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Mahatma Gandhi
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<td>APCI</td>
<td>Atmospheric-pressure chemical-ionisation interface</td>
</tr>
<tr>
<td>DGE</td>
<td>German Nutrition Society</td>
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<tr>
<td>EI</td>
<td>Electron impact</td>
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<tr>
<td>EIC</td>
<td>Extracted ion chromatogram</td>
</tr>
<tr>
<td>ESI</td>
<td>Electrospray interface</td>
</tr>
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<td>FA</td>
<td>Folic Acid</td>
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<tr>
<td>GC</td>
<td>Gas chromatography</td>
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<td>HILIC</td>
<td>Hydrophilic interaction chromatography</td>
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<td>HPLC</td>
<td>High performance liquid chromatography</td>
</tr>
<tr>
<td>LC</td>
<td>Liquid chromatography</td>
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<tr>
<td>LOQ</td>
<td>Limit of quantification</td>
</tr>
<tr>
<td>MS</td>
<td>Mass spectrometry</td>
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<td>NP</td>
<td>Normal phase</td>
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<td>5-MTHF</td>
<td>5-Methyltetrahydrofolic acid</td>
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<tr>
<td>ODS</td>
<td>Octyldecyl-silica</td>
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<tr>
<td>QTOF</td>
<td>Quadrupole time of flight</td>
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<td>RP</td>
<td>Reversed phase</td>
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<tr>
<td>S/N</td>
<td>Signal to noise ratio</td>
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<td>THF</td>
<td>Tetrahydrofolic acid</td>
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1 INTRODUCTION

Folates belong to the group of water soluble vitamins and were first isolated in 1941. However in the past few years they have become the centre of scientific attention. It is desirable to improve the folate intake in the general population, as they play an important role in a number of metabolic processes [HOFFBRAND & WEIR, 2001]. Decreased status of folate can result in neural tube defects in foetus and has also been associated with dementia, cognitive impairment, and many different forms of cancer. Therefore it is essential to accurately detect the folate status in food, serum and blood [LIANG et al, 2009].

Several methods of detection have been applied to identify folate in food. Relying on the bacterial growth of Lactobacillus rhamnosus, the microbiological assay has been the “gold standard” for a very long time. Unfortunately this method can be time consuming and only the total folate concentration can be detected. The more commonly used method to detect folate vitamers is by using HPLC for separation and a UV detector or mass spectrometer for detection. In this way not only folates can be analysed more rapidly, but the metabolites can also be identified and quantified simultaneously [KALMBACH et al. 2011, McMasters, 2005; NELSON et al., 2006; NELSON et al., 2004; SHANE, 2011].

Reversed phase HPLC is by far the most frequently used choice of analysis especially for chromatographic separation of organic low molecular mass compounds such as pharmaceuticals, peptides, pesticides, food constituents and additives. It has its limitations with the retention and analysis of very polar compounds like sugars, amino acids, nucleosides and folates [LÄMMERHOFER et al., 2008; WU et al., 2008].

Hydrophilic liquid interaction chromatography, in short HILIC, can be seen as an alternative approach to effectively separate these polar compounds on polar stationary phases. It uses an aqueous-organic mixture as the mobile phase, with water being the stronger solvent. It is believed that the retention of analytes in HILIC is caused by partitioning of the analyte into a water layer, formed and
immobilised on the surface of the column packing. With HILIC, eluent preparation is supposed to be less complicated and separation should be faster as higher flow rates can be set due to lower viscosity of the mobile phase. Literature describes HILIC to be a powerful tool to detect and identify a wide range of polar compounds [GUO & GAIKI, 2005; KARATAPANIS et al., 2009; SNYDER et al., 2010]. Unfortunately only limited attention has so far been paid to the separation efficiencies of HILIC. Especially when it comes to folate, also to be known as a very polar vitamin, hardly any analysis have been performed yet.

The aim of this thesis is to analyse limits of detection for 3 folate metabolites (folic acid, tetrahydrofolic acid and 5-methyltetrahydrofolic acid) by using a hydrophilic liquid interaction chromatography column in a liquid chromatography/mass spectrometry system. The obtained results are then compared to those of a reversed phase column, using the same LC/MS system. The objective is to find out with which column more sensitive results can be achieved. Literature describes HILIC to be a promising method to detect folate vitamers. As hardly any literature is available on HILIC analysis coupled to a time of flight mass spectrometer, detection is carried out with micrOTOF-QII from Bruker Daltonics. Additionally, storage stability tests on all three folate vitamers are performed and compared to define at which temperatures the folate vitamers are most stable and how the stock solutions should be stored.
2 LITERATURE REVIEW

2.1 GENERAL

Folic acid and its derivates, referred to as folates, belong to the group of water soluble vitamins and are an essential element in the human diet. In the past few years folates have become the centre of scientific attention when it comes to improving the amount of folate intake in the population [HUMPHREY et al., 2008; NELSON et al., 2004]. The term folate describes a group of several different vitamin forms that differ from each other with regard to the oxidation state of the pteridine ring, number of glutamate residues and C1 residues [HORN, 2009]. But all in all they have a related biological activity, same basic structure and are quite sensitive to oxygen, light and heat [HOFFBRAND & WEIR, 2001; PATRING et al., 2005].

Folate vitamers play an important role in a number of metabolic processes, especially as acceptors of single carbon units. They are involved in the remethylation of homocysteine to methionine and in the DNA synthesis. Low intake is, amongst others, linked to neural tube defects in foetus as well as cardiovascular diseases. It can even be connected with some forms of cancer [INSEL et al., 2011].

2.2 HISTORY

The discovery of folates goes back to 1931 when Lucy Wills monitored pregnant women in India and found out that anaemia could be positively influenced by the administration of yeast extract [LEDCHOWSKI, 2010]. Baker’s yeast is now known to produce folate vitamers [HJORTMOA et al., 2005]. However folates were first isolated in 1941 out of 4 tons of spinach leaves. Therefore its name derives from the Latin word “folium” which means “the leaf”.
Its correct chemical structure was found in 1945 when it was also first synthesised. In 1961 Victor Herbert performed a milestone experiment and found evidence of the correlation between folate and mood disorders. In 1964 Brian Hibbard first discovered a correlation between folate status and neural tube defects. With the medical research council trial in 1991 final proof was found. In 1998 it was found that folate deficiency could be related with higher risk of colonic cancer [HOFFBRAND & WEIR, 2001].

2.3 CHEMICAL CHARACTERISTICS

While folate was discovered in 1931, its correct chemical structure was developed in 1945 when it was also first synthesised [HOFFBRAND & WEIR, 2001]. As demonstrated in Figure 1, naturally occurring folates consist of a pteridine ring and a para-aminobenzoic acid ring where up to 8 glutamate residues can be conjugated through an amid bond.

![Figure 1: Pteroylmonoglutamate](NELSON et al., 2004)
Therefore folate vitamers are chemically correctly known as pteroylglutamates. When containing only one glutamate the folate vitamer is referred to as pteroylmonoglutamate, in case of more than one glutamate residue pteroylpolyglutamate would be the correct synonym [BIESALSKI et al., 2004; PIETRZIK et al., 2008].

While naturally occurring food folate vitamers are pteroylpolyglutamates, the synthetic folic acid which is used for food fortification and supplementation, is a monoglutamate [LEDOCHOWSKI, 2010].

Folate species differ from each other with respect to

- the oxidation state of the pteridine ring
- the number of glutamate residues connected to the para-aminobenzoic acid moiety
- substitution of the nitrogen atoms in carbon position 5 and/or 10 with C1 residues (most notably methyl- and formyl-groups) [HORN, 2009].

### 2.4 STABILITY

Folate vitamers are sensitive against oxidation, light and heat. The rate of folate degradation can vary due to

- pH of the medium
- reducing agents in buffer
- different kinds of folate metabolites
- type of buffer and
- food system [ARCOT & SHRESTHA, 2005].
Long storage and reheating of groceries result in a large decrease of these vitamin metabolites. During the cooking process the amount of monoglutamates can decrease up to 70% whereas polyglutamates decrease on average by 50% [LEITZMANN et al., 2009].

Because of their instability it is necessary to stabilise folate vitamers during sample preparation, analysis and also storage. A way to reduce folate degradation is by using dimmed light and to purge the vitamin with nitrogen. As these methods do not preserve against all losses it is essential to add stabilising agents such as ascorbic acid or 2-mercaptoethanol to the analytes, so accurate analysis can be performed.

Patring et al. analysed the stability of 5-methyltetrahydrofolic acid and tetrahydrofolic acid in yeast extracts. Over a period of 4 weeks the folate metabolites were frozen at -22°C. The study showed quite different results for these two folate vitamers. While 5-methyltetrahydrofolic acid did not show any significant decrease, tetrahydrofolic acid was less stable and showed losses up to 53% after storage for 4 weeks at -22°C. The right handling is an important step in folate analysis. Long-term storage, heat treatment as well as repeated freezing and defrosting cycles can substantially influence stability [PATRING et al., 2005].

Johansson et al. analysed the folate content in frozen vegetarian ready meals before and after reheating them. The study group found out that 3 different cooking processes (with stove, oven and microwave) reduced folate content of some meals by up to 25%. However no decision could be made which one of the 3 reheating methods is preferable for folate stability, as results were inconsistent [JOHANSSON et al., 2008].

2.5 ROLE IN METABOLIC PROCESSES IN HUMANS

Folate vitamers play an important role in the one-carbon metabolism where they serve as an acceptor for C1 units. Serine for example is a large source for these
one carbon units. These C1 units are then used for a number of metabolic processes in humans including the biosynthesis of purine and pyrimidine for DNA- and RNA synthesis, cell division and cell renewal as well as the remethylation of homocysteine to methionine [KOETZKO & PIETRZIK, 2004; LEITZMANN et al., 2009].

In folate metabolism, 5-methyltetrahydrofolate, 5-formyltetrahydrofolate, tetrahydrofolate and unmetabolised dietary folic acid are the main folate derivates [LAZAROU & KAPSOU, 2010; LIANG et al., 2009]. The methyl group for pyrimidine derives from 5, 10-methylenetetrahydrofolic acid while it transforms to dihydrofolate. Dihydrofolate can then be converted to tetrahydrofolate through dihydrofolate reductase. 10-formyltetrahydrofolate supplies the single-carbon unit for purine. Certain antibiotics and cytostatics like methotrexate can interfere in the folate metabolism and inhibit the reduction from DHF to THF through dihydrofolate reductase and the availability of THF for metabolic processes is then blocked. During the transformation of serine to glycine, serine donates C1 units to THF which then transforms to 5, 10-methylenetetrahydrofolate. The speed of the purine and pyrimidine synthesis is closely related to the folate metabolism and shows their important role. When folate deficiency occurs, it may result in cellular dysfunction.

With methylenetetrahydrofolate reductase, 5, 10-methylenetetrahydrofolate can be reduced to 5-methyltetrahydrofolate. The methyl group of this folate vitamer is used for the vitamin B12 dependant remethylation of homocysteine to methionine. This process is irreversible. Through this cycle 5-methyltetrahydrofolic acid is transformed back to tetrahydrofolic acid [INSEL et al., 2011]. Therefore a low folate status can result in the accumulation of homocysteine. High levels of homocysteine are correlated with coronary heart disease, cerebrovascular disease, and peripheral vascular disease [HUMPHREY et al., 2008; NELSON et al., 2004].
2.6 FOLATE DEFICIENCY

It has been shown that decreased folate status can result in serious diseases. Low levels of folate status increase the risk of neural tube defects in foetus. Neural tube defects occur when the neural tube does not form and close correctly during foetal development. This disease can cause disorders like spina bifida and anencephaly.

Because defects can occur already 30 days after conception it is essential, especially for women who want to conceive, to have a sufficient amount of folic acid supply. Folic acid supplementation before pregnancy can not only reduce the risk of first occurrence of neural tube defects, but also its recurrence [INSEL et al., 2011; TALAULIKAR & ARULKUMARAN, 2011]. Low folic acid intake has also
been associated with psychological symptoms and mood disorders like dementia, cognitive impairment and depression [LIANG et al., 2009].

In 1961 Victor Herbert conducted an experiment that has been famous in the history of folate research. For a period of 4 months he only consumed food deficient in folate vitamers and monitored the effects and symptoms. The diet resulted in insomnia, irritability and depressive mood. 48 hours later, after taking folate supplements, all symptoms disappeared [HOFFBRAND & WEIR, 2001]. Several different studies like cross-sectional, biochemical, in vitro and retrospective studies give evidence that there is a relationship between decreased folate intake and mood disorders like depression. However most studies have been accomplished with a very specific population and only few with the general population. Also studies indicate that low dietary intake of folate can result in various forms of cancer. Higher intakes can reduce the risk of suffering from colon, pancreas, oesophagus, stomach, and possibly cervix and breast malignancies [LAZAROU & KAPSOU, 2010; ULRICH & POTTER, 2006].

In 2008 Jaszewski et al. performed a randomised chemoprevention trial and investigated whether folic acid supplementation inhibits the recurrence of colorectal adenomas. The study group found out that a supplementation of 5 mg of folic acid per day for a period of 3 years could reduce the recurrence of this type of disease [JASZEWSKI et al., 2008].

2.7 UPPER INTAKE LEVEL

Nutritional supplements and the mandatory fortification of folic acid in certain countries such as Canada or the United States, can lead to elevated intake of folic acid. Therefore the upper limit for folic acid is set to 1000 µg per day. This upper level was basically set to avoid masking a vitamin B12 deficiency. However the clinical significance of a higher intake level of folic acid has not
been well established yet. There have been studies showing that folate can enhance the growth of existing cancers. It is also said that when consuming 15 mg of folate vitamers per day for a period of more than a month may cause sleeping disorders, irritability and hyperactivity. Furthermore the effects of anticonvulsants may decrease with a high folate intake [LEITZMANN et al., 2009; ULRICH & POTTER, 2005].

2.8 BIOAVAILABILITY & DIETARY REQUIREMENT

As folic acid is one of the most stable folate forms it reaches an availability of 100 % whereas folates in foods, which are mostly reduced, have an estimated availability of about 50 %. However food fortified with folic acid shows a bioavailability of nearly 85 % [SHILS et al., 2005].

Due to this varying bioavailability of folate in food the term folate equivalent is used to state folate requirement. 1 µg of folate equivalent equals 1 µg of food folate and 0.5 µg of synthetic folic acid. According to the German nutrition society, the DGE, infants of the age between 0 and 12 months should have a folate intake of 60 to 80 µg of folate equivalents per day. For children between 1 and 7 years the recommended daily intake is 200 to 300 µg equivalents. The recommended folate intake for children above 10 years and adults is set to 400 µg of folate equivalents per day.

Due to the correlation of decreased status of folate vitamers and the risk of neural tube defects in foetus the recommended daily intake for pregnant and breast feeding women should be higher, that is, to round about 600 µg of folate equivalents per day [DGE, 2004; LEITZMANN et al., 2009].
2.9 SOURCES

Folates can only be synthesised by plants and some bacteria species. Naturally nearly all folate vitamers found in foods are pteroylpolyglutamates. Folates can be found in several vegetables as leafy vegetables, cabbage and spinach as well as in yeast, liver, kidney and hen’s egg. Synthetic folic acid is used for food fortification and supplementation [LEDOCHOWSKI, 2010].

Due to unfavourable folate intake and its large impact for the prevention of certain diseases, some countries like USA and Canada have decided to fortify flour with folic acid on a compulsory basis. Since this mandatory fortification in 1998 several studies have reported that blood folate levels have increased and incidents on neural tube defects have decreased by 20 – 30 % [PITKIN, 2007]. While folic acid can naturally almost not be found in plant and animal foods, 5-methyltetrahydrofolate, 5-formyltetrahydrofolate, 10-formyltetrahydrofolate and other reduced folates are more common [SHRESTHA et al., 2011].

In this thesis the focus will be on the following three folates:

- folic acid
- tetrahydrofolic acid
- 5-methyltetrahydrofolic acid
2.10 FOLATES USED IN THIS THESIS

2.10.1 FOLIC ACID

With only one glutamate residue conjugated, folic acid can also be declared as pteroylmonoglutamate. It is the most oxidised, stable and easily absorbable form of this group of vitamins. It is a synthetic yellow-orange crystalline powder with a molecular weight of 441.4 g/Mol [ARCOT & SHRESTHA, 2005]. It is taste- and scentless and does not appear in nature [KOK et al., 2004].

Folic acid is limitedly soluble in water and non-soluble in alcohol. The correct IUPAC term is N-[4(2–Amino–4–hydroxypteridine–6–ylmethylamino) benzoyl]–L(+)–glutamic acid [KOETZKO & PIETRZIK, 2004]. It usually does not appear naturally in food products but is used for food-fortification as well as supplementation especially for childbearing women to reduce the risk of neural tube defect in foetus. Folic acid is most stable to heat compared to the other derivatives [KOK et al., 2004; PATRING et al., 2005].

![Folic acid molecule](image-url)
2.10.2 TETRAHYDROFOLIC ACID

Fig. 4: Tetrahydrofolic acid [NELSON et al., 2004]

Tetrahydrofolic acid is a yellow to light brown powder that has a molecular weight of 445.43 g/Mol, with the molecular formula C\textsubscript{19}H\textsubscript{23}N\textsubscript{7}O\textsubscript{6} and is the biological active form of the folate vitamers. It has an important role as cofactor for enzymes that transfer methyl groups, especially the transformation of serine to glycine. During this process serine donates a one carbon unit to THF which is then transformed to 5, 10-methylenetetrahydrofolate.

Tetrahydrofolic acid can also be regenerated by dihydrofolate reductase out of dihydrofolate. Certain antibiotics and cytostatics can interfere with this reaction. Methotrexate can inhibit dihydrofolate reductase and therefore limit the availability of THF [GARBIS et al., 2001; INSEL et al., 2011; LEDOCHOWSKI, 2010].
2.10.3 5-METHYLTETRAHYDROFOLIC ACID

Fig. 5: 5-Methyltetrahydrofolic acid [NELSON et al, 2004]

Being one of the predominant folate forms in human plasma 5-methyltetrahydrofolic acid is used to accurately diagnose a folate deficiency [NELSON et al., 2004]. Together with vitamin B12, this folate metabolite is involved in the remethylation of the amino acid homocysteine to methionine [INSEL et al., 2011].

2.11 MEASUREMENT

Accurate assessment of folate status in humans has become more important than ever, because a decreased folate status is associated with several diseases. Adequate folate supply can reduce the risk of neural tube defects and prevent cardiovascular diseases. The diversity of the folate forms however complicates the task of assaying endogenous folate. There is a strong need to develop an optimised method that gives reliable information about folate status in humans and its content in foods. However analysis do not turn out to be that easy, due to the diversity of the folate forms,
the low stability of the metabolites, their presence in low concentrations in biological systems and complex extraction and detection techniques. All these features complicate the task of assaying folates [ARKOT & SHRESTHA, 2005; HANNISDAL et al., 2009].

2.12 METHOD OF DETECTION

During the past few years several methods of detection have been applied to identify food folate including microbiological assay, radiobinding assay, radiometric assay as well as electrochemical, spectrophotometric and fluorometric methods.

Relying on the bacterial growth of Lactobacillus rhamnosus the microbiological assay has been the “gold standard” for a long time. It was introduced in the 1930s and was one of the first methods of detection for folate analysis. When using microbiological assay for folate measurements only total folate concentration can be identified. It is also known to be time-consuming, requiring a minimum of 24 hours to obtain assay results. This can result in a loss of folates as they are known to be quite instable [KALMBACH et al., 2011, NELSON et al., 2006, NELSON et al., 2004; SHANE, 2011].

There have been various analytical methods based on gas or liquid chromatography developed in the past years for the determination of folates [LIANG et al., 2009]. Although gas chromatography coupled with electron impact mass spectrometry (GC/EI-MS) allows sensitive detection to evaluate low molecular weight metabolites, it is not well suited to analyse metabolites with low volatility and thermal instability. Also GC/MS requires complex sample preparation including extraction and chemical derivatisation before injection. This can lead to experimental errors and increases the probability of folate degradation in the oven of the gas chromatography system [GARBIS et al., 2001].
The newer and more common method for rapid and quantitative analysis of folates in food, serum and blood is by using high performance liquid chromatography for separation and subsequent spectrometric detection. This can amongst others be done via UV or mass sensitive detectors. With these methods folate metabolites can be simultaneously and accurately identified and quantified [LIU et al., 2011].

As the importance of folate assessment increases, Puwastiena et al. evaluated inter-laboratory performance of food folate assay using 3 test materials (soybean flour, fish powder and breakfast cereal). The test materials were then sent to 34 laboratories in various countries. These were asked to use their routine method of analysis for total folate measurement. From the 34 laboratories, 26 sent back their results. 22 laboratories used microbiological assays and 4 used HPLC with UV detection, LC or radiobinding assays. The results for the 3 food materials differed from each other from 24 to 35 %. Therefore comparison was difficult. The finding suggests that there should be a uniform method of folate detection amongst laboratories worldwide so accurate results can be achieved [PUWASTIENA et al., 2005].

2.13 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

2.13.1 HISTORY

After Mikhail Tswett invented chromatography in 1902, the first separation with columns took place in the 1920s with the purification of petroleum [WELLINGS, 2006]. The first paper on high performance liquid chromatography was published in 1966 and since then the number of publications has grown exponentially. With the introduction of suitable columns for the separation of proteins in 1980 a complete new and facilitated application area in biochemistry has been opened.
It is primarily since the 1990s, that the understanding of the separation process taking place in HPLC has grown and suitable equipment and columns for all kinds of bio-molecules are now available [Snyder et al., 2010].

2.13.2 GENERAL

High performance liquid chromatography is known to be well accepted and is an analytical technique that is often used in different kinds of laboratories. The system is able to separate, identify, and quantify compounds in samples. The preparation time is usually short and the assessed qualitative and quantitative data can be recorded in one run even for samples that widely vary in their polarity. HPLC is often used in the pharmaceutical industry and a large number of laboratories use this type of chromatography for drug discovery, development and production [Ahuja & Dong, 2005; Karcher et al., 2005].

2.13.3 PARTS

The main components of an HPLC system are

- the solvent reservoir
- the mobile phase
- the pump
- the injector or the auto sampler
- the column (with the stationary phase)
- the detector and
- the data system with which data can be recorded and analysed [Snyder et al., 2010].
2.13.3.1 THE SOLVENT RESERVOIR

The mobile phase is usually held by the reservoir which is one of the essential parts of the HPLC system. The reservoir containers are mostly made out of glass. The solvent is filtered when leaving the reservoir through a tube and flows to the pump of the HPLC system.

There are two basic elution modes than can be used with an HPLC system. With isocratic elution either a pure solvent or a premixed mobile phase, which does not change throughout the whole run and therefore remains constant, is used [SYNDER et al., 2010]. Usually this elution mode is used to separate simple mixtures of compounds [McMASTER, 2005].

![Diagram of HPLC System](image)

Fig. 6: HPLC System with one solvent as mobile phase [WATERS, 2011]

The second type of elution, the gradient elution, requires a mobile phase that changes composition during analysis. This mode can be very useful for samples with molecules that vary a lot in their polarity. Therefore a better resolution and
decreased analysis time can be obtained. However it should be noted, that re-equilibration to the start is indispensable before the next sample can be injected [SNYDER et al., 2010; McMASTER, 2005].

![HPLC System with two solvents as mobile phase](image)

*Fig. 7: HPLC System with two solvents as mobile phase* [WATERS, 2011]

### 2.13.3.2 THE MOBILE PHASE

Using the correct composition and type of mobile phase is an important choice as it is one of the essential components to obtain a good separation [SNYDER et al., 2010]. The mobile phase should always be degassed before usage to eliminate bubbles that may lead to pump-delivery problems in the HPLC system. The most common and effective method of degassing the mobile phase is by filtration though a special kind of filter. Another method would be to flush the solvent with an inert gas like helium [McMASTER, 2005].
2.13.3 THE PUMP

The pump takes the solvent out from the reservoir, generates sufficient pressure and drives the mobile phase forward to the injector and later to the column. Most operators of a HPLC pumping system use pressures at about 150 to 200 bar [McMASTER, 2005; SNYDER et al., 2010].

2.13.3.4 THE INJECTOR / AUTO SAMPLER

Through the injector the sample is introduced in the system and therefore added to the flowing and pressurised mobile phase. For large analysis hundreds of samples and therefore injections are required. In this case it is essential to have an auto sampler that injects the samples automatically. An auto sampler is supposed to be more precise and accurate and the analysis can be left unattended. This is the reason why auto samplers have nearly replaced all manual injection procedures. The operator just places a sample on a tray which will then be automatically injected after starting a run. This is usually done computer-assisted [SYNDER et al., 2010].

2.13.3.5 COLUMN OVENS

According to Snyder et al. it is strongly recommended to use a column oven to keep temperature under control. Column temperature influences retention time and affects column selectivity. Usually temperatures of 30 – 50 °C are used to improve retention time [AHUJA & DONG, 2005; SYNDER et al., 2010].
2.13.3.6 COLUMN

After the sample has been injected into the system, a continuously flowing mobile phase that is pressurised by the pump drives the sample to the HPLC column. The column is the heart of the whole HPLC system, where the actual separation takes place [McMASTER, 2005]. Since 1960 HPLC systems have largely improved with regard to speed, efficiency as well as stability and reproducibility. The early columns were made out of glass but nowadays, due to high pressures which are used for analysis, more resistant materials such as metal are required [SNYDER et al., 2010].

![Diagram of HPLC column separation](image)

*Fig. 8.: HPLC column separation model [McMASTER, 2005]*

The sample that is injected into the HPLC system is separated in the column using either a polar stationary phase and a less polar mobile phase, as in normal phase liquid chromatography or the other way round as in reversed phase chromatography. With the first mentioned “mode” of separation polar compounds are more strongly retained than the less polar ones. These are therefore eluted first [SNYDER et al., 2010].
Nowadays HPLC columns are made out of stainless steel [McMASTER, 2005]. A column of an HPLC system is packed with a support and an attached stationary phase. Till the year 2000 particles with diameters of about 5 µm were used for routine separation. However now particle sizes of 3 µm and smaller are becoming more popular due to benefits such as shorter run time. The most common columns are silica based columns. The big advantage of silica is, that it can be bonded with different ligands as for example C8, C18, phenyl and cyano [SNYDER et al., 2005]. According to McMaster about 80 % of HPLC separations are carried out on a C18 column. It consists of a nonpolar organic phase chemically bonded to an underlying silica surface [McMASTER, 2005]. However the big disadvantage of silica based columns is, that silica dissolves in the mobile phase at pH above 8.0, at higher temperatures, as well as high salt concentrations and therefore shortens the column life dramatically. For analysis at high pH other materials than silica can be used to increase the stability of the column [McMASTER, 2005; SNYDER et al., 2010]. The column stationary phase does not only influence retention and selectivity, it also determines stability, reproducibility, peak shape, and column efficiency. Basically there are two major groups of stationary phases:

- normal phase and
- reversed phase.

However in the past few years hydrophilic liquid interaction chromatography has attracted attention. It uses a polar stationary phase with an aqueous organic mobile phase. This combination can be a useful technique for separation of small polar compounds [GUO & GAIKI, 2005; IKEGAMI et al., 2008; SNYDER et al., 2010].
2.13.3.7.1 NORMAL PHASE

Being the first type of column available for HPLC, normal phase columns are untreated silica based columns [McMASTER 2005]. A polar stationary phase, like unbonded silica, is used with a less polar, highly organic mobile phase consisting of either single or mixed organic solvents [IKEGAMI et al, 2008]. More polar components of the sample are stronger retained while the more nonpolar molecules are eluted first [McMASTER, 2005]. Normal phase liquid chromatography has lost in popularity in the past few years due to long equilibration times, poor reproducibility and asymmetric peaks. However by improving the quality of the stationary phase, all these disadvantages have been eliminated, so that the typical normal phase is gaining in popularity again [LORBACH, 2009].

2.13.3.7.2 REVERSED PHASE

After the introduction of reversed phase columns for high performance liquid chromatography in the 1970s the use of normal phase chromatography became far less common. These reversed phase columns separate the sample with a nonpolar bound phase and a polar mobile phase by retaining the nonpolar compounds longest on the column packing. Very polar molecules interact with the mobile phase, are therefore less retained and leave the column first. With time the reversed phase column can change its characteristics due to hydrolysis of the bonded phases. Hence calcium and magnesium ions from glass reservoirs holding the mobile phases could modify its performance [McMASTER, 2005]. Silica based stationary phases with C8 or C18 chains attached are the typical separation media for reversed phase liquid chromatography due to better peak shape for small molecules [LÄMMERHOFER et al, 2008]. The first bonded-phases columns were the C18-silica columns or ODS (Octyldecyl-silica). The C18
side chains are attached to the silica support by Si-O-Si links. Whereas the C8 columns (Octyl) have, as the name, a C8 side chain attached to the silica by Si-O-Si linkage [McMASTER, 2005].

2.13.3.7.3 HILIC

As mentioned before several liquid chromatography separation methods can be used to analyse certain samples. Reversed phase is by far the most frequently used method [WU et al., 2008]. RPLC has become the method of choice for the chromatographic separation of organic low molecular mass compounds such as pharmaceuticals, peptides, pesticides, food constituents and additives. It is also known for its exceptionally broad applicability for organic molecules as well as its high efficiency and compatibility with ESI-MS [LÄMMERHOFER et al., 2008]. However RPLC has limitations particularly for the retention and analysis of very polar compounds like sugars, amino acids, nucleosides and folates [WU et al., 2008]. Small polar compounds are often very challenging to analyse due to lack of retention on conventional reversed phase columns. For this purpose hydrophilic liquid interaction chromatography, short HILIC, could be the preferred choice of method. HILIC can be seen as a complementary technique to reversed phase liquid chromatography with the benefit of being able to retain polar analytes. Instead of using a non-aqueous phase as in normal phase liquid chromatography an aqueous-organic mixture with water is being used with HILIC. This is the reason why it can also be called “aqueous normal-phase chromatography” [SNYDER et al., 2010]. The problem with NPLC is the difficulty in interfacing with mass spectrometric detectors [GUO & GAIKI, 2005]. As the aqueous organic mobile phase is compatible to ESI-MS, this combination gives the scientist the opportunity to detect and identify a variety of polar compounds. HILIC has attracted the interest of many researchers who study the separation of polar compounds [IKEAGAMI et al., 2008].
2.13.3.7.3.1 RETENTION MECHANISM

Hydrophilic liquid interaction chromatography provides an alternative approach to effectively separate polar compounds on polar stationary phases [GUO & GAIKI, 2005]. It acts as a counterpart to traditional RP liquid chromatography with an elution order being more or less the opposite of reversed phase separation [HEMSTROM & IRGUM 2006; KARATAPANIS et al., 2009]. Similar as in normal phase liquid chromatography polar compounds are more strongly retained in HILIC. However the non-aqueous mobile phase in NPLC is replaced in HILIC with an aqueous-organic mixture with water being the stronger solvent. This is the reason why HILIC is more suitable for MS detection than NPLC and it also improves the MS sensitivity [GUO & GAIKI, 2005].

By adding water to the organic mobile phase, the water forms a surface on the polar stationary phase. It is believed that the retention of analytes in HILIC is caused by partitioning of the analyte into a water layer formed and immobilised on the surface of the column packing [KARATAPANIS, 2009]. There are several advantages with HILIC as for example:

- very good retention of polar analytes
- less complicated eluent preparation, which can be a huge time-saver, especially for large sample batches
- enhanced sensitivity in ESI-MS due to the high organic content in the mobile phase
- fast separation due to lower viscosity of the mobile phase within the limits of the system, higher flow rates can be set
- the elution order of the analytes is usually the opposite of that in reversed phase separation which can give useful alternative selectivity.
Various stationary phases have been developed for HILIC to separate peptides, proteins, oligosaccharides, drugs, metabolites and many others, some of which are mentioned subsequently.

- underivatised silica
- amino propyl silica: initially used for carbohydrate separation. Their advantage is, that they prevent the formation of double peaks.
- nonsilica-based amino packing
- amide silica [HEMSTROM & IRGUM, 2006; JANDERA, 2011; McCALLEY, 2010; SNYDER et al.; 2010].

All these stationary phases are also traditionally used in NPLC. However, unbonded silica appears to be the method of choice when using HILIC and mass spectrometric detection [SNYDER et al., 2010]. Lately there have been some new inventions on the market for HILIC columns. Different manufacturers have designed their own stationary phases specifically for HILIC. The company Dichrom (formerly SeQuant) has invented a zwitterionic HILIC phase called the ZIC®-HILIC or the US company Waters that has launched the Atlantis, HILIC Silica column. Providing scientists with more choices to meet their specific needs, it makes it more and more challenging to choose the “right” column for the compound being analysed. As most studies about HILIC use only one column, there are only a few literature reports discussing the various columns. Finding the right one for their purpose can thus be quite difficult. For sure more studies are required in this field to find out more about the different types of polar stationary phases and HILIC separation [GUO & GAIKI, 2005; HEMSTRÖM et al., 2011].
2.13.3.7.3.2 APPLICATION

The application of HILIC for the analysis of small polar molecules has expanded in the last few years and goes from carbohydrates to nucleosides, nucleotides, amino acids, peptides and proteins. It also includes many small polar compounds such as drugs, toxins, plant extracts and other compounds important to food and pharmaceutical industries. Therefore it can be used by fields like the pharmaceutical chemistry, proteomics, metabolomics and glycomics [GUO & GAIKI, 2005; IKEGAMI et al., 2008]. But unfortunately only limited attention has so far been paid to the separation efficiencies of HILIC.

Although the acronym HILIC was introduced by Alpert in 1990 the number of publications regarding HILIC has substantially increased since 2000 [HEMSTROM & IRGUM, 2006]. The first generation of HILIC separation started in 1975 when Linden et al. separated carbohydrates by using an amino-silica phase in a mixture of 75 % of acetonitrile and 25 % of water [IKEGAMI et al., 2008]. Early HILIC applications mainly focused on carbohydrates and peptides analysis. But in the recent years analysis with HILIC has shown that it is a useful technique for separation of all kinds of small polar compounds including folates.

In 2001 Garbis et al. published an article about the determination of folates using HILIC coupled to a tandem mass spectrometer. In 2006 Bajad et al. analysed the separation and quantification of water soluble cellular metabolites by HILIC coupled to tandem mass spectrometry.

Ikeagami et al. reviewed the separation efficiencies in hydrophilic liquid interaction chromatography. But considering the growing importance and the notable potential of HILIC, there have been surprisingly few reviews dedicated to this topic [BAJAD et al., 2006; GARBIS et al., 2001].
2.13.3.7.3.3 PROBLEMS WITH HILIC COLUMNS

As HILIC has been introduced more recently and has not yet been used as often as RP liquid chromatography there have been some problems reported. Because the exact separation mechanism is less well understood compared to RPLC it is difficult to understand certain effects and changes in separation and analysis [McCALLLEY, 2010]. Fronting and tailing of peak shape seem to be two of the problems often reported. However as HILIC is such a new method of choice further improvements in HILIC columns as well as a better understanding of how to control these peak shape problems seem to be necessary. Some samples may only require an increase of buffer in the mobile phase. Also a change in column or mobile-phase pH may solve the problem. Silica columns or different kind of bonded phases should be used to avoid column bleeding, a common problem with bonded phases [SNYDER et al., 2010]. Another drawback of HILIC is the high consumption of expensive and environmentally less friendly organic solvent. Also columns can take very long to equilibrate with the mobile phase, in some cases up to 1 hour.

HILIC does not have the broad applicability as reversed phase liquid chromatography because some compounds are not soluble in the high fraction of organic solvent and neutral or non-polar analytes show hardly retention. This can result in precipitation and unsatisfactory peak shapes [RUTA et al., 2010]. McCally compared several different kinds of HILIC columns and found out that the results varied a lot. Some of the HILIC stationary phases had far better performance and others obtained nearly same or worse results than with reversed phase liquid chromatography. It was shown that different stationary phases achieved different selectivity when always using the same mobile phase. The mechanisms of how separation works in HILIC are very complex and it seems that the type of stationary phase has a large impact on retention behaviour [McCALLY, 2010].
Despite all problems, that not necessarily have to show up, HILIC plays an important role in modern pharmaceutical development and has also been used for determination of folates [HEMSTRÖM & IRGUM; 2006]. The analysis of polar compounds by hydrophilic liquid interaction chromatography coupled with a mass spectrometric detector can be used complementary to reversed phase liquid chromatography as recent analysis with HILIC has shown that it is a useful technique for separating such small polar compounds [NUJJS, 2011].

2.13.3.8 THE DETECTOR

Compounds from the HPLC system which are separated by the analytical column need to move to a detector for further analysis. A chromatographic detector converts a physical or chemical output of an eluted analyte into an electrical signal.

Fig. 9: HPLC system diagram [SNYDER et al., 2010]
Positioned right after the column, the detector analyses the effluent coming from the LC. After the detection the mobile phase stays in a liquid state and is transferred to a waste container, fraction collector or another detector like a mass detector [SNYDER et al., 2010]. To choose the right detector one has to include the sample specific characteristics in the decision making process. Therefore different detectors can be distinguished [SNYDER et al., 2010].

### 2.13.3.8.1 UV-VISIBLE DETECTORS

Most commonly used in HPLC are photometers based on the absorption of ultraviolet and visible light. They are insensitive to flow and temperature changes and are known to be reliable and easy to operate. UV-visible detectors can measure a variety of compound types throughout varying wave-lengths. Ideally UV detectors are used for gradient elution. The 3 commonly used UV detectors are:

- **Fixed-wavelength detectors**: while they are hardly used today, these detectors were once the mainly used UV detectors.

- **Variable-wavelength detectors**: coupled to HPLC, it is the most common used UV-visible detector.

- **Diode array detectors**: also called photodiode-array [SNYDER et al., 2010].

### 2.13.3.8.2 MASS SPECTRAL DETECTORS – LC/MS

Liquid chromatography coupled with mass spectrometry application shortly referred to as LC/MS application has been used since the 1980s in analytical
laboratories and is now well known to be a routine analytical method [GEY, 2008].

Fig. 10: Basic LC/MS System model [McMASTER, 2005]

2.14 MASS SPECTROMETER

Mass spectrometry is an essential analytical tool in pharmacy, chemistry, medicine and many related fields. The basic principal of a mass spectrometer is to create ions out of a sample, separate these ions and detect them qualitatively and quantitatively. This is done by giving information about the mass and weight of the molecule and therefore the ratio of molecule mass and ionic charge (m/z). This way the compounds can be identified by comparing the results with mass spectra in a data base.

Basically a mass spectrometer consists of 3 important parts

- the ionisation interface
- the mass analyser
- the detector [GROSS, 2011].
As a mass spectrometer can detect ions in the gaseous phase, the mobile phase coming from the HPLC must be evaporated and ions must be created out of the sample. This is the main function of an ionisation interface [SNYDER et al., 2010]. A high vacuum is produced by the vacuum pumping system of the mass spectrometer to prevent collision of ions with air [McMASTER, 2005].

In most LC/MS systems the ionisation interface can produce both either positive or negative charges, resulting in positive or negative ions. Electrospray interface, short ESI, and atmospheric-pressure chemical-ionisation interface, short APCI, are both methods to add a charge to the analyte and to get rid of the mobile phase. ESI uses heated drying gas that flows against the stream of the ions and therefore reduces the flow of neutral and unwanted ions.

![Diagram of the electrospray interface (ESI)](image)

Fig. 11: Schematic of the electrospray interface (ESI) for the LC/MS detector [SNYDER et al., 2010]

With APCI the mobile phase is first vaporised and then a charge is added to the analyte in gas phase [SNYDER et al., 2010]. As APCI is not well suited for folate analysis due to the high temperatures required, ESI is therefore more frequently
applied [QUINLIVAN et al, 2006]. ESI is used for polar species as well as compounds with high molecular weight. Whereas APCI is mostly used for small less polar compounds, that do not ionise well with ESI, like steroids and carotenoids [PRAMANIK et al., 2002].

Fig. 12: Schematic of the atmospheric-pressure chemical-ionisation interface (APCI) for the LC/MS detector [SNYDER et al., 2010]

Once the sample is ionised it can proceed to the analysing part of the mass spectrometer where the ions are fragmented [McMASTER, 2005]. Most commonly used MS systems are:

- quadrupole (as one of the oldest types)
- quadrupole ion trap
- time-of-flight tube
2.14.1 QUADRUPOLE ANALYSER

After the sample is charged in the ionising interface it is moved to the higher-vacuum area of the analyser. Being one of the first mass spectrometry systems and nowadays the most commonly used and cheapest mass spectral detectors, the quadrupole analyser uses four rods and an electric field to isolate selected ions from the sample [McMASTER 2005, SNYDER et al., 2010]. However before passing the analyser rods, a repeller plate of same charge state as the sample ions, forces the ions to the focusing lens. There again charges of same polarity drive the ions into a stream before entering the most important part of this mass detector, the quadrupole analyser. A dc and RF voltage is applied to four rods arranged, generating the electromagnetic field. This dc/RF field can be adjusted computer-controlled to address a single fragment mass. These selected fragments are being forced down the quadrupole between the rods. The other ions end up colliding with the rods by neutralisation. After passing the analyser the fragments leave the path by a lens and hit the detector that produces an amplified signal. This signal is converted and forwarded to a computer system where spectrums as well as current signals can be monitored [McMASTER, 2005].
2.14.2 TANDEM MS: TRIPLE QUADRUPOLE

Tandem mass spectrometry is a method that includes at least two stages of analysis. Most commonly it is used to isolate a precursor ion which is then fragmented. A frequently used instrument of this type using quadrupoles as analysers is the triple quadrupole detector [HOFFMANN & STROOBANT, 2007].

![Schematic of a triple quadrupole mass analyser](image)

*Fig. 12: Schematic of a triple quadrupole mass analyser [SNYDER et al., 2010]*

With this kind of detector additional selectivity can be obtained. 3 quadrupoles are therefore set up one after the other. Another part then analyses the product ions [SNYDER et al., 2010]. The first quadrupole acts as a kind of filter that allows only selected ions of a given m/z (parent ions) to pass and proceed to the second quadrupole, which is filled with a heavy inert gas such as argon. Here the ions are fragmented in the collision cell with optimal collision energy for producing fragment ions, also called daughter ions, and moved forward to the third quadrupole. This quadrupole isolates the specific, desired fragments and sends them to the multiplier for measurement. This unique fragmentation from parent ion to daughter ion can be allocated to one specific analyte and therefore increase the selectivity of the triple quadrupole in comparison to the single quadrupole detector [SNYDER et al., 2010].
2.14.3 ION TRAP

According to McMaster Laboratories, ion trap mass spectrometers are 10 to 100 times more sensitive than a quadrupole. Ion traps are based on the principle that the preferred product ion is fragmented and isolated in the same physical space. After ionisation in the interface either ions of a certain m/z or all ions can be accumulated in the ion trap between the electrodes with voltage. By increasing or decreasing the voltage only the desired fragments can escape through the traps exit. Either the ions are then sent to the multiplier for detection or further fragmentation and isolation can follow [McMASTER, 2005; SNYDER et al., 2010].

Fig. 13: ion trap analyser model [McMASTER, 2005]
2.14.4 QUADRUPOLE TIME OF FLIGHT ANALYSER

A quadrupole time of flight mass spectrometer is based on the theory that particles of varying mass fly with a varying speed. After ionisation only selected compounds are allowed to enter the collision cell. After accelerating the ions with a specific energy they are moved to the time-of-flight tube. Each fragment of a certain m/z ratio has its own flight time. Whereas lighter fragments reach the detector first, the heavier ones arrive last. Large flight tubes are supposed to increase the sensitivity. To avoid big time of flight instruments electrical “mirrors” are used to reflect and accelerate the fragment [McMASTER, 2005; SNYDER et al., 2010].

Fig. 15: Schematic of a Q-TOF system: S, ion source; Q₁, Quadrupole 1; Q₂, Quadrupole 2; IP, ion pusher; R, reflectron; D, detector [LAVAGNINI et al., 2006]
2.14.5 MASS SPECTRUM

A mass spectrum represents the signals intensity as well as m/z ratio. The so-called peak is a signal that reflects the m/z ratio of an ion that has been created from the analyte with the ion source. The intensity of the peak correlates with the abundance of the desired ion [GROSS, 2011].

Fig. 16: Example of a peak [MEYER, 2010]

The entity of all peaks is called a chromatogram. The retention time gives qualitative information about a component. It describes the time between injection of the sample and detection after passing through the column.
However the area as well as the height of a peak gives quantitative information of the sample. They are both proportional to the amount of the compound of interest [MEYER, 2010]. These intensity measures can be influenced by noise that can result from the electronics of the instrument.

The signal to noise ratio (S/N ratio) gives a quantitative measure of a signals quality by putting the intensity of a signal in relation to the noise. Accordingly a peak with a high signal has a better S/N ratio than a less intensive signal. The most intense ion of the mass spectrum is also called base peak [GROSS, 2011].
## 3 MATERIAL & METHODS

### 3.1 REAGENTS

<table>
<thead>
<tr>
<th>CHEMICALS</th>
<th>DESCRIPTION &amp; STRUCTURE</th>
<th>COMPANY</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetonitrile (HPLC gradient grade)</td>
<td>C₂H₃N</td>
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</tr>
<tr>
<td>Water (HPLC gradient grade)</td>
<td>H₂O</td>
<td>Carl Roth GmbH + CoKG, Germany</td>
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<tr>
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<td>HCO₂H</td>
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<td>C₄H₁₀O₂S₂</td>
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<tr>
<td>Folic acid</td>
<td>C₁₉H₁₉N₇O₆</td>
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<td>Merck &amp; Cie, Schaffhausen, Switzerland</td>
</tr>
<tr>
<td>Tetrahydrofolate</td>
<td>C₁₉H₂₃N₇O₈</td>
<td>Merck &amp; Cie, Schaffhausen, Switzerland</td>
</tr>
</tbody>
</table>

*Table 1: Reagents used in this thesis*
3.2 DEVICES

<table>
<thead>
<tr>
<th>EQUIPMENT</th>
<th>DESCRIPTION</th>
<th>COMPANY</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pipettes</td>
<td>Eppendorf Reference variable (10 µL, 100 µL and 1000 µL)</td>
<td>Eppendorf AG (Hamburg, Germany)</td>
</tr>
<tr>
<td>Amber glass vials</td>
<td>2 ml vials</td>
<td>Agilent Technologies, Böblingen Germany</td>
</tr>
<tr>
<td>Weighing scale</td>
<td>Sartorius ISO 9001 Modell KB BA 100</td>
<td>Sartorius AG (Göttingen, Germany)</td>
</tr>
<tr>
<td>HPLC System</td>
<td>Dionex Ultimate 3000 micro-HPLC-system</td>
<td>Dionex Corporation, Vienna, Austria</td>
</tr>
<tr>
<td>Mass spectrometer</td>
<td>Bruker Daltonics micrOTOF-QII (MS)</td>
<td>Bruker Daltonics, Bremen, Germany</td>
</tr>
<tr>
<td>HILIC column</td>
<td>Acclaim® HILIC-10 (Using 3µm 120Å 3.0 x 150 mm)</td>
<td>Dionex Corporation, Vienna, Austria</td>
</tr>
<tr>
<td>Reverse phase column</td>
<td>Atlantis T3 3µm 2,1x150mm</td>
<td>Waters, Vienna, Austria</td>
</tr>
</tbody>
</table>

Table 2: Devices used in this thesis
3.3 SOFTWARE

“25 Hystar” Version 3.2 from Bruker Daltonics GmbH (Bremen, Deutschland) and “Compass™” Version 1.3 software was used for HPLC/MS system control, data acquisition as well as data processing.

3.4 SOLUTIONS FOR HILIC ANALYSIS

10 mg of each folic acid, tetrahydrofolic acid and 5-methyltetrahydrofolic acid were weighed into a 100 ml amber vial and dissolved in 100 ml of buffer solution resulting in a stock solution of 100 µg/ml. The buffer solution contained 20 mmol/l of each Monopotassiumdihydrogenphosphate (KH$_2$PO$_4$) and Dipotassiummonohydrogenphosphate (K$_2$HPO$_4$). 0.1 % of DDT and 1 % of sodium ascorbate were added as anti-oxidants to prevent oxidative loss. The stock solution was flushed with argon and then immediately frozen at -20°C until analysis.

The stock solution was diluted to 5 dilution levels with a mixture of 80 % acetonitrile and 20 % buffer solution:

- 10 µg/ml
- 5 µg/ml
- 2.5 µg/ml
- 1 µg/ml
- 0.5 µg/ml

This way the samples were diluted in the same solution as the mobile phase. Each dilution level was injected in triplicate onto the LC/MS system to ensure reproducible results.
To ensure that none of the folate derivates decrease in their concentration during measurements, one dilution level (2.5 µg/ml) was measured before the whole sample batch and at the end of it, to ensure that concentrations were the same at the beginning and at the end of analysis. To ensure accurate results, measurements were done 3 times on one day (intraday variability) as well as on 3 different days (interday variability) per metabolite, giving 9 different results for each metabolite and each dilution level.

A blank level, consisting of 80 % of acetonitrile and 20 % of water was analysed between each dilution level to wash the column and in order to verify if there are any components retained on the column which could result in a memory effect.

### 3.5 SOLUTIONS FOR RPLC ANALYSIS

1.03 mg of folic acid, 1.07 mg of 5-methyltetrahydrofolic acid and 1.04 mg of tetrahydrofolic acid were separately weighed into an amber vial and dissolved in 100 ml of buffer containing 20 mmol/l of each KH$_2$PO$_4$ and K$_2$HPO$_4$, 0.1 % of DDT and 1 % of sodium ascorbate. The stock solution was diluted in 4 dilution levels for folic acid and 5-methyltetrahydrofolic acid

- 10 µg/ml
- 5 µg/ml
- 1 µg/ml
- 0.5 µg/ml

and 3 dilution levels for tetrahydrofolic acid

- 10 µg/ml
- 5 µg/ml
- 1 µg/ml
These results were then compared to measurements done with hydrophilic liquid interaction chromatography.

3.6 HPLC METHODS FOR HILIC AND RPLC ANALYSIS

Column oven temperature was set to 25°C, sampler temperature to 4°C and the mobile phase was driven at a flow rate of 0.300 ml/min.

3.7 MOBILE PHASE FOR HILIC ANALYSIS

As hydrophilic liquid interaction chromatography uses highly organic mobile phases which can contain up to 40 % of water or buffer, one can adjust optimal sample retention by increasing the concentration of acetonitrile. The level of organic solvent in the mobile phase probably has the largest influence on retention [JANDERA, 2011].
As the HILIC column used in this thesis was compatible with up to 20 % aqueous mobile phase, the mobile phase consisted of A: 80 % acetonitrile and 20 % water and B: 100 % acetonitrile to ensure that not more aqueous phase is applied to the column. 0.1 % of formic acid was added to the mobile phases in order to improve ionisation.
After experimenting with several different gradients the mobile phase was set to the following multi step gradient time program:

<table>
<thead>
<tr>
<th>Minute</th>
<th>% B</th>
<th>Flow</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>100 %</td>
<td>0.300 ml/min</td>
</tr>
<tr>
<td>5.0</td>
<td>100 %</td>
<td>0.300 ml/min</td>
</tr>
<tr>
<td>5.1</td>
<td>5 %</td>
<td>0.300 ml/min</td>
</tr>
<tr>
<td>11.0</td>
<td>5 %</td>
<td>0.300 ml/min</td>
</tr>
<tr>
<td>11.1</td>
<td>0 %</td>
<td>0.300 ml/min</td>
</tr>
<tr>
<td>25.0</td>
<td>0 %</td>
<td>0.300 ml/min</td>
</tr>
<tr>
<td>25.1</td>
<td>100 %</td>
<td>0.300 ml/min</td>
</tr>
<tr>
<td>45.0</td>
<td>100 %</td>
<td>0.300 ml/min</td>
</tr>
</tbody>
</table>

Table 3: Multi step gradient time program for HILIC analysis

### 3.8 MOBILE PHASE FOP RPLC ANALYSIS

The mobile phase for RPLC analysis consists of water (A) and acetonitrile (B). 0.1 % formic acid was added to the mobile phases in order to improve ionisation. The system was set up to the following multi step time program:

<table>
<thead>
<tr>
<th>Minute</th>
<th>% B</th>
<th>Flow</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.1 %</td>
<td>0.300 ml/min</td>
</tr>
<tr>
<td>7</td>
<td>10 %</td>
<td>0.300 ml/min</td>
</tr>
<tr>
<td>10</td>
<td>20 %</td>
<td>0.300 ml/min</td>
</tr>
<tr>
<td>14</td>
<td>40 %</td>
<td>0.300 ml/min</td>
</tr>
<tr>
<td>20</td>
<td>0.1 %</td>
<td>0.300 ml/min</td>
</tr>
</tbody>
</table>

Table 4: Multi step gradient time program for RPLC analysis
3.9   STABILITY TEST

To define how the stock solution should be stored stability tests were performed. A stock solution was filled in plastic vials as well as glass amber vials and concentrations were measured by liquid chromatography coupled with time of flight mass spectrometer. They were then stored for 1 month at 3 different temperatures:

- +4°C
- -20°C
- -80°C

After the month had passed the 6 samples were analysed using the same liquid chromatography/mass spectrometry system. Each sample was measured 5 times and an average area has been calculated. To compare the different results of each sample the average area was calculated in per cent of the average area obtained before storage of the stock solution.
4 RESULTS & DISCUSSION

4.1 RESULTS STABILITY TEST

4.1.1 RESULTS AFTER ONE MONTH STORAGE AT -80°C

<table>
<thead>
<tr>
<th>AVERAGE AREA BEFORE STORAGE</th>
<th>PLASTIC</th>
<th>GLASS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AVERAGE AREA</td>
<td>% REMAINING</td>
</tr>
<tr>
<td></td>
<td>-80 °C</td>
<td></td>
</tr>
<tr>
<td>FA</td>
<td>9 269 788</td>
<td>99 %</td>
</tr>
<tr>
<td>5-MTHF</td>
<td>35 223 049</td>
<td>100 %</td>
</tr>
<tr>
<td>THF</td>
<td>29 837 395</td>
<td>99 %</td>
</tr>
</tbody>
</table>

Table 5: Results of 3 folate derivat after one month storage at -80°C

4.1.2 RESULTS AFTER ONE MONTH STORAGE AT -20°C

<table>
<thead>
<tr>
<th>AVERAGE AREA BEFORE STORAGE</th>
<th>PLASTIC</th>
<th>GLASS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AVERAGE AREA</td>
<td>% REMAINING</td>
</tr>
<tr>
<td></td>
<td>-20 °C</td>
<td></td>
</tr>
<tr>
<td>FA</td>
<td>9 269 788</td>
<td>99 %</td>
</tr>
<tr>
<td>5-MTHF</td>
<td>35 223 049</td>
<td>94 %</td>
</tr>
<tr>
<td>THF</td>
<td>29 837 395</td>
<td>95 %</td>
</tr>
</tbody>
</table>

Table 6: Results of 3 folate derivat after one month storage at -20°C
4.1.3 RESULTS AFTER ONE MONTH STORAGE AT +4°C

<table>
<thead>
<tr>
<th></th>
<th>AVERAGE AREA BEFORE STORAGE</th>
<th>PLASTIC</th>
<th></th>
<th>GLASS</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>AVERAGE AREA +4 °C</td>
<td>% REMAINING</td>
<td>AVERAGE AREA +4 °C</td>
<td>% REMAINING</td>
</tr>
<tr>
<td>FA</td>
<td>9 269 788</td>
<td>8 277 805</td>
<td>89 %</td>
<td>8 729 708</td>
<td>94 %</td>
</tr>
<tr>
<td>5-MTHF</td>
<td>35 223 049</td>
<td>32 514 707</td>
<td>92 %</td>
<td>32 073 019</td>
<td>91 %</td>
</tr>
<tr>
<td>THF</td>
<td>29 837 395</td>
<td>13 282 813</td>
<td>45 %</td>
<td>24 592 120</td>
<td>82 %</td>
</tr>
</tbody>
</table>

Table 7: Results of 3 folate derivates after one month storage at +4°C

As can be seen in Table 4, the results achieved after storage at -80°C for 4 weeks show, that there was no significant loss of either one of the three folate vitamers. Also hardly any difference can be seen between storage in plastic or glass vials at this temperature. However when comparing the results of other storage temperatures with the measurement before storing the metabolites (Table 5 and 6), the highest loss was achieved with tetrahydrofolic acid at +4°C in plastic vials. Glass seems to be a better material concerning degradation of the folate vitamers, when working at about +4°C. It can be thus said, that it is not recommended to store any of the three folate metabolites at +4°C regardless of the reservoir material being plastic or glass.

Folic acid confirms to be one of the most stable metabolites. This experiment shows that storage of the metabolites at -20°C with a maximum loss of only calculated 4 % in glass amber vials is sufficient for this thesis. However these results differ from those of Patring et al. who analysed the stability of 5-methyltetrahydrofolic acid and tetrahydrofolic acid in yeast extracts frozen at -22°C. While 5-methyltetrahydrofolic acid did not show significant decrease tetrahydrofolic acid was less stable and showed losses up to 53 % after storage for 4 weeks [PATRING et al., 2005].
Liu et al. performed ultra-high-performance liquid chromatography tandem mass spectrometry to quantify tissue folate. They found out that especially tetrahydrofolate was very unstable when the tissue was stored at room temperature and exposed to light for 1 hour. When stored on ice in the dark, folate degradation was adequately prevented. In the study by Liu et al. the stability data show that the presence of antioxidants did not entirely protect folate from degradation [LIU et al., 2011].

4.2 RESULTS OF DETERMINATION OF FOLATE VITAMERS WITH HILIC

When the HILIC column was eluted with a hydrophobic mobile phase consisting of more than 80 % of acetonitrile the retention time for the highly hydrophilic folates exceeded 45 minutes. These results were also found when Garbis et al. determined folates in human plasma while using hydrophilic liquid interaction chromatography coupled to tandem mass spectrometry. With a mobile phase consisting of more than 80 % of acetonitrile the retention of folates increased dramatically to 60 – 80 minutes [GARBIS et al., 2001]. Therefore the time program in Table 1 was used for HILIC analysis. To make sure that the concentration of the folate derivates does not decrease during measurements, one dilution level (2.5 µg/ml) was measured before the whole sample batch and at the end of it. This test showed that there was no decrease of either one of the folate metabolites during analysis.

As shown in the Appendix the RSD of folic acid values vary from 0.30 % to 2.10 % and for tetrahydrofolic acid and 5-methyltetrahydrofolic acid from 0.01 % to 1.48 % and 0.70 % to 5.87 %, respectively. Calibration curves were calculated for all three folate species. They all showed a linear response with correlation coefficients ($R^2$) for folic acid of 0.9853, for tetrahydrofolic acid of 0.9895 and for 5-methyltetrahydrofolic acid of 0.9985. The measurements showed that for a concentration below 0.5 µg/ml, no further detection was possible.
Therefore the limit of detection for all 3 folate metabolites can be set to 0.5 µg/ml with HPLC using Acclaim® HILIC-10 coupled to a Bruker Daltonics micrOTOF-QII mass spectrometer. As can be seen in the Appendix peak shapes were not ideal as they showed tailing. This is known to be a common problem with HILIC columns.

4.3 RESULTS OF DETERMINATION OF FOLATE VITAMERS WITH RPLC

RSD for folic acid varied from 2.34 – 8.27 % when using the reversed phase column. For tetrahydrofolic acid and 5-methyltetrahydrofolic acid values ranged from 6.51 to 6.84 % and 0.81 to 17.12 %, respectively. Again calibration curves were calculated for all three folate vitamers. They all showed a linear response with correlation coefficients (R²) of 0.9653 for folic acid, 0.9978 for tetrahydrofolic acid and 0.997 for 5-methyltetrahydrofolic acid. Folic acid and 5-methyltetrahydrofolic acid showed a limit of detection of about 0.5 µg/ml and tetrahydrofolic acid at about 1 µg/ml.

When comparing the results of the HILIC column to those of the RP column all 3 folate metabolites achieved nearly the same limit of detection. The HILIC column used in this thesis combined with a quadrupole time of flight mass spectrometer as detector did not show satisfactory results as it was supposed to achieve better sensitivity than the reversed phase column.

Gabris et al. achieved much better results using hydrophilic interaction chromatography coupled to negative ion electrospray-tandem mass spectrometry with multiple reaction monitoring. Analysis was done with human plasma, however the limit of detection for FA was at 37.5 pmol/L, for THF at 425 pmol/L and for 5-MTHF at 165 pmol/L [GABRIS et al., 2001].

Sanchez et al. analysed the determination of folate metabolites in human bio fluids using hydrophilic liquid interaction chromatography together with tandem mass spectrometry. This study group achieved much higher sensitivity with a
limit of detection of 0.3 to 8.3 ng/ml. Their stock solution contained of 10 mg of standard that was dissolved in 10 ml of 0.2 M potassium phosphate also containing 0.03 % ascorbic acid. The standard solutions were diluted in deionised water or 80:20 acetonitrile:water. These standard solutions were also stable while storing them at -20°C for at least a month, which is comparable with the results shown in this thesis. However chromatographic separation took only 14 minutes with a mobile phase consisting of 20 mM ammonium formate in 80:20 acetonitrile:water. Flow rate was set much higher with 1 ml/minute and the column oven was adjusted to a temperature of 35°C [SANCHEZ et al., 2010].

Bajad et al. showed that by paying closer attention to pH of the standard solutions during analysis HILIC performance can improve – a possible error for the inefficient performance of the Acclaim® HILIC-10 column in this thesis.

Grumbach et al. pointed out the retention benefits of HILIC by analysing allantoin, a substance used in cosmetic creams, with a HILIC and a reversed phase column. They showed that allantoin was not retained during RPLC analysis, however nearly reached a retention factor of 1 when analysing the substance under HILIC conditions. This experiment shows the retention benefits of this technique for polar analytes in general [GRUMBACH et al., 2004].

Liang et al. developed an analytical method for the simultaneous measurement of sixteen compounds involved in the folate metabolic pathway. This was done by hydrophilic liquid interaction chromatography coupled to a tandem mass spectrometry detector. The study group also tested the influence of certain parameters such as temperature, pH, and flow rate to obtain optimal conditions. The limit of detection for folic acid and 5-methyltetrahydrofolic acid was at 0.1 ng/ml and 1 ng/ml for tetrahydrofolic acid. All three folate vitamers achieved far better results than in this thesis [LIANG et al., 2009].
5 CONCLUSION, OUTLOOK AND FUTURE REMARKS

The analysis of highly hydrophilic and polar compounds by hydrophilic liquid interaction chromatography coupled with mass spectrometry is supposed to be an approach additional to reversed phase liquid chromatography [NUIJJS, 2011]. This however could not be proven in this thesis, as the reversed phase column achieved nearly as good results as obtained with the HILIC column. Although HILIC has already been proven in several studies to be a powerful tool to detect small polar compounds, such as folates, this could not be confirmed in this thesis.

The usage of hydrophilic liquid interaction chromatography still seems to be rather seldom. This is probably also the reason why literature concerning this topic is not ubiquitous. Maybe this is a reflection of the still existing problems that come along with this rather new technology. Further studies have to be done in this field in order to get reliable results, especially when working with such a complicated method analysing folates. This tends to be rather challenging, especially concerning stability, solubility and analysis.

One approach would be to test the different materials that are used for HILIC columns, because the type of stationary phases can have a large impact on retention behaviour [McCALLY, 2010]. Columns from different manufacturers vary in their reproducibility, stability and compatibility with MS-systems (e.g. column-bleeding). Most researchers performing analysis with HILIC use only one single column. As several different kinds of stationary phases for HILIC are available (silica, amino, cyano, amide, aspartamide phases etc.), multiple columns should be examined and compared to find the appropriate column that would provide the best results in the field of folate analysis [GUO & GAIKI, 2005]. Once the right stationary phase is found the mobile phase needs to be adjusted. To do so experiments should be performed starting with a high concentration (up to 40 %) of buffer or water. After that the concentration of acetonitrile should be increased to find an acceptable sample retention [JANDERA, 2011]. Therefore
another approach to improving HILIC sensitivity towards folates is by varying the elution gradient. Liang et al. achieved great results using a linear elution gradient when the mobile phase consisted of up to 95 % of acetonitrile [LIANG et al., 2009]. Ruta et al. found out, while investigating the effects of sample dilution on peak shape in HILIC, that with a high initial concentration of acetonitrile, the sample should not be diluted in more than 10 % of water in order to achieve sharp peaks [RUTA et al., 2010]. As peak shapes were not ideal in this thesis, the dilution of the sample should be changed and results should be compared to find out which sample dilution is best for folate analysis with HILIC.

In this thesis better results might also have been achieved by paying more attention and varying several factors such as column temperature or the pH of the buffer solutions. A further field for experimenting, and therefore enhancing folate analysis, is by comparing results achieved at different pH. It is said that the pH is one of the most powerful ways to improve selectivity in HILIC separation [HEMSTRÖM, 2011].

Coupling ion traps or quadrupole analyser as mass spectral detectors to the LC system might also improve sensitivity. It is also necessary to examine and compare different kinds of detectors coupled to hydrophilic liquid interaction chromatography, as hardly any literature could be found on this topic.

As can be seen, there are several different approaches on how to find a more accurate method for folate analysis with hydrophilic liquid interaction chromatography. The right circumstances and conditions are still to be found, so that HILIC might once be used in the routine analysis of folic acid and its metabolites.
6 ABSTRACT

It is essential to detect folate concentration in food, serum and blood accurately to improve the populations supply of it and to avoid the occurrence of several diseases. Therefore a newer, not so commonly used HILIC column (Acclaim® HILIC-10) for HPLC/MS was used in this thesis to analyse 3 folate metabolites (folic acid, tetrahydrofolic acid and 5-methyltetrahydrofolic acid). The results were then compared with the results of the analysis with a reversed phase column. Additionally stability storage tests were performed, in order to verify at what storage temperature folates are most stable. Stability tests have shown that folic acid confirms to be the most stable metabolite and that storage of the vitamers at -20°C and in glass amber vials is sufficient for this thesis. Literature assures of achieving better results with HILIC columns than the more commonly used reversed phase columns. However in this thesis this could not be proven. The reversed phase column showed nearly the same sensitivity concerning all folate metabolites, which resulted in nearly the same limits of detection as the Acclaim® HILIC-10 column.

Better performance might have been achieved by varying factors such as column temperature, buffer pH, type of stationary phase or composition of the mobile phase. Also coupling an ion trap or a quadrupole analyser as a mass spectral detector to the LC system instead of the time of flight mass spectrometer might also improve sensitivity. Further studies should be done to find the right circumstances and conditions so that accurate and better results can be obtained when using hydrophilic liquid interaction chromatography for folate analysis.
7 ZUSAMMENFASSUNG

### RESULTS OF FA DETERMINATION USING HILIC

<table>
<thead>
<tr>
<th>DILUTION LEVEL</th>
<th>MEASUREMENT</th>
<th>AVERAGE AREA INTRADAY</th>
<th>AVERAGE AREA</th>
<th>STANDARD DEVIATION</th>
<th>RSD</th>
<th>TARGET CONCENTRATION [µg/ml]</th>
<th>CALCULATED CONCENTRATION [µg/ml]</th>
<th>ACCURACY</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 µg/ml</td>
<td>Day 1</td>
<td>2 419 335</td>
<td></td>
<td></td>
<td></td>
<td>2.10 %</td>
<td>10</td>
<td>10.06</td>
</tr>
<tr>
<td></td>
<td>Day 2</td>
<td>2 325 911</td>
<td>2 382 914</td>
<td>49 997</td>
<td>2.10</td>
<td>10</td>
<td>10.06</td>
<td>100.59 %</td>
</tr>
<tr>
<td></td>
<td>Day 3</td>
<td>2 403 497</td>
<td></td>
<td></td>
<td></td>
<td>2.10 %</td>
<td>10</td>
<td>10.06</td>
</tr>
<tr>
<td>5 µg/ml</td>
<td>Day 1</td>
<td>1 182 806</td>
<td>1 177 141</td>
<td>13 213</td>
<td>1.12</td>
<td>5</td>
<td>4.99</td>
<td>99.89 %</td>
</tr>
<tr>
<td></td>
<td>Day 2</td>
<td>1 164 121</td>
<td>1 177 141</td>
<td>13 213</td>
<td>1.12</td>
<td>5</td>
<td>4.99</td>
<td>99.89 %</td>
</tr>
<tr>
<td></td>
<td>Day 3</td>
<td>1 184 497</td>
<td></td>
<td></td>
<td></td>
<td>1.12 %</td>
<td>5</td>
<td>99.89 %</td>
</tr>
<tr>
<td>2,5 µg/ml</td>
<td>Day 1</td>
<td>573 833</td>
<td>581 114</td>
<td>10 671</td>
<td>1.84</td>
<td>2.5</td>
<td>2.49</td>
<td>99.65 %</td>
</tr>
<tr>
<td></td>
<td>Day 2</td>
<td>588 924</td>
<td>581 114</td>
<td>10 671</td>
<td>1.84</td>
<td>2.5</td>
<td>2.49</td>
<td>99.65 %</td>
</tr>
<tr>
<td></td>
<td>Day 3</td>
<td>580 584</td>
<td></td>
<td></td>
<td></td>
<td>1.84 %</td>
<td>2.5</td>
<td>99.65 %</td>
</tr>
<tr>
<td>1 µg/ml</td>
<td>Day 1</td>
<td>229 489</td>
<td>227 874</td>
<td>1 359</td>
<td>0.60</td>
<td>1</td>
<td>1.01</td>
<td>100.77 %</td>
</tr>
<tr>
<td></td>
<td>Day 2</td>
<td>227 567</td>
<td>227 874</td>
<td>1 359</td>
<td>0.60</td>
<td>1</td>
<td>1.01</td>
<td>100.77 %</td>
</tr>
<tr>
<td></td>
<td>Day 3</td>
<td>226 566</td>
<td></td>
<td></td>
<td></td>
<td>0.60 %</td>
<td>1</td>
<td>100.77 %</td>
</tr>
<tr>
<td>0.5 µg/ml</td>
<td>Day 1</td>
<td>114 659</td>
<td>113 678</td>
<td>337</td>
<td>0.30</td>
<td>0.5</td>
<td>0.53</td>
<td>105.62 %</td>
</tr>
<tr>
<td></td>
<td>Day 2</td>
<td>115 135</td>
<td>113 678</td>
<td>337</td>
<td>0.30</td>
<td>0.5</td>
<td>0.53</td>
<td>105.62 %</td>
</tr>
<tr>
<td></td>
<td>Day 3</td>
<td>111 242</td>
<td></td>
<td></td>
<td></td>
<td>0.30 %</td>
<td>0.5</td>
<td>105.62 %</td>
</tr>
</tbody>
</table>

*Table 8: Results of FA determination using HILIC*
### RESULTS OF THF DETERMINATION USING HILIC

<table>
<thead>
<tr>
<th>DILUTION LEVEL</th>
<th>MEASUREMENT</th>
<th>AVERAGE AREA</th>
<th>AVERAGE AREA INTRADAY</th>
<th>STANDARD DEVIATION</th>
<th>RSD</th>
<th>TARGET CONCENTRATION [µg/ml]</th>
<th>CALCULATED CONCENTRATION [µg/ml]</th>
<th>ACCURACY</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 µg/ml</td>
<td>Day 1</td>
<td>894 185</td>
<td>894 185</td>
<td>885 086</td>
<td>0.99%</td>
<td>10</td>
<td>10.07</td>
<td>100.71%</td>
</tr>
<tr>
<td></td>
<td>Day 2</td>
<td>884 438</td>
<td>876 634</td>
<td>865 086</td>
<td>1.48%</td>
<td>5</td>
<td>4.85</td>
<td>97.06%</td>
</tr>
<tr>
<td></td>
<td>Day 3</td>
<td>415 162</td>
<td>423 289</td>
<td>421 328</td>
<td>0.09%</td>
<td>2</td>
<td>2.48</td>
<td>99.25%</td>
</tr>
<tr>
<td>5 µg/ml</td>
<td>Day 1</td>
<td>427 856</td>
<td>423 289</td>
<td>421 328</td>
<td>0.85%</td>
<td>1</td>
<td>1.06</td>
<td>105.99%</td>
</tr>
<tr>
<td></td>
<td>Day 2</td>
<td>421 933</td>
<td>423 289</td>
<td>421 328</td>
<td>0.85%</td>
<td>1</td>
<td>1.06</td>
<td>105.99%</td>
</tr>
<tr>
<td></td>
<td>Day 3</td>
<td>214 119</td>
<td>212 933</td>
<td>214 319</td>
<td>1.07%</td>
<td>0.5</td>
<td>0.54</td>
<td>108.14%</td>
</tr>
<tr>
<td>2.5 µg/ml</td>
<td>Day 1</td>
<td>213 201</td>
<td>213 201</td>
<td>213 201</td>
<td>1.07%</td>
<td>0.5</td>
<td>0.54</td>
<td>108.14%</td>
</tr>
<tr>
<td></td>
<td>Day 2</td>
<td>214 119</td>
<td>214 119</td>
<td>214 119</td>
<td>1.07%</td>
<td>0.5</td>
<td>0.54</td>
<td>108.14%</td>
</tr>
<tr>
<td></td>
<td>Day 3</td>
<td>214 119</td>
<td>214 119</td>
<td>214 119</td>
<td>1.07%</td>
<td>0.5</td>
<td>0.54</td>
<td>108.14%</td>
</tr>
<tr>
<td>1 µg/ml</td>
<td>Day 1</td>
<td>87 788</td>
<td>87 626</td>
<td>88 045</td>
<td>0.85%</td>
<td>0.5</td>
<td>0.54</td>
<td>108.14%</td>
</tr>
<tr>
<td></td>
<td>Day 2</td>
<td>86 812</td>
<td>87 626</td>
<td>88 045</td>
<td>0.85%</td>
<td>0.5</td>
<td>0.54</td>
<td>108.14%</td>
</tr>
<tr>
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<td>Day 3</td>
<td>88 279</td>
<td>88 279</td>
<td>88 279</td>
<td>0.85%</td>
<td>0.5</td>
<td>0.54</td>
<td>108.14%</td>
</tr>
<tr>
<td>0.5 µg/ml</td>
<td>Day 1</td>
<td>41 286</td>
<td>41 286</td>
<td>41 286</td>
<td>0.85%</td>
<td>0.5</td>
<td>0.54</td>
<td>108.14%</td>
</tr>
<tr>
<td></td>
<td>Day 2</td>
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<td>41 596</td>
<td>41 596</td>
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<td>0.54</td>
<td>108.14%</td>
</tr>
<tr>
<td></td>
<td>Day 3</td>
<td>42 163</td>
<td>42 163</td>
<td>42 163</td>
<td>0.85%</td>
<td>0.5</td>
<td>0.54</td>
<td>108.14%</td>
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Table 9: Results of THF determination using HILIC
### RESULTS OF 5-MTHF DETERMINATION USING HILIC

<table>
<thead>
<tr>
<th>DILUTION LEVEL</th>
<th>MEASUREMENT</th>
<th>AVERAGE AREA INTRADAY</th>
<th>AVERAGE AREA</th>
<th>STANDARD DEVIATION</th>
<th>RSD</th>
<th>TARGET CONCENTRATION [µg/ml]</th>
<th>CALCULATED CONCENTRATION [µg/ml]</th>
<th>ACCURACY</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 µg/ml</td>
<td>Day 1</td>
<td>1186 765</td>
<td>1202 800</td>
<td>20 411</td>
<td>1.70%</td>
<td>10</td>
<td>10.11</td>
<td>101.12%</td>
</tr>
<tr>
<td></td>
<td>Day 2</td>
<td>1225 776</td>
<td>1155 899</td>
<td>23 122</td>
<td>1.95%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Day 3</td>
<td>1195 859</td>
<td>1150 896</td>
<td>22 122</td>
<td>1.92%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 µg/ml</td>
<td>Day 1</td>
<td>1152 765</td>
<td>114 751</td>
<td>4 122</td>
<td>0.70%</td>
<td>5</td>
<td>4.88</td>
<td>97.56%</td>
</tr>
<tr>
<td></td>
<td>Day 2</td>
<td>591 721</td>
<td>587 051</td>
<td>4 122</td>
<td>0.70%</td>
<td></td>
<td>5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Day 3</td>
<td>583 921</td>
<td>587 051</td>
<td>4 122</td>
<td>0.70%</td>
<td></td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>2.5 µg/ml</td>
<td>Day 1</td>
<td>300 703</td>
<td>308 624</td>
<td>11 202</td>
<td>3.63%</td>
<td>2.5</td>
<td>2.51</td>
<td>100.46%</td>
</tr>
<tr>
<td></td>
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<td>316 546</td>
<td>308 622</td>
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<td>3.63%</td>
<td></td>
<td>2.51</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Day 3</td>
<td>308 622</td>
<td>308 622</td>
<td>11 202</td>
<td>3.63%</td>
<td></td>
<td>2.51</td>
<td></td>
</tr>
<tr>
<td>1 µg/ml</td>
<td>Day 1</td>
<td>129 673</td>
<td>137 741</td>
<td>8 080</td>
<td>5.87%</td>
<td>1</td>
<td>1.07</td>
<td>105.89%</td>
</tr>
<tr>
<td></td>
<td>Day 2</td>
<td>137 717</td>
<td>137 741</td>
<td>8 080</td>
<td>5.87%</td>
<td></td>
<td>1.07</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Day 3</td>
<td>145 833</td>
<td>137 741</td>
<td>8 080</td>
<td>5.87%</td>
<td></td>
<td>1.07</td>
<td></td>
</tr>
<tr>
<td>0.5 µg/ml</td>
<td>Day 1</td>
<td>73 475</td>
<td>75 879</td>
<td>2 769</td>
<td>3.65%</td>
<td>0.5</td>
<td>0.53</td>
<td>106.61%</td>
</tr>
<tr>
<td></td>
<td>Day 2</td>
<td>75 256</td>
<td>75 879</td>
<td>2 769</td>
<td>3.65%</td>
<td></td>
<td>0.53</td>
<td></td>
</tr>
<tr>
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<td>Day 3</td>
<td>78 907</td>
<td>75 879</td>
<td>2 769</td>
<td>3.65%</td>
<td></td>
<td>0.53</td>
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</tr>
</tbody>
</table>

*Table 10: Results of 5-MTHF determination using HILIC*
8.4 FA CALIBRATION CURVE USING HILIC

\[ y = 0.0042x + 50.648 \]
\[ R^2 = 0.9853 \]

*Fig. 17: FA calibration curve using HILIC*

8.5 THF CALIBRATION CURVE USING HILIC

\[ y = 0.0113x + 69.707 \]
\[ R^2 = 0.9895 \]

*Fig. 18: THF calibration curve using HILIC*
8.6 5-MTHF CALIBRATION CURVE USING HILIC

$y = 0.0085x - 111.9$

$R^2 = 0.9985$

*Fig. 19: 5-MTHF calibration curve using HILIC*
8.7 EIC OF FA USING HILIC

Fig. 20: EIC of FA at different concentrations using HILIC
8.8 EIC OF THF USING HILIC

Fig. 21: EIC of THF at different concentrations using HILIC
8.9 EIC OF 5-MTHF USING HILIC

![Graphs showing EIC of 5-MTHF at different concentrations using HILIC](image)

Fig. 22: EIC of 5-MTHF at different concentrations using HILIC
<table>
<thead>
<tr>
<th>DILUTION LEVEL</th>
<th>AREA INTRADAY</th>
<th>AVERAGE AREA</th>
<th>STANDARD DEVIATION</th>
<th>RSD</th>
<th>TARGET CONCENTRATION [µg/ml]</th>
<th>CALCULATED CONCENTRATION [µg/ml]</th>
<th>ACCURACY</th>
</tr>
</thead>
<tbody>
<tr>
<td>10.30 µg/ml</td>
<td>337 192</td>
<td>343 788</td>
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<td>10.30</td>
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<td>92.34 %</td>
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<td></td>
<td>352 729</td>
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<td>341 443</td>
<td></td>
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<tr>
<td>5.15 µg/ml</td>
<td>237 787</td>
<td>23 1 533</td>
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<td>5.15</td>
<td>6.35</td>
<td>123.22 %</td>
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<td></td>
<td>230 976</td>
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</tr>
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<td>35 224</td>
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<td>1.03</td>
<td>0.81</td>
<td>78.63 %</td>
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<td></td>
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<td>37 302</td>
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<tr>
<td>0.51 µg/ml</td>
<td>18 815</td>
<td>17 413</td>
<td>1 440</td>
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<td>0.51</td>
<td>0.31</td>
<td>59.73 %</td>
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<tr>
<td></td>
<td>17 487</td>
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*Table 11: Results of FA determination using RPLC*
Table 12: Results of THF determination using RPLC

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<th>STANDARD DEVIATION</th>
<th>RSD</th>
<th>TARGET CONCENTRATION [µg/ml]</th>
<th>CALCULATED CONCENTRATION [µg/ml]</th>
<th>ACCURACY</th>
</tr>
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<tbody>
<tr>
<td>10.40 µg/ml</td>
<td>129 476</td>
<td>120 431</td>
<td>7 835</td>
<td>6.51 %</td>
<td>10.40</td>
<td>10.50</td>
<td>100.95 %</td>
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<tr>
<td></td>
<td>116 042</td>
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<td>115 774</td>
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</tr>
<tr>
<td>5.20 µg/ml</td>
<td>58 090</td>
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<td>8.39 %</td>
<td>5.20</td>
<td>4.95</td>
<td>95.16 %</td>
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<td></td>
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<tr>
<td>1.04 µg/ml</td>
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<td>10 258</td>
<td>702</td>
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<td>1.19</td>
<td>114.35 %</td>
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<td></td>
<td>10 126</td>
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Table 12: Results of THF determination using RPLC
## 8.12 RESULTS OF 5-MTHF DETERMINATION USING RPLC

<table>
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<tr>
<th>DILUTION LEVEL</th>
<th>CALCULATED CONCENTRATION [µg/ml]</th>
<th>TARGET CONCENTRATION [µg/ml]</th>
<th>RSD</th>
<th>STANDARD DEVIATION</th>
<th>AVERAGE AREA</th>
<th>AREA INTRADAY</th>
</tr>
</thead>
<tbody>
<tr>
<td>10.70 µg/ml</td>
<td>572.755</td>
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<td>0.81 %</td>
<td>4 643</td>
<td>572 480</td>
<td>572 755</td>
</tr>
<tr>
<td>5.35 µg/ml</td>
<td>576.979</td>
<td>10.70</td>
<td>0.81 %</td>
<td>8 724</td>
<td>312 110</td>
<td>311 591</td>
</tr>
<tr>
<td>1.07 µg/ml</td>
<td>312 619</td>
<td>5.35</td>
<td>2.80 %</td>
<td>8 724</td>
<td>320 044</td>
<td>320 044</td>
</tr>
<tr>
<td>0.53 µg/ml</td>
<td>51 081</td>
<td>1.07</td>
<td>0.89 %</td>
<td>461</td>
<td>51 197</td>
<td>51 597</td>
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Table 13: Results 5-MTHF determination using RPLC

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<th>CONCENTRATION [µg/ml]</th>
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<th>5.72</th>
<th>5.35</th>
<th>0.96</th>
</tr>
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<tbody>
<tr>
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<td>576 979</td>
<td>312 619</td>
<td>51 197</td>
</tr>
<tr>
<td>AREA INTRADAY</td>
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<td>572 480</td>
<td>312 110</td>
<td>51 597</td>
</tr>
<tr>
<td>RSD</td>
<td>0.81 %</td>
<td>2.80 %</td>
<td>0.89 %</td>
<td>0.96</td>
</tr>
<tr>
<td>STANDARD DEVIATION</td>
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<td>8 724</td>
<td>8 724</td>
<td>461</td>
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<tr>
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<td>5.35</td>
<td>1.07</td>
</tr>
<tr>
<td>CALCULATED CONCENTRATION [µg/ml]</td>
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<td>5.72</td>
<td>5.35</td>
<td>0.96</td>
</tr>
<tr>
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<td>572 480</td>
<td>576 979</td>
<td>312 619</td>
<td>51 197</td>
</tr>
<tr>
<td>AREA INTRADAY</td>
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<td>572 480</td>
<td>312 110</td>
<td>51 597</td>
</tr>
<tr>
<td>RSD</td>
<td>0.81 %</td>
<td>2.80 %</td>
<td>0.89 %</td>
<td>0.96</td>
</tr>
<tr>
<td>STANDARD DEVIATION</td>
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<td>8 724</td>
<td>8 724</td>
<td>461</td>
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<tr>
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<td>5.35</td>
<td>1.07</td>
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<td>5.72</td>
<td>5.35</td>
<td>0.96</td>
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<td>576 979</td>
<td>312 619</td>
<td>51 197</td>
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<tr>
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<td>572 480</td>
<td>312 110</td>
<td>51 597</td>
</tr>
<tr>
<td>RSD</td>
<td>0.81 %</td>
<td>2.80 %</td>
<td>0.89 %</td>
<td>0.96</td>
</tr>
<tr>
<td>STANDARD DEVIATION</td>
<td>4 643</td>
<td>8 724</td>
<td>8 724</td>
<td>461</td>
</tr>
<tr>
<td>TARGET CONCENTRATION</td>
<td>10.70</td>
<td>5.35</td>
<td>5.35</td>
<td>1.07</td>
</tr>
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<td>10.49</td>
<td>5.72</td>
<td>5.35</td>
<td>0.96</td>
</tr>
<tr>
<td>AVERAGE AREA</td>
<td>572 480</td>
<td>576 979</td>
<td>312 619</td>
<td>51 197</td>
</tr>
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<td>572 480</td>
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</tr>
<tr>
<td>RSD</td>
<td>0.81 %</td>
<td>2.80 %</td>
<td>0.89 %</td>
<td>0.96</td>
</tr>
<tr>
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<td>461</td>
</tr>
<tr>
<td>TARGET CONCENTRATION</td>
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<td>5.35</td>
<td>5.35</td>
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<tr>
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<td>10.49</td>
<td>5.72</td>
<td>5.35</td>
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</tr>
<tr>
<td>AVERAGE AREA</td>
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<td>312 619</td>
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<td>AREA INTRADAY</td>
<td>572 755</td>
<td>572 480</td>
<td>312 110</td>
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</tr>
<tr>
<td>RSD</td>
<td>0.81 %</td>
<td>2.80 %</td>
<td>0.89 %</td>
<td>0.96</td>
</tr>
<tr>
<td>STANDARD DEVIATION</td>
<td>4 643</td>
<td>8 724</td>
<td>8 724</td>
<td>461</td>
</tr>
<tr>
<td>TARGET CONCENTRATION</td>
<td>10.70</td>
<td>5.35</td>
<td>5.35</td>
<td>1.07</td>
</tr>
<tr>
<td>CALCULATED CONCENTRATION [µg/ml]</td>
<td>10.49</td>
<td>5.72</td>
<td>5.35</td>
<td>0.96</td>
</tr>
<tr>
<td>AVERAGE AREA</td>
<td>572 480</td>
<td>576 979</td>
<td>312 619</td>
<td>51 197</td>
</tr>
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<td>AREA INTRADAY</td>
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<td>312 110</td>
<td>51 597</td>
</tr>
<tr>
<td>RSD</td>
<td>0.81 %</td>
<td>2.80 %</td>
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<td>0.96</td>
</tr>
</tbody>
</table>
8.13 FA CALIBRATION CURVE USING RPLC

![Graph](https://example.com/graph1.png)

\[ y = 0.0282x - 183.44 \]
\[ R^2 = 0.9653 \]

*Fig. 23: FA calibration curve using RPLC*

8.14 THF CALIBRATION CURVE USING RP CHROMATOGRAPHY

![Graph](https://example.com/graph2.png)

\[ y = 0.0845x + 322.45 \]
\[ R^2 = 0.9978 \]

*Fig. 24: THF calibration curve using RPLC*
8.15 5-MTHF CALIBRATION CURVE USING RP CHROMATOGRAPHY

Fig. 25: 5-MTHF calibration curve using RPLC

\[
y = 0.0183x + 17.571
\]

\[
R^2 = 0.997
\]

[c] in ng/ml

Area
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                french:  basic knowledge