

Nitrogen fertilisation increases biogenic amines and amino acid concentrations in *Vitis vinifera* var. *Riesling* musts and wines

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Abstract

BACKGROUND: Wines rich in biogenic amines can cause adverse health effects to the consumer. Being nitrogen-containing substances, the amount of amines in wines might be strongly influenced by the rate of nitrogen fertiliser application during grape production. The aim of this work was to evaluate the effect of nitrogen fertilisation in the vineyard on the formation of biogenic amines in musts and wines.

RESULTS: In a field experiment which compared unfertilised and fertilised (60 and 150 kg N ha⁻¹) vines over two separate years, the total amine concentrations in must and wine increased. The latter was due to an increase of individual amines such as ethylamine, histamine, isopentylamine, phenylethylamine and spermidine in the musts and wines with the nitrogen application. Furthermore, the fermentation process increased the concentration of histamine and ethylamine in most of the treatments, while spermidine, spermine and isopentylamine concentrations generally decreased. Throughout both vintages, the concentrations of tyramine and histamine of the investigated musts and wines never reached detrimental levels to the health of non-allergenic people.

CONCLUSIONS: Nitrogen fertilisation has a significant effect on amines formation in musts and wines. Furthermore, during fermentation, ethylamine and histamine increased while other amines were presumably serving as N sources during fermentation.

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Keywords: phenylethylamine; histamine; ethylamine; amino acids; nitrogen fertilisation; Riesling

INTRODUCTION

Biogenic amines are low-molecular-weight organic bases, which might reduce the tolerability of foods, especially of alcoholic beverages.¹ Amines have aliphatic, aromatic or heterocyclic structures and are formed mainly by the decarboxylation of amino acids in foods.² Besides decarboxylation, other synthesis pathways are the amination or transamination of aldehydes and ketones.³ The wine production process is currently also the focus of several other studies, since biogenic amines are especially found in food products which are strongly influenced by microbiological processes during their manufacture.^{4,5}

After the ingestion of the cyclic amines histamine (HIN), phenylethylamine (PEA) and tyramine (TRN), allergy-sensitive people can develop various symptoms such as headache, nausea, vomiting, diarrhoea, skin rashes, migraine and hypotension or hypertension. The aliphatic amines cadaverine (CAD) and putrescine (PUT), on the other hand, are known to potentiate the toxicity of other amines.⁶ The concentrations in which TRN triggers incompatibilities can vary: according to Soufleros *et al.*⁷ 25–40 ppm TRN in foods is critical. Lüthy and Schlatter⁸ reported that the ingestion of 5 mg of PEA administered in apple juice caused volunteers participating in their study to subsequently complain of headaches, nausea and dizziness. However, in the same study,

the ingestion of the same amount of PEA by the consumption of wine caused no significant effect. Although no tolerance limit has been established for PEA, formerly in Switzerland, a tolerance value of 10 mg L⁻¹ HIN in wine existed.⁹

Besides the aromatic and heterocyclic amines, aliphatic monoamines [isopentylamine (IPA) and ethylamine (ETH)], and

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aliphatic polyamines [agmatine (AGM), CAD, 1,3-diaminopropane (DAP), PUT, spermidine (SPD) and spermine (SPN)] can be found in grape must and wine.^{10–12} Aliphatic polyamines are ascribed as having numerous functions in plants in the terms of growth (e.g. cell division, fruit ripening) and stress tolerance;^{2,13} therefore, these amines are already present in berries and grape musts prior to the fermentation process.¹⁰

A number of processes during wine production might cause the formation of biogenic amines in the finished product. Among these processes, N fertilisation has a direct impact on the formation of aliphatic polyamines in grapes.¹¹ In addition to N fertilisation, microorganisms living on grape berries, such as yeasts, moulds and bacteria, may be involved in the synthesis of several amines in the mature berries (e.g. PEA, SPD, TRN, AGM and isobutylamine).¹² Other causes of amine formation are the wine processing steps, during which yeasts and bacteria may decarboxylate amino acids at some stage in the alcoholic and malolactic fermentation. Although previous studies have reported no remarkable amine formation due to alcoholic fermentation, undesirable amine formation such as HIN, TRN and PUT by *Saccharomyces cerevisiae*, which is the most important fermenting microorganism during alcoholic fermentation, cannot be completely excluded.¹⁴ After alcoholic fermentation, most red (but also some white wines) will pass through malolactic fermentation. Nowadays, this process can be better controlled by inoculation with commercially available bacterial strains of the species *Oenococcus oeni*, which are selected because they produce low amounts of HIN, PUT or TRN, in contrast to other bacterial genera (e.g. *Lactobacillus* and *Pediococcus*).¹⁵

The aim of this study was to evaluate the effect of N fertilisation in the vineyard on the spectrum and quantity of biogenic amines. In addition, amino acids were investigated to unravel any possible relationships between the alteration of amines and their precursors during fermentation to the final wines.

EXPERIMENTAL

Experimental set-up

Grapes from a long-term field trial located in the German wine-growing region Rheingau, close to the 50th latitude, were used for the production of musts and wines. The vines (*Vitis vinifera* var. *Riesling*) had been planted in 1977 on a loamy sandy soil. The experiment was started in 1985 with three nitrogen fertilisation treatments of (bud break/after blooming) 0/0 (0 kg N), 0/60 (60 kg N), and 60/90 (150 kg N) kg N ha⁻¹. The fertilisation treatments were continued until 2007. The nitrogen was applied as granular ammonium nitrate (27.5% N) at bud break and after blooming each year. The experiment was set out in a completely randomised design with four replicates on a total area of approximately 6000 m². Pest and disease control (against *Plasmopara viticola* and *Uncinula necator*) and further vineyard management were similar for the whole experimental field.

The present study deals with the vintages 2005 and 2007. The vintage 2006 is not included in the analysis because in that year a high infestation with botrytis bunch rot occurred. In 2005, to avoid the spread of botrytis bunch rot, infested grape bunches were removed from the field. The respective harvest dates were 17 October 2005 and 22 October 2007. The bud break, blooming and veraison for these years were recorded by the German Weather Service (Deutscher Wetterdienst) exemplary for the Rheingau region as being 23 April 2005 and 14 April 2007, 17 June 2005 and 25 May 2007, 13 August 2005 and 3 August 2007, respectively.

Table 1. Meteorological data between bud break and harvest in the two experimental years, 2005 and 2007, recorded by the German Weather Service

Year and month	Rainfall (mm)	Mean temperature (°C)
2005		
April	61.3	11.1
May	51.8	14.5
June	33.5	19.0
July	43.5	20.2
August	41.7	17.4
September	38.3	16.9
October	35.0	12.3
2007		
April	0.3	14.2
May	62.8	16.0
June	48.4	19.0
July	74.7	18.4
August	41.9	18.2
September	39.3	13.9
October	6.3	10.4

The meteorological data were recorded by the German Weather Service (Table 1).

Vinification

Destemmed and crushed grapes were pressed with a commercial pneumatic press (WP 100; Willmes, Lorsch, Germany) without further processing. After a sedimentation time of approximately 24 h at 14 to 16°C, the must was separated from the deposits. The clarified juices were inoculated with a commercial yeast strain [Oenoferm Tipico (Erbslöh, Geisenheim, Germany) in 2005 and Lalvin EC 1118 (Lallemand, Montréal, Canada) in 2007]. No information is available about the potential of these strains to synthesise amines. The fermentation took place at 16 to 18°C in 10-L glass flasks and was controlled by a daily measurement of density using a portable density meter (DMA 35n; Anton Paar, Ostfildern-Scharnhausen, Germany). Once the fermentation was finished, a sulfuring with 50 mg L⁻¹ of SO₂ and a pre-filtration (Seitz K 250; Pall Filtersystems, Bad Kreuznach, Germany) were conducted. The wines were sterile filtered prior to bottling (Seitz EK; Pall Filtersystems, Bad Kreuznach, Germany).

Sample collection, preparation and storage

Soil samples were collected in spring before the annual fertiliser treatment at a depth of 0 to 90 cm for determination of nitrate-N. Leaf samples for nitrogen analysis were collected each year after blooming, from the vines opposite to the first developing bunch of grapes. The leaves were then cleaned with distilled water and dried at 90°C. The sampling of the musts took place after pressing of the grapes and self clarification by sedimentation. Deposits from the musts were separated via filtration through a laboratory filter (Filter N/37; Munktell & Filtrak GmbH, Bärenstein, Germany). The wines were sampled immediately after bottling. The must and wine samples were stored at -18°C.

Extraction and derivatisation of biogenic amines

The sample preparation followed the method of Smith and Davies¹⁶ and Bleser.¹¹ For amine extraction, 2 mL of must and

wine samples were added to 4 mL 0.2 mol L⁻¹ perchloric acid (Merck, Darmstadt, Germany). The samples were stored in the dark for 1 h at 4°C and vortexed every 20 min. During this procedure, biogenic amines are protonated. After extraction, 300 µL of the samples were transferred to reaction tubes (Safe-Lock Tubes 2.0 mL; Eppendorf, Hamburg, Germany) for the derivatisation procedure. Since most biogenic amines neither show a natural UV absorption nor a native fluorescence, a chemical derivatisation is necessary for their detection. This derivatisation took place before the chromatographic separation (pre-column derivatisation) with 1-dimethylamino-naphthalene 5-sulfonic acid chloride (DNS-Cl) as a fluorescent reagent. In the reaction vessel, 400 µL DNS-Cl solution (10 mg DNS-Cl) dissolved in 1 mL of acetone and 200 µL of saturated Na₂CO₃ solution were added to 300 µL of the sample and shaken for 1 h at 60°C in a thermo mixer (Thermo Mixer comfort; Eppendorf) under exclusion of light at 550 rpm. The addition of saturated Na₂CO₃ solution is necessary to create a strong alkaline environment (pH > 9.5). During the pre-column derivatisation, both primary and secondary amines are converted to dansylamines.

A solid phase extraction with the Baker SPE system (J.T. Baker, Deventer, Netherlands) was used for the purification of the samples, whereby the amine-containing organic phase was separated from the aqueous phase (i.e. that containing sugar). Initially, the C18 separation columns of the Baker SPE system had to be conditioned with 100% methanol (gradient grade; Merck), followed by a washing step with bi-distilled water. Subsequently, the sample was placed on the column and was sucked through it with a vacuum pressure of 5–6 bar. After a second washing step with bi-distilled water, the dansylated and purified amines were eluted with 100% methanol (gradient grade; Merck).

Chromatographic system for biogenic amine determination

For the analysis of the biogenic amines, a HPLC system (Agilent 1100 Series; Hewlett-Packard, Böblingen, Germany) was used. Fifteen microlitres of the prepared sample was injected on a C18 column (PerfectSil Target ODS-3 5 µm, 250 × 3.0 mm; MZ-Analysentechnik GmbH, Mainz, Germany). The temperature of the column oven was kept constantly at 35°C during the separation. For the fluorescence detection, the excitation wavelength was set at 254 nm and the emission wavelength at 510 nm (PTM-gain 14).

Chromatographic conditions for biogenic amine determination

The separation of the amines was carried out with modifications according to González de Llano *et al.*¹⁷ using a binary gradient containing acetonitrile (J.T. Baker) as solvent A and Tris buffer (gradient grade; Merck) as solvent B. Tris buffer (0.1 mol L⁻¹) was prepared with bi-distilled water; a pH of 8.5 was adjusted using acetic acid. Finally, the Tris buffer was diluted in the ratio 1:2 with bi-distilled water. The gradient elution program (percentage of solvent A) and flow rates were as follows: 0 min, 25%, 1 mL min⁻¹; 2 min, 50%, 0.3 mL min⁻¹; 12 min, 54.4%, 0.3 mL min⁻¹; 13 min, 55%, 1 mL min⁻¹; 25 min, 60%, 1 mL min⁻¹; 32 min, 60%, 1 mL min⁻¹; 37 min, 100%, 1 mL min⁻¹; 41 min, 100%, 1 mL min⁻¹; 42 min, 25%, 1 mL min⁻¹; 43 min, 25%, 1 mL min⁻¹.

Identification and quantification of biogenic amines

Eleven different biogenic amines (AGM, DAP, CAD, ETH, HIN, IPA, PEA, PUT, SPD SPN and TRN) could be separated within 45 min. The presence of amines was confirmed by comparison with the

retention times of reference compounds. A calibration curve of peak area versus concentration (mg L⁻¹) was calculated for each standard reagent.

Determination of nitrate, total nitrogen, amino acids, residual sugar and organic acids

Nitrate-N in the soil was determined according to the N_{min} method as described by Löhnertz.¹⁸ For the analysis of total N in the leaves, musts and wines, the Kjeldahl method for wet digestion was performed as described by Schaller.¹⁹ After chemical decomposition, total N was determined using flow injection analysis (Tecator; Foss, Rellingen, Germany). Ammonia (NH₄⁺) and amino acids were analysed using ion-exchange chromatography and post-column derivatisation with ninhydrin on an automated amino acid analyser, A200 (AminoSys, Hennigsdorf, Germany). The following 21 amino acids were determined: alanine (ALA), arginine (ARG), asparagine, aspartic acid, γ-aminobutyric acid, glutamine, glutamic acid, glycine, histidine (HIS), isoleucine, leucine, lysine (LYS), methionine, ornithine (ORN), phenylalanine (PHE), proline, serine, threonine, tryptophane, tyrosine and valine. The general enological parameters of the grape must and wine were analysed using Fourier transform spectroscopy (WineScan FT 120; Foss) according to Patz *et al.*²⁰

Statistical methods

The mean values and standard deviations were calculated with Excel® (Microsoft Office 2007), while SPSS Version 16.0.1 (1989–2007; SPSS Inc., Chicago, IL, USA) was used for further statistical analyses. The data were analysed by one-way ANOVA considering the N fertilisation as main factor. A Tukey test was used to compare the means. If assumptions of normality and homogeneity of variance were not met, transformations were employed.²¹ Significant differences between means are indicated by the use of different letters. Linear regression curves were used to describe the relationship between parameters and the coefficient of determination (*R*²) was calculated. The level of significance is indicated by an asterisk (**P* ≤ 0.05).

RESULTS AND DISCUSSION

Soil nitrogen (nitrate-N), yield and N nutritional status of vines

To evaluate the N-fertiliser accumulation, the soil nitrate-N was analysed after winter before fertiliser application. The nitrate-N showed a significant fertiliser response in both experimental years (2005 and 2007) when comparing the plots that had been highly fertilised (150 kg N ha⁻¹) annually since 1985 with the moderately fertilised ones (60 kg N ha⁻¹) or the unfertilised ones (0 kg N ha⁻¹) (Table 2). The soil nitrate-N of the moderate fertiliser treatment and unfertilised plots was below 30 kg N ha⁻¹, which can be described as being inadequate.¹⁸ The yield was significantly reduced in the 2005 vintage in the highly fertilised variant, which can be explained by the thinning of the botrytised grape bunches in this year. For the same reason, the yields in 2007 were twice as high as those in 2005.

Total N in leaves and total N in musts and wines are parameters characterising the N nutritional status. These varied according to the different rates of N application to the experimental vineyard plots. Table 2 shows that in both years (2005 and 2007) mainly the highest fertilisation level led to enhanced total N concentrations in the leaves as well as increased total N in the musts and wines. In contrast, the moderate fertilisation level mostly did not differ

Table 2. Influence of fertilisation level (0, 60, 150 kg N ha⁻¹) on nitrate-N of the soil (in spring before the annual fertiliser treatment in 0 to 90 cm), yield, total nitrogen in leaves (after blooming) and total nitrogen in musts and wines

Year and nitrogen in musts and wines	Fertilisation level		
	0 kg N ha ⁻¹	60 kg N ha ⁻¹	150 kg N ha ⁻¹
Vintage 2005			
Nitrate-N soil (kg NO ₃ -N ha ⁻¹)	25.2 (6.1) ^a	27.8 (5.4) ^a	57.4 (30.7) ^b
Yield (kg vine ⁻¹)	2.4 (0.3) ^b	2.4 (0.4) ^b	1.5 (0.6) ^a
N leaves (%)	2.0 (0.1) ^a	2.0 (0.1) ^a	2.3 (0.03) ^b
N must (mg L ⁻¹)	259.0 (19.7) ^a	273.4 (17.0) ^a	408.0 (32.4) ^b
N wine (mg L ⁻¹)	84.9 (5.9) ^a	100.6 (9.1) ^b	160.0 (5.2) ^c
Vintage 2007			
Nitrate-N soil (kg NO ₃ -N ha ⁻¹)	18.1 (6.4) ^a	19.1 (2.9) ^a	47.4 (7.8) ^b
Yield (kg vine ⁻¹)	4.3 (0.4)	4.0 (0.7)	4.9 (0.3)
N leaves (%)	1.9 (0.1) ^a	2.0 (0.05) ^a	2.3 (0.05) ^b
N must (mg L ⁻¹)	175.8 (19.8) ^a	212.8 (43.9) ^a	472.5 (38.0) ^b
N wine (mg L ⁻¹)	40.8 (8.2) ^a	53.3 (17.4) ^a	160.3 (21.7) ^b
Results are given as mean values with standard deviations in parentheses (<i>n</i> = 4). Significant differences (Tukey test; <i>P</i> ≤ 0.05) between fertiliser levels are indicated by different letters.			

significantly from the unfertilised control. Earlier studies in the years from 1990 to 2005 by Linsenmeier and Löhnertz²² and 1994 to 1995 by Bleser,¹¹ which had been conducted in the same field trial, also showed a strong correlation between N fertilisation level and the total nitrogen content of different plant organs (leaves, lignified parts, developing and ripe berries). Linsenmeier and Löhnertz²² summarised the results over the entire experimental period: according to the authors, the leaf N contents of unfertilised vines showed a stable low N status over 20 years, with small and light-coloured leaves of the unfertilised vines being indicative of N deficiency.

Influence of N fertilisation on N-containing compounds and general enological parameters

The quantity of N-containing compounds is an important criterion for further processing. An insufficient supply can lead to a lagging or stuck fermentation, resulting in reduced wine quality.^{23,24} According to Bell and Henschke,²³ the largest proportion of N is found in the forms of proteins, free amino acids and ammonium in grape must. Ammonium and amino acids, which occur in varying concentrations in the musts, are utilised by yeasts as nitrogen sources, with the exception of proline which is poorly used by yeast and only when the other amino acids and ammonium are exhausted.

For the assessment of yeast assimilable nitrogen (YAN) (Table 3), the amino acid proline and all high-molecular peptides and proteins were excluded as they cannot be used as nitrogen sources by yeasts.²³ According to Bell and Henschke,²³ the practical minimal limit of YAN in musts is considered to be 140 mg N L⁻¹. In the current study, the N supply of grape must from the unfertilised control and the moderate fertilisation level has to be characterised as being sub-optimal as the YAN was less than 140 mg N L⁻¹. Most of the highly fertilised varieties (150 kg N) reached a YAN higher than 140 mg N L⁻¹ (Table 3). The proportion of amine N within

Table 3. Influence of fertilisation level (0, 60, 150 kg N ha⁻¹) on nitrogen-containing organic compounds (amino acids and amines) in musts and wines

Year and nitrogen compounds	Fertilisation level		
	0 kg N ha ⁻¹	60 kg N ha ⁻¹	150 kg N ha ⁻¹
Vintage 2005			
Amino acids must (mg L ⁻¹)	780.8 (150.3)	754.6 (22.1)	1052.6 (170.1)
Amino acids wine (mg L ⁻¹)	153.2 (22.6) ^a	196.8 (68.2) ^a	515.9 (32.2) ^b
YAN must (mg L ⁻¹)	104.4 (19.8) ^a	102.8 (5.1) ^a	175.0 (26.5) ^b
Amines must (mg L ⁻¹)	3.2 (0.2)	3.6 (0.7)	4.5 (0.8)
Amines wine (mg L ⁻¹)	2.7 (0.7) ^a	2.6 (0.7) ^a	4.8 (0.5) ^b
Amine N must (µg L ⁻¹)	0.9 (0.1) ^a	1.0 (0.2) ^{ab}	1.2 (0.2) ^b
Amine N wine (µg L ⁻¹)	0.8 (0.2) ^a	0.8 (0.2) ^a	1.4 (0.1) ^b
Vintage 2007			
Amino acids must (mg L ⁻¹)	621.6 (114.3) ^a	830.6 (245.1) ^a	1635.8 (260.5) ^b
Amino acids wine (mg L ⁻¹)	315.7 (43.7) ^a	359.3 (56.9) ^a	539.7 (46.2) ^b
YAN must (mg L ⁻¹)	88.1 (19.4) ^a	124.5 (26.4) ^a	295.7 (37.6) ^b
Amines must (mg L ⁻¹)	3.1 (0.6) ^a	3.5 (0.6) ^a	4.9 (0.7) ^b
Amines wine (mg L ⁻¹)	0.4 (0.1) ^a	0.5 (0.1) ^a	1.5 (0.4) ^b
Amine N must (µg L ⁻¹)	0.9 (0.2) ^a	1.0 (0.2) ^{ab}	1.3 (0.2) ^b
Amine N wine (µg L ⁻¹)	0.1 (0.03) ^a	0.2 (0.03) ^a	0.5 (0.1) ^b
Results are given as mean values with standard deviations in parentheses (<i>n</i> = 4). Significant differences (Tukey test; <i>P</i> ≤ 0.05) between fertiliser levels are indicated by different letters.			

the whole nitrogen pool of the musts in the current study was low with an average of about 3.7‰.

From the results, it can be said that the total amine content in the musts increased significantly with enhanced N fertiliser application. During the fermentation, the amine N was in general depleted by 39%, while the average depletion of total N and YAN was 67% and 92%, respectively. The fertiliser impact on total N, amino acids and amines remained obvious also after fermentation. However, the N status of the musts was rather low during the experimental period, hence, keeping the significant depletion in mind, the amine N was presumably used by microorganisms during fermentation.

The enological parameters of the musts differed only in 2007: the pH and malic acid concentrations were highest in the highly fertilised variants, whereas the reducing sugar concentrations decreased with increasing N fertilisation (Table 4). While the N-fertiliser effect on sugar concentrations and pH of grapes have shown conflicting trends in several studies, the increases in malic acid concentrations with enhanced fertiliser application are in agreement with the majority of previous investigations.²³ Bell and Henschke²³ supposed that an increase of vine vigour leads to a higher degree of canopy and shaded leaves and consequently, an altered microclimate in the bunch zone (lower light interception and temperature). This has a crucial effect on photosynthates leading to lower total soluble solids concentration and pH due to higher malic acid concentration in grapes. A lack of fertiliser effects on the enological parameters in 2005 may be explained by the thinning of the botrytised grape bunches in this vintage. In contrast to the 2007 vintage, the fermentations in 2005 were

Table 4. Influence of fertilisation level (0, 60, 150 kg N ha⁻¹) on the general enological parameters of musts

Vintage and parameter	Fertilisation level		
	0 kg N ha ⁻¹	60 kg N ha ⁻¹	150 kg N ha ⁻¹
Vintage 2005			
Reducing sugars (g L ⁻¹)	203.7 (6.0)	202.7 (1.7)	209.3 (11.7)
pH	3.1 (0.03)	3.1 (0.01)	3.2 (0.03)
Malic acid (g L ⁻¹)	2.0 (0.2)	1.8 (0.2)	2.2 (0.2)
Lactic acid (g L ⁻¹)	ND	ND	ND
Total acids (g L ⁻¹)	6.7 (0.6)	6.6 (0.4)	7.0 (0.2)
Vintage 2007			
Reducing sugars (g L ⁻¹)	200.9 (4.4) ^a	201.1 (7.5) ^{ab}	197.3 (5.5) ^b
pH	3.0 (0.03) ^a	3.1 (0.02) ^{ab}	3.1 (0.1) ^b
Malic acid (g L ⁻¹)	3.3 (0.1) ^{ab}	3.2 (0.4) ^a	4.0 (0.5) ^b
Lactic acid (g L ⁻¹)	ND	ND	ND
Total acids (g L ⁻¹)	9.1 (0.2)	9.2 (0.6)	9.8 (0.8)

Results are given as mean values with standard deviations in parentheses ($n = 4$), Significant differences (Tukey test; $P = 0.05$) between fertiliser levels are indicated by different letters. ND, not detected.

Table 5. Influence of fertilisation level (0, 60, 150 kg N ha⁻¹) on the general enological parameters of wines

Vintage and parameter	Fertilisation level		
	0 kg N ha ⁻¹	60 kg N ha ⁻¹	150 kg N ha ⁻¹
Vintage 2005			
Reducing sugars (g L ⁻¹)	14.6 (1.5)	14.4 (3.3)	12.8 (4.7)
pH	3.0 (0.1)	3.0 (0.01)	3.0 (0.03)
Malic acid (g L ⁻¹)	2.5 (0.1)	2.3 (0.1)	2.3 (0.3)
Lactic acid (g L ⁻¹)	0.8 (0.1)	0.8 (0.1)	0.8 (0.1)
Total acids (g L ⁻¹)	7.4 (0.4)	7.1 (0.1)	7.3 (0.2)
Ethanol (g L ⁻¹)	12.1 (0.4)	12.1 (0.1)	12.5 (0.4)
Vintage 2007			
Reducing sugars (g L ⁻¹)	7.4 (2.5) ^b	4.7 (1.8) ^{ab}	1.4 (0.2) ^a
pH	2.6 (0.03)	2.6 (0.1)	2.7 (0.1)
Malic acid (g L ⁻¹)	3.2 (0.1) ^b	3.0 (0.01) ^a	3.1 (0.1) ^{ab}
Lactic acid (g L ⁻¹)	0.5 (0.1)	0.5 (0.2)	0.6 (0.1)
Total acids (g L ⁻¹)	8.9 (0.2)	9.0 (0.3)	9.0 (0.6)
Ethanol (g L ⁻¹)	12.0 (0.3)	12.2 (0.4)	12.0 (0.2)

Results are given as mean values with standard deviations in parentheses ($n = 4$). Significant differences (Tukey test; $P \leq 0.05$) between fertiliser levels are indicated by different letters.

generally incomplete with residual sugar levels ranging from 12.8 to 14.6 g L⁻¹ (Table 5). This may be explained by the use of two different yeast strains in these years. In the 2007 vintage, the wines from the unfertilised variant had significantly higher residual sugar concentrations than those from the highly fertilised variant due to insufficient must-YAN resulting in incomplete fermentation. The effect of YAN concentration in the must on the fermentation was obvious: in both years, the fermentation of the must was faster in the flasks of the highly fertilised vines (data not shown), which is in agreement with the findings of Bisson.²⁴ Spontaneous malolactic fermentation occurred in both vintages: on average, the formation of lactic acid was about 0.6 g L⁻¹. This process occurred regardless of the fertiliser levels. However, the reduction of malic acid was low compared to the investigation by Moreno-Arribas *et al.*²⁵ who found up to 2.6 mg L⁻¹ lower malic acid contents in wines with increased TRN formation after malolactic fermentation.

Impact of N fertilisation on individual amines

Of all the 11 amines examined, only the following eight were detected in both vintages: CAD, ETH, HIN, IPA, PEA, PUT, SPD and SPN (Fig. 1). AGM could not be found in any of the samples, and the aromatic monoamine TRN (which derives from tyrosine via decarboxylation¹) was detected only in few samples in small amounts where the highest value (0.4 mg L⁻¹) was reached in a must of a low fertilised variant. Moreover, DAP could only be detected in few must samples in the 2007 vintage.

Within the aliphatic polyamines, only the SPD concentrations in the wines were influenced by N fertilisation, but not the SPD concentrations of the musts nor the concentrations of PUT, SPN and CAD in either the musts or wines. These results are in agreement with those of Bleser¹¹ who investigated the same vineyard in the vintages 1994 and 1995, and who suggested that the sum of AGM, CAD, DAP and SPN concentrations in musts and wines are not significantly influenced by N fertilisation. Nevertheless, a trend for an increase in PUT, SPN and CAD was recognisable in our results, but due to the high standard deviations no statistical significances

could be obtained (Fig. 1). In contrast to our results, Bleser¹¹ found a significant fertiliser effect on SPD concentrations in musts and PUT concentrations in musts and wines.

The effect of enhanced N supply on amines was clearer for ETH, HIN, IPA and PEA (Fig. 1). PEA and ETH concentrations of musts and wines were significantly influenced in 2005, whereas the IPA concentrations were affected in the musts of the 2007 vintage. In the case of HIN, the N fertilisation effect was significant only after fermentation in 2005, although critical levels of HIN which would provoke health problems were not reached. Similar to our results, Ancín-Azpilicueta *et al.*²⁶ found increased HIN concentrations in wines with rising foliar N applications, though at levels that could be detrimental to human health.²⁶ In another study, Bertrand *et al.*²⁷ investigated the effect of soil N fertilisation on amines in Merlot wines in which HIN reached levels above the former tolerance value in Switzerland. These high HIN values may be explained by the higher N status of the grape musts found by Ancín-Azpilicueta *et al.*²⁶ and Bertrand *et al.*²⁷ in comparison to the N values found in our musts.

While HIN and ETH concentrations increased during fermentation in most of the experimental variants, the amines PEA, PUT and CAD were decreased in the wines of the 2007 vintage (Fig. 1). The highest decrease during fermentation was found for IPA, SPD and SPN, which could be recovered only in small amounts in the wines of both years. Similar results have been reported by Sass-Kiss and Hajós,²⁸ who examined a reduced SPN content in Hungarian sweet wines after fermentation. Also, Bleser¹¹ observed a decrease of the SPN, SPD and PUT concentrations in the final wines after fermentation. Torrea-Goñi and Ancín-Azpilicueta,²⁹ who studied the effect of different *S. cerevisiae* strains on amine concentrations of wines, suggest that non-volatile as well as volatile amines can serve as nitrogen sources for yeasts during alcoholic fermentation. In addition to yeasts, lactic acid bacteria may also have participated in the amine degradation³⁰ in the current study, since the incidence of lactic acid bacteria cannot be excluded.

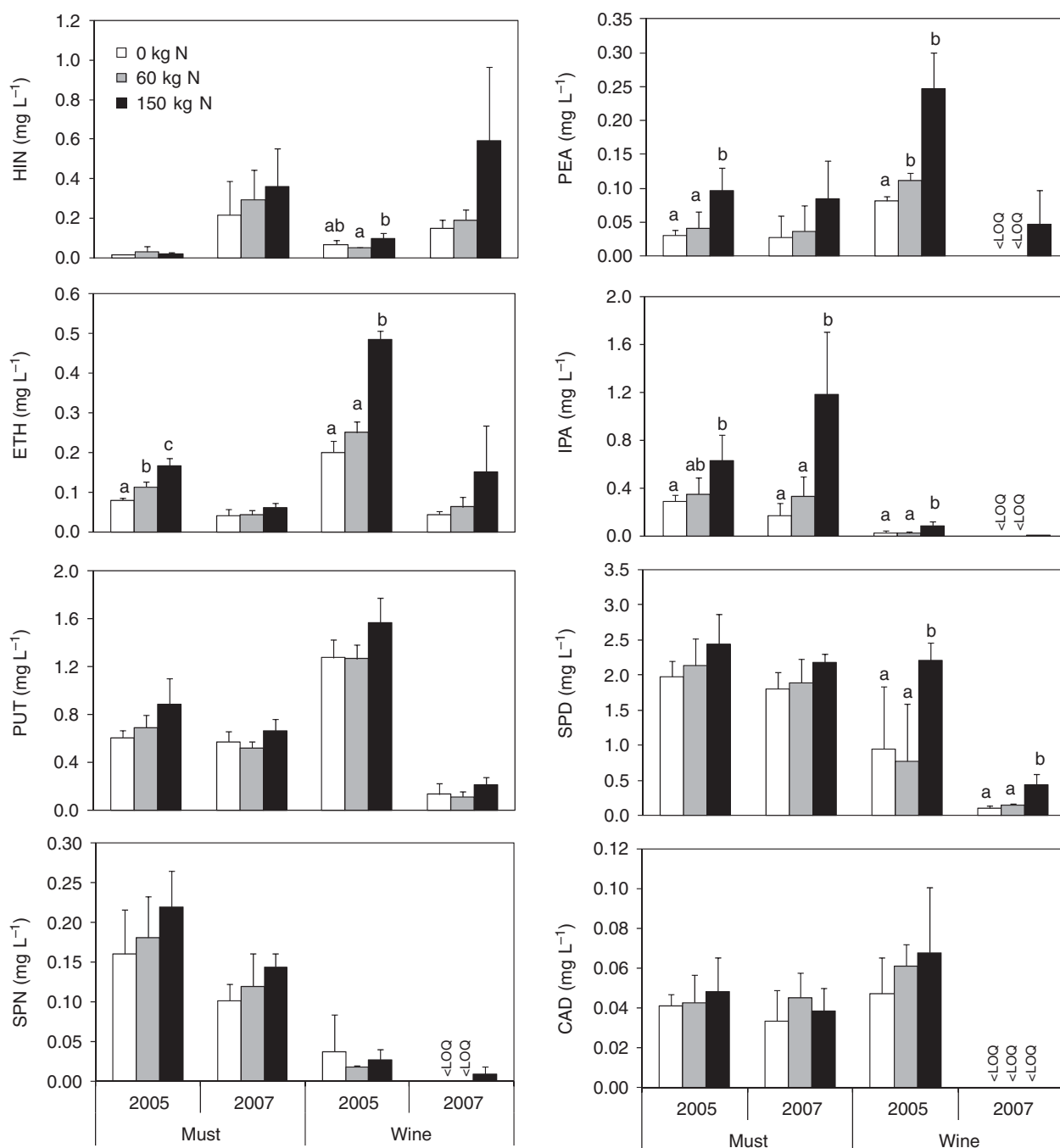


Figure 1. Impact of fertilisation level (0, 60, 150 kg N ha⁻¹) on the amine concentrations (CAD, ETH, HIN, IPA, PEA, PUT, SPD and SPN) in musts and wines (mean values $\pm$ standard deviations; $n = 4$) in two years: 2005 and 2007. Significant differences (Tukey test; $P \leq 0.05$) between fertiliser levels are indicated by the use of different letters. LOQ limit of quantification.

Influence of fermentation on amines and its relation to precursor amino acids

The three amines – IPA, SPD and SPN – that were generally being degraded account for more than 70% of the total amine concentration of musts, but only 39% of the total amine concentration of wines. Therefore, the balance, Δ (where Δ = amine concentration of wines minus amine concentration of musts), of total amines after fermentation was negative in most variants (Fig. 2), which indicates that the amines were generally being degraded during fermentation. Four exceptions marked in Fig. 2 were found in one unfertilised, one medium fertilised and

two highly fertilised variants in the first experimental year. The total amine degradation in 2005 was generally lower than in 2007, which may be attributed to a generally higher amount of N sources for fermenting microbes in the form of YAN in the musts in this year (Table 3). Furthermore, these variations may be due to the different yeast strains which were used, although no information is available about the ability of these specific yeast strains to degrade or synthesise biogenic amines.

In the current investigation, no significant relationship was found between the balance of total amino acid content of the must and the balance of total amines in the individual vintages

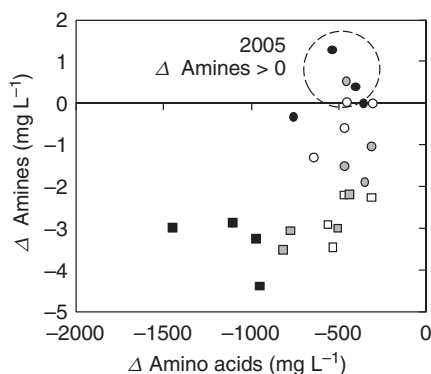


Figure 2. Linkage of alterations in the amino acid content with alterations in the biogenic amine content during fermentation. Positive or negative Delta (Δ) represents an increase or decrease of compounds. The fertiliser levels 0 kg N ha^{-1} , 60 kg N ha^{-1} and 150 kg N ha^{-1} are displayed by the use of white, grey and black filling of symbols, respectively ($\circ$ 2005, $\square$ 2007). Positive Δ values in 2005 are highlighted by a dashed circle.

(Fig. 2). Therefore, the relationships between the balances of single amines which were enriched during fermentation in one vintage or both vintages and their specific precursor amino acids seemed

to be useful to explore. These amines are CAD, ETH, HIN, PEA, PUT and DAP (Fig. 3); however, DAP is not shown because it was found only in small amounts. For CAD, PEA and PUT the balances were positive though mainly only in 2005, while for HIN and ETH they were positive in both vintages.

In most fermentation flasks, the Δ of precursor amino acids was negative except for HIS in a highly fertilised variant from 2005, ORN in some flasks in both vintages and LYS in almost all variants. In agreement with our studies, LYS excretion was observed by Torrea-Goñi and Ancín-Azpilicueta,²⁹ who tested different yeast strains according to their amino acid degradation capacity and the formation of amines. LYS can be decarboxylated to CAD, whereas PUT can be formed either from ORN directly or via the decarboxylation of ARG to ORN¹. The latter pathway may explain the previously mentioned increases in ORN. ARG can also be decarboxylated to AGM¹ which could not be detected either in the musts or wines. PUT itself is the precursor compound of SPD and SPN, which are formed via spermidine synthase and spermine synthase. DAP can be synthesised from both SPD and SPN¹. These complex biochemical pathways might explain the lack of significant correlations for Δ PUT with Δ ORN or Δ ARG. Relationships between PUT and its precursor amino acids were also not found by Torrea-Goñi and Ancin-Azpilicueta.²⁹

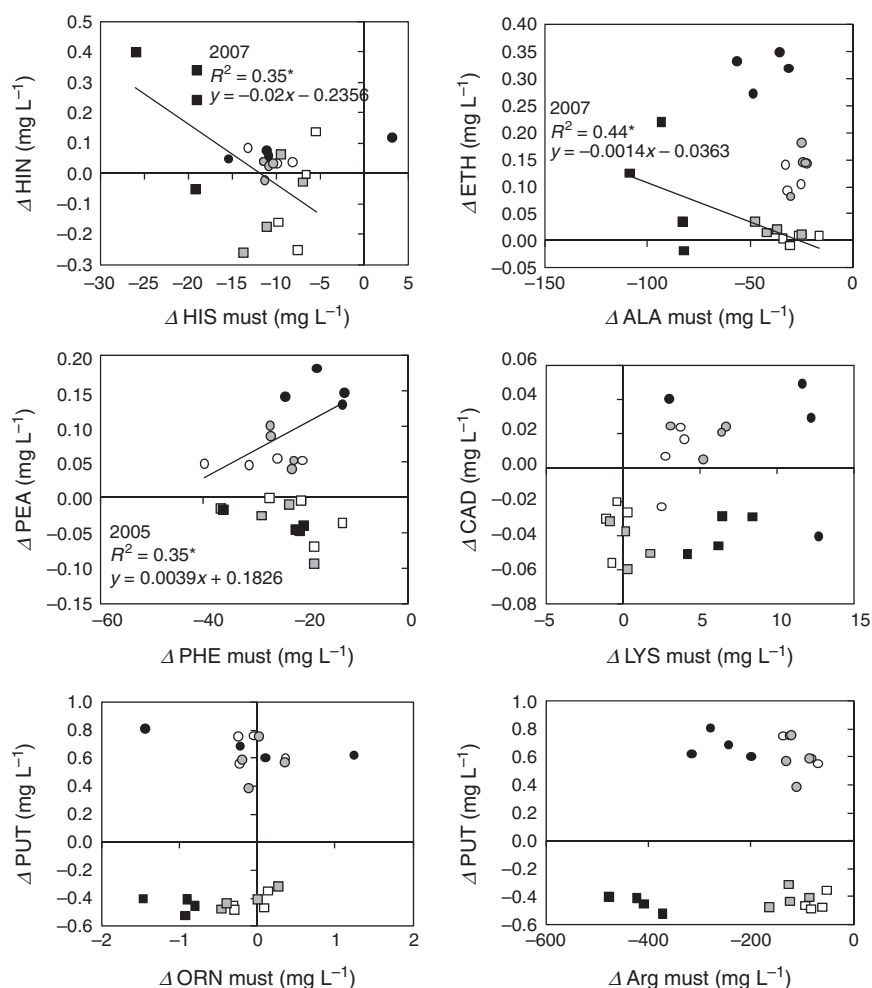


Figure 3. Impact of distinct amino acid content alterations on specific biogenic amine content alterations during fermentation in 2 years (2005 and 2007). Positive or negative Delta (Δ) represents an increase or decrease of compounds. Fertiliser levels 0 kg N ha^{-1} , 60 kg N ha^{-1} and 150 kg N ha^{-1} are displayed by the use of white, grey and black filling of symbols, respectively ($\circ$ 2005, $\square$ 2007). Significant coefficients of determination (R^2) are specified by the use of an asterisk * significant at $P \leq 0.05$. Relationships ($n = 12$) are shown by linear regression in the case of a significant R^2 .

Significant relationships were found between HIN formation and HIS degradation in 2007 (Fig. 3). Ough already suspected in 1971 that an over-supply of N prevents the degradation of HIN by yeasts during fermentation.³¹ Moreover, a high HIS concentration could induce the formation of HIS decarboxylase and, thereby, contribute to HIN formation. Even though he studied wines derived from different origins and processing methods, this author found a positive correlation between the N concentrations of wines with HIN. Bach *et al.*⁹ found increases of HIN in wines after the addition of N sources to musts (diammonium hydrogen phosphate and inactivated dry yeast). These authors found that the HIN formation was due to malolactic fermentation and not due to alcoholic fermentation. In the current study, we could not determine whether the HIN formation could be explained by yeasts or bacteria. ETH formation and ALA degradation correlated significantly in 2007, while PEA formation and PHE formation correlated significantly in 2005 (Fig. 3). The negative correlations observed for HIN and ETH indicate that more amines were synthesised when more precursor amino acids were present and so were available for degradation. On the other hand, the positive correlation between Δ PEA and Δ PHE in 2005 indicates that the more PEA was synthesised the less PHE was degraded, which leads to the assumption that PEA formation during fermentation is not only an effect of PHE availability but must also involve other biochemical reactions. According to Askar and Treptow,¹ ETH can be either formed by decarboxylation of the amino acid ALA or by transamination of acetaldehyde or of other nitrogenous compounds. Our results suggest that ETH was synthesised from its precursor ALA during fermentation (Fig. 3). The ETH contents were increased during fermentation and the ALA contents were decreased especially in the variants with a high N supply. However, no negative health effects caused by the consumption of ETH are known.³²

CONCLUSIONS

Generally, it can be concluded that soil N fertilisation has a significant impact on the total N and YAN of musts as well as on the total amines of grape musts and wines. The individual amines ETH, HIN, IPA, PEA and SPD increased in musts and wines with enhanced N fertilisation. The fertilisation effect on amine formation in grapes was observed in the grape must. In wines the fertilisation effect on amine formation in grapes may be overlaid by the activity of fermenting microorganisms. During fermentation, mainly ETH and HIN were increased while the other investigated amines were decreased, presumably serving as N sources during fermentation. ETH formation was significantly related to the depletion of its precursor ALA during fermentation.

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