Determination of Free and Conjugated Indole-3-Acetic Acid, Tryptophan, and Tryptophan Metabolites in Grape Must and Wine

Katrin Hoenicke,[†] Thomas J. Simat,*[,†] Hans Steinhart,[†] Hans J. Köhler,[‡] and Arnold Schwab[‡]

University of Hamburg, Institute of Biochemistry and Food Chemistry, Grindelallee 117, D-20146 Hamburg, Germany, and Bayerische Landesanstalt für Weinbau und Gartenbau, D-97209 Veitshöchheim, Germany

Tryptophan (Trp) and its metabolites, especially indole-3-acetic acid (IAA), are considered to be potential precursors of 2-aminoacetophenone (AAP), an aroma compound that causes an "untypical aging off-flavor" (UTA) in *Vitis vinifera* wines. In this study, RP-HPLC with fluorescence detection was used for the qualitative and quantitative analysis of Trp and Trp metabolites in grape musts and wines to which different viticultural measures had been applied (time of harvest, soil treatment, leaf plucking, vine prune). An alkaline hydrolysis was developed to release bound IAA and Trp. A sensitive and selective determination of different Trp metabolites was achieved after solid phase extraction using a strong anion exchange material. In the examined grape musts, more than 95% of the total IAA was bound either as ester conjugate or as amide conjugate. Free IAA and other Trp metabolites were below the detection limit (<3 μg/L) or could be determined only in traces. Their amounts increased significantly during fermentation, whereas the amount of Trp decreased. It could be shown that the different viticultural measures applied (except the vine prune) as well as the climatic conditions of the vintage exhibited significant influences on the amounts of Trp and Trp metabolites in grape musts or wines.

Keywords: *Indole-3-acetic acid; tryptophan; alkaline hydrolysis; grape must; wine; Vitis vinifera; HPLC analysis; untypical aging off-flavor (UTA); viticultural measures*

INTRODUCTION

Since 1988, an off-flavor in V*itis vinifera* white wines, formed within a few months after fermentation, has been frequently reported (*1*−*5*). This off-flavor is described by aroma descriptors such as "acacia blossom", "furniture polish", "wet wool", "mothball", or "fusel alcohol", combined with a loss of the typical bouquet of the grape variety (*2*, *6*, *7*). The so-called "untypical aging off-flavor" (UTA) has been associated with the aroma compound 2-aminoacetophenone (AAP) (*1*), which has an odor threshold of 1 μg/L in the wine (*8*). Particularly dry-stress for the vines caused by low rainfall or intensive solar radiation and nutrient deficiencies are supposed as the main cause of UTA formation (*3*, *4*, *9*). Additionally, high yield and an early grape vintage seem to be related to the occurrence of UTA (*10*−*14*). The detailed mechanism of AAP formation in wine has not been elucidated to date. Tryptophan (Trp) and its metabolites, especially the phytohormone indole-3-acetic acid (IAA), are considered to be potential precursors of AAP (*15*−*18*). Studies by Christoph et al. (*19*) have shown that AAP can be formed by an oxidative degradation of IAA, which is triggered by a sulfuration, a measure indispensable for white wine making.

Several investigations have been carried out regarding the amounts of Trp and IAA in different matrixes but only a few have dealt with the amount of IAA in grape must or wine (*20*−*22*). However, to the best of the authors' knowledge, there are no papers regarding the amount of bound IAA in grape musts and wines, although in plants most of this growth hormone exists bound in its storage forms (ester conjugates of sugar moieties or amide conjugates of amino acids) (*23*).

It has been established that alkaline hydrolysis releases conjugated IAA in seeds of different cereals and legumes (*24*, *25*), in plant tissues of *Arabidopsis* (*26*), in tomato fruits (*27*), and in tobacco leaves (*28*). In these studies, an alkaline hydrolysis was carried out in 7 M NaOH for 3 h at 110 °C. Acidic hydrolysis is usually avoided because of the instability of the indole ring at low pH. At higher pH, the indole ring is relatively stable, but the phenomenon of an increase in the IAA level, which is probably due to an oxidative conversion of some other abundant indolic components, such as the precursor Trp itself, has been reported (*25*). Enzymatic hydrolysis of bound IAA is not effective since a wide range of commercial enzymes expected to cleave amide or ester linkages are inactive toward IAA conjugates (*23*).

In this paper, the amounts of Trp, IAA, and several other Trp metabolites such as tryptophol (TOH), *N*-formylkynurenine (NFK), anthranilic acid (AA), indole-3-lactic acid (ILA), and 1-methyl-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid (MTHCC), the condensation product of Trp and acetaldehyde, are analyzed in different grape musts and wines. A method for the determination of bound IAA after alkaline hydrolysis in must and wine is reported that allows the determination of peptide bound Trp, too. The influences of yeast metabolism and different viticultural measures on the amounts of Trp and Trp metabolites are investigated,

* To whom correspondence should be addressed (telephone +49 40 42838 4359; fax +49 40 42838 4342; e-mail thomas_simat@public.uni-hamburg.de).

[†] University of Hamburg.

[‡] Bayerische Landesanstalt für Weinbau und Gartenbau.

10.1021/jf010575v CCC: $20.00
Published on Web 10/10/2001

and the role of Trp and Trp metabolites as precursors of the aroma compound AAP, causing UTA, are discussed.

MATERIALS AND METHODS

Reagents. Analytical grade AA, IAA, ILA, TOH, and the internal standards (IS), 5-methoxy-tryptophan (5-MeO-Trp) and indole-3-propionic acid (IPA), respectively, were obtained from Sigma (Deisenhofen, Germany), *N*-(3-indolylacetyl)-DL-aspartic acid (IAA-Asp) was from Aldrich (Deisenhofen, Germany), and 2,6-di-*tert*-butyl-*p*-kresol (BHT) was from Fluka (Deisenhofen, Germany). Trp was donated by Degussa (Hanau, Germany), H-Ala-Trp-Ala-OH (LWL) was purchased from Bachem Biochemica (Heidelberg, Germany). NFK and MTHCC were synthesized as described by Simat et al. (*29*). Acetonitrile (gradient grade), methanol (gradient grade), and trifluoroacetic acid (TFA) (Uvasol) were purchased from Merck (Darmstadt, Germany). Deionized water was purified by a bidistillator (Heraeus-Destamat Bi 18E, Kleinostheim, Germany). All other chemicals used were of analytical purity.

Materials. The grape musts and wines were provided by the Bayerische Landesanstalt für Weinbau und Gartenbau (Veitshöchheim, Germany), where the vines were cultivated using different viticultural conditions (crop load, leaf plucking, soil treatment, time of harvest).

Grapes. Thirty-nine grape samples (*Vitis vinifera* cv. Kerner) of the vintage 1996, harvested at four degrees of ripening, were destemmed and immediately frozen at −18 °C. After thawing the samples, the grapes were extracted by a commercially available juice extractor (Vita compact, Krups, Solingen, Germany). The resulting juices were centrifuged at 4000*g* for 5 min, and aliquots of the supernatant were used for analysis.

Grape Musts. Forty-eight must samples (*V. vinifera* cv. Kerner) of the vintages 1996−1999 were destemmed, crushed, and pressed in a spindle press. The resulting musts were immediately frozen at −18 °C. After thawing and centrifugation of the samples at 4000 g for 5 min, aliquots of the supernatant were used for analysis.

Wines. Forty-eight wine samples were produced by the classical white wine vinification from the above-mentioned must samples. After addition of SO_2 (50−55 mg/L SO_2) to the samples, the wines were bottled in wide, rounded bottles (Bocksbeutel) and stored at 13−14 °C before analyzing.

Determination of Free Trp and Trp Metabolites. For the determination of free Trp and Trp metabolites, including AA, IAA, ILA, MTHCC, NFK, and TOH, 40 μL of IS solution (5-MeO-Trp, 500 mg/L; IPA, 5 mg/L) was added to 2 mL of grape juice, must, or wine. The samples were adjusted to neutral pH (pH 6−7) using 1 M NaOH. A strong anion exchange (SAX) solid-phase extraction (SPE) was performed according to Hoenicke et al. (*21*) to separate the nonanionic compounds from the acidic compounds. One milliliter of the pretreated samples were applied to a Merck LiChrolut SAX SPE cartridge (500 mg, 3 mL). Subsequently, Trp and the nonanionic Trp metabolites (IS = 5-MeO-Trp) were eluted with 4 mL of acetonitrile/water (5:95). The anionic Trp metabolites (IS = IPA) were eluted with 3 mL of 2 M acetic acid/acetonitrile (95:5). Both solutions were subjected to HPLC analysis. The determination was performed by IS calculation.

Experimental Design for Hydrolysis Optimization. For the release of ester and amide bound IAA, an alkaline hydrolysis was performed. Hydrolysis experiments were carried out under different conditions with 1 mL of must, spiked with 20 μL of IS solution (5-MeO-Trp, 500 mg/L; IPA, 5 mg/L) in 15 mL polypropylene vials (Wheaton Redi-PakStarline, Zinsser, Frankfurt, Germany). The vials were transferred into a flat flange reaction vessel (155 × 75 mm, PTFE coated O-Ring, Schott, Mainz, Germany) that was evacuated (10 mbar) and purged three times with nitrogen. After the samples were aerated and cooled, the alkaline solutions were diluted with 5 mL of bidistilled water and acidified by addition of 700 μL of 85% phosphoric acid (pH 3−3.5).

The hydrolysis parameters were optimized using two different experimental designs. In the first phase, the effects of an antioxidant addition, the NaOH concentration, and the hydrolysis time were estimated in a full factorial design at two levels ($2^{(3-0)}$). Using this design, BHT (0, 0.005, and 0.01%) was added to the must, and NaOH concentrations in a wide range of molarity (3, 5, and 7 M) were tested at three different hydrolysis times (3, 5, and 7 h). The hydrolysis efficiency was estimated by analyzing the amount of IAA in the must after hydrolysis at 100 °C.

In the second phase, the objective of the experiment was to obtain the optimum hydrolysis conditions (NaOH concentration, hydrolysis time, and temperature) by using the Box-Behnken design (*30*). On the basis of the results of the first experiment, narrower ranges of NaOH concentration (4, 5, and 6 M) and hydrolysis time (5, 6, and 7 h) were tested at three different temperatures (80, 100, and 120 °C). The efficiency of the IAA release from its amide conjugate was estimated by analyzing the recovery of free IAA, added as IAA-Asp to the must before hydrolysis. For this, experiments were carried out with a must without and with addition of 150 μg/L IAA-Asp (corresponds to 90 μg/L IAA), respectively. Finally, the release of Trp from Trp peptides at the optimized hydrolysis condition was investigated by hydrolysis of LWL.

Determination of Free Plus Bound IAA and Trp. After hydrolysis and neutralization, the obtained solution was applied to a Varian Bond Elute C18/OH SPE cartridge (500 mg, 10 mL, Darmstadt, Germany) preconditioned with 5 mL of methanol and 5 mL of 1 M acetic acid. Subsequently, the column was washed with 5 mL of bidistilled water and the elution was carried out with 5 mL of methanol/water (50:50). The eluate was neutralized with 1 M NaOH and applied to a Merck LiChrolut SAX cartridge (500 mg, 3 mL), which had been conditioned as mentioned above (*21*). Trp and 5-MeO-Trp (IS) were not retarded and were eluted directly into a 10-mL volumetric flask with 3 mL of bidistilled water. The volumetric flask was diluted to volume with bidistilled water and was directly used for HPLC analysis of Trp. After the SPE cartridge was washed with 10 mL of acetonitrile/water (5:95), IAA and IPA (IS) were eluted with 3 mL of 2 M acetic acid/acetonitrile (95:5). The eluate was directly used for HPLC analysis of IAA. The quantification of Trp and IAA was performed by IS calculation.

HPLC Analysis. The HPLC system consisted of a TSP AS 100 autosampler with integrated thermostat (Thermo Separation Products, Egelsbach, Germany), a Merck L 6200 A high-pressure gradient pump and a Merck F 1080 programmable fluorescence detector. System control, data acquisition, and processing were performed with Kroma system 2000 software, version 1.8 (Bio-Tek Kontron Instruments, Neufahrn, Germany). Chromatographic separations were carried out on a Nucleosil 120-3 C18 (250 × 4 mm) column equipped with a Nucleosil 120-3 C18 (20 × 4 mm) precolumn (CS, Langerwehe, Germany) using a binary gradient (solvent A: 0.1% TFA in bidistilled water, solvent B: acetonitrile). The following gradient was used: 0 min, 5% B; 15 min, 30% B; 20 min, 50% B. The column was washed for 2 min with 50% B after each run and equilibrated for 8 min at the starting conditions. The flow rate was set to 1 mL/min, the injection volume was 20 μL, and the temperature of the column thermostat was 35 °C. The fluorescence excitation was set to 255 nm and the emission to 435 nm for the detection of NFK or AA (retention times < 12 min). After 12 min, the excitation and emission were changed to 270 and 360 nm, respectively, for the detection of Trp, MTHCC, ILA, IAA, or TOH (retention times > 12 min).

Statistical Analysis. The experimental designs of the statistical experiment and their evaluations were performed using STATISTICA software, release 5.1 (StatSoft Inc., Hamburg, Germany). To determine the factors significantly affecting the hydrolysis of IAA, an analysis of variance (ANOVA) was carried out. For studying the influence of the individual viticultural measures on the amounts of Trp and its metabolites, an ANOVA was carried out with SPSS for Windows, release 9.0.1 (SPSS Inc., Germany).

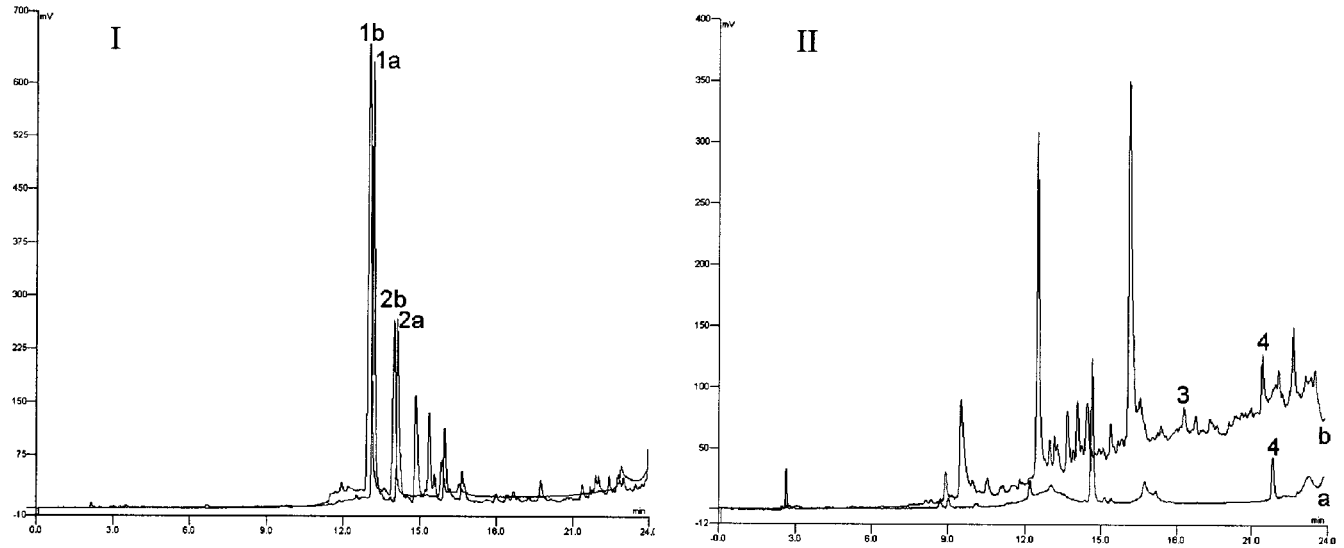

Figure 1. HPLC-Fl chromatograms of a must before (a) and after hydrolysis (b), obtained from the Trp-eluate (I), and the IAA-eluate (II) after solid-phase extraction using a strong anion exchange material; Trp **1**, 5-MeO-Trp (IS) **2**, IAA **3**, IPA (IS) **4**.

RESULTS AND DISCUSSION

Alkaline Hydrolysis Optimization. In the first phase of the statistical experiment, the ANOVA effects for the three factors antioxidant addition, NaOH concentration, and hydrolysis time on the amount of IAA after hydrolysis were estimated. The molar value of the NaOH as well as the hydrolysis time significantly affected the amount of IAA, while an addition of the antioxidant BHT had no effect. However, using 7 M NaOH, usually applied for hydrolysis of conjugated IAA (*24*−*28*), the amount of IAA determined after hydrolysis was at the minimum both by hydrolysis of 3 and 7 h. This effect may be due to a spontaneous degradation of released IAA under the strong alkaline conditions. Using 3 M NaOH, the hydrolysis of amide bound IAA was found to be not complete after 3 h. On the other hand, too long hydrolysis times enhance the risk of an oxidative degradation of released IAA or an oxidative conversion of other indolic compounds to IAA.

The results of the first experiment showed that for hydrolysis of bound IAA the optimum range of the NaOH concentration was between 4 and 6 M with hydrolysis times of longer than 3 h. To achieve a maximum hydrolysis efficiency, a Box-Behnken design was constructed by hydrolysis of IAA-Asp based on the results of the first experiment using three different hydrolysis temperatures. The evaluation of the experimental design revealed that variations in the settings of the temperature and time significantly affected the recovery of IAA released from its amide during hydrolysis, while a variation of the NaOH concentration between 4 and 6 M exhibited no significant effect. The effect of hydrolysis time and temperature on the hydrolysis efficiency of amide bound IAA was not simple, due to many artifacts. Maximum hydrolysis efficiency was achieved using 5 M NaOH at 120 °C for 5 h. Using a hydrolysis temperature of 120 °C, a longer hydrolysis time had a negative effect on the recovery of IAA. However, at lower temperatures the hydrolysis time had no effect. The temperature exhibited a quadratic effect. At 100 °C, the recovery of IAA was found to be at the minimum. It was increased by setting the temperature at 80 °C and especially by setting the temperature at 120 °C.

Alkaline Hydrolysis Validation. Owing to the results of the experimental design, the alkaline hydrolysis was carried out in 5 M NaOH at 120 °C for 5 h. To verify the chosen hydrolysis conditions, IAA was determined after hydrolysis of musts spiked with 0, 50, 100, 150, and 200 μg/L IAA-Asp (corresponds to 0, 30, 60, 90, and 120 μg/L IAA) ($n = 3$). A recovery rate for released IAA of 91% with a coefficient of variation (CV) of 17.6% was considered to be adequate. Furthermore, hydrolysis of LWL revealed a complete release of Trp.

To verify the stabilities of free IAA and Trp during hydrolysis, a must was spiked with 30 μg/L IAA and 5 mg/L Trp ($n = 3$) prior to hydrolysis, and their recoveries were examined after hydrolysis. The method exhibited good results with recoveries of 87% for IAA (CV = 5.6%) and 105% for Trp (CV = 6.4%). The detection limits were 12 μg/L for free plus bound IAA and 50 μg/L for free plus bound Trp, respectively. For statistical control, the amount of free and bound IAA of one must was permanently determined during the ongoing investigations and monitored using a Shewhart control chart which plots center line (mean), warning and control limits (double and triple standard deviation). From the control chart, a CV of 9.2% could be read. The chromatograms of the obtained solutions (Trp-eluate, IAA-eluate) before and after hydrolysis of a must are shown in Figure 1.

Determination of IAA, Trp, and Trp Metabolites in Grape Must and Wine. The values of free and bound IAA and Trp determined in the different musts and wines of the vintages 1996−1999 are shown in Figure 2. Only traces of free IAA (<3 μg/L) could be determined in the examined grape musts, whereas the amount of bound IAA ranged from <12 to 120 μg/L. In the wines, the amount of free IAA varied between <3 and 90 μg/L, while the concentration of bound IAA decreased to around less than 40 μg/L. Trp was analyzed in significantly higher amounts. In dependence on the vintages, 2−80 mg/L of unbound Trp were determined in the grape musts, and <0.012−24 mg/L in the wines. The concentrations of conjugated Trp in the grape musts were nearly the same as in the wines, on average 4 and 4.6 mg/L, respectively. Consequently, in musts Trp existed chiefly unbound, whereas in wines (except for

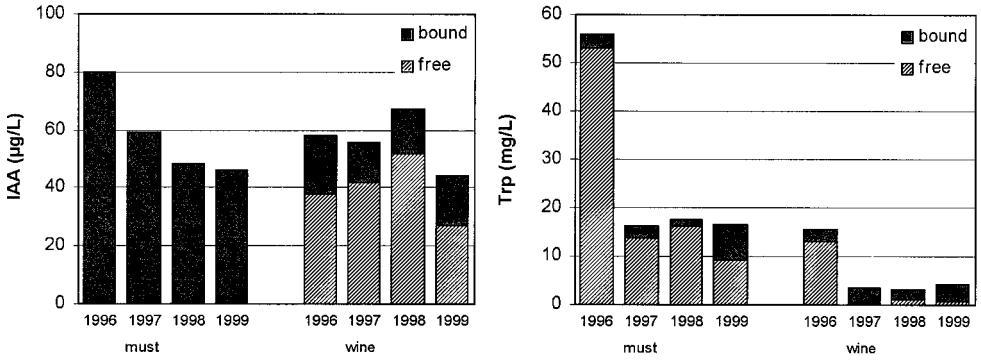

Figure 2. Free and bound IAA and Trp in grape musts and wines of 1996−1999 vintages (arithmetic means; $n = 12$).

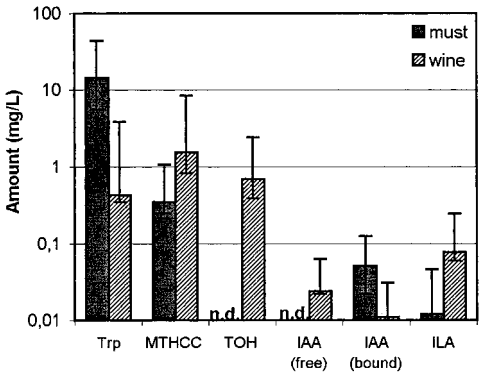

Figure 3. Trp and its metabolites prior to and after fermentation of the 1996−1999 vintages; nd: not detectable, TOH median <0.002 mg/L, IAA median <0.003 mg/L (medians, lower/upper quartiles; logarithmic scale; $n = 48$).

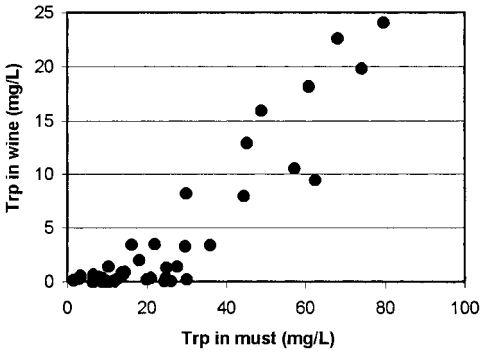

Figure 4. Correlation between the Trp concentration of a must and the Trp concentration of the corresponding wine ($n = 48$).

the vintage 1996) Trp was mainly conjugated. By way of contrast, in the musts the growth hormone IAA existed mainly bound in its storage forms. This conjugation of IAA to sugar moieties or amino acids can be attributed to an immobilization of the phytohormone in the plant, since free IAA is immediately utilizable in growth (*23*). The analyzed small range of the IAA content in the grape musts, as compared with that of Trp, indicates a regulation of the growth hormone by the plant. An ester hydrolysis of a must according to Bandurski and Schulze (*24*) in 1 M NaOH at room temperature for 1 h revealed that most of the IAA in the must was conjugated to amino acids; ester conjugates of sugar moieties amounted to about 20% of the total bound IAA.

The contents of Trp and Trp metabolites in the examined grape musts and wines are shown in Figure 3. In the grape musts in addition to Trp and conjugated IAA the Trp metabolite ILA was analyzed (<0.004−0.1 mg/L). Furthermore, MTHCC, the condensation product of Trp and acetaldehyde could be detected (<0.002−3.16 mg/L). TOH, NFK, and AA were not detectable (<0.002, <0.24, and <0.011 mg/L); however, the detection limit for NFK was relatively high. In the wines under study, partly higher amounts of Trp metabolites were analyzed. The amount of TOH ranged between <0.002 and 13.1 mg/L and for ILA <0.004−0.59 mg/L were detected. The amount of MTHCC in the wines was 0.13−24.6 mg/L. However, NFK and AA were not detectable under the chosen conditions (<0.24 and <0.011 mg/L, respectively).

Influence of Yeast Metabolism on Trp and Trp Metabolites. In all batches, the level of free Trp decreased during fermentation, while bound Trp was detected in the same or even in higher concentrations in the wines. This effect may be due to an autolysis of the yeast cells at the end of fermentation. The consumption of free Trp by the yeast was between 60 and 100%. This utilization of Trp by the yeast is in accordance with the results of Bergner and Haller (*31*), Polo and Llaguno (*32*), and Sponholz (*33*). However, the utilization of Trp by the yeast depended on the amount of Trp in the must. If the Trp content of the must was low, nearly 100% was utilized. At Trp concentrations above a threshold of about 15 mg/L in the must, its utilization was between 65 and 100%, at an average of 85% (cf. Figure 4). Thus, it seems that a Trp minimum of 15 mg/L is required for yeast growth. This finding indicates the important role of Trp in yeast growth and development. However, Jones and Pierce (*34*) classified Trp as one of the last amino acid consumed by the yeast from wort, which was not utilized before depletion of the amino acids aspartate, asparagine, arginine, glutamate, glutamine, lysine, serine, and threonine. By way of contrast, in grape must Trp seems to be readily utilized as the preferred nitrogen source. This result corresponds with studies of Bisson (*35*). According to this author, in grape juice all amino acids appeared to be taken up at the early time of fermentation and especially amino acids originally present in low concentrations, such as Trp, were consumed prior to the onset of the growth phase.

In accordance with the results of Kradolfer et al. (*36*) and Shin et al. (*37*), TOH was identified as the main degradation product of Trp in *Saccharomyces*. Between the concentration of Trp in the must and TOH in the wine, a significant correlation ($\alpha < 0.01$) was observed, although the range of the TOH formation rate was very wide (cf. Figure 5). Between 0.04 and 57 mol-% (on average 12 mol-%) of the metabolized Trp was degraded

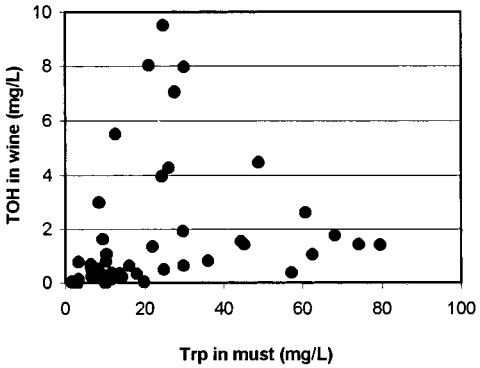

Figure 5. Correlation between the Trp concentration of a must and the TOH concentration of the corresponding wine ($n = 48$).

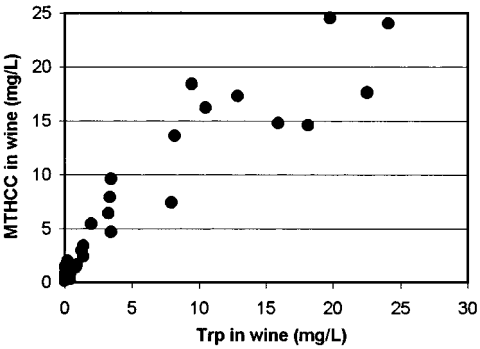

Figure 6. Correlation between the concentration of Trp and MTHCC in wine ($n = 48$).

to TOH. Since TOH was not detectable in musts before fermentation, it can be assumed as a marker for microbiological activity. The degradation pathway of Trp to TOH occurs through the "Ehrlich-pathway", which includes an oxidative transamination to indole-3-pyruvic acid, a decarboxylation to indole-3-acetaldehyde, and a hydrogenation to TOH (*36*−*39*). According to Shin et al. (*37*), IAA and as a byproduct indole-3-aldehyde were formed from indole-3-pyruvate, too, but to a much smaller extent. In this study, no significant correlation was observed between the concentration of Trp in the must and the concentration of IAA in the wine ($\alpha > 0.05$). Regarding the formation of IAA, it has to be clarified whether IAA is formed by the yeast metabolism from Trp and/or by a release from its conjugates, since about 70% of conjugated IAA was hydrolyzed during fermentation. Furthermore, the total amount of free plus conjugated IAA in the wine was mostly the same or even lower than the total amount of IAA in the must (cf. Figure 2).

The metabolic pathway of Trp to AA by *Candida lipolytica* was reported by Schindler and Zähner (*40*). However, in this study AA was not detectable (<0.011 mg/L). This finding is in accordance with studies of Shin et al. (*41*). After incubation of *Saccharomyces* with radiolabeled Trp in a broth medium, they found that only 2% of added Trp went through the kynureninase flux (alanine liberation) and they did not detect AA.

Besides AA, in the examined grape musts and wines NFK was not detectable (<0.24 mg/L). This may be due to its relatively high detection limit, since Dollmann et al. (*20*) detected up to 94 μg/L of the deformylation product kynurenine in different musts using HPLC with electrospray ionization tandem mass spectrometry detection (HPLC-ESI-MS-MS).

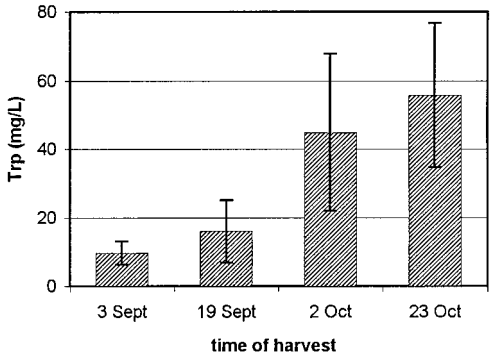

Figure 7. Trp content in grape juices, extracted from grapes harvested at four different times during grape ripeness (arithmetic means, standard deviations; $n = 39$).

Besides TOH, MTHCC was found as another abundant degradation product of Trp. Unlike TOH, MTHCC is not a typical fermentation product formed by yeast metabolism. MTHCC is formed by reaction of Trp with acetaldehyde, which is produced by the yeast during fermentation. Thus, the concentration of MTHCC in the wine was generally higher than in the corresponding must. A significant correlation ($\alpha < 0.01$) was found between the concentration of Trp and MTHCC in the wine (Figure 6). If the Trp content of the wine was low, only traces of MTHCC could be observed. With increasing Trp content, the amount of MTHCC increased as well. However, at high Trp concentrations the formation of MTHCC seems to reach a plateau. This may be due to the concentration of available acetaldehyde, which limited the formation of MTHCC.

Influences of Different Viticultural Measures on Trp and Trp Metabolites. To study the influences of different viticultural measures on the amounts of Trp and Trp metabolites in must and wine, an ANOVA was carried out. Except for the vine prune (10 or 20 buds/vine), the different applied measures partly exert significant influences ($\alpha < 0.01$) on the amounts of IAA and Trp in the musts or wines and on the amounts of the examined Trp metabolites formed during fermentation.

With the stage of maturity, the amounts of bound IAA and free and bound Trp in the grapes increased significantly ($\alpha < 0.01$). In the wines produced from late harvested grapes, significantly higher ($\alpha < 0.01$) amounts of Trp metabolites were analyzed. By analysis of 39 grape juices, extracted from grapes harvested at four different times during grape ripeness (3 Sept; 19 Sept; 2 Oct; 23 Oct) a significant increase ($\alpha < 0.01$) in the Trp content with advanced ripeness could be observed (Figure 7). This finding confirms the well-known fact that the nitrogen or amino acid contents of the grape musts and wines increase with the ripeness of the harvested grapes (*10*, *33*, *42*−*45*).

A reducing of the leaf area to 50% by leaf plucking resulted in significantly lower concentrations of bound IAA in the musts ($\alpha < 0.01$) (Figure 8). The amounts of free and bound Trp in the musts or the examined Trp metabolites in the wines decreased as well. According to Löhnertz et al. (*45*), a defoliation leads to a loss of amino acids stored in the leaves and partial movement to the berries during veraison. Moreover, a defoliation reduces the protection of the grapes against sun rays. The resulting higher temperatures in the grapes lead to a higher protein synthesis and therefore to lower amounts of free amino acids.

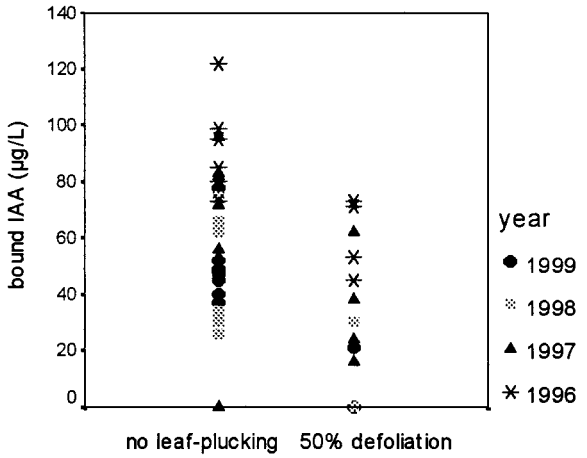

Figure 8. Influence of a leaf plucking on the amount of bound IAA in musts ($n = 48$).

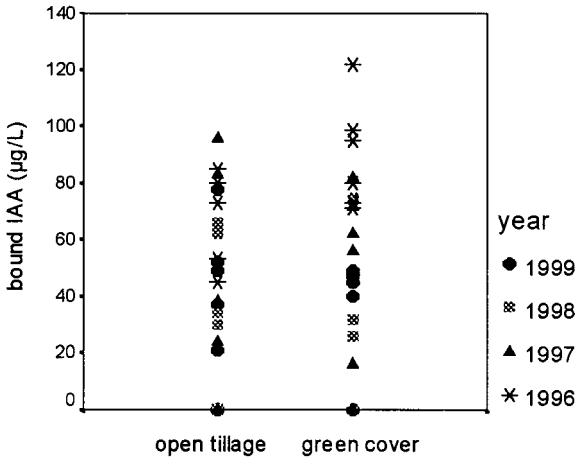

Figure 9. Influence of the soil treatment on the amount of bound IAA in musts ($n = 48$).

The effect of the soil treatment (open tillage, permanent green cover crop) varied and depended on the respective climatic conditions of the vintage. In the examined musts, only the Trp content was significantly affected ($\alpha < 0.01$). For IAA, there was a significant interaction ($\alpha < 0.05$) between the soil treatment and the vintage of the respective grapes (Figure 9). Especially in dry years, such as 1999, higher amounts of bound IAA or free and bound Trp were detected in the musts produced from vines from an open tillage. However, in the vintage 1996 (a relatively damp year), the open tillage led to significantly lower amounts of bound IAA or free and bound Trp. This interaction between the cultivation system and the climate may be due to the following factors: Besides the nitrogen and water competition by the plants, the permanent green cover crop leads to a lower water deposition in the deeper layers of the soil. Since the nitrification is lower in dry soils, the nitrate concentration in soils of a permanent green cover crop is usually small. Especially in dry years, these lower nitrogen conditions lead to a lower storage of nitrogen in the grapes (*12*, *45*−*47*). However, in damp years with a high rainfall, the washing out of the water-soluble nitrate is higher in open tilled soils than in green covered soils. Therefore, in these years, the storage of nitrogen in the grapes is higher in vines cultivated by a permanent cover crop.

Since the climatic conditions of a year play an important part in the storage of nitrogen compounds in the grapes (*43*, *44*, *48*), a significant influence ($\alpha < 0.01$) of the vintage on the amounts of IAA and Trp in the musts and the examined Trp metabolites in the wines was observed. Musts and wines of the 1996 vintage, a year with a relatively high and constant rainfall, contained significantly higher amounts of Trp than those of 1997−1999 (cf. Figure 2). In this damp year, the intake of nitrogen compounds from the soil in the grapes was increased and the protein synthesis was reduced. On the other hand, in the dry year 1999, the amount of free Trp in the must was low, but the fraction of bound Trp was high as compared with that of 1996−1998.

Relation between Trp and Trp Metabolites and the Occurrence of UTA. As the main causes of UTA formation, dryness, high yield, and an early grape vintage are under consideration (*12*, *14*). However, if the nutritional supply for the vines is sufficient, a high crop load does not increase the potential of a wine to express UTA (*3*). Regarding different viticultural measures, it turned out that UTA-supporting measures led to comparatively lower amounts of the AAP-precursor IAA, Trp, or the examined Trp metabolites in musts and wines. For example, lower amounts of Trp, IAA, and other Trp metabolites were analyzed in musts and wines of a dry vintage as well as in musts and wines from an early grape vintage. However, the level of yield (10 or 20 buds/vine) did not significantly affect the amounts of Trp and Trp metabolites.

Therefore, it seems that the occurrence of UTA is related to a low content of nitrogen substances in the must or wine as discussed by Löhnertz (*12*) and Löhnertz et al. (*45*). Consequently, the appearance of UTA seems not to be directly caused by a higher amount of the precursor IAA in the must or wine but rather to be connected with a nitrogen deficiency of the harvested grapes. In this study, it could be shown that the Trp utilization during fermentation was nearly 100% if the Trp concentrations of the must were low. Therefore, it has to be investigated whether the metabolic fate of Trp in *Saccharomyces cerevisiae* is influenced by the nitrogen supply of the must. However, according to Köhler et al. (*49*), a legally permitted addition of 30 g/hL diammoniumphosphate has no significant effect on UTA formation.

Regarding the development of UTA, it was shown that the aroma impact substance AAP can be formed by an oxidative degradation of IAA after fermentation and sulfuration of the wine (*19*). Therefore, the metabolic synthesis of IAA by the yeast and the finally resulting IAA concentration in the wine before sulfuration have to be further researched.

In summary, it can be stated that the amounts of Trp and bound IAA in the must or the amounts of Trp metabolites in the wine are affected by the climatic conditions of the vintage and by different viticultural measures such as the time of harvest, the soil treatment, and leaf plucking. However, UTA-supporting measures led to comparatively lower amounts of Trp and the examined Trp metabolites in musts and wines. Therefore, the formation of UTA seems more likely to be related to a low content of nitrogen substances and not to a formation of higher amounts of IAA in the grapes.

ABBREVIATIONS USED

AA, anthranilic acid; AAP, 2-aminoacetophenone; ANOVA, multifactorial analysis of variance; BHT, 2,6-

di-*tert*-butyl-*p*-kresol; IAA, indole-3-acetic acid; IAA-Asp, *N*-(3-indolylacetyl)-DL-aspartic acid; ILA, indole-3-lactic acid; IPA, indole-3-propionic acid; IS, internal standard; LWL, H-Ala-Trp-Ala-OH; 5-MeO-Trp, 5-methoxy-tryptophan; MTHCC, 1-methyl-1,2,3,4-tetrahydro-*β*-carboline-3-carboxylic acid; NFK; *N*-formylkynurenine; SPE, solid-phase extraction; SAX, strong anion exchange; TFA, trifluoroacetic acid; Trp, tryptophan; TOH, tryptophol; UTA, untypical aging off-flavor.

ACKNOWLEDGMENT

We thank the Landesanstalt für Weinbau und Gartenbau, Veitshöchheim, for providing the must and wine samples.

LITERATURE CITED

(1) Rapp, A.; Versini, G.; Ullemeyer, H. 2-Aminoacetophenon: Verursachende Komponente der "Untypischen Alterungsnote" ("Naphthalinton", "Hybridton") bei Wein. *Vitis* **1993**, *32*, 61−62.

(2) Christoph, N.; Bauer-Christoph, C.; Geβner, M.; Köhler, H. J. Die "Untypische Alterungsnote" im Wein, Teil I: Untersuchungen zum Auftreten und zur sensorischen Charakterisierung der "Untypischen Alterungsnote". *Rebe Wein* **1995**, *48*, 350−356.

(3) Rapp, A.; Versini, G. Fehlaroma: Die untypische Weinalterung. *Dtsch. Weinbau* **1995**, *18*, 18−22.

(4) Hühn, T.; Sponholz, W. R.; Gafner, J. Einflussfaktoren der vorzeitigen und untypischen Weinalterung. *Schweizer. Z. Obst Weinbau* **1997**, *133*, 636−638.

(5) Eder, R. Mängel, Fehler und Krankheiten der Weine. Ursachen, Vermeidung und Behebung. Teil 10: Untypische Alterungsnote (UTA). *Winzer* **1998**, *54*, 13−14.

(6) Geβner, M.; Köhler, H. J.; Christoph, N.; Bauer-Christoph, C.; Miltenberger, R.; Schmitt, A. Die "Untypische Alterungsnote im Wein, Teil II: Beschreibende Verkostung von UTA-Weinen; Beziehungen zwischen Sensorik und chemisch-physikalischen Analysenwerten. *Rebe Wein* **1995**, *48*, 388−394.

(7) Fischer, U.; Sponholz, R. Die sensorische Beschreibung der Untypischen Alterungsnote. *Dtsch. Weinbau* **2000**, *3*, 16−21.

(8) Rapp, A. Den Geheimnissen der Alterung des Weines auf der Spur. *Winzer-Z.* **1995**, *10*, 32−35.

(9) Müller, E.; Peternel, M.; Löhnertz, O. Aktuelle Forschungsergebnisse: Stickstoff und Qualität. *Dtsch. Weinbau* **1998**, *7*, 14−17.

(10) Köhler, H. J.; Christoph, N.; Geβner, M.; Bauer-Christoph, C. Die "Untypische Alterungsnote" im Wein, Teil III: Zusammenhänge zwischen dem Auftreten der "Untypischen Alterungsnote" und dem Reifestadium der Trauben (Lesetermin). *Rebe Wein* **1995**, *48*, 424−430.

(11) Wohlfahrt, P. Untypische Alterungsnote: Erfahrungen aus den vergangenen drei Jahren. *Badische Winzer* **1995**, *20*, 383−388.

(12) Löhnertz, O. UTA und Rebenernährung. Streβ macht alt. *Dtsch. Weinmagazin* **1996**, *18*, 18−23.

(13) Schwab, A.; Peternel, M.; Köhler, H. J.; Heigel, K. P. Die "Untypische Alterungsnote" im Wein, Teil IV: Beeinflussung durch weinbauliche Maβnahmen. *Rebe Wein* **1996**, *49*, 181−187.

(14) Schwab, A.; Christoph, N.; Köhler, H. J.; Geβner, M.; Simat, T. J. Einfluβ weinbaulicher Maβnahmen auf die Ausprägung der Untypischen Alterungsnote bei Weiβweinen. Teil I: Einfluβ des Lesezeitpunktes. *Vitic. Enol. Sci.* **1999**, *54*, 114−120.

(15) Rapp, A.; Versini, G.; Ullemeyer, H.; Engel, L. Nachweis und Bestimmung von 2-Aminoacetophenon in vergorenen Modellösungen. *Vitis* **1995**, *34*, 193−194.

(16) Christoph, N.; Bauer-Christoph, C.; Geβner, M.; Köhler, H. J. Die "Untypische Alterungsnote" im Wein, Teil VI: Untersuchungen zur Bildung von *o*-Aminoacetophenon aus Produkten des Tryptophan-Stoffwechsels vor der alkoholischen Gärung. *Rebe Wein* **1996**, *49*, 246−250.

(17) Dollmann, B.; Wichmann, D.; Schmitt, A.; Köhler, H. J.; Schreier, P. Formation of the "untypical aging off-flavour" in wine: Generation of 2-aminoacetophenone in model studies with *Saccharomyces cerevisiae*. *Vitic. Enol. Sci.* **1996**, *51*, 122−125.

(18) Geβner, M.; Köhler, H. J.; Christoph, N.; Bauer-Christoph, C. Die "Untypische Alterungsnote" im Wein, Teil VII: Untersuchungen zur Bildung von o-Aminoacetophenon aus Produkten des Tryptophan-Stoffwechsels bei der alkoholischen Gärung. *Rebe Wein* **1996**, *49*, 251−255.

(19) Christoph, N.; Bauer-Christoph, C.; Geβner, M.; Köhler, H. J.; Simat, T. J.; Hoenicke, K. Bildung von 2-Aminoacetophenon und Formylaminoacetophenon im Wein durch Einwirkung von schwefliger Säure auf Indol-3-essigsäure. *Vitic. Enol. Sci.* **1998**, *53*, 79−86.

(20) Dollmann, B.; Richling, E.; Herderich, M.; Köhler, H. J.; Schmitt, A.; Schwab, A.; Schreier, P. High Performance liquid chromatography-electrospray ionization tandem mass spectrometry (HPLC-ESI-MS-MS) for the quantification of L-kynurenine and indole-3-acetic acid in grape must by isotope dilution assay. *Vitis* **1997**, *36*, 97−101.

(21) Hoenicke, K.; Simat, T. J.; Steinhart, H.; Christoph, N.; Köhler, H. J.; Schwab, A. Determination of tryptophan and tryptophan metabolites in grape musts and wine. *Adv. Exp. Med. Biol.* **1999**, *467*, 671−677.

(22) Mattivi, F.; Vrhovšek, U.; Versini, G. Determination of indole-3-acetic acid, tryptophan and other indoles in must and wine by high-performance liquid chromatography with fluorescence detection. *J. Chromatogr. A* **1999**, 855, 227−235.

(23) Cohen, J. D.; Bandurski, R. S. Chemistry and physiology of the bound auxins. *Annu. Rev. Plant Physiol.* **1982**, *33*, 403−430.

(24) Bandurski, R. S.; Schulze, A. Concentration of Indole-3-acetic Acid and its Derivates in Plants. *Plant Physiol.* **1977**, *60*, 211−213.

(25) Bialek, K.; Cohen, J. D. Quantification of Indoleacetic Acid Conjugates in Bean Seeds by Direct Tissue Hydrolysis. *Plant Physiol.* **1989**, *90*, 398−400.

(26) Ilic, N.; Normanly, J.; Cohen, J. D. Quantification of Free Plus Conjugated Indoleacetic Acid in *Arabidopsis* Requires Correction for the Nonenzymatic Conversion of Indolic Nitriles. *Plant Physiol.* **1996**, *111*, 781−788.

(27) Riov, J.; Bangerth, F. Metabolism of Auxin in Tomato Fruit Tissue. *Plant Physiol.* **1992**, *100*, 1396−1402.

(28) Sitbon, F.; Östin, A.; Sundberg, B.; Olsson, O.; Sandberg, G. Conjugation of Indole-3-Acetic Acid (IAA) in Wild-Type and IAA-Overproducing Transgenic Tobacco Plants, and Identification of the Main Conjugates by Frit-Fast Atom Bombardment Liquid Chromatography−Mass Spectrometry. *Plant Physiol.* **1993**, *101*, 313−320.

(29) Simat, T. J.; Meyer, K.; Steinhart, H. Synthesis and analysis of oxidation and carbonyl condensation compounds of tryptophan. *J. Chromatogr. A* **1994**, *661*, 93−99.

(30) Box, G. E. P.; Behnken, D. W. Some new three level designs for the study of quantitative variables. *Technometrics* **1960**, *2*, 455−475.

(31) Bergner, K. G.; Haller, H. E. Das Verhalten der freien Aminosäuren von Weiβwein im Verlauf der Gärung, bei Ausbau, Lagerung und Umgärung. *Mitt. Klosterneuburg* **1969**, *19*, 264−288.

(32) Polo, M. C.; Llaguno, C. Changes in free amino acids during the alcoholic fermentation of grape juice by flower yeasts. II: Composition of wines and yeast cells. *Connaiss. Vigne Vin*. **1974**, *8*, 321−342.

(33) Sponholz, W. R. Nitrogen Compounds in Grapes, Must, and Wine. In *Proceedings of the International Symposium on Nitrogen in Grapes and Wine*; Rautz, J. M., Ed.; Seattle, WA, 18−19 June 1991; pp 67−77.
(34) Jones, M.; Pierce, J. S. Adsorption of amino acids from worth by yeast. *J. Instit. Brew.* **1964**, *70*, 307−315.
(35) Bisson, L. F. Influences of Nitrogen on Yeast and Fermentation of Grapes. In *Proceedings of the International Symposium on Nitrogen in Grapes and Wine*; Rautz, J. M., Ed.; Seattle, WA, 18−19 June 1991; pp 78−89.
(36) Kradolfer, P.; Niederberger, P.; Hütter, R. Tryptophan degradation in *Saccharomyces cerevisiae*: Characterisation of two aromatic aminotransferases. *Arch. Microbiol.* **1982**, *133*, 242−248.
(37) Shin, M.; Shinguu, T.; Sano, K.; Umezawa, C. Metabolic Fates of L-Tryptophan in *Saccharomyces uvarum* (*Saccharomyces carlsbergensis*). *Chem. Pharm. Bull.* **1991**, *39*, 1792−1795.
(38) Ehrlich, F. Über Tryptophol (*β*-Indolyläthylalkohol), ein neues Gärprodukt der Hefe aus Aminosäure. *Ber. Dtsch. Chem. Ges.* **1912**, *45*, 883.
(39) Lingens, F.; Goebel, W.; Uesseler, H. Regulation der Biosynthese der aromatischen Aminosäuren in *Saccharomyces cerevisiae*. 2. Repression, Induktion und Aktivierung. *Eur. J. Biochem.* **1967**, *1*, 363−374.
(40) Schindler F.; Zähner, H. Stoffwechselprodukte von Mikroorganismen. Tryptanthrin, ein von Tryptophan abzuleitendes Antibioticum aus *Candida lipolytica*. *Arch. Microbiol.* **1971**, *79*, 187−203.
(41) Shin, M.; Sano, K.; Umezawa, C. Metabolism of Tryptophan to Niacin in *Saccharomyces uvarum*. *J. Nutr. Sci. Vitaminol.* **1991**, *37*, 269−283.
(42) Kliewer, W. M. Changes in the concentration of free amino acids in grape berries during maturation. *Am. J. Enol. Vitic.* **1968**, *19*, 166−174.
(43) Schrader, U.; Lemperle, E.; Becker, N. J.; Bergner, K. G. Der Aminosäure-, Zucker-, Säure- und Mineralgehalt von Weinbeeren in Abhängigkeit vom Kleinklima des Standortes der Rebe. II. Mitteilung Aminosäuregehalt. *Vitic. Enol. Sci.* **1976**, *31*, 9−24.
(44) Tusseau, D.; Benoit, C.; Valade, M. Amino acids contents during grape ripening. In *Actualities Oenologique 89, Comptes Rendus de 4. Symposium International d'œnologie;* Ribereau-Gayon, P., Lonvaud, A., Eds.; Bordeaux, France, 15−17 Juin 1989; pp 20−24.
(45) Löhnertz, O.; Prior, B.; Bleser, B.; Linsenmeyer, A. Einfluss von weinbaulichen Ma*β*nahmen auf die Aminosäuregehalte in Trauben und Mosten der Sorte Riesling. In *Intervitis Interfructa. 5. Internationales Symposium: Innovationen in der Kellerwirtschaft. Mikroorganismen und Weinbereitung*; Stuttgart, Germany; 11−12 June 1998; pp 1−23.
(46) Redl, H. Gärungsprobleme und Beeinträchtigungen der Weinqualität durch Weingartenbegrünung? *Winzer* **1999**, *55*, 6−11.
(47) Schaller, K. Influence of different soil tillage systems on uptake of N, P, K, Mg, Ca, and organic N in berries during growth and development of the variety white Riesling. *Bull. de l'O.I.V.* **1999**, *72*, 602−629.
(48) Flancy, D.; Poux, C. Notes sur la teneur en acides aminés du moût de raisin et du vin function de conditions de l'année. *Ann. Technol. Agric.* **1965**, *14*, 87−88.
(49) Köhler, H. J.; Christoph, N.; Bauer-Christoph, C.; Ge*β*ner, M.; Curschmann, K. Die "Untypische Alterungsnote" im Wein, Teil V: Einflu*β* kellerwirtschaftlicher Ma*β*nahmen auf die Ausprägung der UTA. *Rebe Wein* **1996**, *49*, 213−218.

Received for review May 3, 2001. Revised manuscript received August 4, 2001. Accepted August 4, 2001. This research project was supported by the Weinbauring Franken e.V. and the FEI (Forschungskreis der Ernährungsindustie e.V., Bonn), the AIF and the Ministry of Economics and Technology. AIF Project No. 11770 N.

JF010575V