# Kinetic Evaluation of 3β-Hydroxycholest-5-en-7-one (7-ketocholesterol) Stability During Saponification

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**ABSTRACT:** To clarify conflicting information regarding 7-ketocholesterol (7-KC) recovery from saponification, we evaluated the stability of 7-KC in methanolic alkaline medium. We added 1 N alcoholic KOH to 7-KC or lard spiked with 7-KC and held the mixtures at 45, 55, 65, and 75°C for different times to simulate various saponification conditions. Gas-chromatographic determination of residual 7-KC with 5α-cholestane as the internal standard (IS) showed that the higher the saponification temperature, the greater the 7-KC degradation. Hot saponification at 75°C for 30 min caused extensive destruction and left only 31% 7-KC. 7-KC loss during saponification could be described by pseudo first-order kinetics, and the dependence of degradation rate on temperature ($T$, K) by $k$ ($h^{-1}$) = ($2.6 \times 10^{17}$) exp ($-1.36 \times 10^4/T$). As predicted by the kinetic equation, 7-KC loss during room-temperature saponification (21°C) was practically negligible; following the exposure of 7-KC or lard spiked with 7-KC to 1 N alcoholic KOH for 18 h, about 97% 7-KC was recovered. 6-Ketocholestanol, when used as an IS, should be looked at carefully because of potential tautomerization, leading to the formation of two enol isomers when in extended contact with trimethylsilyl derivatization reagents.
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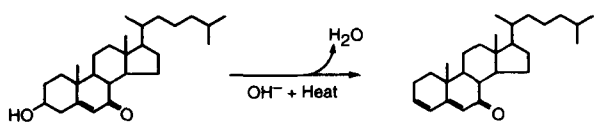
**KEY WORDS:** Degradation, first-order kinetics, gas chromatography, 6-ketocholestanol, 7-ketocholesterol stability, mass spectrometry, saponification, tautomerization, trimethylsilyl ether.

Cholesterol, as an unsaturated lipid, undergoes autooxidation similar to unsaturated fatty acids, leading to the production of various cholesterol oxides (CO). Some CO have received much attention due to their undesirable biological implications, such as cytotoxicity, atherogenicity, sterol metabolism interference, mutagenicity, and carcinogenicity. A number of reviews have been written on cholesterol oxidation (1–3), biological effects of CO (4–7), and analysis of CO (8,9). Among some 60 CO (1), the more commonly occurring CO in foods include cholesterol α-epoxide (α-EP), cholesterol β-epoxide (β-EP), 7α-hydroxycholesterol, 7β-hydroxycholesterol, and 7-ketocholesterol (7-KC, 3β-hydroxycholest-5-en-7-one). While 7-KC is usually most predominant, 25-hydroxycholesterol and 5α-cholestane-3β,5,6β-triol are seldom reported (10). Because CO are minor components of the lipid fraction, analysis of CO requires enrichment by further isolating or purifying them from the bulk of accompanying lipids, mostly triacylglycerols and phospholipids. To this end, saponification or chromatographic fractionation has been used frequently (8,9). Although saponification may be harsher than chromatographic means, it enables the enrichment of CO resulting from the oxidation of both free and esterified cholesterol, whereas the latter, in itself, concentrates CO from the oxidation of free cholesterol.

With regard to saponification, the structural integrity of two types of CO, EP and 7-KC, has been a concern. Contrary to the earlier observation of no structural alteration of β-EP (11), an extensive loss of α-EP, up to 75%, after conventional hot saponification was reported, implying that the ring opening was caused by the hot alkaline reaction (12). Park and Addis (13) were the first to report that the recovery of α-EP was virtually complete when saponification was conducted at ambient temperature for 18 h (i.e., cold saponification). The full recovery of EP has been confirmed after not only cold (14–16) but also hot saponification (17,18). Despite these results, a recent paper (19) still stated that saponification would degrade EP and was avoided. The instability of 7-KC in hot alkaline medium has long been known, with its dehydration product, cholesta-3,5-dien-7-one (Scheme 1), being the predominant degradation product (11,20,21). Smith *et al.* (22) used cold saponification to concentrate CO, including 7-KC, in human liver and plasma samples. Park and Addis (13) confirmed the negligible loss of 7-KC (96% recovery) after cold saponification. While this virtually full recovery was reconfirmed by many researchers (14–16,18,23), others documented extensive destruction of 7-KC during cold saponification, leaving only 11–40% (24) or 80% (25). Although 7-KC was not included, Hwang and Maerker (26) reported the




**SCHEME 1**

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instability of similar ketone derivatives of cholesterol during cold saponification, with the recovery being about 70%. In contrast, Osada *et al.* (27) stated that CO standards, including 7-KC, were stable during hot saponification in ethanolic KOH (70°C for 1 h). Following similar hot saponification (70°C for 30 min), 7-KC was also the predominant CO detected in oxidized low-density lipoprotein (LDL) (28).

Because 7-KC is frequently the predominant product of cholesterol oxidation, it is important to accurately determine 7-KC to evaluate the extent of cholesterol oxidation in food and biological specimens. Therefore, we considered it necessary to reinvestigate the stability of 7-KC during saponification. We subjected 7-KC in alkaline methanol to various temperatures for different reaction times to simulate cold and hot saponifications. We report the kinetic data, which demonstrate the extensive destruction of 7-KC during conventional hot saponification, but its virtually full recovery from cold saponification. Initially, we tried 6-ketocholestanol (6-KCA) as internal standard (IS). However, it was unsuccessful because of enolization upon trimethylsilylation, which is also reported in the present study.

## EXPERIMENTAL PROCEDURES

*Reagents.* All reagents used were Certified ACS grade. Diethyl ether, methanol, and potassium hydroxide were from EM Science (Gibbstown, NJ). Ethyl acetate, methylene chloride, and anhydrous sodium sulfate were from Fisher Scientific (Pittsburgh, PA). Anhydrous pyridine was from Aldrich Chemical Co. (Milwaukee, WI). Sylon BTZ was from Supelco, Inc. (Bellefonte, PA). 5α-Cholestane and 6-KCA were purchased from Sigma Chemical Co. (St. Louis, MO). 7-KC and cholesta-3,5-dien-7-one were from Steraloids, Inc. (Wilton, NH).

*Saponification of 7-KC.* A stock solution, containing 7-KC and 5α-cholestane, was prepared with ethyl acetate. Aliquots of the stock solution, equivalent to 250 μg of 7-KC and 118 μg of 5α-cholestane, were transferred into a Teflon-lined screw-cap tube (26 × 150 mm) to prepare five groups of samples (duplicates). After evaporating ethyl acetate under a nitrogen stream, 10 mL of 1 N KOH in methanol was added. After capping the tube, one group was left at room temperature (21°C) for 18 h for cold saponification (13). The remaining four groups were placed in water baths at 45 ± 1, 55 ± 1, 65 ± 1, and 75 ± 1°C, respectively. After heating for various times, each test tube was withdrawn and placed in iced water to stop the reaction. Nonsaponifiables [i.e., 7-KC and internal standard (IS)] were extracted as previously described (13). The organic phase was collected, and the aqueous phase was re-extracted twice. The combined extract was washed with 5 mL of 0.5 N KOH, followed by two portions of 5 mL distilled water, and dried over anhydrous sodium sulfate. After filtration and solvent removal, the dried extract was obtained.

*Saponification of spiked lard.* Five grams of lard was dissolved into 25 mL methylene chloride to which 6.25 mg of 7-KC and 2.95 mg of 5α-cholestane (IS) were added. One-milliliter aliquots were transferred to a screw-cap tube, and the solvent was evaporated. Saponification was conducted at room temperature for 18 h after two different treatments. First, spiked lard samples ($n$ = 5) were redissolved in 2 mL methylene chloride and then mixed with 10 mL of 1 N methanolic KOH. Crystallized lard particles were redissolved by intermittent sonication for 10 min in a water bath at 20°C. Second, spiked lard samples ($n$ = 5) were directly mixed with 10 mL of 1 N methanolic KOH. The mixture was warmed in a water bath at 65°C for about 4 min to solubilize the lard and then placed at room temperature. Nonsaponifiables were extracted, dried, and freed of solvent as described above.

*Gas-chromatographic (GC) determination of 7-KC.* The dried extracts (nonsaponifiables) were redissolved in 50 μL pyridine to which 50 μL Sylon BTZ was added to derivatize CO to corresponding trimethysilyl (TMS) ethers for GC determination (13). Capillary GC was performed with a Varian (Palo Alto, CA) Vista 6000 gas chromatograph, equipped with a flame-ionization detector as described previously (29) with modification in temperature programming; the oven temperature was programmed from 210 to 250°C at 1.8°C/min. The column was a fused-silica capillary column with 100% methyl silicone stationary phase, DB-1 (0.232 mm × 15 m, 0.1 μm film thickness) from J&W Scientific, Inc. (Folsom, CA). The retention of 7-KC from various saponification conditions was calculated by dividing the amount of 7-KC recovered after saponification by the initial amount of 7-KC prior to saponification.

*GC/mass spectrometry (MS).* A Hewlett-Packard (Avondale, PA) 5890 Series II gas chromatograph interfaced to a 5971A Mass Selective Detector was used with a DB-5 column (0.25 mm × 30 m, 0.1 μm film thickness, J&W Scientific, Inc.). The column oven was kept at 270°C, the injector at 280°C, and helium was the carrier gas at 8 psi. Injection was made at 20:1 split ratio. The electron impact ionization was set at 70 ev, and the source temperature was 190°C. Mass spectra scanned between mass/charge (*m/z*) 100 and 600 were recorded on a computer (Vectra QS/20; Hewlett-Packard) and identified by utilizing an on-line computer library (Wiley).

*Statistical analysis.* All samples were prepared at least in duplicate. Excel (Microsoft Corporation, Redmond, WA) software version 5.0 was utilized for regression analysis and $t$-test of the data.

## RESULTS AND DISCUSSION

Because 7-KC was detected as the predominant CO in human liver and plasma samples exposed to alkaline methanol at room temperature overnight by Smith *et al.* (22), we were prompted to conduct the quantitative investigation of 7-KC recovery after cold saponification and document its full recovery (13). While many researchers (14,16–18,23) confirmed the full recovery after cold saponification, others claimed extensive destruction of 7-KC (24–26). Contrary to the long-known instability of 7-KC in hot alkaline medium (11,20,21), 7-KC was thought to be stable during hot saponi-

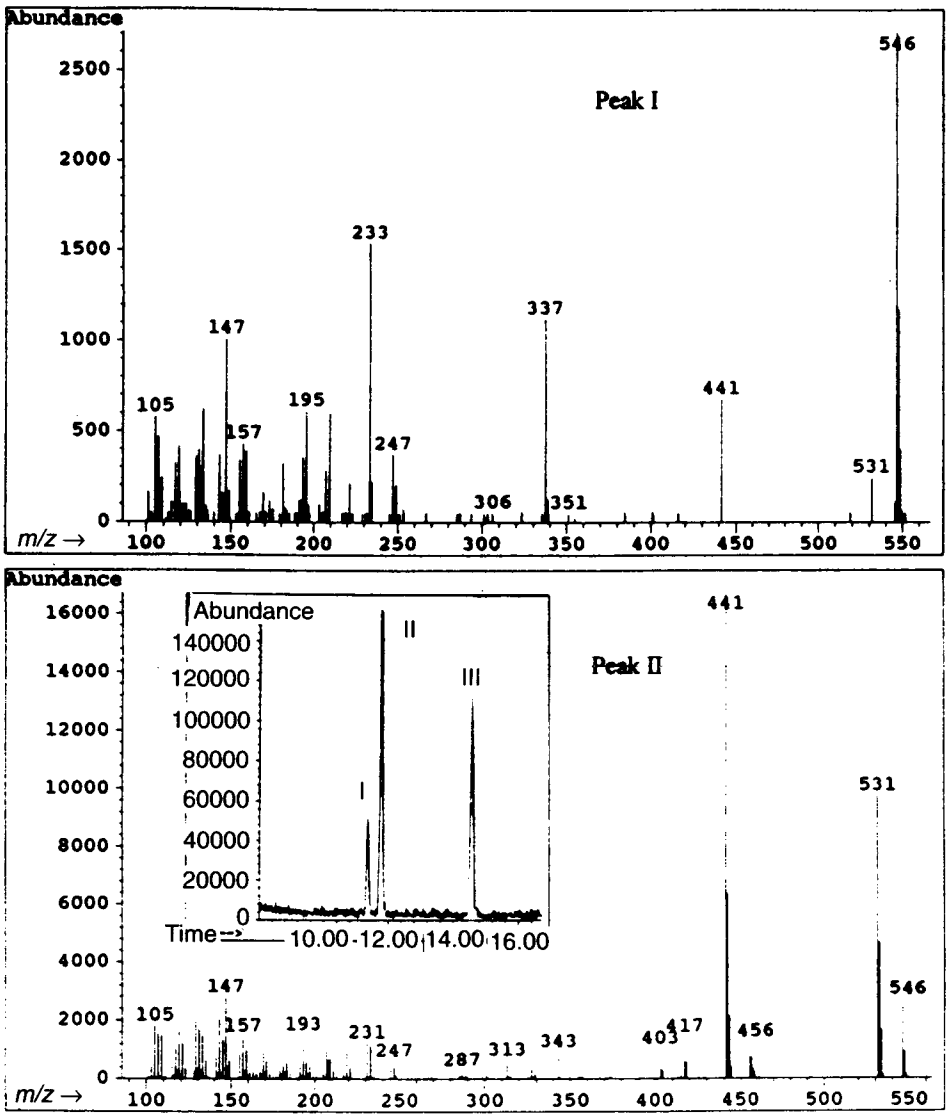



**FIG. 1.** Total ion chromatogram (inset) of 6-ketocholestanol left in trimethylsilyl derivatization reagents for 45 d at 4°C and electron impact mass spectra of artifact peaks I (top) and II (bottom). As trimethylsilyl ethers, peaks I and II are isomeric 3,6-dihydroxycholestanol, and peak III is 6-ketocholestanol.

fication (27,28). To address whether cold saponification causes degradation and whether conventional hot saponification causes no degradation, we examined 7-KC recovery after mixing a standard mixture (consisting of 7-KC and IS) with 1 N methanolic KOH and then exposing the reaction mixture to various temperatures. Initially, we tried 6-KCA as the IS, taking advantage of its structural similarity to 7-KC and stability during saponification. However, when we analyzed 6-KCA, stored for one day at 4°C after TMS derivatization, we observed two new peaks (I and II) that eluted ahead of the 6-KCA TMS ether (peak III of the inset in Fig. 1). Moreover, the two new peaks grew larger as 6-KCA was left in prolonged contact with TMS derivatization reagent (Table 1).

The mass spectra of peaks I and II shared some diagnostically important ions of TMS ether sterols (Fig. 1), indicating that they may be positional isomers. If 6-KCA tautomerized during TMS derivatization, the formation of two enol isomers is possible, leading to *bis*-TMS ethers with molecular weight of 546, in addition to the intended 6-KCA TMS ether. Indeed,

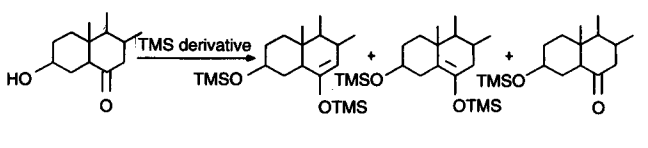
**TABLE 1**
**Residual 6-Ketocholestanol (%) Due to Its Conversion to Enol Isomers**

| | Storage temperature (°C) | | |
|---|---|---|---|
| Time (h)[a] | 21 | 4 | −25 |
| 4 | 96.3 | ND[b] | ND |
| 8 | 92.5 | ND | ND |
| 12 | ND | 90.7 | ND |
| 24 | 84.8 | 85.6 | 88.3 |
| 48 | 76.7 | 81.7 | 82.3 |

[a]In contact with trimethylsilylation reagents after initial holding at 21°C for 0.5 h.
[b]ND, not determined.

*m/z* 546 was observed and suggested as the molecular ion (M) peak in both spectra. Also, *m/z* 531 (M-$CH_3$) and 441 (M-$CH_3$-90) were clearly seen, supporting *m/z* 546 as the M. Alkali-catalyzed enolization proceeds *via* proton abstraction from the α-carbon of carbonyl compounds. Due to lower C-H bond strength in tertiary than in secondary carbons, the abstraction of α-hydrogen at $C_5$ of 6-KCA would be preferred to that at $C_7$ favoring the formation of $\Delta^5$ over $\Delta^6$. Because peak II was always larger than peak I, we therefore postulate that peaks I and II would have isomeric structures as shown in Scheme 2 (partially presented); however, we did not continue the structure assignment study further. What is more important about the potential tautomerization of 6-KCA is that enol isomers were evident in as short a time as 4 h at room temperature (Table 1). This observation deserves attention because 6-KCA was frequently used as an IS for determination of CO as TMS ethers (19,30–32); aged samples (i.e., derivatized and kept) may contain an amount of IS which differs from the added amount unless excess TMS derivatization reagents were removed (19,32). Accordingly, we replaced 6-KCA with 5α-cholestane as the IS throughout the study.

The usual technique for predicting chemical stability has been the application of the Arrhenius equation, which enables the calculation of activation energy from the temperature dependence of reaction rates: $k = k_0 \exp(-E_a/RT)$, where $k$ = measured rate constant, $k_0$ = pre-exponential factor, $E_a$ = activation energy, $R$ = ideal gas constant, and $T$ = absolute temperature. As far as the loss of 7-KC during saponification is concerned, the chemical reaction may be described by the second-order reaction: $-d[7\text{-KC}]/dt = k[7\text{-KC}][\text{Base}]$. However, the degradation reaction may be analyzed by pseudo first-order reaction kinetics because the base reagent is present in excess. Then, the rate equation for the first-order reaction becomes $\log [7\text{-KC}/7\text{-KC}_0] = -(k/2.303)t$, where $7\text{-KC}_0$ is the initial concentration of 7-KC. Accordingly, the detrimental effects of saponification on 7-KC were studied by monitoring the retention of 7-KC in alkaline methanol exposed to four different temperatures for different reaction times. As expected, higher temperatures and prolonged exposure promoted higher degradation of 7-KC; cholesta-3,5-dien-7-one (confirmed by mass spectra comparison) emerged as the dominant reaction product (Fig. 2). The semilog plots of residual 7-KC at each temperature vs. reaction time are shown in Figure 3. Even brief heating (5 min) at 65–75°C, the usual temperature range for refluxing, was sufficient to destroy almost 10% of the 7-KC. On the other hand, at a lower temperature, such as 45°C, it took about 2 h to destroy a similar amount of 7-KC. The saponification at 45°C for 30 min




**SCHEME 2**

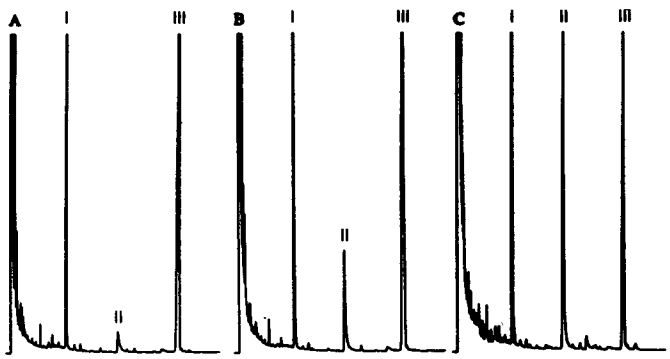



**FIG. 2.** Gas chromatograms of 7- ketocholesterol (as trimethylsilyl ether) after exposure to 1 N methanolic KOH at (A) 21°C for 18 h, and at (B) 65°C for 5 and (C) 60 min, respectively. Peaks I, II, and III are 5α-cholestane (internal standard), cholesta-3,5-dien-7-one, and 7-ketocholesterol, respectively. (A) Peak II of the chromatogram was a contaminant in the standard 7-ketocholesterol used and (B and C) that of the two chromatograms augmented as a result of the hot saponification of 7-ketocholesterol.

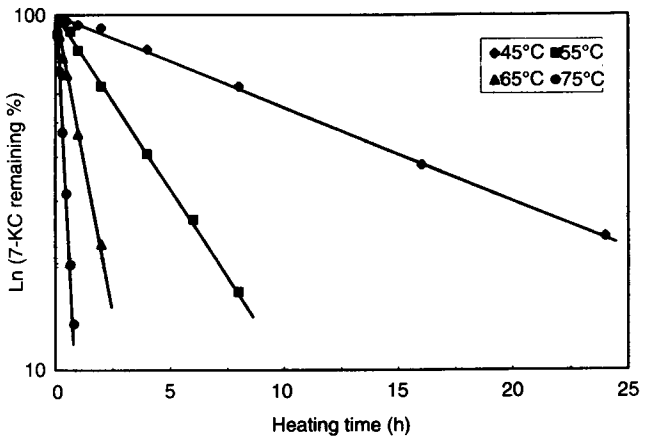



**FIG. 3.** Effects of time and temperature on the retention of 7-ketocholesterol (7-KC) in alkaline solution.

resulted in about 96% retention of 7-KC, whereas the same length of reaction time at 75°C resulted in severe destruction of 7-KC, *ca.* 31% retention. All of these results indicate how influential saponification temperature and duration are on 7-KC stability. The reaffirmation of 7-KC instability under hot saponification in the present study strongly suggests that lymphatic absorption of 7-KC (27) and 7-KC level in oxidized LDL (28) were likely grossly underestimated because lipid extracts were subjected to hot saponification.

The semilog plots of heating time vs. 7-KC retention at the four saponification temperatures (Fig. 3) yielded straight lines, indicating that the destruction of 7-KC during saponification can be described by first-order reaction kinetics. Regression analysis of the semilog plots of residual 7-KC concentration vs. exposure time showed high correlation with $r^2 = 0.99$ (Table 2). From the slopes of the regression lines of the semilog plots, first-order rate constants were obtained for

**TABLE 2**
**First-Order Rate Constants of 7-KC Destruction in 1 N Methanolic KOH**

| Temperature (°C) | $k$ ($h^{-1}$) | $y$-Intercept | $r^2$ |
|---|---|---|---|
| 45 | 0.0604 | 101.0 | 0.99 |
| 55 | 0.2264 | 101.6 | 0.99 |
| 65 | 0.7311 | 97.4 | 0.99 |
| 75 | 2.4911 | 106.7 | 0.99 |

the loss of 7-KC; the results are summarized in Table 2. The relationship between the saponification temperature ($T$, K) and the measured rate constant ($k$) was investigated from the Arrhenius plot (Fig. 4), in which the semilog plot of log $k$ vs. $1/T$ yields a straight line. The slope of the regression line is −13,645 (K) and therefore, the activation energy was estimated to be about 27 kcal/mol. The $Q_{10}$ value (the increase in reaction rate per 10°C increase) was about 3.2–3.7. These data imply that the degradation of 7-KC is heavily influenced by a small fluctuation in temperature during saponification. This may explain why variable degrees of low recovery of 7-KC after cold saponification were experienced (24,25). Total lipid extracts consist largely of nonpolar, neutral lipids, such as triacylglycerols, and therefore are not freely soluble in methanol, a polar solvent for saponification. Application of a brief heating helps to dissolve nonpolar lipid extracts in methanolic alkaline solution. However, it is likely that such warming, depending on its temperature and duration, might have already caused 7-KC degradation before the implementation of cold saponification. To avoid warming of lipid extracts in methanolic alkaline solution, we previously solubilized the extracts in 5 mL methanol by warming. Then, after cooling it below 35°C, we added an equal volume of 2 N methanolic KOH for the cold saponification, which successfully recovered about 96% 7-KC (13).

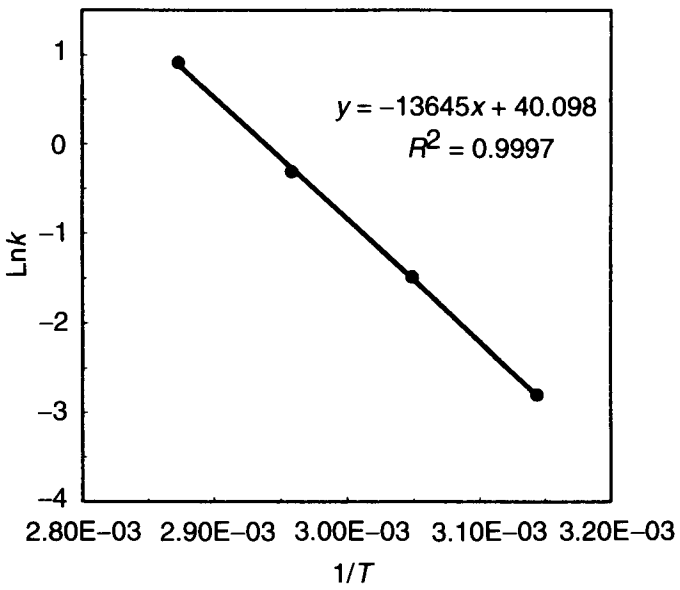



**FIG. 4.** Arrhenius plot of 7-ketocholesterol degradation in alkaline solution.

From the regression plot (Fig. 4), the degradation rate of 7-KC during saponification at temperatures other than those tested may be predicted. For instance, for saponification at room temperature (21°C), a rate constant of 0.0018/h is predicted. This indicates that the half-life of 7-KC would be 385 h at room temperature. This also implies that the retention of 7-KC during cold saponification, i.e., at room temperature for 18 h, would be about 97%, suggesting that the degradation is practically negligible. To test the validity of this predicted recovery, we prepared nonsaponifiables from lard spiked with 7-KC in two different ways. In the first procedure, we dissolved the lard in methylene chloride, followed by the addition of methanolic alkaline solution. Prior to cold saponification, crystallized lard particles were redissolved by sonication in a water bath below 20°C. In the second procedure, we placed lard directly in methanolic KOH and warmed the mixture at 65°C for 4 min to dissolve the lard. After cooling to room temperature rapidly, both mixtures were left overnight for cold saponification. The cold saponification with no use of heating resulted in a 96.1 ± 3.1% ($n$ = 5) recovery, which agreed with the above predicted value and also reaffirmed our previous report (13). The second approach with brief warming resulted in a recovery of 89.3 ± 4.0% ($n$ = 5), which was significantly lower ($P < 0.05$). Nevertheless, this recovery was still much higher than the 11–40% reported by Van de Bovenkamp *et al.* (24).

Some studies conducted saponification at 30–37°C for 18 h, describing it as mild saponification, but presented no recovery data (33–35). Although such a temperature range may be viewed as slightly elevated compared with room temperature, the regression plot of Figure 4 predicts that the reaction rate constant would be 0.0072/h at 30°C and 0.0199/h at 37°C, and therefore 12–30% of 7-KC would be lost after 18 h reaction. Hodis *et al.* (36) performed cold saponification by treating lipid extracts from atherosclerotic aorta tissues with 20% KOH in methanol and ether at room temperature (22°C) for 3 h and found cholesta-3,5-dien-7-one the highest among CO detected; they found little 7-KC. One may presume that cholesta-3,5-dien-7-one and not 7-KC was in fact the major CO. However, such inference requires clarification because of possible derivation of cholesta-3,5-dien-7-one from 7-KC during saponification. We found that 7-KC stability was also affected by the strength of alkaline reagents used for saponification. When we saponified 7-KC at 65°C for 1 h with 1 N and 2 N methanolic KOH, respectively, we observed a drastic decrease in its recovery, i.e., 48 vs. 18%. The effect of alkali strength on 7-KC conversion to cholesta-3,5-dien-7-one was also noticeable during cold saponification. When we exposed 7-KC to 20% methanolic KOH (about 3.6 N) at 22°C for 3 h, we observed about 10% loss of 7-KC and the emergence of cholesta-3,5-dien-7-one. This observation suggests that the finding of cholesta-3,5-dien-7-one by Hodis *et al.* (36) may be due at least in part to its derivation from 7-KC. Because such an occurrence could be a consequence of the use of concentrated alkali rather than saponification temperature, it is implied that not all cold saponification practices, i.e., by

merely conducting base-catalyzed hydrolysis at room temperature, will qualify for satisfactory recovery of 7-KC. Our observations in the present study suggest that 7-KC recovery be carefully evaluated when cold saponification is to be carried out at conditions other than those reported previously (13) and again in the present study, i.e., at room temperature for about 18 h after dissolving lipid extracts in 1 N methanolic KOH without the use of heating.

7-KC is one of the more common and often the predominant CO in many food products. We studied the stability of 7-KC during saponification. Time–temperature relationships showed that the destruction of 7-KC can be described by a pseudo first-order reaction. We demonstrated that the stability of 7-KC was sensitive to a small change in temperature during saponification, as implied by a large activation energy, about 27 kcal/mol, and that both a brief heating of the lipid extracts containing 7-KC, to dissolve in methanolic alkaline solution, and the use of concentrated alcoholic KOH could be sufficiently harsh to cause considerable destruction of 7-KC. We reaffirmed that careful implementation of cold saponification, i.e., avoidance of heating 7-KC in hot alkaline solution, results in 7-KC recovery of about 96%. Care must be given to the potential enolization of 6-KCA in TMS derivatization reagents when one uses it for quantitating CO as TMS ethers.

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