Thesis Biobased Chemistry and Technology

Biobased nitrile production from amino acids: influence of side-chain and halide on reactivity

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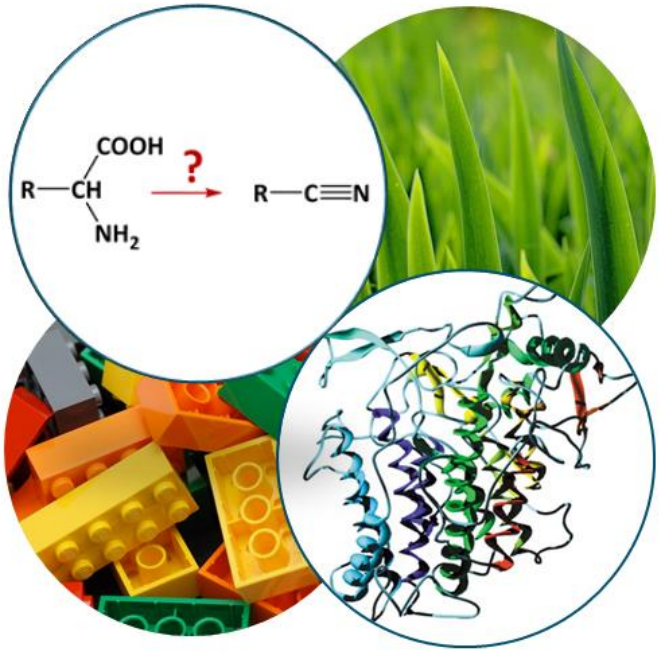

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WAGENINGEN UNIVERSITY
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Biobased nitrile production by enzymatic oxidative decarboxylation of amino acids

Influence of side-chain and halide on reactivity

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1 Background

The definition of biorefinery is "the sustainable processing of biomass into a spectrum of marketable products and energy", as formulated by the International Energy Agency (IEA) Bioenergy Task 42[1]. This efficient use of biomass can already be successfully applied to various types of biomass[2]. However, not all biomass components are yet considered starting materials for high-value co-products. For instance, the bioethanol industry yields vast amounts of a protein-rich residue, also referred to as 'distillers dried grains with solubles' (DDGS) which is currently used as animal feed and has a relatively low economic value[2]. A substantial amount of amino acids could be generated by fractionation of the proteins contained in DDGS or other protein-rich rest streams. Indeed, a few million metric tonnes of each amino acid are expected to become available worldwide in this way[3]. As many amino acids are non-essential, this fraction is not essential for the food- and feed industry, which makes amino acids suitable as alternative feedstock for the production of nitrogen-containing chemicals, particularly due to the presence of the amino functionality in amino acids. Additionally, the N-functionalised chemicals would exceed animal feed in economic value and the common energy-intensive functionalization of nitrogen-free fossil precursors with ammonia would be circumvented[4].

Nitriles (R-CN) are an important class of such N-functionalised chemicals with a wide-spread use in industry. Indeed, nitriles are used as starting material or intermediate in the synthesis of different polymers (nitrile butadiene rubber), solvents (acetonitrile), adhesives (cyanoacrylate)[5], Lego® bricks and chemicals like the key intermediate acrylonitrile used in acrylic fibres and acrylonitrile-butadiene-styrene (ABS) resins[6]. A broad range of nitriles can already be produced in a biobased manner by the oxidative decarboxylation of amino acids, which could thus be obtained from protein-rich rest streams, as depicted in Figure 1.

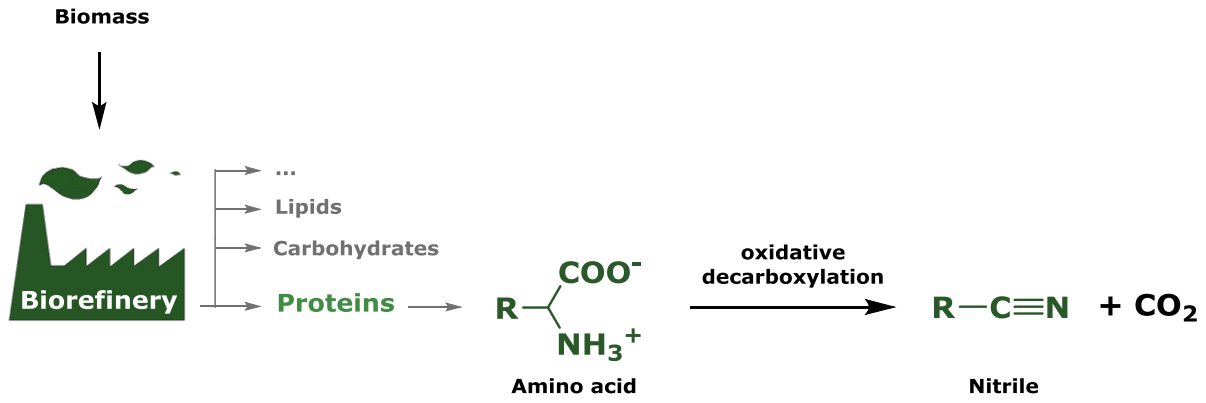

Figure 1: Biobased route for industrial nitriles formation from amino acids obtained from biomass

1.1 Oxidative decarboxylation of amino acids

Amino acids can undergo oxidative decarboxylation under different reaction conditions depending on whether a halide is used or not, and on the approach chosen.

Firstly, research on the oxidative decarboxylation of amino acids using halides has been going on for almost a century and it shows that an activated halogenating species is required. Halogens suitable for this reaction are bromine and chlorine, and are denoted by X. The activated halogenating species (X^+), is formed by the *in situ* oxidation of a halogen source (X^-: bromide or chloride salt). The exact nature of X^+ remains unknown; suggestions from literature include HOX[7], $^-$OX[8, 9], H_2O^+Br[10], NaOX[11], Enzyme-X[12] and X_2[12]. Different approaches exist to acquire this activated halogenating species:

- Addition of stoichiometric amounts of reagent (chemical approach)
- Electrochemistry
- Heterogeneous catalysis
- Biocatalysis

All four approaches are described briefly in the following section and a summary on the use of a halide for the oxidative decarboxylation of amino acids to nitriles is given in Figure 2.

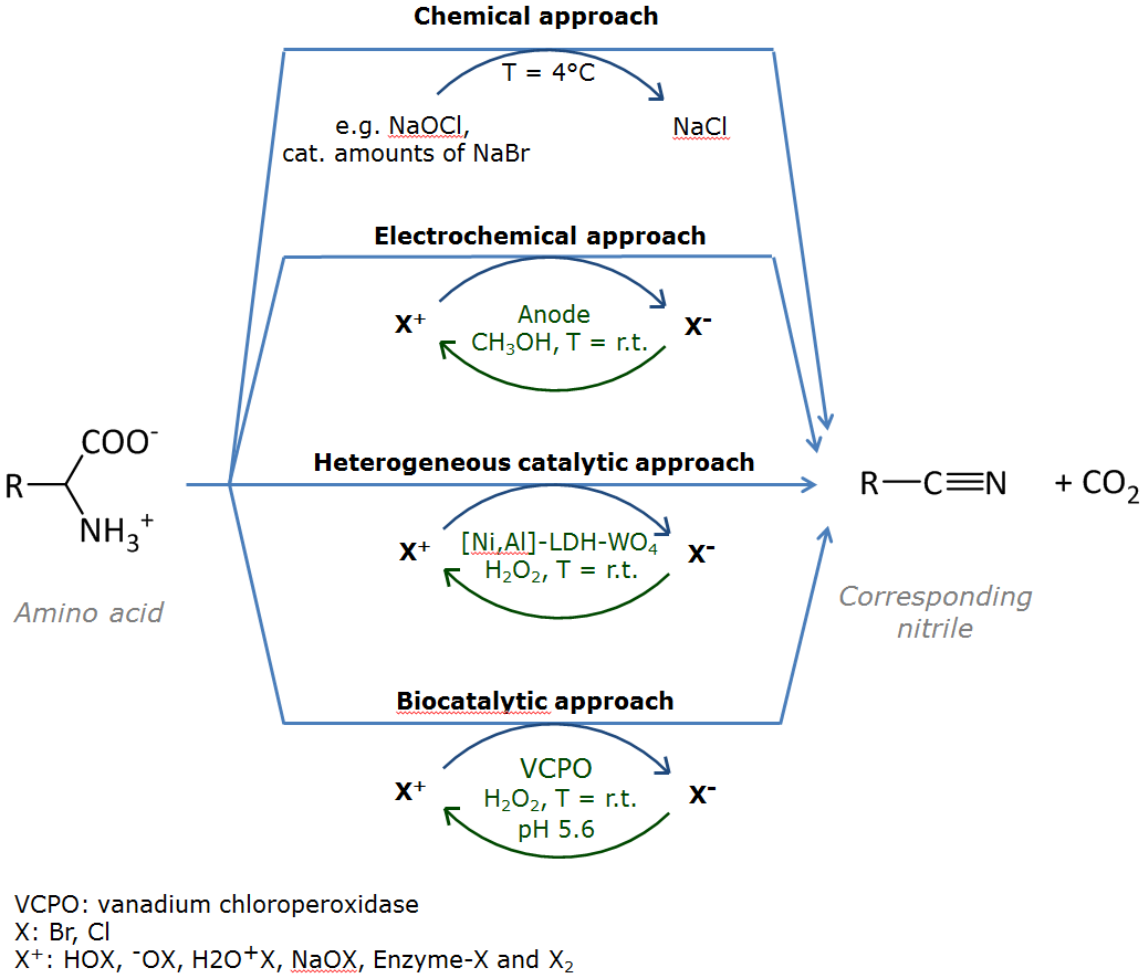

Figure 2: Four approaches for the oxidative decarboxylation of amino acids to their corresponding nitriles involving halides.

In the chemical approach, the activated halogenating species can be readily added to the reaction mixture, examples of such reagents include chloramine T[13], trichlorocyanuric acid[14, 15] and N-bromosuccinimide[10, 16]. The use of such hazardous chemicals was avoided by the use of sodium hypobromite (NaOBr)[11] in the reaction mixture, or by the *in-situ* oxidation of a halide (Br^-) by sodium hypochlorite[4] to form the halogenating agent. As the reactions of the amino acids with the halogenating agent were exothermic in this chemical approach, the reactions were generally carried out at low temperature, which required cooling, making the reaction energy-intensive. A second disadvantage of the chemical approach is its high salt formation. Both disadvantages are circumvented in the electrochemical approach, in which bromide is oxidised continuously to BrO^- at the anode of an anode-cathode system[9]. However, methanol is used as main solvent and electricity has to be supplied to the system continuously. All disadvantages listed above can be circumvented in both catalytic approaches, as the reactions can be performed at room temperature, only catalytic amounts of the halogen source are required, water is used as solvent and only electricity for stirring has to be supplied. In the heterogeneous catalytic approach, the formation of the activated halogenated species is catalysed by peroxotungstate immobilised on a layered double hydroxide[8], whereas the biocatalytic approach involves an enzyme: a haloperoxidase[3]. The catalysts are not directly involved in the conversion of the amino acid to the nitrile, but only serve to generate the activated halogenating species *in situ*. Both catalytic approaches require the use of hydrogen peroxide for the regeneration of the hypohalous acid after reacting with the amino acid.

Secondly, the oxidative decarboxylation of amino acids to the corresponding nitriles without the use of a halide was described recently[17]. The approach chosen is based on the use of a heterogeneous ruthenium-based catalyst using molecular oxygen as terminal oxidant. However, this approach requires a temperature of 100°C, an oxygen pressure of 30 bar, making a special corrosion-resistant reactor necessary, and a reaction time of 24 hours, which make the process energy-intensive. Although this approach has the advantage of producing very little waste, the use of a halide can circumvent the drawbacks of long reaction times and high energy requirements. Therefore, the present study focussed on the use of a halide, and more specifically, on the enzymatic production of the activated halogenating species.

1.2 Oxidative decarboxylation by biocatalysis

For the present study, the enzymatic approach is chosen due to both its simplicity and the favourable characteristics of the enzyme involved, the haloperoxidase.

Two types of enzymes are known to be capable of oxidising halides (X^-) to their corresponding hypohalous acid (HOX), by using hydrogen peroxide as oxidising agent: myeloperoxidases (found in human eosinophils and neutrophils[18]) and haloperoxidases[19]. The latter enzyme family can be split into sub-families depending on their prosthetic group: an iron-heme complex[20] or a vanadium oxide moiety[19]. Three types of vanadium-dependent haloperoxidases (VHPO) exist and they are named after the most electronegative halogen they are capable of oxidising[21] (Table 1).

Table 1: The three different types of vanadium haloperoxidases and their distribution in nature.

Haloperoxidase (abbreviation)	Substrate	Wild type source[19]
Vanadium chloroperoxidase (VCPO)	I^-, Br^- and Cl^-	Terrestrial fungi (e.g. *Curvularia inaequalis*)
Vanadium bromoperoxidase (VBPO)	I^- and Br^-	Brown and red seaweeds
Vanadium iodoperoxidase (VIPO)	I^-	Some brown seaweeds

Vanadium chloroperoxidase (VCPO) is the most versatile enzyme of the three types of VHPO due to its wide substrate range. The wild-type enzyme originates from the terrestrial fungus *Curvularia inaequalis*, which can be found in decaying lignocellulose[19, 22]. *C. inaequalis* produces VCPO, an enzyme which is responsible for the natural formation of halogen-containing complex organic molecules. Moreover, VCPO catalyses the oxidation of halides to the antimicrobial agents HOBr and HOCl, which can degrade lignin by oxidative attack for penetration into the host[22].

VCPO has more favourable characteristics than only its substrate range: high thermostability (up to 90°C), low activity loss even when stored at -20°C for several years, very low K_m for bromide oxidation, very high resistance against oxidising agents (H_2O_2 and singlet oxygen, which will be discussed later) and high stability in organic as well as aqueous solvents[3].

1.2.1 Mechanisms

Under slightly acidic conditions, haloperoxidases convert a halogen source into the activated halogenating species by using hydrogen peroxide as oxidising agent (Equation 1).

$$H_2O_2 + X^- + H^+ \longrightarrow X^+ + H_2O \qquad (1)$$

$X^- = Br^-$ or Cl^-,
$X^+ = HOX$[7], ^-OX[8], H_2O^+X[10], $NaOX$[11], Enzyme-X[12], X_2[12], X^+[16],...

Although literature suggests several possibilities for the nature of X^+ (Equation 1), mechanistic information related to VCPO points to the formation of a hypohalous acid (HOX). The mechanism of the oxidation of the halide by VCPO (Figure 3) shows that the vanadate moiety plays a crucial role in the catalytic cycle. The different key steps of the mechanism are listed below[22].

A. Approach of H_2O_2 to the vanadium oxide moiety
B. H_2O is released upon binding of the H_2O_2
C. Formation of a peroxo intermediate. Hydrogen bonding with the amine of Lys_{353} activates the peroxo intermediate, inducing a strong polarisation of the O-O bond[a]
D. Nucleophilic attack of the halide on the peroxo intermediate, yielding an enzyme-hypohalous species.
E. H_2O enters, and after a rearrangement, HOX is formed and released.
F. The catalytic cycle ends.

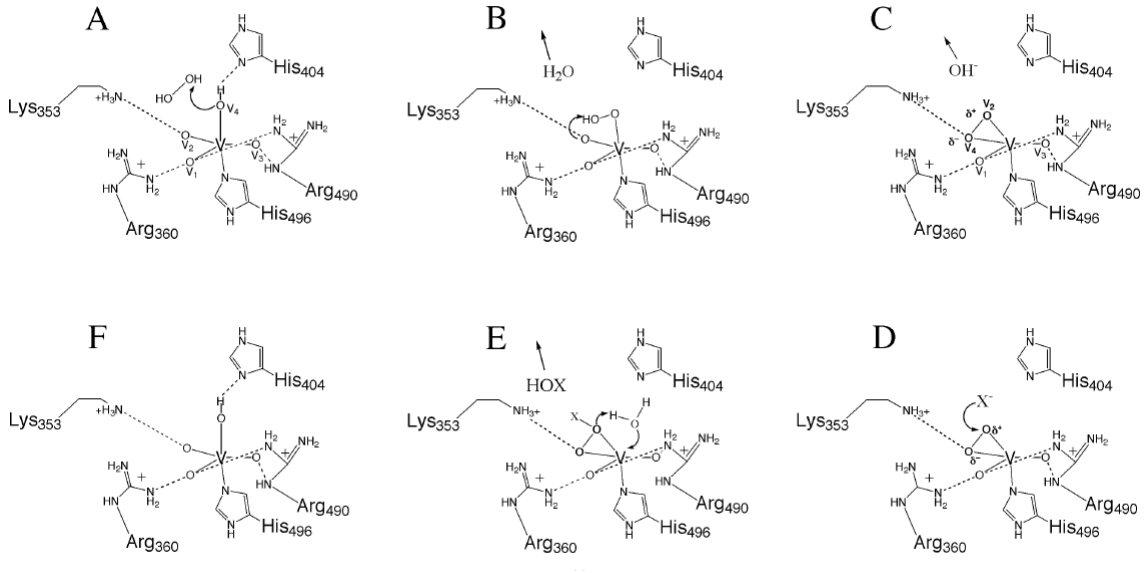

Figure 3: Mechanism of the production of HOX from H_2O_2, X^- and H^+ by vanadium chloroperoxidase, as published by Wever (2012).

The HOX produced by VCPO reacts with the amino acids to form the corresponding nitriles according to a mechanism that is believed to be independent of the approach chosen for the generation of HOX. The exact mechanism by which this oxidative decarboxylation occurs is currently still under debate. However, most literature[11, 18, 23] points to the mechanism in which the first step consists of halogenation of the amine to yield an N-monohalogenated amino acid. Another study, however, stated that the halogenation takes place at the α-carbon rather than at the amine[16], suggesting that the exact mechanism could be dependent on the halogen source. Even so, it was stated in several articles that the reaction is steered towards nitrile formation only if the amine reacts with two equivalents of HOX under slightly acidic conditions[8, 11, 23]. During this reaction, the mono-halogenated amino acid is once more halogenated, after which carbon dioxide is split off, forming a product with one fewer carbon. If however, the conditions are slightly alkaline, the amino acid is halogenated only once and the reaction is steered towards aldehyde formation together with splitting off of carbon dioxide and ammonia[11]. This last statement, however, was not supported in all literature available[15, 16]. Nevertheless, this side-reaction will be included in the proposed mechanism, because it is believed to occur in very recent work[8]. An overview of the proposed mechanism can be seen in Figure 4.

[a] For VCPO only, in the case of the presence of chloride ions, one of the peroxide oxygens is protonated before breakage of the O-O bond. This protonation is believed not to happen in the case of bromide ions.

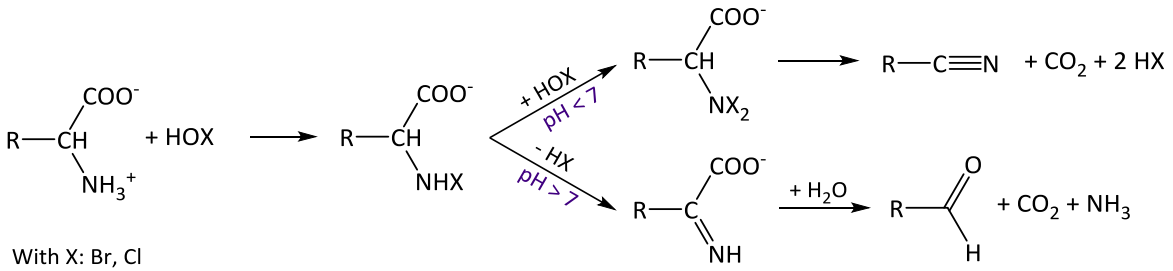

Figure 4: Proposed mechanism for the oxidative decarboxylation of amino acids to their corresponding nitriles (top pathway) and aldehydes (bottom pathway).

1.2.2 Parameters influencing the conversion and selectivity towards the nitrile

The conversion and selectivity towards the nitrile can be influenced by different parameters such as the pH of the reaction mixture, temperature, the nature of the amino acid and the nature of the halide. The effects of pH and temperature on the reactivity of the amino acids have already been reported in literature. Indeed, regarding the effect of pH, slightly acidic conditions are required for nitrile formation instead of aldehyde formation[7, 10, 11, 16], and the optimal pH for VCPO was found to be around pH 5 [19, 24]. Moreover, it was showed that, for phenylalanine and glutamic acid, the optimal working pH is 5.6-5.8[3]. Regarding the temperature, it was shown that the enzymatic decarboxylation of amino acids can be done at room temperature[3], and it was stated that the tendency to form the aldehyde increases with increasing temperature[11]. The influence of the nature of the halide and the nature of the amino acid have been less well studied. Indeed, although differences in reactivity of HOBr and HOCl with H_2O_2 to form singlet oxygen were found[18], the effect of the nature of the halide on the oxidative decarboxylation of amino acids remains widely unknown. The same holds true for the influence of the nature of the amino acid.

2 Aim of the research

This research focusses on two main aspects of the oxidative decarboxylation of amino acids to the corresponding nitriles: the concentration of the halide and the nature of the amino acid. Specifically, the influence of the length and functionality of the side-chain on the reactivity of the amino acids is focussed on. Regarding the halide, it would have been very interesting to investigate the difference between bromide and chloride, but this was not feasible time-wise within this study. Therefore, only bromide was considered and the influence of the concentration of bromide was investigated. Both main aspects of the study are elaborated on in the following sections.

2.1 Nature of the side-chain of the amino acid

The main aim of this study is to investigate how the side chain of amino acids determines their reactivity towards nitrile formation by enzymatic oxidative decarboxylation. This is based on the suggestion of different studies that the structure of the amino acid itself appears to influence the conversion and selectivity of the reaction towards the corresponding nitrile[11, 14, 16, 23]. It was shown, for instance, that the time required to reach full conversion as well as the selectivity towards the nitrile is very different for valine and alanine[23]. Furthermore, it was shown that amino acids often require different amounts of bromide for the conversion of a mole of amino acid to the corresponding bromide[11], indicating that the side-chain of the amino acid influences its reactivity. Interestingly, this early work showed that the reactivity of glutamic- and aspartic acid differed much[11] and this observation was confirmed recently[17], as well as in recent unpublished work. Indeed, where glutamic acid (Glu) exhibited near full conversion and high selectivity towards the nitrile after 60 min, the opposite was observed at the same bromide concentration for aspartic acid (Asp). A higher bromide concentration appears to be required for Asp to reach conversions similar to those found for Glu at low bromide concentration after 60 min. As these amino acids only differ by a single carbon in the side chain, it is likely that the length of the side chain influences the reactivity of the amino acid. This leads to the first research question:

- *What is the influence of the length of the side-chain of an amino acid on the conversion and selectivity towards the corresponding nitrile?*

Furthermore, both glutamic acid and aspartic acid have a carboxyl moiety as side-chain functionality. A second research question therefore arises:

- *What is the influence of the side-chain functionality of an amino acid on the conversion and selectivity towards the corresponding nitrile?*

Literature suggested that the tendency to form nitriles rather than aldehydes is increased when the length of the side chain is increased[11]. However, to our knowledge, no literature is available reporting the effect of the side chain functionality on the reactivity of the amino acid for this reaction.

A hypothesis related to both research questions is that the difference in reactivity between Glu and Asp may be (partially) determined by the occurrence and strength of intra- and intermolecular interactions.

Firstly, ring formation could occur due to intramolecular H-bonding between the two carboxyls of Glu or Asp[25, 26] or between the amine and side chain carboxyl[26, 27]. This is possible for both Glu and Asp, but the difference in ring stability could imply that more halide is required to break the stronger ring of Asp than the weaker ring in Glu. Secondly, the amine of Asp molecules are thought to be more hindered than the amine of Glu due to interactions with other Asp or Glu molecules, implying that more halide would be required to break the interactions in Asp than for Glu.

These hypotheses were tested by investigating the reactivity of different natural and synthetic amino acids which differ in either the length or functionality of the side-chain. An overview of these amino acids is given in Table 2. The choice of the substrates is not only based on their chemical structure, but also on the availability of the amino acids and the corresponding nitriles, the ease of analysis by HPLC and lastly, the conditions under which the substrate is sold (supplied purified or as HCl salt).

Table 2: Overview of the amino acids investigated during this study.

Amino acid	Number of carbons	Side-chain functionality	Corresponding nitrile
Aspartic acid (Asp)	4	-COOH	Cyanoacetic acid (AspCN)
Glutamic acid (Glu)	5	-COOH	3-cyanopropanoic acid (GluCN)
2-aminoadipic acid (2AAA)	6	-COOH	4-cyanobutanoic acid (2AAACN)
Serine (Ser)	3	-OH	glycolonitrile (SerCN)
Homoserine (HSer)	4	-OH	3-hydroxypropionitrile (HSerCN)
2-aminobutanoic acid (2Aba)	4	$-CH_3$	Propionitrile (2AbaCN)
2-aminopentanoic acid (norvaline, NVal)	5	$-CH_3$	Butyronitrile (NValCN)
2-aminocaproic acid (norleucine, NLeu)	6	$-CH_3$	Valeronitrile (NLeuCN)

2.2 Concentration and role of the halide

Not only the nature of the amino acid seemed to influence the conversion and selectivity towards nitrile formation, but also the concentration of the halide source as suggested by unpublished work. This was not completely new, as Friedman and Morgulis already showed in 1936 that different amino acids require different amounts of bromide for the conversion of one mole of amino acid to the corresponding nitrile. This leads to the third research question:

- *What is the influence of the concentration of the bromide on the conversion and the selectivity towards the nitrile?*

For each amino acid selected (Table 2), a screening for the optimal concentration of bromide was made (NaBr as halogen source), followed by a time-course reaction at a set bromide concentration. The samples were analysed by HPLC.

The role of the bromide was also studied by doping the reaction mixture with another anion. This is based on the hypothesis that intra- and intermolecular interactions must first be broken by bromide before bromination of the amine of the amino acid can occur. If another anion is present in solution, it could break these interactions instead of bromide. As a consequence, it is possible that more bromide is then available for oxidation by VCPO. This could lead to higher conversion rates and enhanced nitrile production. Fluoride was chosen for this purpose as it was a halide, like bromide, and it was not known to inhibit VCPO, unlike nitrate[28] or phospate[29].

3 Results and discussion

The influence of the length of the side chain as a function of halide concentration or time on the reactivity of the amino acids will be evaluated first by discussing the results for the amino acids having the same side-chain functionality. This will be followed by a discussion on the role of the side-chain functionality in the oxidative decarboxylation of amino acids to nitriles. This will be done by comparison of the results of the three groups of amino acids under study.

3.1 Aliphatic amino acids: 2-aminobutanoic acid, norvaline and norleucine

2-Aminobutanoic acid (2Aba, 4 carbons), norvaline (NVal, 5 carbons) and norleucine (NLeu, 6 carbons) are three amino acids with a linear aliphatic moiety at the end of the side-chain and their corresponding nitriles are propionitrile (2AbaCN), butyronitrile (NValCN) and valeronitrile (NLeuCN) (Figure 5). First, NaBr screening was done to find the optimal concentration of NaBr and to investigate how sensitive the reaction is to NaBr. Then, the course of the reactions in time was followed for the three amino acids at 2 mM NaBr and the reaction rate was calculated for the conversion of the amino acids.

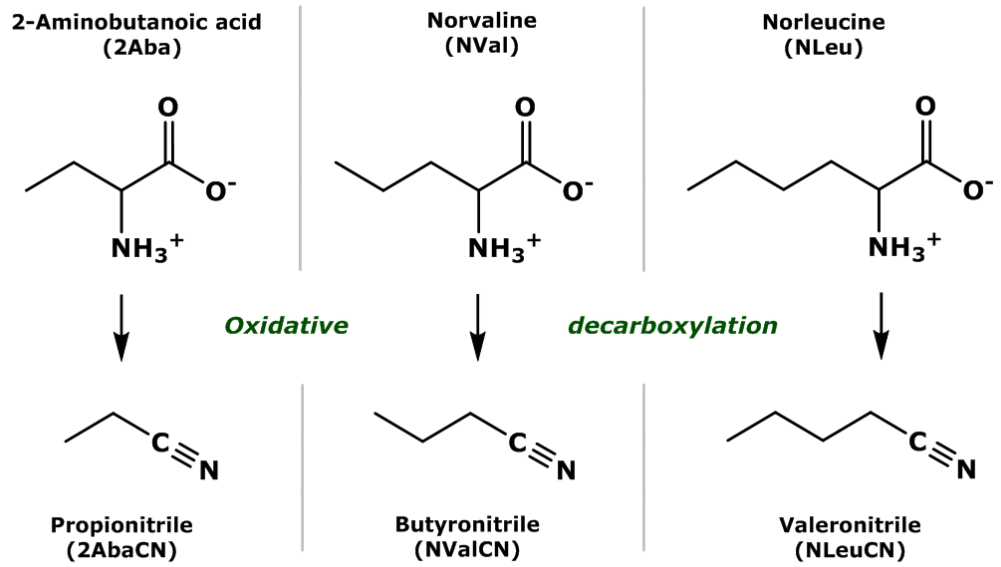

Figure 5: Aliphatic amino acids under study, depicted in their zwitterionic state at pH 5.6, based on the pKa values for the different functionalities.

3.1.1 NaBr screening

In Figure 6, the conversion and the selectivity towards the nitrile after 60 min is shown for the three amino acids, at NaBr concentrations varying between 0.5 and 20 mM. The first interesting result is the big difference between the conversions and selectivities observed at 0.5 mM NaBr and the conversions and selectivities at 2 mM NaBr or higher. In fact, low conversions and low selectivities are obtained for all three amino acids only at 0.5 mM NaBr. Even though only catalytic amounts of bromide are required according to glutamic acid conversion at the same concentration of NaBr (unpublished results), 0.5 mM NaBr is insufficient to reach complete conversion within 60 min. Somehow a bromide concentration 'threshold' is not yet reached. This is a remarkable finding as, 0.5 mM NaBr is well above the K_m of VCPO for bromide oxidation (~9 μM)[30], so VCPO should be oxidising bromide at around maximum rate. Further research on this finding is therefore definitely recommended. At higher NaBr concentrations however, the minimum bromide requirement seems to be met as complete conversions are observed at 2 and 5 mM bromide for all three amino acids. This is analogous to the findings for NLeu using the [Ni,Al]-LDH-WO_4 catalyst, where equal amounts of bromide as amino acid were used[8]. Furthermore, these results confirm an early finding stating that the bromide requirements for aliphatic amino acids (NLeu and alanine) are the same[11].

The second and possibly most general observation that can be made from this graph is that the conversion is clearly NaBr dependent and the selectivity towards the nitrile after 60 min is not. Indeed, regarding conversions, at NaBr concentrations equal or higher than 2 mM the conversion for all three amino acids decreases with increasing NaBr concentration; from 100% at 2 mM NaBr to less than 50-85% at 20 mM NaBr. This indicates that the VCPO must be somehow inhibited by the excess of bromide. This observation was supported by the K_i of 0.5 mM for bromide for VCPO from *Curvularia inaequalis*[30], indicating that significant substrate inhibition occurs at bromide concentrations between 2 and 20 mM.

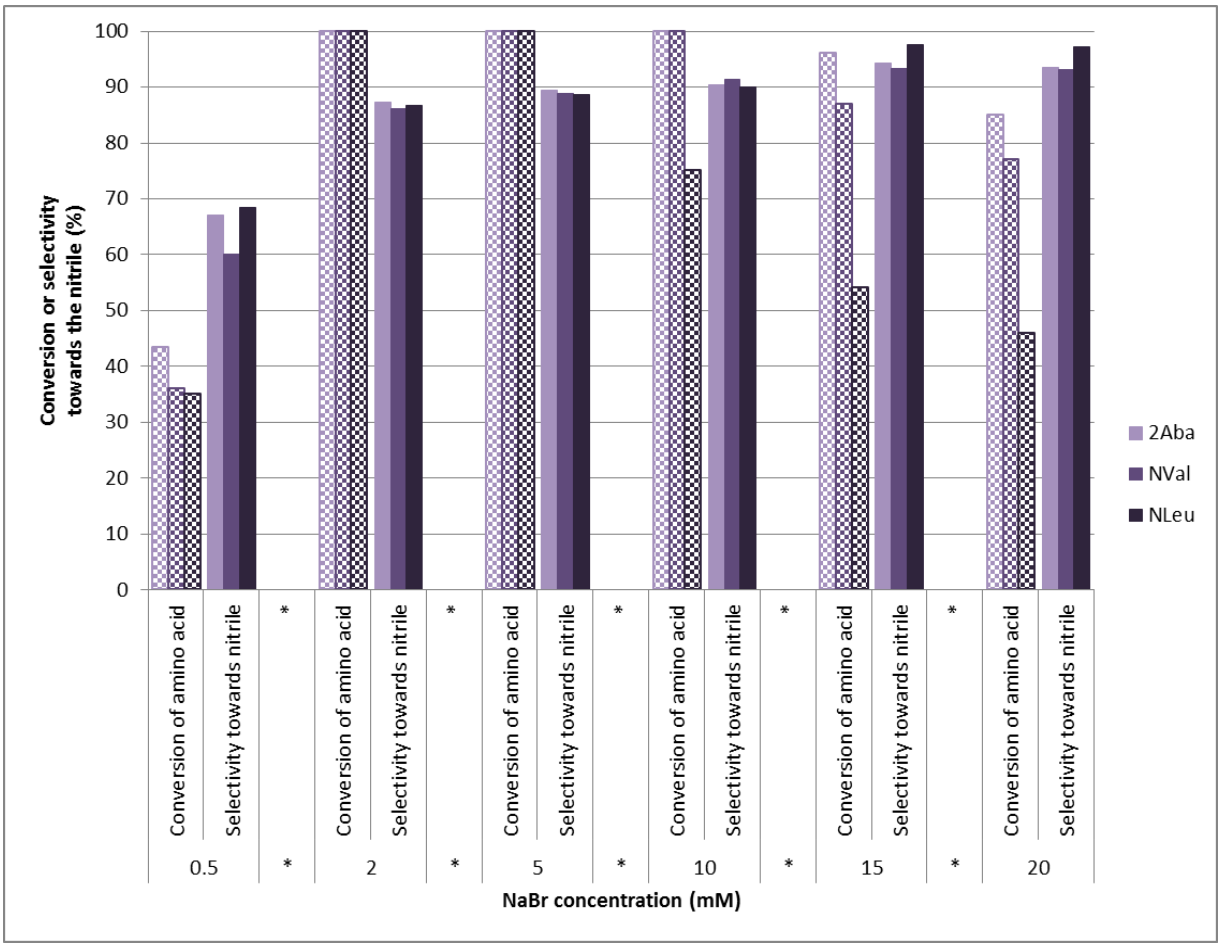

Figure 6: Conversion and selectivity towards the nitrile at different NaBr concentrations for 2Aba, NVal and NLeu. Starting concentration of amino acid: 5 mM. Reaction time: 60 min.

Interestingly, the decrease in conversion at higher NaBr concentrations is by far most pronounced for NLeu, which has the *longest* side-chain of the three amino acids. This is followed by NVal, the second biggest amino acid, which also shows a lower sensitivity to the NaBr than 2Aba. These different sensitivities to NaBr imply that, not only VCPO is inhibited by bromide, but in some manner the side-chain plays a role by interacting with the bromide, leading to differences in conversion. As ring formation is not possible for any of these amino acids and as intermolecular interactions are not expected to differ much for these amino acids, another explanation must exist. It could be speculated that the bromide is less available for the VCPO in the case of NLeu than of 2Aba due to the longer side-chain. Indeed, a study by Lund et al. (2008)[31] states that large polarisable anions like iodide can be attracted to a hydrophobic surface by hydrophobic interactions. Alternatively, a smaller anion such as fluoride, is more strongly solvated and therefore interacts much less with hydrophobic surfaces. As bromide is more similar to iodide than to fluoride, it could be speculated that it would also interact with hydrophobic surfaces. So, as NLeu has a hydrophobic side-chain of 4 carbons, it might be hydrophobic enough for hydrophobic interactions with bromide.

At concentrations equal to or higher than 2 mM, the selectivity towards the nitrile after 60 min, as mentioned before, does not seem to be influenced much by the NaBr concentration for any of the three amino acids. A possible explanation for this finding is that, as long as the bromide concentration is kept

above a certain 'threshold', di-bromination will take place at the amine of the amino acid leading to nitrile formation. If in turn this threshold is not reached, relatively more mono-bromination may take place at the amine leading to more aldehyde formation, as explained in Section 1.2.1, Figure 4.

3.1.2 Time course reactions

From the NaBr screening results only data for a reaction time of 60 min were obtained. If complete conversion is attained within this time frame, it might as well be attained earlier. This information about the reactions kinetics is provided by time course reactions at a certain bromide concentration.

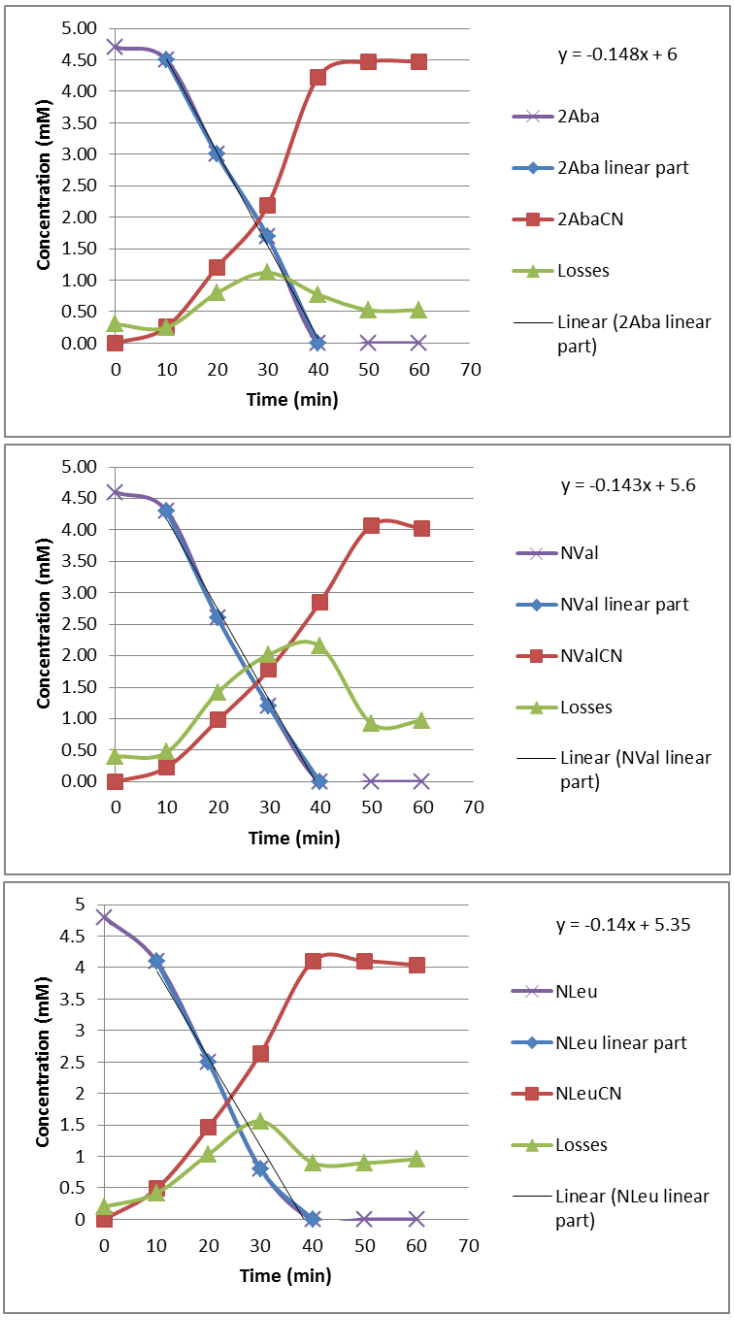

Figure 7: Time course reactions for 2Aba (top), NVal (middle) and NLeu (bottom) at 2 mM NaBr. Starting concentration of amino acid: 5 mM.

In Figure 7, the time course reaction for each amino acid at 2 mM NaBr is shown. From these graphs can be seen that the conversion is complete after 40 min for all three amino acids and that nitrile formation continues for another 10 min, after which a nitrile concentration of 4.48, 4.07 and 4.11 mM is reached for 2Aba, NVal and NLeu. These yields are somewhat lower than obtained for NLeu by oxidative decarboxylation with a [Ni,Al]-LDH-WO_4 catalyst (99% conversion and selectivity towards the nitrile)[8], but higher than obtained for NLeu by Ru-catalyzed aerobic oxidative decarboxylation (89% conversion, 79% selectivity towards the nitrile)[17]. It was in the same range though as obtained for NLeu by oxidative decarboxylation using trichloroisocyanuric acid (~85% nitrile)[14]. Furthermore, very interesting observations can be made regarding the reaction order and the losses.

Firstly, the straight lines seen for the conversion of the amino acids indicates a zero-order reaction, implying that the reaction rate is independent of the concentration of amino acid. This means that the bromination of the amino acid is not the rate limiting step in the reaction and thus that another reaction step must be proceeding more slowly (e.g. bromide oxidation by VCPO). This could be supported by the fact that 2Aba and NVal [b] were converted much more slowly at 0.5 mM NaBr but also via a zero-order reaction (see Figure 8 for these time course reactions) than at 2 mM NaBr. As for the reaction rate, it was calculated for the conversion of the amino acids only, and it was found to be similar at 2 mM NaBr for the three amino acids: ~0.14 mM min^{-1}. The hydrogen peroxide supply rate equalled 0.26 mM min^{-1}, roughly twice as fast as the amino acid conversion, indicating that about two moles of hydrogen peroxide are required for the conversion of one mole of amino acid.

[b] No 0.5 mM time course reaction was done for NLeu due to a lack of time.

Secondly, the losses are calculated as the difference between the theoretical starting concentration of amino acid (5 mM) and the concentration of amino acid and nitrile at a specific reaction time. The losses therefore include all possible by-products. This method was opted for because the amino acid analysis for some reactions showed a lower concentration of amino acid at t=0 than of nitrile later on in the reaction (this is clearly visible for the time course reactions for Glu and 2AAA in section 3.3.1.2, Figure 18). This might be due to the different HPLC analysis method for the amino acids than for the nitriles. In either way, the losses appeared to increase around 20-30 min and to decrease after 30-40 min again. This observation could be explained by the formation of mono- and di-brominated reaction intermediates, which are then converted further into the corresponding nitriles, thereby lowering the losses again. These brominated compounds could not be identified on the HPLC chromatograms because the isolated brominated compounds were not available commercially and time did not allow for MS analysis. After the maximum nitrile concentration was reached, some losses remained. These can be accounted for by the formation of by-products, as an extra peak was found on the chromatograms for all three amino acids (Appendix 1, 2 and 3). These extra peaks are likely to correspond to the aldehyde as the peaks have a slightly higher retention time than the nitrile. This theory was supported by a Purpald colour test (see Experimental section), which showed the presence of an aldehyde in the reaction mixture with NLeu (results not shown).

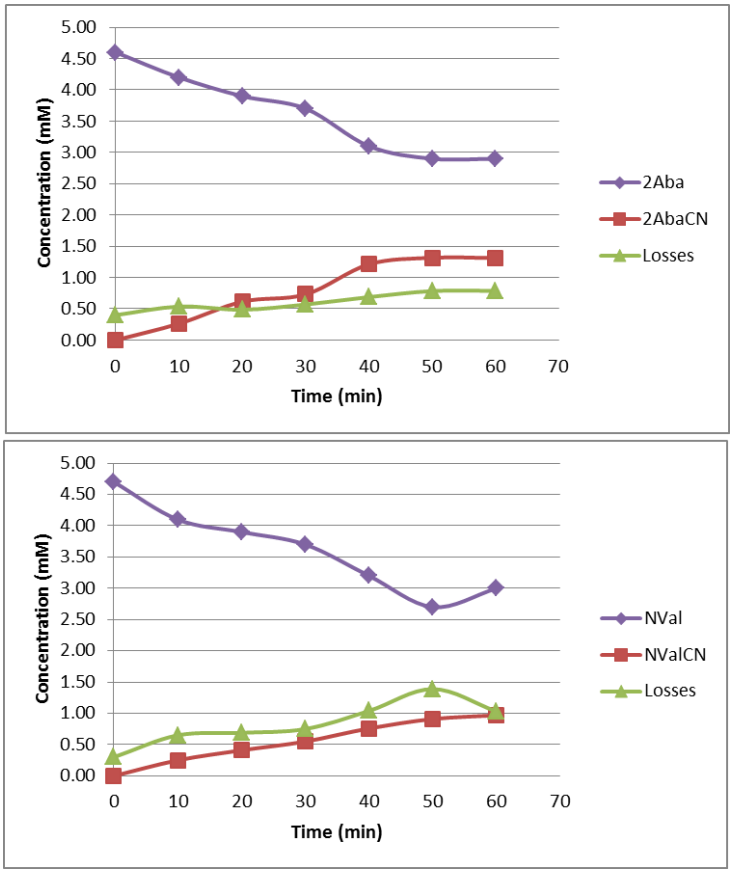

Figure 8: Time course reactions for 2Aba (top) and NVal (bottom) at 0.5 mM NaBr. Starting concentration of amino acid: 5 mM.

Finally, the time course reactions could further shed some light on the low conversions and selectivities found for the three amino acids at 0.5 mM NaBr. Indeed, they indicate that the reactions might not be complete after 60 min at 0.5 mM NaBr. At 0.5 mM NaBr, conversions of around 37% for both 2Aba and NVal were reached after 60 min, with a selectivity of 77% for 2Aba and 57% for NVal[c]. These values roughly correspond to the values found at t=20 min in the time course reactions at 2 mM NaBr. From this time point on, the conversion and selectivity still increased significantly at 2 mM NaBr, and this was also thought to happen if samples had been taken at time points higher than 60 min at 0.5 mM NaBr.

[c] These values do not correspond exactly to the values given in Figure 6, as the values were obtained from the time course reactions at 0.5 mM, which was a different set of experiments than the NaBr screening.

3.2 Amino acids with hydroxyl side-chain functionality: serine and homoserine

Serine (Ser, 3 carbons) and homoserine (HSer, 4 carbons) are two nucleophilic amino acids because of the –OH functionality at the end of the side-chain. The nitrile corresponding to Ser is glycolonitrile (SerCN) and the nitrile corresponding to HSer is 3-hydroxypropionitrile (HSerCN), as shown in Figure 9. The synthetic amino acid 5-hydroxynorvaline (5 carbons) would have been very interesting to include in the study, but it was not available commercially.

Similar to the hydrophobic amino acids, NaBr screening and a time course reaction at 2 mM NaBr were done for Ser and HSer.

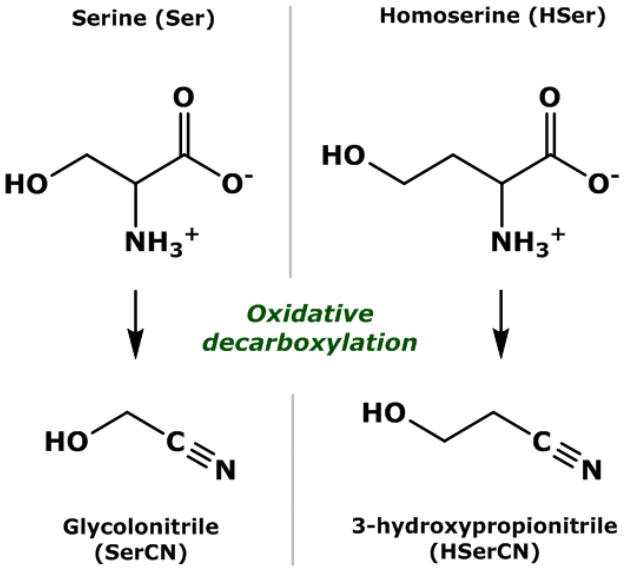

Figure 9: Amino acids with hydroxyl side-chain functionality under study, depicted in their zwitterionic state at pH 5.6, based on the pKa values for the different functionalities.

3.2.1 NaBr screening

In Figure 10, the conversion and the selectivity towards the nitrile after 60 min is shown for the Ser and HSer, at NaBr concentrations varying between 0.5 and 20 mM. The results have both similarities and differences with the results obtained for the aliphatic amino acids. The observations regarding the conversion of the amino acids will be explained first, followed by the observations related to the selectivity towards the nitrile.

The first obvious observation is that the conversions of both Ser and HSer are dependent on the concentration of NaBr, similar to the aliphatic amino acids. Additionally, lower conversions are obtained for 0.5 mM NaBr than at higher NaBr concentrations. Similarly to the aliphatic amino acids, the bromide concentration 'threshold' is not reached at 0.5 mM NaBr. At higher NaBr concentrations, the minimum bromide requirements for VCPO are met as complete conversions are observed at 2, 5 and 10 mM NaBr for both amino acids. Interestingly, the Ru-catalyzed aerobic oxidative decarboxylation of HSer gave only ~50% conversion (and ~80% selectivity towards the nitrile)[17], which is only half of the conversion obtained in this study. Moreover, the conversion decreases with increasing NaBr concentration; from 100% at 2 mM NaBr to 66% for Ser and 92% for HSer at 20 mM NaBr. This phenomenon was also seen for the aliphatic amino acids (2Aba, NVal and NLeu), pointing to inhibition of VCPO by excess of bromide. For Ser and HSer, the decrease in conversion was very different from each other and opposite to the results from the aliphatic amino acids. Indeed and remarkably, the decrease in conversion at higher NaBr concentrations is by far most pronounced for Ser, which has the *shortest* side-chain of the two amino acids.

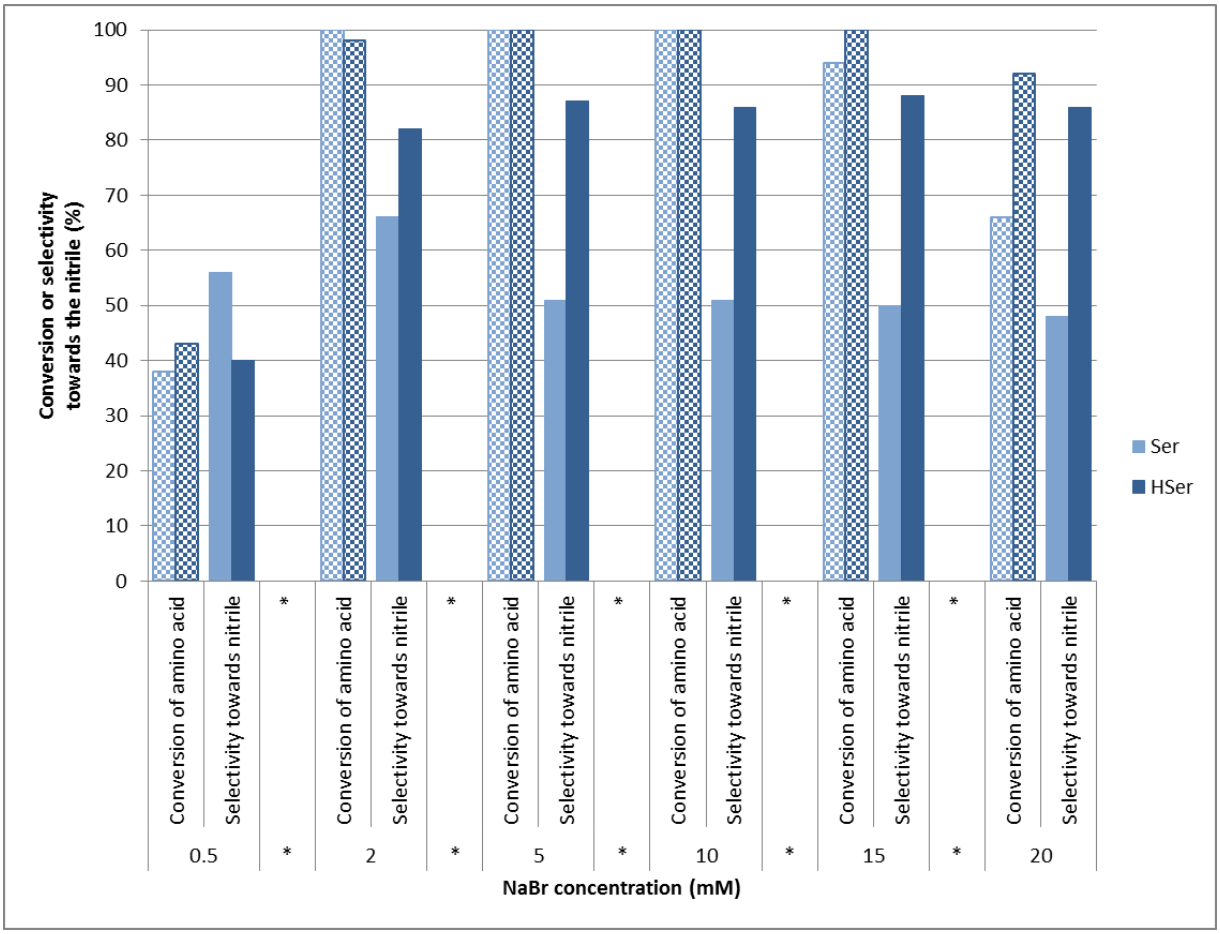

Figure 10: Conversion and selectivity towards the nitrile at different NaBr concentrations for Ser and HSer. Starting concentration of amino acid: 5 mM. Reaction time: 60 min.

The differences in reactivity of amino acids with aliphatic or hydroxyl groups as a function of NaBr concentration imply that, not only the VCPO is inhibited by bromide, but in some manner the side-chain plays a role by interacting with the bromide, leading to different conversions.

The selectivity towards the nitrile after 60 min does not seem to be influenced much by the NaBr concentration at concentrations equal to or higher than 2 mM for either Ser or HSer. This is the same finding as for the aliphatic amino acids, so see Section 3.1.1 for a possible explanation. Also similar to the results for 2Aba, NVal and NLeu is that the selectivity at 0.5 mM for HSer is lower than the selectivity at higher NaBr concentrations. This is, however, not the case for Ser, which shows fairly similar selectivity at all tested NaBr concentrations. In order to get more detailed information about the selectivity towards the nitrile, it would be interesting to look at the selectivity at lower conversions. For this, time course reactions should be carried out at the different NaBr concentrations. Furthermore, instead of observing very similar selectivity for both Ser as HSer, much lower selectivity is observed for Ser than for HSer, especially at NaBr concentrations higher than 2 mM (~ 50% for Ser and ~ 85% for HSer). This stresses the importance of the position of the hydroxyl moiety, so of the length of the side-chain. These findings were remarkable though as much more similar selectivity (94% for Ser and 99% for HSer) was found at complete conversion of Ser and HSer using the [Ni,Al]-LDH-WO_4 catalyst[8] for the oxidative decarboxylation of the amino acids[d]. This implies that the catalyst used for bromide oxidation may play a more important role than expected.

[d] Equal amounts of amino acid as bromide were used.

3.2.2 Time course reactions

In Figure 11, the time course reaction for Ser and HSer at 2 mM NaBr is shown. Firstly, the conversion of the amino acids proceeds differently for Ser than for HSer. Where a clear zero-order reaction is seen for the conversion of Ser, the conversion of HSer seems to proceed via a zero-order reaction first, followed by a possibly first-order reaction starting at around t=30 min.

This would, interestingly, imply that the concentration of HSer matters for the reaction rate at the end of the reaction, whereas the concentration of Ser does not. As for the reaction rate, it was calculated for the conversion of the amino acids only, and it was found to be similar at the linear parts for Ser and HSer: ~-0.16 mM min^{-1}.

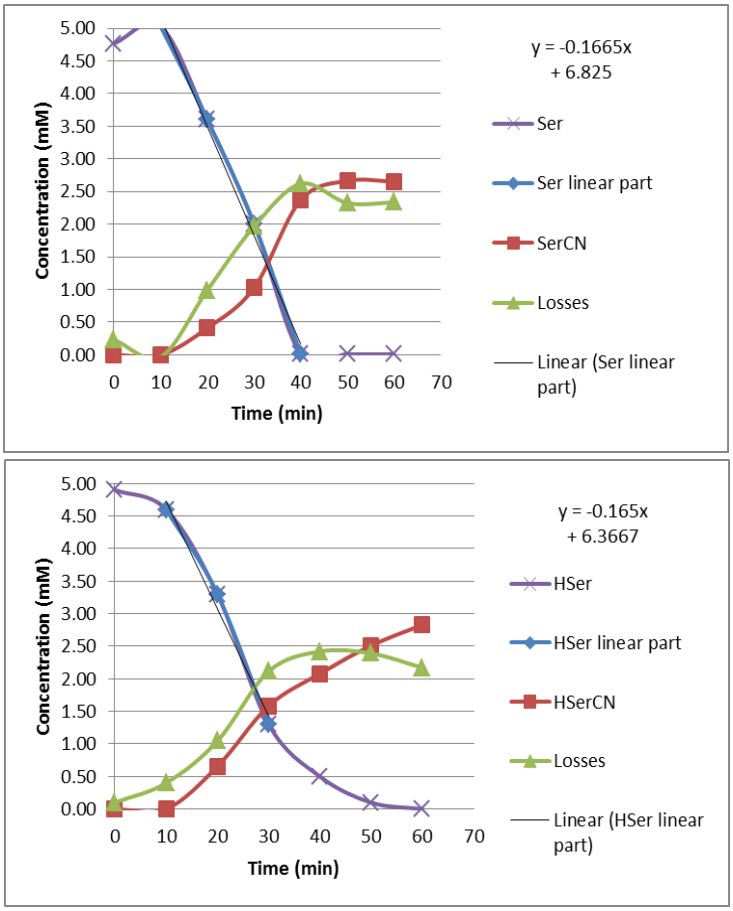

Figure 11: Time course reactions for Ser (top) and HSer (bottom) at 2 mM NaBr. Starting concentration of amino acid: 5 mM.

Comment: Firstly, the time course reaction for Ser shows a strangely high Ser concentration at t=10 min, higher than the theoretical starting concentration of 5 mM also seen at t=0 min. This can only be explained by a pipetting error for this particular reaction time point as the same Ser stock solution was used for all the other reaction time points.

Secondly, it can be seen that the final nitrile concentration for HSer at 2 mM NaBr (3.95 mM) is significantly different for the NaBr screening results than for the time course reaction at 2 mM NaBr (2.83 mM). Therefore, it would be wise to repeat these experiments in order to obtain more reliable data for HSer at this NaBr concentration.

Thirdly, time course reactions for Ser and HSer at 0.5 mM (Figure 12) show much lower conversion rates for the amino acids than at 2 mM (~-0.03 mM min^{-1} at 0.5 mM NaBr versus ~-0.16 mM min^{-1} at 2 mM NaBr), indicating that the bromide oxidation rate by VCPO is influenced by the concentration of bromide. Furthermore, it could indicate that the reactions were simply not finished within 60 min, as was also stated for the aliphatic amino acids.

The final observation is that the losses appear to increase until around 40 min and to decrease after that time point again. This is clearly less pronounced than for the aliphatic amino acids and could be explained by the formation of relatively much by-product, and relatively few reaction intermediates. As by-product (aldehyde) formation is promoted by monobrominated intermediates, it is likely that the second bromination step of the amino acid is slower than for the aliphatic amino acids. Moreover, an extra peak with considerable area was seen on the chromatograms for both Ser and HSer (Appendix 4 and 5). Similar to the hydrophobic amino acids, the extra peaks are likely to correspond to the aldehyde as the peaks have a lower retention time than the nitrile[e]. This theory is supported by a set of experiments that was done to test the stability of SerCN. Under the same reaction conditions as for the other experiments involving Ser, SerCN served as starting material to investigate if it is converted into something else due to the presence of hydrogen peroxide and/or HOBr. No Ser was present in the reaction mixture. HPLC analysis revealed that SerCN is

[e] For 2Aba, NVal and NLeu, an Acquity UPLC® BEH C18 column was used whereas for Ser and HSer a Rezex ROA Organic acid H+ (8%) column was used (see Experimental section). Where the aldehyde has more affinity for the C18 column than the nitrile, it is expected to have less affinity for the Organic acid column than the nitrile, explaining the opposite order of retention times.

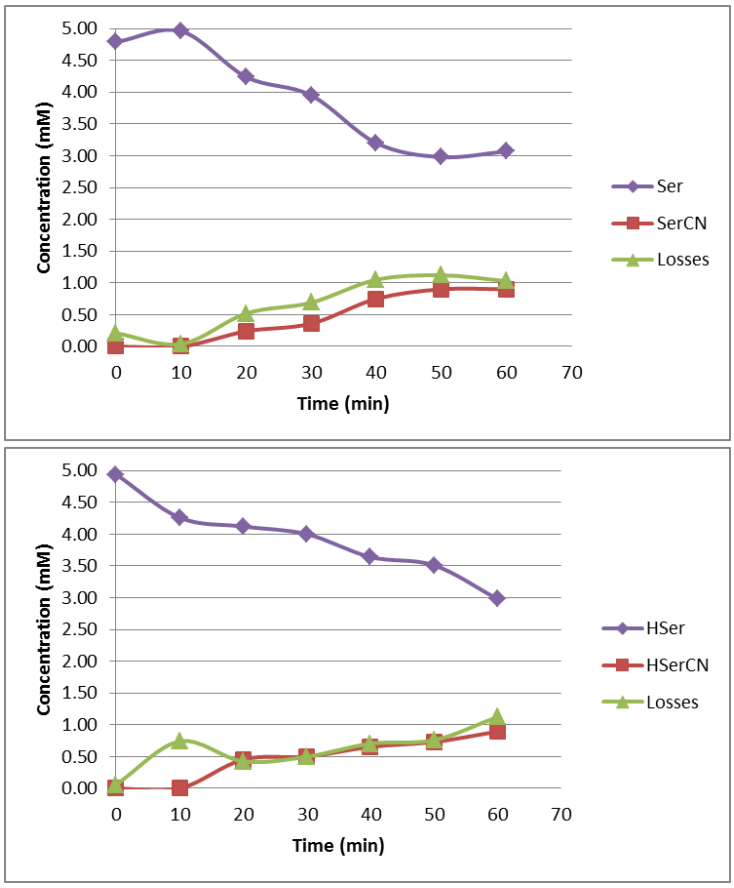

Figure 12: Time course reactions for Ser (top) and HSer (bottom) at 0.5 mM NaBr. Starting concentration of amino acid: 5 mM.

completely stable under the reaction conditions given in the Experimental section (see Appendix 6 for the HPLC chromatograms). If the by-product had been formed from SerCN, then it could not have been the aldehyde that was formed, as it is likely formed by monobromination of Ser.

Coming back to a hypothesis for the differences in reactivity of different amino acids, an important factor that could influence the reactivity of amino acids is possible occurrence of inter- and intramolecular interactions. In the case of Ser and HSer, the most relevant type of interactions is the ability to form a ring by intramolecular H-bonding, as shown in Figure 13: Possibilities for ring formation for Ser and HSer. It was hypothesised that the more stable the ring is, the more bromide is required to reach high conversion and selectivity. For HSer, the most likely structure is structure (2), as the hydroxyl is closer to the amine than to the carboxyl. For Ser, structure (3) is thought to be more likely as H-bonding with the amine (structure (1)) yields a 5-membered ring. Although a 5-membered ring is more stable than a 6-membered ring, the 5-membered ring has more ring strain, which could make it more likely that H-bonding with the carboxyl occurs. The environment around the amine would therefore be different for Ser and HSer, which could be an indication for the differences in reactivity of the two amino acids. However, in order to be more conclusive, not only the lowest energy states of the Ser and HSer should be calculated but also the energy barriers of ring formation (by means of computational modelling).

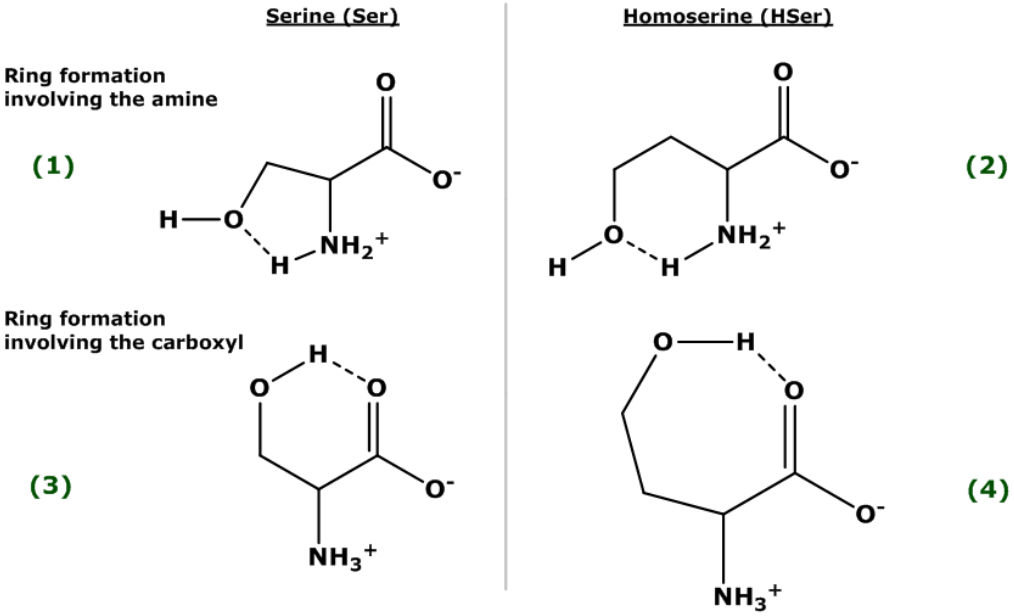

Figure 13: Possibilities for ring formation for Ser and HSer.

Even so, Ser seems to be different than the other amino acids discussed so far. Where 2Aba, the smallest aliphatic amino acid, showed very high conversion and selectivity at higher NaBr concentrations, Ser did not. The hydroxyl, due to its proximity to the amine, may make the amino acid more prone to by-product formation than in HSer. However, in order to be able to draw conclusions, more research is needed. It would, for instance, have been interesting to study the reactivity of 5-hydroxynorvaline (5 carbon atoms) for nucleophilic amino acids and alanine (3 carbon atoms) for the hydrophobic amino acids. This is because Ser and HSer have respectively 3 and 4 carbon atoms, whereas 2Aba, NVal and NLeu have respectively 4, 5 and 6 carbons. The question remains now if the results obtained for Ser and HSer are really comparable with the results obtained for 2Aba, NVal and NLeu.

3.3 Acidic amino acids: aspartic acid, glutamic acid and 2-aminoadipic acid

Aspartic acid (Asp, 4 carbons), glutamic acid (Glu, 5 carbons) and 2-aminoadipic acid (2AAA, 6 carbons) are three amino acids with a –COOH functionality at the end of the side-chain. The corresponding nitriles are cyanoacetic acid (AspCN), 3-cyanopropanoic acid (GluCN) and 4-cyanobutanoic acid (2AAACN) as depicted in Figure 14.

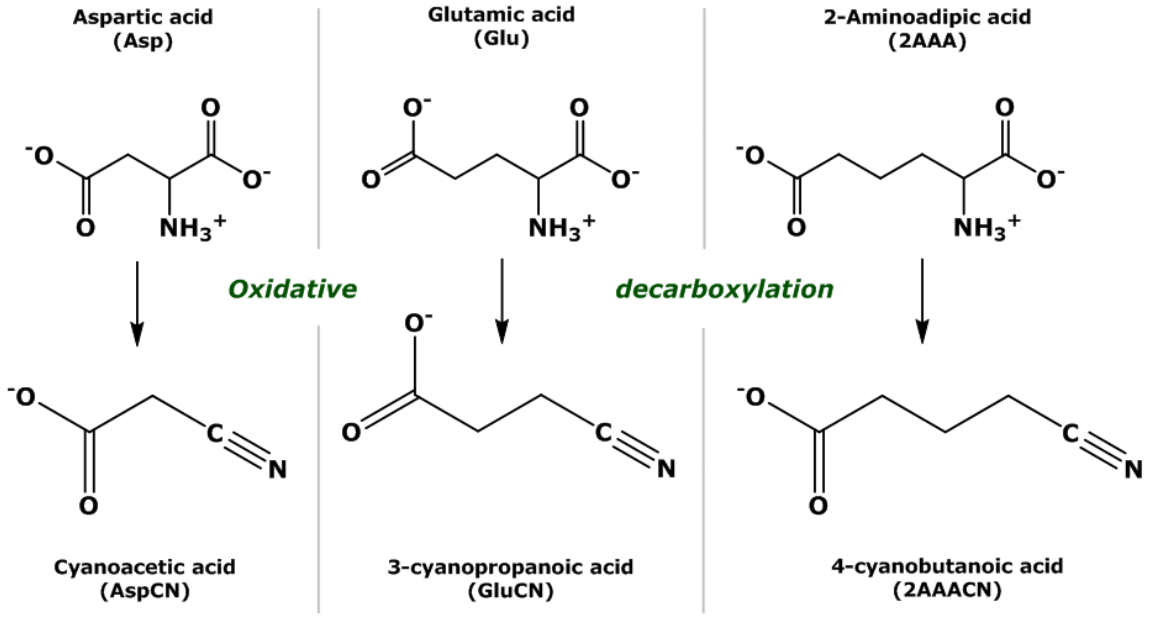

Figure 14: Acidic amino acids under study, depicted with their three charged functionalities based on the pKa values for the different functionalities.

With the purpose of obtaining results comparable to the other two groups of amino acids, the same NaBr screening was done for Asp, Glu and 2AAA and is shown in Figure 15. Already at first glance it is obvious that these three amino acids do not show any clearly visible trend, meaning that they probably do not behave similarly to either the aliphatic or the nucleophilic amino acids. Therefore, it was chosen to not discuss the results obtained for the three amino acids together. Glu and 2AAA will be discussed first, as these two amino acids appear to have more similarities than Asp has for either Glu or 2AAA.

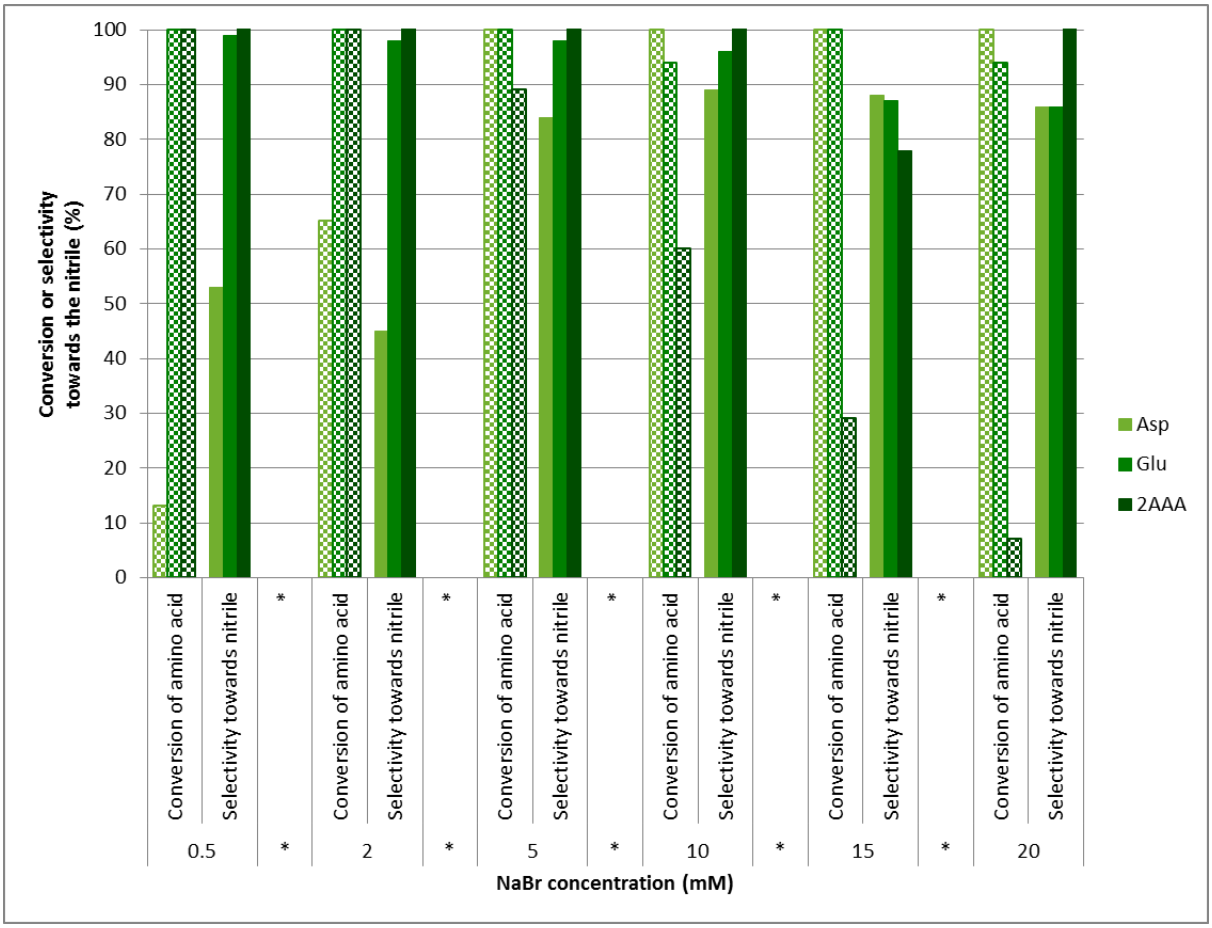

Figure 15: Conversion and selectivity towards the nitrile at different NaBr concentrations for Asp, Glu and 2AAA. Starting concentration of amino acid: 5 mM. Reaction time: 60 min.

3.3.1 Glutamic acid and 2-aminoadipic acid

Similar to the other amino acids studied, the NaBr screening will be discussed first, followed by the time course reactions.

3.3.1.1 NaBr screening

In Figure 16, the conversion and the selectivity towards the nitrile after 60 min is shown for Glu and 2AAA, at NaBr concentrations varying between 0 and 20 mM. The observations regarding the conversion of the amino acids will be explained first, followed by the observations related to the selectivity towards the nitrile.

Firstly, in contrast to the observations for the hydrophobic and amino acids with a hydroxyl side-chain functionality, no bromide concentration 'threshold' was observed for Glu and 2AAA. In fact, where the other amino acids exhibited poor conversion (below 50%) at 0.5 mM NaBr, Glu and 2AAA show 100% conversion, confirming earlier findings for Glu (5 mM) at 2 mM NaBr[3]. What's more, even at 0.2 mM NaBr Glu and 2AAA showed 100% conversion, but it can also be seen in Figure 16 that at least some bromide is required for conversion as no Glu nor 2AAA was converted in the absence of bromide. This indicates that, at least for Glu and 2AAA, only catalytic amounts of bromide are required for full conversion.

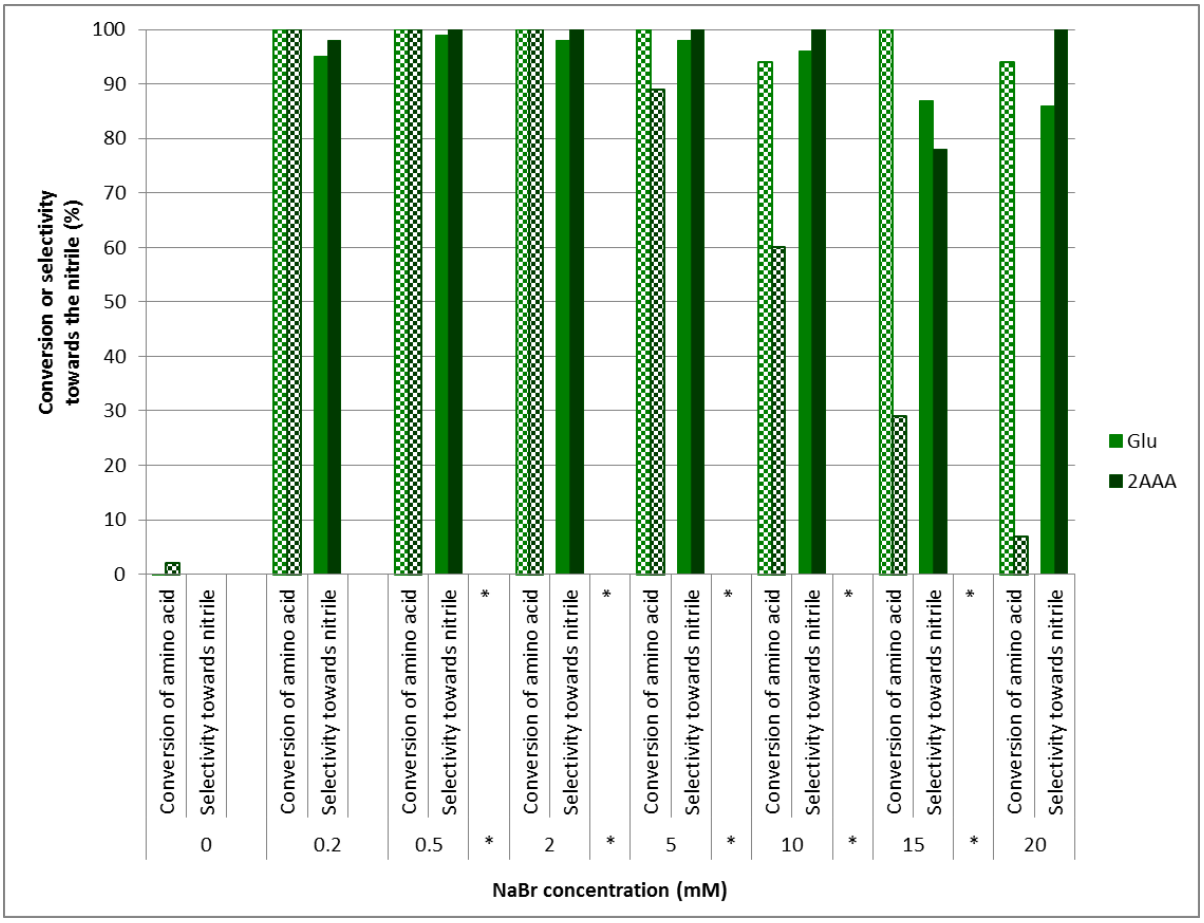

Figure 16: Conversion and selectivity towards the nitrile at different NaBr concentrations for Glu and 2AAA. Starting concentration of amino acid: 5 mM. Reaction time: 60 min. The selectivity seen for 2AAA at 15 mM NaBr is somewhat low, probably due to experimental error, as no extra peaks were found on the HPLC chromatograms. The conversion seen for 2AAA at 0 mM NaBr is also due to experimental error as no product was seen at all.

Secondly, the results related to 2AAA show bromide inhibition at higher NaBr concentrations, similar to the other two groups of amino acids. However, this is not the case for Glu, which shows almost complete conversion at all NaBr concentrations investigated. It could be speculated that bromide is somehow trapped by Glu at high bromide concentrations and not by 2AAA, making less bromide available for inhibition of VCPO. Referring back to the ring formation hypothesis, it is judged likely that ring formation can occur for Glu but not for 2AAA due to distance between the side-chain carboxyl and the backbone in 2AAA. Therefore, the bromide might be somehow trapped in the ring of Glu and not by the linear 2AAA.

Finally, similar selectivity towards the nitrile are observed at all NaBr concentrations for both Glu and 2AAA, indicating that double bromination of the amine may occur fast, resulting in only nitrile formation.

3.3.1.2 Time course reactions

In Figure 17, the time course reaction for Glu and 2AAA at 2 mM NaBr is shown. It can be seen that both Glu and 2AAA are fully converted to the corresponding nitrile within 40 min. Remarkably, conversion of the amino acids starts immediately after starting the reaction, unlike for the other amino acids studied so far, which started to be converted only after 10 min. Furthermore, the conversion of the amino acids is, again, a zero-order reaction, and the conversion rate is very similar for Glu and 2AAA: ~-0.14 mM min^{-1}.

Finally, a time course reaction was also done at 0.5 mM NaBr for Glu (Figure 18), and a very similar graph as at 2 mM NaBr was obtained. This shows that 0.5 mM NaBr is sufficient to reach full conversion within 40 min.

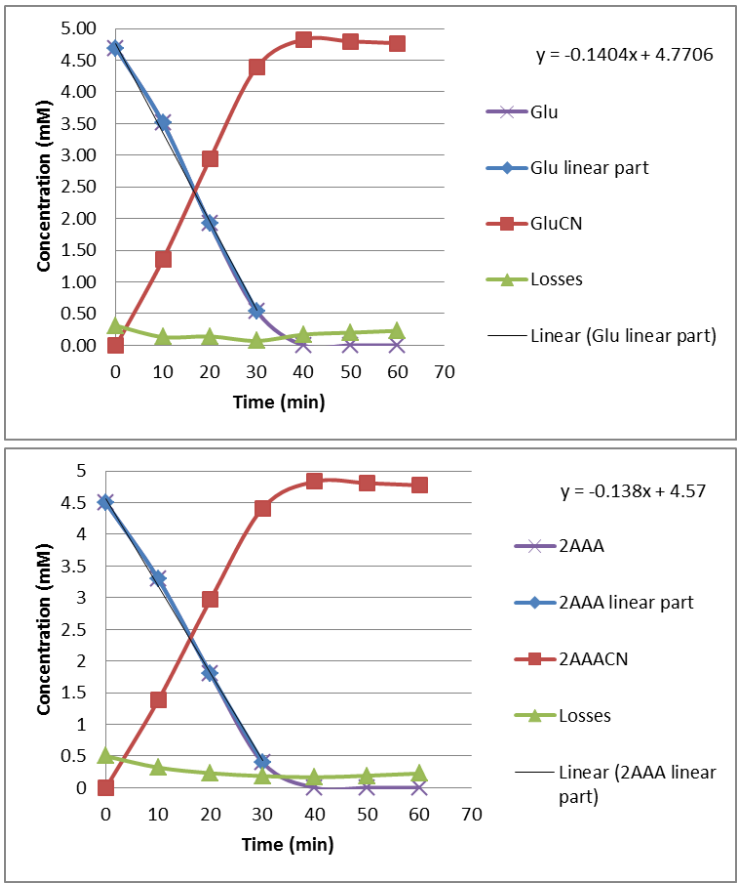

Figure 18: Time course reactions for Glu (top) and 2AAA (bottom) at 2 mM NaBr. Starting concentration of amino acid: 5 mM.

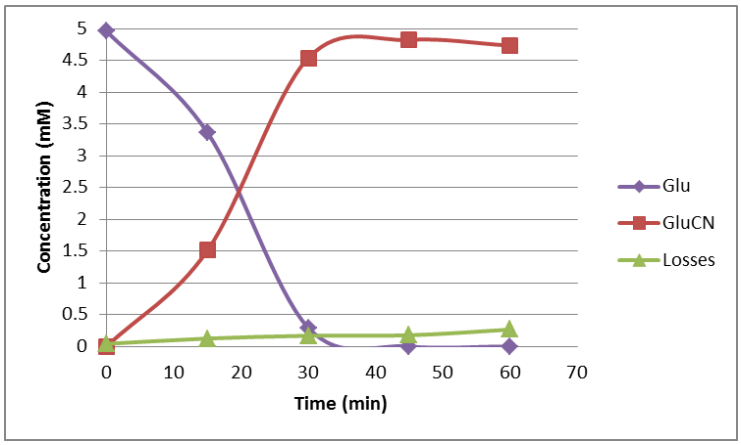

Figure 17: Time course reactions for Glu at 0.5 mM NaBr. Starting concentration of amino acid: 5 mM.

3.3.2 Aspartic acid

As stated earlier, almost eighty years ago already, Asp was reported to behave differently than Glu[11]. This was confirmed by the NaBr screening on Asp, Glu and 2AAA. Therefore, this small and interesting amino acid will be elaborated on in the following sections.

3.3.2.1 NaBr screening

In Figure 19, the conversion and the selectivity towards the nitrile after 60 min is shown for Asp, at NaBr concentrations varying between 0.5 and 20 mM. Firstly, the conversion is very low (13% and 65%) at 0.5 and 2 mM NaBr, in contrast to the complete conversions observed for Glu and 2AAA at these NaBr concentrations. It seems that, again, a bromide concentration 'threshold' is not reached, like for the aliphatic amino acids and the ones with hydroxyl side-chain functionality. The 'threshold', however, seems to be higher than for the other amino acids, indicating that another phenomenon is likely involved. Furthermore at these concentrations of NaBr, the selectivity towards the nitrile is also low (53% and 45%), indicating that reactions might not yet be complete, as observed for the other two groups of amino acids. Interestingly, no decrease in conversion is seen at higher NaBr concentrations, indicating a possible interaction between Asp and bromide ('trapping', like for Glu) preventing bromide inhibition of VCPO.

The low conversions of Asp compared to Glu at 0.5 and 2 mM NaBr might be explained by two hypotheses: the occurrence of intramolecular interactions leading to ring formation, and intermolecular interactions. Both types of interactions are elaborated on below.

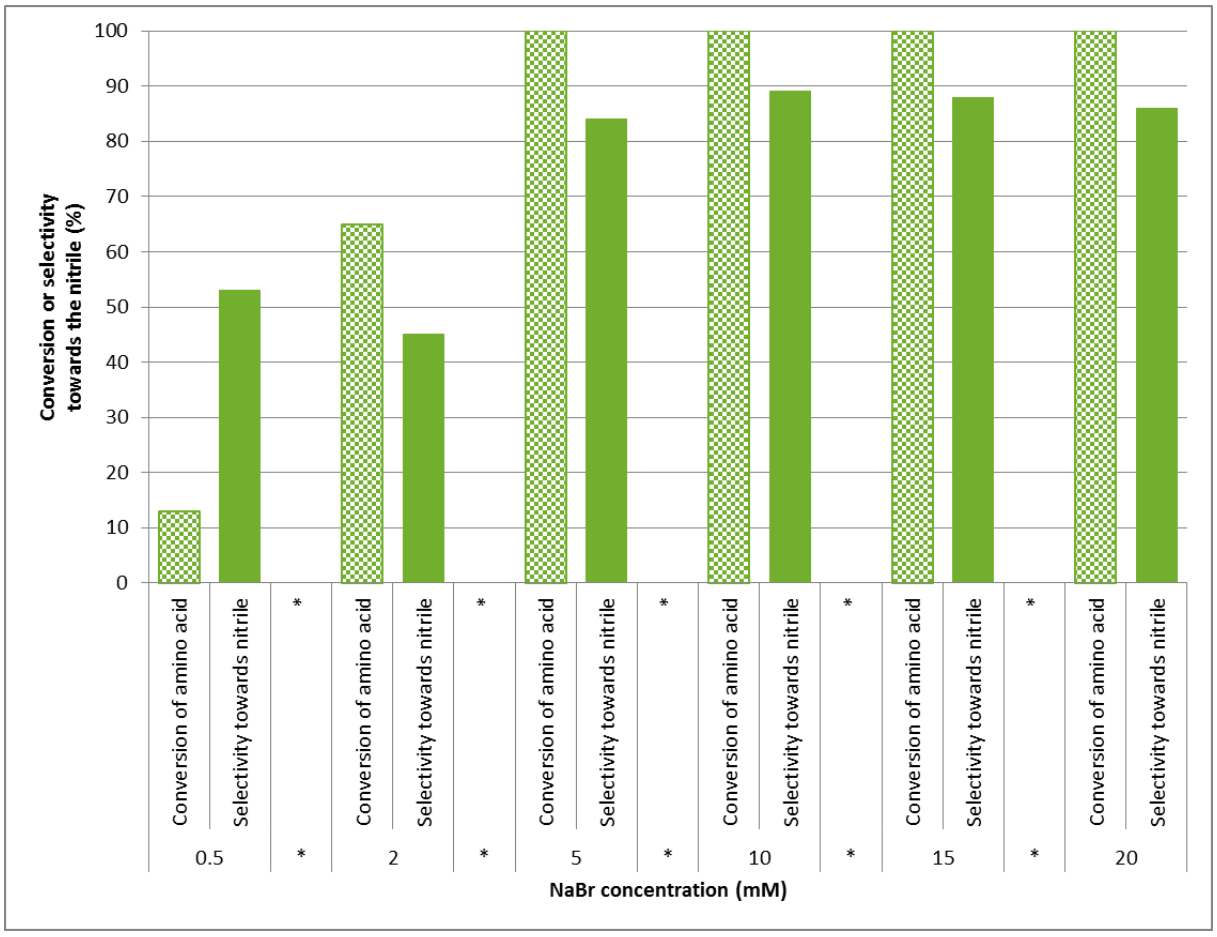

Figure 19: Conversion and selectivity towards the nitrile at different NaBr concentrations for Asp. Starting concentration of amino acid: 5 mM. Reaction time: 60 min.

Firstly, ring formation could occur due to intramolecular H-bonding between the two carboxyls of Glu or Asp[25, 26] or between the amine and side chain carboxyl[26, 27]. This was shown in different studies after calculation of the lowest energy structures of Glu and Asp by different techniques (infrared photodissociation spectroscopy[27], density functional theory[25-27] and molecular orbital theory[26]). Although the calculations were done in the gas phase, it is clear that the nature of the H-bonding is dependent on the protonation state of the amino acids. In this work, both Glu and Asp are in slightly acidic aqueous solution. This implies that both amino acids have deprotonated carboxyl groups and a protonated amine. Therefore, the most probable intramolecular H-bonding in both amino acids will be between the amine and the side-chain carboxyl. This H-bonding leading to ring formation can occur for both Glu and Asp, but the stability of the ring is different for the two amino acids. Indeed, ring formation in Asp yields a six-membered ring as shown in Figure 20, which is both entropically and thermodynamically more stable than the resulting seven-membered ring in Glu. This intramolecular interaction must first be broken to enable the activated halogenating species to halogenate the amine. As the six-membered ring in Asp is more stable than the ring in Glu, more halide is thought to be required for Asp than for Glu.

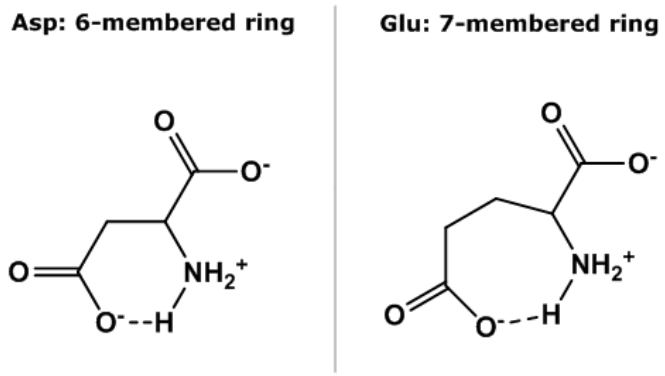

Figure 20: Intramolecular interactions for Asp (left) and Glu (right)

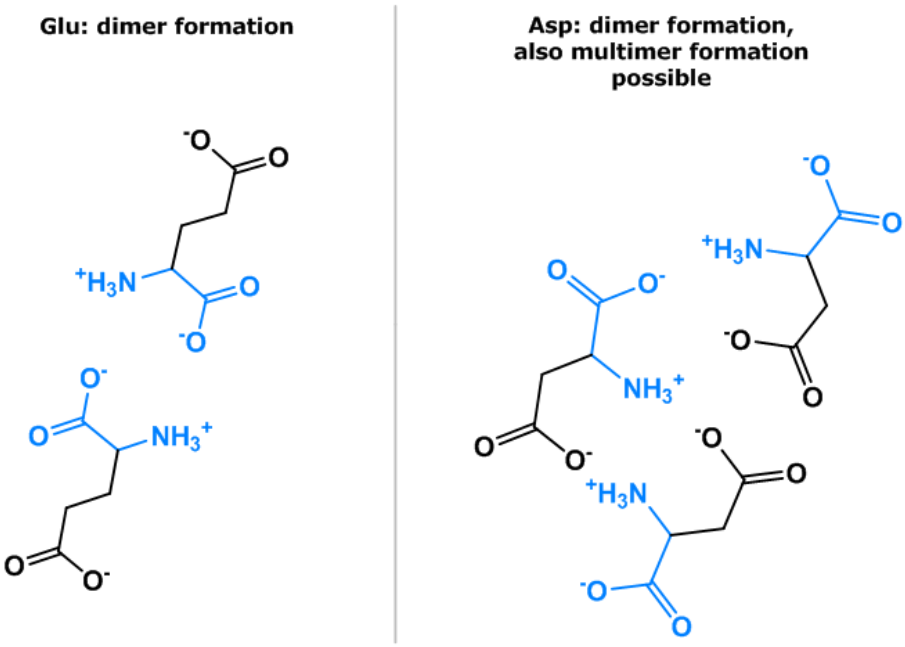

Figure 21: Intermolecular interactions for Asp (right) and Glu (left)

Secondly, intermolecular interactions could further explain the difference in reactivity between Glu and Asp. Whereas 'dimer' formation is believed to happen for both Glu and Asp, the formation of intermolecular interactions with more than one molecule of amino acid is much more probable for Asp than for Glu. This is due to the length of the side chain of the amino acids and therefore the possibilities of interactions with moieties of other molecules, as depicted in Figure 21. As a consequence, the amines of Asp molecules are much more hindered than the amines of Glu, implying that more halide would be required to break the interactions in Asp than for Glu.

The last interesting finding obtained from the NaBr screening was that different by-products were formed from Asp, among which malonic acid, depicted in Figure 22, which can be formed by oxidation of the aldehyde corresponding to Asp (3-oxopropanoate). Different by-products could be formed during the oxidative decarboxylation of Asp, an overview is given in Figure 25, page 27. Only malonic acid was identified as by-product by comparison with a standard by HPLC.

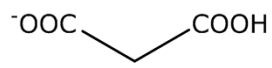

Figure 22: Malonic acid

3.3.2.2 *Time course reaction*

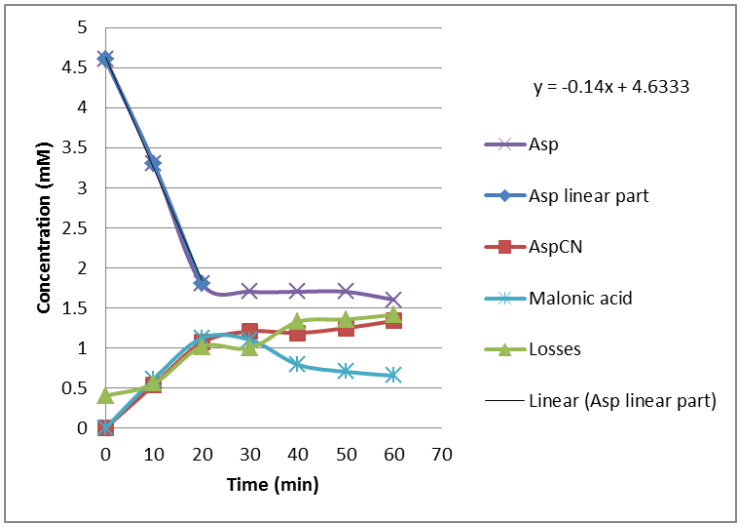

Figure 23: Time course reaction for Asp at 2 mM NaBr. Starting concentration of amino acid: 5 mM.

The time course reaction for Asp at 2 mM NaBr (Figure 23) sheds some light on the low conversion and selectivity observed at low NaBr concentration in the screening. Remarkably, the conversion of Asp and the formation of products proceeded well until 30 min, at which point the reaction stopped almost completely: only the by-product malonic acid is still being converted into another by-product/s. This is a clear indication that the bromide in solution may be depleted after 30 min. Two different tests were done to investigate the hypothesis of bromide depletion: addition of extra NaBr after 40 min, and an adapted monochlorodimedone (MCD) assay as a means of quantification of the concentration of bromide in solution after 40 min.

3.3.2.3 *Bromide depletion tests*

NaBr doping

The first test to investigate if bromide was indeed depleted in the time course reaction of Asp at 2 mM NaBr was simply the addition of extra NaBr to the reaction mixture after 40 min. The concentration in the reaction mixture was increased by 3 mM NaBr to a total NaBr concentration of 5 mM, and the results are depicted in Figure 24. It is shown that the addition of extra NaBr induces a continuation of the reaction. Full conversion and much higher selectivity than without extra NaBr is obtained, implying that bromide was indeed depleted after 40 min. Furthermore, it is also shown that the conversion and selectivity at 2+3 mM NaBr after 40+40 min is in the same range as at 5 mM NaBr after 60 min. This indicates that the reaction at 2+3 mM NaBr probably proceeded very similarly to the reaction at 5 mM. Finally, although the bromide was thought to be needed in catalytic amounts for the oxidative decarboxylation of amino acids, it is clearly only true in the case of Glu and 2AAA as, in the case of Asp, NaBr is consumed somehow.

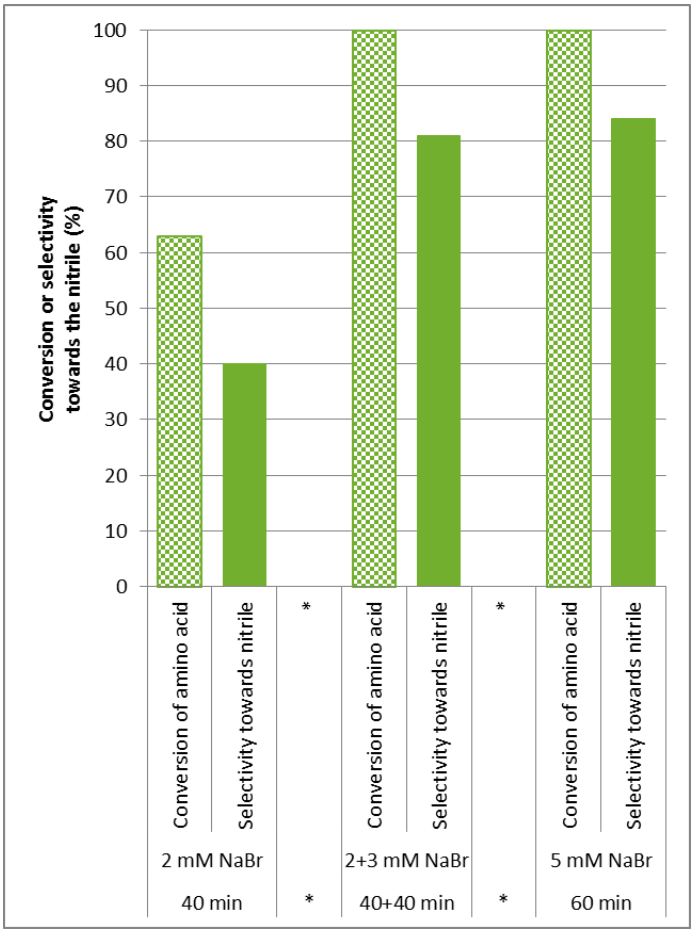

Figure 24: Comparison between the oxidative decarboxylation of Asp at 2 mM NaBr after 40 min (left), at 2 mM NaBr with 3 mM NaBr extra after 40 min (middle) and at 5 mM NaBr after 60 min (right) for comparison with the 2+3 mM NaBr results.

NaBr quantification by MCD assay

The second test was meant to quantify the concentration of bromide in solution after 40 min reaction time. For this purpose, the MCD assay was adapted to a bromide quantification assay and then carried out. An MCD assay is an experiment usually meant for the assessment of the activity of a haloperoxidase by measuring the decrease in UV-absorbance of the MCD[32]. This test is based on the correlation between the concentration of bromide and the consumption of MCD resulting in a certain final UV-absorbance. A calibration line was made from the UV-absorbance of MCD at known bromide concentrations after 25 min and it can be found in Appendix 16. Then, samples from the oxidative decarboxylation of Asp at 2 mM NaBr at t=0, 10 and 40 min were prepared fresh. These samples served as the only bromide source for the assay. The NaBr concentrations calculated from the absorbance values obtained after 25 min are given in Table 3. Although the calculated concentration for t=0 is higher than the theoretical concentration (2.6 mM instead of 2 mM), it can be concluded that the bromide concentration is indeed depleting over time. As the error made is not known, the final bromide concentration at t=40 min is estimated to be lower than 1 mM.

Table 3: UV-absorbances and corresponding bromide concentrations obtained from the MCD assay for the t=0, 10 and 40 min reaction time samples of the oxidative decarboxylation of Asp. All experiments in the MCD assay were done in duplo.

Sample	UV-absorbance after 25 min (mAbs)	Calculated corresponding NaBr concentration (mM)	Theoretical NaBr concentration (mM)
t=0 min	0.41	2.6	2
t=10 min	0.80	1.6	2
t=40 min	1.11	0.7	2

In any case, if the bromide is depleted after 40 min, a brominated by-product must have been formed. Of course, mono- or di-brominated intermediates are always formed in the reactions under study, but these compounds are unstable[23]. They are then converted further into the nitrile or aldehyde hereby releasing the bromide into solution again.

From the chromatograms obtained for each time point of the time course reaction of Asp at 2 mM (Appendix 7 and 8), it becomes clear that several different by-products are formed in time. Three clear extra peaks can be observed. Regarding the nature of these by-products, an overview of the possible by-product formation pathways is given in Figure 25.

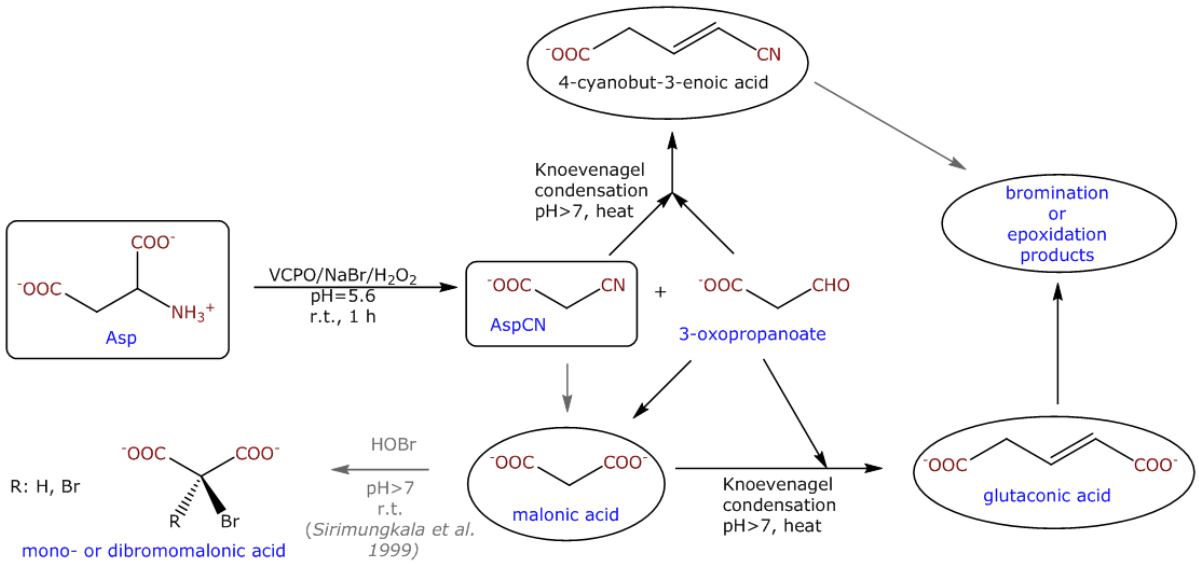

Figure 25: Possible by-product formation pathways in the oxidative decarboxylation of Asp at 2 mM NaBr. The most likely by-products are marked with an ellipse.

In this overview, some pathways are depicted in grey instead of black, implying that these pathways are less likely to occur than the pathways depicted in black. The reasoning behind this is as follows. Similarly to SerCN, the stability of AspCN and malonic acid under the reaction conditions of interest was studied (see Appendix 13 and 14 for HPLC chromatograms). Both AspCN and malonic acid turned out to be stable at different concentrations of NaBr, in the presence of VCPO and hydrogen peroxide. Therefore, it is unlikely that by-products such as mono- or dibromomalonic acid[33] were formed with only AspCN or malonic acid as starting material.

Furthermore, the aldehyde corresponding to Asp (3-oxopropanoate) could be involved in two Knoevenagel condensation reactions: one with AspCN and one with malonic acid. However, for this type of condensation reaction to take place, basic conditions and heat are required, two conditions that are not met in this study. However, after quenching the reactions with sodium thiosulfate ($Na_2S_2O_3$), the pH is increased from 5.6 to 7-8. Unfortunately, as the 3-oxopropanoic acid was not available commercially, it was not possible to investigate if this reaction is possible under these reaction conditions. It could be tested by reacting a known aldehyde with malonic acid under the same reaction conditions, and analyse the reaction mixture by HPLC with the theoretical condensation products as calibration standards. If these condensation reactions turn out to be possible, bromination or epoxidation products could be formed from the two condensation products.

To make the story even more complex, when the HPLC analysis of AspCN was done two weeks later, a different pattern of extra peaks was observed, as can be seen in Appendix 9 and 10. Obviously, the quenching with $Na_2S_2O_3$ may stop the oxidative decarboxylation of Asp, but it does not stop side-reactions from occurring. Finally, the formation of by-products becomes even more difficult to explain as the extra peak pattern for unquenched samples is again different than the first two patterns mentioned (Appendix 11 and 12). Indeed, different peaks as well as peaks with different area ratios were observed for unquenched samples.

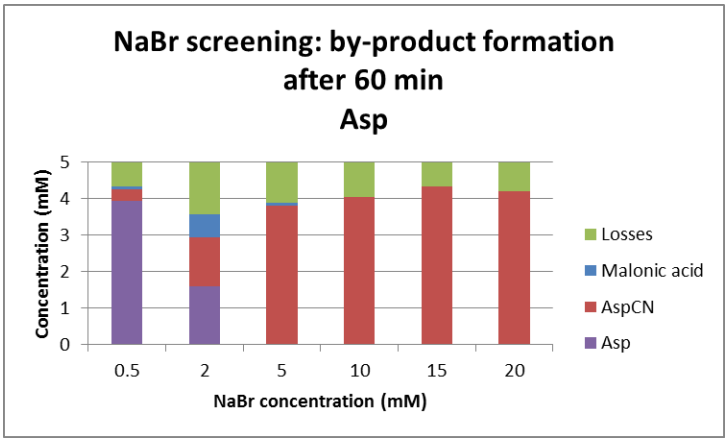

Figure 26: Overview of the different compounds detected by HPLC analysis of the NaBr screening of Asp after 60 min reaction time

It must be noted that the by-product peak patterns discussed above were all obtained at 2 mM NaBr. At 0.5, 5 and 10 mM NaBr, only one extra peak was observed after 60 min: the peak with the retention time of ~25 min. At 15 and 20 mM NaBr, two extra peaks were seen at ~13 min and at ~25 min. The formation of the nitrile and malonic acid from Asp at all NaBr concentrations studied is shown in Figure 26.

Concluding, the formation of by-products during the oxidative decarboxylation of Asp is dependent on the reaction time, the time of analysis, the addition of $Na_2S_2O_3$ inducing a change of pH, and the concentration of NaBr. It is clearly a complex issue and requires further research before it is understood completely.

3.4 Role of bromide in oxidative decarboxylation of amino acids

Bromide is required for the VCPO to produce the reactive species, HOBr, necessary for the oxidative decarboxylation of amino acids for the production of nitriles. HOBr brominates twice the amine group of the amino acid, after which decarboxylation takes place and the nitrile is formed. VCPO, however, can only generate a certain amount of HOBr from the available bromide per time unit. Additionally, bromide is regenerated during decarboxylation, implying that the concentration of HOBr in solution is always low. The different results for the amino acids at different NaBr concentrations must therefore be the result of different concentrations of bromide in solution instead of HOBr.

It was speculated that intra- and intermolecular interactions (Figure 20 and 21) could be broken by the negatively charged bromide, allowing HOBr to reach the amine more easily. In order to test the role of the bromide in the oxidative decarboxylation of amino acids capable of ring formation, another halide, fluoride, was added to the reaction mixtures of Asp and Glu, both at 0.5 mM NaBr. Fluoride has the same charge as bromide, and it cannot be oxidised by VCPO to hypofluorous acid. It was hypothesised that the addition of fluoride to the reaction mixture would lead to a higher availability of the bromide for the VCPO and thus potentially to a higher conversion of Asp at 0.5 mM NaBr. Glu yielded high conversion at 0.5 mM NaBr, and therefore serves as positive control.

First, in order to test the inhibitory effect of fluoride on VCPO, the MCD assay was done in presence of different concentrations of fluoride (Appendix 16). It has to be kept in mind that the MCD assay involves a different bromination reaction than the oxidative decarboxylation of the amino acids. Nevertheless, the assay can show possible inhibition of the enzyme by fluoride. From the MCD assay, it became clear that fluoride inhibits the VCPO at concentrations higher than 1 mM; the enzyme activity dropped by ~30% at 5 mM NaF, and ~67% at 10 mM NaF. This has to be kept in mind for the experiment discussed below.

In Figure 27 and 28[34], the results of the fluoride addition reactions with Asp and Glu at 0.5 and 10 mM NaBr are shown, with concentrations of NaF varying between 0.5, 5 and 10 mM. From these experiments became clear that the fluoride had no influence at all on the reactivity of Asp and Glu despite the inhibition of VCPO by high concentrations of fluoride. Apparently, the VCPO was concentrated enough in the reaction mixture to still allow the oxidative decarboxylation of Asp and Glu to proceed at normal rate. If the fluoride does not increase the conversion of Asp by making more bromide available for the VCPO, then bromide itself must be responsible for the differences in reactivity observed in all NaBr screenings. These results do not support the theory of intra- and intermolecular interactions or fluoride may not be a suitable anion to break these if the interactions exist.

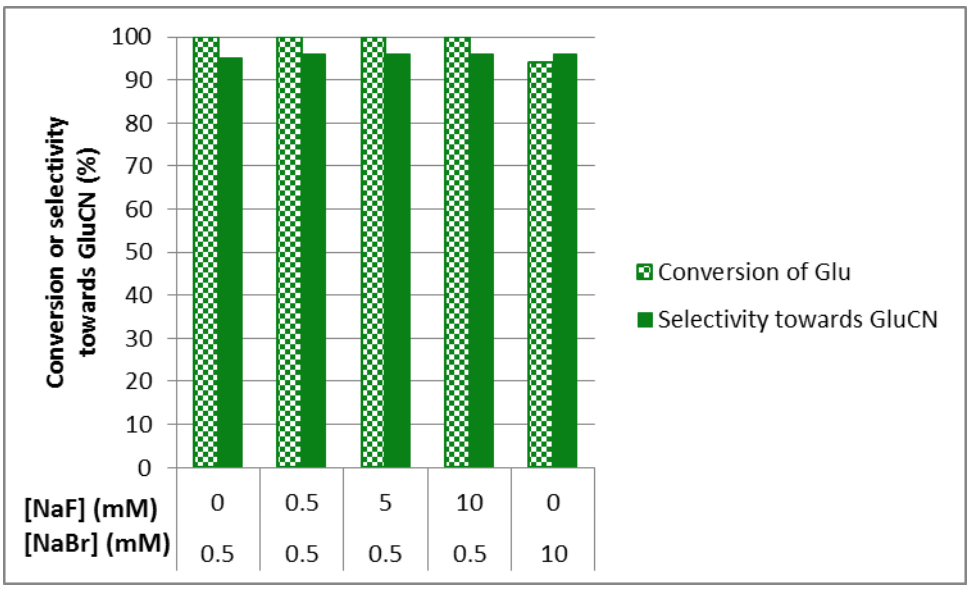

Figure 27: Conversion and selectivity towards the nitrile at different NaBr and NaF concentrations for Glu. Starting concentration of amino acid: 5 mM. Reaction time: 60 min.

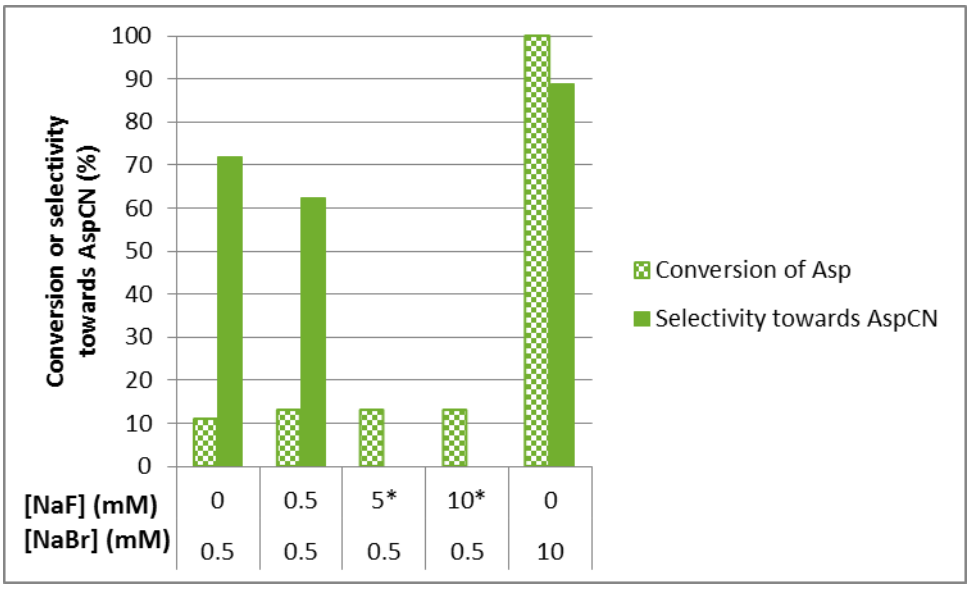

Figure 28: Conversion and selectivity towards the nitrile at different NaBr and NaF concentrations for Asp. Starting concentration of amino acid: 5 mM. Reaction time: 60 min. *The selectivity towards the nitrile could not be calculated due to interference of NaF with the HPLC chromatograms. It was tried to decrease the NaF interference by adding NaF to the eluent, as suggested by Langemeier and Rogers (1995), but the interference did not decrease. In spite of this, the selectivity was estimated to be very similar to the other two NaF concentrations based on the peak areas of malonic acid and an unidentified by-product.

3.5 Influence of the side-chain functionality on the reactivity of amino acids to the corresponding nitriles by oxidative decarboxylation

From all the results discussed above, it is clear that the length of the side-chain influences the reactivity of amino acids, but this influence on the reactivity was less pronounced for the side-chain functionality. In Appendix 17, 18 and 19, the NaBr screenings classified per number of carbon atoms can be seen, making it easier to evaluate the influence of the side-chain functionality. From the three graphs can be seen that the side-chain functionality only influences much the reactivity of the amino acids at low NaBr concentrations (0.5 and 2 mM NaBr). At higher NaBr concentrations, the differences between the compared amino acids are much less pronounced. Indeed, at high NaBr concentrations, the bromide is present in such a large excess that there is always sufficient bromide present in solution to compensate for the interactions with the bromide induced by the side-chain functionality. At lower NaBr concentrations, these interactions play a much more important role.

Generally, the side-chain functionality appeared to determine the sensitivity to the concentration of NaBr. Where a bromide 'threshold' is observed for the aliphatic amino acids and the ones with hydroxyl functionality, this is not the case for Glu and 2AAA, with Asp probably being an exception to the rule. Furthermore, the selectivity towards the nitrile after 60 min seems to be affected by the side-chain functionality. This is illustrated by the somewhat higher selectivity for Glu and 2AAA in comparison to the aliphatic amino acids. These, in turn, show higher selectivity after 60 min than Ser and HSer. This could imply that the second bromination step is influenced by the side-chain functionality.

Additionally the conversion rate of all amino acids at 2 mM NaBr appeared to be affected to a minor extent by the nature of the side-chain functionality: ~-0.14 mM min^{-1} for the acidic and aliphatic amino acids, and ~0.16 mM min^{-1} for the amino acids with hydroxyl side-chain functionality.

4 Conclusions

The influence of the length and functionality of the side-chain on the reactivity of amino acids towards nitrile formation by enzymatic oxidative decarboxylation was evaluated, as well as the influence of the concentration of bromide. For this purpose, NaBr screening and time course reactions at 2 mM NaBr were done for all amino acids.

Generally, all amino acids under study were successfully converted to their corresponding nitriles within 60 min and the optimal bromide concentration was elucidated for all of them. Furthermore, all three factors under study (length of side-chain, functionality and bromide concentration) appeared to influence the reactivity of amino acids in different ways.

Aliphatic amino acids

Firstly, the aliphatic amino acids (2Aba, NVal and NLeu) were most successfully converted to nitrile at 2 and 5 mM NaBr, at which bromide concentration complete conversion was observed for all, as well as high selectivity towards the nitrile (85-90%).

Furthermore, the length of the side-chain did not influence much the selectivity towards the nitrile after 60 min, except at 0.5 mM NaBr where small differences were observed. The conversion of amino acid, however, was influenced significantly by the side-chain length between 10 and 20 mM NaBr: the longer the side-chain, the lower the conversion. This may be explained by hydrophobic interactions between the side-chain and the bromide. However, a factor independent on the length of the side-chain is the conversion of the three amino acids, which showed zero-order kinetics and equalled ~-0.14 mM min^{-1}.

Moreover, the concentration of NaBr also appeared to influence the reactivity of the amino acids. Interestingly, low conversion (35-45%) and selectivity towards the nitrile (60-70%) was seen at 0.5 mM NaBr and much higher conversion (100% at 2 and 5 mM NaBr) and selectivity (85-95%) at higher NaBr concentrations (2-20 mM). This indicates that a bromide concentration 'threshold' may not yet have been reached at 0.5 mM NaBr. Furthermore, the higher the NaBr concentration, the lower the conversion was, probably due to bromide inhibition of VCPO. Moreover, at higher NaBr concentration (5-20 mM), the selectivity towards the nitrile after 60 min appeared to be independent not only of the side-chain length but also of the bromide concentration. However, it has to be kept in mind that the selectivity at lower conversions might be different at different bromide concentrations. What's more, only one by-product was detected, in low concentrations, at all NaBr concentrations tested: the aldehyde corresponding to the amino acids.

Amino acids with hydroxyl side-chain functionality

Secondly, the amino acids with hydroxyl functionality (Ser and HSer) were most successfully converted to nitrile at 2 mM NaBr, at which bromide concentration complete conversion was attained within 60 min with a selectivity towards the nitrile of 65% for Ser and 82% for HSer.

The length of the side-chain influenced the reactivity of Ser and HSer but probably in a different way than for the aliphatic amino acids. The conversion was affected only at 15 and 20 mM NaBr, in a way opposite to the aliphatic amino acids. Indeed, for Ser and HSer, the longer the side-chain, the higher the conversion. Whereas the influence of the length on the conversion was minor, the influence on the selectivity towards the nitrile after 60 min was considerable (~50% for Ser and ~85% for HSer), possibly due to different environments around the amine of the amino acid. However, the conversion rate of the amino acids appeared to be independent of the side-chain length and equalled ~-0.16 mM min^{-1}.

Additionally, the concentration of NaBr influenced the reactivity of Ser and HSer in a similar way as for the aliphatic amino acids: low conversion at 0.5 mM NaBr (~40% for both amino acids) and complete conversion at higher concentrations, with a decline in conversion observed at 15 and 20 mM due to bromide inhibition of VCPO.

Moreover, only one by-product was formed from Ser and HSer, but in different concentrations: high concentration for Ser and low concentration for HSer, indicating that the rates of the second bromination of the amine are different for Ser and HSer. More clarity could be gained by investigating the behaviour of the synthetic amino acid 5-hydroxynorvaline.

Acidic amino acids

Thirdly, it was shown that the acidic amino acids (Asp, Glu and 2AAA) were three different complex cases, with different optimal conditions for each of them. Indeed, 2AAA attained complete conversion and 95-100% selectivity towards the nitrile after 60 min at NaBr concentrations between 0.2 and 2 mM. Glu, on the contrary, attained complete or nearly complete conversion at all bromide concentrations investigated (0.2-20 mM), with the highest selectivity between 0.2 and 10 mM NaBr (~95%). Asp, the third and shortest acidic amino acid, showed even different optimal conditions: complete conversion and the highest selectivity towards the nitrile (~85%) at 5-20 mM NaBr. From these screening results, it became clear that Glu and 2AAA showed reactivities more similar to each other than to Asp. The conversion rates of the amino acids, in turn, appeared to be independent of the length of the side-chain and equalled ~-0.14 mM min^{-1}.

Glu and 2AAA

For Glu and 2AAA, the length of the side chain had a significant effect on the conversion at higher NaBr concentrations. Indeed, the longer the side-chain, the lower the conversion at high concentrations of NaBr, possibly due to the occurrence of intramolecular interactions in Glu leading to trapping of bromide. Moreover, although the differences were very small, the longer the side-chain, the higher the selectivity towards the nitrile after 60 min.

Additionally, the concentration of NaBr had no influence on the reactivity at 0.2-2 mM NaBr. However, at higher NaBr concentrations, bromide inhibition occurs for 2AAA, and thus not for Glu. The selectivity towards the nitrile after 60 min is not much influenced by the concentration of NaBr.

Asp

Even though Asp is only a small amino acid, it showed a complex behaviour towards oxidative decarboxylation to AspCN. Indeed, at 0.5 and 2 mM NaBr, low conversion and selectivity towards the nitrile was observed. At 2 mM NaBr, the bromide turned out to be depleted (less than 1 mM left in solution) after 40 min, stopping the reaction from proceeding further. At higher NaBr concentrations (5-20 mM), however, complete conversion and high selectivity was observed, indicating that sufficient bromide was present in solution, and no bromide inhibition of VCPO seemed to occur. Furthermore, no proof has been found so far for intra- and intermolecular interactions to play an important role. Instead, one or more bromide scavenging by-products seem to be formed at low NaBr concentrations, possibly accounting for the high NaBr requirement for high conversion.

Furthermore, the formation of by-products at 2 mM NaBr seemed to be the most complex of all NaBr concentrations tested. By-product formation appeared to be dependent on the reaction time, the time of analysis, the addition of $Na_2S_2O_3$ inducing a change of pH, and the concentration of NaBr. An explanation for the complexity of the reactions involving Asp could be the formation of malonic acid, which is very reactive due to its two carboxyl groups and two acidic protons, making the compound prone to side-reactions.

Side-chain functionality

Additionally, the influence of the side-chain functionality on the reactivity of amino acids to nitrile formation by enzymatic oxidative decarboxylation was evaluated. Generally, aliphatic amino acids gave the most straightforward results due to the lack of reactivity of the aliphatic functionality. The amino acids with hydroxyl side-chain functionality gave more difficult results to interpret due to both the choice for the small amino acid Ser, which appeared to be prone to side-reactions, and the unavailability of 5-

hydroxynorvaline. The acidic amino acids gave the most complex results due to both the negatively charged side-chain carboxyl and the choice for the small amino acid Asp, which induces a complex pattern of by-products. Furthermore, the biggest influence of the side-chain functionality was that a bromide 'threshold' between 0.5 and 2 mM NaBr was observed for the aliphatic amino acids, the ones with hydroxyl functionality and Asp, and not for Glu and 2AAA. At higher NaBr concentrations, the differences between the types of amino acids were much less pronounced, except for Glu and Asp, possibly due to trapping of bromide by the amino acids. The selectivity towards the nitrile was found to be highest for the acidic amino acids, followed by the aliphatic ones. The amino acids with hydroxyl functionality gave the lowest selectivity after 60 min. This indicates that the functionality may influence the second bromination step of the amine. Additionally, the conversion rate of all amino acids at 2 mM NaBr appeared to be affected to a minor extent by the side-chain functionality: ~0.14 mM min^{-1} for the acidic and aliphatic amino acids, and ~0.16 mM min^{-1} for the amino acids with hydroxyl side-chain functionality.

Role of the bromide

Lastly, no proof was found for the occurrence of intra- and/or intermolecular interactions, nor for the implication of bromide herein. However, it became clear from the doping of the reaction mixture with fluoride that bromide itself determines the reactivity of amino acids, and not a halide in general.

5 Recommendations

Firstly, the amino acids alanine (aliphatic, three carbon atoms) and 5-hydroxynorvaline (hydroxyl functionality, 5 carbon atoms) would be interesting to include in the project. These amino acids would make comparison of the side-chain functionalities easier, as there would be more amino acids with the same number of carbon atoms. However, 5-hydroxynorvaline was not easily available commercially and a suitable analysis method should first become available for alanine.

Secondly, further research should be done regarding the reactivity of amino acids at low bromide concentration. The fact that a bromide 'threshold' is seen for all amino acids except Glu and 2AAA is not yet understood.

Additionally, a complex pattern of by-products was obtained for Asp at 2 mM NaBr. In order to determine whether brominated by-products were formed, mass spectrometry could be done for the different by-products as these were all UV-active. This analysis should be done directly after the reaction and the first HPLC analysis of the reaction products. Additionally, the Knoevenagel condensation of malonic acid or cyanoacetic acid (AspCN) with the aldehyde (3-oxopropanoate) could be tested by reacting any known small aldehyde with either malonic acid or the nitrile under the standard reaction conditions given in the Experimental Setup. However, it is known that basic conditions and heat are required for Knoevenagel condensations to be possible, so it seems unlikely to happen in the reactions with Asp.

Furthermore, in order to obtain more clarity about the occurrence of intramolecular interactions for the acidic amino acids as well as for the ones with hydroxyl functionality, different tests could be done. In fact, interactions between a proton from the amine and an oxygen from the side-chain carboxyl/hydroxyl group could be shown by 2D ^{1}H NMR and proven by computational modelling, using methods like density functional theory. For instance, it was shown for Asp that proton transfer takes place between the amine and the side-chain carboxyl, indicating that ring formation occurred. Unfortunately, these calculations have only been done for the gas phase, and not yet including the water solvent nor bromide, which are likely to be important factors. Additionally, Raman or near infrared spectroscopy could further show changes in vibrations for the different functionalities for the acidic amino acids especially, in the absence and presence of bromide, because it can be measured in aqueous solution.

Lastly, HOX not only reacts with the amino acid, it can also react with hydrogen peroxide to form singlet oxygen (Equation 2)[18, 24, 35-38]. Singlet oxygen ($^{1}O_2$) is molecular oxygen in its first excited state, and often can be formed from different compounds upon exposure to visible light[37]. In the case of haloperoxidases, when hydrogen peroxide is present in excess and is not consumed directly by the enzyme, it can react spontaneously with the formed HOX as shown in Equation (2). Moreover, VCPO has even been reported to be capable of producing singlet oxygen from hydrogen peroxide under mildly acidic conditions[24].

$$HOX + H_2O_2 \rightarrow {}^{1}O_2 + X^- + H^+ + H_2O \quad (2)$$

Singlet oxygen is a highly reactive species and it has been shown to react with amino acids in two ways: by direct chemical reaction or physical quenching[37]. Examples of a direct chemical reaction are oxidation of the side-chain, as reported for methionine and cysteine, and formation of endo-peroxides with subsequent (ring) cleavage[37, 38], as reported for histidine, tryptophan, tyrosine at physiological pH. At higher pH, arginine and lysine were also shown to react, but only in their deprotonated form. Physical quenching, on the other hand, implies energy transfer and loss of the excited state without any chemical reaction occurring[37]. Only tryptophan has been reported to be capable of physical quenching.

In conclusion, although singlet oxygen can react with several amino acids, it is deemed unlikely that this factor is of much importance in the present study for two reasons. Firstly, the amino acids under investigation will not be any of the ones mentioned above. Secondly, the working pH will be slightly acidic, reducing the risk of interaction between singlet oxygen and the chosen amino acids. Despite this, singlet oxygen formation has to be kept in mind since there is, to our knowledge, not much known about the effect of singlet oxygen on other amino acids than the ones mentioned.

6 Experimental section

Materials

L-glutamic acid (98.5+%), L-aspartic acid (≥98%), L-serine (≥99%),L- norvaline (≥99%), L-norleucine (≥98%), malonic acid (99%), 2-cyanoacetic acid (≥99% pure), glycolonitrile (70%), 4-cyanobutanoic acid (98% pure), NaBr (99% pure), H_2O_2 (35 wt%), citric acid, Na_3VO_4, and Trizma® base were purchased from Sigma-Aldrich. Butyronitrile (99%), propionitrile (99%), 3-hydroxypropionitrile (99%) were purchased from Acros Organics and L-homoserine (99%), L-2-aminobutanoic acid (98+%), L-2-aminoadipic acid (98%) and valeronitrile (99%) from Alfa Aesar. 3-cyanopropanoic acid (95.9%) was provided by Interchim and monochlorodimedone by BioResource Products.

Enzyme

Vanadium chloroperoxidase plasmid was expressed in *E. coli* using a protocol described elsewhere[39]. The obtained VCPO (40-60% purified) was concentrated and stored in 100 mM Tris/H_2SO_4 buffer pH=8.2 containing 100 µM Na_3VO_4 at -20°C and has an activity of 92 U/mL (based on MCD assay). It was supplied by the group of Biobased Chemistry and Technology, Wageningen University.

MCD assay

1. *For fluoride inhibition assay:*

In a UV disposable cuvette were added 1 mL of MCD mix (50 µM MCD, 1 mM H_2O_2, 0.5 mM NaBr, 100 µM Na_3VO_4, 50 mM citrate buffer pH 5.0) and 10µL of 100x diluted VCPO sample. The bromination of monochlorodimedone (MCD) was followed in time at 290 nm on a UV-1650PC spectrophotometer from Shimadzu.

2. *For bromide depletion test:*

In a UV disposable cuvette were added 1 mL of MCD mix (75 µM MCD, 1 mM H_2O_2, 100 µM Na_3VO_4, 20 mM citrate buffer pH 5.6) and 2 µL undiluted VCPO sample. The bromination of monochlorodimedone (MCD) was followed in time at 290 nm on a UV-1650PC spectrophotometer from Shimadzu.

Standard procedure for oxidative decarboxylation of amino acids by VCPO

A typical procedure is as follows: in a glass vial 5 mM of amino acid, 0.5-20 mM of NaBr, 0.36 U/mL (90 nM) of VCPO and 20 mM citrate buffer at pH 5.6, contained in a total volume of 2 mL, were stirred at 400 rpm, at room temperature (21°C). To this reaction mixture, 16 mM H_2O_2/h (66 µL of 0.5 M H_2O_2/h) was added continuously, using a NE-1600 syringe pump from ProSense. The reaction was stopped at the different time points by adding $Na_2S_2O_3$. Samples were taken from the reaction mixture for HPLC analysis.

Purpald test

In glass reaction tubes, 300 µl of freshly prepared 34 mM Purpald, dissolved in 2 M NaOH, and 300 µl sample from the reaction mixture were added[40]. The reaction mixture was incubated at room temperature for at least 30 minutes until colour development.

HPLC

Amino acids were analysed by derivatisation as previously described[3]. Nitriles were analysed without dilution or derivatisation by using an UltiMate 3000 from Thermo Scientific. Detection was achieved using a RI-101 detector from Shodex set at 35°C. The columns used were a Rezex ROA Organic acid H+ (8%) column (7.8 × 300 mm) from Phenomenex, at 35°C with a flow of 0.5 mL/min for 3-cyanopropanoic acid, 2-cyanoacetic acid, 4-cyanobutanoic acid, glycolonitrile and 3-hydroxypropionitrile; and an Acquity UPLC® BEH C18 column (2.1 × 150 mm, 1.7 µm particle size) at 80°C with a flow of 0.3 mL/min for valeronitrile, butyronitrile and propionitrile. The elution was carried out using 12 mM H2SO4 and the quantification was performed by external standard method.

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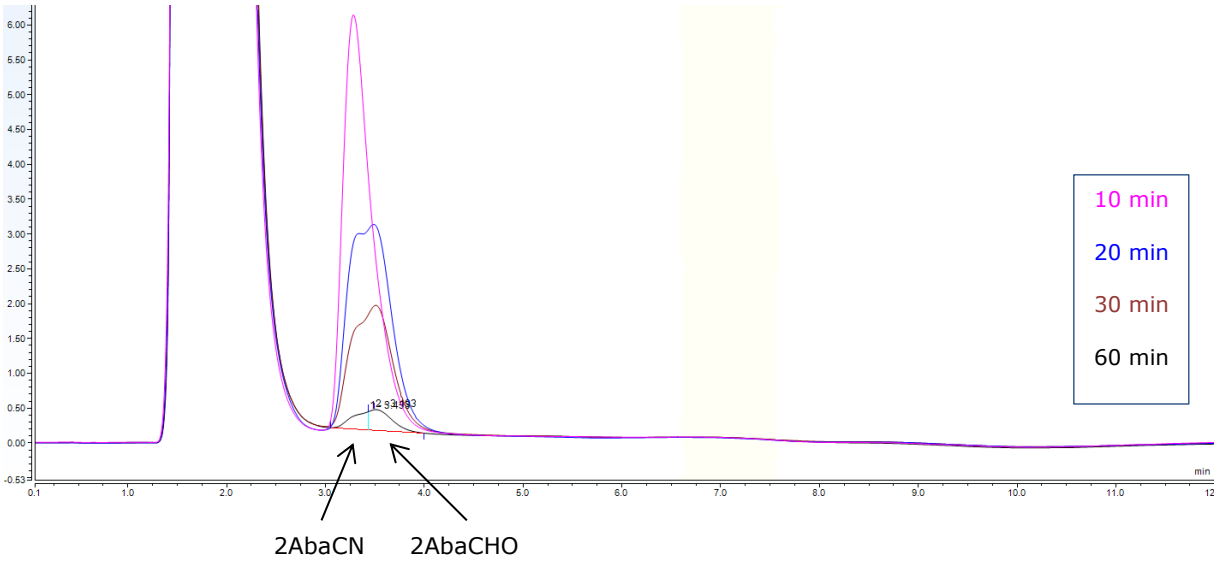
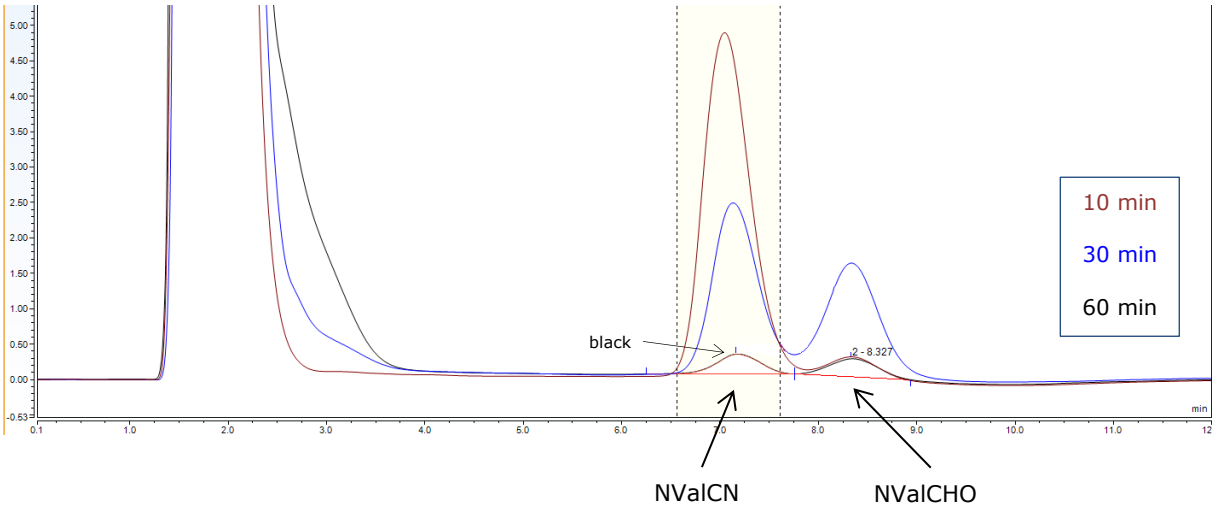
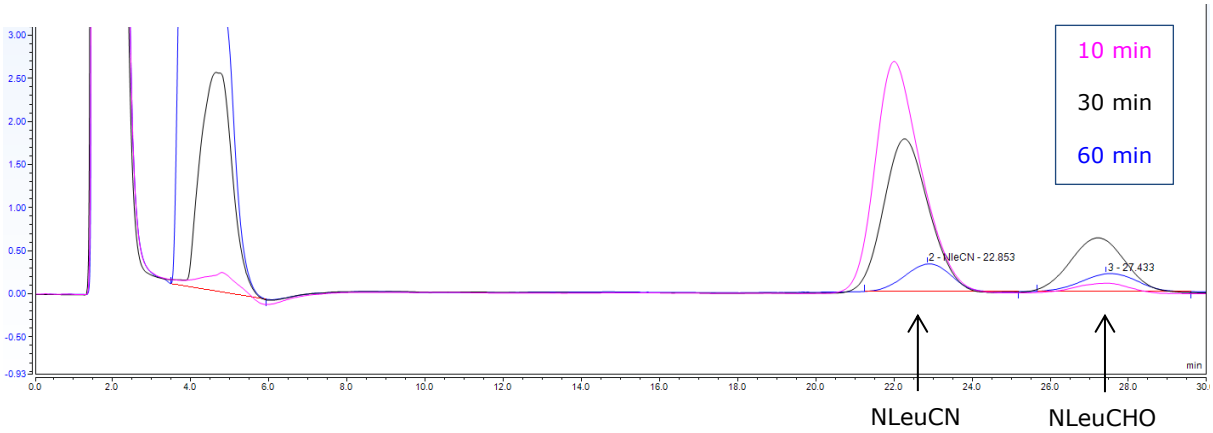
Appendix I: HPLC chromatograms - aliphatic amino acids



Appendix 1: HPLC chromatograms for the time course reaction of 2Aba at 2 mM NaBr. Reaction times: 10, 20, 30 and 60 min.

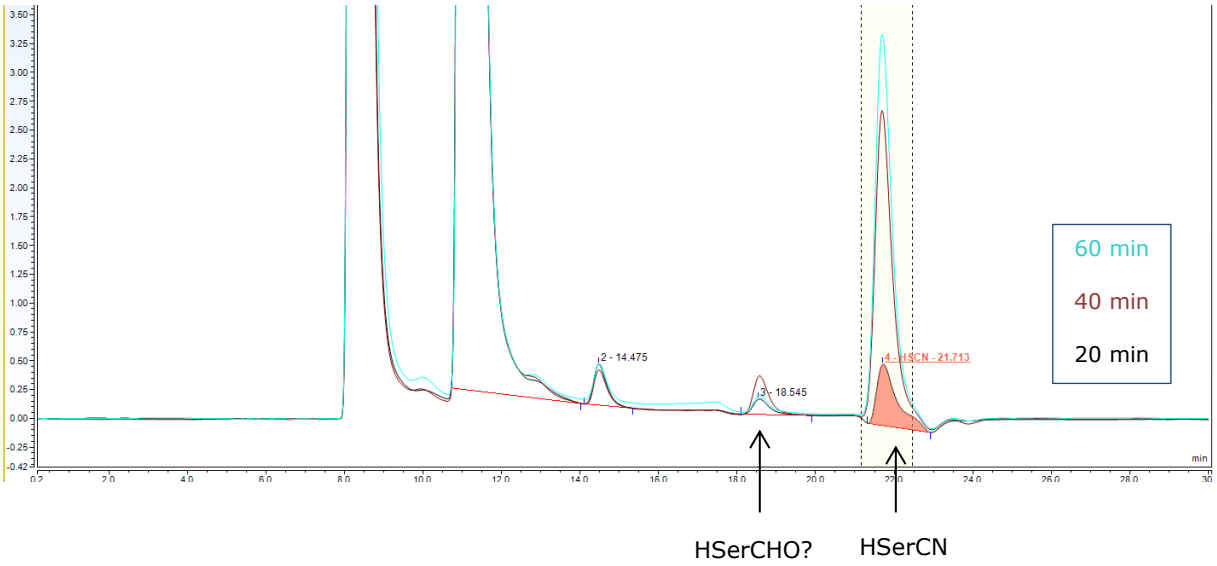


Appendix 2: HPLC chromatograms for the time course reaction of NVal at 2 mM NaBr. Reaction times: 10, 30 and 60 min.

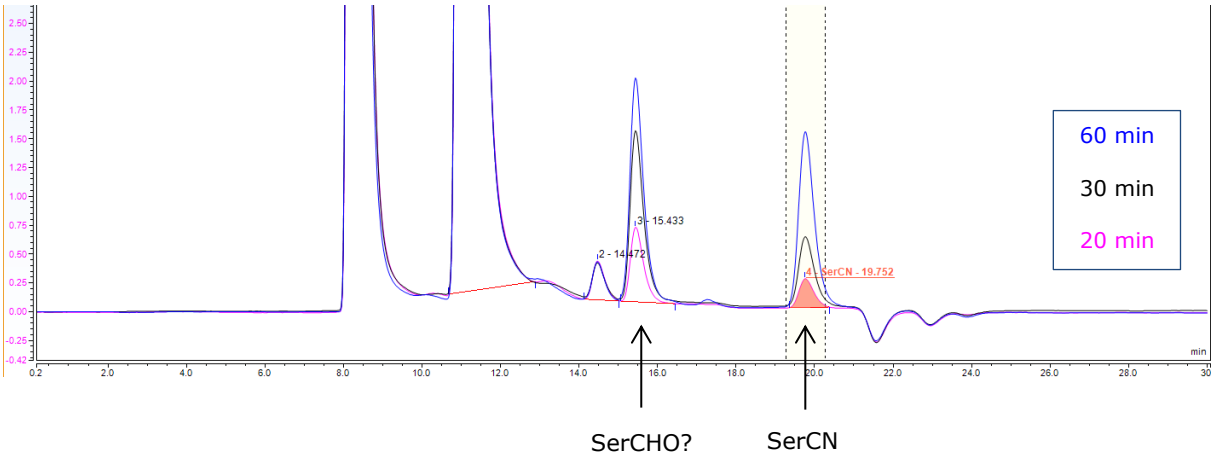


Appendix 3: HPLC chromatograms for the time course reaction of NLeu at 2 mM NaBr. Reaction times: 10, 30 and 60 min.

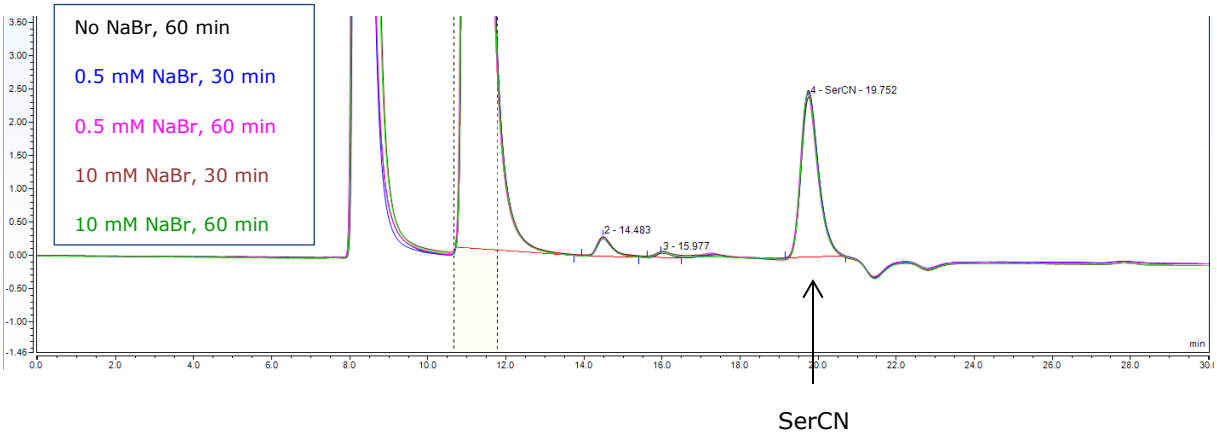
Appendix II: HPLC chromatograms - amino acids with hydroxyl functionality



Appendix 4: HPLC chromatograms for the time course reaction of HSer at 2 mM NaBr. Reaction times: 20, 40 and 60 min.

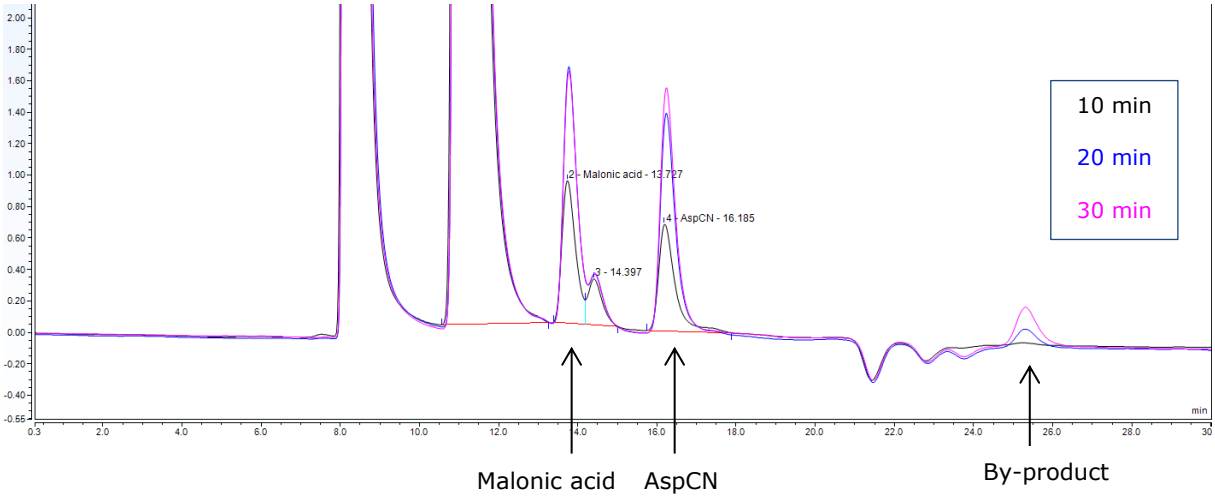


Appendix 5: HPLC chromatograms for the time course reaction of Ser at 2 mM NaBr. Reaction times: 20, 30 and 60 min.

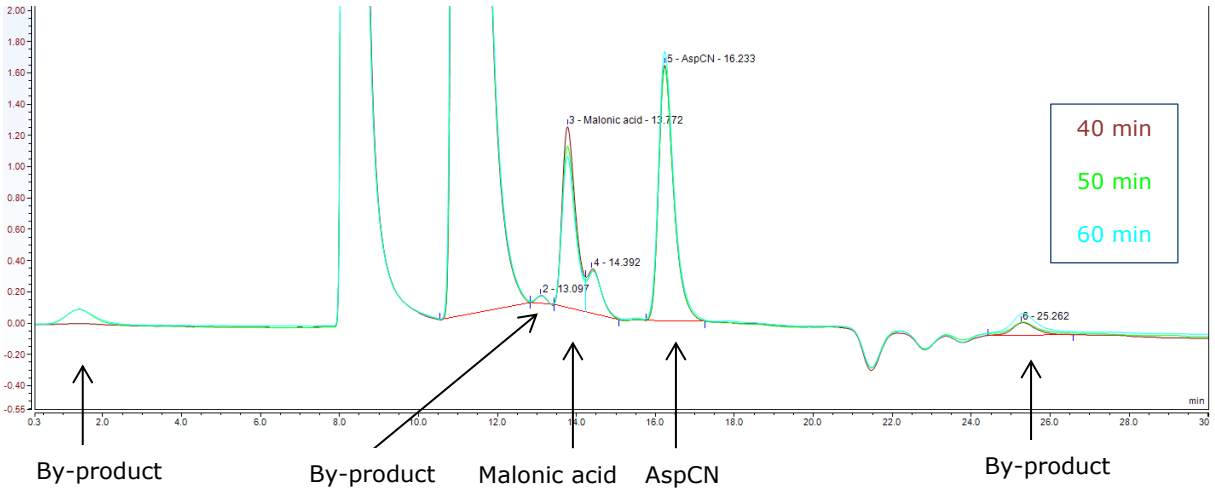


Appendix 6: HPLC chromatograms for the stability test of SerCN under different conditions.

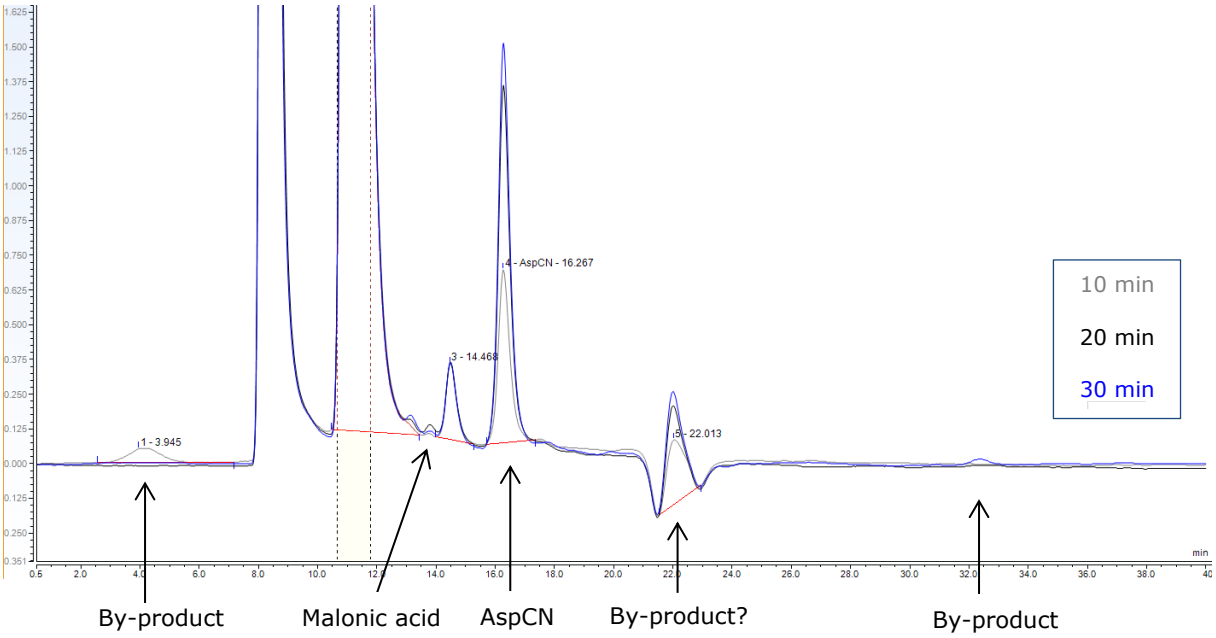
Appendix III: HPLC chromatograms - acidic amino acids



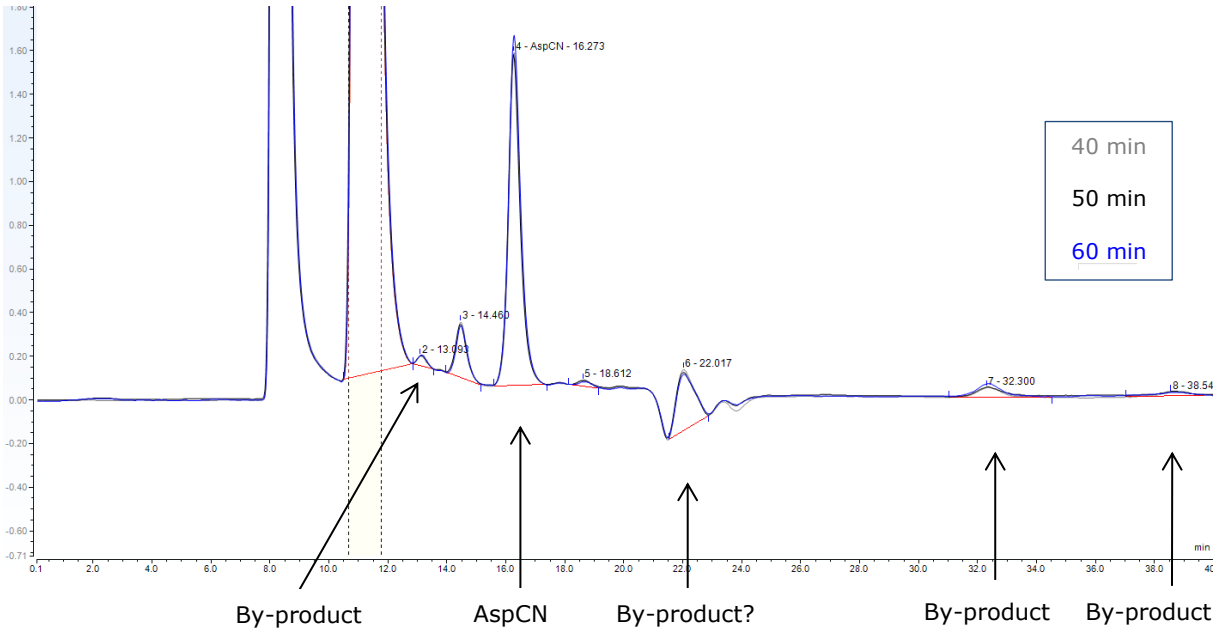
Appendix 7: HPLC chromatograms for the time course reaction of Asp at 2 mM NaBr. Reaction times: 10, 20 and 30 min.



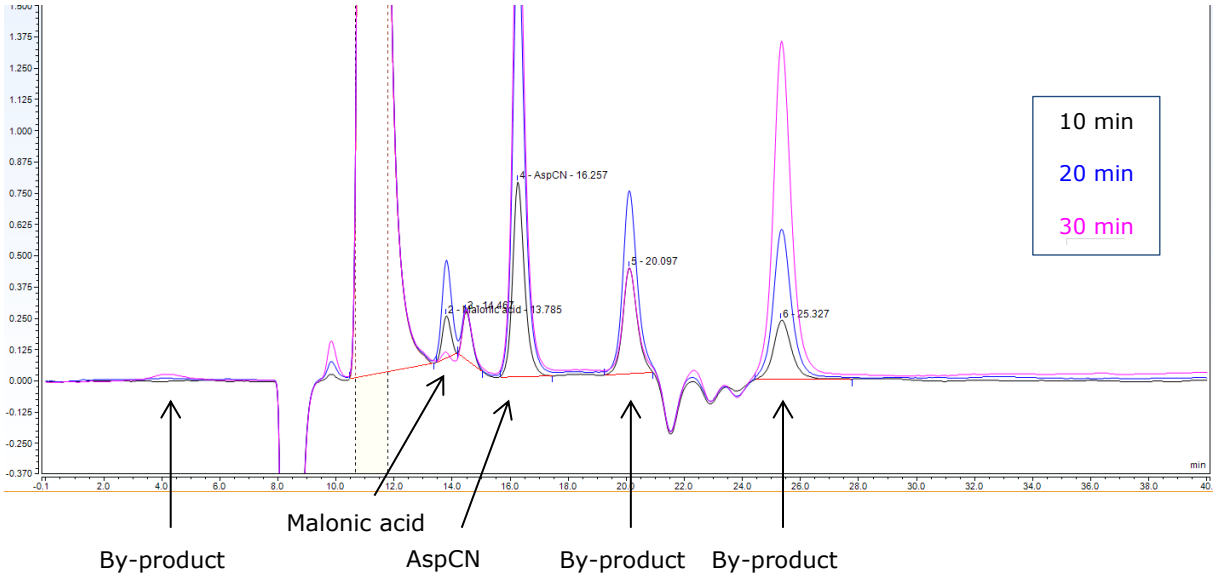
Appendix 8: HPLC chromatograms for the time course reaction of Asp at 2 mM NaBr. Reaction times: 40, 50 and 60 min.



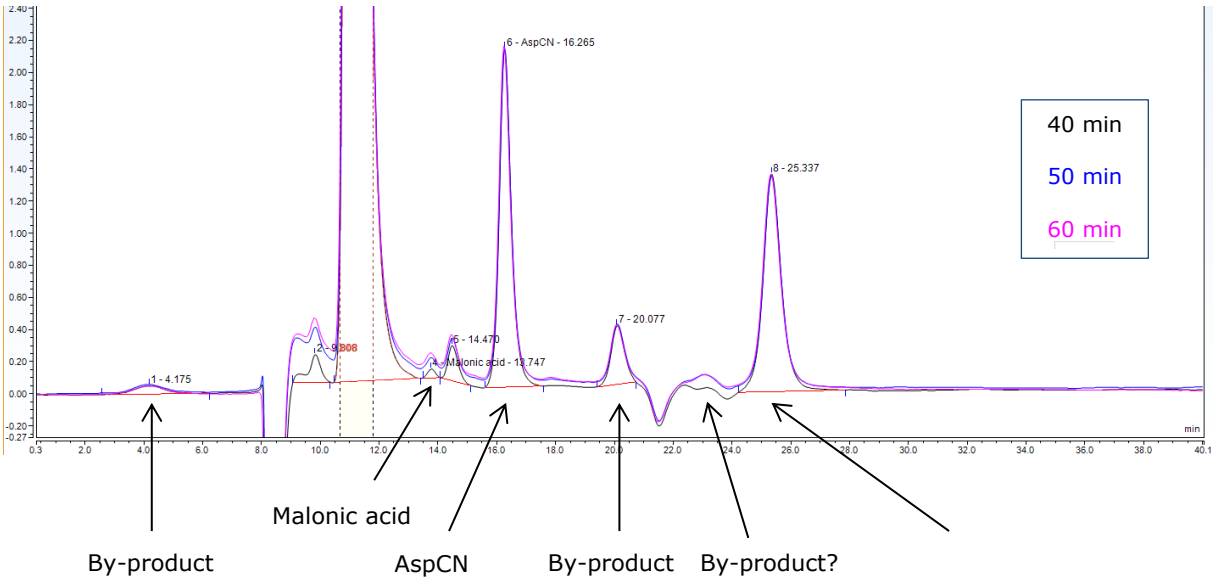
Appendix 9: HPLC chromatograms for the time course reaction of Asp at 2 mM NaBr, 2 weeks after reaction. Reaction times: 10, 20 and 30 min.



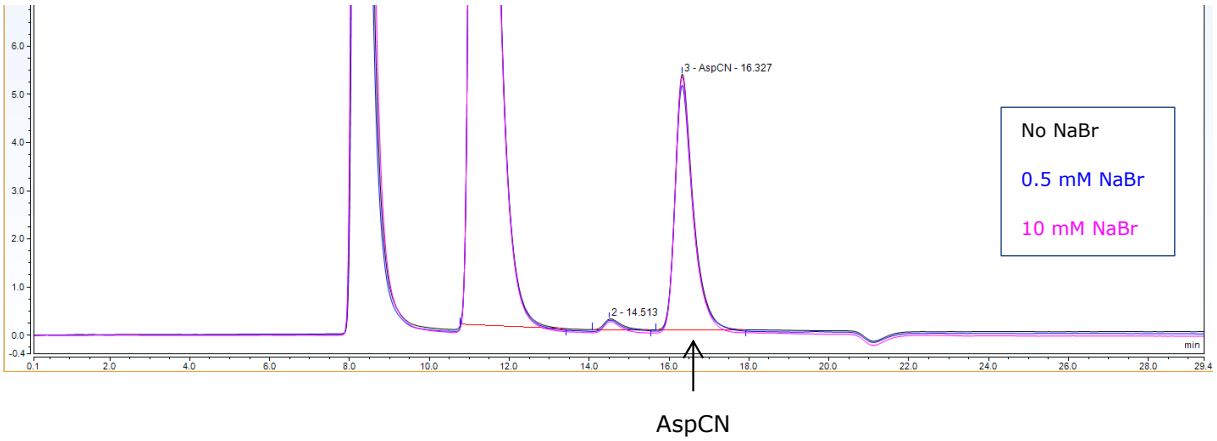
Appendix 10: HPLC chromatograms for the time course reaction of Asp at 2 mM NaBr, 2 weeks after reaction. Reaction times: 40, 50 and 60 min.



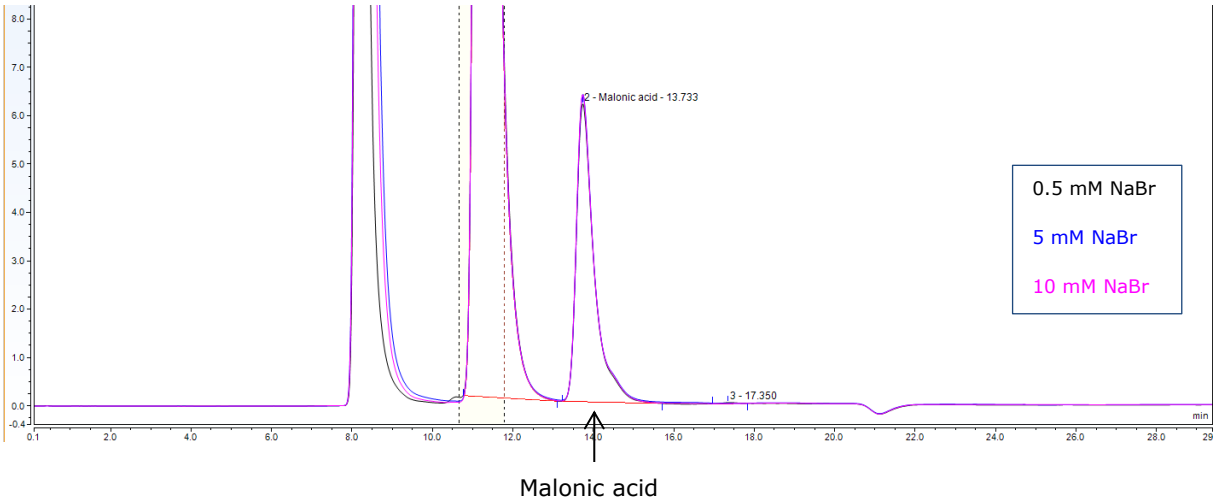
Appendix 11: HPLC chromatograms for the time course reaction of Asp at 2 mM NaBr, directly after reaction, no quenching. Reaction times: 10, 20 and 30 min.



Appendix 12: HPLC chromatograms for the time course reaction of Asp at 2 mM NaBr, directly after reaction, no quenching. Reaction times: 40, 50 and 60 min.

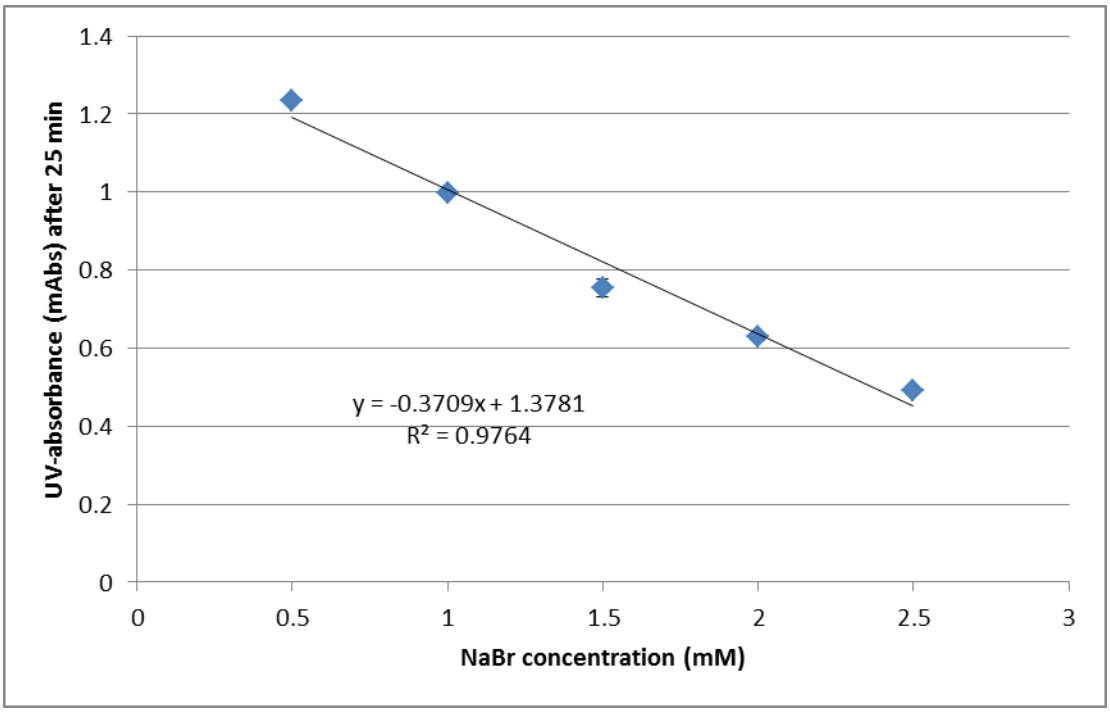


Appendix 13: HPLC chromatograms for the stability test of AspCN under different conditions. Reaction time: 60 min.

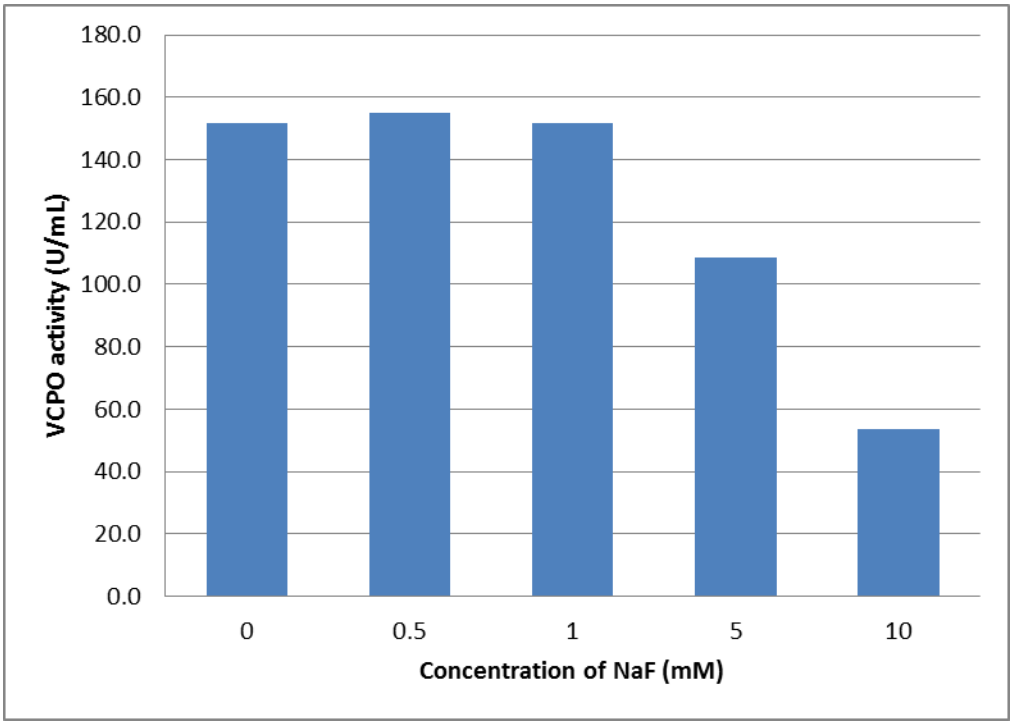


Appendix 14: HPLC chromatograms for the stability test of malonic acid under different conditions. Reaction time: 60 min.

Appendix IV: MCD assays

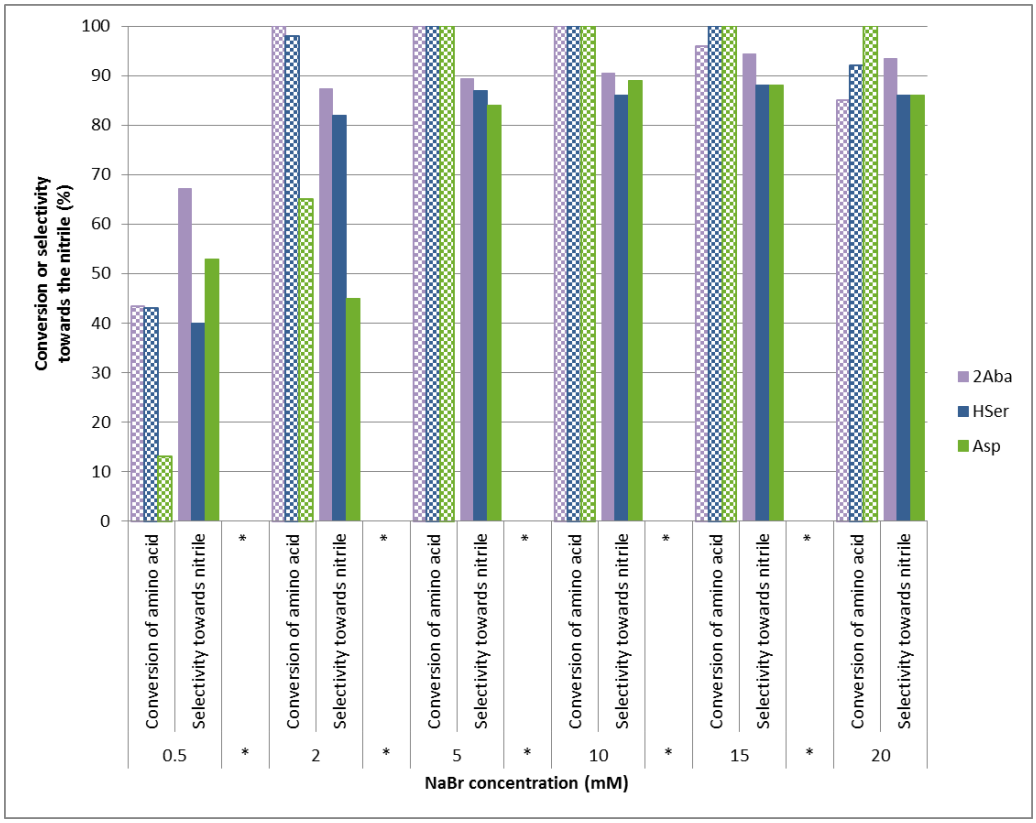


Appendix 15: Calibration line MCD assay for bromide depletion test. UV-absorbance after 25 min at known NaBr concentrations.

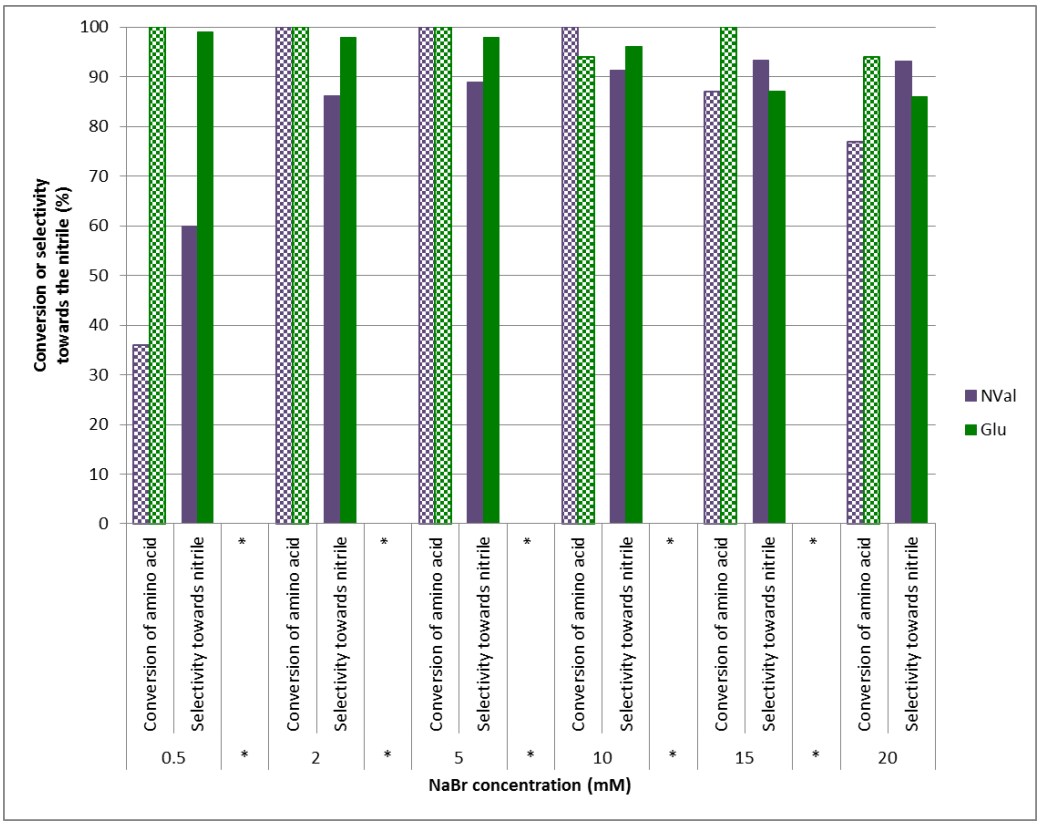


Appendix 16: MCD assay: effect of fluoride on the activity of VCPO.

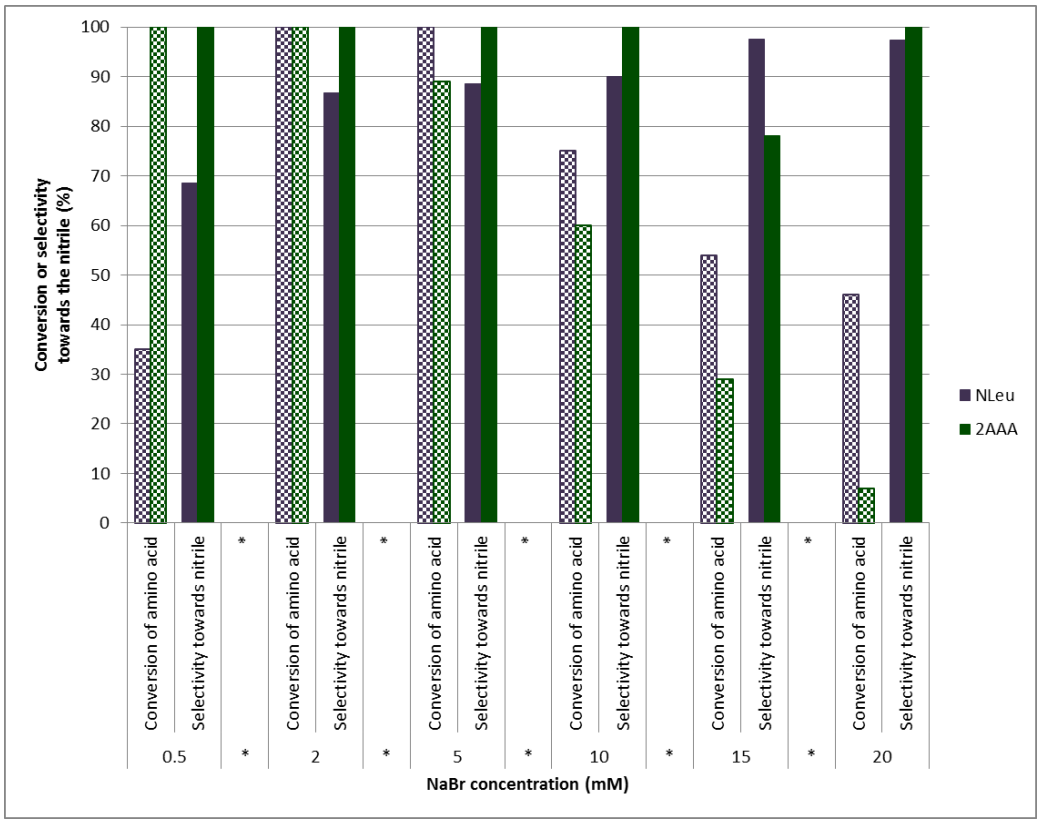
Appendix V: NaBr screening graphs for comparison of side-chain functionality



Appendix 17: Conversion and selectivity towards the nitrile at different NaBr concentrations for 2Aba, HSer and Asp, all containing four carbon atoms. Starting concentration of amino acid: 5 mM. Reaction time: 60 min.



Appendix 18: Conversion and selectivity towards the nitrile at different NaBr concentrations for NVal and Glu, both containing five carbon atoms. Starting concentration of amino acid: 5 mM. Reaction time: 60 min.



Appendix 19: Conversion and selectivity towards the nitrile at different NaBr concentrations for NLeu and 2AAA, both containing six carbon atoms. Starting concentration of amino acid: 5 mM. Reaction time: 60 min.