UNIVERSITY OF NOVA GORICA
GRADUATE SCHOOL

DEVELOPMENT OF NEW METHODS FOR DETERMINATION OF BIOGENIC AMINES AND INVESTIGATION OF RELATED ENZYMATIC PROCESSES

DISSERTATION

Sara Budal

Mentor: prof.dr. Mladen Franko

Nova Gorica, 2013

SUMMARY

Awareness concerning the importance of food safety, and related impacts on human health has grown significantly in recent years due to potential toxicological and physiological effects of food ingredients on individuals. Much emphasis has been given to biogenic amines (BAs), as toxic substances usually present in fermented food and used as indicators for food subjected to poor hygienic conditions during food processing.

The toxicity of biogenic amines led to considerable interest in the development of methods, and consequently also analytical technologies, for their determination. High performance liquid chromatography (HPLC) is the most frequently used analytical tool, for this purpose, but a derivatization step, necessary for BAs determination, makes this technique time consuming in sample preparation steps. Therefore, for screening purposes, an analytical method which enables rapid detection of trace amounts of biogenic amines is very much needed.

In this work we used the thermal lens spectrometry (TLS) for development of an analytical method, combined with an enzymatic reaction and a colorimetric reaction, to avoid in this way the derivatization step.

In the enzymatic reaction, transglutaminase (TGase) catalyses an acyl transfer reaction between the γ-carboxamide group of protein bound glutamine residue (Gln), and the amino group of a primary amine, diamine or polyamine in peptides or proteins; in our case the amino groups of BA. During this reaction ammonia is released, and in our case detected with a colorimetric reaction known as indophenol blue reaction. The presence of indophenol is visible when a particular blue coloration is formed with a characteristic absorption band between 550 and 750 nm, with a maximum at around 650 nm.

Novel dual beam TLS spectrometers based on the excitation from a He-Ne (632.8 nm) and a Kr-laser (647 nm) were constructed, tested and used for the detection of indophenol. For the first time a probe beam at 543.5 nm was used as a second He-Ne laser. This facilitates the alignment of the pump and probe beams, and their

separation before reaching the detector, which would be much more demanding in case of two read beams (632.8 and 647 nm). In addition, the shorter wavelength of the probe beam contributes to a 16.4% higher sensitivity of the TLS measurement for the given wavelengths.

The influence of two organic solvents, acetonitrile and ethanol, on indophenol reaction was tested. Organic solvents were used as additives to water in thermal lens spectrometry in order to obtain higher enhancement of the TLS signal, due to generally better thermooptical properties of organic solvents in comparison with water. The TLS enhancement factor, when using 25% acetonitrile/water mixture, was 1.62 times higher in comparison with the enhancement factor in water. Decision to choose acetonitrile instead of ethanol is entirely due to the higher TLS signal achieved with acetonitrile that results from a greater enhancement factor.

Batch mode measurement on a He-Ne TLS detection unit was initially performed to test the performances and applicability of the described methods for the determination of ammonia by the indophenol blue method. The limit of detection achieved by TLS in water with 25% of added acetonitrile, after indophenol blue method optimization, was 8 ng/mL, and is in fact 9.8 times lower when compared to spectrophotometric measurements in water. In comparison to spectrophotometric detection in 25% acetonitrile the improvement was even higher (16 times) which was due to the effect of acetonitrile on spectral properties and the reaction rate of indophenol blue formation.

The TGase enzymatic reaction was tested in combination with indophenol blue reaction after its optimization. Tested BAs are putrescine, cadaverine, tyramine and histamine. At this point, with this TLS setup, the achieved improvement are expressed in a 44 to 135 fold lower LOD, for biogenic amines investigated, compared to the reported enzymatic method in the literature. Limits of detection ranged from 5.2×10^{-7} M to 2.0×10^{-6} M for putrescine and tyramine, respectively.

To improve achieved limits of detection for BAs, in batch mode, with the He-Ne TLS detection unit, a new TLS system was constructed exploiting the higher power

of Kr-laser (200 mW), that also better matches the absorbance of indophenol at 650 nm. In a previous system the excitation laser emission line was at 632.8 nm, in this case the emission line of Kr-laser is 647 nm, much closer to the absorption maximum of indophenol blue at 650 nm. This contributes to a higher absorbance by 1.08 times. The limits of detection achieved in this case for the four investigated biogenic amines are between 12 to 32 times lower compared to those achieved by the He-Ne detection unit, and ranged from 3.6×10^{-8} M to 1.3×10^{-7} M from cadaverine to tyramine, respectively. Improvement was partially due to the seven-times higher excitation laser power provided by the Kr-laser and to better matching of the excitation wavelength with the absorption maximum of indophenol blue. Additional improvement can only be attributed to better alignment of the system, which was not carefully investigated for the He-Ne TLS system, which was constructed primarily for testing purposes.

Developed batch mode method was also used for analysis of real samples of the white wine to test applicability of the method in real samples. The method is not specific for the identification of single biogenic amine, but allows the determination of the total biogenic amines present. For this reason the quantity of biogenic amines was expressed as equivalents of histamine, the only BA for which the maximum contamination level is regulated. Tested samples were two different types of white wine: home made wine from mixed white grapes and Rebula wine provided by the Centre of Wine Research at the University of Nova Gorica. Based on the addition of histamine and from the difference of TLS signals from spiked and original wine samples, a concentration of BAs corresponding to $(6.4\pm0.3)\times10^{-7}$ M which corresponds to 0.069±0.003 mg/L equivalents of histamine was estimated for home made wine, and $(1.8\pm0.9)\times10^{-6}$ M concentration which correspond to 0.2±0.1 mg/L equivalents of histamine for Rebula wine. Results indicate that in the selected samples of wine the concentrations of BA were up to five times lower when compared to the reported values in the literature. This allows us to consider the developed TLS method as a highly sensitive and rapid tool for semi-quantitative screening of BAs presence in real samples. However for the ultimate confirmation of the presence of BAs in investigated samples and for final validation of the method, a

comparative analysis with an independent analytical method such as HPLC is necessary.

A flow injection analysis system (FIA) was constructed to facilitate sample handling and improve sample throughput. A FIA manifold consisted of two different HPLC pumps, one injection valve and a bioanalytical column with immobilized enzyme, a reaction coil and Kr-TLS detection unit. Measurements demonstrated a complete loss of activity of immobilized TGase. It was also clearly evident, that the reaction of indophenol blue reaction proceeds even without the use of MTGase, and has at the same time confirmed that the indophenol blue method suffered from BAs interference. This, however, also confirmed that for screening purposes, the FIA-TLS method can be used without the need of TGase, and provides comparable or lower LODs (2.6×10^{-6} M for histamine and 7.7×10^{-6} M for tyramine), as an enzymatic method reported in literature.

As known from the literature, indophenol blue reaction suffers from interference in the presence of aminoacids that can cause errors in ammonia determination. For this reason, three structurally different amino acids (asparagine, glycine, and histidine), which cover almost entire spectrum of positive interferences by amino acids (0.8 – 56 %) as known from the literature, were tested in order to estimate possible margins of positive errors from interferences. Determined relative error due to the interference from aminoacids was found to be 6% for asparagines, 12% for glycine and 20% for histidine, when the amino acids were present in concentrations equimolar to those of ammonia entering the indophenol reaction. Based on these results and known concentrations of free amino acids in natural waters (10^{-7} M) it was concluded that significant positive errors from interfering amino acids can only be expected at extremely low concentrations of ammonium (below 10^{-6} M). At concentrations of ammonium ten times higher than the LOD of the TLS method developed in this work the errors due to the presence of amino acids in natural waters are estimated at 1-2% or less. However, the presence of amino acids shall not affect the determination of biogenic amines in real samples, which requires extraction of

biogenic amines. During this process the amino acids are separated and removed because of their solubility in the aqueous phase.

CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

BA = Biogenic amine

BAs = Biogenic amines

TGase = Transglutaminase

MTGase = Microbial transglutaminase

GTGase = Transglutaminase from guinea pig liver

CBZ = N-carbobenzoxy

TLS = Thermal lens spectrometry

CPG = Controlled pore glass

FIA = Flow-injection analysis

LOD = Limit of detection

PUT = Putrescine

CAD = Cadaverine

HIS = Histamine

TYR = Tyramine

SD = standard desviation

RSD = relative standard deviation

SPE = solid phase extraction

1 Introduction

An awareness of the importance of a healthy diet has increased considerably in recent years, and hence, any issue related to food safety has a considerable impact on consumers' behaviour and official policy. Demand for good quality and healthier food products have prompted the research on compounds with toxic effect and harmful impact on human health. Among others, many studies have focused on the presence of biogenic amines (BAs) in foodstuffs and their adverse effect of on humans.

BAs are formed mainly by microbial decarboxylation of amino acids, through a fermentative process (Wantke F., 1996) or due to microbial contamination and poor hygienic conditions during aging or storage and their content in food depends on protein content in food (Leuschner R.G., 1998; Eerola S., 1997).). In some cases microbial contamination and related formation of BAs can also result from environmental pollution by municipal wastewaters and sewage systems. The non-observance of the optimal conditions of storage and conservation which determine the speed of protein hydrolysation and at the same time, the start of microbial degradation, and indicate the absence of hygiene during the processing, storage, transport and distribution of the food products. Therefore, BAs are frequently used as indicators of food quality and hygienic conditions during food processing.

The presence of BAs in different fermented food such as cheese (Novella-Rodríguez S., 2003), fish (Lehane L., 2000), sausages (Suzzi G., 2003), meat (Loovas E., 1991) and in beverages as beer (Izquiredo-Pulido M., 2000) and wine (Manfroi L., 2009), can cause different adverse effects, such as headaches, respiratory problems, heart palpitations, hyper- or hypotension and several allergic reactions. These symptoms can be amplified in individuals with respiratory and coronary problems, people with intestinal problems (gastritis, stomach and colon ulcers) and individuals in treatment with monoamine oxidase (MAO) or diamines oxidase (DAO) inhibitors used for stress and depression therapy or in Alzheimer's or Parkinson's diseases.

The toxicity and potential use of BAs as food quality markers are the two main reasons for the need of developing novel analytical methods for the determination of BAs. The third reason for the growing demand for improved analytical methods is the long time of analysis for all known methods and in some cases insufficient sensitivity or selectivity.

Various methods for the determination of BAs exist at present and are mainly based on chromatography (HPLC, GC-MS) that requires time-consuming derivatization procedures for BAs, which last up to one hour (Proestos J., 2008). In HPLC, pre- or post-column derivatization are commonly used for individual separation and detection of BAs. Derivatization is necessary because the majority of BAs do not possess chromophoric or fluorophoric moieties. *O*-phthalaldehyde (OPA) is one of the preferred derivatising agents, giving rise to highly fluorescent derivatives, but it can react only with primary amines. Dansyl-chloride, another widely used derivatising reagent, can react also with secondary and tertiary amines (Proestos J., 2008). Derivatives are stable and fluorescent and detectable in the UV region. Gas chromatography (GC) is another widely used technique; the derivatization step is needed to obtain the corresponding (*o*-heptafluorobutyryl) volatile derivatives that allow the determination by gas chromatography (Fernandes J.O., 2000).Capillary electrophoresis (CE) is attractive due to the short analysis time, without need of a derivatization step and high specificity, but the lack of sensitivity is the main problem in this case.

Other non chromatographic methods include ELISA tests, electrochemical biosensors, and enzymatic methods.

ELISA tests were developed for histamine determination. The time required for complete analysis is about 20 minutes, 10 for incubation, and rest for complete enzyme activation and deactivation, before the absorbance is measured at 450 nm (Marcobal A., 2004). However, by these test only histamine can be determinined. To the best of our knowledge, ELISA tests for other biogenic amines were not reported in literature.

Approximately same time (20 minutes) is also needed in the case of electrochemical biosensor for determination of biogenic amines (Lange J., 2002). The drawback of

this method is that unlike in microplate readers used in ELISA, where up to 96 samples can be analysed simultaneously, each sample must be analyzed individually. Enzymatic methods are often used and are commonly based on peroxidase enzymes and detection of the produced hydrogen peroxide by an amperometric method (Male K.B., 1996; Frébort I., 2000). Recently another enzymatic method was reported for detection of biogenic amines, which utilizes transglutaminase and optical detection (Punakivi K., 2006) and requires two hours of reaction time.

Relatively low sample throughput is in general the main drawback of the available methods for detection of BAs. Therefore, rapid analytical methods, which enable highly sensitive and selective detection of trace amounts of biogenic amines are needed. In order to reduce the number of samples analysed by time consuming methods, fast screening methods are highly desired as well. In this case, selectivity can be sacrificed by determining total concentration instead of concentrations of individual BAs, in order to gain higher sample throughput.

1.1 Objectives

This work focused on the development of a sensitive and high sample throughput analytical method for the detection of biogenic amines in food samples.

From this perspective it is our general objective to investigate the applicability of bioanalytical assays to introduce required selectivity, highly sensitive laser techniques such as thermal lens spectrometry in order to provide appropriate sensitivity and higher throughput as well as flow injection analysis to facilitate sample handling and improve sample throughput.

Furthermore, our specific objectives include immobilization and incorporation of transglutaminase into a flow injection system, construction and testing of a novel thermal lens spectrometer as a detection system for batch mode as well as flow injection measurements, and finally testing of the developed methods for analysis of real samples.

2 Theoretical background

2.1 Biogenic amines

Biogenic amines (BAs), are organic bases of low molecular weight which include aliphatic, monoamines, diamines and polyamines, catecolamines, as well as indolyl and imidazolyl amines (*Figure 1*). They are also the sources of nitrogen and precursors for the synthesis of hormones, alkaloids, nucleic acids and proteins (Silla-Santos M.H., 1996).

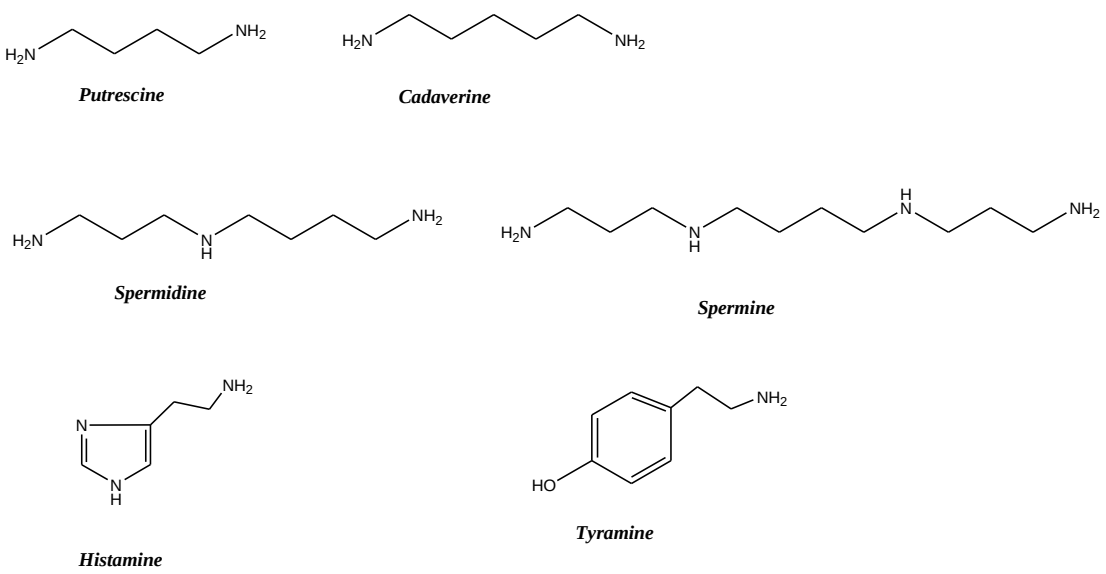

Figure 1: *Structures of the most common biogenic amines.*

Their formation occurs in living organisms (biosynthesized in animal and plant cells) and they are present in a variety of foods, primarily as a consequence of microbial decarboxylation of corresponding aminoacids. This process, called proteolytic process, usually takes place during preparation, ripening and storage of high-protein content and fermented food (fish, meat, dairy products, sausage, wine, beer etc.).

The amount and type of biogenic amines formed is strongly influenced by the food composition, microbial flora and other parameters, which promote bacterial growth during food storage, such as pH, salt concentration, bacterial activity as well as humidity, storage temperature, and ripening time (Halász A., 1994; Alberto M.R., 2002). The formation of biogenic amines can change during food processing and

storage, and can also be influenced by the hygienic conditions (Draisci R., 1998) in the food production process. Their formation has been proposed as an index (Mietz J.L.K., 1977) of chemical freshness of various foodstuffs such as fish, olives, and fruits.

They are usually used as quality markers due to their impact on human health if present in food at high concentration levels.

2.1.1 Toxicity of BAs

Low levels of BAs in food are not considered a serious risk. However, if the amount of ingested BAs is high enough various physiological effects such as hypotension (histamine, putrescine, cadaverine are interested) or hypertension (tyramine), nausea, headache, rash, dizziness, cardiac palpitation and in some cases anaphylactic shock syndrome and death in very extreme cases (Rawles D.D., 1996) can occure.

BAs are also considered precursors of carcinogens such as *N*-nitrosamines (Križek M., 1998a). Reaction of nitrosating agents with primary amines produces short-lived alkylating species that react with other components in the food matrix and generate products (mainly alcohols) with toxic activity. The nitrosable secondary amines (spermine, spermidine) can form nitrosamines through reaction with nitrite, while tertiary amines produce a range of labile *N*-nitroso products (Halász A., 1994).

Normally, ingested BAs are metabolized by the natural detoxification system, present in the gastro-intestinal tract of mammalians (Silla-Santos M.H., 1996). Under normal conditions, exogenous amines adsorbed from food are rapidly detoxified by the action of amine oxidase or conjugation. Enzymes involved in detoxification system are monoamino oxidase (MAO, EC 1.4.3.4) and diamino oxidase (DAO, EC 1.4.3.6). MAO and DAO are present in gut epithel and thus oxidation products of BA are introduced in blood (Križek M., 1998a). Polyamines are usually first acetylated and then oxidized by DAO (Ascar A., 1986; Stratton J.E., 1991).

In the case of allergic individuals, or those taking drugs acting as monoamine inhibitors, or when the intake of BAs is high, the detoxification process is inefficient and BAs accumulate in the body (Halász A., 1994). People with gastrointestinal problems (gastritis, irritable bowel syndrome, Crohn's disease, stomach and colonic ulcers) are also at risk because the activity of oxidases in these individuals is lower than in healthy individuals. Patients treated with inhibitors of MAO and DAO

(antihistamines, antimalaria agents, and psychopharmaceutics) have altered metabolism of BAs that can also cause health problems (Silla-Santos M.H., 1996; Halász A., 1994).

2.1.2 Function of BAs

In plants, polyamines are involved in a number of cell processes (cell division and differentiation, synthesis of nucleic acids and proteins, membrane stability, stress responses and delay in senescence). Different studies (Kalač P., 2002b) have demonstrated that the polyamines putrescine, spermine and spermidine are practically present in all vegetables at levels less than mg/100g of fresh weight.

In plants, diamines, like putrescine and polyamines spermine and spermidine are engaged in physiological processes such as cell divisions, flowering, fruit development and response to stress (Halász A., 1994).

Polyamines are responsible for growth, renovation and metabolism of every organ in the body and are essential for maintaining the high metabolic activity and immunological system of the gut (Silla-Santos M.H., 1996; Bardocz S., 1995). This role diversity of polyamines in cellular metabolism and growth is recognized in tumour growth, and for this reason inhibition of polyamine biosynthesis in tumour-baring individuals is one of the targets in tumour therapy research (Bardocz S., 1995).

BAs as putrescine, cadaverine, and spermine also act as free radical scavengers. Tyramine also has an antioxidative activity that increases with its content, and depends on amino and hydroxyl groups (Halász A., 1994).

Diamines such as histamine, putrescine and cadaverine are decomposition products of histidine, ornithine and lysine.

2.1.3 Presence in food

Food can naturally contain potentially harmful substances such as BAs. Different authors have reported intensive hypertensive crises following consumption of food rich in tyramine or other amines from aged cheese, wine, beer and yeast extracts but also vegetables such as sauerkraut, broad bean, banana peel and avocado (McCabe B.J., 1986).

2.1.3.1 Seafood

The most frequent intoxication involves histamine and this poisoning is known as "scombroid fish poisoning", because it is often associated with the ingestion of scombroid fish like tuna, mackerel and sardines (Halász A., 1994). Therefore, the maximum permitted level of histamine in fish samples indicated, and adopted by European Commission regulations, is 200 mg kg^{-1}. Histamine poisoning occurs frequently in Asia. Histamine was reported in extremely high levels in some salted and dried fermented products. Other countries outside Asia have also reported cases of histamine poisoning (Lehane L., 2000). The largest outbreak (2656 cases) was recorded in Japan in 1973 (Lehane L., 2000). USA FDA, has established 500 ppm as a hazardous level for histamine (FDA, 2001). So, this is considered an indicator of earlier microbial decomposition of seafood and a guidance level of 50 ppm is considered as the chemical index for fresh fish spoilage. Histamine is generally not uniformly distributed in a decomposed food. The histamine level of a decomposing fish varied from 50 ppm in one location to exceeding 500 ppm in another location of the tissue (FDA, 2001; Lehane L., 2000). The fish and fishery products with high histamine level are prohibited from being sold for human consumption (Ben-Gigirey B., 1998). A more detailed review in the oral toxicity to humans of histamine in fish muscle suggested that histamine induced slight poisoning at 80-400 mg/kg (ppm) fish, moderate poisoning at ›400 mg/kg and severe posoning at ›1000 mg/kg. Based on the assessment of poisoning cases, the guidance levels suggested for histamine content for seafood are for safe consumption ‹50mg/kg, toxic and unsafe for human consumption ›1000 mg/kg (Lehane L., 2000).

2.1.3.2 Beer

Beer has been reported to be a health risk for some consumers, resulting from BAs content. The first symptom is hypertensive crises after consumption in patients treated with different drugs (painkillers, drugs for stress and depression treatment and in case of Parkinson's disease), that inhibit the natural detoxification enzyme monoamine oxidase (MAO E.C 1.4.3.4.) (Shulman K.I., 1997). The BAs that cause this effect is tyramine. Tyramine intakes exceeding 6 mg in a period of 4-h or beers

with more than 10mg of tyramine per litre have been considered dangerous for this type of patients (Tailor S.A., 1994).

2.1.3.3 Wine

BAs may be formed by yeasts during the alcoholic fermentation; by lactic acid bacteria (LAB) during malolactic fermentation (MLF) and during maturation of wines. Biogenic amines can also be present in the must. Putrescine in grapes is associated with potassium deficiencies in the soil. The main biogenic amines in wine are histamine, tyramine, putrescine and cadaverine.

High levels of biogenic amines correlate fairly well with other wine spoilage components for example butyric acid, lactic acid, acetic acid, ethylacetate and diethyl succinate. Red wines also have higher levels of BAs than white wines, mainly due to vinification practices and maturation (Manfroi L., 2009).

An increase in the levels of biogenic amines usually occurs towards the end of the MLF or during maturation, when lactobacilli and pediococci are the main culprits.

At this stage there are no legal limits, but certain countries have recommended maximum limits with regard to histamine levels (mg/L) that are applicable to imported wines:

- Switzerland - 10 mg/L
- France - 8 mg/L
- The Netherlands - 3 mg/L
- Belgium - 5-6 mg/L
- Germany - 2 mg/L
- Austria - 10 mg/L

Factors influencing biogenic amine formation in wines are:

- pH
- levels of SO_2

presence of precursor amino acids

- number of decarboxylase positive LAB
- duration of the initial fermentation phase
- time of skin contact
- spontaneous MLF
- turbidity of wine during barrel maturation (lees contact)

2.1.3.4 Meat, fruits and vegetables

"Red meat" (adult bovine) and "white meat" (chicken) are particularly susceptible to protein degradation under appropriate conditions. In fatty foods (bacon), a high temperature and the presence of water can contribute to the formation of carcinogen N-nitrosopyrrolidine from putrescine or spermidine (Loovas E., 1991).

Free biogenic amines in fruits and vegetables form the typical and characteristic taste of mature foods and precursors of certain aroma compounds (Askar A., 1989). BAs presence at relatively high concentrations in vegetables was also associated with spoilage due to prolonged storage time and high temperature (Cerutti G., 1989; Yen G.C., 1992).

Fruits and fruit juices are particularly rich in putrescine (Maxa E., 1993; Shalaby A.R., 1996), while green vegetables are rich in spermidine (Valero D., 2002). It was described (Cirilo M.P.G., 2003; Shalaby A.R.,2000) that cooking processes and heat treatments can influence polyamine contents.

Tyramine and other aromatic amines are less widespread than polyamines, they can reach particularly high concentrations in some vegetables (e.g. in *Acacia berlandieri*) which seem to have a defensive role against insects and herbivores. In most fruits and vegetables, the reported mean tyramine contents of 0.7, 2.3, 1.0, and 0.4 mg/100g, were respectively for banana pulp, avocado, orange pulp and tomato (Udenfriend S., 1959). High mean levels of tyramine were reported by Tarjan and Janossy (1978) for vegetables such as potato (84.0 mg/100g), paprika (26.6 mg/100g), tomato (25.0 mg/100g) and cabbage (67.0 mg/100mg). Mean tyramine levels of 0.7 and 3.7 mg/100g were found in concentrated tomato paste and ketchup samples respectively (Kalač P., 2002b).

2.1.3.5 Cheeses

The BAs of major interest present in cheeses are cadaverine, histamine, spermidine, spermine and tyramine. They are responsible for aroma and taste, the most abundant BAs in cheeses is tyramine, and is the principal cause of the so called "cheese reaction".

2.2 Determination of biogenic amines

Analysis of certain BAs in food is a necessity in order to assess potential health hazards before consumption.

The highest drawbacks in the analysis of BAs in food are:

a. Complexity of the sample matrix
b. Low concentration levels at which the compounds are present in the samples (Busto O., 1994)

Trichloroacetic acid, hydrochloric acid or a methanol-water mixture have been used for the extraction of biogenic amines from solid food samples (Lange J., 2002). A purification and preconcentration process usually occur before BAs determination and consist of liquid-liquid extraction (LLE) or solid phase extraction (SPE). SPE has been preferred over liquid-liquid extraction because of the practical advantages of no emulsions, better recoveries, cleaner extracts achievable, and the ability to remove many interferences and matrix components selectively. Of course, SPE was also prefered because of the much lower quantities of solvents required, and the corresponding decrease in the volume of waste solvents produced. Another advantage for SPE over LLE is that the LLE process requires at least two and usually three successive solvent equilibrium, according to chromatographic theory and practice, in order to achieve the highest recoveries. What has become one of the most important advantages for SPE is that it is much more capable of being automated. Such an advantage is very desirable during these times of tight timelines, abundant needs for maximizing throughput while minimizing cost and optimizing the use of scarce and expensive human resources.

Several studies confirm the suitability of C-18-based sorbent cartridges for BAs extraction (Zotou A., 2003).

2.2.1 Analytical techniques

Several methods for BAs analysis in food are based on thin layer chromatography (Naguib K., 1995), amino acid analysers (Simon-Sarkadi L., 1994a), liquid chromatography (HPLC) (Moret S., 1996), gas chromatography (Rogers P.L., 1997), biochemical assays (Zeng K., 2000), conventional capillary electrophoresis (CE) (Santos B., 2004) with UV or fluorescence detection (Lange J., 2002), reversed phase

high performance liquid chromatography (HPLC) with pre-column (Reggiani R., 1990), post-column (Hyvönen T., 1992) or on-column derivatization techniques, coupled with UV-DAD or fluorescence detectors (Saito K., 1992). Alternative approaches are based on HPLC tandem mass spectrometry (Gosetti F., 2007) and ion-exchange chromatography with pulsed amperometry (Hoekstra J.C., 1998) or conductometric detection (Cinquina A.L., 2004).

HPLC with pre- or post-column derivatization are commonly used for individual separation and detection of BAs. Derivatization is necessary because the majority of BAs do not possess chromophoric or fluorophoric moieties. Derivatization reagents are *o*-phthalaldehyde, dansylchloride or dabsylchloride. The first one reacts only with primary amines and its fluorescent derivatives are instable, the other two react with primary and secondary amines and their derivatives are stable.

Recently electrochemical biosensors (Bouvrette T., 1997; Compagnone D., 2001; Lange J., 2002; Niculescu M., 2000) or bioreactors (Tombelli S., 1998) for BAs detection on commercial or home-purified enzymes have been used. The favourite enzyme is the diamine oxidase (DAO) obtained from microorganisms, plants or animal tissues that showed very different enzymatic activity.

However, HPLC methods require complicated and expensive instrumentation, extensive sample pre-treatment and time consuming operation. Alternatively, biosensor technology allows fast, cost effective and specific detection of BAs (histamine) in seafood spoilage (Malle P., 1996).

For a more specific, simple and fast determinations of biogenic amines, the enzymatic methods have become very common recently.

The most widely used methods are based on biogenic amine conversion to the corresponding aldehydes by the amine oxidase that catalyzes a fast oxidative deamination (*Figure 2*). Hydrogen peroxide and ammonia are produced in this reaction.

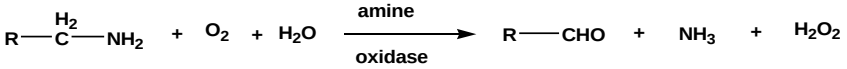

Figure 2: *Reaction of BAs with amine oxidase enzyme.*

In this method the hydrogen peroxide was detected with a colouring reaction using a peroxidase enzyme and a chromogen.

Another promising enzyme that can be used for biogenic amine determination is the transglutaminase, belonging to the group of acyltransferases. This enzyme catalyses an acyl transfer reaction between a donor, the γ-carboxamide group of protein bound glutamine residue (Gln), and the acceptor, the amino group of primary amine, diamine or polyamine in peptides or proteins, in our case, the amino groups of BA. In this reaction a cross-linkage is formed and ammonia is produced as a byproduct (*Figure 3*) (Punakivi K., 2006).

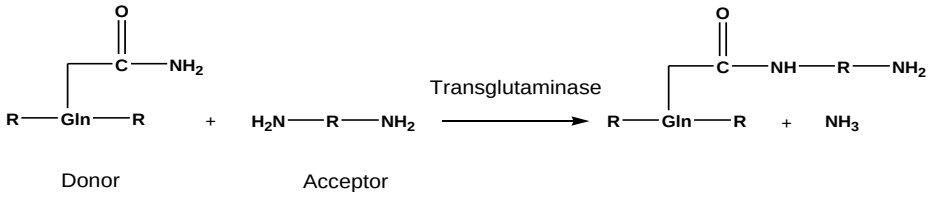

Figure 3: *Reaction of a biogenic amine with the acyl groups donor, catalized by transglutaminase.*

2.2.1.1 Ammonia determination

Ammonia is a significant alkaline pollutant which can be found in the atmosphere, soil and water. Ammonia is released into the environment by industrial processes, usage of natural or synthetic fertilizers or animal excreta. Ammonia emitted into the troposphere is captured by acidic cloud droplets and neutralizes their acidity by forming ammonium salts or reacting with acidic gases to form aerosol (Tanabe S., 1988). Frequent presence of ammonium (or ammonia) in a vide variety of environmental, clinical and industrial samples have stimulated the development of a large number of methods for its determination. One of the most widely used methods for the determination of ammonium (or ammonia) is the spectrophotometric indophenol blue method (Clescieri L.S., 1998).

The indophenol blue method is based on a particular reaction known as the Berthelot reaction. In this reaction the hypochlorite is necessary as a source of chlorine. Under an alkaline condition, up to pH 10 (Prenter J., 2004), ammonium and hypochlorite

form a monochloroamine, this reacts with two molecules of salicylic acid and forms the indophenol (*Figure 4*).

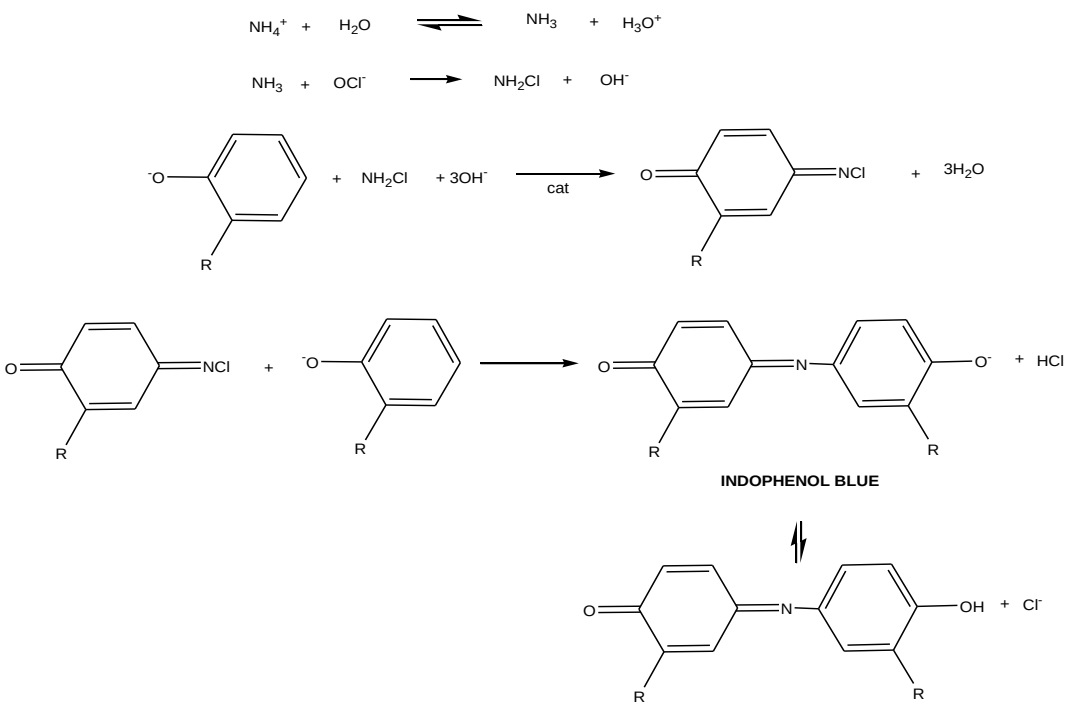

Figure 4: *The Berthelot reaction*

The presence of indophenol is visible when a particular blue coloration is formed and its absorbance is measured with a spectrophotometer. The indophenol complex has an absorption band between 550 and 750 nm, with a maximum at around 650 nm.

The order of reagent additions is also very important, as well as the mixing process especially in batch mode measurements. The reagent which introduces the aromatic ring, salicylic acid, must be added before the chlorine donor. Inversion of the reagent or inadequate mixing should cause erratic results (Prenter J., 2004). The catalyst widely used to promote the time of reaction is nitroprusside. However, this is a toxic substance and was replaced by manganese sulfate (Kurama H., 2002). In order to prevent interference by the reaction of other ions present in solution sodium potassium tartrate is used as a complexing agent.

2.3 Transglutaminase

Transglutaminases (TGases, EC 2.3.2.13) are a family of calcium-dependent enzymes that catalyse the posttranslational modification of a wide range of proteins involving protein crosslinking, polyamination and deamidation (Lorand L., 2003).

Heinrich Waelesh and collegues (Sarkar N.K., 1957) identified an amine-incorporating activity in liver homogenates, where amines are attached to the γ-glutamyl moieties after the release of ammonia and this activity has been named "transglutamination".

Until now nine homologous transglutaminase genes have been identified in humans and mice, all, except one, code active transglutaminases (TGases 1-7 and factor XIIIa) which are variably expressed in multiple organs and cell types of organisms (Grenard P., 2001).

2.3.1 Transglutaminase catalysing reactions

TGase active site (Cys277) catalyses a nucleofilic attack on delta carbon of the glutamine side chain (cystein protease-like catalytic mechanism). Thiol-ester bound enzyme-substrate intermediate is formed and a release of ammonia takes place (Folk J.E., 1983). Formed γ-glutamyl-enzymes thioester intermediately transfers the γ-glutamyl moiety to (*Figure 5*):

I. another amine –*transamidation* (small BAs or the ε-amine group in a lysine)
II. an aliphatic alcohol - *esterification*
III. water - *glutamine hydrolysis; deamidation*

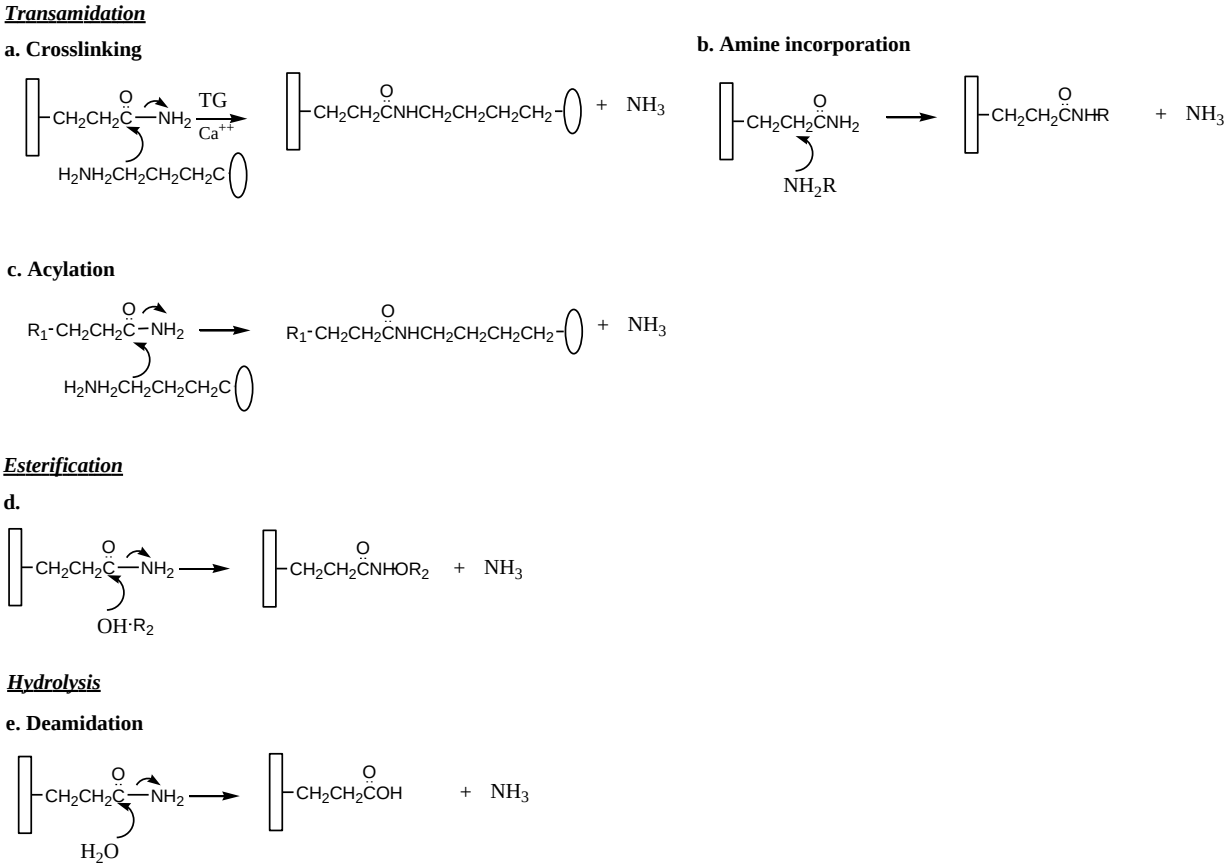

Figure 5: *Transglutaminase post-translational reactions.*

TGases are widely distributed in the most mammalian tissues and body fluids (Aeschlimann D.,1994). Factor XIII and TGase from guinea pig liver (GTGase) have been well characterized as Ca^{++}-dependent enzymes (Folk and Chung, 1973).

Ca^{++}-independent microbial transglutaminase (MTGase) was firstly isolated from the culture broth of *Streptoverticillium* Bacteria (Ando H., 1989) and the primary structure was determined by Kanaju and coworkers (1993). They found that MTGase and GTGase have low sequence similarity, even in active site regions, but the hydrophobic environment of the catalytic site was similar.

MTGase has, in recent years, acquired interest due to the potential application in food industry (Zhou L.D., 2000). Guinea pig liver transglutaminase was the unique source of commercial transglutaminase for decades. The scarce source and the complicated separation and purification processes resulted in an extremely expensive enzyme, which makes it difficult to apply in food processing on an industrial scale.

Many efforts have led to production of transglutaminase from micoorganisms (Ando H., 1989).

Commercial use of microbial transglutaminase in the food industry started with the manufacturing of surimi in Japan. Addition of transglutaminase increases the elasticity and firmness of surimi gel, and can minimise wastage by stabilising fluctuations in raw material quality (Armbrust C., 2003). Transglutaminase is used world-wide for preparation of meat, dairy, bakery, soy products, pasta etc. to improve the texture and to modify the properties of prepared foods in general (Kuraishi C., 2001).

Ajinomoto Company Japan, commercialises various "ready-to-use" food enzyme preparations. These mainly consist of sugar matrix in combination with transglutaminase in order to simplify its use (Ajinomoto Company Information).

Deamidation activity of these two different TGases differs. It was proven that MTGase had little deamidation activity (on CBZ-Gln-Gly as substrate) and less than that of GTGase (Nonaka M.., 1996), but MTGase is more stable than GTGase (Motoki M., 1997); therefore, on longer incubation; MTGase might achieve high efficiency in deamidation. The optimum pH for deamidation of CBZ-Gln-Gly was around pH 6. MTGase can catalyze deamidation over wider range of pH than GTGase, but the activity was lower. In the reaction of primary amines, an optimum pH of TGase is 6.0 -8.0 (Motoki M., 1997), but deamidation occurred at a slightly acidic pH. This result was due to the ionization state of the substrate, because only unprotonated forms are reactive (Folk J.E., 1973).

In deamidation, the rate-limiting step is the nucleophilic attack by H_2O. In primary amine incorporation the deacylation of MTGase, after nucleofilic attack by NH_2OH, occurs much faster than that of other TGase. At a low concentration of amine substrate, water can act as the acyl acceptor and protein-bound glutamyl residues are formed (Folk J.E., 1966a).

However, MTGase remains the most desiderate at the moment for many applications (meat and poultry products especially). It is most stable in activity and is effective over a wide range of temperatures and pH.

2.3.2 Transglutaminase and interaction with amines

In vitro, many amines, diamines, polyamines, and alcohols are capable of interaction with the protein-γ-glutamyl-enzyme intermediate. However, in vivo, only lysine ε-amino groups and polyamines are the available amine substrates (Lorand L., 1984), and coupling of amine neurotransmitters (Walther D.J., 2003), or membrane lipids (Nemes Z., 1999) to proteins is possible in cells with specialized pathways for producing such substrate compounds.

Due to the drastic and disruptive effects of uncontrolled protein cross-linking in living cells, the enzymatic activity of vertebrate TGases are controlled by the availability of calcium ions, which are essential cofactors for the operation of catalytically active conformational states.

2.3.3 Transglutaminase and diseases

Tissue transglutaminase has been identified as a contributor to the formation of cataracts and to Celiac disease, and is suspected to be involved in atherosclerosis, inflammation, fibrosis, diabetes, cancer metastases, autoimmune diseases, psoriasis (Kim S.Y., 2002). It is also suspected to have a role in neurodegenerative diseases, such as Huntington's disease, Alzheimer disease, Parkinson disease, associated with an increase in polyglutamine-containing peptides in the brain (Cooper A.J.L., 2002; Singer S.M., 2002; Karpuj M.V., 2002).

2.3.4 Transglutaminase and its applications

Use of enzymes in many industrial, biomedical and biotechnological applications requires new biocatalysts capable of working in extreme physicochemical conditions. Several strategies have been evaluated for preparing highly stable enzymes, including: isolation from thermo-philic organisms (Sunna A., 1997), site-directed mutagenesis (Minagawa H., 2000); immobilization in solid supports (Saito T., 1997); surface covalent modification with low-molecular-weight compounds (Murphy A., 1996) as well as with water soluble polymers (Darias R., 2001). Among these different approaches, enzyme chemical modification seems particularly promising because it is simple, inexpensive, and allows for the preparation of water-soluble biocatalysts with functional stability (Gòmez L., 2000).

Several disadvantages are associated with the toxicity of reagents commonly used for modifying enzymes through chemical procedures (Lalibertè G., 1994), which are inappropriate for catalysts in biochemical use and food applications. Addition of chemicals often reduces the catalytic effectiveness of the enzymes (Darias R., 2001).
TGase produces either intra- or inter-molecular isopeptide bonds with the γ-carboxamide group of endprotein glutamine residue as an acyl donor substrate and the ε-amino groups of endprotein lysine residues as an acyl acceptor (Aeschlimann D., 1994). In addition, reactive lysines may be substituted by several low-molecular-weight compounds containing primary amino groups, giving rise to a variety of protein-(γ-glutamyl) derivatives (Folk J.E., 1985). For these reasons, TGase was used as a biotechnological tool for the modification of biological activities of peptides (Esposito C., 1999; Esposito C., 1995; Mancuso F., 2001) and proteins (Bechtold U., 2000) with covalent linking polyamines to their reactive endo-glutamine residues.

2.3.5 Transglutaminase in foods

TGase is expected to improve the nutritional value and rheological properties of food proteins and as source of gelatinization of food products, for improving solubility, water-holding capacity or thermal stability of food proteins or to improve food flavour, nutritional value appearance or texture (Wijngaards G., 1997). The use of TGase to increase the quality for a wide range of food products is increasing since large quantities of MTGase have become commercially available (Yokoyama K., 2004).
In the baking industry it is used to improve the functional properties of bread, pastry and croissant dough. Recent studies in the molecular mechanism of celiac disease suggest the possibility that TGase in baked products act upon gliadin proteins in dough to generate epitope associated with celiac disease. Effects of TGase in foods are largely due to its crosslinking activity. Food treated with transglutaminase potentially contains deamidated protein residues and proteins conjugated to any free amines in the food matrix. The exact product mix usually depends on particular processing conditions.
A beneficial effect of transglutaminase in cereal products has been reported (Gerrard J.A., 2000; Bauer N., 2003a-b; Collar C., 2004; Rasiah I.A., 2005).

In general, TGase strengthens dough and also the final product. It has proved a beneficial action in protecting frozen doughs from damage, leading to a higher quality product during thawing and baking.

2.4 Thermal Lens Spectrometry (TLS)

TLS is a highly sensitive detection method based on an indirect measurement of absorbance by a photothermal effect, which originates from a non radiative relaxation of excited molecules in the sample that result in defocusing of the laser beam. During the deexcitation that might involve vibrational relaxation, internal and external conversion and intersystem crossing, the absorbed energy is converted into heat. In the case of a non fluorescent sample it is possible to assume that the amount of released heat is equal to the energy absorbed by the sample.

A laser beam with a Gaussian profile is used for excitation and the heat results in the formation of a radial temperature distribution with its maximum at the center of the beam. Consequently a lens-like element is formed in the irradiated sample, due to the change in the refractive index. Due to the thermal conductivity on the samples part, the heat will be continuously dissipated into the environment. For this reason the thermal lens effect is time dependent and reaches a maximum value when the amount of heat released during the radiationless relaxation processes, is in equilibrium with the heat dissipated into the environment (steady-state thermal lens).

Different theoretical models (Weimer W.A., 1985; Dovichi N.J., 1984) have described mathematically the time and position dependent changes in beam centre intensity during the thermal lens experiment. The theoretical models range from relatively simple formulations to complex and sophisticated derivations. These models cover thermal lens effects, generated under different excitation conditions (pulsed or continuous wave excitation), different pump/probe geometries (single and dual beam, collinear and crossed beam configurations) and different sample conditions (stationary and flowing samples).

These derivations have often been simplified. One approximation is used for most analytical applications, where the magnitude of the thermal lens effect is usually measured as relative change in the beam centre intensity.

The relative change in the probe beam intensity is described in this relationship:

$$\frac{\Delta Ibc}{Ibc} = \frac{2.303 \times P \times (-dn/dT) \times A}{\lambda \times k} \qquad \text{(Eq.1)}$$

Where :

$\frac{\Delta Ibc}{Ibc}$	is the relative change in the beam centre intensity
P	is the excitation laser power
$\delta n / \delta T$	is the sample's temperature coefficient of the refractive index
A	is the absorbance of the sample
λ	is the probe beam wavelength
k	is the thermal conductivity of the sample

From this relationship we can see that the sensitivity of the technique could be increased simply by increasing the laser power and/or improving the thermo-optical properties of the sample medium, i.e., the thermal conductivity and temperature coefficient of refractive index.

Higher TLS signals can be obtained when experiments are performed in solvents with more favourable thermo-optical properties than water. As seen from the equation (Eq.1) high temperature-dependence of refractive index and low thermal conductivity are preferred for TLS detection. Water is the most common medium used in analytical chemistry, but due to relatively low temperature-dependence of refractive index and high thermal conductivity, water provides a low enhancement factor (E), which is defined in equation 2 (Eq.2). For this reason water is not preffered for TLS detection. Organic solvents induce much higher enhancements and are for this reason more suitable for TLS measurements.

$$E = -\frac{dn}{dT} / 1.91\lambda \times k \qquad \text{(Eq. 2)}$$

Using adequate laser power, absorbances on the order of 10^{-7} absorption units have been measured by TLS (Dovichi N.J., 1987).

2.5 Flow Injection Analysis (FIA) system

The concept of flow injection analysis (FIA) was introduced in the mid-seventies. It was preceded by the success of segmented flow analysis, mainly in clinical and environmental analysis. Flow Injection Analysis (FIA) is an analytical technique based on fluid manipulation of samples and reagents. Samples are injected into a carrier/reagent solution which transports the sample zone into a detector while chemical or biochemical reactions take place. The detector continuously records the changes in absorbance, electrode potential or other physical parameters resulting from the passage of the sample material through the flow cell.

The general FIA protocol comprises of the following steps (*Figure 6*) :

A) injection of an exact volume of analyte solution into a flowing stream of reagent.

B) sample (red zone) moves downstream and the sample solution disperses into reagent forming a reaction product (yellow). The grade of mixing and the length of reaction time are controlled by the flow rate, by channel volume and geometry.

C) the reaction mixture flows through the detector producing a response.

D) the peak height recorded by the detector is proportional to the analyte concentration.

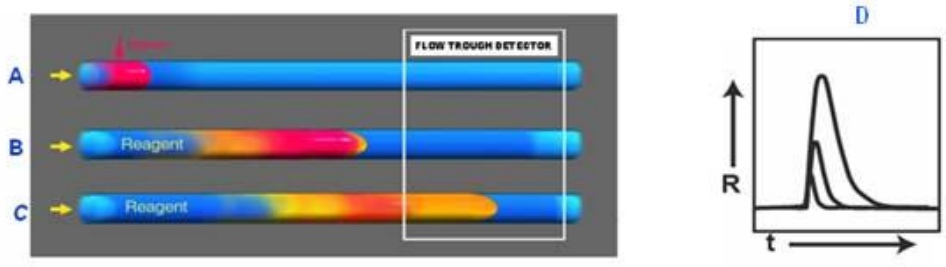

Figure 6: *FIA sample steam and resulting peak diagram.*
(Taken from: FIAlab, principles of flow injection: www.flowinjection.com)

The fast and intensive development of the FIA methodology was due to several factors essential for routine analytical determinations, such as very limited sample consumption, the short analysis time based on a transient signal measurement in a flow-through detector and an on-line carrying out difficult operations of separation, preconcentration or physicochemical conversion of analytes into detectable species.

3 Experimental

3.1 Materials

Four biogenic amines (BA) were used in our studies: tyramine hydrochloride (98%) and histamine dihydrochloride (98%) purchased from Alfa Aesar (Karlruhe, Germany), putrescine hydrochloride (98%) purchased from AppliChem (Darmstadt, Germany), cadaverine hydrochloride (98%) from Sigma-Aldrich (Seelze, Germany).

Transglutaminase (E.C. 2.3.2.13, 1670 nkat/g) was purchased from Ajinomoto Co. INC., Japan (Europe Sales GMBH, Hamburg, Germany), transglutaminase from guinea pig liver (E.C. 2.3.2.13, 2 UN) and N-carbobenzoxy-L-glutaminylglycine (CBZ-Gln-Gly) were purchased from Sigma-Aldrich (Seelze, Germany).

In the solid phase extraction (SPE) studies cartridges C18 from Sigma-Aldrich (Seelze, Germany) were used. For cartridge conditioning a pure methanol solution (Sigma-Aldrich Seelze, Germany) and deionized water (18.0 MΩ-cm^{-1} deionized water; Nanopure, Barnstead, Germany) was used for phenylalquinlamines (histamine and tyramine) and pure methanol solution (Sigma-Aldrich Seelze, Germany) and HCO_3 solution at pH 12 was used for alquilamines (putrescine and cadaverine). For sample elution pure methanol solution (Sigma-Aldrich Seelze, Germany) was used in case of phenylalquinlamines and 2% of glacial acetic acid solution (Fluka, Seelze, Germany) in the other case.

For the enzyme immobilization controlled-pore glass (CPG 240, 80-120 mesh) was purchased from Sigma Chemicals and Sigma-Aldrich (Seelze, Germany), 50% glutaraldehyde solution and 3-amminopropyltriehoxysilane were obtained from Merck.

For the indophenol blue method reagents used were sodium salicylate (99.5 %) purchased from Sigma-Aldrich (Seelze, Germany), sodium hydroxide from Riedel-de-Haën (Seelze, Germany), solution of sodium hypochlorite (Pejo, Slovenia), potassium sodium tartrate tetrahydrate (99 %) and manganese sulfate monohydrate (98 %) from Fluka (Seelze, Germany).

Organic solvents, acetonitrile and ethanol, used for TLS measurements were purchased from Sigma-Aldrich (Seelze, Germany).

3.2 Preparation of solutions

3.2.1 Solution for indophenol reaction

All solutions used were prepared using deionized water (18.0 MΩ-cm-1), prepared using a Nanopure system (Barnstead, Germany).

A stock solution containing 100 mg/L NH_4^+ was prepared by dissolving 0.297g of ammonium chloride in 1L of deionized water (18.0 MΩ-cm^{-1}). Working solutions were prepared daily by diluting a stock solution of ammonium chloride accurately.

Reagents preparations are described in *Table 1*:

Table 1: Concentration of used reagents.

REAGENTS	***CONCENTRATION***
Sodium salicylate	1.5 M
Sodium potassium tartrate tetrahydrate	30 g/L
Manganese sulphate monohydrate	2.5×10^{-3} M
Sodium hydroxide	0.5 M
Hypochlorite	Commercial bleach (40g/L of active Cl^-)

3.2.2 Solution for enzymatic reaction

The standard solutions of biogenic amines were prepared by dissolving each of them separately in a water solution. These standard solutions were stored in glass containers at 4° C.

Enzyme substrate (2mM) N-carbobenzoxy-L-glutaminylglycine (CBZ-Gln-Gly) was dissolved in 0.5 M NaOH.

Enzymes (MTGase and GTGase) were opportunely dissolved in cold water in order to obtain enzyme activity 16.7 nkat/mL (1 U/mL).

3.3 Indophenol blue method for biogenic amines determination exploiting enzymatic reaction

Two mixtures of reagents were prepared for the indophenol blue method. Mixture A contained 10 mL of sodium salicylate, 10 mL of potassium sodium tartrate and 2 mL of manganese sulphate prepared with appropriate dilution of corresponding stock aqueous solutions. Mixture B, which contained 10 mL of sodium hydroxide and 10 mL of hypochlorite was also prepared with an appropriate dilution of corresponding stock aqueous solutions. Each mixture was freshly prepared daily.

One mL of each mixture (A and B) was introduced in a 10 mL flask with one mL of CBZ-Gln-Gly, one mL of enzyme solution, one mL of BA of interest, and a mixture of acetonitrile/water (1/1; v/v) or other solvent,in order to obtain the final volume of 10 mL. Then the solution was transferred in a quartz cuvette (batch mode measurement) and introduced either in a UV-Vis spectrophotometer (absorbance measured at 650nm) or exposed in a TLS cuvette holder.

3.4 Thermal lens detection system

In the study two dual-beam detection units were built and tested:

3.4.1 He-Ne detection unit

A high power He-Ne laser (*Figure 7*) operating at 632.8 nm (35mW, MellesGriot, Carlsbad, California, USA) used as an excitation source and a relatively weaker He-Ne laser operating at 543.5 nm (5mW, MellesGriot, Carlsbad, California, USA) as a source of the probe beam. Separate lenses were used to focus the excitation beam directly onto the sample and to mismatch the beam waists of the pump and the probe beams. A variable-speed mechanical chopper modulated the pump laser beam at 30 Hz. In order to achieve optimal sensitivity a good spatial overlapping of both beams inside the sample is necessary. The alignment of the two beams was facilitated by a dichroic mirror. The generated thermal lens produces fluctuations in the radial intensity distribution of the probe beam that is monitored by a PIN photodiode, placed behind a filter which filters out the excitation laser beam. The photodiode is connected to a lock-in amplifier (Stanford Research System, Sunnyvale, California, USA) and a personal computer for data acquisition.

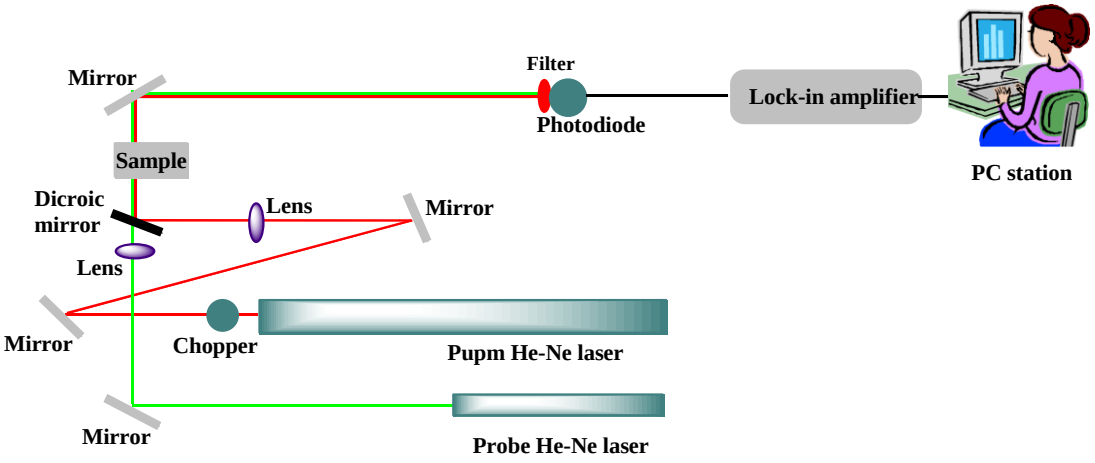

Figure 7: *Schematic representation of the He-Ne detection unit.*

3.4.2 Kr-detection unit

A powerful Kr-laser (*Figure 8*) operating at 647 nm (200mW,) as excitation source and a relatively weaker He-Ne laser operating at 543.5 nm (5mW, MellesGriot, Carlsbad, California, USA) as a source of the probe beam was used. Separate lenses are used to focus the excitation beam directly onto the sample and to mismatch the beam waists of the pump and the probe beams. A variable-speed mechanical chopper modulated the pump laser beam at 30 Hz. For optimal sensitivity a good spatial overlapping of both beams inside the sample is necessary. A dichroic mirror facilitates the alignment of the two beams. The fluctations generated by the thermal lens is monitored by a PIN photodiode, behind a filter which filters out the excitation laser beam. A lock-in amplifier (Stanford Research System, Sunnyvale, California, USA) is connected to photodiode and a personal computer is linked for data acquisition.

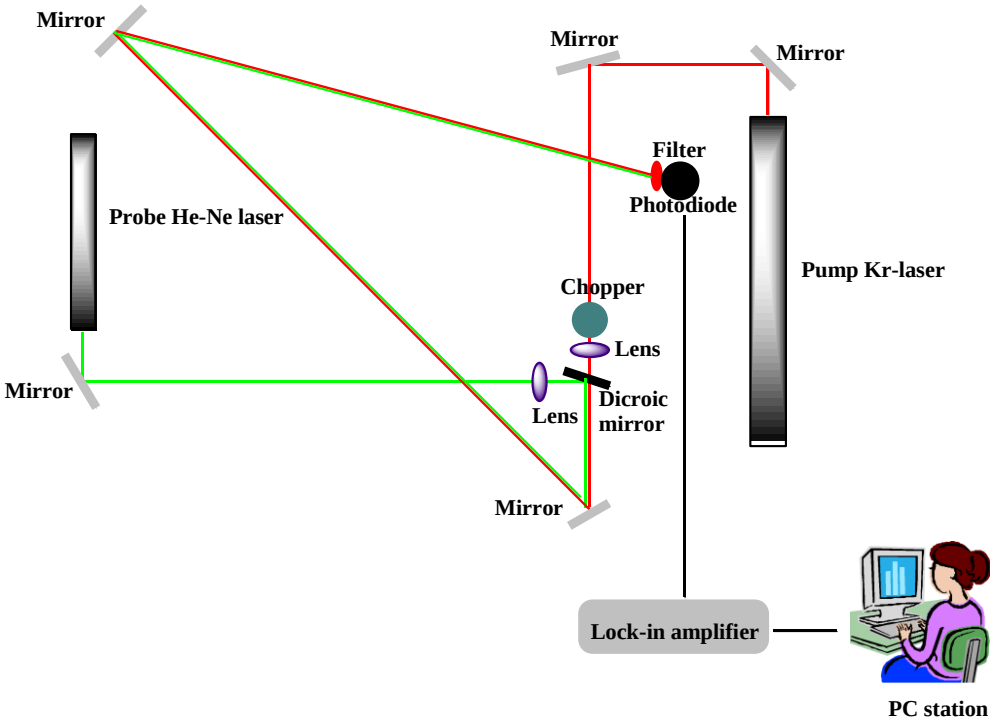

***Figure 8**: Schematic representation of Kr-detection unit.*

3.5 Bioanalytical system

3.5.1 Enzyme immobilization on glass beads

TGases enzymes were immobilized using the method based on cross-linking with glutataldehyde and binding to the activated controlled porosity glass – CPG (Pogačnik and Franko, 2001).

Immobilization consists of 4 fundamental steps:

Pre-cleaning step: CPG-240 was boiled 5% nitric acid for 30 min. The CGP-240 was filtered on a sintered glass filter, washed with deionized water and dried in an oven at 95° C. The whole procedure with water washing was repeated twice.

Aminoalkylating step: diluted 3-aminopropyltriethoxysilane (98%) was adjusted to pH 3.5 with HCl acid. The dried CGP-240 was added to the aminoalkylating solution and swirled for 150 min in a water bath at 75° C. The beads were filtered, washed with deionized water and dried at 95° C until the moisture was completely

evaporated. The alkylamination process was repeated again to ensure complete activation of the glass.

Cross-linking: the cross-linking agent was a solution of glutaraldehyde. A solution of glutaraldehyde was prepared in a phosphate buffer. Aminoalkilated glass was added to glutaraldehyde-buffer solution in a vessel, which was put under nitrogen gas atmosphere for 1 hour in order to remove air from CPG-240. Glass was then washed well in deionized water and the cross-linking process was repeated once more.

Enzyme immobilization: TGases were dissolved in a cold (4°C) phosphate buffer (0.1 M, pH 6.5) and then added to the pre-treated glass. Solutions were again put under the nitrogen atmosphere for 1 hour. Immobilized enzymes were washed with a cold phosphate buffer in order to ensure the removal of any unlinked enzyme. The glass beads with immobilized enzymes were stored at 4° C in a phosphate buffer.
No decrease in activity of stock immobilized enzymes was observed after storage for 3 months. Immobilized enzyme was packed into a bioanalytical column as needed.

3.5.2 Protein quantization – Bradford reaction

To ensure that the enzyme was linked onto glass beads a simple method was used.
Bradford method was used to verify and approximately evaluate the presence of protein on the glass beads. It is based on the observation that the absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465 nm to 595 nm when binding to protein occurs. 200μL of dissolved enzyme linked on glass-beads and 800μL of Bradford reagent were mixed in a plastic cuvette. With a spectrophotometer the absorbance of the sample was measured at 595nm against a mixture of Bradford reagent and buffer without enzyme (blank). The concentration of protein was calculated by a calibration curve made with the same enzyme.

3.5.3 Transglutaminase activity

Transglutaminase (TGase) activity was measured by the colorimetric hydroxamate procedure with N-carbobenzoxy-L-glutaminylglycine (CBZ-Gln-Gly). The test was made in a plastic cuvette in a final volume of 1.2 mL of 1000mM Tris Buffer pH 6.0,

containing 200mM of hydroxylamine, 20mM glutathione reduced form, 1000mM $CaCl_2$, 35mM CBZ-Gln-Gly. After 10 minutes of incubation with the enzyme at 37°C, 12% (v/v) trichloroacetic acid reagent and 5% (w/v) ferric chloride solution was added, and the resulting red colour was measured at 525 nm. An enzyme unit is defined as the amount of enzyme which catalyzed the formation of 1.0 μmole of hydroxamate per minute from N-carbobenzoxy-L-glutaminylglycine (CBZ-Gln-Gly) and hydroxylamine at pH 6.0 at 37°C. (L-Glutamic acid γ-monohydroxamate is the standard).

In the activity test for TGase in organic media like acetonitrile and ethanol these solvents were added at the end and we only look for spectral shift.

3.5.4 Bioanalytical FIA setup

Experiments were performed using flow-injection analysis (FIA) manifold with and without immobilized TGases. The "in-house" constructed FIA manifold consisted of two HPLC pumps (two Knauer Smartline pump or one Shimatzu LC-10Ai), one injection Knauer valve, a bioanalytical column with immobilized enzyme, a reaction coil and the TLS detection unit with electronics (Lock-in amplifier and computer, *Figure 9*).

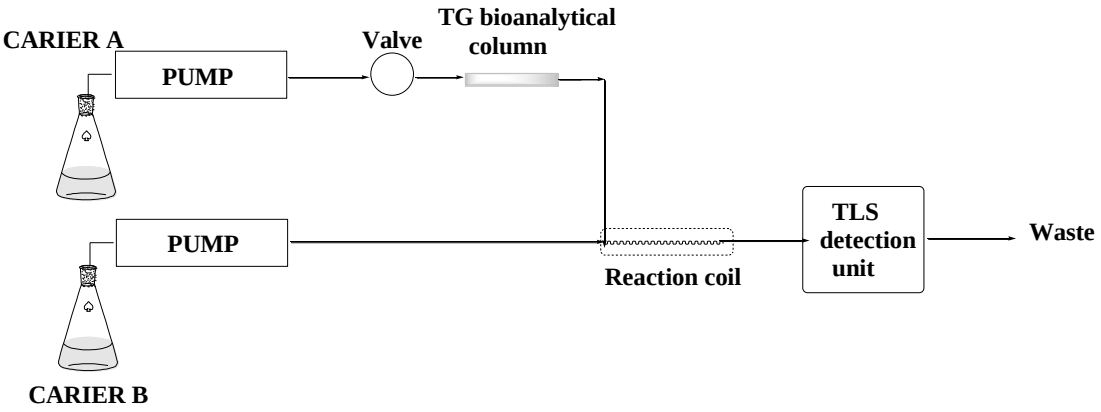

***Figure 9**: Bioanalytical FIA setup, consisting of two HPLC pumps, one injection valve (200 μL inj.loop), bioanalytical column with immobilized TGase enzyme, reaction coil and the TLS detection unit.*

Repeated sample injections (at least thrice) with a 200 µL injection loop were performed in order to verify the repreciabilty and minimize measurement errors.

Each bioanalytical column filling was used for several experiments in one day. Around 20-30 mg of glass beads with immobilized TGase enzyme were usually used to fill the column.

4 Results and discussion

4.1 Detection of ammonia by formation of indophenol

Our aim was to detect biogenic amines based on the formation of indophenol in Berthelot reaction for the determination of ammonia, which is released in an enzymatic reaction between biogenic amines and transglutaminase (TGase). The enzyme catalyses the reaction between biogenic amines and the enzyme substrate (CBZ-Gln-Gly), in which ammonia is released as a product.

The formation of indophenol was found to be time dependent, as evidenced by the increase in absorbance with time as already reported (Molins-Legua C., 2006). The highest rate of increase was observed during the first 500-600 seconds of reaction, as is evident from the slope of the curve (*Figure 10*). Based on this observation we decided to select 600 seconds time as sufficient for incubation in the indophenol formation to enable sufficiently rapid and sensitive determination of ammonia.

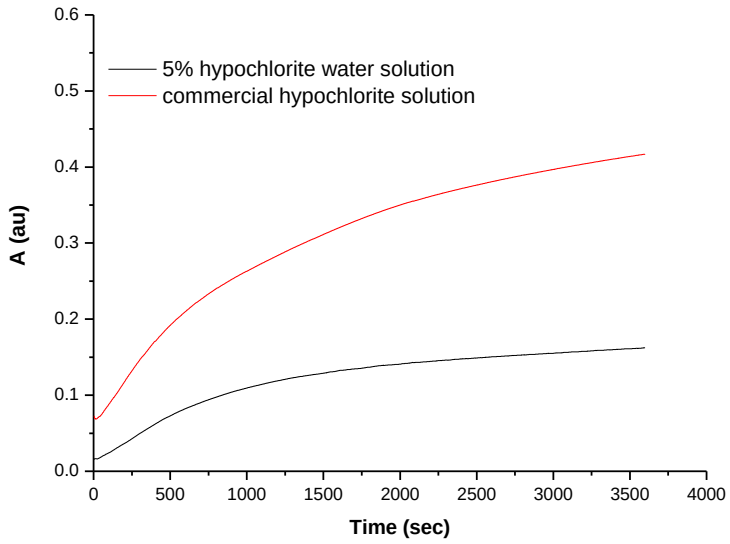

Figure 10: *Kinetic curves of the indophenol formation with 5% hypochlorite water solution and using commercial hypochlorite solution (50 mg/L NH_4Cl).*

Under such conditions the spectrophotometric detection of ammonia was attempted and the related calibration curve is shown in *Figure 11*. The achieved limit of detection was 78 ng/mL which compares favourably, and is in fact 2 times lower in

comparison to the LOD reported by Molins-Legua C. and coworkers (2006) (Table 2) which is based on the same reaction but using a lower concentration of hypochlorite.

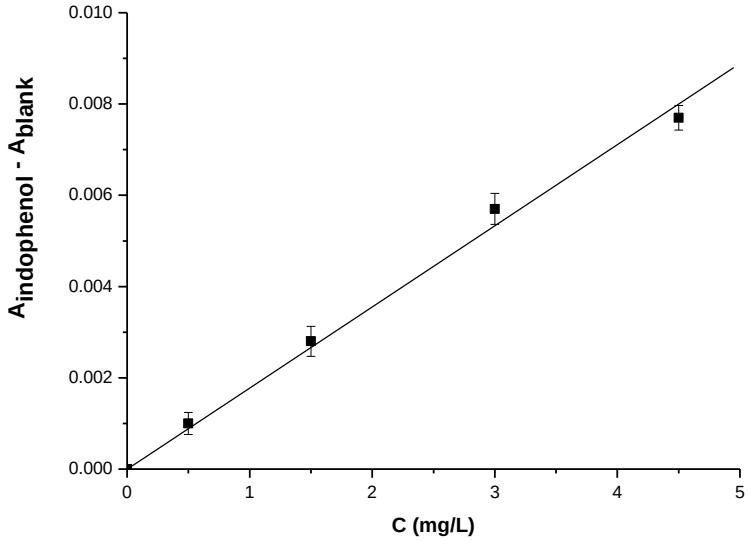

Figure 11: *Typical calibration curve for the spectrophotometric determination of ammonia based on indophenol formation in water after 10 minutes of incubation time (R= 0.997; k=0.178 A×L/mg).*

The improvement of LOD was presumably entirely due to use of a higher concentration of hypochlorite as evident from the differences in the kinetic curves for Berthelot reactions at different concentration of hypochlorite (*Figure 10*).

4.1.1 Influence of organic solvents on the Berthelot reaction (indopehnol UV-Vis spectra)

In the following section the indophenol blue method was tested in different organic solvents to exploit, in a best possible way, the thermooptical properties of solvents and the resulting enhancement of the thermal lens signal. Spectrophotometric measurements were performed initially to observe possible effects of organic solvents on spectral properties of indophenol.

The influence of acetonitrile and ethanol, as the two most frequently applied organic solvents as additives to water in thermal lens spectrometry was investigated (see

paragraph 4.1.2.). Ammonium chloride was used as a standard in measurements of eventual spectral shifts caused by the organic solvent (*Figure 12*).

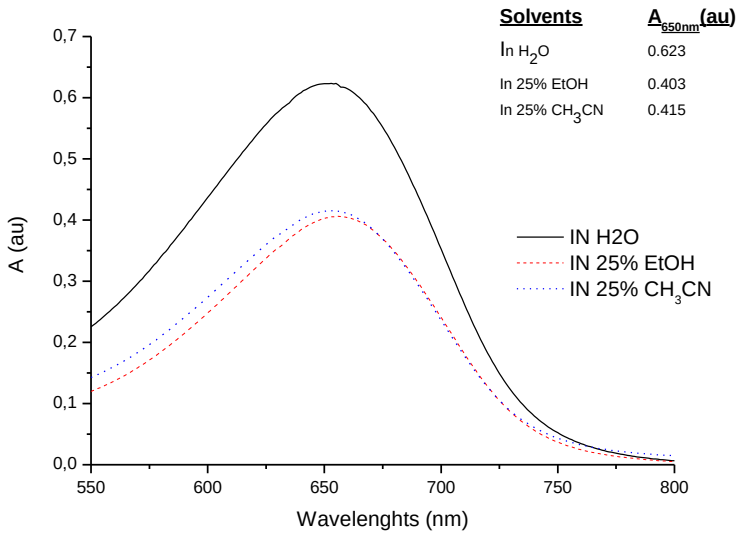

Figure 12: *Absorbance spectrum of indophenol in water, in 25% ethanol and in 25% acetonitrile/water mixture (concentration of NH_4Cl = 250 mg/L) after 600 seconds of incubation.*

The measurements of the absorbance spectra of indophenol in the presence of organic solvents were performed by running the Berthelot reaction in aqueous solution and in both organic solvents.

No significant change in the position of λ_{max} (650 nm) was observed, while the absorbance of indophenol in 25% acetonitrile/water mixture and in 25% ethanol/water mixture after 10 minutes of incubation was 1.5-times lower when compared to absorbance in water respectively (*Figure 12*).

The difference in the absorbance spectrum is mainly due to different reaction rates in different solvents, as visible in *Figure 13*, where the slope of the curve showing indophenol formation is lower in acetonitrile in comparison with the slope in water.

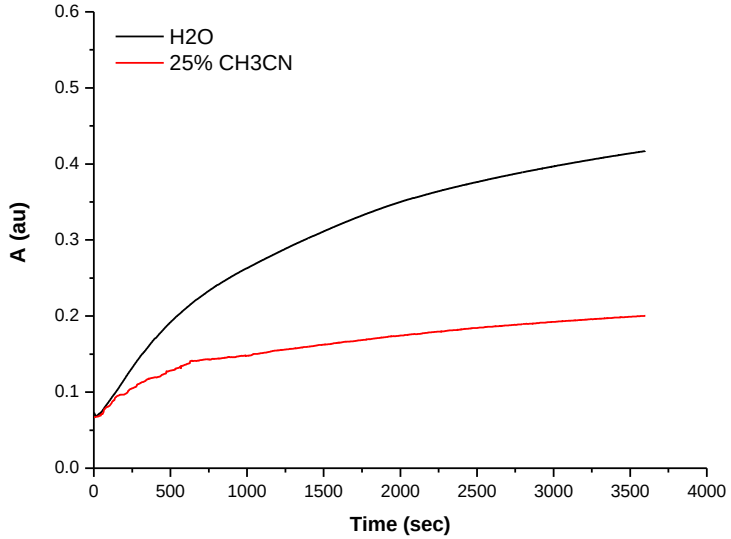

Figure 13: *Kinetic curves of indophenol formation in water and in 25% acetonitrile water solution (NH_4Cl 50 mg/L).*

The limit of detection (*Table 2*), which was observed in water in comparison to the LOD in 25% acetonitrile/water mixture was 1.68 times lower, in accordance with the difference in absorbance maxima (*Figure 12*).

Table 2: *Table of calculated LOD for indophenol formation on the spectrophotometer.*

	THIS WORK	***MOLINS-LEGUA METHOD***
LOD IN WATER (ng/mL)	78	160
LOD IN 25 % ACETONITRILE (ng/mL)	131	--

4.1.2 Enhancement (E) factors in TLS

As was already discussed in the introductory section, the TLS signal is considerably affected by the solvent used in the measurements. Different organic solvents have different thermo-optical properties [temperature coefficient of refractive index (dn/dT) and thermal conductivity (k)], and these affect the TLS signal. The lowest signal is obtained with the use of water. For this reason we studied two different

solvents and their effect on indophenol reaction. The enhancement of the thermal lens signal in organic solvents, with respect to that in water, was due to higher dn/dT and a lower k.

For calculation of the enhancement factor the values for thermo optical properties, i.e. dn/dT and k of pure solvents were taken from Bialkowski S.E. (1996), and are presented for acetonitrile and ethanol (*Table 3*). For mixtures of liquids or solutions, the information on their thermo-optical properties is usually unavailable, and these values should be measured or calculated for each mixture. Most frequently, the enhancement factors for liquid mixtures are estimated assuming that dn/dT and k are linear functions of the volume fraction of each solvent or in case of *k* by using some empirical relations for calculation of its value from the thermal conductivities of pure solvents (Weimer W.B., 1986).

As shown for the case of acetonitrile, in this work the following relation (Eq.3) was used:

$$\frac{dn/dT}{k} = \frac{F_{H_2O}(dn/dT)_{H_2O} + F_{CH_3CN}(dn/dT)_{CH_3CN}}{F_{H_2O}k_{H_2O} + F_{CH_3CN}k_{CH_3CN} - 0.72F_{H2O}F_{CH_3CN}(k_{CH_3CN} - k_{H_2O})} \qquad \text{(Eq.3)}$$

where F is the volume fraction of the specified solvent. The calculated values for the enhancement factor are given in *Table 3*, *Table 4* and *Table 5*.

Table 3: *Thermooptical properties of water, acetonitrile and ethanol.*

SOLVENT	k /(W m^{-1} K^{-1})*	(dn/dT)/(10^{-4} K^{-1})*	E (W^{-1})
Water (100%)	0.598	-0.91	146
Acetonitrile(100%)	0.188	-4.50	2306
Ethanol (100%)	0.169	-4.00	2280

*Thermooptical properties were taken from Bialkowski S.E. 1996. Enhancement factors were calculated for 543.5 nm.

Table 4: Calculated thermooptical properties of acetonitrile/water mixtures.

SOLVENT	$-\frac{dn/dT}{k}(m/W)^{*}$	E (W^{-1})	E (W^{-1}) × C^{**}
Acetonitrile(10%)	2.17×10^{-4}	209	188
Acetonitrile(25%)	3.28×10^{-4}	315	236
Acetonitrile(40%)	4.65×10^{-4}	448	269
Acetonitrile(50%)	5.79×10^{-4}	558	279
Acetonitrile(60%)	7.25×10^{-4}	698	279
Acetonitrile(80%)	1.19×10^{-3}	1146	229
Acetonitrile(90%)	1.62×10^{-3}	1561	156

*Thermooptical properties were taken from Bialkowski S.E. 1996. Enhancement factors were calculated for 543.5 nm.

** C= dilution factor

Table 5: *Calculated thermooptical properties of ethanol/water mixtures.*

SOLVENT	$-\frac{dn/dT}{k}(m/W)^{*}$	E (W^{-1})	E (W^{-1}) × C^{**}
Ethanol (10%)	2.11×10^{-4}	203	183
Ethanol (25%)	3.06×10^{-4}	295	221
Ethanol (40%)	4.30×10^{-4}	414	248
Ethanol (50%)	5.33×10^{-4}	513	256
Ethanol (60%)	6.66×10^{-4}	722	253
Ethanol (80%)	1.11×10^{-3}	1069	214
Ethanol (90%)	1.54×10^{-3}	1483	148

*Thermooptical properties were taken from Bialkowski S.E. 1996. Enhancement factors were calculated for 543.5 nm.

** C= dilution factor

The calculated enhancement factors show the expected increase with volume fraction of organic solvent. The highest effective enhancement factor, which is reflected in the final sensitivity of the method, is however, predicted for volume fraction of 0.5 acetonitrile and 0.6 in the case of ethanol (*Figure 14*), because at a higher volume fractions the inevitable dilution of the sample contributes to the decreased sensitivity more than is the increase from the enhancement factor.

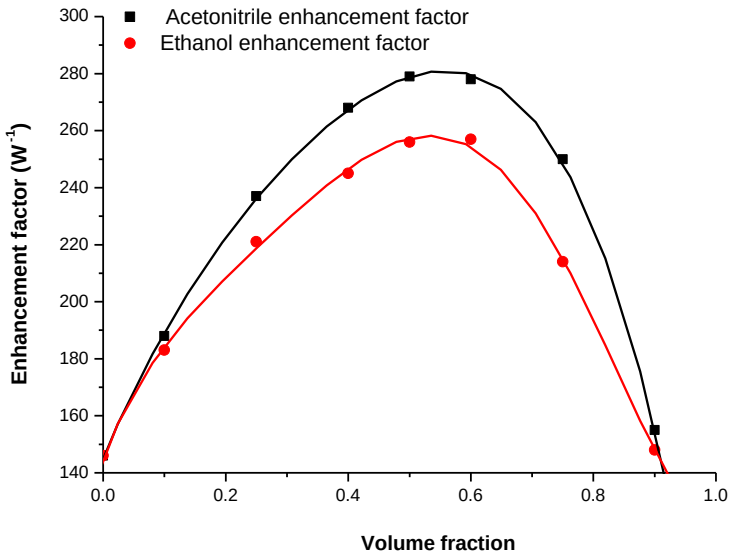

Figure 14: *Plot of the calculated effective enhancement factor as a function of the acetonitrile or ethanol volume fraction. The curve is a plot of E values calculated from Eq. (1) and multiplied by dilution factors.*

Based on the enhancement factors of two investigated solvents, we have chosen acetonitrile/water mixture that assures a higher TLS signal due to the higher enhancement factor.

4.1.3 TLS determination of ammonia

As already discussed in the previous section, an addition of organic solvents increases the TLS signal. A higher enhancement factor was predicted for 25% acetonitrile solution (0.236 mW^{-1}) and 25% ethanol (0.221 mW^{-1}) when compared to water (0.146 mW^{-1}). This 1.62-times higher enhancement factor actually cancels out

over a 1.5-times decrease in sensitivity due to changes in absorbance and reaction rate of indophenol formation, discussed earlier. Therefore, improvement in sensitivity, compared to spectrophotometry, can still be predicted for laser powers higher than 7 mW.

As in spectrophotometric measurements, in this case also the calibration curve was prepared in order to compare the limit of detection for both methods (*Figure 15*). The application of TLS detection resulted in 9.8 times lower limit of detection as compared to spectrophotometric measurements in water. In comparison to spectrophotometric detection in 25% acetonitrile the improvement was even higher (16 times) which is due to the effect of acetonitrile on spectral properties and the reaction rate of indophenol blue formation and increased standard deviation of blank measurement (*Table 6*) in case of spectrophetometry.

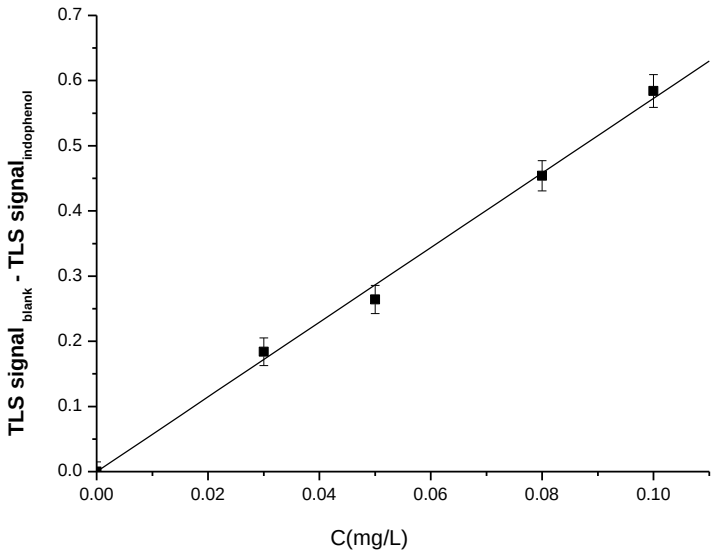

Figure 15: *Calibration curve for the determination of ammonium by TLS with He-Ne as excitation laser, based on indophenol formation in 25% acetonitrile/water mixture after 10 minutes of incubation time (R=0.998; k= 5.73 mV*×L*/mg).*

In addition, TLS provides about a 10 times higher signal to noise ratio, which seems to be the main reason for the significant improvement of LOD in the case of TLS.

Table 6: *Table of calculated LOD for indophenol formation on the He-Ne TLS detection unit.*

	Spectrophotometer in H_2O	***Spectrophotometer in 25% CH_3CN***	***He-Ne TLS detection unit***
LOD	78 ng/mL	131 ng/mL	8 ng/mL
SD of blank	4.55×10^{-3} au	6.5×10^{-3}au	0.015 mV
Average blank signal	0.023 au	0.032 au	0.758 mV
S/N	5.05	4.92	50.5

4.1.3.1 Recovery test

Accuracy of the method was tested by analysing water samples fortified with different levels of NH_4^+. Results are presented in *Table 7* and demonstrate comparable agreement of measured concentrations with true values.

Table 7: *Different concentration of fortified samples and recovery obtained.*

NH_4^+ concentration in the sample (µg/L)	***measured NH_4^+ (µg/L)***	***RECOVERY (%)***
30	32 ±3	104 ±10
50	46 ± 4	91.6 ± 8
100	99 ± 2	98.9 ± 4

4.1.3.2 Interferences in ammonia determination

The presence of amino acids in natural waters, which originate from microalgae and bacteria, interferes with determination of ammonia using the indophenol blue reaction. As demonstrated by Jüttner (1999), naturally occurring nitrogenous compounds interfere with modified indophenol blue reaction using salicilate, as in

this work. Among them L-aspargine, L-histidine, and glycine, which were observed to give between 3 and 42% positive interference in the determination of ammonium, respectively, at equimolar concentrations of interferents and ammonium. For this reason these three structurally different amino acids, available in the laboratory (asparagine, glycine, and histidine), which cover an almost entire spectrum of possible positive interferences by amino acids (0.8 – 56 %) (Jüttner F.,1999) were tested, in order to determine possible margins of errors due to interferences in the indophenol blue method applied in this work.
In all measurements the concentration used to estimate possible positive errors in the determination of ammonia due to the presence of amino acids was 0.5 mM for all species.

The relative errors due to the interference from amino acids, determined from the ratio of measured TLS signals for amino acids and the TLS signal for equimolar concentration of ammonium, were found to be 6% for asparagine, 12% for glycine and 20% for histidine.

Similarly to the results of Jüttner this work confirms the lowest contribution of asparagine to the interference. Overall the observed interferences from the selected amino acids show over 30% smaller contribution to positive errors, compared to the method described in the literature (Jüttner F., 1999). In particular the contribution of glycine is about 4 times lower than previously reported, while interference from histidine is about 2 times higher than reported. These differences are most probably due to the use of different catalysts in the Berthelot reaction (manganese sulphate instead of nitroprusside).

In natural waters the concentrations of all free amino acids on the order of 10^{-7} M can be expected. (Thuman E.M., 1985). This is comparable or lower than the LOD for determination of ammonium by this method. Therefore, significant positive errors from interfering amino acids can only be expected at extremely low concentrations of ammonium (below 10^{-6} M). At concentrations of ammonium ten times higher than the LOD of this TLS method the errors due to the presence of amino acids in natural waters are estimated at 1-2% or less.

The problem of the interferences can be in principle resolved also by using the indophenol reaction based on the phenol instead of salicilate. However, due to the toxicity of the phenol, this option was not exploited in this work.

However, in view of the final application of the newly developed method for determination of biogenic amines, the presence of amino acids in analysed samples, does not pose any limitation in terms of interferences, since during the extraction of biogenic amines the amino acids are separated due to their solubility in aqueous phase.

4.2 Detection of biogenic amines based on indophenol blue reaction

Previously optimized indophenol blue reaction was used as colorimetric method for the detection of biogenic amines.

During the MTGase enzymatic reaction with substrate (Cbz-Gln-Gly) and biogenic amine, ammonia is released and detected by exploiting indophenol blue reaction. Detection of structurally different biogenic amines (putrescine, cadaverine, histamine and tyramine) was investigated.

4.2.1 Influence of buffers an MTGase (UV-Vis measurements)

The optimum pH for MTGase is between 6 and 7 (specified by the producer). For this reason a study of buffer influence on indophenol blue reaction in the pH range 6-7 was necessary, since the recommended pH for indophenol formation is between 7-10.5 (Aminot, 1997).

Tested buffers were: phosphate buffer (pH 7), PBS buffer (pH 7), acetate buffer (pH 6) and Tris- buffer (pH 7) (usually used in TGase enzymatic activity test protocols). As is clearly visible in *Figure 16* the highest sensitivity of biogenic amine determination by MTGase and indophenol blue reaction is obtained in water and in a phosphate buffer at pH 7. Reactions in acetate and PBS buffer showed lower absorbance after 10 minutes of reaction compared to the phosphate buffer. This can be attributed to the presence of bivalent ions, that have a negative influence on the MTGase enzymatic activity and thermal stability, in our case the addition of $MgCl_2$ (Kütemeyer C., 2005) in PBS buffer. This effect was the strongest in case of tyramine. For acetate buffer the reason of a lower enzymatic activity is the acidic pH which is not favorable for indophenol blue reaction, where the alkaline environment

is of crucial importance for monoamine formation and subsequently for indophenol formation. For TRIS buffer, no absorbance was observed at 650 nm during the course of reaction (*Figure 17*).

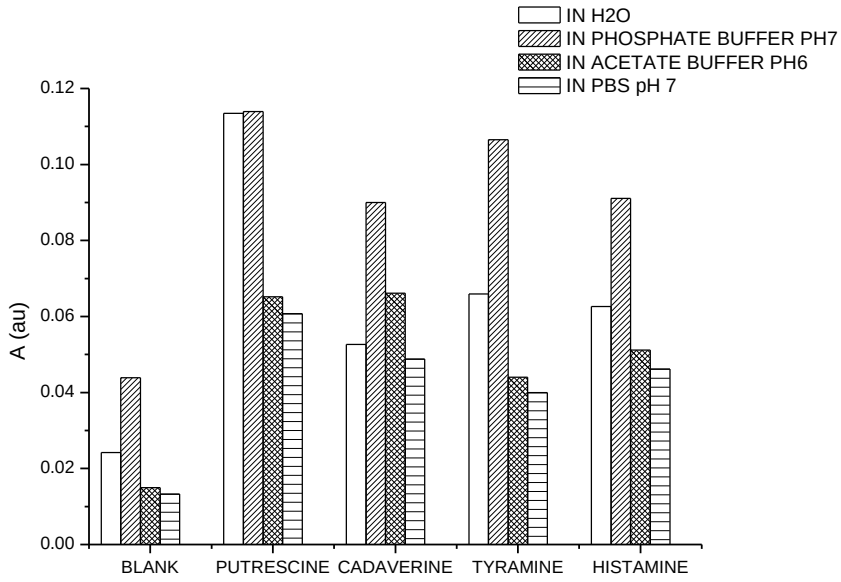

Figure 16: *Measured absorbances for indophenol blue formation after MTGasee enzymatic reaction in different buffer solutions (BAs concentration 2mM).*

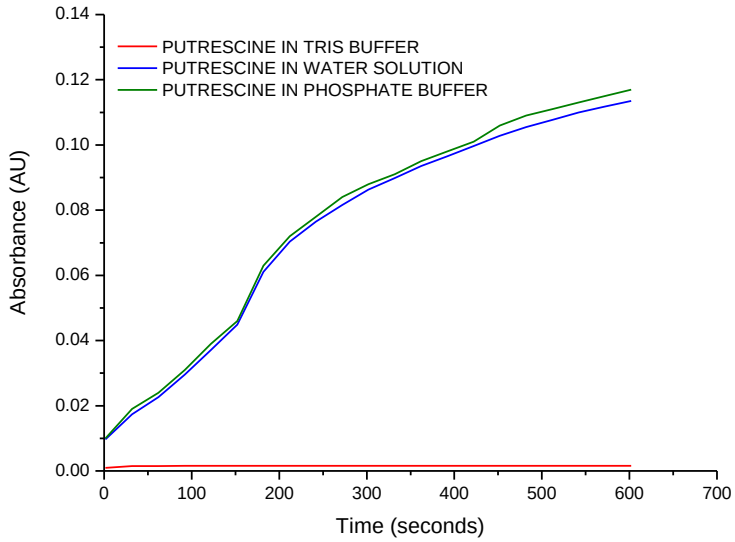

Figure 17: *Kinetic curves of indophenol formation using putrescine as representative biogenic amine (BA concentration 2mM) in different buffer solutions at 650nm.*

In all of the cases, the blank sample was prepared by introducing deionized water into the mixture of reagents instead of the BA of interest. The detected signal was due to the non specificity of MTGase, which is also capable of reacting with water in the absence of biogenic amines (Folk, 1983). In such a case the ammonium originates from the CBZ substrate.

4.2.2 Influence of organic solvents on MTGase activity

Enzyme activity can also be influenced by the presence of organic solvents (Gekko K., 1998). To evaluate TGase activity in the presence of organic solvents added to incease the sensitivity of TLS detection, activity test for transglutaminases (TGase) was performed in ethanol/water and acetonitrile/water mixtures according to published procedures (Folk J. E., 1966a and 1966b). TGase activity is usually measured in a TRIS buffer, but since the TRIS buffer was shown to interfere with the indophenol formation (section 4.2.1) the effects of ethanol and acetonitrile were compared to TGase activity in water. Solutions of TGase were prepared in water, in 25% ethanol/water mixture and in 25% acetonitrile/water mixture. Measurements of the TGase-substrate reaction product (Cbz-Gln-Gly hydroxamate) showed comparable absorbances at 525nm (Chung, S.I., 1970) in case of water and acetonitrile/water mixture and an about 25% increase in activity and consequently absorbance in ethanol/water mixture. Similar effects for ethanol were already described in the literature (Cui L., 2008).

4.2.3 Determination of BAs with He-Ne-TLS detection unit (batch mode)

The magnitudes in TLS signals resulting from the formed indophenol were plotted against the concentration of biogenic amines (*Figure 18* and *Figure 19*) in order to obtain the calibration curve for each of investigated BAs. It is clearly visible, that the calibration curves are not linear. The nonlinearity is mainly attributed to the saturation of the TLS signal, which is caused by the high blank signal originating from ammonium (and/or amino compounds) present in deionized water, as confirmed by ion chromatography (25 ng/mL). The nonlinearity for the given

detection system is observable at signals above 0.6 mV and does not affect the determination of cadaverine, for which the lowest sensitivity was achieved. In any case, the nonlinearity does not hinder the applicability of the developed method for the purpose of fast screening, where only a binary response is required (BAs present or not present at a concentration above selected limit). However, for the calculation of the LODs the slopes of calibration curves were required and for this purpose only the concentrations within the linearity range for a given BA were considered. The resulting curves have similar, and acceptable correlation, coefficient between 0.980 and 0.996. Limits of detection calculated for each of biogenic amines are listed in *Table 8* and are in good agreement with LODs achieved for the determination of ammonia (section 4.1). Actually, the LODs are just slightly higher than those achieved for the determination of ammonium (expressed in molar concentrations), when considering the formation of one ammonium per molecule of a biogenic amine.

Table 8: Table of merit for batch mode determination of BAs with He-Ne TLS detection unit.

	Putrescine	***Cadaverine***	***Histamine***	***Tyramine***
LOD (M)	5.2×10^{-7}	1.4×10^{-6}	1.5×10^{-6}	2.0×10^{-6}
Average blank signal (V)	2.1×10^{-3}	2.1×10^{-3}	2.1×10^{-3}	2.1×10^{-3}
RSD of blank (%)	4.1%	4.1%	4.1%	4.1%
Linearity range (M)	1.0×10^{-6} 2.5×10^{-6}	1.0×10^{-6} 2.5×10^{-6}	1.5×10^{-6} 2.5×10^{-6}	1.5×10^{-6} 2.5×10^{-6}

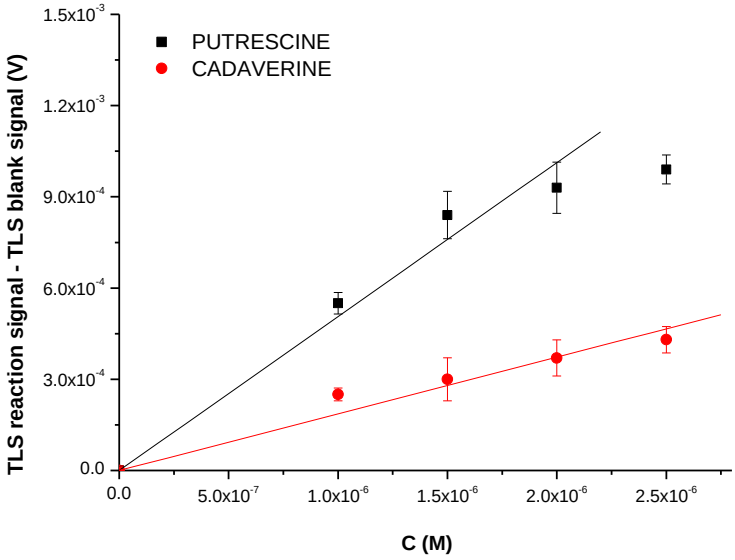

Figure 18: *Calibration curves for putrescine and cadaverine recorded by the He-Ne detection unit ($R_{putrescine}$=0.987, $k_{putrescine}$=506 V,; $R_{cadaverine}$=0.980; $k_{cadaverine}$=186 V/M).*

(Data for putrescine at 2.5×10^{-6} M was omitted in the linear fit.)

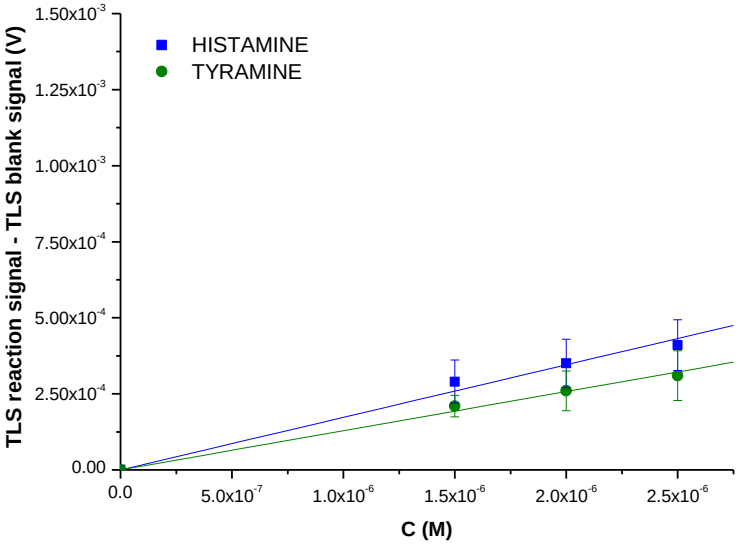

Figure 19: *Calibration curves for histamine and tyramine detected by the He-Ne detection unit ($R_{histamine}$=0.993, $k_{histamine}$=173 V/M; $R_{tyramine}$=0.996; $k_{tyramine}$=129 V/M).*

When compared to a previously reported method for BA determination using MTGase (Punakivi K., 2006), the LODs achieved in this work represent a 44 fold improvement in case of histamine, 30 fold improvement in case of tyramine and 71 fold improvement in case of cadaverine, while in case of putrescine this improvement is even 135 fold. In addition to the TLS enhancement of sensitivity by 4.4 times for the 30 mW excitation power, such improvement in LOD is attributed also to 13.5-times higher extinction coefficient of indopehnol at 650 nm compared to the extinction coefficient of NADH absorption maximum at 340 nm which was related to a concentration of BA in the method developed by Punakivi. Besides the significant improvement of the LODs it has to be pointed out that previously reported TGase method requires two hours of reaction time, to achieve the indicated LODs, while the newly developed method described in this work requires just 10 minutes of reaction time, thus reducing the time of analysis by 12 times.

4.2.4 Determination of BAs with Kr-TLS detection unit (batch mode)

To further improve the limits of detection for BAs the determination achieved with the He-Ne TLS detection unit, a new powerful TLS system was constructed exploiting the higher power of a Kr-laser (200 mW) which also better matches the absorbance of indophenol at 650 nm. With the previous system the excitation laser emission line was at 632.8 nm, in this case the emission line of the Kr-laser was 647 nm, much closer to the absorption maximum of indophenol blue at 650 nm. This contributes to higher absorbance by 1.08 times. This setup in general allows detection of at least 10 times lower concentrations (10^{-8} M concentration range) of BAs compared to the He-Ne detection unit (*Figure 20* and *Figure 21*).

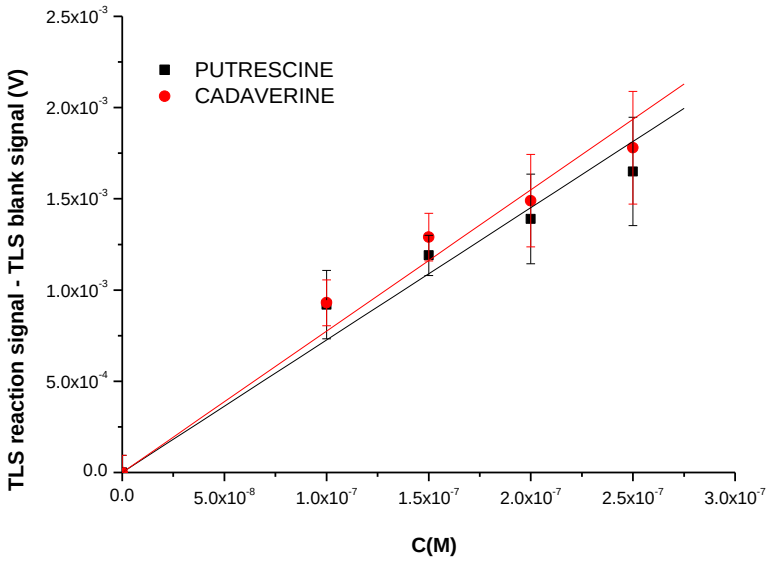

Figure 20: *Calibration curves for putrescine and cadaverine detected by the Kr-TLS detection unit ($R_{putrescine}$=0.964, $k_{putrescine}$=7258 V/M; $R_{cadaverine}$=0.976; $k_{cadaverine}$=7745 V/M).*

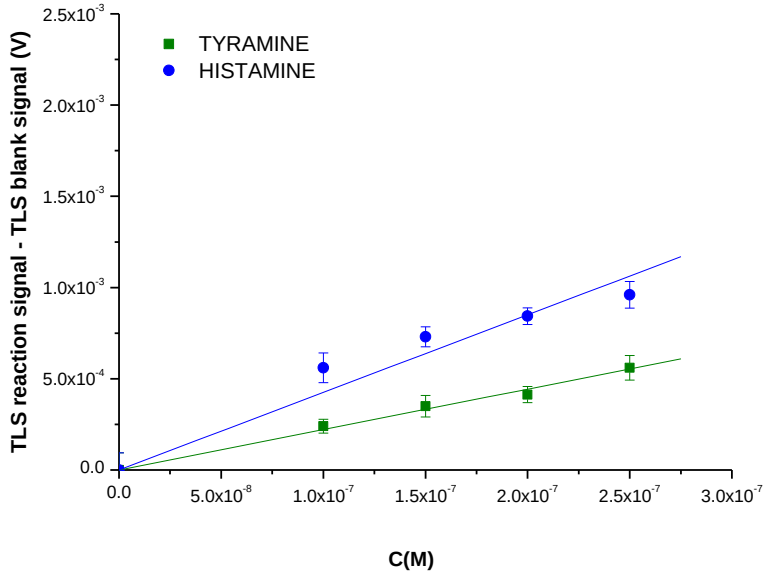

Figure 21: *Calibration curves histamine and tyramine detected by the Kr-TLS detection unit ($R_{histamine}$=0.975, $k_{histamine}$=4253 V/M; $R_{tyramine}$=0.996; $k_{tyramine}$=2215 V/M).*

The LODs (*Table 9*) reported for the four investigated biogenic amines are between 12 to 32 times lower compared to LODs achieved by the He-Ne detection unit. Part of this improvement is due to a 7-times higher excitation laser power provided by the Kr-laser and to better matching of the excitation wavelength with the absorption maximum of indophenol blue. Additional improvement can only be attributed to better alignment of the system, particularly to the proper positioning of the sample cell with respect to the waist of the probe beam. This was not carefully investigated for the He-Ne TLS system, which was constructed primarily for testing purposes.
Better performance from the Kr-TLS instrument is also evident from the lower RSD of the measurements, as shown for the blank signals in *Table 8* and Table 9.

Table 9: *Table of merit for batch mode determination of BAs with Kr-TLS detection unit.*

	Putrescine	***Cadaverine***	***Histamine***	***Tyramine***
LOD (M)	4.0×10^{-8}	3.6×10^{-8}	6.6×10^{-8}	1.3×10^{-7}
Average blank signal (V)	9.23×10^{-3}	9.23×10^{-3}	9.23×10^{-3}	9.23×10^{-3}
RSD (%)	1.0 %	1.0 %	1.0 %	1.0 %
Linearity range (M)	1.0×10^{-7} 2.5×10^{-7}	1.0×10^{-7} 2.5×10^{-7}	1.0×10^{-7} 2.5×10^{-7}	1.0×10^{-7} 2.5×10^{-7}

4.2.4.1 Determination of biogenic amines in real samples

The newly developed method was tested in real samples with complex matrices to verify its applicability. Wine was selected as a complex matrix sample for this purpose.
Before the sample analysis, an extraction method was tested using standard solution of biogenic amines in order to determine the recoveries for each studied amine. The extraction of BAs was based on the already developed extraction method (Molins-Legua C., 2005). Amine standard solutions were prepared as described in caption

3.2.2 and extracted with the Molins-Legua procedure**.** As a blank solution synthetic wine was used, as decribed by Peña-Gallego and co-workers (2009): an aqueous solution at pH 3.5, containing 10% (v/v) ethanol and 5 g L^{-1} of tartaric acid.

Limits of detection achieved after the SPE procedure for each biogenic amine are illustrated in *Table 10*, with corresponding calibration curves (*Figure 22, Figure 23*) obtained after extraction. Limits of detections for each biogenic amine are similar to those obtained in direct determination of BAs in standard solutions (previous chapter).

Table 10: *Table of merit for batch mode determination of BAs with the Kr-TLS detection unit after extraction.*

	Putrescine	***Cadaverine***	***Histamine***	***Tyramine***
LOD (M)	5.0×10^{-8}	5.5×10^{-8}	8.4×10^{-8}	1×10^{-7}
Average blank signal (V)	9.89×10^{-3}	9.89×10^{-3}	0.01017	0.01017
Linearity range (M)	5×10^{-8} 1.5×10^{-7}	5×10^{-8} 1.5×10^{-7}	1×10^{-7} 2.5×10^{-6}	1×10^{-7} 2.5×10^{-6}

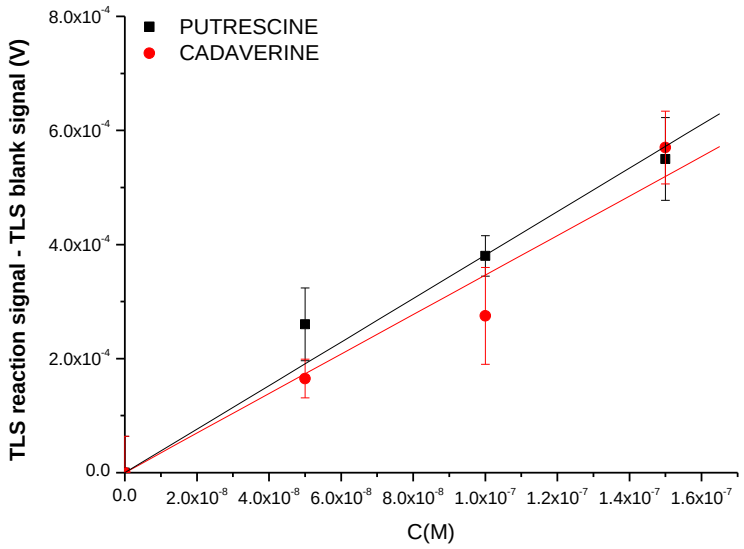

Figure 22: *Calibration curves for putrescine and cadaverine for the Kr-TLS detection unit after extraction ($R_{putrescine}$=0.988, $k_{putrescine}$=3814 V/M; $R_{cadaverine}$=0.979; $k_{cadaverine}$=3464 V/M).*

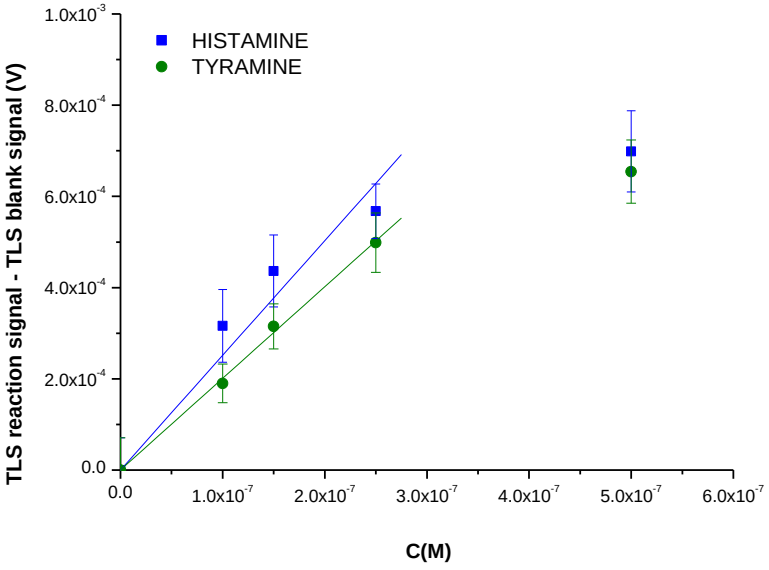

Figure 23: *Calibration curves for histamine and tyramine for the Kr-TLS detection unit after extraction ($R_{histamine}$=0.975, $k_{histamine}$= 2515 V/M,; $R_{tyramine}$=0.999; $k_{tyramine}$=2010 V/M).(*Data for histamine and tyramine at 5×10^{-7}M were omitted in the linear fit).

As in the case of ammonia determination (section 4.1.3) accuracy of the method was tested by analysing aqueous solutions with different concentration levels of a single biogenic amine. Results demonstrate good agreement with true values in the case of putrescine (*Table 11*), histamine (*Table 13*) and tyramine (*Table 14*). In case of cadaverine (*Table 12*) the measurements at 1×10^{-7} M showed only 70% recovery, while at 1.5×10^{-7} M recovery of over 130% was observed.

Table 11: *Recoveries for determination of putrescine.*

PUTRESCINE CONCENTRATION IN THE SAMPLE (M)	***MEASURED PUTRESCINE (M)***	***RECOVERY (%)***
$\boldsymbol{5\times10^{-8}}$	$5.9\times10^{-8}\pm 1\times10^{-8}$	118±20
$\boldsymbol{1\times10^{-7}}$	$1.1\times10^{-7}\pm0.5\times10^{-8}$	110±5
$\boldsymbol{1.5\times10^{-7}}$	$1.5\times10^{-7}\pm0.9\times10^{-8}$	100±6

Table 12: *Recoveries for the determination of cadaverine.*

CADAVERINE CONCENTRATION IN THE SAMPLE (M)	***MEASURED PUTRESCINE (M)***	***RECOVERY (%)***
$\boldsymbol{5\times10^{-8}}$	$4.4\times10^{-8}\pm 1\times10^{-8}$	88±20
$\boldsymbol{1\times10^{-7}}$	$7\times10^{-8}\pm1.5\times10^{-8}$	70±15
$\boldsymbol{1.5\times10^{-7}}$	$2\times10^{-7}\pm0.4\times10^{-8}$	133±3

Table 13: *Recoveries for the determination of histamine.*

HISTAMINE CONCENTRATION IN THE SAMPLE (M)	***MEASURED PUTRESCINE (M)***	***RECOVERY (%)***
$\boldsymbol{1\times10^{-7}}$	$1.1\times10^{-7}\pm0.5\times10^{-8}$	110±5
$\boldsymbol{1.5\times10^{-7}}$	$1.4\times10^{-7}\pm0.8\times10^{-8}$	93±5
$\boldsymbol{2.5\times10^{-7}}$	$2.8\times10^{-7}\pm0.7\times10^{-8}$	112±2

Table 14: *Recoveries for the determination of tyramine.*

TYRAMINE CONCENTRATION IN THE SAMPLE (M)	***MEASURED PUTRESCINE (M)***	***RECOVERY (%)***
1×10^{-7}	$8.8\times10^{-8}\pm1.0\times10^{-8}$	88±10
1.5×10^{-7}	$1.6\times10^{-7}\pm0.22\times10^{-8}$	107±2
2.5×10^{-7}	$2.7\times10^{-7}\pm0.55\times10^{-8}$	108±3

The assay is not specific for identification of single biogenic amines, but allows the determination of the total biogenic amines present. Therefore, for analysis of the wine samples, we decided to express the quantity of biogenic amines as equivalents of histamine, the only BA for which the maximum contamination level in wine is regulated. Tested samples were two different types of white wine for which different levels of BAs were expected due to different composition of grapes: home made wine from mixed white grapes (sample 1) and wine sample produced from Rebula grapes and provided by the Centre of Wine Research at the University of Nova Gorica (sample 2). Both wines were produced using the classic procedures of crushing, pressing, fermentation, filtration and bottling.

As it is visible from the comparison of the signals in *Figure 24*, the presence of BAs in both samples can be predicted. The higher signal in sample 2, suggests higher concentration of biogenic amines. However, based on the addition of histamine and from the difference of TLS signals from spiked and original wine samples, a concentration of BAs corresponding to $(6.4\pm0.3)\times10^{-7}$ M which corresponds to 0.069±0.003 mg/L equivalents of histamine was estimated for sample 1, and $(1.8\pm0.9)\times10^{-6}$ M concentration which correspond to 0.2±0.1 mg/L equivalents of histamine was estimated for sample 2. Due to a relatively large standard deviation in the measurement of spiked sample 2 the difference in concentrations of BAs in the two samples could not be confirmed. Nonetheless, these results indicate, that in the selected samples of wine the concentrations of BA are up to 5 times lower compared to the reported values (recalculated as histamine equivalents) in Greek wine (c_{His}= 0.34 mg/L by Proestos C., 2008). But even concentrations below 0.1mg/L can still be reliably detected due to the high sensitivity of the TLS method.

Definitive confirmation of the presence of BAs and their concentrations however requires determination of these compounds with a second standardised method such as HPLC, which would also be necessary for final validation of the proposed enzymatic method.

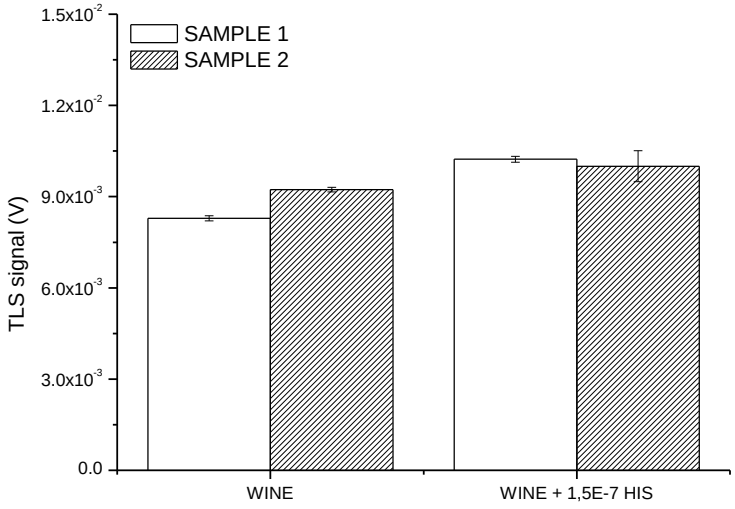

Figure 24: *Signals of sample 1 and sample 2 with the Kr-TLS detection unit.*

4.3 FIA-TLS bioanalytical system

In the following section the performances of two different TLS detection systems were analyzed and optimized. In order to achieve best performance for the FIA system in terms of sensitivity and LOD experimental variables that affect the performance were studied.

4.3.1 Influence of sample volume

Influence of different sample volumes was examined. 2mM solutions of each biogenic amine were injected using 4 different sample loops (10µL; 50µL; 100µL, 200µL).

Different flow rates were chosen for the 2 pumps in order to ensure higher residence time in the bioanalytical column. The objective was to ensure a sufficient incubation time (10 minutes), as determined in batch mode measurements.

The peak height increased with increasing sample injection volume in the range 10µL - 200µL. Increasing the injection volume, caused broadening of the peaks, for investigated sample injection volumes in the range 10µL - 200µL. The highest TLS signal was obtained with a 200µL sample volume and was selected as the sample volume for injection of BAs in the flow steam (*Figure 25*).

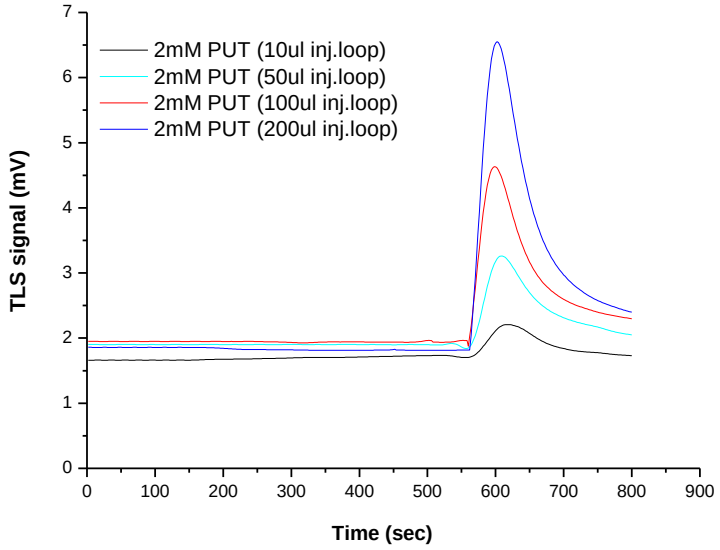

Figure 25: *Influence of the sample volume on the peak height (conditions: 0.2 mL/min pump A; 0.3 mL/min pump B; 2mM putrescine; excitation at 647 nm, P=200mW).*

4.3.2 Determination of BAs with Kr-TLS detection unit

Two structurally different biogenic amines were used for this study: putrescine as a representative of diamines and histamines as a representative of heterocyclic monoamines. The resulting graphs of the TLS signal as function of time (*Figure 26*), where the signal heights correspond to the different concentration of BAs injected, clearly differ in the peak width and height.

These differences could be attributed to different structures of investigated BAs. The observed longer retentions of putrescine in the column can be explained by differences in the structure of putrescine (aliphatic) and histamine (heterocyclic) which can contribute to stronger interaction with the material of the bioanalytical column and its packing in case of putrescine.

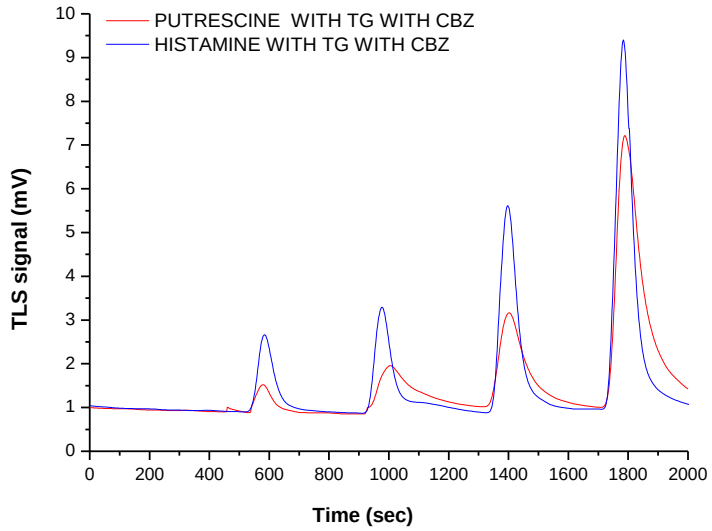

Figure 26: *FIA-Kr-TLS diagram of different BAs with the use of immobilized MTGase and with CBZ-Gln-Gly (conditions: 0.2 mL/min pump A; 0.3 mL/min pump B; BAs concentration: 0.5mM; 1mM; 2mM; 5mM; excitation at 647 nm, P=200mW).*

Another immediate observation can be made regarding the sensitivity of the method. This is substantially lower compared to the previously discussed determination of BAs by MTGase in batch mode, where concentrations at the level of 10^{-6} M were easily detected, while in the FIA mode at least two orders of magnitude higher concentrations are required for detection.

The observed low sensitivity could be due to the loss of MTGase activity during the immobilization procedure. To elucidate this effect another set of measurements was conducted under similar conditions but without immobilised MTGase and without the addition of CBZ-Gln-Gly.

From *Figure 27*, it is clearly evident that the reaction of indophenol blue formation proceeds even without the use of MTGase and CBZ-Gln-Gly. Similar peak heights, such as those in the presence of MTGase, show that TGA completely lost the activity upon immobilization. This has, concurrently, confirmed that the indophenol blue method suffers from BAs interference. However, taking into account the high concentrations of BAs used in this test, we have estimated that the contribution of

BAs to direct formation of indophenol is only 1% compared to the signal generated by the ammonium released from BAs (in batch mode).

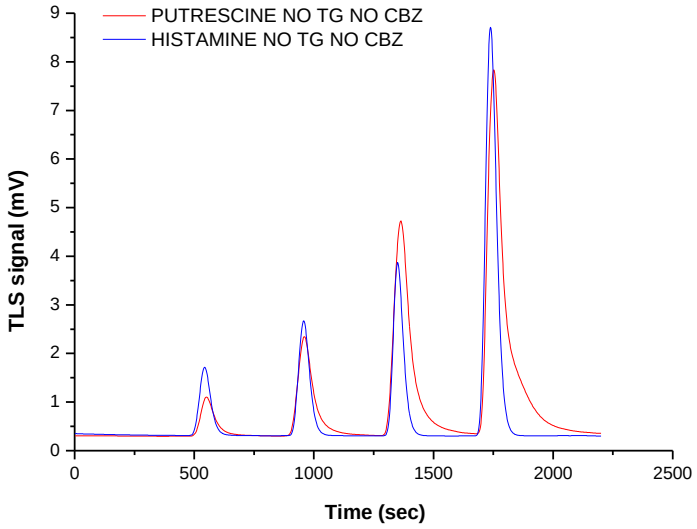

Figure 27: *FIA-Kr-TLS diagram of different BAs without the use of MTGase and without CBZ-Gln-Gly (conditions: 0.2 mL/min pump A; 0.3 mL/min pump B; BAs concentration: 0.5mM; 1mM; 2mM; 5mM; excitation at 647 nm; P=200mW).*

As a last option we have tried to change the type of enzyme immobilized on CPG, always a TGase, but this time from guinea pig liver. Even upon changing the enzyme the sensitivity of the method did not improve as illustrated in *Figure 28* and *Figure 29*.

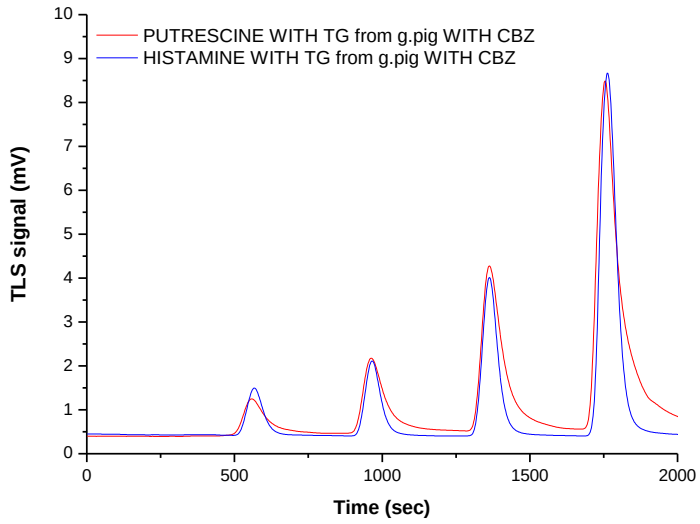

***Figure 28**: FIA-Kr-TLS diagram of different BAs with the use of GTGase and with CBZ-Gln-Gly (conditions: 0.2 mL/min pump A; 0.3 mL/min pump B; BAs concentration: 0.5mM; 1mM; 2mM; 5mM; immobilized enzyme GTGase; excitation at 647 nm; P=200mW).*

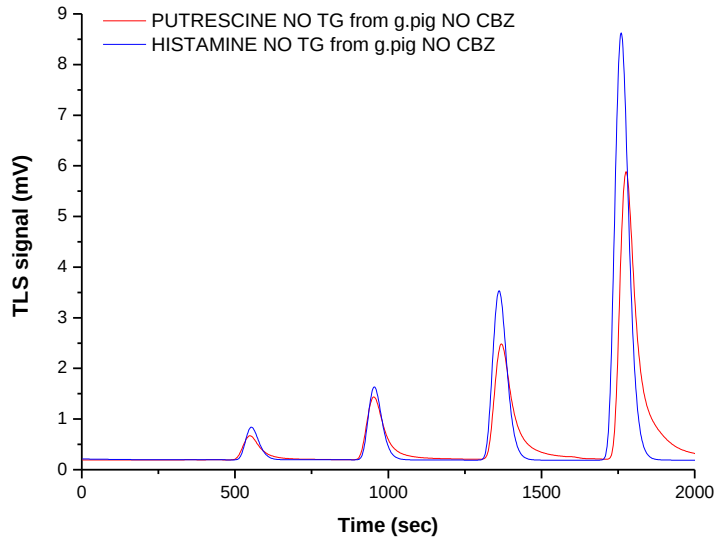

***Figure 29:** FIA-Kr-TLS diagram of different BAs without the use of GTGase and without CBZ-Gln-Gly (conditions: 0.2 mL/min pump A; 0.3 mL/min pump B; BAs concentration: 0.5mM; 1mM; 2mM; 5mM; excitation at 647 nm; P=200mW).*

4.3.2.1 Addition of acetonitile for BAs determination in the FIA-system

As already discussed in previous chapters, the addition of organic solvents, in our case 25% acetonitrile added to carrier solution B, improves the sensitivity for the detection of biogenic amines. With this addition we conducted our investigations using 10 times lower concentrations of BAs injected in the FIA system in comparison to concentrations when using only water solutions (*Figure 30*).

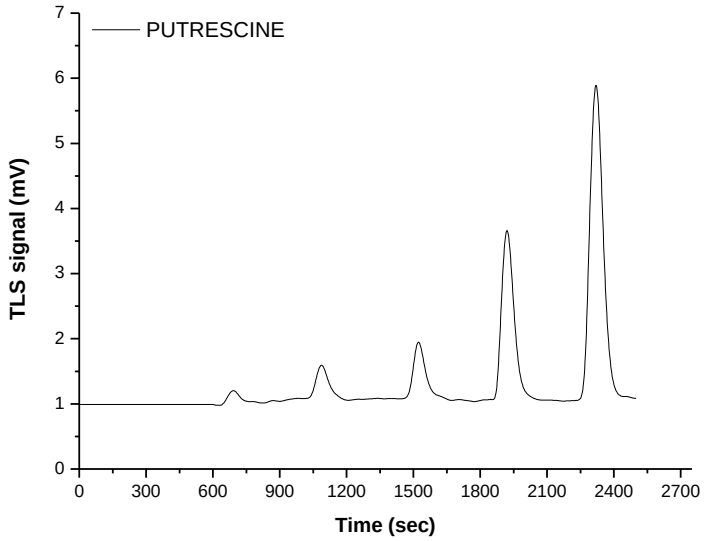

Figure 30: *FIA-TLS diagram of different BAs without the use of TGase and without CBZ-Gln-Gly (conditions: 0.2 mL/min pump A; 0.2 mL/min pump B; BAs concentration: 0.05mM;0.1mM;0.25mM;0.5mM;1mM; excitation at 647 nm; P=200mW).*

Despite a much lower sensitivity in FIA-TLS compared to the batch mode measurements, the limits of detection were still 10-times lower (*Table 15*) in comparison with the bioanalytical method reported in the literature.

Table 15: *Table of merit for detection of BAs by the FIA-Kr-TLS system with an addtion of 25% acetonitrile into the aqueous carrier solution.*

	Putrescine	***Cadaverine***	***Histamine***	***Tyramine***
Sensitivity (mV/M)	6.91	4.53	11.8	4.04
R^2	0.985	0.957	0.993	0.940
LOD (M)	4.5×10^{-6}	6.8×10^{-6}	2.6×10^{-6}	7.7×10^{-6}
Linearity range (M)	5.5×10^{-5} – 1.0×10^{-3}	5.5×10^{-5} – 2.5×10^{-4}	1.0×10^{-4} – 5.0×10^{-4}	1.0×10^{-4} – 5.0×10^{-4}

Direct determination of BAs with indophenol blue method is, however, subject to strong interference from ammonium ions, which is also present when using TGase, unless ammonium is separated by extraction of BAs or some other procedure. The method in the present state is, therefore, only useful for fast screening of BAs. The suggested screening procedure is as follows:

1) analysis of the sample by FIA-TLS indophenol blue method. This gives an indication of the presence of BAs and/or ammonium. The results for ammonium can be quantified.
2) Batch mode analysis of the sample using TGase and indophenol blue method, This gives the sum of concentrations of ammonium and BAs.

As demonstrated in this work, the sensitivity of direct BA determination by the indophenol method (step 1) is about 10 times lower when compared to the determination of ammonium and one mole of BA produces about one mole of ammonium when reacting with TGase. Therefore, the difference in molar concentrations of ammonium determined in step 2 and the concentration determined in step 1 gives a good estimation of the concentration of BAs in the sample.

However, in order to eliminate entirely the interference of ammonium and some other constituents of the sample the extraction of BAs was proposed, investigated and discussed in more detail in the previous chapter (4.1.3.2).

5 Conclusions

Novel dual beam TLS spectrometers based on the excitation from a He-Ne (632.8 nm) and a Kr laser (647 nm) were constructed, which for the first time utilize a green probe beam at 543.5 nm from a second He-Ne laser. This facilitates the alignment of the pump and probe beams, and their separation before reaching the detector, which would be far more demanding in case of two read beams (632.8 and 647 nm). In addition, the shorter wavelength of the probe beam (543.5 nm) contributes to a 16.4% higher sensitivity in the TLS measurement compared to the usually used probe beam from a He-Ne laser (632.8 nm).

Indophenol blue reaction was proven as a suitable alternative tool for the determination of ammonia by TLS. The application of TLS resulted in over 9.8 times lower LODs, compared to spectrophotometric measurements in water. Improvement was even higher (16 times) in 25% acetonitrile solutions in which the LODs were lowered down to 8 ng/mL in this work.

The indophenol blue-TLS method was further successfully employed for the detection of biogenic amines, which was based on the release of ammonium from the reaction between the biogenic amine and the enzyme transglutaminase.

Comparison of the developed method based on TLS detection with He-Ne excitation, to the enzymatic method previously reported in the literature, showed an improvement expressed in 44 to 135 fold lower LODs, for the different biogenic amines investigated. The calculated LODs were dependent on the biogenic amine and ranged from 5.2×10^{-7} M to 2.0×10^{-6} M.

A new powerful TLS system was constructed with two fundamental characteristics to further improve the LODs was utilised: higher laser power (Kr-laser, 200 mW), and excitation wavelength (647 nm) similar to the absorbance maximum of indophenol, close to 650 nm. The achieved LODs (3.6×10^{-8}M to 1.3×10^{-7}M) were 12 to 32 times lower compared to He-Ne detection unit and in the range of those obtained with RP-HPLC with UV detection. However, the method developed in this work does not require the derivatization step needed for BAs prior determination by HPLC, which

is time-consuming and lasting from half an hour to one hour. It must, however, be pointed out that the TGase-TLS method can not discriminate between individual BAs and is therefore only applicable as a screening method for determining the presence or absence of BAs. In this respect the high sample throughput is of more importance than just the selectivity of the method.
In this context, owing to the high sensitivity of TLS detection, the previously required two hours reaction time for the enzymatic method (Punakivi K., 2006) was reduced to only 10 minutes. This allows an increase in sample throughput to at least 6 samples per hour.

In view of a possible flow injection method for rapid screening for BAs, it was, however, found that during the immobilization of MTGase or GTGase the enzyme's activity was not preserved. Our experiments on a FIA-TLS manifold, however, confirmed, that for screening purposes, the indophenol blue FIA-TLS method can be used without the need of TGase, and provides comparable or lower LODs (2.6×10^{-6} M for histamine and 7.7×10^{-6} for tyramine), as enzymatic method reported in literature, while offering about 10 times higher sample throughput. In such a case the relative error due to the interference from aminoacids, which are known to interfere with the indophenol-blue reaction, was found to be 6% for asparagines, 12% for glycine and 20% for histidine, when the amino acids were present in concentrations equimolar to those of ammonia entering the indophenol reaction.

The developed batch mode method was also used for analysis of real samples of white wine in order to test the applicability of the method to real samples where it allows the determination of the total biogenic amines present. For this reason the quantity of biogenic amines is expressed as equivalents of histamine, the only BA for which the maximum contamination level in wine is regulated.

Based on the standard addition of histamine, a concentration of BAs corresponding to $(6.4\pm0.3)\times10^{-7}$ M or 0.069±0.003 mg/L equivalents of histamine was estimated for home made wine, and $(1.8\pm0.9)\times10^{-6}$ M or 0.2±0.1 mg/L equivalents of histamine was for the Rebula wine. Results indicated that in the selected samples of wine the concentrations of BA are up to 5 times lower compared to the reported

values in literature. This allows us to consider the developed TLS method as a highly sensitive and rapid tool for semi-quantitative screening of BAs presence in real samples. However for the ultimate confirmation of the presence of BAs in investigated samples and for final validation of the method, a comparative analysis with an independent analytical method such as HPLC is necessary.

However, additional work shall be focused on immobilization procedures for TGase, which should provide higher specificity of the method and enable the analysis in the FIA mode. This is the main prerequisite for further development of microfluidic bioassay systems which can further increase the speed of diffusion controlled reactions, due to increased interface surface to volume ratio. Under microfluidic thermal lens microscopic detection sample throughputs of up to 30/hour are predicted.

6 References

Aeschlimann D., Paulsson M. (1994). Transglutaminases: Protein cross-linking enzymes in tissues and body fluids. Thromb. Haemostasis **71**: 402-415.

Alberto M.R., Farias M.E., Manca de Nadra M.C. (2002). Effect of wine phenolic compounds on *Lactobacillus hilgardii* 5w viability. J. Food Prot. **65**: 211-213.

Ando H., Adachi M., Umeda K., Matsuura A., Nonaka M., Ucio R., Tanaka H.,

Armbrust C., Werlein H.D., Watkinson B. (2003). Transglutaminase – Einsatz und Wirkung in Fischprodukten. Deut. Lebens. Rundsch. **99**: 181-187.

Askar A., Trepton H. (1989). Biogene Amine in Fleischprodukten. Ernahrung/Nutrition **14**: 425-429.

Bardocz S. (1995). Polyamines in food and their consequences for food quality and human health. Trends Food Sci. Technol. **6**: 341-346.

Bauer N., Koehler P., Wieser H., Scieberle P. (2003a). Studies on effects of microbial transglutaminase on gluten proteins of wheat. I. Biochemical analysis. Cereal Chem. **80**: 781-786.

Bauer N., Koehler P., Wieser H., Scieberle P. (2003b). Studies on effects of microbial transglutaminase on gluten proteins of wheat. II. Rheological properties. Cereal Chem. **80**: 787-790.

Bechtold U., Otterbach J.T., Pasternack R, Fuchsbauer H.L. 2000. Enzymic preparation of protein G-peroxidase conjugates catalysed by transglutaminase. J. Biochem. **127:**239–245.

Ben-Gigirey B., Craven C., An H. (1998). Histamine formation in Albacore mucle analyzed by AOAC and enzymatic methods. J.Food Science **63:** 210-214.

Bialkowski S.E. (1996). Photothermal spectroscopy methods for chemical analysis. In: Winefordner (Ed.), Chemical Analysis, Vol. 134, New York.

Ben-Gigirey B., Craven C., An H. (1998). Histamine formation in Albacore mucle analyzed by AOAC and enzymatic methods. J.Food Science **63:** 210-214.

Bouvrette P., Male K.B., Luong J.H.T., Gibbs B.F. (1997). Amperometric biosensor for diamine using diamine oxidase purified from porcine kidney. Enzyme Microb. Technol. **20**: 32-38.

Busto O., Guasch J., Borrull F. (1996). Biogenic amines in wine: A review of analytical methods. J. Inter. Sci. Vigne Vin **30**: 85-101.

Cerutti G., Finoli C. Vecchio A. (1989). Non-volatile amine biogenesis from wort to beer. Monatsschr. Brauwiss. **42**: 246-248.

Chung, S.I. (1970). Mechanism of action of guinea pig liver transglutaminase. VII. Chemical and stereochemical aspects of substrates binding and catalysis. J. Biol. Chem. **245**: 6424-6435,

Cinquina A. L., Longo F., Calì A., De Santis L., Baccelliere R., Cozzani R. (2004). Validation and comparison of analytical methods for the determination of histamine in tuna fish samples. J.Chrom. A **1032**: 79-85.

Cirilo M.P.G., Coelho A.F.S., Araujo C.M., Goncalves F.R.B., Nogueria F.D., Gloria M.B.A. (2003). Profile and levels of bioactive amines in green and roasted coffee**.** Food Chem. **82**: 397-402.

Clescieri L.S., Arnold E., Deaton A.D. (1998). Standard Methods for the Examination of Water and Wastewater, 20th ed. American Public Health Association, American Water Works Association, and Water Environment Federation, Baltimore.

Collar C., Bollain C. (2004). Impact of microbial transglutaminase on the viscoelastic profile of formulated bread doughs. European Food Res. Technol. **218**: 139-146.

Compagnone D., Isoldi, G., Moscone D., Palleschi G. (2001). Amperometric detection of biogenic amines in cheese using immobilised diamine oxidase. Anal. Lett. **34**: 841-854.

Cooper A. J. L., Jeitner T.M., Gentile V., Blass J.P. (2002). Cross linking of polyglutamine domains catalyzed by tissue transglutaminase is greatly favored with pathological-length repeats: does transglutaminase activity play a role in $(CAG)_n/Q_n$-expansion diseases? Neurochem. Int. **40**: 53-67.

Cui L., Guocheng D., Dongxu Z., Xuerong F., Jian C. (2008). Stability and conformational changes of transglutaminase from *Streptomyces hygroscopicus* in ethanol–aqueous medium. Process Biochem. **43**: 887-891.

Darias R., Villalonga R. (2001). Functional stabilization of cellulase by covalent modification with chitosan. J. Chem. Technol. Biotechnol. **76:** 489-493.

Dovichi N.J., Nolan T.G., Weimer W.A. (1984). Theory for laser-induced photothermal refraction. Anal. Chem. **56:** 1700-1704.

Draisci R., Volpe G., Lucentini L., Cecilia A., Federico R., Palleschi G. (1998). Determination of biogenic amines with electrochemical biosensor and its application to salted anchovies. Food Chemistry **62**: 225-232.

Eerola S., Roig-Sagues A.X., Lilleberg L., Aatto H. (1997). Biogenic amines in dry sausages during shelf-life storage. Z. Lebensm.Unters. For. A **205**: 351-355.

Esposito C., Costa C., Amoresano A., Mariniello L., Sommella M.G., Caputo I., Porta R. (1999). Transglutaminase-mediated amine incorporation into substance P protects the peptide against proteolysis in vitro. Regul. Peptides, **84:** 75–80.

Esposito C., Mancuso F., Calignano A., Di Pierro P., Pucci P., Porta R. (1995). Neurokinin receptors could be differentiated by their capacity to respond to the transglutaminase- synthesized γ-(glutamyl^{5}) spermidine derivative of substance. P. J. Neurochem.**65:**420–426.

FDA (Food and Drug Administration) 2001. Fish and fisheries products hazards and controls guidance. Third Edition.
http://www.fda.gov/Food/GuidanceComplianceRegulatoryInformation/GuidanceDocumants/Seafood/FushandFisheriesProductsHazardsandcontrolsGuidance/ucm091091.htm.

Fernandes J.O., Ferreira M.A. Combined ion-pair extraction and gas chromatography-mass spectrometry for the simultaneous determination of diamines, polyamines and aromatic amines in Port wine and grape juice. J.Chrom. A. **886**: 183-195.

Folk J.E. (1983). Mechanism and basis for specificity of transglutaminase-catalyzed epsilon-(gamma-glutamyl) lysine bond formation. Adv. Enzymol. Relat. Areas Mol. Biol. **54**: 1-56.

Folk J.E., Cole P. W. (1966a). Mechanism of action of Guinea Pig Liver Transglutaminase. J. Biol. Chem. **241**: 5518-5525.

Folk J. E., Cole P.W. (1966b). Transglutaminase: Mechanistic features of the active site as determined by kinetic and inhibitor studies. Biochim. Biophys. Acta **22**: 244-264.

Folk J.E., Chung S.I. (1985) Transglutaminases. Methods Enzymol. **113:.**358–364.

Folk J.E., Chung S.I. (1973). Molecular and catalytic properties of transglutaminase. Adv. Enzymol. Relat. Areas Mol. Biol. **38**: 109-191.

Franko M. (2008). Thermal Lens Spectrometric Detection in Flow Injection Analysis and Separation Techniques. Appl. Spectrosc. Rev. **43**: 358-388.

Frébort I., Skoupá L., Pec P. (2000) Amine oxidase-based flow biosensor for the assessment of fish freshness. Food Contr. **11**: 13-18.

Gekko K., Ohmae E., Kameyama K., Takagi T. (1998). Acetonitrile-protein interactions: amino acid solubility and preferential salvation. Biochim. Biophys. Acta **1873**: 195-205.

Gerrard J. A., Newberry M.P., Wilson A.J., Ross, M., Fayle S.E., Kavale S. (2000). Pastry lift and croissant volume as affected by microbial transglutaminase. J. Food Science **65**: 312-314.

Grenard P., Bates M.K., Aeschlimann D. (2001). Evolution of transglutaminase genes: identification of a transglutaminase gene cluster on human chromosome 15q15. Structure of gene encoding transglutaminsae X and a novel gene family member, transglutaminase Z. J.Biol. Chem. **276**: 33066-33078.

Gómez L., Villalonga R. (2000). Functional stabilization of invertase by covalent modification with pectin. Biotechnol. Lett. **22:**1191–1195.

Gosetti F., Mazzucco E., Gianotti V., Polati S., Gennaro M.C.(2007). High performance liquid chromatography/tandem mass spectrometry determination of biogenic amines in typical Piedmont cheeses. J.Chromatogr.A. **1149:**151–157.

Halász A., Baráth A., Simon-Sakadi L., and Holzapfel W. (1994). Biogenic amines and their production by microorganisms in food. Trends Food Sci. Technol. **5**: 42-49.

Hoekstra J. C., Johnson D.C. (1998). Comparison of Potential-Time Waveforms for the Detection of Biogenic Amines in Complex Mixtures following Their Separation by Liquid Chromatography. Anal. Chem. **70**: 83-88.

Hyvönen T., Keinänen T.A., Khomutov A.R., Eloranta T.O. (1992). Monitoring of the uptake and metabolism of aminooxy analogues of polyamines in cultured cells by high-performance liquid chromatography. J.Chromatogr. **574**: 17-21.

Izquiredo-Pulido M., Mariné-Font A., Vidal-Carou M.C. (2000). Effect of tyrosine on tyramine formation during beer fermentation. Food Chem **70**: 329-332.

Jüttner F. (1999). Interference with ammonium determination by the indopohenol-type reaction of salicylate and dichloroisocyanurate. Fresenius' J. Ana.Chem. **363**: 128-129.

Kalač P., Šavel J., Křižek M., Pelikánová T., Prokopová M. (2002a). Biogenic amine formation in bottled beer. Food Chem.**79**: 431-434.

Kalač P., Švecová S., Pelikánová T. (2002b). Levels of biogenic amines in typical vegetable products. Food Chem. **77**: 349-351.

Kanaji T., Ozaki H.,. Takao T., Kawajiri K., Ide H., Motoki M., Simonishi Y. (1993). Primary structure of microbial transglutaminase from Streptoverticillium sp. strain s-8112. J.Biol. Chem. **268**: 11565-11572.

Karpuj M.V., Becker M.W., Steinman L. (2002). Evidence for a role for transglutaminase in Huntington's disease and the potential therapeutic implications. Neurochem. Int. **40**: 31-36.

Kim S. Y., Jeitner T.M., Sternert P.M. (2002). Transglutaminases in disease. Neurochem. Int. **40**: 85-103.

Krìžek M., Kalač P. (1998a). Biogenic amines in foods and their roles in human nutrition. Czech J.Food Sci. **16**: 151-159.

Krìžek M., Pelikánová T.. (1998b). Determination of seven biogenic amines in foods by micellar electrokinetic capillary chromatography. J.Chromatogr. A. **815**: 243-250.

Kuraishi C., Yamazaki K., Susa Y. (2001). Transglutaminase: its utilization in the food industry. Food Rev. Int. **17**: 221-246.

Kurama H., Poetzschke J., Haseneder R. (2001). The application of memebrane filtration for the removal of ammonium ions from potable water.

Kütemeyer C., Froeck M. ,Werlein H.-D., Watkinson B.M. (2005). The influence of salts and temperature on enzymatic activity of microbial transglutaminase. Food Contr. **16**: 735-737.

Laliberte G., Proulx D., De Pauw N., De La Noue J. (1994). Algal technology in wastewater treatment. In: Algae and water pollution, Rai, LC., Gaur, JP. And Soeder, CJ. (Eds.), Archiv fur hydrobiology,Vol. 42. Berlin, 283-302.

Lange J., Wittmann C. (2002). Enzyme sensor array for the determination of biogenic amines in food samples. Anal. Bioanal. Chem. **372**: 276-283.

Lehane L., Olley J. (2000). Histamine fish poisoning revisited. Int. J.Food Microbiol. **58**: 1-37.

Leuschner R.G., Heidel M., Hammes W.P. (1998). Histamine and tyramine degradation by food fermenting microorganisms. Int. J.Food Micorbiol. **39**: 1-10.

Lovaas E. (1991). Antioxidative effects of polyamines J.O.A.C.S. **68**: 353-358.

Lorand L., Conrand S.M. (1984). Transglutaminases. Mol. Cell. Biochem. **58**: 9-35.

Lorand L., Graham R.M. (2003). Transglutaminases: crosslinking enzymes with pleiotropic functions. Nat. Rev. Mol. Cell Biol. **4**: 140-156.

Malle P., and Valle' M. (1996). Assay of biogenic amines involved in fish decomposition. J. Ass. Off. Analy. Chem **79**: 43-49.

Male K.B., Bouvrette P., Luong J.H.T., Gobbs B.F. (1996) Amperometric biosensor for total histamine, putrescine and cadaverine using diamine oxidase. J. Food Sci. **61:**1012–1016.

Mancuso F., Porta R., Calignano A., Di Pierro P., Sommella M.C., Esposito C. (2001). Substance P and its transglutaminase-synthesized derivatives elicit yawning behavior via nitric oxide in rats. Peptides. **22:** 1453–1457.

Manfroi L., Silva P.H.A., Rizzon L.A., Sabaini P.S., Glória M.B.A. (2009). Influence of alcoholic and malolacticstarter cultures on bioactive amines in Merlot wines. Food Chem. **116:** 208-213.

Marcobal A., Polo M.C., Martín-Álvarez P.J., Moreno-Arribas M.V. (2004) Biogenic amine content of red Spadish wines: comparison of a direct Elisa and an HPLC method for the determination of histamine in wine. Food Res. Int. **38:** 387-394.

Maxa E., Brandes W. (1993). Biogene amine in fruchtsäften. Mitt. Klosterneuburg **86**: 101-106.

McCabe, B.J. (1986). Dietary tyramine and other pressor amines in MAOI regimens: a review. J. Am. Diet. Assoc. **86**: 1059-1064.

Mietz J.L.K., Karmas E. (1977). Chemical quality index of canned tuna as determined by high-pressure liquid chromatography. J. Food Sci. Technol. **42**: 155-158.

Minagawa H., Kaneko H. (2000). Effect of double mutation on thermostability of lactate oxidase. Biotechnol. Lett. **22:** 1131-1133.

Molins-Legua C., Campins-Falcò P. (2005) Solid phase extraction of amines. Anal. Chim. Acta **546:** 206-220.

Molins-Legua C., Meseguer-Lloret S., Moliner-Martinez Y., Campíns-Falcó P. (2006). A guide for selecting the most appropriate method for ammonium determination in water analysis. Trends Anal. Chem. **25:** 282-290.

Moret S., Conte L.S. (1996). High-performance liquid chromatographic evaluation of biogenic amines in foods. An analysis of different methods of sample preparation in relation to food characteristics. J. Chromatogr.A **729**: 363-369.

Motoki M. (1997). Gelation of proteins. In: Gel Technology. Tokyo: Science forum, 80.

Murphy A, Ò-Fágáin C. (1996). Stability characteristics of chemically modified trypsin. J. Biotechnol. **49:**163–171.

Naguib K., Ayesh A.M., Shalaby A.R. (1995). Studies on the determination of biogenic amines in foods. 1. Development of a TLC method for the determination of eight biogenic amines in fish. J.Agric. Food Chem. **43**: 134-139.

Nemes Z., Marekov L.N., Fèsus L., Steinert P.M. (1999). A novel function for transglutaminse 1: Attachment of a long-chain ω-hydroxyceramides to involucrin by ester bond formation. Proceedings of the National Academy of Sciences **96**: 8402-8407.

Niculescu M., Nistor C., Frébort I., Pec P., Mattiasson, B., Csöregi, E. (2000). Redox hydrogel-based amperometric bienzyme electrodes for fish freshness monitoring. Anal.Chem. **72**: 1591-1597.

Nonaka M., Sawa A., MatsuuraY., Motoki M., Nio N. (1996). Deamidation of several food proteins using free and immobilized Ca^{2+}-independent microbial transglutaminase. Biosci. Biotech. Bioch. **60**: 532-533.

Novella-Rodríguez S., Veciana-Nogués M.T., Izquierdo-Pulido M., Vidal-Carou M.C. (2003). Distribution of biogenic amines and polyamines in cheese. J.Food Sci **68**: 750-755.

Peña-Gallego A., Hernández-Orte P., Cacho J., Ferreira V. (2009). Biogenic amine determination in wines using solid-phase extraction: A comparative study. J.Chromatogr.A **1216**: 3398-3401.

Pogačnik L., Franko M. (2001). Optimisation of FIA system for detection of organophosphorus and carbamate pesticides based on cholinesterase inhibition. Talanta **54:** 631-641.

Prenter J., MacNeil C., Dick J.T.A., Riddell G.E., Dunn A.M. (2004). Lethal and sublethal toxicity of ammonia to native, invasive and parasitised freshwater amphipods. Water Res. **38:** 2847-2850.

Proestos C., Loukatos P., Komaitis M. (2008). Determination of biogenic amines in wines by HPLC with precolumn dansylation and fluorimetric detection. Food Chem. **106**: 1218-1224.

Punakivi K., Smolander M., Niku-Paavola L., Mattinen J., Buchert J. (2006). Enzymatic determation of biogenic amines with transglutaminase. Talenta **68:** 1040-1045.

Rasiah I. A., Sutton K.H., Low F.L., Lin H.M., Gerrard J.A. (2005). Crosslinking of wheat dough proteins by glucose oxidase and the resulting effects on bread and croissants. Food Chem. **89**: 325-332.

Rawles D.D., Flick G.J., Martin R.Y. (1996). Biogenic Amines in Fish and Shellfish. In: Advances in Food & Nutrition Research, Vol. 39, p. 329-365.

Reggiani R., Giussani P., Bertani A. (1990). Relationship between the Accumulation of Putrescine and the Tolerance to Oxygen-Deficit Stress in Gramineae Seedlings. Plant cell Physiol. **31**: 489-494.

Rogers P.L., Staruszkiewicz W. (1997). Gas chromatographic method for putrescine and cadaverine in canned tuna and mahimahi and fluorometric method for histamine (minor modification of AOAC official method 977.13). J.AOAC Int. **80**: 591-602.

Romero R., Bagur M., Sánchez-Viñas M., Gázquez, D. (2000). Optimization of experimental variables in the dansyl chloride derivatization of biogenic amines for their determination by RP-HPLC. Chromatographia **51**: 404-410.

Saito K., Horie M., Nose N., Nakagomi K., Nakazawa H. (1992). High-performance liquid chromatography of histamine and 1-methylhistamine with on-column fluroescence derivatization. J. Chromatogr.A. **595:** 163-168.

Saito T., Arsenault A.L., Yamauchi M., Kuboki Y., Crenshau M.A. (1997). Mineral induction by immobilized phosphoproteins. Bone **21:** 305-311.

Santos B., Simonet B.M., Ríos A., Varcálcel M. (2004). Direct automatic determiantion of biogenic amines in wine by flow injection-capillary electrophoresis-mass spectrometry. Electrophoresis **25:** 3427-3433.

Sarkar N. K., Clarke D.D., Waelsch H. (1957). An Enzymically Catalyzed Incorporation of Amines Into Proteins. Biochim. Biophys. Acta **25**: 451-452.

Shalaby A. R. (1999). Simple, rapid and valid thin layer chromatographic method for determining biogenic amines in foods. Food Chem.**65**: 117-121.

Shalaby A. R. (2000). Changes in biogenic amies in nature and germinating legume seeds and their behaviour during cooking. Die Nahrung **44**: 23-27.

Shulman K. I., Tailor S.A.N., Walker S.E., Gardner D.M. (1997). Tap (draft) beer and monoamine oxidase inhibitor dietary restrictions. Can.J.Psychiatry **42**: 310-312.

Silla Santos, M. H. (1996). Biogenic amines: their importance in foods. Int. J. Food Microbiol. **29**: 213-231.

Simon-Sarkadi L., Holzapfel W., Halasz A.J. (1994). Biogenic amine content and microbial contamination of leafy vegetables during storage at 5 °C. J.Food Biochem. **17**: 407-418.

Simon-Sarkadi L., Holzapfel W.H., (1994). Determination of biogenic amines in leafy vegetables by amino acid analyser. Z. Lebensm.Unters. For. A **198**: 230-233.

Singer S. M., Zainelli G.M., Norlund J.M., Lee N.A. (2002). Transglutaminase bonds in neurofibrillary tangles and paired helical filament tau early in Alzheimer's disease. Neurochem. Int. **40**: 17-30.

Stratton J. E., Hutkins R.W., Taylor S. L. (1991). Biogenic amines in cheese and other fermented foods. A review. J. Food Prot. **54**: 460-470.

Stratton J. E., Taylor S.L. (1991). Scombroid poisoning. In: Microbiology of Marine Food Products. Ward, D. and C. Hackney, Van Nostrand Reinhold, New York. New York, USA, Van Nostrand Reinhold Co., p. 331-347.

Sunna A., Tokajian S., Burghardt J., Rainey F., Antranikian G., Hashwa F. (1997). Identification of *Bacillus kaustophilus*, *Bacillus thermocatenulatus* and *Bacillus* strain HSR as members of *Bacillus thermoleovorans*. Syst. Appl. Microbiol. **20**: 232-237.

Suzzi G., Gardini F. (2003). Biogenic amines in dry fermented sausages: A review. Int. J. Food Microb. **88**: 41-54.

Tailor S. A., Shulman H.I., Walker S.E., Moss J., Gardner D. (1994). Hypertensive episode associated with phenelzine and tap beer a reanalysis of the role of pressor amines in beer. J. Clinic. Psychopharmacol. **14**: 5-14.

Tanabe S., Matsumoto H. (1988). Effect of preliminary treatment with ammonia on the reduction of CuY zeolite. Appl.Catal. **45:** 27-37.

Tarjan V., Janossy G.P.Y. (1978). The role of biogenic amines in food. Die Nahrung **22**: 285-289.

Thuman E.M. (1985). Organic Geochemistry of Natural Waters, Kluver Academic Publishers, Dordrecht.

Tombelli S., Mascini M. (1998). Electrochemical biosensors for biogenic amines: a comparison between different approaches. Anal. Chim. Acta **358**: 277-284.

Udenfriend S., Lovenberg W., Sjoderma A. (1959). Physiologically active amines in common fruits and vegetables. Archives Biochem. Biophys. **85**: 487–490.

Valero D., Martínez-Romero D., Serrano M. (2002). The role of polyamines in the improvement of the shelf life of fruit. Trends Food Sci. Tech. **13**: 228-234.

Vyas N.K., Vyas M.N., Quiocho, F.A. (1988). Sugar and signal transducer binding sites of the *Escherichia coli* galactose chemoreceptor protein. Science **242:** 1290-1295.

Walther D. J., Peter J.U., Winter S., Holtje M., Paulmann N., Grohmann M., Vonwinckel J., Alamo-Bethencourt V., Wilhelm C.S., Ahnert-Hilger G., Bader M. (2003). Serotonylation of small GTPases is a signal transduction pathway that triggers platelet α-granule release. Cell **115**: 851-862.

Wantke F., Hemmer F., Haglmuller T., Gotz M., Jarisch R. (1996). Histamine in wine. Int. Arch. Allergy Immun. **110**: 397.

Weimer W.B., Dovichi N.J. (1986). Simple Model for the Time Dependence of the Periodically Excited Crossed-Beam Thermal Lens. J. Appl. Phy, **59**: 225.

Wijngaards G., Zhu Y., Maagd J.M., Bol J. (1997) Transglutaminase as a potential tool in developing new protein ingredients and foods. Med. Fac.Landbouw Univ. Gent. **62**: 1381-1383.

Wu M. L., Yang C.C., Yang G.Y., Ger J., Deng J.F. (1997). Scrombroid fish poisoning: an overlooked marine food poisoning. Vet. Hum.Toxicol. **39**: 236-241.

Yen G. C. (1992). Effects of heat treatment and storage temperature on the biogenic amine contents of straw mushroom (Volvariella volvacea). J. Sci. Food Agr. **58**: 59-61.

Yokoyama K., Nio N., Kikuchi Y. (2004). Properties and applications of microbial transglutaminases. Appl. Microbiol. Biot. **63**: 447-454.

Zeng K., Tachikawa H., Zhu Z., Davidson V.L. (2000). Amperometric detection of histamine with a methylamine dehydrogenase polypyrrole based sensor. Anal. Chem. **72**: 2211-2215.

Zhou L.D., Chen J., Du G.C. (2000). Functional properties of microbial transglutamianse and its application method in food processing. China. Food additives **1**: 54-59.

Zotou A., Loukou Z., Soufleros E., Stratis I. (2003). Chromatography with Pre-column Dansylation and Ultraviolet Detection. Chromatographia **57**: 429-439.