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Application of Solid Phase Extraction with Gas Chromatography - Mass Spectrometry in Geographical Profiling and Characterization of Volatile Organics in Kenyan Honey

Fredrick Ng'ang'a[1,2,*] Antony Gachanja[1] Anam Onditi[1] Elijah Ngumba[1]

1. Chemistry department, Jomo Kenyatta University of Agriculture and Technology, P.O Box 57483-00200, Nairobi Kenya.
2. Coffee Research Foundation,P.O Box 4, Ruiru, kenya

* E-mail of the corresponding author mungaf@yahoo.com

Abstract

Honey from different regions fetch different market prices, this makes it prone to mislabeling in terms of origin. This study aimed at identifying markers specific to Kenyan honey from different regions so as to develop a fast reliable profile that can be used in the geographical profiling of honey. Solid-Phase Extraction followed by Gas Chromatography-Mass Spectrometry was used to extract and identify volatile organic compounds in honey from various regions in Kenya. Various volatile organic compounds were identified, they were classified into the following classes; esters, carboxylic acids, aldehydes ketones, and hydrocarbons. It was established that the presence or absence of certain compounds and their variation in concentration can be used to classify honey from different geographical regions in Kenya.

Keywords: Honey, Volatile Organics, Gas Chromatography-Mass Spectrometry (GC-MS), Kenya

1. Introduction

Honey as a natural product is greatly appreciated by consumers, not only for its nutritive properties, but also for its characteristic aroma and sweet taste. Aroma is caused by the presence of many different volatile compounds in it (Soria, *et al* 2003).

The composition and flavor of honey varies, this mainly depends on the source of the nectar(s) from which it originates and to a lesser extent on certain external factors - climatic conditions and beekeeping practices in removing and extracting honey (White, 1975). A large number of organic compounds have been described in different types of honey. Some of the compounds have been described as characteristic of the floral source whereas other compounds like some alcohols, branched aldehydes and furan derivatives may be related to microbial purity or processing and storage of honey (Bouseta, *et al* 1995).

Because of the high price of certain honey types based on botanical and geographical origin, adulteration with low cost and nutritional value substances (Cotte, *et al* 2007) or mislabeling regarding the botanical or geographical origin (Alissandrakis, *et al* 2007) sometimes occurs. Nonetheless, the discrimination between different types of honey is important for honeys that possess discrete aroma, taste and special nutritional properties (Lusby, *et al* 2005; Ma˘rghitas *et al* 2009) which make them preferable for consumers. Thus, the profiling and identification of organic compounds which could be used as markers for the discrimination and classification of honey based on their geographical origin is of high importance.

Aroma compounds are present in honey at very low concentrations as complex mixtures of volatile components of different functionality and relatively low molecular weight. Gas Chromatography – Mass Spectrometry (GC-MS) is usually the technique of choice for the determination of volatile organic compounds in honey this is due to its high separation efficiency and sensitivity and also it provides qualitative and quantitative data for these compounds. However, GC-MS requires the prior removal of sugars and water (Soria, *et al* 2003).

In this study solid-phase extraction followed by gas chromatography-mass spectrometry (GC-MS) was used to extract and identify volatile organic compounds in honey obtained from various geographical origins in Kenya. Solid phase Extraction technique was employed as it offers the advantage of eliminating, by washing with water, some interfering substances such as sugars and acids thus making it possible to obtain the honey volatile fraction without

the need of applying heat; however optimization of several parameters is necessary before applying this technique (V´azquez *et al* 2006). Extraction conditions were optimized in order to obtain the highest yields of volatile substances.

2.Experimental

2.1 Method development and optimization

2.1.1 Choice of eluting solvent

10 ml each of a 30 % honey solution was passed through the two preconditioned Sep-Pak (waters) C18 SPE cartridges at a flow rate of 1ml/min , each of the cartridge was washed with 5ml of distilled water . One was eluted with 5ml of hexane while the other one with 5ml dichloromethane. Both eluent were concentrated to 1ml in a MiVac (GeneVac) vacuum sample concentrator at 25 ^{O}c and spiked with 100µl of 100ppm Internal Standard (Benzophenone) followed by GC-MS analysis.

2.1.2 Sample throughput (Volume) optimization

5, 10, 15 and 20ml of the 30 % honey solution was passed through a precondition C18 SPE cartridge . the cartridge was washed with 5 ml of distilled water, allowed to dry and later each eluted with 5ml of DCM (flow rate of 1ml/min). The eluent were further pre-concentrated to a 1ml, spiked with 100µl of 100ppm of internal standard and analyzed by GC-MS.

2.1.3 Honey amount optimization

10 ml each of 4 honey solutions i.e. 10%, 20%, 30% and 40% were passed through a preconditioned C18 SPE cartridge, washed with 5 ml distilled water and eluted with 5ml of DCM. The eluent was further pre-concentrated to a volume of 1ml and spiked with 100µl of 100ppm internal standard and latter analyzed by GC-MS.

2.1.4 Blank preparation

10ml of water was passed through a preconditioned cartridge and allowed to dry. The cartridge was eluted with 5ml of Dichloromethane. The eluent was further pre-concentrated to 1ml and analyzed by GC-MS.

2.1.5 Chromatographic conditions

GC-MS analyses were performed in a GC8000 Top series (CE instruments) coupled to a Voyager-Finnigan quadrupole mass spectrometer detector. 1µl of each extracts were injected into the split less mode in a DB5 (Cross-linked 5% Phenyl-95% Methyl Siloxane) capillary column (30m × 0.25mm i.d × 0.1µm film thickness) The injection temperature was maintained at 200^{0}c, while the oven temperature was kept at 60^{o}c for 3min and programmed to rise at 3^{o}c/min to 240 ^{o}c where it would be held for 5min. Helium was used as the carrier gas at flow rate of 1ml/min.Mass spectra were recorded in the Electron Ionization mode at 70 eV scanning the 40-450m/z range, the ion source and transfer line temperature were maintained at 200^{o}c and 250^{o}c respectively.

2.1.6 Sampling

Stratified geographical sampling was used in this stage. In obtaining the samples the existing provincial and district boundaries were used, a target of 4 samples per district was set. Samples were obtained through existing networks of bee farmers. a total of 33 samples were obtained in this study. Table 1 shows the geographical distribution of the samples.

2.1.7 Sample collection transportation and storage

Approximately 250g of the fresh unprocessed honey acquired from the farmers was placed in glass containers with air tight plastic lids. The glass containers used had been thoroughly cleaned with distilled water and dried in an oven at 100 ^{o}C prior to them being sealed for use in the sampling stage. Once the samples were obtained they were transported to the laboratory in Jomo Kenyatta University of Agriculture and Technology where they were stored in a refrigerator at 4 ^{0}C.

2.1.8 Analysis of Volatile compounds in honey from various geographical regions in Kenya.

20 grams of honey obtained were dissolved in 100ml of distilled water. 10ml were passed through a preconditioned cartridge at a rate of 1ml/min. the volatile fraction was eluted with 5ml of Dichloromethane. The DCM eluent was further pre-concentrated in a vacuum sample concentrator at 30 ^{o}c to a volume of 1ml and spiked with 100µl of

100ppm internal standard followed by GC-MS analysis.

2.1.9 Data analysis

The chromatographic peaks were each studied and individual mass spectra were obtained and compared with the spectral data from the National Institute of Standards and Technology (NIST) library, where there was no conclusive identity from the NIST library, the Golm Metabolome (http://csbdb.mpimp-golm.mpg.de/csbdb/gmd/msri/gmd_sspq.html) an on-line database was used. Peaks which were present in the blank were not considered. Quantification was done by comparing the peak areas of individual compounds identified with that of the internal standard used (100µl of Benzophenone).In developing the geographical profile compounds which were identified to be present in all the sampling points within the various sampling blocks were grouped together. The profile developed was subjected to principal component analysis (MS-Excel) to assess its discriminating power

3. Results and Discussion

3.1 Optimisation Studies

In the solvent optimization step two solvent i.e. Hexane and Dichloromethane were used. From figure 1 the Dichloromethane eluent was found to have higher concentrations of the volatile organic compound as compared to the Hexane eluent, thus it was chosen as the best elution solvent. 10 compounds identified (Table 2) to be present in both eluents were used so as to establish the trend; these 10 compounds identified were further used in subsequent optimization studies.

C18 cartridges are reverse phase SPE catridges, retention of organic analytes from polar solutions (e.g. water) onto these SPE material is due primarily to the attractive forces between the carbon-hydrogen bonds in the analyte and the functional groups on the silica surface (van der Waals forces). To elute an adsorbed compound from a C18 reversed phase cartridge, a nonpolar solvent is used to disrupt the forces that bind the compound to the packing (Supelco, 1998). Despite the fact that hexane is considered to be a strong reverse phase elution solvent due to the fact that its highly non polar than dichloromethane, it however showed the least ability to elute the organic compounds as compared to dichloromethane, this could perhaps be attributed to the mid polarity of the organic compounds present in honey thus dichloromethane is able to efficiently breakdown the forces of attraction between the organic compounds and the polymeric material packed in the cartridge.

The variation in quantities eluted by both solvents could also be attributed to the differences in elution power between the two solvent. According to Snyder's empirical eluant strength parameter (ε^{of}) which arranges solvents in increasing elution strength, hexane has an eluant strength of 0.01 while DCM is 0.42 this implies that as an elution solvent DCM is much more powerful than hexane (Snyder ,1978)

In the sample optimization stage it was found that the optimum sample throughput volume was 10ml. Figure 2 shows how the concentration of the 10 compounds varied with increasing sample volume. In the honey amount optimization stage, a 20 % honey solution was found to give the highest concentrations of the 10 compounds analyzed in this stage. Figure 3 shows the variation in concentration of the volatile organic compounds with varying sample concentration.

3.2 Volatile compounds present in honey from different geographical regions in Kenya.

Chromatographic analysis of the extracts obtained by solid phase extraction enabled the identification of 57 different compounds from samples collected in Kenya. A typical honey VOC chromatogram (figure 4) exhibited from 15 to 25 peaks. Differences in chromatographic profiles were observed when comparing honey samples from the different geographical regions. The volatile compounds identified were classified into 5 groups. These were terpenes and derivatives, aldehydes, ketones, carboxylic acids and esters. This was in agreement with reports from researchers in other parts of the world who have been profiling volatile organic compounds in honey from different floral sources (Soria *et al*., 2003; Baroni *et. al.,* 2006; Wolski, *et al.,* 2006).

The carboxylic acids, 2-(2-hydroxyethoxy)ethyl octadecanoic acid (Aqua Cera) and n-hexadecanoic acid (palmitic acid) were found to be present in all the samples analyzed and were therefore disregarded in the development of the profile. These two compounds have been found to be constituent of bees wax (Christie, 2008). The presence or absence of certain compounds from honey obtained from various geographical regions enabled the identification

of various markers specific to the honeys geographical origin.

From the profile developed (table 3), Principal Component Analysis (PCA) was used to assess the extent of clustering, the discriminating power of the profile and also in explaining the variance in the profile. PCA represents one of the most frequently used chemometric tools mainly due to its very attractive features. PCA allows relatively easy projecting of data from a higher to a lower dimensional space and then reconstructing them without any preliminary assumptions about their distribution (Stanimirova *et al* 2007).

From figure 5 Principal Component (PC) 1 contributed to 38 .53 % of the variance while PC 2 contributed to 31.03%. From the PCA cluster plot there is a distinct discrimination of the samples based on their geographical origin i.e. no clustering is evident thus as a discriminating tool it's effective.

Other compounds which were identified from this study but could not be used in the development of the profile are classified in table 4.

Nonanoic acid has been reported as a marker present in Eucalyptus honey extracted by SPME (Piasenzotto, *et al* , 2003), it has also been found to be present in heather, lime-honey dew honey, and buckwheat honey from Poland (Wolski, *et al* 2006). Compounds such as Tricosane, tetracosane,9-Octadecanoic acid and Trans-e(sup 9)-octadecenoic acid which were found to be present in honey have been previous reported by to be characteristic markers of *Prunus mahaleb* Honey in Croatia **(**Jerković, *et al,* 2011). In this study only one honey sample was found to have a furan derivative 5-(hydroxymethyl)-2-furaldehyde). This could be attributed to the fact that fresh unprocessed honey obtained from farmers was used. Certain volatile compounds, especially furan derivatives (furfural, methyl furoate), may be formed by heating or during prolonged storage of honey (V´azquez, *et al*, 2006). In practice, Hydroxymethylfurfural (HMF) is used as a marker of excessive heating, and maximum limits are regulated by law. To the best of our knowledge this is the first attempt in Kenya to profile locally produced honey based on its volatile compound constituents.

4. Conclusion

Solid-phase extraction proved to be a good method to isolate volatile compounds in honeys. The optimum operating were found to be 10 ml of a 20 % honey solution (1:5 W/V) and eluted with 5ml of dichloromethane. Solid-phase extraction technique followed by gas chromatography-mass spectrometry enabled efficient characterization of various Volatile Organic compounds present in Kenyan honey from various geographical regions, the presence or absence and variation in concentration of the various compounds enabled the development of volatile organic compound profile of honey obtained from farmers in different geographical regions in the country.

5. Acknowledgments

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Region	**Number of samples**
Lower Eastern Province (LEP)	8
Upper Eastern Province (UEP)	8
Central Rift Valley (CRVP)	6
South rift valley (SRVP)	5
Nairobi and Central Province (NCP)	6
Total	**33**

Table 1: Geographical distribution of honey samples collected for analysis

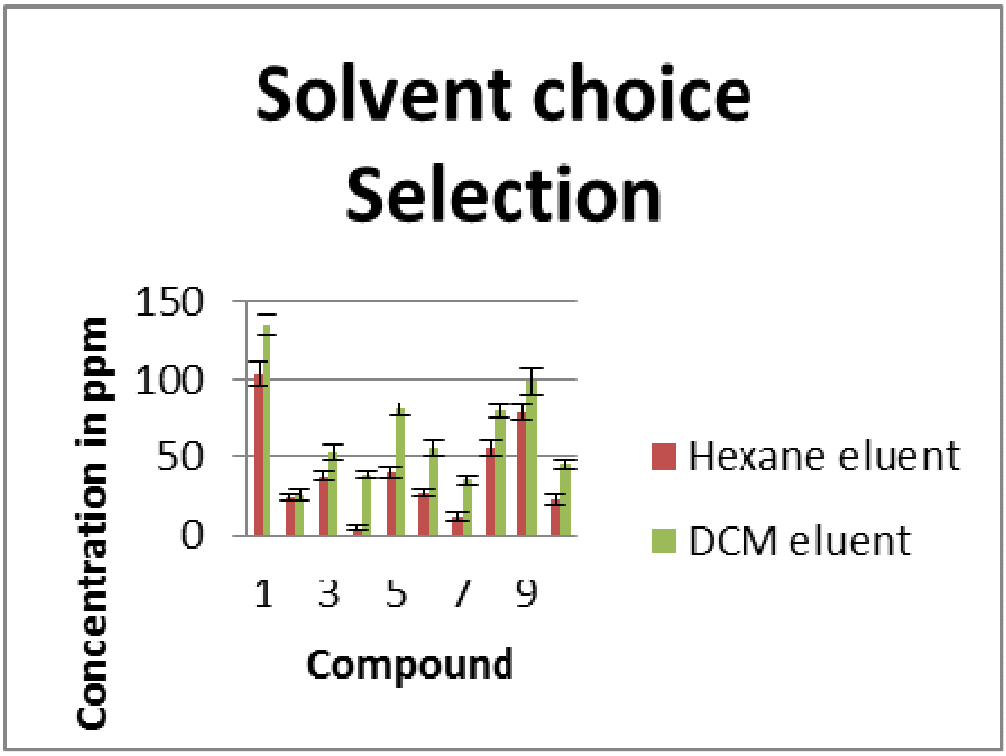

Figure 1: Comparison of the concentration of Compounds eluted from SPE cartridge by Hexane and DCM

Compound Number	Identity
1	4-Ethyltetradecane
2	10-Methyl Dodecanoic Acid
3	2,6,10-Trimethyltetradecane
4	E-15-Heptadecenal
5	n-Hexadecanoic Acid
6	Methyl Hexadecanoate
7	Methyl 14-Methyl Hexadecanoate
8	Methyl Octadecanoate
9	Methyl Icosanoate
10	2,2'-Methylenebis(4-Methyl-6-Tert-Butylphenol)

Table 2: Identity of the compounds used in the optimization study of the solid phase extraction.

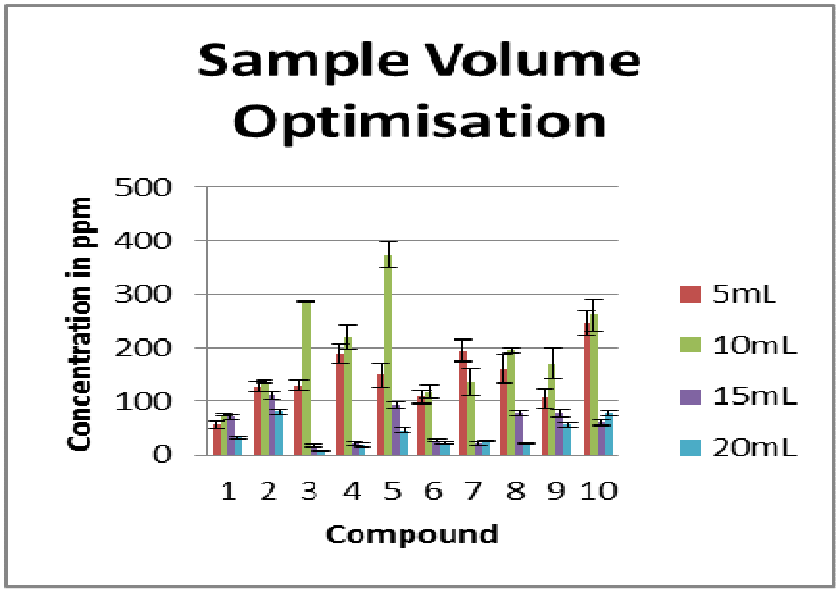

Figure 2: Comparison of concentration of compounds eluted with DCM with varying sample volume

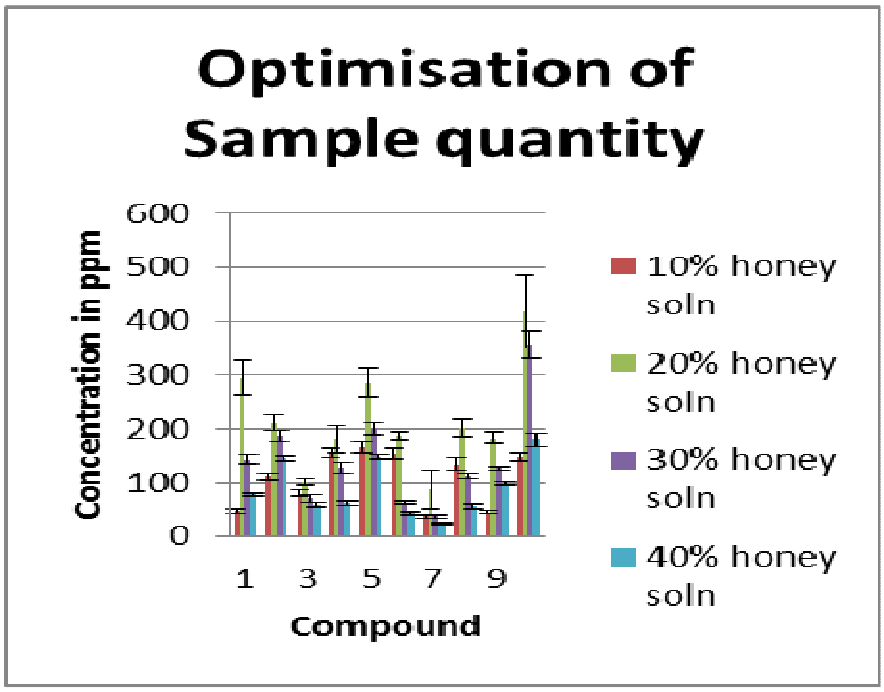

Figure 3: Comparison of concentration of compounds eluted with DCM with varying sample concentration.

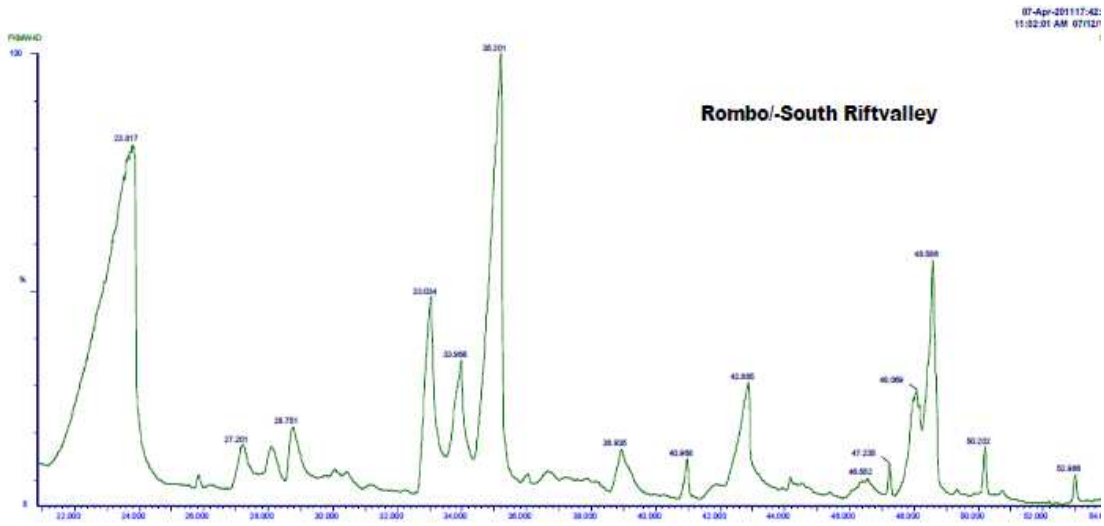

Figure 4. A typical chromatogram obtained after running the samples in the GC-MS

Compound	**Region**				
	LEP	**UEP**	**CRVP**	**SRVP**	**NCP**
Carboxylic Acids	Mwingi	Kitui			
2-decanynoic acid	ND	ND	ND	53.45±9.98	ND
14-methyl pentadecanoic acid	ND	ND	ND	ND	109.15±23.45
Trans-e(sup 9)-octadecenoic acid	ND	ND	11.78±4.35	ND	29.41±6.78
16.methylheptadecanoic acid	ND	ND	ND	ND	34.47±8.78
Ketones and Aldehydes					
2-pentadecanone	ND	66.2±14.56	ND	ND	ND
E-14-Heptadecenal	35±12.34	14.94±2.43	ND	61.23±25.5	32.78±8.89
3-heptadecenal	ND	ND	ND	13.86±4.71	ND
Esters					
1-Methylethyl ester tetradecanoic acid	ND	27.34±7.45	ND	ND	ND
Oleic acid,methyl ester	ND	ND	23.98±6.4	ND	ND
Stearic acid, methyl ester	ND	ND	17.45±4.34	ND	ND
hydrocarbons					
2,6,10,15-tetramethylheptadecane	ND	26.11±3.13	ND	216.98±39.7	ND
2,4-dimethyleicosane	ND	44.30±7.70	ND	ND	ND
10-MethylEicosane	ND	ND	ND	68.03±13.61	ND
n-nonadecane	38.67±6.34	15.65±5.63	ND	ND	ND

7-n-Hexyleicosane	ND	ND	ND	ND	12.89±3.56
n-docosane	ND	ND	ND	56.51±9.78	37.06±7.98
Others	Mwingi	Kitui			
Sandoracopimar-15-en8á-yl acetate	ND	66.03±15.70	ND	ND	ND

Table 3: Concentrations in ppm of the various compounds identified from samples obtained from different geographical regions

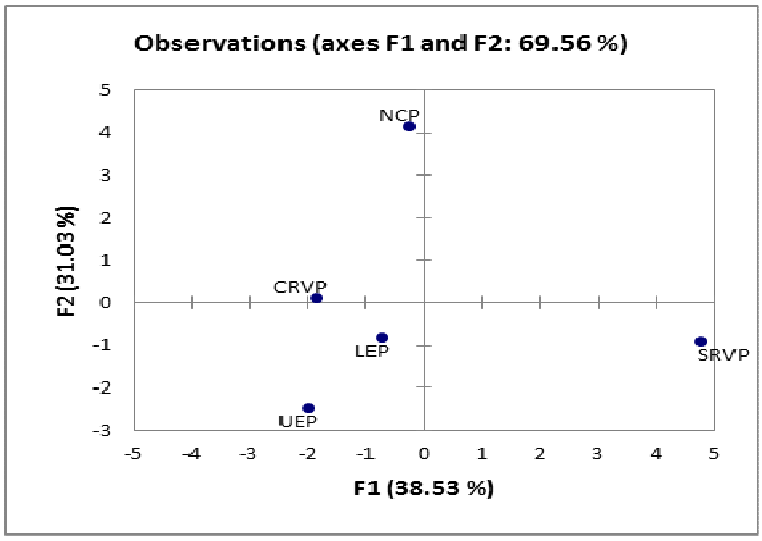

Figure 5: PCA clustering of the profile developed

Class	Compound
Hydrocarbons	6-tridecene ,Tricosane, 2,3dimethyl decane, 10-methylEicosane, Phylloclade-15-ene, 2-pentylcyclohexane, Tetracosane, 3,butyl-4-hexylBicyclo[4.3.0]nonane, 1-Methoxy-13-methyl pentadecane, 1-Acetoxynonadecane
Ketones& Aldehydes	Dodecen-2-one, 3-heptadecanal, Ethanone, 5-(hydroxymethyl)-2-furaldehyde, 3-Acetyl-2-pentanone,cyclopentadecanone, 4-hydroxy-3,5,6-trimethyl-4-[3-oxo-1-butenyl]-2-cyclohexene-1-one
Esters	Ethylpentadecanoate, 6-dodecanolAcetate, Methyl-6,8-dodecadienyl ester
Carboxylic acids	Nonanoic acid, 9-Octadecanoic acid,
Others	2,3,6-trimethylhept-3-en-1-ol,2-butyl-1-octanol,E-2-methyl-3-13-octadecadiene-1-ol, 1-decylsulfonyl-d-manitol, 3,4-Dimethylpentyl-3s-ethyl4s-methylpentanoate

Table 4. Classification of other Volatile organic compounds identified from the SPE eluent but not used in the development of the profile.