CHROMATOGRAPHIC DETERMINATION OF AMINES IN FOOD SAMPLES

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Abstract

In the first part of this thesis literature about the determination amines in food samples of the past decade (2007 – 2017) has been reviewed. The sample preparation methods and chromatographic determination methods have been reviewed. The review is focused on biogenic amines (BA) since BAs are the most relevant in food samples. Monitoring the concentration levels of BAs in foods is important because elevated levels of amine concentrations in food products can indicate spoilage which can lead to food poisoning.

Food samples are complex matrices therefore sample preparation is required prior to analysis. Amine extraction methods are reviewed in more detail, including conventional solid-liquid extraction (SLE) and solid phase extraction (SPE), and novel and miniaturized methods: solid phase micro extraction (SPME), liquid phase micro extraction (LPME) and dispersive liquid-liquid micro extraction (DLLME). The derivatization methods of Bas have also been reviewed including derivatization with o - phthaldialdehyde (OPA), dansyl chloride, benzoyl chloride and diethyl ethoxymethylenemalonate (DEEMM).

Chromatographic methods are well researched tools in determination of amines in food samples. In the past decade only few application were found of thin layer chromatography (TLC). The gas chromatography (GC) has been used more often, especially in the analyses of beverages. However, the high performance liquid chromatography (HPLC) is the main method of choice in determination of amines in food samples as demonstrated by the large numbers of research articles. Recently also the ultra-high performance liquid chromatography (UHPLC) has been gaining popularity.

In this master's thesis experimental part several sets of experiments were performed. Adsorbing materials were synthesized using suspension polymerization and silica gel functionalization. Compositions of materials were estimated by ATR-FTIR. The materials were characterized in terms of their suitability for amine adsorption. Ion exchange capacity was determined by titration. Static and dynamic binding capacity was determined by HPLC-UV. Derivatization studies of atmospheric amines by 9-Fluorenylmethoxycarbonyl chloride (Fmoc-Cl) were carried out by HPLC-UV. Potential imine formation was investigated by HPLC-UV. The most promising adsorbing material was a hydrolyzed copolymer of divinylbenzene and methacrylic anhydride (DVB-$(MAA)_2O$). Its ion exchange capacity was 4.8 meq/g, static binding capacity was 0.95 mmol/g of tertiary amine and dynamic binding capacity was 2.0 mmol/g for primary amine and 0.8 mmol/g for tertiary amine.

Keywords

Amines, biogenic amines, sample preparation, extraction, derivatization, food samples, chromatographic analysis, HPLC, TLC, GC, suspension polymerisation, FTIR, adsorbents, ion exchange capacity, imines

Where deposited

E - thesis

Additional information

Preface

The experimental part of this thesis was carried out in the Laboratory of Analytical Chemistry in University of Helsinki, Finland during the period from December 2014 to May 2015. The literature part was done during autumn 2017 to spring 2018.

I wish to express my endless gratefulness to my supervisor Norbert Maier for the incredible amount of knowledge I have gained during this work.

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I sincerely wish to thank all my loved ones, friends and family for all the encouragement, love and support.

Table of contents

Abbreviations

(DVB-$(MAA)_2O$)	Copolymer of divinylbenzene and methacrylic anhydride
(EDMA-BMA-AA)	Copolymer of ethylene dimethacrylate, butyl methacrylate and acrylic acid
(EDMA-MAA)	Copolymer of ethylene dimethacrylate and methacrylic acid
$(MAA)_2O$	Methacrylic anhydride
(Sty-DVB-$(MAA)_2O$)	Copolymer of styrene, divinylbenzene and methacrylic anhydride
AA	Acrylic acid
ACN	Acetonitrile
AGM	Agmatine
AIBN	α,α´-azobisbutyronitrile
anh	Methacrylic anhydride
ATR	Attenuated total reflectance
ATR-FTIR	Attenuated total reflectance Fourier transform Infra-red
BA	Biogenic amines
BMA	Butyl methacrylate
CAD	Cadaverine
CE	Capillary electrophoresis
CI	Interval of confidence
DA	Dopamine
DAD	Diode array detector
DAO	Diaminooxidase
DEEMM	Diethyl ethoxymethylenemalonate
DLLME	Dispersive liquid-liquid micro extraction
DVB	Divinylbenzene
EDMA	Ethylene dimethacrylate
ELSD	Evaporative light scattering detection
ESI	Electrospray ionization
FL	Fluorescence
Fmoc-Cl	9-Fluorenylmethoxycarbonyl chloride
GC	Gas chromatography
HAAs	Heterocyclic aromatic amines

HF-LPME	Hollow fiber liquid phase microextraction
HIS	Histamine
HPLC	High performance liquid chromatography
IEC	Ion exchange capacity
IR	Infra-red
IS	Internal standard
LLE	Liquid – liquid extraction
LPME	Liquid phase micro extraction
MAA	Methacrylic acid
MAE	Microwave assisted extraction
MAO	Monoaminooxidase
MRM	Multiple reactions monitoring
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
MSPD	Matrix solid phase dispersion
NE	Norepinephrine
NME	Normetanephrine
NOR	Noradrenaline
OCT	Octopamine
OPA	o-phthaldialdehyde
PA	Primary amine, 3-phenyl propylamine
PAD	Photodiode array detector
PAO	Polyaminooxidase
PDMS	Polydimethylsiloxane
PEA	Phenylethylamine
Primary amine	3-phenyl propylamine
PUT	Putrescine
PVA	Polyvinyl alcohol
PVP	Polyvinylpyrrolidone
RP	Reversed phase
RSD	Relative standard deviation
SALLE	Salting-out assisted liquid-liquid extraction
SD	Standard deviation

SDME	Single drop microextraction
SER	Serotonin
SFO	Solidification of floating organic droplets
SH-Silica	Mercaptopropyl-silica
SIM	Selective ion monitoring
SLE	Solid-liquid extraction
SO_3H-Silica	Silica supported sulphonic acid
SPD	Spermidine
SPE	Solid phase extraction
SPM	Spermine
SPME	Solid phase microextraction
Sty	Styrene
TA	Tertiary amine, N,N-Dimethylbenzylamine
TCA	Trichloroacetic acid
Tertiary amine	N,N-Dimethylbenzylamine
TFA	Trifluoroacetic acid
TFAA	Trifluoroacetylacetone
TLC	Thin layer chromatography
TMA	Trimethyl amine
TMA-N	Trimethylamine nitrogen
TRP	Tryptamine
TVB-N	Total volatile base nitrogen
TYR	Tyramine
UA	Ultrasound assisted
UHPLC	Ultra-high performance liquid chromatography
UV	Ultra violet
VOC	Volatile organic compounds

1 Introduction

‘’Amines’’ is a group of basic compounds containing at least one amino group. Amines that are found in food samples most often are biologically active or biogenic. Among the most commonly detected biogenic amines (BA) are cadaverine, spermine, spermidine, histamine, dopamine, and serotonin. Biogenic amines in organisms have such functions as neural transmission, regulation of blood pressure and temperature, and cell growth regulation. Biogenic amines originate mostly from decarboxylation of amino acids either by endogenous enzymes or microbial activity, and transamination reactions of aldehydes and ketones. In food samples biogenic amines are found in high concentrations in fermented products such as wine, beer, cheese and in low concentrations in non-fermented products such as vegetables, fruit, meat and milk.[1-3]

Monitoring of biogenic amines in food samples is important because they are indicators of food quality. Overly high levels of BAs can lead to food poisoning. For example, maximum allowed concentration of histamine in foods is 50 – 100 mg/kg and above 1080 mg/kg histamine becomes toxic[1]. Detoxification of BAs is done by oxidase ferments (monoaminooxidase (MAO), diamine oxidase (DAO) and polyaminooxidase (PAO))[2]. This should be especially taken into account by people who are taking MAO inhibitor containing drugs such as anti-depressants. If metabolic degradation of BAs is slowed down their concentrations can reach toxic levels that can cause, for example, migraines[4]. BAs also affect the organoleptic properties and can be used to indicate the authenticity of foods[2-3].

Food samples can be particularly challenging matrices of analytes hence sample preparation is almost always implemented prior to analysis. Sample preparation include steps like homogenization, degreasing, degassing and analyte extraction from the matrix. Most common extraction methods include solid phase extraction (SPE), liquid-liquid extraction (LLE) and solid-liquid extraction (SLE)[4]. More recently used methods have been developed with a focus on cost efficiency and green practices. Therefore miniaturized methods such as solid phase micro extraction (SPME), dispersive liquid-liquid micro extraction (DLLME), and hollow fiber liquid phase microextraction (HF-LPME) are gaining popularity over the traditional extraction methods[2].

Analyses of BAs in food samples can be performed by several techniques such as capillary electrophoresis (CE), gas chromatography (GC)[5-6] and high performance liquid chromatography (HPLC). However the conventional methods are HPLC coupled with ultraviolet (UV) or fluorescence (FL) detectors. Mass spectrometry (MS) and tandem mass spectrometry (MS/MS)[7-8] have been used instead of the traditional detection methods to improve characteristics such as sensitivity, selectivity and linearity as well as identification of the analytes. Among the most powerful techniques is ultra-high performance liquid chromatography (UHPLC)[9] coupled with mass spectrometry due to its high sensitivity, selectivity and applicability. [7-8]

Nevertheless even the most powerful techniques can have problems like insufficient retention times and separation. These can be overcome by using derivatization - reaction of analyte molecules with the derivatization agent. The intention of the reaction is to introduce specific functional groups that the detectors are more sensitive to. Derivatization can be done before or after chromatographic separation in the column. Among the most commonly used derivatization agents are dansyl chloride, 9-fluorenylmethyl chloroformate (Fmoc-Cl), o - phthaldialdehyde (OPA), benzoyl chloride and diethyl ethoxymethylenemalonate (DEEMM).[7]

In the literature part of this thesis the field of chromatographic determination of amines in food samples will be reviewed. This field is rather well established and conventional methods have been developed several decades ago. To narrow down the field of research only chromatographic determination of biogenic amines was assessed in more detail as well as research that has emerged in the last decade (2007 – 2017). The general trend is to move away from the conventional analysis and sample preparation methods and develop and use new methods that would be more focused on green practices, miniaturization, cost efficiency, and improvement of analytical characteristics.

2 Amines in foodstuffs

‘’Amines’’ is a class of chemicals that contains the amino group. Amines are basic compounds with low molecular weight. The most abundant amines in foodstuffs are biogenic amines (BA). They are highly important in food quality assessment. Biogenic amines are defined as basic organic low molecular weight compounds containing at least one amino group with biological activity. BAs originate in foods mainly from microbial decarboxylation of amino acids[10]. Biogenic amines and their properties, origins in foods and toxicological effects will be discussed in more detail in the following chapters.

There is also a group of amines that occurs in protein and other nitrogenous compound rich foods that have been exposed to high temperatures – heterocyclic aromatic amines (HAAs). HAAs are potentially carcinogenic and mutagenic compounds consisting of two to five condensed aromatic rings with nitrogen atoms in them. Occurrence of HAAs in heated foods is in the level of ng/g[11]. For the most part HAAs are forming in meat and fish in temperatures above 150°C. While the level of formation of HAAs is low in boiled foods (100°C), it becomes significant during baking, grilling and frying, where temperatures easily exceed 150°C[12]. However, since HAAs are not forming in foods under normal conditions, they will not be discussed further.

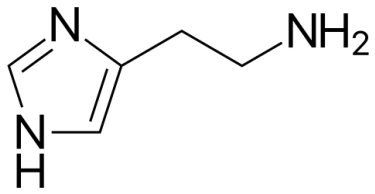
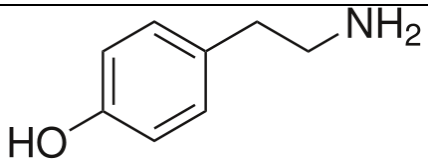
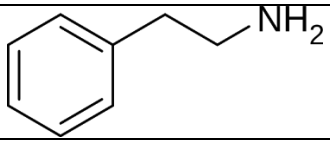
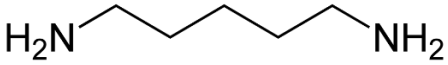
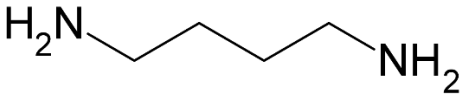
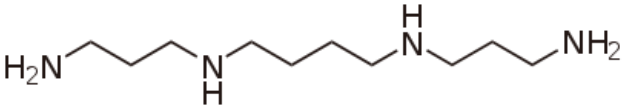
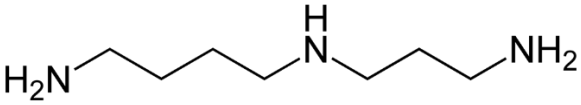
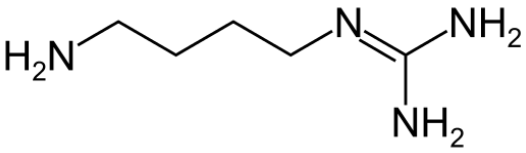
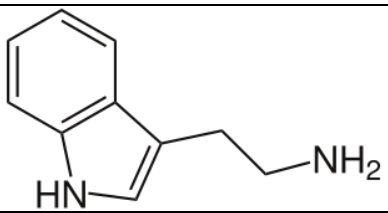
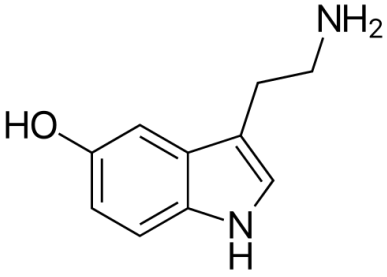
Other amines that are chromatographically analyzed in food samples include volatile amines, short-chain aliphatic amines and primary aromatic amines (PAA). Volatile amines such as methylamine, diethylamine and trimethylamine originate in foods, especially fish, during the spoilage process and can also indicate microbial growth[13]. Short-chain aliphatic amines such as diethanolamine, morpholine, 2-dimethylaminoetanol and 3-metoxypropylamine do not occur in foods by themselves but are added artificially. These are used, for example, as additives in glazing agents for fruit coating. Since short-chain aliphatic amines are a potential health hazard, their use is forbidden in many countries thus it must be monitored[14]. PAAs are highly toxic and potentially carcinogenic compounds that are used in producing food packaging materials. Therefore it is of high importance that the levels of PAAs in foods are being monitored[15]. Nevertheless assessment of analyses of these amines is beyond the scope of this thesis.

2.1 Biogenic amines (BA)

The research on biogenic amines begun in the 1950s as histamine was discovered. Shortly after putrescine, cadaverine and tyramine were discovered and up to date there are more than 20 known BAs that have been found in foods. As the name indicates biogenic amines are amines that possess biological activity. Among the most commonly analyzed BAs are cadaverine (CAD), putrescine (PUT), spermine (SPM), phenylethylamine (PEA), spermidine (SPD), agmatine (AGM), tryptamine (TRP), histamine (HIS), tyramine (TYR), serotonin (SER), dopamine (DA), octopamine (OCT), norepinephrine (NE), noradrenaline (NOR) and normetanephrine (NME). They affect such physiological processes as growth regulation (cadaverine, spermine, spermidine), neural transmission (catecholamines, serotonin), as mediators of inflammation (histamine, tyramine), cell membrane stabilization and cell proliferation (spermine and spermidine).[1]

Biogenic amines can be classified in several ways. Based on the number of amine groups in the molecule BAs are monoamines, for example, tyramine and phenylethylamine, diamines - cadaverine and putrescine, or polyamines – spermine and spermidine. When classifying based on the chemical structure of the molecules BAs are aliphatic – agmatine, putrescine, spermine and cadaverine, aromatic – tyramine and phenylethylamine, or heterocyclic – tryptamine and histamine. BAs can also be classified based on their biological activity – vasoactive BAs are for example, tryptamine, tyramine and phenylalanine, and psychoactive BAs are histamine, cadaverine and putrescine.[16] Names, abbreviations and structures of the most commonly analyzed BA molecules and food matrices in which they are found can be seen in **Table 1**.

Table 1. Structures of the most commonly analyzed biogenic amines in food samples.

No	Analyte	Structure	Matrix	Ref
1	Histamine (HIS)		crab fish cheese	17 8-9, 13 3, 5
2	Tyramine (TYR)		crab fish cheese chocolate	17 8-9 3, 5 18
3	Phenylethyl amine (PEA)		cheese fish	3 9, 19
4	Cadaverine (CAD)		crab fish cheese	17 8, 13 5
5	Putrescine (PUT)		crab fish cheese	17 8, 13 3, 5
6	Spermine (SPM)		cheese wine	3 20
7	Spermidine (SPD)		cheese wine	3 20
8	Agmatine (AGM)		fish wine	9, 19 20
9	Tryptamine (TRP)		crab fish	17 9, 19
10	Serotonin (SER)		chocolate cocoa, broad bean wine	18 18 20

2.2 Origin of biogenic amines in foods

Biogenic amines are widely met in many different food samples. Among these are foods such as meat, fish, vegetables, fruit and milk, where BAs have originated endogenously in low concentrations. Foods with higher concentrations of BAs have generally been suppressed to microbial activity and mostly involve fermented products such as cheese, beer, wine and sauerkraut. Apart from naturally occurring BAs in food, higher concentrations of BAs can indicate microbiological contamination. The mechanism of creation of BAs by microbial activity is through thermal or enzymatic decarboxylation of amino acids. Bacteria involved in these processes are among others Bacillus, Hafnia, Morganella morganii, Proteus, Lactobacillus, Enterobacteriaceae and Enterococcus.[1]

Metabolism of biogenic amines is a natural process in the cells of living organisms. BAs are created from respective amino acids. Therefore tyramine is produced from tyrosine, tryptamine and serotonin from tryptophane, phenylethylamine from phenylalanine, histamine from histidine, cadaverine from lysine and agmatine from arginine. Exception involves physiological polyamines spermine and spermidine that are created by de novo synthesis from putrescine which in turn is created form agmatine[1]. When amounts of BAs in living organisms become too high, catabolism takes turn. Biogenic amine molecules are broken down by enzymes respectively monoamines by monoaminooxidase (MAO), diamines by diaminooxidase (DAO) and polyamines by polyaminooxidase (PAO)[2].

2.3 Biogenic amines as food quality indicators

Amines can indicate the level of quality of food in several ways. First and foremost it is the freshness of food. As the demand for high quality food rises so does the necessity to be able to estimate the freshness of foods. Elevated amounts of certain amines can indicate spoilage of the food product. Due to the generally unpleasant odor of amines they can easily be detected even organoleptically. However, before amine concentrations have reached organoleptically noticeable levels they can be detected by analytical methods. Due to the enzymatic and microbiological activity amounts of amines increase with the time of storage and temperature of the storage space. This way it can be estimated whether the conditions of storage and processing of the foods have been appropriate.[17, 21]

There are several different amines that can indicate the freshness of food. Most often assessed are contents of biogenic amines and volatile amines expressed as total volatile base nitrogen (TVB-N) and trimethylamine nitrogen (TMA-N). TVB-N and TMA-N are spoilage indices that are used in quality determination of meat[22], fish[13, 21] and crab[17]. Allowable initial levels of TVB- N and TMA-N before starting freezing have been set. For example, for most frozen-stored fish species allowed amounts of TVB-N is 10-15 mg/100g[21]. However some argue that TVB-N is not an adequate index to evaluate freshness of fish stored in ice, namely European sea bass, since the levels of TVB-N noticeably start rising only after its shelf time[23].

Determination of concentrations of biogenic amines is considered a more reliable way of indicating food freshness than just volatile amines. Firstly, the technique used for analysis of biogenic amines is generally chromatography while for TVB-N and TMA-N titrations and spectroscopy. Most often analyzed BAs in regard to freshness are tyramine, putrescine, cadaverine, tryptamine and histamine. Depending on the type of the food levels of some BAs will be rising more prominently than others as spoilage occurs. For example, concentrations of histamine rise fastest in Chinese mitten crab and some fish species. Putrescine and cadaverine are also good indicators of spoilage.[13, 17]

A new approach in assessing food quality in terms of authenticity also involves determination of biogenic amines. Producers of some foods (for example, wines) might add lesser quality wines or sugar to the product in order to lower the manufacturing costs. If one wants to detect this kind of fraudulent behavior, typically a group of experts would be doing sensory tests on the product. While this approach works for limited number of samples, there still is need for a cost efficient method applicable to routine analyses. Chromatographic analyses of biogenic amines could be developed as part of the authentication measures.[24]

2.4 Biogenic amine toxicity

Once the levels of biogenic amines in foods increase enough, they become toxic. The effects on humans after consuming foods with elevated amounts of BAs include one or more of the following: hypotension or hypertension, nausea, rash, dizziness, cardiac palpitations and emesis or even death[13]. However serious the consequences of BA poisoning are there are only few clearly defined regulations concerning allowable intake and toxicity levels of BAs. The reason of this is particularly difficult estimation of toxic levels of BAs because they differ from person to person. Also synergetic effects of BAs are noticeable. BAs like cadaverine may enhance histamine poisoning effects because they work as diaminooxidase inhibitors. In general it is presumed that accumulation of BAs in food products is undesirable. Since histamine poisoning has been studied the most, there is a clear limit of allowable histamine in fish products. In other foods however only recommendations are available, for example, 100 mg of histamine per kg of food and 2 mg per liter of alcoholic beverage[16].

Toxicity of biogenic amines can stem from several processes. For one, BAs can be precursors of carcinogenic N-nitrosamines[13]. Spermine and spermidine might take part in cancer growth because of their function in cell proliferation and membrane stabilization[2]. Usage of monoaminooxidase inhibitor (MAOI) drugs such as some antidepressants can also increase the concentrations of BAs in human body causing poisoning effects as BAs are not being decomposed by normal metabolic pathways[4]. Another problem associated with toxicity of biogenic amines is that it does not decrease when food is treated by freezing, cooking, smoking or canning[17].

3 Food sample preparation

Food samples generally are problematic to analyze and have complex matrices. In order to obtain a sample of analytes suitable for chromatographic analysis the initial food sample must be pre-treated by several methods. Different chromatographic methods will require differently prepared samples, but generally all sample pre-treatment is done to improve analyte separation and detection, decrease the limits of detection (LOD), and remove interferences. Amines are analytes that are present in food samples in very low concentrations so sample preconcentration step should be included. Food sample preparation consists of sample clean-up, enrichment and derivatization.

Analytes should be removed from the matrix since it consists of compounds that will greatly hinder the analysis process. The importance of this is based on the food matrix. For liquid samples like beverages simple filtration might be enough to prepare sample for HPLC analysis. If suspended particles are present, centrifugation can be employed. Solid food samples will require procedures like cutting, grinding, degreasing, homogenization, degassing, centrifugation, filtration, and dilution. Afterwards extractions are used to eliminate unwanted matrix compounds, and to collect analytes from the sample.[2, 4]

Analyte sample should also be purified of interfering compounds. In case of amines major interfering compounds are amino acids. In derivatization process amines as well as amino acids react with the derivatizing reagent by the amino group producing derivatives whose peaks might be overlapping in chromatograms. Polyphenols are also affiliated with amines in their occurrence in foods. Polyphenols can compromise detection of amines as they are spectroscopically and fluorometrically active. Impact of the interfering compounds can be eliminated either by removing them from the sample or specifically extracting analytes. However, separation of amines from amino acids is highly challenging.[2]

Food sample preparation methods used in the past decade are reviewed in the following chapters. Derivatization of amines is also an essential part of amine analysis in food samples and is therefore discussed separately. An overview of the sample preparation and extraction methods can be seen in **Table 2** where their uses and characteristics are noted for specific analytes and matrices.

Table 2. Food sample preparation and biogenic amine extraction methods.

No	Analyte	Matrix	Sample preparation	Extraction method	Extraction procedure	Recovery, ± RSD %	Ref
1	HIS, PUT, CAD	fish (Hammour, Negrule, Zubaidi)	Grinding	SLE	Sample homogenised with 6% (w/v) trichloroacetic acid (TCA). Homogenate centrifuged, filtered and dissolved in TCA	-	[13]
2	AGM, CAD, HIS, PEA, PUT, TRP, TYR	fish (mackerel, mahi-mahi, salmon) tuna	Thawed, homogenised, spiked with internal standard	MSPD	Sample ground in a mortar with washed and dried MSPD sorbent (BulkBondesil 40 µm CN-U). Eluted with 20:80 (v/v) 50mM ammonium formate buffer (pH 3,0) in ACN.	80 - 109 ± 0.2 - 9.5 83.4 –112.5 ± 2.5 – 9.1	[9] [19]
3	HIM, TYR, PUT, CAD	wine	Centrifuged, and ultra-filtered (membrane pore size 10.000Da)	SPE	Oasis MCX resin conditioned by methanol and Milli-Q water. 0.6 mL of sample were percolated, and washed with 2mL 10mM H_3PO_4:MeOH (90:10); then 2mL 10mM NaOH:MeOH (70:30); then 2mL of 10mM $CaCl_2$:MeOH (70:30). Analytes eluted with 1.2mL 100mM NaOH:MeOH (65:35). Eluate collected in a vial with 100µL 1.2 M HCl.	64.8 – 103± 3.54 – 12.5	[25]
4	HIS, TYR, SPM, SPD, AGM, PUT, CAD, PEA, TRP	cheese, sausage, fish wine, red wine, beer	Wine and beer homogenized in ultrasound bath and filtrated. Others homogenized in ultrasound bath and centrifuged with 0.1 M HCl	In situ derivat. ultrasound assisted DLLME	Sample solution, internal standard and $NaHCO_3$–Na_2CO_3 buffer (pH 9.2) placed in a tube. 50 mM 4′-carbonyl chloride rosamine (CCR)(der. reag.) in ACN and bromobenzene (extractant) were injected. Tube was capped and shaken vigorously. Then solution placed in ultrasound bath (1 min, 40 kHz). Afterwards centrifuged. Sedimented extractant droplets withdrawn and diluted with ACN.	83 - 111	[7]
5	PUT, CAD, HIS, TYR	fish (anchovy)	Washed with tap water, then Milli-Q water, triturated and homogenized	SLE	Sample centrifuged with 0.6 M perchloric acid. Suspension was vacuum filtrated. Residue was extracted again, filtrates combined and diluted with 0.6 M perchloric acid.	71 - 108	[8]

Table 2 continues. Food sample preparation and biogenic amine extraction methods.

No	Analyte	Matrix	Sample preparation	Extraction method	Extraction procedure	Recovery, ± RSD %	Ref
6	TRP, PUT, CAD, HIS, TYR, SPM	shrimp sauce, tomato ketchup	Vortexed with water until homogenous. Centrifuged, supernatant collected, spiked with BAs and diluted with water.	Hollow fibre LPME with in situ derivatizat.	Sample, sat. NaOH carbonate (pH 9.5) and dansyl chloride (der. reag) stirred. 25µL of HCl (0.1M) withdrawn by a syringe. Syringe needle inserted in hollow fibre segment and assembly immersed in organic solvent to impregnate pores of hollow fibre. Then acceptor phase (HCl) injected into the lumen of hollow fibre. Hollow fibre placed immediately in sample solution and sample vial agitated. Acceptor phase withdrawn into syringe and hollow fibre discarded.	86.7 – 104 ± 3.1 – 6.9	[26]
7	PUT, HIS, TYR, PEA, SPM, SPD, TRP	sufu (fermented food)	Ground and homogenized	SLE and SPE	SLE: homogenate extracted twice with 6 % TCA, centrifuged, filtrated and diluted with TCA. Derivatized with CNBF and aqueous phase collected. SPE: Waters Oasis MCX cartridge, activated with methanol and water. Sample passed through and washed with water/acetone (80/20, v/v). Eluted with ACN, eluate evaporated to dryness and reconstituted in ACN.	83.3 –103.5 ± 1.26–5.24	[27]
8	HIS, TYR, PUT, CAD, TRP, PEA, SPM, SPD	cheese	Grated with a cheese grater	SLE	5g sample was suspended in 12mL 0.6M perchloric acid, shaken, and centrifuged. Repeated 3 times with fresh 0.6M perchloric acid each. Extracts combined and filled up to 50mLwith 0.6M perchloric acid. 1.5 mL of extract was centrifuged, and 0.5mL of supernatant neutralized with NaOH.	-	[28]
9	HIS, CAD, PUT, TYR, SPM, SPD, PEA, TRP, AGM	fish (mackerel, beltfish, pomfret, croaker)	Fish tissue free from bones, skins and organs homogenized in blender	bead-beating disruption extraction	20 mg fish tissue + 300 µL 5 mg/mL 5-sulfosalicylic acid solution + 100 µL 4.5 µg/mL IS + 100 µL 20% ACN + Zirconia beads into 2mL centrifuge tube. Simultaneous homogenization and extraction in a mixed grinding apparatus at 20 Hz for 1 min twice. Then centrifuged, 100 µL supernatant diluted with 900 µL 50 mM sodium tetraborate buffer and vortexed.	74.9 –119.3 ± 1.2 – 8.6	[29]

3.1 Amine extraction

Extraction is an especially important step in the sample preparation process. Several different extraction techniques have been developed. Among the most commonly used are solid-liquid extraction (SLE), liquid-liquid extraction (LLE), and solid phase extraction (SPE). These techniques have been known for decades and have been researched extensively. However well-known and conventional extraction techniques they might be, there is still space for improvement. More recent research has focused on making these techniques quicker, less laborious, more environmentally friendly and cost efficient. Therefore new, more efficient methods have been developed, such as dispersive liquid-liquid micro extraction (DLLME), solid phase micro extraction (SPME), matrix solid phase dispersion (MSPD), salting-out assisted liquid-liquid extraction (SALLE), and liquid phase micro extraction (LPME).[2]

3.1.1 Liquid-liquid extraction (LLE)

LLE is based on an extraction of analytes from a liquid matrix by a liquid extractant. For LLE to be successful, analyte affection for the extractant has to be greater than that for the matrix solution. Liquid-liquid extraction is a common extraction method, however it hardly meets the modern day requirements. Traditional LLE produces large amounts (hundreds of milliliters) of toxic and expensive solvent waste. Low recoveries of LLE and emulsion appearance resulting in long procedure times are also not unusual.

Despite the long extraction times, LLE was successfully used by La Torre et al.[30] in sample treatment for determination of biogenic amines in donkey milk by HPLC with mass spectrometric detection. At first protein were precipitated from the donkey milk samples, the samples were centrifuged and filtrated. Then derivatization of amines (TRY, PEA, PUT, CAD, HIS, TYR, SPM and SPD) was done by dansyl chloride. The dansyl derivatives were extracted three times with ethyl acetate. The organic phases were pooled together, washed with water, evaporated to dryness, reconstituted in ACN and filtered. Finally aliquots of filtrates were analyzed by HPLC-APCI-MS. The recoveries of the method were also satisfactory (from 77.7 to 99.4%).

3.1.2 Solid-liquid extraction (SLE)

Solid-liquid extraction is the most well-known and straight forward extraction method from solid samples. In SLE solvent is applied directly to the solid sample and analytes are extracted. To improve the extraction efficiency, surface area of the sample should be as large as possible. This can be achieved by grinding, pulverizing or homogenizing the sample. The choice of solvent used should be based on the analyte solubility in it. However, in case of amines extractant can be an acid such as trichloroacetic acid (TCA), hydrochloric acid or perchloric acid thus employing the basic characteristics of amines.

The general procedure of SLE starts with homogenizing the solid sample and mixing the homogenate with extraction solvent. After that centrifugation or filtration takes place and supernatant or filtrate is collected. Extraction is then repeated several times with fresh portions of solvent, and collected supernatants are pooled together. Finally the extraction solvent is evaporated, and residue is reconstituted in a solvent appropriate for further analysis. This is a time consuming and laborious procedure requiring large amounts of solvents that are wasted.[31]

The extraction efficiency of biogenic amines by different acidic extractants was compared by Spizzirri et al.[32] They used 0.1 M HCl, 0.1 M $HClO_4$ and 5% (w/w) TCA to extract eight biogenic amines, PEA, PUT, CAD, HIS, TYR, SPD, SER and SPM, from tea and infusion samples. They applied extractants over tea leaves, then homogenized, centrifuged, filtered, purified by SPE (C18), dried the eluate with nitrogen and reconstituted in extraction solvent. Then the extracted analytes were derivatized by dansyl chloride and analyzed by HPLC-UV. The results showed that all extractants are suitable for biogenic amine extraction from tea samples as the recoveries were above 88%. TCA 5% (w/w) however showed excellent recovery of 95% and was concluded as the most efficient extractant.

3.1.3 Solid phase extraction (SPE)

Solid phase extraction in regards to amine analysis in food samples is used both as a clean-up technique and analyte extraction technique. SPE is a method where compounds of interest get adsorbed from a solution on a solid adsorbent and then eluted. As a method SPE has multiple benefits over LLE, the largest of which is the significantly reduced consumption of toxic and costly solvents. SPE procedure is also quicker to execute and has higher recoveries. The shortcomings of SPE include time consumption and laboriousness, while automated SPE techniques are costly[26].

As a clean-up technique for amine containing food samples most often used SPE adsorbents are based on non-polar silica C18. This allows the removal of polyphenols and other interfering organic compounds often present in food samples. However, when SPE is used for extraction of amines themselves, adsorbents of choice are acidic to best utilize the basic nature of amines.

An impact of SPE usage in determination of amines in foods was demonstrated by Oguri et al.[33] They employed SPE as a clean-up technique for selective determination of histamine in fermented Japanese foods. Normally in fermented foods a wide variety of biogenic amines such as cadaverine, putrescine, serotonin and tyramine are present. When analyzing such sample by HPLC with UV detection the peaks of amines might overlap. Therefore, if only the content of histamine is of interest, it is required to remove the interfering compounds. When this is done by ion exchange column chromatography it may take two to three hours which is excessive for routine analyses. Usage of SPE Sep-Pack Plus C-18 cartridge allowed the removal of unwanted compounds in a simple manner. Chromatogram of a solution that contained five biogenic amines (HIS, CAD, PUT, SER and TYR) was compared to a chromatogram of the same solution with the SPE clean-up. In the cleaned-up solution chromatogram only the histamine peak was remaining while in the untreated solution chromatogram large peaks of other BAs are present. This indicates that SPE clean-up is an effective way to free sample from interfering compounds.

3.1.4 Novel and improved extraction techniques

Recent trends in sample preparation methods are focused towards miniaturization, simplification, automation and overall improvement of analytical outcomes. Methods are developed to be more environmentally friendly, cost efficient, and rapid while not compromising the high selectivity and recoveries. Novel extraction techniques include single drop micro extraction (SDME), stir bar sorptive extraction (SBSE) microwave assisted extraction (MAE), cloud point extraction (CPE), dispersive liquid-liquid micro extraction (DLLME), solid phase micro extraction (SPME), matrix solid phase dispersion (MSPD), hollow fiber liquid phase microextraction (HF-LPME), salting-out assisted liquid-liquid extraction (SALLE) and liquid phase micro extraction (LPME). SPME, MSPD, DLLME, LPME and SALLE will be discussed in more detail in the following chapters because these have been used more frequently in biogenic amine extraction from food samples.[34]

Sample preparation methods developed in the last decade tend to increasingly incorporate the use of ultrasonication. Ultrasonic radiation is a sound with a frequency above 16 kHz. When used in assistance of extraction, ultrasonication decreases the time required for establishment of extraction equilibrium. Therefore ultrasound assisted (UA) extraction methods are proven to be fast and efficient. Ultrasound assistance has been used with extraction methods like DLLME[7, 35-36] and liquid-liquid microextraction[37]. Ultrasound has also been used in homogenization of food samples[7].

3.1.4.1 Liquid phase micro extraction (LPME)

Liquid phase microextraction is a miniaturized method of LLE. LPME requires solvents only at the microliter range unlike LLE where hundreds of milliliters are needed. Most prominent divisions of LPME include single-drop microextraction (SDME), hollow fiber microextraction (HF-LPME) and dispersive liquid-liquid extraction (DLLME). In amine extraction from food samples DLLME is utilized the most, while HF-LPME has also found its applications.[38]

HF-LPME is a method where extraction is performed with assistance of a porous hollow fiber. The fiber is immersed in a water immiscible organic solvent to create a supported liquid membrane (SLM) on the walls of the pores. Then an acceptor solution is filled in the lumen of the fiber. Finally the whole assembly is immersed in the aqueous sample solution. Analytes are then extracted though the SLM and collected in the acceptor solution in the lumen of the fiber. The collected solution can then be directly analyzed by HPLC or GC.[39]

HF-LPME was used in determination of BAs (TRP, PUT, CAD, HIS, TYR and SPM) in shrimp sauce and tomato ketchup by HPLC- UV[26]. In situ derivatization by dansyl chloride was also performed. Acceptor phase was 0.1 M HCl. Method optimization showed the highest extraction efficiencies at room temperature with 30 min extraction time. Obtained recoveries were in the range of 86.7% – 104% for all amines.

3.1.4.2 Salting-out assisted liquid-liquid extraction (SALLE)

Salting-out assisted liquid-liquid extraction (SALLE) is an improved LLE technique. The major drawbacks of conventional LLE include excessive use of hazardous solvents and poor extraction efficiency of polar compounds as only water-immiscible solvents can be used. In amine extraction higher extraction efficiency is achieved by using polar solvents because amines are polar compounds. However, polar organic solvents are miscible with water making it difficult to separate the phases as needed in LLE. Addition of inorganic salts to the extraction medium improves separation of two phases enabling the use of water-miscible solvents in LLE. Often used water-miscible organic solvents are acetonitrile (ACN), ethyl acetate, acetone and ethanol.[40-41]

Ramos et al.[42] used SALLE in single-stage derivatization with dansyl chloride and extraction of BAs from wine samples. The organic phase in extraction was acetonitrile and salting-out was done by NaCl. The combined sample preparation step was reported as fast – doable in 25 min. Jain et al.[41] developed a method of biogenic amine determination in fruit juices and alcoholic beverages involving SALLE. They also used single stage derivatization and extraction. Derivatization was done with 1-naphthylisothiocyanate, extraction of derivatives with water-miscible ACN, and phase separation with assistance of ammonium sulphate. The obtained method was simple and rapid.

3.1.4.3 Dispersive liquid-liquid extraction (DLLME)

Dispersive liquid-liquid extraction is a novel extraction technique first introduced by Rezaee in 2006[43]. Since then it has been gaining popularity within a wide spectrum of applications, including use in biogenic amine containing food sample preparation. DLLME is based on LLE but as a miniaturized technique uses significantly less solvent as a receptor phase (in microliter range). Organic extractant and dispersant solvents are injected in an aqueous sample solution and cloudy solution appears as extractant droplets are dispersed. Then cloudy solution is centrifuged and extractant with analytes sediments at the bottom. Since the surface area of the extractant is so large extraction occurs very rapidly becoming the most prominent improvement of DLLME.

DLLME can be used in sample preparation for high performance liquid chromatography (HPLC). Single-step derivatization and extraction is a regular praxis when employing DLLME. Wu et al.[35] employed DLLME in their development of a one-step fluorescence labeling and ultrasound assisted (UA) DLLME for determination of seven biogenic amines in food samples by HPLC-FL. It was successfully applied to different food samples: cheese, beer, rice wine yoghurt, and ham sausage. Huang et al.[36] also used UA-DLLME together with HPLC-FL to determine biogenic amines (tyramine, octopamine and phenylethylamine) in rice wine. Both methods showed good results in terms of linearity, sensitivity and recovery. UA-DLLME was used also by He et al.[7] in determination of BAs in food samples by UHPLC tandem mass spectrometry (MS/MS).

DLLME is often the most appropriate method for gas chromatography (GC) analysis. Almeida et al.[44] developed a method for simultaneous determination of 18 biogenic amines in beer by DLLME GC-MS. Derivatization was performed in a single-step with extraction. Resulting method was fast, reliable and the obtained results were comparable with those from previously reported methods. Similar DLLME/GC-MS method with single-step derivatization was used by Plotka-Wasylka et al.[6] in determination of 13 BAs in home-made fermented alcoholic drinks.

Combination of DLLME with other extraction methods have been reported. Jia et al.[45] developed a DLLME method based on solidification of floating organic droplets (SFO). Nine BAs were determined in alcoholic beverages (white wine, red wine, rice wine and beer) by LC-UV and in situ benzoylation. In DLLME-SFO organic droplets containing analytes are

solidified by freezing, therefore it is easier to remove them completely. DLLME-SFO has advantages over just DLLME as the extraction is performed more precisely and accurately, and organic solvents used are less toxic.

3.1.4.4 Solid phase micro extraction (SPME)

Solid phase micro extraction was first introduced in the 1990. SPME is based on placing an adsorbent, usually polymer coated fiber, in the sample or its headspace. Analytes are then adsorbed on it. SPME has many advantages over other extraction techniques. For starters, extraction does not require solvents, and desorption is simple and effective. SPME is a miniature method and can be made portable and applicable to field sampling. However, the shortcomings of the method include limited range of operating temperatures (240° – 280°C) as well as fiber breakage, cost, stripping of coatings, and swelling in organic solvents.[38]

On-fiber derivatization SPME is a modification of SPME. This method is very fast, simple and environmentally friendly as organic solvents and reagents are used only in microliter amounts. Huang et al.[46] used on-fiber derivatization SPME with direct insertion in GC-MS in determination of four nonvolatile BAs in fish samples. A schematic representation of the method can be seen in **Figure 1**. The polydimethylsiloxane divinylbenzene (PDMS/DVB) fiber was dipped in a derivatization reagent and extractant solution, forming a thin membrane coating. Afterwards the fiber was immersed in the analyte containing solution for extraction. Finally the fiber was directly inserted in a GC injector for thermal desorption.

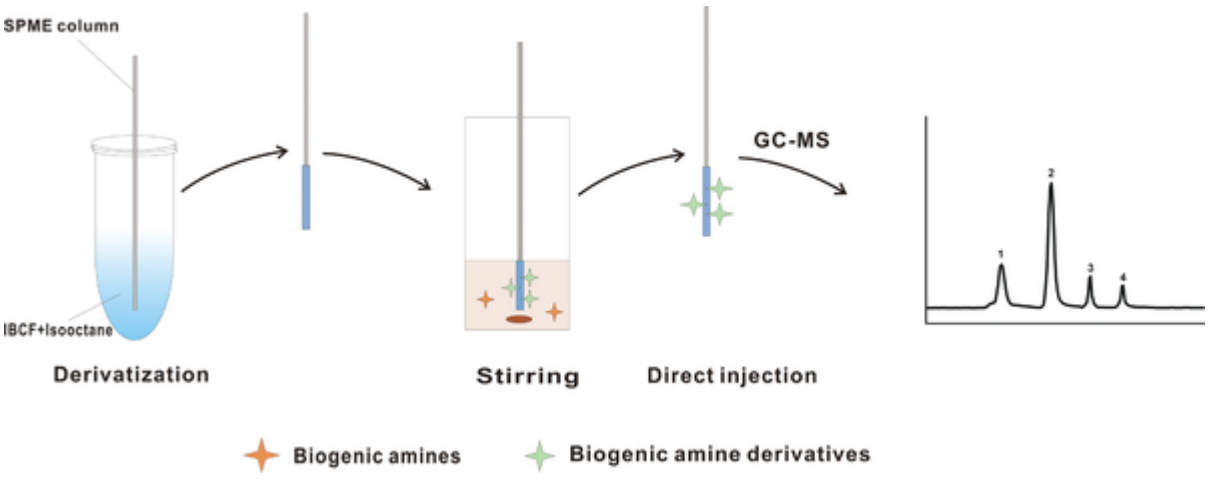


Figure 1. Schematic representation of on-fiber derivatization SPME method. Reprinted with permission from [46].

On-fiber derivatization SPME was also used by Awan et al. [47] in determination of BAs in cheese, meat, and vegetables. However, there derivatization of analytes and extraction on fiber occurred in gas phase. A vial containing analyte solution in ethanol and trifluoroacetylacetone (TFAA) was placed in the agitator of the autosampler of GC-MS. Then (PDMS/DVB) SPME fiber was inserted in the headspace of the vial. Contents of the vial were evaporated by heating from 90°C to 120°C, and derivatization and extraction took place in the gas phase. Thermal desorption was done in injector of GC-MS system.

3.1.4.5 Matrix solid phase dispersion (MSPD)

Matrix solid phase dispersion (MSPD) is essentially the homogenization step of SLE combined with extraction. In MSPD solid sample is ground together with an abrasive substance such as sand in a mortar. This facilitates more thorough breakdown of the matrix, and allows the analytes to be reached by the solvent easier. MSPD is also known to be simple, robust and rapid sample preparation technique.

MSPD was compared with conventional homogenization in tomato product sample preparation[31]. It was found that both methods have good recoveries that are rather dependent on individual characteristics of the method, such as concentration or volume of the solvent used for extraction. Therefore optimization of the method has important role. MSPD has also been used in tuna[19] and sea food product[9] sample preparation for biogenic amine determination by UHPLC. There a 40 µm CN-U sorbent was used as the abrasive substance and the medium for solid phase extraction. Food sample was ground together with the sorbent in a glass mortar and then eluted with ammonium formate buffer. This method also showed good recoveries.

3.2 Amine adsorbents

There is a variety of adsorbents suitable for amine adsorption. These adsorbents are used in SPE or SPME. The properties of adsorbents used are based on the properties of compounds to be extracted. SPE adsorbents can be cationic, anionic or non-polar. They can employ either the basic nature of amines resulting in acidic adsorbent, or the organic backbone of the biogenic amine molecules. Among the most commonly observed are silica based and polymeric adsorbents based on divinylbenzene such as polydimethylsiloxane divinylbenzene (PDMS/DVB).

There are commercially available adsorbents that can be used for amine adsorption from food samples. For example, Pena-Gallego et al.[25] used Oasis MCX resins to extract histamine, putrescine, cadaverine and tyramine from wine samples. Oasis MCX resins are made in combination of sulphonic groups and reverse-phase (C18) ion exchange adsorbents. They concluded that mixed mode sorbents are efficient in biogenic amine extraction from complex matrices.

If the commercially available adsorbents are lacking selectivity their properties may be chosen by synthesizing adsorbents specifically tailored for the amine adsorption. One approach is to synthesize ligands on to the silica backbone to obtain specific amine adsorbents. Saaid et al.[48] used it to create three different mesoporous silica based sorbents with immobilized crown ether ligands. All three sorbents were found to be selective towards spermidine. They tested the performance of the sorbents also on real food samples such as cheese, tomato ketchup, fish sauce, canned tuna and soy sauce. The recoveries of spermidine were varying between 71.2% to 99.8%. Tameem et al.[49] employed a similar approach by immobilizing a hydrazone ligand (4-hydroxy-N´-[(E)-(2hydroxyphenyl)methylidene benzohydrazide) in to a silica sol-gel matrix. This sorbent proved to be highly selective of aliphatic and heterocyclic biogenic amines: putrescine, cadaverine, histamine and spermidine. Sorbent viability was tested in food samples (ketchup, orange juice and soy sauce) and the recoveries found were ranging from 69.1% to 99.7%.

Custom made sorbents can also be utilized in micro SPE, where a small amount of sorbent is placed inside a porous membrane envelope which is then placed inside a sample solution for analyte adsorption. Basheer et al.[50] synthesized sol-gel sorbents with eight immobilized hydrazone ligands with different functional groups for micro SPE. The most hydrophobic ligand (benzophenone 2.4-dinitrophenylhydrazone) showed the largest extraction capability. It was able to successfully extract both aliphatic and aromatic biogenic amines. Basheer et al also compared the performance of micro SPE technique against SPE with commercially available Oasis-HLB-SPE sorbents. While the recoveries were higher for SPE than micro SPE, they were comparable and all higher than 71%. Micro SPE bared also the advantages of having a high pre-concentration, reusability of the sorbent as well as reduced solvent consumption and time.

4 Amine derivatization

Derivatization is a reaction of analytes with derivatizing reagent in order to create derivatives with desired characteristics. Since amines do not have chromophores, derivatization can be used to introduce ligands that are visible by the UV or fluorescence detectors, thus increasing selectivity and sensitivity of detection. Derivatization can also enhance the separation of the analytes and help with the tailing problem of amines in separation on reversed phase HPLC columns. Amines are small, basic compounds, and therefore interactions with the free silanol groups of the silica based C18 adsorbent material will occur, resulting in peak tailing. This can be overcome by preliminary reaction with the amino group to decrease the basicity of the molecule. Making the derivative molecule more sterically hindered by reaction of amine with a large derivatization reagent will prevent it from reaching the free silanol groups as well. Derivatization will also help in circumstances where analytes are present in very low concentrations compared to the matrix and other interfering compounds.[51]

4.1 Derivatization of BAs from food samples

Prior to derivatization analytes should be made available for the reaction. For liquid matrices such as wine, beer and liquors sample pre-treatment can be rather simple. Often a filtration is enough. Polyvinylpyrrolidone (PVP) is used as an additive to eliminate the interfering polyphenols from beer and wine samples. For the solid samples a more elaborate sample pre-treatment procedure is necessary. It can involve homogenization and extraction of the analytes with mineral or organic acids or organic solvents.[52]

In food sample preparation before HPLC or GC analysis analytes need to be extracted from the matrix and derivatized. Derivatization of the biogenic amines can be beneficial for a wide range of food samples. An overview of the derivatization reagents and procedures used for derivatization of biogenic amines from food samples can be seen in **Table 3.**

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Table 3. Biogenic amine derivatization methods for chromatographic analysis.

No	Analyte	Matrix	Derivatization reagent	Derivatization process	Analysis method	Ref
1	HIS, PUT, CAD	fish	Benzoyl chloride	1 mL of 2 M NaOH to 50 µL of amine solution + 10 µL benzoyl chloride. Mixed, incubated for 20 min in RT, quenched with 2 mL saturated NaCl. Extraction of derivatives with 4 mL diethyl ether. Mixture centrifuged, organic layer evaporated to dryness, residue dissolved in 500 µL MeOH.	HPLC-PAD	[13]
2	HIS, TRP, PEA, TYR, PUT, SPD, SPM	beer	4-Chloro-3,5-dinitrobenzo trifluoride (CNBF)	200 µL 0.5 mM BA solution + 300 µL H_3BO_3–$Na2B_4O_7$ buffer (pH 9.5) + 100 µL 18.5 mM CNBF methanol solution. Then whole solution diluted to 1.0 mL with water, incubated at 60◦C for 30min, and quenched with 10 µL 2 M HCl.	RP-HPLC-UV	[53]
3	CAD, HIS, SPM, SPD, TRP, TYR	cheese	dansyl chloride	5.0 mL 0.25 M $NaHCO_3$ + 1,0 mL 0.02 M dansyl chloride + 0.5 mL BA solution + acetone up to 10.5 mL. Incubated at 60 °C for 20 min. Brought to 25 °C before analysis.	HPLC-MS/MS	[54]
4	HIS, PEA, TYR, TRP	wine	2-chloro-1,3-dinitro-5-(trifluoromethyl)-benzene (CNBF)	Extracted and dried analytes + CNBF + diisopropylethylamine + ethanol. Mixture refluxed for 2h. Extraction of derivatives with ethyl acetate. Solvent evaporated, residue dissolved in methanol.	HPLC-DAD	[55]
5	TRP, PEA, PUT, CAD, HIS, SER, TYR, SPD, SPM	dry fermented meat	4-dimethylamino azobenzene-40-sulfonyl chloride (dabsyl chloride)	2 mL amine extract + 4 mL dabsyl chloride in ACN (5 mg / mL) + 150 – 500 µL 2M NaOH (pH 7.4 – 10.6) + 600 µLof 0.95 M $NaHCO_3$. Incubated at 70° and 80°C for 20 min. Ice bath cooling to stop the reaction.	RP-HPLC-UV	[56]
6	PEA, PUT, CAD, HIS, TYR, SPM, SPD	wine	dansyl chloride	0.5 mL wine sample + 150 µL of sat. NaOH carbonate solution (pH 11.5) + 2 mL of Dns-Cl 0.5% w/v in acetone. Incubated for 45 min at 40°C with agitation. Residual Dns-Cl removed with ammonia solution. Mixture cooled to room temperature in dark for 30 min and filtered through nylon 0.45 µm filters.	HPLC-PAD	[57]
7	SPM, SPD, HIM, TYR, CAD, PUT, AGM, DA, TRP, PEA	wine	dansyl chloride	50 – 100 µL sample + 10 µL norvaline (IS) + 100 µL dansyl chloride + 0.2 M $Na_2B_4O_7$ x $10H_2O$ (pH 9.3) up to 1 mL. Incubated for 30 min at 40°C in ultrasonic bath. Centrifuged, supernatant diluted with methanol (1:1, v/v).	HPLC-FL	[58]

Table 3 continues. Biogenic amine derivatization methods for chromatographic analysis.

No	Analyte	Matrix	Derivatization reagent	Derivatization process	Analysis method	Ref
8	HIS, TYR, PEA, TRP	wine	1-fluoro-2-nitro-4(trifluoromethyl) benzene (FNBT)	Analyte extract in ethanol + 330 µL FNBT + 30 µL diisopropylethylamine stirred for 2 h. 10 mL of water added and derivatives extracted with ethyl acetate (3 x 15 mL). Solvent evaporated, residue dissolved in methanol.	HPLC-DAD	[59]
9	TRP, PEA, PUT, CAD, HIS, SPM, SPD, TYR, SER, OCT	pork, beef, carp	dansyl chloride	1 mL degreased BA extract in 5% TCA + 200 µL 2 M NaOH + 300 µL sat. Na_2CO_3 solution + 4 mL of 5 mg/L dansyl chloride. Incubated for 15 min at 60°C and shaken once every 5 min. Quenched with 200 µL ammonia and cooled to room temperature for 20 min. Mixture evaporated to 5 mL with nitrogen flow at 40°C.	HPLC-FL	[60]
10	PEA, CAD, PUT, TYR, HIS, iso amylamine	fruit (grapes, physalis), wine, honey	o-phthaldialdehyde (OPA)	Derivatization in automatic injector. 1µL borate 0.2 M, pH 9.5. + 2µL BA sample + 2µL OPA (5mg/mL in MeOH) / N-acetyl-l-cysteine (40mg/mL in borate buffer, pH 9.5) 1:5 with needle wash 5 times between solutions. Mixed 15 times, wait 3min. Added 3µL water, mixed 5 times, and injected.	HPLC-FL	[61]
11	SPM, SPD, PEA, CAD, PUT, TYR, SER; HIS, AGM	wine	diethyl ethoxymethylenemalonate (DEEMM)	430 µL 1 M borate buffer (pH 9.0) + 300 µL MeOH + 400 µL sample, 10 µL IS (2-aminoadipic acid, 1.00 g/L) + 12 µL DEEMM. Incubated for 30 min in ultrasound bath. Then heated for 2 h at 70–80 °C for degradation of excess DEEMM.	RP-HPLC-PAD	[62]
12	TYR, TRP, PUT, CAD, HIS, PEA, SPD	wine	dansyl chloride	1 mL wine sample + 100 µL IS (1.7-diaminoheptane) + 0.5 mL sat. Na_2CO_3 + 1 mL dansyl chloride (10 mg/mL in acetone). Incubated for 45 min at 40°C water bath in dark with an occasional shake. Then cooled to room temperature, and 70 µL ammonia added to precipitate extra reagent.	UPLC – Q-TOF-MS	[63]
13	PUT, CAD, TRP, TYR, HIS, SPM	salami	benzoyl chloride	1mL 2M NaOH + 20 µL benzoyl chloride + 2 mL BA extract. Mixed and incubated in a water bath at 30°C for 40 min. Next, 2 mL of sat. urea solution added, vortexed for 30 s, and placed in water bath at 30°C for 10 min. Derivatives extracted with 3 mL diethyl ether and vortexed for 1 min. Centrifuged for 5 min, and organic phase was dried with nitrogen flow. Dried extract solubilized in 2 mL n-hexane/isopropanol (4:1) solution, and filtered by 0.20 µm filter.	HPLC-dual absorbance detection	[64]

4.2 Derivatization medium

Derivatization of amines can be performed either in aqueous or non-aqueous phase. Therefore amines can be extracted from the food sample matrix into organic phase and then derivatized, or derivatized already in the aqueous matrix phase and extracted into organic phase as derivatives. Derivatization in aqueous phase is often employed, however it possesses such drawbacks as large time consumption for derivatization and extraction, and low reproducibility based on instability of derivatives and irreproducible extraction results.

Non-aqueous single step derivatization method for the determination of putrescine and cadaverine by GC-MS was developed by Awan[65]. Derivatization reagent used was trifluoroacetylacetone (TFAA) as it increases the volatility of derivatives needed for analysis by ion-trap GC-MS or GC-FID. The derivatization method in non-aqueous solvents (methanol and ethanol) was found to be fast (15 min), sensitive, accurate (recoveries 91.8 – 97.7 %) and cost efficient, as the time consuming and costly solvent extraction and pH control steps were removed. Awan et al.[47] also used simultaneous derivatization by TFAA and extraction by on-fiber SPME in the gas phase combined with GC-MS method for determination of PUT and CAD in food samples (cheese, meat, and vegetables).

4.3 Derivatization reagents

There is a plethora of reagents suitable for derivatization of amines. The choice of reagent depends on several factors, most important of which is the mode of derivatization. Automated and on-line derivatization methods require reagents that are capable of reacting quickly and under normal conditions. For derivatization reactions executed off´-line it is possible to use reagents that require longer reaction times and elevated temperatures. Molecular structures of often used derivatization reagents can be seen in **Figure 2**.

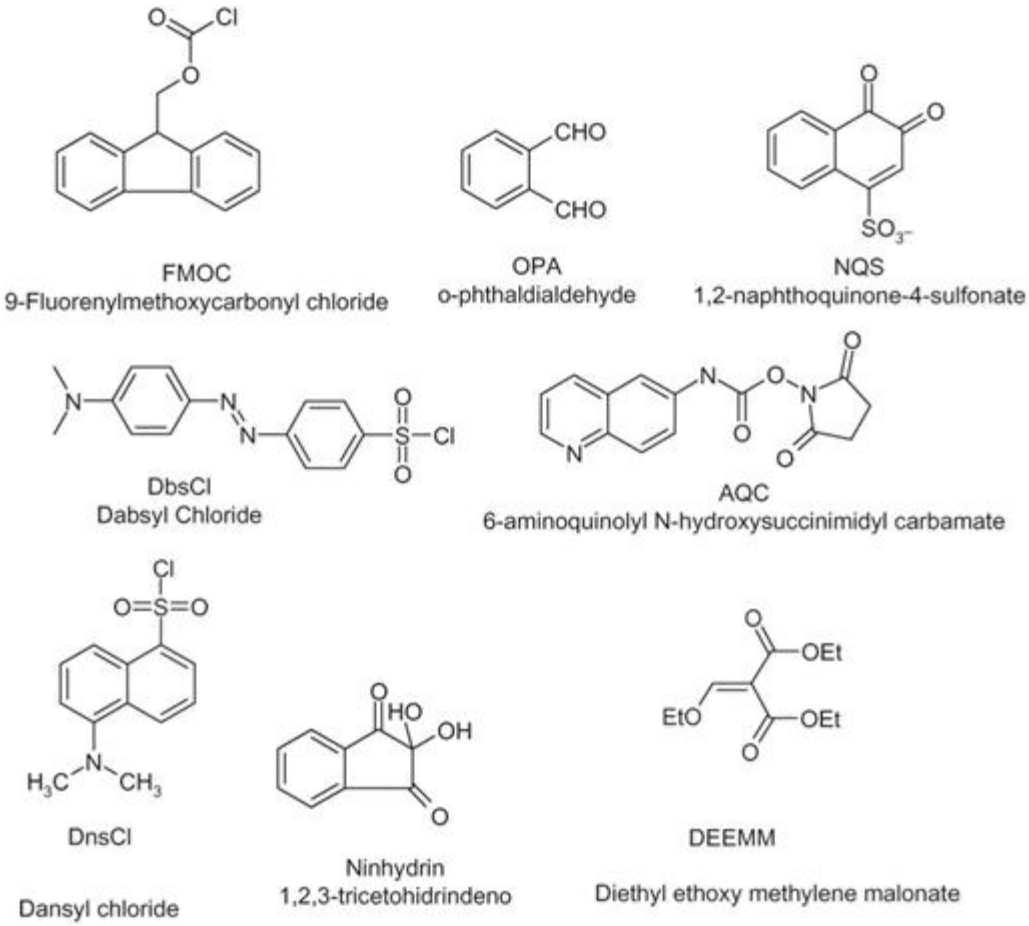

Figure 2. Molecular structures of the most often used derivatization reagents for biogenic amines from food samples. Reproduced with permission from [66].

Among the most often used derivatization reagents are o-phthaldialdehyde (OPA), dansyl chloride and benzoyl chloride. OPA is well suited for automated and on-line derivatizations as it reacts with amines rapidly at room temperature. For example, OPA was used for automated pre-column derivatization of histamine in food samples, and derivatization reaction took only 30 seconds[67]. Dansyl chloride, dabsyl chloride, and benzoyl chloride are commonly used for off-line pre-column derivatization. A scheme of reaction between amines and benzoyl chloride can be seen in **Figure 3**. Reaction times for these vary from 15 min to 45 min and temperatures from 30°C to 80°C as demonstrated in methods collected in **Table 3**.

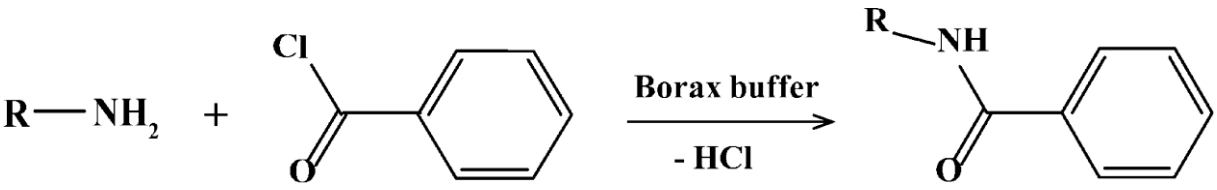

Figure 3. Reaction scheme of amine derivatization with benzoyl chloride. Reprinted with permission from[29].

Diethyl ethoxy methylene malonate (DEEMM) is a reagent often used in derivatization of analytes from wine and cheese, and occasionally beers. Because of the exceptional performance of DEEMM it has been chosen by the International Organization of Vine and Wine to be the official derivatization reagent for wine analysis with HPLC-DAD. Since wines are complex matrices containing both amino acids and biogenic amines, the derivatization reagent has to be able to produce derivatives for simultaneous analysis of the different compounds. Furthermore the use of DEEMM is beneficial because it is straightforward to use, does not create reaction by-products, can react with both primary and secondary amino groups and the aminoenone derivatives are stable.[68]

4.4 Modes of derivatization (on/off-line, pre/on/post-column)

Derivatization can be implemented pre-column, on-column or post-column as well as on-line or off-line. Schematic representation of pre-column, on-column, and post-column derivatization mechanism can be seen in **Figure 4**. However, the pre-column derivatization is the most often employed one because of the higher sensitivity of detection. Off-line procedures are generally highly laborious, time consuming and may result in contamination and losses of analyte. On-line or automated procedures are free from these drawbacks and are especially beneficial for labile compounds and those retaining too strongly on the stationary phase when performed pre-column. Nevertheless the majority of derivatization procedures are performed off-line as automatization can be a high cost process. Examples of more rarely used derivatization methods are described in the following paragraphs.[51]

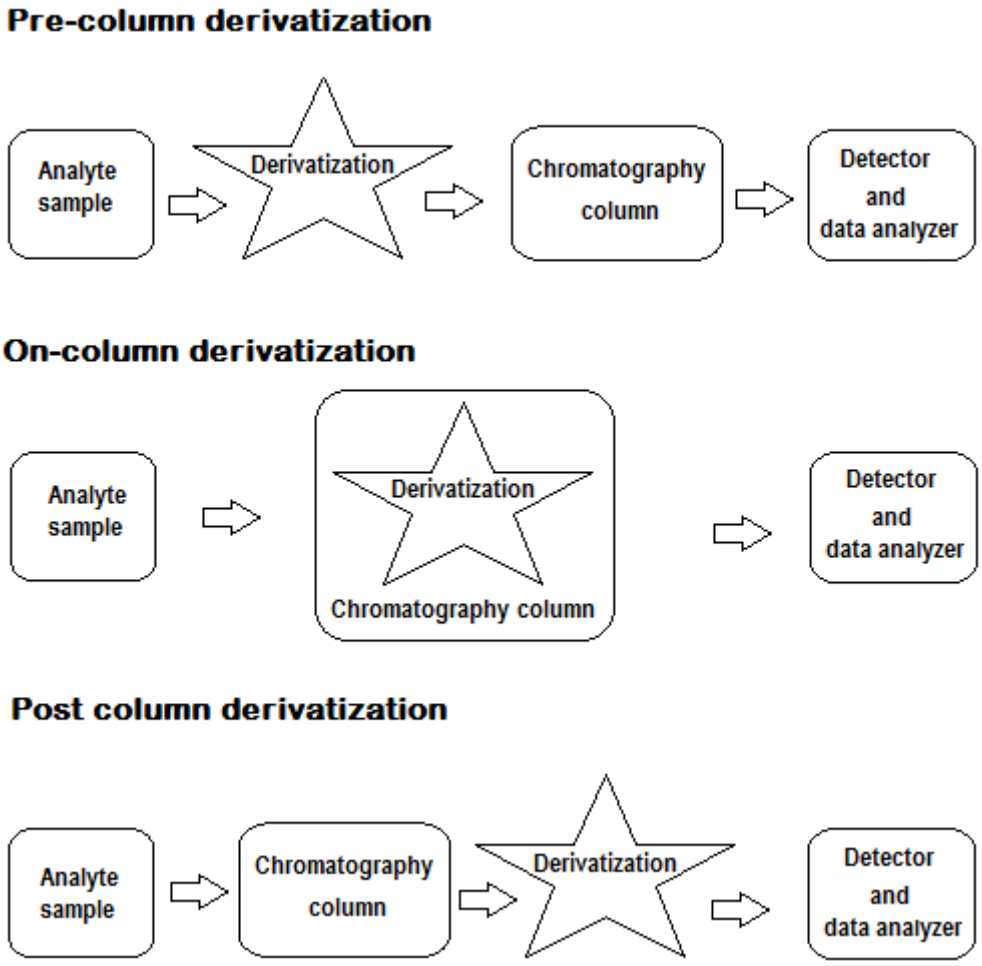

Figure 4. Schematic representation of pre-column, on-column and post-column derivatization modes.

An automated on-line derivatization method was developed by Notou et al.[69]. Zone fluidics were employed in the derivatization. They used naphthalene-2,3-dicarboxaldehyde (NDA) as a labeling reagent. Analytes were separated by HPLC and detected with fluorescence detection. Method was validated for determination of BAs (HIS, PEA, TYR, TRP, isoamylamine and propylamine) in milk samples. Obtained limits of detection were remarkably small at picogram level. Other noticeable benefits of the method were short analysis times (3 min) and low consumption of the expensive derivatization reagent (few microliters).

Another automated pre-column derivatization method was developed by Kelly et al.[61] It was used for determination of 24 BAs and amino acids in wines, grapes, honey and physalis fruit. Derivatization was performed in the loop by o-phthaldialdehyde (OPA) reagent. Separation and detection of analytes was done by HPLC with fluorescence detection. The method was reported to be fast and economical as there is no need for sample preparation other than dilution and total analysis time is 40 min. Since the high sensitivity of the method enabled this, the addition of internal standard step was removed to simplify the method and reduce the chance of contamination.

An automated post-column derivatization method was used in determination of BAs (TYR, PUT, CAD, SPM, SPD, HIS and AGM) in squid and white prawn[70]. Separation on HPLC was done in less than an hour. Internal standard (1,6-diaminohexane) was added to improve the precision of quantification of analytes. Derivatization reagent used was OPA, and derivatives were detected with fluorescence detection.

More recent derivatization approaches involve in situ single-step derivatization, where derivatization and extraction is performed in the same reaction mixture as a combined step. Plotka-Wasylka et al.[6] developed an in situ derivatization by isobutyl chloroformate combined with DLLME for BA determination in home-made fermented alcoholic drinks. Obtained method was simple, fast and highly reproducible.

5 Chromatographic analysis

Chromatographic analyses are by far the most popular methods concerning amine determination in food samples. However, there are also other analysis methods available for determination of BAs in foods. Most prominent of them is capillary electrophoresis (CE). Compared to high performance liquid chromatography (HPLC) capillary electrophoresis can have some benefits. It is faster, more cost efficient, easier to operate, produces less organic solvent waste, has shorter analysis times and higher sensitivities[71-72]. Nevertheless, HPLC has advantages, such as lower limits of detection, compared to other methods. Modern chromatography has developed from thin layer chromatography (TLC). While TLC can be used in biogenic amine analysis, gas chromatography (GC) or, more importantly, high performance liquid chromatography (HPLC) will be the technique of choice nowadays.

Analyses of biogenic amines performed by TLC, GC and HPLC are discussed in the following chapters. State of the art analysis methods are described, and new advances and improvements in the past decade are discussed in more detail. Objectives for developing new analysis methods and improving the old ones are to make them more rapid, reliable, simple, environmentally friendly and cost efficient.

5.1 Thin layer chromatography (TLC)

Thin layer chromatography might be viewed as an analysis method of the past, but it still has its place in the modern day analytical laboratory. In TLC analysis analyte solution is placed on plates (generally silicagel covered) and allowed to evaporate. Plates are then placed vertically in a development chamber containing eluent. Separation of analytes occurs as they are carried by the eluent through the silicagel. Detection of analytes is done by illuminating the plate with a certain wavelength. Analyses performed by TLC compared to HPLC are fast, inexpensive to operate and do not require complicated and expensive machinery or highly trained staff. Additionally, analyses of multiple samples can be performed simultaneously.[73]

There is a large number of research articles about biogenic amine determination by TLC in food samples published prior to 2007. However, in the past decade only a few researches have been offered[73]. The TLC methods are extensively researched and not much can be done to further improve them. Nevertheless, recent advancements in imaging techniques have also enabled TLC to be employed as a quantitative method. TLC might be the method of choice for routine analyses of biogenic amines in food samples such as fish, meat, cheese and wine in laboratories that are not instrumentally equipped.[74]

Romano et al.[74] developed and validated a method for quantitative biogenic amine (PUT, CAD, HIS and TYR) detection in wines by TLC. Dansyl chloride was used as a derivatization reagent and densitometry as a matter of detection. Internal standard used was 1,3-diaminopropane. Derivatization of amine and internal standard mixture was done by adding saturated $NaHCO_3$ solution and dansyl chloride (5 mg/mL in acetone) and incubating in darkness for an hour at 55°C. NaCl was used to stop the process. Extraction was done by shaking for 5 min with iso-hexane. Aliquots of organic phase were placed on silicagel 60 TLC glass plates that were developed in a glass TLC chamber for 90 min. Eluent used was 4/1 chloroform/triethylamine. Detection was done by illuminating TLC plates at 312 nm wavelength, images were taken by Infinity-1000 imaging system and analyzed by BIO-1D software. Results obtained by TLC were compared with those obtained by HPLC method issued by the International Organization of Vine and Wine, and no significant difference was found, concluding that the method is valid to be used.

Quantitative TLC was used also by Ayesh et al.[75] in determination of BAs (PUT, CAD, HIS and TYR) in fish samples. They prepared dansylated derivatives by derivatization of amines by dansyl chloride and separated them on one-dimensional TLC. Development solvent was chloroform: benzene: triethylamine (6: 4.5: 1) and spots were visualized by UV lamp (λ=365 nm). Determination of BAs was done by densitometer (λ=254 nm). Results showed levels of BAs in the range of mg per 100g.

High performance thin layer chromatography (HPTLC) is an improved method of TLC with higher resolution and increased precision of quantitative analysis. Li et al.[76] used HPTLC with densitometry detection to develop a method for simultaneous detection of biogenic amines in aerobically stored beef. Dansyl chloride was used as a derivatizing reagent similarly as for most of the TLC analysis methods of biogenic amines. Automated TLC sampler was used in application of derivative solution and plates were developed in a twin through chamber by different solvent systems containing chloroform, diethyl ether and triethylamine. Linearity of the method was in the range from 0.5 to 40 µg/ml and levels of detection (LOD) ranged from 0.03 to 0.37 µg/mL.

5.2 Gas chromatography (GC)

Gas chromatography is an analysis method where analyte solution is injected into the GC apparatus, analytes are evaporated and introduced to a capillary column where separation of analytes occurs as they are moved through the capillary by a carrier gas. At the exit of the capillary analytes are detected most often by mass spectrometry (MS). For amine analysis by GC the highest sensitivity and selectivity will be achieved with nitrogen-selective detection, electron capture detection, nitrosamine-specific detection and mass spectrometry[51]. The use of gas chromatography in food analyses is not as uncommon as TLC but is still limited. Most often GC is used in analyses of beverages such as beer, wine and fermented alcoholic drinks. It can also be used in analyses of foods like cheese and fish.

Biogenic amines in beverages originate from microbiological activity during fermentation processes. Cunha et al.[77] used a GC-MS method in determination of BAs in liqueurs. Even though liqueurs are not produced by fermentation, the strong alcohols that liqueurs are made of are produced using fermentation, resulting in presence of BAs in liqueurs. With this method BAs (HIS, TYR, CAD and morpholine) were detected along with volatile amines. Gas chromatograph with split/split-less injector port, DB 5-MS capillary column, and single quadrupole inert mass selective detector was used. Quantitative analysis was based on selective ion monitoring (SIM) mode. In the study of 27 liqueurs it was found that BAs are present in quantities that do not possess a health concern in the amounts that liqueurs usually are consumed.

A GC-MS method was used in determination of BAs (HIS, CAD, TYR and PUT) in fish samples[46]. Stationary phase was RXi-5MS column (30 m, 0.25 mm, 0.25 µm film thickness), and carrier gas helium at a flow rate 1.33 mL/min. The GC injection port temperature was 270°C, split-less mode was used and total run time was 17.3 min. Ion source temperature was 230°C and interface temperature 280°C. Electron impact ionization mode (70 eV) was used in operating the mass spectrometer and SIM mode in quantification of analytes. Obtained LOD was in the range from 2.98 to 45.3 µg/L.

5.2.1 Separation

Separation of BA analytes from food samples was performed by several capillary columns. Most stationary phases were composed of 5% phenyl siloxane and 95% methyl polysiloxane. These are low polarity and low bleed columns suitable for combination with MS detection. Most often chosen dimensions of the capillary columns were 30 m, 0.25 mm I.D, and 0.25 µm film thickness. Injections were done either by split-less or pulsed split-less mode. Helium was generally used as a carrier gas. Temperature programs used were dependent on the chosen method. An overview of the stationary phases used can be seen in **Table 4**.

Table 4. Stationary phases used in GC separation of biogenic amines from food samples.

Nr	Column	Composition	Dimensions	Ref
1	Rxi-5MS	Crossbond diphenyl dimethyl polysiloxane	30 m, 0.25 mm I.D, 0.25 µm film thickness	46
2	HP-5 MS	5% Phenyl siloxane / 95% methyl polyorganosiloxane	30 m, 0.25 mm I.D, 0.25 µm film thickness	5, 78
3	DB 5-MS	(5%-Phenyl)-methylpolysiloxane	30 m, 0.25 mm I.D, 0.25 µm film thickness	77
4	DB 5-MS	(5%-Phenyl)-methylpolysiloxane	20 m, 0.18 mm I.D, 0.18 mm film thickness	44
5	Varian CPSil-8	5% Phenyl / 95% methyl	30 m, 0.25 mm I.D, 0.25 µm film thickness	47
6	ZB 5-MS	5% Phenyl-Arylene, 95% Dimethylpolysiloxane	30 m, 0.25 mm I.D, 0.25 µm film thickness	6

5.2.2 Mas spectrometric detection

Mass spectrometric detection is the most often employed detection method for GC of biogenic amines. Mass spectrometer consists of three major elements – ion source, mass analyzer, and detector. There are different ion sources available and they can be chosen depending on the physical state of the sample or the extent of fragmentation required. Electron impact ionization (EI) is a hard ionization method and will produce more fragmentation than the soft chemical ionization (CI). Gas and vapor phase analytes can be ionized either by electron ionization or chemical ionization. For liquid and solid samples electron spray ionization (ESI) and matrix-assisted laser desorption ionization (MALDI) are common ionization methods.

In amine analysis the electron impact ionization mode at 70 eV is the ionization method of choice. While quadrupole is the most commonly used mass analyzer the triple-axis detector has also been utilized. The selective ion monitoring (SIM) mode has been employed in quantitative studies.

Cunha et al.[78] used GC-MS in simultaneous quantification of 22 amines in port wine and grape juice samples. Parameters of MS were: electron impact ionization (70 eV), ion source at 230°C, and MS quadrupole at 150°C. Mode of MS operating was selective ion monitoring (SIM). The method had high separation, good sensitivity, LOD and linearity.

GC-MS method was also used by Huang et al.[46] in determination of BAs (PUT, CAD, HIS and TYR) in fish samples. Plotka-Wasylka et al.[6] used GC-MS in determination of BAs in homemade alcoholic drinks. Awan et al.[47] determined PUT and CAD in vegetable, cheese and meat samples by GC-MS. Mohammadi et al.[5] used GC-MS in determination of CAD, PUT, HIS and TYR in cheese samples.

5.3 High performance liquid chromatography (HPLC)

An overview of HPLC methods used in recent research can be seen in **Table 5** where such characteristics of methods are collected as solid phase or used column, mobile phase composition and flow, detector and limit of detection (LOD).

Table 5. HPLC conditions for determination of biogenic amines in food samples.

No	Analytes	Matrix	Solid phase	Mobile phase	Detection	LOD	Ref
1	TRP, PEA, HIS, CAD, SPD, TYR	cheese, yoghurt, rice wine, beer, ham sausage	C18 (4.6 × 200 mm, 5 μm)	Gradient elution: (A) 5 % ACN and (B) 100 % ACN. 65–70 % (B) 0-10 min, 70–78 % (B) 10-16 min, 78–80 % (B) 16-20 min, 80–100% (B) 20-25min and keeping constant until 32 min. Flow rate: 1 mL/min	FL (λex=279 nm; λem=380 nm)	1.1 – 7.8 ng/mL	[35]
2	AGM, PEA, PUT, CAD, HIS, SER, TYR, SPD, SPM	wine, fruit nectar	Kinetex C18 core–shell particle column (4.6 × 100 mm, 2.6 μm)	Gradient elution: (A) 65% ACN and (B) 35% ACN. Total run time 11 min. Flow rate 0.6 mL/min.	UV (λ=254 nm), FL (λex=320 nm; λem=523 nm)	UV: 13–112 ng/mL FL: 2 – 23 ng/mL	[20]
3	TYR, HIS, PEA, PUT, CAD, TRP, AGM, SPD, SPM	meat, meat products	Pickering PCX 3100 cation-exchange column (K^+, 4 x 150 mm, 10 μm); pre-column (K^+, 3 × 20 mm, 10 μm)	Gradient elution: (A) 11% 2-propanol, 0.9% K_2HPO_4, 0.3% acetic acid, 87.8% water, pH 6.00; (B) 5% KCl, 4% 2-propanol, 0.9% K_2HPO_4, 0.3% acetic acid, 89.8% water, pH 5.63; (C) 0.7% KCl, 4% 2-propanol, 0.5% KOH, 94.8% water, pH 13.00. Total run time 31 min. Flow rate 0.5 mL/min.	FL (λex=330 nm; λem=465 nm)	30 – 100 ng/mL	[79]
4	PUT, CAD, SPD, SPM, AGM, HIM, TYR, SER	fermented tofu, stinky brine; Chinese rice wines	column: inertsil ODS-3 (4.6 × 250 mm, 5 μm); guard column: Hypersil ODS-3	Gradient elution: (A) 10 mM ammonium acetate and (B) ACN. Flow rate 1 mL/min	UV (λ=254 nm)	-	[80] [81]
5	TRP, PUT, HIS, TYR, SPD	fish, meat products, fruit juice, canned vegetables, fruits, soy bean products	column: Spherisorb ODS2 (4.5 × 250 mm, 5 μm)	Isocratic elution: ACN / water (67/33, v/v). Total run time 30 min. Flow rate 1.2 mL/min.	UV/VIS (λ=254 nm)	4.43 – 7.34 ng/mL	[82]
6	HIS, PUT, CAD	fish (Hammour, Negrule, Zubaidi)	C18 reversed-phase (4.6 × 250 mm)	Gradient elution of water/ACN from 40% to 80% in 20 min at 1.0 mL/min, then ACN to 100% in 5 min, finally ACN to 40% in 5 min. Total run time 30 min.	PAD (λ=254 nm)	-	[13]

Table 5 continues. HPLC conditions for determination of biogenic amines in food samples.

No	Analytes	Matrix	Solid phase	Mobile phase	Detection	LOD	Ref
7	TYR, SER, DA, NE, NME	cocoa, chocolate, broad beans	pre-column: RP-18e (4 x 4 mm, 5-µm), column: RP-18e (125 × 3 mm, 5 µm)	Gradient elution: (A) acetate buffer (pH 4.66); (B) methanol. 0 min 100 % A and flow rate 0.5 mL/min, 30 min 90 % A, 10 % B. Flow rate 1,0 mL/min.	simultaneous FL (λex=285 nm; λem=315 nm) and DAD (λ=275 nm)	10 ng/mL	[18]
8	HIS, PEA, PUT, CAD, SPD	wine	Phenomenex Gemini C18 column (4.6 × 250 mm, 5 µm)	Gradient elution: (A) acetate buffer (pH 4.0); (B) ACN. Total run time 40 min. Flow rate 1.0 mL/min.	FL (λex=320 nm; λem=523 nm)	3 – 220 ng/mL	[42]
9	CAD, HIS, SPM, SPD, TRP, TYR, PUT	cheese	Waters Atlantis HILIC column (2.1 × 150 mm, 3 µm)	Gradient elution: (A) acetonitrile; (B) ammonium formate 50.0mM in ultrapure (pH 4,00). Total run time 23 min. Flow rate 0.27 – 0.32 mL/min.	MS/MS with positive polarity APCI, and hybrid triple quad/linear ion trap	1.1 – 3.5 ng/mL	[83]
10	HIS, SPD, SPM, TYR, PUT, CAD, AGM, PEA	cheese	column: RP C18 (4.6 × 250 mm, 5 µm) guard-pak: C18 (4.6 × 10 mm, 5 µm)	Gradient elution: (A) ACN/water 20/80 (v/v) mixture containing TFA (0.05%, v/v); (B) ACN/water 20/80 containing TFA (0.35%, v/v). Total run time 40 min. Flow rate 0.7 mL/min.	ELSD	1400 - 3600 1000 – 3200 ng/mL	[3] [55]
11	TRP, PEA, PUT, HIS, TYR, SPD	orange juice	COSMOSIL 5C18-MS-I column (4.6 × 250 mm, 5 µm)	Gradient elution: ACN/water (70/30). Flow rate: 1 mL/min (0–11,0 min), 1.5 mL/min (11.1–19.0 min), 1.0 mL/ (19.1–20.0 min). Run time 20 min.	UV (λ=254 nm)	0.0038 – 0.0313 ng/mL	[50]
12	PUT, CAD, TRP, PEA, HIS, TYR, AGM	red and white wine	Hichrom C18 (3.9 × 300 mm, 10 µm)	Gradient elution: (A) 0.05M acetate buffer: methanol 60:40 (pH 6, 7 or 8); (B) methanol. Total run time 32 min.	UV-vis (λ=254 nm)	200 - 2500 ng/mL	[84]

5.3.1 HPLC separation

Separation of analytes by HPLC is done either on a monolithic column or column packed with porous particles. Amine analytes most often are separated by reversed phase (RP) octadecylsilyl (ODS) columns. Hydrophobic interaction liquid chromatography (HILIC) columns and ion exchange columns have also been used successfully. The most commonly used reversed phase column dimensions are 4.6 × 250 mm with particles 5 micrometer in diameter. However, columns with smaller particle diameter size, such as 3 µm, 2.6 µm, and 1.8 µm have also been utilized.

Usage of smaller diameter particle columns is beneficial as with the increased separation efficiency of the analytes the total analysis times decrease. For regular 5 µm particle columns run times vary between 20 and 40 minutes, while for smaller particle sizes the run times usually are shorter than 10 minutes. However, the decrease in particle size increases the backpressure significantly. Typically HPLC backpressures are lower than 200 bar but with smaller particle sizes they can go up to 700 – 800 bar.

Since a regular HPLC system can not withstand these kinds of pressures, new kind of apparatuses have been developed – ultra high pressure liquid chromatography (UHPLC). UHPLC will be discussed in more detail in section 5.4. The main drawback of the UHPLC is that the equipment is very expensive and not widely available. Therefore there is need for methods that can utilize smaller particle size columns with the HPLC equipment.

Conventional HPLC methods of 5 µm columns were adapted for the use of the small diameter particle size (1.8 – 3 µm) columns[85]. They were applied for determination of nine BAs in seafood samples. The use of HPLC system with the small particle size columns was possible because the backpressures remained under 170 bar. The new methods showed decrease of analysis times up to 59% and eluent consumption up to 62%. In terms of linearity, repeatability and recovery the performance of the new methods remained comparable to the 5 µm ones.

5.3.2 HPLC detection

The most often used HPLC detection methods are ultraviolet (UV)[86] and fluorescence (FL)[87]. Other methods involve diode array detector (DAD) (also called photodiode array detector (PAD) at different suppliers) and evaporative light scattering detection (ELSD)[3]. However, methods developed in recent years mostly use mass spectrometric (MS) detection.

UV detector can only detect one wavelength at a time. DAD is essentially a UV-vis detector but consisting of many diodes that allow scanning through a wide range of wavelengths. While DAD is more costly it is less time consuming, especially in the method development phase as the best wavelength can be selected for analysis. DAD is also beneficial in routine analyses as it can show possible impurities, co-eluting peaks or changes in method.

An excellent detection method for HPLC is mass spectrometry. Equipment of MS is more expensive, however it has many advantages over UV and DAD detection methods. For one it can operate without derivatization, which is time consuming and may result in poor recoveries, analyte losses and contamination. Nevertheless the majority of researchers still employ derivatization as it can improve some characteristics of analysis[88]. MS also does not require complicated sample preparation. For example, because of the high selectivity of mass spectrometry, the analyte extracts do not need to be as ‘’clean’’ as for other detection methods. Therefore less laborious extraction techniques can be used. However, in detection of biogenic amines in food samples a matrix effect can be a problem as addressed in some cheese sample analyses[54, 83, 89].

Tandem mass spectrometry (MS/MS) has even more benefits as it can be used in confirmation of analytes. Consequently different kinds of analytes such as biogenic amines and preservatives can be detected simultaneously[90]. Tandem MS detection methods also have higher sensitivities and precision.[89] Performance of tandem MS was well demonstrated in a method for determination of biogenic amines in licorice[91]. The method showed to be highly sensitive as LODs of nine BAs (PUT, CAD, HIS, SPM, SPD, TYR, TRP, AGM and PEA) were between 1.4 to 2.7 ng/mL. MS/MS is a great tool for identification of analytes and their structural properties. Therefore the derivatization step may be omitted which is simplifying the sample preparation and reducing the probability of contamination and analyte loss.

Evaporative light scattering detector (ELSD) is a detector that has the benefit of not needing the derivatization, thus excluding the drawbacks associated with it. Compared to MS detection that also can be done without derivatization, ELSD is less costly and can be combined with different types of solvents. Limits of detection (LOD) however are much higher than for MS as the intrinsic limit of detection for ELSD method is 100 ng/mL. While this might require pre-concentration step of analytes, biogenic amines in food samples are usually present in high enough quantities to be detected by ELSD. Additional shortcoming of the ELSD is also the lack of linearity between concentration of analyte and detector response.[3, 92]

5.4 Ultra-high performance liquid chromatography (UHPLC)

Ultra-high performance liquid chromatography (UHPLC) is a new and improved separation technique based on HPLC. The biggest difference between HPLC and UHPLC is the particle size in separation columns. While in HPLC often used particle sizes are 5 µm, in UHPLC they are less than 2 µm. Decrease of particle size in this magnitude means significant increase of pressure (up to 1000 bar), therefore UHPLC requires adjusted chromatographic systems that can handle the pressure. Detection of UHPLC has been done either by UV, fluorescence, mass spectrometry or tandem mass spectrometry (MS/MS).[93]

The most prominent improvements of UHPLC over HPLC are the reduced analysis times. UHPLC run can be up to 10 times faster than a conventional HPLC run. Short analysis times also mean that smaller amounts of the expensive and toxic solvents will be consumed. Therefore UHPLC is a more environmentally friendly and cost efficient method, while the efficiency of the separation method is not compromised.[94]

In biogenic amine analysis UHPLC can be used as well as HPLC. However, short analysis times allow more samples to be analyzed, which is beneficial when large numbers of samples need to be analyzed. In recent years utilization of UHPLC has increased. An overview of the conditions for determination of biogenic amines in food samples by UHPLC can be seen in **Table 6**.

Table 6. UHPLC conditions for determination of biogenic amines in food samples.

No	Analytes	Matrix	Solid phase	Mobile phase	Detection	LOD	Ref
1	TYR, PUT, SER, CAD, HIS; PEA, AGM, SPD, SPM, TRP, DA, OCT	wine, fish, cheese, dry fermented sausage	UHPLC BEH C18 column (2.1×50 mm, 1.7 μm)	Linear gradient elution: (A) 0.1M sodium acetate and 10mM sodium octanesulphonate adjusted to pH 4.8 with acetic acid; (B) 0.2M sodium acetate and 10mM sodium octanesulphonate adjusted to pH 4.5 with acetic acid /ACN (6.6/3.4). Total run time 7 min. Flow rate 0.8 mL/min.	FL (λex=340 nm; λem=445 nm)	50 – 200 ng/mL	94
2	PUT, CAD, SPM, SPD, PEA, HIS, TYR, TRP	pork, beef, chicken, fish, cheese, edible mushrooms	Zorbax Eclipse XDB – C18 column (4.6×50 mm, 1.8 μm)	Linear gradient elution: (A) 100% ACN; (B) 50% ACN in water. Total run time less than 6 min. Flow rate 1.0 mL/min.	UV (λ=225 nm)	32 – 98 ng/mL	93
3	AGM, TRP, PEA, PUT, CAD, HIS, SER, TYR, SPD, SPM, DA, NOR	fish, fish products	BEH C18 column (2.1×50 mm, 1.7 μm)	Elution: (A) 0,1% formic acid in deionized water (25%); (B) 0,1% formic acid in ACN (75%). Flow rate 0.25 mL/min.	triple quadrupole MS/MS. Ionization: ESI+	20 – 200 ng/mg	95
4	PEA, CAD, TRP, PUT, TYR, HIS, AGM,	cheese (gouda, emmental, burgos, cabrales)	Waters Acquity UPLC BEH C18 (2.1×100 mm, 1.7μm)	Ternary gradient elution: (A) 25 mM acetate buffer pH 6.7 and 0.02% sodium azide; (B) methanol; (C) ACN. Total run time under 10 min. Flow rate 0.45 mL/min.	PAD (λ=280 nm)	10 – 150 ng/mL	96
5	AGM, CAD, HIS, PEA, PUT, TRP, TYR	fish (mackerel, mahi-mahi, sockeye salmon)	Waters Acquity UPLC BEH HILIC (2.1×150 mm, 1.7μm)	Gradient elution: (A) 20:80 (v/v) ammonium formate buffer in ACN, pH 3.00; (B) ACN. Run time 18 min. Flow rate 0.75 mL/min.	orbitrap MS, positive ion APCI	36 – 370 ng/mg	9
6	PUT, CAD, HIS, TYR	fish (anchovy)	Waters Acquity UPLC BEH C18 (2.1×100 mm, 1.7μm)	Gradient elution: (A) methanol; (B) aqueous solution of formic acid 0.1% (v/v). Total run time 8.5 min. Flow rate 0.2 mL/min.	tandem quadrupole MS/MS Ionization: ESI+	7.5 ng/mg	8
7	TRP, PUT, HIS, CAD, PEA, TYR,	rice wine	Eclipce Plus C18 (2.1×50 mm, 1.8 μm)	Gradient elution: (A) 5% ACN containing 0.1% formic acid; (B) 0.1% formic acid in ACN. Total run time 8 min. Flow rate 0.2 mL/min.	MRM triple quad MS/MS Ionization: ESI+	0.15 – 0.27 μM	97

A UHPLC method was used in determination of biogenic amines in Korean wine, Makgeolli[98]. Amines (AGM, TYR, PEA, PUT, CAD, HIS, TRP, SPD and SPM) were derivatized by dansyl chloride prior to analysis. Despite using the UHPLC system, researchers chose a C18 column with particle size of 5 µm which is commonly used in HPLC. Nevertheless it was 50 mm short like typical columns used for UHPLC. Elution times were 21 minutes which is untypically long for UHPLC. Analytes were detected using the tandem mass spectrometry with multiple reaction monitoring (MRM) mode and electrospray ionization in positive mode. However, the LODs of derivatized analytes were significantly lower than those in the methods observed in **Table 6** varying between 0.1 to 4.6 ng/mL.

An outstanding performance of the UHPLC technique was demonstrated by Redruello et al.[68] in their method of simultaneously determining biogenic amines, amino acids and ammonium ions in beers. Since simultaneous analysis of different amino compounds can be challenging, analytes were derivatized with DEEMM beforehand. They used a UPLC BEH C18 (2.1×150 mm, 1.7µm) column for aminoenone derivative separation and DAD for detection. Using this method researchers managed to separate 31 compounds including 9 biogenic amines, 21 amino acids and ammonium ions in under 14 minutes. The LODs, precision and accuracy of the method also showed good results. LODs for BAs were in the range of 100 to 130 ng/mL which is common for diode array detection.

A method of simultaneous determination of biogenic amines and amino acids by UHPLC has also been established by Fiechter et al.[99] The method involved analyte derivatization with 6- aminoquinolyl-N-hydroxy succinimidyl carbamate (AQC) and detection by UV. They used it for ripened acid-curd cheese samples. This UHPLC method also verified the greatest advantage of UHPLC – separation of large amount of analytes in a very short time. Accordingly, 15 amines and 23 amino acid derivatives were separated within nine minutes.

6 Summary of literature part

Amines in food mostly are appearing from amino acid microbial decarboxylation, hence their presence in food is a good indicator of freshness. Consumption of foods with elevated levels of biogenic amine concentrations can cause negative health effects and their presence can indicate spoilage of a food product. Therefore it is important to monitor the levels of Bas in foods. Chromatographic methods are excellent tools for determination of BAs.

Sample preparation plays significant role in determination of BAs originating from such complex matrices as foodstuffs. Extraction of amines being the most crucial step. Therefore many different extraction methods have been developed. The well-established conventional techniques such as SLE and SPE are still widely used even though new and improved methods have been developed. Often the conventional methods are chosen because they are straightforward to use and typically do not require special equipment that would not be available in a typical laboratory. The new methods however are focusing on miniaturization, greenness and cost efficiency while not compromising the performance. Among the more often used newer methods are LPME, SPME, SALLE, DLLME and MSPD.

Derivatization of biogenic amines is a common step to increase sensitivity and selectivity of the detection methods. Derivatization can be performed on or off-line, pre, post or on-column. Since automated on-line methods can be expensive, the most common derivatization mode still is off-line pre-column derivatization. The most often used derivatization reagents include dansyl chloride, benzoyl chloride, DEEMM and OPA, latest of which is commonly used in on-column derivatization because of the short reaction times.

Chromatographic methods have been well researched over time. However, in the past decade only few application were found of TLC use in determination of amines in food samples. The use of gas chromatography has been more prominent, especially in the analyses of beverages. GC often has been coupled with mass spectrometric detection. Nevertheless, HPLC is the main method of choice in determination of amines in food samples as demonstrated by the large numbers of research articles. HPLC has been successfully coupled to various detectors such as UV, fluorescence, DAD and mass spectrometry. Recently also the ultra-high performance

liquid chromatography (UHPLC) has been gaining popularity creating one of the most powerful analytical tools when coupled to tandem mass spectrometry.

EXPERIMENTAL PART

7 Introduction to experimental part

Amines are a class of chemicals that contains the NH group. Low molecular weight aliphatic amines such as methylamine (MA), dimethylamine (DMA), trimethylamine (TMA) etc. are highly volatile, thus their presence in the atmosphere is notable. There are amines in the ambient air, coming from the anthropogenic as well as biogenic sources. Some examples of anthropogenic amine sources are factories, exhaust fumes, tobacco smoke[100] and cattle farms[101]. Their presence in the air is affecting human health and environmental states. Therefore amine levels in the atmosphere should be monitored. [102]

When assessing the quality of air amine presence must be evaluated. Consequent exposure to even low concentrations of amines has shown to be a health hazard. Therefore in many countries the allowed concentrations of amines in the air are very low. Odour threshold for volatile amines and hygienic threshold values for many amines are in the low ppm ranges. For methylamine recommended value is 0.03 ppm and for trimethylamine only 0.002 ppm. [103]

Many of the amines in the atmosphere pose danger to the human health because of their toxicity. One way to access their chronic toxicity is threshold limit value - time weighted average index. It takes into account not only the inherent toxicity of the compound but also its lifetime in the atmosphere. While most amines will have degraded within 2 – 20 hours, there are numerous compounds that survive for more than 3 days. Trifluoroethylamine can survive in the atmosphere for up to 13 days. The majority of amines will degrade in the oxidation reactions with NO_3, O_3 and mainly OH radical. Amine impact on the environment is notable. Amines can behave as the cloud condensation nuclei (CCN). Thus they are affecting the climate by changing the amount of precipitation.[104]

Analysis methods for air amines are generally divided into direct analysis and off-line analysis methods. One of the difficulties lying in amine analysis is their low concentrations in the air. Amines can be analysed directly from the air by methods such as PTR-MS[105]. Off-line analysis methods require sampling, after which the analysis is done in the laboratory. Amines from the air can be sampled either by grab or binding methods. First works by collecting the air as is and then transporting it to the location where the analysis is performed. The second uses adsorbents to trap the amines physically or chemically.[106]

Sorbents are materials capable of retaining molecules either from gas or liquid phases. There are several ways how sorbents can be classified. First, classification can be done by the mechanism of trapping. It is either by surface forces in adsorbents or by chemical reactions with the surface of the sorbent material. Second, sorbents are classified by the method used to release the adsorbates. These can be either thermal desorption or solvent desorption. Third, sorbents are classified by their composition and polarity. Adsorbing compounds can be carbon based - usually hydrophobic and non-polar, oxygen containing – hydrophilic and polar, and polymer based - hydrophilic or hydrophobic, depending on the functional groups in the porous polymer matrix. [107]

The goal of the experimental part of this thesis was to prepare and characterize new materials for amine adsorption from the air.

8 Experimental

8.1 Used chemicals and equipment

A list of chemicals used in this work are collected in **Table 7.** Information about supplier and purities is provided where possible. A list of instrumentation used can be seen in **Table 8**.

Table 7. List of used chemicals, their purities and suppliers.

Chemicals	**Purity**	**Supplier**	**Product No.**
Methacrylic anhydride ($(MAA)_2O$)	94 %	Sigma-Aldrich	276685
1,4-Butanediol	99%	Sigma-Aldrich	493732
1-Dodecanol	98 %	Merck-Schuchardt	803462
1-Propanol	>99%	Merck	1.01024.1000
3-Mercaptopropyl trimethoxy silane (3-MPTS)	95%	Aldrich	175617
Acrylic acid (AA)	99 %	Sigma-Aldrich	147230
Acetone	tech.	VWR	20063.365
Acetonitril (ACN)	>99.9%	Sigma-Aldrich	34851
α,α´-Azobisbutyronitrile (AIBN)	98 %	Sigma-Aldrich	441090
Butylmethacrylate (BMA)	99 %	Sigma-Aldrich	235865
Cyclohexanol	99 %	Sigma-Aldrich	105899
di-n-butylphtalate	>99%	BDH Chemicals Ltd	28073
Poly(vinylalcohol) (PVA)	87-89% hydrolysed.	Aldrich	36,308-1
divinylbenzene (DVB)	80 %	Sigma-Aldrich	414565
Ethylenedimethacrylate (EDMA)	98 %	Sigma-Aldrich	335681
Fmoc-Cl **(9-**Fluorenylmethoxycarbonyl chloride**)**	99%	Sigma-Aldrich	23186
Hydrogen chloride solution (1.0 M)		FF-Chemicals Ab Oy	FF226
Hydrogen peroxide solution	technical, 33%	VWR	23613.297
Isooctane	99.5 %	Fluka	
Methacrylic acid (MAA)	99 %	Aldrich	
Methanol	HPLC/MS quality	Fisher Chemical	A456-212
Sodium chloride (solid)	Baker analysed	J T Baker	278
Sodium hydroxide solution (1.0 M)		Fisher Chemical	J/7620/15
Sodium hydroxide (solid)	Baker analysed	J T Baker	402
Neutral aluminium oxide		Woelm Pharma	02084
Phenolphtalein (solid)	ACS Reagent	Sigma-Aldrich	105945
3-Phenyl-propylamine (PA)	98%	Fluka	79010
p-Toluol sulphonic acid Hydrate (PTSA)	>99%	Merck	9631
Poly(vinylalcohol) (PVA)	87–89% hydrolysed	Aldrich	36, 308-1

Table 7 continues. List of used chemicals, their purities and suppliers.

Silica gel	0.063-0.200 mm	Merck	1734
Styrene	≥ 99 %	Aldrich	S4972
N,N-Dimethylbenzylamine (TA)	>99%	Aldrich	185582
Toluene	HPLC quality	VWR	83625.320
Trifluoroacetic acid (TFA)	Reagent Grade	Fisher Chemical	T/3256/PB05
Trimethylamine.HCl (TMA)	98%	Sigma Aldrich	T72761
Nitrogen gas			

Table 8. List of used equipment, their models and manufacturers.

Equipment	**Model and manufacturer**	**Notes**
10 mL Burette		± 0.02 mL
Mohr pipettes		3, 5, 10, 20 mL
Analytical balance	Mettler, AE 200	± 0.0001 g
GC oven	Hewlett Packard 5890, Series II	± 1^{0}C
HPLC instrument	Hewlett Packard, Series 1100	Binary Pump G1312A Degasser G1322A Autosampler G1313A Diode Array Detector G1315A Column Oven G1315A
Chromatographic Column	Zorbax C8 (150 x 4 mm i.d., 5μm)	
Magnetically stirrer	IKA, C-MAG HS7	
ATR-FTIR Spectrometer	Platinum-ATR, model Alpha, Bruker	Single reflection diamond crystal
Microscope	Nikon, ALPHAPHOT-2 YS2	
Standard Test Sieves	Retsch	106 and 250 μm
Soxhlet apparatus	Lenz	30 mL
SPE device	Biotage, VacMaster™ 10 and 20	
Extraction thimbles	Whatman 603	22 x 800 mm
pH meter	Radiometer, PHM220	
Ultrasonic bath	Branson, 5510	
Rotation evaporator	RII, Buechi	

8.2 Preparation of polymeric adsorbents

Two different types of polymers were prepared by suspension polymerisation - ethylene dimethacrylate (EDMA) polymers and anhydride polymers. In EDMA polymers acids were incorporated directly, while in anhydride polymers methacrylic anhydride was incorporated instead of acid. Afterwards anhydride polymers were hydrolysed to obtain free acid groups on the surface.

8.2.1 Suspension polymerization

Polymeric adsorbents were prepared by suspension polymerization. Procedure was adapted from Saito et al (2006)[108] and variables were adjusted to obtain desired results. Polymerization mixture consisted of continuous and organic phase. Continuous phase was prepared by dissolving 3.0 g of dispergent polyvinyl alcohol (PVA) and 3.0 g of NaCl in 100 g of water. Organic phase consisted of monomer mixture and initiator. A,α´-azobisbutyronitrile (AIBN) was used as a polymerisation initiator.

Composition of polymerisation mixture can be seen in **Table 9**. All monomers except for acrylic acid (AA), methacrylic acid (MAA) and methacrylic anhydride ($(MAA)_2O$) were purified beforehand from inhibitors on aluminium oxide column. Polymerisation was done in nitrogen atmosphere because the presence of oxygen inhibits the activity of the initiator. Polymerisation mixture was heated first to 70°C and then to 85°C. A plot of the heating programme is shown in **Figure 5**. The schematic representation of polymerisation reactor can be seen in **Figure 6**.

Table 9. Composition of the polymerization mixture for 5 g batches.

Compound	**m, g (DVB-$(MAA)_2O$)**	**m, g (Sty-DVB-$(MAA)_2O$)**	**m, g (EDMA-BMA-AA)**	**m, g (EDMA-MAA)**
Continuous phase	106.00	106.00	106.00	106.00
DVB	4.00	3.00	-	-
Styrene	-	1.50	-	-
MAA	-	-	-	0.5
BMA	-	-	3.00	-
AA	-	-	1.00	-
$(MAA)_2O$	2.0	1.50	-	-
EDMA	-	-	3.00	6.00
AIBN	0.12	0.12	0.12	0.12
di-n-butylphtalate	6.00	6.00	6.00	6.00
Stirring speed	2	2	2	2
Reference	Bratkowska(2012)[109]	Svobodova(2011)[110]	Fekete (2006)[111]	Saito (2006)[108]

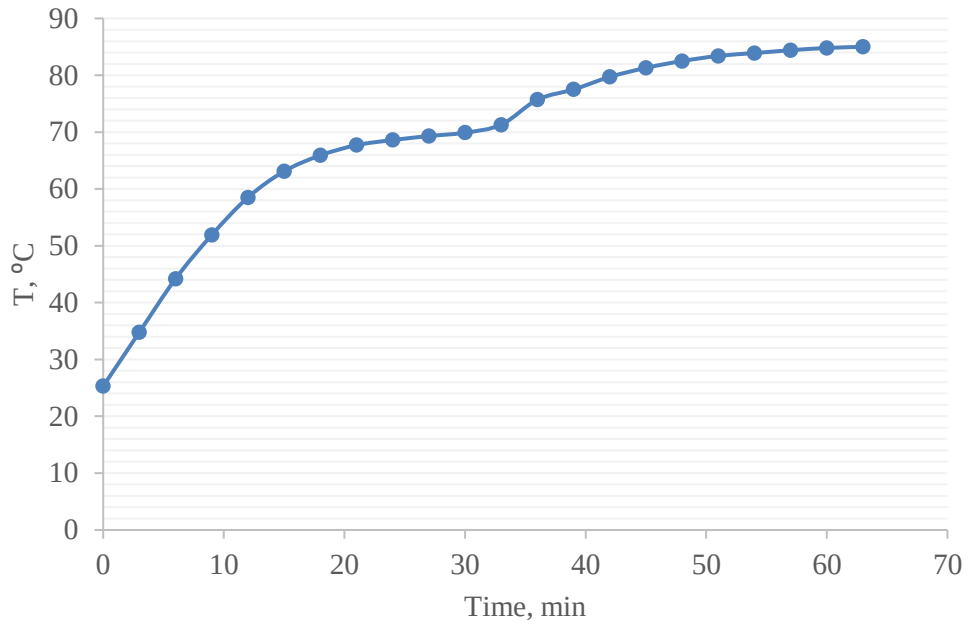

Figure 5. Temperature of the water bath during the heating program for the suspension polymerisation.

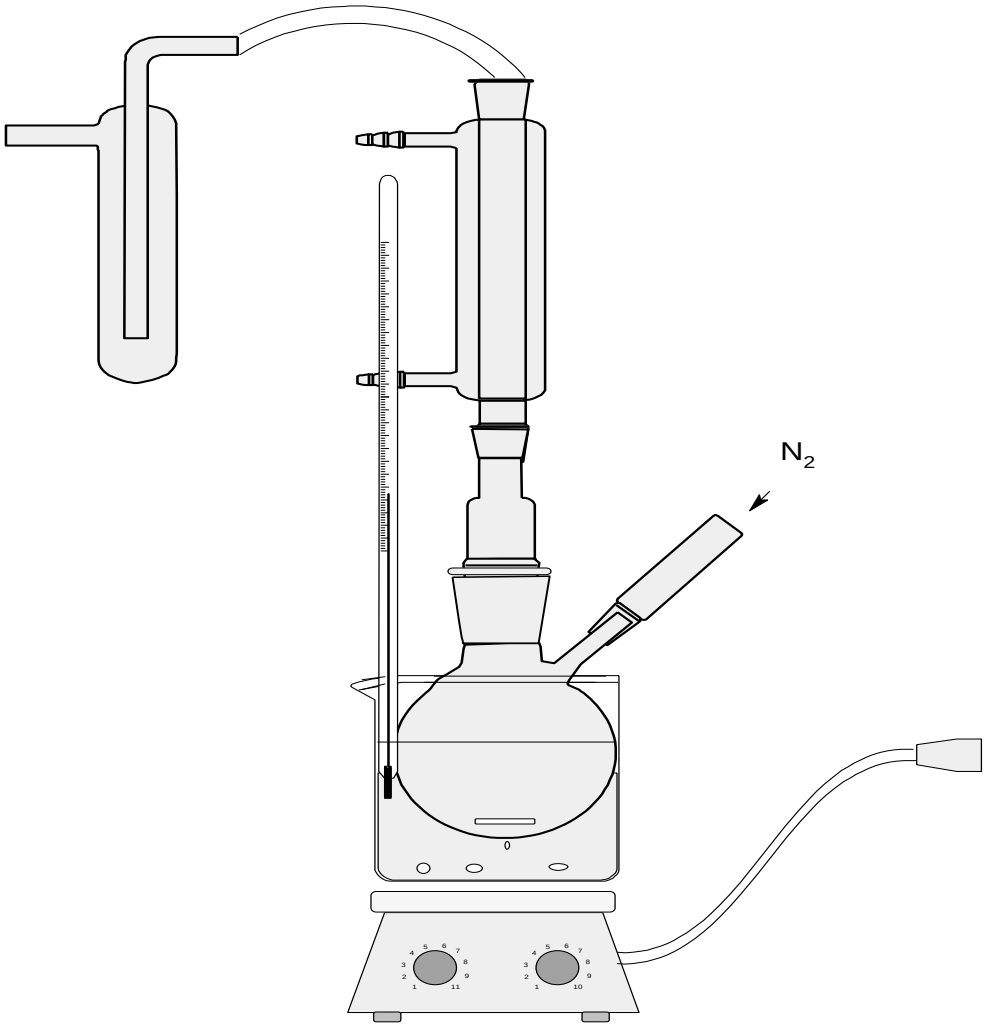

Figure 6. Schematic representation of polymerisation reactor.

Polymer processing after polymerisation reaction was performed the following way. The round-bottom flask was removed from heating and supernatant was decanted. Then particles were transferred to a conical flask where they were washed with MilliQ water. The conical flask was placed in an ultrasonic bath for 2-3 minutes. After particles had settled the supernatant was decanted again. Filling up the beaker with MilliQ water, sonication, particle settling and decanting was repeated five times or until the supernatant was clear. Afterwards particles were quantitatively transferred to an extraction thimble. The thimble was placed in a soxhlet apparatus for exhaustive extraction with acetone overnight. Finally the thimble was placed in an oven at 70ºC degrees to dry.

8.2.2 Hydrolysis of anhydride polymers

Hydrolysis of the anhydride polymers (DVB-$(MAA)_2O$) and (Sty-DVB-$(MAA)_2O$) was performed in a closed round bottom flask using 3 M NaOH. First, 3.0 g of dry polymer were weighed in a flask. Then 60.0 mL of methanol were added to the polymer and the flask was placed in an ultrasonic bath for a while to remove the air from the pores and introduce methanol in them. Afterwards 60.0 mL of 3 M NaOH were added. Flask was kept at 70ºC in a water bath for 20 hours with rotation stirring. Then polymers were washed with water and HCl in a glass column. Finally polymers were washed neutral with water, then with acetone and placed in an oven at 70ºC degrees to dry.

8.3 Synthesis of silica functionalized with sulphonic acid groups

Synthesis procedure was adapted from Shylesh et al (2004)[112]. First, 15 g of silica gel were suspended in 125 g of toluene and refluxed with magnetic stirring under nitrogen atmosphere. Then 3 mL of 3-mercaptopropyl trimethoxy silane (3-MPTS) were added to the suspension. As a catalyst 0.1 g of p-toluol sulphonic acid was added. The mixture was refluxed overnight. The next day it was filtered and washed with toluene and methanol. Then the silica gel was refluxed with methanol and filtered two more times. Afterwards it was dried in an oven.

Oxidation of mercapto propyl groups on the silica surface was done by weighing 5.00 g of silicagel and adding excess of 30% H_2O_2 in a round bottom flask. Magnetic stirring was added and mixture was left at room temperature overnight. Afterwards it was filtered, washed with water and dried in an oven. Schematic representation of the reactions can be seen in **Figure 7.**

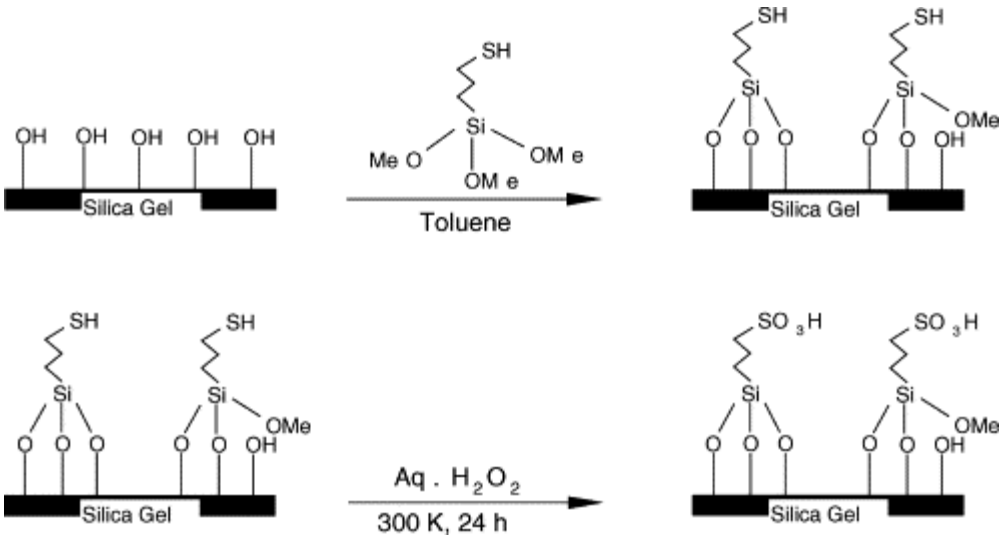

Figure 7. Schematic representation of reactions in preparation of propylsulfonic acid modified silica gel. Reprinted with permission from [112].

8.4 ATR-FTIR spectroscopy

Attenuated total reflectance Fourier transform Infra-red (ATR-FTIR) spectra were recorded for all the monomers, porogen and polymers before and after the hydrolysis. Information from ATR-FTIR spectra was used to evaluate the composition of the polymers. Monomer samples were analysed as is. Polymer samples were prepared by grinding in a porcelain mortar. ATR-FTIR spectra were recorded by placing a small amount of sample on the attenuated total reflectance (ATR) disc and setting up the measurement.

8.4.1 Polymer composition estimation

Calibration was done by recording six ATR-FTIR spectra and plotting the responses. First, four DVB and methacrylic anhydride or methacrylic acid mixtures (1:1 – 5:1) were prepared by combining required amounts of monomers by micropipettes. Composition ratio was calculated per mass. Densities of the monomers were used for calculating the volumes required for mixture

preparation. ATR-FTIR spectra of pure DVB, methacrylic anhydride and methacrylic acid were also recorded.

8.5 Determination of ion exchange capacity

Ion exchange capacity (IEC) of the adsorbents was determined by titrations. Because of agglomeration in aqueous solutions EDMA polymers had to be analysed with solutions containing methanol (MeOH). Anhydride polymers and functionalized silica gel could be analysed using water solutions.

8.5.1 Weak ion exchangers – polymers

Prepared solutions: 0.1 M HCl, 0.35 M HCl, 0.5 M HCl, 1 M HCl/MeOH (1:1), 0.4 M NaOH/MeOH (1:1), 0.2 M NaOH 0.5 M NaOH, 0.8 M NaOH, 0.1% phenolphthalein in MeOH.

Dry polymer was quantitatively introduced to a 100 mL glass column. Then it was consequently exposed to different solvents allowed to drop out at a rate 1 drop per sec. For anhydride polymers aqueous solutions were used in determination of IEC. For EDMA polymers reagent solutions in methanol were used. The solutions used and masses of the polymers are specified in the **Table 10.**

Table 10. Used solutions for IEC determination of polymers.

Polymer	**c NaOH, M**	**c HCl, M**	**m poly, g**	**V aliq, mL**	**V NaOH, mL**
EDMA-BMA-AA	0.3	0.1	2.0	10	10
EDMA-MAA	0.2	0.1	3.0	10	20
DVB-$(MAA)_2O$	0.35	0.5	3.0	10	20
DVB-$(MAA)_2O$ (50%)	0.8	0.5	3.0	10	20
Sty-DVB-(MAA)2O	0.35	0.5	3.0	10	20

The procedure of IEC determination was performed as follows. At the beginning the polymer was washed consequently with water or MeOH/water, then 0.1 M HCl or HCl/MeOH, and washed neutral with water or MeOH/water. Then for the ion exchange on polymer 20.0 mL of

NaOH solution were taken to the column and effluent (first elution) was collected in 50 mL volumetric flask. Additionally 15.0 mL water were taken to the column and effluent was collected in the same flask. Next, polymer was washed with 0.1 M HCl or HCl/MeOH and then washed neutral with water or MeOH/water. The ion exchange on polymer was repeated once more to collect the effluent from second elution. Finally polymer was washed with 0.1 M HCl or HCl/MeOH and then washed neutral with water or MeOH/water.

Solution for titration was prepared in erlenmeyer flask and consisted of 10.0 mL of effluent sample or NaOH solution, 20 mL water and couple of drops of phenolphthalein solution. Burette (10 ± 0.02 mL) was filled with the HCl solution. Solution was titrated by HCl solution until the pink colouring vanished. The consumed volume of HCl solution was written down. Titration was repeated three times.

The volumes of consumed HCl were used in IEC calculations. Amount of NaOH in 50 mL volumetric flask was calculated by equation 1. Ion exchange capacity of the polymer was calculated by equation 2 from the difference between the amount of NaOH in a 50 mL volumetric flask of NaOH solution and effluent.

$$n_{NaOH} = \frac{c_{HCl} * V_{HCl} * V_{flask}}{V_{aliq}} \quad (1)$$

Where n_{NaOH}– amount of NaOH in a volumetric flask, mmol; c_{HCl}– concentration of HCl used for titration, mol/l; V_{HCl}– volume of HCl consumed during titration, mL; V_{flask}– volume of the volumetric flask, mL; V_{aliq}– volume of sample solution used in titration, mL.

Example $n_{NaOH} = \frac{0.1 \frac{mol}{L} * 4.02\ mL * 50\ mL}{10\ mL} = 2.01\ mmol$

$$IEC = \frac{n_{NaOH} - n_{eluted}}{m_{polym}} \quad (2)$$

Where IEC– ion exchange capacity, meq/g; n_{NaOH}– amount of NaOH in a NaOH solution volumetric flask, mmol; n_{eluted}– amount of NaOH in the eluted solution volumetric flask, mmol; m_{polym}– mass of the polymer in the column, g.

Example $IEC = \frac{2.01\ mmol - 0.13\ mmol}{1.0\ g} = 1.88\ meq/g$

8.5.2 Strong ion exchanger- silica supported sulphonic acid

Ion exchange on functionalised silica gel was done the following way. Precisely about 1 g of silicagel was weighed in washed and dried SPE cartridge. It was conditioned by washing with MilliQ water, 1 M HCl and water again until neutral reaction. Then 0.1 M NaCl solution was applied to the cartridge and acidic reaction was observed. Eluted solution was collected in 10 mL volumetric flask until neutral reaction and filled up with MilliQ water till the mark. Afterwards silica gel was washed with 1 M HCl and water until neutral reaction. Then again 0.1 M NaCl solution was applied to check, whether ion exchange capacity remains the same in second and third elution. The eluent was collected as before two more times with thorough washing afterwards.

For titrations burette was filled with standardized 0.1 M NaOH solution. Sample solution was prepared in erlenmeyer flask and consisted of 3.0 mL of eluted sample, about 50 mL of water and couple of drops of phenolphthalein solution. Solution was titrated by NaOH solution until stable pink colouring. The consumed volume of NaOH solution was written down and calculations were done. Titration was repeated three times for every eluted sample.

8.6 Particle size distribution

Spherical particles with various diameters were obtained by suspension polymerization. Wet sieving was used to separate fractions of different particle size. Dried polymer was weighed and quantitatively transferred into an erlenmeyer flask. Then acetone was added to the polymer and suspension was sonicated for 5 minutes to introduce the solvent to the pores. Polymer was allowed to swell for an hour to avoid swelling during the sieving.

Suspension was sieved through 100 µm and 250 µm sieves by applying acetone. Particle sample from the largest and middle fraction were observed in a microscope to estimate the particle size. If smaller particles were still present, sieving was continued. Three fractions (<100 µm; 100 µm - 250 µm; >250 µm) were collected in weighed vials and dried overnight. Afterwards polymer fractions were weighed and a particle distribution plot was created.

8.7 Amine binding experiments

8.7.1 Amine analysis by HPLC

Primary (3-phenyl propylamine) (PA) and tertiary (N,N-Dimethylbenzylamine) amine (TA) solutions were analysed for the linearity estimation. All HPLC separations were performed on a Zorbax C8 (150 x 4 mm i.d., 5μm) column. The HPLC analysis conditions were: isocratic run, mobile phase – ACN/water/TMA/TFA, 20/80/0.1/0.1 (V/V), degassed by sonication, 0.5 μL injection, room temperature (23°C), flow rate – 1 mL/min, 10 min per run, 3 repetitions of every standard solution, washing between runs with ACN. Obtained chromatograms were analysed and analyte peaks were integrated. Statistical evaluation of the peak areas was done. To observe the linearity region of the amine analysis the peak areas were plotted against the amine concentration and linear fit was made.

8.7.2 Static binding (Isotherms)

Seven PA and TA methanol solutions were prepared as calibration solutions. PA solutions were made in the concentration range from 0.17 – 10.20 mg/mL. TA solutions were made in the concentration range from 0.15 – 12.07 mg/mL.

Procedure of the static amine binding experiments was performed as follows. First, 100 mg of the adsorbent were weighed in sealable test tubes. Then, 2.5 mL of each of the amine calibration solutions were pipetted in the test tubes and placed in a shaker to equilibrate for two to three hours. Afterwards supernatants of the adsorbents and calibration solutions were transferred to the HPLC vials and analysed by HPLC.

Parameters of HPLC runs were: mobile phase – ACN/water/TMA/TFA, 20/80/0.1/0.1 (V/V), isocratic elution mode, injection volume – 1.0 μL, flow rate – 1 mL/min, room temperature (23°C), 6 min per run, detection wavelengths 210 nm and 254 nm. Obtained chromatograms were analysed and integrated peak areas were written down. Calibration curves were made by plotting amine peak areas against concentrations. Amine binding was calculated as the difference between offered amount of amine and remaining after the binding. Isotherms were made by plotting remaining amount of amine against the bound.

8.7.3 Dynamic binding (break-through curves)

Amine break-through of the adsorbent was estimated by applying amine solution to the adsorbent and monitoring the appearance of the amine in the effluent. Adsorbents used were DVB-$(MAA)_2O$ polymer and sulphonated silica gel ($Si\text{-}SO_3H$). PA and TA solutions were used. Concentrations of both solutions were 0.025 M.

First, 1.00 g of the adsorbent was placed in a solid phase extraction (SPE) cartridge. Adsorbent was protonated by applying mixture of 1 M HCl and MeOH. Then, adsorbent was washed neutral with MeOH. Amine solution in portions was applied to the adsorbent without stirring the adsorbent bed. The flow rate was kept at 1.0 mL/min. Effluent fractions were collected in volumetric flasks. The experimental setup can be seen in **Figure 8**.

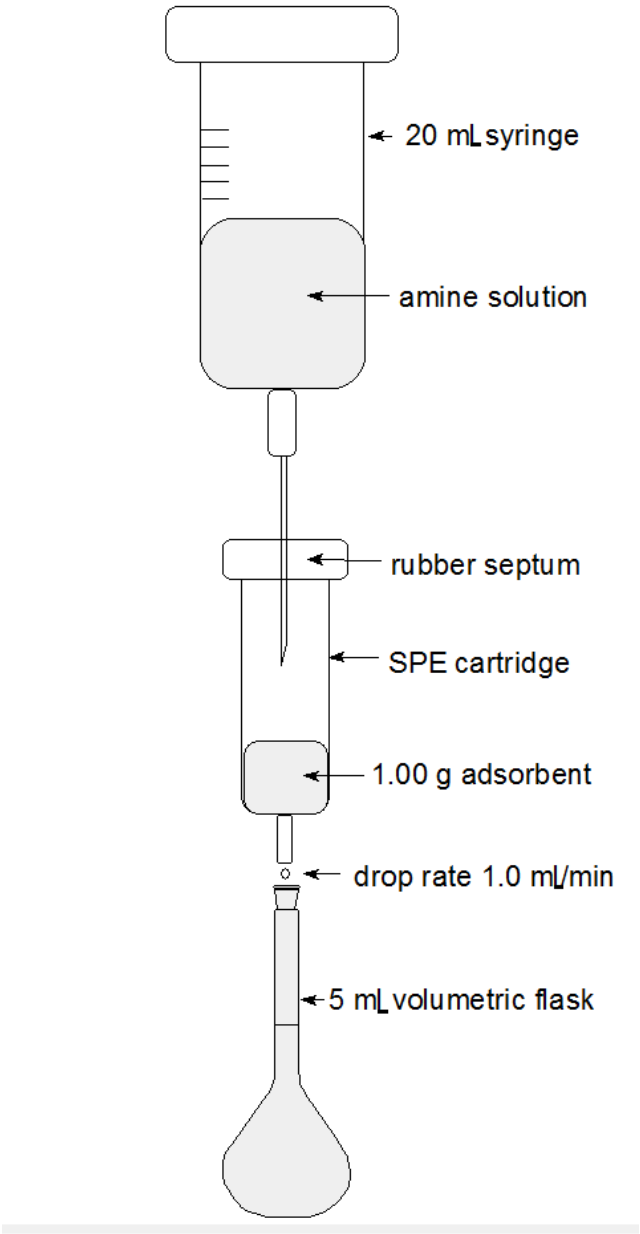

Figure 8. Schematic representation of the setup for dynamic binding experiments.

Fraction samples were transferred to HPLC vials. If the estimated fraction sample concentrations were too high, samples were diluted by MeOH. Samples were analysed by HPLC. The HPLC run parameters were the same as in static binding experiments: mobile phase – ACN/water/TMA/TFA, 20/80/0.1/0.1 (V/V), isocratic elution mode, injection volume – 1.0 µL, flow rate – 1 mL/min, room temperature (23°C), 6 min per run, injection 3 times, detection wavelengths 210 nm and 254 nm. Obtained chromatograms were analysed and integrated peak areas were written down.

Calibration curves were made by plotting amine peak areas against concentrations. Break-through curves were made by plotting the break-through percentage against the amount of amine offered to the 1 g of polymer. Break-through percentage was calculated from the areas of the amine peaks. Each fraction area was divided by the corrected peak area of the calibration solution that was offered to the polymer.

8.8 Amine derivatization

Following solutions were prepared for derivatization: 10 mM Fmoc-Cl in ACN, 60 mM $NaHCO_3$ in water, ISTD 10mM methyl benzyl cyanide in ACN, 100 mM Glycine in water, 100 mM TRIS in water. Derivatized amine solutions: Primary amine, Dimethylamine (DMA) 15 mM in water, Ethylamine (EA) 15 mM in water, Methylamine (MA) 15 mM in water, Diethylamine (DEA) 15 mM in ACN, Isobutylamine 15 mM in ACN, Trimethylamine TMA, 15 mM in water, Tertiary amine (N-N-dimethylbenzylamine) 15 mM in ACN.

Derivatization was done in HPLC vials by pipetting together 100 µL amine solution, 500 µL Fmoc-Cl solution and 300 µL $NaHCO_3$ solution. Mixture was shaken and analysed by HPLC according to parameters in **Table 11**. Kinetics of derivatization reaction was studied and results showed the reaction is almost immediate. Solutions were analysed separately to establish the retention times.

Table 11. Parameters of HPLC runs for derivatization experiments.

Parameters	**Conditions**
Mobile phase A	ACN:water: TFA, 20 : 80 :0.2 (V:V)
Mobile phase B	ACN: 0.2 % TFA (V:V)
Mobile phase for isocratic runs	MeOH/ACN/water/TFA/TEA, 350/400/250/0.3/0.3, (V/V)
Elution mode	Isocratic or Gradient
Injection volume	1.0 µL
Needlewash	MeOH
Flowrate	1 mL/min
Temperature	23°C
Wavelength	210, 254nm
Run time for isocratic runs	50 min

8.9 Imine formation experiments

Kinetics of imine formation from PA and acetone was investigated by HPLC. Methanol and acetonitrile were used as solvents to mimic different reaction conditions. Impact of water and acid addition to the reaction mixture was studied.

8.9.1 Imine formation kinetics in MeOH

Investigation of imine formation kinetics in methanol was performed as follows. A series of solutions was prepared: 17.1 mM PA solution in MeOH; 1710 mM acetone solution in MeOH; 171 mM acetone solution in MeOH; 17.1 mM acetone solution in MeOH. Reaction mixtures were prepared in 10 mL volumetric flasks by transferring 5 mL of 17.1 mM PA solution and adding 5 mL of one of the acetone solutions. Reference solution was prepared the same way but adding MeOH instead of acetone solution.

Reaction mixtures were transferred to HPLC vials and analysed right away (injection done 2 - 3 min after mixing). The same mixture was consequently injected and analysed for about 20 hours. Obtained areas of amine and imine peaks were integrated and plotted against time to obtain kinetic plot. Parameters of HPLC runs were: mobile phase – ACN/water/TFA, 20/80/0.2 (V/V), isocratic elution mode, injection volume – 3.0 µL, flow rate – 1 mL/min, stop time – 12 min, needle wash with MeOH for 1 min, room temperature (23°C).

Kinetic experiments in MeOH in presence of acid and water

To investigate the impact on kinetics of imine formation in presence of trifluoroacetic acid (TFA) the following actions were performed. Experiment was done by adding 10-fold excess of TFA in terms of primary amine to the reaction mixture. Reaction mixture consisted of 5 mL 17.1 mM PA in MeOH, 65 µL TFA and 4.935 mL neat acetone. Control sample was prepared the same way but methanol was added instead of acetone.

Investigation of the kinetics of imine formation in methanol in presence of TFA and water was done the same way as with kinetic experiments in presence of just TFA. Reaction mixture consisted of 5 mL 17.1 mM PA in MeOH, 65 µL TFA, 1 mL water and 3.935 mL neat acetone. Control sample was prepared the same way but methanol was added instead of acetone.

Reaction mixtures were transferred to HPLC vials and analysed right away (injection done 2-3 min after mixing). Analysis conditions were the same as for kinetic experiments in MeOH without TFA. Areas of amine and imine peaks were integrated and plotted against the time to obtain the kinetic plot.

8.9.2 Imine formation kinetics in ACN

To investigate the kinetics of imine formation in acetonitrile (ACN) the following actions were performed. A series of solutions was prepared: 16.9 mM PA in ACN; 1680 mM Acetone solution in ACN; 168 mM acetone solution in ACN; 16.8 mM acetone solution in ACN. Series of reaction mixture solutions were prepared in 10 mL volumetric flasks by transferring 5 mL of 16.9 mM primary amine solution and adding 5 mL of ACN or acetone solution.

Reaction mixtures were transferred to HPLC vials and analysed repeatedly for 24 hours. Parameters of HPLC runs were: mobile phase – ACN/water/TFA, 20/80/0.2 (V/V), isocratic elution mode, injection volume – 3.0 µL, flow rate – 1 mL/min, stop time – 12 min, needle wash with MeOH for 1 min, room temperature (23°C). Areas of amine and imine peaks were integrated and plotted against time to obtain kinetic plot.

9 Results and discussion

9.1 Preparation of adsorbing media

In order to be favourable to use adsorbing media have to possess a number of specific qualities. Adsorbents have to have a high surface area to make it possible for a higher amount of analyte to adsorb. This can be achieved by increasing the porosity of the material. Adsorbents also have to be adjusted based on the type of analyte of interest. In this case the focus was on adsorbing basic amines. Thus acidic group incorporation in the material can be favourable.

9.1.1 Bulk polymerisation

At first bulk polymerisation was chosen as the method for preparation of polymers because of several reasons. The main point is the ease of preparation. In bulk polymerisation monomer mixture is placed in a vessel and left for polymerisation. This provides high monomer incorporation rates up to 90 – 100 %. Another important aspect is the ease of material porosity control. The amount of porogen in the monomer mixture can be increased easily which in turn will increase the porosity.

Unfortunately bulk polymerisation possesses numerous drawbacks, therefore it was decided to change the strategy of polymer preparation. The main disadvantage was the poor mechanical stability of the ready polymer. This caused difficulties in handling the material. It had to be ground in a mortar and sieved to obtain particles in the desired size range (100 – 250 µm). Due to their electrostatic behaviour both actions were problematic. Aggregation of the particles was observed so it was problematic to separate particles in the desired size range. Because of the poor mechanical stability polymers were fragmenting severely. This in turn led to low yields of the particles in the desired size range.

9.1.2 Suspension polymerisation

The more successful approach for polymeric adsorbent preparation was suspension polymerisation. It has numerous benefits compared to bulk polymerisation. First is the high mechanical stability of the polymer caused by the spherical shape of the particles. For packed bed adsorbents spherical is the most favourable shape for particles. This is due to the closest packing and stability of the particles. The size of the spherical particles can be altered by controlling suspension polymerisation parameters. Irregular shape particles will not provide the closest packing. They can eventually dislocate from the initial place thus increasing the void volumes. Also fragmentation of the less rigid particles can cause blockage of the system.

The procedure of adsorbent preparation by suspension polymerisation was adopted from Saito et al [108]. Two types of polymers were prepared inspired by this procedure: ones containing ethylene dimethacrylate (EDMA) and others containing divinylbenzene (DVB). EDMA polymers containing methacrylic acid (MAA) were prepared directly how described by Saito et al [108]. For the EDMA – butyl methacrylate (BMA) – acrylic acid (AA), the monomer mixture composition as adapted from Fekete et al [111]. Preparation of EDMA polymers by this method was successful.

The DVB polymer preparation created several issues. Composition of the monomer mixture for DVB – MAA polymer preparation was inspired by Bratkowska et al [109] and the DVB – styrene – MAA by Svobodova et al [110]. The problems related to this approach were caused by the preference of MAA to remain in the aqueous continuous phase. The polymerisation of MAA begun already in the continuous phase by producing fine polymeric mass without forming spherical particles. This led to low incorporation of MAA into polymers. Isolation of the formed material was difficult due to its overly fine nature. Thus a new approach was chosen for the DVB polymers.

The use of MAA had to be substituted for DVB polymers. This was done by utilisation of methacrylic anhydride ($(MAA)_2O$). The new approach led to better incorporation of the anhydride. This was investigated by infra-red spectroscopy (ATR-FTIR) in chapter 9.2.1. The amount of anhydride used was varied to obtain the highest incorporation rate. Since monomer mixture was spending less time in the aqueous continuous phase, polymer particles were formed

more readily. Formed spherical particles were relatively easy to isolate after the reaction because the major fraction was in the size range larger than 100 µm in diameter.

The free acid groups on the polymer were obtained by hydrolysis of the incorporated anhydride functional groups. Hydrolysis was performed by placing the polymer particles in a 3 M NaOH/water solution and heating the mixture at 70°C for 20 hours. It was observed in the microscope that because of the milling effect the mechanical stirring caused fragmentation of the particles, thus rotational stirring was used instead. After the hydrolysis polymer obtained slightly yellow colour. The completion of the hydrolysis reaction was checked by determining the ion exchange capacity (IEC) of the polymer (chapter 9.2.3.). When IEC did not change after additional hydrolysis for 24h at 75°C, it was concluded that the former hydrolysis conditions are sufficient to complete the reaction.

9.1.3 Silica supported sulphonic acid

Silica gel was chosen as an adsorbent for numerous reasons. First, it is robust and does not swell in solvents. These factors are important when material is packed and high pressures of different solvents are applied. Second, it has established surface chemistry and high surface area. Third, it has controlled porosity. The downside of the silica supported sulphonic acid is that it possesses self-hydrolysis in water. Thus the estimated ion exchange capacity will decrease over time. Self-hydrolysis is also undesirable because the removed ligands will appear as material bleeding and contaminate the sample.

Particles in the desired size range (100 – 250 µm) were obtained by sieving the irregular shape silica gel from commercial material prior to reaction. Mercaptopropyl-silica (SH-Silica) was obtained by reaction of silica with 3-mercaptopropyl trimethoxy silane. Due to the use of magnetic stirring a lot of fine particles were created. Oxidation of the mercaptopropyl silica to SO_3H-Silica was done by hydrogen peroxide. Overall the preparation of silica supported sulphonic acid was highly laborious.

9.2 Characterization of adsorbing media

A number of parameters of the prepared adsorbing media were characterised by using several analytical methods. Infra-red (ATR-FTIR) spectroscopy was used to estimate the composition of the polymers. Titrations were used to determine the ion exchange capacity (IEC). Also the particle size distribution was estimated.

9.2.1 Polymer composition estimation by ATR-FTIR

Calibration in ATR-FTIR spectroscopy was done to estimate the composition of the polymers. Several assumptions were made to utilize this concept. First, polymers act the same as the liquid monomer mixture. Second, there is no reaction between the monomers. Third, only monomers are present in the mixture.

Pure monomer spectra were observed to locate characteristic peaks for comparison of the ratios. For DVB it was a peak at 706.7 cm^{-1}, for anhydride 1778,5 cm^{-1} and for MAA 1689.3 cm^{-1} and 1632 cm^{-1}. The two MAA peaks were used to compare which gives higher linearity. The 1689.3 cm^{-1} turned out to give higher linearity. Intensities of these peaks were measured in the monomer mixture spectra and the ratio between them was calculated. The ATR-FTIR spectra of the monomers and polymers can be found in the Appendix.

Ratios of anhydride vs DVB were plotted against the percentage of anhydride in the mixture to obtain the calibration curve for DVB-$(MAA)_2O$ polymer (**Figure 9**). The calibration curve of DVB-MAA polymer can be seen in **Figure 10** where ratios of MAA vs DVB were plotted against the percentage of MAA in the polymer. Linear equation from the calibration curve was used to calculate the percentage of anhydride or MAA in the polymer as showed in the following example.

Example. (Anhydryde) / (DVB) = 39 mm /37 mm = 1.054

W_{anh} = ((1.054 – 0.0608) / 3.2324) * 100 % = 30.7 % (DVB, Anh 50%)

(Anhydryde) / (DVB) = 38 mm / 49 mm = 0.776

W_{anh} = ((0.776 – 0.0608) / 3.2324) * 100 % = 22.1 % (DVB, Anh 33%)

Here it was observed that the higher the percentage of anhydride was in the monomer mixture, the higher the anhydride incorporation percentage. For DVB-$(MAA)_2O$ polymer with anhydride content 33 % in the monomer mixture, 22.1 % of anhydride was found in the polymer. While for 50 % content in the monomer mixture – 30.7 % were found.

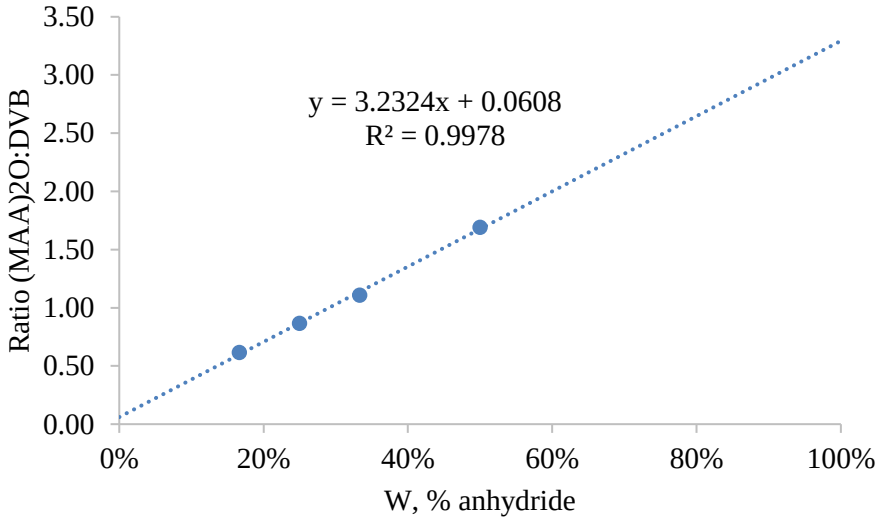

Figure 9. Calibration curve for ratios of the monomer mixtures, peak intensities vs anhydride percentage.

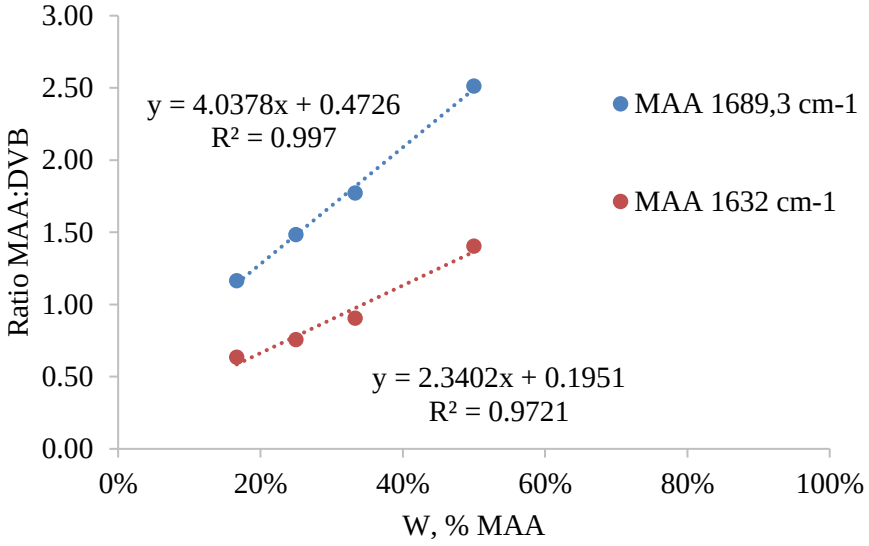

Figure 10. Calibration curve for ratios of the monomer mixtures, peak intensities vs MAA percentage.

9.2.2 Particle size distribution

Suspension polymerization produces spherical particles with various diameters. Classification of the particle size is needed because it is a measure of repeatability of polymerization. Desired particle size range was 100 – 250 µm in diameter. Polymerisation parameters were adjusted in such way that the largest fraction (over 50 %) would be within the desired particle size range. Parameters affecting the particle size the most were stirring rate and the amount of dispergent. The size of the particles can be increased by decreasing the stirring rate and the amount of dispergent. Fractions of different particle size were separated by wet sieving.

Graphical display of the particle size distribution can be seen in **Figure 11**. The desired particle size (100 – 250 µm) fraction is larger than 50 % for all the polymers with the highest for DVB-anhydride polymer – 69.4 %. Fractions with particle size larger than 250 and smaller than 100 are roughly equal around 20 %.

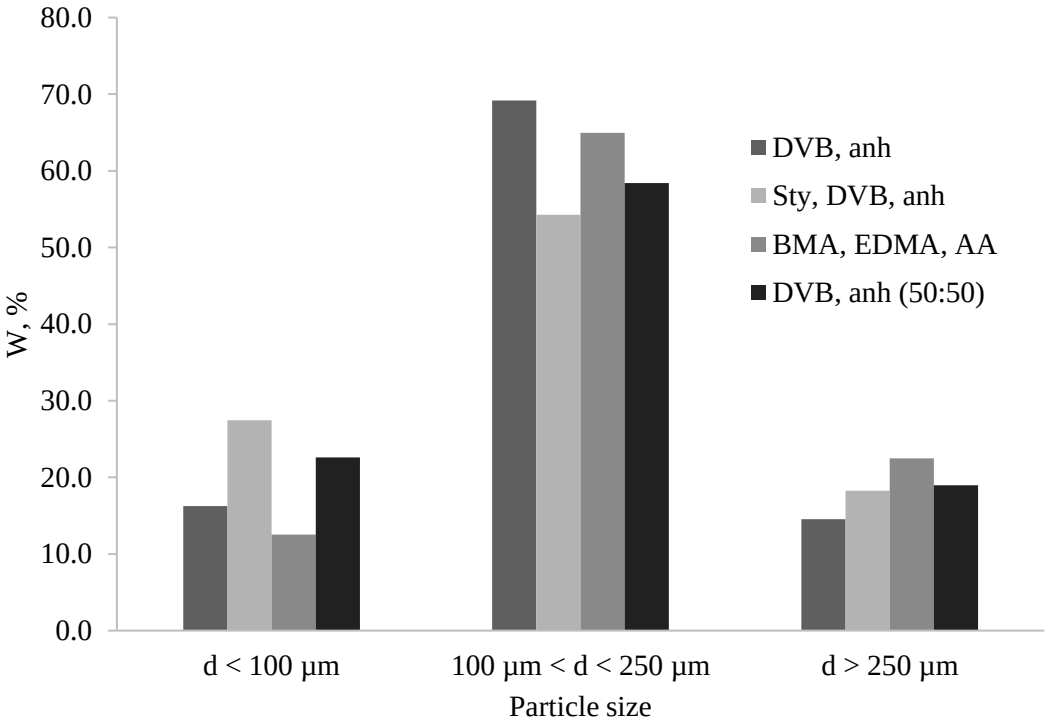

Figure 11. Particle size distribution of polymers.

9.2.3 Ion exchange capacity

Ion exchange capacity (IEC) is a measure of material ability to exchange counter-ion equivalents per specified quantity of the material[113]. The unit used in this work is milliequivalents exchanged per gram of dry material (meq/g). The higher the value, the more ions the material can exchange.

IEC was determined by titrations for all the adsorbents. The ion exchange capacities of the polymers and SO_3H-Silica are collected in **Table 12**. Average value of IEC of the two elutions was calculated. Elutions were done two times to check whether the material is capable of recovering after the ion exchange was performed. If the values stay consistent, the material is suitable for repeated ion exchange.

Table 12. Ion exchange capacities of 100 – 250 µm particle size polymers and SO_3H-Silica.

Adsorbent	IEC, meq/g, 1. elution	IEC, meq/g, 2. elution	IEC, meq/g, average
EDMA-BMA-AA	1.28	1.38	1.33
EDMA-MAA	0.92	0.72	0.82
DVB-$(MAA)_2O$	2.79	2.84	2.82
DVB-$(MAA)_2O$ (50%)	4.66	4.92	4.79
Sty-DVB-$(MAA)_2O$	2.37	2.29	2.33
SO_3H-Silica	0.39	0.38	0.38

Calculated ion exchange capacity of the strong ion exchanger – functionalized silica gel was found about 0.4 milliequivalents to 1 g of dry silica gel. The results stay consistent also in second and third elution. This IEC value is significantly lower than those of the weak ion exchangers – polymers.

For polymers the highest IEC values were observed for DVB polymers. Especially for DVB-$(MAA)_2O$ polymer with original anhydride content 50 % (4.79 meq/g). IEC of EDMA polymers were slightly lower – 0.82 – 1.33 meq/g. The IEC stays relatively consistent for all the polymers during first and second elution. The reason why observed IEC values are lower than the expected ones could be the incomplete incorporation of acid or anhydride in the polymer.

9.3 Amine binding studies

9.3.1 Amine analysis by HPLC

Prior to beginning the analysis on high performance liquid chromatography (HPLC) instrument its performance needed to be checked. Since most of the following analyses were done using primary amine (3-phenyl-propylamine) and tertiary amine (N,N-Dimethyl-3-phenyl-propylamine) solutions, the linearity range of these amines was investigated. Statistical evaluation of the peak areas was done and can be seen in **Table 13** for the primary amine and in **Table 14** for the tertiary. The plot of the peak areas versus the amine concentration for the primary amine can be seen in **Figure 12** and for tertiary in **Figure 13**.

Table 13. Statistical evaluation of the primary amine analysis.

Primary Amine						
c, mg/mL	0.019	0.082	0.408	1.037	1.657	2.051
Area, 1	22.3551	114.803	618.212	1495.95	2678.24	2749.08
Area, 2	28.3574	118.561	613.174	1550.16	2483.7	3057.37
Area, 3		112.794	601.398	1583.73	2386.51	3144.5
Average	25.36	115.39	610.93	1543.28	2516.15	2983.65
SD	4.24	2.93	8.63	44.29	148.55	207.76
RSD, %	16.74	2.54	1.41	2.87	5.90	6.96
CI	4.80	3.31	9.76	50.12	168.10	235.11

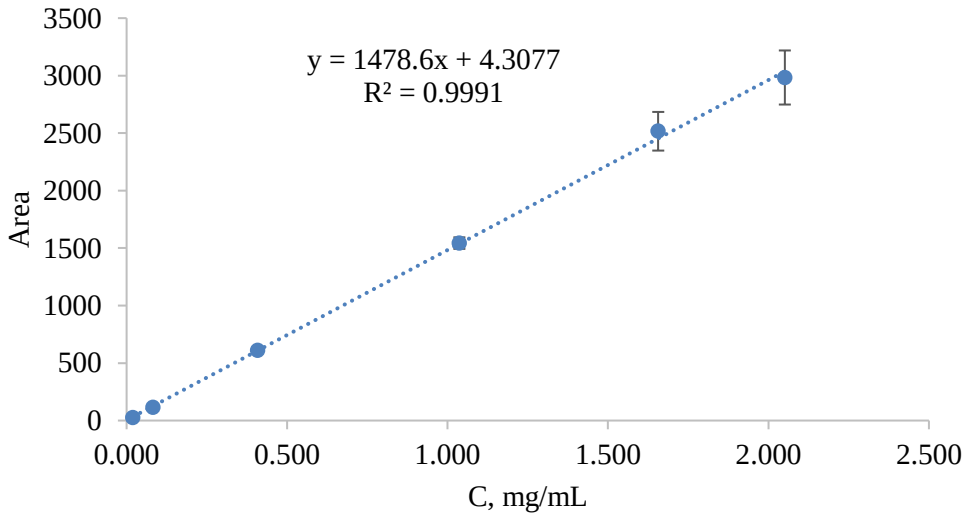

Figure 12. Linearity region in HPLC for the primary amine.

Table 14. Statistical evaluation of the tertiary amine analysis.

Tertiary Amine						
c, mg/mL	0.02	0.081	0.419	1.043	1.665	2.087
Area, 1	34.4354	127.063	608.8	1649.96	2548.71	3105.54
Area, 2	31.5854	130.028	643.498	1619.89	2588.77	3136.16
Area, 3	32.191	128.156	632.895	1473.9	2549.84	3288.57
Average	32.74	128.42	628.40	1581.25	2562.44	3176.76
SD	1.50	1.50	17.78	94.18	22.81	98.04
RSD, %	4.59	1.17	2.83	5.96	0.89	3.09
CI	1.70	1.70	20.12	106.57	25.81	110.94

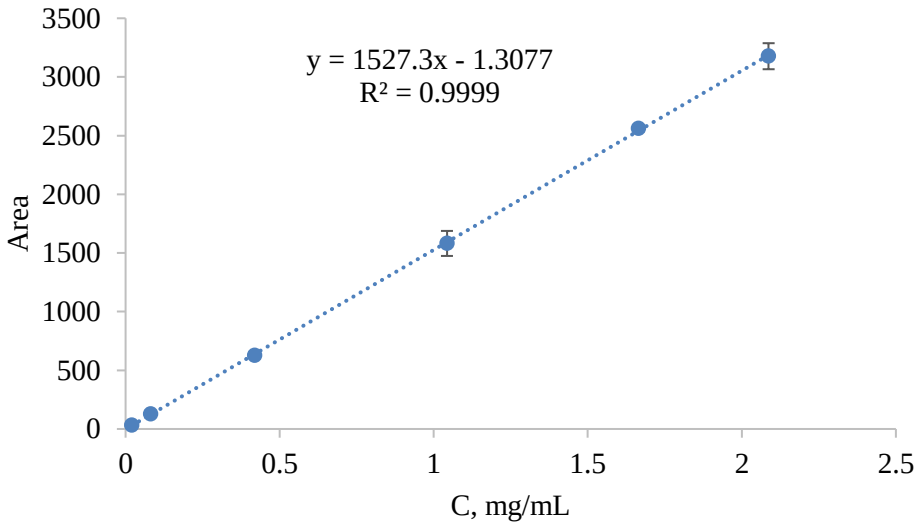

Figure 13. Linearity region in HPLC for the tertiary amine.

It can be seen from the linearity plots in **figures 12** and **13** that for both amines the concentration range is linear over two orders of magnitude (0.02 – 2.00 mg/mL). The coefficient of determination is higher than 0.999 for both amines which is a good measure of linearity. The measurements are relatively precise as the relative standard deviation (RSD) is lower than 5 % for most cases of three peaks of subsequent analyses of the same sample.

9.3.2 Static binding (isotherms)

9.3.2.1 Silica functionalised with sulphonic acid groups

Sulphonated silica gel demonstrates not only specific but also non-specific binding ability. Ideally material would adsorb only at the functional group binding sites. In practice this is not true since the backbone of the material possesses a binding ability too. In case of sulphonated silica gel it was possible to observe the non-specific adsorption on the material by investigating the amine binding on silica gel and Si-SO_3H precursor mercaptopropyl silica (SH-silica). Specific and non-specific binding of amines on the silica based materials can be seen from the isotherms (**Figure 14** for primary amine and **Figure 15** for tertiary).

Binding capacity of the sulphonated silica was as high as 22-99% of the solution concentrations offered to the material. Non-specific binding of amines was seen on the SH-silica and silica gel. Binding capacity of the SH-silica was very low compared to the sulphonated silica. It was in the range of 5-57%. The silica gel binding capacity was even lower for tertiary amine (8-39%), but higher for the primary amine (21-72%).

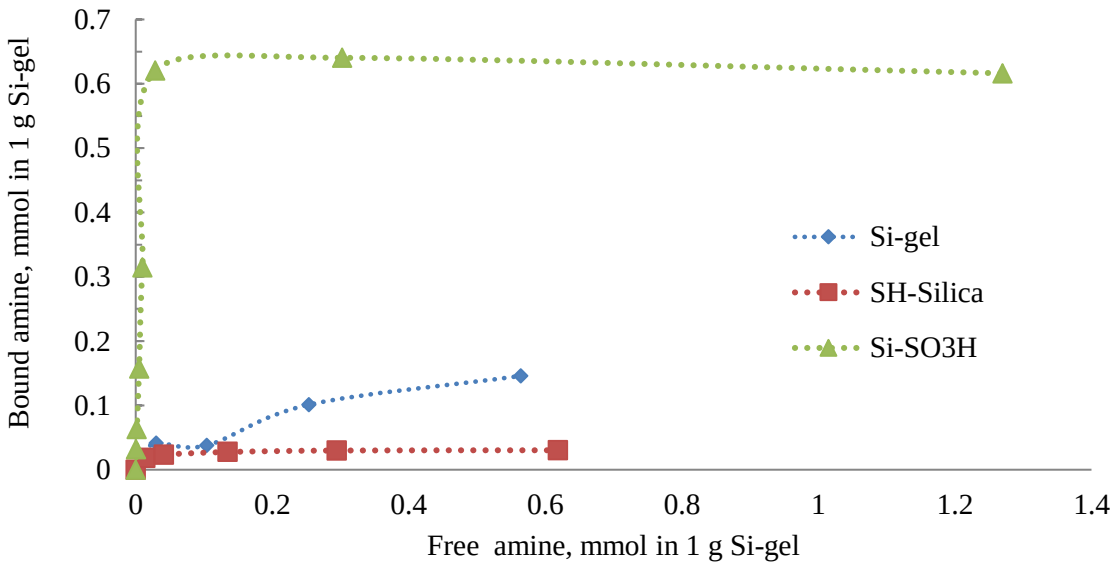

Figure 14. Primary amine isotherms for Si-SO_3H, SH-Silica and Silica gel.

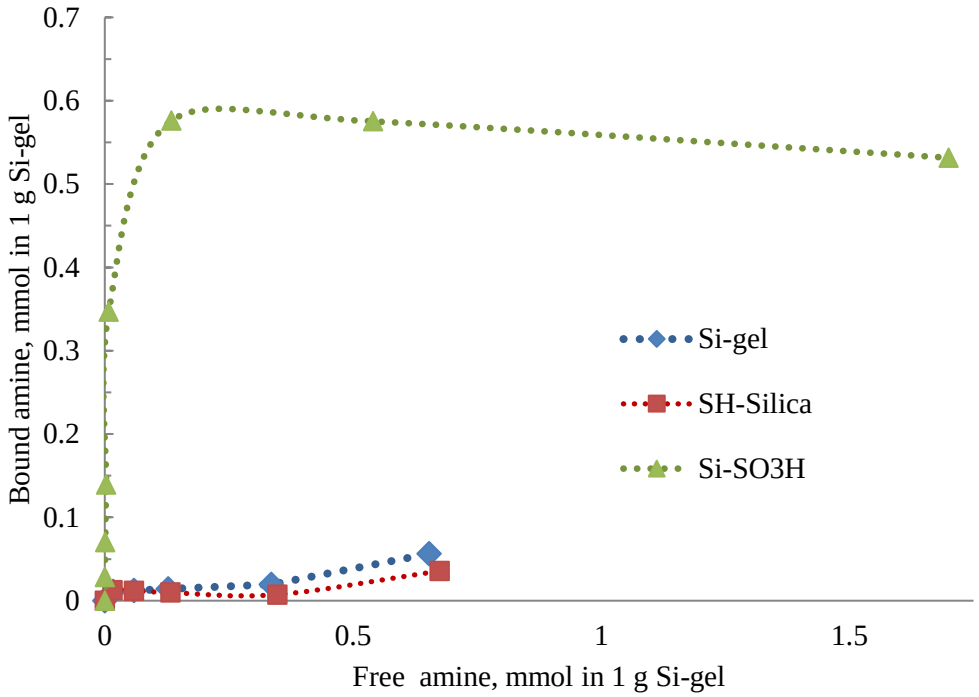

Figure 15. Tertiary amine isotherms for Si-SO_3H, SH-Silica and Silica gel.

After longer incubation periods (6 h, 18 h) the binding capacity of the sulphonated silica started to decrease. This could be explained by the loss of ligands from the material due to self-hydrolysis. Thus in actual applications it would be better to use shorter sampling times to avoid losses in capacity.

The saturation of the sulphonated silica occurs at about 0.63 mmol primary amine and 0.58 mmol tertiary amine per 1 g of material. These values correlate with the dynamic binding experiment results and the ion exchange capacity. It can be concluded from the equilibrium of the static binding experiments that sulphonated silica material has a sufficient amine binding capacity. The non-specific binding capacity arising from the parent material is minor compared to the total binding capacity.

9.3.2.2 Polymeric adsorbents

Polymer static binding capacity was determined by experiments of primary and tertiary amines with HPLC. The tertiary amine experiments were successful and provided consistent results. Obtained isotherms can be seen in **Figure 16**. The static binding capacities of the materials are noted in the paragraph under the isotherm plot.

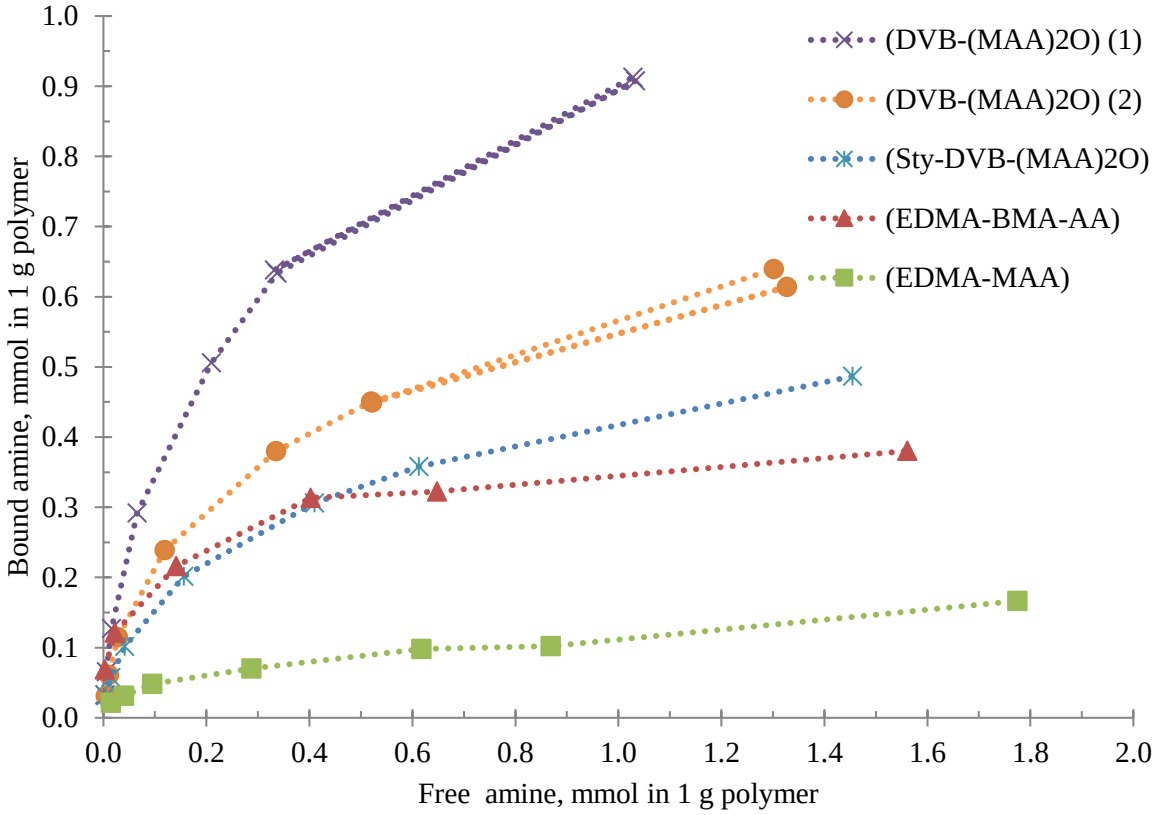

Figure 16. Tertiary amine isotherms for polymers.

The results of tertiary amine saturation capacity of the polymers correlate with the IEC determined by titrations. Like in the IEC data the DVB polymers have the highest static binding capacities and EDMA polymers have lower ones. The DVB-$(MAA)_2O$ (50%) has the highest capacity of over 0.95 meq/g, followed by DVB-$(MAA)_2O$ of 0.65 meq/g and Sty-DVB-$(MAA)_2O$ of 0.5 meq/g. EDMA-BMA-AA polymer has the capacity of 0.37 meq/g and EDMA-MAA of 0.2 meq/g.

All of the static binding capacity values for polymeric adsorbents show significantly lower values than IEC determined by titrations. IEC for the DVB polymers are in the range from 2.33 – 4.79 meq/g which is about five times higher than the dynamic binding capacity. For EDMA polymers the IEC values are 0.82 – 1.33 meq/g, four times higher than dynamic binding capacity.

While making the static binding experiments with primary amine a specific chromatographic pattern was observed. A second peak next to the amine peak appeared with intermediate peak in between. In additional experiments it was found that the extra peak was imine and intermediate peak was imine undergoing exchange to amine during the analysis. Imine formation occurred due to the residual acetone presence in pores of the polymers. The imine formation on materials was investigated in more detail and results can be found in chapter 9.5.

Since imine formation was present in analysis of primary amines for the static binding capacity determination experiments, the obtained amine concentrations in sample solutions were smaller than the actual ones. Hence the results of these experiments had to be rejected. The primary amine experiments have to be repeated in complete absence of acetone to obtain the actual primary amine static binding capacity of polymers.

9.3.3 Dynamic binding (break-through curves)

Dynamic binding experiments were carried out for the most promising materials with the highest ion exchange capacities (IEC) of each group. From weak ion exchangers it was the DVB-$(MAA)_2O$ polymer (IEC 4.6 - 4.9 meq/g) and from strong ion exchangers the sulphonated silica gel ($Si\text{-}SO_3H$) (IEC 0.8 meq/g). Both primary and tertiary amine solutions were used to test the break-through of the materials. The goal of the dynamic binding experiments was to determine the amount of amine one gram of adsorbent can adsorb from a dynamic flow before 10 % break-through occurs – 10 % of the initial amine concentration is found in the effluent. The value at 10 % break-through is considered the dynamic binding capacity.

9.3.3.1 Weak ion exchanger - DVB-$(MAA)_2O$

Several experiments of dynamic adsorption for the high capacity DVB-$(MAA)_2O$ polymer were carried out. Various amine solution concentrations and portion volumes were used to optimize the appearance of the break-through curve. The most successful case was to use 0.025 M amine solution and 5 mL fraction volumes as well as keep the elution flow rate at 1,0 mL/min.

Primary amine break-through curve for the polymer is shown in **Figure 17** as the blue dotted line with the square markers. Tertiary amine break-through curve is shown in **Figure 17** as the red dotted line with the triangle markers. It can be seen that the primary amine 10 % break-through occurs at about 2.0 mmol of amine for 1 g of the polymer. For the tertiary amine the 10 % break-through occurs at about 0.8 mmol of amine for 1 g of the polymer.

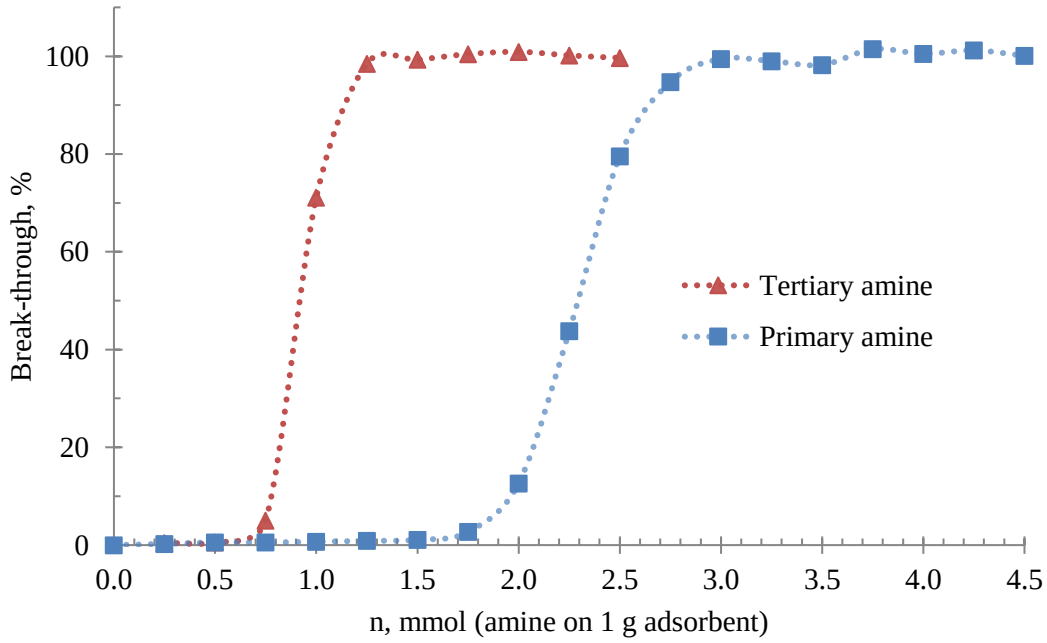

Figure 17. Primary and tertiary amine break-through curves for the DVB-$(MAA)_2O$ polymer.

The break-through experiments show that the dynamic capacity of the polymer is lower than static binding capacity value (0.95 meq/g) for tertiary amine. Dynamic binding capacity is lower than ion exchange capacity determined by titrations (4.79 meq/g) for both primary and tertiary amines. Primary amine shows higher binding and thus more of the amine can be introduced to the polymer before the 10 % break-through occurs. For primary amine the dynamic capacity is approximately two times lower than static ion exchange capacity while for the tertiary amine it is five times lower.

9.3.3.2 Strong ion exchanger – sulphonated silica gel (Si-SO_3H)

The experimental setup used for the sulphonated silica gel was the same as optimized for the polymeric adsorbent. Primary amine break-through curve is shown in **Figure 18** as the blue dotted line with the square markers. Tertiary amine break-through curve is shown in **Figure 18** as the red dotted line with the triangle markers.

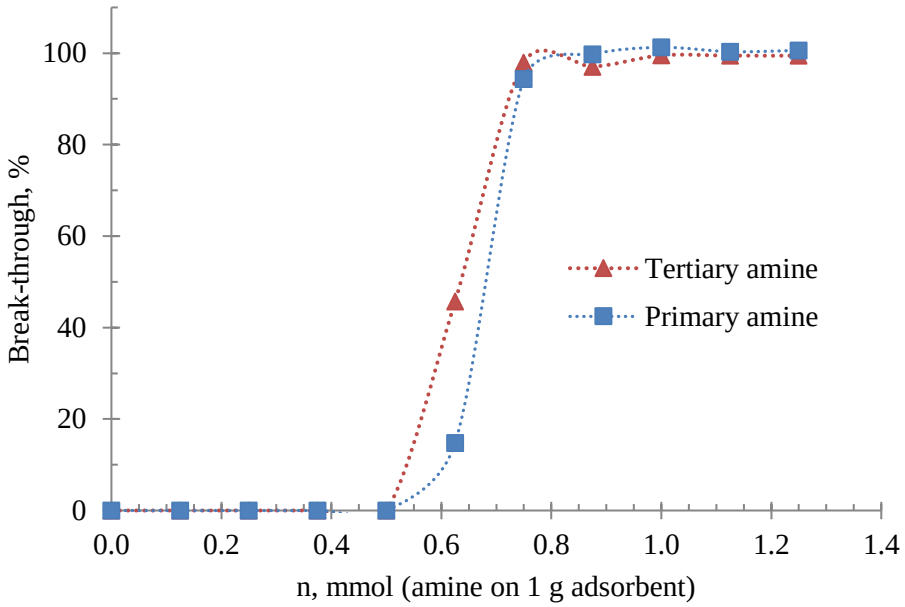

Figure 18. Primary and tertiary amine break-through curves for the high capacity Si-SO_3H adsorbent.

It can be seen from the break-through curves that the profiles of both curves are virtually the same. The 10 % break-through occurs at 0.6 mmol of primary amine and at 0.55 mmol of tertiary amine for 1 g of the polymer. The ion exchange capacity determined by titrations for both amines was 0.8 meq/g, the static binding capacity for primary amine was 0.63 mmol/g and for tertiary amine 0.58 mmol/g. It can be concluded from the break-through experiments that while the dynamic capacity of the sulphonated silica gel adsorbent is lower by 0.2 mmol/g than ion exchange capacity, it is essentially the same as static binding capacity value for both primary and tertiary amines.

9.4 Derivatization of amines for HPLC analysis

Since atmospheric amines mostly do not contain any chromophores, they need to be derivatized in order to analyze with HPLC-UV. Derivatization of amines was performed by Fmoc-Cl in presence of $NaHCO_3$ to neutralize the forming HCl. Since leftover Fmoc-Cl peak is overlapping with some amine peaks, it needed to be removed. Quenching of leftover Fmoc-Cl was performed by excess (10-fold in molarity) of glycine or TRIS solutions. Drawback of TRIS is it might not be stable enough. Glycine on the other hand forms quenching product that is overlapping with the internal standard. A chromatogram of 12 amine mixture derivatives can be seen in **Figure 19**.

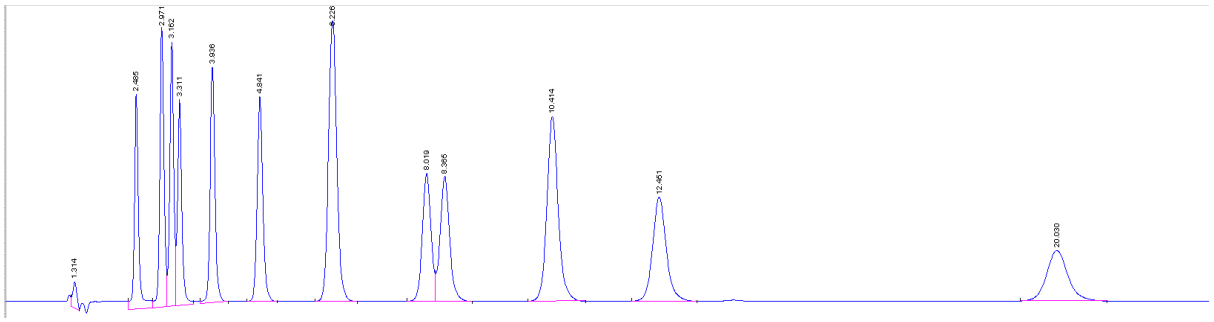

Figure 19. Chromatogram of derivatized 12 amine mix.

9.5 Imine formation studies

While performing amine binding experiments a specific peak pattern was observed in the HPLC chromatogram. Next to the amine peak a smaller peak with intermediate species in between them was observed. It was proposed that it could be imine formation from the amine and acetone according to the equation in **Figure 20**. Since polymers were washed with the acetone prior to drying it could still be remaining the pores of the polymers.

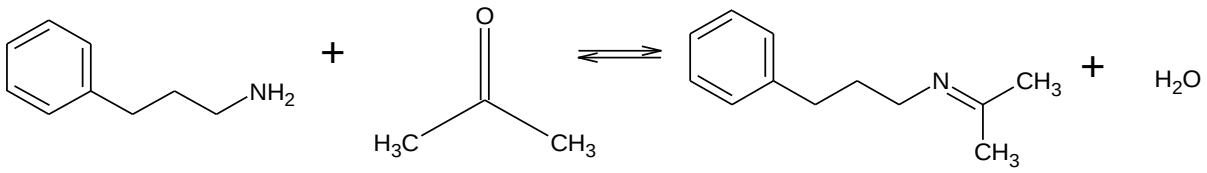

Figure 20. Equation of imine formation from 3-phenyl propylamine and acetone.

To investigate the possible formation of imine from amine and keto-compound on a non-polar polymer surface ATR-FTIR spectra were measured for toluene, cyclohexanone, acetone, 3-phenyl propylamine and their mixtures (amine/cyclohexanone/toluene – 5/5/90 (w/w)) and (amine/acetone/toluene – 5/5/90 (w/w)). Analysis of these compounds were chosen, because toluene mimics non polar surface of polymers. Cyclohexanone is a keto-compound with higher boiling point, thus significantly less volatile than acetone. Primary amine, 3-phenyl propylamine, was chosen because of the lower volatility.

Obtained ATR-FTIR spectra of the compounds were used to identify specific peaks. These were then used to compare to the ATR-FTIR spectra of the mixtures. Imine formation was clearly observed in the amine/cyclohexanone/toluene mixture. The same pattern was observed also in the spectrum of amine/acetone/toluene mixture (**Figure 21**). Thus it could be concluded that on a non-polar surface there would be imine formation from a primary amine and acetone.

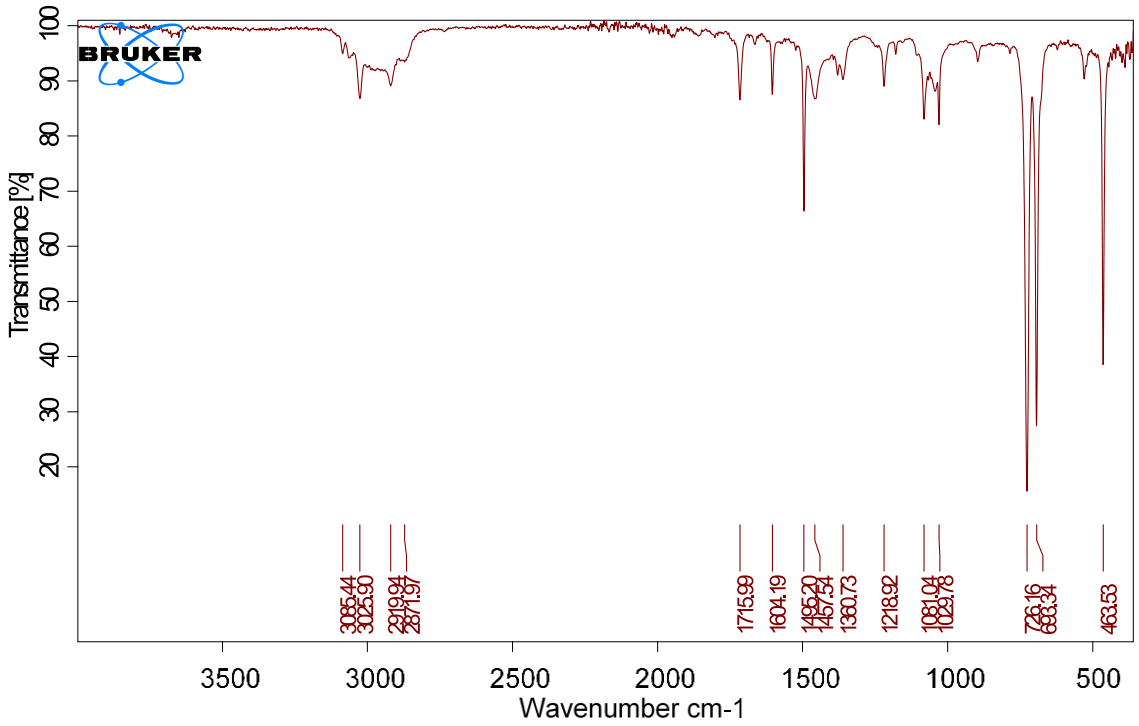

Figure 21. ATR-FTIR spectrum of acetone mixture (3-phenyl propylamine/acetone/toluene – 5/5/90 (w/w).

Imine formation from the amine on the surface of polymeric adsorbent would lead to incorrect estimation of amount of adsorbed amine. Amount of amine found on the surface of adsorbent would be lower than the actual. The amount of imines formed could not be used to estimate the initial amounts of amine because imines would not be adsorbed on an acidic adsorbent. To estimate the extent to which imine formation could be affecting amine analysis, further investigation was done on the imine formation kinetics. Primary amine and acetone mixtures in different ratios and solvents were analyzed by HPLC over extended period of time.

9.5.1 Kinetics of imine formation in MeOH

The most appropriate mixture was chosen for kinetics analysis. Most appropriate mixture was mixture, where concentrations of primary amine and acetone are 1:1, because it was relatively slow thus more points could be observed before establishment of equilibrium. Mixture where concentrations of primary amine and acetone are 1:800 was also analysed to compare the reaction rate with that in a high excess of acetone. Data of PA : acetone mixture 1:1 can be seen in **Figure 22** and of mixture 1:800 in **Figure 23**. The zero time area on the plot is taken from the reference mixture where no acetone was added.

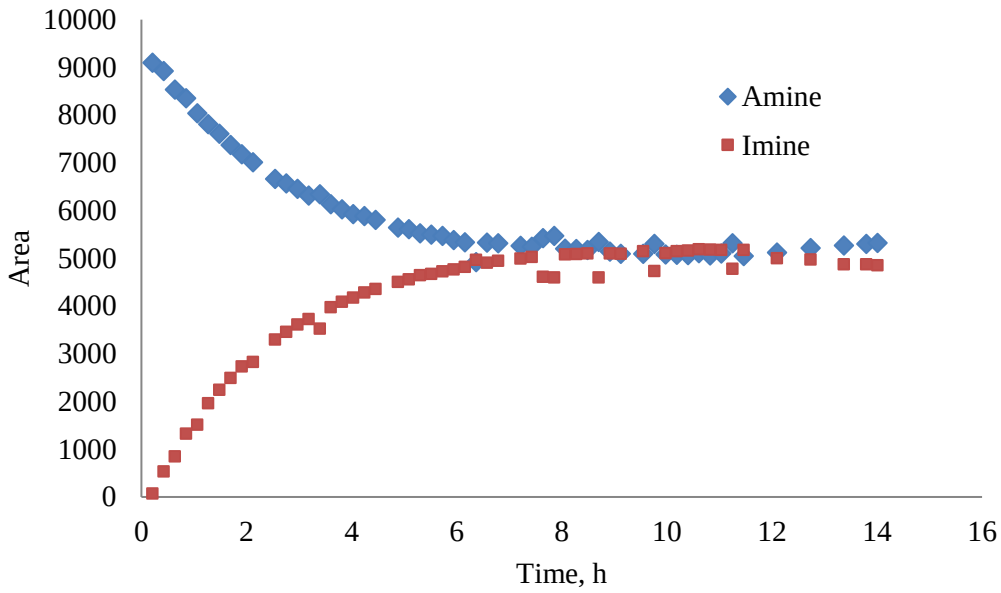

Figure 22. Kinetic plot of imine formation in MeOH, (PA/Acetone, 1/1).

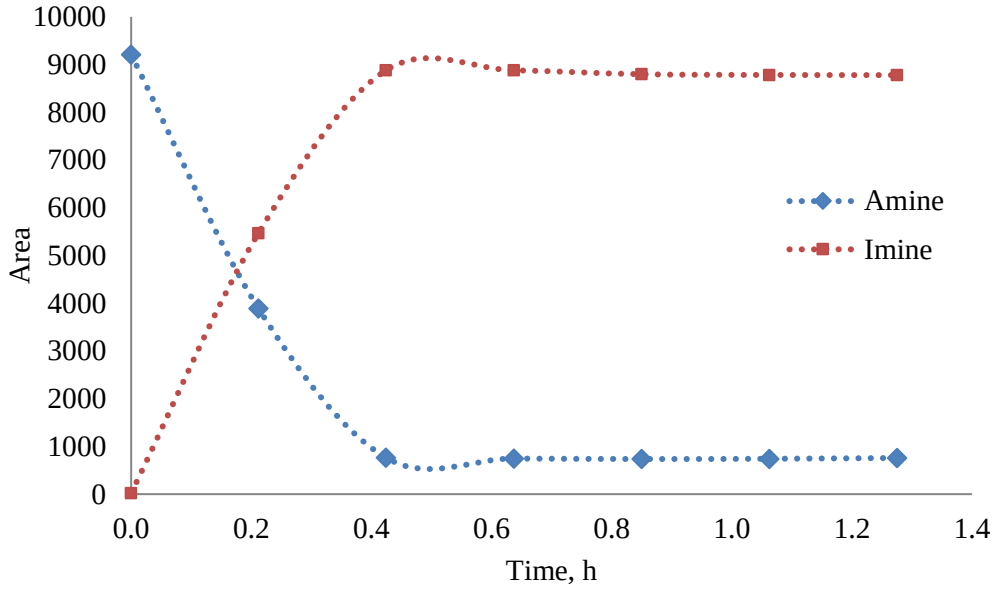

Figure 23. Kinetic plot of imine formation in MeOH, (PA/Acetone, 1/800).

It can be seen from the plots that the areas of amine peaks decrease with time and imine peak areas consequently increase until equilibrium is reached. For PA : acetone mixture 1:1 equilibrium is reached in about 8 hours with approximately half of the initial amine concentration left. In the mixture with high acetone excess (PA : acetone 1:800) equilibrium is reached much sooner - in about half an hour with approximately 10% of initial amine concentration left.

Imine formation is notable in both solutions. The equilibrium is established more rapidly in the mixture with a higher acetone concentration. Also smaller fraction of initial amine is left after establishment of equilibrium. If imine formation reaction would occur at the measuring site on the surface of the adsorbent, it is clear that the observed amount of amine would be erroneous. Several actions could be performed to decrease the loss of amine. For example, making measurements as soon as possible and moving the equilibrium more towards amine formation by the presence of acid and water.

9.5.2 Impact of water and acid presence

Addition of an acid to the reaction mixture would help shift the equilibrium towards amine. Addition of an acid and water would help shift the equilibrium towards amine even more so. This would be beneficial for amine adsorption in atmospheric conditions, because mostly water is present in abundance in the atmosphere and so are acidic conditions.

The results of the experiment though still show the formation of imines. The kinetic plot of imine formation in PA/acetone 1/680 in MeOH in presence of trifluoro acetic acid is shown in **Figure 24**. It can be seen that equilibrium is reached after about 8 hours with about half of the initial amine left. These results are comparable with those of PA/acetone 1/1. Even though the acetone was in large excess, the presence of acid has slowed down the reaction to the rate of PA/acetone 1/1. Unfortunately these results are still notable and imine formation at such extent cannot be neglected.

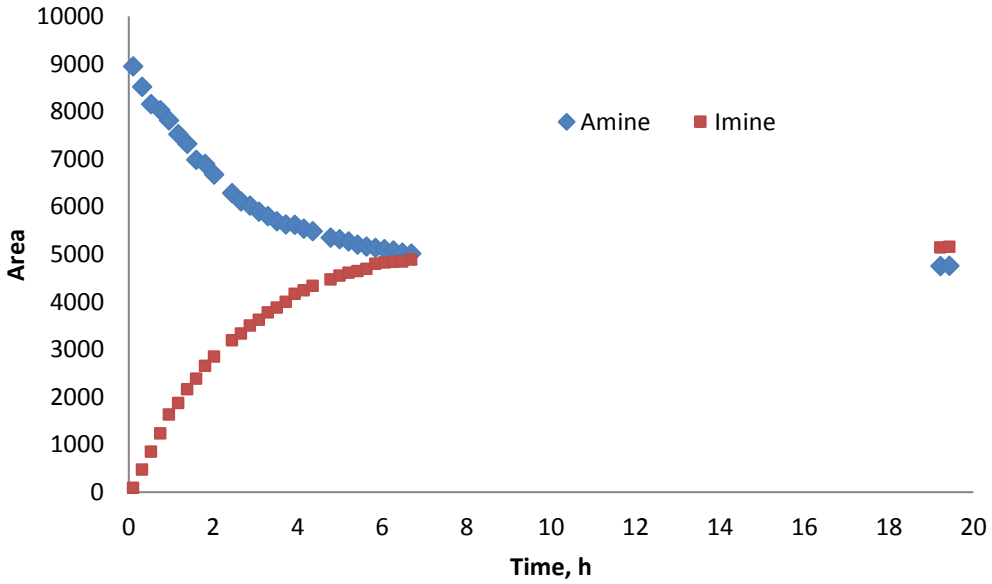

Figure 24. Kinetic plot of imine formation in PA/Acetone, 1/680 in MeOH in presence of TFA.

In the experiments with water addition, as expected, the rate of reaction slows down even more. Unfortunately it still does not stop the imine formation completely. Already after half an hour 3% of initial amine was lost. The reaction is slower at lower concentrations that would be more common in atmospheric conditions, but the loss of amine still should be accounted for.

9.5.3 Kinetics of imine formation in ACN

Kinetic plots of different composition reaction mixtures in ACN were obtained. The collected results from the kinetic plots can be seen in **Table 15**. In ACN imine formation is slower than in MeOH but still notable. For example, in 1/800 mixture in ACN the equilibrium is reached after 3 hours while in MeOH it was already reached after half an hour. For the 1/1 PA/acetone mixture in MeOH, equilibrium is established after 8 hours, while in ACN in 22 hours only 3 % of amine is lost.

Table 15. Equilibrium points of kinetic plots of PA/acetone mixtures in ACN.

Mixture PA/acetone in ACN	PA fraction	Imine fraction	Time when equilibrium is reached
1/800	10 %	90 %	3 h
1/800 (repeated)	10 %	90%	2.5 h
1/100	10 %	90 %	not clearly reached in 22 h
1/100 (lower concentration)	25 %	75 %	not clearly reached in 40 h
1/10	70 %	30 %	not reached in 22 h
1/1	97 %	3 %	not reached in 22 h

Kinetic plot of imine formation in PA/acetone 1/800 mixture in ACN can be seen in **Figure 25** and of PA/acetone 1/100 in ACN in **Figure 26**. Clearly imine formation reaction is more rapid in the mixture with higher excess of acetone. The concentration of the amine and acetone in the reaction mixture is also important. Even though the ratios between primary amine and acetone may stay the same, reaction will be slower in the reaction mixture with the lower concentrations. Although the equilibrium is not yet clearly reached after 40 hours in the PA/acetone 1/100 reaction mixture, there are 25 % of the amine left, while in the higher concentration mixture, already after 22 hours only 10 % are left.

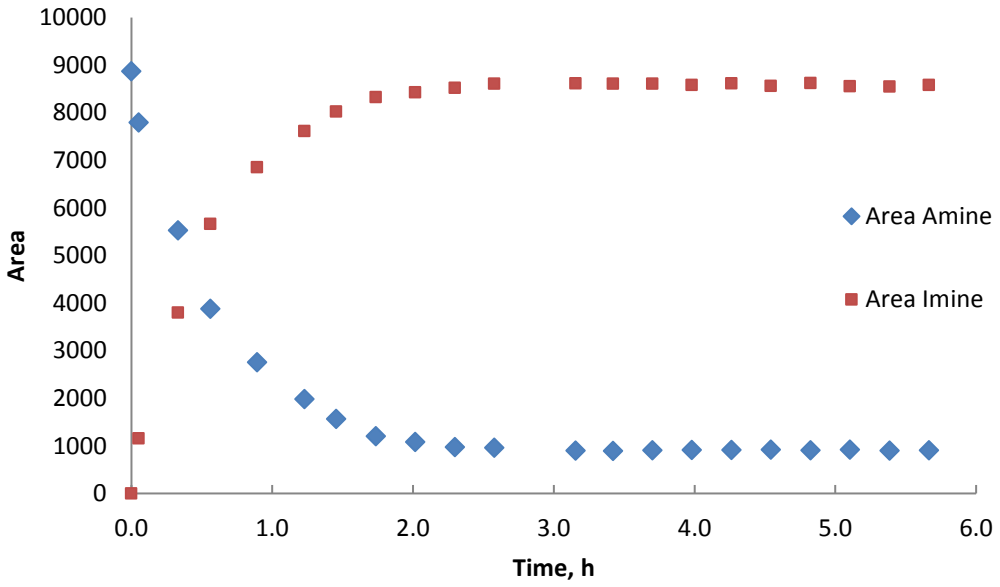

Figure 25. Kinetic plot of imine formation in mixture PA/Acetone, 1/800 in ACN.

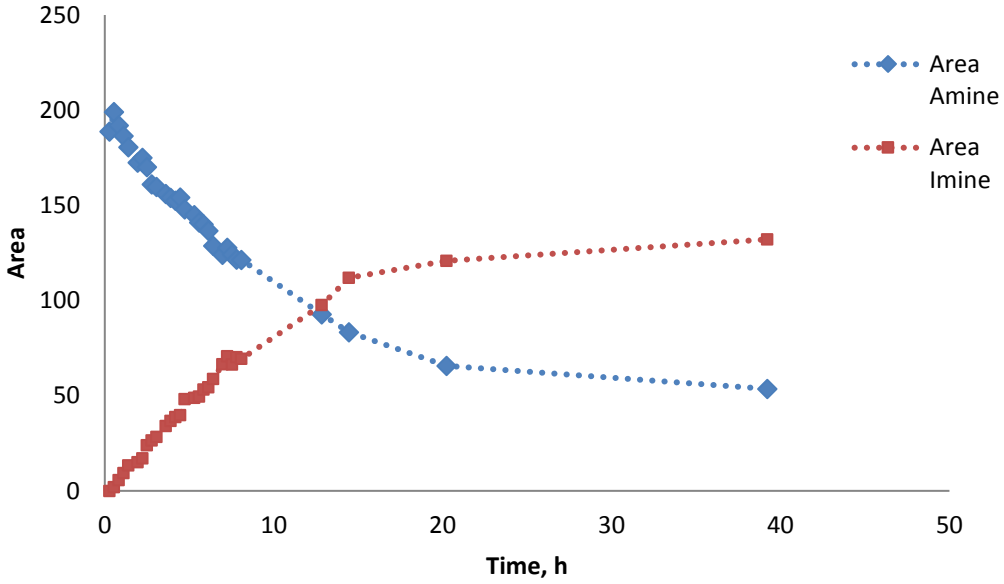

Figure 26. Kinetic plot of imine formation in mixture PA/Acetone, 1/100 in ACN.

10 Conclusions

The aim of this thesis experimental part was to synthesize and characterize materials that would be capable of adsorbing amines from the air. This goal has been reached successfully. Five adsorbents were prepared by using different approaches inspired by methods found in literature. Properties of adsorbents that are relevant to preparation of materials and adsorption of amines were estimated by different techniques.

A strong ion exchanger adsorbent was prepared by incorporating propylsulfonic acid ligands on the surface of silica gel. A number of weak ion exchangers were synthesized by means of suspension polymerization. There the neutral monomers such as DVB, styrene and ethylene dimethacrylate were copolymerized with acidic monomers such as acrylic acid and methacrylic acid. The following polymers were obtained: copolymer of divinylbenzene and methacrylic anhydride (DVB-$(MAA)_2O$), copolymer of ethylene dimethacrylate, butyl methacrylate and acrylic acid (EDMA-BMA-AA), copolymer of ethylene dimethacrylate and methacrylic acid (EDMA-MAA), and copolymer of styrene, divinylbenzene and methacrylic anhydride (Sty-DVB-$(MAA)_2O$). The polymers made with methacrylic anhydride were then hydrolyzed to obtain acidity.

The composition of the (DVB-$(MAA)_2O$) polymers was assessed by ATR-FTIR. It was found that if 33 % of the monomer mixture for polymerization was composed of methacrylic anhydride, then 22.1 % of it would be incorporated in the polymer. While for 50 % anhydride content in the monomer mixture – 30.7 %. A particle size distribution revealed that over 50 % of all obtained polymeric adsorbent particles were in the desired size range (100 – 250 µm) with the highest of 69.4 % for (DVB-$(MAA)_2O$) polymers.

Ion exchange capacities of the adsorbents were estimated by titrations. For SO_3H-Silica it was 0.8 meq/g, and for polymers ranging from 0.8 to 4.8 meq/g with the highest value for (DVB-$(MAA)_2O$) polymers. The static binding capacities of the SO_3H-Silica were 0.63 mmol of primary amine and 0.58 mmol of tertiary amine per 1 g of material. For polymers it ranged between 0.2 mmol/g to 0.95 mmol/g for tertiary amine, with the highest result being for (DVB-$(MAA)_2O$). The dynamic binding capacities of the SO_3H-Silica were 0.6 mmol/g for primary amine and 0.55 mmol/g for tertiary amine. For (DVB-$(MAA)_2O$) polymer the dynamic binding

capacities were 2.0 mmol/g for primary amine and 0.8 mmol/g for tertiary amine. The static and dynamic capacities had virtually the same values for SO_3H-Silica and, while higher, the ion exchange capacity was also close to that. However for the (DVB-$(MAA)_2O$) polymer the ion exchange capacity was eight times higher than static and six times higher than dynamic binding capacities for tertiary amine. For primary amine IEC was more than two times higher than dynamic binding capacity.

The most promising adsorbing material of the ones prepared and characterized was the (DVB-$(MAA)_2O$) polymer. It had the highest results in ion exchange capacities, static binding and dynamic binding capacities. It also had the largest fraction of particles in the desired size range.

Formation of imines was observed on the surface of polymeric adsorbents due to leftover acetone in the pores after soxhlet extraction. This phenomenon was further explored as similar conditions might appear in the atmosphere, where the prepared adsorbents were intended to perform in, leading to incorrect assessment of amine concentrations. It was found that imine formation is significant in both methanol and acetonitrile in a large excess of acetone over primary amine. In atmospherically more relevant conditions, such as in presence of water, acidic conditions, and lower concentrations of carbonyls, imine formations was slower but notable nevertheless.

11 References

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12 Appendix

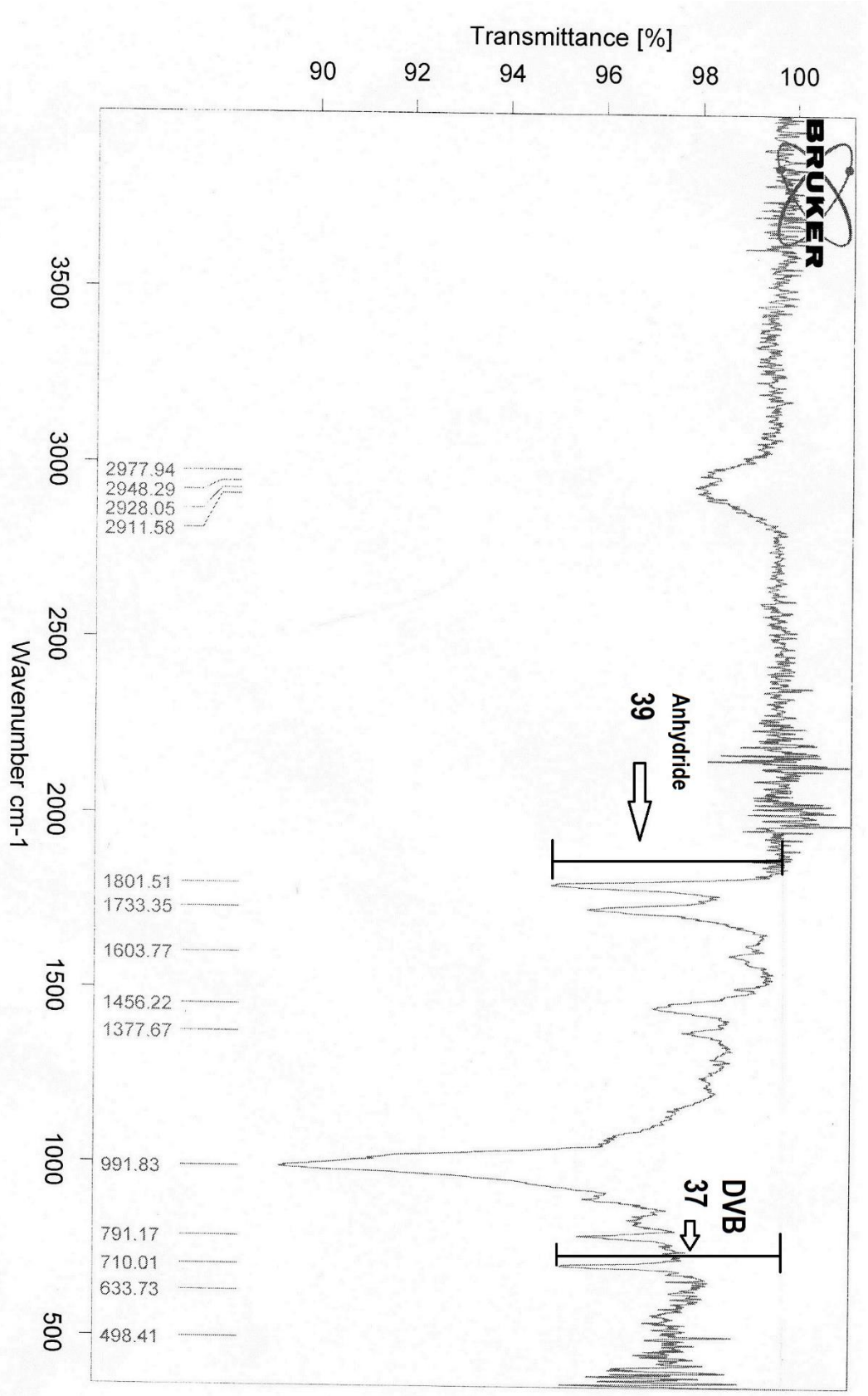

Figure 27. ATR-FTIR spectrum of (DVB-(MAA)2O) polymer with characteristic bands of anhydride (1801 cm^{-1}) and DVB (710 cm^{-1}) measured.

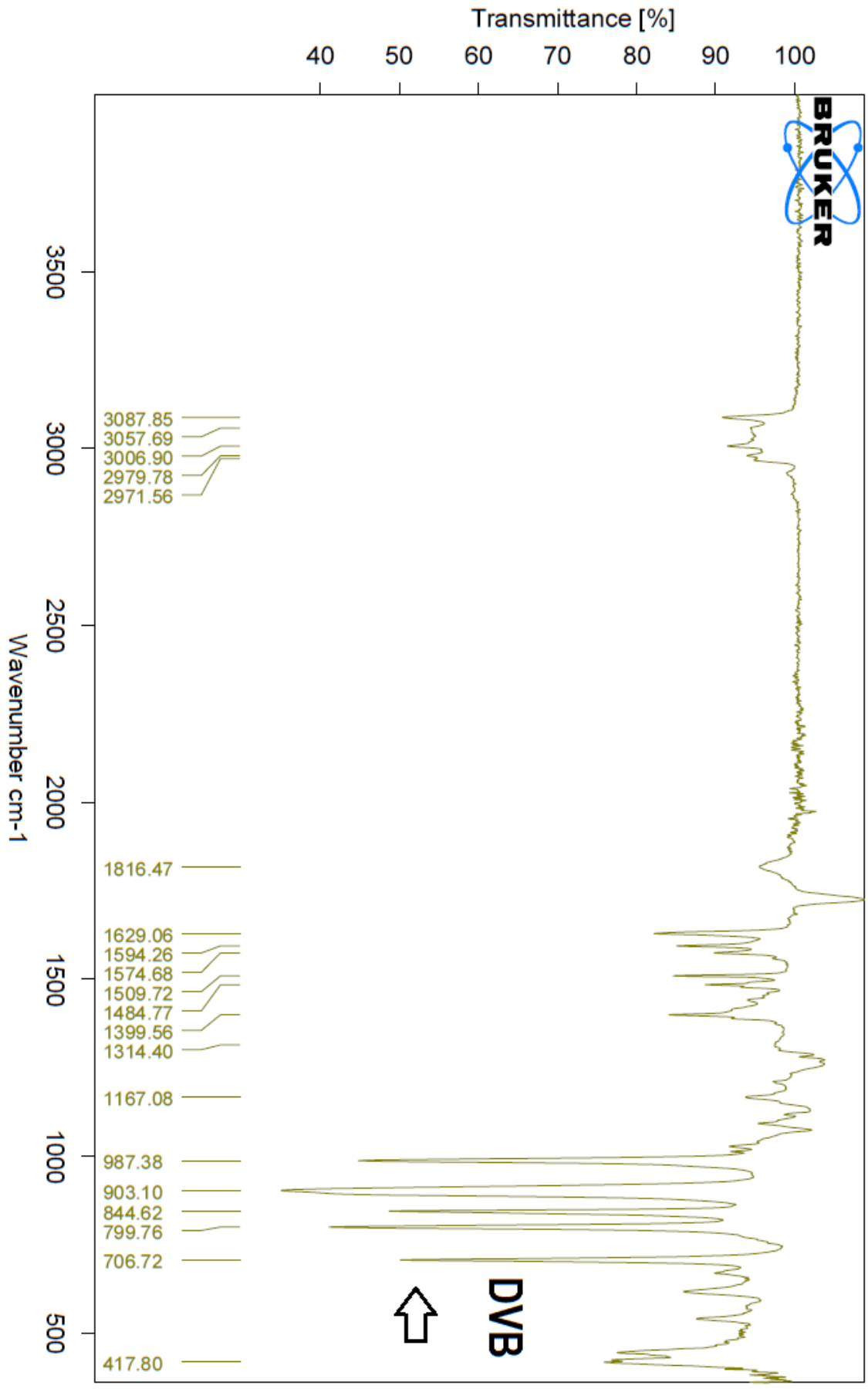

Figure 28. ATR-FTIR spectrum of DVB with characteristic band at 706.7 cm^{-1}.

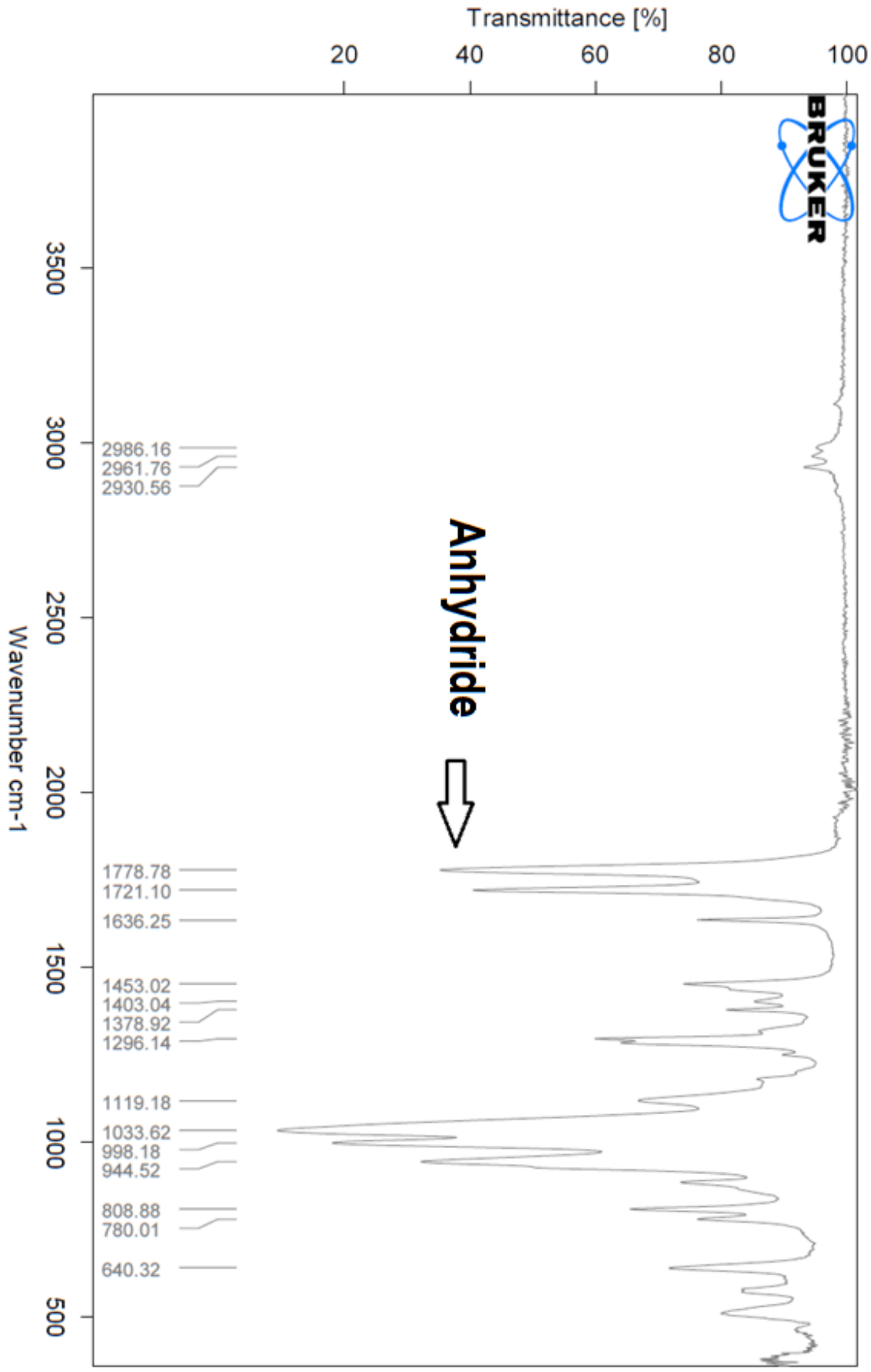

Figure 29. ATR-FTIR spectrum of anhydride monomer with characteristic band at 1778.8 cm^{-1}

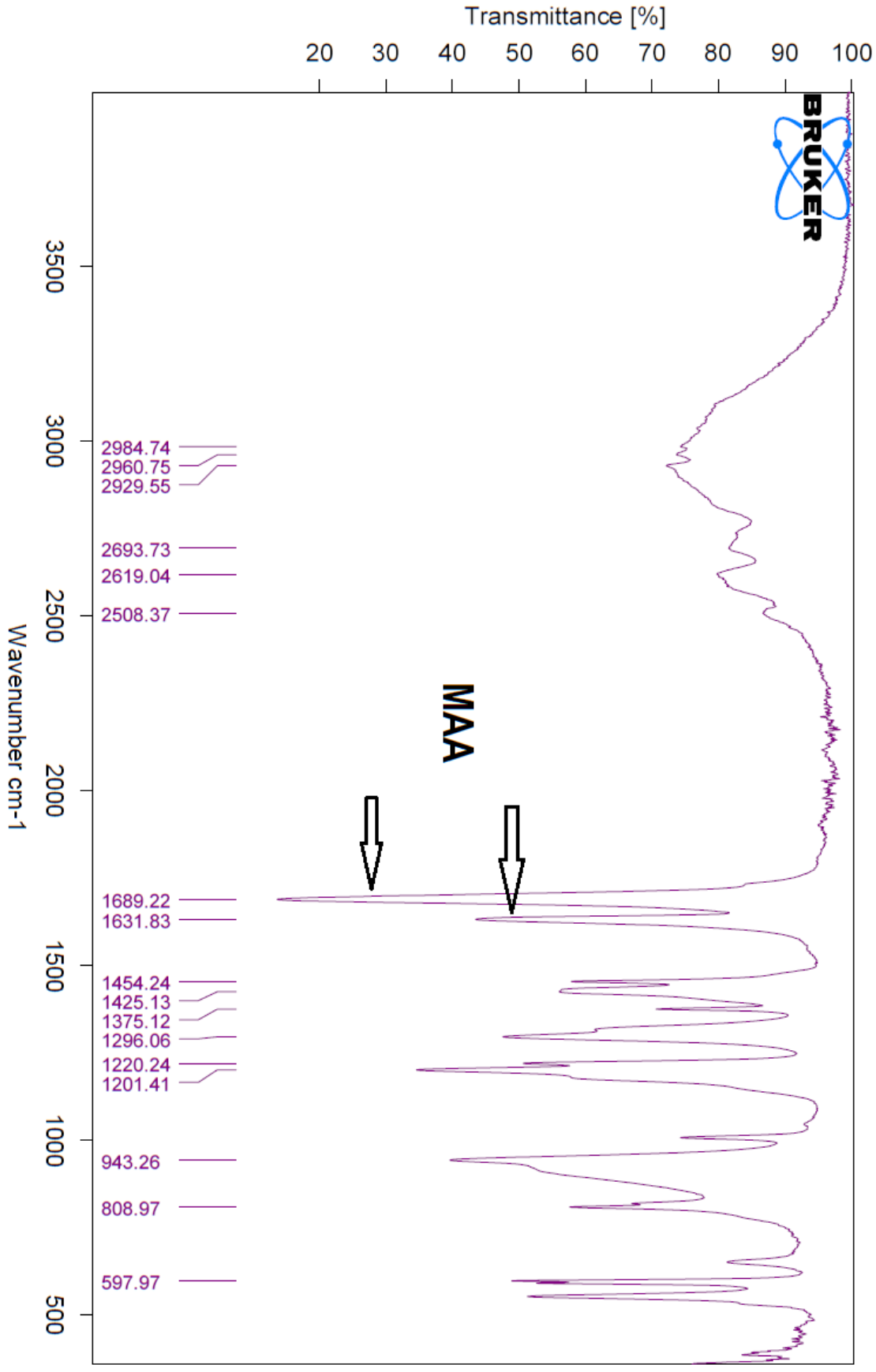

Figure 30. ATR-FTIR spectrum of MAA with characteristic bands at 1689 cm^{-1} and 1632 cm^{-1}.

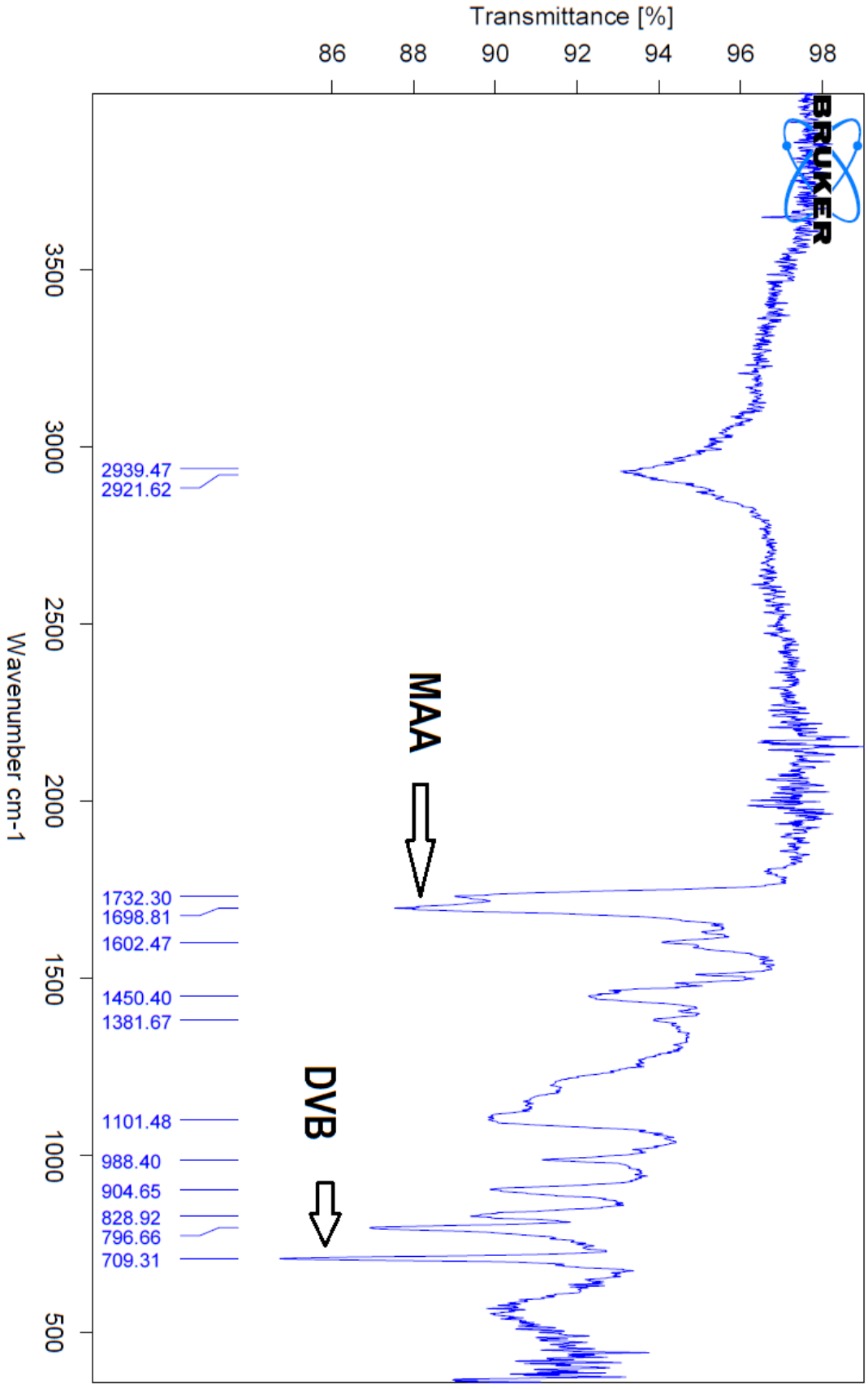

Figure 31. ATR-FTIR spectrum of hydrolyzed (DVB-$(MAA)_2O$) polymer with characteristic bands of MAA (1699 cm^{-1}) and DVB (709 cm^{-1}) measured.