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ADVANCES IN CHROMATOGRAPHIC AND SPECTROSCOPIC CHARACTERIZATION OF BLUE PEA PIGMENTS AND PHENOLICS

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Abstract

Advances in chromatographic and spectroscopic techniques have transformed blue pea (Clitoria ternatea L.) into a model system for studying natural blue colorants and complex phenolic matrices. Flowers, teas, and seedscontain structurally diverse ternatintype anthocyanins, flavonols, and phenolic acids, the accurate characterization of which requires highresolution separation and detection platforms. Conventional colorimetric assays for "total anthocyanins" or "total phenolics" have therefore been superseded by integrated UHPLC–DAD, UHPLC–QTOF–HRMS, and tandem MS/MS workflows that resolve individual pigments, define their glycosylation and acylation patterns, and relate these to color expression, stability, and bioactivity. In parallel, greener extraction strategies, such as ultrasound and microwaveassisted extraction, often optimized by chemometric or responsesurface methodologies, have significantly improved the recovery of anthocyanins and coextracted polyphenols while reducing solvent use and processing time, enabling the scalable production of standardized, foodgrade extracts. Spectroscopic approaches, particularly UV–Vis with pHdifferential methods and CIELAB color analysis, provide rapid, non destructive monitoring of pigment equilibrium, thermal degradation, and formulation behavior, while NMR and advanced MS^n experiments deliver higher-order structural confirmation of newly reported anthocyanins and associated flavonols and phenolic acids. Collectively, these analytical and process innovations support a shift from bulk estimations to molecule level understanding, underpinning the rational design of blue peabased colorants and multifunctional phytochemical ingredients for foods, nutraceuticals and emerging smart packaging and delivery systems.

INTRODUCTION

Recent advances in chromatographic and spectroscopic techniques have transformed Clitoria ternatea L. (blue pea) from an ornamental plant into a model system for studying natural colorants and bioactive phytochemicals [1].Rich in polyacylated anthocyanins (ternatins), flavonols, and phenolic acids, its chemical complexity demands highresolution analytical tools for accurate profiling [2]. Early work based on UV–Vis and conventional HPLC provided only bulk estimates of "total anthocyanins" or "total phenolics," However, current research integrates UHPLC–QTOF–HRMS, UHPLC–ESI–MS/MS, and optimized HPLC–DAD with complementary spectroscopic methods to identify individual molecular species linked to color stability and bioactivity [3].

Distinctive ternatin anthocyanins, characterized by multiple glycosylation and acylation on a delphinidin core, exhibit remarkable pH-dependent color expression and thermal resistance, features now resolved through advanced LC–MS workflows. In addition to flower studies, UHPLC–QTOF–HRMS profiling of seeds and fruits has revealed the abundance of rutin, quercetin derivatives, catechin, and diverse phenolic acids, linking developmental variations to their antioxidant potential [3].

Fig 01.Clitoria ternatea Flower(Blue pea)

Concurrently, green extraction strategies, such as ultrasound and microwaveassisted techniques, have been optimized via multivariate designs to yield anthocyaninrich extracts with high recovery, reduced solvent use, and strong antioxidant activity. Spectroscopic analyses, particularly UV–Vis and NMR, complement chromatography by monitoring pH and temperature dependent pigment transformations and confirming structural features. Together, these integrated analytical advances have enabled the molecular level characterization of C. ternatea pigments and phenolics, supporting their emerging applications in functional foods, nutraceuticals, and intelligent packaging systems [4].

EXTRACTION

Ultrasound and microwave assisted extraction

Ultrasound and microwaveassisted extraction of blue pea flowers generally starts with cleaned, fresh, or dried petals that are ground to increase the surface area and then mixed with a selected solvent system, most commonly water, ethanol–water, or more recently, glycerol–water (60:40) or choline chloride–glycerol natural deep eutectic solvents for greener processing. In ultrasoundassisted extraction (UAE), the petal–solvent mixture (typical solid–liquid ratios around 1:10–1:20 g/mL or optimized petal/solvent ratios of 2.5–7% w/v) is placed in an ultrasonic bath or probe system operating at frequencies of approximately 40–42 kHz and power ≈400–500 W, with temperature controlled between 40 and 80 °C and extraction time usually 30–60 min. Cavitation bubbles formed during sonication collapse near plant tissues, generating localized shear forces and microjets that rupture cell walls and enhance mass transfer, allowing anthocyanins, phenolic acids and flavonoids to diffuse rapidly into the solvent, this translates into significantly higher total anthocyanin and phenolic yields and improved antioxidant capacity compared with conventional hotwater maceration. In comparative studies on butterfly pea, UAE performed at approximately 70 °C for 5–45 min increased total anthocyanin content (TAC) by approximately 15% relative to conventional extraction and produced extracts with superior ferricreducing antioxidant power, confirming that ultrasoundinduced cell disruption is particularly effective for this matrix [5].

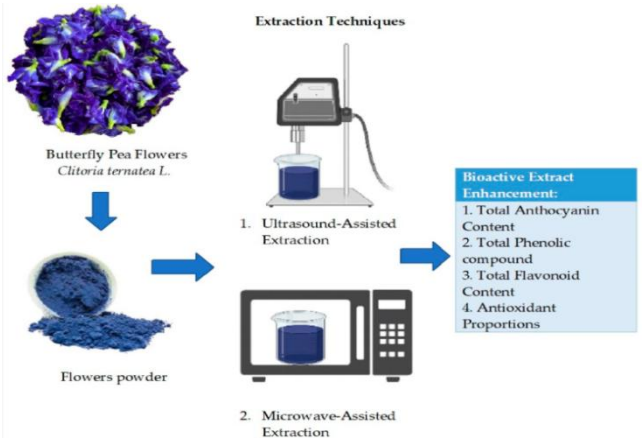

Fig 02: The Non-Conventional Extraction Processes Of Butterfly Pea Flowers

When glycerol-water (60:40) is used instead of simple water or hydroalcoholic mixtures, the resulting extracts show even higher phenolic and flavonoid contents and better anthocyanin stability, yielding viscous, foodgrade colorants that are especially suitable for beverages, gels, and films [6]. Microwave assisted extraction (MAE) follows a similar sample preparation strategy but uses microwave energy to heat the solventsolid system volumetrically, typically at 60–80 °C for short times (3–5 min) in sealed microwave vessels or laboratory microwave reactors. Rapid heating increases cell permeability and solvent penetration, giving anthocyanin yields approximately 14% higher than conventional extraction under optimized conditions while using less solvent and much shorter extraction times. However, because microwaves can cause localized overheating and degradation of thermolabile pigments, extraction times longer than about 5 min tend to reduce TAC;therefore, MAE protocols emphasize brief exposures and careful control of solvent dielectric properties [7]. Overall, current literature indicates that both UAE and MAE markedly improve extraction efficiency and process sustainability for Clitoria ternatea, with UAE (especially in glycerol–water or other green solvents) generally offering the best balance of high anthocyanin yield, preserved antioxidant activity and scalability for food applications [8].

Table 01: Extraction approaches for Clitoria ternatea pigments

Extraction method	Typical conditions (time, T, equipment)	Solvent system(s)	Main advantages
Conventional solvent extraction	60–90 °C, 1–3 h, heating with stirring; solid–liquid 1:10–1:20 g/mL	Water or ethanol–water	Simple; low equipment cost; easy to scale up
Ultrasound-assisted extraction (UAE)	40–80 °C, 30–60 min; ultrasonic bath/probe at ≈40–42 kHz, 400–500 W; 1:10–1:20 g/mL	Water, ethanol–water, glycerol–water (60:40), NADES	Higher total anthocyanin and phenolic yields; enhanced antioxidant capacity; reduced extraction time and solvent; green solvents give food-grade, stable colourants.
Microwave-assisted extraction (MAE)	60–80 °C equivalent, 3–5 min irradiation in sealed microwave vessels	Water or ethanol–water	Very short extraction time; ≈14% higher anthocyanin yield vs conventional under optimized conditions; lower solvent and energy use.

CHROMATOGRAPHIC TECHNIQUES HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC/UPLC)

High-performance liquid chromatography (HPLC) and its miniaturized variant, ultrahigh- performance liquid chromatography (UHPLC/UPLC), are the core chromatographic techniques used to separate, identify, and quantify anthocyanins and associated phenolics from blue pea (Clitoria ternatea) for research and quality control [9]. These techniques exploit differences in polarity and hydrophobicity between polyacylated delphinidin/cyanidin derivatives and co extracted phenolics, enabling resolution of complex pigment mixtures prior to spectroscopic or mass spectrometric detection [10].

Column chemistry

Chromatographic separation of blue pea anthocyanins primarilyrelies on reversephase C18 columns, which provide strong retention for aromatic, moderately non polar pigments, such as delphinidin and cyanidin based ternatins. The long C18 chains interact with the conjugated π systems of the flavonoid rings through hydrophobic and π–π interactions, allowing fine discrimination between pigments that differ only slightly in the number or position of glycosyl and acyl substituents. In conventional HPLC setups, columns are commonly 150–250 mm long and packed with 3–5 µm particles, which offer a balance between resolution, analysis time, and backpressure suitable for routine laboratory use. In contrast, ultrahigh-performance liquid chromatography(UPLC) systems use shorter columns (often 50–100 mm) with sub 2 µm particles, dramatically increasing the plate number and peak capacity and enabling high-throughput analyses without sacrificing selectivity [11]. Endcapped C18 phases are preferred to minimize secondary interactions with residual silanols, which can otherwise lead to peak tailing, especially for more polar flavonols and phenolic acids that co-elute with anthocyanin. Because ternatins are large, heavily acylated molecules, their retention is very sensitive to subtle changes in the stationaryphase characteristics; column age, lot to lot variability, and even slight differences in bonding density can alter selectivity [12]. Therefore, method validation routinely includes checks for retention reproducibility and resolution between critical pairs of ternatin isomers. The column temperature (typically 25–40 °C) is also treated as an active parameter rather than a fixed setting, as it influences both the solvent viscosity and analytestationaryphase interactions.small increase in temperature can sharpen peaks and reduce backpressure but may slightly reduce retention, requiring coordinated adjustment of gradient conditions to maintain separation quality [13].

Mobile phases and elution conditions

Most methods employ binary mobile phases consisting of acidified water and an organic solvent, usually acetonitrile or methanol, operated under gradient elution to cope with the wide polarity range of blue pea phenolics [14]. The aqueous component is acidified with 0.1–5% formic acid, trifluoroacetic acid (TFA), or similar acids to maintain anthocyanins predominantly in their flavylium cation form, which stabilizes the colored species, improves peak symmetry, and reduces the transformation to colorless chalcones or degradation products. A typical gradient might begin at 90–95% aqueous phase to elute very polar compounds (small phenolic acids, sugars), then gradually increase the organic proportion to 30–40% over 15–30 minutes to sequentially elute less polar flavonols and progressively more hydrophobic, heavily acylated ternatins [15]. The gradient profile was carefully tuned a gradient that is too steepcompresses the elution window so that multiple ternatin isomers co-elute, whereas a gradient that is too shallow lengthens the run times and can broaden the peaks due to prolonged residence on the column. Flow rates were adjusted according to the column dimensions and pressure limitsaround 0.8–1.0 mL/min for 4.6 mm i.d. HPLC columns and 0.2–0.4 mL/min for narrower UPLC columns, ensuring that the backpressure remained within the instrument specifications. In some applications, acetonitrile is preferred over methanol because of its lower viscosity (reducing backpressure) and different elution strength, which can sharpen the resolution between closely eluting anthocyanins and co-extracted flavonols(20). Method developers also evaluate alternative acid modifiers (e.g,formic vs TFA) to balance MS compatibility with chromatographic performance, as stronger ion-pairing acids such as TFA can suppress MS signals despite improving peak shapes in purely DADbased methods [16].

HPLC/UPLC with diode array detection (HPLC–DAD/UPLC–DAD)HPLC/UPLC

Diode array detection (HPLC–DAD/UPLC–DAD) is one of the most widely used analytical platforms for profiling blue pea (Clitoria ternatea) pigments and co occurring phenolics because it combines good separation efficiency with spectrally rich, yet simple, detection. In this setup, anthocyanin rich extracts (often preenriched on macroporous resins such as Amberlite XAD 16) are injected onto a reversephase C18 column and eluted with an acidified water–organic gradient, while the diodearray detector continuously records absorbance at both a diagnostic wavelength (typically 520–530 nm for anthocyanins) and across a full spectral range (≈200–600 nm) for each eluting peak. Monitoring at 520–530 nm yields sensitive chromatograms for anthocyanins, allowing accurate peak integration and quantification, whereas the full UV–Vis spectra provide qualitative information: anthocyanins display intense visible bands in the 500–600 nm region together with characteristic band I and band II signals in the UV region, while flavonols and phenolic acids show dominant absorption below 400 nm with weaker or no visible bands.

This spectral distinction enables rapid, non MS based discrimination between anthocyanins and co eluting flavonols or phenolic acids and can give preliminary hints about structural features, such as acylation (often associated with shoulder peaks and slight bathochromic shifts) or different glycosylation patterns that subtly change λmaxmax and band ratios [17]. Method development for UPLC–DAD in C. ternatea has shown the power of this combination for comprehensive profiling. A recent two dimensional UPLC–DAD–MS/MS approach for flower extracts first used UPLC–DAD to screen macroporousresinenriched fractions, then applied MS/MS to confirm structures, ultimately identifying 11 anthocyanins and 7 flavonoids with percentage peak areas >1% in the firstdimension chromatogram]18]. In study, cyanidin 3 glucoside and quercetin 3 rutinoside served as calibration

standards for total anthocyanin (TA) and total flavonoid (TF) quantification, and full method validation (linearity, LOD/LOQ, precision, accuracy) demonstrated that the UPLC–DAD method was sufficiently enough for routine quantitative analysis of blue pea pigments and flavonoids. Earlier UPLC–DAD methods, such as the study that first identified five major anthocyanins (delphinidin 3 (6″ p coumaroyl) rutinoside, [19].

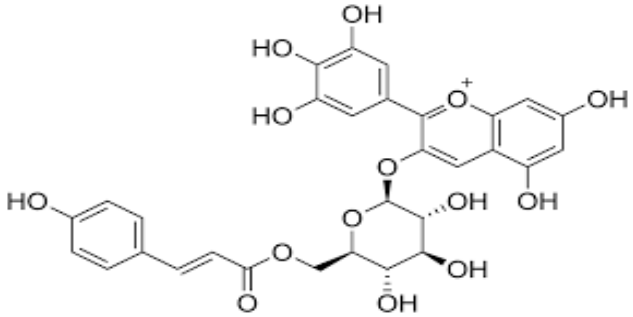

Fig 02: Delphinidin 3 (6″ p coumaroyl) rutinoside.

cyanidin 3 (6″ p coumaroyl) rutinoside, delphinidin 3 (p coumaroyl) glucoside cis/trans isomers, cyanidin 3 (p coumaroyl) glucoside and delphinidin 3 pyranoside), used the same detector to generate both quantitative chromatograms and peak specific spectra for each compound [20].

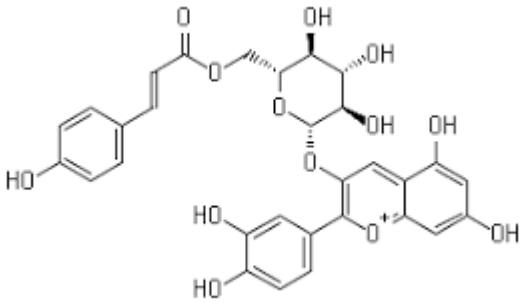

Fig 03: Cyanidin 3 (p coumaroyl) glucoside

Studies showed the relative peak intensities and spectra revealed that cyanidin 3 (p coumaroyl) glucoside was the most abundant anthocyanin, followed by its rutinoside and the corresponding delphinidin derivatives, illustrating how DAD data alone can already provide meaningful compositional insights before MS based confirmation.Beyond pure analytical characterization, HPLC/UPLC–DAD is frequently applied in the extractionoptimization and stability studies of butterfly pea extracts(9)(14). For example, the optimization of extraction conditions (solvent strength, temperature, and time) uses DAD chromatograms at 520 nm to compare total peak areas and individual anthocyanin peaks under different conditions, enabling researchers to select parameters that maximize pigment recovery without extensive degradation [21].

In green extraction studies where anthocyaninrich edible extracts are obtained (e.g,. using optimized temperature and time in acidified solvents), UPLC–DAD is used to quantify the concentrations of multiple anthocyanins and non anthocyanin phenolics (often 13 anthocyanins and 9 non anthocyanins or similar numbers) and to monitor changes during storage or processing [22]. The combination of high resolution UPLC separation with DAD spectral fingerprints thus provides a powerful, relatively low cost and MS independent way to perform routine quality control, assess extraction efficiency, and follow pigment stability in blue pea based colorants, beverages, gels, and other functional products, with MS or MS/MS added when deeper structural confirmation is required [22].

Advantages of UPLC

UPLC methods uses shorter columns and smaller particles, which increases the peak capacity and shorten the analysis time while preserving or improving the resolution. This is particularly advantageous for highthroughput screening of raw materials, anthocyaninrich fractions, and formulated products such as beverages, emulsions, or intelligent packaging films, where multiple anthocyanins and flavonoids must be quantified simultaneously(6)(27). Validated UPLC–DAD or UPLC–MS/MS methods for C. ternatea typically demonstrate low limits of detection, good precision, and strong performance under routine conditions, making them essential tools for both academic studies and industrial quality control of blue peabased colorants and nutraceutical ingredients [23].

Spectroscopic Characterization

UV–Visible spectroscopy

UV–Vis spectroscopy remains a fundamental tool for anthocyanin characterization because flavonoid chromophores exhibit distinct absorption maxima and band shapes that respond sensitively to substitution patterns and pH changes. In blue pea the extracts, visible λmaxmax typically appears in the 570–620 nm region, with shifts in peak position and intensity reflecting variations in the glycosylation and acylation of the delphinidin or cyanidin core, as well as the relative proportions of flavylium, quinonoidal, and chalcone forms under different pH conditions. For example, studies comparing water, ethanol, and hydroalcoholic extracts have shown that more highly acylated anthocyanins give stronger, narrower bands in the visible region and maintain an intense blue color over a broader pH range, whereas less substituted pigments show greater hypsochromic shifts and loss of absorbance upon neutralization [24]. In practice, UV–Vis spectra (200–700 nm) are routinely recorded for each chromatographic peak or crude extract, and λmaxmax, band ratios, and spectral shoulders are interpreted alongside retention time to support preliminary peak assignment before detailed mass spectrometric analysis. The pHdifferential method uses absorbance at the visible maximum at two pH values (e.g. 1.0 and 4.5), is also widely applied to calculate total monomeric anthocyanin content and to track pigment degradation during thermal and storage stability studies [25].

MASS SPECTROMETRY (MS AND MS/MS)

When hyphenated with HPLC or UPLC, mass spectrometry provides molecularlevel resolution that complements UV–Vis data by enabling accurate mass determination, fragmentation analysis and tentative structural elucidation of individual anthocyanins and coextracted polyphenols. In positive ion electrospray mode, blue pea pigments typically produce protonated molecular ions [M+H]+[M+H]+, whose m/z values reflect the overall degree of glycosylation and acylation, while MS/MS and MS^n experiments yield characteristic product ions corresponding to sequential loss of sugar residues (e.g. 162 Da for hexose, 146 Da for deoxyhexose) and acyl groups (e.g. 146 Da for p coumaroyl, 86 Da for malonyl) [26]. This allows distinction between closely related glycosides and acylated derivatives that may have similar UV–Vis spectra and

overlapping chromatographic behaviour, such as delphinidin 3 (6″ p coumaroyl) rutinoside versus its glucoside analogues. Recent LC–MS studies on C. ternatea have used systematic MS/MS fragmentation to map ternatin families (B , C and D type), assigning complex substitution motifs like glucose–p coumaroyl–glucose–p coumaroyl (G C G C) chains on the delphinidin core based on observed neutral losses and diagnostic fragment ions. In advanced workflows, highresolution MS and MS^n are further combined with UV–Vis and retention data to propose structures for previously unreported anthocyanins and to differentiate them from coeluting flavonols and phenolic acids, providing a robust platform for comprehensive pigment profiling in blue pea extracts and derived food systems [27].

Table 02: chromatographic and spectroscopic tools

Technique	Platform / conditions (from review)	Information obtained
HPLC/UPLC–DAD	Reverse-phase C18 columns (HPLC: 150–250 mm, 3–5 µm; UPLC: 50–100 mm, sub-2 µm); acidified water–acetonitrile/methanol gradients; monitoring at 520–530 nm and 200–600 nm.	Separation and quantification of individual anthocyanins, flavonols, phenolic acids; UV–Vis spectral fingerprints.
UHPLC–QTOF–HRMS / UPLC–ESI–MS/MS	Reverse-phase C18 with sub-2 µm particles; positive-ion ESI; MS/MS and MS^n fragmentation.	Exact mass, elemental composition, diagnostic fragment ions and neutral losses (162 Da hexose, 146 Da deoxyhexose or p-coumaroyl, etc.).
UV–Vis spectrophotometry (including pH-differential)	Spectra 200–700 nm; visible λmax typically 570–620 nm; measurements at pH 1.0 and 4.5.	λmax, band shapes, colour intensity; total monomeric anthocyanin content; pH-dependent colour changes and degradation.
CIELAB colour analysis	Measurement of L*, a*, b* and ΔE in jellies, beverages, films containing blue pea extracts.	Objective colour coordinates and overall colour difference.

Phenolic Constituents Beyond Anthocyanins

Beyond anthocyanins, blue pea (Clitoria ternatea) contains a diverse suite of phenolic constituents, notably flavonols and phenolic acids, that substantially contribute to its antioxidant capacity and overall phytochemical profile. Recent HPLC/UPLC–DAD–ESI–MS/MS studies (2022–2025) have identified quercetin, kaempferol, myricetin, rutin and their glycosides (e.g. quercetin 3 rutinoside, quercetin 3 glucoside, hyperoside, kaempferol 3 neohesperidoside) as major non anthocyanin flavonoids in flowers, teas and optimized flavonoid extracts, alongside seed profiles rich in myricetin, quercetin, catechin derivatives and rutin [28].

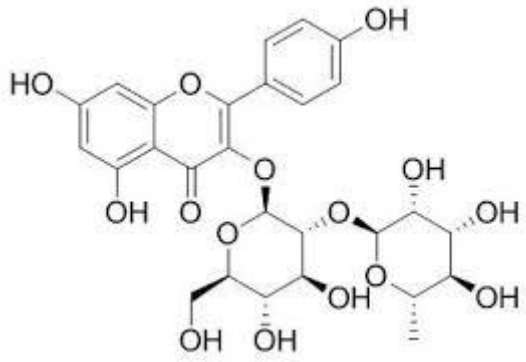

Fig 03: Kaempferol 3 neohesperidoside

UHPLC–ESI–MS/MS characterisation of seeds has also revealed a panel of at least 14 phenolic acids, including gentisic, protocatechuic, o and p coumaric and sinapic acids, whose levels vary with developmental stage and correlate with antioxidant and cytotoxic activities [29]. Chromatographic techniques separate these phenolics using reverse phase gradients, while UV–Vis signatures (band I/II positions and intensities) and diagnostic MS/MS fragments (neutral losses of 162 Da for hexose, 146 Da for deoxyhexose, 146 Da for p coumaroyl, etc.) are combined with retention characteristics to assign individual peaks and build comprehensive phenolic fingerprints for C. ternatea flowers, teas and seed extracts [30]. Collectively, these flavonols and phenolic acids act synergistically with ternatin anthocyanins to enhance radical scavenging, metal chelating and antiinflammatory potential, and they also modulate sensory attributes such as astringency in blue pea infusions through interactions with salivary proteins [31].

CONCLUSION

Advances in chromatographic and spectroscopic techniques have substantially deepened the understanding of blue pea (Clitoria ternatea L.) pigments and phenolic constituents, shifting the focus from bulk colorimetric estimations to detailed molecular characterization. High resolution platforms such as UHPLC–DAD, UHPLC–QTOF–HRMS, and tandem MS/MS now enable precise separation, identification, and quantification of complex ternatin anthocyanins and co occurring flavonols and phenolic acids, revealing how specific glycosylation and acylation patterns govern colour expression, stability, and bioactivity. In parallel, ultrasound and microwave assisted extraction, often optimized through chemometric and response surface approaches, have improved extraction yields while reducing solvent consumption and processing time, supporting greener production of

standardized, food grade anthocyanin rich extracts. Spectroscopic methods, particularly UV–Vis with pH differential analysis, remain central for rapid monitoring of pigment equilibria and degradation, while NMR and complementary techniques provide higher order structural validation when coupled with LC–MS workflows. Comprehensive profiling of non anthocyanin phenolics from flowers, teas, and seeds further links compositional fingerprints with antioxidant, antibacterial, and antibiofilm activities, positioning C. ternatea as a versatile model for natural blue colorants and multifunctional phytochemicals. Collectively, these integrated analytical and extraction advances underpin the rational development of blue peabased ingredients for applications in foods, nutraceuticals, and emerging smartpackaging systems.

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CONFLICTS OF INTEREST
The author declares no conflicts of interest.

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