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Temperature affects the kinetics but not the products of the reaction between 4-methylbenzoquinone and lysine

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Abstract

Quinones are electrophilic compounds that can undergo Michael addition or Schiff base reaction with nucleophilic amines, but the effect of temperature has not been systematically studied. The aim of this study was to characterize how temperature affects the reaction mechanism and kinetics of 4-methylbenzoquinone (4MBQ) with lysine (Lys), N\textsubscript{\alpha}-acetyl Lys or N\textsubscript{\varepsilon}-acetyl Lys. The products were identified and characterized by LC-MS/MS, which revealed formation of Michael addition products, Schiff base, and a di-adduct in Lys and N\textsubscript{\varepsilon}-acetyl Lys-containing reaction mixtures. The product profiles were not affected by temperature in the range 15–100 °C. NMR analysis proved that Michael addition of N\textsubscript{\varepsilon}-acetyl Lys occurred on the C5 position of 4MBQ. Rate constants for the reactions studied by stopped-flow UV-vis spectrophotometry under pseudo-first-order conditions where the amines were present in excess in the range 15 °C to 45 °C showed the n-amo groups of Lys are more reactive than the ε-groups. The kinetics results revealed that the temperature dependence of reaction rates followed the Arhenius law, with activation energies in the order: Lys > N\textsubscript{\varepsilon}-acetyl Lys < N\textsubscript{\alpha}-acetyl Lys. Our results provide detailed knowledge about the temperature dependence of the reaction between Lys residues and quinones under conditions relevant for storage of foods.

Keywords: Michael addition, Schiff base, Polyphenol, Amino acid modification, Rate constant, Oxidation

1. Introduction

Polyphenols are widely present in plants and foods and their antioxidative properties are well-studied (Lemos et al., 2007; Van Den Berg & Labadie, 1989). Polyphenols with catechol moieties are easily oxidized by endogenous compounds (oxidizing enzymes, transition metal ions, and reactive oxygen species) in foods to generate quinones which are highly reactive electrophiles. Quinones react with themselves forming polymers or by addition to nucleophiles, such as thiol, amine, amide, indole, and imidazole functional groups in proteins, peptides, or amino acids (Yaylayan, 2003). In foods, quinone-protein conjugates have been shown to affect functional properties of proteins, e.g. by altering textural properties and water-holding capacity of meat products (Jongberg, Terkelsen, Miklos, & Lund, 2015), and to be involved in beer haze formation (Jongberg, Andersen, & Lund, 2020). Thus, food quality may be adversely affected if the reaction between quinones and proteins is not controlled (Lund, 2021). Nevertheless, the reaction of quinones with the thiol group of glutathione in wine results in colorless thiol-quinone adducts and can be used to avoid polyphenol polymerization and undesired browning (Singleton, 1987; Waterhouse & Laurie, 2006).

Primary products of reactions between quinones and amino acid residues include Michael addition products and Schiff bases (including amino- and thioquinone derivatives) (Scheme 1) (Kroll & Rohn, 2003; Li, Jongberg, Andersen, Davies, & Lund, 2016; Yaylayan, 2003; Yin, Hedegaard, Skibsted, & Andersen, 2014). The Michael addition is initiated by the addition of a nucleophile to a carbon–carbon double bond, forming a catechol-amino acid Michael addition product (Compound B, Scheme 1) through the formation of an intermediate adduct (Compound A, Scheme 1). The catechol-amino acid Michael addition product can be further oxidized in the presence of atmospheric oxygen to a colored substituted aminquinone (Compound C, Scheme 1). The Schiff base formation takes place by the condensation of the amino group to a carbonyl group of a quinone (Compound E, Scheme 1), through the formation of a gem-amino alcohol intermediate (Compound D, Scheme 1). Both the Schiff base and the oxidized Michael addition product may undergo further reactions with amino acids and form di-
adducts (Compound F, Scheme 1) (Hurrell, Finot, & Cuq, 1982; Yin et al., 2014). The type of reaction product formed depends on the structure of the catechol, the nucleophilicity of amino acids, and reaction conditions (pH and presence of metal ions) (Buzio & Waite, 2000; Faure et al., 2012; Hurrell et al., 1982; Nematollahi, Afkhami, Mosaed, & Rafiee, 2004; Tian, Cao, Wang, Yang, & Feng, 2013; Yang, Stuart, & Kamperman, 2014). Moreover, temperature may also affect the product formation. For example, previous studies have reported the formation of Schiff bases at high temperatures, e.g. at 70 °C (Yin et al., 2014) and 120 °C (Guerra & Yaylayan, 2014), whereas only Michael addition products were observed at low temperatures, e.g. 25 °C (Li et al., 2016). Rate constants of amine-quinone reactions have been reported to decrease with decreasing pH (Li et al., 2016; Nematollahi et al., 2004). However, the influence of temperature on product distribution has not been studied systematically, and the effects of temperature on the kinetics of amino acids reacting with quinones are unclear.

4-Methylbenzoquinone (4MBQ), formed by oxidation of 4-methylcatechol (4MC), is a highly reactive electrophile. It has been reported that 4MBQ is kinetically preferred to react with thiol groups, with significantly higher rate constants (>500,000 times) than for reactions with amine groups (Li et al., 2016). Nevertheless, when thiol groups are not available or blocked (e.g. oxidized or buried in the protein), amines have been suggested to become the main targets for quinones (Chen & Li, 2019). In addition, Lys residues are typically present in foods in much higher concentrations than Cys residues, e.g. milk contains amine groups in the mM range, while free thiol groups are only present in μM range (Poojary, Hellwig, Henle, & Lund, 2022; Jansson et al., 2017). Lys is an essential amino acid with a structure consisting of two amino groups (α- and ε-amino groups) (Rose, Borman, Coon, & Lambert, 1955). Among the nucleophilic amine groups on proteins, the ε-amino group of Lys is often the most abundant group, while the α-amino group is reactive when located in the N-terminal of proteins or if Lys is present as the free amino acid. It is therefore important to understand the reactivity of both amino groups of Lys. Also, understanding the kinetics and mechanism of reactions that potentially may lead to losses of Lys, such as the reaction with quinones, is necessary in order to control these reactions during food processing.

In the present study, the kinetics and the temperature dependence of the reaction of 4MBQ with Lys, Nα-acetyl Lys, and Nε-acetyl Lys was studied in a temperature range of 15–45 °C. This temperature range is relevant for storage of foods and covers “cool,” “ambient,” “warm,” and “excessive heat” conditions at 15, 25, 35, and 45 °C, respectively (Kim, Alrefaei, Bushlaibi, Ndegwa, Kaseloo, & Wynn, 2019). In addition, the reaction products formed at 100 °C were also examined to cover conditions relevant for food cooking. The mechanism of the reaction was further examined based on the characterization of product distributions by LC-MS/MS and NMR spectroscopy.

2. Materials and methods

2.1. Chemicals and reagents

4-Methyl catechol (4MC) (≥95 %), lysine (Lys) (≥98 %), Nα-acetyl lysine (Nα-acetyl Lys) (≥98 %), Nε-acetyl lysine (Nε-acetyl Lys) (≥98 %),
and acetonitrile (HPLC grade) were purchased from Sigma-Aldrich Denmark A/S (Copenhagen, Denmark). Ultra-pure water produced from a Milli-Q water purification system (Millipore, Bedford, MA) was used for all the experiments.

2.2. Generation of 4MBQ

4MBQ was generated according to the method reported by Li et al. (2016) with slight modifications. Briefly, 4MC (2 mM) was dissolved in phosphate buffer solution (0.1 M, pH 4.5). Cyclic voltammograms of this solution were obtained by using a voltammetric analyzer (CV-50 W, BAS Co., Ltd.) with the same electrodes used in the previous study (a glassy carbon working electrode, a platinum coil counter electrode, and an Ag/AgCl (reference electrode, Metrohm, Switzerland). The bulk electrolysis was performed at an initial potential of 460 mV versus the reference electrode under nitrogen by using the same voltammetric analyzer with a working electrode of reticulated vitreous carbon tube. Electrolysis was terminated when the amount of charge passed was equivalent to two electrons per molecule. The 4MBQ yield was estimated to be ca. 75 % (1.5 mM) by UV-vis spectrophotometry (Cintra 40, GBC Scientific Equipment Pty. Ltd., Australia); the absorbance of the sample was measured at 395 nm, and the 4MBQ concentration was determined by the Beer-Lambert law using an extinction coefficient ε (395) of 1350 M⁻¹ cm⁻¹ (Whitaker, Voragen, & Wong, 2002).

2.3. Identification of reaction products by LC-MS/MS

To ensure that pH of the reaction mixtures of 4MBQ and amino acids reached 7.0, the amino acids were dissolved in 0.2 M of phosphate buffer with different pH. Reaction products of 4MBQ (0.2 mM) and amino acids (20 mM) were prepared at 15, 25, 35, and 45 °C, and analyzed by LC-ESI-MS/MS as previously described (Zhu, Poojary, Andersen, & Lund, 2019) with some modifications. All the reaction solutions were stirred for 2 min at the corresponding temperature, and then filtered through 0.22 μm syringe filters and injected (10 μL) into an Ultimate 3000 UHPLC system (Thermo Scientific, CA USA) equipped with a Zorbax Eclipse Plus C-18 column (Agilent Technologies, Glostrup, Denmark; 100 mm length, 2.1 mm ID, 1.8 μm particle size). The mobile phase consisted of 0.1 % formic acid in water (mobile phase A) and 100 % acetonitrile (mobile phase B) with a flow rate of 0.25 mL/min. The gradient program was as follows, 0–14.1 min: 5 % B; 14.1–14.2 min: 5 %–95 % B; 14.2–15.8: 95 % B; 15.8–15.9 min: 95 %–5% B; 15.9–21.4: 5 % B. The column was kept at 25 °C. Mass spectra were obtained using an Orbitrap Q Exactive mass spectrometer (Thermo Scientific, CA, USA). The negative mode was used for 4MBQ-N-acetyl Lys adduct identification and positive mode was applied to 4MBQ-Lys and 4MBQ-N-acetyl Lys adduct identification. The spray voltage for negative and positive modes were −3.0 and 3.8 kV, respectively The other MS parameters were as follows, capillary temperature: 300 °C; sheath gas flow rate: 28 arbitrary units; auxiliary gas flow rate: 15 arbitrary units; sweep gas flow rate: 4 arbitrary units; S-lens RF level: 60 %. The full MS scans were acquired from 70 to 1000 m/z at a resolution of 70,000. The MS/MS fragmentation patterns of the adducts were obtained by high-energy N₂ collision induced dissociation-based MS/MS mode (resolution: 17,500), setting a normalization collision energy of 30 %.

2.4. Analysis of reaction products formed at 100 °C by UHPLC

4MBQ and amino acids were pre-heated individually at 100 °C in a metallic block heater for 1 min. Subsequently, aliquots of 0.5 mL of 4MBQ (0.2 mM) and 0.5 mL of N-acetyl lys (20 mM) were mixed in a 2 mL Eppendorf tube and incubated at 100 °C for 2 min. The reaction mixtures were also prepared at room temperature and included as control samples. All samples were filtered through 0.22 μm syringe filters and injected (10 μL) into a UHPLC instrument with a Dionex Ultimate 3000 system (Thermo Scientific, CA, USA) coupled with a Diode Array Detector (DAD, with acquisition wavelengths of 285 nm, 401 nm and 500 nm, according to the UV–vis spectra of the reaction mixtures). A Zorbax Eclipse Plus C-18 column (Agilent Technologies, Glostrup, Denmark; 100 mm length, 2.1 mm ID, 1.8 μm particle size) operated at 25 °C was used to separate analytes. The mobile phase consisted of 0.1 % trifluoroacetic acid in water (mobile phase A) and 100 % acetonitrile (mobile phase B) with a flow rate of 0.25 mL/min. The gradient program was as follows, 0–14.1 min: 5 % B; 14.1–14.2 min: 5 %–95 % B; 14.2–15.8: 95 % B; 15.8–15.9 min: 95 %–5% B; 15.9–20.4: 5 % B.

2.5. Purification and characterization of reaction products by NMR spectroscopy

The reaction product of 1.5 mM of 4MBQ and 100 mM of Nα-acetyl Lys resulting in the highest peak intensity from LC-MS analysis (Peak 1 of LC-MS chromatogram in Supplementary materials Fig. S1) was purified with a semi-preparative LC system equipped with an Eclipse Plus C18 column (Agilent Technologies, Glostrup, Denmark; 100 mm length, 4.6 mm ID, 3.5 μm particle size). The mobile phase consisted of 0.1 % formic acid in water (mobile phase A) and 100 % acetonitrile (mobile phase B) with a flow rate of 1.4 mL/min. The injection volume was 0.1 mL. The isotopic program was 15 % B for 10.0 min, and the column was operated at 25 °C. The chromatograms and the UV–vis spectra of each fraction were recorded by UHPLC-DAD (as described in 2.4) and the chromatograms were compared to the chromatograms obtained from LC-MS/MS (as described in 2.3). Thereafter, the peak at a retention time of 1.4–1.6 min (Supplementary materials Fig. S2) was identified as Peak 1 shown in Fig. S1 in Supplementary materials. After 500 injections, the target fraction was collected and freeze-dried (Heto Drywinner, Thermo Fisher). Subsequently, 5 mg of the sample was dissolved in 700 μL D₂O. Characterization by liquid-state NMR spectroscopy was performed using a Bruker instrument equipped with a non-inverse cryoprobe at 500 MHz (1H NMR) or 126 MHz (13C NMR). 13C NMR spectra were recorded in the attached proton test (APT) mode. Two-dimensional homonuclear quantum-quantum correlation spectroscopy (COSY), heteronuclear single quantum coherence (HSQC) and heteronuclear multiple bond correlation (HMBC) experiments were recorded to facilitate the assignment of the resonances. The deuterium-proton exchange experiment was performed by freeze-drying the D₂O-dissolved sample with MilliQ water twice, and acquiring the 13C NMR spectrum in H₂O, containing a closed lock capillary tube with D₂O. 1H spectra were referenced to the HDO signal (4.79 ppm), while the 13C spectra were referenced to the N-acetyl CH₃ signal (21.7 ppm). All spectra were processed using Mnova software (Mestrelab Research, Spain).

The oxidized Michael addition product (Compound C shown in Scheme 1) displayed the following peaks in 1H NMR (500 MHz, D₂O): δ 6.39 (s, 1H; H6), 5.74 (s, 1H, exchangeable, partly overlapped by HDO; H3), 4.28–4.24 (m, 1H; H7), 3.51 (t, J = 7 Hz, 1H; H8), 2.24 (s, 3H; CH3), 2.03 (s, 3H; Ac), 1.92–1.84 (m, 1H, H9), 1.79–1.70 (m, 3H; H8 and H9), 1.50–1.42 (m, 2H; Hδ). 13C NMR (126 MHz, D₂O): δ 186.0 (C2), 177.6 (COO), 173.9 (C1 and CON), 161.0 (C4), 146.6 (CS), 130.3 (C6), 53.8 (C3), 43.9 (C4), 30.6 (C7), 27.0 (C8), 22.6 (C9), 21.7 (Ac), 17.0 (CH2). 13C NMR (126 MHz, H₂O) δ 185.8 (C2), 178.9 (COO), 173.7 and 173.4 (C1 and CON), 160.9 (C4), 146.5 (CS), 130.0 (C6), 95.3 (C3), 54.7 (C4), 43.8 (C3), 31.0 (C8), 26.9 (C9), 22.4 (C10), 21.7 (Ac), 16.8 (CH3).

2.6. Kinetic investigation of the reaction between 4MBQ and amino acids

The reaction between 4MBQ and amino acids was investigated by measuring absorbance in the UV/Vis range (195–740 nm) at 15, 25, 30, 35 and 45 °C by using a SX20 stopped-flow spectrophotometer containing two drive syringes (Applied Photophysics, London, UK). The kinetic investigation was only performed up to 45 °C as it was not possible to increase the temperature in the instrument further and still obtain reliable results. Reactants (4MBQ and amino acids) were dissolved in 0.2 M phosphate buffer and loaded individually into the two
syringes. Equal volumes of each reactant were injected into the optical cell and UV–visible spectra recorded over time. The pH of the final mixture was 7.0. The concentrations of amino acids were at least 12.5 times higher than the 4MBQ concentrations to obtain pseudo-first-order kinetic conditions, which is a common approach for determination of rate constants (Van Boekel, 2008). Single (Eq. (1)) or double exponential curves (Eq. (2)) were fitted to the absorbance changes over time. For each sample, the type of exponential fitting was evaluated for each experiment and chosen to ensure $R^2 > 0.9700$.

\[ y = y_0 + A_1 e^{-x/t_1} \]  
\[ y = y_0 + A_1 e^{-x/t_1} + A_2 e^{-x/t_2} \]

Where $y$ is the absorbance at time $x$, $y_0$ is the initial absorbance, $A_1$, $A_2$, $t_1$, and $t_2$ are fitting parameters. In decay fitting, $A_1$, $A_2$ are positive, whereas in growth fitting, $A_1$, $A_2$ are negative. $k_{obs}$ and $k_{obs}'$ were calculated from the reciprocal value of $t_1$ and $t_2$, respectively.

The Arrhenius equation (Eq (3)) was used to evaluate the temperature dependence of the experimentally determined rate constants.

\[ \ln k = \ln A - \left( \frac{E_a}{R} \right) \left( \frac{1}{T} \right) \]  

Where $k$ is the reaction rate constant, $A$ is the pre-exponential factor, $E_a$ is the activation energy, $R$ is the gas constant, and $T$ is the temperature. $R^2$ values were $> 0.981$.

### 2.7. Statistical data analysis

Analysis of variance (ANOVA) was carried out by Microsoft Excel 2007. Significance was defined as $P < 0.05$. Curve fitting and the calculation of rate constants were performed using the OriginPro 2016 software (OriginLab Co., Northampton, USA).

### 3. Results and discussion

The products formed upon reaction between 4MBQ and amino acids were investigated by UHPLC-DAD and LC-MS/MS. In all samples, the peak with retention time ($R_T$) of 8.56 min was identified as 4MC.

![Chromatograms obtained by UV detection at 285 nm of 4MC (A), the reaction solution of 4MBQ (B), and adducts from the reaction of Lys with 4MBQ at 15 ℃ (C), 25 ℃ (D), 30 ℃ (E), 35 ℃ (F), and 45 ℃ (G). The retention time of the 4MC absorbance peak was at ~ 8.6 min, and the peak intensities are reported in the figure. The adduct peaks are marked as Peak 1 ($R_T = 1.94$ min) and Peak 2 ($R_T = 2.51$ min).](image)

Fig. 1.
The solution of 4MC oxidized by electrolysis showed a characteristic peak of 4MBQ at $R_T = 5.20$ min together with the peak of 4MC (Fig. 1B). 4MBQ was not detected when it was incubated with Lys and its derivatives.

### 3.1. Identification of reaction products formed from 4MBQ and Lys by LC-MS/MS

The HPLC-DAD chromatograms of the reaction mixture of 4MBQ and Lys incubated at temperatures between 15 and 45 °C showed two product peaks with $R_T$ of 1.94 (Peak 1) and 2.51 min (Peak 2) (Fig. 1C-G). The MS spectra revealed that Peak 1 was corresponding to $m/z$ 395.2294 [M + H]$^+$, which was tentatively identified as a di-adduct derived from one molecule of 4MBQ and two molecules of Lys (Compound A, in Fig. 2), where one of the Lys was added through Michael addition, and the other by a Schiff base formation reaction. The MS/MS spectra of the corresponding compound had product ions of $m/z$ 351.2402 (44 mass units lower than the precursor ion, suggesting a carboxylic group was lost), $m/z$ 268.1660 (127 mass units lower than the precursor ion), and $m/z$ 205.1339 (Table 1 and Fig. 2; see Supplementary materials Fig. S3 for MS/MS spectra). Hurrell et al. (1982) also proposed that besides mono adducts formed by either Michael addition or Schiff base conjugation, further reactions lead to complexes between Lys and polymerized quinones forming di-adducts. Peak 2 corresponded to a molecular ion $m/z$ 267.1341 [M + H]$^+$, which was tentatively identified as an oxidized Michael addition product (Compound B, in Fig. 2). The MS/MS spectra had a distinct fragmentation pattern with product ions of $m/z$ 251.1390, $m/z$ 250.1390, $m/z$ 206.1181, and $m/z$ 178.0865, which corresponded to the loss of a hydroxyl group, an amine group, a molecule of ammonia and a carbonyl group, and an ethylamine and a carboxyl group, respectively (Supplementary materials Fig. S4). Lys-moiety derived product ions, $m/z$ 84.0815 and $m/z$ 128.0710 (Compound A and B in Supplementary materials Fig. S5 and Table 1), were observed in the MS/MS spectra of both the compounds corresponding to Peak 1 and Peak 2. The identification of a Michael addition product was in agreement with the previous studies of Yin et al. (2014) and Li et al. (2016), however, they did not report the presence of di-adducts (Michael addition/Schiff base). In addition to the 1:1 quinone-Lys adduct and 1:2 quinone-Lys adduct, the formation of the adducts such as 1:3 quinone-Lys adducts was also explored, however, such adducts were not found.

### 3.2. Identification of reaction products formed from 4MBQ and $N^\varepsilon$-acetyl Lys by LC-MS/MS

The chromatogram of the reaction mixture of 4MBQ and $N^\varepsilon$-acetyl Lys had two peaks corresponding to reaction products, with $R_T$ of 4.71 (Peak 1) and 5.42 (Peak 2) (Supplementary materials Fig. S6). According to the MS spectra, Peak 1 and Peak 2 both corresponded to a molecular ion of $m/z$ 309.1449 [M + H]$^+$, which is matched to an oxidized Michael addition product (Compound C, Fig. 2). The MS/MS spectra and the fragmentation patterns of the oxidized Michael addition products related to Peak 1 and Peak 2 are shown in Supplementary materials Fig. S7 and Fig. S8 accordingly.
Table 1

<table>
<thead>
<tr>
<th>Compound</th>
<th>Exact mass</th>
<th>Accurate mass</th>
<th>Mass error (ppm)</th>
<th>Retention time (min)/Peaks</th>
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</thead>
<tbody>
<tr>
<td>4MBQ-Lys adduct*</td>
<td>395.2289</td>
<td>395.2289</td>
<td>0</td>
<td>395.2294 (3) 84.0815 (90), 128.0710 (60), 205.1339 (2), 268.1660 (24), 351.2402 (3) 1.94/Peak 1 (major)</td>
</tr>
<tr>
<td></td>
<td>267.1339</td>
<td>267.1344</td>
<td>1.87</td>
<td>267.1341 (1) 84.0815 (1 0 0), 128.0710 (40), 178.0865 (5), 206.1181 (18), 251.1390 (3) 2.51/Peak 2 (minor)</td>
</tr>
<tr>
<td>ε-acetyl Lys-adduct*</td>
<td>309.1445</td>
<td>309.1456</td>
<td>3.56</td>
<td>309.1449 (7) 84.0817 (1 0 0), 126.0919 (24), 138.0551 (0.5), 250.1070 (3), 265.1547 (3) 4.71/Peak 1 (minor)</td>
</tr>
<tr>
<td></td>
<td>309.1445</td>
<td>309.1451</td>
<td>1.94</td>
<td>309.1449 (8) 84.0816 (1 0 0), 126.0919 (30), 138.0551 (0.5), 250.1070 (4), 265.1551 (4) 5.42/Peak 2 (major)</td>
</tr>
<tr>
<td>4MBQ-N</td>
<td>307.1299</td>
<td>307.1303</td>
<td>1.30</td>
<td>307.1294 (7) 126.0552 (22), 263.1402 (14), 289.1194 (10) 5.58/Peak 1 (major)</td>
</tr>
<tr>
<td></td>
<td>477.2355</td>
<td>477.2361</td>
<td>1.26</td>
<td>477.2361 (9) 126.0552 (22), 183.0772 (8), 251.1394 (2), 435.2258 (12) 6.25/Peak 2 (minor)</td>
</tr>
</tbody>
</table>

* means negative mode was used. Exact mass is theoretical mass; Accurate mass is the experimentally determined mass.

The MS analysis performed in positive ionization mode did not result in specific spectral patterns for the products of the reaction of 4MBQ with N-acetyl Lys, therefore, negative mode was applied. The UHPLC-DAD profiling of the reaction mixture resulted in two distinct peaks with R_t of 5.58 (Peak 1) and 6.25 min (Peak 2) (Supplementary materials Fig. S1). According to the MS spectra, Peak 1 had a molecular ion m/z 307.1394 [M – H] , which was tentatively identified as an oxidized Michael addition product (Compound D, Fig. 2). The MS/MS spectra and fragmentation pattern of the corresponding compound is shown in Supplementary materials Fig. S9. The MS spectra corresponding to Peak 2 had a molecular ion of m/z 477.2361 [M – H] , which was tentatively identified as a di-adduct, presumably formed when a molecule of 4MBQ reacted with two molecules of N-acetyl Lys (Compound E, Fig. 2, Supplementary materials Fig. S10).

3.3. Identification of reaction products formed from 4MBQ and N-acetyl Lys by LC-MS/MS

The regiochemistry of the Michael addition product was investigated by using HMBC NMR spectroscopy, which also provided the key to the complete assignment of the 1H and 13C signals for the oxidized Michael addition product. Specifically, connectivity between the Lys H2 and the benzoquinone ipso position (5-position of 4MBQ) of the Michael addition product was established, together with connectivity through the benzoquinone ring of the product, such as, e.g., C4-to-(H30)-(H31)-to-H6-to-C1 (Fig. 3). Due to deuterium exchange, the connectivity for H3 could not be established from HMBC, however, strong 3-bond

3.4. NMR analysis of oxidized Michael addition product

It was possible to separate and purify the major product of the reaction mixture of 4MBQ and N-acetyl Lys (identified as an oxidized Michael addition product according to the LC-MS/MS analysis; Peak 1 shown in Supplementary materials Fig. S1; and Compound D shown in Fig. 2). The NMR analysis was employed to confirm the chemical structure of the product. The product was found to be quasi-stable during the freeze-drying step used during the NMR sample preparation, but was stable in D2O under the NMR experimental conditions for at least one week. Repeated freeze drying and dissolution degraded the compound substantially.

The 1H spectrum had peaks characteristic of N-acetyl Lys with a slightly downfield shifted ε-CH2 chemical shift value of 3.51 ppm (Supplementary materials Fig. S11A), compared to a previous report that the ε-CH2 was around 3.0 ppm (Knerr, Pischetsrieder, & Severin, 1994). N-acetyl Lys assignments were corroborated by COSY (Supplementary materials Fig. S12), HSQC and HMBC data. Additional singlets were visible in the 1H spectrum, obtained 6 h after dissolution in D2O, appearing at 6.39 (one proton), 5.74 (approx. one proton), and 2.4 ppm (three protons). These three signals had HSQC correlations in the 13C direction at 130.3, 95.3, and 17.0 ppm, respectively (Supplementary materials Fig. S13). The proton signal at 5.74 ppm, however, was susceptible to proton-deuterium exchange; after 3 days it had been completely exchanged for deuterium, whereas the other two signals remained unchanged (Supplementary materials Fig. S11B and S13). The signal at 95.3 ppm was also absent in the D2O-equilibrated 13C NMR spectrum, likely due to the loss in polarization transfer for deuterated carbon, as well as signal broadening from C-D coupling. A deuterium-proton exchange experiment was conducted, confirming that the signal at 95.3 ppm reemerged in the 13C NMR spectrum upon protonation in H2O (Supplementary materials Fig. S14). The 13C NMR spectrum in H2O displayed four downfield C=O signals (185.8, 178.9, 173.7, and 173.4 ppm), while two of these signals were overlapped in the corresponding spectrum in D2O. Furthermore, two CH and one CH3 signal from the benzoquinone were visible, in addition to the aliphatic N-acetyl Lys signals. Collectively, these observations correlate with the product being an oxidized Michael addition product from nucleophilic attack of the Lys ε-amino on the 5- or 6-position of 4MBQ.
7 correlations of C4 with both H(CH3), H6, and H ε unequivocally positioned C3 by exclusion of any other possibilities. These assignments, proton-deuterium exchange, and chemical shift values, matching the structure shown in Fig. 2 (Compound D), are in agreement with a related, recently published structure (Marmelstein, Lobba, Mogilevsky, Maza, Brauer, & Francis, 2020).

Unfortunately, adducts of the reaction between 4MBQ and Lys were eluted early during the chromatographic run, and could not be separated properly from co-eluting salts to obtain a successful identification by NMR.

In a previous study, the thiol group of Cys was found to react on the C6 position of 4MBQ as evidenced by two-dimensional NMR spectroscopy (Arsad et al., 2020). The difference in reaction position of 4MBQ between the study on Cys and the present study could be derived from the different reaction conditions used in the two studies; the thiol adduct was synthesized at pH 4.5 by including Cys during electrolysis (Arsad et al., 2020), while the 4MBQ-amine reaction in the present study was conducted at pH 7.0 after 4MBQ was generated by electrolysis.

3.5. Effect of temperature on reaction products formed from 4MBQ and Lys or N-acetylated-Lys

The UHPLC-DAD chromatographic profiling (detection wavelength, 285 nm) of the reaction mixture of 4MBQ and Lys or N-acetylated Lys revealed that the peak intensity of 4MC decreased with increasing temperature, with an apparent loss of intensity observed from 30 °C to 35 °C (Fig. 1, Supplementary materials Fig. S1 and Fig. S6). It seemed that the 4MBQ-amine reaction inhibited the reduction of 4MBQ to yield 4MC and the inhibition was enhanced from 30 °C to 35 °C. On the other hand, the peak intensities corresponding to the reaction products did not change irrespective of reaction temperature (15–45 °C), suggesting the reaction between quinones and Lys/N-acetylated Lys derivatives are independent of the tested reaction temperature (Fig. 1, Supplementary materials Fig. S1 and Fig. S6). An additional experiment was conducted to verify if the nature of products vary at the reaction temperature of 100 °C, with a result showing no difference in the peak intensities compared to that obtained for reaction carried out at 22 °C (Supplementary materials Fig. S15). These observations further confirmed that temperature did not affect the nature of the products and reaction products were stable at high temperatures. Many previous studies have confirmed the formation of Michael addition products upon reaction between quinones and amines at low temperatures (Li et al., 2016). Guerra et al. (2014) identified the Schiff base formation by LC-MS/MS when catechin and glycine was incubated at 120 °C for 70 min until the sample was dried. In another study, Schiff base products derived from Lys and epicatechin (pH 8.0, 70 °C heating for 10 min in the presence of ferrous ion) were identified by LC-MS (Yin et al., 2014). A few studies have shown that benzoquinone reacted with amines to form Schiff bases in organic solutions (MeOH/THF) (Klein, Bargas, Horak, & Navarro, 1988). Based on these studies, it appears that formation of Schiff base products require high temperature or anhydrous condition. This can be explained by the fact that removal of water molecules from the reaction mixture shifts the equilibrium towards the side of the

Fig. 3. HMBC NMR spectrum of oxidized Michael addition product with the assignment of observed C/H correlations indicated by arrows. The spectrum was recorded 32 h after the sample was dissolved in D2O. Connectivity is established from Lys H ε to C1, showing unequivocally that the Michael addition has taken place by the attack of Lys N ε at C4. Connectivity for H3/C3 is lacking due to proton-deuterium exchange.

Fig. 4. UV-visible spectra of 2 mM 4MC at 25 °C, pH 4.5 and 0.75 mM 4MBQ generated from 4MC by bulk electrolysis. The absorption maximum for 4MC was around 285 nm, while it was at 401 nm for 4MBQ.
products (Yin et al., 2014) (reaction 5 in Scheme 1). In the present study, both products of Schiff base formation and Michael addition were tentatively identified when 4MBQ was incubated with Lys and N\textsubscript{\alpha}-acetyl Lys in the temperature range of 15–45 °C. The distribution of products generated at 100 °C were found to be the same as seen at 15–45 °C. However, it is essential to purify and quantify both Schiff base and Michael addition products before concluding on whether the formation of Schiff base products is quantitatively substantial in the tested reaction conditions.

3.6. Kinetics of 4MBQ-amine reactions

The rate constants for the reactions were determined by applying pseudo-first order conditions, where the concentrations of the amino acids could be considered as being constant due to the large excess compared to 4MBQ. 4MC had a characteristic maximum UV-absorption at around 285 nm, whereas 4MBQ had a specific absorption band at 401 nm (Fig. 4) (Li et al., 2016). The aqueous solution of 4MBQ was unstable, as evidenced by a decrease of absorption intensity at 401 nm over time together with an increasing absorbance at 285 nm (Fig. 5A). In the presence of excess Lys, N\textsubscript{\varepsilon}-acetyl Lys, or N\textsubscript{\alpha}-acetyl Lys, the 4MBQ absorption peak at 401 nm decreased, whereas concurrent absorption increases were seen at 285 nm, 308 nm, and 491 nm. The steady increases at 308 nm and 491 nm were assigned to absorption spectra of products of the reactions between 4MBQ and the amino acids. The absorption spectra of 4MBQ-Lys and 4MBQ-N\textsubscript{\varepsilon}-acetyl Lys mixtures had two isoabsorbent points at around 340 nm and 450 nm, indicating that the UV–vis active reaction intermediates did not accumulate during the reactions (Fig. 5B and Fig. 5C). In the case of 4MBQ-N\textsubscript{\alpha}-acetyl Lys, only one isoabsorbent point at 450 nm was observed (Fig. 5D).

The kinetics of the absorption changes at 285 and 401 nm were characterized by fitting to double exponential decay curves. The spectral changes at 308 nm and 491 nm had two stages of change, an increase followed by a decrease, and the kinetics of the initial increase in absorption were determined by fitting to single exponential curves (Supplementary materials Fig. S16A and Fig. S16B). When the concentration of N\textsubscript{\alpha}-acetyl Lys was over 30 mM and the temperature was over 25 °C, the spectra at 401 nm showed two stages of decrease with an initial rapid decrease followed by a slower decrease. The first stage of rapid decrease was used for the single exponential fitting (Supplementary materials Fig. S16C). The subsequent slower decrease was not included in the fitting. The first stages of the change in absorption were more likely related to the immediate reactions (reactions 1–5 in Scheme 1), including the mono Michael addition and Schiff base formation. The following stage of change is likely to refer to the further reactions to form di-adducts (reactions 6 and 7 in Scheme 1).

The exponential fits allowed the calculation of pseudo-first-order rate constants (k\textsubscript{obs} and k\textsuperscript{′}\textsubscript{obs}) for the reactions (Fig. 6). For the rate constants determined from the double exponential decay fitting of absorption, k\textsubscript{obs} was dependent on the amino acid concentration while the k\textsuperscript{′}\textsubscript{obs} values obtained at different reactant concentrations were nearly constant (data not shown). Thus, apparent second-order rate constants (k\textsubscript{a}) for the reactions could be determined from the slopes of linear correlations of k\textsubscript{obs} values as a function of concentration of amino acids. The k\textsubscript{a} values of the reaction of 4MBQ with Lys, N\textsuperscript{\alpha}-acetyl Lys, and N\textsuperscript{\varepsilon}-acetyl Lys are shown in Supplementary materials Table S1, S2, and S3, respectively. The plots of 4MBQ reacting with Lys was shown as examples of determination of k\textsubscript{a} in Fig. 7. For the reaction of 4MBQ with
Fig. 6. Absorption changes for the reaction of 4MBQ (0.2 mM) with Lys (10 mM) were recorded at 285 nm (A), 308 nm (B), 401 nm (C), and 491 nm (D). Solid lines show first-order fits by exponential curves. Double exponential fitting was applied to the plots at 285 nm and 401 nm, while single exponential fitting was used for absorptions at 308 nm and 491 nm.

Fig. 7. Pseudo-first-order rate constants ($k_{\text{obs}}$) for the reaction of 4MBQ (0.2 mM) with Lys determined at 285 nm (A), 308 nm (B), 401 nm (C), and 491 nm (D) at 25 °C in aqueous phosphate buffer (0.1 M) at pH 7.0 and plotted against Lys concentration (2.5–50 mM). Second-order rate constants ($k_2$) were determined as the slope of the linear regressions.
Table 3
Second-order rate constants ($k_2$) for reaction of 4MBQ with Lys, N$^\alpha$-acetyl Lys, N$^\alpha$-acetyl Lys at pH 7.0 and determined using absorbance changes at 285 nm, 308 nm, 401 nm, and 491 nm.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Temp. (°C)</th>
<th>$k_2^{obs}$ (M$^{-1}$s$^{-1}$)</th>
<th>$k_2^{obs}$ (M$^{-1}$s$^{-1}$)</th>
<th>$k_2^{obs}$ (M$^{-1}$s$^{-1}$)</th>
<th>$k_2^{obs}$ (M$^{-1}$s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lys</td>
<td>15</td>
<td>1.2 ± 0.1</td>
<td>1.2 ± 0.1</td>
<td>1.9 ± 0.0</td>
<td>1.2 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>3.2 ± 0.1</td>
<td>3.2 ± 0.1</td>
<td>4.5 ± 0.1</td>
<td>3.0 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>3.6 ± 0.1</td>
<td>3.7 ± 0.1</td>
<td>5.7 ± 0.1</td>
<td>3.2 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>35</td>
<td>5.6 ± 0.1</td>
<td>5.5 ± 0.2</td>
<td>8.6 ± 0.2</td>
<td>5.3 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>45</td>
<td>10.8 ± 0.2</td>
<td>10.9 ± 0.1</td>
<td>16.1 ± 0.2</td>
<td>10.4 ± 0.3</td>
</tr>
<tr>
<td>N$^\alpha$-acetyl Lys</td>
<td>15</td>
<td>0.8 ± 0.0</td>
<td>0.8 ± 0.0</td>
<td>1.0 ± 0.1</td>
<td>0.7 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>1.5 ± 0.0</td>
<td>1.6 ± 0.0</td>
<td>1.8 ± 0.1</td>
<td>1.4 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>2.9 ± 0.1</td>
<td>2.8 ± 0.1</td>
<td>3.5 ± 0.1</td>
<td>2.3 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>35</td>
<td>4.3 ± 0.3</td>
<td>3.4 ± 0.2</td>
<td>4.2 ± 0.1</td>
<td>2.9 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>45</td>
<td>7.7 ± 0.2</td>
<td>7.8 ± 0.3</td>
<td>7.7 ± 0.1</td>
<td>7.0 ± 0.2</td>
</tr>
<tr>
<td>N$^\alpha$-acetyl Lys</td>
<td>15</td>
<td>0.2 ± 0.0</td>
<td>0.2 ± 0.0</td>
<td>0.7 ± 0.0</td>
<td>0.3 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>0.5 ± 0.0</td>
<td>0.5 ± 0.0</td>
<td>1.4 ± 0.1</td>
<td>0.4 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>0.7 ± 0.0</td>
<td>0.7 ± 0.0</td>
<td>2.5 ± 0.1</td>
<td>0.7 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>35</td>
<td>1.3 ± 0.1</td>
<td>1.1 ± 0.0</td>
<td>3.7 ± 0.2</td>
<td>0.9 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>45</td>
<td>2.7 ± 0.2</td>
<td>2.6 ± 0.2</td>
<td>6.5 ± 0.6</td>
<td>2.5 ± 0.1</td>
</tr>
</tbody>
</table>

(Standard deviations given as 0.0 means the values are below 0.05.)

Fig. 8. Arrhenius plots for the reactions of 4MBQ with Lys, N$^\alpha$-acetyl Lys and N$^\alpha$-acetyl Lys according to the observations at 491 nm.

Lys, $k_2$ was 4.5 ± 0.1 M$^{-1}$ s$^{-1}$ determined at 401 nm (25 °C) (Fig. 7C), which was higher than the rate constants for product formation, where $k_2$ determined at 308 nm and 491 nm were 3.2 ± 0.1 M$^{-1}$ s$^{-1}$ and 3.0 ± 0.1 M$^{-1}$ s$^{-1}$, respectively (Fig. 7B and 7D). Similarly, for the 4MBQ reaction with N$^\alpha$-acetyl Lys the $k_2$ for the 4MBQ decay determined at 401 nm was higher than $k_2$ determined at the other three wavelengths, suggesting that other reactions apart from the product formation contribute to absorption changes at 401 nm. This could likely be the concurrent decay of 4MBQ to 4MC (Li et al., 2016) and quinone polymerization (Bittner, 2006). For the reaction of 4MBQ with N$^\alpha$-acetyl Lys, $k_2$ determined at all four wavelengths were almost similar at all examined temperatures (Table 2).

Based on the second-order rate constants the order of reactivity towards 4MBQ was: Lys > N$^\alpha$-acetyl Lys > N$^\alpha$-acetyl Lys (Table 2). Interestingly, the value of $k_2$ of Lys reacting with 4MBQ was almost equal to the sum of rate constants of N$^\alpha$-acytelyLys and N$^\alpha$-acetyl Lys reacting with 4MBQ. This may be explained by two amino groups in Lys reacting independently with 4MBQ, with rate constants very similar to the corresponding rate constants of the acetylated forms of Lys. The rate constants observed for the reaction of 4MBQ with N$^\alpha$-acetyl Lys was higher than that of N$^\alpha$-acetyl Lys, indicating that the ε-amino group was more reactive than the ε-amino group, which was also found by Pierpoint (1969) and in our previous study (Li et al., 2016). As discussed previously this could be due to the lower pKa value of the ε-amino group compared to that of the ε-amino group (8.90 vs 10.28), and thus a higher concentration of the deprotonated amine at pH 7.0, which is a better nucleophile than the protonated amine. This is in agreement with previous studies showing lower rate constants at lower pH (Li et al., 2016; Nematollahi et al., 2004).

As expected, $k_2$ increased when the temperature was increased from 15 to 45 °C (Table 2). The second-order rate constants $k_2$ in the range of 15 to 45 °C was fitted to the Arrhenius equation (Fig. 8), which gave acceptable linear fits ($R^2 = 0.981-0.998$) (Table 3). The activation energy ($E_a$) is the threshold kinetic energy for a reaction to occur, and was found to be similar for the four wavelengths used to calculate $k_2$ (Dai, Luo, Luo, Zheng, & Zhang, 2021; Menzinger & Wogang, 1969). The $E_a$ of N$^\alpha$-acetyl Lys was found to be slightly higher than those of N$^\alpha$-acetyl Lys and Lys, indicating that the ε-amino group had a higher energy barrier for reaction than the ε-amino group. The $E_a$ of the 4MBQ reaction with Lys was higher than that of the reaction between the 3,4-quinone of bisphenol A with N-acytely Cys and glutathione, which were 10.8 and 19.9 kJ/mol, respectively (Stack, Conrad, & Mahmud, 2018). These results indicate that the reaction between quinones and thiol groups has

Table 3
Arrhenius parameters for reactions of 4MBQ with Lys, N$^\alpha$-acetyl Lys, N$^\alpha$-acetyl Lys at pH 7.0 determined from second-order rate constants determined from absorbance changes at 285 nm, 308 nm, 401 nm, and 491 nm. The data are presented as mean ± standard errors.

<table>
<thead>
<tr>
<th>Amino acids</th>
<th>Arrhenius parameters</th>
<th>285 nm</th>
<th>308 nm</th>
<th>401 nm</th>
<th>491 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lys</td>
<td>$E_a$ (kJ/mol)</td>
<td>54.3 ± 3.6</td>
<td>55.2 ± 3.6</td>
<td>54.2 ± 1.9</td>
<td>54.0 ± 4.3</td>
</tr>
<tr>
<td></td>
<td>lnA</td>
<td>16.0 ± 1.4</td>
<td>16.4 ± 1.4</td>
<td>16.4 ± 0.8</td>
<td>15.9 ± 1.7</td>
</tr>
<tr>
<td></td>
<td>$R^2$</td>
<td>0.987</td>
<td>0.988</td>
<td>0.996</td>
<td>0.981</td>
</tr>
<tr>
<td>N$^\alpha$-acetyl Lys</td>
<td>$E_a$ (kJ/mol)</td>
<td>57.8 ± 4.4</td>
<td>57.3 ± 2.8</td>
<td>52.6 ± 4.2</td>
<td>56.1 ± 4.1</td>
</tr>
<tr>
<td></td>
<td>lnA</td>
<td>17.0 ± 1.7</td>
<td>16.8 ± 1.1</td>
<td>15.1 ± 1.7</td>
<td>16.2 ± 1.6</td>
</tr>
<tr>
<td></td>
<td>$R^2$</td>
<td>0.993</td>
<td>0.981</td>
<td>0.984</td>
<td>0.981</td>
</tr>
<tr>
<td>N$^\alpha$-acetyl Lys</td>
<td>$E_a$ (kJ/mol)</td>
<td>62.8 ± 4.4</td>
<td>64.3 ± 1.5</td>
<td>75.8 ± 3.8</td>
<td>61.7 ± 4.4</td>
</tr>
<tr>
<td></td>
<td>lnA</td>
<td>17.8 ± 1.8</td>
<td>18.3 ± 0.6</td>
<td>17.0 ± 1.5</td>
<td>17.2 ± 2.0</td>
</tr>
<tr>
<td></td>
<td>$R^2$</td>
<td>0.985</td>
<td>0.998</td>
<td>0.987</td>
<td>0.981</td>
</tr>
</tbody>
</table>
a lower activation energy barrier compared to the reaction of amine groups. The identified products of the reactions demonstrated competing Michael addition and Schiff base pathways, and the determined second order rate constants must therefore be a sum of the kinetics of the initial rate determining steps of both pathways. Although the rate constants were found to follow the usual Arrhenius temperature dependency, the distribution of the identified products did not seem to be temperature dependent, which indicate similar activation energies for the two pathways. According to the kinetics, N²-acetyl Lys was more reactive than N²-acetyl Lys (Table 3), indicating that the α-amino group is more reactive than the ϵ-amino group towards quinones. An interesting result was also the identification of di-adducts formed by a combination of Michael addition and Schiff base pathways. The di-adduct distribution was also not found to be affected by temperature (described above). The formation of di-adduct between nucleophilic groups and quinones (Cao & Xiong, 2015; Nikolantoniak, Jourdes, Shinoda, Teissedre, Quideau, & Darriet, 2012; Stack et al., 2018; Tang et al., 2017) may play an important role by cross-linking proteins in the presence of polyphenols (Jongberg, Torngren, Gunvig, Skibsted, & Lund, 2013) and may thereby change food properties, such as textural stability (Jongberg et al., 2015; Tang et al., 2017) and haze formation in beverages (Jongberg, Andersen & Lund, 2020).

4. Conclusion

The reaction products identified by LC-MS/MS analysis show that 4MBQ reacts with Lys by both Michael addition and Schiff base formation. NMR analysis proved that the Michael addition of Lys primarily takes place at the C5 position of 4MBQ. Similar distributions of reaction products were found to be generated at 15–45 °C and at 100 °C. The second order rate constants for the reactions of 4MBQ with the amino acids followed the Arrhenius law between 15 and 45 °C, and the rate constants shown at that pH 7.0 the Lys α-amino group is more reactive than the ϵ-amino group due to the presence of a higher amount of non-protonated amino groups.

CRediT authorship contribution statement

Jingyuan Liu: Conceptualization, Investigation, Formal analysis, Visualization, Writing – original draft. Maheshma H. Poojary: Methodology, Validation, Writing – review & editing, Supervision. Mikkel B. Thygesen: Investigation, Formal analysis, Visualization, Writing – review & editing. Mogens L. Andersen: Conceptualization, Validation, Writing – review & editing, Supervision. Marianne N. Lund: Conceptualization, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Marianne Nissen Lund reports financial support was provided by Independent Research Fund Denmark. Jingyuan Liu reports financial support was provided by China Scholarship Council.

Data availability

Data will be made available on request.

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