MS.ID-IJPBS-4004
International Journal of Pharmacy and Biological Sciences-IJPBS™ (2026) 16 (4): 29-38
Online ISSN: 2230-7605, Print ISSN: 2321-3272
Review Article | Pharmaceutical Sciences | OA Journal | MCI Approved | Index Copernicus

Contemporary Analytical Perspectives on the Characterization Quantification and Validation Challenges of Natural Isothiocyanates

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Received: 19 May 2026/ Accepted: 8 Jun 2026 / Published online: 1 Oct 2026
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Abstract

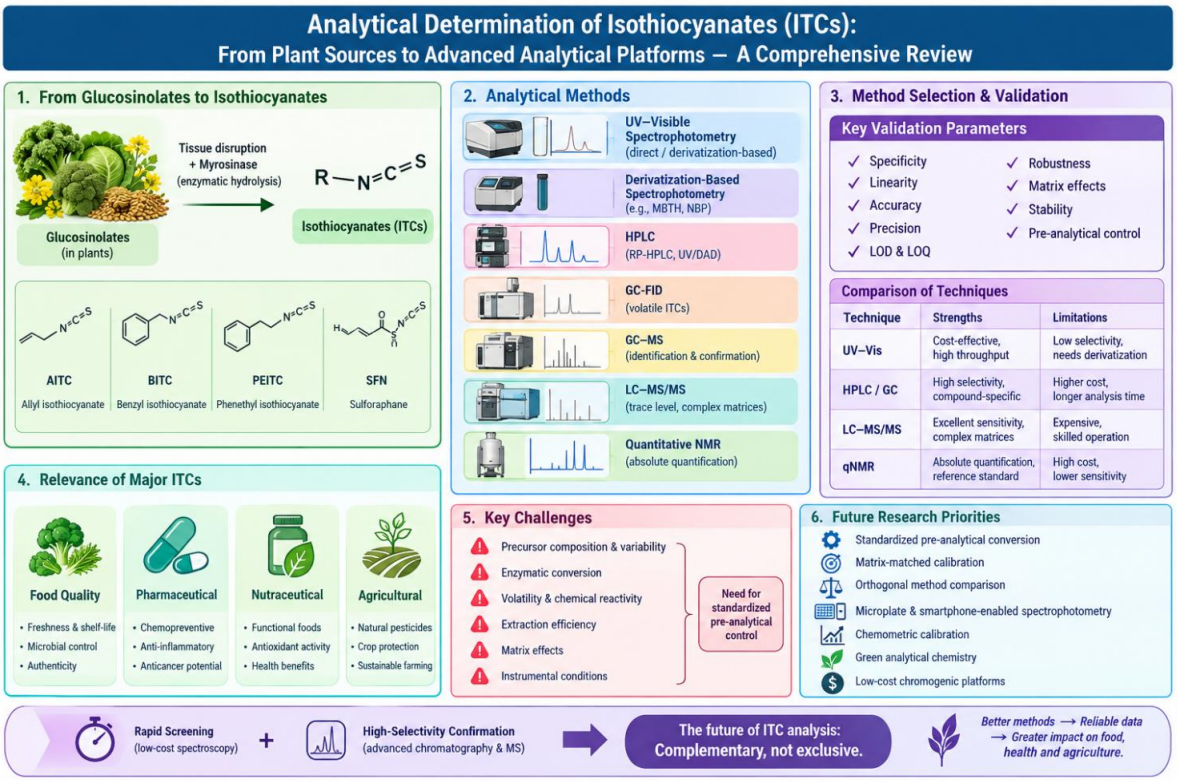
Isothiocyanates (ITCs) are electrophilic sulfur-containing phytochemicals generated predominantly from glucosinolates following tissue disruption and myrosinase-mediated hydrolysis. Among the most investigated members, allyl isothiocyanate (AITC), benzyl isothiocyanate (BITC), phenethyl isothiocyanate (PEITC) and sulforaphane (SFN) occupy an important interface between plant biochemistry, food quality, nutraceutical development and pharmaceutical research. From an analytical chemistry perspective, however, ITCs are challenging targets because their measured concentrations are strongly influenced by precursor composition, enzymatic conversion, volatility, chemical reactivity, extraction efficiency, matrix effects and instrumental conditions. This review critically examines the analytical determination of AITC, BITC, PEITC and SFN using UV–visible spectrophotometry, derivatization-based spectrophotometry, HPLC, GC-FID, GC–MS, LC–MS/MS and quantitative NMR. Particular emphasis is placed on method selection, pre-analytical control and validation parameters including specificity, linearity, accuracy, precision, limit of detection, limit of quantification, robustness, matrix effects and analyte stability. UV–visible methods offer important advantages in cost and throughput but generally require a chemically justified derivatization strategy when direct absorbance is weak or non-selective. Chromatographic and mass-spectrometric approaches provide superior compound-specific information and remain essential for confirmatory analysis, complex matrices and trace-level biological investigations. The review further discusses the food, pharmaceutical, nutraceutical and agricultural relevance of the four major ITCs, while separating established analytical evidence from biological hypotheses that remain predominantly preclinical. Finally, research priorities are proposed, including standardized pre-analytical conversion, matrix-matched calibration, orthogonal method comparison, microplate and smartphone-enabled spectrophotometry, chemometric calibration, green analytical chemistry and the experimental development of low-cost chromogenic platforms. The overall perspective is that future ITC analysis should combine rapid

screening with high-selectivity confirmation rather than treat low-cost spectroscopy and advanced chromatography as mutually exclusive technologies.

Keywords
Allyl isothiocyanate; Benzyl isothiocyanate; Phenethyl isothiocyanate; Sulforaphane; analytical validation.

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Graphical Abstract:



1. INTRODUCTION

Isothiocyanates constitute a chemically diverse family of plant-derived secondary metabolites defined by the electrophilic –N=C=S group. They are especially abundant as hydrolysis products of glucosinolates in Brassicaceae plants. The parent glucosinolates are relatively stable storage forms, while tissue disruption allows endogenous myrosinase to contact the substrate and generate an unstable aglycone that may rearrange into an ITC or other products. This biochemical architecture has direct analytical consequences: the concentration of an ITC in a processed sample is not necessarily a fixed property of the plant material, but an outcome of substrate abundance, enzyme activity, tissue disruption, temperature, pH, processing history, extraction and storage. [1,2]

The supplied manuscript identifies AITC from sinigrin, BITC from glucotropaeolin, PEITC from gluconasturtiin and SFN from glucoraphanin as representative precursor-product relationships. That framework is consistent with methodological reviews showing that sample preparation and myrosinase activation are among the major determinants of ITC recovery. A dedicated methodological assessment concluded that extraction and analytical pipelines vary substantially across ITCs and matrices, making method standardization a central challenge.[3]

The analytical literature has consequently evolved from simple total-ITC spectrophotometry toward compound-specific chromatography, tandem mass spectrometry, qNMR and, more recently, sensor and chemometric approaches. At the same time, there remains a practical need for inexpensive and rapid methods that can screen large numbers of food, botanical and nutraceutical samples before more selective techniques are applied.[4]

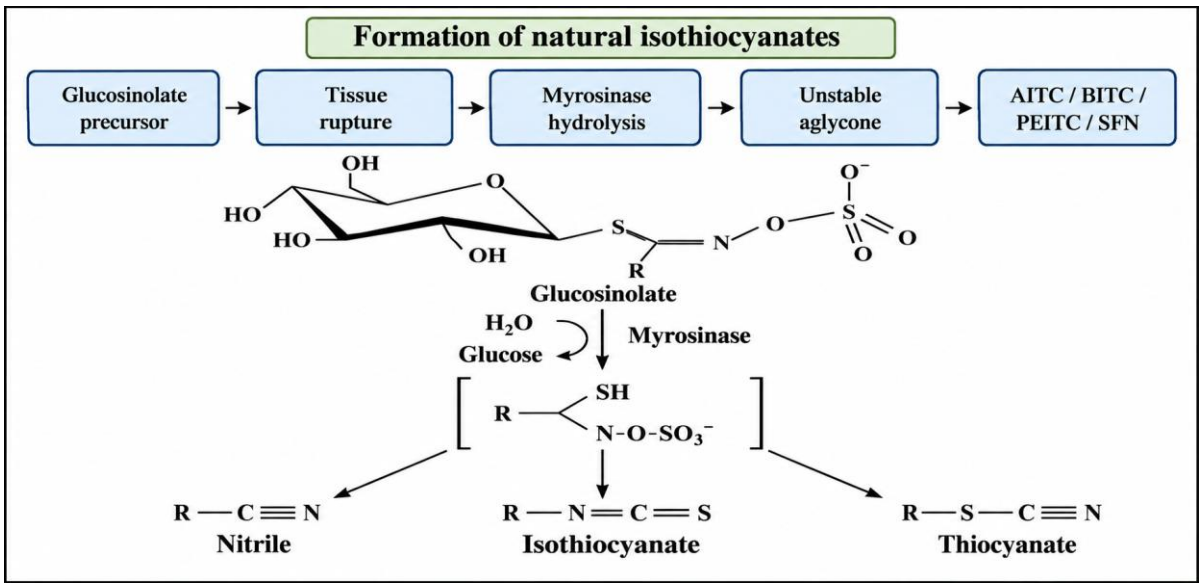


Figure 1: Formation Of Natural Isothiocyanates from Glucosinolate Precursors.

2. Chemical and biological profiles of major ITCs

ITC	Representative precursor	Major natural sources	Analytical characteristics	Principal research areas
AITC	Sinigrin	Mustard, horseradish, wasabi	Relatively volatile; susceptible to handling and extraction losses	Food flavor, antimicrobial activity, preservation, agriculture
BITC	Glucotropaeolin	Garden cress, nasturtium, mustard	Reactive aromatic ITC; matrix-dependent recovery	Antimicrobial, pharmacological and nutraceutical research
PEITC	Gluconasturtiin	Watercress, Brassica vegetables	Aromatic ITC; studied extensively in biological models	Chemoprevention, inflammation and pharmacological research
SFN	Glucoraphanin	Broccoli, broccoli sprouts	Reactive; direct UV response is limited; analytical stability is critical	Nrf2 biology, nutraceuticals, chemoprevention and functional foods

2.1 Allyl isothiocyanate

AITC is responsible for much of the characteristic pungency of mustard, horseradish and wasabi. Its chemical reactivity and volatility contribute to both its sensory properties and its analytical difficulty. Analytically, AITC is well suited to GC-based methods when volatility is controlled, while HPLC and spectrophotometric methods provide alternatives for formulations and total-ITC screening. Recent work has also explored AITC as a natural antimicrobial and as an active component for food and agricultural applications.

From a pharmaceutical-analysis perspective, AITC should not be treated merely as a 'bioactive compound'. The analytical question should specify whether the objective is assay of intact AITC, total ITC-generating capacity, degradation products, or metabolites. These are different measurands and require different methods. [5,6]

2.2 Benzyl isothiocyanate

BITC is generated primarily from glucotropaeolin and is found in garden cress and related plants. Its aromatic structure and chemical reactivity make it attractive for pharmacological investigation, but the same reactivity can complicate quantitative analysis because free BITC can interact with matrix nucleophiles. LC–MS/MS methods have been particularly useful for following BITC and its glutathione/cysteine-derived metabolites in biological systems, illustrating the transition from food analysis to pharmacokinetic and mechanistic research.[7]

2.3 Phenethyl isothiocyanate

PEITC is associated with watercress and other Brassicaceae sources and has been studied extensively in chemoprevention and cellular signaling. From an analytical standpoint, PEITC illustrates the importance of distinguishing parent

ITC concentration from metabolite exposure. In biological matrices, the relevant analytical targets may include conjugates formed through the mercapturic-acid pathway rather than free PEITC alone. [8,9]

2.4 Sulforaphane

SFN is generated from glucoraphanin and is arguably the most extensively investigated ITC in modern nutraceutical and cancer-prevention research. Its analytical determination is complicated by limited UV chromophoric strength and instability under certain conditions. Published reviews document the use of GC, GC–MS, HPLC, UHPLC, LC–MS and HPLC–MS/MS for SFN, while noting that high-temperature GC conditions can cause decomposition. [10]

3. Analytical challenges associated with ITCs

ITC analysis can be divided into four stages: formation, extraction, separation and detection. Errors introduced during the first two stages may dominate the final result even when the instrumental method is highly precise.[11]

Stage	Major risk	Analytical consequence	Control strategy
Formation	Variable Myrosinase activity	Under/over-estimation of ITC	Standardize tissue disruption, hydration, time and temperature
Extraction	Volatility, reaction with matrix components, solvent dependence	Low recovery or selective loss	Optimize solvent, extraction time and temperature; use recovery studies
Separation	Adsorption, precipitation, degradation	Peak loss or distorted quantification	System suitability; evaluate analyte stability in mobile phase
Detection	Weak UV response or non-specific color	Poor sensitivity/selectivity	Derivatization, PDA/DAD, MS or orthogonal detection
Storage	Oxidation, hydrolysis or volatilization	Time-dependent concentration drift	Stability study and controlled storage

4. Methods of estimation

4.1 UV–visible spectrophotometry

UV–visible spectrophotometry is attractive for routine laboratories because it requires relatively inexpensive instrumentation, has high sample throughput and can be readily adapted to microplate formats. However, direct measurement is limited by the weak or non-selective absorption of several ITCs, particularly SFN. Published reviews identify UV-based total-ITC assays and derivatization approaches as useful screening tools but emphasize their lower compound specificity compared with chromatographic methods. [12,13]

A classic example is cyclo-condensation of ITCs with 1,2-benzenedithiol to generate a strongly absorbing 1,3-benzodithiole-2-thione derivative. The reaction is useful for estimating total ITC-type reactivity but does not identify the side chain and therefore cannot distinguish AITC from BITC, PEITC or SFN without additional separation.

This distinction is critical for the proposed NBP strategy in the supplied manuscript. The draft proposes NBP as a chromogenic reagent and describes optimization of reagent concentration, time, temperature, solvent, pH and wavelength. However, before the method can be claimed as selective, the NBP–ITC reaction product must be experimentally demonstrated, its spectral properties established, and non-ITC matrix reactions excluded.[14-16]

4.2 HPLC

RP-HPLC with UV/PDA detection is among the most accessible compound-specific methods. It is especially useful for relatively nonvolatile ITCs and formulated products. Published SFN methods commonly use C18 reversed-phase columns and low-wavelength UV detection, although the weak UV chromophore of SFN can limit sensitivity. Alternative detectors such as ELSD and MS can improve detection capability. [17]

An important limitation is that chromatographic separation does not guarantee complete analyte recovery. Published studies have reported losses of ITCs during RP-HPLC associated with precipitation and concentration-dependent behavior. Such effects reinforce the need to establish recovery and stability rather than assuming quantitative transfer from extraction vial to chromatographic peak.[17]

4.3 GC-FID

GC-FID remains valuable for volatile ITCs such as AITC because of its quantitative reproducibility and relatively low cost compared with MS. It is suitable for food quality control and reference-method development when injection and oven conditions are carefully controlled.[18]

4.4 GC–MS

GC–MS provides mass spectral information in addition to chromatographic retention. It is useful for identification and confirmation of volatile ITCs and degradation products. Nevertheless, elevated injector and oven temperatures can cause thermal degradation, particularly for SFN. Method-development studies have therefore emphasized temperature optimization and careful interpretation of degradation peaks. [19]

4.5 LC–MS/MS

LC–MS/MS provides high selectivity and sensitivity and is especially valuable for biological matrices, trace-level quantification and metabolite studies. It can distinguish parent ITCs from glutathione, cysteine and mercapturic-acid pathway conjugates, which is essential when studying absorption, metabolism and pharmacokinetics.[20]

4.6 Quantitative NMR

qNMR offers a complementary strategy for purity assessment, reference-standard characterization and orthogonal quantification. Because quantitative NMR is based on signal integration rather than chromatographic detector response, it can be useful for assigning the purity of reference materials that are subsequently used in GC or LC calibration. [21,22]

Table 1: Various techniques used for estimation of ITC

Technique	Primary advantage	Major limitation	Recommended role
UV–Vis	Very low cost; rapid	Low specificity unless derivatized	Screening and high-throughput ranking
Derivatization UV–Vis	Enhanced optical response	Reaction selectivity must be demonstrated	Potential low-cost quantitative method
HPLC-UV/PDA	Accessible compound separation	Limited sensitivity for weak chromophores	Routine QC and target-specific assays
GC-FID	Good quantitative response for volatile ITCs	Thermal/volatility-related losses	AITC and volatile ITC QC
GC–MS	Identification + quantification	Thermal degradation possible; higher cost	Confirmation and profiling
LC–MS/MS	Excellent sensitivity/selectivity	High cost and method complexity	Trace analysis and biological matrices
qNMR	Orthogonal quantification and purity	Capital intensive; lower throughput	Reference standards and confirmation

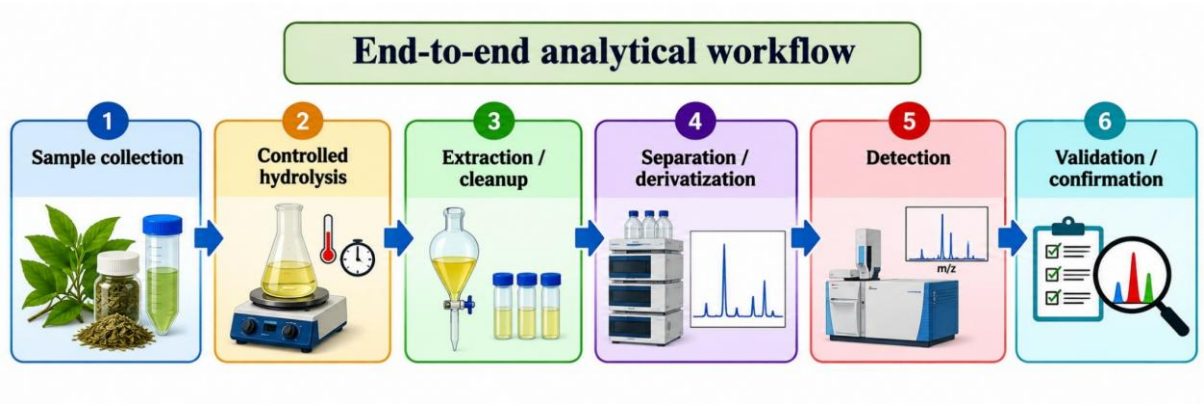


Figure 2: End-to-end analytical workflow for ITC determination.

5. Analytical decision framework

No single platform is optimal for every ITC or every matrix. The most rational selection depends on the analytical question. If the objective is rapid screening of hundreds of foods or botanical extracts, UV–Vis can be attractive. If the objective is routine assay of a specific ITC in a formulation, HPLC may be more appropriate. If the analyte is volatile, GC-FID or GC–MS becomes attractive. If the objective is trace-level measurement of an ITC or its metabolites in plasma or urine, LC–MS/MS is generally preferable.[23]

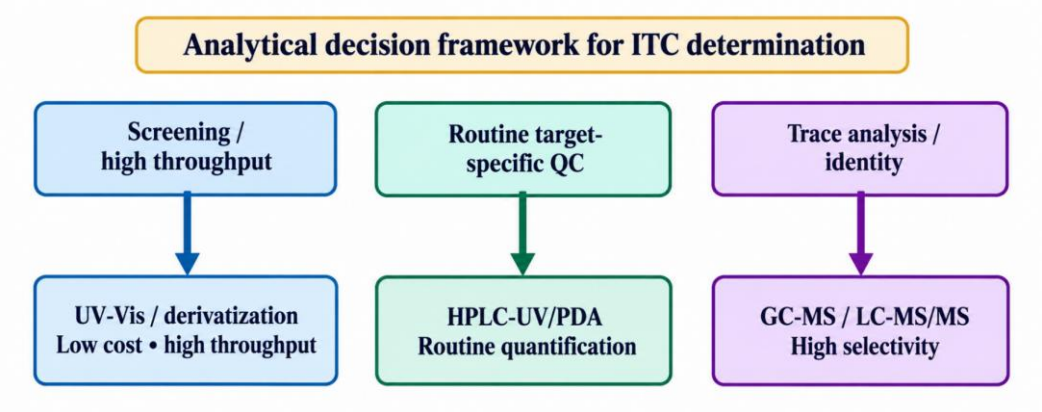


Figure 3: Analytical decision framework positioning UV–Vis relative to chromatographic and mass-spectrometric approaches.

6. Validation framework for ITC analytical methods

Validation should be aligned with the intended analytical claim and, where appropriate, the principles of ICH Q2(R2). For natural-product assays, validation should cover not only the detector but the entire workflow from extraction through measurement.[24]

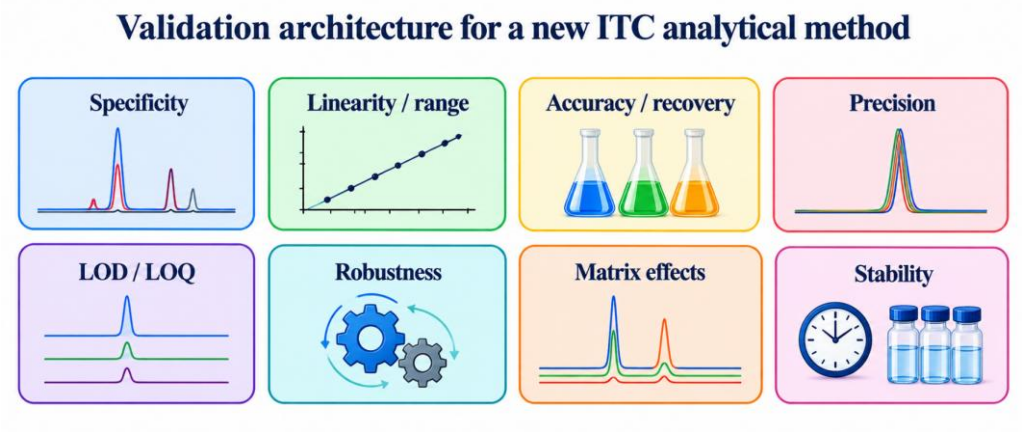


Figure 4: Validation architecture for a new ITC analytical method.

6.1 Specificity and selectivity

Specificity should be evaluated using reagent blanks, solvent blanks, matrix blanks, individual ITC standards, mixed standards and representative interfering compounds. For a derivatization assay, specificity must demonstrate that the measured color or absorbance originates predominantly from the target ITC reaction. This is particularly important for NBP because a chromogenic reagent may react with other electrophilic or nucleophilic constituents. [25,27]

6.2 Linearity and range

At least five concentration levels should normally be investigated across the intended working range. Regression should include slope, intercept, confidence intervals and residual assessment. A high correlation coefficient alone is insufficient evidence of an appropriate calibration model.[26]

6.3 Accuracy

Accuracy should be assessed through recovery experiments using low, medium and high concentrations in representative matrices. Matrix-matched recovery is preferable to solvent-only recovery when plant extracts, nutraceuticals or formulations are the intended sample types.[27]

6.4 Precision

Repeatability should be established using replicate preparations under the same conditions. Intermediate precision should incorporate changes in day, analyst, reagent preparation or instrument where appropriate. Results should be expressed as %RSD with predefined acceptance criteria.[28]

6.5 LOD and LOQ

LOD and LOQ should be derived using a statistically justified procedure appropriate to the calibration model and analytical purpose. For spectrophotometric methods, visual detection of

color should not be used as a substitute for quantitative estimation of detection capability.[29]

6.6 Robustness

Robustness is particularly important for chemical derivatization. Deliberate small variations in reagent concentration, pH, temperature, reaction time, wavelength, solvent composition and extraction time should be introduced to determine which variables materially affect response.[30]

6.7 Matrix effects

Matrix effects should be studied by comparing standards prepared in solvent with standards added to extracted blank matrices and with naturally occurring samples. This is essential for cruciferous vegetables and nutraceutical extracts because pigments, phenolics and other reactive compounds can influence optical and chromatographic response.[31]

6.8 Stability

Stability should be examined for standards, extracts and developed chromophores. Short-term, long-term, freeze–thaw, autosampler and post-derivatization stability may be relevant depending on the method. The stability of the analyte under actual analytical conditions is especially important for ITCs because they can undergo chemical transformation.[32]

7. Applications of AITC, BITC, PEITC and SFN

ITC	Food applications	Pharmaceutical/nutraceutical research	Agricultural/other applications
AITC	Flavour, preservation, antimicrobial packaging	Antimicrobial, anti-inflammatory and chemopreventive research	Biofumigation and pest-management research
BITC	Mustard/cress products; natural antimicrobial systems	Cancer biology, cellular defense and antimicrobial research	Natural-product research
PEITC	Watercress and functional foods	Chemoprevention and pharmacological research	Limited compared with AITC
SFN	Broccoli, sprouts, functional foods and supplements	Nrf2/cytoprotective research, chemoprevention, inflammation	Plant/food processing optimization

These applications are analytically distinct: food QC may prioritize throughput and reproducibility, whereas pharmacokinetic research requires high selectivity and sensitivity. [33-35]

8.Biological activities: current evidence and limitations

ITCs are frequently discussed in terms of antioxidant, antimicrobial, anti-inflammatory and anticancer activity. Mechanistically, their electrophilicity allows covalent modification of thiol-containing biomolecules, including glutathione and protein cysteine residues. This chemistry can influence cellular signaling pathways, redox homeostasis and transcriptional responses.

SFN has received particular attention for activation of the Keap1–Nrf2 pathway and induction of cytoprotective responses. AITC, BITC and PEITC have also been investigated in cellular and animal models for antimicrobial, inflammatory and chemopreventive effects. However, a rigorous review should distinguish mechanistic/preclinical findings from established clinical efficacy and limitations like :

Absence of a universally standardized sample-preparation protocol across AITC, BITC, PEITC and SFN.

Insufficient harmonization of myrosinase activation and glucosinolate-to-ITC conversion conditions.

Limited inter-method comparability because different studies use different standards, extraction procedures and calibration models.

Need for better characterization of ITC degradation and transformation during extraction, evaporation, injection and storage.

Direct UV methods remain constrained by weak or non-selective chromophores for several ITCs.

Derivatization methods require stronger evidence for reaction stoichiometry, product identity and matrix selectivity.

Limited availability of well-characterized certified or high-purity reference materials for routine quantitative work.

Need for inter-laboratory validation before low-cost spectrophotometric assays can support regulatory or compendial applications.

Biological studies increasingly require simultaneous measurement of parent ITCs and metabolites rather than free ITC alone. [39,40]

Future ITC analysis is likely to move toward integrated workflows combining inexpensive screening with selective confirmation. A validated derivatization UV–Vis assay could be particularly

valuable for high-throughput ranking of plant extracts, food products and nutraceutical batches, provided that its chemical selectivity is demonstrated. [41,43]

10. CONCLUSION

AITC, BITC, PEITC and SFN are important natural products whose analytical measurement is inseparable from their chemical reactivity and biological formation pathways. The major methodological challenge is therefore not simply detector sensitivity. It is control of the complete analytical chain from glucosinolate conversion and extraction to separation, detection, calibration and interpretation.[47]

UV–visible spectrophotometry has an important practical role because of its low cost and high throughput, but direct measurement is often limited by poor specificity. Derivatization can overcome part of this limitation, although the reaction chemistry must be demonstrated and validated. HPLC provides a practical routine platform; GC-FID and GC–MS are valuable for volatile ITCs; LC–MS/MS provides the strongest option for trace-level target and metabolite analysis; and qNMR offers valuable orthogonal quantification and reference-standard characterization.

The most promising future strategy is therefore an integrated analytical hierarchy: rapid UV–Vis screening, chromatographic confirmation, and MS/qNMR when high selectivity or reference-level confidence is required. For the proposed NBP approach, the decisive research gap is the experimental establishment of a selective, reproducible and stable NBP–ITC chromogenic reaction. If successfully demonstrated and validated, such a platform could provide a meaningful low-cost addition to the current analytical toolbox for food, nutraceutical and natural-product laboratories.

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