000 ng/mL concentrations. These samples for calibration were also spiked with 50 ng/mL isotope-labeled internal standards of 3',5'-cAMP and 3',5'-cGMP.

### 4.2.5 Instrumentation

For LC-MS/MS analysis an Alliance 2690 (Waters, Milford, MA, USA), equipped with an autosampler, column oven and binary pump was coupled to an API 3000 triple quadrupole mass spectrometer (Applied Biosystems, Toronto, Canada) with a Turbo Ion Spray (electrospray) source. The MS was operated in the negative-ion multiple reaction monitoring mode (MRM). Data acquisition was performed with Analyst 1.4.1 software.

HPLC and MS conditions and settings were as previously described [61]. A Zorbax SB-C18 column 150 mm × 3.0 mm i.d., 3.5 µm particle size (Agilent Technologies, Brussels, Belgium), coupled to a C18 Security Guard column 4 mm × 3.0 mm i.d. (Phenomenex, Torrance, CA, USA) was used for separations. Solvent A consisted of 0.1% formic acid and solvent B of acetonitrile/methanol/water in a 1/2/4 volume ratio. The flow rate was 0.35 mL/min. Linear gradient: initial conditions 100% A; then B from 0% to 50% in 10 min; next, B went to 100% B in 1 min and was maintained for 1 min, followed by column regeneration for 10 min. Total analysis time was 22 min. The column temperature was 25 °C and the autosampler temperature was set at 10 °C. MS instrument parameters were optimized for maximum sensitivity in MRM mode. Negative voltage and temperature were respectively set on -4200 V and 470 °C. Nebulizer gas was set at 15 psi, curtain gas at 6 psi and collision gas at 3 psi. The dwell time was 150 ms. The Q1 resolution was set on unit, the Q3 resolution was set on low.

## 4.3 Results and discussion

The LC-MS/MS approach for the quantification of 3',5'-cAMP, 3',5'-cGMP, 2',3'-cAMP and 2',3'-cGMP based on an SPE procedure on native silica (allowing selective cyclic NMP

accumulation and simultaneous protein and phospholipid removal) followed by reversed phase separation (allowing cNMP retention while eluting the salt in the void) was introduced in the previous chapter [61]. This methodology was developed specifically for analysis of these solutes in limited amounts of plasma and tissue samples. The detection limits (LOD, limit of detection) in 200 µL plasma were 0.023 and 0.038 ng/mL corresponding to 3.5 and 5.5 fmol (for 50 µL injections), for 3',5'-cAMP and 3',5'-cGMP, respectively. The LOD's of the isomers were 2.0 and 5.5 fmol, for 2',3'-cAMP and 2',3'-cGMP respectively. As this sensitivity proved to be about an order of magnitude higher than what was reached before for the detection of 3',5'-cAMP and 3',5'-cGMP in plant tissue by LC-MS/MS [34], the applicability of the developed methodology to assess the cNMP levels in vegetative tissue was investigated. *Arabidopsis* was selected as testing material because its importance as a model system for plant research. Moreover, given the previously reported implication of 3',5'-cNMPs in stress responses, I investigated whether a change in 2',3'-isomers is measurable.

A chromatogram representing the separation of 10 ng/mL (0.5 ng/50 µL injections) of a mixture of 3',5'-cAMP/cGMP and the corresponding 2',3'-isomers, as well as their respective MS/MS transitions is shown in Figure 4.1A. In Figure 4.1B the elution and transitions of the 2 isotopically labeled (3',5') standards, eluting slightly later than the lighter isotopic compositions (*i.e.* non-labeled 3',5'-derivates), are shown. Note that the combination of reproducible chromatography and the addition of the isotopically labeled internal standards ($^{2}D_{1}{}^{15}N_{3}$-3',5'-cGMP and $^{13}C_{10}$,$^{15}N_{5}$ -3',5'-cAMP) allowed unequivocal peak assignment at all times, as the 2',3'-isomers were eluting more than a minute before the labeled and natural 3',5'-cNMPs.

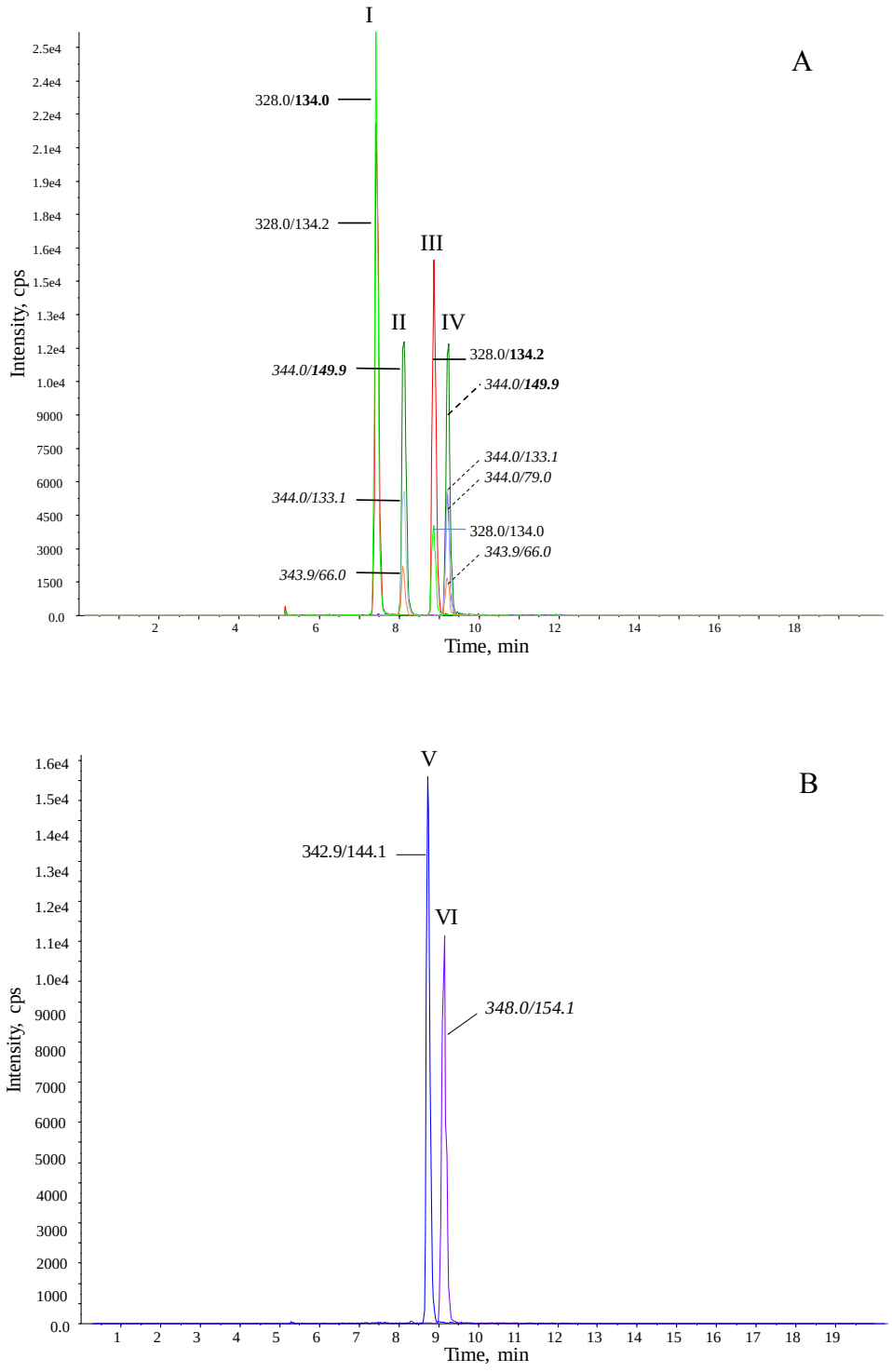


*Figure 4.1. MRM chromatogram of a mixture of: A/ I. 2',3'-cAMP, II. 2',3'-cGMP, III. 3',5'-cAMP and IV. 3',5'-cGMP and B/ V. Internal Standard 3'5'-cAMP and VI. Internal Standard 3',5'-cGMP at a concentration of 10 ng/mL for each analyte using the validated LC–MS/MS method. Multiple MRM transitions for the same molecule (quantification- and validation product ions) are shown. Quantification product ions are marked in bold.*

The previously developed sample preparation procedure for careful extraction of the cyclic nucleotides from tissue samples was applied for extraction of these solutes from plant leaves. This was based on the rationale that if the developed HILIC-SPE approach allowed high solute recovery while effectively removing co-eluting ion-suppressing solutes affecting LC-MS quantitation, similar method performance could be expected when applying this method for the analysis of cNMPs in vegetative tissue.

First, the frozen plants samples were homogenized manually with mortar and pestle with addition of liquid nitrogen. Exhaustive cyclic nucleotide extraction was obtained when extracting 25 mg plant tissue samples with consecutively 600µL 4% acetic acid in water and with 1200 µL acetonitrile. The recovery increased when raising the acidic content from 1 to 4%, but no further increase of the latter was obtained when more acid was used. The next steps were identical to the earlier described sample preparation method [61].

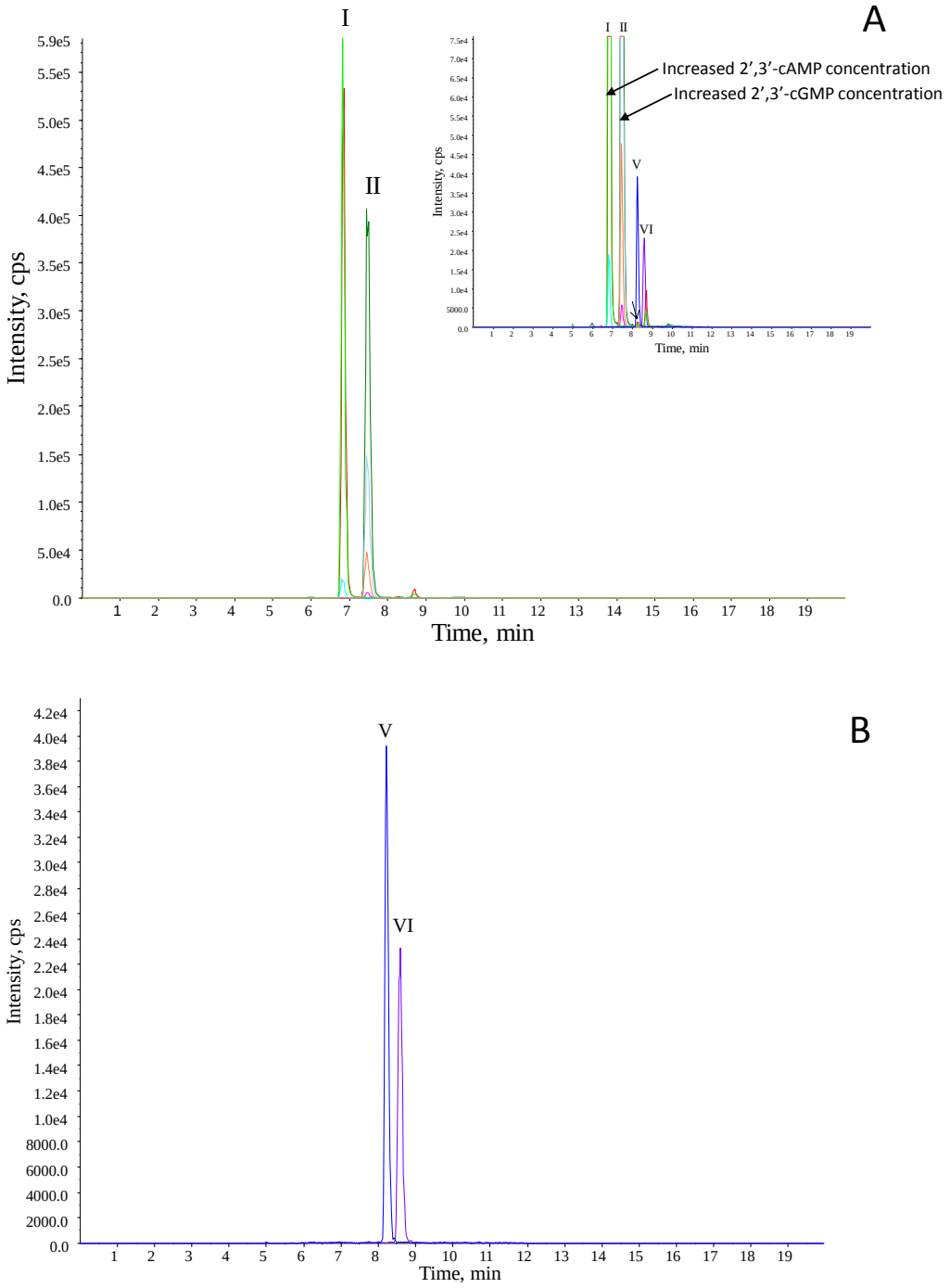


*Figure 4.2. A/ Representative MRM chromatogram of an Arabidopsis plant extract (25mg) stressed by scalpel after 30 min, after SPE on silica and analysis by LC–MS/MS. I. 2',3'-cAMP, II. 2',3'-cGMP. Spiked with 50 ng/mL internal standard of 3'5'-cAMP (V) and 3',5'-cGMP (VI). Measured concentrations are 548 pg/mg and 571 pg/mg for respectively 2',3'-cAMP and 2',3'-cGMP. Also a zoom of the chromatogram is displayed, showing the two 2',3'-isomers as well as the internal standards 3'5'-cAMP and 3'5'-cGMP. B/ MRM chromatogram of the same sample displaying only the internal standards 3'5'-cAMP and 3'5'-cGMP.*

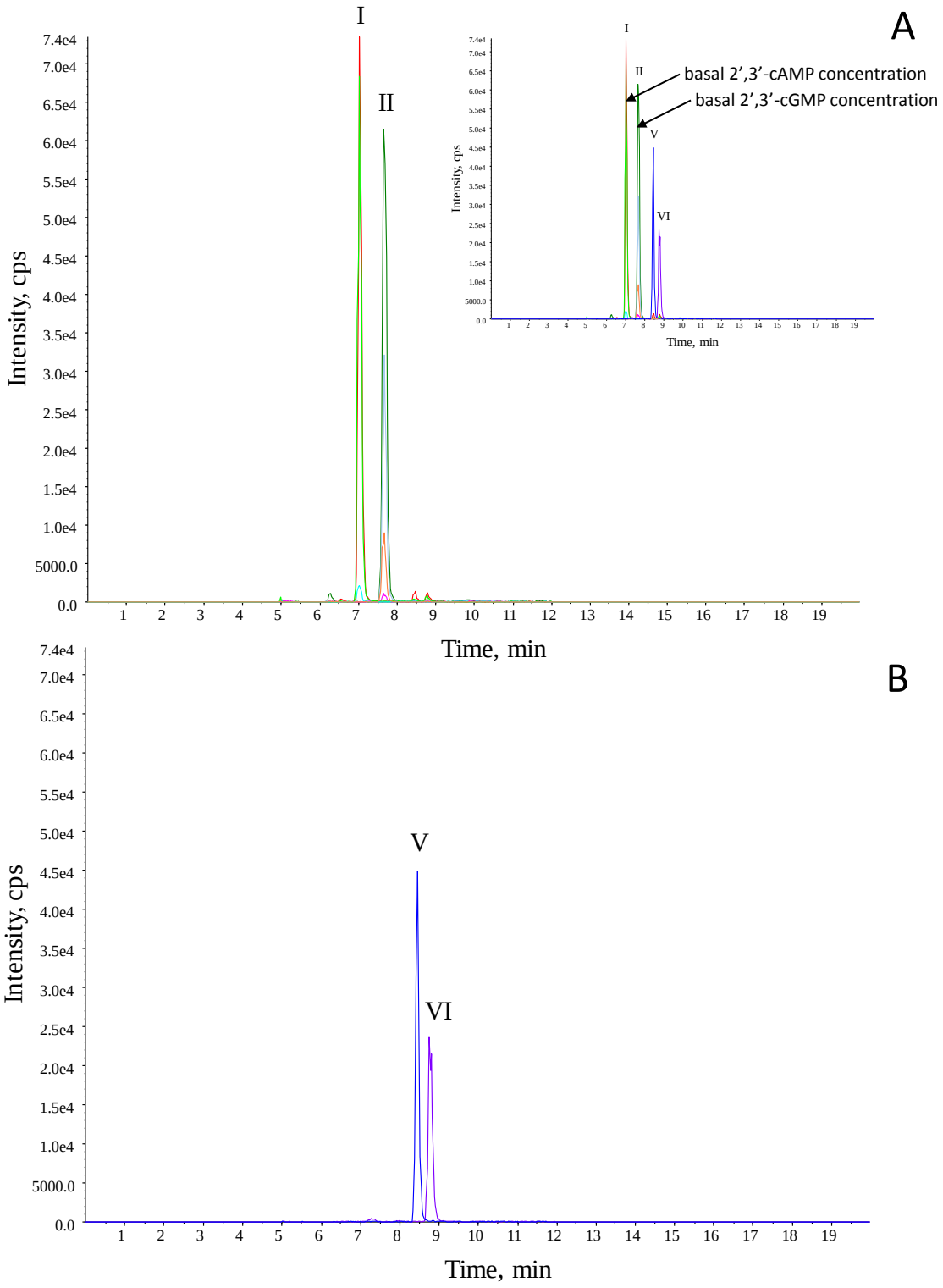


*Figure 4.3. A/ Representative MRM chromatogram of an unstressed Arabidopsis control plant extract (25mg), after SPE on silica and analysis by LC–MS/MS. I. 2',3'-cAMP, II. 2',3'-cGMP (respective concentrations: 84 pg/mg and 107 pg/mg). B/ representation of the specific transitions of the isotopically labeled 3'5'-cAMP (V) and 3',5'-cGMP (VI), added at 50 ng/mL concentrations.*

In Figure 4.2 chromatograms of the cNMP signals extracted from 25 mg stressed *Arabidopsis* leaves, are shown. The wounded leaves were removed from the plant 30 minutes after wounding. Intense signals for 2',3'-cAMP (I) and 2',3'-cGMP (II) became apparent (Figure 4.2.A). The 3',5'-cAMP (III) and 3',5'-cGMP (IV) signals were below the limit of detection and coeluted with the isotopically labeled standards (V and VI), represented in Figure 4.2B, at 8.4 and 8.6 min, respectively. Note that a small transition of 328.0/134.0, corresponding to the 3',5'-cAMP is visible (see arrow in Figure 4.2.A). Again this is based on the combination of reproducible chromatography and the addition of the isotopically labeled internal standards. However, as the intensity of the confirmatory transitions 328/107.2 and 328/92.2 was too low, these signals were not withheld as sufficiently positive 3',5'-cAMP identification allowing unambiguous quantification. For comparison, in Figure 4.3, corresponding chromatograms of the control samples are represented. No wounding stress was applied to these leaves, which were removed from the plants, followed by immediate quenching of the metabolism in liquid nitrogen. Peak areas from the 2',3'-cAMP (I) and 2',3'-cGMP (II) in the stressed leaves were over fivefold higher as compared to the unstressed leaves, pointing towards an accumulation of 2',3'-cAMP/cGMP in leaves in response to wounding stress.

This qualitative information was converted to quantitative data by internal standard calibration. $^{2}D_{1}{}^{15}N_{3}$-3',5'-cGMP and $^{13}C_{10}$,$^{15}N_{5}$ -3',5'-cAMP were thereby used as IS for 2',3'-cGMP and 2',3'-cAMP, respectively. Correlation coefficients ($R^2$) of 0.9953 and 0.9958 were obtained for both solutes. Several plant extracts of different batches were tested. The corresponding quantitative results, measured concentrations and relative standard deviations (RSD in %), are shown in Table 4.1. The quantitative data confirmed the significant increase in signal intensity of 2',3'-cAMP and 2',3'-cGMP, 30 minutes after wounding, between wounded and non-wounded *Arabidopsis* leaves, with an increase by a factor of 6.4 (± 0.2) and 5.2 (± 0.2) , respectively.

Table 4.1 shows prolonged application of the wounding stress up to 60 minutes resulted in a further increase in 2',3'-cGMP and 2',3'-cAMP concentration. These results were also confirmed after stressing the leaves with forceps across the middle of the leaf for 30 and 60 minutes [62]. An increase with a factor 3.8 (± 0.1) and 3.5 (± 0.1) for respectively 2',3'-cAMP and 2',3'-cGMP, as compared to the control plants could be observed after half an hour. Because the changes in concentration between stressed and control plant extracts are

significant, the need for a full validation study is less critical and was beyond the scope of this work.

*Table 4.1. Measured concentrations, with precision data, of 2',3'-cAMP and 2',3'-cGMP in plant extracts by LC-MS/MS.*

| Plant extract sample | Type of injury | Time of injury (min) | Conc. 2',3'-cAMP (pg/mg) | Precision (RSD %) | Conc. 2',3'-cGMP (pg/mg) | Precision (RSD %) |
|---|---|---|---|---|---|---|
| *Arabidopsis* | Scalpel | 30 | 552 | 7.4 | 570 | 5.9 |
| | | 30 (control) | 86 | 6.8 | 110 | 4.3 |
| | | 60 | 1024 | 6.4 | 1144 | 8.6 |
| | | 60 (control) | 546 | 4.2 | 598 | 8.5 |
| | Forceps | 30 | 1470 | 7.1 | 2020 | 7.8 |
| | | 30 (control) | 392 | 5.6 | 576 | 7.3 |
| | | 60 | 1442 | 6.9 | 2038 | 6.5 |
| | | 60 (control) | 992 | 7.3 | 1574 | 6.8 |

Note that a mild increase in the 2',3'-cNMP concentrations was also observed in the control samples, which were leaves of other plants from the same batch, possibly suggesting plant communication warranting further study. The observations are less distinct in *Arabidopsis* leaves after 60 minutes after wounding. The measured concentration fluxes can therefore be considered as an early response to plant wounding.

As described earlier, 2',3'-cNMP can arise by RNase activity [52]**.** These RNases could be activated by wounding the plants, which is suggested by the current observations. Further investigation is required concerning the functionality of the induction of 2',3'-derivates. The selective expression of the 2',3'-cAMP pathway *versus* the 3',5'-cAMP pathway for animals

and humans has already been described in the literature [63] as well as the fact that 2',3'-cAMP proves to inhibit cell proliferation [56,64]. Since growth reduction is also a typical response of plants after onset of stress [65], 2',3'-cNMP accumulation may be involved in cellular signaling controlling the inhibition of cell proliferation and perhaps involved in cell death, as suggested for animal systems [9].

## 4.4 Conclusion

A sensitive and accurate method to quantitate 2',3-cAMP and 2',3-cGMP concentrations in plant material was developed. The method consists of a HILIC-SPE sample preparation technique and a selective LC-MS/MS separation-detection method. *Arabidopsis thaliana* (Col-0) plants were selected as testing material. Concentrations in pg/mg of control and stressed plant material were compared. A significant increase in signal intensity of 2',3'-cAMP and 2',3'-cGMP was observed for wounded plants, especially for plants stressed after 30 min. A correlation between plant stress and both 2',3'-cAMP and 2',3'-cAMP concentrations in plant material was indicated. These observations and the method presented can be of use for future biological research to unravel the role of these cyclic nucleotides in plants under normal and stress conditions.

During this work, one of the persistent challenges was the ubiquitous presence of a high number of competing molecules in biological matrices for ionization in the soft ionization sources used. Although some of the issues were already successful addressed as described in chapter 3, the relevance of fundamentally tackling some of these issues, like due to the presence of phospholipids in blood or plasma, remained high. Because reversed phase combined with ESI LC-MS/MS is one of the most widely used methods for analysis in pharmaceutical and biochemical applications, the research in the next chapter was initiated as it could be beneficial for a large variety of applications.

## 4.5 References

[1] K. A. Lucas, G. M. Pitari, S. Kazerounian, I. Ruiz-Stewart, J. Park, S. Schulz, and S. A. Waldman, "Guanylyl cyclases and signaling by cyclic GMP," *Pharmacological Reviews*, vol. 52, pp. 375-413, Sep 2000.

[2] E. W. Sutherland, G. A. Robison, and R. W. Butcher, "Some aspects of biological role of adenosine 3',5'-monophosphate (cyclic AMP)," *Circulation*, vol. 37, pp. 279-306, 1968.

[3] J. L. Botsford and J. G. Harman, "Cyclic-AMP in prokaryotes," *Microbiological Reviews*, vol. 56, pp. 100-122, Mar 1992.

[4] M. Chinkers and D. L. Garbers, "Signal transduction by guanylyl cyclases," *Annual Review of Biochemistry*, vol. 60, pp. 553-575, 1991.

[5] E. W. Sutherland and T. W. Rall, "The properties of an adenine ribonucleotide produced with cellular particles, ATP, $Mg^{++}$, and epinephrine or glucagon," *Journal of the American Chemical Society*, vol. 79, pp. 3608-3608, 1957.

[6] A. T. Bender and J. A. Beavo, "Cyclic nucleotide phosphodiesterases: Molecular regulation to clinical use," *Pharmacological Reviews*, vol. 58, pp. 488-520, Sep 2006.

[7] E. Stokvis, H. Rosing, and J. H. Beijnen, "Stable isotopically labeled internal standards in quantitative bioanalysis using liquid chromatography/mass spectrometry: necessity or not?," *Rapid Communications in Mass Spectrometry*, vol. 19, pp. 401-407, 2005.

[8] M. Montminy, "Transcriptional regulation by cyclic AMP," *Annual Review of Biochemistry*, vol. 66, pp. 807-822, 1997.

[9] E. K. Jackson, J. Ren, and Z. Mi, "Extracellular 2 ',3 '-cAMP Is a Source of Adenosine," *Journal of Biological Chemistry*, vol. 284, pp. 33097-33106, Nov 27 2009.

[10] E. Boadu, S. Vaskinn, E. Sundkvist, R. Jaeger, and G. Sager, "Inhibition by guanosine cyclic monophosphate (cGMP) analogues of uptake of H-3 3',5'-cGMP without stimulation of ATPase activity in human erythrocyte inside-out vesicles," *Biochemical Pharmacology*, vol. 62, pp. 425-429, Aug 2001.

[11] E. Molokanova, B. Trivedi, A. Savchenko, and R. H. Kramer, "Modulation of rod photoreceptor cyclic nucleotide-gated channels by tyrosine phosphorylation," *Journal of Neuroscience*, vol. 17, pp. 9068-9076, Dec 1 1997.

[12] F. S. Gragasin, E. D. Michelakis, A. Hogan, R. Moudgil, K. Hashimoto, X. C. Wu, A. Haromy, and S. L. Archer, "The neurovascular mechanism of clitoral erection: nitric oxide and cGMP-stimulated activation of BKCa channels," *Faseb Journal,* vol. 18, pp. 1382-1391, Sep 2004.

[13] M. R. Vossler, H. Yao, R. D. York, M. G. Pan, C. S. Rim, and P. J. S. Stork, "cAMP activates MAP kinase and Elk-1 through a B-Raf- and Rap1-dependent pathway," *Cell,* vol. 89, pp. 73-82, Apr 4 1997.

[14] R. A. Levine, S. E. Lewis, J. Shulman, and Washingt.A, "Metabolism of cyclic adenosine 3',5'-monophosphate-8-14C by isolated, perfused rat liver," *Journal of Biological Chemistry,* vol. 244, pp. 4017-&, 1969.

[15] D. Schild and D. Restrepo, "Transduction mechanisms in vertebrate olfactory receptor cells," *Physiological Reviews,* vol. 78, pp. 429-466, Apr 1998.

[16] T. Misaka, Y. Kusakabe, Y. Emori, T. Gonoi, S. Arai, and K. Abe, "Taste buds have a cyclic nucleotide-activated channel, CNGgust," *Journal of Biological Chemistry,* vol. 272, pp. 22623-22629, Sep 5 1997.

[17] C. Gehring, "Adenyl cyclases and cAMP in plant signaling - past and present," *Cell Communication and Signaling,* vol. 8, p. 5, Jun 2010.

[18] W. Ma, Z. Qi, A. Smigel, R. K. Walker, R. Verma, and G. A. Berkowitz, "$Ca^{2+}$, cAMP, and transduction of non-self perception during plant immune responses," *Proceedings of the National Academy of Sciences of the United States of America,* vol. 106, pp. 20995-21000, Dec 8 2009.

[19] Y. Ma, R. K. Walker, Y. Zhao, and G. A. Berkowitz, "Linking ligand perception by PEPR pattern recognition receptors to cytosolic $Ca^{2+}$ elevation and downstream immune signaling in plants," *Proceedings of the National Academy of Sciences of the United States of America,* vol. 109, pp. 19852-19857, Nov 27 2012.

[20] J. Durner, D. Wendehenne, and D. F. Klessig, "Defense gene induction in tobacco by nitric oxide, cyclic GMP, and cyclic ADP-ribose," *Proceedings of the National Academy of Sciences of the United States of America,* vol. 95, pp. 10328-10333, Aug 18 1998.

[21] L. Donaldson, N. Ludidi, M. R. Knight, C. Gehring, and K. Denby, "Salt and osmotic stress cause rapid increases in *Arabidopsis thaliana* cGMP levels," *Febs Letters,* vol. 569, pp. 317-320, Jul 2 2004.

[22] M. Tunc-Ozdemir, C. Rato, E. Brown, S. Rogers, A. Mooneyham, S. Frietsch, R. Malho, and J. E. Harper, "Cyclic Nucleotide Gated Channels 7 and 8 Are Essential for Male Reproductive Fertility," *Plos One,* vol. 8, Feb 12 2013.

[23] M. Tunc-Ozdemir, C. Tang, M. R. Ishka, E. Brown, N. R. Groves, C. T. Myers, R. Mittler, and J. F. Harper, "A Cyclic Nucleotide-Gated Channel (CNGC16) in Pollen Is Critical for Stress Tolerance in Pollen Reproductive Development," *Plant Physiology,* vol. 161, pp. 1010-1020, Feb 2013.

[24] F. J. M. Maathuis and D. Sanders, "Sodium uptake in *Arabidopsis* roots is regulated by cyclic nucleotides," *Plant Physiology,* vol. 127, pp. 1617-1625, Dec 2001.

[25] S. P. Penson, R. C. Schuurink, A. Fath, F. Gubler, J. V. Jacobsen, and R. L. Jones, "cGMP is required for gibberellic acid-induced gene expression in barley aleurone," *Plant Cell,* vol. 8, pp. 2325-2333, Dec 1996.

[26] D. Bridges, M. E. Fraser, and G. B. G. Moorhead, "Cyclic nucleotide binding proteins in the *Arabidopsis thaliana* and Oryza sativa genomes," *Bmc Bioinformatics,* vol. 6, pp. 1-12, Jan 2005.

[27] N. Amrhein and P. Filner, "Adenosine 3'-5'-cyclic monophosphate in *Chlamydomonas-reinhardtii* - Isolation and characterization," *Proceedings of the National Academy of Sciences of the United States of America,* vol. 70, pp. 1099-1103, 1973.

[28] D. Salomon and J. P. Mascaren, "Auxin-induced synthesis of cyclic 3',5'-adenosine monophosphate in Avena coleoptiles," *Life Sciences Pt-2 Biochemistry General and Molecular Biology,* vol. 10, pp. 879-885, 1971.

[29] E. G. Brown, M. J. Edwards, R. P. Newton, and C. J. Smith, "Plurality of cyclic nucleotide phosphodiesterase in *Spinacea oleracea*: subcellular distribution, partial purification, and properties ," *Phytochemistry,* vol. 18, pp. 1943-1948, 1979.

[30] R. P. Newton, N. Gibbs, C. D. Moyse, J. L. Wiebers, and E. G. Brown, "Mass-spectrometric identification of adenosine 3'-5'-cyclic monophosphate isolated from a higher-plant tissue," *Phytochemistry,* vol. 19, pp. 1909-1911, 1980.

[31] E. G. Brown and R. P. Newton, "Occurrence of adenosine 3'-5'-cyclic monophosphate in plant-tissues," *Phytochemistry,* vol. 12, pp. 2683-2685, 1973.

[32] L. A. Lomovatskaya, A. S. Romanenko, N. V. Filinova, and L. V. Dudareva, "Determination of cAMP in plant cells by a modified enzyme immunoassay method," *Plant Cell Reports,* vol. 30, pp. 125-132, Jan 2011.

[33] A. Spiteri, O. M. Viratelle, P. Raymond, M. Rancillac, J. Labouesse, and A. Pradet, "Artefactual origins of cyclic-AMP in higher-plant tissues," *Plant Physiology,* vol. 91, pp. 624-628, Oct 1989.

[34] E. Witters, K. Vanhoutte, W. Dewitte, I. Machackova, E. Benkova, W. Van Dongen, and H. A. Van Onckelen, "Analysis of cyclic nucleotides and cytokinins in minute

plant samples using phase-system switching capillary electrospray-liquid chromatography-tandem mass spectrometry," *Phytochemical Analysis,* vol. 10, pp. 143-151, May-Jun 1999.

[35] R. P. Newton, L. Roef, E. Witters, and H. Van Onckelen, "Tansley Review No. 106 - Cyclic nucleotides in higher plants: the enduring paradox," *New Phytologist,* vol. 143, pp. 427-455, Sep 1999.

[36] M. K. Haddox, J. H. Stephens, and N. D. Goldberg, "Cyclic GMP in meristematic and elongating regions of bean root," *Federation Proceedings,* vol. 33, pp. 522-522, 1974.

[37] I. H. Ames, R. A. Richman, and J. P. Weiss, "Is cyclic-GMP involved in the regulation of tumorigenesis in the *Nicotiana* genetic tumor system," *Plant and Cell Physiology,* vol. 21, pp. 367-372, 1980.

[38] R. P. Newton, E. E. Kingston, D. E. Evans, L. M. Younis, and E. G. Brown, "Occurrence of guanosine 3',5'-cyclic monophosphate (cyclic-GMP) and associated enzyme systems in *Phaseolus vulgaris*," *Phytochemistry,* vol. 23, pp. 1367-1372, 1984.

[39] R. Walden, "The alphabet soup of plant intracellular signalling: enter cyclic nucleotides," *Current Opinion in Plant Biology,* vol. 1, pp. 419-423, Oct 1998.

[40] H. Ehsan, J. P. Reichheld, L. Roef, E. Witters, F. Lardon, D. Van Bockstaele, and H. Van Onckelen, "Effect of indomethacin on cell cycle dependent cyclic AMP fluxes in tobacco BY-2 cells," *Febs Letters,* vol. 422, pp. 165-169, Jan 30 1998.

[41] R. P. Newton and C. J. Smith, "Cyclic nucleotides," *Phytochemistry,* vol. 65, pp. 2423-2437, Sep 2004.

[42] B. Kaplan, T. Sherman, and H. Fromm, "Cyclic nucleotide-gated channels in plants," *Febs Letters,* vol. 581, pp. 2237-2246, May 25 2007.

[43] Y. Teng, W. Xu, and M. Ma, "cGMP is required for seed germination in *Arabidopsis thaliana*," *Journal of Plant Physiology,* vol. 167, pp. 885-889, 2010.

[44] S. Pfeiffer, B. Janistyn, G. Jessner, H. Pichorner, and R. Ebermann, "Gaseous nitric oxide stimulates guanosine-3',5'-cyclic monophosphate (cGMP) formation in spruce needles," *Phytochemistry,* vol. 36, pp. 259-262, May 1994.

[45] A. J. Millar, R. B. McGrath, and N. H. Chua, "Phytochrome phototransduction pathways," *Annual Review of Genetics,* vol. 28, pp. 325-349, 1994.

[46] A. Moutinho, P. J. Hussey, A. J. Trewavas, and R. Malho, "cAMP acts as a second messenger in pollen tube growth and reorientation," *Proceedings of the National*

*Academy of Sciences of the United States of America*, vol. 98, pp. 10481-10486, Aug 28 2001.

[47] L. V. Bindschedler, F. Minibayeva, S. L. Gardner, C. Gerrish, D. R. Davies, and G. P. Bolwell, "Early signalling events in the apoplastic oxidative burst in suspension cultured French bean cells involve cAMP and $Ca^{2+}$," *New Phytologist*, vol. 151, pp. 185-194, Jul 2001.

[48] J. H. Payne and R. G. Cooke, "Effects of beta-adrenergic agonists and other putative transmitters on progesterone production by dispersed ovine luteal cells," *Animal Reproduction Science*, vol. 35, pp. 91-97, Mar 1994.

[49] G.-T. Huang, S.-L. Ma, L.-P. Bai, L. Zhang, H. Ma, P. Jia, and Z. F. Guo, "Signal transduction during cold, salt, and drought stresses in plants," *Molecular Biology Reports*, vol. 39, pp. 969-987, Feb 2012.

[50] J.-C. Isner and F. J. M. Maathuis, "Measurement of cellular cGMP in plant cells and tissues using the endogenous fluorescent reporter FlincG," *Plant Journal*, vol. 65, pp. 329-334, Jan 2011.

[51] H. Richards, S. Das, C. J. Smith, L. Pereira, A. Geisbrecht, N. J. Devitt, J. van Geyschem, A. G. Brenton, and R. P. Newton, "Cyclic nucleotide content of tobacco BY-2 cells," *Phytochemistry*, vol. 61, pp. 531-537, Nov 2002.

[52] J. Ren, Z. Mi, N. A. Stewart, and E. K. Jackson, "Identification and Quantification of 2 ',3 '-cAMP Release by the Kidney," *Journal of Pharmacology and Experimental Therapeutics*, vol. 328, pp. 855-865, Mar 2009.

[53] E. K. Jackson, "The 2 ',3 '-cAMP-adenosine pathway," *American Journal of Physiology-Renal Physiology*, vol. 301, pp. F1160-F1167, Dec 2011.

[54] E. K. Jackson, J. Ren, D. Cheng, and Z. Mi, "Extracellular cAMP-adenosine pathways in the mouse kidney," *American Journal of Physiology-Renal Physiology*, vol. 301, pp. F565-F573, Sep 2011.

[55] E. K. Jackson, J. Ren, and D. G. Gillespie, "2 ',3 '-cAMP, 3 '-AMP, and 2 '-AMP inhibit human aortic and coronary vascular smooth muscle cell proliferation via A(2B) receptors," *American Journal of Physiology-Heart and Circulatory Physiology*, vol. 301, pp. H391-H401, Aug 2011.

[56] J. D. Verrier, T. C. Jackson, R. Bansal, P. M. Kochanek, A. M. Puccio, D. O. Okonkwo, and E. K. Jackson, "The brain in vivo expresses the 2 ',3 '-cAMP-adenosine pathway," *Journal of Neurochemistry*, vol. 122, pp. 115-125, Jul 2012.

[57] J. Vercammen, H. Pham-Tuan, I. Arickx, D. Van der Straeten, and P. Sandra, "Monitoring of isothiocyanates emanating from *Arabidopsis thaliana* upon paraquat spraying," *Journal of Chromatography A*, vol. 912, pp. 127-134, Mar 30 2001.

[58] Q. Gu, F. David, F. Lynen, K. Rumpel, J. Dugardeyn, D. Van Der Straeten, G. W. Xu, and P. Sandra, "Evaluation of automated sample preparation, retention time locked gas chromatography-mass spectrometry and data analysis methods for the metabolomic study of *Arabidopsis* species," *Journal of Chromatography A*, vol. 1218, pp. 3247-3254, May 27 2011.

[59] E. R. Santarem, B. Pelissier, and J. J. Finer, "Effect of explant orientation, pH, solidifying agent and wounding on initiation of soybean somatic embryos," *In Vitro Cellular & Developmental Biology-Plant*, vol. 33, pp. 13-19, Jan-Mar 1997.

[60] N. C. Soares, R. Francisco, J. M. Vielba, C. P. Ricardo, and P. A. Jackson, "Associating Wound-Related Changes in the Apoplast Proteome of Medicago with Early Steps in the ROS Signal-Transduction Pathway," *Journal of Proteome Research*, vol. 8, pp. 2298-2309, May 2009.

[61] T. Van Damme, Y. H. Zhang, F. Lynen, and P. Sandra, "Determination of cyclic guanosine- and cyclic adenosine monophosphate (cGMP and cAMP) in human plasma and animal tissues by solid phase extraction on silica and liquid chromatography-triple quadrupole mass spectrometry," *Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences*, vol. 909, pp. 14-21, Nov 2012.

[62] C. C. C. Chang, L. Ball, M. J. Fryer, N. R. Baker, S. Karpinski, and P. M. Mullineaux, "Induction of ascorbate peroxidase 2 expression in wounded *Arabidopsis* leaves does not involve known wound-signalling pathways but is associated with changes in photosynthesis," *Plant Journal*, vol. 38, pp. 499-511, May 2004.

[63] J. D. Verrier, J. L. Exo, T. C. Jackson, J. Ren, D. G. Gillespie, R. K. Dubey, and E. K. Jackson, "Expression of the 2 ',3 '-cAMP-adenosine pathway in astrocytes and microglia," *Journal of Neurochemistry*, vol. 118, pp. 979-987, Sep 2011.

[64] R. K. Dubey, M. Rosselli, D. G. Gillespie, Z. Mi, and E. K. Jackson, "Extracellular 3 ',5 '-cAMP-Adenosine Pathway Inhibits Glomerular Mesangial Cell Growth," *Journal of Pharmacology and Experimental Therapeutics*, vol. 333, pp. 808-815, Jun 2010.

[65] C. Granier and F. Tardieu, "Water deficit and spatial pattern of leaf development. Variability in responses can be simulated using a simple model of leaf development," *Plant Physiology*, vol. 119, pp. 609-619, Feb 1999.

# Chapter 5. Solid phase extraction based on hydrophilic interaction liquid chromatography with acetone as eluent for eliminating matrix effects in the analysis of biological fluids by LC-MS

Adapted from T. Van Damme, M. Lachova, F. Lynen, R. Szucs, and P. Sandra, "Solid-phase extraction based on hydrophilic interaction liquid chromatography with acetone as eluent for eliminating matrix effects in the analysis of biological fluids by LC-MS," *Analytical and Bioanalytical Chemistry*, vol. 406, pp. 401-407, Jan 2014. The practical work related to this chapter has been performed on an equal basis by Thomas Van Damme and Mirka Lachova.

## *Summary*

Analysis of drugs and metabolites in biological matrices such as blood or plasma by LC-MS is routinely challenged by the presence of large quantities of competing molecules for ionization in soft ionization sources, such as proteins and phospholipids. While the former can easily be removed by protein precipitation, pre-analytical extraction of the latter is necessary because they show very high retention in reversed phase LC resulting in long analysis times or in ion suppression effects when not eluted before the next runs. A novel HILIC based SPE approach making use of silica cartridges and of acetone as organic solvent, is introduced in this chapter as a potent alternative to current commercial methods for phospholipid removal. The methodology was developed and tested for a broad polarity range of pharmaceutical solutes (log P from 0 to 6.6) and broad applicability can therefore be envisaged.

## 5.1 Introduction

Quantitative analysis of solutes in biological fluids for pharmaceutical drug metabolism studies relies today almost entirely on the use of LC-MS/MS [1]. The main reason thereof is the unique ability of this technique to quantify a broad variety of solutes, in acceptable volumes of complex matrices such as blood, urine, cerebrospinal fluid, *etc*., in a selective and reliable way down to the ng/mL and even pg/mL quantities as required by regulatory

instances. Because of the need for faster analysis on shorter columns combined with the wish to decrease solute detection limits and sample volumes and because of the inherent sensitivity of state-of-the-art LC-MS/MS instrumentation, an increasing disparity appears between the quantities of samples to be analyzed and on-column matrix concentrations. This evolution is unfavourably affecting ion suppression phenomena taking place in atmospheric pressure ionisation sources, negatively influencing the figures of merit of quantitative methods, whereby trace analysis methods are especially disturbed [2-7]. In this process, first described by Tang and Kebarle in 1993 for electrospray ionisation [8], matrix components ubiquitously present in biological samples, such as phospholipids in plasma, tend to out-compete the solute of interest for ionisation [9,10]. Extensive overviews of matrix effects in LC-MS were *e.g.* issued by Cappiello *et al*. and by Michotte *et al*. whereby detailed descriptions are provided on when these phenomena might be expected, how they can be evaluated and which strategies are currently used to overcome them [11,12]. Special emphasis is thereby also set on sample-preparation procedures and recent improvements on chromatographic and mass spectrometric conditions. Note that ESI has thereby been shown to depict a higher susceptibility to this phenomenon compared to atmospheric pressure chemical ionization (APCI) [9,13,14]. Competition for the available charges and for access to the droplet surface [9,15] droplet viscosity changes affecting the surface tension and in this way the efficacy of ion formation [16], the formation of solid salt micro-particles and of neutral ion pairs in the droplets [16-20] have been described as the main causes of these ion suppression problems.

Typically proteins and particularly phospholipids (such as phosphatidylcholines and lyso-phosphatidylcholines), are interfering in one of those ways, although the precise mechanism at hand for a given problem is sometimes hard to assess. This because the phenomenon strongly depends on the character of the analytes, of the co-eluting components and of the mobile phase effluent composition as well as on the flow rate at that particular time during the analysis. However, whereby in reversed phase LC the elution of proteins and also of salts is fairly easily controllable, phospholipids accumulate on-column and often elute only during later analyses, affecting MS sensitivity in an uncontrolled way.

This phenomenon is typically taking place in a high throughput environment where analysis times should be as short as possible. Straightforward selectivity tuning to avoid peak overlap

[21], extensive column flushing with strong solvents for complete phospholipid elution and the use of ion suppression free micro- and nano-LC-MS techniques are thereby considered time-consuming or less favorable approaches due to regulatory concerns, respectively. As the use of stable isotopically labeled internal standards (SIL), when available, has also been shown not to correct appropriately for the ion suppression effects [22], the only viable current approach involves phospholipid removal by sample preparation.

Although in some cases conventional protein precipitation followed by dilution is sufficient to supress the phenomenon [13,23], liquid-liquid extraction (LLE) [14,24-26], solid phase extraction (SPE) and dispersive SPE [27,28] approaches have been developed for phospholipid removal. SPE based approaches are thereby preferred as they are considered the most straightforward, generic and automatable [14,29-34]. In the last few years, various SPE strategies have been developed for phospholipid removal, often combined with on-cartridge protein precipitation, including HybridSPE (Supelco, St. Louis, MO, USA) based on zirconia-bonded silica particles which exhibits strong but selective affinity for phospholipids [35-39], Phree-SPE (Phenomenex, Torrance, CA, USA) Evolute (Biotage, Uppsala, Sweden) and Ostro (Waters, Milford, MA, USA). The use of automated SPE approaches, in-line with HPLC, where phospholipids are removed via on-line trapping approaches have also been introduced [40,41].

In this contribution an alternative, cheap and fast silica based HILIC SPE approach is developed for selective phospholipid removal and protein precipitation from a broad polarity range of selected pharmaceuticals. The peculiar benefits of using acetone as HILIC-SPE solvent are thereby demonstrated.

## 5.2 Experimental

### 5.2.1 Chemicals and materials

Zidovudine, sulfadimethoxine, carbamazepine, risperidone, propranolol, amitriptyline, trifluoroperazine and terfenadine were purchased from Sigma-Aldrich (Bornem, Belgium). Sulfamethazine and indomethacine were from Fluka (St. Louis, MO, USA). The stock solutions (1 mg/mL) were prepared in methanol and stored at -18°C. The stock solutions were

diluted in water or blank human serum (University Hospital, Ghent University, Belgium) at 25 ng/mL (ppb). Methanol, water, acetonitrile, ammonium acetate and formic acid were obtained from Biosolve (Valkenswaard, the Netherlands) and acetone from Sigma-Aldrich (Bornem, Belgium). 2-propanol was purchased from Fisher Scientific (Erembodegem, Belgium). HyperSep Si SPE cartridges (50 mg/1 mL) were purchased from Thermo Fisher Scientific (Erembodegem, Belgium) and Hybrid SPE – Phospholipid ULTRA cartridges (30 mg/1 mL) from Sigma-Aldrich (Bornem, Belgium). PVDF 0,45 µm microfilters from Grace Division (Lokeren, Belgium) were used. For SPE, a Visiprep SPE vacuum manifold 12-port model from Supelco (Bellefonte, PA, USA) was used.

### 5.2.2 Instrumentation

All experiments were performed on an Alliance 2690 Separation Module (Waters, Milford, MA, USA) equipped with autosampler, column oven and binary pump. The LC system was coupled to an API 3000 triple quadrupole mass spectrometer (Applied Biosystems, Toronto, Canada) with Turbo Ion Spray (electrospray) source. Data acquisition was performed with Analyst 1.4.1. software.

### 5.2.3 LC-MS/MS conditions

Reversed phase LC separations were undertaken using two columns. The first column was a Zorbax SB C18, 50 mm x 2.1 mm i.d., 3.5 µm particle size (RPLC 1). The second column was a Zorbax SB C18, 50 mm x 2.1 mm i.d., 1.8 µm particle size (RPLC 2). The columns were purchased from Agilent Technologies (Brussels, Belgium). The mobile phase consisted of solvent A 0.1% formic acid in water, B 0.1% formic acid in methanol and C 2-propanol. The gradient profile of RPLC 1 and RPLC 2 is outlined in Table 5.1A and B, respectively.

*Table 5.1 The gradient profiles of RPLC 1 (A) and RPLC 2 (B).*

| Time (min) | Solvent A (%) | Solvent B (%) | Solvent C (%) |
|---|---|---|---|
| 0 | 80 | 20 | 0 |
| 1 | 0 | 100 | 0 |
| 1.5 | 0 | 55 | 45 |
| 7.5 | 0 | 55 | 45 |
| 7.7 | 0 | 100 | 0 |
| 7.9 | 80 | 20 | 0 |

A

| Time (min) | Solvent A (%) | Solvent B (%) | Solvent C (%) |
|---|---|---|---|
| 0 | 80 | 20 | 0 |
| 1 | 0 | 100 | 0 |
| 6 | 0 | 100 | 0 |
| 6.5 | 0 | 50 | 50 |
| 8.5 | 0 | 50 | 50 |
| 9 | 80 | 20 | 0 |

B

In both methods, the flow was 200 µL/min, the column temperature was set at 35°C and the autosampler temperature at 20°C. Injection volume was 2 µL. Both columns were equipped with a C18 Security Guard column 4 mm x 2.0 mm i.d. (Phenomenex, Torrance, CA, USA).

HILIC separations were performed on a Zorbax RX-SIL, 100 mm x 3 mm i.d., 1.8 µm particle size. The mobile phase was composed of solvent A 5 mM ammonium acetate in water, B acetonitrile and C acetone. The following mobile phase compositions were applied in isocratic runs: 10% A and 90% B, 10% A and 90% C, 5% A and 95% B.

The Turbo Ion Spray voltage and temperature were at 5000 V and 400°C, respectively. The gas settings were: nebulizer gas 15, curtain gas 8; collision gas 6. Declustering potential, focusing potential, entrance potential, collision energy and collision cell exit potential were optimized for each pharmaceutical compound. All analytes were monitored by multiple reaction monitoring (MRM). MRM transitions and compound-dependent parameters are summarized in Table 5.2. Entrance potential and dwell time for all compounds were set at 10

V and 150 ms, respectively. The mass spectrometer was operated in the positive mode. Log P values were calculated by the KOWWIN™ software version 1.68 from the United States Environmental Protection Agency (EPA) website.

*Table 5.2 MRM transitions and compound-dependent parameters.*

| Nr. | Compound | Log P | Precursor ion (m/z) Q1 | Product ion (m/z) Q3 | Declustering potential (V) | Focusing potential (V) | Collision energy (V) | Collision cell exit potential (V) |
|---|---|---|---|---|---|---|---|---|
| 1 | Sulfamethazine | 0.3 | 279.2 | 186.1 | 56 | 230 | 23 | 12 |
| 2 | Zidovudine | 0 | 268.2 | 127.2 | 41 | 180 | 19 | 8 |
| 3 | Risperidone | 2.7 | 411.3 | 191.1 | 56 | 240 | 43 | 12 |
| 4 | Sulfadimethoxine | 1.6 | 311.2 | 156.2 | 31 | 140 | 29 | 10 |
| 5 | Propranolol | 3.0 | 260.2 | 116.2 | 56 | 220 | 25 | 6 |
| 6 | Carbamazepine | 2.5 | 237.2 | 194.3 | 51 | 150 | 29 | 12 |
| 7 | Amitriptyline | 5.0 | 278.3 | 232.9 | 51 | 300 | 21 | 16 |
| 8 | Terfenadine | 6.6 | 472.2 | 436.1 | 31 | 90 | 33 | 12 |
| 9 | Trifluoroperazine | 5.0 | 408.2 | 141.1 | 101 | 80 | 35 | 8 |
| 10 | Indomethacin | 4.3 | 358.1 | 138.9 | 66 | 270 | 25 | 8 |
| Lyso PC | Lyso-phosphatidylcholine (16:0), (18:2), (18:1) | | 496.6<br>520.7<br>522.6 | 184.2 | 40 | 170 | 25 | 10 |
| PC | Phosphatidylcholine (16:0/20:4), (16:0/18:2), (16:0/18:1), (18:0/18:2) | | 758.7<br>760.7<br>782.7<br>786.7 | 184.2 | 40 | 170 | 25 | 10 |

## 5.2.4 Sample preparation

During the course of this study, several modifications on a series of SPE procedures were performed. Only the most successful procedure using silica particles (HILIC SPE) is described in detail.

Human blood plasma, stored at -20°C was thawed in a water bath at room temperature, vortexed for 1 min and filtered through the microfilter. 100 µL plasma spiked at the 25 ppb level was mixed with 300 µL 0.1% formic acid in acetone. The sample was vortexed for 1 min and centrifuged at 3000 rpm for 3 min. The supernatant was diluted with 600 µL acetone. The SPE cartridge was preconditioned by flushing the cartridge with 1 mL methanol and 2

mL 5 mM ammonium acetate in water/acetone (5/95). A collection vial was inserted into the SPE vacuum manifold and the complete sample (*ca*. 1 mL) was loaded on the cartridge and vacuum was applied. The cartridge was then flushed with 2 mL of 5 mM ammonium acetate in water/acetone (5/95). The effluent of loading and flushing was evaporated to dryness under a nitrogen flow and reconstituted in 200 µL water 0.1% formic acid/methanol (50/50). 2 µL was injected. Preconditioning, loading and flushing of the SPE cartridge was performed with vacuum at 10 mmHg.

## 5.3 Results and discussion

Both reversed phase LC methods developed (see 5.2.3) allow the analysis of most active pharmaceutical ingredients (log P from 0 to 6.6). 25 ng/mL (50 pg on column) of each solute was thereby analyzed by LC-MS/MS in the MRM mode as ion suppression phenomena are occurring at those concentrations levels and below. A very important (and uncommon) aspect in routine RPLC methods is that a ternary gradient is applied whereby 2-propanol is replacing methanol at the end of the gradient [42-44]. This allows for swift phospholipid elution and visualization of these important ion suppressors. This was part of the novelty research conducted in this thesis. Figure 5.1A and 5.1B show MRM chromatograms obtained for the analysis of the pharmaceuticals as such and when spiked to plasma deproteinized with acetonitrile. In the latter the most prevalent phospholipids in plasma (Table 5.2) are visible, and it can be seen that the elution window of the pharmaceutical species seems not to overlap with the pharmaceutical compounds. Note, however, that the MS signal decreases the closer the solutes are eluting to the lyso-phospholipds. In Figure 5.1C the full scan Q1 MRM chromatogram of the same sample as in Figure 5.1B is presented illustrating the presence of an early eluting polar fraction (salts) and the late eluting highly retained fraction. The latter fraction spans from 5 to 10 min explaining its direct influence on the ionization efficiency of the solutes 3 to 10. Altering the chromatographic parameters in such a way that target solutes are not eluting within regions where ion suppression is occurring, is unrealistic. On the other hand, the selecting conditions in which the lipids are not eluting within the timeframe of the "target" chromatogram is not applicable as well as they will slowly elute in subsequent chromatograms. Moreover, the concentration of the phospholipids varies from sample to

sample resulting in different ionization suppression profiles decreasing the consistency of the analytical data. The strong influence of the phospholipid zone on the ionization efficiency of the solutes is also nicely illustrated via post-column infusion, a technique developed by Bonfiglio *et al*. [45]. A diluted solution of terfenadine (10 ng/mL methanol) was added post-column to the column effluent at 3 µL/min. 2 µL of neat solvent *i.e.* 0.1% formic acid/methanol (50/50) (Figure 5.2A), of deproteinized plasma reconstituted in 0.1% formic acid/methanol (50/50) (Figure 5.2B) and of the HILIC SPE extract (Figure 5.2C) were thereby injected on the column, respectively. Drops in the MRM trace of the pharmaceutical are only visible in Figure 5.2B corresponding to the elution time of the salts (not often interfering in RPLC) and especially of the phospholipids [46].

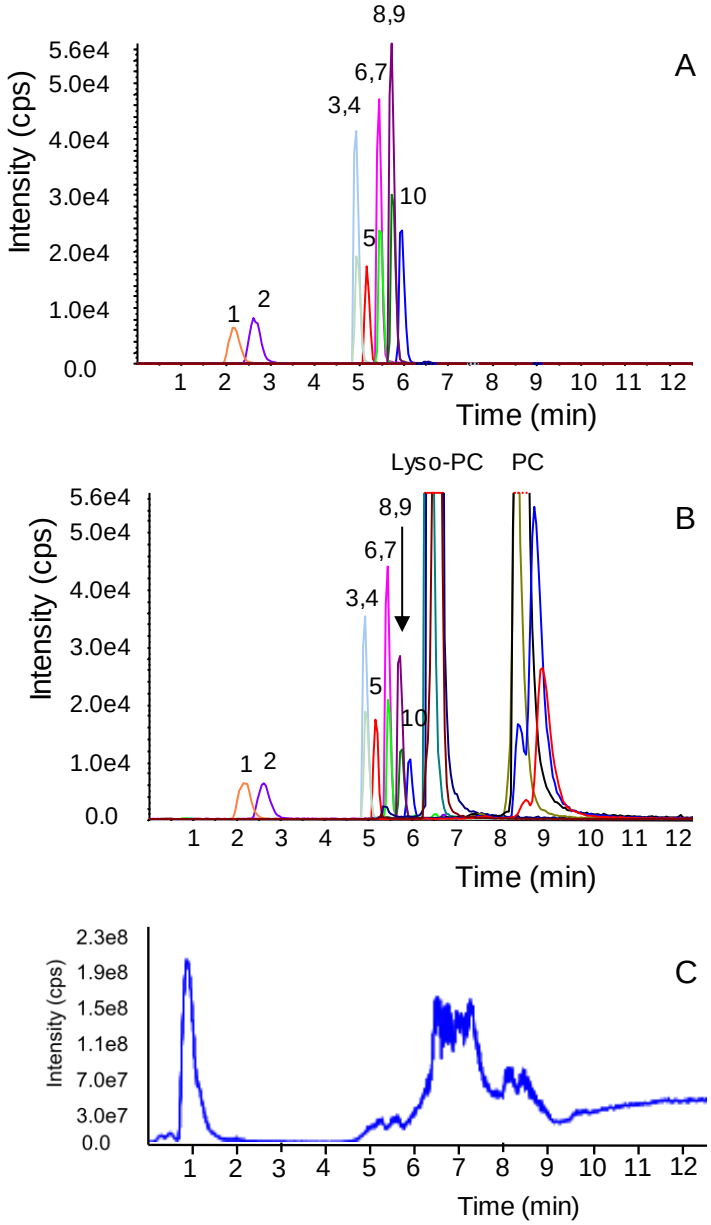


*Figure 5.1. MRM chromatograms of the standard mixture at 25 ng/mL (A) and spiked supernatant at 25 ng/mL of a protein precipitated plasma sample (B). Full scan Q1 MRM analysis of the same sample (C). Conditions method RPLC 1. Peak numbering as in Table 2.*

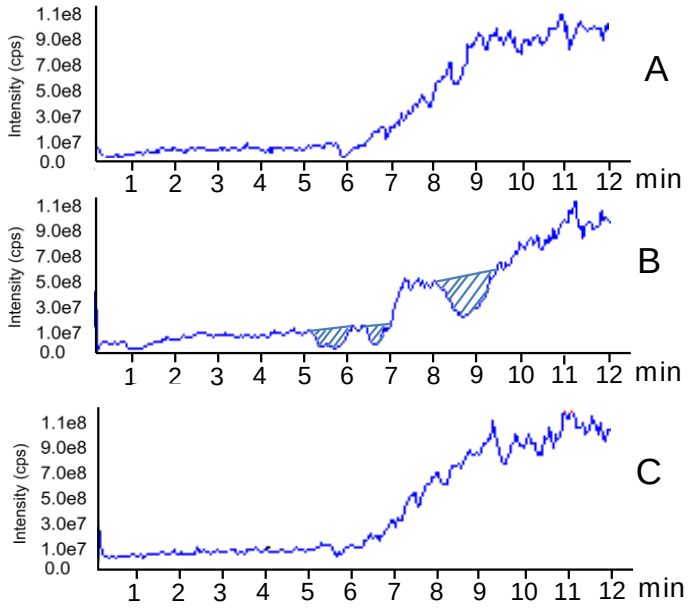


*Figure 5.2. Post-column infusion traces of MRM of a standard solution of terfenandine (10 ng/mL) at 3 µL/min for A. Neat solvent, B. Blank protein precipitated plasma supernatant and C. Hybrid SPE treated plasma sample. Conditions method RPLC 1.*

Although various SPE approaches for phospholipid (mainly phosphatidylcholines and lyso-phosphatidylcholines) removal have recently been commercialized, none seems to be exploiting high zwitterionic retention typically observed in HILIC analysis of biomolecules [47], where solutes such as, for example nucleoside phosphates, are often highly retained. Before using HILIC SPE, the separation/fractionation/elimination of phospholipids from plasma was studied by conventional HILIC LC. The same spiked and deproteinized plasma sample was analyzed on a neat silica column in the isocratic mode with different solvent compositions. Figure 5.3A shows the separation with 10% 5 mM ammonium acetate in water/90% acetonitrile which is more or less the standard mobile phase composition for HILIC. Some of the pharmaceuticals elute very early, others later and some of them are overlapping with the phosphatidylcholines. The latter have only half of the retention compared to the more polar lyso-phosphatidylcholines. Note that the elution order in RP LC is reversed. Although acetone is considered a weaker solvent in HILIC, my experience with selectivity changes in HILIC when shifting to other solvents pushed me to evaluate the selectivity of acetone as alternative solvent to better fractionate the pharmaceuticals from the phospholipids. Figure 5.3B shows the separation with 10% 5 mM ammonium acetate in

water/90% acetone. The selectivity differences compared to Figure 5.3A are remarkable. The pharmaceuticals exhibit a much shorter analysis time and are completely, whatever their log P value, separated from the phosphatidylcholines and lyso-phosphatidylcholines. When utilizing acetone in combination with reducing the aqueous content to 5% (Figure 5.3C), very strong phospholipid retention was obtained (no elution in 80 min) with only a small retention increase for the pharmaceuticals.

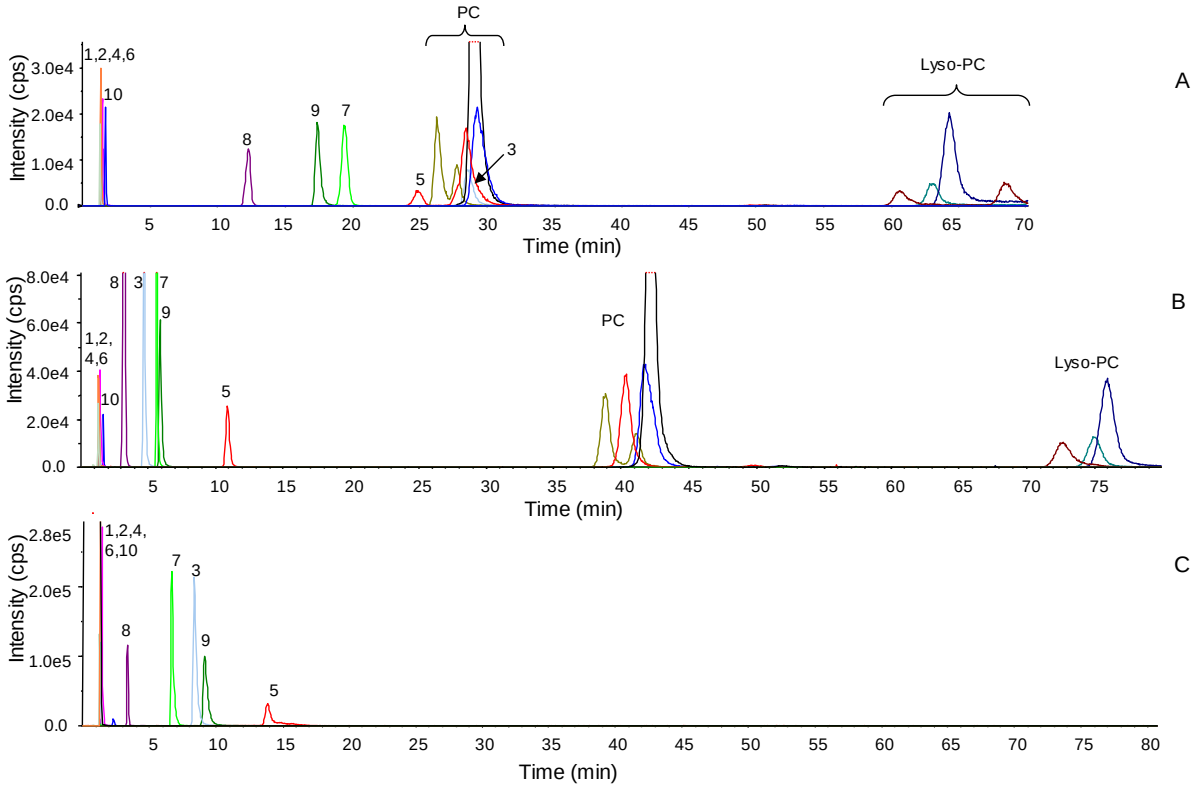


*Figure 5.3. HILIC MRM chromatograms of a spiked supernatant at 25 ng/mL of a protein precipitated plasma sample. A. 10% 5 mM ammonium acetate in water:90% acetonitrile. B. 10% 5 mM ammonium acetate in water: 90% acetone, C. 5% 5 mM ammonium acetate in water: 95% acetone. Conditions method HILIC. Peak numbering as in Table 5.2.*

Based on these observations a procedure for protein precipitation and phospholipid removal by HILIC SPE was developed. In order not to split denaturation and sample preparation for HILIC SPE such that extra evaporation and reconstitution steps are mandatory, deproteinization with acetone containing 0.1% formic acid was compared to classical protein precipitation with acetonitrile containing 0.1% formic acid as often applied in other SPE protein/phospholipid procedures such as the hybrid SPE. Differences between the two

methods in terms of protein removal and pharmaceutical recoveries were only marginal. The complete supernatant was then diluted with acetone to provide a solution of 10% water/90% acetone that was loaded on the cartridge. The solvent combination 5% water/95% is used to quickly elute all pharmaceutical solutes while retaining the phospholipids. After (fast) evaporation of the collected eluent fractions the solutes were reconstituted in 50/50 water 0.1% formic acid/methanol for LC-MS/MS analysis.

In Figure 5.4, the MRM chromatograms of the pharmaceuticals as such (A), spiked before the HILIC SPE method in plasma (B) and spiked after the HILIC SPE method in plasma (C), at 25 ppb are presented. No lyso-phosphatidylcholines and phosphatidylcholines are detected in Figure 5.4B and 5.4C. The post-column infusion experiment in Figure 5.2C also showed that a serum sample treated HILIC SPE does not show any ion suppression in the region where the phospholipids elute. By comparing the MS response in the samples after SPE with the MS response for the analytes in the standard solution, the matrix effect was calculated. The mean value for n=3 with the RSD% are given in Table 5.3.

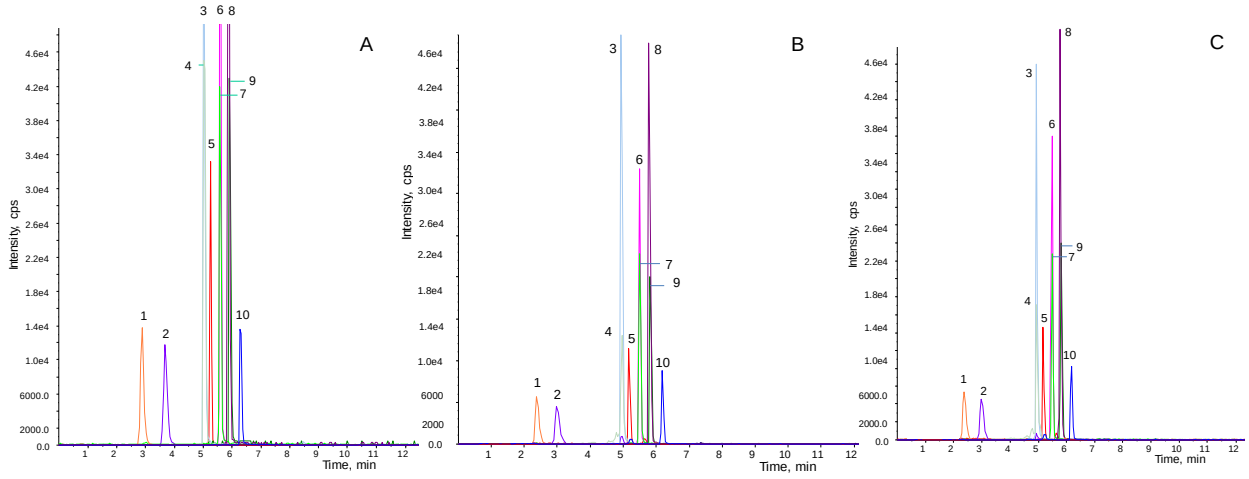


*Figure 5.4. RPLC MRM chromatograms of A. standard mixture B. HILIC SPE plasma extract spiked before SPE and C. HILIC SPE plasma extract spiked after SPE, all spiked at 25 ng/mL. Dilution factor of 2 comparing A with B and C. Conditions method RPLC 2. Peak numbering as in Table 5.2.*

*Table 5.3 The mean value for n=3 with the RSD%.*

| | Recovery | | Matrix Effect | |
|---|---|---|---|---|
| Compound | Measured (%) | RSD (%) | Measured (%) | RSD (%) |
| 1 Sulfamethazine | 89.7 | 3.2 | 84.3 | 3.8 |
| 2 Zidovudine | 81.6 | 3.7 | 103.0 | 3.6 |
| 3 Risperidone | 110.3 | 3.4 | 88.6 | 3.4 |
| 4 Sulfadimethoxine | 86.0 | 4.1 | 98.1 | 3.9 |
| 5 Propranolol | 91.5 | 3.8 | 88.4 | 3.3 |
| 6 Carbamazepine | 87.2 | 3.7 | 103.3 | 3.3 |
| 7 Amitriptyline | 95.1 | 3.3 | 104.0 | 3.5 |
| 8 Terfenadine | 83.7 | 3.9 | 109.3 | 3.7 |
| 9 Trifluoroperazine | 91.5 | 3.7 | 108.2 | 3.0 |
| 10 Indomethacin | 86.8 | 3.5 | 101.5 | 3.4 |

The recovery of analytes by HILIC SPE was calculated by comparing the MS response of analytes in plasma samples spiked before and after SPE. The data are included in Table 5.3. The recovery was between 80 and 110 % with a standard deviation of less than 3%.

The HILIC SPE method has been intensively and successfully used in our laboratories in recent years [48]. Moreover, the whole procedure can easily be automated in an off-line set-up. Over the course of several years no reproducibility issues were observed with the described sample preparation approach, illustrating the higher reliability of analyses on silica cartridges in the aqueous normal phase mode when compared to the conventional (non-aqueous) normal phase mode, an approach more prone to occasionally depict poorer reproducibility data.

In the last years, more and more SPE methods with similar goals *i.e.* removing endogenous components from plasma and serum samples negatively impacting the accuracy and precision of LC-MS/MS pharmaceutical target determinations, have been introduced. The HILIC SPE method presented is in no way inferior to the commercial approaches, to the contrary. As an example, the HybridSPE procedure combining on-cartridge protein precipitation with selective retention of phospholipids on zirconia particles and carried out exactly as described

in the guidelines (www.sigmaaldrich.com/.../hybridspe-ppt.html - page 10) was giving the same matrix data but less recovery. Adding an extra washing step as described in ref [49] for the determination of serotonin along with dopamine and melatonin in serum resulted in high recoveries but substantial breakthrough of the phospholipids. Further optimization of HybridSPE for the given range of solutes was outside the scope of the study. It illustrates, however, that all methods have their specific characteristics and should be evaluated as a function of the targets and selected chromatographic conditions.

## 5.4 Conclusion

An effective SPE procedure for phospholipid removal from plasma was developed. Based on the observation that phospholipids are well retained in HILIC on native silica and on the fact that the retention of the former can be significantly increased when replacing acetonitrile by acetone, with a concomitant decrease in the retention of pharmaceuticals, a new method for phospholipid removal could be proposed. Phospholipid removal was ascertained by LC-MS/MS analysis and removal of the ion suppression phenomena was confirmed by post-column infusion experiments and by comparing the peak areas obtained upon analysis of spiked plasma with the analysis of standard solutions. Moreover, the recovery was from 80 to 110% for a broad range of selected pharmaceuticals (log P 0 to 6.6). In general aceton based HILIC can be an interesting new development. However this needs more study in relation to the reliability and robustness.

## 5.5 References

[1] C. Prakash, C. L. Shaffer, and A. Nedderman, "Analytical strategies for identifying drug metabolites," *Mass Spectrometry Reviews,* vol. 26, pp. 340-369, May-Jun 2007.

[2] O. A. Ismaiel, M. S. Halquist, M. Y. Elmamly, A. Shalaby, and H. T. Karnes, "Monitoring phospholipids for assessment of ion enhancement and ion suppression in ESI and APCI LC/MS/MS for chlorpheniramine in human plasma and the importance of multiple source matrix effect evaluations," *Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences,* vol. 875, pp. 333-343, Nov 2008.

[3] G. D. Zhang and C. E. Wujcik, "Overcoming ionization effects through chromatography: A case study for the ESI-LC-MS/MS quantitation of a hydrophobic therapeutic agent in human serum using a stable-label internal standard," *Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences,* vol. 877, pp. 2003-2010, Jul 2009.

[4] J. C. Van De Steene, K. A. Mortier, and W. E. Lambert, "Tackling matrix effects during development of a liquid chromatographic-electrospray ionisation tandem mass spectrometric analysis of nine basic pharmaceuticals in aqueous environmental samples," *Journal of Chromatography A,* vol. 1123, pp. 71-81, Aug 2006.

[5] E. Chambers, D. M. Wagrowski-Diehl, Z. L. Lu, and J. R. Mazzeo, "Systematic and comprehensive strategy for reducing matrix effects in LC/MS/MS analyses," *Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences,* vol. 852, pp. 22-34, Jun 2007.

[6] H. John, M. Eddleston, R. E. Clutton, F. Worek, and H. Thiermann, "Simultaneous quantification of the organophosphorus pesticides dimethoate and omethoate in porcine plasma and urine by LC-ESI-MS/MS and flow-injection-ESI-MS/MS," *Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences,* vol. 878, pp. 1234-1245, May 2010.

[7] O. Gonzalez, R. M. Alonso, N. Ferreiros, W. Weinmann, R. Zimmermann, and S. Dresen, "Development of an LC-MS/MS method for the quantitation of 55 compounds prescribed in combined cardiovascular therapy," *Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences,* vol. 879, pp. 243-252, Feb 2011.

[8] L. Tang and P. Kebarle, "Dependence of ion intensity in electrospray mass-spectrometry on the concentration of the analytes in the electrosprayed solution," *Analytical Chemistry,* vol. 65, pp. 3654-3668, Dec 1993.

[9] R. King, R. Bonfiglio, C. Fernandez-Metzler, C. Miller-Stein, and T. Olah, "Mechanistic investigation of ionization suppression in electrospray ionization," *Journal of the American Society for Mass Spectrometry,* vol. 11, pp. 942-950, Nov 2000.

[10] L. L. Jessome and D. A. Volmer, "Ion suppression: A major concern in mass spectrometry," *LCGC North America,* vol. 24, pp. 498-510, May 2006.

[11] H. Trufelli, P. Palma, G. Famiglini, and A. Cappiello, "An overview of matrix effects in liquid chromatography-mass spectrometry," *Mass Spectrometry Reviews,* vol. 30, pp. 491-509, May-Jun 2011.

[12] A. Van Eeckhaut, K. Lanckmans, S. Sarre, I. Smolders, and Y. Michotte, "Validation of bioanalytical LC-MS/MS assays: Evaluation of matrix effects," *Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences,* vol. 877, pp. 2198-2207, Aug 2009.

[13] R. Dams, M. A. Huestis, W. E. Lambert, and C. M. Murphy, "Matrix effect in bio-analysis of illicit drugs with LC-MS/MS: Influence of ionization type, sample preparation, and biofluid," *Journal of the American Society for Mass Spectrometry,* vol. 14, pp. 1290-1294, Nov 2003.

[14] S. Souverain, S. Rudaz, and J. L. Veuthey, "Matrix effect in LC-ESI-MS and LC-APCI-MS with off-line and on-line extraction procedures," *Journal of Chromatography A,* vol. 1058, pp. 61-66, Nov 2004.

[15] N. B. Cech and C. G. Enke, "Practical implications of some recent studies in electrospray ionization fundamentals," *Mass Spectrometry Reviews,* vol. 20, pp. 362-387, Nov-Dec 2001.

[16] A. Cappiello, G. Famiglini, P. Palma, and H. Trufelli, "Matrix effects in liquid chromatography-mass spectrometry," *Journal of Liquid Chromatography & Related Technologies,* vol. 33, pp. 1067-1081, 2010.

[17] J. Eshraghi and S. K. Chowdhury, "Factors affecting electrospray ionization of effluents containing trifluoroacetic acid for high-performance liquid chromatography/mass spectrometry," *Analytical Chemistry,* vol. 65, pp. 3528-3533, Dec 1993.

[18] A. Apffel, S. Fischer, G. Goldberg, P. C. Goodley, and F. E. Kuhlmann, "Enhanced sensitivity for peptide mapping with electrospray liquid- chromatography mass-spectrometry in the presence of signal suppression due to trifluoroacetic acid-containing mobile phases," *Journal of Chromatography A,* vol. 712, pp. 177-190, Sep 1995.

[19] S. A. Gustavsson, J. Samskog, K. E. Markides, and B. Langstrom, "Studies of signal suppression in liquid chromatography-electrospray ionization mass spectrometry using volatile ion-pairing reagents," *Journal of Chromatography A*, vol. 937, pp. 41-47, Dec 2001.

[20] M. Holcapek, K. Volna, P. Jandera, L. Kolarova, K. Lemr, M. Exner, and A. Cirkva, "Effects of ion-pairing reagents on the electrospray signal suppression of sulphonated dyes and intermediates," *Journal of Mass Spectrometry*, vol. 39, pp. 43-50, Jan 2004.

[21] Z. Q. Ye, H. Tsao, H. Gao, and C. L. Brummel, "Minimizing matrix effects while preserving throughput in LC-MS/MS bioanalysis," *Bioanalysis*, vol. 3, pp. 1587-1601, Jul 2011.

[22] S. Wang, M. Cyronak, and E. Yang, "Does a stable isotopically labeled internal standard always correct analyte response? A matrix effect study on a LC/MS/MS method for the determination of carvedilol enantiomers in human plasma," *Journal of Pharmaceutical and Biomedical Analysis*, vol. 43, pp. 701-707, Jan 2007.

[23] A. Kruve, I. Leito, and K. Herodes, "Combating matrix effects in LC/ESI/MS: The extrapolative dilution approach," *Analytica Chimica Acta*, vol. 651, pp. 75-80, Sep 2009.

[24] I. Fu, E. J. Woolf, and B. K. Matuszewski, "Effect of the sample matrix on the determination of indinavir in human urine by HPLC with turbo ion spray tandem mass spectrometric detection," *Journal of Pharmaceutical and Biomedical Analysis*, vol. 18, pp. 347-357, Nov 1998.

[25] Wahajuddin, S. P. Singh, and G. K. Jain, "Determination of lumefantrine in rat plasma by liquid-liquid extraction using LC-MS/MS with electrospray ionization: Assay development, validation and application to a pharmacokinetic study," *Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences*, vol. 877, pp. 1133-1139, Apr 2009.

[26] M. Larsson and F. Han, "Determination of rifalazil in dog plasma by liquid-liquid extraction and LC-MS/MS: Quality assessment by incurred sample analysis," *Journal of Pharmaceutical and Biomedical Analysis*, vol. 45, pp. 616-624, Nov 2007.

[27] F. Plossl, M. Giera, and F. Bracher, "Multiresidue analytical method using dispersive solid-phase extraction and gas chromatography/ion trap mass spectrometry to determine pharmaceuticals in whole blood," *Journal of Chromatography A*, vol. 1135, pp. 19-26, Nov 2006.

[28] G. Stubbings and T. Bigwood, "The development and validation of a multiclass liquid chromatography tandem mass spectrometry (LC-MS/MS) procedure for the

determination of veterinary drug residues in animal tissue using a QuEChERS (QUick, Easy, CHeap, Effective, Rugged and Safe) approach," *Analytica Chimica Acta,* vol. 637, pp. 68-78, Apr 2009.

[29] W. J. Zhou, L. Ding, G. L. Xu, Y. Q. Wang, L. N. Sun, J. C. He, and X. P. Chen, "Determination of bencycloquidium bromide in human urine using weak cation-exchange solid-phase extraction and LC-ESI-MS: Method validation and application to kinetic study of urinary excretion," *Journal of Pharmaceutical and Biomedical Analysis,* vol. 50, pp. 35-40, Aug 2009.

[30] R. N. Rao, D. D. Shinde, M. Talluri, and S. B. Agawane, "LC-ESI-MS determination and pharmacokinetics of adrafinil in rats," *Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences,* vol. 873, pp. 119-123, Sep 2008.

[31] M. G. Williams, E. M. Shobe, B. E. Bothwell, and W. Z. Zhong, "Development of a sensitive bioanalytical method for determination of PNU-83757 in rat, monkey and human plasma: from LC-UV to LC-MS/MS," *Journal of Pharmaceutical and Biomedical Analysis,* vol. 30, pp. 429-441, Oct 2002.

[32] C. W. Hu, B. H. Lin, and M. R. Chao, "Quantitative determination of urinary N3-methyladenine by isotope-dilution LC-MS/MS with automated solid-phase extraction," *International Journal of Mass Spectrometry,* vol. 304, pp. 68-73, Jul 2011.

[33] Y. N. Babu, M. Nemec, P. Solich, and V. Wsol, "Liquid chromatographic-electrospray mass spectrometric determination (LC-ESI-MS) of phase II metabolites of flobufen in rat liver micro somes - Chiral discrimination," *Talanta,* vol. 75, pp. 494-502, Apr 2008.

[34] C. Chin, Z. P. Zhang, and H. T. Karnes, "A study of matrix effects on an LC/MS/MS assay for olanzapine and desmethyl olanzapine," *Journal of Pharmaceutical and Biomedical Analysis,* vol. 35, pp. 1149-1167, Sep 2004.

[35] V. Pucci, S. Di Palma, A. Alfieri, F. Bonelli, and E. Monteagudo, "A novel strategy for reducing phospholipids-based matrix effect in LC-ESI-MS bioanalysis by means of HybridSPE," *Journal of Pharmaceutical and Biomedical Analysis,* vol. 50, pp. 867-871, Dec 2009.

[36] O. A. Ismaiel, T. Y. Zhang, R. G. Jenkins, and H. T. Karnes, "Investigation of endogenous blood plasma phospholipids, cholesterol and glycerides that contribute to matrix effects in bioanalysis by liquid chromatography/mass spectrometry," *Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences,* vol. 878, pp. 3303-3316, Dec 2010.

[37] K. Ardjomand-Woelkart, M. Kollroser, L. Li, H. Derendorf, V. Butterweck, and R. Bauer, "Development and validation of a LC-MS/MS method based on a new 96-well Hybrid-SPE (TM)-precipitation technique for quantification of CYP450 substrates/metabolites in rat plasma," *Analytical and Bioanalytical Chemistry,* vol. 400, pp. 2371-2381, Jun 2011.

[38] C. Ferreiro-Vera, F. Priego-Capote, and M. D. L. de Castro, "Comparison of sample preparation approaches for phospholipids profiling in human serum by liquid chromatography-tandem mass spectrometry," *Journal of Chromatography A,* vol. 1240, pp. 21-28, Jun 2012.

[39] W. Zeng, Y. Xu, M. Constanzer, and E. J. Woolf, "Determination of sitagliptinin human plasma using protein precipitation and tandem mass spectrometry," *Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences,* vol. 878, pp. 1817-1823, Jul 2010.

[40] O. A. Ismaiel, T. Y. Zhang, R. Jenkins, and H. T. Karnes, "Determination of octreotide and assessment of matrix effects in human plasma using ultra high performance liquid chromatography-tandem mass spectrometry," *Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences,* vol. 879, pp. 2081-2088, Jul 2011.

[41] D. Thibeault, N. Caron, R. Djiana, R. Kremer, and D. Blank, "Development and optimization of simplified LC-MS/MS quantification of 25-hydroxyvitamin D using protein precipitation combined with on-line solid phase extraction (SPE)," *Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences,* vol. 883, pp. 120-127, Feb 2012.

[42] A. S. Vila, A. I. Castellote-Bargallo, M. Rodriguez-Palmero-Seuma, and M. C. Lopez-Sabater, "High-performance liquid chromatography with evaporative light-scattering detection for the determination of phospholipid classes in human milk, infant formulas and phospholipid sources of long-chain polyunsaturated fatty acids," *Journal of Chromatography A,* vol. 1008, pp. 73-80, Aug 2003.

[43] F. Gao, X. Tian, D. Wen, J. Liao, T. Wang, and H. Liu, "Analysis of phospholipid species in rat peritoneal surface layer by liquid chromatography/electrospray ionization ion-trap mass spectrometry," *Biochimica et Biophysica Acta,* vol. 1761, pp. 667-676, Jul 2006.

[44] X. Wu, Y. Li, Q. Wang, W. Li, and Y. Feng, "Effects of berberine and pomegranate seed oil on plasma phospholipid metabolites associated with risks of type 2 diabetes mellitus by U-HPLC/Q-TOF-MS," *Journal of Chromatography B,* vol. 1007, pp. 110-120, Dec 2015.

[45] R. Bonfiglio, R. C. King, T. V. Olah, and K. Merkle, "The effects of sample preparation methods on the variability of the electrospray ionization response for model drug compounds," *Rapid Communications in Mass Spectrometry,* vol. 13, pp. 1175-1185, 1999.

[46] M. J. Bogusz, E. Al Enazi, H. Hassan, J. Abdel-Jawaad, J. Al Ruwaily, and M. Al Tufail, "Simultaneous LC-MS-MS determination of cyclosporine A, tacrolimus, and sirolimus in whole blood as well as mycophenolic acid in plasma using common pretreatment procedure," *Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences,* vol. 850, pp. 471-480, May 2007.

[47] W. Goutier, P. A. Spaans, M. A. W. van der Neut, A. C. McCreary, and J. H. Reinders, "Development and application of an LC-MS/MS method for measuring the effect of (partial) agonists on cAMP accumulation in vitro," *Journal of Neuroscience Methods,* vol. 188, pp. 24-31, Apr 2010.

[48] T. Van Damme, Y. H. Zhang, F. Lynen, and P. Sandra, "Determination of cyclic guanosine- and cyclic adenosine monophosphate (cGMP and cAMP) in human plasma and animal tissues by solid phase extraction on silica and liquid chromatography-triple quadrupole mass spectrometry," *Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences,* vol. 909, pp. 14-21, Nov 2012.

[49] M. Moriarty, A. Lee, B. O'Connell, M. Lehane, H. Keeley, and A. Furey, "The Application and Validation of HybridSPE-Precipitation Cartridge Technology for the Rapid Clean-up of Serum Matrices (from Phospholipids) for the Clinical Analysis of Serotonin, Dopamine and Melatonin," *Chromatographia,* vol. 75, pp. 1257-1269, Nov 2012.

# General conclusions and future perspectives

Analysis of polar secondary metabolites in complex matrices has proven to be challenging, throughout this research. Fundamental research and testing had to be performed to develop successful approaches for the analysis of polar molecules in the various studied biological samples. The most commonly used separation method, RPLC, was the method of choice for the analysis of the polar solutes studied in this work. This separation mode is also preferred in pharmaceutical analysis because of its high robustness compared to the other LC modes. High aqueous fractions in the mobile phase were thereby required to give the polar solutes sufficient retention on C18 based stationary phases. The electrospray ionization source was selected as it allowed for sub-ppb level detections and the development of validated methods. These low limits of detection were also reached by the exploitation of fragmentation and MRM capabilities of an MS/MS system together with the development of powerful sample preparation approaches based on HILIC SPE.

Cyclic nucleotides are second messengers and are a group of small polar signaling molecules in eukaryotes. In particular the biochemically very relevant cyclic nucleotides cAMP and cGMP were selected for analysis in biological matrices. The 3',5' isomers of these solutes play a crucial role in signal transduction processes and are of great interest by the pharmaceutical and biochemical industry for successful detection methods and consequently quantitative results. The most commonly and widely used quantitative method for analysis of this type of nucleotides, enzyme-linked immunosorbent assay (ELISA), is hindered by its single solute approach and cost. Because these polar solutes had to be analyzed in complex matrices that suffer from ion suppression phenomena, the development of adequate sample preparation approaches proved to be essential. The matrix components, ubiquitously present in biological samples, tend to out-compete the solutes of interest for ionization, with a detrimental impact on the sensitivity of detection. These matrix components could be subdivided in three main contributors: salts, proteins and phospholipids. The contribution of each of these groups to the detrimental matrix effects in RPLC-ESI MS/MS was investigated and addressed.

Therefore the presence of cyclic nucleotides 3',5'-cAMP and 3',5'-cGMP in human plasma was investigated first (chapter 3). One of the innovative approaches proposed in this thesis

was solid phase extraction on silica, exploiting the HILIC mechanism. It proved to be very effective in enrichment of the polar target solutes and in fractionation of interferences. HILIC-SPE was relatively unexploited, it is more challenging concerning sample loading capacity compared to the well-used RP SPE.

It proved, however, a very suitable pre-concentration tool, also removing the proteins by precipitation in an acetonitrile solution whereby they remained denaturated on the cartridge. Salts eluted early in the RPLC analysis and where therefore of no concern as the solutes of interest were well retained. Phospholipids could experience some partial breakthrough during the SPE process because of the elution with 100% water, but they are subsequently completely retained on the (C18) guard column under the highly aqueous mobile conditions used. Stable isotope-labeled internal standards, with identical chemical and physical properties as the analytes were thereby added prior to the sample preparation to ensure sufficient precision and accuracy. This HILIC-SPE RPLC-MS/MS method allowed for detection in the low ppb ranges for small sample sizes of representative biological samples. Basal plasma concentrations for fifteen healthy human patients were determined for 3',5'-cAMP and for 3',5'-cGMP, in the 0.15 – 10 ng/mL range. The figures of merit were determined and confirmed the potential of this newly developed method for analysis of polar solutes in complex matrices.

The scope of this newly developed method was further broadened to analyze more challenging complex matrices like animal tissues as well. The sample preparation method was thereby slightly modified to successfully handle such samples and to extract the molecules of interest. The tailor made HILIC-SPE RPLC-MS/MS method was applied to small volumes of pancreas and kidney of rabbits (chapter 3). During this study on animal tissues, the naturally occurring 2',3' isomers of cAMP and cGMP were also detected as two additional signals depicting the same MRM transitions as the 3',5'-cyclic nucleotides. These isomers had been reported to occur in animal tissues, although limited quantitative data was available. The structures were confirmed by high resolution MS (time-of-flight MS) and subsequently by comparison of the retention times with standards, also allowing an estimation of their concentrations.

The HILIC-SPE RPLC-MS/MS method was subsequently adapted for detection of the second messenger molecules cAMP and cGMP in plant tissues as well (chapter 4). At the time of this

research almost no quantitative information was available on the 2',3' isomers of cAMP and cGMP in plants material. Cyclic nucleotide concentrations were measured in *Arabidopsis thaliana* (Col-0) leaves before and after the application of wounding stress. A significant (~5-fold) up-regulation of 2',3'-cAMP and 2',3'-cGMP was thereby measured in wounded *Arabidopsis* leaves compared to the control samples. The results indicated a thus far unreported strong correlation between plant stress and 2',3'-cAMP and 2',3'-cGMP levels in plant material, opening possible new insights in understanding the role of cyclic nucleotides in plants.

However, although studied in chapter 3, the question from the pharmaceutical industry to solve the presence of phospholipids in complex matrices still required the development of further simplified procedures. A broad polarity range of pharmaceutical solutes was therefore analyzed in human plasma under various conditions (chapter 5). An uncommon aspect in RPLC methods, applied during this research, was the use of a ternary gradient whereby 2-propanol is replacing methanol at the end of the gradient. This allowed for swift phospholipid elution and visualization of these important ion suppressors in RP-LC. This strong influence of a phospholipid zone on the ionization efficiency of the solutes was also clearly illustrated via post-column infusion. The experience with selectivity changes in HILIC when shifting to other solvents asked for an evaluation of the selectivity of acetone as alternative solvent, although considered a weaker solvent in HILIC. Based on the observation that phospholipids are well retained in HILIC (LC) on native silica and on the fact that the retention of the former can be significantly increased when replacing acetonitrile by acetone, with a concomitant decrease in the retention of the selected pharmaceuticals, a new method for protein precipitation and phospholipid removal by HILIC-SPE could be developed. Recovery and matrix effects were calculated while a comparison with a commercial available SPE method, HybridSPE, for removing endogenous components from plasma was performed. This comparison indicated the power and broad applicability of this newly developed method at a fraction of the cost of the commercial method.

It can be concluded that a combination of a well-designed and effective sample preparation method, HILIC-SPE, in combination with a robust and sensitive chromatographic method like RPLC-ESI MS/MS is a powerful tool for the analysis of polar metabolites in complex biological matrices. Moreover, the methods allow for multiple solute detection and

quantification, which is of high relevance in pharmaceutical research, with the benefits of pre-concentration of the target solutes, tackling the matrix effect issues and allowing compatibility small sample volumes.

The developed methods can be applied to tackle industrial challenges in polar solute analysis in complex matrices, whereby exploitation in thus far unstudied classes (such as the isomers of cCMP and cUMP, oligonucleotides, saccharides or other polar metabolites) can be envisaged. This could pave the way in the discovery of thus far non elucidated biochemical processes. More broadly other biological matrices like cerebrospinal fluid (CSF) and higher plant extracts can be investigated with the newly developed methods. Other up- or downregulation studies of polar metabolites on plant material can be foreseen, like *e.g.* the influence of drought or salt stress. Quantitative results can thereby help understanding and explaining the different pathways and biological roles of the secondary metabolites in these challenging matrices. Also interesting is further comparison of novel and broader commercial developed methods for phospholipid removal (*e.g.* Phree-SPE, Ostro) with the in this work developed procedure. The benefits of the use of acetone and of other solvents in HILIC SPE and chromatography, based on the interesting selectivity changes observed in this thesis, can also form a strong basis for further research and for further strengthening of the methods.

The developments could also allow for implementation in high throughput environments if the compatibility with the mass flow sensitive APCI source can be demonstrated, which is able to handle higher flow rates. In general several of the developments in this work are expected to stimulate novel biological, pharmaceutical, chromatographic and mass spectrometric research at various levels.

# Summary

In this thesis the problem of effective analysis of polar solutes in complex biological samples was analyzed through the development of quantitative and qualitative analytical methods for the enhanced analysis of secondary metabolites in biological extracts. The occurrence of an ubiquitous amount of competing solutes in the ionization source, leading to so called matrix effects, is affecting the ionization efficiency in mass spectrometry and hence the quality of quantitative methods. A combination of a well-designed and effective sample preparation method, HILIC-SPE, in combination with a robust and sensitive chromatographic method like RPLC-ESI MS/MS is a powerful tool for the analysis of polar metabolites in complex biological matrices. The methods allow for multiple solute detection and quantification, with the benefits of pre-concentration of the target solutes, tackling the matrix effect issues and allowing compatibility small sample volumes.

In chapter 1, the importance of messenger molecules in organisms is explained. These molecules are playing a crucial role in signal transduction. An overview of the most relevant type of secondary messengers is presented, with extra focus on the cyclic nucleotides cAMP and cGMP and their role in biological functions. Because quantitative measurement of levels of second messengers could serve as mechanism biomarkers for the pharmacological modulation by 'specific' inhibitors, there is a need for more sensitive and more robust quantitative determinations, driven by the pharmaceutical and biochemical industry.

In chapter 2 an overview of *in vivo* metabolite quantification approaches for secondary metabolite analysis is described. Chromatographic and biochemical strategies for analysis of these secondary messengers in biological samples are explained. A literature study is included, describing the quantitation of these secondary messengers through various techniques.

In chapter 3, a new tailor made sample preparation method, based on HILIC technology, together with a robust and sensitive LC-MS/MS method is developed for the determination and quantification of 3',5'-cGMP and 3',5'-cAMP and its naturally occurring 2',3 isomers in human plasma and animal tissue extracts. The power of tandem mass spectrometry (MS/MS) is applied for more selective, more sensitive and simultaneous high throughput detection and quantitation of multiple molecules of interest in the sub-ppb range. SPE on silica was used as

sample preparation method for the enrichment of the targets and for the removal of interferences leading to MS ion suppression. Basal plasma concentrations for fifteen healthy human patients in the low ppb range were thereby determined.

In chapter 4, the presence of cyclic second messengers cAMP and cGMP is investigated in plant material. The HILIC-SPE RPLC-MS/MS method was adapted to measure 2',3'-cAMP and 2',3'-cGMP concentrations in *Arabidopsis thaliana* leaves because no quantitative information was available regarding these 2',3'-cyclic isomers in plant material at that moment. A correlation between plant stress and a rapid increase in both 2',3-cAMP and 2',3-cGMP concentration was thereby quantitatively observed for the first time, opening possible new insights in understanding the role of cyclic nucleotides in plants.

In chapter 5, the detrimental matrix effects from the ubiquitous presence of a high number of competing molecules in biological matrices on LC-MS/MS methods when using soft ionization sources are investigated. In particular the presence of phospholipids in complex matrices required the development of further simplified procedures. A broad polarity range of pharmaceutical solutes was therefore analyzed in human plasma under various conditions. A novel phospholipid removal approach via SPE, based on acetone based hydrophilic interaction liquid chromatography was developed. Post column infusion experiments confirmed the removal of the ion suppression phenomena.

# Samenvatting

Het doel van deze thesis, zoals beschreven in het deel ‘Algemene inleiding en doelstelling, was het ontwikkelen van gevoelige analytische methodieken voor de analyse van polaire moleculen in biologische stalen, en in het bijzonder secundaire boodschappermoleculen. Ook dienden er methodes te worden ontwikkeld welke de analyse toelaten van relatief kleine monster volumes. Nieuwe monster voorbereiding methodes werden daartoe ontwikkeld, gebaseerd op hydrofiele interactie vloeistof chromatografie-vaste fase extractie (HILIC-SPE), in combinatie met gevoelige en selectieve analyse via LC-MS/MS.

In hoofdstuk 1 werd het belang van secundaire boodschappermoleculen beschreven. Deze moleculen spelen een cruciale rol bij het doorgeven van signalen in organismen. Een overzicht van de meest relevante types van dergelijke metabolieten wordt besproken, waarbij nadruk gelegd wordt op twee specifieke cyclische nucleotiden, namelijk cAMP en cGMP, welke in detail geanalyseerd worden in deze thesis. Omdat dergelijke secundaire boodschappermoleculen een belangrijke rol spelen in vele biologische en farmaceutische processen, en omdat ze mogelijks kunnen ingezet worden als biomarker, is er een hoge nood aan de ontwikkeling van verbeterde en meer gevoelige kwantitatieve manieren om deze moleculen te bepalen.

In hoofdstuk 2 wordt een overzicht gegeven van de huidige methoden voor de kwalitatieve en kwantitatieve analyse van deze secundaire metabolieten. De nadruk wordt daarbij gelegd op de analyse van monsters zoals bloed, plasma, dierlijke weefsels en planten extracten. Eerst worden verschillende chromatografische strategieën beschreven, waarbij de succesvolle combinatie van HPLC en massa spectrometrie met meer aandacht besproken wordt. Daarna worden de meest gebruikte immuno analysemethoden beschreven. Met deze informatie kan de lezer een beter idee vormen van de moeilijkheden en uitdagingen welke overwonnen moeten worden bij het analyseren van deze moleculen in representatieve biologische monsters. In een laatste deel van dit hoofdstuk worden de gepubliceerde analyse technieken voor de kwantificatie van cyclische nucleotiden beschreven.

In het volgende en eerste experimentele hoofdstuk (3) wordt een alternatieve monster voorbereidings methode ontwikkeld en beschreven voor de analyse van cGMP en cAMP in biologische monsters, namelijk in menselijk bloed en dierlijke weefsels. De strategie maakt

gebruik van vaste fase extractie methode (SPE) gebaseerd op de hydrofiele interacties met silica en bleek efficiënt in het verrijken van de specifieke doelmoleculen en voor het verwijderen van de nadelige interferenties welke voorkomen door het matrix effect bij dergelijke monsters. Een nieuwe ontwikkelde LC-MS/MS methode sloot hier goed bij aan wat toeliet een robuuste, reproduceerbaar en specifieke detectie methode voor deze metabolieten te ontwikkelen. De kracht van een drievoudige quadrupool systeem moest daarbij worden gebruikt om de meest gevoelige en selectieve detectie toe te laten. Hierdoor kon niet alleen van 15 gezonde mensen de basale plasma concentraties worden gemeten met een hoge precisie en accuratesse, voor 3',5'-cAMP en 3',5'-cGMP, maar konden ook de 2',3' natuurlijk voorkomende isomeren van cAMP en cGMP bepaald worden in dierlijke weefsels.

In hoofdstuk 4 werden deze cyclische nucleotiden geanalyseerd en bestudeerd in planten. De nieuw ontwikkelde HILIC-SPE LC-MS/MS methode werd daarvoor aangepast om ook 2',3'-cAMP en 2',3'-cGMP concentraties kwantitatief te kunnen bepalen in plant materiaal. Merk op dat er tot op dat stadium geen kwantitatieve informatie over deze 2',3' cyclische isomeren beschikbaar was in planten. Concentraties werden bepaald in *Arabidopsis thaliana* planten voor en na het aanbrengen van snijwonden op de bladeren. De resultaten toonden een tot dan toe ongekend verband aan tussen deze stress factoren en de 2',3'-cAMP en 2',3'-cGMP concentraties in planten. Deze resultaten kunnen bijdragen in het beter begrijpen van de rol van secundaire messengers in planten.

In hoofdstuk 5 werd het onderzoek verder toegespitst op het verwijderen van de nadelige matrix effecten bij de analysen van biologische monsters met de wijd verspreide LC-MS techniek. Hiervoor werd een nieuwe vaste fase extractie methode ontwikkeld gebaseerd op de principes van hydrofiele interactie vloeistof chromatografie (HILIC), enigszins aansluitend bij de eerder vermelde HILIC-SPE LC-MS/MS methode. De nieuw ontwikkelde techniek baseerde zich op het feit dat fosfolipiden, die verantwoordelijk zijn voor een groot deel van de matrix effecten, goed weerhouden worden in HILIC op silica. Uit verder onderzoek bleek dat deze retentie nog veel verder kan worden verhoogd door het vervangen van acetonitril door aceton als mobiele fase. De combinatie van deze nieuw ontwikkelde monster voorbereiding techniek samen met de mogelijkheid tot gevoelige en specifieke scheiding door LC-MS/MS was succesvol en toonde ontegensprekelijk de verwijdering aan van fosfolipiden in biologische moleculen alsook van andere ionensuppressie componenten.

# Bibliography

## Publications

**T. Van Damme**, Y. H. Zhang, F. Lynen, and P. Sandra, "Determination of cyclic guanosine- and cyclic adenosine monophosphate (cGMP and cAMP) in human plasma and animal tissues by solid phase extraction on silica and liquid chromatography-triple quadrupole mass spectrometry," *Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences*, vol. 909, pp. 14-21, Nov 2012.

**T. Van Damme**, M. Lachova, F. Lynen, R. Szucs, and P. Sandra, "Solid-phase extraction based on hydrophilic interaction liquid chromatography with acetone as eluent for eliminating matrix effects in the analysis of biological fluids by LC-MS," *Analytical and Bioanalytical Chemistry*, vol. 406, pp. 401-407, Jan 2014.

**T. Van Damme**, D. Blancquaert, P. Couturon, D. Van Der Straeten, P. Sandra, and F. Lynen, "Wounding stress causes rapid increase in concentration of the naturally occurring 2 ',3 '-isomers of cyclic guanosine- and cyclic adenosine monophosphate (cGMP and cAMP) in plant tissues," *Phytochemistry*, vol. 103, pp. 59-66, Jul 2014.

## Oral presentation

**T. Van Damme**, Y. H. Zhang, F. Lynen, and P. Sandra, “Immunoassay Enhanced LC/MS/MS Method for the Determination of Nucleotides,” Chemistry Conference for Young Scientists (ChemCys), 2010.

## Posters

**T. Van Damme**, Y. H. Zhang, F. Lynen, and P. Sandra, "Determination of cyclic guanosine- and cyclic adenosine monophosphate (cGMP and cAMP) in human plasma and animal tissues by HILIC SPE in combination with LC-MS/MS," *International Symposium on Hyphenated Techniques for Sample Preparation (HTSP-2 and HTC-12)*, 2012.

**T. Van Damme**, Y. H. Zhang, F. Lynen, and P. Sandra, "Determination of cyclic guanosine- and cyclic adenosine monophosphate (cGMP and cAMP) in human plasma and animal tissues by HILIC SPE in combination with LC-MS/MS," Chemistry Conference for Young Scientists (ChemCys), 2012.

Best poster presentation in the field of Analytical Chemistry.

**T. Van Damme**, M. Lachova, F. Lynen, R. Szucs, and P. Sandra, "Acetone based HILIC SPE as an alternative approach for eliminating matrix effects in LC-MS," HPLC 2013.

# Dankwoord

*Ik wil graag van deze gelegenheid gebruik maken om een aantal mensen te bedanken welke het mede mogelijk maakten om deze thesis tot een goed einde te brengen. Vooreerst wil ik graag Em. Prof. Dr P. Sandra bedanken voor de mogelijkheid die hij mij gaf om een doctoraat te starten binnen zijn succesvolle onderzoeksgroep, in samenwerking met de Pfizer groep. Deze wil ik dan ook bedanken voor de beurs welke voor dit onderzoek werd vrijgemaakt en voor hun in-house kennis en ervaring. Even grote dank gaat naar Prof. Dr. F. Lynen voor de begeleiding, ondersteuning, analytische kennis en het nalezen van het vele schrijfwerk. Ook ben ik hun dankbaar voor de kansen welke ik kreeg om mij verder te ontwikkelen binnen de onderzoeksgroep, zowel op wetenschappelijk als persoonlijk vlak. Ook de kansen welke ik kreeg om deel te nemen aan nationale en internationale congressen werden enorm gewaardeerd. Al deze ervaring en kennis heeft mij gesterkt als onderzoeker en persoon, welke reeds tot hun recht zijn gekomen in mijn huidige professionele uitdaging bij PVS Chemicals Belgium (zwavelzuur productie op industriële schaal in havengebied Gent) als operations manager.*

*Ook de collega doctoraatstudenten en het personeel binnen de onderzoeksgroep en S4 wil ik bedanken voor de technische, wetenschappelijke en persoonlijke hulp bij dit onderzoek. Marc, Maarten, Bram, Anna, Mirka, Stefan, Beatrix, Hanna, Michaël, Sander, Seppe, Pjotr, Dries, Stijn, Pieter Jan, Mario en zoveel andere, merci! Bedankt ook aan de mensen welke vrijwillig bloed gedoneerd hebben in functie van dit onderzoek. Een woordje van dank ook aan de faculteit diergeneeskunde voor het ter beschikking stellen van verschillende dierlijke weefsels. Ook het laboratorium van functionele plant biologie (FPB) onder leiding van Prof. D. Van Der Straeten wil ik graag bedanken voor de vele plant monsters en de specifieke vakkennis, nodig om deze thesis tot een goed einde te brengen.*

*Hierbij komt een einde aan mijn studie- en onderzoek periode aan de universiteit Gent. Het minste wat je kan zeggen is dat dit een bewogen en interessante periode is geweest, in een ongewoon traject. Het afstuderen als laatste licentiaat in de scheikunde in 2008 was een speciaal moment. In de periode erna, tijdens dit thesis onderzoek en verdere professionele carrière is er veel gebeurd in mijn persoonlijk leven. De absolute hoogtepunten waren de geboortes van onze dochters Anna en Lena. Ook dit waren bewogen gebeurtenissen waar ik*

*jullie graag meer uitleg over geef indien gewenst. Mijn vriendin, Kristin, is gedurende deze volledige periode een ongelofelijke steun en toeverlaat geweest, en nog altijd. Merci! Ook mijn ouders hebben een belangrijke rol gespeeld in de mogelijkheid om mij te ontwikkelen, zowel op professioneel als persoonlijk vlak, waarvoor veel dank.*

*Ik draag dit werk graag op aan mijn prachtige dochters Anna en Lena.*

*Thomas*