

Comparative Evaluation of Spectrophotometric and HPLC-PDA Methods for Carotenoid Quantification in Cold Pressed *Capsicum annuum* Oil

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Abstract

Accurate and validated analytical methods for the measurement of carotenoids in plant oils are needed for quality control, regulatory compliance and product development in the food, nutraceutical, and pharmaceutical industries. In this work, the methods of UV–Vis spectrophotometry and high-performance liquid chromatography with photodiode array detection (HPLC–PDA) were evaluated for the quantification of carotenoids in cold-pressed *Capsicum annuum* oil under harmonized validation conditions. Both techniques were validated according to ICH Q2 (R2) principles, which include predetermined analytical target profiles, risk-based validation criteria and measurement uncertainty estimation. The spectrophotometric approach exhibited good linearity ($R^2=0.9999$), a recovery of 97.66–100.96% and an interday precision of $\leq 3.34\%$ RSD, indicating its suitability for quick screening of apparent total carotenoids represented as β -carotene equivalents. Greater specificity for β -carotene was obtained with the developed HPLC-PDA method, yielding $R^2 = 0.9992$, with 82.75–95.14% recovery and LODs and LOQs of 5.41 and 9.00 $\mu\text{g/mL}$, respectively. The apparent total carotenoid content was estimated at 161.36 ± 0.83 mg/g oil by spectrophotometric analysis, and a β -carotene concentration of 8.5 mg/g oil was determined by HPLC-PDA measurements. This example demonstrates that the two methods do not measure the same endpoint but rather address different analytical concerns. Overall, the two approaches are complementary: spectrophotometry, as a high-throughput low-cost approach, is good for quality control, whereas HPLC-PDA is suitable when compound-specific identification and regulatory evidence are needed.

Keywords: *Capsicum annuum*, Carotenoids, β -carotene, UV–Vis spectrophotometry, HPLC-PDA, Quality control

1. Introduction

Carotenoids are a class of lipophilic pigments that play various biological and ecological roles and are thus important for plant physiology and human health (Metibemu & Ogungbe, 2022; Polyakov et al., 2023). Despite being mostly known for the yellow, orange and red colors that they impart to many fruits and vegetables, these molecules are structurally integrated within cellular architectures to enable light capture, provide photoprotection and regulate signaling cascades (Sun et al., 2022). Other dietary carotenoids, such as β -carotene, lutein, zeaxanthin, lycopene and capsanthin, are also thought to have more general physiological benefits in humans beyond their provitamin A and antioxidant activities (Bas, 2024; Kim et al., 2023). These phytochemicals are most commonly found in the diet and industry in *Capsicum annuum* L. Specifically, red pepper and paprika matrices have yielded complex profiles of capsanthin, capsorubin, β -carotene, β -cryptoxanthin, lutein and zeaxanthin. The relative amounts of these constituents, however, are still highly variable and are dependent on plant genotype, maturity at harvest and processing parameters (Guzman et al., 2021; Hornero-Méndez et al., 2000; Tang et al., 2024; Wang et al., 2024).

In this context, one should highlight cold pressing, whereby, together with the avoidance of external heat stress and organic solvents during the preparation of oils by this method, lipophilic pigments and other sensitive oil-soluble components are better maintained (Yılmaz et al., 2015). However, the analysis of carotenoids in such oil matrices is associated with several well-known analytical difficulties. They are highly labile under light, oxygen, heat and alkaline conditions, which rapidly induce isomerization, while coextracted bulk lipids (and their breakdown products) often conceal target signals during detection. Ultraviolet–visible (UV–Vis) spectrophotometry is a rapid and inexpensive screening approach for determining the apparent total carotenoid concentration but is nonselective and hence limits further profiling. High-performance liquid chromatography–photodiode array detection (HPLC–PDA) provides compound-specific separation and definitive retention-time and spectrum confirmation and addresses these problems but is slower because of its long duration, high solvent usage, and complex sample preparation techniques (Kopec et al., 2012; Luterotti et al., 2013; Scott, 2001; Xu et al., 2023).

The release of ICH Q2 (R2) and ICH Q14 has moved analytical validation to predefined analytical target profiles, science and risk-based development and life-cycle management of analytical processes (ICH, 2024a; ICH, 2024b). These notions are useful for comparing methods that do not measure exactly the same end point, as long as the intended application and acceptance criteria are explicitly defined. Spectrophotometric and HPLC approaches have been utilized for carotenoid analysis, but very few studies have compared the two methods directly under the same experimental settings for cold-pressed *C. annuum* oil. This limitation is of practical importance since laboratories typically have to select between a rapid and low-cost total pigment test or a slower and compound-specific chromatographic assay for quality control, labeling or research (Dias et al., 2010; Rivera & Canela-Garayoa, 2012). Therefore, in this study, UV–Vis spectrophotometry and HPLC–PDA were used to determine the presence of carotenoids in the same *C. annuum* oil matrix according to harmonized validation criteria. The contribution of this work does not lie merely in confirming the expected higher selectivity of HPLC–PDA over UV–Vis spectrophotometry, but in defining the analytical scope of both techniques under harmonized validation criteria, clarifying their calculation bases, and providing practical decision guidance for laboratories analyzing pigment-rich *Capsicum* oil.

2. Materials and Methods

2.1 Chemicals and reagents

The chemicals employed were of analytical or HPLC grade to ensure optimal purity and analytical dependability (USP, 2024). Standard β -carotene (purity > 97%) was acquired from Sigma–Aldrich (St. Louis, Missouri, USA) and stored under nitrogen at -20°C in the dark to prevent degradation according to a previously documented stability regimen (Olennikov & Kashchenko, 2022). Isopropyl alcohol (analytical grade, Burdick & Jackson (Korea)) hexane, dichloromethane, ethyl acetate, cyclohexane, diethyl ether and methanol were purchased from Fisher Scientific UK Ltd. (Leicestershire, UK). HPLC-grade acetone and acetonitrile were purchased from Dunkans Pure Chemicals, Gyeonggi-do, South Korea. Potassium hydroxide (KOH) of analytical grade was used for the saponification because it is aligned with green chemistry principles (Rodríguez-Rodríguez et al., 2024). Deionized water was obtained from an Econos purification system (Bangkok, Thailand) and used throughout the experiments. All the solvents were filtered through $0.45\text{-}\mu\text{m}$ membrane filters before being used to achieve superior chromatographic performance (Loescher et al., 2014).

2.2 Sample collection and storage

Cold-pressed *C. annuum* oil was obtained from a certified organic cooperative in Lopburi Province, Thailand, chosen to reduce geographic variability while guaranteeing good agricultural practices. For the extraction of oil, a mechanical screw press was used at ambient temperature, ensuring that no external heat or chemical solvent (which would alter the bioactive constituents and baseline analytical integrity) was applied. The samples were immediately transferred to amber glass vials, purged with nitrogen to minimize oxidative degradation as described in standard stability protocols described by Schmidt et al. (2021), and stored in the dark between 4 and 8°C . We conducted physical–chemical checks to confirm sample stability, and all the analytical

characterizations were completed within 30 days after extraction to ensure that robust and reliable data were obtained.

2.3 Analytical target profile development

In accordance with the ICH Q2(R2) and Q14 guidelines, ATP was established for both methods prior to validation (ICH, 2024a; ICH, 2024b). For spectrophotometric analysis, an ATP-specific rapid total carotenoid quantification method with a precision $\leq 5\%$ RSD, accuracy within 95–105% recovery, and a linear response across the expected working range was used. For HPLC-PDA analysis, ATP was used to define selective β -carotene quantification with precision $\leq 10\%$ RSD, accuracy within 80–110% recovery, and chromatographic resolution of >1.5 for critical peak pairs following systematic method development approaches (TentaConsult, 2024). These profiles guided method development and established acceptance criteria for validation parameters.

2.4 Spectrophotometric analysis of total carotenoids

UV–Vis spectrophotometric analysis was performed using a Shimadzu UV–1800 spectrophotometer (Shimadzu Corporation, Kyoto, Japan). A primary stock solution of β -carotene was prepared in isopropanol at 10.0 mg/mL and stored under nitrogen at -20°C in the dark. Working standards were prepared fresh by serial dilution to obtain concentrations of 0.08–2.5 mg/mL. The analytical target of this procedure was the rapid estimation of apparent total carotenoids as β -carotene equivalents rather than selective quantification of individual carotenoids. Sample preparation was carried out by weighing 0.40 g of *C. annuum* oil (which was diluted in isopropanol in a volumetric flask under light protection). Solutions were diluted more when required to ensure that the measured absorbance was kept only within the validated limits of calibration. The absorbance spectra were recorded in the range from 300 to 600 nm using standard 1-cm quartz cuvettes, and quantification was performed at 449 nm. The apparent total carotenoid content (TCCapp, mg/g oil) was determined according to the following equation: $\text{TCCapp} = C \times V \times \text{DF}/m$, where C is the concentration of β -carotene equivalents obtained through the calibration curve (mg/mL), V is the final volume (mL), DF represents the dilution factor used in this study and m corresponds to the sample mass. Owing to the nonselective nature of detection at 449 nm, which denotes UV–Vis absorption where many carotenoids coelute, the analytical output is reported herein as apparent total carotenoids expressed as β -carotene equivalents and thus may falsely incorporate contributions from other overlapping carotenoids and/or coabsorbing chromophores in the sample matrix.

2.5 HPLC–PDA analysis of β -carotene

Measurements were conducted with a photodiode array detector (Agilent Technologies, Santa Clara, California, USA) on an Agilent 1260 Infinity II within a temperature-controlled and vibration-isolated room for stability and repeatability assurance. Through the use of double sequential multisolvent extraction, lipid interference was eliminated, and carotenoids were retained (Breithaupt & Bamedi, 2002). Briefly, 1.0 g of *Capsicum* oil was dissolved in 50 mL of isopropanol, and 10 mL of 20% (w/v) KOH in methanol was added. The mixture was agitated well on a vortex and incubated in the dark at room temperature for 60 min with occasional shaking for complete saponification. Carotenoids were subsequently isolated by serial extraction with 3×20 mL of diethyl ether, hexane, dichloromethane, ethyl acetate and cyclohexane, following the recognized procedure designed for the isolation of carotenoids (Stinco et al., 2014). The combined organic phases were washed with deionized water to a neutral pH, dried with anhydrous sodium sulfate and concentrated on a rotary evaporator under reduced pressure at 40°C . The dried residue was reconstituted in 2.0 mL of HPLC-grade acetone and filtered through 0.22 μm PTFE syringe filters before analysis.

Chromatographic separation was accomplished with a Poroshell EC-C18 column (2.1×150 mm, 4 μm particle size; Agilent Technologies) held at 30°C according to previously reported chromatographic systems (García-Plazaola & Esteban, 2021). Although C30 columns possess superior resolution for geometrical isomers, as shown in previous comparative studies (Gupta et al., 2015; Sander et al., 2000), the C18 column was chosen in this work for practical reasons related to its commercial availability, cost, and sufficient separation of the target compounds. The mobile phase was composed of acetone (solvent A) and water (solvent B) and was delivered in gradient mode as follows: 0–5 minutes, 75% A; 5–10 minutes, 75–95% A; 10–17 minutes, 95% A

isocratic; 17–22 minutes, 95–100% A linear gradient; 22–27 minutes, 100% A isocratic; and 27–30 minutes, 100–75% A returning to initial conditions. The flow rate was set at 0.35 mL/min, with an injection volume of 10 μ L and an equilibration time of 5 minutes between injections, according to robustness testing (Ornelas-Paz et al., 2007). Detection was performed at 450 nm with PDA spectral acquisition from 250 to 700 nm for peak identification and purity assessment (de Rosso & Mercadante, 2007). β -Carotene standards prepared in acetone over the concentration range of 3.125–200 μ g/mL were used for external calibration. β -Carotene identification was based on three criteria: (i) retention time agreement with the authentic β -carotene standard, (ii) matching of the PDA spectral maxima and fine structure, and (iii) acceptable peak purity with no coeluting matrix peaks in the blank or stressed sample chromatograms.

2.6 Method validation according to the ICH Q2 (R2)

Both analytical methods were comprehensively validated following the ICH Q2(R2) guidelines, with an increased emphasis on risk-based approaches and measurement uncertainty assessment (ICH, 2024a; Swartz & Krull, 2012). The validation included specificity, linearity, accuracy, precision, sensitivity, robustness, and system suitability testing according to modern validation frameworks (Voigtman, 2017).

2.6.1 Specificity and selectivity

Spectrophotometric specificity was evaluated by analyzing solvent blanks, oil matrix blanks, and standard solutions to identify potential interferences following established protocols (Eurachem, 2024). For HPLC analysis, specificity was assessed through peak purity analysis via photodiode array spectral data; forced degradation studies under stress conditions (light, heat, acid, base, and oxidation); and analysis of matrix blanks to confirm the absence of coeluting peaks (Scott et al., 1996).

2.6.2 Linearity

Calibration curves were constructed using a minimum of six concentration levels spanning 80–120% of the target analytical range following statistical guidelines (European Committee for Standardization, 2020). Linear regression analysis was performed with evaluation of correlation coefficients (R^2), residual analysis, and statistical assessment of linearity assumptions. Lack-of-fit testing was conducted to confirm the linearity validity according to ISO standards (ISO, 2019-2023).

2.6.3 Accuracy

Recovery studies were performed at three concentrations (low, medium, and high), representing 80%, 100%, and 120% of the target concentrations, respectively, following regulatory requirements (FDA, 2023). β -Carotene standards were spiked into representative matrix samples and analyzed in triplicate. Recovery percentages were calculated and evaluated against predetermined acceptance criteria established in the analytical target profiles.

2.6.4 Precision

Repeatability (intraday precision) was assessed through six replicate analyses of quality control samples at three concentrations on a single day (Tangri et al., 2012). Intermediate precision (interday precision) was evaluated through analyses on three separate days by different analysts following best practices for method transfer (Compliance Online, 2024). Relative standard deviation (%RSD) values were calculated and compared against acceptance criteria.

2.6.5 Sensitivity

The limits of detection (LODs) and limits of quantitation (LOQs) were calculated by signal-to-noise procedure for chromatographic methods and by statistical methods based on calibration curve parameters as described in established protocols (AOAC, 2012; CLSI, 2012). The LOD was defined as the concentration that yielded a signal three times the noise level, and the LOQ was defined as the concentration that gave a signal-to-noise ratio of 10:1.

2.6.6 Robustness

The robustness of the method was tested by systematic variation of analytical parameters, including column temperature ($\pm 2^{\circ}\text{C}$), flow rate ($\pm 0.03\text{ mL/min}$), mobile phase composition ($\pm 2\%$) and analyst-to-analyst variation (Thompson et al., 2002). To determine the statistical significance of the parameter effects, analysis of variance (ANOVA) was performed after extensive validation procedures (Horwitz, 1982).

2.6.7 Measurement uncertainty

An extended uncertainty analysis was performed according to ISO/IEC Guide 98-3, considering contributions from calibration standards, sample preparation, instrument precision and the environment (Majcen et al., 2010). Calculations were based on a bottom-up approach, and uncertainty propagation was conducted according to state-of-the-art methods via Monte Carlo simulation (Majcen et al., 2011).

2.6.8 Statistical analysis

Statistical analyses were performed using IBM SPSS Statistics (version 29.0). The validation data are presented as the means, standard deviations, relative standard deviations, recovery percentages, regression equations, coefficients of determination, LODs, LOQs, and estimates of measurement uncertainty. Linearity was assessed by least-squares regression and residual inspection. When appropriate, comparisons of robustness conditions were tested using one-way ANOVA, with $p < 0.05$ considered to indicate statistical significance.

2.7 Cost and practical performance assumptions

The cost per analysis was approximated on the basis of the consumption of laboratory-scale reagents and solvents, disposable consumables (cuvettes, syringe filters, HPLC vials, and pipette tips), approximate equipment operating/depreciation costs, and analyst time needed for routine sample preparation and analysis. These data points are for relative comparison across methodologies under the authors' laboratory settings and should not be considered to be universal pricing.

3. Results

3.1 Analytical target profile achievement

Both analytical methods were successfully validated according to their predefined analytical target profiles and were found to be appropriate for their intended purpose. The spectrophotometric approach satisfies the needs for quick screening of apparent total carotenoids with interday precision of $\leq 3.34\%$ RSD, recoveries of 97.66–100.96% and good linearity. The HPLC-PDA approach was selective for the quantification of β -carotene, with an interday precision of $< 7.3\%$ RSD, recovery of 82.75–95.14% and sufficient chromatographic performance for the β -carotene peak.

3.2 Method validation performance

The complete validation findings showed that the method was fully compliant with the ICH Q2(R2) analytical standards, demonstrating its applicability for its intended applications (Swartz & Krull, 2012). Table 1 provides the major performance metrics.

3.2.1 Linearity and range

The spectrophotometric method exhibited linearity over 0.08–2.5 mg/mL, with $y = 1.0212x + 0.0117$ and $R^2 = 0.9999$. The HPLC-PDA method demonstrated linearity for β -carotene concentrations over 3.125–200 $\mu\text{g/mL}$, with $y = 9.5465x - 45.906$ and $R^2 = 0.9992$. Residual inspection did not indicate systematic curvature within the validated ranges. The calibration curves for both procedures are shown in Figure 1.

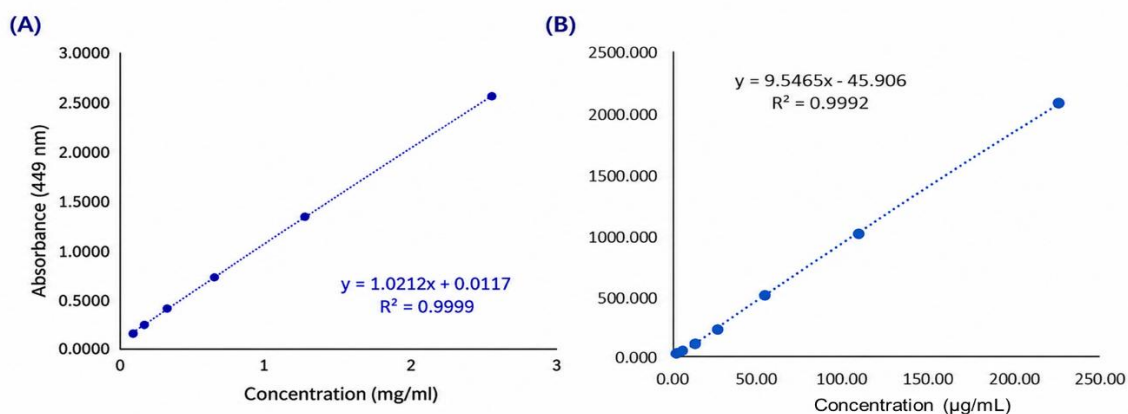


Figure 1. Calibration curves for carotenoid analysis by UV–Vis spectrophotometry and HPLC–PDA. (A) β -Carotene-equivalent calibration for spectrophotometric quantification at 449 nm. (B) β -Carotene calibration for HPLC-PDA analysis at 450 nm.

3.2.2 Accuracy and recovery

Recovery experiments indicated that both methods are sufficiently accurate within the respective acceptance criteria on the basis of the established ATP (ICH, 2023b). Recovered values at low (97.66%), medium (100.96%), and high (100.71%) concentrations ranged from 95–105% according to the spectrophotometric method. HPLC recovery rates of 82.75%, 92.30%, and 95.14% at the indicated concentrations were obtained within the acceptance criteria of 80–110%. Indeed, some release of carotenoids might occur during saponification and multisolvent extraction (Lv et al., 2023). The slightly lower HPLC recoveries are associated with a certain degree of carotenoid degradation, as suggested by previous work on carotenoid analysis in complex lipid matrices (Lv et al., 2023).

3.2.3 Precision and reproducibility

Both approaches achieved high precision according to established acceptance criteria (Tangri et al., 2012). This was due to the excellent repeatability of the spectrophotometric method, with an intraday RSD < 0.015% and an interday precision $\leq 3.34\%$, demonstrating excellent method precision. HPLC analysis revealed acceptable precision (intraday coefficients of variation < 5.3%, interday < 7.3%), which is in accordance with the international guidelines for bioanalytical methods (FDA, 2023).

3.2.4 Sensitivity and detection capability

The HPLC-PDA assay displayed excellent sensitivity, with an LOD of 5.41 $\mu\text{g/mL}$ and a LOQ of 9.00 $\mu\text{g/mL}$, which appeared to be suitable for quantifying trace-level carotenoids with the desired sensitivity. On the other hand, the spectrophotometric method presented an LOD of 0.10 mg/mL and an LOQ of 0.22 mg/mL, which, although higher in absolute terms, were suitable for determining total carotenoids in high-concentration matrices. The sensitivity of HPLC-PDA is improved significantly, which is very advantageous for the measurement of single carotenoid concentrations at low concentrations or in diluted samples.

3.2.5 Robustness and system suitability

Both methods were robust under the selected conditions. With respect to spectrophotometry, the solvent source, measurement delay, and room temperature variation ($\pm 2^\circ\text{C}$) did not significantly affect the analytical results. The β -carotene stock solution remained suitable for at least 7 days at 4–8 $^\circ\text{C}$ in the dark, with degradation < 5%. For HPLC-PDA, no significant changes in peak area or retention time were observed after minor changes to column temperature (28–32 $^\circ\text{C}$) or flow rate (0.32–0.38 mL/min) (ANOVA, $p > 0.05$). These results support routine use provided that light exposure, oxygen contact, and prolonged alkaline treatment are minimized.

3.2.6 Measurement uncertainty assessment

The expanded measurement uncertainty was $\pm 4.2\%$ for spectrophotometric analysis and $\pm 8.7\%$ for HPLC-PDA analysis at approximately 95% confidence. The greatest contributions came from standard preparation, sample preparation and instrumental precision. The increased uncertainty for HPLC-PDA was indicative of the multistep saponification, extraction, concentration, reconstitution and filtration procedure. These values are appropriate for the intended quantitative goals and provide practical boundaries for the interpretation of discrepancies between batches or techniques.

Table 1. Summary of the analytical validation parameters for the spectrophotometric and HPLC–PDA methods used in the determination of carotenoids in *Capsicum annuum* oil.

Validation parameter	Spectrophotometry	HPLC–PDA (β -Carotene)
Analyte	Total carotenoids (as β -carotene equivalents)	β -Carotene
Concentration range	0.08–2.5 mg/mL	3.125–200 μ g/mL
Linearity (R^2)	0.9999	0.9992
Regression equation	$y = 1.0212x + 0.0117$	$y = 9.5465x - 45.906$
Limit of detection (LOD)	0.10 mg/mL	5.41 μ g/mL
Limit of quantitation (LOQ)	0.22 mg/mL	9.00 μ g/mL
Recovery (%)	97.66–100.96	82.75–95.14
Precision (%RSD)	Intraday: $< 0.015\%$ Interday: $\leq 3.34\%$	Intraday: $< 5.3\%$ Interday: $< 7.3\%$
Measurement uncertainty (95% CI)	$\pm 4.2\%$	$\pm 8.7\%$
Robustness	Stable under minor solvent/source variations	No significant variation with $\pm 2^\circ\text{C}$ or ± 0.03 mL/min
Analysis time	< 5 minutes/sample	~ 30 minutes/sample
Specificity	Nonselective; total absorbance at 449 nm	Peak confirmed by retention time and UV spectrum
Cost per analysis	\$0.12	\$3.50
Practical suitability	Routine QC, rapid screening, high- throughput	Regulatory testing, nutritional labeling, research

Notes: Validation data according to ICH Q2 (R2) The numbers for measurement uncertainty are the expanded uncertainty at approximately the 95% confidence level. Cost estimates are based on laboratory-scale solvents/reagents, consumables, instrument operating/depreciation cost and analyst time under the authors' laboratory settings and are meant for relative comparison rather than universal pricing.

3.3 Quantification of carotenoids in *Capsicum annuum* oil

Both validated methods were successfully used to estimate the carotenoid content in the cold-pressed oil of *Capsicum annuum*, resulting in complementary analytical data that are in line with recent work on *Capsicum* carotenoid diversity (Gong et al., 2024; Haile et al., 2023).

3.3.1 Total carotenoids by spectrophotometry

The total apparent carotenoid content was 161.36 ± 0.83 mg/g oil ($16.14 \pm 0.08\%$ w/w), represented as β -carotene equivalents. The value was computed using the β -carotene calibration curve with $\text{TCCapp} = (C \times V \times \text{DF})/m$, where C was obtained from the absorbance at 449 nm, V was the final dilution volume, DF was the dilution factor and m was the oil mass in grams. The word ‘apparent’ is employed because spectrophotometry is used to measure the total absorbance of all the chromophores at 449 nm. Therefore, the value should not be considered the chromatographic sum of resolved carotenoids. The UV–Vis spectrum showed a typical carotenoid absorption profile with a λ max at ~ 449 nm (Figure 2).

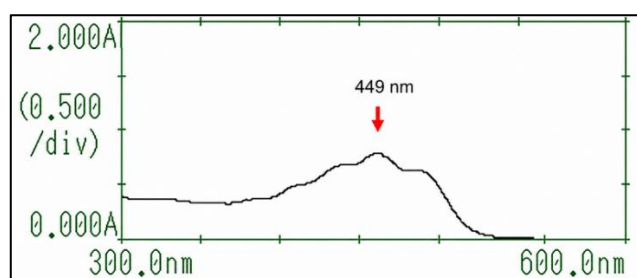


Figure 2. UV–Vis absorption spectrum of cold-pressed *Capsicum annuum* oil diluted in isopropanol. The marked absorbance maximum at approximately 449 nm was used for apparent total carotenoid quantification as β -carotene equivalents.

3.3.2 β -Carotene quantification by HPLC-PDA

The β -carotene peak was identified at 14.9 ± 0.1 min and verified by matching the retention time with that of the authentic standard and the PDA spectrum (Figure 3). The PDA spectra revealed characteristic β -carotene absorption maxima of ca. 453 and 478 nm with adequate fine structures, and the peak-purity assessment did not indicate relevant coelution. The β -carotene concentration was measured and recorded as 8.5 mg/g oil. To prevent confusion between analytical precision and sample heterogeneity, the value is now given independently of technique precision. Table 1 indicates the verified analytical precision (intraday < 5.3%, interday < 7.3%), and any larger between-aliquot or between-bottle variance, if seen, must be ascribed to sample heterogeneity rather than the precision of the instrument.

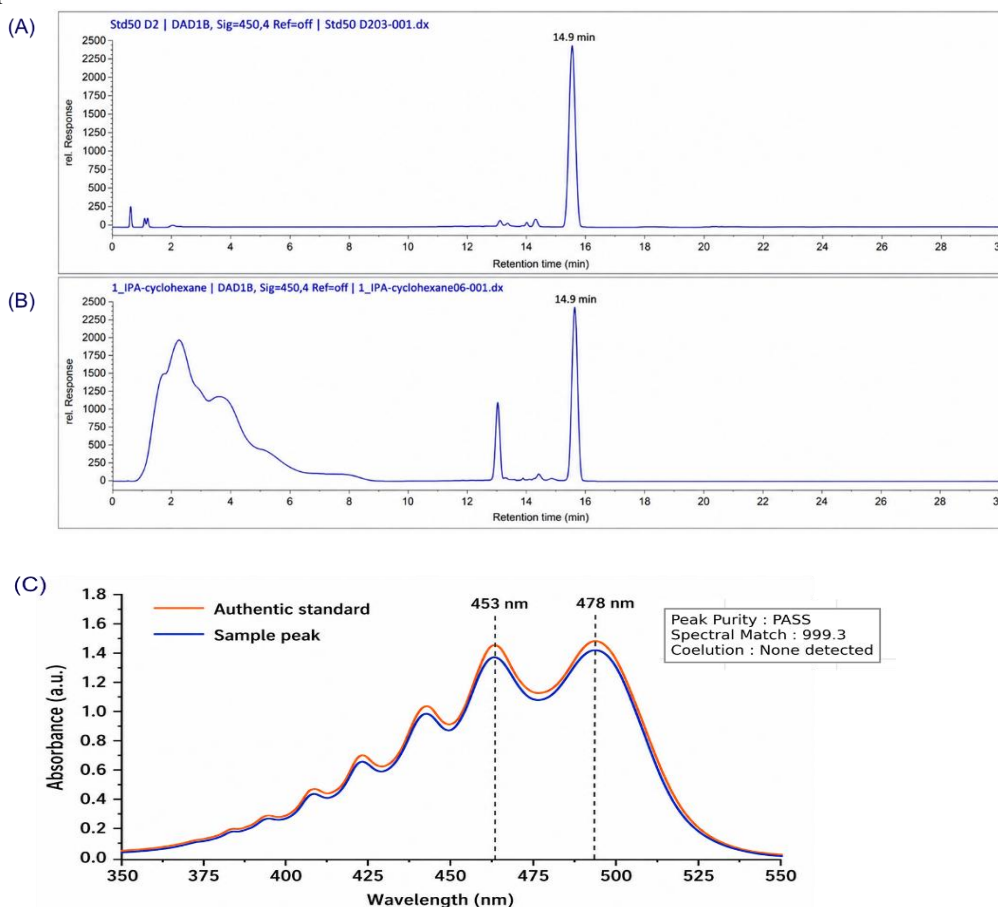


Figure 3. HPLC-PDA chromatograms and spectral confirmation of β -carotene. (A) Authentic β -carotene standard. (B) *Capsicum annuum* oil sample. (C) PDA spectrum and peak purity assessment of the β -carotene peak.

3.3.3 Basis for complementary method comparison

The two methodologies have different analytical goals and therefore should not be expected to produce the same numerical results. The apparent total carotenoids were estimated as β -carotene equivalents using UV–Vis spectrophotometry, whereas the HPLC–PDA method provided chromatographic β -carotene quantification. The comparison is therefore based on the intended use, validation performance, selectivity, cost, analysis time, and practical suitability. In routine quality control, spectrophotometry can rapidly screen for batch-to-batch pigment consistency, while HPLC-PDA can confirm the content of a specified carotenoid for labeling, regulatory, or research purposes. The difference between apparent total carotenoids (161.36 mg/g) and β -carotene (8.5 mg/g) is consistent with the known diversity of *Capsicum* carotenoids, particularly the presence of capsanthin, capsorubin, β -cryptoxanthin, lutein, and zeaxanthin.

3.4 Practical method performance comparison

A practical comparison confirmed the expected trade-off between specificity and rapidity. Spectrophotometry was fast (<5 min/sample), used a minimum amount of solvent, and was inexpensive but provided a nonselective estimate of total chromophores. HPLC-PDA was more labor intensive in sample processing and time consuming (~30 min/chromatographic run) but allowed compound-specific identification and quantification. In this study, principal component analysis and multivariate plots were excluded because the dataset was limited to validation and practical performance metrics. This eliminated overstatement of the statistical evidence and sharpened the comparison to analytically significant criteria.

4. Discussion

The validation results indicate that spectrophotometry and HPLC-PDA are fit for different analytical applications within the framework of the ICH Q2 (R2) guidelines. The spectrophotometric method is excellent for rapid screening of apparent total carotenoids because of its linearity and precision. However, owing to the nonselective absorbance at 449 nm, the results must be interpreted as β -carotene-equivalent total chromophores rather than chromatographically confirmed total carotenoids. HPLC-PDA has reduced throughput but higher specificity because β -carotene is isolated from matrix components and is identified by retention time and PDA spectral features. The poorer recoveries of HPLC-PDA than those of spectrophotometry might be associated with saponification, solvent partitioning, concentration, reconstitution and filtration in the chromatographic process. Every step may lead to the loss of analytes, oxidation or isomerization, particularly for light- and oxygen-sensitive carotenoids. Therefore, HPLC-PDA is the method of choice for compound-specific quantification, but sample preparation must be carefully monitored and, when possible, further refined to improve recovery and reduce preparation time. The apparent total carotenoid value should be interpreted with caution. The carotenoid levels quoted for fresh or dried *Capsicum* fruit are often on a fresh or dry weight basis and are therefore not directly comparable with those of a concentrated oil or oleoresin-like matrix. Spectrophotometric measurements expressed as β -carotene equivalents may be higher than those of HPLC-resolved β -carotene because capsanthin, capsorubin, xanthophyll esters, degradation products or other coabsorbing chromophores contribute to the absorbance at 449 nm. Therefore, the spectrophotometric results should be used as a quick pigment indicator for quality control, while HPLC-PDA should be employed when exact carotenoid levels are needed.

For routine quality control in the manufacture of food, nutraceuticals, or natural colors, the spectrophotometric approach is effective when the goal is rapid monitoring of pigment uniformity from batch to batch. It is a good technique for screening large numbers of samples because it is affordable, simple in terms of sample preparation and short in analysis time. However, the method cannot differentiate between β -carotene and capsanthin, capsorubin, xanthophylls or degradation products and should thus not be used for compound-specific claims. To answer queries about nutritional labeling, regulatory paperwork or research that requires a specific carotenoid, HPLC-PDA is more appropriate. Selective quantification of β -carotene enables laboratories to support provitamin A-related interpretation and to document compound identity with chromatographic retention and PDA spectral data. Therefore, the updated comparison should be seen as a judgment framework rather than a straightforward verification of equivalence between two metrics. Both procedures are more acceptable for

regulatory purposes when the validation concepts of ICH Q2(R2), analytical target profiles, and risk-based approaches are utilized (Swartz & Krull, 2012). A review of all sources of uncertainty is essential for the interpretation of results and quality by design principles during the stage of analytical development (European Commission, 2010). These features are in line with upcoming legislation requiring science-based approaches and life cycle management of analytical methods (TentaConsult, 2024).

Some technical issues define when the two strategies are feasible. The spectrophotometric method remains a constrained Beer–Lambert-based procedure that demands very careful and controllable dilution, matrix background and blank correction. Inherent coextracted components such as chlorophyll derivatives, oxidized lipids or synthetic additives can interfere with the absorbance at 449 nm and therefore overestimate the carotenoid output. Hence, the spectrophotometric values need to be reported explicitly as apparent total carotenoids in β -carotene equivalents. Multistep sample preparation is important for HPLC analysis because of the numerous unique sources of analytical uncertainty. Saponification parameters such as temperature, duration and alkaline strength substantially affect the recovery of carotenoids and their structural stability. Sequential solvent extraction can be a useful technique for bulk lipid elimination, but certain polar carotenoids can be selectively removed, and artifacts of isomerization can form. Thus, simplified preparation strategies (e.g., direct injection or solid-phase extraction) that generally improve recovery and reduce analysis duration are often good candidates for future method development (Rodríguez-Rodríguez et al., 2024). The C18 and C30 stationary phases are crucial factors during HPLC optimization. In this work, a C18 column was used for practical constraints unrelated to the structural separation of carotenoids, but C30 phases provide increased the baseline separation of carotenoid geometrical isomers (Gupta et al., 2015; Sander et al., 2000). This enhanced selectivity is particularly useful for more detailed chemoprofiling or when thermally treated samples are likely to be enriched in heat-induced *cis*-isomers (Ivane et al., 2024).

Carotenoid stability is essential for the interpretation of both techniques. Amber glass containers, nitrogen flushing, low-temperature storage (4–8°C), subdued-light sample preparation, fresh working standards and short analysis windows were employed in this work to decrease the risk of deterioration. However, β -carotene and other *Capsicum* carotenoids can be oxidized, photodegraded and isomerized (*cis-trans*) during saponification, solvent partitioning, evaporation and reconstitution. These processes might partially explain the worse HPLC-PDA recovery than that of the spectrophotometric results. Future studies should include formal stability-indicating assays, such as time-course exposure to light, oxygen, heat and alkaline conditions, to assess carotenoid losses during sample preparation and storage.

The environmental impact of analytical procedures is becoming increasingly relevant in ordinary laboratories. The spectrophotometric approach has many advantageous green analytical features, such as minimal solvent volume, limited waste generation and less instrument time. These characteristics make it a useful first-line screening approach where chemical specificity is not needed. HPLC-PDA has higher solvent consumption and results in more waste. However, the ecological impact can be decreased by solvent recycling, shorter columns, columns with smaller internal diameters, UHPLC transfer and a greener choice of mobile phase. Such advances must be weighed against the need for sufficient carotenoid separation and spectral confirmation.

Future efforts should be directed toward the development of a comprehensive carotenoid profiling technique with an enhanced sample preparation strategy and a rigorous stability-indicating validation protocol. Improved separation of closely related carotenoid isomers and of more complex esterified forms can be achieved by the use of C30 stationary phases or liquid chromatography–tandem mass spectrometry systems. Switching to simpler extraction methods, solid-phase clean-up or direct-dilution procedures can also reduce oxidative loss and increase sample throughput. Newer technologies such as hyperspectral imaging and chemometric modeling are proving effective for screening purposes, whereas traditional chromatographic validation is still needed to support any chemical-specific claims. Accurate carotenoid analysis is still crucial for food quality control, nutrition labeling and general public health monitoring despite the introduction of new technologies. In practice, the most appropriate procedure depends on the analytical purpose. Spectrophotometry is appropriate for rapid high-throughput pigment screening for day-to-day quality control, whereas HPLC-PDA delivers the specific and accurate β -carotene data needed for nutritional analysis, bioavailability studies and regulatory submissions.

5. Conclusion

This work presents a comparative analysis of UV–Vis spectrophotometry and HPLC–PDA for the detection of carotenoids in cold-pressed *C. annuum* oil. Both procedures were validated in line with the principles of ICH Q2(R2) comprising established analytical target profiles, risk-based acceptance criteria and measurement uncertainty estimation. The spectrophotometric approach exhibited excellent linearity ($R^2 = 0.9999$), strong recovery, and high precision and is appropriate for quick screening of apparent total carotenoids as β -carotene equivalents. The HPLC-PDA approach showed better specificity and sensitivity for β -carotene measurement, which was confirmed by the retention time and PDA spectrum. The numerical difference between the apparent total carotenoids (161.36 mg/g oil) and β -carotene (8.5 mg/g oil) should be taken as an indicator that the two techniques are aimed at different analytical end points. Spectrophotometry provides a total chromophore index useful for general quality control; HPLC-PDA provides the compound-specific data needed for labeling, regulatory documentation or mechanistic studies. Therefore, the comparison in this study provides more practical guidance for technique selection than a straightforward comparison of equivalence does. Future work includes chromatographic profiling of numerous carotenoids in *Capsicum*, formal stability investigations and enhanced sample-preparation techniques for better recovery and shorter analysis times. A practical way to ensure the quality of pigment-rich oil from *Capsicum* and related functional foods or nutraceutical products is to combine rapid spectrophotometric screening with specific validation by HPLC-PDA.

Conflict of interest

None.

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Data availability statement

All the data produced or analyzed during this research are included in this published publication. Additional raw data supporting the findings are available from the corresponding author upon reasonable request and subject to proper data sharing agreements.

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