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Research Article

A Kinetic Degradation Study of Curcumin in Its Free Form and Loaded in Polymeric Micelles

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Abstract. Curcumin, a phenolic compound, possesses many pharmacological activities and is under clinical evaluation to treat different diseases. However, conflicting data about its stability have been reported. In this study, the kinetic degradation of curcumin from a natural curcuminoid mixture under various conditions (pH, temperature, and dielectric constant of the medium) was investigated. Moreover, the degradation of pure curcumin at some selected conditions was also determined. To fully solubilize curcumin and to prevent precipitation of curcumin that occurs when low concentrations of co–solvent are present, a 50:50 (*v*/*v*) aqueous buffer/methanol mixture was used as standard medium to study its degradation kinetics. The results showed that degradation of curcumin both as pure compound and present in the curcuminoid mixture followed first order kinetic reaction. It was further shown that an increasing pH, temperature, and dielectric constant of the medium resulted in an increase in the degradation rate. Curcumin showed rapid degradation due to autoxidation in aqueous buffer pH=8.0 with a rate constant of 280×10^{-3} h^{-1}, corresponding with a half–life ($t_{1/2}$) of 2.5 h. Dioxygenated bicyclopentadione was identified as the final degradation product. Importantly, curcumin loaded as curcuminoid mixture in *ω*–methoxy poly (ethylene glycol)–*b*–(*N*–(2–benzoyloxypropyl) methacrylamide) (mPEG–HPMA–Bz) polymeric micelles and in Triton X–100 micelles was about 300–500 times more stable than in aqueous buffer. Therefore, loading of curcumin into polymeric micelles is a promising approach to stabilize this compound and develop formulations suitable for further pharmaceutical and clinical studies.

KEY WORDS: curcumin; degradation; polymeric micelles; stability.

INTRODUCTION

Curcumin ([1,7–bis–(4–hydroxy–3–methoxyphenyl)–1,6–heptadiene–3,5–dione] (diferuloyl methane)), a phenolic compound (1), is present in many kinds of medicinal plants, especially in *Curcuma longa* (turmeric), and was first isolated by Vogel *et al*. (2). Curcumin possesses many pharmacological activities including antioxidant, anti–infection, anti–inflammation, anti–Alzheimer and anticancer (3–5). Most of the commercial curcumin products contain the structurally related compounds demethoxycurcumin and bisdemethoxycurcumin (Fig. 1). The bis–α,β–unsaturated β–diketone form of curcumin and also of demethoxycurcumin/bisdemethoxycurcumin exists in a nonpolar environment, whereas the enol tautomer predominates in both aqueous solution and in polar protic or aprotic solvents as well as in a biological media (6). Curcumin has a very low aqueous solubility particularly at acidic and neutral pH. It has been reported that curcumin solubility in aqueous buffer (pH= 5.0) was only 11 ng/mL (7). Curcumin in the form of commercially available curcuminoid mixture is presently under investigation in more than 100 clinical trials (8). Capsules containing curcumin as powder were administered to healthy volunteers (9) as well as to cancer patients (10) in a high dose of 4–12 g/day. However, curcumin has a very low bioavailability after oral administration (10). Furthermore, in a preclinical study it was shown that only 0.6 μmol/L of free curcumin and its conjugates were detected in the rat's serum after oral administration of diets containing 0.5% of curcumin (11). The major factors of its low bioavailability are the poor absorption, rapid metabolism especially by glucuronidation conjugation and rapid elimination (12). The chemical instability of curcumin under alkaline pH is well documented. Schneider *et al*. published a number of interesting papers in which the degradation of curcumin in aqueous buffer was studied (8). They also studied the degradation mechanism and identified the formed products.

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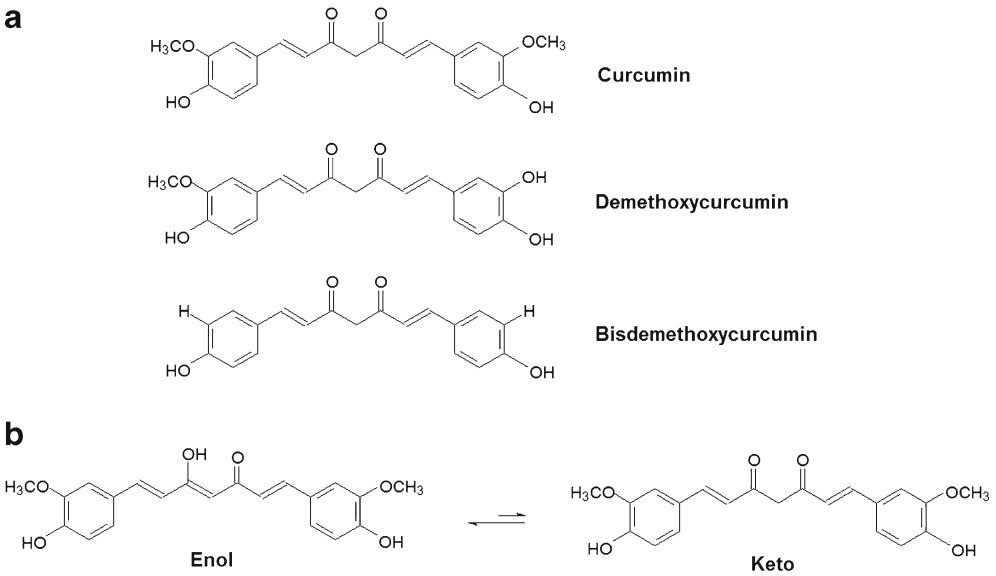

Fig. 1. Chemical structures of curcumin, demethoxycurcumin, and bisdemethoxycurcumin (**a**) and the diketone and enol tautomeric forms of curcumin (**b**)

They concluded that curcumin does not degrade via a hydrolytic process resulting in chain scission, as assumed in the older literature (7,13,14), but via oxidation yielding a bicyclopentadione final product. They convincingly showed that degradation of 1 mol of curcumin is associated with the consumption of 1 mol of O_2 (15). Further, by degrading curcumin in media with $^{18}O_2$ and $H_2{}^{18}O$, they showed that the final degradation product contains one oxygen atom of O_2 and one from water (Supplemental 1) (16). In a recent paper, they proposed a reaction scheme of the oxidative conversion of curcumin via 15 intermediate compounds to a deoxygenated bicyclopentadione (17). A landmark paper of Wang *et al.* (>400 Citations) demonstrated that curcumin in PBS pH=7.2 with 2% methanol almost completely degraded within 30 min at 37°C (13). However, we found that this rapid "degradation" was not due to chemical decomposition but likely due to precipitation of curcumin, demethoxycurcumin, and bisdemethoxycurcumin (see results section "Solubility of Curcumin in the Form of Curcuminoid Mixture in Aqueous Buffer/Methanol Mixtures"). The low bioavailability, together with its rapid degradation under physiological or alkaline conditions is a major limitation for the clinical application of curcumin. Therefore, particularly innovative nanoformulations have been developed to overcome these problems. Interestingly, it has been reported that encapsulation of curcumin in the form of curcuminoid mixture in micelles or nanoparticles retards the degradation of curcumin (18–23). In this present study, we focused on the curcuminoid mixture because the anticancer and anti-inflammatory activities of this mixture were stronger than that of pure curcumin alone and because the curcuminoid mixture has been used in most clinical trials (5,24). The degradation of curcumin in the curcuminoid mixture in its free form was performed in mixture of water and methanol (varying volume fractions) and at different pH and temperatures to get insight into the mechanism of enhanced stability of curcumin in the form of curcuminoid mixture loaded in polymeric micelles.

MATERIALS AND METHODS

Materials

Curcumin in the form of a curcuminoid mixture extracted from *Curcuma longa* (Curcumin) was purchased from Sigma–Aldrich (C1386,>65% purity, St. Louis, MO, USA). Pure curcumin was purchased from Sigma–Aldrich (78246, ≥99.5% purity, St. Louis, MO, USA). *ω*–Methoxy poly(ethylene glycol)–*b*–(*N*–(2–benzoyloxypropyl) methacrylamide) (mPEG–HPMA–Bz) was synthesized as described previously (25,26). Methanol, acetonitrile, acetic acid, acetone and Triton X–100 were purchased from Merck (Darmstadt, Germany). All chemicals were the highest grade available.

Reversed–Phase High Performance Liquid Chromatography (RP–HPLC)

HPLC analysis was carried out on a Waters system using an analytical C18 column, SunFire™ (5 μm, 150×4.6 mm). A gradient system was run from 5:95 (*v*/*v*) acetonitrile/water as eluent A and acetonitrile as eluent B. Both eluents were adjusted by addition of acetic acid to pH=3.0. The gradient was run from 45% A to 60% B in 10 min. The injection volume was 20 μL, the flow rate was 1.2 mL/min and the detection wavelengths were 425 and 254 nm. Peak areas were processed with Empower software (Waters Corporation).

Solubility of Curcumin in the Form of Curcuminoid Mixture in Aqueous Buffer/Methanol Mixtures

First, we studied the solubility of curcumin in the form of curcuminoid mixture in aqueous buffer with 2% (*v*/*v*) methanol at room temperature. In detail, 20 μL of a stock solution of curcuminoid mixture in methanol (curcumin concentration was 1.25 and 2.50 mg/mL (3.4 and 6.8 mM)) was added to 980 μL of 100 mM ammonium acetate buffer, pH=5.0 (final concentration of curcumin in the curcuminoid mixture in the

buffer solution was 25 and 50 μg/mL (or 68 and 136 μM). Next, the solubility of curcumin in the curcuminoid mixture in different aqueous buffer/methanol mixtures was studied as follows. The curcuminoid mixture was dissolved in methanol at a curcumin concentration of 5 mg/mL and 5 μL of this stock solution was added to 995 μL of 100 mM ammonium acetate buffer pH=5.0 that contained 10–50% (*v*/*v*) of methanol (final concentration in the buffer solution was 25 μg/mL (68 μM)). All samples were separately kept at room temperature for 6 h, and for 3 and 7 days in tightly closed vials. At different time points, samples of 1 mL were taken and centrifuged at 5000×*g* for 20 min in a Sigma 4K15 laboratory centrifuge (Osterode, Germany), rotor no. 11156) and 200 μL of the supernatant were mixed with 800 μL of methanol and stored at −20°C prior to the HPLC analysis (see "Reversed–Phase High Performance Liquid Chromatography (RP–HPLC)" Section). Calibration was done using stock solutions of the curcuminoid mixture in methanol (curcumin concentration was 0.0625–10 μg/mL, 0.17–27 μM) with an injection volume of 20 μL.

Dynamic light scattering (DLS) analysis was performed to investigate the possible formation of (nano) precipitates. In detail, 20 μL of stock solutions of the curcuminoid mixture in methanol (curcumin concentration was 37 μg/mL (100 μM) and 50 μg/mL (136 μM)) was added to 980 μL of 100 mM ammonium acetate buffer, pH=5.0 or 100 mM phosphate buffer, pH=8.0, or buffer (pH=5.0 or 8.0) containing 50% (*v*/*v*) of methanol. These mixtures were incubated at 25 and 37°C. The appearances of the mixtures were visually inspected. The scattering intensity of the solutions/dispersions as well as the size of the particles was measured using a particle size analyzer (Malvern, UK) at 0 and 24 h. The size measurements were taken at a fixed angle of 173°.

To investigate the degradation and precipitation of curcumin in curcuminoid mixture at low concentration of co-solvent (2% (*v*/*v*) of methanol), 22 μL of curcuminoid mixture stock solution in methanol (curcumin concentration was 1.8 mg/mL (5 mM)) was added to 1078 μL of phosphate buffer pH=7.2 and incubated for 2 h at 37°C (13). Thereafter, a sample of 100 μL was immediately added to 900 μL of methanol and adjusted the pH to 5.0 prior. After 2 h incubation, the samples were centrifuged at 5000×*g* for 20 min, 4°C to separate the precipitates. The supernatant of each sample was removed and 100 μL of the supernatant was added to 900 μL methanol. Next, 1 mL of methanol was added to solubilize the precipitates and 100 μL was added to 900 μL of methanol. The samples were analyzed by HPLC as described in the previous section.

Degradation of Curcumin in the Curcuminoid Mixture in Media of Different pH

A stock solution of the curcuminoid mixture in methanol (50 μL with curcumin concentration of 5 mg/mL (13.6 mM)) was added to 10 mL of aqueous buffer/methanol mixture (the final concentration of curcumin in the curcuminoid mixture was 25 μg/mL (68 μM)). The buffers used were ammonium acetate (pH=5.0), phosphate (pH=7.0 and 8.0), borate (pH=9.0 and 10.0), and ammonium buffers (pH=11.0 and 12.0). The buffer concentrations were 100 mM and the methanol volume fraction was 50% (*v*/*v*). The samples were incubated at 37°C for at least 24 h or until no curcumin was detected. Samples of 200 μL were withdrawn at regular time intervals and added to 800 μL of methanol. The pH of the solution was checked regularly and adjusted if necessary. The withdrawn samples were stored at −20°C prior to the analysis using RP–HPLC. The reaction rate constant (k_{obs}) was calculated from the slope of the plot of the logarithm (log) of the curcumin concentration versus time.

Degradation of Curcumin in the Curcuminoid Mixture as a Function of Temperature

The influence of temperature on the degradation of curcumin in the curcuminoid mixture was studied using the curcuminoid mixture solution in a 50:50 (*v*/*v*) mixture of phosphate buffer/methanol pH=8.0. The final concentration of curcumin in the curcuminoid mixture was 25 μg/mL (68 μM). The samples were incubated at 37, 50, and 60°C for at least 24 h or until no curcumin was detected. Samples of 200 μL were withdrawn at different time points, added to 800 μL of methanol, and stored at −20°C prior to analysis using RP–HPLC.

Degradation of Curcumin in the Curcuminoid Mixture in Media of Different Dielectric Constants

A stock solution of the curcuminoid mixture in methanol (curcumin concentration was 5 mg/mL, 13.6 mM) was diluted to a concentration of 25 μg/mL (68 μM) with solutions of 100 mM phosphate buffer pH=8.0 with different volume fractions of methanol (25:75, 40:60, 50:50, 60:40, and 75:25 (*v*/*v*)). The pH of the buffer/methanol mixtures was adjusted prior to the degradation study. Dehydration of the pH electrode by methanol was prevented by soaking it in water between the measurements. The pH of the mixture was checked regularly and the change in pH was less than 0.1 during the study. The samples were incubated at 37°C and for at least 5 days or until no curcumin was detected. Samples of 200 μL were withdrawn at different time points, added to 800 μL of methanol, and stored at −20°C prior to analysis using RP–HPLC. The dielectric constants (Є) of the buffer/methanol mixture were calculated according to the formula $Є=[(Є_{methanol}\times\text{methanol}\ (\%))+(Є_{water}\times\text{water}\ (\%))]/100$ with $Є_{methanol}=32.7$ and $Є_{water}=78.5$ (27,28).

Degradation of Pure Curcumin

A stock solution of pure curcumin (Sigma–Aldrich Product; see "Materials" Section) in methanol (2.5 mg/mL, 6.8 mM) and curcumin in the curcuminoid mixture in methanol (5 mg/mL, 13.6 mM) were diluted to a concentration of 25 μg/mL (68 μM) and 5 μg/mL (13.6 μM) in a solution of 50:50 (*v*/*v*) of 100 mM phosphate buffer/methanol mixture pH=8.0. The samples were incubated at 37°C and for at least 5 days or until no curcumin was detected. Samples of 200 μL were withdrawn at different time points, added to 800 μL of methanol, and subsequently stored at −20°C prior to analysis using RP–HPLC.

Degradation of Curcumin (as Curcuminoid Mixture) Loaded in Micellar Formulations of mPEG–HPMA–Bz and Triton X–100

mPEG–HPMA–Bz polymer with a molecular weight of 28 kDa was synthesized by a free radical polymerization method and characterized by ^{1}H–NMR (24,25). Curcumin (in the form of the curcuminoid mixture) loaded mPEG–HPMA–Bz micelles were prepared by a nanoprecipitation method (25,26). Forty mg of polymer was dissolved in 300 μL of acetone to which 200 μL of the curcuminoid mixture in acetone (curcumin concentration was 1 mg/mL (2.7 mM) was added. The solution of curcuminoid mixture and polymer was slowly dropped into 2 mL of 100 mM ammonium acetate buffer (pH=5.0) under stirring for 2 h. Subsequently, non–entrapped curcuminoid mixture were removed by centrifugation 5000×*g* for 20 min in a Sigma 4 K15 laboratory centrifuge (Osterode, Germany), rotor no. 11156) and the supernatant was filtered using a 0.45 μm nylon membrane (Phenex™, Phenomenex Inc, USA). Triton X–100 was also used to solubilize the curcuminoid mixture. Solid curcuminoid mixture (curcumin concentration was 5 mg) was added to 50 mL of 2% (*w*/*w*) of Triton X–100 in 100 mM ammonium acetate buffer, pH=5.0. Under stirring, a clear solution was obtained in a few minutes. For degradation study, the curcuminoid mixture formulations (1 mL) were added to 3 mL of 100 mM of phosphate buffer pH=8.0 with a final concentration of curcumin in the curcuminoid mixture of 25 μg/mL (68 μM) and incubated at 37°C for at least 7 days or until no curcumin was detected. At regular time points samples of 200 μL were withdrawn, added with 800 μL of methanol, and stored at −20°C prior to analysis using RP–HPLC.

Determination of Degradation Products by LC–MS Analysis

Samples with the curcumin concentration of 250 μg/mL (680 μM) in the curcuminoid mixture in 100 mM ammonium buffer/methanol mixtures (50:50 (*v*/*v*)) pH=9.0, were incubated at 37°C and at 0, 4, 24, and 168 h, samples of 200 μL were added with 800 μL of methanol. The final concentration of curcumin in the curcuminoid mixture was 50 μg/mL (136 μM). The samples were analyzed using an electrospray ionization mass spectrometer (ESI–MS) (Bruker, Bremen, Germany). An HPLC column (5 μm, 150×4.6 mm, SunFire™) was coupled to the mass spectrometer. A gradient system was run from 5:95 (*v*/*v*) acetonitrile/water as eluent A and acetonitrile as eluent B. Both eluents were adjusted by addition of acetic acid to pH=3.0 (0.125% (*v*/*v*) of acetic acid). The gradient was run from 90% A to 70% B in 15 min with a flow rate of 1.2 mL/min and the injection volume of 100 μL. The scan range was 140–415 m/z.

RESULTS AND DISCUSSION

Solubility of Curcumin in the Form of Curcuminoid Mixture in Aqueous Buffer/Methanol Mixtures

The solubility of curcumin from a natural curcuminoid mixture in an aqueous buffer with 2% (*v*/*v*) methanol was firstly studied because the solubility of curcumin in water is too low to be accurately detected. Further, the pH of the buffer was 5.0 because it has been shown that at this slightly acidic pH curcumin has a good stability (13,29). Table I shows that amount of curcumin in the curcuminoid mixture solubilized in aqueous buffer (with 2% (*v*/*v*) of methanol) decreases in time reaching solubility values of 0.3–0.4 μg/mL (0.8–1.1 μM) at day 7 which is in reasonable agreement with the solubility of curcumin in water reported by Kurien *et al.* (0.6 μg/mL) (30). However, this low concentration is not suitable for performing an accurate kinetic stability study. Therefore, we investigated the solubility of curcumin in the form of the commercial curcuminoid mixture in different solvent mixtures of aqueous buffer and methanol (volume fraction of methanol ranging from 10 to 50% (*v*/*v*), Table II). This table shows that at 40 and 50% (*v*/*v*) methanol, the curcumin concentration remained stable for 7 days (24–25 μg/mL or 65–68 μM) whereas at lower methanol volume fractions (10–30% (*v*/*v*)), the curcumin concentration dropped in time. This demonstrates that in these mixtures the initial curcumin concentration was above its maximum solubility leading to precipitation of curcumin and also curcuminoid mixture in time. Further investigation showed that the curcuminoid mixture at curcumin concentration of 37 μg/mL (100 μM; concentration used by Wang *et al.* (13)) and 50 μg/mL (136 μM) in 2% (*v*/*v*) methanol/buffer pH=5.0 and 8.0 gave turbid aqueous dispersions and precipitates which were observed after 24 h for samples incubated both 25 and 37°C (Supplemental 2). DLS analysis showed that small particles at both pH and temperatures (800–1000 nm, scattering intensity=130–200 kilo counts per second (kcps)) were detected directly after addition of the curcuminoid mixture in methanol stock solution to the aqueous buffer. The particles grew in size of >1000 nm (scattering intensity=300–500 kcps) during 24 h of incubation (Supplemental 3). In contrast, 100 and 136 μM of curcumin in curcuminoid mixture in 50% (*v*/*v*) methanol/buffer pH=5.0 and 8.0 at 25 and 37°C were clear solutions and neither precipitates were seen (visual inspection) nor nanoparticles were detected using DLS.

After the addition of the curcuminoid mixture to 2% (*v*/*v*) methanol/phosphate buffer mixture pH=7.2, a turbid dispersion was obtained (initial concentration curcuminoid mixture was 100 μM, similar as that used in reference 13). The HPLC chromatograms of the samples at 0 and 2 h are shown in Supplemental 4 and the percentage recovery of curcumin in the curcuminoid mixture at 0 and 2 h are shown in Supplemental 5. The HPLC chromatogram revealed the peaks of curcumin, demethoxycurcumin and bisdemethoxycurcumin in the curcuminoid mixture at 0 h. The recovery of curcumin was 97±8%. After 2 h of incubation and centrifugation, the concentration of curcumin in the supernatant was very low (1±1% recovery). On the other hand, the HPLC

Table I. The Concentration of Curcumin in the Curcuminoid Mixture in 2% (*v*/*v*) of Methanol in Buffer Solution, pH=5.0 at Different Times (Mean±SD; *n*=3)

	Solubility (μg/mL)		
Initial concentration (μg/mL)	6 h	Day 3	Day 7
25	2.1±0.7	1.4±0.7	0.3±0.0
50	1.7±0.2	0.9±0.6	0.4±0.2

Table II. The Concentration of Curcumin in the Curcuminoid Mixture in Buffer/Methanol Mixtures at pH=5.0 (Mean±SD; n=3). Initial Concentration of Curcumin Was 25 μg/mL

	Solubility (μg/mL)		
Buffer:methanol (% (v/v))	6 h	Day 3	Day 7
90:10	17.0±0.3	0.3±0.0	0.3±0.0
80:20	19.0±0.3	1.4±0.0	1.0±0.0
70:30	24.3±0.5	4.3±0.1	8.6±0.1
60:40	23.9±0.7	23.4±0.7	23.7±0.6
50:50	25.0±0.7	24.8±0.7	25.0±0.4

chromatogram of the dissolved precipitates from the curcuminoid mixture at 2 h showed that all three compounds were present and the recovery of curcumin was 83±8%. It can thus be concluded that the fast "degradation" of curcumin from the curcuminoid mixture under the applied condition (buffer plus 2% (v/v) methanol, concentration of the curcuminoid was 100 μM) is mostly due to precipitation (total recovery is 85%, so 15% degradation). It is therefore concluded that the concentration of organic co–solvents used in previous studies (e.g., in ref 13) was insufficient to fully dissolve the curcuminoids resulting in precipitation. Based on these results, 50% (v/v) of methanol in aqueous buffer was selected as the appropriate solvent to study the degradation of curcumin in the form of the curcuminoid mixture.

Degradation of Curcumin in the Curcuminoid Mixture in Media of Different pH

The HPLC chromatogram of the commercial curcumin product from a natural curcuminoid mixture shows three separated peaks which can be assigned to curcumin and two curcumin derivatives, demethoxycurcumin, and bisdemethoxycurcumin (Fig. 2). The identification of curcumin, demethoxycurcumin, and bisdemethoxycurcumin was done by MS detection (see "Determination of Degradation Products by LC–MS Analysis" Section). Figure 2a shows the chromatograms of the curcuminoid mixture incubated under basic condition (pH=9.0) and at 37°C in aqueous buffer/methanol (50:50 (v/v)) for 0, 4, and 24 h, respectively. This figure shows that the concentration of curcumin (as well as that of demethoxycurcumin) decreased in time due to chemical degradation. It should be pointed out that detection was done at 425 nm at which wavelength the degradation products are not detected. Compared to the chromatograms of Fig. 2b, the peaks corresponding to curcumin have a lower intensity which can be ascribed to the lower absorbance of curcumin at 254 compared to 425 nm. Clearly, Fig. 2b shows that degradation products with lower retention times than that of curcumin are present, in line with previous publications (13,17). The degradation of curcumin in the form of the curcuminoid mixture was also studied as a function of the pH while the other parameters (volume fraction methanol and temperature) were fixed. The pH dependent degradation of curcumin is shown in Fig. 3 which exhibits that the curcumin concentration decreased according to the first order kinetics. The calculated k_{obs} values at pH=7.0, 8.0, 9.0, 10.0, 11.0, and 12.0 at 37°C were $(3.2\pm0.3)\times10^{-3}$, $(7.6\pm0.4)\times10^{-3}$, $(76.0\pm0.4)\times10^{-3}$, $(219\pm22)\times10^{-3}$, $(309\pm2)\times10^{-3}$, and $(693\pm11)\times10^{-3}$ h^{-1}, respectively, whereas at pH=5.0, curcumin is stable, in line with previous findings (29). Supplemental 6 and 7 show the pH–log k_{obs} plots for curcumin in the curcuminoid mixture. The fact that degradation does not occur at pH=5.0 but only above pH=7.0 indicates that the first step in the degradation process of curcumin is deprotonation of one of the three hydroxyl groups of curcumin. However, different pK_a values of curcumin have been reported in several studies. Tønnesen *et al.* reported that they were 7.8, 8.5, and 9.0 (29). Bernabé–Pineda *et al.* reported values of 8.38, 9.88, and 10.51 (31) whereas the values reported by Leung

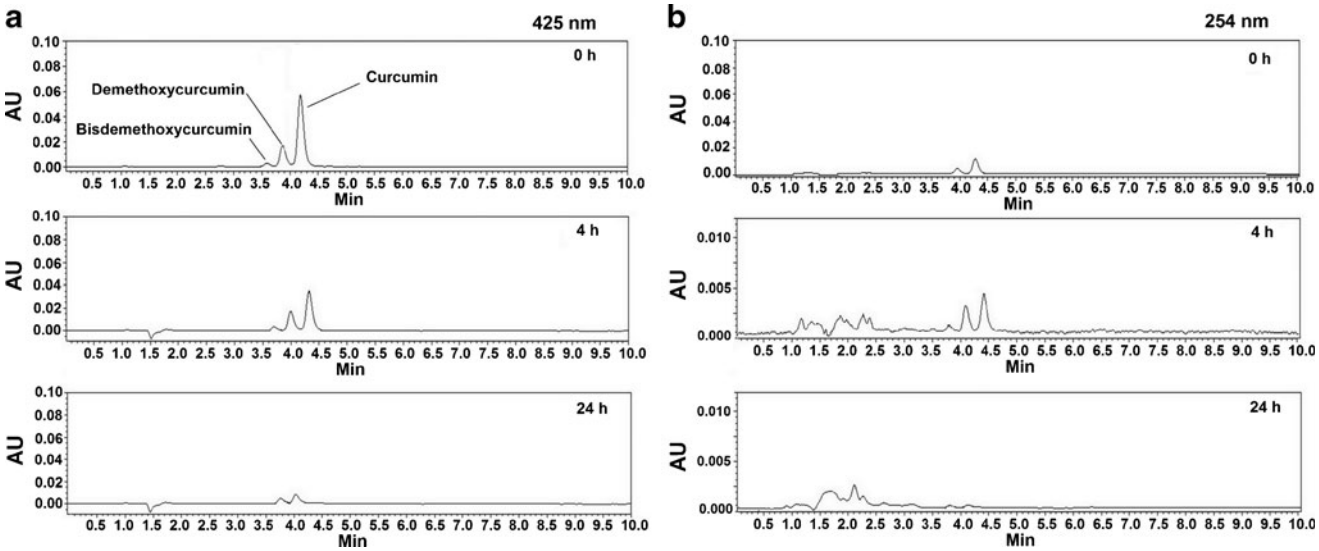

Fig. 2. Chromatograms of the curcuminoid mixture in 50:50 (v/v) buffer/methanol pH=9.0 in different stages of its degradation at a wavelength of 425 (**a**) and 254 nm (**b**)

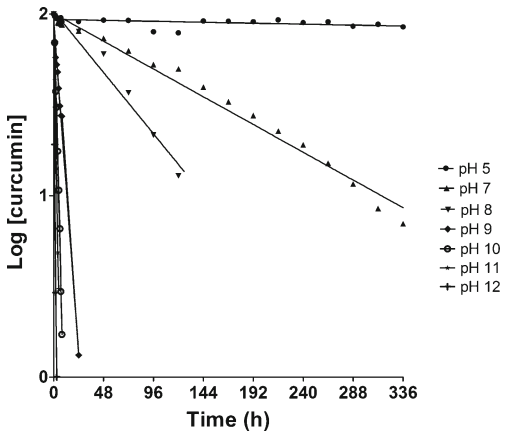

Fig. 3. The first order degradation plot of curcumin in the curcuminoid mixture as a function of pH and at 37°C in 50:50 (*v*/*v*) buffer/methanol (n=3)

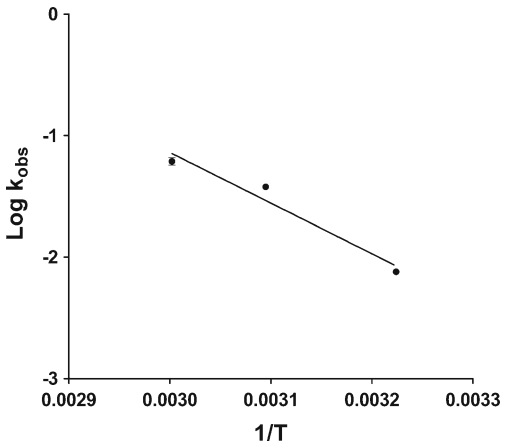

Fig. 4. Arrhenius plot for curcumin in the curcuminoid mixture degradation at pH=8.0, 37°C in 50:50 (*v*/*v*) buffer/methanol (n=3)

et al. were 8.3, 10.0, and 10.2 (32). Although there is disagreement about the real pK_a values of curcumin, it can be concluded that going from pH=7.0 to 10.0, curcumin is essentially converted from an uncharged to a (highly) negatively charged molecule. The next step in the degradation process of curcumin is autoxidation. Indeed, it has been reported that a phenolic antioxidant donates an electron once the hydroxyl group is deprotonated (33). The proton–donating potential increased and the phenolic compound easily formed a phenoxyl radical as a function of pH in the presence of oxygen (34). Also, an increased antioxidant activity of phenolic compounds and a standard antioxidant, butylhydroxytoluene (BHT), was observed with an increasing pH corresponding to the deprotonation and ionization of the hydroxyl group (35–37). Although not the main aim of this study the degradation of the log concentration versus time plot of demethoxycurcumin at pH=8.0 and 9.0, 37°C (Supplemental 8) yielded lower k_{obs} (2.9 (±0.2)×10^{-3} and 30.4 (±2.5)×10^{-3} h^{-1}) than that of curcumin (7.6 (±0.4)×10^{-3} and 76.0 (±0.4)×10^{-3} h^{-1}). This demonstrates that degradation rate of demethoxycurcumin is slower than that of curcumin, in line with literature data of Gordon *et al.* (38). It should be mentioned that the peak areas of bisdemethoxycurcumin in the different chromatograms were too small for an accurate calculation of k_{obs}.

Degradation of Curcumin in the Curcuminoid Mixture as a Function of Temperature

The influence of temperature on the degradation kinetics of curcumin in the curcuminoid mixture was determined between 37–60°C in buffer/methanol mixture (50:50 (*v*/*v*)) at pH=8.0. The log of curcumin concentration versus time plots are shown in Supplemental 9. In line with expectations, the results clearly demonstrate that an increasing temperature resulted in an increasing degradation rate of curcumin. The calculated k_{obs} values were used for the Arrhenius plot which shows a linear relationship between log k_{obs} and 1/T (r^2=0.958) (Fig. 4). The E_a was calculated to be 79.6±2.2 kJ/mol using the Arrhenius equation: $k_{obs}=Ae^{-E_a/RT}$ where k_{obs} is the reaction constant (h^{-1}), A is the frequency factor (h^{-1}), E_a is the activation energy (J/mol), R is the gas constant (8.314 J/mol K), and T is the absolute temperature (K).

Degradation of Curcumin in the Curcuminoid Mixture in Media of Different Dielectric Constants

The degradation of curcumin in the curcuminoid mixture in buffer/methanol mixtures at pH=8.0 with different methanol volume fractions was performed in order to investigate the influence of the dielectric constant on the degradation kinetics and to calculate by extrapolation the stability of curcumin in aqueous buffer only. The log of curcumin concentration versus time plot for curcumin degradation in these different aqueous buffer/methanol mixtures is shown in Supplemental 10. Figure 5 reveals that the log k_{obs} values linearly increases with increasing dielectric constant and thus with increasing aqueous buffer–volume fraction of the mixture. Likely, with increasing volume fraction of methanol, the pKa values of curcumin shift to higher values because of a

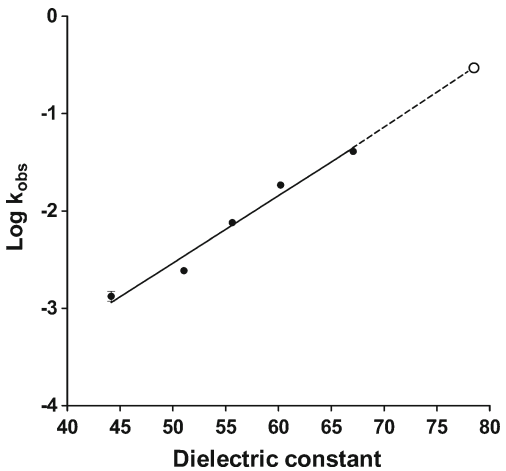

Fig. 5. Effect of the dielectric constant on the k_{obs} at pH=8.0, 37°C. The open symbol represents the calculated k_{obs} in aqueous buffer pH=8.0 by extrapolation (n=3)

decreasing polarity of the medium, and consequently stabilization of the formed phenolic anion is less favorable. Because of the very low solubility of curcumin in water, no accurate degradation study could be performed. Therefore, the k_{obs} of curcumin in water of pH=8.0 was calculated using Fig. 5 by extrapolating to the dielectric constant value of water (78.5). The calculate k_{obs} in aqueous buffer from the extrapolation is 280×10^{-3} h^{-1} (Fig. 5, open symbol) corresponding with a half–life ($t_{1/2}$) of 2.5 h. In the study of Wang *et al.*, the stability of curcumin was investigated in buffer solutions with only 2% (*v*/*v*) of methanol and at 37°C (13). The authors reported a $t_{1/2}$ of 1.05 min (or 0.018 h) at pH=8.0. We, however, found an about 139 times longer half–life in aqueous buffer which might be ascribed to the fact that Wang *et al.* used a buffer with 2% (*v*/*v*) methanol at a curcumin concentration of 0.1 mM (or 37 μg/mL) (13). However, this is far above the saturation concentration (<1 μg/mL; see Table I) and therefore, besides degradation also precipitation of curcumin occurred resulting in an overestimation of the k_{obs}.

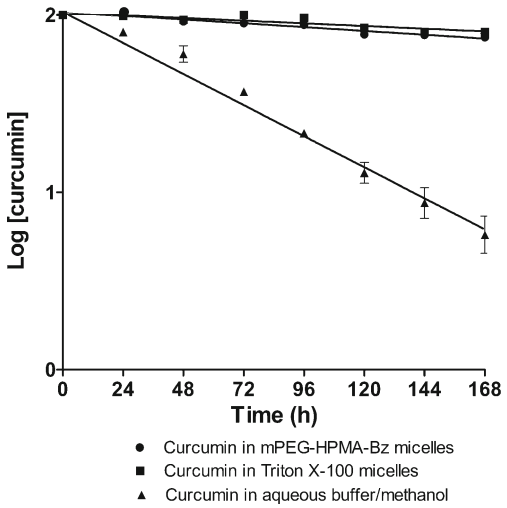

Fig. 6. The first order degradation plot of curcumin as the curcuminoid mixture formulations at pH=8.0, 37°C (n=3)

Degradation of Pure Curcumin

The degradation of pure curcumin was investigated and compared with that of curcumin in the natural curcuminoid mixture. Supplemental 11 shows that the degradation of pure curcumin (25 μg/mL (68 μM) and 5 μg/mL (13.6 μM)) in 50% (*v*/*v*) of phosphate buffer / methanol pH=8.0 at 37°C was faster than that of curcumin in the natural mixture of curcuminoids. The calculated k_{obs} values for pure curcumin and curcumin in the mixture of curcuminoids at 5 μg/mL (13.6 μM) were $(11.0\pm0.9)\times10^{-3}$ and $(7.9\pm0.1)\times10^{-3}$ h^{-1}, respectively. At 25 μg/mL (68 μM), the k_{obs} values were $(7.8\pm0.2)\times10^{-3}$ and $(7.6\pm0.4)\times10^{-3}$ h^{-1}, respectively. These results demonstrate, as also reported previously, that pure curcumin particularly at low concentration is slightly more susceptible for oxidative degradation than that in its natural mixture. This can likely be ascribed to the stabilizing effect of the two derivative curcuminoids, bisdemethoxycurcumin and demethoxycurcumin as suggested by Gordon *et al* (38).

Degradation of Curcumin (as Curcuminoid Mixture) Loaded in Micellar Formulations of mPEG–HPMA–Bz and Triton X–100

It has been reported that the stability of curcumin increases when bound to plasma proteins or loaded in nanoformulations (39–41). We therefore studied the degradation of curcumin in two different formulations, namely loaded in mPEG–HPMA–Bz micelles and in Triton X–100 micelles. It is remarked that curcumin was loaded as curcuminoid mixture because this mixture is used frequently in other pharmaceutical formulations used in (pre)clinical studies because of the synergistic pharmacological effects of the compound in the mixture (5,24). Figure 6 shows that the degradation of curcumin in both micellar formulations is substantially slower than that of curcumin in a 50:50 (*v*/*v*) mixture of phosphate buffer/methanol, pH=8.0, 37°C. The calculated k_{obs} values of curcumin solubilized in mPEG–HPMA–Bz and in Triton X–100 micelles were 0.9 $(\pm0.1)\times10^{-3}$ and 0.6 $(\pm0.1)\times10^{-3}$ h^{-1}. The dielectric constant values (calculated using Fig. 5) of the hydrophobic cores of mPEG–HPMA–Bz and Triton X–100 micelles were 42.6±0.7 and 40.4±0.6, respectively. This demonstrates that as expected the core of micelles is very hydrophobic which stabilizes curcumin against oxidative degradation. The $t_{1/2}$ of curcumin in mPEG–HPMA–Bz and Triton X–100 micelles (777±87 and 1100±95 h, respectively) is about 300–500 times higher than that of free curcumin in aqueous buffer (2.5 h). This shows that in line with reported data nanoformulations substantially protect curcumin against degradation (42–44).

Determination of Degradation Products by LC–MS Analysis

Figure 7a–d shows the LC chromatograms of curcumin as curcuminoid mixture before and after degradation in 50:50 (*v*/*v*) ammonium buffer/methanol pH=9.0 at 37°C for 0, 4, 24, and 168 h. Detection was done at 254 nm.The individual LC–UV peaks were collected and analyzed by LC–MS. The peaks with a retention time between 13.8–14.1 min correspond with compounds with *m*/*z* at 367, 337, and 307 which can be assigned to curcumin, demethoxycurcumin, and bisdemethoxycurcumin, respectively. The retention times of curcumin, demethoxycurcumin, and bisdemethoxycurcumin were prolonged because the elution gradient was adjusted to allow better separation and identification of the degradation products. The peak from the LC chromatogram with a retention time of 1.4 min was assigned to a sodium acetate cluster (*m*/*z* at 305) from the ammonium buffer which was used as a medium in this study. After 4 h of degradation (50% of curcumin remained), degradation peaks are clearly detectable. The samples after 24 and 168 h incubation had 20 and 0% of curcumin remaining and correspondingly the degradation peaks increased as a function of time. LC–ESI–MS analysis at 4 h shows the presence of compounds with molecular

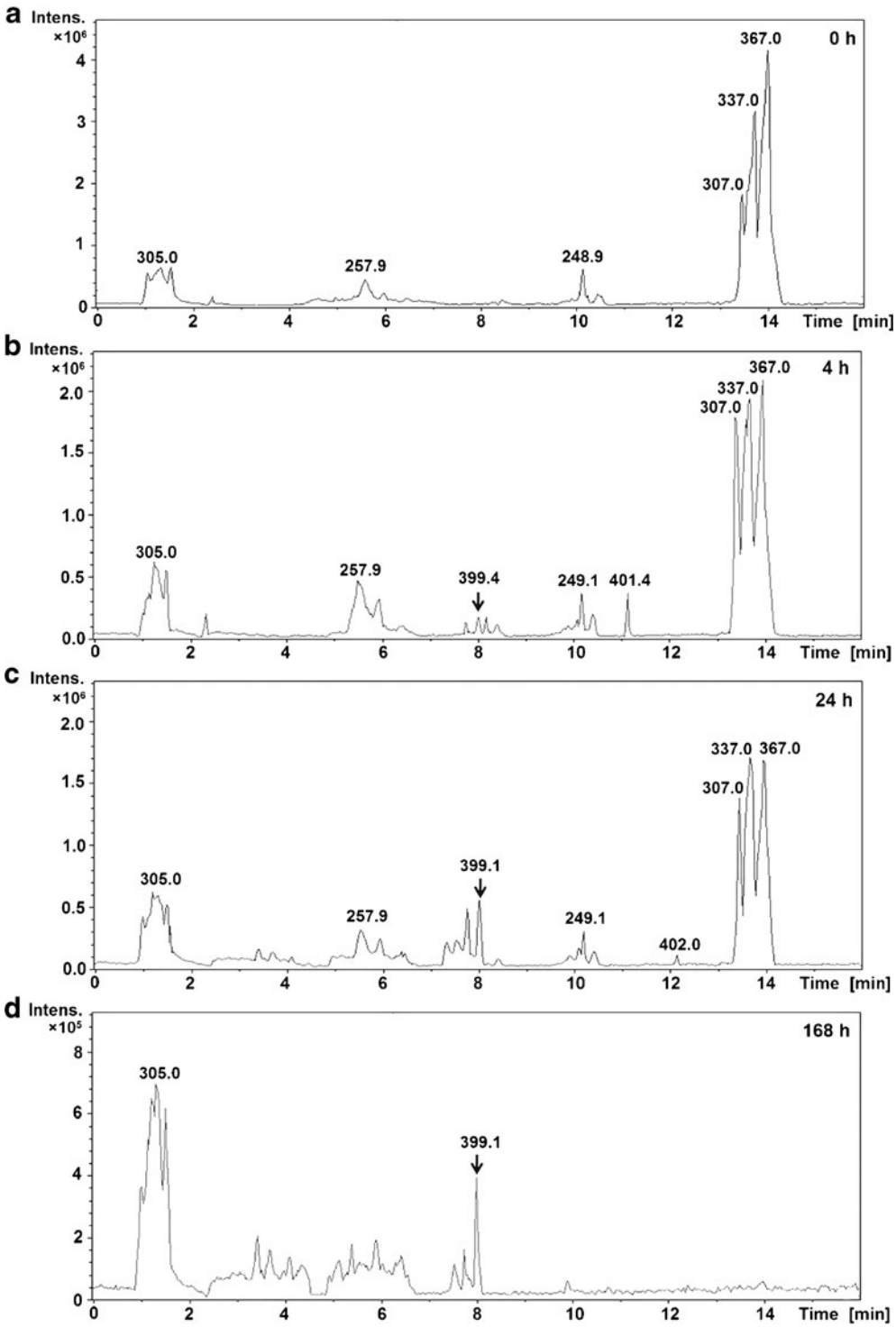

Fig. 7. Chromatograms of the curcuminoid mixture and its degradation products in 50:50 (*v*/*v*) buffer/methanol pH=9.0, 37°C at 0 (**a**), 4 (**b**), 24 (**c**) and 168 h (**d**) obtained by LC–ESI–MS

ion $[M–H]^-$ at *m*/*z* of 367, 337, 307, 401, 399, 249, 258, and 305 (Fig. 7b). The peaks in LC chromatograms with *m*/*z* at 249, 258, and 401 were not clearly identified, but they might be assigned as the reaction intermediate compounds from the autoxidation of curcumin. The *m*/*z* at 399 peak in LC chromatogram with the retention time of 8.0 min is a bicyclopentadione (structure shown in Supplemental 1) as demonstrated by Gordon *et al.* (17) and originates from Griesser *et al.* (15). The MS spectrum of the molecular ion *m*/*z* 399 and the fragment ions at *m*/*z* 249 which were from the *m*/*z* 399 (bicyclopentadione) peak in the LC chromatogram is shown in Fig. 8a. Similarly to the previous report,

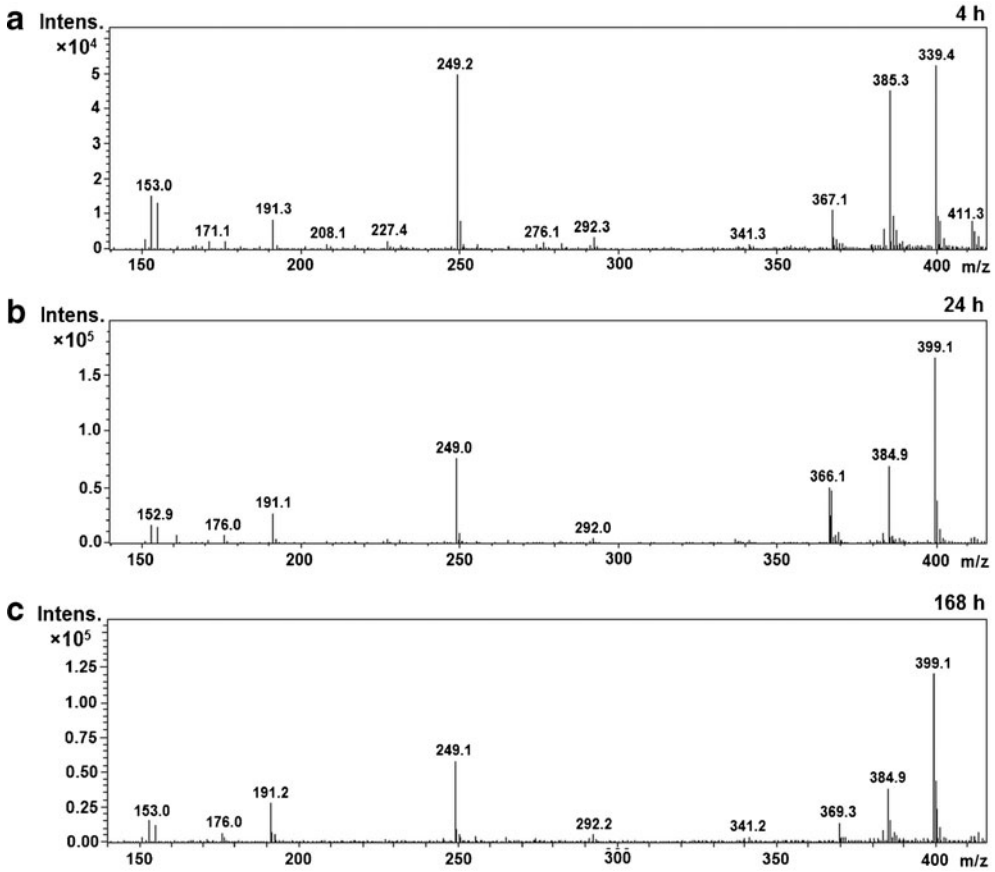

Fig. 8. The MS spectra at *m/z* 399 of the major degradation product of curcumin in the curcuminoid mixture (bicyclopentadione) at 4 (**a**), 24 (**b**), and 168 h (**c**) obtained by LC–ESI–MS

the fragment ions at *m/z* 249 might be assigned to the lack of one carbon bicyclic ring attached with the one of phenolic ring (Supplemental 12) (15). The peak of a bicyclopentadione was also detected after 24 and 168 h (Fig. 7c–d) as a final degradation product and the MS spectra are shown in Fig. 8b–c. The other degradation peaks detected by HPLC analysis after 24 and 168 h are likely intermediates from curcumin oxidative transformation and their isomers that proceeds through 15 intermediates (17). In line with previous studies, ferulic acid (Mw=194), vanillin (Mw=152), and feruloylmethane (Mw=192) are hardly formed as degradation products (15,17).

CONCLUSION

This study shows that in order to investigate the degradation of a practically water–insoluble compound like curcumin (in the form of the commercially available curcuminoid mixture), an appropriate aqueous organic solvent mixture has to be identified to solubilize the present compounds. This study confirms that autoxidation, and not chain scission, is the main pathway for degradation of curcumin. Loading in mPEG–HPMA–Bz and Triton X–100 micelles substantially stabilizes curcumin against oxidative degradation likely because deprotonation of one of the hydroxyl groups, the first step in the degradation process, in the hydrophobic core of the micelles is prevented. Therefore, loading into polymeric micelles is a promising approach for the stabilization of curcumin making such formulations suitable for further pharmaceutical development and ultimately clinical translation.

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