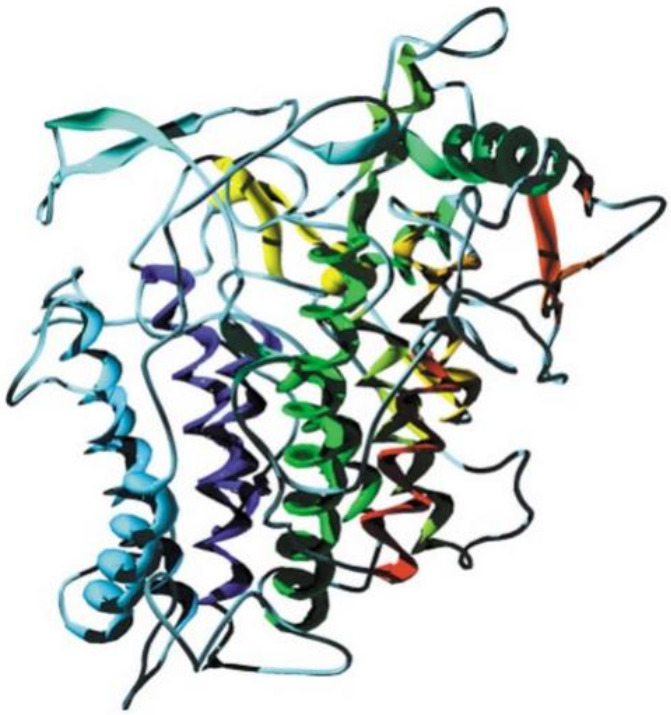
Biobased Chemistry and Technology

Thesis Biobased Chemistry and Technology

# Vanadium chloroperoxidase mediated conversion of amino acids and other biobased compounds

Sven Hemming



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# Vanadium chloroperoxidase mediated conversion of amino acids and other biobased compounds

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# 1. Introduction

Due to increasing environmental and geopolitical issues of fossil resources, the transition towards biobased processes for the production of chemicals and materials has become inevitable[1]. This conversion of biobased components for the creation of fuels, power, heat and value-added chemicals is described with the term biorefinery. The valorisation of biomass to industry relevant compounds is already applied to various types of biomass streams.[1,2] However a lot of abundantly available biomass is not utilized yet.[3] Nitrogen-rich waste streams such as poultry feather meal and dried distillers grains with soluble (DDGS) could be used as starting material for nitrogen-containing chemicals due to the presence of amine groups.[3] However based on the unavailability of process methods and/or poor cost and energy efficiency of existing methods[4], this is not done on a commercial scale. An approach to tackle these issues is the application of biocatalysts in biorefinery.

Biocatalysts offer the advantage of energy and material efficiency due to mild, near-ambient reaction conditions. Additionally, they offer high selectivity and an abundance of possible reactions due to the variety of enzymatic reactions existing in nature. The challenges regarding biocatalysis are the isolation of useful enzymes or the development of whole cell approaches, their stability and turnover rates in desired media and finally the price and development cycle of these biocatalysts.[5]

A highly versatile biocatalyst for conversion of biobased compounds to industry relevant chemicals is vanadium chloroperoxidase (VCPO) which will be the focus of this research.

## 1.1. Vanadium chloroperoxidase

VCPO belongs to the enzyme family of haloperoxidases which consist of two sub-families characterised by their prosthetic group: an iron-heme complex or a vanadium oxide moiety.[6] Three types of vanadium-dependent haloperoxidases can be distinguished by the most electronegative halide the enzyme is capable of oxidizing[7] which also inspired the names of the enzymes. Fluoride oxidation is not possible due to the higher redox potential of fluoride in comparison to hydrogen peroxide.

In nature, VCPO mediates the oxidation of halides to the bactericidal and oxidising agents HOBr and HOCl which are proposed by previous research to be able to degrade lignin by oxidative attack[6], thus enabling the penetration of lignocellulosic cell walls. This may be used as an attack mechanism by phytopathogenic hyphomycetes like *Curvularia inaequalis*. Furthermore, HOBr and HOCl react with a diverse set of complex organic molecules yielding halogenated compounds.[6]
For biotechnological application VCPO offers advantageous enzymatic properties like a high thermostability[8], strong resistance towards oxidants[9], stable activity in partly organic solvents[8,10,11], low loss of activity when stored at -20°C and very low Michaelis constant for bromide oxidation.[12]

### 1.1.1. Mechanism

Haloperoxidases catalyse the two-electron oxidation of a halide ($X^-$) to a corresponding reactive species ($X^+$) by using hydrogen peroxide as an oxidising agent.

$$H_2O_2 + X^- + H^+ \rightarrow X^+ + H_2O$$

$$X^- = Br^- \, or \, Cl^- \, or \, I^-$$
$$X^+ = HOX, \, {}^-OX, \, X_2 \; {}^{7,13,14}$$

This reaction requires slightly acidic conditions. Which reactive species is generated in the active site of VCPO is still under debate. Most literature points to hypohalous acid which is released from the active site into the reaction mixture[6] (Figure 1).

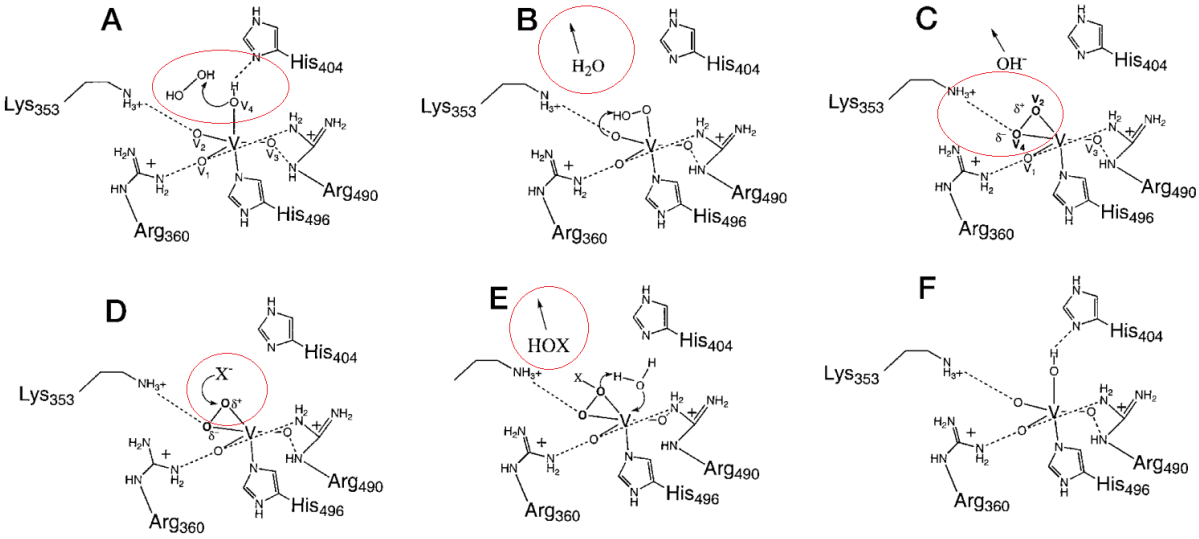


*Figure 1. Proposed minimal reaction scheme of vanadium chloroperoxidase catalysis, taken and modified from Hemrika et al.[7]*
*A. Approach of hydrogen peroxide to the vanadium oxide moiety. B. Release of water upon binding. C. Formation of a peroxo intermediate. Introduction of strong polarity due to hydrogen bonding with the amine of Lys353 activates the intermediate. D. Nucleophilic attack of the halide on the peroxo intermediate, resulting in an enzyme-hypohalous species. E. Water reacts and subsequent release of HOX. F. Catalytic cycle ends, native structure is restored.*

Hypohalous acid rapidly deprotonates and can form other reactive species which are further depicted as $'Br^{+'}$.These reactive species solemnly seem to be responsible for further nonspecific reactions with organic compounds. This makes the VCPO mediated conversions a unique reaction system where an enzymatic conversion, formation of activated halogenating species, is coupled with a subsequently chemical reaction, halogenation of organic molecules via a reactive species. A further advantage is the absence of the much more reactive and harmful $X_2$ in the conversion. An application of these reactive species is the conversion of amino acids to their corresponding nitriles.

## 1.2. Oxidative decarboxylation of amino acids towards nitriles

As mentioned earlier, nitrogen-rich waste streams are an underutilized source of amine groups for nitrogen-containing chemicals. Nitriles are a class of nitrogen-containing chemicals with great importance and use in industry. They are used as starting materials or intermediate in the synthesis of different polymers, solvents, adhesives and chemicals like the key intermediate acrylonitrile. Amino acids can undergo oxidative decarboxylation (Figure 2) under vastly different reaction conditions depending on the chosen means of conversion and the usage of a halide.

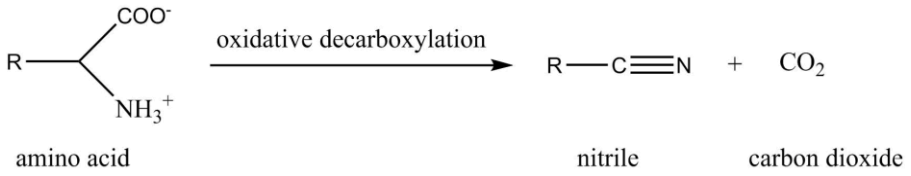


*Figure 2. General reaction of producing nitriles from amino acids via oxidative decarboxylation.*

Previous research demonstrated the conversion of amino acids to their corresponding nitriles mediated via VCPO.[11] The exact mechanism by which this oxidative decarboxylation occurs is still under debate. Two branches of explanations can be distinguished due to the attacking point of the bromide.

Most research points to a mechanism in which the first step is the halogenation of the amine to yield an N-monohalogenated amino acid.[15–17] It is suggested that the reaction is steered towards the nitrile formation only if the amine reacts with two equivalents of HOX under slightly acidic conditions. During this proposed reaction, the N-monohalogenated amino acid amine reacts a second time with HOX. Carbon dioxide is then split of and a product with one carbon less is formed. If the reaction conditions are slightly alkaline, the reaction is steered towards the aldehyde formation with splitting off of carbon dioxide and ammonia (Figure 3).

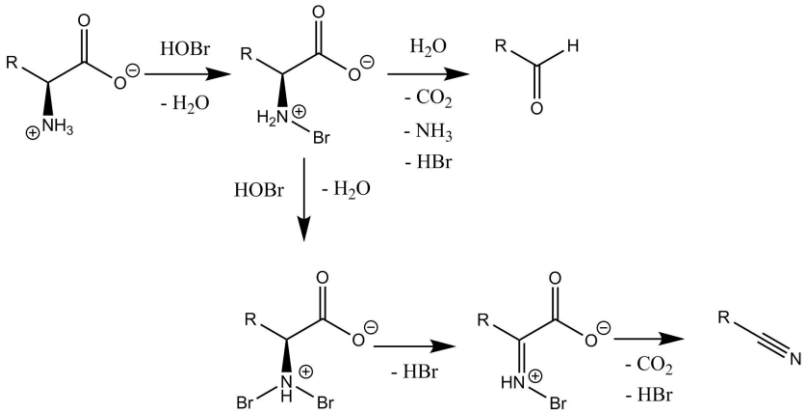


*Figure 3. Proposed mechanism of nitrile and aldehyde formation dependent on the number of halogenations occurring at the nitrogen of the amine group. Under slightly acidic conditions an amino acid is halogenated at the amine forming an N-monohalogenated amino acid. In neutral or slightly acidic pH a second bromination occurs and a nitrile is formed through the release of two bromide ions and carbon dioxide from an N,N-dibromo amino acid intermediate (lower path). In alkaline conditions bromide elimination is preferred and the aldehyde is obtained by release of carbon dioxide, ammonia and bromide. (upper path). (Taken and modified from Cleas et al., 2015 [15])*

The second branch of explanation favours the halogenation at the alpha carbon of the amino acid. This is based on the observation that an N,N-dialkylated amino acid was still able to be converted to an aldehyde using N-Bromosuccinimide.[18,19] As these results were only reported once, the explanation approach using the bromination of the nitrogen of the amine group is favoured in further discussion.

## 1.3. Influencing parameters for the conversion of amino acids

Total conversion and selectivity towards the nitrile are influenced by several reaction conditions and substrate parameters.

Reaction conditions like the temperature, pH of the reaction mixture and the halide used were shown to have a significant effect. The conversion towards the nitrile can be conducted at mild temperatures around 23 °C[11] and an increase in temperature increases the tendency towards the formation of the aldehyde rather than the nitrile.[16] Regarding the pH of the reaction mixture it was shown that a slightly acidic environment is favourable for the nitrile and increased pH leads to aldehyde formation.[13,16] The exact optimal pH value for conversion of a specific amino acids seems to be amino acid dependent.[11] The optimal pH value for VCPO activity was determined to be around pH 5.[6] Research on the nature of the halide was not extensively conducted but it was suggested that usage of chloride rather than the bromide seems to favour the formation of the aldehyde. The concentration of the bromide is influencing the conversion and selectivity significantly depending on the nature of the amino acid.[20] A general trend is that a bromide threshold between 0.5 and 5 mM seems to be present for all amino acids but glutamic acid and 2-aminoadipic acid. The reactivity and selectivity for almost all amino acids is highest in between these concentrations whereas higher bromide concentrations usually lower the reactivity and selectivity towards the nitrile.[20] Aspartic acid holds a unique position by showing the highest reactivity and selectivity in the higher concentrations of bromide around 5-20 mM.[11,20]

Research regarding the influence of substrate parameters on the conversion towards the nitrile presented complex results regarding the length of the side chain and the functional group attached to it. It was shown that amino acids with acyclic alkyl substituent at the side chain (2-aminobutanoic acid, norvaline and norleucine) react well towards the nitrile in low bromide concentration.[20] Hydroxy side chained amino acids (serine and homoserine) differ slightly in reactivity. Acidic amino acids are the group with the most significant differences. Aspartic acid shows poor reactivity and selectivity in low bromide concentration whereas glutamic acid and 2-aminoadipic acid show very good conversion results.[20] Therefore a monocausal approach seems to be not appropriate to explain the differences in reactivity of different amino acids towards the nitrile.

As the length of the functional chain and nature of the functional group did not explain the differences fully, a novel approach to explain the differences in reactivity and selectivity is the consideration of the functional group at the beta carbon of the amino acid.

# 2. Aim of the research

The aim of the research presented here focuses on two aspects of VCPO mediated conversion: the influence of the functional group at the beta carbon of amino acids on their conversion towards the nitrile and the possibility of novel substrates for VCPO mediated conversion.

## 2.1. Influence of the functional group(s) at the beta carbon of amino acids in the conversion towards their respective nitrile

The main goal of this study is to examine how the functional groups(s) at the beta carbon influence the conversion of an amino acid towards the corresponding nitrile. It was shown that amino acids differ in reactivity and selectivity and as well in the concentration of sodium bromide needed for full conversion in a VCPO mediated conversion.[11,16] This suggests that the structure of an amino acids itself influences their reactivity and selectivity. Therefore, former studies focused on the effect of length and functionality of the amino acid side chain in a VCPO mediated conversion. In a W-catalyst conversion system, a significant differences of reactivity and selectivity of serine and threonine was reported.[15] As both amino acids only differ in the presence of a methyl group at the beta carbon a new hypothesis was formed. The first research questions reads as:

> *What is the influence of the functional group(s) at the beta carbon of an amino acid on the reactivity and selectivity in the VCPO mediated conversion towards the corresponding nitrile?*

## 2.2. Conversion of α-hydroxy acids

Another point of interest is the possibility of novel substrates for the conversion mediated by VCPO. It is suggested that VCPO is only responsible for the creation of the reactive species, thus the substrates for the reactive species are not limited due to enzymatic interaction. α-Hydroxy acids, which are structurally similar compounds to amino acids, were suggested as a suitable substrate for VCPO mediated conversion. Therefore, the second research question was formed:

> *Is it possible to use a VCPO mediated conversion system for the oxidative decarboxylation of α-Hydroxy acids?*

## 2.3. Conversion of Limonene via VCPO

Another novel substrate for VCPO mediated conversion is R-limonene. Former studies have shown that the stereospecific oxidation of R-limonene to its epoxide and diol without the appearance of halogenated forms via a haloperoxidase is possible.[21] Thus, the third research questions was formed:

> *What is the influence of a VCPO mediated conversion on R-limonene and is it possible to produce specifically its epoxide and diol?*

# 3. Results

The research conducted on VCPO mediated conversions is presented in three parts. First, the research on the influence of the beta carbon functional group(s) on the conversion of amino acid towards their corresponding nitrile is presented. This part (Chapter 3.1) aims to extend the knowledge about the differences in reactivity and selectivity for amino acids by focusing on this structural feature.
The following two parts are focussing on the extension of usability of VCPO mediated conversion by exploring novel substrates. More precisely, the second part (Chapter 3.2) covers the conversion of α-hydroxy acids mediated by VCPO, while the third part (Chapter 3.3) of this chapter deals with the possibility of limonene conversion mediated by VCPO.

## 3.1. Influence of functional group(s) at the beta carbon of amino acids on the conversion towards their respective nitrile

The research conducted on the influence of the functional group at the beta carbon was split into two subchapters depending on the changes made at the beta carbon.

Firstly, the influence of the addition of an extra methyl group at the beta carbon was investigated. Therefore, four amino acids pairs that differ only in the presence of an additional methyl group at the beta carbon were chosen (see Table 1, P.7) and analysed.

In the second part of this subchapter, the influence of the functional groups at the beta carbon on conversion towards the nitrile was examined. Two amino acids triplets that differ only in the presence of a methyl ($-CH_3$), hydroxy (-OH) or carboxyl (-COOH) group at the beta carbon were chosen and analysed (see Table 2, P.20).

For all the amino acids and pairs/triplets the analysis followed the same procedure.
First, a NaBr sensitivity screening was conducted in five different concentrations from 0 to 20 mM. This was done to determine the sensitivity of the conversion of an amino acids towards its nitrile in dependence of the bromide concentration present. The sensitivity was determined in terms of reactivity and selectivity. Reactivity was expressed as the reduction of amino acids in comparison to the starting concentration. Selectivity was measured by the formation of the nitrile in comparison of amino acid consumed. Both values are depicted in relative percentage.
In a second step the course of the reaction over time was examined. A 60 minutes conversion at 5 mM NaBr was stopped at five time points to measure reactivity and selectivity towards the nitrile. From the reactivity of that five time points, a consumption rate between each subsequent point was calculated.

The results presented in Chapter 3.1. are single measurements. The data shown in the next chapters is the only complete data set which was obtained by the same enzyme fraction and therefore same activity. Duplicates sets were conducted with different enzyme fraction which activities may differ and were therefore excluded. This issue and decision will be further elaborated upon in the discussion section about the enzyme characterisation.

### 3.1.1. Addition of a methyl group at the beta carbon

In this section the conversion of amino acid pairs which differ in the addition of an extra methyl group at the beta carbon are compared. This implies that a hydrogen atom at the beta carbon in the first amino acid was substituted by a methyl group in its comparison partner. Table 1 shows the compared amino acid pairs, their corresponding nitriles as well as their substituents at the beta carbon labelled with R1 to R3. A generalized amino acid in its zwitterion state and side chain groups in green is depicted in Figure 4.

*Table 1. Overview of amino acids pairs investigated in this chapter. R1 to R3 indicate the functional groups/atoms at the beta carbon of the amino acid side chain (see Figure 4).*

| Amino acid | R1 | R2 | R3 | Nitrile | Chapter |
|---|---|---|---|---|---|
| 2-aminobutanoic acid | $-CH_3$ | -H | -H | Propionitrile | 3.1.1.1. |
| Valine | $-CH_3$ | $-CH_3$ | -H | Isobutylnitrile | 3.1.1.1. |
| | | | | | |
| Serine | -OH | -H | -H | Glycolonitrile | 3.1.1.2. |
| Threonine | -OH | $-CH_3$ | -H | Lactonitrile | 3.1.1.2. |
| | | | | | |
| Aspartic acid | -COOH | -H | -H | Cyanoacetic acid | 3.1.1.3. |
| Methyl aspartic acid | -COOH | $-CH_3$ | -H | 2-cyanopropanoic acid | 3.1.1.3. |
| | | | | | |
| Valine | $-CH_3$ | $-CH_3$ | -H | Isobutyronitrile | 3.1.1.4. |
| Threonine | -OH | $-CH_2$ | -H | Lactonitrile | 3.1.1.4. |

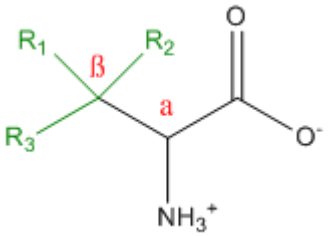


*Figure 4. Generalized amino acids in zwitterion state at working pH 5.6. Functional groups are indicated in green. The alpha and beta carbon are marked with red letters.*

### 3.1.1.1. 2-aminobutanoic acid and valine

2-aminobutanoic acid (2ABA) and valine (Val) are non-polar amino acids with an acyclic alkyl moiety on the side chain. 2-aminobutanoic acid has two hydrogen atoms and a single methyl group situated on the beta carbon. Valine differs to 2-aminobutanoic acid in the presence of a second methyl group instead of a hydrogen at the beta carbon (Figure 5). The corresponding nitriles are propionitrile (2ABACN) and isobutyronitrile (ValCN).

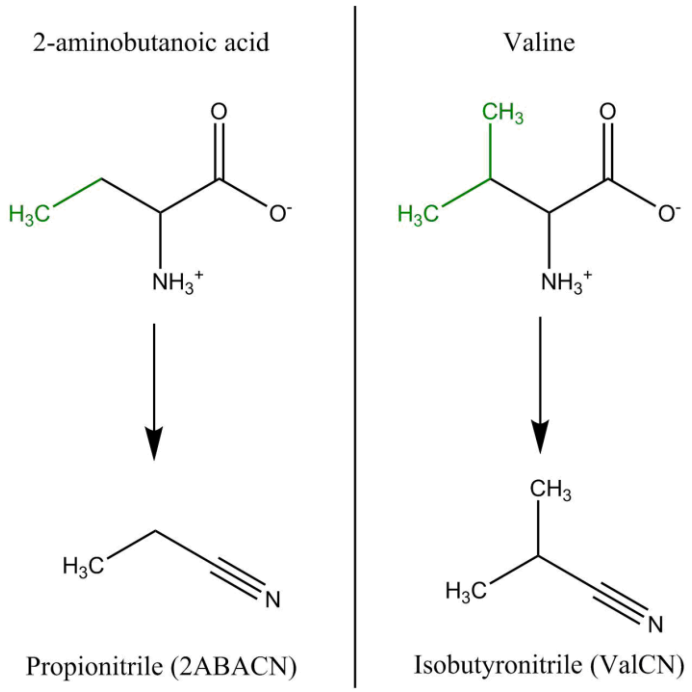


*Figure 5. Skeletal formula of 2ABA and Val in zwitterion state at working pH 5.6. Functional groups at the beta carbon are marked in green. Their corresponding nitriles are depicted below.*

First a sodium bromide sensitivity screening was conducted in five different NaBr concentrations from 0 to 20 mM. The reaction was stopped at 60 minutes and the starting concentration of the amino acid was 5 mM. The result of the sensitivity screening in terms of reactivity are shown in Figure 6. The influence of bromide concentration on selectivity is shown in Figure 7.

The NaBr sensitivity screening showed the same trend for both amino acids. Without the presence of bromide, no conversion happened. When bromide was present, the reactivity and selectivity were dependent on the concentration of bromide. In the lowest NaBr concentrations of 0.5 mM, the reactivity was also lowest with 30% (2ABA) and 22% (Val). Increasing the NaBr concentration, the reactivity peaked for both amino acids at 5 mM NaBr concentration with 85% (2ABA) and 55% (Val). The increase in reactivity from 0.5 mM to 5 mM was the biggest change in reactivity observed for this pair. Increasing the NaBr concentration further led to a decrease in reactivity for both amino acids (Figure 6).

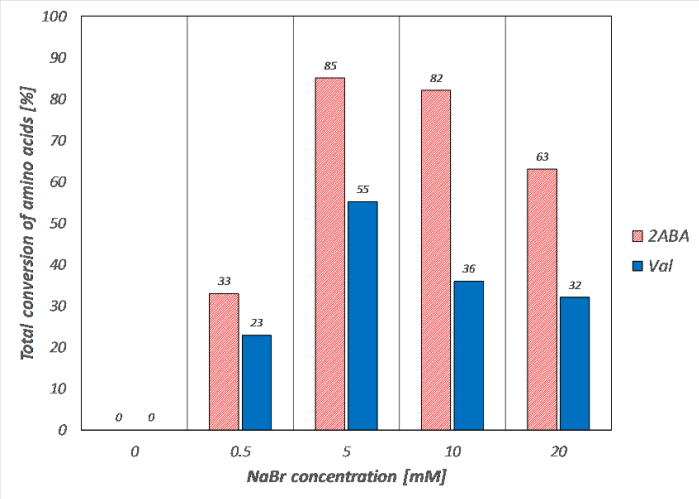


*Figure 6. Reactivity screening of amino acids 2ABA and Val at different sodium bromide concentrations. Reaction time was 60 minutes.*

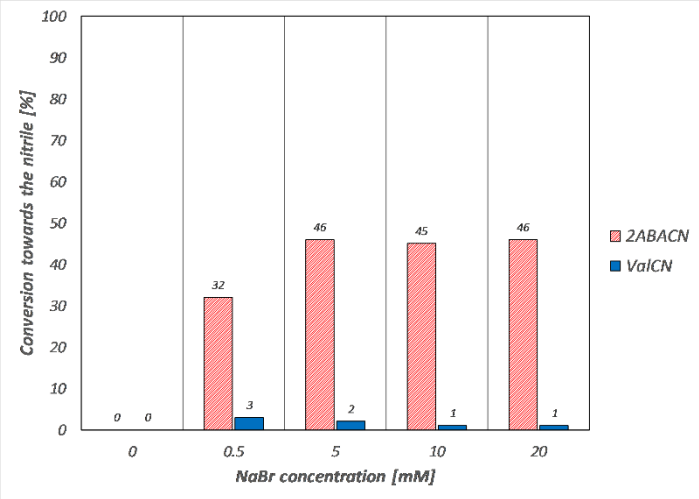


*Figure 7. Selectivity test of amino acids 2ABA and Val towards their corresponding nitrile at different sodium bromide concentrations. Reaction time was 60 minutes.*

Comparing the reactivity of both amino acids, it was prominent that the reactivity of 2ABA was always higher than the reactivity of Val. The difference was smallest in the 0.5 mM NaBr concentration with 10% and biggest in 10 mM with 47%.

Comparing the selectivity (Figure 7), it can be seen that the selectivity of 2ABA to 2ABACN varied only in the beginning. The change of 0.5 to 5 mM NaBr increased the selectivity by 14%. At 0.5 mM the selectivity of 2ABA was with 32% of consumed amino acid towards the nitrile. Increasing the bromide concentrations led to an initial increase of selectivity by 14% to around 46%. Further increase in halide concentration did not change the selectivity towards the nitrile.

Valine showed almost no conversion towards the nitrile independent from the NaBr concentration.

Comparing the selectivity of the two amino acids, it was salient that the selectivity of 2ABA towards its nitrile was always higher than that of Val. The biggest difference could be seen at the 20 mM bromide concentration with 45%. The smallest difference was visible in the 0.5 mM bromide concentration with 29%.

To obtain further information about reaction kinetics and selectivity, a reaction time course at a fixed bromide concentration of 5 mM was conducted. The time duration was set to 60 minutes and the starting concentration of amino acid to 5 mM.

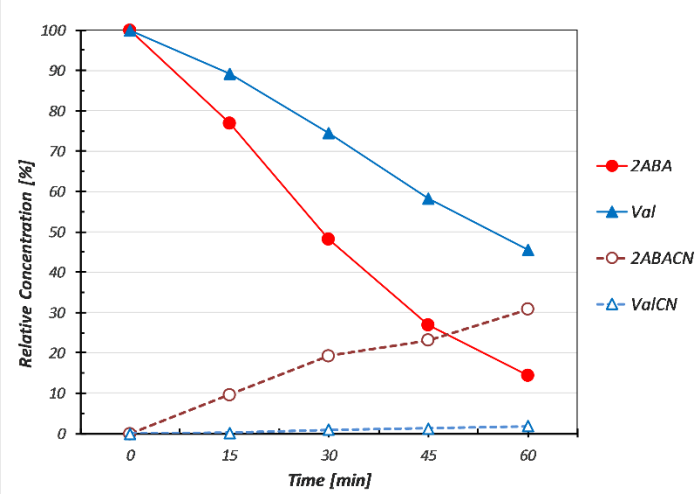


*Figure 9. Reaction time course for 2ABA (circles) and Val (triangles) to their corresponding nitriles at 5 mM NaBr. Starting concentration 5 mM and reaction time 60 minutes.*

Figure 9 shows the course of the conversion of 2ABA and Val in time with five measurements. The concentration of amino acid and corresponding nitrile produced is given in relative terms.

It can be seen that the conversion was not complete for neither of the amino acids after 60 minutes. In the end 14% of 2ABA and 45% of Val remained in solution. The corresponding nitriles 2ABACN and ValCN were formed with 30% and 2% respectively. It can be seen that the consumption of 2ABA and the formation of 2ABACN was higher than the conversion of Val. Almost no ValCN was formed whereas up to 55% of Val was consumed.

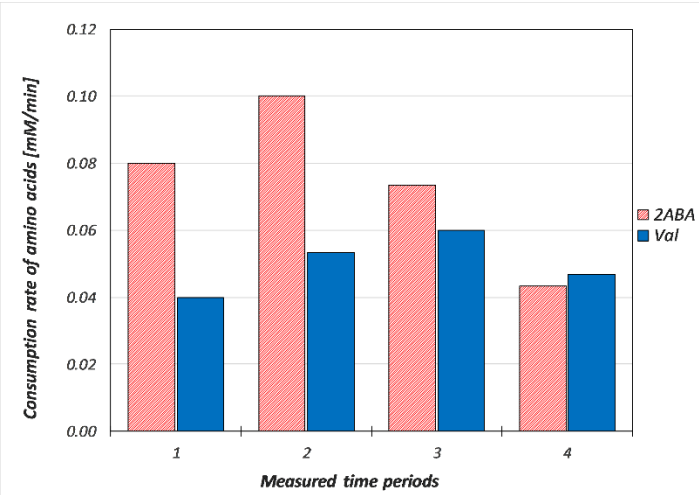


*Figure 8. Consumption rate of 2ABA (striped bar) and Val (filled bar) in the time periods between measurements.*

Figure 8 shows the consumption rate of both amino acids in four time periods. Each time period is defined as the time in between two of the measured points from the reaction time course.

The decrease of 2ABA started with 0.08 mM*min$^{-1}$. In the second time period the consumption rate increased and peaked at 0.10 mM*min$^{-1}$. From this point, the consumption rate decreased until the end reaching 0.04 mM*min$^{-1}$.

The consumption rate of Val showed a similar trend. The consumption rate was increasing in the first two time periods from 0.04 to 0.05 mM*min$^{-1}$. In the third time period it peaked with 0.06 mM*min$^{-1}$. Afterwards it decreased again. It can be seen that the consumption rate of 2ABA was almost twice as high in the beginning in comparison to the consumption rate of Val. The difference decreased in the third time period and was almost gone in the end.

### 3.1.1.2. *Serine and threonine*

Serine (Ser) and threonine (Thr) are polar amino acids with a hydroxy (-OH) functional group at the beta carbon. Serine has a single hydroxy group and two hydrogen atoms situated on the beta carbon. Threonine differs to serine in the presence of an additional methyl group. The corresponding nitriles are glycolonitrile (SerCN) and lactonitrile (ThrCN) (Figure 10).

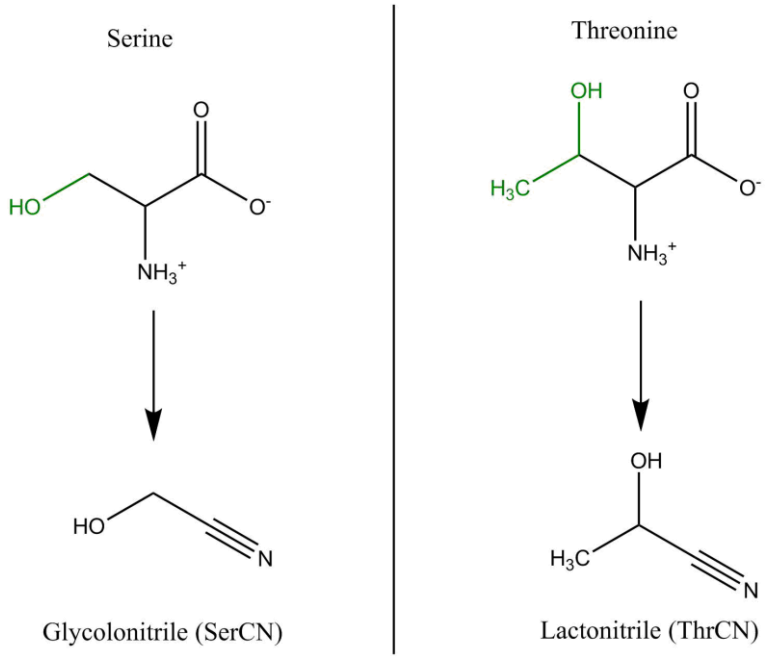


*Figure 10. Skeletal formula of Ser and Thr in zwitterion state at working pH 5.6. Functional groups at the beta carbon are marked in green. Their corresponding nitriles are depicted below.*

The bromide sensitivity screening was also conducted in five different NaBr concentrations between 0 and 20 mM. The reaction time was set to 60 minutes and the starting concentration of the amino acid to 5 mM. The results of the sodium bromide sensitivity screening are depicted in Figure 11 and Figure 12.

Without bromide present, no conversion or nitrile formation was visible. Figure 11 shows that the reactivity of both amino acids varied with different bromide concentrations. Ser showed a 56% conversion at the lowest NaBr concentration whereas Thr showed a 26% conversion. Increasing the bromide concentration to 5 mM increased conversion for both amino acids. At the same time the reactivity peaked with 78% for Ser and 63% for Thr.

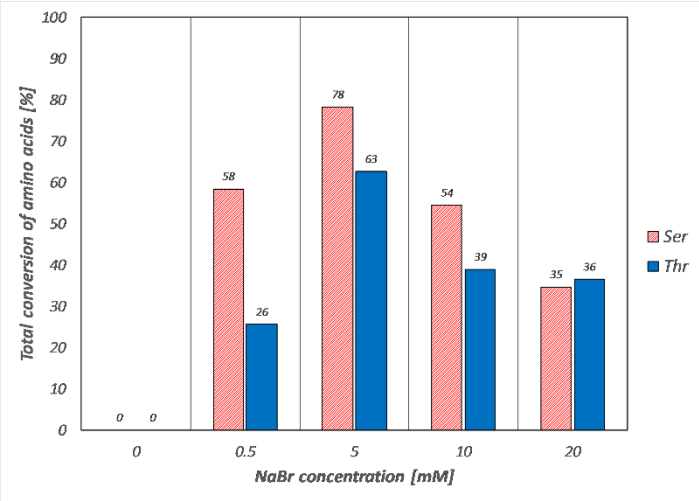


*Figure 11. Reactivity of amino acids Ser and Thr at different sodium bromide concentrations. Reaction time was 60 minutes.*

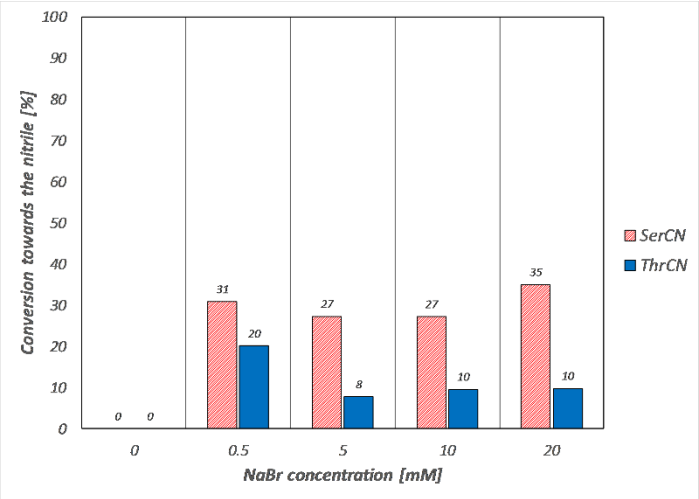


*Figure 12. Selectivity of amino acids Ser and Thr towards their corresponding nitrile at different sodium bromide concentrations. Reaction time was 60 minutes.*

The change in in reactivity of both amino acids from 0.5 mM to 5 mM bromide was the biggest seen in the sensitivity screening. Further increasing bromide concentration led to a drop in total conversion to 54% in Ser and 39% of Thr. At 20 mM bromide the total conversion is only 35% for Ser and 36% for Thr.

Comparing both conversions, it is visible that the conversion of Ser was almost always greater than the conversion of Thr. Only in the highest bromide concentration the conversion of Thr was bigger by 1%. The difference in reactivity shrunk with the increase of bromide concentration.

The initial selectivity at 0.5 mM was 31% for SerCN and 20% for ThrCN. Increasing the bromide concentration lowered the selectivity for both amino acids.

The difference in selectivity in a single amino acid between 5 and 10 mM is very small. Increasing to 20 mM bromide concentration gave a rise in selectivity for both amino acids.

Comparing the consumption rates showed the smallest difference at the lowest bromide concentration with 11%. The biggest consumption rate difference was seen in the 20 mM bromide concentration with 22%.

To obtain further information about reaction kinetics and selectivity, a reaction time course at a fixed bromide concentration of 5 mM was conducted. The time duration was set to 60 minutes and the starting concentration of amino acid to 5 mM.

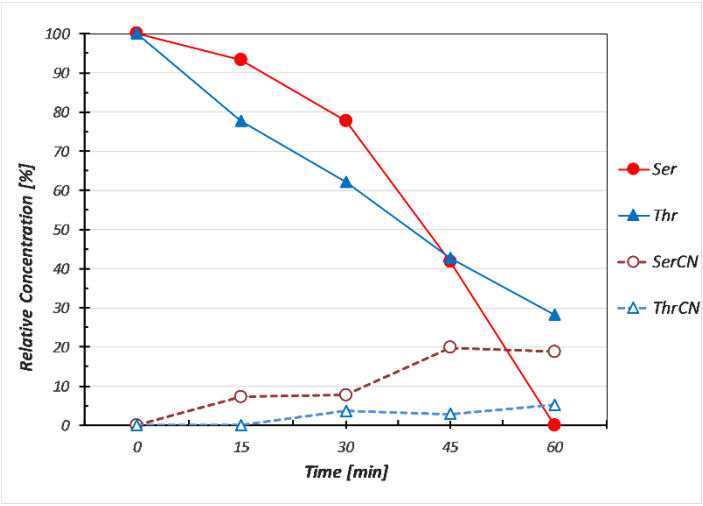


*Figure 13. Reaction time course for Ser (circles) and Thr (triangles) to their corresponding nitriles at 5 mM NaBr. Starting concentration 5 mM and reaction time 60 minutes.*

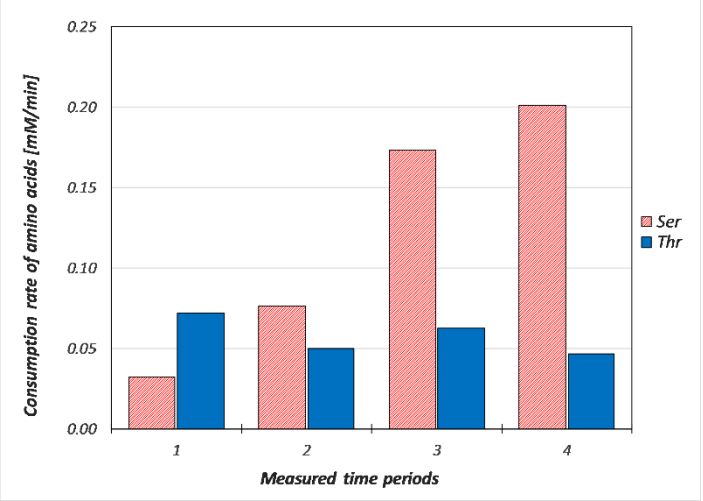


*Figure 14. Consumption rate of Ser (striped bar) and Thr (filled bar) in the time periods between measurements.*

Figure 13 depicts the time course of the reaction and Figure 14 the consumption rates. The reaction time course of Ser showed full conversion after 60 minutes. In contrast to this, 28% of the starting concentration of Thr was still in solution. The corresponding nitriles were formed with 19% for SerCN and 5% for ThrCN. Both nitriles showed an unsteady formation over time whereas the amino acids showed a steady decline.

The consumption rate of Ser started off low with ~ 0.03 mM*min$^{-1}$. In the coming time periods the consumption rate increased continuously to a maximum in the last period of ~ 0.20 mM*min$^{-1}$.

The consumption rate of Thr started at ~0.05 and in the following time period fluctuated around 0.05 and 0.07.

Comparing both rates the difference of both was not very big in the beginning with ~ 0.04 mM*min$^{-1}$ in the first two periods. In the third and the fourth time period Ser showed a much higher consumption rate than Thr with as much as 0.15 mM*min$^{-1}$ more consumption than Thr.

### 3.1.1.3. *Aspartic acid and threo-β-methylaspartic acid*

Aspartic acid (Asp) and threo-β-Methylaspartic acid (MAA) are polar acidic amino acids. Asp has a carboxyl group and two hydrogen atoms at the beta carbon. MAA is an amino acid derivate of aspartic acid where a hydrogen atom is replaced with a methyl group at the beta carbon. The corresponding nitriles are cyanoacetic acid (AspCN) and 2-cyanopropanoic acid (MAACN) (Figure 15). As 2-cyanopropanoic acid was not easily commercially available the product of MAA conversion could not be identified via comparison of retention time to the pure substance. Nevertheless the retention time of the conversion product was close to the conversion product of aspartic acid and therefore seemed to be similar in structure. It will therefore be called MAA product rather than MAACN.

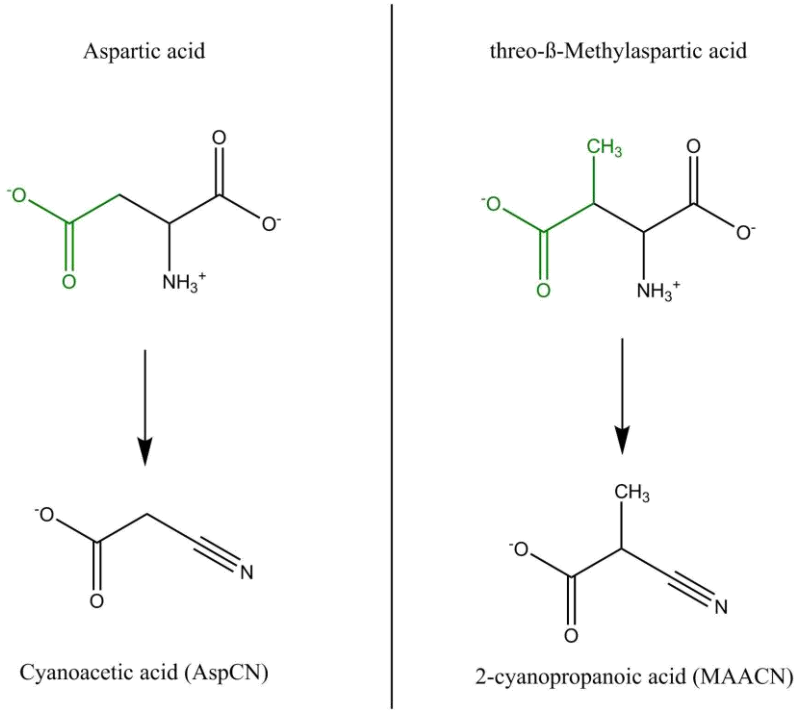


*Figure 15. Skeletal formula of Asp and MAA in zwitterion state at working pH 5.6. Functional groups at the beta carbon are marked in green. Their corresponding nitriles are depicted below.*

A sodium bromide sensitivity screening was conducted in five different NaBr concentrations between 0 and 20 mM. The reaction time was 60 minutes and the starting concentration of the amino acid 5 mM. The results of the sodium bromide sensitivity screening are depicted in Figure 16 and Figure 17.

The first observation was that without bromide no conversion was possible. The second observation was that the conversion of the both amino acids was dependent on the bromide concentration in solution. Nevertheless Asp and MAA showed different conversion properties than the other amino acids. MAA had its peak of conversion in the lowest bromide concentration with 83% total conversion.

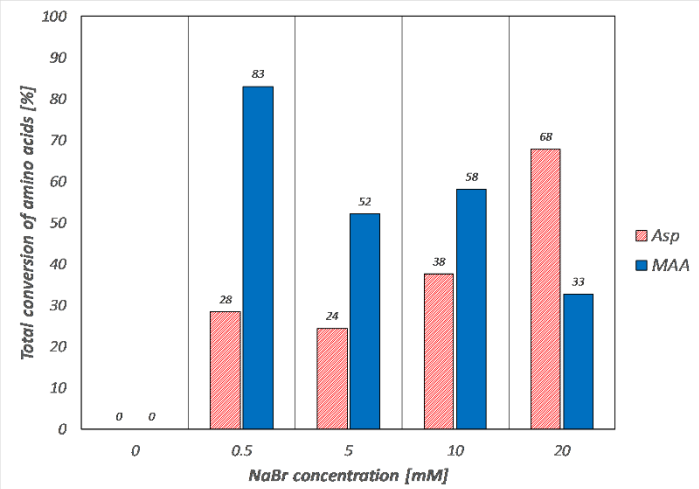


*Figure 16. Reactivity of amino acids Asp and MAA at different sodium bromide concentrations. Reaction time was 60 minutes.*

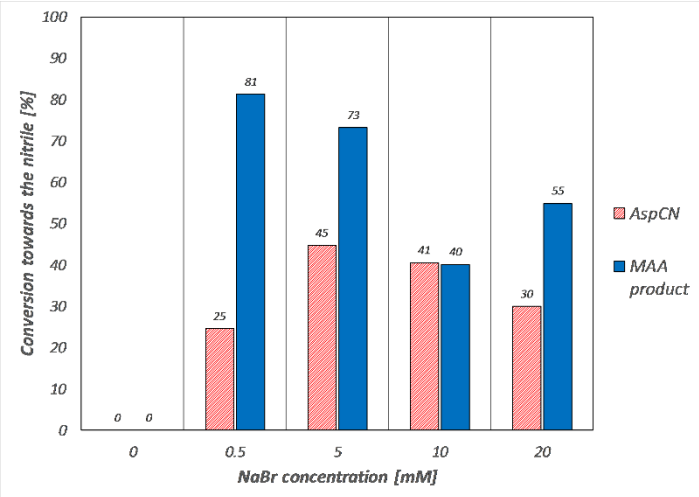


*Figure 17. Selectivity screening of amino acids Asp and MAA at different sodium bromide concentrations. Reaction time was 60 minutes.*

With increasing bromide concentration, the conversion of MAA decreased (Figure 16). The jump from 0.5 to 5 mM bromide was accompanied by a decrease of ~31% reactivity. In the highest bromide concentration the reactivity was minimal with 33%.

Aspartic acid showed low conversion in the lowest bromide concentration (28%) but increasing conversion with the increase of bromide from 5 to 10 mM. The peak conversion of Asp was reached at 20 mM bromide concentration with 68% total conversion.

The differences in reactivity ranged from 55% at 0.5 mM to 20% at 10 mM bromide.

Moreover, the selectivity of both amino acids towards the nitrile (Figure 17) showed a dependence on the bromide concentration. The highest selectivity of MAA was reached at 0.5 mM bromide. Further increasing bromide concentration, decreased the selectivity. At 10 mM the selectivity towards MAA product reached a minimum with 40% and with 20 mM bromide the selectivity increased again to ~ 55%. The selectivity of Asp started with a minimum of 25% at 0.5 mM bromide, peaked at 5 mM with 45 and decreased slowly with further increase of bromide. Comparing the selectivity, it can be seen that the selectivity of MAA was almost always greater than the selectivity of Asp. The biggest difference in selectivity was visible in the 0.5 mM bromide concentration with 58%. Further increasing bromide concentration reduced the differences in selectivity. However MAA showed almost always a higher selectivity. Only in the 10 mM NaBr concentration, selectivity was almost equal.

Similar to the conversions before, to obtain further information about reaction kinetics and selectivity, a reaction time course at a fixed bromide concentration of 5 mM was conducted. The time duration was set to 60 minutes and the starting concentration of amino acid to 5 mM.

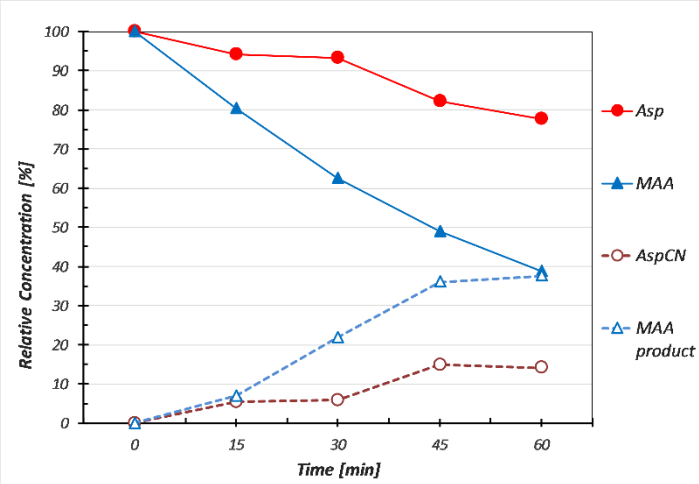


*Figure 19. Reaction time course for Asp (circles) and MAA (triangles) to their corresponding nitriles/product at 5 mM NaBr. Starting concentration 5 mM and reaction time 60 minutes.*

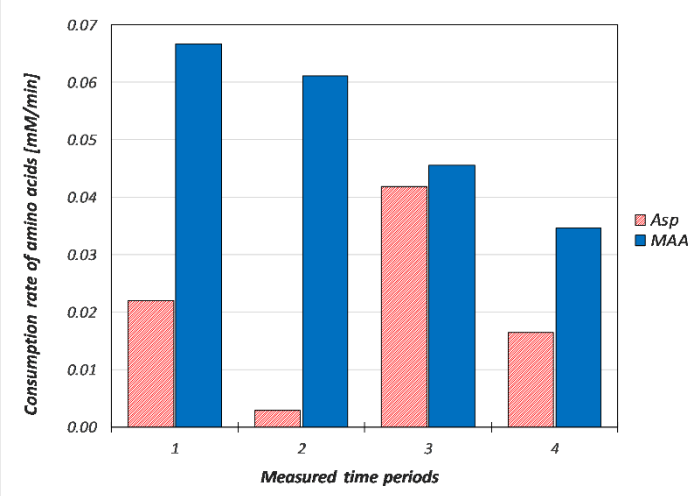


*Figure 18. Consumption rate of Asp (striped bar) and MAA (filled bar) in the time periods between measurements.*

It is visible that both conversions were not completed after 60 minutes. 40% of MAA and 84% of Asp remained in solution. MAA was consumed in a steady and high rate. The consumption rate was highest in the beginning (0.07 $mM*min^{-1}$) and decreased over time with its minimum in the last time period (~0.035 $mM*min^{-1}$). The consumption of Asp showed no clear trend. It started off with ~0.02 $mM*min^{-1}$, and then decreased in the second time period. It reached its peak in the third period and decreased again.

The conversion product of MAA was formed with a relative concentration of 38%. The formation of product stalled at the end of the conversion.

AspCN showed an equally fast formation rate as MAA product in the beginning. In the second period the formation stalled. In the third period it was the fastest with ~ 0.04 $mM*min^{-1}$.

In comparison it can be stated that the consumption of MAA and the formation of MAA product was always higher in the chosen conversion setting. The biggest difference in consumption rate could be seen in the first and second period with ~0.04 and 0.05 $mM*min^{-1}$. The smallest difference was visible in the third period with ~0.005 $mM*min^{-1}$.

### 3.1.1.4. Valine and threonine

Valine (Val) and threonine (Thr) is a special amino acid pair as in comparison to the other pairs not a hydrogen is substituted but a hydroxy group. Both have a methyl group at the beta carbon plus an additional functional group and a single hydrogen. In the case of valine it is a second methyl group, and in the case of threonine it is a hydroxy group. Their corresponding nitriles are isobutyronitrile (ValCN) and lactonitrile (ThrCN) (Figure 20).

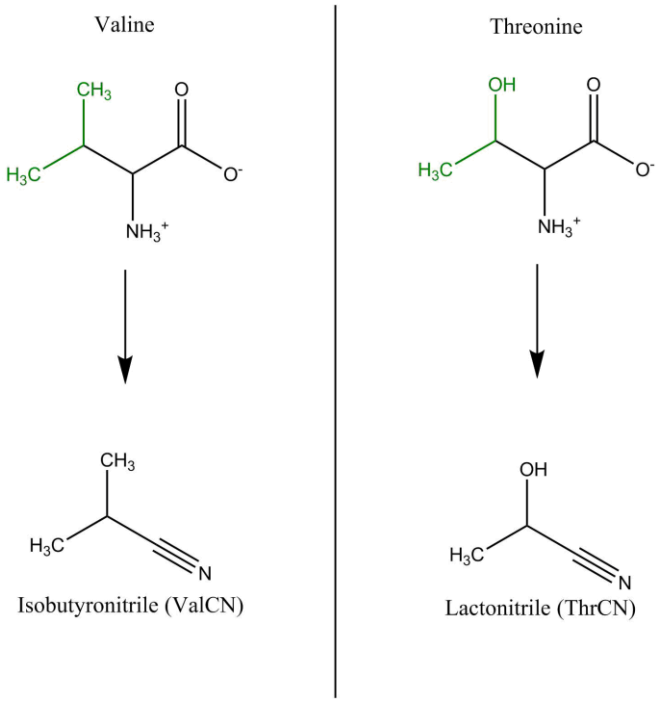


*Figure 20. Skeletal formula of Val and Thr in zwitterion state at working pH 5.6. Functional groups at the beta carbon are marked in green. Their corresponding nitriles are depicted below.*

First a sodium bromide sensitivity screening was conducted in five different NaBr concentrations from 0 to 20 mM. The reaction was stopped at 60 minutes and the starting concentration of the amino acid was 5 mM. The result of the sensitivity screening in terms of reactivity are shown in Figure 21. The influence of bromide concentration on selectivity is shown in Figure 22.

As observed before, no conversion was visible at a concentration of 0 mM bromide. The reactivity of both amino acids varied with different bromide concentrations. Both amino acids showed their lowest reactivity at the 0.5 mM bromide concentration with 23% for Val and 26% for Thr. Increasing the bromide concentration to 5 mM, peaked the reactivity for both amino acids at 55% for Val and 63% for Thr. Further increase of bromide led to a decrease in conversion.

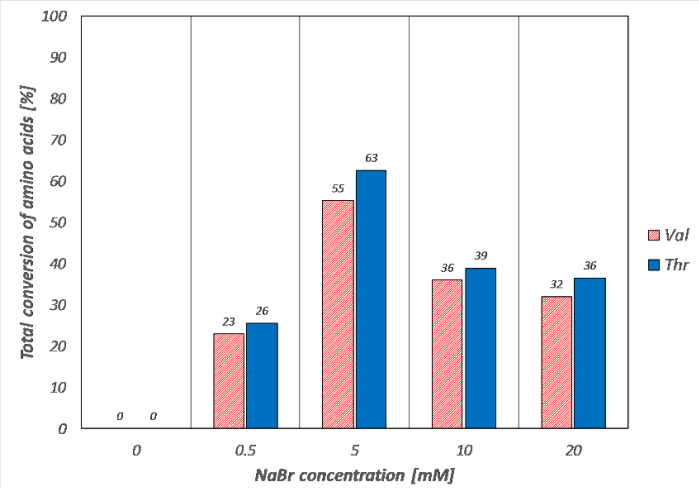


*Figure 21. Reactivity of amino acids Val and Thr at different sodium bromide concentrations. Reaction time was 60 minutes.*

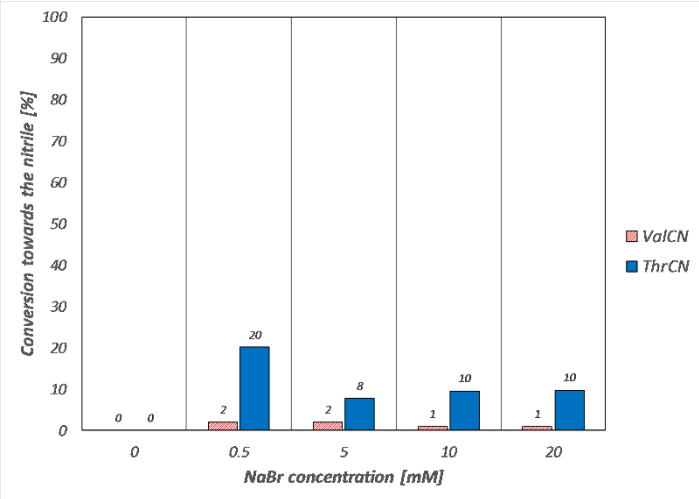


*Figure 22. Selectivity screening of amino acids Val and Thr towards their nitriles at different sodium bromide concentrations. Reaction time was 60 minutes.*

Comparing both reactivities it can be seen that the conversion of Val was always lower than the conversion of Thr. Nevertheless the difference of reactivity was not big as it ranged from 3 - 8%.

ThrCN was formed maximally at 0.5 mM bromide concentration with 20% conversion towards the nitrile. Increasing the bromide concentration to 5 mM and 10 mM, initially decreased selectivity (8% and 10%). The highest bromide concentration led to steady selectivity (10%).

Valine showed almost no conversion towards the nitrile independent from the NaBr concentration.

Comparing the selectivity of the two amino acids, it was salient that the selectivity of Thr towards its nitrile was always higher than that of Val. The difference in selectivity was in a range of 6 – 18%.

Similar to the conversions before, a reaction time course at a fixed bromide concentration was conducted to obtain further information about reaction kinetics and selectivity. The time duration was set to 60 minutes and the starting concentration of amino acid to 5 mM. The concentration of bromide was chosen to be 5 mM as well.

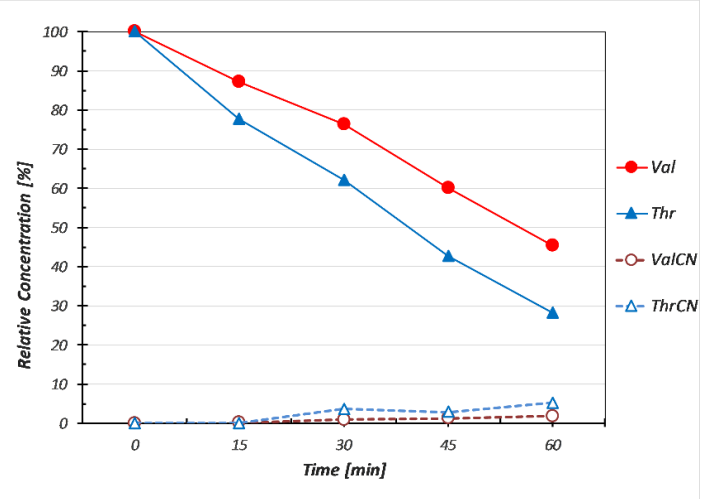


*Figure 23. Reaction time course for Val (circles) and Thr (triangles) to their corresponding nitriles at 5 mM NaBr. Starting concentration 5 mM and reaction time 60 minutes.*

Figure 23 shows the reaction time course of Val and Thr. It can be seen that the conversion of both amino was not completed after 60 minutes. Val remained with 45% and Thr with 29%. Very small amounts of corresponding nitriles were formed (3% ValCN and 5% ThrCN).

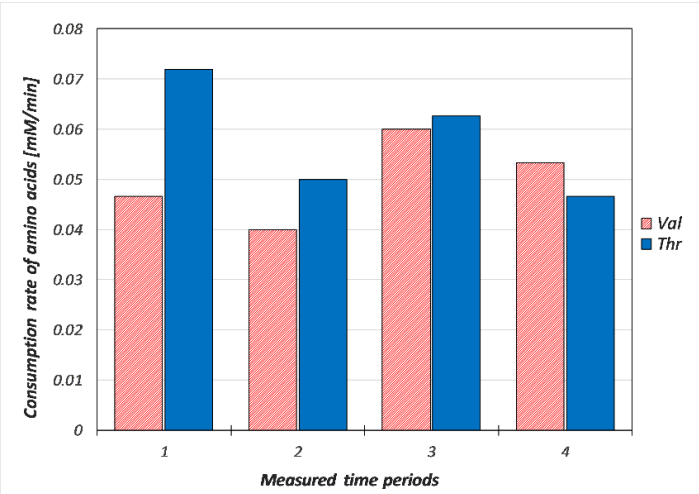


*Figure 24. Consumption rate of Val (striped bar) and Thr (filled bar) in the time periods between measurements.*

The decrease of Val started off fast (0.048 mM*min$^{-1}$) but decreased in the second period. Then it peaked in the third period with ~ 0.06 mM*min$^{-1}$ and decreased again in the fourth period. A similar trend could be seen for the Thr consumption. It peaked starting with ~ 0.07 mM*min$^{-1}$, decreased in the second period. Similar to Val, Thr consumption rate rose again in the third period and then reached minimum in the fourth period with ~ 0.048 mM*min$^{-1}$.

Comparing the two conversions, Thr showed a greater consumption rate in the first three time periods. The advantage diminished over time and in the fourth period Val consumption was greater than Thr consumption.

### 3.1.2. Comparison of –$CH_3$,-OH and –COOH functionality

In this section, the second comparison of amino acid conversion is focusing on the exchange of the functional group at the beta carbon. Therefore, the influence of a methyl (–$CH_3$), hydroxy (-OH) or carboxyl (–COOH) functional group at the beta carbon on the conversion of an amino acid towards its nitrile is examined. Table 2 shows the compared amino acid triplets, their corresponding nitriles as well as their substituents at the beta carbon labelled with R1 to R3. A generalized amino acid in its zwitterion state and side chain groups in green is depicted in Figure 25.

The first group are three amino acids with different functional groups and two hydrogen atoms (2-aminobutanoic acid, serine and aspartic acid). The second group are three different amino acids with the functional groups, a methyl group and a hydrogen atom at the beta carbon (Valine, threonine and methyl aspartic acid).

*Table 2. Overview of amino acids pairs investigated in this chapter. R1 to R3 show the functional groups/atoms at the beta carbon of the amino acid side chain.*

| Amino acid | R1 | R2 | R3 | Nitrile |
|---|---|---|---|---|
| 2-aminobutanoic acid | -$CH_3$ | -H | -H | Propionitrile |
| Serine | -OH | -H | -H | Glycolonitrile |
| Aspartic acid | -COOH | -H | -H | Cyanoacetic acid |
| | | | | |
| Valine | -$CH_3$ | -$CH_3$ | -H | Isobutyronitrile |
| Threonine | -OH | -$CH_3$ | -H | Lactonitrile |
| Methyl aspartic acid | -COOH | -$CH_3$ | -H | 2-cyanopropanoic acid |

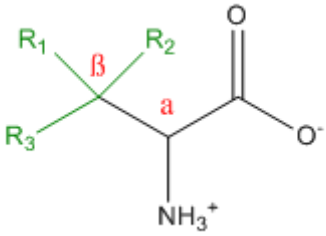


*Figure 25. Generalized amino acids in zwitterion state at working pH 5.6. Functional groups are indicated in green. The alpha and beta carbon are marked with red letters.*

### 3.1.2.1. 2-aminobutanoic acid, serine and aspartic acid

2ABA, Ser and Asp and their corresponding nitriles have been introduced before. The same data collected is presented in a different context here.

Figure 26 and Figure 27 shows the results of the reactivity screening of the three amino acids in dependence of the bromide concentration. The general trend of the conversion is described in chapter 3.1.1.

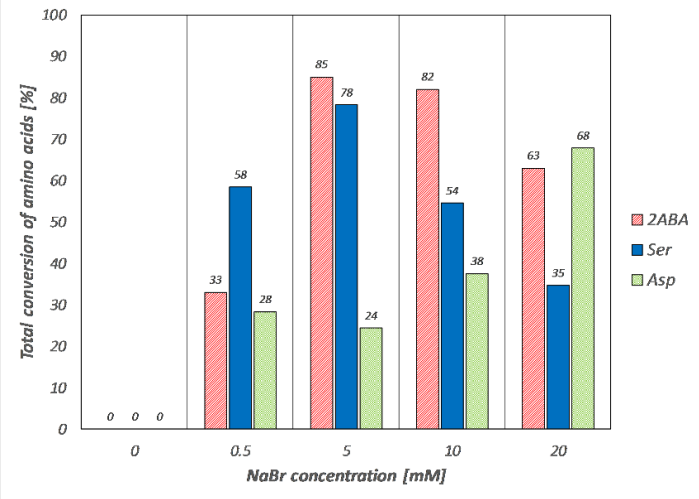


*Figure 26. Reactivity screening of amino acids 2ABA, Ser and Asp at different sodium bromide concentrations. Reaction time was 60 minutes.*

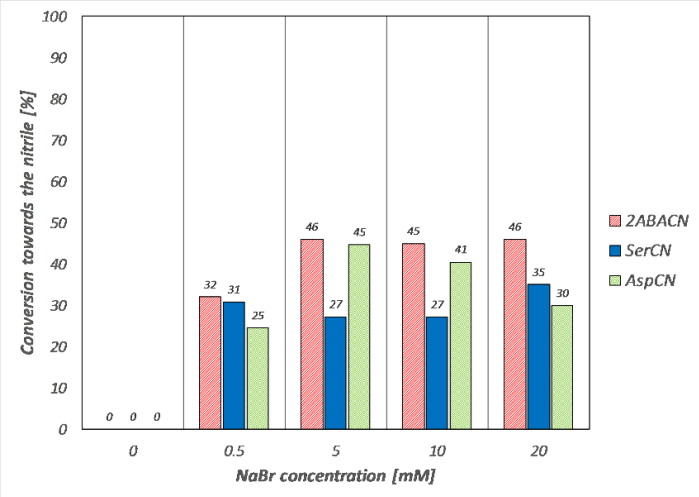


*Figure 27. Selectivity screening of amino acids 2ABA, Ser and Asp towards their nitriles at different sodium bromide concentrations. Reaction time was 60 minutes.*

Comparing the three conversions, it is visible that Ser showed the highest conversion in the 0.5 mM bromide concentration. Increasing the bromide concentration led to an increase in conversion in 2ABA (to 85%) and Ser (to 78%). 2ABA conversion at 5 mM bromide was greater than Ser conversion. Further increasing the bromide concentration led to the decrease in reactivity in 2ABA and Ser.

Asp showed low conversion in the low NaBr concentrations. Increasing the halide concentration led to an increase in conversion with a maximum of 68% at 20 mM NaBr.

Figure 27 shows the selectivity towards the nitriles. In low bromide concentrations the selectivity of the amino acids was rather similar. 2ABA showed the highest selectivity. Increasing the bromide concentration led to an initial increase of selectivity in 2ABA which stayed constant with increasing bromide concentration. Selectivity of Ser dropped with 5 and 10 mM bromide concentration and increased at 20 mM again but stayed in a narrow range. Selectivity for Asp started the lowest in low bromide concentrations with 25%. It peaked at 5 mM with 45% and dropped with further bromide concentration increase.

Similar to the conversions before, the time course of the reactions is put into a new context here.

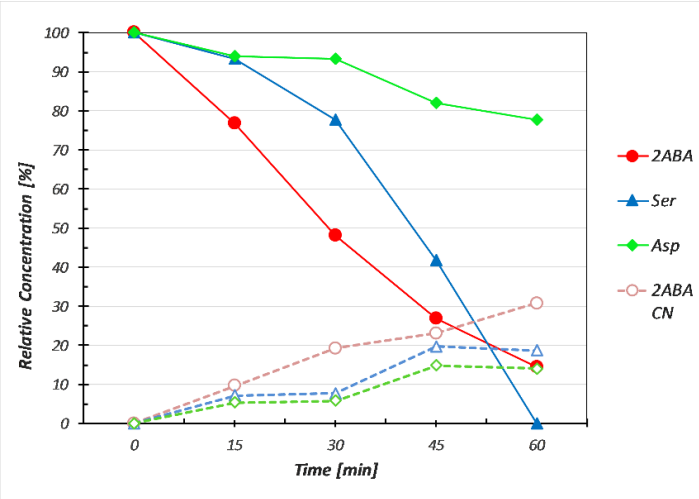


*Figure 28. Reaction time course for 2ABA (circles), Ser (triangles) and Asp (diamonds) to their corresponding nitriles at 5 mM NaBr. Reaction time was 60 minutes.*

Figure 28 shows the reaction time course of 2ABA, Ser and Asp. It can be seen that the conversion of 2ABA started off quite fast and was almost bigger for the whole time. The conversion of Ser started very low with ~ 0.03 mM*min$^{-1}$. In the following time periods, the consumption rate increased and peaked in the last period. Ser was the only amino acid converted completely after 60 minutes.

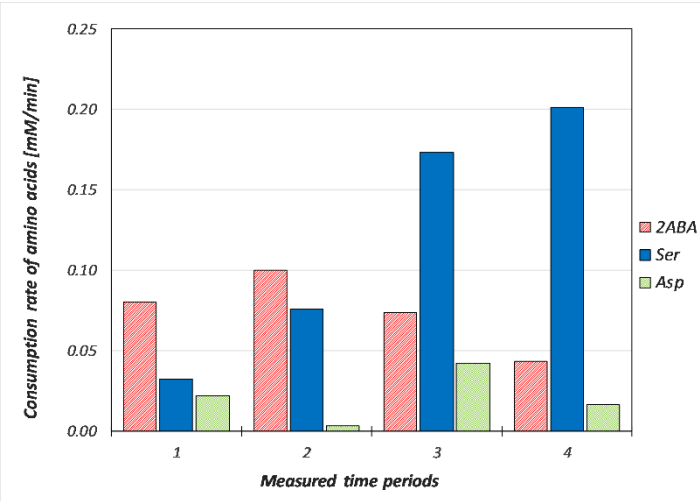


*Figure 29. Consumption rate of 2ABA (striped bar), Ser (filled bar) and Asp (checkered bar) in the time periods between measurements.*

Asp showed an initial rate of ~0.025 mM*min$^{-1}$. Further time periods showed a fluctuation over time. The maximal reaction rate was reached between 30 and 45 minutes.

The formation of nitrile showed that 2ABACN was formed the most with a final percentage of ~30%. SerCN and AspCN were formed less with around 20% and 15% respectively.

Comparing the consumption rates of the three amino acids, it was visible that Ser showed the highest difference in consumption rate to the two other amino acid rates in the third and fourth period with a difference up to ~ 0.17 mM*min$^{-1}$ (Asp consumption rate vs. Ser consumption rate) in the fourth period.

### 3.1.2.2. *Valine, threonine and threo-β-methylaspartic acid*

2ABA, Ser and Aspartic acid and their corresponding nitriles have been introduced before. The same data collected is presented in a different context here.

Figure 31 and Figure 30 shows the results of the reactivity screening of the three amino acids in dependence of the bromide concentration. The general trend of the conversion is described in chapter 3.1.1.

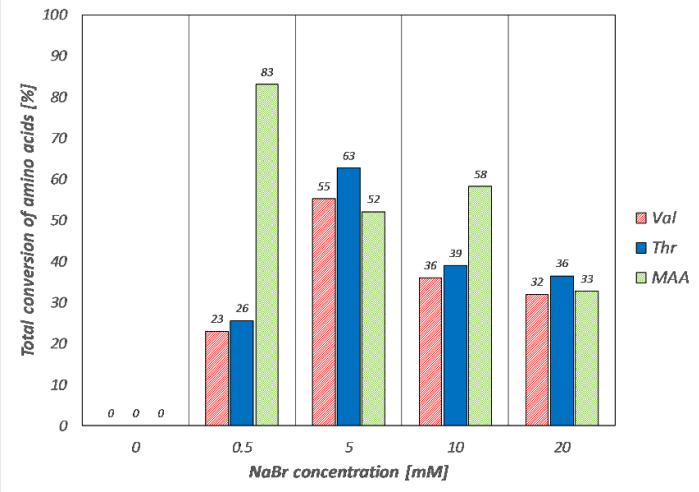


*Figure 31. Reactivity screening of amino acids Val, Thr and MAA at different sodium bromide concentrations. Reaction time was 60 minutes.*

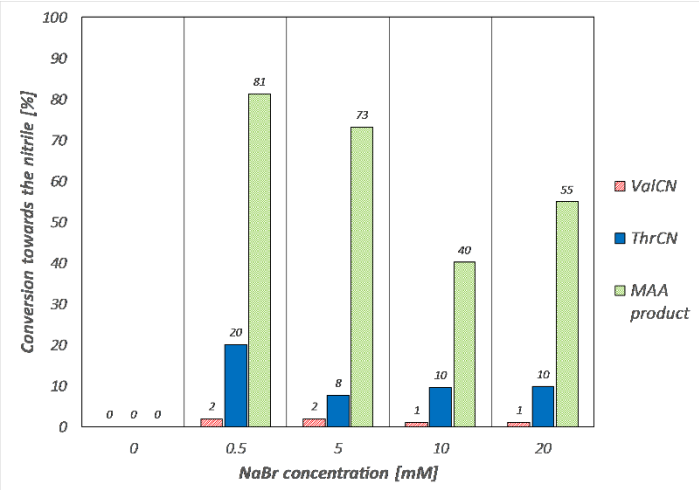


*Figure 30. Selectivity screening of amino acids Val, Thr and MAA towards their nitriles at different sodium bromide concentrations. Reaction time was 60 minutes.*

Figure 31 shows the total conversion of Val, Thr and MAA. Comparing the reactivity in dependence on bromide concentration it can be seen that the bromide concentration has an influence on the reactivity. MAA showed almost full conversion (83%) at 0.5 mM NaBr concentration. The two other amino acids showed a very low reactivity with 23% for Val and 26% for Thr.

Increasing the NaBr concentration resulted in the increase of reactivity for Val (to 55%) and Thr (to 63%) only. The reactivity of MAA dropped significantly to 52%. The reactivity here was similar. Further increasing the bromide concentration led to a reduction of reactivity in Val and Thr but a slight increase in MAA. In the highest concentration of bromide, the reactivity of all three amino acids is further decreased.

Figure 30 shows the sensitivity of selectivity of the three amino acids towards their corresponding nitrile/product. No ValCN could be detected for all bromide concentrations. Both Thr and MAA showed the highest selectivity at 0.5 mM with 20% and 83% towards the nitrile respectively. Increasing the bromide concentration led to the decrease in selectivity. It can be noted that the selectivity of MAA is in all cases much higher. The biggest difference between selectivity towards ThrCN and MAA product could be seen at the 5 mM bromide concentration with 65%.

Similar to the conversions before, the time course of the reactions is put into a new context here.

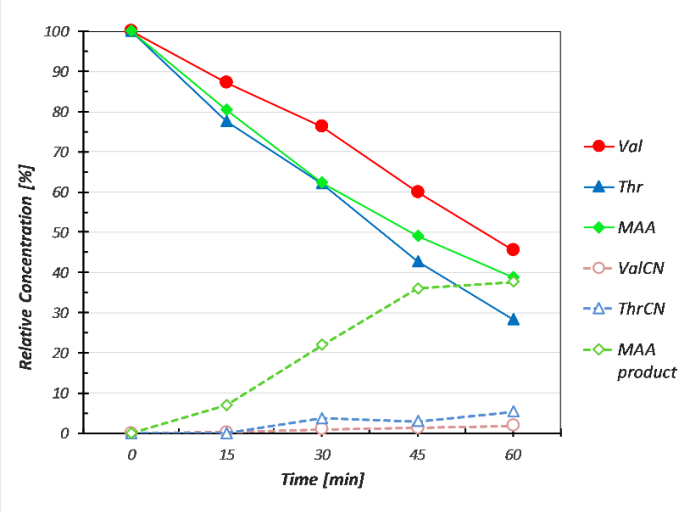


*Figure 32. Reaction time course for Val (circles), Thr (triangles) and MAA (diamonds) to their corresponding nitriles/products at 5 mM NaBr. Reaction time was 60 minutes.*

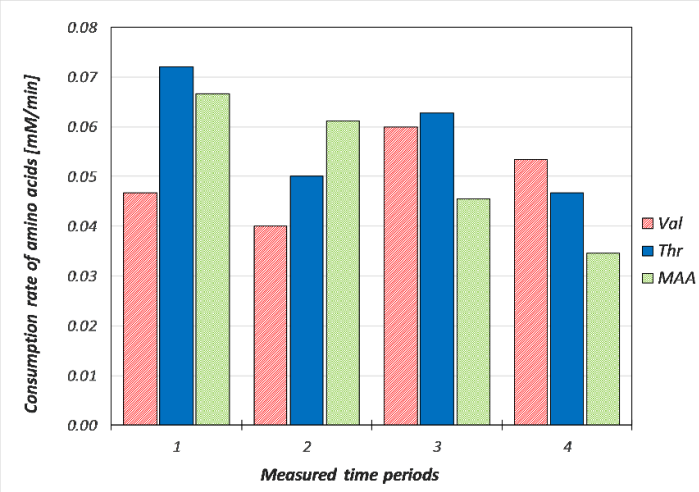


*Figure 33. Consumption rate of Val (striped bar), Thr (filled bar) and MAA (checkered bar) in the time periods between measurements.*

Figure 32 shows the reaction course over time. Comparing Val, Thr and MAA, it can be seen that Thr was converted maximally with ~29% Thr remaining after 60 minutes. MAA was converted less with 40% of MAA remaining in solution. Val remained with 45% in solution after 60 minutes. The profile of consumption did not differ a lot. Thr was consumed the most.

The selectivity however was completely different. ThrCN and ValCN were hardly formed (5% and 3% respectively) whereas the selectivity of MAA was around 39% in the end.

Comparing it can be said that the reactivity of the three amino acids was quite similar. The selectivity for MAA was higher than for Thr and Val.

## 3.2. α-Hydroxy acid conversion

The α-hydroxy acids mandelic and Gluconic (Figure 34) acid were tested for their reactivity in a conversion mediated by VCPO. The experimental setup conditions of the amino acid conversion were used. The reaction time was set to 60 minutes after which the concentration of substrate was measured via HPLC analysis and comparison to external standards of the pure substances. At the start the α-hydroxy acid concentration was 5 mM. Halides used were chloride and bromide as sodium chloride and sodium bromide in concentrations from 0 to 20 mM. The results of this conversion are depicted in Table 3 below.

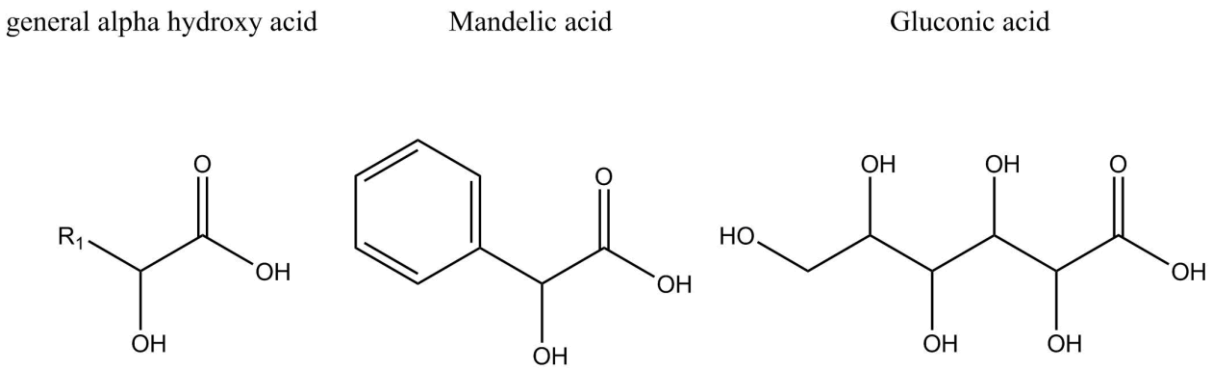


*Figure 34. Skeletal structure of a general α-hydroxy acid with the substituent R1 at the beta carbon. Next to the general form, are the two AHA depicted which were used in this research, mandelic and gluconic acid.*

*Table 3. Overview of α-hydroxy acid studied with the corresponding halide and the tried concentrations.*

| Substrate | Halide source | Halide Concentration | Conversion possible? |
|---|---|---|---|
| Mandelic acid | NaBr | 0-20 mM | No conversion of substrate |
| Mandelic acid | NaCl | 0-20 mM | No conversion of substrate |
| Gluconic acid | NaBr | 0-20 mM | No conversion of substrate |
| Gluconic acid | NaCl | 0-20 mM | No conversion of substrate |

As it can be seen, there was no conversion of any of the α-hydroxy acid in any combination of halide or concentration.

## 3.3. R-Limonene conversion

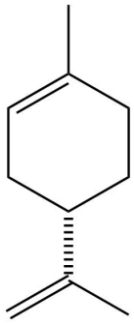

*Figure 35. Skeletal structure of R-limonene*

The conversion of limonene was conducted in the same reactions condition as the amino acids. The reaction media was changed to contain 10% v/v acetonitrile to increase the solubility of limonene.

The reaction time was set to 60 minutes and a concentration of 5 mM NaBr was used. In the beginning, 5 mM of limonene were in solution.

The reaction products were compared to pure samples of limonene, limonene epoxide and limonene diol. Further analyses of the reaction products were conducted by GC-MS.

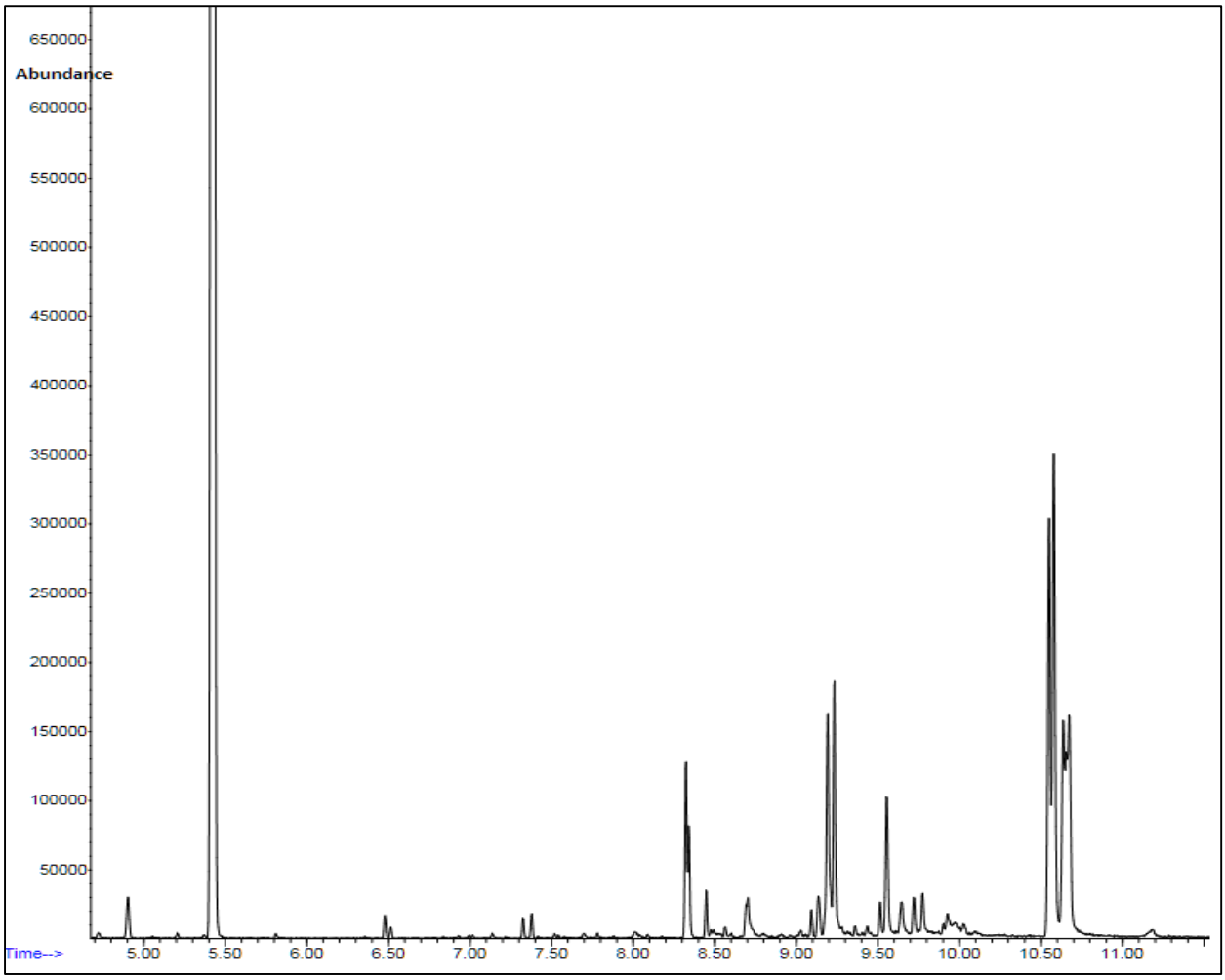


*Figure 36. Total ion count of the reaction mixture of D-limonene conversion mediated by VCPO after 60 minutes and 5 mM NaBr concentration.*

The result of the 60 minute conversion is depicted in Figure 36. The reaction mixture was analysed using GC-MS. The biggest peak at 5.49 minutes corresponded to pure limonene. This was the fraction which was not converted and appeared with an abundance of 1.23E+08. Figure 36 therefore shows a

zoomed in version of the total ion count to make the reaction products visible. These products ranged in abundance from 2.5E+05 to 3.40E+06 at a retention time of 10.578 minutes. However just the ten most abundant products were further analyzed.

None of the reaction products formed could be identified via comparison of retention time and spectrum to pure samples of limonene epoxide and limonene diol. To further make statements about the reaction mediated by VCPO, the mass spectrum of the reaction products was analyzed for hints of their structure. Table 4 shows the abundance of the ten most abundant reaction products, their molecular ion and its abundance in the spectrum, an M-2 and M+2 ion as well as their abundance if applicable. Unfortunately, no high resolution mass spectrum of the reaction products was obtained. Therefore, the mass-to-charge ratio of the substances was given with an accuracy of two decimals.

*Table 4. Retention time of main product (Figure 36) of R-limonene conversion and their corresponding mass-to-charge-ratio. Additionally if applicable M-2 and M+2 ion.*

| Retention time | 8.325 | 8.344 | 8.447 | 9.196 | 9.234 | 9.556 | 10.551 | 10.578 | 10.64 | 10.672 |
|---|---|---|---|---|---|---|---|---|---|---|
| Product Abundance | 2.49E+06 | 1.28E+06 | 6.89E+05 | 2.08E+06 | 2.04E+06 | 1.24E+06 | 2.61E+06 | 3.40E+06 | 1.49E+06 | 1.66E+06 |
| Molecular Ion [m/z] | 215.90 | 219.00 | 152.10 | 214.10 | 250.00 | 249.90 | 296.90 | 297.01 | 296.90 | 296.90 |
| Abundance M | 900 | 200 | 200 | 400 | 400 | 250 | 800 | 800 | 650 | 600 |
| M-2 [m/z] | 213.90 | 217.00 | | 216.10 | 248.00 | 247.90 | 294.90 | 295.01 | 294.90 | 294.90 |
| Abundance M-2 | 800 | 190 | | 390 | 390 | 240 | 600 | 500 | 200 | 300 |
| M+2 [m/z] | | | | | | | 298.90 | 299.01 | 298.90 | 298.90 |
| Abundance M+2 | | | | | | | 600 | 500 | 200 | 300 |

Of these ten reaction products, the product at the retention time of 8.447 minutes was the only one under a mass-to-charge-ratio of 200. The reactions products at a retention time of 8.325, 8.344, 9.196, 9.23 and 9.556 minutes showed a mass-to-charge-ratio of ~214 to 250 and all of them had a M-2 peak in the spectrum which was roughly in the same intensity as the molecular ion.

Another group which could be distinguished is the reaction products with a mass-to-charge ration of ~297. The products appeared at the 10.551 and 10.578 minute mark are the most abundant products in the whole conversion with as much as 2.61E+06 and 3.40+06. The second pair showed a lower abundance but also in the same range. All of these products showed an M, M+2 and M-2 ion. Three of the four products showed these ions in an intensity ratio of roughly 1:2:1 (M-2,M,M+2). Only the product at 10.551 minutes showed a different intensity ration with 0.8:1:0.8 (M-2,M,M+2).

# 4. Discussion

Before discussing the results of the research, several factors and issues of VCPO characterisation have to be discussed which shed light on the reliability and reproducibility of the results. The exact procedure of enzyme isolation and characterization is given in the experimental section and the data of the characterization is given in the Appendix 9.1.

After extraction and heat inactivation of unwanted enzymes, VCPO was purified by weak anion chromatography using a DEAE resin. The loaded resin was washed several times and then VCPO was eluded with a gradient of NaCl solution. The fractions of this elution were stored separately and VCPO activity was tested with a quick activity test based on a phenol red assay. The so determined active fractions were further analysed in depth by using a monochlorodimedone (MCD) assay (see experimental section) which gave insight on the activity of the enzyme fraction and a Bradford-Protein assay which yielded the protein content of a fraction. Both values were used to determine the specific enzyme activity. The fractions were frozen and then used in consecutive order during experiments. Problems arose as one characterised enzyme fraction showed no activity in the conversion of amino acids towards their nitriles although the chosen specific activity was correct. Further experiments revealed that more than one enzyme fraction did not show any activity in amino acids conversion but in the MCD assay. The most likely issue here is that the MCD assay itself is not the best tool to determine haloperoxidase activity. As recent research has shown the problem of the assay is that its determining aspect is based on the reduction of substrate and not on the formation of the halogenated compound, thus it is likely to produce false positives when other enzymes are present.[22]

From this point on all the activity of enzyme fractions was determined by the conversion of glutamic acid. A standardized assay with 0.5 mM NaBr and 5 mM glutamic acid was used to verify the activity of the enzyme fractions. This is a valid approach to determine general activity but no differences in between. The chosen concentration of bromide in combination with glutamic acid resulted in full conversion after 60 minutes in all positive enzyme fraction and thus allows no comparison of activity. This fact was not realized until the end of the research. Therefore, the data presented here is the only complete data set which was gathered using a single enzyme fraction and thus should have the same enzyme activity. This means that the reliability of the data gathered is low as they have to be seen as single measurements.

## 4.1. Influence of functional group(s) at the beta carbon of amino acids on the conversion towards their respective nitrile

The collected data showed several notable trends in the conversion of amino acids towards their nitriles.

The conversion of 2ABA and Val showed that there is a significant increase in reactivity between 0.5 and 5 mM bromide concentration. This jump in reactivity was observed before for a plethora of amino acids.[20] It was hypothesised that a certain threshold of bromide is needed for a high reactivity and

that this threshold is between 0.5 and 5 mM bromide.[20] However what the mechanistic foundation of this threshold is, remains still not known. The continuous decrease of reactivity in higher bromide concentrations is a phenomenon which was also observed before.[20] This phenomenon can be explained by enzyme inhibition by bromide as the inhibitor constant of VCPO by bromide is rather low with 0.5 mM. Therefore, increasing the NaBr concentration is very likely to lead to lower reactivity. Additionally the steady sensitivity of selectivity of amino acids with acyclic alkyl functional groups was reported before as well.[20]

The novel observation is that the reactivity and the selectivity of Val was generally lower than the reactivity and selectivity of 2ABA. This is further supported by the reaction time course which showed that 2ABA is more rapidly consumed and 2ABACN formed. After the 60 minutes of conversion 14% of 2ABA and 45% of Val remained but in relative percentages 30% 2ABACN was formed in comparison to 2% ValCN. The difference in selectivity is immense at 5 mM. As both amino acids only differ in the additional methyl group at the beta carbon, it can be hypothesized that the methyl group shows two effects on the conversion of amino acids. First it reduces the reactivity of conversion in general. Second it reduces the selectivity towards the nitrile.

Moreover, one trend which continues in the following data becomes obvious. Comparing the reactivity and yield of nitrile with previous research on VCPO mediated conversion of amino acids[11,20] reveals that the general reactivity and selectivity towards the nitrile is in general lower than in experiments with similar conditions[20]. This may be due to two facts: first the bromide concentration used in this series of experiments was 5 mM instead of 2 mM[20]. This concentration was chosen to avoid full conversion before 60 minutes which was observed for several amino acids in the 2 mM bromide concentration[20]. As the chosen bromide concentration is ten times bigger than the inhibitor constant of VCPO by bromide[12], which is 0.5 mM, enzyme inhibition very likely occurred and lowered the reactivity and the yield. The second reason is that the enzyme used in this series of experiments seemed to show lower activity as expected.

The conversion of Ser and Thr displays some of the patterns observed before but also yields differences. As observed before, the biggest reactivity increase in the bromide screening for both amino acids occurs in the change from 0.5 to 5 mM bromide. In general Ser shows a higher reactivity and selectivity towards the nitrile than Thr. Previous studies using a W-catalyst observed similar differences in reactivity and selectivity for Ser and Thr.[15] The interesting finding here is that the difference in reactivity is shrinking with increasing bromide concentration. This phenomena has not been observed before.

The influence of bromide concentration on selectivity was observed before.[20] Previous research showed the maximum selectivity in the low bromide concentrations and a decreased but steady selectivity for higher bromide concentrations similar to the results in this study.[20] This rise of selectivity in the higher bromide concentrations observed in this study has not been observed before.

According to 2ABA and Val comparison, it seems that an additional methyl group negatively influence the reactivity strongest in low bromide concentrations. The higher the bromide concentrations, the

less pronounced is the inhibitory effect. In terms of selectivity an additional methyl group was decreasing the selectivity constantly.

The periodic formation of SerCN is very likely the result of an experimental mistake as such a time course is very hard to explain. Additionally the fluctuation of the consumption rate of Thr are also very likely an error due to experimental mistakes.

As the results for Asp and MAA are quite unique, they will be discussed later in this chapter.

In the comparison of Val and Thr a reoccurring pattern was observed again but also a new finding was revealed. The biggest increase in reactivity was in in between 0.5 and 5 mM bromide concentration. Similar to previous pairs, the amino acid with the additional methyl showed lower conversion and selectivity in both the NaBr screening as well as in the reaction time course. However, this effect in reactivity was much smaller than before whereas the effect on selectivity was still very pronounced.

The hypothesis is that the addition of a methyl group decreases the reactivity and the selectivity of an amino acids. This mechanism could work due to steric hindrance or due to electronic effects of the additional methyl group. For the change in reactivity, the difference between a hydroxy group and a methyl group at the beta carbon seemed to be almost the same in the conversion of Val and Thr. This may indicates that the effect of the additional group at the beta carbon is more of steric rather than electronic nature. This is due the fact that a methyl group is much more similar to a hydroxy group in terms of size than in electric effects and the reactivity difference is quite small. Nevertheless a methyl group takes up more space due to its three hydrogen atoms than a hydroxy group.

The general influence of the methyl group at the beta carbon may be explained by taking conformers of amino acids into account. Due to the flexibility of the side chain, steric hindrance of the halogenation at the amine group could occur and therefore lead to a decreased reactivity and selectivity. Assuming the reaction pathway proposed by Claes *et al*.[15] is correct, the conversion of amino acids to their corresponding nitrile requires two subsequent halogenations at the nitrogen of the amine group. As the side chain of amino acids is flexible in it torsions angle, the side chain methyl group could interfere with the halogenation. The methyl group is much larger than a single hydrogen atom and therefore prone to steric hindrance. If this theory applies, the first bromination would be more likely than the second one. After the first bromination, a large bromine ion is bound to the nitrogen of the amine. This would further decrease the possibility of a second bromination of the nitrogen. Therefore, the effect of an additional methyl group on the selectivity should be higher than on the general reactivity.

This trend is also visible in the collected data. It can be seen in the examples of 2ABA and Val, Ser and Thr and Val and Thr conversion that the influence of the methyl on selectivity seems to be greater than on the reactivity. For reactivity a single halogenation, and for selectivity two halogenations are needed.

Supporting this explanation approach is research using density functional theory and experimental data to determine the likelihood of conformers in amino acids. For the polar amino acids with a hydroxy group Ser and Thr, it was shown that they may be present in several conformers coexisting in aqueous solutions.[23] Some of the more likely non-standard conformers of Thr show a localisation of the methyl group close to the nitrogen of the amine group. The energy barriers between these conformers are quite low and hydrogen-bonds with water molecules are proposed to even lower the energy barrier of these conformers. It is hypothesised that conformer formation is possible in the other amino acids as well and contributes to the seen influence of the methyl group.

The only weakness of this explanation approach is the big difference in selectivity towards the nitrile of Val and Thr. Val has two methyl groups whereas Thr has a methyl and hydroxy group at the beta carbon. If conformers are possible in all amino acids and steric hindrance of functional groups at the beta carbon is a main contributor to the decrease in selectivity, the differences in selectivity should not be very big. However the size of the methyl group is just similar to the hydroxy group. Carbon has an atomic radius of around 70 pm. As the methyl group is $sp^3$ hybridized the average C-H bond length is around 109 pm and the angle 109.5° which methyl has three of.[24] In comparison to a 60 pm oxygen atom radius and a single O-H bond of ~ 95.4 pm, the methyl group takes up more volume which could become crucial after the first bromination and thus prohibit further bromination of the amine.

The only exception of this trend, is aspartic acid and methyl aspartic acid. Here the addition of a methyl group led to higher reactivity in lower bromide concentrations. Only in the highest concentrations Asp was more reactive than MAA. The selectivity was also increased for MAA in comparison to Asp. The uniqueness of Asp was among the first results observed in the conversion of amino acid toward nitriles[16] and was reported multiple times.[20] This unique property is even more puzzling as acidic amino acids in general seem to be converted in the lowest NaBr concentrations as glutamic acid can be converted fully using 0.5 mM NaBr (data not shown here) which was also shown by previous work.[11,20] The unique behaviour of Asp was explained by the intra- and intermolecular interactions which decrease the reactivity and selectivity in low bromide concentrations and the occurrence of side reactions depleting the bromide in solution.[20] Most remarkably, the spike of a depleted Asp conversion solution with additional bromide was able to continue the conversion of Asp towards its nitrile.[20]

The here observed change of almost full conversion of MAA in low bromide concentration of 0.5 mM points to the reduction of brominated side product formation. The additional methyl group at the beta carbon seems to add a new structural component to Asp which hinders the formation of these brominated by-products. Unfortunately these by-products were not identified nor isolated before.[20] Therefore, this hypothesis can only be supported by the fact that an almost full conversion of MAA is possible at 0.5 mM bromide concentration.

With the additional methyl group, MAA shows similar patterns of reactivity and selectivity as other acidic amino acids like glutamic acid and 2-aminoadipic acid[20]. This is remarkable as it seems to contradict the hypothesis made before that a methyl group reduces the reactivity and selectivity

towards the nitrile. An explanation could be that the second carboxyl group at the side chain makes the formation of a conformer which puts the methyl next to the nitrogen at the amine thermodynamically unfavourable.

However it has to be mentioned that the dip in reactivity at 5 mM and the dip in selectivity at 10 mM bromide of MAA is an unusual occurrence which is very likely due experimental variation. Fluctuations in the consumption rate of Asp are also very likely experimental mistakes. The differences in these rates are too big as to be explainable otherwise.

As a lot of reactions in this study show a high by-product formation, it has to be mentioned that the most logical by-product is the corresponding aldehyde of the amino acids. Previous studies reported the presents of aldehydes in the reaction solution via an aldehyde test.[20] In the used analysis setup the aldehyde should have a shorter retention time then the nitrile. In appendix 9.2. several HPLC chromatograms are depicted. However a test for aldehydes was not repeated and therefore no further information regarding the by-products has been gathered.

Comparing the differences in the two different groups of amino acids triples it could be seen that the influence of the functional group is depending on the presence of another bigger functional group at the beta carbon.

The first triplet shows the biggest difference in its general reactivity. Asp is barely consumed leaving 80% Asp in solution. 2ABA and Ser show a rapid reactivity which results in full conversion of Ser after 60 minutes and roughly 18% of 2ABA in solution. However the selectivity towards the nitrile after 60 minutes is still comparable with 30% 2ABACN, 20% SerCN and ~15% AspCN.

These results indicate that the presence of a single methyl and hydroxy group at the beta carbon has no big influence in reactivity and selectivity. However a lone carboxyl group reduces reactivity and selectivity a lot. This is in agreement with research previous made.[20] The differences in methyl group side chain amino acid and hydroxy group amino acids is barely visible. However Asp shows a different reaction profile. This seem to be attributed to the formation of brominated by-products and intra- and intermolecular interactions which may hinder the reactivity of Asp in general.

In the second triplet, the influence of the side chain functionality is different. At 5 mM the reactivity of all the three amino acids is comparable after 60 minutes with 55% to 30% of the amino acid remaining in solution. The selectivity however shows very big differences. ValCN and ThrCN are almost not formed with ~3% and 5% respectively. MAA product however is formed with up to 40%. It can be concluded that the presence of the methyl group seems to effect the influence of the second functional group at the beta carbon in terms of selectivity towards the nitrile.

## 4.2. α-Hydroxy acid conversion

The conversion of α-hydroxy acids mediated by VCPO was not possible. As previous research showed oxidative decarboxylation of α-hydroxy acids is possible.[25,26] However, Pink et al. showed that the conversion of an α-hydroxy acid via hypobromous acid is only possible if the organic acid is in its undissociated form.[25] In the present study, the working pH of 5.6 was set around the optimum for VCPO activity which is pH 5. As the pKa of mandelic acid is around 3.41 and the pKa of Gluconic acid is 3.86, virtually all of the organic acid was present in its dissociated form, therefore making a conversion impossible. Lowering the pH to a value where oxidative decarboxylation of α-hydroxy acids is possible, would very likely lead to a substantial decrease of VCPO activity and stability. Thus, it has to be concluded that is not possible to use a VCPO mediated conversion for the oxidative decarboxylation of α-hydroxy acid.

## 4.3. Limonene conversion

The conversion of limonene mediated by VCPO was possible. Several reaction products could be observed, but not identified. The specific formation of limonene epoxide and limonene diol as indicated by other studies using a similar enzyme[21] could not be confirmed. An explanation for this discrepancy may be that the enzyme used in the other study is just similar in its basic function, but not in its enzymatic structure and mechanism. The heme-containing chloroperoxidase (CPO) by *Caldariomyces fumago* catalyses hydroxylations and epoxidation of olefins.[27] Also oxygen transfer to carbon-carbon double bonds catalysed by CPO have been described.[21] All of these enzymatic function were not postulated for VCPO. Nevertheless, the reaction products of VCPO mediated limonene conversion seem to be explainable by the enzymatic activity of VCPO. Most of the reaction products are brominated species of R-limonene.

The only reaction product not showing a bromination is the product at 8.447 minutes. No M+2 or M-2 were observable. The mass-to-charge-ratio is equivalent to limonene oxide but as all the reaction product were compared to pure samples of limonene oxide and diol, it can be stated that it is not limonene oxide. Therefore, it is very likely a hydroxy-limonene species.

Four of the reaction products show an M and M-2 ion in an intensity ratio of about 1:1 and a mass-to-charge-ratio over 210. This is a very strong indication of a single brominated R-limonene species. Examining the charge-to-mass-ratio of the M and M-2 ions further gives a further indication on what products were probably formed. The reaction product at 8.325 and 9.196 minutes is very likely a monobromo substituted R-limonene as the mass-to-charge ratio is the sum of limonene (136.2) plus a bromine ion (79/81) and minus a hydrogen (1). The analysis of the further products is very difficult as it seems that the mass spectrometer used was not well tuned to get reliable mass-to-charge-ratios.

The four most abundant reaction products also show an M+2 and M-2 ion with 1:2:1 intensity. This indicates an addition of $Br_2$ to R-limonene. The expected mass-to-charge-ratio would be around 294/296/298.

Another fact that supports the theory of brominated limonene products in the VCPO mediated conversion is the large presence of unconverted limonene. The bromination of limonene removes bromide from the reaction mixture which leads to the stopping limonene conversion.

Thus is has to be concluded that the VCPO mediated conversion produces brominated R-limonene species and is not suited for producing specifically limonene epoxide and diol.

# 5. Summary

## 5.1. Influence of functional group(s) at the beta carbon of amino acids on the conversion towards their respective nitrile

The influence of the functional group(s) at the beta carbon of an amino acid in oxidative decarboxylation mediated by VCPO was determined. This was done by a screening of reactivity and sensitivity in different bromide concentrations and a reaction time course at 5 mM NaBr.

Generally the conversion of amino acids towards the nitriles was possible but the yield was lower than documented in previous research. In different studies, the addition of a methyl group at the beta carbon of an amino acid appeared to influence the reactivity of the amino acids regardless of other functional groups. The general trend was that the addition of a methyl group reduced the reactivity and the selectivity of the conversion. The selectivity seemed to be negatively influences slightly higher.

In the amino acids with a methyl group at the side chain the effect is strongest and constant over changing bromide concentrations in the total conversion and selectivity. This could be seen in 2ABA showing one of the highest conversions after 60 minutes with 85% and a selectivity of roughly 35% whereas Val showed only 45% reactivity with 2% selectivity.

In the amino with hydroxy side chain functionality a similar trend was visible. However the negative effect of the methyl group is reduced with increasing bromide concentration. The influence on the selectivity was constant.

The effect of the additional methyl group on the Asp was completely unique. MAA showed as the only amino acid with an additional methyl group higher reactivity from 0.5 to 10 mM bromide and higher selectivity in all but one case than Asp.

The exchange of a hydroxy group to a methyl group in Val and Thr had almost no effect on the reactivity but an influence on the reactivity.

These results led to the hypothesis that the effect of the methyl group is due to steric hindrance and the formation of conformers of amino acids which inhibit the bromination of the nitrogen of the amine group.

## 5.2. α-Hydroxy acid conversion

Using the same reaction conditions as the amino acid conversion, it was shown that the conversion of α-hydroxy acid using a VCPO mediated conversion was not possible. This is very likely due to the deprotonated state of dissociated form the α-hydroxy acid were in which did not allow for a oxidative decarboxylation with HOBr.

## 5.3. Limonene conversion

Using similar reaction conditions as the amino acid conversion, it was shown that a conversion of R-limonene via VCPO is possible. However the expected the reaction products could not be completely identified. Comparison with pure samples of limonene epoxide and limonene diol resulted in no positive match. Analyses of the reaction products via GC-MS indicate mostly single and double brominated reaction products derived from R-limonene in the solution.

# 6. Recommendation

For future studies with VCPO it is recommend to change the isolation/purification protocol to include a mandatory second purification step like a second anion purification. Additionally all gather VCPO fractions should be mixed to obtain a large quantity of homogeneous VCPO enzyme. The so produced batch should be characterized using MCD assay, Bradford and a test of glutamic acid conversion at 5 mM NaBr to see if there is activity in amino acids conversion. After verifying activity in both test, the batch should be split in smaller sample. Before using a fresh sample a single glutamic acid conversion test should be conducted to see if changes in activity occurred during storage.

Further testing regarding the conversion of amino acids should be focused on the mechanism of conversion itself. Even as there are theories and proposed mechanism on what reactive species is produced by VCPO and how the oxidative decarboxylation works, it is still not sufficiently explained.

A promising approach would be a test of reactivity of an N-methyl-amino acid like N-Methyl-L-glutamic acid. Furthermore N,N-dimethyl-amino acids could be tested to develop an understanding if the reaction mechanism of oxidative decarboxylation of amino acids really occurs with the halogenation of the amine.

Furthermore, as almost all amino acids showed a distinct change of reactivity in between 0.5 and 5 mM bromide, the reactivity between these smaller bromide concentrations should be further investigated. Narrowing down the exact value or range where the biggest change of reactivity happens, might shed light on a mechanistic property of the oxidative decarboxylation of amino acids.

To further support or clarify the results and theories gathered for intramolecular and intermolecular interactions as well as conformers of amino acids, methods of computational chemistry like density functional theory should be deployed to see if these interactions are possible and feasible at the given reaction parameters.

# 7. Experimental

## 7.1. Materials

L-Glutamic acid (98%), L-Threonine (99%), L-allo-Threonine (99%), L-Valine (99%), L-aspartic acid (99%), L-Serine (99%), DL-threo-β-Methylaspartic acid (97%), 2-Hydroxypropanenitrile (ThrCN) (≥ 97%), 2-Hydroxyacetonitrile (SerCN) (~70%), 2-Methylpropanenitrile (ValCN) (99%), 2-Cyanoacetic acid (AspCN) (≥ 99%), R-Limonene (98%), mixture of (+)-Limonene oxide, mixture of cis and trans (97%), (1S,2S,4R)-(+)-Limonene-1,2-diol (> 97%), mandelic acid (99%), gluconic acid (98%), sodium bromide (99%), sodium chloride (99%), sodium iodide (97%), citric acid, sodium thiosulfate ($Na_2S_2O_3$) (98% anh.), Sodium orthovanadate ($Na_3VO_4$)(95%), DEAE-Sepharose resin and hydrogen peroxide (35 wt%) were purchased from Sigma Aldrich. Propionitrile (2ABACN) (99%) and L-2-aminobutanoic acid (98%) were provided from Alfa Aesar. 3-Cyanopropanoic acid (GluCN) (95,9%) was purchased from Interchim and monochlorodimedone (MCD) by BioResource Products.

## 7.2. Vanadium chloroperoxidase (VCPO) production and characterisation

VCPO was expressed in *E.coli* containing a plasmid carrying the VCPO gene.[28] Bacteria were lysed using sonication for 5 minutes. Non-VCPO enzymes were partially inactivated by heat treatment at 70°C for 10 minutes. Weak anion chromatography using DEAE resin from Sephacel was used to separate VCPO from denaturated enzymes. Elution was performed stepwise with 100 mM Tris/$H_2SO_4$ buffer pH 8.2 with 0 M to 1 M NaCl. Fractions were concentrated using a 30 kDa membrane and stored in 100 mM Tris/$H_2SO_4$ buffer pH 8.2 containing 100 mM $Na_3VO_4$ at -20 °C. The enzyme was supplied by the Biobased Chemicals and Technologies (BCT) group, Wageningen University & Research. Enzymatic activity and the protein content of the obtained enzyme fractions were determined via MCD assays[29] and Bradford tests.[30]

### 7.2.1. MCD assay

A disposable UV cuvette was filled with 1 mL of MCD mix (50 µL MCD, 1 mM $H_2O_2$, 100 µM $Na_3VO_4$, 20 mM citrate buffer pH 5.6) and 2 µL of properly diluted VCPO sample. The reduction of MCD was followed over time at 290 nm on a UV-1650PC spectrometer from Shimadzu for 10 minutes. If the slope of change in absorption was higher than -250 mAbs/min, the VCPO sample was diluted further and the dilution noted. The equation below was used to determine the enzymatic activity of a enzyme fraction.

$$A_{ez} = \frac{Slope * Vc * Df}{Vez * \varepsilon_{MCD} * l}$$

$A_{ez} = enzymatic\ activity\ [U * mL^{-1}]$

$Slope = slope\ of\ the\ linear\ part\ [Abs/min]$

$Vc = total\ cuvette\ volume\ [mL]$

$V_{MCD} = volume\ of\ MCD\ mix\ added\ in\ cuvette$

$Df = dilution\ factor$

$V_{ez} = volume\ of\ enzyme\ sample\ added\ in\ the\ cuvette\ [mL]$
$\varepsilon_{MCD} = molar\ extinction\ coefficient\ if\ MCD\ [mM^{-1} * cm^{-1}]$
$l = lengt\ of\ the\ cuvette\ [cm]$

Using the protein concentrations from the Bradford-Protein assay the following formula was used to determine the specific activity.

$$SA = \frac{A_{Ez}}{C_{protein}}$$
$$SA = specific\ activity\ [U/mg]$$

$$C_{protein} = protein\ concentration\ [mg/mL]$$

## 7.3. Standard procedure of conversion using VCPO

A 5 mL volume vial with 5 mM substrate, 0.5-20 mM NaBr, 0.36 U/mL of VCPO and 20 mM citrate buffer pH 5.6 was stirred at 400 rpm at room temperature. Using a NE-1600 syringe pump from ProSense 33 µL of 1 M $H_2O_2$ solution was added per hour. After reaching the desired reaction time, the reaction was stopped by adding 50 µL 1 M $Na_2S_2O_3$.

## 7.4. Analysis

### 7.4.1. Amino acids and nitriles

Amino acid concentration was analysed after derivatisation[11] based on the work of Hanczko *et al*. [31] The method uses the derivatisation of amino acids using o-Phthalaldehyde (OPA) and 9-Fluorenylmethyl (FMOC) to make them easily to separate and visible in UV at 338nm/263nm. External standards of all stock amino acids solution and an internal standard of Norleucine were used for quantification.

Nitriles were analysed via an Ultimate 3000 HPLC from Thermo Fisher Scientific. Separation was archived using a Rezex ROA Organic acid H+ (8%) column (7.8x300 mm) from Phenomenex. Temperature of the column oven was set to 35 °C with a flow rate of 0.5 mL per hour of 12 mM $H_2SO_4$ over 25 minutes. Detection was done via a RI-detector from Shodex at 35 °C. The quantification of reaction products was done via external standards.

### 7.4.2. α-Hydroxy acid

α-Hydroxy acid analysis was done using the same procedure as for the nitriles with addition of UV detection. External standard were used for quantification.

### 7.4.3. R-Limonene

Limonene and its reaction products were extracted by adding 2 mL of DCM to the reaction mixture. The separated organic layer was transferred to a HPLC vial via pipetting. The samples were analysed via GS-MS. Agilent 7890A GS in combination with Agilent 5975 MS was used. Starting temperature was 60 °C, with a ramp up of 8.5 °C/ min to 100 °C and a further faster ramp up to 240 with a rate

of 30 °C/ min. The total runtime was 23 minutes. Details of the method used are shown in the table below.

*Table 5. Operation specification for GC.*

| | |
|---|---|
| Gas Chromatograph | Agilent 7890A GC system |
| Analytical column | HP-5MS (30 m x 0.25 mm x 0.25 µm) |
| Injector-Port Temp | 250°C |
| Injector Type | Split 50:1 |
| Syringe volume | 10 µL |
| Injection volume | 1 µL |
| Rinse solvent | Acetone |
| Carrier gas | 1.05 mL per min |

*Table 6. Operation specification for MS.*

| | |
|---|---|
| Mass spectrometer | Agilent 5975C VL MSD |
| GC inlet Temp | 250°c |
| Ion source Temp | 250°C |
| Function type | Full scan |
| Full scan range | m/z 35-300 |
| Solvent delay | 3 min |

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# 9. Appendix

## 9.1. VCPO characterisation data

| protein concentration via bradford-protein assay | | | | | MCD assay test | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| sample | sample volume [mL] | protein conc. [ug/mL] | protein mass [ug] | | sample | total volume [mL] | enzyme volume [uL] | dilution factor [Df] | NaBr conc [mM] | slope [mAbs/min] | A_ez (enzyme activity ) [U/mL] | [U/uL] | specific activity [U/mg] | Activity in amino acid conversion? |
| vslow | 5 | 96.34 | 481.70 | | vslow | 2.02 | 20 | 1 | 0.5 | >324 | | | | |
| slow | 5 | 127.57 | 637.86 | | vslow | 2.01 | 10 | 1 | 0.5 | 0.148 | 1.487 | 0.001 | 15.439 | No |
| medium | 5 | 226.20 | 1131.01 | | slow | 2.01 | 10 | 1 | 0.5 | 0.167 | 1.678 | 0.002 | 13.156 | No |
| F25 | 0.6 | 132.00 | 79.20 | | ok | 2.01 | 10 | 1 | 0.5 | >399 | | | | |
| F26 | 0.6 | 1050.70 | 630.42 | | ok | 2.01 | 10 | 2 | 0.5 | 250 | | | | |
| F27 | 0.6 | 1267.70 | 760.62 | | ok | 2.01 | 10 | 2 | 0.5 | 0.234 | 4.703 | 0.005 | 20.793 | No |
| F28 | 0.6 | 1918.00 | 1150.80 | | F25 | 2.01 | 10 | 10 | 0.5 | 0.135 | 13.568 | 0.014 | 102.784 | No |
| F29 | 0.8 | 969.00 | 775.20 | | F25 | 2.01 | 10 | 10 | 0.5 | 0.134 | 13.467 | 0.013 | 102.023 | |
| F30 | 0.8 | 1086.00 | 868.80 | | F26 | 2.01 | 10 | 10 | 0.5 | 390 | | | | |
| F31 | 0.25 | 2985.00 | 746.25 | | F26 | 2.01 | 10 | 20 | 0.5 | 0.182 | 36.582 | 0.037 | 34.817 | Yes |
| | | | | | F27 | 2.01 | 10 | 20 | 0.5 | 266 | | | | |
| | | | | | F27 | 2.01 | 10 | 30 | 0.5 | 0.235 | 70.853 | 0.071 | 55.891 | Yes |
| | | | | | F28 | 2.01 | 10 | 40 | 0.5 | 0.123 | 49.446 | 0.049 | 25.780 | Yes |
| | | | | | F29 | 2.01 | 10 | 30 | 0.5 | 0.9 | 271.350 | 0.271 | 280.031 | No |
| | | | | | F30 | 2.01 | 10 | 30 | 0.5 | 0.12 | 36.180 | 0.036 | 33.315 | No |
| | | | | | F31 | 2.01 | 10 | 30 | 0.5 | 0.191 | 57.587 | 0.058 | 19.292 | No |

## 9.2. Amino acid HPLC chromatograms

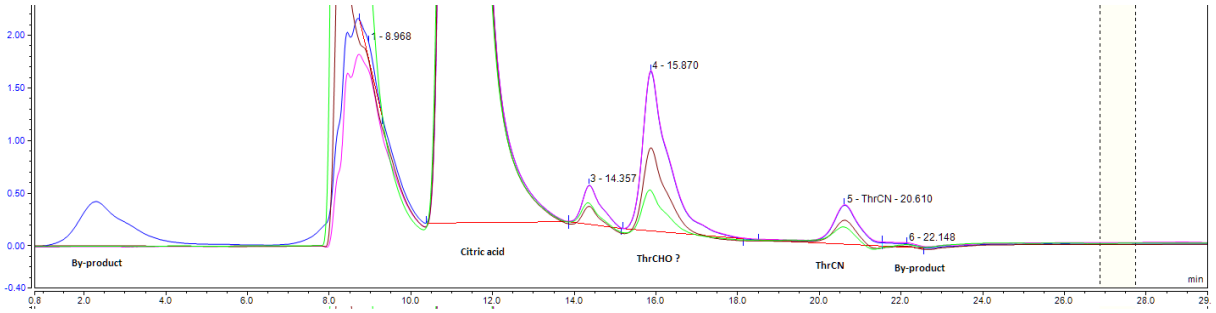


*Figure 37. HPLC chromatogram for the NaBr sensitivity screening of Thr. Pink and blue (overlapping) 0.5 and 5 mM NaBr. Brown 10 mM NaBr and Green 20 mM NaBr.*

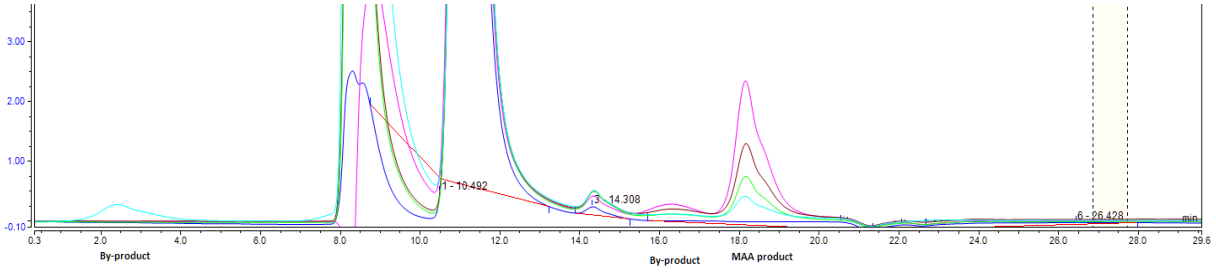


*Figure 38. HPLC chromatogram for the NaBr sensitivity screening of MAA. Pink 0.5 mM. Brown 5 mM. Green 10 mM and light blue 20 mM.*

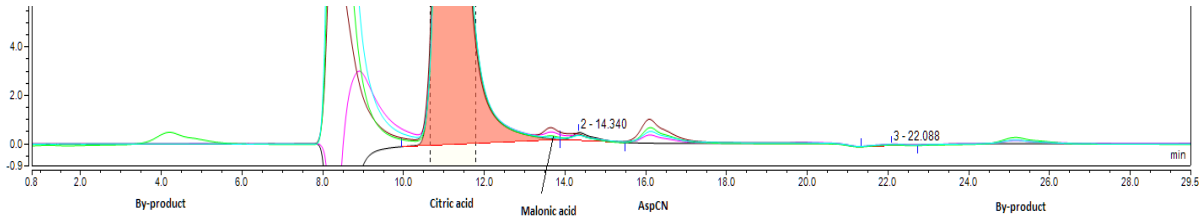


*Figure 39. HPLC chromatogram of the NaBr sensitivity screening of Asp. Brown 20 mM. Green 10 mM. Light blue 5 mM and pink 0.5 mM NaBr.*

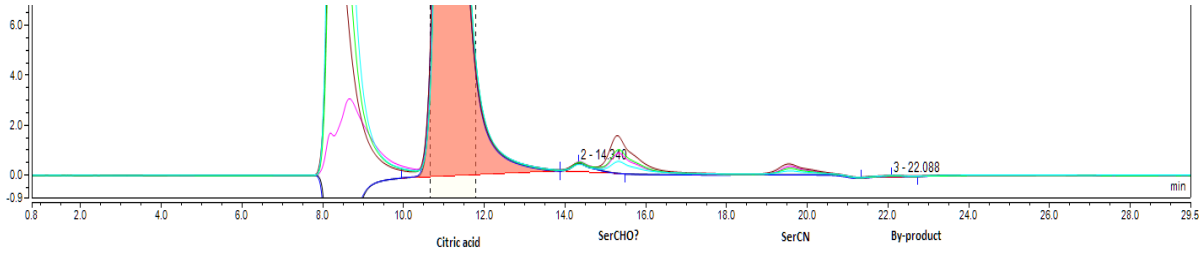


*Figure 40. HPLC chromatogram of the NaBr sensitivity screening of Ser. Light blue 20 mM. Green 10 mM. Brown 5 mM. Pink 0.5 mM.*

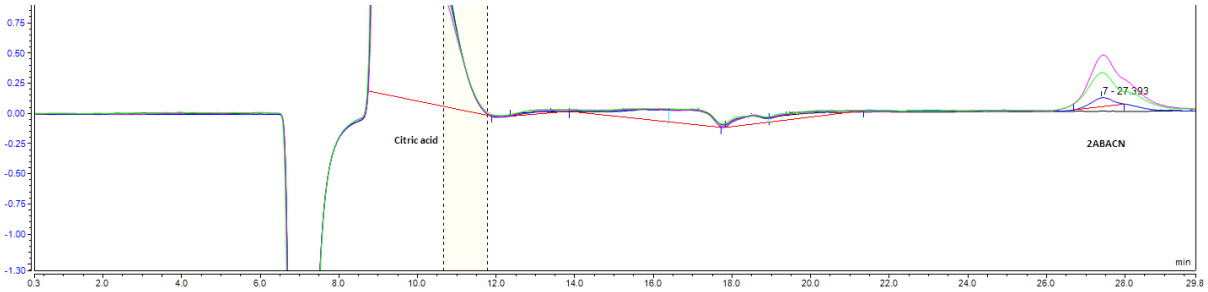


*Figure 41. HPLC chromatogram of the NaBr sensitivity screening of 2ABACN. Pink 5 mM. Green 10 mM. Blue 20 mM. Black 0.5 mM.*

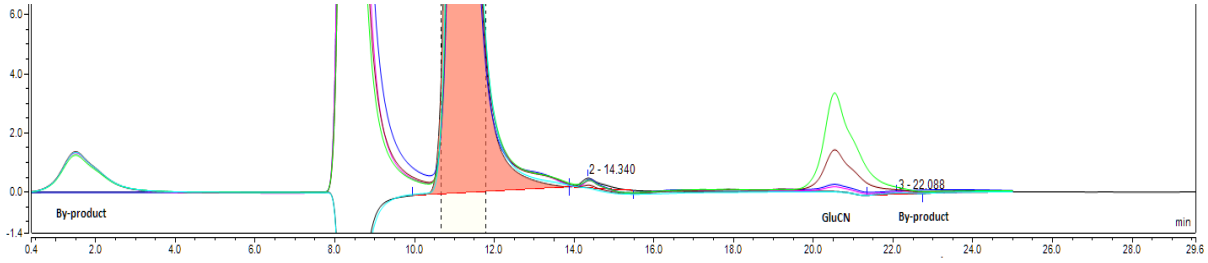


*Figure 42. HPLC chromatogram of the NaBr sensitivity screening of Glu. Green 0.5 mM. Brown 5 mM. Blue 10 mM and Pink 20 mM. Light blue 0 mM.*