International Journal of Biochemistry Research & Review

18(2): 1-9, 2017; Article no.IJBCRR.35180
ISSN: 2231-086X, NLM ID: 101654445

Different Approaches for Quantification of the Bioactive Metastable Thiosulfinates in Garlic Using a Validated High Performance Liquid Chromatography Method

Florence A. Opondo[1*], Were L. L. Munyendo[2], Patrick M. Shem[3] and Raphael O. Nyayieka[1]

[1]Department of Chemistry and Biochemistry, Moi University, P.O.Box 3900 – 30100 Eldoret, Kenya.
[2]School of Pharmacy and Health Sciences, United States International University – Africa, P.O.Box 14634 – 00800 Nairobi, Kenya.
[3]Department of Chemical Sciences and Technology, Technical University of Kenya, P.O.Box 52428 – 00200 Nairobi, Kenya.

Authors' contributions

This work was carried out in collaboration between all authors. Author FAO designed the study, wrote the protocol and wrote the first draft of the manuscript. Authors WLLM and PMS performed the statistical analysis and managed the analyses of the study. Author RON managed the literature searches and methodology. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/IJBCRR/2017/35180
Editor(s):
(1) Sultan Ayesh Mohammed Saghir, Discipline of Pharmacology, School of Pharmaceutical Sciences, Universiti Sains Malaysia, Penang, Malaysia.
Reviewers:
(1) Mosad Ahmed Ghareeb, Theodor Bilharz Research Institute (TBRI), Egypt.
(2) Birsa Mihail Lucian, Alexandru Ioan Cuza University of Iasi, Romania.
Complete Peer review History: http://www.sciencedomain.org/review-history/20228

Original Research Article

Received 30th June 2017
Accepted 21st July 2017
Published 26th July 2017

ABSTRACT

Aims: In this study *Allium sativum* extracts *were* evaluated for antibacterial activity, High Performance Liquid Chromatography method was developed and validated for direct quantification of alliin and indirect quantification of metastable bioactive thiosulfinates.
Study Design: The experimental research design composed of bioassay using Disc-diffusion method and chemical assay which involved differential quantification of bioactive metastable thiosulfinates in *Allium sativum* using a validated HPLC method.

**Corresponding author: E-mail: fopondo07@gmail.com, munyendow@gmail.com;*

Place and Duration of Study: Chemical Engineering Laboratory, School of Engineering, Moi University, Eldoret, Kenya. The study duration was from June 2015 to August 2016.
Methodology: Antibacterial activity conducted for gram-positive *Staphylococcus aureus* and *Pseudomonas aeroginosa* while *Escherichia coli* was bioassayed as the gram-negative bacteria. The bioactive metastable thiosulfinates were differentially quantified using HPLC method through direct quantification of alliin.
Results: The highest percent yield of 0.32% was realized from a garlic cloves blend marc of 25 g. The activity of the extracts was noted to be dependent on the duration of time after reconstitution but before assaying. This was revealed from evaluation of antibacterial activity constancy which indicated that the garlic extract exhibited a pattern of decreasing zone of inhibition from above 14 mm at 3 hours reducing to 6 mm after 24 hours. The HPLC method developed enabled elution of alliin at 2.5 minutes illustrating high levels of accuracy from the calculated mean percent recovery ± SD that ranged from 99.06 ± 0.08 to 99.56 ± 0.11 and 99.08 ± 0.12 to 99.34 ± 0.03 for the inter-day and intra-day respectively which is regarded optimum for the method application. The data obtained for quantification is in agreement with the results of bioactivity constancy evaluation in that as the bioactivity of the garlic extracts diminishes with time after extraction, so the % bioactive thiosulfinates falls along the time intervals from 22.9% at 0 hour to 10.0% after 24 hours.
Conclusion: The devised method for differential quantification of bioactive thiosulfinates proved to be valid and accurate hence applicable for evaluating the metastable bioactive constituents of garlic extracts.

Keywords: Thiosulfinates; garlic; antibacterial; high performance liquid chromatography.

1. INTRODUCTION

The antimicrobial efficacy and other healing properties displayed by *Allium sativum* (Garlic) are attributed to thiosulfinates as well as other bioactive secondary metabolites compounds which are formed through an enzymatic reaction that occur when garlic cloves are crushed [1]. These thiosulfinates are not naturally present in an intact garlic bulb, but it is the crushing of the bulb that triggers release of the enzyme alliinase required for enzymatic reaction which produces thiosulfinates [2]. During this rapid process, various kinds of the thiosulfinates are formed of which allicin and alliin are produced in the greatest abundance. The amounts of alliin and allicin present in different strains of garlic have been studied and considerable variations reported ranging from 2.8 to 7.7 mg/gram [3].

The transformation of alliin into the biologically active allicin molecule upon crushing of a garlic clove is extremely rapid. This efficiency ensures that the clove defense mechanism is only activated in a very small location and for a short period of time, whereas the rest of the alliin and alliinase remain preserved in their respective compartments and are available for interaction in case of subsequent microbial attacks [4].

Although garlic extracts have continually been used as herbal therapy for a variety of bacterial, fungal and viral infections; safety issues have been highlighted regarding ingestion of garlic extracts [5]. The literature cautions against use of garlic herbal medications thereby leaving a gap in knowledge regarding appropriate exploitation of *Allium sativum* extracts. These gaps regards garlic chemical constituents' stability and biotransformation alongside unexplained contraindications like topical garlic burns, anaphylaxis and platelet dysfunction not studied could continue to be magnified. Therefore extensive investigation into garlic and its medicinal value has led to an improvement in the quality and yield of fresh garlic production. Several garlic food supplements ranging from tablets to powder are now formulated based on either alliin content or on the potential to produce allicin [6].

Consequently, a number of analytical methods are currently being used to determine S-alkenylcysteine sulfoxides in garlic. These include; electrophoresis, spectrophotometric methods, gas chromatography, micellar electrokinetic capillary chromatography, thin layer chromatography, high performance ion-pair chromatography, and high performance liquid chromatography. The methods are categorized into direct methods that allow determination of S-alkenylcysteine sulfoxides content before their enzymatic hydrolysis to form allicin or indirect methods that are employed in the determination of diverse products arising from the enzymatic reaction [7].

Most of these methods are realized to require external standards which have very low resolution during quantitative analysis. On the other hand Gas Chromatography (GC) remains less suitable, the metastable thiosulfinates have to be converted to more stable compounds which are able withstand high temperatures in the GC column. Usually by spectrophotometric techniques, quantification of thiosulfinates targets thiols as a probe. In a direct approach, thiols are reacted with the disulfide bond present in thiosulfinates and the resulting thiol concentration is monitored.

Alternatively the excess thiol could be reacted with a disulfide acid compound in an indirect approach [1]. Both spectrophotometry and chromatography have also been previously combined to specifically quantify allicin. This involved a reversed phase HPLC with UV and electrochemical detection (ED) coupled with on-line post-column photochemical reaction [8].

Since allicin is electrochemically inactive, post-column irradiation at 254 nm reduces the responsiveness of allicin to UV detector but allow it to be detected using electrochemical detector. As the quantification of allicin, alliin and other bioactive metastable thiosulfinates in garlic continue to present a challenge. This paper therefore reports for the first time a differential approach in which a direct determination of the more stable alliin using a validated HPLC method yields reliable data that is applied to quantify the bioactive metastable thiosulfinates in garlic more accurately and precisely. A cross-validation of the results in this paper is also illustrated by the concurrent quantification of results to the evaluation of antimicrobial activity constancy with time after garlic extraction both by infusion and soxhlet techniques.

2. MATERIALS AND METHODS

2.1 Chemicals and Apparatus

The extraction process was done on a Soxhlet system model 345 (England) with 0.45 µm microbibre filters and Whitman thimbles sourced from Ultra lab Nairobi Kenya while Chloroform, ethyl acetate, hexane, ethanol, methanol all HPLC grade and Dimethyl Sulfoxide (DMSO) were sourced from Gelsap Laboratory supplies Nairobi Kenya. Standard Allyl sulfonic acid (alliin) was purchased from Sigma Aldrich St Louis USA. Bacterial strains for bioactivity evaluation were obtained from Microbiologics, Canada; Mueller Hinton agar and Amoxicillin clavulanate impregnated discs were from Cypress Diagnostics, Belgium. The HPLC analytical work was done on a LC-10AT Liquid Chromatography System with SPD-10A UV-Vis detector, (Shimadzu Japan), using Luna^{TM} ODS C_{18} column (250 mm x 4.6 mm, 5 µm). All the solvents and reagents were of analytical grade and used without any further purification.

2.2 Sample Collection, Processing and Extraction

Allium sativum (Garlic) plant sample cloves were collected from Kimalel Location specifically at 0°28'0.01'' N 35°58' 0.01'' E in Baringo County, Kenya. The fresh cloves were carried in polythene bags to the laboratory, then washed with clean water to remove soil before aerating to avoid rotting. Garlic juice was produced aseptically from peeled cloves using a juice extractor which afforded a smooth paste. The fresh garlic paste (50 ml) was immediately centrifuged at 4000 × g for 10 min at 24°C and decanted in readiness for extraction.

Hot continuous extraction method was adopted for extractions. In a soxhlet apparatus, 25 g of the blended garlic paste was extracted with 100 ml of analytical grade methanol solvent cycling for 2 hours over a heating mantle at 30°C. Excess solvent was removed by rota evaporation under vacuo and extract immediately stored in a freezer at 40°C to minimize loss of volatile constituents. Percentage yields for the extraction was then calculated by the following expression adopted from [9].

$$\text{Percentage yield} = \frac{crude\ extract}{blended\ garlic\ cloves}\ X\ 100 \qquad (1)$$

2.3 Evaluation of Extracts' Antibacterial Activity Constancy

The rapid bioconversions of allyl thiosulfinates in garlic extracts impacts the bioactivity. This was evaluated by antibacterial activity testing at time intervals using the disc diffusion method [10] Based on NCCL's recommendation, the gram-positive *Staphylococcus aureus* (ATCC 25923) and *Pseudomonas aeroginosa* (ATCC 27853) while gram-negative *Escherichia coli* (ATCC 25922) were used as test microorganisms representing most common infectious diseases pathogens. To obtain freshly growing strains, from the purchased isolates bacterial strains were sub-cultured onto Muller Hinton agar and incubated at for 37°C for 4 hours.

To evaluate antibacterial activity constancy at set time intervals of 0, 3, 6, 12 and 24 hours, a 10^8 colony forming units (cfu/mL) was prepared in sterile distilled water and inoculated uniformly onto the agar. A disc of 5mm diameter was then impregnated with 10 µL of the test extract (100 mg/mL reconstituted in DMSO) and aseptically placed on the inoculated petri dish before incubating at 37℃ for 24 hours. This was repeated in triplicate for all the set time intervals with zones of inhibition being measured at respective 24^{th} hour for each. In the experimental set up the negative controls were methanol and DMSO solvents while positive control was the Amoxicillin clavulanate impregnated discs. The trend of extract antibacterial activity with time was then graphically traced to describe the activity constancy.

2.4 Metastable Thiosulfinates Quantification Method Development and Validation

Given that allyl thiosulfinates (alliin) is the predominant precursor in garlic cloves which interacts with the enzyme alliinase to produce metastable thiosulfinates that are the main biologically active components, a HPLC method was developed and validated for the determination of allyl sulfonic acid to be applied for quantification of the thiosulfinates by difference.

2.4.1 Chromatographic conditions optimization

The HPLC system comprised of a Shimadzu LC-10AT pump, a SPD-10A UV detection at 224 nm, on a Luna™ ODS-2 RP-18 column (250 mm x 4.6 mm, 5 µm). Two mobile phase systems were assessed in terms of peak resolution and retention times, the first comprised of aqueous glacial acetic acid: methanol (50:50) (v/v) while the second 0.01% sodium dodecylsulfate solution: methanol (30:70). The flow rate was optimized by altering rate of flow from 0.5 mL/min to 0.75 mL/min and finally 1.0 mL/min. Effect of the column temperature on the resolution was also studied at 25℃, 35℃ and 55℃.

2.4.2 Calibration curves

A series of standard solutions were prepared by diluting allyl sulfonic acid stock standard solution (100.0 mg/mL) with the mobile phase to obtain two sets of concentration ranges of 0.2 –1.0 mg/mL and 0.5 mg/mL – 8.0 mg/mL. 25 µL were then injected for each concentration in triplicate and chromatographed under the optimized conditions. The calibration curve was constructed by plotting the peak area against the corresponding concentration of standard solutions ranges each separately but on the same axis for comparison based on the computed regression coefficients.

2.4.3 Accuracy and precision

The accuracy of the method was ascertained by determination of the analytical recoveries. This entailed intra-day and inter-day evaluations by analysis of alliin at three concentration levels (0.2 mg/mL, 0.6 mg/mL and 1.0 mg/mL) in quality control samples (n=9) on the same day and on three consecutive validation days. Computed Relative Standard Deviation (RSD) in precision statistics was used to establish precision.

2.4.4 Robustness and limit of quantification / detection

The robustness of the developed method was assessed by purposely altering experimental conditions. Effect of the flow rate on peak resolution was studied by changing stepwise from a flow rate of 0.6 mL/min to 1.5 mL/min while mobile phase composition remained the same. The column temperature impact on the peak resolution was also studied at 45℃ and 65℃.

Similar to previous studies [11], the Limits of quantification and detection were calculated by the simultaneous analysis of alliin in prepared solutions and using the SD values from inter-day and intra-day recoveries precision statistics.

2.5 Differential Quantification of the Metastable Bioactive Thiosulfinates

Samples of garlic preparations were made mimicking the formulations usually applied in alternative medicine products during its utilization as a therapeutic. These entailed an infusion prepared by gentle boiling the blended cloves in distilled water for 15 minutes. The second preparation was a soxhlet extract of blended extract using ethanol and methanol solvents.

The content of alliin in all the samples were determined at set time intervals as 0, 3, 6, 12 and 24 hours. The bioactive metastable bioactive thiosulfinates were then quantified using the following expression that was devised by the Author.

$$\%\ \text{Bioactive thiosulfinates}_{\text{ith hour}} = \frac{Alliin\ (mg/ml)_{0th\ hour} - Alliin\ (mg/ml)_{ith\ hour}}{Alliin\ (mg/ml)_{0th\ hour}} X\ 100 \quad (2)$$

Where,

0th hour is the time immediately after blending the garlic cloves
ith hour is at the set time interval (3rd, 6th, 12th and 24th hour) after blending garlic cloves

3. RESULTS AND DISCUSSION

3.1 Sample Processing and Extraction

The obtained extract was quantified by comparing to the blended garlic cloves gel that was packed into soxhlet apparatus for extraction. The highest percent yield of 0.32% was realized from a garlic cloves blend marc of 25 g. This could be regarded as being low in respect to the efficiency of soxhlet as an extraction method. Nevertheless the low yield is orchestrated by the duration for the extraction which was set cognizant to the nature of the targeted constituents of garlic that are volatile in nature.

3.2 Evaluation of Extract's Antibacterial Activity Constancy

The activity of the extracts was noted to be dependent on the duration of time after reconstitution but before assaying. The evaluation of antibacterial activity constancy could be illustrated by Fig. 1 indicating the realized zones of inhibition on performing the antibacterial assays after set time intervals post reconstitution for the controls and garlic extract. The solvent DMSO used as the negative control illustrated a minimal zone of inhibition equivalent to the disc diameter. This was realized to remain constant for all the duration. The pattern was also evident with the positive control amoxicillin clavulanate discs that maintained a specific stable zone of inhibition even on assaying at 24 hours post reconstitution. Strikingly the garlic extract for all strains of bacterial assays exhibited a pattern of decreasing zone of inhibition.

There was a consistent reduction in zones of inhibition with increase in time after reconstitution but before assay. This could be explained from the point that the antimicrobial activity of garlic extract constituents declines on standing which could be linked to the enzymatic transformation of bioactive thiosulfinates as indicated in previous findings [12]. This is therefore a confirmation of the bioactive constituents in garlic extract being metastable which is in line with reports of [13], who showed that extracted compounds from garlic usually undergo some sort of change in consequence of conditions like photochemical irradiation.

Direct quantification of bioactive constituents thus presents challenges with this non-constancy witnessed which therefore calls for alternative analytical strategies to exactly elaborate on duration of activity for dosage regimens clarity.

3.3 Metastable Thiosulfinates Quantification

3.3.1 HPLC method development and validation

Based on the presented challenges for the direct quantification of bioactive thiosulfinates, this study aimed at developing a HPLC method that determines alliin which is the key precursor envisaged to act on pro-antibacterial constituents in garlic extract as a reliable differential quantification technique. The standard dimethyl sulfoxide (alliin) was found to be stable in the diluent for more than 24 hours hence allowing for effective elution and separation.

This guided adoption of the mobile phase as aqueous acetic acid: methanol in the ratio 50:50 (v/v). An increase in the percentage of methanol led to poor resolution while increasing the percentage of distilled water resulted in longer run time. The flow rate of 0.75 mL/min ensured a comparatively short run time while the column was thermostated at 35℃. Under these conditions, alliin was eluted at 2.5 minutes as shown in the chromatogram (Fig. 2), a peak that was absent on elution of a blank.

The alliin peak areas responses were plotted against the respective concentrations from which calibration curve was built using up to six concentrations in the range 0.2 mg/mL to 1.0 mg/mL. This set of standard solutions gave satisfactory linearity as demonstrated from the obtained regression coefficient (R^2) of above 0.999, thus sufficient for quantification of alliin.

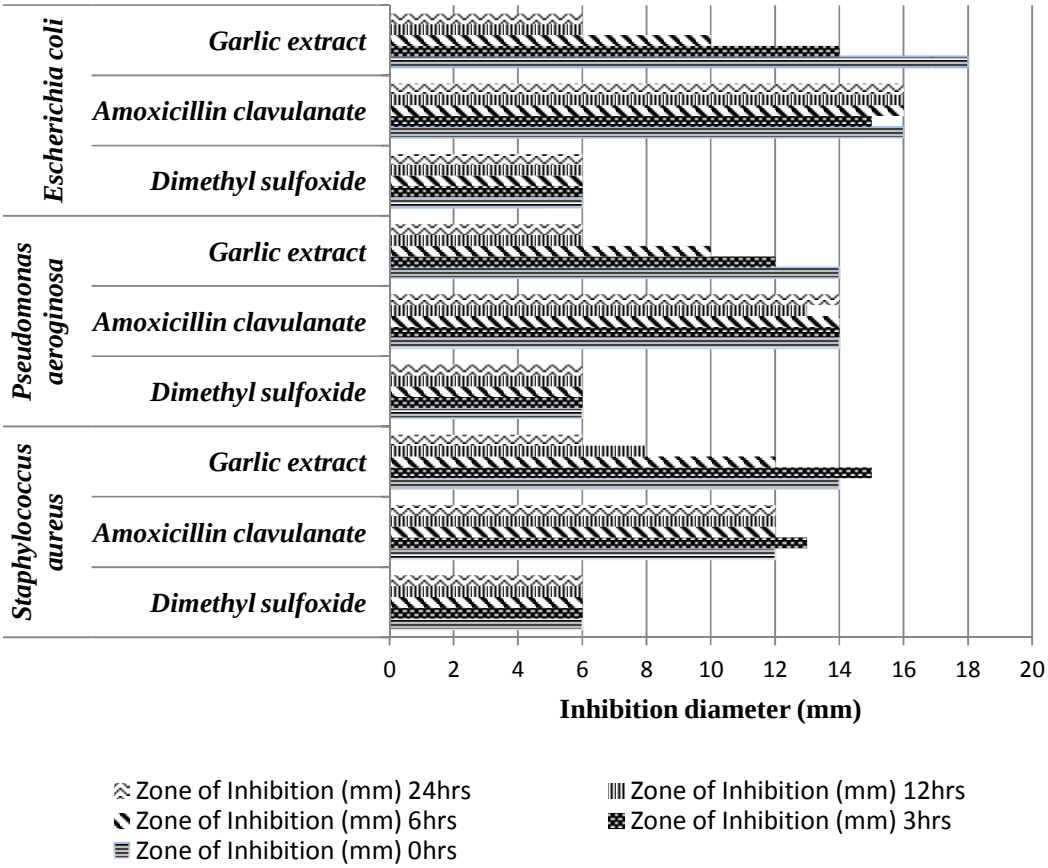

Fig. 1. Variation of zone of inhibition with time after reconstitution in DMSO

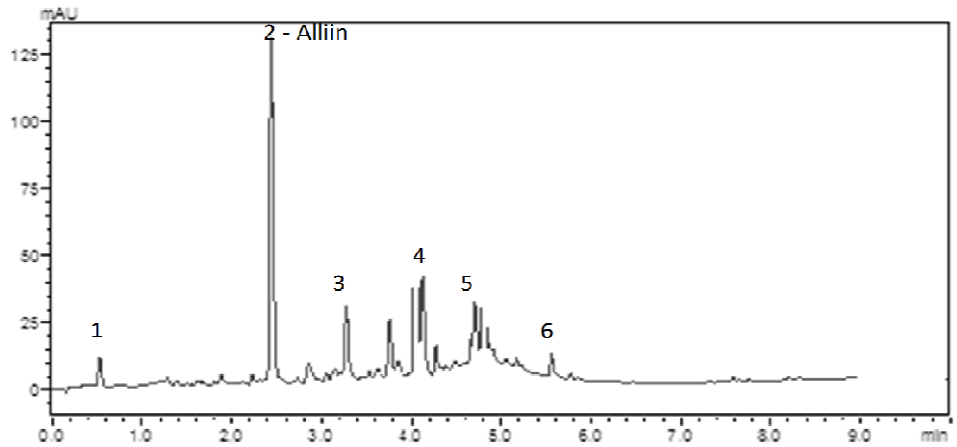

Fig. 2. HPLC chromatograph for standard alliin elution

The developed method was validated for determination of alliin through evaluation of accuracy and precision through intra-day and inter-day determinations whose precision statistical data is represented in Table 1.

High level of accuracy was illustrated by the developed method as seen from the calculated mean percent recovery ± SD that ranged from 99.06 ± 0.08 to 99.56 ± 0.11 and 99.08 ± 0.12 to 99.34 ± 0.03 for the inter-day and intra-day

respectively which is regarded optimum for the method application.

Satisfactory precision was also noted from the quite minimal values for the RSD% was just 0.0082 and 0.0299 for inter-day and intra-day respectively. Chromatographic conditions variations did not result in significant change in values of the peak area. Alteration of the flow rate affected only the retention time with an indirect proportionality fashion in that increasing flow rate resulted in a respective reduction in the retention time and vice versa but not the peak area. Elution at 45℃ or 65℃ did not have any recognizable variations in assay values. This could be related to the fact that usually, column temperatures affect mainly dissolution of the analyte in the mobile phase [11]. Robustness of the method was thus confirmed hence the method fit for utilization for determination of alliin in matrices.

The lowest concentration of alliin that could be determined was evaluated in terms of Limit of Quantification (LOQ) and found to be 0.186 mg/mL while the Limit of Detection (LOD) was 0.492 mg/mL.

As per the recommendations of International Conference for Harmonization [14] on validation of analytical procedures, LOD ought to be 3.3 times lower than the LOQ. This condition was found to have been fulfilled by the developed method in this study.

The developed method attributes were wholesomely found to satisfy the criteria for use in the determination of alliin and on that basis it deemed valid for application in the quantification of alliin and hence forming a basis for the metastable thiosulfinates quantification by difference.

3.4 Differential Quantification of the Metastable Bioactive Thiosulfinates

The developed and validated HPLC methods was applied for the direct determination of alliin in the garlic samples. The peak area realized was correlated to the concentration of the prepared standard solution and by the equation of the straight line calibration curve concentrations of alliin was found to be as indicated in Table 2 for the respective time interval. This was then utilized as the requisite data for the quantification of the metastable bioactive thiosulfinates applying the equation (ii). In the application of equation (ii) the 0^{th} hour was considered the initial time following the set time for a particular time interval. This provided accurate duration determination for the bioconversion events that yielded the bioactive thiosulfinates in the extract.

Calculations were therefore appropriately carried out considering the 0^{th} for 3^{rd} hour to be 0 hours; for 6^{th} hour to be 3 hours; for 12^{th} hour 6 hours and for 24^{th} hour 12 hours. This enabled computation of the percent bioactive thiosulfinates in the extracts at the set time intervals which gave the quantities shown in Table 3.

The results for the quantification of bioactive thiosulfinates indicated that infusion as an extraction method yields a high percentage of bioactive thiosulfinates as compared to the soxhlet extraction. This could be attributed to the metastability of the active constituents in garlic that may be affected by the repeated heating during the soxhlet extraction method.

Table 1. Precision statistics results for intra-day and inter-day analysis of alliin standard

Conc. mg/mL	Day1	Day2	Day3	Mean	S.D	RSD	RSD%
0.2	98.95	98.85	99.39	99.06	0.082533	**0.000833**	**0.0833**
0.4	99.52	99.71	99.46	99.56	0.106562	**0.001070**	**0.1070**
0.6	98.52	99.23	99.18	98.98	0.323557	**0.003269**	**0.3269**
0.8	98.94	99.16	99.57	99.22	0.261066	**0.002631**	**0.2631**
1.0	99.19	99.30	99.11	99.20	0.077889	**0.000785**	**0.0785**
1.2	99.34	99.33	99.35	99.34	0.008165	**0.000082**	**0.0082**
Mean	99.08	99.26	99.34				
S.D	0.124667	0.077587	0.029667				
RSD	**0.001258**	**0.000782**	**0.000299**				
RSD%	**0.1258**	**0.0782**	**0.0299**				

Table 2. Alliin concentration at set time intervals

Sample	Alliin (mg/mL) at time intervals				
	0th hour	3rd hour	6th hour	12th hour	24th hour
Infusion extract	2.35	1.81	1.47	1.29	1.16
Ethanolic soxhlet extract	1.66	1.42	1.26	1.13	1.04
Methanolic soxhlet extract	1.72	1.44	1.23	1.09	0.99

Table 3. Calculated % bioactive thiosulfinates at set time intervals

Sample	% Bioactive thiosulfinates at time intervals				
	0th hour	3rd hour	6th hour	12th hour	24th hour
Infusion extract	0.00%	22.9%	18.7%	12.2%	10.0%
Ethanolic soxhlet extract	0.00%	14.4%	11.3%	10.3%	7.96%
Methanolic soxhlet extract	0.00%	16.2%	14.5%	11.4%	9.17%

The data obtained for quantification is in agreement with the results of bioactivity constancy evaluation in that as the bioactivity of the garlic extracts diminishes with time after extraction, so the % bioactive thiosulfinates falls along the time intervals. This is a clear justification and further support for the previous explanation that the alliin is enzymatically converted to other thiosulfinates which are responsible for the antimicrobial efficacy [9]. The devised method for differential quantification of bioactive thiosulfinates is also proved to be valid and accurate hence applicable for evaluating the metastable constituents of garlic extracts.

4. CONCLUSION

The accurate quantification of the efficacious constituents in garlic extracts involves a series of analytical steps through complex instrumentation with extraneous experimental processes. This continues to be a drawback in the exploitation of Allium sativum plant phytochemical principles fully into therapeutic agents. In bid to contribute to simplification of this analysis a HPLC method was successfully developed exhibiting satisfactory accuracy and precision and with recommended Limits of Detection and Limits of Quantification desirable for analysis of bioactive thiosulfinates even in bulk. The antimicrobial activity constancy evaluation revealed a reduction in bioactivity of garlic extracts with time after extraction which was in agreement with the results of the devised method for differential quantification of the bioactive thiosulfinates in the extracts. Therefore a confirmation and proof of the validity and applicability of the devised method for quantification of bioactive thiosulfinates in garlic extracts.

ACKNOWLEDGEMENTS

The authors are sincerely grateful and appreciate Mr. Kenneth Werimo, the Director Kenya Marine and Fisheries Research Institute, Kisumu (KEMFRI, Kisumu) for giving us the opportunity to conduct the bioassay. The authors are greatly indebted to the other Researchers Mr. Ouko and Mr. Odada for their invaluable advice on bioassay. Many thanks also go to Technicians Mr. Odhiambo Gilbert and Madam Mary Akinyi for their assistance during the research process at KEMFRI, Kisumu, Kenya.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Mansor N, Herng HJ, Samsudin SJ, Sufian S, Uemura Y. Quantification and characterization of allicin in garlic extract. J. of Medical and Bioengineering. 2016;5(1):24–27.
2. Block ES, Ahmad JL, Catalfamo MK, Apitz-Castro R. The chemistry of alkyl thiosulfinate esters: Antithrombotic organosulfur compounds from garlic: Structure, mechanistic and synthetic studies. J of the American Chemical Society. 1986;108:7045-7055.
3. Olech Z, Zaborska W. A spectrophotometric assay for total garlic thiosulfinates content. Pol. J. Food Nutr. Sci. 2012;62(1):23–29.
4. Shen CH, Xiao H, Parkin KL. *In vitro* stability and chemical reactivity of

thiosulfinates. J. Agric Food Chem. 2002;50(9):2644–2651.
5. Camargo CR, Galmarini PW, Simon CK. Effect of cooking on garlic (*Allium sativum L*.), anti-platelet activity and thiosulfinates content. J Agric Food Chem. 2007;55: 1280-1288.
6. Miron T, Feigenbalt G, Weiner L, Mirelman D, Wichek M, Rabinkov A. A spectrophotometric assay for allicin, alliin and alliinase (alliin-lyase) with a chromogenic thiol: Reaction of 4-mercaptopyridine with thiosulfinates. J. Anal. Biochem. 2002;307:76–83.
7. Apawu KA, Chu-Ngi Ho CK, Wardeska JD, Peng Sun W. Reversed-phase HPLC determination of alliin in diverse varieties of fresh garlic and commercial products. J. of Chromatography. 2009;83:634–805.
8. Han J, Lawson L, Han G, Han P. Spectrophotometric method for quantitative determination of allicin and total garlic thiosulfinates. J. Anal. Biochem. 1995;225:157–160.
9. Ilic D, Nikolic´ DV, Nikolic´ BL, Stankovic´ ZM, Stanojevic´ PL, Cakic´ DM. Allicin and related compounds: Biosynthesis, synthesis and pharmacological activity. J of Physics Chemistry and Technology. 2011;1:9–20.
10. National Committee for Clinical Laboratory Standards. Performance standards for antimicrobial disk susceptibility tests: Approved standards NCCLS document M2-A4, Villanora. 2013;4:28-33.
11. Munyendo WL, Baraza LD, Sowayi G. Stability and kinetics studies using an RP-HPLC-UV method developed for assays of salvianolic acid A degradation as a therapeutic. Int. Research J. of Pure and Applied Chem. 2015;7(3):99–109.
12. Benavides GA, Squadrito GL, Mills RW, Patel HD, Isbell TS, Patel RP, et al. Hydrogen sulfide mediates the vasoactivity of garlic. Proc Natl Acad Sci USA. 2007;104(46):17977–17982.
13. Bocchini, P, Andalò, C, Pozzi, R, Galletti, GC, Antonelli, A. Determination of diallyl thiosulfinate (allicin) in garlic (*Allium sativum L*.) by high-performance liquid chromatography with a post-column photochemical reactor. Analytica Chimica Acta. 2001;441:37–43.
14. ICH Harmonised Tripartite guideline, validation of analytical procedures: Text and methodology Q2 (R1); 2003. Available:http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q2R1 Guideline.pdf (Accessed 15 November 2016)

Peer-review history:
The peer review history for this paper can be accessed here:
http://sciencedomain.org/review-history/20228