1-1-2001

Development of a solid phase micro-extraction-gas chromatographic method for the analysis of volatile components in vanilla extracts and flavourings

Tomislav Sostaric
Edith Cowan University

Edith Cowan University

Copyright Warning

USE OF THESIS

The Use of Thesis statement is not included in this version of the thesis.

DEVELOPMENT OF A SOLID PHASE MICRO-EXTRACTION-GAS CHROMATOGRAPHIC METHOD FOR THE ANALYSIS OF VOLATILE COMPONENTS IN VANILLA EXTRACTS AND FLAVOURINGS.

TOMISLAV SOSTARIC B.Sc.

Thesis submitted in partial fulfillment of the requirements for the award of

MASTER OF BIOLOGICAL SCIENCES

SCHOOL OF NATURAL SCIENCES

EDITH COWAN UNIVERSITY

ABSTRACT

Vanilla is a very important flavouring agent, it is used as a major ingredient in a number of food products. The taste and aroma results from a specific blend of components present in the extract. There are over 170 volatile components, which all contribute to the flavour of the extract. These volatile components can be present in trace amounts or in relatively high concentrations. The range and concentration of volatile components is somewhat characteristic of the vanilla extract and its origin. Due to the high cost and low availability of natural extract, nature-identical and synthetic flavourings are often used to flavour foods and beverages. As natural extracts are very expensive, compared to nature-identical and synthetic vanilla flavourings, there have been many attempts to adulterate them.

There are many different methods available for the characterisation of vanilla extracts. These include high performance liquid chromatography (HPLC), isotope ratio mass spectrometry (IRMS), gas chromatography (GC) and thin layer chromatography. However, traditional methods for the extraction of volatile components from non-volatile components for GC analysis, in particular, are time-consuming and prone to sample loss and degradation.

Solid phase micro-extraction (SPME) is a relatively new separation technique, which can be used in conjunction with HPLC or GC. The analytes can be extracted from a variety of matrices using a fused silica fibre exposed to the headspace of the sample. This provides a simple and effective technique for the

selective extraction of volatile and semi-volatile components from a sample containing non-volatile components.

A SPME-GC method was developed to extract and analyse a range of natural extracts, nature-identical extracts and synthetic flavourings. Using a polyacrylate (PA) fibre, the volatile components were extracted at room temperature. No sample preparation was required (other than dilution). The SPME-GC method was used to qualitatively and quantitatively analyse a range of extracts and flavourings as well as food products. The method was also successfully compared to an existing HPLC method.

DECLARATION

I certify that this thesis does not, to the best of my knowledge and belief:

(i) incorporate without acknowledgement any material previously submitted for a degree or diploma in any institution of higher education;

(ii) contain any material previously published or written by any other person except where due reference is made in the text; or

(iii) contain any defamatory material.

Tomislav Sostaric

11 July, 2001

ACKNOWLEDGEMENTS

I would like to thank all the people who have helped me through this thesis. Foremost, a sincerest thank you to Dr Mary C. Boyce for her guidance, enthusiasm and unfailing support throughout the entire course of this project. I would like to thank the Academic staff and all my fellow students at Edith Cowan University, especially Dr Evadne E. Spickett, Mark A Bannister, John Poland, Bryan Duff, Julia Wilson and Anetta Cutler.

I would like to acknowledge Dr John Hall of Varian Australia, for his technical insight and expertise in the use of the Gas Chromatography-Mass Spectrometery. Monica Witsch of Peters Brownes group of companies for her insight into Vanilla extracts and Vanilla related products.

A special thanks to the suppliers of the Vanilla extracts and products, who allowed me to analyse their products.

Finally, I would like to acknowledge the support and continued encouragement of my family and friends throughout the duration of this thesis. In particular, thanks to Paul, Maria, Jelena and Ivana.

DEDICATION

I would like to dedicate this thesis to all my family. Without their love and encouragement this thesis would not have been possible.

PUBLISHED MATERIAL.

Part of the content in this thesis has been published in a refereed journal article; The article (details below) is supplied in Appendix A.

Sostaric, T., Boyce, M.C., & Spickett, E.E. (2000). Analysis of the volatile components in vanilla extracts and flavourings by solid-phase micro extraction and gas chromatography. Journal of Agricultural Food Chemistry, 48 (12), 5802-5807.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1. INTRODUCTION.

1.1. Introduction.

Vanilla, an extract obtained from the bean of a tropical orchid, is widely used as a flavouring agent in the food industry. For example, vanilla is used to flavour ice cream, chocolates and beverages (Archer, 1989). It is not easy to obtain the natural vanilla extract, as the vanilla orchid is an exotic plant with strict growing conditions. Cultivated crops are grown in a small portion of the globe including Mexico, Tahiti, Martinique, Madagascar and the Bourbon islands (Belay & Poole, 1993). Each plant requires at least two years to flower, and hand pollination is necessary for a sufficient crop yield (Lampretch *et al.*, 1994). Therefore, harvesting and curing of the beans takes place over an extended time period and requires the work of a considerable number of people. Currently, the global yield is approximately 1.5 tonnes, far short of global demand (Martin *et al.*, 1981).

From a chemical perspective, over 200 components have been identified in natural vanilla extracts. This knowledge has led to the manufacture of products, which closely match natural extracts and are produced at a fraction of the cost (Riley, 1989). The demand for natural extracts and their high monetary value has led to the practice of adulterating natural extracts (Belay & Poole, 1993).

The detection of adulteration is important for a number of reasons. Firstly, the deception of consumers is prohibited in many countries (Martin *et al.*, 1977).

Additives are substances that are not normally present in foods, but enhance their characteristics by providing certain flavours and aromas or increasing shelf life (Klimes *et al.* 1976). Artificial additives have been the subject of considerable public alarm and many people now avoid foods that contain them. This does not necessarily mean that such action is supported by scientific evidence, but public perception is an important aspect of the food marketplace (Ranadive, 1992). The presence of synthetic vanilla flavour in food must be declared on the product label to inform consumers, in many parts of the world. It is also important for the supplier and the producer to know the origin or authenticity of a vanilla extract (Bricout, 1982).

A number of suitable analytical techniques have been developed for the analysis of vanilla extracts, these include isotope ratio mass spectrometry (IRMS), high performance liquid chromatography (HPLC), and capillary electrophoresis (CE). These techniques often require laborious sample preparation steps prior to analysis and when used in isolation may not always be able to identify adulterated samples. When comparing capillary GC to HPLC and CE, the HPLC method reports a lower separation power, and the CE technique observes lower sensitivity. Solid phase micro-extraction (SPME) is a developing extraction technique, which both extracts and concentrates the analytes in a single step without the use of solvents. SPME coupled with GC may provide an alternative technique that is faster, cheaper and more sensitive to subtle differences between extracts (Steffan & Pawliszyn, 1996).

1.2. Vanilla flavour.

The aroma and flavour, are the defining characteristics of vanilla extracts. This has led to its widespread use in the food, beverage and confectionery industry (Guarino & Brown, 1985). The vanilla flavour obtained from the cured, unripe fruit of the *Vanilla Planifolia and Vanilla Tahitensis* plant is formed by a number of biochemical transformations and results in the production of over 170 volatile aromatic compounds (Hoffman & Salb, 1979). Vanillin is the major flavour constituent in natural vanilla extract, although the presence and relative concentrations of other flavour constituents give the extract its distinct flavour (Fayet *et al.,* 1987). Fats, water, waxes and sugars make up 80-90% of the content in the extract, the remaining 10-20% consist of several hundred flavour compounds (Ranadive, 1992).

The distinct flavour and aroma of the extract is a result of natural enzymatic reactions which occur during the curing process (Riley, 1989) which also has a large effect on the quality of the product. The green bean when picked, does not have the characteristic flavour and odour associated with vanilla, due to the vanillin being present as a glycoside. However, after the maturation processes of drying and warming, the glycosidic linkages are enzymatically hydrolysed to release glucose and vanillin (Guarino & Brown, 1985).

There are two common curing processes; the Madagascan and Mexican processes. In the Madagascan curing process, the vanilla pods are first placed in hot water, which destroys chlorophyll and increases enzymatic activity. The pods

are then spread out on blankets and exposed to the sun during the day. At night, the pods are placed in boxes and covered with blankets. The process is repeated daily for approximately 2-8 weeks. During this time the hydrolysis of the vanillin glycoside and the release of vanillin takes place. The chocolate coloured pods are then placed in trays and stored in holding warehouses until they develop a black colour due to dehydration (Riley, 1989).

The Mexican curing process involves initially storing the pods outside until they shrivel then transferal to large wooden "sweating" boxes. Mats are placed around the boxes so that warm temperatures are maintained and the enzymatic hydrolysis can take place. The process is repeated until the colour of the pods turns dark brown (McCormick, 1988).

In flavouring applications, a dilute ethanolic vanilla extract is used, rather than the ground vanilla pods, due to their inherent instability. Also, presence of the unsaturated fatty components in the pod can result in rancidity. The vanilla pods are finely chopped and the flavour extracted by solvent extraction. When considering vanilla extracts, the concentration of vanillin is an important factor. The term 'fold' is used to indicate the concentration of the vanilla extract (Wallace, 1983).

1.3. Synthetic vanilla flavourings.

Demand for natural extracts exceeds supply and therefore drives up the market value of natural extracts (Butehorn and Pyell, 1996). As a consequence, there has

been a great increase in the demand for synthetically produced vanillin. Vanillin can be readily synthesised from lignin, quiacol or eugenol. Commercially, it is often produced from lignin present in concentrated sulphite waste liquors in paper mills (Hocking & Martin, 1997). The lignin, as a lignosulfonic acid, is treated with a lime solution to form a calcium lignosulfate compound. Sodium hydroxide is added and heat applied, converting it to the sodium lignosulphate form. In the final stage, sodium lignosulphate is oxidised to vanillin (Leong *et al.,* 1989). The synthetic product lacks the associated compounds that are present in natural vanilla, therefore, while the synthetic vanilla flavour exhibits a characteristic vanilla like note, it lacks some of the aromatic factors present in natural vanilla (McCormick, 1988).

Another synthetic product, ethyl vanillin, has three to four times the strength of flavour when compared to vanillin and is used mainly in the formulation of imitation vanilla. This product has reached a high level of quality, and can be used as a flavour additive in a wide variety of products (Martin, 1977). However, at high concentrations, the unacceptably harsh chemical character becomes evident. The imitation product gives an acceptable flavour quality at a considerable cost saving compared to the natural counterpart.

1.4. Adulteration and the blending of natural vanilla extracts.

A quality natural vanilla extract such as Bourbon or Mexican contains no added flavour compounds and is not blended with other natural extracts. Vanilla extracts containing no artificial additives are labelled on the packaging as natural

vanilla. There are also blended natural extracts, which consists of two or more vanilla extracts, to enhance its flavour characteristics (Bricout, 1974). Nature identical flavourings can contain synthetic vanillin, which are added to an extract derived from plant material or are solely derived from plant material. Lignin derived vanillin and ethyl vanillin, are common sources of synthetic vanilla flavouring.

The high price of natural vanilla extracts has resulted in frequent attempts at adulteration (Pyell, 1996;Yang & Peppard, 1994). Inexpensive synthetic vanillin substitutes have become an unwanted nuisance in the authentic extraction industry. Though artificial or imitation vanilla is heavily used in products requiring non-authentic vanillin, a problem occurs when a non-authentic extract/flavouring is presented as a natural extract.

Synthetic vanillin marketed as a natural vanilla extract is not difficult to identify with simple screening tests, as the level of vanillin is characteristically high and the chromatographic profile relatively simple (Bricout, 1974; Hermann & Stockli, 1982). A nature identical extract can be more of a challenge as it is plant based it contains an array of minor components (Lampretch, 1994). Apart from the marketing of synthetic or nature identical extracts as authentic natural vanilla extracts, synthetic vanillin can be added to inferior natural vanilla extracts to increase the percentage of vanillin (Lampretch, 1994).

1.5. Existing sample techniques.

The adulteration of natural vanilla extracts in the commercial market is a major concern. The need for quality control of flavouring agents claiming to be authentic vanilla extracts has given rise to several publications dealing with the analysis of vanilla extracts and flavourings (Belay, 1993; Pyell, 1996; Steffen, 1996). Several methods have been employed to characterise and help identify the origin of vanilla extracts, including IRMS, HPLC, GC and the relatively new technique CE.

1.5.1. High performance liquid chromatography.

Chromatography is a separation technique that can be used to analyse both organic and inorganic compounds. A common feature is the use of two immiscible phases: a stationary phase and a mobile phase. Substances to be separated distribute themselves between the mobile phase and the stationary phase in proportion to their partition coefficients. There are several separation mechanisms employed in chromatography common ones include: adsorption, partition, size exclusion, affinity and ion exchange (Jagerdeo *et al.*, 2000).

In HPLC, the mobile phase is a liquid pumped under high pressure through a column, which is packed with a stationary phase. A large percentage of HPLC separations involving organic compounds employ a non-polar stationary phase and a relatively polar mobile phase. This is generally referred to as reverse-phase chromatography. The non-polar stationary phase is commonly octadecyl bonded

to silica. The separation mechanism used in reverse phase HPLC is quite complex, and is best described as the combination of adsorption and partition processes (Boyce & Spickett, 2000). The mobile phase usually involves a water/organic solvent mixture. In general, early eluting solutes tend to be more polar preferring the mobile phase, whereas the non-polar solutes are retained longer on the column (Lampretch *et al.*, 1994).

HPLC has been used to separate and identify key natural components in vanilla extracts and to authenticate vanilla extracts. Ranadive (1992), developed a HPLC method, which utilized a C18 column and a methanol acidified water mobile phase. The major flavour components present in a number of natural extracts including Madagscan, Indonesian, Mexican, Tongan and Tahitian were quantified. The levels of *p*-hydroxybenzoic acid, *p*-hydroxybenzaldehyde, vanillin, and vanillic acid were measured in the different types of extracts. There was no apparent correlation between the levels of *p*-hydroxybenzaldehyde and *p*-hydroxybenzoic acid and the geographical origin of the extract (Jurgens, 1981). However, the Tahitian species was found to contain *p*-hydroxybenzoic acid at much higher concentrations than the other extracts and also contained anisic acid, anisic aldehyde and heliotropin, which were absent in other vanilla extracts, which are derived from *Vanilla Planifolia* (Ranadive, 1992).

HPLC has also been used to analyse synthetic extracts. Wallace (1983), successfully separated and quantified vanillin and several other phenolic components produced during the manufacturing of vanillin from pulp mill

effluent. This method is useful for the determination and analysis of synthetic vanillin derived from lignin.

1.5.2. Stable Isotope ratio mass spectrometry.

Stable isotope ratio analysis is a technique that uses a very different approach to HPLC in characterising vanilla extracts and flavourings. Isotope ratio mass spectrophotometer "IRMS" measures the relative abundance of isotopes such as $^{12}C/^{13}C$ or $^{14}N/^{15}N$. For carbon isotope analysis the sample is first combusted to produce CO_2 which enters a mass spectrometer where it is bombarded with electrons to produce the molecular ion CO_2^+. Most of the CO_2^+ will have a molecular weight of 44, however, a small amount of the CO_2^+ will contain ^{13}C and will, therefore, have a molecular weight of 45. The different weights are separated as the molecular ions travel through the mass spectrometer and are subsequently detected. The intensity and the relative abundance of both are measured (Riley, 1989; O'Malley, 1997). Approximately 98.89% of all carbon in nature consist of the carbon 12 isotope and 1.08% of all carbon occurs as the carbon 13 isotope. The ratio of these two stable isotopes in natural materials varies slightly because of isotopic fractionation during physical, chemical and biological processes (Hoffman & Salb, 1979). Photosynthesis also initiates fractionation of the isotopes (Riley, 1989; Lampretch *et al.*, 1994).

Plants can fix CO_2 by one of three mechanisms: Calvin synthesis, Hatch Slack synthesis and Crassulacean acid metabolism. Crassulacean acid metabolism is a combination of Calvin and Hatch-Slack synthesis. This is the pathway both

Vanilla Planifolia and Vanilla Tahitensis undertake during respiration, carbon assimilation occurs via the carboxylation of phosphenolpyruvate. Malic acid is accumulated which is then decarboxylated in the presence of light. The CO_2 liberated from the decarboxylation is fixed by ribulose-1,5-diphosphate which inturn yields 3-phosphoglycerate, (Calvin synthesis). During this process, the heavier isotope ^{13}C is discriminated against and the $^{12}C/^{13}C$ ratio in the plant is altered. As each photosynthetic mechanism discriminates against ^{13}C to a different extent, determination of the $^{12}C/^{13}C$ ratio for plant tissue indicates the mechanism used by the plant (Bricout *et al.*, 1981).

The $^{12}C/^{13}C$ ratio for vanillin is dependent on its source. For example, the $^{12}C/^{13}C$ ratio for naturally derived vanillin is typically –18 to –21 on the $\delta\ ^{13}C$ scale (Hoffman *et al.*, 1979). Table 1.1 lists the $\delta\ ^{13}C$ values for a number of vanilla extracts (Hoffman *et al.*, 1979). Lignin producing plants use the Calvin cycle for photosynthesis therefore synthetic vanillin derived from lignin has a higher $\delta\ ^{13}C$ value (-23 to –31), so extracts labeled as "natural" with $\delta\ ^{13}C$ values greater than –21 have been supplemented with synthetic vanillin to enhance the vanillin concentration (Riley, 1989). IRMS is a powerful technique for identifying adulterated samples where the vanillin is isolated from the extract using preparative HPLC. This particular method is limited to a few laboratories, as the instrumentation is not readily available and tends to be expensive.

Table 1.1. δ ^{13}C values for vanillin isolated from natural extracts and synthetic vanilla flavour, *Hoffman and Salb (1979)*

Sample	*Δ ^{13}C carbon values*
Madagascan natural extract	-20.4
Javan natural extract	-18.7
Mexican natural extract	-20.3
Tahitian natural extract	-16.8
Lignin derived vanillin	-27.0

1.5.3. Capillary electrophoresis.

In Capillary electrophoresis (CE), the column is a thin fused silica capillary usually ranging from 25 to 100 μm in diameter and 25-80 cm in length. Both ends of the capillary tube are placed in buffer solution with a detector placed at one of the ends. A negatively charged electrode is placed at the detector end of the capillary tube and a positively charged electrode to the injection end. A potential difference is applied across the capillary to (1) generate electroosmotic flow and as the result flow to the bulk solution through the capillary, and (2) separate the analytes by differences in their migration in the electric field. Charged analytes, introduced into the mobile phase, will be either attracted to or repelled from the electrodes depending on their charge. The difference in the migration speed of the analytes allows them to be separated (Boyce, 1999).

The most commonly used CE techniques are capillary zone electrophoresis (CZE) and micellar electrokinetic capillary chromatography (MEKC). CZE is used to separate charged ions while MEKC is used to separate neutral/uncharged compounds. MEKC uses a surfactant in the buffer solution. When a surfactant, such as sodium dodecyl sulfate (SDS) is present at high enough concentration (above critical micellar micellar concentration) it forms micelles. Separation of neutral and charged organic species is achieved as the analytes distribute bulk between the aqueous electrolyte and the organic micellar phase (Kuhn *et al.*, 1993; Boyce & Spickett, 2000). Since the introduction of MEKC in 1985, a wide range of organic compounds present in food, have been analysed by MEKC (Pyell, 1996).

Pyell developed an MEKC method for the analysis of vanilla extracts. Twelve flavour compounds found in vanilla extracts were separated using a buffer system which consisted of 100 mmol/l SDS, 10 mmol/l disodium borate and 100 mmol/l boric acid adjusted the pH to 8.7. The method was applied to real vanilla extracts (including Bourbon, Mexican, Tahitian, Madagascan, Indonesian and Tongan), which were used as flavour additives in bakery products and vanilla flavoured beverages. The major components found in the natural extract were quantified. Prelimary analysis indicated that the natural extracts did not contain any ethyl vanillin, therefore, it was used as the internal standard. Using peak height ratios, the concentrations of key constituents were determined with good reproducibility (Pyell, 1996).

The work did not however attempt to characterise the extracts based on quantitative differences. A range of food and beverages were also analyzed (including flavoured milk, coffee and ice cream). Vanillin was the only flavour compound detected in the coffee sample, however, key components characteristic of natural extracts were detected in the flavoured milk and ice cream. This CE method provides rapid alternative to HPLC for the screening of vanilla extracts.

1.5.4. Capillary gas chromatography.

In capillary Gas Chromatography (GC) components are separated as they are carried through an open tubular column, typically 30 m long, by an inert carrier gas. The relative interactions of the gaseous analyte molecules with the stationary phase (usually liquid) bonded to the wall of the tube, influences the separation of components. The separated components are then fed into a detector sensitive to the analytes of interest. GC is used for the analysis of a wide range of complex mixtures due to its high separation capabilities, (which far exceed HPLC), however, it requires the sample to be in the gas phase. Hence liquid samples are vaporised on entering the injection port prior to transportation to the separation column and are limited to volatile, heat stable compounds. If non-volatile components are to be analysed derivatisation has to be performed. This involves chemically modifying non-volatile components making them more volatile (Steffan, 1996).

While GC is ideal for the analysis of complex mixtures, the lengthy preparation steps often precludes its use. This is certainly the case for the routine sampling of

vanilla extracts (Steffen, 1996). Vanilla extracts contain both volatile flavour compounds and non-volatile compounds. Static headspace sampling can be employed to extract the volatiles, however, the methods are time-consuming and require specialised equipment. Klimes and Lamparsky (1996) used GC to identify a large number of flavour compounds in Bourbon extracts. Several sample preparation steps have been used to isolate the components and this included absorption of the volatiles onto a charcoal filter, a high vacuum distillation technique and solvent extraction. However, the complex preparation steps required for the analysis of vanilla extracts has precluded the use of GC for the routine sampling of extracts (Martin *et al.*, 1973; Schlack *et al.*, 1974).

1.6. Solid phase microextraction.

Solid phase micro-extraction (SPME) is a developing extraction technique. It utilizes a short (approximately 1 cm) fibre made of fused silica. The fibre is coated with a polymeric or extracting material. As the material is similar to that used in GC capillary column, the fibre is stable at higher temperatures (Yang, 1994). The coated fibre is attached to a wire, which runs through the open bore of a stainless steel needle of a syringe. The needle has a wider bore than the fibre, enabling it to be retracted into the needle protecting it from breakage (Figure. 1.1.). During an extraction the fibre is removed from the protective sleeve and exposed to the sample. When extraction is complete the fibre is retracted back into the needle (Wercinski, 1999). In the case of GC analysis, the needle (with the fibre inside) pierces the septum of the GC injector inlet. Once past the inlet the

fibre is exposed and the analytes are thermally desorbed from the fibre and swept onto the separation column (Steffen, 1996; Pawliszyn, 1997).

1.6.1. Extraction modes.

The sample (liquid or solid) is placed in a glass vial and sealed with a cap containing a septum. The protective sheath of the SPME pierces the septum and the fibre is then immersed directly into the aqueous sample (direct immersion (DI)) or exposed to the headspace (HS) (Figure. 1.2.). Direct extraction is ideal for extracting non-volatile components such as pesticides, high molecular weight components and for water analysis. However, it does rely on the sample being relatively clean. The sample is generally agitated to bring the analyte in contact with the fibre.

Headspace analysis is useful for analysing volatile components. It is less sensitive than direct immersion for all but the most volatile components. However, a major advantage is that fibre damage due to the sample matrix is avoided. Extraction is complete when equilibrium has been established between the fibre, the headspace, and the sample. The headspace mode also allows for modifications of the matrix, such as a change in pH, without any damage to the fibre (Pawliszyn, 1997). It also allows the extraction of target volatile components from a complex sample containing both volatile and non-volatile components. Therefore it is ideal for natural vanilla extracts, which contain both volatile and non-volatile components. In particular, they contain fats, waxes, and sugars that are not only

potentially damaging to the fibre but also to the separation column in the case of GC analysis.

Figure 1.1. SPME assembly device.

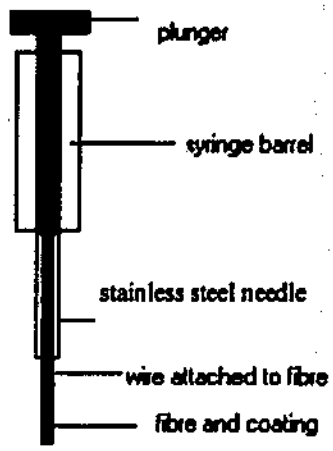
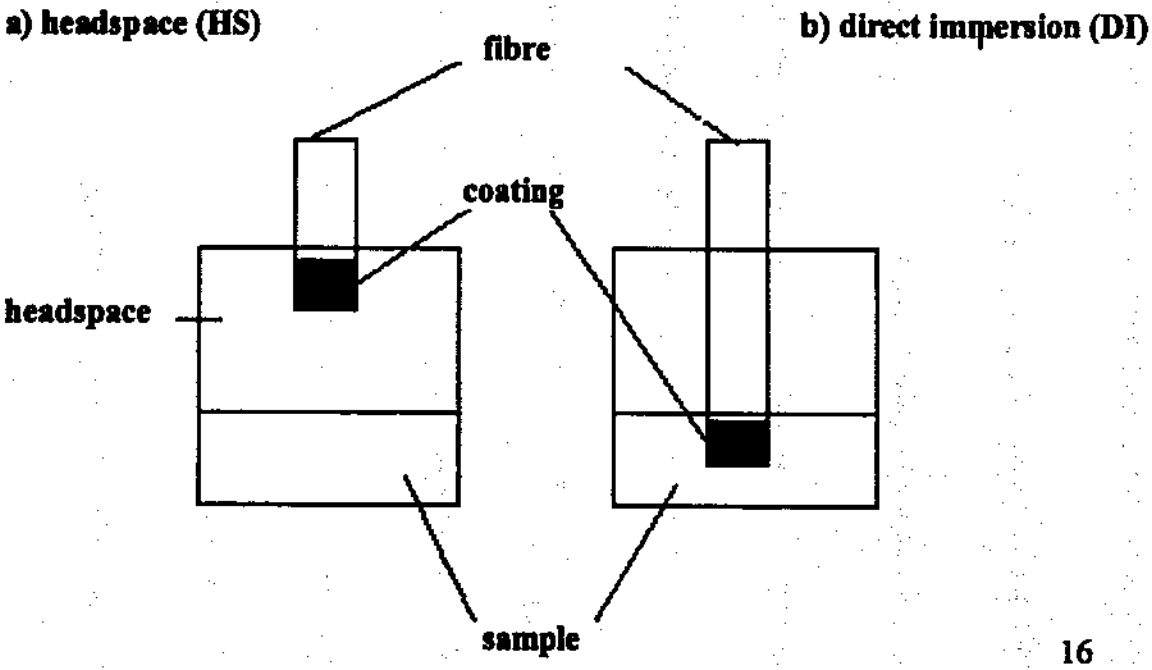


Figure 1.2. Main SPME extraction modes a) headspace extraction. b) direct immersion



1.6.2. Polymeric coatings.

The fused silica fibre is coated with an extracting polymeric material. There are several materials commercially available. Typically, the chemical nature of the target analyte determines the type of coating used, with the general rule "like dissolves like" applying. Fibre coatings are generally classified by their polarity and film thickness. The most common non-polar phase is the poly (dimethylsiloxane) (PDMS). It is available in three different film thickness of 100 μm, 30 μm and 7 μm. The thicker coating extracts higher concentrations of analyte, but the extraction time is longer as more analytes penetrate a larger volume (Steffan and Pawliszyn, 1996).

More polar phases include polyacrylate (PA) and carbowax (CW). The PA coating is not in liquid form at room temperature, and due to its rigidness, the migration of the analytes in and out of the fibre coating is slower. Therefore, extraction times are longer and desorption temperatures are higher when compared to other liquid coatings. The CW phase just like the carbowax GC column is ideal for polar compounds, however, a major draw back of this phase is its tendency to swell or to be stripped from the fibre. To overcome this problem, a highly crosslinked CW phase, CW/ poly(divinylbenzene) (DVB), has been synthesized (Wercinski, 1999).

Mixed phase coatings or porous particle blends involve a porous material such as DVB or carboxen, which has the ability to absorb and physically retain analytes. As the porous material is a solid it is suspended in a liquid such as CW or PDMS

to coat it onto the fibre. Their coatings tend to increase the extraction efficiency particularly of analytes that are of the correct size to be physically retained by the pores. They also have the advantage of extending the extraction possibilities of single phases by introducing more polarity (Pawliszyn, 1999).

The coating's ability to bond and crosslink determines its stability; three different classifications are available to describe the stability of the coating; nonbonded, bonded and crosslinked. Bonded coatings are described as a chemically bonded phase or crosslinked to the fibre or fused silica. They are very stable and can withstand washing and exposure to organic solvents. However, only very thin coatings are truly bonded such as the 7 μm PDMS coating. For thicker coatings, the phase is not bonded to the silica. The 30 μm and 100 μm coatings are examples of non-bonded phases (Pawliszyn, 1997).

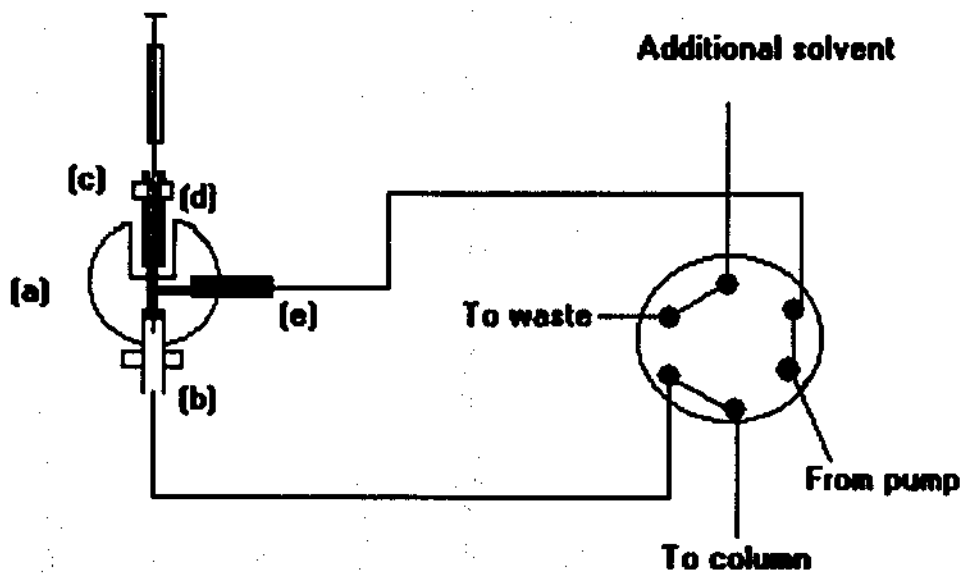
1.6.3. Interfaces with GC and HPLC interfaces.

As SPME is solvent free, it can be easily interfaced to most analytical instruments. Only the extracted components are introduced to the system, therefore, matrix contaminants, large volumes of solvent or vapour do not have to be dealt with (Jinno *et al.*, 2000). The most common analytical instrument used in conjunction with SPME has been the GC. The standard capillary GC injector can be applied to SPME as long as the injector liner has an inside diameter, which is close to the outside diameter of the fibre needle being used. The narrow inserts increase the linear flow around the actual fibre, which inturn removes desorbed analytes at higher efficiency (Kataoka *et al.*, 2000). The analytes are desorbed in

the injector and mixed with the carrier gas. During desorption which may take seconds or several minutes the injector is operated in the splitless mode. To avoid broad solvent peaks the temperature of the separation column is kept low during desorption to focus the sample and solvent at the top of the column (Caude & Rivasseau, 1995). The sample is then separated and analysed in the normal manner.

The typical SPME-HPLC interface consists of a desorption chamber and an injection valve system as shown in Figure. 1.3. The start of injection loop is enlarged to fit the initial section of the SPME syringe. The initial tubing of the SPME derivative (as seen in Figures 1.3.) holding the SPME device is sealed to withstand solvent pressures as high as 4500 psi. The desorption chamber is placed in between the injection loop and the injection valve. As the injection valve is placed in the load position, it allows the fibre to be introduced in the desorption chamber. A heater can also be installed at this point to assist in the desorption process. The desorption volume is similar to the volume of the typical injection loop (Jinno, 2000).

Figure 1.3. Schematic outline of a SPME device interfaced with a HPLC, a) Stainless steel 1/16" Tee piece peek tubing, b) 1/16" Stainless steel tubing, c) 1/16" Stainless steel peek tubing, d) Stainless steel peek union and e) with one piece peek union.



1.6.4. Optimising a SPME-GC method.

1.6 4.1. Fibre choice and extraction mode.

The selection of the appropriate fibre to extract a sample is a key parameter. Ideally one wants to match the polarity of the analytes and the coating to maximise extraction recovery and hence sensitivity. However, when choosing the fibre, its ruggedness and the sample matrix are also important considerations. The PDMS coating is rugged, resistant to swelling and has been well researched for SPME applications making it an attractive option for new applications. The sample matrix may preclude the use of some fibres and this has been illustrated in the analysis of drug formulations. Large proportions of drug formulations are present in hydrophobic solvents, (e.g. ointments). As non-bonded fibres tend to swell when exposed to hydrophobic solvents, the bonded 7μm PDMS is the best fibre to use in fingerprinting a wide range of pharmaceuticals (Yang & Peppard, 1994; Pawliszyn, 1999; Marsili, 2000a).

The extraction mode is also an important parameter to consider early on in method development. It depends on the analytes of interest, the method of analysis (GC or HPLC) and the sample matrix. As stated earlier, headspace is ideal for volatile compounds including those associated with flavour and aroma (Pawliszyn, 1997). However, direct immersion (DI) is necessary to analyse non-volatile components. For example, non-volatile acids such as *p*-hydroxybenzoic acid and vanillic acid in vanilla extracts, the non-volatile sulfur compounds in wine (Mestre *et al.*, 2000a) and aroma analytes from cheese products (Jaillais *et*

al., 1997) are important flavour compounds that are not easily extracted by SPME-HS. While DI is ideal for non-volatiles in relatively clean samples, it is not suitable for dirty samples and extra preparation steps may be necessary.

The sample matrix may also contain compounds that can damage the fibre such as high concentrations of sugar in wine must (Mestre *et al.*, 1999a), oil, protein and undissolved solids (Wercinski, 1998). Another consideration when deciding the mode of extraction is the method of analysis. For GC analysis, when no pretreatment such as derivitisation of the analytes is desired, the extraction of compounds that may be irreversibly retained on the GC column and/or the fibre, needs to be avoided. HS sampling is generally the preferred option as matrix effects, fibre contamination and irreversible retention are minimized (Pawlisyn, 1999).

1.6.4.2. Optimising sensitivity.

The ideal SPME method is one that provides the desired sensitivity in the minimum amount of time. Having decided on the fibre and the mode of extraction the major parameters that effect sensitivity may need to be considered. These include: mixing the sample, heating the sample during extraction, saturating the sample with salt, and maximising the ratio of liquid to headspace volumes in the vials (Lord, 1999; Wercinski, 1998).

Mixing is a widely used technique in both HS and DI-SPME. The agitation accelerates the transfer of analytes from the solution to the fibre coating. In

general, the equilibrium time progressively decreases with an increasing agitation rate. However, reproducible stirring rates are essential if good precision is desired (Lord & Pawliszyn, 2000). Sonication is a widely used and efficient agitation technique. The technique provides very short extraction times, which frequently approach the theoretical limits calculated for perfectly agitated samples. The only drawback is that care has to be taken as a large amount of energy is introduced into the system, which can raise the extracting temperature and hence influence extraction (Yang *et al.*, 1999).

Heating the sample can also increase extraction efficiency of some analytes. Heating increases the concentration of analytes, particularly semi-volatiles, in the headspace and therefore the amount available for absorption onto the fibre. However, in HS analysis, three phases exist (the headspace, fibre and liquid), any increases in extraction temperature (and hence the fibre) shifts the equilibrium between the fibre and the headspace in favour of the headspace. For volatile components this can result in a loss of extraction efficiency (Pawlisyzn & Zhang, 1993). Zhang and Pawliszyn (1993) cooled the fibre while simultaneously heating the sample to overcome this and achieved greater sensitivity. Elevated extraction temperature was successfully used in the analysis of nineteen varieties of freshly grated cheeses including swiss, cheddar, and romano. Compounds contributing to cheese aroma are medium molecular weight flavour compounds, which range from volatile to semi volatile compounds. However, the main components of interest are the non-polar compounds (Jaillais, 1999). In that experiment, the samples were initially analysed at room temperature but the

reproducibility was poor. At 60°C both reproducibly and sensitivity were improved.

The addition of salt to the sample is used to drive non-polar compounds into the headspace, while leaving polar compounds largely unaffected (Steffen & Pawliszyn, 1996). When salt is added to the sample, water molecules are tied up in hydration spheres around the salt ions. This reduces the availability of water molecules to dissolve the analyte molecules increasing the availability of the analyte for the fibre (Pawliszyn, 1999). For example, in the determination of barbituates the addition of inorganic salt to the sample increased the amount of undissociated drug extracted by the 65 μm Carbowax-DVB coating (Pawlinzyn, 1999; Rasmussen, 1997). Yang (1994) added NaCl at concentrations of 0.15 g/mL to increase the extraction efficiency of flavour compounds in coffee and fruit juices (Yang *et al.*, 1997).

The sensitivity of the SPME method is proportional to the number (concentration) of analytes, n, extracted from the original sample. The sample volume has a direct relationship with the number of moles in a solution. As the volume of the sample increases, the amount of analyte extracted also increases. The extraction amount will increase to a point where the sample volume becomes larger than the volume capacity of the fibre coating, which will cause a constant analyte volume in the fibre coating (Pawliszyn, 1996).

1.6.4.3. Absorption and Desorption

It is desirable for absorption or sampling time to be as short as possible to maximise productivity. For good precision it is best to sample when equilibrium conditions have been achieved. For very volatile compounds, equilibrium times are quick and occur in minutes. However, for some volatiles and semivolatiles equilibrium may take several hours. Higher sampling temperatures or mixing the sample can be used to speed up equilibrium times (Matich, 1999). However, if sampling time can be accurately controlled, precision is usually acceptable under non-equilibrium conditions (Wercinski, 1998). Desorption of the analyte is closely related to the efficiency of the separation and the precision. Desorption times are very rapid for volatile compounds but can be several minutes for more polar and high molecular weight compounds. For good precision and accuracy desorption needs to be reproducible and complete. High injector temperatures and a steady flow of mobile phase promote quick and effective desorption (Popp, *et al.*, 1999).

1.6.5. Applications of SPME

A very useful feature of SPME is the applicability to food, drug analysis, and the ability to extract substances from products without opening packages. The flavours contained in wine can be checked before its purchase by the insertion of a SPME fibre through the cork of the bottle into the headspace of the wine. As only an insignificant amount of flavour is extracted, the actual composition of the product does not change. The other big plus is, that the product does not become

contaminated by an extracting solvent. This similar process can be applied using on line monitoring during or, after the manufacturing of each item individually to ensure optimum product quality (Muller, 1999; Brunton *et al.*, 2000; Bicchi *et al.*, 1997; Miller *et al.*, 1999; Hall & Brodbelt, 1997; Ligor & Buszewski, 1999).

SPME has also been used to analyse flavour components in a variety of foods and beverages. Alcoholic beverages that have received attention include, wine (Olivia *et al.*, 1999), beer (Jelen *et al.*, 1999; Scarlata & Ebeler 1999), vodka (Ng *et al.*, 1996) and brandy (Ebeler *et al.*, 2000). Various coffees (Bicchi *et al.*, 1997) and cola beverages (Elmore *et al.*, 1997) have also been examined by SPME-GC to identify characteristic flavour and aroma characteristics. Volatiles in fruit, fruit juices and vegetables have been widely examined. Studies of these include orange juice (Steffen & Pawliszyn 1996; Jia *et al.*, 1998; Bazemore *et al.*, 1999) Brazilian fruit (Augusto *et al.*, 2000), apples (Song & others 1997), tomatoes and strawberries (Song *et al.*, 1998), berry fruits, mango and banana (Ibanez *et al.*, 1998) and fermented cucumbers (Marsili & Miller 2000b). Off flavours in wine (Mestre *et al.*, 2000b) and meat (Brunton *et al.*, 2000) have also been investigated as it provides valuable information on the life of the product. Less obvious foods such as cereals (Zhou *et al.*, 1999) and ham (Ruiz *et al.*, 1998). have been investigated by SPME to characterise the flavours produced. A recent comprehensive review illustrates the main food applications that have been assessed to date (Kataoka *et al.*, 2000). This suggest that SPME can be a very versatile quality control tool in beverage production (Guidotti & Panzironi, 2000).

1.7. Basis for research.

The application of SPME-GC for the analysis of a variety of volatile flavour compounds has recently been reported. In many instances this work has concentrated on the developing and optimising a SPME method for qualitative analysis. The complex sample matrix of natural extracts and the need for simple effective methods to screen and authenticate vanilla extracts and flavouring makes it an ideal sample base for SPME analysis.

The purpose of the study is to:

1. develop a SPME-GC method for the qualitative analysis of vanilla extracts and flavourings.
2. develop a SPME-GC method for the quantitative analysis of vanilla extracts and flavourings.
3. to evaluate the effectiveness of the method for discriminating between extracts and flavourings.

CHAPTER 2. EXPERIMENTAL

2.1. Reagents.

The reagents used in all experimental procedures were of analytical reagent grade. Vanillin, ethyl vanillin, cuminyl aldehyde, piperonal, *m*-methoxy benzaldehyde, *p*-methoxy benzaldehyde, ethyl-*m*-benzoate, ethyl octanoate, ethyl nonanoate, ethyl decanoate, 4-hydroxybenzyl alcohol, 4-hydroxy-3-methoxybenzyl alcohol, 4-hydroxy benzaldehyde, coumarin, ethanol, acetic acid, ethyl acetate, were purchased from Sigma Alldrich, Australia, and used upon receival.

2.2. Standards.

2.2.1. Preparation of a standard mixture for the development and optimisation of GC separation.

A standard mixture comprising the compounds listed in Table 2.1. was prepared in ethyl acetate. All standard components were present at concentrations of 1 $mg.L^{-1}$.

2.2.2. Preparation of standard mixture for the development and optimisation of a SPME-GC method.

A standard mixture comprising the substances listed in Table 2.2 was prepared in 95:5 water:ethanol. Ethyl-*m*-benzoate, piperonal and *m*-methoxybenzaldehyde were present at concentrations of 0.1 $mg.L^{-1}$ while all other standards were present at concentrations of 10 $mg.L^{-1}$. The lower concentrations of ethyl-*m*-benzoate, piperonal and *m*-methoxybenzaldehyde in the standard mix were necessary as these volatile components overload the capillary column at higher concentrations. Individual standard solutions for each of these compounds were prepared in a similar manner.

2.2.3. Preparation of calibration standards for quantitative analysis by SPME-GC.

A stock standard solution was prepared containing the components listed in Table 2.3. The concentration of the components varied and is detailed in Table 2.3. For example the concentration of vanillin was 1000 $mg.L^{-1}$ while the concentration of ethyl-*m*-benzoate was 0.5 $mg.L^{-1}$. A 20 fold dilution of the stock solution was used for qualitative analysis. For external standards quantitation, three working standards were prepared by taking 5 mL, 10 mL and 25mL of the stock solution and diluting them to 100 mL with 5% ethanol in water. Calibration standards were prepared in a similar way for internal standards. However, prior to making up to volume, 10 mL of a 1000 $mg.L^{-1}$ cuminyl aldehyde internal standard (prepared in 5% ethanol in water) was added to each standard. Aliquots (0 mL, 5

mL, 10 mL and 25 mL) of the stock solution were added to separate vials each containing 100mL of the diluted extract to prepare the standards for standard additions.

2.2.4. Preparation of calibration standards for quantitative HPLC analysis.

A stock solution comprising of substances listed in Table 2.4 were prepared in a 95:5 water:ethanol. 4-hydroxybenzyl alcohol, 4-hydroxy-3-methoxybenzyl alcohol, vanillin, ethyl vanillin and coumarin were present at 10 $mg.L^{-1}$ while 4-hydroxybenzaldehyde and *p*-methoxybenzaldehyde were present at 0.1 $mg.L^{-1}$. The aldehydes tend to absorb strongly at the selected wavelength and therefore lower concentrations of the aldehydes were used. Aliquots (0 mL, 2.5 mL, 5 mL and 10mL) of the stock solution were added to separate vials each containing 100 mL of the diluted extract to prepare standards for standard addition. A 1 in 20 dilution of the stock solution was used for qualitative analysis.

Table 2.1. Components and concentrations, present in the standard mixture method used to optimise the GC separation.

Analyte	*Concentration* (mgL^{-1})
m-Methoxybenzaldehyde	1.000
Protacatechuic acid	1.000
Ethyl-*m*-benzoate	1.000
Piperonal	1.000
Vanillin	1.000
Coumarin	1.000
Ethyl vanillin	1.000

Table 2.2. Components and their concentration present in the standard mixture used to develop and optimise a SPME-GC method.

Analyte	*Concentration* (mgL^{-1})
m-Methoxybenzaldehyde	0.100
Ethyl-*m*-benzoate	0.100
Piperonal	0.100
Vanillin	10.000
Coumarin	10.000
Ethyl vanillin	10.000

Table 2.3. The components, and their concentration, present in the stock solution used to prepare calibration standards for SPME-GC.

Analyte	*Concentration* (mgL^{-1})
Ethyl octanoate	0.500
m-Methoxybenzaldehyde	10.000
p-Methoxybenzaldehyde	10.000
Ethyl-*m*-benzoate	0.500
Ethyl nonanoate	0.500
Piperonal	10.000
Ethyl decanoate	0.500
Vanillin	1000.000
Coumarin	1000.000
Ethyl vanillin	1000.000
Cuminyl aldehyde*	1000.000

***Note, present in the stock solution used to prepare the calibration standards.**

Table 2.4. The components and the concentration present in the stock solution used to prepare calibration standards for HPLC analysis.

Analyte	*Stock solution* (mgL^{-1})
Vanillic acid	1.000
4-hydroxy-benzoic acid	1.000
4-hydroxy-benzaldehyde	0.100
Vanillin	10.000
Ethyl vanillin	10.000
p-Methoxybenzaldehyde	0.100
m-Methoxybenzaldehyde	0.100
Piperonal	1.000
Coumarin	10.000
Protocatucic acid	1.000

2.3. Samples.

The natural vanilla extracts and vanilla flavourings were supplied by a number of flavour houses and a local dairy food manufacturer. The suppliers names have been replaced with letters e.g. Bourbon A. Several of the natural extracts were certified and came with certification documents (see Appendix 1 for a typical example). The extracts varied in fold strength or concentration of vanillin and were typically too concentrated for direct analysis by HPLC or GC. The extracts were diluted using 5% ethanol in water to avoid column overload and the dilution details for each extract and flavouring are supplied in Table 2.5. Food samples including yogurt, ice cream and custard powder were purchased locally. The yogurt and ice cream samples were diluted by a factor of two using water prior to analysis.

Table 2.5. The dilution factor required for each extract or flavouring prior to HPLC and GC analysis.

Sample	*Dilution (SPME) ($mg.L^{-1}$)*	*Factor (HPLC) ($mg.L^{-1}$)*
A Bourbon natural extract	10.000	10.000
B Bourbon natural extract	10.000	10.000
C Bourbon natural extract	10.000	10.000
A Indonesian natural extract	10.000	10.000
B Indonesian natural extract	10.000	10.000
D Indonesian natural extract	10.000	10.000
B Tongan natural extract	10.000	10.000
A Mexican natural extract	10.000	10.000
A Tahitian natural extract	10.000	10.000
D Madagascan natural extract	10.000	10.000
A Nature identical extract	50.000	40.000
F Nature identical extract	50.000	40.000
A Synthetic vanilla flavour	50.000	40.000
F Synthetic vanilla flavour	50.000	40.000

2.4. Instrumentation.

2.4.1. GC-MS conditions.

For direct injection, 2.5 µL of the sample was injected directly onto the column. The gas chromatographic analysis was carried out using a Varian 3400 GC, and a Varian 2000 mass spectrometer (MS) detector. Helium was used as the carrier gas with a flow rate of 1.0 $mL.min^{-1}$. All the components were separated using a 30 m x 0.25 mm column with a 0.25 µm film of (5% phenyl)-95% Methyl polysiloxane (AT5) stationary phase (Alltech, Australia). The injector temperature was set at 250°C , the column was maintained at 80°C for 2 minutes then ramped to 200°C at $8°C.min^{-1}$ and further ramped to 250°C at $50°C.min^{-1}$ unless stated otherwise. The NIST '98 MS Library was used to identify key components in the samples.

SPME-GC analysis was carried out using a Varian 3400 GC fitted with a split/split-less injector suitable for SPME analysis, a Varian 2000 mass spectrometer detector and a Varian 9200 auto-sampler. Helium was used as the carrier gas with a flow rate of 1.0 $mL.min^{-1}$. The components were separated on a 30 m x 0.2 mm column with a 0.25 µm film of (5% phenyl)-95% Methyl polysiloxane (AT5) stationary phase (Alltech, Australia). The injector temperature was set at 250°C and operated in the split-less mode for 2 minutes unless otherwise stated. The column was maintained at 40°C for 2 minutes then ramped to 200°C at $8°C.min^{-1}$ and further ramped to 250°C at $50°C.min^{-1}$ unless

stated otherwise. The NIST '98 MS Library was used to identify key components in the samples.

2.4.2. HPLC conditions.

High performance liquid chromatography analysis was performed using a Varian 9010 gradient pump, a Varian 9050 variable wavelength UV-VIS detector and a Varian autosampler fitted with a 10 μL Rheodyne loop. Separation was achieved on an Altima C18 (250 mm x 4.6 mm) with 5 μm particles supplied by Alltech, Australia. A two solvent gradient elution method was employed. Solvent A was methanol while solvent B was a acetic acid/water solution (5:95 v/v). The gradient range was 0-1 minutes, isocratic 18% A in B; 1-8 minutes,18-50% A in B; 8-20 minutes, 50- 75% A in B; 20-30 minutes, 75% A in B. The flow rate was 1.5 $mL.min^{-1}$ and the wavelength of detection was 280 nm, (Lampretch, 1994).

2.4.3. Solid phase micro-extraction (SPME) conditions.

The SPME fibres, supplied by Supelco Australia, were conditioned as recommended by the manufacturer and the details are indicated in Table 2.6. The sample or standard mixture (200 μL) was transfed to a vial, which was sealed with a screw capped top containing a teflon lined septum. The fibre was exposed to the headspace of the sample for 40 minutes, unless otherwise stated. The fibre was then retracted and inserted immediately into the inlet of the GC. For non-ambient temperature extractions a heating block (Thermoline, BTC 9000) was

used to heat the vial and its contents. Each sample was analysed in triplicate, using a fresh vial and aliquot for each replicate.

Table 2.6. Maximum operating conditioning temperatures recommended by the manufacturer " Supelco " for a number of SPME fibres.

Fibre type	*Hub colour*	*Maximum Temperature (°C)*	*Conditioning Temperature (°C)*	*Time (hours)*
100 µm Polydimethylsiloxane	Red	280	250	1
85 µm Polyacrylate	White	320	300	2
65 µm Carbowax/divinylbenzene	Orange	265	250	0.5
65 µm Polydimethylsiloxane/ divinylbenzene	Blue	270	260	0.5

CHAPTER 3. DETERMINING GC SEPARATION CONDITIONS.

3.1. Introduction.

SPME has the advantage that it can be combined with GC without any major changes to the hardware. The injector insert is one of the few modifications necessary. It should be narrow to optimise desorption of the analytes from the fibre and to aid in the quick transport of the analyte by the carrier gas onto the column. The separation conditions and detector conditions generally remain unchanged. In this chapter the GC separation method is determined as well as the typical background scan for SPME-GC.

3.2. Results and Discussion.

3.2.1. Developing a suitable GC separation method.

A standard solution (see Table 2.1.) containing the volatile and semivolatile components known to be present in natural and synthetic extracts and introduced into the GC by direct injection, was separated using a method developed previously in the laboratory (Boyce, 1997). All the components in the extract were well resolved and separated within 20 minutes (Figure. 3.1.). The standard mixture was subsequently extracted using a PDMS fibre in the HS mode, the analytes were then desorbed from the fibre in the injector at 250°C for three minutes with the injector operating in the splitless mode. All other chromatographic conditions were unchanged. The component peaks were broad

and in some cases split. Clearly, the sample was not entering the column as a narrow plug (data not shown). The initial starting temperature of the column was 80°C, which was ramped at 5°C.min^{-1}. Under these conditions the volatile compounds were rapidly desorbed from the fibre and entered the column as narrow plug which were separated efficiently. However, less volatile components were desorbed gradually from the fibre and entered the separation column as a wide band. Components at the head of the band travelled through the column before the component at the end of the band leading to broad peaks and in more severe case split peaks. In an attempt to avoid this the column temperature was reduced to 40°C and kept at this temperature during the desorption process (5 minutes) and the experiment repeated. All the components, with the exception of protocatechuic acid, eluted from the column as narrow bands and within 15 minutes (Figure. 3.2.). Protacatechuic acid eluted after 25 minutes as a broad band, protocatechuic acid is non-volatile and for effective analysis by GC it requires derivitisation.

3.2.2. Determination of the background for SPME-GC.

The background scan for a SPME-GC run was investigated through a series of steps. Backgrounds scans were obtained under each of the following:

1. column temperature-programmed run without an injection.
2. column temperature-programmed run with a clean SPME fibre only in the injector port.
3. column temperature-programmed run with a clean SPME fibre and an empty vial.

The chromatogram obtained when no injection occurred is supplied in Figure. 3.3. The spectrum showed a flat baseline with no extraneous peaks, indicating that the GC system used in this study was clean. Figures. 3.4. and 3.5. shows chromatograms recorded when a clean fibre was placed in the injected port. The large broad band in Figure. 3.4. is characteristic of a fibre that has not been conditioned fully. Figure. 3.5. shows the background scan for a well conditioned new fibre. The spectrum has a flat baseline similar to that of Figure. 3.3. where no injection occurred. Fibres that have been left unused and not reconditioned tend to provide extraneous peaks and were more prevalent as the fibre aged.

The background scan for a SPME-GC method using an empty vial contained a large number of peaks, some of them at very high intensities. The peaks were identified as siloxanes and were attributed to the vial and/or septum. The vial and septum from another supplier were tested and a similar background was observed. The vials (and not the septa) were washed in ethyl acetate and dried in the oven at 100°C for 48 hours. The siloxane peaks were reduced significantly (Figure. 3.6). Wercinski reported the source of these peaks to be from the septa and not from the vials. However, any attempt to heat-teat the septa, even at low temperatures, distorted them reducing the effectiveness of the seal with the vial.

A Bourbon extract was analysed by SPME-GC to determine if the siloxane peaks co-eluted with key flavour components. The siloxane peaks were well resolved from any of the sample peaks (Figure. 3.7.). In addition, the intensity of the siloxane peaks was very much reduced as the components in the sample competed effectively for the fibre. No further work was carried out to remove the siloxanes. The vials were washed in ethyl acetate and dried at 100°C for 48 hours for the remainder of the study.

Figure 3.1 Separation of key flavour components by GC using direct injection.

1. *m*-Methoxybenzaldehyde **2. Ethyl-*m*-benzoate** **3. Piperonal**

4. Vanillin **5. Coumarin** **6. Ethyl vanillin**

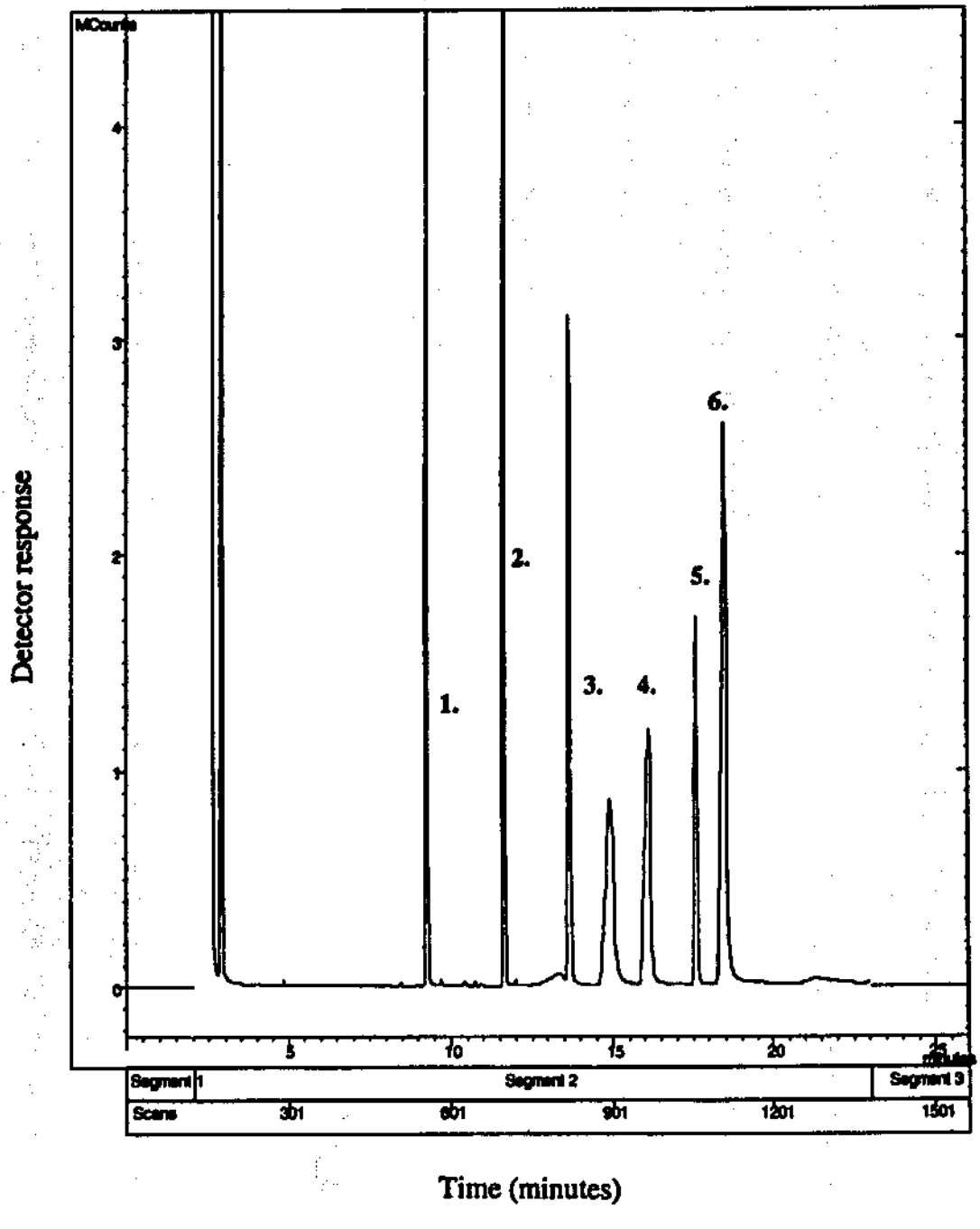


Figure 3.2 Separation of flavour components by SPME-GC.

1. Ethyl octanoate
2. *m*-Methoxybenzaldehyde
3. *p*-Methoxybenzaldehyde
4. Ethyl-*m*-benzoate
5. Ethyl nonanoate
6. Piperonal
7. Ethyl decanoate
8. Vanillin
9. Coumarin
10. Ethyl vanillin

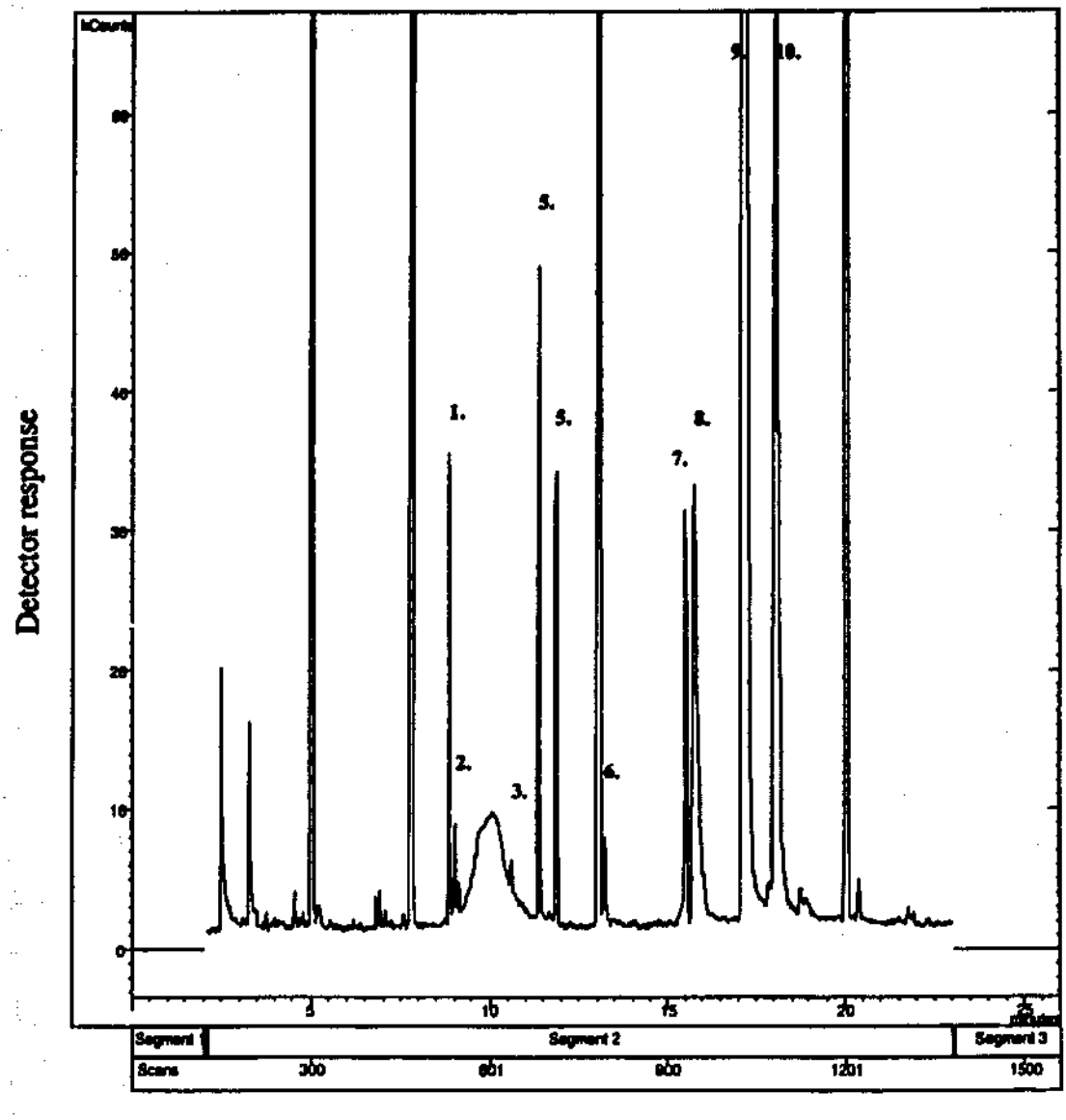


Figure 3.3 Typical background scan for GC mass analysis.

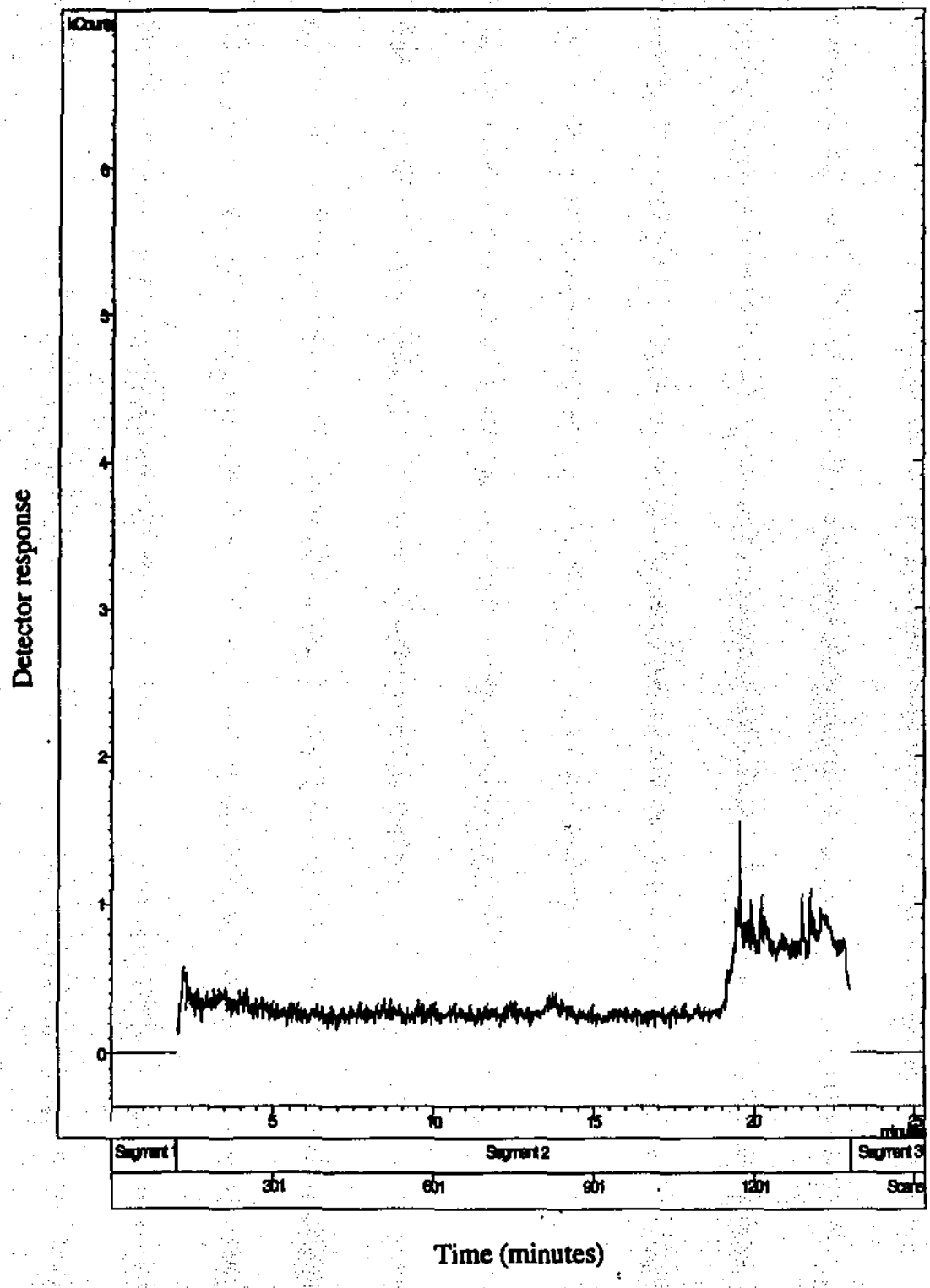


Figure 3.4.Typical background scan for SPME-GC-MS, when the fibre was not fully conditioned.

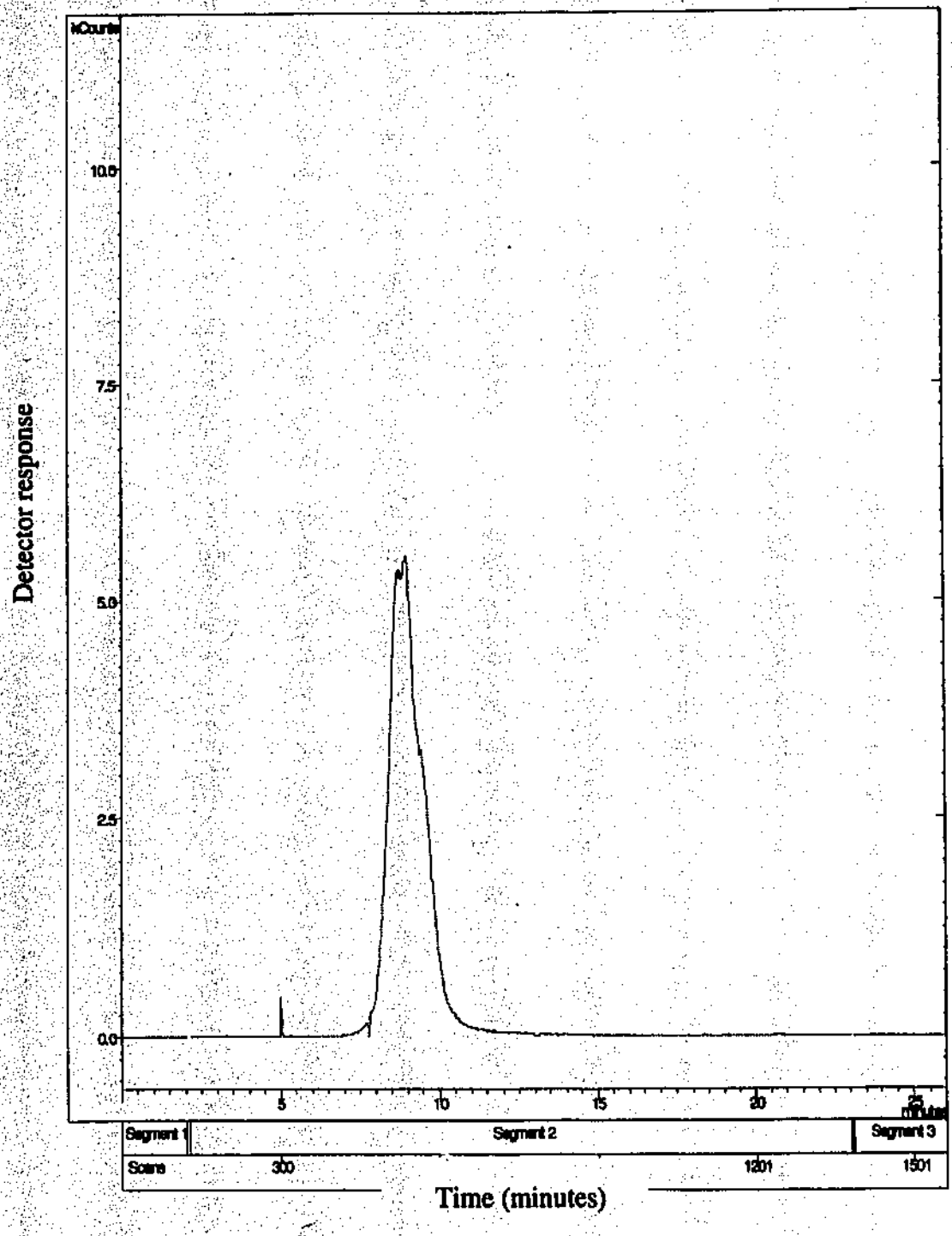


Figure 3.5 Typical chromatogram SPME-GC analysis when the fibre is fully conditioned and desorbed.

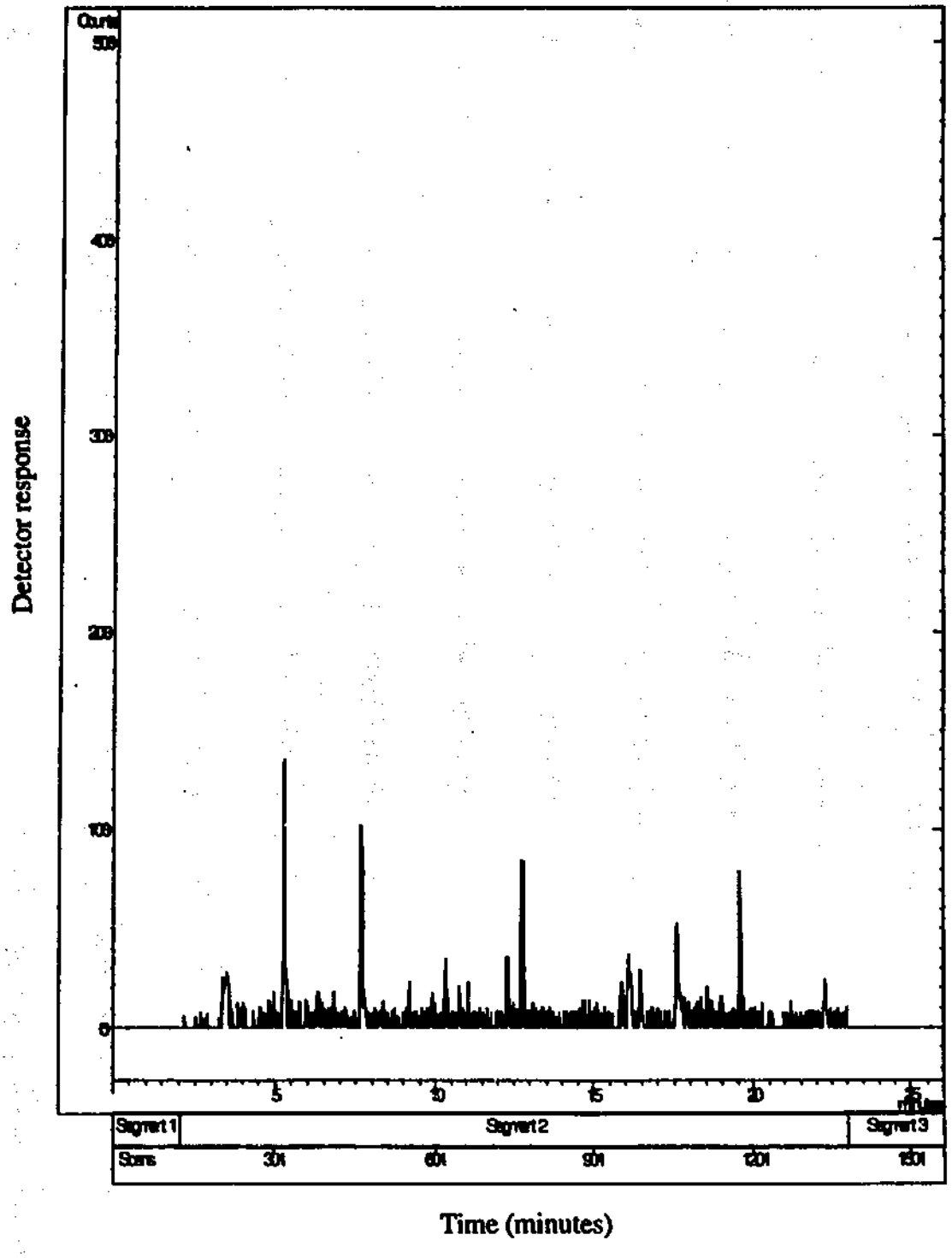


Figure 3.6 The concentration of residual siloxanes in the vials: unwashed: after washing with ethyl acetate.

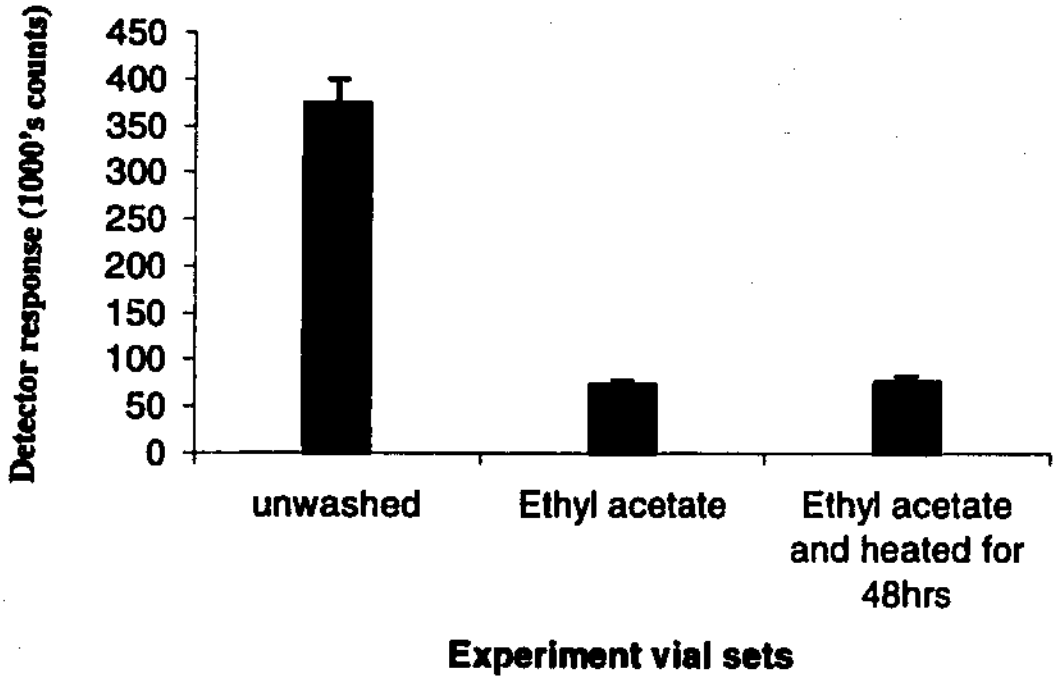
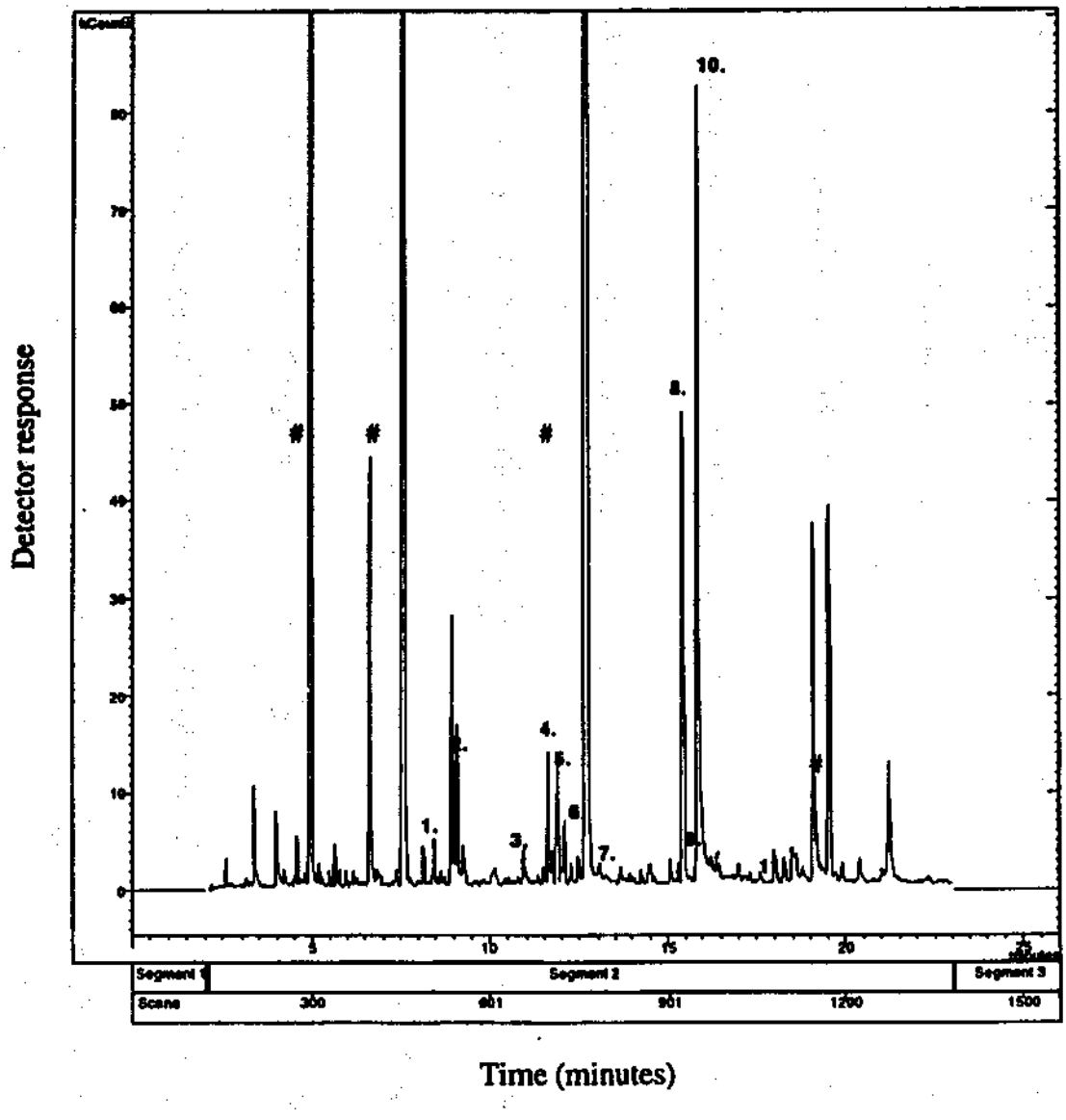


Figure 3.7 Separation of volatile components extracted by SPME and analysed by GC-MS. The siloxane peaks are indicated as #.

Legend.

1.	Ethyl hexanoate.	7.	Piperonal.
2.	*m*-Methoxybenzaldehyde.	8.	3-phenyl-2-propenoic acid methyl ester.
3.	*p*-Methoxybenzaldehyde.	9.	Ethyl decanoate.
4.	Ethyl-*m*-benzoate.	10.	Vanillin.
5.	5-propenyl-1,3-benzodioxole.	11.	Coumarin.
6.	Ethyl nonanoate.		



CHAPTER 4.DEVELOPING A SPME METHOD.

4.1. Introduction.

There are several factors that can influence fibre extraction. Key parameters include: extraction mode, fibre type, extraction time and temperature, desorption time, vial volume, ratio of liquid to headspace volume, ionic strength and pH of the sample (Pawliszyn, 1999, Kataoka *et al.*, 2000; Mayer & Fritz, 1997). The fibre type and the extraction mode are generally the first parameters to be determined. The absorption and desorption conditions such as extraction temperature, absorption time and desorption times are generally included in any SPME optimisation (Roberts *et al.*, 2000; Ruiz *et al.*, 1998). Other factors, such as ionic strength and temperature are optimised when sensitivity or extraction times is an issue. This chapter reports the development of an optimised fibre extraction method. The parameters investigated (fibre choice, absorption time and temperature, desorption time and pH) and their influence on the extraction will be discussed.

4.2. Major factors influencing fibre extractions.

4.2.1. Fibre choice.

The fibre coating is an important parameter as it influences component selectivity. Several coatings are commercially available and more continue to come on to the market. The first available fibres, and, in particular, the PDMS and PA fibres, have been experimentally trailed for a range of applications. The non-polar PDMS coating has been shown to be best at extracting non-polar

volatiles including essential oils from hops (Field, 1996), terpenes in wine (De la Calle Garcia *et al.*, 1998b; Mestre *et al.*, 1999b; De la Calle Garcia & Reichenbacher, 1998a), aroma compounds in cheese (Jaillais, 1999; Dufour, 2001), fruit (Radovic *et al.*, 2001; Song *et al.*, 1997; Miller & Stuart, 1999; Tia *et al.*, 1998; Song *et al.*, 1997; Ibanez *et al.*, 1998; Song *et al.*, 1998), and Brazilian nuts (Augusto *et al.*, 2000). The PA fibre was developed primarily for polar compounds but is also excellent for extracting semi volatiles such as flavour acids and alcohols in tobacco (Clark *et al.*, 1997). It has also been shown to be ideal for extracting complex mixes which range in polarity such as the profiling of flavour components in roasted coffee and coffee beverages (Bicchi, 1997). Other coatings including DVB and carbowax (CW) are also finding uses (Yang, 1994). The CW-DVB and PDMS-DVB fibres have been applied to a wide range of flavours and contaminants in food samples (Kataoka, 2000), and can expand the selective range of the fibre (Wercinski, 1998). Mixed component coatings, particularly those containing carboxen, are proving to be more sensitive than single coatings such as PDMS. For example, it is more sensitive in the extraction of volatile sulfur compounds present in wine (Mestre *et al.*, 2000a) and the determination of barbiturates (Pawliszyn, 1999). However, carboxen containing coatings were not commercially available at the start of this study and, therefore, have not been investigated here.

4.2.2. Adsorption and desorption conditions.

In order to extract components reproducibly from a sample, it is desirable to do so when the system is at equilibrium (Pawliszyn, 1997). In SPME headspace

analysis of the analytes, show equilibration between three phases, the liquid phase, the headspace and the polymeric fibre coating. A plot of extraction time versus amount extracted can be used to determine the time taken for the components to reach equilibrium between the phases. The point where the curve plateaus or levels off is considered to be the equilibrium time (Steffan & Pawliszyn, 1996). Highly volatile components tend to plateau or equilibrate in minutes, whereas lower volatility compounds can take up to an hour or longer (Steffen & Pawliszyn, 1996).

Once extraction is complete, the fibre containing the analytes is ready for desorption into a GC. Desorption is closely related to the efficiency of the chromatographic separation as the process involves inserting the fibre into a hot GC injector. A constant flow of carrier gas within the injector helps to carry the desorbed analytes from the injector onto a cool separation column. As the desorption may occur over several minutes, focussing of the sample at the top of the column by keeping the initial column temperature low is essential to avoid broad tailing peaks.

The efficiency of thermal desorption of the analyte in a GC injection port is dependent on the injector temperature and desorption time. The optimal desorption temperature should be approximately equal to the boiling point of the least volatile analyte.

4.2.3. Extraction temperature.

Increasing the extraction temperature enhances diffusion of the analyte into the fibre (which is often the limiting diffusion process), speeding up the equilibration times. The increased temperature also influences extraction sensitivity; it shifts the equilibrium between the fibre and the HS in favour of the HS and it increases the concentration of semi volatiles in the headspace (Dean & Hancock, 1999)

4.2.4. pH.

Adjusting the pH of the sample can improve the sensitivity of the method for basic and acidic analytes. In solution, dissociated and undissociated forms of the acid coexist. The dissociated form is very hydrophilic and is poorly extracted by a hydrophobic coating while the neutral species is more effectively extracted (Chee *et al.*, 1999). Lowering the pH of the sample, protonates acids, increasing affinity for the fibre particularly in DI mode. This was successfully applied to the flavour analysis undertaken (Yang, 1994).

4.3. Results and discussion.

We explored the effect of several variables on the extraction of volatiles from vanilla extracts. These included fibre choice, absorption and desorption time, temperature, and pH. The extraction mode, vial size and sample volume were not experimentally determined.

HS was selected as the extraction mode because of the nature of the sample matrix. Natural vanillin extracts are a complex mixture of volatiles, semi-volatiles and non-volatile flavour components. In addition, the matrix consists of fats, sugars and waxes. In order to successfully couple SPME with GC, with no sample pretreatment, such as derivitisation, the sugars, fats and non-volatile components must not be extracted, as these will damage the GC column. In DI mode the fats, sugars, and high molecular weight compounds would also compete with volatiles and semi-volatile components for the fibre. Furthermore, the waxes and high molecular weight compounds may irreversibly bind to the fibre (Arthur & Pawliszyn, 1990). To avoid both fibre and column damage, SPME-HS was chosen.

The sample vial volume was not varied and was fixed at 2.0 mL, because this vial size is compatible with the auto-sampler available in the laboratory and permitted the automated SPME extractions to be performed at room temperature. The volume of sample in the vial was also fixed at 200 µL, which ensured that HS, and not DI, occurred. The vials had a maximum volume of 2000 µL, however the autosampler inserted the fibre at 80% vial depth. Therefore, the volume was kept at 200 µL to ensure that only HS occurred.

4.3.1. Fibre choice.

Three types of fibre coatings were investigated for use in SPME-HS: poly(dimethylsiloxane), (PDMS), carbowax/poly(divinylbenzene), (CW/DVB), and poly(acrylate) (FA). Figure. 4.1. shows a comparison of the extraction

efficiencies of the fibre coatings for the analytes extracted from the standard mixture. The components in the standard mixture (Table 2.2) were chosen because (a) they are known to be present in vanilla extracts; (b) they include component characteristics of natural extracts and synthetic vanilla flavourings (e.g. ethyl vanillin); and (c) because these compounds have a range of volatility and polarity. Each fibre was effective at extracting six of the nine target components, however, none of the fibres extracted *p*-hydroxybenzaldehyde, protocatechuic acid or vanillic acid at concentrations of 10 $mg.L^{-1}$.

All the fibres extracted the early eluting (see Table 4.1 for retention time data) more volatile components (e.g. ethyl-*m*-benzoate, *m*-methoxybenzaldehyde and piperonal) in the greatest amounts, however, the PA fibre was superior in that it extracted more of each component. For the less volatile later eluting components, the PA fibre was also superior. For example, the PA fibre extracted over 50% more vanillin then either the PDMS or CW/DVB fibre. The greater sensitivity reported for multi-component coating was not observed here. The experiment was repeated using a natural Bourbon extract and the efficiencies of the different fibres for extracting the main volatiles are shown in Figure 4.2. The target compounds were selected because they were either in the standard mixture or were present in high concentrations in the natural extract. As observed with the standard mixture, the PA coating was the most efficient at extracting vanilin and ethyl-*m*-benzoate. It was also as efficient as the other fibres at extracting three major esters identified in the extract. The PA fibre was, therefore, used for the remainder of the study.

4.3.2. Adsorption and desorption conditions.

The optimal desorption time which gave maximum signal intensity for the analytes of interest was determined. The GC injector temperature was fixed at 250°C. The desorption time was varied from 0.5 to 3 minutes, with the column temperature held at 40°C for the selected desorption time, and the detector response for some target compounds recorded (Figure 4.3.). The signal intensity increased as the desorption time was increased from 0.5 to 1 or 2 min. Longer desorption times resulted in a lower response signal. Therefore, 1 or 2 minutes was considered as a suitable desorption time. A 2 minute desorption time, i.e. the injector operated in the splitless mode for 2 minutes and the column temperature held at 40°C for two minutes was used for subsequent work.

To determine the optimal extraction time (when the SPME-HS system is at equilibrium), the PA fibre was exposed to the standard mixture listed in Table 2.2. for differing amounts of time between 5 and 100 minutes at 25°C. The extraction time was plotted against the amount extracted and the plateau used to indicate equilibrium. From the graph it appears that equilibrium conditions were achieved for each component after 40 minutes (Figure. 4.4). It is evident from the graph that the more volatile components equilibrated faster and the leveling off was very defined. The less volatile components, such as vanillin, plateaued after 40 minutes but the change in slope was not as dramatic and may indicate that vanillin was not fully equilibrated. The experiment was repeated using a natural vanilla Bourbon extract and similar results were obtained (Figure. 4.5). For example 3-propenoic propyl ester, which is a volatile component, achieved

equilibrium after 30 minutes, and vanillin, which is a semi-volatile component, reached equilibrium after 40 minutes.

The precision of the extraction method was then investigated. The standard mixture was extracted several times using an absorption time of 40 minutes and a desorption time of 2 minutes. The percent relative standard deviation (% RSD) for all the compounds was excellent and ranged between 2.5 and 6.4% (Table 4.1) for seven extractions. When the experiment was repeated using a natural extract the % RSD for the main components gave similar values (2.6-8%) (Table 4.1).

4.3.3. pH

The pH of the standard mixture was varied to determine if lower pH might increase the extraction efficiency of the non-volatile acids to detectable levels. The natural extracts have a pH of approximately 4.7. The standard solution was prepared at three different pH's: pH 4.7, pH 2 and pH 1. At low pH the acids are protonated, making them more organic-like, reducing their hydrophilic nature and attraction for the aqueous phase. However, PA fibre did not extract detectable levels of vanillic acid or protocatechuic acid at either pH 1 or pH 2. The extraction of the other analytes in the standard mixture did not vary with pH (see Figure. 4.6). This is not surprising since they do not have ionisable hydrogen atoms.

4.3.4. Extraction temperature.

The effect of using higher temperatures to reduce the equilibration time was investigated. In addition, the effect of temperature on the extraction of the non-volatile acids was also of interest. The standard mixture, containing the same nine targets, was extracted using the PA fibre at different temperatures (ambient, 40, 60, 80 and 100°C). The high temperatures were not sufficient to increase the headspace concentration of the acids for their detection. As temperature increased the extraction efficiency increased for all detected components with the exception of ethyl-*m*-benzoate. The largest increases in extraction were observed for the less volatile components such as vanilla, coumarin and ethyl vanillin as their concentration in the headspace increased significantly. Similar results were recorded by Wercinski for the analysis of semivolatiles including vanillin (Wercinski, 1999). For the volatile ethyl-*m*-benzoate, the higher temperatures caused a fall in its extraction efficiency. The increase in temperature did not increase its already high concentration in the headspace as dramatically.

Furthermore, the shift in equilibrium between the fibre and the headspace favoured the headspace, reducing fibre absorption. The overall effect was a fall in the absorption of ethyl-*m*-benzoate. This effect was exacerbated at higher temperatures. 80°C was chosen as a compromise considering the extraction efficiency for the volatile components was still relatively high. The steady state sampling conditions were determined at 80°C by exposing the fibre to the standard mixture for different periods between 5 and 80 minutes. In general, equilibrium conditions were achieved in a shorter time. For example, equilibrium

conditions were achieved within 20 minutes for the more volatile components (*m*-methoxybenzaldehyde and piperonal) and achieved after 30 minutes for the less volatile components (ethyl vanillin and vanillin) (Figure 4.7.). While this method results in shorter absorption time, the error (expressed as standard deviation) incurred among replicates was greater than for the same experiment conducted at room temperature.

The precision of the elevated extraction method was also investigated. The standard mixture was extracted several times using a 30 minutes absorption time. The percent relative standard deviation for all the compounds ranged between 6.4 and 15.5% for seven extractions (Table 4.2.) indicating that the reproducibility of the method was poorer than for the same experiment conducted at room temperature. The poorer reproducibility between replicates for the higher temperature experiment is not surprising since the extraction process was done manually and involved quickly removing the fibre from the vial (held at 80°C) and inserting it into the GC inlet to minimise temperature changes.

4.3.5. Mixing the sample.

The effect of mixing the sample on equilibration times was investigated. The standard mixture was sonicated at room temperature for varying periods of time (2, 5, 10 and 20 minutes). These initial experiments showed large changes in the temperature of the sample. After just five minutes of sonicating the sample the temperature had increased substantially, in some cases by as much as 20°C. Therefore, it was necessary to thermostat the sample during sonication in order to

determine the effect of sonication on the extraction (Eisert, 1997). As we were unable to adequately control the temperature during sonication, these experiments had to be abandoned.

4.4. Concluding remarks.

Having completed the temperature work, it was decided that the extra sampling time required to achieve steady state conditions at room temperature was preferable to the extra labour required to process the samples manually at higher temperature. In addition, the automated process resulted in better precision composed to the manual process. A PA fibre, using a desorption time of 2 minutes, an extraction temperature of 25°C and an absorption time of 40 minutes was used to extract volatile components from vanilla extracts and flavourings.

Figure 4.1 **Comparisons of the extraction efficiencies of the PDMS, PA, and CW/DVB fibers, using a prepared standard mixture. The means were calculated from three replicates and the vertical bars show standard deviation.**

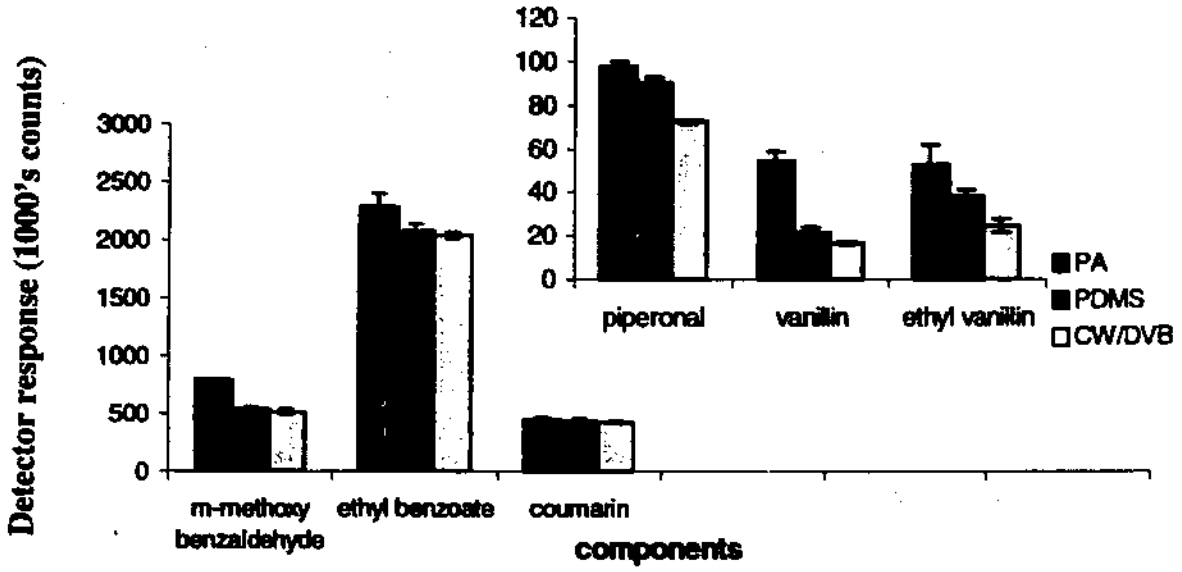


Figure 4.2 Comparisons of the extraction efficiency of the PDMS, PA, and CW/DVB fibers using a Bourbon natural vanilla extract. The means were calculated from three replicates and the vertical bars show standard deviation.

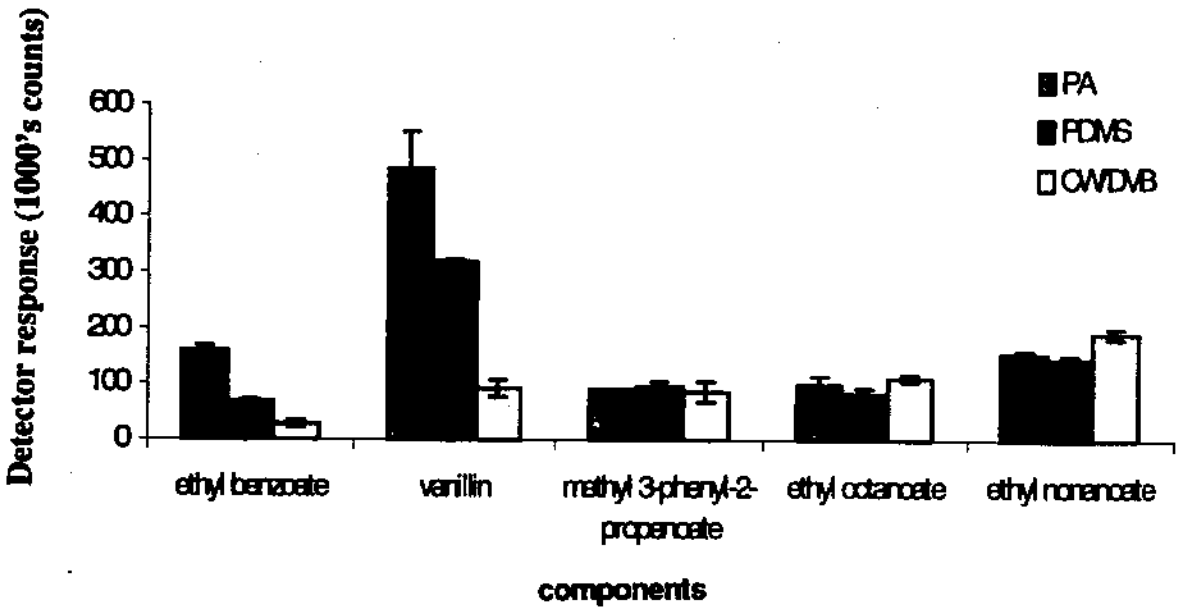


Figure 4.3 Comparisons of four different desorption times using a PA fibre and a prepared standard mixture. The means were calculated from three replicates and the vertical bars show standard deviation.

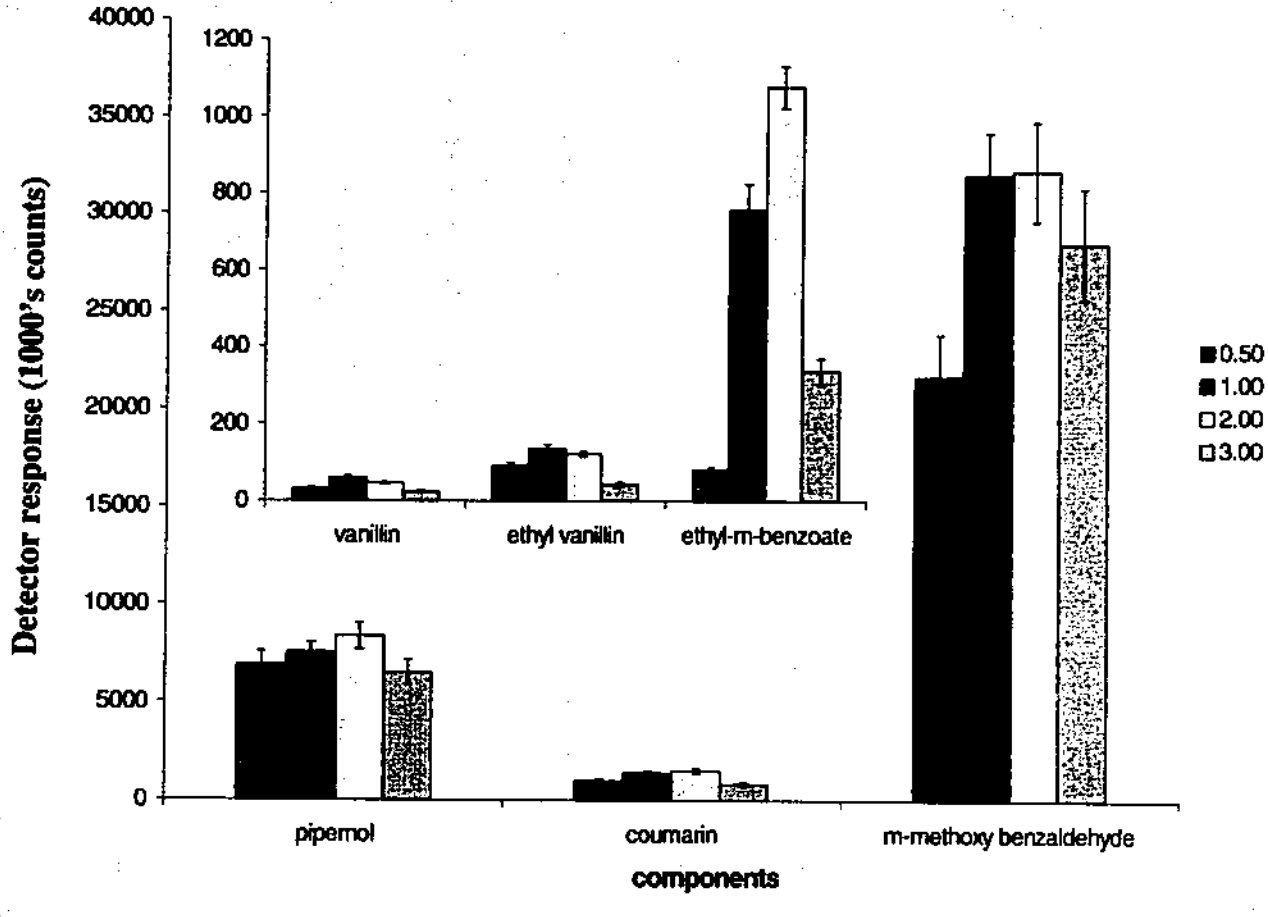


Figure 4.4 Effect of absorption time at room temperature on the extraction efficiency of the PA fibre using a prepared standard mixture. The means were calculated from three replicates and the vertical bars show standard deviation.

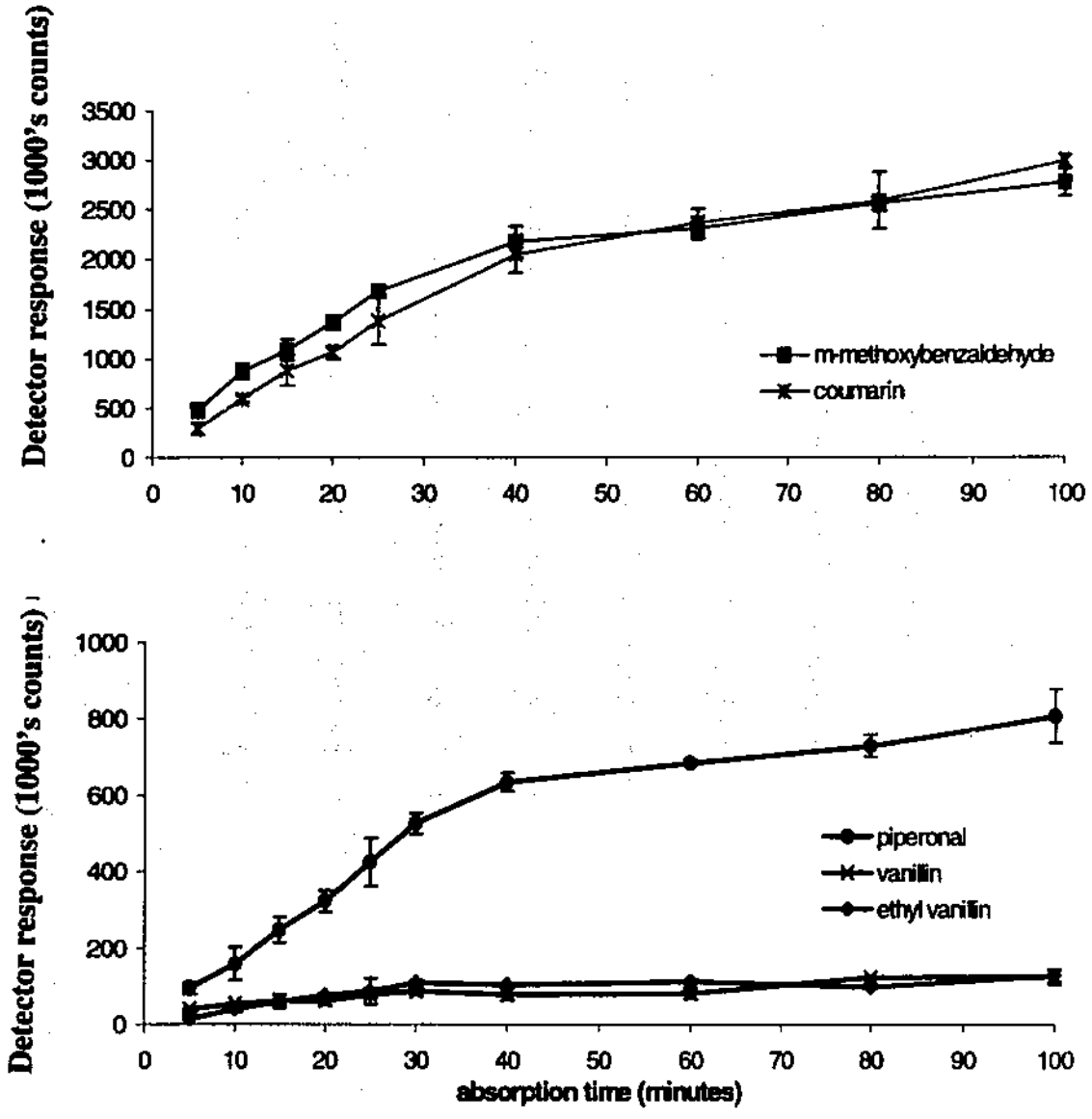


Figure 4.5 The effect of absorption time at room temperature on the extraction efficiency of the PA fibre using a Bourbon natural vanilla extract. The means were calculated from three replicates and the vertical bars show standard deviation.

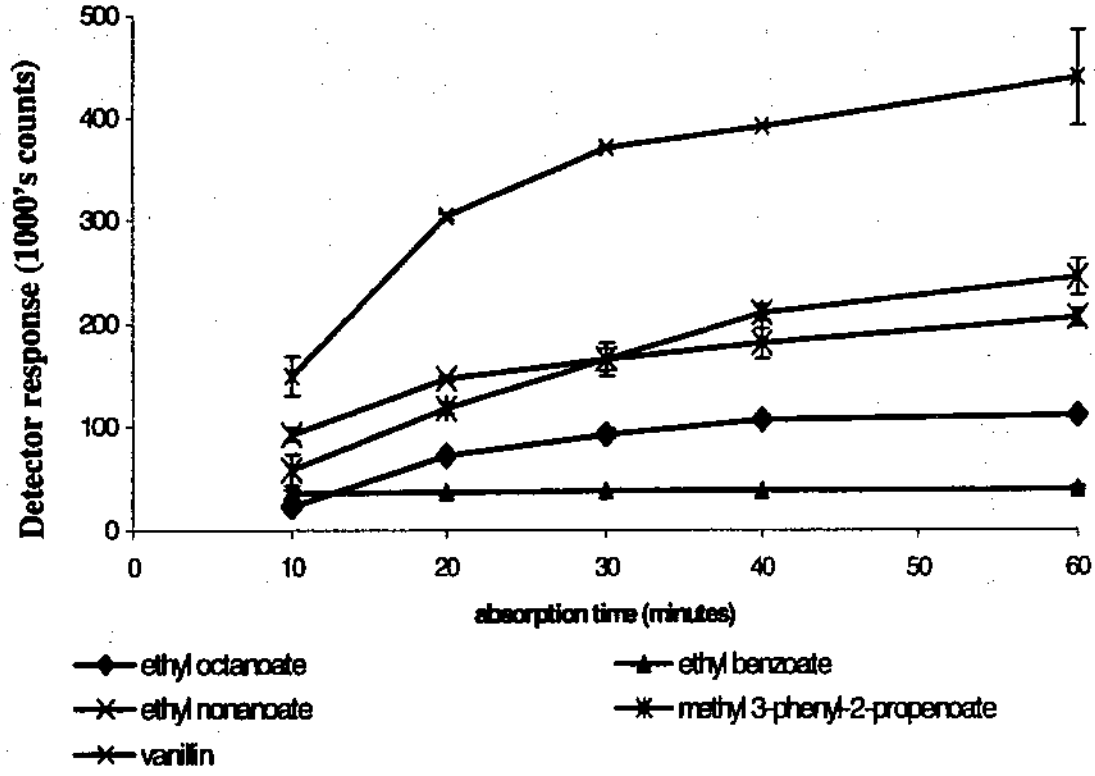


Figure 4.6 **The effect of adjusting the pH at room temperature, using a PA fibre and a prepared standard mixture. The means were calculated from three replicates and the vertical bars show standard deviation.**

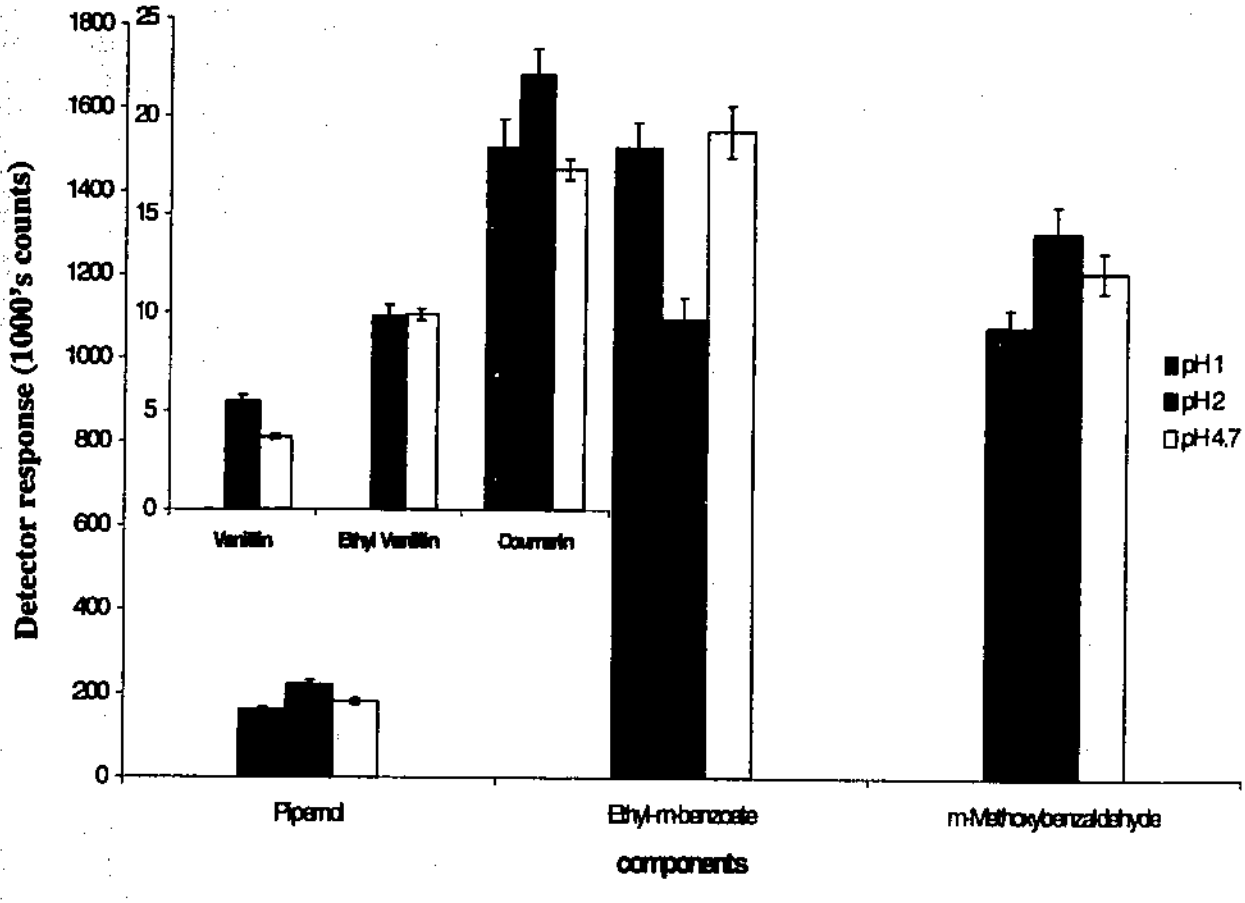


Figure 4.7 Effect of absorption time at 80°C on the extraction efficiency of the PA fibre using a prepared standard mixture. The means were calculated from three replicates and the vertical bars show standard deviation.

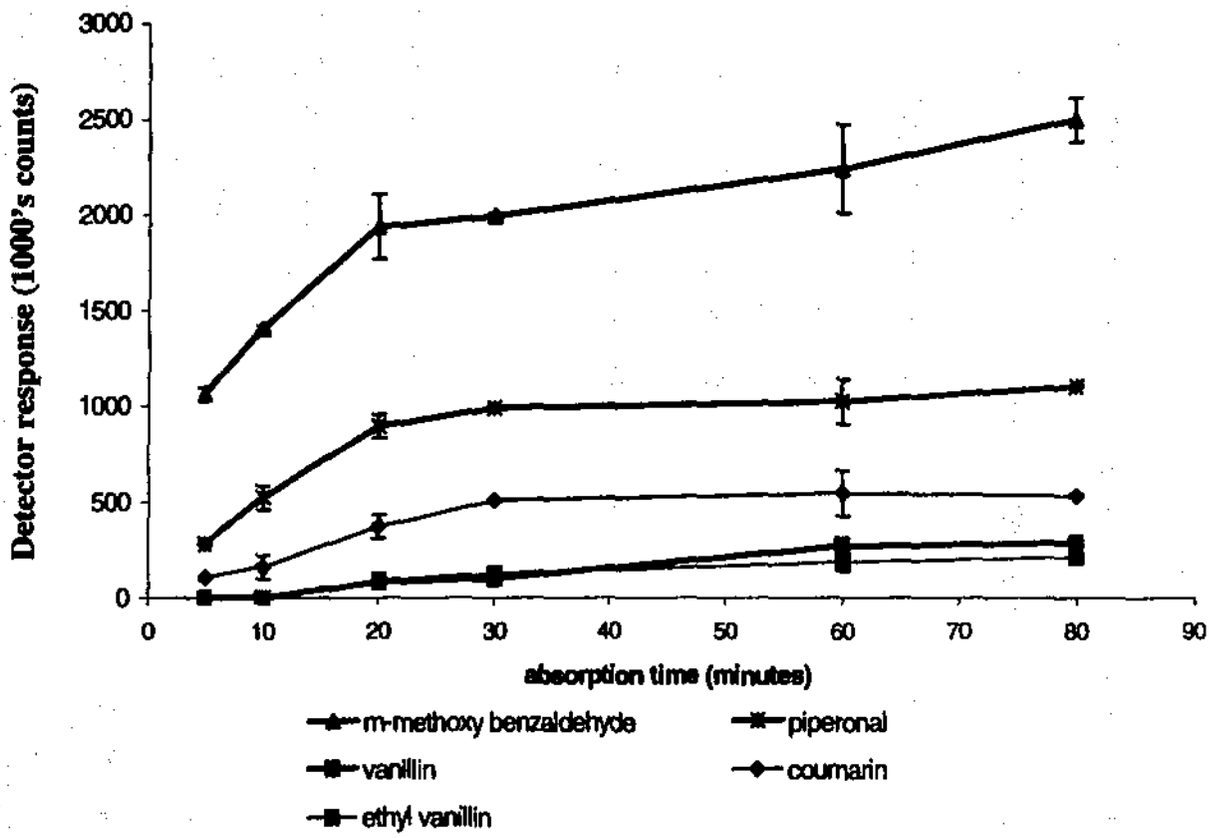


Table 4.1 Concentration, GC retention and precision data for components in the prepared standard mixture at room temperature.

Analyte	*Concentration* ($mg.L^{-1}$)	*Retention time* (*minutes*)	*Precision* (*% RSD*) 25°C
Cuminyl aldehyde	100.0	10.6	3.90
Ethyl octanoate	0.05	8.9	4.27
m-methoxybenzaldehyde	1.0	9.1	6.76
p-methoxybenzaldehyde	1.0	10.4	5.32
Ethyl-*m*-benzoate	0.05	11.6	2.85
Ethyl nonanoate	0.05	12.4	9.56
Piperonal	1.0	13.3	9.83
Ethyl decanoate	0.05	14.8	5.71
Vanillin	100.0	15.9	3.12
Coumarin	100.0	17.9	2.80
Ethyl vanillin	100.0	18.2	2.27

Table 4.2 Concentration, precision data for components in the prepared standard mixture at 80°C.

Analyte	*Concentration* ($mg.L^{-1}$)	*Precision* (*% RSD*) *80°C*
m-methoxybenzaldehyde	0.1	8.03
Ethyl-*m*-benzoate	0.1	6.39
Piperonal	0.1	13.50
Vanillin	10.0	15.51
Coumarin	10.0	10.75
Ethyl vanillin	10.0	14.65

CHAPTER 5. QUALITATIVE AND QUANTITATIVE ANALYSIS

5.1. Introduction.

5.1.1. Qualitative analysis.

Qualitative analysis provides a rapid method that may be used to identify the origin or nature of a sample. For example, plant species have been identified by analysing their essential oil or their polyphenol profile (Field *et al.*, 1996). The method relies on the presence of characteristic components or fingerprint regions unique to the sample. Qualitative analysis of vanilla extracts has been carried out using HPLC. The HPLC chromatograms can easily distinguish natural extracts from synthetic extracts, however, discrimination between natural extracts is limited (Ranadive, 1993). In this chapter we investigated if qualitative analysis of SPME-GC-MS generated chromatograms were useful in discriminating against natural extracts and synthetic extracts and, in particular, in discriminating between natural extracts. The extracts were also analysed by HPLC and the two methods compared.

5.1.2. Quantitative analysis.

SPME was initially considered as a screening tool for impurities with limited quantitative capabilities. However, its use as a quantitative tool down to ppt levels has been well documented (Clark & Bunch, 1997; Christoph & Levsen, 1999; Pawliszyn, 1997). For example, pesticides were sampled using SPME and subsequently analysed and quantified by GC (Volante *et al.*, 1998; Vitali *et al.*,

1998). There are several quantitative methods available including: external standards, internal standards and multiple additions. The method employed depends on the accuracy required, the background matrix and the presence of potential interferences. Generally, internal standards or multiple additions are employed when the sample matrix is complex and may influence the analysis. The multiple addition method has been used to qualify for a wide range of sample types. This particular method successfully quantified components from a wide range of sample matrices including fruit juices, essential oils and polluted water samples (Page & Lacroix, 2000; Field *et al.*, 1996; Yang & Peppard, 1994; Bazemore *et al.*, 1999). In this chapter the best quantitation method was determined and used to quantify the key components in natural extracts and flavourings. The results were compared with HPLC.

5.2. Results and discussions.

5.2.1. Qualitative results.

Six different types of natural extracts were analysed, each extract produced a unique chromatographic profile (Figures 5.1.-5.6.). Key components which were identified and their intensities for each extract are tabulated in Table 5.1. using selected ion chromatograms. The selected ion chromatograms incorporated single mass ions which identified each key component. For example a mass of 151 was selected for vanillin. The key components for each extract were determined and the selected ion chromatograms generated (Figures 5.7.-5.12.). The spectra were simpler as the siloxane peaks and unidentified minor peaks were removed. The

components determined as key were those that were unique to the extract and/or present in minor or greater amounts or had very different concentrations in different extracts. For example, *p*-methoxybenzaldehyde was determined as a key component because it is unique to Tahitian and 5-propenyl-1,3-benzodioxle was selected as it was the highest peak relative to the other components in the Bourbon extract, but was present in relatively lower concentration in the other extracts.

As expected all the natural extracts contained vanillin as a major component. Most of the extracts contained the esters in trace or minor amounts. However, Bourbon contained relatively high amounts of ethyl hexanoate when compared to vanillin and this can be seen when the simpler selected ion chromatograms are compared (Figures 5.7. – 5.12.). Ethyl-*m*-benzoate was also present in all the extracts in either trace or minor amounts (Table 5.1.) (Sostaric *et al.*, 2000).

The Bourbon, Tahitian, Mexican and Indonesian extracts were easily identifiable particularly when the key components were compared in the selected ion chromatograms (see Figures 5.7., 5.11., 5.12. and 5.8. respectively). The Tahitian extract (see Figure 5.11.) was readily distinguishable from the other extracts because of the presence of large amounts of *p*-methoxybenzaldehyde and of *p*-methoxybenzoic acid methyl ester which were present in trace amounts in the other extracts. The presence of components unique to the Tahitian extracts is not surprising since it is derived from *Vanilla Tahitensis*, the other extracts are all derived from *Vanilla Planifolia* (Sostaric *et al.*, 2000).

The Bourbon extract (see Figure 5.7.) had a complex chromatogram as most of the key components were present, with the exception of those that were unique to Tahitian presented in Figure 5.11. There were also a large number of unidentified compounds present (see Figure 5.1). The Bourbon extract was distinguished from the Indonesian extract by the different relative amounts of ethyl hexanoate, 5-propenyl-1,3-benzodioxole and ethyl nonanoate (compare Figures. 5.7. and Figures. 5.8.). The Mexican extract, Figure 5.12, was identifiable because of the absence of some key components including ethyl octanoate, *m*-methoxybenzaldehyde and the presence of piperonal. The relatively simple selected ion chromatogram (see Figure 5.6.) was also a distinguishing feature.

The Tongan and Madagascan extracts (Figures 5.9. and 5.10. respectively) had more similar profiles but differences were also evident on closer examination, particularly when the selected ion chromatograms were investigated. The Tongan extract, Figure 5.9, produced a relatively simple selected ion chromatogram as several key components (including piperonal, coumarin and *m*-methoxybenzaldehyde) were absent, and most components were present in trace amounts with the exception of vanillin and the unidentified compound. The Madagascan extract, see Figure 5.10, differed from the Tongan extract due to the presence and/or absence of compounds present in trace amounts when compared to the Tongan. The Madagascan extract contained *m*-methoxybenzaldehyde, 5-propenyl-1,3-benzodioxole and *p*-methoxybenzoic acid methyl ester, which were all absent from the Tongan extract.

Differences between the extracts varied from very distinctive to minimal. The distinctive features of Tahitian make it particularly easy to identify. The main differences between the Tongan and Madagascan, which are the presence or absences of trace components is unlikely to be sufficient to identify these extracts when supplied by a range of different flavour houses. There were subtle differences in the preparation of the ethanolic extracts would lead to losses of some trace components. In addition, the natural variation expected for plant material may contribute strongly to any differences outlined for these extracts.

The variation between natural extracts of the same origin was investigated. Three Bourbon extracts and three Indonesian extracts from different flavour houses were analysed. The selected ion chromatograms for the three Bourbon extracts, supplied by Company A, B and C and labelled as A, B and C are supplied in Figures 5.13.-5.15. The three bourbons recorded similar profiles. To highlight differences between the extracts, the presence and absence of key components are indicated in Table 5.2. The Bourbon extract supplied by Company A did not contain any coumarin but as it is present in only trace amounts in the other extracts, it is at lower than the detectable level of the instrument.

The chromatograms recorded for the three Indonesian extracts are supplied in the selected ion chromatograms, Figures 5.16.-5.18, and labeled A, B and D to reflect the companies that supplied them. The three extracts were similar, though some minor differences in the presence and absence of trace components were noted. All three extracts had piperonal and coumarin missing, (Table 5.2). Our study of extracts of the same origin but supplied by different flavour houses was limited

due to the difficulty in obtaining pure (non blended and certified) extracts. However, this small study does suggest that the variation in the profile of the extract is quite small. The quantitative analysis discussed in later sections will highlight more significant differences.

Nature identical (two samples) and synthetic vanilla flavouring (two samples) were analysed in triplicate. Profiles of the nature identical extracts and the synthetic extracts are provided in selected ion chromatograms, Figures 5.19-5.21 respectively. The nature identical extract was easily identified when compared to the synthetic extract as it had a relatively complex chromatogram and contained a number of flavour components characteristic of natural extracts such as *m*-methoxybenzaldehyde, *p*-methoxybenzaldehyde, ethyl-*m*-benzoate, piperonal and several esters. It was also easily recognisible when compared to the natural extracts as the vanillin content relative to the other components was very high. One interesting component contained in one of the nature identical extracts, was ethyl vanillin. This particular component is synthetically produced and does not occur naturally. Therefore, it is not accurate to label it as nature identical. The synthetic extract was characterised by a large ethyl vanillin peak. Other naturally occurring components including *p*-methoxybenzaldehyde, ethyl-*m*-benzoate, ethyl nonanoate, ethyl decanoate and vanillin are contained in the profile. These may have been added to enhance the flavour of the synthetic extract and to mask the harsh flavour of ethyl vanillin .

Qualitative analysis was sufficient to distinguish between natural extracts, nature identical extracts and synthetic flavourings. Our study also showed differences

between different natural extracts were evident and possibly significant enough to characterise an extract, particularly in discriminating between Mexican, Bourbon, Tahitian and Indonesian (Sostaric *et al.*, 2000). However, a study involving a large number of samples of a given extract would need to be completed, possibly using chemometric methods, to determine whether the differences were greater than the variation introduced due to processing and natural variation. This study was outside the scope of the project and would require the cooperation of the flavour houses. For more similar extracts such as Tongan and Madagascan, other data such as quantitative data might be useful for better discrimination.

5.2.2. Quantitative results.

5.2.2.1. Determination of a suitable quantitative method.

Three quantitative methods were investigated for the analysis of key components in natural extracts and vanilla flavourings: internal standards, multiple additions and external standards.

The method of internal standards was used to analyse a natural Bourbon extract. Cuminyl aldehyde was picked as the internal standard (I.S.) because it was available in the laboratory, it is volatile, it has a similar chemistry to the other key components, and it was well resolved from the other components in the extracts. Cuminyl aldehyde was added at concentrations of 10 $mg.L^{-1}$ to all standards and the sample. A stock standard mixture containing the components to be quantified in the natural extract, including vanillin, the esters and *p*-methoxybenzaldehyde,

was prepared and the appropriate dilutions made to generate a series of working standards (Table 2.3). The concentration of the analytes in any given standard varied due to the differing volatility of these components and their affinity for the fibre. For example, the standard mixture containing 50 $mg.L^{-1}$ vanillin, contained only 0.025 $mg.L^{-1}$ of each of the esters and 0.5 $mg.L^{-1}$ of the methoxybenzaldehydes. The standards and the Bourbon extract (in triplicate) were extracted by SPME and analysed by GC-MS. A calibration plot was generated on plotting the ratio of peak areas (IS:analyte) vs ratio of concentration (IS:analyte) and the response factor for each component generated. The response factor was then used to calculate the concentration of each component. However, the response factor should be constant over the concentration range studied. The response factor varied with concentration. For example, the correlation coefficient of the slope (response factor) was 0.52 for vanillin and 0.90 for ethyl vanillin. Repeating the experiment produced similar non-linear results. It is not clear as to why such non-linear results were recorded. Therefore, reliable quantitative data could not be obtained.

The key components in A Bourbon extract were quantitatively determined using the method of standard additions. A stock standard mixture similar to that used for the internal standards work was employed. The relative concentrations of the components were also unchanged. Four aliquots of the bourbon extract were spiked with varying amounts of the standard mixture to give final concentrations of (expressed for vanillin); 0, 50, 75, 100 $mg.L^{-1}$, and the experiment was carried out in triplicate (see section 2.2.4 for details). The expected linear increase in peak area with increase in concentration of the analyte was not observed, with the

exception of coumarin. The correlation coefficients (R^2) are tabulated in Table 5.3. The non-linear results may be the best result of the simultaneous addition of several analytes. Therefore, the experiment was repeated using the same Bourbon extract, although in this instance only vanillin at concentrations of 0, 50, 75, 100 $mg.L^{-1}$ was added to the aliquots of Bourbon extract. Similar results were observed and R^2 values are recorded in Table 5.3. To determine if the non linear results were due to the matrix, the experiment was repeated using a standard mixture (see Table 2.2.4. for components), instead of the Bourbon extracts. The mixture was spiked as before and again non-linear results were recorded. The experiment was repeated but in this instance the standard mixture was spiked with varying amounts of a diluted standard mixture to give final concentrations of 0, 25, 75, 100 $mg.L^{-1}$ (expressed for vanillin). The results did not differ from the previously acquired results with again non-linear results recorded. We were unable to determine the cause or reason for these results.

The method of external standards was tested. A stock standard solution was diluted to prepare a set of working standards in the concentration range 0-100 $mg.L^{-1}$ (expressed for vanillin) (see section 2.2.4. for more detail). The relative concentration of the components in the mixture is similar to that used in internal standards and multiple additions. The standards were exposed to the fibre and analysed as normal. The calibration curve was linear for each component and the slope R^2 values were recorded in Table 5.3. The key components in the Bourbon extract were then subsequently quantified. Cuminyl aldehyde was added to the extract to give a final concentration of 10.0 $mg.L^{-1}$ to determine if there were significant matrix effects that may influence the quantitative result. The

concentration of cuminyl aldehyde recovered from the extract was measured at 10.35 $mg.L^{-1}$. The standard deviation was 3.9%, which is within the variation experienced for SPME (see Table 4.1 in Chapter 4.). The concentration determined for vanillin was 1840 $mg.L^{-1}$, which was within the manufacturers specifications. The external standards was the method of choice. This was somewhat surprising, as given the complexity of the natural extract an internal standards method or standards addition method would seem more appropriate.

5.2.2.2. Quantitative analysis of natural extracts and flavourings analysed using SPME-GC-MS.

The concentration of the main components in the natural extracts were determined using the method of external standards and are tabulated in Table 5.4. Certified samples were supplied with a vanillin concentration. The experimentally determined vanillin concentration for these certified samples fell within manufacturer specifications (Table 5.4.), except for the Tongan extract which is lightly lower.

The concentration of vanillin in the two certified Bourbon extracts were very different with 3200 $mg.L^{-1}$ vanilla reported for B and 1840 $mg.L^{-1}$ for A. However, the values were within the suppliers specifications. A Bourbon was reported as being a 2-fold extract and as B Bourbon is almost double it may be a single fold. The C Bourbon extract, which was not certified had a similar vanillin content to the A Bourbon extract. The *p*-methoxybenzaldehyde concentration varied between all three extracts. For example the concentration found in A (0.19

mg.L^{-1}) was approximately double that found in C (0.083 mg.L^{-1}) which was approximately double that found in B (0.03 mg.L^{-1}). The concentrations of the other minor components were similar across all three extracts. There was nothing to suggest that the non-certified Bourbon was not the genuine article (Table 5.5.).

Both certified Indonesian extracts reported similar vanillin concentrations (2710 mg.L^{-1} for B and 2924 mg.L^{-1} for A). The non-certified Indonesian extract had a much lower vanillin concentration, approximately three times lower (1070 mg.L^{-1}), and *m*-methoxybenzaldehyde was detected at relatively high concentrations (0.12 mg.L^{-1}). This is in contrast to the certified Indonesian extracts, which contained no detectable levels of *m*-methoxybenzaldehyde. The low concentration of vanillin but the relatively high concentration of *m*-methoxybenzaldehyde suggests that the sample is unlikely to be a true Indonesian extract but, possibly a blend or an inferior Indonesian extract that has been spiked with selected flavouring components including *m*-methoxybenzaldehyde to improve its aroma (Table 5.6.).

The certified Tongan extract had a vanillin concentration of 1626 mg.L^{-1}, again this was consistent with the manufacturer's specifications. As indicated in the qualitative section, the Tongan extract reported a very similar chromatographic profile as the Madagascan extract. The quantitative data did not highlight any major differences other than the high concentration of vanillin for the Madagascan extract. However, since the fold of the extracts is not known the large difference in vanillin concentration may be due only to a difference in fold strengths (Table 5.7.).

The Tahitian was a certified extract contained 1140 $mg.L^{-1}$ vanillin. This value was consistent with the manufacturers specifications. The relatively large amounts of *p*-methoxybenzaldehyde a unique characteristic of the extract and highlighted during qualitative analysis was also confirmed here. The Tahitian extract contained 18.33 $mg.L^{-1}$ *p*-methoxybenzaldehyde, which was 100 times the amount present in the other extracts (Table 5.7).

The Mexican extract was not a certified sample, and the concentration of vanillin was experimentally determined as 988 $mg.L^{-1}$, the lowest of all the analysed extracts. It has previously been reported that Mexican extracts typically contain less vanillin when compared to other extracts (Ranadive, 1993). Individual component concentrations show no significant differences between the extracts however, the Mexican extract is one of only three extract to contain piperonal (Table 5.7).

The vanillin concentration of the uncertified Madagascan was determined as 3526 $mg.L^{-1}$, which is the highest for all six extract types. The Madagascan also contained 0.12 $mg.L^{-1}$ of *p*-methoxybenzaldehyde, the only other extract to report anything close was the Bourbon extract at 0.2 $mg.L^{-1}$. There was no piperonal component recorded in the Madagascan (Table 5.7).

Quantitative analysis provides useful information on fold strength and vanillin concentration, however, the differences in concentration of many of the other flavour components is not sufficient to highlight differences not already deduced

by qualitative analysis, with the exception of *p*-methoxybenzaldehyde and *p*-methoxybenzoic acid methyl ester in the Tahitian extract.

Nature identical and synthetic flavour extracts were also analysed and key components quantified. None of the nature identical or flavour extracts came with any certification documents. Both extracts labelled as nature identical had characteristically high vanillin concentrations in the range of 80,000 $mg.L^{-1}$ (Table 5.8.) which was approximately 100 times higher than the concentrations found in natural extracts. Both extracts contained a range of minor flavouring components including ethyl octanoate, ethyl-*m*-benzoate, ethyl nonanoate, piperonal and ethyl decanoate. This was expected since nature identical use a plant based extract to which vanillin is added. However, the piperonal concentration was very high in the nature identical extract supplied by Manufacturer A. In fact, it was approximately 500 times higher than the concentration found in natural extracts and in the other nature identical extract, indicating that it was added to the extract. The extract supplied by Manufacturer A also contained large amounts ethyl vanillin a synthetic substitute for vanillin indicated that this extract is in fact a synthetic extracts should be labelled appropriately (Table 5.8.).

Two vanilla flavourings labelled as synthetic flavourings were analysed and shown to contain vanillin and ethyl vanillin (see Table 5.8.). The flavour extract supplied by Manufacturer A contained just vanillin and ethyl vanillin, and the latter was present in very high concentrations (approximately 50000 $mg.L^{-1}$). The flavouring extract supplied by Manufacturer F contained relatively more vanillin

and less ethyl vanillin. It also contained other flavouring components including a range of esters and ethyl-*m*-benzoate. The lower concentration of ethyl vanillin and the relatively higher concentration of vanillin and the presence of other flavouring compounds would provide a more subtle vanilla flavouring and also help mask the harsher flavour of ethyl vanillin (Belay & Poole, 1993).

5.2.3. Applications to real samples.

The SPME method was also investigated for the potential of the technique to identify the source of vanilla flavouring used in common food products. The samples included yogurt, natural vanilla ice cream and custard powder. The chromatogram recorded for the yogurt is supplied in Figure 5.22, and showed the presence of ethyl vanillin, indicating that the vanilla yogurt was flavoured using a synthetic flavour extract. This experiment also highlighted one of the major advantages of SPME analysis ie. The minimal sample preparation required. The sample preparation was negligible and involved exposing the fibre to the headspace of a freshly opened tub.

The fibre was exposed to melted natural vanilla ice-cream. The resulting chromatogram (see Figure 5.23.), indicated the presence of *p*-methoxybenzaldehyde, ethyl decanoate and small amounts of vanillin. A comparison to the chromatogram recorded of the original extracts and the absence of ethyl vanillin it indicates that a natural vanilla extract was used. The relative large amounts of *p*-methoxybenzaldehyde

compared to vanillin also indicated a Tahitian extract was use to flavour the ice cream.

Vanilla custard powder was analysed to determine if the method could be extended to solid samples. At room temperature SPME-GC analysis recorded a blank baseline, indicating that the concentrations of the volatiles in the headspace were minimal. The high temperature SPME method (absorption time 30 min at 80°C) described in Chapter 4 was employed. Ethyl vanillin and vanillin were detected under these conditions (see Figure 5.24). The presence of ethyl vanillin indicates that the custard was flavoured with a synthetic vanilla flavouring.

The real sample analysis displayed the possibility of screening food samples without the associated time consuming (4 to 24 hrs) and laborious extractive procedures. Such as, vacuum or steam distillation (Olivia *et al.*, 1999; Jelen *et al.*, 1998; Shafer *et al.*, 1997). As liquid and solid food samples can be easily analysed. The volatile components can be simply extracted by waving the SPME fibre over a ice cream or yoghurt tub, with no adverse loss in sample amount. The technique can qualitatively distinguish to a high degree of accuracy if the sample used a synthetic or natural extract. It is also possible to determine the origin of the more identifiable natural extracts used in flavouring the food product.

5.2.3. Analysis of extracts by HPLC

The extracts were also analysed by HPLC to (a) compare existing HPLC data for extracts with our extracts, (b) to determine what common components in the

extract would be detected by both HPLC and SPME and (c) to compare quantitative data.

The extracts were separated on a C18 column using a methanol/acidified water mobile phase and the chromatograms are supplied in Figures 5.25-5.38. The key components were identified by using retention time and by comparing diode array spectra of standards and the components in the extract (see Appendix C for diode array scan of vanillin). The major components identified in the extracts by HPLC included: vanillic acid, *p*-hydroxybenzoic acid, *p*-hydroxybenzaldehyde, vanillin, ethyl vanillin and *p*-methoxybenzaldehyde. Many of these components are not detected by SPME-GC-MS because of their non-volatile nature. Components which, were detected by both SPME-GC and HPLC, included vanillin, ethyl vanillin and *p*-methoxybenzaldehyde. Coumarin and piperonal, which were, separated and detected in the standard mixture were not detected in any of the extracts (Appendix B). The natural concentration of these components in the extracts is low and as the extracts were typically diluted by a factor of 10 to ensure that vanillin did not overload the column, the two components were possibly below the detection limit of the instrument. As vanillin and ethyl vanillin were detected by both HPLC and SPME-GC, their concentrations were determined by both methods and the results compared.

The HPLC data obtained for vanillin in the certified natural extracts was compared with the vanillin range quoted by the supplier and also with the data generated by SPME-GC (Table 5.5.). The HPLC results did not always support the suppliers specifications or vanillin obtained from SPME-GC analysis. For

example, the Indonesian B extract contained 3350 $mg.L^{-1}$ vanillin, which was well above the concentration range quoted by the manufacturer (1800-2700 $mg.L^{-1}$) and very different to the value determined by SPME-GC (2710 $mg.L^{-1}$), which lies within the manufacturers specifications. In contrast, the HPLC result obtained for vanillin in the Indonesian A extract (2920 $mg.L^{-1}$) was very similar to that determined by SPME-GC (2880 $mg.L^{-1}$) and both these values fell within the concentration range specified by the manufacturer range. As HPLC is a well developed and recognised quantitative method the conflicting results were not immediately attributed to poor HPLC data (Hermann & Stockli, 1982). Initially the higher value recorded by HPLC for the Indonesian B extract was attributed to co-elution, however, an examination of the diode array spectra for vanillin ruled this out. Analysis of the certified Bourbon extract also provided some interesting results. The HPLC and SPME-GC quantitative data for vanillin in the Bourbon A extract were in close agreement with values 1850 $mg.L^{-1}$ and 1840 $mg.L^{-1}$ respectively recorded. These values also sat with in the range (1800-4000 $mg.L^{-1}$), which was very wide, specified by the manufacturer. However, the same data recorded for the Bourbon B extract was very different. The HPLC and SPME-GC data varied significantly (2720 and 3200 $mg.L^{-1}$ respectively) and again the HPLC data did not support the data supplied by the manufacturers range. To explain the conflicting data, the age and shelf life of the samples were investigated, as the concentration of some flavour components will decrease slightly overtime, particularly if stored in light conditions. However, over the shelf life of the sample, the change in concentration of vanillin should not fall dramatically reduced and certainly not fall below the lower concentration range (pers. Comm.). Both extracts were of similar age, less than one year old when

analysed (shelf life of 2-5 yrs) and both were stored under similar conditions (at room temperature and in the dark). Therefore, the low result obtained from the HPLC was unlikely to be due to sample degradation.

The certified Tongan extract recorded similar HPLC and SPME-GC results for vanillin concentration (1930 and 1630 $mg.L^{-1}$ respectively) and the values were within the manufacturers specifications (1600- 2700 $mg.L^{-1}$). The HPLC result recorded for vanillin content in the Tahitian extract was outside the manufacturers specifications and also different for the value determined by the SPME-GC.

The SPME-GC results supported the concentration range quoted by the manufacturer for all the six certified samples. The HPLC results for three of these extracts did agree with the certification data and in these instances the data closely agreed with the SPME data. The HPLC data for the other three extracts did not agree with the certification data. These results suggested the SPME-GC is reliable quantitative method and can be used to check HPLC data or indeed as an alternative to HPLC analysis.

The vanillin concentration of other non-certified natural extracts, nature identical and synthetic extracts were determined by both HPLC and SPME-GC and compared (Table 5.5.). In many cases the HPLC vanillin concentration was much lower than the SPME-GC results. For example, HPLC reported a vanillin concentration of 2250 $mg.L^{-1}$, for the Madagascan extract, which was 1000 $mg.L^{-1}$ lower than the SPME-GC results. The consistently lower vanillin concentration

determined by HPLC for several extracts may suggest that the vanillin is undergoing degradation in the mobile phase. The concentration of ethyl vanillin in the synthetic extract was determined by both methods and there was good agreement, less than 10 % deviation.

Qualitative analysis of the HPLC chromatograms recorded for the natural extracts and synthetic flavouring clearly identified the synthetic flavourings. Like the SPME-GC generated spectra, the HPLC chromatogram for the synthetic flavourings were very simple and contained few peaks in contrast to the natural extracts. The chromatograms of the natural extracts were very similar with the exception of Tahitian. The relatively high concentration of *p*-methoxybenzaldehyde was a distinguishable feature for the Tahitian extract, which was also reported by Ranadive. He also reported very high concentrations of *p*-hydroxybenzoic acid for the *Vanilla Tahitensis* derived extracts compared to the *Vanilla Planifolia* derived extracts, typically 10 times more *p*-hydroxybenzoic acid for the *Vanilla Tahitensis* than the *Vanilla Planifolia* derived extracts, Jurgen in 1981 also reported similar findings. The quantitative data recorded for the natural extracts largely supported this trend. The Tahitian extract had 9390 $mg.L^{-1}$ *p*-hydroxybenzoic acid while the other extracts had concentrations that ranged from 25 – 1140 $mg.L^{-1}$ (Table 5.5).

5.2.4. Concluding remarks.

SPME-GC has been shown to be an effective tool for both qualitative and quantitative analysis of vanilla extracts and flavourings. Most of the extracts

provided a unique chromatogram after sampling by SPME and analysis by GC. Using SPME-GC volatile components not usually scrutinised and manipulated by would be adulterators can be used to characterise extracts. To confidently determine these compounds a study of a large number of natural extracts need to be analysed for in-depth chemometics analysis. Such a study, which was outside the scope of this project, would identify subtle but consistent differences between the extracts.

HPLC was also used to screen and characterise the extracts, however, it was not very effective at discriminating between many of the extracts, with the exception of the Tahitian extract. In addition, adulterators have been known to alter the concentration of key compounds, typically analysed by HPLC, making it even more difficult to discriminate between the extracts.

SPME-GC also provided an excellent method for quantifying the vanillin concentration in extracts and flavourings. This work indicated that SPME-GC is superior and more reliable than HPLC. In addition, SPME-GC is rapid and requires no sample pre-treatment other than dilution. It is perfect for on-line automated sampling, where the fibre can be exposed to the sample prior to packaging.

However, the true power of SPME-GC is probably best experienced when used in combination with HPLC. The two techniques are very complimentary. Used in tandem, they provide a complex profile of an extract. The HPLC fingerprints the non-volatiles while SPME-GC provides a volatile/semi-volatile profile, which is

difficult to obtain by traditional GC techniques. The detailed set of data on an extract would make it very difficult to mimic and adulterate samples.

Table 5.1. The presence or absence of key components in a range of vanilla extracts and flavourings.

Component	*Bourbon*	*Indonesian*	*Madagascan*	*Tahitian*	*Tongan*	*Mexican*	*Nature identical*	*Artificial*
Ethyl hexanoate	++	+	++	+	++	++	++	+
Ethyl octanoate	+	+	+	+				
m-Methoxybenzaldehyde	+			+	+		++	
p-Methoxybenzaldehyde	+	+	+	++++	+	+	+	+
Ethyl-*m*-benzoate	+	+	+	+	+	+	+	+
5-propenyl-1,3-benzodioxole	+++	+	+	++		+	+	
Ethyl nonanoate	++	+	+	+	+	++	+	+
Unidentified compound		++	++	++++	+++	+++	++	++
Piperonal	+			+		+	+	
p-methoxybenzoic acid methyl ester			+	+++		+	+	
3-phenyl-2-propenoic acid methyl ester	+	+	++	+	++	++		
Ethyl decanoate	+	+	+	+	+	+	+	+
Vanillin	+++	+++	+++	+++	+++	+++	++++	+
Coumarin	+							
Ethyl vanillin								++++

LEGEND

PEAK DESCRIPTION	SYMBOL
Trace	+
Normal	++
Major	+++
Extreme	++++

Table 5.2. The presence or absence of key components in 3 Bourbon and 3 Indonesian extracts.

Component	*A.bourbon*	*B.bourbon*	*C.bourbon*	*A.Indonesian*	*B.Indonesian*	*D.Indonesian*
Ethyl hexanoate	+++	++	+	+	+++	+
Ethyl octanoate	+		+		+	+
m-Methoxybenzaldehyde	+	+	+			+
p-Methoxybenzaldehyde	+	+	+	+	+	+
Ethyl-*m*-benzoate	+	++	++	+	+	+
5-propenyl-1,3-ber zodioxole	+++	++	+	++	++	
Ethyl nonanoate	++	+	+	+	+	+
Unidentified compound			++	++	+++	+++
Piperonal	+	+	+			
p-methoxybenzoic acid methyl ester						
3-phenyl-2-propenoic acid methyl ester	++	+	+	++	++	++
Ethyl decanoate	+	++	+	+	+	+
Vanillin	+++	+++	+++	+++	+++	+++
Coumarin		+	+			
Ethyl vanillin						

LEGEND

PEAK DESCRIPTION	SYMBOL
Trace	+
Normal	++
Major	+++
Extreme	++++

Table 5.3. Comparison of correlation coefficients for each quantitation method.

COMPONENTS	*EXTERNAL STANDARD* (R^2)	*STANDARD ADDITION* (R^2)	*INTERNAL STANDARD* (R^2)
Cuminyl aldehyde	0.9449	0.8420	
Ethyl octanoate	0.9837	0.8410	
m-Methoxybenzaldehyde	0.9713	0.2510	
p-Methoxybenzaldehyde	0.9994	0.8395	
Ethyl-*m*-benzoate	0.9846	0.8365	
Ethyl nonanoate	0.9196	0.5482	
Piperonal	0.9983	0.5632	
Ethyl decanoate	0.9910	0.5843	
Vanillin	0.9023	0.4133	0.5160
Coumarin	0.9998	0.9914	
Ethyl vanillin	0.9303	0.1624	0.8996

Table 5.4. Quantitation data for vanillin determined by SPME-GC and HPLC and the concentrations quoted by the manufacturer.

Extract flavouring type	*Concentration SPME (mgL^{-1})*	*Concentration HPLC (mgL^{-1})*	*Concentration range (mgL^{-1}) quoted by manufacturer*
A Bourbon	1840	1850	1800-4000
B Bourbon	3200	2750	3200-4100
A Indonesian	2920	2880	1800-3000
B Indonesian	2710	3350	1800-2700
B Tongan	1626	1930	1800-2700
A Tahitian	1140	1850	900-1500
C Bourbon	1618	2210	
D Indonesian	1070	2000	
A Mexican	988	860	
D Madagscan	3530	2250	
A N.Identical	80750	26120	
F N.Identical	75501	48080	
A Flavour	66	0	
F Flavour	51180	11370	

Table 5.5. Quantitation data determined by SPME-GC for key components in three Bourbon extracts.

Components (SPME)	*A Bourbon* (mgL^{-1})	*B Bourbon* (mgL^{-1})	*C Bourbon* (mgL^{-1})
Ethyl octanoate	<0.01	0	<0.01
m-Methoxybenzaldehyde	0.01	0.02	0.03
p-Methoxybenzaldehdye	0.2	0.03	0.08
Ethyl-*m*-benzoate	<0.01	<0.01	<0.01
Ethyl nonanoate	0.03	<0.01	0.01
Piperonal	0.06	0.05	0.06
Ethyl decanoate	0.01	<0.01	<0.01
Vanillin	1840	3200	1620
Coumarin	0	<0.01	<0.01
Ethyl vanillin	0	0	0

Table 5.6. Quantitation data determined by SPME-GC for key components in three Indonesian extracts.

Components (SPME)	*A Indonesian* (mgL^{-1})	*B Indonesian* (mgL^{-1})	*D Indonesian* (mgL^{-1})
Ethyl octanoate	0	<0.01	<0.01
m-Methoxybenzaldehyde	0	0	0.1
p-Methoxybenzaldehyde	0.03	0.04	0.04
Ethyl-*m*-benzoate	<0.01	<0.01	<0.01
Ethyl nonanoate	<0.01	0.03	<0.01
Piperonal	0	0	0
Ethyl decanoate	<0.01	0.01	<0.01
Vanillin	2920	2710	1070
Coumarin	0	0	0
Ethyl vanillin	0	0	0

Table 5.7. Quantitation data determined by SPME-GC for natural identical extracts and synthetic vanilla flavourings.

Components (SPME)	*A Nature Identical (mgL^{-1})*	*F Nature Identical (mgL^{-1})*	*F Synthetic Vanilla (mgL^{-1})*	*A Synthetic Vanilla (mgL^{-1})*
Ethyl octanoate	<0.01	0	0	0
m-Methoxybenzaldehyde	0	0.03	0	0
p-Methoxybenzaldehyde	0	0.04	0	0.5
Ethyl-*m*-benzoate	<0.01	<0.01	0	<0.01
Ethyl nonanoate	<0.01	<0.01	0	<0.01
Piperonal	270	0.09	0	0
Ethyl decanoate	<0.01	<0.01	0	0.01
Vanillin	80750	75500	50180	66
Coumarin	0	0	0	0
Ethyl vanillin	11500	0	16350	15040

Table 5.8. Quantitation data determined by SPME-GC for natural vanilla extracts.

Components (SPME)	*B Tongan* (mgL^{-1})	*A Mexican* (mgL^{-1})	*A Tahitian* (mgL^{-1})	*D Madagascan* (mgL^{-1})
Ethyl octanoate	0.01	0	<0.01	0.01
m-Methoxybenzaldehyde	0	0	0.01	0
p-Methoxybenzaldehyde	0.04	0.06	18	0.1
Ethyl-*m*-benzoate	<0.01	<0.01	<0.01	<0.01
Ethyl nonanoate	0.05	0.04	0	0.06
Piperonal	0	0.03	0.02	0
Ethyl decanoate	0.02	0.01	<0.01	0.01
Vanillin	1630	990	1140	3530
Coumarin	0	0	0	0
Ethyl vanillin	0	0	0	0

Table 5.9. Bourbon extract component concentration using HPLC.

Components (HPLC)	*A Bourbon* ($mg\ L^{-1}$)	*B Bourbon* ($mg\ L^{-1}$)	*C Bourbon* ($mg\ L^{-1}$)
Vanillic acid	30	60	40
p-Hydroxybenzoic acid	40	40	270
p-Hydroxybenzaldehyde	100	2370	350
Vanillin	1850	2750	2210
Ethyl vanillin	0	0	0
p-Methoxybenzaldehyde	40	3	40

Table 5.10. Indonesian extract component concentration using HPLC.

Components (HPLC)	*A Indonesian* ($mg\ L^{-1}$)	*B Indonesian* ($mg\ L^{-1}$)	*D Indonesian* ($mg\ L^{-1}$)
Vanillic acid	140	140	260
p-Hydroxybenzoic acid	570	1140	350
p-Hydroxybenzaldehyde	660	880	420
Vanillin	2880	3350	2000
Ethyl vanillin	0	0	0
p-Methoxybenzaldehyde	110	110	190

Table 5.11 Other natural extracts component concentration in HPLC

Components (HPLC)	*D Madagascan* ($mg\ L^{-1}$)	*A Mexican* ($mg\ L^{-1}$)	*B Tongan* ($mg\ L^{-1}$)	*A Tahitian* ($mg\ L^{-1}$)
Vanillic acid	320	20	840	28
p-Hydroxybenzoic acid	1060	25	30	9390
p-Hydroxybenzaldehyde	660	1060	370	1140
Vanillin	2250	860	1930	1850
Ethyl vanillin	0	0	0	0
p-Methoxybenzaldehyde	3	80	420	870

Table 5.12. Nature identical and synthetic extracts component concentration HPLC

Components (HPLC)	*F Nature Identical* ($mg\ L^{-1}$)	*A Synthetic Flavour* ($mg\ L^{-1}$)	*F Synthetic Flavour* ($mg\ L^{-1}$)
Vanillic acid	70	0	0
p-Hydroxybenzoic acid	140	0	0
p-Hydroxybenzaldehyde	90	0	6
Vanillin	48080	0	11370
Ethyl vanillin	0	12740	11320
p-Methoxybenzaldehyde	530	0	0

Figure 5.1 GC separation of flavour components present in Bourbon A extract extracted using SPME.

Legend.

1. Ethyl hexanoate.
2. Ethyl octanoate.
3. *m*-Methoxybenzaldehyde.
4. *p*-Methoxybenzaldehyde.
5. 5-propenyl-1,3-benzodioxole.
6. Ethyl-*m*-benzoate.
7. Ethyl nonanoate.
8. Unidentified compound.
9. Piperonal.
10. *p*-Methoxybenzoic acid methyl ester.
11. 3-phenyl-2-propenoic acid methyl ester.
12. Ethyl decanoate.
13. Vanillin.
14. Coumarin.
15. Ethyl vanillin.

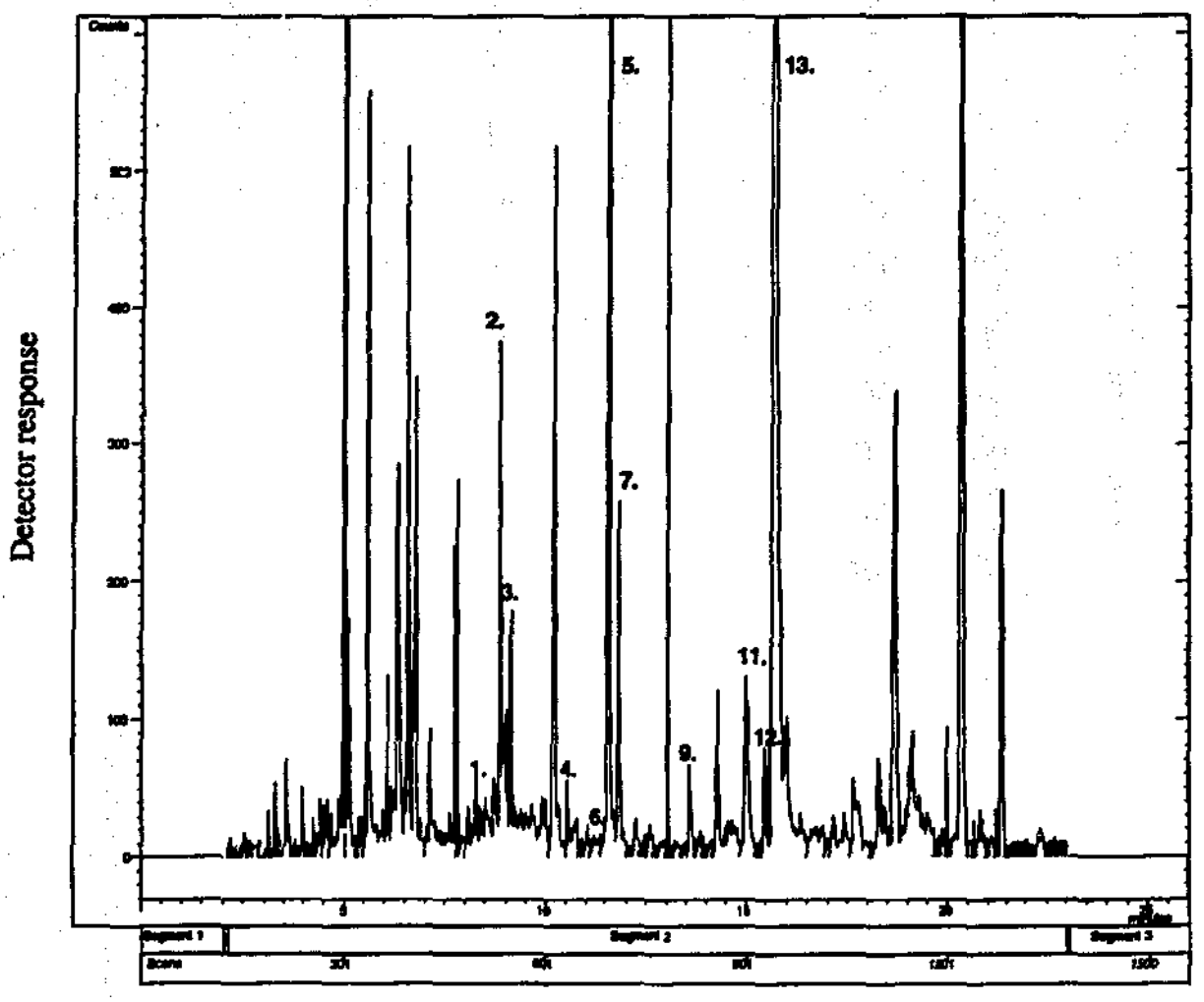


Figure 5.2 GC separation of flavour components present in Indonesian A extract extracted using SPME.

Legend.

1. Ethyl hexanoate.
2. Ethyl octanoate.
3. *m*-Methoxybenzaldehyde.
4. *p*-Methoxybenzaldehyde.
5. 5-propenyl-1,3-benzodioxole.
6. Ethyl-*m*-benzoate.
7. Ethyl nonanoate.
8. Unidentified compound.
9. Piperonal.
10. *p*-Methoxybenzoic acid methyl ester.
11. 3-phenyl-2-propenoic acid methyl ester.
12. Ethyl decanoate.
13. Vanillin.
14. Coumarin.
15. Ethyl vanillin.

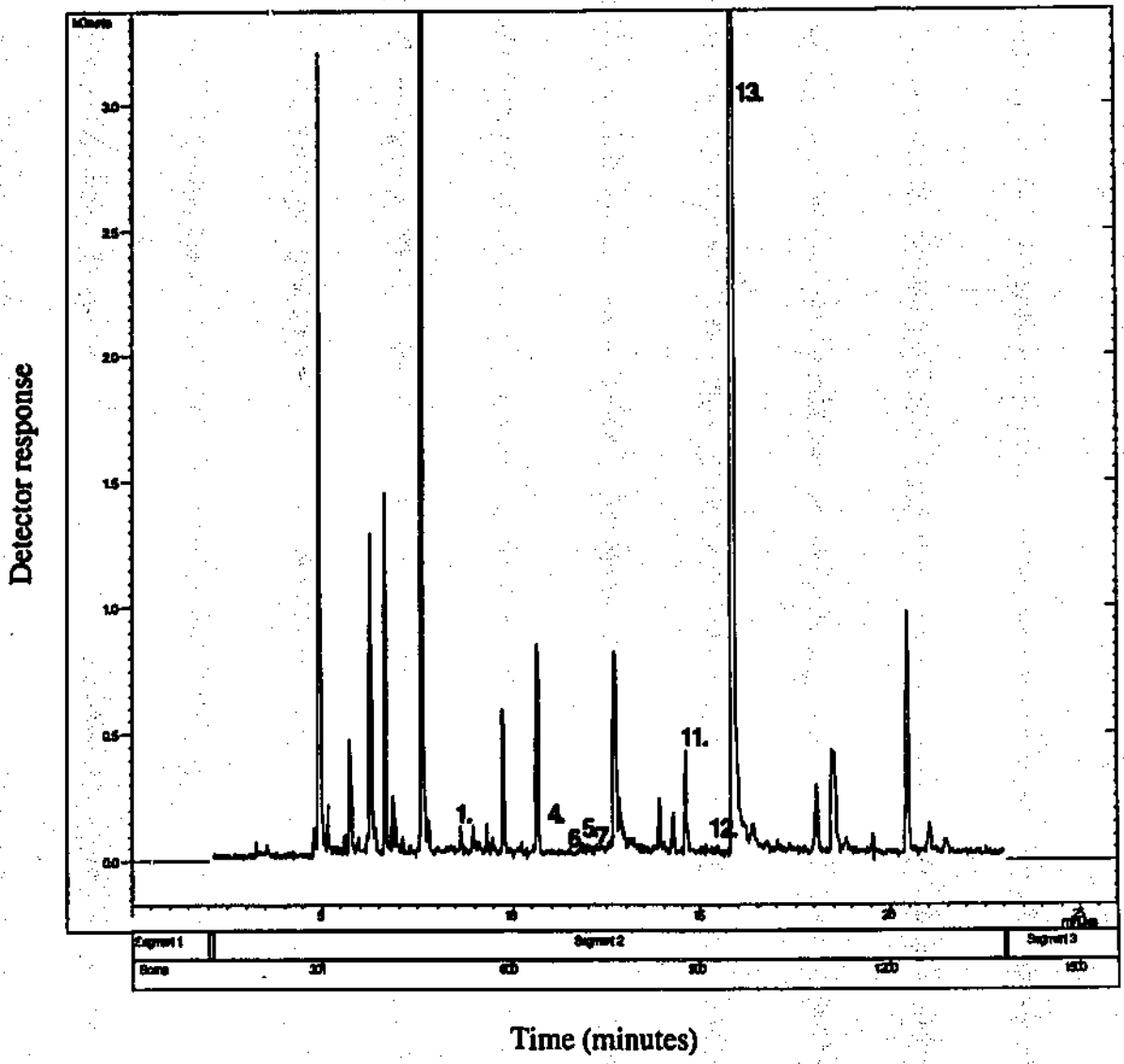


Figure 5.3 GC separation of flavour components present in Tongan B extract extracted using SPME.

Legend.

1. Ethyl hexanoate.
2. Ethyl octanoate.
3. *m*-Methoxybenzaldehyde.
4. *p*-Methoxybenzaldehyde.
5. 5-propenyl-1,3-benzodioxole.
6. Ethyl-*m*-benzoate.
7. Ethyl nonanoate.
8. Unidentified compound.
9. Piperonal.
10. *p*-Methoxybenzoic acid methyl ester.
11. 3-phenyl-2-propenoic acid methyl ester.
12. Ethyl decanoate.
13. Vanillin.
14. Coumarin.
15. Ethyl vanillin.

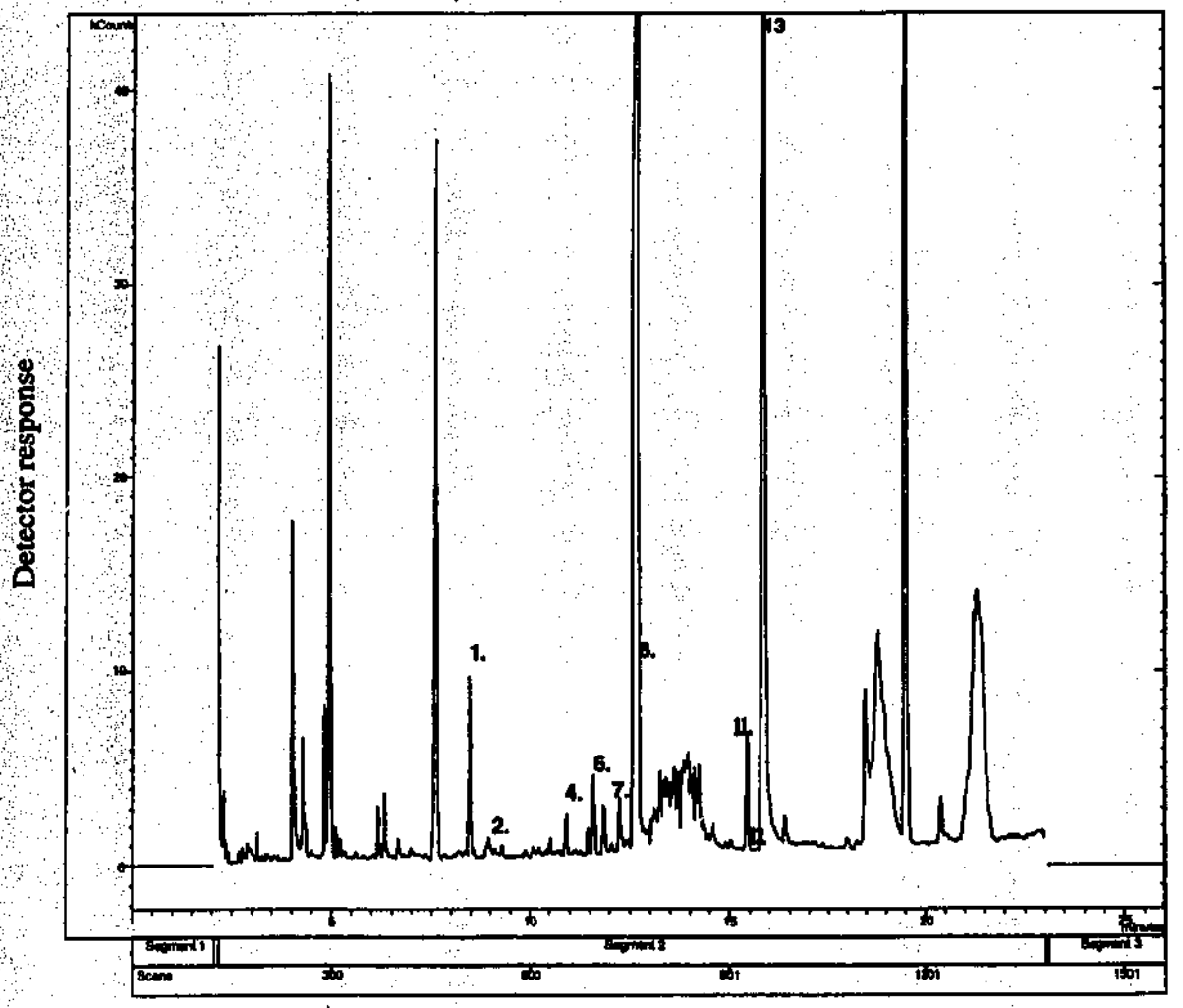


Figure 5.4 GC separation of flavour components present in Madagascan D extract extracted using SPME.

Legend.

1. Ethyl hexanoate.
2. Ethyl octanoate.
3. *m*-Methoxybenzaldehyde.
4. *p*-Methoxybenzaldehyde.
5. 5-propenyl-1,3-benzodioxole.
6. Ethyl-*m*-benzoate.
7. Ethyl nonanoate.
8. Unidentified compound.
9. Piperonal.
10. *p*-Methoxybenzoic acid methyl ester.
11. 3-phenyl-2-propenoic acid methyl ester.
12. Ethyl decanoate.
13. Vanillin.
14. Coumarin.
15. Ethyl vanillin.

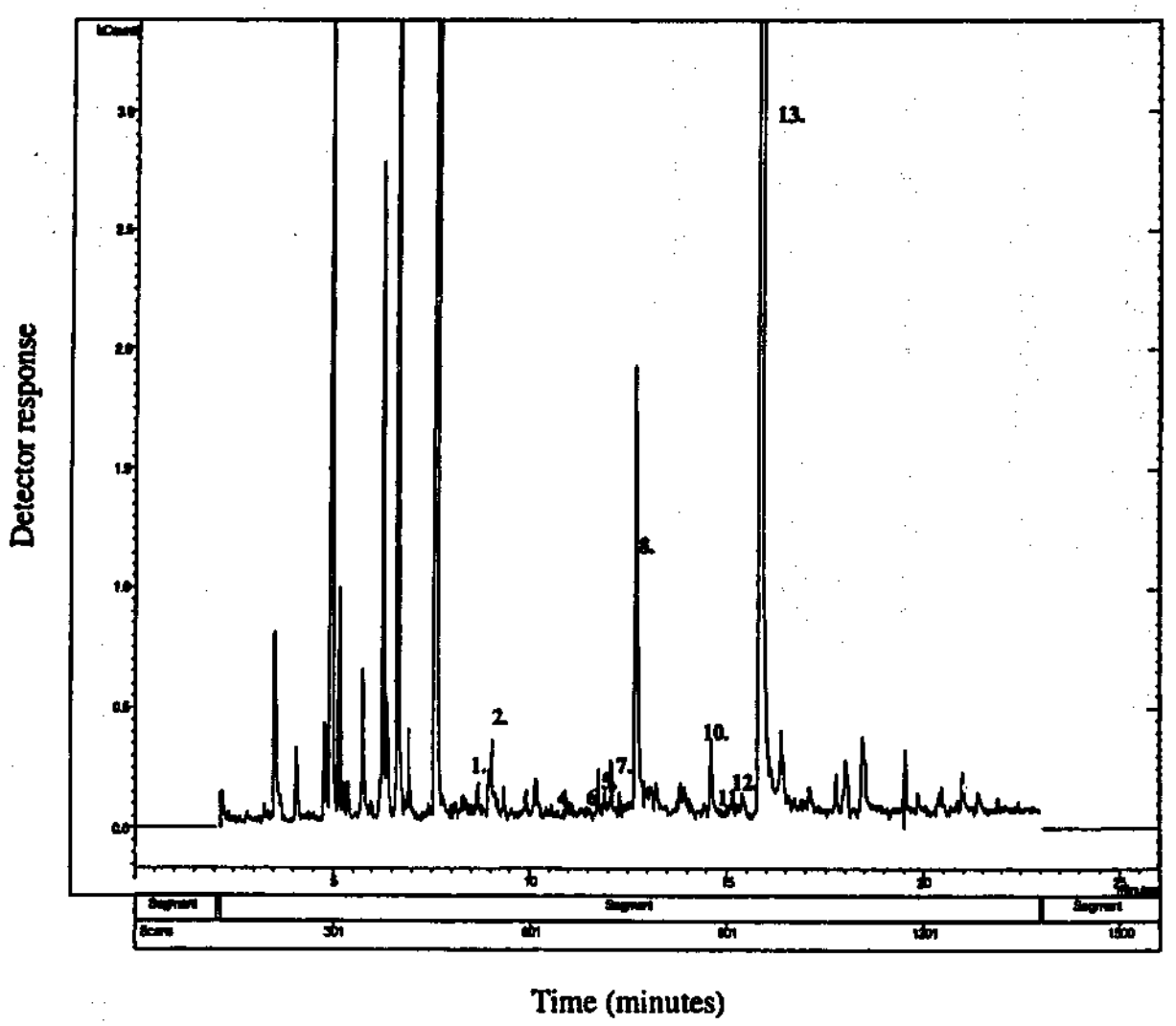


Figure 5.5 GC separation of flavour components present in Tahitian A extract extracted using SPME.

Legend.

1. Ethyl hexanoate.
2. Ethyl octanoate.
3. *m*-Methoxybenzaldehyde.
4. *p*-Methoxybenzaldehyde.
5. 5-propenyl-1,3-benzodioxole.
6. Ethyl-*m*-benzoate.
7. Ethyl nonanoate.
8. Unidentified compound.
9. Piperonal.
10. *p*-Methoxy[illegible]oic acid methyl ester.
11. 3-phenyl-2-propenoic acid methyl ester.
12. Ethyl decanoate.
13. Vanillin.
14. Coumarin.
15. Ethyl vanillin.

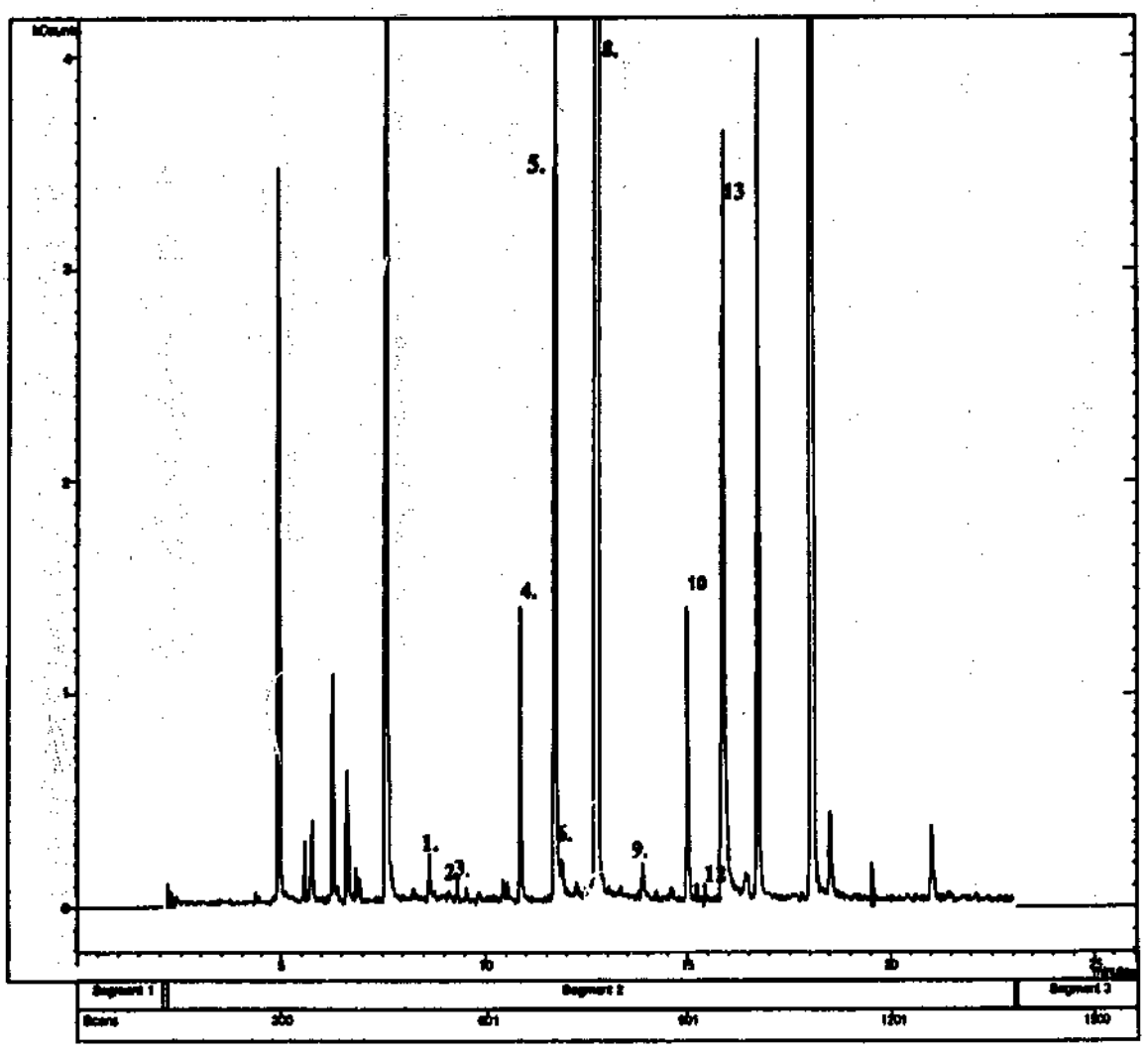


Figure 5.6 GC separation of flavour components present in Mexican A extract extracted using SPME.

Legend.

1. Ethyl hexanoate.
2. Ethyl octanoate.
3. *m*-Methoxybenzaldehyde.
4. *p*-Methoxybenzaldehyde.
5. 5-propenyl-1,3-benzodioxole.
6. Ethyl-*m*-benzoate.
7. Ethyl nonanoate.
8. Unidentified compound.
9. Piperonal.
10. *p*-Methoxybenzoic acid methyl ester.
11. 3-phenyl-2-propenoic acid methyl ester.
12. Ethyl decanoate.
13. Vanillin.
14. Coumarin.
15. Ethyl vanillin.

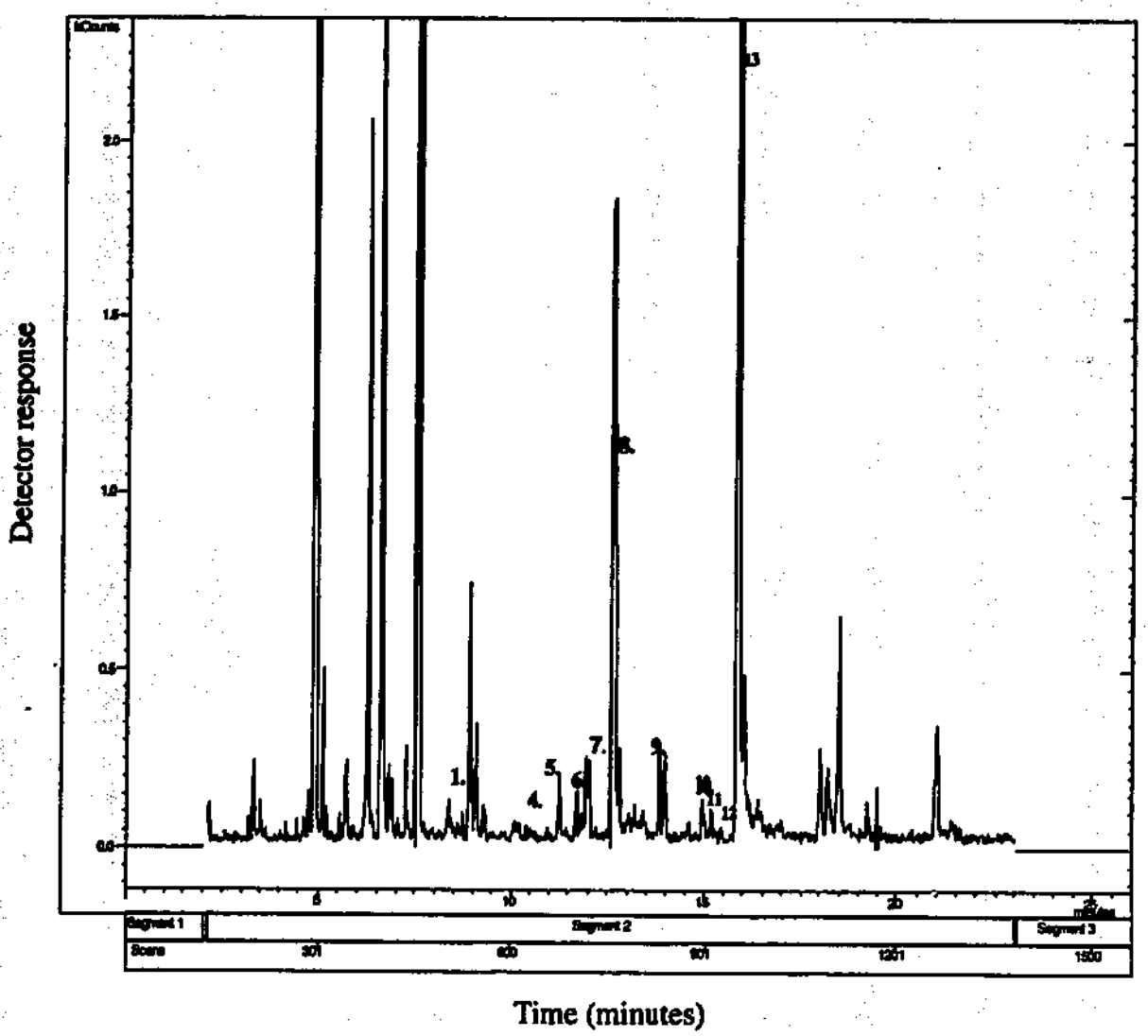


Figure 5.7 GC separation (using selected ion mode) of Flavour components present in Bourbon A extract extracted using SPME.

Legend.

1. Ethyl hexanoate.
2. Ethyl octanoate.
3. *m*-Methoxybenzaldehyde.
4. *p*-Methoxybenzaldehyde.
5. 5-propenyl-1,3-benzodioxole.
6. Ethyl-*m*-benzoate.
7. Ethyl nonanoate.
8. Unidentified compound.
9. Piperonal.
10. *p*-Methoxybenzoic acid methyl ester.
11. 3-phenyl-2-propenoic acid methyl ester.
12. Ethyl decanoate.
13. Vanillin.
14. Coumarin.
15. Ethyl vanillin.

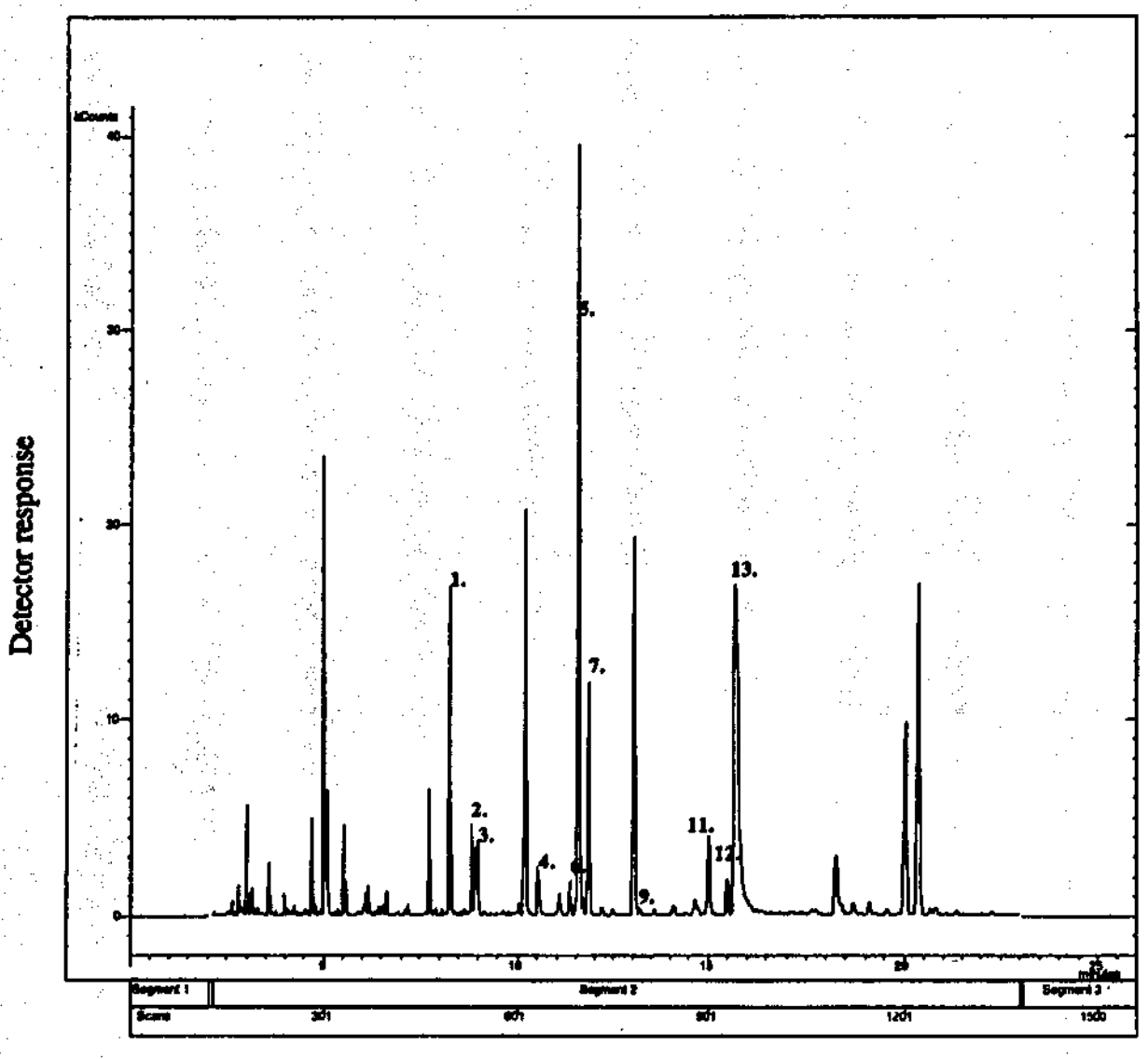


Figure 5.8 GC separation (using selected ion mode) of Flavour components present in Indonesian A extract extracted using SPME.

Legend.

1. Ethyl hexanoate.
2. Ethyl octanoate.
3. *m*-Methoxybenzaldehyde.
4. *p*-Methoxybenzaldehyde.
5. 5-propenyl-1,3-benzodioxole.
6. Ethyl-*m*-benzoate.
7. Ethyl nonanoate.
8. Unidentified compound.
9. Piperonal.
10. *p*-Methoxybenzoic acid methyl ester.
11. 3-phenyl-2-propenoic acid methyl ester.
12. Ethyl decanoate.
13. Vanillin.
14. Coumarin.
15. Ethyl vanillin.

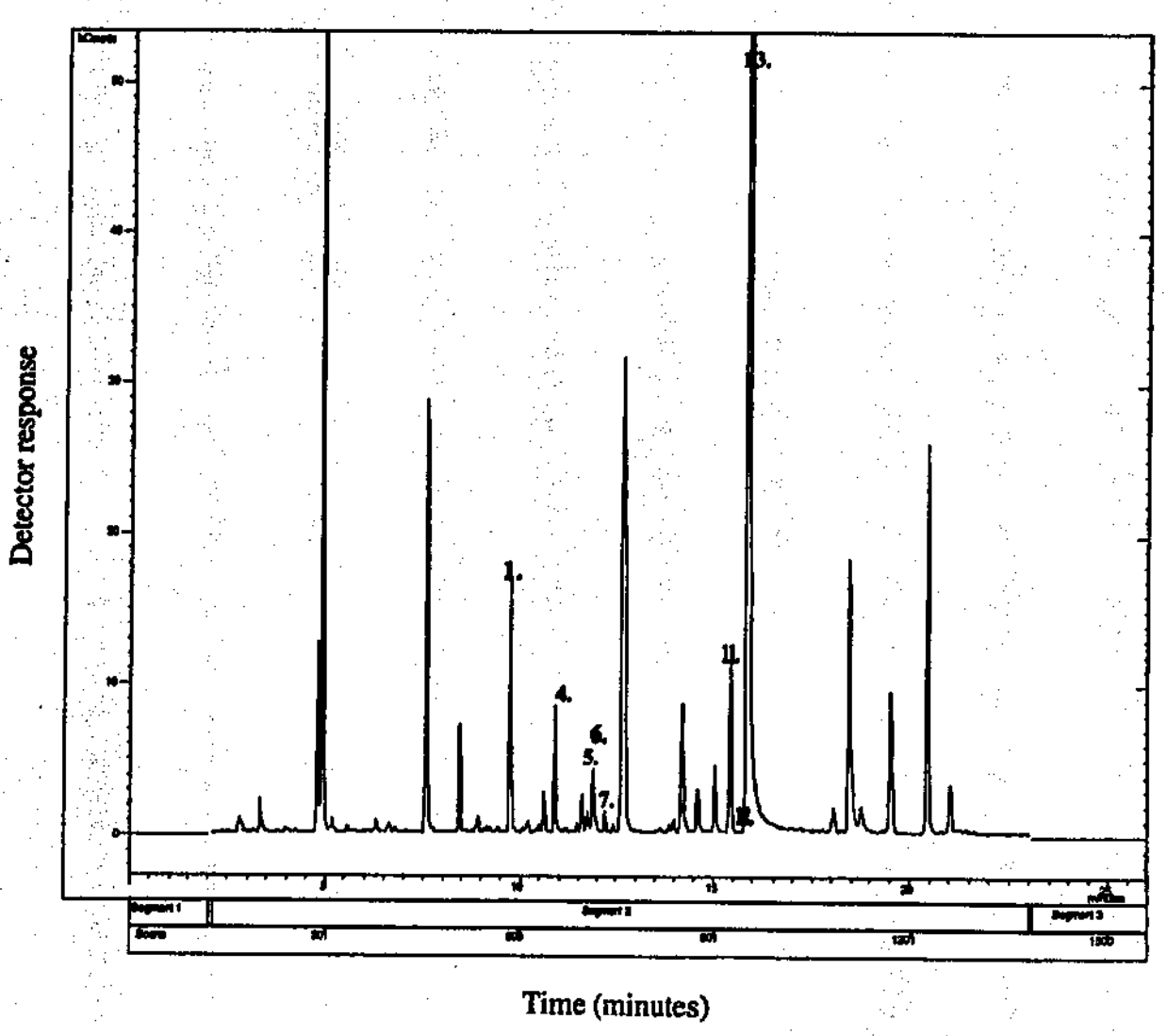


Figure 5.9 GC separation (using selected ion mode) of Flavour components present in Tongan B extract extracted using SPME.

Legend.

1. Ethyl hexanoate.
2. Ethyl octanoate.
3. *m*-Methoxybenzaldehyde.
4. *p*-Methoxybenzaldehyde.
5. 5-propenyl-1,3-benzodioxole.
6. Ethyl-*m*-benzoate.
7. Ethyl nonanoate.
8. Unidentified compound.
9. Piperonal.
10. *p*-Methoxybenzoic acid methyl ester.
11. 3-phenyl-2-propenoic acid methyl ester.
12. Ethyl decanoate.
13. Vanillin.
14. Coumarin.
15. Ethyl vanillin.

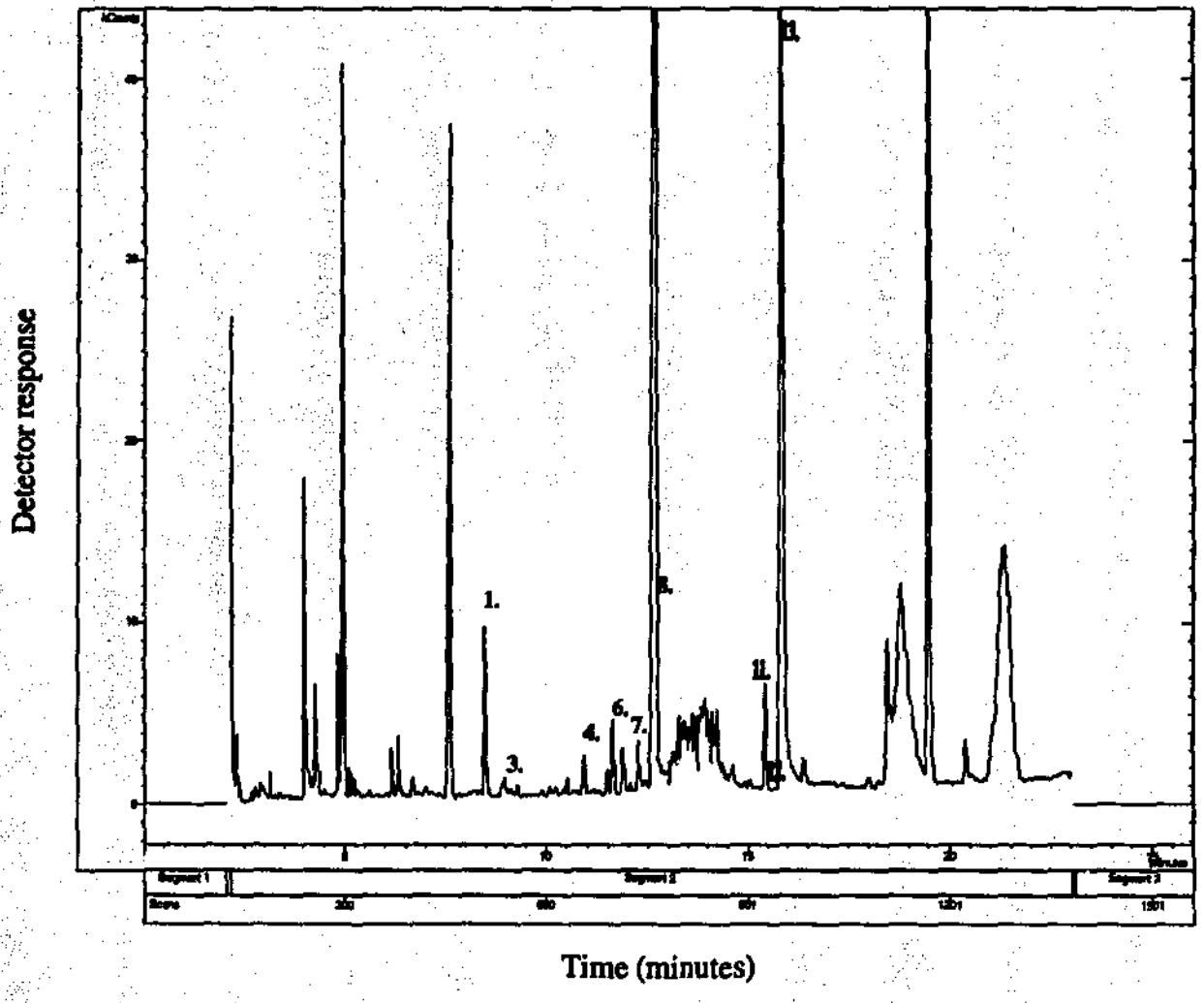


Figure 5.10 GC separation (using selected ion mode) of Flavour components present in Madagascan D extract extracted using SPME.

Legend.

1. Ethyl hexanoate.
2. Ethyl octanoate.
3. *m*-Methoxybenzaldehyde.
4. *p*-Methoxybenzaldehyde.
5. 5-propenyl-1,3-benzodioxole.
6. Ethyl-*m*-benzoate.
7. Ethyl nonanoate.
8. Unidentified compound.
9. Piperonal.
10. *p*-Methoxybenzoic acid methyl ester.
11. 3-phenyl-2-propenoic acid methyl ester.
12. Ethyl decanoate.
13. Vanillin.
14. Coumarin.
15. Ethyl vanillin.

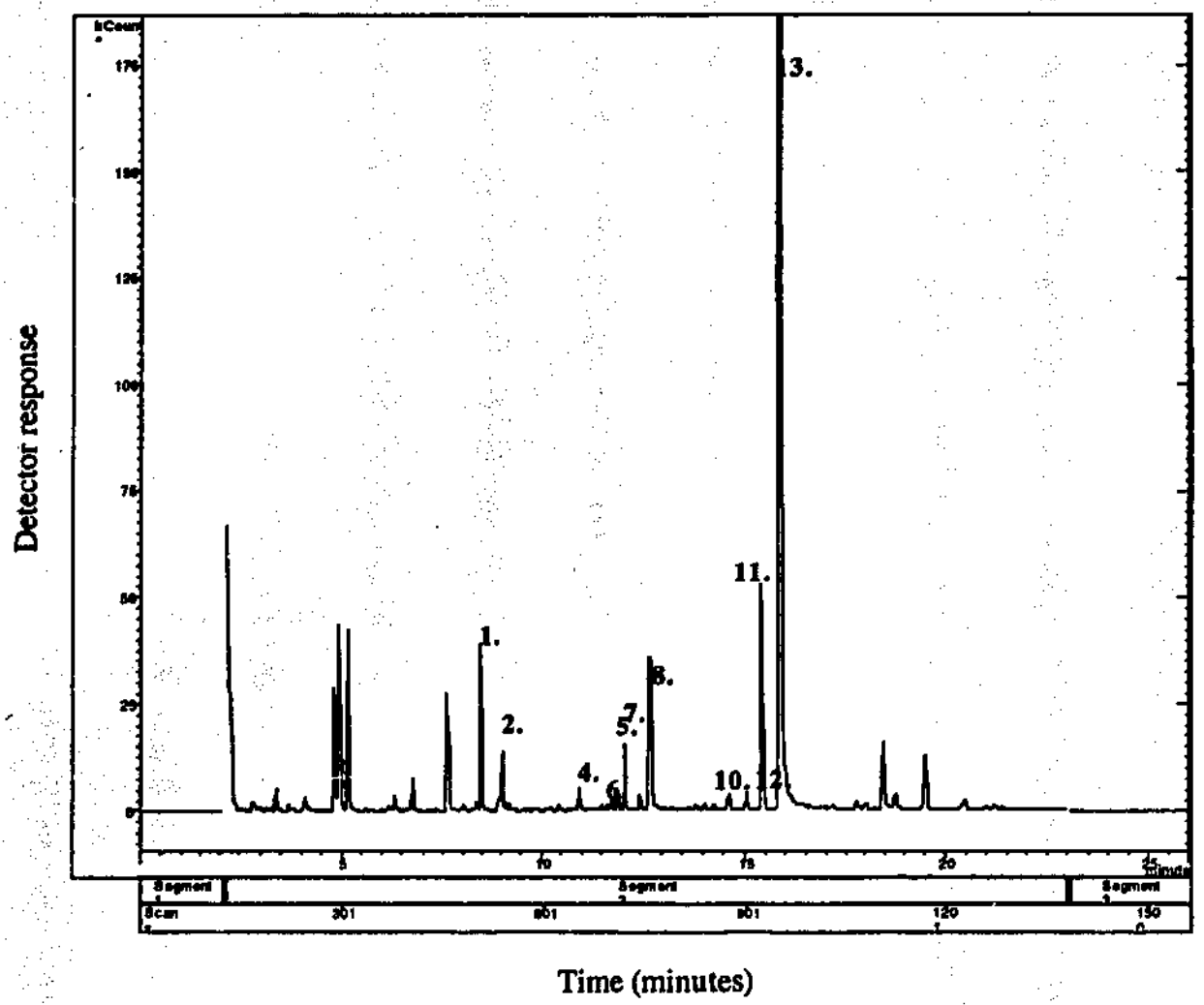


Figure 5.11 GC separation (using selected ion mode) of Flavour components present in Tahitian A extract extracted using SPME.

Legend.

1. Ethyl hexanoate.
2. Ethyl octanoate.
3. *m*-Methoxybenzaldehyde.
4. *p*-Methoxybenzaldehyde.
5. 5-propenyl-1,3-benzodioxole.
6. Ethyl-*m*-benzoate.
7. Ethyl nonanoate.
8. Unidentified compound.
9. Piperonal.
10. *p*-Methoxybenzoic acid methyl ester.
11. 3-phenyl-2-propenoic acid methyl ester.
12. Ethyl decanoate.
13. Vanillin.
14. Coumarin.
15. Ethyl vanillin.

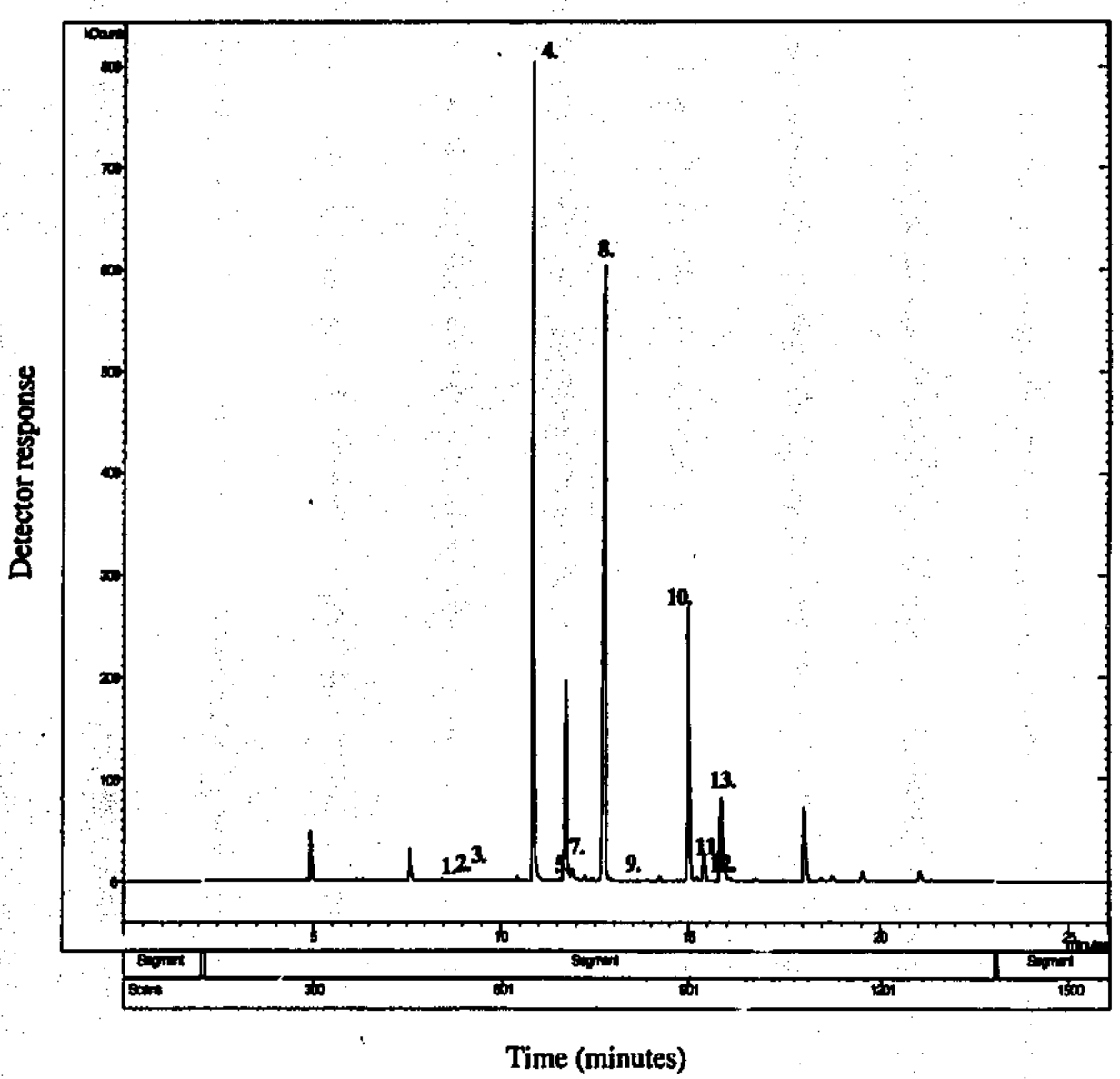


Figure 5.12 GC separation (using selected ion mode) of Flavour components present in Mexican A extract extracted using SPME.

Legend.

1. Ethyl hexanoate.
2. Ethyl octanoate.
3. *m*-Methoxybenzaldehyde.
4. *p*-Methoxybenzaldehyde.
5. 5-propenyl-1,3-benzodioxole.
6. Ethyl-*m*-benzoate.
7. Ethyl nonanoate.
8. Unidentified compound.
9. Piperonal.
10. *p*-Methoxybenzoic acid methyl ester.
11. 3-phenyl-2-propenoic acid methyl ester.
12. Ethyl decanoate.
13. Vanillin.
14. Coumarin.
15. Ethyl vanillin.

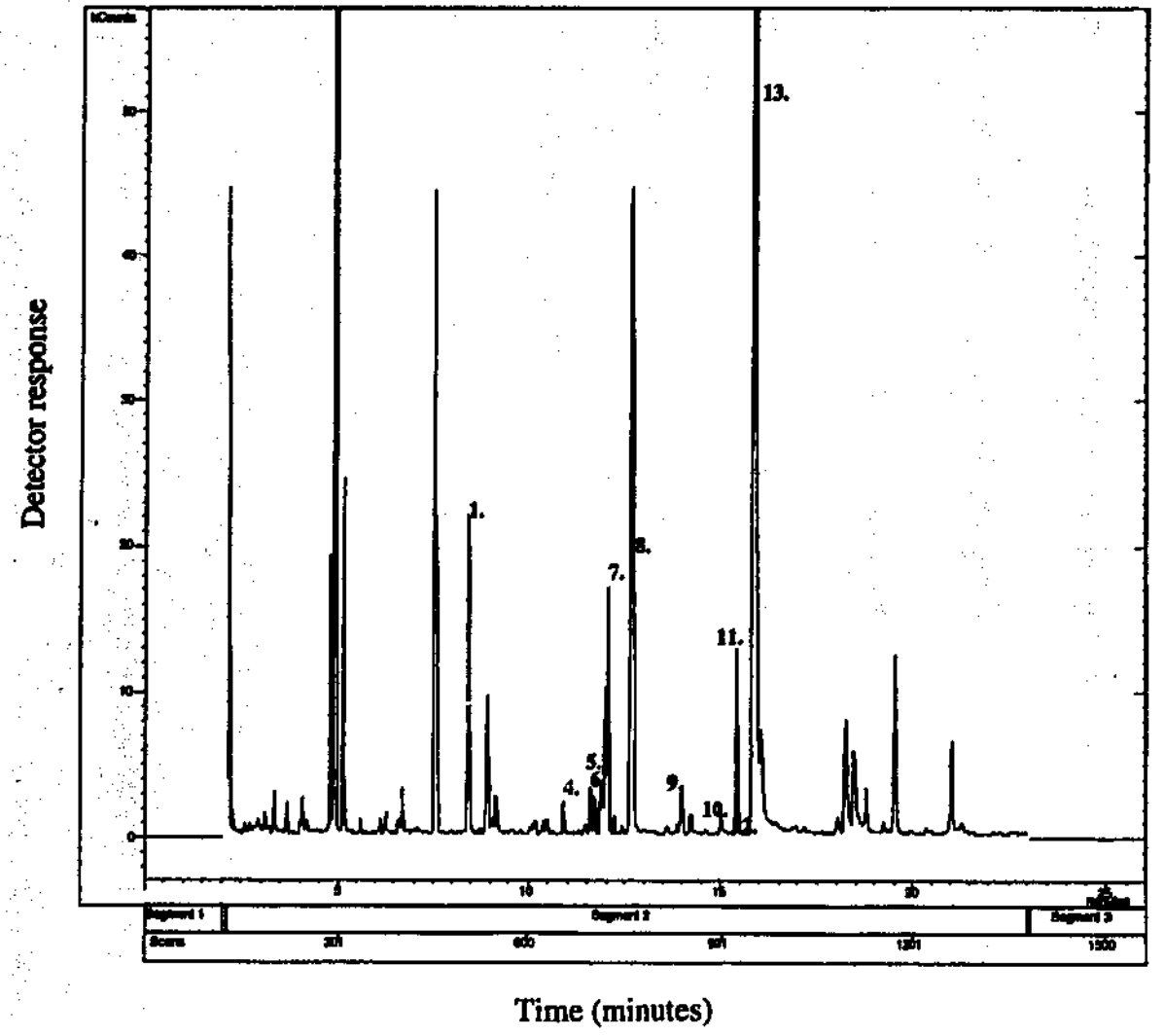


Figure 5.13 GC separation (using selected ion mode) of Flavour components present in Bourbon A extract extracted using SPME.

Legend.

1. Ethyl hexanoate.
2. Ethyl octanoate.
3. *m*-Methoxybenzaldehyde.
4. *p*-Methoxybenzaldehyde.
5. 5-propenyl-1,3-benzodioxole.
6. Ethyl-*m*-benzoate.
7. Ethyl nonanoate.
8. Unidentified compound.
9. Piperonal.
10. *p*-Methoxybenzoic acid methyl ester.
11. 3-phenyl-2-propenoic acid methyl ester.
12. Ethyl decanoate.
13. Vanillin.
14. Coumarin.
15. Ethyl vanillin.

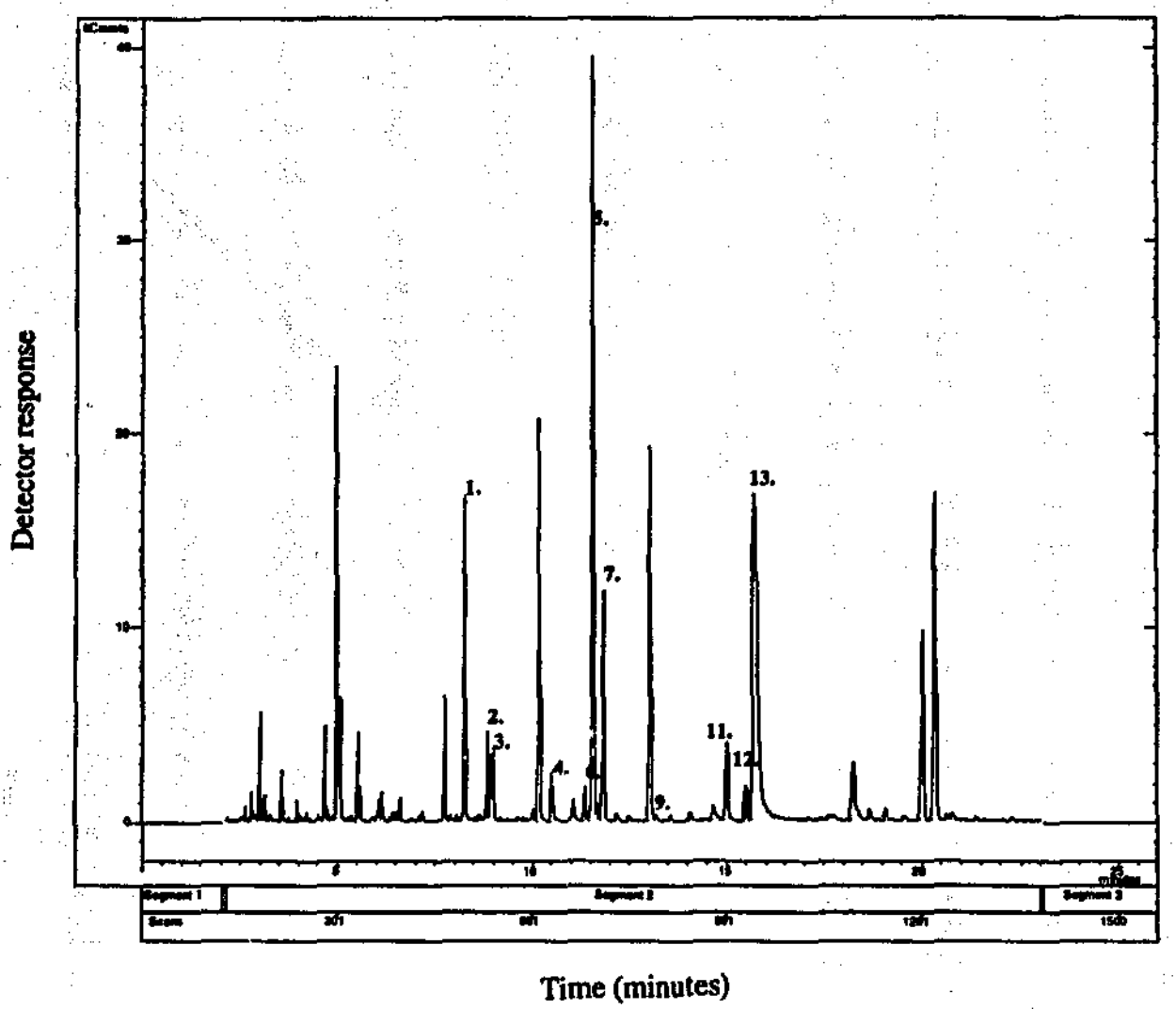


Figure 5.14 GC separation (using selected ion mode) of Flavour components present in Bourbon B extract extracted using SPME.

Legend.

1. Ethyl hexanoate.
2. Ethyl octanoate.
3. *m*-Methoxybenzaldehyde.
4. *p*-Methoxybenzaldehyde.
5. 5-propenyl-1,3-benzodioxole.
6. Ethyl-*m*-benzoate.
7. Ethyl nonanoate.
8. Unidentified compound.
9. Piperonal.
10. *p*-Methoxybenzoic acid methyl ester.
11. 3-phenyl-2-propenoic acid methyl ester.
12. Ethyl decanoate.
13. Vanillin.
14. Coumarin.
15. Ethyl vanillin.

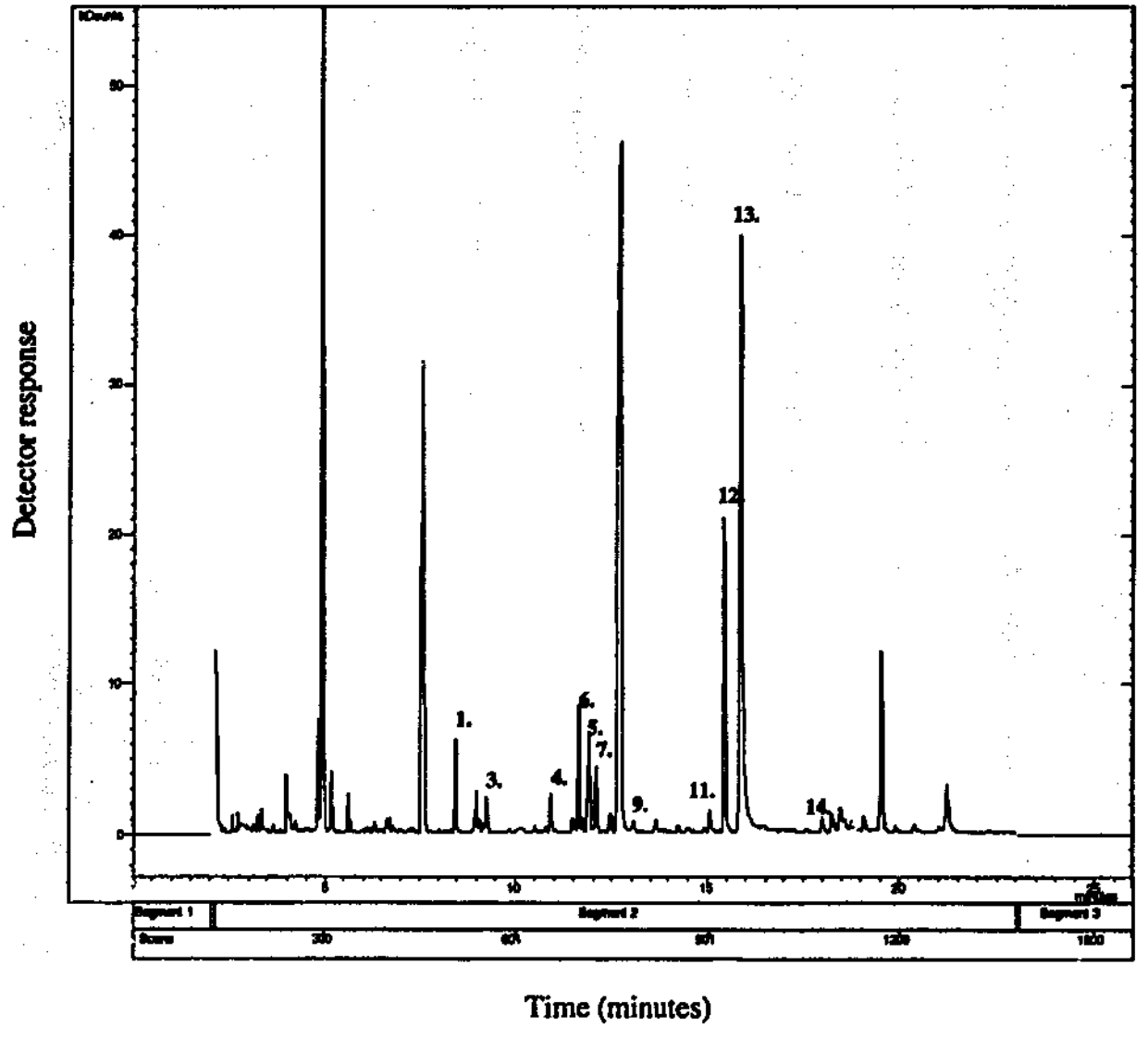


Figure 5.16 GC separation (using selected ion mode) of Flavour components present in Indonesian A extract extracted using SPME.

Legend.

1. Ethyl hexanoate.
2. Ethyl octanoate.
3. *m*-Methoxybenzaldehyde.
4. *p*-Methoxybenzaldehyde.
5. 5-propenyl-1,3-benzodioxole.
6. Ethyl-*m*-benzoate.
7. Ethyl nonanoate.
8. Unidentified compound.
9. Piperonal.
10. *p*-Methoxybenzoic acid methyl ester.
11. 3-phenyl-2-propenoic acid methyl ester.
12. Ethyl decanoate.
13. Vanillin.
14. Coumarin.
15. Ethyl vanillin.

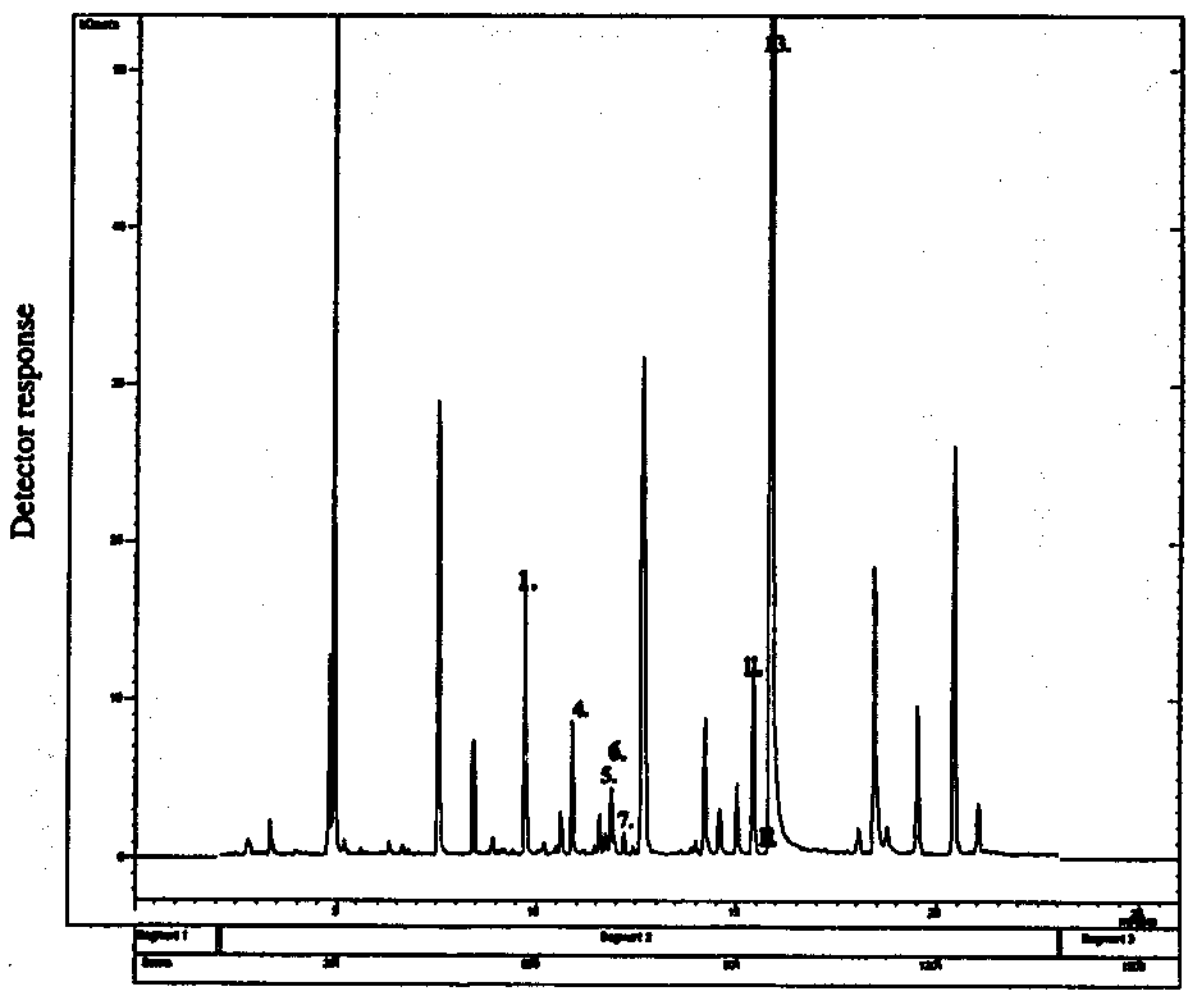


Figure 5.17 GC separation (using selected ion mode) of Flavour components present in Indonesian B extract extracted using SPME.

Legend.

1. Ethyl hexanoate.
2. Ethyl octanoate.
3. *m*-Methoxybenzaldehyde.
4. *p*-Methoxybenzaldehyde.
5. 5-propenyl-1,3-benzodioxole.
6. Ethyl-*m*-benzoate.
7. Ethyl nonanoate.
8. Unidentified compound.
9. Piperonal.
10. *p*-Methoxybenzoic acid methyl ester.
11. 3-phenyl-2-propenoic acid methyl ester.
12. Ethyl decanoate.
13. Vanillin.
14. Coumarin.
15. Ethyl vanillin.

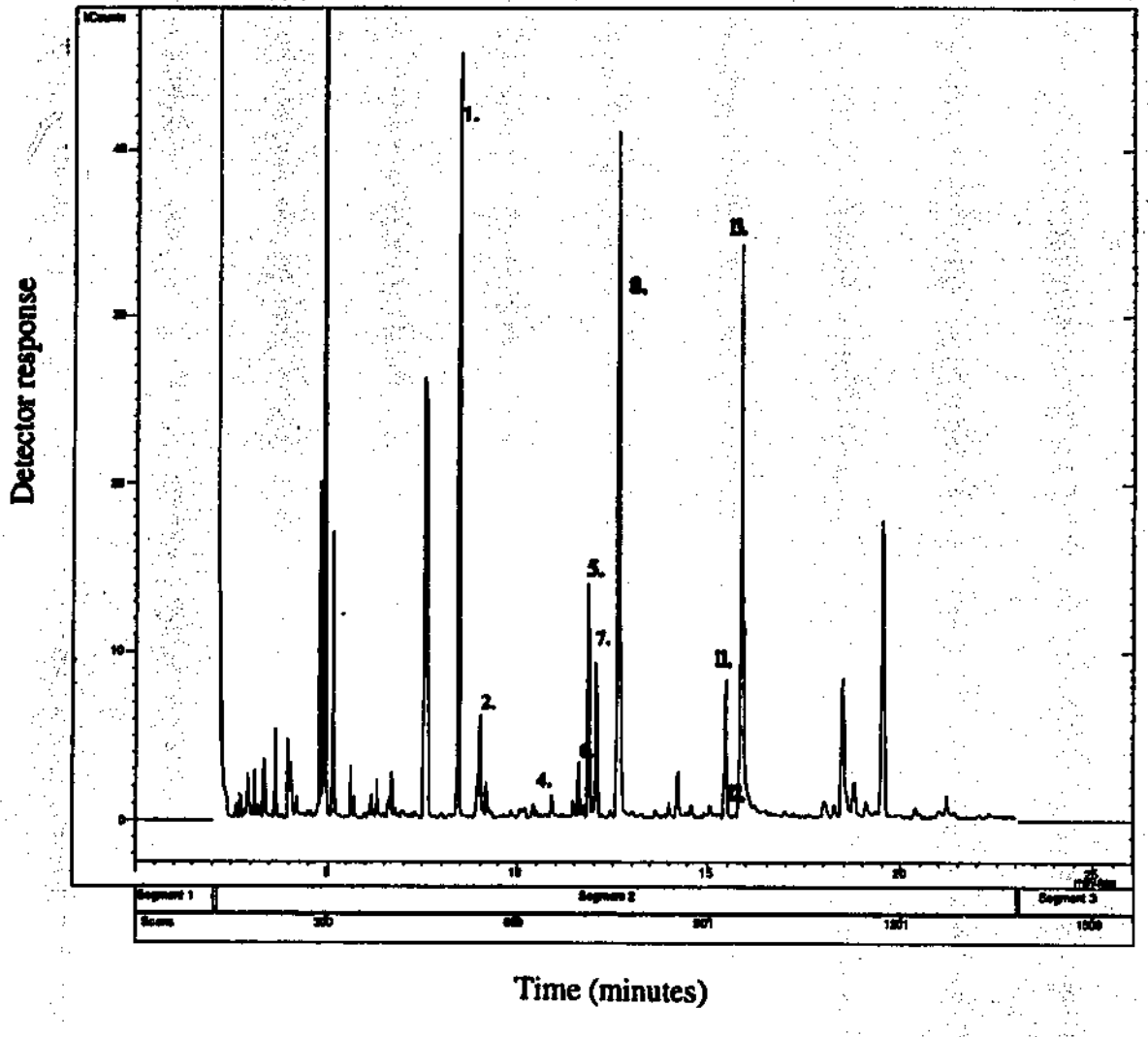


Figure 5.18 GC separation (using selected ion mode) of Flavour components present in Indonesian D extract extracted using SPME.

Legend.

1. Ethyl hexanoate.
2. Ethyl octanoate.
3. *m*-Methoxybenzaldehyde.
4. *p*-Methoxybenzaldehyde.
5. 5-propenyl-1,3-benzodioxole.
6. Ethyl-*m*-benzoate.
7. Ethyl nonanoate.
8. Unidentified compound.
9. Piperonal.
10. *p*-Methoxybenzoic acid methyl ester.
11. 3-phenyl-2-propenoic acid methyl ester.
12. Ethyl decanoate.
13. Vanillin.
14. Coumarin.
15. Ethyl vanillin.

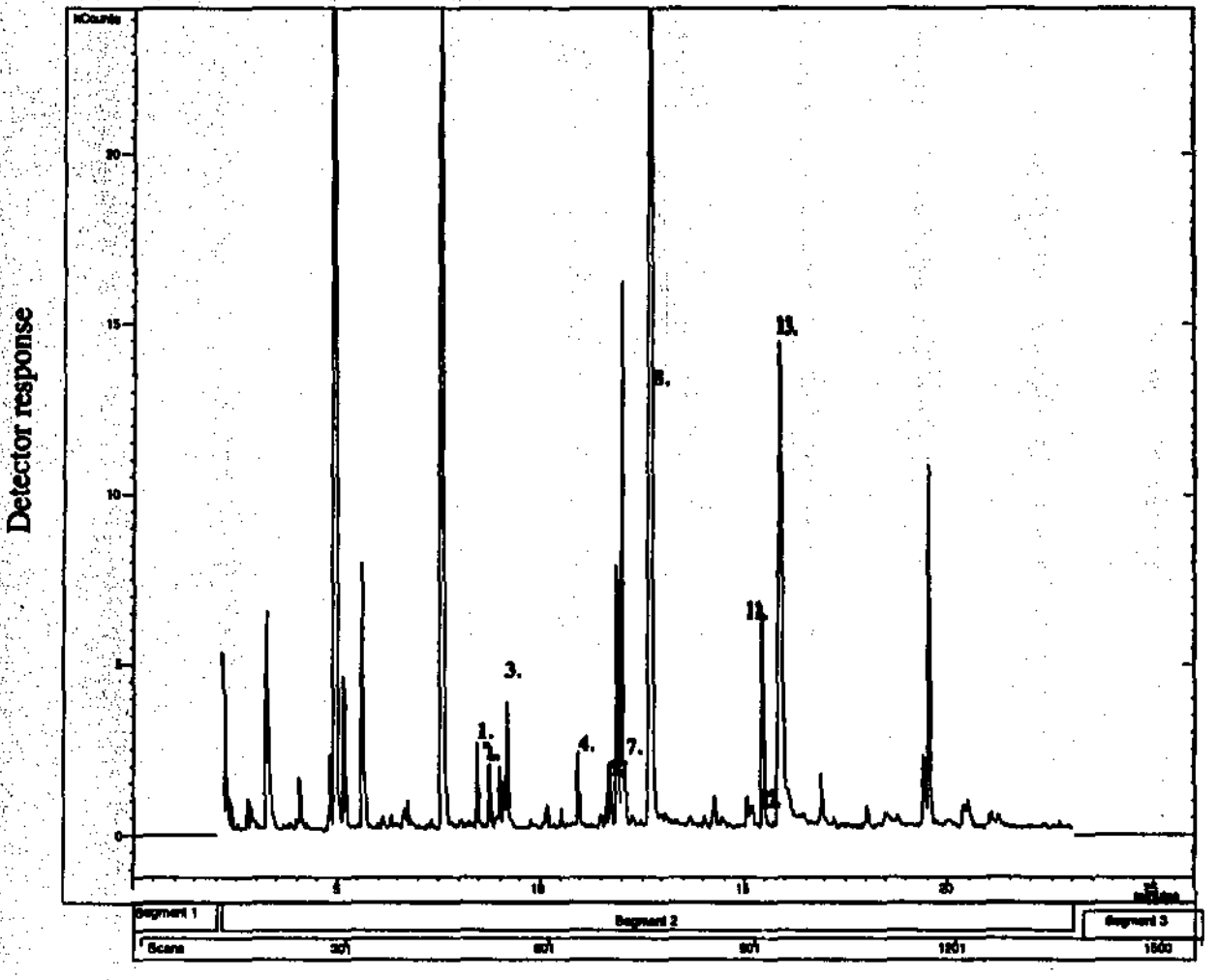


Figure 5.19 GC separation (using selected ion mode) of Flavour components present in Nature identical F extract extracted using SPME.

Legend.

1. Ethyl hexanoate.
2. Ethyl octanoate.
3. *m*-Methoxybenzaldehyde.
4. *p*-Methoxybenzaldehyde.
5. 5-propenyl-1,3-benzodioxole.
6. Ethyl-*m*-benzoate.
7. Ethyl nonanoate.
8. Unidentified compound.
9. Piperonal.
10. *p*-Methoxybenzoic acid methyl ester.
11. 3-phenyl-2-propenoic acid methyl ester.
12. Ethyl decanoate.
13. Vanillin.
14. Coumarin.
15. Ethyl vanillin.

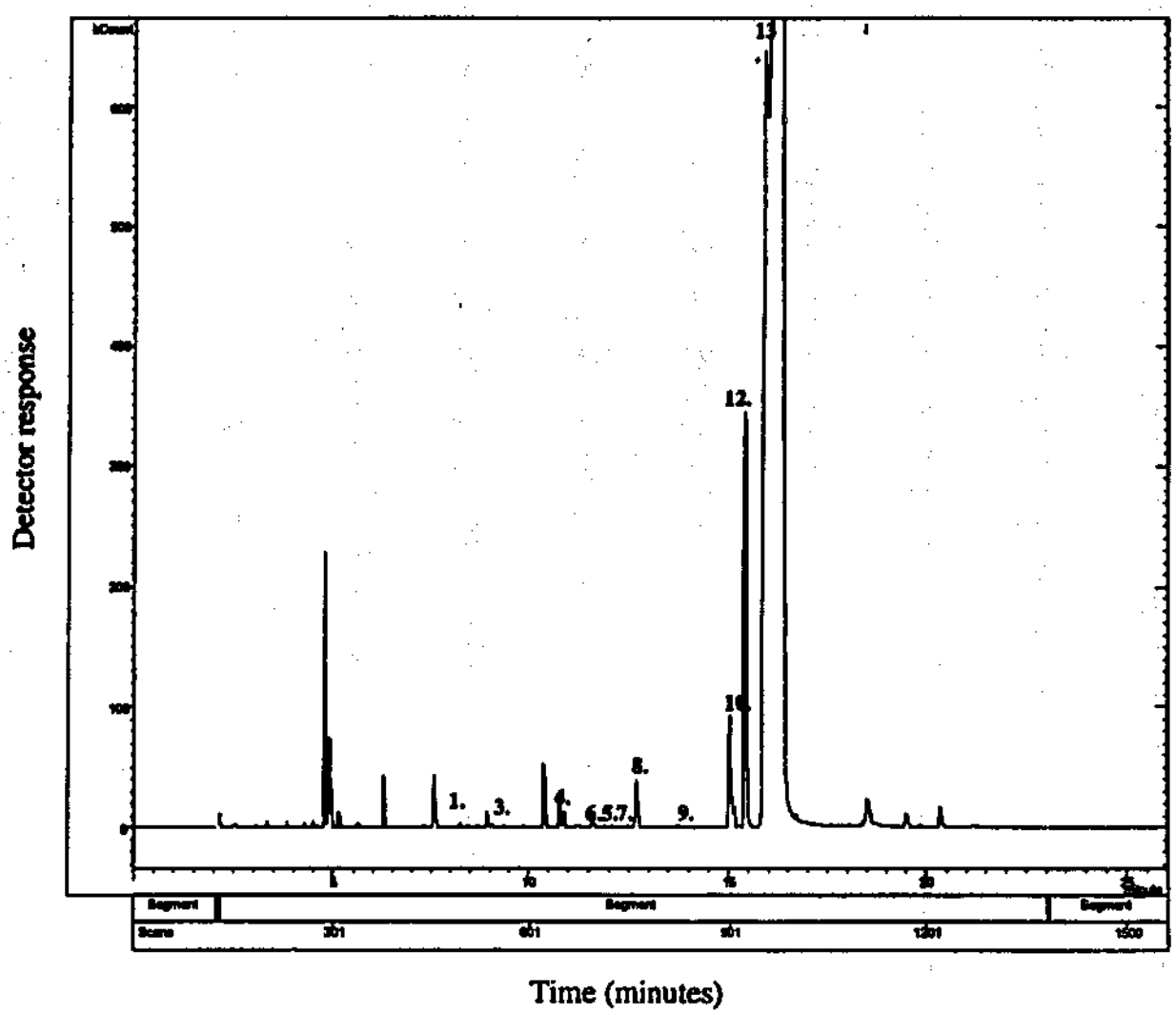


Figure 5.20 GC separation (using selected ion mode) of Flavour components present in Synthetic flavour F extract extracted using SPME.

Legend.

1. Ethyl hexanoate.
2. Ethyl octanoate.
3. *m*-Methoxybenzaldehyde.
4. *p*-Methoxybenzaldehyde.
5. 5-propenyl-1,3-benzodioxole.
6. Ethyl-*m*-benzoate.
7. Ethyl nonanoate.
8. Unidentified compound.
9. Piperonal.
10. *p*-Methoxybenzoic acid methyl ester.
11. 3-phenyl-2-propenoic acid methyl ester.
12. Ethyl decanoate.
13. Vanillin.
14. Coumarin.
15. Ethyl vanillin.

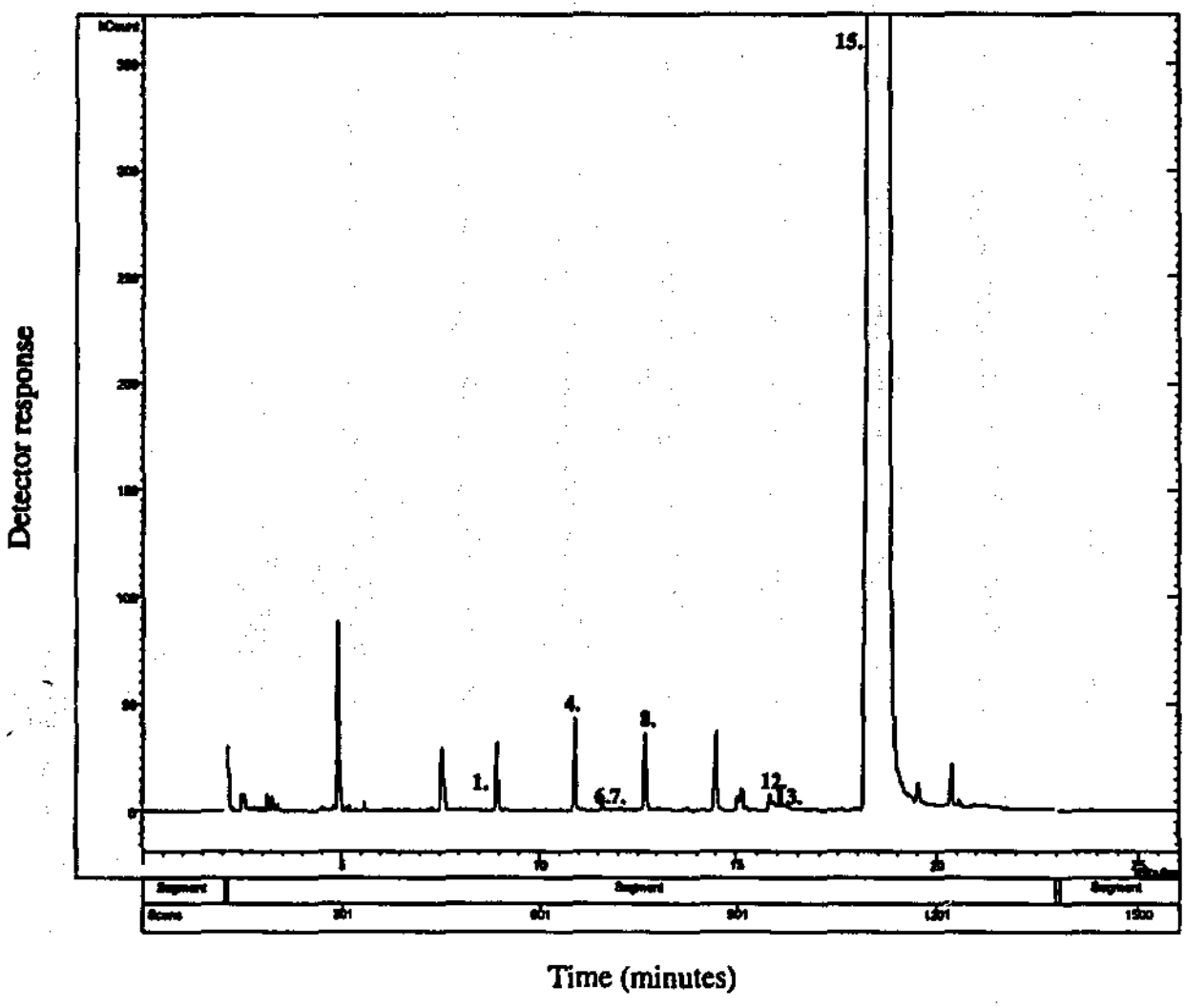
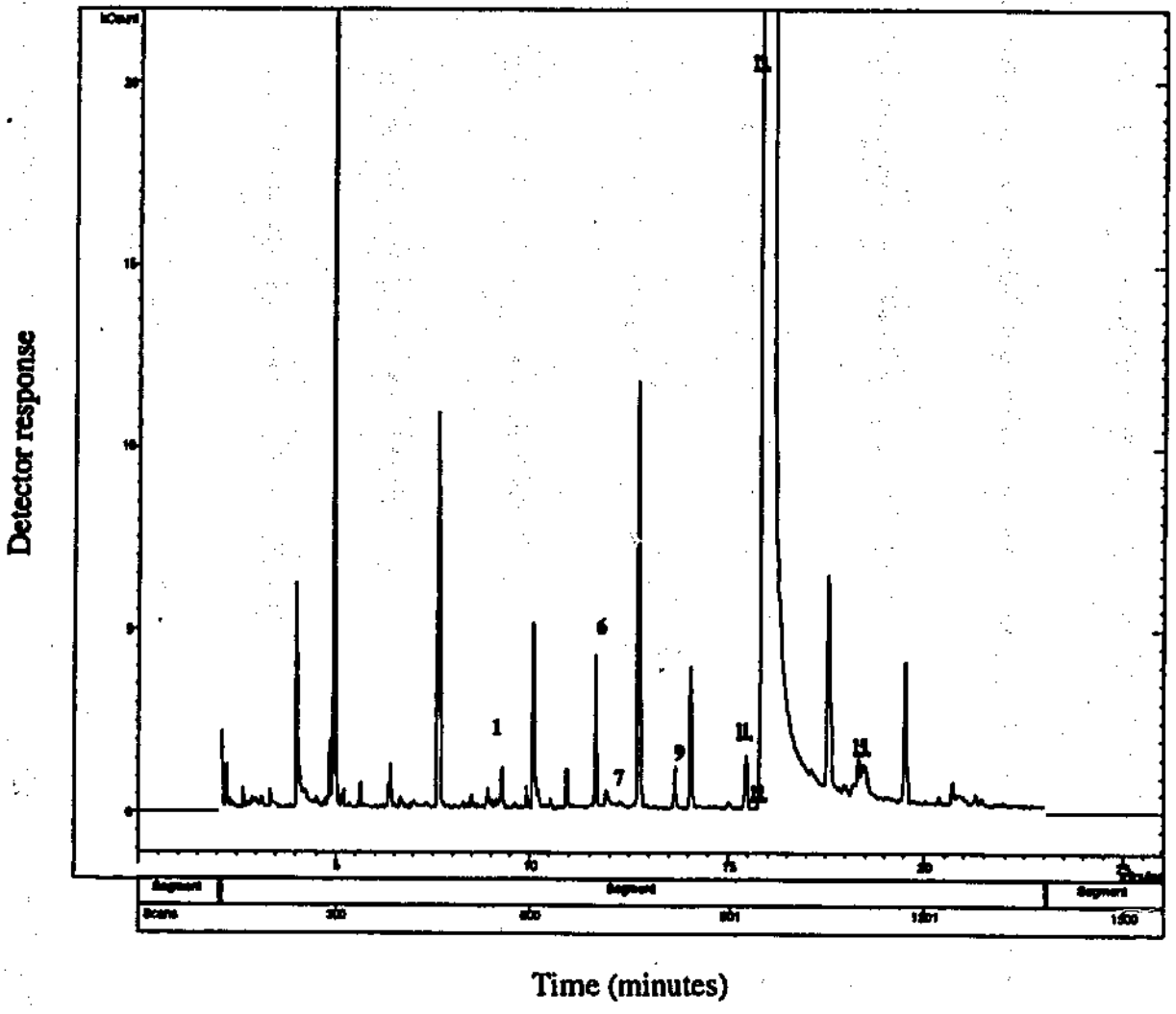


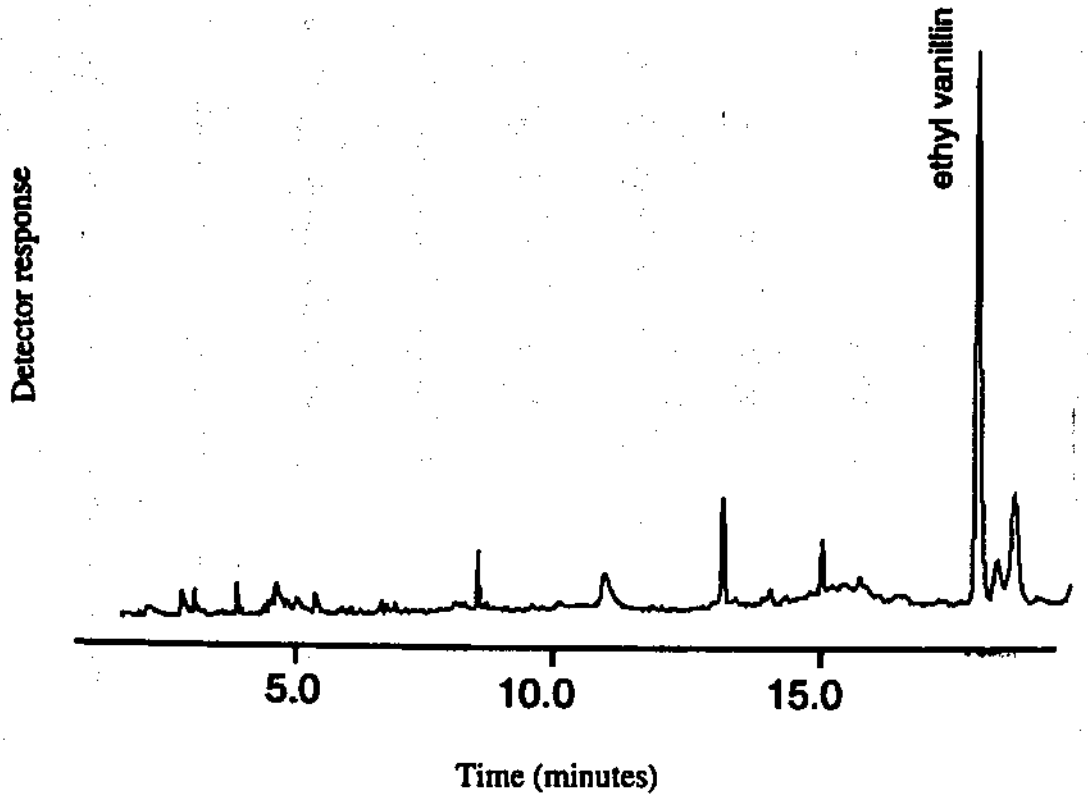
Figure 5.21 GC separation (using selected ion mode) of Flavour components present in Nature identical A extract extracted using SPME.

Legend.

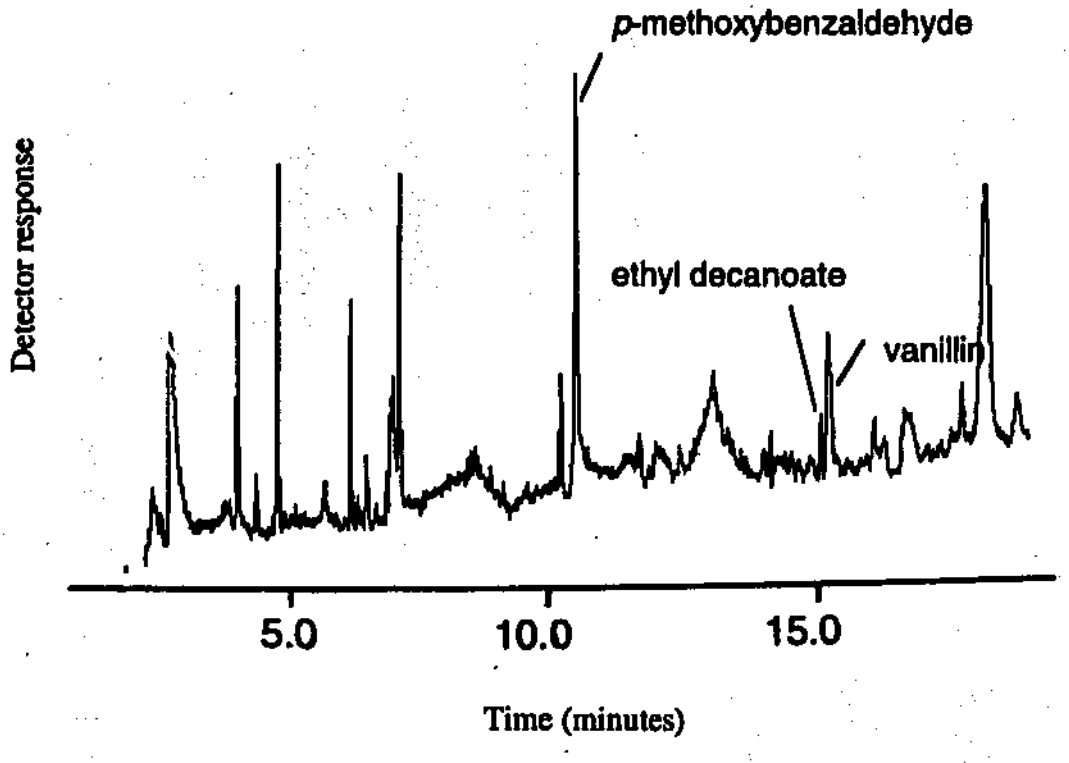
1. Ethyl hexanoate.
2. Ethyl octanoate.
3. *m*-Methoxybenzaldehyde.
4. *p*-Methoxybenzaldehyde.
5. 5-propenyl-1,3-benzodioxole.
6. Ethyl-*m*-benzoate.
7. Ethyl nonanoate.
8. Unidentified compound.
9. Piperonal.
10. *p*-Methoxybenzoic acid methyl ester.
11. 3-phenyl-2-propenoic acid methyl ester.
12. Ethyl decanoate.
13. Vanillin.
14. Coumarin.
15. Ethyl vanillin.



Figures 5.22 GC separation of components extracted from yogurt using SPME.



Figures 5.23 GC separation of components extracted from natural vanilla ice-cream using SPME.



Figures 5.24 GC separation of components extracted from vanilla custard powder using SPME.

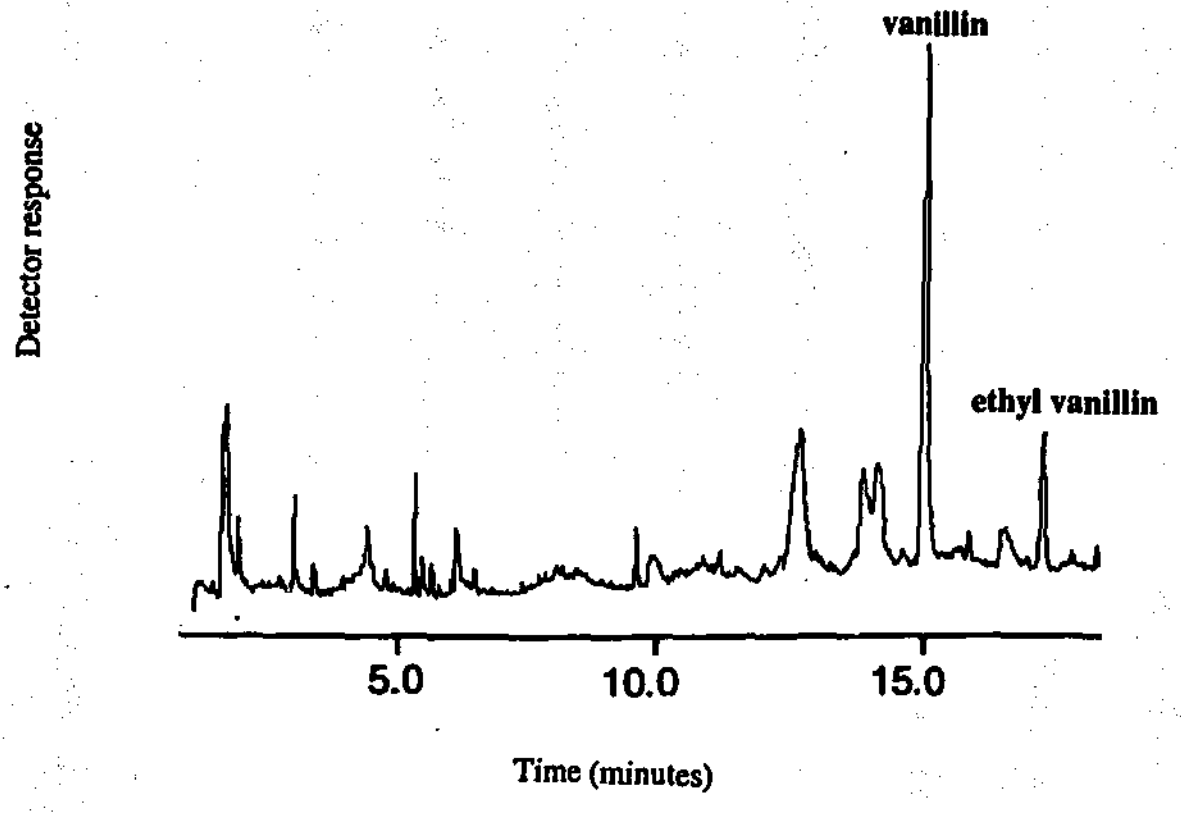


Figure 5.25 Separation of Flavour components in A Bourbon by HPLC.

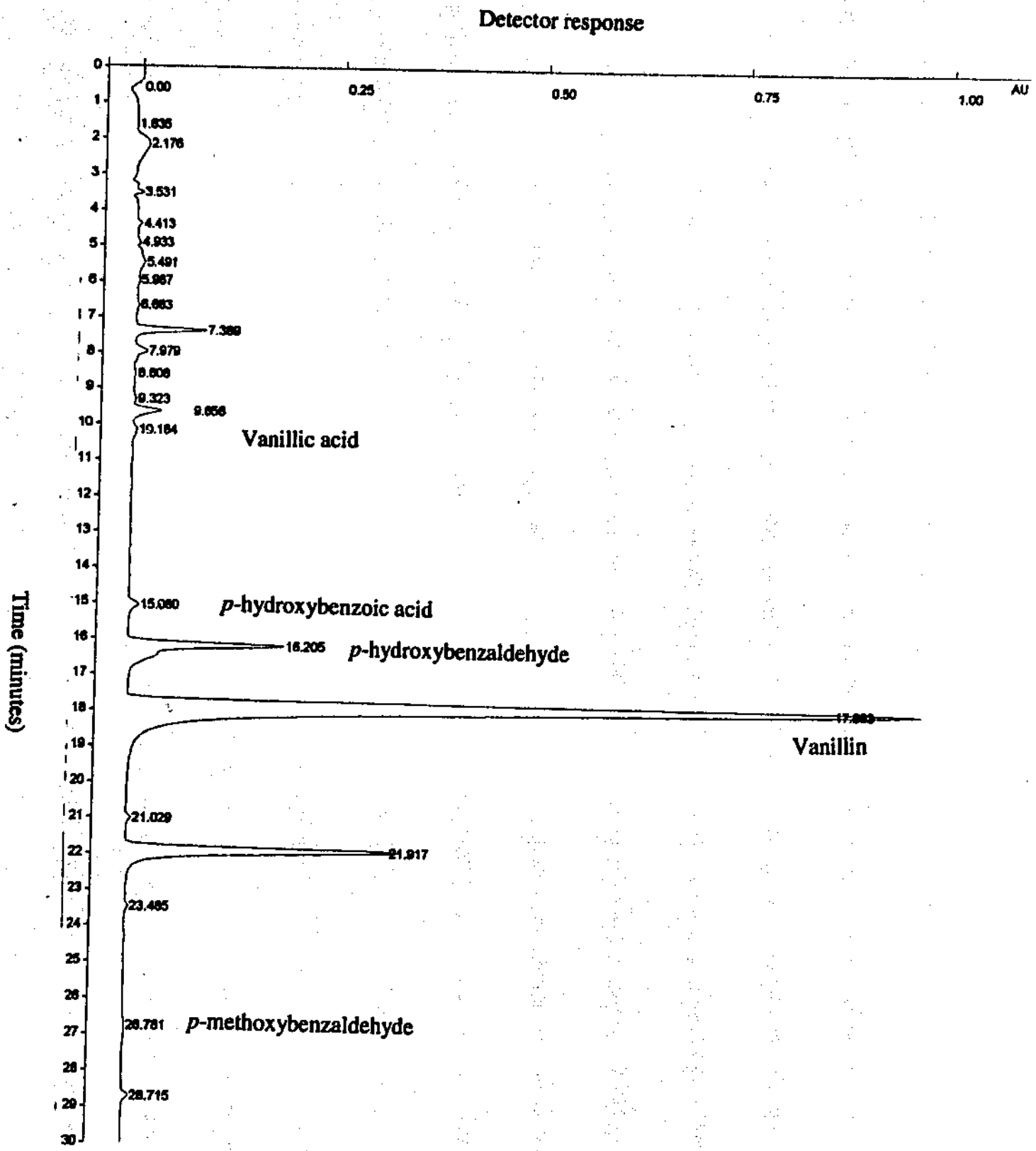


Figure 5.26 Separation of Flavour components in B Bourbon by HPLC.

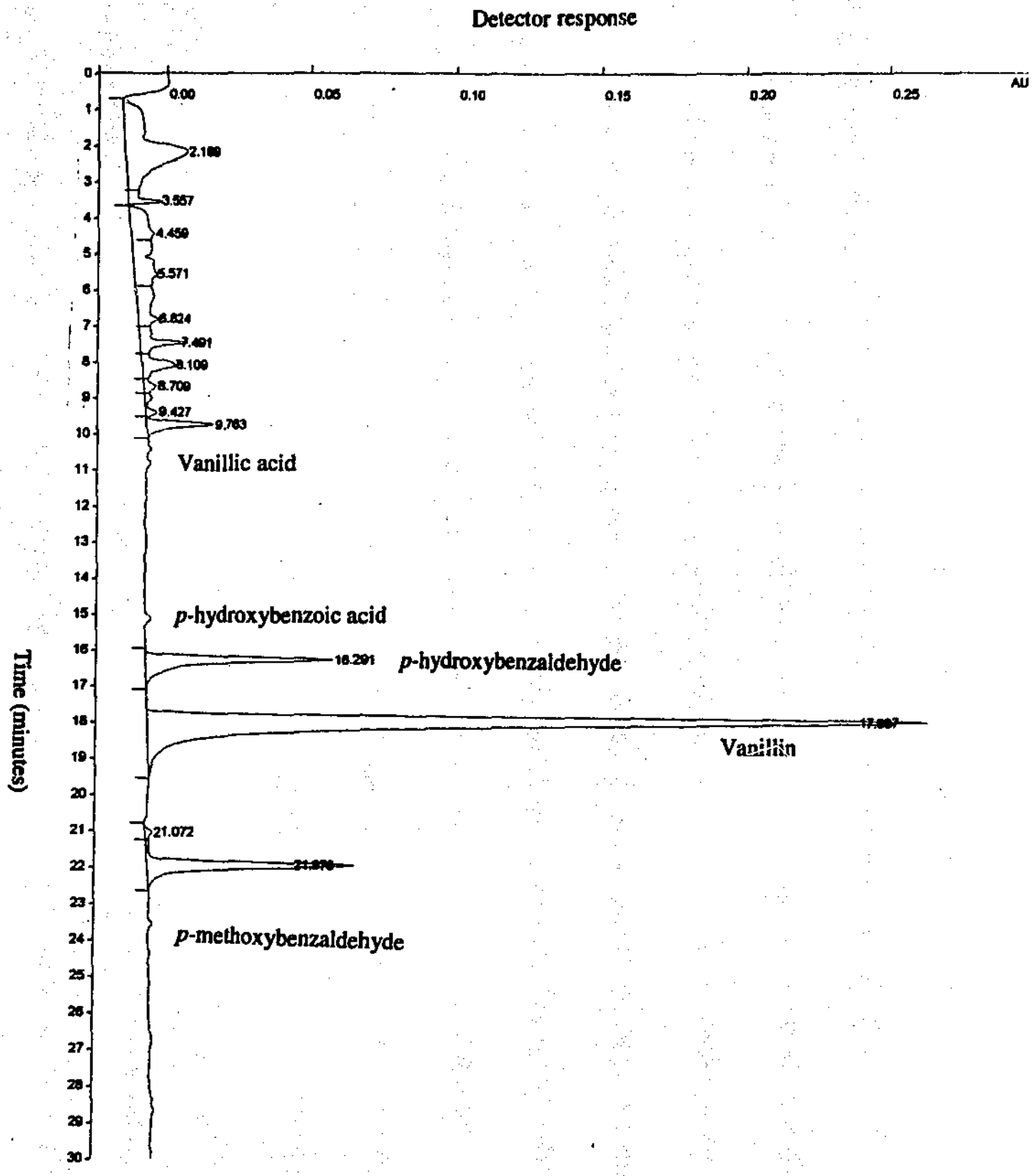


Figure 5.27 Separation of Flavour components in C Bourbon by HPLC.

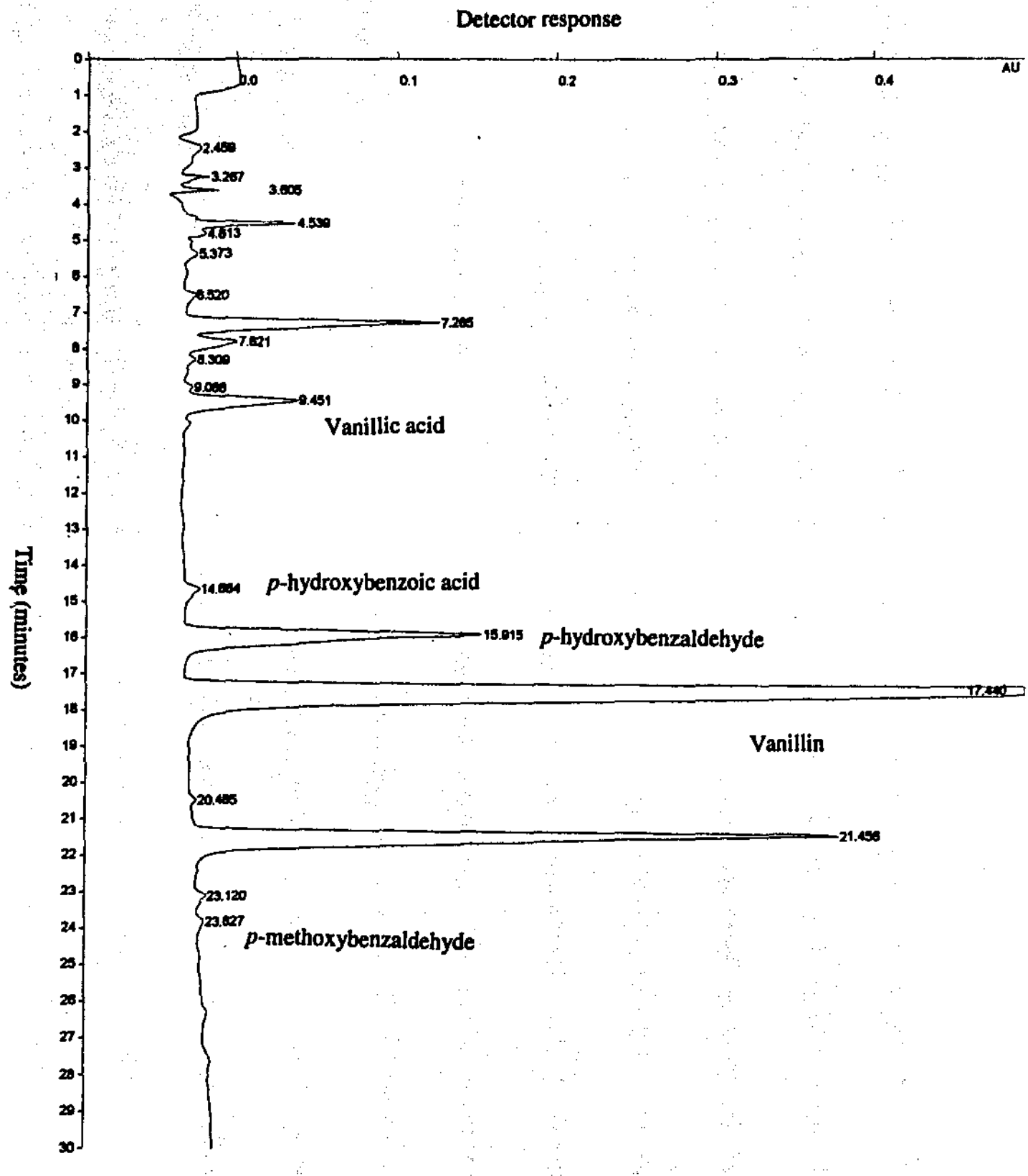


Figure 5.28 Separation of Flavour components in A Indonesian by HPLC.

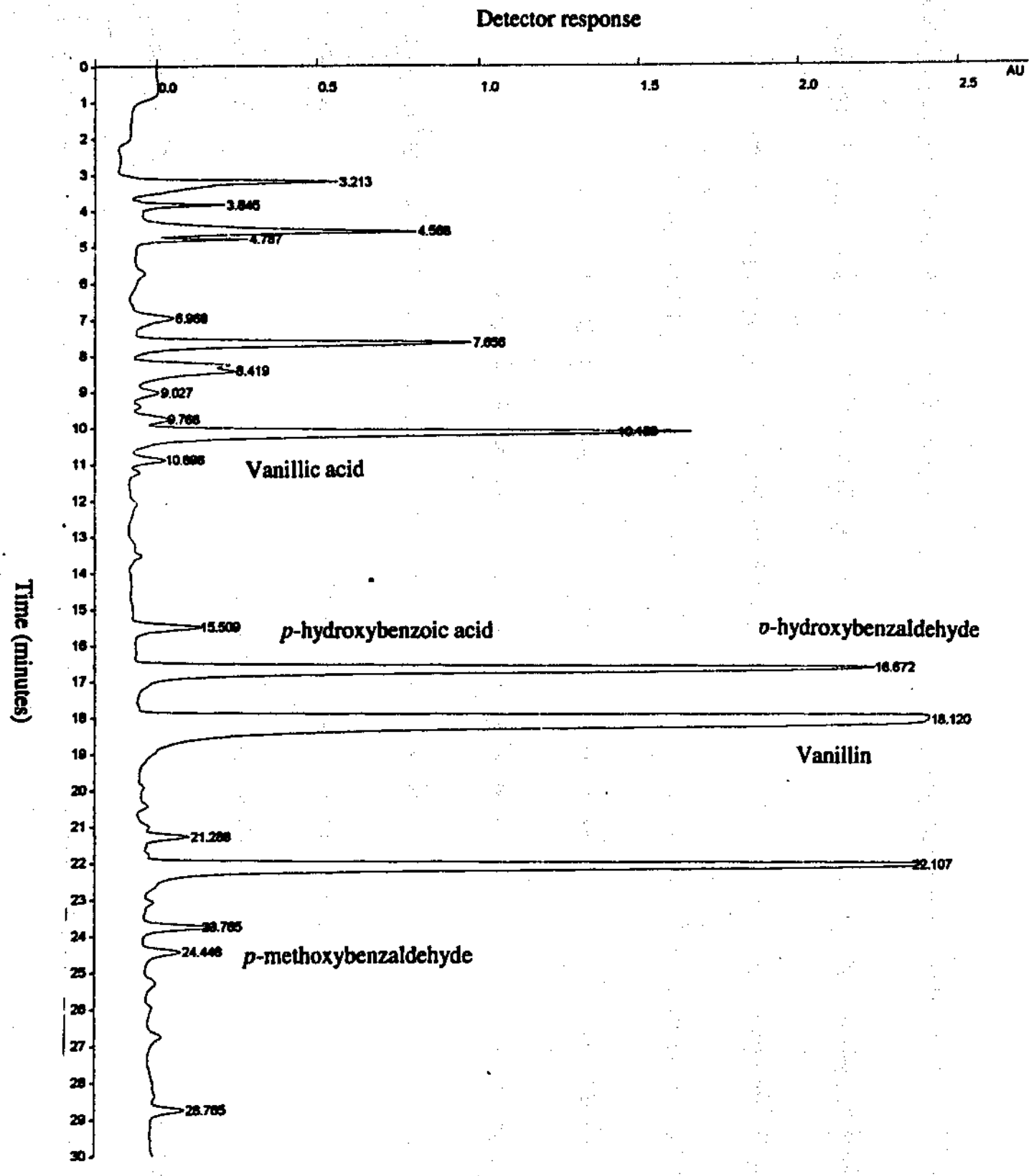


Figure 5.29 Separation of Flavour components in B Indonesian by HPLC.

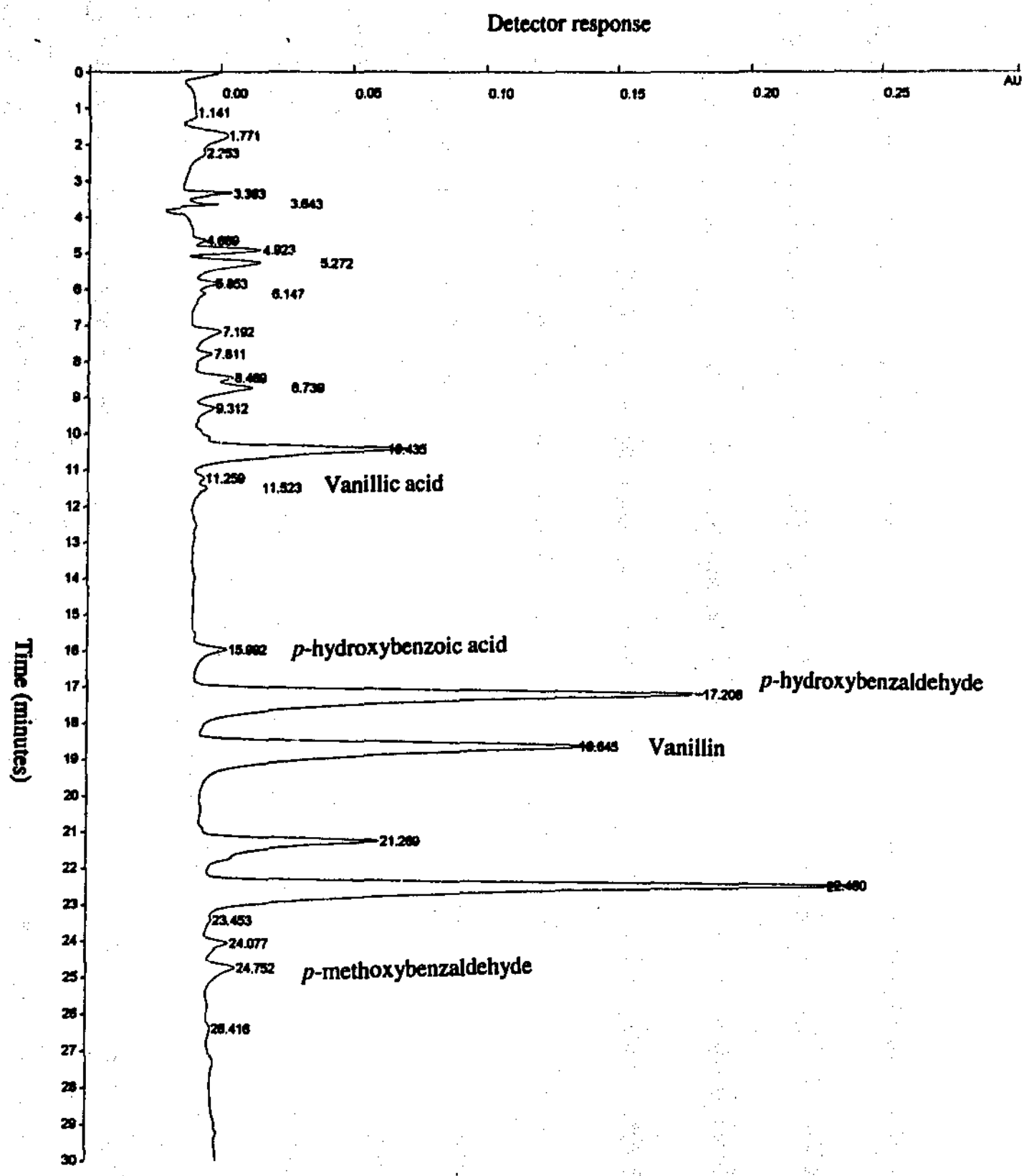


Figure 5.30 Separation of Flavour components in D Indonesian by HPLC.

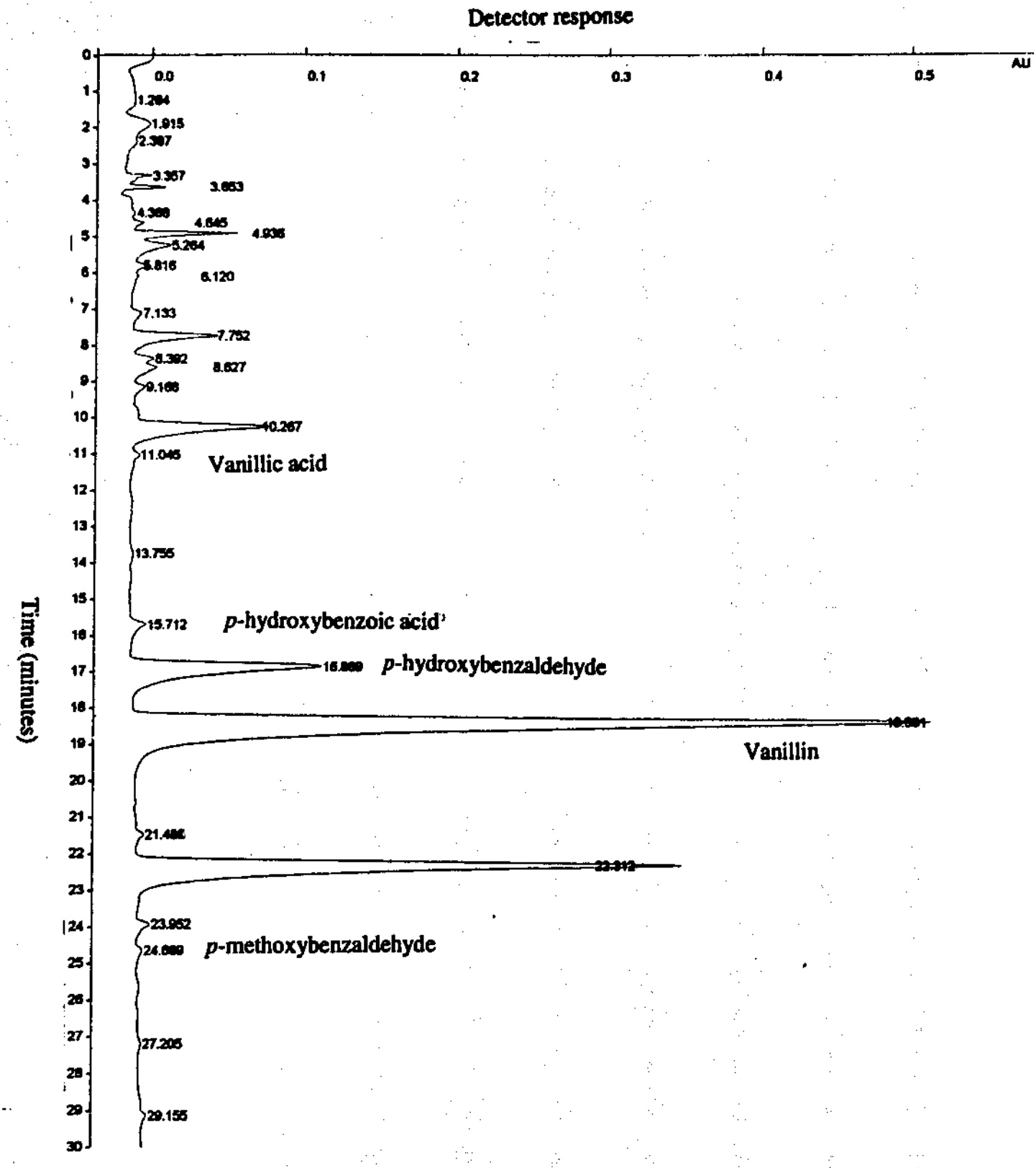


Figure 5.31 Separation of Flavour components in B Tongan by HPLC.

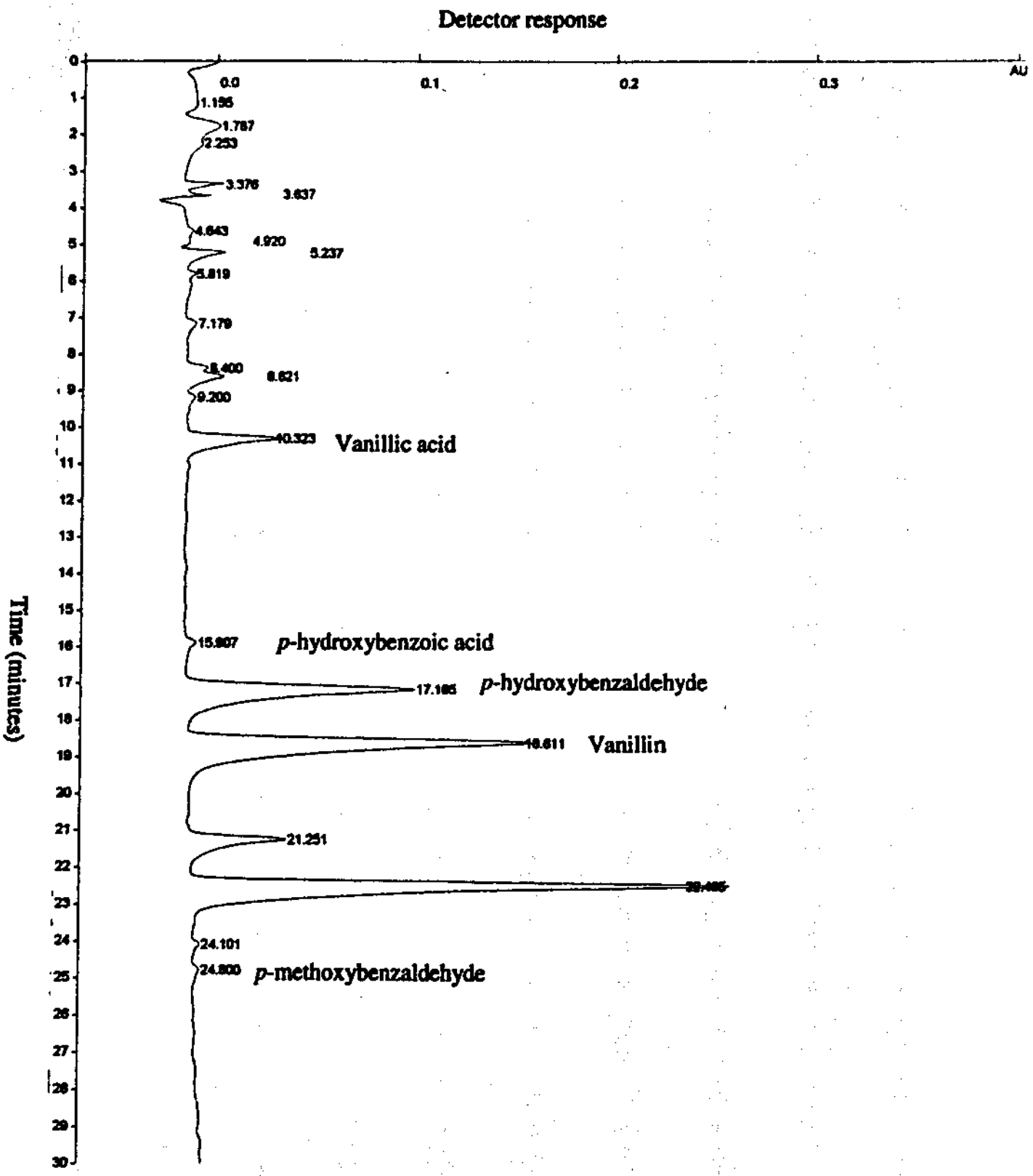


Figure 5.32 Separation of Flavour components in A Mexican by HPLC.

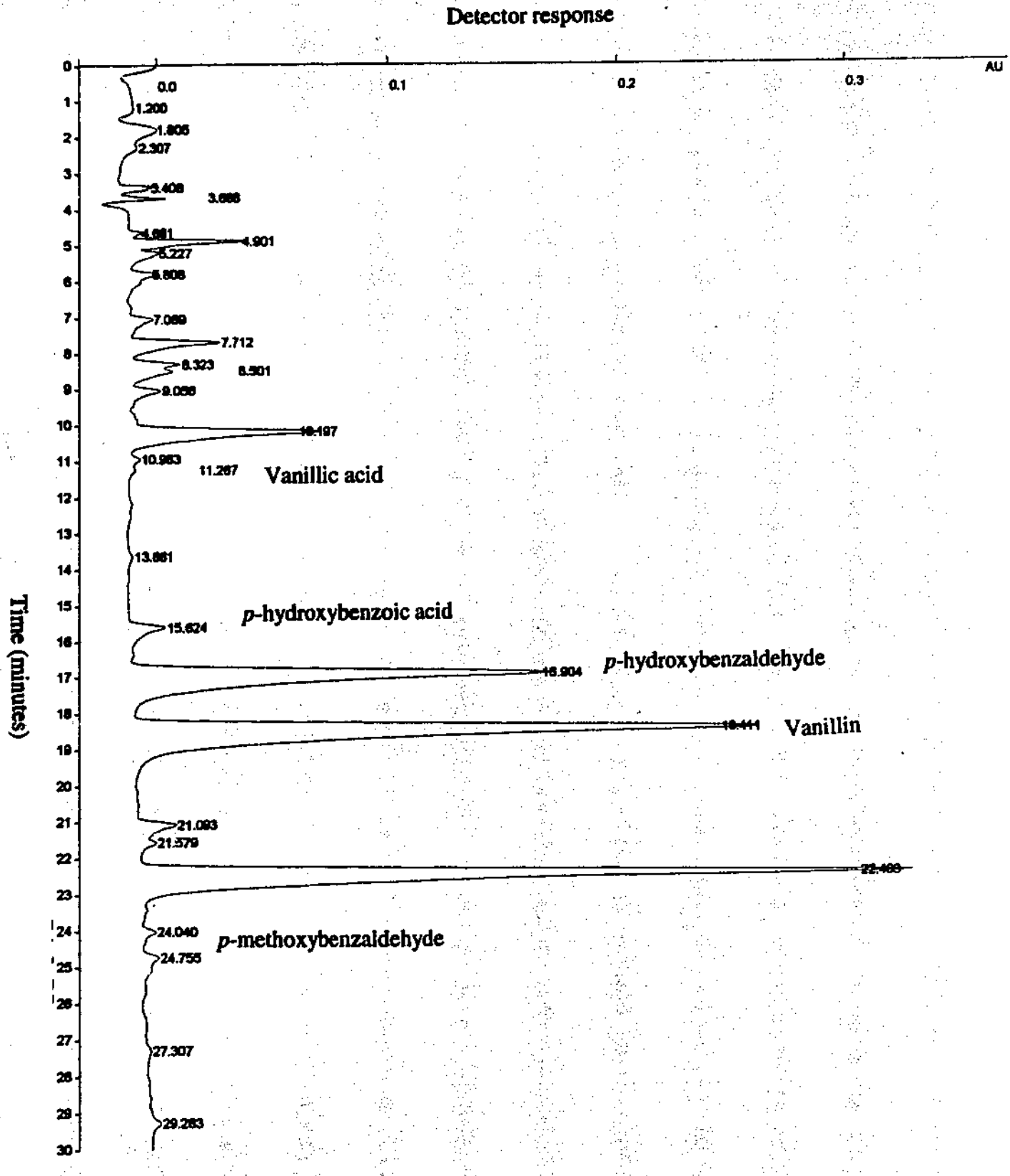


Figure 5.33 Separation of Flavour components in D Madagascan by HPLC.

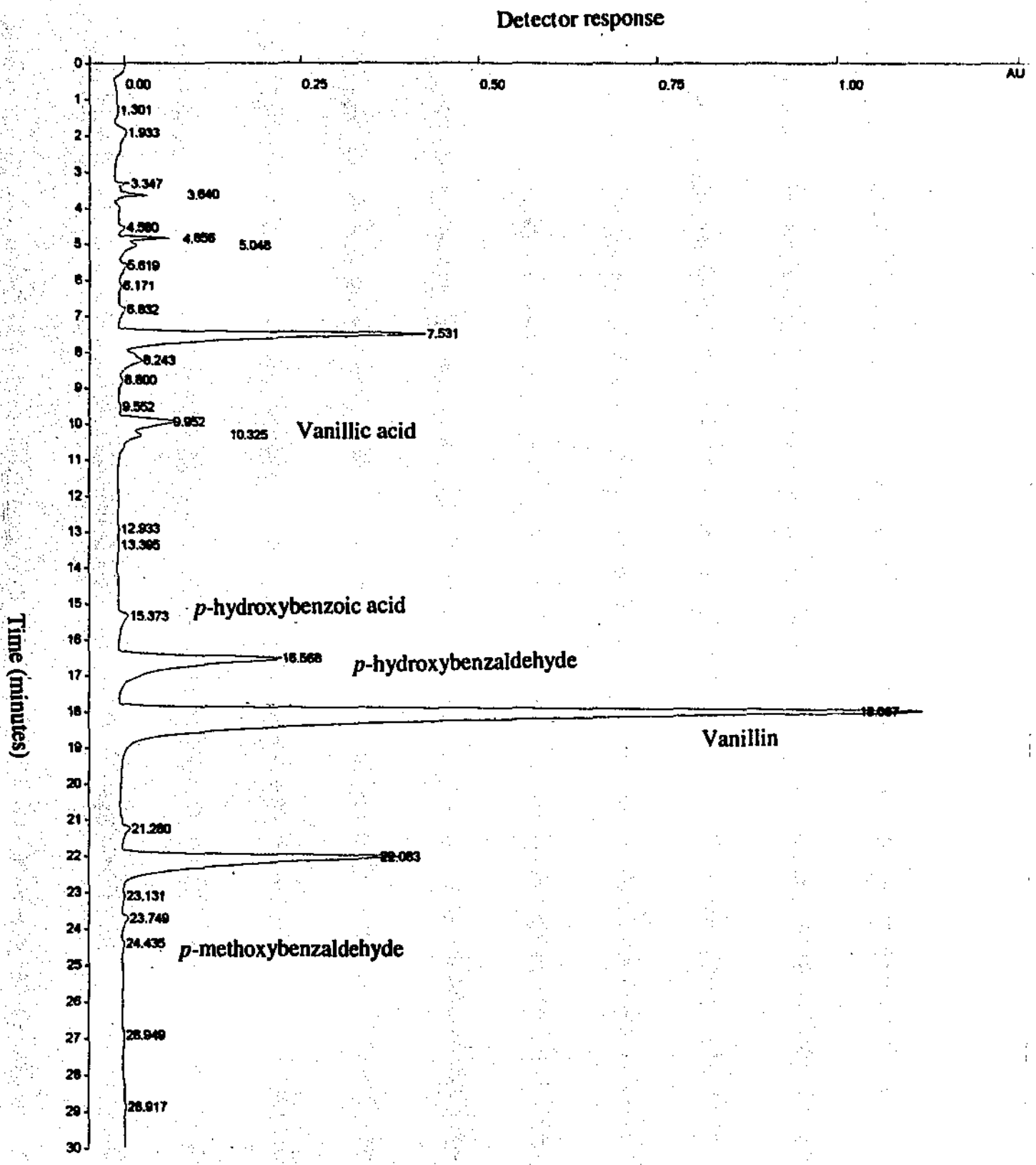


Figure 5.34 Separation of Flavour components in A Tahitian by HPLC.

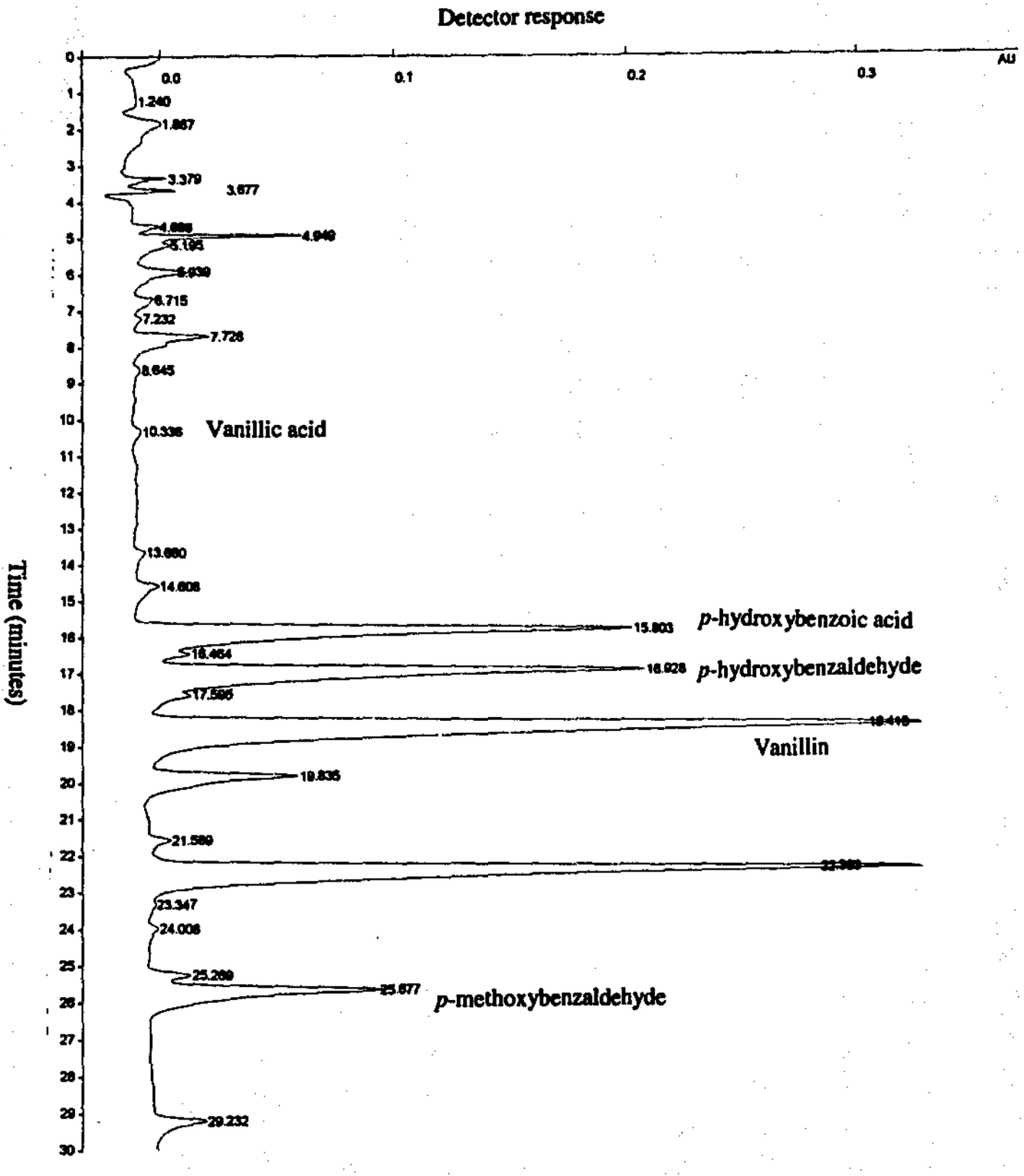


Figure 5.35 Separation of Flavour components in F Nature identical by HPLC.

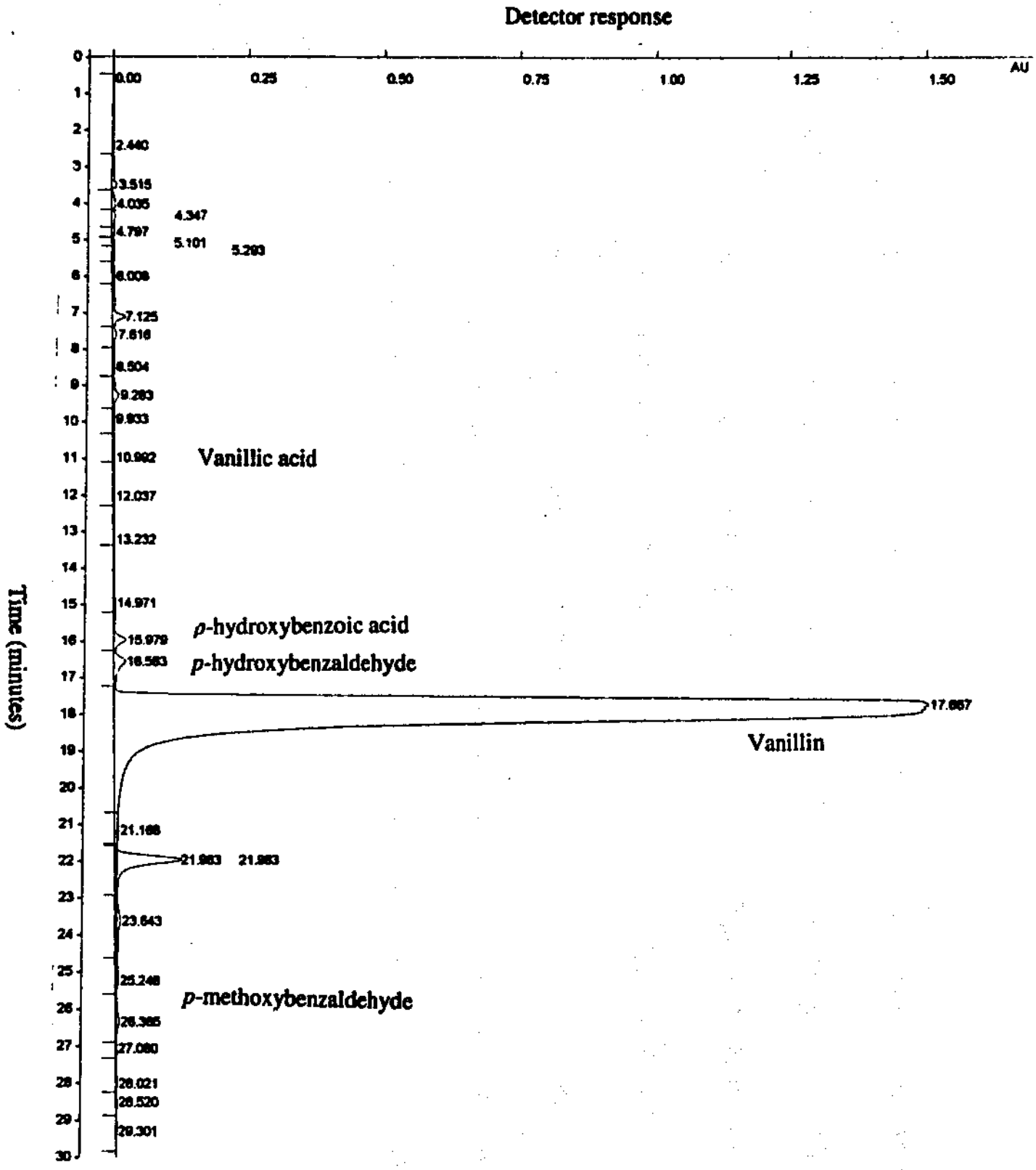


Figure 5.36 Separation of Flavour components in A Synthetic flavour by HPLC.

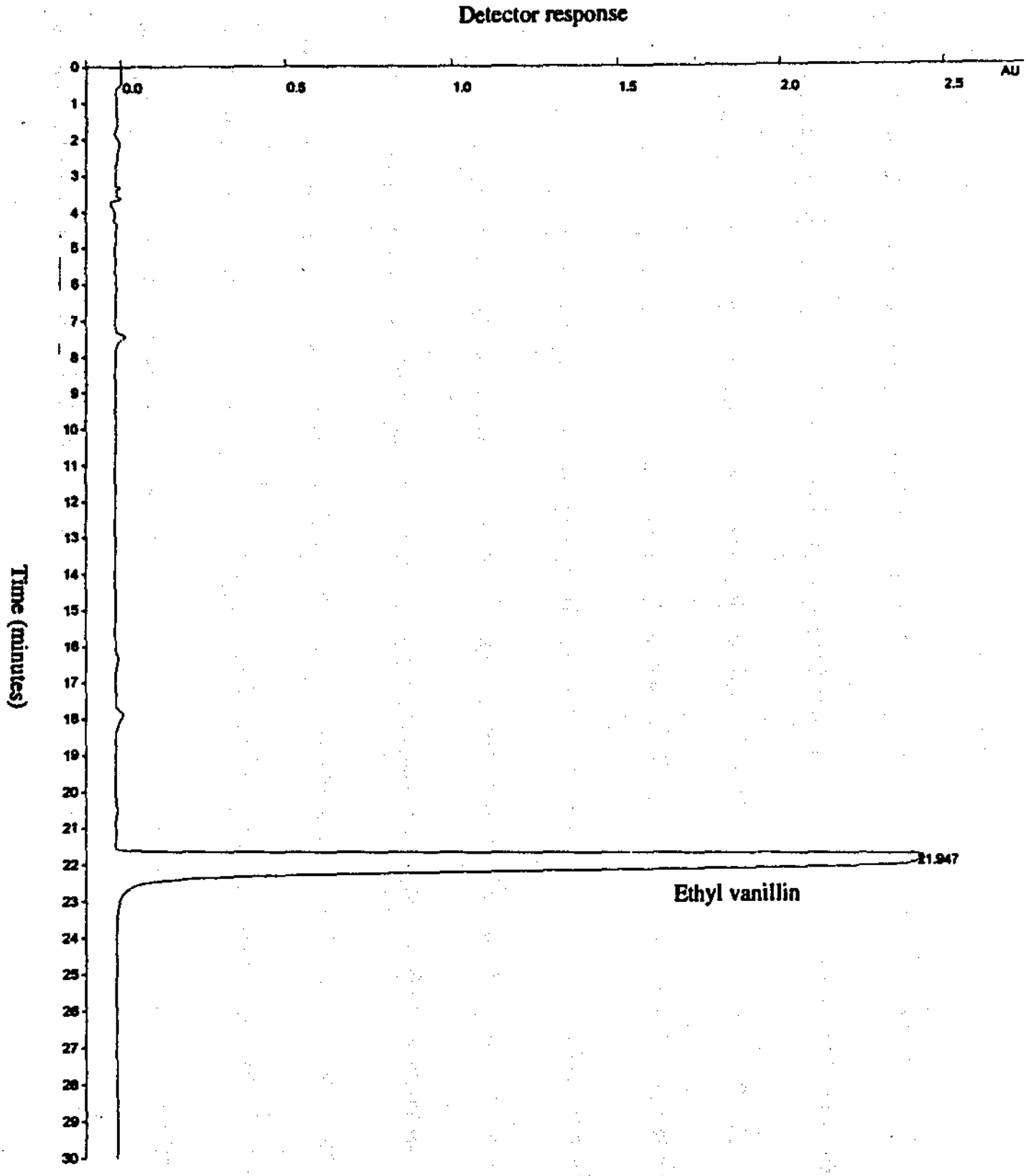
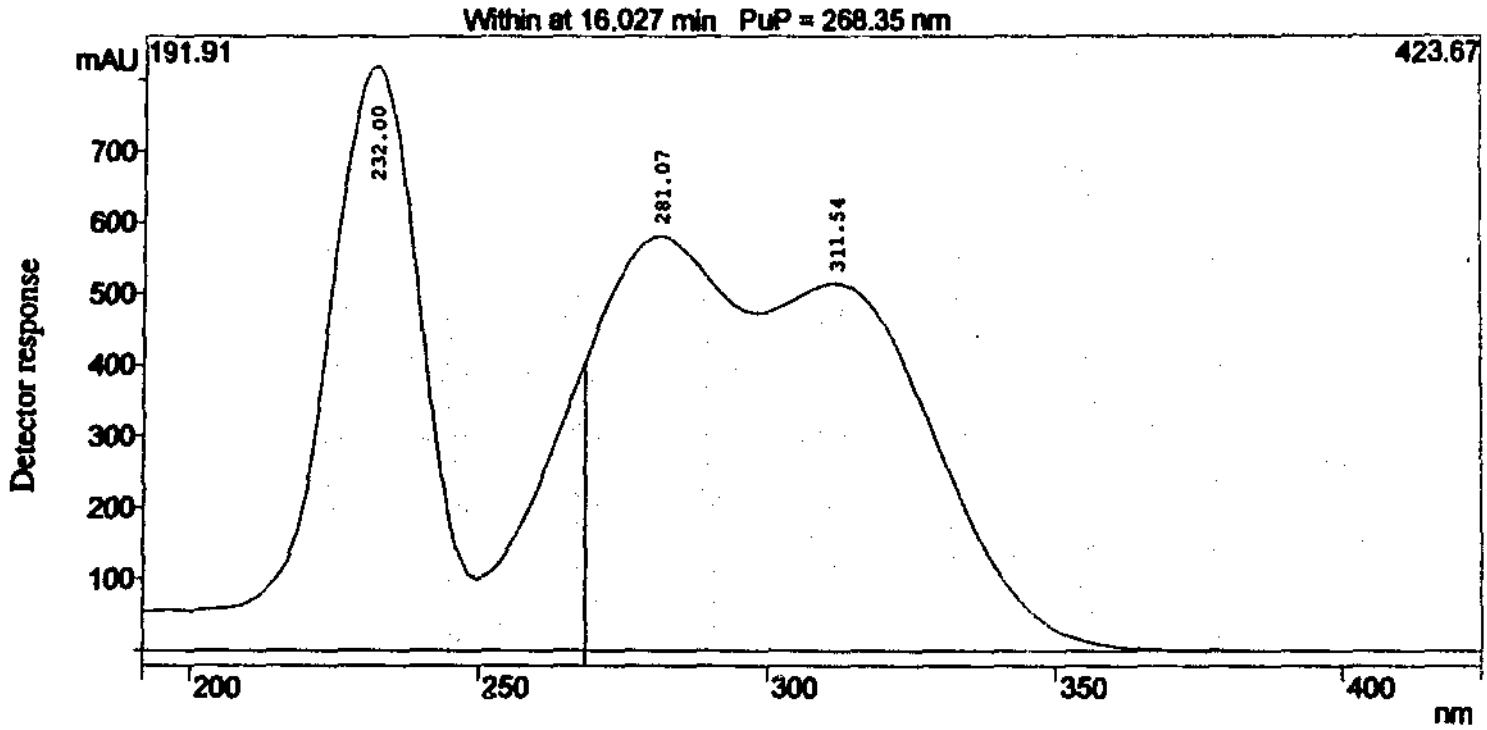


Figure 5.37. Multiple wavelength scan for vanillin using a diode array detector

Scan Rate: 0.625 Hz Bunch: 1 Data Rate: 0.625 Hz
Detector Range: 191.910->423.670 nm Valid Range: 191.910->423.670 nm
Spectrum Type: Within Correction Type: Baseline

Channel Range: 191.91 to 423.67 nm Absorbance Range: -0.0470 to 821.04 mAU

Max Wavelength(nm):	232.00	281.07	311.54
Percent of Max Abs.:	99.8%	70.8%	62.7%



nm	mAU	nm	mAU	nm	mAU	nm	mAU	nm	mAU	nm	mAU
191.91	54.422	196.44	56.720	200.19	55.126	202.91	57.678	205.80	59.464	208.88	63.768
211.33	74.976	213.02	85.460	214.80	101.35	216.63	123.88	218.55	167.96	220.52	244.65
222.60	366.29	224.74	519.15	226.38	628.73	227.52	694.97	228.69	750.76	229.88	792.17
231.10	816.36	232.33	821.03	233.61	804.60	234.88	765.89	236.22	705.20	237.58	624.53
238.97	529.22	240.38	427.18	241.83	328.45	243.33	242.50	244.86	175.89	246.41	131.18
248.02	107.01	249.66	99.674	251.35	105.46	253.08	120.15	254.86	141.53	256.69	168.34
258.57	200.07	260.50	236.45	262.48	277.06	264.51	321.28	266.63	368.16	268.79	416.43
271.04	464.14	273.32	508.32	275.72	545.19	278.17	570.51	280.07	580.35	281.35	581.44
282.67	578.47	284.00	571.71	285.35	561.73	286.73	549.17	288.14	535.18	289.57	520.74
291.01	506.85	292.51	494.56	294.01	484.45	295.54	477.42	297.10	473.77	298.72	473.50
300.32	476.49	302.00	482.06	303.69	489.41	305.41	497.50	307.17	505.18	308.95	511.41
310.79	514.84	312.66	514.46	314.57	508.90	316.51	497.35	318.50	479.26	320.54	454.31
322.60	423.07	324.73	386.14	326.92	344.85	329.14	300.68	331.42	255.43	333.75	210.88
336.14	168.84	338.57	130.82	341.07	97.984	343.66	70.894	346.29	49.583	349.00	33.550
351.76	21.999	354.63	14.010	357.54	8.6630	360.57	5.2494	363.66	3.1392	366.85	1.8784
370.13	1.1465	373.50	0.7505	376.98	0.5338	380.54	0.4009	384.25	0.3094	388.07	0.2579
392.01	0.2097	396.07	0.1344	400.26	0.1109	404.63	0.0688	409.14	0.0220	413.79	0.0133
418.64	-0.013	423.67	-0.047								

References.

Archer, A.W., (1989). Analysis of vanilla essences by high-performance liquid chromatography. Journal of Chromatography, 462, 461-466.

Arthur, C.L., & Pawliszyn, J. (1990). Solid phase microextraction with thermal desorption using fused silica optical fibres. Journal of Analytical Chemistry, 62, 2145-2148.

Augusto, F., Pires Valente, A.L., Santos Tada, E., & Rivellino, S.R. (2000). Screening of Brazilian fruit aromas using solid-phase microextraction-gas chromatography-mass spectrometry. Journal of Chromatography A, 873, 117-127.

Baker, D.R. (1995). Capillary electrophoresis. New York: Wiley.

Bazemore, R., Goodner, K., & Rousetf, R. (1999). Volatiles from unpasteurised and excessively heated orange juice analysed with solid phase microextraction and GC-Olfactometry. Journal of Food Science, 64, 800-803.

Belay, M., & Poole, C., (1993). Determination of vanillin and related flavour compounds in natural vanilla extracts and vanilla flavoured foods by thin-layer chromatography and automated multiple development. Chromatographia, 37, 365-373.

Bicchi, C.P., Panero, O.M., Pellegrino, G.M., & Vanni, A.C. (1997). Characterization of roasted coffee and coffee beverages by solid phase microextraction-gas chromatography and principal component analysis. Journal of Agricultural and Food Chemistry, 45, 4680-4686.

Boyce, M.C., & Spickett, E.E. (2000). Determination of additives in cosmetics by micellar electrokinetic capillary chromatography. Journal of Liquid Chromatography and Related Technologies, 23 (11), 1689-1697.

Boyd-Boland, A. (1998). Solid phase microextraction. Chemistry in Australia, 65 (4), 10.

Bricout, J., Fontes, J.-C., & Marlivat, L. (1974). Detection of synthetic vanillin in vanilla extracts by isotopic analysis. Journal of Associative and Official Analytical Chemistry, 57, 713-715.

Bricout, J., Koalrt, J., Derbesy, M., & Beccat, B. (1981). New possibilities with analysis of stable isotopes of carbon in quality control of vanillas. Annales des Falsifications at de l'Expertise Chimique, 74 (803), 891.

Bricout, J.(1982). Possibilities of stable isotope analysis in the control of food products. Amsterdam: Elvisier Science Publishers.

Brunton, N.P., Cronin, D.A., Monahan, F.J., & Durcan, R. (2000). A comparison of solid-phase microextraction (SPME) fibers for measurement of hexanal and pentanal in cooked turkey. Journal of Agricultural and Food Chemistry, 68, 339-345.

Butehor, U., & Pyell, U. (1996). Micellar electrokinetic chromatography as a screening method for the analysis of vanilla flavourings and vanilla extracts. Journal of Chromatography A, 736, 321-332.

Chee, K.K., Wong, M.K., Lee, H.K. (1999). SPME for the determination of organochlorine pesticides in natural waters. In J. Pawliszyn (Ed.), Applications of solid phase microextraction (pp. 212-226). Cambridge, UK: The Royal Society of Chemistry.

Clark, T.J., & Bunch, J.E. (1997). Qualitative and quantitative analysis of flavour additives on tobacco products using SPME-GC-mass spectroscopy. Journal of Agricultural and Food Chemistry, 45, 844-849.

De la Calle Garcia, D., & Reinchenbacher, M. (1998a). Use of solid-phase microextraction-capillary-gas chromatography (SPME-GC) for the volatile characterisation of wines by means of chemometrical methods. Journal of Analytical Chemistry,360, 784-787.

De la Calle Garcia, D., Reichenbacher, M., & Danzer, K. (1998b). Analysis of wine bouquet components using headspace solid-phase microextraction-capillary gas chromatography. Journal of High Resolution Chromatography,21 (7), 373-377.

Dean, J.R., & Hancock, P. (1999). Direct analysis of solids using SPME. In J. Pawliszyn (Ed.), Applications of solid phase microextraction (pp. 248-257). Cambridge, UK: The Royal Society of Chemistry.

Dufour, J.P., Delbecq, P., Albela, L.P. (2001). Solid-phase microextraction combined with gas chromatography-olfaction for analysis of cheese aroma. Acs Symposium Series, 782, 123-137.

Ebeler, S.E., Terrrien, M.B. & Butzke, C.E. (2000). Analysis of brandy aroma by solid-phase microextraction and liquid-liquid extraction. Journal of Agriculture and Food Science, 45, 625-630.

Elmore, S., Erbahadir, M.A. & Mottram, D.S. (1997). Comparison of dynamic headspace concentration on Tenax with solid-phase microextraction for the analysis of aroma volatiles. Journal of Agriculture and Food Science, 45, 2638-2641.

Fayet, B., Tisse, C., Gosrera, M., & Estlenno, J. (1987). New analytical specifications in the study of vanilla beans. Analusia, 15 (5), 217.

Field, J.A., Nickerson, G., Derryl, D.J., & Heider, C. (1996). Determination of essential oils in hops by headspace solid-phase microextraction. Journal of Agricultural and Food Chemistry, 44, 1768-1772.

Grote, C., & Levsen, K. (1999). The applications of SPME in water analysis. In J. Pawliszyn (Ed.), Applications of solid phase microextraction (pp. 169-187). Cambridge, UK: The Royal Society of Chemistry.

Guarino, P.A., & Brown, S.M. (1985). Liquid chromatographic determination of vanillin and related flavour compounds in vanilla extract: Cooperative study. Journal of Associative and Official Analytical Chemistry, 68, 1198-1201.

Guidotti, M., & Panzironi, L. (2000). Caffeine determination in beverages by SPME and GC-MS. Industrie Delie Bevande, 29 (170), 595-597.

Hall, B.J., & Brodbelt, J.S. (1997). Determination of barbiturates by solid-phase microextraction (SPME) and ion trap gas chromatography-mass spectrometry. Journal of Chromatography A, 777, 275-282.

Herrmann, A., & Stockli, M. (1982). Rapid control of vanilla-containing products using high-performance liquid chromatography. Journal of Chromatography, 246, 313-316.

Hocking, M.B. (1997). Vanillin: Synthetic flavoring from spent sulfite liquor. Journal of Chemical Education, 74 (9), 1055.

Hoffman, P.G., & Salb, M. (1979). Isolation and stable isotope ratio analysis of vanillin. Journal of Agricultural and Foods Chemistry, 27, 352-355.

Ibanez, E., Lopez-Sebastian, S., Ramos, E., hera, J., & Reglero, G. (1998). Analysis of volatile fruit components by headspace solid-phase microextraction. Journal of Agricultural and Food Chemistry, 63 (2), 245-248.

Jagerdeo, E., Passetti, E., Dugar, D.M. (2000). Liquid chromatographic determination of vanillin and related aromatic compounds. Journal of Aoac International, 83 (1), 237.

Jaillais, B., Bertraud, V., & Auger, J. (1999). Cryo-trapping/SPME/GC analysis of cheese aroma. Talanta, 48, 747-753.

Jelen, H.H., Wlazly, K., Wasowicz, E., & Kaminski, E. (1998). Solid-phase microextraction for the analysis of some alcohols and esters in beer: Comparison with static headspace method. Journal of Agricultural and Food Chemistry,46, 1469-1473.

Jia, M., Zhang, Q.H., & Min, D.B. (1998). Optimization of solid-phase microextraction for headspace flavour compounds of orange juice. Journal of Agricultural and Food Chemistry, 46, 2744-2747.

Jinno, K., Morishima, Y., Saito, Y., & Fujimoto, C. (2000). SPME/HPLC using PEEK as extraction medium. Journal of Chromatography, 21 (4), 362-363.

Jurgens, U. (1961). The vanillin/p-hydroxybenzaldehyde ratio in bourbon vanilla. Lebernamittalchemie und Gerichtliche Chemie, 35 (5), 97

Kataoka, H., Lord, H.L., & Pawlisyzn, J. (2000). Applications of solid-phase microextraction in food analysis. Journal of Chromatography A, 880, 35-62.

Klimes, I., & Lamparsky, D. (1976). Vanilla volatiles – A comprehensive analysis. International Flavors of Food Additives, Nov/Dec, 272-276.

Kuhn, R., & Hoffestetter-Kuhn, S. (1993). Capillary electrophoresis: Principles and practice. New York: Springer Verleg.

Leong, G., Archavilis, A., & Derbesy, M. (1989). Research on the glycoside fraction of the vanilla bean. Journal of Essential Oil Research, 1, 33.

Ligor, M., & Buszewski, B. (1999). Determination of menthol and menthone in food and pharmaceutical products by solid-phase microextraction-gas chromatography. Journal of Chromatography A, 847, 161-169.

Lord, H. (1999). Optimization of drug analysis by SPME. In J. Pawliszyn (Ed.), Applications of solid phase microextractions (pp. 540-556). Cambridge, UK: The Royal Society of Chemistry.

Lord, H. & Pawliszyn, J. (2000). Evolution of solid-phase microextraction technology. Journal of Chromatography A, 885, 153-193.

Marsili, R.T. (2000a). Shelf-life prediction of processed milk by solid-phase microextraction, mass spectrometry, and multivariate analysis. Journal of Agriculture and Food Chemistry, 48, 3470-3475.

Marsili, R.T., & Miller, N. (2000b). Determination of major aroma impact compounds in fermented cucumbers by solid-phase microextraction chromatography-mass spectrometry-olfactometry detection. Journal of Chromatographic Science, 38, 307-314.

Martin, G.E., Guinand, G.G., & Figert, D.M. (1973). Comparison of gas-liquid. Gas-solid, liquid-liquid, and liquid-solid chromatographic techniques in analysis of vanillin and ethyl vanillin. Journal of Agricultural and Food Chemistry,21,544-547.

Martin, G.E., Alfonso, F.C., Figert, D.M., & Burggraff, J.M., (1981). Stable isotope ratio determination of the origin of vanillin in vanilla extracts and its relationship to vanillin/potassium ratios. Journal of Associative and Official Analytical Chemistry, 64, 1149-1153.

Matich, A.J. (1999). Analysis of food and plant volatiles. In J. Pawliszyn (Ed.), Applications of solid phase microextractions (pp. 349-363). Cambridge, UK: The Royal Society of Chemistry.

Mayer, D.L., & Fritz, J.S. (1997). Semi-micro solid-phase extraction of organic compounds from aqueous and biological samples. Journal of Analytical Chemistry, 71 (1), 23-27.

McCormick, R. (1988). Vanilla and vanillin remain over-versatile ingredients. Prepared Foods, 157 (2), 123.

Merrory, J. (1988). Natural flavourings from vanilla beans. In Food flavourings: Composition, manufacture, and use, (2nd ed., pp. 163). Westport: Avi Publishing.

Mestres, M., Marti, M.P., Busto, O., & Guasch, J. (1999a). Simultaneous analysis of thiols, sulfides and disulfides in wine aroma by headspace solid-phase microextraction-gas chromatography. Journal Chromatography A,849, 293-297.

Mestres, M., Sala, C., Marti, M.P., Busto, O., & Guasch, J. (1999b). Headspace solid-phase microextraction of sulfides and disulfides using carboxen-polydimethylsiloxane fibres in the analysis of wine aroma. Journal of Chromatography A, 835, 137-144.

Mestres, M., Busto, O., & Guasch, J. (2000a). Analysis of organic sulfur compounds in wine aroma. Review. Journal of Chromatography, 881 (1/2),569.

Mestres, M., Marti, M.P., Busto, O., & Guasch, J. (2000b). Analysis of low-volatility organic sulfur compounds in wines by solid-phase microextraction and gas chromatography. Journal of Chromatography A, 881, 583-590.

Miller, K., Poole, C., & Pawlowski, T. (1996). Classification of the botanical origin of cinnamon by solid-phase microextraction and gas chromatography. Journal of Chromatographia 42, 639-646.

Miller, M.E., & Stuart, J.D. (1999). Comparison of gas-sampled and SPME-sampled static headspace for the determination of volatile flavour components. Journal of Analytical Chemistry, 71 (1), 23-27.

Muller, L., Gorecki, T., & Pawliszyn, J. (1999). Optimization of the SPME device design for field applications. Fresenius' Journal of Analytical Chemistry, 346 (7), 610.

Ng, L.K, Hupe, M., Harnois, J. & Moccia, D. (1996). Characterisation of commercial vodkas by solid-phase microextraction and gas chromatography/mass spectrometry analysis. Journal of Agriculture and Food Science 70, 380-388.

O'Malley, V.P., Burke, R.A., & Schlotzhauer, W.S. (1997). Using GC-MS/Combustion/IRMS to determine the $^{13}C/^{12}C$ ratios of individual hydrocarbons produced from the combustion of biomass materials-applications to biomass burning. Organic Geochemistry, 27 (7), 567-581(15).

Oliva, J., Navarro, S., Barba, A., & Navarro, G. (1999). Determination of chloropyrifos, penconazole, fenarimol, vinclozolin and metalaxyl in grapes, must and wine by on-line microextraction and gas chromatography. Journal of Chromatography A, 883, 43-51.

Page, B.D. & Lacroix, G. (2000). Analysis of volatile contaminants in vegetable oils by headspace solid-phase microextraction with carboxen-based fibres. Journal of Chromatography A, 873, 79-94.

Pawliszyn, J. (1997). Solid-phase microextraction-theory and practice. New York: Wiley-VCH.

Popp, P., Moder, M., & McCann, I. (1999). Determination of sulfur-containing compounds in wastewater. In J. Pawliszyn (Ed.), Applications of solid phase microextractions (pp.227-237). Cambridge, UK: The Royal Society of Chemistry.

Radovic, B.S., Careri, M., Mangia, A., Musci, M., Gerboles, M., & Anklam, E. (2001). Contribution of dynamic headspace GC-MS analysis of aroma compounds to authenticity testing of honey. Food Chemistry, 72 (4), 511-520(10)

Rivasseau, C., & Caude, M. (1995). Comparison of on-line SPE-HPLC and SPME-GC for the analysis of microcontaminants in water. Chromatographia, 41 (7/8), 462-469.

Roberts, D.D., Pollien, P., & Milo, C. (2000). Solid-phase microextraction method development for headspace analysis of volatile flavour components. Journal of Agricultural and Food Chemistry, 48, 2430-2437.

Ruiz, J., Cava, R., Ventanas, J., & Jensen, M.T. (1998). Headspace solid phase microextraction for the analysis of volatiles in a meat product: Dry-Cured Iberian ham. Journal of Agricultural and Food Chemistry, 46, 4688-4694.

Scarlata, C.J., & Ebeler, S.E. (1999). Headspace solid-phase microextraction for the analysis of dimethyl sulfide in beer. Journal of Agricultural and Food Chemistry, 47, 2505-2508.

Schafer, B., Henning, P., & Engewald, W. (1997). Methodological aspects of headspace SPME: Application of the retention index system. Journal of High Resolution Chromatography, 20, 217-221.

Scheppers Wercinski, S.A. (1999). Solid phase microextraction: A practical guide. New York: Marcel Dekker.

Schlack, J.E., & Diccero, J.J. (1974). Gas-liquid chromatographic quantitative determination of vanillin and ethyl vanillin in foods. Journal of Associative and Official Analytical Chemistry., 57, 329-331.

Song, J., Gardner, B.D., & Beaudry, R.M. (1997). Rapid analysis of volatile flavour compounds in apple fruit using SPME and GC/Time-of-flight mass spectrometry. Journal of Agricultural and Food Chemistry, 45 (5), 1801-1807.

Song, J., Fan, L., & Beaudry, R.M. (1998). Application of solid phase microextraction and gas chromatography/Time-of-flight mass spectrometry for rapid analysis of flavour volatiles in tomato and strawberry fruits. Journal of Agricultural and Food Chemistry, 46, 3721-3726.

Sostaric, T., Boyce, M.C., & Spickett, E.E. (2000). Analysis of the volatile components in vanilla extracts and flavorings by solid-phase microextraction and gas chromatography. Journal of Agricultural Food Chemistry, 48 (12), 5802-5807.

Steffen, A., & Pawliszyn, J. (1996). Analysis of flavour volatiles using headspace solid-phase microextraction. Journal of Agricultural and Food Chemistry, 44, 2187-2193.

Vitali, M., Guidotti, M., Giovinazzo, R., & Cedrone, O. (1998). Determination of pesticides residues in wine by SPME and GC/MS for consumer risk assessment. Journal of Food Additives and Contaminants, 15, (3), 280-287.

Volante, M., Cattaneo, M., Bianchi, M., & Zoccola, G. (1998). Some applications of solid phase microextraction (SPME) in the analysis of pesticide residues in food. Journal of Environmental Science and Health, B33, (3), 279-292.

Wallace, E.M. (1983). HPLC of vanillin and related phenols. Journal of Chromatographic Science, 21, 139.

Yang, K.W., Eisert, R., Lord, H., & Pawliszyn, J. (1999). Determination of pesticides in foods by automated SPME-GC-MS. In J. Pawliszyn (Ed), Applications of solid phase microextractions (pp. 435-447). Cambridge, UK: The Royal Society of Chemistry.

Yang, M.J., Orton, M.L., & Pawliszyn, J. (1997). Quantitative determination of caffeine in beverages using a combined SPME-GC/MS method. Journal of Chemical Education, 74 (9), 1130-1132.

Yang, X., & Peppered, T. (1994). Solid-phase microextraction for flavour analysis. Journal of Agricultural and Food Chemistry, 42, 1925-1930.

Zhou, M., Robards, K., Glennie-Holmes, M., & Helliwell, S. (1999). Analysis of volatile compounds and their contribution to flavor in cereals. Journal of Agricultural and Food Chemistry, 47, 3941-3957.

Appendix A

Journal Paper

Analysis of the Volatile Components in Vanilla Extracts and Flavorings by Solid-Phase Microextraction and Gas Chromatography

Tomislav Sostaric, Mary C. Boyce, and Evadne E. Spickett

Faculty of Communications, Health and Science, Edith Cowan University, 2 Bradford St., Mt. Lawley, Perth, Western Australia, 6050, Australia

Journal of Agricultural and Food Chemistry®

Reprinted from
Volume 48, Number 12, Pages 5802–5807

Analysis of the Volatile Components in Vanilla Extracts and Flavorings by Solid-Phase Microextraction and Gas Chromatography

Tomislav Sostaric, Mary C. Boyce,* and Evadne E. Spickett

Faculty of Communications, Health and Science, Edith Cowan University, 2 Bradford St., Mt. Lawley, Perth, Western Australia, 6050, Australia

The development and application of a solid-phase microextraction (SPME) method in the analysis of vanilla extracts and vanilla flavorings was studied. The SPME method was developed to be used in conjunction with gas chromatography mass spectrometry (GC−MS). The optimized SPME sampling parameters for the determination of the volatile components included a poly(acrylate) fiber, a 40-min sampling time at room temperature, and a 2-min desorption time. The reproducibility of the method was good, with a percent relative standard deviation between 2.5 and 6.4% for the target compounds. The data suggest that the origin of natural extracts can be readily determined from the GC profile and that differences exist between nature-identical and synthetic flavorings and the natural extracts. The method also has potential for identifying the type of vanilla extract/flavoring used to flavor food.

Keywords: *Solid-phase microextraction; vanilla extracts; flavor analysis; gas chromatography*

INTRODUCTION

Vanilla is one of the most widely used flavoring ingredients in food. Several vanilla flavoring agents are used, the most prized being natural extracts derived from the vanilla orchid. The specific taste and aroma properties of the different agents result from the blend of components present. Over 170 volatile components that contribute to flavor have been identified in natural extracts, some being present in minute amounts (Klimes and Lamparsky, 1976; Ranadive, 1992). Vanillin, *p*-hydroxybenzaldehyde, vanillic acid, *p*-methoxybenzaldehyde, and piperonal are some of the components found in the highest quantitites. High quality natural extracts are expensive and their supply is limited, therefore, nature-identical and synthetic vanilla flavorings are frequently used to flavor food. Nature-identical flavorings contain only components that are found in nature. Synthetic vanilla flavorings usually contain vanillin and/or ethylvanillin that has been synthetically produced. As natural extracts are expensive compared to nature-identical and synthetic vanilla flavorings, there have been many attempts at adulterating natural extracts or substituting less expensive vanilla flavorings for natural extracts (Riley and Kleyn, 1989; Lamprecht et al., 1994).

Many different methods have been developed to characterize vanilla extracts including high-performance liquid chromatography (HPLC), isotope ratio mass spectrometry (IRMS), gas chromatography (GC), and thin-layer chromatography (Ranadive, 1992; Belay and Poole, 1993; Lamprecht et al., 1994). HPLC allows the relative concentrations of the main components such as vanillin, *p*-hydroxybenzoic acid, and *p*-hydroxybenzaldehyde to be compared and can be used to determine the origin of the extract. Vanillin extracted from the vanilla orchid has a characteristic carbon isotope signature (−16 to − 21‰) which is very different to that of vanillin derived from lignin (−26 to −32‰) (Lamprecht et al., 1994). However, the analysis involves isolation of pure vanillin from the extract prior to isotope analysis. In addition, the IRMS equipment is expensive and not available in many laboratories. GC is potentially ideal for the analysis of complex mixtures such as natural extracts. However, traditional methods for extracting volatile components from the nonvolatile components (fats, sugars, and waxes) are both time-consuming and prone to sample loss and degradation. An alternative has been to sample the headspace only; however, conventional headspace methods such as static headspace GC or dynamic purge and trap GC methods require concentration steps and specific sampling equipment and are time-consuming (Steffan and Pawliszyn, 1996).

Solid-phase microextraction (SPME) is a relatively new solventless extraction technique that can be used in conjunction with HPLC or GC. The analytes are extracted from a variety of matrixes by partitioning them from a liquid or gaseous sample into an immobilized stationary phase. The stationary phase, which is coated onto a fused silica fiber, is exposed to the headspace or liquid. The extracted analytes can then be thermally desorbed in the injector of the GC and subsequently swept onto the column where they are separated. This provides a simple and effective method for the selective extraction of volatile and semivolatile components from a matrix containing nonvolatile high molecular weight components. The technique has been successfully used for the analysis of volatiles from apples (Song et al., 1997), cinnamon (Miller et al., 1996), orange juice (Steffen et al., 1996), ground coffee (Yang and Peppard, 1994), and hops (Field et al., 1996).

* Corresponding author (telephone 61 8 93706328; e-mail m.boyce@cowan.edu.au; fax 61 8 93706103).

10.1021/jf000515+ CCC: $19.00
Published on Web 10/21/2000

Table 1. Concentration, GC Retention, and Precision Data for Components Present in the Standard Mixture

analyte	concentration ppm	retention time, min	precision (% RSD) 25 °C	80 °C
m-methoxybenzaldehyde	0.10	9.1	3.32	8.03
ethyl benzoate	0.10	11.6	4.30	6.39
piperonal	0.10	13.3	6.40	13.50
vanillin	10.0	15.9	2.51	15.51
coumarin	10.0	17.9	5.22	10.75
ethylvanillin	10.0	18.2	5.22	14.65
p-hydroxybenzaldehyde	10.0			
vanillic acid	10.0			
protocatechuic acid	10.0			

We report here the development of a qualitative method for the analysis of flavor volatiles present in vanilla extracts/flavorings using SPME. This had been applied to the analysis of vanilla extracts (natural and synthetic) and food samples.

MATERIALS AND METHODS

Chemicals. Coumarin, ethyl vanillin, ethyl benzoate, *p*-hydroxybenzaldehyde, *m*-methoxybenzaldehyde, piperonal, protocatechuic acid, vanillin, and vanillic acid were purchased from Sigma, Australia, and used as received. Ethanol, HPLC grade, was purchased from Aldrich, Australia.

Standards and Samples. A standard mixture comprising the substances listed in Table 1 was prepared in 95:5 water: ethanol. This matrix was chosen to match the ethanol content of the diluted (1 in 10) natural extracts. Ethyl benzoate, piperonal, and *m*-methoxybenzaldehyde were present at a concentration of 0.1 ppm, while all other standards were present at concentrations of 10 ppm. The lower concentrations of ethyl benzoate, *m*-methoxybenzaldehyde, and piperonal in the standard mix were necessary as these volatile components overload the capillary GC at higher concentrations. Individual standard solutions for each of these compounds were prepared in a similar manner. Certified Bourbon, Indonesian, and Tahitian vanilla extracts (0.2% v/v in 35% ethanol), nature-identical vanilla flavoring, synthetic vanilla flavoring, and vanilla flavored food products were obtained locally. The natural extracts were diluted (1 in 10) with water prior to analysis.

General GC–MS Analysis Conditions. Gas chromatographic analysis was carried out using a Varian 3400 GC fitted with a split/splitless injector suitable for SPME analysis, a Varian 2000 mass spectrometer (MS) detector, and a Varian 9200 autosampler. Helium was used as the carrier gas with a flow rate of 1.0 mL/min. The components were separated on a 30 m × 0.2 mm column with a 0.25 μm film of DB5 stationary phase (Alltech, Australia). The injector temperature was set at 250 °C and operated in the splitless mode for 2 min unless otherwise stated. The column was maintained at 40 °C for 2 min then ramped to 200 °C at 8 °C·min^{-1} and further ramped to 250 °C at 50 °C·min^{-1}. The NIST '92 MS Library was used to identify key components in the samples.

General Conditions for SPME Extraction. Three SPME fibers were used in this study: poly(dimethylsiloxane) (PDMS), poly(acrylate) (PA), and carbowax/poly(divinylbenzene) (CW/DVB). The thickness of the polymeric coating varied with the fiber type: the PDMS fiber coating was 100 μm thick, the PA coating was 85 μm thick, and the CW/DVB coating was 65 μm thick. All the fibers were supplied by Supelco (Australia and Canada) and were conditioned as recommended by the manufacturer. The sample or standard mixture (200 μL) was transferred to a 2.0 mL vial, which was sealed with a screw capped top containing a Teflon-lined septum. The fiber was exposed to the headspace of the sample for 40 min, unless otherwise stated. The fiber was then retracted and inserted immediately into the inlet of the GC. For nonambient temperature extractions a heating block (Thermoline, BTC 9000) was used to heat the vial and its contents. Each sample was analyzed in triplicate, using a fresh vial and aliquot for each replicate.

RESULTS AND DISCUSSION

There are several factors that influence headspace analysis by SPME. They include fiber type, extraction temperature, adsorption time, and desorption conditions. Therefore, we explored the effect of these variables on the extraction of volatiles characteristic of vanilla extracts. The sample vial volume was not varied and was fixed at 2.0 mL. This vial size is compatible with the autosampler available in our laboratory and allowed us to perform automated SPME extractions at room temperature. The components in the standard mixture (Table 1) were chosen because (a) they are known to be present in vanilla extracts, (b) they include components characteristic of natural extracts and synthetic vanilla flavorings (e.g., ethylvanillin), and (c) they have a range of volatilities and polarity.

Determination of the Best Fiber Coating for SPME. The fiber coatings used in this study were the PDMS, PA, and CW/DVB fibers. PDMS was trialed as it has been used successfully for the analysis of both polar and nonpolar volatile components and the phase is similar to the stationary phase coating on the GC column used for this study (Miller et al., 1996; Steffan and Pawliszyn, 1996). The PA fiber was investigated as it has been used successfully for the extraction of more polar analytes (Steffan and Pawliszyn, 1996). The mixed coating was trialed as it was considered suitable for the analysis of polar semivolatiles (Pawliszyn, 1997).

Figure 1 shows a comparison of the extraction efficiencies of the fiber coatings for the analytes extracted from the standard mixture. Each fiber was effective at extracting six of the nine target components; however, none of the fibers extracted *p*-hydroxybenzaldehyde, protocatechuic acid, or vanillic acid at concentrations of 10 ppm. All the fibers extracted the early eluting (see Table 1 for retention time data) more volatile components (e.g., ethyl benzoate, *m*-methoxybenzaldehyde and piperonal) in the greatest amounts; however, the PA fiber was superior in that it extracted more of each component. For the less volatile, later eluting components, the PA fiber was also superior. For example, the PA fiber extracted over 50% more vanillin than either the PDMS or CW/DVB fiber. The experiment was repeated using a natural Bourbon extract and the efficiencies of the different fibers for extracting the main volatiles are shown in Figure 2. The target compounds were selected because they either were in the standard mixture or were present in high concentrations in the natural extract. As observed with the standard mixture, the PA coating was the most efficient at extracting vanillin and ethyl benzoate. It was also as efficient as the other fibers at extracting three major esters identified in the extract. The PA fiber was, therefore, used for the remainder of the study.

Absorption and Desorption Conditions. A desorption temperature of 250 °C with the injector operating in the splitless mode for 2 min was sufficient to quantitatively transfer all the components from the fiber to the separation column. During desorption the column temperature was held at 40 °C for 2 min to focus the sample onto the top of the column. The fiber was thermally desorbed prior to each run by putting the fiber in the injector with the split open for approximately 5 min.

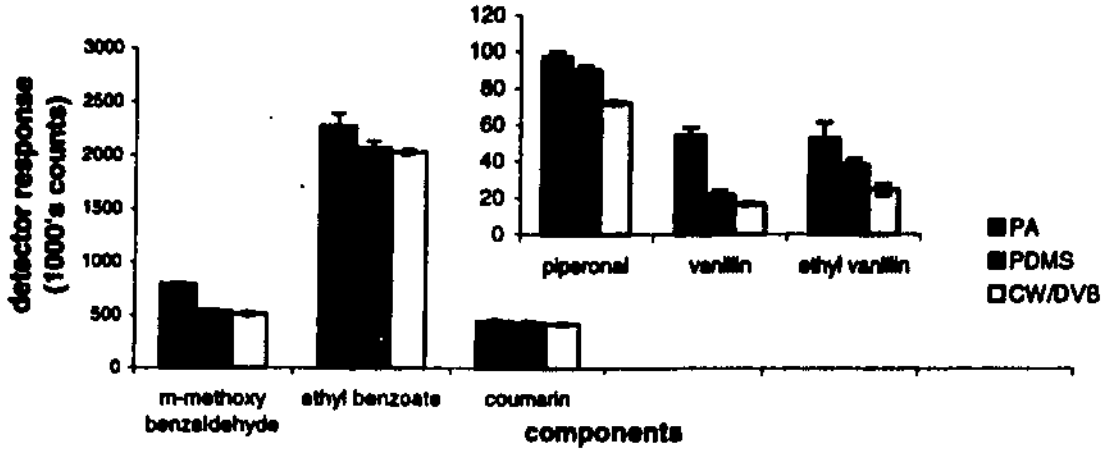


Figure 1. Comparison of the extraction efficiencies of the poly(dimethylsiloxane) (PDMS), poly(acrylate) (PA), and carbowax/poly(divinylbenzene) (CW/DVB) fibers using a prepared mixture.

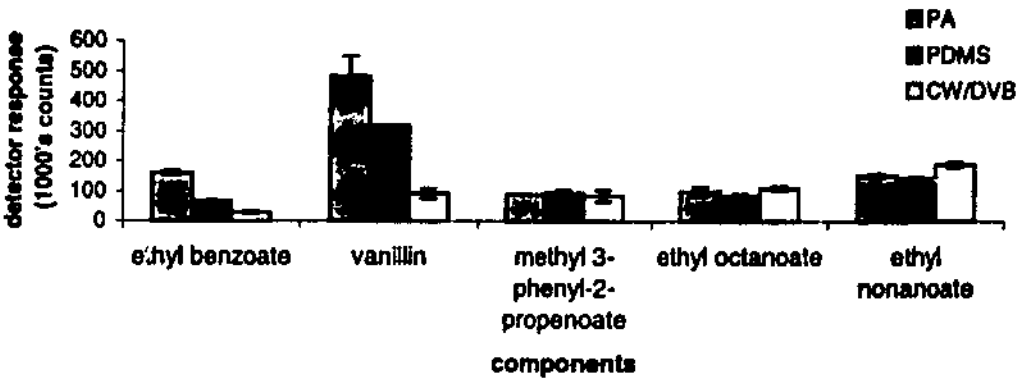


Figure 2. Comparison of the extraction efficiencies of the poly(dimethylsiloxane) (PDMS), poly(acrylate) (PA), and carbowax/poly(divinylbenzene) (CW/DVB) fibers using a natural Bourbon vanilla extract.

To extract components reproducibly from a sample it is desirable to do so when the system is at equilibrium (Pawliszyn, 1997). For SPME headspace analysis the analytes equilibrate between three phases, the liquid phase, the headspace, and the polymeric fiber coating. A plot of extraction time versus amount extracted can be used to determine the time taken for the components to reach equilibrium between the phases. The point where the curve plateaus or levels off is considered to be the equilibration time (Steffan and Pawliszyn, 1996). Therefore, to determine equilibrium or steady-state sampling conditions at room temperature, the PA fiber was exposed to the standard mixture for differing amounts of time between 5 and 100 min. Equilibrium conditions were achieved for all components in 40 min (Figure 3). The experiment was repeated using a natural vanilla Bourbon extract and similar results were obtained, with equilibrium conditions being achieved for all target components in 40 min (Figure 4).

The precision of the method was then investigated. The standard mixture was extracted several times using an absorption time of 40 min. The percent relative standard deviation (% RSD) for all the compounds was excellent and ranged between 2.5 and 6.4% for seven extractions (Table 1). When the experiment was repeated for a natural extract the % RSD for the main components gave similar values (2.6–8%).

Stirring or sonicating the sample during absorption or employing a higher extraction temperature will generally increase the rate at which steady-state conditions are achieved (Pawliszyn, 1997). We investigated the effect of using higher temperatures to reduce the equilibration time.

The standard mixture was extracted using the PA fiber at different temperatures (ambient and 40, 60, and 80 °C). As temperature increased the extraction efficiency increased for all components and was greatest at 80 °C. The largest increases in extraction were observed for the less volatile components such as vanillin, coumarin, and ethylvanillin (data not shown). Therefore, the steady-state sampling conditions were determined at 80 °C by exposing the fiber to the standard mixture for different time periods between 5 and 80 min. In general, equilibrium conditions were achieved in a shorter time. For example, it was achieved within 20 min for the more volatile components (*m*-methoxybenzaldehyde and piperonal) and achieved after 30 min for the less volatile components (ethylvanillin and coumarin) (Figure 5). While this method results in shorter absorption times, the error (expressed as standard deviation) incurred between replicates was greater than for the same experiment conducted at room temperature. The precision of the method was also investigated. The standard mixture was extracted several times using a 30-min absorption time. The percent relative standard deviation for all the compounds ranged between 6.4 and 15.5% for seven extractions (Table 1), indicating that the reproducibility of the method was poorer than for the same experiment conducted at room temperature. The poorer reproducibility between replicates for the high-temperature experiment is not surprising since the extraction process was done manually and involved quickly removing the fiber from the vial (held at 80 °C) and inserting it into the GC inlet to minimize temperature changes.

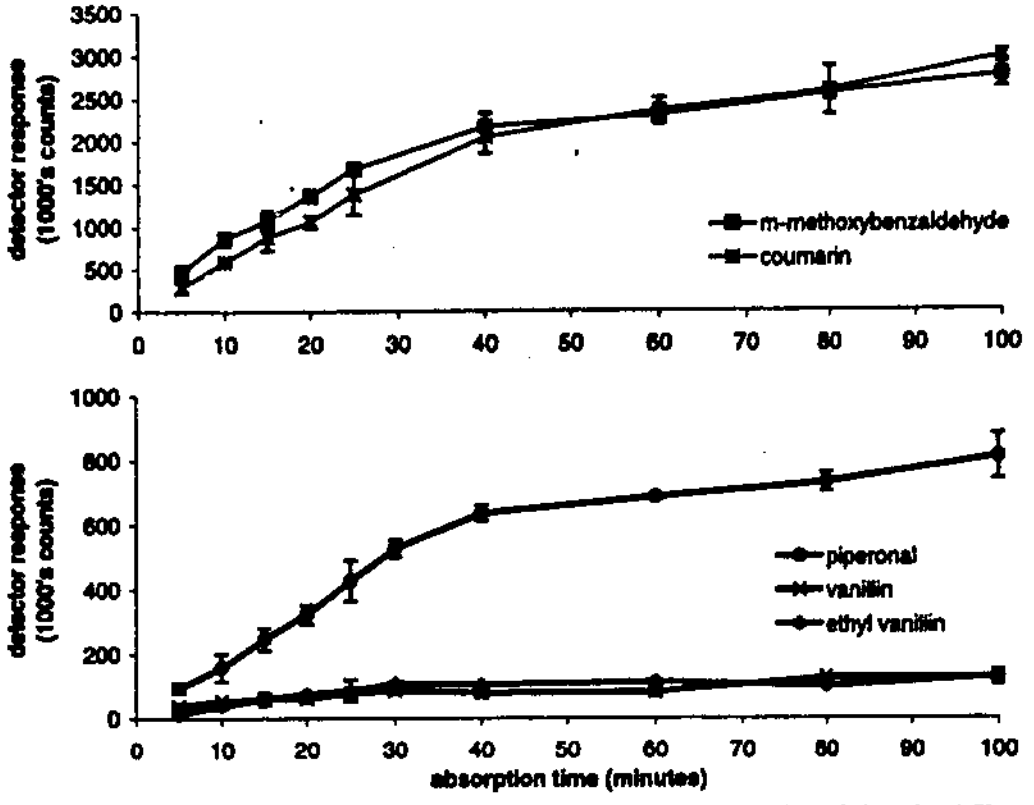


Figure 3. Effect of absorption time at room temperature on the extraction efficiency of the poly(acrylate) fiber using a standard mixture.

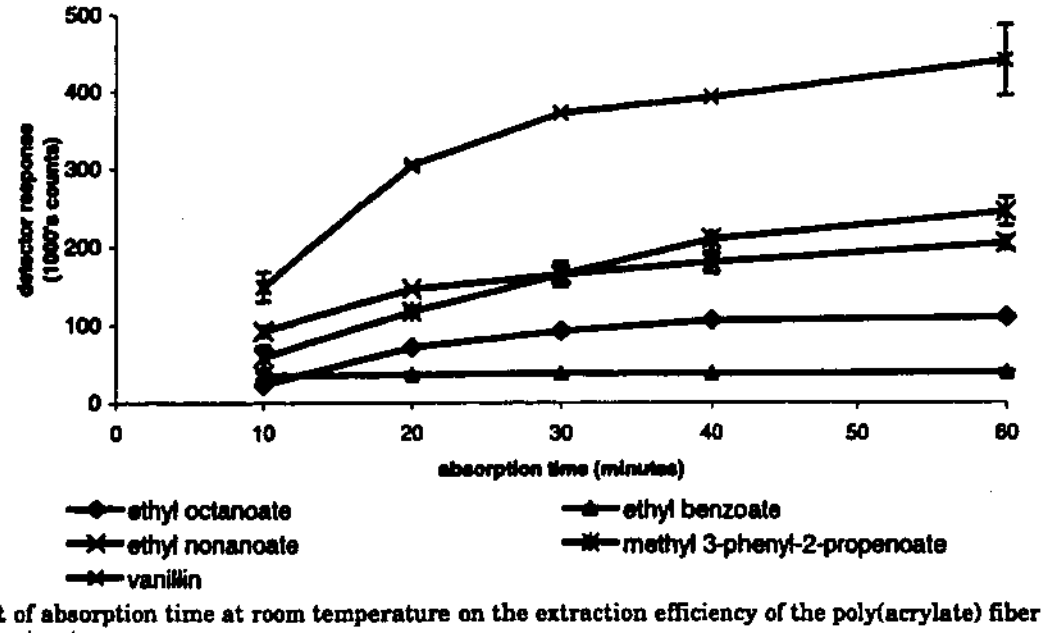


Figure 4. Effect of absorption time at room temperature on the extraction efficiency of the poly(acrylate) fiber using a natural Bourbon vanilla extract.

Having completed the temperature work, we felt that the extra sampling time required to achieve steady-state conditions at room temperature was preferable to the extra labor required to process the samples manually at higher temperature. In addition, the automated process gave better precision over the manual process. Therefore, a PA fiber, using a desorption time of 2 min, an extraction temperature of 25 °C, and an absorption time of 40 min was used to extract volatile components from vanilla extracts and flavorings.

Application of SPME to Real Samples. A preliminary investigation was carried out to determine if SPME-GC–MS could potentially be used to discriminate between different types of extracts and flavorings. A Bourbon, Tahitian, and Indonesian extract (from a common supplier) were each analyzed in triplicate. They were distinguishable from each other by the presence of key components or fingerprint regions unique to the extract (Figure 6). For example, the Tahitian extract was distinguishable because of the presence of large amounts of *p*-methoxybenzaldehyde and an unidentified aromatic component having a retention time of 12.8 min, which were absent from the other extracts. The large amount of *p*-methoxybenzoic acid methyl ester present compared to the trace amount found in the Indonesian and Bourbon extracts was also a distinguishing feature. The Bourbon and Indonesian extracts were distinguishable from each other by the different relative amounts of key components such as hexanoic acid, 5-propenyl-1,3-benzodioxole, and ethyl nonanoate.

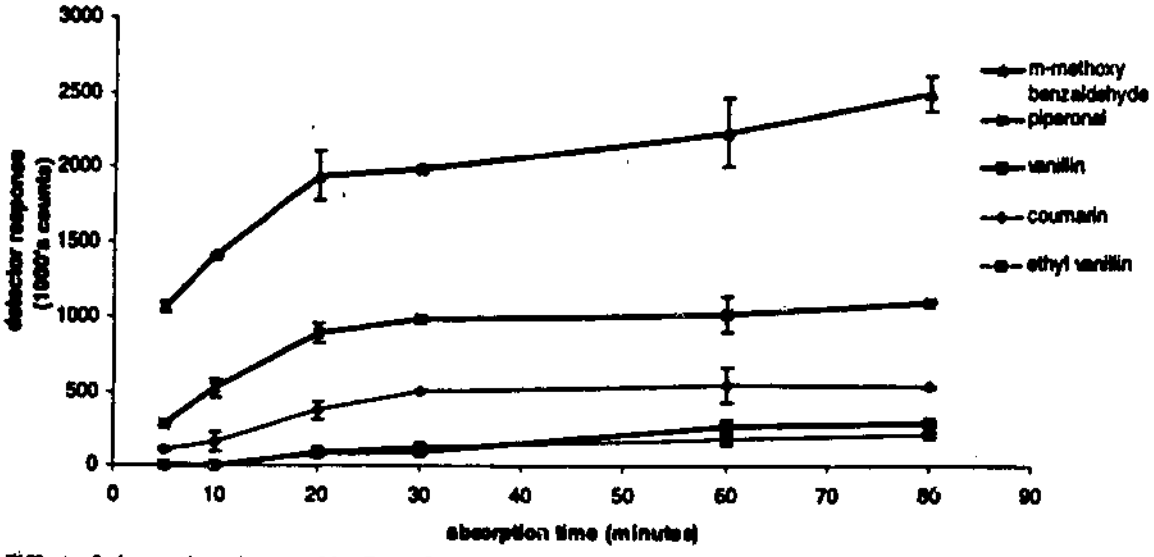


Figure 5. Effect of absorption time at 80 °C on the extraction efficiency of the poly(acrylate) fiber using a standard mixture.

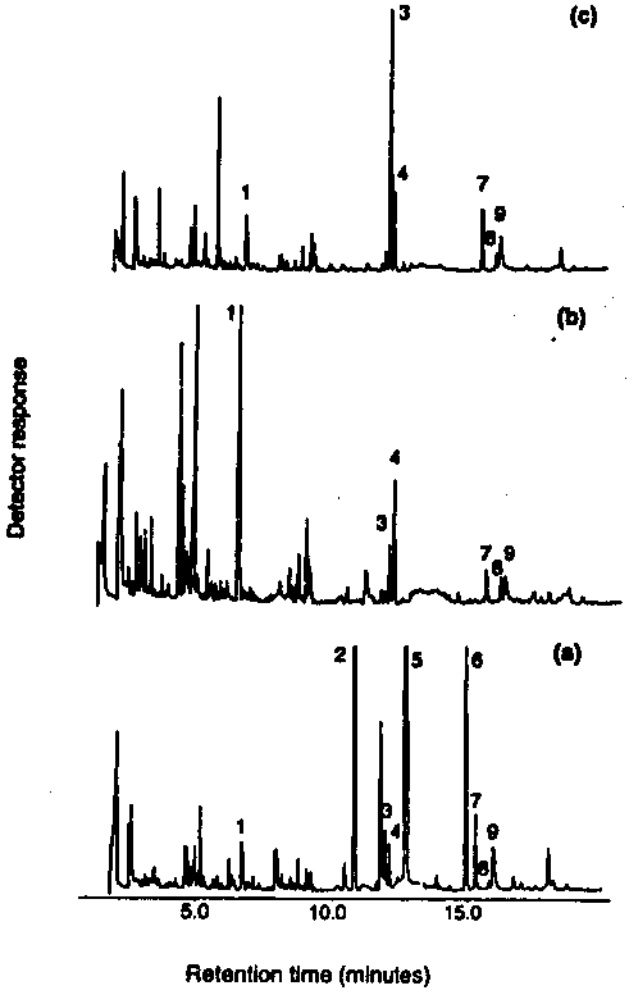


Figure 6. Gas chromatographic profile of headspace volatile components sampled by solid-phase microextraction at room temperature from (a) Tahetian natural vanilla extract; (b) Indonesian natural vanilla extract, and (c) Bourbon natural vanilla extract. The optimized conditions are given in the Experimental Section. Peak identification: 1 = ethyl hexanoate; 2 = *p*-methoxybenzaldehyde; 3 = 5-propenyl-1,3-benzodioxole; 4 = ethyl nonanoate; 5 = unidentified component; 6 = *p*-methoxybenzoic acid methyl ester; 7 = 3-phenyl-2-propenoic acid methyl ester; 8 = ethyl decanoate; 9 = vanillin.

However, as these are natural extracts, natural variation between extracts is to be expected. Whether the differences observed here, particularly for Bourbon and

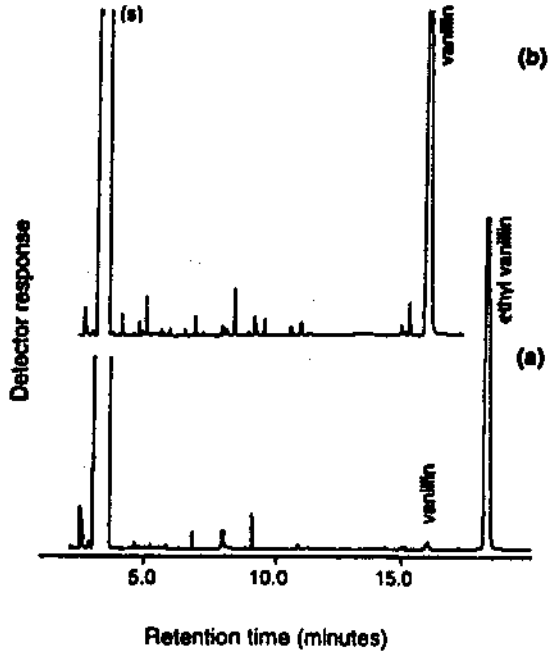


Figure 7. Gas chromatographic profile of headspace volatile components sampled by solid-phase microextraction at room temperature from (a) synthetic vanilla flavoring and (b) nature-identical Bourbon flavoring. The optimized conditions are given under Materials and Methods. (s) = solvent.

Indonesian, are sufficient to distinguish between these extracts from a range of suppliers is currently under study. The relative concentrations of key components and the differences in the profiles of the natural extracts from a number of sources are being measured by using this SPME-GC–MS method.

Samples of nature-identical Bourbon flavoring and synthetic vanilla flavoring were analyzed next and compared against each other and the natural extracts. The synthetic vanilla flavoring was easy to identify, in that it had a relatively simple chromatogram with only a few major components present (Figure 7a). Another key difference was the presence of ethylvanillin, which does not occur naturally in vanilla extracts. The nature-identical Bourbon flavoring was clearly different than the natural Bourbon extract. The vanillin content was extremely high, and the straight chain esters such as ethyl nonanoate and ethyl decanoate, characteristic of natural vanilla extracts including Bourbon, were absent (Figure 7b).

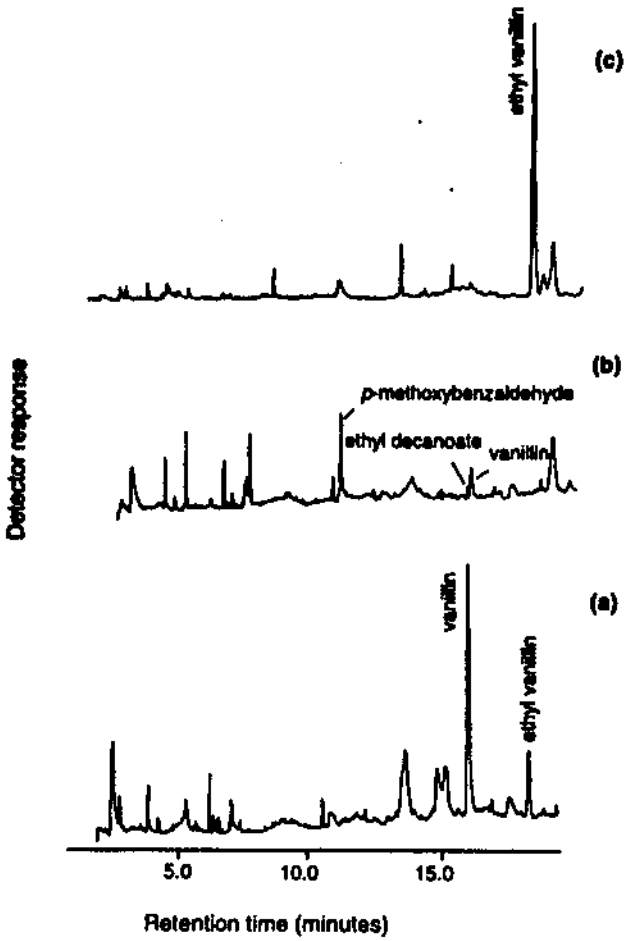


Figure 8. Gas chromatographic profile of headspace volatile components sampled by solid-phase microextraction at room temperature from (a) yogurt and (b) ice cream and at 80 °C from (c) custard powder. The optimized conditions are given under Materials and Methods.

The SPME-GC–MS method was also used to tentatively identify the source of vanilla flavoring used in some common food products. Locally produced yogurt, ice-cream, and custard powder were sampled. The chromatogram of the volatiles emitted by yogurt showed the presence of ethylvanillin, indicating that synthetic flavoring was used (Figure 8a). Ice cream, labeled as containing natural vanilla extract, was similarly analyzed. The presence of *p*-methoxybenzaldehyde and the straight chain ethyl decanoate and the absence of ethylvanillin indicated that a natural extract was used to flavor the ice cream (Figure 8b). Custard powder was also analyzed to see if the method was applicable to solid samples. At room temperature the concentrations of volatiles given off was minimal so the high-temperature method (absorption time 30 min at 80 °C) was employed. The presence of ethylvanillin indicated that a synthetic vanilla flavoring was used (Figure 8c).

In summary, using SPME as the extraction tool, GC can be used to analyze the complex mixture of components present in natural extracts. The key components (e.g., vanillin, ethyl benzoate, piperonal, *m*-methoxybenzaldehyde) routinely identified by HPLC are extracted, as well as a range of esters.

Of the fibers tested the PA fiber was superior for extracting the flavor volatiles. The volatile components were sufficiently concentrated on the coated fiber to be analyzed directly; therefore, no sample preparation is required. At 25 °C the automated SPME method was suitable for the analysis of extracts and most food samples. The high-temperature SPME was suitable for analyzing samples with low concentrations of volatiles.

ACKNOWLEDGMENT

We thank Monica Witsch of Peters & Brownes Group for useful discussions.

LITERATURE CITED

Belay, M.; Poole, C. Determination of vanillin and related flavor compounds in natural vanilla extracts and vanilla flavored foods by thin-layer chromatography and automated multiple development. *Chromatographia* **1993**, *37*, 365–373.

Field, J. A.; Nickerson, G.; Derryl, D. J.; Heider, C. Determination of essential oils in hops by headspace solid-phase microextraction. *J. Agric. Food Chem.* **1996**, *44*, 1768–1772.

Klimes, I.; Lamparsky, D. Vanilla volatiles–A comprehensive analysis. *Int. Flavors Food Addit.* **1976**, *Nov/Dec*, 272–276.

Lamprecht, G.; Pichlmayer, F.; Schmid, E. Determination of the authenticity of vanilla extracts by stable isotope ratio analysis and component analysis by HPLC. *J. Agric. Food Chem.* **1994**, *42*, 1722–1727.

Miller, K.; Poole, C.; Pawlowski, T. Classification of the botanical origin of cinnamon by solid-phase microextraction and gas chromatography. *Chromatographia* **1996**, *42*, 639–646.

Pawliszyn, J. *Solid-phase microextraction-theory and practice*; Wiley-VCH: New York, 1997.

Song, J.; Gardner, B.; Holland, J.; Beaudry, R. Rapid analysis of volatile flavor compounds in apple fruit using SPME and GC/time-of-flight mass spectrometry. *J. Agric. Food Chem.* **1997**, *45*, 1601–1807.

Steffen, A.; Pawliszyn, J. Analysis of flavor volatiles using headspace solid-phase microextraction. *J. Agric. Food Chem.* **1996**, *44*, 2187–2193.

Ranadive, A. Vanillin and related flavor compounds in vanilla extracts made from beans of various global origins. *J. Agric. Food Chem.* **1992**, *40*, 1922–1924.

Riley, K.; Kleyn, D. Fundamental principles of vanilla/vanilla extract processing and methods of detecting adulteration in vanilla extracts. *Food Technol.* **1989**, *Oct*, 64–77.

Yang, X.; Peppard, T. Solid-phase microextraction for flavour analysis. *J. Agric. Food Chem.* **1994**, *42*, 1925–1930.

Received for review April 26, 2000. Revised manuscript received July 31, 2000. Accepted August 1, 2000.

JF000515+

Appendix B

Sample of a Certified document stating vanillin content.

SPECIFICATION SHEET

Product Name	- TAHITIAN VANILLA EXTRACT (2X)	
Description	- Pure Vanilla Extract	
Color	- Darkish Brown	
Aroma	- Typical Vanilla-like	
Ingredient Line	- Made from 26.70 ounces of selected, choice Tahitian vanilla beans produced by our unique cold process method of extraction, 35% natural corn alcohol, .8 pounds of sugar per gallon and water.	
Shelf Life	- At least one year.	
Storage	- Keep at room temperature, away from direct sunlight and heat.	
Packaging	- High density polyethylene gallon bottles	
Analytical	- Lead Number	- 1.54 - 1.80
	Vanillin	- .09 - .15
Microbiological	- Plate Count	- <10,000
	- Yeast	- <1/ ML
	- Mold	- <1/ ML
	- MPN Coliform	- <0.3/ML
	- E. Coli	- <0.3/ML
	- CP Stapylococci	- <1/ ML
	- Salmonella	- Negative