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Development of a UPLC-Q-ToF-MS Method for the Determination of Sulforaphane and Iberin in Cruciferous Vegetables †

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Abstract: Sulforaphane (1-isothiocyanato-4-(methylsulfinyl)-butane) and iberin (1-isothiocyanato-3-methylsulfinylpropane) have attracted greatest attention due to their anti-inflammatory and cancer-preventive properties. These isothiocyanates are products of the enzymatic hydrolysis of the glucosinolates glucoraphanin and glucoiberin, contained only in the plants of the order *Brassicales*. Cruciferous vegetables such as broccoli, cabbage and cauliflower, belong in the order *Brassicales* and specifically in the *Brassicaceae* family. Our aim was to develop an efficient and accurate method for the simultaneous determination of sulforaphane and iberin in cruciferous vegetables using Ultra-high Performance Liquid Chromatography-Quadrupole Time-of-Flight Mass Spectrometry (UPLC-Q-ToF-MS). The method was applied for the quantitative determination of these compounds in a variety of cruciferous vegetables (green and purple broccoli, white and purple cabbage, radish, turnip, arugula, watercress and cauliflower). The results showed that green and purple broccoli contained the highest levels of sulforaphane (660.14 ± 34.29 to 210.11 ± 9.76 μg g^{-1} dry weight) while the highest concentration of iberin was detected in purple broccoli (144.98 ± 3.56 μg g^{-1} dry weight). The lowest concentrations of sulforaphane and iberin were measured in watercress and radish. The differences in content of these compounds can be attributed to the variability among *Brassicaceae* species, geography, season and various environmental factors.

Keywords: sulforaphane; iberin; Brassica; broccoli; cabbage; vegetables; liquid chromatography; mass spectrometry

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1. Introduction

Cruciferous vegetables are plant foods belonging to the *Brassicaceae* family (order *Brassicales*). Consumption of cruciferous vegetables has been correlated with a reduction in the incidence of several non-communicable diseases such as cancer, diabetes and cardiovascular disease [1]. These benefits are attributed to the presence of glucosinolates which upon enzymatic hydrolysis by myrosinase, release isothiocyanates, highly beneficial for human health. Among these, sulforaphane (1-isothiocyanato-4-(methylsulfinyl)-butane) and iberin (1-isothiocyanato-3-methylsulfinylpropane) (Figure 1), derived from the enzymatic hydrolysis of glucoraphanin and glucoiberin glucosinolates, have been reported to present considerable anti-inflammatory capacity [2–6]. The analytical determi-

nation of sulforaphane and iberin presents several problems due to the lack of chromophores, the high volatility and the precipitation of these unstable oils in the liquid chromatography column [7]. Herein we present a reliable method for the determination of these compounds in cruciferous vegetables using Ultra Performance-Liquid Chromatography-Quadrupole-Time of Flight-Mass Spectrometry (UPLC-Q-ToF-MS).

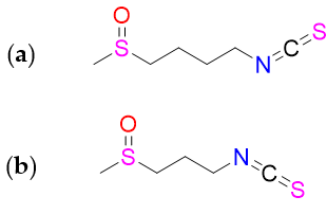

Figure 1. Molecular structures of (**a**) sulforaphane and (**b**) iberin.

2. Materials and Methods

2.1. Reagents

Sulforaphane was synthesized according to D'Souza et al. [8]. Iberin, dichloromethane (CH_2Cl_2) (analytical grade) and methanol (MeOH) (LC-MS grade) were obtained from Sigma-Aldrich Chemical Co (St. Louis, MO, USA). Ultra-pure water was provided by MilliQ purification system (Millipore Direct-Q, Bedford, MA, USA).

2.2. Standard Solutions

Stock solutions (1000 mg L^{-1}) of sulforaphane and iberin were prepared in MeOH and stored in dark glass containers at −20 °C. A solution of 10 mg L^{-1} in MeOH was used for the full scan and MS/MS experiments. Calibration curves of sulforaphane and iberin were constructed using the standard concentrations of 0.1, 0.5, 1.0, 3.0, 5.0, 8.0, 10.0 and 12.0 mg L^{-1} by appropriate dilution of stock solution with MeOH.

2.3. Sampling

Fresh samples of green broccoli, purple broccoli (sample 1), white cauliflower, white cabbage, red cabbage, watercress and radish, were originated from Chalkida (38°28′02.5″ N 23°38′13.4″ E), Greece and one purple broccoli sample (sample 2) was originated from Achaia (38°05′31.6″ N 21°31′24.0″ E), Greece. Samples were collected in February 2017. Broccoli and cauliflower florets, cabbage and watercress leaves, and roots of radish were used for the preparation of extracts. Samples were lyophilized, immediately after received, and ground to a fine homogenous powder using a mortar and pestle.

2.4. Preparation of Extracts

The preparation of extracts was performed according to literature [9] with the following modifications: 25 mL of distilled water (pH 7.0) were added into 1 g of dry vegetable and incubated in a water bath for 3 h at 45 ± 3 °C. The mixture was left outside the water bath for 30 min to reach room temperature. After addition of 30 mL CH_2Cl_2, the mixture was stirred for 15 min and then filtered using a Buchner funnel with Whatman filter paper grade 1 (Whatman Ltd., Maidstone, UK). The solid residue was extracted twice with 30 mL CH_2Cl_2. The filtrates were combined into a separation funnel for the removal of excess water and were dried with 1 g anhydrous sodium sulfate. The solvent was then evaporated to dryness at 35 °C, on a Heidolph II rotary evaporator (Heidolph Instruments GmbH and Co.KG, Schwabach, Germany). The residue was dissolved in 1 mL MeOH. The extract was then injected to the LC-MS system after a 10-fold dilution with MeOH. The measurements were performed in triplicates.

2.5. UPLC-Q-ToF-MS

The high resolution mass spectrometry spectra were recorded on an Agilent 6530 Quadrupole Time of Flight LC-MS system (Q-ToF-MS), with an ESI source, coupled with Agilent 1290 Infinity UPLC system and an autosampler (Agilent Technologies, Santa Clara, CA, USA). Nitrogen was used as the collision gas and positive electrospray ionization (ESI) was used for the MS experiments. The data acquisition was carried out with Agilent MassHunter software (version B.06.00). The following Q-TOF conditions were used: drying gas, 12 L/min; gas temperature, 300 °C; fragmentor, 150 V; skimmer, 65 V; capillary voltage, 4000 V; nebulizer gas, 45 psi, acquisition rate, 1 spectra/s (threshold 200 Abs, 0.01% rel.); MS scan range, 50–1500. For the MS/MS experiments, an auto-MS/MS method was developed with the following parameters: MS/MS acquisition rate, 1 spectra/s (threshold 5 Abs, 0.01% rel.); MS/MS scan range, 50–1500; collision energy slope, 5 V; offset, 2.5 V; preferred charge state, 2, 1, unknown. The mass accuracy of the Q-ToF-MS was calibrated before each analysis using a calibrant solution for scanning up to *m*/*z* 1500. Mass calibration of the Q-ToF MS was controlled by constant infusion of a reference mass solution (obtained from Agilent Technologies) into the source of the Q-ToF-MS during the analysis with the reference ions 121.0509 and 922.0098. The raw data files were processed with Agilent MassHunter Qualitative Analysis software (version B.07.00).

2.6. Chromatographic Study

Chromatographic study was performed with an Agilent Zorbax C18 (50 × 2.1 mm, 1.8 μm) column. The mobile phase was ultra-pure water/0.1% formic acid (A) and MeOH/0.1% formic acid (B) with the following gradient: 0 min: 5% B; 1 min: 5% B; 8.5 min: 95% B; 9.5 min: 95% B; 11.5 min: 5% B; 26.5 min: 5% B. The total run time including column equilibration was 26.5 min. The injection volume was 2 μL and the flow rate was 0.4 mL min^{-1}. The column oven temperature was set at 27 °C.

3. Results and Discussion

3.1. Mass Spectrometry Study

Sulforaphane and iberin were studied in positive ESI mode with flow injection analysis (FIA), to record the full scan and MS^2 spectra (Figure 2). The full scan spectrum of sulforaphane showed the ion $[M + H]^+$ at *m*/*z* 178.0354 (Δ 0.56 ppm), while the ion $[M + Na]^+$ was observed at *m*/*z* 200.0172 (Δ 1.00 ppm) (Figure 2a). In the MS^2 spectrum of the ion $[M + H]^+$, an ion at *m*/*z* 114.0372 was detected (Figure 2b) in accordance with previous literature study [10]. The full scan spectrum of iberin showed the ion $[M + H]^+$ at *m*/*z* 164.0197 (Δ 1.22 ppm), while the ion $[M + Na]^+$ was observed at *m*/*z* 186.0017 (Δ 0.54 ppm) (Figure 2c). In the MS^2 spectrum of the ion $[M + H]^+$, a characteristic ion at *m*/*z* 105.0364 was detected (Figure 2d).

3.2. Method Validation

The peak area of the extracted ion chromatograms was utilized for the quantification of sulforaphane and iberin in cruciferous vegetables. The linearity of the new UPLC-Q-ToF-MS method was determined by the construction of a calibration curve at different concentrations (Figure 3). Limit of detection (LOD) and quantification (LOQ) for sulforaphane were 1.19 mg L^{-1} and 3.61 mg L^{-1} while for iberin the LOD and LOQ were calculated at 1.11 mg L^{-1} and 3.35 mg L^{-1}, respectively.

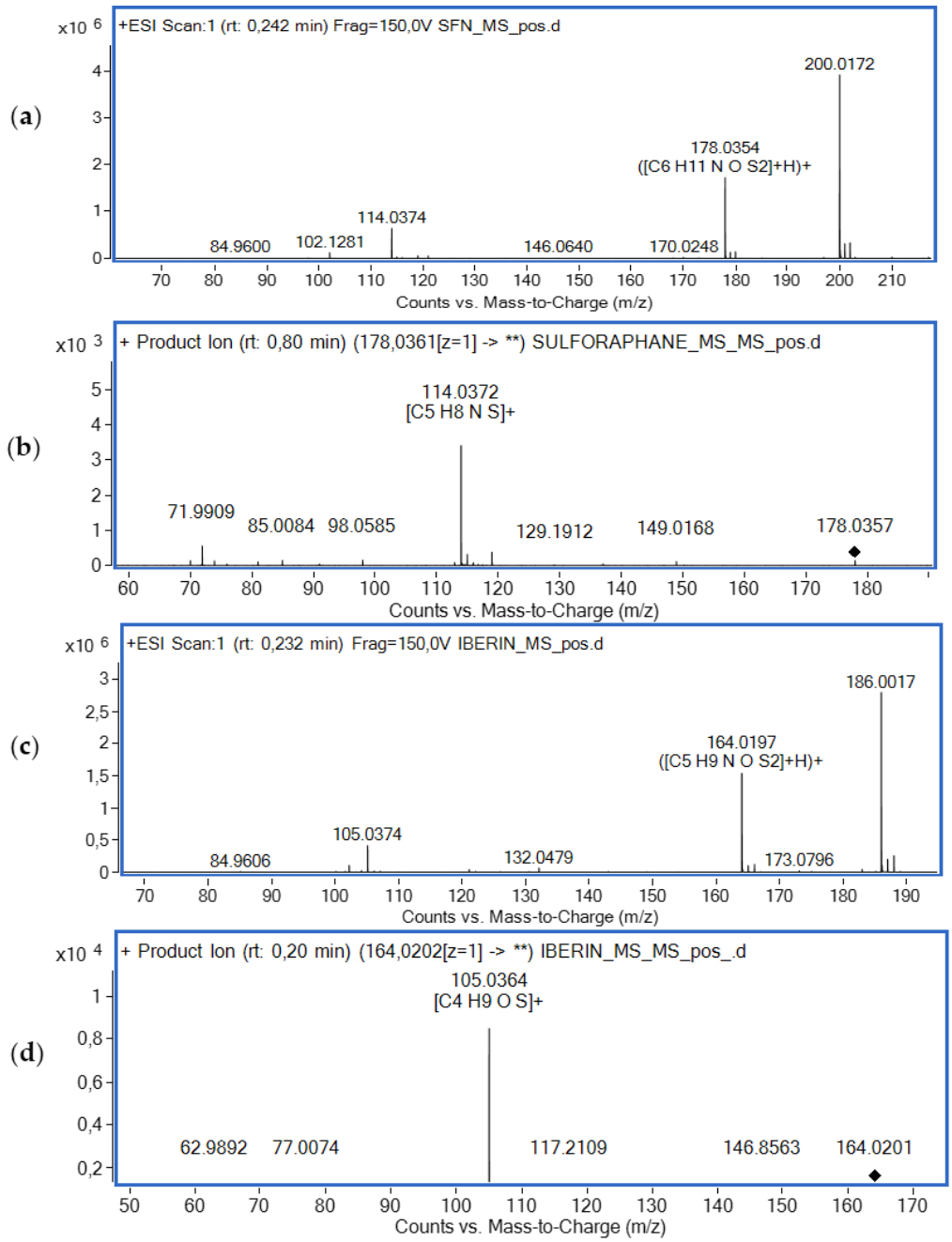

Figure 2. (**a**) Full scan mass spectrum of sulforaphane; (**b**) MS^2 mass spectrum of sulforaphane; (**c**) Full scan mass spectrum of iberin; (**d**) MS^2 mass spectrum of iberin.

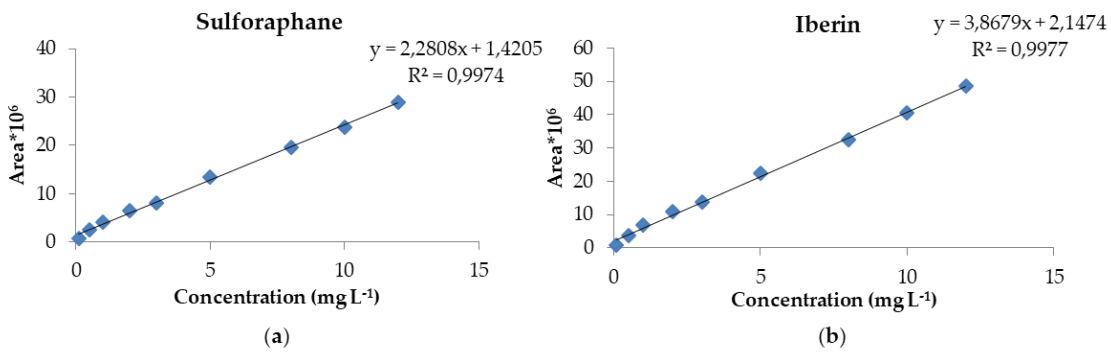

Figure 3. Calibration curves for: (**a**) Sulforaphane; (**b**) Iberin.

3.3. Analysis of Extracts

Table 1 presents the content of sulforaphane and iberin in various cruciferous vegetables. Green broccoli was found to contain the highest amount of sulforaphane (660.14 ± 34.29 μg g^{-1} dry weight) while the highest concentration of iberin was measured at the purple broccoli sample 1 (144.98 ± 3.56 μg g^{-1} dry weight) originated from Chalkida. The results are in accordance with literature [7]. A representative extracted ion chromatogram of sulforaphane and iberin is presented at Figure 4a. In the mass spectra of the corresponding retention times of each compound the characteristic ions that reported earlier in this study (Figure 2), at *m*/*z* 105.0371 (Figure 4b) and *m*/*z* 114.0372 (Figure 4c) were detected, confirming the presence of these compounds in the vegetable extracts.

Table 1. Content of sulforaphane and iberin in various cruciferous vegetables in μg g^{-1} dry weight ± S.D.

Compound	Green Broccoli	Purple Broccoli 1	Purple Broccoli 2	White Cauliflower	White Cabbage	Red Cabbage	Radish	Watercress
Sulforaphane	660.14 ± 34.29	15.05 ± 0.43	210.11 ± 9.76	14.89 ± 1.62	73.71 ± 1.27	143.83 ± 3.44	9.25 ± 0.14	4.44 ± 0.53
Iberin	20.95 ± 0.67	144.98 ± 3.56	<LOD	47.48 ± 5.07	84.57 ± 0.20	30.12 ± 0.13	0.83 ± 0.09	<LOD

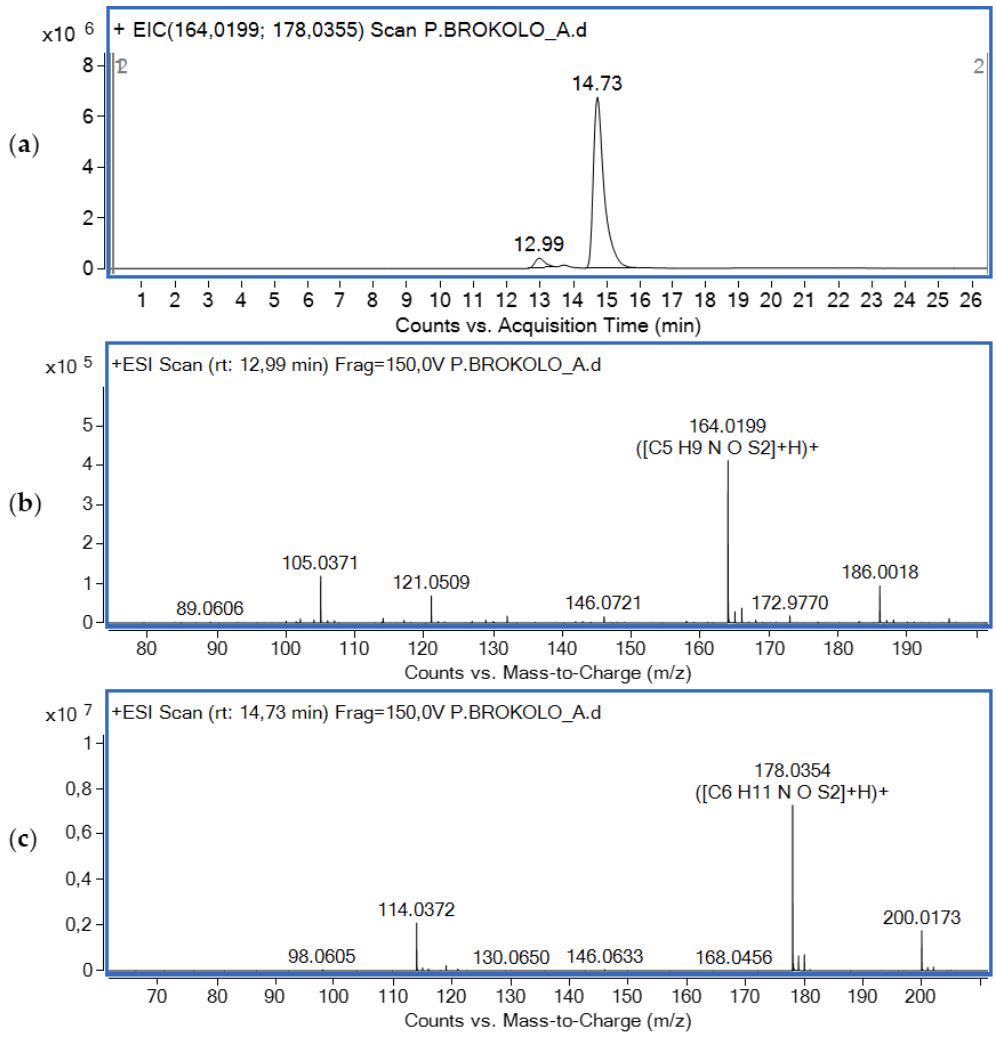

Figure 4. (**a**) Extracted ion chromatograms of sulforaphane and iberin in green broccoli extract; (**b**) Mass spectrum of iberin in green broccoli extract; (**c**) Mass spectrum of sulforaphane in green broccoli extract.

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