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Metabolism of tryptophan and indole-3-acetic acid formation during vinification and its influence on the formation of 2-aminoacetophenone

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The tryptophan (Trp) metabolite indole-3-acetic acid (IAA) is considered to be a potential precursor of 2-aminoacetophenone (AAP), an aroma compound supposed to cause the "untypical aging off-flavour" (UTA) in wine. The Trp metabolism during the vinification of grapes from Vitis vinifera *cv. 'Müller Thurgau' harvested at two different ripeness stages was investigated. For the fermentations free run juice, a fraction extracted under light pressure as well as the mashes were inoculated with three different commercial yeasts and two wild yeast strains, respectively. Fermentations were performed without or with addition of nutrient supplements and at two different temperatures. Trp and its metabolites IAA (unbound and conjugated), indole-3-ethanol (tryptophol, TOH), indole-3-lactic acid (ILA), as well as 1-methyl-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid (MTHCC) were determined at seven different stages of vinification by RP-HPLC and fluorescence detection. AAP was determined in the wines after ten months of storage and a heat treatment of 72 h at 40 °C by GC-MS. It could be shown that Trp is almost completely consumed by the yeast within the first phase of fermentation. Of the initial amount of Trp 1 to 10 % reacted with acetaldehyde yielding MTHCC, 6 to 22 % were metabolised yielding TOH, whereas less than 5 % were metabolised yielding ILA and IAA, respectively. IAA is shown to be formed by the yeasts both by hydrolysis of conjugated IAA from the must and by neosynthesis. The amount of unbound IAA prior to sulphuration was significantly lower in the samples from the late harvested grapes, mash fermentations, inoculation with commercial yeast strains and addition of a nutrient supplement produced from inactive yeasts, respectively. The amount of AAP increased with early harvested grapes and inoculation with wild yeast strains. A significant but low correlation was found between the content of unbound IAA prior to sulphuration and the amount of AAP.*

Key Words: *Vitis vinifera*, untypical aging off-flavour (UTA), Trp metabolism, indole-3-acetic acid, tryptophol, indole-3-lactic acid, 1-methyl-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid, fermentation, ripeness, yeast

Stoffwechsel des Tryptophans und die Bildung von Indol-3-essigsäure während der Weinbereitung und deren Einfluss auf die Entstehung von 2-Aminoacetophenon. *Der Tryptophanmetabolit Indol-3-essigsäure (IAA) ist eine potenzielle Vorstufe von 2-Aminoacetophenon (AAP), das die Hauptaromakomponente der "Untypischen Alterungsnote" (UTA) in Weißweinen ist. Gegenstand dieser Untersuchung ist der Tryptophanstoffwechsel während der Weinbereitung. Trauben von* Vitis vinifera *cv. 'Müller Thurgau' wurden in zwei unterschiedlichen Reifestadien geerntet. Von diesen wurden Moste, Pressmoste sowie die Maischen zur Fermentation nach Inokulation mit drei verschiedenen Kultur- und zwei Wildhefen eingesetzt. Die Gärung wurde bei zwei verschiedenen Temperaturen sowie mit und ohne Zusatz von zwei Hefenährstoffen durchgeführt. Tryptophan (Trp) und seine Metabolite Indol-3-essigsäure (ungebunden und konjugiert), Indol-3-ethanol (Tryptophol, TOH), Indol-3-milchsäure (ILA) sowie 1-Me-*

thyl-1,2,3,4-tetrahydro-β-carbolin-3-carbonsäure (MTHCC) wurden zu sieben Zeitpunkten der Gärung sowie nach Lagerung mittels RP-HPLC und Fluoreszenzdetektion bestimmt. AAP wurde nach zehnmonatiger Lagerung und Wärmebehandlung (72 h, 40 °C) mittels GC-MS quantifiziert. Trp wurde bereits in der ersten Phase der Gärung von den Hefen vollständig verbraucht. Von der Ausgangsmenge reagierten 1 bis 10 % mit Acetaldehyd unter Bildung von MTHCC, 6 bis 22 % wurden zu TOH umgesetzt, während weniger als 5 % zu ILA und IAA metabolisiert wurden. IAA wurde während der Gärung sowohl aus der mosteigenen konjugierten IAA freigesetzt als auch von den Hefen synthetisiert. Die Menge der ungebundenen IAA in dem Gärmedium vor der Schwefelung war signifikant geringer bei Verwendung spät gelesener Trauben, bei der Maischegärung, bei Verwendung von Reinzuchthefen sowie nach Zusatz eines Hefenährstoffes. Signifikant höhere Gehalte an AAP waren in den frühgelesenen Weinen und bei Vergärung mit Wildhefen aufzufinden. Der Gehalt ungebundener IAA vor der Schwefelung des Gärmediums zeigte eine geringe, jedoch signifikante Korrelation mit dem AAP-Gehalt der korrespondierenden Weine.

Schlagwörter: *Vitis vinifera*, Untypische Alterungsnote (UTA), Tryptophanmetabolismus, Indol-3-essigsäure, Tryptophol, Indol-3-milchsäure, 1-Methyl-1,2,3,4-tetrahydro-β-carbolin-3-carbonsäure, Gärung, Reife, Hefe

Le métabolisme du tryptophane et la formation d'acide indole-3-acétique au cours de la vinification et leur influence sur la formation de 2-aminoacetophenone. *Le métabolite du tryptophane (Trp), l'acide indole-3-acétique (IAA), est un précurseur potentiel du 2-aminoacetophenone (AAP), qui est la composante principale de l'arôme du « vieillissement atypique» (UTA) dans les vins blancs. Le métabolisme du Trp au cours de la vinification a fait l' objet de la présente recherche. Les raisins de* Vitis vinifera *cv. 'Müller Thurgau' ont été récoltés dans deux états de maturité différents. Leurs moûts, leurs moûts de pressage et leurs vendanges foulées ont été utilisés aux fins de fermentation après inoculation avec trois différentes levures de culture et deux levures sauvages. La fermentation a été effectuée à deux températures différentes, avec et sans addition de deux substances nutritives pour levures. Le Trp et ses métabolites, IAA (libre et conjuguée), indole-3-éthanole (tryptophole, TOH), l'acide indole-3-lactique (ILA) ainsi que l'acide 1-méthyl-1,2,3,4-tétrahydro-β-carboline-3-carboxylique (MTHCC) ont été déterminés à sept moments au cours de la fermentation et du stockage au moyen du RP-HPLC et de la détection par fluorescence. Le AAP a été quantifié après un stockage de dix mois et traitement thermique (72 h, 40 °C) au moyen de GC-MS. Le Trp a été entièrement consommé par les levures au cours de la première étape de fermentation déjà. De la quantité initiale, 1 à 10 % ont réagi avec l'acétaldéhyde en formant du MTHCC, 6 à 22 % ont été transformés en TOH, tandis que moins de 5 % ont été métabolisés en ILA et en IAA. Au cours de la fermentation, IAA a été libéré de IAA conjugué propre au moût, mais a également été synthétisé par les levures. La quantité d'IAA libre dans le milieu de fermentation avant le sulfitage était significativement moins élevée que lors de l'utilisation de raisins vendangés tardivement, lors de la macération, lors de l'utilisation de levures sélectionnées et après l'addition d'une substance nutritive pour levures. Des teneurs significativement plus élevées en AAP ont été trouvées dans les vins vendangés précocement et en cas d'une fermentation avec des levures sauvages. La teneur en IAA avant le sulfitage du milieu de fermentation présentait une corrélation faible, mais significative avec la teneur en AAP des vins correspondants.*

Mots clés : *Vitis vinifera*, vieillissement atypique (UTA), métabolisme de tryptophane, acide indole-3-acétique, tryptophole, acide indole-3-lactique, acide 1-methyl-1,2,3,4-tetrahydro-β-carboline-3-carboxylique, fermentation, maturation, levure

Tryptophan (Trp) and its metabolites, especially the phytohormone indole-3-acetic acid (IAA), are considered to be potential precursors of 2-aminoacetophenone (AAP), an aroma compound which is associated with the so called "untypical aging off-flavour" (UTA) in *Vitis vinifera* white wines (Christoph et al., 1996; Gessner et al., 1996; Rapp and Versini, 1995; Rapp et al., 1993). The off-flavour is described by aroma descriptors such as "acacia blossom", "naphthalene", "furniture polish", "wet wool" or "fusel alcohol". Depending on the intensity of the wine flavour, UTA can be realized organoleptically at 0.5 to 1.5 µg/l AAP (Fischer and Sponholz, 2000; Christoph et al., 1995; Gessner et al., 1995). Studies on the formation of UTA have revealed a significant correlation between sensorily detectable UTA and high AAP-concentrations, and wines from grapes which were grown under stress by insufficient water or nitrogen supply, as well as wines produced from high yield or early harvested grapes (Schwab et al. 1996; Köhler et al., 1995; Rapp and Versini,

1995). Investigations on the mechanism of AAP formation have shown that AAP can be formed by an oxidative degradation of IAA, which is triggered by a sulphuration of the young wine. However, no significant formation of AAP was observed for the ester or amide conjugated IAA, indicating that only unbound IAA is susceptible to this oxidative degradation (CHRISTOPH et al., 1998). Grape musts contain very low amounts of unbound IAA (< 3 µg/l) indicating a strong physiological regulation of this phytohormone (HOENICKE et al., 2001a). About 95 % of total IAA were bound either as ester or amide conjugates (HOENICKE et al., 2001a; COHEN and BANDURSKI, 1982). The amount of unbound IAA increases significantly during fermentation, resulting in higher amounts of unbound IAA in the wine (<3 to 90 µg/l) (HOENICKE et al., 2001a and b).
In this paper, the metabolism of Trp and the formation of unbound IAA and other Trp metabolites was investigated during fermentation of musts resulting from both early and late harvested grapes and different enological parameters (e.g. nutrient supply, fermentation temperature, yeast strain). An examination was made of whether conjugated IAA can be hydrolysed by the yeast yielding unbound IAA and/or unbound IAA is synthesized by the yeast. Finally, an evaluation was made of whether the amount of unbound IAA prior to and after sulphuration correlates with the amount of AAP formed during storage of the corresponding wines.

Material and methods

Musts and mash

The grapes (*Vitis vinifera* cv. 'Müller-Thurgau') were grown and vinificated at the Bayerische Landesanstalt für Weinbau und Gartenbau (LWG, Veitshöchheim, Germany). The vines were cultivated under usual viticultural conditions (natural green cover crop, yield: 90 hl/ha) and harvested at two different dates (early harvest; late harvest); free-run juice and a fraction obtained under light pressure (pressure juice) were separated. Fermentation of both juice fractions and fermentation of the crushed grapes (mash fermentation) were conducted. Basic chemical analyses were carried out by the LWG Veitshöchheim (Tab. 1).

Fermentations

Fermentations were performed in 2.5 l flasks. For inoculation three different commercial yeast strains (*Saccharomyces cerevisiae* cv. Uvaferm CM, *Saccharomyces cerevisiae* cv. Lalvin W, *Saccharomyces cerevisiae* var. *bayanus* cv. Lalvin E) as well as two different wild yeast strains (*Kloeckera apiculata, Metschnikovia pulcherima*) were used (10 g/hl). For each experiment an initial volume of 2 l of must or mash was used. With mash fermentation the pomace was removed at the end of fermentation.

Table 1:
Analytical parameters of the musts used for fermentation

	Early harvest (12th Sept. 2000)			Late harvest (4th Oct. 2000)		
	Free run juice	Pressure juice	Mash (after 20h)	Free run juice	Pressure juice	Mash (after 20h)
°Oechsle	75	77	73	75	75	77
Sugar (g/l)	170	177	166	180	178	183
Total acids (g/l)	8.9	7.5	8.5	6.4	6.6	6.6
pH-value	2.96	3.10	2.87	3.35	3.41	3.40
Conductivity (µS/cm)	2640	2730	2070	2860	3080	2490
Polyphenols (mg/l gallic acid)	215	234	246	293	333	387
Total nitrogen (mg/l)	352	483	n.d.	462	630	n.d.

n.d. = not determined

For nutrient supplementation either diammoniumhydrogenphosphate (DAP) or Fermaid® (Lallemand) were added to the must. DAP provides the yeast with ammonia as nitrogen source and phosphate as phosphorous source and was supplemented in amounts of 1 g/l. Fermaid® is produced from inactive yeasts and other ingredients. It is to provide the yeast with nitrogen, vitamins, minerals and lipids (sterols, fatty acids) and was added in amounts of 0.4 g/l. Fermentations were carried out at two different temperatures, 12 to 14 °C and 22 to 24 °C, respectively. After fermentation samples were sulphurized with 120 mg/l potassium bisulphite and stored at 14 °C for five days. After that the yeast was separated from the young wine by filtration. Free sulphur dioxide was adjusted to 50 mg/l, and the wines were stored at 14 °C in the dark.
Samples for the determination of the extract (°Oe) were taken daily during fermentation; all other parameters were only analysed at the following eight stages: 1. must, 2. beginning fermentation, 3. turbulent fermentation, 4. middle of fermentation, 5. fading fermentation, 6. end of fermentation, 7. after sulphuration, 8. after three months of storage.

Chemicals

Indol-3-acetic acid (IAA), indole-3-ethanol (tryptophol, TOH), indole-3-lactic acid (ILA), and the internal standards 5-methoxy-Trp (5-MeO-Trp) and indole-3-propionic acid (IPA) were obtained from Sigma (Deisenhofen, Germany).
Tryptophan (Trp) was donated by Degussa (Hanau, Germany), 1-methyl-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid (MTHCC) was synthesized as described by Simat et al. (1994). Acetonitrile (gradient grade) and trifluoroacetic acid (Uvasol) were purchased from Merck (Darmstadt, Germany). Deionized water was purified by a bidestillator (Heraeus-Destamat Bi 18E, Kleinostheim, Germany). All other chemicals used were of analytical-grade purity.

Analysis of free and conjugated tryptophan metabolites and 2-aminoacetophenon

Tryptophan and its metabolites IAA, TOH, ILA, the carbonyl condensation compound MTHCC as well as conjugated Trp and conjugated IAA were analysed by RP-HPLC with fluorescence detection according to Hoenicke et al. (2001a). AAP was analysed after 10 months of storage at 14 °C in the dark and a heat treatment of 72 h at 40 °C by GC-MS according to Christoph et al. (1998).

Results and discussion

Influences on the time course of the fermentation of sugars

Although the musts of the early harvested grapes contain only about 76 % of the nitrogen content and 50 % of the Trp content (352 mg N/l, 8.5 mg unbound Trp/l) of those of the late harvested grapes (462 mg N/l, 17.4 mg unbound Trp/l), the time course of sugar fermentation is similar (Fig. 1). The initial fermentation velocity of the early harvested grapes seems to be slightly higher than that of the later harvested grapes. The fermentation velocity is highly dependent on the temperature (14 or 24 °C). A decrease of 10 °C doubles the duration of fermentation. The yeasts Lalvin E and Lalvin W tend to a slightly lower fermentation velocity than the Uvaferm CM yeast. The addition of nutritional supplements to the musts accelerated the fermentation: while the addition of Fermaid® exhibited a strong effect on the time course of alcoholic fermentation, only a slight effect was observed for the addition of DAHP (early harvest > late harvest, data not shown).

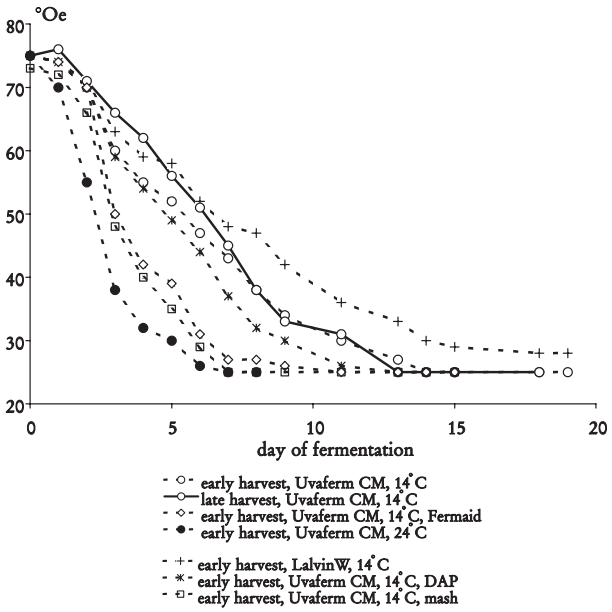

Fig. 1: Influences of grape ripeness and different vinification treatments on the time course of the fermentation of sugars

This indicates that components of Fermaid® (amino acids, vitamins, sterols, fatty acids) and to a lesser extent DAHP (inorganic nitrogen and phosphate) are limiting factors in the fermentation of sugars. The mash fermentation revealed a similar effect on the fermentation velocity to that of the Fermaid® supplementation.

Tryptophan utilization

Yeasts regulate their amino acid uptake by permeases (general amino acid permease (GAP) and specific permeases) in their plasma membrane. In the cells the amino acids may be incorporated in proteins, catabolized or stored/compartimentalized in vacuoles (WALKER, 1998).

The musts under investigation differ significantly in their Trp content with reference to the time of harvest (8.5 mg or 17.4 mg unbound Trp/l). During all fermentations using commercial yeast strains more than 95 % of the initial amount of Trp disappeared, indicating its efficient utilization for the production of biomass (structural proteins and functional enzymes). During the onset of fermentation 2 to 9 mg Trp/l are taken up by the yeasts per day. The Trp consumption is predominantly dependent on the fermentation temperature, which rules the growth of the microorganisms. Both yeasts from Lalvin (E and W) exhibit a slower Trp consumption than the Uvaferm CM yeast (Fig. 2a). JIRANEK et al. (1995) determined the Trp demand of eight different *S. cerevisiae* strains when all amino acids were in excess with 11 to 46 mg Trp/l in a medium containing 200 g/l glucose. In nitrogen limited media they found a 50 % depletion of the initial amount of Trp within 25 h, which is in the range of our results. A comparison of the consumption of Trp by the yeasts and the fermentation of sugars shows that Trp decrease and the connected formation of yeast biomass precedes the degradation of the carbohydrates. In the must of early harvested grapes with low content of Trp (8.5 mg/l) more than 95 % of the initial Trp is taken up by the yeast before a notable loss of sugar is detectable. In the must of late harvested grapes with a high content of Trp (17.4 mg/l) more than 95 % of the initial Trp is taken up by the yeast ahead of the turbulent phase of fermentation (Fig. 2b). In agreement with BISSON (1991) reported that amino acids present in low concentrations like Trp are generally utilized within the first drop of must weight (°Brix). After ethanol treatment of the yeast cells in order to permeabilise the plasma membrane they found high values of amino acids released

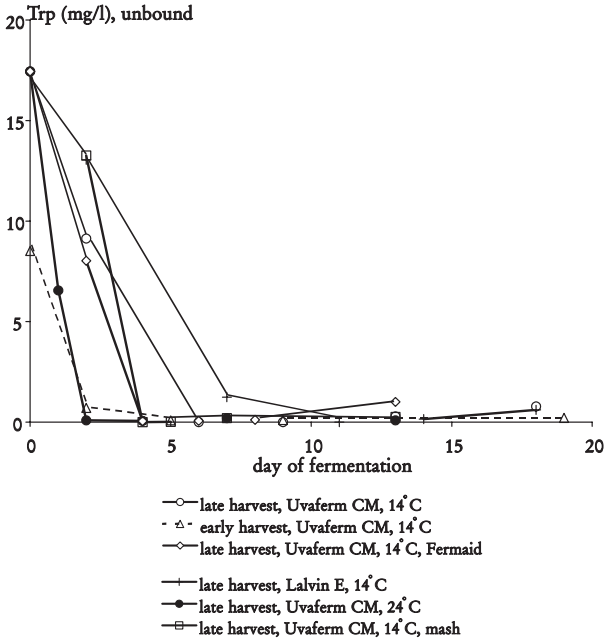

Fig. 2a: Consumption of unbound Trp. Influences of grape ripeness and different vinification treatments

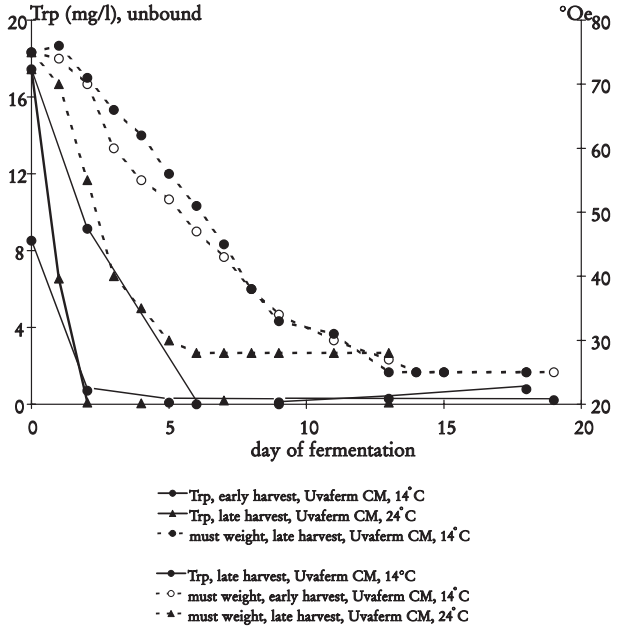

Fig. 2b: Consumption of unbound Trp. Time course of Trp depletion and fermentation of sugars

in the early fermentation, while at mid-fermentation amino acids could not be extracted due to low cellular levels, which indicates the utilization of free amino acids for the production of biomass in this stage of fermentation.
In contrast to DAP supplementation the addition of Fermaid® to the musts accelerated the Trp consumption, indicating again that a growth limiting factor in the musts but not ammonium nor phosphate is a constituent of Fermaid®. This effect seems to be stronger than the known ammonium ion inhibition of GAP expression (WALKER, 1998). Also mash fermentation of the grapes under investigation led to a faster Trp consumption. ANCIN et al. (1996) stated that chemical properties of the fermentation medium may influence the uptake and metabolism of nitrogen compounds: particularly, grape tissue particles stimulate the fermentation by providing nutritional factors like nitrogen substances, lipids and trace metals.
"Growth Factors" of yeasts may include vitamins, purines and pyrimidines, amino acids, fatty acids, sterols (in particular ergosterol) and other compounds. In particular biotin and panthothenic acid are known growth factors of *S. cerevisiae* (WALKER, 1998). BISSON (2001) investigated gene expression of yeasts in nitrogen limited cultures. She found these nitrogen deficient cultures to exhibit a higher expression of genes involved in the expression of biotin and of genes that are involved in the degradation of cellular compounds containing nitrogen, so that these can be reutilized in the syntheses of other nitrogen containing compounds.
At the end of fermentation the Trp content of the fermentation medium increased again, especially in those of late harvested grapes. MONTEIRO and BISSON (1991) also reported the liberation of amino acids in the last stage of fermentation, presumably due to release from yeast cells. At the end of fermentation free amino acids were once again available for release, probably as a consequence of protein turnover.

Formation of 1-methyl-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid

MTHCC is a stable product of the condensation reaction between Trp and acetaldehyde. Irrespective of the time of harvest low amounts of MTHCC (0.2 mg/l) were found in the musts prior to fermentation. Additional MTHCC is formed in the beginning of the fermentation parallel to the consumption of Trp and then remains stable even after three months of storage of the wine (Fig. 3a). Depending on the conditions of fermentation 1 to 10 mol% of the initial amount of free Trp react with acetaldehyde to yield MTHCC and are no longer utilizable by the yeast. The longer Trp is available during fermentation, the more MTHCC is formed: e.g. more MTHCC is formed during the relatively slower fermentation of the yeasts Lalvin E and Lalvin W, and during the fermentation of the musts of late harvested grapes (high Trp content) (Fig. 3b).

Deamination of tryptophan

Formation of tryptophol, indole-3-lactic acid, and indole-3-acetic acid

The deamination of amino acids, which is a common pathway in yeasts, utilizes the α-amino group as common nitrogen source in order to synthesize the nitrogen substances which are required for growth (BISSON, 1991). Thereby Trp is metabolized via indole-3-pyruvate (oxidative deamination) yielding ILA, and via indole-3-acetaldehyde (decarboxylation) yielding TOH (reduction) or IAA (oxidation) (SHIN et al., 1991; MARTENS and FRANKENBERGER, 1993) (Fig. 4).

Tryptophol

No TOH (< 10 µg/l) was detected in the musts prior to fermentation. TOH was released into the must by the yeasts later in the fermentation well after Trp utilization (Fig. 5a, b). In the low Trp containing musts of early harvested grapes the TOH formation occurred earlier than in the Trp rich musts, despite the lower concentration of the precursor. All fermentation batches of the Trp rich musts (late harvested grapes) showed a noticeable release of TOH measured after sulphuration in the young wine.
Highest TOH concentrations (>2 mg/l) were observed within all mash fermentations, and with most fermentations of late harvested grapes at higher temperatures (22 to 24 °C). This elucidates the influence of the fermentation temperature and the composition of the must on this pathway. Six to 22 mol% of the initial amount of Trp (without mash fermentations since "mash extractable" Trp could not be determined) are converted to TOH during the fermentations under investigation. Consequently, in yeasts Trp is mainly metabolised to TOH, besides incorporation into proteins, which was also reported by SHIN et al. (1991).
The higher alcohols like TOH are thought to be waste products of the yeasts, which are not further metabolized. During storage, however, a significant decrease of

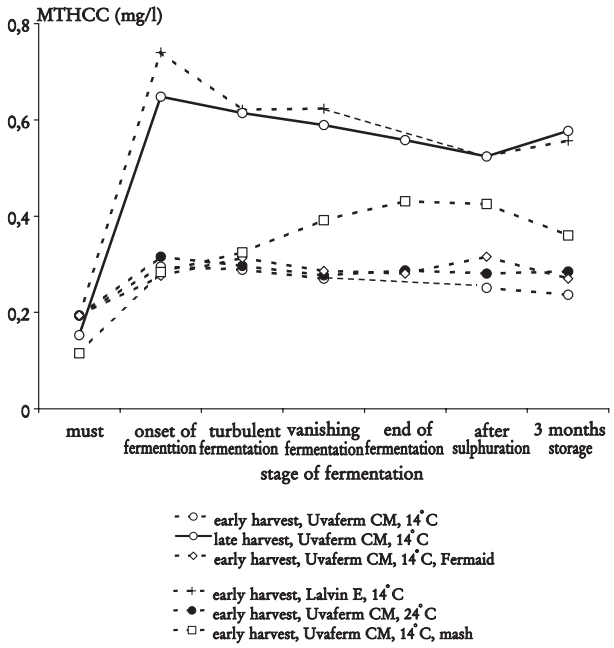

Fig. 3a: Formation of MTHCC from Trp. Influences of grape ripeness and different vinification treatments

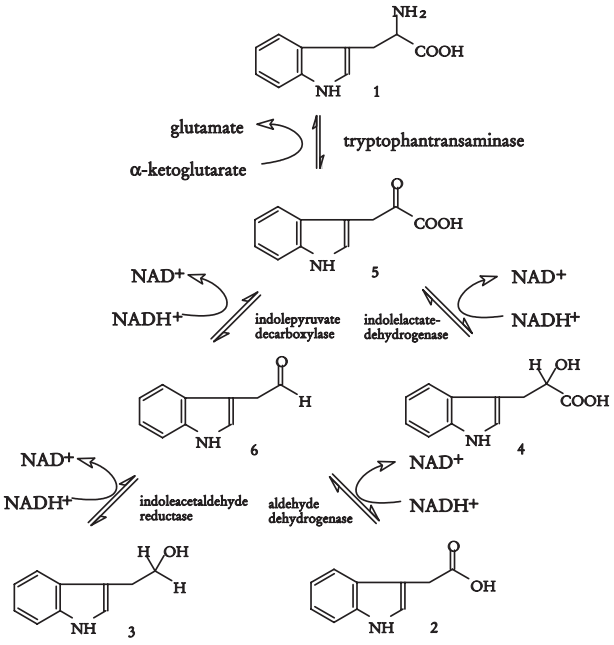

Fig. 4: Deamination pathway of Trp. 1: Trp, 2: IAA, 3: TOH, 4: ILA, 5: indole-3-pyruvate, 6: indole-3-acetaldehyde

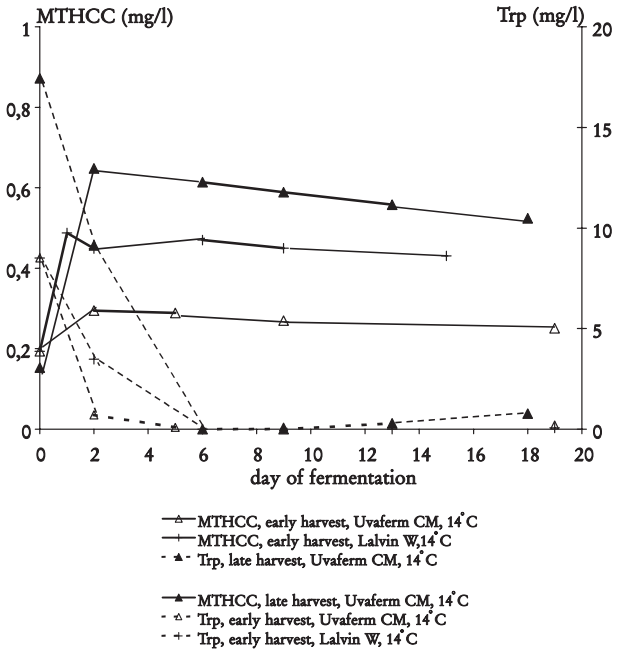

Fig. 3b: Formation of MTHCC from Trp. Time course of Trp depletion and MTHCC formation during fermentation

TOH could be observed in all samples. From the experiments it is not clear whether this degradation is non-specific chemical or enzymatic although TOH is a relatively stable substance. It could be of interest to see if the TOH decrease during three months of storage may be enzymatically catalysed.

Indole-3-lactic acid (ILA)

ILA is, like TOH, a metabolite of the Trp de-amination pathway. In contrast to TOH, low amounts of ILA are detectable in the musts (early harvest: 0.1 mg/l; late harvest: 0.25 mg/l). During the fermentations of the early harvested grapes no marked synthesis of ILA was detectable (only formation up to 0.1 mg/l) except for the mash fermented samples (0.3 to 0.4 mg/l). Similarly, low amounts of ILA were formed in the beginning of the fermentation of the late harvested grapes whereas relatively high amounts (> 0.1 mg/l) were released into the medium at the end of fermentation and after sulphuration, respectively. In accordance with the results of the early harvested grapes, mash fermentation and to a lesser extent "pressure extract" formed the highest ILA values (0.4 to 2.0 mg/l). Neither addition of DAP nor of Fermaid® was able to induce ILA forma-

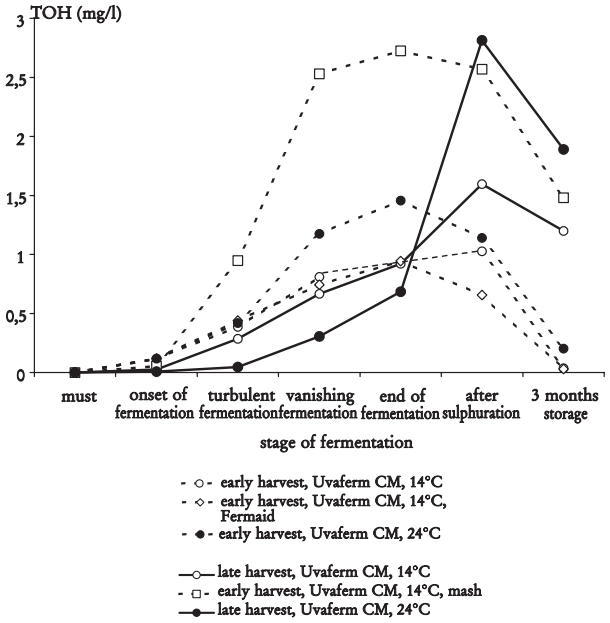

Fig. 5a: Formation of tryptophol (TOH) from Trp. Influences of grape ripeness and different vinification treatments

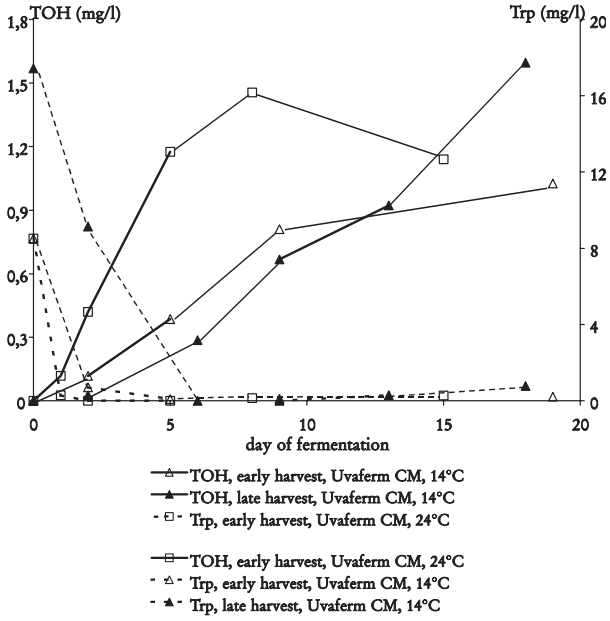

Fig. 5b: Formation of tryptophol (TOH) from Trp. Time course of Trp depletion and TOH formation during fermentation

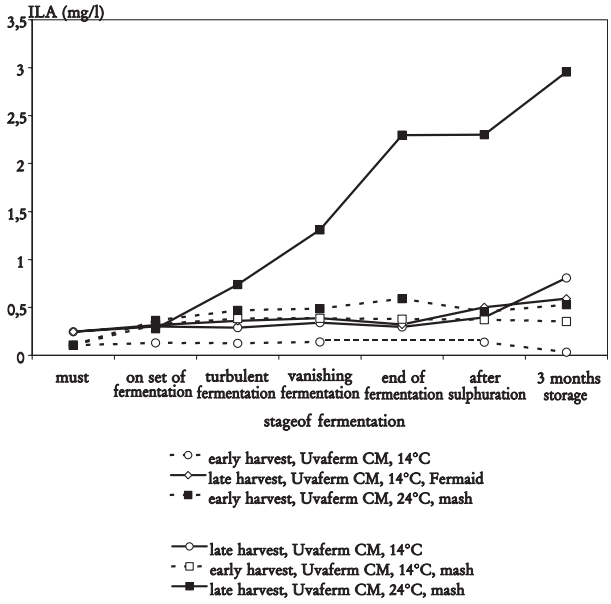

Fig. 6: Influences of grape ripeness and different vinification treatments on the time course of the formation of indole-3-lactic acid (ILA)

tion like that observed during mash fermentation, indicating certain extractable substances in the skin of the grapes that can alter metabolism either by themselves or by influencing the yeasts gene expression.
After sulphiting during three months of storage a significant decrease of ILA was detected in wines made from early harvested grapes. By contrast, a significant increase of ILA during storage was observed in wines made from late harvested grapes. Similarly in the case of TOH the activity of different enzymes after sulphiting of the wine may be speculated upon (Fig. 6).

Indole-3-acetic acid (IAA)

IAA is, like TOH and ILA, a metabolite of the Trp deamination pathway of *Saccharomyces* (SHIN et al., 1991). In the musts only traces of unbound IAA were detectable (< 3 µg/l), which might be due to a regulation of this phytohormone by the *V. vinifera* plant. The regulation might be facilitated by conjugation of IAA with amino acids (COHEN and BANDURSKI, 1982). In the examined musts sodium hydroxide hydrolysable IAA was determined in amounts of 35 to 90 µg/l.
In the musts of the late harvested grapes 80 to 90 µg/l conjugated IAA were determined, while lower amounts were detected in the early harvested grapes (35 to 45 µg/l). During fermentation of the musts the conjugated

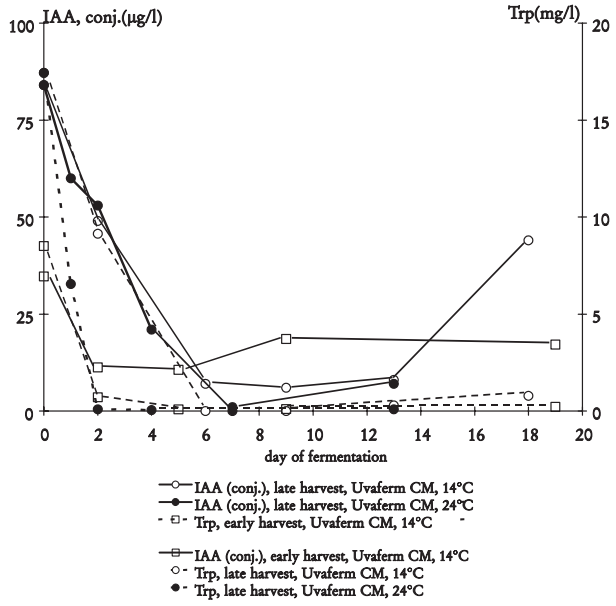

Fig. 7: Consumption of bound indole-3-acetic acid (IAA) and unbound Trp during fermentation

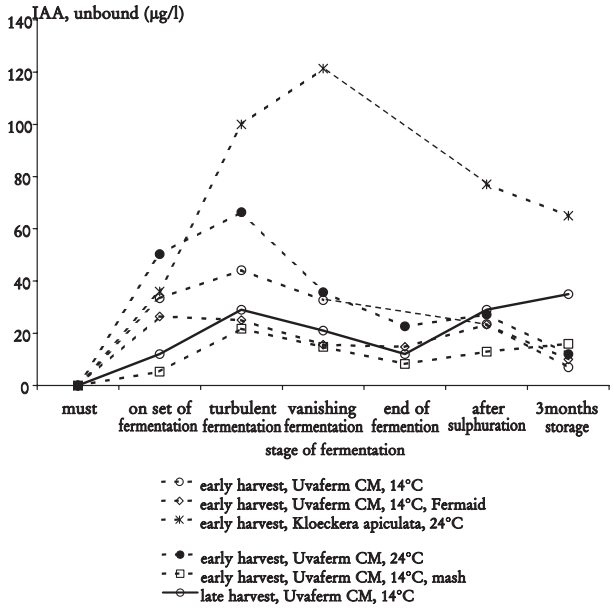

Fig. 8a: Influences of grape ripeness and different vinification treatments on the formation of unbound IAA.

IAA disappeared from the fermentation solution, reaching a minimum concentration of about 20 µg/l in the first stages of fermentation parallel to the Trp uptake (Fig. 7). If the IAA in the musts is conjugated to amino acids or peptides (which is likely) it could be actively transported into the yeast cell, where the yeast can utilize the amino acids after hydrolysis. BECKER and NAIDER (1980) reported a single broad specific transport system in *S. cerevisiae,* which is capable of taking up di- and tripeptides (preference for hydrophobic peptides) without prior hydrolysis. The relatively fast decrease of conjugated IAA in parallel with the Trp consumption makes this hypothesis rather probable. However, the wild yeast strains *K. apiculata* and *M. pulcherima* did not exhibit the uptake of the conjugated IAA to the described extent.

BECKER and NAIDER (1980) also state that oligopeptides require extracellular hydrolysis to smaller peptides and free amino acids by secreted peptidases, which are commonly not attributed to *S. cerevisiae*. Only low amounts of unbound IAA (< 30 µg/l) are detectable until sulphiting , indicating that hydrolysis of IAA cannot be facilitated by extracellular hydrolysis by the yeasts under investigation. The release of unbound IAA into the wine after sulphiting indicates that the excess of stored IAA in the yeasts might permeate

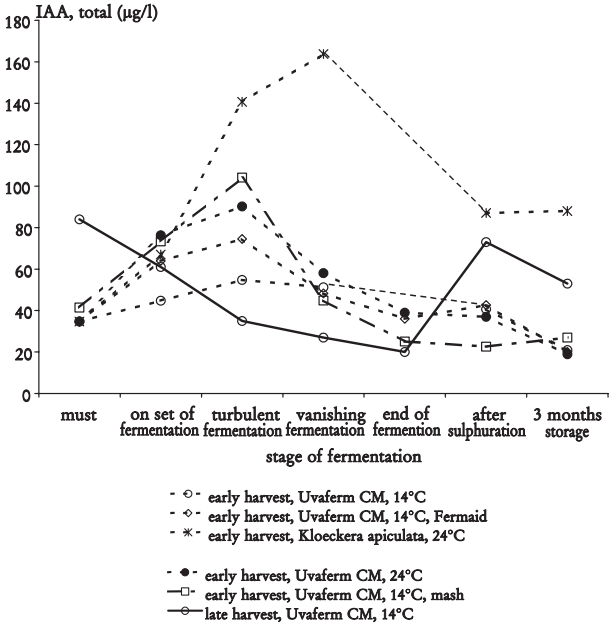

Fig. 8b: Influences of grape ripeness and different vinification treatments on the time course of total IAA, unbound and conjugated)

through the membrane because of the high alcohol concentration at the end of fermentation.
The IAA metabolism of the yeasts depended on the ripeness of the fermented grapes (Fig. 8a, b). In the fermentation media of early harvested grapes an increase of unbound and total IAA (unbound + conjugated) can be observed with a maximum at the middle of fermentation, indicating a biosynthesis and secretion of unbound IAA by the yeasts (20 to 120 µg/l). Unlike the fermentation of early harvested grapes, in the fermentation media of late harvested grapes only the amount of unbound IAA increases slightly, while total IAA decreases during fermentation. This could be traced back to an intensive deamination catabolism of Trp in the nitrogen deficient fermentation medium, which might be used for the synthesis of urgently needed nitrogen substances for the yeasts.
After the turbulent fermentation of both early and late harvested grapes the IAA content decreases continuously, which indicates an enzymatic or chemical degradation. At the end of fermentation and after sulphiting an increase of IAA compounds can be observed only in the wines resulting from late harvested grapes. Further, it has to be evaluated whether the release of IAA after sulphiting in production scale fermentations is comparable to 2 l model fermentations. From our experiments it remains unclear which point of time in the course of fermentation (prior to or following sulphiting) should be taken for the measurement of unbound IAA as precursor of AAP.

Yeast metabolism and formation of 2-aminoacetophenone

IAA was evaluated as a potential precursor of the UTA aroma impact compound AAP. AAP can be formed from IAA after sulphiting of the young wine (Christoph et al., 1998). Additionally the occurrence of UTA was correlated with water deficient vegetation periods, a lack of nitrogen sources and unripe harvested grapes. It was shown by Hoenicke et al. (2001a), however, that the IAA content of late harvested grapes was higher than in early harvested grapes.
Our fermentation experiments have shown that the IAA content in the fermentation media varies widely during the different stages of fermentation and is influenced by the ripeness of the fermented grapes, nutritional supplements, the fermentation temperature and the yeast strain (Fig. 8a, b). Data were evaluated by the statistical software SPSS (SPSS Inc., Germany) for significant factors by multifactorial univariate analysis of variance and the Scheffé-post-hoc-test. Ripe grapes contained higher amounts of bound IAA, but during their fermentation significantly lower amounts of unbound IAA were released compared to the fermentation of unripe grapes ($\alpha < 0.01$). A decreased amount of unbound IAA prior to sulphiting was also observed for musts supplemented with Fermaid® ($\alpha < 0.01$) as well as for mash fermented media ($\alpha < 0.01$). Both could indicate that IAA formation depends on the nutritional supply of the yeasts and that Fermaid® or mash components might resolve this deficiency in the must of early harvested grapes, but not DAP (at least in the musts under investigation). Fermentation performed at higher temperatures revealed a tendency to a higher formation of unbound IAA (not significant, $\alpha > 0.05$). The highest amounts of unbound IAA (> 100 µg/l) were formed in the course of the fermentation with the wild yeast strains ($\alpha < 0.01$).
AAP responsible for UTA should be formed within one year after fermentation. Its formation, however, depends on the storage temperature. After ten months of storage at 14 °C in the dark the amounts of AAP in the samples were all below 0.4 µg/l. After 72 h of storage at 40 °C simulating the "normal" storage at room temperature over several months a significant increase of AAP concentrations (< 0.05 to 1.9 µg/l) was analysed in single wines. Due to the low quantity of the model wines a sensory evaluation by a skilled panel was not possible to perform. Multifactorial univariate analysis of variance of AAP data revealed that the use of unripe grapes and wild yeast strains led to significantly higher amounts of AAP ($\alpha < 0.05$). No significant effect of the nutritional supplements (DAP, Fermaid®) could be observed on AAP formation.
Since the amount of unbound IAA at the time of sulphiting should reflect best the amount of IAA as precursor, the amount of unbound IAA prior to and directly after sulphiting was correlated with the amount of AAP determined after ten months of storage and a further storage at elevated temperatures (40 °C) for 72 h (Fig. 9a, b). We found a significant correlation ($\alpha < 0.02$) of the amounts of unbound IAA prior to and after sulphiting and AAP. The coefficients of determination r^2 were 0.54 and 0.27, respectively. This might indicate that about 30 to 50 % of the variation of AAP can be traced back to the concentration of its precursor IAA. This, however, also indicates that other relevant factors beside the amount of unbound IAA must influence AAP formation.

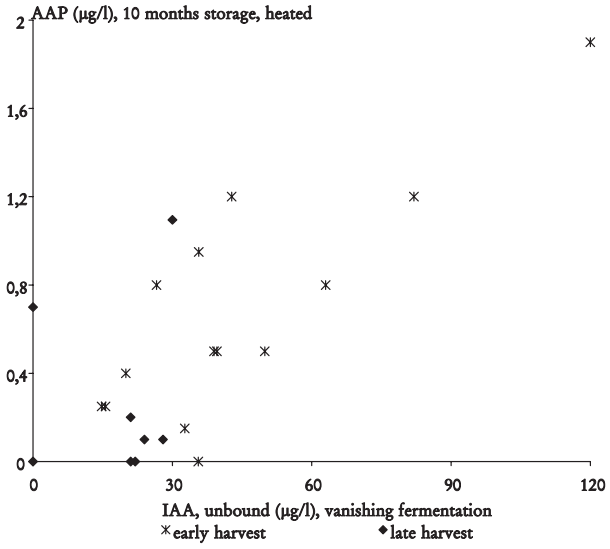

Fig. 9a: Correlation of the amount of unbound indole-3-acetic acid (IAA) at vanishing fermentation and 2-aminoacetophenone (AAP) after 10 months storage and heat treatment ($\alpha < 0.001$, $r^2 = 0.54$, n = 22).

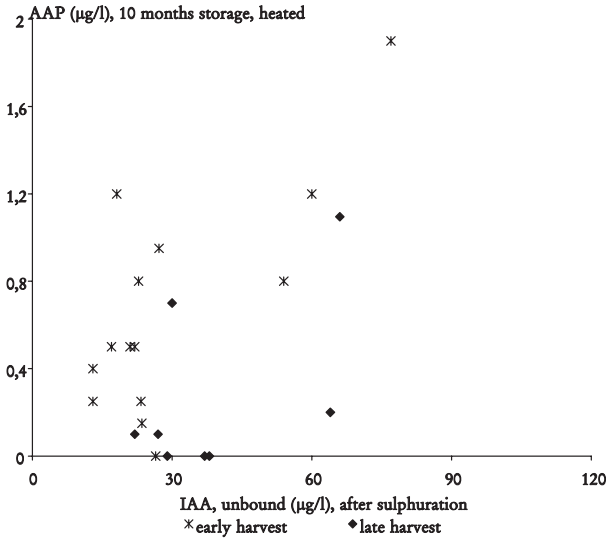

Fig. 9b: Correlation of the amount of unbound indole-3-acetic acid (IAA) after sulphiting and 2-aminoacetophenone (AAP) after 10 months storage and heat treatment ($\alpha = 0.014$, $r^2 = 0.27$, n = 22)

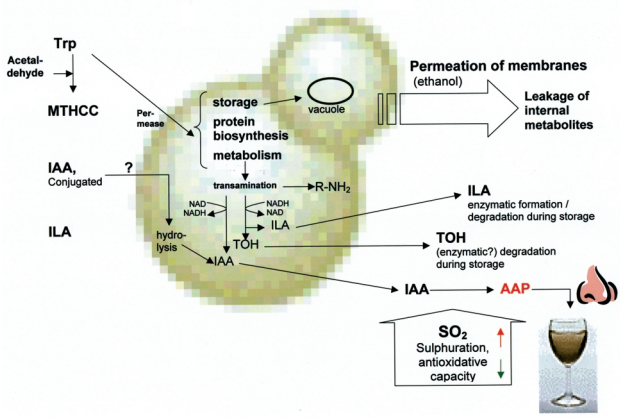

Fig. 10: Overview of the proposed metabolism of tryptophol (TOH) and 2-aminoacetophenon (AAP) formation

Conclusions

The metabolism of the amino acid Trp was investigated during vinifications of different musts using diverse enological techniques. Tryptophan (8.5 to 17.4 mg/l) was completely consumed in all fermentation batches prior to the turbulent phase of fermentation (< 0.2 mg/l) within all different experimental conditions. It was either used for the production of biomass or catabolized to different compounds, of which MTHCC, TOH, ILA, and IAA were investigated (Fig. 10). Depending on the velocity of fermentation MTHCC is formed in a Pictet-Spengler-Reaction (1 to 10 mol% of the initial amount of Trp), which cannot be utilized by the yeasts. Most of the Trp was metabolised, yielding TOH (6 to 22 mol%), which indicates that the α-amino nitrogen of Trp was used for the formation of other nitrogen containing substances after deamination. Following the same deamination pathway, IAA (0 to 1 mol%) and ILA (0 to 5 mol%) were also formed (Fig. 11).

The amount of conjugated IAA dropped during fermentation to about 20 µg/l. It may be speculated whether IAA is bound to peptides which could be transferred into the yeast cell by specific peptide transport systems. Unbound IAA - a potential precursor of AAP - was released during fermentation, predominantly in the fermentation media of early harvested grapes. A neosynthesis of IAA by the yeasts could be observed. Although the total amount of IAA was higher in the late harvested grapes than in early harvested (85 and

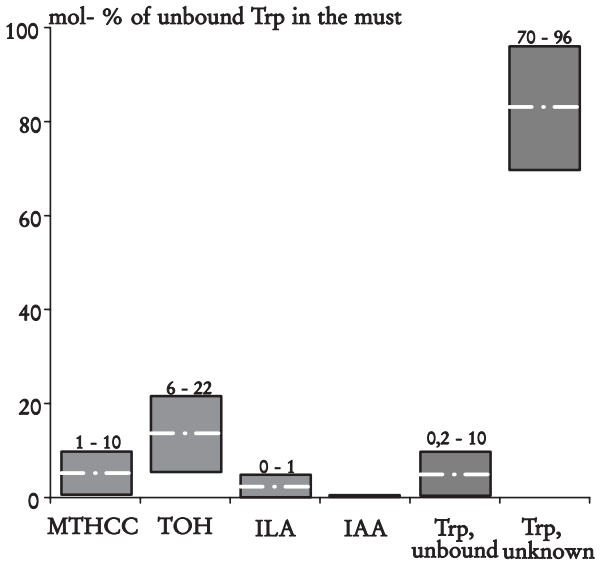

Fig. 11: Metabolic fates of the unbound tryptophan (Trp) in must during fermentation (Trp, unknown = difference of the sum of metabolites and the initial amount of unbound Trp)

35 µg/l), the amount of unbound IAA prior to sulphiting was higher in the fermentation media of early harvested grapes. A marked decrease for the concentration of free IAA was observed during three months of storage.

UTA relevant amounts of AAP (up to 1.9 µg/l) in single wines were measured after a simulation of a longer storage at room temperature by using a 72 h storage at 40 °C. The amount of unbound IAA prior to and after sulphiting and the AAP concentration after heat treatment correlated significantly, indicating that about 30 to 50 % of the variance of the AAP values might be traced back to the amount of its precursor. The amount of IAA prior to sulphiting is significantly influenced by the ripeness of the grapes used, the nutritional supply of the yeasts and the yeast strain. However, only the ripeness of the grapes and the use of wild yeast strains revealed a significant influence on AAP formation in this investigation. This demonstrates that other factors than the amount of the precursor IAA contribute to the formation of AAP, factors which are still unknown. Additionally, skilled sensory panels judged wines with high intensities of UTA descriptors, which did not contain sensory relevant amounts of AAP. This indicates that other odorous compounds might also contribute to UTA, which have to be identified by sensory and analytical investigations in future.

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