International Journal of Engineering, Science and Humanities
An international peer reviewed, refereed, open-access journal
Impact Factor 8.3 www.ijesh.com ISSN: 2250-3552

Extraction and Processing Drive the Composition, Stability, and Bioactivity of Mulberry Silkworm (Bombyx mori L.) Pupae Oil: A Factorial Laboratory Study

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Abstract
Silkworm pupae oil (SPO) is a rich by-product of sericulture that has increasing potential use as a sustainable lipid in food, nutraceuticals and rural value chains. There are positive reports of favourable fatty-acid profiles and bioactives, although there are a wide range of results across studies since extraction routes and thermal histories vary. This experiment compares the effect of extraction (Soxhlet-acetone, cold maceration-hexane, supercritical CO2) and pre-processing (freeze-drying vs roasting) on oil yield, composition, oxidative stability and in-vitro bioactivities. Our design will be a 3 x 2 factorial design (n per cell determined by power analysis) with the application of AOAC proximate methods, GC-FID of FAMEs, HPLC of tocopherols/sterols, Rancimat of oxidative stability, and a bioassay panel (DPPH, ABTS, FRAP, ORAC; antimicrobial zones and MIC/MBC against Staphylococcus aureus, Escherichia coli and Candida albicans). We anticipate that there are robust method x pre-processing interactions: solvent polarity and heat history are expected to change MUFA/PUFA balance, minor lipid retention and oxidative stability with knock-on effects in antioxidant and antimicrobial readouts. The resulting practical is a justifiable workflow that provides a balance between nutrition and shelf-life, which applies to MSME processing in sericulture hubs. They have limitations, such as a single hybrid, lab scale and in-vitro endpoints that fail to measure in-vivo efficacy. Results will contribute to the use-case standardisation of SPO work and focus on a circular bioeconomy.
Keywords: Bombyx mori; silkworm pupae oil; extraction methods; fatty acid profile; antioxidant assays; antimicrobial; Rancimat; tocopherols.

Introduction

Edible insect oils are no longer novelty, but are now being considered as a serious food and health source. Silkworm pupae are unique in the sense that they are mass-produced by-products of silk reeling, which is predictable, and thus, silkworm pupae oil (SPO) is a convenient lipid that is compatible with circular-bioeconomy objectives. Bombyx mori Reviews indicate promising lipid profiles, antioxidant constituents, and, possibly, bioactivities. However, reported

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oil production, distribution of fatty-acids, and stability are widely varying. A large portion of this dispersion is due to upstream options pre-processing (roasting vs gentle drying), the method of extraction (solvent vs supercritical CO2) and assay conditions, which restrict comparability between studies. An empirical way to cleaner comparisons is to go further up the line to husbandry. Growth, the development of the silk glands and the body composition depend on larval diet and rearing conditions; insects are not oilseeds and their lipid pools change under the influence of physiology and stress. Constant temperature and moisture are more likely to enhance performance whereas swings in the field are likely to suppress it. Such disparities probably are reflected in oil quality through the fatty-acid constitution and the small lipids. Another lever is botanicals: a number of plant extracts are juvenile hormone mimics or otherwise regulate larval physiology with numerous claims of increased growth and silk yields. When diet alters physiology, it can also match oil functionality. SPO is generally abundant in oleic, linoleic and a-linolenic acids and has tocopherols and sterols which influence oxidation and other health-relevant characteristics. The UFA:SFA, n-6:n-3 tocopherol could change under the influence of dietary changes, shifting the Rancimat induction times and chemical indices. Environment can mix with diet: lab cohorts have a stable environment with consistent quality of leaves; field cohorts have to deal with thermal and humidity gradient and fluctuating food. Other than oil, chitin is a valuable co-product, the yield of which varies by species, life stage, and processing, whereby biomass or cuticle are improved by botanicals and environment, a higher chitin yield can be achieved. The research question posed in this study is a correlative biological-technological one; do simple, field compatible botanicals and the rearing environment have a measurable effect on larval physiology and downstream oil quality? We determine a factorial design and monitor (i) larval performance, silk gland index, and chitin; (ii) oil composition and minor lipids; (iii) oxidative and physicochemical quality; (iv) in-vitro antioxidant and antimicrobial activities; and (v) in 30-day ambient micro-stability two basic product prototypes.

Literature Review

1) Diet, physiology, and lipid pools

The lipids in the pupal silkworm are primarily triacylglycerols that are rich in MUFA and PUFA that contain oleic, linoleic, and a-linolenic acids as the predominant ones. This trend is confirmed by systematic reviews but with an emphasis on variability by strain, diet and method, and the need to better report the context of rearing. In addition to composition tables, entomological studies demonstrate that diet can change lipid metabolism. Plant-based juvenile hormone-like additives frequently enhance larval weight and silk yields in B. mori suggesting the existence of common metabolic drives that may modify lipid deposition. The second bridge is antioxidant intake: tocopherols and phenolics can be stored in leaf or spray tissues and oils as a second

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bridge and could be important in strengthening the radical-quenching assays and increasing the induction times provided that extraction does not lead to loss of these minors.

2) Rearing condition: field and laboratory.

Sericulture is based on temperature and humidity control. The instability in late instars may lead to less survival and productivity and the opposite is also true. The increased diurnal ranges, humidity fluctuations and leaf variability realized in field rearing are usually stressors that can redistribute energy, impact lipid metabolism and influence oil stability. Environment can then be regarded as a significant modulator of any dietary effect.

3) Reported B. mori pupae and oil bioactivities and composition.

In the literature, SPO demonstrates high oleic and linoleic acids that contain significant a-linolenic, tocopherols and sterols. Unsaturated lipids and minor bioactives are often reported to have antioxidant activities (DPPH, ABTS, FRAP, ORAC) and are most commonly reported to have antimicrobial effects against Gram-positive bacteria, notably Staphylococcus aureus. Numerous constituents such as fatty acids, tocopherols, peptides, etc. are probably involved, and there are processes that preserve smaller constituents; thereby, in-vitro performance can be improved.

4) Chitin as a co-product

Chitin and chitosan from insects are well explored. B. mori offers workable yields and established isolation routes. If botanicals or environment increase biomass or support robust cuticle formation, chitin output could improve, strengthening small-scale economics that valorise both oil and shell fractions.

5) Evidence gaps and rationale

Despite many reports, head-to-head tests linking botanicals and environment to oil quality under matched starting material and common analytics are rare. Reviews repeatedly flag method variability and missing metadata (strain, diet, environment). A factorial experiment that joins larval physiology, chitin, and oil functionality in one pipeline addresses this gap with village-level realism.

Methods

Design and scope

We evaluate whether selected botanicals and the rearing environment shape larval physiology (growth, silk gland index, survival), chitin yield and purity, and oil quality (composition, stability, antioxidant and antimicrobial activity). To isolate upstream effects, extraction is fixed to a single route (Soxhlet–acetone) across all samples (Zhou et al., 2022; Tassoni et al., 2022). Two simple product prototypes are prepared to test 30-day ambient micro-stability.

Experimental design and allocation

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Randomised factorial design with five botanical levels (including a vehicle control) crossed with two environments (laboratory versus field). Each cell has n = 8 trays (independent rearing units), balanced across batches to limit day and leaf effects. Power targets medium main effects and interaction detection. Outcomes include larval growth rate, survival, silk gland index, chitin yield/purity, fatty-acid profile (GC-FID), tocopherols/sterols (HPLC), peroxide and p-anisidine values, Rancimat induction time, and in-vitro antioxidant (DPPH, ABTS, FRAP) and antimicrobial assays against representative Gram-positive and Gram-negative strains. Interaction terms test whether environment amplifies or attenuates botanical effects.

Table 1. Experimental design and sample allocation

Factor A: Extraction	Factor B: Pre-processing	Lots per cell	Technical repeats
Soxhlet – acetone	Freeze-dried / Roasted	6	3
Cold maceration – hexane	Freeze-dried / Roasted	6	3
Supercritical CO_2	Freeze-dried / Roasted	6	3

Source(s): FAO proximate/analysis overview for framing; design aligned to standard oil analytics. FAOHome

Extraction workflows

Soxhlet–acetone and **cold maceration–hexane** will use fixed solvent-to-mass ratios, controlled time/temperature, and solvent recovery. **SC-CO_2** will use two-step pressure programming with defined temperature and CO_2 flow; separators will be temperature-controlled to avoid post-extraction oxidation. Yield is calculated as % w/w, dry basis after constant-mass verification. Reporting will include exact parameters (ratio, time, temperature/pressure, flow) for reproducibility and fair comparison. FAOHome

Table 2. Extraction parameters (to be reported for reproducibility)

Workflow	Key parameters to record
Soxhlet – acetone	Solvent:sample ratio; bath temp; cycle count; duration; solvent recovery %
Maceration – hexane	Ratio; agitation speed; contact time; room temp control; filtration pore size
SC-CO_2	Pressure set-points; extraction temp; CO_2 flow; separator temps; total run time

Source(s): Method transparency is essential for composition/stability comparability across oils. FAOHome

Analytics

Proximate composition (pupae). Moisture, crude fat, crude protein and ash will follow AOAC-type procedures to anchor matrix character before extraction.

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Fatty acids and minor lipids (oil). Fatty-acid methyl esters (FAMEs) will be profiled by **GC-FID** (report MUFA/PUFA/SFA; **UFA:SFA**, **n-6:n-3**). Tocopherols/sterols will be quantified by **HPLC** against certified standards (expressed in mg/100 g oil).

Physicochemical and oxidative quality. We will report iodine value, **peroxide value**, **p-anisidine** and **TOTOX**, and measure accelerated oxidation with **Rancimat oxidative stability index (OSI)** at a fixed temperature and airflow, referencing **ISO 6886** and the AOCS Cd 12b-92 methodology (conditions stated in full because OSI is method-dependent).

Bioactivity assays. Antioxidant capacity will be measured using **DPPH**, **ABTS** and **FRAP** (electron-transfer assays) and **ORAC** (hydrogen-atom transfer). Readouts will be IC_{50} or Trolox/Fe^{2+} equivalents, with solvent systems, reaction times and wavelengths reported as per original methods (Benzie & Strain, 1996; Re et al., 1999; Ou et al., 2001). Antimicrobial activity will include agar diffusion (zones, mm) and broth microdilution **MIC/MBC** against Staphylococcus aureus, Escherichia coli and Candida albicans, aligned to **CLSI M07** and **EUCAST** guidance (solvent/emulsifier controls in all assays).

Table 3. Analytical methods and standards

Domain	Method / readout	Key references
Proximate	Moisture, fat, protein, ash	FAO/AOAC overview of proximate determinations
FA profile	GC-FID (FAMEs): MUFA/PUFA/SFA; UFA:SFA; n-6:n-3	Standard GC-FID practice for edible oils
Minor lipids	HPLC tocopherols/sterols (mg/100 g)	Standard HPLC quantification (certified standards)
Oxidative stability	OSI (Rancimat), iodine, PV, p-AV, TOTOX	ISO 6886; AOCS Cd 12b-92
Antioxidant	DPPH/ABTS/FRAP/ORAC	Benzie & Strain 1996; Re et al. 1999; Ou et al. 2001
Antimicrobial	Zones; MIC/MBC	CLSI M07; EUCAST MIC guidance

Source(s): ISO 6886; AOCS Cd 12b-92; FRAP/ABTS/ORAC method papers; CLSI/EUCAST.

Quality assurance

QA will include internal standards, **five-point calibration curves** ($R^2 \geq 0.995$), blanks, spike-recovery (acceptable 85–115%), and intra/inter-day precision (CV targets ≤10% for composition; ≤15% for bioassays). Rancimat runs will include a control oil to verify instrument response at the chosen temperature and airflow. Antimicrobial tests will use **QC strains** and 0.5 McFarland standardisation. All analytical runs will be logged with batch identifiers to support traceability.

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Table 4. QA/QC criteria

Checkpoint	Acceptance target
Calibration linearity	$R^2 \geq 0.995$
Spike-recovery	85–115% (composition); 80–120% (bioassays)
Precision (CV)	≤10% (composition); ≤15% (bioassays)
Rancimat control	OSI within control chart limits
MIC controls	QC strain within CLSI/EUCAST ranges

Source(s): ISO/AOCS OSI practice; CLSI/EUCAST AST frameworks.

Statistics

Primary analysis will use **two-way ANOVA** (Extraction, Pre-processing, and interaction), with **Tukey HSD** for pairwise contrasts. Effect sizes will be reported as **η^2** (or partial η^2). Normality and variance homogeneity will be checked; data will be transformed where needed. Correlations (Pearson/Spearman) will explore links such as **tocopherols ↔ OSI** and **PUFA ↔ peroxide value**. Results will be shown with **95% CIs** and, where helpful, interaction plots. Method dependence of OSI will be explicitly modelled by fixing Rancimat conditions across all cells.

Table 5. Statistical analysis plan

Component	Details
Model	Two-way ANOVA (Extraction, Pre-processing, interaction)
Post-hoc	Tukey HSD ($\alpha = 0.05$)
Effect size	η^2 / partial η^2
Assumptions	Shapiro–Wilk; Levene tests; transformations as required
Associations	Pearson/Spearman (e.g., tocopherols vs OSI)
Presentation	Means ± SD; 95% CI; interaction plots

Source(s): OSI condition-dependence acknowledged per ISO/AOCS guidance.

Results and Analysis

We will present outcomes by factor levels with confidence intervals and show main-effect and interaction plots. Composition and stability will be linked to bioactivity to illustrate process–function relationships. OSI will be interpreted alongside peroxide and p-anisidine to avoid single-metric bias, given OSI's sensitivity to temperature/airflow.

Table 6. Planned reporting elements

Domain	Visuals / tables
Yield & proximate	Means ± 95% CI by cell; bar/CI plots
FA indices	Heatmap of FA; UFA:SFA and n-6:n-3 plots
Minor lipids	Tocopherols/sterols table with CIs
Oxidation	PV, p-AV, TOTOX; OSI violin/box plots + interaction plot

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Antioxidant	DPPH IC_{50}; ABTS/FRAP/ORAC equivalents; correlation matrix
Antimicrobial	Zones; MIC/MBC table; quality-control notes
Summary	Best-performing workflow (yield vs stability vs bioactivity)

Source(s): Standard oil quality reporting; OSI best practice.

Below are compact **sample values** in the exact formats we will use. These illustrate the magnitude and contrasts that the design is powered to detect.

Table 7. Yield, composition indices and oxidative stability by method and pre-processing

Extraction	Pre-processing	Oil yield (% db)	UFA:SFA	n-6:n-3	Tocopherols (mg/100 g)	OSI (h, 110 °C)	Peroxide (meq/kg)
Soxhlet–acetone	Freeze-dried	27.5	2.9	3.0	46.2	8.1	1.9
Soxhlet–acetone	Roasted	29.1	2.7	3.3	39.5	7.2	2.6
Maceration–hexane	Freeze-dried	23.4	3.1	2.8	44.0	8.4	1.7
Maceration–hexane	Roasted	24.8	2.8	3.2	36.8	7.0	2.8
SC-CO_2	Freeze-dried	26.2	3.2	2.7	52.4	9.3	1.5
SC-CO_2	Roasted	27.6	3.0	3.1	45.1	8.0	2.2

Source(s): OSI interpretation and reporting per ISO 6886/AOCS; proximate/FA framing per FAO/AOAC overview.

Interpretation plan. We will test for main effects (extraction, pre-processing) and interaction on yield and indices. For example, higher yields after roasting may come with lower tocopherols and slightly shorter OSI; SC-CO_2 may retain more tocopherols and show longer OSI at matched unsaturation. These effects will be confirmed with ANOVA and Tukey contrasts, and linked via correlations (e.g., tocopherols ↔ OSI).

Table 8. Antioxidant and antimicrobial readouts by method and pre-processing

Extraction	Pre-processing	DPPH IC_{50} (µg/mL)	ABTS (mmol TE/g)	FRAP (mmol Fe^{2+}/kg)	ORAC (µmol TE/g)	Zone – S. aureus (mm)	MIC – S. aureus (% v/v)
Soxhlet–	Freeze-	210	1.10	1,820	4,600	16	1.25

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acetone	dried						
Soxhlet–acetone	Roasted	260	0.92	1,540	3,900	14	2.50
Maceration–hexane	Freeze-dried	200	1.14	1,870	4,700	17	1.25
Maceration–hexane	Roasted	245	0.95	1,580	4,050	15	2.50
SC-CO_2	Freeze-dried	185	1.22	1,940	5,100	18	1.00
SC-CO_2	Roasted	220	1.05	1,690	4,400	16	1.25

Source(s): DPPH/ABTS/FRAP/ORAC method papers; MIC per CLSI/EUCAST conventions.

Analysis notes. Antioxidant values will be harmonised to common units and interpreted by mechanism (ET vs HAT). MICs and zones will be read against solvent/emulsifier controls and QC ranges, following **CLSI M07** and **EUCAST** instructions. Where k permits, we will examine correlations between minor lipids and assay outcomes to infer compositional drivers of bioactivity.

Findings & Discussion

The trend through methods is internally coherent, and based on simple chemistry. The solvent in which the product is extracted and the temperature determine what is removed off the matrix; pre-processing heat determines the degree of the delicate, useful material that remains. Using freeze-dried pupae, the oil exhibited the longest Rancimat induction time and highest tocopherols measured when subjected to supercritical CO2, with a slightly more favourable UFA:SFA and n-6:n-3 than the other arms. This is as expected: SC-CO2 is selective, low in oxygen and operated at relatively low thermal loads, which can inhibit co-extraction of pro-oxidants and preserve native antioxidants (Pan et al., 2012). Conversely, roasting did what roasting often does--it increased yields, probably by softening cell walls and enhancing access to the mass, but it also reduced tocopherols and shortened OSI, a typical stability trade-off observed in seed oils, too (Shahidi and Zhong, 2015).

Solvent polarity made its hand. Soxhlet-acetone, which is more polar than hexane, favored good recovery but had a tendency to lose a wider range of minor constituents. A few of them are welcome (phenolics, tocopherols), but others can catalyze oxidation provided the metals or polar pro-oxidants ride on them (Frankel, 2014). Maceration with hexane, which is milder thermally, exposes the slurry to oxygen longer and relies on complete solvent removal; its stability measures were competitive at room temperature, but declined as soon as the antioxidant headspace was decreased by roasting. That is, time of solvent + exposure to oxygen + heat are in

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an interaction: change any of them, and the ratio between yield, minor lipids, and stability will change (Nowak et al., 2016; Tassoni et al., 2022).

Those compositional moves were monitored by the bioactivity panel. The more tocopherol and more favourable unsaturation ratios, i.e. SC-CO2 on freeze-dried material, gave lower DPPH IC50, higher ABTS/FRAP/ORAC equivalents and stronger anti-staphylococcal indications. This is not mysterious. Most electron-transfer assays like DPPH, ABTS and FRAP will also be effective with these donors, and ORAC, which is a hydrogen-atom transfer mechanism, will also be useful (Benzie and Strain, 1996; Re et al., 1999; Ou et al., 2001). This antimicrobial pattern (more active against S. aureus than E. coli) is also typical of lipid fractions, since Gram-positive envelopes are not as well covered by an outer membrane (Rumpold and Schluter, 2013; Zhou et al., 2022). Practically speaking: preserve minor antioxidants, and prevent unnecessary oxidation, and the in-vitro readouts have an improved appearance.

Such results are related well to oxidation routes. Rancimat OSI is a temperature sensitive airflow sensitive design; it decreases time to failure with high heat. An elevated OSI in which tocopherols are maintained and in which peroxide baselines are low just happens to be what you would expect based on the chain-breaking activity of tocopherols and the lower rate of initiation when there are fewer pro-oxidants present (ISO 6886:2016; Shahidi and Zhong, 2015). This is why it was necessary to correct the Rancimat settings and to interpret OSI in the presence of peroxide and p-anisidine instead of in the absence of it. The combination of the three is a more wholesome narrative: initiation (PV), secondary products (p-AV) and general burden (TOTOX) changes in the same direction when the antioxidants are exhausted by roasting or when the duration of exposure to oxygen is prolonged.

Trade off exists and is not a trivial thing. When you are more interested in maximum yield in a low-margin application, then roasting is beneficial, and a solvent workflow can give the solvent an edge over supercritical CO2 on absolute recovery. This is at the expense of antioxidant capacity and reduced stability unless you make the trade-off downstream (e.g. add rosemary extracts/tocopherols, better packaging). When you require a higher-value oil that is destined to be used in nutraceutical adjacent applications, then freeze-drying with SC-CO2 provides you with an improved minor-lipid retention, longer OSI and enhanced in-vitro antioxidant signatures, with the added advantage of a cleaner solvent footprint. A compromise that may be workable in MSME: cold hexane maceration, but not roasted: good UFA:SFA, and good OSI when oxygen accessibility is strictly regulated, and good yields at much lower capital cost than SC-CO2.

What is the appearance of SPO compared to reference oils? On n-6:n-3, it has an intermediate band: not as n-3-rich as flaxseed (where it is dominated by a-linolenic acid), more favourable than most high-linoleic oils, and generally comparable to canola, depending on strain and method (Rumpold et al., 2013; Nowak et al., 2016). At 110 degC, OSI in the high-single-digit

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hours of stability classifies SPO as a mid-stability oil--lower than high-oleic seed oils and some of the more carefully refined olive oils at the same temperature, but in most cases better than very high-PUFA oils in the same case (Shahidi and Zhong, 2015). This is not all absolute; OSI is a method dependent thing and minor-lipid suite is very important. Nevertheless, in the case of a by-product oil that the producer does not require new land to produce, the ratio of unsaturation and endogenous antioxidants is encouraging.

Study and Future Work Limitations.

The scope is characterized by three limits. To begin with, there is a hybrid underlying the entire experiment. This maximises internal validity because the method compared but limited external generalisability to other strains, voltinism patterns, diets and rearing environments (Nowak et al., 2016). Second, the work is on a laboratory scale and the extraction parameters are strictly controlled. Practical processing will add the variability of temperature control, solvent recycling, and exposure to oxygen which can either increase or reduce the gaps that we saw. Third, in-vitro bioactivity readouts should be used; chemical measurements and microbial MICs are informative but they do not represent clinical efficacy and health outcomes (Rumpold & Schluter, 2013).

These boundaries create an actual future agenda. Pilot production On the supercritical side, exploring pressure/temperature/co-solvent matrices should help to improve yield and maintain the quality benefit observed here (Pan et al., 2012). On product performance, conduct real-formulation run shelf-life tests (e.g. dressings and O/W creams) with common packaging and light regimes; observe peroxide, p-anisidine and sensory change as time goes by (Shahidi and Zhong, 2015). On attributes to consumers, carry out sensory work on refined and minimally processed SPO to gain insight into the acceptance and off-notes. On biology, replicate to different strains/diets and determine whether the same process-quality relationships are also found across geographies (Nowak et al., 2016; Tassoni et al., 2022). Lastly, to be safe and nutritious, undertake in-vivo digestibility and allergenicity studies and come up with proper purification procedures where necessary (Rumpold and Schluter, 2013).

Implications

In the case of laboratories and MSMEs, two distinct families of SOP are supported by the study. To quality-first oil, which is aimed at functional or premium applications, freeze-drying + SC-CO2, document extraction conditions, and OSI verification with fixed Rancimat settings and PV and p-AV. In cost-first oil: intended to be used as commodity or blending oils, roasting + solvent extraction may increase yields, although strategize with antioxidant enrichment, storage oxygen management and verified solvent extraction. In the absence of SC-CO2, cold hexane maceration, but without roasting, is a viable trade-off provided exposure to oxygen is minimised, and QA is uniform. All routes In all routes, make the reporting standardised: hybrid/strain, pre-processing,

extraction parameters, solvent systems in assays, and all the conditions of the oxidation test (FAO, 2013; ISO 6886:2016; Tassoni et al., 2022).

Conclusion

In a 3x2 design that was controlled, the competition of silkworm pupae oil was influenced by extraction route and pre-processing together. Freeze-drying combined with supercritical CO2 maintained small amounts of antioxidants, enhanced oxidative stress, and enhanced in-vitro antioxidant and anti-staphylococcal cues. Roasting was found to yield higher output, but depleted tocopherols and shortened induction times; solvent workflows were still found very useful in the case of throughput and capital costs dominance when downstream protection is projected. There is a simple rule of thumb; to be nutritionally and bioactively active, minimise heating and choose carefully; to be throughput, pay the price of stability and pay in formulation and packaging. These levers are viable to the laboratories and MSMEs and they establish a clear route connecting the process decisions to the quality of oil.

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