Food Analytical Methods
https://doi.org/10.1007/s12161-023-02544-y

RESEARCH

Development of Liquid Chromatography-Mass Spectrometry (LC-MS) Method for Quantification of Broccoli Sulforaphane

Ali Ali Redha[1,2] · Hung T. Hong[3] · Luciana Torquati[1] · Geoffrey R. Nash[4] · Michael J. Gidley[2] · Daniel Cozzolino[2]

Received: 28 September 2023 / Accepted: 18 October 2023
© The Author(s) 2023

Abstract

Sulforaphane (SFN) is an isothiocyanate and the product of the hydrolysis of glucoraphanin (GRA) by myrosinase. Broccoli is one of the rich sources of GRA and thus SFN. SFN possess a wide range of bioactivities and is considered an anti-cancer phytochemical. Most of the current common methods used to quantify SFN are based on high-pressure liquid chromatography (HPLC) with diode array detection (DAD) — also known as HPLC-DAD. Although this technique has shown encouraging results, the detection of SFN by DAD is relatively weak and affected by high interference of sample matrices. Therefore, the aim of this study was to develop a liquid chromatography-mass spectrometry (LC-MS) method in which SFN is identified by molecular mass to give more accurate results. The developed method demonstrated a highly reproducible retention time (7.204 ± 0.008 min), producing a sharp, symmetrical and well-defined sulforaphane peak in standard and test samples. The most dominant ion of sulforaphane in the pure and test samples was 178 m/z ($[M + H]^+$). In terms of linearity, the calibration curve had a coefficient of determination (R^2) of 0.9963. The limit of detection of this method is 1.3 ng/mL, and the limit of quantification is 3.9 ng/mL, indicating high sensitivity. The uniformity of peak shape and retention time in both pure and test samples were the same suggesting excellent selectivity. Overall, the developed method showed promising results in identifying and quantifying broccoli SFN.

Keywords Sulforaphane · Broccoli · Liquid chromatography · Mass spectrometry · LC-MS · quantification

Introduction

Sulforaphane (SFN) is a natural isothiocyanate with nutraceutical properties (Bahoosh et al. 2022). SFN is the product of the enzymatic hydrolysis of a glucosinolate called glucoraphanin (GRA). GRA is the most dominant glucosinolate in broccoli (Ali Ali Redha et al. 2023a, 2023b). Evidence from *in vitro* and *in vivo* studies has shown promising anti-cancer effects of SFN in cell and animal models (Gasmi et al. 2023). This has led to an increase in the number of clinical studies evaluating the anti-cancer potential of SFN in the past years (Gasmi et al. 2023). With the rise in the evidence about the bioactive potential of SFN, it is expected that nutraceutical companies would explore producing new products rich in SFN or GRA. Nutraceutical companies may consider cruciferous vegetables as a good source of GRA and SFN. Among these vegetables, broccoli (*Brassica oleracea* var. *italica*) is one of the rich sources of GRA (the precursor of SFN) (Li et al. 2022). The concentration of GRA in common broccoli can range between 30 and 850 mg/100 g DW (Langston et al. 2023). GRA becomes converted into SFN upon the breakdown of tissues and release of the enzyme myrosinase when broccoli is processed, which is responsible for the conversion reaction (Ali Redha et al. 2023a, 2023b).

✉ Ali Ali Redha
aa1249@exeter.ac.uk

1 The Department of Public Health and Sport Sciences, University of Exeter Medical School, Faculty of Health and Life Sciences, University of Exeter, Exeter EX1 2LU, UK

2 Centre for Nutrition and Food Sciences, Queensland Alliance for Agriculture and Food Innovation (QAAFI), The University of Queensland, Brisbane, QLD 4072, Australia

3 School of Agriculture and Food Sciences, The University of Queensland, Brisbane, QLD 4072, Australia

4 Natural Sciences, Faculty of Environment, Science and Economy, University of Exeter, Exeter EX4 4QF, UK

Published online: 02 November 2023

The amount of GRA and thus SFN could vary in different broccoli samples and products due to environmental, agricultural and industrial factors. Most of the analytical methods used to determine broccoli SFN are chromatographic and include gas chromatography (Chiang et al. 1998) and liquid chromatography (Ares et al. 2013; Campas-Baypoli et al. 2010; Celik et al. 2014). It has been reported that SFN undergoes thermal degradation in the injection ports of gas chromatography equipment, making this technique unfavourable and not robust for SFN quantification (Campas-Baypoli et al. 2010). Liquid chromatographic methods such as high-pressure liquid chromatography (HPLC) with diode array detection (DAD), also known as HPLC-DAD, seem to be the most commonly used techniques for SFN quantification (Campas-Baypoli et al. 2010; Celik et al. 2014). Although HPLC-DAD is generally considered a robust technique, it is associated with limited sensitivity and is not capable of providing information about the molecular mass of the analysed compounds which is important for accurately identifying the compound of interest. On the other hand, chromatographic techniques hyphenated with mass spectrometry, such as liquid chromatography-mass spectrometry (LC-MS), can accurately identify compounds based on their molecular mass. This can become very important when dealing with plant extracts and plant-based products since they contain a wide range of compounds. Overall, techniques such as LC-MS have high sensitivity, selectivity and accuracy in comparison to HPLC-DAD and can provide a comprehensive analysis of the product of interest (De Girolamo et al. 2022; Pitt 2009).

The current study aimed to develop a fast and robust LC-MS method to quantify the broccoli SFN and apply the method for quantifying SFN content of several broccoli-based supplements from our labs.

Materials and Methods

Materials

A total of 11 different food-grade broccoli-based supplements (labelled A-K), in form of dried powder, obtained from different experiments in our labs, were used as test samples in this study. Extraction materials included phosphate-buffered saline (PBS) prepared from tablets purchased from Thermo Fisher Scientific (Waltham, USA) and dichloromethane which was purchased from Merck (Darmstadt, Germany). Pure sulforaphane (25 mg/mL) was purchased from Sapphire Bioscience Pty Ltd (Redfern, Australia), and acetonitrile (HPLC grade) was purchased from Merck (Warsaw, Poland).

Extraction of Sulforaphane

An amount of 0.5 g of samples A-K was mixed with 3.0 mL of PBS (10×, pH = 7.4). The samples were sonicated for 90 min at 37 °C with 100-W power in an ultrasonic bath (500 TD, SONICLEAN™, Australia). Then, sulforaphane was extracted with 10-mL dichloromethane. The samples were vortexed and shaken for 10 min at 250 strokes/min using a reciprocating shaker (SSL2, Stuart, UK). Then, the samples were centrifuged for 10 min at 3500 rpm and 15 °C (Centrifuge 5810 R, Eppendorf, Germany). The water was discarded and then the extract was collected. The extraction was repeated in triplicate for each sample. A volume of 1 mL of the combined extract was evaporated under reduced pressure at 35 °C using a vacuum concentrator (SpeedVac SPD140DDA, Thermo Fisher Scientific, USA). The resulting extract was then dissolved in 0.5 mL of pure HPLC grade acetonitrile and filtered through a 0.22-µm syringe filter.

Instrumentation and Operating Conditions

SNF was identified and quantified using a certified Shimadzu LC-MS-2020 system (Shimadzu, Kyoto, Japan). The Nexera X2 UHPLC system consisted of a system controller (CBM-20A), two pumps (LC-30AD), an autosampler (SIL-30AC), a valve unit (FCV-20AH^2), a column heater (CTO-20AC) and a degasser (DGU-20A5R). The Nexera X2 UHPLC system was coupled to an MS-2020 quadrupole mass spectrometer (Shimadzu, Kyoto, Japan), and the DUIS source was operated with a nebulizer gas flow of 1.5 L/min, drying gas flow of 17 L/min, desolvation line (DL) temperature of 300 °C, interface temperature of 350 °C and heat block temperature of 500 °C. Selective ion monitoring (SIM) in the positive ion mode of the ion m/z 178 was used to identify and quantify sulforaphane. Labsolutions LCMS software Ver.5.96 (Shimadzu, Kyoto, Japan) was used for instrument control and data processing.

Separation Conditions

Chromatographic separation was carried out on a reverse-phase Acquity UPLC BEH C18 column (100 × 2.1 mm i.d., 1.7 particle size; Waters, Dublin, Ireland) at a flowrate of 0.2 mL/min and a column oven temperature of 30 °C. The 11.5-min gradient elution started isocratic at 0% mobile phase B (acetonitrile, 0.1% formic acid) and 100% mobile phase A (MQ-Water, 0.1% formic acid) for 2 min, increasing to 70% B in 8 min before increasing sharply to 100% in 0.5 min, then holding for 1.5 min, conditioning for 0.2 min and finally re-equilibrating for 1.3 min. Sulforaphane concentrations

were determined using external calibration curves within a working range of 0.5–50 µg/mL

Preparation of Standard Solutions

Standard solutions were prepared from the pure SNF stock solution (25 mg/mL) in ethanol. The dilutions were made with ethanol to final concentrations of 0.5, 1.0, 1.5, 2.0, 2.5 and 5.0 µg/mL.

Method Validation

The linearity of an analytical method refers to its capacity, within a specified range, to produce test outcomes that exhibit a consistent proportional relationship with the concentration or quantity of the substance being analysed in the sample.

The sensitivity of the developed method was determined by calculating the limit of detection (LOD) and limit of quantification (LOQ). The LOD is the lowest concentration of the analyte that can be detected with signal-to-noise ratio of at least 3:1, while the LOQ is determined as the lowest concentration level at which the peak height maintains a signal-to-noise ratio exceeding 10. Additionally, the achieved precision (measured as relative standard deviation (RSD%)) and accuracy (measured as percentage bias) were assessed within a range of ± 10%.

The specificity of an analytical technique can be described as its capacity to accurately identify the analyte, even in the presence of impurities, degradation products, and matrix components, is present. This attribute was assessed by contrasting the chromatograms obtained from standard solutions and sample solutions.

The precision and accuracy of the method were evaluated by assessing the repeatability of one tested sample over seven repetitions. This was done by calculating the degrees of freedom, which measures the random variability in the results. A lower degree of freedom indicates better precision and accuracy of the method. It also means that the results are more consistent and less affected by random fluctuations.

Results and Discussion

Extraction of Sulforaphane

The SNF extraction method proposed by Liang et al. (2006) has been widely used and reproduced (Liang et al. 2006). The current study has modified this method by controlling the pH to further enhance the hydrolysis of GRA into SFN and the extraction process. The enzymatic conversion of GRA into SFN is pH dependent. GRA is hydrolysed into SFN in the pH range of 5 to 8, while at lower pH levels (2–5), GRA is hydrolysed into sulforaphane nitrile, and at higher pH levels (> 8), it is hydrolysed into sulforaphane thiocyanate. Sulforaphane nitrile and sulforaphane thiocyanate are considered unfavourable products due to lack of bioactivity. The current method has replaced water in the hydrolysis step with PBS. PBS is a water-based salt buffer with a pH of 7.4 which is considered a favourable pH for the conversion of GRA into SFN. In fact, PBS is isotonic (has the same osmolarity and ion concentration) with most living cells. The solubility of SFN in PBS is approximately 10 mg/mL (Cayman Chemical 2023). The hydrolysis process was allowed to take place in a 37 °C ultrasonic bath for 90 min. Ultrasonic waves are capable of breaking down the plant cell wall (Babaei-Ghaghelestany et al. 2020), and thus assist in releasing GRA and enhancing the conversion rate. After hydrolysis, the samples were extracted with dichloromethane in which SFN has a solubility of 25 mg/L (González et al. 2021).

Quantification of Sulforaphane

The quantified amount of SFN in the formulations ranged between 8 and 22 mg/kg (Table 1). The average retention time of SFN was 7.204 ± 0.008 min (average of 63 runs) in this method. This is a relatively short time in comparison to one of the common HPLC-DAD methods that have reported an average retention time of 14.18 ± 0.08 min (Campas-Baypoli et al. 2010). The retention time was similar to that obtained with another less commonly used method that reported 7.20 min ± 0.03% (Celik et al. 2014). The sulforaphane peak, in standards and test samples, was symmetrical, sharp and well-resolved (Figs. 1a and 2a). The most dominant ion was 178 m/z ($[M + H]^+$) in both the pure SFN and test samples (Figs. 1b and 2b); this was also

Table 1 Sulforaphane content (mg/kg) of broccoli-based supplements with varying formulations determined by the developed LC-MS method

Sample	Sulforaphane content (mg/kg)	SD	RSD%
A	20.11	1.58	7.86
B	22.46	1.79	7.96
C	17.13	0.65	3.77
D	8.35	1.56	18.64
E	14.22	3.44	24.22
F	19.70	5.30	26.89
G	18.63	2.85	15.27
H	16.71	3.37	20.14
I	15.17	0.44	2.90
J	17.07	1.98	11.61
K	11.26	0.99	8.79

SD standard deviation, *RSD%* relative standard deviation

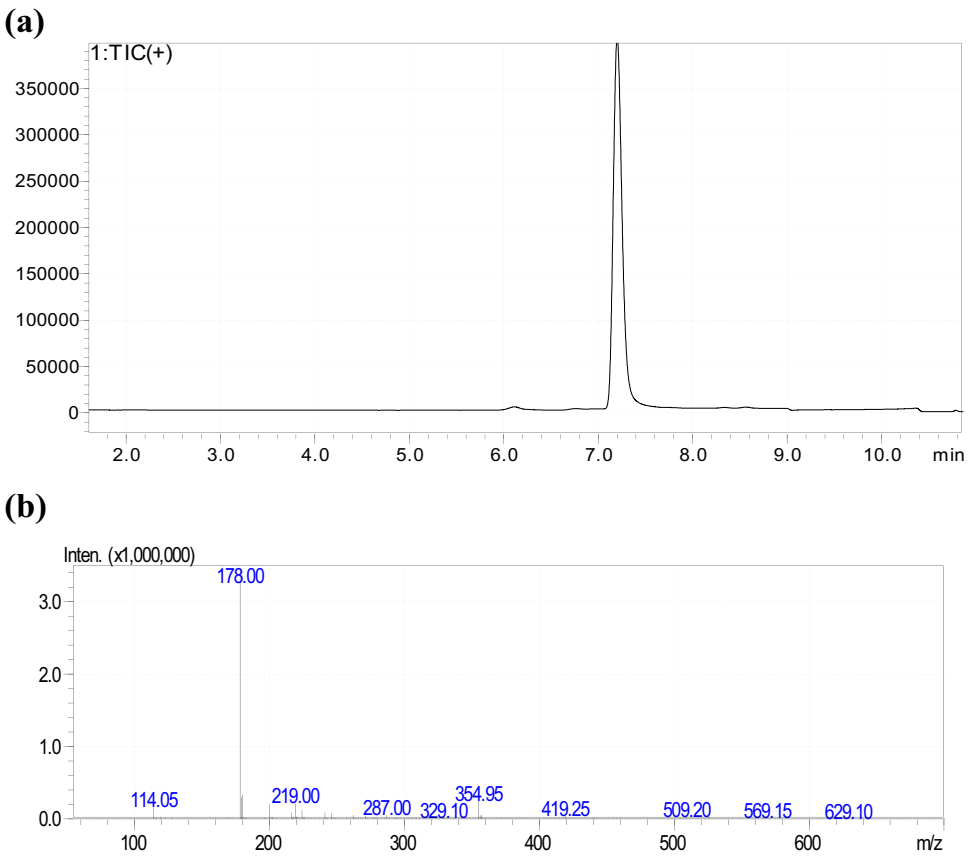

Fig. 1 Chromatograph (**a**) and mass spectrum (**b**) of pure sulforaphane

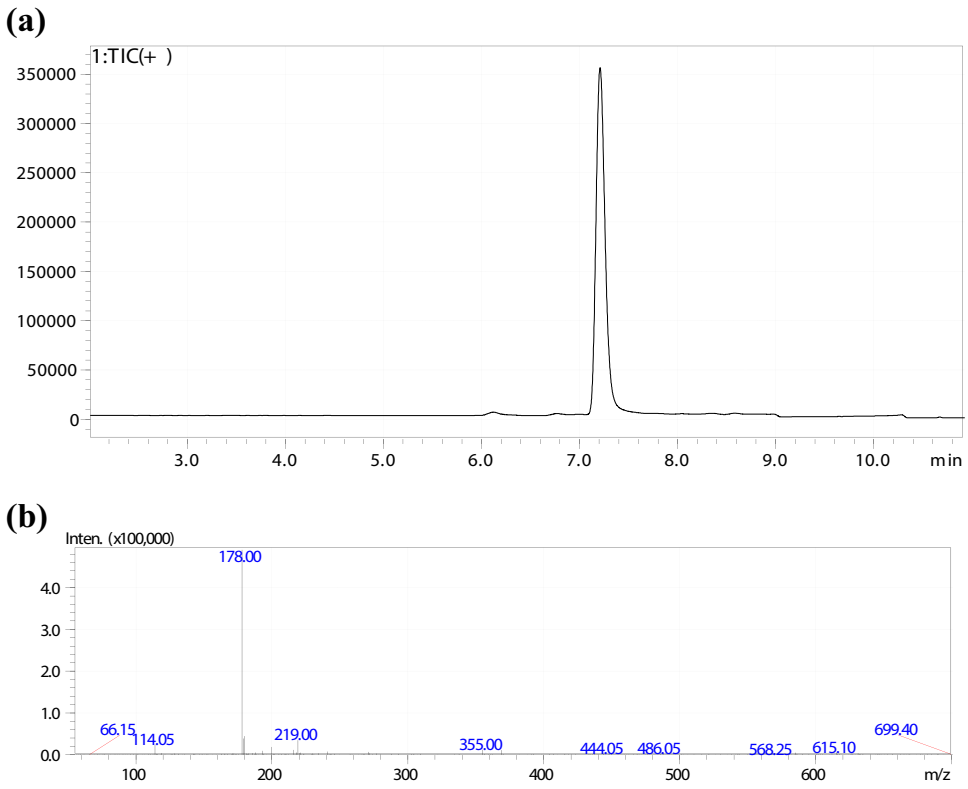

Fig. 2 Chromatograph (**a**) and mass spectrum (**b**) of a broccoli-based supplement formulation

reported by a previous study analysing the MS spectrum of SFN (Ares et al. 2013).

A C18 column was selected for this method for several reasons. SFN contains polar functional groups including a sulfinyl group (–SO–) and an isothiocyanate group (–N=C=S), which make it polar due to the electronegativity differences between the sulphur and nitrogen atoms. These polar functional groups contribute to an uneven distribution of electron density within the molecule, resulting in a net dipole moment. Considering this property of SFN, it can be difficult to retain on some columns. However, C18 columns have a high hydrophobicity and provide strong retention for polar analytes. C18 columns possess their unique bonded phases that provide excellent selectivity for isothiocyanate compounds and allow for a high degree of separation between isothiocyanate compounds and other polar or non-polar compounds in broccoli sample matrix. C18 columns can operate over a wide pH range from 1 to 12, which makes them suitable for analysing isothiocyanate compounds in a variety of complex sample matrices. Moreover, C18 columns are highly durable and stable, even under harsh conditions as high temperature, pH and high pressure (Teutenberg et al. 2009). Lastly, C18 columns have superior separation power and longevity even when frequently used for analysing complex and challenging samples (Harrieder et al. 2022).

The developed standard curve had a linear equation of $y = 858{,}251x + 226{,}811$ for pure sulforaphane standards ranging between 0.25 and 5.00 µg/L. The curve had a coefficient of determination (R^2) of 0.9963 suggesting a strong correlation between the area under the curve and the concentration of sulforaphane. This was close to HPLC-DAD methods which reported 0.99985 (Celik et al. 2014) and 1 (Campas-Baypoli et al. 2010). In terms of sensitivity, the method had an LOD of 1.3 ng/mL and an LOQ 3.9 ng/mL which are considered very low suggesting the high sensitivity of this method. The LOD and LOQ of this method are remarkably lower than those reported by previous studies using HPLC-DAD: LOD = 29.7 ng/mL, LOQ = 90 ng/mL (Celik et al. 2014); and LOD = 0.2 µg/mL, LOQ = 0.6 µg/mL (Ares et al. 2013). In terms of specificity, the chromatograms of the pure samples (e.g., Fig. 1a) and test samples (e.g., Fig. 2a) were identical in shape and had the same retention time. In fact, other compounds/peak were retained at that retention time.

The quality control analysis approach involved repeating the same standards at the beginning and end of the analysis batch, with at least one standard introduced after every ten samples. These standards were included to verify the accuracy and precision of the method and ensure the reliability of the results. The obtained data were evaluated using statistical tools such as relative standard deviation (RSD) and percentage difference. The acceptability of the results was determined based on the established precision. Any deviations from the set benchmarks were probed and remedied where necessary. The use of quality control measures ensured that the analytical method produced trustworthy and precise results for the identification and quantification of the analyte in the sample.

Conclusions

The proposed LC-MS method was successful in accurately identifying and quantifying SFN. Its efficiency can be attributed to the short retention time of the analyte. The standard curve demonstrated an excellent correlation with $R^2 > 0.99$. The LOD and LOQ values were lower when compared to other liquid chromatography-based methods, further enhancing its effectiveness. In addition, the method exhibited a good selectivity. Quality control was thoroughly implemented throughout the process to ensure the validity and accuracy of results.

Author Contribution Ali Ali Redha: conceptualization; data curation; formal analysis; investigation; methodology; software; writing — original draft; writing — review and editing. Hung T. Hong: conceptualization; data curation; formal analysis; investigation; methodology; software; writing — review and editing. Luciana Torquati: writing — review and editing; supervision. Geoffrey R. Nash: writing — review and editing; supervision. Michael J. Gidley: writing — review and editing; supervision. Daniel Cozzolino: writing — review and editing; supervision.

Funding Financial support for this study was provided by the QUEX Institute, a partnership between The University of Queensland and the University of Exeter. Funding support has also been made available from PepsiCo, Inc.

Data Availability The data that support the findings of this study are available from the corresponding author upon reasonable request.

Declarations

Conflict of Interest Ali Ali Redha declares that he has no conflict of interest. Hung T. Hong declares that he has no conflict of interest. Luciana Torquati declares that she has no conflict of interest. Geoffrey R. Nash declares that he has no conflict of interest. Michael J. Gidley declares that he has no conflict of interest. Daniel Cozzolino declares that he has no conflict of interest.

Disclaimer The views expressed in this report are those of the authors and do not necessarily represent the position of policy of PepsiCo, Inc.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will

need to obtain permission directly from the copyright holder. To view a copy of this licence, visit http://creativecommons.org/licenses/by/4.0/.

References

Ali Redha A, Langston F, Nash GR, Bows JR, Torquati L, Gidley MJ, Cozzolino D (2023a) Determination of glucosinolates in broccoli (Brassica oleracea var. italica) by combining mid-infrared (MIR) spectroscopy with chemometrics. Int J Food Sci Technol. https://doi.org/10.1111/ijfs.16664

Ali Redha A, Torquati L, Langston F, Nash GR, Gidley MJ, Cozzolino D (2023b) Determination of glucosinolates and isothiocyanates in glucosinolate-rich vegetables and oilseeds using infrared spectroscopy: a systematic review. Crit Rev Food Sci Nutr:1–17. https://doi.org/10.1080/10408398.2023.2198015

Ares AM, Bernal J, Martín MT, Bernal JL, Nozal MJ (2013) Optimized formation, extraction, and determination of sulforaphane in broccoli by liquid chromatography with diode array detection. Food Anal Methods 7(3):730–740. https://doi.org/10.1007/s12161-013-9766-6

Babaei-Ghaghelestany A, Alebrahim MT, MacGregor DR, Khatami SA, Farzaneh HN, R. (2020) Evaluation of ultrasound technology to break seed dormancy of common lambsquarters (Chenopodium album). Food Sci Nutr 8(6):2662–2669. https://doi.org/10.1002/fsn3.1547

Bahoosh SR, Shokoohinia Y, Eftekhari M (2022) Glucosinolates and their hydrolysis products as potential nutraceuticals to combat cytokine storm in SARS-COV-2. Daru 30(1):245–252. https://doi.org/10.1007/s40199-022-00435-x

Campas-Baypoli ON, Sanchez-Machado DI, Bueno-Solano C, Ramirez-Wong B, Lopez-Cervantes J (2010) HPLC method validation for measurement of sulforaphane level in broccoli by-products. Biomed Chromatogr 24(4):387–392. https://doi.org/10.1002/bmc.1303

Chemical C (2023) L-sulforaphane product information. C. C. Company

Celik H, Ariburnu E, Baymak MS, Yesilada E (2014) A rapid validated HPLC method for determination of sulforaphane and glucoraphanin in broccoli and red cabbage prepared by various cooking techniques. Anal Methods 6(13):4559–4566. https://doi.org/10.1039/c4ay00112e

Chiang WCK, Pusateri DJ, Leitz REA (1998) Gas chromatography/mass spectrometry method for the determination of sulforaphane and sulforaphane nitrile in broccoli. J Agric Food Chem 46(3):1018–1021

De Girolamo A, Lippolis V, Pascale M (2022) Overview of recent liquid chromatography mass spectrometry-based methods for natural toxins detection in food products. Toxins 14(5). https://doi.org/10.3390/toxins14050328

Gasmi A, Gasmi Benahmed A, Shanaida M, Chirumbolo S, Menzel A, Anzar W, Arshad M, Cruz-Martins N, Lysiuk R, Beley N, Oliinyk P, Shanaida V, Denys A, Peana M, Bjorklund G (2023) Anticancer activity of broccoli, its organosulfur and polyphenolic compounds. Crit Rev Food Sci Nutr:1–19. https://doi.org/10.1080/10408398.2023.2195493

González F, Quintero J, Del Río R, Mahn A (2021) Optimization of an extraction process to obtain a food-grade sulforaphane-rich extract from broccoli (Brassica oleracea var. italica). Molecules 26(13):4042. https://doi.org/10.3390/molecules

Harrieder EM, Kretschmer F, Bocker S, Witting M (2022) Current state-of-the-art of separation methods used in LC-MS based metabolomics and lipidomics. J Chromatogr B Anal Technol Biomed Life Sci 1188:123069. https://doi.org/10.1016/j.jchromb.2021.123069

Langston F, Ali Redha A, Nash GR, Bows JR, Torquati L, Gidley MJ, Cozzolino D (2023) Qualitative analysis of broccoli (Brassica oleracea var. italica) glucosinolates: Investigating the use of mid-infrared spectroscopy combined with chemometrics. J Food Compos Anal 123. https://doi.org/10.1016/j.jfca.2023.105532

Li H, Xia Y, Liu H-Y, Guo H, He X-Q, Liu Y, Wu D-T, Mai Y-H, Li H-B, Zou L, Gan R-Y (2022) Nutritional values, beneficial effects, and food applications of broccoli (Brassica oleracea var. italica Plenck). Trends Food Sci Technol 119:288–308. https://doi.org/10.1016/j.tifs.2021.12.015

Liang H, Yuan QP, Dong HR, Liu YM (2006) Determination of sulforaphane in broccoli and cabbage by high-performance liquid chromatography. J Food Compos Anal 19(5):473–476. https://doi.org/10.1016/j.jfca.2005.11.005

Pitt JJ (2009) Principles and applications of liquid chromatography-mass spectrometry in clinical biochemistry. Clin Biochem Rev 30(1):19–34

Teutenberg T, Hollebekkers K, Wiese S, Boergers A (2009) Temperature and pH-stability of commercial stationary phases. J Sep Sci 32(9):1262–1274. https://doi.org/10.1002/jssc.200800712

Publisher's Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.