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Thermal Stability and Transformation of Chlorogenic Acids in Stevia (*Stevia rebaudiana*) Products: Degradation Pathways, Acyl Migration, Metal-Ion Catalysis, and Processing Implications

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Abstract

Stevia (*Stevia rebaudiana*) is processed industrially mainly for steviol glycosides, whereas its co-occurring chlorogenic acids—especially dicaffeoylquinic acids (diCQAs)—are emerging value-added ingredients. The stability of a commercial stevia chlorogenic acid product (total acids 61.39%, comprising 53.8% diCQAs and 7.4% mono-CQAs) was evaluated in the solid state and in 80% aqueous methanol under heat (80–180 °C), light, oxygen, metal ions (Fe^{3+}, Cu^{2+}), pH (3.0–8.0), and solvent conditions. In solids, total acid loss was 1.2–2.4% (80–100 °C, 24 h), 9.2/9.0% (120 °C, 24 h/140 °C, 4 h), and 44.9/92.9% at 160/180 °C (4 h; $n = 3$, SD ≤ 0.38%); as holding times differed, these values define thresholds for the specific conditions rather than a kinetic envelope. LC–MS tentatively assigned methylation, acetylation, and lactonization products that accumulated below 160 °C and degraded above 180 °C. In solution, total acid content varied by less than ±7% at 60–80 °C, with acyl migration of 4,5-diCQA and 5-CQA. Reversible insoluble Fe^{3+}/Cu^{2+}–diCQA complexes (97.89–99.90% EDTA recovery, $n = 3$) accelerated light- and oxidation-induced loss (67.6%/48.6% vs. 36.5% under light; 27.9% vs. 9.3% under oxidation), Cu^{2+} more strongly. pH-related deficits were buffer-related analytical effects; only pH 5.0 lost significantly (−3.77%). The solid was light-stable (3.76%/25 d), whereas concentrated process liquid lost 11.1% (80 °C/10 h) vs. 1.1% in the dilute stream, providing practical processing windows for quality control.

Keywords: stevia; dicaffeoylquinic acid; thermal stability; degradation pathway

Academic Editor: Matteo Guidotti

Received: 23 September 2026
Revised: 5 November 2026
Accepted: 19 November 2026
Published: 21 November 2026

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1. Introduction

Stevia (*Stevia rebaudiana* (Bertoni) Hemsl.) is a perennial herb of the family Asteraceae native to the alpine grasslands bordering Paraguay and Brazil in South America. Stevia was introduced to China in 1977 and is currently cultivated as a specialty crop; the planting area has reached 20,000–25,000 ha, with an annual output exceeding 100,000 tons. Stevia is best known for its diterpene glycosides (steviol glycosides), whose sweetness is 200–300 times that of sucrose; owing to their non-cariogenic properties and suitability for diabetic consumers, steviol glycosides are widely used in

the food, pharmaceutical, and personal-care industries, with a global market volume of ~10,000 tons [1].

In addition to steviol glycosides and flavonoids, stevia leaves contain approximately 7% (HPLC) chlorogenic acids, of which the dicaffeoyl-substituted isochlorogenic acids (diCQAs) account for up to ~5% [2,3]. The diCQA content in stevia is markedly higher than that in traditional medicinal/industrial plants such as *Lonicera japonica* and *Eucommia ulmoides* [3]. However, the content and composition of chlorogenic acids vary considerably among cultivars [2]. Pre-harvest factors further shape this variability: cultivation conditions, water and nutrient supply, and abiotic stress (temperature, light, salinity) alter stevia growth, total phenol and antioxidant status, and stevioside content, and therefore determine the batch-to-batch consistency of the starting material and, in turn, of the finished chlorogenic acid product [4,5,6. Isochlorogenic acids (dicaffeoylquinic acids) comprise three positional isomers—isochlorogenic acid A (3,5-diCQA), B (3,4-diCQA), and C (4,5-diCQA) [7]—and exhibit multiple activities, including regulation of glucose metabolism [8,9], hepatoprotection [10,11], anti-inflammatory [12], and antibacterial effects [13,14]. Because of the higher degree of caffeoylation and the o-dihydroxyphenol (catechol) moieties, diCQAs generally display stronger antioxidant, anti-inflammatory, and antiviral activities than mono-caffeoyl chlorogenic acids [2,7,12]. Stevia-derived isochlorogenic acids have been approved in China as both a new food ingredient and a new feed additive and are now produced at an industrial scale, where stevia-derived chlorogenic acid is a byproduct/co-product of steviol glycoside production [3].

Caffeoylquinic acids are intrinsically labile. The o-dihydroxyl structure of chlorogenic acids is prone to decomposition under high temperatures, light, and pH changes, which lowers the extraction yield and product integrity [15–20]. Quantitative studies on mono-caffeoyl chlorogenic acids have established that 5-CQA degrades in aqueous solution under heating [15], that its thermal stability is strongly pH-dependent [16], that alcoholic extraction can generate esters and amine derivatives [17,18], and that roasting drives CQA degradation and lactone formation in coffee [19–21]. For diCQAs, Deshpande et al. demonstrated that acyl migration among the mono- and dicaffeoylquinic acid isomers occurs under aqueous basic, aqueous acidic, and dry-roasting conditions via hydrated intermediates [22], and Clifford et al. established LC–MS^n diagnostic rules that discriminate the six diCQA isomers [23]. Xue et al. reported the stability and degradation of CQAs under different storage conditions [24]. Within our own group, earlier work screened the influence of light, temperature, dissolved oxygen, pH, and metal ions on stevia chlorogenic acid content [25], and a subsequent UPLC-QTOF MS/MS study identified dehydration, degradation, hydrolysis, and isomerization as the main degradation routes of stevia CQAs under solvent, light, heat, and pH stresses [26].

Nevertheless, three aspects remain unresolved for industrial practice, and each has consequences that the existing literature does not resolve. (i) A temperature–time envelope for the solid diCQA product under realistic processing conditions (drying, coating, extrusion) has not been established; the earlier stability screens from our group [25,26] were qualitative screening studies and did not quantify loss at defined temperatures, times, and replicate numbers; (ii) the complete solid-state transformation network (degradation, methylation, acetylation, lactonization) and its temperature dependence, including the transient accumulation and subsequent degradation of these derivatives, are not described quantitatively; and (iii) the interplay between metal-ion complexation (a common unavoidable contaminant in processing water and vessels) and light/oxidative loss, and its mechanistic basis, has not been reported for stevia products.

In this study, a commercial stevia isochlorogenic acid product (total acids 61.39%,

diCQAs 53.79%) was subjected to solid heat treatment at 80–180 °C and solution treatment at 60–80 °C, metal ions, light, oxygen, pH, and solvent variations following our standardized stability protocols. Transformation products were identified using UPLC-QTOF MS/MS. The results provide a quantitative stability map—thermal thresholds, transformation pathways, and metal-ion catalytic behavior—that guides processing, storage, transport, and end-use of stevia chlorogenic acid ingredients.

2. Results

2.1. Thermal Stability of the Solid Product: A Quantitative Processing Window

The total acid content of the solid product decreased over three well-separated heat-load regimes (Figure 1c). Heating at 80 °C or 100 °C for 24 h removed 1.2% and 2.4% of the total acids, 120 °C/24 h and 140 °C/4 h removed 9.2% and 9.0%, whereas 160 °C/4 h and 180 °C/4 h removed 44.9% and 92.9%. The boundary between the second and the third regime is therefore sharp and lies between 140 and 160 °C: within the processing window the loss stayed below 10% even after 24 h at 120 °C, but a 4 h excursion to 160 °C already destroyed almost half of the chlorogenic acids, and at 180 °C only 4.4% of the product remained as chlorogenic acids. The diCQA pool, which accounts for 53.8% of the product and 87.6% of the total acids, lost 1.3%, 2.5%, 9.8%, 10.3%, 52.3% and 97.6% of its initial content at the six temperatures, whereas the mono-CQA pool (7.4% of the product) lost only 0.9–2.4% up to 140 °C and was still 4.6% above its initial level at 160 °C. The decrease in the diCQA pool thus accounts for essentially the whole total-acid loss, and the diesters are the degradation hotspot of the solid: at 140 °C the diesters lose 5.5% and the total acids 5.5, and at 160 °C 28.2 against 27.6. Only above 160 °C did the mono-CQA pool begin to shrink (−61.7% at 180 °C), and even then it remained far more resistant than the diCQA pool (−97.6%), in line with the general rule that a higher degree of caffeoylation lowers the thermal stability of the ester chain [27–29]. Unless stated otherwise, every value is the mean ± SD of three independent replicate preparations, and treatments that do not share a letter differ significantly (Tukey HSD, $p < 0.05$) as detailed in the Supporting Information.

Superimposed on this net loss, the mono-CQA pool was redistributed between 140 and 160 °C, and in a direction opposite to that found in solution: 5-CQA rose from 4.67% to 4.84% at 140 °C (+3.7%) and to 5.37% at 160 °C (+15.1%), whereas 3-CQA (−13.6% and −17.8%) and 4-CQA (−6.1% and −10.1%) declined, while the pool as a whole was conserved (+4.6% at 160 °C). This is consistent with positional acyl migration within the monoester pool [22], with a net shift towards the 5-isomer in the dry solid; because migration proceeds through hydrated intermediates [22], which are scarce in an anhydrous matrix, the solid-state direction need not coincide with the aqueous one (Section 2.3). The 0.70 percentage-point gain in 5-CQA amounts to only 2.5% of the 28.2% of diCQA lost at 160 °C, so conversion of diCQAs into mono-CQAs is a minor pathway. 5-CQA then collapsed at 180 °C (1.86%, −60.2%). Free caffeic acid, the smallest pool (0.25% of the product), was by far the most labile component (−28.2% at 100 °C, −76.0% at 120 °C and −89% to −92% at ≥140 °C), so that ester hydrolysis was not accompanied by accumulation of the free aglycone.

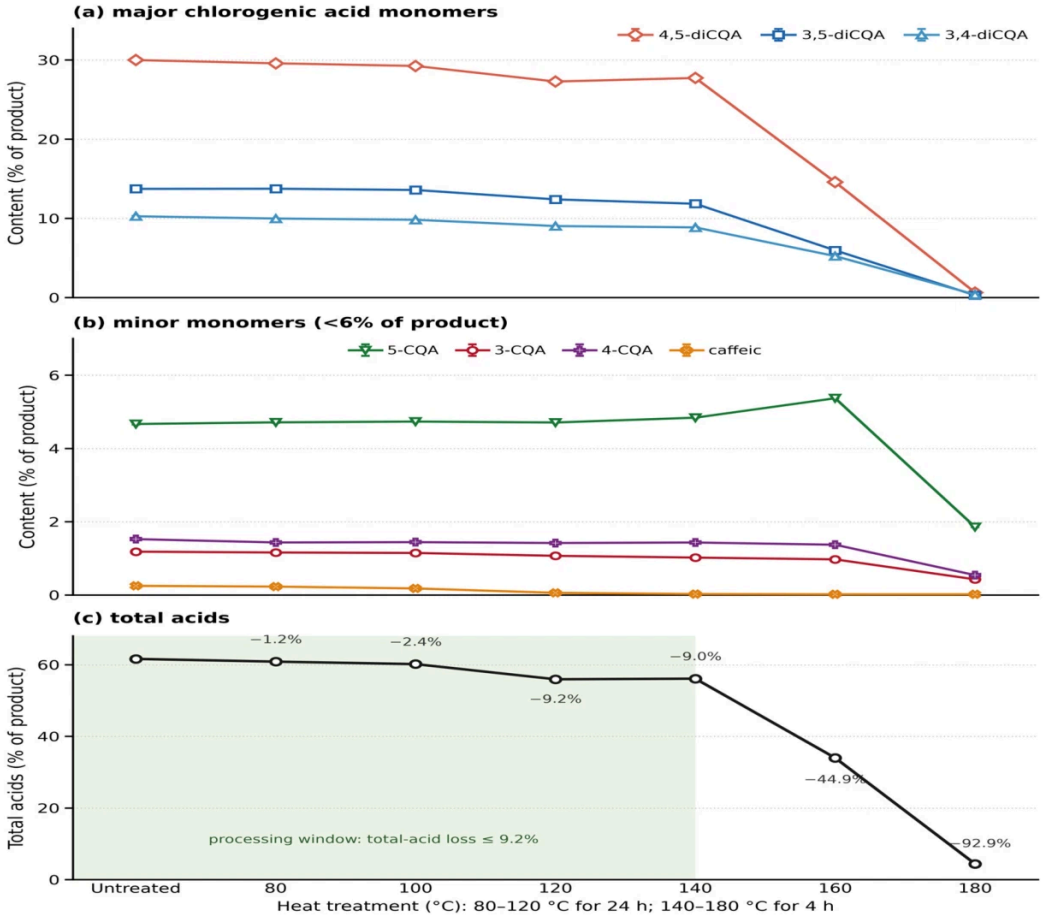


Figure 1. Thermal stability of the solid chlorogenic acid product. (**a**) Major monomers (4,5-diCQA, 3,5-diCQA and 3,4-diCQA); (**b**) minor monomers (5-CQA, 3-CQA, 4-CQA and caffeic acid, <6% of the product); (**c**) total acids. The solid was heated at 80, 100 and 120 °C for 24 h and at 140, 160 and 180 °C for 4 h. Contents are expressed as % of the product; the shading in (**c**) marks the processing window in which the total-acid loss remained ≤9.2%, and the percentages give the total-acid loss relative to the untreated product. Values are means ± SD of three independent determinations (n = 3); where no error bar is visible it is smaller than the symbol.

2.2. Identification of Solid-State Thermal Transformation Products and Pathways

Untreated, mid-loss (160 °C/4 h), and near-complete-loss (180 °C/4 h) samples were profiled using UPLC-QTOF MS/MS (Figure 2 and Table 1). After heating, quinic acid and acetylquinic acid increased strongly, whereas CQA, diCQA, triCQA, caffeic acid, and steviol glycosides decreased. Ferulic acid, feruloyl-caffeoylquinic acid, acetyl-dicaffeoylquinic acid, and the dicaffeoylquinic acid lactone first increased (160 °C) and then decreased or disappeared (180 °C). The observed transformation network (Figure 3) comprises (i) hydrolytic degradation of CQA and diCQA to caffeic acid and quinic acid, and (ii) features tentatively assigned as methylation (caffeic acid → ferulic acid), acetylation (quinic acid → acetylquinic acid), and methylation, acetylation, and lactonization of diCQA (feruloyl-caffeoylquinic acid, acetyl-diCQA, and diCQA lactone). These assignments are based on accurate precursor masses and MS^2 product-ion patterns only. No methyl or acetyl donor was identified in the dry solid, and no authentic standards or NMR data were obtained for these products, so alternative origins (pre-existing ferulic acid and feruloylquinic acids released from the matrix) cannot be excluded (Section 5 and Conclusions). Heat exerted a dual effect in the present tests: below 160 °C, the assigned methylated/acetylated/lactonized features accumulated, whereas at 180 °C these

features themselves decreased. This pattern is consistent with, but does not by itself prove, sequential formation and degradation of these intermediates.

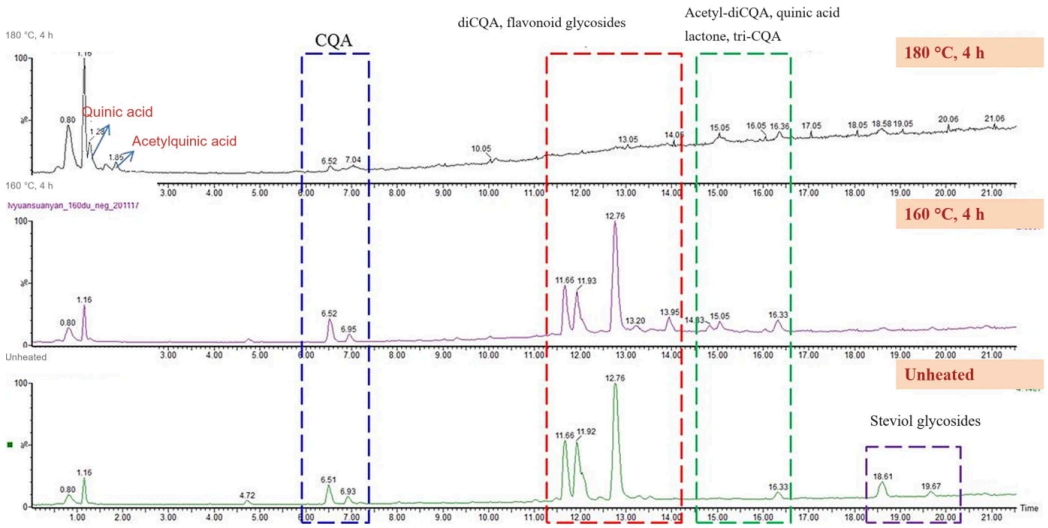


Figure 2. Total ion chromatograms (ESI⁻) of the stevia chlorogenic acid product before heating and after heating at 160 °C/4 h and 180 °C/4 h.

Table 1. Compounds identified by UPLC-QTOF MS/MS (ESI⁻) in the product before and after heat treatment; peak areas are relative (semiquantitative).

tR (min)	[M–H]⁻/Adduct	Compound (Assigned)	Formula	Original	160 °C/4 h	180 °C/4 h
1.28	191.0543	Quinic acid	$C_7H_{12}O_6$	7.37×10^4	2.58×10^5	1.30×10^6
1.65	233.0636	Acetylquinic acid	$C_9H_{14}O_7$	–	1.56×10^3	1.45×10^4
1.86	233.0636	Acetylquinic acid	$C_9H_{14}O_7$	–	1.16×10^4	9.40×10^4
4.76	353.0881	CQA	$C_{16}H_{18}O_9$	1.77×10^5	9.35×10^4	5.34×10^3
6.51	353.0881	CQA	$C_{16}H_{18}O_9$	5.57×10^5	4.74×10^5	2.91×10^4
6.93	353.0881	CQA	$C_{16}H_{18}O_9$	3.43×10^5	2.34×10^5	6.27×10^3
7.21	179.0339	Caffeic acid	$C_9H_8O_4$	5.05×10^4	2.06×10^3	–
11.66	515.1195	diCQA	$C_{25}H_{24}O_{12}$	2.77×10^6	1.76×10^6	4.63×10^3
11.92	515.1195	diCQA	$C_{25}H_{24}O_{12}$	2.16×10^6	1.14×10^6	2.86×10^3
12.76	515.1195	diCQA	$C_{25}H_{24}O_{12}$	3.39×10^6	3.04×10^6	1.26×10^4
13.42	193.0487	Ferulic acid	$C_{10}H_{10}O_4$	3.15×10^3	1.17×10^5	9.19×10^3
13.95	529.134	Feruloyl-caffeoylquinic acid	$C_{26}H_{26}O_{12}$	2.43×10^3	5.85×10^5	1.72×10^3
14.83	557.1277	Acetyl-dicaffeoylquinic acid	$C_{27}H_{26}O_{13}$	1.23×10^4	1.27×10^5	1.10×10^3
15.05	497.108	Dicaffeoylquinic acid lactone	$C_{25}H_{22}O_{11}$	–	9.88×10^4	–
16.33	677.1492	tri-CQA	$C_{34}H_{30}O_{15}$	1.78×10^5	1.48×10^5	–
18.61	641.3154	Stevioside	$C_{32}H_{50}O_{13}$	5.69×10^4	4.85×10^3	–
18.68	849.3749	Stevioside (formate adduct [M+HCOO]⁻)	$C_{39}H_{60}O_{18}$	1.12×10^5	7.82×10^3	–
18.99	1011.4252	Rebaudioside A (formate adduct [M+HCOO]⁻)	$C_{46}H_{74}O_{23}$	1.95×10^5	8.10×10^3	1.20×10^3
19.67	355.2108	Unidentified feature (tentative formula $C_{18}H_{30}O_4$)	$C_{18}H_{30}O_4$	6.22×10^4	3.84×10^4	3.45×10^3

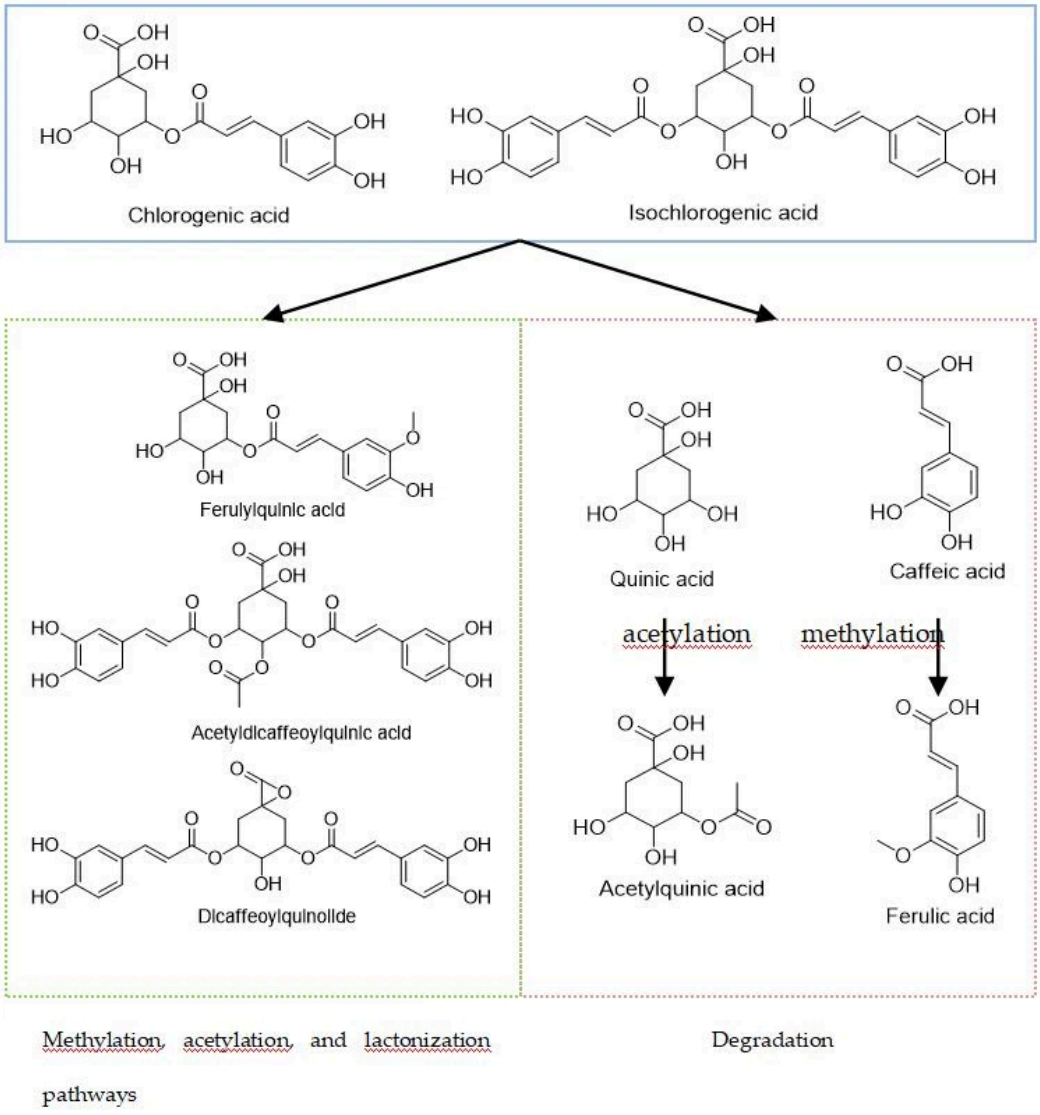


Figure 3. Proposed thermal transformation network of stevia chlorogenic acids, based on accurate-mass and MS^2 assignments (dashed steps are tentative and not confirmed with authentic standards or NMR). degradation (CQA/diCQA → caffeic acid + quinic acid), methylation (caffeic acid → ferulic acid), acetylation (quinic acid → acetylquinic acid), and diCQA methylation/acetylation/lactonization; dashed arrows denote temperature-dependent (dual-effect) steps.

2.3. Solution Thermal Stability: Near-Conserved Total Acids and Acyl-Migration-Driven Isomer Interconversion

In 80% aqueous methanol, heating at 60–80 °C for up to 8 h only slightly changed the total acid content (0.32–0.35 g/100 mL; −4.1% to +5.4% relative to the stock; Figure 4), and the sums of diCQAs and CQAs were conserved within ±7% (sheets E2 and E3 of the Supporting Information). Monomer-level changes were highly consistent across temperatures: 4,5-diCQA decreased (−12.8% to −16.6%), while 3,4- and 3,5-diCQA increased progressively (up to +35.4% and +16.3%, respectively); 5-CQA decreased (−3.0% to −5.8%), while 3-CQA and 4-CQA increased (up to +41.9% and +47.3%, respectively). The same overall pattern was obtained in 80% ethanol (Figure 5; 4,5-diCQA −8.9% to −14.3%, 3,4-diCQA up to +21.0%, 3-CQA up to +24.9%, 4-CQA up to +46.8%, 5-CQA down to −17.1%), showing that the interconversion is solvent-type-independent; the only deviation is 3,5-diCQA, which decreased slightly in ethanol (−4.0% to −6.3%) instead of in-

creasing. These results indicate the conversion of 4,5-diCQA to 3,4- and 3,5-diCQA and of 5-CQA to 3- and 4-CQA during thermal treatment, that is, isomer interconversion toward thermodynamically more stable structures (Figure 6). All values are means of three independent determinations (n = 3); the SD of the relative changes was ≤4.2% in methanol (≤2.9% excluding caffeic acid) and ≤1.2% in ethanol (sheets E2 and E3 of the Supporting Information).

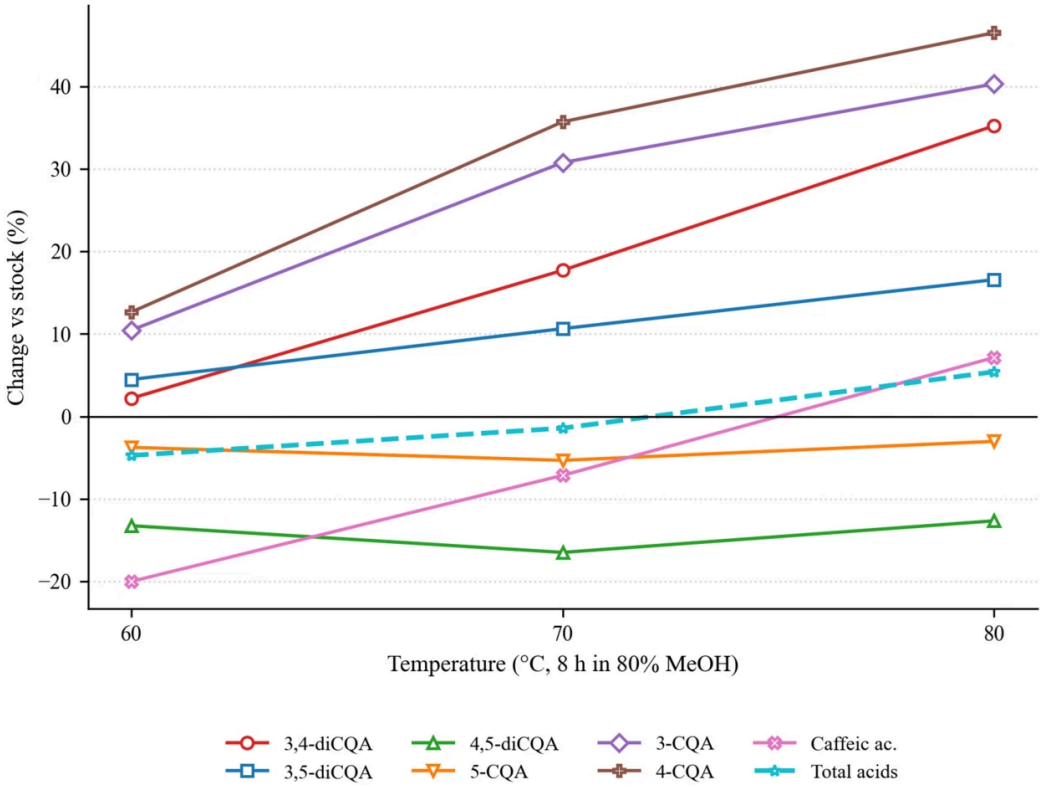


Figure 4. Change in monomer contents in 80% aqueous methanol (60–80 °C, 8 h) relative to stock.

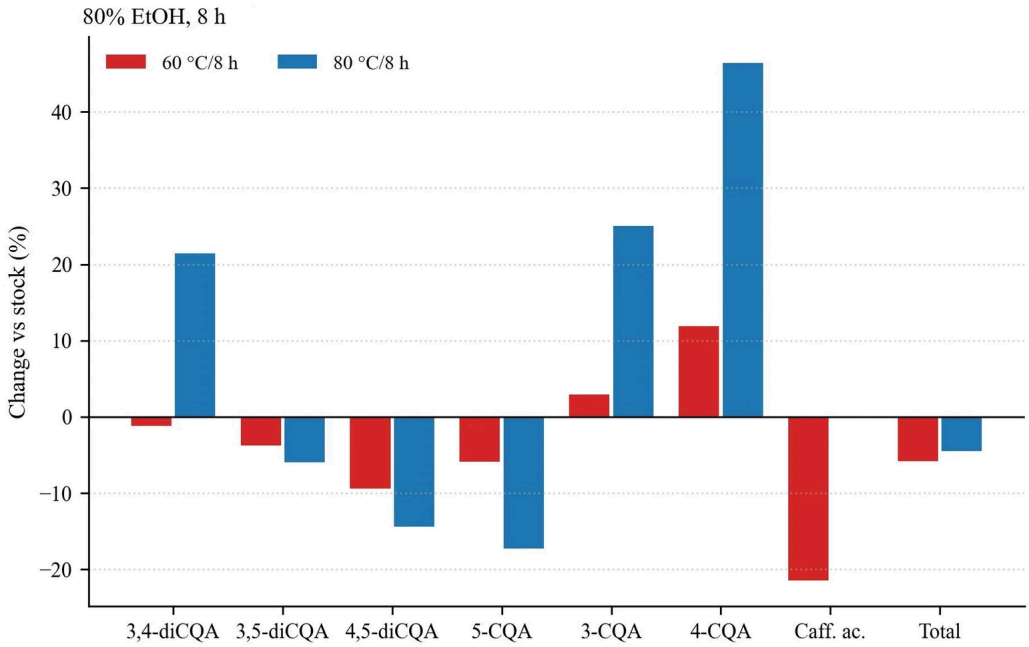


Figure 5. Change in monomer contents in 80% aqueous ethanol (60–80 °C, 8 h) relative to stock.

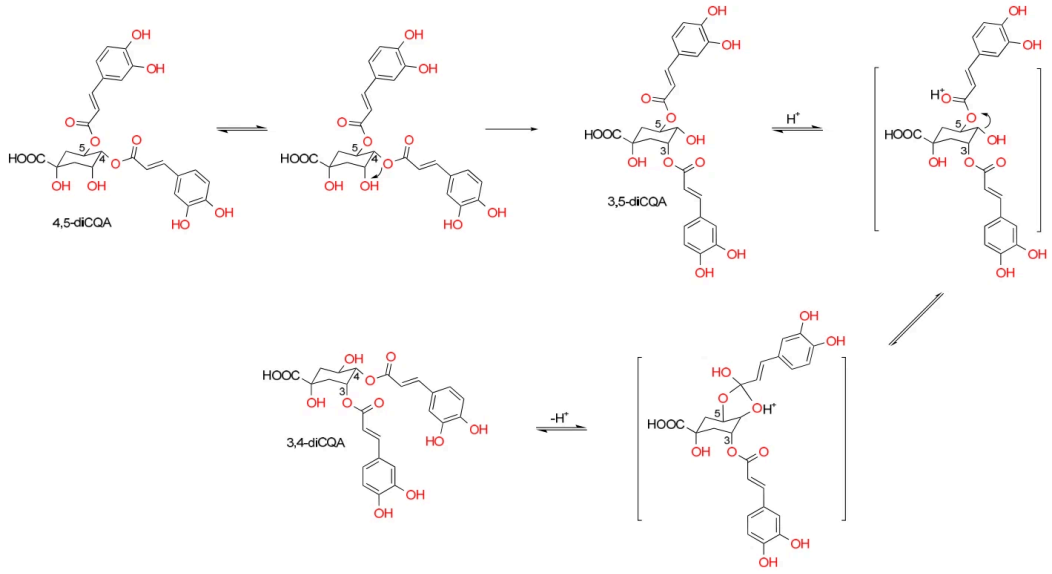


Figure 6. Monomer interconversion in 80% aqueous methanol at 60–80 °C: 4,5-diCQA → 3,4- and 3,5-diCQA.

2.4. Metal Ions: Complexation Without Direct Loss, but with Catalytic Acceleration of Light/Oxidative Loss

Addition of Fe^{3+} or Cu^{2+} lowered the measured contents of diCQAs and CQAs with increasing metal concentration. However, after EDTA-2Na decomplexation, the detected contents recovered to 97.89–99.90% of the untreated sample, with per-monomer recoveries of 96.79–108.18% relative to the EDTA-only control (Table S4 of the Supporting Information); the EDTA-only control itself recovered 99.90 ± 0.11% ($p < 0.001$), demonstrating that complexation itself did not destroy the active ingredients (Figure 7). After the addition of metal ions, the solutions darkened, trace insoluble particles adhered to the vial wall, and filtration markedly reduced absorbance; the maximum absorption wavelength remained near 325 nm throughout, and metal addition did not shift λmax (Table 2). The absorbance decrease in Table 2 (6.6–12%) should therefore be read as a turbidity/precipitation effect of the colloidal metal–chlorogenate complexes (Table 2 being a semi-quantitative comparison), consistent with the small (<2.1%) HPLC-determined losses, rather than a chemical loss; the light and oxidation tests below used a metal excess (ca. 2:1 metal:diCQA) and are not directly comparable with the 1:20–1:5 complexation ratios of Figure 7.

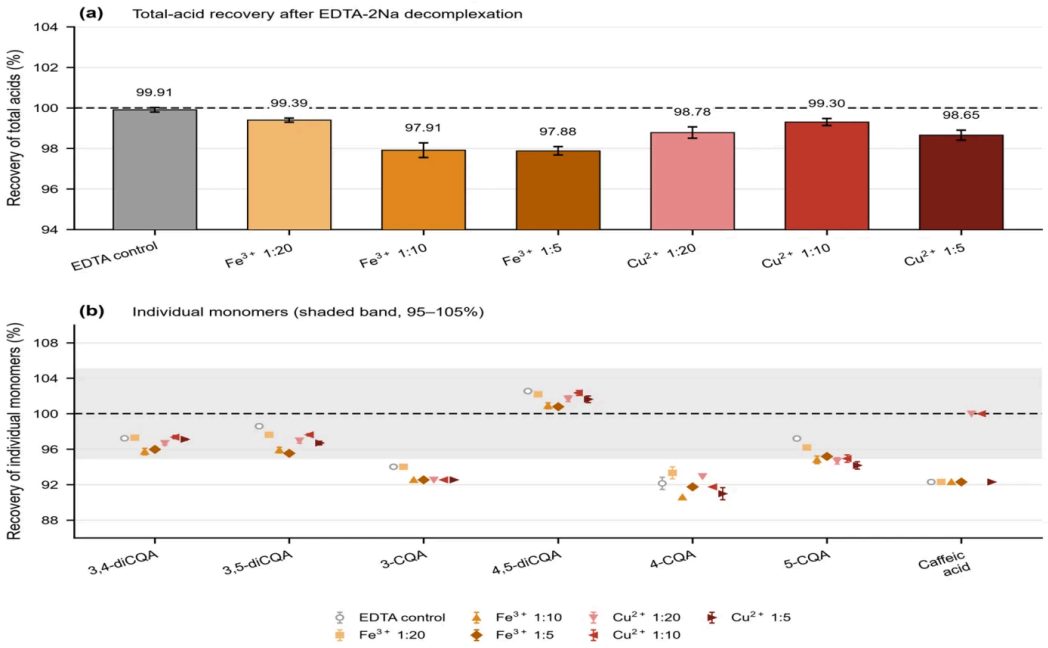


Figure 7. Reversibility of metal–chlorogenic acid complexation probed by EDTA-2Na decomplexation. (**a**) Recovery of total acids after decomplexation in the EDTA-only control and in the six metal treatments (Fe^{3+} or Cu^{2+} at metal:acid ratios of 1:20, 1:10 and 1:5), expressed relative to an untreated sample (dashed line, 100%); (**b**) recovery of the seven individual monomers (shaded band, 95–105%; a few individual monomer values lie slightly outside the band, see sheet Table S4 of the Supporting Information). Values are means ± SD (n = 3); total acids = sum of the seven quantified monomers.

Table 2. Full-wavelength absorption before and after filtration and metal-ion addition.

Sample	λmax (nm)	Abs(330 nm) Before Filtration	Abs After Filtration	Δ After Filtration (%)	Δ After Metal Addition (%)
No metal ions	325	2.38	2.41	+1.26	–
+Fe^{3+}	326	2.27	2.12	−6.61	−12.03
+Cu^{2+}	324	2.32	2.25	−3.02	−6.64

The complexes are not inert intermediates. Under light exposure for 20 d (values relative to the day-1 content of the same group), the dark control lost 28.21 ± 0.52% and the illuminated metal-free control 36.46 ± 0.39% of total acids, while the parallel metal-free run lost 40.38 ± 0.19%; addition of Fe^{3+} (48.59 ± 0.66%) and especially Cu^{2+} (67.63 ± 0.29%) accelerated the loss (Figure 8), with diCQA losses exceeding 70% in the Cu^{2+} group (3,4-: −71.81%; 4,5-: −77.62%). The acceleration was time-resolved and metal-specific: Cu^{2+} had already diverged from the parallel control by day 8 (−45.98% vs. −22.64%), whereas Fe^{3+} separated only at day 20 (−48.59% vs. −40.38%). Under oxidative conditions (15 d of air bubbling; values relative to day 0), the metal-free control lost 9.31 ± 0.26% of total acids (4,5-diCQA −16.11%; caffeic acid −16.75%); Fe^{3+} partly suppressed the loss (5.52 ± 0.22%), whereas Cu^{2+} tripled it (27.92 ± 0.15%; 4,5-diCQA −34.51%; caffeic acid −60.01%; Figure 9). Fe^{3+} thus shows a mild protective effect on oxidative loss, while Cu^{2+} is a strong pro-oxidant/catalyst; Cu^{2+} catalyzes both photo- and oxidative degradation more strongly than Fe^{3+}.

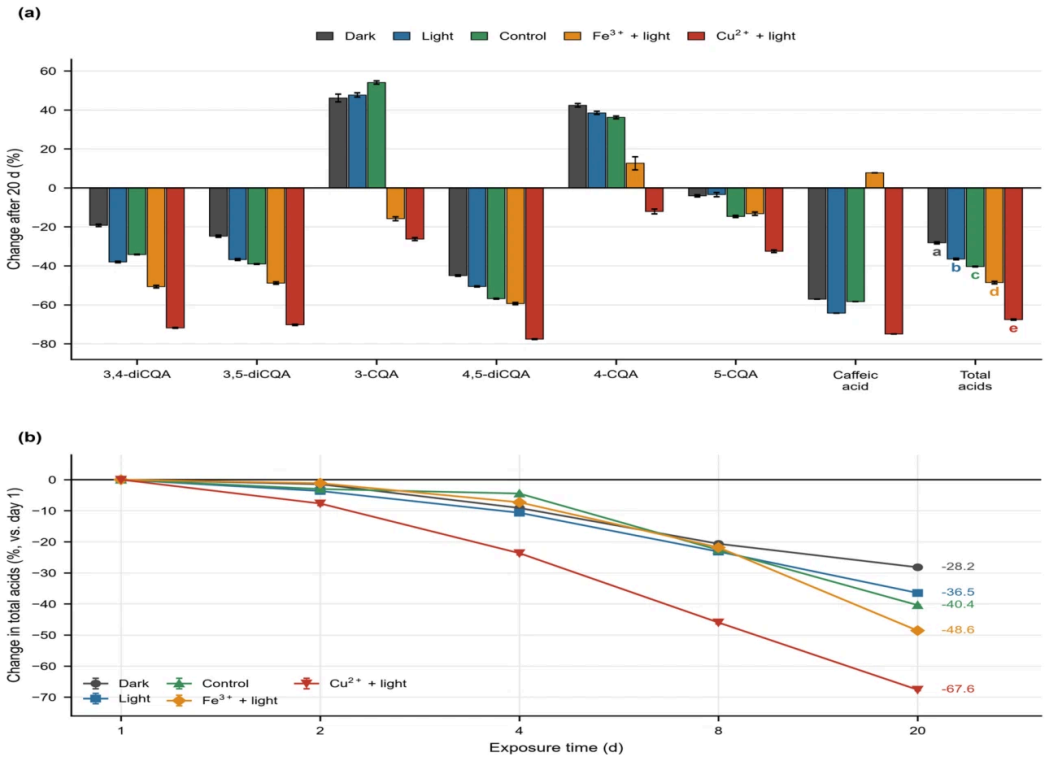


Figure 8. Light-induced degradation of the product solution (12,000 lx; days 1, 2, 4, 8 and 20) without metal ions (dark and light) and with Fe^{3+} or Cu^{2+} (10 mM metal, a ca. 2-fold molar excess over diCQA; not directly comparable with the 1:5 complexation ratios of Figure 7). (**a**) Change in the individual chlorogenic acids and in total acids after 20 d (different letters denote significant differences among the five groups; one-way ANOVA followed by Tukey HSD, $p < 0.001$); (**b**) time course of the total-acid change. Values are relative to the day-1 content of the same group (means ± SD, $n = 3$); the solid-state light-exposure data (25 d) are reported in Section 2.6.

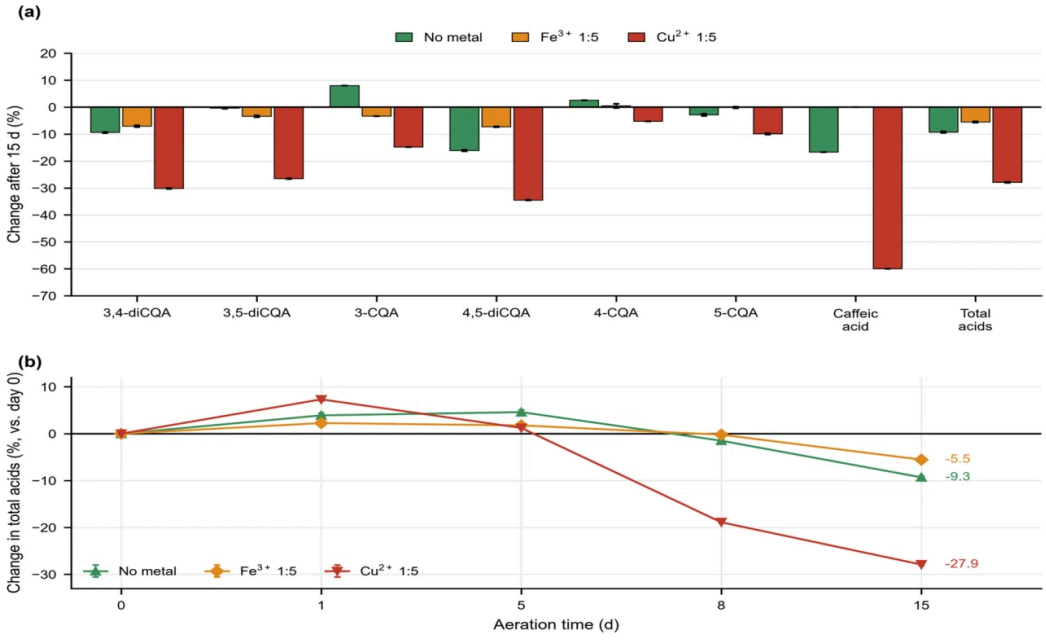


Figure 9. Oxidative degradation of the product solution (15 d of air bubbling) without metal ions and with Fe^{3+} or Cu^{2+} (10 mM metal, a ca. 2-fold molar excess over diCQA). (**a**) Change in the individual chlorogenic acids and in total acids after 15 d; (**b**) time course of the total-acid change. Values are relative to day 0 of the same group (means ± SD, $n = 3$).

2.5. pH Dependence of Hydrolysis and Interconversion

Relative to the original solution (80% aqueous methanol), the total acid content of the unheated samples differed significantly across pH ($p < 0.001$): it was lowest at pH 3.0 and 4.0 (losses of 4.67% ± 0.10% and 6.18% ± 0.10%) and highest at pH 5.0 and 8.0 (+2.58% ± 0.37% and +4.22% ± 0.18%; $p \leq 0.016$). Because this offset existed before heating and shifted nearly all components in the same direction (−8.4% to +7.7%), it is attributed to buffer-related effects on the chromatographic determination (matrix effects, possibly together with partial precipitation at pH 3.0–4.0) rather than to a real loss or formation of acids—isomerization cannot create acid, so the apparent gains at pH 5.0–8.0 are analytical offsets of the same origin. After heating at 80 °C for 2 h, the total acid change relative to the matched unheated sample was statistically insignificant at pH 3.0, 4.0, 6.0 and 8.0 (−0.25% to −0.52%, $p \geq 0.076$) and −0.84% at pH 7.0 ($p = 0.0085$); the only clear loss occurred at pH 5.0 (−3.77% ± 0.25%, $p < 0.001$), where all major components declined in parallel, indicating a non-selective loss process rather than interconversion (Figure 10). In the pH 6.0–8.0 range, heating promoted the conversion of 4,5-diCQA (−2.0% to −3.6%) toward 3,5-diCQA (+2.4% to +3.7%) and toward the monoesters, preferentially 4-CQA (+2.7% to +5.0%), while 3,4-diCQA, 3-CQA and 5-CQA remained essentially unchanged: the strong interconversion buffer kept the total acid content almost constant.

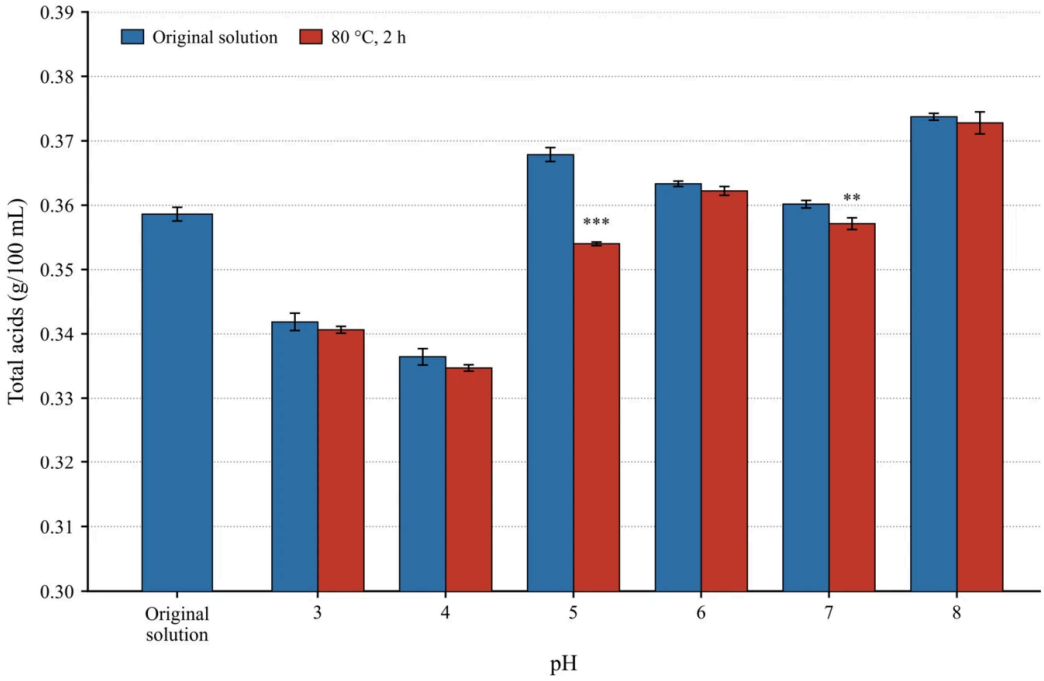


Figure 10. Total acid content (g/100 mL) of the product in 80% aqueous methanol at pH 3.0–8.0 before (blue) and after heating at 80 °C for 2 h (red). Values are means ± SD of three independent determinations ($n = 3$); asterisks indicate significant differences between heated and unheated samples at the same pH (*t*-test, ** $p < 0.01$, *** $p < 0.001$).

2.6. Solvent and Process-Liquid Behavior

Solid-phase light exposure for 25 days caused only 3.76% total acid loss (3,4-diCQA −5.95%; 3,5-diCQA −3.63%; 4,5-diCQA −3.89%; 4-CQA −4.33%; 3-/5-CQA < 1.0%), indicating good light stability of the solid product, with strong direct light to be avoided during storage.

For process liquids, refluxing the pre-concentration liquid at 55 °C/8 h changed the total acid content by only −0.18% ± 0.19% (mean ± SD, $n = 3$; t-test $p = 0.60$) and at 80 °C/10 h by −1.10% ± 0.27% ($p = 0.017$), whereas the post-concentration liquid lost 2.65% ±

0.33% and 11.09% ± 0.07% under the same conditions (both $p < 0.001$) (Figure 11). The concentrated stream was therefore about one order of magnitude more heat-labile than the dilute stream, and its losses were dominated by the diCQAs (ΣdiCQA −2.7% at 55 °C and −11.3% at 80 °C, against ΣCQA −0.1% and −8.3%); in the dilute liquid the diCQAs were almost unchanged and the mono-CQAs were slightly enriched at 55 °C (ΣCQA +2.9%) but decreased at 80 °C (ΣdiCQA −1.1%, ΣCQA −1.7%). Among individual components, 3,4-diCQA was the most labile in the concentrated liquid at 80 °C (−15.5%), followed by 3,5-diCQA (−12.1%) and 4,5-diCQA (−10.2%), whereas 3-CQA was the most resistant (−3.1%). HPLC comparison revealed a peak near 15 min in the process liquid—tentatively assigned, from accurate mass and retention behaviour, to methylated/ethylated chlorogenic acid derivatives—that was absent both before and after heating of the purified product in methanol or ethanol; its identification requires confirmation with authentic standards, and the ethyl acetate/ethanol-containing process matrix is the most plausible source of the alkyl donor. Taken together, these results indicate that the process matrix promotes alkylation of chlorogenic acids at elevated temperature, and that the concentrated stream is markedly more susceptible to thermal loss than either the dilute stream or the purified isochlorogenic acid product. All values are means of three independent determinations ($n = 3$); the SD of the relative changes was ≤0.54% (Table S8 of the Supporting Information).

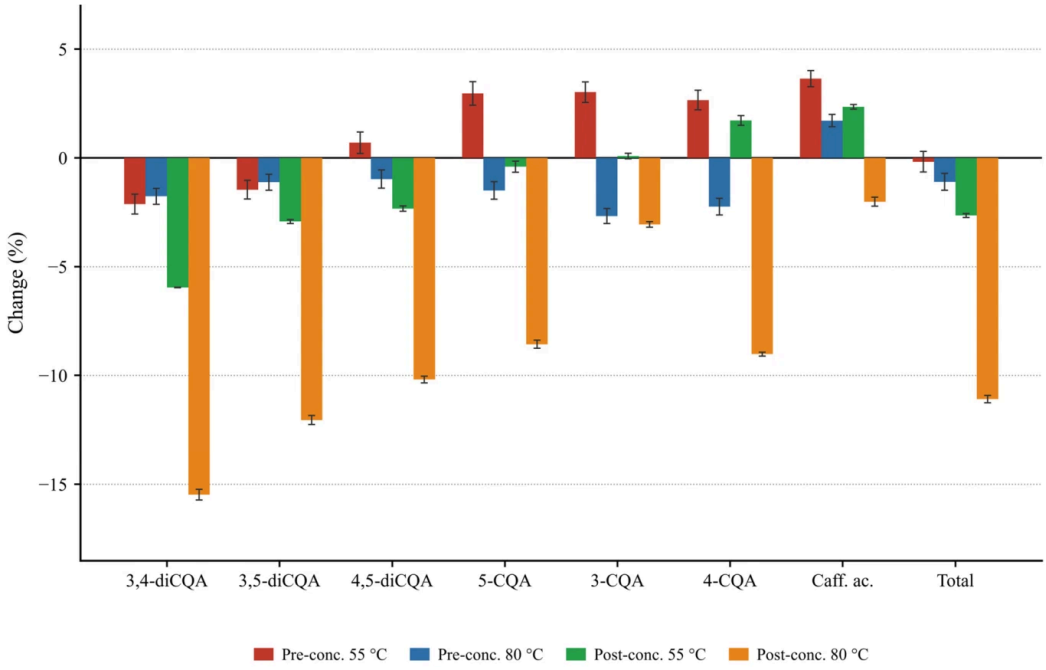


Figure 11. Thermal stability of the process liquids before and after concentration. Relative changes (%) of the individual chlorogenic acids and of the total acids after refluxing at 55 °C for 8 h and at 80 °C for 10 h, each referred to the untreated liquid of the same stage. Values are means ± SD of three independent determinations ($n = 3$); total acid changes differed significantly from the untreated control at both temperatures in the concentrated liquid ($p < 0.001$) and at 80 °C in the dilute liquid ($p = 0.017$), but not at 55 °C in the dilute liquid ($p = 0.60$; *t*-test).

3. Discussion

3.1. The Solid-State Transformation Network and Its Temperature Dependence

The solid-state results define a practically exploitable processing window: total acid loss <10% at ≤140 °C/4 h, but ~45% loss at 160 °C/4 h. The LC–MS data show that the losses arise not only from hydrolytic degradation to caffeic and quinic acids [15,19,20]

but also from thermally driven covalent modifications—methylation, acetylation, and lactonization—that have a pronounced temperature dependence. The dual (promoting–degrading) temperature effect below 160 °C versus above 180 °C has, to our knowledge, not been reported quantitatively for commercial stevia-derived chlorogenic acid products under dry-heat processing conditions, although related behaviour is described for coffee roasting and for purified chlorogenic acids [27–30]. Recent reviews of caffeoylquinic acid chemistry and of processing effects position this behavior within a general structural lability—the higher the degree of caffeoylation, the more readily the acyl ester chain degrades thermally or photochemically—and attribute the bulk of CQA loss during thermal processing to lactonization, polymerization, and hydrolysis [27–29]. Under milder conditions, the process is real but slow, with CGA half-lives of ca. 4–5 years estimated for green coffee during long-term storage [30]. Methylated and acetylated derivatives (ferulic acid, acetylquinic acid, feruloyl-caffeoylquinic acid, acetyl-diCQA) and the diCQA lactone behave as metastable pools: they accumulate at moderate temperature and are themselves consumed at higher temperature. The transient rise of 5-CQA reflects acyl migration within the mono-CQA pool (Section 2.1), and direct conversion of diCQAs into mono-CQAs is minor (≤2.5% of the diCQA loss at 160 °C); the higher loss rates of the diCQA pool therefore reflect the lower stability of its two ester bonds, echoing the mono/diCQA interconversion reported under aqueous and roasting conditions [22].

3.2. Solution Behavior: Acyl-Migration-Driven Structural Stabilization

In solution, total acid and class sums (ΣdiCQA, ΣCQA) varied by less than ±7% of the stock even at 80 °C (sheets E2 and E3 of the Supporting Information); however, positional isomers re-equilibrate. The observed conversions—4,5-diCQA → 3,4-/3,5-diCQA and 5-CQA → 3-/4-CQA—are fully consistent with the intramolecular acyl-migration mechanism (via hydrated intermediates) established for caffeoylquinic acids under neutral–weakly basic aqueous conditions [22,28,31,32]. The net direction of conversion is consistent with the relative stability of the positional isomers reported for acyl migration under aqueous and roasting conditions [22,28,31,32], in which the 4,5-diCQA and 5-CQA forms are the less stable isomers and the 3,4-/3,5-diCQA and 3-/4-CQA forms accumulate upon heating. The only deviation is 3,5-diCQA in ethanol, which decreased slightly (−4.0% to −6.3%) instead of accumulating, i.e., the net direction of its change is solvent-dependent. No thermodynamic quantity (equilibrium constant or computed Gibbs energy) was measured in this study, so this ranking is presented as an interpretation consistent with the published acyl-migration behaviour rather than as an experimentally determined order. This "unstable-to-stable" interconversion is an internal quality-preserving buffer: it minimizes total acid loss during mild processing and storage, consistent with the near-conserved (within ±7%) total acid in Figures 4 and 5; it likewise agrees with the degradation kinetics quantified for isochlorogenic acid A in aqueous solution [33] and with the preparative conversion of chlorogenic acid into its more stable isomers during processing [34].

3.3. Metal Ions: Complexation, Catalysis, and the Fe^{3+}/Cu^{2+} Asymmetry

The immediate content drop upon metal-ion addition is a detectability artifact of insoluble complex formation (recovered by EDTA), not an actual loss—consistent with the well-established catecholate/gallate chelation chemistry of hydroxycinnamic acids, whereby the caffeic-acid catechol coordinates Fe^{3+} and Cu^{2+} into poorly soluble adducts that are dissociated by strong chelators such as EDTA [27,35]. The long-term danger, however, is catalytic: complex intermediates accelerate photodegradation and oxidative

loss; given the very low UV fraction of the light source (Section 4.6), this photodegradation is attributable mainly to the visible fraction of the irradiation. Caffeic acid's catechol and its phenolate anion coordinate Cu^{2+}, and the resulting complex can undergo electron transfer to generate o-quinone and superoxide radical anions, which inflict oxidative damage and lower the energy barrier for active-component reactions [36]. This mechanism explains why Cu^{2+} exceeds Fe^{3+} in accelerating photo- and oxidative loss: Cu^{2+} cycles readily between Cu^{2+} and Cu^{+}, generating reactive oxygen species, whereas Fe^{3+} binds the catechol moiety strongly but is largely redox-inactive under these conditions and can act as a sink for oxidizable centers [35,36]. The higher catalytic efficiency of Cu^{2+} is an empirical observation of this study and cannot be rationalized by the Irving–Williams series, which applies only to divalent ions; direct verification (stability constants, spin-trapping EPR or ROS probes, and pH-controlled experiments) is left for future work. In practical terms, avoid introducing metal ions (processing vessels, water, packaging) and avoid combining acidic conditions (pH < 4) with metals, since hydrolytic and metal-catalyzed routes then superimpose (Sections 2.4 and 2.5).

3.4. Integrated Stability Map and Industrial Recommendations

Combining all factors, the influence ranking for solid product loss is dominated by temperature (≈160 °C onset), followed by prolonged strong-light exposure (which nevertheless caused only ~3.8% total acid loss in the solid over 25 days; Section 2.6); for liquid product, the loss drivers in increasing order are pH, oxidation, light, storage time, and metal ions. Recommended and testable operating rules: (1) solid handling at ≤140 °C with minimal time (extrusion ≤ 160 °C as an upper bound for short processes); (2) avoid metal-ion introduction at every stage; (3) sealed, opaque, cool, and dry storage (as supported by packaging-dependent CQA retention in stored roasted coffee and by the slow, multi-year CGA degradation estimated for stored green coffee [30,37]); (4) use formulated aqueous/alcoholic solutions promptly, avoid open storage and strong direct light; (5) for concentrated process streams, minimize thermal hold time and control conditions that promote alkylation.

3.5. Limitations

Results are now reported as the mean ± SD of three independent determinations per condition (n = 3), and the study remains descriptive in scope. Several design limitations should be kept in mind when interpreting the data. First, the solid heat treatments used different holding times at low (24 h) and high (4 h) temperature, so temperature and time effects are confounded and no kinetic parameters (rate constants, half-lives, activation energies) are reported. Second, LC–MS components were quantified as relative peak areas (semi-quantitative) and the methylated, acetylated, and lactonized products were assigned from accurate masses and MS^2 product-ion patterns only, without authentic standards or NMR confirmation, so they remain tentative. Third, the light and oxidation tests used a suprastoichiometric metal loading (about a 2:1 metal:diCQA molar ratio) and the metal-ion solutions were not pH-adjusted, so the two metal-related experiments are not directly comparable. Fourth, the solution tests were performed in aqueous organic solvents rather than food-grade media; LC–MS components were quantified as relative peak areas (semi-quantitative), whereas total and monomer acid contents (%) were obtained by the group's standard HPLC-UV protocol. The regiochemistry of the methylated, acetylated, and lactonized products was assigned from the exact masses and MS^2 product-ion patterns, according to published rules [23]; however, no authentic standards, NMR data, or isotope-labelling experiments were available, so these assignments and the underlying reaction mechanisms remain tentative. Metal-complex stoichiometry

and stability constants were not determined, and no thermodynamic measurements were made. Fifth, the solid heat treatments were performed in a vented electric oven under ambient air, and the oxygen level of the heating atmosphere was not controlled or monitored. The replicates are independent preparations of a single production batch; batch-to-batch variation was not replicated.

4. Materials and Methods

4.1. Materials and Reagents

Stevia chlorogenic acid product (batch no. 2-0969-200613; total acid content 61.39%; diCQA content 53.79%) was supplied by Chenguang Biotech Group Co., Ltd. (Handan, China). Reference standards of 3,5-diCQA, 4,5-diCQA, 3,4-diCQA, 3-CQA, 4-CQA, 5-CQA, and caffeic acid (purity > 95%) were obtained from Chengdu Mansite Biotechnology Co., Ltd. (Chengdu, China). Acetonitrile, formic acid, and trifluoroacetic acid were of chromatographic grade. Methanol and ethanol were of analytical grade. $FeCl_3$ and $CuSO_4$ (analytical grade) were dissolved in ultrapure water to prepare 1 mol/L stock solutions of each. EDTA-2Na (analytical grade) was used at a concentration of 1 mol/L for decomplexation. Citric acid (0.1 mol/L) and disodium hydrogen phosphate (0.2 mol/L) were used to prepare pH-adjusting buffer solutions. For every condition three independent replicate preparations were made from the same production batch; each replicate was separately weighed, separately carried through the complete procedure of that experiment and injected once. The three values therefore represent independent replicate preparations, not repeated injections of a single extract.

4.2. Instrumentation

Electric blast drying oven (101-1AB, Tianjin Taiste Instrument Co., Ltd. Tianjin, China); artificial climate chamber (Shanghai Yiheng Scientific Instrument Co., Ltd. Shanghai, China); peristaltic pump; thermostatic water bath; analytical balance (AUW220, Shimadzu, Kyoto, Japan); pH meter (Shanghai Sanxin Instrument Factory, Shanghai, China); peristaltic pump (LongerPump BT 100-1L, Baoding, China); lux meter (calibrated TES-1332A, Taishi Electronic Industry Co., Ltd., Taipei City, China); UVA-365 ultraviolet radiometer (UVA-365, Taiwan Lu Chang Electronics Enterprise Co., Ltd., Taipei City, China); HPLC system (e2695, Waters, Milford, MA,); and UPLC-QTOF MS/MS system (Xevo G2-XS, Waters, Milford, MA,) equipped with a Waters BEH C18 column (2.1 mm × 100 mm, 1.7 μm).

4.3. Solid-State Heat Treatment and Content-Loss Calculation

Chlorogenic acid samples (60 g/tray) were spread evenly on stainless-steel trays (32 × 22 cm; bed depth ≤ 4 mm; exposed bed area 380–400 cm^2) and heated in the oven at 80, 100, and 120 °C for 24 h, and at 140, 160, and 180 °C for 4 h. Each condition was performed in three independent replicates (n = 3). After each treatment, the content was determined by HPLC (Section 4.10). The content loss rate was calculated as follows:

$$\text{Loss (\%)} = (A_1 - A_2)/A_1 \times 100 \quad (1)$$

where A_1 is the total acid content of the untreated original sample, and A_2 is that of the treated sample.

4.4. Solution Heat Treatment

Product (0.5164 g) was dissolved in 100 mL of 80% methanol and heated at 60, 70, and 80 °C for 8 h (three independent replicates, n = 3). The 80% ethanol solution was heated at 60 and 80 °C for 8 h for comparison. After heating, the solutions were cooled

and weight-replenished with the corresponding solvent before HPLC analysis.

4.5. Metal-Ion Treatment and EDTA Decomplexation

0.5164 g of product was dissolved in 80% methanol to 100 mL. $CuSO_4$ or $FeCl_3$ stock solution was added slowly to reach metal-ion-to-diCQA molar ratios of 1:20, 1:10, and 1:5, and samples were left standing for 24 h. To distinguish complexation-induced detectability loss from genuine degradation, an equimolar amount of 1 mol/L EDTA-2Na was added to the 1:5 samples to decomplex; after mixing, the solutions were allowed to stand for 24 h and re-analyzed. In addition, solutions before and after metal-ion addition were filtered through a 0.45 μm membrane and subjected to full-wavelength scanning (300–400 nm) to examine λmax shifts.

4.6. Light-Exposure Tests

Solid: The product was spread on stainless-steel trays (bed depth ≤ 4 mm); after 1, 2, 4, 8, and 25 days of exposure in the climate chamber (37 °C, 35% RH, 12,000 lx), samples from four corners and the center were mixed for analysis (three independent replicates, n = 3). The light source was a cold white fluorescent (6500 K) lamp; light intensity was verified at 12,000 lx with a calibrated TES-1332A lux meter, and the spectral distribution and UV contribution of the source were characterized: 320–400 nm UVA radiation, measured with a UVA-365 ultraviolet radiometer, accounted for approximately 1–2% of the total irradiance.

Solution: 0.5164 g product/100 mL (80% methanol) was sealed; the dark control was wrapped in aluminum foil; both groups were placed in the same climate chamber under the same conditions (37 °C, 35% RH, 12,000 lx) and sampled at 1, 2, 4, 8, and 20 days, with weight replenishment before each sampling. Parallel solution groups containing 1 mL of 1 mol/L $CuSO_4$ or $FeCl_3$ (per 100 mL) were run under the same light conditions.

4.7. Oxidative-Loss Tests

Solutions (as in Section 4.6, ± metal ions) were sealed and protected from light; air was bubbled through at a fixed rate of 1.7 mL/min using a peristaltic pump. Samples were taken at 1, 5, 10, and 15 days with weight replenishment; a metal-ion-free solution under identical conditions served as the control.

4.8. pH-Stability Tests

Citric acid (0.1 mol/L) and disodium hydrogen phosphate (0.2 mol/L) stock solutions were added to methanol to adjust the pH to 3.0, 4.0, 5.0, 6.0, 7.0, and 8.0. The product (0.5164 g) was brought to 100 mL with each pH-adjusted solution; each was divided into two portions, one left untreated and the other heated at 80 °C for 2 h, then analyzed.

4.9. Process-Liquid Tests

The stevia isochlorogenic acid process liquid was sampled at two stages of the industrial workflow: the ethyl acetate extract before concentration (total acids ca. 1.45% (g/100 g); solids content ca. 2%, w/w) and the same stream after concentration (total acids ca. 19.0% (g/100 g); solids content ca. 30%, w/w), i.e., a ca. 13-fold concentration of the target acids. Each liquid was refluxed at 55 °C for 8 h and at 80 °C for 10 h, cooled to room temperature, and weight-replenished with ethyl acetate to its original gravimetric weight. Because the reflux temperatures approach the boiling point of ethyl acetate (77 °C), evaporative solvent loss would otherwise concentrate the analytes and inflate the apparent contents; the gravimetric correction restores the initial solvent mass, so that each heated sample is compared with an untreated aliquot of the same liquid on an

identical mass basis. Each condition was performed in three independent replicates (n = 3) and analysed by HPLC as described in Section 4.10.

4.10. HPLC Quantification and UPLC-QTOF MS/MS Identification

The HPLC method was validated for linearity, accuracy, precision, and detection capability: the intra-day precision was 0.07% RSD for peak areas and 0.11% RSD for retention times (six replicate injections), the inter-day precision was 0.67–1.25% RSD (three batches analysed in duplicate on six days, ≥24 h apart). Samples with absorbance above the detector linear range were diluted before measurement. HPLC conditions (330 nm): column temperature, 40 °C; mobile phase A, 0.1% trifluoroacetic acid in water; mobile phase B, acetonitrile; gradient, 0–2 min 95% A, 2–10 min 95–80% A, 10–20 min 80–65% A, 20–20.5 min 65–50% A, 20.5–45 min 50–0% A, 45–48.5 min 0% A, 48.5–49.5 min 0–65% A, 49.5–50 min 65–95% A, 50–55 min 95% A; flow rate, 0.2 mL/min (LC–MS) or 1.5 mL/min (HPLC-UV).

The UPLC-QTOF MS/MS conditions (ESI^-) were as follows: scan range, 50–2000 Da; collision gas, argon; collision energy, 20–40 V; capillary voltage, 2.5 kV; cone voltage, 40 V; source temperature, 120 °C; desolvation gas, nitrogen at 300 °C and 700 L/h; cone gas, 50 L/h. Compounds were assigned based on the exact $[M-H]^-$/adduct masses and MS^2 fragment patterns, following the $LC–MS^n$ rules of Clifford et al. [23].

4.11. Statistical Analysis

All stability experiments were performed in three independent replicates (n = 3) under the group's standardized protocols. Results are reported as mean ± SD. Differences among treatments were evaluated by one-way analysis of variance (ANOVA) followed by Tukey's honestly significant difference (HSD) post hoc test, with $p < 0.05$ considered statistically significant; pairwise comparisons between heated and unheated samples at the same pH (Section 2.5) and between stage-matched process liquids (Section 2.6) used two-sided Student's t-tests. Error bars in Figures 1, 4, 5 and 7–11 represent the SD of three independent determinations. All contents are reported to four decimals together with the relative standard deviation (RSD), because SD values below 0.0005 were previously rounded to 0.000 and could be misread as zero dispersion. For each analyte and each comparison set, a one-way analysis of variance was performed on the three replicate values, followed by Tukey's honestly significant difference test at $\alpha = 0.05$ on the pooled within-group mean square ($df = N - k$); conditions sharing a letter in the compact letter display do not differ significantly. All F, df and exact p values and all letter groupings are given in the Supporting Information (Tables S1–S9).

5. Conclusions

In the solid state, the stevia chlorogenic acid product showed low total acid loss at ≤140 °C (≤9.2%) but severe loss at 160–180 °C (4 h; 44.9% and 92.9%), carried almost entirely by the diCQA pool (−52.3% at 160 °C while the mono-CQA pool remained 4.6% above its initial level). Tentatively assigned methylated, acetylated, and lactonized derivatives accumulated at intermediate temperatures and require confirmation with standards or NMR. In solution at 60–80 °C, total acid content varied by less than ±7%, with acyl migration of 4,5-diCQA and 5-CQA. Metal ions did not destroy the actives directly (EDTA recovery 97.89–99.90%, n = 3) but accelerated light- and oxidation-induced loss, most strongly with Cu^{2+} (67.6% under light vs. 36.5% without metal). pH 3.0–4.0 deficits were buffer-related analytical effects; only pH 5.0 lost significantly (−3.77%); pH 6.0–8.0 interconversion buffered total acids. The solid was light-stable (3.76%/25 d), whereas the concentrated process liquid lost 11.1% (80 °C/10 h)—an order of magnitude more than

the dilute stream (−1.1%)—so thermal load after concentration should be minimised. Subject to the limitations in Section 3.5 (confounded temperature–time design, tentative assignments, suprastoichiometric metal loading, unmonitored pH, non-food-grade media), these results support practical guidance: ≤140 °C solid handling for ≤4 h and ≤80 °C, metal-ion-free, sealed, opaque liquids.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/doi/s1.

Author Contributions: Conceptualization, X.Y.; methodology, M.-L.X.; software, F.L.; validation, R.T.; investigation, F.L.; writing—original draft preparation, M.-L.X.; writing—review and editing, M.-L.X.; supervision, L.H.; project administration, L.H.; funding acquisition, H.L. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Development of Key Technologies for the Preparation and Industrial Application of High-Quality Isochlorogenic Acid (grant number 254Z2805G).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are contained within the article and its supplementary material. Raw chromatographic records (HPLC chromatograms and UPLC-QTOF MS/MS data files), replicate-level measurements for all conditions, and the method-validation data are available in the Supplementary Materials and from the corresponding author upon reasonable request.

Acknowledgments: During the preparation of this manuscript, the authors used the open-source AI coding assistant opencode (version 1.15.12, npm package opencode-ai; https://opencode.ai), powered by a large language model (model ID: opencode/big-pickle). The tool was used solely for revising the paper and checking the content. All scientific opinions, study design, data analysis, and conclusions are the sole responsibility of the authors, who independently verified and revised all generated content.

Conflicts of Interest: Authors Mei-li Xu, Lijuan Hou, Fuyong Lu, and Rumeng Tang were employed by Chenguang Biological Technology Group Co., Ltd. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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