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Multifactorial stress modulation of secondary metabolism in Theobroma cacao and Coffea arabica: from growth chamber to field and human bioavailability

Modulación multifactorial del estrés sobre el metabolismo secundario en Theobroma cacao y Café arábico: del invernadero al campo y la biodisponibilidad humana

Byron Oviedo-Bayas

Abstract: Theobroma cacao and Coffea arabica accumulate secondary metabolites (SMs) that determine crop resilience, beverage quality, and consumer health outcomes. A Box-Behnken response-surface design applied four simultaneous stress factors soil water status (30–70% field capacity), atmospheric CO_2 (400–800 µmol mol^{-1}), UV-B irradiance (0–4 kJ m^{-2} d^{-1}), and foliar methyl jasmonate (0–200 µM) to seedlings of both species for 21 days. The optimal combination (55% field capacity, 600 µmol mol^{-1} CO_2, 2 kJ m^{-2} d^{-1} UV-B, 100 µM methyl jasmonate) raised total polyphenols by 38% in cacao (42.3 to 58.4 mg GAE g^{-1} DW) and 44% in coffee (68.1 to 98.1 mg GAE g^{-1} DW). Chlorogenic acid gains in coffee reached 52%; procyanidin gains in cacao, 31%. Methylxanthines were unaffected (<12% variation). Field validation at two Ecuadorian sites (250 m and 1,100 m a.s.l.) recovered 68–72% of laboratory increments. A 24-participant randomized cross-over pilot demonstrated significantly higher plasma Cmax (0.82 vs. 0.54 µM; P = 0.004) and AUC0–24 (8.4 vs. 5.7 µM·h; P = 0.009) for chlorogenic acid catabolites after stress-enhanced coffee versus control. These results provide a translational framework for agronomic protocols that enrich bioactive SM profiles in cacao and coffee.

Keywords: abiotic stress; bioavailability; Coffea arabica; polyphenols; Theobroma cacao

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Resumen

Theobroma cacao y Café arábico acumulan metabolitos secundarios (MS) que determinan la resiliencia del cultivo, la calidad de la bebida y la salud del consumidor. Se aplicó un diseño de superficie de respuesta Box-Behnken con cuatro factores de estrés simultáneos contenido hídrico del suelo (30–70% de la capacidad de campo, CC), CO_2 atmosférico (400–800 µmol mol^{-1}), irradiancia UV-B (0–4 kJ m^{-2} d^{-1}) y metil jasmonato foliar (0–200 µM) a plántulas de ambas especies durante 21 días. La combinación óptima (55% CC, 600 µmol mol^{-1} CO_2, 2 kJ m^{-2} d^{-1} UV-B, 100 µM MeJA) incrementó los polifenoles totales en un 38% en cacao (42.3 a 58.4 mg EAG g^{-1} peso seco) y un 44% en café (68.1 a 98.1 mg EAG g^{-1} peso seco). Las ganancias de ácido clorogénico en café alcanzaron el 52% y las de procianidinas en cacao el 31%. Las metilxantinas no resultaron afectadas (<12% de variación). La validación de campo en dos sitios ecuatorianos (250 m y 1.100 m s.n.m.) recuperó entre el 68 y el 72% de los incrementos obtenidos en laboratorio. Un ensayo cruzado aleatorizado doble ciego con 24 participantes demostró Cmax plasmático (0.82 vs. 0.54 µM; P = 0.004) y AUC0–24 (8.4 vs. 5.7 µM·h; P = 0.009) significativamente superiores para catabolitos del ácido clorogénico tras la ingesta de café enriquecido por estrés frente al control. Estos hallazgos proporcionan un marco traslacional para protocolos agronómicos que enriquezcan los perfiles de MS bioactivos en cacao y café.

Palabras clave: biodisponibilidad; Café arábico; estrés abiótico; polifenoles; Theobroma cacao

Introduction

Theobroma cacao L. and Coffea arabica L. represent two of the most economically significant perennial tropical crops, collectively sustaining the livelihoods of approximately 14 million farmers worldwide and generating combined global market values exceeding US$1.5 trillion annually. Their commercial and nutritional value is directly tied to their secondary metabolite (SM) complement: in cacao, theobromine, procyanidins, and epicatechin oligomers serve dual roles as pathogen-defense compounds and as sources of cardioprotective and neuroprotective activities recognized in human consumers (Lahive et al., 2019). In coffee, chlorogenic acids chiefly 5-caffeoylquinic acid (5-CQA) drive cup quality and underpin documented associations with lower all-cause mortality and reduced incidence of type 2 diabetes and hepatic steatosis (Farah & Donangelo, 2006; Poole et al., 2017). Underpinning SM accumulation in both species is a phenylpropanoid backbone whose biosynthetic rate responds measurably to environmental perturbation, a characteristic well-documented in

medicinal plants (Pant et al., 2021; Isah, 2019) yet rarely examined at the multifactorial systems level in perennial tropical crops.

The theoretical basis for stress-driven SM enrichment is the over-reduction hypothesis of Selmar & Kleinwächter (2013): when an abiotic stressor limits growth and curtails photosynthetic electron consumption, the resulting excess of reducing equivalents is redirected toward SM biosynthesis. Hartmann (2007) situated this within an evolutionary framework, arguing that SMs represent molecular capital accumulated through prolonged plant–pathogen coevolution, and that sub-lethal abiotic stressors can co-opt these biosynthetic pathways to amplify phytochemical output. Recent studies on C. arabica have confirmed this principle at the metabolite level: Sarzynski et al. (2024) demonstrated that drought applied during the flowering stage significantly modulates chlorogenic acid content and isomer composition in green coffee beans, underscoring the agronomic relevance of stress timing and intensity. Concurrently, Guzman et al. (2021) synthesized evidence that climatic variation temperature, rainfall, and altitude-driven UV exposure systematically alters both quantitative SM levels and sensory attributes across Coffea species, confirming that agronomic management of environmental stress is a viable lever for quality enhancement. Oviedo-Bayas (2021) applied this mechanistic framework specifically to T. cacao and C. arabica, providing a species-level inventory of how individual abiotic stressors modulate key metabolite titers. However, three analytical shortcomings remain unaddressed: no prior investigation has applied a formal multivariate design to decompose interaction effects among concurrently applied stressors; controlled-environment optima have rarely been transferred to tropical production systems; and an increased SM concentration does not guarantee proportional human bioavailability, because colonic microbial transformation substantially modifies systemic metabolite exposure (Mayorga-Gross & Esquivel, 2019).

The present study addresses these three gaps through nested objectives: (i) mapping SM response surfaces for four simultaneously applied stress factors using a Box-Behnken design in seedlings of both species; (ii) transferring the laboratory-identified optimal treatment to field plots at two contrasting Ecuadorian altitudes; and (iii) conducting a randomized cross-over pilot study to characterize plasma pharmacokinetics of selected SMs in healthy volunteers who consumed

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beverages prepared from stress-enhanced versus conventional material. Together, these objectives extend the physiological framework of Oviedo-Bayas (2021) into a translational roadmap relevant for agroindustry and functional food development.

Materials and methods

Plant material and controlled-environment setup

Seeds of T. cacao (clone CCN-51, the commercially dominant genotype in Ecuador representing >80% of national production) and C. arabica (variety Typica, widely cultivated in Andean highland systems) were surface-decontaminated with 0.5% sodium hypochlorite for 10 min, rinsed three times under deionized water, and germinated on moistened filter paper at 28 ± 1°C with 80% relative humidity in darkness. At the two-true-leaf stage, uniform seedlings were transplanted individually to 2-L containers filled with a 3:1 (v/v) perlite–compost mixture (pH 6.2; electrical conductivity 0.4 dS m^{-1}). All containers were acclimated for four weeks in a controlled-environment chamber under standardized conditions (12/12 h photoperiod, 400 ± 20 µmol m^{-2} s^{-1} PPFD provided by full-spectrum LED arrays, 28/22°C day/night, 70% relative humidity) before stress treatments commenced.

Multifactorial stress design

A Box-Behnken response-surface design with four factors at three levels was deployed as specified in Table 1. Soil water status was regulated gravimetrically by weighing containers daily and restoring them to target field-capacity percentages with deionized water. Atmospheric CO_2 was maintained using a CO_2 controller (GreenPower, Bluelab, NZ) connected to a gas cylinder supply within sealed growth chambers. UV-B irradiance was supplemented using Philips TL 20W/12 lamps (peak emission 313 nm) calibrated weekly with a UV-B sensor (SolarLight PMA2110). Foliar methyl jasmonate was applied by fine-mist spray to runoff every three days. The design generated 29 experimental runs per species with five biological replicates (n = 290 containers per species). Treatments lasted 21 consecutive days, a duration validated in pilot trials as sufficient to produce measurable sub-lethal SM responses without inducing irreversible chlorosis or growth arrest (Pant et al., 2021; Isah, 2019).

Table 1. *Box-Behnken design factors and levels applied to Theobroma cacao and Coffea arabica seedlings.*

Factor	Code	Low (−1)	Centre (0)	High (+1)
Soil water status (% FC)	A	30	50	70
Atmospheric CO_2 (µmol mol^{-1})	B	400	600	800
UV-B irradiance (kJ m^{-2} d^{-1})	C	0	2	4
Methyl jasmonate (µM)	D	0	100	200

FC = field capacity.

Field validation

The optimal treatment combination identified by response-surface optimization was installed at two Ecuadorian field sites representing contrasting production environments. Site 1 (lowland cacao): Universidad Técnica Estatal de Quevedo experimental farm, Los Ríos Province (coordinates 1°01′S, 79°28′W; 250 m a.s.l.; mean annual temperature 24.5°C; annual rainfall 1,800 mm; Tropaquepts inceptisol; CCN-51 adult trees 8 years old). Site 2 (highland coffee): Pedernales district, Manabí Province (coordinates 0°04′N, 79°57′W; 1,100 m a.s.l.; 18.2°C; 1,200 mm annual rainfall; Humitropepts inceptisol; Typica trees 6 years old). Five adult trees per treatment per site were monitored across two consecutive growing seasons (October 2022–March 2023 and April 2023–September 2023). Water deficit was imposed using portable polypropylene shelter canopies combined with regulated drip irrigation controlled by tensiometers. CO_2 enrichment was omitted due to field engineering constraints. UV-B supplementation and foliar jasmonate applications followed growth-chamber protocols. Seeds and beans were collected at commercial maturity for phytochemical analysis.

Phytochemical quantification

Lyophilized seed powders (500 mg) were extracted three times under probe sonication (25 W, 2 min per cycle) with 80% (v/v) aqueous methanol. Pooled extracts were filtered through 0.45-µm PVDF membranes and concentrated under reduced pressure at 40°C. Total polyphenol content was determined by the Folin–Ciocalteu assay and expressed as mg gallic acid equivalents (GAE) per g dry weight (DW). Total flavonoids were measured by aluminum chloride colorimetry. Individual chlorogenic acid isomers (3-CQA, 4-CQA, 5-CQA, 3,4-

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diCQA, 3,5-diCQA) were resolved by HPLC-DAD (Shimadzu LC-20AT, C18 column, gradient elution 0.1% formic acid/acetonitrile) against certified external standards. Procyanidin profiling followed the MS-assisted protocol of Cerri et al. (2019). Methylxanthines (theobromine, caffeine) were quantified by reverse-phase HPLC-DAD. Antioxidant capacity was determined by DPPH radical-scavenging and FRAP assays. All determinations were performed in analytical triplicate.

Human bioavailability pilot study

Twenty-four healthy, non-smoking, medication-free adults (12 male, 12 females; mean age 31 ± 6 years; BMI 22.4 ± 2.1 kg m^{-2}) participated in a randomized double-blind four-arm cross-over design with 14-day washout periods. Participants avoided polyphenol-rich foods and beverages for 48 h before each intervention. Each participant consumed in counterbalanced order: (i) a cacao beverage prepared from stress-enhanced material (25 g cocoa powder per 200 mL water, 45°C); (ii) a matched control cacao beverage; (iii) a coffee beverage from stress-enhanced material (150 mL freshly prepared instant coffee, 2.5 g per 150 mL); and (iv) a matched control coffee beverage. Venous blood was collected into EDTA-K2 tubes at 0, 0.5, 1, 2, 4, 6, and 24 h post-ingestion. Plasma was separated by centrifugation (1,500 × g, 10 min, 4°C) and stored at −80°C. Chlorogenic acid catabolites (ferulic acid, dihydroferulic acid, 3-(3-hydroxyphenyl) propionic acid) and methylxanthines were quantified by UHPLC-MS/MS using a triple-quadrupole instrument (Waters Xevo TQ-S) with stable-isotope internal standards. Pharmacokinetic descriptors (Cmax, Tmax, AUC0–24, t½) were estimated by non-compartmental analysis (Phoenix WinNonlin v. 8.4). Ethics approval was obtained from the institutional review board (protocol EC-UTEQ-2024-007) in full compliance with the Declaration of Helsinki; all participants provided written informed consent prior to enrolment.

Statistical analysis

Second-order polynomial regression was performed in Statistica v. 13.5 (TIBCO Software, Inc.). Model adequacy was evaluated through lack-of-fit F-tests, Shapiro–Wilk tests for residual normality, and Levene tests for variance homogeneity. Response surfaces and contour plots were generated to visualize factor interactions and identify optimal-response regions using canonical analysis. Field trial data were analyzed by mixed-effects ANOVA with site and treatment as fixed effects and individual tree identity as a random factor. Pharmacokinetic

comparisons between stress-enhanced and control arms used paired t-tests or Wilcoxon signed-rank tests as appropriate, with Bonferroni correction for multiple comparisons. All analyses adopted $P \leq 0.05$ as the criterion for statistical significance.

Results

Response-surface models and optimal stress treatment

Second-order polynomial models adequately described all SM endpoints in both species (adjusted R^2 0.82–0.96; lack-of-fit $P > 0.05$ throughout). In cacao, the total polyphenol model (adjusted $R^2 = 0.94$) identified water deficit (linear and quadratic terms), UV-B irradiance, and the water-deficit × methyl jasmonate interaction as dominant predictors ($P < 0.001$ each). In coffee, CO_2 concentration, UV-B irradiance, and their interaction term dominated polyphenol variation, consistent with the interpretation that elevated CO_2 supplies additional carbon substrate that UV-B-activated phenylpropanoid transcription can exploit (Verdaguer et al., 2017). Canonical analysis located the ridge of maximum SM response within a narrow central region of the factor space, confirming that intermediate rather than extreme stress levels maximize SM output a pattern consistent with the over-reduction hypothesis of Selmar & Kleinwächter (2013).

Under the globally optimal combination (55% FC, 600 µmol mol^{-1} CO_2, 2 kJ m^{-2} d^{-1} UV-B, 100 µM MeJA), cacao total polyphenols increased from 42.3 ± 1.8 to 58.4 ± 2.3 mg GAE g^{-1} DW (+38%; $P < 0.001$) and coffee polyphenols from 68.1 ± 3.1 to 98.1 ± 3.9 mg GAE g^{-1} DW (+44%; $P < 0.001$). At the metabolite level, coffee 5-CQA reached 18.9 ± 1.1 vs. 12.4 ± 0.9 mg g^{-1} DW (+52%) and cacao procyanidins reached 3.7 ± 0.3 vs. 2.8 ± 0.2 mg CE g^{-1} DW (+31%). Methylxanthine concentrations varied by no more than 12% across all 29 treatment combinations, consistent with the constitutive genetic regulation of purine alkaloid pathways in both species (Bae et al., 2008; Kim et al., 2010). A comprehensive summary of phytochemical results appears in Table 2.

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Table 2. *Phytochemical responses under optimal multifactorial stress in Theobroma cacao and Coffea arabica.*

Variable	Species	Control	Optimal stress	Change (%)	P
Total polyphenols (mg GAE g^{-1} DW)	T. cacao	42.3 ± 1.8	58.4 ± 2.3 a	+38	<0.001
	C. arabica	68.1 ± 3.1	98.1 ± 3.9 a	+44	<0.001
5-CQA (mg g^{-1} DW)	C. arabica	12.4 ± 0.9	18.9 ± 1.1 a	+52	<0.001
Procyanidins (mg CE g^{-1} DW)	T. cacao	2.8 ± 0.2	3.7 ± 0.3 a	+31	<0.001
Methylxanthines	Both	Baseline	±12% max	n.s.	>0.05

a Significantly different from control ($P < 0.05$, Wilcoxon signed-rank test with Bonferroni correction). GAE = gallic acid equivalents; CE = catechin equivalents; DW = dry weight; 5-CQA = 5-caffeoylquinic acid; n.s. = not significant.

Field-scale recovery of SM increments

At Site 1 (Los Ríos, 1°01′S, 79°28′W, 250 m a.s.l.), regulated deficit irrigation at 60% FC recovered 68% of the polyphenol increment predicted by the response-surface model. Supplemental UV-B raised chlorogenic acid content a further 19% above water-deficit-only plots ($P = 0.023$), confirming that UV-B-driven phenylpropanoid induction persists under full-canopy field conditions. At Site 2 (Manabí, 0°04′N, 79°57′W, 1,100 m a.s.l.), where ambient UV-B is inherently elevated at altitude, water deficit alone recovered 72% of the laboratory-predicted SM increment. At both sites, methylxanthine concentrations deviated no more than 11% from paired control trees, reinforcing genetic determinism of alkaloid titers (Cerri et al., 2019; Lahive et al., 2019). No statistically significant differences in yield (bean weight per tree) or visual quality grade were observed between stressed and control trees at either site across both seasons (data not shown), indicating that the stress protocol induces metabolic responses without economically significant agronomic penalties.

Plasma pharmacokinetics in study participants

Participants who ingested stress-enhanced coffee displayed significantly higher plasma Cmax (0.82 ± 0.11 vs. 0.54 ± 0.08 μM; P = 0.004) and AUC0–24 (8.4 ± 1.2 vs. 5.7 ± 0.9 μM·h; P = 0.009) for pooled chlorogenic acid catabolites relative to the control coffee arm. Tmax did not differ between arms (1.8 ± 0.3 vs. 2.0 ± 0.4 h; P = 0.19), indicating that the pharmacokinetic advantage reflects higher initial substrate load rather than altered gastric emptying or intestinal transit. In the cacao arms, epicatechin plasma concentrations were significantly elevated at 1 h post-ingestion (0.34 ± 0.05 vs. 0.22 ± 0.04 μM; P = 0.018) but converged by 6 h, consistent with capacity-limited colonic fermentation of procyanidins as characterized by Mayorga-Gross & Esquivel (2019). Methylxanthine pharmacokinetics did not differ between stress-enhanced and control arms (P > 0.10 for all comparisons), matching the limited harvest-time methylxanthine variation described above. Pharmacokinetic parameters are summarized in Table 3.

Table 3. Non-compartmental pharmacokinetic parameters of plasma analytes following beverage consumption in 24 healthy adults (mean ± SD).

Parameter	Enhanced coffee	Control coffee	P	Enhanced cacao (1 h)	Control cacao (1 h)	P
Cmax (μM)	0.82 ± 0.11 a	0.54 ± 0.08	0.004	0.34 ± 0.05 a	0.22 ± 0.04	0.018
Tmax (h)	1.8 ± 0.3	2.0 ± 0.4	0.19	—	—	—
AUC0–24 (μM·h)	8.4 ± 1.2 a	5.7 ± 0.9	0.009	—	—	—

a Significantly different from corresponding control arm (P < 0.05, paired t-test, Bonferroni corrected). Cmax = peak plasma concentration; Tmax = time to peak; AUC0–24 = area under the curve from 0 to 24 h.

The central finding of this study is that a formally optimized combination of four concurrently applied stressors produces additive to synergistic SM enrichment in both T. cacao and C. arabica at scales ranging from seedling phytochemistry to human plasma

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pharmacokinetics. The Box-Behnken design revealed synergies among concurrent stressors that would not be detected by conventional one-factor-at-a-time approaches: water deficit and UV-B engage convergent but temporally distinct signaling arms drought elevates apo plastic ROS and activates abscisic acid signaling (Laxa et al., 2019), whereas UV-B activates UVR8-dependent upregulation of MYB-mediated phenylpropanoid gene clusters (Verdaguer et al., 2017) and methyl jasmonate amplifies both cascades via jasmonate–abscisic acid crosstalk. The identification of an intermediate-stress optimum rather than a monotonically increasing response is consistent with the over-reduction hypothesis (Selmar & Kleinwächter, 2013) and with the principle that moderate water limitation improves SM quality in spice and medicinal crops (Kleinwächter & Selmar, 2015), now extended to two commercially dominant tropical perennial species.

Field validation at two contrasting Ecuadorian sites demonstrated that controlled-environment optima transfer to production conditions at 68–72% recovery rates, without detectable yield penalties across two full growing seasons. The partial attenuation relative to chamber predictions most likely reflects canopy buffering of UV-B penetration, greater root-volume buffering of soil water potential dynamics, and soil microbiome interactions with jasmonate signaling — all legitimate targets for multi-site, multi-genotype validation studies (Guzman et al., 2021). These recovery rates compare favorably with analogous translational studies in spice crops, where 50–70% recovery of SM increments from greenhouse to field is commonly reported. The drought-flowering interaction reported by Sarzynski et al. (2024) for coffee chlorogenic acids highlights that stress timing relative to phenological stage is an additional variable warranting systematic investigation in future Box-Behnken studies that include developmental stage as a treatment factor.

The pharmacokinetic arm provides, to our knowledge, the first direct evidence that an agronomically applied stress protocol elevates circulating concentrations of bioactive SM catabolites in human consumers. The 47% increase in pooled chlorogenic acid AUC0–24 from stress-enhanced coffee is biologically meaningful in the context of the dose–response relationships underpinning the epidemiological associations between habitual coffee consumption and reduced chronic disease risk documented in umbrella meta-analyses (Poole et al., 2017). The capacity-limited colonic fermentation of cacao procyanidins, evidenced by convergent epicatechin plasma levels at 6 h, is consistent with established mechanistic models (Mayorga-Gross & Esquivel,

2019) and indicates that further SM enrichment may require exploration of depolymerized procyanidin fractions with more favorable intestinal absorption kinetics. The absence of any pharmacokinetic difference in methylxanthines confirms that purine alkaloid titers are governed primarily by genetic determinism rather than agronomic stress (Kim et al., 2010; Bae et al., 2008), limiting the scope of stress-based enhancement to polyphenolic SM classes.

Several limitations warrant transparent acknowledgement. The pilot enrolled 24 participants and was statistically powered only for primary pharmacokinetic endpoints; larger randomized trials with clinical biomarker outcomes inflammatory cytokine panels, post-prandial glycaemia, lipid oxidation markers are required before nutritional guidance can be formulated. Field trials covered two seasons at two sites in a single climatic zone; multi-environment, multi-genotype validation across Ecuador's heterogeneous agroecological gradient and extension to equatorial African and Southeast Asian production zones would substantially strengthen external validity. CO_2 enrichment, which strongly modulated coffee SM accumulation in the growth chamber, could not be replicated under open-field conditions; free-air CO_2 enrichment facilities in tropical agroecosystems would be required. Finally, the fate of stress-induced metabolite surpluses through the post-harvest processing chain fermentation and drying for cacao; wet-milling and roasting for coffee and their interaction with the thermal degradation of labile phenolics during roasting remain to be quantified to confirm that the agronomic enrichment effect survives until the point of consumer ingestion.

Conclusions

Simultaneous multifactorial stress modulation reliably increases SM concentrations in T. cacao and C. arabica at controlled-environment and field scales, and these increments translate into measurably higher systemic exposure of bioactive chlorogenic acid catabolites in human consumers. The Box-Behnken response-surface approach efficiently identified a four-factor optimum that raised total polyphenols by 38–44% and key individual SM classes by 31–52%, with 68–72% field recovery and no detectable yield penalty. The 47% increase in chlorogenic acid AUC0–24 after stress-enhanced coffee consumption provides a direct mechanistic link between agronomic intervention and human bioactive exposure. These findings establish a scientifically grounded framework for cultivation protocols designed to yield

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functional food ingredients with enriched bioactive profiles and motivate adequately powered clinical trials to quantify the long-term consumer health consequences of stress-enhanced cacao and coffee consumption.

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