

Stability of Curcumin in Buffer Solutions and Characterization of its Degradation Products

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ABSTRACT

The degradation kinetics of curcumin under various pH conditions and the stability of curcumin in physiological matrices were investigated. When curcumin was incubated in 0.1 M phosphate buffer and serum-free medium, pH 7.2 at 37 (C, about 90% decomposed within 30 min. A series of pH conditions ranging from 3 to 10 were tested and the result showed that decomposition was pH-dependent and occurred faster at neutral-basic conditions. It is more stable in cell culture medium containing 10% fetal calf serum and in human blood; less than 20% of curcumin decomposed within 1 h, and after incubation for 8 h, about 50% of curcumin is still remained. Trans-6-(4'-hydroxy-3'-methoxyphenyl)-2,4-dioxo-5-hexenal was predicted as major degradation product and vanillin, ferulic acid, feruloyl methane were identified as minor degradation products. The amount of vanillin increased with incubation time.

Introduction

Although use of medicinal plants and their active principles in the prevention and treatment of chronic diseases is based on the experience of traditional systems of medicine from different ethnic societies, their use in modern medicine is limited by the lack of scientific data. Few medicinal plants have attracted the interest of scientists and been the subject of scientific investigations. One plant that has been investigated is *Curcuma longa* Linn. The powdered rhizome of this plant, turmeric, has been extensively used to color and flavor foods. The yellow color it imparts is primarily caused by curcumin, a phenolic pigment 1.

Curcumin is an antioxidant that inhibits lipid peroxidation in rat liver microsomes, and a scavenger of reactive oxygen species that decreases the formation of inflammatory compounds such as prostaglandins and leukotrienes 2-5. In addition, chemopreventive properties in skin and forestomach carcinogenesis and various pharmaceutical applications have been reported 6-8. It is interesting to note that the clinical trials of curcumin in human cancer patients are in progress. The pharmacokinetics and toxicity of curcumin has also been studied. When administered orally, 75% of curcumin is excreted in the faeces while only traces appear in the urine. Measurements of blood plasma levels have shown that curcumin is poorly absorbed from the gut 9-10. Three hours after gavage, curcumin could be detected in the plasma of 1 of 4 animals. No apparent toxic effects have been seen with doses up to 5 g/kg 9. With oral doses of 0.6 mg [3H]curcumin per rat, 89% of the radioactivity was excreted in the faeces, and 6% of the radioactivity was excreted in the urine 11. From these data, it is unlikely that a substantial concentration of curcumin occurs in the body after ingestion. Nevertheless, the fate of the remaining curcumin not excreted in the faeces is unclear.

The initial aim of this investigation, which is a part of a large-scale research project to study the chemopreventive properties of curcumin in Taiwan, was to re-evaluate the pharmacokinetic properties of curcumin using a new sensitive HPLC method 1. Previous studies have demonstrated that curcumin is absorbed poorly by the gastrointestinal tract and/or undergoes presystemic transformation 8. Indeed, when administered orally in doses of 1 g/kg in rats, we could not detect curcumin in blood between 2 and 12 hrs after administration (data not shown). Conjugate-formation of curcumin with the constituents of plasma was considered to be one of the reasons that curcumin could not be detected in plasma after absorption. The reaction of curcumin with albumin or glutathione was conducted in 0.1 M phosphate buffer, pH 7.2 at 37

(C for 30 min. UV-visible spectra absorbance of curcumin and the reaction mixtures were monitored at 420 nm. From this, we found that curcumin alone in phosphate buffer undergoes loss of the absorbance at 420 nm (data not shown), indicating that it is unstable at physiological condition in vitro. The degradation kinetics of curcumin under various pH conditions and the stability of curcumin in physiological matrices were investigated. In addition, the major degradation product and some of the minor products were investigated and characterized.

Experimental Section

1. Materials

Curcumin (from *Curcuma longa*, Turmeric), vanillin, vanillic acid, and ferulic acid were purchased from Sigma Chemical Co., St. Louis, MO. Ferulic aldehyde and feruloyl methane were purchased from Aldrich Chemical Co., Milwaukee, WI. Bis(trimethylsilyl)-trifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane was obtained from Pierce Chemical Co., Rockford, IL. Pyridine, acetic acid, citric acid and other chemicals for the preparation of buffer systems were purchased from E. Merck Chemical Co., Darmstadt. HPLC grade methanol, acetonitrile and tetrahydrofuran were obtained from BDH Laboratory Supplies (Poole, BH15 1TD, England). Synthetic curcumin was synthesized and provided by Yung Shin Pharmaceutical Ind. Co., Taiwan, R.O.C. RPMI medium 1640 was obtained from Gibco BRL (Grand Island, N.Y. USA). Human blood was obtained from healthy volunteers. Recipes for the pH of buffer solutions from 3 to 10 are derived from Methods in Enzymology¹².

2. Analysis of curcumin by HPLC

HPLC was performed with a Jasco liquid chromatograph equipped with a PU-980 intelligent pump, a variable wavelength UV-975 UV/Vis detector. The procedure described by Cooper et al.¹ was used for the determination of curcumin on a C18 column (150(3.9 mm, 5- μ m particle size, Waters). Aliquots of 20 μ l of 5 mM curcumin (dissolved in methanol) were added to 980 μ l 0.1 M solutions of different buffers or serum-free medium, pH 7.2. Samples were incubated at 37 $^{\circ}$ C for indicated times. After incubation, 100 μ l of reaction mixtures were added to 900 μ l of HPLC mobile phase (40% THF, 60% water, 1% citric acid, pH 3.0). Samples of curcumin in RPMI 1640 medium containing 10% fetal calf serum or in human plasma were acidified to pH 3.0 with 6N HCl and extracted two times with equal volumes of ethyl acetate-

propanol (9:1, v/v). The organic layer was combined together, dried under a stream of nitrogen, diluted with HPLC mobile phase, and filtered through a 0.45 μ m PVDF membrane filters. The recovery of curcumin in this assay was between 87-95% (data not shown).

3. Degradation products analysis by HPLC

The filtrate of curcumin incubated at 37 °C, in 0.1 M phosphate buffer, pH 7.2 (or in serum-free medium) were analyzed with HPLC and GC/MS. Vanillin, vanillic acid, ferulic acid, ferulic aldehyde and feruloyl methane were used as standards. Separations were performed on a C18 column. One mobile phase was 35% methanol and 1% acetic acid in water (C18 column: 150(3.9 mm, 5- μ m particle size, Waters) 13, the other was 12% THF, 5% acetonitrile, 2% acetic acid in water, adjusted to pH 3.0 or 4.0 with concentrated KOH solution. The system was run isocratically at a flow rate of 1 ml/min (C18 column: 250(4.6 mm, 5- μ m particle size, J. T Baker). Sample detection was achieved at 280 nm and injection volumes were 20 μ l. Samples were injected for HPLC analysis without further dilution. Chromatographic peaks from incubation samples were identified by spiking with authentic standards.

4. Degradation products analysis by mass spectrometry

The major degradation product was isolated and collected using HPLC with 12% THF, 5% acetonitrile and 2% acetic acid as mobile phase. The combined collection was extracted two times with an equal volume of ethyl acetate. The combined organic phase was dried over Na₂SO₄ and concentrated by rotar-vapor. Structure elucidation was conducted in National Science Council Taipei Regional Analytical Instrument Center by VG Platform Electrospray (ESI) mass spectrometry and TSQ-46C Electron Impact (EI) mass spectrometry.

The minor degradation products were characterized by gas chromatography mass spectrometry (GC/MS), the reaction mixture of curcumin in 0.1 M phosphate buffer at 37 °C, pH 7.2, 1 h was acidified with 6N HCl to pH 3.0 and extracted two times with an equal volume of ethyl acetate-propanol (9:1, v/v). The combined organic phase was dried over Na₂SO₄ and the solvent was evaporated with N₂ and analyzed after derivatization with BSTFA/pyridine (2:1, v/v) as described by Tuor, et al.¹⁴. GC/MS was performed on a Hewlett-Packard Model 5890 microprocessor-controlled gas chromatograph interfaced to a Hewlett-Packard Model 5971 A mass selective detector. Electron impact ionization was performed at a high ionization voltage of 70 eV. GC separations were carried out on a DB-5 capillary column (15 m(0.25mm inner

diameter, 0.25 (m film thickness, J&W Scientific, Folsom, CA) with helium as the carrier gas at an inlet pressure of 30 kPa. The injection port was kept at 250 (C, the GC/MS interface was maintained at 280(C. The column temperature was increased from 80 (C to 240 (C at a rate of 8 (C per min after 4 min at 80 (C and held at 240 (C for 1 min. An aliquot (1 (l) of each derivatized sample was injected without any further treatment into the injection port in the splitless mode. Characteristic ions of the trimethylsilyl (Me₃Si) derivatives of degradation products were monitored individually.

Results

When curcumin was added to 0.1 M phosphate buffer, pH 7.2 (physiological condition in vitro), more than 90% of curcumin degraded. A series of pH values from 3 to 10 in buffer solutions were assayed for this degradation.

Figure 1 shows the kinetics of curcumin degraded at various pH values, 37(C, using reversed-phase HPLC. Logarithmic plots of the residual curcumin concentration versus time were reasonably linear at all pH values tested, indicating that degradation followed apparent first-order kinetics at 37(C and constant ionic strength. The pH dependence of the overall first-order degradation rate constant of curcumin is shown in Table 1. The catalytic effect of the buffer system used in the kinetic studies was determined at constant pH (7.2) and various buffer concentrations (0.1 M, 0.05 M, 0.025 M). No appreciable buffer catalytic effect on the degradation of curcumin was observed. (Table 1).

Figure 2 shows the stability of curcumin in different physiological matrices. Curcumin degraded rapidly not only in 0.1 M phosphate buffer but also in serum-free medium after 37(C incubation for 1 h. In medium containing 10% fetal calf serum and in human blood, less than 20% of curcumin decomposed within 1 hr, and after incubation for 8 hrs, more than 50% of curcumin still remained.

The degradation products analysed by different HPLC systems are shown in Figures 3. Figure 3A and 3B shows the chromatogram of standard mixture and the analysis of buffer mixture of curcumin in 0.1 M phosphate buffer reacted at 37(C for 1 h respectively. Vanillin, ferulic acid and feruloyl methane were found in this analysis. Vanillin was the major degradation product in this assay. However, when another mobile phase system and longer column was used, the vanillin peak separated into two peaks. A major unknown peak was eluted

at the retention time just next to vanillin. Vanillin was the smaller and the unknown compound the larger peak (Figure 3C, 3D). When the incubation time of curcumin in 0.1 M phosphate buffer at pH 7.2 was increased, we found that the abundance of the major unknown peak decreased and the abundance of vanillin increased (Figure 4A). The same result was found with curcumin in serum-free medium (Figure 4B). Figure 4C shows the chromatogram of curcumin at zero incubation time with different HPLC conditions.

Large scale reactions were conducted in order to isolate and confirm the structure of the major unknown degradation product. Preliminary data shows that the isolated unknown compound is unstable in phosphate buffer (especially at higher temperature, eg. 60(C) and could be converted to vanillin (data not shown). Structure elucidation were performed by electrospray (ESI) and electron impact (EI) mass spectrometry. The results show a molecular ion $[M+H]^+$ of 249 (Figure 5A) in ESI/MS and a M^+ of 248 in EI/MS (Figure 5B) indicating that the molecular weight of this major degradation product is 248. The structure was predicted as trans-6-(4'-hydroxy-3'-methoxyphenyl)-2,4-dioxo-5-hexenal.

Some of the minor degradation products was further confirmed by GC/MS with authentic standards derivatized by trimethylsilylation. Table 2 shows the mass spectra of derivatized products and GC retention times. Vanillin, ferulic acid and feruloyl methane were characterized in this assay as the same result of HPLC analysis.

Discussion

It has been shown that curcumin has a poor light stability. About a 5% decrease in absorbance due to curcumin has been measured during the time for typical sample preparation when clear rather than amber glassware is used ¹. Curcumin decomposes when exposed to sunlight, both in ethanolic and methanolic extracts and as a solid, vanillin, vanillic acid, ferulic aldehyde and ferulic acid have been identified as the degradation products ¹⁵. However, little is known about the fate of curcumin in physiological conditions in vitro.

In the present work, we found that more than 90% of curcumin decomposed rapidly in buffer systems at neutral-basic pH conditions. The more stable of curcumin in acidic pH condition may be contributed by the conjugated diene structure. However, when the pH is adjusted to neutral-basic conditions, proton removed from the phenolic group, leading to the destruction of this structure. During our investigation, the similar result was also reported by

Commandeur. et al.¹⁶, which indicated that curcumin is unstable in phosphate buffer at pH 7.4 measured by spectrophotometry. The stability of curcumin was strongly improved by lowering the pH or by adding glutathione, N-acetyl-L-cystein, ascorbic acid and rat liver microsomes. However, further investigation was conducted in this study. We determined the kinetics of curcumin degraded at various pH values, characterized some of the degradation products and investigated the stability of curcumin in physiological matrices.

The major degradation product predicted in this study was identified by mass spectrometry. Further characterization by IR or NMR and even the biological effect of this compound deserves to be studied. However, it is somewhat difficult to get large amount and high purity of this compound because of the low solubility of curcumin in aqueous solutions and the instability of this compound during sample preparation (which can be converted to vanillin and other compounds). When the incubation time of curcumin in buffer solution at 37°C increased, vanillin will become the major degradation product. (Figure 4)

From the fact that curcumin decomposes rapidly in serum-free medium, precautions must be taken during cell culture experiments. In addition, the biological effects caused by the degradation products of curcumin, especially vanillin, must be taken into consideration. Vanillin, a naturally occurring flavoring, has been reported to inhibit mutagenesis in bacterial and mammalian cells. It may act as antimutagen by modifying DNA replication and DNA repair systems after cellular DNA damage by mutagens¹⁷. Vanillin is also a powerful scavenger of superoxide and hydroxyl radicals. It inhibits iron-dependent lipid peroxidation in rat brain homogenate, microsomes and mitochondria¹⁸. The ability of curcumin to inhibit mutagenesis, lipid peroxidation and free radical generation has been well documented¹⁹⁻²¹. It would be valuable and interesting to compare the potency of vanillin and curcumin on these aspects.

In summary, the present study indicated that curcumin decomposed rapidly in buffer solutions at 37 °C, neutral-basic pH conditions. Trans-6-(4'-hydroxy-3'-methoxyphenyl)-2,4-dioxo-5-hexenal was predicted as major degradation product and vanillin, ferulic acid, feruloyl methane were identified as minor degradation products at short-time reaction. However, further studies are needed to confirm the unidentified components. When cell culture experiments are conducted to evaluate the biological effects of curcumin, treatment of culture cells in serum-free medium must be avoided and the effects of vanillin might be taken into consideration.

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Acknowledgement

This study was supported by National Science Council grants NSC 84-2622-B-002-007 and NSC 85-2331-B-002-084. We thank Mr. C. M. Chiou and C. K. Chen for the helpful discussions and suggestions during this work.

Table 1

Buffer systems, observed rate constants and $t_{1/2}$ for the degradation of curcumin at 37°C

pH	Buffer system	Buffer concentration (M)	k_{obs} , $\text{min}^{-1} \times 10^3$	$t_{1/2}$, min
3.0	Citrate-phosphate	0.1	5.842	118.63
5.0	Citrate-phosphate	0.1	3.481	199.08
6.0	Phosphate	0.1	3.541	195.69
6.5	Phosphate	0.1	4.529	153.02
6.8	Phosphate	0.1	39.755	39.75
7.2	Phosphate	0.1	73.715	9.40
7.2	Phosphate	0.5	72.645	9.54
7.2	Phosphate	0.025	73.218	9.47
8.0	Phosphate	0.1	656.65	1.05
10.0	Carbonate	0.1	49.328	14.05

Table 2

Mass spectra and GC retention times of some degradation products of curcumin

TMS derivatives of products	Retention time (min)	Mass spectrum m/z (rel intensity)
1 Vanillin (mono-TMS ether)	12.40	224 (M^+ , 26.9%), 209 (42.5), 194 (100), 104 (7.7), 73 (20.3)
2 Vanillic acid (di-TMS ether)	15.94	312 (M^+ , 50.5%), 297 (86.4), 267 (68.0), 253 (44.7), 223 (66.0), 193 (27.2), 126 (75.7), 73 (100)
3 Ferulic aldehyde (mono-TMS ether)	16.89	250 (M^+ , 75.7%), 235 (32.0), 220 (100), 192 (47.6), 102 (16.5), 73 (68.9)
4 Feruloyl methane (mono-TMS ether)	17.75	264 (M^+ , 100%), 249 (78.6), 234 (90.3), 219 (88.3), 117 (30.1), 102 (43.7), 88 (23.3), 73 (99)
5 Ferulic acid (di-TMS ether)	20.09	2338 (M^+ , 65%), 308 (35.9), 293 (22.3), 249 (45.6), 219 (22.3), 154 (15.5), 147 (31.1), 73 (100)

Figure Legends

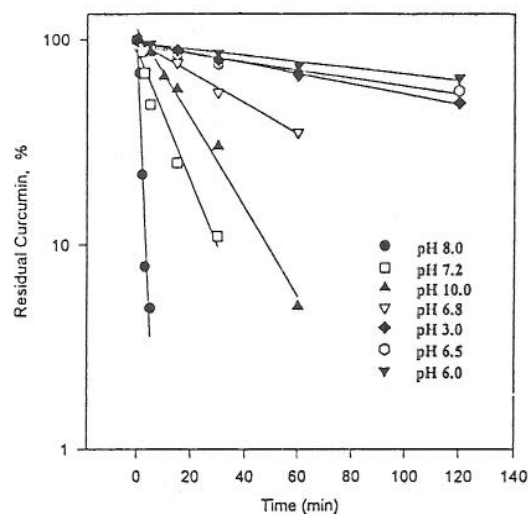


Figure 1. Apparent first-order plots for the degradation of curcumin at various pH values. The data are normalized to a value of 100 at zero time. Points represent the experimental data and the solid lines were drawn using linear least-squares regression analysis.

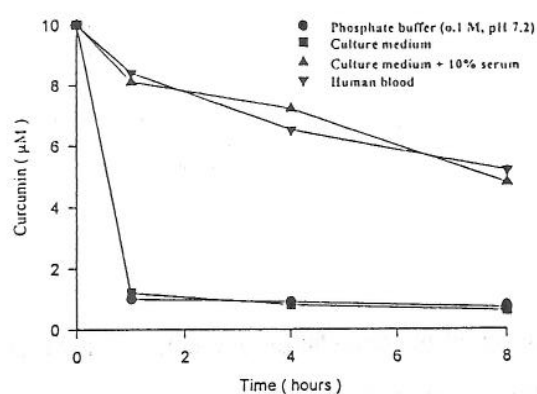


Figure 2. Effect of different physiological conditions in vitro on the stability of curcumin incubated at 37°C for 1, 4, and 8 hrs. Each value represents the mean of duplicate samples.

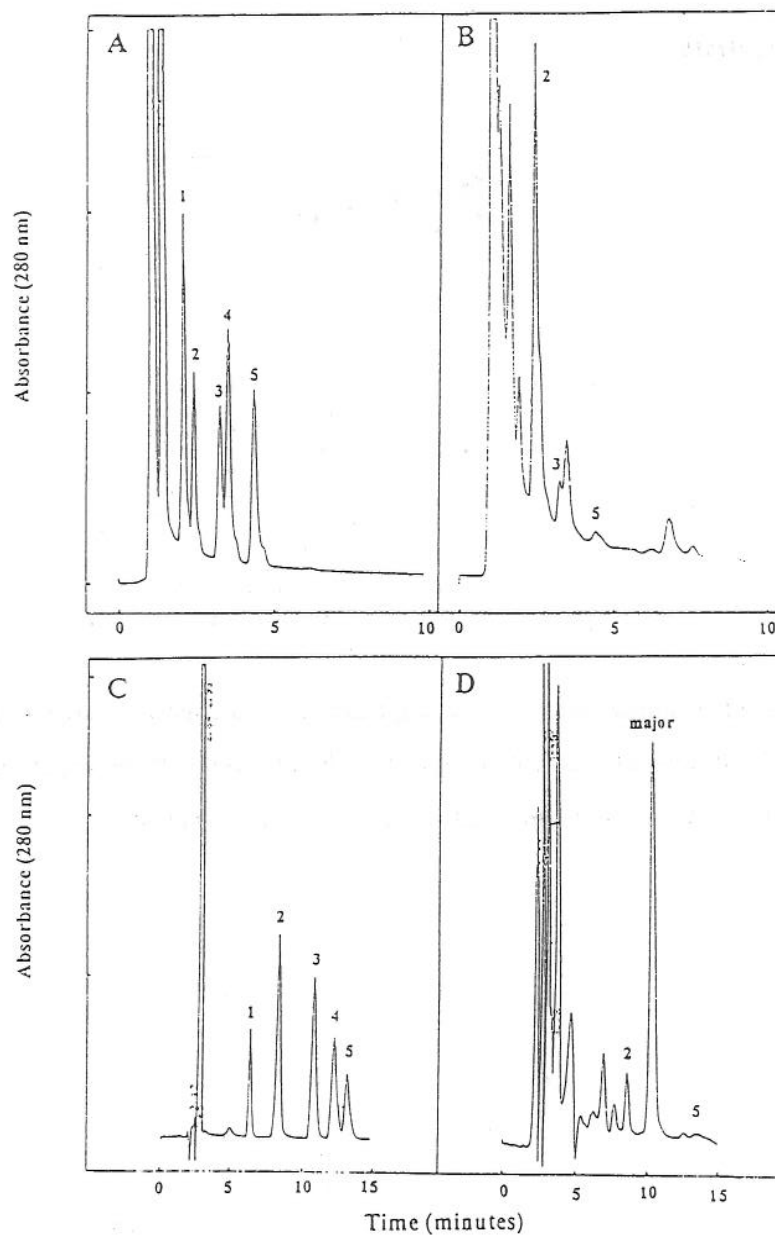


Figure 3. HPLC analysis of the degradation products of curcumin in 0.1 M phosphate buffer, at 37°C for 2 hrs. A: Standards mixture with the mobile phase of 35% methanol and 1% acetic acid. 1: vanillic acid, 2: vanillin, 3: ferulic acid, 4: ferulic aldehyde, 5: feruloyl methane. B: Buffer mixture with the same mobile phase as in Figure 3A. C: Standards mixture with the mobile phase of 12% THF, 5% acetonitrile and 2% acetic acid in water adjusted to pH 4.0 with concentrated KOH solution. 1: vanillic acid, 2: vanillin, 3: ferulic acid, 4: ferulic aldehyde, 5: feruloyl methane. D: Buffer mixture with the same mobile phase as in Figure 3C.

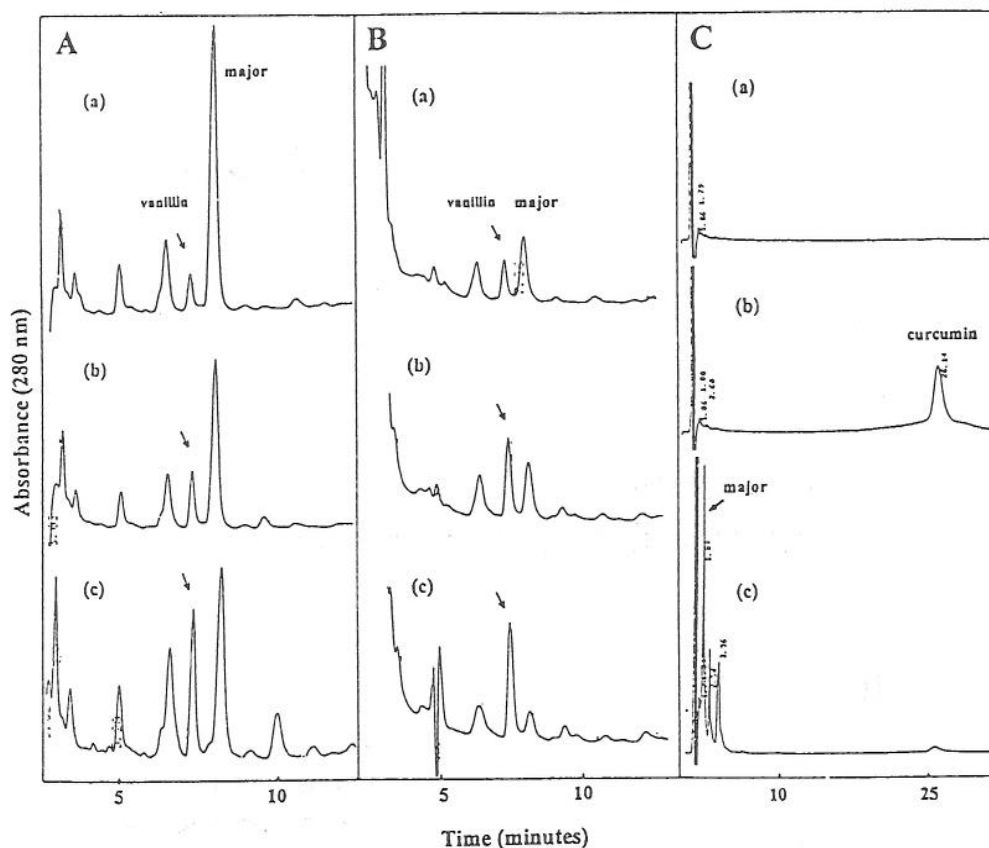


Figure 4. HPLC chromatograms of the degradation products of curcumin at different incubation times. A: Curcumin in 0.1 M phosphate buffer, pH 7.2 at 37°C for (a) 2 hrs, (b) 12 hrs, (c) 24 hrs. B: Curcumin in RPMI 1640 serum-free medium, pH 7.2 at 37°C for (a) 1 hrs, (b) 4 hrs, (c) 8 hrs. Chromatographic conditions: As described in Fig. 3C and the Experimental Section. The mobile phase was adjusted to pH 3.0. C: Curcumin in 0.1 M phosphate buffer, pH 7.2 at 37°C for (a) 0 hrs, Chromatographic conditions: As described in Figure 4A. (b) 0 hrs, Chromatographic condition: 22.5% THF, 5% acetonitrile, 1% acetic acid, pH 4.5. (c) 0.5 hrs, Chromatographic condition: As described in Figure 4C (b).

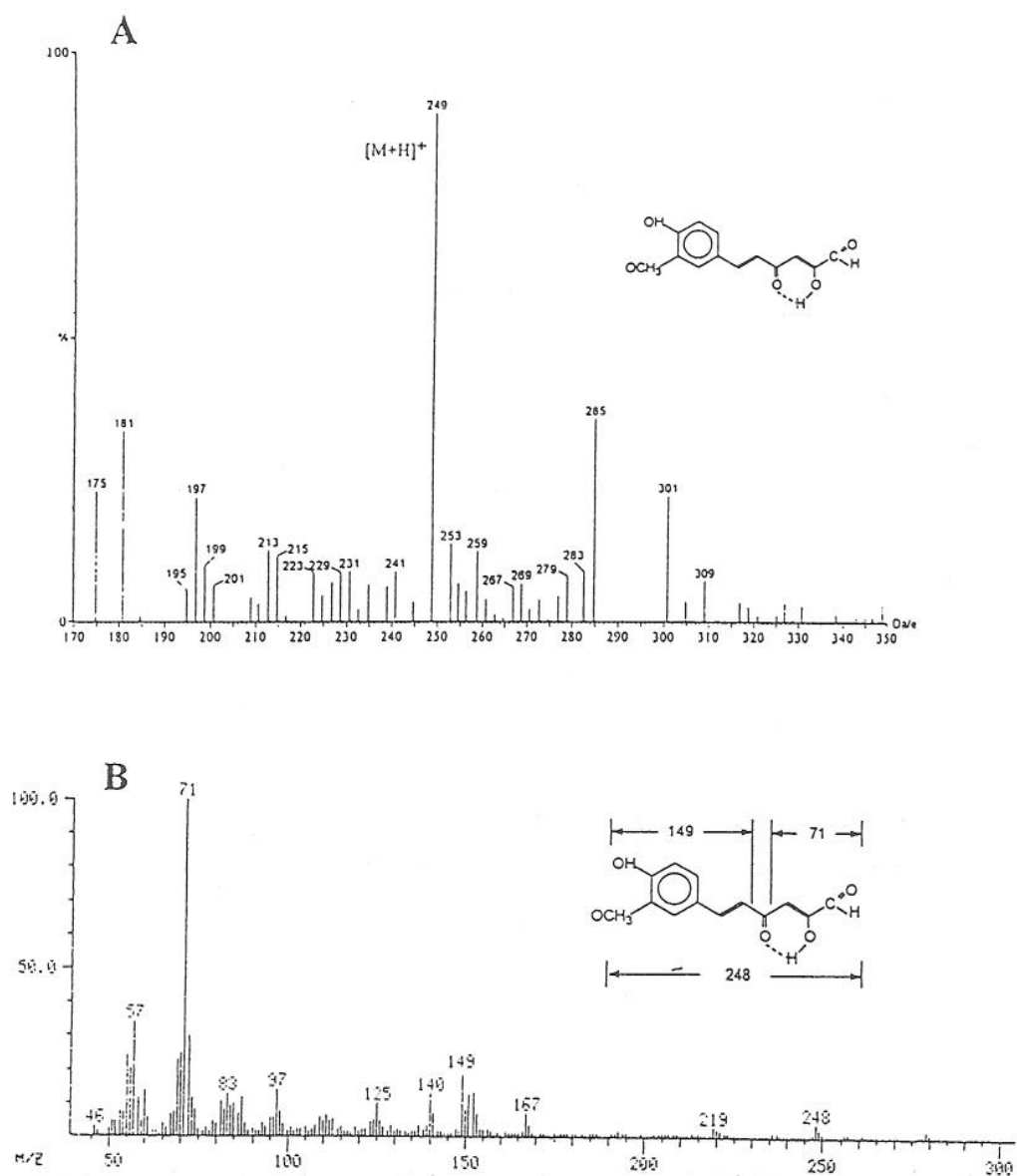


Figure 5. A: ESI/MS of m/z 249, the protonated molecule of the major degradation product of curcumin in 0.1 M phosphate buffer. B: EI/MS of m/z 248. The spectra shows the major fragment ion of 71 and 149. The structure was predicted as trans-6-(4'-hydroxy-3'-methoxyphenyl)-2,4-dioxo-5-hexenal.

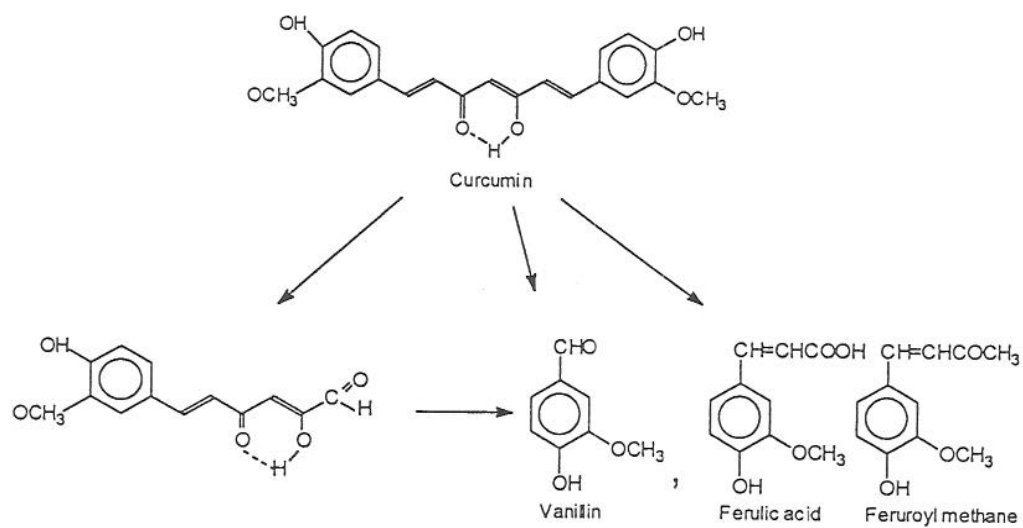


Figure 6. Chemical structures of products obtained from the degradation of curcumin in 0.1 M phosphate buffer, pH 7.2 at 37°C .

