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Photooxidation of Tryptophan Leading to 2-Aminoacetophenone – A Possible Reason for the Untypical Aging Off-flavor in Wine

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ABSTRACT

2-Aminoacetophenone (AAP) was recognized as the key compound for the so-called untypical aging off-flavor (UTA) in *Vitis vinifera* wines. In this study, it was shown that AAP can be formed by photooxidation of free and protein-bound tryptophan (TRP) in combination with a subsequent storage in model wine. Solutions of TRP and lysozyme were exposed to artificial sunlight both in the presence and in the absence of the photosensitizer riboflavin. Aliquots of the irradiation batches were stored in model wine solutions containing tartaric acid, sulfite and ethanol in different combinations. AAP formation could be identified from both free and bound (lysozyme) TRP, while free TRP resulted in higher yields. The presence of riboflavin during irradiation generally favored the AAP formation. AAP formation increased with increasing irradiation times, but AAP was not detectable, if TRP was directly incubated in model wine. Not only the irradiation time but also the storage time of model wines favored the formation of AAP. Concerning the model wine composition, it became evident that the presence of tartaric acid resulted in the highest AAP formation during storage.

INTRODUCTION

The untypical aging (UTA) in wine is an off-flavor, which is described as floor polish, wet wool, fusel alcohol, naphthalene or acacia blossom (1,2). 2-Aminoacetophenone (AAP) was identified by Rapp *et al.* (3) as the key compound mainly being responsible for UTA. Already AAP concentrations of 0.5–1.5 μg L^{-1} and >1.5 μg L^{-1} in white and red wines, respectively, can result in UTA (1). Masking effects by other wine flavors and wine compounds could be responsible for higher odor thresholds in strong white wines and red wines (1). Reasons for the formation of AAP in wine were extensively studied. Because of structural relations, tryptophan (TRP) and its metabolites were regarded as the precursors of AAP, when the performed studies differentiated in enzymatic and nonenzymatic pathways. Starting from indole-3-acetic acid (IAA), reaction pathways of oxidative degradation by radical cooxidation of sulfite were discussed by Christoph *et al.* (4), resulting in AAP in wine (Fig. 1A). The mechanisms were supported by the additional detection of skatole (SKA) and N-formyl-2-aminoacetophenone (FAP) as degradation products. Experiments with TRP under the same conditions also yielded FAP and AAP, but to a lower extend, whereas the formation of FAP and AAP could not be observed from kynurenine (KYN) and N'-formylkynurenine (NFK). The sulfite depending formation of AAP from IAA was later confirmed by Hoenicke *et al.* (5), while they also excluded KYN as a precursor of AAP. During yeast fermentation experiments, IAA spiked to model wines at levels far above the natural occurrences revealed AAP in rather low yields (5–7). Therefore, this way of formation was considered not being relevant. Concerning KYN, the fermentative formation of AAP was reported both as not relevant (5,6) and highly detectable (7). Different experimental conditions, especially the type and amount of yeast, may explain the discrepancies.

Since the influence of sunlight on the formation of AAP from IAA was already shown (8), the photooxidation of TRP was of major concern in literature. The published studies strongly differed in the experimental conditions, as for the form of TRP (free or bound), source of light, kind of medium (buffer, pH), presence of oxygen as well as photosensitizers (9,10). Thus, a direct comparison of the results is difficult. Some of the major reported photoproducts are NFK and KYN (Fig. 1B), which were identified after irradiation of both free and protein-bound TRP (11–15). Summaries of the pathways of the photooxidation of TRP residues in proteins generally propose two major mechanisms (9,10,16). Direct UV absorption generates excited state species and radicals, resulting in C-3 peroxyl radicals after reaction with oxygen (Fig. 1B). The singlet oxygen-mediated pathway leads to a dioxetane or a hydroperoxide, which can decompose to NFK yielding KYN by hydrolysis. As mentioned above, the chemical relationship of NFK and KYN with FAP and AAP led authors assume that they could be potential precursors of AAP. The fact that NFK and KYN were identified as photoproducts of TRP encouraged us to fill the gap between the well-known TRP photochemistry and wine processing on the way of AAP formation. Therefore, the aim of this study was to show, if UV light irradiation of free or bound TRP in combination with a subsequent storage in model wine yields AAP to corroborate the hypothesis that the exposure of extra- or intracuticular wine berry proteins to sunlight results in potential precursors of AAP. After extraction into the must and during wine storage, the formation of AAP takes place. However, light exposure could also occur at a later stage as during storage of white wines in clear bottles. Thus, intrinsic wine peptides or residues of fining proteins like lysozyme, or even free TRP could undergo photodegradation in the presence of riboflavin in bottled

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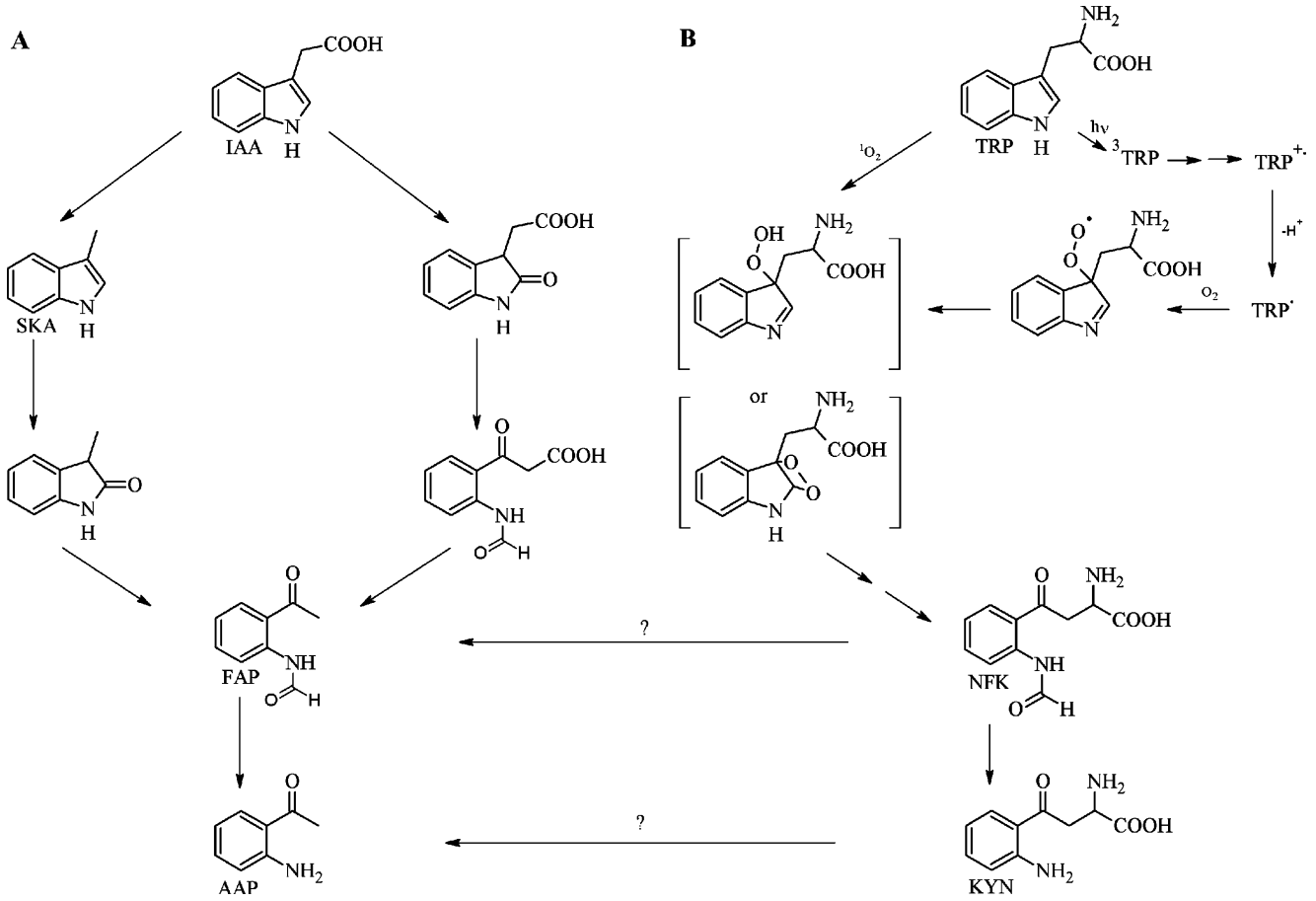

Figure 1. (A) Postulated pathways of AAP formation from IAA (4); indole-3-acetic acid (IAA), skatole (SKA), N-formyl-2-aminoacetophenone (FAP), 2-aminoacetophenone (AAP). (B) Mechanism of sensitized photooxidation of tryptophan (TRP) (10); N'-formylkynurenine (NFK), kynurenine (KYN).

wines, which also is associated with the oxygen management during the vinery process (17).

MATERIALS AND METHODS

Materials. 2-Aminoacetophenone (AAP) (98%), L-tryptophan (≥99.5%), ethyl acetate (≥99.8%), and ethanol (≥99.8%), d_4-methanol (99.96 atom% D), deuterochloroform ($CDCl_3$, 99.8 atom% D) and formic acid (98–100%) were purchased from Sigma-Aldrich (Steinheim, Germany). Diethyl ether (≥98%) was purchased from AppliChem (Darmstadt, Germany) and freshly distilled prior use. Potassium pyrosulfite was obtained from Hefereinzucht Schlag (Aalen, Germany), and L-(+)-tartaric acid (pure) from AppliChem (Darmstadt, Germany). Riboflavin (98%), sodium tert-butylate (98%), sodium hydroxide pellets (≥99%), acetic anhydride (99%), sodium hydrogen carbonate (≥99%), sodium sulfate (>99%) and carbon tetrachloride (>99.8%) were from Merck (Darmstadt, Germany). Carbon tetrachloride was distilled twice. Water was purified by a Synergy ultrapure water system (Merck Millipore, Schwalbach, Germany). Lysozyme from chicken egg white (81989 U mg^{-1}) was obtained from Sigma-Aldrich; according to (18), the TRP content was determined to 7.3 g/100 g (n = 2; %RSD = 0.5).

Synthesis of reference substances. The synthesized reference substances were identified by ^{1}H-NMR and GC-MS. NMR spectra were recorded on a Varian (Darmstadt, Germany) Unity Inova-300 spectrometer at 300 MHz. The samples were dissolved in $CDCl_3$, or CD_3OD. The chemical shifts were referenced to the residual solvent signals at δ 7.26 ($CDCl_3$) and 3.31 (CD_3OD).

d_3-2-Aminoacetophenone (d_3-AAP). AAP (3 mg, 0.022 mmol) was dissolved in 500 µL of d_4-methanol, and catalytic amounts of sodium tert-butylate (2 mg) were added. After stirring for 4 h, 15 µL of formic acid (2.6 M) was added. The solvent was removed under a stream of nitrogen, and the residue taken up in 10 mL ethanol. The solution was diluted 1:100 with ethanol, to be used as internal standard solution. The residual nonconverted AAP, determined by GC-MS/MS, was below the limit of detection (LOD). For NMR analysis, AAP (5 mg, 0.037 mmol) was treated as described for synthesis. After stirring for 4 h, d_4-methanol was slowly removed under a stream of nitrogen. Carbon tetrachloride (500 µL) was added and removed under a stream of nitrogen, which was repeated three times. The residue was taken up in 500 µL of $CDCl_3$, transferred through a syringe filter into an NMR tube and filled up to with 600 µL $CDCl_3$. MS (EI, 70 eV): m/z (%) 138 (92), 120 (100), 92 (56), 65 (29), 46 (10). ^{1}H-NMR (300 MHz, CD_3OD): δ = 7.74 (dd, J = 8.1 Hz, J = 1.3 Hz, 1 H, 6-H), 7.23 (ddd, J = 8.2 Hz, J = 7.0 Hz, J = 1.4 Hz, 1 H, 4-H), 6.72 (br d, J = 8.2 Hz, 1 H, 3-H), 6.58 (ddd, J = 8.1 Hz, J = 7.0 Hz, J = 1.0 Hz, 1 H, 5-H). ^{1}H-NMR (300 MHz, $CDCl_3$): δ = 7.71 (dd, J = 8.2 Hz, J = 1.4 Hz, 1 H, 6-H), 7.26 (ddd, 4-H, overlaid by $CDCl_3$), 6.61-6.68 (m-like, 2 H, 3-H and 5-H), 6.25 (br s, 2 H, -NH_2).

N-Formyl-2-aminoacetophenone (FAP). Following a method described in literature (5,19), AAP (0.9 g, 6.7 mmol) was added to a ice-cooled mixture of acetic anhydride (1.4 mL) and formic acid (2.2 mL). After stirring on ice for 30 min and further for 3.5 h at ambient temperature, diethyl ether (30 mL) and saturated sodium hydrogencarbonate solution (15 mL) were added. Extraction was performed by shaking in a separatory funnel and repeated once with 30 mL diethyl ether. The combined organic extracts were dried over sodium sulfate and rotatory evaporated. The residue was recrystallized from ethanol, dried in a desiccator and yielded FAP as colorless crystals (0.8 g, 4.9 mmol, 74%). GC-MS analysis confirmed the absence of AAP. MS (EI): m/z (%) 163 (26), 148 (10), 135 (61), 120 (100), 92 (35), 65 (23), 43 (28). ^{1}H-NMR (300 MHz, $CDCl_3$): δ = 11.60 (br s, 1 H, –CHO), 8.75 (br d, J = 8.3 Hz, 1 H, 6-H), 8.50 (s, 1 H, -NH-), 7.92 (br d, J = 7.9 Hz, 1 H, 3-H), 7.57 (br t, J = 7.9 Hz, 1 H, 4-H), 7.17 (br t, 1 H, J = 7.5 Hz, 5-H), 2,65 (s, 3 H, -CO-CH_3).

Irradiation experiments. L-Tryptophan (0.046 g, 0.23 mmol) or lysozyme (0.64 g; 0.045 mmol) was dissolved in 50 mL water. For the catalyzed batches, 1 mg (2.7 μmol) or 12.5 μg (0.033 μmol) riboflavin was added. Irradiation was performed with 25 mL of the solutions in 50 mL quartz beakers (diameter 38 mm, Geyer, Renningen, Germany) with teflon caps, which were placed in a self-constructed cooling chamber made from quartz glass (12 × 5 × 5 cm) operated by a chiller (thermostat model WK 230, Lauda, Lauda-Königshofen, Germany). Temperature of the solutions was kept at 25 ± 0.5°C under constant stirring with a magnetic stirrer, Variomag Micro (Thermo Scientific). The light source was a sun simulator SOL 500 (Dr. Hönle, Gräfelfing, Germany) with an integrated metal halogen lamp (UVA 9.7 mW cm^{-2} and UVB 0.25 mW cm^{-2}). The front filter glass was replaced by an aluminum plate with two windows (4 × 4 cm^2 each) to hold WG 295 glass filters (Schott, Mainz, Germany). The cooling chamber was directly positioned in front of the windows to completely expose the solutions to UV light.

Storage experiments. Irradiated and nonirradiated solutions were transformed into different model wines. Depending on the aimed compositions, 4 mL of sample solution was spiked with 50 μL of an aqueous L-(+)tartaric acid solution (270 g L^{-1}), 50 μL of an aqueous potassium pyrosulfite solution (8.66 g L^{-1}) and 650 μL of ethanol. The pH of the model wines was adjusted to 3.3 with sodium hydroxide solution (1 M), which finally were filled up with water to 5 mL. Solutions of TRP, which were transformed into model wine W4, were stored at their native pH values, which were between 3.6 and 3.8. Thus, following model wines were obtained: model wine W1 (according to (20): 2.7 g L^{-1} L-(+)-tartaric acid, 50 mg L^{-1} SO_2, 13 Vol% ethanol), model wine W2 (2.7 g L^{-1} L-(+)-tartaric acid, 50 mg L^{-1} SO_2), model wine W3 (2.7 g L^{-1} L-(+)-tartaric acid, 13 Vol% ethanol) and model wine W4 (50 mg L^{-1} SO_2, 13 Vol% ethanol). The model wines were transferred into 10 mL screw-capped vials and stored protected from light at 40 or 18°C. After defined periods of storage (0, 1, 3, 5 and 20 weeks), aliquots were sampled and prepared for GC-MS/MS analysis.

Sample preparation. Samples of the stored model wines (2 mL) were pipetted into 5 mL screw-capped centrifuge tubes, spiked with 100 μL of the internal standard solution, followed by the addition of 1 mL ethyl acetate. After extraction for 1 min, the emulsions were centrifuged at 10 000 U min^{-1} for 20 min. The organic layer was drawn into a syringe, transferred through a 0.45-μm filter tip (VWR International, Darmstadt, Germany) into a GC vial. The extraction was performed in duplicates and the mean was calculated. Samples of irradiated free TRP, which were stored in model wine W1 at 40°C, were diluted 1:10 in model wine before extraction.

Calibration. For calibration, stock solutions of AAP and FAP (*ca* 40 mg L^{-1}) were prepared in water and diluted 1:20 and 1:2000 with water. The obtained standard solutions with concentrations of *ca* 0.02 mg L^{-1} (standard 1) and 2 mg L^{-1} (standard 2) were transformed into model wines. Therefore, 500 μL of the standard 1 solution and 25, 60, 125, 250 and 1250 μL of the standard 2 solution were transformed into the respective model wine, as described in Storage Experiments, resulting in AAP and FAP concentrations of 2–500 μg L^{-1}. Aliquots (2 mL) were treated as described for sample preparation. Thus, calibration points were expressed as model wine concentrations. The extraction was performed in duplicates and the mean was calculated.

Gas chromatography–mass spectrometry (GC-MS/MS and GC-MS). An Agilent (Waldbronn, Germany) 7890A gas chromatograph equipped with an ALS 7693 autosampler and coupled to a GC-MS Triple Quad 7000 (Agilent) was used for GC-MS/MS analysis. Separation was performed on an HP5-ms Ultra Inert column (30 m × 0.25 mm × 0.25 μm) (Agilent). Injector settings: pulsed splitless; inlet temperature program: 60°C (1 min hold) to 250°C (10 min hold) (ramp of 900°C min^{-1}), injection volume 2 μL; oven temperature program: 60°C (0.2 min hold) to 150°C (1 min hold) (ramp of 10°C min^{-1}) to 250°C (1 min hold) (ramp of 50°C min^{-1}); carrier gas: He, constant pressure 15 psi; ion source: 230°C, electron energy 70 eV; multiple reaction monitoring (MRM) mode: collision gas N_2; quench gas He. Retention times, collision energy and MRM transitions are summarized in Table 1. Agilent MassHunter qualitative analysis B.06.00 was used for data acquisition.

Mass spectra for identification of reference substances were recorded on an Agilent (Waldbronn, Germany) 6890 gas chromatograph coupled to an 5973 MSD (Agilent) equipped with an HP5-ms Ultra Inert column (30 m × 0.25 mm × 0.25 μm) (Agilent). Injector settings: splitless; inlet temperature: 60°C, injection volume 2 μL; oven temperature program: 60°C (0.2 min hold) to 150°C (1 min hold) (ramp of 10°C min^{-1}) to 250°C (1 min hold) (ramp of 50°C min^{-1}); carrier gas: He, constant flow (0.8 mL min^{-1}); ion source: 230°C, electron energy 70 eV; scan mode: 30–800 amu. Agilent MSD ChemStation E.02.00.493 was used for data acquisition.

Statistics. Statistics were performed using IBM SPSS Statistics for Windows, Version 19.0. Armonk, NY: IBM Corp. released 2010. The significance level was chosen to be 0.05 and was tested with two sample *t*-test.

RESULTS AND DISCUSSION

Determination of AAP and FAP by GC-MS/MS

The difficulty of AAP quantification in wine is due to strong matrix effects, caused by numerous volatile wine flavors, and due to the low concentrations in wines. In literature, several methods of the analysis of AAP in wine are described, mainly using gas chromatography with mass spectrometric detection (GC-MS) (20–24), when one-dimensional as well as multidimensional GC methods (20,21) were applied. Extraction of AAP was performed by liquid–liquid extraction with trichlorofluoromethane (4,21) or n-pentane (22), LiChrolut EN supported solid–liquid extraction (20), and by direct immersion (23) or headspace solid-phase microextraction (24). Besides ethyl vanillin (4), 2, 4-dichloroaniline (21), d_8 -acetophenone (23), d_3-AAP (20,22), or d_5-AAP (24) were used as internal standards. As we performed our experiments only in model wine solutions, strong matrix effects were not to be expected. To develop a fast and simple method for the quantitation of AAP and FAP, a simple liquid–liquid extraction with ethyl acetate was chosen. In literature, however, an influence of pH and sulfurous acid on the extraction yields of AAP was discussed (20,22,24), but the obtained results were inconsistent. Therefore, we decided to prepare calibration standards in model wines to take the extraction process into account, which also may refer to a kind of matrix-matched standards. Fan *et al.* (23) also used model wines for calibration, to be extracted by solid-phase microextraction. Replicates of extraction ($n = 6$) from a model wine W1 (199 μg L^{-1} AAP) resulted in a relative standard deviation of 1.9%. The determination of AAP and FAP was performed by GC-MS/MS in the highly selective multiple reaction monitoring (MRM) mode (Table 1), when, according to (22), d_3-AAP was used as internal standard. The obtained calibration graphs with high coefficients of correlation are exemplarily shown in Fig. 2. Following the DIN 32645 method (25), LODs of 0.5 μg L^{-1} and

Table 1. Retention times, collision energy, MRM transitions for quantifiers and qualifiers and sensitivity of the GC-MS/MS method; LODs and LOQs were calculated from the calibration data according to DIN 32645 (25).

Compound	Retention time (min)	Collision energy (eV)	Quantifier MRM	Qualifier MRM	LOD (μg L^{-1})	LOQ (μg L^{-1})
d_3-AAP	9.62	20	138 > 120	138 > 92	–	–
AAP	9.66	20	135 > 120	135 > 92	0.5	1.5
FAP	12.37	20	135 > 120	163 > 120	0.8	2.0

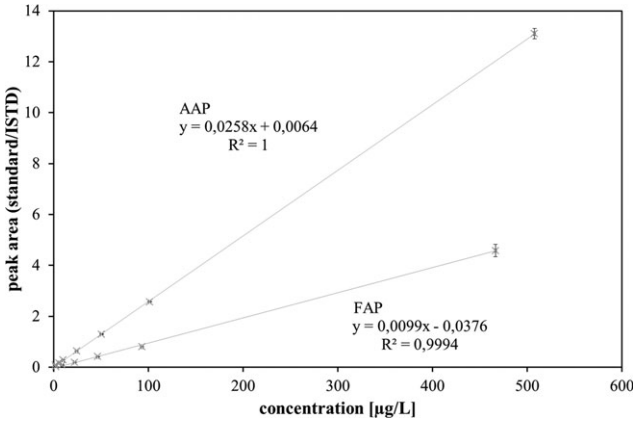

Figure 2. Linear calibration graphs for AAP and FAP (duplicates of extraction).

0.8 µg L^{-1} and limits of quantitation (LOQs) of 1.5 and 2.0 µg L^{-1} were achieved for AAP and FAP, respectively (Table 1).

AAP formation from free and bound tryptophan

The formation of AAP by photooxidation of TRP in combination with a subsequent storage under model wine conditions was first studied with free TRP in the presence of the photosensitizer riboflavin (2.7 µmol/50 mL), when the irradiation time was 15 h. After storage for 1 week at 40°C in model wine W1, AAP clearly could be detected (Table 2). Under the same conditions, equimolar amounts of protein-bound TRP (lysozyme) also resulted in the formation of AAP, although to a nearly ten-fold lower extent (Table 2). This is to be explained by a lower degree of photooxidation of the lysozyme-bound than of free TRP. Edwards and Silva (26) already showed for lysozyme and α-lactalbumine that the accessibility of the TRP residues to the solvent, thus exposure to UV light, influences their photosensitivity. Increasing accessibility led to a higher degree of photooxidation. They reported that only two of the six TRP residues of lysozyme are exposed (26). Besides the accessibility of TRP residues, the yield of AAP from photooxidized TRP residues is another variable, which altogether will explain our results. However, in model wines prepared from nonirradiated TRP solutions and stored under the same conditions, AAP formation was below the LOD for free and lysozyme-bound TRP. From these first results, it clearly could be concluded that a former photooxidation is a prerequisite for the formation of AAP during the incubation under model wine conditions. FAP that is discussed to be a direct precursor of AAP (4,5) could also be identified in the stored model wines prepared from irradiated TRP and lysozyme solutions, but was below the LOQ and LOD, if nonirradiated free and lysozyme-bound TRP was applied. As compared to AAP, the FAP concentrations were clearly lower both from free and protein-bound TRP (Table 2). Calculating the sum of AAP and FAP, *ca* 0.09 and 0.009 mol% of free and lysozyme-bound TRP, respectively, were productively transformed, resulting in final AAP concentrations of 432 and 45 µg L^{-1} in the studied model wines.

Influence of storage time and storage temperature

The influence of storage time and storage temperature on the formation of AAP was studied with nonirradiated and irradiated samples of lysozyme, transformed into model wine W1 that was extracted immediately and after storage for 1–20 weeks at 40°C and 18°C. Meanwhile, it did not come as a surprise that AAP formation increased with both storage time and storage temperature. As compared to 18°C, storage at 40°C significantly enhanced the formation of AAP (Fig. 3). Only after 1 week at 40°C, AAP strongly exceeded the value obtained after 5 weeks at 18°C. It is known from the literature that a higher temperature and increasing storage time enforces the formation of AAP (4,8). Studies on the existence of AAP at different stages of vinification showed that in the wine berry, in the must and in the wine direct after fermentation, AAP concentrations were mostly below the odor threshold (1,7,35). Higher concentrations were only found after fermentation in the course of storage, which is in accordance with our results. In model wines immediately extracted after preparation, AAP could well be determined in samples from irradiated free TRP, but were below LOQ in samples from irradiated lysozyme (Table 2). Thereafter, AAP concentration further increased with increasing storage time. Concerning FAP, the highest concentrations were found immediately after the irradiation process (Table 2), which decreased with storage time, especially at the higher temperature of 40°C (Table 2, Fig. 3), when already after 3 weeks it was below the LOQ. At the lower temperature of 18°C, FAP only decreased by *ca* 60% within the longer storage time of 5 weeks (Fig. 3). These findings are well in agreement with the results published by Christoph *et al.* (4). They also reported an enormous thermal instability of FAP. Under the influence of heat—also in the absence of sulfite—FAP degraded rapidly. In solutions of FAP stored in model wine for 4 days at 45°C and further 4 weeks at 20°C, 90 mol% of the added FAP was transformed to AAP.

Table 2. AAP and FAP formation from free and lysozyme-bound TRP, irradiated for 15 h in the presence of riboflavin (2.7 µmol/50 mL) and transformed into model wines stored at 40°C.

Model wine	Storage time (weeks)	Free TRP		Lysozyme	
		AAP ± SD (µg L^{-})	FAP ± SD (µg L^{-})	AAP ± SD (µg L^{-})	FAP ± SD (µg L^{-})
W1	0	24.9 ± 3.5 (n = 3)	581 ± 88.3 (n = 3)	<LOQ (n = 3)	27.9 ± 0.9 (n = 3)
W1	1	432 ± 4.5 (n = 2)	142 ± 10.6 (n = 2)	45.0 ± 1.3 (n = 3)	14.5 ± 0.8 (n = 3)
	3	444 ± 3.3 (n = 2)	<LOQ (n = 2)	71.9 ± 7.5 (n = 5)	<LOQ (n = 5)
	5	824 ± 10.2 (n = 2)	<LOQ (n = 2)	92.7 ± 5.0 (n = 6)	<LOQ (n = 6)
W4	1	232 ± 7.6 (n = 2)	572 ± 47.8 (n = 2)	28.5 ± 0.7 (n = 2)	9.8 ± 2.3 (n = 2)
	3	377 ± 2.2 (n = 2)	204 ± 10.8 (n = 2)	50.1 ± 0.8 (n = 2)	3.7 ± 0.4 (n = 2)
	5	382 ± 9.1 (n = 2)	115 ± 16.3 (n = 2)	54.5 ± 1.6 (n = 2)	<LOQ (n = 2)

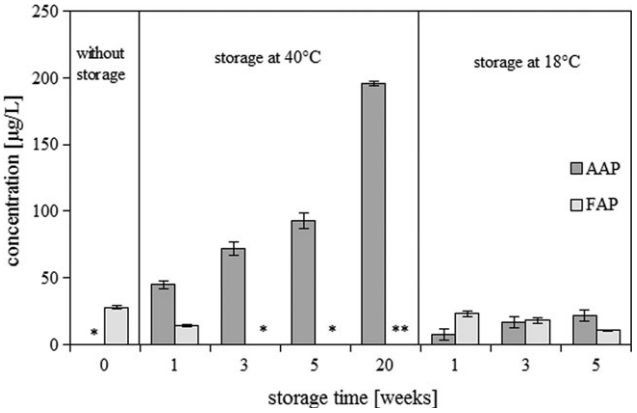

Figure 3. Influence of storage time and temperature on AAP and FAP formation. Solutions of lysozyme were irradiated for 15 h in the presence of riboflavin (2.7 µmol/50 mL) and transformed into model wine W1. Samples were analyzed without storage and after storage at 40°C or 18°C for 1, 3, 5 and 20 weeks; means ± SD, $n = 3$ for the nonstored samples, $n = 3$, 5, 6 and 2 for the samples stored at 40°C for 1, 3, 5 and 20 weeks, respectively, $n = 4$ (AAP) and $n = 2$ (FAP) for the samples stored at 18°C; * <LOQ; **<LOD.

Influence of irradiation time

To demonstrate the influence of light exposure on the formation of AAP, the irradiation time was varied from 0 to 45 h for solutions of lysozyme in the presence of riboflavin (2.7 µmol/50 mL). After the irradiations, W1 model wines were prepared and stored for 3 weeks at 40°C. As to be expected, time of irradiation highly correlated with the formation of AAP in model wines (Fig. 4). Already an irradiation time of 3 h led to an AAP formation of 12.9 µg L^{-1}, while it reached 99.8 µg L^{-1} after 45 h. Nonirradiated samples, however, did not result in detectable AAP amounts. The formation of FAP was below the LOD up to 3 h and remained below the LOQ above 6 h of irradiation. FAP was nearly completely degraded after a storage time of 3 weeks, which was only 1 week during the first experiments.

The influence of UV light on UTA is obvious, as UTA often occurred in years with high insolation and low water supply (27–29). Accordingly, the longer lysozyme was being exposed to UV light the more AAP was formed in model wines. Since we used the model protein lysozyme for our experiments, an appropriate TRP source has to be identified to transform the results to real conditions. Both intrinsic wine proteins and proteins present on the surface of wine berries as well as free TRP may be involved. Thus, exudates of micro- or macroorganisms exemplarily come into question, or the biological insecticide *Bacillus thuringiensis* (Bt), which is used for vine protection. The Cry1Ab toxin, for example, which is produced during sporulation of Bt (30), owns 17 TRP residues (31,32). The sunlight-mediated destruction of up to 65% of TRP residues in endotoxin crystals from Bt was already shown in literature (33,34).

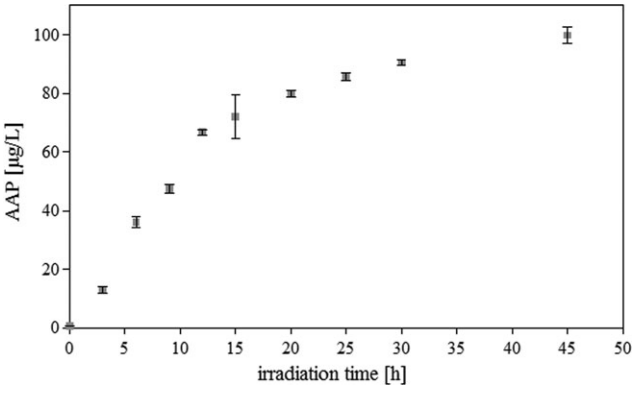

Figure 4. Influence of irradiation time on AAP formation. Solutions of lysozyme were irradiated in the presence of riboflavin (2.7 µmol/50 mL) for 0–45 h and stored in model wine W1 for 3 weeks at 40°C; means ± SD, $n = 2$ except 0 h and 15 h ($n = 5$).

Influence of model wine composition

The so far presented results were obtained by the transformation of irradiated solutions of free and bound TRP into the well-established model wine W1 and stored. To study the effects of acid, SO_2 and ethanol during storage on the formation of AAP, samples of lysozyme, irradiated in the presence of riboflavin (2.7 µmol/50 mL), were transformed into three additional model wines. As compared to model wine W1, model wine W2 was free of ethanol, model wine W3 free of SO_2 and model wine W4 free of acid, while the other respective components remained constant. The thus obtained model wines were generally stored at the optimal conditions of 5 weeks and 40°C. The highest AAP amounts were found in model wines containing all three components, tartaric acid, SO_2 and ethanol, *i.e.* model wine W1 (Fig. 5). The absence of ethanol (model wine W2) or SO_2 (model wine W3) generally had the consequence of significantly lower AAP formation, but the presence of only SO_2 (W2) or ethanol (W3) under acidic conditions did not turn out a significant difference concerning the formation of AAP. Although the highest AAP amounts could be determined, if tartaric acid was added to the model wines (W1, W2 and W3), the presence of tartaric acid was not essential for AAP formation. Samples transformed into model wine W4 (without tartaric acid) also revealed AAP, but the found AAP amounts were significantly lower than in the presence of acid (model wine W1, W2 and W3) (Fig. 5). As to

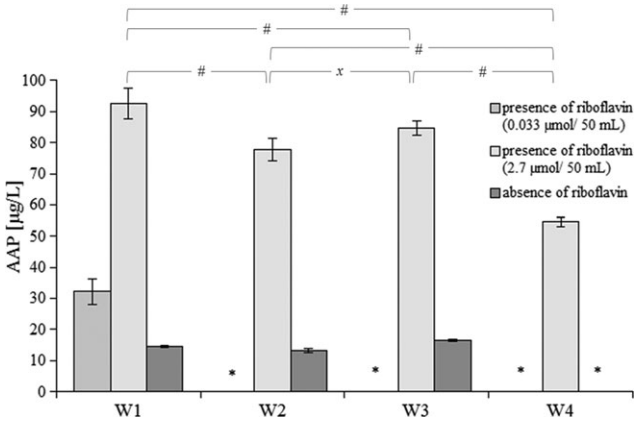

Figure 5. Influence of model wine composition on AAP formation during storage. Model wines were prepared from lysozyme solutions irradiated for 15 h in the presence of riboflavin (2.7 µmol/50 mL) and stored for 5 weeks at 40°C. In addition, the influence of the photosensitizer riboflavin during irradiation is exemplarily shown. Means ± SD, $n = 3$ except the samples irradiated in the presence of riboflavin and stored in W1 ($n = 6$) or W4 ($n = 2$); *Not studied; #Significant ($P < 0.05$), *x* not significant.

be expected from the former results, the formation of FAP could not be detected during these experiments (high temperature, long storage time). To show the influence of acid on the formation of FAP and its transformation to AAP, further experiments with the following modifications were undertaken: (1) irradiated samples were prepared without storage ($t = 0$) and storage time was additionally shortened to 1 week and 3 weeks; (2) besides lysozyme, irradiated solution of free TRP was additionally applied; (3) storage was performed in the acid and nonacid model wines W1 and W4. As mentioned above, AAP formation increased with extended storage times. This also held true for the present experiments with model wines W1 and W4 prepared from irradiated solutions of both free and lysozyme-bound TRP (Table 2). In model wines prepared from irradiated solutions of free TRP, significantly higher AAP concentrations could be determined in the presence (W1) than in the absence of acid (W4) (Table 2). After storage of 5 weeks, more than twofold of AAP was determined in W1 compared to W4. The same trend could be shown at each stage of storage of model wines W1 made from irradiated lysozyme, whereas the AAP values were approximately half of them in model wines W4 (Table 2). FAP was generally well detectable in model wines W1 and W4 freshly prepared (without storage) from samples of both irradiated free and lysozyme-bound TRP, but FAP was less stable during storage under the acidic conditions of W1 (Table 2). In model wines prepared from irradiated solutions of free TRP, FAP decreased more than fourfold in W1 only after 1 week, whereas it was still detectable after 3 and 5 weeks in W4 (Table 2). Concerning irradiated lysozyme, FAP also more rapidly disappeared under the acidic conditions of W1 than in W4 (Table 2).

Influence of the photosensitizer riboflavin during irradiation

It is known that the presence of a photosensitizer during irradiations increases the photochemical degradation of TRP. Due to the formation of TRP-riboflavin adducts, especially for lysozyme, a high efficiency of riboflavin as photosensitizer was described (36,37). Riboflavin is present in most living systems, in the free form or as flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) (41). Thus, its presence on the surface of wine berries is not unlikely. To guarantee optimal conditions for the formation of AAP, the so far chosen riboflavin concentrations (2.7 μmol/50 mL) were rather high as compared to those, which are typically present in grapes (38) and wines (39,40). Therefore, additional irradiations of lysozyme were exemplarily performed in the presence of more relevant riboflavin concentrations (0.033 μmol/50 mL) and without riboflavin, when the irradiation time was 15 h, and the storage conditions were 5 weeks at 40°C. AAP could successfully be detected also in model wine W1 prepared from nonsensitized irradiation batches, but the AAP concentration was about two-fold and six-fold, if 0.033 μmol and 2.7 μmol riboflavin, respectively, was present during the irradiation (Fig. 5). Concerning the presence and the absence of riboflavin during lysozyme irradiations, the model wines W2 and W3 provided nearly the same results.

Pathways of AAP formation

As reported in literature (4,5), FAP was also identified in our experiments as a precursor of AAP. During storage in model wines, reinforced in the presence of acid (model wine W1) and at higher temperatures (40°C), FAP degradation occurred, while the AAP concentration increased. However, FAP obviously was not exclusively transformed into AAP. In model wines prepared from TRP, steady losses of FAP occurred, while AAP did not correspondingly increase (Table 2). Therefore, the dissipation of FAP must follow at least one other route than hydrolysis, which, however, could not be identified. In addition, AAP still increased in model wine W1, when FAP was already disappeared. Thus, there obviously was another AAP precursor different from FAP. With regard to the known TRP photochemistry (Fig. 1B) (13–17), NFK should provide the first access to AAP, followed by a possible hydrolysis to KYN (Fig. 6). The ongoing formation of AAP or FAP from KYN or NFK requires an elimination of the amino acid head group. Thereby, the hydrolysis of NFK presumably proceeds more rapidly than the elimination, still providing KYN as AAP precursor, when NFK and FAP already disappeared (<LOQ). This could explain why AAP increased during the storage of model wines, although FAP was not detectable anymore. In the case of lysozyme, FAP was detectable after irradiation, but AAP also steadily increased, when FAP was below the LOQ, and the finally determined AAP strongly exceeded the original FAP concentrations (Table 2). Here, protein-bound KYN and NFK have additionally to be taken into account as precursors of FAP and AAP. Exploring the mechanisms in more detail is surely highly interesting, but was out of scope of this study.

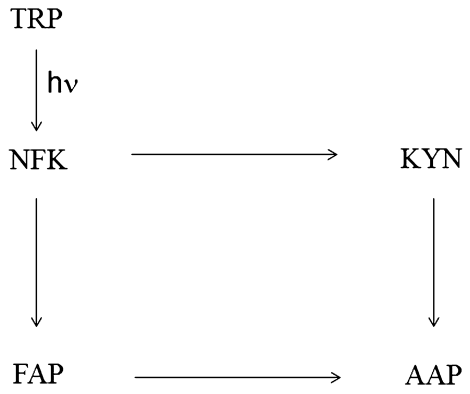

Figure 6. Proposed pathways of AAP formation through free and protein-bound NFK and KYN.

CONCLUSIONS

AAP, the character impact compound of the untypical aging off-flavor (UTA) in wine, was clearly formed by UV light irradiation of TRP (free or bound) in combination with a subsequent storage in model wine. Extending the irradiation time increased the AAP formation, but nonirradiated samples did not yield AAP during storage in model wines. A clear conclusion is that AAP formation was photochemically initiated, and TRP can be regarded as its primary precursor. However, lower AAP amounts were formed from lysozyme-bound TRP than from the free amino acid. Furthermore, we could show that the AAP formation mechanism proceeded via FAP, however, not exclusively, and is affected by the set storage conditions. A faster transformation was observed in the presence of acid and at the higher temperature of 40°C. Further research will be focused on appropriate TRP sources and to prove its formation—according to this model—under real conditions.

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REFERENCES

1. Christoph, N., C. Bauer-Christoph, M. Geßner and H. J. Köhler (1995) Die "Untypische Alterungsnote" im Wein Teil I: Untersuchungen zum Auftreten und zur sensorischen Charakterisierung der "Untypischen Alterungsnote". *Rebe & Wein* **48**, 350–356.
2. Fischer, U. and R. Sponholz (2000) Die sensorische Beschreibung der Untypischen Alterungsnote. *Dtsch. Weinbau* **3**, 16–21.
3. Rapp, A., G. Versini and H. Ullemeyer (1993) 2-Aminoacetophenone: Causal component of "untypical aging flavour" ("naphthalene note", "hybrid note") of wine. *Vitis* **32**, 61–62.
4. Christoph, N., C. Bauer-Christoph, M. Geßner, H. J. Köhler, T. J. Simat and K. Hoenicke (1998) Formation of 2-aminoacetophenone and formylaminoacetophenone in wine by reaction of sulfurous acid with indole-3-acetic acid. *Vitic. Enol. Sci.* **53**, 79–86.
5. Hoenicke, K., O. Borchert, K. Grüning and T. J. Simat (2002) "Untypical aging off-flavor" in wine: Synthesis of potential degradation compounds of indole-3-acetic acid and kynurenine and their evaluation as precursors of 2-aminoacetophenone. *J. Agric. Food Chem.* **50**, 4303–4309.
6. Geßner, M., H. J. Köhler, N. Christoph and C. Bauer-Christoph (1996) Die "Untypische Alterungsnote im Wein" Teil VII Untersuchungen zur Bildung von o-Aminoacetophenon aus Produkten des Tryptophan-Stoffwechsels bei der alkoholischen Gärung. *Rebe & Wein* **49**, 251–255.
7. Dollmann, B., A. Schmitt, H. Köhler and P. Schreier (1996) Formation of the "untypical aging off-flavour" in wine: Generation of 2-aminoacetophenone in model studies with *Saccharomyces cerevisiae*. *Vitic. Enol. Sci.* **51**, 122–125.
8. Christoph, N., C. Bauer-Christoph, M. Geßner and H. J. Köhler (1996) Die "Untypische Alterungsnote" im Wein Teil VI Untersuchungen zur Bildung von o-Aminoacetophenon aus Produkten des Tryptophan-Stoffwechsels vor der alkoholischen Gärung. *Rebe & Wein* **49**, 246–250.
9. Creed, D. (1984) The photophysics and photochemistry of the near-UV absorbing amino acids - I. Tryptophan and its simple derivatives. *Photochem. Photobiol.* **39**, 537–562.
10. Pattison, D. I., A. S. Rahmanto and M. J. Davies (2012) Photo-oxidation of proteins. *Photochem. Photobiol. Sci.* **11**, 38–53.
11. Bolte, M. and J. Lemaire (1989) Monohydrogen chromate photosensitized oxidation of tryptophan exposed to near-UV-visible light. *J. Photochem. Photobiol., A* **46**, 285–294.
12. Benassi, C. A., E. Scoffone, G. Galiazzo and G. Iori (1967) Proflavine-sensitized photooxidation of tryptophan and related peptides. *Photochem. Photobiol.* **6**, 857–866.
13. Pirie, A. (1971) Formation of N'-formylkynurenine in proteins from lens and other sources by exposure to sunlight. *Biochem. J.* **125**, 203–208.
14. Gomyo, T. and M. Fujimaki (1970) Studies on changes of protein by dye sensitized photooxidation Part III. On the photodecomposition products of lysozyme. *Agr. Biol. Chem.* **34**, 302–309.
15. Asquith, R. S. and D. E. Rivett (1971) Studies on the photooxidation of tryptophan. *Biochim. Biophys. Acta* **252**, 111–116.
16. Kerwin, B. A. and R. L. Remmele (2007) Protect from light: Photodegradation and protein biologics. *J. Pharm. Sci.* **96**, 1468–1479.
17. Morozova, K., O. Schmidt and W. Schwack (2014) Impact of headspace oxygen and copper and iron addition on oxygen consumption rate, sulphur dioxide loss, colour and sensory properties of Riesling wine. *Eur. Food Res. Technol.* **238**, 653–663.
18. European Commision (2009) Commission Regulation (EC) No 152/2009 of 27 January 2009 laying down the methods of sampling and analysis for the official control of feed. *Off. J. Eur. Commun.* **54**, 32–37.
19. Fürstner, A. and D. N. Jumbam (1992) Titanium-induced syntheses of furans, benzofurans and indoles. *Tetrahedron* **48**, 5991–6010.
20. Schmarr, H.-G., S. Ganß, W. Sang and T. Potouridis (2007) Analysis of 2-aminoacetophenone in wine using a stable isotope dilution assay and multidimensional gas chromatography-mass spectrometry. *J. Chromatogr. A* **1150**, 78–84.
21. Rapp, A., G. Versini and L. Engel (1995) Determination of 2-aminoacetophenone in fermented model wine solutions. *Vitis* **34**, 193–194.
22. Dollmann, B., D. Wichmann, A. Schmitt, H. Koehler and P. Schreier (1996) Quantitative analysis of 2-aminoacetophenone in off-flavored wines by stable isotope dilution assay. *J. AOAC Int.* **79**, 583–586.
23. Fan, W., I.-M. Tsai and M. C. Qian (2007) Analysis of 2-aminoacetophenone by direct-immersion solid-phase microextraction and gas chromatography-mass spectrometry and its sensory impact in Chardonnay and Pinot gris wines. *Food Chem.* **105**, 1144–1150.
24. Gulan, S. and U. Arzberger (2008) Quantification of 2-aminoacetophenone in white wine by headspace solid phase micro extraction and gas chromatography-mass spectrometry. *Dtsch. Lebensm.-Rundsch.* **104**, 41–46.
25. Deutsches Institut für Normung e.V. (2008) DIN 32645 Chemical analysis – Decision limit, detection limit and determination limit under repeatability conditions - Terms, methods, evaluation. Beuth, Berlin.
26. Edwards, A. M. and E. Silva (1985) Photochemical reactivity of the homologous proteins α-lactalbumin and lysozyme. *Radiat. Environ. Biophys.* **24**, 141–148.
27. Schwab, A., M. Peternel, J. Köhler and K.-P. Heigel (1996) Die Untypische Alterungsnote im Wein Teil IV: Beeinflussung durch weinbauliche Maßnahmen. *Rebe & Wein* **49**, 181–187.
28. Sponholz, W. R., T. Hühn, A. Engelmann and A. Siben (1997) Possible influence of some viticultural parameters on the incidence of "untypical" off-flavours in Riesling wines. *Vitic. Enol. Sci.* **52**, 41–50.
29. Schwab, A. and M. Peternel (2001) Ungenügende Traubenreife: Ursache der "Untypischen Alterungsnote (UTA)" im Wein. *Rebe & Wein* **49**, 14–17.
30. Schnepf, E., N. Crickmore, J. Van Rie, D. Lereclus, J. Baum, J. Feitelson, D. R. Zeigler and D. H. Dean (1998) Bacillus thuringiensis and its pesticidal crystal proteins. *Microbiol. Mol. Biol. Rev.* **62**, 775–806.
31. The Universal Protein Resource (UniProt) UniProt Knowledgebase (UniProtKB) P0A372 (CR1AB_BACTA). Available at: http://www.uniprot.org/uniprot/P0A372. Accessed on 21 January 2014.
32. The Universal Protein Resource (UniProt) UniProt Knowledgebase (UniProtKB) P0A370 (CR1AB_BACTK). Available at: http://www.uniprot.org/uniprot/P0A370. Accessed on 21 January 2014.
33. Pozsgay, M., P. Fast, H. Kaplan and P. R. Carey (1987) The effect of sunlight on the protein crystals from *Bacillus thuringiensis* var. *kurstaki* HD1 and NRD12: A raman spectroscopic study. *J. Invertebr. Pathol.* **50**, 246–253.
34. Pusztai, M., P. Fast, L. Gringorten, H. Kaplan, T. Lessard and P. R. Carey (1991) The mechanism of sunlight-mediated inactivation of *Bacillus thuringiensis* crystals. *Biochem. J.* **273**, 43–47.
35. Köhler, H., N. Christoph, C. Bauer-Christoph, M. Geßner and K. Curschmann (1996) Die "Untypische Alterungsnote" im Wein Teil V: Einfluß kellerwirtschaftlicher Maßnahmen auf die Ausprägung der UTA. *Rebe & Wein* **49**, 213–218.
36. Silva, E. and J. Gaule (1977) Light-induced binding of riboflavin to lysozyme. *Radiat. Environ. Biophys.* **14**, 303–310.
37. Silva, E. (1979) Rate constants studies of the dye-sensitized photoinactivation of lysozyme. *Radiat. Environ. Biophys.* **16**, 71–79.
38. Souci, S. W., W. Fachmann and H. Kraut (2000) *Food Composition and Nutrition Tables* (6th revised and completed ed.), p. 930. Medpharm, Stuttgart.
39. Andrés-Lacueva, C., F. Mattivi and D. Tonon (1998) Determination of riboflavin, flavin mononucleotide and flavin-adenine dinucleotide in wine and other beverages by high-performance liquid chromatography with fluorescence detection. *J. Chromatogr. A* **823**, 355–363.
40. Mattivi, F., A. Monetti, U. Vrhovsek, D. Tonon and C. Andrés-Lacueva (2000) High-performance liquid chromatographic determination of the riboflavin concentration in white wines for predicting their resistance to light. *J. Chromatogr. A* **888**, 121–127.
41. Choe, E., R. Huang and D. B. Min (2005) Chemical reactions and stability of riboflavin in foods. *J. Food Sci.* **70**, R28–R36.