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ORIGINAL ARTICLE

# Quantitative Analysis of Benzyl Isothiocyanate in *Salvadora persica* Extract and Dental Care Herbal Formulations Using Reversed Phase C18 High-Performance Liquid Chromatography Method

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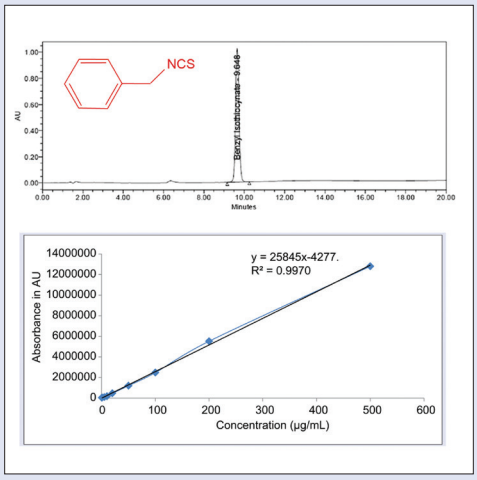
## ABSTRACT

**Context:** Benzyl isothiocyanate is the active antimicrobial agent in *Salvadora persica* (siwak) widely used in Islamic countries for oral hygiene. **Aims:** Quantification of benzyl isothiocyanate in the ethanol extract of *S. persica* and some dental care herbal formulations labeled to contain siwak. **Settings and Design:** Simple and sensitive high-performance liquid chromatography method was designed. **Subjects and Methods:** Separation was achieved on reverse phase $C_{18}$ (250 mm × 4.6 mm, 5 µ) column with a mobile phase comprising acetonitrile and water (1:1). The detection was carried out at 190 nm using ultra violet-visible detector. The flow rate was kept at 1 mL/min. **Results:** A sharp and well-defined peak was obtained at the retention time of 9.322 ± 0.3 min. Linear regression analysis data for the calibration plot showed a good linear relationship between response and concentration in the range of 0.5–500 µg/mL with a regression coefficient ($r^2$) of 0.9977. The method was validated for accuracy, precision, robustness, and sensitivity. All the parameters examined met the current recommendations for the International Conference on Harmonization guidelines for method validation. **Conclusions:** The method was applied for the quantification of benzyl isothiocyanate in siwak extract, dental care powder, mouth wash, and toothpaste claimed to contain siwak. The developed method was found specific, simple, selective, and reliable for routine use in quality control analysis of different commercially available herbal care products.

**Key words:** Benzyl isothiocyanate, dental care products, International Conference on Harmonization guidelines, reversed phase C18 high-performance liquid chromatography, *Salvadora persica*

## SUMMARY

- A simple, accurate and precise method was developed for the analysis of the antimicrobial agent benzyl isothiocyanate in Salvadora persica (Siwak) extract and selected dental care herbal formulations using RP18 HPLC
- Amount of benzyl isothiocyanate will indicate the efficacy of Siwak products
- The method subject to ICH validation guidelines.




**Abbreviations used:** RP18: Reversed phase C18, HPLC: High performance liquid chromatography, UV: Ultra violet, $r^2$: regression coefficient, ICH: international conference on harmonization, TLC: Thin layer chromatography, $CHCl_3$: Chloroform, v/v: volume/volume, RSD: Relative standard deviation, LOD: Limit of detection, LOQ: Limit of quantification.

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## INTRODUCTION

Family *Salvadoraceae* comprises three genera *Azima*, *Dobera*, and *Salvadora*.[1] *Salvadora persica* (siwak) is one of the most popular medicinal plants throughout the Muslim world.[2-4] *S. persica* with its fibrous branches have been promoted by the World Health Organization for oral hygiene.[5] The spread of Islamic culture had a significant influence on the use of *S. persica*.[6] Among 182 plant species suitable for preparing tooth brushing sticks, *S. persica* is the most extensively used.[7] The extracts of *S. persica* roots have significant antimicrobial activity.[8-10] The main antimicrobial component is the sulfur-containing volatile component benzyl isothiocyanate.[11]

We report here on the development of a high-performance liquid chromatography (HPLC) method for the quantification of benzyl isothiocyanate in *S. persica* and dental care products labeled to contain siwak using reversed phase C18 column. This method provides a tool to evaluate the medical value of any siwak product based on its benzyl isothiocyanate contents.



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## SUBJECTS AND METHODS

### Samples, chemicals, solvents, and standards

Standard benzyl isothiocyanate [Figure 1] was purchased from Sigma-Aldrich, St. Louis, MO, USA. HPLC grade acetonitrile and methanol (E. Merck, Darmstadt, Germany) were used for the analysis. Deionized water was purified by Milli-Q system (Millipore, Bedford, MA, USA). Orthophosphoric acid ($H_3PO_4$) 88% was purchased from Fisher Scientific Company (UK).

### Plant material and dental care herbal formulations

The roots of *S. persica*, family *Salvadoraceae* were purchased from the local market at Al-Kharj city in March 2016. The plant material was identified by Dr. Mohammad Atiqur Rahman, Taxonomist of the Medicinal, Aromatic and Poisonous Plants Research Center, College of Pharmacy, King Saud University, Riyadh, Saudi Arabia. Voucher specimen (#9011) was deposited at the herbarium of this center. Dental care herbal products labeled to contain siwak extract were purchased from the local market at Al-Kharj city, Saudi Arabia. Others formulations were purchased from Hyderabad, India.

### Extractions procedure for plant materials

The fresh roots of *S. persica* (20 g) were cut into small pieces and extracted with ethanol (3 mL × 100 mL) by maceration. Extracts were combined, evaporated under reduced pressure using rotary vacuum evaporator, transferred to 20 mL volumetric flask, volumes were completed using ethanol. Solutions were kept in refrigerator till the time of analyses.

### Preparation of dental care herbal products for analysis of benzyl isothiocyanate

Accurately weighed 10 g from each product labeled to contain siwak extract was separately extracted with ethanol (3 mL × 70 mL) for 30 min. The ethanol extracts from each sample were combined and separately concentrated under reduced pressure using rotary vacuum evaporator. The concentrates of each sample were separately reconstituted in accurately measured 10 mL of ethanol and stored under refrigeration until the chromatographic analysis. A volume of 200 mL of mouth wash product labeled to contain siwak extract were extracted with $CHCl_3$ three times, 100 mL each. The combined $CHCl_3$ soluble fractions were evaporated under reduced pressure and transferred to 10 mL volumetric flask with ethanol.

### High performance liquid chromatography chromatographic conditions

The HPLC analysis was performed on a Waters Alliance e2695 separating module (Waters Co., MA, USA) using ultra violet detector (Waters 2998) with autosampler and column oven. The instrument was controlled using "EMPOWER" software installed with equipment for data collection and acquisition. Compounds were separated on a $C_{18}$ reverse phase column (250 mm × 4.6 mm, particle size 5 µm) maintained at room temperature. Acetonitrile and water in the ratio of 1:1 were used as the mobile phase. The flow rate was kept as 1 mL/min. The detection wavelength was 190 nm and the injection volume was 10 µL. All chromatographic operations were carried out at ambient temperature.

### Method validation

The guidelines of the International Conference on Harmonization (ICH) were applied for the validation of the proposed HPLC method.[12]

### Linearity

A stock solution containing 1000 µg/mL of benzyl isothiocyanate was prepared in ethanol and different aliquot were prepared to get known concentrations starting from 0.1 to 500 µg/mL. The calibration graph was plotted using peak area versus drug concentration [Figure 2]. For assessing the linearity, the least square regression equation was used to calculate the correlation coefficient [Table 1].

### Accuracy

Recovery of benzyl isothiocyanate was determined by the standard addition method where extra amounts of benzyl isothiocyanate standard (0, 50, 100, and 150%) were added to preanalyzed samples and the mixtures were reanalyzed using the proposed method [Table 2].

### Precision

To evaluate precision, repeatability and intermediate precision were carried out. Repeatability of the method was evaluated by carrying out six independent assays of a test sample against benzyl isothiocyanate standard and calculating the percent relative standard deviation (RSD). Intra- and inter-day precisions were done by repeating the analysis on the same day (intra-day precision) and on three consecutive days (inter-day

**Table 1:** Linear regression data for the calibration curve of (*n*=3)

| Parameters | HPLC |
|---|---|
| Linearity range (ng/spot) | 0.5-500 µg/mL |
| Regression equation | Y=25,845X–4277 |
| Correlation coefficient | 0.9970 |
| Slope±SD | 2584±25.25 |
| Intercept±SD | 4277.7±202.5 |
| SE of slope | 134.16 |
| SE of intercept | 19.61 |

HPLC: High performance liquid chromatography; SD: Standard deviation; SE: Standard error

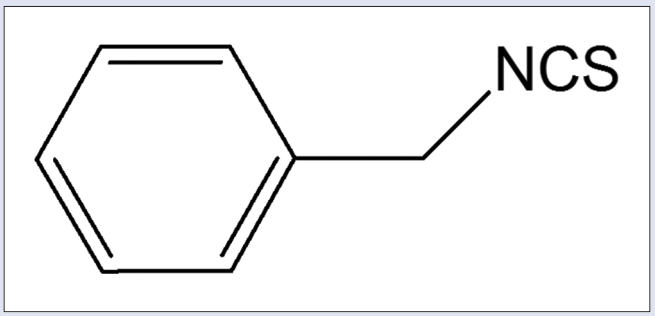


**Figure 1:** Structure of benzyl isothiocyanate

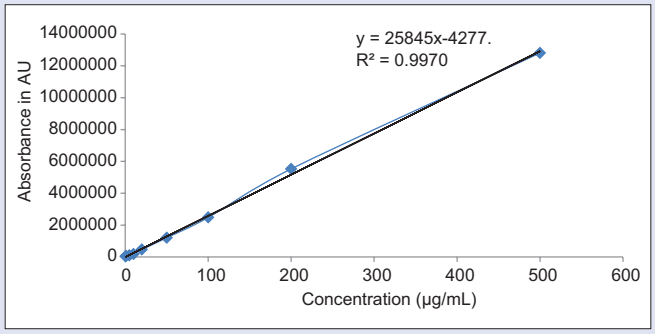


**Figure 2:** Linearity graph of benzyl isothiocyanate

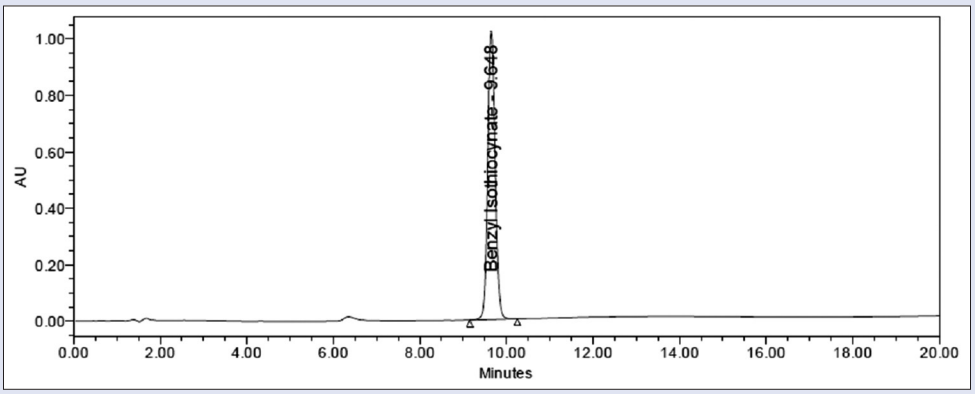


**Figure 3:** High-performance liquid chromatography chromatogram of benzyl isothiocyanatein standard at 190 nm

**Table 2:** Accuracy of the method (*n*=3)

| Percentage of standard spiked to the sample | Theoretical content (µg/mL) | Amount of drug recovered (µg)±SD | Percentage of drug recovered | Percentage of RSD |
|---|---|---|---|---|
| 0 | 142 | 141.3±1.15 | 99.5 | 1.17 |
| 50 | 213 | 215.7±2.52 | 101.3 | 1.07 |
| 100 | 284 | 281.0±3.00 | 98.9 | 1.55 |
| 150 | 355 | 355.7±5.51 | 100.2 | 0.82 |

SD: Standard deviation; RSD: Relative standard deviation

**Table 3:** Repeatability of the method (*n*=6)

| Concentration (µg/mL) | Peak area | | Retention time | |
|---|---|---|---|---|
| | Mean peak area±SD | Percentage of RSD | Mean $R_t$±SD | Percentage of RSD |
| 100 | 2,336,154±26,533 | 1.14 | 9.537±0.08 | 0.85 |
| 200 | 4,844,610±40,285 | 0.83 | 9.614±0.01 | 0.07 |
| 500 | 12,071,199±57,747 | 0.48 | 9.668±0.05 | 0.55 |

SD: Standard deviation; RSD: Relative standard deviation

precision). Assay for each analysis was calculated and %RSD was determined [Tables 3 and 4].

## Robustness

The robustness of the method was carried out by purposefully altering the chromatographic conditions and observing the changes. Here, we made variations in the flow rate of the mobile phase and the detection wavelength and observed the variation in retention time and area of the peak [Table 5].

## Limit of detection and limit of quantification

Limit of detection (LOD) (signal-to-noise ratio = 3.3) and limit of quantification (LOQ) (signal to noise ratio = 10) for the method has been determined based on signal-to-noise ratio for both compounds as per the ICH guidelines.

# RESULTS AND DISCUSSION

## Method development

To find out a suitable mobile phase for the analysis of benzyl isothiocyanate in the ethanol extract of siwak and selected dental care herbal formulations, different trials had been carried out. Different compositions of mobile phase with methanol and water; acetonitrile and water have been applied in different ratios. Since the absorbance maxima suitable for quantitation of benzyl isothiocyanatein was found as 190 nm, buffers were avoided from the mobile phase, by considering the fact that wavelengths below 210 can suffer from interference due to impurity present in buffer, solvents which may obscure the peak of interest. Finally, chromatographic separation was carried out successfully in the isocratic mode using a mixture of acetonitrile: Water (50:50, v/v) as mobile phase. Retention time of benzyl isothiocyanatein was found as 9.322 ± 0.3 min [Figure 3]. The column temperature was ambient. The column was equilibrated with the mobile phase flowing at 1.0 mL/min for about 30 min before injection.

## Linearity

Calibration graph was plotted on the basis of triplicate analysis of each calibration solutions using peak area against concentration and was found linear in the range of 0.5–500 µg/mL with a good linear relationship of 0.9977 ± 0.0011 [Figure 2]. The linear regression data for the calibration plot are indicative of a good linear relationship between peak area and concentration over a wide range [Table 1].

## Accuracy

Recovery study for the proposed method was conducted by spiking previously analyzed test solution with benzyl isothiocyanate standard. The recovery of the method was found in the range of 98.9–101.3%. The values of % recovery, standard deviation and %RSD are listed in Table 2.

## Precision

The repeatability and intermediate precisions were calculated and reported in terms of %RSD in Tables 2 and 3. Intermediate precision include data of intra- and inter-day. The low values of %RSD indicate the reproducibility of the method, which can be adopted in any laboratory for the routine analysis of benzyl isothiocyanate in crude drug and different herbal formulations [Tables 3 and 4].

## Robustness

There was no significant change in the retention time and area of benzyl isothiocyanate was found when the flow rate of the mobile phase and detection wavelength were slightly changed. The low values of the RSD, shown in Table 5, indicated the robustness of the method.

**Table 4:** Precision of the method (*n*=6)

| Concentration (µg/mL) | Interday precision | | Intraday precision | | Inter system precision | |
|---|---|---|---|---|---|---|
| | Mean peak area±SD | Percentage of RSD | Mean peak area±SD | Percentage of RSD | Mean peak area±SD | Percentage of RSD |
| 100 | 2,329,414±41,059 | 1.76 | 2,358,918±39,164 | 1.66 | 2,355,918±50,040 | 2.12 |
| 200 | 4,831,277±51,906 | 1.07 | 4,843,080±45,321 | 0.94 | 4,819,747±23,964 | 0.50 |
| 500 | 12,104,532±100,667 | 0.83 | 12,181,199±162,601 | 1.33 | 12,257,866±227,455 | 1.86 |

SD: Standard deviation; RSD: Relative standard deviation

**Table 5:** Robustness of the method by changing detecting wavelengths and temperature of column (*n*=6)

| Parameters | Actual | Used | Mean area±SD | Percentage RSD of area | Mean $R_t$±SD | Percentage RSD of $R_t$ |
|---|---|---|---|---|---|---|
| Detecting wavelength (nm) | 254 | 252 | 2,305,512±8598 | 0.37 | 9.469±0.12 | 1.26 |
| | | 254 | 4,784,123±54,456 | 0.55 | 9.544±0.11 | 1.11 |
| | | 256 | 12,091,199±75,728 | 1.87 | 9.640±0.04 | 0.44 |
| Flow rate (mL/min) | 1 | 0.8 | 2,343,178±32,047 | 1.37 | 9.483±0.07 | 0.75 |
| | | 1 | 4,748,790±58,450 | 1.23 | 9.540±0.12 | 1.26 |
| | | 1.2 | 12,075,832±64,704 | 0.54 | 9.597±0.08 | 0.87 |

SD: Standard deviation; RSD: Relative standard deviation

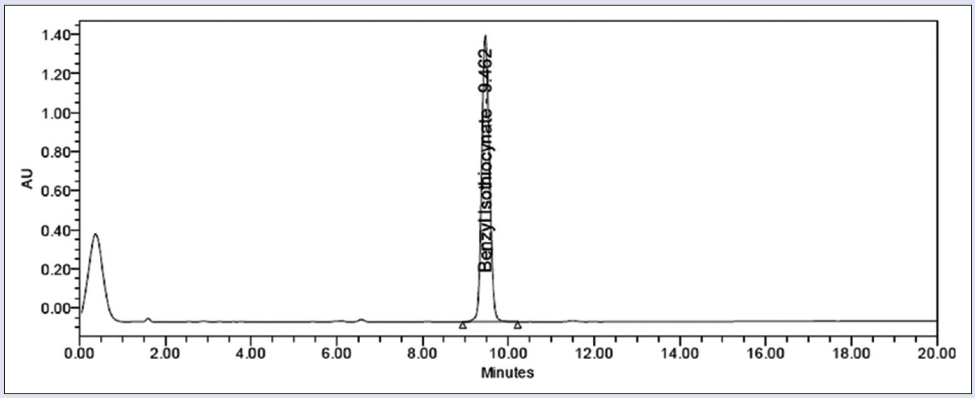


**Figure 4:** High-performance liquid chromatography chromatogram of benzyl isothiocyanatein in siwak sample at 190 nm

## Limit of detection and limit of quantification

The LOD and LOQ were determined by signal to noise ratio method and found to be 0.15 µg/mL and 0.55 µg/mL, respectively.

## Analysis of benzyl isothiocyanate in samples

The proposed validated method was applied for analysis of benzyl isothiocyanate in the ethanol extract of siwak [Figure 4] and selected dental care herbal formulations labeled to contain siwak extract. The peak areas of triplicate samples were analyzed by regression equation obtained from calibration plot to get the content of benzyl isothiocyanate in samples. The benzyl isothiocyanate content in the ethanol extract of siwak was found as 0.071% w/w. At the same time, in dental care formulations, the peak of benzyl isothiocyanate was not detected, and hence, it was reported as absent. It was also demonstrated that peaks of benzyl isothiocyanate in siwak extract was well resolved and did not merged with any impurity or any other constituent of drug.

# CONCLUSION

This HPLC method reported here is accurate, precise, reproducible, and specific with good sensitivity. The mobile phase used in the method was simple without using buffer and resulted in symmetric peaks at reasonable retention time. Extraction procedure was straight forward and no prior treatment was required. The method can be successfully used for routine analysis of benzyl isothiocyanate in crude drug and in herbal formulations without interference.

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## Conflicts of interest

There are no conflicts of interest.

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