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Nitrosation and Analysis of Amino Acid Derivatives by Isocratic HPLC

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Abstract

The objective of this study was to characterize the nitrosation of the classical amino acids by N_2O_3. Nitrosation of amino acids results in the formation of mainly α-hydroxy-acids that are suitable for isocratic HPLC analysis and subsequent quantification of amino acids in biological samples. The method is particularly suitable for detection of amino acids in e.g. fermentation media as the α-hydroxy-acids can be quantified in parallel to a variety of other organic substrates and products. The amino acids were transformed into their corresponding α-hydroxy-acids in acidic KNO_2 solutions. The reactions were terminated by NaOH addition and the α-hydroxy-acids separated by isocratic HPLC and quantified by refractive index or UV absorption detection. Nitrosation of 18 of the classical amino acids; glycine, L-alanine, L-valine, L-leucine, L-isoleucine, L-methionine, L-serine, L-threonine, L-asparagine, L-glutamine, L-aspartic acid, L-glutamic acid, L-proline, L-cysteine, L-phenylalanine, L-lysine, L-tyrosine, and L-tryptophane formed detectable nitrosation products. L-lysine, however, needed incubation in 96 mM formic acid to produce a detectable product, while L-phenylalanine had to be incubated in120 mM HNO_3 and 100 mM HCl. Optimal reaction conditions for most amino acids included 40 min of incubation of up to 5 g L^{-1} amino acid in 160 mM KNO_2 in 100 mM HCl at 45°C to maximize product yields.

Keywords*:* van Slyke reaction; nitrosation; dinitrogen trioxide; α-hydroxy acids; analysis

1. Introduction

Primary amino acids can be transformed into their corresponding α-hydroxy acids by nitrosation of their amino group by dinitrogen trioxide, N_2O_3, formed from nitrite under acidic conditions. The reaction progresses via the intermediate formation of firstly a nitrosamine and secondly a diazonium group.[1-4] The diazonium group is released as N_2 via an intramolecular lactone formation with a carboxyl group.[5] The reaction can be stopped by addition of NaOH and the lactones are hydrolysed into α-hydroxy acids.[6] The reaction is named after van Slyke who measured the release of N_2 to quantify peptide concentrations in biological samples.[7]

Pleissner et al. demonstrated that the α-hydroxy acids formed by the van Slyke reaction could be used to quantify individual amino acids in e.g. fermentation media.[6] After nitrosation with nitrite, the α-hydroxy acids were subjected to isocratic separation on an Aminex (Bio-Rad) column under acidic conditions followed by refractive index (RI) detection. This is one of the most versatile analytical methods for quantification of small biological molecules. On the same column, disaccharides, hexoses, pentoses, organic acids, ketones, alcohols, polyols and more different molecules can be separated and simultaneously quantified in the same sample. The method is well established in microbial biotechnology[8] and has been used to quantify substrate uptake and metabolite synthesis in cultures of bacteria,[9,10] filamentous fungi,[11,12] and microalgae.[13,14] The method has also been used to analyse metabolites in e.g. foods,[15] beverages,[16,17] human body fluids,[18,19] and plant extracts.[20] The separation of individual molecules is, however, restricted to uncharged molecules and therefore cannot be used directly to analyse amino acids as these will be positively charged in the acidic mobile phase. In contrast will α-hydroxy acids be neutral at low pH. Nitrosation of samples prior to analysis therefore enables simultaneous quantification of amino acids along with other small molecules in one sample. So far has this method[6] been used to quantify the amino acids, glycine, L-alanine, and L-glutamic acid after transformed into their corresponding α-hydroxy acids, glycolic acid,

lactic acid, or α-hydroxyglutaric acid, respectively, in parallel to glucose, acetic acid, and phosphate (measured as phosphoric acid) in cultures of the dinoflagellate *Crypthecodinium cohnii*, a heterotrophic microalga that depends on organic nitrogen sources.[6,21]

Reactions between amino acids and nitrosating agents may deviate from the course of reaction described above. Proline is a secondary amine and forms a stable nitrosamine upon reaction with N_2O_3.[1] For some of the other amino acids more than one product is formed in reactions with N_2O_3. Glycine reacts for example with nitrous acid via different parallel reactions[22] forming glycolic acid and one additional product.[6] N_2O_3 may also cause nitration of the ring structure in aromatic amino acids.[23] L-tryptophane can be nitrated at several positions on the aromatic ring[24] and 5 products from nitrosation of histidine have previously been identified.[25] In cysteine is also the sulfhydryl group a target for nitrosation in a reaction with NO^+.[26] The end product of the reaction between N_2O_3 and glutamic acid is α-hydroxyglutaric acid under alkaline conditions while it is a γ-lactone under acidic additions.[6, 27] Similarly is aspartic acid converted into malic acid at low pH, while probably a β–lactone is the end product under alkaline conditions.[6] Therefore have only 13 of the 20 classical amino acids so far been transformed by nitrite into products that are detectable by RI detection and applicable for amino acid quantification.[6]

Nitrosation of the different amino acids by nitrite is not yet well characterised and kinetic models of the nitrosation reactions that can be used to evaluate and optimise the reaction conditions are still lacking. Spectrophotometric quantification of N_2O_3 concentrations in reaction mixtures has demonstrated that nitrosation of a number of amino acids are 3'order reactions, being 1' and 2'order with respect to amino acid and nitrite concentrations, respectively.[28] If, however, the initial concentration of nitrite is substantially higher than the amino acid concentration, as would be desirable when the purpose of the reaction is the rapid formation of a detectable nitrosation product, the course of the nitrosation reaction can expectantly be described by a pseudo 1'order expression

$$-r = k' \cdot c_{aa} \tag{1}$$

where *-r* is the reaction rate, *k'* is a pseudo 1'order rate constant, and c_{aa} is the concentration of amino acid. The magnitude of *k'* depends on the 3'order rate constant for the particular amino acid and the concentration of nitrite. If reactions are performed in the presence of nucleophilic species, additional nitrosating agents may be formed. Chloride, for example, forms nitrosyl chloride in presence of nitrite. These nitrosating agents act as catalysts and increase the reaction rates.[29-31] In batch reactions, the concentration of amino acid will then decrease exponentially

$$c_{aa} = c_0 \cdot e^{-k' \cdot t} \tag{2}$$

where c_0 is the initial amino acid concentration and *t* is the time of the reaction. The amino acid concentration, c_{aa}, is related to the degree of conversion of the amino acid, *X*

$$c_{aa} = c_0(1 - X) \tag{3}$$

Since only the concentration of α-hydroxy acid (or other detectable products), c_p, is quantified experimentally,[6] the right hand terms of Eqs. 2 and 3 are combined, *X* is isolated, and c_p is described by

$$c_p = Y \cdot c_0 \cdot X = Y \cdot c_0\left(1 - e^{-k' \cdot t}\right) \quad (4)$$

where Y represents the yield of detectable product relative to the amount of amino acid converted. Y is expected to have a value of 0-1 and may depend on the amino acid, the product, and the reaction conditions. Before reactions are started by HCl addition, $X = 0$. Reactions are completed when all amino acids are consumed[7] and the degree of conversion at this point, X_∞ will approach a value of 1.

In this study, we have characterized and evaluated the reactions between the 20 classical amino acids and nitrite in hydrochloric acid, including reaction conditions, product yields, and their pseudo 1'order reactions kinetics. We have also modified the prior analytical protocols and increased the number of classical amino acids that provide detectable nitrosation products from 13 to a total of 18.

2. Materials and Methods

2.1. Reaction conditions

Reactions were, in most cases, carried out in 2.5 mL polypropylene tubes (Eppendorf) at 45°C for 40 min, containing 1 mL of amino acid solution (0-5 g L^{-1}) mixed with 0.2 mL of 1 M KNO_2. Reactions were started by addition of 0.04 mL 3 M HCl and stopped by addition of 0.2 mL 2 M NaOH. The reaction mixtures contained 0-66.6 mM amino acid, 160 mM KNO_2, and 100 mM HCl. Deviations from this protocol are described in Results and Discussion.

2.2. HPLC analysis of α-hydroxy acids and metabolites

All α-hydroxy acids and other products produced by reactions with N_2O_3 were analysed using isocratic HPLC: 25 µL of reaction mixture was added on an Aminex HPX-87H column (Bio-Rad) and eluted

with 0.4 mL min^{-1} of 5 mM H_2SO_4 at 27°C. Detection was performed by a Knauer K-2300 RI detector at room temperature as well as by a Shimadzu SPD-10A UV/VIS detector.

2.3. Determination of gas evolution

Gas formation from nitrosation of amino acids was measured by adding 50 mL of 10 g L^{-1} amino acid solution into a 100 mL glass flask placed on a magnetic stirrer. The flask was sealed by a lid with 3 ports. Two ports fitted with on-off valves were used to add 10 mL of 1 M KNO_2 as well as 0.5-2 mL of 3 M HCl to start reactions and to equilibrate the pressure in the flask at the time of HCl addition. Through the third port, a Teflon tubing (i.d. = 2 mm) provided a connection to the head-space of a second, 250 mL lidded flask, filled with water. When pressure was build up in the headspace, water was pushed out of the second flask via a second Teflon tubing and into a 100 mL glass measuring cylinder where the volume of displaced water was read. Reactions were carried out at room temperature and the gas equation was used to estimate the amount of produced gas from the volume of displaced water.

2.4. Reaction analysis

For three amino acids, glycine, L-alanine, and L-aspartic acid were their corresponding α-hydroxy acids, glycolic acid, lactic acid, and malic acid, available in pure form and used to prepare standard solutions of known concentrations. The concentrations of these α-hydroxy acids produced by nitrosation of glycine, L-alanine, or L-aspartic acid were quantified in reaction mixtures by HPLC by comparison of their respective peak areas to those of the corresponding standard solutions. Yields of these α-hydroxy acids, *Y*, were determined as

$$Y = \frac{c_p}{c_0 \cdot X_\infty} = \frac{c_p}{c_0} \tag{5}$$

where c_p was measured after reactions had been completed ($X_\infty \approx 1$).

Since most of the α-hydroxy acids produced from nitrosation of amino acids were not available in pure form, the course of nitrosation reactions were described by a modified version of Eq. 4

$$A = Y_A \cdot c_0\left(1 - e^{-k' \cdot t}\right) \tag{6}$$

where product concentration is replaced by product peak area in chromatograms, A, and product yield is replaced by the yield in terms of product peak area, Y_A. Pseudo 1'order rate constants, k' for the nitrosation of the amino acids were estimated by fitting Eq. 6 to experimentally determined peak areas by simultaneous optimization of the values of k' and Y_A in order to obtain the smallest overall discrepancy (evaluated as root mean square error) between Eq. 6 and the measured peak areas.

The time needed for 90% conversion of amino acids into their respective nitrosation products, τ_{90} was calculated as

$$\tau_{90} = \frac{\ln\left(\frac{Y_A \cdot c_0 \cdot X_\infty - 0.9 Y_A \cdot c_0}{Y_A \cdot c_0 \cdot X_\infty}\right)}{-k'} = \frac{\ln 0.1}{-k'} \tag{7}$$

as $X_\infty \approx 1$. Reactions were subsequently carried out for periods of time longer than τ_{90} to allow the formation of maximal concentrations of detectable products in the reaction mixtures.

3. Results and discussion

3.1. Detection of amino acid derivatives

We optimized reaction conditions for nitrosation of amino acids in solutions of HCl and KNO_2, and were able to reproduce RI detectable products from the same 13 amino acids as Pleissner et al.,[6] i.e. glycine, L-alanine, L-valine, L-leucine, L-isoleucine, L-methionine, L-serine, L-threonine, L-asparagine, L-glutamine, L-aspartic acid, L-glutamic acid, and L-proline. Also nitrosation of L-cysteine resulted in a product that was detected by RI at 28.7 min.

Nitrosation of the aromatic amino acids, L-tyrosine and L-tryptophane resulted in brownish products as they were nitrated at the aromatic ring.[23] At least 3-4 products were formed from both amino acids, as would also be expected.[24] Products from nitrosation of L-tyrosine eluted after 9.9, 20.2, 25.5, and 34.7 min, respectively and were detectable by UV absorption at 310-410 nm although their absorption maxima differed. Only the products eluting at 9.9 and 34.7 min were also seen in RI chromatograms. Three products from L-tryptophane were seen in UV chromatograms recorded at 310 nm after 4.3, 26.1, and 30.3 min, respectively, but not in RI chromatograms. The yields of the different products depended on the reaction conditions as well as the time of incubation.

Nitrosation of L-lysine, L-phenylalanine, L-arginine, and L-histidine in HCl and KNO_2 did not give products that were detectable by neither RI nor UV-absorption at wavelengths higher than 310 nm. However, when 120 mM HNO_3 was included in the reaction mixture, a product from L-phenylalanine was detectable by RI at 10.7 min. The product developed a brownish colour suggesting that modifications of the aromatic part of the molecule had taken place. Most likely was one or more nitro-groups added to the aromatic ring by HNO_3 [32] in a parallel reaction to the nitrosation of the amino

group by N_2O_3. When HCl was replaced by formic acid, a product from L-lysine was detectable by RI at 24.6 min. It is possible that the amino group at the ε-position in lysine is formylated[33] and the amino group on the α-position then undergoes nitrosation and produces a detectable product. In the end, it was therefore only from L-arginine and L-histidine we did not find nitrosation products that were detectable by the HPLC analysis.

3.2. Acid concentration

The acidity of the reaction mixture affects the yield of products from nitrosation of amino acids. In most experiments, we used HCl to create low pH values and start the nitrosation reactions. Product yields in terms of peak areas from 18 amino acids after 40 min of reaction at 45°C with 100 mM KNO_2 in different concentrations of HCl (except L-lysine in formic acid and L-phenylalanine in HCl and HNO_3) are seen in Fig. 1. For all the amino acids, 50-100 mM acid was needed to maximize product yields. HCl concentration higher than 100-200 mM had a negative effect on the product yields from glycine, L-alanine, L-valine, L-leucine, L-proline, the aromatic amino acids, L-phenylalanine, L-tyrosine, and L-tryptophane, and L-lysine. Product formation from the remaining amino acids, all carrying different functional groups that may favour the release of N_2 and formation of α-hydroxy acids, a hydroxyl group in L-serine and L-threonine, a carboxylic acid group in L-aspartic acid and L-glutamic acid, a carboxamid group in L-asparagine and L-glutamine, a thiol group in L-cysteine, and a thio-ether group in L-methionine, was unaffected by high concentrations of HCl. Subsequent nitrosation reactions were therefore carried out in 100 mM HCl (L-lysine in 96 mM formic acid, L-phenylalanine in 100 mM HCl and 120 mM HNO_3) where product yields from all the amino acids were close to maximal. This concentration is 4 times below the HCl concentration of 400 mM used by Pleissner et al.[6] The lower HCl concentration had no apparent influence on which products that were

formed, but resulted in higher yields of the detectable products used in the analysis and with retention times shown in Table 1.

Nitrosation of amino acids have previously been carried out in different acid solutions. The original nitrosation procedure used by van Slyke[7] used acetic acid while other authors used nitrous acid,[34] sulphuric acid,[32] perchloric acid,[26,35] butyric acid,[6] or HCl.[6,36] We used HCl. Organic acids may interfere with the HPLC analysis as their retention times may be similar to some of the α-hydroxy acids or other products formed from nitrosation of the amino acids. Chloride ions from HCl are not retained by the column and will therefore not elute at the same time as the nitrosation products. The chloride ions may also form nitrosyl chloride and speed up reaction rates[29-31] allowing for shorter incubation times.

3.3. Initial KNO_2 concentration

A surplus KNO_2 concentration is needed for complete transformation of amino acids into α-hydroxy acids or other nitrosation products and is a prerequisite for using pseudo 1'order kinetics to describe reaction rates (Eq. 1) and product concentrations in the reaction mixtures (Eq. 4). Product yields from 18 amino acids after 40 min of incubation at 45°C in 100 mM HCl (96 mM formic acid for L-lysine, 100 mM HCl and 120 mM HNO_3 for L-phenylalanine) and 0 to 194 mM KNO_2 are shown in (Fig. 2). Nitrosation products were detected at initial KNO_2 concentrations above 23-46 mM. Product yields increased at increasing KNO_2 concentrations up to 88-125 mM. Higher KNO_2 concentrations resulted in no further increases in product yields. Based on the results in Fig. 2, subsequent nitrosation reactions were carried out at an initial KNO_2 concentration of 160 mM. The yields of all nitrosation products detectable by RI were close to maximal at this initial KNO_2 concentration.

Nitrite itself gave rise to a peak in the chromatograms at 16.4 min, probably created by N_2O_3, reformed from unused nitrite in the acidic mobile phase. When initial KNO_2 concentrations had been

above 25 mM, excess nitrite was still present in all reaction mixtures after the reactions had been stopped.

3.4. Reaction rates and incubation time

Product formation from nitrosation of 18 amino acids in reaction mixtures incubated 0-110 min at 45°C in 160 mM KNO_2 and 100 mM HCl (96 mM formic acid for L-lysine, 100 mM HCl and 120 mM HNO_3 for L-phenylalanine) is shown in Fig. 3. Eq. 6 was fitted to these data in order to model the progression of the reactions and estimate *k'* and τ_{90} (Table 1). For 16 amino acids, τ_{90} was between 3 and 24 min while longer incubation periods were needed to maximise the degree of conversion of the aromatic amino acids, L-tyrosine and L-tryptophane. Some of the nitrosation products from the aromatic amino acids were not stable, but rather intermediate products (Fig. 3C), and therefore less suitable for use as a measure of the initial amino acid concentration.

With the exception of L-tyrosine and L-tryptophane, the nitrosation of all amino acids were almost completed (*X* well above 0.9) after 30 min of incubation. Subsequent nitrosation reactions were therefore carried out for 40 min before stopped by the addition of NaOH to ensure almost complete conversion of the amino acids. When Pleissner et al. followed the nitrosation of glycine and L-alanine at 45°C in 160 mM KNO_2 and 370 mM butyric acid the reactions were not completed after 120 min.[6] Reactions therefore do progress at higher rates in the presence of chloride ions, as also expected.[29-31]

3.5. Yields of α-hydroxy acids

According to the van Slyke reaction,[1-5] the nitrosation of glycine, L-alanine, and L-aspartic acid will result in the formation of the α-hydroxy acids, glycolic acid, lactic acid or malic acid, respectively. Chromatograms of the same α-hydroxy acids in standard solutions confirmed their identities as products in the reaction mixtures and were used to determine their concentrations. Fig. 4 shows

concentrations of glycolic acid, lactic acid or malic acid after 40 min of nitrosation at 45°C in 160 mM KNO_2 and 100 mM HCl as function of the initial concentrations of glycine, L-lactic acid or L-aspartic acid, respectively. At this point, these reactions have almost been completed (Fig. 3, $X_\infty \approx 1$), and the slopes of the regression curves (Eq. 5) will represent the yields of the α-hydroxy acids, Y. Nitrosation of these 3 amino acids resulted in 76-87% conversion into their corresponding α-hydroxy acids when performed in 100 mM HCl. L-alanine was transformed into lactic acid with a yield of 87% which is considerably higher than the 73% yield previously obtained in 400 mM HCl.[6] The higher yield is a result of the lower HCl concentration (Fig. 1A).

We also measured the evolution of gas after 60 min of incubation (incubation times were prolonged to better allow time for transfer of dissolved gases into the gas phase) of 10 g L^{-1} L-alanine, glycine, L-glutamic acid, or L-valine in 160 mM KNO_2 and 30, 120 or 480 mM HCl (Fig. 5). Highest gas yields (0.82-1.15 moles of gas per mole of amino acid) evolved from nitrosation reactions taken place in 120 mM HCl. This observation seems to confirm the results in Fig. 4, namely that HCl concentration close to 100 mM favours the van Slyke reaction with the predominant synthesis of α-hydroxy acids and N_2.

All 20 classical amino acids were subsequently screened for gas evolution during nitrosation reactions in 100 mM KNO_2 and 120 mM HCl. Nitrosation of L-proline and L-tyrosine did not result in gas formation (Table 1). L-proline forms a stable nitrosamine without releasing N_2 [1] and L-tyrosine is transformed to nitrotyrosine while its primary amino group is apparently not reacting.[23]

Quit different gas yields, Y_{gas}, were seen from the two amino acids, L-arginine and L-histidine that did not produce detectable nitrosation products (Table 1). Nitrosation of L-arginine resulted in a gas yield of only 0.31 moles of gas per mole arginine, while the gas yield from nitrosation of L-histidine was as high as 1.31 moles of gas per mole histidine, possibly because also its guanidinium group is a potential target for nitrosation.[37] Nevertheless, both amino acids did react with N_2O_3, but if

their products remained charged at low pH, they will not be retained by the column and cannot be separated from other charged species in the reaction mixtures.

Nitrosation of the remaining 16 amino acids resulted in gas yields of 0.73-1.26 mole of gas per mole amino acid. It is not new that nitrosation of some amino acids may result in gas yields greater than 1 mole N_2 per mole amino acid.[34] NO from decomposition of nitrous acid may also contribute to the total gas production, especially at low pH.[35] Still, the gas yields were close to 1 mole of gas per mole amino acid. This indicates that the degree of conversion of most of the amino acids into their corresponding α-hydroxy acids have been high. The reaction conditions seem therefore to have been close to optimal for the conversion of amino acids into their corresponding α-hydroxy acids (or other nitrosation products) that can be separated and subsequently detected individually by the HPLC procedure.

3.6. Standard curves

Quantification of amino acids from RI or UV chromatograms of their corresponding α-hydroxy acids depends on standard curves based on solutions of known concentrations of the amino acids.[6] Although the need for UV detection in order to quantify nitrosation products from L-tyrosine and L-tryptophane reduces the possibility for simultaneous quantification of these two amino acids and other metabolites, UV chromatograms were useful for the characterization of the reactions between N_2O_3 and these two amino acids. The relationships between product peak areas and original amino acid concentrations from 0-5 g L^{-1} (0-2 g L^{-1} for tryptophane) were linear for all the 18 amino acids that were transformed into detectable products. Regression analysis showed that the initial amino acid concentrations explained 97-100% ($r^2 \geq 0.97$) of the recorded peak areas. Since the slopes of the regression curves represent product yields in terms of peak area, Y_A (see Eq. 6), these are only comparable for nitrosation products

detected by the same detector. L-isoleucine gave rise to the highest Y_A, 5.7 times higher than Y_A for L-lysine and is therefore measured with almost 6 times higher sensitivity than L-lysine.

4. Conclusion

We have characterized the nitrosation of amino acid by N_2O_3 in acidic solutions. Quantitative analysis of amino acid derivatives after reaction with N_2O_3 by isocratic HPLC and RI detection allowed quantification of 14 out of the 20 classical amino acids when up to 5 g L^{-1} amino acid is reacted with 160 mM KNO_2 in 100 mM HCl at 45°C for 40 min, i.e. glycine, L-alanine, L-valine, L-leucine, L-isoleucine, L-methionine, L-serine, L-threonine, L-asparagine, L-glutamine, L-aspartic acid, L-glutamic acid, L-proline, and L-cysteine. When HCl was replaced by formic acid or HNO_3 included in the reaction mixture, also L-phenylalanine and L-lysine formed nitrosation products that were retained and could be used to quantify also these amino acids. Multiple products from nitrosation of L-tyrosine and L-tryptophane could be detected by UV absorption and used to quantify also these two amino acids.

Versatile analytical procedures for quantification of amino acids are essential. A number of derivatization procedures are already well established, which add chromophores onto the amino acids either before or after chromatographic separation allowing for UV absorbance or fluorescence detection.[38] Nitrosation of amino acids results in the formation of products that can be separated by isocratic chromatographic principles compatible with RI quantification and supplements thereby existing analytical procedures. The established methodologies, as well as novel chromatographic methods based on MS/MS detection of either amino acids[39] or amino acid derivatives[40] may offer higher sensitivities than can be achieved by RI-detection.[41] Amino acids are, however, regularly present in more than sufficient concentrations for RI quantification, e.g. in microbial cultures where they in combination with other organic molecules may be used as substrates.[6,21] The nitrosation of amino acids

into their corresponding α-hydroxy acids and subsequent HPLC analysis have so far therefore been a particularly useful analytical procedure in microbial cultivations for simultaneous quantification of amino acids, additional organic substrates, and metabolic products.

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Table **1**. The 20 classical amino acids. Principle for detection of corresponding α-hydroxy acids (refractive index, RI, UV absorption, UV, retention time, RT, pseudo 1'order rate constant, *k'*, time needed for 90% conversion of amino acids into their respective nitrosation products, τ_{90}, yield of α-hydroxy acid, *Y*, pH of reaction mixtures, gas yield, Y_{gas}, initial amino acid concentrations resulting in confirmed linear standard curves from nitrosation reactions, slopes of standard curves, Y_A, coefficient of determination for standard curves, r^2. Reactions were carried out at 45°C in 160 mM KNO_2 and 100 mM HCl.

Amino acid	Detector	RT min	k' min^{-1}	τ_{90} min	Y	pH	Y_{gas}	c_o $g\ L^{-1}$	Y_A $L\ g^{-1}$	r^2
Glycine	RI	12.4	0.34	6.9	0.78	2.7	1.03	0-5	213.2	0.9996
L-alanine	RI	12.4	0.10	23.9	0.87	2.8	0.89	0-5	239.3	0.9998
L-valine	RI	18.2	0.21	11.0	-	2.8	0.99	0-5	264.3	0.9987
L-leucine	RI	26.7	0.17	13.8	-	2.7	1.12	0-5	301.2	0.9837
L-isoleucine	RI	25.5	0.25	9.1	-	2.7	1.06	0-5	315.7	0.9975
L-serine	RI	10.0	0.25	9.4	-	2.7	1.13	0-5	261.5	0.9977

L-threonine	RI	10.7	0.38	6.1	-	2.7	1.18	0-5	255.3	0.9991
L-methionine	RI	22.0	0.29	7.8	-	2.7	1.11	0-5	176.0	0.9945
L-cysteine	RI	28.7	0.47	4.9	-	2.1	1.16	0-5	82.4	0.9987
L-aspartic acid	RI	8.4	0.32	7.2	0.76	2.7	0.92	0-5	279.6	0.9969
L-glutamic acid	RI	10.0	0.30	7.6	-	2.1	1.25	0-5	98.6	0.9954
L-aspargine	RI	11.8	0.81	2.9	-	2.7	1.23	0-5	297.2	0.9993
L-glutamine	RI	10.0	0.36	7.1	-	2.7	1.11	0-5	120.6	0.9959
L-proline	RI	20.2	0.14	16.5	-	2.7	0.00	0-5	247.4	0.9987
L-phenylalanine[a]	RI	10.6	0.10	22.2	-	2.7	1.18	0-5	313.3	0.9656
L-tyrosine	UV	20.0	0.03	82.6	-	2.5	0.00	0-5	40.9	0.9761
L-tryptophane	UV	30.2	0.02	102.2	-	2.7	1.03	0-2	119.2	0.9954
L-lysine[b]	RI	24.4	0.07	34.6	-	3.8	0.73	0-5	55.2	0.9920
L-arginine	-	-	-	-	-	3.2	0.33	-	-	-
L-histidine	-	-	-	-	-	3.3	1.31	-	-	-

Legends to figures

Fig. 1. Product peak areas of nitrosation products from 18 amino acids after 40 min of reaction with 160 mM KNO_2 at 45°C in different acid concentrations, i.e. HCl if other acids are not specified. Initial amino acid concentrations were 2 g L^{-1}. A. glycine □, L-alanine ○, and L-valine ◊. B. L-leucine □, L-isoleucine ○, and L- serine ◊. C. L-threonine □, L-methionine ○, and L-cysteine ◊. D. L-aspartic acid □, L-glutamic acid ○, and L-asparagine ◊, L-glutamine Δ. E. L-proline □, L-phenylalanine in 100 mM HCl and 120 mM HNO_3 ○, and L-tyrosine ◊. F. L-tryptophane □, L-lysine in 96 mM formic acid ○.

Fig. 2. Product peak areas of nitrosation products from 18 amino acids after 40 min of reaction at 45°C in 100 mM HCl if other acids are not specified, and in different concentrations of KNO_2. Initial amino acid concentrations were 2 g L^{-1}. A. glycine □, L-alanine ○, and L-valine ◊. B. L-leucine □, L-isoleucine ○, and L- serine ◊. C. L-threonine □, L-methionine ○, and L-cysteine ◊. D. L-aspartic acid □, L-glutamic acid ○, and L-asparagine ◊, L-glutamine Δ. E. L-proline □, L-phenylalanine in 100 mM HCl and 120 mM HNO_3 ○, and L-tyrosine ◊. F. L-tryptophane □, L-lysine in 96 mM formic acid ○.

Fig. 3. Product peak areas of nitrosation products from 18 amino acids after reaction with 160 mM KNO_2 at 45°C in 100 mM HCl if other acids are not specified, after different times of incubation. Initial amino acid concentrations were 2 g L^{-1}. A. glycine □, L-alanine ○, and L-valine ◊. B. L-leucine □, L-isoleucine ○, and L- serine ◊. C. L-threonine □, L-methionine ○, and L-cysteine ◊. D. L-aspartic acid □, L-glutamic acid ○, and L-asparagine ◊, L-glutamine Δ. E. L-proline □, L-phenylalanine in 100 mM HCl and 120 mM HNO_3 ○, and L-tyrosine ◊. F. L-tryptophane product peak with RT = 30.2 min □ and intermediate product peak with RT = 26.2 min Δ, L-lysine in 96 mM formic acid ○.

Fig. 4. Relationship between final concentrations of the α-hydroxy acids, glycolic acid □, lactic acid ○, or malic acid ◊, and initial concentrations of their corresponding amino acids, glycine, L-alanine, and L-aspartic acid, respectively. Reactions were incubated for 40 min at 45°C in 160 mM KNO_2 and 100 mM HCl. Slopes of regression lines correspond to yields, *Y* of α-hydroxy acid (Table 1).

Fig. 5. Gas yields from nitrosation of L-alanine, glycine, L-valine, and L-glutamic acid after 60 min of incubation at 45°C in 160 mM KNO_2 and 3 concentrations of HCl.

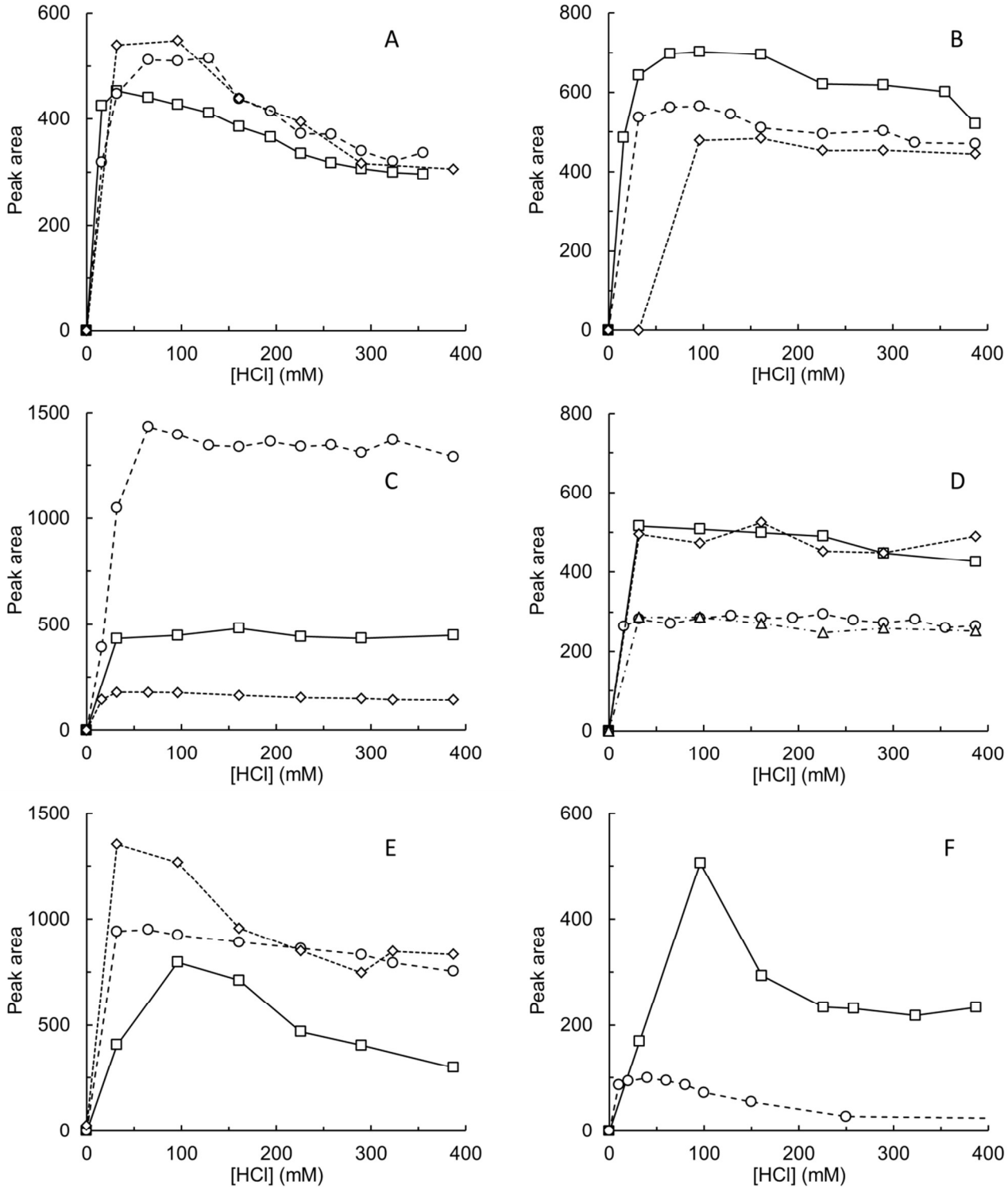


Fig. 1.

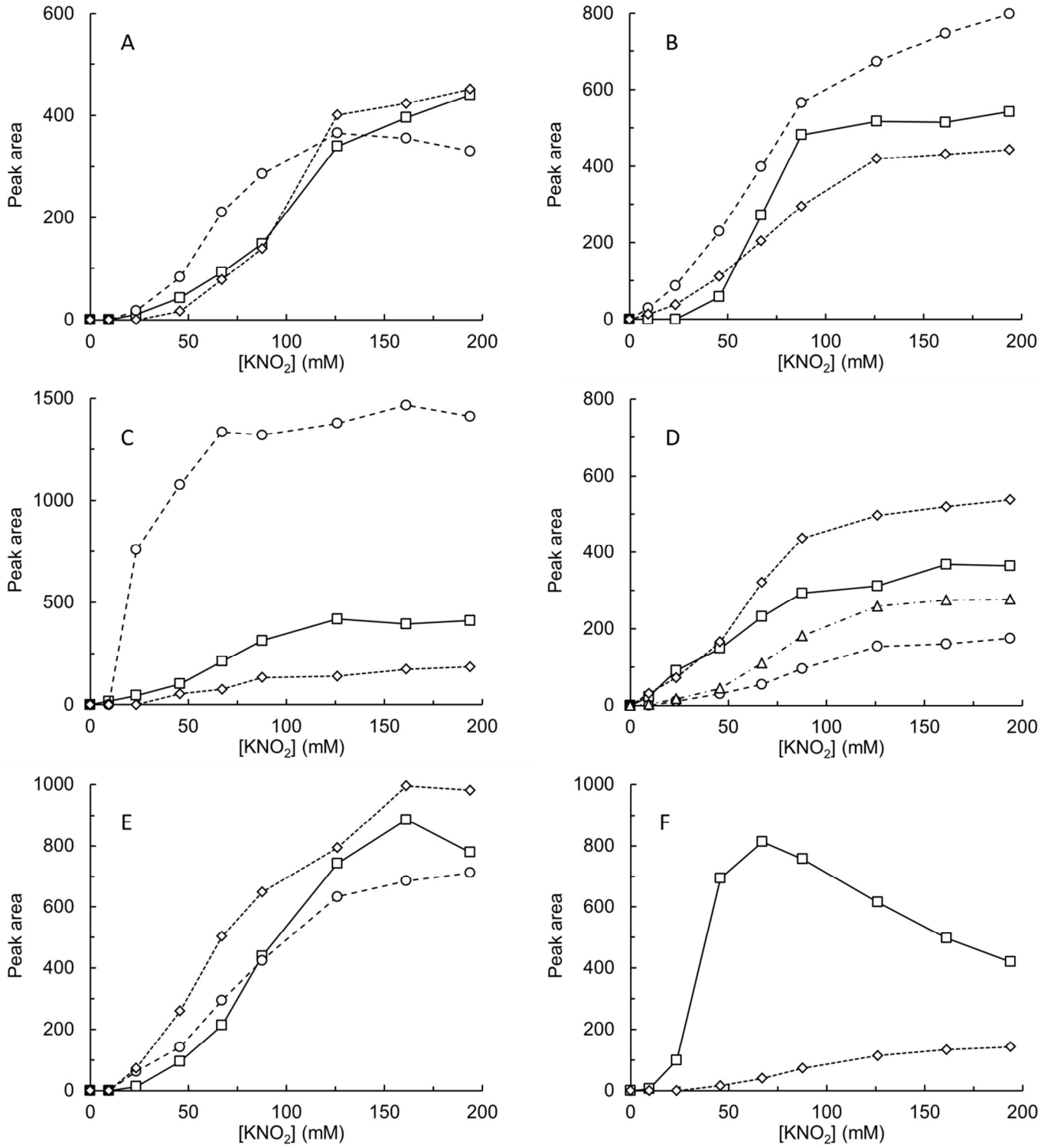


Fig. 2

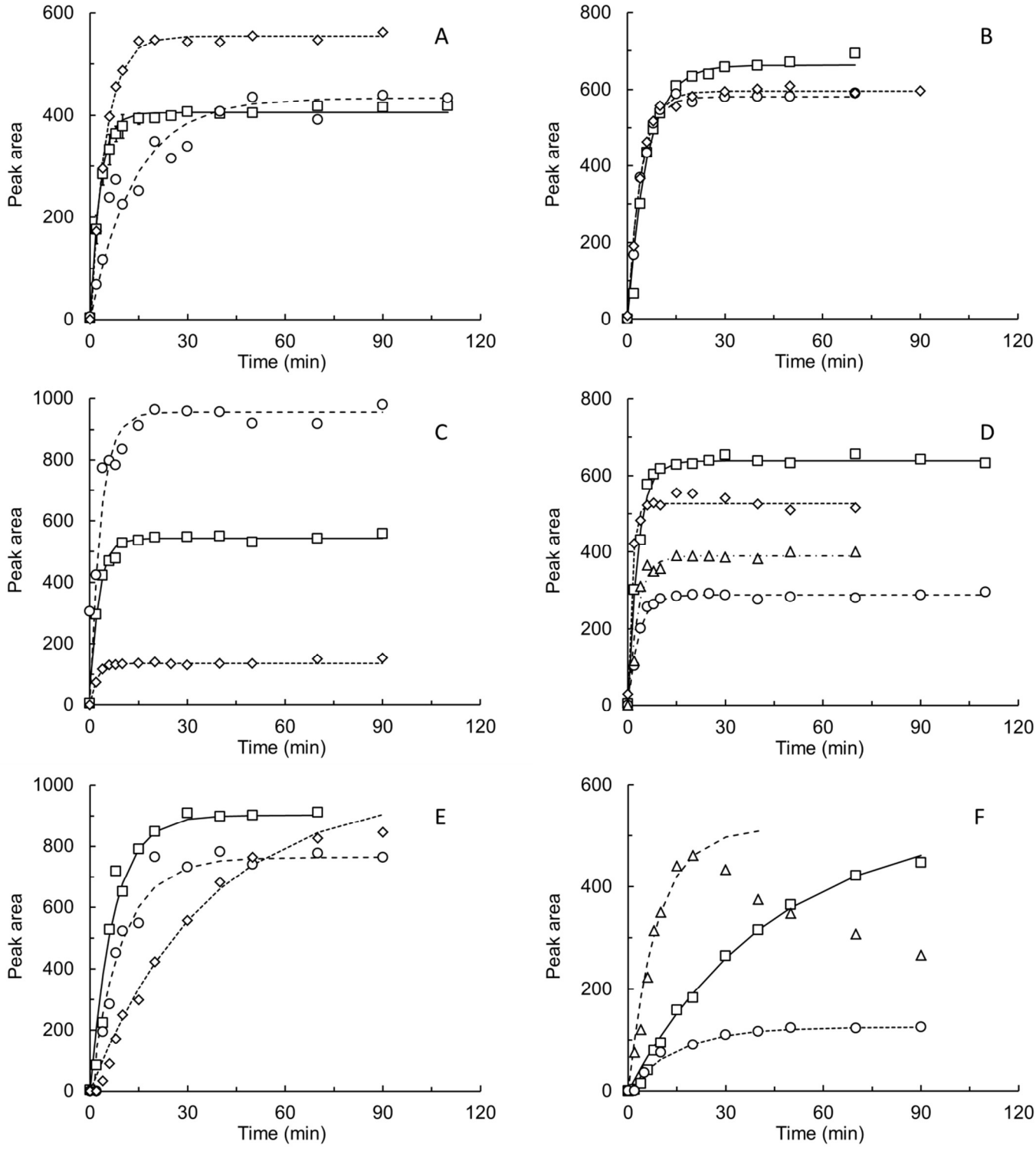


Fig. 3

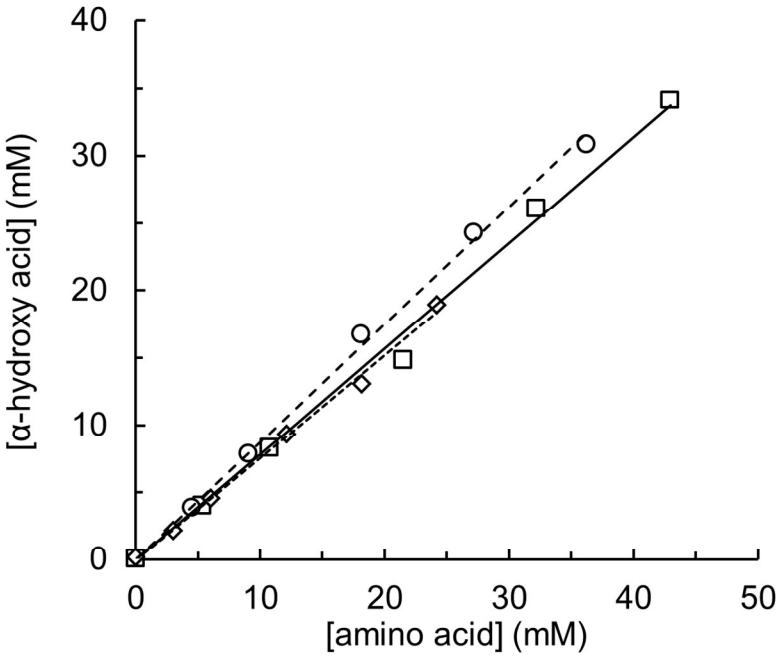


Fig. 4

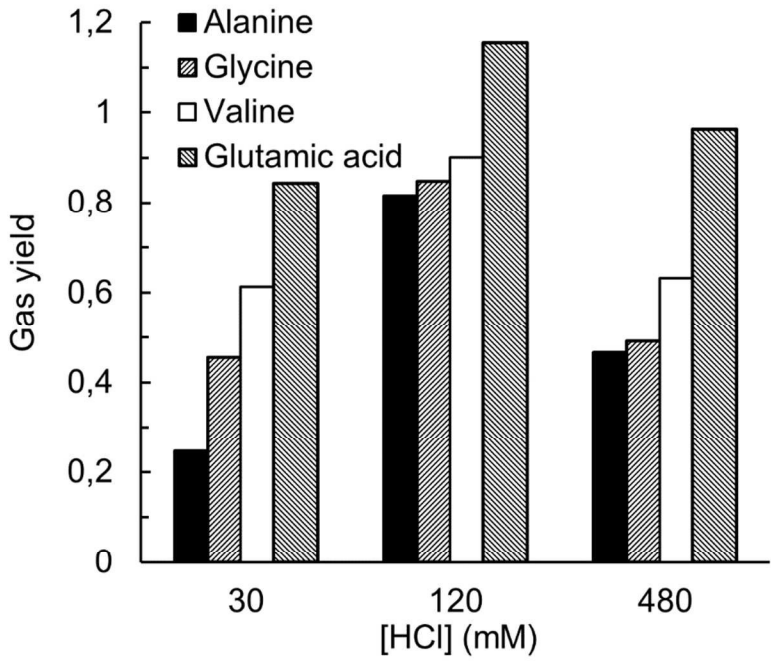
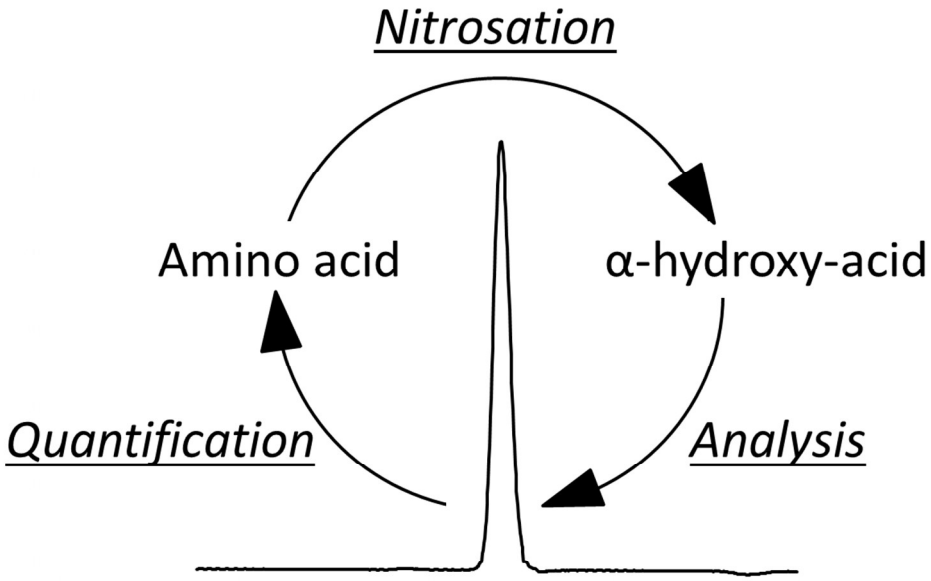


Fig. 5



Graphical abstract.

Amino acids are transformed by nitrosation with dinitrogen trioxide into their corresponding α-hydroxy acids, which are separated and analysed by HPLC, and used to quantify the original amino acid concentration in samples.