Extraction, Purification and Identification of The Bioactive Compound Sulforaphane from Broccoli (*Brassica Oleracea* Var. *Italica*) By HPLC

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KEYWORDS

Myrosinase; Broccoli; Sulforaphane; purification; HPLC

ABSTRACT

The study included extraction, purification and diagnosing of the bioactive compound sulforaphane from broccoli (*Brassica oleracea* var. *Italica*) and dietary supplement of sulforaphane (Double Wood) by HPLC using different solvents including DMSO, HPLC water, acetonitrile, and dichloromethane. Sulforaphane an isothiocyanate widely distributed in *Brassicaceae* vegetables that comes from the enzymatic hydrolysis of glucoraphanin through myrosinase. The results of the HPLC chromatogram of the purified sulforaphane appearing 4 peaks including sulforaphane at high percentage 82.715%, HPLC chromatogram of extracted sulforaphane by HPLC water where appearing 8 peaks including sulforaphane at low percentage 4.542%, and HPLC chromatogram of the sulforaphane supplement (Double Wood) extracted by acetonitrile where appearing 4 peaks including 2 peaks of sulforaphane at percentage 5.545% and 39.038% respectively, while HPLC chromatograms of all extracts by DCM contain sulforaphane at percentage ranging from 99.904%-100%. The results of HPLC chromatogram are compared to the standard sulforaphane which diagnosed at retention time 2.640 min. and wavelength 254 nm.

1. Introduction

Broccoli (*Brassica oleracea* var. *Italica*) comeback to *Brassicaceae*, known since the time of the Romans, which is morphologically similar to cauliflower culturing in cold seasons, broccoli have a good therapeutic value as regulating blood sugar, lowers cholesterol and high blood pressure, as well as considered a rich source of sulforaphane which having anticarcinogenic effects. In Iraq, broccoli cultivation is still not widely known, and cultured with cauliflower (1). Myrosinase is an important enzyme found in broccoli, cabbage, and cauliflower, members in the *Brassica* family that have different nutritional importance due to its high content of bioactive compounds, particularly glucosinolates (2). The system of glucosinolate-myrosinase represented the entrance of defense mechanism in all Brassica vegetables, when the plant tissue is damage, myrosinase release and hydrolysis the glucosinolates to formation bioactive compounds such as isothiocyanates, which are displaying variety of healthy properties like anticancer activity (3,4). Sulforaphane as well as the derived isothiocyanates from glucosinolates contribute in the detoxification enzymes modulation and protection the cells from risk of the chronic diseases such as cancer (5,6). Myrosinase plays an essential role in the glucoraphanin hydrolysis into the active compound sulforaphane, which has anticancer and antioxidant properties (7). Isothiocyanates (ITCs) are the volatile phytochemicals that belong to the category of highly reactive organosulfur compounds and are identified as the main compounds responsible for the characteristic pungent aroma and bitter taste of the cruciferous vegetables e.g. broccoli, radish, watercress, cabbage, cauliflower and mustard (8), which are not present in the plants in their native form, but are formed when glucosinolates get hydrolyzed by myrosinase enzyme. Glucosinolates (GSLs) are the chemically stable and biologically inactive thioester compounds that are the precursors of isothiocyanates (9). Upon exposure to physical conditions such as cell disruption or mastication, these plants activate the myrosinase enzyme that hydrolyzes the glucosinolates into glucose and several unstable intermediates that undergo rearrangements and transform into secondary metabolites such as isothiocyanates, indoles, thiocyanates and nitriles (10). Sulforaphane an isothiocyanate widely distributed in *Brassicaceae* vegetables, its chemical structure consists of a sulfoxide group wherein the sulfur is a chiral center with four different groups around the sulfur: oxygen, a four-carbon chain terminating in an

isothiocyanate, a methyl group and the electron pair solitary, because of this, the isothiocyanates are strongly electrophilic compounds that can react with nucleophilic groups such as thiol, hydroxyl, and amino groups (11). Sulforaphane has health promoting properties, which are related with its high capacity to induce Phase II detoxifying enzymes, additionally, SFN exhibits the highest bioavailability among well-known antioxidant phytochemicals, about 20-fold higher than quercetin, and 80-fold higher than curcumin (12,13). SFN comes from the enzymatic hydrolysis of glucoraphanin, occurs through myrosinase, which is compartmentalized in the vegetable inside myrosin cells (14). The aim of this search to extraction, purification and identification of the bioactive compound sulforaphane from the local broccoli by HPLC and choose the best solvent for extraction with high recovery.

2. Methodology

Materials. Broccoli purchased from the local markets in Basra, Iraq. Cleaned and washed with distilled water and kept at the refrigerator temperature at 4 °C in polyethylene bags until use.

Chemicals. Standard glucoraphanin from Santa Cruz Biotech.Co., USA, standard sulforaphane and bovine serum albumin (BSA) from Sigma-Aldrich Co., USA. All solvents from HPLC grade including water, dichloromethane (DCM), acetonitrile from BHD Co., England. Dimethyl sulfoxide (DMSO) from Fluka Co., Germany.

Apparatuses. High Liquid Performance Chromatography (HPLC), Shimadzu Co., Kyoto, Japan.

Enzyme extraction. Crude myrosinase extract was prepared according to (15) with some modifications by using sodium phosphate buffer (pH 6.5) in a ratio of 1:3 (w/v), put about 50 g of broccoli, added 150 ml from the buffer solutions in the electrical blender to damage the plant tissue and releasing myrosinase, then leaving the mixture for 10 min. to complete enzyme reaction, then filtration with 4 layers of cheesecloth to get rid of the big parts of the plant, centrifuged at 5000 rpm/min. for 30 min., discard the sediment and keep the supernatant for determination of enzyme activity later. All steps were performed at 4 °C.

Enzyme assay. Myrosinase activity was determined according to (16) that described by (17) with some modification, a reaction mixture consisting of 0.5 ml of broccoli extracted by 33 mM potassium phosphate buffer (pH 6.5) containing 0.5 mM ascorbic acid., 0.5 ml extracted glucoraphanin (from the best procedure), and 1ml of glucose reagent (kit GOD/POD ready to use), incubated in a water bath at a temperature of 37 °C for 15 minutes, the reaction was stopped at 95 ◦C for 5 min, enzyme activity was measured at 546 nm according to glucose kit procedure, the supernatants were used as a crude enzyme in the later purification steps.

Protein assay. A concentration of protein was measured using bovine serum albumin (BSA) as standard (18).

Sulforaphane extraction and purification. Sulforaphane was extracted and purified according to (19) that described by (20) with some modifications. About 25 g of broccoli sprouts were homogenized with 200 ml of distilled water, then hydrolyzed at 37 °C for 3 h in a water bath. After that, 50 ml was extracted three times with 112.5 ml of dichloromethane. The dichloromethane fraction was dried at 35 °C under vacuum on a rotary evaporator. The residue was dissolved in 2 ml of 10% acetonitrile and filtered through a 0.45 µm Millipore filter. The purified extracts were diagnosing by HPLC column C18 (5 µm particle size, 250 × 4.6 mm). HPLC conditions are run out according to method of (21) with some modification, the solvent consisted of 20% acetonitrile with HPLC-grade water 80%, column temperature 30 °C, flow rate 0.5 ml/min, injection volume 10 µl, and total run 10 min. at wavelength 254 nm. The concentration of the extracted sulforaphane was calculated from the equation of the sulforaphane standard curve comparing with standard sulforaphane.

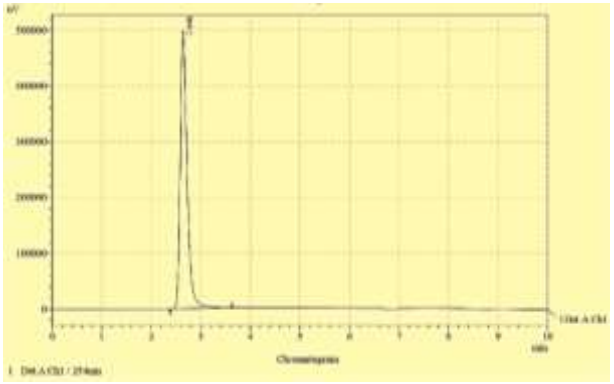
Sulforaphane dietary supplements. The dietary supplement of sulforaphane that was used in the

study is from brand Double Wood (DW) diagnosing by HPLC as the same conditions mention above after extraction with water, DCM, and acetonitrile to identify sulforaphane in this supplement.

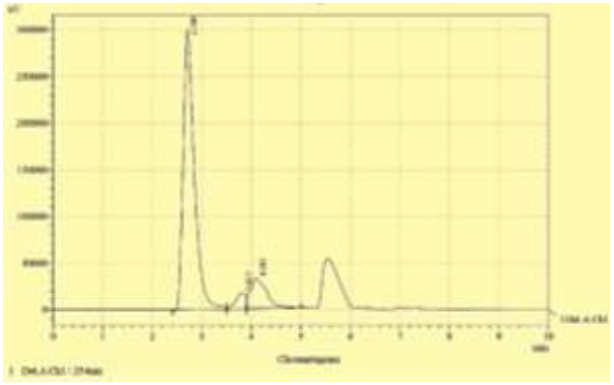
Diagnosing standard sulforaphane by HPLC. Standard sulforaphane was prepared by dissolving 25 mg in dimethyl sulfoxide (DMSO) as stock solution was injected in the HPLC as the same conditions mention above. The standard curve of sulforaphane was prepared from sulforaphane stock solution at concentrations (5, 10, 15, 20, and 25) mg/ml with DMSO, drawing the relationship between standard sulforaphane concentration and sub-curved area to finding the equation of the standard curve.

Result and Discussion

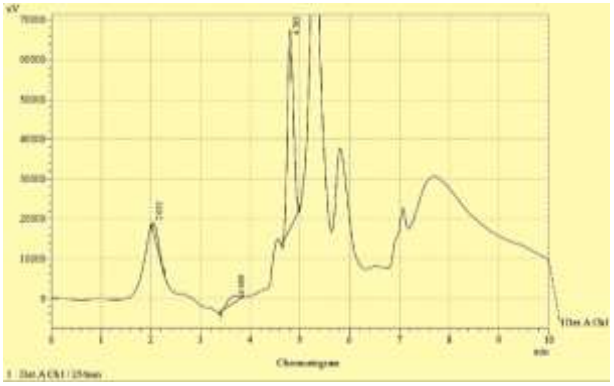
Diagnosing standard, extracted and supplements of sulforaphane by HPLC. The results shown in the figure (1-A) refers to the HPLC chromatogram of the standard sulforaphane that diagnosed at retention time 2.640 min. and wavelength 254 nm. The result in the figure (1-B) indicates to the HPLC chromatogram of the purified sulforaphane appearing 4 peaks including sulforaphane at high percentage 82.715%, the figure (1-C) indicates to the HPLC chromatogram of extracted sulforaphane by HPLC water where appearing 8 peaks including sulforaphane at low percentage 4.542%, the figure (1-D) indicates to the HPLC chromatogram of extracted sulforaphane by acetonitrile where appearing 7 peaks including sulforaphane at percentage 22.648%, and the figure (1-E) indicates to the HPLC chromatogram of extracted sulforaphane by DCM where noting only one peak for sulforaphane at percentage 100%. The result in the figure (1-F) indicates to the HPLC chromatogram of the sulforaphane supplement (Double Wood) extracted by HPLC water where appearing 7 peaks don't contain sulforaphane, the figure (1-G) indicates to the HPLC chromatogram of the sulforaphane supplement (Double Wood) extracted by acetonitrile where appearing 4 peaks including 2 peaks of sulforaphane at percentage 5.545% and 39.038% respectively, and the figure (1-H) indicates to the HPLC chromatogram of the sulforaphane supplement (Double Wood) extracted by DCM where noting 2 peaks including sulforaphane at percentage 99.904%. DMSO can dissolve both polar and nonpolar substances, this property makes it effective in solubilizing a wide range of organic compounds, which allows to extract sulforaphane efficiently from complex plant matrices (22), also regarded as safe solvent due to it used in biological and pharmaceutical applications, but using high concentrations, may induce cellular toxicity, which may limit its use in certain sensitive applications (23). Water is essential for hydrolysis myrosinase where converts glucoraphanin into sulforaphane, the reaction occurs naturally in an aqueous environment, making water indispensable in extraction that rely on enzymatic activity, as well as water is widely compatible with various analytical techniques, including HPLC due to its polar nature that makes it an effective solvent for polar compounds like glucosinolates and their derivatives, where facilitates the extraction of sulforaphane and related compounds from plant tissues (24,25). Acetonitrile is less polar than water but still effective in extracting a wide range of organic compounds, its moderate polarity allows it to solubilize both polar and nonpolar substances, making it a versatile solvent for the extraction of sulforaphane (26). It can extract sulforaphane more efficiently from both polar and nonpolar compounds because it is effective in combination with other solvents or as part of a solvent gradient in extraction protocols, leading to higher overall yields of sulforaphane (27), while dichloromethane despite its efficiency, safety concerns may limit its widespread use because it is more toxic than DMSO and acetonitrile, it is classified as a potential carcinogen solvent, so requires more careful handling and proper ventilation to prevent exposure to harmful vapors (28).



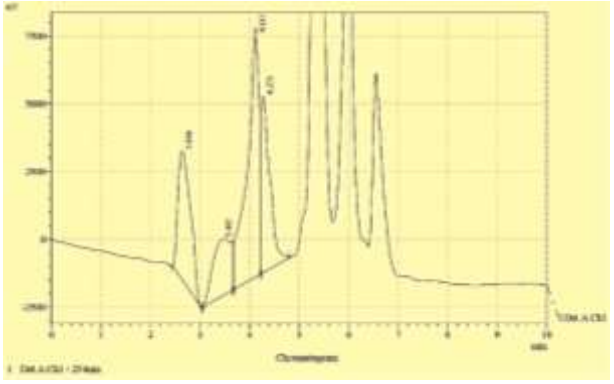
A: Standard sulforaphane



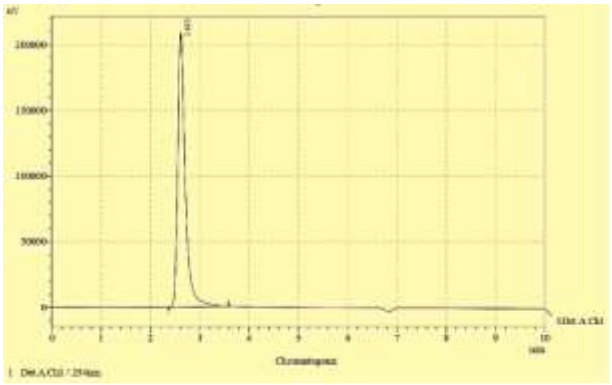
B: Purified sulforaphane



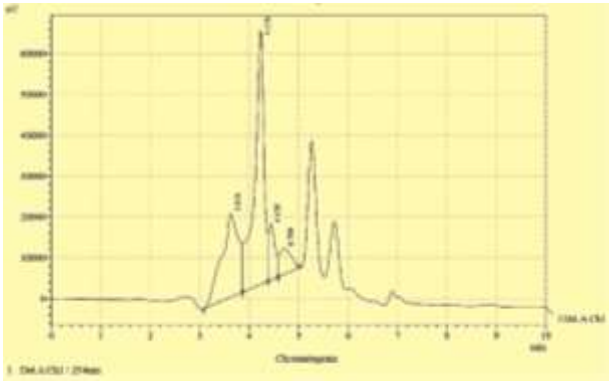
C: Extracted sulforaphane by HPLC water



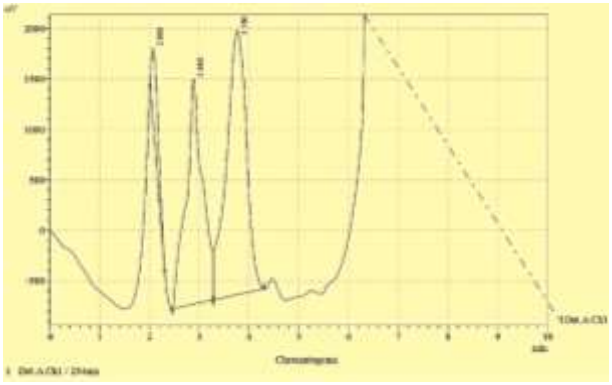
D: Extracted sulforaphane by acetonitrile



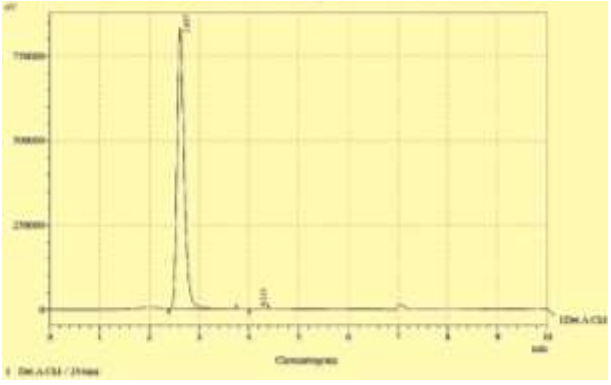
E: Extracted sulforaphane by DCM



F: Sulforaphane supplement (DW) with HPLC water



G: Sulforaphane supplement (DW) with acetonitrile



H: Sulforaphane supplement (DW) with DCM

Figure 1 (A-H): HPLC chromatograms of standard, purified, extracted, dietary supplement (DW) of sulforaphane with different solvents at wave length 254 nm and retention time 10 min.

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