HS-SPME-GC-TOFMS Methodology for Verification of Geographical Origin and Authenticity Attributes of Coffee Samples

by

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Author's declaration

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

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Abstract

Increasing consumer awareness of food safety issues requires the development of highly sophisticated techniques for the authentication of food commodities. The food products targeted for falsification are either products of high commercial value or those produced in large quantities. For this reason, the present investigation is directed toward the characterization of coffee samples according to geographical origin attributes. In addition, the current examination is focused on the identification of particular marker compounds that compose the volatile and semivolatile aroma fraction of flavoured and dessert coffees. The conducted research involved the development of a rapid headspace solid phase microextraction (HS-SPME) – gas chromatography – time-of-flight mass spectrometry (GC-TOFMS) method for the verification of geographical origin traceability of coffee samples. As opposed to the utilization of traditional univariate optimization methods, the current study employs the application of multivariate experimental designs to the optimization of extraction-influencing parameters. Hence, the two-level full factorial first-order design aided in the identification of two influential variables: extraction time and sample temperature. The optimum set of conditions for the two variables was 12 min and 55 ^{o}C, respectively, as directed by utilization of the Doehlert matrix and response surface methodology. The high-throughput automated SPME procedure was completed under optimized conditions by implementing a single divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) metal fiber with excellent properties of durability, which ensured the complete analysis of coffee samples in sequence. The coffee sample originating from an authentic Brazilian coffee producing region and characterized by rich volatile and semivolatile chromatographic profiles was selected as a reference starting point for data evaluation. The combination of the retention index (RI) system using C_8-C_{40} alkanes and the mass spectral library search was utilized for the confirmation of analyte identity in this reference sample. Twenty-nine volatile and semivolatile compounds selected across the wide range of GC chromatogram were then evaluated in terms of chromatographic peak areas for all samples that are to be submitted to this classification study. The semiquantitative results were submitted to

statistical evaluation, namely principal component analysis (PCA) for the establishment of corresponding geographical origin discriminations.

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List of Acronyms

Analysis of variance	ANOVA
Bovine spongiform encephalopathy	BSE
Carbowax/divinylbenzene	CW/DVB
Carboxen/polydimethylsiloxane	CAR/PDMS
Direct immersion-solid phase microextraction	DI-SPME
Divinylbenzene/carboxen/polydimethylsiloxane	DVB/CAR/PDMS
Electron impact	EI
Environmental Protection Agency	EPA
European Commission	EC
European Union	EU
Food and Agriculture Organization of the United Nations	FAO
Gas chromatography	GC
Gas chromatography-mass spectrometry	GC-MS
Gas chromatography - time-of-flight mass spectrometry	GC-TOFMS
Headspace-solid phase microextraction	HS-SPME
High performance liquid chromatography	HPLC
Internal diameter	ID
Liquid chromatography-mass spectrometry	LC-MS
Mid-infrared	MIR
National Institute of Standards and Technology	NIST
Near-infrared	NIR
Organochlorine pesticides	OCPs
Part per million	ppm
Part per trillion	ppt
Polyacrylate	PA
Polycyclic aromatic hydrocarbons	PAHs
Polydimethylsiloxane	PDMS
Polydimethylsiloxane/divinylbenzene	PDMS/DVB
Polytetrafluoroethylene	PTFE

Principal component analysis	PCA
Principal component 1	PC1
Principal component 2	PC2
Protected designation of origin	PDO
Relative standard deviation	RSD
Retention index	RI
Solid phase microextraction	SPME
Static headspace	SH
Supelco low bleed	SLB
Time-of-flight	TOF
Time-of-flight mass spectrometer	TOFMS
Total ion current	TIC
Traditional speciality guaranteed	TSG
Ultraviolet	UV
Vintners Quality Alliance	VQA
World Health Organization	WHO

1.0. Introduction

1.1. The importance of food analysis

Increasing consumer awareness of food safety issues has led to the development of new and increasingly sophisticated techniques for the verification of food quality. Accordingly, research dedicated to the assessment of food quality has been actively conducted in recent years and various objectives underlying the importance of food analysis were introduced. The focus of the current section is to briefly introduce those aspects of modern food analysis that are most significant.

Primarily, food analysis is significant for the evaluation of nutritional value and the quality control of fresh and processed products [1]. As it is well known, most foods have a finite shelf-life or the time period between production and when a food product is deemed unacceptable [2]. For this reason, knowing the original and modified chromatographic patterns of fresh and processed/stored food commodities, respectively, is an asset in determining the compositional changes that many food commodities are prone to. The major deteriorative reactions that influence the shelf-life stability of food commodities can be classified into the following categories: *i)* enzymatic changes; *ii)* biological changes; *iii)* physical changes; and *iv)* chemical changes. The enzymatic reactions are highly correlated with biochemical processes in plant and animal tissues and occur as a result of the natural presence of enzymes in foods. The occurrence of enzymatic changes influences colour, flavour, texture and nutritional characteristics of foods, the extent of which is greatly dependent on temperature, water activity and oxygen content, as these conditions affect the rate of enzymatic reactions.

Biological changes occur in food primarily as a result of microbial activity. While in specific instances, such as food fermentations, the bacterial growth enhances the shelf-life stability, in others it initiates food spoilage and corresponding bacterial foodborne infections, which in the most severe cases lead to serious disease outbreaks, illnesses and deaths. From the analytical chemistry point of view, the detection of foodborne pathogens in food commodities has been mainly conducted by monitoring volatile metabolic compounds produced by microorganisms in growth media. Such an approach was utilized in one of the previous studies, which focused on monitoring

headspace volatile metabolites of *Salmonella* and *Escherichia coli* [3], two foodborne pathogens known to induce severe foodborne disease outbreaks. In addition, another screening study was performed to monitor the volatiles emitted by potatoes infected by *Ralstonia solanacearum* and *Clavibacter michiganensis* bacteria [4].

Physical changes occur as a result of improper selection of packaging material. For this reason, the characterization of food packaging materials has become increasingly important within the food sector in recent years due to the application of active materials in food packaging [5]. With regard to this, active packaging systems were defined as materials that "perform a role other than an inert barrier to the outside environment." In addition to their critical functions of increasing the shelf-life stability and preserving the quality, sensory and safety properties, the active packaging materials sometimes initiate the undesirable depletion of aroma constituents from packaged food commodities.

Chemical changes, on the other hand, are mainly initiated by auto-oxidation processes of polyunsaturated fatty acids that are present in variable amounts in most foods [6]. This process, also referred to as "lipid oxidation" or "oxidative degradation" is one of the well-recognized factors leading to severe nutritional quality losses, flavour deteriorations, decreased shelf-life stability and generation of toxic substances. The research dedicated to the quality assessment of lipid containing foods has been mainly conducted from a toxicological perspective, as a majority of secondary oxidation products are toxic and their intake through food leads to potential negative effects on human health [7]. With regard to this, such examinations are focused on the determination of specific indicators of lipid oxidation, an aspect in which aldehydes play a crucial role. The research focused on the determination of secondary oxidation products as markers of oxidative degradation has included up to this point various types of food commodities such as: infant foods [6-8], rapeseed oil [9] and meat products [10-12].

In addition, one of the objectives underlying the importance of food analysis is the determination of impurities or involuntary additives that are considered to be "extraneous substances introduced to food accidentally" such as metals released from devices or vessels, pesticides and solvents [13]. Some of these impurities are frequently toxic and impose negative effects on human health such as for example, the presence of organic

solvent residues caused by the improper use of technology and constituents released into food during transportation processes. Occasionally, food production procedures do not initiate the generation of impurities in final processed food products, as these are likely to be existent in raw materials at the time of purchase. For instance, the quality of processed fruit and vegetable products is very much altered by the initial presence of pesticides in fresh/raw materials. The research dedicated to the determination of organochlorine pesticides (OCPs) and their less persistent and more commonly utilized organophosphorous counterparts has been extensive for years, due to the serious effects of their increased bioaccumulation throughout the food chain [14]. Several authors presented publications on the determination of pesticides in the most commonly affected food commodities such as olive oil [15,16], milk [17,18], honey [19], fruit and fruit juices [20-22].

Another aspect for which food analysis needs to be conducted is directed toward the determination of food additives, which constitute one of the major groups of food constituents [13]. As directed by the Codex Alimentarius Commission, which is jointly prescribed by the Food and Agriculture Organization of the United Nations (FAO) and World Health Organization (WHO), food additive is defined as

> "any substance not normally consumed as a food by itself and not normally used as a typical ingredient of food, whether or not it has nutritional value, the intentional addition of which to food for a technological (including organoleptic) purpose in the manufacture, processing, preparation, treatment, packaging, transport or holding of such food results or may be reasonably expected to result (directly or indirectly), in it or its by-products becoming a component of or otherwise affecting the characteristics of such foods" [2].

Accordingly, the utilization of food additives in food processing and production procedures is conducted so that the following two basic functions can be performed: *i)* preservation of food quality and *ii)* improved flavour and colour characteristics of foods. The contents of such additive components are highly correlated with product quality; however, it is important to note that their standard values should not be exceeded. Their positive effects from a production point of view can, in the instances of extreme overdosing, result in unfavourable effects to human health.

The processing and packaging procedures are conducted to increase the shelf-life stability of food commodities. In addition to preservation of food quality through the utilization of food additives, numerous traditional and novel food processing technologies have been employed for this purpose. The worldwide restriction of hazardous chemical use in food preservation led to the utilization of irradiation for this purpose [23]. Food irradiation has been associated with the most clean and safe method of food preservation, as it improves the quality and safety of foods through increased shelf-life stability, microorganism sterilization and delayed ripening processes. However, due to the unproven risks associated with consumption of irradiated foods, food irradiation has been banned in some countries, whereas in others irradiated food is required to be labelled by legislation. For these reasons, the detection of irradiated food commodities, usually achieved by monitoring radiolytic volatile marker compounds, has become another important aspect of food analysis.

In addition to the aforementioned aspects of food analysis, which are focused on the detection of deteriorative processes and the determination of various quality influencing food constituents, research in the field of food analysis has also been directed toward the determination of natural constituents. The majority of compounds that constitute a food's natural composition contribute to the specific flavour that a particular commodity is known for. Having in mind that flavour composition is a critical factor in consumer acceptance of food [24], it is obvious that examination of those compounds that are correlated with it is crucial for those divisions within the food sector that are associated with quality determinations of food commodities.

1.2. Food authenticity and traceability

The characteristic flavours of food and beverage products are generally the result of extremely complex multisubstance mixtures, containing several hundred compounds characterized by different chemical structures and physico-chemical properties [25]. Therefore, in most cases, the flavour impression that is perceived as a single sensation is the result of complex sensory impression of many individual substances in specific concentration ratios. These volatile constituents are usually present in only minor

amounts (ppm to ppt range); however, as already mentioned in Section 1.1 of this document, their presence decisively influences the acceptance of foodstuffs. For example, among numerous compounds comprising the wine bouquet, aldehydes, esters and ethers were exclusively identified to provide the characteristic and desirable fragrances of this alcoholic beverage [13]. On the other hand, the high consumer acceptance of wines processed in oak wood barrels as opposed to stainless steel ones is attributed to the presence of wood-related compounds such as syringaldehyde, *cis*- and *trans*-oak lactones, eugenol and guaiacol [26]. Similarly, volatile sulfur compounds, namely, methanethiol, hydrogen sulfide and dimethyl sulfide have been detected as important contributors to cheddar cheese flavour, as their contents increase during the aging process of this particular cheese [27]. Final acceptable coffee aroma, on the other hand, is mainly attributed to the presence of pyrazines formed during the roasting procedure, which is responsible for the transformation of green coffee beans into the roasted ones [28,29].

Flavour composition of food commodities is greatly affected by numerous parameters, among which the following are most significant: *i)* geographical origin declaration; *ii)* type of food product; *iii)* nature of raw materials such as crop/plant variety; *iv)* processing technology and *v)* storage conditions [13]. These parameters independently or in combination with each other influence the presence or absence of compounds, which are potentially associated with the flavour composition of a particular food product. Furthermore, the dependence of volatile and semivolatile flavour constituents on the above mentioned quality-influencing production and processing factors gives rise to the possibility of characterizing food commodities through performance of qualitative screening comparison studies. This aspect of food analysis has been especially significant for assessment of food authenticity and successful utilization of traceability systems.

With regard to food authentication, it is worth noting that this direction in food analysis has become significant due to the foodborne disease outbreaks in different parts of the world. The bovine spongiform encephalopathy (BSE), Salmonella and avian influenza outbreaks have caused increased consumer awareness of food safety issues, thus creating a demand for certification of food quality [30]. Such certified verification

requires the provision of genuinity assurance and adulterations' recognition patterns. Once submitted to consumers, producers and other groups within the food sector, it ensures protection against fraud, eliminating harmful economic consequences and exposing the products' provenance under both compositional and geographical aspects.

Even though food authenticity and traceability are very closely associated as they are both thoroughly examined in the overall quality control system, it is important to emphasize at this point that traceability is another aspect of food authenticity assessment. For example, food authentication procedures might be directed toward the determination of contaminants, storage/packaging/processing-induced degradation products and potentially toxic substances. On the other hand, the assessment of food authenticity through utilization of traceability systems involves finding the historical specifications of a particular food commodity [30]. Accordingly, the European Union (EU) Commission presented the following detailed definition of traceability:

> "the possibility to find and follow the trace, throughout all the stages of production, processing and distribution of a foodstuff, feedstuff, an animal destined for food production or a substance destined to be incorporated in foodstuff or feedstuff or with a probability of being used as such"

(REGULATIONS (EC) No 1830/2003 & 882/2004) [31]. The improvement and development of current and novel traceability systems, respectively, have become crucial requirements of the overall authentication process especially due to increasing globalization of food markets and easiness associated with import and export of food commodities [32].

Even though food adulteration has been practiced since biblical times [33], the occurrence of more sophisticated fraudulent practices as pertaining to the food sector has been prominent since the beginning of the 19th century [34]. From a historical perspective, various food commodities have been adulterated: pepper has been adulterated with gravel, leaves and twigs; tea with re-dried tea leaves; milk and wine have been diluted with water; and roasted grains have been added to coffee. In general, authenticity issues fall into at least one of the following categories: *i)* noncompliance with legal requirements (product standards, geographical origin of the product, max/min water content); *ii)* erroneous addition of specific ingredients (illegal addition of food

additives and preservatives or addition of legal ones in higher than prescribed concentrations) and *iii)* noncompliance regarding the use of certain technological processes (improper implementation of preservation and packaging procedures) [31,35]. According to the detailed characterization of the authentication aspects mentioned above, it can be concluded that for reliable food authenticity verifications and successful utilization of traceability systems, the certification of geographical origin attributes is a must. The food product geographical declaration has especially become an important aspect of traceability verification procedure since the authenticity and quality parameters are often associated with a particular geographical origin and/or production area [36]. For this reason, all divisions within the food sector are in high demand for the development of analytical strategies capable of tracing back the food commodities to their production area.

In order to protect the rights of consumers and genuine food processors in terms of food adulteration and fraudulent or deceptive practices in food processing, the European Union recently established a series of regulations regarding food safety and traceability [37]. The following European Union systems were mainly announced for the promotion and protection of food products of recognized quality and origin: *i)* protected designation of origin, PDO (aimed at protecting foodstuffs that are produced, processed and prepared in a given geographical area using recognized know-how) and *ii)* traditional speciality guaranteed, TSG (aimed at protecting foodstuffs possessing a traditional character, either in the composition or means of production). Various types of food commodities have been already protected through PDO legislation in order to guarantee that their quality is closely linked to their geographical origin. Such products include Mediterranean extra virgin olive oil, French "Olive de Nice" olive oil, Mediterranean wine, Kalamata olives from Greece, Slovakian, Polish and Romanian bryndza cheeses and 'Mozzarella di Bufala Campana' cheese from Italy [32, 36-40]. A particular example illustrating quality differences between PDO labelled and highest-quality commercially available olive oils is presented in Figure 1. This study, conducted by Berlioz *et al*. was directed toward qualitative determination of volatile and semivolatile compound compositions in French olive oils in order to establish the differentiation according to quality parameters [40]. As far as the TSG protective labelling is concerned, the

following types of food commodities have been protected: mozzarella cheese from Italy and Sahti beer from Finland [37].

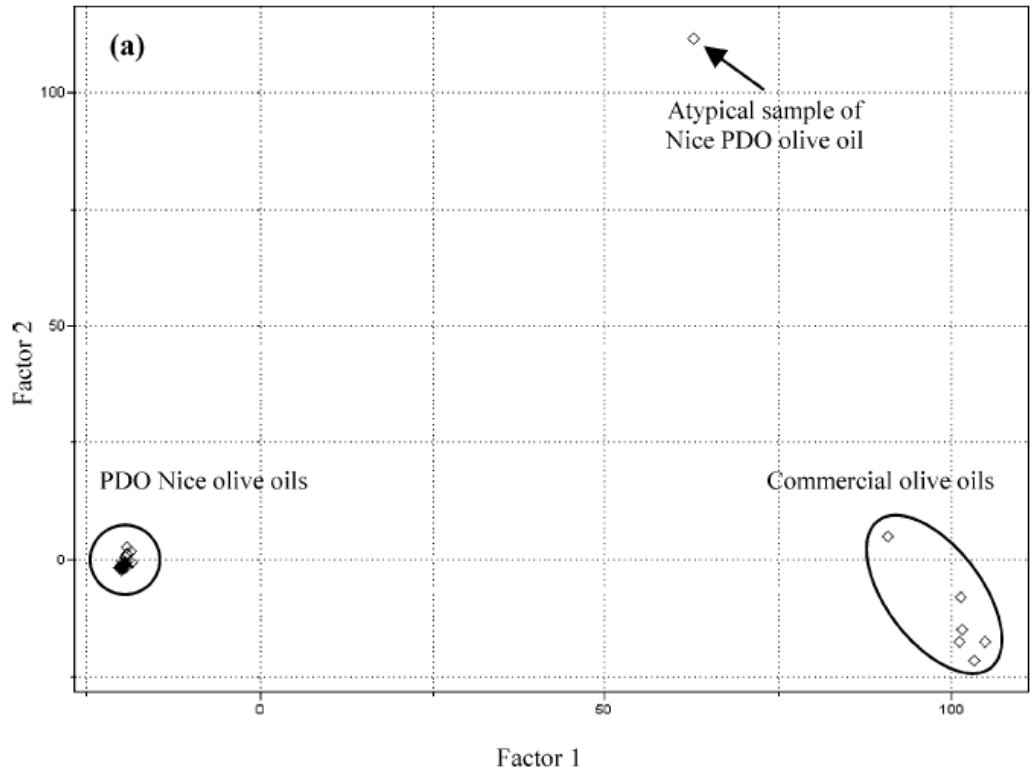

Figure 1. Scatter plot of principal component analysis scores associated with differentiation of PDO labelled and commercially available olive oils from France [40].

Up to this point, this document provided specifics associated with current European legislations designed to emphasize genuine products' geographical origin and production attributes. However, it is important to emphasize that quality control and legislative labelling procedures are undertaken in North American countries as well. For instance, only ice wines pre-approved by the Appellation of Origin system called Vintners Quality Alliance (VQA) are allowed to be produced in Canada [26]. Among the well-established VQA standards, the prohibition of artificial freezing and sugar addition during the entire ice wine production process are main requirements differentiating this regulation from the ones established in other countries where this high commercial value

commodity is produced. Therefore, the development and utilization of highly sophisticated techniques capable of allowing qualitative differentiation of ice wines based on various quality influencing parameters such as grape variety, geographical declaration and production-associated specifics are crucial quality control requirements. One of the characterization aspects of such qualitative comparison study recently conducted by Setkova *et al*. is provided in Figure 2, illustrating the ice wine discrimination according to geographical origin declaration [26].

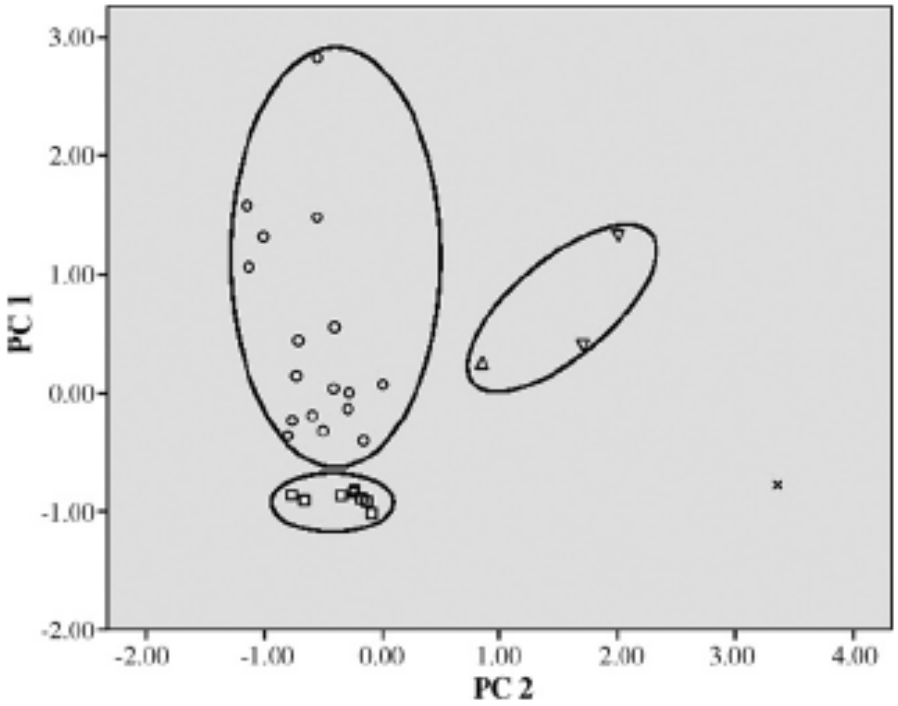

Figure 2. Scatter plot of principal component analysis scores associated with differentiation of ice wines according to geographical indication [26]. Sample code labels ○ and □ represent ice wines originating from Ontario and Czech Republic, respectively. Sample code labels Δ and ∇ represent ice wines from British Columbia of Riesling and Ehrenfelser grape varieties, respectively. The outlier sample coded with "x" symbol most likely belongs to different grape variety according to the results illustrated by previous classification studies [26].

As it was emphasized previously, various genuine food commodities possessing recognized quality, geographical origin and qualified national declarations have been protected by legislative authorities, so that the authenticity of these valuable products is ensured. These denominations are established for those products that are produced in particular geographical regions, with typical characteristics linked to natural factors, to the environment and to traditions of the region [36]. At this point, it is also important to emphasize that food commodities most likely to be targets for adulteration include those that are of high-value and that undergo a number of processing steps before they appear on the market [33]. For this reason, the present investigation is directed toward the characterization of coffee as one of the enormously important food commodities.

1.3. Coffee

Coffee is the world most popular beverage after water, considering that over 400 billion cups are consumed annually [41]. In addition, coffee is one of the most important agricultural products in the international trade, whose production is responsible for putting into motion approximately 35 billion US dollars on annual basis. The coffee aroma is characterized by the presence of a wide range of volatiles belonging to several classes of compounds such as furans, pyrazines, ketones, alcohols, aldehydes, esters, pyrroles, thiophenes, sulphur compounds, benzenic compounds, phenols, pyridines, thiazoles, oxazoles, lactones, alkanes, alkenes and acids [28]. These components are present in variable concentrations and each of them contributes uniquely to the final aroma quality.

Coffee, both as a beverage and a plant, originates from north-eastern African countries, where it was first consumed as a drink and from where its use widely expanded to Arabia, then Constantinople and Venice [28,29]. By the middle of the 17^{th} century, the consumption of coffee beverage had spread all over Europe [28]. Today, coffee represents one of the most popular beverages in the world, and it is classified as a food commodity with enormous social and commercial importance.

Research dedicated to the recognition of coffee adulteration practices has been conducted for years. In addition to the point emphasized in Section 1.2., regarding the

adulterate addition of roasted grains to coffee, the following complete list of ways to perform fraudulent practices as pertaining to coffee was composed by Tzouros *et al*.:
i) adulteration with coffee substitutes; *ii)* mixing two coffee bean varieties and *iii)* mixing of high commercial value coffee beans from one region with lower commercial value ones originating from other regions [42].

One of the most commonly utilized strategies in this field of coffee adulteration recognition patterns is the possibility to discriminate coffees based on the coffee bean variety. Even though at least 66 species of the genus *Coffea L.* have been identified so far, only two varieties with pronounced economical and commercial importance are extensively cultivated in coffee producing regions: *C. arabica L.* (*arabica* coffee) and *C. canephora Pierre* (*robusta* coffee) [43]. *Arabica* coffee beans are generally characterized by more pronounced quality and consumer acceptance, consequently they command higher commercial value prices and are therefore subject to fraudulent practices [29,44]. Even though the raw beans of the two species are easily distinguishable due to significant size differences, such visual differentiation is not possible after the completion of the roasting process, which initiates a 33% and 18-22% decrease in volume and weight, respectively [28]. The characterization of coffee samples according to the bean variety has been established by several authors who accomplished this task either by focusing on the metal content [44-45] or volatile/semivolatile compound compositions [29,46-50]. As an illustrative example of the possibility to conduct such characterization study aimed at discriminating different coffee bean varieties, the work presented by Bicchi *et al*. is emphasized here [50]. Based on the statistical evaluation to which the volatile and semivolatile compound compositions were submitted within this study, the coffee blends constituting various proportions of high-quality *arabica* and low-quality *robusta* coffee beans were successfully discriminated (please refer to Figure 3). The benefits provided by the development of methods to ensure the establishment of such fingerprinting profiles are clearly illustrated here, since the authenticity as pertaining to the proportion of higher and lower quality beans of any unknown commercially available coffee sample can be assessed based on its clustering with other examined samples.

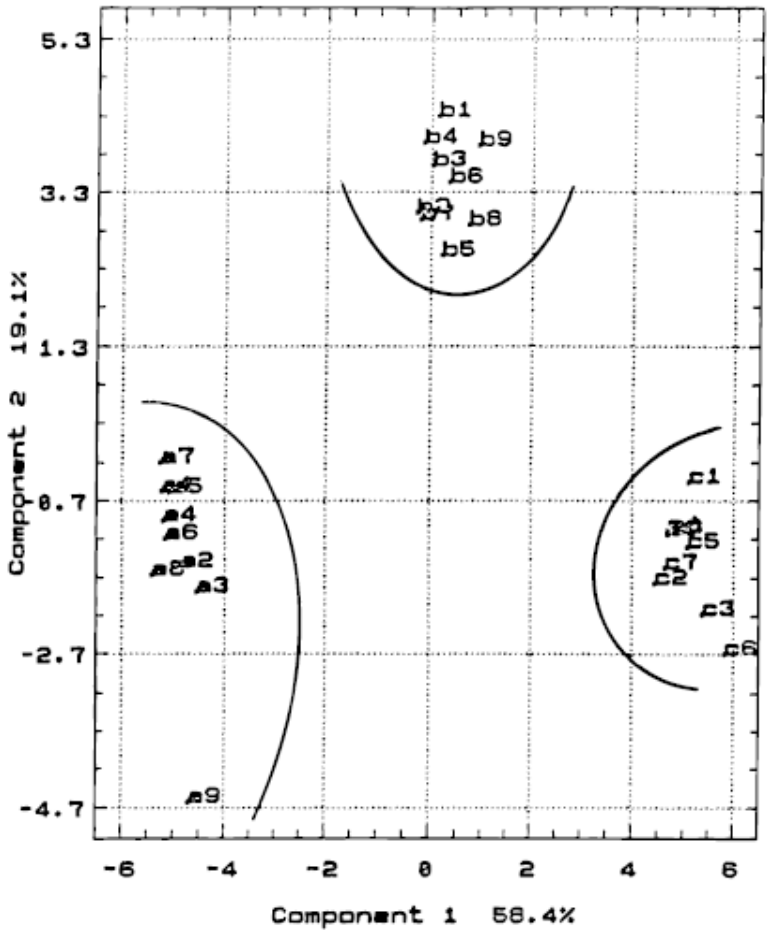

Figure 3. Scatter plot of PCA scores associated with the differentiation of coffee blends according to the proportion of *arabica* and *robusta* coffee beans [50]. (a) 50% *arabica* - 50% *robusta*; (b) 40% *arabica* - 60% *robusta*; (c) coffee blends.

Other classification studies aimed at discriminating between these two bean varieties of differing quality were targeted at determination of chlorogenic acid, caffeine, fatty acid, sterolic, diterpenic alcohol and triglyceride profiles, and subsequent submission of data to statistical evaluation [43]. In addition to utilizing the qualitative comparison procedure based on the compositions of volatile/semivolatile, metal and other aforementioned chemical constituents and submitting the semiquantitative results to statistical analysis, several authors performed the differentiation studies based on the determination of characteristic marker components that are predominant in one of the two

species of interest. Accordingly, 2-methylisoborneol was identified as a key aroma compound in r*obusta* coffee [51] and trigonelline (*N*-methylnicotinic acid), nicotinic acid and caffeine were also assessed in terms of representing the marker compounds responsible for the differentiation between the two varieties [52]. As far as the addition of fraudulent components is concerned, various speculations have become announced on the potential adulteration of *arabica* coffee beans with lower price adulterants such as cereals, coffee twigs, brown sugar and corn [53]. Jham *et al*. examined the possibility of detecting *arabica* coffee bean adulteration with corn and hence suggested γ-tocopherol as a potential marker of fraudulent practices associated with erroneous addition of this less expensive adulterant to coffee, as the contents of this compound are higher in corn as compared with authentic, uncontaminated coffee samples [53]. In this published study, the quantitative analysis of tocopherols in several commercially available coffee samples indicated only one commercially available sample with unusually elevated levels of γ-tocopherol.

The major producers of coffee beans are South American countries such as Brazil and Colombia; however, coffee beans are produced to a large extent in Central America, Africa and Asia. The principal consumers of coffee beans are, on the other hand, European and North American countries, where at this point it is important to emphasize the significance of quality certification procedures based on patterns and historical specifications for both the internal and external coffee markets. Considering that in recent years the increasing practices associated with commanding coffee prices on the basis of their geographical origin and falsifying the product declaration have been detected, the need for analytical methodologies capable of verifying the geographical origin of coffee is therefore outstanding [45]. In order to meet this demand, several authors' publications were directed toward meeting this objective [47, 49-50, 54]; however, the relatively small number of publications in this area suggests there is a need for development of highly sophisticated and modern techniques capable of ensuring the geographical origin authenticity of coffee. Regarding this aspect of coffee authenticity assessment through verification of geographical attributes, two publications are mentioned at this point due to the success in meeting the outlined objectives. After evaluating the qualitative results associated with 20 compounds having detectable and

measurable peak areas in all samples that are to be submitted to statistical study, Bicchi *et al*. were able to establish the geographical origin discriminations between Kenyan, Colombian and Guatemalan *arabica* variety coffees [50]. A similar study was conducted by Costa Freitas *et al*., the exception being in the collection of samples considered within this particular discrimination study, which in this case consisted of Brazil, Costa Rica and Honduras *arabica* samples (please refer to Figure 4) as well as India, Angola, Ivory Coast, Cameroon and Uganda *robusta* samples. This study also indicated volatile and semivolatile marker compounds responsible for the conducted geographical origin classifications, among which 2-methylpyrazine, furfuryl acetate, gamma-butyrolactone and 2-pentylfuran were most important [54].

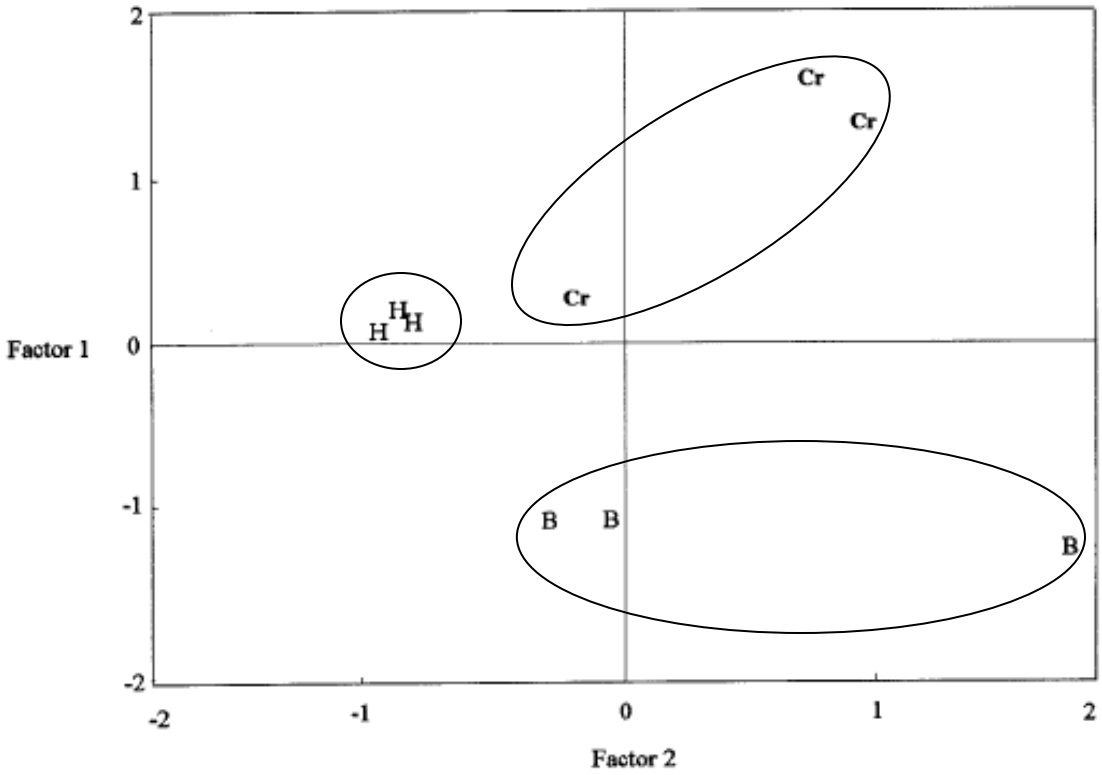

Figure 4. Scatter plot of PCA scores associated with the differentiation of coffee samples according to the geographical origin attributes [54].
(B) Brazil; (H) Honduras; (Cr) Costa Rica.

As was already emphasized earlier in this document, the producers of coffee beans are South American, Central American, African and Asian countries. The two already mentioned and previously conducted studies directed toward the determination of coffee authenticity from the perspective of geographical origin verification included samples from Italian and Portuguese markets. Keeping in mind that in addition to European import markets, the principal consumers of coffee beans are North American countries as well, the current study was conducted to provide the geographical origin verification of coffee samples commercially available in Canada through the development and utilization of an appropriate methodology discussed in future sections of this document. Furthermore, the research presented in the current document was also directed toward the comparison of volatile and semivolatile compound profiles between the commercially available samples in Canada and those received directly from authentic coffee producing regions. Finally, specific volatile and semivolatile marker compounds exclusively predominant in dessert coffee types were detected. To the best of this author's knowledge, no such studies involving the geographical origin and authenticity verifications of North American, namely Canadian, imported coffee commodities were previously conducted and published.

1.4. Methods for verification of food authenticity

When considering the selection of an appropriate method for food analysis, it is important to realize that food commodities are generally very complex mixtures composed of *i)* organic (such as carbohydrates, lipids, proteins and vitamins) and *ii)* inorganic substances (such as water and minerals) [55]. The analytical procedure for such complex samples consists of several steps typically including sampling, sample preparation, separation, quantitation, statistical evaluation and decision making [56]. Conducting each of these procedures in an appropriate way is crucial for obtaining correct and accurate results. The sampling step includes the selection of an appropriate sampling location such that the samples acquired properly define the object or problem under investigation. This step is also associated with acquiring samples in correct amounts. The objective of the sample preparation step is to isolate the components of

interest from a sample matrix, since most analytical instruments can not handle the matrix directly. The sample preparation step involves the performance of extraction procedures that bring the analytes of interest to a suitable concentration level for detection. The separation step of the analytical procedure is intended to divide the isolated complex mixture containing target analytes into its constituents. The quantitation step is associated with the determination of amounts of the target analytes. However, regarding the objective of the present investigation, which is directed toward nontarget coffee analysis and a subsequent classification procedure based on the volatile and semivolatile compound compositions, it is important to emphasize that either a polar or nonpolar and volatile or semivolatile component(s) could be the potential marker compound(s) responsible for differentiation [57]. Hence, due to the lack of previous knowledge about marker compound(s) allowing such a discrimination study and considering that this information is deducible only after conducting statistical analysis, the quantitative analytical procedure for the nontarget analysis of such a complex sample as coffee constituting several hundred compounds is impractical and almost impossible. Therefore, when meeting the objectives of such a classification study as presented in the present document, the qualitative or semiquantitative analysis involving the deduction of chromatographic peak areas is sufficient. As already specified, the next step of the analytical procedure, namely statistical evaluation, is implemented to study the main sources of variability between the samples and detect the potential relationships/variables responsible for differentiation. After the completion of the aforementioned steps, the conclusive decision making step is supported by data acquired and it may be followed by further investigations.

The definitive authentication of food products requires the use of highly sophisticated analytical techniques, as the perpetrators of this type of fraud employ methods of adulteration and misinterpretation that are increasingly difficult to detect. In this aspect, the methodologies successfully applied to food authentication include spectroscopy (ultraviolet (UV), near-infrared (NIR), mid-infrared (MIR), visible and Raman), isotopic analysis and chromatographic techniques [37]. However, as was already emphasized in previous sections of this document, the flavour composition of food commodities, including coffee, is attributed to the presence of volatile and

semivolatile organic compounds. These mixtures comprise a wide range of organic chemicals that are characterized by varying polarities and volatilities, usually occur in trace concentrations and likely are included in other complex organic and inorganic matrices [25]. The analysis of naturally volatile and semivolatile organic compounds in food samples is generally carried out by implementing gas chromatographic (GC) methods [55]. Since most aroma compounds are volatile and semivolatile, they are generally excellent candidates for GC analysis. The determination of volatile and semivolatile coffee constituents by utilizing GC methods has been extensively reported in the literature [28-29, 46-47, 49-50, 54, 59-72].

Another aspect for which GC methods are suitable for performance of food authenticity verifications is related to the convenient submission of GC data to multivariate statistical evaluations [37]. Multivariate analytical techniques (chemometrics) have been widely applied in industry, government and university-related centres over the past 30 years [31]. In order to deal with complex daily tasks, analytical chemists are faced with large data sets that are produced by modern multielement and multicomponent analytical apparatus, such as gas chromatography. The acquired results require the application of chemometric methods in order to be able to detect the essential influencing factors from many possible influences, to quantify their relationships and to extract the latent information from immense amounts of data. With respect to the objectives outlined within the current study, namely directed toward authenticity confirmation of complex food commodities such as coffee, it is important to emphasize that chemometrics has been successively applied to the following: *i)* determination of geographical origin, based on flavour and aromatic components, trace elements, etc. and *ii)* detection of frauds in case of product adulteration, contamination and substitution [35]. Furthermore, chemometric techniques are suitable to handle large number of objects (such as samples and cases) and variables (such as measurements and properties). Classification is one of the basic methodologies in chemometrics that utilizes the construction of a mathematical model capable of evaluating the membership of a sample to a particular class [36]. Once the classification model has been built, the membership of new unknown objects can be predicted based on their association with other previously characterized objects. Among the chemometric methods applied to the classification of

food commodities and food quality assessments, principal component analysis (PCA) is one of the most common classifying techniques. PCA reduces the dimensionality of a data set in which there is a large number of inter-correlated variables, while retaining as much as possible of the information present in the original data [31]. The data dimensionality is reduced to a smaller set of orthogonal factors of easier interpretation, which when plotted against each other demonstrate the clustering of individual samples on the basis of similarity among their data [47]. PCA has been successfully applied to GC data in order to discriminate coffees based on previously specified quality-influencing factors [46-47, 49-50, 54].

As it can be concluded from previous section on chemometric tools, these techniques are capable of handling large data sets that are produced by modern multielement and multicomponent analytical methodologies [31]. In particular, reliable PCA classifications can be established successfully for those circumstances involving a large number of objects (coffee samples in this case) and variables (volatile and semivolatile coffee constituents in this study). Statistical evaluation of data leads to the detection and deduction of those particular marker components, which are most responsible for the establishment of a classification model. Therefore, the reliable identification of volatile and semivolatile constituents in a particular food commodity is absolutely necessary as it informs analytical community on the identity of these marker constituents. This aspect leads to additional suitability of GC methods toward meeting the objectives of the current study.

Many procedures have been used as criteria for the identification of compounds resolved by gas chromatography, not all of which were entirely satisfactory [73]. Identification of an unknown component is a complicated problem, in spite of the great progress made in the theory and practice of chromatographic methods [74]. Briefly, the confirmation of analyte identity can be based on one or the combination of the following methods: *i)* injection of pure standards followed by retention time comparison; *ii)* comparison between experimentally obtained mass spectra and those available in mass spectral library and *iii)* utilization of retention index (RI) approach. The approach based on the injection of pure standards and subsequent retention time comparison is exclusively suitable for target study involving a reasonably acceptable number of

analytes (a number of analytes is in the range of 10-20). Since the objective of the current examination is directed toward nontarget screening of volatile and semivolatile coffee constituents, this approach is highly unsuitable, as the purchase of such a large number of standards is impractical. The analyte identification procedure based on the comparison between the experimental and library archive mass spectra is more practical; however this approach is insufficient when utilized alone. On the other hand, the concept of the retention index, introduced by Kovats in 1958, is the most reliable way of expressing chromatographic retention data. For this reason, it has firmly established itself in many techniques of column chromatography and a large volume of accepted values has been gathered in the literature for comparative purposes. This retention index system reports the retention behaviour of a compound relative to that of the *n*-paraffin hydrocarbons [73]. As it has been well documented in previously published works, the collection of GC retention indices represents an extremely useful tool for the confirmation of an analyte's identity in complex food matrices [75]. These indices are independent from all the experimental conditions, except the polarity of the GC stationary phase utilized in a particular study. For this reason, their utilization is fundamental for interlaboratory comparison. Furthermore, their use eliminates labour-intensive and time-consuming procedures associated with the identification performed based on the injection of pure compounds and corresponding retention time comparison [75]. When dealing with complex aroma of food and flavour matrices, it is quite common for a number of compounds to exhibit similar fragmentation patterns, in which case, the identification of compounds based on retention index system is crucial for the analyte identity conformation [76]. Due to the aforementioned advantages associated with the utilization of the retention index system and the fact that the mass selective detection was available, confirmation of analyte identity in the current study was established based on the combination of these two methods.

While the nontarget determination of aroma-contributing coffee constituents has been successfully achieved through implementation of gas chromatographic methods, recently the major objective of any GC method is the total separation of the most critical sample components in the minimum time [55]. Even though it is evidently preferable to deliver entirely resolved analytes to the detector system and employ 25-30 m capillary

columns to ensure satisfactory separations and meet these objectives, this approach is characterized by a substantial disadvantage: cost in analytical time. The entire resolution of all sample components in a typical simple-to-medium complexity food sample would generally require 0.5-1.5 hour long analysis times, the time cost which is impractical for laboratories with high daily sample throughput requirements. Provided that the most selective GC column for a specific application is being utilized, the most evident route toward reducing gas chromatographic run times is to increase the gas flow rate and/or the temperature program ramp. However, this option can only be implemented until the lowest degree of acceptable analyte separation is attained. For this reason, the members of the chromatographic community realized that the development and utilization of high-speed GC methods are crucial requirements to be met in order to increase the throughput of analytical procedures.

The primary aim of any fast GC technique is to maintain sufficient resolving power for the separation between the analytes of interest. The most commonly exploited approach allowing reduced GC run times, faster food analyses and subsequent high sample throughput characteristics involves the utilization of narrow-bore capillaries [55]. These capillary columns have substantially reduced internal diameter and stationary phase thickness. A decrease in a column internal diameter reduces resistance to mass transfer in the gaseous phase and hence column band broadening, which leads to an increase in resolving power. Nowadays, narrow-bore column fast GC work is mainly carried out with internal diameter and stationary phase thickness dimensions of approximately 0.1 mm and 0.1 μm, respectively, and this approach was also utilized in the current study.

At this point, it is worth emphasizing that the idea of utilizing narrow-bore capillary columns to increase analysis speed is not new. The advantages offered upon implementing such an approach have been demonstrated since the beginning of the 1960s [55]. However, the widespread employment of reduced-I.D. columns as a route toward faster separations was significantly delayed by the absence of appropriate GC systems. For this reason, the efficient exploitation of narrow-bore capillaries has become achievable exclusively in the last decade due to the introduction and development of the

following instrumental requirements: *i)* rapid oven heating/cooling cycle capabilities; *ii)* fast detectors and *iii)* fully automated rapid sample preparation and injection procedures.

As far as the choice of the temperature program or oven heating/cooling cycle capabilities is concerned, theoretical studies have shown that a rate of 10 ^{o}C per minute should be utilized in order to attain an optimum separation [55]. In those circumstances when this value is exceeded, satisfactory component resolution is sacrificed due to the insufficient period of time in which the volatiles and semivolatiles are partitioned in the stationary phase. On the contrary, if lower than optimum program rates are applied, resolution becomes unaltered while on the other hand elution times are significantly longer hence decreasing the speed of analysis.

Narrow-bore fast GC techniques generate, typically, very narrow analyte bands. Consequently, detector capabilities become very important as high-speed elution necessitates fast acquisition rates. The application of an insufficient acquisition rate initiates incorrect peak reconstruction and hence unreliable peak quantification. With regard to the choice of an appropriate detection system, it is important to emphasize that one of the most powerful tools for the determination and identification of organic compounds in complex samples is the combination of a chromatographic separation technique with mass spectrometric detection (GC-MS) [77]. The benefits offered upon utilizing mass spectrometric detection are especially crucial in those circumstances in which the nontarget qualitative profiling study is conducted, as is the case in the current document. The comparison between experimental and reference/library mass spectra is by no mistake one of the powerful techniques for the confirmation of analyte identity. The use of extracted ion traces allows reliable quantitation of even those peaks that are not fully separated. As far as the utilization of a mass detector in combination with narrow-bore column fast GC is concerned, this approach has often been reported in the literature and characterized by many advantages. Among different types of mass spectrometers that are available for coupling to GC systems, such as the sector instrument, the ion trap, the quadrupole and the time-of-flight (TOF), the latter offers numerous advantages and is most compatible for use in combination with fast gas chromatographic methods [55]. High-speed time-of-flight mass analyzer offers many advantages: *i)* wide mass acquisition range for which the upper mass value is dictated by

the boiling point temperatures of target analytes of interest and *ii)* very fast spectral data acquisition rates that form an essential requirement accompanied by the use of fast gas chromatographic techniques. The additional sophisticated features of the high-speed TOFMS instrument were thoroughly summarized by Setkova *et al*. in the project directed toward the classification of ice wine samples according to various quality-influencing attributes such as grape variety and geographical origin [26, 57]. The total GC run time was 5.7 min per ice wine sample; the length of chromatographic run time which falls into the category of fast gas chromatographic analyses. The large data set acquired in the previous ice wine characterization study was easily processed by utilizing the benefits of a fully automated background subtraction, baseline correction, peak find and mass spectral deconvolution algorithms. The ChromaTOF software provided automatic assignment of most unique and least coeluting quantification mass to a particular peak. Furthermore, the "compare to reference" feature incorporated within the ChromaTOF software allowed the comparison of the particular sample undergoing processing procedure to the preselected reference sample, such that the comparison results are sorted in the following fashion: "match," "out of tolerance," "contaminant" and "not found" [57]. For all the aforementioned aspects and unique features of this instrument, it is obvious that this experimental set-up was compatible to follow fast chromatographic analysis so that the GC separation is no longer the limiting step in food analysis. Furthermore, the full exploitation of benefits provided by the ChromaTOF software offered the possibility to distinguish between peaks with poor chromatographic resolution. An example of the performance of such a sophisticated procedure is illustrated in Figure 5. This illustration demonstrates that seven different compounds are possible to be assigned with peak apexes very close to each other under the 2.5 sec segment of the total ion current (TIC) chromatogram.

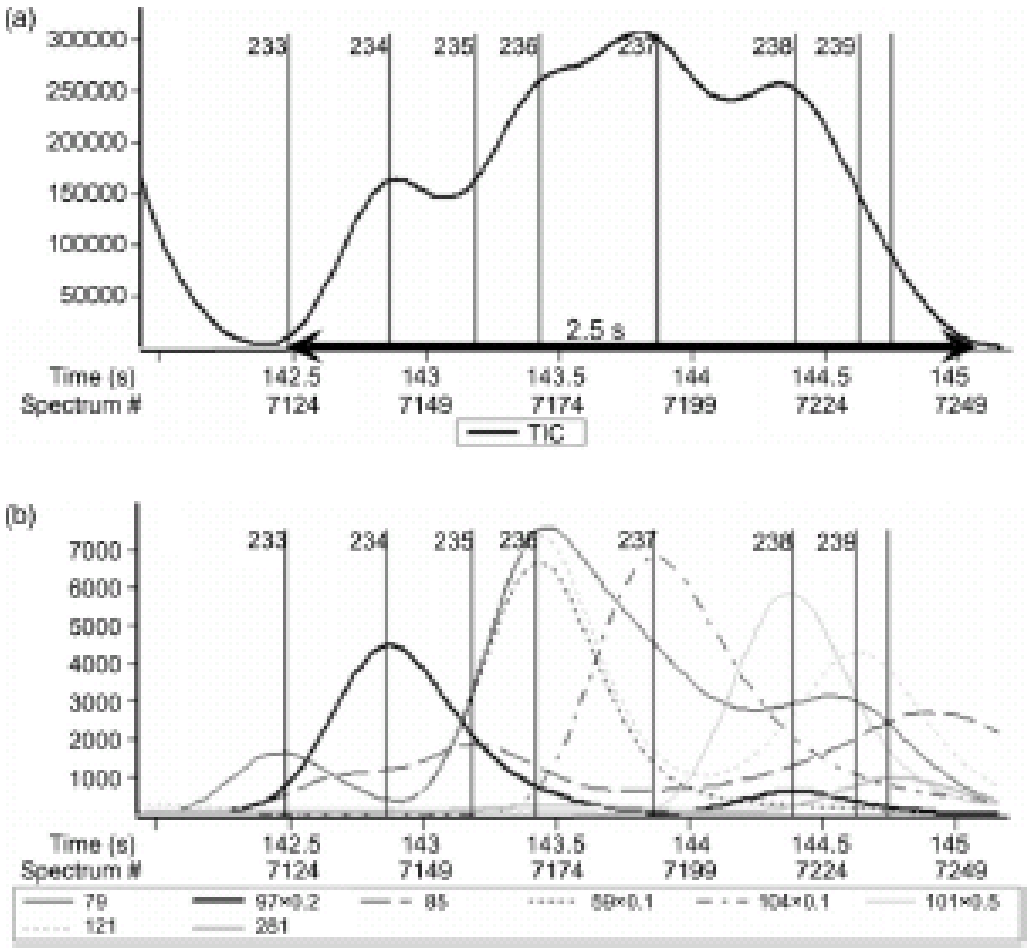

Figure 5. Utilization of automated peak find algorithm feature provided by the ChromaTOF software [57]. (a) total ion current (TIC) chromatogram; (b) automatic assignment of seven different compounds under TIC.

It must be emphasized, however, that some chromatographic separation is required for the deconvolution algorithm to recognize the presence of two or more compounds in one peak. The results published by Deursen *et al*. demonstrate this principle (refer to Figure 6) [77]. The illustration here shows the identification of the coeluting compounds octane and *cis*-1,4-dimethylcyclohexane. The deconvolution procedure was successfully initialized due to 10 msec (equivalent to 5 spectra at 500 spectra/sec data acquisition rate) separation between the apexes of coeluting compounds.

Accordingly, GC-TOFMS system with high-speed data acquisition rate option is perfectly suitable to be used in combination with fast chromatographic methods. This

instrumental equipment has been utilized for high-speed chromatographic analyses of complex matrices in previously published literature findings. In addition to the aforementioned analysis of ice wines, the sample matrices examined up to this point include mostly oil samples such as lemon, lime and orange oil samples [78-79]. To the best of this author's knowledge, no previously published documents focusing on the determination of volatile and semivolatile coffee constituents and subsequent assessment of coffee authenticity using high-speed GC-TOFMS system are available in literature.

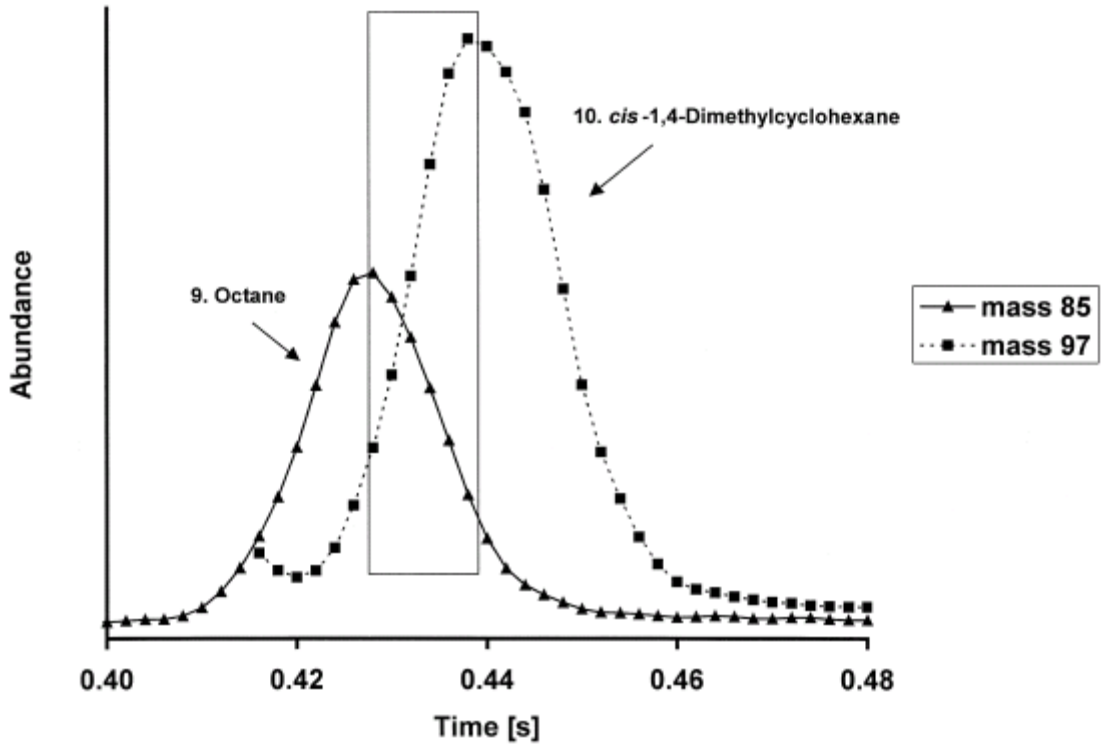

Figure 6. Deconvolution and "unique" mass assignment for octane and *cis*-1,4-dimethylcyclohexane [77].

It was already emphasized previously in this document that in addition to the absence of rapid oven temperature programming capabilities and fast detectors, another

reason limiting the utilization of high-speed chromatographic methods is the incompatibility of conventional sample preparation methods. At this point it is important to emphasize that the combination of any fast GC method with an extensively long and tedious sample preparation procedure has little or no practicality. A modern and very effective tendency is to combine automated sample preparation systems with GC instrumentation. Such an approach increases the throughput of analysis drastically as it allows the performance of simultaneous procedures associated with sample preparation and gas chromatographic analysis. In other words, the sample preparation of the next sample in sequence is carried out in parallel with the chromatographic analysis of the previously prepared sample. In these circumstances, the presence of an operator is not necessary, which initiates the possibility of completing overnight batch analyses. The next section of this document will therefore focus on the relevant discussion of sample preparation methods in food analysis.

1.5. Sample preparation methods in food analysis

As it was already emphasized in Section 1.4 of this document, the analytical procedure for complex sample matrices consists of several steps namely, sampling, sample preparation, separation, quantitation, statistical evaluation and decision making [56]. At this point, however, it is important to note that analytical steps follow one after another, and a subsequent step can not begin until the preceding one has been completed. For this reason, the statement that the overall speed of the analytical process is determined by the slowest step in this sequence is obvious.

There have been major breakthroughs in the development of improved instrumentation especially toward the hyphenation of different steps into one system. Such aspirations have been focused on the development of ideal instruments capable of performing all the analytical steps without human intervention. Today's sophisticated instruments, such as gas chromatograph-mass spectrometer (GC-MS) and liquid chromatograph-mass spectrometer (LC-MS) can perform such challenging operations by separating and quantifying target analytes from complex mixtures as well as applying chemometric methods to evaluate results statistically [56]. The previously mentioned

hyphenation of fast gas chromatographic separation with high-speed acquisition rate option time-of-flight mass analyzer represents yet another illustration of such sophisticated developments.

On the other hand, the hyphenation of sampling and sample preparation steps is not easily achievable, primarily because conventional sample preparation techniques utilize multi-step procedures and use of organic solvents. For this reason, the development of an automated method that integrates sampling and sample preparation with separation methods is greatly hindered and, as a consequence, over 80% of the analysis time is usually spent on conventional sampling and sample preparation procedures [56]. As already emphasized previously in this document, such an approach is highly unsuitable in the industrial sector as it drastically decreases the throughput of analysis and moreover is highly incompatible with the utilization of fast chromatographic techniques. Keeping in mind that more than 80% of the analysis time is spent on sampling and sample preparation steps, such as extraction, concentration, fractionation and isolation of analytes, the statement that the selection of an appropriate sample preparation method is the most critical step in the entire analytical process of the investigation of volatiles is not an exaggeration [1, 25].

Focusing on the sample preparation techniques from the food analysis perspective, it was already elaborated previously in this document that aroma composition of food commodities including coffee is attributed to a vast number of compounds present at variable concentrations. As far as the analysis of coffee constituents is concerned, it is worth emphasizing that the list of identified compounds in such a complex matrix is far from completion. In addition to the presence of a large number of compounds having varying physico-chemical properties, it is important to note that due to the relatively low concentrations of volatile and semivolatile materials that can affect the acceptance or rejection of a particular food commodity, an appropriate sample preparation procedure is normally required to extract and concentrate sample constituents of interest prior to instrumental analysis [25].

Sample preparation in food analysis is complicated by a number of factors such as: *i)* concentration level; *ii)* matrix effects; *iii)* complexities of aromas; *iv)* variation of volatility and *v)* instability [25]. The effect of the concentration level is attributed to the

fact that the levels of aroma-contributing compounds are generally low, typically in the ppm, ppb or ppt range. For this reason, the component isolation procedure alone is insufficient and it must be accompanied with the concentration of the analytes of interest by several orders of magnitude. The matrix effects in food analysis must be carefully considered and eliminated as they represent the competition between the interfering substances and analytes. Volatile compounds constituting the composition of a particular food commodity are frequently intracellular and must be liberated by disruption. As was previously emphasized in this document, food samples are frequently composed of nonvolatile components such as lipids, proteins or carbohydrates. The presence of these components leads to complications as far as the isolation of target analytes of interest is concerned. Complexity of food aroma, being another significant factor complicating sample preparation, is attributed to the presence of a vast number of compounds belonging to various chemical classes covering a wide range of polarities and solubilities. Coffee, for instance, has almost 800 identified components (refer to Table 1) [25]. Variation of volatility is induced by the wide range of volatilities of present components. The instability aspect of components comprising food composition rises from the fact that many aroma-influencing components are unstable and may undergo oxidation by air or degradation by heat or extreme pH levels.

Table 1. Classes of aroma compounds in coffee [25].

chemical class	*no of identified compounds*
hydrocarbons	74
alcohols	20
aldehydes	30
ketones	73
acids	25
esters	31
lactones	3
phenols (and ethers)	48
furans	127
thiophenes	26
pyrroles	71
oxazoles	35
thiazoles	27
pyridines	19
pyrazines	86
amines and miscellaneous nitrogen compounds	32
sulfur compounds	47
miscellaneous	17
total	**791**

In order to achieve a practical and reliable method for the analysis of complex food matrices, several sample preparation methods have been developed and previously utilized including direct injection of the sample, steam distillation, extraction with organic solvents and headspace methods [1, 25]. Direct injection of the sample is one of the most convenient techniques and it has been found to perform well for essential oils [25]. In certain circumstances, the sample is required to be diluted with a solvent prior to sample injection, such that the appropriate response within the detection limits can be achieved. In addition to essential oils, direct injection of the sample can also be utilized for concentrated aqueous samples. However, the aqueous phase may be injected only if the sample is sufficiently concentrated and even under these circumstances, certain complications may arise. First, upon conversion of water into steam, the volume increases dramatically (1 μL of water becomes more than 1000 μL of steam) to the level significantly larger than the injector volume of many current gas chromatographs. Furthermore, if the aqueous sample contains dissolved solutes such as carbohydrates or

proteins, additional problematic complications may arise upon injection of the sample. Accordingly, the nonvolatile components may decompose, leaving a nonvolatile residue in the injector and at the head of the column. One potential solution to this problem relies on the use of a guard column consisting of deactivated fused silica tubing between the injector and the analytical column. This tubing does not affect the separation characteristics or retention times of target analytes. As already emphasized previously, the direct injection of the sample is highly unsuitable when the aqueous phase is too dilute as the most modern measuring techniques are not sensitive enough to allow direct analysis of food constituents present at trace levels without the necessity of analyte isolation and preconcentration [13]. Therefore, alternative sample preparation methods may be considered.

Direct solvent extraction of aqueous samples can be performed when relatively large amounts of the aqueous samples are available. In such circumstances, separatory funnels or commercial liquid-liquid extractors may be utilized. The suitability of different organic solvents is an important factor to be considered when utilizing this particular approach of analyte isolation. In general, the following solvents have been most commonly utilized in food analysis: diethyl ether, diethyl ether/pentane mixtures, hydrocarbons, freons and methylene chloride [25]. Generally, diethyl ether and methylene chloride are good general purpose solvents. Ether can form explosive peroxides, and hence contains inhibitors that will show up in GC-MS analysis. Methylene chloride is on the other hand toxic and characterized as an animal carcinogen. After extraction, solvent concentration is usually attained by drying it over sodium sulfate or magnesium sulfate and concentrating it on a steam bath.

One of the most common sample preparation techniques in food analysis utilizes steam distillation followed by solvent extraction techniques. The distillation step separates the volatiles from the nonvolatiles. Some of the advantages of this approach include simplicity of operation, no need for complex apparatus, reproducibility, rapidity and the wide range of samples that can be handled by this set up. Distillation of food samples can be carried out directly, indirectly or under vacuum [25]. The advantages of indirect and vacuum steam distillations are associated with a lower degree of sample decomposition as the sample is not heated directly under these circumstances. As far as

the objectives of the current document are concerned, it is worth emphasizing that distillation techniques (steam and vacuum) have been widely used for the isolation and concentration of volatile constituents in coffee [80-81]. However, the utilization of high temperatures and organic solvents associated with the distillation leads to significant degree of artefact production and generation of the extracts misrepresentative of the original sample composition.

After the announcement of United States Environmental Protection Agency (EPA) regulation on the reduction and potential elimination of solvent use in residue analysis, headspace extraction techniques have become widely employed in many analytical chemistry fields including food analysis. It was already emphasized in one of the previous sections of this document that most aroma-influencing volatile and semivolatile food constituents may be solvent extracted, distilled and that in some rare cases the direct injection of the sample may be suitable as well. On the other hand, it is frequently preferable to take advantage of more prominent volatility characteristics of aroma-contributing compounds and hence rely on the techniques of headspace analysis.

Headspace sampling techniques are frequently divided into three broad categories: static headspace, dynamic headspace, and purge and trap. Each of these techniques is based on the same fundamental principle of sampling analytes from a solid or liquid material by investigation of the atmosphere adjacent to the sample [25]. As already emphasized previously, one of the benefits of utilizing headspace techniques is elimination of organic solvent use. Elimination of organic solvent use is particularly important from an environmental point of view as it does not necessitate further procedures of solvent disposal or solvent reduction by evaporation. Furthermore, once solvents are not employed to remove the analytes from the sample matrix, the resulting chromatogram has no solvent peak. This may be especially important when the target analytes of interest are early eluters, as the possibility of solvent peak masking the analyte peaks is eliminated.

Static headspace involves heating an aliquot of a liquid or solid sample in a sealed vial at a given temperature for a given time period and then injecting a small portion of the headspace above the sample matrix (usually about 1 mL) directly into the GC [78]. Besides the elimination of organic solvent use, the utilization of static headspace

sampling offers other advantages such as easy automation, simplicity of operation and reasonable cost per analysis. As far as the objectives of the current document are concerned, it is important to emphasize that static headspace sampling has also been utilized for the isolation of coffee aroma constituents [46, 59]. Maeztu *et al*. reported the utilization of an automated static headspace gas chromatography – mass spectrometry (SH-GC-MS) method for the analysis of various espresso samples followed by statistical evaluation to characterize different espresso blends [46]. In this particular method, a 6 mL portion of espresso drink was introduced into a 10 mL vial, equilibrated for 20 min at 60 ^{o}C after which 3 mL of coffee headspace was injected into GC injector port. Seventy seven volatile compounds were identified by headspace analysis of the espresso samples, among which thirteen had already been detected as key odourants in coffee. Furthermore, this particular static headspace sampling methodology combined with GC-MS allowed the reliable establishment of appropriate espresso sample statistical characterizations. In another study presented by Sanz *et al*., the effect of static headspace sampling conditions, namely temperature and time was examined so that it can be concluded that the optimum sample temperature was 90 ^{o}C, while the equilibration time was varying for different chemical classes of compounds [59]. In this particular study, 2 g portions of roasted ground coffee were transferred to 10 mL vials. The reported number of tentatively identified volatile compounds was 122, while this study was not directed toward the classification of coffee samples according to the quality-influencing attributes.

While the utilization of static headspace sampling offers numerous advantages as previously mentioned, it is also crucial to emphasize the drawbacks of this headspace sampling methodology. First of all, any static headspace analysis can inject only a fraction of the target analyte into the gas chromatograph, since the concentration in the headspace is in equilibrium with the one in the sample matrix and only a portion of the headspace is withdrawn and transferred for analysis. For this reason, static headspace is highly unsuitable and may lack sensitivity in those circumstances when the analyte concentration in the original sample is very low [25].

Dynamic headspace utilizes the movement of analytes away from the sample matrix in the headspace phase [25]. Instead of allowing the sample volatiles to establish

equilibrium between the sample matrix and the surrounding headspace, this technique involves continual sampling of the gas phase above the sample by flushing with an inert gas [82]. The atmosphere around the sample material is constantly swept away by a flow of carrier gas, which takes the volatile analytes with it leading to essentially quantitative removal of the analytes from the sample matrix. Due to the increased amount of volatile analytes in the headspace, the entire dynamic headspace sample is transferred to the gas chromatograph for a single analysis. This is accomplished by venting the carrier gas of the dynamic headspace through a collection trap, which retains the organic compounds while letting the carrier gas pass through. Hence, the analytes from a large headspace volume are concentrated in the trap and this technique is also referred to as purge and trap due to the sample purging with a flow of carrier followed by analyte trapping. The utilization of this sampling technique offers similar advantages to those available when using static headspace, namely, solvent use elimination, easily accomplishable automation and sample preparation simplicity. On the other hand, the introduction of a trapping stage and subsequent sorbent selection leads to increased sensitivity and selectivity characteristics.

Purge and trap has been previously employed in food component analysis and flavour profiling [82]. Purge and trap techniques have also been applied in the analysis of coffee aroma constituents. Costa Freitas *et al*. described the use of purge and trap combined with GC-MS for determination of volatile patterns in green and roasted *arabica* and *robusta* varieties [54]. In addition to the completion of geographical origin classifications (some of them are illustrated in Figure 4), this study was also directed toward the discriminations of coffee samples according to the bean variety and qualitative profiling of volatile and semivolatile green coffee constituents.

Even though dynamic headspace or purge and trap methods have been reported in many food analysis applications, it is important to emphasize potential drawbacks associated with these sampling methods. First of all, purge and trap instrumentation is complex and it requires careful monitoring of several sections such as valving and heating zones. For this reason, the purchase of such sampling and sample introduction material may be expensive. The utilization of this technique gives rise to many opportunities for malfunctions, including valve leaking and contamination and hence

numerous sources of errors including sample storage and purging efficiency may be introduced. For this reason, even though large concentration factors are possible, any interferants present in the sample are also concentrated, the reason for which the increased sensitivity for target analytes of interest may not be as significant [82]. Most important, purge and trap technique requires long preparation times due to the performance of purging and trap drying procedures.

As can be seen from the illustrations presented in the previous sections, the utilization of all the mentioned sampling techniques introduces various advantages and drawbacks. As was already emphasized previously in this document, complex food mixtures comprise a wide range of organic chemicals that possess varying volatilities and polarities, usually occur in trace concentrations and likely are included in other organic and inorganic sample interferences [25]. Fortunately, most aroma chemicals are volatile or semivolatile, and procedures for their isolation from foods have been established to take advantage of this volatility. Not so advantageous is the length of time usually required when utilizing the aforementioned conventional sample preparation techniques to obtain an isolate that is representative of the original aroma or flavour of the sample. The selection of steam distillation, solvent extraction, and purge and trap techniques might require several hours before the chemist can begin the chromatographic separation. The simple act of isolation may itself introduce artefacts from impurities in the solvent used, or through decomposition of the matrix or the aroma chemicals themselves. For this reason, no single sample preparation technique mentioned so far will prove optimal in every aspect of reliable sample preparation procedure. Many of these sampling methods require large sample volumes, the use of toxic organic solvents and necessitate long sample preparation times [1]. Furthermore, the automation of these labour-intensive techniques might be problematic giving rise to their incompatibility with fast chromatographic analysis whose importance was emphasized in one of the previous sections of this document. Solid phase microextraction (SPME) was developed to eliminate these drawbacks and for this reason the objective of the next section of this document is directed toward the introduction of this newly developed technique as well as the emphasis on its importance in food analysis.

1.5.1. Solid phase microextraction

Solid phase microextraction (SPME) is a fairly recent sample preparation technique developed by Pawliszyn *et al*. in 1989 in an attempt to address the need to facilitate rapid sample preparation both in the laboratory and on-site where the investigated system is located [83]. SPME has been used routinely in combination with GC and GC-MS, and successfully applied to a wide variety of compounds, especially for the extraction of volatile and semivolatile organic compounds from environmental, biological and food samples [1]. SPME was also introduced for direct coupling with high-performance liquid chromatography (HPLC) and liquid chromatography – mass spectrometry (LC-MS) in order to analyze weakly volatile or thermally labile compounds not amenable to GC or GC-MS. Briefly, the SPME process involves the performance of two basic steps: *i)* partitioning of analytes between the coating and the sample matrix and *ii)* desorption of concentrated extracts into an analytical instrument [84].

The SPME apparatus consists of a fiber holder and a fiber assembly, the latter containing a 1-2 cm long retractable SPME fiber [85]. The SPME fiber itself consists of a thin, fused-silica fiber coated with a thin polymeric coating (refer to Figure 7 for the illustration of commercial SPME device developed by Supelco). Now that a schematic illustration of commercially available SPME device has been provided, the principle of SPME will be emphasized again with reference to helpful graphical illustrations. First, the sample is placed in a vial, which is sealed with a septum-equipped cap. When the SPME needle pierces the septum of the vial and the fiber is extended through the needle into the sample, the target analytes immediately start partitioning from the sample matrix into the stationary phase. In other words, a small amount of extracting phase associated with a solid support is placed in contact with the sample matrix or its headspace for a predetermined amount of time. After a suitable extraction time, the fiber coating is withdrawn into the needle, the needle is removed through the septum of the sample-containing vial, and is then inserted directly into the injection port of the gas chromatograph. This basic principle of the SPME extraction/desorption cycle is illustrated in Figure 8 [1].

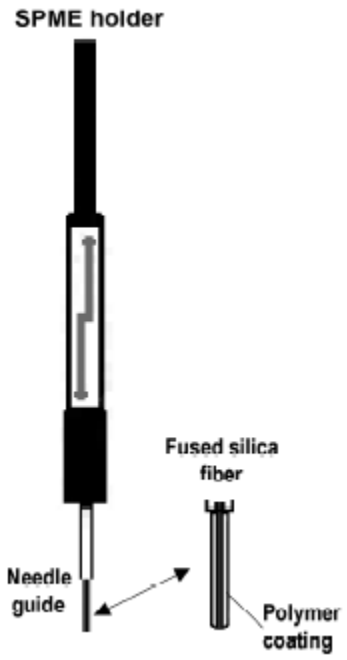

Figure 7. Schematic diagram of a commercial SPME device [86].

Figure 8 also demonstrates that depending on whether the polymeric coating is exposed directly to the sample matrix or to the headspace above the sample, the SPME extraction can be carried out in either direct or headspace configuration mode, respectively. The more detailed comparison between these two modes will be provided in later sections of this document. As can be seen in Figure 8, the extraction procedure is accompanied by parallel sample agitation utilized to enhance the mass transport from sample matrix to the fiber coating.

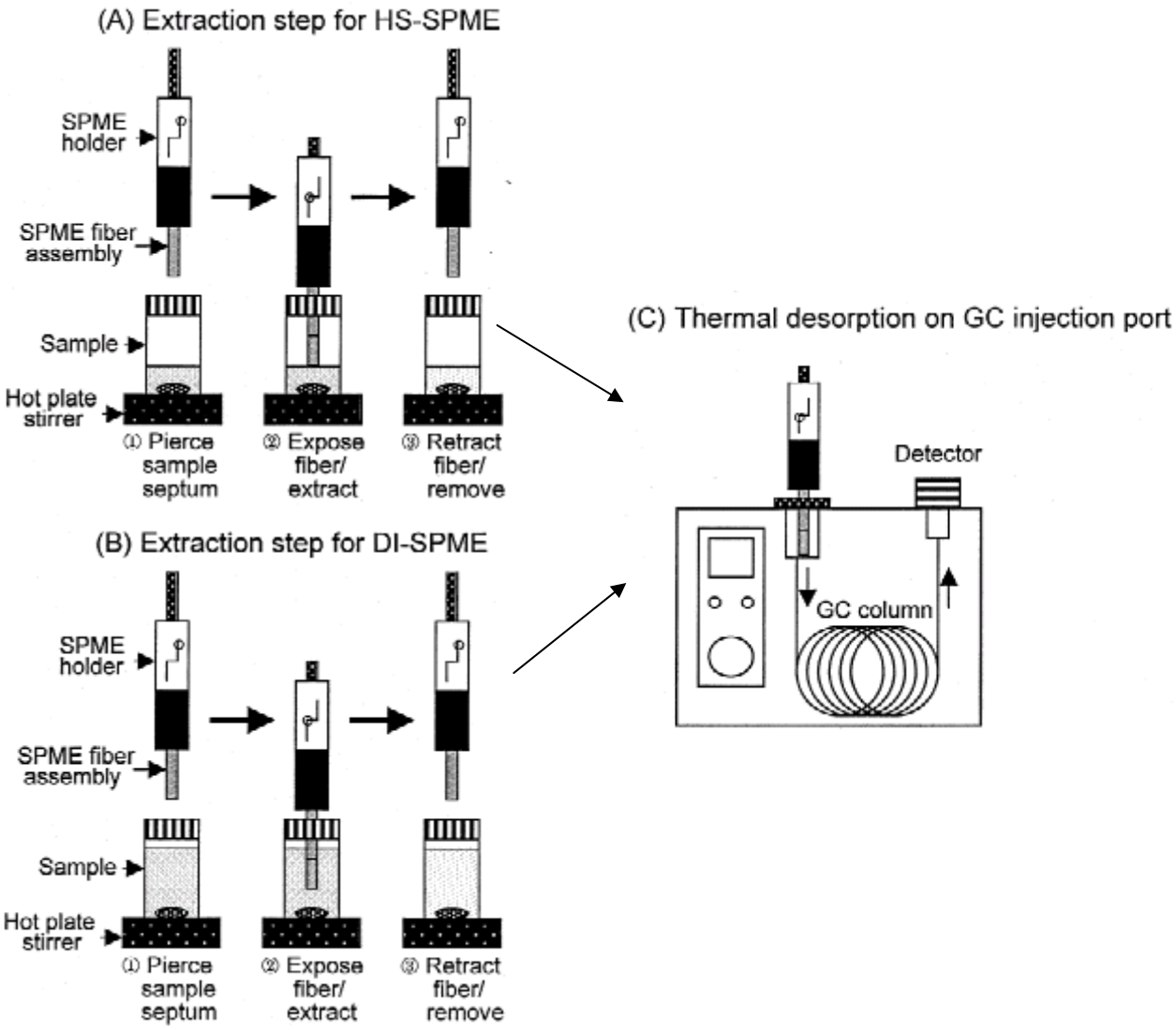

Figure 8. SPME extraction processes when utilizing headspace (A) and direct-immersion (B) modes of extraction. Thermal desorption into GC injector port illustrated in (C) [1].

As already emphasized previously, the transport of analytes from the matrix into the coating begins as soon as the coated fiber has been placed in contact with the sample. This concept is illustrated in Figure 9 which will also be a good reference for some of other SPME principles.

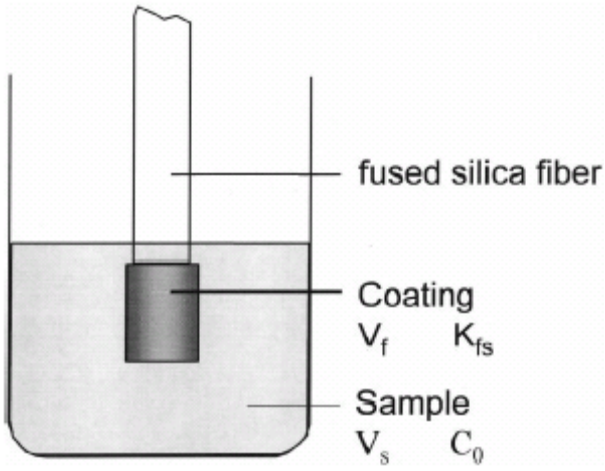

Figure 9. Microextraction with SPME. V_f, volume of fiber coating; K_{fs}, fiber coating/sample matrix distribution constant; V_s, sample volume; C_o, initial concentration of analyte in the sample [83].

Typically, SPME extraction is considered to be complete when the analyte concentration has reached distribution equilibrium between the sample matrix and the fiber coating [84]. In practice, this statement implies that once equilibrium is reached, the amount of analyte extracted by the polymeric coating is constant within the limits of experimental error and it is independent of further increase in extraction time. For this reason, when utilizing SPME as an extraction technique, maximum sensitivity is obtained under equilibrium conditions. However, the extraction can be interrupted prior to reaching an equilibrium state, in which case, constant timing and agitation conditions are strictly necessary.

The equilibrium conditions are described by the following equation:

$$n = \frac{K_{fs} V_f V_s C_o}{K_{fs} V_f + V_s} \qquad \text{(Equation 1)}$$

where n is the amount extracted by the coating, K_{fs} is a fiber coating/sample matrix distribution constant, V_f is the fiber coating volume, V_s is the sample volume, and C_o is the initial concentration of a given analyte in the sample [84].

The most dramatic advantages of SPME exist at the extremes of sample volumes. Since the SPME set-up is small and convenient, coated fibers can be used to extract analytes from very small samples. It is very difficult to utilize small sample volumes by using conventional sample preparation techniques. This is particularly important for food analysis applications involving limited sample sizes. Furthermore, the fact that SPME is an equilibrium technique and does not extract target analytes exhaustively enables its introduction in a living system without significant disturbance effects. Another advantage associated with sample volume use which is offered upon utilizing SPME leads to simplification of Equation 1. Namely, when utilizing large sample volumes, the resulting relationship $K_{fs} V_f << V_s$ leads to the following simplification for the amount of analyte extracted:

$$n = K_{fs} V_f C_o \qquad \text{(Equation 2)}$$

This approach emphasizes the suitability of the SPME technique for field applications, as the amount of extracted analyte is independent of the volume of the sample [83]. In practice, this relationship enables the fiber direct exposure to the ambient air, water or production stream, and there is no need to collect a defined sample prior to analysis.

Equations 1 and 2 indicate that the efficiency of the extraction process is dependent on the fiber coating/sample matrix distribution constant. This is a characteristic parameter that describes properties of a coating and its selectivity toward the analyte of interest versus other matrix components [84]. Equations 1 and 2 also demonstrate that coating volume is another parameter affecting the sensitivity of the SPME method. However, the use of thicker coatings to compensate for this effect will also result in longer equilibration times. The following SPME fiber coatings of varying thicknesses, lengths and polarity characteristics are commercially available up to this date: *i)* polydimethylsiloxane (PDMS, available in 100, 30 and 7 μm coating

thicknesses); *ii)* polyacrylate (PA); *iii)* polydimethylsiloxane/divinylbenzene (PDMS/DVB); *iv)* carboxen/polydimethylsiloxane (CAR/PDMS);
v) divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS);
vi) carbowax/divinylbenzene (CW/DVB). These polymeric coatings can further be categorized into liquid and solid ones. The performance characteristics and extraction mechanisms of liquid and solid coatings are substantially different [56]. With liquid coatings, the analytes partition into the extraction phase, in which the molecules are solvated by the coating molecules. For this reason, the liquid coatings extract the target analytes of interest via the process of absorption. The diffusion coefficient in the liquid coating enables the molecules to penetrate the whole volume of the coating within a reasonable extraction time. On the other hand, solid or mixed-phase coatings consist of porous solid particles imbedded into a PDMS or CW layer [87]. In the case of solid sorbents, the coating has a glassy or well defined crystalline structure, which if dense, substantially reduces the diffusion coefficients within the coating structure [83]. Within the time span of the experiment, therefore, sorption occurs only on the porous surface of the coating. During extraction by use of a solid phase, after long extraction times, compounds with poor affinity toward the extracting phase are frequently displaced by analytes characterized by stronger binding, or those present in the sample at high concentrations [56]. The reason for the existence of such an effect is caused by a limited surface area available for adsorption. If this area is substantially occupied, competition or displacement effects take place, and the equilibrium amount extracted can vary with the concentrations of both the target and other analytes. Therefore, one substantial disadvantage of adsorption extraction is the existence of nonlinear isotherms when the surface coverage is substantial. In the case of extraction of analytes with liquid coatings, partitioning between the sample matrix and extraction phase occurs. Therefore, the principle advantage of absorption extraction is a linear isotherm over a wide range of analyte and interference concentrations. Under these conditions, equilibrium extraction amounts vary only if the bulk coating properties are modified by the extracted components [83]. This occurs only when the amount extracted is a substantial portion of the extraction phase, resulting in a possible source of nonlinearity. This situation is rarely

encountered, because SPME extraction is typically used to determine analytes present at trace levels [56].

As was already emphasized previously in this document, SPME extraction can be carried out by utilizing either direct-immersion (DI-SPME) or headspace (HS-SPME) extraction modes [1]. In the direct-immersion extraction mode, the coated fiber is inserted into the sample and the analytes are transported directly from the sample matrix to the extracting phase [83]. To facilitate rapid extraction, some level of agitation is required to transport analytes from the bulk of the solution to the vicinity of the fiber [84]. For aqueous matrices, this can be accomplished by stirring, sonication and rapid fiber or vial movements. These conditions are necessary to reduce the effect caused by the "depletion zone" produced close to the fiber as a result of fluid shielding and slow diffusion coefficients of analytes in liquid matrices. When utilizing headspace mode of extraction, the analytes need to be transported through the barrier of air before they can reach the coating. This modification serves primarily to protect fiber coating from damage by high molecular weight and other nonvolatile interferences present in the sample matrix. The headspace mode of extraction also allows the performance of matrix modifications such as pH adjustment and salt addition without damaging the fiber. Amounts of analyte extracted into the coating from the same vial at equilibrium using direct and headspace SPME sampling modes are identical as long as sample and gaseous headspace volumes are the same. This is caused by the fact that the equilibrium concentration is independent of fiber location in the sample/headspace system.

Another important advantageous SPME aspect is the ease of automation of sample preparation steps corresponding to this technique. As was already elaborated in Section 1.4 of this document, the utilization of high-speed chromatographic methods must be accompanied by fully automated rapid sample preparation and injection procedures. Most conventional sample preparation methods demonstrate either weak compatibility or no compatibility at all to be automated and integrated with separation methods. On the other hand, due to the substantial similarity between SPME fiber and liquid injection syringe, all steps within the SPME procedure conducted prior to GC analysis can be fully automated [56]. In addition to fully automated steps toward the generation of concentrated extracts, the rapid sample introduction by means of desorbing

the fiber into the GC injector port is fully automated as well. One of the highly sophisticated, fully developed and most commonly used SPME-compatible autosamplers for GC was introduced by CTC Analytics in 1999 [83]. This is a robotic system with a great deal of flexibility for programming SPME analyses (refer to Figure 10). Samples are loaded onto trays accommodating different vial sizes, and samples are heated and agitated in a separate sample preparation chamber during the incubation and extraction processes. To facilitate agitation, the sample preparation chamber or agitator tray is rotated at a programmable rotation speed during incubation and extraction procedures. The fiber conditioning station is also included in this package, thus allowing "bake-out" or fiber cleaning procedures to be performed after each extraction/desorption cycle outside of the injection port.

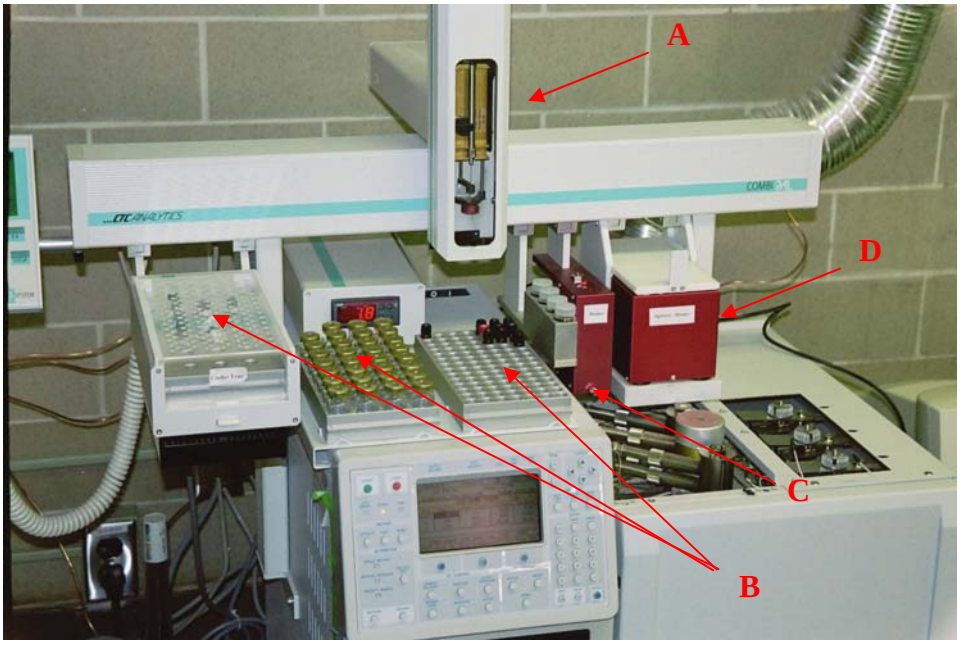

Figure 10. Commercial SPME-GC autosampler (CTC Analytics CombiPAL): A – sample preparation/injection arm; B – sample trays; C – needle heater; D – agitator tray

As it can be seen from the aforementioned illustrations, the development of SPME has addressed the need for a rapid sample preparation technique, as the following procedures are integrated into a single solvent-free step: *i)* sampling; *ii)* extraction; *iii)* concentration and *iv)* sample introduction. The following advantages are offered upon utilizing SPME as a sample preparation method: short sample preparation times; small sample volumes; analyte concentration from liquid, gaseous and solid samples; solvent-free extraction technique; and easily automated to allow high-throughput analysis.

With regard to the objectives of the current document, which are directed toward semiquantitative determination of volatiles and semivolatiles in complex food matrices, namely coffee and utilizing an appropriate sample preparation technique for these purposes, it is important to emphasize that SPME is perfectly suitable to meet these objectives. For all the advantages offered upon utilizing this technique, it is obvious that SPME represents an ideal approach to aroma isolation since it provides an extract whose composition is representative of the original sample mixture and that is free of solvents and other impurities. Furthermore, in most cases, the SPME sample preparation does not require previous sample treatment and can be completed within a few minutes, which makes it ideally appropriate for utilization in combination with high-throughput fast gas chromatographic techniques. Furthermore, the easiness of SPME automation and generation of rapid injection autosampling devices demonstrate SPME applicability in high-speed analyses as in this way sharper injection zones and faster separation times are facilitated. For these reasons, the number of SPME applications in food analysis is rapidly increasing [88]. Briefly, this technique has been applied to study aroma profiles and various contaminants/off-flavours in dairy products such as cheese, milk and butter; meat products; fruits and vegetables; olive oils; honey; alcoholic beverages and cereals [6-8, 10-12, 14-22, 26-27, 56-57, 89]. SPME has also been successfully utilized for the extraction of volatile and semivolatile coffee aroma constituents, which was followed in certain cases by performance of discriminating statistical data evaluation studies [28-29, 47-50, 61, 62-70].

1.5.1.1. Stages in SPME method development

Several steps must be properly followed when developing an SPME method, including selection of the fiber coating, selection of the sampling mode, optimization of agitation conditions, selection of the sample volume and optimization of the extraction and desorption conditions [56]. The following section will provide the discussion of the most significant method development steps and those procedures that were associated within the scope of the current study.

Selection of the fiber coating

Selection of a suitable fiber coating is the first step in SPME method development. As was already deduced from Equations 1 and 2, the efficiency of the extraction process is dependent on the fiber coating/sample matrix distribution constant. This is a characteristic parameter that describes the properties of a coating and its selectivity toward the analyte of interest versus other matrix components. As in the case of chromatographic column stationary phases, fiber suitability for the specific compounds of interest is determined by coating polarity [56]. For this reason, the affinity of the fiber for an analyte depends on the principle of 'like dissolves like' [1]. Single-phase or absorption-based coatings such as nonpolar polydimethylsiloxane (PDMS) and polar polyacrylate (PA) provide high capacity for the extraction of apolar (such as many volatile flavour compounds) and polar (such as phenols and alcohols) compounds, respectively [1,85]. Mixed-phase or adsorption-based coatings as listed in Section 1.5.1 are suitable for the extraction of volatile low-molecular mass and polar analytes [1]. These coatings provide satisfactory selectivity towards a wide range of analytes that have different physico-chemical properties. In addition, as opposed to the principle of extraction employed with absorption fibers, diffusion of the analytes into the adsorption fiber coating does not occur. For this reason, one advantage offered upon utilizing adsorption-based coatings is that the typical extraction times are significantly shorter as compared with absorption-based fibers. Nevertheless, adsorption fibers have a smaller linear dynamic range and displacement and carryover effects are likely to occur [56].

Selection of the sampling mode

As already emphasized previously in this document, SPME extraction can be carried out by utilizing either direct-immersion (DI-SPME) or headspace (HS-SPME) extraction modes [1]. Both DI and HS sampling modes of SPME are used extensively in SPME-GC applications [56]. While DI-SPME is more suitable for gaseous or simple liquid sample matrices, HS-SPME is preferentially used for extraction from complex liquid and solid samples. HS extraction is more suitable for analytes of high to medium volatility and low to medium polarity. The opposite is true for DI extraction, as it should be utilized for extraction of compounds having low to medium volatility and high to medium polarity. Since the analyte affinity for the matrix also plays an important role, HS extraction might become more complicated for some analytes in dirty samples. In such cases, matrix modifications, such as pH adjustment or salt addition, should be conducted in order to improve transfer of the target compounds from the matrix to the headspace above the sample. Furthermore, in those circumstances when the analytes are extracted from complex matrices, two potential complications may arise [90]. One is associated with competition among different phases for the analyte and the other with the 'fouling' of the extraction phase, due to the adsorption of macromolecules such as proteins and humic materials at the interface. This is especially important due to the well known and already mentioned (refer to Section 1.5) matrix interferences associated with the composition of food samples. The typical approach used to reduce 'fouling' of the extraction phase involves introduction of a barrier between the sample matrix and the extraction phase so that the transport of high molecular weight interferences is restricted. This barrier may be in the form of a porous membrane having pores smaller than the size of the interfering macromolecules (refer to Figure 11a) [90]. A gap made of gas is also a very effective separation barrier since the analytes must be transported through the gaseous barrier to reach the coating, thus resulting in exclusion of nonvolatile components of the matrix (refer to Figure 11b). This approach is practically implemented by placing the extraction phase in the headspace above the sample or by utilizing HS-SPME. For this reason, for very dirty samples, only HS mode should be used to ensure protection of the fiber coating from damage by high molecular weight and other nonvolatile compounds present in the sample matrix.

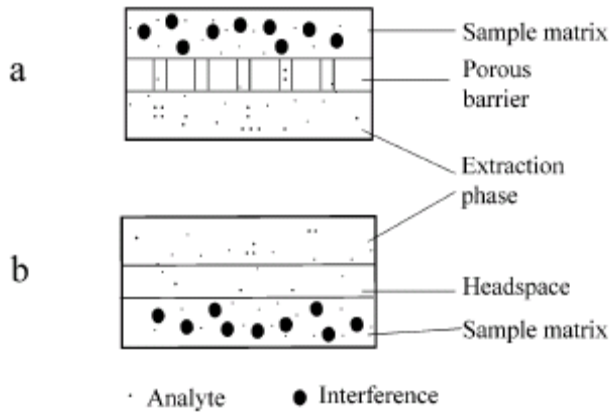

Figure 11. Extraction using selective barrier approaches based on size exclusion with a porous membrane (a) and introduction of a headspace gap (b) [90].

Optimization of agitation conditions

Agitation of the sample assists the mass transport between the sample and the fiber coating [56, 84]. The time required to reach equilibrium can be reduced by using an agitation method. The more effective the stirring, the shorter the extraction times required to achieve equilibrium or enhance sensitivity in pre-equilibrium conditions. This agitation effect and its influence on the extraction time profile obtained for polycyclic aromatic hydrocarbons (PAHs) are illustrated in Figure 12. By examining these two graphs, it is obvious that as the rotational speed of the magnetic stirrer increases, the time required to reach equilibration state decreases from 8 min to 3 min and from 25 min to 10 min for more volatile analytes, naphthalene and acenaphthene, respectively [83]. For less volatile analytes, phenanthrene and chrysene, the equilibrium is not reached during the examined time profile in either case. However, it is obvious that the increased agitation efficiency in this case resulted in increased sensitivity under nonequilibrium conditions since the amount of analyte extracted after 70 min at low agitation (refer to Figure 12 a) is approximately the same as the amount extracted after 45 min at higher level agitation.

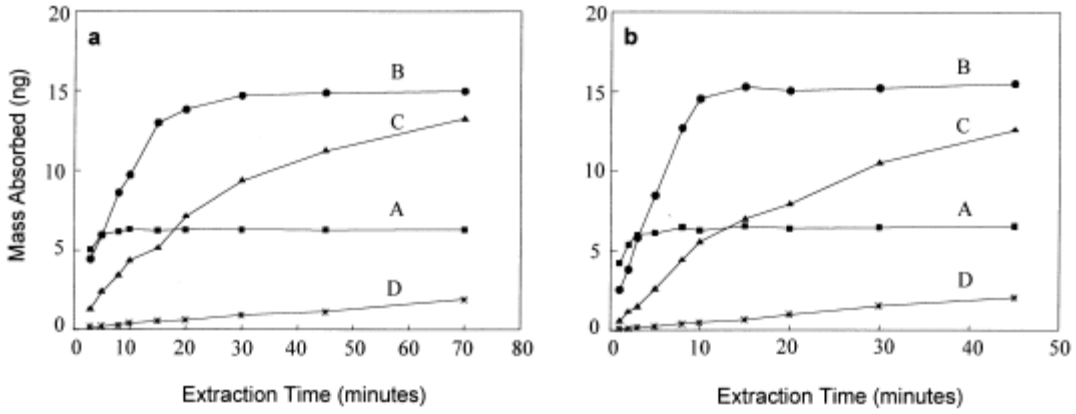

Figure 12. Time extraction profiles obtained for HS-SPME of several PAHs from aqueous samples at (a) 75% and (b) 100% stirring rates. A – naphthalene; B – acenaphthene; C – phenanthrene; D - chrusene [83].

Selection of the sample volume

The sensitivity of the method is directly proportional to the number of moles (n) extracted from the analyzed sample at equilibrium [56]. As the sample volume (V_s) increases, so does the amount of analyte extracted, until the volume of the sample becomes significantly larger than the product of the distribution constant and coating volume (K_{fs} V_f) [84]. At this point, the K_{fs} $V_f << V_s$ relationship predominates resulting in the amount of analyte extracted being independent of any further increase in sample volume. In practice however, the experimental arrangement, namely the size of the sample is often limited by sample vial sizes or by the availability of the sample volume. In the case of laboratory experiments in which the size of the sample vial is usually dictated by the autosampler characteristics, the size of the sample volume typically influences the amount of analyte extracted. On the other hand, in those applications utilizing SPME for in-field applications, the amount of analyte extracted is independent of V_s, as in these cases the analyst is dealing with extremely large sample volumes. When dealing with complex multiphase systems typically encountered in HS-SPME mode of extraction, the situation is more complex since the analytes partition to the

gaseous phase as well as to the coating. Under these circumstances, volatile analytes prefer to accumulate in the headspace, resulting in a substantial loss of sensitivity when the headspace volume is very large. For this reason, the volume of the gaseous phase should be minimized for high sensitivity headspace extraction [84].

Optimization of extraction conditions

In most studies, sample extraction represents the time-limiting step of the SPME procedure since this process is usually more time consuming than sample incubation, analyte desorption or fiber bake-out [56]. Therefore, selection of the optimum extraction time is one of the most crucial steps in SPME method development. One of the most popular SPME approaches involves reaching a partitioning equilibrium between the sample matrix and extraction phase [83]. The equilibration time is defined as the time after which the amount of extracted analyte remains constant and corresponds within experimental error to the amount extracted at infinite extraction time [84]. Even though, equilibrium extraction provides the highest sensitivity, extraction time selection is always a compromise between the length, sensitivity and repeatability of the analytical method. Both equilibrium and pre-equilibrium extractions need precise and perfectly repeatable timing, although for the latter procedures, timing is more critical. At this point, it is important to emphasize that perfectly repeatable timing is guaranteed by using an autosampler. In the case of pre-equilibrium extraction, the longer the extraction times and the less steep the extraction profile curve slope, the smaller the relative errors that occur [56].

Extraction temperature or the temperature of the sample during the extraction is another parameter that needs to be carefully considered when optimizing SPME methods. Two opposite effects are taking place if the extraction temperature is raised [56]. The positive effect of increasing the extraction temperature is derived from the kinetic viewpoint. Therefore, an increase in temperature during the SPME extraction process results in increased headspace capacity and/or analyte diffusion coefficient, which leads to increase in the rate of extraction or rate of mass transfer into the fiber [84]. Consequently, increasing the extraction temperature reduces the equilibration time and makes the overall SPME procedure faster [56]. On the other hand, according to

thermodynamic theory, an increase in extraction temperature results in decreased analyte distribution constant between the sample matrix and the fiber coating, which decreases analyte recovery at equilibrium and method sensitivity [83]. This effect is illustrated in Figure 13 [83]. As shown in Figure 13, the lowest extraction temperature produces a very long equilibration time, but ultimately the highest amount extracted. On the other hand, the highest extraction temperature tested produces a very fast equilibration time but the lower equilibrium amount extracted.

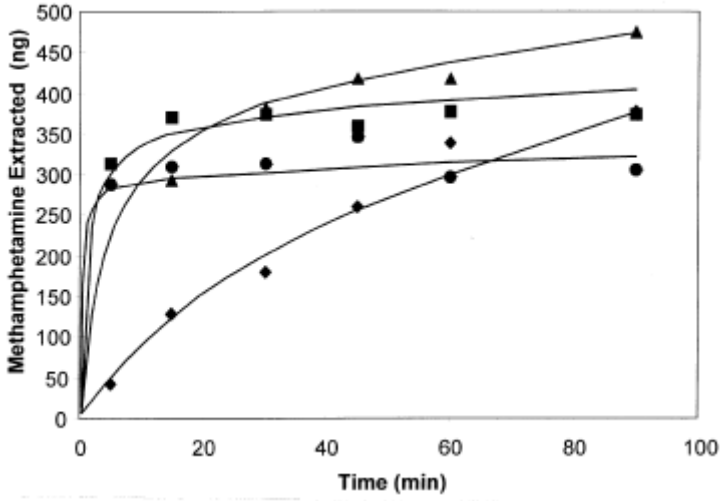

Figure 13. Effect of extraction temperature on equilibrium time and amount extracted for methamphetamine analysis [83]. Key: (♦) 22 ^{o}C; (▲) 40 ^{o}C; (■) 60 ^{o}C and (●) 73 ^{o}C

Optimization of desorption conditions

There are several major factors influencing the time required to desorb analytes of interest and allow their transfer onto the front of the GC column. In the case of thermal desorption into the GC inlet, these include carrier gas flow rate defined by the geometry of the injector or injector insert and injector temperature. The conventional solvent injections require large-volume inserts/liners to accommodate the expansion of the evaporated solvent and split valve opening to ensure the removal of remaining solvent vapours [84]. On the other hand, SPME procedure does not initiate the solvent

introduction and, therefore, the activated split flow is unnecessary. In fact, for optimum sensitivity, the split flow needs to be closed during desorption so that all analytes can be transferred onto the front of the GC column. Another important point in this aspect involves the necessary rapid removal of desorbed analytes from the injector. This is accomplished by generating high liner flow rates of the carrier gas around the coating and hence reducing the diameter of the injector insert to achieve this requirement. For this reason, narrow bore GC injector inserts are highly recommended for SPME use. When emphasizing the injector/desorption temperature effects, it is important to clarify that the analyte desorption is ensured since the gas-coating distribution constant decreases as the injector temperature increases [56].

1.5.1.2. Univariate and multivariate optimization designs

The systematic method development approaches discussed so far are all traditional univariate approaches. The traditionally utilized univariate optimization design involves the performance of "one factor at a time" experiments, thus offering the possibility to examine the effect of one variable at a time since all other variables are kept constant during a particular optimization experiment, except the one being studied [91]. For this reason, this traditional method might overlook the possibility of potential variable interaction, the issue which arises when the variables kept constant in previously performed optimization experiments change. In the presence of such interactions, a true optimal value for the two interacting variables is unlikely to be attained using a univariate optimization approach [56]. Consequently, after the completion of "univariate" SPME method development, some SPME parameters might require re-examination followed by repetition of corresponding optimization experiments [84]. This is particularly required after performing matrix modifications such as salt addition, pH adjustment and sample temperature optimization to the value which was not utilized during previous method development steps. In those circumstances, the analyst is strongly recommended to make sure that for instance, previously selected fiber coating is compatible with the new matrix or that the extraction time profile has not been altered at some point during the optimization process. As a result of these disadvantageous requirements, which in particular cases necessitate the performance of labour intensive procedures and in order

to obtain truly optimal method performance while performing a minimum number of optimization experiments, the utilization of multivariate optimization designs has emerged [56].

Multivariate designs offer the simultaneous variation of several control variables, consequently reducing the number of experiments to be performed in the SPME optimization procedure [92]. Furthermore, multivariate designs offer the possibility of distinguishing those interactions among variables that would not be detectable by classical experimental design [93]. Multivariate optimization is typically performed in two stages [56]. The first step of such optimization procedure most commonly involves the utilization of first order two-level full factorial designs in order to assess the importance of each variable on the effectiveness of particular analytical procedure. In addition to detecting the highly influential factors, this preliminary step is also capable of identifying the potential interactions between variables. The second step of multivariate optimization procedure involves the performance of second order designs to locate the optimum set of conditions for those factors which were previously identified as significant based on first-order design. Among various second order designs available to meet the aforementioned objectives, Doehlert design which was proposed by Doehlert in 1970 is by far most useful [92]. Doehlert designs are easily applied to variable optimization. Most commonly they are utilized in conjunction with a response surface methodology to yield the true optimum values for the influential factors [56]. Doehlert optimization of two variables involves the construction of the Doehlert matrix, which consists of one central point and six points forming a regular hexagon. Consequently, the number of variables to be optimized does not equate the number of levels at which the variables are examined. For instance, in a two-variable Doehlert design, one variable is studied at five levels, while the other is studied at only three levels. The general and widely accepted rule is to choose the variable with the stronger effect as the factor with five levels [92].

In summary, the use of multivariate optimization design methods combining first-order (to detect influential variables) and second-order (to optimize influential variables) experimental designs has become increasingly popular due to all the advantageous aspects emphasized above. For this reason, the multivariate design combining two-level

full factorial and Doehlert matrix design accompanied by response surface methodology for first-order and second-order designs, respectively, was employed in the current study as well. To the best of this author's knowledge, there are no published literature findings on the utilization of this combination of multivariate optimization designs for SPME method development associated with the extraction of volatile and semivolatile coffee aroma constituents.

2.0. Experimental

2.1. Samples

The coffee samples considered for geographical origin discrimination were collected from: *i)* producing regions such as Brazil and Colombia and *ii)* Canada (where the geographical specifications were acquired from coffee distributing markets). The total number of samples submitted to geographical origin classification is summarized in the following way: *i)* production area, Brazil - 11 samples; *ii)* production area, Colombia - 8 samples; *iii)* Canada collection comprised of South American samples (Brazil - 2 samples and Colombia - 12 samples), Central American samples (Costa Rica - 3 samples and Guatemala - 4 samples), African samples (Ethiopia - 3 samples) and Asian samples (Indonesia - 4 samples).

2.2. Analytical reagents and supplies

The alkane mixtures containing C_8 to C_{20} and C_{21} to C_{40} straight-chain alkanes of 40 mg/L concentration in hexane and toluene, respectively, were purchased from Fluka (Buchs, Switzerland). Helium of purity 5.0 (Praxair Canada, Mississauga, Ontario, Canada) was utilized as the GC carrier gas. The SPME fiber optimization step within the SPME method development was carried out by testing commercially available silica SPME fibers obtained from Supelco (Bellefonte, PA, USA) and coated with the following polymers: polydimethylsiloxane (PDMS, 100 μm and PDMS, 30 μm), polydimethylsiloxane/divinylbenzene (PDMS/DVB, 65 μm), divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS, 50/30 μm), carboxen/polydimethylsiloxane (CAR/PDMS, 75 μm), polyacrylate (PA, 85 μm), and carbowax/divinylbenzene (CW/DVB, 70 μm). All fibers were conditioned according to the manufacturer's recommendations prior to their first use. Clear glass crimp cap 10 mL SPME vials (22 x 46 mm) and caps equipped with polytetrafluoroethylene (PTFE)/silicone septa (20 mm) were purchased from MicroLiter Analytical Supplies, Inc. (Suwanee, GA, USA) and Canadian Life Science (Peterborough, Ontario, Canada), respectively.

2.3. Equipment

A CombiPAL SPME autosampler (Leap Technologies, Carrboro, NS, USA) equipped with an agitator and fiber conditioning station was utilized to allow for a completely automated SPME procedure. The autosampler was employed in combination with a gas chromatograph 6890 (Agilent Technologies, Palo Alto, CA, USA) coupled to a Pegasus III mass spectrometer (LECO, St. Joseph, MI, USA). The mass spectrometer was equipped with a time-of-flight (high-speed data acquisition rate option) mass analyzer. The separation of volatile and semivolatile compounds was achieved by utilizing the 5% diphenyl/95% dimethylpolysiloxane SLB-5 column (10 m length, internal diameter 180 μm, 0.18 μm film thickness) purchased from Supelco (Bellefonte, PA, USA). A high-pressure Merlin Microseal septumless injection kit (Merlin Instrument Co., Half Moon Bay, CA, USA) was obtained from Chromatographic Specialties (Canada). After the completion of SPME fiber optimization experiments, the 23-gauge needle size super elastic SPME fiber coated with best suitable polymer was used in combination with this septumless injector for the completion of overall sequence of coffee samples.

2.4. Experimental conditions

The fiber optimization experiment was accomplished by testing the efficiency of commercially available fiber coatings listed in Section 2.2. For this particular optimization experiment, the samples were prepared by transferring 1-g portions of ground coffee (Van Houtte, 100 % Colombian dark roast coffee) obtained in grocery supermarket to 10-mL vials. The incubation and extraction procedures were completed by utilizing sample temperature and agitation speed of 60 ^{o}C and 500 rpm, respectively. The employed incubation and extraction times were 10 min and 20 min, respectively, whereas the thermal desorption of analytes in the GC injector port was enabled for 1.5 min at 250 ^{o}C. After each extraction-desorption cycle, the fiber was cleaned in the fiber conditioning station for 5 min. The desorption optimization experiments were carried out by utilizing the same conditions, with exception of desorption time and temperature. After selecting the best performance fiber coating and optimizing desorption conditions,

multivariate extraction optimizing experiments were carried out (full description provided in one of the future sections).

Fiber selection, optimization of desorption conditions and multivariate experimental design extraction optimization experiments were carried out by employing the same GC and MS conditions. The splitless mode injections were carried out at constant injector temperature of 250 ^{o}C (except for the experiment involving optimization of desorption conditions, when the temperature was variable). The carrier gas, helium was used at a constant flow rate of 1.5 mL/min. The oven temperature program applied in the analysis consisted of utilizing 40 ^{o}C initial temperature for 1.5 min, after which the temperature was raised at a rate of 40 ^{o}C/min to 295 ^{o}C, for a total GC run time of 7.9 min. The transfer line temperature was held at 295 ^{o}C. The column eluent was submitted to electron impact (EI) ionization, while the ion source temperature was kept at 220 ^{o}C. The detector voltage was 1700 V. The data acquisition rate was 10 spectra/sec and mass fragments were collected in an *m/z* range of 35-450.

2.5. Autosampler, data acquisition and statistical analysis software

The CombiPAL autosampler was operated using the PAL Cycle Composer with Macro Editor software (version 1.4.0.). After the collection of GC-MS data, the total ion current (TIC) chromatograms were processed using the LECO ChromaTOF automated data processing software (version 2.32). The compounds considered for method optimization were identified by comparing their mass spectra to the mass spectra of reference compounds in the US National Institute of Standards and Technology (NIST), Terpene, and Flavor libraries (the latter two were included in the ChromaTOF software). With regard to the compounds identified in actual coffee samples submitted to differentiation study, the identity confirmation was based on: *i)* the mass spectral comparison described above and *ii)* the calculation of experimental retention indices and their comparison with the literature retention index data. The multivariate optimization design step composed of: *i)* two-level full factorial and *ii)* Doehlert matrix which was utilized for SPME method development was completed by employing Statistica software. The characterization of coffee samples according to geographical specifications was

established using PCA evaluation included within SPSS software for Windows (version 15.0).

3.0. Results and discussion

3.1. Method optimization

The following sections will describe the SPME method development steps conducted in the current study. After the completion of one of the preliminary optimization steps, namely selection of the fiber coating, the desorption and the extraction conditions were optimized by utilization of univariate and multivariate optimization designs, respectively.

3.1.1. Fiber selection experiment

As emphasized previously, the sensitivity of the SPME extraction technique depends on the value of the distribution constant of analytes partitioned between the sample matrix and the fiber coating material [13]. For this reason, the extraction efficiency of all commercially available SPME fiber coatings described in Section 2.2 was tested such that the coating having the highest affinity toward the volatile and semivolatile coffee constituents can be selected for the analysis of real coffee samples. For this purpose, 15 analytes (see corresponding peak numbers 3, 7, 9, 13, 15, 17, 23, 36, 43, 68, 83-84, 89, 93 and 102 in the list of tentatively identified compounds illustrated in Table 6) being characterized by different volatilities and polarities were selected across the GC chromatogram and the total sum of their peak areas was used to identify the SPME coating having best performance characteristics. The results (refer to Figure 14) illustrated that CAR/PDMS and DVB/CAR/PDMS fibers were most suitable for this study, as the total sum of the peak areas was highest for these two fibers.

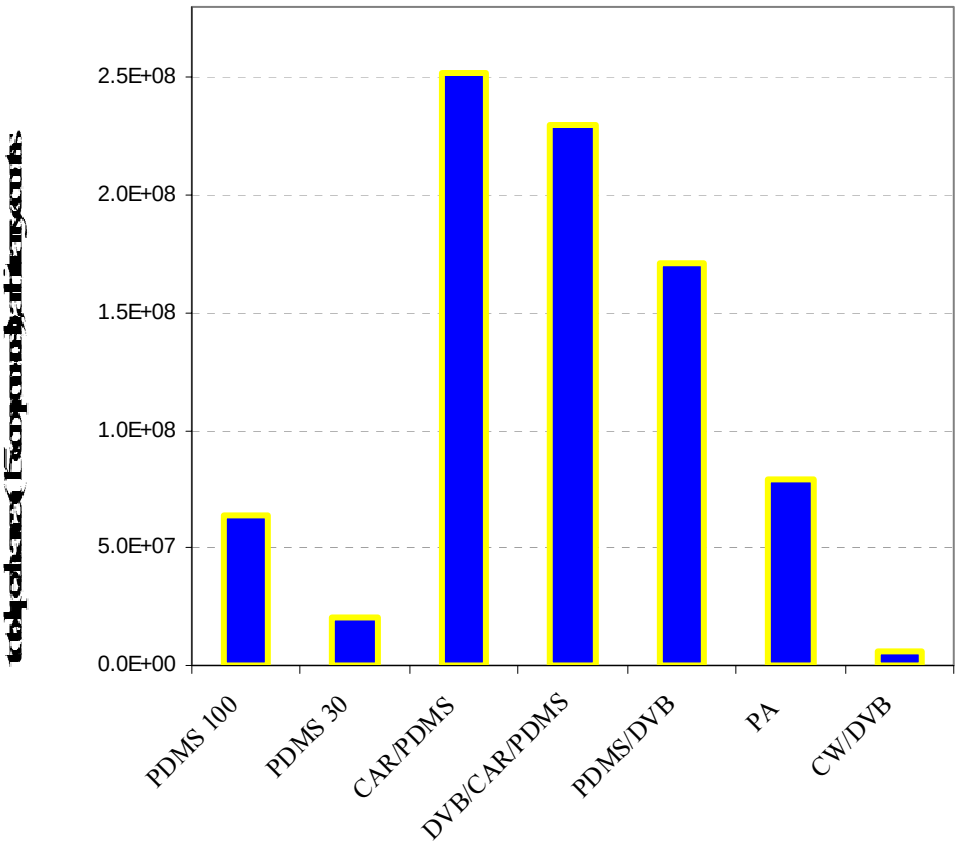

Figure 14. Performance characteristics of different commercially available SPME fiber coatings.

These two fiber coatings are commonly used in flavour analysis [62] and for this reason, the more detailed comparison of these two fibers was conducted and results are illustrated in Figure 15, leading to the conclusion that the CAR/PDMS fiber assembly exhibits better sensitivity for low molecular weight analytes. On the other hand, the triple phase DVB/CAR/PDMS coating material proved to have better performance characteristics for sufficient isolation of analytes having a wide range of physico-chemical properties. The similar outcomes were concluded by Mondello *et al.* [28] and Akiyama *et al.* [63] in their studies associated with the optimization of the SPME conditions for isolation of coffee aroma constituents. Based on the data evaluation completed within this particular optimization experiment, DVB/CAR/PDMS fiber was utilized in all further optimization/real analysis experiments.

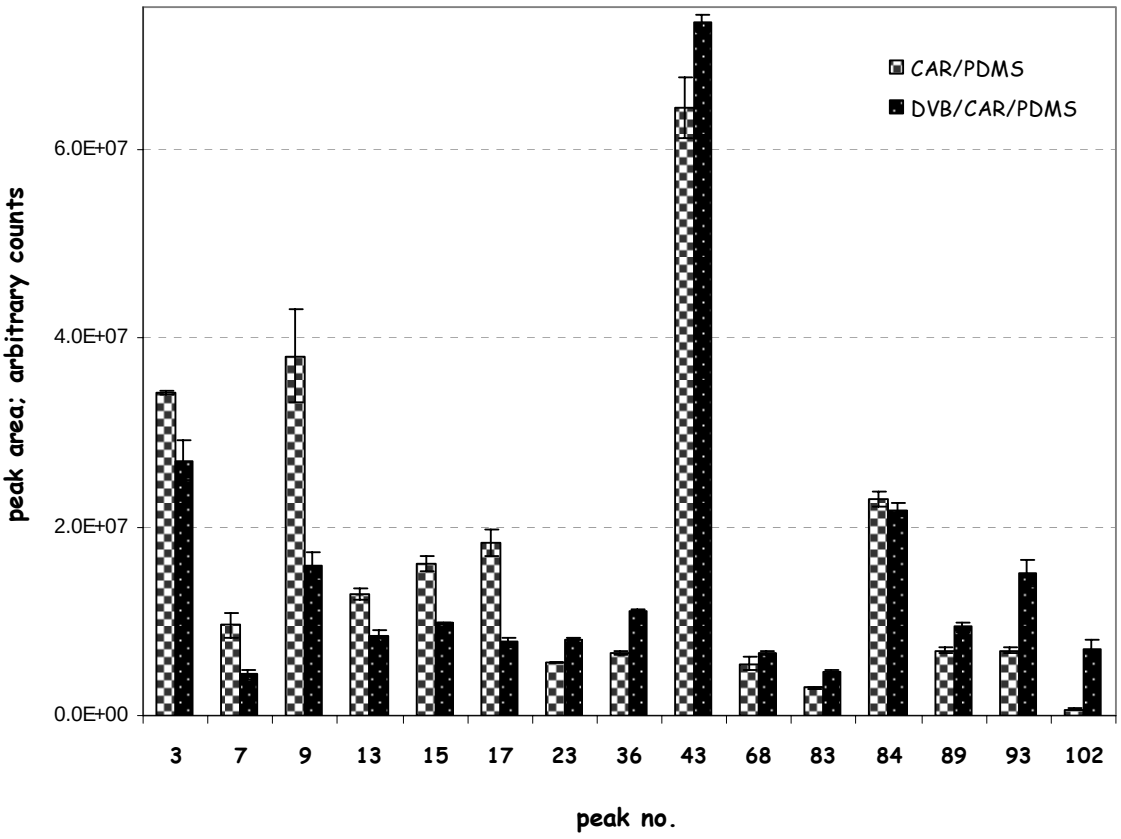

Figure 15. Performance characteristics of CAR/PDMS and DVB/CAR/PDMS fibers for isolation of volatile and semivolatile constituents. The peak numbers are associated with the compounds presented in Table 6

3.1.2. Optimization of desorption conditions

As emphasized in Section 1.5.1.1 of this document, desorption conditions are required to be optimized carefully to ensure optimum desorption efficiency of the analytes in the injector port of the gas chromatograph. In addition, these parameters need to be appropriately selected to allow elimination of any potential analyte carryovers after completion of each extraction-desorption cycle.

The previous section of this document indicated that DVB/CAR/PDMS coating possesses the best performance characteristics for a wide range of analytes selected across the entire GC chromatogram and being characterized by various physico-chemical

characteristics. The manufacturer's recommendation on the maximum allowable temperature range for this particular fiber coating was used as a reference point for the range of desorption temperatures that needed to be examined within the desorption temperature optimization experiment. Accordingly, desorption temperatures in the range 230-270 ^{o}C were tested. After evaluation of the data corresponding to the desorption temperature effects on desorption efficiency of several analytes of various physico-chemical properties, it was concluded that for most of the selected analytes, higher desorption temperatures (250-270 ^{o}C) proved to be most suitable. However, 270 ^{o}C was immediately rejected as the optimum desorption temperature since this temperature is equivalent to the upper/maximum allowable temperature limit for this particular fiber coating. Potential deterioration of fiber coating at such a high temperature after several consecutive injection cycles resembling a real analysis scenario is a valid reason for rejection of 270 ^{o}C as a desorption temperature value. Furthermore, the desorption efficiencies obtained when utilizing 250 ^{o}C and 260 ^{o}C were equivalent. Consequently, 250 ^{o}C was selected as the optimum desorption temperature value and it was utilized in all optimization and real sample coffee analysis experiments.

The next step of desorption optimization procedure involved the selection of optimum desorption time. Consequently, the following desorption times were examined so that the most appropriate desorption conditions can be selected: 0.5, 1, 1.5 and 2 minutes. To aid in identifying the optimum desorption time, the carryover effects of analytes were examined in this particular optimization experiment by injecting the blank DVB/CAR/PDMS fiber in the GC injection port immediately after the completion of each extraction-desorption procedure of coffee sample. From the list of analytes studied and selected for optimization purposes, 2,3-butanedione and caffeine were the only compounds that appeared in blank runs. As opposed to 0.5 and 1 min long desorption processes, the carry-over percentages were significantly minimized after employing 1.5 min desorption time (refer to Figure 16).

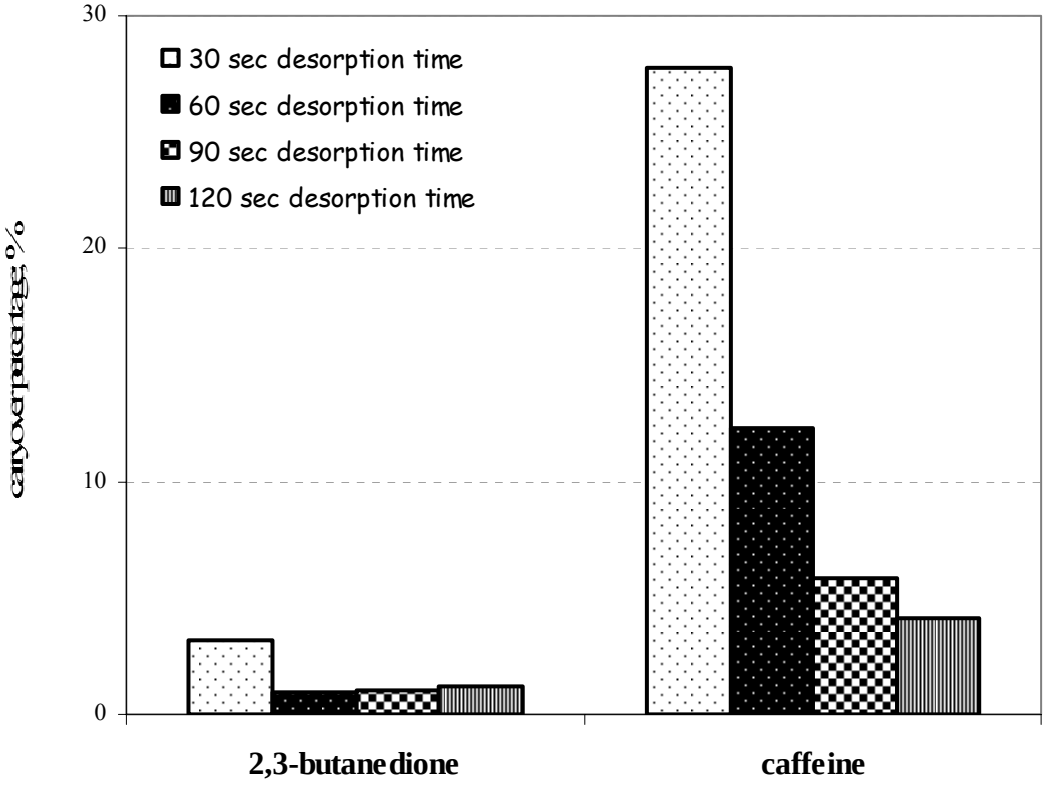

Figure 16. Selection of optimum desorption time based on carryover percentages.

In addition to being too long and impractical for high-speed analyses, a 2 min desorption time did not lead to a significant elimination of the carryover effects. Therefore, the optimum desorption time was fixed to 1.5 min with the possibility of conducting 'baking' procedures to completely remove residual carryover analytes before the start of the next extraction procedure. This was accomplished by baking the fiber coating for 5 min at 250 ^{o}C in the autosampler fiber conditioning station (component C in Figure 10) after the completion of each desorption process. To demonstrate successful elimination of the carryover effects for 1.5 min long desorption after which the baking procedure was applied, the total ion current (TIC) chromatograms of the coffee sample and corresponding blank fiber are overlaid in Figure 17.

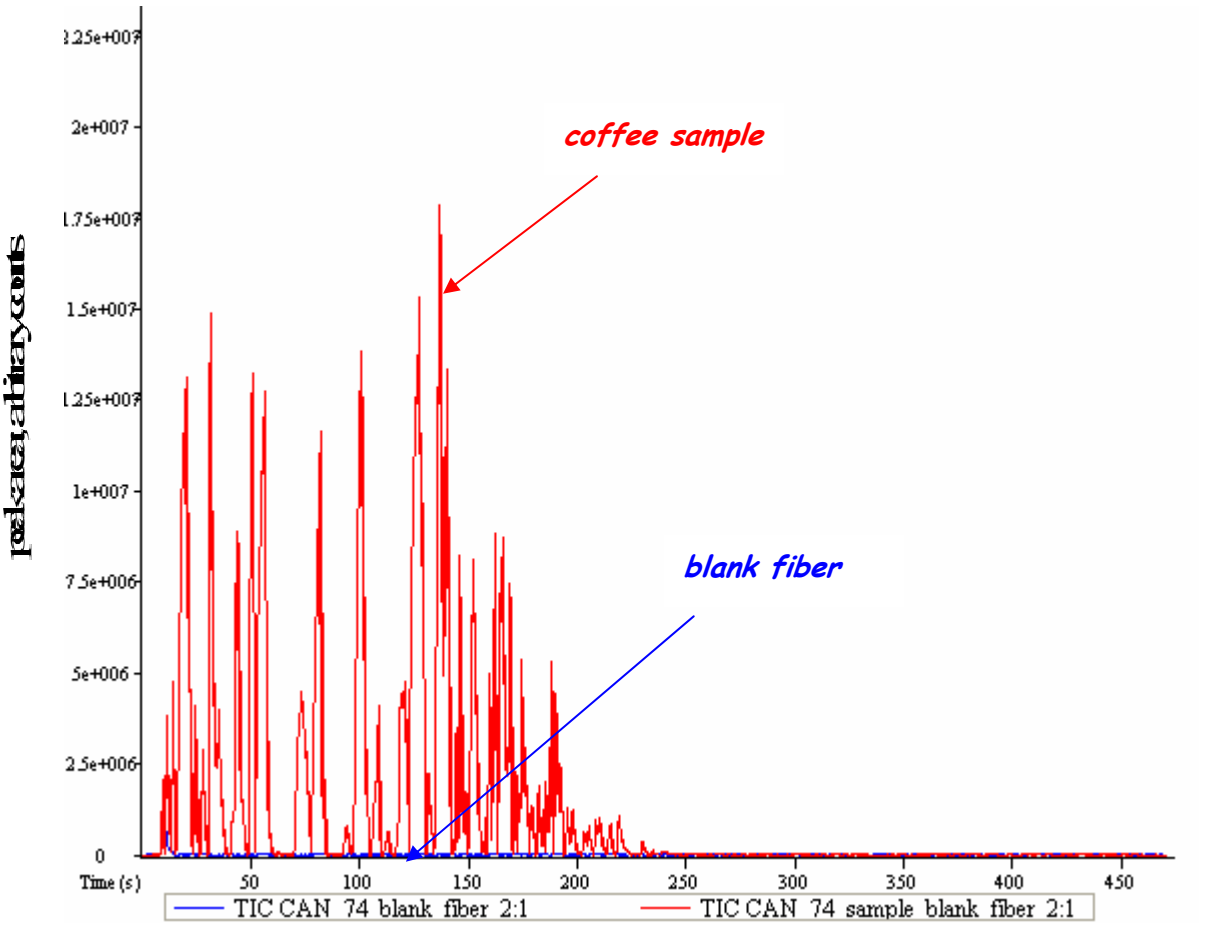

Figure 17. Total ion current (TIC) chromatograms for coffee sample and the corresponding blank fiber. Desorption conducted for 1.5 min after which the baking procedure was performed for 5 min in fiber conditioning station.

3.1.3. Multivariate optimization design

The effectiveness of analyte preconcentration using the SPME technique depends on the following parameters: *i)* amount of sample; *ii)* incubation time; *iii)* extraction time; *iv)* incubation/extraction temperature and *v)* agitation speed [33]. For this reason, the extent to which each of these variables affects the efficiency of the SPME procedure was examined via application of multivariate optimization design.

3.1.3.1. Two-level full factorial first-order design

The first step of the optimization procedure involved the utilization of two-level full factorial design to assess the importance of each variable (from the list illustrated in Section 3.1.3) on the SPME extraction efficiency. Consequently, a 2^5 full factorial design was applied to identify the most important variables and potential variable interactions.

Each of the five studied variables was examined at two levels (minimum and maximum ones), for which the values were arbitrarily selected to cover the wide range of experimental conditions. A 2^5 factorial design required the performance of 32 experiments, which were done in duplicates, for a total number of 64 experiments. Based on the selection of minimum and maximum levels, the variables were also examined in the corresponding central points of a factorial design. The central point experiments were performed in four replications, leading to the overall number of 68 experiments required for this particular optimization step. Table 2 illustrates the minimum, maximum and central point ranges for each variable of interest.

Table 2. The minimum, maximum and central point ranges selected for each variable of interest

Variables	***Levels***		
	minimum	*central point*	*maximum*
Amount of coffee sample (g)	0.5	1.0	1.5
Incubation time (min)	0.5	10.25	20
Extraction time (min)	0.5	15.25	30
Incubation/Extraction temperature (^{o} C)	40	60	80
Agitation speed (rpm)	0	250	500

The data evaluation pertaining to this particular experiment was accomplished by integrating and evaluating the peak areas corresponding to 30 compounds having different retention times and being characterized by various polarity and volatility characteristics, which were selected across the entire GC chromatogram. This is a common approach being employed when developing methods to allow nontarget analysis

of complex food matrices such as coffee in this particular case. In addition, this technique considers reasonable assumption that either a polar or a nonpolar, volatile or semivolatile compound could be the marker for establishing the geographical origin discrimination of food samples [57]. The response based on the sum of the peak areas is one of the most useful parameters for the optimization of SPME conditions, and hence it was considered as the end point in this study as the sum of the peak areas corresponding to 30 analytes selected for method optimization. An analysis of variance (ANOVA) was performed to assess whether the experimental variables examined in this study were affecting the performance of HS-SPME procedure in a significant way. The variable effects and interactions are summarized in the Pareto chart illustrated in Figure 18.

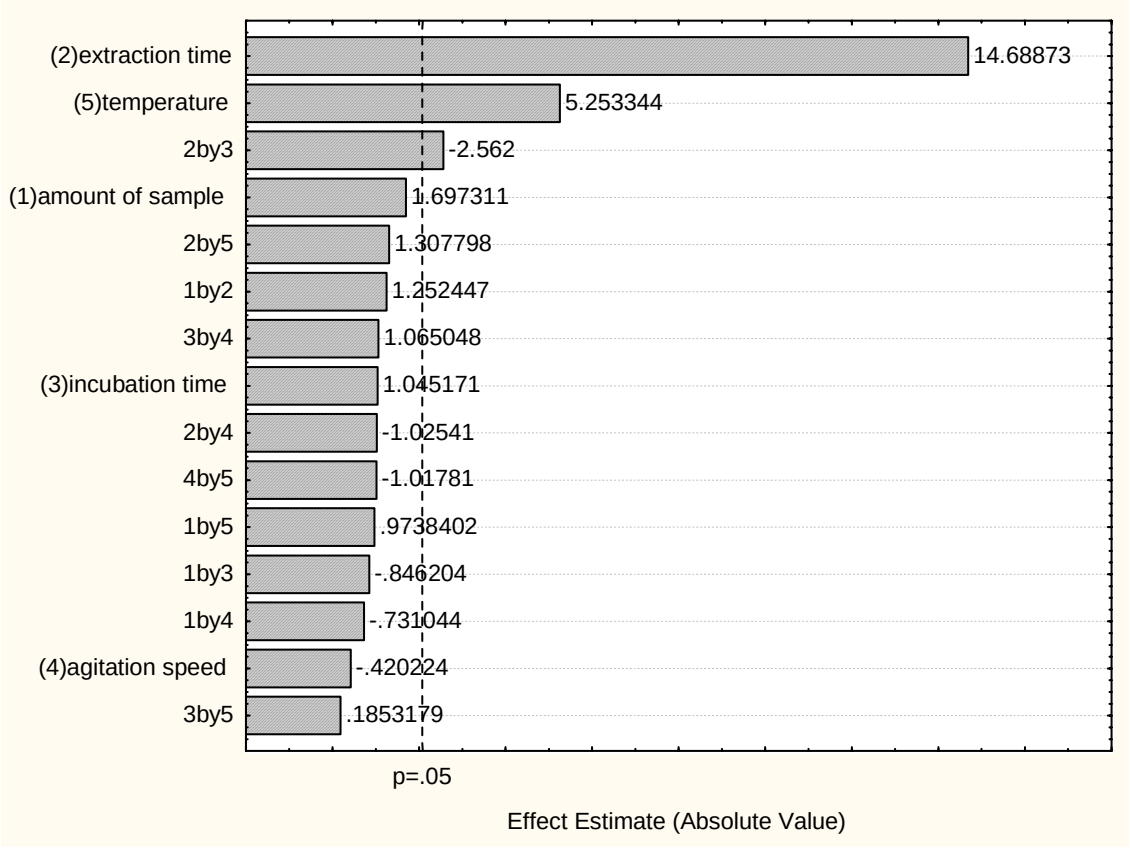

Figure 18. Pareto chart of standardized effects of 2^5 factorial design for total chromatographic peak area corresponding to 30 analytes selected for SPME method optimization. The numbers indicated in parentheses on y-axis were assigned to each variable so that potential interactions between variables can be identified.

As demonstrated in Figure 18, extraction time and sample temperature are statistically significant at the 95% confidence level. Furthermore, the results summarized in the Pareto chart indicate that increasing these two factors will increase the analytical signal as well. The positive effect of extraction time increase can be attributed to the fact that the equilibrium is not reached even after 30 min for the less volatile compounds evaluated in this study. With regard to the sample temperature, two opposite phenomena occur upon increasing the sample temperature. Higher extraction temperature enhances the release of analytes from the sample matrix into the headspace, hence the rate of analyte transfer toward the fiber is increased [57]. On the other hand, by increasing the sample temperature, the distribution constant of the analyte between the headspace and fiber coating decreases, hence decreasing the method sensitivity [84]. The Pareto chart from Figure 18 illustrates that upon increasing the sample temperature, the effect of more prominent analyte transfer toward the fiber is more significant than the one associated with the decrease of distribution constant.

The effect of sample temperature was consequently evaluated in more detail by considering the sum of the peak areas of the first 15 more volatile compounds as the response for Pareto chart illustrated in Figure 19. This finding is contrary to the one presented in Figure 18, as the higher significance of sample temperature as compared to extraction time is observed. As expected, the extraction time was not identified as most influential variable this time due to the shorter equilibration times of more volatile analytes. Furthermore, increasing the sample temperature has a negative effect on the SPME extraction efficiency in this case, which is in accordance with the fact that the effect of decreasing distribution constant is more critical for lighter compounds.

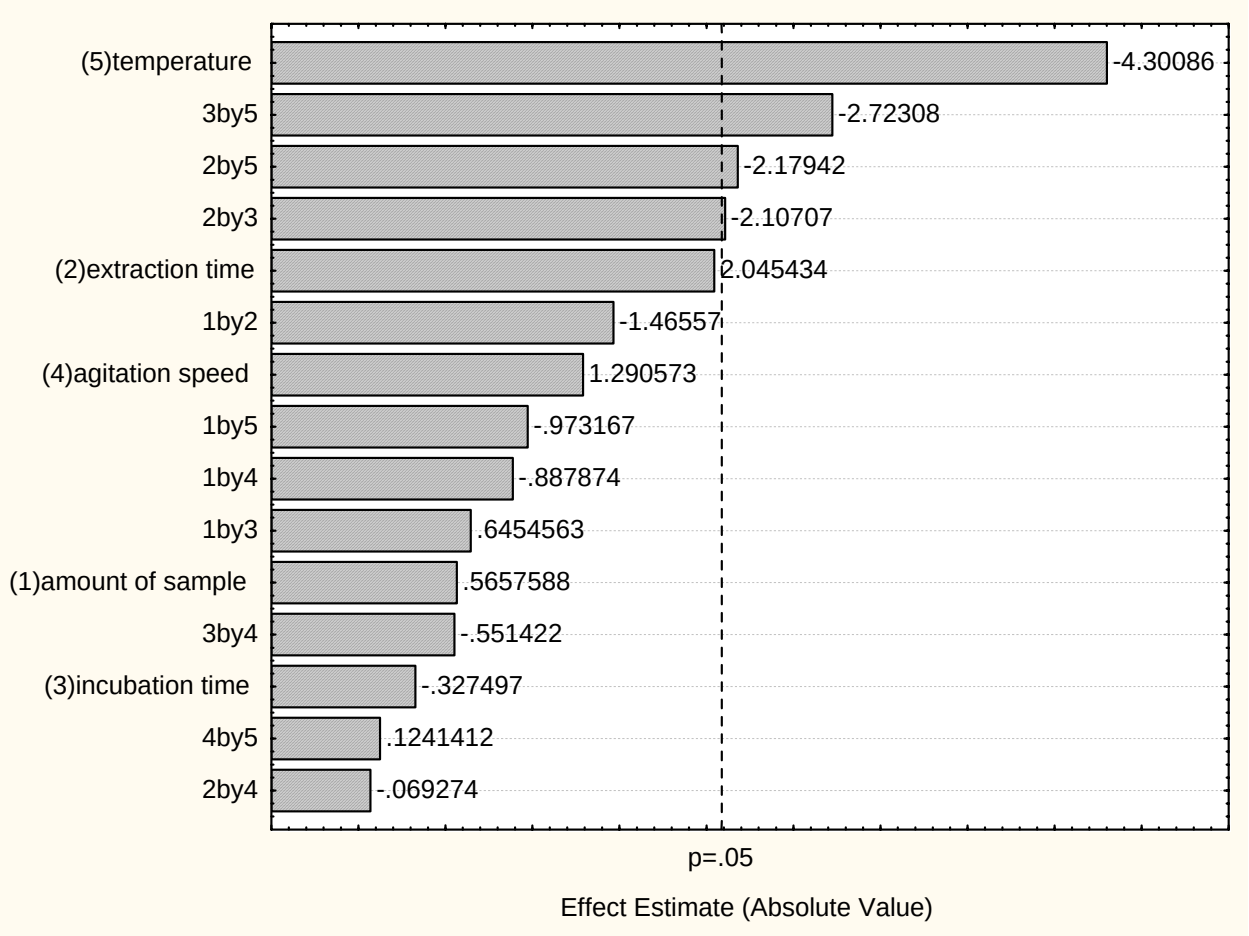

Figure 19. Pareto chart of standardized effects of 2^5 factorial design for total chromatographic peak area corresponding to 15 more volatile analytes selected for the SPME method optimization. The numbers indicated in parentheses on y-axis were assigned to each variable so that potential interactions between variables can be identified.

3.1.3.2. Doehlert design

The results obtained by the implementation of the full factorial design demonstrate that at the 95% confidence level, the sample amount, incubation time and agitation speed are not affecting the SPME extraction efficiency to a significant extent. For this reason, the values associated with these three parameters were fixed in accordance to previously selected ranges in Table 2 to 1 g, 5 min and 500 rpm,

respectively. The highly influencing variables, extraction time and sample temperature needed to be further optimized through the utilization of the second-order Doehlert design.

The two-variable Doehlert matrix was generated to allow the five-level and three-level examinations of extraction time and sample temperature, respectively. The experimental fields composing the Doehlert matrix are summarized in Table 3. Seven experiments were required to be performed in duplicates for this particular two-variable matrix having the shape of a regular hexagon with a central point.

Table 3. Experimental fields for two-variable Doehlert matrix

	Experimental variables	
	Incubation/Extraction Temperature (^{o} C)	*Extraction Time (min)*
1	40	5
2	40	13
3	55	9
4	55	1
5	55	17
6	70	13
7	70	5

The sum of the peak areas corresponding to the same 30 preselected analytes in combination with the experimental fields presented in Table 3 were used to obtain the response surface plots. The response surface plot associated with the sum of the peak areas corresponding to both more volatile and less volatile compounds is illustrated in Figure 20. From these results, it can be concluded that the optimum set of conditions for all evaluated analytes consists of 12 min and 65 ^{o}C.

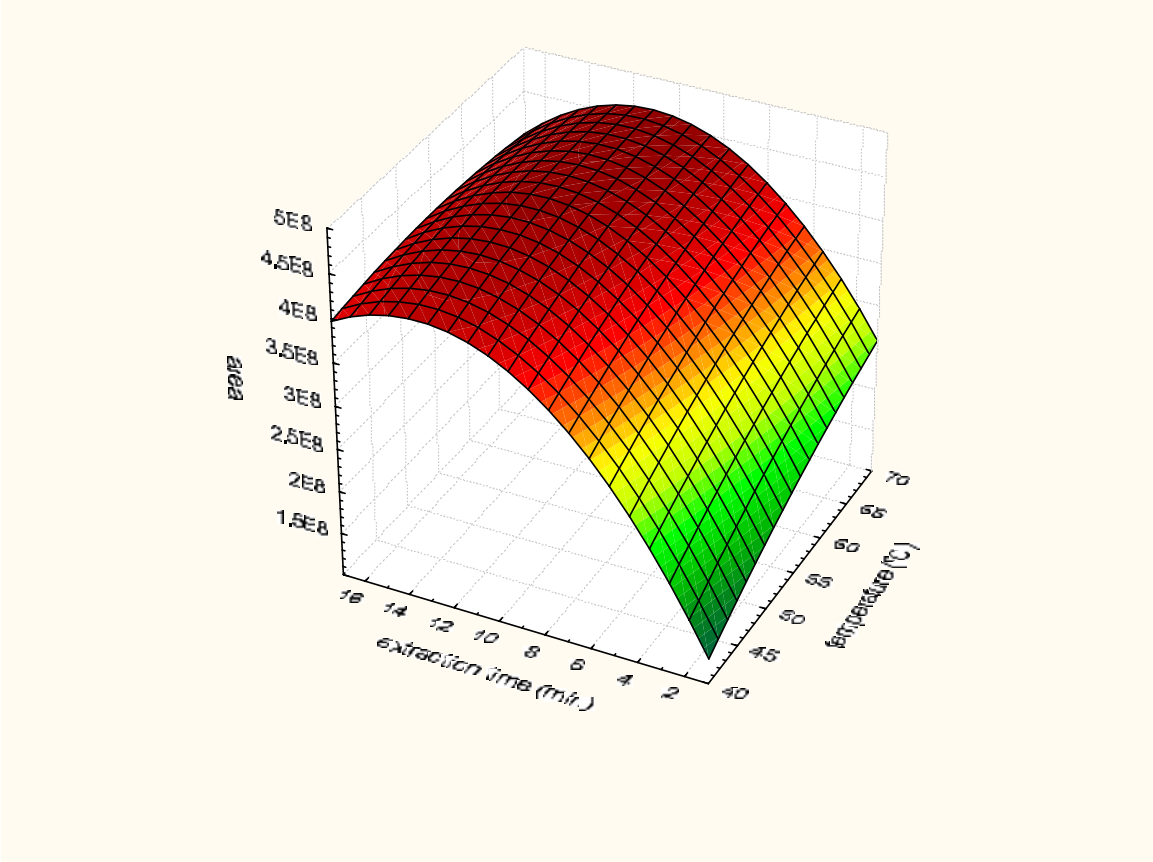

Figure 20. Response surface associated with both more volatile and less volatile analytes selected for method optimization

The effect of these two variables was further investigated by building a response surface plot (Figure 21) for 15 more volatile analytes, exclusively. The optimum value for the extraction time parameter resulting from the response surface plot in Figure 21 is 12 min in this case as well. However, the effect of sample temperature on the extraction efficiency is quite different than when considering all analytes, since the optimum temperature for the extraction of more volatile analytes is lower than 40 ^{o}C. Accordingly, the results observed through full factorial design are confirmed by utilizing the response surface methodology as well, since it is clear that the optimal temperature condition is

affected by analyte volatility. Since the objective of this study is to perform nontarget screening of coffee constituents across the entire GC chromatogram, the sample temperature of 55 ^{o}C was selected as a compromise condition to ensure the efficient extraction of both the more volatile and less volatile analytes.

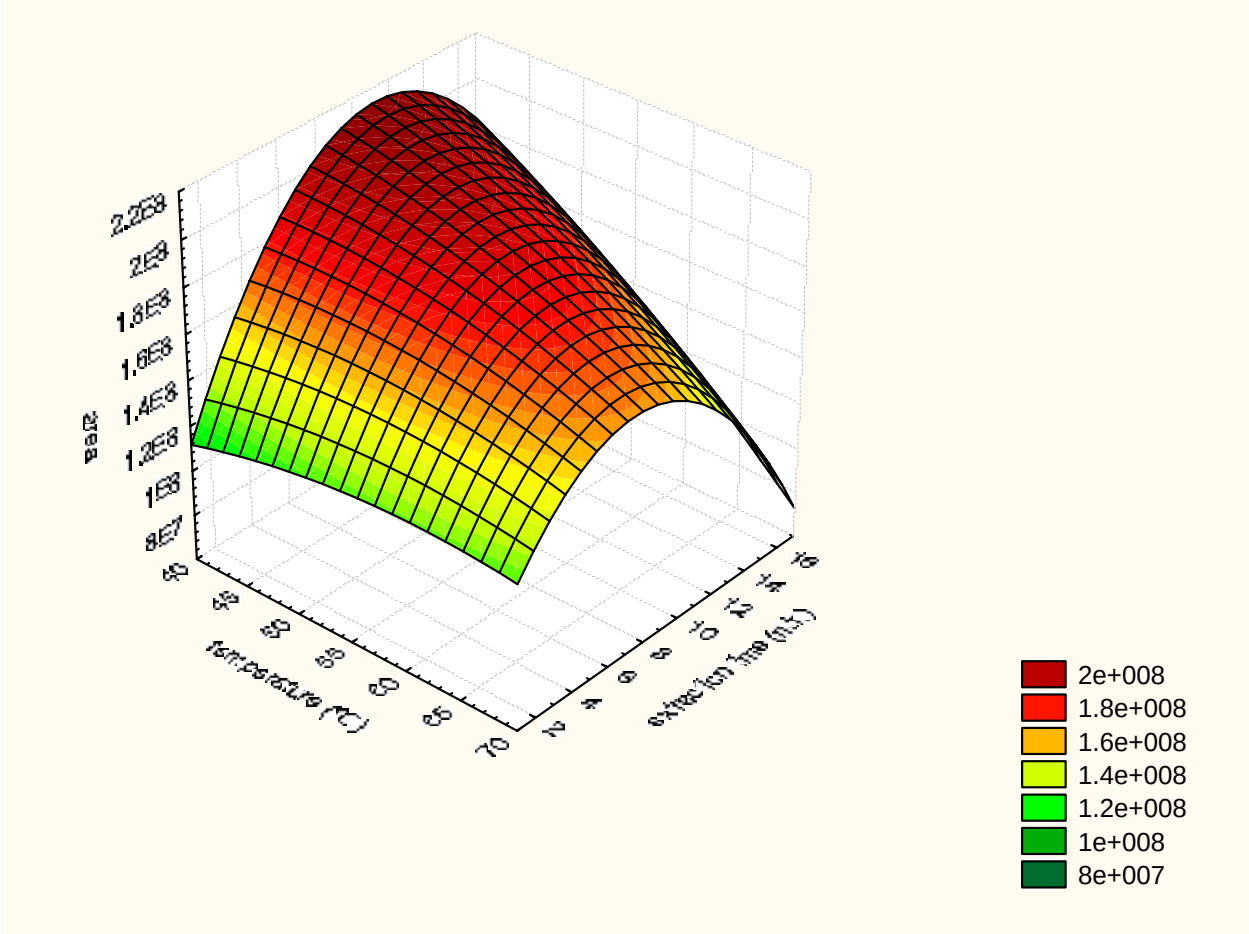

Figure 21. Response surface associated with 15 more volatile analytes selected for method optimization

3.2. Method validation

Seven replicates of the coffee sample that was utilized for method development and optimization (Van Houtte, 100% Colombian dark roast coffee) were injected to evaluate the repeatability of the analytical method with the optimized experimental conditions (refer to Table 4). The results indicate that the developed method meets several standard parameters of analytical quality of the data. The repeatability of the optimized HS-SPME-GC-TOFMS method for coffee analysis, expressed as relative standard deviation (RSD, %), ranged from 1.6 (1-furfurylpyrrole) to 15.1% (caffeine) for all the method optimization compounds.

Table 4. Method repeatability expressed in terms of relative standard deviation

peak no.	compound name	RI (exp)	RI (lit)	RSD; %
1	2,3-butanedione	599	584 [a)]	*5.4*
2	2-methylbutanal	667	645 [b)]	*10.1*
3	2,3-pentanedione	697	702 [a)]	*5.4*
4	1-methylpyrrole	740	731 [b)]	*5.2*
5	pyridine	762	726 [b)]	*3.2*
6	2,3-hexanedione	788	781 [a)]	*6.1*
7	2-methylpyrazine	833	820 [a)]	*2.4*
8	2,3-dimethylpyrazine	902	916 [a)]	*4.9*
9	5-methylfurfural	973	960 [a)]	*2.8*
10	furfuryl acetate	1004	996 [a)]	*4.1*
11	2-formyl-1-methylpyrrole	1020	na	*3.3*
12	N-acetyl-4(H)-pyridine	1038	na	*4.3*
13	2-ethyl-3,5-dimethylpyrazine	1094	1083 [a)]	*2.7*
14	2-furfuryl-5-methylfuran	1184	na	*3.3*
15	1-furfurylpyrrole	1189	1183 [a)]	*1.6*
16	4-ethyl-2-methoxyphenol	1285	1275 [a)]	*7.9*
17	2-methoxy-4-vinylphenol	1325	1309 [a)]	*7.8*
18	caffeine	1936	1837 [a)]	*15.1*

The literature retention indices displayed in the table and confirmatory retention indices were obtained from literature sources [37, 73, 94-119]

[a)] Retention indices from literature sources when HP-5 stationary phase utilized

[b)] Retention indices from literature sources when HP-1 stationary phase utilized

3.3. Analysis of volatile and semivolatile coffee constituents

After the completion of the method development and validation sections, the optimized HS-SPME-GC-TOFMS method was applied to the analysis of real coffee samples (described in Section 2.1. of this document) that are to be submitted to statistical discrimination study on geographical origin. The utilization of a high-speed TOFMS instrument ensured the completion of one GC-MS run of a complex coffee sample in 7.9 min. For this reason, the SPME extraction and preconcentration procedures were the only time-limiting steps of the coffee analysis. The complete list of benefits provided by the ChromaTOF software included fully automated background subtraction, baseline correction, peak find and mass spectral deconvolution algorithms. This was cost-effective and beneficial for the current nontarget screening study, whose objective is the identification of suitable markers for geographical origin coffee traceability.

Having this in mind, the coffee sample originating from a Brazilian authentic production region having a rich volatile and semivolatile chromatographic profile was selected as the reference sample, to which all the other coffee samples involved in this characterization study were compared (TIC chromatogram of a reference coffee sample is illustrated in Figure 22). This data evaluation strategy allowed the exploitation of the "compare to reference" feature incorporated within the ChromaTOF software (refer to Section 1.4). The compound identification was performed by utilizing the following two procedures: *i)* the comparison between experimental and reference/library mass spectra and *ii)* the comparison between experimental and literature retention indices.

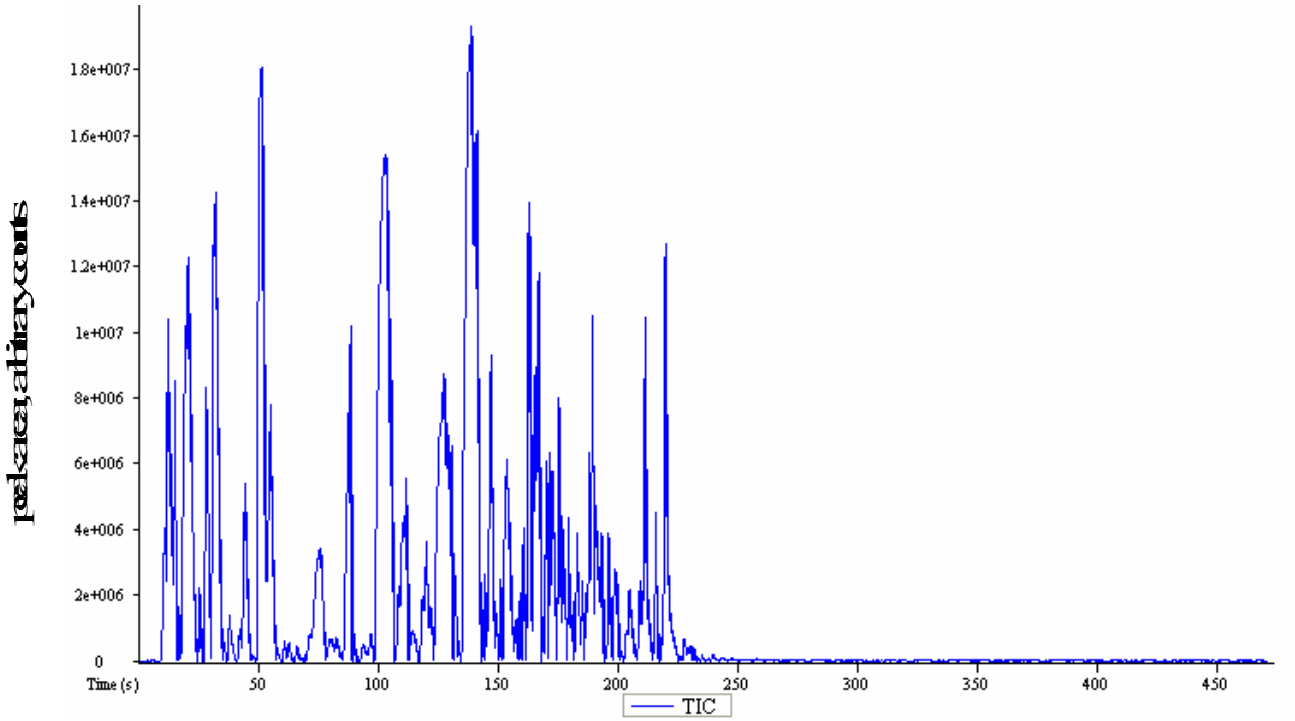

Figure 22. TIC chromatogram of reference coffee sample originating from an authentic coffee-producing Brazilian region

By utilizing the two identification methods specified here, the coffee aroma associated with an authentic reference sample from Brazil is described by 102 volatile and semivolatile analytes with specifications illustrated in Table 5 (identification procedure based on the comparison between experimental and reference/library mass spectra) and Table 6 (identification procedure based on the comparison between experimental and literature retention indices). The abundant pyrazine fraction, which is formed during the roasting procedure transformation of green coffee beans into the roasted ones, is described by 25 positively identified compounds. In addition, the coffee aroma associated with this reference sample can be attributed to extensive ketone, aldehyde, furan, pyridine and pyrrole fractions.

Table 5. Volatile and semivolatile compounds identified in the reference coffee sample. Identification procedure based on the comparison between experimental and reference/library mass spectra

peak no.	compound name	R.T. (s)	base ion	confirmation ions
1	acetaldehyde	10.531	44	43, 42, 41
2	2-propanone	11.631	43	58, 39
3	2,3-butanedione	14.230	43	86
4	2-methylfuran	14.730	82	53, 81, 39, 51
5	1,2-dimethylhydrazine	17.030	45	60, 42, 59
6	3-methylbutanal	18.030	44	39, 71
7	2-methylbutanal	18.830	57	41, 39, 86
8	acetic acid	20.230	43	45, 60
9	2,3-pentanedione	21.830	43	57, 100
10	2,5-dimethylfuran	22.630	96	95, 53, 67
11	3-hydroxy-2-butanone	24.830	45	43, 88, 73
12	2-vinylfuran	25.130	94	65, 66, 39
13	1-methylpyrrole	27.730	81	39, 51, 78, 66
14	pyrazine	28.430	80	53, 52, 50
15	pyridine	31.730	79	52, 50, 78, 75
16	pyrrole	33.030	67	41, 40, 64
17	2,3-hexanedione	37.530	43	71, 41, 114
18	2-methyl-5-ethylfuran	40.329	95	110, 67, 81
19	hexanal	41.229	44	56, 39, 72
20	3,4-hexanedione	41.729	57	114, 69
21	2-methyltetrahydrofuran-3-one	44.229	43	72, 100, 44, 55
22	4-methylthiazole	47.829	99	71, 72, 39
23	2-methylpyrazine	51.229	94	67, 39, 53, 42, 37
24	3-furaldehyde	54.529	95	39, 38, 50
25	furfural	55.729	96	95, 39, 67
26	2-allylfuran	60.429	108	77, 39, 107, 51
27	2,5-dimethylpyrrole	65.728	94	95, 53, 67
28	3-methylpyridine	69.328	93	66, 92, 40, 54, 63
29	acetol acetate	73.428	43	74
30	furfuryl alcohol	88.128	98	41, 81, 53, 70
31	furfuryl formate	96.627	81	53, 126, 80, 39
32	2,6-dimethylpyrazine	99.627	108	42, 39, 81, 67
33	2,5-dimethylpyrazine	100.127	42	108, 40, 81, 52, 66
34	2-acetylfuran	100.727	95	110, 96, 111
35	2-ethylpyrazine	104.327	107	80, 53, 96
36	2,3-dimethylpyrazine	104.927	67	108, 40, 52, 93
37	gamma-butyrolactone	105.527	42	86, 56, 39
38	2(5H)-furanone	111.527	55	84, 54, 83
39	2-ethylpyrrole	113.526	80	53, 78, 67
40	2,5-hexanedione	113.926	43	99, 71, 57
41	benzaldehyde	122.826	77	105, 77, 51

42	3-ethylpyridine	123.426	92	107, 65, 79
43	5-methylfurfural	126.526	110	109, 53, 81
44	4-methyl-3-pentanone	129.226	57	43, 100, 71
45	1-acetoxy-2-butanone	130.126	43	57, 101, 73
46	3-ethyl-2,4-dimethylpyrrole	131.226	108	93, 67, 79
47	2-pentylfuran	131.826	81	82, 53, 95
48	furfuryl acetate	136.226	81	98, 43, 140, 69
49	2-ethyl-6-methylpyrazine	139.125	121	39, 94, 66, 107
50	2-ethyl-3-methylpyrazine	139.625	121	39, 81, 107
51	trimethylpyrazine	140.525	42	122, 81, 54
52	2-formyl-1-methylpyrrole	141.225	109	108, 53, 80, 110
53	2-propionylfuran	141.925	95	124, 67, 55
54	2-butyltetrahydrofuran	143.225	71	43, 41, 39
55	2-ethenyl-6-methylpyrazine	144.225	120	52, 119, 39, 94
56	2-acetylpyrazine	146.325	43	122, 67, 108
57	N-acetyl-4(H)-pyridine	146.825	80	123, 43, 81
58	3,6-heptanedione	147.825	43	57, 99, 71
59	2-acetylpyridine	149.125	79	121, 93, 50
60	2,2'-bifuran	149.625	134	105, 63, 95
61	3,3,5-trimethylcyclohexene	150.425	109	67, 41, 82
62	2-formylpyrrole	153.825	95	94, 66
63	2,3,4-trimethyl-2-cyclopenten-1-one	157.325	109	124, 81, 41
64	2,6-diethylpyrazine	161.725	135	108, 53, 80, 66
65	2,3-diethylpyrazine	162.824	121	136, 80, 107
66	2-furfurylfuran	163.324	91	148, 120, 81, 94
67	3-ethyl-2,5-dimethylpyrazine	164.224	135	136, 42, 56, 108
68	2-ethyl-3,5-dimethylpyrazine	164.724	135	136, 42, 39, 96
69	furfuryl propanoate	165.224	81	98, 154, 69
70	2-acetylpyrrole	165.924	94	66, 39, 80
71	2-methoxyphenol	167.024	109	124, 53, 65, 77
72	2-methyl-6-[(1E)-1-propenyl]pyrazine	168.324	133	134, 39, 66
73	2-acetyl-3-methylpyrazine	173.524	43	136, 93, 67, 108
74	maltol	176.424	126	55, 97, 80
75	2-isobutyl-3-methylpyrazine	177.724	108	135, 93, 150
76	5H-5-methyl-6,7-dihydrocyclopentapyrazine	178.924	119	134, 78, 92, 65
77	2-methyl-3,5-diethylpyrazine	182.524	149	150, 39, 53, 122, 107
78	2-formyl-4,5-dimethylpyrrole	183.824	122	123, 94, 67
79	2-allyl-6-methylpyrazine	184.424	133	134, 39, 66, 78
80	2-acetyl-3-ethylpyrazine	184.924	135	150, 149, 67, 94, 107
81	2-heptylfuran	186.124	81	95, 166, 53
82	spiro[4.5]decan-2-one	186.924	81	94, 67, 152
83	2-furfuryl-5-methylfuran	187.823	162	43, 147, 119, 53
84	1-furfurylpyrrole	189.023	81	147, 53, 39, 67
85	ethyl maltol	190.223	140	139, 71, 125, 55
86	1-methylpyrrolo[1,2-a]pyrazine	195.023	132	104, 51, 91, 117
87	furfuryl isovalerate	198.123	81	98, 57, 182
88	2-isopentyl-6-methylpyrazine	204.623	108	121, 149, 66
89	4-ethyl-2-methoxyphenol	210.923	137	152, 122, 91, 77, 51
90	2-[(2-furylmethoxy)methyl]furan	215.422	81	53, 39, 97, 178, 69
91	indole	217.622	117	90, 63, 51

92	2,6-dimethyl-3(2-methyl-1-butyl)pyrazine	218.122	122	163, 53, 80
93	2-methoxy-4-vinylphenol	219.422	135	150, 107, 77, 63, 89
94	carvacrol	219.722	135	150, 77, 107, 117
95	eugenol	229.622	164	103, 149, 77, 55
96	<alpha-> copaene	230.122	161	41, 204
97	<beta-> damascenone	231.622	69	121, 41, 105, 77
98	difurfuryl sulfide	248.221	81	53, 194, 126
99	(+)-δ-cadinene	257.521	161	134, 41, 81, 189
100	<cis-> calamenene	258.221	159	129, 119, 144
101	<alpha-> calacorene	261.921	157	142, 115, 200, 129
102	caffeine	327.118	194	109, 55, 67, 82, 165

Table 6. Volatile and semivolatile compounds identified in the reference coffee sample. Identification procedure based on the comparison between experimental and literature retention indices

peak no.	compound name	R.T. (s)	characteristic mass	CAS no.	RI (exp)	RI (lit)
1	acetaldehyde	10.531	44	75-07-0	477	381 [b]
2	2-propanone	11.631	43	67-64-1	528	471 [b]
3	2,3-butanedione	14.230	86	431-03-8	599	584 [a]
4	2-methylfuran	14.730	62	534-22-5	608	606 [a]
5	1,2-dimethylhydrazine	17.030	80	540-73-8	645	na
6	3-methylbutanal	18.030	44	590-86-3	658	654 [a]
7	2-methylbutanal	18.830	57	96-17-3	667	645 [b]
8	acetic acid	20.230	75	64-19-7	682	na
9	2,3-pentanedione	21.830	100	600-14-6	697	702 [a]
10	2,5-dimethylfuran	22.630	94	625-86-5	704	703 [a]
11	3-hydroxy-2-butanone	24.830	42	513-86-0	721	681 [b]
12	2-vinylfuran	25.130	39	1487-18-9	723	na
13	1-methylpyrrole	27.730	114	96-54-8	740	731 [b]
14	pyrazine	28.430	96	290-37-9	744	729 [a]
15	pyridine	31.730	75	110-86-1	762	726 [b]
16	pyrrole	33.030	40	109-97-7	768	739 [b]
17	2,3-hexanedione	37.530	81	3848-24-6	788	781 [a]
18	2-methyl-5-ethylfuran	40.329	110	1703-52-2	799	791 [b]
19	hexanal	41.229	72	66-25-1	802	800 [a]
20	3,4-hexanedione	41.729	70	4437-51-8	804	773 [b]
21	2-methyltetrahydrofuran-3-one	44.229	55	3188-00-9	812	806 [a]
22	4-methylthiazole	47.829	71	693-95-8	823	791 [b]
23	2-methylpyrazine	51.229	37	109-08-0	833	820 [a]
24	3-furaldehyde	54.529	91	498-60-2	842	862 [a]

25	furfural	55.729	112	98-01-1	845	852 [a]
26	2-allylfuran	60.429	39	75135-41-0	856	na
27	2,5-dimethylpyrrole	65.728	42	625-84-3	867	na
28	3-methylpyridine	69.328	54	108-99-6	874	871 [a]
29	acetol acetate	73.428	122	592-20-1	882	na
30	furfuryl alcohol	88.128	67	98-00-0	906	860 [a]
31	furfuryl formate	96.627	39	13493-97-5	875	na
32	2,6-dimethylpyrazine	99.627	74	108-50-9	885	886 [b]
33	2,5-dimethylpyrazine	100.127	107	123-32-0	886	912 [a]
34	2-acetylfuran	100.727	96	1192-62-7	888	913 [a]
35	2-ethylpyrazine	104.327	96	13925-00-3	900	914 [a]
36	2,3-dimethylpyrazine	104.927	67	5910-89-4	902	916 [a]
37	gamma-butyrolactone	105.527	43	96-48-0	904	941 [a]
38	2(5H)-furanone	111.527	84	497-23-4	924	na
39	2-ethylpyrrole	113.526	85	1551-06-0	930	941 [a]
40	2,5-hexanedione	113.926	42	110-13-4	932	na
41	benzaldehyde	122.826	42	100-52-7	961	960 [a]
42	3-ethylpyridine	123.426	57	536-78-7	963	955 [a]
43	5-methylfurfural	126.526	104	620-02-0	973	960 [a]
44	4-methyl-3-pentanone	129.226	142	565-69-5	982	na
45	1-acetoxy-2-butanone	130.126	86	1575-57-1	985	na
46	3-ethyl-2,4-dimethylpyrrole	131.226	122	517-22-6	988	na
47	2-pentylfuran	131.826	81	3777-69-3	990	991 [a]
48	furfuryl acetate	136.226	91	623-17-6	1004	996 [a]
49	2-ethyl-6-methylpyrazine	139.125	86	13925-03-6	1014	1000 [a]
50	2-ethyl-3-methylpyrazine	139.625	102	15707-23-0	1015	1001 [a]
51	trimethylpyrazine	140.525	43	14667-55-1	1018	1002 [a]
52	2-formyl-1-methylpyrrole	141.225	108	1192-58-1	1020	na
53	2-propionylfuran	141.925	95	3194-15-8	1022	988 [b]
54	2-butyltetrahydrofuran	143.225	39	1004-29-1	1026	na
55	2-ethenyl-6-methylpyrazine	144.225	53	13925-09-2	1030	na
56	2-acetylpyrazine	146.325	108	22047-25-2	1036	1023 [a]
57	N-acetyl-4(H)-pyridine	146.825	124	67402-83-9	1038	na
58	3,6-heptanedione	147.825	101	1703-51-1	1041	na
59	2-acetylpyridine	149.125	39	1122-62-9	1045	1032 [a]
60	2,2'-bifuran	149.625	105	5905-00-0	1047	na
61	3,3,5-trimethylcyclohexene	150.425	98	503-45-7	1049	na
62	2-formylpyrrole	153.825	87	1003-29-8	1060	1043 [a]
63	2,3,4-trimethyl-2-cyclopenten-1-one	157.325	65	28790-86-5	1071	na
64	2,6-diethylpyrazine	161.725	145	13067-27-1	1085	na
65	2,3-diethylpyrazine	162.824	42	15707-24-1	1088	1080 [a]
66	2-furfurylfuran	163.324	59	1197-40-6	1090	na
67	3-ethyl-2,5-dimethylpyrazine	164.224	56	13360-65-1	1093	1079 [a]
68	2-ethyl-3,5-dimethylpyrazine	164.724	132	13925-07-0	1094	1083 [a]
69	furfuryl propanoate	165.224	154	623-19-8	1096	na
70	2-acetylpyrrole	165.924	141	1072-83-9	1098	1074 [a]
71	2-methoxyphenol	167.024	91	90-05-1	1102	1104 [a]
72	2-methyl-6-[(1E)-1-propenyl]pyrazine	168.324	38	18217-81-7	1107	na
73	2-acetyl-3-methylpyrazine	173.524	66	23787-80-6	1128	1082 [a]
74	maltol	176.424	87	118-71-8	1139	1108 [a]

75	2-isobutyl-3-methylpyrazine	177.724	66	13925-06-9	1144	1134 [a)]
76	5H-5-methyl-6,7-dihydrocyclopentapyrazine	178.924	123	23747-48-0	1149	1139 [a)]
77	2-methyl-3,5-diethylpyrazine	182.524	134	18138-05-1	1163	na
78	2-formyl-4,5-dimethylpyrrole	183.824	105	53700-95-1	1168	na
79	2-allyl-6-methylpyrazine	184.424	153	55138-64-2	1170	na
80	2-acetyl-3-ethylpyrazine	184.924	91	32974-92-8	1172	1158 [a)]
81	2-heptylfuran	186.124	166	3777-71-7	1177	1193 [a)]
82	spiro[4.5]decan-2-one	186.924	168	3643-16-1	1180	na
83	2-furfuryl-5-methylfuran	187.823	115	13678-51-8	1184	na
84	1-furfurylpyrrole	189.023	39	1438-94-4	1189	1183 [a)]
85	ethyl maltol	190.223	65	4940-11-8	1193	1199 [a)]
86	1-methylpyrrolo[1,2-a]pyrazine	195.023	190	64608-59-9	1214	na
87	furfuryl isovalerate	198.123	69	13678-60-9	1228	1222 [a)]
88	2-isopentyl-6-methylpyrazine	204.623	121	91010-41-2	1257	na
89	4-ethyl-2-methoxyphenol	210.923	79	2785-89-9	1285	1275 [a)]
90	2-[(2-furylmethoxy)methyl]furan	215.422	83	4437-22-3	1305	na
91	indole	217.622	108	120-72-9	1316	1297 [a)]
92	2,6-dimethyl-3(2-methyl-1-butyl)pyrazine	218.122	122	56617-70-0	1318	na
93	2-methoxy-4-vinylphenol	219.422	53	7786-61-0	1325	1309 [a)]
94	carvacrol	219.722	146	499-75-2	1326	1317 [a)]
95	eugenol	229.622	178	97-53-0	1374	1366 [a)]
96	<alpha-> copaene	230.122	204	3856-25-5	1377	1375 [a)]
97	<beta-> damascenone	231.622	70	23696-85-7	1384	1379 [a)]
98	difurfuryl sulfide	248.221	81	13678-67-6	1468	1463 [a)]
99	(+)-δ-cadinene	257.521	161	483-76-1	1517	1518 [a)]
100	<cis-> calamenene	258.221	159	483-77-2	1521	1525 [a)]
101	<alpha-> calacorene	261.921	157	Na	1541	1544 [a)]
102	caffeine	327.118	194	58-08-2	1936	1837 [a)]

The literature retention indices displayed in the table and confirmatory retention indices were obtained from literature sources [37, 73, 94-119]

[a)] Retention indices from literature sources when HP-5 stationary phase utilized

[b)] Retention indices from literature sources when HP-1 stationary phase utilized

Underlined compounds were evaluated in terms of chromatographic peak areas in all the samples that are to be submitted to PCA evaluation.

3.4. Statistical evaluation of data and geographical origin discrimination of coffee samples

Prior to utilizing the statistical evaluation tools for verification of geographical origin attributes, 29 coffee aroma contributing compounds (see the underlined compounds in Table 6) were evaluated in all samples that are to be submitted to the classification study by performing automatic integration procedure. In addition, the automatic integration and peak assignment steps were inspected, leading to the necessity to perform corresponding manual procedures in some cases. The average peak areas were calculated based on three replicates for which the analysis of real samples was performed. In accordance with previously published study by Zambonin *et al*. [47], the data were re-processed by employing an "internal normalization" procedure such that the areas of each of the underlined 29 peaks in Table 6 were expressed as a percentage of the sum of their total average peak areas. PCA statistical processing was then performed to study the main sources of variability between the coffee samples originating from various cultivation areas and detect the potential relationships/variables responsible for differentiation.

3.4.1. Authentic sample collections

As already stated in the Introduction section of this document, the major producers of coffee beans are South American countries such as Brazil and Colombia. For this reason, the preliminary objective associated with the geographical origin characterization of coffee samples was directed toward submitting the normalized data corresponding to samples received from authentic/production regions to statistical analysis. Overall, the authentic Brazilian and Colombian collections were comprised of 11 and 8 samples, respectively.

The final PCA analysis led to the extraction of two principal components (PCs) having the initial eigenvalues > 1, which contributed to 80% of the total variance of the data set. The first principal component (PC1) identified as a linear combination of 5-methylfurfural, 2,3-dimethylpyrazine and 2-methylpyrazine accounted for 53% of the variance. The second principal component (PC2) was mainly characterized by variable

2,3-diethylpyrazine, and this extracted factor contributed to 27% of the variance. The scatter plot of PCA scores corresponding to this discrimination is illustrated in Figure 23.

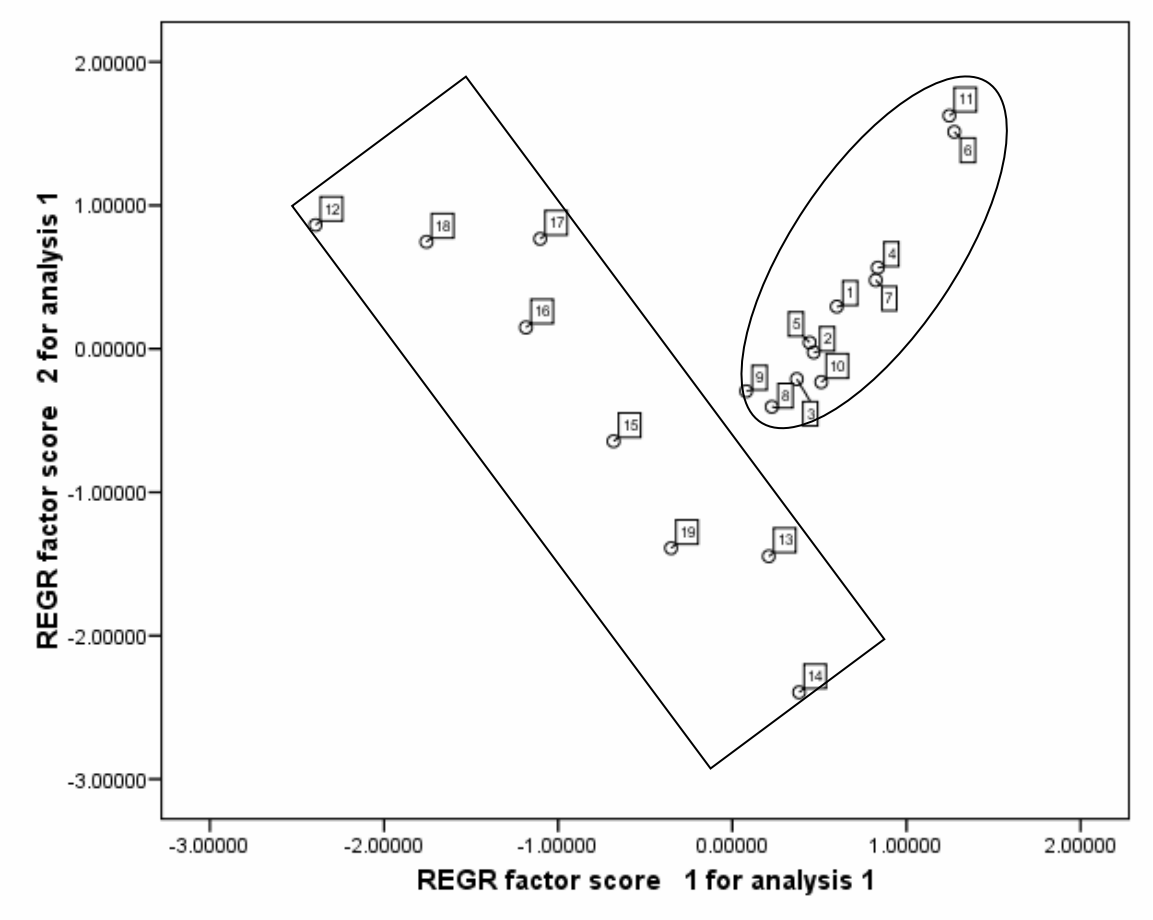

Figure 23. Scatter plot (PC1 and PC2 plotted on the x- and y-axis, respectively) of PCA scores associated with differentiation of authentic Brazilian (sample codes 1-11) and Colombian (sample codes 12-19) coffee samples

The conclusion that can be drawn from the illustrated classification study is that the two authentic coffee sample collections originating from Brazil and Colombia can be distinguished according to the geographical origin specifications. As opposed to the Brazilian samples, which are very well grouped together, the samples originating from

the Colombian production regions are more scattered. At this point, however, it should be noted that in addition to geographical origin, several other parameters were identified to influence the chemical composition of coffee such as: *i)* soil and climate conditions; *ii)* coffee bean processing methodology and *iii)* length and temperature of roasting process [29,66]. Unfortunately, the standardization of such conditions is not easily established for samples originating from various production areas and was not achievable in the current study either. The less prominent grouping of Colombian samples can be attributed to any of the aforementioned factors. However, the outcome of the current study illustrates that the two authentic sample collections from Brazil and Colombia can be characterized based on geographical origin attributes despite the potential influences and variation causes initiated by mentioned unstandardized factors.

3.4.2. Comparison between authentic and imported sample collections

The reliable authenticity verification of imported food commodities including coffee requires the implementation of quality certification procedures capable of tracing the product back to its production area. Furthermore, the assessment of authenticity within the imported food sector requires the identification of the potential changes undergone by a particular food commodity, especially if these changes are associated with nutritional, flavour and quality deteriorations during storage/transportation. For this reason, the objective within this particular characterization study was to compare the volatile coffee aroma profiles of authentic and nonauthentic Brazilian and Colombian coffee samples.

The results from finalized PCA evaluation indicated the extraction of two PCs representing 81% of the total variance of the data set. PC1 represented 48% of the variance and was strongly characterized by the following variables: 2,3-diethylpyrazine, 2-methylpyrazine and 2,3-dimethylpyrazine. PC2 on the other hand was represented as the linear combination of furfuryl acetate and 5-methylfurfural, and it accounted for 33% of the variance. The results of the current classification study are summarized in the scatter plot illustrated in Figure 24.

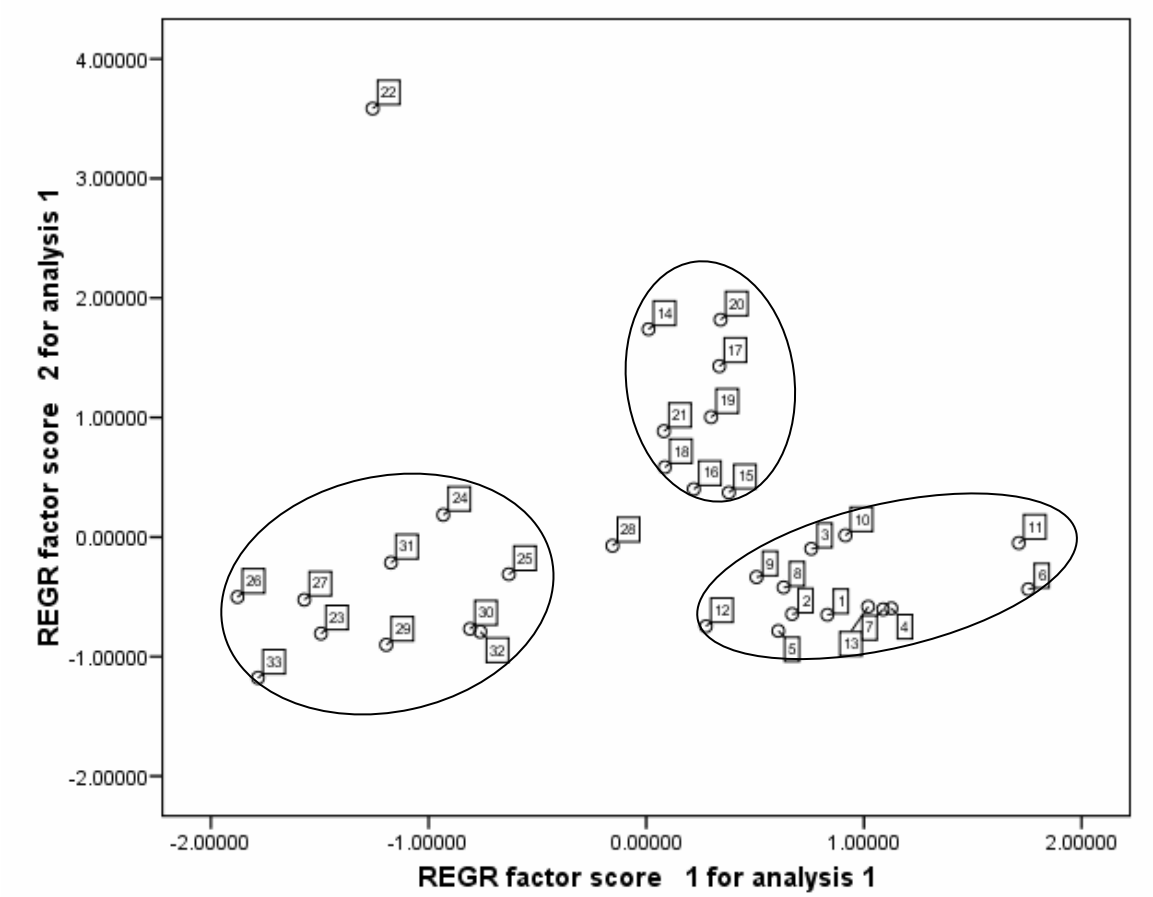

Figure 24. Scatter plot (PC1 and PC2 plotted on the x- and y-axis, respectively) of PCA scores associated with differentiation of authentic and nonauthentic Brazilian (authentic sample codes 1-11; nonauthentic sample codes 12-13) and Colombian (authentic sample codes 14-21; nonauthentic sample codes 22-33) coffee samples

The results represented in Figure 24 demonstrate more prominent discrimination of authentic samples from Brazil and Colombia, which in this case is probably due to the larger number of samples submitted to this particular PCA evaluation as compared with the one presented in Section 3.4.1. It is well established that the overall success of the PCA classification study is highly correlated with the number of samples submitted to a particular evaluation.

The outcome scatter plot illustrated in Figure 24 also demonstrates that the two nonauthentic samples corresponding to the Brazilian origin specification (sample codes 12 and 13) and collected from Canadian coffee import industries are grouped very well with the authentic samples from Brazil. However, the similar finding can not be confirmed for the Colombian nonauthentic samples (sample codes 22-33) belonging to the Canada import collection. These samples deviate strongly from the corresponding authentic collection, except for the sample labelled with code 28, which in this case is closest to the Colombian authentic collection grouping.

Furthermore, except for the sample 28, which is more associated with the authentic sample grouping and outlier sample 22, which is located on the highly positive range of PC2, the remaining nonauthentic Colombian samples are strongly correlated with each other. Considering that these samples form a distinct cluster on the negative ranges of PC1 and PC2, it can be concluded that their volatile and semivolatile compositions were potentially altered during transportation or storage conditions. It has been previously documented that both green and roasted coffee types are prone to compositional changes during storage [120]. The amount of published studies in this field is scarce for the definite conclusion concerned with the nature of changes to be drawn [121]. However, it is known that the storage conditions decrease the roasting coffee aroma and lead to the production of stale and off-flavour notes. The extent to which the shelf-life stability of coffee is altered during storage is strongly correlated with temperature conditions as well as moisture and headspace oxygen contents [120]. The influence of storage on deterioration of true aroma quality was studied by Reed *et al*. who reported a gradual decrease and increase of pyrazine and off-flavour fractions, respectively during storage of peanuts [122]. The conflicting finding was presented by Warner *et al*. who reported constant levels of pyrazines and increasing levels of aldehydes with storage of peanuts [123].

3.4.3. Geographical origin assessment of nonauthentic sample collections

Recently, the cultivation of coffee beans has been very prominent in Central America, Africa and Asia. Due to the detection of increasing practices of selling coffees on the basis of their geographical origin, the reliability of the current methodology for verification of geographical origin traceability was assessed. For this reason, the nonauthentic sample collections having Central American, African and Asian product declarations together with nonauthentic South American samples were submitted to statistical evaluation. The objective of the current examination was the identification of variables responsible for clustering and discrimination of samples originating from the following regions: *i)* South America, Colombia (12 samples); *ii)* Central America, Costa Rica and Guatemala (3 and 4 samples, respectively); *iii)* Africa, Ethiopia (3 samples) and *iv)* Asia, Indonesia (4 samples).

The results of the finalized PCA scores are illustrated in the scatter plot demonstrated in Figure 25. Two principal components were extracted explaining 64% of the total variance of the data set. PC1 represented 35% of the variance and was strongly characterized by 2-ethyl-3,5-dimethylpyrazine and furfuryl acetate. PC2 explained 29% of the variance and was mainly represented by the linear combination of the following variables: 2,6-diethylpyrazine, 2-methylpyrazine, 2,3-dimethylpyrazine and 2,5-dimethylpyrazine. The results represented in the scatter plot in Figure 25 demonstrate a clear classification of coffee samples according to the geographical origin attributes. The only outlier that was detected in this particular study is the sample coded with number 1, which was already identified in Section 3.4.2. (in Section 3.4.2. this sample was coded with number 22) as being incorrectly grouped. The results showed in Figure 25 illustrate that this sample for which the Colombian product declaration and origin specification were claimed by importers is grouped together with the Ethiopian samples on the highly positive range of PC1. The grouping of this particular sample, which is not established in accordance with its geographical origin declaration, demonstrates an example of potential fraudulent practices that are likely to occur during the distribution of imported food commodities. The possibility that this sample was mislabelled during the sample collection procedure might also explain the incorrect clustering associated with it.

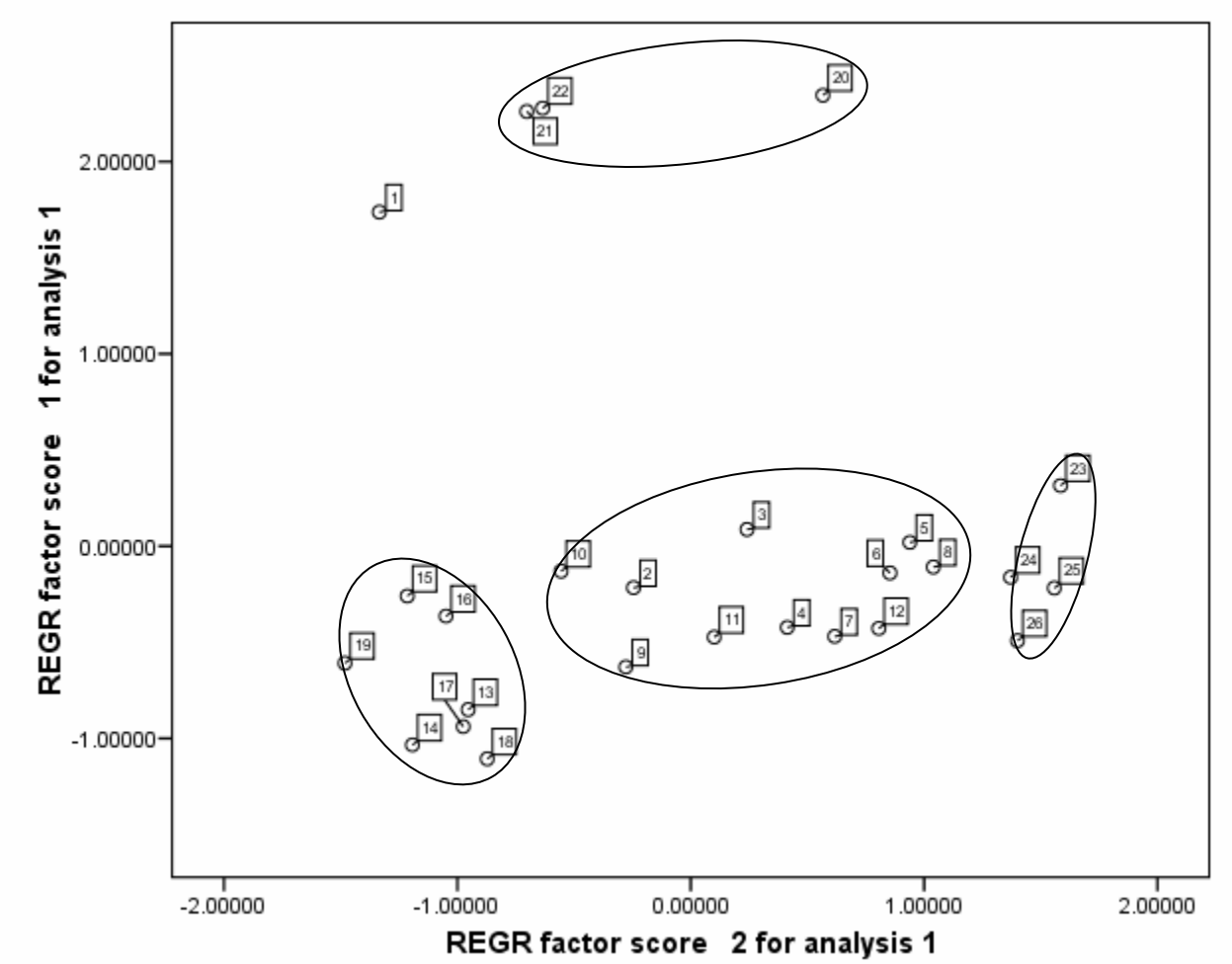

Figure 25. Scatter plot (PC2 and PC1 plotted on the x- and y-axis, respectively) of PCA scores associated with differentiation of nonauthentic South America, Colombia (sample codes 1-12); Central America, Costa Rica and Guatemala (sample codes 13-15 and 16-19, respectively); Africa, Ethiopia (sample codes 20-22); and Asia, Indonesia (sample codes 23-26) coffee samples

In accordance with the previous literature findings, the discrimination of coffees on the basis of their geographical origin was established after detecting 5-methylfurfural among the variables responsible for differentiation [49]. In the current study, the characterization of Ethiopian coffee samples (sample codes 20-22) and their clustering on the highly positive side of PC1 was strongly influenced by 2-ethyl-3,5-dimethylpyrazine and furfuryl acetate. In previously published literature sources, 2-ethyl-3,5-

dimethylpyrazine was already identified as one of the most potent odorants in coffee [46], whereas furfuryl acetate was identified as a marker in coffee origin classification studies [54].

3.4.4. Case study - geographical origin determination of an unknown sample

The following case study is focused on the determination of the geographical origin declaration for the coffee sample belonging to a nonauthentic Canadian imported sample collection. This sample, for which the Latin American geographical origin denomination was claimed by the Canadian distributors, is characterized by highly prominent consumer acceptance and enormously high consumption rates. Since Latin America comprises a vast geographical entity, and includes both the South American and Central American regions, this case study was aimed at confirming either a South American or Central American origin denominations for this particular coffee sample.

The preliminary step of the case study was directed toward establishing the geographical origin differentiations between South American and Central American coffee samples belonging to the nonauthentic imported Canadian sample collection. In other words, the appropriate classification model was built by including samples for which the geographical origin attributes were known. Overall, the nonauthentic South American and Central American collections were comprised of 12 and 7 samples, respectively. The results from the PCA evaluation indicated the extraction of two PCs representing 89% of the total variance of the data set. PC1 represented 50% of the variance and was strongly characterized by the following variables: 5-methylfurfural, 2,5-dimethylpyrazine and 2-ethyl-3,5-dimethylpyrazine. PC2 on the other hand was represented as the linear combination of 2,3-diethylpyrazine and 2-methylpyrazine, and it accounted for 39% of the variance. The results of this model classification study are summarized in the scatter plot illustrated in Figure 26.

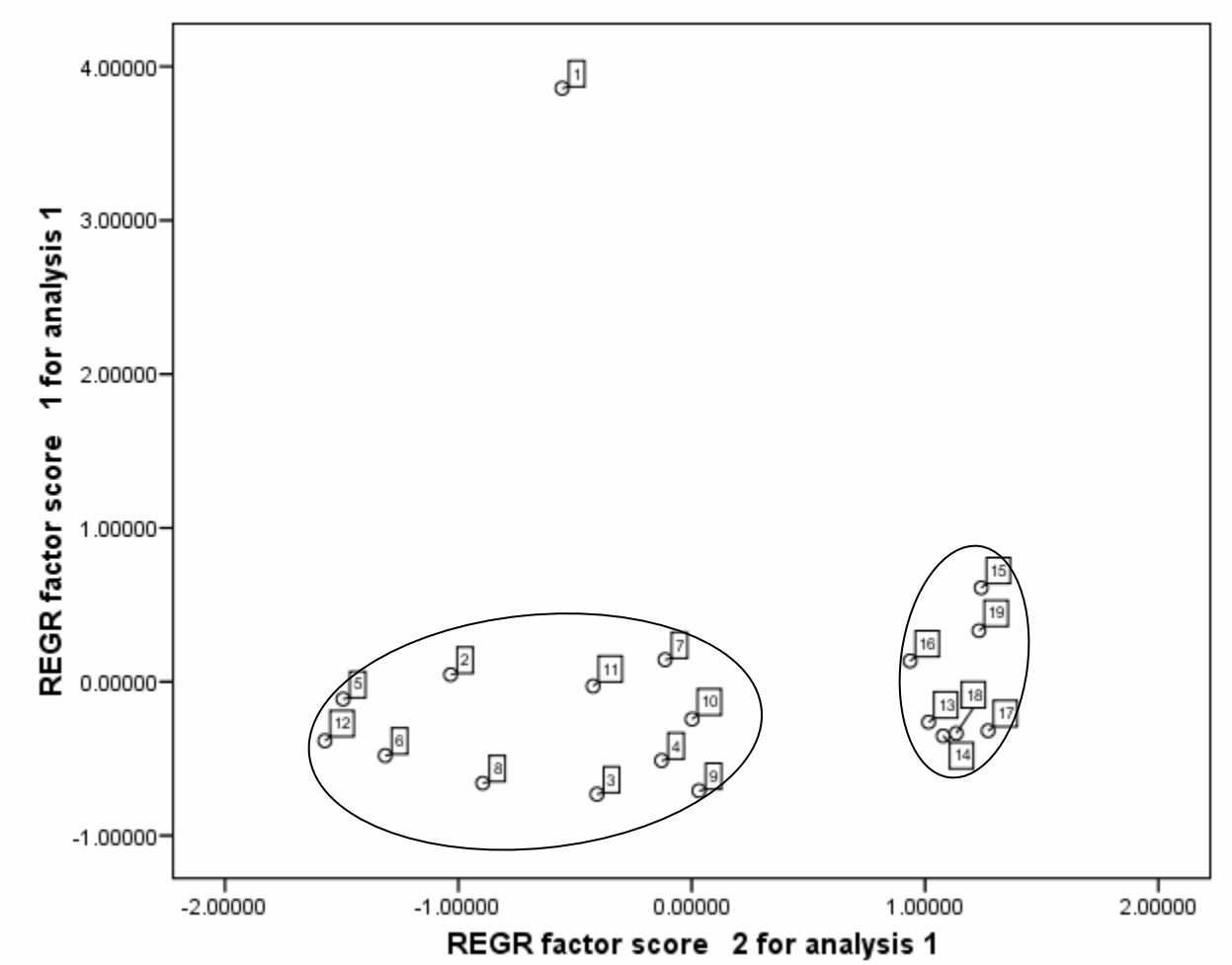

Figure 26. Scatter plot (PC2 and PC1 plotted on the x- and y-axis, respectively) of PCA scores associated with differentiation of nonauthentic South American (sample codes 1-12) and Central American (sample codes 13-19) coffee samples.

The next step of the current case study involved the submission of normalized chromatographic data expressed in terms of volatile and semivolatile constituents' peak areas to the constructed classification model illustrated in Figure 26. This PCA evaluation resulted in extraction of two principal components, accounting for 89% of the total variance of the data set. The results shown in Figure 27 illustrate that this characterization model was capable of evaluating the membership of an unknown sample

(coded with number 20) to a particular class, namely Central American geographical indication. These results are in accordance with the principles illustrated in Section 1.4 of this document as they demonstrate that the developed HS-SPME-GC-TOFMS method was properly optimized to allow the construction of statistical models directed to predict the membership of unknown samples.

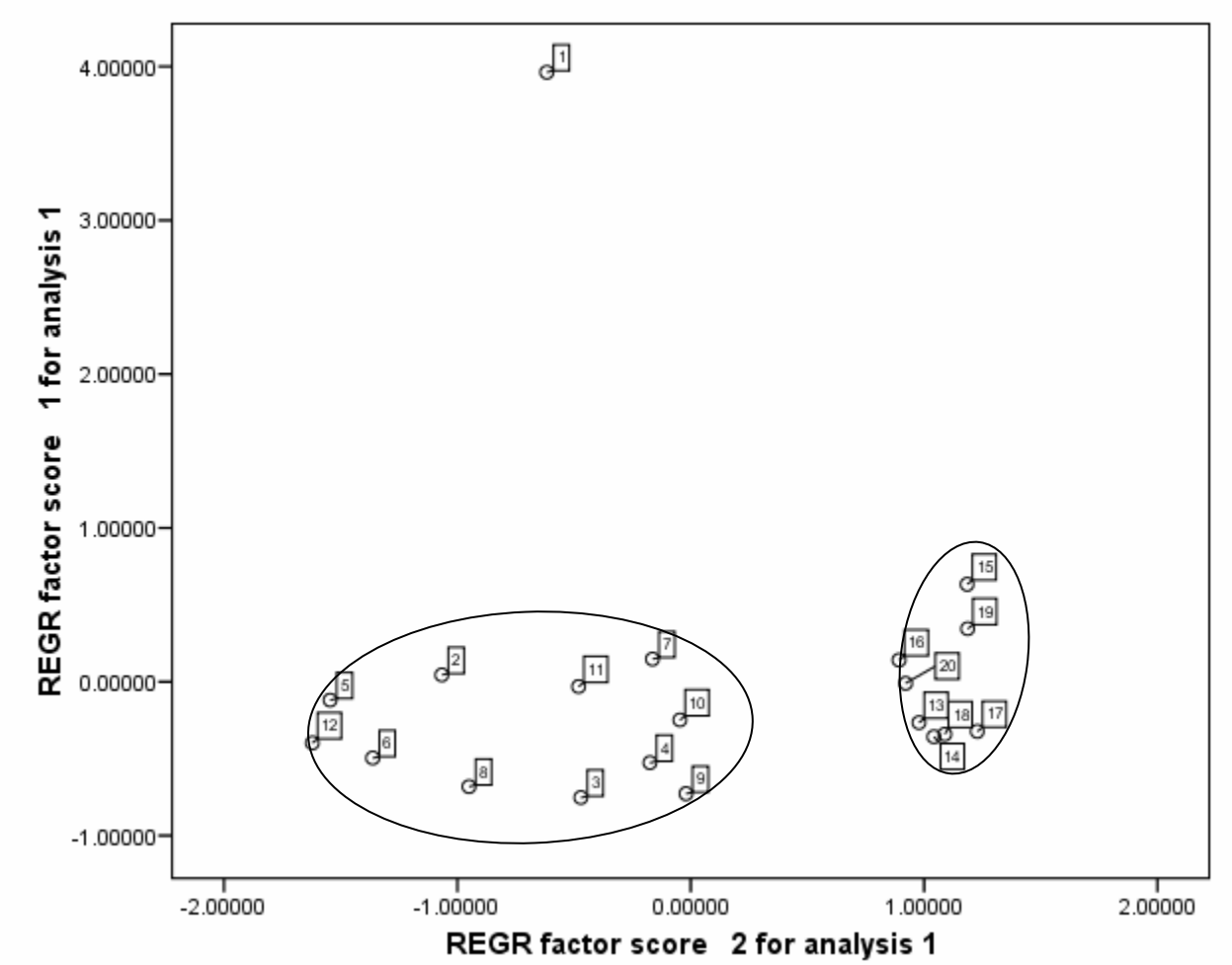

Figure 27. Scatter plot (PC2 and PC1 plotted on the x- and y-axis, respectively) of PCA scores associated with differentiation of nonauthentic South American (sample codes 1-12) and Central American (sample codes 13-19) coffee samples to predict the membership of the unknown sample (code 20).

3.5. Volatile and semivolatile aroma fraction of dessert coffees

As opposed to the production procedure associated with regular coffees, flavoured coffee is produced in a slightly modified way. In this particular production process, the flavouring is poured evenly over warm coffee beans immediately after the completion of the roasting process. As a consequence, the volatile and semivolatile aroma profiles of dessert coffees are altered, as compared to the regular coffees, since it has been already established that the chemical composition of coffee is dependent on coffee bean processing methodology. Since this particular processing technology involves the addition of flavouring substitutes (such as, for example, the addition of vanilla and caramel type flavourings), the objective of the current study was directed towards identification of those flavour volatile and semivolatile constituents that are exclusively predominant in dessert coffees. Such an investigation is particularly significant due to the aforementioned aspects (refer to Section 1.3) on increasing practices associated with the addition of erroneous components aimed at improving the flavour characteristics of coffee and increasing the consumer acceptance of commercial coffee products, the aspects both of which are significant from the production/distribution point of view.

After examining the volatile and semivolatile aroma composition of flavoured coffees, it was concluded indeed that these samples are characterized by additional components, which were not detectable in regular coffees. The list of these volatile and semivolatile constituents is provided in Table 7, together with the specifics as pertaining to the identification procedure (based on the comparison between the experimental and library mass spectra and comparison between experimentally obtained retention indices and those that are available in literature sources [113]).

Table 7. Volatile and semivolatile compounds identified in the dessert coffee sample.

Compound name	R.T. (s)	unique mass	similarity	CAS no.	RI (exp)	RI (lit)	base ion	confirmation ions
piperonal	227.422	145	883	120-57-0	1364	1364	149	121, 63, 91
gamma-nonalactone	230.922	85	853	104-61-0	1381	1362	85	55, 99, 71
delta-nonalactone	236.522	55	928	3301-94-8	1408	1389	99	71, 42, 55, 114
vanillin	241.221	153	931	121-33-5	1432	1394	152	81, 109, 51, 65, 137
gamma-decalactone	250.521	128	941	706-14-9	1480	1469	85	43, 128, 100, 69
delta-decalactone	255.521	71	928	705-86-2	1506	1494	99	71, 42, 55, 114

The extracted ion chromatogram of the dessert coffee sample (scaled to incorporate these additional flavour compounds, exclusively) is illustrated in Figure 28.

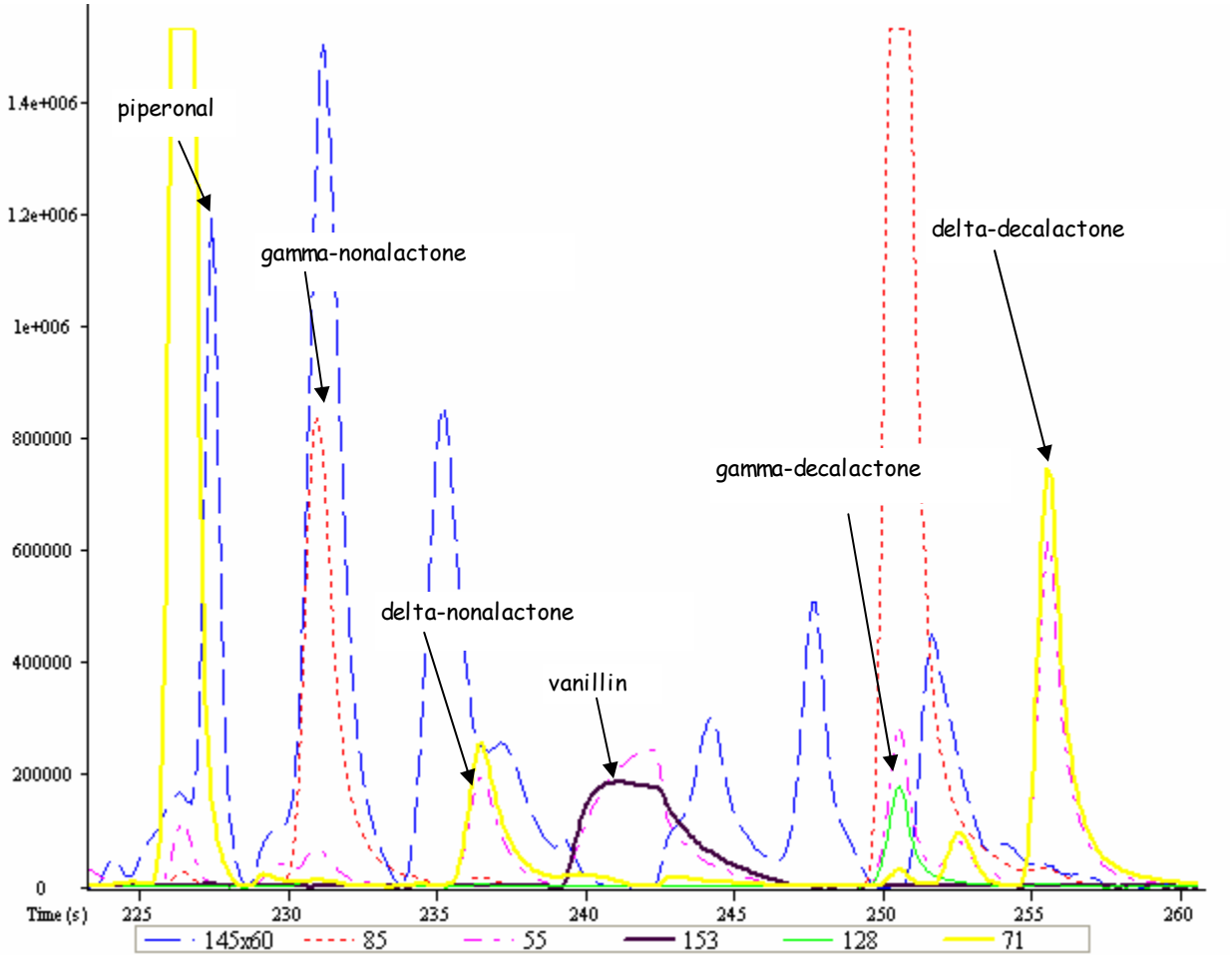

Figure 28. The extracted ion chromatogram of the dessert coffee sample.

4.0. Conclusions

In the present investigation, HS-SPME-GC-TOFMS methodology was developed and optimized for the purpose of verifying the geographical origin attributes of coffee samples originating from both authentic and importing regions. Among the tested commercially available fiber coatings, the mixed-phase DVB/CAR/PDMS fiber demonstrated best performance characteristics for a wide range of analytes having different physico-chemical characteristics and hence this coating was used in superelastic metal fiber assembly form for the completion of overall sequence of coffee samples. The SPME method optimization was completed by the utilization of multivariate experimental design and accordingly the optimum set of extraction conditions for the two identified influential parameters was 12 min and 55 ^{o}C for extraction time and temperature, respectively. The utilization of a high-speed data acquisition rate option offered by the Pegasus III TOFMS instrument ensured the completion of one GC-MS run of a complex coffee sample in 7.9 min. The complete list of benefits provided by the ChromaTOF software including fully automated background subtraction, baseline correction, peak find and mass spectral deconvolution algorithms was exploited during the data evaluation procedure. Finally, the acquired data set was submitted to a principal component analysis and the corresponding geographical origin discriminations of coffees originating from South and Central America, Africa and Asia were successfully established. At this point it is important to emphasize that in addition to the successful geographical discrimination of: *i)* authentic sample collections from Brazil and Colombia and *ii)* nonauthentic sample collections from South America, Central America, Africa and Asia, this classification study was also successful in detecting potential compositional changes that coffee undergoes due to the limited shelf-life stability over extensive storage conditions. Finally, the conducted geographical origin verification of the collected samples proved that this rapid analytical methodology demonstrates great potential for the assessment of quality and detection of adulterations in worldwide coffee industry.

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