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CLASSIFICATION OF CALCIUM SUPPLEMENTS THROUGH APPLICATION OF PRINCIPAL COMPONENT ANALYSIS: A STUDY BY INAA AND AAS

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Different types of Ca supplements are available in the local markets of Pakistan. It is sometimes difficult to classify these with respect to their composition. In the present work principal component analysis (PCA) technique was applied to classify different Ca supplements on the basis of their elemental data obtained using instrumental neutron activation analysis (INAA) and atomic absorption spectrometry (AAS) techniques. The graphical representation of principal component analysis (PCA) scores utilizing intricate analytical data successfully generated four different types of Ca supplements with compatible samples grouped together. These included Ca supplements with $CaCO_3$ as Ca source along with vitamin C, the supplements with $CaCO_3$ as Ca source along with vitamin D, Supplements with Ca from bone meal and supplements with chelated calcium.

Keywords: Atomic absorption spectrometry (AAS), Calcium supplements, Instrumental neutron activation analysis (INAA), Principal component analysis (PCA), Scores

1. Introduction

It is a well established fact that our daily intake of diets cannot cater for the recommended intakes for Calcium (Ca) in human beings as described in 2000 Dietary Guidelines for Americans [1]. For this reason several supplements and complementary diets are available in the markets that can be consumed to satisfy the Ca requirements of our body. In 2002, calcium supplements were supposed to be the number one selling mineral supplement in the world and the 3rd highest in USA [2]. In Pakistan tremendous enhancement of bone related diseases are being registered like osteoporosis and low bone mineral density (BMD) in general population and therefore, the use of supplements has become a common practice. Absorption of Ca by the body is directly related to its total amount consumed at one time and whether it has been taken on an empty stomach or with food [3]. The absorption of calcium decreases with increase in its concentration in the supplement [2, 3]. Different type of calcium compounds are used in the supplements formulations where Ca citrate and Ca carbonate are most common. The Ca compounds used in these supplements, apart from Ca as their main component, may also contain other major, minor and trace minerals. These supporting minerals on one hand are important since Ca in the presence of these mineral elements forms more effective supplement for bone related disorders, while on the other hand their elemental contents if high may pose toxic effects in the body. These naturally occurring Ca salts are used by many pharmaceutical companies without proper quality control procedures where they may contain many undesirable trace elements. Unfortunately such supplements especially in developing countries are sold without any safety check by the regulatory bodies. It is therefore, important to study the elemental composition of these supplements to monitor and control their levels in supplements to avoid any health effects especially due to high doses [4]. A study was therefore, designed to check the trace element contents of different national and multinational Ca supplements available in the market using instrumental neutron activation analysis (INAA) and Atomic Absorption Spectrometry (AAS) techniques.

Scientists have used many mathematical approaches for the analysis of acquired analytical data to abridge and examine such intricate results [5, 6]. It is also sometimes difficult to extract appropriate information from such raw data due to its enormity and complexity. Principal components analysis (PCA) is one such data analysis approach of multivariate mathematical, statistical and graphical technique that easily generates its potential patterns. PCA provides a low dimensional data review and is generally used as a visualization tool and helps to identify outliers, and perform quality control [7]. Significant information can be gathered from confounding data sets where most suitable, simple and non-parametric PCA

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method helps to reduce intricate dataset to a lower dimension and expose all covert parameters that often underlie the data. PCA-based methods have been successfully applied for medicinal and health related issues for different classification purposes [8, 9]. In this study PCA has been applied to the Ca supplement elemental data to generate scores patterns and to visualize and classify these supplements and gain understanding of their composition.

2. Experimental

2.1. Sampling

Nineteen national and multinational calcium supplements (CAC 1000, CAC Plus, Lophos, Multical 1000, Multical Plus, Calceplex, Calfo-V, High-C 1000, High-C Plus, Calcee Plus, Casan, Aqua Vit, Decalce, Abocal, Cal-C Plus, VC-Cal, Chewcal, Decalc Plus, Qalsan) were collected from the local markets. These included supplements in the form of tablets and powder in sachet with calcium ranging from 125 to 1250 mg per tablet/dose. Most of the supplements were flavored and colored. Information regarding their potency, composition, color, flavor and weight of dose of each supplement was documented. The samples were processed at the Pakistan Institute of Nuclear Science and Technology (PINSTECH) sample preparation laboratories of INAA and AAS. Table 1 shows the details of these supplements.

2.2. INAA Procedures

All supplement samples were grounded to fine powder and stored in pre-cleaned and clearly marked polyethylene bottles and tightly screw caped. The homogeneity of these samples was determined by quantifying their Mn contents for randomly collected samples of weight about 150 mg using INAA. The variation in the results was ~6% around the mean values depicting the homogeneity of the samples. Approximately 150 mg of supplement samples and reference materials (RMs) (IAEA H-5 and IAEA SL-1) from the International Atomic Energy Agency (IAEA) as control materials were separately packed and in pre-cleaned polyethylene capsules. All capsules were heat sealed. Multiple sample batches were prepared in accordance with three different irradiation protocols. All prepared targets in reactor rabbits were irradiated in a 27 kW tank-in-pool Miniature Neutron Source Reactor (MNSR), Pakistan Atomic Research Reactor -2 (PARR-2) which with thermal neutron flux of 1x 10^{12} $cm^{-2}s^{-1}$. The radio-assay scheme was carefully optimized for cooling and counting times in accordance with the half-life of the elemental isotope desired. All active targets were analyzed using Canberra Model AL-30 high purity germanium detector linked to a PC-based Intertechnique Multichannel Analyzer (MCA). The system resolution was 1.9 keV for ^{60}Co peak at 1332.5 keV with peak to Compton ratio was 40:1. "Intergamma, version 5.03" software was used for data acquisition. Our in-house computer programs GammaCal was applied for all calculations [10].

2.3. AAS Procedures

All supplements were digested before analysis by taking 0.5 g of sample in 100 ml digestion flasks fitted with air condenser, 25 cm long. Distilled 5.0 ml HNO_3 was added to the sample and heated at 80 ^{0}C for 40 minutes with occasional shaking. A Clear solution was obtained, cooled and transferred to a 10 ml measuring flask. The volume of this solution was made up with de-ionized water. A blank was also prepared under similar conditions. Reference material (IAEA H-5, animal bone) was also digested using the same procedure. Hitachi model Z-2000 polarized Zeeman atomic absorption spectrophotometer was employed for all measurements. Hollow cathode lamp of Cd, Ni and Cu from Hitachi were used as radiation source. Cd, Ni and Cu were analyzed using Graphite Furnace and Flame Furnace Atomic Absorption Spectrometry modes of polarized Z-2000 AAS.

2.4. Principal Component Analysis (PCA)

Principal Components Analysis (PCA) is unsupervised pattern recognition that is applied to a data matrix to study the relationship between the sample and the variable. Through application of PCA large variable data matrices is reduced to a new variable of principal components (PCs) but containing complete information from the raw data [11, 12]. PCA transforms the raw data set as vector samples into a new set of vector samples with derived dimensions that can reduce this data into small dimensions with insignificant loss of information. The aim of PCA is to decompose nonlinearity data into PCs. The following equation explains basic model for PCA follows:

$$X = T \cdot P + E \quad (1)$$

Where T is the scores matrix, P is the loadings matrix and E is the matrix of residuals. The scores describe the samples in the new data space along columns and loadings explain the weights of original variables in determining the new projection axis and residuals show the selected number of

Table 1. Details of studied calcium supplements.

Brands	Country/city	Form	Weight per dose
Calcium Supplements With Vitamin C Formulation			
Abocal	USA	Tablets	7.10 gm
Cal-C Plus	Pakistan /Karachi	Sachet	7.91 gm
VC-Cal	Pakistan /Karachi	Sachet	7.93 gm
Calcium Supplements With Vitamin D Formulation			
Chewcal	UK	Tablet	1.34 gm
Decalc Plus	Pakistan /Lahore	Tablets	1.37gm
Qalsan	Switzerland	Tablet	2.20 gm
Supplements with Ca-phosphate formulation			
Decalce	Pakistan /Lahore	Tablets	1.01 gm
Casan	Pakistan /Karachi	Tablets	1.13 gm
Chelated Ca Supplements			
CAC 1000	Switzerland	Tablets	5.20 gm
CAC Plus	Switzerland	Tablet	6.57 gm
Lophos	Pakistan /Karachi	Tablets	0.73 gm
Multical 1000	Pakistan /Karachi	Sachet	10.5 gm
Multical Plus	Pakistan /Karachi	Sachet	10.5 gm
Calceplex	Pakistan /Islamabad	Sachet	6.77 gm
Calfo-V	Pakistan /Hattar	Sachet	6.94 gm
High-C 1000	Pakistan /Islamabad	Sachet	8.54 gm
High-C Plus	Pakistan /Islamabad	Sachet	6.37 gm
Aqua Vit	Pakistan /Islamabad	Sachet	5.0 gm
Calcee Plus	Pakistan /Islamabad	Sachet	6.30 gm

components that explain the model [13]. The data is reduced through PCA and provides visual information on multidimensional data through new smaller variables.

In this study elemental data is the data matrix. The available data on elemental composition of Ca supplements consists of the 17 elements. In the scores matrix each row represents each sample and contain as many rows as the original data. The loadings consist of series of row vectors and contain as many columns as the original data matrix; therefore each row represents each element. Different preprocessing steps are involved to overcome the inconsistency in the pattern of the data mostly related to the analytical techniques of analysis before the final application of PCA. Most important processing step is standardization as this puts all variables on an equal scale. It is possible that some variables will have intensities that are orders of magnitudes greater than others. Standardization scales each variable so the mean value is 0, and the standard deviation is 1, thus all variables are given equal importance. It is estimated that PCs account for approximately 75% of the total variance [14]. Variables with eigenvalue < 1 form the relevant components as it is predicted that maximum variation in the data set is in the first few transformed attributes, while the remainders are discarded. Groups of components with most similar variance values are clustered in different PCs. In this study the multivariate technique of PCA was

Table 2. Eigenvalues for PCA (Standardized).

N. PCs	Eigenvalues	
	E-value	%
Total SS	323	100
#1	80.59988	24.95352
#2	42.97694	13.30555
#3	38.67616	11.97404
#4	37.68996	11.66872
#5	28.13991	8.712047

applied to the elemental data of 19 supplements to classify and understand the similarities and disparities in supplement sample with respect to their elemental concentrations as variables and predict their groupings.

3. Results and Discussion

19 Ca supplements from national and multinational pharmaceutical companies were analyzed for different elements using INAA and AAS techniques. PCA using excel spreadsheets was applied to the acquired data to look for some patterns and correlations in the analytical data and samples. Multivariate analysis tool was employed to generate PCA. The standardized data output gave Eigen values, Scores and Loadings as dimensionless numbers in each case [15]. Total variation of our results was explained by the first four eigen values (Table 2) therefore, the these principal components were sufficient to effectively describe the data. In this work only scores have been exploited to obtain any similarities and dissimilarities between the studied supplements.

3.1. PCA Analysis of Scores

The scores consist of different column vectors and is basically an abstract mathematical transformation of the original data. The scores contain as many rows as the original data matrix. Each row in Table 3 represents each sample and therefore, column 1 of the scores in PCA contains the sample number while the following columns called principal components (PC) represent its scores. These scores PCs have been graphically explained through different XY scattered plots that facilitate to explain any relationship between the samples or the possible outliers. First three score factors have been used to plot the scores in various formations (Figures 1a and 1b). Representative Figure 1a shows 2 PC scores plotted between PC1 and PC2 while Figure 1b represents scores plotted between PC2 and PC3. Both figures show some common groupings with 5 prominent clusters in Figure 1a and 6 clusters in Figure 1b. Supplements namely Abocal, CalcePlus and Vc-Cal are clustered together as one group. The composition of all three supplements showed that their similarity is ascribed to the presence of $CaCO_3$ as source of Ca along with vitamin C, however it can be seen that Abocal is slightly shifted from the other two supplements predicting some dissimilarities in its composition. It was found that this supplement is in the form of tablet while CalcePlus and Vc-Cal are in powdered form. The weight per dose of Abocal is slightly lower (7.1 g) and does not contain any flavor, while weight of Cal-C Plus and Vc-Cal is 7.9 g each and are both orange flavored. There are multiple compounds of Ca sources in CalcePlus and Vc-Cal while $CaCO_3$ is the only source of Ca in Abocal. These supplements are usually taken as diet food and are frequently used as drinks, because of vitamin C. Another common cluster in both figures consists of Chewcal, Qalsan and Decalc Plus. The composition of all three supplements shows $CaCO_3$ along with vitamin D. These Ca supplements administered with vitamin D promote maximum absorption of calcium. Although all three supplements are grouped together however their larger distances indicate some compositional variations. Qalsan and Chewcal are multinational while Decalc Plus is a local brand, therefore, the differences could be due to diversity in their flavors, weight per dose, quality of $CaCO_3$ and other additives apart from vitamin D contents. Plot of PC1 and PC2 show two supplement samples Decalc and Casan far apart however in plot of PC2 and PC3 they group together. It was found that

Table 3. PCA scores table.

Supplements	PC1	PC2	PC3	PC4	PC5
CAC 1000	-2.01943	0.631267	0.350422	-0.33566	0.021639
CAC Plus	0.047569	-2.33459	1.525478	-1.58117	0.140319
Lophos	-0.03372	0.668132	-1.50049	-0.88446	0.243372
Multical 1000	-2.03631	-0.07721	0.43154	2.026347	-2.01065
Multical Plus	-3.10795	2.247418	0.996358	-0.74423	1.826484
Calceplex	-0.39702	0.322432	-1.00761	0.114816	0.562739
Calfo-V	-1.90922	-1.27177	0.922342	3.211094	-1.43361
High-C 1000	-2.30633	2.090082	-0.55792	-0.17884	0.532168
High-C Plus	-1.20039	-0.87282	0.889678	0.868127	0.039089
Aqua Vit	-1.48987	0.087969	0.776833	0.068326	0.174081
Calcee Plus	-0.39986	-1.66222	1.645222	-3.88573	-1.95154
Decalce	3.466571	-2.44515	1.48589	1.094401	2.705792
Casan	5.129497	3.807793	2.789034	0.468716	-1.01524
Abocal	0.304488	0.204483	-1.74716	-0.64194	-0.40803
Cal-C Plus	0.178541	-0.59419	-0.91642	0.488261	1.30427
VC-Cal	0.141281	-0.25825	-0.27663	0.203336	1.330335
Chewcal	2.522237	-0.77159	-2.53731	0.767381	-1.2797
Decalc Plus	1.332348	0.583984	-2.25887	-0.81477	-0.03448
Qalsan	1.777579	-0.35577	-1.01039	-0.24402	-0.74704

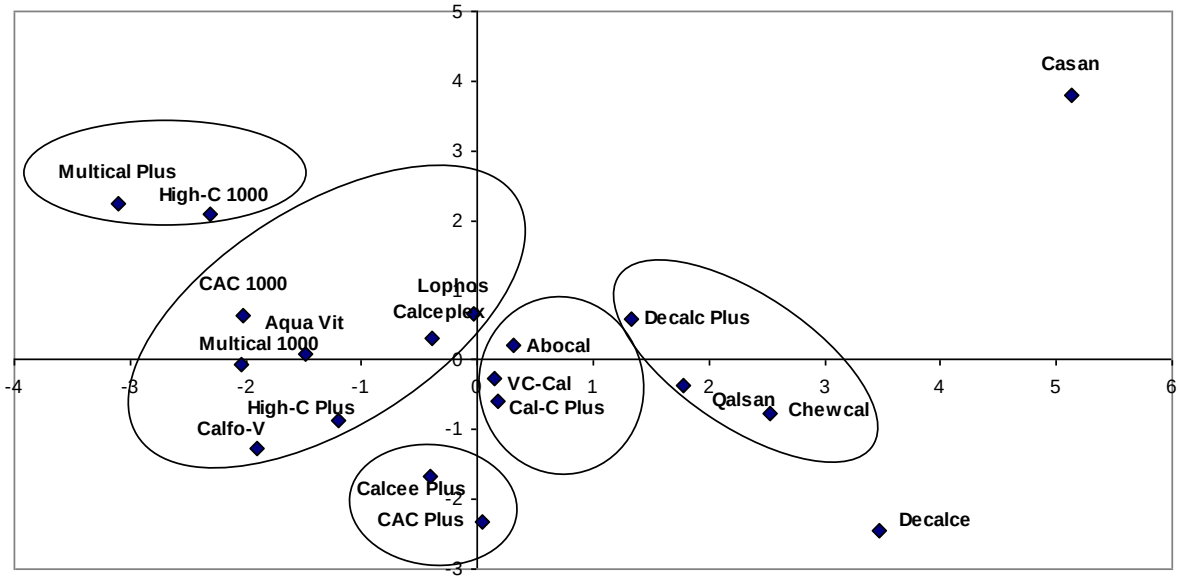

Figure 1a: Score plot of PC1 and PC2 for Ca supplements.

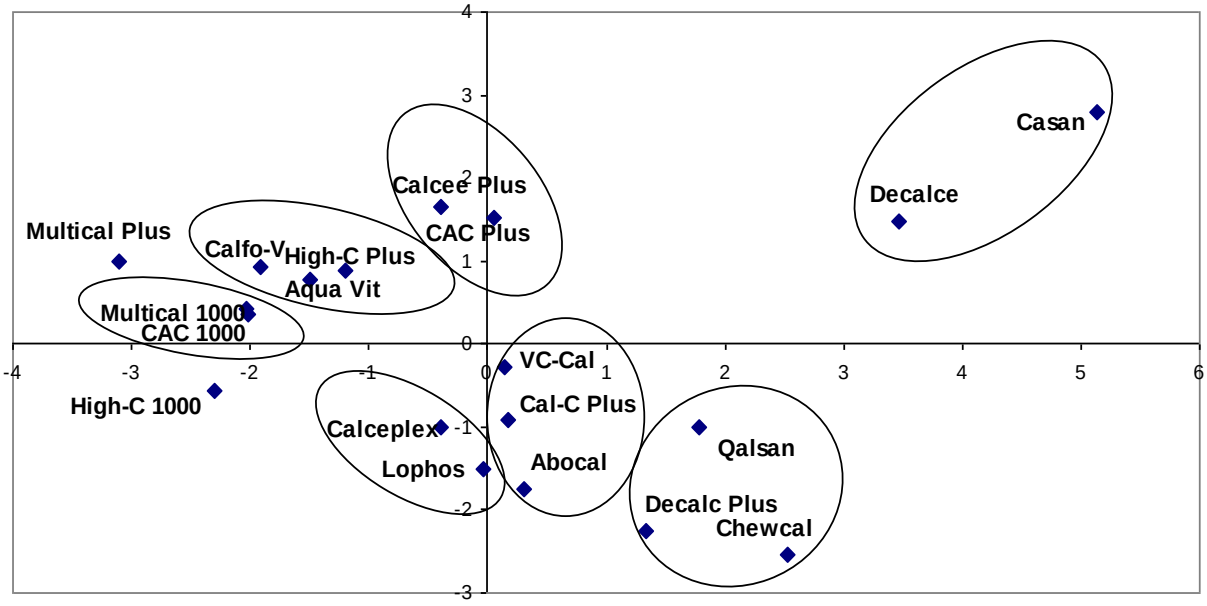

Figure 1b. Score plot of PC2 and PC3 for Ca supplements.

these two samples are prepared from bone meal Ca which is basically Ca phosphate. Ca from this category is not well absorbed therefore these supplements are rarely recommended by doctors and dieticians. They differ in a sense that Decalc contains very small inventory of detected trace elements amounting to only 607 μg/g while in Casan the quantified elements amount to approximately 6833 μg/g. Relatively large cluster is seen in Figure 1a with Cac-1000, Laphos, Multical-1000, Calceplex, Calfo V, High-C Plus and Aqua Vit along with two small groups of 2 samples each with High-C-1000 and Multical Plus as one set and Cac Plus and Calcee Plus as second set. All these supplements on investigation were characterized as ones that have been prepared from Ca bounded with amino acid (chelated calcium). e.g. gluconate, citrate, lactate, acetate etc. These chelated Ca supplements increase the mobility and are highly soluble and absorbed easily where the trace minerals are bound to advanced organic chelates for superior absorption. Since these samples in gigantic cluster were found to correlate with one or two of its type predicting that there are additional ingredients in their formulae that are not compatible with other supplements, therefore these samples were further scrutinized and separate PCA was applied to look for their possible behavior as a chelated Ca group.

Figures 2a and 2b represent 2 PC scores plots between PC1 and PC2 and PC1 and PC3 respectively. Both plots show similarity in CaC 1000 and Aqua Vit as one group and High-C Plus and Calcee Plus as another group. The probable relationship between CaC 1000 and Aqua Vit is those both have been administered from $CaCO_3$ along with vitamin C and are orange flavored with almost same weight per dose of 5.2 and 5.0 g respectively. Similarly the composition of High-C Plus and Calcee Plus shows that both supplements are in the form of sachet prepared by national pharmaceutical company. The chemical composition is almost identical with $CaCO_3$ 156.7 mg/sachet), Ca Pantothenate (15 mg/sachet), Ca Glycerophosphate (373.3 mg/sachet), Nicotinamide (50mg/sachet), Riboflavin (Vit B2) (15 mg/sachet), with compatible weight per dose of 6.37 and 6.3 g respectively and both are orange flavored. Relatively week link is observed for Multical 1000 and High-C 1000 in Figure 2a while they show close relationship in Figure 2b. Both national brands in sachet and orange flavored contain $CaCO_3$ (327 mg/sachet), Calcium Lactate Gluconate (1000mg/sachet), Ascorbic acid (500 mg/sachet) in glucose base. Satellite clusters of CaC Plus and Multical Plus identified in Figure 1and b. Multical Plus, orange flavored sugar free local brand in the form of sachet and CaC 1000 Plus, a multinational pineapple flavored sugar free supplement are also observed to form a link in Figure 2b. Apart from $CaCO_3$ they contain aspartame and vitamin B. CaC Plus, Calceplex and Laphos are found to lie far away from these

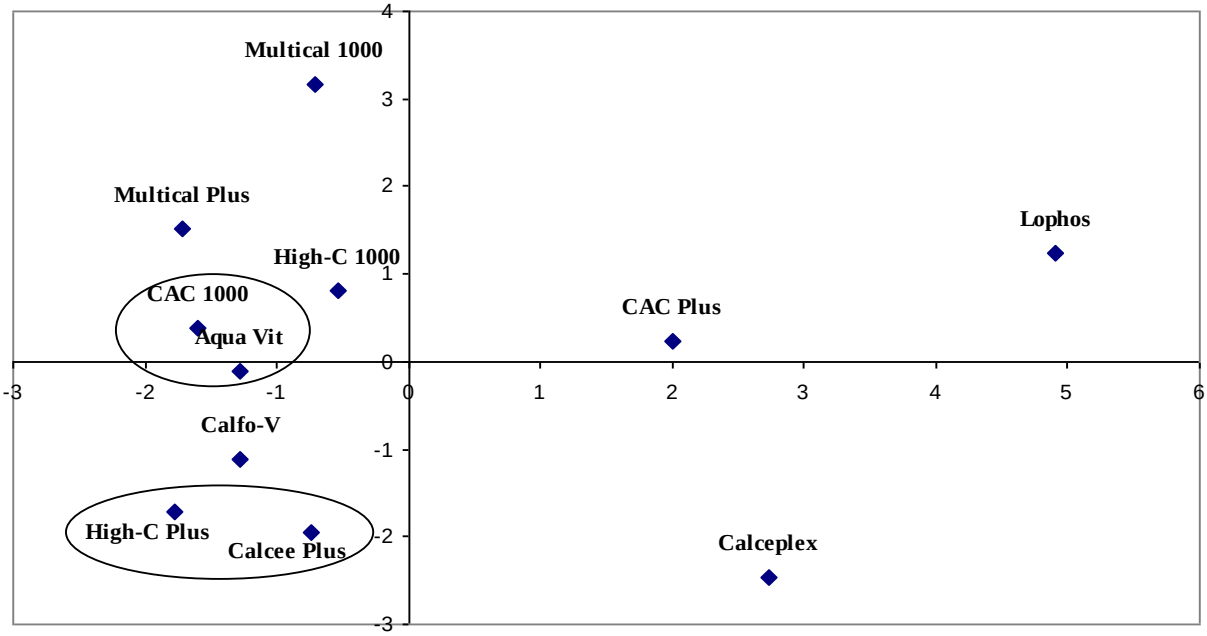

Figure 2a. Score plot of PC1 and PC2 for chelated Ca supplements.

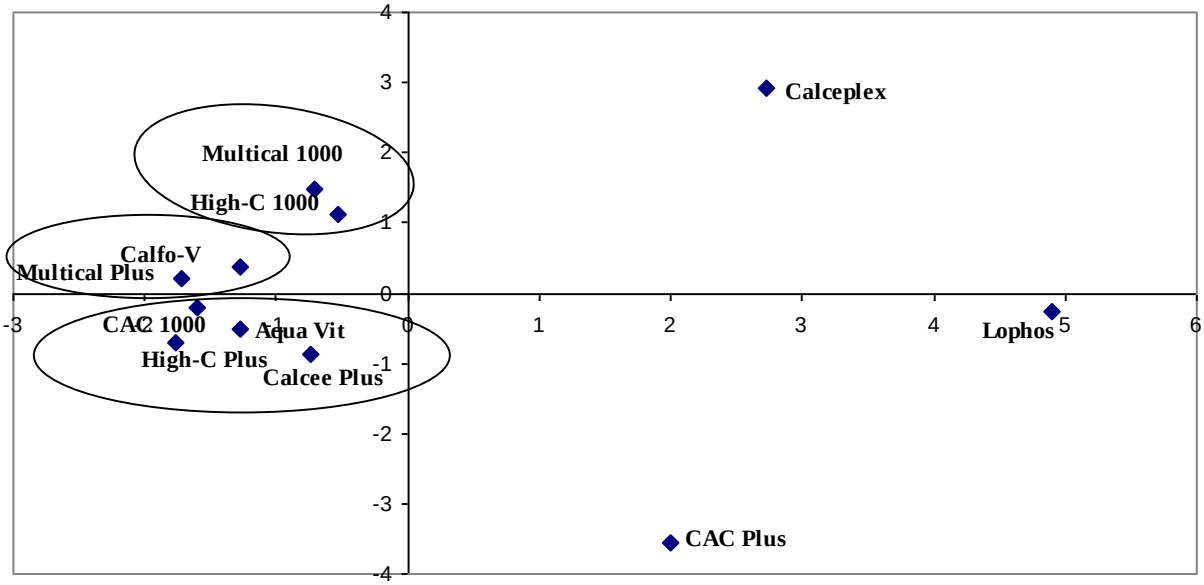

Figure 2b. Score plot of PC1 and PC3 for Chelated Ca supplements.

clusters indicating that these samples represent anomalous elemental pattern. On investigation it was found that multinational brand CaC 1000 effervescent tablets and Calceplex, a national brand in the form of sachet are orange flavored and contain 327 mg $CaCO_3$ and vitamin C, therefore it is perceived that they show different behaviors due to the quality and quantity of its ingredients and other quality control measures taken by the pharmaceutical company. The Na content of CaC 1000 was found to be very high while highest Zn and Ni are present in Calceplex. The third chelated Ca supplement that was found to show erratic behavior was Laphos. Its composition shows that the main source of Ca in this supplement in not $CaCO_3$ but Calcium acetate

[$(Ca(CH_3COO)_2$]. Ca acetate is also natural water soluble mineral that limits phosphate levels in blood of subjects with critical kidney related diseases and dialysis patients. Both figures also show some affinity between Calfo V and Aqua Vit. Upon securitization of the elemental data it was found that both samples contained highest Sb and As contents.

4. Conclusions

Multivariate principal component analysis technique was applied to classify different Ca supplements available in the market on the basis of their elemental data obtained using INAA and AAS. Significant principal components were selected considering first four Eigen values. The graphical representation of this mathematical and statistical technique gave some logical, traceable and feasible patterns for intricate analytical data set. Since the aim was to easily classify the supplements according to their compatibility with others therefore only PCA scores were exploited. The scores plot identified four types of supplements as:

1. The supplements with $CaCO_3$ as Ca source along with vitamin C.
2. The supplements with $CaCO_3$ as Ca source along with vitamin D.
3. Calcium from bone meal (Ca phosphate).
4. Calcium bounded with amino acid (chelated calcium). e.g. gluconate, citrate, lactate, acetate.

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