A Chemometrics Study of Antimicrobial Activity in Garlic Extract

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ABSTRACT

Combining ^{13}C NMR spectroscopy and a multivariate statistical analysis such as principal components regression (PCR), the identification of the probable compounds which makes one mixture different from another is possible. The Ilocos and Taiwan varieties of garlic (*Allium sativum*) were extracted and fractionation, profiled by ^{13}C NMR, and assayed for antibacterial activity against *Escherichia coli*. PCR analysis was done using the ^{13}C NMR shifts and their antibacterial activity in both lag and log growth phases. Results show that the Ilocos and Taiwan varieties are different from each other.

PCR analysis revealed that the ^{13}C NMR shifts of the compounds responsible for antibacterial activity are δ = 61, 63, 70 and 104.5 ppm—corresponding to an unsaturated organic molecule with a –C–S=O functional group.

Keywords

Garlic, *Allium sativum*, nuclear magnetic resonance (NMR) spectroscopy, chemometrics, principal components analysis (PCA), principal components regression (PCR).

1. INTRODUCTION

Garlic extracts are known to be effective against Gram-negative bacteria. Various active principles containing sulfur ethers and sulfoxides have been isolated, namely allicin, ajoene and a number of allyl sulfides [2,3]. However, each compound individually has been found to exhibit weak activity as compared to the crude extract [4]. The isolation and purification of a number of garlic compounds have been reported to be difficult because of the instability of the main parent compound, allicin, from which a large number of other components such as ajoene, allyl sulfides and disulfides derive themselves [5]. Allicin is itself produced only when the bulb is bruised or injured: it is part of the plant's defense mechanism against infection upon injury [6].

Chemometrics is the chemical discipline that uses mathematical and statistical methods for designing optimal experimental procedures and for providing maximum chemical information through the analysis of chemical data, collapsing them into a more comprehensible format [7,8]. The concept of pattern recognition—where sets of preprocessed data are correlated and their underlying patterns determined by statistical methods—is an integral part of chemometrics. Its end-result is a model, which can represent the data accurately and predict or validate other related data. Principal components analysis (PCA) and principal components regression (PCR) are two of the many available statistical methods of chemometric analysis which is based on pattern recognition.

2. PCA, PCR AND UNSCRAMBLER®

Principal Component Analysis (PCA) is one of the multivariate methods of analysis. It has been used widely with large multidimensional data sets and the use of PCA allows the number of variables in a multivariate data set to be reduced, while retaining as much as possible the variation present in the data set. PCA concerns itself with only one data matrix while Principal Component Regression (PCR) concerns two matrices, the X matrix which contains the independent variables and the Y matrix containing the dependent variables [9].

Given a set of **p** variables $\mathbf{X_1, X_2,\ldots, X_p}$, this reduction is achieved by taking the **p** variables and finding the combinations of these variables to produce eigenvectors called principal components **(PCs)** $\mathbf{PC_1, PC_2,\ldots, PC_p}$, which are uncorrelated. PCs are ordered so that PC_1 exhibits the greatest amount of the variation, PC_2 exhibits the second greatest variation, and so on.

Principal components (PCs) are linear functions of the original variables estimated to contain the main structured information in the data.

To obtain the PCs from the p variables, the following equation for squared covariance or correlation matrix is used:

(Eqn. 1.)

$$Cov(X_j, X_k) = \frac{\sum_{i=1}^{n}(X_{ij} - \overline{X}_j)(X_{ik} - \overline{X}_k)}{(n-1)}$$

where

$$\overline{X}_j = \frac{\sum_{i=1}^{n} X_{ij}}{n}$$

and $j, k = 1, 2, \ldots, p.$

The covariance matrix then has the following form:

(Eqn. 2):

$$S = \begin{bmatrix} s_{11} & s_{12} & s_{13} & \dots & s_{1p} \\ s_{21} & s_{22} & s_{23} & \dots & s_{2p} \\ . & . & . & & . \\ . & . & . & & . \\ s_{p1} & s_{p2} & s_{p3} & \dots & s_{pp} \end{bmatrix}$$

where S is the covariance matrix, s_{jk} is the covariance of variables X_j and X_k when j is not equal to k.

When the variables are measured on comparable scales, the covariance matrix is used; however, when the variables have different units or widely different scales, a correlation matrix where variables are standardized should be used.

PCA is concerned with finding the variances and coefficients of the data set or finding the eigenvalues and the eigenvectors of the sample correlation matrix. One way to calculate eigenvectors (PCs) and their associated eigenvalues from the correlation matrix is to use an iterative process.

Unscrambler® 9.1 is a comprehensive multivariate software developed by CAMO, Inc. (www.camo.com) that can be used to do PCA and PCR [10].

3. METHODOLOGY

3.1 Preparation of crude garlic extracts

Ilocos and Taiwan garlic bulbs were obtained from Shopwise Araneta Cubao. One hundred grams of Ilocos garlic cloves (IGC) and Taiwan garlic cloves (TGC) were peeled and crushed using mortar-and-pestle in 50 mL of deionized water. Large solid particles were filtered out, and the remaining mixture was freeze-dried (Labconco Freezone 4.5 Freeze Dry System). One gram of the freeze-dried extract was reconstituted with deionized water in 1-mL volumetric flasks.

3.2 Fractionation of garlic extract

Preparative reverse-phase thin-layer chromatography (RP-TLC) analysis showed the presence of four spots. Ten fractions were isolated isocratically from TGC/IGC through a packed reverse-phase silica column (Merck Silica Gel 60 RP-18, 40-63 μm), with 1:1 methanol:water as eluent. RP-TLC analysis on the fractions revealed the occurrence of overlaps, reducing the number of spots from four to two (fraction A: 1-4; B: 7-10). Fractions containing similar RP-TLC profiles were grouped into two—TG/IGF12 and TG/IGF34—and freeze-dried.

3.3 NMR Profiling

Freeze-dried TG/IGC and TG/IGF samples were reconstituted with deuterated water (D2O) in 1-mL volumetric flasks and were subjected to one-dimensional ^{1}H and ^{13}C NMR analysis (JEOL Lambda 400 MHz NMR spectrometer). The chemical shifts of ^{13}C NMR peaks for all samples were stored in individual peaktable.peak files. The reconstituted samples were stored in 1.5-mL microfuge tubes and stored at -20 °C.

3.4 Bioassay

E. coli (ATCC 25922) slant culture was purchased from the Microbiological Services division of the University of the Philippines - Natural Science Research Institute (UP-NSRI). Subcultures were grown in liquid LB media from the provided slant. All flasks were placed in a shaking water bath set at 37°C and incubated for 7 hours. Absorbances at 600 nm from each culture were taken per hour using UNICO 1100 UV-Vis spectrophotometer.

3.4.1 Extract at lag phase

Sterilized 50-mL erlenmeyer flasks containing LB broth were inoculated with E. coli from starter cultures grown for 12-14 hours in a shaking water bath (37°C). Each flask contained one of the following: 100 μL of reconstituted IGC, TGC, IGF12, TGF12, IGF34 and TGF34. Positive and negative controls were flasks inoculated with the same culture, one containing 100 μL 0.05g/mL ampicillin.

3.4.2 Extract at log phase

The sterilized culture flasks containing LB broth were inoculated with E. coli and incubated for 3 hours. The reconstituted extracts and/or fractions were then added, and their absorbances monitored per hour at 600 nm for 4 more hours.

4. RESULTS AND DISCUSSION

4.1 Primary Analysis

Bioactivity is quantified as the ratio of %transmittance-per-gram of extract/fraction and AMP, in a scale of 0 to 5—0 corresponding to zero activity and 5 to AMP's activity. For the Ilocos variety, both sets of IGF34's have the highest ratios. Based on the modified growth curves of the extracts and the fractions for both lag and log phases, there is no significant difference between IGC and TGC in both sets at lag phase (Table 1).

Table 1. Bioactivity Table of crude and fractionated Ilocos and Taiwan garlic

Sample Code	*LAG*	*LOG*
S1-IGC	2.45	2.48
S1-TGC	2.44	2.24
S2-IGC	2.44	3.43
S2-TGC	2.43	2.58
S1-IGF12	0.99	0.20
S1-TGF12	0.97	0.21
S2-IGF12	0.98	0.21
S2-TG12	0.97	0.27
S1-IGF34	6.24	4.02
S1-TGF34	3.44	2.03
S2-IGF34	6.22	1.58

The isolated fractions F12 and F34 possess different activities compared to the crude extracts. The low bioactivity of F12 per gram compared to either TGC or IGC and the increased bioactivity of F34 per gram indicate that there may be a negative synergistic effect caused by the first fraction. TGF12 and IGF12 both display possible inhibitory activity against TGF34 and IGF34, respectively, yet one cannot discount the possibility of the F12 fractions of both varieties having lower antimicrobial garlic compounds gram-per-gram as compared to the F34 fractions.

The ^{13}C NMR spectrum of IGF12 does not have all of the signature peaks for garlic sulfo-ethers and sulfoxides. Since the bioactivities of both IGF12 and TGF12 are the same in the lag and log phases of bacterial growth, this suggests either or both of the following possibilities: first, that other antimicrobial compounds are active, which are not organosulfur compounds; or second, that the suspect garlic organosulfur compounds may be present in very small amounts to be rendered undetectable by ^{13}C NMR.

The ^{13}C NMR spectra of IGF34 do not have the signature peaks for the allyl sulfides, disulfides and trisulfides (δ33.30, 41.72 and 42.49 ppm, respectively), but its narrow range of chemical shifts (δ57.001, 58.087, 61.246, 63.155, 63.945, 65.031 ppm) correspond to the expected chemical shift range for the –C-S=O functional group of allicin.

4.2 Chemometric Analysis

The ^{13}C NMR and bioassay data underwent PCR analysis using **Unscrambler® 9.1**. The results show that the two varieties of garlic have little difference in terms of their ^{13}C NMR profiles and antibacterial activities, as shown in the primary analysis and the PCR score and loading plots in Figures 1 and 2. Based on bioactivity ratio alone, IGC and TGC have no significant difference with each other (see Table 1), but when their bioactivities are factored in with their individual ^{13}C NMR spectra, chemometric analysis shows that there is indeed a small difference between the two varieties.

For the analysis of mixtures, that is, the fractionated components of garlic, PCR analysis indicates that the compounds present in the different fractions should have the following ^{13}C NMR shifts in order to make the mixture active against bacteria: δ = 61, 63, 70 and 104.5 ppm. This means that the suspect compounds should be unsaturated (δ = 104.5 ppm) organic molecules with a –C–S=O functional group (δ = 61, 63 and 70 ppm)—from the actual and predicted ^{13}C NMR shifts, allicin and ajoene fit the criteria.

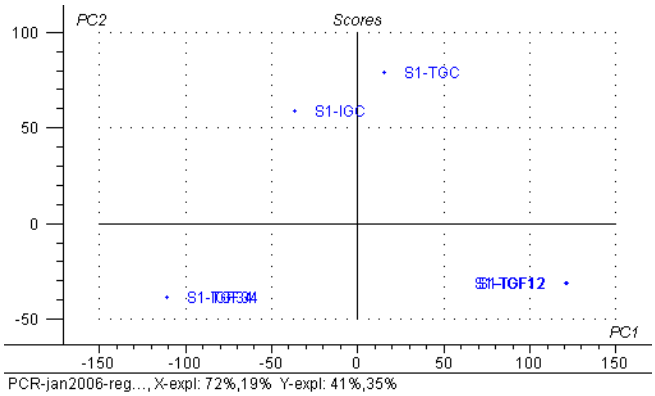

Figure 1. PC score plot for PC2 vs PC1

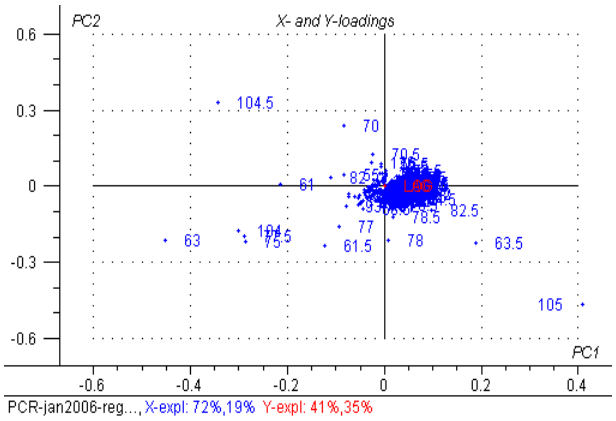

Figure 2. PC loadings plot for PC2 vs PC1

5. CONCLUSIONS

In profiling mixtures, whose components are difficult to isolate due to their relative instability, ^{13}C NMR spectroscopy can be effectively used, especially in the determination of the key carbon skeleton or functional groups of the compounds in question, since the spectra produced is less complex and has better resolution than that of ^{1}H NMR. The disadvantage, however, is the lower sensitivity of ^{13}C NMR as compared with ^{1}H NMR. Combined with a multivariate statistical analysis such as principal components regression (PCR), the identification of the probable compounds which makes one mixture different from another is possible. This study effectively illustrates this analytical technique.

The two varieties of garlic (*Allium sativum*), Ilocos and Taiwan varieties, have been subjected to extraction and fractionation, taking their ^{13}C NMR spectra for each crude extract and partially-purified fractions, as well as their antibacterial activity against E. coli, as raw data for PCR analysis, with the ^{13}C NMR shifts as the

X-variables and their antibacterial activity in both lag and lag phases as the Y- variables. The results show that the Ilocos and Taiwan varieties are different from each other. However, their partially-purified fractions are very similar for both varieties, signifying that the compounds which make Ilocos and Taiwan garlic different from each other are lost during fractionation. From the PCR loadings and regression coefficient charts, the ^{13}C NMR shifts of the compounds responsible for antibacterial activity are seen (δ = 61, 63, 70 and 104.5 ppm)—corresponding to an unsaturated organic molecule with a (–C–S(=O)-) functional group.

6. RECOMMENDATIONS

For further elaboration of the identity of the compounds responsible for garlic's activity, a combination of ^{13}C NMR and infrared (IR) absorbances of the mixtures may be used for chemometric analysis.

To confirm the correlation of ^{13}C NMR chemical shifts with bioactivity, known compounds must be analyzed using similar chemometric techniques.

7. ACKNOWLEDGEMENTS

The authors would like to thank DOST-PCASTRD for the research grant and the Ateneo de Manila University's Department of Mathematics for The Unscrambler™ 9.1 software.

8. REFERENCES

[1] Wilkinson, J. (2002). Synergistic effects in herbal medicine. *Phytopharmacognosy Lecture and Networking Event*. London.

[2] Ross, I. "Medicinal Plants of the World". © 1999, Humana Press, Totowa, NJ. pp. 26-63.

[3] Duke, J. http://www.ars-grin.gov/duke Last accessed 11 August 2005.

[4] Lawson, L. D. "Garlic: a review of its medicinal effects and indicated active compounds." *Phytomedicines of Europe: Chemistry and Biological Activity*; Lawson, L. D., Bauer, R., Eds.; ACS Symposium Series, American Chemical Society; ©1998, Washington, DC. Vol. 691, pp. 176-209.

[5] Ross, Z. M.; O'Gara, E. A.; Hill, D. J.; Sleightholme, H. V.; Maslin, D. J. (2001) Antimicrobial properties of garlic oil against human enteric bacteria: evaluation of methodologies and comparisons with garlic oil sulfides and garlic powder. *Appl. Environ. Microbiol*. 67(1): 475-480.

[6] Cavallito, C. J.; Bailey, J. H. (1944) Allicin, the antibacterial principle of Allium sativum. I. Isolation, physical properties and bacterial action. *J. Am. Chem. Soc*. 66: 1950-1951.

[7] Beebe, K. R.; Pell, R. J.; Seasholtz, M. B. "Chemometrics: A Practical Guide". ©1998, John Wiley and Sons, Inc., New York, NY. pp. 81-85.

[8] Otto, M. "Chemometrics: Statistics and Computer Application in Analytical Chemistry". ©1999, Wiley-VCH Verlag GmbH, Weinheim, Germany. pp. 124-132, 196-198.

[9] "Principal Component Analysis," University of Otago. http://neon.otago.ac.nz/chemlect/chem306/pca/Theory_PCA/index.html Last accessed 7 February 2006.

[10] The Unscrambler by Camo, Inc. http://www.camo.com/ Last accessed 7 February 2006.