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## Application of Principal Component and Hierarchical Cluster Analysis to Classify Different Spices Based on In-Vitro Antioxidant Activity and Individual Polyphenolic Antioxidant Compounds

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## **Application of principal component and hierarchical cluster analysis to classify different spices based on *in-vitro* antioxidant activity and individual polyphenolic antioxidant compounds**

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### **Abstract**

This study investigated the variations in antioxidant profiles between spices using pattern recognition tools; classification was achieved based on the results of global antioxidant activity assays (2,2-diphenyl-1-picrylhydrazyl [DPPH], oxygen radical absorbance capacity [ORAC], ferric reducing antioxidant power [FRAP], microsomal lipid peroxidation [MLP] and 2,2'-azinobis(3-ethylbenzothiazoline-6-sulphonic acid) [ABTS]), levels of different polyphenolic compounds (gallic acid [GA], carnosol [CAR], carnosic acid [CRA], caffeic acid [CA], rosmarinic acid [RA], luteolin-7-*O*-glucoside [LOG], apigenin-7-*O*-glucoside [APOG]) and total phenols [TP]) of spices namely rosemary, oregano, marjoram, sage, basil, thyme, fennel, celery, cumin and parsley, commonly consumed in Ireland were analysed. Rosemary showed the highest antioxidant activity measured by the DPPH (11.02 g Trolox/g DW) assay whereas oregano had the highest activity in ORAC (28.31 g Trolox/g DW) test. In contrast, parsley showed the lowest antioxidant activity in both of the assays. Interrelationships of these assays and the spices were investigated by principal component analysis (PCA) and hierarchical cluster analysis (HCA). PCA revealed that the first two components represented 73% of the total variability in antioxidant activity and different antioxidant groups. HCA classified samples into four main groups on the basis of the measured parameters.

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## **1. Introduction**

Spices have long been recognized to possess medicinal properties and have been effectively used in the indigenous systems of medicine in India and also in other countries (Nadkarni, & Nadkarni, 1976). Apart from their traditional use, a host of beneficial physiological effects have been reported by extensive animal studies during the past three decades. Among these are their beneficial influence on lipid metabolism (Srinivasan et al., 2004), efficacy as anti-diabetic (Srinivasan, 2005), ability to stimulate digestion, anti-carcinogenic, anti-atherosclerotic, and anti-inflammatory capacity (Lampe, 2003). Spices are a particularly good source of polyphenols with high antioxidant activities. The demand for healthy ingredients and a natural way of preventing diseases are contributing to the increased use of spices. World production of spices increased by approximately 24% in the year 2008 from 2000 (FAO, 2010). The American Spice Trade Association (ASTA, 2000) reported that in USA within the last twenty years there had been a significant increase in spice consumption with overall spice consumption being doubled. Many of the beneficial effects on health were found to be related to their high polyphenolic content. Polyphenols are secondary metabolites; they often are differentially distributed among limited taxonomic groups within the plant kingdom. Taxonomically linked spices might show considerable similarity in qualitative polyphenolic profile. However, quantity of individual polyphenols could vary widely in spices of the same family. Both qualitative and quantitative polyphenolic profiling together with total

antioxidant activity measured by different methods could be used to classify spices. Classification of spices is needed for dietary guidance materials to help people select appropriate types of these foods to meet their need for a healthy diet (Pennington, & Fisher, 2009). Many countries have food guides with graphic depictions of the food groups and subgroups, along with recommendations for consumption (Painter et al., 2002). The application of chemometric tools (Alonso-Salces et al., 2006; Arvanitoyannis et al., 1999; Chia-Hui, & Zhi-Kai, 2005; Downey et al., 2003; Forina et al., 2004; Kamimura et al., 2000; Woodcock et al., 2007) to the characterisation, determination of geographic origin and quality control of food products has recently become a very active research area. Çam et al. (2009) applied chemometrics to classify pomegranate juices on the basis of their antioxidant activity and reported the main determinant of this parameter to be cultivar. Wang et al. (2009) also carried out principal component analysis overview of the similarities and differences among 10 algal species and also investigated the relationships between total phenolic content and different antioxidant activity assays. PCA is a mathematical tool which performs a reduction in data dimensionality and allows the visualisation of underlying structure in experimental data and relationships between data and samples. Multivariate mathematical approaches are powerful tools which often permit a relatively simple representation of similarities between samples on the basis of more-or-less complex analytical data. The present study aims to use chemometric tools to gain insights into variations in the complex antioxidant profiles between selections of spices and to classify them based on antioxidant activity and levels of individual antioxidant polyphenols.

## **2. Materials and methods**

### *2.1. Samples and reagents*

Three batches of dried and powdered rosemary, oregano, marjoram, sage, basil, thyme, fennel, celery, cumin and parsley were sourced from AllinAll Ingredients Ltd., Dublin, Ireland. Folin-Ciocalteu Reagent, sodium carbonate, 2,2-diphenyl-1-picrylhydrazyl, anhydrous sodium acetate, acetic acid, ferric chloride hexahydrate, 2,4,6-tri(2-pyridyl)-S-triazine, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, fluorescein, 2,2'-azobis(2-amidinopropane) dihydrochloride, rat liver microsomes, ascorbic acid, thiobarbituric acid, anhydrous ferrous sulfate, 2,2'-azinobis(3-ethylbenzothiazoline-6-sulphonic acid, gallic acid, caffeic acid, luteolin-7-*O*-glucoside, apigenin-7-*O*-glucoside, carnosol, carnosic acid and rosmarinic acid were purchased from Sigma-Aldrich, Wicklow, Ireland.

### *2.2. Conventional solid/liquid extraction*

Solid/liquid extractions were carried out according to the method of Shan et al. (2005) with slight modifications. Briefly, dried and ground samples (0.5 g, particle size range: 500 to 600  $\mu\text{m}$ ) were homogenized for 1 min at 24,000 rpm using an Ultra-Turrax T-25 Tissue homogenizer (Janke & Kunkel, IKA<sup>®</sup>-Labortechnik, Saufen, Germany) in 25 mL of 80% methanol at room temperature ( $\sim 23$  °C). Methanol (80%) has been reported to be a highly efficient solvent for the extraction of phenolic antioxidants from spices (Shan et al., 2005). Therefore, this solvent was used in the present study. The homogenized sample suspension was shaken overnight with a V400 Multitude Vortexer (Alpha laboratories, North Yori, ON, Canada) at 1,500 rpm at room temperature ( $\cong 25$ °C). The sample suspension was then centrifuged for 15 min at 2,000 g (MSE Mistral 3000i, Sanyo Gallenkamp, Leicestershire, UK) and immediately filtered through 0.22  $\mu\text{m}$

polytetrafluoroethylene (PTFE) filters. The extracts were kept at -20 °C for 10 days and the analyses were carried out within this time period. The experiment was performed in triplicates.

### *2.3. Determination of total phenolic content*

The total phenolic content was determined using Folin-Ciocalteu Reagent (FCR) as described by Singleton et al. (1999). The experiment was performed in triplicates. Gallic acid solutions in methanol (10-400 mg/L) were used as standards. In each replicate, 100 µL of the appropriately diluted sample extract, 100 µL methanol, 100 µL FCR and finally 700 µL Na<sub>2</sub>CO<sub>3</sub> (20 %) were added together and vortexed. The mixture was incubated for 20 min in the dark at room temperature. After incubation the mixture was centrifuged at 10,000g for 3 min. The absorbance of the supernatant was measured at 735 nm using a spectrophotometer. The total phenolic content was expressed as gallic acid equivalents [g GAE/100 g dry weight (DW)] of the sample.

### *2.4. Determination of radical scavenging activity (DPPH)*

DPPH scavenging activity assay was performed as described by Goupy et al. (1999) with a slight modification. 2, 2-Diphenyl-1-picrylhydrazyl was dissolved in methanol (0.238 mg/mL). The reagent was prepared 2 hours prior to use to ensure all the DPPH has dissolved. The flask containing DPPH solution was covered with aluminum foil to protect it from light and stored in a refrigerator. For the actual measurement a 1 in 5 dilution of the DPPH stock solution with methanol was made in a 50 mL volumetric flask. Trolox dissolved in methanol in appropriate dilution was used as a standard. In each replicate 500 µL from the appropriately diluted sample extract were added to 500 µL DPPH solution. Preliminary experiments were carried out to determine the exact dilutions

required. In the control, 500  $\mu\text{L}$  of methanol were added to equal volume of DPPH solution. As a blank 500  $\mu\text{L}$  sample extract was mixed with 500  $\mu\text{L}$  methanol. The absorbance was read at 515 nm using a spectrophotometer. The radical scavenging activity was expressed as g Trolox/100 g DW of the sample.

### *2.5. Oxygen radical absorbance capacity (ORAC)*

ORAC assay was conducted using fluorescein ( $\text{C}_{20}\text{H}_{10}\text{Na}_2\text{O}_5$ ) as the fluorescent probe, according to a previously described procedure (Huang et al., 2002). The final assay mixture (200  $\mu\text{L}$ ) contained 150  $\mu\text{L}$  of fluorescein (10 nM), 25  $\mu\text{L}$  of AAPH (2, 2'-azobis (2-amidinopropane) dihydrochloride, 240 mM), 25  $\mu\text{L}$  of sample extracts or phosphate buffer (10 mM phosphate buffer, pH 7.4) as the blank. The fluorescence of the assay mixtures were recorded every 30 seconds cycle with the automated BMG FLUOstar Omega microplate reader system (Offenburg, Germany). The complete run was comprised of 250 cycles. This resulted in a fluorescence decay curve due to the oxidative degradation of fluorescein by AAPH. The difference in area between the fluorescence decay curve of the blank and the sample extract was used to calculate the ORAC values of the samples. Trolox in different concentrations (5-20  $\mu\text{M}$ ) was used to obtain a standard curve which was used to compare ORAC values of various samples. The data were analysed with the data analysis software, MARS linked with Omega reader control software.

### *2.6. HPLC analysis of the extracts*

Reverse phase high performance liquid chromatography (RP-HPLC) of the filtered sample extracts were carried out according to the method of Tsao and Yang (2003). The chromatographic system (Shimadzu-Model no SPD-M10A VP, Kyoto, Japan) consisted



of a pump, a vacuum degasser, a diode-array detector and was controlled through EZ Start 7.3 software (Shimadzu) at 37 °C. An Agilent C18 column (15 cm × 4.6 mm, 5 µm, Agilent Technologies., Santa Clara, CA, USA) was utilized with a binary mobile phase of 6 % acetic acid in 2 mM sodium acetate (final pH 2.55, v/v, solvent A) and acetonitrile (solvent B). The flow rate was kept constant at 1.0 mL/min for a total run time of 80 min. The following gradient program was carried out: 0-15% B in 45 min, 15-30% B in 15 min, 30-50% B in 5 min, 50-100% B in 5 min and 100-0% B in 10 min. The injection volume for all the samples was 10 µL. All the standards for quantification purposes were dissolved in methanol. Identification of the compound was achieved by comparing their retention times and UV-Vis spectra with those of authenticated standards by using the inline diode array detector (DAD) with a 3D feature.

### 2.7. Data analysis

Pattern recognition methods were applied to the data collection; these were principal component analysis (PCA) as an unsupervised classification method and hierarchical cluster analysis (HCA) as an unsupervised learning method. PCA and HCA were applied to data set as described by Patras et al. (2010). PLS regression was also used for the prediction of total antioxidant activity, based on the parameters analysed, using an equation of the form.

$$Y_i = b_0 + \sum_{j=1}^m b_j x_{ij} \quad (1)$$

where  $Y_i$  is response (antioxidant activity),  $b_0$  was the y intercept, and  $b_j$  was the regression coefficient for the  $j$ th prediction parameters ( $X_j$ ) in the model. The contribution of each variable to the prediction of the antioxidant was evaluated using the regression coefficients obtained for the standardised variables.

## 2.8. Model validation

The predictive performance of the derived model was validated in a separate set of experiments. The mathematical predictive model assessments were carried out (Jagannath, & Tsuchido, 2003) by calculating the model performance indices, accuracy factor (AF), bias factor (BF) (Ross, 1996; Patras et al., 2009).

$$AF = 10^{\frac{\sum \log|V_p/V_E|}{n_e}} \quad (2)$$

$$BF = 10^{\frac{\sum \log(V_p/V_E)}{n_e}} \quad (3)$$

The criterion used to characterize the fitting efficiency of the data to the model was the multiple correlation coefficients ( $R^2$ ) and their average mean deviation (Patras et al., 2009).

$$E(\%) = \frac{1}{n_e} \sum_{i=1}^n \left\| \frac{V_E - V_P}{V_E} \right\| \times 100 \quad (4)$$

where,  $n_e$  is the number of experimental data,  $V_E$  is the experimental value and  $V_P$  is the predicted value.

## 3. Results and discussion

### 3.1. Total antioxidant activity and levels of antioxidant polyphenols

In-vitro antioxidant activity of spices was measured by DPPH and ORAC assays. It should be noted that previously published (Hossain et al., 2008) data on the antioxidant activity of spices measured by FRAP, ABTS and MLP assays were also used in the present study for classification purposes and partial least square regression analysis. DPPH radical scavenging assay showed a wide variation among the spices examined with rosemary having the highest antioxidant activity (Table 1) due to its high content of

antioxidant polyphenols namely rosmarinic acid, carnosol and carnosic acid. Other spices with high antioxidant activity were oregano, marjoram, sage, basil and thyme. Previous studies (Cuvelier et al., 1994; Zheng, & Wang, 2001; Dorman et al., 2004; Hossain et al., 2008) also reported that these spices had very strong antioxidant activity. The spices with relatively low antioxidant activity included celery, cumin, fennel and parsley. ORAC values ranged from 5.76 to 28.31 g Trolox/100 g DW with oregano having the highest (28.31 g Trolox/100 g DW). The phenolic content of the tested spice extracts varied significantly, ranging from 0.78 to 8.37 g GAE/100 g DW (Table 1). The spices having high antioxidant activity as measured by DPPH and ORAC assays also had high total phenol content. The total phenol (TP) contents were highly correlated with all the antioxidant activity assays with Pearson correlation coefficient ( $r$ ) range of 0.911 to 0.978. The results emphasized the importance of phenolic compounds in the antioxidant behaviour of spice extracts and indicated that the phenolic compounds contributed significantly to the total antioxidant activity. The major phenolics identified by RP-HPLC in the extracts of spices examined were rosmarinic acid, caffeic acid, gallic acid, carnosic acid, carnosol, apigenin-7-*O*-glucoside and luteolin-7-*O*-glucoside. These polyphenols were differentially distributed among different spices (Table 1). The highest rosmarinic acid content was observed in marjoram (16.91 mg/g DW) followed by rosemary (16.42 mg/g DW). Marjoram also showed highest level of gallic acid (2.28 mg/g DW). Highest amount of caffeic acid was found in cumin (0.42 mg/g DW). Apigenin-7-*O*-glucoside and luteolin-7-*O*-glucoside, two major flavonoids present in spice extracts, were detected in parsley (7.51 mg/g DW) and celery (6.54 mg/g DW) respectively in highest concentration

among the spices examined. A typical HPLC chromatogram of different spices is illustrated in Figure 1.

### *3.2. Principal component and Hierarchical Cluster Analysis*

PCA was applied to the data set of 10 different spices after standardisation (the mean of the values for each variable is subtracted from each variable value and the result is divided by the standard deviation of the values for each variable). After standardisation, each parameter contributes equally to the data set variance and carries equal weight in principal component calculation. PC1 explained 59% of the total variance in the data set while PC2 explained 14%. The cumulative explained variance for each additional PC is shown graphically in Fig 2c. PC1 is generally better correlated with the variables than PC2. This is to be expected because PCs are extracted successively, each one accounting for as much of the remaining variance as possible. The sample score plot for PC1 vs PC2 is shown in Figure 2a & 2b and a number of observations may be made. Firstly, cumin, fennel and celery are located on the left half of the plot while, with the exception of sage which is situated on the right-hand side of the figure i.e. to the right of the PC1 zero point. Interestingly, marjoram was located some distance away from all of the other sample types. This suggests that its composition in terms of some of the antioxidant parameters differs significantly from the other samples. Pennington and Fisher (2009) used PCA to classify a range of fruits and vegetables. Classification was based on physical and chemical characteristics. Figure 2b illustrates the relationships between the parameters studied in the present work i.e. (total antioxidant activity and individual polyphenolic antioxidant compounds). Not surprisingly, total antioxidant activity measured by (MLP, DPPH, FRAP, ORAC & ABTS) are clustered together on the right

hand side of the loading plot. These parameters are significantly correlated as evidenced by their Pearson correlation coefficients (data not shown). CA, APOG are found in opposition to MLP, RA, DPPH, FRAP, ABTS, ORAC, and CRA while LOG and GA occupied a unique location at the very top of the figure. By using the plots in Figure 2a & 2b, it is possible to suggest reasons for the location of the spices on the basis of their chemical composition. The location of parsley in the lower left-hand quadrant of Figure 2a may be explained by their high values of APOG (Table 1) which are co-located in this region of the PC space. In contrast, basil, parsley, has low total antioxidant activity (ABTS) and content of CRA so they are located diametrically opposite to oregano and rosemary. Marjoram occupies a space between these two clusters on PC1 and contains significant quantities of LOG and GA (4.60 & 2.12mg/100g, respectively). Sage exhibits high total antioxidant activity as measured by MLP assay. In contrast, celery, cumin & fennel exhibit very low total antioxidant activity as measured by MLP, FRAP, DPPH & ORAC assays and they are located diametrically opposite to oregano and rosemary. It is possible to suggest that the contribution of CRA, CAR and RA on antioxidant activity in spices is greater than that of CA and APOG. To understand more about the relationship between the different variables and sample groups, some other PCs. PC4, which later accounted for 10% of the total data variance. Some observations are quite apparent from score and loading plots as illustrated in Figures 3a and 3b. The location of sage in the lower right hand quadrant of Figure 3a may be explained by its carnosol (Table 1); CAR is co-located in this region of the PC space. Score and loading plots of PC1 and PC4 also suggest that thyme, oregano and rosemary form discrete groups and are well

characterised by total antioxidant activity (FRAP, DPPH, ORAC, ABTS) (data not shown).

The results obtained following HCA are shown as a dendrogram (Figure 4) in which four well-defined clusters are visible. Samples will be grouped in clusters in terms of their nearness or similarity. A group of samples (A) is clearly discernible which is composed of rosemary, oregano and sage. These species are associated with high antioxidant activity as measured by DPPH, FRAP, ABTS, MLP & ORAC. A second cluster (B) consists of marjoram alone because of the highest levels of GA and RA (Table 1) and fairly high in antioxidant activity as measured by DPPH and FRAP and ORAC assay. This is in agreement with the results of the PCA in which marjoram samples lay at some distance from the others. A third cluster (labelled C) includes thyme and basil while cluster D consists of cumin fennel, parsley and celery. Biglari et al. (2009) studied the effect of long-term cold storage on antioxidant compounds in dates using cluster analysis and found it to be a quite useful technique for classification. It is possible that Cluster A and Cluster D are well separated due to variations in total antioxidant activity and individual polyphenolic antioxidant compounds.

### *3.3. Prediction of total antioxidant activity by partial least squares regression*

A multivariate partial least squares regression analysis was performed taking the antioxidant activity of the spices as dependent variables ( $Y_i$ ) and their phenolic profiles ( $X_n$ ) as predictor ones. The linear models were constructed as:

$$Y_i = b_0 + b_1X_1 + b_2X_2 + \dots + b_nX_n \quad (5)$$

PLS extracts a few linear combinations (PLS factors) of polyphenolic antioxidants data that predict as much of the systematic variation in the sample data as possible. Data were

centered prior to PLS regression so that all results were interpretable in terms of variation around the mean. A final data matrix containing eight phenolic compounds (gallic acid, carnosol, carnosic acid, caffeic acid acid, rosmarinic acid, luteolin-7-*O*-glucoside, apigenin-7-*O*-glucoside and total phenols).

For total antioxidant activity by FRAP, ABTS, DPPH, ORAC & MLP assay, the predicted response models were found to fit well with the experimental data with high regression coefficients ( $R^2$ ) (Fig 5). The values were closely correlated with the experimental data as demonstrated by regression coefficient ( $R^2$ ) values 0.99, 0.98, 0.97, 0.96, 0.99 for total antioxidant activity by FRAP, ABTS, DPPH, ORAC & MLP assay respectively. This study also dealt with the validation of the developed model using a set of data obtained from additional test runs, exclusive of those performed in the elaboration of the model, as recommended by Ross (1996). The linear equations are shown as below:

$$\text{FRAP} = 1.13 + \text{RA} (-0.0372) + \text{CA} (-0.706) + \text{GA} (-1.249) + \text{CRA} (-0.849) + \text{CAR} (0.453) + \text{APOG} (-0.214) + \text{LOG} (-0.054) + \text{TP} (2.467)$$

$$\text{ABTS} = -0.161 + \text{RA} (-0.236) + \text{CA} (2.44) + \text{GA} (-1.746) + \text{CRA} (0.98) + \text{CAR} (0.134) + \text{APOG} (0.041) + \text{LOG} (0.169) + \text{TP} (1.708)$$

$$\text{DPPH} = -0.71 + \text{RA} (0.098) + \text{CA} (2.12) + \text{GA} (0.35) + \text{CRA} (0.32) + \text{CAR} (-0.074) + \text{APOG} (-0.034) + \text{LOG} (0.046) + \text{TP} (0.827)$$

$$\text{ORAC} = 13.85 + \text{RA} (0.239) + \text{CA} (-25.094) + \text{GA} (0.825) + \text{CRA} (0.419) + \text{CAR} (0.263) + \text{APOG} (0.228) + \text{LOG} (0.0451) + \text{TP} (0.872)$$

$$\text{MLP} = -0.160 + \text{RA} (0.19) + \text{CA} (3.48) + \text{GA} (-0.305) + \text{CRA} (0.0352) + \text{CAR} (0.486) + \text{APOG} (0.231) + \text{LOG} (0.412) + \text{TP} (-0.215)$$

To confirm the adequacy of the fitted models, studentised residuals versus run order were tested and the residuals were observed to be scattered randomly, suggesting that the variances of the original observations were constant for all responses. The applicability of

the models was also quantitatively evaluated by comparing the bias and accuracy factors for each of the parameter (Equations. (2) and (3)). In most cases, as shown in Table 2, the accuracy factor (AF) values for the predicted model were close to 1.00, except for MLP (1.44), ABTS (1.22). The bias factor (BF) values for the predicted models were also close to 1.00, ranging from 0.89 to 1.05 for all the parameters studied. Despite some variations, results obtained from the validated predicted model and actual experimental values showed that the established models reliably predicted total antioxidant activity by FRAP, ABTS, DPPH, ORAC and MLP assays. The predicted values were found to be not significantly ( $p>0.05$ ) different from experimental values using a paired t-test (Table 3). In addition variations between the predicted and experimental values obtained for total antioxidant activity by FRAP, ABTS, DPPH, ORAC and MLP assay were within the acceptable error range as depicted by average mean deviation (Table 3). Therefore, the predictive performance of the established model may be considered acceptable.

#### **4. Conclusions**

Considerable variations were observed between different spice samples in terms of total antioxidant activity and different antioxidant polyphenolic compounds. Unsupervised pattern recognition techniques enabled visualization of this complex dataset and underlying relationships responsible for clustering observed. Rosemary and oregano exhibited the highest DPPH radical scavenging activity and ORAC value, respectively. In addition Marjoram contained high levels of gallic acid and rosmarinic acid. In contrast, cumin fennel, parsley and celery had low levels of phenolic constituents which gave them low scores on PCA plot in terms of total antioxidant activity. The combination of chemical characterization and multivariate data analysis allows easy interpretation of



similarities and differences in spices on their antioxidant activity and content of polyphenolic antioxidant compounds. This data analysis technique provides powerful insights into the variations in the antioxidant profiles between different spices; information from this study may be useful for promoting optimum spice consumption in order to prevent lifestyle-related diseases in consumers.

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Table 1. Quantity of different polyphenols and antioxidant capacity of a range of spices<sup>a</sup>.

Parameter	Rosemary	Oregano	Marjoram	Sage	Basil	Thyme	Fennel	Celery	Cumin	Parsley
<b>Polyphenols (mg/g DW<sup>b</sup>)</b>										
Rosmarinic acid	16.42±0.36	6.61±0.30	16.91±0.18	14.98±0.26	4.19±0.03	3.37±0.01	ND <sup>c</sup>	ND	ND	ND
Caffeic acid	0.08±0.00	0.06±0.00	0.10±0.00	0.13±0.00	0.07±0.00	0.10±0.00	0.29±0.01	0.17±0.00	0.42±0.01	0.07±0.00
Gallic acid	0.55±0.01	0.45±0.02	2.28±0.15	0.46±0.02	0.36±0.02	0.27±0.03	0.66±0.03	0.76±0.01	0.56±0.03	0.46±0.01
Carnosic acid	9.97±0.40	4.72±0.09	3.01±0.05	6.37±0.04	ND	6.41±0.09	ND	ND	ND	ND
Carnosol	8.19±0.41	5.82±0.04	1.76±0.07	15.08±0.24	1.38±0.07	3.99±0.11	ND	ND	ND	ND
Apigenin-7- <i>O</i> -glucoside	0.50±0.01	2.54±0.01	0.83±0.03	0.53±0.01	0.18±0.01	0.16±0.01	0.43±0.01	1.56±0.07	1.46±0.02	7.52±0.17
Luteolin-7- <i>O</i> -glucoside	0.71±0.02	3.01±0.01	4.61±0.07	4.95±0.01	1.27±0.01	1.04±0.02	2.11±0.04	6.54±0.08	2.24±0.07	1.25±0.08
<b>Antioxidant activity (g Trolox/ 100 g DW)</b>										
ORAC <sup>d</sup>	26.90±0.20	28.31±0.15	25.36±0.10	25.84±0.11	17.57±0.10	20.64±0.07	6.64±0.08	10.50±0.15	5.76±0.07	13.25±0.12
DPPH <sup>e</sup>	11.02±0.10	8.52±0.06	8.21±0.18	6.39±0.12	2.46±0.07	4.34±0.06	0.70±0.01	1.64±0.08	0.88±0.02	0.29±0.01
FRAP <sup>f</sup>	14.54±0.25	18.86±0.11	12.26±0.03	14.28±0.26	5.83±0.08	8.80±0.02	1.52±0.00	2.29±0.13	1.83±0.01	1.28±0.00
ABTS <sup>g</sup>	18.34±0.20	18.09±0.10	8.14±0.17	14.79±0.34	2.87±0.03	15.31±0.10	1.23±0.02	1.84±0.03	1.19±0.01	1.35±0.02
MLP <sup>h</sup> ((g/L) <sup>-1</sup> )	3.05±0.03	2.26±0.02	2.47±0.04	9.82±0.12	1.59±0.01	1.48±0.02	0.37±0.01	1.22±0.01	0.63±0.00	0.28±0.01
TP <sup>i</sup> (g GAE <sup>j</sup> /100 g DW)	8.05±0.21	8.37±0.16	6.76±0.12	5.46±0.10	2.15±0.06	4.65±0.07	0.78±0.02	1.28±0.04	0.78±0.01	0.99±0.01

<sup>a</sup> Data are expressed as means ± SD (n=3)

<sup>b</sup> DW, dry weight

<sup>c</sup> ND, not detected

<sup>d</sup> ORAC, oxygen radical absorbance capacity

<sup>e</sup> DPPH, 2,2-diphenyl-1-picrylhydrazyl

<sup>f</sup> FRAP, ferric reducing antioxidant power

<sup>g</sup> ABTS, 2,2'-azinobis(3-ethylbenzothiazoline-6-sulphonic acid); <sup>h</sup> MLP, microsomal lipid peroxidation

<sup>i</sup> TP, total phenol; <sup>j</sup> GAE, gallic acid equivalent

Table 2. Bias and accuracy factor for the responses studied

Parameters	No of observations	Bias factor	Accuracy factor
FRAP	10	1.01	1.07
ABTS	10	1.05	1.22
DPPH	10	0.98	1.18
ORAC	10	1.01	1.11
MLP	10	0.89	1.46

Table 3. Actual, predicted and average mean deviation for the responses

	<i>Response</i>	<i>Rosemary</i>	<i>Oregano</i>	<i>Marjoram</i>	<i>Sage</i>	<i>Basil</i>	<i>Thyme</i>	<i>Fennel</i>	<i>Celery</i>	<i>Cumin</i>	<i>Parsley</i>
	FRAP										
Experimental		14.57	18.84	12.29	14.37	5.91	8.81	1.53	2.40	1.83	1.29
Predicted		13.95	18.73	12.24	14.33	5.82	8.35	1.93	2.65	1.96	1.07
E%		4.26	0.60	0.36	0.27	1.50	5.22	26.25	10.68	7.11	16.56
	ABTS										
Experimental		18.23	18.04	8.15	15.14	2.58	15.26	1.22	1.96	1.31	1.35
Predicted		20.21	18.01	7.85	14.27	3.08	13.92	1.10	2.28	1.63	1.45
E%		10.86	0.15	3.66	5.76	19.35	8.79	9.83	16.32	24.42	7.86
	DPPH										
Experimental		10.99	8.56	8.12	6.52	2.39	4.52	0.71	1.61	0.89	0.30
Predicted		10.32	8.22	8.61	6.73	1.91	5.54	0.85	1.21	1.10	0.23
E%		6.12	3.90	6.06	3.27	19.83	22.56	20.25	24.84	22.59	23.16
	ORAC										
Experimental		26.83	28.15	25.39	25.87	17.68	20.72	6.61	10.48	5.68	13.12
Predicted		29.70	25.59	24.91	26.02	15.91	20.28	8.17	11.60	4.60	14.89
E%		10.65	9.12	1.89	0.57	9.99	2.16	23.55	10.74	19.11	13.56
	MLP										
Experimental		3.87	2.27	2.51	9.71	1.60	1.48	0.37	1.23	0.62	0.39
Predicted		4.14	2.42	2.72	9.72	1.05	1.45	0.35	1.47	0.76	0.48
E%		7.02	6.69	8.28	0.15	34.37	2.03	5.40	20.34	22.47	23.07



Figure 1. HPLC profiles of methanolic extracts from rosemary, oregano, basil and sage at 320 nm (1, gallic acid; 2, caffeic acid; 3, luteolin-7-*O*-glucoside; 4, apigenin-7-*O*-glucoside; 5, rosmarinic acid; 6, carnosol and 7, carnosic acid)

Figure 2. Principal component analysis (PCA) plots. a) PCA scores plot for different spice samples, b) loading plots for different variables on PC1 and PC2, c) Cumulative variance

Figure 3. Principal component analysis (PCA) plots. a) PCA scores plot for different spice samples, b) loading plots for different variables on PC1 and PC4

Figure 4. Dendrogram of hierarchical cluster analysis of spices.

Figure 5. Predicted and actual (experimental values) for (a) FRAP, (b) ABTS, (c) DPPH, (d) ORAC (e) MLP

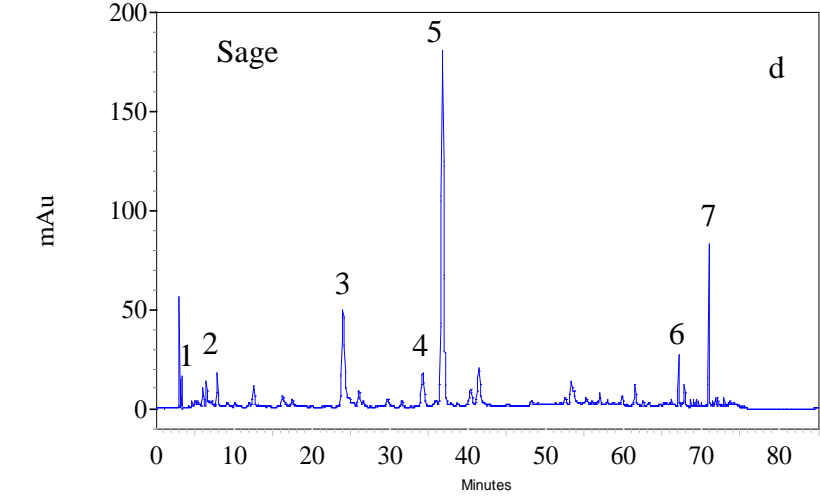
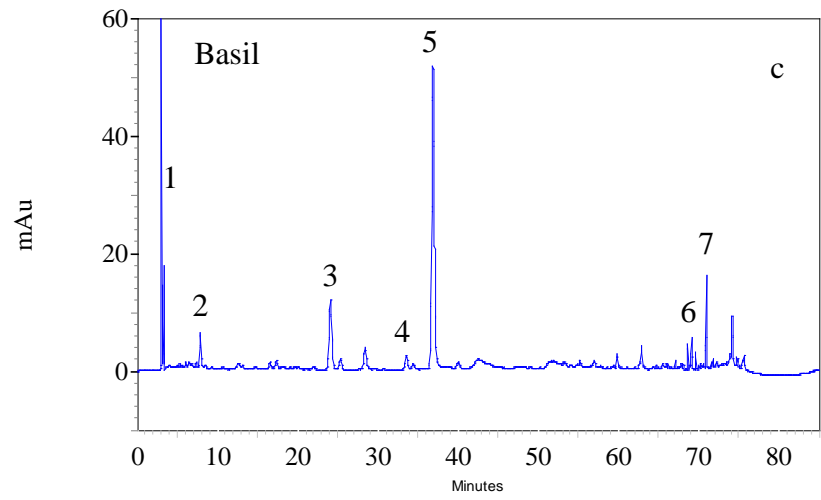
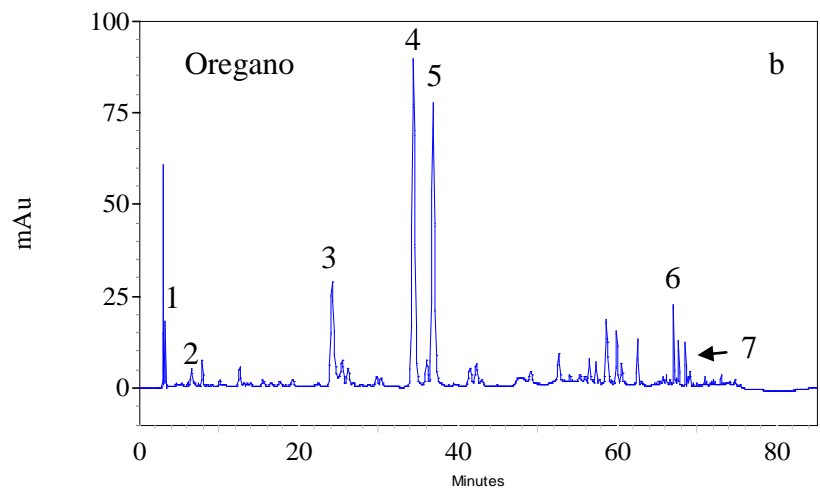
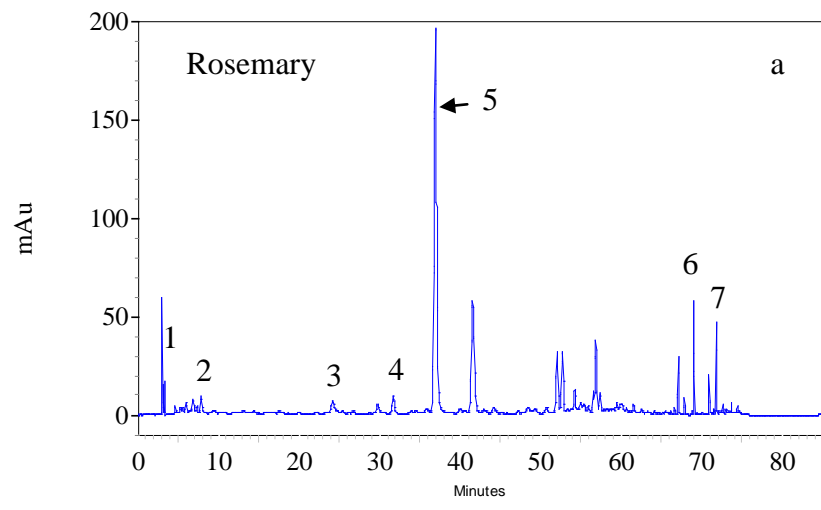


Figure 1

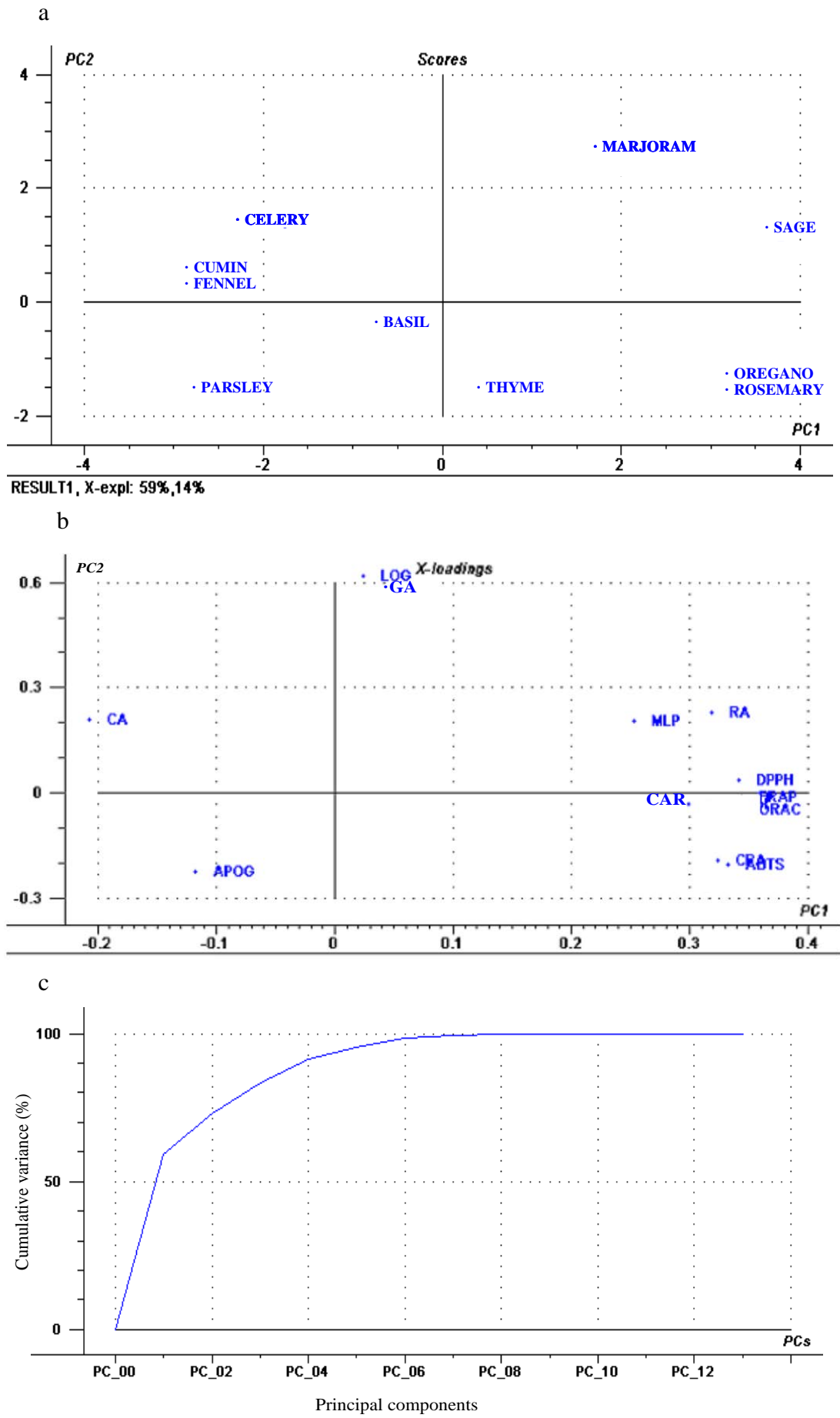


Figure 2

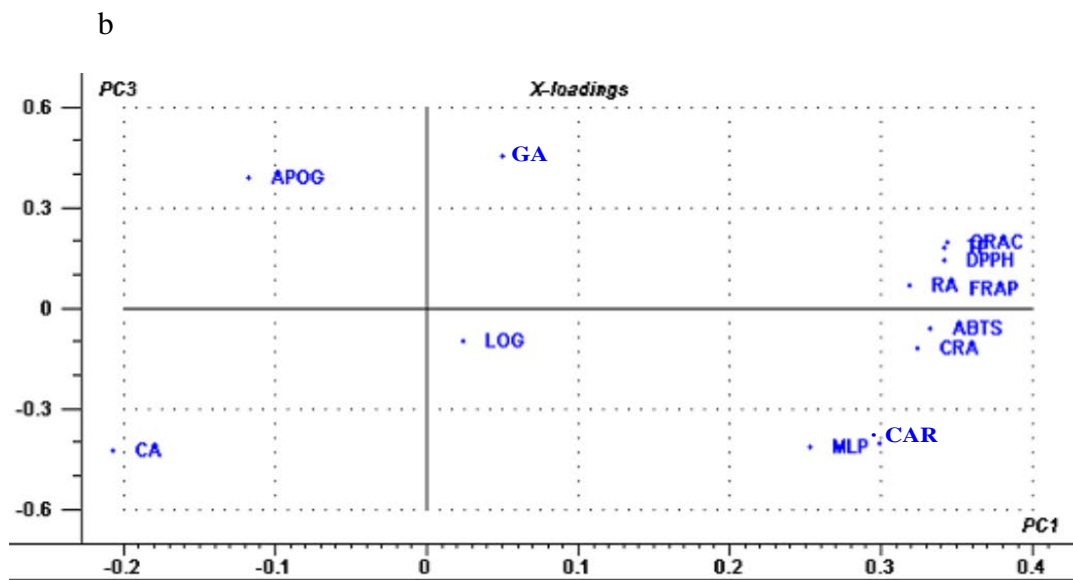
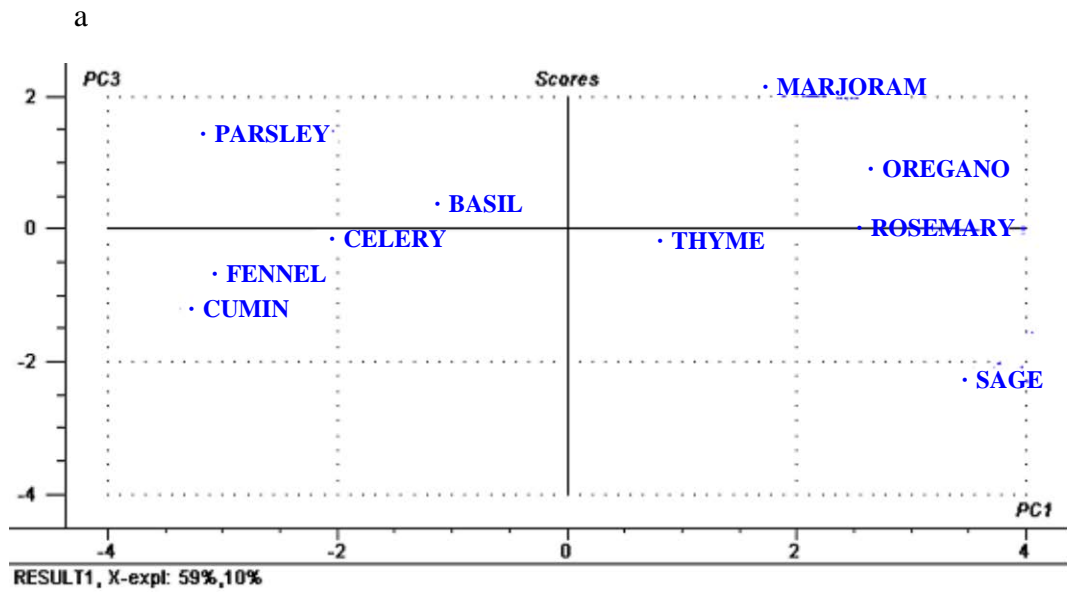


Figure 3

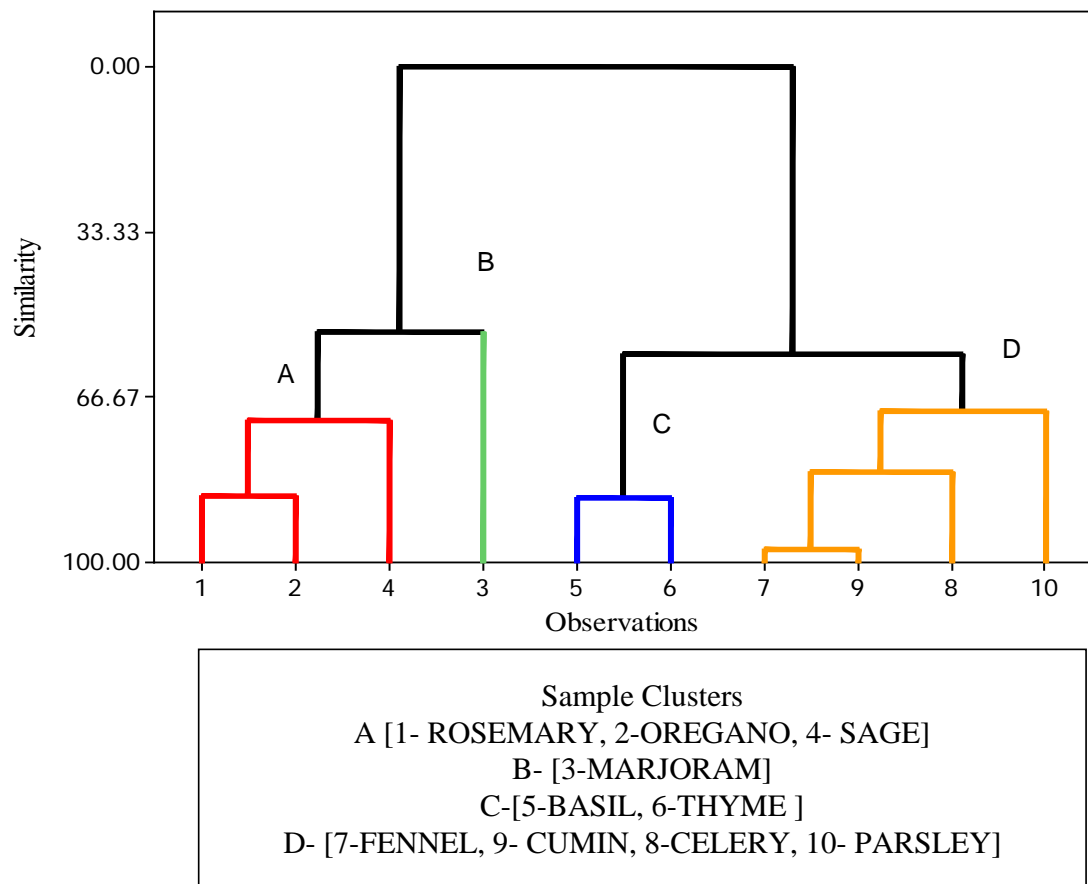


Figure 4

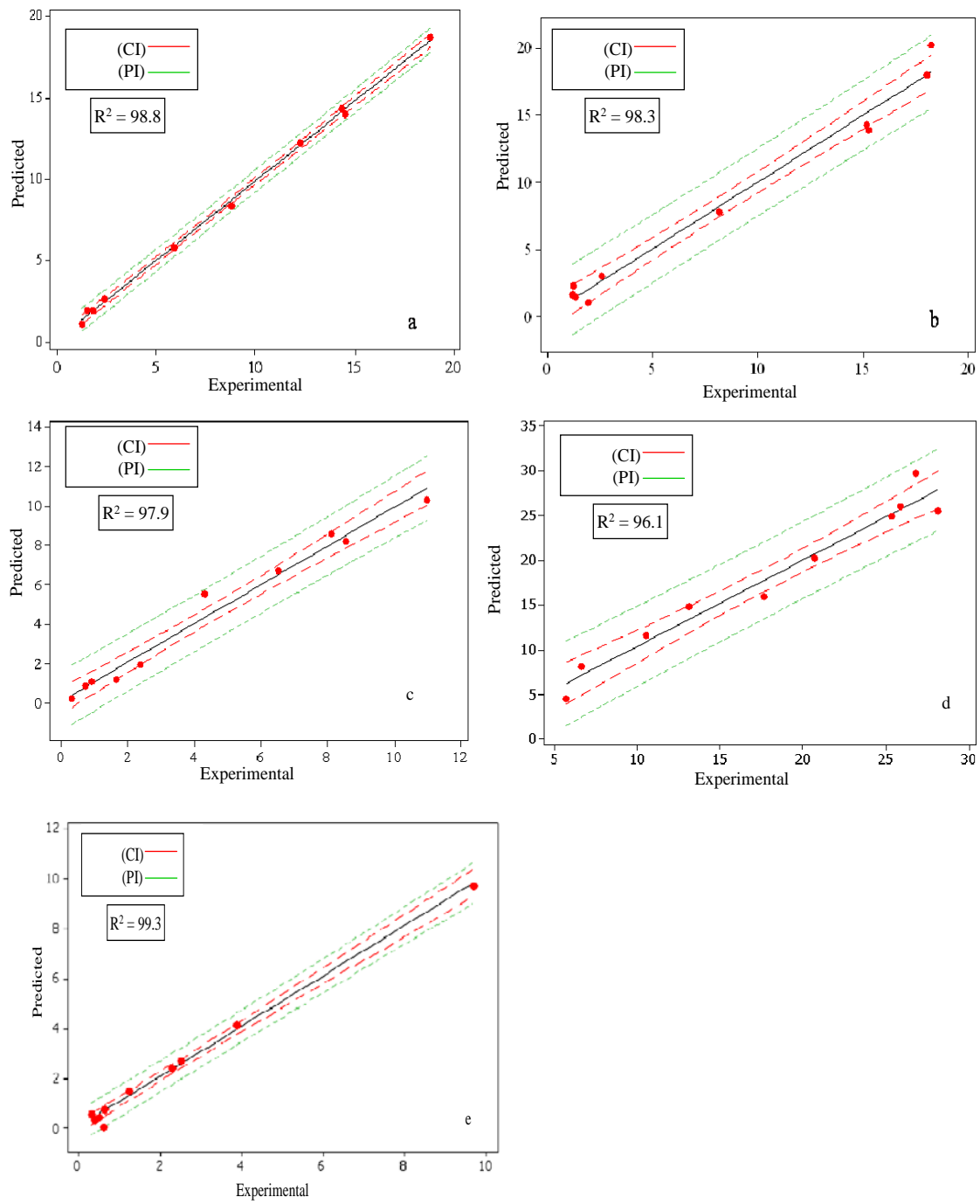


Figure 5